Microfluidic Systems for Cancer Diagnosis
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Microfluidic Systems for Cancer Diagnosis

Edited by

Jose L. Garcia-Cordero

Institute of Human Biology (IHB), Roche Pharma Research and Early Development, Roche Innovation Center, Basel, Switzerland

Alexander Revzin

Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, MN, USA
Despite tremendous financial investments and significant scientific advances, cancer remains a leading cause of mortality and morbidity around the world. There remains a strong need for technological innovation to better diagnose, monitor, and treat cancer. Our methods collection provides a reader with some of the recent, exciting microfluidics technologies for cancer diagnosis and monitoring. Contributions in this collection fit into broad categories: (1) liquid biopsy for cancer diagnosis and (2) platforms for precision oncology or personalized medicine.

Liquid biopsy refers to querying biological fluids, such as blood, for tumor-derived materials. Some of the more common liquid biopsy biomarkers are extracellular vesicles, cell-free DNA, and circulating tumor cells. Liquid biopsies are exciting for early diagnosis of cancer because they can contain a signature of both the tissue of origin and its malignant state. There is, therefore, a tremendous promise to use liquid biopsy for early detection of cancer before symptoms develop. As highlighted by the contributions to this methods collection, microfluidic technologies hold considerable promise for liquid biopsy applications. These devices may be portable and inexpensive, can integrate sophisticated multi-step workflows, and may leverage interesting transport phenomena not easily accessible on the macro scale (e.g., inertial forces). Our collection also highlights that microfluidics may be integrated with bioanalytical modules for electrical or optical interrogation of liquid biopsy biomarkers like extracellular vesicles.

Precision oncology is a broad term that implies tailoring treatment regimen to an individual cancer patient. There are scenarios where a panel of targeted therapeutics may be selected based on patient-specific mutations. However, in the majority of cases related to administering front-line chemotherapy or immunotherapy, an oncologist makes therapy selection using general (NCCN) guidelines based on the patient’s age and performance status, a semi-quantitative score of patient’s ability for self-care, daily activity, and physical state. After a therapy regimen is chosen, the patient’s response is evaluated by imaging every 2–3 months as the primary metric of success. Only a fraction of patients (percentage varies depending on the cancer type) respond to the first therapy selection while non-responders lose precious time. There is increasing realization that this one-size-fits-most approach is not acceptable and that functional tests tailored to individual patients are necessary.

The challenge is in creating effective patient avatars for testing anti-cancer drugs. Contributions to this methods collection highlight several benefits of microfluidic technologies for precision oncology. Microfluidic cancer cultures require minimal amount of starting cellular material and may be used to test therapies based on small amounts of patient tumor tissue. These cultures may be engineered to better reflect tumor heterogeneity (e.g., by adding stromal cells) or may contain intact tumor tissue. Furthermore, as highlighted by one of the contributions, microfluidic devices may be integrated with a gradient generator and drug dispenser to test different types and concentrations of drugs.

In this book, we have compiled an interesting collection of microfluidic technologies for cancer diagnostics. As expected from current research efforts around the world, most chapters focus on separating, detecting, releasing, and characterizing circulating tumor cells and clusters. Other chapters focus on assessing the effects of cancer drugs in 2D and 3D cultures and human cancer cells cultured in microfluidic devices. A new technology for
fine-needle aspiration used to collect cystic samples at the point-of-care is presented in this book. There are also chapters on cell free-DNA extraction from plasma as well as the detection and isolation of extracellular vesicles. Another area of research is immunophenotyping cells based on their cytokine secretion profile.

Overall, we are excited by the diversity of technologies represented in this book and are thankful to all the authors for their willingness to contribute. It is also worth noting geographic diversity with contributions coming from Asia, Australia, Europe, and North and South America. This underscores the global importance of the problem being tackled. We hope that a reader will find this book helpful and will be able to use protocols detailed here to further their scientific goals. It is our wish that the detailed protocols we have collected in this book will be helpful to either replicate the construction of microfluidic devices specifically developed for cancer diagnosis or to catalyze development of new and better cancer diagnostic devices.

Basel, Switzerland

Jose L. Garcia-Cordero

Rochester, MN, USA

Alexander Revzin
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Contributors

AYNUR ABDULLA  •  State Key Laboratory of Oncogenes and Related Genes, Institute for Personalized Medicine, School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai, China

SAM H. AU  •  Department of Bioengineering, Imperial College London, London, UK; Cancer Research UK Convergence Science Centre, London, UK

LOURDES BASABE-DESMONTS  •  Microfluidics Cluster UPV/EHU, BIOMICs microfluidics Group, Lascaray Research Center, University of the Basque Country UPV/EHU, Vitoria-Gasteiz, Spain; Basque Foundation of Science, IKERBASQUE, Bilbao, Spain; Bioaraba Health Research Institute, Microfluidics Cluster UPV/EHU, Vitoria-Gasteiz, Spain

FERNANDO BENITO-LOPEZ  •  Bioaraba Health Research Institute, Microfluidics Cluster UPV/EHU, Vitoria-Gasteiz, Spain; Microfluidics Cluster UPV/EHU, Analytical Microsystems & Materials for Lab-on-a-Chip (AMMa-LOAC) Group, Analytical Chemistry Department, University of the Basque Country UPV/EHU, Leioa, Spain

DANIEL D. BILLADEAU  •  Department of Immunology, Mayo Clinic College of Medicine and Science, Rochester, MN, USA

JEFFREY T. BORENSTEIN  •  Draper, Bioengineering Division, Cambridge, MA, USA

MERT BOYA  •  School of Electrical and Computer Engineering, Georgia Institute of Technology, Atlanta, GA, USA

KANGFU CHEN  •  Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, ON, Canada

DAHEUI CHOI  •  Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, MN, USA

ALVARO J. CONDE  •  Micronit B.V., Enschede, Netherlands; Heriot-Watt University, Edinburgh, UK

YAO DAI  •  Department of Radiation Oncology, College of Medicine, University of Florida, Gainesville, FL, USA

MUHAMMEDIN DELIORMAN  •  Division of Engineering, New York University Abu Dhabi (NYUAD), Abu Dhabi, UAE

LIN DING  •  School of Biomedical Engineering, University of Technology Sydney, Sydney, NSW, Australia

XIANTING DING  •  State Key Laboratory of Oncogenes and Related Genes, Institute for Personalized Medicine, School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai, China

HAMIDREZA ABOULKHEYR ES  •  School of Biomedical Engineering, University of Technology Sydney, Sydney, NSW, Australia

Z. HUGH FAN  •  Interdisciplinary Microsystems Group (IMG), Department of Mechanical and Aerospace Engineering, University of Florida, Gainesville, FL, USA; J. Crayton Pruitt Family Department of Biomedical Engineering, University of Florida, Gainesville, FL, USA; Department of Chemistry, University of Florida, Gainesville, FL, USA
Contributors xi

William P. Mann • Department of Mechanical & Aerospace Engineering, Herbert Wertheim College of Engineering, University of Florida, Gainesville, FL, USA

Alex Markoski • Draper, Bioengineering Division, Cambridge, MA, USA; School of Engineering, Center for Biomedical Engineering, and Legoretta Cancer Center, Brown University, Providence, RI, USA

Filipe Marques • Division of Micro and Nanosystems, KTH Royal Institute of Technology, Stockholm, Sweden

Chen Ni • School of Mechanical Engineering, Jiangsu Key Laboratory for Design and Manufacture of Micro-Nano Biomedical Instruments, Southeast University, Nanjing, China

Caroline Y. N. Nicoliche • Brazilian Nanotechnology National Laboratory, Brazilian Center for Research in Energy and Materials, Campinas, SP, Brazil; Institute of Chemistry, University of Campinas, Campinas, SP, Brazil

Christine M. O’Keeffe • Johns Hopkins University, Whiting School of Engineering, Department of Biomedical Engineering, Baltimore, MD, USA

Roisin M. Owens • Department of Chemical Engineering and Biotechnology, Philippa Fawcett Drive, Cambridge, UK

Dimitri Pappas • Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, TX, USA

Karl Paterson • Centre for Microsystems and Photonics, EEE Department, University of Strathclyde, Glasgow, UK; ScreenIn3D Limited, Technology and Innovation Centre, Glasgow, UK

Edward Phelps • Department of Biomedical Engineering, Herbert Wertheim College of Engineering, University of Florida, Gainesville, FL, USA

Mohammad A. Qasaimeh • Division of Engineering, New York University Abu Dhabi (NYUAD), Abu Dhabi, UAE; Tandon School of Engineering, New York University, Brooklyn, NY, USA

Payar Radfar • School of Biomedical Engineering, University of Technology Sydney, Sydney, NSW, Australia

Eduardo Reátegui • William G. Lowrie Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, OH, USA; Comprehensive Cancer Center, The Ohio State University, Columbus, OH, USA

Alexander Revzin • Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, MN, USA

Xilal Y. Rima • William G. Lowrie Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, OH, USA

Roberto Rodriguez-Moncayo • Laboratory of Microtechnologies Applied to Biomedicine, Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional (Cinvestav), Monterrey, NL, Mexico; Research Laboratory of Electronics, Massachusetts Institute of Technology (MIT), Cambridge, MA, USA

Niclas Roxhed • Division of Micro and Nanosystems, KTH Royal Institute of Technology, Stockholm, Sweden

Janire Saez • Microfluidics Cluster UPV/EHU, BIOMICS microfluidics Group, Lascaray Research Center, University of the Basque Country UPV/EHU, Vitoria-Gasteiz, Spain; Basque Foundation of Science, IKERBASQUE, Bilbao, Spain; Bioaraba Health Research Institute, Microfluidics Cluster UPV/EHU, Vitoria-Gasteiz, Spain

Maria Sancho-Albero • Department of Chemical and Environmental Engineering, University of Zaragoza, Zaragoza, Spain; Department of Molecular Biochemistry and
Contributors

Pharmacology, Instituto di Richerche Farmacologiche Mario Negri IRCCS, Milan, Italy; Instituto de Nanociencia y Materiales de Aragón (INMA), CSIC-Universidad de Zaragoza, Zaragoza, Spain; Networking Research Center on Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN, Madrid, Spain; Laboratorio de Microscopías Avanzadas, Universidad de Zaragoza, Zaragoza, Spain; Instituto de Investigación Sanitaria de Aragón (IIS-Aragón), Zaragoza, Spain

A. FATIH SARIOGLU • School of Electrical and Computer Engineering, Georgia Institute of Technology, Atlanta, GA, USA; Parker H. Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA, USA; Winship Cancer Institute, Emory University, Atlanta, GA, USA; Institute for Electronics and Nanotechnology, Georgia Institute of Technology, Atlanta, GA, USA

ACHILLEAS SAVVA • Department of Chemical Engineering and Biotechnology, Philippa Fawcett Drive, Cambridge, UK

GABRIEL R. SCHLEDER • Brazilian Nanotechnology National Laboratory, Brazilian Center for Research in Energy and Materials, Campinas, SP, Brazil; John A. Paulson School of Engineering and Applied Sciences, Harvard University, Cambridge, MA, USA

VICTOR SEBASTIÁN • Department of Chemical and Environmental Engineering, University of Zaragoza, Zaragoza, Spain; Instituto de Nanociencia y Materiales de Aragón (INMA), CSIC-Universidad de Zaragoza, Zaragoza, Spain; Networking Research Center on Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN, Madrid, Spain; Laboratorio de Microscopías Avanzadas, Universidad de Zaragoza, Zaragoza, Spain; Instituto de Investigación Sanitaria de Aragón (IIS-Aragón), Zaragoza, Spain

BLANKA SHARMA • Department of Biomedical Engineering, Herbert Wertheim College of Engineering, University of Florida, Gainesville, FL, USA

PEIKE SHENG • Department of Biochemistry and Molecular Biology, College of Medicine, University of Florida, Gainesville, FL, USA

DIETMAR W. SIEMANN • Department of Radiation Oncology, College of Medicine, University of Florida, Gainesville, FL, USA

GIULIA S. DA SILVA • Croda do Brasil Ltda, Campinas, SP, Brazil

GULNAZ STYBAEVA • Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, MN, USA

MAI TANAKA • Department of Radiation Oncology, College of Medicine, University of Florida, Gainesville, FL, USA

XIN TANG • Department of Mechanical & Aerospace Engineering, Herbert Wertheim College of Engineering, University of Florida, Gainesville, FL, USA

MADISON TEMPLES • Department of Biomedical Engineering, Herbert Wertheim College of Engineering, University of Florida, Gainesville, FL, USA

MATT TRAU • Centre for Personalized Nanomedicine, Australian Institute for Bioengineering and Nanotechnology (AIBN), The University of Queensland, Brisbane, QLD, Australia; School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, QLD, Australia

SARAH VILLAREAL • Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, TX, USA

JING WANG • Key Laboratory of OptoElectronic Science and Technology for Medicine of Ministry of Education, Fujian Provincial Key Laboratory of Photonics Technology, Fujian Normal University, Fuzhou, China

TZA-HUEI JEFF WANG • Johns Hopkins University, Whiting School of Engineering, Department of Biomedical Engineering, Baltimore, MD, USA
MAJID EBRABHIMI WARKIANI • School of Biomedical Engineering, University of Technology Sydney, Sydney, NSW, Australia; Institute for Biomedical Materials and Devices (IBMD), University of Technology Sydney, Sydney, NSW, Australia; SUStech-UTS Joint Research Centre for Biomedical Materials and Devices, Southern University of Science and Technology, Shenzhen, People’s Republic of China; Institute of Molecular Medicine, Sechenov University, Moscow, Russia

ADRIENNE E. WIDENER • Department of Biomedical Engineering, Herbert Wertheim College of Engineering, University of Florida, Gainesville, FL, USA

IAN Y. WONG • School of Engineering, Center for Biomedical Engineering, and Legoretta Cancer Center, Brown University, Providence, RI, USA

ALAIN WUETHRICH • Centre for Personalized Nanomedicine, Australian Institute for Bioengineering and Nanotechnology (AIBN), The University of Queensland, Brisbane, QLD, Australia

NAN XIANG • School of Mechanical Engineering, Jiangsu Key Laboratory for Design and Manufacture of Micro-Nano Biomedical Instruments, Southeast University, Nanjing, China

MINGYI XIE • Department of Biochemistry and Molecular Biology, College of Medicine, University of Florida, Gainesville, FL, USA

YIJIA YANG • Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, TX, USA

MICHELE ZAGNONI • Centre for Microsystems and Photonics, EEE Department, University of Strathclyde, Glasgow, UK; ScreenIn3D Limited, Technology and Innovation Centre, Glasgow, UK

BO ZENG • Institute of Cardiovascular Research, Southwest Medical University, Luzhou, China

JIAO ZHAI • State Key Laboratory of Analog- and Mixed-Signal VLSI, Institute of Microelectronics, University of Macau, Macau, China

JINGJING ZHANG • William G. Lowrie Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, OH, USA

ZHENG ZHOU • School of Mechanical Engineering, Jiangsu Key Laboratory for Design and Manufacture of Micro-Nano Biomedical Instruments, Southeast University, Nanjing, China

ZHIXIAN ZHU • School of Mechanical Engineering, Jiangsu Key Laboratory for Design and Manufacture of Micro-Nano Biomedical Instruments, Southeast University, Nanjing, China
Lateral Filter Array Microfluidic Devices for Detecting Circulating Tumor Cells

Kangfu Chen, Thomas J. George, and Z. Hugh Fan

Abstract

Circulating tumor cells (CTCs) are an important liquid biopsy biomarker for next-generation cancer diagnosis and prognosis. However, their clinical usage is hindered by the rarity of CTCs in patient’s peripheral blood. Microfluidics has shown unique advantages in CTC isolation and detection. We have developed lateral filter array microfluidic (LFAM) devices for highly efficient CTC isolation. In this chapter, we describe in detail the design and fabrication of the LFAM devices and their applications for CTC enumeration from clinical blood samples.

Key words Circulating tumor cells, Microfluidics, Filtration, Immunoaffinity, Pancreatic cancer

1 Introduction

Cancer spreads from the primary tumor to secondary organs through metastases, which represent the leading cause of cancer deaths [1]. The process of metastases includes tumor cells’ intravasation, circulation, extravasation, and colonization in a distant site [2]. Circulating tumor cells (CTCs) play an important role in metastases, and they are defined as tumor cells that penetrate the blood or lymphatic vessels and enter the circulating system. The appearance of CTCs in the circulating system can be an indicator of an underlying malignancy [3]. As a result, CTCs are an important liquid biopsy biomarker for cancer diagnosis, prognosis, and treatment monitoring [4]. However, CTCs are extremely rare in the blood, and normally only a few to tens of CTCs exist in a billion of healthy blood cells [5]. This rarity makes their isolation and detection a technological challenge [6, 7]. Microfluidics has been widely used to address the challenge due to smaller diffusion distance in microstructures than in conventional containers, leading to more opportunities to interact between CTCs and capture agents on the microstructure surfaces and accordingly higher CTC capture
efficiency (which is defined by the number of tumor cells captured divided by the number of tumor cells present). A variety of CTC isolation approaches have been developed, and they can be roughly categorized as biological-property-based (e.g., affinity-based) and physical-property-based methods. Affinity-based methods make use of biomarkers specifically expressed on CTCs, such as epithelial cell adhesion molecule (EpCAM). Antibodies or aptamers targeting these biomarkers are used to capture CTCs and isolate them from healthy blood cells [8, 9]. The advantages of affinity-based methods include high specificity, which is often characterized by CTC cell purity that is defined by the number of target tumor cells captured divided by the number of all cells captured (i.e., both target cells captured by specific interactions and nontarget cells captured by nonspecific interactions). However, the variation in biomarker expression levels on CTCs due to epithelial-mesenchymal transition (EMT) make these affinity-based methods imperfect [10]. On the other hand, physical-property-based methods, which differentiate CTCs from normal blood cells by their size [11, 12], elasticity [13, 14], electrical properties [15, 16], etc., are considered as “label-free.” Such methods usually offer high throughput and produce high CTC capture efficiency for those CTCs that are significantly different from normal blood cells in physical properties. However, CTCs are very heterogenous in clinical samples, and some of them overlap with normal blood cells in physical properties. Such a dilemma frequently makes the capture efficiency and cell purity a “trade-off.” A higher CTC selection threshold for better cell purity may cause significant CTC loss, while a lower CTC selection threshold for higher capture efficiency can result in the capture of many nontarget cells.

To overcome the shortcomings of both affinity-based and physical-property-based approaches, we have developed lateral filter array microfluidic (LFAM) devices that combine the merits of both methods [6, 17]. The LFAM device consists of a serpentine main channel and an array of lateral filters [17]. The filters allow small blood cells to pass through while trapping large tumor cells via filtration. Furthermore, the lateral filters were functionalized with antibodies to capture small CTCs via immunoaffinity. With LFAM devices, we were able to isolate CTCs with high capture efficiency and good cell purity. Our protocols are detailed below.

2 Materials

2.1 Silicon Master Fabrication

2.1.1 Instruments

1. Computer-aided design (CAD) software (e.g., AutoCAD) (see Note 1).
2. Acid bench.
3. Suss Delta 80 Spinner.
4. Hot plate.
5. Karl Suss MA-6 Contact Aligner.
6. STS deep reactive ion etch (DRIE) system.

2.1.2 Master Fabrication
1. Silicon wafers (diameter: 100 mm; thickness: 500 μm).
2. Photoresists: AZ1512 positive photoresist (MicroChemicals GmbH).
3. AZ 300 MIF developer (EMD Performance Materials Corp.).
4. Photoresist liftoff solution: PRS-3000 (J.T. Baker).
5. Teflon tweezers for silicon wafer handling.
6. Acetone.
7. Isopropanol (IPA).
8. Piranha etch solution (H$_2$SO$_4$: H$_2$O$_2$ = 3: 1).
9. Hexamethyldisiloxane (HMDS).
10. Deionized (DI) water.
11. Filtered, pressurized inert gas pistol (air or nitrogen).
12. Desiccator with vacuum connection.

2.2 Device Fabrication
1. Trichloro(1H,1H,2H,2H-perfluorooctyl)silane (TPOS).
2. Polydimethylsiloxane (PDMS) Sylgard 184 kit: silicone elastomer base and curing agent (Dow Corning, Midland, MI).
3. Weighing scale with resolution of 0.1 g.
4. Weighing container.
5. Petri dish (150 mm) or tin foil.
6. Oven.
7. Razor blade or scalpel.
8. Micro-punches.
9. Glass microscope slides, 75 mm × 25 mm.
10. 70% ethanol.
11. DI water.
12. Inert gas pistol (air or nitrogen).
13. UV-ozone cleaner.

2.3 Device Functionalization
1. Programmable syringe pumps.
2. Sterile 3 mL Luer-lock syringes.
3. Female Luer-to-barb adapters.
4. Tubing: 0.0625 in. outer diameter, 0.008 in. inner diameter.
5. 99% ethanol.
6. Dulbecco's phosphate buffered saline (DPBS) without Ca\textsuperscript{2+} / Mg\textsuperscript{2+} ions.
7. Bovine serum albumin (BSA).
8. Pipettes.
9. Avidin.
10. Biotinylated anti-EpCAM (anti-human CD326, clone 1B7, eBioscience, San Diego, CA).

2.4 Processing Clinical Samples
1. Sterile 50 mL centrifuge tubes.
2. DPBS without Ca\textsuperscript{2+}/Mg\textsuperscript{2+} ions.
3. 70% ethanol.
4. Sterile 5 mL Luer-lock syringe.
5. Polytetrafluoroethylene (PTFE) micromagnetic stir bar, 2.5 mm in length and 1 mm diameter.
6. Magnetic stirring plate.
7. Paraformaldehyde (PFA), 96%.
8. Triton X-100, 98%, molecular biology grade.
9. Anti-CK-FITC (cytokeratin antibody conjugated with fluorescein isothiocyanate, clone CAM 5.2, BD Biosciences, San Jose, CA).
10. Anti-CD45-PE (CD45 antibody conjugated with phycoerythrin, clone HI30, BD Biosciences, San Jose, CA).
11. DAPI (4′,6-diamidino-2-phenylindole, Invitrogen, Carlsbad, CA).

3 Methods

3.1 Silicon Master Fabrication
1. Draw the layout of the LFAM device using CAD software (see Note 1).
2. Submit the CAD file to a commercial supplier for a chrome mask fabrication at resolution of 1 μm (see Note 2).
3. Start the washing step of the fabrication process in Fig. 1a. Bathe a silicon wafer in a glass beaker containing acetone for 5 min to remove organic materials. The beaker can be placed in an ultrasonic bath cleaner or on a shaker for optimized cleaning performance (see Note 3).
4. Spray wash the silicon wafer with IPA to remove acetone residues on the silicon wafer.
5. For deep cleaning of organic materials, immerse the wafer into a piranha solution in a glass dish for 5 min. After being taken out, wash the wafer with running DI water for at least 2 min to
Fig. 1 (a) Illustration of the fabrication process of a silicon master. (b) Fabrication of LFAM devices. (c) Functionalization of LFAM device surfaces using antibodies.

remove piranha solution, followed by drying the silicon wafer with a nitrogen gun. Transfer the dry wafer to a buffered oxide etch (BOE) solution in a polyethylene beaker for 20 s to remove silicon oxide on the silicon wafer surface. Rinse the wafer thoroughly for 30 s with DI water. Dry the wafer with a nitrogen gun (see Note 4).

6. Bake the wafer at 130 °C for 10 min until fully dry, and cool it down to room temperature.

7. Vaporize HMDS onto the silicon wafer to create hydrophobic surfaces (see Note 5).

8. Spin-coat a layer of 2 μm thick AZ1512 positive photoresist.

9. Soft bake the wafer on a hotplate at 112 °C for 1 min.

10. Expose the wafer using Karl Suss MA-6 Contact Aligner (see Note 6).

11. Develop exposed photoresist in AZ MIF300 developer for 75 s.

12. Hard bake the wafer at 125 °C for 1 min.

13. Use the DRIE system to vertically etch the silicon wafer for 45 μm (see Note 7).

14. Strip of the photoresist layer using a PRS-300 solution at 70 °C.

15. Rinse the wafer with IPA.
3.2 LFAM Device Fabrication

1. Treat the silicon master with 30 μL of TPOS in vacuum for 30 min (see Note 8).

2. Place the silicon master in a 150 mm petri dish or a wrapped bowl made of tin foil.

3. Mix 20 g of PDMS prepolymer (base/curing agent = 10:1) in a weighing boat.

4. Place the PDMS mixture in vacuum for 15–30 min to drive out bubbles (see Note 9).

5. Cast PDMS on the silicon master, as illustrated in the first step of Fig. 1b.

6. Incubate PDMS in the oven at 75 °C for 2–4 h (see Note 10).

7. Separate the polymerized PDMS from the silicon master, and cut the PDMS substrate into the size of a microscope slide.

8. Punch holes at the inlet and outlet of the PDMS substrate using micro-punches (Fig. 2a).

9. Rinse and air dry a glass microscope slide.

10. Treat the PDMS substrate and the microscope slide with UV-ozone (UVO) for 5 min (Fig. 1b) (see Note 11).

11. Bind the PDMS with the microscope slide. A picture of a completed LFAM device is shown in Fig. 2a (see Note 12).
3.3 LFAM Device Functionalization

1. Fill a 3 mL Luer-lock syringe with 99% ethanol.
2. Cap the syringe with a female Luer-lock adaptor.
3. Connect one end of the tubing to the tip of the Luer-lock adaptor.
4. Connect the other end of the tubing to the inlet of the LFAM device.
5. Fix the syringe on the programmable syringe pump.
6. Infuse 200 μL of ethanol through the LFAM device to clean the microchannels (see Note 13).
7. Repeat steps 1–6 using another syringe filled with DI water to wash away ethanol in the device.
8. Repeat steps 1–6 using another syringe filled with DPBS to prepare the device for the surface functionalization (see Note 14).
9. Introduce 50 μL of 1 mg/mL avidin from the inlet of the LFAM device using a pipette to initiate the immobilization of antibodies onto the channel surfaces as illustrated in Fig. 1c.
10. Incubate the avidin solution in the device at room temperature for 15 min (see Note 15).
11. Infuse 200 μL of DPBS to wash away the avidin solution.
12. Introduce 50 μL of 10 μg/mL biotinylated EpCAM antibody (anti-EpCAM) from the inlet of the device using a pipette (see Note 16).
13. Incubate the solution in the device for 15 min.
14. Infuse 200 μL of 1% BSA in DPBS to the device to wash away the antibody solution (see Note 17).
15. Incubate the solution in the device for 15 min. The device is then ready for CTC isolation.

3.4 Processing Clinical Samples Using LFAM Devices

1. Functionize two LFAM devices by following the protocol detailed in 3.3 (see Note 18).
2. Add 4 mL of a whole blood sample collected in an EDTA (ethylenediaminetetraacetic acid) tube (“purple top”) to a 15 mL tube (see Note 19).
3. Add 4 mL of DPBS to the tube to double the original sample volume (see Note 20).
4. Mix the sample by manually inverting the tube for eight to ten times.
5. Prepare two 5 mL syringes and add a mini magnetic stir bar (2.5 mm in length and 1 mm in diameter) to each syringe (see Note 21). Then load 4 mL of the diluted blood sample to each syringe.
6. Cap the syringe with a female Luer-lock adapter.
7. Connect the syringe with the device inlet using tubing filled with DPBS (see Note 22).
8. Fix both syringes on the syringe pump in parallel and place a magnetic stirring plate under the syringes. Allow magnetic bars to rotate and mix the blood sample, preventing cells from settling down (see Note 21).
9. Infuse samples at 3.6 mL/h using the syringe pump for about 1 h (see Note 23).
10. Prepare two 3 mL syringes and load 3 mL of 1% BSA to each syringe. After sample infusion, disconnect the tubing of samples from the inlet of the LFAM device, and connect the BSA syringe with the device by following steps 6–7.
11. Wash the device with 500 μL of 1% BSA at an infusion flow rate of 3.6 mL/hr.
12. Prepare two 1 mL syringes and load 500 μL of 4% PFA to each syringe. Infuse 100 μL of 4% PFA through the devices at a flow rate of 3.6 mL/hr. (see Note 24).
13. Incubate the PFA solution in the devices at room temperature for 10 min for cell fixation.
14. Prepare two 1 mL syringes and load 500 μL of 0.1% Triton X-100 solution to each syringe. Infuse 100 μL of 0.1% Triton X-100 through the devices at a flow rate of 3.6 mL/hr.
15. Incubate the Triton X-100 solution in the devices at room temperature for 10 min for cell membrane permeabilization.
16. Wash the devices by infusing 200 μL of 1% BSA at 3.6 mL/hr.
17. Prepare 120 μL of a staining/conjugation cocktail containing 80 μL of 500 nM DAPI in water, 20 μL of 10 μg/mL anti-CK-FITC, and 20 μL of 10 μg/mL anti-CD45-PE (see Note 25).
18. Infuse 60 μL of the cocktail into each device using a pipette.
19. Incubate the cocktail in the devices at room temperature for 20 min (see Note 26).
20. Wash the devices by pumping 600 μL of DPBS at 3.6 hr./mL.
21. Enumerate CTCs under the fluorescent microscope. CTCs are identified using the definition of DAPI+/CK+/CD45− as shown in Fig. 3 (see Note 27).

4 Notes

1. In addition to a common CAD software (AutoCAD), L-Edit is a specialized photomask design software for integrated circuits. A freeware, LayoutEditor, can also be used for designing
Fig. 3 (a) Pictures of a CTC after three fluorescent filters as well as under bright field (BF). CTCs are DAPI+/CK-/CD45-. (b) Picture of a white blood cell (WBC) captured non-specially by the device, showing DAPI+/CK-/CD45+

microfluidic devices. Also note that the default file format from AutoCAD is DWG, but the DXF file extension should be used. DXF will be converted to GDSII that can be read by a photomask fabrication machine.

2. If a chrome mask writer (e.g., Heidelberg Laser Writer DWL 66FS) is available, a photomask can be fabricated in house. For chrome mask fabrication, a 5” × 5” glass substrate coated with a chromium film and a positive photoresist was purchased and used. The drawing in the CAD file was first written on the photoresist layer by the mask writer. The substrate was then developed with corresponding photoresist developer. Afterward, the substrate was placed in a chrome etchant to transfer the pattern from the photoresist layer to the chrome film. Then the photoresist was stripped off and the chrome mask was ready for use.

3. This washing step is not required for brand-new, clean silicon wafers. Note that this washing step must be carried out in a chemical fume hood to prevent acetone inhalation.

4. The washing step using a piranha solution is optional. However, it is required if the wafer is dusty or contaminated. Also, this step must be done in a certified general acid bench. Face shields, non-flammable protection suites, and safety-grade
extra-thick rubber gloves are required. Safety rules must be followed strictly as the piranha solution and BOE are highly corrosive. When the piranha solution is being made, strong heat and large amount of oxygen will be produced. As a result, the piranha solution should be made away from any flammable materials. The disposal of the piranha solution should be taken with extra caution. The solution should be left on the acid bench for at least 45 min to cool down before disposal. The cap of the waste bottle for the piranha solution should be kept loosen for leftover oxygen release, while plastic waste bottles are preferred. Mishandling of piranha solution disposal may cause explosion. Similarly, BOE can etch silicon oxide. Therefore, BOE should not be handled in glass beakers. Also, the waste bottles for BOE disposal must be made of plastics.

5. This HMDS step is to prime the silicon wafer surface for promoting the adhesion of photoresist. It is recommended to have 30 s for HMDS vapor generation by heating it and 1 min for vapor deposition.

6. The exposure dose is dependent on the type and amount of the photoresist used in the spin-coating step.

7. It is recommended to take out the wafer after every 10 μm etch to cool it down. Overheat on the wafer surface will affect the masking effectiveness of the photoresist layer during etching.

8. This TPOS step is to facilitate the separation of PDMS from the silicon master. The silane should be kept in a small weighing boat placed next to the silicon master, both of which are housed in a desiccator, followed by applying a vacuum for promoting the evaporation of TPOS and coating it onto the silicon master.

9. This bubble removal step is important for device transparency and for preventing potential cross-talking among microchannels.

10. The oven temperature and curing time can vary. At a higher temperature, the curing step generally requires less time. Please follow the recommended protocol from the manufacturer of the Sylgard 184 kit.

11. The UV-ozone treatment is to prime the surfaces of both PDMS substrate and microscope slide to enhance their bonding. Other methods such as oxygen plasma or corona discharge may be used.

12. A variety of filters in a size from 10 to 6 μm is created in one device to address the heterogeneity of CTCs, as shown in Fig. 2c and d. The device contains five filter zones, with a decrease of 1 μm in the filter size for each subsequent zone. The 10 μm filters are near the inlet and the 6 μm filters are close to the outlet.
13. The ethanol flushing of microchannels makes surface less hydrophobic and drives out air bubbles. Leave an extra drop of ethanol to cover the inlet to avoid bubble formation during the initiation of the subsequent step.

14. Similar to Note 13 above, add an extra drop of DPBS to cover the inlet of the device to avoid bubble formation.

15. Avidin is physically absorbed onto the surfaces of microchannels and filters. An alternative approach for immobilizing antibodies is to attach antibodies via covalent bonding, in which either glass or PDMS surfaces are chemically modified for antibody conjugation.

16. Antibodies are biotinylated to facilitate the conjugation with avidin on the surfaces of microchannels and filters.

17. The function of BSA is to passivate the surfaces to reduce nonspecific binding of tumor cells.

18. Two devices are prepared to process two portions of clinical samples, increasing the sample throughput and CTC enumeration reproducibility.

19. Clinical specimens should be processed as soon as possible or on the same day of the collection. If the samples need to be shipped or cannot be processed on the same day, specialized tubes (rather than low-cost EDTA tubes) should be used to preserve the properties of CTCs.

20. The 1:1 dilution of the blood sample is to reduce the blood viscosity and diminish the effects of the viscosity variation among different cancer patients. The equal volume dilution is also used in the commercially available Ficoll-Paque kits for mononuclear cell isolation from a blood sample.

21. The magnetic stirring is used to keep cells in suspension. Otherwise, cells tend to settle down to the bottom of the syringe, causing inhomogeneous cell solution infusion.

22. Similar to Note 13, avoid bubble formation in the connection between the syringe tip and the tip of tubing and in the connection between the tip of tubing and the inlet of the LFAM device.

23. The flow rate of syringe pumps will affect CTC capture efficiency and cell purity. In general, the capture efficiency decreases with increasing flow rates due to (1) the reduced interaction time between tumor cells and capture agents and (2) the increased shear stress at a higher flow rate. However, the cell purity increases with increasing flow rates due to the increased shear force to dislodge those nontarget cells bound nonspecifically. The specific flow rate was selected after our detailed device characterization [17].
24. The function of PFA is to preserve cells for subsequent immunocytochemistry by fixing them.

25. The ratio of DAPI and fluorescently labeled antibodies may change based on their concentrations of stock solutions as well as the biomarker expression level of CTCs.

26. To reduce photobleaching of fluorescent dyes, the devices are covered by tin foil or other materials to avoid light exposure.

27. CTCs isolated by the LFAM devices can also be EpCAM+ because some of them are captured by anti-EpCAM.

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Circulating Tumor Cell Cluster Sorting by Size and Asymmetry

Sam H. Au

Abstract

Circulating tumor cell (CTC) clusters are rare yet potent initiators of metastasis and may be useful as clinical biomarkers. Numerous techniques have been developed to isolate individual circulating tumor cells from the blood, but these techniques are often ineffective at capturing CTC clusters and may cause cluster damage or dissociation during processing or recovery. This chapter describes methods for fabricating and operating a two-stage continuous microfluidic chip that isolates and recovers viable CTC clusters from blood or biological fluids using deterministic lateral displacement.

Key words Microfluidics, Circulating tumor cell clusters, Aggregates, Isolation, Sorting

1 Introduction

Multicellular aggregates of CTCs (CTC clusters) may be 100-fold more metastatic competent than single CTCs [1–3]. In mouse models, CTC clusters are responsible for seeding half or more of secondary metastatic tumors [1, 2], and in humans, the presence of even a single CTC cluster in sampled blood is correlated with significantly reduced progression-free survival rates in patients with prostate [2, 4], colorectal [5], breast [2, 6], pancreatic [7], and lung [8, 9] cancers.

Numerous methods for sorting individual CTCs have been developed, yet very few have been developed to isolate only CTC clusters from blood or other biological fluids [10–12]. The technique described here captures clusters using deterministic lateral displacement (DLD), a reliable, low-cost, highly scalable, and label-free method of isolation. This device is unique in that it isolates clusters based on two physical parameters: size and asymmetry (Fig. 1a-d). Stage 1 was designed to sort clusters based on size, while Stage 2 sorts by asymmetry (Fig. 1d). The incorporation of sorting by asymmetry helps to overcome the major limitations of
size-based sorting for CTCs: overlap in the sizes of target and nontarget cells. Because the sizes of individual CTCs vary dramatically (e.g., one group reported CTCs with diameters ranging from 4 to 30 μm within a single patient [13]) and most clusters exist as two to four cell aggregates [11], size-based sorting alone is insufficient to sort CTC clusters from individual CTCs or the far more numerous white blood cells (diameters ~6–18 μm) found in blood. Asymmetry is a useful sorting methodology for clusters because unlike individual CTCs and white cells, clusters are by nature asymmetrical.

The fabrication and operation of the device are described in this chapter. The Notes section details where the computer assisted design file can be downloaded for free (see Note 1). It is assumed that you have prepared a sample biofluid from which you wish to isolate clusters and have all safety and ethical approvals in place.

## 2 Materials

### 2.1 Master Mold Fabrication

1. Spin coater capable of operating at ≥3500 rpm.
2. Oxygen plasma asher.
3. UV mask aligner.
4. 2× hot plates.
5. Optical or contact profilometer.
6. Tweezers.
7. Glass petri dishes capable of holding 102 mm diameter wafers.
8. Photomask (see Notes 2 and 4).

2.1.2 Consumables
1. Precleaned 102 mm diameter silicon wafer.
2. SU-8 2025 negative photoresist, 10–15 mL.
3. SU-8 2050 negative photoresist, 10–15 mL.
4. SU-8 developer, 100–200 mL.
5. Acetone in squirt bottle, 20–30 mL.
6. Isopropanol in squirt bottle, 20–30 mL.
7. Compressed oxygen.
8. Compressed filtered nitrogen.
9. 150 mm diameter petri dish.
10. Semiconductor-grade tape.

2.2 Soft Lithography
1. Oxygen plasma oven.

2.2.1 Equipment
2. Hot plate.
3. Oven.
4. Vacuum desiccator.
5. 1.2 mm or 1.0 mm diameter biopsy punchers.
6. Scalpel.

2.2.2 Consumables
1. 76 × 26 mm unfrosted and uncoated glass microscopic slides.
2. Sylgard 184 polydimethylsiloxane (PDMS) prepolymer, 100–120 g, and its cross-linker, 10–12 g.
3. Compressed oxygen.

2.3 Device Preparation and Operation
1. Three syringe pumps capable of infusion rates 8–40 μL/min (it is recommended that pumps be compatible with syringes of at least 50 mL volume).

2.3.1 Equipment
2. HEPA-filtered laminar flow hood (optional, if culture or long-term analysis of isolated clusters is desired).

2.3.2 Consumables
1. Flexible polymeric tubing (Tygon or similar) with outer diameters approximately slightly larger than the diameter of biopsy punchers used above, 2–3 m per device.
2. 23 gauge blunt tipped needles.
3. 10 mL and 30 mL volume syringes.
4. Tubing pinch clamps, four per device.
5. Cluster-laden samples:
   - If using whole blood, at least 10 m collected into acid citrate dextrose or ethylenediaminetetraacetic acid containing tubes is recommended to be split evenly for processing in two devices.
   - If using blood fractions or spiked samples, it is recommended to use highly concentrated samples without dilution to reduce processing time.

6. Phosphate buffered saline (PBS).
7. Pluronic F68.
8. Isopropanol.
9. Distilled water.
10. Collection vessels for isolated clusters.

3 Methods

For best results, carry out all microfabrication steps (3.2–3.2) in an ISO 6 class 1000 cleanroom environment (see Note 3) and device preparation and operation steps (3.3–3.4) in a laminar flow cabinet or equivalent using sterile technique (see Note 4). Each soft lithography process creates two identical devices.

3.1 Wafer Fabrication

1. Heat two hot plates, one to 65 °C and another to 95 °C, allowing sufficient time for temperatures to reach stable set point.

2. Prepare silicon wafer by first baking at 95 °C for 10 min, followed by oxygen plasma treatment at ~300 mmTor O₂ at 50 W for 35 s. The wafer should then be centered onto a spin coater and secured. Deposit ~4 mL of SU-82025 onto the center of the wafer and then spin at 500 rpm for 10 s with an acceleration of 100 rpm/s, followed by 2750 rpm for 30 seconds with an acceleration of 300 rpm/s. Transfer the photoresist-coated wafer to 65 °C hot plate, and bake for 1 min followed by 5 min on the 95 °C hot plate.

3. Load the photomask labeled “Stage 2, H = 30um” onto the mask aligner ensuring the inked side is facing downward toward the silicon substrate. Load the photoresist-coated wafer and expose with UV light with incident energy of 150 mJ/cm² ensuring that appropriate eye protection is worn.

4. Remove the exposed wafer and bake at 65 °C for 1 min followed by 5 min at 95 °C.

5. Deposit ~4 mL of SU-82050 onto the center of the wafer, and then spin at 500 rpm for 10 s with an acceleration of 100 rpm/s, followed by 2500 rpm for 30 seconds with an
acceleration of 300 rpm/s. Transfer the wafer onto the 65 °C hot plate and bake for 5 min followed by 15 min on the 95 °C hot plate.

6. Load the photoresist-coated wafer and the photomask labeled “Stage 1, H = 90μm” onto the mask aligner ensuring the inked side is facing downward toward the silicon substrate. Align the photomask to the substrate by adjusting the x-y and rotation of the stages using the six alignment marks on the periphery of the wafer and photomask (see Note 5). Expose with UV light with total incident energy of 220 mJ/cm² ensuring that appropriate eye protection is worn.

7. Remove the exposed wafer and bake at 65 °C for 5 min followed by 10 min at 95 °C.

8. Allow the wafer to cool for 5 min at room temperature, and then submerge the wafer in 50 mL of SU-8 developer in a glass petri dish. Gently agitate or swirl the developer for 10 min. Remove and submerge a second time into 50 mL of fresh SU-8 developer in a new glass petri dish. Gently agitate or swirl until wafer is fully developed, approximately 5 min (see Note 6).

9. Remove the wafer using tweezers, and gently rinse with acetone for 10 seconds and then isopropanol for a further 15 seconds. Gently dry the water using filtered nitrogen gas. Bake the wafer at 65 °C for 5 min followed by 15–20 min at 95 °C. Allow to cool at room temperature, and then securely tape petri dish, applying pressure using tweezers to the tape to ensure air bubbles are removed underneath all taped surfaces.

10. Measure the height of SU-8 features using an optical profilometer. Stage 1 features should be 90 μm high, and Stage 2 features should be 30 μm high, with generally acceptable deviations of ±5% from set point (see Note 7).

3.2 Soft Lithography

1. Thoroughly mix PDMS prepolymer with cross-linker at a ratio of 10:1 (w/w), and fill the wafer-container petri dish to a height of ~1 cm (approximately 175 mL). Cover the dish and place into a vacuum desiccator until bubbles in the prepolymer are no longer visible (approximately 1–2 h). Then place into an oven set to 60 °C for at least 8 h.

2. Clean glass microscopic slides by rinsing with acetone for 10 s followed by isopropanol for 10 s. Dry by filtered nitrogen gas and store under cover.

3. Using a scalpel, cut through the PDMS ensuring the scalpel blade meets the silicon wafer underneath in the shape of a ring around the devices. Remove the circular PDMS slab; invert and place onto a clean surface. Cut along the indicated rectangular outlines of each of the two devices.
4. Using a biopsy punch, vertically punch to remove a cylindrical plug from the circular ports of each inlet and outlet (Fig. 1, ports a–f).

5. Treat rectangular PDMS slabs feature side up alongside equal numbers of glass microscopic slides with oxygen plasma at \( \sim 300 \text{ mmTorr O}_2 \) at 50 W for 35 s.

6. Immediately invert rectangular PDMS slabs and place onto the glass microscopic slides. Apply gentle pressure on top of the assembled devices ensuring air bubbles are removed. Bake assembled devices, glass side down, on a hotplate at 85 °C for 15–20 minutes.

3.3 Device Preparation

1. Insert tubing into all outlets and Stage 2 buffer ports (Fig. 1, ports c–f) leaving the unconnected ends of the ports to terminate into tubes, vials, or plates as desired. Ensure that the unconnected ends of the outlet tubing are always maintained at the same height as the device to prevent flow in devices until desired.

2. Load and flush with \( \sim 5 \text{ mL} \ 70\% \text{ isopropanol in distilled water (v/v)} \) by filling two syringes and connecting them to \( \sim 7 \text{ cm}-\text{long tubing via blunt needles. Connect these to Stage 1 inlet ports (Fig. 1, ports a and b), and infuse half the volume by applying gentle pressure to syringe plungers. Pinch to close the tubing connected to ports c and d using tubing pinch clamps and then infusing the remaining isopropanol. Perform a wash by replacing syringes with new syringes containing \( \sim 10 \text{ mL PBS} \) each (be careful not to introduce air bubbles when exchanging syringes; see Note 8), and infuse half the volume using gentle pressure. Then, unpinch tubing connected to ports c and d before infusing the remainder of the buffer.

3. Load and flush devices, as described in step 2, but with \( \sim 5 \text{ mL} \) of 3% Pluronic F68 in PBS (w/v) instead of isopropanol. Allow Pluronic to remain in chip for 45 min at room temperature before washing with buffer as described above. Move the end of tubing connected to ports c and f to new tubes or vials in preparation of collection of clusters.

3.4 Device Operation

1. Prepare three syringes, one containing cluster-laden biofluid and the other two containing PBS. The PBS syringes must contain at least 5× greater volumes of fluid than the biofluid syringe (i.e., for 10 mL of biofluid, each PBS syringe should contain at least 50 mL). Connect the three syringes to \( \sim 1\text{-m-long tubing via blunt needles and load them into syringe pumps. Slowly infuse from all pumps at a rate of } \sim 5 \mu\text{L/min and pause when all air has been evacuated from tubing. Remove existing tubing from port d. Insert the end of tubing} \)
containing cluster-laden biofluid into port a, and the tubing containing PBS into ports b and d, making sure not to introduce any air bubbles.

2. Set the infusion rates for ports a, b, and d to 8.3, 31.6, and 40 μL/min, respectively (see Notes 9 and 10). Engage all pumps simultaneously and run until all the biofluid has been infused. Large clusters should be enriched in the effluent of port c, small clusters from the effluent of port f, and unsorted cells in the effluent of port e (see Note 11).

### 4 Notes

1. Computer assisted design files are available for download at https://biomicrotech.science/downloads. Layers should be printed on separate photomasks. By downloading the design, you agree to cite the original manuscript that describes this technique if you use or modify the design or any of its constituent parts in any associated publications [10]: https://doi.org/10.1038/s41598-017-01150-3.

2. For most users, photomasks are best obtained by submitting design files to a commercial mask printing service. Chrome on glass format is recommended, although any format capable of generating features of 10 micron size or better should work.

3. If a cleanroom is not available, lower cost laminar enclosures are available. Biosafety cabinets can also be modified with activated carbon filtration to create class 1000 clean air that is safe for use with solvents.

4. Operation in HEPA-filtered laminar biocabinets and sterile techniques, including the use of autoclaved or sterilized reagents, tubing, and collection vessels, is strongly recommended if culture or long-term analysis of clusters is desired.

5. If alignment marks on the wafer are not discernible after the first post-exposure bake, dab a small amount of SU-8 developer onto alignment marks using the end of a cotton swab, and dry before proceeding with alignment.

6. Development should be complete when opaque SU-8 photoresist no longer lifts off from wafer and device features appear sharp under magnification and the naked eye.

7. If SU-8 features are not within acceptable ranges, repeat the process with faster/slower spin speeds to lower/raise feature heights, respectively, until appropriate heights are achieved. Recommended spin speeds and baking conditions can be found in manufacturer specification sheets for SU-8.
8. When exchanging tubing or syringes, it is vital that air bubbles are not permitted to enter the system. Always purge trapped air before making connections by applying gentle positive pressure to syringe plungers, and if a bubble is suspected to have entered a port, refill the port with excess buffer, and use a sterile fine tip needle to manually displace any bubbles.

9. Clusters may settle within syringes over time due to gravity. This can be addressed by gently rocking syringe pump infusing the biofluid on a rocker platform or by operating the pump on its side such that the clusters settle toward syringe outlets.

10. Each device has been tested to be capable of processing whole blood at rates up to 0.5 mL/hr. (8.3 µL/min) without apparent damage to CTC clusters, but it is possible that higher processing rates are feasible without device modification. If higher processing rates are attempted, it is suggested that the ratio of buffers feeds is increasing to match (i.e., a 10% increase in buffer flow rates should match a 10% increase in biofluid sample flow rate).

11. Because of the continuous operation of the platform, the “waste” stream (port e) can theoretically be linked to downstream processing, such as devices that isolate individual CTCs. However, be careful if direct downstream connections are attempted since these will add resistance to upstream elements, and therefore careful recalibration of outlet resistances is necessary.

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Digital Microfluidics with an On-Chip Drug Dispenser for Single or Combinational Drug Screening

Caiwei Li, Jiao Zhai, and Yanwei Jia

Abstract

Rapid and accurate cancer drug screening is of great importance in precision medicine. However, the limited amount of tumor biopsy samples has hindered the application of traditional drug screening methods with microwell plates for individual patients. A microfluidic system provides an ideal platform for handling trace amounts of samples. This emerging platform has a good role in nucleic acid-related and cell related assays. Nevertheless, convenient drug dispensing remains a challenge for clinical on-chip cancer drug screening. Similar sized droplets are merged to add drugs for a desired screened concentration which significantly complicated the on-chip drug dispensing protocols. Here, we introduce a novel digital microfluidic system with a specially structured electrode (a drug dispenser) to dispense drugs by droplet electro-ejection under a high-voltage actuation signal, which can be conveniently adjusted by external electric controls. With this system, the screened drug concentrations span up to four orders of magnitude with small sample consumption. Various amounts of drugs can be delivered to the cell sample with desired amount in a flexible electric control. Moreover, single drug or combinatorial multidrug on-chip screening can be readily achieved. The drug response of normal MCF-10A breast cells and MDA-MB-231 breast tumor cells to two chemotherapeutic substances, cisplatin (Cis) and epirubicin (EP), was tested individually and in combination for proof-of-principle verification. The comparable on-chip and off-chip results confirmed the feasibility of our innovative DMF system for cancer drug screening.

Key words Digital microfluidics, Cancer drug screening, Drug dispenser, MCF-10A cell, MDA-MB-231 cell, Cisplatin, Epirubicin

1 Introduction

Clinically, chemotherapy has become the most accepted method for cancer treatment. Antitumor drugs in chemotherapy can control and inhibit the growth of tumors in order to shrink them to the point where surgery can be performed. However, chemotherapy is also toxic for healthy cells, causing severe side effects, and different patients may have distinct responses to the same antitumor drug [1, 2]. These facts lead to a high demand for drug screening before the prescription of drugs in precision medicine. Conventional drug
screening tests operate on a 384-well plate to reduce the sample consumption. However, manually adding drugs to reach a preset concentration in the numerous wells on the plate is an error-prone process. In addition, the automatic dosing machine, like a brand named Opentrons, that is on the market is bulky and expensive. Moreover, the minimum sample volume of 100 μL on microplates also makes the biopsy process stressful. As an alternative platform, microfluidics offers a great potential by manipulating trace amounts of samples.

Microfluidic technology generally includes flow-based channel microfluidics and electric-based digital microfluidics (DMF) [3]. Channel microfluidics has been applied in drug screening for various cancers, including blood cancer, pancreatic cancer, and prostate cancer [4, 5]. However, a channel microfluidic system requires bulky tubing connections, valves, and pumps for drop manipulation. Adding various concentrations of drugs to the cell samples either prolongs the preparation process (off-chip addition) or complicates the drug concentration adjustment (on-chip addition by merging two similarly sized drops) [6, 7]. More critically, combinational multidrug screening with different drug concentrations faces big obstacles in channel microfluidics due to the increase in sample volume when new reagents are added.

Unlike channel microfluidics, DMF is a cutting-edge technique that manipulates individual drops on-chip based on the electrowetting on dielectric (EWOD) force with convenient external electric controls [8, 9]. The elimination of the tubing, pumps, and valves makes it well suited for clinical precision medicine. Here we present a novel DMF system with flexible on-chip drug dispensing for single drug and combinational multidrug screening. With the system we design, we can freely control the drug concentration on-chip by electronic control; moreover, the concentration range can span up to four orders of magnitude.

Figure 1a shows the schematics of the digital microfluidic system. It contains three modules: (i) the electronic control system with a field programmable gate array (FPGA) and a printed circuit board (PCB) for power supply, actuation signal generation, data collection, and data transfer; (ii) the customized software control system for human-computer interaction; and (iii) the DMF chip for a cell culture with various drug combinations and concentrations.

The drug dispenser, a specially structured electrode, is designed to achieve flexible on-chip drug dispensing. As shown in Fig. 1b, when a drug stock drop, a concentrated drug drop, is placed on the drug dispenser electrode and actuated, a desired amount of the drug is ejected by adjusting the actuation control parameters, including the actual voltage, the actuation frequency, the ejection time, and the jetting bar width [10]. These ejected droplets are picked up by a drop containing cells for cell culture with various drug concentrations at designed cell culture positions. Using breast
cancer as a model system, we tested the drug responses of two types of cells, MDA-MB-231 breast cancer cells and normal MCF-10A breast cells, to two cancer drugs, cisplatin (Cis) and epirubicin (EP), individually and in combination. The on-chip results were also compared with the off-chip results to test the reliability of our DMF system for cancer drug screening.

2 Materials

2.1 MCF-10A Cell Culture

1. 70% ethanol: dissolved in pure water, stored at 25 °C.
2. MCF-10A cells: stored in liquid nitrogen.
3. Culture medium for MCF-10A cells: Dulbecco’s modified eagle medium (DMEM) supplemented with 5% (v/v) horse serum, 100 ng/mL cholera toxin, 0.5 mg/mL hydrocortisone, 10 μg/mL insulin, and 100 U/mL penicillin-streptomycin; stored at 4 °C.
4. Trypsin-ethylene diaminetetraacetic acid (EDTA): stored at 4 °C.
5. Phosphate-buffered saline (PBS) solution: stored at 25 °C.

2.2 MDA-MB-231 Cell Culture

1. 70% ethanol: dissolved in pure water, stored at 25 °C.
2. MDA-MB-231 cells: stored in liquid nitrogen.
3. Culture medium for MDA-MB-231 cells: DMEM supplemented with 10% (v/v) FBS and 100 U/mL penicillin-streptomycin; stored at 4 °C.
4. Trypsin-ethylene diaminetetraacetic acid (EDTA): stored at 4 °C.
5. Phosphate-buffered saline (PBS) solution: stored at 25 °C.

2.3 DMF System Setup and Chip Fabrication

1. DMF chip with drug dispenser (jetting bar).
2. Chromium (Cr) electrodes.
3. ITO glass.
4. Conductive tape.
5. DMF control system, consisting of software control and hardware control.
6. Ethanol at room temperature.
7. Acetone at room temperature.
8. Isopropyl alcohol (IPA) at room temperature.
9. SU-83000 photoresist stored at 4 °C, defrosted before use.
10. SU-8 developer stored at room temperature.
11. Teflon amorphous fluoropolymer (AF) solution: dissolved in FC-40 to generate a 0.5% solution for hydrophobic coating.
12. Silicone oil: stored at room temperature.
13. Pluronic F127 solution: 10% solution in water.

2.4 On-Chip and Off-Chip Drug Screening Tests

1. 50% DMSO solution: dissolved in DI water.
2. Epirubicin hydrochloride (EP) stock solution: 5.8 mg EP dissolved in 2 mL 50% DMSO for a 5 mM EP stock solution.
3. Cisplatin (Cis) stock solution: 2.5 mg Cis dissolved in 2 mL 50% DMSO for a 4 mM EP stock solution.
4. Cy3 solution: a fluorescence dye, stored at −20 °C.
5. Calcein-AM solution: 20 μM in a PBS solution.
6. Propidium iodide (PI) solution: 50 μM in a PBS solution.
7. 0.1% Gelatin solution: stored at 4 °C.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Cell Culture and Passage

1. Spray the laminar flow hood and lab bench with 70% ethanol and wipe it down with a paper towel.
2. Thawing cells (MDA-MB-231 cells and MCF-10A cells): rapidly (< 1 min) thaw the frozen cells in a vial in a 37 °C water bath before transferring the cells into the centrifuge tube.
containing the desired amount of pre-warmed growth medium (see Note 1). Centrifuge the cell suspension at 150–300 xg for 3–5 min. After the centrifugation, gently decant the supernatant without disturbing the cells. Resuspend the cells with the complete growth medium before transferring them into the cell culture plate (six-well plate) and the incubator.

3. Cell culture: change the growth medium in the six-well plate every day, each well containing 2 mL of the culture medium.

4. Cell passaging (see Note 2): remove the cell culture medium from the plate. Wash the cells with PBS solution. Add 200 μL of trypsin to each well of the six-well plate, and incubate at 37 °C until the cells are fully detached from the dish (3–5 min for MCF-10A cells, 5–10 min for MDA-MB-231 cells). Resuspend the cells in fresh growth medium to stop the detaching method. Pipette thoroughly but gently to get a single cell solution. Transfer the cell suspension into the centrifuge tube and centrifuge it at 150–300 xg for 3–5 min. After the centrifugation, gently decant the supernatant without disturbing the cells. Resuspend the cells with the complete growth medium. Plate the cells onto a new plate.

3.2 DMF Chip Design

1. Design the structure of the DMF chip, including a glass bottom plate with patterned chromium (Cr) electrodes, a top plate made of ITO glass, and a 200 μm conductive tape as a spacer (see Fig. 2).

2. Draw the specific circuit design on the bottom plate with electrodes using CAD software.

3. Find a reliable manufacturer to make the bottom plate with patterned chromium (Cr) electrodes on it. The thickness of chromium electrode is 90 nm.

3.3 Dielectric Layer (10 μm SU-83010 Photoresist) Coating on the Bottom Plate

1. Take the SU-83010 photoresist out of the refrigerator 12 h before using it.

2. Clean the bottom plate with DI water, acetone solution, and IPA solution (see Note 3), and use a N₂ purging gun to blow dry it.

3. Dehydrate the bottom plate on a hot plate at 150 °C for 10 min, and then transfer it from the hot plate and wait until its temperature cools down to room temperature (see Note 4).

4. Put the clean and dehydrated bottom plate in the plasma machine for a 2-min plasma surface treatment.

5. Transfer the bottom plate to the spin coater machine as soon as possible, pour an appropriate amount of SU-83010 photoresist onto the bottom plate, and set the parameters of the machine in the following program (see Note 5): first step: 500 rpm, ramp 5 s, hold 10 s; second step: 3000 rpm, ramp 8 s, hold 30 s.
6. Bake the bottom plate on a hot plate at 65 °C for at least 3 min, and then adjust the temperature of the hot plate to 95 °C for 5 min. Naturally cool down to room temperature.

7. Put the bottom plate under a UV-exposure machine for 12 s (this parameter can be changed according to the specific light intensity).

8. Bake the bottom plate on a hot plate at 65 °C for at least 3 min, and gradually adjust to 95 °C for 5 min. Slowly cool down to room temperature.

9. Rinse the bottom plate with SU-8 developer in a proper glass container for 1 min. Spray with IPA solution to get rid of the developer residue. Use a N₂ purging gun to blow dry.

10. Bake the bottom plate on a hot plate at 150 °C for 10 min and slowly cool down to room temperature.

3.4 Hydrophobic Layer (Teflon) Coating

1. Prepare the bottom plate with the dielectric layer (SU-83010 photoresist) and an appropriately shaped top plate for use.

2. Clean with DI water, acetone solution, and IPA solution in turn. Use a N₂ purging gun to blow dry.

3. Dehydrate the top plate and bottom plate on a hot plate at 150 °C for 10 min. Cool down to room temperature.

4. Transfer the plates to the spin coater machine and pour the desired amount of 0.5% Teflon solution on the surface of the top and bottom plates. Start the spin coater with the following parameters: 500 rpm, ramp 5 s, hold 10 s; 1000 rpm, ramp 5 s, hold 60 s.

5. Bake the plates on a hot plate at 65 °C for at least 5 min and gradually adjust to 95 °C for 5 min. Slowly cool down to room temperature.
3.5 DMF System Setup

1. Spray the laminar flow hood and lab bench with 70% ethanol and wipe it down with a paper towel.
2. Place the bottom plate and top plate on a designed 3D-printed chip holder, and fix them by two clips (see Fig. 3).
3. Connect the DMF chip to an electronically controlled printed circuit board (PCB) as shown in Fig. 4. The electronically controlled PCB can be driven by commands of custom-written control software in a computer via a Bluetooth connection.
4. Place a florescence microscope next to the control system for monitoring the activity of the DMF chip.
5. Use CellSens software to detect the florescence intensity in the on-chip drug screening test.

3.6 Drug Concentration Quantification

1. Prepare the drug stock solution: prepare a 4 mM Cis stock solution by mixing 2.5 mg Cis powder and 2 mL 50% DMSO solution. Add an appropriate amount of Cy3 solution to the Cis stock solution to make a 4 mM Cis stock solution containing 10 μM Cy3 (see Note 6). Prepare a 5 mM Cis stock solution by mixing 5.8 mg EP powder and 2 mL 50% DMSO solution.
2. Make a series of drug concentrations (from 0.1 to 100 μM) by serial dilution: dilute both the EP stock solution and the Cis stock solution (containing 10 μM Cy3) with DEME solution. Carry out the operation on a 96-well plate or with centrifuge tubes. Normally, store the drug stocks in centrifuge tubes.

Fig. 3 The designed 3D-printed chip holder with two clips to fix the top plate with bottom plate
3. Add 10% Pluronic F127 solution (v/v = 1:100) to both drug solutions with various concentrations (see Note 7).

4. Preload the drug solution with a series of concentrations (6 μL) on the DMF chip, and place them under a fluorescence microscope to detect their fluorescence intensities (the recommended exposure time is less than 5 s when adjusted to gray field mode) (see Note 8).

5. Use Excel or Origin software to construct the drug (both Cis and EP) concentration calibration curve according to their fluorescence intensities as shown in Fig. 5.

6. Preload a 0.6 μL drug stock (4 mM Cis with 10 μM Cy3/5 mM EP) drop on the DMF chip. Transport it to the drug dispenser position.

7. Set the system in jetting mode. Adjust and vary the actuation control parameter (including the frequency, the ejection actuation voltage, the ejection time, and the jetting bar width) until the drug stock drop starts to eject various amounts of tiny droplets, and the volume varies from 0.1 to 1.5 nL during each actuation (see Note 9).

8. Set the system in transportation mode. Load a 0.6 μL DEME drop on the DMF chip, and transport it to a specific position (the drug dispenser electrode) to pick up the ejection that was ejected by the drug stock drop. Mix the tiny, ejected drug drop with the DMEM drop. Transport the mixture drop to a specific position to measure its fluorescence intensity.

9. Fit the fluorescence intensity of the mixture drop to the drug concentration calibration curve mentioned in step 5 in order to determine the precise drug concentration in the mixture drop.
3.7 On-Chip Single Drug Screening

1. Prepare a fresh cell suspension solution (MDA-MB-231 breast cancer cells and normal MCF-10A breast cells), a drug stock (4 mM Cis with 10 μM Cy3/5 mM EP) solution, and a set of DMF systems for a drug screening test.

2. Add an appropriate amount of Pluronic F127 solution (final 0.01%) to all the reagents.

3. Use pipette tips to dip a little bit of gelatin, and place it at the center of the targeted cell culture electrode spot on the DMF chips (see Note 10). Wait until air dried.

4. Assemble the top plate and bottom plate together. Add a sufficient amount of silicon oil through a specific hole to make sure that the silicon oil can fully cover all the electrodes on the chip (see Note 11).

5. Connect the electronic control system to the DMF chip. Adjust the control parameters.

6. Load a 0.6 μL Cis stock drop (4 mM Cis with 10 μM Cy3) or a 5 mM EP stock drop onto the drug dispenser electrode on the DMF chip. Set the electronic control system in jetting mode, and actuate the drug stock drop to eject the desired amount of tiny drug droplets.

7. Set the system in transportation mode. Load a 0.6 μL cell suspension drop onto the DMF chip, and transport it to pick up the ejected drug droplets (see Note 12). The mixture solution contains both the cells and the drug. After several ejections and pickups, the mixture drop will contain different concentrations of the drug (see Note 13).

8. Place the DMF chip under a fluorescence microscope to measure the fluorescence intensity of the cell-drug mixture drop.

9. Fit the fluorescence intensity of the mixture drop to the standard drug fluorescence-concentration curve to determine the specific drug concentration in the mixture drop.

10. Place the DMF chip into a sterile cell culture dish. Put some moist paper towels inside the dish (see Note 14).
11. Transfer the cell culture dish into the incubator and incubate it at 37 °C and 5% CO₂ for 24 h.

12. After 24-h incubation, take the cell culture dish containing the DMF chip out of the incubator.

13. Connect the DMF chip to the electronic control system. Adjust the electronic parameters (including the frequency, the ejection actuation voltage, the ejection time) on electronic control board to set the system in transportation mode. Load 0.6 μL of cell viability indicator reagent (20 μM calcein–50 μM PI mixture solution) (see Note 15). Transport it to mix it with the cell-drug mixture drop on the DMF chip. Place the chip inside the cell culture dish and put it into the incubator for 10 min.

14. Place the DMF chip under a fluorescence microscope to observe the green or red fluorescence of the cells. Count the number of live and dead cells (green and red fluorescent cells, respectively) to calculate the cell viability.

15. Save the data as more as possible (at least ten data points each group). Use Origin software to construct the cell viability-drug concentration curve, and calculate the IC50 (half maximal inhibitory concentration) of the drug.

### 3.8 On-Chip Multidrug Screening

1. Carry out steps 1–5 of the on-chip single drug screening method.

2. Preload a 0.6 μL Cis stock drop (4 mM with 10 μM Cy3) onto a drug dispenser electrode, and load a 0.6 μL 5 mM EP stock drop onto another drug dispenser electrode on the same DMF chip.

3. Set the system in jetting mode. Actuate the EP stock drop once or several times at the appropriate position of the jetting bar to get the expected amount of drug ejection.

4. Load a 0.6 μL cell suspension solution to pick up the EP stock ejection. Place the ejection under a fluorescence microscope to measure the fluorescence intensity of the cell-EP mixture solution. Fit the intensity to the standard EP fluorescence-concentration curve to determine the precise EP concentration in the drop.

5. Set the system in jetting mode. Actuate the Cis stock drop once or several times to get the expected amount of drug ejection. Transport the cell-EP mixture drop to pick up the Cis ejection (see Fig. 6).

6. Measure the fluorescence intensity of the cell-EP-Cis mixture solution, and then subtract it from the fluorescence of the cell-EP drop. Fit the subtracted value to the standard Cis fluorescence-concentration curve to determine the precise Cis concentration in the mixture drop.
Fig. 6 Schematics of combinatorial drug screening on a digital microfluidic (DMF) chip. (a–c) The dynamic schematics of cell droplet moving and mixing with jetted drug A; (d–f) the dynamic schematics of cell-drug mixed droplet moving and mixing with jetted drug B.

7. Carry out steps 10–13 of the on-chip single drug screening method.

8. Use statistic software to construct the expected chart.

3.9 Off-Chip Drug Screening

1. Obtain the targeted cell suspension solution (MDA-MB-231 cells and MCF-10A cells) after cell passage.

2. Use an automated cell counting system to count the cells. Dilute the cell suspension by cell culture medium to the expected concentration (1.0 × 10^4 cells/mL). Transfer the diluted cell suspension solution to a 96-well plate. Make sure that each well contains 100 μL of cell suspension solution. Place the 96-well plate into an incubator (37 °C and 5% CO₂) for at least 8 h. Observe the plate under a microscope for the appearance of cell adherence (see Note 16).

3. If the cell adherence is successful, place the 96-well plate back into the incubator for overnight incubation until the cells reach around 80% confluence.

4. Take the 96-well plate out of the incubator. Prepare a series of Cis (0–100 μM) or EP (0–50 μM) concentrations or different Cis-EP combinatorial concentrations, which are all dissolved in DMEM cell culture medium. Use a pipette to draw the cell culture medium out of the wells of the 96-well plate, and transfer the 100 μL drug-DMEM mixture solution into each well (see Note 17). Each concentration has five replicates. Set up a negative control: the wells with cells contain only 100 μL 0.1% (v/v) DMSO solution. Set up a blank control: the wells without cells contain only 100 μL DMEM cell culture medium.

5. Place the plate, to which the drug solution is added, into the incubator for 24-h drug treatment.
6. Use a pipette to draw the drug solution out of the wells of the 96-well plate. Transfer 90 μL fresh culture medium and 10 μL CCK-8 solution into each well. Incubate the cells in the cell incubator for 30 min.

7. Place the 96-well plate into a microplate reader, and measure the absorbance value of each well at 450 nm.

8. Collect the experimental data and use statistic software to analyze it. Subtract the absorbance value of the blank control. Normalize to the control wells. Construct a cell viability-drug concentration curve. Combine the on-chip result and the off-chip result for comparison.

4 Notes

1. MDA-MB-231 cells and MCF-10A cells have their own appropriate growth medium.

2. Cell passage refers to the diluting method of cells that have already reached high confluence (80–90% confluence) in order to enable continuous culture propagation. For adherent cells, such as MDA-MB-231 cells and MCF-10A cells, the specific procedure of the cell passaging is slightly different from that of suspension cells.

3. Acetone solution has the ability to remove some organic impurities, and IPA solution is mainly used to remove acetone residues.

4. The dehydrating process is important: water residues will form polar OH-bonds on the substrate of the bottom plate, making it hydrophilic and causing bad adhesion with nonpolar substances.

5. The specific parameters of the spin coater machine can be adjusted depending on the different requirements of the thickness of the coating layer.

6. The concentration of the fluorescent dye Cy3 added to the Cis solution is 1000 times lower than the Cis concentration, so the influence of this amount of additive on the cells for drug screening is negligible.

7. Pluronic F127 solution can promote the drop to move smoothly through the silicon oil on the chip and can also prevent drops from early evaporation.

8. With an exposure time of over 5 s, the fluorescence of a drug with a high concentration may be saturated, which will interfere with the observation.
9. The amount of ejection of droplets that is placed on the drug dispenser position is related to the actuation parameter, so we can get the desired amount of ejection by adjusting the actuation parameter (see Fig. 7).

10. Gelatin is placed at the targeted cell culture electrodes, which can promote cell adhesion and prevent the cell drops from floating freely on the chip.

11. Silicon oil is added to promote smooth drop transportation on the DMF chip.

12. Different control parameters can lead to different movements of a drop on the DMF chip. There are two modes in our system: the transportation mode and the jetting mode. For drop transportation, normally we choose a fixed frequency of 1000 Hz with a low-voltage (50 V) sine wave signal. For tiny droplet ejection (jetting), the frequency was normally fixed at 800 Hz, and the drop was actuated by a square wave with a high pulse voltage (Vp–p from 556 to 1020 V).

13. By adjusting the jetting parameter, we can achieve the desired amount of drug ejection for subsequently picking up a cell drop to get a mixture solution with a series of drug concentrations.

14. Moist paper towels are mainly used for maintaining a humid environment inside the culture dish in order to ensure that the drops on the DMF chip will not evaporate too quickly.

15. The live cells will be stained green by the calcein solution, and the dead cells will be stained red by the PI solution [11] (see Fig. 8).

16. Cell adherence means that the cells adhere to the bottom of the wells. Unsuccessful cell adherence indicates a problem with the activity of the cells, so another batch of cells is immediately required.
Fig. 8 (a) Breast cancer cells MDA-MB-231 and (b) normal breast cells MCF-10A after 24 h of culture with various concentrations generated by the drug dispenser. The live cell will be stained green by calcein solution, and the dead cell will be stained red by PI solution.

17. Make sure that the tips do not touch the cells that adhere to the bottom of the wells when drawing the cell culture medium out of the plate.

18. EP solution is intrinsically red, which will affect the measurement of the absorbance at 450 nm.

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Affinity-Based Microfluidics Combined with Atomic Force Microscopy for Isolation and Nanomechanical Characterization of Circulating Tumor Cells

Muhammedin Deliorman, Ayoub Glia, and Mohammad A. Qasaimeh

Abstract

In this chapter, we present the materials and methods required to isolate and characterize circulating tumor cells (CTCs) from blood samples of cancer patients based on our newly developed microfluidic technologies. In particular, the devices presented herein are designed to be compatible with atomic force microscopy (AFM) for post-capture nanomechanical investigation of CTCs. Microfluidics is well-established as a technology for isolating CTCs from the whole blood of cancer patients, and AFM is a gold standard for quantitative biophysical analysis of cells. However, CTCs are very scarce in nature, and those captured using standard closed-channel microfluidic chips are typically inaccessible for AFM procedures. As a result, their nanomechanical properties largely remain unexplored. Thus, given limitations associated with current microfluidic designs, significant efforts are put toward bringing innovative designs for real time characterization of CTCs. In light of this constant endeavor, the scope of this chapter is to compile our recent efforts on two microfluidic technologies, namely, the AFM-Chip and the HB-MFP, which proved to be efficient in isolating CTCs through antibody-antigen interactions, and their subsequent characterization using AFM.

Key words  Microfluidics, Microfluidic device, Microfluidic probe, Circulating tumor cells, Antibody-antigen capture, Multiplexing, Atomic force microscopy, Nanomechanical characterization, Herringbone micromixer, Hydrodynamic flow confinement

1 Introduction

Circulating tumor cells (CTCs) and their isolation emerge as a promising minimally invasive approach for tracking tumor progression and treatment in real time [1], in addition to the more invasive “gold standard” tissue biopsies. Typically ranging from 1 to 100 cells per milliliter of blood, CTCs have the ability to gain access to and disseminate within the bloodstream at an early stage of tumorigenesis, constituting a biomarker for successful tumor vascular invasion [2]. Recently, it has become evident that alongside genetic mutations, alterations in cellular mechanical properties,
such as stiffness and bio-adhesiveness, are also associated with malignant cell transformation in tumors [3, 4]. Cellular stiffness, or cellular deformability under an applied load, is postulated as a causative factor in determining cancer cell invasiveness [5]. Meanwhile, intercellular adhesive events mediated by different classes of biomolecules on the host cell surface (adhesins) and surface of CTCs (receptors) are acknowledged as an important link to initiate cancer metastasis [6]. Therefore, equally important to understanding genetic mutations in CTCs is realizing their nanomechanical attributes that underlie their tendencies toward tumorigenesis and metastasis. This could be accomplished by efficiently isolating viable CTCs from clinical blood samples and subsequently investigating how their distinct adaptive mechanophenotypes (e.g., cellular stiffness) and surface receptor expression levels (e.g., bio-adhesiveness) contribute to a higher risk of cancer metastasis.

Over the past two decades, closed-channel microfluidics has been extensively used to enrich CTCs from whole blood samples of cancer patients by deploying various approaches including label-free and affinity-based methods [7–9]. In these approaches, it is aimed to isolate viable CTCs with high purity and throughput, test their susceptibility to various physical/chemical stimuli, and analyze their genome content [9]. Here, high purity rate refers to the specific separation of CTCs from overwhelmingly tense background of interfering cells such as white blood cells, and high throughput rate refers to the ability of processing a large volume of blood sample in a short amount of time to achieve a high number of isolated CTCs. Among the several methods, isolation of CTCs through affinity chromatography benefits from many microfluidic-associated phenomena. Pioneered by HB-Chip [10], in these devices CTCs are either captured by targeting one of their distinct surface antigens (positive selection) or separated by targeting antigens specific to background cells (negative selection). In both cases, capture antibodies bound to device surfaces are used in the selection. With its higher purity [9], the positive CTC selection is typically achieved through epithelial cell-specific markers. Yet, the reliance on epithelial markers alone, which not always is highly expressed on the surface of CTCs, commonly lowers the isolation rate with risk of not detecting CTCs of different phenotypes [11]. Moreover, with regard to probing mechanical properties of CTCs, the closed channels present an important limitation; that is, the inability to externally access captured CTCs due to permanent covalent bonding between PDMS and the commonly used glass bottom substrate [12]. Although in some cases cells can be released and collected from an outlet tube (e.g., via ligand exchange [13]), the scarcity and fragility of CTCs make it difficult to probe their mechanical characteristics. Another important challenge originates from the small-scale nature of the channels that limits processing
larger volumes of blood to isolate higher number of CTCs and prevents multiplex cell capture [14].

To date, several techniques have been employed to study the mechanics of cancer cells, such as micropipette aspiration [15], optofluidics [16], microplates [17], acoustic microscopy [18], and atomic force microscopy (AFM) [5, 19, 20]. Due to its capability for localized force measurement, AFM is an ideal technique to probe the stiffness and bio-adhesiveness of the entire cell surface and provide their distributions at the nanoscale. The simplicity of the sample preparation in the technique also enables in situ observation of physiological events at the cellular and molecular levels with remarkable spatial and temporal resolutions. Importantly, AFM offers versatility to control the forces exerted on cells allowing a gentle interaction with cells in a viable physiological environment (medium), prolonging their viability for sufficient quantitative measurements. In addition, it allows for direct multi-parametric quantification of cancer cell biomechanics [21].

To allow seamless integration of affinity-based microfluidic devices with AFM, first we introduced the AFM-Chip [12, 22]. We showed that with its refined linker chemistry for antibody immobilization and unique reversible physical assembly, the AFM-Chip is efficient for isolating prostate cancer CTCs and their subsequent characterization using AFM (Fig. 1a, top panel). Notably, unlike other devices where CTCs are captured on all walls of the PDMS channel, in the AFM-Chip, CTCs are exclusively captured only on the bottom glass slide. This brings advantage in terms of a higher sample size (i.e., number of captured CTCs) when glass slides are transferred to an AFM stage [12]. For high throughput, however, multiplex capture of CTCs in this method is only feasible by simultaneously processing blood in separate AFM-Chips. As such, the large array of channels (typically 16, each ~900 μm wide) in the AFM-Chip is designed to process high throughput under optimized conditions, with up to 1.2 mL of whole blood per 1 h. Patterning individual channels with antibody cocktails is possible but not practical due to multiple reasons: first, it will reduce the effective capture area for each antibody set within the channels. Second, an additional PDMS replica mold with an array of multiple capillaries will be needed to covalently immobilize different antibodies on repeated patterns of stripes. Third, enlarging the width of microfluidic channels to accommodate patterns of different antibodies may result in channel collapse due to the soft structural nature of PDMS. Therefore, in case of prostate cancer CTCs, 3 AFM chips are needed to capture CTCs while targeting 3 different cell surface receptors during the same experiment: epithelial cellular adhesion molecule (EpCAM), prostate specific membrane antigen (PSMA), and prostate specific antigen (PSA) (Fig. 1a, bottom panel). Regarding AFM integration, the transition from CTC capture to single-cell characterization in the AFM-Chip
is also susceptible to cell loss, which is associated with the shearing involved in manually peeling off the PDMS layer.

Thus, and utilizing our expertise in microfluidic probes [23–25], we next introduced the HB-MFP [14]. With its novel “open microfluidic” design, our aim was to overcome the limitations related to closed-channel microfluidic devices and allow for (1) multiplex cell capture in a single run, (2) high throughput by processing larger blood volumes for higher CTC capture, (3) complete cell recovery by minimizing cell loses and damage, and (4) seamless external access to captured cells. The HB-MFP, which stands for herringbone (HB)-microfluidic probe (MFP), is a 3D printed device that works in an open-space (“channel-less”) microfluidic configuration, where the probe is fully decoupled from the glass CTC-capture substrate (Fig. 1b, left panel). For its operation, HB-MFP is placed in a parallel configuration over the activated glass slide with a small separation distance (~50 μm) in between, where the hydrodynamic flow confinement (HFC) region provides “virtual walls” to confine the processed blood [14]. Here, the parallel alignment, separation distance, and scanning speed and direction are precisely controlled with a programmable XYZ
micropositioner. With this, spatial flexibility is introduced, where entire antibody-activated CTC-capture glass slide is efficiently scanned in a 2D printing mode and localized areas are easily targeted. The contactless configuration also allows to independently handle the glass substrate for patterning with stripes of different capture antibodies prior to the capture experiments (Fig. 1b, right panel). Noteworthy, being a contactless CTC-capture technology, HB-MFP excels AFM-Chip in its integration with AFM through its seamless retraction from the glass slide by controlled pull-up, thus leaving fully recovered, captured live CTCs for further nanomechanical characterization.

In this chapter, we describe materials and methods for the design and fabrication of AFM-Chip and HB-MFP. We also provide protocols for using them in affinity-based multiplex capture of prostate cancer CTCs and integration with commercial AFM setup for post-capture nanomechanical analysis of captured cells. The reader should note that, due to flexibility in chemical modifications, the AFM-Chip and HB-MFP can also be used to target isolation of CTCs from other epithelial cancers (e.g., breast, lung, pancreas, colon) and non-epithelial cancers (e.g., multiple myeloma) by simply activating bottom glass slides with the appropriate capture antibodies. Nevertheless, because AFM-Chip is fabricated using soft lithography in PDMS and HB-MFP is fabricated using stereolithography 3D printing, in the following subsections we describe the materials and methods that are either common or specific to each approach. In each subsection, we additionally provide notes regarding concepts in the methods. Finally, we discuss the technologies by highlighting their advantages and limitations, summarize findings, and give future directions on their use for research of CTCs.

2 Materials

Important Suppliers and catalog numbers of materials provided below are given as reference. Required materials are necessary for carrying out the experimental methods presented in this chapter. Optional materials, on the other hand, are for the additional experimental methods marked as “Optional” in the Methods section.

2.1 Materials Common to all Protocols

2.1.1 Reagents

1. Sterile 10× Dulbecco’s phosphate buffered saline (PBS), pH 7.4.
2. Deionized (DI) water.
3. Absolute ethanol.
4. Bovine serum albumin (BSA).
5. Antihuman monoclonal PSMA antibodies conjugated to Alexa Fluor 488 (green) and antihuman monoclonal CD45 antibodies conjugated to Alexa Fluor 647 (red) (Santa Cruz Biotechnology, catalog numbers: sc-516,606 and sc-1178).

6. Antihuman monoclonal cytokeratin 5 (CK5) antibodies conjugated to Alexa 647 (red) and 4′,6-diamidino-2-phenylindole (DAPI) dye (blue) (Abcam, catalog numbers: ab28106 and ab228549).

7. Sylgard 184 silicone elastomer kit.

Optional

8. 5/6-carboxyfluorescein succinimidyl ester (NHS-fluorescein).
9. Dimethylformamide (DMF).

2.1.2 Equipment

Required

1. Pre-cleaned microscope slides (75 mm × 25 mm × 1 mm).
2. Sterile 1.5 mL Eppendorf tubes.
3. Sterile 150 mm Petri dishes.
4. Sterile 200 μL pipette tips.
5. Sterile 50 mL centrifuge tubes.
6. 20–200 μL adjustable-volume pipette.
7. 1 mL plastic transfer pipettes.
8. Spatula.
9. Weighing paper.
10. Microbalance.
11. Vortex mixer.
12. Ultrasonic cleaner.
13. Benchtop ultraviolet (UV)-ozone cleaner.
14. Dry oven.
15. Vacuum desiccator.
16. Timer.
17. Parafilm.
18. Aluminum foil.
19. Razor blade.
20. 2 mm biopsy punch.
21. Wafer-handling tweezers.
22. Disposable plastic cups and plastic stirrers.
23. Disposable 10 mL plastic syringes and 38.1 mm needles.
24. 180 mL glass crystallizing dishes.
25. 10 mL glass vials.
26. Diamond point marker.
27. Fume hood.
28. Class II laminar airflow biosafety cabinet.
29. Liquid nitrogen tank.
30. Polyethylene tubes with 0.8 mm and 4.00 mm inner diameters.
31. 1 mL glass syringes with tubing connectors.
32. Low pressure syringe pump (Cetoni neMESYS, catalog number: 290n).
33. Digital rocker.
34. Ethylenediaminetetraacetic acid (EDTA) tubes.
35. Blood transportation box.
36. Gel refrigerant packs.
37. Stereo microscope.
38. Inverted fluorescence microscope.
39. AFM colloidal tips (NanoAndMore, catalog number: CP-PNPL-SiO-C-5).
40. AFM equipped with inverted fluorescence microscope (AFM Workshop, USA).

Optional

41. Clear plastic tape.
42. Stirring bottle (Cetoni neMIX, catalog number: A3927000008).
43. AFM liquid sample heater (AFM Workshop, USA).

2.2 Materials

2.2.1 Reagents

1. Anhydrous toluene.
2. 3-Aminopropyltriethoxysilane (APTES).
3. N-[β-Maleimidopropoxy]-succinimide ester (BMPS).
4. Antihuman monoclonal 9C4, YPSMA-1, and C-19 antibodies against EpCAM, PSA, and PSMA antigens, respectively (Santa Cruz Biotechnology, catalog number: sc-21,792, sc-7638, and sc-59,674).
5. Soft lithography reagents, including SU8 2050 photoresist, SU8 developer, chromium etchant, AZ351B developer, acetone, and isopropyl alcohol. These reagents are crucial for mask fabrications of AFM-Chip microfluidic channels and HB elements, as well as the master mold. Refer to reference [22] for detailed procedures.
2.2.2 Equipment
1. CAD software (Autodesk AutoCAD, USA).
2. Clean room soft lithography equipment, including silicon wafers, spin coater, hot plate, UV mask aligner, and profilometer. These equipment are necessary for the mask fabrications of AFM-Chip microfluidic channels and HB elements and its master mold fabrication. Refer to reference [22] for detailed procedures.

2.3 Materials
Specific for HB-MFP
2.3.1 Reagents
1. 3-Mercaptopropyl trimethoxysilane (MPTMS).
2. 4-Maleimidobutyric acid N-hydroxysuccinimide ester (GMBS).
3. NeutrAvidin.
4. Biotinylated antihuman monoclonal 9C4 and LNI-17 antibodies against EpCAM and PSA, respectively (Biolegend, catalog numbers: 324216 and 342,510), and biotinylated antihuman monoclonal C-19 antibodies against PSMA antigens (Novus Biologicals, catalog number: NBP2-33194B).
5. Photopolymer resin (PlasClear V2, Asiga).

2.3.2 Equipment
1. CAD software.
2. Stereolithography 3D printer (MAX X27, Asiga).
3. XYZ linear motorized stage with built-in controller (Thorlabs, catalog number: MT402).

3 Methods
3.1 Methods for AFM-Chip
3.1.1 Device Design
1. Use AutoCAD to design a microfluidic chip with 16 channels, each measuring 900 μm wide, 85 μm deep, and 15 mm long, and HB elements, each 25 μm wide and 40 μm deep, oriented at 45° with respect to the longitudinal axes of the channels and have 30 μm gaps in between them (Fig. 2a–c) (see Note 1).

3.1.2 Device Fabrication Using Soft Lithography in PDMS
1. Carry out the device fabrication on a 100 mm silicon wafer (master mold) with two layers of high-viscosity negative photoresist SU-8 using a standard soft lithography protocol [22, 26]: one layer for the microfluidic channels and the other layer for the HB elements (Note 2).
2. After master development, use a stereo microscope to visually verify the developed channels and HB elements. Optional. Measure the height of the developed structures using a profilometer.
3. Firmly tape all the edges of the wafer on a 150 mm Petri dish bottom. Seal the Petri dish with parafilm and store the wafer in a vacuum desiccator (see Note 3).
Fig. 2 Methods for AFM-Chip design, assembly, activation, and blood processing. (a) A CAD design of 16 parallel microfluidic channels incorporated with HB elements (red) and common inlet and outlet ports (purple). Inset: Close-up view of the two channel entrances shows the pattern design of HB elements. The pattern repeats (17 total) across each channel. (b) A micrograph shows a typical PDMS replica mold (75 mm × 25 mm) with punched inlet and outlet ports. (c) A micrograph (top) and cross-sectional schematics (bottom) show the geometry, periodicity, and size of HB elements residing on top of one of PDMS channels. (d) A cartoon featuring the mechanism of reversible physical bonding between PDMS chip and APTES-silanized glass substrate is via stable NH-O hydrogen bonds. (e) After microfluidic device assembly, the linker chemistry steps involve the reaction of APTES silane molecules with a modular crosslinker BMPS to covalently immobilize antibodies on the device glass substrate. (f) A whole blood sample of a prostate cancer patient transferred in a glass syringe, where no air bubbles are trapped in the syringe. The blood is processed in AFM-Chip within 3–4 h of blood draw. (g) A micrograph shows the experimental setup for multiplex capture of CTCs of prostate cancer from whole blood, where for high throughput, blood passes through 3 separate antibody-activated AFM-Chips to target EpCAM, PSMA, and PSA markers. (h) Once captured, antigen-specific immunomarkers are used for the identification of CTCs (green dots pointed by arrows). A magnified single captured intact CTC is shown in the inset (scale bar: 8 μm). (Reproduced with permission from Ref. [12])

3.1.3 PDMS Replica Molding

1. Use a plastic cup and a plastic stirrer to mix the PDMS base elastomer (Sylgard 184) and the curing agent at a 10:1 (w/w) ratio. Stir the mixture homogeneously for 5–10 min (see Note 4).

2. Slowly pour the mixture over the master wafer till 5–10 mm thickness to form a PDMS replica mold.

3. Place the wafer in a vacuum desiccator for 1 h to remove the air bubbles generated within the PDMS during the mixing step.

4. Cure the PDMS mold in a 60 °C dry oven for 2 h. When fully cured, the PDMS mixture will solidify and become free of air bubbles.

5. After the PDMS is cured, remove the mold from the oven, allow it to cool to room temperature, and carefully cut it along the edges of the master wafer. When cutting, keep the pressure minimum to prevent wafer from breaking.
6. Slowly peel off the PDMS. Leave the master wafer inside the Petri dish with surrounding PDMS and store it safely for future PDMS molding.

7. Cut the PDMS mold into 75 mm × 25 mm chips using a razor blade, and remove all uneven edges. When cutting, ensure that microfluidic channels of each chips are well-centered with respect to chip edges.

8. Observe the channels and HB elements of the PDMS chips under stereo microscope. Discard the ones with distorted features.

9. Punch the inlet and outlet ports of the PDMS chips straight down using a 2 mm biopsy punch. Ensure that the punched holes are completely free of PDMS (Fig. 2b).

10. Place the PDMS chips in a glass dish with the channel surfaces facing up. Clean them ultrasonically in DI water and in ethanol for 3 min to remove large particles from the channel surfaces. Cover the glass dish with aluminum foil during cleaning.

11. Hold the PDMS chips using tweezers, wash them thoroughly with ethanol, and dry them with nitrogen gas.

12. Place the cleaned PDMS chips in a Petri dish with channels facing upward. Seal the Petri dish with parafilm, and store the PDMS chips in a vacuum desiccator. Optional. Cover the top surfaces of PDMS chips with clear plastic tape. Although this is very common approach, it is not recommended prior to follow-up chemical modifications due to residues that tape may leave on chip surfaces.

3.1.4 Glass Cleaning and Silanization

**Important** All glass cleaning and silanization/annealing steps must be carried in clean glass crystallizing dishes covered with aluminum foil. Glass slides (75 mm × 25 mm) must be handled with tweezers.

1. Leave a small cross mark on far end corner of one of the surfaces on glass slides using a diamond point marker. Ensure that the marked surfaces always face up during chemical modifications and AFM-Chip assembly.

2. Ultrasonically clean glass slides in DI water and ethanol for 3 min to remove large particles from their surfaces. Dry the slides with nitrogen gas.

3. Clean the glass slides under UV-ozone for 30 min (see Note 5).

4. Soak the glass slides in an anhydrous toluene solution containing 0.5% wt. APTES molecules for 45 min at room temperature (see Note 6).
5. After the silanization, immerse the glass in ethanol and sonicate for 3 min to remove loosely bound molecules.

6. Wash thoroughly the glass slides with ethanol and dry them with nitrogen gas.

7. Place the glass slides in a 100 °C dry oven for 30 min to allow APTES annealing (see Note 7).

3.1.5 Reversible AFM-Chip Assembly

1. Bring the cleaned PDMS chips (with the channel sides facing down) and APTES-silanized glass slides into contact at room temperature and under atmospheric pressure to enable reversible AFM-Chip assembly (Fig. 2d) (see Note 8).

2. Gently press on the PDMS chips to ensure that the surfaces of the AFM-Chip are well-bonded (see Note 9).

3. Place the AFM-Chips in a vacuum desiccator for 15 min to allow any trapped gas bubbles between the surfaces to escape naturally.

3.1.6 Antibody Immobilization on Glass

**Important** (a) After each modification step, manually flush the AFM-Chip channels with 100 μL PBS using a syringe (connected through polyethylene tubes) so that unbound molecules/antibodies are removed. Also, pipette PBS droplets on the inlet and outlet ports of the AFM-Chips to prevent liquid evaporation during incubation times. (b) For multiplex capture of prostate cancer CTCs, repeat the below steps 1–6 three times, each time using separate AFM-Chips, to immobilize anti-EpCAM, anti-PSMA, and anti-PSA antibodies. (c) The linker chemistry used to activate the glass slide is depicted in Fig. 2e. The glass activation steps are as the following:

1. Prepare a 3 mg/mL solution of BMPS in PBS in a glass vial. Mix the solution using a vortex mixer for 5 sec.

2. Fill a glass syringe with 100 μL BMPS solution for each AFM-Chip, and manually pass it through their channels using polyethylene tubes. Incubate the silanized surfaces for 30 min at room temperature (see Note 10).

3. Dilute concentrated antibody solutions (EpCAM, PSMA, and PSA) to concentration of 10 μg/mL using PBS in Eppendorf tubes. Mix the solution using a vortex mixer for 5 sec (see Note 11).

4. Fill a glass syringe with 100 μL antibody solution for each AFM-Chip, and manually pass it through their channels using polyethylene tubes. Incubate the maleimide-activated surfaces for 45 min at room temperature (see Note 12).

5. Prepare 1% (v/v) BSA solution in PBS in a 50 mL centrifuge tube. Transfer it to a 100 μL syringe, and manually pass it through AFM-Chip channels through polyethylene tubes.
Allow for 20 min to block the nonspecific binding sites on microfluidic channel surfaces (see Note 13).

6. Store the AFM-Chips at room temperature until the cell capture experiments.

3.1.7 Antibody Coverage Characterization

**Important** This subsection is optional. However, antibody coverage characterization is recommended to verify the chemisorption and orientation of each antibody functional group to maleimide-activated glass substrate as control.

1. Manually pass 1-mg NHS-fluorescein in 400 μL DMF through AFM-Chip channels, and allow incubation for 60 min in the dark at room temperature (see Note 14).

2. Manually flush the channels with 100 μL PBS to remove unbound dyes. Detach the glass substrate from the PDMS and immerse them in Petri dishes filled with dye-free PBS.

3. Carry out the imaging of glass slides under inverted fluorescence microscope using fluorescein isothiocyanate (FITC) filter cube and through 10× air objective.

4. Calculate the corrected total fluorescence of images using an algorithm that scales and shifts each pixel value of the input image to match its mean and standard deviation to those of target images (corrected) (see Note 15).

3.1.8 Blood Processing Using AFM-Chip

**Important** (a) Obtain ethical approval from your institution’s IRB and the collaborating hospital’s ethics committee. (b) Obtain written consent from volunteer subjects after explaining the study to them. (c) Collect blood samples in EDTA tubes and process them within 3–4 h of collection. (d) Place the EDTA tubes on a digital rocker at a slow mode during cell capture experiments. (e) Wear personal protective equipment (e.g., gloves, lab coat, goggles) at all times when handling blood. (f) For multiplex capture of prostate cancer CTCs, repeat the below steps 1–8 at once using separate AFM-Chips activated with anti-EpCAM, anti-PSMA, and anti-PSA antibodies. The blood processing steps are as the following:

1. **Fill the glass syringe with 100 μL BSA solution in PBS (1%, v/v), and inject it through the tubes to prevent cell settling through sedimentation.**

2. **Transfer 1 mL of whole blood from EDTA tubes into a glass syringe under a biosafety cabinet using a disposable needle syringe (Fig. 2f).** If air bubbles are trapped in the process, slowly discharge them with the syringe end pointing upward.

3. **Connect polyethylene tubes to the syringe and inject the blood slowly until it reaches the exit end of the tubes.**
4. Place an antibody-activated AFM-Chip on an inverted microscope stage. Mount the syringe to a syringe pump and connect it to the AFM-Chip using polyethylene tubes. Connect the exit port of the AFM-Chip to a waste container with another set of polyethylene tubes (Fig. 2g). Optional. Use a stirring bottle between the syringe and the AFM-Chip to prevent cell sedimentation.

5. Adjust the flow rate of the syringe pump to 20 μL/min and inject the blood completely into microfluidic channels.

6. Replace the empty blood syringe with a glass syringe filled with 100 μL PBS. Wash channels at a 20 μL/min flow rate (for 5–10 min) to remove any unattached cells within the channels.

7. Replace the empty PBS syringe with a glass syringe filled with 100 μL of anti-PSMA antibodies conjugated to Alexa Fluor 488. Pass the antibodies through channels at a 20 μL/min flow rate (for 5–10 min) and allow incubation for 20 min in the dark to immunostain captured CTCs.

8. Wash the channels with 100 μL PBS at a 20 μL/min flow rate (for 5–10 min) to remove any unbound dye.

9. Repeat steps 6–8 with 100 μL of anti-CD45 antibodies conjugated to Alexa Fluor 647 to immunostain nonspecifically bound white blood cells (WBCs). Note here that the different staining antibodies can be mixed for staining different cell types at the same time/run; however, this may cause cross-staining and compromise specificity.

10. Follow with fluorescence imaging of stained cells (see Fig. 2b and refer to Subheadings 3.3.1 and 3.3.2).

### 3.2 Methods for HB-MFP

#### 3.2.1 Device Design

1. Use SolidWorks to draw the HB-MFP and its tip surface (see Fig. 3a, left panel) (see Note 16).

2. Optional. Use COMSOL Multiphysics (COMSOL, USA) to produce 3D numerical models of the HB-MFP for specific applications (see Fig. 3a, right panel).

#### 3.2.2 Device Fabrication Using Stereolithography 3D Printing

1. Print the HB-MFP with a stereolithography 3D printer using a photopolymer resin (see Note 17).

2. Observe the printed HB-MFP tip surface under a stereo microscope to visually verify the structure, dimensions, and surface finish of HB elements (Fig. 3b).

3. Wash thoroughly the printed HB-MFP with DI water and ethanol. Dry it with nitrogen gas and clean its surfaces under UV-ozone for 2 min.

4. Store the cleaned HB-MFP in a vacuum desiccator for further use.
**Fig. 3** The HB-MFP device for multiplex capture of CTCs on patterned antibody glass slides. (a) Left panel: schematic shows the bottom surface of HB-MFP (the mesa region) with its central circular injection aperture (in), two peripheral crescent-shaped aspiration apertures (Asp-1 and Asp-2), and radially staggered HB elements in between. Apertures are linked to 3 fluidic tube channels, and HB elements provide enhanced cell capture efficiency. Right panel: schematic shows the fluid flow swirling pattern (shown in salmon) induced by HB elements as the fluid flow is propagating from the central injection aperture toward the peripheral aspiration apertures. The confined fluid (HFC) is shown in pink. (b) Micrographs show the tip surface of a 3D printed HB-MFP. Zoomed images of dashed boxes show the radial structure and surface finish of the HB elements (scale bar: 0.5 mm). (c) HB-MFP at work: the distribution of PC3 (prostate cancer cell line, green) and control MCF-7 (breast cancer cell line, red) cells on EpCAM, control (no antibody), PSMA, and PSA stripes. Using HB-MFP, PC3 and MCF-7 cells (mixed at equal concentrations) were both captured on EpCAM stripes, whereas only PC3 cells were captured on PSMA and PSA stripes (scale bar: 350 μm). Once captured, antigen-specific immunomarkers are used for the identification of cells (green and red dots) (Reproduced with permission from Ref. [14])

3.2.3 Microfluidic Chip for Antibody Patterning

1. Use SolidWorks to draw a microfluidic chip with 3 channels, each 1 mm wide, 100 μm deep, 21 mm long, and with separate inlet and outlet ports (see Note 18).
2. Print the microfluidic chip with a stereolithography 3D printer using a photopolymer resin (see Note 17).
3. Use the cleaned microfluidic chips (master mold) for further PDMS replica molding following steps 1–12 in Subheading 3.1.3.

3.2.4 Glass Cleaning and Activation

1. Reference instructions in steps 1–3 of Subheading 3.1.4 to clean glass surfaces, and in steps 1–3 of Subheading 3.1.5 to reversibly bond the cleaned PDMS chips (channel sides down) and glass slides (crossed surfaces up).
2. Transfer the microfluidic chips to a 60 °C dry oven for 2 h for high strength reversible bond between their surfaces.

3. Reference instructions in steps 1 and 2 of Subheading 3.1.6 to chemically modify glass surfaces in 3 of the channels using a solution of ethanol containing 1% wt. MPTMS, followed by a solution of ethanol containing 1% wt. GMBS, and a solution of PBS containing 1% wt NeutrAvidin. Allow their activation for 30 min at room temperature (see Note 19).

4. Reference instructions in steps 3–4 of Subheading 3.1.6 to immobilize biotinylated anti-EpCAM, anti-PSMA, and anti-PSA antibodies (10 μg/mL concentration in PBS) in 3 of the NeutrAvidin-activated channels. Allow their immobilization for 1 h at room temperature.

5. Reference instructions in step 5 of Subheading 3.1.6 to block nonspecific binding sites on microfluidic channel surfaces using 1% (v/v) BSA solution in PBS for 20 min.

6. Optional. Reference instructions in steps 1–4 of Subheading 3.1.7 to characterize the antibody patterns as control for their density and orientation.

7. Slowly peel off the PDMS and immerse the antibody patterned glass slides in PBS/medium in a Petri dish for immediate cell capture experiments. Optional. The glass slides can also be stored in refrigerator at 4 °C for prolonged durations. However, it should be noted that this may result in lowering the cell capture efficiency due to conformational changes of antibodies on glass surface [12].

### 3.2.5 Blood Processing Using HB-MFP

1. Reference instructions in Steps 1-3 of Subheading 3.1.8 to fill a glass syringe with 1 mL of blood.

2. Integrate an XYZ linear motorized stage with a stage of inverted fluorescence microscope.

3. Mount the filled blood syringe to a syringe pump and connect it to the injection aperture of the HB-MFP using polyethylene tubes. Mount two additional empty glass syringes to each of the aspiration apertures of the HB-MFP using another set of polyethylene tubes (see Fig. 1b, left panel). Note here that the tubing connected to the empty syringes should be pre-filled with buffer to avoid air in the system.

4. Connect the HB-MFP to the motorized stage via its implemented twist-lock arm. Position the HB-MFP on top of 1× or 2× objective lens so that the whole processing area can be observed. Place antibody patterned glass slide on inverted microscope stage under the HB-MFP (see Fig. 1b, right panel).

5. Run the injection through the HB-MFP until a small blood droplet is formed in the MFP’s head “mesa” region, then
aspirate using both aspiration syringes. This is to ensure that the injection and aspirations are fully operational and to eliminate any remaining air bubbles.

6. Use the built-in controller of the motorized stage to align the base of the HB-MFP parallel to the glass surface. Afterward, start approaching the glass surface until the HB-MFP reaches a gap of 50 μm (see Note 20).

7. Adjust the blood injection flow rate of a syringe pump to 1.2 mL/h while keeping the aspiration flow rate at 3.6 mL/h (see Note 21).

8. Process the blood completely by hovering the HB-MFP over antibody patterns in a zig-zag manner at a speed of 0.35 mm/s (see Note 22).

9. Reference instructions in steps 6–9 of Subheading 3.1.8 to remove any unattached cells on glass surface and immunostain captured CTCs and nonspecifically bound WBCs (see Note 23). Note here that the timing mentioned in Subheading 3.1.8 may be different in the case of the HB-MFP depending on the scanning area and speed.

3.3 Methods
Common for AFM-Chip and HB-MFP

3.3.1 CTC Capture and Purity Evaluation

1. Investigate the CTC capture by manually counting captured CTCs under inverted fluorescence microscope in the dark using FITC filter cube and through 20× air objectives (Figs. 2h and 3c).

2. Investigate the isolation purity by counting nonspecifically bound WBCs under inverted fluorescence microscope in the dark using tetramethylrhodamine (TRITC) filter cube and through a 20× air objective.

3. Calculate the purity of the device by determining the ratio of captured CTCs to the total number of bound cells, including WBCs.

4. Optional. Dedicate another set of experiments for live/dead investigations of captured CTCs using Live/Dead assay kit for mammalian cells (Invitrogen).

3.3.2 Further CTC Verification

1. After AFM measurements (see Subheading 3.3.3), further immunostain captured CTCs (single and cluster) and WBCs (background) for DAPI and CK in the dark to verify, under inverted fluorescence microscope using DAPI and TRITC filter cubes and through a 60× air objective, that captured CTCs are DAPI+/CK+ (Fig. 4, top panel) and WBCs are DAPI+/CK- (Fig. 4, bottom panel).

Important Perform the AFM measurements using commercially available systems. Ideally, the AFM paired with an inverted fluorescence microscope and enclosed in a vibration enclosure will suit best for identifying immunomarked CTCs and achieving a high
degree of sound absorptions during the sensitive force measurements. Moreover, ensure that the tip is attached to the cantilever for proper engagement with the cell surface.

**Force Measurements**

1. Slowly peel off the PDMS in the AFM-Chip or pull up the HB-MFP and transfer the glass slides with immunostained captured CTCs onto AFM stage for their downstream nanomechanical characterization (Fig. 5a).

2. Use a custom-made PDMS gasket as the “AFM liquid cell” to hold the cell medium in the glass slides [12, 22] (Fig. 5b) (see Note 24). Optional. Use an AFM liquid sample heater to maintain the cell medium temperature at 37 °C.

3. Mount a 6 μm-diameter colloidal tip with a nominal spring constant of 0.06 N/m on AFM liquid tip holder.

4. Determine the cantilever spring constants from the power spectral density of the thermal noise fluctuations.

5. Define a clean area (i.e., free of cells) on the glass surface, and approach the tip to engage with the surface. Calibrate the sensitivity of the photodiode by indenting the tip against the glass surface.

6. Disengage the tip and position it above the center of a single captured intact CTC using the AFM integrated camera (Fig. 5c). Approach the tip to engage with the cell surface.

7. Perform the force measurements either in single force mode or in force volume mode. In the latter, repeat the tip approach-retraction process over a 10 μm² cell surface area at a resolution of 8 × 8 pixels.
Fig. 5 AFM setup and force measurements of prostate cancer CTCs captured using AFM-Chip or HB-MFP. (a) The cartoon shows an AFM tip interacting with a single captured intact prostate CTC. (b) After peeling off the PDMS from glass slide, a custom-made AFM liquid cell was used to confine the cell medium. (c) The micrograph shows an AFM tip positioned above the center of a stained prostate CTC for follow-up force measurements (scale bar: 30 µm). (d) The force curve demonstrates the interaction between the AFM tip and the CTC, where tip’s approach to and retraction from the cell surface are shown by black arrows. After initial tip-cell surface contact, the cell experiences an amount of surface deformation “D” due to the applied constant loading force. During tip retraction, the cell experiences a number of adhesion forces “F” (peaks 1–7 in the retraction process) due to its interactions with the AFM tip surface. In the process, the work of detachment “WD” (shaded gray area below the zero-force line) indicates the amount of work needed to completely detach the tip from the cell surface. (e) The measured adhesion values are tabulated for all peaks in Fig. 5d. (f) After the initial tip-cell surface contact, force data (black circles) were fit (red line) to surface indentation (dashed blue ellipse in Fig. 5d) to estimate the local Young’s modulus “E.” (Reproduced with permission from [12])

8. Set the trigger force (i.e., applied constant force) to 12 nN, reflecting cell surface indentations of ~0.4 µm. Set the tip approach and retraction velocities to 4 µm/s.
9. Repeat steps 6–8 until all captured CTCs are characterized.
10. Disengage the tip and remove the glass slide from the AFM stage.

**Force Analysis**

1. Exclude the force curves with unclear approaches and/or retraction curves from the analysis (see Note 25).
2. For the cell surface deformations measurements, measure the distance from cell contact point till trigger force is reached (Fig. 5d).
3. For the adhesion measurements, define the maximum adhesion forces from each data point in the retraction curves and assign as minima if they were less than their five nearest neighbors to the right and left. Choose 10 pN as the cutoff force value due to experimental noise during measurements (Fig. 5d and e).

4. For the work of detachment measurements, use a trapezoidal integral to calculate the area confined between the zero-force line and the retraction curve (Fig. 5d).

5. For the elasticity measurements, use classic Hertz model of contact mechanics. Choose 100 nm fit range when applying the model (Fig. 5f) (see Note 26).

1. Properly dispose the glass slides along with all other experimental components used in the capture/characterization of CTCs. Clean thoroughly the glass syringes with ethanol and safely store them in labeled containers for further use.

2. In case of blood spill, ensure that the infected surface is thoroughly wiped with ethanol.

**AFM-Chip**

1. AFM-Chip assembly occurs via reversible physical PDMS-to-glass bonding using silane molecules (e.g., APTES). Such bonding is stable, creating firm and high-performance microfluidic channels that could withstand up to 150 μL/min flow rates with no leakage of blood, and reversible, allowing—thanks to highly hydrophobic nature of silanized glass substrate—opportunities for conducting fundamental studies on intact captured CTCs by simply peeling off the PDMS.

2. The reversible AFM-Chip assembly further allows the use of intermediate linker molecules (e.g., BMPS) for site-specific covalent immobilization of antibodies exclusively on glass surfaces. This provides a high density of oriented (active) antibodies, thus improving the specific capture of CTCs and significantly reducing the capture of other blood cells.

3. The antibody loading capacity in AFM-Chip is independent of the choice of antibodies due to highly site-specific coupling between intermediate linker molecules and antibodies. This gives great flexibility in targeting different surface markers of CTCs for their specific capture.

**HB-MFP**

1. HB-MFP is modular (can be easily redesigned to fit the specific requirements of any experiment), cheaply 3D printed (can be reproduced within an hour), and reusable (can be autoclaved).
2. HB-MFP allows for functionalizing bottom substrate with stripes of different capture antibodies and scanning the fluidics delivery system across the substrate in a 2D printing style. This promotes multiplex CTC capture in a single run.

3. HB-MFP offers high throughput blood processing and efficient cell capture and preserves cell integrity and viability due to low shear stresses, gives full access to captured CTCs for post-processing steps without loss or dislocation thanks to its independent blood delivery system, and provides capability to capture CTC clusters of any size thanks to its “channel-less” configuration.

3.4.2 Limitations

AFM-Chip

1. Inherent limitations of AFM-Chip mainly stem from:
   
   (a) Its fabrication method that is very time consuming, expensive, and labor intensive due to soft lithography procedures.

   (b) The narrow channel dimensions that limit the throughput, make the channels susceptible to clogging, jeopardize cell integrity and viability, and prevent capturing clusters of CTCs due to high shear stresses.

   (c) The dependency on distinct CTC surface antigens that limits the enrichment of CTCs due to highly heterogeneous subpopulations.

   (d) The extra steps needed to detach bound CTCs from glass for further genetic analysis, which limits the cell recovery due to their fragile nature.

2. In AFM-Chip, the transition from capturing CTCs to characterizing them using AFM may lead to cell loss. This is because of two factors: a) annually peeling off the PDMS may cause cell loss, which can be reduced by slowly peeling off the PDMS starting from its inlet side, and b) after removing the PDMS, the AFM liquid cell should be immediately placed and the glass slide should be filled with cell medium to prevent the surface from drying and additional cell loss.

HB-MFP

1. The fabrication of HB-MFP, and in particular of its herringbone elements, inextricably depends on the resolution of 3D printer being used and the type of resin. This limits the universality of the HB-MFP fabrication protocol.

2. Other limitations related to HB-MFP include the requirement of an XYZ micropositioner for functioning, dependency on distinct antigens for CTC capture, the need of extra steps to release bound CTCs from glass, susceptibility to clogging (although rare), and being prone to contamination (since this is an open-space microfluidic configuration).
3.4.3 Data Analysis

1. Our simulation results revealed that the shear stress gradient generated within the channels of AFM-Chip was less than 2 Pa at flow rate of 20 μL/min and within 80% of HB-MFP processing area was between 0.1 and 0.4 Pa at flow rate of up to 3.6 mL/h. These shear stress values are expected not to jeopardize the intactness and viability of captured CTCs.

2. We were able to efficiently isolate CTCs from whole blood samples of prostate cancer patients through their specific EpCAM, PSMA, and PSA antigens with counts ranging from as low as 1 CTCs/mL (AFM-Chip) and 6 CTCs/mL (HB-MFP) to as high as 17 CTCs/mL (AFM-Chip) and 280 CTCs/mL (HB-MFP) for localized and metastatic cancer, respectively. Moreover, with HB-MFP, we were also able to show that capturing CTC clusters comprising as many as 50 cells is achievable. Such clusters are of a high research importance due to their association with metastasis in many reports and are difficult to capture using the standard closed-channel microfluidic devices.

3. Multiplex profiles of CTCs revealed certain cellular phenotypes based on PSMA and PSA expression levels. As such, capturing CTCs through their PSMA and PSA provided information on invasiveness, treatment response, and metastatic activity of CTCs and helped in differentiating the cancer grades between patients. Moreover, when nanomechanically characterized, CTCs originating from metastatic cancer demonstrated decreased elasticity and increased deformability compared to those originating from localized cancer. While the average adhesion of CTCs to the AFM tip surface remained the same in both groups, there were more multiple adhesion events in metastatic CTCs than there were in their counterparts.

3.5 Future Directions

AFM-Chip

1. It will be ideal to 3D print the master mold for the AFM-Chip. 3D printers are rapidly developing, and this could easily be accomplished with ones having pixel resolution of <20 μm.

2. AFM-Chips with larger channel dimensions can be designed and fabricated to increase the throughput while still avoiding potential channel collapse.

3. AFM-Chips functionalized with different capture antibodies (e.g., EpCAM, PSMA, and PSA in case of prostate cancer) can be connected sequentially for multiplex capture of CTCs, extending the exposure of blood content by three times longer capture path.
**HB-MFP**

1. By varying the design of its apertures and their separation distances, HB-MFP could provide flexibility in adjusting the size and shape of its processing area, thus larger processing areas could be obtained without compromising flow resistance. Processing areas can be easily utilized for scaling up throughput and multiplex capture, where large blood volumes can be processed with significantly higher flow rates but without exposing the blood content to high shear stresses and evading saturation.

2. Multiplex capture using HB-MFP can be further designed to include tens of other recognition molecules in any desired patterns. Moreover, for rigorous clinical use, complete automation can be implemented to the functioning of HB-MFP. For example, with simple modification in the design, HB-MFP can operate with a wash-on-the-fly mode, thus eliminating the separate washing process. This could be accomplished by injecting the blood from one of the crescent-shaped apertures, while the washing fluid (e.g., PBS) is injected from the other, and both fluids are aspirated from the central aperture.

3. Advancing the parallel alignment sensitivity of the HB-MFP, as well as its bottom surface finish, will enable higher blood processing throughput while maintaining higher capture efficiency due to minimized shear stresses. As a result, faster processing of blood samples will allow more cancer material to be retrieved, widening the time frame for downstream analysis using AFM.

## 4 Notes

1. The design of the PDMS chip is adapted from our previously developed device [27]. Here, the large array of microfluidic channels, along with their dimensions, aim to achieve high throughput, thus reducing the blood processing time. On the other hand, the HB elements aim to serve as a fluidic chaotic mixer to enhance the cell-substrate interactions, thus improving the CTC-capture yield within the channels [10].

2. (a) The master mold fabrication must be conducted in a fully equipped clean room. The design can be submitted to a soft lithography vendor for mask printing. (b) Three devices can be master molded on a 100 mm silicon wafer. Refer to reference [22] for detailed procedures.

3. The master wafer can be kept in the desiccator for extended periods and reused multiple times.
4. If PDMS is not mixed thoroughly or the amount of catalyst is not adequate, PDMS will not cure completely and will make the master wafer unusable.

5. A small but persistent amount of carbon will always be present on the glass surfaces as an indication of environmental contamination. Exposure to air makes it impossible to prevent such trace contamination.

6. The silanization step must be conducted under fume hood. Toluene rapidly produces toxic concentrations at room temperature; therefore, direct inhalation must be avoided.

7. The annealing process will result in ~0.9-nm silane monolayer film thickness.

8. Assembling the platform is through reversible physical bondage of silanols on PDMS with amines on silanized glass slide via NH-O hydrogen bonds.

9. (a) Avoid pressure on the channel areas, which could damage the structure of HB elements and collapse the shallow channels. (b) Misaligning in this step may cause the channels to leak or not to fit into glass slide properly.

10. BMPS is an intermediate linker molecule that provides an excellent route to covalently tether antibodies to silanized glass surfaces in an oriented fashion and at high concentrations through site-specific coupling: succinimide group to couple with the reactive silane amino groups, and maleimide group to couple with the sulphydryl groups of antibodies.

11. 10 μg/mL antibody concentration used in functionalizing the AFM-Chip is sufficient to fully cover the glass surface within its 16 channels in an oriented fashion. Here, the high density and activity of antibodies aim to increase the CTC capture yield, while minimizing the nonspecific binding of other cells, thus increasing the CTC purity rate. However, it should also be noted that proper antibody concentration is subject to characterization and biological testing.

12. (a) During incubation time, keep the chips in a humidity chamber (e.g., a Petri dish containing small amount of water droplets) to prevent evaporation of the antibody solution. (b) The specific binding of antibodies is only on the glass substrate (through maleimide interaction). The top and side walls of the channels are expected to have minimal amount of antibodies physically adsorbed through nonspecific interactions.

13. BSA is used to block the remaining nonspecific binding sites on the substrate surface, primarily available due to protonated amines formed during the APTES silanization process.
14. The succinimide groups of NHS-fluorescein bind specifically with the N-terminal groups of antibodies, which are located at the ends of their fab arms.

15. In processing the fluorescence images, use a G'MIC (gmic.eu) color transfer filter to transfer the color characteristics of one image to another, and analyze images in ImageJ (https://imagej.nih.gov/ij) to calculate the corrected total fluorescence of images [12].

16. (a) The HB-MFP consists of 3 fluidic ports: one circular injection aperture surrounded by two symmetrical crescent-shaped aspiration apertures with ~4 mm radius separation distance, which represents the blood processing area. Additionally, a twist-lock mechanism is introduced in the design to connect the HB-MFP to an XYZ micropositioner with built-in controller so that its translational and rotational motions are feasible to adjust the alignment, separation distance, and direction of the device. (b) The HB micromixers are radially implemented across the processing area between the two aspiration apertures, where each is 100 μm wide, 40 μm deep, and oriented at 80° with 60 μm gaps between them. Note that the 80° angle in the HB structure was designed to maximize the angular displacement (i.e., twisting) of the radial flow, thus improving cell-surface interactions.

17. When printing the parts, strictly follow the protocols provided by 3D printer manufacturer.

18. The channel number is custom, and it depends on the number of capture antibodies targeted in the patterning. For example, for the multiplex capture of prostate cancer CTCs, 3 channels are assigned to be patterned with anti-EpCAM, anti-PSMA, and anti-PSA antibodies. Also optional is to have one more channel in between as control (no antibody).

19. Chemical modification steps are part of avidin-biotin chemistry, which is widely used to strongly bond antibodies to glass surfaces via affinity-based linkages.

20. The ~50 μm separation distance between HB-MFP and glass surface represents a standard channel height in HB-based microfluidic chips, which we also proved to be the optimal separation distance at which the capture efficiency is highest [10].

21. To achieve HFC between the tip surface of the HB-MFP (called mesa) and the glass surface, the ratio of the fluid aspiration to that of injection flow rates (α = Q_{Asp}/Q_{Inj}) is maintained above the theoretical limit (i.e., α = 1.5 based on fluidic dipolar configuration).
22. Scanning speeds of as high as 2.35 mm/s could be achieved with $\alpha = 10$ along with ratio of mesa/processing area ($\varphi$) = 1.75 and as low as 0.35 mm/s with $\alpha = 2$ and $\varphi = 1.25$. To put these values into perspective, the HB-MFP turns capable to fully scan a 75 mm $\times$ 25 mm glass slide in 1 to 4 min, and with $\alpha > 10$, in less than a min.

23. The washing and immunostaining process can be accomplished in two ways: using HB-MFP with a \textit{wash-on-the-fly} configuration, or manually after pulling up the HB-MFP and gently replacing the cell medium with fresh one using plastic transfer pipette.

24. The cell medium choice can range from PBS to cell culture medium of choice (e.g., DMEM, RPMI) to help in maintaining the viability of the CTCs.

25. One way to analyze AFM force curves is by extracting raw data as ASCII files. Numerical computing environments, such as MATLAB (MathWorks, USA) or R (\url{r-project.org}), can then be utilized to process the raw data.

26. According to the model, the loading force ($F_L$) applied by the AFM tip required to indent a distance ($\delta$) into the cell surface is given by

$$F_L = \frac{4}{3} \frac{E}{(1-\nu^2)} R^{1/2} \delta^{3/2}$$  \hspace{1cm} (1)

where $E$ is the elasticity modulus of the cell, $R$ is the radius of the AFM tip, and $\nu$ is the Poisson ratio of the cell, which was set to 0.5 assuming cell’s incompressibility.

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Capture and Selective Release of Viable Circulating Tumor Cells

Xilal Y. Rima, Jingjing Zhang, and Eduardo Reátegui

Abstract

Selectively capturing and releasing viable circulating tumor cells (CTCs) from the peripheral blood of cancer patients is advantageous for investigating the molecular hallmarks of metastasis and developing personalized therapeutics. CTC-based liquid biopsies are flourishing in the clinical setting, offering opportunities to track the real-time responses of patients during clinical trials and lending accessibility to cancers that are traditionally difficult to diagnose. However, CTCs are rare compared to the breadth of cells that reside in the circulatory network, which has encouraged the engineering of novel microfluidic devices. Current microfluidic technologies either extensively enrich CTCs but compromise cellular viability or sort viable CTCs at low efficiencies. Herein we present a procedure to fabricate and operate a microfluidic device capable of capturing CTCs at high efficiencies while ensuring high viability. The microvortex-inducing microfluidic device functionalized with nanointerfaces positively enriches CTCs via cancer-specific immunoaffinity, while a thermally responsive surface chemistry releases the captured cells by raising the temperature to 37 °C.

Key words Circulating tumor cells (CTCs), Microfluidics, Nanocoating, Cell release, Immunoaffinity, Surface chemistry, Cancer diagnosis

1 Introduction

Metastasis is the leading cause of cancer-related deaths [1], which initiates from the dissemination of circulating tumor cells (CTCs) from primary tumors into premetastatic niches [2]. Therefore, investigating the molecular signature of CTCs can provide essential information on the influence of cellular heterogeneity on the metastatic cascade, such as the phenotypic evolution of CTCs to survive hostile circulatory stresses [3], evade immune responses [4], become dormant [5], or accelerate metastatic growth [6], which can enhance prognoses and uncover therapeutic targets. In the clinical setting, sampling CTCs from peripheral blood provides a noninvasive alternative to tissue biopsies for epithelial cancers [7], which can facilitate clinical trials by monitoring patient responses to
treatments via serial monitoring [8, 9]. On the other hand, harvesting CTCs for ex vivo culture can enhance screening practices to develop personalized therapeutics [10].

Although liquid biopsies are a promising avenue in oncology, CTCs are rare among the erythrocytes and leukocytes present in the blood [11]. Therefore, techniques to sort and enrich CTCs are warranted for exploratory studies, therapeutic screenings, and diagnostic developments. Microfluidic devices are encouraging candidates to selectively sort CTCs from whole blood given the highly controllable and reproducible conditions microsystems provide [12]. A common microfluidic method for CTC enrichment is the separation of tumor cells from non-tumor cells through physical properties via inertial forces [13, 14]. Although microfluidic technologies based on physical separation ensure high viability, a hindrance in translation stems from the fact that the sizes of cells are temporally dependent on the cell cycle [15], CTCs are highly heterogeneous [16, 17], continual shear stresses can promote the survival of certain phenotypic subpopulations [18], and sorting efficiencies tend to be low. Capturing viable CTCs based on molecular features through positive immunoselection results in greater efficiencies [19], which can be significantly enhanced by overcoming the laminar flow regime imposed by microfluidic devices by inducing microvortices with a herringbone design [20]. The capture efficiency can be further enhanced by providing three-dimensional nanostructures to increase the surface area of capture [21].

Herein, we provide a facile protocol to enrich and release viable CTCs for ex vivo culture through positive immunoselection and temperature-dependent release [22]. The CTCs are discriminated from non-tumor cells by decorating the microfluidic device through layer-by-layer (LbL) assembly with antibodies against upregulated antigens on tumor cells, such as epidermal growth factor receptor (EGFR), epithelial cell adhesion molecule (EpCAM), and human epidermal growth factor receptor 2 (HER2). The use of a herringbone design for the microfluidic device and the addition of nanoparticles allows for increased efficiency in capturing CTCs. On the other hand, the gentle release of the immobilized CTCs via the temperature-dependent dissociation of gelatin incorporated in the nanoassembly at physiological temperatures allows for highly efficient and viable harvesting of CTCs. The microfluidic device was optimized for universal capture and validated by simulating breast and prostate CTCs by spiking SKBR3 and PC3 cells into whole blood, where efficiencies of 94.3 ± 4.39% and 88.2 ± 3.81% and viabilities of 84.1 ± 4.21% and 81.9 ± 4.62% were obtained, respectively.
2 Materials

2.1 Solution Preparation

1. Solutions for cleaning: acetone, isopropanol, ethanol, phosphate-buffered saline (PBS).

2. Gelatin-biotin solution: functionalize a 4% gelatin from bovine skin (~ 225 g Bloom, Type B) diluted in PBS with EZ-Link™ Sulfo-NHS-Biotin according to the manufacturer’s instruction at a mass ratio of 3.5:1, respectively. Adjust the pH to 7.4 and allow the solution to react at room temperature for 2 h. Dialyze the solution for 48 h against distilled water (DI) at a volume ratio of 1:1000, respectively. Replace the water every 12 h. Freeze dry the purified solution for 1 week. The solution can be kept at 4 °C for long-term storage.

3. Biotinylated antibodies: biotinylate the desired antibodies with the EZ-Link™ Micro Sulfo-NHS-Biotinylation Kit according to the manufacturer’s instructions.

4. Antibody buffer: 1% bovine serum albumin (BSA) diluted in PBS.

5. Blocking solution: 3% BSA and 0.05% polyoxyethylene sorbitan monolaurate (Tween® 20) diluted in PBS.

2.2 Microfluidic Fabrication

1. Equipment: vacuum oven, oxygen plasma chamber, vacuum pump, programmable spin coater, programmable hot plate, UV-LED masking system, Karl Süss MJB-3 Contact Aligner, vacuum chamber, and a scale.

2. Reagents: acetone, isopropanol, SU-8 50, SU-8 25, SU-8 Developer, ethanol, (3-aminopropyl)triethoxysilane, SYLGARD™ 184 Silicone Elastomer Base, and SYLGARD™ 184 Silicone Elastomer Curing Agent.

3. Supplies: AutoCAD® to design the mask, chrome mask, 4” silicon wafer, alignment tool, Petri dishes, aluminum foil, weighing dish, plastic forks, surgical blades, wafer tweezers, adhesive tape, 1.2 mm biopsy punch, and glass slides.

2.3 Device Functionalization

1. Reagents: ethanol, PBS, gelatin-biotin, streptavidin, 90-nm streptavidin-coated polystyrene nanoparticles, antibody buffer, α-EGFR (IMC-C225; ImClone Systems, New York City, NY), α-EpCAM (AF960; R & D Systems, Minneapolis, MN), and α-HER2 (AF1129; R & D Systems).

2. Supplies: 3 mL and 10 mL Luer lock syringes, 18-gauge and 30-gauge blunt needle tips, Kimwipes, and parafilm.


2.4 Selective CTC Capturing

1. Equipment: 50 mL conical tube rocker, air compressor with a pressure line, and a programmable syringe pump.
2. Reagents: PBS, blocking solution, ethanol, and whole blood patient samples.
3. Supplies: 3 mL and 10 mL Luer lock syringes; 18-gauge, 25-gauge, and 30-gauge blunt needle tips; Kimwipes; 15 mL and 50 mL conical tubes; 0.02″ inner-diameter Tygon tubing; custom-made snorkel cap; 22 mm medium/hard O-rings; custom-made stabilizer; custom-made compression fittings; BD Vacutainer® Safety-Lok™ blood collection sets; and BD Vacutainer® EDTA Tubes.

2.5 Release of Viable CTCs

1. Equipment: programmable syringe pump, programmable hot plate, and a Nikon® Ti2 microscope.
2. Reagents: PBS.
3. Supplies: 10 mL Luer lock syringe, 25-gauge blunt needle tips, glass slides, ProPlate® Multi-Well Chamber, and ImageJ for data analysis.

3 Methods

3.1 Microfluidic Fabrication

Before fabricating microfluidic devices, sanitize all workspaces with 70% ethanol. Furthermore, ensure all instruments are particle-free by introducing a nitrogen gas airflow in an outward spiraling direction.

3.1.1 Two-Layer Herringbone Master Mold Fabrication

The following processes should take place within a cleanroom or inside a particle-free fume hood.

1. Wet the surface of the 4″ silicon wafer with acetone and subsequently with isopropanol, and dry the surface with nitrogen gas.
2. Dehydrate the wafer in the oven for 30 min at 200 °C.
3. Turn on the plasma chamber, open the valve to the oxygen tank (ensure the regulator is set to 6 psig), and turn on the vacuum pump.
4. Set the plasma time to 1 min and begin the plasma treatment.
5. During the gas stabilization phase, set the flow rate of oxygen to at most 10 cc/min. Note that once the plasma treatment begins, a blue hue should appear, which confirms that the plasma treatment is effective.
6. Center the wafer onto the spin coater with the plasma-treated side facing upward using the alignment tool, and deposit enough SU-8 50 to cover approximately two-thirds of the surface without introducing any bubbles.
Fig. 1 Schematic of microfabrication. (a) The first layer of SU-8 is exposed to UV light to photopattern the microchannel. (b) The second layer of SU-8 is exposed to UV light to structurize the herringbone features on the microchannel. (c) The master mold is fabricated and can be used repeatedly for soft photolithography. (d) The PDMS mixture is poured onto the master mold. (e) After crosslinking the mixture, the PDMS retains the negative structure of the master mold with the herringbone design. (f) The micropatterned PDMS is bound to a glass slide to complete the microfluidic device. (g) A scanning electron microscopic (SEM) image demonstrates the distinct microstructures on the PDMS.

7. Spin the wafer for 40 s at 300 rpm to resist, 15 s at 770 rpm to spread, and 30 s at 2650 rpm to spin. The setting should result in a layer with a thickness of 50 ± 4 μm.

8. Prebake the wafer at 65 °C for 20 min.

9. Ramp up the temperature to 100 °C at a rate of 1 °C/min, and bake the wafer for 55 min once the temperature reaches 100 °C.

10. Allow the wafer to cool down to room temperature after baking.

11. Center the wafer in the UV masking system, and place the particle-free chrome mask facing downward, and set the substrate distance to 500 μm (the wafer is 450 μm and the desired height is 50 μm) with a separation distance of 10 μm.

12. Expose the wafer to 11 mW/cm² of 365-nm light for eight exposures at 10-s intervals for 4 s (Fig. 1a).

13. Bake the wafer at 65 °C for 3 min and subsequently at 100 °C for 7 min.

14. As previously described, deposit SU-8 25 onto the wafer and spin the wafer for 5 s at 600 rpm to spread and 30 s at 1250 rpm to spin. The setting should result in a layer with an additional thickness of 40 ± 4 μm.
15. Prebake the wafer at 65 °C for 20 min, ramp up the temperature to 100 °C at a rate of 1 °C/min, bake the wafer for 15 min once the temperature reaches 100 °C, and allow the wafer to cool down to room temperature after baking.

16. Align the wafer to the previous design with a Karl Süss MJB-3 Contact Aligner.

17. As previously described, expose the wafer to 11 mW/cm² of 365-nm light for ten exposures at 10-s intervals for 4 s (Fig. 1b).

18. Bake the wafer at 65 °C for 3 min and subsequently at 100 °C for 7 min.

19. Immerse the wafer into SU-8 Developer for 15 min.

20. Rinse the wafer with isopropanol. If the SU-8 becomes opaque, the development is not complete in which case the wafer should be reimmersed in the developer.

21. Bake the wafer at 65 °C for 3 min, ramp up the temperature to 100 °C at a rate of 1 °C/min, and bake the wafer for 15 min once the temperature reaches 100 °C.

22. Coat the developed master mold with (3-aminopropyl) triethoxysilane through chemical vapor deposition overnight by placing 200 μL into an aluminum foil-covered Petri dish within a vacuum chamber.

23. The master mold is complete (Fig. 1c) and can be reused (see Note 1).

3.1.2 Poly(Dimethylsiloxane) (PDMS) Preparation

1. Pour 20 g of the elastomer base and 2 g of the curing agent for each master mold onto a weighing dish. The weights can be changed but must follow a 10:1 ratio of the elastomer base to the curing agent.

2. Mix the elastomer mixture with a particle-free fork until a uniform milky-white consistency is achieved (~ 5 min).

3. Carefully pour the well-mixed elastomer mixture (PDMS) onto the master mold (Fig. 1d).

4. Place the silicon PDMS-covered master mold in a vacuum chamber, and degas the PDMS for at least 45 min or until no bubbles remain.

5. Turn off the pump and gradually allow the vacuum chamber to equilibrate to atmospheric pressure.

6. Remove the remaining bubbles and apparent particles with a particle-free fork.

7. Place the PDMS-covered master mold in the oven at 65 °C for no less than 10 h. Usually, this step is performed overnight.
8. Once fully cured, cut the PDMS with a surgical blade in one continuous circular motion. Failing to adhere to the technique may result in scratching of the wafer.

9. Slowly and gently remove the cut out region of the PDMS with wafer tweezers to avoid tearing the PDMS or the underlying SU-8 structures. The PDMS will retain the negative micropattern of the SU-8 structures (Fig. 1e).

10. Place the micropatterned side of the PDMS facing downward, and cut the PDMS into individual devices. Avoid touching the micropatterned side. If needed, immobilize the surrounding area of the PDMS using strips of adhesive tape.

11. Flip the PDMS so that the micropatterned surface is facing upward, and punch holes at the inlets and outlets of each device with a 1.5 mm biopsy punch.

12. Remove any residual fragments on the PDMS micropatterned surface using adhesive tape.

13. Inspect and clean the silicon wafer dish with nitrogen gas, and pour new PDMS in the wafer dish following steps 1–7 for long-term storage or producing new devices.

3.1.3 Device Bonding

1. Sonicate an equal number of glass slides corresponding to the number of PDMS devices in 70% ethanol for 5 min, followed by sonication in DI water for 5 min. Repeat the cleaning steps a total of three times. Dry the glass slide with nitrogen gas and store the clean slides in a particle-free container.

2. Preheat the hot plate to 65 °C.

3. Open the plasma chamber, and place the glass slides and PDMS segments facing upward.

4. As previously described, set the plasma time to 1 min and begin the plasma treatment.

5. Upon completion of the plasma treatment, open the chamber, carefully remove the glass slide, and place it on a clean surface with the plasma-treated side facing upward.

6. Similarly, carefully remove the PDMS without touching the micropatterned surface, and place the PDMS onto the surface of the glass with the micropatterned side facing downward. If any air bubbles are present, gently press on the PDMS. Excessive force may interfere with the natural bonding process.

7. Heat the device on the hotplate at 65 °C for 5 min to strengthen the bond. Note that pre-functionalized glass can be used as well to fabricate devices, whereby plasma treatment of 15 s and a temperature of 45 °C for 20 min is recommended [23].
8. Store the fully bonded microfluidic devices in a particle-free glass container for future use (Fig. 1f).

This two-layer microfluidic fabrication protocol results in a sharp herringbone design (Fig. 1g) that increases the turbulence within the microfluidic channel to increase the surface contact of CTCs.

3.2 Device Functionalization

3.2.1 Lbl Nanocoating

1. Pre-wet the inlet and outlet of the device by introducing ethanol using a syringe with a 30-gauge blunt needle until droplets of ethanol form on the top of the holes. Remove all bubbles within the ethanol.

2. Exchange the needle tip for an 18-gauge blunt needle tip, produce a small droplet of ethanol on the tip, and insert the tip into the inlet so that both droplets coalesce. The liquid-to-liquid connection helps avoid bubbles within the device.

3. Prime the device with 400 \( \mu \text{L} \) of 100% ethanol at \( \sim 1 \text{ mL/min} \). Harsh and fast handling may detach the PDMS from the glass. Use a Kimwipe to wipe the excess liquid exiting the device, and monitor the flow through the device. Meanwhile, check for leaks on the sides of the device or the formation of bubbles within the device.

4. Flush the device with 400 \( \mu \text{L} \) of PBS into the inlet following steps 1–3.

5. Introduce 400 \( \mu \text{L} \) of gelatin-biotin into the inlet and the outlet following steps 1–3, and incubate the device for 15 min at room temperature (see Note 2). Ensure the presence of droplets on the inlet and outlet to avoid drying during the incubation period.

6. Flush the device with 400 \( \mu \text{L} \) of PBS into the inlet following steps 1–3 to remove the excess gelatin-biotin.

7. Introduce 400 \( \mu \text{L} \) of 100 \( \mu \text{g/mL} \) streptavidin into the inlet and the outlet following steps 1–3, and incubate the device for 15 min at room temperature while ensuring the presence of droplets on the inlet and the outlet.

8. Flush the device with 400 \( \mu \text{L} \) of PBS into the inlet following steps 1–3 to remove the excess streptavidin.

9. Repeat steps 5–8 until a total of four layers of gelatin-biotin functionalization are complete (the top layer should be gelatin-biotin). Note that gelatin-biotin and streptavidin are introduced through both the inlet and outlet during each functionalization layer to ensure the homogenization of the layer throughout the device. Flush PBS through the inlet only throughout the process.
10. Introduce 400 μL of 90-nm streptavidin-coated nanoparticles into the inlet following **steps 1–3**, and incubate the device for 15 min at room temperature.

11. Reintroduce 400 μL of 90-nm streptavidin-coated nanoparticles into the outlet following **steps 1–3**, and incubate the device for 15 min at room temperature.

12. Flush the device with 400 μL PBS into the inlet following **steps 1–3** to remove the excess streptavidin-coated nanoparticles, and fill the device with PBS for storage.

13. Store the functionalized devices in a hydrated compartment with a wetted Kimwipe to maintain humidity, and seal with parafilm at 4 °C for future use or immediately start the antibody functionalization.

### 3.2.2 Antibody Functionalization

1. For single antibody use (e.g., α-EpCAM alone), the biotinylated antibody concentration is 20 μg/mL within the antibody buffer. For an antibody cocktail (e.g., a mixture of α-EpCAM, α-HER2, and α-EGFR), the biotinylated antibody concentration is 10 μg/mL for each antibody within the antibody buffer. Do not vortex the antibody solution to avoid the formation of bubbles and to protect the quality of the antibodies, but gently mix the solution with a pipette.

2. Flush the device with 400 μL of PBS into the inlet as previously described.

3. Cut a 200 μL pipette tip at a ~ 45° angle using a blade. The angle facilitates the insertion and extrusion of fluid into the microfluidic device.

4. Slowly load 200 μL of the antibody solution into the inlet hole of the device using the precut tip. Leave half of the volume inside the tip before releasing the tip from the pipette, and incubate the device for 30 min at room temperature. The antibody solution will be in constant motion due to hydrodynamic forces.

5. Gently reinstall the pipette onto the tip and slowly inject the remaining volume into the device.

6. Load 200 μL of the antibody solution into the outlet following **steps 3–5**.

7. Flush the device with 400 μL of PBS into the inlet as previously described to remove the excess antibodies, and fill the device with PBS for storage.

8. Store the functionalized devices in a hydrated compartment with a wetted Kimwipe to maintain humidity, and seal with parafilm at 4 °C for up to 72 h or immediately process blood.
3.3 Selective CTC Capturing

3.3.1 Device and Sample Preparation

1. Equilibrate all working solutions to room temperature (see Note 2).

2. Flush the device with 400 μL of PBS into the inlet as previously described.

3. Introduce at least 250 μL of the blocking solution via the inlet and the outlet, and incubate for a minimum of 60 min at room temperature (or overnight at 4 °C).

4. Punch a hole on a 15 mL conical tube lid using tweezers for waste collection.

5. Cut two pieces of Tygon tubing for the inlet and the outlet at ~45 ° angles using a blade.

6. Wipe the outside of the tubing with a Kimwipe containing 70% ethanol to remove any external debris, and flush the blocking solution through the tubing to remove any internal debris.

7. Prepare the particle-free snorkel cap by adding two O-rings to the sides, threading the inlet tubing through the stabilizer via the 45 ° side, attaching the stabilizer to the compression fitting, and twisting it into the snorkel cap (Fig. 2a). These materials can be reused (see Note 3).

3.3.2 Whole Blood Processing

1. Blood specimens were drawn with a BD Vacutainer® Safety-Lok™ blood collection set via standard phlebotomy and were collected in BD Vacutainer® EDTA Tubes. Note that the samples were processed within an hour; however, samples can be stored for at most 12 h at 4 °C (see Note 4).

2. Transfer 3.5–5 mL of whole blood into a 50 mL conical tube. Note that the capacity of the snorkel cap-sealed conical tube is 9 mL.

3. Slowly mix the blood by pipetting up and down at the slowest setting. Mixing vigorously can decrease the capture efficiency and increase nonspecific binding by producing cell debris.

4. Seal the 50 mL conical tube containing blood with the assembled snorkel cap at an angle to avoid getting blood into the pressure line.

5. Place the snorkel cap-sealed conical tube into the rocker assembly, and attach the pressure lines to the respective fitting.

6. Attach the 45 ° side of the inlet tubing that was threaded through the stabilizer and the compression fitting into the inlet.

7. Introduce the blocking solution through the outlet and allow the solution to navigate through the inlet tubing and into the snorkel cap until a droplet of the solution is observed within the snorkel cap-sealed conical tube.

8. Slowly invert the 50 mL conical tube so that the tubing is facing downward (Fig. 2b), and place on a 50 mL conical tube rocker.
Fig. 2 Assembly and processing of whole blood. (a) Separate custom-made components are required to introduce whole blood into the microfluidic device. (b) The individual pieces are assembled to constitute the snorkel cap-sealed conical tube, which is rocked and pressurized to introduce whole blood into the microfluidic device. (c) A photograph of the transparent microfluidic device displays the whole blood that is processed. (d) The surfaces of the microfluidic device are functionalized with four alternating layers of gelatin (yellow) and streptavidin (blue) via LbL assembly with biotin (brown) as indicated by the dotted line. Polystyrene nanoparticles (green) conjugated with streptavidin are deposited onto the top layer of the LbL assembly, which attach antibodies against tumor-specific antigens (pink). (e) The CTCs are immobilized onto the microfluidic surface via interactions with the antibody and the CTC antigens (orange). (f) The CTCs attached to the nanoassembly are released with the dissociation of gelatin.

9. Activate the 50 mL conical tube rocker and set the pressure to 0.1 psig via the air compressor. Ensure air bubbles do not form within the tubing. If so, re-prime the device with the blocking solution following step 7. Note that once the blood runs into the device, the device cannot be re-primed as the shear stresses on the cells will generate cell debris.

10. Connect the 45° side of the outlet tubing to the outlet and through the hole punched into the 15 mL conical tube lid until the middle of the conical tube is reached. This step should be performed once the blood starts to flow through the tubing but prior to entering the device.

11. Adjust the flow rate to 1.25 mL/h by changing the pressure on the air compressor or by changing the height differential with the outlet tubing for more subtle changes.

12. Allow the sample to run through the device for 4 mL.
13. Once the sample is processed, turn off the air compressor, remove the pressure line, turn off the 50 mL conical tube rocker, rotate the 50 mL conical tube so that the inlet tubing is pointing upward, and remove and disassemble the snorkel cap.

14. Connect a syringe with PBS to Tygon tubing using a 25-gauge blunt needle tip. Employing the liquid-to-liquid connection, insert the tubing into the outlet of the device, and flush 2.5 mL of PBS at a flow rate of 2.5 mL/h as controlled by a syringe pump to wash out residual cells.

The transparent microfluidic device will turn red during sample processing (Fig. 2c). Within the confines of the microchannel, CTCs that encounter the nanoassembly (Fig. 2d) adhere via antibody-antigen interactions and are immobilized (Fig. 2e).

### 3.4 Release of Viable CTCs

1. Preheat the hot plate to 37 °C.
2. Cut a piece of Tygon tubing at a ~ 45 ° angle and insert it into the outlet of the device.
3. Set the device onto the hot plate for 10 min.
4. As previously described, use a syringe pump to flow PBS into the device at a flow rate of 1.5 – 2.5 mL/h and collect the CTCs.
5. The CTCs collected from the microfluidic device were centrifuged for 5 min at 1000 rpm to separate the cells from surface functionalization reagents (e.g., gelatin, nanoparticles, antibodies), plated on 16-well ProPlate® Multi-Well Chamber attached to a glass slide, imaged with a Nikon® Ti2 microscope, and analyzed with ImageJ.

The increase in temperature dissociates the gelatin in the LbL assembly, which disrupts the entire surface chemistry and releases the viable CTCs into suspension (Fig. 2f).

### 3.5 Results

The following results were obtained to demonstrate the utility of the microfluidic device by simulating the capture of breast and prostate CTCs by spiking SKBR3 and PC3 cells into whole blood, respectively.

#### 3.5.1 Selective CTC Capturing

Tumor cells were captured on the microfluidic with relatively minimal remnants of nonspecific leukocyte adhesion as rendered with the immunofluorescence of cytokeratin, a biomarker for CTCs originating from epithelial cancers, and CD45, a biomarker expressed on nucleated hematopoietic cells (Fig. 3a). The tumor cells were immobilized and adhered to the nanoassembly upon interaction with the microstructures (Fig. 3b). Most of the tumor cells within the blood were captured, lending efficiencies of 94.3 ± 4.39% for SKBR3 and 88.2 ± 3.81% for PC3 (Fig. 3c). Furthermore, the specificity of the nanoassembly to sort tumor cells
was demonstrated with positive predictive values (PPVs) for cell capture of 48.7 ± 5.01% for SKBR3 and 49.1 ± 2.24% for PC3 (Fig. 3d).

3.5.2 Release of Viable CTCs

Cells that were released by dissociating the nanoassembly in the microfluidic device were mainly tumor cells as demonstrated with cytokeratin and CD45 (Fig. 4a). The released cells could be expanded under cell culture conditions (Fig. 4b). A majority of the cells that were captured were released with efficiencies of 94.6 ± 1.65% for SKBR3 and 95.0 ± 1.86% for PC3. Furthermore, the cells that were released were highly viable with cellular viabilities of 84.1 ± 4.21% for SKBR3 and 81.9 ± 4.62% for PC3.
Fig. 4 Release of viable tumor cells from the microfluidic device. (a) SKBR3 cells are released from the microfluidic device, where cytokeratin is stained in red, CD45 in green, and DAPI in blue. The arrows indicate the presence of leukocytes. (b) The enriched SKBR3 cells are viable and expand after 10 days of culture. (c) Of the captured tumor cells, the SKBR3 and PC3 released from the microfluidic device provide efficiencies of $94.6 \pm 1.65\%$ and $95.0 \pm 1.86\%$, respectively ($n = 4$ for SKBR3 and $n = 3$ for PC3, the error bars indicate the standard deviation). (d) The viability of the released cells is $84.1 \pm 4.21\%$ and $81.9 \pm 4.62\%$, respectively ($n = 4$ for SKBR3 and $n = 3$ for PC3, the error bars indicate the standard deviation).

4 Notes

1. While much of the protocol can be performed in any lab, the master mold fabrication requires more advanced machinery that many labs may not possess. However, the master mold fabrication is not routinely performed and is included for completeness.

2. When referring to room temperature, please ensure that the temperature does not exceed $37 \degree C$ as the gelatin coating dissociates at $37 \degree C$ and all LbL will be compromised.

3. All reusable material considered a biohazard should be sterilized after the conclusion of an experiment with 70% ethanol.

4. Given the necessity to process whole blood to capture CTCs, the described experimental protocol can only be performed in labs that adhere to Biosafety Level 2 (BSL2).
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Chapter 6

Single-Response Electronic Tongue and Machine Learning Enable the Multidetermination of Extracellular Vesicle Biomarkers for Cancer Diagnostics Without Recognition Elements

Caroline Y. N. Nicoliche, Giulia S. da Silva, Leticia Gomes-de-Pontes, Gabriel R. Schleder, and Renato S. Lima

Abstract

Platforms based on impedimetric electronic tongue (nonselective sensor) and machine learning are promising to bring disease screening biosensors into mainstream use toward straightforward, fast, and accurate analyses at the point-of-care, thus contributing to rationalize and decentralize laboratory tests with social and economic impacts being achieved. By combining a low-cost and scalable electronic tongue with machine learning, in this chapter, we describe the simultaneous determination of two extracellular vesicle (EV) biomarkers, i.e., the concentrations of EV and carried proteins, in mice blood with Ehrlich tumor from a single impedance spectrum without using biorecognizing elements. This tumor shows primary features of mammary tumor cells. Pencil HB core electrodes are integrated into polydimethylsiloxane (PDMS) microfluidic chip. The platform shows the highest throughput in comparison with the methods addressed in the literature to determine EV biomarkers.

Key words Nonselective sensor, Impedance, Artificial intelligence, Breast cancer, Point-of-care, Accuracy

1 Introduction

On-field and mass testing kits play a pivotal role at the front line to combat epidemics and pandemics as they decentralize diagnostics from clinical laboratories and provide comprehensive feedback on the health of a large number of individuals, thus decreasing the population infection risks and assisting the decision-makers against virus spread [1]. Besides, these devices can provide in-home monitoring of chronic diseases and medical assays in resource-poor settings, further contributing for lifesaving procedures by remotely providing physicians with rapid physiological data, hence aiding...
clinical prognoses, early treatments, and personalized medicine [2, 3].

While biosensors are attractive for point-of-care assays, the recognition elements imply some drawbacks against their translation into daily practice. First, these biomolecules (e.g., proteins and antibodies) usually require a complex and costly synthesis, being further limited when produced on a large scale [4]. Second, the recognition elements can undergo denaturation, which impairs their long-term robustness and capability for specifically recognizing the targets, with the ensuing incidence of false negatives and positives [5–7]. To date, the poor stability of the electrode-anchored biomolecules (usually until 3 days when stored at 4 °C in electrolyte [8]) along with their complex and low-yield synthesis has contributed to impair the commercialization and clinical adaptation of biosensors due to drawbacks such as improper handling, transportation, and storage and high cost [6].

In this chapter, we demonstrate that low-cost, fast, accurate, and scalable diagnostics can be achieved by converging an impedimetric electronic tongue (nonselective sensing array) with machine learning (ML) for providing the simultaneous determination of two extracellular vesicle (EV) biomarkers, the concentrations of EV (C_V) and carried proteins (C_P) [9]. EVs are lipid nanoparticles (50 to 200 nm in diameter) released by cells into circulation, being a frontier type of circulating biomarkers for the diagnosis and prognosis of diseases such as cancer and Alzheimer’s disease [10]. Such lipid structures are a powerful alternative to improve the clinical accuracy as they afford a plethora of biomarkers, including the quantity of EVs themselves and the content of EV-attached proteins, which either are inherited from parent cells or occur as membrane-bound molecules [11].

After isolation of the EVs (10 min) using a low-cost technique (<$9.00 per sample) through size exclusion chromatography, blood samples have been analyzed by an impedimetric microfluidic device (<6 min) bearing ready-to-use and cross-reactive probes. Broadly available at a low-cost, scholar-pencil graphite cores (HB scale) have been reversibly inserted into single-piece polydimethylsiloxane (PDMS) microfluidic chips and acted as parallel electrical double-layer capacitors. In contrast to traditional electronic tongues [9], our fingerprints have been recorded from a single scan of differential capacitance (C_d) because of the chemical diversification of the frequency-function impedance responses (Bode plots) and heterogeneous areas and composition of the graphite cores [9, 12]. Three parameters govern the sensor capacitive outcomes, namely, the electrical polarization of charged entities in the electrical double-layer (<10^2 Hz), the electrode material properties (10^2 to 10^4 Hz), and the geometric or solution capacitance (C_S) as the charged particles are not subjected to the action of electric at very high frequencies (>10^5 Hz) [13, 14]. C_S varies with
dimensional parameters of the electrodes (area and gap) and with the sample dielectric constant. These characteristics along the range of applied frequencies show the diversified nature of the Bode plots as aforesaid.

Concentrations of both EVs ($C_V$) and extravasicular proteins ($C_P$) can be attained from a single impedance spectrum with the aid of simple mathematical equations fitted by the supervised sure independence screening and sparsifying operator (SISSO), without using recognition elements. As advantages, SISSO assures accurate predictions from simple mathematical equations and a small number of training samples. In practice, the $C_d$ data (primary features) as a function of the ac voltage frequency are converted into low-dimensional descriptors with only a few features. This ML method thus favors the deployment of ML-aided sample-to-answer systems on mobile phones, facilitating detection at the point-of-care because no result interpretation and data treatment by the user are required. Our data further show the potentiality in employing ML for smartly picking up features from whole sensing spectra to improve the analytical performance when compared with conventional routines using univariate responses.

Assuming the data by the nanoparticle tracking analysis (NTA) and Bradford assay methods as the true (expected) results, the mean absolute error (MAEs) were found out to be $0.2 \times 10^8 \text{ P mL}^{-1}$ and $1.0 \mu g \mu L^{-1}$ for $C_V$ and $C_P$, respectively. These analyses were performed in mice blood samples bearing Ehrlich tumor that is a neoplasm from female mice breast carcinoma, showing major features of mammary tumor cells.

2 Materials

Prepare all solutions employing ultrapure water (deionized water obtained with resistivity of $0.48 \text{ M}\Omega \text{ cm}$) and analytical grade reagents. Prepare and store all reagents at room temperature (the exceptions are indicated along the chapter). Waste disposal regulations should be thoroughly followed. After opening the molecular exclusion chromatography column for EV isolation, you should clean it to remove the sodium azide that derives from the column storage. Follow diligently the manufacturer’s instructions for cleaning before use. Afterward, add the blood sample and make the buffer elution.

2.1 Device Microfabrication

1. Preparing 15 g of poly(dimethylsiloxane) (PDMS): monomer in the curing agent.

2. Scaffold for the electrode channels: 3D-printed cylinders in poly(lactic acid) (PLA) with diameter of 2.0 mm shaped by an extrusion printer of fused deposition modeling.
3. Scaffold for the sample channels: stainless-steel capillaries with 700.0 μm diameter.

4. Pool-like pieces, i.e., rectangular hollow pieces, to accommodate PDMS: two 3D-printed rectangular pieces of PLA with 19 mm × 59 mm × 15 mm (intern dimensions) with (i) five lateral 2.0 mm holes each side to fit the PLA cylinders (electrode channel scaffolds) and (ii) two openings to insert the stainless-steel capillary in opposed side (sample channel scaffold).

5. Electrodes: scholar HB graphite cores with 1.7 mm diameter (provided by Faber-Castell, São Carlos, Brazil). These cores show an intermediate graphite-to-clay (G/C) ratio in relation to the extreme cases, 9H and 9B, of the HB scale (see Note 1).

### 2.2 EV Isolation from Blood Samples

1. Commercial exclusion chromatography columns packed with porous polysaccharide resin: store at 20 °C.

2. Centrifuge with rack suitable for tubes containing 1.5 mL of blood.

3. Phosphate buffered saline (PBS) buffer.

4. Filter paper with 0.22 μm pores (see Note 2).

### 2.3 EV Electrochemical Analysis

1. Plastic syringes to manually insert the samples in the microfluidic chip.

2. Potentiostat to perform impedance analyses at stationary conditions.

3. 0.1 mmol L⁻¹ KCl solution, used to confirm the absence of contamination on the probes.

4. Vesicle samples: dilute the sample 1:3 v/v in deionized water.

### 2.4 Machine Learning Analyses

1. Principal component analysis (PCA): implemented in the scikit-learn software using python programming language [23].

2. SISSO: software implemented in the FORTRAN programming language [24].

### 3 Methods

Perform all procedures at room temperature unless otherwise specified. Regarding the machine learning (ML) method, we follow a general workflow, evaluating the different components required (see Note 3).
3.1 Device Microfabrication

1. Deposit photoresist (AZ® 50XT) on a stainless-steel capillary by dipping it into the photoresist and then performing centrifugation at 2000 rpm for 10 s three times, followed by drying at room temperature for 20 min (see Note 4).

2. Assemble the scaffolds (Fig. 1). The 3D-printed pieces (electrode scaffolds) were placed on both below and above the photoresist-coated capillary (sample scaffold).

3. Pour the PDMS mixture (10:1 w/w monomer in the curing agent) slowly into the scaffold (see Note 5).

4. Put the system in a turned off laboratory oven and then heat it to 60 °C for 1 h to cure PDMS.

5. Allow the system for cooling to room temperature.

6. Pour acetone over the PDMS for partially removing the photoresist coated along all the stainless-steel capillary surface (see Note 6).

7. Remove manually the electrode and sample scaffolds.

8. Put the PDMS device back to the oven for a hard backing for 30 min at 120 °C.

9. Cool it down at room temperature.
**Fig. 2** Sensing principle and capacitor structure. (a) PDMS device with pairs of HB cores. The top and bottom electrodes were short-circuited utilizing copper pieces for attaining an association of five capacitors in parallel. As a result, the measured single response of $C_d$ shows chemical diversification (fingerprint-like response) as it encompasses contributions of the five individual $C_d$ outputs as indicated by the equivalent circuit on the side of the device optical image. (b) Stereoscopy image of the sample channel (horizontal channel) in contact with two electrode channels (cross sections filled with red dye). Such a structure corresponds to one double-layer capacitor of the electronic tongue array.

10. Insert 5 cm-length HB cores (electrodes) into the device above and below the sample channel (700.0 μm in diameter).

11. To make the short circuit of the five pairs of pencil cores using copper pieces to attain an association of five polarizable capacitors in parallel (Fig. 2), each one comprised of a pair of HB pencil cores above and below the sample channel (see Note 7).

### 3.2 EV Isolation from Blood Samples

1. Centrifuge samples prior to loading on the column (see Note 8).

2. Centrifuge samples at 1500 rpm for 10 min to remove any cells and large particles.

3. Gently move the supernatant to a new tube and centrifuge again at 10,000 rpm for 10 min.

4. For EV isolation, use lower g-forces over the second centrifugation step.

5. Reserve 750 μL of blood sample to run into the column.

6. Prepare the PBS solution at 7.4 pH: add 800 mL of distilled water in a suitable container, 8.000 g of sodium chloride, 0.200 g of KCl, 1.440 g of sodium phosphate dibasic, and 0.245 g of potassium phosphate monobasic to the solution. Next, add distilled water until the total solution volume is 1 L. Store this solution at 20 °C.

7. Add 3 mL of PBS into the exclusion chromatography column.
8. Allow the blood sample (750 μL) to run into column. The column will stop flowing when all of the sample has entered the loading frit.

9. Top up the column with PBS solution (see Note 9).

10. Collect the initially added volume of PBS (3 mL).

11. Then, start collecting the EV with fractions in 500 μL microtubes.

12. Discard fractions 1 (500 μL) and 2 (500 μL).

13. Collect fraction 3 (500 μL) for the analyses using the electronic tongue (see Note 10).

### 3.3 EV Electrochemical Analysis

1. Accomplish the measurements at room temperature.

2. Clean the microfluidic device with the aid of plastic syringe using 3 mL of ethanol and then 3 mL of deionized water.

3. Insert 0.1 mmol L$^{-1}$ KCl solution into the sensor through plastic syringe.

4. Perform impedimetric assays applying sine wave voltage signal amplitude (peak-to-peak potential) of 25 mV ac and integration time of 2 s at 100 Hz. Wait until the impedance signal stabilizes, i.e., approximately 3 min. Start the spectrum mode analyses ($n = 8$) with frequencies ranging from $10^0$ to $10^6$ Hz to achieve Bode plots (complex impedance vs. frequency).

5. Clean the chip with 3 mL of deionized water.

6. Add the sample into the sensor using plastic syringe. The device was applied to 12 samples of mice blood with [6] and without [6] Ehrlich tumor.

7. Perform impedimetric assays adopting the same prior conditions.

8. For each sample change, a new cleaning with water must be previously conducted.

9. Analyses of the KCl solution between measurements to the samples can be made to check any possible electrode fouling by observing variations in the impedances achieved for KCl. No significant changes have been obtained in our group.

10. Save and convert the impedances into capacitance values using equation relative to polarizable electrodes, with a negligible polarization resistance [9].

### 3.4 Machine Learning Analyses

1. Define the target property to be predicted. In this case, we train supervised regression machine learning models for multioutput predictions of both $C_V$ and $C_P$ (Fig. 3).

2. Obtain and organize the available data in a computer-readable table format. The data table has dimensions of $N x (T + F)$,
where $N$ is the number of examples (samples), $T$ is the number of target properties, and $F$ is the number of input features. We have two target properties, and as data features, we use measurements of differential capacitances measured at 15 different frequencies, from $10^1$ to $10^6$ Hz. Thus, $T = 2$ and $F = 15$.

3. Data preprocessing and cleaning by plotting graphs of targets vs. features for checking and correcting formatting errors and data outliers.

4. Preliminary screening: normalize each feature vector, by transforming $x_{\text{normalized}} = (x - x_{\text{minimum}})/(x_{\text{maximum}} - x_{\text{minimum}})$. Then, verify if the examples are distinguishable by the available features, by using PCA. We plot the examples’ first two principal components, also evaluating if they together describe a large percentage of the dataset variance.

5. Divide the complete dataset into two subsets: one training and one test dataset at an approximately 80/20 ratio. The first will be used only to train the machine learning predictive model and, the second, only to verify the accuracy and generalization of the trained model on unseen data.

6. Select an appropriate machine learning algorithm for small to medium datasets (see Note 11). We select to train models using the SISSO method, which translates to finding the coefficients of the linear equation using the best combination of the input features discovered by the SISSO algorithm. We chose a 2D model, namely, two trainable coefficients.

7. Using the training dataset, train the model with the selected algorithm. First, estimate the training error and accuracy using the k-fold cross-validation strategy (see Note 12) for training

$$C_V = a_1 \left| \frac{(C9/C5) - (C13/C9)}{C9/C5} \right| + a_2 \left| \frac{(C8/C7) - (C13/C6)}{C13/C6} \right| + b_1$$

$$C_P = a_3 \left| \frac{(C8/C1) - (C15/C9)}{C8/C1} \right| + a_4 \left| \frac{(C9/C2) - (C14/C6)}{C9/C2} \right| + b_2$$
Fig. 4 Analysis of EVs in mice blood. Fittings of predicted vs. expected (A) $C_V$ and (B) $C_P$ from SISSO descriptors. In both the cases, the red dashed lines mean the ideal behavior, whereas their insets display average confidence intervals (units: $10^8$ P mL$^{-1}$ for $C_V$ and $\mu g \mu L^{-1}$ for $C_P$) to EVs from samples without (blue) and with the Ehrlich tumor (red). Adapted from Ref. [9] with permission from American Chemical Society (ACS)

and validation. We use fivefold cross-validation. Then, train using the complete training dataset.

8. Assess both training and test performance of the predictive models by calculating the relevant quantitative statistical metrics. We calculate the mean absolute error (MAE) and the coefficient of determination $R^2$, which measure the quality of the trained predictive model against the true data (Fig. 4).

9. Having the model achieved adequate performance, it can be employed to obtain the target properties of new data.

4 Notes

1. The G/C ratios define the core hardness in accordance with 20 HB scales, 9H up to 9B. As the core becomes softer through the increase in the quantity of graphite (higher G/C ratios as indicated by the B scale; the letter “B” designates the pencil’s mark blackness), it deposits more graphite leaving darker marks onto paper. In contrast, an increase in the amount of clay (lower G/C ratios as indicated by the H scale; the letter “H” indicates the hardness of the pencil core) creates harder cores that leave lighter marks onto paper. It is noteworthy that even cores of the same scale (our case) show heterogeneous G/C ratio and roughness, thus acting as cross-reactive sensing probes by providing distinct impedance signals (fingerprints) to each sample.

2. Filter paper dimensions depend on the used filtering bulb.
3. The creation of predictive machine learning models requires four interdependent components [25], each demanding careful examination: a) problem definition, b) data (input), c) numerical representation (descriptor), and d) algorithms, model training, validation, and finally, predictions.

4. The photoresist deposited on the stainless-steel capillary (sample scaffold) assured an adhesive contact with the 3D-printed pieces (sample scaffolds). This procedure was intended to avoid the filling of the scaffold junctions (capillary/3D-printed pieces) by PDMS, ultimately assuring the contact between sample and electrode in the final microfluidic device (see Fig. 2b).

5. To eliminate the air bubbles formed during mixing of the PDMS reagents, degas the mixture under vacuum during 20 min before its addition into the pool-like pieces with attached scaffolds.

6. This procedure allows the detachment of the stainless-steel capillary (sample scaffold) from 3D-printed pieces (sample scaffolds) in the junction regions by partially removing the photoresist coated on the capillary surface, ultimately facilitating the manual removal of the scaffolds.

7. Diversified single responses are obtained from the HB cores in contact with the sample as the equivalent $C_d$ that is attained by the impedimetric sensor (with short-circuited polarizable capacitors) covers contributions of the individual $C_d$ data.

8. To avoid clogging of the column filters, it is recommended to filter or centrifuge the biological sample to remove large particulate matter.

9. To collect accurate volumes, only load the required volume to the top of the column, wait for the volume to run through, and repeat such a protocol.

10. This routine is intended to achieve the highest concentration of EVs while showing high size homogeneity as well [26].

11. Particularly in the small to medium (approximately $<10^5$) dataset size regime, the algorithm choice must suit the problem dataset size and representation, mainly because of the number of trainable coefficients, which should be proportional to the dataset size (number of samples) [27]. Otherwise, the model will overtrain, i.e., describe only the data (including noise) from the available examples instead of learning the general pattern of the problem. SISSO is robust to overfitting due to having only a small number of coefficients to be trained, chosen by the user. It combines the input features in a nonlinear way to achieve better predictive capabilities.
12. *k*-fold cross-validation is a model validation technique for estimating the performance and generalization of models using out-of-sample testing. It divides the dataset into *k* parts and executes *k* rounds of training. Each round considers a different part as the test set for calculating performance metrics while using the other *k*-1 parts for training. The validation performance is then calculated as the average of all folds.

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Chapter 7

Functional Interrogation of Ca\(^{2+}\) Signals in Human Cancer Cells In Vitro and Ex Vivo by Fluorescent Microscopy and Molecular Tools

Chenyu Liang, Miao Huang, Mai Tanaka, Suzanne Lightsey, Madison Temples, Sharon E. Lepler, Peike Sheng, William P. Mann, Adrienne E. Widener, Dietmar W. Siemann, Blanka Sharma, Mingyi Xie, Yao Dai, Edward Phelps, Bo Zeng, and Xin Tang

Abstract

Genetically encoded calcium indicators (GECIs) and high-resolution confocal microscopy enable dynamic visualization of calcium signals in cells and tissues. Two-dimensional and 3D biocompatible materials mimic the mechanical microenvironments of tumor and healthy tissues in a programmable manner. Cancer xenograft models and ex vivo functional imaging of tumor slices reveal physiologically relevant functions of calcium dynamics in tumors at different progression stages. Integration of these powerful techniques allows us to quantify, diagnose, model, and understand cancer pathobiology. Here, we describe detailed materials and methods used to establish this integrated interrogation platform, from generating transduced cancer cell lines that stably express CaViar (GCaMP5G + QuasAr2) to in vitro and ex vivo calcium imaging of the cells in 2D/3D hydrogels and tumor tissues. These tools open the possibility for detailed explorations of mechano-electro-chemical network dynamics in living systems.

Key words  Genetically encoded calcium indicator (GECIs), In vitro and ex vivo calcium imaging, Optogenetics, Retrovirus and lentivirus transduction, 2D and 3D hydrogels, CRISPR/Cas9 gene editing, Cancer xenograft model, Mechanobiology

1 Introduction

In tumor cells, calcium (Ca\(^{2+}\)) signals regulate essential cellular activities that impact tumor progression including proliferation, metabolism, migration, epithelial-mesenchymal transition (EMT), and apoptosis [1–4]. The aberrantly elevated or reduced expression of Ca\(^{2+}\) channels and active transporters (including pumps and

Chenyu Liang and Miao Huang contributed equally to this work.
exchangers) in cancer cells has recently been reported [5–7]. However, existing data on the dynamic Ca\(^{2+}\) signals in cancer cells are still insufficient to decode the characteristics of the Ca\(^{2+}\) language to understand their genetic and biological influences. Multicolor genetically encoded Ca\(^{2+}\) indicators (GECIs) including GCaMP (green), CyCaMP (cyan), YCaMP (yellow), and RCaMP (red) have been widely used to detect cytoplasmic Ca\(^{2+}\) transients in neurons and cardiac myocytes [8], for their advantages of (1) long-term, noninvasive Ca\(^{2+}\) imaging in living systems, (2) targeted trafficking to subcellular domains, and (3) real-time measurement of Ca\(^{2+}\) dynamics without any invasive loading procedure [9–12]. Here, leveraging techniques from molecular biology [13–16] and all-optical functional interrogation [15–19], we demonstrate that stable cancer and normal cell lines that express CaViar (GCaMP5G Ca\(^{2+}\) indicator + QuasAr2 voltage indicator) can be generated for real-time monitoring of intra- and intercellular Ca\(^{2+}\) signals under commercially available fluorescent microscopy.

To investigate how the physiologically relevant biophysical and mechanical microenvironments could interact with and influence the intra- and intercellular Ca\(^{2+}\) signals in cancer cells, we employ 2D polyacrylamide (PAA) gels [20–26] and 3D polyethylene glycol (PEG) gels [27–29] with programmable mechanical stiffnesses and chemical composition. To elucidate the in vivo functions of Ca\(^{2+}\) dynamics in cancer development, we inoculate human cancer cells into nude mice to generate xenograft models and perform ex vivo Ca\(^{2+}\) imaging of tumor slices. In the following sections, we elaborate on the materials and methods used throughout the investigating processes, including production of transduced human cancer cell lines expressing genetically encoded calcium indicator (GEVI), culture and maintenance of the cell lines, preparation of 2D and 3D hydrogel substrates, in vitro calcium imaging, generation of cancer cell xenograft model, and ex vivo calcium imaging. These integrated techniques enable the systematic and quantitative dissection of physiological Ca\(^{2+}\) network in tumor cells, shedding light on its mechanistic effects on tumor progression and metastasis. These data will potentially bring insights into the development of next-generation cancer therapies to detect, suppress, and treat tumors.

## 2 Materials

### 2.1 Molecular Biology

1. Plasmid XT023 containing QuasAr2-TS-GCamP5G-ER2 (in molecular biology grade water).
2. NEB Stable Competent *E. coli* (with SOC medium (Super Optimal broth with Catabolite repression)).
3. LB-Agar (+Ampicillin) plate.
4. LB-Broth (+Ampicillin) solution.
5. 1.5 mL microcentrifuge tube.
6. 15 mL centrifuge tube.
7. Water bath device.
8. Bacterial incubator with shaker.
9. Parafilm.
10. 4 °C fridge.
11. 1000 μL/100 μL/10 μL/1 μL pipette guns and tips.
12. QIAprep Spin Miniprep Kit.
13. ZymoPURE II Plasmid Midiprep Kit.
14. Q5 High-Fidelity 2× Master Mix.
15. 100 bp/1 kb DNA Ladder.
16. Gel Loading Dye.
17. Monarch DNA Gel Extraction Kit.
18. Retroviral plasmid pBabe-puro (in molecular biology grade water).
19. Lentiviral expression plasmid (in molecular biology grade water).
20. EcoRI (in 1× NEBuffer™ EcoRI/SspI).
21. Gibson Assembly Master Mix.
22. Phoenix cells.
23. HEK 293T cells.
24. Lentiviral packaging plasmids pMD2.G and psPAX2 (in molecular biology grade water).

2.2 Cancer and Normal (Control) Cell Lines

1. Human colon adenocarcinoma HCT-8 cell line.
2. Human prostate carcinoma DU145 cell line.
3. Embryonic African green monkey kidney MA-104 cell line.

2.3 Two-Dimensional (2D) and Three-Dimensional (3D) Biocompatible Hydrogels

2.3.1 2D

1. Acrylamide.
2. N, N′-methylene bis-acrylamide.
3. TEMED.
4. Ammonium persulfate.
5. HEPES.
6. Fibronectin.

2.3.2 3D

1. PEG (Polyethylene Glycol)-DA (3.4 kDa) (see Note 1).
2. PEG-dMMP (degradable matrix metalloproteinase peptide)-PEG (made with 3.4 kDa PEG-SVA for a final MW of ~8 kDa).
3. PEG-RGD (arginine-glycine-aspartate)/PEG-RAD (made with 3.4 kDa PEG-SVA for a final MW of ~4 kDa).
4. 2-Hydroxy-4′(2-hydroxyethoxy)-2-methylpropiophenone (photoinitiator).
5. 70% ethanol.
6. Phosphate buffered saline (PBS) reagent.
7. UV light device.

2.4 Confocal Microscopy System
1. Fluorescent confocal microscope system.
2. Monochrome camera.
3. Stage-top incubator (to maintain constant temperature 37 °C, sufficient flow of CO₂, and 95% humidity).
4. Software for confocal microscope system and camera.

2.5 Inoculation of Cancer Cells into Nude Mice
1. 1 mL disposable sterile syringe.
2. 25-gauge sterile injection needle.
3. Sterile cotton swab.
4. 75% ethanol.
5. 1.5 mL centrifuge tube.
6. Wet ice.

2.6 Tumor Tissue Slicing
1. Equipment.
   (a) Electronic scale.
   (b) Digital caliper.
   (c) Vibrating blade microtome.
   (d) Forceps.
   (e) Scissors.
   (f) Razor blade.
   (g) Line pen.
   (h) Super glue.
2. Solution.
   (a) Normal saline solution (room temperature).
   (b) Pentobarbital sodium solution (1%, 100 μL/20 g, 2–8 °C).
   (c) Slicing solution (–20 °C, precool for 5–10 min to become a mixture of ice and water): NaCl 116, NaHCO₃ 26.1, KCl 5.4, NaH₂PO₄ 1.2, MgSO₄ 0.8, CaCl₂ 1.8, HEPES 25.2, and glucose 16.7 (all in mM), pH = 7.2 (NaOH adjusted).
3 Methods

3.1 Production of Retrovirus and Lentivirus Encoding CaViar Components (GCaMP5G and QuasAr2) and Transduction of Human Cancer Cells

3.1.1 Plasmid Isolation

Goal

To isolate (from bacteria) and amplify XT023 plasmid.

Steps

Day 0: Heat Shock Transformation Protocol; start this after 3 p.m

1. Pre-warm SOC medium at room temperature and LB-Agar (+Amp) selective plates at 37 °C for 1 h.
2. Thaw E. coli on ice (about 10 min) and mix the cells by flicking (see Note 2).
3. Transfer 50 μl of E. coli and 2 μl of plasmid to a chilled 1.5 ml microcentrifuge tube. Mix by flicking the tube four to five times.
4. Place the mixture on ice for 30 min.
5. Heat shock in a water bath at exactly 42 °C for exactly 30 s. DO NOT MIX.
6. Place on ice for 5 min. DO NOT MIX.
7. Add 950 μL of pre-warmed SOC medium to the cell/plasmid mixture.
8. Shake vigorously (250 rpm) or rotate at 37 °C for 1 h in a bacteria incubator with shaker.
9. Plate 100 μL transformed E. coli onto a pre-warmed LB-Agar (+Amp) plate. Also make dilutions of 1:10 and 1:100 using SOC medium.
10. Incubate plates overnight (no longer than 16 h) at 37 °C in a bacterial incubator.

Day 1

1. First thing in the morning, take out the plates and apply parafilm to the edges to seal the plates. Store the plates at 4 °C until use.
2. In the afternoon, pick five colonies from each plate and inoculate each colony in 5 mL LB-Broth (+Amp). Use 200 μL pipette tips to pick colonies, and then drop it into the 15 mL centrifuge tube containing LB-Broth (+Amp).
3. Incubate tubes overnight at 37 °C with constant shaking at 250 rpm. Keep the tube cap loose during incubation.

Day 2

1. In the morning, take out the tubes. Check to see if the LB-Broth solution in the tubes is cloudy (see Note 3).
2. Miniprep protocol (see Note 4).
(a) Preheat the Elution Solution to 50 °C.

(b) Transfer 1 mL of an overnight recombinant *E. coli* culture to a microcentrifuge tube and pellet cells at 12,000 × *g* for 1 min. Discard the supernatant.

(c) Resuspend the pellet in 200 μL of Resuspension Solution. Vortex and pipette up and down until cells are thoroughly homogeneous (see Note 5).

(d) Add 200 μL Lysis Solution. Immediately mix the contents by gentle inversion (six to eight times) until the mixture is clear and viscous. DO NOT VORTEX or PIPETTE UP AND DOWN (see Note 6)!

(e) Add 350 μL Neutralization/Binding Solution. Gently invert the tube six to eight times. DO NOT VORTEX or PIPETTE UP AND DOWN!

(f) Pellet cell debris at 12,000 × *g* for 10 min.

(g) Insert a GenElute Miniprep Binding Column into a provided microcentrifuge tube.

(h) Add 500 μL of the Column Preparation Solution to each column and centrifuge it at 12,000 × *g* for 1 min. Discard the flow-through.

(i) Transfer supernatant (cleared lysate) from step f into the column. Centrifuge it at 12,000 × *g* for 1 min. Discard the flow-through.

(j) Add 750 μL of the Wash Solution to the column. Centrifuge it at 12,000 × *g* for 1 min. Discard the flow-through.

(k) Centrifuge at max speed for 2 min without additional Solution. This removes excess EtOH.

(l) Transfer the column to a fresh, new collection tube. Add 100 μL of preheated Elution Solution. Incubate it for 10 min at room temperature.

(m) Centrifuge the tube at 12,000 × *g* for 1 min.

(n) Nanodrop eluted plasmid to measure the concentration and quality (see Note 7).

3. Send multiple colonies of the prepared plasmid out to Genewiz for Sanger sequencing (see Note 8).

**Day 3**

After the genetic sequences of all plasmids are completed, identify the “correct” plasmid and continue the following steps:

1. Inoculate 2 mL of bacterial culture from Day 2 into a new 50 mL LB-Broth (+Amp).

2. Incubate the tubes overnight at 37 °C with constant shaking at 250 rpm.
Day 4

1. In the morning, take out the tubes from incubator.

2. Use all the culture for Midiprep (Zymogen). Determine DNA concentration (please refer to step n of the Miniprep protocol in Day 2) (see Note 9).
   
   (a) Buffer preparation: add 88 mL of 95% ethanol to the 23 mL ZymoPURE Wash 2 (Concentrate) before use.

   (b) The ZymoPURE P2 and ZymoPURE Binding Buffer may have precipitated. If this occurs, dissolve the precipitate by incubating the bottles at 30–37 °C for 10–20 min and mix by inversion. DO NOT MICROWAVE!

   (c) Before starting: centrifuge up to 50 mL of bacterial culture at $\geq 3400 \times g$ for 10 min to pellet the cells in a 50 mL conical tube. Discard the supernatant.

   (d) Add 8 mL of ZymoPURE P1 (Red) to the bacterial cell pellet, and resuspend completely by vortexing or pipetting.

   (e) Add 8 mL of ZymoPURE P2 (Green) and immediately mix by gently inverting the tube six times. DO NOT VORTEX! Let the tube sit for 2–3 min (see Note 10).

   (f) Add 8 mL of ZymoPURE P3 (Yellow) and mix gently but thoroughly by inversion. DO NOT VORTEX (see Note 11)!

   (g) Ensure that the plug is attached to the Luer Lock at the bottom of ZymoPURE Syringe Filter. Place the syringe filter upright in a tube rack and load the lysate into the ZymoPURE Syringe Filter, and then wait 5–8 min for the precipitate to float to the top.

   (h) Remove the Luer Lock plug from the bottom of the syringe, and place it into a clean 50 mL conical tube. Place the plunger in the syringe, and push the solution through the ZymoPURE Syringe Filter in one continuous motion until approximately 20 mL of cleared lysate is recovered. SAVE THE CLEARED LYSATE!

   (i) Add 8 mL of ZymoPURE Binding Buffer to the cleared lysate from step h, and mix thoroughly by inverting the capped tube eight times.

   (j) Ensure the connections of the Zymo-Spin III-P Column Assembly are finger-tight.

   (k) Remove the 50 mL Reservoir from the top of the Zymo-Spin III-P Column Assembly, and place the assembly into a 50 mL conical tube.
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(l) Add 10 mL of the mixture from step i into the 15 mL Conical Reservoir/Zymo-Spin III-P Column Assembly, and centrifuge at $500 \times g$ for 2 min. Empty the 50 mL conical tube, and repeat step l until the entire sample has passed through the column.

(m) Add 5 mL of ZymoPURE Wash 1 to the Zymo-Spin V-PS Column Assembly, and centrifuge the column at $500 \times g$ for 2 min. Discard the flow-through.

(n) Add 5 mL of ZymoPURE Wash 2 to the Zymo-Spin V-PS Column Assembly, and centrifuge at $500 \times g$ for 2 min. REPEAT THIS WASH STEP.

(o) Remove and discard the 15 mL Reservoir-X from the Zymo-Spin V-PS Column. Place the Zymo-Spin V-PS Column in a collection tube, and centrifuge at $\geq 16,000 \times g$ for 1 min, in a microcentrifuge, to remove any residual wash buffer.

(p) Transfer the Zymo-Spin V-PS Column into a clean 1.5 mL tube, and add 200 $\mu$L of ZymoPURE Elution Buffer directly to the column matrix. Incubate the column at room temperature for 2 min, and then centrifuge at $\geq 16,000 \times g$ for 1 min in a microcentrifuge.

(q) Optional: for removal of residual endotoxins, place the EndoZero II Spin-Column in a clean 1.5 mL microcentrifuge tube. Add the entire eluate from step p into the EndoZero II Spin-Column, wait 2 min, and then centrifuge at $10,000 \times g$ for 1 min in a microcentrifuge. Store the eluted plasmid DNA at $\leq -20^\circ C$.

3.1.2 Polymerase Chain Reaction (PCR)

Goal To amplify target genes and prepare for Gibson Assembly.

Gene: QuasAr2-TS-GCamP5G-ER2.

Steps

1. Design and order primers. The primers should include two parts: one part anneals to the 5’ ends of the target gene insert for PCR amplification, and another part anneals to the sticky ends of the retroviral backbone cut by restriction enzyme (RE) in preparation for Gibson Assembly (requires 20–40 base pairs of overlap between the DNA fragments for anneal).

(a) Primer design: NEB (NEBuilder) website https://nebuilder.neb.com/#!/

(i) New fragment → load file → select virus backbone → select restriction digest for production of a linearized fragment → select enzyme(s). When selecting enzyme(s), only those with a single cut site in the sequence
should be considered. The target gene fragment can either be inserted or replace the original gene fragment after the promoter (such as CMV, EF1α, or CAG) in the virus backbone.

(ii) New fragment → load file → select template sequence for the target gene fragment → select PCR for production of a linearized fragment → enter the start and end base of the target gene fragment.

(iii) Settings → Product/Kit: Gibson ASSEMBLY Master Mix → PCR Polymerase/Kit: Q5 High-Fidelity 2× Master Mix/Hot Start Taq DNA polymerase.

(b) Order primers: IDT (Integrated DNA Technologies) website https://www.idtdna.com/pages
   (i) Custom DNA oligos → DNA oligos → single-stranded DNA (order now) → scale: 25 nanomole DNA Oligo → copy and paste the designed primer sequence from NEB website → formulation: none (powder).

2. PCR protocol: available on NEB website (https://www.neb.com/tools-and-resources/search?type=Protocol), depending on the PCR Polymerase/Kit used. Take Q5 High-Fidelity 2× Master Mix as an example:
   (a) Reaction Setup.
   Assemble all reaction components (Table 1) on ice, and quickly transfer the reactions to a thermocycler pre-heated to the denaturation temperature (98 °C). All components should be mixed prior to use (see Note 12).
   Transfer PCR tubes to a PCR machine and begin thermocycling. Thermocycling Conditions for a Routine PCR (Table 2).
   *Use the NEB Tm Calculator (https://tmcalculator.neb.com/#!/main) for exact temperature.

| Component                  | 50 μL reaction | 50 μL reaction | Final concentration |
|----------------------------|----------------|----------------|---------------------|
| Q5 high-Fidelity 2× master mix | 12.5 μL       | 25 μL          | 1×                  |
| 10 μM forward primer       | 1.25 μL        | 2.5 μL         | 0.5 μM              |
| 10 μM reverse primer       | 1.25 μL        | 2.5 μL         | 0.5 μM              |
| Template DNA               | Variable       | Variable       | < 1000 ng           |
| Nuclease-free water        | To 25 μL       | To 50 μL       |                     |
Table 2
Thermocycling conditions for PCR

| Step                        | Temp       | Time         |
|-----------------------------|------------|--------------|
| Initial denaturation        | 98 °C      | 30 s         |
| 25–35 cycles                | 98 °C      | 5–10 s       |
|                             | *50–72 °C  | 10–30 s      |
|                             | 72 °C      | 20–30 s/kb   |
| Final extension             | 72 °C      | 2 min        |
| Hold                        | 4–10 °C    |              |

Table 3
Recommended amounts of DNA template

| DNA             | Amount        |
|-----------------|---------------|
| DNA genomic     | 1 ng–1 μg     |
| Plasmid or viral| 1 pg–10 ng    |

(b) General Guidelines.

(i) Template.
Use of high quality, purified DNA templates greatly enhances the success of PCR. Recommended amounts of DNA template for a 50 μL reaction are as Table 3.

(ii) Primers.
Oligonucleotide primers are generally 20–40 nucleotides in length and ideally have a GC content of 40–60%. The best results are typically seen when using each primer at a final concentration of 0.5 μM in the reaction.

(iii) Mg$^{2+}$ and additives.
The Q5 High-Fidelity Master Mix contains 2.0 mM Mg$^{2+}$ when used at a 1× concentration. This is optimal for most PCR products generated with this master mix.

(iv) Deoxynucleotides.
The final concentration of dNTPs is 200 μM of each deoxynucleotide in the 1× Q5 High-Fidelity Master Mix. Q5 High-Fidelity DNA Polymerase cannot incorporate dUTP and is not recommended for use with uracil-containing primers or templates.
(v) Q5 High-Fidelity DNA Polymerase concentration.

The concentration of Q5 High-Fidelity DNA Polymerase in the Q5 High-Fidelity 2× Master Mix has been optimized for best results under a wide range of conditions.

(vi) Denaturation.

An initial denaturation of 30 s at 98 °C is sufficient for most amplicons from pure DNA templates. Longer denaturation times can be used (up to 3 min) for templates that require it.

During thermocycling, the denaturation step should be kept to a minimum. Typically, a 5–10-s denaturation at 98 °C is recommended for most templates.

(vii) Annealing.

Optimal annealing temperatures for Q5 High-Fidelity DNA Polymerase tend to be higher than for other PCR polymerases. The NEB $T_{m}$ Calculator should be used to determine the annealing temperature when using this enzyme. Typically use a 10–30 s annealing step at 3 °C above the $T_{m}$ of the lower $T_{m}$ primer. A temperature gradient can also be used to optimize the annealing temperature for each primer pair.

For high $T_{m}$ primer pairs, two-step cycling without a separate annealing step can be used (see Note 10).

(viii) Extension.

The recommended extension temperature is 72 °C. Extension times are generally 20–30 s per kb for complex, genomic samples but can be reduced to 10 s per kb for simple templates (plasmid, $E. coli$, etc.) or complex templates <1 kb. Extension time can be increased to 40 s per kb for cDNA or long, complex templates, if necessary.

A final extension of 2 min at 72 °C is recommended.

(ix) Cycle number.

Generally, 25–35 cycles yield sufficient products. For genomic amplicons, 30–35 cycles are recommended.

(x) Two-step PCR.

When primers with annealing temperatures ≥72 °C are used, a two-step thermocycling protocol (combining annealing and extension into one step) is possible.
(xi) Amplification of long products.
When amplifying products >6 kb, it is often helpful to increase the extension time to 40–50 s/kb.

(xii) PCR product.
The PCR products generated using Q5 High-Fidelity 2× Master Mix have blunt ends. If cloning is the next step, then blunt-end cloning is recommended. If T/A-cloning is preferred, the DNA should be purified prior to A-addition, as Q5 High-Fidelity DNA Polymerase will degrade any overhangs generated.

The addition of an untemplated -dA can be done with Taq DNA Polymerase (NEB #M0267) or Klenow exo- (NEB #M0212).

3.1.3 Running Gel

Goal To purify the target gene fragment amplified by PCR.

Steps

1. Weigh 1.7 g of agarose and 170 mL of TAE (proper pH buffer for DNA) for 170–200 mL of cast. See formula for 1 L 20× TAE (ssd) in Table 4.
2. Use magnetic hot plate to completely mix agarose and TAE in a beaker.
3. Cover the top of the beaker and use a microwave to boil the solution and dissolve the agarose completely.
4. Cool the agarose solution down to about 50 °C, and pour it into a gel cast with the well comb in place.
5. After the gel solidifies, remove the comb and add TAE into the cast until the gel surface is covered.
6. Use a pipette to carefully load 100 bp/1 kb DNA Ladder into the first well of the gel.
7. Use a pipette to carefully transfer the PCR products and Gel Loading Dye into the other wells of gel.

Table 4
Formula for 1 L 20× TAE (ssd)

| Tris base                  | 96.88 (g) |
|---------------------------|-----------|
| Na2EDTA                   | 7.44      |
| Trihydrate Na acetate     | 13.01     |
| pH adjusts to 7.6 using acetic acid (about 50 mL) |          |
| QS adjusts to 1 L with ddH2O |          |
8. Run the gel at 80–150 V and 0.3–0.4 A until the pink line of loading dye is approximately 75–80% of the way down the gel. A typical run time is about 1–1.5 h, depending on the gel concentration and voltage (see Note 13).

9. Turn OFF power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.

10. Using a UV illumination device, visualize and cut off the DNA bands from the gel. Use long-wavelength UV and expose the gel for as little time as possible to minimize damage to the DNA. Use the DNA ladder in the first column as a guide for the size of the DNA in the sample columns (see Note 14).

11. Extract and purify the target DNA from the agarose gel using Monarch DNA Gel Extraction Kit. Protocol:
   (a) Transfer the gel slice containing the target DNA into a 1 mL Eppendorf tube and weigh the gel slice.
   (b) Add four volumes of Monarch Gel Dissolving Buffer to the tube with the gel slice (e.g., 400 μL buffer per 100 mg agarose). If the gel slice is >150 mg, consider reducing the amount of Gel Dissolving Buffer to 3 or 3.5 volumes to minimize the guanidine salt present in the workflow.
   (c) Incubate the sample between 37 and 55 °C (typically 50 °C) and vortex every 2–3 min until the gel slice is completely dissolved (generally 5–10 min) (see Note 15).
   (d) Insert the column into the collection tube and load the sample onto the column. Centrifuge at 16,000 × g for 1 min, and then discard flow-through.
   (e) Reinsert the column into the collection tube. Add 200 μL DNA Wash Buffer and centrifuge at 16,000 × g for 1 min. Discarding flow-through is optional (see Note 16).
   (f) Repeat wash (step e).
   (g) Transfer the column to a clean 1 mL Eppendorf tube. Use care to ensure that the tip of the column has not come into contact with the flow-through. If in doubt, recentrifuge the column for 1 min before placing into clean tube.
   (h) Add ≥6 μL of DNA Elution Buffer to the center of the matrix. Wait for 1 min, and centrifuge at 16,000 × g for 1 min to elute DNA (see Note 17).
   (i) Nanodrop the eluted plasmid to measure the concentration and quality.

3.1.4 Gibson Assembly

**Goal** To insert the target gene fragment into virus backbone (Fig. 1a).
Fig. 1 Generation of transduced cancer cell lines that stably express CaViar (GCaMP6F + QuasAr2). (a) QuasAr2-TS-GCaMP6F-ER2 gene being inserted into a transfer plasmid. (b) Production of lentivirus/retrovirus that contains the QuasAr2-TS-GCaMP6F-ER2 gene. (c) Transduced cell expressing CaViar

Table 5
Reaction components for restriction digest

| Component                        | 10 μM L reaction |
|----------------------------------|------------------|
| DNA                              | 1 μg             |
| 10× NEBuffer EcoRI/SspI          | 5 μL (1×)        |
| EcoRI                            | 1.0 μL (20 units)|
| Nuclease-free water              | To 50 μL        |

Steps

1. Use restriction enzyme (RE) to cut the virus backbone (retroviral plasmid pBabe-puro; lentiviral expression plasmid). Restriction digest protocol: available on NEB website (https://nebcloner.neb.com/#!/redigest), depending on the enzyme used. Take EcoRI as an example:
   (a) Set up reaction as Table 5.
   (b) Incubate at 37 °C for 5–15 min.

2. Gibson Assembly Protocol: available on NEB website (https://www.neb.com/tools-and-resources/search?type=Protocol), depending on the Assembly Product/Kit used. Take Gibson Assembly Master Mix as an example:
**Table 6**
Reaction components for Gibson Assembly

|                              | Recommended amount of fragments used for assembly |
|------------------------------|---------------------------------------------------|
|                              | Two to three fragment assembly | Four to six fragment assembly | Positive control** |
| Total amount of fragments    | 0.02–0.5 pmols* X μL | 0.2–1 pmols* X μL | 10 μL |
| Gibson assembly master mix   | 10 μL | 10 μL | 10 μL |
| (2×)                         |       |       |       |
| Deionized H₂O                | 10-X μL | 10-X μL | 0 |
| Total volume                 | 20 μL*** | 20 μL*** | 20 μL |

(a) Set up the following reaction on ice (Table 6).

*Optimized cloning efficiency is 50–100 ng of vector with 2–three-fold molar excess of each insert. Use five-fold molar excess of any insert(s) less than 200 bp. To achieve optimal assembly efficiency using four to six fragment assemblies, use a 1:1 molar ratio of each insert vector. Total volume of unpurified PCR fragments in the assembly reaction should not exceed 20%.

**Control reagents are provided for five experiments.

***If greater numbers of fragments are assembled, additional Gibson Assembly Master Mix may be required.

(b) Incubate samples in a thermocycler at 50 °C for 15 min when two or three fragments are being assembled or 60 min when four to six fragments are being assembled. Following incubation, store the samples on ice or at –20 °C for subsequent transformation.

(c) Transform the DNA construct into *E. coli* for amplification and Miniprep. Send the construct to Genewiz for Sanger sequencing.

3.1.5 Retroviral Transduction

**Goal** To make virus with target gene and use it to transfect target cell line (Fig. 1b, c).

**I. Transfection into Phoenix Cells**

**Day 1**

1. Plate Phoenix cells at a 1:5 split in a T-75 flask, and incubate it overnight with 10 mL of complete media (*see Note 18*). Use a low passage of Phoenix cells (P2–4), split them at 90% confluency, and plate at no less than 1:5.
Day 2
1. Refeed Phoenix cells with 6 mL of serum-free DMEM per T-75 flask.
2. Make the following Lipofectamine mix (for 1 T-75 flask):
   - 970 μL Serum-free DMEM
   - 30 μL Lipofectamine 3000 (Invitrogen)
3. Make the following transfection mix dropwise with gentle vortexing, and incubate it for 20 min at room temperature. Dilute the DNA in 1 mL serum-free media to give a final of 2 mL transfection mix.
   - 5 μg Plasmid in 1 mL serum-free media
   - 1 mL Lipofectamine mix from step 2.
4. Add all the 2 mL Lipofectamine/DNA mix to the T-75 flask; rock back and forth.
5. Incubate the flask overnight.

Day 3
1. Aspirate the medium and feed the Phoenix cells with 20 mL of complete medium.
2. Incubate the flask overnight.

II. Transduction of Target Cells (HCT-8 & DU145)

Plate the target cells in T-25 flasks so that at the time of transduction, the cells are about 50% confluence.

Day 4
1. Collect the supernatant from the Phoenix cells in a conical tube and refeed cells.
2. Add sterile polybrene (Sigma-Aldrich) to the supernatant at a final concentration of 10 μg/mL.
   - Add 20 μL of polybrene to 20 mL of supernatant.
3. Filter the virus through a sterile 0.45-um Steriflip (Sigma-Aldrich).
4. Remove the medium from target cells, and replace that with 6 mL of virus prepared in step 3.
5. Incubate the cells for 24 h.

Day 5
1. Repeat steps 1–5 from Day 4 (see Note 19).
Day 6–∞

1. Refeed the transduced cells (and kill control) with the appropriate complete medium and 5 μg/mL puromycin (Thermo Fisher) (see Note 20).

2. Change the medium as needed and grow the cells to confluence.

3. Split the cells into more flasks and maintain the puromycin selection.

III. Characterization/Verification

1. Check the signals of tagged fluorescent protein (GCaMP5G) under microscopy to verify the expression of the target gene and membrane trafficking of the functional protein.

**Once confirmed, grow up cells in a bigger flask and freeze down cells.

Day 1

1. Plate HEK 293 T cells at 1.5–2 × 10^6 cells/well in six-well plates with 3 mL of DMEM (night) (see Note 21).

Day 2

<Transfection>

12 h later, aspirate the medium and add 1.5 mL of fresh complete DMEM.

1. Dilute 2 μg expression plasmids (containing the target gene) with 0.8 μg of pMD2.G and 1.2 μg of psPAX2 in 125 μL of OPTI-MEM (This serum-free medium can also be replaced by DMEM without FBS.) and 5 μL P reagent (from Lipofectamine kit).

2. Dilute 5 μL of Lipofectamine 3000 in 125 μL OPTI-MEM.

3. Incubate the dilutions at room temperature for 5 min.

4. Combine the dilutions of Lipofectamine and plasmids into 250 μL transfection mix.

5. Incubate the mix for 20 min at room temperature.

6. Apply the transfection mixtures (250 μL/well) to six-well plates (9:30 a.m.).

Day 3–5

<Collection of viral particles>

1. After 6–8 h of transfection, aspirate the medium and add 2 mL of warm freestyle 293 (gibco) medium/well (This medium can also be replaced by DMEM with FBS.) (night).
2. At the 30th, 48th, 60th, and 72nd hours post transfection, collect and store the supernatant in a 15 mL falcon tube.
3. Centrifuge the tube for 5 min at 2000 × \( g \) to precipitate cell debris.
4. Filter the supernatant through 0.45 μm filters to remove residual cell debris. Wrap falcon tubes with aluminum foil and store them at 4 °C.

<Transduction of target cell line (MA-104)>

1. Plate 2.5 × 10^5 cells/well with 1 mL of complete Eagle’s Minimum Essential Medium (EMEM) in a 12-well plate (8:00 p.m.).
2. After 22 h, dilute 10 mg/mL of polybrene to 4 μg/mL (2500×) in 1000 μL of supernatant containing virus and 300 μL of complete EMEM (6:00 pm) (50% confluence) (see Note 22).
3. Aspirate the old complete EMEM and apply the 1300 μL mixture to MA-104 cells in each well.
4. 15 – 16 h post transduction, aspirate the mixture and add 1 mL of complete EMEM/well (9:00 a.m.).
5. 48 h post transduction, trypsinize and pool the cells to 10 cm plates, and start hygromycin (100 μg/mL, Thermo Fisher) selection.
6. Grow the cells to confluence and freeze down the cells.

3.2 Culture and Maintenance of Human Cancer Cells

3.2.1 Subculture of Adherent Cells (for T-75 Flask)

1. Take out the cell culture flask from incubation, and check the cell confluence under microscope. Make sure that the cell confluence is about 60–70% or more before subculture.
2. Warm up the PBS, trypsin, and complete cell culture medium in a water bath at 37 °C (see Note 23).
3. Turn on the biosafety cabinet (BSC) and the UV light for at least 15 min prior to the subculture. Put gloves on and use 70% ethanol to wipe the gloves, the working surface inside the hood, and the bottles for PBS, trypsin, and culture medium. Only open the bottles inside the hood.
4. Turn the vacuum pump on. Remove the medium in the flask using an aspirating pipette connected to the vacuum pump. Only touch the end of the pipette that will not touch the medium.
5. Use 5–10 mL of sterile PBS to wash the cells. Do not aim for the cells adhering to the bottom of the flask. Shake the flask very gently to completely cover the cells with PBS, and then aspirate the PBS. This step completely removes the residual medium in the flask to avoid deactivating trypsin.
6. Add 2–3 mL of trypsin into the flask (aim for the cells), and shake the flask very gently to completely cover the cells with trypsin. Put the flask into the 37 °C incubator for 5–10 min until the cells are all detached (see Note 24).

7. Gently knock the bottom of the flask to help cells detach. Check if all the cells are detached using a microscope.

8. Add at least 6–9 mL of fresh medium to the flask (medium/trypsin = 3:1 or more). Wash the bottom of the flask with the medium/trypsin mixture using the serological pipette to collect all the cells. Transfer the cells to a sterile 15 mL centrifuge tube.

9. Centrifuge the tube at 300 × g for 3 min at room temperature. A cell pellet should be visible at the bottom of the tube after centrifugation.

10. Aspirate the supernatant. Keep the pipette away from the cell pellet.

11. Resuspend the cells in the complete culture medium, mix them well with pipetting, and add an appropriate volume of cells to a new flask. Fill the flask with complete medium to reach a total volume of 11–13 mL, and label the flask with the cell line type, the passage number, the split ratio, your name, and the date (see Note 25). For a flask with a close cap, partially open the cap during culture in the incubator.

12. Put the new flask into the 37 °C incubator to maintain cell growth. Discard the excess cells.

13. Apply 70% ethanol to wipe the working surface of the hood. Turn on the UV light and turn off the vacuum pump.

14. Check the cells regularly and change the medium every 2–3 days.

3.2.2 Cryopreservation of Cells

1. Follow steps 1–10 in the subculture protocol.

2. Resuspend the cells in the complete culture medium, mix them well with pipetting, and perform cell counting to determine the total cell numbers.

3. Centrifuge the cells at 300 × g for 3 min at room temperature. A cell pellet should be visible at the bottom of the tube after centrifugation.

4. Aspirate the supernatant. Keep the pipette away from the cell pellet.

5. Resuspend the cells in freezing medium at the concentration of 1 million cells/mL, and mix them well with a pipette (see Note 26).

6. Aliquot 1 mL of the cell suspension into sterile cryovials. Label the cryovials with cell line type, passage number, and date.
7. Put the cryovials into a −80 °C freezer and freeze them overnight.

8. Transfer the cryovials to a liquid N₂ storage tank.

3.2.3 Thawing of Frozen Cells

1. Take the cryovial containing the frozen cells from liquid N₂ storage, and immediately put it into a 37 °C water bath.

2. Quickly thaw the cells (< 1 min) in the 37 °C water bath until there is just a small bit of ice left in the vial.

3. Wipe the outside of the cryovial with 70% ethanol and put the vial into the BSC.

4. Transfer the thawed cell suspension to a sterile 15 mL centrifuge tube, and add 10 mL of pre-warmed complete culture medium dropwise.

5. Centrifuge the cells at 300 × g for 3 min at room temperature.

6. Aspirate the supernatant. Keep the pipette away from the cell pellet.

7. Resuspend cells in the complete culture medium, mix them well with pipetting, and transfer the cells into a cell culture flask. Fill the flask with complete medium to reach a total volume of 11–13 mL (for T-75), and label the flask with the cell line type, the passage number, “Thaw,” your name, and the date.

8. Put the new flask into the 37 °C incubator to maintain cell growth.

9. Change the medium next day, and then follow the subculture protocol to maintain the cell culture. Typically, the cells are ready to use for experiment after two passages.

3.2.4 Cell Viability and Counting

1. Mix the cell suspension well by gentle manual pipetting.

2. Take an appropriate volume of cell suspension sample into a sample tube, and mix it with an equal volume of 0.4% Trypan Blue Solution.

3. Count the blue staining cells (nonviable) and the number of total cells using either an automatic cell counter or a hemacytometer. Count cells using a hemocytometer: https://www.abcam.com/protocols/counting-cells-using-a-haemocytometer

4. Determine cell viability. Cell viability should be at least 95% for healthy log-phase cultures.

3.3 Preparation of 2D and 3D Biomaterials

3.3.1 PAA Gel Preparation

Polyacrylamide (PAA) gels were prepared following the protocols described elsewhere [20–23]. Briefly, the PAA gels were made with varied relative concentrations of acrylamide and N,N'-methylene bis-acrylamide to obtain different cross-link percentages resulting in different elastic moduli. The volume percentages of acrylamide...
and N,N′-methylene bis-acrylamide were, respectively, 12.5% and 0.5% (250 Pa), 20% and 6.5% (10 kPa), 20% and 13.5% (20 kPa), and 20% and 24% (40 kPa). All the substrates were covalently coated with 25 mg/mL fibronectin.

### Abbreviations Contained in This Protocol

PEG: poly(ethylene) glycol; dMMP: degradable matrix metalloproteinase peptide; RGD: arginine-glycine-aspartate; MW: molecular weight

### Hydrogel Composition *(see Note 27)*.

**“Medium” Stiffness Hydrogel (Median E = 20–25 kPa).**

- 50:50 ratio of PEG-DA (3.4 kDa) and PEG-dMMP-PEG (8 kDa).
- 5 mM of PEG-RGD (4 kDa).
- PEG-DA and PEG-dMMP-PEG at 10% w/v.

### Procedure *(see Notes 28–31)*

1. Turn on the UV light.
2. Determine the amount of each material needed to make the hydrogels using 10 μL of precursor solution per hydrogel.
3. Weigh the needed amount of each PEG material in 1.5 mL amber centrifuge tubes.
   - (a) Weigh PEG-RGD first.
   - (b) After you weigh the PEG-RGD, adjust the weights needed for PEG-dMMP-PEG and PEG-DA to account for any excess PEG-RGD material weighed.
     - (i) PEG-RGD is a difficult material to weigh—weigh amount as close as possible, and adjust the amount needed of PEG-dMMP-PEG and PEG-DA accordingly.
   - (c) Weigh PEG-dMMP-PEG and PEG-DA.
4. Prepare the photoinitiator at a 10% (w/v) solution.
   - (a) Weigh the material non-sterilely in 1.5 mL amber centrifuge tube.
   - (b) Add the necessary amount of 70% ethanol (made fresh, with synergy water) and vortex to mix.
   - (c) For sterile gels, filter sterilize the photoinitiator into a new tube in the BSC using a 0.22 μm filter.
5. Make the precursor solution by adding PBS to the different PEGs to make PEG-DA and PEG-dMMP-PEG at 10% (w/v), and keep PEG-RGD at 5 mM.
   - (a) Add the necessary amount to PEG-DA and to PEG-dMMP-PEG to keep the concentration at 10% (w/v).
After adding PBS to each vial, vortex the solutions before mixing.

Take the same volume from PEG-DA and PEG-dMMP-PEG solution, and add to the vial with PEG-RGD keeping it at 5 mM with a 50:50 ratio of PEG-DA and PEG-dMMP-PEG.

After adding the PEG-DA and PEG-dMMP-PEG solutions to PEG-RGD, vortex and keep the mix on ice until use.

6. Add the photoinitiator to the final precursor solution at a concentration of 0.05%.
   (a) For each 1 mL of PEG hydrogel solution, add 5 mL of the photoinitiator solution.
   (b) Vortex the solution and keep it on ice until use.

7. Adjust the UV light to the desired conditions.
   (a) Use UV light safety goggles to directly look at the light; never look at it without eye protection.
   (b) Use a radiometer to read the intensity of the UV light output. Adjust it up or down until the meter reads 4.15 mW/cm².

8. Count and collect the needed number of HCT-8 colon cancer cells (densities of 0.2–5 million cells/ml were used).

9. Centrifuge the cells to form a pellet and then aspirate the media.

10. Resuspend the cells in the hydrogel precursor solution, and ensure that any cell clumps are gently broken up using a micropipette.

11. In a sterile, 35 mm Petri dish, use an ImmEdge pen to draw a quadrant with hydrophobic barriers.

12. In each quadrant, add one 10 μL droplet of the hydrogel solution using a micropipette.
   (a) Ensure the droplets are close enough together to all be under the UV light.
   (b) There should be four droplets per Petri dish.

13. After all the droplets are formed on the Petri dish, mix each droplet up and down using a micropipette to evenly mix the cells in each droplet and prevent cell settling.

14. Put the lid on the Petri dish, place it directly under the strongest part of the UV light, and photopolymerize the hydrogels at 4.15 mW/cm² for 3 min.

15. Rinse the hydrogels two times with 70 mL of PBS.

16. Add 150 mL of media and place in the incubator for culture.
3.4 Ca\(^{2+}\) Imaging In Vitro

3.4.1 Hardware and Software of Microscopy

The hardware systems used for imaging include a commercial fluorescent confocal microscope system (Nikon, A1R HD25), a monochrome camera (FLIR, BFS-U3-70S7M-C), and a desktop computer that is installed with the 64-bit Microsoft\(\copyright\) Windows 10 Pro operating system. The Nikon confocal microscope system consists of multiple components: the Ti2-E inverted microscope, the LU-N4 laser units (405 nm, 488 nm, 561 nm, and 640 nm laser channels), the confocal controller, a standard fluorescence detector (four photomultiplier tubes (PMT)), and a scan head (two Galvano scanners and one resonant scanner). The Ti2-E inverted microscope comprises an LED Lamp-house for illumination, a motorized XY stage, six motorized epi-fluorescence filters, a condenser turret, six motorized nosepieces, a stage joystick, and a stage-top incubator (TOKAI HIT, INU-TIZB-F1) including the control of temperature (37 °C), humidity, and premixed gas (95% air and 5% CO\(_2\)) flow.

3D image stacks and videos are acquired by the confocal microscope system. The Ti2-E inverted microscope works independently to acquire bright-field (BF) images via the monochrome camera. The DiaLamp (a white LED equipped on the Ti2-E microscope) serves as a light source for bright-field imaging.

Two software systems are involved to coordinate these devices: (1) SpinView, which controls the BFS-U3-70S7M-C camera, and (2) Nikon NIS-Elements, which controls the whole confocal system and the operation of the Ti2-E microscope.

3.4.2 Live Cell Ca\(^{2+}\) Imaging

The following steps detail how to achieve a 3D z-stack image and a time-lapse image acquisition using the confocal microscope system to observe calcium dynamics of the transduced HCT-8, DU145, and MA-104 cell lines (Fig. 2).

1. Turn on the Nikon A1R confocal microscope system following a specific sequence: first, the LU-N4 laser units; next, the confocal controller; then, the Ti2-E microscope controller; and, finally, the Ti2-E inverted microscope.

2. In the Ti2-E inverted microscope, turn on the DiaLamp, and switch to the 10× objective (CFI Plan Apochromat \(\lambda\) 10×/0.45) and the light-path on the right side for BF imaging to identify the cells of interest. Using the 10× magnification, move the XY motor stage using the joystick, and adjust the focus to find appropriate FOVs containing cell colonies/spheroids that grow well on/in the substrate.

3. Close the DiaLamp and switch to the light-path on the left side for fluorescent imaging. Launch Elements, open the FITC channel (e.g., 488; Em: 525/50), and switch to the resonant scanner for high-speed imaging. Adjust the focus to get the maximum intensity of fluorescent signals in the whole FOV. Fluorescent images captured in the FITC channel display the Ca\(^{2+}\) dynamics of cells that express GCaMP5G.
Fig. 2 In vitro and ex vivo imaging of Ca\(^{2+}\) signal. (Left) Multiwavelength lasers are controlled by acousto-optic tunable filter (AOTF) to generate excitation light with desired wavelength, frequency, patterns, and intensity. (Right) Both in vitro cells and ex vivo CRISPR/Cas9-engineered or virus-infected tissues can be imaged under fluorescent microscopy in parallel with patch-clamp electrophysiology recording. Fluorescence that indicates Ca\(^{2+}\) signals from the in-focus imaging plane passes through the microscope objective and pinhole. Photomultiplier tube (PMT) collects the in-focus emission light and converts it into functional imaging. Abbreviations: DAQ data acquisition device, PBS polarizing beam splitter, HWP half-wave plate, DM dichroic mirror, CRISPR clustered regularly interspaced short palindromic repeats

4. For 3D z-stack imaging.
   
   (a) Slowly adjust the knob of the Z-plane and record the highest and the lowest Z position to form a z-stack that covers the overall z-height of the target cell colonies/spheroids. Input an appropriate step size of Z-plane to generate enough frames for a 3D z-stack image.

   (b) Switch to the galvano scanner for high-resolution imaging. To avoid photobleaching of fluorophore and capture images with low noise, an exposure time of 4 s is set.

   (c) Specify a directory to save the data and start z-stack image acquisition. When finished, switch back to the light-path on the right side and turn on the DiaLamp that allows SpinView to take a BF image.

5. For time-lapse imaging.

   (a) Switch to the galvano scanner for high-resolution imaging. To capture images with low noise and high temporal resolution, an exposure time of 2 s and a frame interval of 3 s are set. Set the duration as 30 min.
(b) Specify a directory to save the data, and start time-lapse image acquisition. When finished, switch back to the light-path on the right side, and turn on the DiaLamp that allows SpinView to take a BF image.

6. Once the experiment is finished, shut down the Nikon A1R confocal microscope system following a specific sequence: first, the Ti2-E inverted microscope; next, the Ti2-E microscope controller; then, the confocal controller; and, finally, the LU-N4 laser units.

7. Post-process and analyze the images using NIS-Elements, ImageJ, and custom MATLAB programs. Each imaging condition is listed below:
   
   (a) Bright-field image: magnification: 10×; DiaLamp intensity: 10%; exposure time: 14 ms.
   
   (b) 3D z-stack imaging: magnification: 10×; laser intensity: 40%; gain of photomultiplier tube: 70; exposure time: 4 s; step size: 2 mm; the range of Z-plane: 50 ~ 100 mm; pixel size: 1024 × 1024
   
   (c) Time-lapse imaging: magnification: 10×; laser intensity: 20%; gain of photomultiplier tube: 70; exposure time: 2 s; frame interval: 3 s; duration: 30 min; pixel size: 1024 × 1024.

3.5 HCT-8 Cell Xenograft Model Generation

1. Culture the tumor cells to 80–90% confluency in a 10 cm Petri dish. Aspirate the medium and add 5 mL of PBS to wash it gently. Add 3 mL of 0.25% trypsin and incubate the flask at 37 °C for 5 min. Use a 1 mL pipette to detach the adherent cells by pipetting solution toward the adherent cells, add 10 mL of neutralizing medium, and transfer the cells to a 15 mL centrifuge tube. Centrifuge the tube at 1500 rpm for 3 min at room temperature, discard the supernatant, and resuspend the cells in 5 mL PBS. Centrifuge the cells again and repeat this washing step twice. Resuspend cells in 0.3 mL PBS, and check the cell density using a hemocytometer or an automatic cell counter.

2. Adjust the cell density in the suspension to 2 × 10^7 cells/mL with PBS, put it on ice to keep the cells in a relatively low metabolic state, and transfer them to the SPF animal room. The inoculation process should be completed within half an hour.

3. Use 4–5-week-old nude mice, pinch the skin on the back of the neck of the nude mouse with the thumb and index finger of the left hand, and clamp the tail of the nude mouse with the little finger. Select the flank of nude mice as the inoculation site. Before inoculation, spray 75% ethanol on a sterile cotton swab, and wipe the skin of the needle insertion site for disinfection.
4. To maintain the integrity of the cancer cells, install a 25-gauge injection needle that has a larger inner diameter onto a 1 mL disposable sterile syringe. After gently pipetting and mixing the cells, immediately aspirate the cells from the centrifuge tube into the syringe. Expel the air in the needle, puncture about 1 cm forward subcutaneously from the needle insertion site, and slide the needle left and right under the skin several times to facilitate cell seeding into clusters and reduce the possibility of leakage and contamination. The injection volume of 0.2 mL (\(4 \times 10^6\) cells) per nude mouse is used for subcutaneous injection.

5. After the injection, slowly withdraw the needle, and gently press the needle hole with a cotton swab to avoid leakage as much as possible.

6. Generally, about 1–2 weeks after inoculation, subcutaneous lumps can be observed.

3.6 \(\text{Ca}^{2+}\) Imaging Ex Vivo

3.6.1 HCT-8 Tumor Tissue Slicing

1. Intraperitoneally inject nude mice with 0.25 mL of 1% pentobarbital sodium, and anesthetize them for 5–10 min. This dosage will normally euthanize the animal; however, it is recommended to perform cervical dislocation immediately after surgery.

2. Fix the nude mice on a foam board and expose the tumor growth site. Gently dissect the tumor with forceps and scissors, and weigh and record the tumor tissue on an electronic scale. Use a digital caliper to measure and record the size (major and minor axes) of the tumor tissue (see Note 32).

3. Put the tumor tissue into a 6 cm Petri dish filled with 3 mL of normal saline, and put the Petri dish into a foam box with ice.

4. Take off the buffer tray from the microtome, and add super glue at the center of the specimen disc. Use forceps to take out the tumor tissue from the Petri dish, and place it at the glue center to fix the tissue.

5. Pour the precooled (\(-20\ °\text{C}\)) slicing solution into the buffer tray (if necessary, room temperature or pre-warmed slicing solution can be used) (see Note 33).

6. Cut the blade into half, install the blade in the blade holder, and fix the holder on the microtome.

7. Power on the microtome and set the parameters:
   (a) Slice thickness: 250 \(\mu\text{m}\) (can be adjusted as needed).
   (b) Push the button in the “UP” direction to raise the buffer tray to adjust the distance between the blade and the tissue. Stop when the distance between the blade and the top of the tissue is about 0.5 cm. Push the button in
the “FORW” direction to advance the blade to the front
of the tissue; click the double arrow button and the indicator light flashes; push the button in the “REV” direction to withdraw the blade to the rear edge of the tissue, and click the double arrow button and the indicator light flashes.

8. Click “SINGLE CONT” to select continuous sectioning mode, and press the “START STOP” button to start sectioning.

9. If the tissue slice curls during the sectioning process, one can gently brush the edge of the tissue slice with a line pen to stretch it. Gently take out the sectioned tissue slice with a line pen, and place it in a 6 cm Petri dish with 4 mL of slicing solution. The dish also needs to be placed in a foam box with ice. Discard the first sectioned tissue slice and start collecting from the second slice. Press the “START STOP” button to stop the sectioning after enough slices are obtained.

10. If one needs to adjust the slice thickness, directly adjust the thickness parameter, repeat steps 8–9, and put the collected slices into a new Petri dish.

11. After sectioning, push the button in the “DOWN” direction to minimize the height of the buffer tray. Take the blade holder off and put away the blade. Take off the buffer tray and pour the liquid into the waste tank. Use the blade to remove and discard the residual tissue. Rinse the buffer tray with water and set it aside to dry.

1. Stain the tumor slice with 5 μM Fluo-4 AM in the slicing solution for 30 min at 37 °C in a humidified incubator with 5% CO₂.

2. Transfer the tumor slice into a glass-bottom imaging dish with 1 mL of slicing solution. Put one or two 12 mm glass coverslips on the tumor slice to press it down to the glass bottom for the best imaging quality. Avoid bubbles between the tumor slice and coverslip.

3. Perform Ca²⁺ imaging of the tumor slice in the slicing solution at 25 °C using a 20× objective and a sCMOS camera (ORCA-Flash4.0 V2, Hamamatsu, Japan) mounted on a Nikon Eclipse Ti-E inverted microscope operated by the NIS-elements software (Nikon, Japan). Time-lapse Fluo-4 fluorescence is excited through a FITC filter set with an exposure time of 200 ms and a frame interval of 2 s.

4. Save the imaging results as *.nd2 files, and export them as serial TIF images for analyses in custom MATLAB codes.
4 Notes

1. Synthesis of the precursor polymers of 3D hydrogel is detailed in Temples et al. (2020) [27].
2. Do not vortex to avoid damaging the cells.
3. Cloudy and nontransparent LB-Broth indicates growth of bacteria.
4. All the solutions and column used in this step are from QIAprep Spin Miniprep Kit.
5. Resuspension Solution is kept at 4 °C.
6. Do not let the lysis reaction exceed 5 min.
7. Do not use DNA with A260/A280 lower than 1.75. A260/A280 of 2.00 is considered the “best.”
8. Design sequencing primers according to the length of interested gene section, and order the primers from IDT in advance.
9. All the solutions, filter, and columns used in this step are from ZymoPURE II Plasmid Midiprep Kit.
10. Cells are completely lysed when the solution appears clear, purple, and viscous.
11. Neutralization is complete after the sample turns yellow and a yellowish precipitate forms.
12. Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.
13. Black is negative; red is positive. The DNA is negatively charged and will run toward the positive electrode. ALWAYS RUN TO RED.
14. When using UV light, protect your eyes and skin by wearing safety goggles or a face shield, gloves, and a lab coat.
15. For DNA fragments >8 kb, an additional 1.5 volumes of water should be added after the slice is dissolved to mitigate the tighter binding of larger pieces of DNA (e.g., 100 mg gel slice/400 μL Gel Dissolving Buffer/150 μL water). Failure to dissolve all the agarose will decrease the recovery yield due to incomplete extraction of the DNA and potential clogging of the column by particles of agarose.
16. Add ethanol to Monarch DNA Wash Buffer prior to use (4 volumes of ≥95% ethanol per volume of Monarch DNA Wash Buffer).
17. Typical elution volumes are 6–20 μL. Nuclease-free water (pH 7–8.5) can also be used to elute the DNA. The yield may slightly increase if a larger volume of DNA Elution Buffer
is used, but the DNA will be less concentrated. For larger size DNA ($\geq 10$ kb), heating the elution buffer to 50 °C prior to use can improve the yield. Care should be used to ensure the elution buffer is delivered onto the matrix and not the wall of the column to maximize elution efficiency.

18. Phoenix cells are weakly adherent. Handle them gently.

19. Aspirate the old virus medium and add new virus to the same target cells in Day 4 for two round transductions. Phoenix cells no longer produce virus 3 days after transfection.

20. Transduction takes no more than 2 days, and the medium needs to be changed to remove virus.

21. The HEK 293 T cells used to make the virus need to be split every 2 days. Cells were seeded at $2 \times 10^6$ for 10 cm plates. After 48 h, the cells will reach about $1 \times 10^7$. The status of cell growth is critical to making virus. Cells with 80–90% confluence can be used for transfection.

22. Add 0.52 $\mu$L of 10 mg/mL polybrene into 1300 $\mu$L of mixture to have final concentration of 4 $\mu$g/mL.

23. Check ATCC website (https://www.atcc.org/) for the formula of complete culture medium for specific cell lines.

24. Long time treatment under trypsin may damage the cells.

25. The appropriate split ratio could differ between different cell lines and can be found on ATCC website (https://www.atcc.org/).

26. Check ATCC website (https://www.atcc.org/) for the formula of freezing medium for specific cell lines.

27. The concentrations, MW, and ratio of these materials may be altered based on the desired hydrogel properties.

28. Take the materials out of the freezer and let them come to room temperature before opening them.

29. When using materials, keep them on the top layer of a bucket of ice (not submerged).

30. Autoclave all equipment prior to the experiment if you are making sterile hydrogels.

   (a) 0.5 mL Eppendorf caps, amber 1.5 mL tubes, forceps, and spatulas, etc.

31. Turn on the UV light so that it has an adequate amount of time to warm up and reach a stable optical output prior to starting the experiment (about 20 min).

32. This step requires gentle and rapid operation.

33. The tissue must be completely submerged in the solution throughout the experimental period.
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Microfluidic Protocols for the Assessment of Anticancer Therapies in 3D Tumor-Stromal Cocultures

Karla Paterson and Michele Zagnoni

Abstract

Microfluidic technologies allow the generation of large datasets using smaller quantities of cells and reagents than with traditional well plate assays. Such miniaturized methods can also facilitate the generation of complex 3D preclinical models of solid tumors with controlled size and cell composition. This is particularly useful in the context of recreating the tumor microenvironment for preclinical screening of immunotherapies and combination therapies at a scale, to reduce the experimental costs during therapy development while using physiologically relevant 3D tumor models, and to assess the therapy’s efficacy. Here, we describe the fabrication of microfluidic devices and the associated protocols to culture tumor-stromal spheroids for assessing the efficacy of anticancer immunotherapies as monotherapies and as part of combination therapy regimes.

Keywords Microfluidic immunoassay, Combination immunotherapy, Spheroid viability, Microfluidic cocultures, 3D tumor models

1 Introduction

The development of microfluidic technology has enabled the production of large volumes of data to assess the anticancer effects of drugs on 3D human tumor models using miniaturized methods [1]. Lab-on-a-chip devices can be fabricated using several polymeric and elastomeric materials, often created as a replica of a master plate produced by photolithography [2]. Polydimethylsiloxane (PDMS) is the most common material used in academic laboratories, as a result of its biochemical characteristics and low cost manufacturing [3]. This material is highly suited for cell culture applications due to its gas permeability and optical transparency. Thermoplastics and glass substrates are instead most commonly used for commercial microfluidic products that can be more easily mass-produced. Microfluidic devices are typically developed as 2D platforms and can be, therefore, straightforwardly interfaced with
microscopy equipment for visualization of micro-flows, single cell tracking, and 3D model imaging, as well as using various spectroscopy techniques for readouts [4–6]. The development of various microfluidic platforms has advanced the use of tumor spheroids for miniaturized screening applications and aided in the expansion of their use for a variety of cancer studies [7]. Advantages of microfluidics, compared to standard 2D and 3D cell culture techniques using well plate or trans-well platforms, include the decreased quantities of samples and reagents required, reduced experimental costs and time to results, higher-throughput potential, and greater control over experimental parameters, including culture of multiple cell types in defined spatial and temporal configurations [4, 8]. Notably, the miniaturization capabilities of microfluidic technology can offer new opportunities for screening limited amounts of live patient samples, creating 3D tumor models and microfluidic drug gradients [1, 9–11]. Many anticancer therapies are now developed as combination therapies, as these can enhance anticancer effects in comparison with individual monotherapies [12–17]. Therefore, there is a need for the development of microfluidic technologies suited to miniaturize drug combination studies.

Using the microfluidic device described in this protocol, we validated miniaturized chemotherapy and immunotherapy preclinical assays to test their efficacy in 3D tumor models, mimicking clinical therapy regimes. We assessed combination carboplatin chemotherapy, anti-PD-L1 checkpoint blockade therapy, and CAR-T cell therapy targeting human triple negative breast cancer spheroids in coculture with stromal cell types to reflect aspects of the native solid tumor microenvironment [18]. Here, we describe the microfluidic platform and protocols that provide powerful, cost-effective, and miniaturized methods for the preclinical assessment of anticancer monotherapies and combination therapy regimes.

## 2 Materials and Equipment

### 2.1 Device Fabrication

| Material          |
|-------------------|
| Chemical fume hood|
| PDMS              |
| Curing agent      |
| Pasteur pipette   |
| Silicon wafer     |
| Aluminum foil     |
| Glass petri dish  |
| Plastic weigh boat|
| Vacuum desiccator |

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Vacuum pump
Oven set to 85 °C
Scalpel
Biopsy punch (4 mm)
Low-adhesion tape and methanol

2.2 Preparation of Devices

- Square polystyrene petri dish (120 mm)
- Oxygen plasma asher
- Synperonic F108 surfactant
- Sterile falcon tubes (50 mL)
- Syringe filter (0.22 μm)
- Phosphate buffered saline (PBS)
- Deionized water
- Autoclave
- Incubator set to 37 °C and 5% CO₂
- Biosafety cabinet
- Basal cell culture media
- Amphotericin B
- Penicillin/streptomycin
- Fetal bovine serum

2.3 Cell Seeding and 3D Cocultures in Microfluidic Devices

- Biosafety cabinet
- Incubator set to 37 °C and 5% CO₂
- Inverted microscope
- PBS
- Trypsin
- Centrifuge
- Complete cell culture media

2.4 Labeling Cells with Membrane Dyes

- Biosafety cabinet
- Incubator set to 37 °C and 5% CO₂
- CellTrace stock solution
- PBS
- FBS
- Complete cell culture media
- DMSO
- Trypsin
- Centrifuge
- Water bath set to 37 °C
## 2.5 Microfluidic Viability Assays

- Biosafety cabinet
- Incubator set to 37 °C and 5% CO₂
- Fluorescein diacetate (FDA) powder
- Acetone
- Propidium iodide (PI) powder
- PBS
- Hoechst stock solution
- Complete cell culture media

## 2.6 Immunotherapy and Combination Therapy Microfluidic Assays

- Biosafety cabinet
- Incubator set to 37 °C and 5% CO₂
- Carboplatin stock solution
- Anti-PD-L1 antibodies
- CAR-T cell solution
- Complete cell culture media

## 2.7 Immunofluorescence in Microfluidic Devices

- PBS
- Bovine serum albumin (BSA) powder
- DMSO
- FBS
- Triton-X
- PFA
- Primary and secondary antibodies

## 2.8 Solutions Prepared

- The following reagents are prepared at room temperature and are stored at 4 °C unless otherwise stated.

### 2.8.1 Synperonic F108 Solution

1. In a chemical fume hood, a 1% w/v synperonic solution is prepared by weighing out 0.5 g of Synperonic F108 into a 50 mL falcon tube.
2. 49 mL of deionized water is added.
3. 0.5 mL of penicillin/streptomycin and 0.5 mL of amphotericin B are added.
4. The lid of the falcon tube is screwed on and the tube is inverted.
5. The synperonic solution is left at 4 °C overnight to dissolve.
6. The next day, the solution is filter sterilized using a 0.22 μm filter.
2.8.2 Phosphate Buffered Saline
1. Four phosphate buffered saline (PBS) tablets are added to a clean and empty 500 mL glass bottle.
2. 400 mL of deionized water is added.
3. The bottle is capped without tightening the lid to allow air to escape from the bottle during autoclaving.
4. The lid of the bottle is taped with autoclave tape.
5. The bottle is autoclaved for 15 min and stored at 4 °C once cooled.

2.8.3 Cell Culture Media
1. Cell culture media is prepared in a biosafety cabinet.
2. Basal media is supplemented with 10% FBS, 1% amphotericin B, and 1% penicillin/streptomycin (10,000 units/mL).
3. 50 mL aliquots of complete media are filter sterilized using a 0.22 μm filter.

2.8.4 Membrane Dye Solutions
1. PBS buffer solution is prepared containing 2% FBS by adding 1 mL of FBS to 49 mL of PBS and filter sterilized using a 0.22-μm filter.
2. Quenching solution is prepared containing 20% FBS by adding 10 mL of FBS to 40 mL of PBS and filter sterilized using a 0.22 μm filter.
3. CellTrace stock solution is prepared by adding the manufacturer recommended volume of dimethyl-sulfoxide (DMSO) to a CellTrace stock vial and pipetted up and down to mix (see Note 1).
4. The solution is immediately prepared prior to use in experiments and stored at −20 °C for up to 1 week after preparation.

2.8.5 Viability Staining Solution
1. FDA stock solution is prepared by weighing out 5 mg of FDA powder and dissolved in 1 mL of acetone in a glass vial. Stock solution is wrapped in tinfoil to protect from light and stored at −20 °C.
2. PI stock solution is prepared by weighing out 2 mg of PI powder and dissolved in 1 mL of PBS in a 1.5 mL Eppendorf tube. Stock solution is wrapped in tinfoil to protect it from light and stored at 4 °C.
3. Viability staining solution is always freshly prepared and protected from light. 50 μL of PI stock solution, 8 μL of FDA stock solution, and 1.5 μL Hoechst stock solution (20 mM) are added to 5 mL of cell culture media.
2.8.6 Solutions for Immunofluorescence in Microfluidic Devices

1. In a chemical fume hood, 0.1% PBSB solution is prepared by weighing out 10 mg of bovine serum albumin (BSA) powder into a 15 mL falcon tube and adding 10 mL of PBS. The solution is left in the fridge to dissolve for use the following day.

2. In a chemical fume hood, PBSBDT blocking solution is prepared, (PBS containing 0.5% Triton-X, 1% DMSO, 1% BSA, and 1% FBS) by weighing out 100 mg of BSA powder into a 15 mL falcon tube. 9.75 mL of PBS, 100 μL of DMSO, 100 μL of FBS, and 50 μL of Triton-X are added. The solution is left in the fridge to dissolve for use the following day.

3 Methods

The following instructions describe the fabrication of multilayered microfluidic devices for the coculture of cancer-stromal spheroids using soft lithography techniques, creating a replica mold from a patterned silicon wafer, in accordance with established protocols [1]. Multilayered microfluidic devices consist of a multitude of channels, each connected by two open wells (Fig. 1). Each channel contains arrays (5 × 5 or 8 × 8) of square microwell traps of dimension 150 × 150 × 150 μm³ or 250 × 250 × 200 μm³. All microfluidic devices are for single-use only. Spheroid formation in microfluidic devices is monitored via bright-field microscopy, and images are collected daily or as required. To ascertain spheroid viability, spheroids are stained using a solution of FDA to identify live cells, PI to identify dead cells, and Hoechst33342 to identify cell nuclei. Spheroids are subsequently fixed and stained for proteins of interest using immunofluorescence protocols. The following procedures are conducted at room temperature.

Fig. 1 Schematic diagram of microfluidic device design (left) and image showing array containing 25 microwells (right), each microwell containing a cancer cell and fibroblast 3D coculture
3.1 Device Fabrication

1. In a chemical fume hood, the required volume of PDMS is weighed out into a plastic weighing boat.

2. The scale is reset, and a 1 mL Pasteur pipette is used to dispense the required volume of curing agent into the weigh boat that will result in a 10:1 PDMS to curing agent ratio.

3. A Pasteur pipette is used to thoroughly mix the solution for at least 1 min until bubbles can be seen and the solution looks uniform (see Note 2).

4. The PDMS solution is poured onto a patterned silicon wafer, wrapped in aluminum foil, inside a glass petri dish.

5. The wafer is placed inside a vacuum desiccator, connected to a vacuum pump, and switched on. The surface of the PDMS solution should turn white due to air bubbles arising from the bulk PDMS within minutes.

6. After 10 min, the air influx valve of the desiccator is closed, and the tubing is disconnected from the pump, before switching the pump off.

7. The wafer is left in the desiccator for 20–30 min until all air bubbles have disappeared and then the valve is slowly opened.

8. The wafer is transferred to an oven at 85 °C for a minimum of 3 h to allow curing of the PDMS solution. Oven shelves should be level to ensure an even thickness of PDMS devices (see Note 3).

9. The wafer is removed from the oven and cooled down to room temperature. The wafer is allowed to cool for at least 30 min.

10. PDMS layers are cut from the wafers using a scalpel. Care should be taken to avoid damage to the silicon wafer.

11. Device open wells are cut using a surgical biopsy punch with the desired size (4 mm diameter in this protocol).

12. Methanol rinse and low-adhesion tape are used to remove any debris or fibers from PDMS layers (see Note 4).

13. Care should be taken to keep the PDMS components clean and free from dust and fibers, inside a plastic dish.

14. In a clean room environment, PDMS layers are placed with the sides to be bonded facing upward on a plastic dish.

15. PDMS layers are placed inside an oxygen plasma generator for 6–18 s (depending on device size) at 100 W power.

16. One layer is carefully aligned on top of the other and gently pressed down.

17. Once devices have been bonded together, they are stored in an oven overnight at 85 °C to strengthen the bonding between layers. Devices are kept dry and at room temperature until required.
### 3.2 Preparation of Devices

1. Devices are placed in an appropriate plastic dish and transferred to a cleanroom environment.

2. Devices are exposed to oxygen plasma treatment for 2 min at 200 W power, 4.5 sccm.

3. Immediately after plasma treatment, devices are transferred to a biosafety cabinet.

4. To create ultralow-adhesion conditions, 20 μL of 1% Synperonic F108 solution is injected into one well of each channel and then repeated in the opposite well. This ensures the flow of fluid through microfluidic channels.

5. Devices are transferred to an incubator set at 37 °C and 5% CO₂ and left overnight.

6. The next day, in a biosafety cabinet, synperonic solution is removed from devices, and the channels are washed once with PBS.

7. Devices are washed once with the cell culture media required for the desired assay.

8. Cell culture media is injected in devices and transferred to an incubator prior to cell seeding.

### 3.3 Cell Seeding and 3D Cocultures in Microfluidic Devices

1. Both wells of each channel are emptied prior to cell seeding (see Note 5).

2. For spheroid formation, the desired cell type is prepared at a concentration of 1–7 × 10⁶ cells/mL. If spheroids are to be formed from more than one cell type, cell suspensions are prepared to the required cell density prior to combining them together (see Note 6).

3. A 10 μL pipette is used to inject 5 μL of the cell suspension into one channel well (Fig. 2).

4. To observe the flow of cells through the channel and into microwell traps, the device is moved on an inverted microscope.

5. Once flow has ceased, 35 μL of culture media is added to both channel wells simultaneously so as not to disturb cells (see Note 7).

6. Devices are transferred to an incubator to allow cells to aggregate and form spheroids, as previously described [1].

7. The next day, media is removed from the open wells to dislodge any cell aggregates that may have formed in the bottom of the open wells.

8. The required volume of media is added to one well and then to the other well, to create a slight flow through the channel.

9. Media is exchanged in this manner every 48 h.
### 3.4 Labeling Cells with Membrane Dyes

1. Prior to injection into devices, cells can be fluorescently labeled using CellTrace™ membrane dyes following the manufacturer's protocol.

2. A stock solution of membrane dye is prepared immediately prior to cell staining. The manufacturer recommended volume of DMSO is added to a CellTrace stock vial. The volume of DMSO to add is dependent upon which CellTrace dye is being used.

3. Cells are trypsinized and centrifuged as normal and resuspended in pre-warmed PBS buffer to a maximum concentration of $1 \times 10^6$ cells/mL in a 15 mL falcon tube.

4. 1 μL of CellTrace dye is added per mL of cell suspension, and the tube is inverted.

5. Cells are transferred to a water bath set at 37 °C for 20 min. The tube is inverted every 5 min.

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**Fig. 2** Schematic diagram of sequential cell seeding and spheroid formation in microfluidic devices
6. After the incubation period, ice-cold quenching solution is added at a volume five times greater than the volume of the staining solution.

7. Cells are centrifuged and the supernatant is removed.

8. Labeled cells are resuspended at the required density for use in experiments.

3.6 Immunotherapy and Combination Therapy Microfluidic Assays

1. At least 24 h after cancer cell seeding and if compact spheroids have formed, anticancer treatment can commence.

2. In a biosafety cabinet, carboplatin stock solution is diluted in cell culture media to the desired concentration for experiments.

3. Culture media is removed from both channel wells simultaneously, and 35 μL of carboplatin solution is added to one channel well and 40 μL to the other.
4. The next day, anti-PD-L1 antibody solution is diluted in culture media to obtain a 1:100 ratio.

5. Culture media is removed from both channel wells simultaneously, and 35 μL of anti-PD-L1 antibody is added to one channel well and 40 μL to the other.

6. The following day, a CAR-T cell suspension of $10 \times 10^6$/mL is prepared.

7. Culture media is removed from both channel wells simultaneously, and 3 μL of CAR-T cell suspension is injected (Fig. 2). To observe the flow of CAR-T cells, an inverted microscope is used.

8. Once flow has ceased, 35 μL of CAR-T culture media is added to both channel wells simultaneously, so as not to disturb cells, and incubated for up to 72 h.

1. All culture media is removed from both channel wells simultaneously.

2. Devices are washed three times with PBSB by adding 35 μL to one channel well and 40 μL to the other simultaneously and then are removed.

3. 35 μL of PBSB is added to each channel well simultaneously and incubated on ice for 30 min.

4. PBSB and 25 μL of 4% paraformaldehyde (PFA) are added to one channel well and 20 μL to the other well and incubated on ice for another 30 min.

5. PFA solution is removed and devices are washed with PBSB.

6. 35 μL of PBSBDT is added to one channel well and 40 μL to the other well for 1 h at room temperature.

7. The blocking solution is removed and 25 μL of primary antibody is added to one channel well and 20 μL to the other well for 24–48 h and stored at 4 °C. Primary antibodies are added at the manufacturer recommended concentration (usually a 1:100 ratio).

8. Devices are washed with PBSB and incubated at room temperature for 10 min.

9. 25 μL of secondary antibody is added to one channel well and 20 μL to the other well at the manufacturer recommended concentration (usually a 1:200 ratio).

10. Secondary antibodies are incubated for 2 h at room temperature in the dark.

11. Secondary antibody solution is removed and devices are washed twice with PBSB.
4 Notes

1. The volume of DMSO required for resuspension depends on the CellTrace dye selected and ranges from 18 to 40 μL.

2. PDMS and curing agent should be thoroughly mixed and many bubbles should form. Otherwise, there will be an inconsistent texture throughout the cast device.

3. PDMS should not be cured for less than 2 h as partially cured PDMS can result in the leaching of solvents/uncured solutions into device channels, and this can be detrimental to cell culture.

4. Other solvents, such as isopropanol, can also be used. However, these may be absorbed more by PDMS.

5. Open wells should be entirely empty prior to cell seeding to achieve sufficient flow of cells into microwells.

6. For seeding devices with multiple cell types, cell suspensions can be mixed prior to seeding with device culture media consisting of a 50:50 mix of the recommended media for each cell type. However, for immunotherapy studies, cancer cells should be allowed 24–48 h of culture to form compact spheroids prior to the removal of all culture media and injection of immune cells. At this stage, 35 μL of the recommended culture media for the particular immune cells injected can be simultaneously added to both channel wells.

7. Single cells and spheroids can be displaced from microwells if the difference in media addition to each channel well is too great.

8. Staining solutions should be freshly prepared immediately prior to staining. The staining protocol has been adapted from Ibi-di’s application Note 33 [19].

9. To obtain the best image quality, spheroids should be imaged immediately after viability staining. Bleaching effects can arise over time and FDA can leach out of stained spheroids. If a high background signal is observed, repeat the PBS wash once or twice and image promptly.

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A Microfluidic Approach for Enrichment and Single-Cell Characterization of Circulating Tumor Cells from Peripheral Blood

Payar Radfar, Lin Ding, Hamidreza Aboulkheyr Es, and Majid Ebrahimi Warkiani

Abstract

The emergence of enabling technologies for the analysis of circulating tumor cells has been shedding new lights into cancer management in the recent years. However, majority of the technologies developed suffer from excessive cost, time-consuming workflows, and reliance on specialized equipment and operators. Herein, we propose a simple workflow for the isolation and characterization of single circulating tumor cells using microfluidic devices. The entire process can be operated by a laboratory technician without relying on any microfluidic expertise and can be completed within few hours of sample collection.

Keywords Single cell, Circulating tumor cells, Rare cell, Droplet microfluidics, Static droplets, Spiral microfluidics, Enrichment

1 Introduction

Cancer is now the second major cause of death globally, and the prominence of this disease has been increasing in the past decades [1]. Greater than 90% of cancer-associated mortality is caused by metastasis that is defined as the spread of cancer cells from the primary tumor to other tissues of the body [2]. During metastasis, cancer cells enter and translocate through the lymph and bloodstream to microvessels of a distant tissue where the cell penetrates and facilitates proliferation to form a secondary tissue [3]. Early diagnosis and treatment of cancer plays a critical role in the management of the disease and would lead to a better treatment outcome. However, early diagnosis and choosing appropriate treatment for cancer are often a difficult task, often relying on invasive collection of tissue biopsy from patients [4]. Taking tissue sample from patients causes discomfort, clinical risk of damage/
infection, surgical complication, and economic burden. Furthermore, some tumor sites are inaccessible surgically which may not allow for biopsy taking [5, 6].

An alternative to conventional sample taking approaches is isolation and analysis of circulating tumor cells (CTCs) from patient’s blood for diagnosis, monitoring, and prediction of cancer [7]. While CTCs can provide valuable clinical information about primary tumor, CTCs are as rare as one per billions of blood cells in cancer patients, and consequently isolation of them is a difficult task [8]. Herein, we demonstrate a workflow for enrichment, isolation, and single-cell analysis of CTCs using novel microfluidic techniques with minimum dependency on skilled operators and expensive equipment.

2 Materials

2.1 Sample Collection and RBC Lysis

1. 10 mL EDTA tube.
2. RBC lysis buffer (10×).
3. Dulbecco’s phosphate-buffered saline (DPBS), pH 7.4.
4. Low-binding pipette tips.

2.2 CTC Enrichment Using Trapezoidal Spiral Microfluidics

1. Bovine serum albumin
2. Conventional 5 or 10 mL syringe.
3. Syringe pump.
4. 2 mL microcentrifuge tube.
5. Silicone tubing (inner diameter: 0.50”; outer diameter: 0.90”)
6. Adhesive Dispensing Ltd., Syringe Tips (0. 50” Long Tip, Adhesive Dispensing Ltd).

2.3 Static Droplet Microfluidic Device Loading

1. Mineral oil, for molecular biology.

2.4 On-Chip Staining, Enumeration, and Live Cell Imaging

1. Hoechst 33342 Solution.
2. CD45 Monoclonal Antibody (H130), APC.
3. CD326 (EpCAM) Antibody (323/A3), FITC.

2.5 Retrieval and Molecular Studies

1. NEBNext® Single Cell/Low Input complementary DNA Synthesis and Amplification Module—Catalog Number E6421L.
2. Invitrogen SuperScript™ III Platinum™ SYBR™ Green One-Step qRT-PCR Kit—Catalog Number 11736059.
3 Methods

3.1 Sample Collection and RBC Lysis

1. Collect 6–10 mL of patient blood sample inside an EDTA blood tube (see Note 1).

2. Lyse the red blood cells by using an RBC lysis buffer as per suppliers’ instruction.

3. Briefly, dilute the 10× RBC lysis buffer to 1× with deionized water and warm it to room temperature.

4. Remove the sample to a tube where you can add 20 times the volume of whole blood—i.e., 20 mL of 1× RBC lysis buffer per 1 mL of whole blood (see Note 2).

5. Gently vortex the tube(s) and incubate at room temperature and in a dark environment for 15 min.

6. Centrifuge the sample at 500 × g for 5 min. Then aspirate the supernatant carefully without disturbing the pellet.

7. Resuspend the pellet in 4.5 mL of PBS using low-binding pipette tips.

3.2 CTC Enrichment Using Trapezoidal Spiral Microfluidics

This section uses a microfluidic device previously described by Warkiani et al. [9].

1. Firstly, the device is designed using a computer aided design (CAD) software (e.g., SolidWorks—Dassault Systèmes, France). The device used in this application is an eight-loop spiral with 8 mm radius gradually increasing to 24 mm. The cross section of the device was a trapezium with 600 μm base, 130 μm and 80 μm inner and outer heights.

2. Once the CAD design is made, the mold is fabricated by a conventional micro-milling technique on a polymethyl methacrylate (PMMA) sheet for subsequent polydimethylsiloxane (PDMS) casting.

3. PDMS is then prepared in 1:10 mixing ratio with curing agent, poured onto the mold, and degassed subsequently.

4. After degassing, cure the PDMS for 2 h in 65 °C oven until it is fully solidified.

5. Carefully remove the device from the mold by using a tweezer.

6. Punch the inlet and outlets using a biopsy punch with a size matching your tubing.

7. Then the device should be bonded on a substrate of choice—glass or PDMS. While different PDMS bonding approaches can be applied, plasma bonding remains one of the most robust approaches. Plasma bonding is achieved by activating the surface of the chip and substrate using a plasma cleaning machine. Depending on the type of machine used, the treatment would
vary; however, in most cases, 4 min of vacuuming and 2 min of plasma treatment would be sufficient to activate the surface.

8. Once the surface of the chip and substrate is activated, the device is carefully picked up and pressed against the substrate. After firmly pressing away all the visible air bubbles, the device is stored in oven until the experiment.

9. After preparing the device, connect your inlet and two outlet tubes (see Note 3) to the device.

10. To minimize the sample loss during cell adhesion to the PDMS, pre-coat the device by incubating it with 1% bovine serum albumin for 5 min, and then wash it thoroughly using PBS.

11. Load the sample in a 5 mL or 10 mL syringe (see Note 4).

12. By using a syringe pump, inject the sample at 1.7 mL/min flow rate (see Note 5 and Fig. 2a).

13. Collect the enriched sample from the inner outlet inside a 2 mL centrifuge tube (see Note 6). Liquid from outer outlet is waste and can be disposed, unless used for other purposes.

3.3 Static Droplet Microfluidic Device Loading

This section uses a 20 nL static droplet microfluidic device previously described by Ding et al. [10].

1. Firstly, the device is designed using a computer-aided design (CAD) software (e.g., SolidWorks—Dassault Systèmes, France). The device dimensions are shown in Fig. 1c.

2. Once the CAD design is made, it is fabricated by a conventional soft photolithography using spin coated SU8 photoresist on silicon wafer.

3. After the mold is fabricated, PDMS is then prepared in 1:10 mixing ratio with curing agent, poured onto the mold, and degassed subsequently.

4. After degassing, cure the PDMS for 2 h in 65 °C oven until it is fully solidified.

5. Carefully cut out the device from the mold by using a scalpel and tweezer.

6. Punch the inlet and outlets using a biopsy punch with the size of your pipette tips.

7. Centrifuge the sample (prepared in Subheading 3.2) at 500 × g for 5 min, and resuspend in 4 μL of PBS.

8. Using a handheld pipette, aspirate the entire 4 μL sample and inject it into the device through the inlet port (see Note 7).

9. At this stage, the liquid sample has been fractionated into 20-nL droplets by utilizing capillary forces (see Fig. 2b, c). Depending on the downstream application, it is always good
Fig. 1 Overall setup for enrichment of CTCs using a spiral microfluidic device and single-cell analysis of them using a static droplet microfluidic (SDM) device. (a) The general workflow for enrichment and analysis of CTCs. (b) Microfluidic setup of spiral microfluidics under biosafety cabinets for enrichment of CTCs. (c) Single-cell isolation and retrieval of CTCs using a SDM device. Figure 1b(i) [9] and Fig. 1c(i) [10] were reproduced with permission under Creative Commons CC-BY license.
Fig. 2 Experimental outcomes of enrichment, CTC isolation, and molecular studies. (a) Microscopic image of spiral microfluidic device during operation, showing the capturing of CTCs and depletion of peripheral blood cells in the chip. (b) Isolated CTC using static droplet microfluidic (SDM) device. (c) Single-cell isolation rate of SDM device at different sample concentrations. (d) qPCR results comparing the amplification and detection of GAPDH gene using SDM device in comparison with a positive and negative control.

3.4 On-Chip Staining, Enumeration, and Live Cell Imaging

The first and most common type of CTC analysis is counting number of CTCs in human blood which can be indicative of the disease state and progression.

1. For on-chip staining of the cells, prepare a 5 μL cocktail of antibody of your choice as per the manufacturer’s protocols (see Note 8). Typically, Hoechst (nucleus staining), anti-CD45 (leukocyte surface biomarker), and an antibody against a cancer surface biomarker (e.g., EpCAM) are used for CTC enumeration.

2. Remove the oil from the main channel via aspiration using a pipette.
3. Inject the cocktail through the main channel and incubate the chip for 30 min.
4. Replace the liquid in main channel with oil.
5. Image the entire chip over the three fluorescent separate channels corresponding to the used antibodies in step 1.
6. Overlay images of the three fluorescent channels and count the CTC cells. CTC cells can be defined as cells with Hoechst positive, CD45 negative, and EpCAM (or other cancer surface antibody marker) positive.
7. Cells can be incubated and imaged over 48 h or longer by providing fresh media through the main channel. Plasma bonding may enhance the procedure in this step, if overtime culture is needed.
8. Cell viability can be characterized by live and dead staining following the described staining principle in this chapter.

### 3.5 Retrieval and Molecular Studies

For retrieval of the droplet of interest, you should ensure the PDMS chip is temporary bonded to a substrate.

1. In order to retrieve the genetic materials of the droplet containing CTC, first prepare a clean static droplet microfluidic device.
2. Punch the inlet and outlet ports using a 1 mm biopsy punch.
3. Temporarily place the device against a glass slide, and firmly press it.
4. Inject the cell solution sample that was prepared in Subheading 3.2.
5. Follow Subheading 3.4 for staining the cells on chip (see Note 9).
6. Scan the chip to identify droplets containing CTCs, as explained in Subheading 3.4.
7. Simply freeze the chip by placing it on dry ice or an ice block (see Note 10).
8. After 10 min, peel off the PDMS chip.
9. While the glass slide remained on the ice block, using a 2 μL pipette, carefully pick up the droplet of interest and dispose it inside a PCR tube (see Note 11).

Based on the intended downstream analysis—e.g., single-cell real-time PCR—sample can be processed accordingly. In this case, we describe the steps to be taken for single-cell real-time PCR. The kits and reagents used are noted in Subheading 2.5 with their catalog numbers.
Table 1
Detailed program and steps for thermocycling samples

(a) The pre-amplification steps of the reverse-transcribed single-cell cDNA

| Cycle step        | Temperature (°C) | Time (seconds) | Cycles |
|-------------------|-----------------|----------------|--------|
| Initial denaturation | 98              | 45             | 1      |
| Denaturation      | 98              | 10             | 20     |
| Annealing         | 62              | 15             |        |
| Extension         | 72              | 180            |        |
| Final extension   | 72              | 300            | 1      |
| Hold              | 4               | ∞              |        |

(b) The qPCR steps of single-cell pre-amplified cDNA

| Initial denaturation | 95              | 300            | 1      |
| Denaturation         | 95              | 15             | 39     |
| Annealing            | 60              | 30             |        |
| Extension and fluorescence recording | 40          | 60             |        |

10. Add 0.5 μL of 10× lysis buffer, 0.25 μL of RNase inhibitor, and 4.25 μL of nuclease-free water, and incubate at room temperature for 5 min.

11. After that, add 1 μL of Single Cell RT Primer mix and 3 μL of nuclease-free water into the lysed cells, and incubate at 70 °C for 5 min to synthesize first strand of complementary DNA.

12. To make cDNA strands for PCR, reverse transcription must be carried out. Add 5 μL of Single Cell RT Buffer, 1 μL of Template Switching Oligo, 2 μL of Single Cell RT Enzyme Mix, and 3 μL of nuclease-free water into the reaction.

13. Then incubate the solution at 42° for 90 min and 70 °C for 10 min.

14. Lastly, to increase the amount of primary cDNA to be detectable by real-time PCR, pre-amplification should be carried out. Add 50 μL of Single Cell cDNA PCR Master Mix, 2 μL Single cell cDNA PCR Primer, and 28 μL of nuclease-free water to reach a final volume of 100 μL.

15. Sample is then incubated in thermal cycler using the program in Table 1a.

16. Then real-time quantitative PCR can be performed to amplify and detect the expression level of specific genes using the appropriate primers. Add 25 μL of Master Mix, 1 μL of Taq Mix, 1 μL of forward and reverse primers, 2 μL of sample, and 20 μL of nuclease-free water (final volume of 50 μL) to the PCR well plate.
17. PCR to be undertaken using a CFX96 thermal cycler as per
instructions in Table 1b.

18. Results is then extracted and analyzed using CFX Maestro
(see Fig. 2d).

4 Notes

1. Ideally you should process the sample as soon as possible in
order to achieve maximum CTC viability. However, samples
may be stored up to 24 h in fridge for certain applications
including quantification.

2. Depending on the sample volume, you may need to divide it
into different centrifuge tubes. Try using the minimum num-
ber of tubes, as transferring liquid would cause cell loss.

3. The outlet tubes must be the same length in order to achieve
optimum cell enrichment efficiency.

4. When loading the syringes, ensure no sample is left inside the
centrifuged tubes. You may wash the tubes using minimal
volume of PBS to aid with retrieving all the samples.

5. Place the syringe pump vertically at a height above the chip to
avoid cell sediment and consequently sample loss.

6. Depending on your starting volume, use appropriate collection
tube with capacity more than one-third of the processing sam-
ple. For example, if 5 mL of sample was processed in step 3
Subheading 3.2, then use 2 mL centrifuge tubes to collect the
sample safely.

7. After the sample filled the device, firmly inject air to ensure the
entire sample has been fractionated.

8. Depending on the concentration of the supplied antibody, the
volume, final concentration, and incubation time of your anti-
body staining must be optimized to suit the application.

9. Off-chip staining before injection of sample is possible as well.
Follow the instructions provided by the antibody supplier.

10. This step can alternatively be done by placing the chip in a −
20 °C freezer for 10 min. In case the sample is to be processed
later in time, you may use a RNA preserving additive such as
RNA later.

11. In this step, ensure you are using low-binding pipette tips. To
avoid any unnecessary molecular content loss due to melting of
the nanoliter drop inside the pipette tip, aspirate 1 μL of 0 °C
mineral oil and dispose it with the drop into the PCR tube.
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Rapid On-Site Evaluation (ROSE): A Microfluidic Approach

Filipe Marques, Janosch Hauser, and Niclas Roxhed

Abstract

Rapid on-site evaluation (ROSE) increases the diagnostic accuracy of fine-needle aspiration (FNA) samples from cysts, a sack-like fluid-containing tissue that sometimes can be precancerous, but is highly dependent on the skills and availability of cytopathologists. We present a semiautomated sample preparation device for ROSE. The device consists of a smearing tool and a capillary-driven chamber that allow smearing and staining of an FNA sample in a single platform. Here, we show the capability of the device to prepare samples for ROSE, using a human pancreatic cancer cell line (PANC-1) and liver, lymph node, and thyroid FNA model samples. Using microfluidics, the device reduces the equipment needed in an operating room for FNA sample preparation, which may lead to a wider implementation of ROSE in healthcare centers.

Key words Capillary microfluidics, Point-of-care, Cytology, Cystic fluid, Fine-needle aspiration, Rapid on-site evaluation

1 Introduction

Rapid on-site evaluation (ROSE) is a point-of-care service during fine-needle aspiration (FNA) interventions that assesses the adequacy of cystic samples for diagnostics. Sample adequacy is evaluated by the number of relevant cells in a sample that determine the malignancy of a cyst. ROSE reduces the overall number of cyst punctures required for an adequate sample and the number of FNA procedures.[1] ROSE starts by placing a small volume of the FNA sample onto a glass slide. This sample is manually smereed to obtain a thin layer of cells dispersed along the glass slide. The sample is air-dried and placed in a container to be stained, often with a rapid Romanowsky-type stain. Excess stain is washed, and lastly a morphological evaluation of the stained cells under a microscope allows to determine the adequacy of the collected FNA sample [2]. FNA sample properties, such as viscosity, cell concentration, and enzyme concentration, depend on the type of cyst, source organs, and patient [3–5]. Consequently, performing of
ROSE requires expertise and is routinely executed by cytopathologists. However, cytopathology resources are often placed elsewhere, due to time constraints or availability [1, 6]. ROSE sample preparation can be reproduced with ThinPrep® instruments or alternative evaluators (AE), e.g., medical doctors, nurses, or cytotechnicians, educated to implement ROSE [7]. Yet, ThinPrep® are bulky instruments not readily available at the operating room to prepare time-critical samples on-site, and AE take up to a year of training with a diagnostic accuracy ranging from 73.5% to 98% depending on operator skill [7, 8]. In general, microfluidics is cost-effective, portable, efficient, and reliable with several biomedical applications in cancer diagnosis. Capillary-driven microfluidics work on the principle of capillary action with no requirements on energy supply or external pumping apparatus, facilitating the design of ready-to-use devices for point-of-care diagnostics and disposability [9].

Here, we describe the fabrication and experimental procedure for a point-of-care sample preparation device that allows healthcare personnel to perform ROSE, as discussed in previous work [10]. The device comprises of two main components: a smearing tool and a capillary-driven microfluidic chamber with a water-soluble film containing a cell stain, designed to prepare samples for ROSE. Using the device, several FNA model samples with different viscosity and origin, such as pancreatic cells (PANC-1), porcine liver, lymph node, and thyroid samples, can be prepared [10].

## 2 Materials

### 2.1 Instruments for ROSE Preparation Device Fabrication

1. Smearing tool and hemispherical holder fabrication with a computer numerical control (CNC) milling machine with micrometer precision.

2. PVA film solution was mixed with a hot plate stirrer.

3. Paper substrate lamination with a HeatSeal™ H600 Pro laminator.

4. Hydrophilic sheet and PVA layer lamination with a thin-film applicator.

5. Adhesive tape and hydrophilic sheet projection with a cutting plotter.

6. Blotting paper and cell counting area frame cutting with a laser cutter.

### 2.2 Smearing Tool

1. Aluminum.
2.3 **PVA Film with Giemsa Stain**

1. Poly(vinyl alcohol) (PVA) 36,627 (Sigma-Aldrich).
2. Wright-Giemsa Stain, Modified WG16 (Sigma-Aldrich).
3. DI water.
4. Absolute ethanol.
5. Whatman® Anotop® 10 Plus syringe filter (Merck).

2.4 **Blotting Unit**

1. Ahlstrom grade 270 and 601 (Ahlstrom Filtration LLC).
2. Poly(vinyl alcohol) (PVA) 360627 (Sigma-Aldrich).

2.5 **Microfluidic Chamber Fabrication**

1. Xerox multipurpose paper.
2. Laminating pouches A4 100 μm.
3. Hydrophilic sheets composed of Xerox type C laser printing transparency.
4. Double-sided adhesive tape 64620 (Tesa).
5. SuperFrost Plus™ Adhesion slides (FisherScientific).

2.6 **PANC-1 Cell Culture**

1. Dulbecco’s Modified Eagle Medium (DMEM) (high glucose).
2. Fetal bovine serum (FBS).
3. Penicillin-streptomycin (PS).
4. Gibco™ GlutaMAX™.

2.7 **1 cP and 57 cP FNA Model Samples**

1. DI water.
2. White sugar cubes.

2.8 **Data Acquisition**

1. Brightfield microscopy using 5×, 10×, 20× and, 40× objectives.

3 **Methods**

3.1 **Smearing Tool Fabrication**

1. Mill a slope using a 1 mm end mill with each vertical step being 1 μm.
2. Use wet and dry sandpaper to round the edges.

3.2 **PVA Film with Giemsa Stain**

1. Prepare a 4% (w/v) PVA solution on a hot plate stirrer at 500 rpm and 50 °C for 1 h.
2. Prepare 45-mg/mL solution of Giemsa stain in absolute ethanol on the hot plate stirrer at 500 rpm and 40 °C for 1 h.
3. Mix both solutions at a 2:1 ratio (PVA/Giemsa stain).
4. Pass the mixture through a Whatman® Anotop® 10 Plus syringe filter to remove all unsolved particles.
3.3 Blotting Unit

The blotting unit consists of a water-soluble PVA film with a controlled thickness, to act as a passive time delay valve, and a porous paper, to absorb liquid when the PVA film has dissolved. Paper and PVA film are assembled by lamination at 80 °C using a laminator.

The PVA film for creating a passive time delay is fabricated as follows:

1. Prepare a 20% (w/v) aqueous solution from granular polymer.
2. Place primary container with PVA solution into a water bath.
3. Use hot plate stirrer at maximum stirring rate at 80 °C until majority of polymer flakes have dissolved (see Note 1).
4. Pass solution through a Whatman® Anotop® 10 Plus syringe filter to remove unsolved particles.
5. Use either a spin-coater or a thin-film applicator to create thin films.
6. Measure the thickness of the dry film using a thickness gauge, and visually inspect film for artifacts.
7. The dissolving time of the film is influenced by several parameters, such as thickness, molecular weight of the polymer material, temperature, and relative humidity (see Note 2).

3.4 Microfluidic Chamber Fabrication

The microfluidic chamber is fabricated in a layer-by-layer fashion from hydrophilic plastic films and double-sided tapes. The layers are structured individually by laser or blade-cutting, according to a design created using CAD (computer-aided design) software. The individual steps are as follows:

1. Structure layers individually.
2. Using alignment pins and corresponding wholes, assemble layers.
3. Use laminator at room temperature to firmly attach layers to each other.
4. To include the dry reagent film, invert assembled microfluidic chamber and add 300 μL of the stain solution (see 3.2 PVA films with Giemsa stain).
5. Let the solution dry on a hot plate at 40 °C for 1 h.

3.5 Instructions for Use

1. Add 10 μL of an FNA sample to the proximal end of a Superfrost® Plus adhesion glass slide.
2. Place the smearing tool on top of the sample for 5 s.
3. Move the smearing tool at approximately 5 cm/s from the proximal to the distal end of the glass slide.
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**Fig. 1** (a) The five device operation steps and (b) detailed workflow of the portable microfluidic device for preparing samples for ROSE. (i) Place sample aliquot on a glass slide, and bring the smearing tool in contact with the sample. (ii) Smear the sample and allow to dry in air. (iii) Fold the microfluidic staining chamber to cover the smear. (iv) Add water, which fills the microfluidic chamber by capillary action and releases the stain from the dry film. Cell staining proceeds until the dissolvable valve opens and the liquid is absorbed by the downstream blotting paper. (v) Remove the chamber and allow to air-dry before microscopy inspection. (Reproduced with permission from Royal Society of Chemistry [10])

4. Leave the smearing tool at the proximal end of the glass slide and allow air dry for 5 min.
5. Laminate the microfluidic chamber by folding it over on the glass slide (Fig. 1a-iii).
6. Apply 300 μL of DI water to the inlet part of the chamber.
7. Once the water is blotted, remove the chamber from the glass slide.
8. Air-dry for 1 min (see Note 3).

### 3.6 PANC-1 Cell Culture

1. Use an adhesion promoted cell culture flask to culture PANC-1 cells.
2. Add a cell media mixture containing DMEM (high glucose) media with 10% FBS, 0.5% PS, and GLUTAMAX supplement.
3. Split cells and change media two times a week.
4. Wash the cells with PBS and trypsin, to promote cell suspension splitting.
5. Collect cells suspended in 1–2 mL fresh media with suspended cells after every cell splitting (see Note 4).

### 3.7 1 cP and 57 cP FNA Model Samples

Cystic samples present viscosities within 1 mPa·s and 10 mPa·s, with the possibility of highly viscous samples that cannot be aspirated via FNA. As such, we created typical and atypical FNA sample models with viscosities of 1 mPa·s and 57 mPa·s, respectively.
1. Place 1 mL of PANC-1 cell suspension in an Eppendorf tube, finalizing a 1 mPa·s sample.
2. Prepare a sugar solution in small container by adding white sugar to DI water in a 60% (w/w) ratio.
3. With a spatula, mix for approximately 10 min.
4. Centrifuge another 1 mL of PANC-1 cell solution at 11000 G.
5. Carefully remove as much of the supernatant as possible without disturbing the pallet.
6. Add 1 mL of the previously prepared sugar solution to the latter pallet.
7. Homogenize the solution with a pipette, by aspirating and dispensing liquid in 2-s cycles for approximately 2 min (see Note 5).

3.8 Cell Counting Area Frame

1. Design a cell counting area frame with a $1 \times 2$ cm$^2$ window using Adobe Illustrator or similar software compatible with the laser cutter.
2. Cut a PMMA sheet following this design with the laser cutter.

3.9 Smearing Speed Assessment with a Motorized Stage

We used a custom-built motorized stage to characterize a good smearing speed for both of 1 mPa·s (see Fig. 2a) and 57 mPa·s (see Fig. 2b) FNA sample models. We performed the following:

1. Attach the smearing tool to a positioner above a motorized stage.
2. Place a Superfrost® Plus adhesion glass slide on the stage.
3. Place 10 μL of a sample prepared in Methods 3.7, aligned with the smearing tool.
4. Use the motorized stage to smear in the following steps (see Fig. 2c):
   (a) Move the smearing tool in Z until there is contact with the glass slide.
   (b) Wait for 5 s.
   (c) Move 4 cm in Y at 1, 3, 5, and 7 cm/s.
   (d) Return to the original Z position.
5. Air-dry for 5 min.
6. Fill a Coplin jar with Wright-Giemsa stain and Modified WG16 and another with DI water.
7. Place the prepared glass slide in the jar with stain for 30 s.
8. Remove the glass slide from the first jar and dip it ten times in the jar with water.
9. Air-dry for 5 min.
Fig. 2 Measured number of cells per area at different smearing speeds for PANC-1 water-based samples with viscosity of (a) 1 mPa·s and (b) 57 mPa·s. Smear illustrations exemplify the lateral spread of the sample, at smearing speeds ≥5 cm/s and ≥3 cm/s for 1 mPa·s and 57 mPa·s samples, respectively. The blue box indicates the cell counting area (CCA). (c) The chamber filling ratio over time as extracted from video sequences resolves the three stages of the microfluidic staining sequence: chamber filling, outlet valve dissolving, and chamber emptying. The plotted data is calculated as an average from six devices, and error bars represent standard deviations. (d) Areal density of dyed cells after preparation of a low viscosity sample at approximately 5 cm/s versus the cell concentration of the sample and respective linear curve fit (dashed line). (Reproduced with permission from Royal Society of Chemistry [10])

10. Place the glass slide in the microscope stage with cell counting frame above it.

11. Use the 10× objective to manually count the cells within the area.

3.10 Linearity Characterization

We characterized the linearity of the device by preparing samples with three different cell concentrations and assessed the number of cells stained within the cell counting area (see Fig. 2d).

1. Prepare a 1:1 and 1:3 dilution of a PANC-1 cell suspension. Use these dilutions and stock suspension as samples.

2. Prepare these samples according to Methods 3.5, until step 3.5.6.

3. Use Ahlstrom grade 222 for blotting after 45 s.

4. Detach the microfluidic chamber from the glass slide.
5. Air-dry for 1 min.
6. Follow steps 3.9.9 to 3.9.11.

### 3.11 Liver, Lymph Node, and Thyroid FNA Model

A section of the liver and thyroid and lymph node were removed postmortem from a swine and placed in a container with 0.9% saline solution for transport. FNA sample preparation with these organs went as follows:

1. Prepare an Eppendorf tube with 1 mL of DPBS.
2. Wash these organs with tap water for approximately 30 s.
3. Place the organs on a Petri dish.
4. With a scalpel, perform a 1 cm-deep incision on the surface of the organ.
5. Whittle the scalpel against the exposed organ walls, in an up and down movement for approximately 30 s (see Note 6).
6. Transfer the resulting liquid to the Eppendorf tube.
7. Homogenize the solution, following the 3.6.7 procedure (see Note 7).

### 3.12 5× and 40× Imaging of PANC-1, Liver, Lymph Node, and Thyroid Cells

1. Prepare PANC-1, liver, lymph node, and thyroid FNA samples according to Methods 3.5, until step 3.5.6.
2. Use Ahlstrom grade 222 for blotting after 45 s.
3. Detach the microfluidic chamber from the glass slide.
4. Air-dry for 1 min.
5. Place a cover slip at the center of the smear.
6. Image using a 5× objective.
7. Place a droplet of Immersol 518F on top of the coverslip.
8. Image using a 40× objective (see Fig. 3).

### 3.13 Hemispherical Holder

1. Mill a 5 cm in diameter hemisphere into a PMMA cube, using a 1 mm rounded end mill.
2. Use wet and dry sandpaper to round the edges.

### 3.14 Small Intestine Tissue Preparation

To better represent the complexity of a liquid from a cyst, we prepared an FNA sample model containing cells and a tissue fragment. For that, a section of small intestine was removed postmortem from a swine and placed in a container with 0.9% saline solution for transport. Sample preparation went as follows:

1. Place the small intestine in a Petri dish.
2. With a scalpel, cut a 3 cm section of the intestine.
3. Expose the luminal side of this section by performing a longitudinal cut.
Fig. 3 Contrast enhanced images of different cell types prepared with the ROSE sample preparation device: (a) liver cells, (b) thyroid cells, (c) lymph node cells, and (d) PANC-1 cells. Individual stained cells are clearly visible as blue dots on a bright background. (Reproduced with permission from Royal Society of Chemistry [10])

4. Wash for 1 min with tap water, and simultaneously move your fingers along the section as to remove any sustenance remnants.

5. Spread this section in the hemispherical holder with the lumen facing upward.

6. Place 500 μL of DMEM in the holder.

7. Use a Dentalux® rubber interdental brush against the lumen (see Note 8).

8. With a pipette, aspirate 500 μL of the latter solution.

9. Cut a 2 × 1 mm² fragment from the intestinal section and place it in the latter solution.

3.15 Small Intestine Tissue Imaging

1. Use the FNA model just created plus tissue fragment.

2. Prepare these samples according to Methods 3.5, until step 3.5.6.

3. Use Ahlstrom grade 222 for blotting after 45 s.

4. Detach the microfluidic chamber from the glass slide.
5. Air-dry for 10 min.
6. Image with 5× and 20× objectives (see Fig. 4).

3.16 Data Treatment

1. Prepare the data using columns plot with mean and standard deviation.
2. Assess the linearity using a linear regression function.
3. Assess the cell images by determining cell size, contrast in relation to the background, and cell features.

4 Notes

1. Step 3 may take several hours.
2. Store PVA films in a controlled environment to achieve reproducible dissolving times.
3. The device can (theoretically) be used to prepare blood smears. Samples prepared with this device can be reutilized, e.g., genetic analysis. The smearing tool and the microfluidic chamber can be used separately if needed.
4. It is common practice to use DMSO in cell cultures to protect cells from freezing damage. For the purpose of smearing cells, do not add DMSO or cells will not attach to the glass slide. Avoid freezing these cells. We noted that PANC-1 cells can be refrigerated for at least 3 days at 4 °C.

5. Vigorous mixing can create air bubbles that result in wide variations in cell position upon smearing. If so, one could use a vacuum chamber to remove the bubbles. The viscosity of the sugar solution is highly dependent on the room temperature. 57 mPa·s corresponds to a room temperature of 22 °C.

6. A lymph node ranges from 0.1 to 2.5 cm, so it can be simpler to hemisect for better handling.

7. When preparing real clinical samples, be aware of blood clots that can compromise the quality of the smear and staining.

8. One can brush the lumen with any means of brushing as long as the majority of cells are not caught in the brush, e.g., with a toothbrush. In an open cyst model, cells should be freely suspended in the liquid.

Acknowledgments

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Microfluidic Acoustic Method for High Yield Extraction of Cell-Free DNA in Low-Volume Plasma Samples

Alvaro J. Conde, Ieva Keraite, Nicholas R. Leslie, and Maïwenn Kersaudy-Kerhoas

Abstract

Cell-free DNA has many applications in clinical medicine, in particular in cancer diagnosis and cancer treatment monitoring. Microfluidic-based solutions could provide solutions for rapid, cheaper, decentralized detection of cell-free tumoral DNA from a simple blood draw, or liquid biopsies, replacing invasive procedures or expensive scans. In this method, we present a simple microfluidic system for the extraction of cell-free DNA from low volume of plasma samples (≤500 μL). The technique is suitable for either static or continuous flow systems and can be used as a stand-alone module or integrated within a lab-on-chip system. The system relies on a simple yet highly versatile bubble-based micromixer module whose custom components can be fabricated with a combination of low-cost rapid prototyping techniques or ordered via widely available 3D-printing services. This system is capable of performing cell-free DNA extractions from small volumes of blood plasma with up to a tenfold increase in capture efficiency when compared to control methods.

Key words Cell-free DNA, Liquid biopsy, Micromixer, Acoustofluidics, Microfluidics

1 Introduction

Blood-based circulating nucleic acid markers have gained considerable interest in the last few years as tools for detecting and monitoring cancers via minimally invasive blood samples: so-called liquid biopsies. In many studies, due to the very low concentration of the analytes of interest, plasma volumes between 1 mL and 5 mL obtained from venipuncture are usually required. Such large volumes restrict the frequency at which serial samples can be collected. However, there is seminal evidence that small volumes of plasma samples (<500 μL) may provide sufficient cell-free DNA (cfDNA) and circulating tumor DNA (ctDNA) concentrations for longitudinal studies and eventually cancer treatment monitoring. In one study, cfDNA obtained from just 25 μL of plasma was used...
to measure tumor burden in mice [1]. In another study, cfDNA extracted from 200 μL aliquots was used to demonstrate that cytotoxic chemotherapy can produce rapidly observable dynamic effects on cfDNA levels [2]. In another pilot study, whole-genome amplification was performed on finger prick blood samples (75 μL), and comparable allelic fractions of somatic mutations between the finger prick sample and matched venous blood were demonstrated [3]. Finally, a recent study showed that ctDNA can be detected and monitored in dried blood spots (50 μL) and could enable new approaches for sample collection and novel study/trial designs for both patients and in vivo models [4]. There is an urgent and unmet need to develop rapid and affordable technologies to diagnose cancer but also to monitor the patient-specific response to cancer therapies. The rapid isolation of cfDNA and ctDNA could allow monitoring and timely adjustments of cancer therapies, avoiding and under- or overdosing potentially life changing side effects and inappropriate usage of costly treatments. However, the isolation of cfDNA and ctDNA from limited volumes of blood represents a technical challenge due to the limited total number of available molecules.

Microfluidic technology is amenable to the manipulation of small volumes and has been proposed to reduce the time to result in a decentralized healthcare system, in the context of cancer detection and monitoring [5, 6].

Most of the current cfDNA extraction methods are based on silica membranes or silica magnetic microbeads [7]. The latter has been reported to have a higher preference for low molecular weight DNA molecules (up to 300 bp) [8], which helps to circumvent cellular genomic DNA contamination in the sample. However, one of the main challenges of this assay is that after the application of a strong magnetic field, the beads tend to aggregate with each other, forming clusters. This issue can compromise the reproducibility and efficiency of the assays since the cluster population can be quite heterogeneous and less surface area is available for the capture assays. Having less surface area has a significant impact in assays where the target analyte is in very low concentrations, such as cfDNA. Extraction efficiency with magnetic bead-based solutions can be improved by the use of micromixers.

Micromixers are essential components in microfluidic devices and have a direct impact on the efficiency and sensitivity of assays [9]. Mixing in microfluidics is also one of the main challenges since inertial effects are almost irrelevant due to the low Reynolds number in which these systems usually operate. Consequently, researchers endeavor to circumvent the limits of purely diffusive mixing by introducing structures or active mechanisms that disturb the flow with the ultimate objective of diminishing the striation length and increasing the area across which diffusion takes place, thus enabling a rapid homogenization of the solution [10]. Acoustic micromixers
are popular because they can deliver high mixing efficiency without the integration of fragile movable components in opposition to their magnetic counterparts [9, 11–13]. Various acoustic principles have been implemented, such as surface acoustic wave (SAW) [14], ultrasound [15], vibrating membranes [16], sharp-edge structures [17], micropillars [18], and cilia [19]. Nevertheless, a number of these devices are fabricated using photolithographic techniques which are not widely available and can be cost-prohibitive for many groups.

Mixers based on the acoustic actuation of air bubbles have been proposed to circumvent some of the issues encountered by other types of mixers. In this particular type of acoustic mixers, air bubbles trapped within miniaturized devices expand and contract under a sound wave, for example, generated by a piezoelectric device. The high frequency oscillation of the bubbles generates a very powerful microstreaming effect that delivers efficient mixing, with mixing times down to a couple of milliseconds [20]. Another advantage of these mixers is that the actuation drivers are typically economical, consume little power, and do not require significant space.

A number of bubble-based micromixers [21–32] of various shapes and performances have been proposed, but most of them are fabricated using soft lithography, which requires specialized equipment and is difficult to mass-manufacture. While the total cost of microfluidic chips fabricated using soft lithography can be prohibitive and unaffordable for many research groups, the prototyping requirements may also be detrimental to the broader use of such technologies [33]. CNC micro-milling [26, 27], laser-cutting [30], and hot embossing [31] have been explored as cheaper for more accessible alternatives, but current designs share a common limitation along with the soft lithography-based devices: the piezoelectric actuator has to be glued using permanent adhesives to the bulk of the chip, impeding the individual control of more than one micromixer per chip and complicating the assembly process.

In a prior publication [34], we have described a novel bubble-based acoustic micromixer module that overcomes many of the aforementioned limitations. In our approach, the micromixer is fabricated using a hybrid strategy, a combination of rapid prototyping technologies that are low-cost and widely available, for example, laser-cutting or 3D-printing. In our method, a user without previous relevant engineering expertise can order all the necessary parts (including the custom microfluidic components) by mail and assemble all of them in a DIY fashion with minimal tools [33].

The results presented in earlier work show that the micromixer can perform effective homogenization of two solutions, in continuous and static flow conditions, with enhanced performances compared to similar devices [34]. The micromixer has demonstrated sensitive detection of ctDNA from low to medium volume of plasma (10–800 μL) in static flow conditions, something unique to our system.
In this method, we detail the step-by-step approach to set up a static flow micromixer module that can be used to extract cfDNA from low-volume plasma samples using a commercial magnetic bead-based assay. The method consists of six distinct steps starting from Step 1, the manufacturing of a mold to create a silicone slab; followed by Step 2, the casting of that silicone slab; Step 3, the manufacturing of the microfluidic chip using laser-cutting and thermal-assisted solvent bonding; Step 4, the assembly of all parts constituting the mixer; Step 5, the extraction of cfDNA on-chip; and finally Step 6, the detection of cfDNA. The microfluidic mixing system can be made by any lab with a 3D printer (preferably a stereolithography printer) and a laser plotter (recommended minimum power: 30 W), and CAD files for all parts are open source. However, we have made provisions for labs that do not have access to resources like a 3D printer and a laser plotter, and all custom parts can be purchased directly from digital manufacturing service providers.

2 Materials

2.1 Microfluidic Chip and Associated Components

The custom microfluidic chips and associated components can be manufactured by different techniques as described in [34]. In this chapter, we describe the assembly of the module including a custom microfluidic chip made from a low-cost rapid prototyping technique based on the use of CO2 laser plotter and solvent-assisted bonding as described in [35]. All CAD files can be downloaded from the BubbleMicromixer project on GitHub: https://github.com/MaiwennKsKh/BubbleMicromixer. Alternatively, for laboratories without access to prototyping facilities, or wanting a quicker method, we recommend to order the stereolithography 3D-printed version of the parts from online digital manufacturing services suppliers. Furthermore, these parts do not require any fabrication technologies or tools beyond what is available in a typical biological lab. The 3D files (stl format) are freely available on GitHub (https://github.com/MaiwennKsKh/BubbleMicromixer). To navigate different CAD files available, use Table 1.

2.1.1 Description of Fabrication Equipment

1. Stereolithographic 3D printer. At time of writing, most fused deposition modeling does not have a resolution suitable for this part. Most hard polymers available for on bench-top stereolithography printer should be suitable for this step. Here, a Formlab Form2 printer was used.

2. CO2 laser plotter. Most CO2 laser plotters of 30 W minimum power will be suitable for the task. Here an Epilog Legend Laser Engraver-Mini 18 (30 W) was used.

3. A vacuum degassing chamber. Here a BACOENG 2 L Vacuum Degassing Chamber Stainless Steel was used.
Table 1
Summary of CAD files available

|                         | Format | This method (using in-house prototyping) | Alternative method (using external 3D-printed parts) |
|-------------------------|--------|------------------------------------------|-----------------------------------------------------|
| Silicone mold           | STL    | ✓                                        | (use in step 3.1 and 3.2)                            |
| Silicone slab           | STL    |                                          | ✓ (Use instead of step 3.1 and 3.2)                  |
| 2D microfluidic chip layers | Dxf (2D) | ✓                                        | (Use in step 3.3; note: The file includes the piezo holder) |
| Microfluidic chip       | STL    | ✓                                        | (Use instead of step 3.3)                            |
| Piezo holder            | STL    | ✓                                        | (Use instead of step 3.3)                            |

4. A heated press capable of reaching at least a load of 2 metric tons and a temperature of at least 70 °C. A PW100–37, Carver, with heated plates was used here.

2.1.2 Description of Materials

1. Formlab Clear Resin, or appropriate material to specific stereolithography printer.
2. PDMS Sylgard 184, Dow Chemical Company.
3. Small disposable transparent cup of at least 50 mL of capacity.
4. Plastic tweezers.
5. 1 mm PMMA casted sheet, to be cut. Here we used Clarex casted sheet. See Note 1 for choice of PMMA.
6. 0.5 mm PMMA casted sheet. Here we used Clarex casted sheet. See Note 1 for choice of PMMA.
7. Alignment aluminum frame made, for example, of two 2 mm-thick aluminum plates with four 1 mm holes spaced as to form a rectangle with a 26.6 mm width and 77.4 mm length allows a flange of at least 1 cm around this rectangle.
8. Absolute ethanol (> 99.95%).
9. Cleanroom tissue.

2.1.3 Description of Components

1. Mounting screws. 2 mm-diameter, 15 mm-long stainless screws, widely available.
2. Fitting frame for the piezoelectric disc. This part is obtained by laser-cutting a 3 mm-thick sheet of acrylic (polymethylmethacrylate) using CO₂ laser plotter. Alternatively, this part can also be 3D-printed (suggested material: PLA, ABS, Rigid SLA/polyjet material) or ordered from a digital manufacturing service provider. See Note 2.
3. **Piezoelectric disc.** 27 mm-wide piezo with leads (7BB-20-6, Murata Electronics). Components with similar maximum frequency of 6.3 kHz and 1 kOhms impedance and obtained from different suppliers will be suitable.

4. **Silicone slab.** This part is casted from a 3D-printed mold (see detailed description in Subheading 3.1). For the 3D mold, download the GitHub design file “silicone mold.” Alternatively, the part can be purchased from a digital manufacturing service provider using the design file “silicone slab” (for material selection, see **Note 3**).

5. **Microfluidic chip.** This part is obtained by laser-cutting and engraving a different layer of transparent acrylic using CO₂ laser plotter using the design file “Cut layers PMMA.dxf.” Alternatively, the 3D-printed chip can be purchased from a digital manufacturing service provider. GitHub design file name: “Microfluidic chip.STL.” See **Note 4** with regard to the choice of 3D-printing material and **Note 5** regarding optical transparency requirements.

6. **Mounting nuts.** 2 mm-diameter, stainless nuts, widely available.

### 2.2 Actuation Setup

A standard function generator can be used as a driver. See **Note 6** for recommended setup.

### 2.3 cfDNA Extraction and Quantification

#### 2.3.1 Reagents

1. Initial sample consisting of human plasma. Human plasma will be obtained by centrifugation from blood samples collected in anticoagulant (preferably EDTA) tubes and less than 5 days old.

2. Magnetic bead-based MagMAX cfDNA isolation kit (Thermo-Fisher Scientific).

3. 80% ethanol solution.

4. Nuclease-free water.

5. Solution of 2% bovine serum albumin (BSA) in 1× PBS.

6. Nuclease-free water. Store at room temperature.

7. Power SYBR® Green Master Mix 2X (Life Technologies). Store at −20 °C.

8. Forward and reverse LINE-1 (long interspersed nuclear elements) primers to amplify a target of 90 bp (LINE FW 5′-TG CCGCAATAAACATACGTG -3′, LINE RV 5′-GACCCA-GCCATCCCATTAC-3′) at 200 nM final concentration. Keep a master stock at 100 μM and make working stocks of 10 μM in nuclease-free water; store all at −20 °C.

9. Human Genomic DNA (Bioline) to make a standard curve with a linear range over five orders of magnitude for absolute quantification.
10. High Sensitivity D5000 ScreenTape (Agilent).
11. High Sensitivity D5000 Reagents (Agilent).

2.3.2 Lab Equipment and Consumables

1. Vortex.
2. Pipettes and disposable tips.
3. DNA LoBind® Tubes (Eppendorf) or similar.
4. Mx3005P Real-Time PCR System (Agilent).
5. 96-well plate or tubes compatible with the real-time PCR system.
6. Automated electrophoresis tool (2200 TapeStation, Agilent).

2.3.3 Other

1. Neodymium magnet to capture the magnetic beads. Cf See Note 7.
2. Adhesive tape (optional).
3. Absorbing tissue.

3 Method

The entire process involves six distinct steps (see Fig. 1). Carry out all procedures at room temperature unless otherwise specified. We recommend the use of adequate personal protective equipment when carrying out this method. As a minimum, a full Howie style lab coat should be worn, as well as goggles and nitrile gloves.

![Fig. 1 Workflow for cfDNA extraction.](https://github.com/MaiwennKsKh/BubbleMicromixer)
3.1 Preparation of the 3D-Printed Mold

To fabricate the slab of step 2 (Subheading 3.2), a 3D-printed part is needed as a mold. The molds contain an array of posts (Fig. 2a) that serve as negative structures for the formation of the pockets after the casting process.

1. Prepare the Form2 printer with Clear Resin following manufacturer’s guidelines.

2. Upload the file “Silicone mould.STL” on the Form 2 printer. Orient the part with the pillars on the opposite direction of the build platform and launch the print. We recommend to print the part directly on the build platform.

3. After the print, wash the printed mold in isopropyl alcohol and UV cured for 1 h under a 355-nm source (80 W cm\(^{-2}\)). If you use a different resin than the one indicated in this method, follow manufacturer’s instructions specific to the resin used.
4. Ensure that no uncured resin is on the part by a close visual inspection. Repeat previous step if any uncured resin is found. Finally, dry using compressed air and put aside for step 2.

3.2 Preparation of the Silicone Slab

The silicone slab can be purchased directly via a custom supplier. However, we detail here how to fabricate the slab from a 3D-printed mold. A diagram of the full process is shown in Fig. 2.

1. Rinse the 3D-printed mold with isopropyl alcohol and dry using compressed air to remove any contaminants.
2. Measure carefully and mix thoroughly 3 grams of base with 0.3 grams of curing agent (Sylgard 184, Dow Chemical Company) in a small disposable transparent cup of at least 50 mL of capacity.
3. Degas the mixture in the vacuum chamber for 20 min or until no more bubbles can be observed. Pay attention to the expansion of the air bubbles in the mixture that can lead to spillages inside the vacuum chamber. A close control is needed for the first couple of minutes. When the raising of the material is too much, simply open the venting valve of the chamber for a moment to collapse the bubbles and reinitiate the vacuum process.
4. Pour the degassed mixture over the clean 3D-printed molds using a disposable Pasteur pipette or a 1 mL disposable syringe.
5. Immediately after the casting, place a small flat piece of acrylic (any thickness from 0.2 mm to 1 mm) on top of the mold to generate a flat and polished surface on the cured PDMS slab. This particular surface is necessary to ensure intimate contact between the slab and the piezoelectric disc and to avoid the use of coupling mediums. Leave the casted molds to cure overnight at room temperature in a flat surface without significant vibrations.
6. After this period, remove the PMMA piece gently, so as not to tear away any of the PDMS, and cure for an additional 2 h at 60 °C to ensure the PDMS slab is completely dry.
7. Carefully peel the PDMS slab from the mold by using plastic tweezers and peel from one of the corner gently.
8. Store until use at room temperature in a dry and clean container.

3.3 Preparation of the Microfluidic Chip

The microfluidic chip can be purchased directly via a custom supplier. However, in this section, we describe the manufacturing of a PMMA microfluidic chip using a CO2 laser plotter and solvent-assisted bonding. The layers of CAD files are available in https://github.com/MaiwennKsKh/BubbleMicromixer. Representations of the PMMA chip layers and the piezo holder monolayer are available in Fig. 3.
In this step, each four layers constituting the microfluidic chip and the monolayer constituting the piezo holder are cut. The four microfluidic chip layers are then bonded using a previously described thermal-assisted solvent bonding process [35]. A YouTube video describing a general step-by-step guide of the cutting and bonding process is available at https://www.youtube.com/watch?v=JzdkNnLQzMU.

1. Set up the Epilog laser cutter as per manufacturer instructions.
2. Upload the file Cut layers PMMA.dxf onto the laser plotter. The dimensions in the file are in mm.
3. Place a 0.5 mm PMMA layer on the cutter bed. Focus the laser head appropriately and align it at a suitable location on the PMMA sheet.
4. Select the two 0.5 mm layers (Layers 1 and 2) on the CAD drawing, and set up the cutting parameters at a power of 4.2 W and a speed of 21 mm/s. Cut.
5. Remove the part and PMMA sheet from the laser cutter bed and put aside.
6. Place a 1 mm PMMA layer on the cutter bed.
7. Select Layers 3 and 4 on the CAD drawing, and set up the cutting parameters at a power of 15 W and a speed 21 mm/s. Cut.

8. Remove the part and PMMA sheet from the laser cutter bed and put aside.

9. Place a 3 mm PMMA layer on the cutter bed.

10. Select the “piezo holder” part on the CAD drawing, and set up the cutting parameters to raster mode, power, 27 W, speed, 17 mm/s. Engrave the part highlighted in pink in Fig. 3b.

11. On the “piezo holder” drawing on the CAD drawing and set-up the cutting parameters to vector mode, power, 16 W, and speed 21 mm/s. Cut.

12. Remove the part and PMMA sheet from the laser cutter bed and put aside.

13. Clean the PMMA layers with a cleanroom tissue soaked in ethanol, and blow-dry with compressed air to remove dust.

14. Preheat the press at a temperature of 70 °C. The press needs to produce at least 1.6 MPa/5 tons at the ram.

15. Prepare the assembly of the layers following the order described in the CAD file: Layer 1 placed at the bottom of the stack and Layer 4 at the top of the stack as indicated in Fig. 3c.

16. Carefully move apart the layers while maintaining the same layer order, and spread 80 μL of ethanol between each of the layers using a pipette just before bonding. Ethanol will allow for the partial melting of a superficial layer and the formation of a strong bond between the PMMA elements even if operating below PMMA Tg. Press the layers together.

17. Immediately after the ethanol spread, place the assembly inside the custom-made alignment aluminum frames and subsequently the plates of the heated press.

18. Apply a load of 2 metric tons for 3 min at 70 °C.

19. After bonding, remove the chips from the press using heat resistant gloves, let the chip cool down to room temperature, blow dry with compressed air, and store until use.

### 3.4 Micromixer Assembly

Figure 4a and b, respectively, show an exploded view of all necessary components. Figure 4c(i–iv) provides a visual guide for the assembly. To mount all the components, follow these steps:

1. Introduce the silicone slab with the air pockets facing toward the complementary chamber of the microfluidic chip.

2. Introduce the piezoelectric disc in the complementary feature of the fitting frame.
3. Align both parts and insert the screws into the fitting frame.
4. Carefully introduce the nuts into the free end of the screws.
5. Tighten the screws using a compatible screwdriver, starting by a loose fit on all screws first and gradually tightening all screws.

3.5 On-Chip cfDNA Extraction (Plasma Sample 100 μL)

1. Chip preparation. Pipette the BSA solution into the chip and leave for 5 min at room temperature. These steps help to avoid nonspecific adsorption of the cfDNA fragments to the chip walls.
2. Wash the chip with nuclease-free water and allow to dry for a couple of minutes before running the experiments.
3. Connect the audio amplifier to the function generator and piezo actuator as shown in Fig. 5.
4. Pipette 100 μL of the plasma sample into the chip.
5. Pipette 150 μL of the lysis binding solution and 5 μL of beads sequentially into the chip.
6. Actuate the micromixer for 10 min. The actuation voltage should be around 60 Vp-p and the frequency around 4 kHz.
7. Lay the neodymium magnet on top of the piezoelectric disc for 5 min. Do not remove the magnet after this step. Adhesive tape can be used to secure the magnet on its position.
8. Aspirate the supernatant until no liquid is observed in the microchamber. Discard supernatant.
9. Pipette 500 μL of wash solution into the chip. The excess liquid at the outlet can be collected using an absorbing tissue.
10. Remove the magnet.
11. Actuate the micromixer for 10 min.
12. Lay the neodymium magnet on top of the piezoelectric disc for 2 min. Do not remove the magnet after this step. Adhesive tape can be used to secure the magnet on its position.
13. Aspirate the supernatant until no liquid is observed in the microchamber. Discard supernatant.

14. Pipette 500 μL of 80% ethanol solution into the chip. The excess liquid at the outlet can be collected using an absorbing cloth.

15. Remove the magnet.

16. Actuate the micromixer for 30 seconds.

17. Lay the neodymium magnet on top of the piezoelectric disc for 2 min. Do not remove the magnet after this step. Adhesive tape can be used to secure the magnet on its position.

18. Aspirate the supernatant until no liquid is observed in the microchamber. Discard supernatant.

19. Repeat steps xiii to xvi.

20. Aspirate the supernatant and let air-dry in a tube for 10–15 min.

21. Add 20 μL of elution solution to the dried tube from step 20. The elution step needs to be performed off-chip. This is due to the fact that the chamber volume is much larger than the required elution volume (20 μL) and would provide inadequate extraction of the cfDNA in the chamber.

22. Vortex for 5 min.

23. Capture the beads with the magnet for 2 min.

24. Collect the eluate with cfDNA.

### 3.6 Cell-Free DNA Quantification and Fragment Analysis

We here describe a straightforward technique for cell-free DNA quantification of the eluates obtained in the previous step. Cell-free DNA quantification by real-time quantitative PCR (RT-qPCR) is performed using the following protocol:

1. For each reaction of total volume of 12.5 μL, prepare a master mix containing 6.25 μl of 2× Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific), 0.25 μL of each LINE-1 primer to a final concentration of each primer of 200 nM, 1 μL cfDNA elution, and 4.75 μL of nuclease-free water.

2. Set up the thermal cycling conditions with an initial denaturation: 10-min cycle at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C.

3. Perform post-amplification melting curve analysis for each reaction to ensure the presence of single specific products (more details in Note 8).

4. Perform cfDNA fragment size analysis is performed using the High Sensitivity D5000 ScreenTape assay on the Agilent 4200 TapeStation system according to manufacturer’s instructions.
5. Analyze data with TapeStation Analysis Software Version A.02.02 (SR1).

Typical PCR results are shown in Fig. 6. The results show that the micromixer can capture, on average, one order of magnitude more cfDNA than the manual bench protocol (control) for 100 μL of plasma sample, and the total cfDNA amount recovered from 500 μL volume is similar on the manual bench protocol and the microfluidic chip. These results indicate that the micromixer enables enhanced capture efficiency due to the superior mixing capabilities when compared to the control methods for this volume range. To the best of our knowledge, this is the first study where a tenfold improvement in microfluidic-based cfDNA capture efficiency is presented on 100 μL of plasma sample. The described module can be used for any application requiring efficient mixing or the use of strong micro-convective forces, e.g., cell lysis.

### 4 Notes

1. Acrylic (PMMA) brands are not all equal when it comes to the use of our ultrafast bonding method [35]. We have investigated the bonding strength obtained on three major brands (Clarex, Perspex, and Oroglass) and two types of sheets (cast and extruded) [36]. Clarex and Perspex casted sheets perform the best in conjunction with this method.

2. Digital manufacturing service providers are companies providing customers 3D-printing services for their own designs. There are numerous services in almost any countries. We make a few company suggestions here. We have not checked if they provide all services needed for the parts in this

![Total cfDNA amount extracted from plasma samples using the micromixer chip and by manual bench extraction method. 100 μL (sample 1) and 500 μL (sample 2) of plasma from different blood donors were used in three independent extractions. (Reproduced from Ref. [34] with permission from the Royal Society of Chemistry) (Fig. 6)]
method. Alternatively, most UK, US, Chinese, and EU services will ship worldwide—UK: Protolabs, Geomiq; USA: Shapeways, Sculpteo; Nigeria: Stampar3d; India: Imaginarium, Think3D; Brazil: 3D Criar, OALOO Impressao 3d; China: FacFox; and Kenya: ab3d.

3. When ordering the silicone slab from a digital manufacturing service provider, you can choose 3DP Silicone, if using Polyjet technique, or Spectroplast True Silicone, if using stereolithography.

4. When choosing a resin to 3D-print the chips, bear in mind that some of them might have biocompatibility problems with the cfDNA assay. We recommend to use “biocompatible” resins typically available from manufacturers and to use additional washing and curing steps to avoid potential inhibitors [37].

5. If using a digital manufacturing service provider, please note that a 3D file with a “bottomless” version of the microfluidic chip is also available in the GitHub repository. This version can be used to create a closed chip with an optical window by gluing a thin layer of transparent acrylic.

6. We recommend the use of the unit TG215 (Thurlby Thandar Instruments) that can work with voltages up to 20 Vp-p. To achieve higher voltages, an audio transformer is needed. We recommend the use of the NTE10/3 (Neutrik). Low-cost (<30 pounds) alternative generators are available commercially from retail suppliers.

7. A round magnet is preferred. We recommend using 10 mm-diameter and 2 mm-height discs.

8. Each sample should be amplified in duplicates or triplicates. A melting curve should be performed as a control measure for nonspecific amplification, and a single peak should be observed. The standard curve for absolute quantification of cfDNA is created with commercially available human genomic DNA (Bioline) with a linear range over five orders of magnitude. For precise quantification, linear regression analysis of the standard curve is expected to demonstrate $R^2 > 0.98$. If the software of real-time PCR does not support automatic quantification of the samples, standard curve equation can be used to calculate DNA concentration in each sample manually.

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Isolation of Extracellular Vesicles by a Microfluidic Platform to Diagnose and Monitor Pancreatic Cancer

María Sancho-Albero and Victor Sebastián

Abstract

Exosomes are extracellular vesicles that are involved in cell-cell communication. Considering their bioavailability and accessibility in all the body fluids (including the blood, semen, breast milk, saliva, and urine), their use has been proposed as an alternative noninvasive tool for the diagnosis, monitoring, and prognosis of several diseases, including cancer. The isolation of exosomes and their subsequent analysis are emerging as a promising technique in diagnostics and personalized medicine. The most widely employed isolation procedure is differential ultracentrifugation, but this approach is laborious, time-consuming, and expensive and with limited isolation yield. Microfluidic devices are now emerging as novel platforms for exosome isolation, which is a low cost technology and enables high purity and fast treatment of exosome isolation. Our approach describes a microfluidic device that enables inflow capture and separation from whole blood using antibody-functionalized magnetic nanoparticles. This device allows isolation of pancreatic cancer-derived exosomes from whole blood without the need of any pretreatment, resulting in a high sensitivity.

Key words  Extracellular vesicles, Exosomes, Magnetic capture, Microfluidics, Pancreatic cancer

1 Introduction

Exosomes are extracellular vesicles with a diameter from 30 to 150 nm. They are secreted by a wide range of cells and are characterized by exhibiting a cell-type lipidic membrane and a specific cargo of proteins and RNAs [1, 2, 3, 4, 5]. Due to the fact that the exosomal genome, transcriptome, and proteome usually mirror the exosomal parental cell, they have been proposed as excellent biomarkers capable of fingerprinting the molecular composition of their parental cells [6, 7, 8, 9, 10]. In addition, their bioavailability and accessibility in all the body fluids (including blood, semen, breast milk, saliva, and urine) highlight their use as alternative noninvasive tools for the diagnosis, monitoring, and prognostic of several diseases, including cancer [11, 12, 13]. Some of the characteristics that make exosomes excellent biomarker tools for cancer diagnosis through a liquid biopsy are as follows: (a) their
content mirrors not only the composition of the parental cell but also its metabolic state; (b) their cargo molecules are protected by a bilipid membrane; (c) they are stable at pH, temperature, and physiological conditions; (d) they can be stored during long time periods at \(-80^\circ\text{C}\); and (e) cancer cells produce and secrete more amount of exosomes compared with healthy cells, thus being significantly more abundant in fluids from cancer patients. Other interesting characteristics of using exosomes as liquid biopsy elements are the low invasiveness of the technique, its low cost, and the reduction of patient damage and invasiveness compared with conventional biopsies such as a surgery, endoscopic procedures, or deep puncture.

It has been also demonstrated that cancer exosomes serve as detection tools for early diagnosis of cancers. In fact, it is described that their concentration in the blood is significantly more elevated compared with circulant tumoral cells (1–10 CTC/mL and 10 \([9]^{14,15}\) exosomes/mL) \([14,15]\). This abundance of exosomes secreted from tumoral cells allows the detection and the diagnosis of cancer in early stages of the diseases when the amount of circulant tumoral cells is undetectable, favoring the diagnosis of the disease before its dissemination to secondary metastasis and, thus, improving the prognosis and the life quality of the patient. In addition, multiple miRNAs and proteins carried by exosomes are usually elevated in biological fluids from cancer patients, which potentially serve as early diagnosis biomarker for cancer \([16]\).

Differential ultracentrifugation is the most employed conventional method used for the isolation of exosomes \([17,18]\). This strategy is based on the elimination of cells, debris, and organelles before isolating the exosome pellet according to their differential size and density. However, though differential centrifugation is a well-established methodology, it presents several disadvantages as it is laborious, time-consuming, and when isolation exosomes from cancer patients bloods large volumes of samples with expensive processing costs are need but also, leading to yields only between 5% and 40% \([19]\). During recent years, novel alternative methodologies have been investigated for the purification of exosomes (immunoaffinity assays, ultrafiltration, or size exclusion chromatography). However, these techniques also present some disadvantages, limiting their transfer to the clinics (additional solvents and chemicals are usually needed, they require long periods of time and exhibit low reproducibility, and the exosomal isolated fraction can present impurities) \([20]\). The presence of these contaminations (such as microvesicles, lipoproteins, virus, bacteria, DNA, apoptotic bodies, or even proteins) can contribute to the appearance of false positives or false negatives, therefore hindering the use of exosomes as diagnostic agents in the clinics \([21]\).

Compared with the previously mentioned methods, the microfluidic platforms supposed an interesting alternative platform for the isolation and collection of intact exosomes from complex
samples such as blood \[22, 23\]. Some of their advantages are (1) exosome separation with high purity, (2) high uniformity in the exosomal isolated fraction, and (3) easy and rapid approach \[22, 24\]. Among the microfluidic systems, the immunoaffinity approaches with a magnetic capture can be highlighted. They are characterized by the use of magnetic nanoparticles that have been previously covered with an exosomal antibody able of recognizing antigens and proteins present in the membrane of the extracellular vesicles. Although exosome isolation and analysis is a promising diagnosis technique, current strategies based on conventional methods require expensive equipment and highly trained personnel and are not capable of being automated. However, the approach herein proposed is based on an in-flow capture and separation of the exosomes from complex fluids, by using an antibody-functionalized magnetic NPs. The operation principle of the device herein presented is based on feeding the sample and the antibody—nanoparticle streams to a coaxial mixer and collecting the captured exosomes downstream after the antibody recognition and the subsequent capture. This fast device can be operated by personnel with basic skills and gives rise to a highly sensitive platform for detection of tumoral markers enriched in cancer patients \[25\].

To sum up, tumor-derived exosomes exhibit significant advantages for being used in cancer diagnosis. In this study, a microfluidic separation device based on a magnetic capture has been designed. It has been also demonstrated the functionality of the present device in the separation of the exosomes derived from patients’ whole blood with pancreatic cancer. To do that, the protein levels of CA19-9 antigen were measured in the isolated exosomes. CA19-9 protein is the only FDA-approved test to monitor the tumor response and recurrence of pancreatic cancer patients. However, this analysis usually exhibits false negative and positive results, limiting their accuracy in this aggressive cancer.

2 Materials

2.1 Synthesis and Characterization of Fe₃O₄-EDC-NHS Nanoparticles

1. Tri–sodium citrate dihydrate (2 mmol).
2. Sodium hydroxide (8 mmol).
3. Sodium nitrate (0.4 mol).
4. Ferrous sulfate (4 mmol).
5. N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC).
6. N-Hydroxsuccinimide (NHS).
7. MES monohydrate buffer pH 5.5.
8. Phosphotungstic acid.
9. Distilled dH₂O.
10. Three-neck flask.
11. Hot plate stirrer.
12. Magnet.
13. Transmission electron microscope (TEM). Tecnai-FEI T20 operated at 200 kV.
14. TEM grid (Cu-200 Mesh Formvar-Electron Microscopy Sciences).
15. Thermogravimetric (TGA) Analyzer. Mettler Toledo TGA/STDA 851e.
16. Bruker Vertex 70 FTIR spectrometer equipped with a DTGS detector (Golden Gate Heated Single Reflection Diamond ATR, Spectra Tecknoroma).
17. Vibrating sample magnetometer (VSM Lake Shore 7410).
18. X-ray photoelectron spectroscopy (XPS) apparatus. Axis Ultra DLD (Kratos Tech.).
19. Dynamic light scattering (DLS). Brookhaven 90 plus and Zeta-PALS software.

### Exosomal Antibody Coupling

1. Anti-CD9 antibody (EPR2949) (Abcam, cat. no. ab92726; RRID: AB_10561589) (stored at \(-20^\circ\text{C}\)).
2. Phosphate buffer saline (PBS).
3. UV-vis spectrophotometer (Thermo Scientific Nanodrop 2000).
4. Quantitative nanoscale mechanical characterization (QNM peakforce mapping) by AFM in a Multimode 8 (Bruker Co., Billerica, MA, USA). Tipo: SNL model, type C; Bruker.
5. Magnesium chloride solution.
6. Muscovite mica substrate.

### Microdevice Assembly

1. Polyether ether ketone-PEEK polymer tubing. Inner diameter, 150 μm, and outer diameter, 350 μm.
2. Polytetrafluoroethylene-PTFE polymer tubing. Inner diameter, 750 μm, and outer diameter, 1/16”.
3. Polyether ether ketone-PEEK thru-hole Tee assembly for use with 1/16” outside diameter tubing.
4. Four units of ¼-28 short Polyether ether ketone-PEEK Nut to connect 1/16” outside diameter tubing.
5. Four units Flangeless Ferrules Ethylene tetrafluoroethylene for 1/16” outside diameter tubing.
6. One, male Luer to male 1/4–28 flat-bottom adapter.
7. One Luer-to-MicroTight® adapter (Upchurch Scientific).
8. Tubing sleeve.
9. 3D printed microfluidic holder.
2.4 Exosomal Source (Serum and/or Blood) and Exosomal Capture

1. Pierce BCA (bicinchoninic acid assay) protein assay kit (Thermo Scientific, cat. no. 23225).
2. Cancer patients’ blood.
3. ExoELISA-ULTRA CD63 (System Biosciences, USA).
4. BioTek Instrument Synergy HT microplate reader (Izasa, s/n 208853).
5. NanoSight (Malvern Instruments, UK).
6. CA19-9 Human ELISA Kit (Thermo Fisher Scientific, USA).

3 Methods

3.1 Synthesis and Characterization of the Fe₃O₄-EDC-NHS Nanoparticles Covered with the Exosomal Antibody

3.1.1 Fe₃O₄ Nanoparticle Synthesis

1. Mix in a three-neck flask: 2 mmol of tri-sodium citrate dihydrate with 8 mmol of NaOH and 0.4 mol of NaNO₃ in 18 mL of distilled H₂O.
2. Heat the previous solution to 100 °C.
3. Add 2 mL of 2 M ferrous sulfate to the previous solution and keep it at 100 °C for 1 h (see Note 1).
4. Cool down the magnetic colloid to room temperature.
5. Wash the magnetic colloid three times with distilled H₂O, trapping Fe₃O₄ nanoparticles with a magnet.
6. Disperse the purified nanoparticles in distilled H₂O.

3.1.2 EDC/NHS Functionalization

1. Mix the Fe₃O₄ nanoparticles with EDC (1:1, w:w) for 30 min at room temperature in MES buffer (pH 5.5).
2. Activate the Fe₃O₄-EDC nanoparticles with NHS (1:1, w:w) for 10 min at room temperature in MES buffer (pH 5.5).
3. Stop the crosslinking reaction by washing the Fe₃O₄-EDC-NHS nanoparticles three times with distilled H₂O.

3.1.3 Nanoparticle Characterization

1. Carry out TEM analysis by using a T20-FEI microscope to study the morphology of size distribution of EDC/NHS functionalized nanoparticles (Fig. 1; see Note 2).
2. Perform TGA analyses in a temperature range between 30 °C and 850 °C with heating rate of 20 °C min⁻¹ in N₂ atmosphere to determine the citrate/Fe ratio (pre-functionalized nanoparticles) as well as the EDC-NHS/Fe ratio (post-functionalized nanoparticles).
3. Record FT-IR spectra with 200 scans in the 400–4000 cm⁻¹ spectral region at 4 cm⁻¹ resolution.
4. Measure the magnetic properties of the nanoparticles at room temperature with a vibrating sample magnetometer.

5. Evaluate the stability of Fe₃O₄ nanoparticles and Fe₃O₄-EDC-NHS nanoparticles during 30 days by XPS (determine the relative abundance of Fe²⁺ and Fe³⁺ species) and zeta potential.

3.1.4 Anti-CD9 Coupling

1. Add 1 μg of anti-CD9 to 100 μg of nanoparticles in a final volume of 10 μL of PBS.

2. Incubate the solution for 4 h at room temperature by vortexing every 15 to 30 min.

3. Eliminate the unbound anti-CD9 by washing the complexes three times with PBS and a magnet.

3.1.5 Nanoparticle-CD9 Complex Characterization

1. Quantify the bounded anti-CD9 indirectly by measuring in a UV-vis spectrophotometer the non-bounded antibody present in the supernatant and measuring the absorbance of the aromatic rings of the amino acids of the antibody (Fig. 2) (see Note 3).

2. Visualize Fe₃O₄-EDC-NHS nanoparticle-anti-CD9 complexes by AFM.

3.2 Fabrication of the Microdevice

The assembly of the microdevice involves at least seven distinct steps that are described below in detail, and the resulting micro-fluidic device is depicted in Fig. 3a:

1. The PEEK inner capillary was coaxially placed inside the PTFE tubing under an optical microscope to get an axisymmetric flow-focusing device.
Fig. 2 UV-vis characterization of antibody coupling. Nanodrop measurements of the nanoparticle-anti-CD9 complexes (100:1, w/w)

Fig. 3 Description of the microdevice. (a) Left—image of the microfluidic platform: 3D printed holder, inlets, coaxial mixer, magnets, and collection vial. Right—the pipe is lifted from the magnets showing the nanoparticles captured at the junctions between the alternate polarization magnets. (b) Operation principle of the coaxial mixer: capture, washing, and elution. (c) ELISA analysis against CD63 and CA19-9 performed after exosome capture from whole blood. (Adapted from Ref. [25] under CC license)

2. PEEK nuts and flangeless ferrules were tightened to the PEEK thru-hole Tee assembly. Tubing is inserted into the end of the Tee assembly, and the nut is tightened, forcing the ferrule into the fitting body. A sleeve was used to insert the capillary to the Tee assembly.
3. PEEK nuts and flangeless ferrules were tightened to the male Luer to male 1/4–28 flat-bottom adapter to inject exosome-containing fluid (sheath flow).

4. Luer-to-MicroTight® adapter was assembled to the capillary to inject the functionalized magnetic nanoparticles (core flow).

5. The length of the PTFE tubing was 10.5 cm (6.5 cm from the inner capillary to the first magnet).

6. The coaxial capillary system was supported in the 3D printed holder to avoid capillary misalignment and to favor fluid dynamic reproducibility (Fig. 3a left).

7. Magnets with alternate polarization was used to achieve a high capture force due to the high magnetic field gradient created between each magnet and the contiguous one (Fig. 3a, right).

8. The core flow (functionalized magnetic nanoparticles) was injected at a flow rate of 10 mL/min, whereas the sheath flow (exosome-containing fluid) was injected at 50 mL/min. The shear stress mismatch between low and fast velocity streams is able to produce turbulence at the point of confluence, promoting entrainment of the low velocity fluid in the fast stream.

3.3 Isolation of the Exosomes with the Microdevice

1. 500 μL of fresh blood were employed for the exosomal capture in the microdevice. Samples were stored during 2 h at 4 °C until use (see Note 6).

2. 20 μg of Fe₃O₄-EDC-NHS nanoparticles were conjugated to 200 ng of anti-CD9 in 50 μL of PBS during 4 h.

3. Eliminate the excess of non-bounded antibody as previously mentioned, and disperse the complexes in 500 μL of PBS.

4. Exosome capture (Fig. 3b). Introduce the Fe₃O₄-EDC-NHS nanoparticle-anti-CD9 and the blood in the microfluidic chip (see Note 4 and Note 5). The magnetic nanoparticles conjugated to the antibody are fed at a flow rate of 10 μL/min in the internal channel. The blood containing the exosomes are introduced at a flow rate of 50 μL/min in the external channel.

4. Exosome washing (Fig. 3b). Once the exosome sample has been processed, introduce PBS in both the inner and the outer channels to wash the microdevice and the captured exosomes from the rest of blood components.

5. Exosome elution (Fig. 3b). Remove the magnet from the microdevice, and recover the nanoparticle-exosome complexes in an Eppendorf tube by introducing PBS in the microdevice as previously mentioned.
3.4 Characterization of the Isolated Exosomes

1. For TEM characterization, the capture sample is deposited onto a TEM grid and is stained with 3% phosphotungstic acid as a negative contrast agent (Fig. 4).

2. To quantify the number of captured exosomes, the ExoELISA-ULTRA CD63 kit is used following manufacturer instructions (Fig. 3c).

3. The concentration and the diameter of the captured exosomes are measured by NanoSight.

4. The tumoral antigen CA19-9 levels (tumoral pancreatic marker) present in exosomes were isolated from pancreatic cancer patients’ blood, and the captured complexes were isolated with the CA19-9 Human ELISA Kit following manufacturer instructions (Fig. 3c).

4 Notes

1. A color change should be observed from pale white to dark brown, which corresponds with the formation of the Fe₃O₄ nanoparticles.

2. To visualize the organic EDC-NHS organic coupling, stain the sample by adding 3% phosphotungstic acid.
3. To quantify the amount of bounded antibody, the following formula can be used: antibody concentration (mg/mL) = (1.55 \( \text{Abs}_{280} \) – 0.76\( \text{Abs}_{260} \)).

4. As negative control, Fe\(_3\)O\(_4\)-EDC-NHS nanoparticles without being covered with anti-CD9 exosomal antibody can be used.

5. An accumulation of nanoparticles containing the recognized and captured exosomes can be observed in the magnet.

6. For collecting fresh blood vacutainer sodium citrate tubes were employed.

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Chapter 13

High-Throughput Separation and Enrichment of Rare Malignant Tumor Cells from Large-Volume Effusions by Inertial Microfluidics

Chen Ni, Zhixian Zhu, Zheng Zhou, and Nan Xiang

Abstract

Detection of malignant tumor cells (MTCs) in pleural effusions is essential for determining the malignancy. However, the sensitivity of MTC detection is significantly decreased due to the existence of a massive number of background blood cells in large-volume samples. Herein, we provide a method for on-chip separation and enrichment of MTCs from malignant pleural effusions (MPEs) by integrating an inertial microfluidic sorter with an inertial microfluidic concentrator. The designed sorter and concentrator are capable of focusing cells toward the specified equilibrium positions by inducing intrinsic hydrodynamic forces, enabling the size-based sorting of cells and the removal of cell-free fluids for cell enrichment. A 99.9% removal of background cells and a nearly 1400-fold ultrahigh enrichment of MTCs from large-volume MPEs can be achieved by this method. The concentrated high-purity MTC solution can be used directly for cytological examination by immunofluorescence staining, enhancing the accurate identification of MPEs. The proposed method can also be employed for the detection and count of rare cells in various clinical samples.

Key words Malignant pleural effusions, Malignant tumor cells, Microfluidics, Cell separation, Cell enrichment, Immunofluorescence staining

1 Introduction

Malignant pleural effusion (MPE), a common complication of metastatic malignancies (e.g., lung cancer, breast cancer, and lymphoma), refers to the abnormal accumulation of excess fluid in the pleural space with a concomitant presence of malignant tumor cells (MTCs) [1–4]. As the median overall survival time for patients with MPEs is only between 4 and 9 months depending on cancer entity [5], accurate identification of MTCs in large-volume pleural effusions is urgent and essential to clinical treatment. Cytological examination for clinical MTC diagnosis determines malignancy through traditional cell smear [6]. However, due to the large
volume of MPEs (tens to hundreds of milliliters) and the presence of a massive number of background blood cells in MPEs, the sensitivity of cytological examination suffers from a non-negligible loss (over 40%) [7, 8]. In addition, the multiple centrifugation and pipetting procedures make the traditional cell smear very time-consuming and may also cause the cell loss during the manual operation. Therefore, high-purity separation of MTCs from background cells and rapid high-fold enrichment of the sample solution may significantly improve the sensitivity of cytological examinations [9]. As a novel technology for precise cell manipulation with advantages of low device cost, simple operation, high precision, and parallel processing capacity, microfluidics has been extensively developed for the separation and enrichment of rare cells [10–14]. The current microfluidic separation techniques could be divided into two categories (i.e., active and passive techniques) [15–17]. As compared with most active microfluidic techniques based on external force fields [18–20], label-free passive inertial microfluidics manipulates cells based on the intrinsic hydrodynamic forces and thus owns the advantages of high-throughput, autonomy, and simplicity [21].

Herein, we describe a protocol for the high-throughput separation and enrichment of rare MTCs from large-volume effusions by inertial microfluidics (Fig. 1). The employed inertial microfluidic sorter and concentrator are fabricated in low-cost polymer-film materials. Benefiting from the laser cutting and simple stacking methods [22], trapezoidal cross-sectional microchannels can be fabricated flexibly, and the fabrication and assembly of the integrated chip can be accomplished within 30 min. The results indicate that a high recovery rate of approximately 85% and a high purity of over 37% can be realized for the separation of rare MTCs at a high throughput of 3 mL/min by using the inertial microfluidic sorter with trapezoidal cross-sections. After separation, the enrichment of rare MTCs is performed using a multiplexed concentrator. An ultrahigh enrichment factor of approximately 1400 can be achieved at a high throughput.

Fig. 1 Workflow for the separation, enrichment, and identification of rare malignant tumor cells (MTCs) from malignant pleural effusions (MPEs). (Reproduced from Ref. [26] with permission from Royal Society of Chemistry)
of 16 mL/min. Therefore, our strategy by integrating on-chip cell separation and enrichment can remove the vast majority of background blood cells in large-volume MPEs and perform ultrahigh-fold enrichment of the presorted MTCs. The concentrated high-purity MTCs can be directly used for immunofluorescence staining, enhancing the identification sensitivity of MPEs.

## 2 Materials

### 2.1 Materials for Chip Fabrication of Microfluidic Sorter and Concentrator

1. Channel layer: polysiloxane film with a layer of protective film on each side (see Note 1).
2. Cover and bottom layers: polyethylene terephthalate (PET) film of 110 μm thickness with a layer of silicone protective film.
3. Chip fixtures: custom cover and bottom plates of transparent polymethyl methacrylate (PMMA), locating pins, and rubber washers. Fix the four locating pins on the bottom plate (see Note 2). Machine the countersunk holes and through holes at the inlet and outlet regions of the cover plate for installing the rubber washers and dispensing needles, respectively (see Note 3).
4. Deionized (DI) water.
5. Absolute ethanol.
6. Double-sided tape (200 μm thick).

### 2.2 Clinical Sample Preparation

1. Pleural effusion sample: malignant pleural effusions collected from patients with lung or breast cancer (see Note 4).
2. Cell strainer (40 μm).
3. Red blood cell (RBC) lysis solution: add 8.29 g of ammonium chloride (NH₄Cl), 1 g of potassium bicarbonate (KHCO₃), and 2 mL of 0.5 M ethylene diamine tetraacetic acid (EDTA) to 1 L of DI water and mix well. Store at 4°C.
4. PBS solution: phosphate buffered saline (PBS) (1×, pH = 7.4).
5. Pasteur pipette (3 mL).
6. Anti-adhesion buffer solution: add 0.2 g of F-127 Surfactant to 40 mL of PBS solution.
7. Centrifuge tube (50 mL).

### 2.3 Immunofluorescence Staining

1. Immunohistochemistry pen.
2. Microscope glass slide.
3. Microscope cover glass.
4. Methanol solution: store at −20 °C.
5. Air-laid paper.
6. Blocking solution: add 300 μL of 10% bovine serum albumin (BSA) solution (see Note 5), 20 μL of goat serum, and 30 μL of fetal bovine serum (FBS) to 700 μL of PBS solution.

7. Diluted antibody solution: add 5 μL of Anti-Pan Cytokeratin (CK) antibody containing goat F(ab) anti-rabbit IgG H & L (TRITC) fluorophore and 5 μL of Anti-Hu CD45 antibody containing goat F(ab) anti-mouse IgG H & L (FITC) fluorophore to 150 μL of blocking solution.

8. Mounting medium: antifade mounting medium with DAPI. Store at 4 °C.

### 3 Methods

#### 3.1 Fabrication of Microfluidic Sorter

1. Draw the inner-wall and outer-wall outlines of the trapezoidal spiral microchannel of two channel layers with different thicknesses, the inlets and outlets of the cover layer, locating holes and chip outline of each layer by using CAD software (see Note 6). Import the CAD drawings into the UV laser cutting system (15 W, 355-nm laser source) (see Note 7).

2. Prepare the PET films and polysiloxane films with dimensions slightly larger than the desired chip outlines. Remove a layer of protective film from one side of the polysiloxane film.

3. Place the prepared film on the platform of the UV laser cutting system, and perform the positioning calibration. Start the laser cutting system to cut the film. Repeat this step to sequentially pattern the cover layer, the channel layers, and the bottom layer (see Note 8) (Fig. 2a).

4. Remove the waste materials on the film after laser cutting (Fig. 2a). Put the films into an ultrasonic cleaner with absolute ethanol for 10 min to remove the chemical substances produced by laser ablation. Rinse the films with DI water and dry with nitrogen.

5. Put the films into the oxygen plasma cleaner for 3 min with oxygen injection every 20 s (see Notes 9 and 10).

6. Place the bottom layer film on the bottom plate of the fixture. First, bond the bottom layer with the inner-wall channel layer (see Note 11). Peel off the protective film on the remaining side of the inner-wall channel layer with extreme care to prevent the channel from being peeled off together. Then, stack and bond the outer-wall channel layer. Carefully tear off the protective film of the outer-wall channel layer after bonding. Finally, stack and bond the cover layer to obtain the sorter chip (see Note 12) (Fig. 2b). The trapezoidal cross section of the finished spiral microchannel is shown in Fig. 2c.
7. Clamp the fabricated sorter chip with the corresponding cover and bottom PMMA plates (see Note 13). Connect the silicone tube to the dispensing needles at the inlets and outlets of the cover plate.

3.2 Fabrication of Microfluidic Concentrator

1. Different from the sorter chip, the channel cross-section of the concentrator chip is a rectangular, and the concentrator chip only contains three layers of films: one cover layer, one middle channel layer, and one bottom layer. Draw the microchannel outlines of the channel layer (see Note 14), the inlets and outlets of the cover layer, and locating holes and chip outline of each layer by using CAD software (Fig. 3a). Import the CAD drawings into the UV laser cutting system.
Fig. 3 (a) CAD drawing of each layer for the single concentrator chip and photograph of the fabricated single concentrator chip. (b) Photograph of the four concentrator chips (1–4), one flow distribution chip (5), and four pieces of double-sided tape (6). (c) Assembly of integrated microfluidic concentrator with the assistance of a custom fixture. (d) Schematic diagram demonstrating the working principle of the designed microchannel and the distributions of A549 cells showing the cell enrichment effect of the concentrator. (e) Schematic diagram illustrating the reduction of sample volume of A549 cell suspension from 400 mL to about 0.25 mL after the secondary concentration. Microscopy images of the initial sample and the target samples collected after primary concentration and secondary concentration. (Reproduced from Ref. [26] with permission from Royal Society of Chemistry)
2. Fabricate and treat the cover layer, channel layer, and bottom layer of the concentrator chip as in steps 2–5 of Method 3.1.

3. Place the bottom layer film on the bottom plate of the fixture. Bond the bottom layer with the channel layer (see Note 11). Peel off the protective film on the remaining side of the channel layer with extreme care to prevent the channel from being peeled off together. Then, stack and bond the cover layer to obtain the concentrator chip (Fig. 3a).

4. Repeat steps 2–3 to fabricate four identical concentrator chips and one flow distribution chip (see Note 15) (Fig. 3b).

5. Use laser to cut four identical double-sided tapes. Fabricate the through holes in each double-sided tape to connect different channel layers (Fig. 3b).

6. Assemble the integrated concentrator chip. Place the single-layer concentrator chip on the corresponding bottom plate. Align the double-sided tape and the concentrator chip through the locating holes. Tear off the brown base layer of the double-sided tape after bonding with the concentrator chip. Repeat this step to sequentially bond the remaining concentrator chips (see Note 16) (Fig. 3c).

7. Clamp the integrated concentrator chip with the corresponding cover and bottom PMMA plates (see Note 13). Connect the silicone tube to the dispensing needles at the inlets and outlets of the cover plate.

3.3 Sample Preparation

1. Filter the MPE samples into centrifuge tubes using a 40 μm cell strainer to remove the large impurities that may block the channel.

2. Centrifuge the filtered samples at 300 g/min for 5 min using a centrifuge. Remove the supernatant, and add an equal volume of PBS solution to eliminate the effect of viscoelasticity on cell inertial focusing. Resuspend the cell pellet by gently blowing using a Pasteur pipette.

3. For pleural effusion samples with a large number of RBCs, add RBC lysis solution with 5–10 fold sample volume. Let mixture incubate for 5 min after mixing. Centrifuge the lysed mixture at 300 g/min for 5 min. Remove the supernatant, and resuspend the cell pellet by adding an appropriate amount of PBS solution and then gently blowing using a Pasteur pipette (see Note 17).

4. Use a cell counter to measure the cell concentration of the prepared suspension. If the concentration is higher than $5 \times 10^5$ cells/mL, add PBS solution to dilute the cell concentration to ensure the separation performance of the chip.
3.4 MTC Separation and Enrichment Operation

1. Prepare multiple 50 mL syringes to draw all the pretreated samples (see Note 18). Prepare two 50 mL syringes to draw 40 mL of anti-adhesion buffer solution and 20 mL of PBS solution, respectively.

2. Connect the inlet tube of the sorter with the syringe loaded with anti-adhesion buffer solution, and fix the syringe on the syringe pump. Flush the chip channel at a flow rate of 1 mL/min for 20 min to prevent cells from adhering to the channel walls.

3. Replace the syringe to inject the samples, separate the cells at a flow rate of 3 mL/min, and collect the waste and MTCs via the two outlets of the sorter, respectively (see Note 19) (Fig. 2d and e).

4. Replace the syringe and inject the 10 mL PBS solution with the same flow rate of 3 mL/min to collect the cells remaining in the channel and the inlet and outlet tubes.

5. Blow the MTC solution collected from the sorter chip with a Pasteur pipette to make the cells evenly suspended in the solution. Use 50 mL syringes to draw the collected MTC solution.

6. Replace the sorter chip with the integrated concentrator chip and flush the chip as in step 2. Concentrate the MTC sample at a flow rate of 16 mL/min, and collect the waste and MTCs at the outlets of the concentrator, respectively (see Note 20) (Fig. 3d).

7. Inject the collected MTC sample into the integrated concentrator chip again for secondary concentration to achieve an ultrahigh concentration factor of approximately 1400 (Fig. 3e).

3.5 Immunofluorescence Staining

1. Draw a closed square using an immunohistochemistry pen on a microscope glass slide (see Note 21). Utilize a pipette to transfer the entire sample solution of the concentrated high-purity MCT solution into the square area. Place the glass slide in a wet box for 30 min at room temperature (Fig. 4a).

2. Use a piece of air-laid paper to slowly absorb the solution from the square area along one edge. Add five drops of PBS solution into the square area, and then use a new piece of air-laid paper to absorb the solution along one edge of the square. Repeat this step three times to complete the washing (see Note 22) (Fig. 4b).

3. Add five drops of the methanol solution precooled at –20 °C into the square area to fix the cells. Place the slide glass in a wet box for 5 min at room temperature (Fig. 4c).
4. Wash the square area as in step 2. Add five drops of the prepared blocking solution into the square area. Place the glass slide in a wet box for 30 minutes at room temperature (Fig. 4d).
5. Use a piece of air-laid paper to slowly absorb the excess blocking solution from the square area along one edge. Add five drops of the prepared diluted antibody solution into the square area. Place the glass slide in a wet box and incubate in a 4 °C refrigerator for 12 hours (see Note 23) (Fig. 4e).

6. Wash the square area as in step 2. Cover the square area with a microscope cover glass after adding four drops of mounting medium. Wipe off the spilled mounting medium with air-laid paper. Observe the slide glass under a fluorescence microscope (see Note 24) (Fig. 4f).

7. Scan manually and photograph the entire square area. Identify and count MTCs based on the excitation color of the cells in the fluorescence micrographs (see Note 25) (Fig. 4g).

4 Notes

1. For the sorter, use polysiloxane films with thicknesses of 50 μm and 150 μm as the inner-wall channel layer and the outer-wall channel layer, respectively. For the concentrator, use a polysiloxane film with a thickness of 150 μm as the channel layer.

2. The sizes of the sorter and concentrator cover and bottom plates are determined according to the sizes of the sorter and concentrator chips, respectively. The locating holes of the bottom plate and the locating pins are interference fit, and the locating holes of the cover plate and the locating pins are clearance fit.

3. The thickness of the rubber washers should be slightly larger than the thickness of the countersunk heads of the cover plate to prevent leakage under a high pressure. The dispensing needles are fixed with glue and sealed on the inlets and outlets of the cover plate.

4. The experiments require the approval of the Institutional Ethical Committee (IEC) for clinical research and informed consent from volunteers and need to be performed in compliance with the local laws and following the institutional guidelines.

5. A 10% BSA solution is prepared by adding 1.0 g of BSA lyophilized powder to 9 mL of PBS solution. Use a 0.22 μm syringe filter to filter the prepared 10% BSA solution. Store at 4 °C.

6. The inner wall and outer wall of the trapezoidal spiral microchannel are drawn and patterned on different channel layers. The trapezoidal spiral microchannel is formed by stitching the inner-wall and outer-wall channel layers of different thicknesses on the same plane. The five-loop radius of the trapezoidal spiral
microchannel is increased from 6 mm to 26 mm. The inner-wall and outer-wall heights of the trapezoidal spiral microchannel are 50 μm and 150 μm, respectively. The microchannel width is determined to be 500 μm.

7. Other laser cutting systems could also be employed in the fabrication of the sorter and concentrator so long as their cutting accuracy meets the required microchannel width.

8. Cutting films of different thicknesses requires careful adjustment of key parameters of laser cutting system such as laser power, laser frequency, processing time, and cutting speed. Thicker films typically require a higher laser power and a lower laser frequency. The effects of key parameters on cutting performance can be found in ref. [23, 24].

9. The oxygen plasma treatment can modify the surface properties of silicon-based materials, and then the different polymer films can be bonded irreversibly. The films can be placed in the plasma cleaner after being attached to the fixed plate using the double-sided tape to prevent from being blown away by the injected air when the oxygen plasma cleaner is opened.

10. Before putting the outer-wall channel layer into the oxygen plasma cleaner for treatment, evenly smear with a marker pen in the area where the waste materials have been torn off to prevent the protective layer of the outer-wall channel layer from adhering to the inner-wall channel layer.

11. During the bonding process, press slowly from the middle of the film to all sides to prevent air bubbles. Use tools such as rolling and pressing with a centrifuge tube or pressing with a load for a few minutes to ensure uniform adhesion.

12. Due to the different thicknesses of the inner-wall and outer-wall channel layers, it is required to carefully and repeatedly press along the direction of the spiral microchannel with the thumbnail to ensure that the inner-wall channel layer with a thinner thickness and the cover layer are completely bonded.

13. Use tape or clips to provide gripping forces for the cover and bottom plates to clamp the chip.

14. The channel layer of concentrator chip is composed of a spiral microchannel with multiplex transversal cross-flow channels and an asymmetric sinusoidal microchannel in series. For the spiral microchannel, the channel widths of channels I, II, and III are 600 μm, 500 μm, and 400 μm, respectively. Multiplex transverse cross-flow channels with a width of 400 μm and a length of 800 μm connect the adjacent channels. The angle between the cross-flow channel and the main channel is 60°, and the arc length between the adjacent cross-flow channels is 4.25 mm. For the asymmetric sinusoidal microchannel, each unit consists of a large curving turn (with an inner radius of
360 μm, an outer radius of 400 μm, and a maximum turn width of 300 μm) and a small curving turn (with an inner radius of 100 μm, an outer radius of 250 μm, and a constant turn width of 150 μm) (Fig. 3a). The number of units is determined to be 25, and the height of the channel layer is 150 μm.

15. The channel structures of the four concentrator chips are identical. The cover and bottom layers of each chip are perforated selectively at the corresponding positions of the inlets and outlets, enabling four concentrator chips to work simultaneously.

16. Different concentrator chips are carefully bonded using double-sided tapes. To prevent the generation of air bubbles, the multiplexed chip is compressed for five times using the palm or rolling pin after assembling each concentrator chip. The flow distribution chip is set in the middle layer of the multiplexed chip to lessen the flow disparity between different layers.

17. The high number of RBCs may affect the separation performance of the chip. For non-bloody pleural effusion samples, the step of lysing RBCs can be omitted.

18. After drawing the pretreated sample with syringes, add about 2 mL of PBS solution to the centrifuge tube, shake the centrifuge tube to wash its inner wall, and then suck the liquid up with syringe. In doing so, the cell loss caused by the sample adhering to the tube wall can be reduced.

19. Under the effect of inertial lift force $F_L$ and Dean drag force $F_D$ generated by trapezoidal spiral microchannel, MTCs are focused into thin lines close to the inner wall, while WBCs are trapped in the core of the Dean vortex near the outer wall (Fig. 2d). Then, MCTs and WBCs are, respectively, separated and collected from two outlets, achieving the size-based separation (Fig. 2e). More details on the separation principle can be found in ref. [25].

20. Cells are enriched successively in the spiral microchannel with multiplex transversal cross-flow channels and the asymmetric sinusoidal microchannel. In the spiral microchannel, cells are gradually focused near the inner wall of the channel I due to $F_L$ and $F_D$. Subsequently, multiplex transversal cross-flow channels transfer the cells into channel II under a hydrodynamic cross-flow filtration effect, leaving the cell-free fluids in channel I. Similarly, cells are then transferred into channel III, leaving the cell-free fluids in channels I and II. Finally, cells are enriched in the inner branch of the Y-shaped outlet and enter the asymmetric sinusoidal microchannel, while the cell-free fluids are evacuated from the outer branch of the Y-shaped outlet and channels I and II. In the asymmetric sinusoidal microchannel, cells are focused at the centerline of the channel.
under $F_L$ and periodically varying $F_D$ and enter the middle outlet, whereas cell-free fluids are removed from two side outlets (Fig. 3d). This single-layer chip can achieve about 40-fold enrichment of MTC samples at a flow rate of 4 mL/min. Through the integration of the four concentrator chips, the flow rate can be increased to 16 mL/min. More details on the enrichment principle can be found in ref. [26].

21. The shape of the closed area has no effect on the staining of cells. However, the square shape facilitates the rapid scanning after staining.

22. When dripping the PBS solution, the pipette should be placed at a height of about 1 mm directly above the square area. The dripping process should be gentle and slow to prevent the fixed cells on the glass slide from being washed away.

23. The incubation time of antibody should be controlled at about 12 h. A longer incubation time may result in the occurrence of double positives.

24. The stained glass slide should be protected from light to prevent the fluorescence from being quenched prematurely.

25. The cell nuclei are marked with DAPI in the mounting medium and show blue under fluorescence excitation. The membranes of MTCs are labeled with the CK antibody with the TRITC fluorophore and show orange red under fluorescence excitation. The membranes of WBCs are bound to CD45 antibody with FITC fluorophore and show green under fluorescence excitation. Therefore, CD45-/CK+/DAPI+ is identified as MTCs, and CD45+/CK-/DAPI+ is identified as WBCs (Fig. 4g).

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SAIF: Label-Free Separation of Circulating Tumor Cells Using a Self-Amplified Inertial Focusing Microfluidic Chip

Aynur Abdulla and Xianting Ding

Abstract

Circulating tumor cells (CTCs) are rare cells existing in the bloodstream with a relatively low number, which facilitate as a predictor of cancer progression. However, it is difficult to obtain highly purified intact CTCs with desired viability due to their low percentage among blood cells. In this chapter, we demonstrate the detailed steps for the fabrication and application of the novel self-amplified inertial-focused (SAIF) microfluidic chip that enables size-based, high-throughput, label-free separation of CTCs from the patient blood. The SAIF chip introduced in this chapter demonstrates the feasibility of an extremely narrow zigzag channel (with 40 μm channel width) connected with two expansion regions to effectively separate different-sized cells with amplified separation distance.

Key words  Circulating tumor cells (CTCs), Inertial focusing, Size based, Label-free

1 Introduction

Cancer is the second main cause of human mortality, and cancer metastasis is the leading cause of cancer-related mortality. However, circulating tumor cells (CTCs) are one of the main causes correlated with cancer metastasis [1]. CTCs are rare cells existing in the bloodstream in a relatively low number, which are shed from primary or metastasis tumors and flow into peripheral bloodstreams by the epithelial-mesenchymal transition (EMT) process [2]. The number of CTCs in the blood is regarded as a predictor of cancer recurrence risk assessment, efficacy detection, and a “liquid biopsy” for cancer monitoring, prognostics, and diagnostics. Despite the easy access to CTCs in peripheral blood without harmful operations, it is difficult to obtain high purified CTCs due to their low presence among blood cells, while pure CTCs are necessary for further biological research, such as cancer cell protein analysis. Therefore, efficient and accurate methods for CTC separation are in urgent need for facilitating cancer diagnosis, prognosis, and treatment [3].
With the prevalent usage of microfluidic techniques, a great number of CTC separation methods emerged ranging from bio-affinity-based methods (e.g., CellSearch system) to physical-properties-based microfluidic devices (e.g., ClearCell® FXI System) [4, 5]. The physical properties-based methods include inertial focusing [6], acoustics [7], microfluidic filters [8], deterministic lateral displacement (DLD) [9], optics [10], and dielectrophoresis (DEP) [11], which are mainly based on the size and deformability of the tumor cells. Even though the acoustics, optics, and dielectrophoresis (DEP) methods are efficient for CTC separation, the cost of chip fabrication and operation complexity is the obstacle to the wide applications of these methods in the clinic. However, inertial-focused microfluidics takes advantage of the hydraulic phenomenon in microscale channels where different-sized cells occupy different equilibrium positions to achieve separation from each other due to the force balance between inertial lift and Dean drag forces [12]. Therefore, the inertial-focused microfluidic is an efficient method to separate CTCs in a label-free size-based manner [13].

In this protocol, we describe the detailed fabrication and operation process of a new SAIF microfluidic device that enables size-based, high-throughput, label-free separation of CTCs from blood cells. The SAIF chip described in this protocol consists of one inlet, an extremely narrow zigzag channel (with 40 μm channel width), a straight channel with two expansion regions, and three outlets (Fig. 1). In the zigzag part, we take advantage of both the curved channels and the straight channels to achieve cell separation, where the semicircle channels induce Dean flow to promote particle migration, and the straight channels suppress the remaining stirring effects of Dean flow to stabilize the achieved cell focusing. Different-sized cells focus due to the influence of inertial lift force ($F_L$) and Dean drag force ($F_D$) in the zigzag part [14]. In the straight channel, cells are mainly influenced by the inertial lift force ($F_L$), while the inertial lift force includes shear-gradient-induced lift forces ($F_{SG}$) and wall-effect lift forces ($F_{WL}$) [15]. When the cells flow into the first expansion region, big cells are dominated by $F_{SG}$, causing the big cells to move toward the two side walls due to the sudden absence of $F_{WL}$. However, small cells keep their former equilibrium positions near the center of the channel. By applying the second expansion region, the separation distance between big and small cells is amplified for higher separation efficiency and recovery rate. Big cells (mainly CTCs) flow to two outside outlets and small cells (mainly white blood cells).

The process of the CTC separation from a lung cancer patient’s blood is described in detail in this protocol. After red blood cell lysis and cell dilution, the cell sample was injected into the chip at a 0.4-mL/min flow rate, and cells were collected from each outlet.
The collected cells were analyzed by immunofluorescence staining to enumerate separated CTCs. CTCs are defined as EpCAM+/CD45−/DAPI+. The SAIF chip presented in this protocol is simple but effective, and high-throughput CTC separation can be readily achieved without complex structures. However, it will lose some smaller-sized CTCs during the separation process. Furthermore, the effect of a high concentration of background blood cells on the separation process can be improved with the cost of longer operation time.

2 Materials

2.1 Materials for Fabrication of Silicon Masters

1. AutoCAD software for chip design.
2. Photomask with designed structure.
3. Acetone, ethanol, and ultrapure water.
4. 3 inch silicon wafer.
5. SU-8-3050 negative photoresist (see Note 1).
6. Hot plates.
7. Spin coater (see Note 1).
8. SU-8 Developer (see Note 1).
9. Isopropyl alcohol.
2.2 Materials and Equipment for Fabrication of PDMS Devices

1. Sylgard 184 kit: silicone elastomer base and curing agent.
2. Precision electronic scale.
3. Paper cup.
4. Glass stirrers.
5. Vacuum chamber.
6. 100 mm × 20 mm petri dish.
7. Oven or hot plate.
8. Razor blade.
9. Microscope slide (75 mm × 25 mm).
10. Micro puncher.
11. Adhesive tape.
12. Air plasma chamber (Model PDC-001, Harrick Plasma, Ithaca, NY).
13. Flexible Tygon® tubes (inner diameter is 0.6 mm).
14. Stainless-steel tube (outer diameter is 0.6 mm).

2.3 Materials and Equipment for Blood Sample Processing

1. Sterile 15 mL centrifuge tubes.
2. Red blood cell lysis buffer (see Note 2).
3. Shaking platform.
4. Sterile 5 mL disposable serological pipettes.
5. Centrifuge.
6. PBS or 0.09% saline.

2.4 Materials and Equipment for Microfluidic Device Operation

1. 10 mL syringe.
2. Syringe pump (see Note 3).
3. Sterilized flexible Tygon® tubes.
4. Needles (22 G).
5. Microscope (see Note 3).
6. High-speed CCD camera (see Note 3).

2.5 96-Well Plate Treatment

1. Polylsine (see Note 4).
2. Phosphate buffer solution (PBS).
3. Precision electronic scale.
4. Sterile 15 mL centrifuge tubes.
5. Cell incubator.
6. Sterile filter.
7. Sterile operating table.
8. Pipette.
2.6 Immunofluorescence Staining

1. Phosphate buffer solution (PBS).
2. Polysine treated 96-well plate.
3. 4% paraformaldehyde (PFA).
4. 3% bovine serum albumin (BSA).
5. Antibody: antihuman EpCAM (see Note 5).
6. Alexa Fluor 488-conjugated antibodies (see Note 5).
7. APC anti-CD45 (see Note 5).
8. 4′,6-Diamidino-2-phenylindole (DAPI).
9. Fluorescence microscope.

3 Methods

3.1 Fabrication of Silicon Masters

1. Design the 2D projection of the SAIF chip structure with AutoCAD software (see Note 6).
2. Print the photomask by a commercial supplier.
3. Wash a 3 inch silicon mask with acetone, ethanol, and ultrapure water, respectively (see Note 7).
4. Dry the silicon wafer on a hot plate.
5. Place the wafer on the tray of the spinner, and pour SU-8-3050 negative photoresist on the wafer.
6. Run the spinner at 500 revolutions per minute (RPM) for 10 s and 3000 revolutions per minute (RPM) for 30 s to achieve a height of 50 μm (see Note 8).
7. Place the wafer on a hot plate at 65 °C for 2 min, and transfer the wafer to a hot plate at 95 °C for 7 min.
8. Exposed the wafer to the UV light through the mask for 140 s.
9. Place the wafer on a hot plate at 65 °C for 2 min, and transfer the wafer to a hot plate at 95 °C for 7 min.
10. Wash the wafer with SU-8 Developer to remove extra SU-8-3050 on the wafer (see Note 9).
11. Wash the wafer with isopropyl alcohol, acetone, ethanol, and ultrapure water, respectively (see Note 10).
12. Dry the wafer on a hot plate.

3.2 Fabrication of PDMS Devices

1. Bake the attained wafer in an oven at 120 °C for 1 h, and cool down to room temperature (see Note 11, 12).
2. Weigh 25 g of PDMS and 2.5 g of curing agent in a paper cup, and mix them thoroughly.
3. Degas the PDMS mixture in a vacuum chamber.
4. Pour the PDMS mixture into a petri dish containing the wafer with the patterned design.
5. Degas it again in a vacuum chamber to ensure no air bubbles.
6. Put the petri dish in an oven at 80 °C for 1 h to make the PDMS mixture solidified.
7. Carefully peel off the solidified PDMS from the wafer, and cut it into shapes the size of a microscope slide.
8. Punch holes of 0.8 mm in the inlet and three outlets (see Note 13).
9. Carefully clean the PDMS with the channel and the microscope slide with tape to remove dust (see Note 14).
10. Bond the PDMS and the slide via oxygen plasma for 50 seconds.
11. Insert stainless-steel tubes in the inlet and the outlets to connect flexible Tygon® tubes (see Note 15).
12. Connect flexible Tygon® tubes with the stainless-steel tube for inlet and outlets (Fig. 2) (see Note 16).

3.3 Sample Processing

1. Collect 2 mL of lung cancer patient blood with agreements in an EDTA tube (see Note 17).
2. Stir the tube thoroughly and remove the blood to a sterile 15 mL centrifuge tube.
3. Add red blood lysis buffer in a ratio of 1 mL blood to 3 mL lysis buffer.
4. Mix the blood and lysis buffer thoroughly, and put the tube on a shaking platform for 10 min at room temperature.
5. Collect the cells by centrifuging at 450 g for 5 min at room temperature.
6. Discard the supernatant carefully and resuspend the cells with 0.09% saline (see Note 18).
7. Wash the collected cells with 0.09% saline three times to remove remained red blood cells by centrifuging at 450 g for 5 min at room temperature.
8. Resuspend the cells with 0.09% saline making the volume 4 mL to be injected to the SAIF chip (see Note 19).
3.4 96-Well Plate Treatment with Polylysine

1. Weigh 5 mg of polylysine powder with a precision electronic scale, add it to a centrifuge tube, and dissolve it with 5 mL of PBS.
2. Fully dissolve the polylysine powder by a vortex.
3. Filter the obtained polylysine solution (concentration of 1 mg/mL) by a sterile filter with a pore size of 0.22 μm, and then dispense the solution. The following steps are all performed on a sterile bench.
4. Dilute the polylysine solution (concentration of 1 mg/mL) with PBS to obtain a working solution of polylysine with a concentration of 0.1 mg/mL.
5. Add 100 μL of polylysine working solution to each well of a 96-well plate, and incubate it in a cell incubator for 1 h or overnight at room temperature on the sterile bench.
6. After incubation, remove the polylysine solution from the wells with a pipette, and wash with PBS at least three times.
7. After washing, dry the 96-well plate on a sterile bench.
8. It can be used directly after drying.

3.5 Operation of Microfluidic Device

1. Fix the SAIF chip on an upright microscope equipped with a high-speed CCD camera.
2. Fill the collected sample in a 10 mL syringe.
3. Set the injection flow rate of the injection pump at 0.4 mL/hour (see Note 20).
4. Place the syringe filled with the sample in the pump, and connect it to the chip with flexible Tygon® tubes (see Note 21).
5. Switch on the computer and start the software to take videos of cells in the chip.

3.6 CTC Separation

1. Run the syringe pump at the flow rate of at 0.4 mL/hour (see Note 21).
2. Cells flow through the chip, bigger cells flow to two outside outlets, and small cells flow to the middle outlet (Fig. 3).
3. Cells were collected from three outlets in individual 1.5 mL tubes

3.7 Immunofluorescence Staining

1. Add the collected cells from the two outside outlets in the wells of the polylysine treated 96-well plate. Add 100 μL of cell solution to each well.
2. Incubate the cells in the cell incubator for 1 h to make the cells stick to the bottom of the well.
3. Aspirate the liquid and wash the cells carefully with PBS three times (see Note 23).
4. Fix the cells by adding 4% paraformaldehyde (PFA) for 10 min, and incubate at room temperature.
5. Aspirate the liquid and wash the cells carefully with PBS three times (see Note 23).
6. Block the cell surface with 3% bovine serum albumin at room temperature for 1 h.
7. Aspirate the liquid and wash the cells carefully with PBS three times (see Note 23).
8. Incubate cells with antihuman EpCAM antibody solution for 2 h at room temperature or overnight at 4 °C (see Note 24).
9. After gently aspirating the antihuman EpCAM antibody solution, wash the cells three times with PBS.
10. After gently aspirating the PBS, add APC antihuman CD45 antibody, Alexa Fluor 488-conjugated antibody, and nuclear probe DAPI solution.
11. Incubate at room temperature for 2 h in a dark room.
12. After incubation, aspirate the liquid and wash the cells carefully with PBS three times (see Note 22).
13. Take the images of the cells with a fluorescence microscope.

### 3.8 Enumeration of CTCs

1. Turn on the fluorescence microscope and the computer-aided software.
2. Fix the 96-well plate with the cells on the stage of the microscope.
3. Add the fluorescence channel of FITC, APC, and DAPI to the software.
4. Check the cells with 4× magnification under the bright field.
5. Switch to the fluorescent channel, and use the automatic scanning function to image the entire area of the well with 10× magnification.
6. Switch to different wells manually, and take the images of the cells in all the wells containing cells.

7. Count the cells EpCAM+/CD45−/DAPI+ (Fig. 4), which are regarded as circulating tumor cells.

4 Notes

1. The SU-8-3050 negative photoresist was purchased from MicroChem, Newtown, MA. The spin coater WS-650-23NPPB was purchased from Laurell, USA. The
SU-8 Developer was purchased from MicroChem, Newtown, MA.

2. The red blood cell lysis buffer was purchased from Beijing Solarbio Science and Technology Co., Ltd.

3. A 10 mL syringe pump was purchased from LongerPump company. The microscope used to connect the high-speed CCD was Eclipse Ci-S purchased from Nikon company. The high-speed CCD camera was Revealer 5F04M, which was purchased from Qianyanlang, Hefei, China.

4. Polylysine was purchased from BD Biocoat.

5. Anti-human EpCAM (ab223582) was purchased from Abcam. APC anti-CD45 (Cat. No. 304037) was purchased from BioLegend.

6. The SAIF chip consists of one sample inlet, two expansion regions, and three outlets. The zigzag part consists of straight channels and semicircle channels. The width of the zigzag part is 40 μm, and the distance between two adjacent straight channels is 1 mm. At the end of the fifth straight channel is the first expansion region. The angle of the first expansion region is 60° and the width is 0.84 mm. The distance between the first expansion region and the second expansion region is 15 mm. The width of the second expansion region is 1.64 mm with a 60° expansion angle, and the distance to the outlet is 10 mm. The height of the channel is 50 μm. At the end of the channel, it splits into three outlets, and the separation points are set at 540 μm from the two side walls.

7. During the photolithographic process, the silicon wafer should be washed thoroughly to make sure there is no dust on the surface of the wafer.

8. During spin-coating of the SU-8-3050 negative photoresist on the wafer process, there shouldn’t be any bubbles and the SU-8-3050 should cover the whole surface of the wafer.

9. The developing process should be controlled in 3 ~ 5 min to prevent overdeveloping.

10. Wear personal protective equipment, including gloves and makes, and avoid the developer splash to clothes.

11. Prior to pouring the PDMS mixture for the first time, salinize the wafer by evaporating methyltrichlorosilane for 5 min to protect the patterned structure from peeling off.

12. Always wear a mask and gloves during salinizing process, and avoid inhaling methyltrichlorosilane.
Table 1
The concentration of antibodies diluted in 50 μL 1% BSA blocking buffer for each well

| Antibody                        | Final concentration (μg/mL) | Volume(μL) |
|---------------------------------|-----------------------------|------------|
| Antihuman EpCAM antibody        | 10                          | 1          |
| APC antihuman CD45 antibody     | 10                          | 2.5        |
| Alexa Fluor 488-conjugated antibody | 1                         | 0.25       |
| DAPI                            | 5                           | 0.25       |

13. When punching the holes for the inlet and outlets, you should make sure there is no extra PDMS left in the holes, which might cause the failure of the chip.

14. The PDMS with the designed channel and the microscope glass should be cleaned carefully to remove fingerprints and dust, which might affect the bonding results.

15. Seal the inlet and outlets with PDMS mixture after inserting the stainless-steel tubes to prevent sample leaking during the sample injection process.

16. Wash the chip with DI water to check the bonding result.

17. Keep the blood sample in 4 °C before red blood cell lysis.

18. During the red blood cell lysis process, lysis buffer could be applied twice to remove the red blood cells more completely.

19. The concentration of the blood cells could be increased to improve the purity of the CTCs in the two outside outlets.

20. Stir the syringe slightly to prevent the cell from sedimenting on the bottom of the syringe wall.

21. Wash the flexible Tygon® tubes to remove any dust in the tube, and dry them in the oven before use.

22. Use a new SAIF chip in case of the chip leaking during the sample injection process.

23. During immunofluorescence staining process, aspirate the reagents carefully to prevent the removal of cells from the bottom of the 96-well plate.

24. The concentration and volume of antibodies added per well are listed in Table 1.

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Patient-Specific Microfluidic Cancer Spheroid Cultures for Testing Cancer Therapies

Daheui Choi, Alan M. Gonzalez-Suarez, Daniel D. Billadeau, Wen Wee Ma, Gulnaz Stybayeva, and Alexander Revzin

Abstract

The field of oncology increasingly focuses on strategies to predict effectiveness of a given therapy on a patient-by-patient basis. Such precision or personalized oncology has the potential of significantly extending patient survival time. Patient-derived organoids are seen as the main source of patient tumor tissue that may be used for therapy testing in personalized oncology. The gold standard approach for culturing cancer organoids is in standard multi-well plates coated with Matrigel. Despite their effectiveness, these standard organoid cultures have drawbacks, namely, requirement of a large starting cell population and polydispersity of cancer organoid sizes. The latter drawback makes it challenging to monitor and quantify changes in organoid size in response to therapy. Microfluidic devices with integrated arrays of microwells may be used to both decrease the amount of starting cellular material required to form organoids and to standardize organoid size to make therapy assessment easier. Herein, we describe methodology for making microfluidic device as well as for seeding patient-derived cancer cells, culturing organoids, and testing therapies using these devices.

Key words Patient-derived organoids, Personalized therapy, Microfluidic cancer cultures, Cancer spheroids, Chemotherapy

1 Introduction

Tumor heterogeneity creates a diversity of cancer signaling pathways and phenotypic variations, making it challenging to use one-size-fits-all therapy approaches [1, 2]. This motivates a drive to establish patient-derived culture platforms that may be used to individualize therapy regimens and determine which therapy benefits which patient [3]. Patient-derived tumor organoids have emerged as the strategy of choice for isolating and propagating patient tissue for individualized testing of therapy [1, 4, 5]. Despite its wide use, this strategy has drawbacks: (1) success of organoid formation in standard cultureware (e.g., six-well plate) depends on the amount of starting cellular material and proliferative capacity of
cells. This extends the timeline (often to several weeks) for generating organoids in sufficient numbers to carry out testing and biases testing toward most proliferative tissue samples. (2) Organoids formed in standard cultureware are of irregular dimensions which makes it challenging to monitor and quantify changes in organoid size as result of therapy administration. Therefore, it is challenging to generate and propagate patient-derived cancer organoids [6], resulting in difficulties to achieve drug screening research when cell availability is limited.

Microfluidic systems may be used to address the limitations of standard organoid culture systems [7]. Microfluidic devices require lower number of cells for spheroid or organoid formation and may be designed to form cells into arrays of similarly sized organoids for therapy testing.

In this chapter, we describe a protocol to create patient-derived cancer organoids in a microfluidic device [7]. The microfluidic device consists of two PDMS layers: (1) a bottom layer containing cylindrical microwells and (2) a top layer containing transport channels and culture chambers. The top of the device is linked to an inlet and outlet where culture media and cells are injected. The inside chamber has 19 cylindrical microwells that are 250 μm in diameter and 300 μm deep, allowing cell trapping and spheroid formation. Due to the small dimensions of the microfluidic chambers, a single device can contain multiple chambers, each with an array of microwells, and may be used to test different therapies. Methods for making and preparing devices, digesting patient tissue, seeding cancer cells, and culturing cancer organoids in microfluidic devices are described below. In addition, methods for assessing therapy effectiveness are discussed.

2 Materials

All materials for cell culture are sterilized by UV treatment or autoclave. All chemicals were used without further treatment or purification process.

Filter all medium used with a 0.2 μm filter prior to use.

1. T-75 and T-125 cell culture flasks.
2. 1× phosphate-buffered saline (PBS).
3. 0.25% Trypsin-EDTA.
4. L-WRN cells.
5. L-cell medium: 10% fetal bovine serum (FBS) and 1× penicillin/streptomycin (P/S) in 90% Dulbecco’s modified eagle medium (DMEM). Add 0.5 mg/mL G-418 and 0.5 mg/mL hygromycin right before usage when needed [8].
6. Primary culture medium: 20% FBS and 1% P/S in 80% Advanced DMEM [8].

7. Organoid media for pancreatic adenocarcinoma organoids: 50% Advanced DMEM, 50% L-WRN conditioned media, 1× 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1× Glutamax, 1× N2 supplement, 1× B27 supplement, 500 ng/mL epidermal growth factor (EGF), 1 μg/mL fibroblast growth factor 10 (FGF10), 3 μM SB202190, 0.5 μM A83–01, 1 mM N-acetyl-L-cysteine (NAC), 10.25 mM Nicotinamide, 10 nM gastrin, 1× P/S, and 10 μM Y-27632 (ROCK inhibitor) [9, 10].

8. Organoid media for ovarian cancer organoids: DMEM with high glucose and pyruvate, 1× B27 supplement, 50 μg/mL ascorbic acid, 20 μg/mL insulin, 0.25 μg/mL hydrocortisone, 100 ng/mL FGF2, 100 nM all-trans-retinoic acid, 1× P/S, and 10 μM ROCK inhibitor [7].

2.2 Digestion of Cancer Tissues and Biopsies

1. Fresh cancer tissue or biopsies.
2. Tissue storage buffer (MACS® Tissue Storage Solution).
3. Tweezers.
4. 6 mm petri dish.
5. Disposable blade.
6. Krebs-Ringer Bicarbonate Buffer (KRB buffer): NaCl (7.135 g), D-glucose (1.00 g), NaHCO3 (2.10 g), KCl (0.42 g), and HEPES (4.765 g) in 1 L distilled water; then adjust pH to 7.3.
7. Collagenase type IV solution in KRB buffer.
8. P/S.
9. 100 μm cell strainer.
10. Red blood cell (RBC) lysis buffer.
11. FBS.

2.3 Maintenance and Propagation of Cancer Organoids

1. Matrigel (see Note 1).
2. Organoid media.
3. Gentle cell dissociate reagent (GCDR).
4. DMEM/F12.

2.4 Fabrication of Microfluidic Device with Microwells

1. Master molds for microfluidic device: (1) bottom layer mold containing cylindrical microwells (250 μm in diameter, 300 μm deep) and (2) top layer mold containing transport channels and culture chambers [7].
2. SYLGARD® 184 silicone elastomer kit. Composed of polydimethylsiloxane (PDMS) elastomer base and curing agent.
3. 10 mm cloning glass cylinders.
4. Blades.
5. Biopsy punch (1–2 mm diameter).

2.5 Seeding of Cancer Spheroids in Microfluidic Devices

1. Microfluidic device.
2. 1× PBS.
3. 1% Pluronic F-127 solution in PBS.
4. Organoid dissociation solution: TrypLE™ Express Enzyme, 1% DNase I, and 10 mM ROCK inhibitor.
5. DMEM media with 10% FBS and 1% P/S.
6. 100 μm cell strainer.
7. Organoid media for the corresponding cell type to seed.
8. Matrigel.

2.6 Testing Cellular Response Against Drug in the Microfluidic Device

1. Drug of interest (e.g., gemcitabine).
2. Organoid media.
3. LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells.
4. 1× PBS.

3 Methods

3.1 Preparation of Wnt-3A, R-Spondin, and Noggin Conditioned Medium (Denoted as “L-WRN Conditioned Medium”)

3.1.1 L-WRN Cell Culture

1. Thaw L-WRN cells and seed cells in a T-75 flask with 13 mL L-cell media (see Note 2) for 24 h.
2. Exchange media to L-cell medium with G418 and Hygromycin B. Change media every 48–72 h.
3. Once cells reach a confluency of 95%, wash with PBS twice and treat cells with 1 mL Trypsin-EDTA for 3–5 min at 37 °C.
4. Subculture 1:2 ~ 1:4 ratio and culture cells until desired cell number reaches.

3.1.2 Collection of L-WRN Conditioned Medium

1. Seed L-WRN cells in a T-125 flask with 25 mL L-cell media without G418 and Hygromycin B.
2. Culture for 72–96 h until confluency is reached.
3. Wash the cells with 10 mL primary cell medium and aspirate.
4. Add 25 mL primary cell media.
5. Every 24 h, collect media from the flask (L-WRN conditioned media) and add fresh primary cell media until day 12.
6. Store collected L-WRN conditioned media at −20 °C.
3.2 Digestion of Cancer Tissues and Biopsies and Seeding of Extracted Cells on Matrigel (Fig. 1)

Tissue digestion and seeding described below should be performed within 20 min to ensure viability of cells.

1. Put fresh tissues or biopsies in tissue storage buffer.
2. Take the tissue using the tweezers and place it in a 6 mm petri dish resting on ice.
3. Add 2 mL of KRB solution for small biopsies (i.e., 1 mm thick × 5–10 mm long) or 5 mL for larger tissues.
4. Mince tissue to small pieces (<1 mm in thickness) using a disposable blade (Fig. 1; Mince tissue).
5. Collect the chopped tissue in a conical tube or sterilized bottle using a pipet. Rinse the petri dish with KRB solution to collect any remaining tissue (see Note 3).
6. Add collagenase type IV to a final concentration of 2.5 mg/mL (total volume 5 mL) for small biopsies and 0.25 mg/mL (total volume 10–20 mL) for larger tissues, respectively.
7. Add P/S (final concentration of 1× for total volume).
8. Digest tissue by placing the tissue containing tube at 37 °C for 2–4 min for small biopsies or 40 min for larger tissues, under agitation (Fig. 1; Enzymatic digestion).

Fig. 1 Workflow for cancer organoid culture and seeding into the microfluidic device from collected tissue. Schematic illustration of whole workflow from tissue digestion to microfluidic device seeding after organoid expansion. After enzymatic digestion of tissues, the small cell aggregates are filtered using 100 μm strainer and embedded in Matrigel for expansion. (Reproduced from Ref. [7] with permission from Springer Nature)
9. Filter the digested tissue using a 100 μm strainer into a new conical tube to remove undigested cell clumps and debris (Fig. 1; Filter 100 μm pore).

10. Wash the strainer with fresh KRB solution to completely filter out residual cells.

11. Centrifuge the filtered cells at 1500 rpm for 5 min and aspirate supernatant.

12. Treat cells with 3 mL RBC lysis buffer for 3 min at 25 °C if RBCs are present. Deactivate RBC lysis buffer by adding 3 mL of PBS with 1% FBS (Fig. 1; RBC lysis).

13. Centrifuge cells at 1500 rpm for 5 min at 4 °C and aspirate supernatant.

14. Add 80–100 μL of Matrigel to each well in a clean 12-well plate. Ensure that surfaces of all wells are completely covered with Matrigel (see Note 4).

15. Place the well plate at 37 °C for at least 10 min for gelation.

16. Add the desired volume of organoid media depending on cell yield.

17. Seed the cells into the Matrigel-precoated well plate (see Note 5).

18. Culture at 37 °C with 5% CO₂ and exchange media every 3–5 days.

3.3 Maintenance and Propagation of Cancer Organoids (Fig. 1; Matrigel culture)

1. Once the organoids are confluent on Matrigel, aspirate media and add 1 mL GCDR to each well.

2. Gently pipette up and down to break down Matrigel and cells organoids in the well plate.

3. Collect cells in a conical tube and spin cells down at 1500 rpm for 5 min at 4 °C. Aspirate supernatant (see Note 6).

4. Resuspend cells with cold DMEM/F12 and pipette up and down ~30 times to break cell clumps and remove Matrigel.

5. Spin cells down at 1500 rpm for 5 min at 4 °C and aspirate supernatant.

6. Prepare an organoid media-Matrigel solution containing organoid media and Matrigel at 1:1 ratio. Total volume needed depends on the number of wells to seed.

7. Add the prepared organoid media-Matrigel solution to the cell tube and mix well avoiding bubble formation.

8. Seed the cell suspension to the center of each well in the well plate to create Matrigel domes. For this, add 400 μL of the cell suspension in the center of each well of a six-well plate (80–100 μL for 12-well plates; see Note 7) without spreading the suspension.
9. Incubate the well plate at 37 °C for 30–40 min to solidify the Matrigel domes.

10. Gently add 3 mL (for 6-well plate) or 1 mL (for 12-well plate) of organoid media to each well.

11. Change media every 3–5 days, depending on cell density.

12. Subculture organoids every 7–10 days.

3.4 Preparation of Microfluidic Device with Microwells (Fig. 2a and b)

1. Prepare 55 g of polydimethylsiloxane (PDMS) elastomer by mixing base and curing agent at a 10:1 ratio.

2. Pour PDMS mixture to both molds until reaching a height of ~1.5 mm, and place it in a vacuum desiccator for 15–20 min to remove trapped bubbles (see Note 8).

3. Bake the molds in a convection oven for 60 min at 80 °C to solidify PDMS.

4. Peel off the PDMS from the mold and cut each individual device following its edges.

5. Punch inlet and outlet holes in the top layer using the biopsy punch.

6. Place both PDMS slabs with microwells and chambers facing up inside a plasma etcher, and treat both PDMS slabs with oxygen plasma for 1 min.

7. Align the PDMS slabs to form the final microfluidic device under a stereoscope. Make sure that microwells and microfluidic chambers are facing each other.

8. Bond cloning cylinders on top of inlet and outlet of the microfluidic device using uncured PDMS (10:1 ratio) to use as media reservoirs.

9. Bake the device in the oven for 20–30 min at 80 °C.

10. Place the device in a 100 mm petri dish. This helps to later move the microfluidic device between incubator, biosafety cabinet, and microscope.

11. Add 200 µL of 1% Pluronic F-127 solution in both inlet and outlet, and leave the device in the vacuum desiccator for at least 30 min to remove air from chambers and microwells. The Pluronic F-127 solution prevents cell attachment to the surface of the microwells [7] (Fig. 2c: 1. Add Pluronic solution).

12. Place the device at 4 °C for long-term storage and dissolve any remaining air bubbles.

3.5 Digestion of Organoids to Single Cells for Seeding into Microfluidic Device

1. Follow steps 1 to 5 in 3.3.

2. Treat cells with 2 mL of organoid dissociation solution and resuspend well.
3. Place the cell tube in a water bath at 37 °C for 3–5 min. Deactivate TrypLE by adding same volume of DMEM media with 10% FBS.

4. Spin cells down at 1500 rpm for 5 min at 4 °C and discard supernatant.

5. Add 1–2 mL media. If cell aggregates are visible, filter the cell suspension using a 100 μm cell strainer to avoid clogging the microfluidic device.
6. Resuspend cells at a cell density of $5 \times 10^5$ cells/200 μL in organoid media.

We recommend seeding cells using an inverted microscope inside the biosafety cabinet to observe cell trapping inside microwells.

1. Sterilize the microfluidic device under UV light for at least 30 min.
2. Discard Pluronic solution from inlet and outlet media reservoirs in the microfluidic device, and add 200 μL of PBS to the inlet and 50 μL to the outlet to wash out the Pluronic solution from the device.
3. Repeat step 2 a total of three times.
4. Add 100 μL and 20 μL of the cell suspension (prepared in 3.5) to the inlet and outlet reservoirs, respectively. The difference in hydrostatic pressure between inlet and outlet creates flow inside the microfluidic device, moving the cell suspension from inlet to outlet. The flow stops once the volume of inlet and outlet reservoirs is at the same level (Fig. 2c: 2. Add cell suspension).
5. As soon as the cell suspension is placed into inlet and outlet, create a high flow rate inside the microfluidic chamber by completely covering the inlet with a finger and applying pressure. If cells are still flowing after removing the applied pressure, repeat the procedure. Keep applying pressure until cells stop moving completely when no pressure is applied. Cells will fall into the microwells by gravity. Be mindful of the number of media remaining in the inlet, and avoid pushing air into the microfluidic channel.
6. Repeat step 5 until an even layer of cells is covering the surface of all microwells. Avoid overfilling the wells and make sure all microwells have an even number of cells.
7. Remove cell suspension from the inlet and outlet reservoirs and keep in a vial for reuse.
8. Add 200 μL of DMEM media with 10% FBS to inlet and outlet. Pipette up and down several times to resuspend all sedimented cells. Completely discard the resuspended media.
9. Wash the chamber with DMEM media with 10% FBS similarly to step 1 to remove all cells residing outside of the microwells (Fig. 2c: 3. Wash with fresh media).
10. Repeat step 8 until no cells are remaining at the inlet, outlet, or inside the microfluidic device.
11. Discard the media in the reservoirs and add organoid media with 1% Matrigel. The added Matrigel helps cells to aggregate.
12. After 24 h, exchange media to organoid media without Matrigel.

13. Exchange media every 24 h and maintain until the cells have formed compact organoids (Fig. 2d).

3.7 Testing Chemotherapies Using Microfluidic Cancer Cultures (Fig. 2e)

1. Prepare chemotherapy treatment solutions by adding the drug of interest (gemcitabine) to organoid media at the desired concentrations (see Note 9).

2. Remove the organoid media from the reservoirs in the microfluidic device.

3. Add 200 μL of drug containing media to the inlet and 50 μL to the outlet reservoir.

4. Exchange media with fresh drug containing media every other day. On the days that media is not exchanged, recirculate media in the device by moving 50 μL of media from the outlet reservoir into the inlet reservoir.

5. Culture cells until the desired time point (typically 7 days).

3.8 Assessment of Cellular Response against Drug in the Microfluidic Device (Fig. 2e)

1. Take micrographs of the organoids every other day (0, 1, 3, 5, and 7 days after drug treatment).

2. Open images in “ImageJ” software (see Note 10).

3. Find the area of each organoid. This can be done manually by drawing a polygon along the boundaries of the organoids and then using the measuring option (Analyze → Measure) (see Note 11).

4. Normalize the organoids area over time against day 0 of drug treatment.

5. Plot data to determine cancer organoid response to chemotherapy treatment.

3.8.1 Spheroid Size Growth Assessment Using ImageJ Software

1. Remove media from inlet and outlet of the device.

2. Wash the microfluidic chamber with PBS.

3. Prepare live/dead staining solution: 0.5 μL of Calcein AM and 2 μL of Ethidium homodimer-1 in 1 mL PBS.

4. Remove PBS and add Live/Dead staining solution to the device.

5. Let the solution flow to the outlet and leave the device at 37 °C incubator for 20–30 min (see Note 12).

6. Acquire fluorescence micrographs of the cancer organoids (live: green fluorescence/dead: red fluorescence) and export images of individual fluorescence channels (Fig. 3a and b).

7. Open the fluorescence images in ImageJ software.
Cancer Spheroids in Microfluidic Devices for Drug Testing

Fig. 3 (a) Left: image of patient-derived PDAC organoids in microwells at day 9 (scale bar for microfluidic image = 5 mm; scale bar for microwells = 250 μm). Right: live/dead assay after gemcitabine treatment with different concentrations to PDAC organoids for 7 days (scale bar = 250 μm; inserts scale bar = 100 μm). (b) Left: live/dead assay after drug treatments to a patient-derived xenograft (PDX) ovarian cancer line in microwells at day 6. Fluorescence micrographs show live (green) and dead (red) cells after drug exposure with different concentrations of gemcitabine (scale bar = 300 μm; inserts scale bar = 100 μm). Right: doxorubicin IC50 curves for ovarian cancer. Red dotted line indicates IC50 values. Black dotted line represents the viability at day 6. (Figures are reproduced from Ref. [7] with permission from Springer Nature)

8. Select the positive fluorescence area in each channel (Image → Adjust → Threshold color → Brightness control → Select) and measure the area (Analyze → Measure).

9. Count the green/red fluorescence area (A) ratio to assess the viability (viability % = 100 × A_{green}/(A_{green} + A_{red})) (Fig. 3b).

4 Notes

1. Matrigel should be kept at 4 °C (or in ice) after thawing to avoid gelation.

2. Because L-WRN cells are fragile after thawing, centrifugation and excess pipetting should be avoided [8].

3. The sectioned tissue tends to stick inside of the tips. To prevent this, precoat the inside of the pipet tip (1 mL tip) with media (or BSA solution) by dipping the entire tip in media and pipetting up and down.
4. The well plate used may vary depending on the number of cells to be extracted. In case of a 24-well plate, add 30–50 μL to each well. Also, if the well surface is not completely coated with Matrigel, the cells could attach to the uncoated surfaces, preventing the formation of 3D organoids.

5. The extracted cell number from biopsies or tissues varies depending on the tissue conditions. The extracted number of cells from biopsies is normally not many; it is hard to count the cell number. In that case, we recommend the one to two wells in 24-well plate or 12-well plate for seeding. In case of large tissue sectioned, 1–5 × 10^5 cells in 12-well plate are recommended. The culture and subculture timeline can be dependent on how fast cells become organoid.

6. Centrifugation at 4 °C prevents Matrigel gelation and allows for easier removal of Matrigel from cells.

7. In order to prevent spreading of Matrigel domes in the wells, (1) preheat the well plate for 30 min at 37 °C before seeding, or (2) leave the well plate in the biosafety hood for 5 min right after seeding Matrigel to minimize movement. Afterward, carefully move the multi-well plate into incubator to complete gelation process.

8. The bubbles in PDMS interfere with observation/imaging of the device.

9. The chemotherapy drug (gemcitabine) can occur dermal and acute toxicity. When it is used, wear gloves and lab coat. The gemcitabine should be stored at 4 °C avoiding light.

10. Image analysis can be done using any preferred software and methodology. We suggest ImageJ as a simple solution for this purpose.

11. If the image size is set up in inch or pixel in ImageJ, set up the scale before measuring (Analyze → Set scale → input distance and unit data).

12. The flow inside the microfluidic device will stop once level of solution in the inlet and outlet equilibrates. We find that staining works better when staining reagents flow in the device. Therefore, we recommend recirculating staining reagents every 15–20 min. This can be done by collecting 100 μL of solution from the outlet and adding it into the inlet.

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Chapter 16

Isolation of Cancer Cells from Liquid Biopsies Using 3D-Printed Affinity Devices

Yijia Yang, Kitiara Griffin, Sarah Villareal, and Dimitri Pappas

Abstract

Liquid biopsies are examination procedures for deciding the grouping of malignant growth cells tracked down in samples of blood and other body fluids. Liquid biopsies are likewise significantly less intrusive than tissue biopsies as they just require small amount of blood or body fluids from the patient. With the utilization of microfluidics, cancer cells can be isolated from the fluid biopsy and achieve early diagnosis. 3D printing is turning out to be progressively well known for microfluidic devices creation. 3D printing has shown multiple advantages compared to traditional microfluidic devices production, including effortless large-scale manufacturing of precise copies, the fuse of new materials, and execution of additional complicated or drawn-out plans that are hard to execute in conventional microfluidic devices. Combining 3D printing with microfluidics makes for a relatively inexpensive analysis of liquid biopsies with a chip that can be more advantageous to use over traditional microfluidic chips. In this chapter, a method for affinity-based separation of cancer cells in a liquid biopsy using a 3D microfluidic chip will be discussed, along with the rationale behind the method.

Key words Cancer isolation, Bioanalysis, 3D printing, Microfluidics, Liquid biopsies

1 Introduction

Liquid biopsies are analysis techniques for determining the concentration of cancer cells found in samples of blood and other bodily fluids. They are important for detecting early stages of cancer during which treatment would be the most successful [1]. Liquid biopsies are also much less invasive than tissue biopsies as they only require blood or a similar sample from the patient. With the use of microfluidics, cancer cells can be isolated from the liquid biopsy and subsequently analyzed to provide a fast diagnosis.

Microfluidic devices are well suited to this task, as they are designed to be able to conduct complex processes on small fluid volumes. There are a variety of microfluidic chip designs that can be utilized for affinity-based separation and isolation, most notably those that feature herringbone structures, inertial focusing, straight
channel, etc. [5, 7, 8]. The use of microfluidic chips provides many advantages in that they are highly efficient in the separation and isolation of certain cell types, require only a small amount of sample and necessary reagents, can be fabricated at low cost, can be easily manufactured and transported, etc. [2, 3]. Additionally, depending on the experimental design and purpose, these microfluidic chips have the potential to be applied to a wide range of biomedical problems.

3D printing is becoming increasingly popular for microfluidic device fabrication. 3D printing has advantages over glass and poly (dimethyl siloxane) fabrication methods, including facile mass production of exact duplicates, the incorporation of new materials, and implementation of more complex or tedious designs that are difficult to execute in traditional microfluidic devices.

Traditional microfluidic devices have, to date, been fabricated using the molten filament method and stereo low-force photolithography. The latter approach allows for higher resolution, down to 25 mm for many commercial 3D printers. The resin used can also have a variety of properties that can be exploited for the microfluidic device with some resins having tougher properties while others allowing for more flexibility and movement. No matter the resin used, however, the 3D-printed microfluidic device is typically more durable against damage and can be more reusable over the traditional man-made microfluidic device. For some designs, such as an affinity-based approach, the reusability and durability of 3D-printed devices can help ensure accurate measurements in the study of liquid biopsies.

Affinity-based methods are often employed in microfluidics to separate the analyte (i.e., target cell) accurately and effectively from the matrix [4]. Affinity separations exploit differences in cell surface antigen expression, differing from intrinsic separation methods such as size, density, and charge. Affinity methods can achieve either positive cell enrichment (capture target cells) or negative cell enrichment (capture nontarget cells to let target cells go through but final sample has low purity) [5]. This affinity binding has been proven to correlate with capture affinity and antigen expression of target cells [4]. Using affinity separation with 3D microfluidic chips can make for a cell separation that is both easy to perform and a chip that can be mass produced or even reused for replicate experiments. Later in this chapter, a simplified protocol will be provided that explains both how to fabricate a 3D microfluidic chip and how to prepare and utilize the chip for affinity-based separations.

The overexpression of the human transferrin receptor, CD71, in proliferating cancer cells is due to the presence of iron within the cell that is necessary for cellular functions and the proliferation itself [6]. CD71 acts as a biomarker for all cancer types and thus can be utilized in early cancer detection methods regardless of cancer type.
As CD71 is a surface glycoprotein, it can undergo cellular separation. More specifically, an immunoaffinity-based separation method can be used to identify and capture cells expressing CD71 by introducing its affinity ligand, anti-CD71. Once the affinity ligand is introduced, the target cells can be captured and accounted for as the substrate will bind to its individual ligand.

EpCAM, anti-Epithelial Cell Adhesion Molecule, is another surface glycoprotein that can be utilized to effectively capture circulating tumor cells (CTCs) corresponding to various cancer phenotypes, such as colon, breast, and prostate cancers to name a few [6]. Like CD71, anti-EpCAM can be used to capture epithelial cancer cells that contain a significant amount of EpCAM on their cell surface. Other ligands may also be employed in the isolation and capture of different cell types depending on the cell’s distinctive makeup. For example, CD45 may be used in the capture of leukocytes for the purposes of separation or further analysis.

Affinity separation allows for the detection and separation of target cells based upon their antigen expression and morphology [6]. By coating a microfluidic device with the appropriate capture antibodies, target cells can be isolated and captured for further analysis [2]. One such example involves the use of flow cytometry to detect and measure physical and chemical characteristics of various cell types, specifically to quantify cell surface antigen expression [2]. Furthermore, various statistical methods can be employed to ascertain capture efficiency, capture purity, and enrichment values to elucidate the implications and significance of captured target cells relative to nontarget cells.

2 Materials

2.1 3D-Printed Microfluidic Device Fabrication

1. Form 3 3D printer from FormLabs (or other SLA printer).
2. Photopolymer clear resin from FormLabs (or other clear resin compatible with chosen printer).
3. Form Wash from FormLabs (if Form 3 used).
4. Form Cure from FormLabs (if Form 3 used).

2.1.1 Instruments for 3D-Printed Microfluidic Device

1. Autodesk Fusion 360 software.
2. PreForm software from FormLabs (or similar for device used).
3. Isopropyl alcohol (concentration of 90% or higher).

2.2 3D-Printed Microfluidic Device Surface Modification/ Antibody Conjugation

1. Sodium hydroxide: 2 M NaOH solution prepared from 8 g of NaOH solid dissolved in 100 mL DI water.
2. Hydrochloric acid: 0.1 M HCl solution prepared from 8.23 mL of concentrated HCl diluted in 1000 mL DI water. Store at room temperature.
3. MES buffer: 0.078 g of 2-(N-morpholino)ethanesulfonic acid in 400 mL DI water to achieve 100 mM MES buffer with pH 5. Store at room temperature.
4. Concentration isopropyl alcohol.
5. EDC-SulfoNHS solution: 0.09585 g EDC with 0.108565 g Sulfo-NHS in 20 mL MES buffer. Fresh made each run.
6. T50: 50 mM Tris(hydroxymethyl)amino methanol hydrochloride acid with 50 mM sodium chloride. Store at 4 °C.
7. Neutravidin: 0.2 mg/mL neutravidin in T50 solution. Store at 4 °C.

2.3 Detection of Clinical Cancer Cells

1. Clinical cancer cell plasma samples. Store in freezer –5 °C to –30 °C.
2. Phosphate buffered saline (PBS) with pH 7.4. Store at room temperature.
3. MitoTracker Green. Store in freezer –5 °C to –30 °C.

2.4 Imaging of Isolated Cancer Cells

1. CMOS camera (Quantalux, Thorlabs) coupled to an inverted epifluorescence microscope (Olympus IX71).
2. ImageJ software (Version 1.43u, National Institutes of Health).

3 Methods

3.1 3D Printing Microfluidic Device

1. 3D-printed microfluidic devices were designed using Autodesk Fusion 360 with measurements of 25 mm in length, 1 mm in height, and 1 mm in width with 25 microns resolution (Fig. 1).
2. 3D model designs of the microfluidic devices were exported from Autodesk Fusion 360 in OBJ file format.
3. The OBJ printer file should be converted to the printer’s format, such as a FORM file for FormLabs 3D printers.
4. When the printing finished, devices were washed in Form Wash with IPA for 10 min.
5. Devices are air-dried after wash and then placed in Form Cure with clear resin setting at 60 °C for 15 min (Fig. 2).
6. Devices can be stored at room temperature for future use.

3.2 Surface Modification

1. Devices are placed in 2 M NaOH solution at 45 °C for 24 h.
2. Rinse the devices with 0.1 M HCl to quench the reaction.
3. Following, dry with N₂.
4. Fill devices’ channels with the 50 mM of EDC-SulfoNHS solution in 100 mM MES. Buffer at room temperature for 20 min.

5. Rinse the devices with concentrated IPA.

6. Dry with N₂.

7. Incubate channels with neutravidin at room temperature for 15 min.
8. Rinse channels with T50 followed by DI water.
9. Air-dry devices and store at 4 °C for future use (Fig. 3).

### 3.3 Isolation of Cancer Cells with 3D-Printed Microfluidic Device

1. Defrost the frozen clinical cancer plasma and transfer 100 μL into centrifuge tube.
2. Wash cells with 900 μL PBS and centrifuge at 4500 rpm for 5 min.
3. Remove the supernatant and resuspend cell pellets with 1000 μL PBS.
4. Stain cancer cells with 1 μL MitoTracker Green to label all cells for visualization. Optional: replace MitoTracker Green with antibody staining after separation.
5. Incubate cancer cells at 37 °C in 5% CO₂ for 45 min.
6. Wash cancer cells four times post-staining with PBS.
7. Resuspend cell pellets with 1000 μL PBS.
8. Transfer sample into 1 mL syringe.
9. Connect 3D-printed microfluidic device with syringe using a 30-gauge PTFE thin wall tubing.
10. Set the flow rate on syringe pump at 0.6 mL h⁻¹ for the experiment.

### 3.4 Imaging

1. Isolated cancer cell images were taken under fluorescence microscope with appropriate excitation and emission filters.
2. Cell images were viewed and analyzed with ImageJ software (Fig. 4).
4 Notes

1. Having the chips stand up on the side with inlet when printing could eliminate the blockage of channels with resin.
2. Wash thoroughly to make sure there are no excessive resins left on the chips before curing step.
3. Have chips dried completely before the curing procedure to avoid blockage of the channels.
4. Prepare MES buffer at pH 5. MES works well if the isoelectric point of the protein is above the pH of the buffer.
5. Do not mix EDC-Sulfo NHS and MES buffer before having chips ready. Incubate chips in solution right after mix EDC-Sulfo NHS and MES buffer to avoid unstable Sulfo-NHS.

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Chapter 17

A Microfluidic SERS Assay to Characterize the Phenotypic Heterogeneity in Cancer-Derived Small Extracellular Vesicles

Jing Wang, Matt Trau, and Alain Wuethrich

Abstract

Small extracellular vesicles (sEVs) are nanoscopic bioparticles that transport biomolecular cargoes between cells. sEVs have been implicated in many pathological processes such as cancer, rendering them as promising targets for therapeutics and diagnostics. Characterizing phenotypic differences in sEV biomolecular cargos could support understanding their roles in cancer. However, this is difficult due to similar physical properties of sEVs and requirement for highly sensitive analysis. Our method describes the preparation and operation of a microfluidic immunoassay with surface-enhanced Raman scattering (SERS) readouts, termed sEV subpopulation characterization platform (ESCP). ESCP applies an alternating current induced electrohydrodynamic flow to enhance collisions of sEVs with the antibody-functionalized sensor surface. Captured sEVs are labeled with plasmonic nanoparticles to facilitate multiplexed and highly sensitive phenotypic characterization of sEVs by SERS. ESCP is demonstrated for characterizing the expression of three tetraspanins (CD9, CD63, CD81) and four cancer-associated biomarkers (MCSP, MCAM, ErbB3, LNGFR) in sEVs derived from cancer cell lines and plasma samples.

Key words Microfluidics, Biosensor, Cancer diagnostics, Extracellular vesicles, Surface-enhanced Raman scattering

1 Introduction

Small extracellular vesicles (sEVs) are 30–150-nm particles and carry biological molecules from their parental cells to recipient cells [1]. EVs are emerging as a promising therapeutic and diagnostic marker given their essential roles in physiology and pathology [1]. However, it is becoming evident that there is considerable diversity in the cargo and function of sEVs, which presents a significant challenge in sEV isolation and characterization [2]. Ultracentrifugation and size exclusion chromatography are two most commonly used size-based sEV isolation methods due to high sEV yield but are still subject to contaminants such as
protein aggregates and/or lipoproteins [3, 4]. Moreover, growing evidence suggests that sEVs having similar physical characteristics (e.g., sizes) might contain unique composition and subsequent distinct functional populations [5]. Because proteins present on the sEV-surface are readily accessible and not obstructed by the sEV lipid bilayer, the sEV isolation method based on the sEV-surface proteins might allow for a greater understanding of sEV heterogeneity in protein composition.

Given that proteins are critical bioactive constituents of sEV cargo, proteomic analysis is thus appealing to provide insights into the nature of sEV heterogeneity, particularly defining sEV populations enriched in tumor-derived proteins. Traditional methods for sEV proteomic analysis include mass spectrometry, Western blot, and enzyme-linked immunosorbent assay, which lack sufficient sensitivity to analyze sEV heterogeneity in clinical samples (e.g., body fluids) and are labor-intensive. High-sensitivity flow cytometry as an emerging approach for sEV proteomic analysis allows detection of single sEVs but is challenging for multiplex phenotypic characterization. Therefore, it is highly desirable to develop a sensitive, integrated platform for sEV proteomic analysis and facilitate the better understanding of sEV heterogeneity.

In this protocol, we developed a sensitive, high-throughput sEV subpopulation characterization platform (ESCP) for sEV heterogeneity evaluation [6]. The ESCP consisted of a microfluidic chip for sEV capture and surface-enhanced Raman scattering (SERS) spectroscopy for multiplex signal readouts. The microfluidic chip was arrayed with 28 pairs of circular ring-shaped gold electrodes. We functionalized the surface of gold electrodes with antigen-specific antibodies to immune-capture different sEV subpopulations that possessed unique surface protein markers. The captured sEVs were subsequently immuno-labeled with SERS nanotags (i.e., gold nanoparticles coated with antibodies and Raman reporters). For multiplex detection of sEV-surface protein markers, we synthesized SERS nanotags with different characteristic Raman signals. By utilizing such a “sandwich” detection format, we performed the multiplex proteomic analysis on isolated sEV subpopulations classified by the surface protein marker expression. The circular ring-shaped gold electrodes were designed to generate a nanoscaled circulating force under the alternating current electric field to increase collisions between antibody-functionalized gold electrodes and sEVs and SERS nanotags [7].

Figure 1 shows the schematic workflow of using ESCP for sEV heterogeneity characterization. To isolate sEV subpopulations expressing different tetraspanin markers, we functionalized three gold electrodes with antibodies against CD9, CD63, and CD81, respectively. Under the application of nanoscaled circulating force, the CD9-, CD63-, or CD81-expressing sEV subpopulations were specifically isolated and enriched on the surface of gold electrodes.
We performed simultaneous sEV labeling with a cocktail of synthesized SERS nanotags. These SERS nanotags possessed unique Raman signals at 1000 cm$^{-1}$ for 4-mercaptopyridine (MPY), 1075 cm$^{-1}$ for 4-mercaptobenzoic acid (MBA), 1335 cm$^{-1}$ for 5, 5$'$-dithiobis (2-nitrobenzoic acid) (DTNB), and 1375 cm$^{-1}$ for 2,3,5,6-tetrafluoro-4-MBA (TFMBA), respectively. To comprehensively evaluate the sEV heterogeneity, we applied the ESCP to investigate the expression of cancer-associated and canonical sEV
markers, respectively. The cancer-associated markers included melanoma-associated chondroitin sulfate proteoglycan (MCSP), melanoma cell adhesion molecule (MCAM), receptor tyrosine-protein kinase (ErbB3), and low-affinity nerve growth factor receptor (LNGFR); the canonical sEV markers included CD9, CD63, and CD81. The presence of each marker on the sEV surface was identified via antibody-functionalized SERS nanotags. Raman scanning was then performed on gold electrodes for signal readouts. A false-color image was established to reflect the sEV heterogeneity based on the characteristic peak intensities of SERS nanotags. We hypothesized that the average signal intensity of each SERS nanotag in the false-color image was positively correlated to the target marker level in the sEV ensemble.

2 Materials

2.1 Cell Culture, Cell Passage, and sEV Generation

1. Cell culture medium 1: 500 mL RPMI-1640 medium supplemented with 50 mL of fetal bovine serum (FBS), 5 mL of $10^4$ U/mL penicillin-streptomycin, and 5 mL of 100× GlutaMAX.

2. Cell culture medium 2: 500 mL Dulbecco’s modified eagle medium (DMEM) supplemented with 50 mL of FBS, 5 mL of $10^4$ U/mL penicillin-streptomycin, and 5 mL of 100× GlutaMAX.

3. 10 mM phosphate-buffered saline (PBS).

4. Cell lines: melanoma cell line (SK-MEL-28), lung cancer cell lines (NCI-H1703, NCI-H1650, and SK-MES-1), and breast cancer cell line (MCF7).

5. TrypLE™ Express Enzyme.

6. Cell culture flasks.

2.2 sEV Isolation Via Size Exclusion Chromatography

1. Amicon® Ultra-15 centrifugal filter device 100 kDa MWCO.

2. Centricon Plus-70 centrifugal filter device 100 kDa NMWL.

3. Sepharose 4B Resin.

4. Empty chromatography columns with 30 μm top and bottom frits and top and end caps.

5. Vacuum flask with stopper.

6. 0.1 M NaOH.

7. Filtered PBS (0.22 μm).

8. Small beaker.

9. Column rack.
2.3 Fabrication of ESCP

1. Layout editor software, e.g., L-Edit (EDA Solutions, UK).
2. Negative photoresist, e.g., AZ® nLOF 2020 (Microchemicals GmbH, Germany).
3. 5 inch chrome mask, e.g., sodalime chrome mask (Bonda Technology Pte Ltd., Singapore).
4. 4 inch glass wafers, e.g., Schott Borofloat® 33 (Bonda Technology Pte Ltd., Singapore).
5. Direct laser write system, e.g., μPG 101 (Heidelberg Instruments, Germany).
6. Spin coater and hot plate, e.g., SM-150/HP-150 duo (Sawatec, Switzerland).
7. Reactive ion etching instrument, e.g., PlasmaPro 80 (Oxford Instruments, UK).
8. E-beam evaporator, e.g., Temescal FC-2000 evaporator (Ferrotec, USA).
9. Mask aligner, e.g., EVG 620 mask aligner (EV Group, Austria).
10. Developer solution: AZ726 MIF Developer (Microchemicals GmbH, Germany).
11. Lift off solution: Remover PG (Microchemicals GmbH, Germany).
12. Polydimethylsiloxane (PDMS) elastomer kit: Sylgard™ 184 Silicone Elastomer Kit (Dow Chemical Company, USA).
13. Brightfield microscope.

2.4 Gold Nanoparticle Synthesis and SERS Nanotag Preparation

1. Gold chloride solution: 1% w/v gold(III) chloride trihydrate (HAuCl₄) in Milli-Q water (store at 4 °C).
2. Sodium citrate solution: 1% w/v sodium citrate dihydrate in Milli-Q water.
3. DSP solution: 1 mM dithiobis(succinimidyl propionate) (DSP) in dimethyl sulfoxide (DMSO).
4. Raman reporter solution: 1 mM MBA, 1 mM TF MBA, 1 mM DTNB, or 1 mM MPY in ethanol (store at 4 °C).
5. Antibody solution: 0.5 mg/mL anti-CD63 antibody (Novus Biologicals, clone H5C6), 0.5 mg/mL anti-CD9 antibody (Novus Biologicals, clone 5G6), 0.5 mg/mL anti-CD81 antibody (Novus Biologicals, clone 1D6), 0.5 mg/mL anti-MCSP antibody (R & D Systems, clone LHM-2), 0.5 mg/mL anti-MCAM antibody (R & D systems, clone 128,018), 0.5 mg/mL anti-ErbB3 antibody (R & D Systems, clone 66,223), and 0.5 mg/mL anti-LNGFR antibody (R & D systems, clone 74,902) in PBS.
6. BSA solution: 0.1% w/v bovine serum albumin (BSA) in Milli-Q water (store at 4 °C).
7. Milli-Q water (resistivity of 18.2 MΩ·cm at 25 °C).

2.5 ESCP Assay
1. ESCP microfluidic device.
2. Signal and wave generator, e.g., 33510B Waveform Generator (Agilent Technologies, USA).
3. PBS.
4. Blocking solution: 1% w/v BSA in PBS.
5. Biotinylated BSA (bBSA) solution: 200 μg/mL bBSA in PBS.
6. Avidin solution: 100 μg/mL avidin in PBS.
7. Capture antibody solution: 10 μg/mL biotinylated anti-CD9 antibody (Invitrogen, clone MEM-61), 10 μg/mL biotinylated anti-CD63 antibody (Biolegend, clone H5C6), and 10 μg/mL biotinylated anti-CD81 antibody (Biolegend, clone 5A6) in PBS.

3 Methods

3.1 Cell Culture
1. Culture SK-MEL-28, NCI-H1703, NCI-H1650, and MCF7 cells in the cell culture medium 1 and SK-MES-1 cells in the cell culture medium 2.
2. Maintain cells in a humidified incubator in 5% CO₂ at 37 °C.
3. Passage cells every 3 days or when the flask is 80% confluence.

3.2 Cell Passage
1. Remove the old medium and wash cells with PBS.
2. Treat cells with 1× TrypLE™ Express Enzyme for 5 min at 37 °C in a humidified incubator.
3. Check the degree of cell detachment under the microscope.
4. Promote cell detachment by mild mechanical disturbance, such as tapping, etc.
5. Add the fresh medium to dilute the enzyme and decrease activity.
6. Pipette the medium several times to dissociate cells into a single-cell suspension.
7. Centrifuge the medium including cells at 400 g for 4 min, and remove the supernatant.
8. Resuspend cells with the medium and transfer back to the flask.
3.3 sEV Generation

1. Remove the old medium when the cell confluence reaches 70%.
2. Wash cells with PBS twice.
3. Culture cells in the RPMI-1640 medium or DMEM in a humidified incubator in 5% CO₂ at 37 °C.
4. After 24 h, collect the conditioned culture medium (i.e., the medium containing EVs) and centrifuge at 800 g for 10 min.
5. Store the conditioned culture medium at 4 °C for short-term storage or –80 °C for long-term storage.

3.4 Column Preparation for Size Exclusion Chromatography

1. Swirl the Sepharose 4B gently.
2. Add 4 mL of filtered PBS to 12 mL of Sepharose 4B resin in a vacuum flask and swirl gently.
3. Seal the top of the flask and perform the degassing.
4. Prepare the empty chromatography column by putting the frit tightly on the bottom of the column and wetting the sides and frit with a small amount of ultrapure water.
5. Gently transfer the degassed resin into the column, and allow it to pack to a 10 mL volume and fit the top frit.
6. Equilibrate the column with at least 10 mL of filtered PBS, and place the end and top caps on the column.
7. Store the column at 4 °C prior to use.

3.5 sEV Isolation

1. Load the conditioned culture medium to the Centricon Plus-70 centrifugal filter device 100-kDa NMWL and centrifuge at 3000 g for 20 min at 4 °C.
2. Perform reverse centrifugation at 1000 g for 2 min at 4 °C to collect the concentrated samples.
3. Attach the chromatography column in an upright position to the column rack and remove the top and end caps.
4. Flush the column with at least one column volume of filtered PBS buffer.
5. Load less than 500 μL of concentrated conditioned culture media or plasma onto the frit of the chromatography column.
6. Top up the column with 1 mL sample volume of filtered PBS buffer.
7. Load 2 mL of filtered PBS buffer to the chromatography column.
8. Wait until the column stops flowing, load another 1.8 mL of filtered PBS buffer to the chromatography column, and collect this volume (see Note 1).
9. Flush the column with 20 mL of filtered PBS before loading another sample.
10. Transfer the collected sample to an Amicon Ultra-2 centrifugal filter device 10 kDa and centrifuge at 3900 g for 30–40 min at 4 °C.

11. Perform reverse spin at 3900 g for 1 min at 4 °C for sample recovery.

3.6 ESCP Device Fabrication

The asymmetric, circular ring-shaped gold electrode array of ESCP is fabricated by a two-step standard photolithographic process [6]. ESCP consists of 28 gold electrodes; each pair is made of a circular inner electrode (1 mm diameter) and outer ring electrode (0.12 mm thick), and these electrodes are separated by a gap of 1 mm.

1. Clean 4 inch wafer with isopropanol, acetone, and water.
2. Dry wafer with a nitrogen gun.
3. Descum wafer using oxygen plasma (1 min, 50 W).
4. Dehydrate wafer at 150 °C for 20 min and leave to cool to room temperature.
5. Add 3.5 mL of photoresist to the center of the wafer and spin coat for 45 s at 4000 rpm to evenly distribute the photoresist (see Note 2).
6. Bake the coated wafer for 1 min at 110 °C.
7. After the wafer has reached room temperature, insert it into the mask aligner and UV-expose at constant dose of 150 mJ/cm².
8. Place the wafer on the hotplate, bake for 1 min at 110 °C, and remove and leave to cool down.
9. Immerse the exposed wafer in developer solution and develop for 45 s.
10. Immediately remove the developed wafer and rinse thoroughly with water followed by drying under a stream of nitrogen.
11. Inspect the developed structures on the wafer using a bright field microscope (see Note 3).
12. Descum wafer using oxygen plasma (1 min, 50 W).
13. Place developed wafer in e-beam evaporator and evaporate 10 nm Ti (adhesion promoter) and 200 nm Au.
14. Immerse wafer in lift off solution for ~4 h (see Note 4).
15. Rinse wafer thoroughly with isopropanol and water to remove metal debris.
16. Second photolithographic step: use the second photomask carrying the insulation pattern and repeat steps 1 to 12.
17. Prepare 55 g of PDMS elastomer (i.e., 50 g elastomer +5 g curing agent) and cast into a petri dish. Cure PDMS elastomer for 20 min at 80 °C.
18. Remove cured PDMS and cut 28 wells (6 mm diameter) into PDMS that align with the wafer’s electrode array.
19. Align PDMS with wafer and thermally bond for 3 h at 65 °C to complete the ESCP fabrication process (Fig. 2) (see Note 5).

### 3.7 ESCP Device Functionalization

1. Rinse ESCP wells with PBS and remove excess liquid.
2. Add 30 μL of bBSA solution to each ESCP well and incubate for 2 h at room temperature (see Note 6).
3. Wash ESCP wells two times with PBS and remove excess liquid.
4. Add 30 μL of avidin solution to each ESCP well and incubate for 1 h at room temperature.
5. Wash ESCP wells two times with PBS and remove excess liquid.
6. Add 30 μL of capture antibody solution to each ESCP well, and incubate for 1 h at room temperature (see Note 7).
7. Wash ESCP wells two times with PBS and remove excess liquid.
8. Add 60 μL of blocking solution to each ESCP well and incubate for 0.5 h at room temperature.
9. Wash ESCP wells three times with PBS and remove excess liquid.

### 3.8 Gold Nanoparticle Synthesis

1. Add 1 mL of gold chloride solution to 99 mL of Milli-Q water and heat to boiling.
2. Add 0.7 mL of sodium citrate solution to the boiling solution, and magnetically stir for another 20 min at boiling temperature.
3. After 20 min, cool down the gold nanoparticle solution at room temperature and store at 4 °C fridge.
3.9 SERS Nanotag Preparation

SERS nanotags were prepared by functionalizing gold nanoparticles with Raman reporters and antibodies.

1. Incubate 1 mL of the gold nanoparticle solution with 10 µL of 1 mM Raman reporter solution (e.g., MBA, TF MBA, DTNB, or MPY) and 2 µL of DSP solution, and constantly shake for 5 h at room temperature (see Note 8, 9).

2. After incubation, centrifuge the mixture at 800 g for 10 min at room temperature and remove the supernatant (see Note 10).

3. Resuspend the gold nanoparticles in 200 µL of 0.1 mM PBS buffer, and incubate with the antibody solution for 30 min at room temperature.

4. After incubation, centrifuge the mixture at 600 g at 4 °C for 10 min to remove free antibodies, and resuspend in 200 µL of BSA solution for 0.5 h at room temperature to block nonspecific binding sites and stabilize SERS nanotags.

5. Store SERS nanotags at 4 °C (see Note 11).

3.10 ESCP Assay

1. Sample: isolated sEVs from conditioned cell culture media or human plasma (see Note 12).

2. Connect ESCP to the signal generator (see Note 13).

3. Add 50 µL sample to each ESCP well, and program the signal generator to apply an alternating current of 500 Hz and 800 mV (sinus waveform) for 45 min.

4. Wash each ESCP well three times with PBS and remove excess liquid.

5. Add 20 µL of SERS nanotags to each ESCP well, and program the signal generator to apply an alternating current of 500 Hz and 800 mV (sinus waveform) for 20 min.

6. Wash each ESCP well three times with PBS and remove excess liquid.

7. Carefully peel off the PDMS from the wafer. Store the wafer in a sealed container at 4 °C, and perform Raman scanning within 3 days after running the assay.

3.11 Raman Scanning and Data Processing

1. Start the WITec Alpha300 R microspectrometer.

2. Calibrate the system by the peak intensity of a silicon wafer at 520 cm⁻¹.

3. Perform the Raman scanning in an area of 60 µm × 60 µm (60 pixels × 60 pixels) with 1 µm spatial resolution using a 20× microscope objective and a 632 nm laser for excitation. The SERS spectrum from each pixel was generated with 50 ms integration time.
4. Use the WITec Project FOUR software to establish the SERS false-color image based on the characteristic peaks of MPY, MBA, DTNB, and TFMBA at 1000 ± 5, 1075 ± 10, 1335 ± 10, and 1375 ± 10 cm⁻¹, respectively.

5. Use the WITec Project FOUR software to export the average SERS spectrum from each false-color image.

6. Perform the baseline correction on each SERS spectrum to remove background noise using the Vancouver Raman algorithm (a five-order polynomial fitting algorithm) [8].

7. Analyze SERS data using either the GraphPad Prism 8 or OriginPro 2019b.

8. Figure 3 shows an example of Raman spectra and bar graphs of characteristic Raman peaks of phenotypic profiling of sEVs from cancer cell lines captured on an anti-CD63 antibody-functionalized ESCP and sEVs labeled with SERS nanotags against tetraspanins and cancer-associated biomarkers.
4 Notes

1. Different samples might generate sEVs of various size distributions, resulting in different sEV fractions. As such, optimization may be required to obtain optimal sEV yield and purity.

2. If bubbles appear on photoresist, remove these carefully using a disposable pipette.

3. If structures are developed incompletely, repeat development process for another 10–20 s.

4. The evaporated metal should lift off readily. If not, extend the lift off process to overnight.

5. The device can be stored in a sealed container when not used immediately.

6. Seal the ESCP wells during incubation with an adhesive foil to prevent evaporation and contamination.

7. Alternatively, incubate antibody solution overnight at 4 °C.

8. The amount of Raman reporters added to the gold nanoparticle solution can be varied to meet the required signal intensity of SERS nanotags.

9. Before adding the MPY reporter, the pH of the gold nanoparticle solution needs to be adjusted to 10 by adding 20 μL of 0.1 M NaOH.

10. The signals of SERS nanotags can be further improved by optimizing the centrifuge speed and time.

11. SERS nanotags are stable at 4 °C for approximately 2 months.

12. After purification of sEVs by size exclusion chromatography, characterize the sEV morphology, size distribution, sEV concentration, and tetraspanin and cancer-associated biomarker expression. This can be performed by nanoparticle tracking analysis, transmission electron microscopy, nanoflow cytometry, Western blot, and other standard techniques.

13. Use a digital multimeter to check the electrical circuitry of the electrode pattern on the wafer.

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Cluster-Wells: A Technology for Routine and Rapid Isolation of Extremely Rare Circulating Tumor Cell Clusters from Unprocessed Whole Blood

Mert Boya and A. Fatih Sarioglu

Abstract

Isolation of extremely rare circulating tumor cell (CTC) clusters from the bloodstream of patients enables minimally invasive diagnosis and prognosis while providing information on their role in metastasis. A few technologies specifically developed for the enrichment of CTC clusters fail to achieve a high enough processing throughput to be practical in clinical settings or risk damaging large clusters owing to their structural design producing high shear forces. Here, we outline a methodology developed for rapid and effective enrichment of CTC clusters from cancer patients, independent of the cluster size and cell surface markers. Minimally invasive access to tumor cells in hematogenous circulation will be an integral part of cancer screening and personalized medicine.

Key words Circulating tumor cell clusters, Cancer screening, Metastasis, Immunofluorescence imaging, Micromachining, Soft lithography

1 Introduction

Circulating tumor cells (CTCs) are malignant cells that detach from the primary tumor, intravasate into bloodstream through leaky vessels, remain viable in the circulation, and extravasate to initiate a new tumor at a distant site [1]. Isolation and downstream analysis of these cells not only enhance the understanding of metastasis at a cellular level but also provide access to a potential biomarker for both diagnosis and prognosis of cancer [2–5]. Besides single CTCs, multicellular groupings of malignant cells, called CTC clusters, play a crucial role in cancer progression. Although they constitute only 2–5% of all CTCs, their metastatic propensity has been shown to be up to 100 times more compared to single tumor cells in circulation [6, 7]. Furthermore, clinical studies have also illustrated the associ-
ation between the presence of CTC clusters with overall survival of patients [8], highlighting the importance of CTC clusters in clinical management of the disease.

Detection and isolation of CTCs from blood samples have been a technical challenge due to the presence of background healthy blood cells and heterogeneity of CTCs. With the advancement of microfabrication techniques, batch enrichment processes were replaced with more sensitive platforms that allow manipulation of individual cells under flow. Among them, filtration-based technologies offer relatively simple and high throughput operation for enriching CTCs; however, they suffer from contamination of healthy blood cells due to size overlap between CTCs and leukocytes [9, 10]. On the other hand, immunoaffinity-based systems tend to have higher specificity [11, 12]. However, they are limited to capturing only a subpopulation of CTCs expressing the desired marker [13, 14]. To date, various promising technologies have been reported for isolation of single CTCs that utilized biophysical and/or biochemical properties of CTCs. While CTC clusters are also observed with some of these technologies, they have low sensitivity and specificity. Isolation of CTC clusters requires extremely gentle operation for preserving the integrity of clusters and unique device geometries for efficient isolation of CTC clusters. A few recently developed cluster-specific technologies achieved better performance for CTC cluster enrichment; however, they achieve that by either lowering the flow rate to clinically unworkable ranges [15, 16] or by employing thin microfluidic channels that are prone to clogging during operation and risk damaging large clusters [16, 17].

Recently, we introduced a highly sensitive CTC cluster enrichment technology, called Cluster-Wells [18], which overcomes the limitation of current technologies and allows isolation of CTC clusters from whole blood samples that have not undergone any preprocessing. The device utilizes the unique geometry of CTC clusters and physically arrests them independently from their surface antigens under sub-physiological flow speeds. This chapter outlines the protocol developed for device fabrication/preparation and processing of whole blood samples drawn from prostate cancer patients. Reliable isolation, imaging, and analysis of CTC clusters would pave the way for more efficient diagnosis and clinical management of the disease.

2 Materials

2.1 Micromachining of Silicon Wafers

2.1.1 Instruments

1. MLA150 Maskless Aligner (Heidelberg Instruments Mikrotechnik GmbH).
2. Vision 320 Reactive Ion Etching (Advanced Vacuum).
3. LPCVD Nitride Furnace (Tystar Corporation).
4. SCS G3P8 Spin Coater (Specialty Coating Systems).
5. STS HRM ICP—Deep Reactive Ion Etching System (Surface Technology Systems).
6. Hot plate.

### 2.1.2 Materials for Wafer Fabrication

1. Silicon wafers (diameter: 100 ± 0.5 mm; orientation: $<100>$; thickness: 500 ± 25 μm; conductivity type: N; dopant: phosphorus; resistivity range: 0–100 Ohm-cm; University Wafer).
2. Adhesion promoter: MicroPrime (ShinEtsuMicroSi).
3. Photoresists: Microposit SC1813 (Shipley), Megaposit SPR 220–7.0 (Shipley).
4. Resist developer: Microposit MF-319 (Shipley).
5. Solvents: isopropyl alcohol (IPA), acetone.

### 2.2 Fabrication of PDMS Molds

1. Disposable weighing boat.
2. Vacuum desiccator.
3. Miltex biopsy puncher, 1.5 mm.
4. Disposable petri dishes, 150 mm.
5. PDMS resin.
6. Trichloro(octyl)silane, 97%.

### 2.3 Fabrication of Devices

1. FlowEZ vacuum pump (Fluigent).
2. Flood UV exposure system (UV III Systems).
3. Adhesive cleaning film (Ultron Systems).
4. PET sheet.
5. Masterflex Tygon tubing.
6. Fluorolink MD 700 (Solvay).
7. 2-Hydroxy-2-methylpropiophenone (Darocur 1173; Sigma-Aldrich).

### 2.4 Device Preparation and Sample Processing

1. Luer stopcock—one-way male to female (Component Supply Company).
2. Masterflex Tygon tubing.
3. Polycarbonate swin-lok filter holder (Cole-Parmer).
4. Infuse/Withdraw PHD Ultra syringe pump (Harvard Apparatus).
5. 200 proof pure ethanol.
6. 1x phosphate-buffered saline.
7. Bovine serum albumin.
8. 4% paraformaldehyde (PFA) in PBS.
2.5 Immunofluorescence Staining

1. 1× phosphate-buffered saline.
2. Glycine.
3. NP40.
4. Normal goat serum blocking solution.
5. Bovine serum albumin.
6. Triton X-100.
7. Tween 20.
8. Mouse anti-Human CD45 (BD Biosciences).
9. Rabbit anti-Human CK 8/18 (Invitrogen).
10. Rabbit anti-Human PSA (Cell Signaling Technology).
11. Rabbit anti-Human EpCAM (Invitrogen).
12. Rabbit anti-Human Vimentin (Invitrogen).
13. Goat anti-Mouse Alexa Fluor 594 (Invitrogen).
14. Goat anti-Rabbit Alexa Fluor 488 (Invitrogen).
15. 4′,6-Diamidino-2-phenylindole (DAPI; Invitrogen).

2.6 Preparation for Imaging

1. 1× phosphate-buffered saline.
2. 2″ × 3″ Fisherbrand glass microscope slides.
3. Eclipse Ti inverted fluorescence microscope (Nikon).

3 Methods

The entire process consists of five main steps. First, micromachining of silicon master-mold is performed inside a cleanroom. Second, silicon mold is transferred into polydimethylsiloxane (PDMS) and replicated using soft lithography inside a laboratory environment. Third, the resulting PDMS mold is filled with a photocurable polymer (Fluorolink MD700) and crosslinked in the mold to form the device. Fourth, devices are prepared and used for isolating tumor cells from whole blood samples drawn from cancer patients. Lastly, the devices are scanned and investigated under a fluorescence microscope.

Similar to commercial membranes, our device can be fabricated at different sizes. While we recommend 47 mm-diameter device for sample volumes >15 mL which allows processing rates of >25 mL/h, here we outlined a sample processing protocol developed for a 25 mm-diameter device. Please note that volumetric flow rates need to be adjusted depending on the size of the device to ensure that cells experience the same flow speed i.e., shear forces during operation.
3.1 Micromachining of Silicon Wafers

Fabrication of silicon master-mold is a three-mask process (see Fig. 1). Micromachining steps described below take place in a cleanroom environment.

3.1.1 Processing the First Mask Layer

1. Start with 4” (100 mm) <100> silicon wafer. Perform wafer cleaning using piranha solution (3:1 mixture of sulfuric acid and hydrogen peroxide) at 120 °C for 10 min. Rinse the wafers using DI water and blow dry with N₂.
2. Perform dehydration bake at 200 °C for 30 min.
3. Coat the wafer with an adhesion promoter (MicroPrime) by spinning for 15 sec at 500 rpm and then for 60 sec at 5000 rpm (ramp 100 rpm/sec).
4. Bake the wafer at 100 °C for 5 min.
5. Spin the first photoresist layer, Microposit SC1813, for 15 sec at 500 rpm and then for 45 sec at 2500 rpm (ramp 500 rpm/sec).

6. Perform soft bake at 115 °C for 1 min.

7. Using MLA150 maskless aligner, expose the photoresist layer with mask #1 at a dose of 150 mJ/cm² using 375-nm laser.

8. Develop the exposed photoresist by immersing the wafer in Shipley MF-319 resist developer for 45 sec. Apply gentle agitation.

9. Perform hard bake at 150 °C for 5 min to strengthen the patterned resist for the subsequent etching (see Note 1).

10. Perform deep reactive ion etching of silicon using STS HRM ICP to a depth of 8 µm at 10-mTorr pressure. Etching is performed using SF₆ (450 sccm) + O₂ (45 sccm) gas mixture at 2200 W power for 8.5 sec/cycle, and passivation is performed using C₄F₈ gas at 1500 W power for 3 sec/cycle. Set the etch cycle platen power to 45 W. Etch for a total of 12 cycles to achieve the desired depth.

11. Remove the photoresist by immersing in acetone. Rinse with isopropyl alcohol (IPA) and blow dry with N₂.

12. Remove any residual resist and passivation layer deposited during etch process by performing descum (O₂ plasma) for 5 min. Set O₂ flow rate to 50 sccm, chamber pressure to 60 mTorr, and platen power to 150 W.

13. Clean the wafer using piranha solution at 120 °C for 10 min. Rinse the wafers using DI water and blow dry with N₂.

3.1.2 Processing the Second Mask Layer

1. Using Tystar furnace, deposit a thin (300 nm) layer of low stress silicon nitride, using the low-pressure chemical vapor deposition (LPCVD) furnace, to function as a hard mask for a future wet-etch process. Set the ammonia (NH₃) flow rate to 100 sccm, dichlorosilane (SiCl₂H₂) flow rate to 13 sccm, pressure to 150 mTorr, and temperature to 850 °C. Perform deposition for 45 min to achieve desired thickness (see Note 2).

2. Spin Megaposit SPR 220–7.0 photoresist for 15 sec at 500 rpm and then for 45 sec at 1500 rpm (ramp 250 rpm/sec).

3. Perform soft bake at 115 °C for 5 min to solidify the photoresist.

4. Using MLA150 maskless aligner, first align the second mask using the previously patterned alignment marks, and then expose the photoresist with mask #2 at a dose of 500 mJ/cm² using 375-nm laser.
5. Develop the exposed photoresist by immersing the wafer in Shipley MF-319 resist developer for 3 min. Apply gentle agita-
tion (see Notes 1 and 3).

6. Perform hard bake at 150 °C for 5 min to strengthen the patterned resist for the subsequent process.

7. Using the patterned resist layer as a mask, remove the underly-
ing silicon nitride in a reactive ion etching (RIE) chamber (Vision 320). Set the chamber pressure to 40 mTorr, platen power to 250 W, and reactive gas (CHF₃) flow rate to 45 sccm. Perform the etching process for 10 min to ensure no residual nitride layer is remaining on the unmasked area.

8. Remove the photoresist by immersing in acetone. Rinse with isopropyl alcohol (IPA) and blow dry with N₂.

9. Remove any residual resist by performing descum (O₂ plasma) for 5 min. Set O₂ flow rate to 50 sccm, chamber pressure to 60 mTorr, and platen power to 150 W.

10. By utilizing the silicon nitride as a hard mask, perform aniso-
tropic wet etching using 45% potassium hydroxide (KOH) solution at 80 °C for 15 min to form the slanted walls around the square pillars patterned previously (see Note 4). Once etched, rinse the wafers with DI water and blow dry with N₂.

11. Perform a blanket dry etch to remove silicon nitride mask from the silicon surface using reactive ion etching with the same process parameters as step 7.

3.1.3 Processing the Third Mask Layer

1. Spin a 25 μm layer of Megaposit SPR 220–7.0 photoresist by spinning for 30 sec at 200 rpm and then for 1 min at 700 rpm (ramp 100 rpm/sec).

2. Perform soft bake at 115 °C for 5 min to solidify the photoresist.

3. Using MLA150 maskless aligner, first align the third mask using the previously patterned alignment marks, and then expose the photoresist with mask #3 at a dose of 600 mJ/cm² using 375-nm laser.

4. Develop the exposed photoresist by immersing the wafer in Shipley MF-319 resist developer for 7 min. Apply gentle agita-
tion (see Notes 1 and 3).

5. Perform hard bake at 150 °C for 5 min to strengthen the patterned resist for the subsequent etching.

6. Perform deep reactive ion etching of silicon using STS HRM ICP to a depth of 50 μm at 10-mTorr pressure. Etching is performed using SF₆ (450 sccm) + O₂ (45 sccm) gas mixture at 2200 W power for 8.5 sec/cycle, and passivation is performed using C₄F₈ gas at 1500 W power for 3 sec/cycle. Set
the etch cycle platen power to 45 W. Etch for a total of 65 cycles to achieve desired depth.

7. Remove the photoresist by immersing in acetone. Rinse with isopropyl alcohol (IPA) and blow dry with N₂.

8. Remove any residual resist and passivation layer deposited during etch process by performing descum (O₂ plasma) for 5 min. Set O₂ flow rate to 50 sccm, chamber pressure to 60 mTorr, and platen power to 150 W.

9. Clean the wafer using piranha solution at 120 °C for 10 min. Rinse the wafers using DI water and blow dry with N₂.

### 3.2 Fabrication of PDMS Molds

After microfabrication of silicon master-mold inside cleanroom, the rest of the fabrication process takes place in the laboratory. Soft-lithography techniques are utilized for fabrication of PDMS molds (Fig. 2).

1. Following the silicon micromachining, coat the wafers with trichloro(octyl) silane under vacuum conditions for 8 h inside a desiccator by adding 200 μL of silane in a container near the wafer.

2. Place the wafer inside a Petri dish and tape it from the edges.

3. Weigh and mix 50 g of PDMS prepolymer and 5 g of cross-linker inside a weighing boat.

4. Pour the mixture on the wafer and perform degassing by placing it in a vacuum desiccator for 1 h. Make sure there are no bubbles remaining in the mixture.

5. Cure the PDMS inside an oven at 65 °C for 4 h.
6. Cut and peel-off the cured PDMS from the silicon master-mold. This PDMS layer is called primary PDMS and used for the subsequent patterning molding process.
7. Perform surface activation on the primary PDMS mold by using O₂ plasma.
8. Coat the PDMS layer with trichloro(octyl)silane under vacuum conditions for 8 h inside a desiccator.
9. Place the primary PDMS inside a Petri dish and attach it to the Petri dish using a double-sided tape.
10. Weigh and mix 50 g of PDMS prepolymer and 5 g of cross-linker inside a weighing boat.
11. Pour the mixture on the primary PDMS mold and perform degassing by placing it in a vacuum desiccator for 1 h. Make sure there are no bubbles remaining in the mixture.
12. Cure the PDMS inside an oven at 65 °C for 4 h.
13. Cut the cured PDMS and detach the secondary PDMS layer from the primary PDMS layer by gentle warping from the edges.
14. Punch inlet and outlet holes on the secondary PDMS using a 1.5 mm biopsy puncher.

### 3.3 Fabrication of Devices

Lastly, polymer devices are fabricated by utilizing vacuum-assisted micromolding technique (Fig. 3).

1. Clean the fabricated secondary PDMS mold by acetone and isopropyl alcohol (IPA). Place the mold inside an oven to evaporate residual solvents.
2. Remove any dirt and particles remaining on the mold using an adhesive cleaning tape.
3. Place the mold on a polyethylene terephthalate (PET) sheet, and attach the Tygon tubing to the outlet hole, where vacuum will be applied by a high-precision vacuum pump.
4. Prepare the UV-curable solution by mixing Fluorolink MD700 with 4% w/w 2-hydroxy-2-methylpropiophenone (Darocur 1173) (see Note 5).
5. Set the vacuum pressure level to 50 mbar, and add the prepared solution to the inlet of the PDMS mold.
6. Once the mold is filled with the UV-curable solution, expose it with the flood UV exposure system for 6 min.
7. Remove the tubing, and peel-off the PDMS mold from the underlying PET sheet/device stack by warping the PDMS gently from one of the ends until it is fully removed.
8. Cool down the PET sheet/device stack using a thermoelectric cooler at 4 °C, and peel-off the device from the sheet starting from one of the ends (see Note 6).

9. Perform quality check under a microscope to ensure there are no defects/damages on the fabricated devices.

3.4 Device Preparation and Sample Processing

1. Place the device into a filter holder, and attach a one-way male to female Luer stopcock to the bottom of the filter holder.

2. Attach a 10 mL syringe to the top, and wet the filter holder and device with pure ethanol by applying three cycles of push-pull. Visually inspect for any large bubbles trapped inside the filter holder (see Note 7).

3. Replace the syringe with a new one, remove the plunger, and attach to the filter holder.

4. Wash the filter holder/device assembly with pre-filtered 1× phosphate-buffered saline (PBS) solution. Perform this step for two repetitive cycles of 10 mL PBS.

5. Replace the 1× PBS with 3% bovine serum albumin (BSA), and incubate at room temperature for 1 h or overnight at 4 °C prior to use with samples. This step prevents nonspecific adhesion of
protein and cells on the device and filter holder surfaces (see Note 8).

6. Following the BSA incubation, replace the BSA with 1× PBS before introducing the sample.

7. Prepare the syringe pump by setting it to the “withdraw” mode, attaching a syringe, choosing the appropriate size from the system setup, and setting the flow rate to 6.25 mL/h.

8. Attach Tygon tubing to the withdrawing syringe on the pump and to the Luer stopcock previously attached to the filter holder.

9. Pour the blood sample to the syringe on top of the filter holder.

10. Start running the blood sample.

11. Once the blood sample is run fully, add 10 mL of 1× PBS to the top syringe and wash the device at 12.5 mL/h.

12. Fix the cells remaining on the device by introducing 3 mL of 4% paraformaldehyde (PFA) at 12.5 mL/h. Incubate the solution for 10 min at room temperature.

13. Wash the filter holder/device assembly with 5 mL of 1× PBS at 12.5 mL/h flow rate. Following this step, the samples can be stored at 4 °C prior to the immunofluorescence staining, if needed.

### 3.5 Immunofluorescence Staining

Perform the following steps at a cold temperature (4 °C), e.g., in an ice bath. The flow rate is 12.5 mL/h for all the steps below.

1. Wash with 4 mL of 10 mM Glycine in 1× PBS.

2. Incubate the cells for 5 min with 3 mL of 1% NP40 in 1× PBS to permeabilize cell membrane.

3. Wash the cells with 4 mL of IF wash solution (0.1% BSA, 0.2% Triton X-100, 0.05% Tween 20 in 1× PBS).

4. Perform blocking by introducing and incubating cells with 2% normal goat serum in 3% BSA for 30 min.

5. Prepare the primary antibody cocktail in 3 mL of 3% BSA solution using the following dilutions:

   - Mouse anti-Human CD45 (1:500).
   - Rabbit anti-Human CK 8/18 (1:400).
   - Rabbit anti-Human PSA (1:750).
   - Rabbit anti-Human EpCAM (1:400).
   - Rabbit anti-Human Vimentin (1:1000).

   Introduce the cocktail and incubate overnight at 4 °C.

6. Wash the cells with 4 mL of IF wash solution (0.1% BSA, 0.2% Triton X-100, 0.05% Tween 20 in 1× PBS).
7. Prepare the secondary antibody cocktail in 3 mL of 3% BSA solution using the following dilutions:
   - Goat anti-Mouse Alexa Fluor 594 (1:500).
   - Goat anti-Rabbit Alexa Fluor 488 (1:500).

   Introduce the cocktail and incubate for 1 h at 4 °C.

8. Wash the cells with 4 mL of IF wash solution (0.1% BSA, 0.2% Triton X-100, 0.05% Tween 20 in 1× PBS).

9. Stain the nuclei of cells with 4′,6-diamidino-2-phenylindole (DAPI) (1:1000) diluted in 1× PBS for 10 min.

10. Wash the cells with 5 mL of 1× PBS solution.

### 3.6 Preparation for Imaging

1. Clean two microscope slides using acetone and isopropyl alcohol (IPA), and place them on top of a clean bench close to the filter holder.

2. Close the Luer stopcock and remove the syringe from the filter holder.

3. Carefully open the filter holder and transfer the device on top of the microscope slide by holding the device from sides with two tweezers to prevent folding.

4. Add 200 μL of 1× PBS on the device and carefully place the second microscope slide on top (see Note 9).

5. Decontaminate the tweezers with 70% ethanol for future use.

6. Image the device under a fluorescence microscope (Fig. 4; see Note 10).

![Fig. 4](image-url) Fluorescence microscope images of a captured two-cell LNCaP human prostate tumor cell cluster. Captured clusters were stained with established cancer markers (EpCAM, CK8/18, PSA, and Vimentin), and nuclei of cells were stained with DAPI. Scale bars, 20 μm
4 Notes

1. Check the patterned resist layer against particles/defects under an optical microscope before making the pattern permanent by the subsequent processes. Remove the resist and repeat the photolithography steps if necessary.

2. Crack-free nitride is needed for preventing groove formation during wet etch process. Low stress nitride deposition prevents this and improves fabrication yield.

3. Check the alignment accuracy before proceeding with the next step. Any misalignment would be catastrophic. If there is a misalignment, the photoresist can be stripped, and photolithography steps can be repeated at this step.

4. Timing and temperature uniformity are extremely important for preventing over-etched regions. Any mistake at this step would be irreversible.

5. As the solution is UV sensitive, it needs to be protected from light once prepared.

6. During the device peel-off from the PET sheet, cooling the stack is optional but recommended as it facilitates easier release and prevents damage during peel-off.

7. Device wetting with pure ethanol is necessary due to the hydrophobic nature of the device. During this process, make sure the device never gets dry. If it dries, repeat the wetting process from the beginning.

8. If stopped at this stage, devices can be stored in 3% BSA solution at 4 °C up to a week.

9. This step needs to be done fast to prevent dry spots on the device.

10. If stored properly, device can be imaged at a later time. To do so, make sure the microscope slides/device stack is stored in a humid, cold (4 °C), and dark environment which would prevent drying and photobleaching.

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Chapter 19

Secretion Function Analysis of Ex Vivo Immune Cells in an Integrated Microfluidic Device

Roberto Rodriguez-Moncayo and Jose L. Garcia-Cordero

Abstract

Immune cells play a major role in the development of cancer, from being able to inhibit it by secreting pro-inflammatory mediators, to assist in its development by secreting growth factors, immunosuppressive mediators, and ECM-modifying enzymes. Therefore, the ex vivo analysis of the secretion function of immune cells can be employed as a reliable prognostic biomarker in cancer. However, one limiting factor in current approaches to probe the ex vivo secretion function of cells is their low throughput and the consumption of large quantities of sample. Microfluidics provides a unique advantage, by being able to integrate different components, such as cell culture and biosensors in a monolithic microdevice; it can increase the analytical throughput and leverage it with its intrinsic low sample requirement. Furthermore, the integration of fluid control elements also allows this analysis to be highly automatable, leading to increases in consistency in the results. Here, we describe an approach to analyze the ex vivo secretion function of immune cells using a highly integrated microfluidic device.

Key words Ex vivo, Immune cells, Function analysis, Secretion, Cytokines, Immunoassay, Microfluidics, High throughput

1 Introduction

The ex vivo analysis of the immune cell function has been shown to be a better diagnostic and prognostic biomarker, with increased specificity, compared to other clinical markers or physiological variables [1, 2]. Particularly, the analysis of secretion function has made a great impact in diagnostics of infectious diseases, where the impaired or enhanced production of cytokines by ex vivo challenged immune cells may have diagnostic or prognostic value [3, 4].

In the context of cancer, immune cells are known to greatly influence the tumor microenvironment by secreting a plethora of soluble factors that include cytokines, growth factors, and extracellular matrix(ECM)-remodeling enzymes [5, 6]. These biomolecules shape the tumor microenvironment (TEM), impacting in
the development of cancer and contributing to either its progress
ion or elimination. For example, M1 macrophages inhibit tumor
progression through the secretion of pro-inflammatory cytokines
such as TNF-α, IFN-γ, and IL-12 [7]. In contrast, M2 macro-
phages support the tumor microenvironment through the secre-
tion of immunosuppressive cytokines (e.g., IL-4, IL-10, IL-6) and
induce tumor proliferation and vascularization through the pro-
duction of growth factors (e.g., PDGF, TGFβ1, HGF) [8]. There-
fore, the ex vivo functional analysis of tumor-associated immune
cells could be potentially employed as a relevant predictive and
therapeutical biomarker in cancer [8].

Conventionally, the analysis of the ex vivo immune cell secre-
tion function is done by isolating the cells from the sample or tissue
of interest—for example, through density gradient centrifugation
or negative selection via magnetic-activated cell sorting (MACS)—
and plating them in wells, where they are stimulated with different
activators to induce the production of the soluble factors of inter-
est: mainly cytokines. Next, supernatant samples are recovered, and
the soluble factors are quantified via an enzyme-linked immuno-
sorbent assay (ELISA), typically carried out in 96-well plates. This
approach, however, has the limitation of low throughput and mul-
tiplexing capabilities while also requiring a large number of cells
(~10^6) per condition [9] which is particularly limiting during
ex vivo studies, where the amount of sample is limited.

Microfluidic devices have shown to be a great tool for the analysis
of the secretory function of immune cells; among the advantages they
provide are multiplexing and high-throughput capabilities, as well as
the ability to integrate fluid flow control elements and highly sensitive
biosensors. These characteristics allow microfluidic devices to probe
many conditions from a small sample of cells, making them particularly
suited for ex vivo cell function studies.

In this protocol, we describe a microfluidic approach for the
ex vivo analysis of the secretory function of immune cells (Fig. 1)
[10]. We describe the fabrication, setup, and operation of a double-
layered polydimethylsiloxane (PDMS) device that is comprised of
32 pairs of cell culture and biosensing chambers, patterned as an 8x4
array. Each cell culture chamber contains ~500 20 μm traps for the
capture of individual immune cells; this cell capture strategy enables
probing both adherent and nonadherent cells. Cells are introduced via
a dedicated inlet that prevents the injection of cell clumps which could
potentially clog the channels. Once the cells have been captured, four
inlets that address individual rows of cell culture chambers are used to
deliver an equal number of chemical stimulants, obtaining eight tech-
nical replicates per condition. To quantify the cytokines produced by
the immune cells in response to the stimuli, we conduct a fluorescence
sandwich immunoassay in the adjacent biosensing chambers. Each
biosensing chamber has a 250 μm “button” valve that allows spatially
resolved immobilization of the capture antibodies via the mechanically
induced trapping of molecular interaction (MITOMI) method [11–13]. The analytical performance of the immunoassay is enhanced by (i) chemical surface modification of glass with an epoxysilane and (ii) an active mixing protocol created by activation of a valve that flanks the cell culture chamber (mixing valve) and the valve that divides the cell culture and biosensing chambers (neck valve). All fluid flow control is carried out by the actuation of elastomeric (Quake) valves patterned throughout the device, making our approach highly automatable (Fig. 2).

2 Materials

2.1 Master Mold Fabrication

1. 4 inch silicon wafers.
2. Oxygen plasma cleaner.
3. Programmable spin coater.
4. Photoresists: SU-8 negative tone epoxy photoresist; positive tone thick film photoresist.
Fig. 2 Design of the double-layer microfluidic device for the ex vivo analysis of immune cell secretion function. The devices are composed of 32 pairs of cell culture and biosensing chambers patterned in the flow layer (blue). Valves patterned throughout the device in the control layer (red) are used to control the fluid flow within the device. The magnification shows a single pair of cell culture and biosensing chambers, where microwells and button valves are patterned, respectively. All scale bars are 150 μm, except for the microwell inset where scale bar represents 50 μm. (a) Cell culture reagents and cell suspension are loaded to tubes and connected to independent inlets to be sequentially delivered to all the cell culture chambers. (b) Immunoassay reagents are injected to the device through independent inlets. Valves (BSV1-4) can be actuated to address individual biosensing chamber rows, enabling the patterning of different capture antibodies which can be delivered through the unused inlets. Scale bars are 150 μm except for microwell inset which is 50 μm.

5. Developers: propylene glycol monomethyl ether acetate (PGMEA) and isopropanol (IPA) for SU-8 negative photoresist. Positive resist developer diluted in deionized water in a 1:4 (v/v) ratio.

6. Positive tone photoresist adhesion promoter to substrate: 20% hexamethyldisilazane in PGMEA.

7. Hot plate.

8. Maskless exposure photolithography system Micro Pattern Generator.

9. 150 mm × 75 mm crystallizing dish for bath immersion and manual photoresist development.

10. Wafer tweezers.

11. Transfer pipettes.

12. Nitrogen gas with spray gun and hose assembly.
2.2 Device Fabrication and Assembly

2.2.1 PDMS Replica

1. Chlorotrimethylsilane.
2. Fume hood.
3. Variable volume micropipette and tips.
4. 100 mm × 15 mm Pyrex petri dishes.
5. Aluminum foil.
6. Two-part silicone elastomer polydimethylsiloxane (PDMS) base and curing agent.
7. Digital scale for weighing.
8. PDMS base and curing agent mixer and deaerator with compatible 100 mL disposable containers.
9. Vacuum desiccator and vacuum pump.
10. Programmable spin coater.
11. Universal convection oven.
12. #12 hook blade and #15 short blades and compatible scalpel handles.
13. 5 in carbon steel scraper blade.
14. Manual catheter punching machine with 26-gauge (0.032 in OD) TiN-coated punch and pin ejector.
15. 5 in straight serrated tip tweezers.
16. Stereomicroscope.
17. Transparent adhesive tape.

2.2.2 Glass Slide Modification

1. 75 mm × 25 mm plain microscope slides.
2. Rotary tool with grinding accessory.
3. Glass Coplin staining jars.
4. Isopropanol.
5. Oxygen plasma cleaner.
6. Toluene.
7. (3-Glycidyloxypropyl)trimethoxysilane (GPS).
8. 100 mL graduated glass cylinder.
9. 100 mL Pyrex media bottle.
10. 3-cc disposable syringe and needle.
11. Hot plate.
12. Laboratory support stand with clamp and clamp holder.
13. Ultrasonic cleaner water bath.
14. Nitrogen gas with spray gun and hose assembly.
15. Glass vacuum desiccator and vacuum pump.
2.2.3 Chip Assembly

1. PDMS chip.
2. GPS-modified glass slide.
3. Oxygen plasma cleaner.
4. (3-Aminopropyl)triethoxysilane (APTES).
5. Disposable 100 × 15 mm polystyrene petri dish.
6. Transparent adhesive tape Magic tape.
7. Glass vacuum desiccator and vacuum pump.
8. Hot plate.

2.2.4 Microfluidic Control

1. Custom-made pneumatic controller consisting of 3/2-way pneumatic miniature solenoid valve manifolds with push-to-connect fittings, custom-made high-power driver based on the ULN2003 chip, and 24 V constant power source.
2. Arduino development board, USB cable, and laptop with LabVIEW and Arduino and NI VISA drivers installed.
3. Low pressure high-precision 0-6 psi pressure regulator with 0-15 psi digital pressure gauge with 1/4 inch NPT push-to-connect fittings 6 mm OD for pressure-driven fluid flow.
4. Precision 2-25 psi pressure regulator and 0-100 psi dial pressure gauge with 1/4 inch NPT push-to-connect fittings 6 mm OD for elastomeric valve control.
5. Five-port polycarbonate manifold with female Luer Locks.
6. 23-gauge dispensing tip with Luer Lock hub.
7. 6 mm OD and 4 mm OD polyurethane pneumatic tubing.
8. 6 mm to 4 mm OD push-to-connect reductors.

2.2.5 Chip Setup

1. Microbore tubing 0.020 in ID.
2. 23RW-gauge stainless steel hypodermic tubing with deburred ends.
3. 23-gauge dispensing tip with Luer Lock hub.
4. 1-cc and 20-cc disposable syringes.
5. Ultrapure water: deionized water from purification system.
6. DPBS: 1× Dulbecco’s phosphate buffered saline without calcium and magnesium.
7. 2% (w/v) Pluronic F-127 in DPBS, sterile filtered with 0.2 μm syringe filter.

2.3 Immune Cell Isolation and Culture

1. 6 mL K2 ethylenediaminetetraacetic acid (EDTA) tube, 23 G × 0.75 inch Safety-Lok blood collection set, quick release tourniquet, and alcohol swabs.
2. 15 mL polypropylene conical centrifuge tubes.
3. Solution for cell separation by density gradient centrifugation: Histopaque 1.077 g/mL.
4. Disposable transfer pipettes.
5. Programmable centrifuge with swinging bucket rotor.
6. DPBS.
7. Serum-free culture medium: RPMI 1640 cell culture medium and 1% (v/v) penicillin-streptomycin (pen-strep).
8. Sterile 30 × 15 mm polystyrene petri dishes.
9. Trypsin-EDTA, DNase I, and 4% bovine serum albumin (BSA) in DPBS sterile filtered with 0.2 μm syringe filters.
10. Complete culture medium: RPMI 1640 medium, 1% (v/v) pen-strep, and 10% (v/v) fetal bovine serum (FBS).
11. 40 L CO₂ incubator.

2.4 On-Chip Immunoassay
1. Pierce biotinylated BSA reconstituted at 2 mg/mL in UPW.
2. DPBST: DPBS, 0.05 % (v/v) Tween-20.
3. NeutrAvidin biotin-binding protein diluted in DPBS at 100 μg/mL (NA).
4. 1xELISA diluent: DPBS, 1:5 (v/v) 5x ELISA diluent.
5. Biotinylated anti-IL-8 capture antibodies in 1x ELISA diluent.
6. Complete culture medium with varying concentrations of lipopolysaccharide from *E. coli* O128:B12.
7. Phycoerythrin (PE)-labeled detection antibodies in 1x ELISA diluent.
8. 40-L CO₂ incubator.

2.5 Data Acquisition and Analysis
1. Inverted epifluorescence microscope with PE-compatible filter set.
2. Personal computer with MATLAB installed.

3 Methods

3.1 Master Mold Fabrication

3.1.1 Flow Layer Mold Fabrication
1. Unscrew the SU-8 negative photoresist bottle cap to allow nitrogen released from the photoresist to escape.
2. Level and preheat the hotplate to 50 °C.
3. Dehydrate a 4 in wafer by treating it with O₂ plasma for at least 7 min.
4. Deposit a ~25 μm high layer of negative tone photoresist on the dehydrated wafer by spin coating at 1000 rpm for 40 s with 10 rpm/s ramp up and down (see Note 1).
5. Place the coated wafer on a leveled surface, and allow a 1-h relaxation time to improve photoresist layer uniformity.

6. Transfer the coated wafer to the hotplate and perform a pre-exposure bake. Pre-exposure bake is done by increasing the temperature by 5 °C every 3 min. When the 65 °C temperature is reached, maintain the temperature for 10 min. Continue increasing the temperature by 5 °C in 3-min intervals until reaching 95 °C. Hold the temperature at 95 °C for 30 min. Turn off the hotplate and allow it to cool down to room temperature.

7. Load the wafer to the micropattern generator. Upload the culture chamber .GDS file to the micropattern generator control software. Expose the design employing a 10 mm write head (θ) with 70 mW power at 100 % intensity (see Note 2).

8. Perform a postexposure bake. Transfer the exposed wafer to a hotplate preheated to 50 °C. Increase temperature by 5 °C in 3-min intervals. Hold temperature at 65 °C and 95 °C for 15 min and 30 min, respectively (see Note 3).

9. Develop the exposed wafer manually over the crystallizing dish with PGMEA, rinse with IPA, and dry with N₂ gun.

10. Perform a hard bake on a hotplate at 135 °C for 2 h to improve the chemical stability of the patterned structures for further wafer processing.

11. Treat the wafer with oxygen plasma for 30 s.

12. Spin-coat the positive tone photoresist adhesion promoter on the patterned wafer at 3000 rpm for 20 s with a 1000 rpm/s ramp.

13. Bake the coated wafer on the hotplate at 120 °C for 5 min.

14. Deposit a 10 μm high layer of positive tone photoresist by spin coating at 1800 rpm for 40 s with a 1000 rpm/s ramp.

15. Perform a two-step soft bake on a hotplate at 90 °C for 1 min followed by 115 °C for 3 min.

16. Deposit a second 10 μm high layer of positive tone photoresist to achieve a 20 μm-high layer. Spin-coat at 1800 rpm for 40 s with a 1000 rpm/s ramp.

17. Perform a second two-step soft bake on a hotplate at 90 °C for 1 min followed by 115 °C for 6 min.

18. Allow the soft-baked photoresist to rehydrate for 1 h.

19. Load the coated wafer to the micropattern generator. Upload the flow channel .GDS file to the micropattern generator control software. Find the alignment marks patterned on the wafer, and calculate center offset and rotation. Manually enter the center offset and rotation in the control software. Expose the
design employing a 10 mm write head (II) with 70 mW power at 40 % intensity four consecutive times.

20. Manually develop the exposed wafer by immersing it in positive tone photoresist developer diluted 1:4 (v/v) in DI water. Rinse with DI water and dry with the N₂ gun.

21. Perform a thermal reflow to round off the flow channels for proper valve actuation. Bake the developed wafer on a hotplate at 130 °C for 1 h.

22. Perform thermal annealing of patterned structures to increase chemical stability during further wafer processing.

23. Deposit a 40 μm high layer of negative tone photoresist by spin coating at 800 rpm for 40 s with a 100 rpm/s ramp.

24. Place the coated wafer on a leveled surface, and allow a 1-h relaxation time to improve uniformity of the photoresist layer.

25. Carry out a pre-exposure bake. Place the coated wafer on a hotplate preheated at 50 °C. Increase the temperature by 5 °C every 3 min. At 65 °C and 95 °C, maintain temperature for 10 and 30 min, respectively.

26. Load the coated wafer to the micropattern generator. Upload the microwells .GDS file to the micropattern generator control software. Find the alignment marks on the wafer and calculate the center offset and wafer rotation. Manually enter the center offset and rotation in the control software. Expose the design with a 10 mm write head (II) with 70 mW power at 100 %.

27. Perform a postexposure bake. Transfer the exposed wafer to a hotplate preheated to 50 °C. Increase temperature by 5 °C in 3-min intervals. Hold temperature at 65 °C and 95 °C for 15 min and 30 min, respectively.

28. Develop the exposed wafer manually over the crystallizing dish with PGMEA, rinse with IPA, and dry with N₂ gun.

29. Perform a hard bake on a hotplate at 135 °C for 2 h to improve adhesion of the patterned structures to the substrate.

3.1.2 Control Layer Mold Fabrication

1. Dehydrate a new 4 in wafer by treating it with O₂ plasma for at least 7 min.

2. Deposit a ~25 μm high layer of negative tone photoresist on the dehydrated wafer by spin coating at 1000 rpm for 40 s with 10 rpm/s ramp up and down.

3. Transfer the coated wafer to a hotplate preheated to 50 °C and perform a pre-exposure bake. Increase the temperature in 5 °C steps every 3 min. When 65 °C and 95 °C are reached, hold for 10 and 30 min, respectively. Turn off the hotplate and allow the wafer to cool down to room temperature.
4. Load the wafer to the micropattern generator. Upload the control layer .GDS file to the micropattern generator control software and expose the design with a 10 mm write head (II) employing a 70 mW power at 100 % intensity.

5. Perform a postexposure bake. Transfer the exposed wafer to a hotplate preheated to 50 °C. Increase the temperature in 5 °C steps every 3 min. When reaching 65 °C and 95 °C, hold the temperature for 15 min and 30 min, respectively.

6. Develop the exposed wafer manually over a crystallizing dish by adding PGMEA, rinsing with IPA, and drying with an N2 gun.

7. Lastly, on a hotplate at 135 °C, perform a hard bake for 2 h to improve the chemical stability of the patterned structures.

3.2 Device Fabrication

1. Prior to PDMS replica molding, render the master molds hydrophobic to prevent PDSM to adhering to the microstructures. Place the master molds in a plastic container, and add 100 μL of chlorotrimethylsilane inside a fume hood. Subject the master mold to the chlorotrimethylsilane vapor for 30 min (see Note 4).

2. Place the control layer molds in a 10 cm glass petri dish lined with aluminum foil.

3. Prepare a 5:1 (w/w) PDMS mixture by mixing 50 g of base with 10 g of curing agent using the conditional mixer.

4. Cast the 5:1 PDMS mixture on the control layer mold. Degas the PDMS mixture for 10 min in a vacuum desiccator. If any air bubbles remain after the 10 min period, remove them by gently blowing N2 on the surface. If particles are visible in PDMS, remove them with a pipette tip.

5. For the flow layer mold, prepare a 20:1 (w/w) PDMS mixture by mixing 10 g of base with 0.5 g of curing agent in the conditional mixer.

6. Spin-coat the flow layer mold with the 20:1 (w/w) PDMS mixture at 1100 rpm for 40 s and a 1000 rpm/s ramp.

7. Partially cure both control and flow layers in the convection oven at 80 °C for 25 min.

8. Remove the master molds from the convection oven and allow to cool down.

9. Cut the control layer with a #12 hook scalpel and peel off the mold. Cut patterned PDMS slab using the scraper blade.

10. Punch all control inlet holes using the manual punching machine with the 26-gauge punch.

11. Remove reaming PDMS pins with the serrated tip tweezers.

12. Thoroughly clean both the control and flow layers with magic tape.
13. Align the control layer PDMS slab over the flow layer under a stereomicroscope.

14. Bond the flow and control layer in the convection oven at 80 °C for 1.5 h (see Note 5).

15. Remove the bonded devices from the convection oven and allow to cool down.

16. Cut the flow layer with the #15 scalpel along the outline of the control layer, and peel off the device.

17. Punch out the flow layer inlets and outlets using the catheter manual punching machine.

18. Remove leftover PDMS pins with the tweezers.

19. Cover the underside of the device with magic tape until ready to be used (see Note 6).

20. To clean the flow layer mold, prepare a 10:1 (w/w) PDMS mixture, cast on the mold, and cure for 1 h. Gently peel off PDMS slab. Keep both master molds in a dust-free environment.

### 3.3 Glass Slide Modification

1. Using the rotary tool, grind the corner of five new glass slides (see Note 7).

2. Place the glass slides inside a Coplin staining jar with DI water.

3. Sonicate the staining jar for 15 min (see Note 8).

4. Decant the jar and flush twice with DI water.

5. With the tweezers, remove each glass slide and blow dry using N₂.

6. Place the glass slides inside the plasma cleaner. Use the ground corner of the glass slide as reference to identify the treated side.

7. Treat the glass slides with O₂ plasma for 30 s at 0.35 mbar pressure and 100% power.

8. Prepare a 1% (v/v) solution of GPS in toluene in the 100 mL Pyrex media bottle. Place the cap on and mix by shaking gently.

9. Transfer the GPS containing solution to a clean staining jar.

10. Immediately after the O₂ plasma treatment, place the glass slides in the staining jar with the GPS solution.

11. Incubate at room temperature for 20 min.

12. Preheat a hot plate to 125 °C.

13. With tweezers, remove each glass slide from the staining jar and rinse with fresh toluene over a crystallizing dish and blow dry with N₂ (see Note 9).

14. Place the glass slides on the hotplate preheated to 125 °C for 20 min. Cover with a petri dish to prevent dust particles falling onto the modified surface (see Note 10).
15. Transfer the glass slides to a staining jar containing fresh toluene.
16. Sonicate the glass slides for 15 min.
17. Remove the glass slides from the staining jar and rinse them with fresh IPA.
18. Blow dry the glass slides with N\textsubscript{2} and place them inside a clean staining jar.
19. Place the epoxy-functionalized glass slides in a vacuum desiccator until ready to be used in an experiment (see \textbf{Note 11}).

\textbf{3.4 Microfluidic Device Assembly}

1. Treat the underside of a PDMS microfluidic device with O\textsubscript{2} plasma for 30 s.
2. Make loops of magic tape, and adhere them to the inside of a polystyrene petri dish lid.
3. Add 500 μL of APTES to the bottom of the petri dish; use the pipette tip to spread the solution over the surface of the dish.
4. Place the treated PDMS microfluidic device on the tape loops on the petri dish lid.
5. Cover the petri dish containing the APTES solution with the lid (see \textbf{Note 12}).
6. Place the petri dish in the vacuum desiccator for 20 min.
7. Preheat a hotplate to 125 °C.
8. Remove the APTES-modified PDMS device from the petri dish, and mount it on an epoxy-modified glass slide.
9. Place the assembled device on the 125 °C preheated hotplate for 20 min (see \textbf{Note 13}).

\textbf{3.5 Microfluidic Device Setup}

1. Mount the assembled device on the stereomicroscope and connect the control tubing.
2. Run the LabVIEW control application, and use it to activate and deactivate the elastomeric valves (see \textbf{Note 14}).
3. Prime all control lines, except the MITOMI buttons, by injecting DI water. Set the control pressure to 5 psi.
4. Once the control lines are primed, increase the pressure to 25 psi and repeatedly activate and deactivate all the valves. This will prevent the valves to irreversible bond to the epoxy-modified glass (see \textbf{Note 15}).
5. Activate all microvalves to isolate the different modules in the device.
6. Block the surface of the cell culture chambers to prevent undesired cell attachment. Inject the Pluronic solution for 1 min at 1 psi. Activate the cell culture outlet valve, and allow remaining air bubbles to diffuse through the porous PDMS bulk. Lower
the pressure to 0.01 psi and deactivate the outlet valve. Continue injecting Pluronic for 15 min.

7. Wash the Pluronic solution with PBS for 15 min at 0.01 psi.

8. Through a biosensing inlet, deliver biotinylated BSA to the biosensing chambers using 1 psi. Plug the biosensing outlet valves, and allow air bubbles to diffuse through the pores of the PDMS bulk.

9. Once the biosensing chambers have been wetted with biotinylated BSA, prime the MITOMI buttons with DI water using a pressure of 5 psi. After priming, deactivate the MITOMI buttons.

10. Remove the plugs from the biosensing outlets and lower the fluid flow driving pressure to 0.1 psi (see Note 16).

11. Continue injecting biotinylated BSA for 15 min.

12. Perform a wash step with DPBST for 10 min.

13. Inject NA for 15 min and wash with DPBST again for 10 min.

14. Close de MITOMI button valves.

15. Inject once again biotinylated BSA for 15 min followed by a wash step with DPBST for 10 min.

16. Open the MITOMI button valves.

17. Flow the biotinylated capture antibodies for 15 min followed by a 10 min wash step with DPBST.

18. Replace the DBPST in the biosensing chambers with complete culture media.

### 3.6 Monocyte Isolation from Peripheral Blood and Culture (See Note 17)

1. Collect 4 mL of whole blood in an EDTA-containing tube.

2. Deposit 2 mL of Histopaque-1077 in two 15 mL conical tubes.

3. Carefully layer 2 mL of whole blood on top of the Histopaque solution.

4. Centrifuge at 400 g for 30 min at room temperature (see Note 18).

5. Remove the plasma layer using a disposable transfer pipette.

6. Recover the PBMC layers and transfer them to new 15 mL conical tubes.

7. Wash by adding 10 mL of DPBS and centrifuging at 250 g for 10 min.

8. Wash a second time by adding 5 mL of DPBS and centrifuging at 250 g for 10 min.

9. Remove supernatants and resuspend in 1 mL serum-free cell culture medium.

10. Transfer the cells to four 30 × 15 mm polystyrene petri dish.
11. Incubate for 30 min at 37 °C and 5 % CO₂ (see Note 19).
12. Remove supernatant and wash twice with 2 mL of warm DPBS.
13. Remove DPBS and add complete culture medium.
14. Incubate at 37 °C and 5 % CO₂ until ready for experimentation.

3.7 Monocyte Seeding to Microfluidic Device

1. Remove supernatant and wash with 2 mL of warm DPBS.
2. Add 100 μL of Trypsin-EDTA and incubate for 5 min at 37 °C.
3. Add 100 μL of BSA to inactivate Trypsin and prevent cell damage.
4. Add 3 μL of DNase.
5. Load the cell suspension to a UV-sterilized microbore tubing.
6. Connect the tubing to the microfluidic device.
7. Inject cells to the cell culture chambers and monitor cell capture under the microscope.
8. By actuating the outlet valve, stop the flow of cells for 30 s to allow the cells to sediment into the microwells.
9. Repeat steps 3.7.7 and 3.7.8 until the majority of microwells have been filled with cells.
10. Wash remaining cells with complete medium.

3.8 On-Chip Cell Secretory Immunophenotyping

1. Prepare the stimuli solution in complete cell culture media.
2. Load the solution to sterile microbore tubings and connect them to the device via de stimuli inlets.
3. Purge the air by pressurizing the tubings to 1 psi and opening momentarily the purge outlet valves.
4. Close the purge outlet valves, and allow remaining air to diffuse through the PDMS bulk pores.
5. Decrease the pressure to 0.01 psi and open the stimuli inlet valves.
6. Allow the stimuli-containing solution to flow for 5 min.
7. Isolate the culture chambers and incubate the chip for 18 h at 37 °C and 5 % CO₂.
8. Perform active mixing for 30 min to allow the supernatant from the cell culture chambers to equilibrate with the solution in the biosensing chamber.
9. Isolate the biosensing chambers from the cell culture chambers through valve actuation.
10. Close the MITOMI button valves and inject DPBST for 10 min.
Fig. 3 Fluorescence sandwich immunoassay. (a) Representative micrographs showing fluorescence signal intensity for different concentrations of TNF-α. (b) Analysis of fluorescence micrographs. Two masks are applied to the original fluorescence image to determine the intensity level of the fluorescence spot and subtracting the mean intensity of the background. (c) The fluorescence signal intensity correlates with the number of TNF-α molecules (mol) present in the cell culture chambers. All scale bars are 150 μm.

11. Inject fluorescently labeled detection antibodies for 1 min, deactivate the MITOMI button valves, and continue perfusing for 15 min.

12. Wash one last time with DPBST for 10 min.

### 3.9 Image Acquisition and Analysis

1. For each experiment, two sets of images are acquired. The first image set is in brightfield from each cell culture chamber right after seeding, and it is employed to quantify the number of cells trapped. The second set of images is from the MITOMI buttons in the PE channel after immunoassay development, and it is employed to quantify the fluorescence intensity displayed by each biosensing area (Fig. 3).

2. To quantify the fluorescence intensity of the biosensor, export the image set to .TIFF format without compression and save them to a directory.

3. Run the MATLAB script (see Note 14).

### 4 Notes

1. The spin coating parameters should be empirically optimized by each laboratory.

2. Due to the inherent variability between equipment, series exposures should be carried out to determine the optimal dose.

3. Critical step: do not perform in an oven.
4. Perform this every 10–15 replicas to prevent PDMS bonding to the SU-8 structures, particularly to the post that gives rise to the cell trapping microwells.

5. Do not leave the mold for extended periods in the oven as PDMS can stick to the SU-8 structures ruining the mold.

6. Do not use tape other than magic tape, since it is the only tape that does not leave residues on the surface after its removal.

7. Use proper personal protection equipment, including goggles and respirator.

8. Do not let the staining jar touch the bottom of the ultrasonic cleaner; suspend the jar using the support stand and clamp.

9. Follow proper environmental health and safety procedures for hazardous waste disposal.

10. Make sure the upper side of the glass slide is the treated surface.

11. Perform the experiments as soon as possible since the surface chemistry might degrade, hindering the performance of the immunoassay.

12. Make sure that the treated PDMS surface is facing the APTES solution.

13. Use the assembled device immediately for better immunoassay performance.

14. All necessary software can be found on our laboratory’s repository at https://github.com/BioARTS-Lab/Cell_secretion_analysis_in_an_integrated_microfluidic_device.

15. If a valve bonds to the glass slide, identify the tubing that connects to it, and with the 20 mL syringe, withdraw vigorously to generate a vacuum to reconstitute the valve.

16. If the pressure is lowered before removing the plugs, backflow can reintroduce air to the biosensing chambers.

17. This method describes the isolation of human monocytes from peripheral blood and should be substituted for an appropriate method (e.g., density gradient centrifugation followed by MACS selection) to isolate immune cells for other samples of interest (e.g., tumor biopsy).

18. Low temperatures will result in cell clumping and poor recovery. Make sure centrifuge acceleration and break are on lowest setting; harsh breaking can affect the separation layer.

19. Longer incubation periods will result in higher contamination with lymphocytes.
Competing Interests

J.L.G.C is currently employed by F. Hofmann La Roche.

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Dynamic Tumor Perfusion and Real-Time Monitoring in a Multiplexed 3D Printed Microdevice

Alex Markoski, Ian Y. Wong, and Jeffrey T. Borenstein

Abstract

Stereolithography based additive manufacturing (“3D printing”) has become a useful tool for the development of novel microfluidic in vitro platforms. This method of manufacturing can reduce production time while allowing for rapid design iteration and complex monolithic structures. The platform described in this chapter has been designed for the capture and evaluation of cancer spheroids in perfusion. Spheroids are created in 3D Petri dishes, stained, and loaded into these 3D printed devices and imaged over time under flow conditions. This design allows for active perfusion into complex 3D cellular constructs resulting in longer viability while providing results which better mimic in vivo conditions compared to traditional monolayer static culture.

Key words Microfluidics, Cancer, 3D printing, Spheroid, Perfusion, Monolithic

1 Introduction

Additive manufacturing (“3D printing”) of transparent, polymeric materials is highly promising for custom, on-demand fabrication of microfluidic devices [1]. Typically, flat elastomeric parts with raised topographic features are prepared by replica molding against photolithographically patterned substrates, a process known as soft lithography [2]. These elastomeric replicas (e.g., polydimethylsiloxane) can then be tightly adhered against a second substrate to form enclosed networks of microscale channels. However, this process is rate limited by the initial photolithography step, which requires a cleanroom as well as the availability of a custom-designed photomask. Alternatively, hard plastics can be machined with open channel geometries (e.g., polystyrene, cyclic olefin copolymer) and then embossed against a second substrate under elevated temperature and pressure [3]. Most recently, 3D printing has been utilized for bespoke fabrication of master substrates for soft lithography, as well as open channel geometries that can then be adhered to a flat
substrate [4]. All these fabrication methods are further limited by the need for strong adhesion between micromolded features and a substrate [5], without which the channel can leak or detach under high fluid pressures. Moreover, the resulting device geometries are constrained to planar architectures, which complicates the integration of tubing connectors and bubble traps. Nozzle-based 3D printing has been previously demonstrated for monolithic microfluidic devices that do not require bonding, but typically exhibit larger feature sizes with rough surfaces (governed by filament or droplet diameter) and also have difficulty with overhanging channel geometries without additional sacrificial supports [1]. Light-directed stereolithographic 3D printing enables finer feature sizes with smoother surfaces, as well as stronger mechanical cohesion between successively printed vertical layers [1].

Three-dimensional (3D) living tissues can be perfused within microfluidic devices to dynamically control fluid flows and biomolecular transport [6]. In particular, the tumor microenvironment is governed by aberrant interstitial flows as well as oxygen and drug gradients, which can be recapitulated using microfluidic devices [7]. For instance, multicellular spheroids can be prepared by aggregating cancer cells in low-adhesion microwells, resulting in tissue-like architecture and barrier function [8]. Spheroid size and cellular composition can be tightly controlled based on the initial cell dispersion, which facilitates preclinical testing of chemotherapeutic drugs and immunotherapies [9]. Microfluidic culture of multicellular spheroids typically entails (1) mechanical trapping of spheroids within U or V shaped arrays of pillars, (2) perfusion to refresh media while avoiding shear-induced damage for active oxygenation and nutrient supply, and (3) longitudinal monitoring, often using confocal microscopy [10,11]. Our recent work has demonstrated microfluidic perfusion of both 3D spheroids and tissue fragments using this approach [12–15].

Here, we describe our approach for light-directed 3D printing of monolithic microfluidic devices (Fig. 1), spheroid formation and capture within the device, device operation with controlled perfusion for extended viability, and quantitative live cell imaging, based on our recent work (Fig. 2) [15].

## 2 Materials

### 2.1 Cells
- CT26.WT cells (ATCC CRL-2638).

### 2.2 Reagents
- CellTracker Green CMFDA Dye (Thermo Fisher C7025).
- Trypsin EDTA 1X (ATCC 30–2101).
- APC Annexin V (Thermo Fisher A35110). ViaStain AOPI Staining Solution (Perkin Elmer CS2-0106).
- Pure agarose powder (Millipore Sigma).
Fig. 1 Developed device from multiple viewpoints with annotations. (a) Cross-sectional view of the side of the device with labels for critical features. (b) Top-down view of the device with an expanded view of the capture geometry. (c) Isometric view of the device. (d) Top-down view of five devices connected via interlocking the external geometry. Reproduced under Creative Commons Attribution licence (CC-BY, 4.0) from [15]

Fig. 2 Experimental timeline depicting the time from cell culture to the end of the experiment

- Isopropanol.
- RPMI-1640 medium (ATCC 30–2001).
- Fetal bovine serum (FBS) (ATCC 30–2020).
- Deionized water.
- Phosphate-buffered saline (PBS) 10X pH 7.4, RNase-free (Thermo Fisher AM9624) Saline 0.9% Sodium Chloride (Fisher Scientific).
2.3 Equipment

- Asiga Max27.
- Thin metal scraper (used to remove the 3D prints from the build platform) tweezers, abrasive sandpaper (micromesh 3600, 4000, 6000).
- Washer bottles with built-in strainers for isopropanol washes.
- Curing oven (nitrogen gas and UV used to cure the 3D print).
- GR1 UV Resin (Pro3dure Medical GmbH, Dortmund, Germany).
- Weighing dish, 1.5 mL conical tube.
- 100 mL autoclave-safe bottle.
- Nitrogen gas (used for curing the devices and drying the channel).
- Autoclave.
- Incubator.
- Microscope.
- Zeiss LSM 780 Confocal.
- Zeiss EC Plan-Neofluor 10X objective.
- Argon 488-nm laser.
- Teflon tape (placed around connector threads to prevent leakage).
- 3D Petri Dish Large Spheroid Micromold (Catalog # 24–35).
- Automated Cell Counter (e.g. Nexcelom Bioscience Cellometer).
- 12 well plate.
- Tygon tubing ID 0.02” (Cole-Parmer #06419–01) 3 mL Luer lock syringe, 10 μL and 500 μL pipette tips.
- Idex Super Flangeless Fitting (Cole-Parmer).

3 Methods

3.1 Preparation for 3D Printing

1. Acquire paper towels, gloves, and safety glasses.
2. Assess the amount of resin in the resin bath (should be at least 10 mm in height).
3. Start up the 3D printer if it is off by pressing the button on the top of the 3D printer (Fig. 3).
4. From the main screen, go to the LED menu by choosing “controls” and then “LED.”
5. Turn on the LED for ~10 s to produce a cured rectangle in the center of the bath. This helps removal of remnants from previous builds.
Fig. 3 Preparation required prior to 3D printing. (a) The main locations of critical components of the Asiga MAX X27 printer. (b) A depiction of the stereolithography printing process. (c) Screen interface for manually starting the LED for cleaning the resin bath. Turn on the LED for 10 s to create a cured rectangle at the bottom of the resin bath. (d) The removal process of the cured rectangle. Move the thin metal scraper under one of the corners of the submerged rectangle without damaging the bottom of the bath. Slowly push it to the edge of the bath, and wait till a majority of the excess resin drips off before removal.
6. Remove the cured rectangle from the bath via the thin metal remover.
   
   (a) Avoid scraping the bottom of the bath as it can tear or deform the bottom causing issues with prints.
   
   (b) Hold the rectangle over the corner of the bath until a majority of the uncured resin drips back into the bath.

7. Throw away the cured rectangle into the trash, and clean the thin metal remover with a paper towel to remove any excess resin.

### 3.2 3D Printing

1. Create desired build in SolidWorks or equivalent CAD software (see Note 1).
   
   (a) Keep in mind the dimensions of said build cannot exceed the size of the 3D printer’s build plate (51.8 × 29.2 × 75 mm).

2. Save the build as a STL file via File→Save As→STL file type (Fig. 4).

3. Open the Asiga Composer and import the STL file into the program.

4. Select the build and rotate it into the desired orientation using the X Y and Z rotation buttons.

5. Select the “autoplace” feature to finalize the placement of the build.

6. Connect the laptop to the Asiga Max 3D printer via an Ethernet cable.
Fig. 5 Depictions of how to safely remove a build post print. (a) Situate the thin metal scraper at the corner of the build, and angle it ~15° offset from the build plate. (b) Slowly move the thin metal scraper back and forth while applying a gradual amount of force at the corner of the build until the scraper slides between the build and the build plate allowing for removal. (c) A dirty bath on the left for initial washing of the device post removal from the 3D printer. After a wash in the dirty bath, the device is then transferred to the clean bath on the right. (d) When placed in either bath, invert the washer bottles with built-in strainers, so the device is submerged in the cleaning solution. Swirl the bottles in a circular motion to agitate the solution, and clean the devices for 3–5 min.

7. Save the Asiga Composer file and send the build to the printer via the “Build” button.
   (a) If the program doesn’t find the 3D printer, try to close the program and open again.

8. On the 3D printer, select the printing option from the starting screen and choose the “next build” option.
   (a) Once the build has been found on the 3D printer, the Ethernet cable can be detached from the laptop.

9. Confirm the material and start the build if...
   (a) There aren’t any bubbles or contaminants in the bath.
   (b) The build plate is clean.

3.3 3D Printed Device Post Processing

3.3.1 Removal and Cleaning

1. Using new disposable gloves, safety glasses, and the thin metal remover, scrape the new build from the building platform (Fig. 5).
   (a) Place the thin metal remover between the nearest corner of the build and the building platform. Push the remover across the building platform lightly as to not scratch the building platform.
   (b) Once positioned, apply force gradually at a ~15° angle while moving the remover slightly left and right. Keep an open hand underneath the platform to catch the build once it is removed to prevent it from falling into the resin bath below.

2. Take the build and place it in the dirty >97% isopropanol container, and slowly flip the container over till the build is submerged.
3. Manually mix the solution for 3–5 min by keeping the device submerged and swirling the container in a circular motion.
4. Using tweezers, remove the build from the dirty container and place it into the clean >97% isopropanol container and repeat step 3.
5. Remove the build via tweezers and place it onto a paper towel and dab the build to dry it.
6. For devices with internal channels, connect tubing to the inlet and place stopper in the outlet.
7. Fill a 3 mL syringe with 100% isopropanol with a 1 mm tip, and connect it to the inlet side.
8. Open the dirty >97% iso container and place the outlet facing it.
9. Flow ~1.5 mL through the device and then pause and remove the outlet stopper and place it the stopper on the bubble trap. Flow the remaining ~1.5 mL through the device while removing the bubble trap stopper for the last 0.5 mL.
10. Remove the syringe from the connected 1 mm tip and fill with air and then reconnect to the system.
11. Forcibly push air through the device until a majority of the isopropanol is removed.
12. Use a high power air gun to remove the rest of the isopropanol from the channel.
13. Check the channel to make sure that the channel is clean of any visible remnants; if not, repeat steps 9-12 until clean.
14. Label the device with the proper ID.

3.3.2 Polishing Procedure

1. Acquire water, a 3600 Micro Mesh, a 4000 Micro Mesh, a 6000 Micro Mesh, 3D printed devices, gloves, and Kimwipes (Fig. 6).
2. Wet the bottom of the device with water.
3. Using the 3600 Micro Mesh (8 μm), make a circular motion around the capture geometry on the bottom of the 3D printed device for 30–60 s.
4. Clean the bottom of the device by dabbing it with a Kimwipe.
5. Wet the bottom of the device with water.
6. Using the 4000 Micro Mesh (5 μm), make a circular motion around the capture geometry on the bottom of the 3D printed device for 30–60 s.
7. Clean the bottom of the device by dabbing it with a Kimwipe.
8. Wet the bottom of the device with water.
Fig. 6 Device polishing procedure. (a) The highlighted region on the bottom device is the capture region which requires polishing. (b) The progression using three different grit sandpaper to polish the capture region. Each numbered step requires at least 30 s of polishing in a circular pattern, cleaning with a Kimwipe, and then wetting the surface with water. (c) Results from profilometer readings of the bottom of the device pre and post polish resulting in a surface roughness value of 0.35 μm (n=3 samples)

9. Using the 6000 Micro Mesh (4 μm), make a circular motion around the capture geometry on the bottom of the 3D printed device for 30–60 s.

10. Clean the bottom of the device by dabbing it with a Kimwipe.

11. Repeat 2–10 skipping steps 6, 7, and 8.

12. Evaluate the polishing through a profilometer. Surface roughness readings of 0.2 μm to 0.4 μm are sufficient.
3.3.3 Curing

1. Turn on nitrogen gas slowly with the door of the oven open, and stop turning when you hear the hissing indicating gas is flowing.
2. Place the build in the center of the oven and close the door.
3. Wait for 30 s to let the nitrogen fill the chamber.
4. Turn on the power of the oven and input 3 min into the timer (displayed as 3.0).
5. Start the oven.
6. After 3 min, remove the build and turn off the N$_2$.
7. Turn off the power for the oven.

3.3.4 Micromold Creation

1. Acquire a 3D Petri dish catalog # 24–35 master for casting agarose micromolds (has an array of 5 by 7 microwells) (Fig. 7).
2. Clean the 3D Petri dish of debris using deionized water.
3. Place micromolds for casting 3D Petri dishes in an autoclave bag and seal it for sterilization. Place the autoclave bag in an autoclave safe container and place both in the autoclave.
4. Run the autoclave in a 30-min wet cycle followed by a 30-min drying cycle. Remove from the autoclave with heat resistance gloves for safety.
5. Bring the autoclaved mold to a biosafety cabinet, and place the micromold in a six-well plate.
6. Measure out 1 g of high quality pure agarose powder in a weighing dish. Place it into a dry 100 mL autoclave-safe glass bottle. Ensure the bottle doesn’t have any liquid in it and leave the screw on the lid loose.
7. Autoclave the micromolds and bottle with agarose powder for 30 min on a dry cycle.
8. Add 50 mL of sterile saline [0.9% (w/v) NaCl] to the bottle containing sterilized agarose powder in a biosafety cabinet. Once the lid is screwed back on, loosely swirl the bottle to mix the agarose powder until the powder is evenly dispersed.
9. Place the bottle in the center of a microwave to boil and completely dissolve the agarose powder. Stop microwave every 10 s and swirl bottle to help dissolve the agarose. Be sure there are no small bits of translucent undissolved agarose. Typically, this process takes seven 10-s repetitions to completely dissolve the agarose.
10. Caution. Molten agarose is very hot, can boil over when the bottle is swirled, and can cause severe skin burns. Always use appropriate personal protection (gloves, oven mitt, eye protection, and lab coat) when working with molten agarose.
11. Transfer the container using oven mitts or a series of paper towels wrapped around the bottle to a biosafety cabinet.
Fig. 7 Production of CT26 spheroids using agarose micromolds. (a) A 24–35 master is seeded with heated agarose in an “S” shaped pattern. (b) Trypsinized cells are moved to a conical tube before being spun down in a centrifuge at 300 g for 5 min. The supernatant is aspirated and the solution is resuspended in 1 mL of fresh media. 20 μL are removed and placed in a 5 μL tube for cell counting. If the required cellular concentration is met, CellTracker Green is added to the cell solution in the 15 mL conical tube and put in a 4 °C refrigerator for 1 h. After, the solution is once again spun down at 300 g for 5 min and resuspended in 1 mL of fresh media for convenience to create a solution of the required seeding concentration. (c) After two 15 min washes in media, the remaining media is aspirated from the well plate, and the cell solution is added to dish dropwise. Let the cells sit for 10 min before replacing the media surrounding the agarose micromold. The following images represent aggregated spheroids in the agarose micromolds after 48 h.

12. Once the agarose has cooled to about 60–70 °C, slowly pipette 330 μL into the agarose micromold while depositing it in an “S” like pattern to evenly coat the mold (see Note 2).

13. Immediately remove any small bubbles that may be trapped in the small features of the agarose micromold by pipetting or gentle scraping before the agarose fully gels.

14. After about 2–3 min, the agarose micromold should have solidified enough for removal. Prepare a 12-well plate to deposit the agarose micromold into. Carefully flex the outer edges of the micromold while holding it above a well of the 12-well plate to deposit into an open well. Do not over flex the micromold as it can crack (see Note 3).
3.3.5 Spheroid Formation Protocol

1. Equilibrate the 3D Petri dish in complete media (RPMI + FBS) leading up to its immediate use. Add 2.5 mL of complete media into the well containing the 3D Petri dish. Incubate for at least 15 min, aspirate the media after 15 min, replace it with 2.5 mL of fresh media, and incubate for another 15 min (see Note 4).

2. Meanwhile, trypsinize the cells using 2 mL of Trypsin EDTA, and place in an incubator for 5–10 min or until the cells have detached from the surface of the cell culture flask and each other.

3. Add 3 mL of complete media to the flask and wash the flask at least three times to remove any cells left adhering to its surface.

4. Transfer the contents of the flask (3 mL of complete media, cells, and 2 mL of trypsin) into a 15 mL conical tube.

5. Place the conical tube into a centrifuge with an appropriate counterbalance (a 15 mL conical tube filled with 5 mL of liquid on the opposite side of the centrifuge), and run it for 5 min at 300 g.

   (a) When running a centrifuge, be sure to properly balance the rotor and do not leave it unattended.

6. Remove the conical tube from the centrifuge and aspirate the supernatant.

7. Resuspend the cells in 1 mL complete media and remove 20 μL to place in a 1.5 mL conical tube for counting cells.

8. In the 1.5 mL conical tube, add 20 μL AOPI Live Dead stain to create a 1:1 ratio (by volume) between cell and staining solutions.

9. Mix the combination well and leave at room temperature for about 5 min.

10. Pipette 20 μL of the AOPI/cell solution to each side of a Cellometer slide for counting.

11. Insert one side of the slide into the Nexcelom Cellometer cell counter.

12. Load the 11_AOPL_HS_LiveDead file on the cellometer program.

13. Set the “Dilution” option to 2.0.

14. Click “Preview” and adjust focus (cells with clear border and white center).

15. Click “Count” and record total cell count. Repeat this step to gain a second reading of the same side for comparison.

16. Repeat steps 14 and 15 for the other side of the cellometer slide.

17. Average all four total cell values to get the cell count per mL.
18. If the average value of the measurements exceeds 118,000 cells/75 μL, then the cells can be used for 300 μm diameter spheroid creation.

19. Add 2 μL of CellTracker Green to the 15 mL conical tube containing the cells and place in a 4 °C refrigerator for 1 h.

20. Remove the conical tube from the refrigerator and centrifuge it again as described in steps 5 and 6.

21. Resuspend the cells in 1 mL of fresh media, and calculate the volume of cells required to reach the required concentration of 118,000 cells/75 μL.

22. Remove media surrounding outside of agarose micromold, and tilt the 12-well plate so medium from the cell seeding area also drains out for removal.

23. Seed cell suspension of 75 μL dropwise into the cell seeding chamber which is the grid part of the agarose micromold.

24. Let the cells to settle into the wells for about 10 min in the incubator, and then add 2.5 mL of fresh media to the outside of the agarose micromolds within the 12-well plate.

25. Leave the agarose micromolds in an incubator for 48 h to allow for spheroid formation.

### 3.3.6 Platform Setup and Spheroid Loading/Staining

1. Premeasure out tubing from syringe pump to the confocal platform and from the platform to the waste beaker location.

2. Place the premade tubing in autoclave bags for sterilization via 30 min wet and 30-min dry cycle.

3. Post sterilization, bring the closed autoclaved bags to the confocal.

4. Bring one 50 mL beaker, six devices, and the spheroids to a biosafety cabinet for spheroid loading.

5. Block the bubble trap with a stopper and attach tubing to the inlet and outlet. Wash the channel of the device with 3 mL of PBS and then 3 mL of complete media. Use additional media to remove any bubbles.

6. Move the device, spheroids, and a waste beaker to a microscope to begin the loading process.

7. Use a stopper to block the inlet of the device, and attach a small tube to the outlet which leads to the waste beaker.

8. Using a 10 μL pipette tip, suck up about 5 μL media, and just before it fills completely, suck up a spheroid so it is in the last 1 μL of media at the tip (see Note 5).

9. Press the pipette tip to the open bubble trap to prevent any leaking, and slowly inject the spheroid and extra media into the device. Tilting the device at a 45-degree angle after injection can help the spheroid flow into the trap as well (Fig. 8).

10. Validate capture via a microscope.
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**Fig. 8** A depiction of the spheroid loading process. (a) With a 10 μL pipette tip, retrieve 4 μL and retrieve the spheroid with another 1 μL. (b) A side view of the spheroid loading setup. The black threaded stopper is put in the inlet, while a 10 μm pipette tip is used to load the spheroid through the bubble trap. The outlet has tubing leading to a waste beaker. (c) A view of a spheroid in the channel with highlighted capture posts. An arrow indicates the direction of flow used to facilitate capture.

**Fig. 9** General schematics of the testing setup on and off the confocal. (a) The basic flow of the testing setup from the source of flow to the waste container. (b) A progress of images showing how to create the threaded tubing connectors. (c) An example of a single device setup on a confocal where “A” leads to the source of flow, “B” is the device, and “C” leads to the waste beaker.

11. If the spheroid isn’t in the capture geometry, additional media can be injected in through the bubble trap to help push it into the space. If a spheroid still isn’t captured at this point, another spheroid can be injected into the device.

12. Transfer the loaded devices on to a single well plate, and tape the device on to the surface. The well plate acts as a protection device for the confocal in case of leaks. Transfer this setup to the confocal for testing (Fig. 9).
Fig. 10 A flowchart displaying imaging setup in the Zen software. Under the progress arrow at the top is a breakdown of the sections, their subsections, and the tasks for each

13. At the confocal, connect all autoclaved tubing to the devices while ensuring no bubbles enter the devices.
   (a) Tubing will be overfilled with media at connector locations to prevent trapping any bubbles.
   (b) Each syringe will be inverted such that the tip is facing upward to allow any stray bubbles to rise and be removed prior to connecting to the tubing.

3.3.7 Imaging Setup

1. Turn on the confocal and associated heating unit and lasers, and give at least 10 min to allow for them to warm up for use.
2. Open Zen on the computer and choose the “Start System” option.
   (a) If the progress bar doesn’t advance, turn everything off and on again (including the complete confocal setup).
3. In the locate tab, go to the incubator section and make sure “H Unit XL” is checked and set to 37 °C (Fig. 10).
4. Turn on the CO2 at this point via a quarter turn of the knob behind the confocal, and watch the value in the local tab change until at least 5% is reached.
5. In Zen, switch to the acquisition tab and open the experimental manager at the top, and either choose a preset utilizing an Argon laser for 488 nm or create a new channel in the channel section and add a channel for an Argon laser for 488 nm (A488).
6. Click A488, and under the imaging setup section, check the T-PMT box which will add a T-PMT section in the channel.
7. In the acquisition section, change the following from the default settings:
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(a) Frame size—512 × 512.
(b) Change from 8 bit to 12 bit.
(c) Change method from sum to mean.

8. These changes should also result in the following:
   (a) Image size of 212.1 μm × 212.1 μm.
   (b) Pixel size of 0.42 μm.
   (c) Speed of 10.
       (i) Pixel dwell of 1.27 μsec.
       (ii) Scan time of 9.38 s.

9. Unselect the Time Series option in the top left of the acquisition tab if it has been selected.

10. Switch to a 10X objective via the “Microscope” tab in on the external LSM control interface.

11. In the channels section, adjust the following parameters:
   (a) A488 gain = 675, 7.5%, 38.2 pinhole.
   (b) T-PMT gain = 329, digital gain = 1.3 (see Note 6).

12. Ensure only the A488 channels are selected when finding the desired device to image via the positioning joystick.

13. Choose “Live” and move to the desired location for imaging.

14. Use Z stacks menu along with zoom controls to define the distance between the first and last image slice such that the entire sample will be captured (see Note 7).

15. Set the increment size to 10 μm. For 300 μm-diameter spheroids, this typically results in 15 to 20 slices total.

16. Turn off “Live” and press the “Start Experiment” button to collect images.

17. After imaging, save the image by pressing the save icon below the mini version of the picture in the menu on the top right of the screen.

18. The captured spheroids should only need to be exposed to the A488 laser for a maximum of 5 min every 10 h for data collection (see Note 8).

4 Notes

1. The design of the device shown in this chapter is composed of six major components which can be seen in Fig. 1: inlet, bubble trap, outlet, microfluidic channel, capture geometry, and exterior connectors. The inlet, channel, and outlet are critical design features which are required for basic functioning for a flow-based system. The threaded inlets make the device
adaptable to multiple tubing sizes. The channel size and length were determined by the size of the target biology (spheroids and tumor fragments) while accommodating for the length required for inlets, outlets, and the printer’s build plate restrictions. Via stereolithography 3D printing, other complex components can be directly integrated into the device, such as bubble traps to prevent bubbles from filling the channels and impeding proper device function. Additionally, the capture geometry is also built into the channel and is capable of safely holding 3D multicellular spheroids in the channel for testing and imaging. The exterior puzzle piece connectors are a convenient design consideration to allow for flexibility and scalability when testing with multiple replicates. Overall, the device’s dimensions were made to be as small as possible within the 3D printer’s capabilities, while allowing for easy tubing connection and user handling [15].

2. When pipetting the heated agarose, pipette slowly as the viscosity of the solution may prevent then entire 330 μL from entering the pipette tip instantly. Holding it in place for 5 to 10 s should allow the agarose to fill the tip without drawing up any bubbles, as the tip exists the solution. If bubbles end up entering the tip, remove the tip and try again. Immediately after removing the tip from the solution, pipette the agarose into the molds. The longer the agarose remains in the pipette tip, the higher the likelihood of it cooling and solidifying inside the tip preventing it from being pipetted into the mold.

3. By lightly touching the surface of the curing 3D Petri dish in the micromold, you can determine if it has solidified enough for removal. If your glove has any residue from the agarose on it from contact, then give the agarose another minute to solidify.

4. Do not attempt to aspirate media directly from the cell seeding area of a 3D Petri dish as the aspirator can damage the agarose wells if sucked up.

5. The spheroids can be very difficult to see, and placing them on a dark background or surface can make them more visible.

6. You may need to reduce the T-PMT gain (master) if it is too bright when imaging. Similarly, you may need to reduce the A488 gain (master) if the intensity of the CellTracker Green prevents you from seeing individual cells or is causing abnormal artifacts in the image.

7. It is good practice to extend the first and last slice a bit past the desired range to ensure the best image quality.

8. The experiment described here is for the evaluation of flow’s effect on 300 μm-diameter CT26 spheroids. Three devices are run with 5 μL/min of flow and another three are run without flow. All devices are monitored via confocal imaging every 10 h over the course of 3 days (Fig. 2).
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Capture and Release of Cancer Cells Through Smart Bioelectronics

Janire Saez, Maite Garcia-Hernando, Achilleas Savva, Roisin M. Owens, Fernando Benito-Lopez, and Lourdes Basabe-Desmonts

Abstract

Noninvasive collection of target cells such as circulating tumor cells (CTCs) is crucial for biology and medicine research. Conventional methods of cell collection are often complex, requiring either size-dependent sorting or invasive enzymatic reactions. Here, we show the development of a functional polymer film, which combines the thermoresponsive poly(N-isopropylacrylamide) and the conducting poly(3,4-ethylenedioxythiophene)/poly(styrene sulfonate), and its use for the capture and release of CTCs. When coated onto microfabricated gold electrodes, the proposed polymer films are capable of noninvasively capturing and controllably releasing cells while, at the same time, monitoring these processes with conventional electrical measurements.

Key words PEDOT:PSS, NIPAAm, Conducting polymers, Bioelectronics, Capture, Release, Cancer cells, Electrochemical impedance spectroscopy

1 Introduction

Metastasis is the leading cause of deaths in cancer [1]. Metastasis occurs when a cell decides to leave the primary tumor site and intravasate to the bloodstream and lymphatics and extravasate to distant organs. Noninvasive collection of these flowing cells such as circulating tumor cells is fundamental for cell biology, diagnosis, and prognosis of cancer research and drug development [2–4]. Traditional methods for collection of target cells that ensure cell viability include density-gradient centrifugation [5], flow cytometry [6, 7], and immunomagnetism [8], which are arduous as they require size-dependent sorting [9, 10]. Recently, biosensors are developed based on aptamers [11, 12] and DNA ligands [13, 14] for immunocapture of target cells [15, 16], taking advantage of biochemically modified particles. However, these systems operate through invasive enzymatic release, damaging cells during capture and release processes.
Organic electronics deals with the application of carbon-based semiconductors in the form of conjugated polymers (CPs) in electronic and optoelectronic devices. These materials have attracted interest due to their electronic properties, tunability, and low temperature processing [17]. Organic electronic materials exhibit mechanical compliance, negligible cytotoxicity, and ease of functionalization and modification of the material surface. Moreover, in contrast with traditional electronic materials, they are transparent and are therefore compatible with optical microscopy assays [18]. Notably, CPs are mixed conductors (ions and electrons), facilitating their direct coupling with biological systems, where ions are the predominant carriers, via a variety of different device designs [19]. A great example of CPs being used in bioelectronics is the poly(3,4-ethylenedioxythiophene) polystyrene sulfonate (PEDOT:PSS) [19–21]. Bioelectronic devices have already been used to monitor cell capture and cell release based on the functionalization of the electronic device with ferrocene (Fc) and μ-cyclodextrin (μ-CD) [11] or an aptamer [22].

Hybrid functional materials such as the thermoresponsive poly (N-isopropylacrylamide) (pNIPAAm) have been also applied for the noninvasive capture and release of cells. pNIPAAm has a lower critical solution temperature (LCST) phase transition in water at 32 °C [23]. Below the LCST, the polymer is hydrophilic and swollen, but above the LCST, the polymer becomes hydrophobic causing the material to shrink [24]. Due to the LCST compatibility with cellular viability, pNIPAAm is useful to modulate cellular adhesion/detachment [25]. The use of functionalized pNIPAAm networks for the capture and release of cells, triggered by a change in temperature, has been reported before [4]. Therefore, the combination of electroactive materials with pNIPAAm could allow the noninvasive monitoring of cell capture and release, providing real-time information without disturbing cellular viability.

Here we show a bioelectronic device made of microfabricated gold electrodes coated with a smart copolymer made of the conducting PEDOT:PSS and the thermoresponsive pNIPAAm polymers. This PEDOT:PSS/pNIPAAm copolymer enables the noninvasive capture and release of cells and the simultaneous electrical and optical monitoring of the whole process (Fig. 1).

2 Materials

2.1 Polymer Preparation

1. Poly(3,4-ethylenedioxythiophene) polystyrene sulfonate (PEDOT:PSS).
2. (3-Glycidoxypropyl)trimethoxysilane (GOPS).
3. N-Isopropylacrylamide (NIPAAm).
4. N,N'-methylenebis(acrylamide) (MBAAm).
Fig. 1 PEDOT:PSS/pNIPAAm coated microfabricated gold electrodes enable the capture and release of cells at mild temperatures and the electrical and optical monitoring of the processes. (Reproduced from [23] with permission of Elsevier)

5. 2, 2-Dimethoxy-2-phenylacetophenone (DMPA).
6. Weighting scale with resolution of 0.1 mg.
7. Weighting container.
8. 5 mL glass vial.
9. Sonicator.
10. Hot plate.
11. Magnetic stirrer.

2.2 Microfabrication of Gold Electrodes

2.2.1 Instruments

1. Spin coater (Laurell).
2. Mask aligner MA/BA6 (Karl Suss MicroTec).
3. Parylene coater (SCS).
4. Oxygen plasma (100 W, 30% O₂) (Diener etcher).
5. E-beam evaporator (Kurt J. Lesker Company).
6. Reactive ion etcher plasma PROF 80 RIE (Oxford Instruments).

2.2.2 Gold Electrode Microfabrication

1. Glass slides 7 cm × 6 cm.
2. Tweezers.
3. p-Xylene (SCS, Kisko).
4. Titanium crucible for E-beam deposition.
5. Gold crucible for E-beam deposition.
6. Photoresists: AZ4214E, AZ10XT (Microchemicals Gmbh).
7. 3% (v/v) micro-90 soap (Cole Palmer).
8. DI water.
9. 50% (v/v) 2-propanol (Sigma-Aldrich).
10. MIF726 developer (MicroChemicals Gmbh).
11. 3-(Trimethoxysilyl)propyl methacrylate.
12. Acetic acid.
13. Acetone.
2.2.3 PDMS Gasket Fabrication

1. Polydimethylsiloxane (PDMS) (Sylgard™ 184 Silicone Elastomer Kit, Dow).
2. 15 mL Falcon tube.
3. Petri dish 100 × 15 mm size.
4. Pipette.
5. Hot plate.

2.3 Collection of Cells Using the Bioelectronic Device

2.3.1 Media Preparation

1. Dulbecco’s Modified Eagle Medium.
2. Fetal bovine serum (FBS).
3. Glutamax.
4. Penicillin-streptomycin.

2.3.2 Cell Culture

1. Phosphate buffered saline (PBS).
2. Trypsin.
3. P75 flasks.
4. 5 mL, 10 mL pipette.
5. Laminar hood cabinet.
6. Centrifuge.
7. Hemocytometer.
8. Eppendorf tube.
9. Trypan blue.

2.3.3 Cell Capture and Release

1. Microplate shaker.
2. Cell culture media.
3. Hot plate.
4. Pipette.
5. Platinum mesh (counter electrode).
6. Two crocodile clips.
7. Autolab Potentiostat PGSTAT204.

3 Methods

3.1 Polymer Preparation

1. Pipette 62 μL of GOPS into 2 mL PEDOT:PSS and ultrasound for 20 min.
2. Add to the previous mixture 452 mg of pNIPAAm, 30.9 mg of DMPA, and 30 mg of mBAAm and stir for 30 min.
3. Reserve the PEDOT:PSS/pNIPAAm copolymer mixture solution.
3.2 Microfabrication of Bioelectronic Devices

1. Draw the design of the electrodes using CAD software.

2. Submit the CAD file to a commercial supplier for a mask fabrication at resolution of 2 μm.

3. Start the washing step of the fabrication process. Sonicate the glass slide in a glass beaker containing 3% micro-90 soap solution for 20 min, and rinse with DI water. Sonicate in a mix of acetone and 2-propanol (50%, v/v) for 20 min (see Note 1).

4. Dehydrate the glass slide in a hot plate for 10 min at 220 °C. Remove the glass slide from the hotplate and let them to cool down (see Note 2).

5. Spin coat the AZ4214E photoresist at 3000 rpm s⁻¹ for 30 s (Fig. 2).

6. Soft bake the photoresist for 2 min at 110 °C, and photopolymerize it applying six pulses of 5 s of lightening (365 nm) with resting times of 3 s between cycles (see Note 3).

7. Introduce the patterned glass into a bath of MIF726 developer for 20 min to remove the non-polymerized resin.

8. Immerse the glass slide in DI water for 5 min, and dry with compressed air.

9. Prepare the glass slide for gold (Au) deposition. Oxidize the glass slide with oxygen plasma for 1 min.

10. Deposit 10 nm titanium (Ti) and 100 nm Au using an E-beam evaporator (see Note 4).

11. Introduce the metalized sample in acetone for 1 h to lift off the areas covered by the photoresist and Au layer.

12. Activate the metalized pattern surface of the device by oxygen plasma (100 W, 30% O₂) for 2 min.

13. Immerse the substrate into a 3% of 3-(trimethoxysilyl)propyl methacrylate and 0.1% of acetic acid in ethanol solution for 30 min.

14. Rinse the device with ethanol and baked for 1 h at 70 °C to finalize the functionalization.

15. Insulate the device with parylene. Create the first layer of parylene by using 1.5 g of p-xylene using a parylene coater. The first parylene layer should be around 1.8 μm in height.

16. Spin coat 3% v/v of Micro-90 soap at 3000 rpm s⁻¹ for 30 s, and, immediately after, deposit a second layer of parylene by weighting 1.5 g of p-xylene, using a parylene coater. The second parylene layer should be around 1.8 μm in height.

17. Perform a second photolithography. Spin coat the positive photoresist AZ10XT on top of the substrate at 3000 rpm s⁻¹ for 30 s, and, then, soft bake for 2 min at 110 °C.
18. Photopolymerize the substrate by applying five cycles of five soft lightening (365 nm) with resting times of 5 s between cycles, using the mask aligner to photopolymerize the resin through a mask.

19. Develop the patterns by introducing the slides into a bath of MIF726 developer for 5 min to remove the non-polymerized resin, and dry applying compressed air.

20. Introduce the substrate into a reactive ion etcher plasma PROF 80 RIE to remove the two layers of parylene covering the electrodes.
21. Spin coat the prepared PEDOT:PSS/pNIPAAm copolymer mixture for 30 s at 1500 rpm s\(^{-1}\) followed by 30 s at 3500 rpm s\(^{-1}\).

22. Bake the coated electrodes at 70 °C for 2 min in a hot plate.

23. Photopolymerize the coated PEDOT:PSS/pNIPAAm for 1 min at 365 nm, without a photomask.

24. Hard bake the PEDOT:PSS/pNIPAAm coated electrodes for 2 h at 120 °C (see Note 5).

25. Rinse the devices with ethanol and DI water to eliminate the non-polymerized monomers.

### 3.3 Adhesion of the PDMS Gasket

1. Mix PDMS with the curing agent (10:1) in a falcon tube, with total volume of 5 mL.

2. Pour the mixture onto a petri dish.

3. Cure the PDMS at 80 °C for 2 h (see Note 6).

4. Cut the cured PDMS with desired shape. Rectangle shape is preferred.

5. Activate the surface of glass of the gold electrodes and PDMS surface with oxygen plasma (100 W, 30% O\(_2\)) for 1 min.

6. Put in contact the glass of the gold electrodes and PDMS and slightly press with the finger.

### 3.4 Preparation of Cell Suspension

1. Prepare cell culture media mixing Dulbecco’s Modified Eagle Medium in 10% FBS, 1% Glutamax, and 1% penicillin-streptomycin.

2. Remove cell culture media from confluent sw480 colon adenocarcinoma cancer cells in p75 flask.

3. Add 7 mL of PBS with a 10 mL stripette to prepare cell for trypsinization and remove PBS.

4. Add 2.5 mL of 0.25% Trypsin with a 5 mL stripette, and incubate for 5 min at 37 °C in 5% CO\(_2\).

5. Check in the microscope that the cells are detached, and add 3.5 mL of cell culture media to stop trypsinization.

6. Centrifuge the cells for 5 min at 200 G and remove the supernatant (see Note 7).

7. Add 10 mL of media and count the number of cells using a hemocytometer and trypan blue.

8. Add, to an Eppendorf tube, the amount of mL containing 6.5 \(10^5\) cells. If necessary, top with media to 1 mL. In case the number of mL exceeds 1 mL, repeat step 5 and reconstitute in 1 mL of media.
3.5 Collection of Cells Using the Bioelectronic Device

1. Capture and release of cells (Fig. 3). Incubate the device with 500 nM of fibronectin (FN) solution in PBS for 2 h (see Note 8).

2. Rinse the device with PBS and peel off the top layer of parylene by using a piece of kapton tape (see Note 9).

3. Bond the PDMS gasket to the electrodes following the protocol described in Subheading 3.3.

4. Capture of cells. Prepare cell suspension as explained in Subheading 3.4 and obtain $6.5 \times 10^5$ cells mL$^{-1}$. Pipette the cell suspension on the electrodes, which are surrounded by the PDMS gasket. Incubate in circular stirring using a microplate shaker for 3 h at room temperature.

5. Remove suspension of cells and wash the device with PBS three times.

6. Add cell culture media at room temperature and perform electrochemical impedance spectroscopy (EIS) measurements to obtain the impedance data with the cells attached to the sensors.

   Electrical connections for two electrode setup. Connect the PEDOT:PSS/pNIPAAm electrode (working-sensing electrode) with a crocodile clip to the potentiostat. Connect the platinum mesh (counter-reference electrode) with a crocodile to the potentiostat. Introduce the pt. mesh into the cell culture media. Perform impedance measurements applying an AC voltage of 0.01 V and a DC voltage of 0 mV versus open-circuit potential.

7. Cell release. Heat the device at 37 °C for 20 min on a hotplate.
8. Remove the suspension with a micropipette of detached cells, and replace with fresh culture media. Perform EIS measurements.

9. Check viability of released cells. Preconcentrate the cells in 200 μL of cell culture media and 200 μL of trypan blue solution. Analyze cell viability by optical microscopy (see Note 10).

4 Notes

1. All washing steps involving solvents should be performed in a wet hood with gas extraction.
2. It is recommended to dehydrate the glass slides after the cleaning step at 100 °C for 15 min to ensure the complete evaporation of water.
3. The UV light exposure dose is dependent on the type and amount of the photoresist used in the spin-coating step.
4. Ti enhances Au adhesion onto the glass slide surface.
5. The resulting layer thickness should be 600 nm in height, according to the measurements performed using DektakXT (Bruker) profilometer.
6. The hot plate temperature and curing time can vary. At higher temperatures, the curing step generally requires less time. Please follow the recommended protocol from the manufacturer of the Sylgard 184 kit.
7. Cell centrifugation time and force should follow manufacturer’s guidelines.
8. Incubate fibronectin in PBS for 2 h to allow the absorption of the FN into the copolymer through electrostatic interactions.
9. Rinse the device with PBS and peel off the top layer of parylene to avoid having FN adsorbed outside the electrode areas.
10. Live cells dyed with trypan blue will appear white. You must count just live cells.

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Chapter 22

Fabrication of Multilayer Microfluidic Arrays for Passive, Efficient DNA Trapping and Profiling

Christine M. O’Keefe and Tza-Huei Jeff Wang

Abstract

Trace amounts of cell-free DNA containing cancer-specific biomarkers can be found in blood plasma. Detection of these biomarkers holds tremendous potential for applications such as noninvasive cancer diagnostics and therapeutic monitoring. However, such DNA molecules are extremely rare, and a typical patient blood sample may only contain a few copies. Here we describe the fabrication and operation of a microfluidic device to efficiently trap single DNA molecules into chambers for detection of tumor-specific biomarkers through a passive, geometric manipulation strategy.

Key words Microfluidic DNA capture efficiency, Passive microfluidics, Circulating DNA, Heterogeneity, Digital PCR, Digital melt, Molecule-by-molecule profiling

1 Introduction

The discovery that cell-free DNA (cfDNA) containing disease-specific alterations can be readily found in patient plasma has opened new opportunities for molecular diagnostics and prognostics in many diseases [1–4]. These so-called liquid biopsies provide several advantages over traditional techniques, such as by offering sampling that is minimally or noninvasive, heterogeneous, and unbiased by localized resection. Thus, numerous studies have investigated the clinical utility of cell-free DNA for both diagnostic and theranostic applications across many diseases [5–9]. One prominent application of liquid biopsies is aimed at early detection and therapeutic monitoring of cancer, whereby liquid biopsies can provide a simple, noninvasive means of sampling DNA derived from otherwise difficult-to-access tissues throughout the body [10–12]. In fact, several studies have shown that both genetic and epigenetic molecular alterations, such as copy number variations, mutations, and DNA methylation, correlate with cancer progression, even at early stages, and can likewise be found in cfDNA in the plasma [13–16].
However, plasma contains a high background of cell-free DNA from healthy tissue, among which tumor-derived molecules are extremely rare, often occurring in frequencies as low as 0.01% \cite{10}. Therefore, detection of these ultrarare biomarkers requires extremely sensitive and specific analysis techniques. Digital technologies, such as digital PCR (dPCR) and droplet digital PCR (ddPCR), are capable of DNA identification and quantification at very high precision and sensitivity and thus are well-suited for rare molecule detection \cite{17, 18}. However, most devices only assess a small percentage (~10%) of total sample volume \cite{19, 20}, increasing the likelihood that rare molecules will not be detected.

Therefore, we sought to develop a microfluidic device that increases DNA capture efficiency to ensure thorough sample analysis. We sought to engineer a device that also exhibits simplicity in operation without requiring complex external equipment. To this end, we developed a multilayer PDMS-based device architecture to enhance DNA loading efficiency by geometrically manipulating the flow profile in the device \cite{21}. The multilayer design promotes efficient loading through two primary mechanisms. First, a curved, single-channel design constantly redirects fluid flow toward the inlets for the chambers and increases flow resistance in the direction of the channel. Second, a ratio of well height to channel height of \(<1\) adjusts the relative force of the vacuum in the direction of the wells with respect to the channel. Furthermore, the vertical compartmentalization strategy complements the densities of the entrapped fluids and facilitates the removal of air from the device. Here we describe the microfabrication technique for multilayer PDMS alignment to create devices that efficiently trap DNA into an evaporation-resistant pico-array and enable subsequent analysis such as digital PCR and digital melt.

## 2 Materials

Prepare all solutions at room temperature.

### 2.1 PDMS

1. Pattern layer: add 30 g of elastomer base and 2 g of curing agent to a mixing cup. Mix and degas in a planetary centrifugal mixer (see Notes 1, 2).
2. Sacrificial layer: add 24 g of elastomer base and 4 g of curing agent to a mixing cup. Mix and degas in a planetary centrifugal mixer (see Notes 1, 2).
3. Adapter layer: add 45 g of elastomer base and 4.5 g of curing agent to a mixing cup. Mix and degas in a planetary centrifugal mixer (see Note 2).
2.2 **Partitioning Oil**

1. Add 1 g of elastomer base, 0.1 g of curing agent, and 5 g of 100 cst silicone oil to a 50 mL conical tube.
2. Vortex at 2000 rpm for 1 min.
3. Desiccate for 10 min before use.

2.3 **Tubing**

1. To interface with the inlet of the device, we recommend using a Luer lock needle and Tygon tubing. Cut a length of Tygon tubing that reaches from an air pressure source to the thermal cycler on which the device will be placed.
2. Using a Luer lock needle with the same outer diameter as the inner diameter of the tubing (we recommend 0.02 inches), insert the needle with Luer on one end of the tubing.
3. On the other end, insert a needle without a Luer lock into the tubing to interface with the device.

3 **Methods**

Steps should be performed in a cleanroom to minimize dust collection on the microfeatures. Hot plates and the oven should be kept in a hood to reduce exposure to photoresist, developer, and PDMS fumes during baking steps. Follow proper waste disposal procedures when disposing of waste materials such as liquid PDMS and SU-8 developer.

3.1 **Mold Fabrication**

To fabricate multilayer devices, first create two master molds: one for the channel layer and one for the chamber layer. Perform all spinning, heating, and developing steps under a fume hood.

1. Dehydrate two silicon wafers by heating at 200 °C on a hot plate for at least 2 h.
2. Oxygen-plasma treat the wafers at 100 W for 1 min.

*For the chamber layer:*

3. Spin SU-8 3050 onto the wafer at 1800 rpm for 1 min (*see Note 3*).
4. Bake on a hot plate at 65 °C for 1 min, followed by 95 °C for 27 min (*see Note 4*).
5. Expose the wafer to the chamber layer mask at 175 J/cm².
6. Bake on a hot plate at 95 °C for 5 min (*see Note 5*).
7. Develop with SU-8 developer for ~12 min or until all unexposed resist is removed.
8. Bake on a hot plate at 200 °C for 6 h.
For the channel layer:

9. Spin SU-8 3025 onto the wafer at 1800 rpm for 1 min (see Note 6).

10. Bake on a hot plate at 65 °C for 1 min, followed by 95 °C for 14 min.

11. Expose the wafer to the chamber layer mask at 150 J/cm².

12. Bake on a hot plate at 95 °C for 5 min.

13. Develop with SU-8 developer for ~8 min or until all unexposed resist is removed.

14. Bake on a hot plate at 200 °C for 6 h.

### 3.2 Device Fabrication

1. Prepare the adapter layer. Pour the adapter layer PDMS onto a blank wafer, and let sit or desiccate until no air bubbles remain.

2. Bake for 30 min at 80 °C.

3. Peel the PDMS from the wafer and use razor blade to cut into 10 mm x 10 mm squares.

4. Use a needle to punch inlet and outlet holes for each device in the center of the squares (inner diameter of the needle for punching should match the outer diameter of the needle used for the inlet interface).

5. Silanize the channel and chamber master molds. Use a fume hood for this step. Place wafers vertically in a vacuum chamber, and add 15 μL of silane to a cup within the chamber. Seal the chamber from the atmosphere. Turn on the vacuum for 2 min. Turn off the vacuum, leaving the wafers in the sealed chamber for 10 more minutes.

6. Spin the 15:1 PDMS onto each mold at 700 rpm for 1 min (see Note 7).

7. Desiccate for 15 min (see Note 8).

8. Bake in 80 °C oven for 6 min.

9. Spin the 6:1 PDMS onto two blank silicon wafers at 100 rpm for 2 min each.

10. Bake wafers in 80 °C oven for 6 min.

11. Peel the PDMS off the first blank wafer and lay on top of the PDMS-covered wafer containing the channel layer (Fig. 1). Ensure there are no air bubbles between the two PDMS layers (see Note 9).

12. Bond the sacrificial and pattern PDMS layers by baking in an oven at 80 °C for 6 min.

13. Peel the bonded PDMS layers off the channel mold (see Note 9).

14. Repeat steps 11–13 for the second blank PDMS wafer and the wafer containing the chamber layer.
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**Fig. 1** Sacrificial PDMS layer is removed from the wafer and placed on top of the pattern layer. Ensure no air bubbles are between the two PDMS layers.

**Fig. 2** Use a needle to punch holes through the channel layer for inlet(s) and outlet(s) interfacing.

15. Use a needle to punch inlet and outlet holes for the device (inner diameter of the needle for punching should match the outer diameter of the needle used for the inlet interface) through the channel layer (Fig. 2) (see Note 10).

16. Align and bond the channel layer and chamber layers. Oxygen-plasma treat the chamber and channel PDMS surfaces at 45 W for 45 s. Using a microscope, align the channel layer to the chamber layer and bring them into contact.

17. Bake the layers together in an oven at 80 °C for 5 min.

18. Bond a 1 mm-thick glass microscope slide to the bottom of the chamber layer. Oxygen-plasma treat the chamber-side of the PDMS and the blank glass slide at 45 W for 45 s. Bring the layers into contact and bake in an 80 °C oven for 5 min.

19. Carefully remove the top sacrificial layer by peeling it from the channel layer with fingers or tweezers (see Note 11).

20. Bond a 0.1 mm-thick coverglass to the top of the PDMS channel layer (without covering the inlet and outlet holes) by the same method as step 18.

21. Align and bond the inlet and outlet adapters to the top of the channel layer by the same method as step 18 (Fig. 3).

22. Bake the device for at least 2 h in an 80 °C oven.

### 3.3 Device Loading

1. Seal the inlet and outlet with adhesive, and place the device under vacuum for a minimum of 2 h (see Note 12).

2. Prepare your mastermix.

3. Use a syringe to load the prepared partitioning oil into the tubing interface. Load at least three times the total volumetric capacity of the device. Ensure that no air is loaded into the tubing after the oil.
Fig. 3 Aligned multilayer device. The chamber layer is bonded such that the pattern faces upright and the sacrificial layer is bonded to the glass slide. The channel layer sits above the chamber layer and is bonded on the top surface to the inlet and outlet adapters as well as a coverglass to prevent evaporation.

4. Use a syringe to load the mastermix into the prepared tubing interface following the oil (Fig. 4). Ensure that there is no air in the needle and a liquid meniscus is visible prior to loading.

5. Within 10 min of removing the device from the vacuum, puncture the adhesive over the inlet with the interfacing needle to load the mastermix.

6. Pressurize the partitioning oil by connecting the Leur end of the interface tubing to an air pressure source set to 15 psi.

7. If there is any mastermix remaining in the channels after loading, remove the seal over the outlet and continue pressurizing the oil to finish partitioning the device.

8. After partitioning, seal the outlet and maintain pressurization. The device is now ready to be run.

4 Notes

1. Amounts provided are recommended for typical use with 4 inch silicon wafers as the master mold. The amount can be adjusted by maintaining at 15:1 ratio for the pattern layer and 6:1 ratio for the sacrificial layer.

2. If you do not have access to a planetary mixer, you can hand stir the PDMS mixture for 10 min and then degas the mixture in a desiccator for 30 min.

3. All SU-8 processing steps for the chamber layer are set for a design of 80 μm feature height.

4. For molds with thicker features (> 30 μm), we recommend also ramping down the temperature by placing the mold on a 65 °C hot plate for 1 min prior to exposure.

5. After the 95 °C base step, we recommend gradual cooling to room temperature over the course of 20 min.

6. All SU-8 processing steps for the channel layer are set for a design of 30 μm feature height.
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Fig. 4 Tubing interface for loading. Partitioning oil is loaded into Tygon tubing first, followed by the reaction mix. Care should be taken to avoid air bubbles between the fluids and after the reaction mix.

7. This spin speed is recommended for molds with features of 80 μm in height. The PDMS layer will be ultrathin, minimizing the amount of PDMS available to vapor and mitigating evaporation during high-temperature reactions.

8. Ensure there are no air bubbles. If so, continue to desiccate until no air bubbles remain.

9. To prevent tearing, it is helpful to use a razor blade to first separate the PDMS from the wafer around the entire perimeter. Then slowly peel the layer off starting from one side.

10. To make alignment of the adapter layer and the inlet and outlet holes on the channel layer easier, you may use a larger needle size to poke holes through the channel layer PDMS.

11. We find it best to start separating the sacrificial and pattern layers with forceps starting from one of the four corners.

12. The time given is for a vacuum force of $-0.05$ to $-0.08$ MPa.

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