Single cell kinase signaling assay using pinched flow coupled droplet microfluidics

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Droplet-based microfluidics has shown potential in high throughput single cell assays by encapsulating individual cells in water-in-oil emulsions. Ordering cells in a micro-channel is necessary to encapsulate individual cells into droplets further enhancing the assay efficiency. This is typically limited due to the difficulty of preparing high-density cell solutions and maintaining them without cell aggregation in long channels (>5 cm). In this study, we developed a short pinched flow channel (5 mm) to separate cell aggregates and to form a uniform cell distribution in a droplet-generating platform that encapsulated single cells with >55% encapsulation efficiency beating Poisson encapsulation statistics. Using this platform and commercially available Sox substrates (8-hydroxy-5-(N,N-dimethylsulfonamido)-2-methylquinoline), we have demonstrated a high throughput dynamic single cell signaling assay to measure the activity of receptor tyrosine kinases (RTKs) in lung cancer cells triggered by cell surface ligand binding. The phosphorylation of the substrates resulted in fluorescent emission, showing a sigmoidal increase over a 12 h period. The result exhibited a heterogeneous signaling rate in individual cells and showed various levels of drug resistance when treated with the tyrosine kinase inhibitor, gefitinib.

I. INTRODUCTION

Current cell-based assays follow an ensemble approach to determine activity1–3 thereby ruling out differences in biomolecular expression among individual cells, which could be misleading because of the averaging of signals from a subpopulation. However, kinetic studies on individual cells, including research on protease activities2 and kinase signal networks,3 cannot be performed using flow cytometers. Recently, several microscope-based platforms were invented for single cell isolation and analysis through microfluidics. For example, micro-valve technology was demonstrated to perform different applications of intracellular measurements of individual cells with a throughput of ~100 cells per experimental run. The capillary electrophoresis system was automatized to analyze ~3.5 cells/min.4–10 Compared with these methods, the droplet based technology is attractive11–21 because of its ultra high throughput cell encapsulations (~1000 droplets/s)12–21 and accurate fluidic controls. Accordingly, a wide range of droplet base single cell polymerase chain reactions (PCR) were carried out in previous studies. To perform the
protein activity assays, individual cells and sensing reagents were encapsulated in the droplets with pL scale of volume to enable high sensitivity assay. Despite these advantages, the droplet based single cell assay was limited by low cell encapsulation rate due to large population of empty droplets affecting the read out efficiency. In fact, cells randomly distributed in a suspension followed the Poisson encapsulation statistics showing low encapsulation rate ~0.24% in a physiological solution with cell density ~10^4 cells/ml.20,25 Highly efficient single cell encapsulation has been demonstrated using inertial migration in both a straight channel26 (56.5% single cell to multiple cells/droplet ratio) and a spiral channel27 (~77% encapsulation efficiency). In addition, hydrodynamic self-sorting of single cells have also been demonstrated to have a high encapsulation efficiency with successful encapsulation and sorting of 70%–80% of the injected cell population.28 However, these approaches typically require a long channel length (>5 cm) and encounter challenges such as cell sedimentation that lead to poor encapsulation rates and blockages when cells aggregate in the channel. In this study, we describe an approach for cell ordering in a short micro-channel (5 mm) using inertial focusing through pinched structures that consist of contracting and expanding chambers. The cell aggregates are separated into individual cells during their transit through the pinching regions of the channel and are then equally spaced or ordered for single cell encapsulation. In addition, the cells are assayed in the droplets without additional surfactants or enzymes, such as DNases, which are often used to avoid cell clumps, making this technique amenable for performing gene amplification inside the droplets.

Our study focuses on single cancer cell analysis, which relates to disease recurrence as a result of tumor cell heterogeneity and its metastasizing nature.29 Cancer metastasis is associated with the overexpression of tyrosine kinase receptors, such as the epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER-2).30–32 Kinases are an important class of enzymes that trigger cellular signaling cascades in order to regulate essential biological mechanisms, including cell proliferation,33 differentiation,32 growth,34 and metabolism.35 Therefore, development and screening of potential drugs to modulate the kinase activity among heterogeneous subpopulations of cells is of utmost importance. This research encourages the use of different platforms that can isolate and assay the cells individually. Zarrine-Afsar and Krylov6 and Dickinson et al.4 have previously reported capillary electrophoresis-based methods to monitor the activation of protein kinase A (PKA) and protein kinase B (PKB) in single cells. However, these methods involve either the cloning of cells to transiently express the GFP-tagged protein substrate or the use of immunofluorescence antibody staining to monitor kinase activity in trapped single cells.

We demonstrated a live cell signal transduction assay measuring the kinetic activity of tyrosine kinase in an antibody-free format using a fluorescent peptide reporter, Sox substrate.36,37 Human lung cancer cells (PC9) were encapsulated in droplets along with a specific tyrosine kinase substrate with and without a specific modulator (epidermal growth factor, EGF). The binding of EGF to its cell surface receptor, EGFR, triggers receptor dimerization, allowing tyrosine in the cytoplasmic portion of the receptor monomer to be trans-phosphorylated by its partner receptor, thus, aiding in signal propagation.38 The addition of EGF to the droplet triggers EGFR signaling,39 thus phosphorylating the peptide substrate and resulting in an increase in fluorescence over time. With the efficient isolation of individual cells, phosphorylation of the Sox substrate provides an understanding of the rate of intracellular downstream signaling triggered by a surface biomolecular binding event. We used this platform to analyze the potential of the kinase inhibitor, Gefitinib, for suppressing the intracellular enzymatic activity of PC9 cancer cells. Less than 20 pL of cell solution was used to simultaneously probe up to 10 single cell assays while monitoring their kinetic profiles in order to study the cellular heterogeneity and the inhibitor effect. Compared with other methods, this platform enables a straightforward study of the specific reaction pathways in single cells with readily available selective substrates.

II. EXPERIMENTAL

Lung cancer cells (PC-9) were cultured in a T-75 flask with Roswell Park Memorial Institute (RPMI) medium (HyClone, Thermo Scientific, USA). The media was supplemented
with 4.5 g/l glucose, 4 mM L-glutamine, phenol red (Hyclone, USA), 1 mM sodium pyruvate, 10% fetal bovine serum, FBS (Hyclone, USA), 50,000 IU/L penicillin, and 50 mg/l streptomycin. The cells were grown at 37 °C in a humidified incubator maintained at 5% CO2. Once the cells were 80%–90% confluent and 24 h before harvesting, they were synchronized by maintaining them in serum-free culture media. The cells were harvested by a 10–15 min treatment with 2% × 0.5% Trypsin-EDTA (Caisson Labs, USA) and finally re-suspended in 2 ml of serum-free RPMI medium. This suspension was passed through a cell strainer with a 40 µm pore size (BD Biosciences, Singapore) to filter out large cell clusters/aggregates. The cells were then re-suspended uniformly in serum-free culture media with 16% Optiprep (Sigma, Singapore) and collected in a 1 ml plastic syringe (BDTM, Singapore). This density matching solution helped to prevent the cells from settling during the flow experiments. The cells were later counted using a standard hemocytometer, and a final cell density of 15–18 × 10^6 cells/ml was used for the experiments.

The microfluidic device was fabricated using standard soft lithography procedures, in which a poly-dimethylsiloxane (PDMS, Sylgard 184-Dow corning, USA) chip was bonded to a PDMS-coated glass slide. HFE-7500 fluorocarbon oil (3 M NovecTM, Singapore) with 2% Krytox (modified with PEG head) surfactant40–42 was used to generate stable droplets. All the syringes were connected to their respective cell, oil and reagent inlet ports on the microfluidic device with 1 mm (OD) tubing and operated using Harvard PhD ULTRA syringe pumps (Harvard Apparatus, USA). The flow rate of the continuous (oil) phase ranged from 20 to 40 µl/min, whereas the flow rates for the reagent and cell mixture were 2–4 µl/min and 5–20 µl/min, respectively, to allow better single cell encapsulation per droplet. The droplets with encapsulated cells were initially collected in a syringe and later injected into an observation chamber (2 mm width, 80 µm height) immersed in water to prevent evaporation of the oil.

The droplet generation and cell encapsulation were imaged using a Phantom v7.3 high-speed camera at frame rates of 10,000–19,000 frames/s. The device was placed on a Nikon Ti-Eclipse fluorescent microscope, and the droplets were imaged using a Nikon 20× S-fluor (0.75 NA) objective and a Hamamatsu sCMOS camera. An automated excitation light source, CoolLED, was used along with a multiple band pass filter to detect the phosphorylated kinase (365 nm EX and 480 nm EM) and nuclear staining over different periods of time. Confocal imaging of the droplets was performed using a 63× 1.35 NA objective on an Olympus FV1000 confocal system with 405 nm and 633 nm excitation lasers and corresponding filter sets. The fluorescent and bright field images were post-processed using ImageJ software.

A reagent mixture containing the live cell nuclear stain DRAQ5 (BioStatus Ltd., UK), the live cell stain calcein AM (Life Technologies, USA), and the fluorescent tyrosine kinase substrate mix (Omnia Kinase Y peptide 12 assay kit, Life Technologies, Singapore) was injected through the reagent inlet. The nuclear stain was replaced by a dead cell stain, i.e., the ethidium bromide homodimer (EtBr-1, Life Technologies, Singapore), to detect the viability of the cells during the imaging period.

III. PINCHED FLOW CELL ORDERING

We describe an approach that enables an ordered distribution of cells in a short microchannel (5 mm) using inertial focusing through pinched microchannel structures (50 µm height) (Fig. 1(a)). These structures consist of contracting and expanding chambers. Cells traveling through the cell-focusing channel with chambers (90 µm wide) and orifices (30 µm wide) are focused along the center of the channel (Fig. 1(b)). Cell clumps are separated into individual cells due to the shear forces exerted on them during their transit through the pinching regions of the microchannel. Subsequently, the separated cells move in groups through a cell pinching channel with chambers (90 µm wide/orifices (12 µm wide) in a uniform spacing (Fig. 1(c)). The cells are ordered along the short straight channel before entering a laminar stream of assay reagents (Fig. 1(d)), which are encapsulated along with the cells downstream in the continuous oil phase droplet-forming region (Fig. 1(e)).
The high pressure from the side walls induced by the fluid flow patterns along with the lift forces repel the cells away from the wall.\textsuperscript{43,44} Thus, equilibrium is achieved by balancing the lift forces induced by the shear gradient and the effects of the contracting boundary. We observed cell ordering under these circumstances with better focusing at higher flow rates (3–20 $\mu$l/min). Separation zones are formed at the corners of the expansion channels at the higher flow rates induced by vortex formation (Fig. S1 in the supplementary material).\textsuperscript{45} Certain factors, such as surface roughness, flow velocity, and the ratio between the cross section areas and the angle and the roundness of the orifice corner, have been previously reported to influence the vortex formation within the microchannel. The streamline and velocity plot indicates that cells would move only through the center region of the expansion channels for Re > 16, based on their flow rates. However, because the cells are nearly the same size as the pinching region, the force exerted in that region allows the particles to squeeze through the channel, travel in a straight line and order themselves once they reach the long rectangular region of the channel. Our experiments suggest that with the pinched channel as a regulator, cell ordering depends on the cell size, volume fraction, and cell flow rate.

Given the addition of reagent inlets prior to the encapsulation of cells inside the droplets, the encapsulation efficiency was primarily dependent on the volume fraction and the flow rate of the cell and oil inlets. Cell ordering using the pinch flow microchannel resulted in the delivery of more than 55% of the encapsulated droplets with a single cell inside them and a reduced

\begin{figure}
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\caption{(a) Schematic representation of the microchannel design with pinched flow structures and droplet encapsulation of cells. (b) Cells flowing through the cell-focusing channel with chambers (90 $\mu$m wide) and orifices (30 $\mu$m wide), in which the cellular clumps are separated, and the cells flow to the center of the channel. (c) Cells flowing through the cell-pinching channel with chambers (90 $\mu$m wide) and orifices (12 $\mu$m wide), which aids in cell ordering before they reach the droplet-forming region. (d) The cells are ordered in a straight channel before droplet encapsulation. (e) The ordered cells are mixed with a chemical reagent and are encapsulated in droplets for assaying. The scaling bars represent a length of 50 $\mu$m.}
\end{figure}
number of empty droplets (25%) and multiple cells per droplet (19%) (Fig. 2(a)). The droplet diameter increased for higher cell and lower oil flow rates (Fig. 2(b)). The average droplet diameter at our operating flow rates was approximately 55–60 μm, which was at least four times the size of the cell (Fig. S2). Increasing the reagent flow rate resulted in an increased number of empty droplets (Fig. S3); therefore, the flow was maintained at a minimal rate (2 μl/min) while using a concentrated substrate solution, enabling the measurement of the kinase reaction in single cells. The cell and oil flow rates were optimized to produce the best encapsulation efficiency (>55%, Fig. 2(c)), while keeping the reagent flow rate constant. Using the live cell stain calcein AM, the viability of the PC9 cells after 12 h was observed to be greater than 95% (Fig. 2(d)).

IV. SINGLE CELL KINASE SIGNALING ASSAY

We used chelation-enhanced fluorescence (CHEF) with Sox (8-hydroxy-5-(N,N-dimethylsulfonylazo)-2-methylquinoline) peptide substrate, which binds to a group of specific tyrosine kinases. This substrate diffuses into the cell and is specifically phosphorylated by the EGFR when stimulated by the addition of EGF molecules, resulting in an increase in fluorescence (Figs. 3(a) and 3(b)). However, the substrate can also react with other members of the tyrosine kinase family present in the cytoplasm, resulting in a low basal fluorescence that can be clearly distinguished from a ligand-stimulated cell when studying a specific pathway. Single PC-9 cells encapsulated in the droplets with the live cell nuclear stain DRAQ5 and phosphorylated SOX substrates are shown in Fig. 3(c).

The ability to compartmentalize single cells inside picoliter droplets enables the use of concentrated substrate solutions at a very low volume, making long-term observations feasible without depleting the reagents. To observe the reactions in the droplets for an extended time

![Graphs and images](image)

FIG. 2. (a) Plot of the experimental observations and the Poisson distribution (λ = 1.17) for encapsulation rates of zero, single, and multiple cells per droplet. (b) Variation of the droplet diameter by changing the oil flow rates while maintaining constant cell flow rate (3, 5, 7, and 9 μl/min, respectively) and reagent flow rate (2 μl/min). (c) Plot representing the variation in encapsulation efficiency by changing cell and oil flow rates (average number of droplets counted for each measurement, N_{avg} = 560). (d) Cell viability plot representing the percentage of live or dead cells inside the droplet during the observation period. Error bars in figures represent standard deviation over two experimental sets.
(12 h), the inlets of the microfluidics devices were tightly sealed by Epoxy glue, and the devices were carefully placed in a humid chamber to prevent oil evaporation from causing the droplets to move during observation. The droplet compartments enable monitoring of the kinase reactions individually over extended periods in a static system without concern about the diffusion effects of the reagents on these cells due to very low working volumes (~few pL). The kinase molecules in individual cells phosphorylate the peptide substrate, eliciting fluorescence signals which occurs due to the chelation of Mg$^{2+}$ ions that forms a bridge between the SOX moiety and the peptide substrate. The fluorescence intensity in the individual droplets increased with the reaction time due to increase in phosphorylated peptide substrates (Fig. 4(a)). Compared with the use of a well plate system to perform bulk measurements (Fig. S4), the signal obtained from the region of interest drawn around the cells inside each droplet enables tracking of the signal intensity over time with minimal cross-talk between cells (Fig. 4(b)). In general, PC-9 cells express very low levels of EGFR, as indicated by comparing the immunofluorescence results with those from EGFR overexpressing cells, i.e., A431 cells (Fig. S5). This low receptor expression in the PC9 cells might result in reduced peptide phosphorylation activity in relation to the gradual (sigmoidal) increase in the fluorescence intensity over time. However, observation of these changes from bulk measurements using standard microplate readers was difficult, whereas the changes were significantly easier to distinguish at the single cell level.

In addition to the kinase activity experiments, this platform also enables studying the effect of drugs on single cell signaling activity. The response of individual PC-9 cancer cells kinase activities to Gefitinib (Selleckchem, Singapore), a drug which suppresses cancer cell migration, was examined. The effect of gefitinib on the individual cells was characterized by monitoring the reactions over 12 h. Due to the heterogeneity of cancer cells, different suppression levels in kinase activities under the same gefitinib concentration (0.1 μM) were observed leading to a smaller increase in fluorescence in the droplets (Fig. 4(c)). However, a few cancer cells vividly
retained kinase activity, showing high gefitinib resistance (Fig. 4(d)), which might indicate the possibility for these cells to metastasize and cause recurrence of cancer even after drug treatment.

V. CONCLUSIONS

Understanding cell heterogeneity is a key factor in developing a sustainable cure during cancer therapy. Recent advances in single cell encapsulation studies have demonstrated the use of different channel geometries to improve cell ordering. In this study, we reported cell ordering in a short pinched flow microchannel (few mm) for the effective encapsulation of single cells into picoliter droplets (with >55% efficiency). Cell clumps are separated into individual cells by the shear forces acting on them during their transit through the pinched regions of the microchannel without the use of additional surfactants and enzymes, such as DNases. The separated individual cells exiting the pinched regions get uniformly spaced for droplet encapsulation. Using the advantages of effective droplet-based encapsulation, a functional kinase signaling assay resulting from cell surface biomolecular (i.e., EGF) binding to a tyrosine kinase receptor (i.e., EGFR),
was performed on single cells by mixing peptide (SOX) substrates into the droplets. Heterogeneous signaling activities reflecting various levels of drug resistance were observed at the single cell level. With its ability to separate cellular aggregates and to effectively encapsulate individual cells into droplets to assay kinase activities, this device would be a useful platform for systems biology research and future drug development for cancer therapy. In addition, based on the current commercial availability of various peptide (Sox) substrates, our platform opens the door to study several functional effects of biomolecular binding while allowing us to proficiently screen the modulatory effects of drugs in single cells.

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See supplementary material at http://dx.doi.org/10.1063/1.4878635 for simulation of fluidic dynamics in the micro-channels, for droplet size characterization according to the flow rates, for the power spectrum of the intensity time trace, for the bulk well.