Differential detachment of intact and viable cells of different sizes using laser-induced microbubbles

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Abstract: Single cell isolation is a prerequisite for the analysis of rare or small cell subtypes. Here, we selectively detach single cells in a heterogeneous population comprised of different morphological subtypes whose sizes vary in body and extension. Such a cellular environment is first accommodated for by a photomechanical method in which pulsed laser irradiation produces microbubbles from a polymer substrate, thus pushing out and detaching cultured cells in an intact, viable, and spatially tailored way. While this has previously only been used at a very low cell density with lack of quantitative characterization, we determine optimal detachment conditions for different cell sizes in terms of an optical fluence and the number of laser pulses. Importantly, our approach is employed to isolate cancer cells with inherent size variation and elucidate cellular heterogeneity in drug sensitivity: i.e., higher resistance for larger cell size. For cells detached by laser-induced microbubbles, morphology, proliferation, and viability are compared with those of conventional trypsin-treated cells detached without any spatial selectivity. These results support the suitability of our photomechanical method for biochemical screen and secondary analysis of cells with unusual responses.

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1. Introduction

Single cell isolation is a prerequisite for the analysis of rare or small cell subtypes [1–3]. Various methods that are currently available for single cell isolation from heterogeneous cell populations include fluorescence-activated cell sorting and magnetic-activated cell sorting [4,5]. These techniques require additional labels to identify cells.

Methods for physically detaching or isolating a single or rare cell without cell labelling include microfluidics [6–8], infrared laser irradiation [9], and nanosecond pulsed laser beam [10]. Microfluidics is a powerful technique for cell sorting because it provides precise fluid control, low sample consumption, device miniaturization, and low analysis cost [6–8]. However, the yield and purity of the isolated cells need to be improved. Single cell detachment using direct infrared laser irradiation of a carbon nanotube (CNT)-based substrate has been reported, but the cell viability is poor because of heat-induced cell necrosis [11]. Moreover, bubbles that are produced by the laser irradiation were too large to enable differential detachment of single cells. Recently, a photomechanical method has been proposed using nanosecond laser pulses (NLPs) to generate optically driven microbubbles that were capable of breaking cell contacts with a composite film of CNT and polydimethylsiloxane (PDMS) [10,11]. This allowed single-cell detachment without affecting cell viability. However, this approach was limited to a very low cell density with lack of quantitative characterization: for example, detachment of a single cell readily isolated within a microfluidic chamber. It is unclear whether the method is applicable to high cell densities,
confining the mechanical disturbance only to a target cell without affecting the surrounding cells. Moreover, optical irradiation conditions for laser-induced microbubbles (LIMB) and thus cell detachment have not been quantitatively characterized, although a laser fluence and the number of laser pulses critically depend on cellular dimensions in their body and extension. These are essential to determine, considering the variation in cell density and size in tissues [12,13].

Here, using LIMBs, we selectively detach single cells in a mixed population whose cellular dimension significantly varies from a few thousands up to 30,000 μm² in area. LIMBs are produced from a PDMS-coated gold (Au) substrate. NLP irradiation onto the Au layer leads to instantaneous heating of the PDMS overlayer. This forms microbubbles within PDMS that eject through the top surface of PDMS layer, breaking physical contacts with cells. In this environment, NLP irradiation conditions to detach cells are fully characterized in terms of fluence and number of laser pulses. We compare morphology, proliferation, and viability of cells detached by LIMBs with those of conventional trypsin-treated cells detached without spatial selectivity. Finally, we utilize our method to test drug sensitivity for intact and viable cells with a specific morphological subtype among a mixed population of various subtypes. This is performed by spatially isolating target cancer cells. This clarifies cellular heterogeneity in which drug resistance significantly increases for a cell subtype with larger size.

2. Materials and methods

2.1 Cell culture

The MDA-MB-231 triple-negative breast cancer cell line (231 WT) was purchased from the American Type Culture Collection (Bethesda, MD, USA). Doxorubicin (DOX)-resistant MDA-MB 231 (231 R) cells were obtained by perfusing approximately 50,000 cells (231 WT) with a gradient of DOX (1.5 μM) in RPMI-1640 culture medium (HyClone Laboratories, Logan, UT, USA) supplemented with 10% (v/v) fetal bovine serum (HyClone Laboratories), 100 units/mL of penicillin (Life Technologies, Carlsbad, CA, USA) and 100 μg/mL of streptomycin (Life Technologies) for 10 days in a Cancer Drug Resistance Accelerator (CDRA) chip (Fig. 1) [14]. 231 WT and R cells were maintained in the supplemented RPMI-1640 at 37 °C in an atmosphere of 5% CO₂ and 95% relative humidity in the absence of DOX.

![Schematic diagram of the CDRA chip](image1.png)

Fig. 1. A Cancer Drug Resistance Accelerator (CDRA) chip. (A) Schematic diagram of the chip and its dimensions. Filled arrow indicates the flow of 1.5 μM DOX and nutrients, whereas empty arrow represents the flow of nutrients only. (B) An optical image of the concentration gradient with red and blue ink. (C) The actual image of the CDRA chip.
2.2 PDMS-coated Au substrate for cell detachment

The substrate consisted of two layers. The 20-nm-thick bottom Au/Cr layer was deposited on a glass substrate (Korea Advanced Nano Fab Center, Suwon, Korea). Here, the Cr was used as an adhesion promotion layer between Au and PDMS substrate. The top layer of PDMS (Sylgard 184; Dow Corning Co., Auburn, MI, USA) was spin-coated and thermally cured over the bottom layer. The PDMS layer was comprised of PDMS prepolymer and curing agent in a 10:1 (w:w) ratio mixed with hexane (Sigma-Aldrich, St. Louis, MO, USA) in a 1:1 (w:w) ratio [10]. The PDMS-hexane mixture was spin-coated onto the Au/Cr-coated glass slide for 30 s at 6,000 rpm to obtain a 5-μm-thick layer. It was then cured at 80 °C for 2 h.

The PDMS film was naturally porous and included many nanoscale void spaces. The optical absorption of Au/Cr film was ~30%. This agrees with values reported elsewhere [15]. There are various ways to increase optical absorption by preparing thicker absorbing films or using nanostructured composite films with PDMS [10,16]. However, such optical absorption of ~30% in this work was high enough to produce LIMB and thus enable cell detachment.

2.3 Fabrication of cell chamber

A chamber for cell culture was made from a thick PDMS block 15 mm (length) × 15 mm (width) × 10 mm (height) and a single cell loading hole (8-mm diameter) by soft lithography [17]. The polymerized PDMS layer was peeled and cut from the dish and cut into squares that were treated with O2 plasma for 30 s at 90 W. Finally, the chamber was bound with the bottom glass slide coated with PDMS/Au/Cr.

2.4 Cell loading

![Diagram](a) Fig. 2. (A) The pulsed laser setup to generate microbubbles and detach cells. The configuration for laser irradiation was combined with an inverted optical microscope which visualizes an image from the bottom side. (B) A schematic for LIMB generation and cell detachment. Pulsed laser irradiation onto the Au film leads to instantaneous heating of the PDMS overlayer in contact. This merges nanoscale voids readily embedded within PDMS into a single microbubble. Then, the microbubble ejects through the PDMS layer to the overlaying culture medium, thus breaking the cell contact formed physically with the PDMS substrate. In the schematic, the pulsed laser beam is given (green arrow) onto cells initially forming physical adhesion with the PDMS substrate (top left). A LIMB is produced at the location near the right edge of the right cell (top right). Then, the cell loses its contact due to the LIMB ejection. The cell shrinks changing its morphology (bottom right). Due to the subsequent laser irradiation, the cell loses its contact formed at other locations. In this manner, entire cell-substrate contacts can be removed, resulting in cell detachment.

For sanitization, the Au-PDMS substrate was irradiated with ultraviolet light for 10 min. The cell chamber was rinsed with phosphate-buffered saline (PBS) (pH 7.4) through the hole. The surface was then filled with PBS containing 10 μg/mL fibronectin (Sigma-Aldrich) at 37 °C for 1 h to promote cell adhesion to the PDMS surface. The surface was rinsed three times with PBS to remove unbound fibronectin molecules. The coated cell chamber was first filled
with 200 μL RPMI-1640 through the hole and then gently loaded with $2 \times 10^3$ cells through the cell inlet using a pipette. A cover glass (18 mm × 18 mm × 0.16 mm; Paul Marienfeld GmbH & Co.KG, Lauda-Königshofen, Germany) was placed on the cell chamber to block the hole.

2.5 Pulsed laser irradiation setup and cell detachment mechanism

A pulsed laser irradiation setup was combined with a model AE31 inverted microscope (MOTIC, Kowloon, Hong Kong) equipped with a 10 × plan achromat phase objective lens and a 10 × ocular lens (Fig. 2(A)). For NLP irradiation, we used an Nd:YAG laser beam (Litron Lasers, Rugby, England) with a 532-nm wavelength and a 7-ns pulse width. The NLPs were delivered into the PDMS-coated Au film in a 10-Hz repetition rate (i.e., 100-ms pulse-to-pulse interval) or a single pulse with no repetition. The laser beam was focused using a plano-convex lens with a 10-mm diameter and 80-mm focal length to generate microbubbles and shear force photomechanically from the PDMS-coated Au film [17–19]. Here, the PDMS has nanoscale porosity readily within the 5-μm thick film. The PDMS film can be instantaneously heated by the Au film under pulsed laser irradiation. The nanoscale voids within the PDMS can merge into a single microbubble (i.e. LIMB) that ejects through the PDMS layer to the overlaying culture medium. The LIMB ejection breaks physical contacts of cells formed with the PDMS substrate (Fig. 2(B)). In a repeated manner, LIMBs can be generated to detach cells entirely from the substrate.

After cell detachment process, we removed the cover glass and retrieved the detached cells into 96-well plates. The cells were incubated for 24 h and their morphology was examined under the inverted microscope to quantify cell viability. Viability was compared to that of cells detached using 0.05% trypsin-EDTA (Gibco, Gaithersburg, MD, USA).

2.6 Drug resistance experiment

231 WT and 231 R cells were seeded in 96 wells in the absence of DOX or at DOX concentrations of up to 1 μM. After 72 h of incubation, 10 μL of EZ-Cytox (Daewoong Service, Seoul, Korea) reagents containing water-soluble tetrazolium salts (WSTs) was added to each well and incubated for 1 h. The absorbance of the WST-formazan produced by mitochondrial dehydrogenase was measured at 450 nm using a microplate reader (Molecular Devices, San Jose, CA, USA). The cell viability was calculated by dividing the number of viable cells at each DOX concentration by the number of cells cultured without DOX.

2.7 Viability and morphology of cells detached by laser-induced micro-bubbles and trypsin

After laser detachment, the cells were retrieved from the fluidic chamber into the wells of 96-well plates (Eppendorf, Hamburg, Germany). Detached cells were first cultured in 96 for 24 h at the determined cell culture condition and then stained with trypan blue solution (Sigma-Aldrich). Live and dead cells were counted using a haemocytometer. Cell morphology was evaluated by measuring the cell aspect ratio using Image J (NIH, Bethesda, MD, USA).

2.8 Optical and fluorescence imaging after cell detachment

After detaching normal-sized cells (231 RN) from the 231 R cells attached to the PDMS surface, the remaining large cells (231 RL) on the surface were stained with rhodamine-phalloidin (Cytoskeleton Inc., Denver, CO, USA) and 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Waltham, MA, USA) to visualize the actin filaments (F-actin) and nuclei. Briefly, the cells were first rinsed with wash buffer then fixed with 2% formaldehyde and 0.2% glutaraldehyde at room temperature (RT) for 10 min and washed again. They were treated with permeabilization buffer for 5 min at RT. They were incubated with rhodamine-phalloidin (100 nM) and DAPI (100 nM) at RT for 30 min. The 231 RL cells were then rinsed three times with wash buffer. The actin filaments and nuclei were visualized by fluorescence
microscopy (DeltaVision Elite; GE Healthcare, Buckinghamshire, UK) at different wavelengths (535 nm excitation and 585 nm emission for rhodamine-phalloidin; 340 nm and 488 nm for DAPI).

2.9 Statistical analysis

All data are expressed as mean ± standard deviation from three or more independent experiments. We conducted Student’s t-test with two-tailed analysis. Differences within p < 0.05 were considered statistically significant.

![Figure 3](image)

Fig. 3. Optimization of NLP conditions for selective and viable detachment of normal-sized cells (MDA-MB-231 WT). (A) Laser spot diameters (n = 3) at different laser fluences (1.3–19.9 mJ/cm²). (B) Effects of laser fluence and hit number (N) on cell death. Each bar represents the average death rate of cells (n = 30) at each detachment condition. Student’s t-test, **p < 0.001, N: the number of laser pulses. The cell death rate was measured by staining detached cells with tryptophan blue. (C) Microbubble generation capable of detaching a target cell (marked by a dotted line) according to the number (N) of laser pulses. The dashed line in red in Fig. 2(C) (N0) shows the target cell before laser irradiation. The cell was then irradiated with a single laser pulse (N1) in the region near the cell body. From this aiming location, laser irradiation was continued into the upper region of the cellular extension using 18 laser pulses (N18). At N23, microbubbles could push the cells to complete detachment, and the cells were then swept away by the flow. The blue arrow denotes a microbubble surrounding the laser spot in white (scale bar = 50 μm). (D) The aspect ratio of detached cells (n = 20) by either trypsin or LIMBs. Cell doubling time and viability of detached cells (n = 30) were determined. Student’s t-test was used for statistical analysis; ND = not determined.
3. Results and discussion

3.1 Laser spot diameter at different optical fluences

We determined the optimal laser fluence for single cell detachment by measuring irradiated spot diameters (D) at different fluences (F). The laser spot diameter was increased from 10 μm (F = 1.3 mJ/cm²) up to 83 μm (F = 19.9 mJ/cm²) (Fig. 3(A)). This suggests that the spot diameter could be accurately controlled to the micron scale.

3.2 Cell viability at different optical fluences

The death rate of the detached MDA-MB-231 cells was proportional to the fluence. At F = 19.9 mJ/cm², cells were detached with a single laser pulse (pulse number N = 1). However, they did not survive (Fig. 3(B)). The cell death at high fluences was reported elsewhere [20]. Large bubbles generated by such high fluence can damage cells [21].

For increasing the viability of detached cells, we irradiated the PDMS surface with multiple laser pulses at lower fluences. At F = 1.3 mJ/cm², the death rate was greatly decreased to less than 20%, but approximately 150 pulses (N150) were required to detach the cells. Thus, we tuned the laser fluence to 2 mJ/cm² to detach the cells by using relatively fewer laser pulses (N25), simultaneously achieving a high cell viability of ~85%. Multiple pulse irradiations at 2 mJ/cm² enabled microbubbles to be generated with subcellular precision to push out and detach a single cell from the PDMS substrate (Fig. 3(C)).

Then, we evaluated potential harmful effects of the photomechanical method on cells by checking the aspect ratio (the long axis of a cell to its short axis), doubling time, and viability of cells, which are major indicators for cell physiology. These were compared to those of cells detached by the conventional trypsin treatment method. There was no significant difference in the cell aspect ratio, doubling time and viability between cells detached by the trypsin treatment and our photomechanical method (Fig. 3(D)).

It is known that heat stress induces cellular senescence-like cell cycle arrest [22]. Senescence-associated-galactosidase (SA-β-gal) activity is a ubiquitous cellular senescence marker [23]. Cells detached by LIMBs and trypsin were not stained for β-Gal [24], indicating that the cells remained in a healthy state (Figs. 4(A) and 4(B)). This supports that heat stress induced by the photomechanical method can be possibly minimized by the 5-μm-thick PDMS film working as a heat-blocking region between the cultured cells and the light-absorbing layer. We note that PDMS is utilized in multi-functional purposes, serving nanoscale seed bubbles to form a merged microbubble to push out cells, blocking heat transition, acting as a biocompatible substrate for cell culture, and even being a part of the microfluidic building block.

According to the above quantitative investigation, here, we chose the spot diameter of approximately 20 μm at F = 2 mJ/cm² as the optimal condition capable of targeting a cell edge sharply at the single cell level, similar to that of the previous report [25].
Fig. 5. Isolation of very large cells (231 RL) using the photomechanical method. (A) Accumulation of DOX in 231 WT (A) and 231 R (B) cells. Cells were treated with DOX (0.5 μM) for 72 h. DOX intrinsically fluoresced red. 231 R cells consisted of normal-sized cells (231 RN, denoted by blue arrow) and large cells (231 RL, denoted by green arrow). (C) Cell area distribution of 231 R cells (n = 135). The insets are the merged images of optical and fluorescent images (blue: DAPI). (D) Schematic of the subcellular detachment of 231 RL cells from the PDMS layer using multiple NLPs. (E) Number of laser pulses at 2 mJ/cm² for the detachment of cells (n = 23) with different sizes.
3.3 Size variation of drug-resistant cancer cells

DOX-resistant cancer cells (231 R) were induced from 231 WT by the CDRA chip. DOX is known to be fluorescent [26], which are widely available for monitoring of drug uptake. DOX uptake was visualized at 72 h to determine intracellular localization. DOX was taken up into 231 WT in the nucleus (Fig. 5(A)), while 231 R cells did not absorb detectable amounts of DOX (Fig. 5(B)). This suggests that 231 R cells were more resistant to DOX than 231 WT because of their drug uptake. Very large cells were often found in 231 R cells. Many types of cancers have complex karyotypes [27,28], and thus their cell sizes vary. The spatial isolation of these cancer cells is challenging. The area distribution of 231 R cells (n = 135) displayed a relatively broad range from approximately 2,000 μm² to 35,000 μm², with a mean of 4,378 μm² (Fig. 5(C)), compared to that of 231 WT cells (n = 135), which ranged from 1,176 μm² to approximately 7,000 μm² with a mean of 3,007 μm² (Fig. 6). Staining with DAPI revealed multiple nuclei or a single large nucleus in very large (19,642–33,742 μm²) 231 R cells (231 RL, Fig. 5(C)), in contrast to normal-sized cells (231 RN). These aberrant phenotypes are characteristics of polyploidy giant cancer cells (PGCCs) [29]. PGCCs acquire an early embryonic-like program to generate reprogrammed cancer cells for drug resistance and metastasis [30].

![Fig. 6. The cell area distribution of wild type MDA-MB-231 cells (n = 135 and average area = 3,007 μm²).](image)

3.4 Isolation of 231 RL cells from 231 R cells

In order to detach 231 RL cells, we first used a laser spot with a subcellular scale (Fig. 5(D)) in a similar way to detach 231 WT cells. The number of laser pulses for cell detachment was presumably dependent on cell size. This was confirmed by the detachment of cells with different sizes for a fixed laser fluence of F = 2.0 mJ/cm² (Fig. 5(E)). As the cell area increased, the number of laser pulses increased, reflecting the progressively larger area of cell attachment to the surface. A single 231 RN cell with 2,690 μm² required 52 laser pulses, whereas a single 231 RL cell with 28,420 μm² required 410 laser pulses. Interestingly, some large cells (>30,000 μm²) were not detached from the surface, even with more than 400 pulses at F = 2.0 mJ/cm².

For a heterogeneous population of cells with 231 RN and 231 RL, we first detached small 231 RN cells leaving the large 231 RL cell alone using F = 12.1 mJ/cm² and then detached the isolated 231 RL cells by trypsin treatment, as depicted in Fig. 7(A). After the laser detachment, the 231 RL cells remaining on the substrate were stained with rhodamine-phalloidin and DAPI to visualize their actin and nuclei, respectively. The stained images (Fig. 7(B)) showed that the 231 RN cells were completely removed, leaving the 231 RL cells
intact. This result suggests that the detachment of normal-sized cells is more efficient than the subcellular detachment of large cells when large cells need to be isolated.

3.5 Heterogeneity in DOX sensitivity in 231 R cells

Using our photomechanical method, single cells with a specific subtype can be spatially isolated for biochemical analysis. In order to determine whether 231 RL cells are a major contributor to drug resistance, we isolated 231 RL and 231 RN cells separately and then investigated their respective drug resistance. 231 RL cells were isolated by whole cell detachment of the 231 RN cells at $F = 12.1 \text{ mJ/cm}^2$ (Fig. 7(A)). 231 RN cells were isolated by subcellular detachment using $F = 2 \text{ mJ/cm}^2$ as confirmed in Fig. 2(B). The 231 RL cells displayed the highest resistance to DOX with the half maximal inhibitory concentration (IC50) of 0.2 μM, which was approximately two-times higher than the resistance of 231 R (IC50 of 0.1 μM) (Fig. 7(C)). In contrast, the 231 RN cells displayed significantly lower resistance to DOX (IC50 of 0.06 μM) compared to 231 R cells. The results indicated that the

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**Fig. 7.** Isolation of very large cells (231 RL) using the photomechanical method. (A) Accumulation of DOX in 231 WT (A) and 231 R (B) cells. Cells were treated with DOX (0.5 μM) for 72 h. DOX intrinsically fluoresced red. 231 R cells consisted of normal-sized cells (231 RN, denoted by blue arrow) and large cells (231 RL, denoted by green arrow). (C) Cell area distribution of 231 R cells ($n = 135$). The insets are the merged images of optical and fluorescent images (blue: DAPI). (D) Schematic of the subcellular detachment of 231 RL cells from the PDMS layer using multiple NLPs. (E) Number of laser pulses at 2 mJ/cm² for the detachment of cells ($n = 23$) with different sizes.
231 RL cells contributed more to the drug resistance in 231 R cells than did the 231 RN cells. The collective results suggest that cell separation based on cell size is a prerequisite for the analysis of heterogeneity in drug-resistant cancer cells.

### 3.6 Comparison of LIMB with other techniques

Conventional methods for single cell isolation require pre-selection using either fluorescent markers or surface antigens, whereas our method can isolate adherent cells in situ without the pre-selection step. These differences and features of various techniques are summarized in Table 1.

| Features                   | FACS [31,32] | Laser microdissection [33,34] | Raman activated cell ejection [35,36] | LIMB |
|----------------------------|--------------|-------------------------------|-------------------------------------|------|
| Efficiency                 | Low          | High                          | High                                | High |
| Damage to the cell         | Often impairing | Often impairing              | Gentle                              | Gentle |
| Target recognition         | Detect relative size, granularity and fluorescence | Visually identified              | Using Raman spectra (Needs detection) | Visually identified |
| Throughput                 | High (5000 cells/s) | Low                           | Low (1 cell/s)                      | Low (1 cell/s) |
| External labelling         | Fluorescent  | Staining                      | Label-free                          | Label-free |
| Sample condition           | Suspension   | Sectioned tissues             | Suspension, attach                  | Attach |
| Minimal sample volume      | High         | High                          | Low to High                         | Low |

### 4. Concluding remarks

We optimized a photomechanical detachment method to selectively isolate single cells from monolayer cultured cells on an Au-PDMS coated substrate. Due to the use of heat-blocking PDMS layer and a low laser fluence of 2 mJ/cm², our laser detachment method did not affect cell viability and proliferation. Cells with different sizes could be freely isolated by adjusting the fluence of laser irradiation. Visual microscopy indicated the success of the method in yielding pure populations of targeted cells from a wide range of neighboring cells. Conventional methods for single cell isolation require pre-selection using either fluorescent markers or surface antigens, whereas our method can isolate adherent cells in situ without the pre-selection step. Furthermore, our results enable to obtain targeted rare cells that are in close proximity to untargeted cells through microscopic inspection, and also enable the isolation of targeted cells from a heterogeneous population of cells.

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Disclosures
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