Traceable clonal culture and chemodrug assay of heterogeneous prostate carcinoma PC3 cells in microfluidic single cell array chips

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Cancer heterogeneity has received considerable attention for its role in tumor initiation and progression, and its implication for diagnostics and therapeutics in the clinic. To facilitate a cellular heterogeneity study in a low cost and highly efficient manner, we present a microfluidic platform that allows traceable clonal culture and characterization. The platform captures single cells into a microwell array and cultures them for clonal expansion, subsequently allowing on-chip characterization of clonal phenotype and response against drug treatments. Using a heterogeneous prostate cancer model, the PC3 cell line, we verified our prototype, identifying three different sub-phenotypes and correlating their clonal drug responsiveness to cell phenotype. © 2014 AIP Publishing LLC.

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I. INTRODUCTION

Cancer heterogeneity results in a high degree of diversity, within and between tumors, in cell morphology, genotype, and immunophenotype.^{1–3} Even within purified cancer cell lines, recent studies have shown that cells are not identical but heterogeneous in metabolic, proliferative and differentiation potentials, and response to drug treatment.^{4–12} For example, PC3 prostate carcinoma cell line gives rise to a mixture of three clonal phenotypes: holoclones, meroclones, and paraclones.^{9–11} The cellular differences are often identifiable through changes in progeny, differentiation potential, subtle but repeatable differences in proliferation and morphology, or physiological response to various treatments.

More recently, fueling clinical research on cancer heterogeneity, tumorigenic cancer stem-like cells have been identified in a number of cancers and are proposed to renew, differentiate, and persist in tumors as a small distinct sub-population that causes relapse and metastasis. Their identification, characterization, and effective treatment have severe implications on disease stratification, therapy selection, and prevention of relapse.^{9–15} Existing treatment efficacy is often measured by bulk tumor shrinkage, but does not necessarily select for cancer-causing stem cells. Thorough characterization and target treatment against cancer stem-like cells could potentially revolutionize current treatment paradigms.

Conventional cell assays, however, generally lack the capacity for precise clonal characterization. Most assays, such as those performed in Petri-dishes and multi-well plates, give an averaged representation of multiple subtypes, muting the contributions of small yet significant

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populations in a heterogeneous mixture. Single cell approaches, on the other hand, offer a level of discrete observation that is unavailable with traditional averaging methods. Previously, the serial dilution method, which relies on multiple dilution steps into smaller volumes and dispensing of the cells in conventional platforms, has been used to facilitate single cell analysis. Nevertheless, such approaches are labor-intensive with limited single cell loading efficiency and reproducibility. In the last decade, microfluidic chips have received significant attention for enabling single cell assays with small sample volumes in a well-controlled microenvironment.^{16–26} To date, several microfluidic devices have been reported that they allow positioning of single cells using active dielectrophoresis,^{17,22} droplet-based microfluidic devices,^{20,21} microwell arrays,^{16,25,26} or passive hydrodynamic weir structures.^{18,19,24} However, these devices mainly focused on single cell capture and lack the capacity for traceable clonal culture, which is necessary for proliferative and phenotypic characterization. More recently, Rowat *et al.* demonstrated a microfluidic chip capable of tracking lineages of single cells.²⁴ While their line-shaped micro-chamber is appropriate for non-adherent cell expansion, it poses spatial constraints that may restrict growth and introduce phenotypic changes in adherent cells. For adherent culture, Lecault *et al.* presented a breakthrough single cell device capable of tracking hematopoietic stem cell division.²⁵ Yet, the non-directed cell capture method required large sample volumes due to its poor capture rate and efficiency.

Here, we present a microfluidic platform that allows traceable clonal culture of adherent cells and subsequent cellular characterization. The device captures single cells into individual microwells in an array. Each microwell is geometrically designed to promote clonal proliferation; selective surface treatment prevents cell migration out of the well to ensure clonal uniformity within the microwell, thereby enabling traceable lineage expansion and subsequent on-chip characterization of clonal phenotype and response against drug treatments. For easy handling, the device is designed to operate in a stand-alone manner by employing a hydrodynamic cell-trapping method that utilizes a difference of fluidic resistances and passive flow driven by gravity. These implementations allow efficient single cell capture (>80% injected cells) into individual microwells, generate gentle flow ($\sim 2 \mu\text{l/h}$) suitable for long term culture, and obviate the need for external energy source/pump for cell positioning and culturing. To investigate the potential of the system for evaluating heterogeneous cell populations in cancer, we captured and cultured single PC3 prostate cancer cells in microwells, identified three different sub-phenotypes in the cancer model, performed chemotherapeutic treatment, and correlated their clonal drug responsiveness to cell phenotype.

II. RESULTS

A. Design of a clonal culture chip for heterogeneity study

An 8×8 array for single-cell clonal culture was designed to study cellular heterogeneity over multiple cell divisions. The chip captures single cells into separate microwells and cultures them into their respective clones. A migration blocking structure embedded around each microwell prevents cross-contamination and allows progeny tracking over days. Each microwell incorporates an integrated hydrodynamic guiding structure to capture cells automatically and with high efficiency. Flow is driven by gravitational potential energy to provide gentle cell flow and a continuous supply of cell culture media. These implementations eliminate the need for any additional actuating or pumping equipment for cell capture and culture.

Figure 1(a) shows the schematic view for a unit microwell of the array chip, illustrating the hydrodynamic guiding structure including a capture site and the migration blocking structure. The hydrodynamic guiding structure can capture a single cell efficiently using a difference of fluidic resistance.^{27–29} In the chamber, as shown in Figure 1(a), the flow stream bifurcates: path A through the center and path B along the sides (path B consists of two symmetric channels) around a single cell capture site in a microwell. Because path A has a considerably shorter length than path B, path A experiences a smaller flow resistance than path B. This difference in flow resistances causes most of the flow (including cells) to follow path A rather than path B.

FIG. 1. (a) Schematics of the unit microwell: an integrated hydrodynamic guiding structure (using flow resistance difference between path A and path B) enhances capturing efficiency and migration blocking structure confines cells into a microwell. Capturing site has a different height (h_b) from other regions (h_a) to capture only single cells. (b) Fabrication procedure; microfluidic parts are replicated from SU8 patterns and hydrophobic region for migration blocking is generated by the SU8 pattern. Then, the prepared PDMS part is bonded to the substrate permanently. (c) Migration blocking structure coating procedure; (1) hydrophobic surface area is defined by SU8 on a hydrophilic glass surface, (2) Pluronic copolymer is introduced and selectively coated only onto the hydrophobic surface, then (3) collagen is coated.

Path A includes the cell capture site, whose height or width is smaller than a cell diameter. Thus, only a single cell will be sterically captured when it attempts to pass through. Once a cell is captured, path A becomes blocked and flow resistance increases significantly, compared to path B. As a result, remaining cells will follow through path B and be captured in subsequent microwells. This passive hydrodynamic structure improves capturing efficiency, which increases with the flow resistance difference. For optimal dimensions, the height and width of path A were limited to capture a single cell (shown in magnified view in Figure 1(a)) and other dimensions were chosen to increase the resistance difference while maintaining unobstructed cell flow and minimizing microwell pitch (width, length, and height as $10\text{ }\mu\text{m}$, $10\text{ }\mu\text{m}$, and $20\text{ }\mu\text{m}$ for path A and $20\text{ }\mu\text{m}$, $800\text{ }\mu\text{m}$, and $40\text{ }\mu\text{m}$ for path B, respectively). In addition, each microwell was designed to have enough room ($\sim 2\text{ }\mu\text{l}$) for multiple days of cell growth (width, length, and height are designed as $\sim 250\text{ }\mu\text{m}$, $\sim 300\text{ }\mu\text{m}$, and $40\text{ }\mu\text{m}$).

To maintain a homogeneous clone inside each microwell, a migration-blocking pattern was embedded around the microwells; these patterns restrict cell movement in the microwells during proliferation; without the blocking pattern, cells can migrate to adjacent microwells (Figure S1 in the supplementary material).³⁰ There are several reported techniques for blocking cellular migration, namely, physical trenches/walls^{31–33} and chemical surface modification.^{34–37} We tested these methods using human prostate cancer model PC3 cells and found that the surface modification to be the most effective (Figure S2 in the supplementary material).³⁰ We selectively deposited a layer of Pluronic copolymer (F108), a coating material with known anti-fouling properties to repel cells and other proteins,^{38–40} by patterning a hydrophobic photoresist, SU8, on a hydrophilic glass substrate (Figure 1(a)). The hydrophobicity of the SU8 pattern allows selective coating of the F108 as it selectively associates with the photoresist pattern over the glass substrate. Figure 1(b) shows our fabrication procedure that uses a single PDMS layer and a glass substrate (see Sec. III). Prior to cell loading, we coated the inner surfaces by flowing Pluronic copolymer and collagen successively (Figure 1(c)), which confers antifouling to only the SU8-coated-area.

While a key advantage of microfluidic devices over conventional methods is their small volume requirement, the small amount of stagnant fluid in each microwell can soon become nutrient-depleted and carry a large amount of cellular metabolic waste. Periodical or continuous fluid replacement is required to maintain healthy cell growth. Here, we utilized simple gravitational potential to generate continuous fluid flow ($\sim 2\text{ }\mu\text{l/h}$).⁴¹ We implemented reservoirs at the inlet and outlet, respectively (Figure 2(a)). When the liquid levels in the two reservoirs are

FIG. 2. (a) Fabricated chips (32×32 and 8×8 microarrays) for single cell clonal culture. The 8×8 array was mainly utilized to generate experimental data. (b) Stacked microphotography from 9 images (taken individually from 9 adjacent microwells, 1 h after a cell loading process): green dots represent PC3 single cells. (c) Photograph of cultured PC3 cells for 5 days. Cell migration was effectively blocked by selective coating of F108 Pluronic copolymer on the hydrophobic area.

different, a gravity flow is generated and fluid flows continuously from the higher-level reservoir. This approach eliminates the need for actuating equipment (syringe pumps or peristaltic pumps) and connecting tubes, making the device user-friendly and highly portable for biological analyses.

B. Clonal culture from single cells

To validate the functionality of our fabricated device (Figure 2(a)), we introduced and cultured fluorescent prostate carcinoma PC3 cells ($\sim 17 \mu\text{m}$ in a diameter, over 90% of cells are in the range of $14\text{--}20 \mu\text{m}$).⁴² The cell loading was performed by simply adding a cell suspension to the inlet reservoir. Through the use of gravity flow and hydrodynamic guiding structures, the device could automatically and efficiently load single cells into the microwell array (Figures 2(b) and S3 (supplementary material) $\sim 81\%$ occupancy rate by single cells on average, with a standard deviation of 5.6, $n=6$).³⁰ After loading, the captured cells were cultured in the microwell array over several days, with a continuous supply of culture medium in a 37°C incubator. During culture, the average doubling time (the time it takes for the number of cells to be doubled) in the microfluidic chips was 1.7 days, which is comparable to the result obtained from conventional Petri-dish culture (1.5 days). This indicates that our microfluidic chips provide suitable micro-environment for the cells. After 5 days of continuous culture, we observed that the Pluronic copolymer-coated migration blocking area could effectively inhibit cell migration between microwells (Figure 2(c)).

To examine cellular heterogeneity, we employed the device to identify and track clonal phenotypes of the human prostate carcinoma PC3 cell model. The PC3 model was chosen because the cell line has been recently shown to exhibit three different sub-phenotypes based on cellular morphology and proliferation rate. Li *et al.* and Zhang *et al.* successfully culture clones in 96 well plates using a serial dilution method and identified three different types of

clones by their distinct morphology.^{9,43} Here, we replicate the experiment using our platform to demonstrate its ability to perform heterogeneity studies in a robust, user-friendly, and high throughput manner. First, we captured single cells into the microwell array. After capturing single cells, we emptied the inlet and refilled it with fresh culture media, so as to prohibit the introduction of additional cells from the inlet, as well as to provide nutrients and remove waste continuously.

After 4 days of culture of the captured cells, three different sub-phenotypic clones, namely, holo-, mero-, and paraclones, could be identified from the heterogeneous parental PC3 cell line (Figure 3(a)). In the microwells, cells from each sub-phenotypic group exhibited different morphology and growth rate: cells in the holoclones were relatively small and proliferated fast; cells in the paraclones were large and proliferated much slower; and cells in the meroclones showed medium size and growth rate, matching that of a conventional dilution assay. We counted the cell number per microwell to determine the cell density information. Combined with their morphology, the density information was utilized to identify three distinct cell groups with different proliferation rates (Figures 3(c) and 3(d)). After 4 days of culture, microwells that were occupied by paraclones have ~ 1 –2 cells/well; meroclones ~ 5 cells/well; and holoclones ~ 12 cells/well. Based upon the number of microwells occupied by each sub-phenotypic group, we calculated the population distribution on each sub-phenotypes (Figure 3(b)) and found that our parental PC3 sample consists of $\sim 20\%$ holoclones, $\sim 40\%$ meroclones, and $\sim 40\%$ paraclones.

C. Chemodrug responsiveness of three different types of clones

We next utilized the clonal culture chips to investigate the effectiveness of chemotherapeutic agents on different clones from a heterogeneous cancer. PC3 cells were captured as single cells into the microwell array and cultured to identify subclones. Subsequently, we introduced chemotherapeutic agents to all cells and verified drug effectiveness by calculating percentage of

FIG. 3. (a) Different types of subclone outgrowth from prostate cancer PC3 cell line (first column: holoclones, second column: meroclones, and third column: paraclones, which have a different proliferation rate and morphology). First row is photographs taken 1 h after cell loading (each microwell has one cell in a capture site) and second row is photographs taken after 4 days at identical microwells. (b) Percentage of three different subclones. (c) Cell population distribution (e.g., there are 19 microwells which contain 4 cells). (d) Proliferation rate of 3 subclones for 4 days. ((b)–(d)) Cells are counted from 3 chips.

cell death in each sub-phenotype. The well-defined cell and colony location facilitates simple identification, tracking, and analysis of drug effectiveness. 1 day after injecting 40 nM/ml of Docetaxel, a clinically relevant chemotherapeutic agent for prostate cancer, we observed that the drug killed most of the progenies in holoclones (>80%), indicated by a distinct change of cell rounding and detachment, but was less effective in eradicating paraclones (<30%) and mero-clones (~50%) (Figures 4(a) and 4(b)). Similar results were observed with a different chemotherapeutic agent, Etoposide (Figure 4(c) and Figure S4 in the supplementary material).³⁰ These results can be attributed to both Docetaxel and Etoposide's mode of action during cell division and proliferation, possessing high potency against actively dividing cells. These experiments demonstrate the potential clinical applications of the microfluidic clonal chip for *in vitro* drug screen.

III. MATERIALS AND METHODS

A. Microfabrication of device

The clonal culture device consists of a single PDMS layer and a glass substrate. A mold for the microchannels and microwells was made on a silicon wafer using negative photoresists, SU8-10 and SU8-25 (Microchem). To generate two different heights (20 μm and 40 μm), we performed photolithography twice to produce different thicknesses. The first layer was patterned with SU8-10 (20 μm) to generate the gap in the single cell capture sites and the second layer was done with SU8-25 (40 μm) to define all microchannels and microwells. After fabricating the mold, PDMS (Dow Corning) was cast and cured to replicate it. Subsequently, two reservoirs were generated by punching through the PDMS using a 5 mm diameter biopsy punch (Miltex). On a separate glass wafer, we patterned a 5 μm thick photoresist (SU8-5, Microchem) to generate the hydrophobic surface for Pluronic copolymer deposition. Finally, the PDMS replica was aligned and bonded to the separately prepared glass substrate.

B. Chip and cell preparation process for single cell clonal culture experiments

Prior to cell loading, we prepared the chip to an optimal condition for cell culturing. As the first step, we sterilized the chips by treating with UV light for 5 min in a biobench. Then

FIG. 4. (a) Docetaxel test results: 40 nM of the drug was added on day 5 (dead cells appear rounded on day 6). (b)–(c) The percentage represents the ratio of dead cells to total cells among each clone in one chip after 1 day exposure of Docetaxel. (b) Cell viability of three sub-phenotypes after 1 day Docetaxel exposure (40 nM/ml), which effectively killed holoclones rather than paraclones. Error bar represents standard deviations. (c) Cell viability of three sub-phenotypes after 2 days Etoposide exposure (10 μM /ml) and ((b)–(c)) Cell are counted from 3 chips.

the chips were placed in a desiccator for 30 min to depressurize and remove the air trapped in the PDMS channels. Immediately after desiccation, we added 100 μl of 1 wt. % F108 (in deionized water, Pluronic copolymer for migration blocking) to the inlet and aspirated from the outlet using a pipette bulb to initiate the gravity flow. Due to the depressurization in the device, the solution can easily fill the microchannels without forming air bubbles. After allowing the F108 solution to flow through the device to coat the hydrophobic surfaces of SU8 on a bottom surface and PDMS microchannel walls for 10 min, the device was washed by flowing deionized water for 5 min (removing all the liquid from inlet and outlet, then adding 100 μl of deionized water into the inlet). Then, the bottom glass surface in microchannels was coated with 0.01% collagen to promote cell adhesion to the glass surface by adding 100 μl of a collagen solution into the inlet. Collagen can be selectively coated only on to the glass substrate due to the anti-fouling property of F108 that has already been used to coat the SU8 and PDMS. After coating the collagen for 1 h in a 37 °C incubator, we washed out and replaced it with cell culture media (RPMI + 10% FBS) and placed the chip in 37 °C incubator overnight, which allows the cell culture media to condition the microwells.

We also prepared a PC3 cell suspension to load cells into the prepared chips. At first, we detached cells cultured in a 35 mm Petri-dish using trypsinization. All the culture media from the Petri-dish was aspirated and 2 ml of trypsin was added, then it was incubated in 37 °C for 5 min enabling trypsin to detach the cells. After detachment, we centrifuged the cell suspension to separate cells out of trypsin, and then we replaced the trypsin with cell culture media. Cells aggregated in the centrifugation process were separated into single cells through repeating pipetting. The cell concentration from the suspension was checked by a hemocytometer and was adjusted to around 5×10^4 cells/ml by adjusting the culture media volume.

C. Cell loading

After successive preparation steps, we added 100 μl of PC3 cell suspension at 5×10^4 cells/ml into the inlet of the conditioned chip. Gravity flow from the pressure difference between inlet and outlet makes the cell-suspended-solution flow. While they flow through microchannels, the hydrodynamic guiding patterns in each microwell enable automatic single cell capturing with high capturing efficiency ($\sim 80\%$). After capturing single cells, we emptied the inlet and refilled it with fresh culture media in order to (1) prohibit additional introduction of cells to the device, (2) provide nutrients, and (3) remove wastes. Then the device was placed in a 37 °C incubator to culture the captured cells. Culture media from the inlet generated continuous gentle flow for approximately one day; therefore, we replaced the media in the inlet once each day.

IV. CONCLUSION

In summary, we present a microfluidic platform that allows traceable clonal culture and characterization to facilitate cellular heterogeneity study in a low cost and highly efficient manner. The device captures individual single cells into a microwell array, cultures cells to allow clonal expansion and subsequently allows on-chip characterization of clonal phenotype and response against drug treatments. For easy handling, the prototype is designed to operate stand-alone by employing a hydrodynamic cell-trapping method that utilizes a difference of fluidic resistance and a natural flowing mechanism driven by gravity. These implementations allow efficient single cell capture ($>80\%$ injected cells) into individual microwells, generate gentle flow suitable for long term culture, and obviate the need for an external energy source/pump for cell manipulation. Moreover, to ensure clonal expansion and uniformity, we implemented surface modification patterning to confine cell movement inside each microwell. Using a heterogeneous human prostate cancer model PC3, we verified our prototype by capturing, culturing and characterizing single-cell progenies for differences in proliferation and morphology. We identified three different sub-phenotypes in the cancer model and correlated their clonal drug responsiveness to cell phenotype.

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