High-throughput cell cycle synchronization using inertial forces in spiral microchannels†

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Efficient synchronization and selection of cells at different stages of the cell replication cycle facilitates both fundamental research and development of cell cycle-targeted therapies. Current chemical-based synchronization methods are unfavorable as these can disrupt cell physiology and metabolism. Microfluidic systems developed for physical cell separation offer a potential alternative over conventional cell synchronization approaches. Here we introduce a spiral microfluidic device for cell cycle synchronization, using the combined effects of inertial forces and Dean drag force. By exploiting the relationship between cell diameter and cell cycle (DNA content/ploidy), we have successfully fractionated several asynchronous mammalian cell lines, as well as primary cells comprising bone marrow-derived human mesenchymal stem cells (hMSCs), into enriched subpopulations of G0/G1 (>85%), S, and G2/M phases. This level of cell cycle enrichment is comparable to existing microfluidic systems, but the throughput (~15 × 106 cells per h) and viability (~95%) of cells thus synchronized are significantly greater. Further, this platform provides rapid collection of synchronized cells or of diameter-sorted cells post-separation, to enable diverse applications in the study and manipulation of cell proliferation.

Introduction

Cell cycle synchronization is essential for studying cellular properties, biological processes and elucidating genetic regulatory mechanisms and events involved in each phase prior to cell division. In eukaryotic cells, the distinct events leading to proper cell division can be separated into four sequential phases: G1 (gap), S (DNA synthesis), G2 (gap) and M (mitosis). As a cell progresses through the cell cycle, it duplicates its chromosomes during the S phase and segregates the chromosomes in the M phase. The use of a highly synchronized population of cells has facilitated the development of a variety of biological systems. For example, via such synchronization of cancer cells, several key oncogenes have been identified and implicated in specific cell cycle checkpoints. Development of cancer therapeutics has thus employed tumor cell synchronization, because anticancer drugs are known to target cells in different phases of the cell cycle. In stem cell therapies that involve nuclear transfer to the host cells, cell cycle synchronization is critical because stem cells in the G0/G1 phase impart higher nuclear transfer efficiency. Thus, there is great interest in developing efficient techniques to rapidly synchronize and to isolate cells at various phases of the cell cycle.

Typically, cells are synchronized by inhibiting DNA replication in the S phase, via methods including addition of chemicals such as hydroxyurea, methotrexate or aphidicolin to the culture media. Synchronized cultures of G0/G1 phase can also be obtained by withdrawal of growth factors and specific amino acids termed “serum starvation” or growing the cells at very high density to achieve “contact inhibition” for non-transformed cell lines. Although these techniques have the ability to obtain large numbers of synchronized dividing cells, the metabolism and biochemical balance within the cells are often altered, leading to unbalanced growth of cells and disrupted progression through the cell cycle. In severe cases, the metabolic agent reduces cell viability and promotes apoptosis. Other synchronization techniques also rely on separation of cells at specific cell cycle stages, including fluorescence-activated cell sorting (FACS) and counter-flow centrifugal elutriation (CCE). In FACS, cells with
similar fluorescence signal intensity (i.e. DNA content) or light scattering characteristics (size) can be sorted rapidly yielding a relatively pure population of synchronized cells. However, the viability of the cells is usually compromised with very poor recovery of cells for subsequent assays. In CCE, cells are synchronized by taking advantage of the relationship between a cell volume (size) and its phase in the cell cycle. This allows the proliferating unsynchronized cell population to be synchronized in the different phases. During elutriation, the smaller cell elutes first from the chamber followed by the larger cells. Thus, the early elution is comprised mainly of cells in the G0/G1 phase of the cell cycle while the S and G2/M cells are eluted subsequently. This method yields cell populations with the highest synchrony in the G1 phase and moderate dispersion of synchrony, especially for S and G2/M phases. Unlike chemical and growth limiting methods, centrifugal elutriation does not affect the metabolism of cells, but requires large capital investment, large cell numbers for sufficient signal/noise ratios, and imposes significant mechanical stress on the cells.

Microfabricated cell separation systems offer considerable advantages over conventional cell sorting approaches, including reduced sample volume, reduced sample preparation procedures, higher sample throughput, and high spatial resolution. Microfluidic devices for cell cycle synchronization and separation have been reported recently, using active separation techniques such as dielectrophoresis and acoustophoresis. Although such devices are powerful tools for size-based cell separation, they often involve complex and costly setups, and the use of active energy sources for separation could potentially affect the cell viability. Passive separation techniques using hydrophoresis and hydrodynamic filtration for cell cycle synchronization have also been demonstrated, but the low throughput of these methods has limited adoption by the biological community. Hence there is a clear need to develop a simple, high throughput, and high purity technique for cell cycle synchronization, with minimal perturbation to cell physiology.

In this work, we present a microfluidics based approach to synchronize cells using inertial forces in spiral microchannels. Recently, size based particle separation in microfluidic systems has been developed based on the principles of inertial migration. In spiral shaped microchannels, under the Poiseuille flow condition, particles of varying sizes equilibrate at distinct positions along the microchannel cross-section under the influence of inertial lift and Dean drag forces. Using this principle, we have successfully synchronized several mammalian permanent cell lines, including Chinese Hamster Ovary Cells (CHO-CD36) and cancer cells (HeLa and KKU-100) into populations enriched in G0/G1 (>85%), S and G2/M phase cells. The separation principle exploits the relationship between a cell volume (and thus diameter or, more generically, “size”) and its phase in the cell cycle: to maintain the same average cell size over many such cycles, each cell will approximately double in volume before it divides. We also demonstrate here the first application of this technique to synchronize primary bone marrow-derived human mesenchymal stem cells (hMSCs). Our results indicate that the G0/G1 to G2/M ratio of 2.8 : 1 of the asynchronous sample is enriched to 15.7 : 1. Similarly, a fourfold enrichment in the G2/M population is achieved post-synchronization. These results are comparable with those reported using other microfluidic systems but afford significantly increased throughput (~15 x 10⁶ cells per h) of synchronized cells maintaining high viability (~95%). We believe that the passive operating principle, coupled with the simple microchannel design, will enable diverse applications requiring high-throughput and low-cost synchronization of primary cells.

Materials and methods

Cell culture

Human bone marrow-derived mesenchymal stromal or stem cells (hMSCs; Lonza, Switzerland) were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA) together with 1% penicillin–streptomycin. Chinese hamster ovary cells transfected with human CD36, or CHO-CD36 (ATCC, USA), were cultured in RPMI 1640 medium (Invitrogen, USA) supplemented with 10% FBS together with 1% penicillin–streptomycin. Cervical cancer cells HeLa (CCL-2™, ATCC, USA) were cultured in low-glucose DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. The cholangiocarcinoma cell line, KKU-100, was cultured in Ham’s F-12 medium containing 10% FBS, 3% HEPES buffer and 1% penicillin–streptomycin. All cultures were maintained at 37°C in a humidified atmosphere containing 5% (v/v) CO₂. The hMSCs were seeded at 500 cells per cm² and cultured in sterile 175 cm² flasks (Corning) and dissociated after 48 hours with 0.01% trypsin and 5.3 mM EDTA to prevent contact inhibition. The CHO-CD36, HeLa and KKU-100 cell lines were cultured in sterile 25 cm² flasks (Corning) and subcultivated (1 : 4) three times a week; media was replaced every 48 h. Subconfluent monolayers were dissociated with 0.01% trypsin and 5.3 mM EDTA.

Prior to spiral microfluidics-based experiments, the asynchronous cells were diluted to 100 000 cells per mL in buffer containing 1× phosphate buffered saline (PBS), 2 mM ethylendiaminetetraacetic acid (EDTA) supplemented with 1% bovine serum albumin (BSA) (Miltenyi Biotec, Germany) to prevent agglomeration and adsorption to the microchannel walls. The solution density was adjusted to prevent cell sedimentation by supplementing with 3.5% w/v dextran 40 (Appli-Chem Asia, Singapore).

To initiate G1 arrest by contact inhibition, hMSCs were seeded at 20 000 cells per cm² and cultured in DMEM supplemented with 10% FBS for 48 h. For G1 arrest by serum starvation, hMSCs were seeded at 500 cells per cm² and cultured in DMEM without FBS for 48 h. The G1-arrested cells were dissociated with 0.01% trypsin and 5.3 mM EDTA solution before fixing in 70% ethanol for 30 min.

Microchannel fabrication and characterization

Devices were fabricated in polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, USA) using standard soft-lithographic techniques (Fig. 2a). Briefly, 6 inch-diameter silicon wafers were first patterned and etched using deep reactive ion etching (DRIE) to define the channels on the wafer. Following etching, the patterned silicon wafers were treated with trichloro
(1H,1H,2H,2H-perfluorooctyl) silane (Sigma Aldrich, USA) for 2 h to facilitate PDMS mold release. Following silanization, PDMS prepolymer, mixed at a 10 : 1 (w/w) ratio with a curing agent, was poured on the silicon master and cured at 70 °C for 2.5 h. The cured PDMS mold was then peeled from the silicon wafer to serve as a master template for subsequent PDMS casting. Next, the PDMS template was silanized for 2 h to aid release of subsequent PDMS molds. Note that surface roughness due to DRIE of the Si master surface and PDMS replication results in channel wall surface roughness on the order of 1 μm—blue and 10 μm—red) (ITS Science & Medical, Singapore) were suspended in equal proportions in 1 mM HCl and 1% BSA at a total concentration of 3.5 (w/v) dextran with 1% BSA at a total concentration of 105 beads per mL. To characterize the spiral microfluidic device, the bead mixtures and cell suspensions were filled in a 60 mL syringe and injected into the microchannel using a syringe pump (NE-1000, New Era Pump Systems Inc., USA) driven at a flowrate of 2.5 mL min \(^{-1}\). Flow was experimentally observed under an inverted epi-fluorescence microscope (Olympus IX81, Olympus Inc., USA) equipped with a 12-bit EMCCD camera (iXonEM + 885, Andor Technology, USA). Following testing, optical microscopic images of the cell populations collected from each outlet were acquired within a hemocytometer, and the cell diameter calculated from the photographs using Metamorph® software (Molecular Devices, USA).

**Cell cycle analysis**

Flow cytometry analysis was conducted on the sorted samples to analyze the cellular DNA content via propidium iodide (PI).\(^{22}\) The sorted, synchronized cell populations were rinsed in 1 × PBS and fixed in 70% ethanol for 30 min at 4°C. Cells were then centrifuged at 600 g for 5 min and incubated for 30 min in the staining solution containing 1 × PBS, 3.8 mM sodium citrate (Sigma Aldrich, USA), 10 μg mL \(^{-1}\) RNase (i-DNA Biotechnology, Singapore) and 50 μg mL \(^{-1}\) propidium iodide (Sigma Aldrich, USA). The stained cells were then tested for their phase in the cell cycle. The device achieves cell cycle synchronization by exploiting the relationship between cell volume (and thus the general size or specific diameter) and phase within the cell cycle. We have size fractionated human mesenchymal stem cells (hMSCs) into synchronized populations of G0/G1, S and G2/M phase cells.

**Results and discussion**

**Design principle**

Fig. 1 shows a schematic illustration of the spiral microfluidic separators. Size-based cell separations using inertial forces in microfluidic systems have gained interest in recent times due to the high separation resolution combined with high throughput (in cells per hour). Continuous size-based separation using the combined effect of inertial lift forces and the Dean drag force, asynchronous cell populations are size fractionated to obtain relatively pure populations of cells in the G0/G1, S and G2/M phase. The cells in the G2/M phase, due to the larger diameter at this cell cycle stage, equilibrate closest to the microchannel inner wall followed by cells in the S and the G0/G1 phase. Inset: photograph of the spiral microchannel with one inlet and eight outlets fabricated in PDMS. (B) Validation of design principle using fluorescently labeled polystyrene particles. Superimposed images illustrating distribution and position of the 10 μm, 15 μm, and 25 μm diameter particles at the inlet, a 500 μm wide channel section prior to the outlet, and the bifurcated outlet of a 140 μm high microchannel at 2.5 mL min \(^{-1}\) flowrate. The randomly distributed particles at the inlet form ordered focused streams which are then collected separately at outlets 1 (largest), 2 and 3 (smallest).
Cells to equilibrate under the influence of the lift forces. Microchannel lengths were calculated to allow all the particles/ratio for the different cell types. The spiral diameter and the ratio for the different cell types. The spiral diameter and the fixed at 500 μm, 20 μm, 10 μm-diameter fluoro- susceptible to size-fractionated lines were estimated using flow cytometric analysis of DNA fluorescence intensity via propidium iodide (see Methods section). As noted, cells in the G2/M phase typically exhibit twice the DNA fluorescence intensity as compared to cells in the G0/G1 phase. The percentage of cells in each phase was calculated, and doublet and aggregate cells were eliminated via comparison of fluorescent area and width plots. Fig. 2 presents histograms indicating the distribution of the DNA content of the sorted (singlet) cells in the G0/G1, S and G2/M phase after synchronization for the HeLa, KKU-100 and CHO-CD36 cell lines. The CHO-CD36 cells show a larger size distribution (19.1 ± 5.3 μm) as the cells pass through their cell cycle compared to the cancer cells. As the device separates cells based on their size, the CHO-CD36 cells are thus fractionated (synchronized) more efficiently in the G2/M phase than the cancer cell lines. Following separation, high synchrony of cells was achieved in cells collected from outlet 4 with 84% of HeLa, 96% of KKU-100 and 86% of CHO-CD36 cells synchronized in the G0/G1 phase. Concurrently, an enrichment of two- to threefold in G2/M phase was achieved in cells collected from outlet 1 (Table 1). These results are comparable with those reported using other microfluidic systems.4–17 However, the high flow throughput of the present approach can fractionate ~15 × 10^6 cells per h, which is at least fivefold higher than that reported previously for other microfluidics-based methods. The passive sorting principle also ensures >90% cell viability after size-based sorting and synchronization of cell subpopulations. A summary of the various microfluidic cell cycle synchronization systems is presented in Table 2.

Synchronization of human mesenchymal stem cells (hMSCs)

We next tested the ability of our device to synchronize primary cells, and specifically to synchronize human bone marrow derived mesenchymal stromal or stem cells (hMSCs).14 These
hMSCs differ from cancer cell lines or engineered cell lines in two important ways. First, the asynchronous cell population termed hMSCs is actually an enriched population of putative stem cells; the standard methods of purification of these rare cells from human bone marrow result in a broader distribution of cell diameter, morphology, and functionality than that is typical of a cell line or highly purified primary tissue cell.\(^{35,36}\) Second, the hMSCs are highly susceptible to contact inhibition, such that expansion of hMSCs at low or high seeding density can alter the cell cycle and potentially the emergence of dominant subpopulations.\(^{37}\) Our analysis of cellular DNA content for hMSCs seeded at densities of 1500 cm\(^{-2}\) and 3000 cm\(^{-2}\) indicates substantially fewer cells in the S and G2/M phases after two days in culture (data not shown) as compared to fractions obtained for hMSCs seeded at a lower initial density. Thus, to enrich the S and G2/M populations, cells were seeded at a lower density of 500 cm\(^{-2}\) and cultured for two days before sorting. Fig. 3 shows optical micrographs and viability results of the sorted hMSCs collected from outlets 1 through 4 using a 200 μm high channel. Following sorting, 3.8%, 31.2%, 44.9% and 20.1% of the initial asynchronous cell population were collected from outlets 1 through 4 respectively. The hMSCs collected from outlet 1 exhibited a mean cell diameter of 23.5 ± 5.6 μm and were significantly larger than those collected from outlet 4 (approximately 15.5 ± 2.1 μm). Following separation, cell viability was assessed \(vi\) trypa blue exclusion assay and through long-term

### Table 1  Distribution of the sorted cells in the various cell cycle phase post synchronization

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Phase</th>
<th>Distribution%</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>HeLa</td>
<td>G0/G1</td>
<td>70.0</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>G2/M</td>
<td>19.0</td>
</tr>
<tr>
<td>KKU-100</td>
<td>G0/G1</td>
<td>74.2</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td>G2/M</td>
<td>10.4</td>
</tr>
<tr>
<td>CHO-CD36</td>
<td>G0/G1</td>
<td>55.0</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td>G2/M</td>
<td>29.0</td>
</tr>
<tr>
<td>hMSCs</td>
<td>G0/G1</td>
<td>56.4</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>16.6</td>
</tr>
<tr>
<td></td>
<td>G2/M</td>
<td>27.0</td>
</tr>
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The viability of the sorted cells was similar to that of the unsorted (control) hMSCs, with more than 90% of the cells collected from each outlets excluding the dye; this suggests that hMSCs were sorted without incurring gross physical damage (Fig. 3b and S2, ESI†). After 14 days of culture, the morphology of the sorted hMSCs was similar to that of the unsorted (control) cells, further demonstrating the maintenance of cell viability post-sorting (Fig. 3c).

In the unsorted, asynchronous (control) hMSC culture, 56.4% of the cells were in G0/G1, 16.6% in S and 27.0% in the G2/M phase, as shown by the DNA histogram (Fig. 4). Post-synchronization, the cell population collected from outlet 1 comprised 70.4% of cells in the S and G2/M phases, while 86.2% of cells from outlet 4 were synchronized to the G0/G1 phase (Table 1). These results indicate that the G0/G1 to G2/M ratio of 3 : 1 for the asynchronous hMSC population is enriched to 16 : 1 for the hMSC subpopulation collected at outlet 4. Similarly, a fourfold enrichment in the G2/M population is achieved in the hMSC subpopulation collected at outlet 1. Although the cell cycle profiles indicate significant overlap between the outlets, the average cell size (diameter) collected from the four outlets shows significant difference ($p < 0.05$). The overlap between adjacent outlets is attributed to the inherent heterogeneity in the size distribution between the various phases of the cell cycle.

To confirm that we were indeed synchronizing hMSCs in the G0/G1 phase, we compared the synchrony of the hMSCs subpopulations of smallest average diameter (from outlet 4) with the hMSCs arrested in the G0/G1 phase by means of serum starvation and contact inhibition (see Fig. S3, ESI†). We determined that 86.2% of the hMSCs collected from outlet 4 of our device were synchronized in the G0/G1 phase, which exceeded that achieved via contact inhibition (76.4%) and by 48 h serum starvation (77.5%); see Table S1, ESI†. The corresponding diameter distributions of the hMSCs collected from outlet 4 (15.5 ± 2.1 μm) narrowed as compared to serum starved (16.9 ± 4.2 μm) and contact inhibited (23.3 ± 3.8 μm) hMSCs. We note that contact inhibition produced cells with similar amount of DNA, but the cell size of the arrested population was as heterogeneous as in the unsorted asynchronous hMSCs population (21.9 ± 3.5 μm). While the main criterion for successful synchronization is that the DNA content in the synchronized cell subpopulation should be similar, the size distribution of the cells should also be relatively uniform as compared to the initial population distribution. The wide variation in cell diameter of the contact-inhibited cells suggests that hMSCs were merely growth arrested with a similar amount of DNA, but that other cellular processes leading to protein and mass synthesis were not really synchronized. Conversely, withdrawal of serum from culture synchronized the hMSCs with G1 phase amount of DNA and arrested mass synthesis, but the cell diameter distribution was still relatively large as compared to G0/G1-enriched subpopulations synchronized by our device. We also note that the shape of serum starved hMSCs was more irregular and prone to membrane blebbing, suggesting that the normal physiology of the hMSCs was disrupted under serum starvation-induced stress.

We next investigated whether the hMSCs synchronized in this device would undergo synchronized divisions. The underlying assumption is that synchronized cells do not merely have similar diameters and DNA content, but that the cells are capable of

<table>
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<th>Table 2</th>
<th>Comparison of microfluidic separation techniques reported for cell cycle synchronization</th>
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<tr>
<td>Reference</td>
<td>Method</td>
</tr>
<tr>
<td>Kim et al. [14]</td>
<td>Dielectrophoresis</td>
</tr>
<tr>
<td>Thevoz et al. [15]</td>
<td>Acoustophoresis</td>
</tr>
<tr>
<td>Choi et al. [16]</td>
<td>Hydrophoresis</td>
</tr>
<tr>
<td>Migita et al. [17]</td>
<td>Hydrodynamic filtration</td>
</tr>
<tr>
<td>This work</td>
<td>Inertial Lift forces and Dean drag</td>
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$^a$ Estimated from data presented in the paper.
passing through the cell cycle as a relatively uniform cohort. To test this hypothesis, the hMSCs collected from outlet 4 with 82% of G0/G1 synchrony were replated and DNA content was analyzed at 24, 48 and 72 h (Fig. 5). Interestingly, after 24 h of culture, the percentage of cells in the S and G2/M was 79.8%, indicating that most of the G0/G1 cells progressed through the subsequent phases (Table 3). Typically, mammalian cells reside in the G1/S phases for 16–24 h and only about 2–3 h in the G2/M phases. It is therefore expected that the majority of the cells were found in the S and G2/M phases 24 h after culture. However, the synchrony of the cells decayed over time as a result of stochastic variation in interdivision times. The population of G0/G1 hMSCs increased to 69.4% after 74 h of culture, due at least in part to contact inhibition of cell growth. Many chemical methods or "batch treatments" (e.g., aphidicolin, roscovitine and colchicine) have been used to arrest cell cultures at a specific phase of the cell cycle. However, those chemical methods typically disrupt subsequent progression of the normal cell cycle progression. For example, Whitfield et al. employed thymidine-nocodazole block to arrest HeLa cells in the G2 phase. However, 12 hours after release from the arrest procedure, cells from all phases of the cell cycle were present rather than cells from only one or two phases. In contrast, our results show that hMSCs synchronized by our device exhibit relatively synchronized divisions over at least the next 24 h cycle.

Finally, we note that the cell cycle of hMSCs was correlated recently with a small and highly proliferative subpopulation of
Table 3 Distribution of the cell cycle phase of the replated human mesenchymal stromal or stem cells collected from outlet 4 of the spiral microchannel device, indicated at varying timepoints post synchroniza-

Conclusions
In this work, we demonstrate high-throughput and high-resolution separation of cell populations as a function of cell diameter via the application of a spiral microfluidic device. This approach employs the combined effect of inertial forces and Dean drag force to fractionate mammalian cells into different stages of the cell cycle on the basis of corresponding cell diameter. Distinct advantages of this platform over other microfluidic separation methods include continuous operation enabling significantly higher sample throughput (~15 × 10⁶ cells per h) and thus reduced sample processing time. The passive operating principle of flow-based physical sorting of cells eliminates the need to integrate external force fields for functionality or inhibitory chemicals, and thereby preserves the integrity and viability of sorted cells (>90%). As mammalian cell suspensions can be separated and synchronized directly, direct modifications of the cells via molecular labeling steps is not required, further reducing processing time, cost, and potential modification of cell functionality post-sorting. This is the first demonstration of microfluidics enabling high-throughput cell cycle synchronization, while maintaining significantly higher viability for primary cells such as human mesenchymal stem cells. We believe that the simple, high-throughput and minimally disruptive nature of our device, which can be employed to fractionate cell subpopulations as a function of diameter and to synchronize the cell cycle, could find diverse applications in both basic and applied research.

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References