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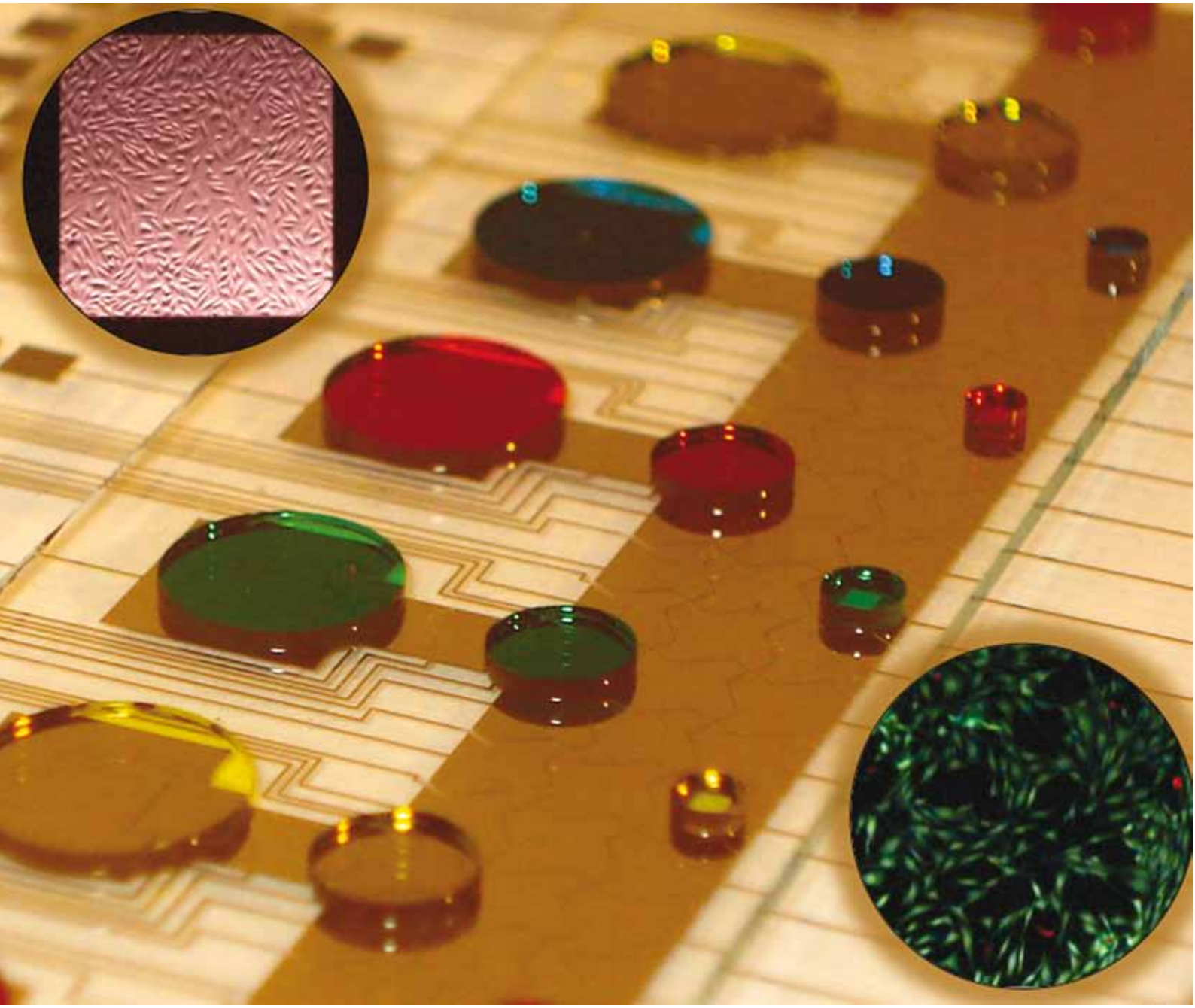

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A microfluidic platform for complete mammalian cell culture†

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We introduce the first lab-on-a-chip platform for complete mammalian cell culture. The new method is powered by digital microfluidics (DMF), a technique in which nanolitre-sized droplets are manipulated on an open surface of an array of electrodes. This is the first application of DMF to adherent cell culture and analysis, and more importantly, represents the first microfluidic platform capable of implementing all of the steps required for mammalian cell culture—cell seeding, growth, detachment, and re-seeding on a fresh surface. Three key innovations were required to implement complete cell culture on a microfluidic device: (1) a technique for growing cells on patterned islands (or "adhesion pads") positioned on an array of DMF actuation electrodes; (2) a method for rapidly and efficiently exchanging media and other reagents on cells grown on adhesion pads; and (3) a system capable of detachment and collection of cells from an (old) origin site and delivery to a (new) destination site for subculture. The new technique was applied to cells from several different lines which were seeded and repeatedly subcultured for weeks at a time in 150 nL droplets. Cells handled in this manner exhibited growth characteristics and morphology comparable to those cultured in standard tissue culture vessels. To illustrate an application for this system, a microfluidic method was developed to implement transient transfection—we propose that the combination of this technique with multigenerational culture allows for "on-demand" generation of transiently transfected cells. Broadly, we anticipate that the automated cell microculture technique presented here will be useful in myriad applications that would benefit from automated mammalian cell culture.

Introduction

In the past decade, applications involving mammalian cells have been extremely popular in the microfluidics and lab-on-a-chip communities.[1–4] For example, microfluidic methods have been developed for cell sorting,[5–9] single cell analysis,[10–14] and cell-based screening assays,[15–20] capitalizing on the advantages of reduced reagent consumption and the favorable scaling of physical phenomena at micron length-dimensions. But despite this intense interest (and thousands of publications), there are no reports of a direct microfluidic analogue to the widely practiced technique of *in vitro* mammalian cell subculture (as observed by Kim *et al.*[21]). In the microfluidic literature, the methods reported have consistently used mammalian cells that were grown and analyzed during a single subculture (*i.e.*, passage), after which the devices and cells were discarded (or, the cells were analyzed outside of the device). While microfluidic systems enable continuous media exchange and hence improved culture conditions, they have no capacity for cell passaging—the experiment is terminated when cells reach confluency.

The closest microfluidic analogue to conventional mammalian cell culture that we are aware of was reported by Lee and coworkers,[22] who developed methods to periodically strip cells from a culture chamber in a microchannel by exposure to trypsin. This is a useful and interesting technique but is not equivalent to conventional *in vitro* culture—in the microfluidic technique, cells are grown, culled, and grown again on the same site, a method likely limited to only a few generations because of build-up of surface contaminants and/or susceptibility to infection. In contrast, conventional (macro-scale) cell culture relies on repeated re-plating of cells in fresh, sterile vessels.[23] We speculate that the conventional format of microfluidics is not an ideal match for complete cell culture, because all of the reagents and cells are positioned in an interconnected network of enclosed microchannels, making it difficult to establish fresh, sterile sites for seeding new generations of cells.

Recently, digital microfluidics (DMF) has emerged as an alternative to the conventional format of enclosed microchannels.[24,25] As depicted in Fig. 1, digital microfluidic devices are formed from an array of electrodes which are used to manipulate discrete fluidic droplets. We recently reported the first application of DMF to cell-based analysis, generating dose–response toxicity data for cells suspended in 150 nL droplets.[26] Under these conditions, cell vitality was shown to be unaffected by digital microfluidic manipulation. Other DMF/cell studies have followed,[27–29] but we are not aware of any reports of the application of DMF to adherent cells (*i.e.*, cells grown on surfaces).

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† Electronic supplementary information (ESI) available: Experimental details on device fabrication and operation, including Supplementary figure 1S, which depicts passive dispensing. See DOI: 10.1039/c002147d

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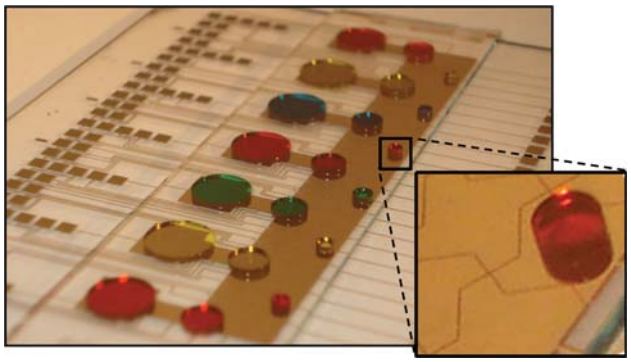

Fig. 1 Photo of a digital microfluidic platform developed for complete cell culture. The device comprised seven reservoirs (left), an array of actuation electrodes (2.22 mm × 2.22 mm) used to manipulate 1 μL droplets (center), and a series of specially designated sites for cell culture in 150 nL droplets (right and inset).

Here, we report the first application of digital microfluidics to adherent cell studies. The system is powered by several key innovations including a heterogeneous device format with dedicated cell culture sites, a passive reagent dispensing mechanism for seeding and feeding cells, and a new scheme for collection and delivery of cells in nanolitre droplets. This work represents the first true microfluidic analogue to macro-scale mammalian cell culture—cells are seeded, grown, and passaged onto fresh culture sites *ad nauseam*. We anticipate that the microfluidic technique presented here will be useful in myriad applications that would benefit from automated, miniaturized mammalian cell culture.

Results and discussion

Cell seeding and growth

Adherent cells are typically cultured on substrates bearing a hydrophilic charged surface, which is required for cell attachment and spreading. For example, commercial cell culture flasks are formed from polystyrene which is treated with plasma oxidation to generate a charged surface. In some cases, this characteristic is enhanced by coating surfaces with extracellular matrix (ECM) proteins like fibronectin or collagen. Surfaces formed in this manner are more biomimetic than those formed by plasma oxidation and such techniques are required for certain cell types including some primary cells,[30] and for the maintenance of (feeder layer-free) undifferentiated embryonic stem cells.[31] In the current work, we used ECM protein coatings to make digital microfluidic devices amenable to adherent cell growth.

Droplet actuation by digital microfluidics requires devices with hydrophobic surfaces. To make DMF devices compatible with both cell growth and droplet actuation, we developed a new strategy in which each device is globally coated with Teflon-AF (which features a contact angle for water of ~115°), and is then locally modified with islands of ECM proteins, forming adhesion pads (Fig. 2a). The adhesion pads were formed by dispensing droplets containing fibronectin and allowing them to dry to the surface. For the purposes of imaging, devices were designed with transparent windows in the electrode array, such that cells growing on adhesion pads could be monitored by microscopy. This had the added advantage of isolating the cells from long-term exposure to electrical fields. Fig. 2b shows HeLa cells on an adhesion pad on a device immediately after seeding, and after 24 h of incubation in a 150 nL droplet of media. As shown, the cells shifted from round to spread morphology, as is observed for cells grown in multiwell plates (Fig. 2c). Similar effects were observed for many different cell lines, including CHO-K1, NIH-3T3 and INS-1 (not shown). During cell growth, evaporation was controlled by storing devices in humidified chambers in a tissue culture incubator.

Media exchange

Cell seeding and growth is the first of many requirements for a complete cell culture platform; the next requirement is a method to remove old cell growth media and replace it with

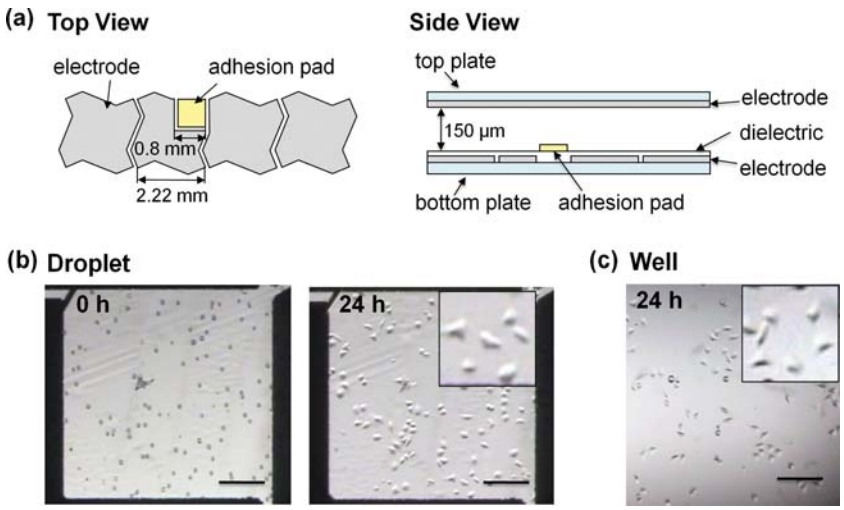

Fig. 2 Digital microfluidic device with adhesion pad for culturing adherent cells. (a) Top- and side-view schematics of a DMF device with an adhesion pad. (b) Pictures of HeLa cells on an adhesion pad immediately after seeding (left) and after 24 h (right). In the pictures, the top plate is not observed because it is transparent. (c) Picture of control HeLa cells after 24 h incubation in a well coated with fibronectin. The cells in droplets showed similar morphology and spreading characteristics to the cells grown in wells. Scale bars are 200 μm and 80 μm for the main panels and insets, respectively.

fresh media. In macro-scale tissue culture, this is accomplished by aspiration and pipetting, and care must be taken to dispense the new media quickly to avoid drying out the cells. Here, we introduce a new DMF-driven method for solution replacement for cell culture, which we have termed "passive dispensing." In this method, solution removal and replacement are simultaneous processes; hence, cells are continuously hydrated, with no concern for cell drying or death between removal and replacement of media.

Passive dispensing is depicted in Fig. 3a. A large source droplet of new media is driven across an adhesion pad bearing a smaller droplet of old media. As the large droplet crosses the adhesion pad, the old media are swept away, leaving a portion of the new media behind. We call this process passive dispensing because the act of splitting the small droplet (which remains on the pad) from the source droplet (which moves away from the pad) is driven by the difference in hydrophilicities of the surface rather than by actuation of electrodes. As depicted in Fig. 1S in the ESI†, this method works both for delivery of an initial droplet to a dry contact pad as well as for exchanging fluid on a pad that already bears a droplet. In the work reported here, passive dispensing was used to generate ~150 nL droplets on the adhesion pads, while active dispensing (*i.e.*, charging adjacent electrodes to dispense a droplet from a reservoir by necking and splitting[32]) was used to generate the 1 μL source droplets actuated on the array of electrodes. We note that passive dispensing is similar to what was developed independently by Yang *et al.*[33] for non-biological applications.

In developing this technique, we hypothesized that the solution transfer efficiency of passive dispensing (*i.e.*, the degree of exchange of new media for old) would be high, because the Reynold's number (Re) in such systems is low (Re < 100 in the device reported here). To test this hypothesis, we evaluated the persistence of fluorescence in droplets containing fluorescein that were replaced with droplets containing phosphate buffered saline (PBS). Fig. 3b shows the results of these experiments, which verify that the solution transfer efficiency is high. After a single passive exchange, less than 3% of the initial fluorescence persists in the droplet on the adhesion pad. And after three passive exchanges, the dye is completely removed. Thus, in the work described here, three passive exchanges were used to deliver new media and other reagents to cells growing on adhesion pads.

Passive dispensing was used to facilitate all aspects of cell culture and growth, including the initial seeding of cells from suspension, and delivery of fresh media to cells growing on adhesion pads. Several different types of cells were cultured in nanolitre droplets for multiple days with media exchange every 24 h (Fig. 3c) with proliferation rates comparable to those observed in macro-scale culture.

Cell subculture

In addition to cell seeding and media exchange, a complete cell culture system must be able to facilitate cell harvesting and passaging (*i.e.* subculture), which comprises separation of a portion of cells from a source population and seeding on a fresh surface. In conventional cell culture, this process is tedious and time-consuming, involving multiple aspiration, dispensing, and centrifugation steps. As described in the introduction, despite intense interest in the lab-on-a-chip community for applications involving cells,[1–4] no methods have been reported that are analogous to conventional mammalian cell passaging.

Here, we introduce the first microfluidic platform for mammalian cell subculture; the four steps in this process are outlined in Fig. 4. Cells were first grown in 150 nL droplets on primary (1°) adhesion pads (Fig. 4a) with regular media exchange *via* passive dispensing. In the second half of the exponential growth phase, cells were detached by passively

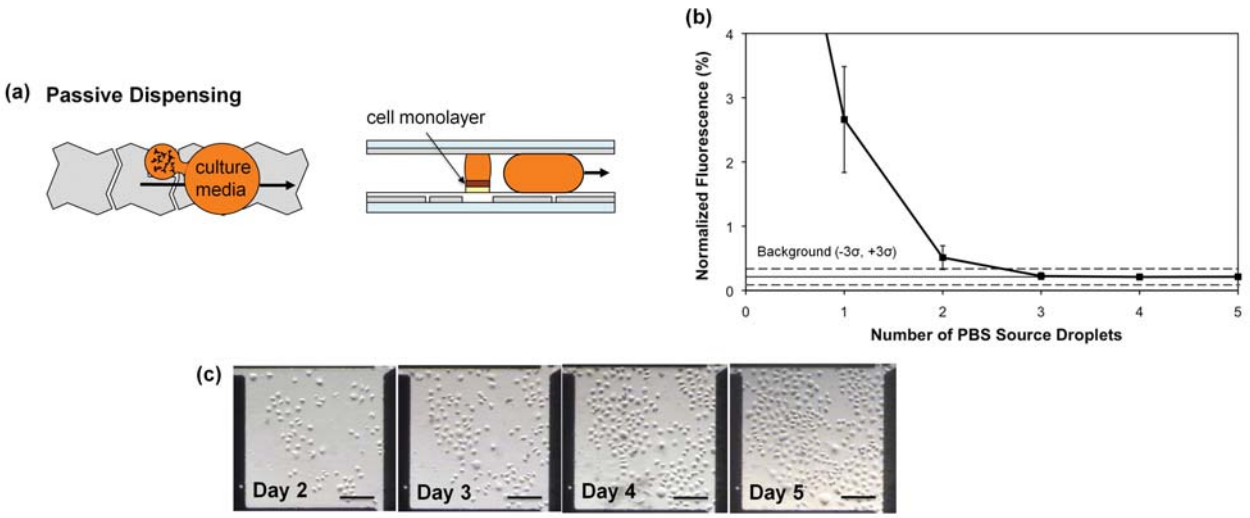

Fig. 3 Passive dispensing for long-term cell culture. (a) Schematics depicting passive media exchange. A new source droplet sweeps the old media away from the culture site while simultaneously delivering fresh media to the cells. (b) Passive dispensing efficiency evaluated by exchanging droplets of 10 nM fluorescein with consecutive droplets of PBS. The fluorescence is reduced to 3% of its initial value after exchange with a single droplet, and is completely extinguished after exchange with three source droplets. (c) Pictures of HeLa cells on a device from day 2 to day 5 of culture, with passive exchange of culture medium every 24 h. Scale bars are 200 μm.

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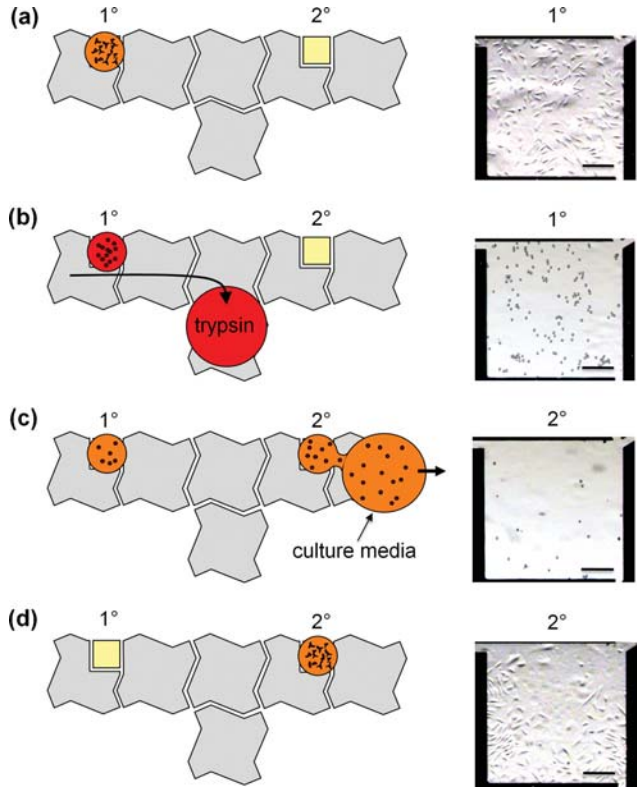

Fig. 4 Schematic and pictures depicting subculture of CHO-K1 cells in droplets by digital microfluidics. (a) Monolayer of the first generation of cells on the primary (1°) adhesion pad. (b) Cells dissociating from the surface of the 1° pad after delivery of trypsin. (c) Trypsinized cells being harvested in a droplet of media containing serum, and then seeded on the secondary (2°) pad. (d) Monolayer of subcultured cells on the 2° pad after 72 h. Scale bars are 200 μm.

exchanging the media with trypsin (Fig. 4b). After cells detached, a fresh droplet of cell culture media was translated over the 1° pad, blocking trypsin and collecting some of the cells. The droplet was translated to the secondary (2°) adhesion pad, where a second passive dispensing step delivered some of the cells in the droplet to the new site (Fig. 4c). In the devices described here, we observed that ~2 to 3% of the 1° cells were delivered to the 2° adhesion pad per droplet; thus, on average four 1 μL droplets were used to collect and deliver ~10% of the 1° cells to the 2° pad. Finally, the subculture of cells was grown on the 2° adhesion pad (Fig. 4d) before they were subcultured again. We applied this technique to several cell lines (CHO-K1, HeLa, and NIH-3T3) for up to 4 subcultures, which suggests that this process is broadly applicable to long-term cell subculture.

In addition to the advantage of being automated, the new microfluidic cell subculture technique is faster and more straightforward than conventional macro-scale methods. These improvements are a result of the efficiency of passive dispensing. For example, the standard cell culture technique of washing cells with PBS before trypsinization can be omitted because passive dispensing efficiently removes the old solution (media) in addition to delivering the new one (trypsin) (see Fig. 3b). Likewise, after exposure to trypsin, separate blocking and washing steps are not needed in the microfluidic technique because as the first droplet of media efficiently replaces the trypsin, most of the cells remain on the surface of the device. This eliminates the requirement for centrifugation.

To evaluate the differences between the new microfluidic method and conventional techniques, two cell lines, CHO-K1 and HeLa, were subcultured on microfluidic devices and in 96 well plates, and their proliferation was monitored for 6–8 days. As shown in Fig. 5, CHO-K1 cell proliferation was nearly identical in the micro- and macro-scale formats but HeLa cell proliferation exhibited differences. In the microfluidic technique, HeLa cells experienced a longer lag phase prior to entering exponential growth, and, once begun, the doubling time in wells (1.28 ± 0.09 days) was shorter than the doubling time in droplets (1.81 ± 0.27 days).

While the data suggest that the proliferation rates in micro- and macro-scale formats are not identical, their similarities are striking, given the many differences in physical parameters. For example, the density of cells per unit area was kept constant in the micro- and macro-scale systems, which led to a volumetric density of cells at seeding in droplets (~133 to 666 cells per μL) ~20 times higher than that in wells (~6 to 32 cells per μL). To compensate for this difference, media in microfluidic devices

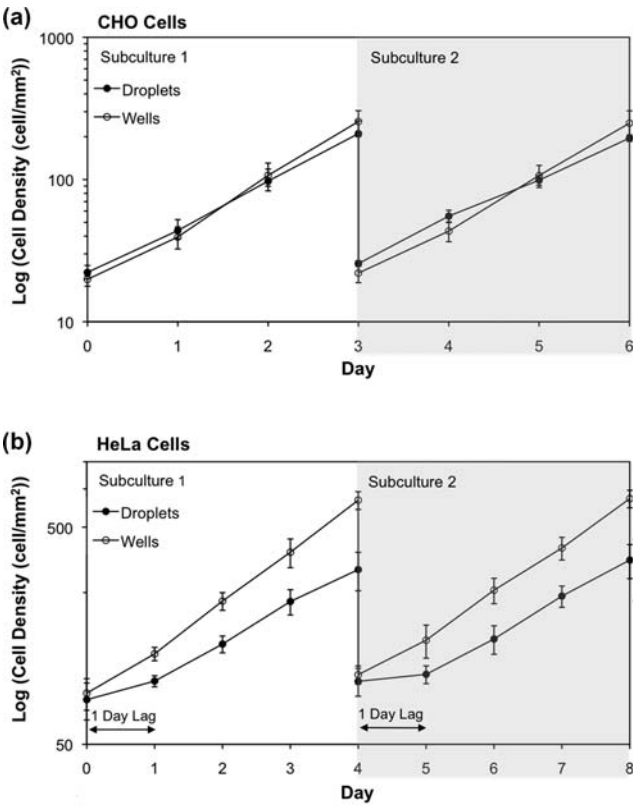

Fig. 5 Growth curves for CHO-K1 and HeLa cells in macro-scale (wells, open circles) and micro-scale (droplets, closed circles) formats. (a) CHO-K1 cells seeded at ~20 cells per mm^2 were cultured for 3 days. Upon reaching a density of 200 cells per mm^2 they were split and cultured in a new well/adhesion pad for another 3 days. (b) HeLa cells seeded at ~100 cells per mm^2 were cultured for 4 days, and then split and cultured for another 4 days. A one-day lag was observed in growth of cells in droplets whereas cells in wells almost immediately entered exponential growth phase. The exponential growth rates in the two systems were similar.

were exchanged more frequently than in wells (every 24 h *vs.* every 3–4 days), but the initial 20-fold difference in nutrients and cell growth factors (as well as differences in accumulated cell excretions) is a likely source of differing growth conditions. In the future, we may design microfluidic devices with volumetric densities closer to that of macro-scale systems; however, we note that this would sacrifice some of the advantages of miniaturization (reduced reagent use, *etc.*). Another potential source of variation between the macro- and micro-scale systems is the integrity of the ECM coating: fibronectin densities and adhesion characteristics may be slightly different on Teflon-AF (microfluidic device) relative to plasma-treated polystyrene (macro-scale). In on-going work, we are evaluating different kinds of local surface treatments for devices, including plasma oxidation and various types of ECM coatings. Regardless, both CHO-K1 and HeLa cells were observed to grow robustly, suggesting that the digital microfluidic environment is suitable for long-term culture and analysis.

On-demand transfection

As an example of an application of the new techniques reported here, we developed means to implement transient transfections in cells grown in the digital microfluidic system. Gene transfer and manipulation of eukaryotic genomes are used widely to develop understanding of gene functions and regulatory sequences, post-translational events, and for the development of gene therapy.[34,35] Most transfections are performed transiently, such that DNA introduced into cells is not passed onto progeny; hence, each time a researcher wants to use such cells, a new batch must be generated. By integrating routine transfections with subculture on an automated digital microfluidic device, we propose that researchers will have access to transfected cells "on-demand."

To illustrate this concept, we performed transient transfection of CHO-K1 cells on microfluidic devices with an expression vector encoding green fluorescent protein (GFP). Prior to transfection, cells were grown and subcultured on the microfluidic platform. Fig. 6a (left) is a picture of CHO-K1 cells 24 h after transfection in a device (in a 150 nL droplet) while Fig. 6a (right) shows CHO-K1 cells similarly transfected in wells (100 μL). As shown in Fig. 6b, the transfection efficiency in the micro-system is comparable to that in wells. While cell transfection in microchannels has been established,[36,37] the work reported here is the first report of transfection by digital microfluidics, and more importantly, represents the first combination of automated multigenerational subculture techniques with transfection (*i.e.*, transfection on-demand) in any microfluidic format.

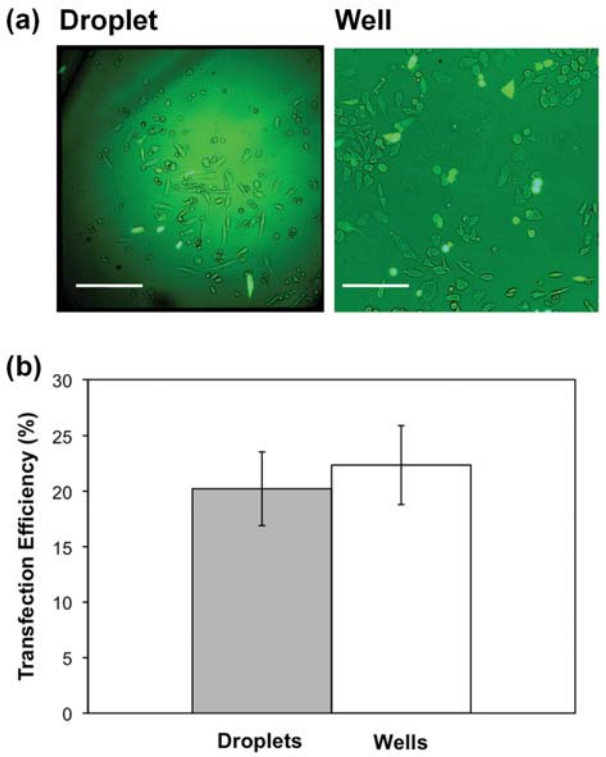

Fig. 6 On-demand transient transfection of CHO-K1 cells in droplets. (a) Pictures of CHO-K1 cells transfected to express GFP in a droplet (left) and in a well (right). Transfected cells appear green as a consequence of GFP emission. (b) Comparison of transfection efficiency in droplets and wells. Approximately 20% of cells were typically transfected in both systems ($n = 3$). Scale bars are 200 μm.

Conclusion

We present the first microfluidic analogue to *in vitro* mammalian cell culture. Development of this system required several key innovations, including techniques for growing cells on adhesion pads on an array of electrodes, a passive system for exchanging media on cells grown on devices, and a method for detachment, collection, and delivery of cells to fresh and sterile sites for subculture. Cells grown using this method exhibit growth characteristics comparable to those found in conventional tissue culture, and are compatible with on-chip genetic transformation. In addition, the new methods developed for media exchange can be used to deliver reagents and staining solutions to cells cultured on DMF devices for cell-based assays. We anticipate that these new methods may be useful for a wide range of applications in cell biology, tissue engineering, -omics, screening and beyond.

Experimental

Reagents and materials

Unless otherwise indicated, general-use reagents were from Sigma-Aldrich (Oakville, ON) and cells and cell culture reagents were from American Type Culture Collection (ATCC, Manassas, VA). Details relating to device fabrication and operation can be found in the ESI†.

Macro-scale cell culture

For macro-scale work, cell lines CHO-K1, NIH-3T3, HeLa, and INS-1 were maintained in cell culture flasks in a humidified incubator (5% CO_2, 37 °C). The growth media included DMEM (NIH-3T3 and HeLa), F-12/DMEM (50/50) (CHO-K1), and RPMI 1640 with 20 μM glucose (INS1). All media formulations were supplemented with 10% fetal bovine serum (Invitrogen Canada, Burlington, ON), and all but F-12/DMEM were

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supplemented with penicillin (100 IU mL^{-1}) and streptomycin (100 μg mL^{-1}). CHO-K1, NIH-3T3, and HeLa were subcultured every 3–4 days at ~5 × 10^5 cells per mm^2, and INS-1 was subcultured every 7 days at the same density. Prior to experiments, cells were harvested and resuspended in fresh media, and cell numbers and viability were quantified using a hemocytometer and trypan blue exclusion (Invitrogen Canada). All reagents, washing solutions and media contained 0.05% Pluronic F68 (w/v).

Microfluidic cell culture

For micro-scale work, CHO-K1, NIH-3T3, HeLa, and INS-1 cells were seeded on adhesion pads in 150 nL droplets of media containing 20–100 cells. For long-term experiments, the media were exchanged by passive dispensing (using three source droplets) every 24 h, and devices were stored in a humidified chamber in an incubator (5% CO_2, 37 °C). For subculture experiments, cells were grown to a high density (~200 to 300 cells) on a primary (1°) adhesion pad. The old media were then replaced with a 150 nL droplet containing trypsin (0.25% vol/vol) and EDTA (1 mM) by passive dispensing (using three source droplets), and cells were allowed to dissociate for 3–5 min. To harvest cells, a droplet of fresh media (1 μL) was translated over the 1° adhesion pad, collecting dissociated cells from the surface. The droplet was then driven to a secondary (2°) pad, where some of the cells were seeded in a 150 nL droplet by passive dispensing. The procedure was repeated four times such that the droplet on the 2° pad contained 20–100 cells. In some cases, the process was repeated for up to four generations of cells (on four adhesion pads). Cell numbers and densities per mm^2 were determined using ImageJ (US National Institutes of Health, http://rsb.info.nih.gov/ij/).

For comparison, some cells were grown and subcultured in 96 well plates (Corning Inc., USA). In these experiments, 50 μL of fibronectin (33 μg mL^{-1} in DI water) was dispensed into each well and then incubated in the tissue culture incubator (1 h). After aspirating and drying, wells were seeded with 20–100 cells per mm^2 in 100 μL of media, and were monitored as described above (densities per mm^2 were measured in three locations per well and then averaged). For subculture experiments, cells were rinsed in PBS, incubated in trypsin/EDTA (3–5 min), rinsed again in cell media, and then split into new fibronectin-coated wells at ~10% of their original density (in 100 μL of media). All solution exchanges in macro-scale experiments were carried out by manual pipetting, and all cell culture experiments in micro- and macro-scale systems were repeated in at least four replicates.

Cell transfection

In transfection experiments, pCAG–GFP plasmid DNA[38] (Addgene, Cambridge, MA) was extracted and purified from *E. coli* using GenElute™ HP Plasmid Miniprep Kits (Sigma-Aldrich, Oakville, ON). A stock solution for transfection was prepared by mixing 0.25 μg DNA and 1 μL Dreamfect cationic lipid (Oz Biosciences, Marseille, France) in 25 μL PBS. That mixture was allowed to complex for 20 minutes and was subsequently diluted 1 : 4 in CHO-K1 culture media for use in experiments (supplemented 0.05% w/v Pluronic F68). CHO-K1 cells were seeded in 150 nL droplets on DMF devices on a 1° adhesion pad, incubated until they reached high density, and then subcultured on a 2° adhesion pad, as described above. After incubating overnight, cell media were exchanged with 150 nL of transfection solution by passive dispensing, such that the cells were exposed to approximately 20 nL lipid reagent and 5 ng DNA. After incubating 3 h, the transfection solution was replaced with fresh media by passive dispensing, and the cells were incubated overnight. For comparison, cells were also seeded in 96 well plates and then transfected using the same reagents according to the manufacturer's protocol for the transfection of CHO-K1 cells.

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