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TECHNICAL NOTE

A digital microfluidic platform for primary cell culture and analysis

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Digital microfluidics (DMF) is a technology that facilitates electrostatic manipulation of discrete nano- and micro-litre droplets across an array of electrodes, which provides the advantages of single sample addressability, automation, and parallelization. There has been considerable interest in recent years in using DMF for cell culture and analysis, but previous studies have used immortalized cell lines. We report here the first digital microfluidic method for primary cell culture and analysis. A new mode of "upside-down" cell culture was implemented by patterning the top plate of a device using a fluorocarbon liftoff technique. This method was useful for culturing three different primary cell types for up to one week, as well as implementing a fixation, permeabilization, and staining procedure for F-actin and nuclei. A multistep assay for monocyte adhesion to endothelial cells (ECs) was performed to evaluate functionality in DMF-cultured primary cells and to demonstrate co-culture using a DMF platform. Monocytes were observed to adhere in significantly greater numbers to ECs exposed to tumor necrosis factor (TNF)-α than those that were not, confirming that ECs cultured in this format maintain *in vivo*-like properties. The ability to manipulate, maintain, and assay primary cells demonstrates a useful application for DMF in studies involving precious samples of cells from small animals or human patients.

Introduction

There are two types of mammalian cells that are commonly used in biomedical research: immortalized cell lines and primary cells. Immortalized cell lines can be grown *in vitro* for many generations, spanning many months-to-years. These cells are straightforward to grow and maintain, but often have phenotypes that differ significantly from those of cells *in vivo*. In contrast, primary cells are used immediately after isolation from animal tissue, and therefore are much closer to *in vivo* phenotype. Unfortunately, primary cells have several limitations for regular use in the laboratory. In long-term studies involving animal models of disease, primary cells are typically available only in limited quantities (*e.g.*, with monthly or yearly isolations). The process of primary cell isolation can be laborious and costly, requiring expensive reagents and hours-to-days of work depending on the cell type. Furthermore, due to their limited number of population doublings, primary cells can only be used for a short period of time in the laboratory. These factors make primary cells an attractive target for miniaturized tools to reduce costs and for automated cell culture and analysis.

Microfluidic channels are the most popular technology used for miniaturization. Primary cell culture in microfluidic channels has been demonstrated repeatedly with applications including cell migration,[1–3] adhesion,[4–6] shear stress,[7–9] cell sorting,[10] and cell-based screening assays.[11] However, microchannel-based systems often require pumps or other external apparatus (with noted exceptions[12]) for applications involving cells. This increases reagent/sample consumption, as such systems require macro-scale tubing and interconnects, which inherently contributes unwanted dead volumes. An additional problem associated with interconnects and other world-to-chip interfaces is the presence of bubbles, which can disturb the local fluid flow within microchannels and can damage cells as a result of the high interfacial energy at the gas–liquid interface. Removing bubbles can be difficult, requiring complex degassing mechanisms or bubble traps.[13]

Digital microfluidics (DMF) is an alternative platform to conventional enclosed microchannels that is capable of manipulating discrete liquid droplets on an array of patterned electrodes.[14] In DMF, droplets can be controlled individually or in parallel to provide precise spatial and temporal control of reagents. Typical volumes for droplets can range from nanolitres to microlitres, and because there is no dead volume, these systems are well suited for minimal reagent/sample consumption. Moreover, because there are no open reservoirs or tubes and interconnects, devices can be readily flipped, allowing for

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convenient use of both sides of each device for imaging. Finally, unlike enclosed microchannels, in non-oil-filled DMF systems, bubble nucleation and growth are non-existent. Previous studies[15–21] have demonstrated that mammalian cells can be cultured and/or analyzed on DMF platforms, but all of the previous work used immortalized cell lines.

Here, we report the first application of DMF to the culture and analysis of primary cells. Three phenotypically different cell types isolated from pig blood vessels (aortic endothelial cells) and heart valves (aortic valve endothelial cells and aortic valve interstitial cells) were cultured and analyzed on a DMF platform. The devices and methods reported here use a new mode of "upside-down" culture in virtual microwells[22] formed by a patterned DMF top plate. Cells were cultured on multiple sites per device for up to one week. With minimal reagent use, primary mammalian cells were fixed, permeabilized and stained on a DMF device. Furthermore, a co-culture system for growing and analyzing endothelial cells and monocytes was developed; this is the first co-culture system that we are aware of in DMF. The co-culture system was used to implement a monocyte adhesion assay, which confirmed that intricate signaling mechanisms were retained by primary cells cultured on this new digital microfluidic platform.

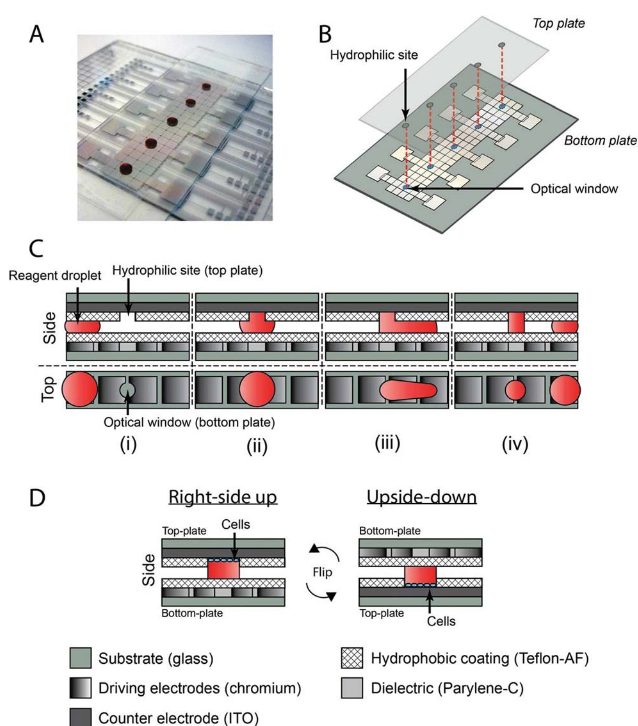

Fig. 1 (A) Photograph of DMF device designed for primary cell culture and analysis. A series of droplets (coloured with red dye for visualization) are positioned at patterned hydrophilic sites on a device. (B) Schematic of device geometry. The top plate is patterned by a liftoff procedure to expose hydrophilic sites. The bottom plate bears an array of individually addressable electrodes with patterned optical windows for imaging. (C) Top and side view schematic of passive dispensing on hydrophilic sites. (i–ii) A droplet is manipulated to the hydrophilic site. By actuation of subsequent electrodes the droplet is (iii) stretched then (iv) passively dispensed, forming a virtual microwell. (D) Side view schematic of device orientation during experimentation. Devices are maintained right-side up during droplet actuation and are positioned upside-down during all incubations.

Methods and materials

Reagents and materials

Unless stated otherwise, materials were purchased from Fisher Scientific Canada (Ottawa, ON, Canada). General-use chemicals were from Sigma Aldrich (Oakville, ON, Canada), fluorescent dyes were from Invitrogen/Life Technologies (Burlington, ON, Canada), and photolithography reagents were from Rohm and Haas (Marlborough, MA). Deionized (DI) water had a resistivity of 18 MΩ·cm at 25 °C.

DMF device fabrication and operation

Digital microfluidic devices were fabricated in the University of Toronto Emerging Communications Technology Institute (ECTI) cleanroom facility, using transparent photomasks printed at 20,000 DPI (Pacific Arts and Designs Inc., Markham, Ontario).

Glass DMF device bottom-plates bearing patterned chromium electrodes were formed by photolithography and etching as described previously.[15] As shown in Fig. 1, the design featured an array of 116 actuation electrodes (2.2 mm × 2.2 mm ea.) connected to 10 reservoir electrodes (4 mm × 4 mm ea.), with inter-electrode gaps of 30–80 μm. The actuation electrodes were roughly square with interdigitated borders (140 μm peak to peak sinusoids). The design also included an array of five 1 mm diameter optical windows (*i.e.*, circular regions free from chromium) with 9 mm between each window. As illustrated in Fig. 1C, each window straddled two actuation electrodes. After patterning the electrodes, the substrates were coated with 7 μm of Parylene-C (Specialty Coating Systems, Indianapolis, IN) and 200 nm of Teflon-AF (DuPont, Wilmington, DE). Parylene-C was applied using a vapor deposition instrument (Specialty Coating Systems), and Teflon-AF was spin-coated (1% wt/wt in Fluorinert FC-40, 3000 rpm, 60 s) followed by post-baking on a hot-plate 165 °C, 10 min). The polymer coatings were removed from contact pads by gentle scraping with a scalpel to facilitate electrical contact for droplet actuation.

DMF device top-plates were formed from indium tin oxide (ITO) coated glass substrates (Delta Technologies Ltd, Stillwater, MN) that were coated with Teflon-AF (200 nm, as above). A lift-off process was used to form an array of 1.5 mm diameter openings of exposed ITO (9 mm between each opening) through the Teflon-AF using methods developed for this purpose.[22] Digital microfluidic devices were assembled with an ITO–glass top plate and a chromium-glass bottom plate. Prior to assembly, the two plates were sterilized by immersing in 70% ethanol (10 min) and then air dried. The hydrophilic sites (exposed ITO) on the top plate were aligned visually to the optical windows on the bottom plate, and the two plates were joined by a spacer formed from four pieces of double-sided tape (total space between plates ~280 μm). Driving potentials (~280 V_{RMS}) were generated by amplifying the sine wave output of a function generator (Agilent Technologies, Santa Clara, CA) operating at 18 kHz and were applied between the top plate (ground) and sequential electrodes on the bottom plate *via* the exposed contact pads. Pluronics F68

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(0.02% wt/vol) was added to all reagents used with digital microfluidics (excluding solutions of Triton X-100) to facilitate droplet movement.[23]

In addition to the standard digital microfluidic operations[24] (*i.e.*, active droplet translation, active droplet dispensing from reservoirs, *etc.*), the devices supported a phenomenon known as passive dispensing.[15] As illustrated in Fig. 1C, in passive dispensing, a source droplet is translated across a hydrophilic site, and surface tension effects result in spontaneous formation of a sub-droplet. In the devices with the dimensions described here, source droplets were 1.4 μL and passively dispensed droplets were 0.5 μL; as reported elsewhere,[22] the volumetric reproducibility for passive dispensing for these dimensions is excellent, with a CV of ~1.2%. As described below, passive dispensing was used for all DMF operations for primary cell culture and analysis.

Primary cell isolation and maintenance

Porcine aortic endothelial cells (PAECs) isolated from pig thoracic aortas were kindly donated from Lowell Langille (University of Toronto).[25] Porcine aortic valve endothelial cells (PAVECs) and porcine aortic valvular interstitial cells (PAVICs) were isolated as described previously.[26,27] PAECs were cultured in M199 (Wisent, St. Bruno, QC, Canada) supplemented with 5% cosmic calf serum (Fisher Scientific Canada), 5% fetal bovine serum (FBS) (Fisher Scientific Canada), and 1% penicillin-streptomycin (*P*-S) (Sigma Aldrich). PAVECs and PAVICs were cultured in M199 and Dulbecco's modified Eagle's medium (DMEM) (Wisent), respectively, each supplemented with 10% FBS and 1% P-S. Cells were cultured in T75 flasks until 80% confluent, then trypsinized, centrifuged, and resuspended at approximately 10^5–10^6 cells mL^{-1} in the appropriate completed culture medium (with M199 or DMEM, as above) to form a cell suspension for use with DMF.

DMF cell culture

Five 5 μL aliquots of cell suspensions were pipetted onto the reservoir electrodes, and then five 1.4 μL droplets (one per reservoir) were actively dispensed by applying potentials to a series of actuation electrodes adjacent to each reservoir. These 1.4 μL cell-containing droplets were driven to the hydrophilic spots patterned on the top plate such that 0.5 μL droplets were generated by passive dispensing (Fig. 1C). The devices were then inverted (with the top plate on the bottom) (Fig. 1D) and were placed in a homemade humidified chamber (a Petri dish containing dampened Kimwipes to prevent evaporation) in an incubator at 37 °C and 5% CO_2 for 12 h. This "incubation state" (*i.e.*, top plate on the bottom in a humidified chamber in a cell culture incubator) was used for all incubation steps described herein. Periodically, devices were removed from the incubator, flipped to orient each device with the ITO top-plate on the top (such that the device was upright) and used for droplet movement. Afterwards, devices were returned to the incubation state. For cell culture, new droplets of media were delivered to cells every 12–16 h until cells were ~70–80% confluent.

DMF staining and microscopy

For imaging without staining, primary cells cultured on DMF were imaged using an inverted CKX41 microscope (Olympus, Markham, ON, Canada) in phase-contrast mode. For comparison, cells were also cultured on tissue culture treated polystyrene (TCPS) flasks and imaged.

For imaging of stained cells, after ~70–80% confluence was reached on DMF devices, primary cells were washed by dispensing at least two 1.4 μL droplets of phosphate buffered saline (PBS) across the virtual microwell sites (displacing the existing droplets with fresh 0.5 μL volumes). Cells were fixed and permeabilized by dispensing and actuating three 1.4 μL droplets across the cells (in series) of (a) 10% (v/v in DI water) neutral buffered formalin (NBF) for 5 min, (b) PBS, and (c) 0.01% (v/v in PBS) Triton X-100 for 5 min. The cells were then washed (two droplets of PBS as above), and 1.4 μL droplets containing FITC-labeled phalloidin (0.1 mg mL^{-1} in PBS) were actively dispensed from reservoirs and actuated across the cell culture site such that 0.5 μL sub-droplets were passively dispensed and then incubated for 45 min at room temperature. The cells were then washed in PBS (as above), and 1.4 μL droplets containing Hoechst (1 μg mL^{-1} in PBS) were driven across the cell culture sites such that 0.5 μL sub-droplets were passively dispensed and then incubated for 5 min at room temperature, and then washed again with PBS (as above). Cells on DMF devices were imaged by flipping them (such that the top plate was on the bottom) using an IX-71 microscope (Olympus) in fluorescence mode.

DMF monocyte adherence assay

THP-1 monocytes (ATCC, Manassas, WA) were cultured in suspension off-chip in RPMI 1640 medium (Invitrogen/Life Technologies) completed with 10% FBS and 1% P-S. Prior to experiments, monocytes were centrifuged, resuspended in media containing Hoechst (0.2 μg ml^{-1} in complete medium), incubated for 30 min, and then centrifuged and resuspended in fresh complete medium at 10^6 cells mL^{-1}.

PAECs grown to confluence on DMF devices were incubated with passively dispensed 0.5 μL droplets containing 0 or 25 ng mL^{-1} tumour necrosis factor alpha (TNF)-α (Invitrogen/Life Technologies) in complete medium for 4 h in the incubation state (see above). Cells were then rinsed by passively dispensing two 0.5 μL droplets of PBS, followed by passive dispensing of one 0.5 μL droplet containing calcien AM (2 μM in PBS containing Ca^{2+} and Mg^{2+}) and storing for 15 min in the incubation state. Cells were then rinsed by passively dispensing two 0.5 μL droplets of PBS, followed by passive dispensing of one 0.5 μL droplet of complete culture medium and incubating for 30 min in the incubation state. 0.5 μL droplets containing Hoechst-labeled monocytes were then delivered to the PAECs by passive dispensing and stored for 10 min in the incubation state. Two droplets of PBS were used to wash the cells (as above), and the cells were then evaluated using an IX-71 microscope for monocyte adhesion. One central image per hydrophilic spot was collected and images were analyzed for monocyte number. Briefly, IMAGEJ software was used to convert images to binary and the "analyze particles" function was used to count the cells.

Results and discussion

Digital microfluidic primary cell culture

We present here the first digital microfluidic platform capable of culturing and analyzing primary cells, shown in Fig. 1. PAECs, PAVECs, and PAVICs were chosen as model cell types because of their importance in cardiovascular biology.[4,26–29] Although these cell types are found in close proximity anatomically, they represent three distinctly different phenotypes.[30] Moreover, PAVECs are an especially interesting target because they are challenging to isolate and culture *in vitro*; under improper culture conditions, they display altered morphologies, function and short-term viability.[26,29,31] We hypothesize that if DMF is useful for culturing, handling, and analyzing these different types of cells (particularly, the sensitive PAVECs), similar methods may be applicable to cells derived from a wide range of tissue types.

PAECs, PAVECs, and PAVICs are adherent cells—that is, they attach, spread, and grow on solid surfaces. There have been three previous reports[15,20,21] of culture of adherent cells on DMF platforms. As listed in Table 1, the new methods reported here share a number of similarities with those reported previously, but also have some differences. The most notable similarity is that each of these systems is capable of supporting a phenomenon known as passive dispensing. Passive dispensing is represented in Fig. 1C; when an aqueous droplet is driven across a hydrophilic site, a smaller droplet, which we call a "virtual microwell,"[22] is spontaneously formed and left behind. Passive dispensing to form virtual microwells is a unique feature of digital microfluidics, and serves as a convenient mechanism to seed, culture, and analyze adherent cells.

The most important difference between the current system and those reported previously[15,20,21] is the new device format and orientation. The methods reported here rely on hydrophilic sites formed on the device top plate, which led us to implement a new method of "upside-down" cell culture in virtual microwells (Fig. 1D). In this scheme, devices are stored for most of the time upside-down (*i.e.*, top plate on the bottom) which allows the cells to adhere, spread, and proliferate. At designated periods, devices are flipped to standard configuration (*i.e.*, ITO plate on the top) for droplet manipulation, but after experiments, the devices are returned to the inverted state. This arrangement is advantageous for a number of reasons. First, it allows for cell growth on hydrophilic sites formed from regions of exposed ITO[22] rather than the adsorbed proteins[15] or peptides[20,21] used previously. In initial experiments with primary cells grown on adsorbed fibronectin on DMF device substrates, we observed that the cells had unexpected morphologies, whereas on ITO surfaces, cells had morphologies that are similar to those grown on conventional TCPS substrates. Second, this device arrangement de-couples the active portion of the digital microfluidic device (*i.e.*, the insulating layer on the bottom plate which allows for the buildup of charge necessary for droplet movement[32]) from the cells. The insulating layers on DMF devices are prone to failure over time because of dielectric breakdown, and the upside-down culture arrangement allows for the possibility of replacing a used/defective bottom plate with a fresh one between experiments (note that this putative feature was not used in any experiments reported here). We propose that this arrangement will be useful for a variety of applications for cell culture and other applications.

Using the methods described here, PAECs, PAVECs, and PAVICs can be reproducibly seeded and grown with high viability. A significant amount of trial-and-error was required for this level of performance,with some of the key points described here. Factors such as cell seeding density and media exchange frequency were critical in maintaining primary cell viability and morphology on device. Seeding densities between 2×10^5–1×10^6 cells mL^{-1} coupled with a media exchange frequency of every 12–16 h maintained viable primary cells with appropriate morphologies. Depending on the assay, the cell seeding densities were altered to vary the duration of culture on device. For example, to demonstrate long-term cell culture, PAECs were cultured for up to 1 week with an initial seeding density of 2×10^5 cells mL^{-1}. For shorter experiments (*e.g.*, those in which microscopy was performed 24 h after staining), primary cells were seeded at 5×10^5–1×10^6 cells mL^{-1}, to achieve the desired level of confluence within 24 h. At densities greater than 2×10^6 cells mL^{-1}, cells displayed rounded morphologies with little spreading, possibly due to overpopulation of the hydrophilic sites and rapid accumulation of cellular waste products. In all experiments, devices were stored in an incubator in humidified chambers with no appreciable evaporation.

Digital microfluidic microscopy, fixation, permeabilization, and staining

As shown in Fig. 2, DMF devices proved to be a useful platform for microscopic imaging of primary cells (in this case, using an

Table 1 Comparison of adherent cell culture using DMF between the new methods reported here and those previously published

	New Methods Reported Here	Methods Reported by Barbulovic-Nad *et al.*[15]	Methods Reported by Lammertyn and Colleagues[20,21]
Type of cells cultured	Primary cells	Immortalized cell lines	Immortalized cell lines
Pattern of hydrophilic sites useful for passive dispensing?	Yes	Yes	Yes
Location of hydrophilic sites	Top plate	Bottom plate	Bottom plate
Hydrophilic site format	Exposed regions of ITO surrounded by Teflon-AF	Spots of adsorbed fibronectin on a Teflon-AF surface	Spots of adsorbed poly-L-lysine on a Teflon AF surface
Device format for droplet movement	Right-side up	Right-side up	Right-side up
Device format for cell culture	Upside down	Right-side up	Right-side up
Maximum duration of cell culture	1 week	2 weeks	3 days
Demonstration of co-culture	Yes	No	No

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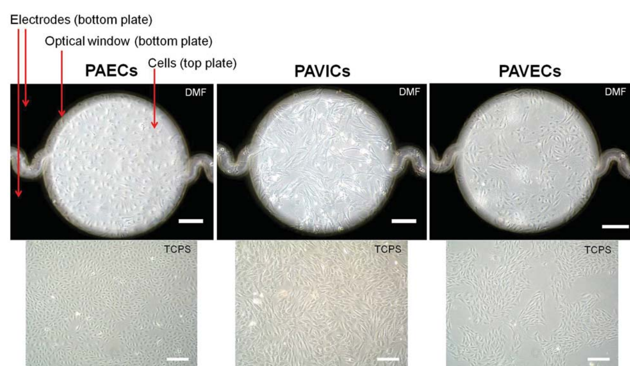

Fig. 2 Phase contrast images of PAECs, PAVICs, and PAVECs cultured on a DMF device (top) and in TCPS flasks (bottom). Scale bar = 200 μm. In the DMF images, the bottom plate is closest to the objective, and the focus is on the layer of cells on the top plate. The cells are viewed through the circular optical window between two electrodes on the bottom plate (which are observable but slightly out of focus).

inverted microscope). For imaging, devices were either positioned with the bottom plate on the bottom (such that the bottom plate was adjacent to the objective) as was the case for the images in Fig. 2, or with top plate on the bottom (such that the top plate was adjacent to the objective). The capacity to use and flip devices to either orientation for imaging is a unique property of digital microfluidic devices, which have no open reservoirs or tubing interconnects that might otherwise interfere. Fig. 2 shows representative phase contrast images of PAECs, PAVECs, and PAVICs grown on DMF devices and for comparison, images of cells grown on conventional TCPS substrates. As shown, the morphologies of cultured primary cells were similar on the two surfaces.

Microscopic imaging of cells is often enhanced by staining with fluorescent dyes, which reveals information about cell state and phenotype. Prior to staining, cells are often fixed to preserve cell state (by exposure to fixatives such as NBF), and permeabilized to allow for deep penetration by dyes and other reagents (by exposure to mild surfactants such as Triton X-100). Before and after these steps and others, the specimen must be repeatedly rinsed as the various reagents can interfere with each other. Here, as demonstrated in Fig. 3, we report the first combination of all of these steps (cell growth, fixation, permeabilization, staining, and rinsing) by DMF. As shown, at 40× magnification, individual actin stress fibers can be observed, demonstrating the compatibility of DMF with high-resolution fluorescent microscopy.

Digital microfluidic monocyte adhesion assay

To evaluate the potential for using digital microfluidic systems for co-culture and multistep assays, we probed their compatibility with endothelial cell/monocyte adhesion experiments. Monocyte adhesion to endothelial cells is an important initiating event in the inflammatory process. Endothelial cells are generally activated prior to adhesion, and this state can be induced by exposure to cytokines such as TNF-α. TNF-α increases monocyte adhesion through upregulation of EC receptors such as E-selectin,[33] intercellular cell adhesion molecule-1 (ICAM-1)[34] and vascular cell adhesion molecule-1 (VCAM-1).[34,35]

PAECs were cultured on DMF devices and then incubated either with or without TNF-α for 4 h. Monocytes pre-labeled with Hoechst were then dispensed from reservoirs and delivered to endothelial cells, which were then rinsed to remove monocytes that did not adhere. As shown in Fig. 4, monocytes had greater adhesion to TNF-α-stimulated PAECs compared to non-stimulated controls, which is consistent with previous studies.[9,36–38] These results demonstrate compatibility of DMF with a fourth cell type (monocytes) and show that primary PAECs cultured using DMF retain *in vivo*-like responses to TNF-α. Moreover, this is the first demonstration of co-culture on a DMF platform. The ability to detect a response with monocytes (*i.e.* adhesion) as a result of endothelial cell activation highlights the potential of DMF to investigate cell-cell interactions.

The assay represented in Fig. 4 required only a 1.4 μL droplet of reagent and cell suspension for each virtual microwell. In comparison, macroscale[39,40] and some microchannel-based adhesion assays[9] require working volumes of tens to hundreds of

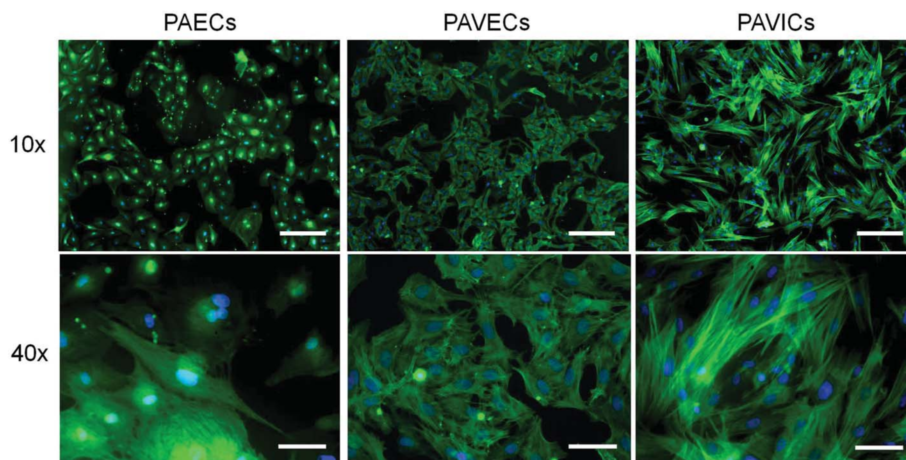

Fig. 3 Fluorescent images of PAECs, PAVECs, and PAVICs after fixing, permeabilizing, and staining on a DMF device. The stains selected for F-actin (FITC-phalloidin, green) and nuclei (Hoechst, blue). Images were taken at both 10× agnification (top row) and 40× magnification (bottom row). Scale bar = 200 μm (top row) and 50 μm (bottom row). In these images, the top plate is closest to the objective.

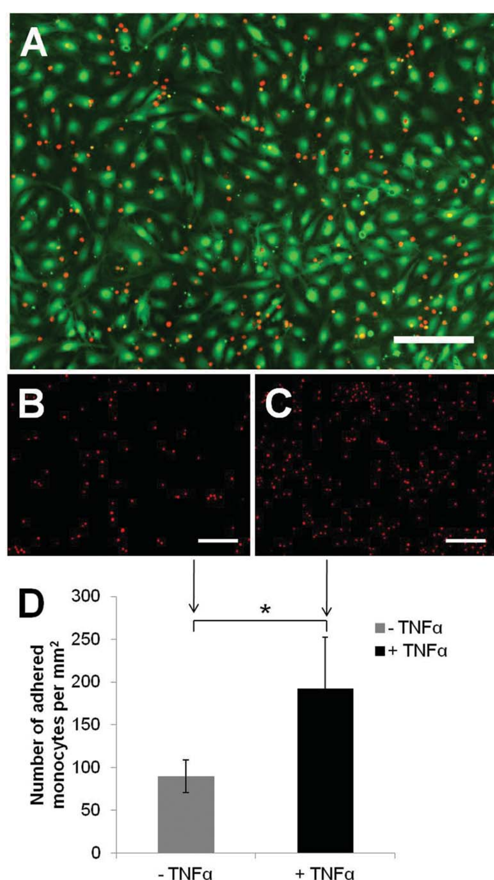

Fig. 4 A monocyte adhesion assay performed on DMF-cultured primary PAECs. (A) Nuclear-stained (Hoechst, red) THP-1 monocytes adhered to PAECs (calcein AM, green). Representative images of nuclear-stained monocytes adhered to (B) non-stimulated and (C) TNF-α-stimulated PAECs. In these images, the top plate is closest to the objective. (D) Monocytes displayed greater adhesion on TNF-α-stimulated PAECs relative to control non-stimulated PAECs. Data presented as mean ± standard deviation. *$P < 0.05$. Scale bar = 200 μm.

microlitres, such that the DMF system facilitates a 10–100-fold reduction in reagents used. The capacity to reduce reagent consumption and increase throughput with DMF is desirable in monocyte adhesion assays or other cases in which precious sample or expensive reagents are used. The potential for combining automated imaging and analysis with DMF in the future is an attractive vision, as such a system would likely be useful for applications ranging from basic biology to drug discovery.

Conclusions

We present the first demonstration of primary cell culture using digital microfluidics. A new mode of "upside-down" culture in virtual microwells was developed to enable primary cell growth with appropriate morphologies and to decouple the cell growth sites from the digital microfluidic driving electrodes. Multi-step cell fixation, permeabilization, and staining processes were demonstrated for the first time on a DMF platform. A monocyte adhesion assay was performed to demonstrate functionality in DMF-cultured primary ECs and to highlight the co-culture capabilities of the device. The combination of DMF and primary cell culture/analysis presented here provides a basis for future studies involving co-culture, high resolution microscopy, and multiplexed experimentation.

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