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Precisely targeted delivery of cells and biomolecules within microchannels using aqueous two-phase systems

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Abstract Laminar and pulsatile flow of aqueous solutions in microfluidic channels can be useful for controlled delivery of cells and molecules. Dispersion effects resulting from diffusion and convective disturbances, however, result in reagent delivery profiles becoming blurred over the length of the channels. This issue is addressed partially by using oil-in-water phase systems. However, there are limitations in terms of the biocompatibility of these systems for adherent cell culture. Here we present a fully biocompatible aqueous two-phase flow system that can be used to pattern cells within simple microfluidic channel designs, as well as to deliver biochemical treatments to cells according to discrete boundaries. We demonstrate that aqueous two-phase systems are capable of precisely delivering cells as laminar patterns, or as islands by way of forced droplet formation. We also demonstrate that these systems can be used to precisely control chemical delivery to preformed monolayers of cells growing within channels. Treatments containing trypsin were localized more reliably using aqueous two-phase delivery than using conventional delivery in aqueous medium.

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1 Introduction

Microfluidic systems have been used extensively for analysis of drug and chemical actions on cells and to reveal mechanisms of cell-cell signaling through soluble factors (Torisawa et al. 2010; Wu et al. 2010; Young and Beebe 2010). Such systems provide a range of capabilities that are not available in conventional petri dish culture including the abilities to precisely manipulate small liquid volumes, localize treatments within cell populations and perform analyses on small numbers of cells (Olofsson et al. 2009; Villa-Diaz et al. 2009; Jovic et al. 2010; Liu et al. 2010). This is usually accomplished through generation of either laminar or pulsatile flow (Gu et al. 2004; Bransky et al. 2008).

Laminar flow is among the most common methods for selective delivery of cells and molecules within microfluidic channels. This method has been used to deliver bacteria, blood cells, and many other mammalian cell types in controlled fashions (Takayama et al. 1999; Regenberg et al. 2004; Lucchetta et al. 2005; Berthier et al. 2011). It has also featured in experiments that probe cell functions through delivery of selective treatments (Sawano et al. 2002). However, for most small molecules and proteins, as well as some larger particles, selective laminar flow delivery is usually limited to several millimeters from the convergence point of the laminar streams. Further downstream, diffusive effects can result in the formation of concentration gradients. It is possible to increase the distance over which selective treatments can be applied by increasing the velocity of flow, but this requires the use of either significantly larger sample volumes or significantly

smaller channel sizes, and may result in undesirable consequences to cells or confounding factors related to increased fluid flow rate (e.g. shear stresses).

Pulsatile flow is also useful for treating cells in microchannels and has been used in a variety of cell signaling experiments (Gu et al. 2004; Jovic et al. 2010). However, dispersion of materials during pulsatile flow is even more problematic than for laminar flow, limiting selectivity of treatments to the time/frequency domain. Furthermore, pulsatile flow is not amenable to cell patterning because most types of cells sediment as they move through channels resulting in rapid loss of pattern fidelity.

Although diffusion gradients and dispersion of materials may be desirable for certain experiments, one can envisage a great number of experiments that could benefit from stable delivery of materials over the entire channel length. Therefore, in order to perform a more extensive repertoire of cell-based experiments in microfluidic channels it is necessary to establish methods for more selective in-channel cell patterning and chemical delivery over greater channel distances.

This need is partially satisfied with biphasic flow, the most commonly used systems consisting of air-and-water or oil-and-water (Zheng et al. 2004; Guillot and Colin 2005; Teh et al. 2008; Brouzes et al. 2009; Vijayakumar et al. 2010). These systems provide high precision chemical separation and delivery; however, neither air nor oil are fully biocompatible with cells and can result in cells death due to mechanical disruption caused by surface stresses or non-biocompatible phase components (Bilek et al. 2003; Wei-Heong Tan 2007). Recently, aqueous two-phase systems (ATPSs) have been incorporated in several cell-based microfluidic applications, including collection of plant and animal cells, separation of erythrocytes and leukocytes from whole blood, and production of polyethylene glycol diacrylate hydrogel microspheres (Yamada et al. 2004; Nam et al. 2005; Soohoo and Walker 2009; Tsukamoto et al. 2009; Ziemecka et al. 2011). Other applications of ATPSs in microfluidic systems include separation of proteins and nucleotides through continuous partitioning or by electrophoresis across the interface between the two polymer solutions (Hahn et al. 2011a, b). In contrast to other biphasic flow systems, ATPSs are fully biocompatible and are composed of phases that differ from each other only by the polymer constituents. The most commonly used ATPS for cell and biomolecule applications is the polyethylene glycol (PEG)-dextran system. Both PEG and dextran can dissolve in aqueous media, including cell culture media, at concentrations low enough to avoid damage to cells (Tavana et al. 2009). Furthermore, the PEG-dextran ATPS is capable of partitioning materials, i.e. the biomolecules or cells of interest can distribute preferentially to either PEG or dextran (Albertsson 1972; Yamada et al. 2004).

Here we report the use of ATPSs in microfluidic channels for delivery of adherent cells and biomolecules to discrete channel regions. We demonstrate that mammalian cells can be directly patterned in precise laminar patterns or as islands by ATPS-mediated delivery. Furthermore we demonstrate that ATPSs can be used to deliver chemical treatments to cells growing within microfluidic channels with higher precision than conventional aqueous buffers.

2 Materials & methods

2.1 ATPS compositions

ATPSs were formed by mixing equal volumes of stock solutions comprised of the following: *i*. 5.0% wt/wt PEG Mr 35,000 kDa (Sigma, St Louis MO) and 6.4% wt/vol dextran Mr 500,000 kDa (Pharmacosmos A/S, Denmark) or *ii.* 14.0% wt/wt PEG Mr 35,000 kDa and 14.0% wt/wt dextran Mr 10,000 kDa (Pharmacosmos). The resulting emulsions contained final concentrations of 2.5% PEG and 3.2% dextran for the dextran Mr 500,000 kDa system and 7.0% PEG and 7.0% dextran for the dextran Mr 10,000 kDa system. The emulsions could be separated into distinct phases by gradual overnight equilibration or by centrifugation at 4000 RCF for 15 min. For cell patterning, polymers were dissolved in DMEM containing 10% FBS by gently triturating the solutions until all polymer particles were completely dissolved. For trypsin delivery, polymers were dissolved in F12K CO_2 independent medium without FBS.

To improve partitioning to the dextran phase, trypsin was mixed with negatively charged carboxymethyl dextran (CM-dextran) (Sigma) prior to addition to the equilibrated dextran phase. The final concentration of CM-dextran in the dextran phase was 0.1 mg/mL. The final concentration of trypsin (Worthington, Lakewood, NJ) was 900 Units/mL.

2.2 Channel designs

Simple microfluidic channel designs were selected to highlight the general utility of ATPS-mediated cell and reagent delivery. Laminar flow was generated using several flow focusing channels of varying geometries that were created using standard photolithographic and soft lithographic processes (McDonald et al. 2000). The first of these designs was a flow focusing channel with three inlet channels and a main channel of 25 mm in length and either 1.5 or 1 mm in width, 0.1 mm in height. The width of the central channel in the hydrodynamically focusing geometry (PEG channel) was either 0.25 or 0.5 mm and could be used to adjust the width of the PEG divider stream in the main channel independent of volumetric flow rate. The second design consisted of seven inlets with a 25 mm-

long main channel, 1.5 mm wide and 0.1 mm in height. This device could be used to generate four laminar dextran streams separated by three PEG streams.

Droplet dispensing devices were created using backside photolithography and contained two dispensing orifices flanked by PEG focusing channels with a central PEG channel separating the two orifices (Futai et al. 2004). PDMS replicas were bonded to 100 μm-thick PDMS membranes to permit Braille actuation at the orifices.

2.3 Cell culture

The C2C12 myofibroblast cell line was used for all experiments. C2C12 cells were maintained in DMEM with 10%FBS in a humidified CO_2 incubator at 37°C. Cells were seeded in fibronectin-coated microfluidic devices at a final density of 20,000 cells/uL. Prior to seeding, cells were labeled with CellTracker dyes or Hoechst 33342 (both from Molecular Probes, Carlsbad, CA) as per manufacturer instructions and washed and centrifuged twice to remove excess dye. Cells were then suspended in dextran solutions and drawn into plastic syringes fitted with Tygon tubing to interface with microfluidic devices.

For laminar cell patterning, cells were seeded in the dextran phase into either three channel or seven channel devices with dextran/cell channels separated by PEG. Once two-phase laminar flow became stable, devices were disconnected from the flow sources and placed in a 37°C CO_2 incubator for 1 h to allow cells to attach. ATPS solutions were then replaced by cell culture medium and cells were incubated for an additional 24 h before microscopic analysis.

Islands of cells were seeded by way of Braille-actuated droplet formation in bell-shaped channels (Futai et al. 2004; Gu et al. 2004). FITC dextran was added to the cell dextran mixture to facilitate alignment of the dextran/cell dispensing orifices with the Braille actuation pins. Initial flow rates of 0.3 mL/h for PEG and 0.05 mL/h for dextran were used to establish laminar flow in Braille devices and clear the channels of loosely adherent cells that entered the channels during flow stabilization. This flow rate was gradually reduced to 0.15 mL for PEG and 0.002 mL/h for dextran to allow droplet formation to occur. Droplet size and spacing were controlled by modifying the Braille pin actuation interval and PEG/dextran flow ratio. After stable droplets containing cells were formed the channels were clamped shut at both upstream and downstream regions to arrest droplet movement. Cells were then allowed to attach to the channels for 1 h before the ATPS medium was replaced with culture medium.

Selective treatment of cells with biomolecules was demonstrated on cells cultured to confluence in laminar flow focusing devices. Trypsin was delivered to cells for 5 min. ATPS/trypsin treated regions were compared to both the non-treated side of the channel and channels treated with biomolecules dissolved in medium without the ATPS.

2.4 Staining, microscopy and statistical analysis

Cell-patterned devices were fixed in 4% paraformaldehyde after 24 h of culture and then washed twice with PBS. Devices used for biomolecule delivery were fixed immediately after treatment. Actin cytoskeleton staining was performed by staining cells with Alexa-568 phalloidin (Molecular Probes) as per manufacturer instructions. Nuclear staining was achieved by labeling cells with Hoechst 33342.

Imaging was performed on a Nikon SMZ 1500 stereoscope and Nikon TE300 inverted microscopes equipment with mercury excitation sources (Nikon, Tokyo, Japan). Image collection and analysis was performed using simple PCI (Hamamatsu, Sewickley, PA), Metamorph (Molecular Devices, Sunnyvale, CA) and Image J software. Statistical analyses were performed using SigmaPlot and SigmaStat (Systat Software, Chicago, IL)

3 Results

3.1 Characteristics of ATPS flow in microchannels

ATPSs could be delivered as laminar streams using simple y-type or t-type channel designs. In contrast to laminar flow of polymer-free buffer solutions such as PBS, ATPS laminar flow required greater relative volumetric flow ratios of dextran to PEG to achieve similar channel occupancy of the laminar streams. The fractional channel occupancy differed for each phase systems due to viscosity differences between dextran and PEG which were dependent on both polymer concentration and molecular weight. Laminar streams of dextran and PEG could be differentiated based on the presence of discrete phase boundaries (Fig. 1(a)). As expected, molecules placed within one phase of the two-phase system were often strongly retained within that phase and displayed little diffusive dispersion in downstream channel regions as compared to PBS controls (Fig. 1(a-d)).

The lack of diffusive dispersion, due to molecule partitioning, is governed by specific energetic and affinity interactions between the molecules of interest and one or both of the phases(Albertsson 1972). Red food colorant and Coomassie G250 both partition almost exclusively to the PEG phase and clearly demonstrate that ATPS delivery in laminar streams can selectively confine chemicals to discrete channel regions more effectively than aqueous buffers alone (Fig. 1(c), (d)). Based on these initial observations, we sought to exploit

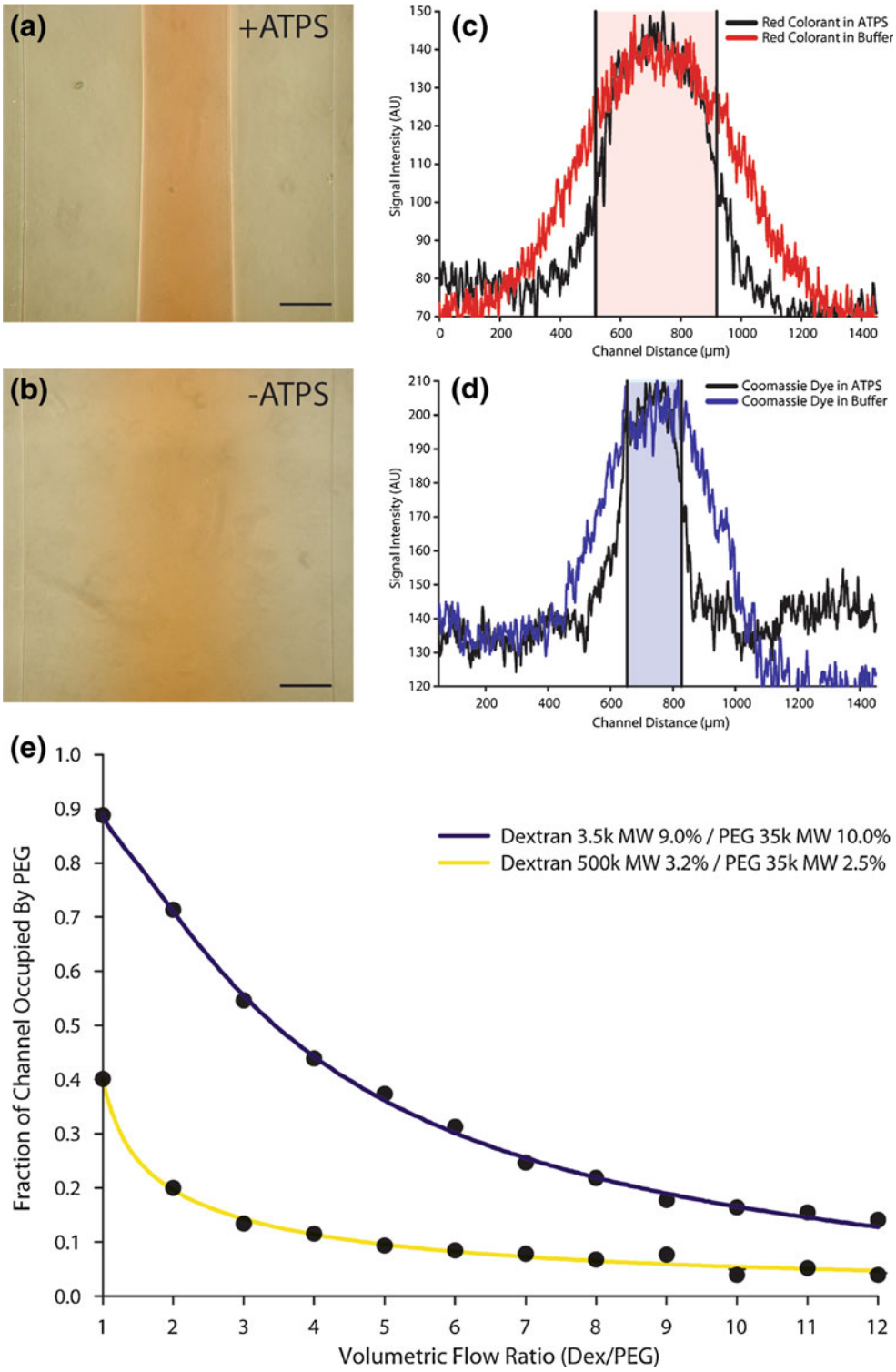

Fig. 1 Characteristics of ATPS flow in Microchannels. (**a**). An ATPS displays a clear phase boundary when delivered as laminar streams within a PDMS-glass microchannel. Food colorants are clearly retained in PEG within the phase boundary. (**b**). Buffer solution do not display partitioning and have no phase boundary. Molecules diffuse laterally as laminar streams move through the channel. The diffusion characteristics of red food colorant (**c**) and Coomassie G250 (**d**) were plotted based on intensity of dye across the channel. The shaded regions of these plots correspond to the actual channel occupancy of the stream immediately downstream from the channel junction. ATPS solutions maintain a strict boundary at regions further downstream whereas buffers allow molecules to diffuse from these regions. (**e**). Example of volumetric flow calibration curve used to determine fractional channel occupancy for different PEG-dextran flow conditions. Scale bar=250 um, n=3 observations

ATPS partitioning properties to more effectively deliver both cells and bioactive molecules within microchannels. In order to more effectively control the delivery of these materials within channels we generated calibration curves for dextran/PEG channel occupancy as a function of volumetric flow ratio (Fig. 1(e)).

3.2 Patterning cells by ATPS laminar flow

Laminar flow of ATPS could be used to establish clear laminar patterns of adherent cell in t-type microchannels (Fig. 2(a-c)). By varying the number of inlet channels it was possible to establish up to four laminar streams for patterning adherent

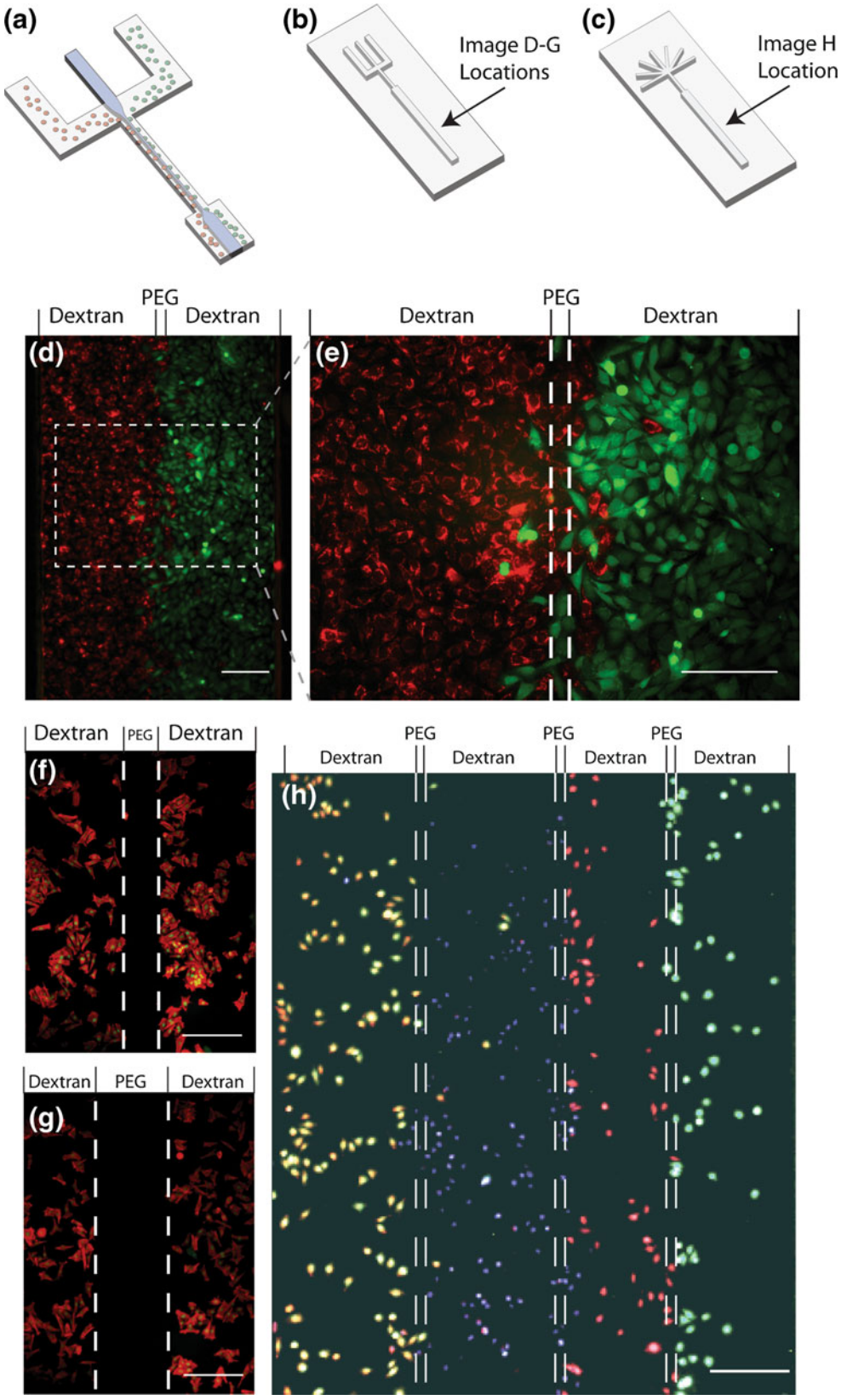

Fig. 2 Precise laminar patterns of adherent cells can be generated through ATPS laminar flow patterning. (**a**). Schematic of cell delivery in dextran with a central PEG spacer. (**b**). A two cell-type device design. (**c**). A four cell-type device design. (**d-e**). Two populations of C2C12 cells labeled with cell tracker green and cell tracker red were patterned using the minimum PEG spacer. (**f-e**). By using devices of different PEG channel width it is possible to change the gap between cell populations using identical volumetric flow conditions as evidence by actin-phalloidin staining of fixed patterned cells. (**h**). it was demonstrated that up to 4 cell populations can be patterned simultaneously. Patterned cells were pre-stained with green cell tracker, red cell tracker, red and green cell tracker, or Hoechst 33342. Scale bar=250 um

cells (Fig. 2(d), (e), (h)). By varying the width of the PEG spacing channel or by decreasing the dextran/PEG flow ratio it was possible to obtain cultures that displayed no gaps (Fig. 2(d), (h)), small gaps (Fig. 2(f)), or large gaps between

cell populations. Using this method multiple populations of cells could be directly seeded into microchannels as laminar patterns. After devices were removed from the syringe lines cells attached to the channel surfaces within 1 h. Cells remained organized in their initial patterns for at least 24 h after seeding. We found that this method of cell delivery was compatible with both active pumping by syringe pump as well as gravity or capillary driven flow. Furthermore, ATPSs retained their patterns even after flow was removed, remaining laminar long enough for cells to sediment and begin attaching to the channels.

Laminar patterns of adherent cells will be useful for experiments that seek to investigate the interactions of multiple cell populations under flow conditions. Additionally, the ability to directly pattern gaps between populations of cells will facilitate in-channel migration assays.

3.3 Patterning islands of cells through forced ATPS droplet formation

Laminar cell patterning can be useful for a variety of migration and co-culture assays in which it is convenient for cells to be patterned on opposite sides of channels. However, some experiments may seek to explore radial migration phenomena and colony migration under flow conditions, or to observe effects of unidirectional signaling of soluble factors. Therefore, it will also be useful to pattern cells in additional configurations such as islands along the length of channels. This cannot be achieved by pulsatile flow of conventional aqueous solutions due to dispersion effects. However, ATPSs permits the formation of controlled dextran droplets surrounded by PEG. Cells are contained within the dextran droplets by the interfacial tension between the two polymer phases.

To control droplet formation, we used a computerized Braille microfluidic platform to valve bell shaped channels by way of pin actuation. This allowed us to control droplet size and spacing and to arrest the droplets for cell attachment to occur (Lai et al. 2011). Using two actuation orifices, we were able to form alternating droplets of dextran within a PEG-filled channel (Fig. 3(a), (b)). After droplets were arrested in the channels, cells remained associated with the dextran phase (Fig. 3(b-d)). By alternating the actuation timing of the dextran channels we could rapidly produce alternating droplets containing different materials as indicated by the presence of alternating droplets containing either FITC or TRITC labeled dextran (Fig. 3(b)). When cell tracker labeled C2C12 cells were incorporated into the dextran phase, alternating colonies of red and green Cell Tracker-labeled cells were observed that readily adhered to the channel surface (Fig. 3(c), (d)).

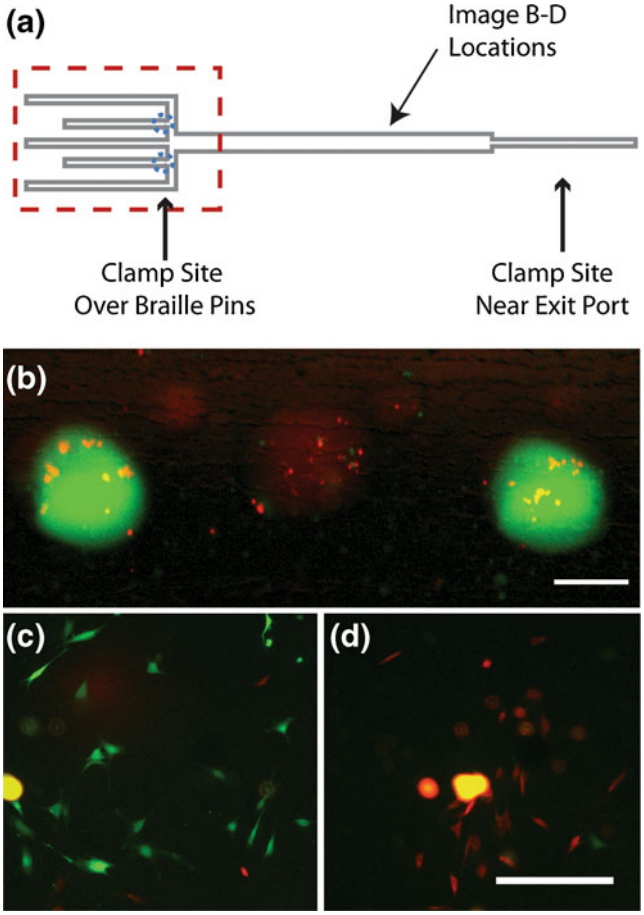

Fig. 3 Braille microfluidics can be used to directly pattern islands of cells by way of forced ATPS droplet formation. (**a**). Schematic of a Braille device with sites of actuation (*blue marks*) and clamp sites for arresting flow (*black arrows*). (**b**). Using alternating actuation of Braille orifices it is possible to deliver alternating droplets of dextran within PEG, as evidenced by several consecutive droplets containing either TRITC-dextran (*red*) or FITC-dextran (*green*) (**c-d**). When cells are incorporate into droplets it is possible to deposit alternating islands of cells that remain patterned for at least 24 h after adhering. Scale bar=100 um

3.4 Selectivity of biomolecule delivery can be improved using ATPSs

Many biomolecules and particles partition within ATPSs to either dextran or PEG. Partitioning is influenced by the size of the molecule or particle, its charge, specific interactions with the phase-forming polymers, and properties related to phase system composition such as molecular weight of the polymers (Albertsson 1972). Cells partition well in the dextran Mr 500,000 phase system; however, many biomolecules partition weakly to dextran in this phase system. Thus, in order to increase specificity for the dextran phase during delivery, we used dextrans of lower molecular weight and incorporated charged dextrans to interact electrostatically with the molecules of interest.

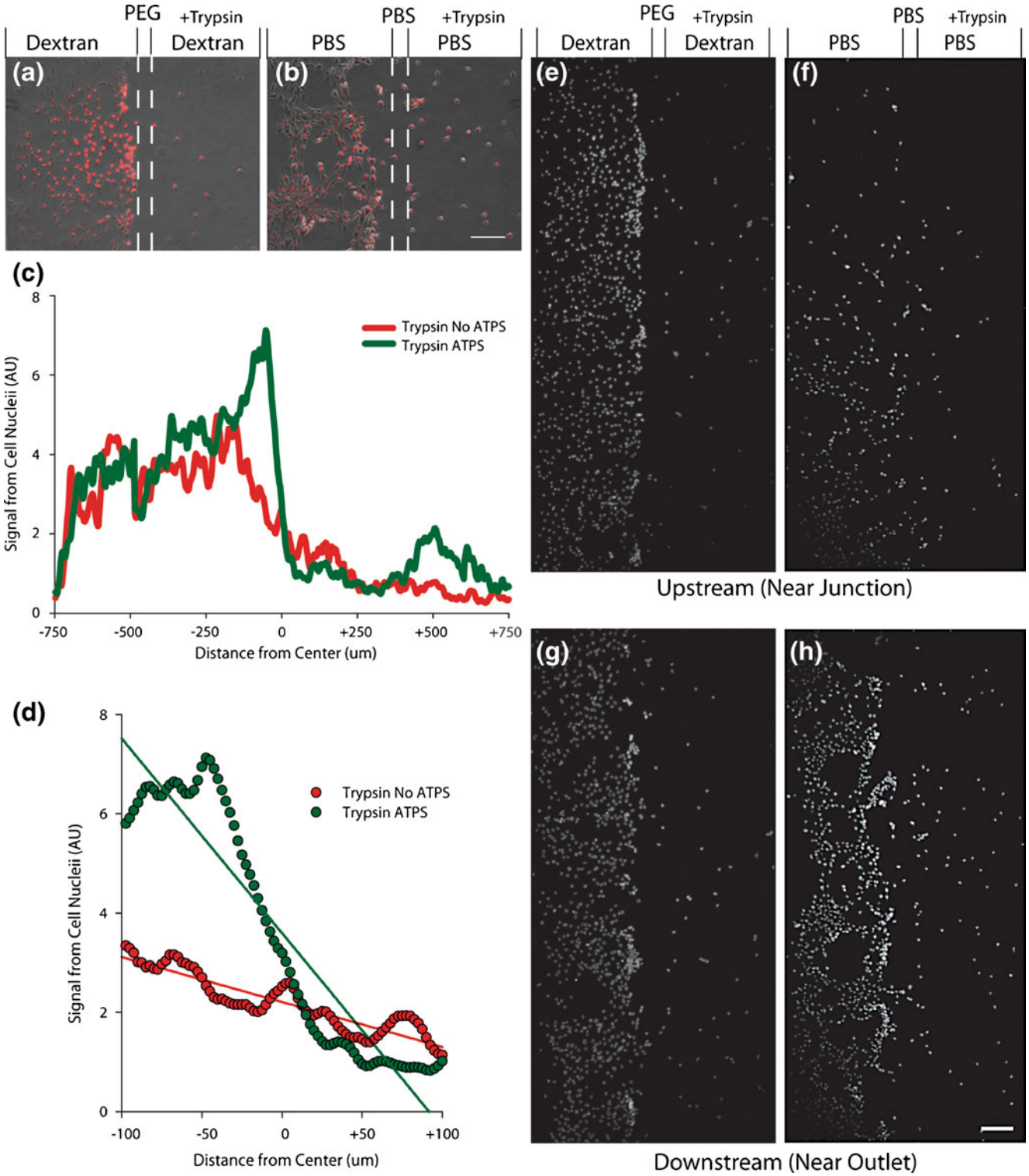

Fig. 4 Trypsin delivery is well confined by ATPSs. (**a**). Representative image of Phase Contrast/Hoechst-labeled cells following ATPS delivery of trypsin on the right side of the channel. (**b**). Representative image of aqueous delivery of trypsin showing effects on both sides of the channel. (**c-d**). Intensity profiles reveal a clear boundary for ATPS delivery and a gradient effect for aqueous delivery. (**e-h**). Boundaries are maintained in both upstream and downstream channel regions for ATPS delivery whereas aqueous delivery shows a gradient effect across the width of the channel that is apparent in all channel regions. Scale bar=200 um, n=3 observations, curves plotted as means

The effects of trypsin were more selective when ATPS was used as a delivery medium. Trypsin, a 23 kDa enzyme, can be used to rapidly dissociate cells from their substrate by cleaving a variety of proteins that mediate cell adhesion

and has been used previously to remove cells within microfluidic channels (Takayama et al. 1999; Nie et al. 2007; Villa-Diaz et al. 2009; van der Meer et al. 2010). Trypsin exists primarily as a cationic protein, and thus we expected its selectivity for dextran to be enhanced through the addition of negatively charged dextran (CM-dextran). When an ATPS was used as the delivery medium, trypsin activity was confined exclusively to the dextran treatment phase (Fig. 4(a), (e), (g)). When a conventional aqueous delivery medium was used, cells on both sides of the channel were affected by trypsin, although cells on the treated side were more thoroughly dissociated (Fig. 4(b), (f), (h)). The intensity profile of Hoechst-labeled cells served as a quantifiable measure of cell dissociation (Fig. 4(c), (d)). ATPS delivery resulted in a strict boundary, whereas conventional aqueous delivery resulted in a gradient effect (Fig. 4(c), (d)). This can be appreciated by measuring the slope of the signal intensity profile in the center of the laminar region of the channel (Fig. 4(d)). The effects of ATPS on trypsin selectivity could be readily observed both in upstream laminar regions (Fig. 4(e)) but also for laminar regions near the end of the channel (Fig. 4(g)).

Collectively these results demonstrate that ATPS delivery in microchannels is capable of more selective confinement of molecule to parts of microfluidic channels than is conventional aqueous delivery. This can result from several factors that influence selectivity. First the higher viscosity of the ATPS results in more limited diffusion than for other less viscous aqueous mediums. However, this factor alone cannot account for the apparent selectiveness of ATPS delivery. The most critical factor is the partitioning behavior of the ATPS. This will vary based on the molecule of interest and the composition of the ATPS, however most molecules partition to one phase or the other. Molecules that partition strongly to the dextran phase will be well maintained regardless of the flow rate or viscosity of the systems. Molecules that favor PEG can also be confined to half of the channel based on the configuration of the phase system. In this case molecules are confined to one side of the channel both by viscous-limitations to diffusion and active partitioning into a thin PEG divider. This concept is illustrated in Fig. 5.

Based on calculation for steady state diffusion from a semi-infinite source, we estimate that ATPS can perform at least twice as well as conventional laminar delivery under slow flow or arrested flow conditions (assuming only modest (kpart of trypsin ~0.6) partitioning of molecules) (Takayama et al. 2003; George et al. 2008). If molecules display an even stronger preference for a single phase, this advantage is further enhance as a function of the partition coefficient. At higher flow rates we would expect the performance of the ATPS to be even better, although the advantage relative to conventional laminar flow becomes less dramatic. However, the only scenario in which ATPS does not provide enhanced selectivity is for molecules that distribute equally among phase, which rarely occurs.

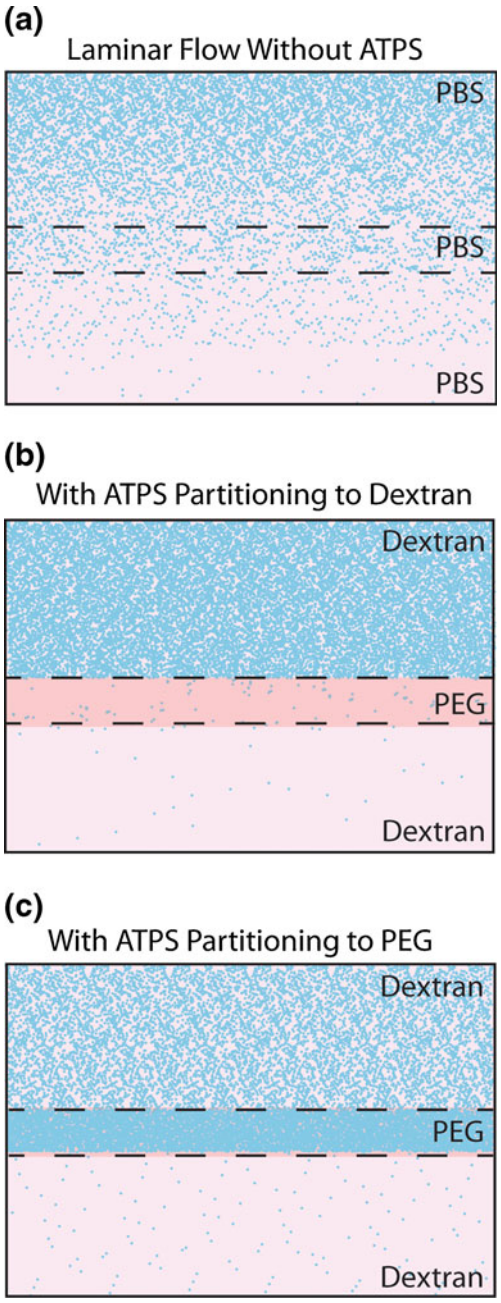

Fig. 5 Molecule or particle localization under various ATPS flow conditions

4 Conclusions

We demonstrate that ATPSs can be used to compliment a variety of microfluidic techniques used to culture mammalian cells. We used both the interfacial and partitioning

properties of the ATPS to enhance delivery of cells and molecules within microfluidic channels. For cell delivery we demonstrated that up to four populations of adherent cells can be patterned simultaneously. We also demonstrated that cells can be encapsulated within dextran droplets and delivered within PEG to produce patterns of small colonies or islands. Additionally, we achieved enhanced selectivity of chemical delivery to cells using an ATPS. This was accomplished for trypsin, and permitted more selective cell harvesting. We propose a model for selective delivery based on the viscosity of ATPS, partitioning behavior and configuration of the phases under laminar flow.

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