COMMUNICATION

Delivery of Proteases in Aqueous Two-Phase Systems Enables Direct Purification of Stem Cell Colonies from Feeder Cell Co-Cultures for Differentiation into Functional Cardiomyocytes

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Stem cells are routinely co-cultured with support cells,[1,2] from which they must be isolated for many downstream applications. Although several feeder-free culture systems have recently emerged,[3–5] feeder culture is currently the method of choice because it offers superior cell viability, better long-term maintenance of stem cell phenotype, is often more cost effective, and produces consistent results among induced pluripotent stem cell (iPSC) lines.[6] However, a challenge for many stem cell researchers is obtaining sufficiently pure iPSC populations from feeder cell co-cultures.

Existing methods for passaging and purifying iPSCs from feeder cultures include mechanical removal[7,8] and protease treatment.[9,10] Unfortunately, these methods tend to carry over contaminating feeder cells. Following feeder-free plating, it is therefore necessary to wait for contaminating cells to die and to weed out spontaneously differentiating areas to obtain sufficiently pure stem cell populations for controlled differentiation. Overall, this is a difficult, inefficient, time consuming and variable process.

We developed a method that uses aqueous two-phase systems (ATPSs)[11,12] for high resolution deliver of enzymes to discrete regions in a cell culture system, thus facilitating selective dissociation of iPSC colonies from MEF feeder cultures. To achieve high resolution protease delivery, we pneumatically dispensed dextran (DEX) droplets into polyethylene glycol (PEG) through a capillary tip (Figure S1, **Figure 1**A–B), where they sank vertically in the PEG medium to contact the cells. These droplets were as small as 8.0 picoliters in volume (Figure 1C–D). Longer pneumatic pulses resulted in larger DEX droplets capable of covering entire stem cell colonies. DEX solutions spread considerably after contacting cells, with DEX 10,000 kDa forming a surface film on the cells (Figure S2). The total number of cells covered by DEX was accounted for by adjusting the volume of the DEX according to its spreading properties. We initially demonstrated our ability to deliver biomolecules on the picoliter scale to cells by delivering plasmid DNA coding for green fluorescent protein (Figure S3).

Proteins typically display an affinity for the DEX phase in the PEG/DEX ATPS;[11] however, protein delivery to cells using ATPS micropatterning has not been demonstrated previously. We initially tested the ability of the DEX 10,000 and 500,000 kDa systems to partition proteases used for cell culture, such as collagenase, dispase and trypsin. Protein blotting of separated DEX and PEG phases revealed that proteases partitioned predominantly to the DEX phase in the DEX 10,000 kDa system (k_{part} of 0.4 or less), indicating that this system was suitable for delivering proteases to cultured cells (Figure S4). In contrast, proteases incorporated into the DEX 500,000 kDa system displayed only modest partitioning to the DEX phase. Using the DEX 10,000 kDa system, we tested our ability to pattern proteases on HEK 293H cells. DEX droplets containing trypsin disrupted cell adhesion and resulted in loss of cell-cell and cell-substrate contact (Figure S5). The smallest DEX droplets (picoliter scale) containing trypsin were capable of disrupting connections between two neighboring cells (Figure S5 B), while larger DEX droplets (nanoliter scale) produced clearings in cell monolayers (Figure S5 C).

To purify stem cells from feeder cultures, we used a robotic micromanipulator to position the capillary needle above iPSC colonies, and then manually triggered pneumatic pulses to dispense the DEX droplets. This allowed us to precisely control both the position and volume of the DEX droplets. We selected an iPSC line[5] that displayed a normal karyotype, expressed stem cell markers and was capable of multiple differentiation potentials (Figure S6) to demonstrate that iPSCs could be released as colonies from their surrounding mouse embryonic fibroblast (MEF) feeder cultures in three simple steps (Figure 1A–B). First, the feeder-conditioned medium was replaced with a solution of PEG and droplets of DEX containing proteases (100 picoliters to 10 nanoliters, depending on colony size) were dispensed onto the center of each colony (step 1). The cells were then incubated for up to 1 hour depending on enzyme concentration/activity (step 2). Finally, the PEG was removed and loose colonies were dislodged by pipetting fresh medium over the surface of the cells (step 3). Our approach, termed localize enzymatic microdissection (LEM) is faster and easier to perform than manual colony picking,[13] more practical than laser-based passaging systems,[7,8] and provides higher purity iPSC cell collection than both manual picking and

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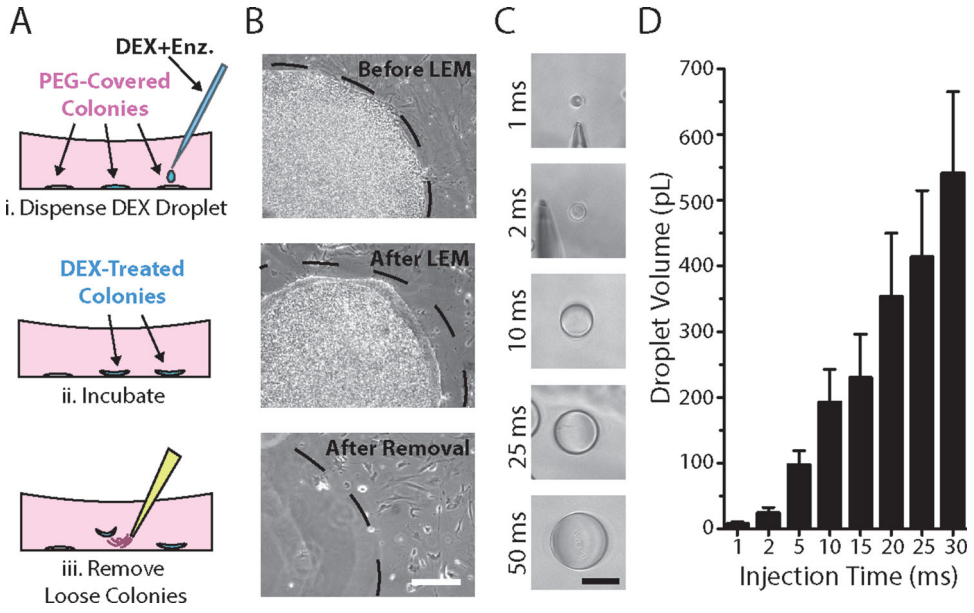

Figure 1. DEX droplets were dispensed in PEG from glass capillary needles. (A) Schematic of the three steps for localized enzymatic microdissection (LEM) iPSC purification. (B) Phase contrast images for each step of the LEM iPSC purification process. Scale bar is 100 µm. (C) Images of DEX droplets produced by various pneumatic pulse lengths from a single capillary. (D) Droplet size varies by injection time.

conventional enzymatic dissociation, thus allowing direct collection of highly pure iPSCs while leaving feeder cells behind.

Collagenase treatment alone could not dissociate stem cells when applied in this format (Figure S7 A). However, dispase and trypsin were highly effective at releasing individual stem cell colonies from MEF co-cultures (Figure S7 A). Dispase was preferred to trypsin based on our observation that growth of dispase-treated cells was more robust after replating. Co-treatment of collagenase and dispase made it easier to break apart large colonies after collection. After replating on matrigel-coated substrates, the fragmented colonies grew to confluence (Figure S7 B-C). Over the course of 3 days, these cells populated the culture vessel, reaching near confluence when 30 colonies or more were seeded into a 35 mm dish. Neither the PEG nor the enzyme treatments had any direct adverse effects on cell viability, as measured by propidium iodide staining (Figure S7 D). The replated iPSCs displayed characteristic stem cell morphologies and expressed several markers of pluripotent stem cells including the transcription factors nanog and oct3/4, and the cell surface antigen ssea-4 (Figure S7 E-F). In contrast to conventional methods for transferring stem cells from feeder culture to feeder-free culture, smooth muscle actin-positive cells were not observed among the LEM purified iPSCs, indicating an absence of contaminating MEFs.

To validate our new technology for isolating stem cells, we confirmed that purified iPSCs were capable of feeder-free differentiation using high efficiency cardiomyocyte differentiation.[14] These protocols require single cell suspensions of iPSCs; therefore, the colony fragments collected by LEM were subjected to Versine treatment followed by trituration. These cells were capable of expansion to 90% confluence in 3 ~ 4 days under feeder-free conditions before application of a second matrigel layer. Beginning 3 ~ 4 days after matrigel application, the cells were treated with activinA, BMP4 and bFGF (for details see[14]). In some cases, beating cells were observed on the 10th day after activinA treatment.

We recorded spontaneous beating after one month and again after 2 months (**Figure 2**A–B, Videos S1 and S2). The beating pattern became stable and well-synchronized by one month. Beating rates of 39.8 and 34.3 beats per minute were observed for the differentiated cardiomyocyte clusters after one and two months, respectively (Figure 2A). These rates were expected for cardiomyocytes under the imaging conditions used to record the beating activity, indicating that our method for collection of iPSCs is sufficiently robust to generate functional differentiated cells without the need for any additional purification steps. Beating cardiomyocytes represented approximately 90% of the total differentiated cells. These differentiated cells expressed the general cardiac markers α-actinin and cardiac troponin T (cTnT), as well as the ventricular marker MLC2v (Figure 2C–D).

The traditional method for differentiating iPSCs into cardiomyocytes and neurons involves culturing iPSCs as embryoid bodies (EBs). This method does not require high iPSC purity; however, the efficiency of cardiomyocyte differentiation is quite low (2 to 20%).[15] Recently, new methods have been developed for generating cardiomyocytes with high efficiency (60 to 90%),[14,16] but these methods demand high purity, single cell iPSC preparations. We used these recently developed differentiation methods with our LEM technology to isolate high purity iPSCs for differentiation without the need for intermediate purification methods prior to differentiation to remove the MEF cells. Thus, our method for selectively capturing iPSC reduces the impact of contaminating MEFs on stem cell differentiation.

Currently, the standard method used by most labs to maintain iPSCs is MEF co-culture. Although feeder-free systems are commercially available and successful, feeder culture still

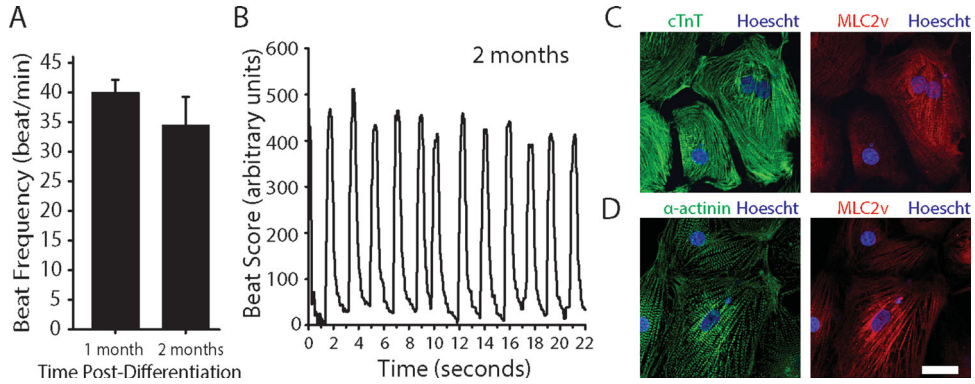

Figure 2. Purified iPSCs were differentiated into functional cardiomyocytes without additional purification. (A) Beat frequency at 1 month and 2 months. Error bars denote mean +/– SEM. (B) Beat features extracted from time sequence images show stable beating patterns. (C) Differentiated cells expressed the general cardiac marker cTnT and the ventricular marker MLC2v. (D) Sarcomeric structures were revealed by α-actinin immunofluorescence in MLC2v-expressing cells. Hoechst was used as a counterstain. Scale bar in (C) and (D) is 20 μm.

prevails as the best method for long-term maintenance of iPSCs because it provides growth conditions that promote high cell viability over many passages. Most labs rely on mechanical scratching to remove iPSC colonies from MEFs during passaging. After removing the colonies with scratching, it is necessary to wait for approximately one week for contaminating MEFs to die, and even then it is still necessary to scratch away differentiated areas under a microscope to obtain the final pure colonies for directed differentiation. This is a time and labor consuming process; however, mechanical scratching offers the advantage that it maintains the connections of stem cells within colonies, just as the LEM approach does. This allows large colonies to grow in feeder-free culture which speeds up cell growth before the single cell dissociation step.

Another commonly used approach is non-localized enzyme treatment (e.g., bath application of dispase, collagenase or Accutase). Bath applied enzyme dissociation is less time consuming than manual scratching. However, enzymes applied in this format not only disconnect stem cells from MEFs, but also destroy the connections among iPSCs within the colonies. After transfer to feeder-free culture, stem cells purified with this method form very small colonies in the presence of contaminating MEFs, resulting in slower cell growth rates. It is also hard to remove differentiated areas from small colonies[17].

The LEM method allows us to obtain cultures of iPSCs from MEF feeder-co-cultures in fewer steps and with higher purity than what can typically be obtained using conventional stem cell harvesting approaches; however, the following considerations will be important for scaling of stem cell technologies that require large numbers of high purity, high quality, feeder-free cells for downstream differentiation experiments or for clinical applications. The components of our system are amenable to automation and scaling such that integration with existing robotic systems[18–20] and computer-aided vision[21–23] could eventually eliminate the need for manual handling of stem cells and other cultures. Integration of the LEM method with automated systems would allow user-free routine colony maintenance such as selection of efficiently induced iPSC colonies, or removal of inefficiently induced iPSC colonies or partially differentiated colonies. Use of similar computer aided systems would also allow precise scaling of droplet volume for various sized colonies.

It should also be emphasized that LEM and the droplet generation method can be applied to a number of other culture systems. For example, this method would be effective for isolating hepatocytes and keratinocytes that grow as colonies on fibroblast feeder layers. Apart from collection of cells for propagation, it would also be possible to use this procedure to collect single colonies free from contaminating cells for clonal analysis of gene expression. In a broader context, this method will enable extracellular patterning of a variety of biomolecules/bioparticles with single cell resolution including proteins, genetic material, microorganisms, and viruses or is some cases drugs.

Finally, the droplet dispensing method used for LEM is capable of generating the smallest user-controlled ATPS droplets yet reported. Our previous reports on cell and biomolecule patterning with ATPS have used liquid handling techniques (such as pin tools, acoustic droplet ejection and micropipettes) that can generate ATPS droplets ranging in size from hundreds of nanoliters to several microliters.[12,24] However, the relatively large size of these droplets limits the resolution of patterning, precluding manipulation of small populations of cells. The development of the capillary ejection system used for LEM relieves these constraints, allowing patterning of ATPSs with single cell resolution. For cells, such as neurons, that extend processes (axons and dendrites) away from their cell bodies, this method could even be used for patterning with subcellular resolution.

Experimental Section

Aqueous Two-Phase Systems: ATPSs were formed using the immiscible polymers PEG (Sigma) and DEX (Pharmacosmos) dissolved in either PBS for droplet characterization or cell culture medium for cell treatment. Two different PEG/DEX systems were used without equilibration between the phases: i. 16% PEG 35,000 kDa/16% DEX 10,000 kDa and ii. 5% PEG 35,000 kDa/6.4% DEX 500,000 kDa. ATPSs were formed with these polymer solutions upon adding droplets of DEX into bulk solutions of PEG, with DEX droplets sinking in PEG due to their slightly greater density. To assess spreading of DEX on cells covered with PEG, FITC-conjugated DEX was added to the DEX phase to a final concentration of 0.1 mg/mL and fluorescence images were

acquired for quantification. Protease partitioning within each ATPS was quantified by measuring total protein with dot blot assays and by measuring enzymatic activities.

Droplet Generation: Picoliter-volume droplets were created by dispensing DEX through glass capillaries, pulled from 0.69 mm ID 1.2 mm OD borosilicate glass tubes (Sutter Instruments) using a Flaming/Brown capillary puller (Sutter Instruments) to create tips with morphologies that resembled "bee stingers". The capillaries were filled with an aqueous solution of DEX by back-loading with rounded gel loading pipette tips. They were then connected to an airline leading to a programmable pneumatic pump (Nordson). The capillary and airline were placed on a holder connected to a micro manipulator (Narishige) affixed to the stage of an inverted microscope (Olympus) (Figure S1). The capillary was lowered into position with the pulled end submerged in PEG and droplets were dispensed from the capillary orifice using pneumatic actuation for various pulse lengths at 18.2 psi constant pressure.

Droplet Characterization: Minimum droplet size was characterized for the phase systems described above for various pneumatic actuation intervals. Capillaries used for these experiments were characterized in terms of orifice size by measuring the electrical resistance of the capillaries. Broken capillaries (useful for dispensing larger DEX volumes) display higher electrical resistance. Images were acquired immediately after dispensing the DEX dAts. Droplets were assumed to be spherical in shape, and thus the droplet volume was estimated from the formula $V = \frac{4}{2}\pi r^2$, where r is the radius of the sphere.

HEK293 Treatments: HEK 293H cells were used for initial gene and enzyme delivery experiments. Lipofectamine 2000 and eGFP plasmids were incorporated into the DEX phase for gene delivery. Cells were maintained under standard culture conditions (DMEM with 10% FBS and 1% antibiotics; 37 °C; 5% CO_2) and switched to DMEM without FBS containing 16% PEG 35,000 kDa prior to enzyme treatment. Droplets of 16% DEX 10,000 kDa containing 0.2 mg/mL trypsin (SAPC Biosciences) were delivered to the cells and images were recorded at the indicated time points.

iPSC Culture: Two human iPSC lines (4' and 5') were cultured on irradiated CF-1 MEF feeders (GlobalStem) in medium containing DMEM/F12 (1:1) supplemented with 20% knockout serum replacement, 1 mM L-glutamine, 0.1 mM non-essential amino acids (all from Gibco), 0.1mM α-mercaptoethanol (Sigma) and 4ng/mL recombinant human basic-FGF (bFGF) (Invitrogen). For feeder-free culture, iPSC colonies were seeded on growth factor reduced matrigel (BD Biosciences)-coated dishes and maintained in MEF-conditioned iPSC medium.

LEM Method: Three to five days after clonal seeding of the iPSCs, the medium was replaced with a solution of DMEM containing 16% PEG and ROCK inhibitor (Y-27632, 10 μM, EMD). Cells were immediately transferred to the microscope with the pneumatic ejection system and a capillary loaded with a solution of DMEM/F12 containing 16% DEX 10,000 kDa, 0.2 mg/mL collagenase (Worthington, 280 U/mg) and 0.2 mg/mL dispase (Worthington, 1.46 U/mg). Droplets of DEX/enzyme solution were dispensed in quick succession above each colony, where they subsequently sank, contacted the colonies and spread over the colony surfaces to deliver the enzymes. Droplet size was adjusted to colony size (for most colonies droplets of 5–30 μm in diameter). Colonies were collected as fragments and plated onto matrigel (as described above for growth analysis), or as single cells for cardiomyocyte differentiation (as described below).

Stem Cell Differentiation: Cytogenetic analysis was performed on 20 G-banded metaphase cells from the human 4' and 5' iPSC lines. All twenty cells demonstrated normal karyotypes. iPSC colonies were identified by immunofluorescence for three pluripotency markers: Oct3/4 (1:100, Santa Cruz), SSEA4 (1:200 DSHB) and Nanog (1:500, Abcam). Fibroblasts were identified by α-smooth muscle actin (1:100 Sigma) immunofluorescence.

Embryoid body (EB) suspension culture was performed in iPSC medium to confirm iPSC pluripotency (ability to differentiate into endoderm, mesoderm and ectoderm). For endoderm differentiation, EBs were first cultured in suspension for 30 days, and then plated for adherent culture in 24-well plates coated with 0.1% gelatin for 14 days. Endodermal cells were identified by α-fetoprotein (1:400, sigma) immunofluorescence. For ectodermal and mesodermal differentiation, EBs were first cultured in suspension for 6 days, and then seeded onto matrigel-coated culture plates for 28 days of adherent culture in neural progenitor medium (DMEM-F12(1:1) supplemented with 1% N2 supplement, 1 mM L-glutamine, 0.1 mM non-essential amino acids (all from Gibco), 20ng/mL bFGF (Invitrogen) and 2 μg/mL heparin (Sigma)). After 28 days, neural rosettes were collected and cultured in suspension in neural progenitor medium for 14 days. After 14 days, neurospheres containing neural progenitor cells were dissociated using Tryple™ Select (Gibco) into single cells and plated on matrigel-coated coverslips for 14 days of culture in neuronal medium (Neurobasal Medium supplemented with 2% B27 supplement, 1mmol/L L-glutamine (all from Gibco) and 10ng/mL BDNF (Peprotech)). Ectodermal neural progenitor cells were identified by Pax6 (1:100, DHSB) immunofluorescence. Neurons were identified by β-tubulin III (1:1000, Eptimics) and TuJ1 (1:1000, Covance) immunofluorescence. Glial cells were identified by GFAP (1:500, DAKO) immunofluorescence. Mesodermal fibroblasts were identified by α-smooth muscle actin (1:100 Sigma) immunofluorescence.

Cardiomyocyte Differentiation: After iPSC collection, colony clumps (from ~180 colonies) were centrifuged at 500 x g, washed once with PBS and centrifuged again at 500 x g. The iPSCs were then treated with 1 ~ 1.5 mL Versene (Gibco) at 37 °C for 5 min. Single cell suspensions were obtained by triturating the colonies in Versene 20 times using a 2 mL serological pipette. The Versene reaction was then quenched with iPSC medium. Finally, the single cell suspension was centrifuged at 1000 × g, and cells were re-suspended in mTeSR1 medium containing 10 μM Y-27632. Cells were plated into one well of a 6-well plate coated with growth factor-reduced matrigel (BD Biosciences). The medium was changed daily. After the cells reached 90% confluence (~3 to 4 days), mTeSR1 medium was replaced with cold (4 °C) mTeSR1 medium containing matrigel (0.9 mg per 6-well plate).

After another 3 to 4 days when the cells reached 100% confluence, differentiation was initiated by replacing mTeSR1 medium with cold (4 °C) RPMI/B27 without insulin (Gibco) containing matrigel (0.5 mg per 6-well plate) and Activin A (100 ng/mL, R&D systems) for 1 day. The next day, the medium was replaced with RPMI/B27 without insulin containing BMP4 (10 ng/mL, R&D systems) and bFGF (8 ng/mL, Invitrogen) for 4 days. On day 6, the medium was replaced with RPMI/B27 complete supplement, which was replenished every 3 days thereafter. Contracting cells were observed on day 10 after Activin A treatment.

Contracting cardiomyocytes were imaged by video microscopy (Leica DMIRB inverted) at days 30 and 60 and the beating rates were recorded. Plots of cardiomyocyte contraction were generated by recording the standard deviation measurements of grey scale images in time-stacks using ImageJ software. For immunofluorescence analysis, beating areas were removed from the plates, dissociated into single cardiomyocytes and plated on coverslips coated with matrigel. Cardiomyocytes were identified by the sarcomeric markers α-actinin (1:800, Sigma), cTnT (1:200, Thermo Scientific) and the ventricular marker MLC2v (1:200, ProteinTech Group). Immunofluorescence images were acquired using a Nikon AR1 confocal microscope.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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