Video Article

Microfluidic Buffer Exchange for Interference-free Micro/Nanoparticle Cell Engineering

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Abstract

Engineering cells with active-ingredient-loaded micro/nanoparticles (NPs) is becoming an increasingly popular method to enhance native therapeutic properties, enable bio imaging and control cell phenotype. A critical yet inadequately addressed issue is the significant number of particles that remain unbound after cell labeling which cannot be readily removed by conventional centrifugation. This leads to an increase in bio imaging background noise and can impart transformative effects onto neighboring non-target cells. In this protocol, we present an inertial microfluidics-based buffer exchange strategy termed as Dean Flow Fractionation (DFF) to efficiently separate labeled cells from free NPs in a high throughput manner. The developed spiral microdevice facilitates continuous collection (>90% cell recovery) of purified cells (THP-1 and MSCs) suspended in new buffer solution, while achieving >95% depletion of unbound fluorescent dye or dye-loaded NPs (silica or PLGA). This single-step, size-based cell purification strategy enables high cell processing throughput (10^6 cells/min) and is highly useful for large-volume cell purification of micro/nanoparticle engineered cells to achieve interference-free clinical application.

Video Link

The video component of this article can be found at https://www.jove.com/video/54327/

Introduction

Engineering cells by agent-loaded micro/nanoparticles (NPs) is a simple, genomic integration-free, and versatile method to enhance bioimaging capability and augment/supplement its native therapeutic properties in regenerative medicine.[1-3] Cellular modifications are achieved by labeling the plasma membrane or cytoplasm with an excess concentration of agent-loaded NPs to saturate the binding sites. However, a major drawback of this method is the significant quantities of unbound particles remaining in solution after cell labeling processes, which can potentially confound precise identification of particle-engineered cells or complicate therapeutic outcomes.[4,5] In addition, exposure to NPs containing transformative agents (growth factors, corticosteroids, *etc.*) at excessively high concentration can cause cytotoxicity and misdirected exposure may induce unintended consequences on non-target cells. Even particulate carriers comprising of "biocompatible" materials [*e.g.*, poly(lactic co-glycolic acid), PLGA] can incite potent immune cell responses under certain conditions as well.[6] This is especially risky in individuals with impaired immunity (*e.g.*, rheumatoid arthritis) which potentially delays systemic nanoparticle clearance.[7] Thus, efficient removal of free particles prior to the introduction of particle-engineered cells is of great importance to minimize toxicity profile and reduce misdirected exposure to agent-loaded particles *in vivo*.

Conventional gradient centrifugation is often used to separate engineered cells from free particles but is laborious and operated in batch mode. Moreover, shear stresses experienced by cells during high-speed centrifugation and the constituents of the density gradient medium may compromise cell integrity and/or influence cell behavior.[8] Microfluidics is an attractive alternative with several separation technologies including deterministic lateral displacement (DLD)[9], dieletrophoresis[10,11] and acoustophoresis[12] developed for small particles separation and buffer exchange applications. However, these methods suffer from low throughput (1-10 µl·min^{-1}) and are prone to clogging issues. Active separations such as dielectrophoresis-based methods also require differences in intrinsic dielectrophoretic cell phenotypes or additional cell labeling steps to achieve separation. A more promising approach involves inertial microfluidics — the lateral migration of particles or cells across streamlines to focus at distinct positions due to dominant lift forces (F_L) at high Reynolds number (Re).[13] Due to its high flow conditions and superior size resolution, it has often been exploited for size-based cell separation[14,15] and buffer exchange applications.[16-18] However, buffer exchange performance remains poor with #10−30% contaminant solution as the separated cells usually remain close to the boundary between original and new buffer solutions.[16-18] More importantly, the size distribution of target cells has to be similar to achieve precise inertial focusing and separation from the original buffer solution which poses an issue especially in the processing of heterogeneous-sized cell types such as mesenchymal stem cells (MSCs).[19]

We have previously developed a new inertial microfluidics cell sorting technique termed Dean Flow Fractionation (DFF) for isolating circulating tumor cells (CTCs)[20] and bacteria[21] from whole blood using a 2-inlet, 2-outlet spiral microchannel device. In this video protocol, we will describe the process of labeling THP-1 (human acute monocytic leukemia cell line) suspension monocytic cells (~15 µm) and MSCs (10-30 µm) with calcein-loaded NPs, followed by fabrication and operation of the DFF spiral microdevice for efficient recovery of labeled cells and removal of unbound NPs.[22] This single step purification strategy enables continuous recovery of labeled suspension and adherent cells suspended in fresh buffer solution without centrifugation. Moreover, it can process up to 10 million cells·ml^{-1}, a cell density amenable for regenerative medicine applications.

Protocol

1. Nanoparticles (NPs) Labeling of Mesenchymal Stem Cells and Monocytes

1. Culture mesenchymal stem cells (MSCs) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics to ≥80% confluency prior to labeling. Similarly, culture THP-1 cells (ATCC) in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS to a density of ~10^6 cells/ml.
2. Load silica NPs (~500 µm) with calcein dye solution (200 µM) using overnight stirring. Fabricate PLGA-calcein AM (CAM) using a protocol described previously.[22]
    1. Dissolve 250 µg CAM and 100 mg PLGA (50:50) in chloroform at 4 °C.
    2. Generate single emulsion NPs using a high speed homogenizer (13,600 x g, 60 sec) at room temperature. Evaporate the chloroform in a chemical hood (≥3 hr) before collection using centrifugation (3,400 x g for 5 min), washing (double distilled water), freeze-drying and storage in -20 °C.
3. Incubate CAM-PLGA NPs (1 mg) or calcein silica NPs (150 µg) in 0.01% poly-L-lysine (PLL) solution at room temperature for 15 - 20 min.
4. Centrifuge at 3,400 x g for 5 min to remove excess PLL supernatant before NPs resuspension in 1 ml of respective culture medium.
5. Incubate the NPs with cells (MSCs or THP-1, ~1 - 2 x 10^6 cells in total) for approximately 24 hr (0.1 mg·ml^{-1} labeling concentration).
6. Dissociate and harvest the labeled adherent MSCs using 2 ml of 0.25% trypsin (5 min, 37 °C) and quench with 6 ml of DMEM. Spin down the cells at (1,000 x g, 4 min) and resuspend to a concentration of 10^5 - 10^6 cells/ml for microfluidic processing. Use labeled THP-1 cells (10^5 - 10^6 cells/ml) directly for microfluidics purification.

2. Microfluidic Device Preparation

1. **Device Fabrication**
    1. Fabricate the microfluidic spiral device (500 µm (w) × 115 µm (h)) with polydimethylsiloxane (PDMS) from a commercial kit using standard soft lithography steps.[23]
    2. Mix 30 g of base prepolymer and 3 g curing agent thoroughly in a weighing boat. De-gas the mixture in a desiccator for 60 min to remove any air bubbles.
    3. Pour the PDMS mixture onto the silicon wafer master mold patterned with the spiral channel design carefully to a height of ~5 - 10 mm.
    4. De-gas the mixture in a desiccator vacuum for 60 min again to remove any air bubbles. Repeat the process until all bubbles are eliminated.
    5. Cure the PDMS mixture in an 80 °C oven for 2 hr until the PDMS is set. Ensure the wafer is not tilted during curing to have a constant device height.
    6. Cut out the PDMS spiral device using a scalpel and carefully peel the PDMS slab from the master mold.
    7. Trim the edges of the device with a scalpel to ensure smooth surface for bonding.
    8. Punch two holes (1.5 mm) for the inlets and two holes (1.5 mm) for the outlets on the PDMS device using a 1.5 mm biopsy puncher.
    9. Wash the device with isopropanol (IPA) to remove any debris and dry the device in an 80 °C oven for 5 min.
    10. Clean the bottom surface of the PDMS device (with channel features) using masking tape.
    11. Clean one side of a glass slide (2" by 3") using masking tape.
    12. Carefully place and expose the cleaned surfaces of the PDMS device and glass slide in the chamber of a plasma cleaner and subject them to vacuum for 60 sec. Next, switch on the plasma power to maximum and lower the chamber pressure until the chamber turns pink in color.
        1. Expose the surfaces to air plasma for 60 sec. The plasma creates reactive species on the exposed surfaces of the PDMS and glass which enables tight bonding when brought into physical contact. Turn off the plasma power and release the pressure from the plasma cleaner to retrieve the device and glass slide.
    13. Bond the PDMS device and glass slide together by pressing the plasma-exposed surfaces tightly and ensuring that no bubbles are trapped between the two surfaces.
    14. Heat the bonded device using a hotplate set at 80 °C for 2 hr to strengthen the bonding.
2. **Device Operation**
    1. Cut two pieces of tubing (1.52 mm OD) of ~15 - 20 cm for the inlet syringes and attach a syringe tip (gauge 23) on one end of each tubing.
    2. Cut two pieces of tubing (1.52 mm OD) of ~5 - 10 cm for the outlets and attach to the outlet holes of the PDMS device.
    3. Prior to sample running, manually prime the device with a syringe containing 70% ethanol until it flows out of the outlet tubing. Allow the ethanol sit for 30 sec to 1 min to sterilize the device.
    4. Load 30 ml of filtered (0.2 µm pore) sheath buffer (Phosphate-Buffered Saline (PBS) with 0.1% Bovine Serum Albumin (BSA)) into the 60 ml syringe and secure the syringe on a syringe pump. Set the pump to the correct settings (Syringe size: 60 ml, Volume: 60,000 µl). Note: The addition of BSA to PBS is to minimize cell-cell binding and non-specific binding between cells and the PDMS device.

5. Load 3 ml of labeled cells into the 3 ml syringe and secure the syringe on a separate syringe pump. Set the pump to the correct settings (Syringe size: 3 ml, Volume: 3,000 µl).
6. Check that no air bubbles are trapped in the syringes to ensure a stable flow. Remove any air bubbles by gently ejecting few drops of liquid out of the nozzle.
7. Connect the inlet syringe tips and tubing to the syringes, and insert them into the respective inlets of the device (sheath and sample inlet). Ensure that there are no bubbles along the tubing.
8. Mount the devices onto an inverted phase-contrast microscope for real time imaging during cell sorting process.
9. Secure a small waste beaker and two 15 ml tubes close to the device on the microscope stage using adhesives.
10. Place the outlet tubings into the waste beaker.
11. Set the flow ratio for sample to sheath buffer to 1:10 and start both syringe pumps to initiate the sorting process (Example: 120 µl/min for sample syringe and 1,200 µl/min for sheath syringe; channel flow velocity ~0.38 m/sec).
12. Run the device for 1.5 min for the flow rate to stabilize. This can be confirmed by presence of inertially focused cells near the channel inner wall under bright-field with phase contrast using a high-speed camera (~5,000 - 10,000 frames per second (fps), exposure time: 10-50 µsec).
13. Position the outlet tubings into different tubes to collect the eluents from the cell outlet and waste outlet.

Representative Results

After labeling the cells with bio imaging agent-loaded NPs overnight, the labeled cells (containing free particles) are harvested and purified by DFF spiral microdevice to remove free NPs in a single step process (**Figure 1A**). The 2-inlet, 2-outlet spiral microchannel is designed by engineering software and microfabricated using SU-8 photoresist. The patterned silicon wafer is then used as a template for PDMS replica molding using soft lithography techniques (**Figure 1B**). To perform cell sorting, the cell sample is pumped into the outer wall inlet of the spiral microdevice while the inner wall inlet runs fresh buffer solution at a higher flow rate (1:10) to pinch the cell sample flow. Separation is achieved by inertial focusing of larger labeled cells at the inner wall and Dean-induced recirculation of small unbound NPs towards the outer wall (**Figure 2A**). The device was first applied to sort labeled suspension monocytic cells (THP-1) from fluorescent silica NPs loaded with calcein (~500 nm in size). High separation efficiencies were obtained as confirmed by high speed imaging (**Figure 2A**) and flow cytometry analysis (**Figure 2B**). Images of eluents collected from device cell outlet also indicated efficient removal of silica and PLGA NPs (~1 - 5 µm) from THP-1 cells. In addition, fluorescence imaging showed that internalized NPs in the labeled THP-1 cells were well retained during DFF purification (insets) (**Figure 3**). Because of MSC size heterogeneity, the spiral microchannel geometry was subsequently modified by changing the channel height and bifurcation design to enable efficient collection of MSCs (**Figure 4**).

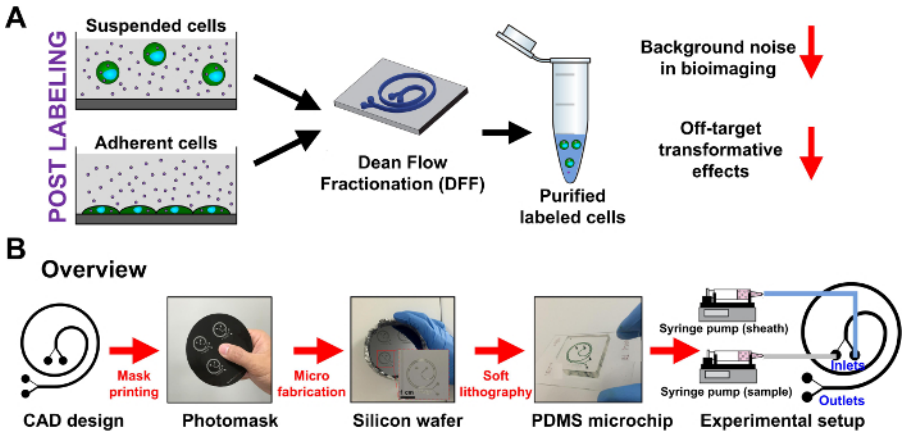

Figure 1. (**A**) Overall workflow for rapid single-step micro/nanoparticle (NPs) removal from suspension and adherent cells post-labeling using Dean Flow Fractionation (DFF) microfluidic technology. (**B**) The DFF spiral device is designed by engineering software and patterned on silicon wafer using microfabrication. PDMS devices are subsequently produced by soft lithography methods. Experimental setup consists of 2 syringe pumps to infuse labeled cells and fresh saline buffer at different flow rates into the device for continuous purification and collection of labeled cells. Please click here to view a larger version of this figure.

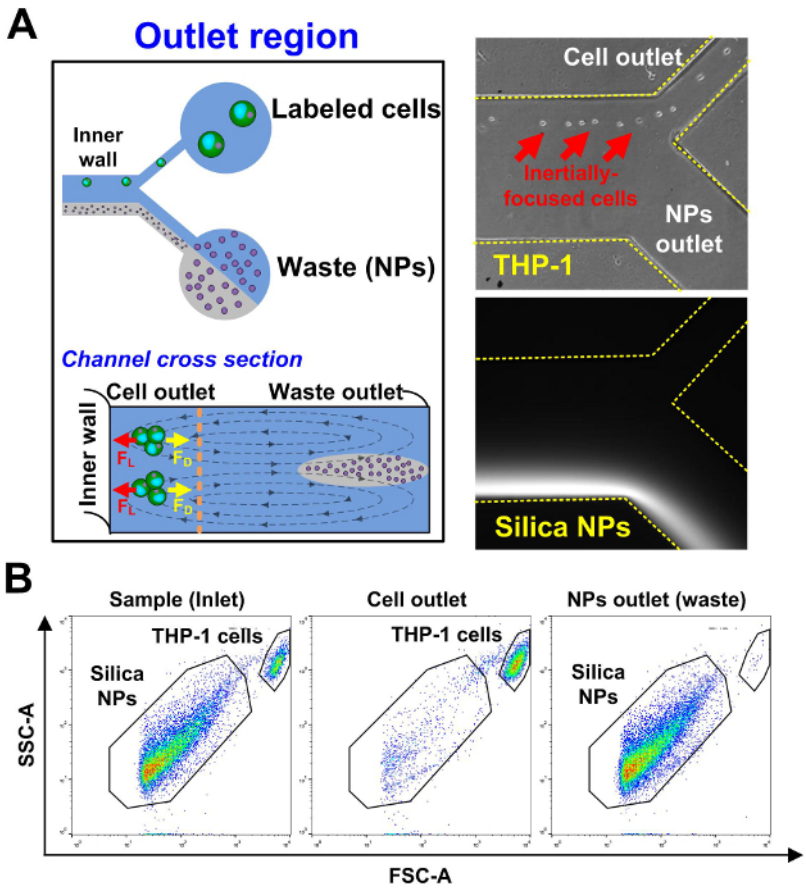

Figure 2. Nanoparticles removal from labeled THP-1 cells via DFF. (**A**) Illustration of DFF separation principle. Larger labeled cells experience strong inertial lift forces (F_L, red arrows) and Dean drag forces (F_D, yellow arrows) and focus near the microchannel inner wall. Smaller NPs and buffer solution are solely affected by Dean drag and recirculate to the outer wall to achieve separation. Representative high speed and fluorescence images indicating equilibrium positions of THP-1 cells (red arrows) and silica NPs loaded with calcein dye at the outlet region. (**B**) Separation performance based on flow cytometry analysis with gating determined by size (FSC-A) and granularity (SSC-A) characteristics. Please click here to view a larger version of this figure.

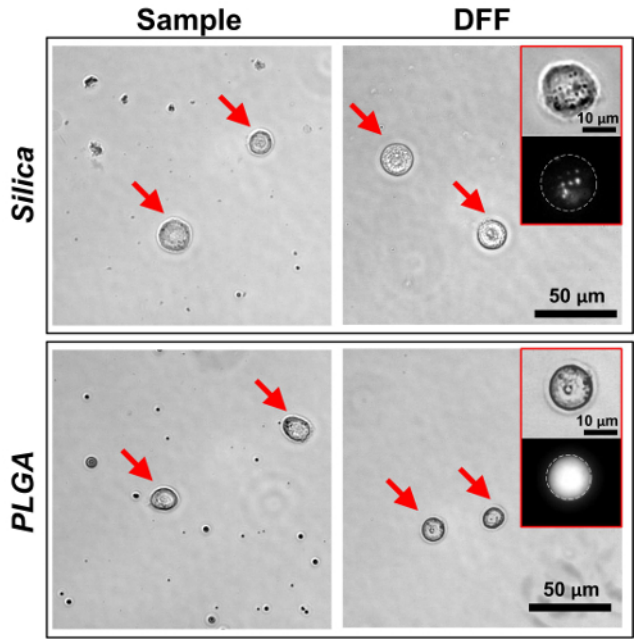

Figure 3. Representative bright field images of purified labeled THP-1 cells (red arrows) and NPs removal efficiency using DFF. Insets show the respective phase contract (above) and fluorescence microscopy images of individual cells. Please click here to view a larger version of this figure.

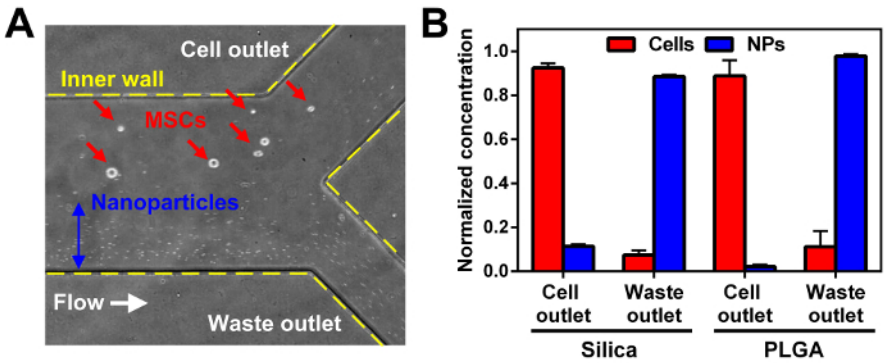

Figure 4. Nanoparticles removal from mesenchymal stem cells (MSCs) via DFF. (**A**) Representative high speed image illustrating efficient separation of MSCs (red arrows) and PLGA NPs (blue arrows) into different outlets. Yellow dashed lines indicate the approximate position of the microchannel walls. (**B**) Separation performance of MSCs from silica and PLGA NPs. Please click here to view a larger version of this figure.

Discussion

The DFF cell purification technology described herein enables rapid and continuous separation of labeled cells in a high throughput manner. This separation approach is ideal for large sample volume or high cell concentration sample processing, and is better than conventional membrane-based filtration which is prone to clogging after extended use. Similarly, affinity-based magnetic separation requires additional cell labelling steps which are laborious and expensive. The purified cells are shown to retain their labeled agents and cell morphology well (**Figure 3**) with minimal flow/shear-induced effects (shear stress τ, ~200-250 dyne/cm^2) due to short residence time within the channel (< 1 sec) [20,24]. In addition, the user can choose the eluted solution of the sorted labeled cells by simply substituting the sheath buffer (saline) with the desired media or buffer solution.

It is critical that there is no dirt or debris in the microchannel prior use which can adversely affect cell focusing behavior. This can be achieved by cleaning the inlet holes thoroughly with IPA, as well as using masking tape to clean the PDMS surface before plasma bonding. Since the microdevice operates under high flow conditions (~1 - 1.5 ml/min), it is also important to heat the bonded PDMS devices on a hotplate to ensure strong bonding between the PDMS and the glass slide. During device operation, if the cells are not focusing well, it means that the flow rates are not sufficient. One can troubleshoot by checking the device inlet region and the syringe tips to ensure that there are no leakages.

Removal of unbound NPs from labeled cells is not trivial since common laboratory separation techniques (*e.g.,* centrifugation) cannot separate particles/cells of different sizes (*e.g.,* unbound particles and labeled cells). While one can argue that repeated washing and changing of culture media can remove free NPs from adherent cells to a certain extent, the process is laborious and ineffective for suspension cell cultures. The DFF microfluidic device utilizes size-based sorting and is highly versatile as it can be used for both suspension and adherent cells with a large size range, as well as removal of NPs of different materials. If the target cells are of different sizes, the DFF device can be optimized by changing the

flow conditions or channel geometry (*e.g.,* channel height and ratio of inner and outer channel widths) to achieve efficient separation. It has been previously demonstrated that DFF is superior compared to conventional centrifugation in NPs removal with minimal cell loss.[22] Furthermore, cell recovery performance remained high (>90%) when the sample concentration was increased to 10^7 cells/ml. This is a key improvement over existing inertial microfluidic cell separation methods which are generally limited to 10^5 cells/ml capacity due to cell-cell interaction and cell overcrowding at equilibrium positions.[25]

While the DFF technology achieves separation based on size difference, it cannot be used for other cell engineering processes such as separation of labeled and unlabeled cells due to negligible change in cell size. Cell surface markers for affinity-based sorting and other common separation modalities (magnetic susceptibility and electrical conductivity) could be considered for such potential applications. DFF technology is also not suitable for multiplexed cell sorting based on fluorescence. A better option would be to use fluorescence activated cell sorting (FACS) for single-step sorting of labelled cells with different fluorescence which operates at high throughput, but requires expensive and bulky equipment.

Finally, this protocol is highly useful for the purification of engineered cells when NPs containing transformative agents (growth factors, drugs, corticosteroids, *etc.*) are used for bioimaging and/or cell therapy. This is because remnant free NPs can exert off-target transformative effects on neighboring cells or immune cells that respond to these foreign particles when injected *in vivo*. It may also unintentionally generate signals that can be misleading during bioimaging. Removal of these free NPs prior to usage thus reduce risk to non-target cells, minimize compilations in immune system, and reduce contamination issues between steps during sequential labeling/engineering of cells. Its successful implementation greatly facilitates cell labeling and enables the interference-free usage of particle based cell engineering in therapy.

Disclosures

The authors have nothing to disclose.

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