Published in final edited form as:

Anal Chem. 2012 December 4; 84(23): 10160–10164. doi:10.1021/ac302855m.

Motility Based Cell Sorting by Planar Cell Chromatography

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Abstract

We report here a new methodology for sorting mammalian cells based on their intrinsic motility on planar substrates, independent of chemoattractants and external fields. This biological analogue of thin layer chromatography consists of arrays of asymmetric adhesive islands on tissue culture dishes that rectify the random movement of cells and direct their migration in a specific direction. We demonstrated the use of planar cell chromatography in the separation of mixtures of 3T3 fibroblasts that express constitutively active Rac1 or RhoA and mixtures of 3T3 fibroblasts and SH-SY5Y neuroblastoma cells.

Keywords

Cell sorting; Cell migration; Cell micropatterning

Cell purification plays a central role in medicine and cell biology, and cell motility is a singularly important index for sorting cells. Proper regulation of cell motility is critical in embryonic development and homeostatic processes such as immunity and wound healing, while its misregulation is implicated in pathologies including chronic inflammatory conditions, vascular diseases, and cancer metastasis. Here we present a biological analogue of thin layer chromatography plates for purifying cells using micropatterned culture dishes on which cells autonomously sort themselves based on their relative motility. As proof of concept, we separated mixtures of 3T3 fibroblasts that express constitutively active Rac1 or RhoA. Requiring no pre-labeling, gradients, external fields, nor mobile phases, planar cell chromatography can be a routine protocol for fractionating cells according to their motility. Cell chromatography, with its ability to fractionate fast and slow cells from an otherwise

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homogeneous cell population, can thus be a powerful tool in conjunction with proteomic analysis for unraveling signaling pathways.

Planar cell chromatography plates rely on the strategic placement of cell adhesive microarray islands to amplify the natural directional persistence (MANDIP) of cells and direct their migration over unlimited distances without external fields or stimulus gradients. (1) Without extracellular cues such as gradients in substrate characteristics or chemoattractants cells migrate with only a small measure of directional persistence. When constrained geometrically by their adhesion microenvironment, cells form focal adhesions and extend lamellipodia preferentially from their extremities. (2,3) On teardrop-shaped islands, cells reorganize their actin bundles and extend lamellipodia from either their sharp tips or blunt ends in directions aligned to the polarity axis defined by their elongated cell bodies. (1) By strategically positioning adhesive islands directly in front of the blunt ends of the teardrop islands and rotationally offsetting islands adjacent to the tips, the MANDIP pattern shown in Figure 1 promotes directional migration upwards whilst limiting downwards migration as the tips of the tear drops point to no attachable landing regions within 60° of the polarity axis.

The area of the teardrops (1000 μm^2) and the distance between islands (3.5 μm) are set to enable extended lamellipodia to span the gaps between islands while momentarily constraining the cells to adopt the shape of the islands. The converging and diverging paths direct cells upwards and offer faster cells a multitude of overtaking opportunities. As proof of concept, we use cell chromatography to separate mixtures of 3T3 fibroblasts that express constitutively active Rac1 (CA-Rac1) or RhoA (CA-Rho).

The Rho superfamily of small GTPases such as Rac1 and RhoA are important regulators of cell migration. (4,5) A mixture of 1500 constitutively active Rac1 and 1500 constitutively active RhoA mutants of NIH 3T3 fibroblasts was randomly plated in the seeding area and the resulting autonomous separation between the two cell populations is shown in Figure 2. Although unnecessary for the separation, the CA-Rac1 mutant here is labelled with green fluorescent protein (GFP) to visualize the outcome. From separate experiments on cyclically arranged teardrops (Figure 3/Table 1) the CA-Rac1 and CA-RhoA mutants have average island hopping periods of 3.0 and 1.4 hrs/hop respectively. With the difference in hopping period, we expect that more rapid moving CA-RhoA cells would move out from the seeding area into the MANDIP micropattern first and the separation would be enhanced with each hopping. Noticeable separation is observed after the first 12 hrs while the cells migrate and proliferate simultaneously. With the close spacing of the adhesive teardrop islands, cells were able to extend across islands and continue to proliferate. This is different from isolated micropatterns wherein cells were observed to switch from growth to apoptosis when confined on islands with decreasing size that restrict cell extension (6). After 72 hrs, quantitative baseline separation is achieved (Figure 2) with the CA-RhoA cells migrating further due to their faster hopping rate. After 72 hrs, cells located beyond 600 μm from the seeding reservoir were collected with a sterile cell scraper and flow cytometry confirmed that >95% of the cells are CA-RhoA mutants.

In contrast, the same 50:50 mixture of CA-RhoA and CA-Rac1 fibroblasts exhibits typical random migration and no practical separation when seeded on an unpatterned area of the same size (Figure 2). The net distance of migration is also smaller when compared to the same cells on the MANDIP array. Differences in the random migration speeds of these two cell populations result in a barely discernable increase in the number of CA-RhoA mutants at farther distances. Flow cytometry of cells located 600 μm and beyond from the seeding area confirmed this with only 54% of the population being CA-RhoA mutants.

Cell chromatography can also be used to separate cells of different phenotype, e.g., 3T3 fibroblasts and SH-SY5Y neuroblastoma cells (Figure 4). Baseline separation of the two cell types is achieved after 72 hrs and FACS analysis of the enriched population of faster cells report >95% 3T3 fibroblasts. Naturally, each cell type will have specific island dimensions and gap distance that maximizes hopping rates and directionality. MANDIP arrays having spatial gradients in island dimensions and gap distances, analogous to mixed-mode chemical chromatography, are expected to yield even more efficient separation for mixtures of different cell types.

Planar cell chromatography differs from traditional Boyden chamber assays of cell migration in two important ways: (1) Boyden chamber assays are end-point determination assays. Only cells that transverse the membrane are observable. In contrast MANDIP-based cell chromatography permits real-time visualization of the entire cell population and requires less than 10^3 cells. This is particularly significant for studies involving rare stem cells or tissue biopsies, for example. (2) Boyden chamber assay migration resulting from a combination of gradient directed chemotaxis and random cell movements (chemokinesis). In contrast, MANDIP-based chromatography is purely the result of directionally rectified chemokinetic cell movements and no chemotactic gradient are necessary. While Boyden chambers assays can be operated without a chemotactic gradient, the random chemokinetic movements alone yield impractically small separation.

Planar cell chromatography has applications in mechanistic studies of cell migration and significant clinical and diagnostic potential as a method for concentrating abnormally motile diseased cells. This approach offers three advantages: 1) It provides sorting based on the intrinsic motility of cells; 2) It utilizes only a substrate with preprinted microarrays and requires no additional equipment; 3) It requires no pre-labeling. Cell chromatography is also a useful complement to flow cytometry, enabling motility-indexed fractionation of cell populations to reduce variability in biological analysis and enhance detection of changes in phenotype. The most obvious disadvantage of cell chromatography is that it is much slower than volume based (7) or density gradient separation (8), magnetic separation, or flow cytometry (9) as the process is limited by the intrinsic cell motility. It may be possible to enhance the cell motility by optimizing the pattern design (10) or utilizing growth factors or chemokines. Advances in the manufacture of micropatterned substrates (11–15) or topographical geometries (16–21) for directing cell migration would enable rapid commercial-scale implementation of cell chromatography on disposable tissue culture dishes and other biocompatible platforms that may become as ubiquitous as TLC plates in chemical laboratories.

Experimental Methods

Cell sorting arrays were imprinted on tissue culture dishes by contact printing. Briefly, silicon wafers with MANDIP patterns were prepared by photolithographic techniques. From this silicon master, complementary polydimethylsiloxane (PDMS) replicas were prepared and used as stamps in subsequent microcontact printing steps to print the background region with cell resistant poly(oligoethyleneglycol methacrylate co methacrylic acid) directly on cell culture dishes (13). Random copolymers of oligoethyleneglycol (OEGMA – 550 MW OEG) and methacrylic acid (MA) from Scientific Polymer Products, NY were prepared by free radical polymerization of 10 wt% methanolic solutions of the two monomers (80:20 OEGMA to MA mass ratio) at 60°C. Polymerizations were initiated with 1 wt% (with respect to monomer) of 2,2'-azobis(2 amidinopropane) dihydrochloride.

The constitutively active Rac1L61 and RhoA L63 NIH 3T3 cells (22) were kind gifts of Dr. Yi Zheng's laboratory (Cincinnati Children's Hospital). NIH 3T3 cells were cultured in Opti

MEM media (Invitrogen) with L-Glutamine, Hepes (25mM), and 10% serum supreme (Cambrex biosciences). RhoAL61 cells were grown by selection with G418 (350 μ g/ml). Human neuroblastoma cells, SH-SY5Y, were cultured in Opti-MEM with 10% fetal bovine serum. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Subconfluent monolayers were dissociated with 0.01% solution of trypsin, resuspended into a fresh basal medium containing 10% serum, and then plated sparsely (~1500 cells) on the seeding area of the cell sorting microarray.

After printing the location of the seeding area is located using an optical microscope and precisely marked at the back of culture dish with a scalpel. Similar markings were made at 600 μ m and 1200 μ m from the seeding area. Tissue culture dishes patterned with marked sorting microarrays were sterilized with UV light for 2 hours then exposed to PSN (penicillin (500 U/ml), streptomycin (0.5 mg/ml) and neomycin (1mg/ml) solution in PBS) for 30 minutes. The microarrays on the tissue culture dish were not precoated with extracellular matrix proteins. Mixtures containing 1500 cells of each type to be sorted were seeded in the marked reservoirs while the seeding area is covered with a thin sheet of PDMS. After 6 hrs the mixture of plated cells was washed twice with PBS then restored with Opti-MEM media containing 10% serum. Mixtures containing constitutive active mutants RhoA were restored with Opti-MEM media containing 10% serum selected with G418 (350 μ g/ml) (Invitrogen) and 10 ng/ml of PDGF. Cell sorting was conducted over 4 days after seeding the cells. Images were recorded at 12 hr intervals and the media was replaced every 24 hrs.

The number of each cell type on the top half of the array after sorting was determined by flow cytometry. Following 4 days of incubation over the cell sorting microarray, cells located between 600 μ m and 1200 μ m from the seeding area were scraped, resuspended in PBS, and passed through a sterile cell strainer. Cells were scraped from parallel sorting experiments on 5 culture dishes to collect ~3000 cells. FACS analysis were performed using a Becton Dickinson FACSCalibur.

Time lapse images were acquired using a Nikon TE 2000 microscope equipped with a CCD camera. At each time interval, phase contrast and fluorescent images were acquired over the entire cell sorting microarray, which spans a much larger area than the field of view of each image. Panoramic images were prepared by manually stitching the phase contrast and fluorescent images then merging these to visualize both GFP and non-GFP labeled cells (Metamorph and Photoshop). The number of cells at each migration distance was counted manually with a resolution/bin size of 75 μ m.

Acknowledgments

This work was supported by the National Institutes of Health (R01EB010043 and R21EB005348) and the National Science Foundation (CBET0928219). We thank Dr. Yi Zheng's laboratory for providing the constitutively active Rac1 and RhoA fibroblasts.

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Figure 1.

MANDIP pattern for planar cell chromatography. Cell-adhesive teardrop-shaped islands on tissue-culture dishes, defined by a background of cell-resistant poly[(oligoethyleneglycol methacrylate)-co-(methacrylic acid)] momentarily constrain the shape of cells. The superimposed image of a fibroblast and its actin filaments (red) and nucleus (blue) shows schematically how fibroblasts constrained on tear-drop islands extend lamellipodia, from both the blunt ends and sharp tips of their teardrop shapes, within envelopes that are preferentially aligned to their extended cell body. The placement of adjacent islands, directly in front of the blunt ends and 60° sideways relative to the tips, acts as a ratchet enhancing the probability that lamellipodia extended from the blunt ends will attach resulting in upwards-biased cell “hopping”. Overall, the hexagonal arrangement of islands in this MANDIP pattern rectifies the random chemokinetic motions of cells in the upwards direction along a multitude of pathways that enable faster cells to overtake slower moving cells.

Figure 2.

Autonomous migration and proliferation of CA-Rac1 (GFP-labelled green) and CA-RhoA 3T3 fibroblast mutants on a culture dish imprinted with the MANDIP cell sorting pattern (Figure 1) and control experiment on an unpatterned culture dish. Mixtures containing 1500 fibroblasts of each type were plated in the seeding area at 0 hr. On the MANDIP pattern, both fibroblast mutants migrate directionally upwards based on their relative motility. More motile CA-RhoA fibroblasts outrun the CA-Rac1 fibroblasts resulting in baseline separation after 72 hrs. The control experiment with no pattern yields no practical separation.

Figure 3.
Autonomous directional migration of CA-Rac1 and CA-Rho NIH 3T3 fibroblasts on circular MANDIP arrays of four tear-drop shaped islands ($1000 \mu\text{m}^2$ area, $3.5 \mu\text{m}$ spacing).

Figure 4.

Autonomous migration and proliferation of CA-RhoA (GFP-labelled green) fibroblasts and SH-SY5Y neuronal cells on a culture dish imprinted with the MANDIP cell sorting pattern (Figure 1). Mixtures containing 1500 cells of each type were plated at the bottom of the pattern at 0 hr. Both cell types migrate directionally upwards based on their relatively motility. More motile fibroblasts outrun the SH-SY5Y cells resulting in baseline separation after 72 hrs.

Table 1

Directionality and hopping speed of CA-Rac1 and CA-Rho NIH 3T3 fibroblasts on MANDIP arrays shown in Supplementary Figure 1.

Cell Type	# Observed Migrations		Average time for single "hop" (hrs)
	CW	CCW	
NIH 3T3-CA-Rac1	58	3	2.95 ± 0.97
NIH 3T3-CA-Rho	78	5	1.35 ± 0.81