Video Article

Assessing Neural Stem Cell Motility Using an Agarose Gel-based Microfluidic Device

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Abstract

While microfluidic technology is reaching a new level of maturity for macromolecular assays, cell-based assays are still at an infant stage[1]. This is largely due to the difficulty with which one can create a cell-compatible and steady microenvironment using conventional microfabrication techniques and materials. We address this problem via the introduction of a novel microfabrication material, agarose gel, as the base material for the microfluidic device. Agarose gel is highly malleable, and permeable to gas and nutrients necessary for cell survival, and thus an ideal material for cell-based assays. We have shown previously that agarose gel based devices have been successful in studying bacterial and neutrophil cell migration[2]. In this report, three parallel microfluidic channels are patterned in an agarose gel membrane of about 1mm thickness. Constant flows with media/buffer are maintained in the two side channels using a peristaltic pump. Cells are maintained in the center channel for observation. Since the nutrients and chemicals in the side channels are constantly diffusing from the side to center channel, the chemical environment of the center channel is easily controlled via the flow along the side channels. Using this device, we demonstrate that the movement of neural stem cells can be monitored optically with ease under various chemical conditions, and the experimental results show that the over expression of epidermal growth factor receptors (EGFR) enhances the motility of neural stem cells. Motility of neural stem cells is an important biomarker for assessing cells aggressiveness, thus tumorigenic factor[3]. Deciphering the mechanism underlying NSC motility will yield insight into both disorders of neural development and into brain cancer stem cell invasion.

Protocol

Procedure

Coating slides with fibronectin

Prior to the assembling of the microfluidic device, sterilize glass slides with fibronectin. Coat the slides in a biohood to maintain sterility. Align a sterile PDMS spacer along the edges of the slide and mark the side that is facing up with a permanent marker. Pipette 1 ml of a 5 µg/ml solution of fibronectin (Sigma) inside the PDMS spacer over the entirety of the glass slide. Leave the slides undisturbed for an hour, then dry completely using an N_2 gun. The slides can be stored at 4ºC for later experiments.

Assembling the device

The entire device assembly protocol is done in a biohood under sterile conditions, using the following procedures:

1. Clean the silicon master with the microchannels patterned on it by wiping it down with 70% ethanol and drying with the N_2 gun. Place a sterile PDMS spacer of 1 mm height using forceps around the relief features of the master.
2. Prepare the agarose gel used in the device by weighing 0.3 g of agarose powder (Fisher Scientific) and 10 ml of CO_2-independent media (Invitrogen) in a 50 ml beaker. Stir the mixture with a spatula and heat in a microwave for 20 seconds on HIGH. If undissolved granules are present, heat the mixture for another 10 seconds and swirl the hot mixture to get rid of the last remaining granules. Repeat the process with a heating time of 8 seconds, and then 5 seconds.
3. Quickly pour the agarose solution onto the silicon master surrounded by the PDMS spacer and immediately cover the agarose mixture with a sterile glass slide. Apply a constant, gentle pressure to the slide for about 2 minutes to ensure that the agarose will gel to the same constant 1 mm height as the PDMS spacer.
4. After the agarose gels, peel off the patterned agarose gel with the PDMS spacer from the silicon master and transfer to a fibronectin coated glass slide with the microchannels in the agarose gel facing the slide. Use a 16 gauge syringe tip to punch holes into the reservoir regions of the microchannels for inlet and outlet accesses. Add 500 µl of CO_2-independent media to the patterned agarose gel on the slide to prevent desiccation of the microchannels.
5. Wash the acrylic manifold used in the device with 70% ethanol and rinse with DI water. Align the holes in the manifold with the holes punched into the agarose gel with the microchannels. Then, align the manifold with the gel, PDMS spacer, and the fibronectin slide with the metal frame of the device. Place screws into device and tighten to the point of resistance using a screwdriver. This is done to ensure that the device is not over-tightened to the extent of deforming the microchannels in the agarose. Tighten the screws in a clockwise order to ensure that the gel inside the device does not shift. Test the device for leaks and blockages in the microchannels using a 1ml syringe. The syringe is fitted with Tygon tubing with a gel-loading pipette tip at the end of the tubing to inject CO_2-independent media in the microchannels. A microchannel is deemed successfully formed when media is observed to only exit from the outlet of the microchannel when media is injected into the corresponding inlet. Now, the device is ready for use.

Preparing and Seeding the cells in the device

When the cells reach 70% confluency (cell density around 100,000 cells/ml), they are prepared to be seeded into the device.

Subject the cells to two DPBS (Invitrogen) washes. Add 200 µl of trypsin-EDTA (Invitrogen) to detach the cells. Transfer the cells to a 15 ml centrifuge tube containing 5ml of M2 media containing serum to inactivate the trypsin. Centrifuged the growth media with the cells at 1000 RPM for 5 minutes. Aspirated off the supernatant and resuspend the pellet of cells 5 ml of DPBS. Centrifuge the DPBS cells again under the same conditions. Aspirated off the supernatant again, and resuspend the pellet of cell in 500 µl of CO_2-independent media.

Seed the resulting suspension of cells into the center microchannel of the device via gravity-driven flow. Add the inlet and outlet of the center microchannel with 60 µl of the cell suspension and 20 µl of CO_2-independent medium, respectively. Observe the center microchannel under a brightfield microscopy to look for cell adhesion. The cells usually adhere after 10 minutes. After the cells adhere to the channel bottom at a proper density, pipeted out the remaining cell suspension in the inlet and the media in the outlet. Place 60 µl of CO_2-independent media in both inlet and outlet of the center channel. Place PDMS covers over the center inlet and outlet to minimize evaporation of media from the center channel.

Imaging the cells in the device

Prior to the assembly of the device, thread the sterile peristaltic tubing through the weather station of the microscope (Olympus X81) and connect with the peristaltic pump (Watson-Marlow 205U/CA). Flush PBS through the tubing at a rate of 4 RPM. After the assembly of the device, prepare appropriate concentrations of epidermal growth factor (EGF) (Sigma) solutions in the CO_2-independent media in a 15 ml centrifuge tubes. Vortex the EGF solutions for 5 seconds and connect with the tubing. Connect the tubings marked with corresponding EGF concentrations to the device. It takes about an hour for the EGF to diffuse across the channel ridge and establish a steady chemical gradient.

An imaging software, Slidebook, is used to take a set of images at every 10 minutes for about 5 hours in an experimental run. The x-y automated stage is programmed such that two sub-areas (each with an area of 400µm x400µm) of the same center channel, and a total of three center channels are imaged at a given time point. Cells in different center channels (typically 3 in one run) have different EGF concentrations, as marked on the tubing.

Materials and Equipment

Cell lines: C17.2 cells are NSCs derived from the external germinal layer of postnatal mouse cerebellum [6, 7]. Using this cell line, we have developed two other murine stem cell lines using retrovirus transfection method. These two stably transfected cell lines are (1) wtEGFR -- that over-expresses the human wild type EGFR, and (2) ΔEGFR - that is a constitutively active EGFR mutant. For ease of detection, retroviruses carry a backbone with the gene of interests followed by an internal site for ribosomal binding (IRES) and the Green fluorescent protein (GFP).

Cell culture: The mouse C17.2 NSCs and their variants were grown in a 6-well plate in M2 media (DMEM (Invitrogen) with 10% fetal bovine serum (FBS) (Invitrogen), 5% horse serum (HS) (Invitrogen), and 1% penicillin/streptomycin (Invitrogen).) Cells were maintained at 37ºC in 80% air and 5% CO_2.

Microfluidic device: A plastic manifold, a PDMS spacer, and a stainless frame for holding the device in place.

Imaging

An inverted fluorescent microscope (Olympus IX-81) is used, in connection with an intensified CCD camera (Orca, Hamamatsu). The microfluidic device is mounted on an automated x-y-z stage, and the stage is enclosed by an environmental chamber that maintains a temperature of 37ºC. There are usually 3-4 devices on the same chip that will be filmed at the same time point using the automated x-y-z stage.

Flow maintaining

A peristaltic pump with 8 parallel lines (Watson-Marlow 205U/CA) auto is used to maintain flows in all of the sink and source channels.

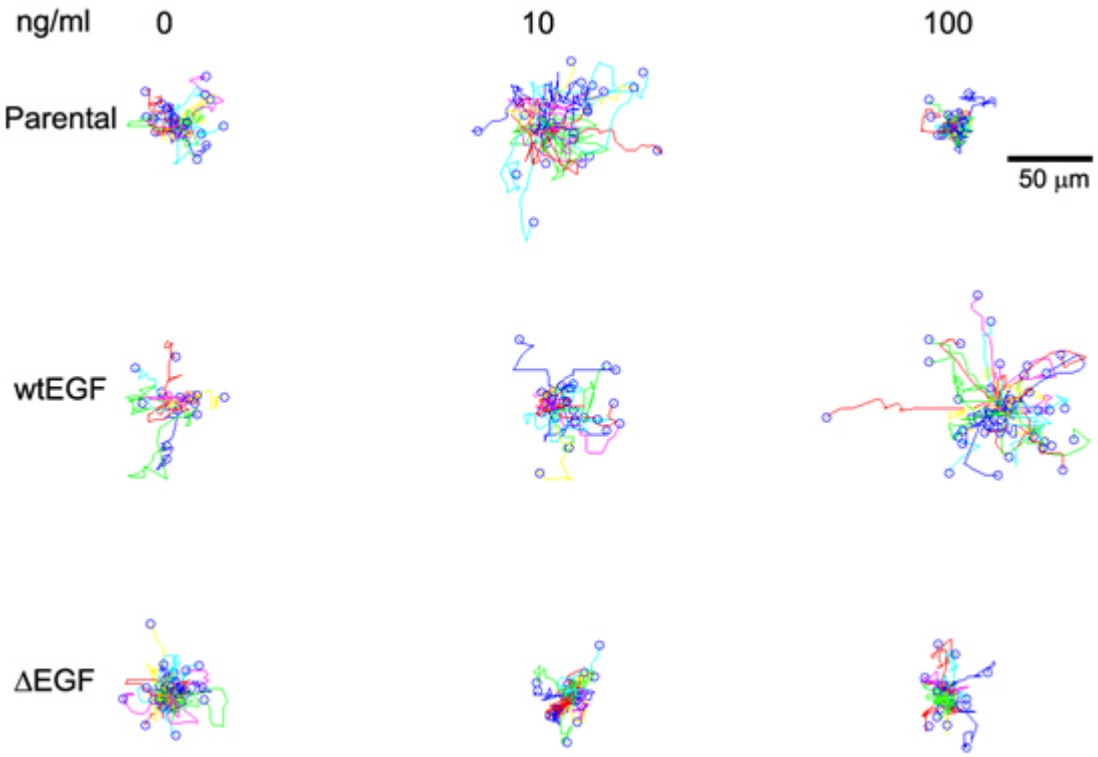

Fig. 1 Motility of three NSC strains (parental, wtEGFR, and DEGFR) in various EGF concentrations All the trajectories are taken from tracking a movie of 5 hours (except for wtEGF 100ng/ml, 4hour), and the origin of all the tracks were repositioned at (0,0) for comparison purpose.

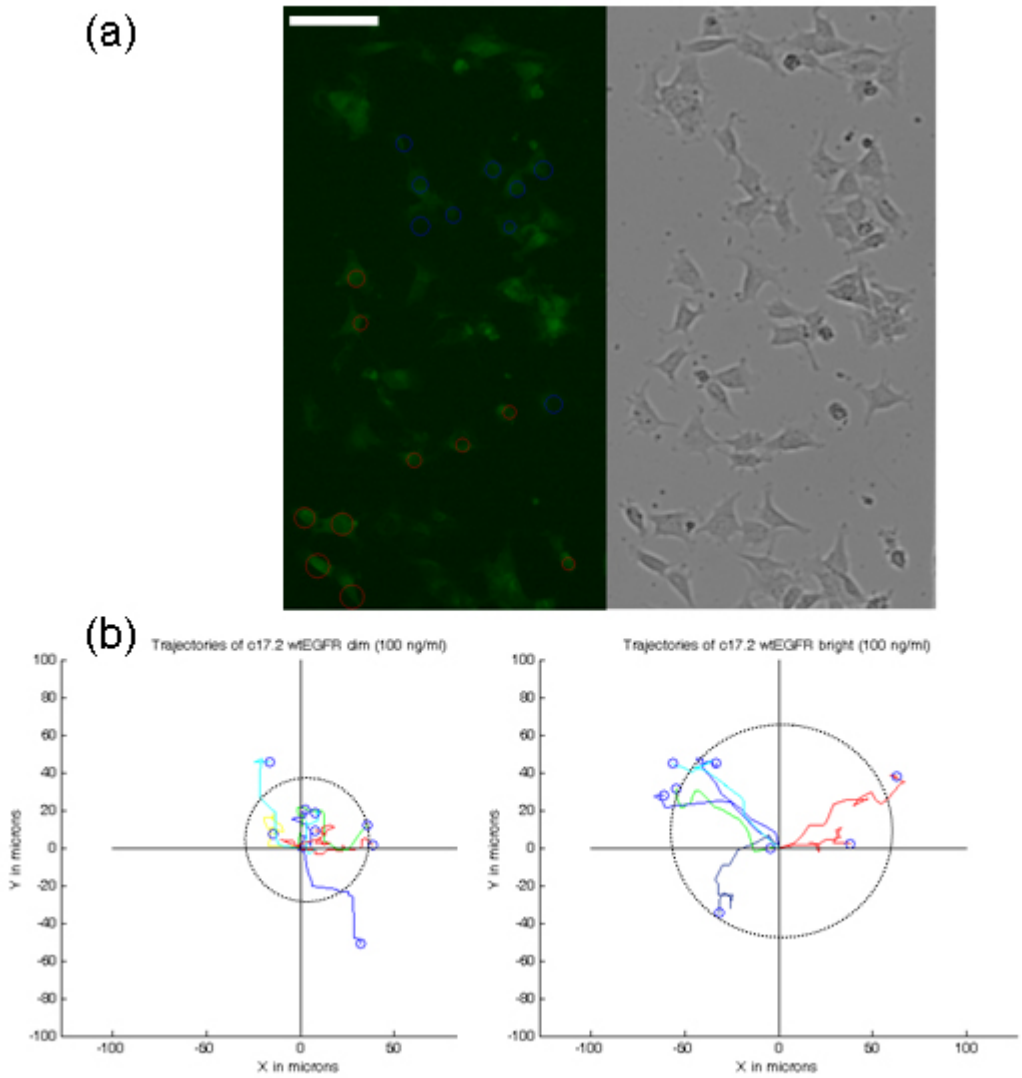

Fig. 2 Correlation of EGFR expression level and motility (a) two images of wtEGF NSCs on the surface of a microfluidic channel with 100ng/ml EGF. The left image is a fluorescent image, and the right is a transmitted bright field image. The scale bar is 100μm, and the Channel width is 400 μm. Cells are separated into two populations by the brightness of the GFP expressed. Cells in the blue circles are dimmer, thus have less EGFR expression level; and cells in red circles are brigher, thus have higher EGFR expression level. (b) Trajectories of cells with low and high EGFR expression level. These trajectories are taken from a four hour-long movie.

Discussion

Fig. 1 shows trajectories of three NSC strains, the parental cells, the wtEGFR and the ΔEGFR (control). Each set of trajectories were obtained from a 5 hours long movie. For parental cells, cell motility peaked when EGF concentration was ~ 10ng/ml; for wtEGFR cells, cell motility peak shifted to 100ng/ml or above; for ΔEGFR cells, the cell motility was independent of EGF concentration as expected.

The wtEGF cells with 100ng/ml were the most motile cells, they moved about 1.5 times faster than the parental cells with 10ng/ml. This indicates that (1) over expression of EGFR enhances cells motility; (2) the number of EGF ligand-receptor biding events has a direct impact on the cell motility. ΔEGFR cell line is a constitutively active EGFR mutant. This mutant appears in ~ 60% of human glioblastoma. It has a truncated extracellular domain that becomes a constitutive active ligand-independent kinase. The intensity of the autophosphorylation is small (approximately 10%) compared with ligand activated wild-type EGFR (wtEGFR) [4]. This underlies the observation that ΔEGF cells have slightly higher motility than the parental and wtEGFR (in the absence of EGF), but the motility is kept a constant in various EGF concentrations.

Since the wtEGFR cells carried same copies of GFP (green fluorescent protein) and EGFR, the fluorescent intensity of the wtEGFR cells reveal the EGFR expression levels. This allows us to directly correlate the EGFR protein expression level (green fluorescent intensity) with the cell motility. Fig. 2 shows the trajectories of two cell population, the cells in red circle are brighter, thus has higher EGFR expression level; and the cells in blue circle are dinner, thus has lower EGFR expression level. The results again demonstrate that the higher the EGFR expression level, the more motile these cells are.

Traditionally, motility of NSCs has been determined using a Boyden chamber assay, where the percentage of cells migrating through a membrane is evaluated[5]. This is a population based assay, where cell behavior can not be assessed individually. The microfluidic device shown here presents an opportunity to study cell motion at a single cell level, and in a well controlled chemical environment. It allows one, for the first time, to correlate directly the EGFR protein expression level with the NSCs motility phenotype in living cells. The small size of the device is especially useful for experiments where cells sources are limited, such as the human stem cells.

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