Video Article

A Simplified System for Evaluating Cell Mechanosensing and Durotaxis *In Vitro*

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Abstract

The composition and mechanical properties of the extracellular matrix are highly variable between tissue types. This connective tissue stroma diversity greatly impacts cell behavior to regulate normal and pathologic processes including cell proliferation, differentiation, adhesion signaling and directional migration. In this regard, the innate ability of certain cell types to migrate towards a stiffer, or less compliant matrix substrate is referred to as durotaxis. This phenomenon plays an important role during embryonic development, wound repair and cancer cell invasion. Here, we describe a straightforward assay to study durotaxis, *in vitro*, using polydimethylsiloxane (PDMS) substrates. Preparation of the described durotaxis chambers creates a rigidity interface between the relatively soft PDMS gel and a rigid glass coverslip. In the example provided, we have used these durotaxis chambers to demonstrate a role for the cdc42/Rac1 GTPase activating protein, cdGAP, in mechanosensing and durotaxis regulation in human U2OS osteosarcoma cells. This assay is readily adaptable to other cell types and/or knockdown of other proteins of interests to explore their respective roles in mechanosignaling and durotaxis.

Video Link

The video component of this article can be found at <http://www.jove.com/video/52949/>

Introduction

The extracellular matrix (ECM) is comprised of a complex array of structural and crosslinking proteins including collagen, fibronectin and laminin. Although it is well established that the ECM provides important structural support for cellular tissues, there is increasing evidence to indicate that cells actively respond to physical changes in their ECM environment to regulate diverse cellular processes including cell survival, differentiation and cell migration. For example, differences in the rigidity of the ECM can drive mesenchymal stem cells towards different lineages, with soft substrates (~1 kPa) promoting neurogenic lineages while stiff (~25 kPa) substrates promote osteogenic differentiation¹. Similarly, an increase in the stromal matrix rigidity has been shown to promote mammary epithelial cell tumorigenesis and invasion into the surrounding tissue^{2,3}.

A particularly interesting aspect of this mechanosignaling activity results in a process known as durotaxis, in which cells migrate preferentially towards a more rigid substrate^{4,5}. Cells constantly sense the physical characteristics of their extracellular environment through integrin receptor binding to the ECM. This, in turn, promotes the accumulation of numerous structural and signaling proteins to their cytoplasmic domains to drive the formation of adhesive structures known as focal adhesions or focal contacts^{6,7}. Since integrins have no inherent enzymatic activity, signals are relayed from the ECM through these accessory proteins to coordinate the cell's response to their changing environment⁸. Accordingly, the identification and characterization of the key proteins involved in regulating mechanosignaling and durotaxis is an important area of investigation.

Various model systems have been developed to study durotaxis *in vitro*, but most have utilized collagen-coated polyacrylamide substrates⁴. However, the preparation of the polyacrylamide substrates can be technically challenging and the collagen used in these assays must be chemically crosslinked to the substrate⁹. Polydimethylsiloxane (PDMS) substrates have been shown to exhibit comparable mechanical properties to the polyacrylamide substrates¹⁰. However, PDMS substrates are prepared by simply mixing a ratio of the base to crosslinker and these substrates can be coated with ECM proteins without the need for chemical crosslinking, thus making PDMS an easier tool to study the effects of rigidity on cell behavior. Herein, we describe how to prepare a simple durotaxis chamber in which a soft PDMS substrate is integrated with a rigid glass coverslip.

The assay, as outlined below, provides a quick and simple method to study durotaxis. For this study we used human U2OS osteosarcoma cells combined with siRNA-mediated knockdown of cdGAP to study the role of this focal adhesion protein in durotaxis¹¹. Importantly, this protocol may be readily adapted to individual requirements. Other cell types may be substituted for the U2OS cells and any protein may be knocked down or overexpressed to determine the effects on cell behavior during durotaxis. Furthermore, this protocol may be adapted to incorporate fluorescently tagged proteins to analyze their dynamics and behavior using FRAP or FRET approaches.

Protocol

1. Preparation of Durotaxis Chambers

1. To prepare one 6-well tissue culture plate, tare the balance with a 50-ml conical tube. Weigh out approximately 10 g of the PDMS base solution in the 50-ml tube (the solution is quite viscous).
2. For a 90:1 substrate (Compliance of ~1 kPa), divide the measured weight of the PDMS base solution in the tube by 90 to determine the correct amount of crosslinker solution needed. Add the calculated amount of the PDMS crosslinker solution to the same tube.
Example: $10 \text{ g} / 90 = 0.11 \text{ g}$. Add 0.11 g of the PDMS crosslinker solution to the PDMS base solution.
NOTE: The base and crosslinker solutions are both provided in the PDMS kit.
3. Vigorously mix the PDMS base/crosslinker mixture for 5 min at RT using a small spatula. At this stage the mixture will contain a large number of air bubbles.
4. Centrifuge the PDMS substrate in a benchtop centrifuge for 5 min at 50 x g at RT to remove the bubbles. If there are still bubbles after the 5 min, centrifuge again.
5. Pipette 1 ml of 90:1 PDMS substrate into each well of the tissue culture treated 6-well plate. Any remaining air bubbles present in the PDMS can be eliminated at this stage by popping them using a 21 G needle. Allow the PDMS to spread for 30 min in the well.
6. Boil 12 mm glass #1 coverslips in distilled water for 5 min. Repeat two times and store the coverslips in distilled water.
7. Place one, dried coverslip into each well of the tissue culture plate by gently touching one side of the coverslip into the PDMS solution then drop the coverslip onto the PDMS. As the coverslip settles, the PDMS will begin to encroach over the edges of the coverslip, but will not completely cover it. This will generate an interface between the PDMS and glass after curing (see **Figure 1A,B**).
8. Incubate the plate at 70 °C in an oven for 16 hr to cure (harden) the PDMS. Place the plate in a cell culture hood and UV sterilize for 10 min.
NOTE: It is best to make the plates within a couple days of use. However, the plates may be wrapped in Parafilm without any buffer and stored at 4 °C for up to 2 weeks without any noticeable decrease in quality.

2. Cell Plating

NOTE: If studying the effect of siRNA-mediated knockdown, perform the knockdown using the manufacturer's instructions or the optimized protocol for the cell type of choice.

1. Coat each durotaxis chamber with 1 ml of 10 µg/ml fibronectin in PBS without calcium and magnesium for 1 hr at 37 °C. Alternatively, the fibronectin may be applied the day prior to analysis by incubating the PDMS with 10 µg/ml fibronectin in PBS for 16 hr at 4 °C. Make sure the entire surface is submerged in the fibronectin in PBS solution.
2. Prepare heat-denatured 1% BSA. Weigh 0.5 g BSA and dissolve it in 50 ml of PBS. Filter sterilize the solution through a 0.22 µm filter and heat at 80 °C for 12 min.
Note: Prepare this solution the day prior to cell plating and store at 4 °C.
3. Aspirate the fibronectin solution and wash 3 times with PBS. Add 1 ml of heat-denatured BSA in PBS to each well and incubate for 30 min at RT.
4. Trypsinize and count cells of choice while the durotaxis chambers are blocking with the heat denatured BSA.
5. Plate 1×10^5 cells in a volume of 2 ml into each well of the durotaxis chamber, using the media required for the particular cell type of choice. Allow the cells to adhere and spread on the substrate for 4 hr in a humidified incubator at 37 °C with 5% CO₂.
NOTE: U2OS cells are routinely maintained in DMEM with 10% FBS, supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 I.U./ml penicillin, 10 µg/ml streptomycin.
NOTE: The cell number used in this example is optimized for U2OS cells. Optimization for other cell types may be required. This density gives the cells enough room to migrate without significant interactions with other cells.

3. Live-cell Imaging

1. Perform live cell imaging on an inverted microscope, using phase contrast with a 10X objective. The microscope should be fitted with an enclosed environmental, humidified chamber, allowing control of temperature at 37 °C and 5% CO₂ during long-term imaging.
2. After the cells have spread for approximately 3.5 hr, assemble the plate in the microscope chamber. Allow the sample(s) to equilibrate in the chamber for 30 min.
3. Set up automated, multi-point visiting on the microscope if available. Focus on the interface between the 90:1 PDMS and the glass coverslip and choose points to image all around the interface with an average of 40 points per durotaxis chamber. Image the cells every 10 min for up to 16 hr.
NOTE: The region of interest will appear as two lines, with the outer line corresponding to the edge of the coverslip and the inner line corresponding to the actual interface between the PDMS and the coverslip. See **Figure 1B**.

4. Data Analysis

1. Generate a spreadsheet as in **Table 1**.
2. Count the number of crossing events from the PDMS to the glass surface and vice versa from each movie generated. Record the number of crossing events in the appropriate column in the excel spreadsheet.
NOTE: A crossing event is defined as the cell nucleus passing over the boundary between the PDMS and glass in either direction.

3. To quantify multiple crossings, count the number of times the cell crossed the interface. That number should be recorded in the excel column corresponding to the substrate on which the cell was located at the end of the movie. Repeat the analysis for every cell that crosses the interface in the movie. Exclude cells that migrate out of the field of view during imaging.
NOTE: It is also important to verify, by cell counting at the beginning of the experiment, that the cells are able to adhere equally to the fibronectin-coated PDMS and glass coverslip surfaces.
4. Calculate the percentage of cells that migrated from PDMS to the glass surface (i.e., underwent durotaxis). Add the number of crossing events from soft to hard and the multiple crossing events that ended on hard and divide by the total number of crossing events.
5. Calculate the percentage of multiple crossings by dividing the number of multiple crossings by the total number of crossings.

Representative Results

A schematic of the durotaxis chamber is shown in **Figure 1A**. Soft PDMS substrate (a 90:1 mixture of PDMS base to crosslinker solutions) is spread in a 6 well dish and a glass coverslip is placed on top of the PDMS, which then partially covers the upper surface of the coverslip, thereby creating an interface between the two substrates of different compliance. The rigidity of the soft PDMS substrate is ~ 1 kPa, which is comparable to the typical compliance of brain tissue, while the rigidity of glass is around 1-2 GPa¹. A phase contrast image of the interface between the PDMS and glass is shown by the white arrowheads in **Figure 1B**. The asterisks denote two control RNAi cells migrating towards the harder substrate. The ragged edge that can be seen (black arrow) corresponds to the edge of the coverslip.

To quantify the data, a Microsoft excel spreadsheet should be generated as in **Table 1**. The number of crossing events should be counted and organized in the corresponding column. Some of the movies generated cannot be used for quantification. A movie may be omitted from quantification if there is a bubble in the PDMS that interferes with a cells progress or if the cells are too densely packed, resulting in collisions that can drastically influence cell directionality. Cell division should also be monitored, as this can also overtly influence cell movement.

Typically, most adherent cell types will preferentially migrate towards a stiffer substrate^{2,4,12}. To begin to understand the molecular mechanism by which cells respond to mechanical cues in their environment, specific proteins can be knocked down using siRNA. In our example, around 70% of the control siRNA-treated cells exhibited durotaxis **Figure 2A**. In striking contrast, when cdGAP was knocked down using RNAi, the cells had no preference as to whether they migrated onto the hard or soft substrate **Figure 2A**. Furthermore, the cdGAP knockdown cells crossed the boundary multiple times, whereas control siRNA treated cells generally only crossed the boundary once, **Figure 2B**. Representative tracks of control siRNA cells show that the cells generally migrate across the boundary once and preferentially stayed on the more rigid surface as compared to cdGAP siRNA-treated cells that did not demonstrate a preference for either rigidity and crossed the boundary multiple times **Figure 2C**.

Figure 1. Schematic of the Durotaxis chamber set up. (A) Soft PDMS is spread in each well of a 6-well dish and a glass coverslip is placed on top of the PDMS. The PDMS will encroach over the sides of the top of the coverslip, before it hardens, to form an interface between the two substrates. Durotaxis is determined by scoring the directionality of the cells crossing the PDMS/glass interface. (B) Representative phase contrast image showing the interface between the soft PDMS and rigid glass substrate. The asterisks show control RNAi-treated U2OS cells undergoing durotaxis. The white arrowheads denote the interface between the PDMS and glass coverslip and the arrow indicates the edge of the glass coverslip under the PDMS. [Please click here to view a larger version of this figure.](#)

Movie number	siRNA	Total number single crosses	Cells crossing from Hard to Soft	Cells crossing Soft to Hard	Total number of Multiple Crosses	Multiple crosses ending on hard	Multiple crosses ending on soft	Total number of crosses
1	Control							
2	Control							
3	Control							
4	Control							
5	Control							
Movie number	siRNA	Total number single crosses	Cells crossing from Hard to Soft	Cells crossing Soft to Hard	Total number of Multiple Crosses	Multiple crosses ending on hard	Multiple crosses ending on soft	Total number of crosses
1	cdGAP							
2	cdGAP							
3	cdGAP							
4	cdGAP							
5	cdGAP							

Table 1. Schematic of Microsoft Excel spreadsheet set up. Columns are established to include the movie number, siRNA used, number of cells that migrated from the soft PDMS to rigid glass substrate or vice versa, as well as the total number of cells analyzed. Columns are also set up for the cells that cross the interface multiple times and end on either the soft PDMS or the glass substrate. If a cell migrates across the boundary multiple times, the number of multiple crossing events of these cells is recorded in the column of the substrate upon which the cell was located at the end of the movie.

Figure 2. Knockdown of cdGAP in U2OS cells causes a loss of durotaxis. (A) Control siRNA treated cells migrate preferentially onto the rigid glass substrate, whereas cdGAP siRNA treated cells did not exhibit any preference. (B) Control siRNA treated cells crossed the boundary between the PDMS and glass once. However, cdGAP RNAi-treated cells migrated back and forth across the boundary multiple times. (C) Tracks of representative cells treated with either control, or cdGAP siRNA were generated using the Manual tracking plugin in ImageJ. The p-values were determined using a student's t-test and errors bars represent the 95% confidence interval. Reproduced by permission from Wormer *et al.* 2014¹¹. [Please click here to view a larger version of this figure.](#)

Discussion

Herein we describe a simple assay to study durotaxis in migrating cells. A major strength of this assay is the ease of preparing the durotaxis chambers using PDMS. The rigidity of the substrates can be easily manipulated by changing the ratio of PDMS base solution to crosslinker to allow the study of various rigidities in the assay. However, one potential limitation of the system is that cells are only exposed to a single change in substrate rigidity as opposed to experiencing a rigidity gradient that is provided by more sophisticated systems⁴. Importantly, unlike polyacrylamide substrates, the ECM components do not have to be chemically cross-linked to PDMS. Indeed, the fibronectin coating is approximately equivalent on both the PDMS and the glass surfaces, as determined by staining with an antibody specific to fibronectin¹¹. Since previous studies have shown that cells behave the same on either PDMS or polyacrylamide substrates, the use of PDMS substrates provides a much easier model with which to study mechanotransduction in cells^{10,12}.

The most important step in the preparation of the PDMS substrates is to ensure that the base and the crosslinker solutions are thoroughly mixed. If they are not mixed well enough, then the substrates will not completely cure upon heat treatment. This will result in the coverslip sinking too far into the PDMS or the coverslip drifting in the PDMS during imaging. If the centrifugation force is too high, the base and crosslinker solutions may separate in the tube. This can be remedied by reducing the centrifugation speed or by degassing the mixture for 30 min using a vacuum chamber. Finally, cell density is critical, as too many cells will influence their ability to spread fully and to durotax unhindered by contact with neighboring cells. The density reported herein was optimized for U2OS cells. However, if other cell types are used, optimal cell numbers will have to be determined empirically.

We have recently used this assay to study how the focal adhesion signaling protein cdGAP regulates mechanosensing and durotaxis¹¹. Clearly, there are numerous cellular components in addition to focal adhesions, such as cytoskeletal elements, protein trafficking components and ion channels that are involved in mechanical signaling and thus may also contribute to the regulation of durotaxis^{13–15}. Each of these components could be readily evaluated in our system using similar RNAi approaches. Additionally, this assay is very amenable to the introduction of various pharmacologic activators or inhibitors. The assay can also be adapted to permit more sophisticated forms of analysis such as the incorporation of fluorescently-tagged proteins, in combination with FRET or FRAP approaches, to study protein dynamics and spatio-temporal activation.

Disclosures

The authors have no conflicts to disclose.

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