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TITLE:

Single Cell Durotaxis Assay for Assessing Mechanical Control of Cellular Movement and Related Signaling Events

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cellular migration, durotaxis, hydrogels, traction force microscopy, extracellular matrix, mechanotransduction, focal adhesions

SHORT ABSTRACT

Mechanical forces are important for controlling cell migration. This protocol demonstrates the use of elastic hydrogels that can be deformed using a glass micropipette and a micromanipulator to stimulate cells with a local stiffness gradient to elicit changes in cell structure and migration.

LONG ABSTRACT

Durotaxis is the process by which cells sense and respond to gradients of tension. In order to study this process in vitro, the stiffness of the substrate underlying a cell must be manipulated. While hydrogels with graded stiffness and long-term migration assays have proven useful in durotaxis studies, immediate, acute responses to local changes in substrate tension allow focused study of individual cell movements and subcellular signaling events. To repeatably test the ability of cells to sense and respond to the underlying substrate stiffness, a modified method for application of acute gradients of increased tension to individual cells cultured on deformable hydrogels is used which allows for real time manipulation of the strength and direction of stiffness gradients imparted upon cells in question. Additionally, by fine tuning the details and parameters of the assay, such as the shape and dimensions of the micropipette or the relative position, placement, and direction of the applied gradient, the assay can be optimized for the study of any mechanically sensitive cell type and system. These parameters can be altered to reliably change the applied stimulus and expand the functionality and versatility of the assay. This method allows examination of both long term durotactic movement as well as more immediate changes in cellular signaling and morphological dynamics in response to changing stiffness.

INTRODUCTION

Over the past few decades, the importance of the mechanical properties of a cell's environment has garnered increasing recognition in cell biology. Different tissues and extracellular matrices have different relative stiffnesses and, as cells migrate throughout the body, they navigate these changes, using these mechanical properties to guide them[1-7]. Cells use the stiffness of a given tissue to inform their motile behavior during processes such as development, wound healing, and

cancer metastasis. However, the molecular mechanisms that allow sensation of and response to these mechanical inputs remain largely unknown[1-7].

In order to study the mechanisms through which cells respond to physical environmental cues, the rigidity or stiffness of the substrate underlying adherent cells must be manipulated. In 2000, Chun-Min Lo, Yu-Li Wang and colleagues developed an assay[8] whereby an individual cell's motile response to changing mechanical cues could be directly tested by stretching deformable extracellular matrix (ECM)-coated polyacrylamide hydrogels on which the cells were plated. Cells exhibits a significant preference for migrating towards stiffer substrates, a phenomenon they dubbed "durotaxis."

Since the original report in 2000, many other techniques have been employed for the study of durotaxis. Steep stiffness gradients have been fabricated by casting gels over rigid features such as polystyrene beads[9] or stiff polymer posts[10] or by polymerizing the substrate around the edges of a glass coverslips[11] to create mechanical 'step-boundaries'. Alternatively, hydrogels with shallower but fixed stiffness gradients have been fabricated by a variety of methods such as gradients of crosslinker created by microfluidic devices[12,13] or side-by-side hydrogel solution droplets of differing stiffness[8], or hydrogels with photoreactive crosslinker treated with graded UV light exposure to create a linear stiffness gradient[14,15]. These techniques have been used to great effect to investigate durotactic cellular movement *en masse* over time. However, typically these features are fabricated in advance of cell plating and their properties remain consistent over the course of the experiment, relying on random cell movement for sampling of mechanical gradients. None of these techniques are amenable to observation of rapid changes in cellular behavior in response to acute mechanical stimulus.

In order to observe cellular responses to acute changes in the mechanical environment, single cell durotaxis assays offer several advantages. In these assays, individual cells are given an acute, mechanical stimulus by pulling the underlying substrate away from the cell with a glass micropipette, thereby introducing a directional gradient of cell-matrix tension. Changes in the motile behavior, such as speed or direction of migration, are then observed by live-cell phase contrast microscopy. This approach facilitates direct observation of cause and effect relationships between mechanical stimuli and cell migration, as it allows rapid, iterative manipulation of the direction and magnitude of the tension gradient and assessment of consequent cellular responses in real time. Further, this method can also be used to mechanically stimulate cells expressing fluorescent fusion proteins or biosensors to visualize changes in the amount, activity, or subcellular localization of proteins suspected to be involved in mechanosensing and durotaxis.

This technique has been employed by groups who study durotaxis[8,16] and is described here as it has been adapted by the Howe Laboratory to study the durotactic behavior of SKOV-3 ovarian cancer cells and the molecular mechanisms that underly durotaxis[17,18]. Additionally, a modified method is described for fabrication of hydrogels with a single, even layer of fluorescent microspheres near the cell culture surface; this facilitates visualization and optimization of

88 micropipette-generated strain gradients and may allow assessment of cell contractility by
89 traction force microscopy.
90
91 **PROTOCOL**
92
93 **1. Fabrication of deformable polyacrylamide hydrogels with embedded fluorescent**
94 **microspheres**
95
96 NOTE: Directions describe polymerization of a 25 kPa hydrogel that is 22 µm in diameter and
97 approximately 66 µm thick. Each or all of these parameters can be modified and directions to do
98 so can be found in **Table 1** and in the notes[17,18].
99
100 **1.1. Activation of glass-bottom dishes or coverslips**
101
102 1.1.1. Prepare the bind silane working solution for activation of a glass-bottom imaging dish or
103 a coverslip that fits into a live-cell imaging chamber. Mix 950 µL of 95% ethanol, 50 µL of glacial
104 acetic acid, and 5 µL of bind silane (γ-methacryloxypropyltrimethoxysilane).
105
106 NOTE: Using a larger bottom coverslip compared to the top coverslip will give additional room to
107 work when preparing the gel and will facilitate positioning the glass micropipette in later steps.
108 Also, if using a coverslip rather than a glass-bottom imaging dish, clean the coverslip as described
109 in the following section.
110
111 1.1.2. Activate the surface of the glass for 20 s with a corona wand and immediately overlay 50
112 µL of the bind silane working solution. Allow the solution to dry for 10 min.
113
114 1.1.3. Rinse two times with 95% ethanol, then two times with isopropanol and then allow the
115 coverslips to airdry for approximately 20 min.
116
117 NOTE: Activated glass can be stored for up to one week in a desiccator.
118
119 1.2. **Cleaning** top coverslips
120
121 1.2.1. Clean 22 mm top coverslips by incubating in 2% HCl at 70 °C for 30 min, then wash in
122 ddH_2O for 10 min two times.
123
124 1.2.2. Incubate the coverslips in a solution of 2% cuvette cleaning concentrate in ddH_2O at 50
125 °C for 30 min, then wash in ddH_2O for 10 min two times.
126
127 1.2.3. Incubate the coverslips in ddH_2O at 90 °C for 30 min, then in 70% ethanol at 70 °C for 10
128 min, and then air dry at 60 °C for a minimum of 2 h.
129
130 NOTE: Cleaned coverslips can be stored indefinitely in a clean desiccator.
131

1.3. Fluorescent microsphere/bead deposition onto top coverslips

1.3.1. Sonicate the stock solution of fluorescent microspheres for 1 h in an ultrasonic water bath. Make a working bead solution by diluting bead stock 1:200 in 100% ethanol and sonicate again for 1 h.

1.3.2. 15 min before the bead solution has finished sonicating, thoroughly clean the coverslips by placing them vertically in a ceramic coverslip holder and treating with room-air plasma for 3 min in a tabletop plasma cleaner.

1.3.3. To facilitate handling and prevent sliding of the coverslip during subsequent steps, place a piece of parafilm in a 60 mm Petri dish lid or a similar container. Place the coverslip in the stabilizer and lightly tap down, ensuring good contact between the parafilm and the coverslip.

1.3.4. For a 22 mm coverslip, add 150 µL of the working bead solution to the top of the coverslip. Immediately aspirate the ethanol solution off from the side of the coverslip, leaving the beads on the coverslip. Allow the coverslip to airdry.

NOTE: The amount of working bead solution added should be ~4 µL/cm^2 and can be scaled to accommodate any size coverslip.

1.4. Casting hydrogels with embedded fluorescent beads

1.4.1. Prepare the hydrogel solution of acrylamide and bis-acrylamide. Mix the solution according to **Table 1**, then add 2.5 µL of 10% APS and 0.5 µL of TEMED. Mix well. Immediately move to the next step.

NOTE: The hydrogel solution mixture can be altered to vary the Young's modulus, or stiffness, of the hydrogel by changing the ratio of acrylamide to bis-acrylamide as shown in **Table 1**. These values have been verified for use in the Howe laboratory using atomic force microscopy but should be confirmed within one's institution.

1.4.2. Immediately after making the hydrogel solution, add a 25 µL drop to the activated side of the glass-bottom dish or bottom coverslip, then immediately place the bead-coated coverslip onto the solution, bead side down. Contacting the drop with the far side of the coverslip followed by slow lowering helps avoid trapping air bubbles within the hydrogel.

NOTE: The height of the hydrogel should be well within the working distance of the objective lens to be used in the later experiment. A hydrogel height of 66 µm works well for most systems. The size of the hydrogel can be scaled by adding more or less hydrogel solution depending on the size of the coverslip. To calculate the appropriate volume of hydrogel solution, use the equation for the volume of a cylinder, $V = \pi r^2 h$ where $\boldsymbol{r}$ is the coverslip radius and $\boldsymbol{h}$ is the desired hydrogel height. Typically, this calculation predicts with fair accuracy the actual height of the hydrogel, as measured by preparing a gel with bead-coated coverslips on both the top and bottom and using

a confocal microscope to measure the distance between the two bead planes. However, it has been observed that the actual height of the hydrogel can deviate from this calculation by ± 20 µm (*e.g.,* depending on the thickness and manufacturer of the top glass coverslip). Direct measurement of gel height using the method described above is recommended.

1.4.3. Allow the gel to polymerize for 30 min, then remove the top coverslip gently with forceps. Adding 50 mM HEPES pH 8.5 to the dish can facilitate removal. Wash for 5 min in 50 mM HEPES pH 8.5 three times.

1.5. Hydrogel activation and extracellular matrix coating

1.5.1. Activate the hydrogel surface by incubating in 0.4 mM Sulfo-SANPAH (sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino) hexanoate) in 50 mM HEPES pH 8.5. Immediately expose to a UV arc lamp in an enclosed area.

NOTE: Protect Sulfo-SANPAH from light prior to activation. For a 400 W lamp, position the gel 10 cm away from bulb within the light box and illuminate for 100 s. The Sulfo-SANPAH solution will change from bright orange to dark brown.

1.5.2. Wash for 5 min in 50 mM HEPES pH 8.5 three times.

NOTE: Hydrated gels may be stored at 4 °C for up to one week.

1.5.3. Incubate the activated hydrogel in 20 µg/mL fibronectin in 50 mM HEPES pH 8.5 for 1 h at 37 °C.

1.5.4. Aspirate the fibronectin solution and wash for 5 min in phosphate-buffered saline (PBS) three times. Sterilize the hydrogel and the lid of the dish for 15 min under UV light in a tissue culture hood with a low volume of PBS. Wash once in sterile PBS.

NOTE: Other types of ECM protein can be used to coat the hydrogel including collagen and laminin.

2. Plating cells

2.1. Add 3 mL of media containing 21,000 cells to fill a 60 mm dish for a final cell density of ~1000 cells/cm^2. Adjust the seeding density as needed to prevent crowding and allow free movement of individual cells.

2.2. Allow the cells to recover at 37 °C for at least 4 h and for up to 18 h before imaging. Prepare for imaging by rinsing with imaging media two times before adding imaging media. Allow the cells to equilibrate for at least 30 min before imaging.

NOTE: Screen media conditions in advance to determine conditions that will optimize migration in the cell line being used. For SKOV-3 cells, DMEM without phenol red, containing 20 mM HEPES and 12.5 ng/mL epidermal growth factor stimulates the most migration. Optimal conditions for Ref52 cells are Ringer's Buffer with 10% fetal bovine serum (FBS) and 25 ng/mL platelet-derived growth factor.

3. Preparation of glass micropipette: pipette pulling and forging

3.1. Pull 100 mm long borosilicate glass micropipettes with a 1.0 mm exterior and 0.58 mm interior diameter in a two-step process to obtain a taper over 2 mm that reduces to ~50 μm in the first millimeter and extends to a long, parallel 10 μm diameter tube in the last millimeter.

3.2. Load pulled pipet into a microforge. Shape the pipet to have a 15 μm blunted tip that is enclosed at the very end of a 250 μm section bent at a ~35° angle from the rest of the pipet. The approximate diameter at the bend should be around 30 μm to lend strength to the tip.

NOTE: Taper and tip dimensions can be adjusted to properly apply desired force (see step 5). Pulling micropipettes at 65 °C for the first step for 3 mm, and 60 °C for the second step produces the dimensions described in step 3.1. Results using different pipet pullers may vary.

3.3. Sterilize the micropipette in 70% ethanol before use.

4. Positioning the micromanipulator and the micropipette

4.1. Remove the dish lid and load the dish onto the microscope stage and center. Use a 10X or similarly low magnification objective. Cover the media with mineral oil to prevent evaporation of the media.

4.2. Inserting pulled pipet

4.2.1. Insert the pulled pipet into the micropipette sheath, pointing the hook down toward the dish. The tip of the hook will be the lowest point when lowered to the gel.

4.2.2. Insert sheath into micromanipulator and adjust until the tip of the pipet is centered over the objective lens in both X and Y directions.

4.2.3. Lower the pipet using coarse manipulator until it just touches the surface of the liquid.

4.3. Using phase contrast or brightfield, focus the microscope on the bead layer at the top of the gel. This will be the reference plane.

4.4. Ensuring that the objective is in no danger of hitting the sample or stage, bring focus above the gel to find the tip of the micropipette, using small adjustments of the coarse

manipulator in the X and Y directions to cast shadows on the focal plane. Only lower the micropipette when certain that the very tip of the pipet is in the field of view.

4.5. Ensure that the blunted tip of micropipette is pointing down by rotating the pipet in the sheath or rotating the sheath in the micromanipulator until the tip is perpendicular to the focal plane. Repeat steps 4.4 and 4.5 as needed. Focus on the tip of the pipet.

4.6. Focus back down to the top bead layer of the gel to gauge how far the pipet is from the gel surface. Focus back up to a plane that is part way between the gel and the tip of the pipet. Slowly lower the pipet to reach the intermediate focal plane.

4.7. Repeat step 4.6 until very faint shadows from the tip of the micropipette can be appreciated when focusing on the hydrogel. Increase to the next highest magnification.

4.8. Lower the micromanipulator until shadows and refractions of the very tip of the micropipette can be appreciated within the bead layer focal plane.

4.9. Increase the magnification to that which will be used in the experiment. Lower the pipet until it hovers just above the surface of the hydrogel.

5. Calibrating the micromanipulator and force generation

5.1. In phase or brightfield, lower the hovering micropipette to touch the surface of the hydrogel. Observe how the pipet looks upon contact with the hydrogel. Continue to lower micropipette in Z until adjustments in X and Y cause pulling and deflection of the hydrogel in those directions. Use the microspheres or nearby cells as fiduciary marks.

NOTE: If the micromanipulator is attached to the phase condenser arm or the bench and not the sample stage itself, always disengage the gel before moving the stage to avoid breaking the pipet or disturbing cells. If the pipet breaks, go back to step 3 and step 4.

5.2. Find an area devoid of cells to engage the gel. Pull it in all directions and get comfortable with the way micromanipulation translates to deformation of the gel.

5.3. Take fluorescent images of the bead field with no manipulation, with the pipet engaging the gel, and with the engaged pipet pulling the gel. Repeat this several times taking good notes regarding the tick marks on the micromanipulator, the way the pipet tip looks in phase or brightfield at each stage of pulling, and the distance the tip moves using that manipulation.

5.4. Use ImageJ as previously described[16,17] to calculate relative bead displacements and force applied to the beads by comparing the null bead field to the bead field without pipet engagement, the bead field with the gel engaged, and the pulled gel.

5.5. To fine tune the tensional stimulus, compare force application using differing micropipette tip dimensions, distances from the cell, or distance pulled by the micromanipulator from initial point of touchdown. The effect of the micropipette tip dimension on force application gives great flexibility to the user but also demonstrates the need to generate force maps for new micropipettes, even when the dimensions and shape closely resemble previously calibrated tips.

6. Conducting the durotaxis assay

6.1. Before performing the experiment, practice engaging the gel near a cell and observe the deformation of the cell when the micromanipulator is repositioned.

6.2. Monitor a group of cells that have clear polarity and appear to be moving for 30 min to identify cells that are moving in a directed manner.

6.3. Choose a cell that is moving in a single, clear direction and monitor it at the desired frame rate for an additional 30 min.

6.4. If determination of forces exerted on the cell or tension exerted by the cell is desired, capture bead field images at each acquisition. If the cell changes its course of direction during monitoring, choose a different cell to monitor as this will make it difficult to determine the effect of stimulation.

6.5. Engage the hydrogel approximately 50 µm away from the cell. Position the pipet in front of the near side of the leading edge and move the micromanipulator such that the gel is deformed orthogonally to the cell's direction of travel. Observe the cell over time as it responds to the acute, local gradient of stiffness.

NOTE: The timing provided here is effective when monitoring SKOV3 or Ref52 fibroblasts, however, the interval and overall time course should be adjusted to suit the cell type and biological event being observed. If pairing with fluorescence microscopy, pause fluorescent acquisition immediately before step 6.5., use phase contrast or brightfield to position micropipette and pull, and restart fluorescent acquisition immediately after.

6.6. If the pipet slips or if the gradient is otherwise relaxed or released, find a new cell by repeating steps 6.2 and 6.3.

7. Determining durotactic migration response

7.1. Using ImageJ[19] or another image analysis program, calculate the turn angle by drawing a line between the middle of the leading edge of the cell at 0 min and 30 min post monitor (reflecting the cell's original trajectory) and another line between the middle of the leading edge just before and 80 min after stimulation and measuring the angle between these two lines.

REPRESENTATIVE RESULTS

By preparing micropipettes (**Figure 1**) and normalizing the force generation of the pulls (**Figure 2** and **Figure 3**) as described above, optimal durotactic conditions have been identified for multiple cell lines. Using this technique, as outlined in **Figure 4**, both SKOV-3 ovarian cancer cells[17,18] and Ref52 rat embryonic fibroblasts (**Figure 5**) move toward increased stiffness in gradients applied by a glass micropipette. In addition to durotaxis, this method can be used to study dynamic signaling events using fluorescent biosensors and markers. For example, the structure of and signaling within focal adhesion structures can be observed upon durotactic stimulation. Vinculin tension sensor (VinTS) is a FRET-based biosensor which localizes to focal adhesions, allowing for fluorescent observation of focal adhesion dynamics and measurement of changes in tension within those structures[20]. Ref52 cells transiently expressing VinTS on 125 kPa polyacrylamide gels show the formation of focal adhesions in the direction of stretch over time period of 40 min (**Figure 6A**). FRET analysis[18,21] reveals that vinculin localized to focal adhesions experiences an immediate change in tension when presented with acute durotactic stimulation (**Figure 6B**) expanding the utility of this assay to the observation of subcellular signaling events in response to durotactic stimulation.

FIGURE LEGENDS:

Figure 1. Diagrams of typical pulled (A) and forged (B) micropipettes. (**A**) Micropipettes are pulled using a two-step protocol to achieve a taper from 1 mm to 10 µm over 2 mm. (**B**) Micropipettes are then loaded into the microforge and their tips are bent, enclosed, and shortened so that the last 250 µm of the micropipette is bent at a ~35° angle and tapers from ~30 µm to a rounded tip that measures ~15 µm.

Figure 2. Improved bead field after ethanol coating as compared to traditional method using poly-L-lysine. Representative hydrogel bead fields from poly-L-lysine and ethanol (EtOH) evaporation coverslip coating methods using yellow-green, red, and dark-red fluorescent beads. Scale bar: 25 µm.

Figure 3. Generation of force map for an example durotactic stretch. (**A**) Position of fluorescent microspheres before and after (pseudo-colored green and red, respectively) deforming the hydrogel with a micropipette (located beyond the right edge of the panel). Scale bar: 25 µm. Displacement vectors (**B**) and displacement heat map (**C**) between null and pulled bead fields generated by Traction Force Microscopy plugins in ImageJ highlight the gradient of bead deflection and hydrogel strain.

Figure 4. Schematic of durotaxis assay and determination of deflection angle. (**A**) A cell is observed for at least 30 min to determine its original trajectory. (**B**) The micropipette is positioned orthogonally to the cell's trajectory, 50 µm from the cell edge. The hydrogel is engaged by the micropipette such that moving the micropipette will exert force on the surface of the hydrogel. (**C**) The micropipette is pulled an additional 20 µm away from the cell, orthogonal to the cell's trajectory which creates an acute, local gradient of tension (denoted in blue) which increases toward the micropipette. (**D**) The cell is observed over time as it navigates the applied gradient. (**E**) In ImageJ or an image analysis program, the original trajectory (dashed line) is

marked by a line drawn from the middle of the cell through the center of the leading edge in the first frame. The final trajectory (solid line) is marked by a line drawn after the cell is allowed to navigate the applied tension gradient. The angle between these two lines toward the stimulus is termed "turn angle," marked here by θ.

Figure 5. Rat Embryonic Fibroblasts move toward regions of increased substrate stiffness in durotaxis. Time course showing durotactic movement of a Ref52 cell 10 min before the pull (panel 1), 1 min before the pull (panel 2), at the time of pull (panel 3), and 1 h after pull (panel 4). Arrow indicates direction of stretch. Scale bar: 50 μm.

Figure 6. Protein localization and activity during durotactic stimulation using fluorescent markers or biosensors. Ref52 cells transiently expressing Vinculin Tension Sensor (VinTS)[20] migrating on 125 kPa polyacrylamide gels are presented with acute durotactic stimulation. (**A**) After stimulation, new focal adhesions form in the direction of stretch as cells re-orient along the stiffness gradient. For two 10 min periods, starting 20 min before mechanical stimulation and 21 min after stimulation, cell morphology (top) and focal adhesion formation (bottom) were monitored. Red color indicates the first timepoint within the time period and green color indicates the timepoint 10 min later. New focal adhesions formed within the 10 min period are shown in green. Before stimulation, new focal adhesions form in the direction of travel. After stimulation, new focal adhesions form in the direction of stretch. Arrow indicates the direction of stretch. Arrowheads indicate areas with focal adhesions formed over that 10 min period. Scale bar: 25 μm. (**B**) FRET analysis of VinTS fluorescence indicates a change in tension within focal adhesions proximal to durotactic stretch. Outline of cell membrane before and after stretch highlight deformation of cell upon stimulation. Arrowheads indicate examples of focal adhesions experiencing changes in FRET ratio upon stretch. Scale bar: 10 μm.

Table 1. Acrylamide gel solutions.

DISCUSSION

Demonstrated here is a repeatable, single-cell durotaxis assay that allows assessment of a cell's ability to alter its migration behavior in response to acute mechanical cues. This technique can also be used in combination with fluorescence microscopy and appropriate fusion proteins or biosensors to examine subcellular signaling and cytoskeletal events within seconds of mechanical stimulation or over a longer timescale during durotactic movement. Understanding a cell's relationship to its environment involves the study of the impact of both the chemical and mechanical aspects of that environment. Though potentially difficult to master, this durotaxis assay can be widely used to understand the cellular response to changes in its mechanical microenvironment.

Significance with respect to existing methods

As mentioned before, this micropipette-based method of durotactic stimulation is highly manipulable, allowing a high degree of spatiotemporal control over mechanical stimuli, a major advantage over other techniques, such as pre-formed linear or step-gradients of rigidity. The

magnitude and direction of the imparted strain gradients can be visualized by tracking the displacement of fluorescent beads embedded in the hydrogel, near the cell culture surface.

Restricting these fiducial markers to a single layer just below the culture surface increases the accuracy of this tracking. Microspheres located below the plane of deflection (imparted either by the micropipette or, for traction force microscopy, by cellular contractility), as would occur with mixing the microspheres evenly throughout the hydrogel, will move less than in-plane microspheres, which can lead to underestimation of applied forces. Also, this modification is easier to perform and more reliable than methods in which beads are overlaid in an extremely thin layer of polyacrylamide cast on top of a pre-formed gel[22] or brought to the hydrogel surface by gravity-assisted settling[23] and produces a more even dispersal of beads across the hydrogel than previously described methods[17,18,24].

Modification and Future applications

The specifics of this assay can be modified to best suit the cell line of interest. For example, a variety of extracellular matrix molecules (*e.g.,* collagen I, collagen IV, laminin) or other adhesive ligands can be used to functionalize the hydrogel. Also, the starting stiffness of the hydrogel can easily be raised or lowered by tuning the ratio of acrylamide to bis-acrylamide (see **Table 1**). By changing the dimensions of the micropipette tip and the magnitude of the pull, this assay can be optimized to impart a repeatable and effective durotactic stimulus for the cell type in question.

Critical Steps and Troubleshooting

Only cells following a steady, linear trajectory of migration prior to hydrogel manipulation should be stimulated to ensure that changes in trajectory are due to the mechanical stimulus and not random fluctuation. Care must be taken to fabricate a glass micropipette that engages the hydrogel surface without slipping but does not tear the gel when pulled. It is important to apply a steady, constant stretch to the hydrogel during the course of the experiment to obtain clean results meaning that the user should be practiced at placing and moving the micropipette before encountering a cell. Any unintentional movement of the micropipette that leads to changes in the tension gradient could affect the cell's ability to durotax. Similarly, manipulation of the gel should be practiced with each new micropipette that is forged as slight changes in pipet shape can cause the pipet/gel interaction to vary.

Failure to position the micropipette within the microscopic field of view before increasing magnification can lead to the accidental breakage of the fragile glass tip. Ensure that the height and X-Y position of the tip is known before lowering the micropipette with the micromanipulator. Always monitor the position of the micropipette to reduce the risk of breakage. It is recommended that the magnification is decreased back down to 10X for each new micropipette loaded into the micromanipulator as slight movements of the micromanipulator and pipet sheath can lead to large apparent changes in the position of the newly loaded micropipette.

Before finding cells to observe, it is important to first test the micropipette to confirm that it will engage the hydrogel as expected and that it is suitable for applying the desired stretch. Finding tip dimensions that suit the experiment and cell type is critical to success in applying durotactic

stimulation. The end of the micropipette should be rounded enough so that it does not break through the gel, but not so rounded that it fails to grip it. If the pipet does not pull gel effectively, it may be sliding along the surface. The shape of the micropipette tip may be too rounded to properly engage with the gel surface. The dimensions at the very tip of the micropipette should be adjusted until firm, steady contact can be achieved consistently. In some cases where the micropipette is slipping across the gel surface, it may be necessary to lower the micropipette further into the hydrogel to gain more traction. If the micropipette tears through gel, the tip may be too fine or too sharp. Gel tearing may also indicate too much force is being applied while pulling. The micropipette should be raised slightly to reduce gel deformation and pulling shorter distances.

Often, if the cell or the edge of the cell is pulled out of focus by the micropipette, the tip of the micropipette is engaged too close to the cell or the stretch is too forceful. Move the micropipette further from the cell, only slightly deforming the cell in the X-Y planes. Moving the cell out of focus will not only make cellular events impossible to monitor and cause optical aberrations, but it will cause the cell to experience more stimulation than the 2-dimensional tension gradient intended.

Most importantly, it is critical to record and analyze only responses that have consistent durotactic manipulation with minimal human error. If the lowering of the tip is imprecise or if the micropipette is repositioned, the results of the experiment will be clouded. Since this assay is complex and many steps are prone to error, care must be taken at every step to avoid unintentional changes in the stimulation of cells. Failure at any step can lead to inconsistent stretch application and unreliable results.

Limitations

There are limitations to this technique that should be considered. Most prominently, accurate forging and manipulation of the glass micropipettes can present a steep learning curve for new users. Additionally, the position and magnitude of the hydrogel pull must be optimized for different cell lines. Examining fluorescent bead displacements before and after hydrogel manipulation can help with this aspect of the technique. Also, while the technique allows high spatiotemporal observation of durotactic behavior in individual cells, this makes it a low-throughput assay. It is therefore important to point out that this assay can also be complemented by other techniques with lower manipulability but higher throughput, such as using hydrogels with pre-formed gradients of rigidity, to analyze the durotactic behavior of larger populations of cells at once. In summary, the high degree of spatiotemporal control of mechanical cues afforded by the single-cell durotactic assay make it very useful for parsing the molecular mechanisms contributing to the durotactic behavior of many different cell types under many conditions.

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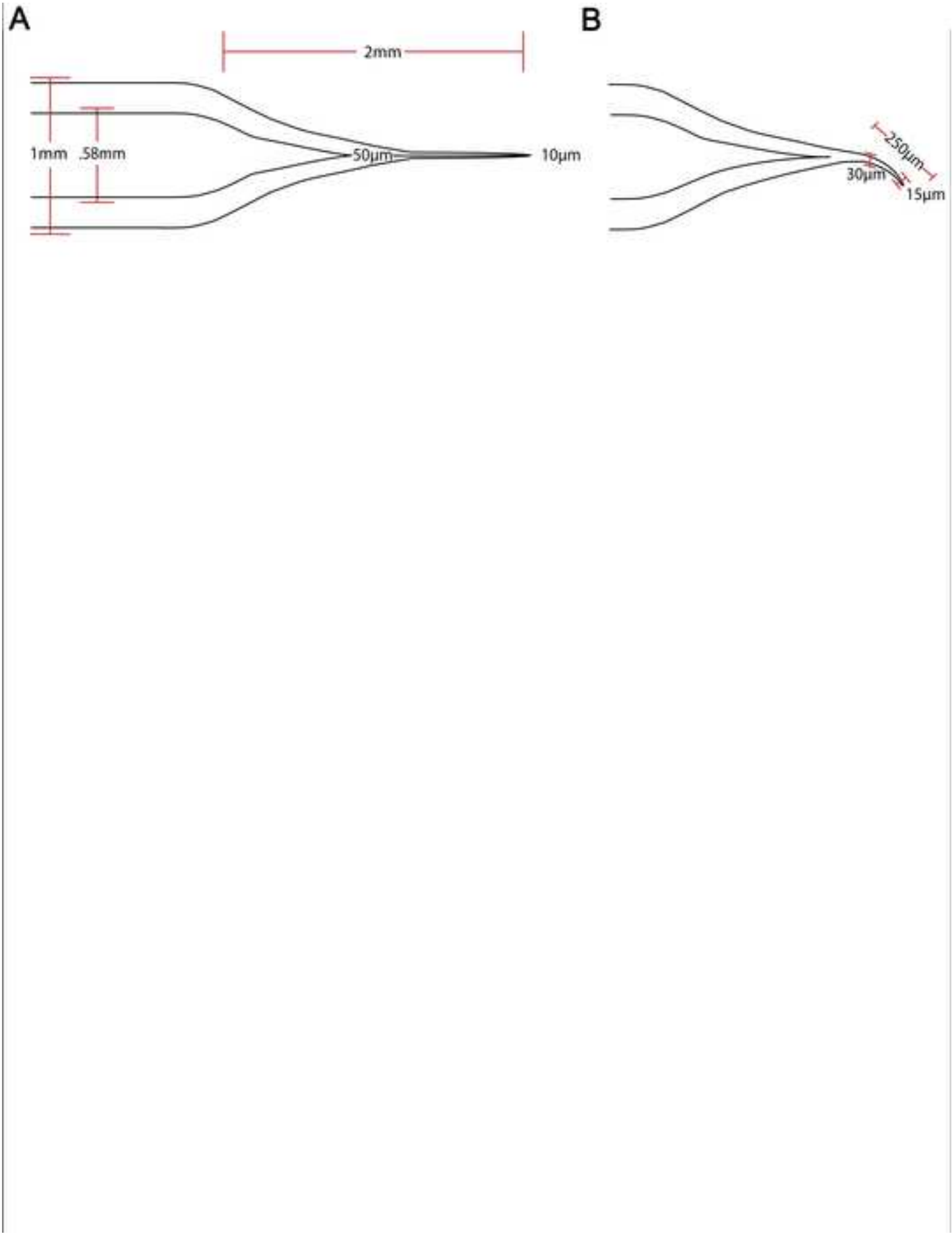
Figure 1



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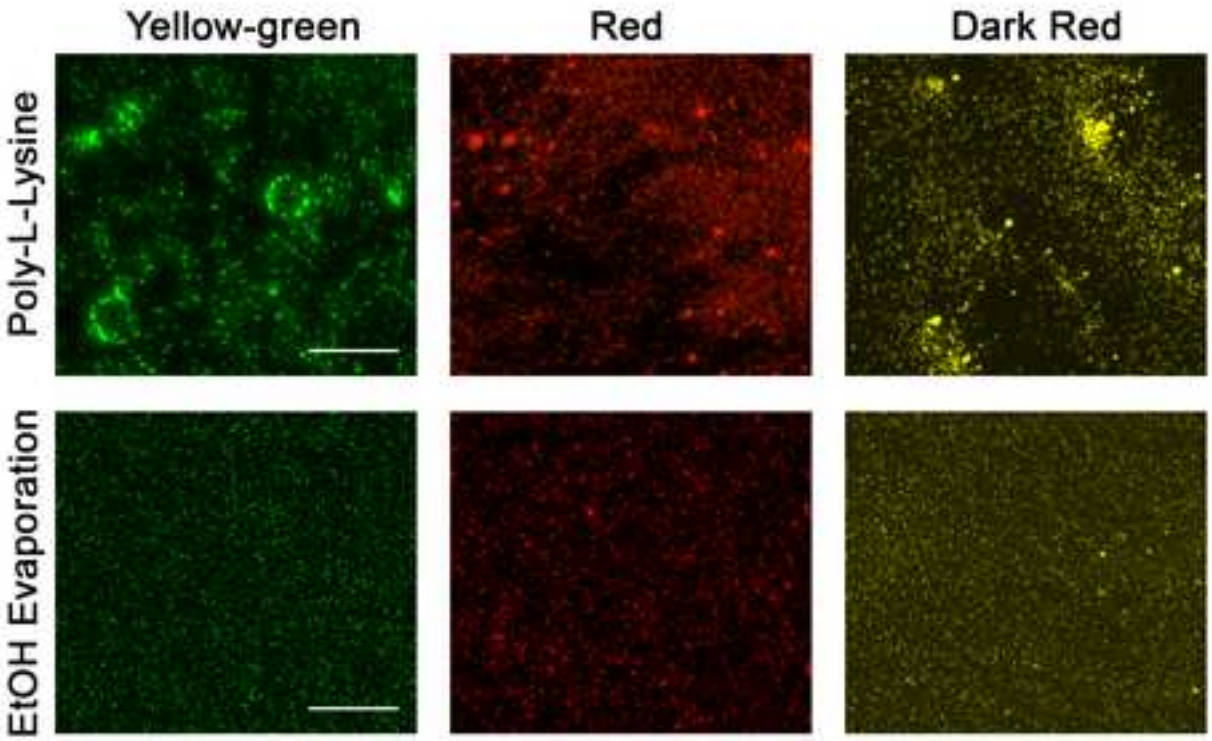

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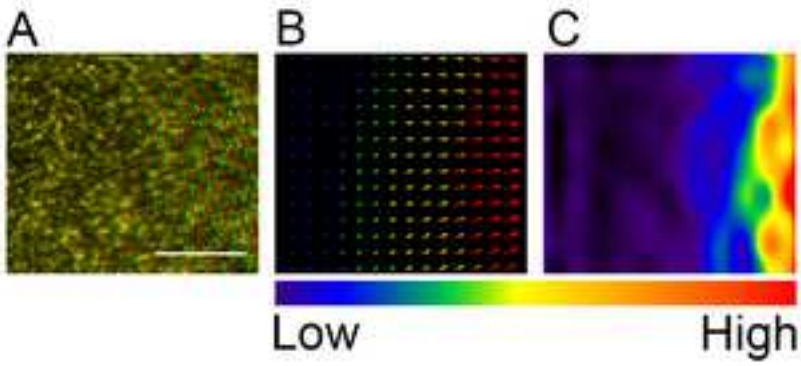

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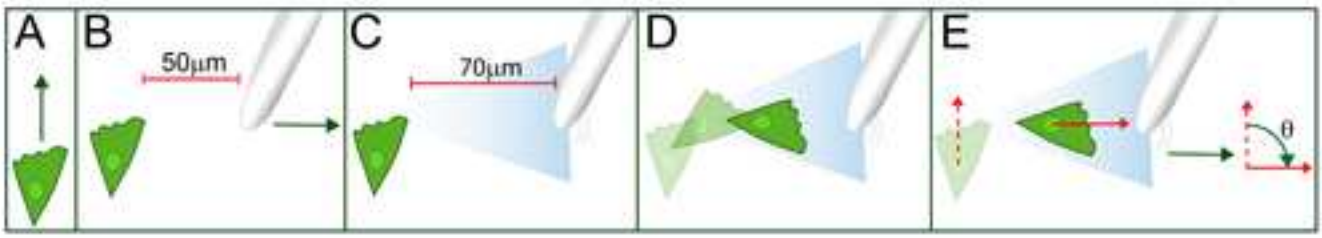

Figure 5

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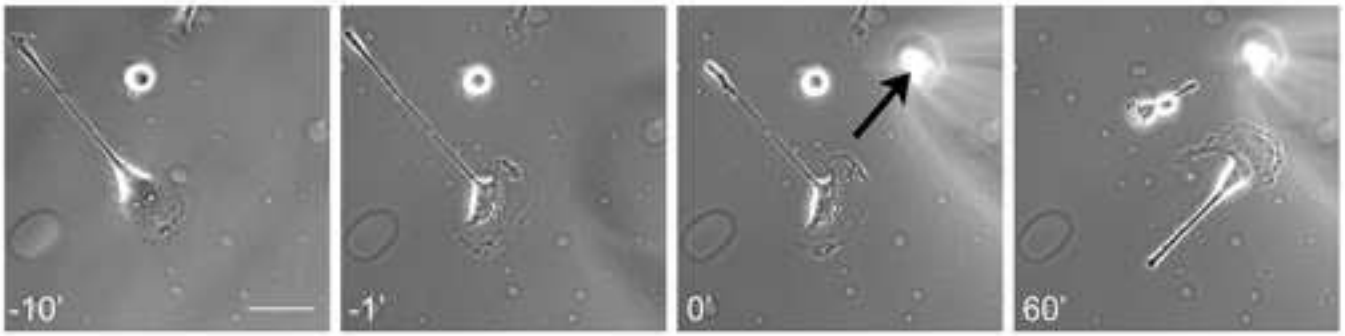

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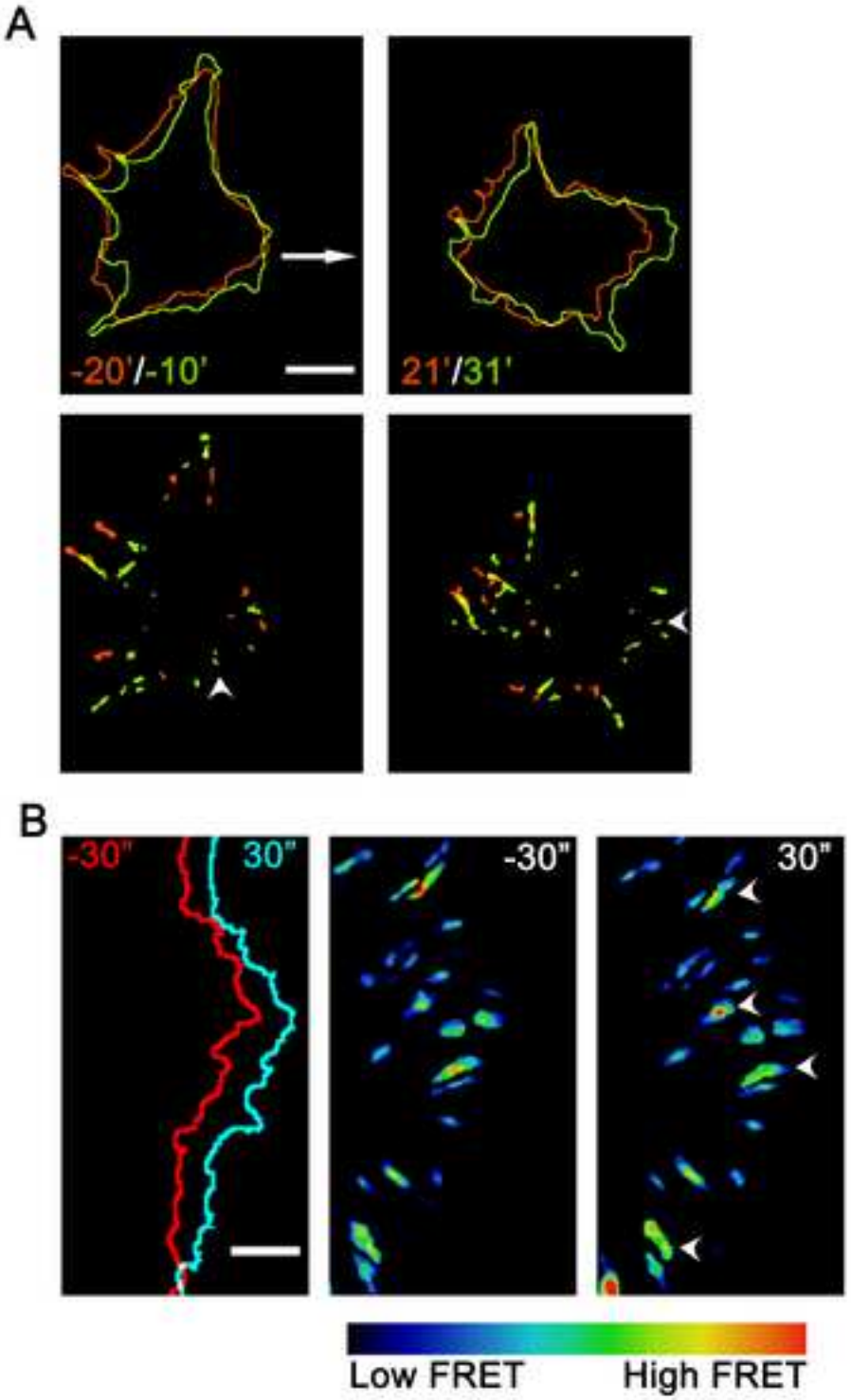

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Table

Desired hydrogel stiffness	3 kPa	25 kPa	125 kPa
7.5% Acrylamide	100 µL	100 µL	160 µL
0.5% Bis-Acrylamide	10 µL	100 µL	100 µL
ddH_2O	287 µL	197 µL	137 µL

Table of Materials

Click here to access/download;Table of Materials;Product List JoVE_Revised_akh.xlsx

Item	Company
Acrylamide 40 %	National Diagnostic
Ammonium Persulfate	Fisher
BD20A High frequency generator	Electro Technic Products
Bind Silane (γ-methacryloxypropyltrimethoxysilane) (	Sigma Aldrich
Bis-acrylamide 2%	National Diagnostic
Borosilicate glass capillaries	World Precision Instruments
Branson 2510 Ultrasonic Cleaner	Bransonic
Coarse Manipulator	Narshige
DMEM	Corning
DMEM without phenol red	Sigma Aldrich
Dual-Stage Glass Micropipette Puller	Narshige
Epidermal Growth Factor	Peprotech
Ethanol	Pharmco-aaper
Fetal Bovine Serum (Qualified One Shot)	Gibco
Fibronectin	EMD Millipore
Fluospheres Carboxylate 0.2 um	Invitrogen
Fugene 6	Roche
Glacial Acetic Acid	Fisher Chemical
Glass Bottom Dish	CellVis
Glass Coverslip	Electron Microscopy Sciences
HCl	JT Baker
Hellmanex III Special cleaning concentrate	Sigma Aldrich
HEPES powder	Sigma Aldrich
Intelli-Ray 400 Shuttered UV Flood Light	Uviton International
Isopropanol	Fisher Chemical
Microforge	Narshige
Micromanipulator	Narshige
Mineral Oil	Sigma Aldrich
Nanopure Life Science UV/UF System	Barnstead
Nikon Eclipse Ti	Nikon
OptiMEM	Invitrogen
Parafilm M	Bemis Company, Inc
PBS	
Platelet Derived Growth Factor-BB (PDGF-BB)	Sigma Aldrich
Ref52	
Ringer's Buffer	
SKOV-3	American Type Culture Collection
Sulfo-SANPAH	Covachem
Tabletop Plasma Cleaner	Harrick Plasma
TEMED	Sigma Aldrich

Catalog Number	Comments
EC-810	
BP179-25	
12011A	115 V - Handheld Corona Wand
M6514	
EC-820	
1B100-4	
	40 kHz frequency
MC35A	
10-013-CV	
D5030	
PC-10	
AF-100-15	
111000200	
A31606-02	
FC010	
F8810, F8807, F8811	
1815091	1.5 ug DNA / 6uL fugene 6 per 35mm dish
A38SI-212	
D60-60-1.5-N	
72224-01	22 mm, #1.5
9535-03	
Z805939	Used at 2% in ddH2O for cleaning coverslips
H3375	Make 50mM HEPES buffer, pH 8.5
UV0338	
A417-4	
MF900	
MHW3	
M5904	
D11931	ddH2O
31985062	
PM-992	
	139 mM NaCl, 2.5 mM KCl, 28.6 mM Na2HPO4, 1.6 mM KH2PO4, pH 7.4
P4056	
	Rat embryonic fibroblast cell line; Culture in DMEM + 10% FBS
	134 mM NaCl, 5.4 mM KCl, 1 mM MgSO4, 2.4 mM CaCl2, 20 mM HEPES, 5 mM D-Glucose, Culture in DMEM + 10% FBS
12414-1	
PDC-32G	
T9281-50	

pH 7.4

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Author(s):	Kathryn V. Svec, Johnathan B. Patterson, Nyla Naim, and Alan K. Howe

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Department:	Pharmacology
Institution:	University of Vermont
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April 17, 2019

To: Bing Wu, Ph.D.
Review Editor, JoVE

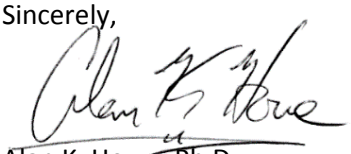
RE: Editorial Revisions of JoVE59995R1

Dear Dr. Wu,

Thank you for your correspondence regarding additional editorial revisions to our manuscript, “Single Cell Durotaxis Assay for Assessing Mechanical Control of Cellular Movement and Related Signaling Events”. We have addressed all of these new editorial comments, as detailed below. We have also appended the original response letter at the end of this one, to ensure that all of our responses and their information are available in one document. We hope that the manuscript is now acceptable for publication in the *Journal of Visualized Experiments*.

Again, we thank you and the reviewers for your time & effort in reviewing this work and we look forward to hearing from you soon.

Sincerely,



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1. "*…thoroughly proofread the manuscript…*" – The text has been proofread and spelling/grammar issues eliminated.

2. *through* 6. "*…provide…sub-heading for steps…*" – Subheadings 1.1, 1.2, 1.3, 1.4, and 1.5 have now been added.

7. "*…avoid long steps/notes…*" – All of the steps, especially all of the highlighted sections, are now four lines or less. All notes, except for the one following step 1.4.2, are also four lines or less. The aforementioned note contains information in direct response to a reviewer's comment; it cannot be shortened to four lines or less without significantly compromising its content.

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To: Bing Wu, Ph.D.
Review Editor, JoVE

RE: Revisions of JoVE59995

Dear Dr. Wu,

Thank you for your correspondence regarding necessary revisions to our manuscript, “Single Cell Durotaxis Assay for Assessing Durotactic Cellular Movement and Related Signaling Events”. We appreciate the positive comments of the reviewers, as well as their detailed and insightful comments and suggestions for improvement. In this revision, we have addressed all of the reviewers’ concerns as well as all of the editorial comments; a summary of the editorial corrections and a detailed response to reviewers’ concerns are appended below. We hope that the manuscript is now acceptable for publication in the *Journal of Visualized Experiments*.

Again, we thank you and the reviewers for your time & effort in reviewing this work and we look forward to hearing from you soon.

Sincerely,

Alan K. Howe, Ph.D.
Associate Professor
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Department of Molecular Physiology & Biophysics
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University of Vermont
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3. "*…12 pt font and single-spaced…*" – This and other formatting guidelines (see below) have been implemented.
4. "*…keywords…*" – Seven keywords/key phrases have been provided.
5. "*…one-line space…*" – This and other formatting guidelines (see above & below) have been implemented.
6. "*…time units…*" - This and other formatting guidelines (see above & below) have been implemented.
7. "*…highlight complete sentences for filming.*" – Done.
8. "*…remove the embedded Table…*" – The table has been removed from the manuscript text and supplied as a separate .xlsx file.
9. "*…revise the Discussion to explicitly cover the following…*" – The Discussion has been extensively revised to address critical steps and troubleshooting, modifications and future applications, comparison to existing methods, and limitations.
10. "*…do not abbreviate journal titles…*" – This formatting has been changed.
11. "*…include a scale bar for all images…*" – Scale bars for micrographs have now been included.

Reviewer #1 – Response

We thank the reviewer for mentioning that "this is a well-written, succinct manuscript", and for their comments and concerns, which are addressed below.

1. "*…perhaps it should be noted what the benefits are of introducing microspheres in this manner…*"
The benefits of the described microsphere handling 'surface-embedding' method, compared to uniform inclusion of beads throughout the hydrogels have been detailed in the revised Discussion section.

2. "*Many common items are not found in the product list…*"
There are no explicit instructions from the publisher as to what to include or exclude. Nonetheless, we have significantly expanded the product list to include items for which we feel the source is of particular importance.

3. "*Perhaps reword 'plasma cleaning'…to be a bit more descriptive.*"
More detailed information for this step (*e.g.* the model, condition, and settings) has been included.

4. "*How exactly are the authors exposing their Sulfo-SANPAH treated coverslips…?*"
More detailed information for this step (*e.g.* the use of a light-fast enclosure surrounding the UV exposure tool) has been included.

5. "*What about using other ECM molecules using this method?*"
We took this comment as a request to *discuss* the use of other ECM molecules, rather than *demonstrate* their use – we hope that this is correct. The ability of the described method to be extended to use different adhesive ligands and different cells has been described in both the protocol text as well as the Discussion section.

6. "*Are the bottom coverslips also in 60 mm Petri dishes?*"
We apologize for the lack of clarity surrounding the bottom coverslips and 60 mm dishes. We routinely use imaging dishes with a 60 mm outer diameter and a central 30 mm glass imaging window, so there is no separate coverslip, *per se* – the hydrogel is cast directly on the silanated glass surface of the dish – so it cannot move or

float. However, the described method is perfectly adaptable to other configurations, such as the use of a smaller diameter bottom coverslip that fits into an appropriate imaging chamber. The appropriate sections have been rewritten to clarify these details.

7. "*Are cells plated in a smaller volume that just covers the bottom coverslips…?*"
The cells are actually plated in a larger volume that covers the entire dish at a density of ~1000 cells/cm^2. This has been indicated in the protocol text.

8. "*Missing period at the end of 5.2.*"
We sincerely thank the reviewer for their detailed reading of the text. A period has been added, and other typographical errors have been corrected.

9. "*Figure 5 is not referenced in the 'Representative Results' section.*"
This was due to a typographical error in the original manuscript that led to us referencing Figure 6 twice. This has been corrected and all figure are now referenced.

10. "*Add labels to the top of the images…in Figure 2...*"
This figure has been labelled for increased clarity.

11. "*It may be difficult for a reader to understand what is being shown in Figure 3A…*"
This figure has been modified in a number of ways to increase clarity. Pseudo-coloring for the null and displaced bead fields have been changed and a labelled scale bar has been added to the vector and heat map panels. The position of the micropipette is outside the boundaries of the depicted bead field, but this has been now clearly indicated in the figure legend. Finally, other aspects of the legend have been re-written for clarity.

12. "*…it may be less confusing for some readers if the turn angle in Figure 4E was …flip[ed] 90 degrees…*"
Flipping the red lines as suggested would depict the 'wrong' angle (*i.e.* an angle we did not measure). However, we agree with the reviewer that the method depicted in the figure could be clearer, so we have changed the lines to arrows and changed the line depicting the original trajectory to a dotted line. We feel that this makes the method much clearer and evident.

13. "*Figure 5 caption does not need to reference '(A)' since the images are not broken down by letters.*"
The '(A)' has been removed.

14. "*In Figure 6, how can the reader identify new focal adhesions in A?*"
We agree with the reviewer regarding the requirement for increased clarity in this figure and have extensively revised both the figure and the legend to simplify and clarify their interpretation.

15. "*The last sentence of the Discussion is unclear (may need a comma).*"
The Discussion, including the last sentence, has been extensively revised to increase content and clarity.

Reviewer #2 – Response

We thank the reviewer for their insightful major concern, as well as the helpful minor concerns. All of these are addressed below.

MAJOR CONCERN

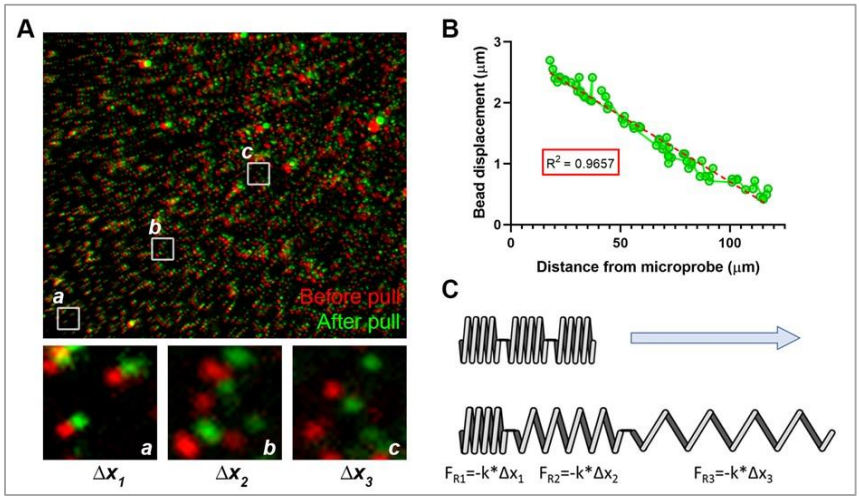
"*I have a general question that I've never understood about this approach. Has anyone ever measured the stiffness of the substrate that is being stretched to confirm that it's actually stiffer? For example's sake,*

*consider a spring that is behaving elastically. We know F = k*x. If we apply a force we get a displacement that is proportional to the spring's stiffness. As long as we are in the elastic regime, if we want the spring to stretch more, we simply apply a larger force. The stiffness of the spring doesn't change. As far as I can see, that's what you're doing here. You're applying a force and getting a strain field, but that doesn't make it stiffer. If the cell wants to deform the matrix further, it still has to apply the same force because the material properties of the gel are the same. This doesn't invalidate the results you see, it just changes how they are interpreted. Could you actually use your AFM to measure the stiffness of the region being pulled?*"

This is a matter of central importance and we appreciate the reviewer bringing it up. Unfortunately, we cannot answer the question in the 'simplest' manner, *i.e.* through experimentation, as there is simply no way to position the micromanipulator within under the AFM we have at our disposal. However, it is important that we still endeavor to address the concern.

The reviewer is correct that the direct proportionality (F_P=k*Δx) of Hooke's Law applies to a linear spring. However, the gels in this assay do not behave in a simple, linear fashion, as can be seen in the nonlinear displacement field generated by stretching the gel with the microprobe (manuscript Fig. 3 and figure below). Consider, then, that the magnitude of the restoring force (i.e. the force required to restore the stretched spring to its original length) is proportional to how far the spring is stretched from its original length; i.e. F_R=-k*Δx. Because the displacement increases with the proximity to the pulled microprobe (e.g. Δx1 < Δx2 < Δx3; see figure below), so then does the restoring force ((F_{R1}=-k*Δx1) < (F_{R2}=-k*Δx2) < (F_{R3}=-k*Δx3)). Functionally, this increased force or counter-tension is perceived by the cell as a stiffer substrate. Specifically, as a cell moves up this gradient (*e.g.* from *a* to *b*), either a 'constant' cell-generated contractile force would produce less and less gel movement, or the cell would have to exert higher force in order to move the gel the same distance. Indeed, this was elegantly & succinctly discussed by Lo *et al* in their original description of durotaxis, in which they state:

> "…the force/deformation caused by the external manipulation will be superimposed on the effects of the cellular probing forces. …Because fibroblasts exert centripetal forces on the substrate, pulling the flexible substrate away from the cell center means that cell-generated forces produce less substrate motion, which may then be interpreted by the cell as being equivalent to a stiffer substrate."



Nonetheless, to clarify the text, we have judiciously replaced certain instances of the term "stiffness" with "tension", as appropriate. We respectfully suggest that this explanation not be included in the final, published manuscript, as (1) we feel it is not needed to appreciate the protocol as presented and (2) the explanation makes certain 'assumptions' (*e.g.* the use of a simple 'spring' to depict a complex pseudo-elastic material) that we feel may draw criticism from readers with formal background or training in engineering and/or physics.

MINOR CONCERNS

1. "*Table 1: Are these stiffness values the Young's modulus?*"
The stiffness values do, indeed, reflect the Young's modulus. Details on their derivation (*e.g.* use of a Hertz model and the AFM probe size & shape) have been added to the appropriate section.

2. "*Have you measured the height of the gel?*"
We have measured the height of our standard gel and found the calculation to be relatively accurate, but we also have found that the height can be influenced by the thickness and manufacturer of the coverglass used for the top. These considerations, as well as a brief method for directly measuring gel height, have been included in this section of the protocol.

3. "*…it might be useful to include the settings that you use successfully in your lab to generate …micropipettes*"
More detailed information for this step (*e.g.* puller model, 1st and 2nd stage pull temperatures, 1st stage pull distance) has been included.

4. "*What is the purpose of the mineral oil?*"
The mineral oil prevents media evaporation during prolonged live-cell imaging sessions. This detail has now been included in the protocol text.

5. "*While…the durotactic response [is described] over a period of 80 minutes, these time points are clearly variable and can be adjusted to whatever experiment the user wants to perform.*"
The reviewer is absolutely correct. This has now been explicitly mentioned in the protocol text.