Cytoskeleton

WILEY

CYTOSKELETON

TECHNIQUES AND PROCEDURES OPEN ACCESS

Double Sided Traction Force Microscopy: A Method to Confine Cells for Physiologically Relevant Force Measurements

Alexia Caillier | Patrick W. Oakes

Department of Cell and Molecular Physiology, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois, USA

Correspondence: Patrick W. Oakes (poakes@luc.edu)

Received: 26 August 2025 | **Revised:** 21 January 2026 | **Accepted:** 2 March 2026

Keywords: cellular confinement | live-cell | traction force microscopy

ABSTRACT

In this paper we describe a technique to make a confined environment of variable stiffness that is suitable for high-resolution live-cell imaging. This versatile and adaptable technique enables cell confinement between soft elastic surfaces made from polyacrylamide gels. The two surfaces retain all their compatibility with multiple approaches to chemically couple adhesion proteins, and additional techniques like micropatterning and traction force microscopy. This method is thus well suited for measuring force production and migration of weakly adherent cells that struggle to migrate in traditional planar environments.

1 | Introduction

Cellular behavior is regulated by signaling which can arise from multiple sources, including molecules in solution, interactions with other cells, or changes in the physical environment (Janmey and Miller 2011; Sala et al. 2024). These signaling cascades are not independent and often overlap (Miller and Davidson 2013; De Belly et al. 2022). While glass and plastic dishes are easy to use and readily available, physiological environments rarely, if ever, resemble these rigid, planar substrates (Yang et al. 2017). In vivo, cells experience complex environments that vary both biochemically and physically (Gaylo et al. 2016; Ventura and Sedzinski 2022; Oudin and Weaver 2016; Devreotes and Horwitz 2015). From combinations of ligands to three-dimensional architectures, these combined environmental factors can strongly influence cellular behavior. There has thus been a concerted effort to develop methods that more strongly resemble physiological environments, while retaining the control of and specificity of in vitro environments (Kim and Hayward 2012; Liu et al. 2025).

When Albert Harris first put cells on an elastic substrate, he noticed that cells would cause the sheet to wrinkle (Harris et al. 1980). This clearly demonstrated that cells were contractile and pulling on the substrate under them. This approach was later refined to enable calculations of forces applied by the cells by embedding fiducial markers in the substrate for the purpose of measuring its deformation (Butler et al. 2002; Dembo and Wang 1999), a technique known as traction force microscopy (TFM) (Munevar et al. 2001). In addition to being able to measure cellular forces, use of elastic substrates has revealed a host of cellular behaviors that are sensitive to the mechanical properties of the environment (Janmey et al. 2020), including cell spreading (Yeung et al. 2005; Oakes 2018), migration (Bangasser et al. 2017; Isomursu et al. 2022; Pelham and Wang 1997), gene expression and differentiation (Saha et al. 2008; Engler et al. 2006; Wolfram et al. 2024; Feng and Nakamura 2025).

Similarly, changes in environmental architecture can strongly impact cell behavior (Muncie and Weaver 2018; Yamada and Sixt 2019). For example, when subjected to a high level of confinement ($<3\,\mu m$), many cells undergo a transformation from a mesenchymal state to a shapeshifting, contractile, and low-adhesive amoeboid migration mode (Liu et al. 2015). Indeed, cell morphology is strongly dependent on the local environment, with cells displaying multiple migration phenotypes in response to the density, organization, and stiffness of extracellular matrix

(ECM) proteins (Charras and Sahai 2014). Simpler approaches confining cells between two parallel surfaces (Liu et al. 2015) or under agar pads (Logue et al. 2015) elicit similar changes in morphology. While many of these studies have been done in strongly adherent cells, confinement also makes it possible to investigate migration of weakly adherent cells. For instance, we recently showed that T cells failed to migrate on 2D fibronectin-coated coverslips but could migrate rapidly and efficiently on the same substrate when confined (Caillier et al. 2024).

Here we describe an approach that combines a previously established method for confining cells (Liu et al. 2015) with tunable elastic substrates. This enables us to modulate both the stiffness of the substrate and the magnitude of confinement to more accurately match what cells might see in vivo, while retaining the experimental control we have in vitro and the ability to combine it with traditional TFM approaches. This approach we hope will be of use for researchers interested in measuring traction forces generated by cells in confinement and for investigating migration behaviors of weakly adherent cells.

2 | Protocol

2.1 | Material Preparation

Reagents and material needed	Brand	Catalog number
22×30 mm #1.5 coverslips	Corning	2,980,223
12 mm round #1.5 coverslips	Chemglass Life Sciences	CLS1763012
2-Propanol	Sigma-Aldrich	190,764-4 L
3-aminopropyltrimethoxysilane	Acros Organics	313,255,000
Glutaraldehyde Aqueous 70%	Electron Microscopy Sciences	16,360
Coverslips racks	Milipore Sigma	Z743685-1EA
Small Coverslips racks (better for 12 mm coverslips)	Milipore Sigma	Z688568-1EA
Sulfo-SANPAH	Covachem	NC2028693
Dimethyl Sulfoxide (DMSO)	Fisher Scientific	BP231
PDMS (Sylgard 184 Silicone Elastometer)	Krayden Dow Corning	DC4019862

2.1.1 | Coverslip Activation

NOTE: This step ensures a covalent bond between the polyacrylamide (PAAm) hydrogels and glass coverslips.

1. Place 22×30 mm #1.5 coverslips and 12 mm round #1.5 coverslips in racks.

   NOTE: For 12 mm round or other small coverslips, holding the coverslips in the rack with tweezers can aid in preventing them from floating.

2. Immerse the rack in a deep glass dish filled with isopropanol. The isopropanol should cover the coverslips completely. Keep track of the volume used for later calculations.
3. Use a glass pipet to add 3-aminopropyltrimethoxysilane to the isopropanol to a final concentration of 2%.
4. Add a stir bar at the bottom of your dish and place on a stir plate at room temperature for 10 min.

   NOTE: When starting the stir plate, pay attention that the stir bar doesn't touch your rack and increase the speed slowly.

5. After 10 min, remove the racks from the dish and dispose of the isopropanol/3-aminopropyltrimethyoxysilane solution in hazardous waste according to your institutional policies.
6. Dip the racks with coverslips slowly six times in deionized water. Replace the solution with fresh deionized water and repeat four times.

   NOTE: When immersing the small round coverslips, it is best to move slowly to prevent the coverslips from floating. Instead of removing the rack entirely from the water, the rack can be oscillated while fully submerged to keep air bubbles from forming.

   NOTE: If you wish to stop at this step, you can let the coverslips dry in a low temperature incubator.
7. Place the racks in the dish and fill with deionized water, making sure the coverslips are fully submerged. Add 1% glutaraldehyde to the solution.
8. Stir on a stir plate at room temperature for 30 min.
9. After 30 min, remove the racks from the dish and dispose of the solution in hazardous waste.
10. Place the racks back in the dish and fill with deionized water and wash on the stir plate for 10 min. Replace the water with fresh deionized water and repeat this wash step two more times.
11. Remove racks from the dish and allow them to dry in a clean environment overnight.

    NOTE: Excess water can be removed using kimwipes to wick away water from the racks.

12. Cover in Aluminum Foil to keep away from dust. Store at room temperature.

2.1.2 | Aliquot Sulfo-SANPAH

NOTE: While we describe here how to coat the gels using the photoactivatable crosslinker Sulfo-SANPAH, there are multiple other approaches to chemically modify the gels with other proteins of interest (Lee et al. 2016; Vignaud et al. 2014; Wolfel et al. 2022; Missirlis et al. 2022; Kumai et al. 2021).

NOTE: Proceed quickly since Sulfo-SANPAH degrades rapidly once it is exposed to moisture. Once frozen, use within 3–4 months.

1. Resuspend the Sulfo-SANPAH in anhydrous DMSO to a concentration of 50 mg/mL. Make 40 μL aliquots in 1.5 mL Eppendorf tubes.
2. Flash freeze the aliquots by dipping them in liquid nitrogen.
3. Store at −80°C.

2.1.3 | Make PDMS Pillar (Refer to Figure 1)

NOTE: The stiffness of the pillar will need to be optimized based on the cell chamber dimension and the dimension of the pillar (Figure 6A–F). As depicted in Figure 6, the length and stiffness of the pillar will affect the curvature of the bottom gel coverslips. Having a curved bottom can still allow for precise measurements and provide a range of different confinement levels, but it does create a gradient of confinement, which can influence cell migration directionality. However, if optimized properly, both gels will rest flat on each other, keeping the confinement uniform (Figure 6E).

1. Mix PDMS (Sylgard 184 elastomer) base with the curing agent at a ratio of 40/1 (for very soft pillars, Figure 6D,E) or 30/1 (for slightly stiffer pillars, Figure 6C).
2. Mix for 1 min.
3. Pour PDMS into the mold with the desired pillar dimension.

   NOTE: We use a custom mold created by drilling 12 mm holes (e.g., the size of the coverslip that is eventually attached to the pillar) into a piece of Delrin and clamping it between two pieces of plexiglass (Figure 1). This is not required though and pillars can easily be fabricated using a 25 mm plastic pipette that is cut to the desired height as a mold. The pillar should be made to match the height of the cell imaging chamber. In our chamber, the space between the bottom coverslip and our lid is 6.6 mm. A 6.4 mm pillar with a 40/1 ratio of PDMS resulted in both gels perfectly resting on each other. A slightly larger or stiffer pillar resulted in a curved bottom coverslip (Figure 6C,D). It is thus critical to optimize this step for your experimental setup to ensure the correct geometry is achieved.
4. Place the mold in a dish to contain any overflow of PDMS before placing the dish in a degassing chamber. Degas for at least 30 min or until no air bubbles are visible.

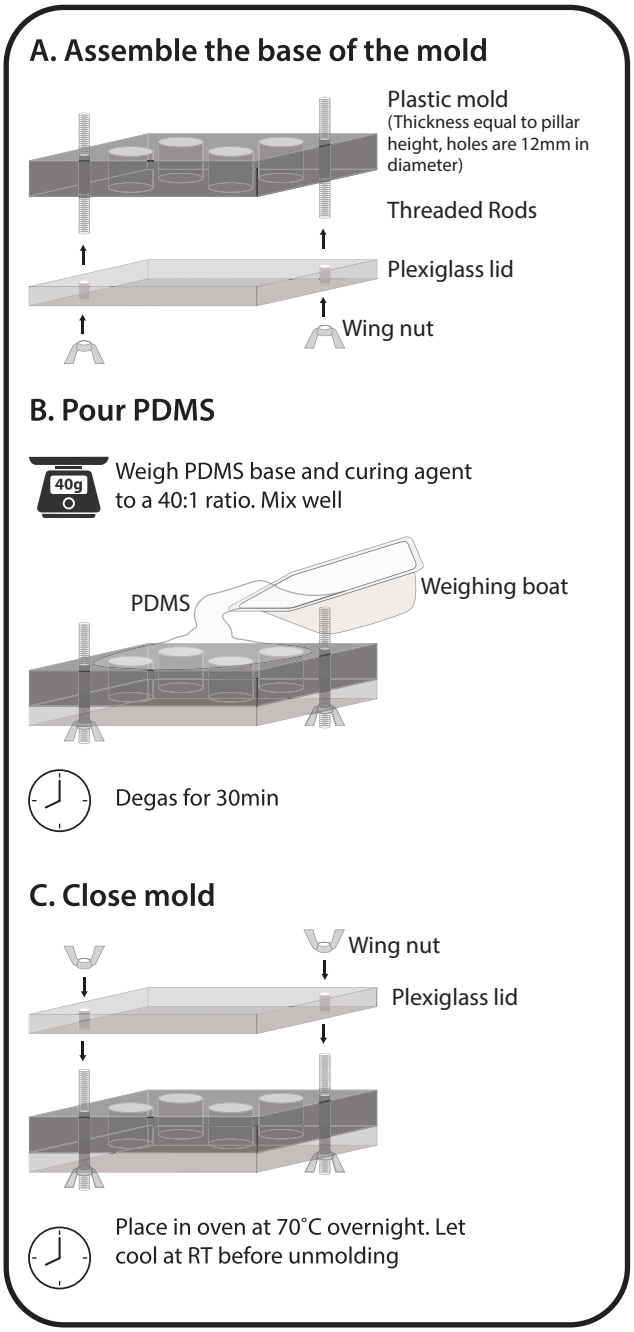

FIGURE 1 | PDMS pillar fabrication. (A–C) PDMS pillar mold set up suggestion. The middle plate has a thickness equal to the desired pillar height. Holes equal to the diameter of the upper coverslip (e.g., 12 mm) were drilled into the plate. We used outer plates made of plexiglass to serve as lids and which are clamped on either side of the mold. Refer to Section 2.1.3 from the text for more detail.

5. Close the mold and place in a 70°C oven overnight.
6. The next day, let cool at room temperature before unmolding.
7. Open the mold using a razor blade and isopropanol to clean up the excess PDMS.
8. Unmold the pillars, clean with isopropanol, and keep in a clean Petri dish for future use.

2.2 | Fabrication of PAAm Hydrogels

Reagents and material needed	Brand	Catalog number
40% Acrylamide solution	Bio-Rad	#1610140
2% Bis solution	Bio-Rad	#1610142
FluoSpheresCarboxylate-Modified Microspheres	Thermofisher Scientific	F8789
TEMED	FisherScientific	BP150-20
Ammonium Persulfate	FisherScientific	BP179-25
Water repellent treatment	Rain-X	800,002,250 W

2.2.1 | Make a Stock Standard Solution (Figure 2A)

NOTE: The stiffness of the gel should be optimized for your cell type. The gels should be soft enough to measure forces, but not so soft that the cells embed in the gels. Cells embedded in the gel will push the gel out of the plane of focus, complicating the analysis. See Table 1 for different stiffness recipes based on previous reports (Yeung et al. 2005).

Example for an 8.6 kPa shear modulus gel:

1. Collect on ice: 40% acrylamide solution, 2% bis-acrylamide solution and miliQ water.
2. In a 15 mL conical mix together: 2.343 mL of 40% acrylamide solution, 1.875 mL of 2% bis-acrylamide solution and 0.781 mL water. Mix well with a pipette.
3. Cover the tube in foil and store at 4°C.

   NOTE: Once made, the stock Standard Solution can be stored at 4°C, protected from light for up to 3 months.

2.2.2 | Make Microscopic Slides Hydrophobic

1. Spray a hydrophobic coating (e.g., Rain-X (ITW Global Brands)), on your microscope slide. Use a tissue to rub the hydrophobic coating onto the microscope slide for 1 min. Once dry, rinse with water and dry the remaining water with Kimwipes.

   NOTE: Multiple coverslips can fit on a single glass slide. For instance, you can fit two 22 × 30 mm and two 12 mm round coverslips on one standard 3″ microscope slide. Therefore, make as many microscope slides as you need according to how many gels you expect to make.
2. To ensure the slide is hydrophobic, repeat step 1 a second time.

   NOTE: Making slides water repellent ensures that it is easy to detach the gels from the slide later.
3. Make sure to remove any streaks or dust from the slides.

   NOTE: It is recommended to make fresh hydrophobic microscope slides each time you make hydrogels, but they can be stored in a sealed container if needed.

2.2.3 | Polymerize PAAm Hydrogels. (Refer to Figure 2)

1. Place the hydrophobic microscope slide in the bottom of a 10 cm dish.
2. In a 1.5 mL Eppendorf tube mix the standard solution, water, and bead components of the gel recipe (Table 1): Using the 16 kPa example, use 300 µL of the Standard Solution, 191.75 µL of MiliQ water, 5 µL of 0.04 µm fluorescent microbeads. Mix well with a pipette (Figure 2B).

   NOTE: It is recommended to use different color beads for the bottom and top gels, though not required. This will help distinguish the interface between gels. The color combination can be chosen to accommodate any fluorophores or stains used in the experiment.
3. Add 0.75 µL TEMED and 2.5 µL of 10% APS and mix the solution quickly by pipetting up and down while on ice.

   NOTE: The TEMED is a catalyst for this interaction while APS initiates the polymerization reaction. By keeping the solution cold on ice, it will limit premature polymerization of the solution, but this step should still be performed quickly. APS should be made fresh for best and most consistent results.
4. For bottom gels: Place a 7 µL drop per gel at the edge of your microscope slide (Figure 2C).
5. Place activated 22 × 30 mm coverslips on the drops, leaving a small overhang to facilitate detachment after the gel has polymerized (Figure 2D).
6. For the top gels: Pipet two 1.98 µL drops in the middle of the microscope slide. These will be the top gels. Make sure to use the correct mixture if using two different color beads (Figure 2C).
7. Place activated round 12 mm coverslips on the drops, leaving a small overhang (Figure 2D).

   NOTE: Keep the remaining PAAm mixture in the tube and place on the bench next to your dish. This will serve as a positive control that the polymerization has worked properly.
8. To prevent the gels from fully drying out during the polymerization process, place a damp kimwipe in the lid of your petri dish. Close the lid and let the gel polymerize for 40 min.

   NOTE: The damp kimwipe needs to be wet enough to keep humidity throughout the polymerization, but not enough for the water to drip from the lid. After ~40 min, confirm that the remaining solution in the tube has polymerized.

   NOTE: Polymerization occurs rapidly once the PAAm solution comes to temperature. While the gel may appear fully polymerized, we allow the reaction to proceed for 40 min to fully ensure that all the monomers have had the

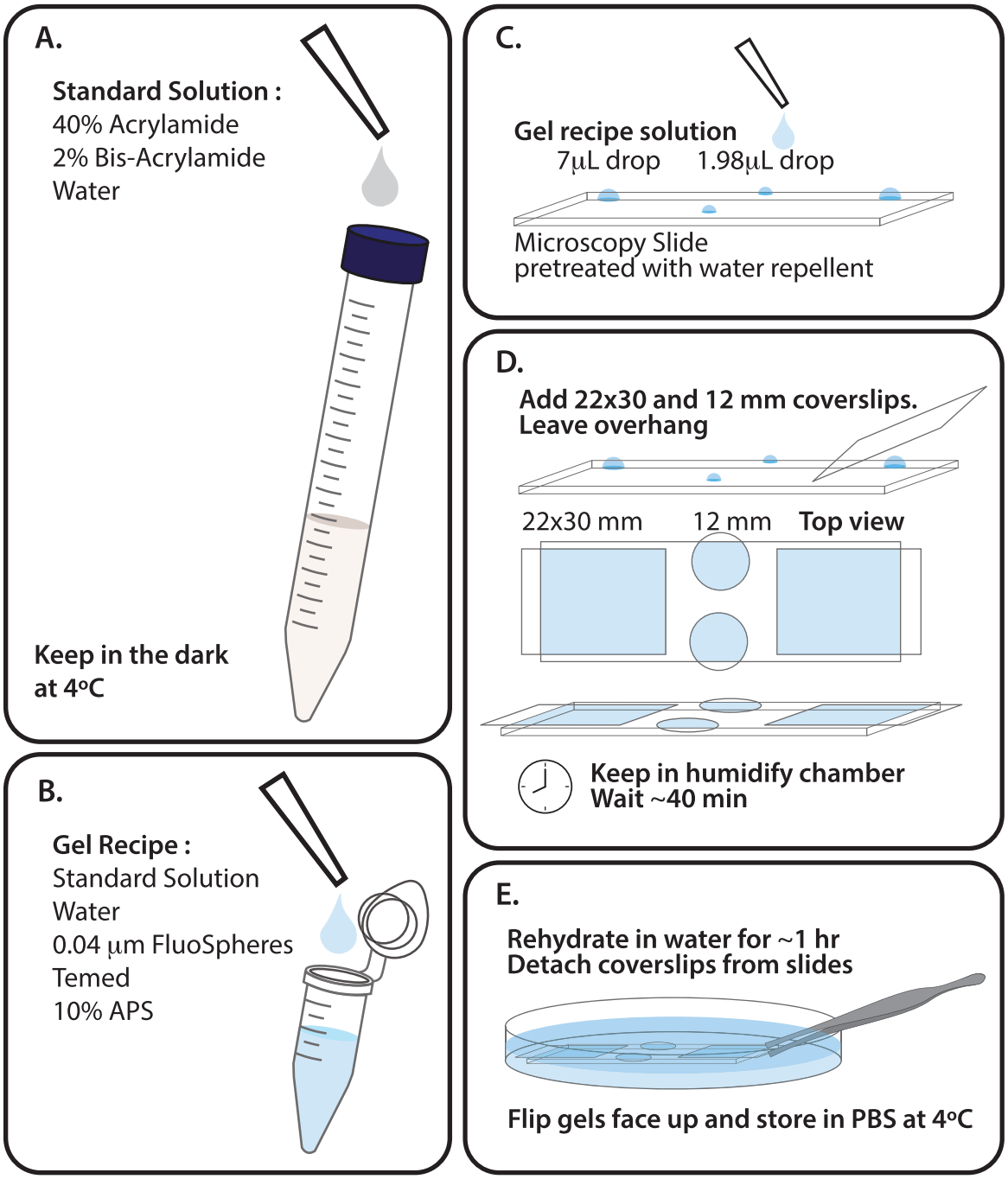

FIGURE 2 | Fabrication of PAAm hydrogels. (A) Make a stock standard solution of desired stiffness based on Table 1 recipes. The standard solution can be stored in the refrigerator for future use for about 3 months. Refer to Section 2.2.1 from the text. (B) Pipette a small volume of Standard solution (refer to Table 1 recipes), add APS, TEMED, fluorescent beads, and complete with water to 500 mL total. (B–E) Polymerize PAAm gels. Refer to Section 2.2.3 from the text for more detail.

opportunity to polymerize. Proceeding before the gel is fully polymerized can affect the stiffness of your gel.

9. Once the gels are polymerized, add water to the dish until the coverslips are fully immersed (Figure 2E).

   NOTE: The gels can be stored at this step for later use by wrapping the dish in parafilm and storing at 4°C. It is recommended to use the gels within the next 48 h.

10. Allow the polymerized gels to rehydrate in water for at least an hour before use.

    NOTE: Softer gels are harder to detach cleanly from the glass slide. A longer hydration makes them detach more easily. For very soft gels (e.g., <400 Pa), an overnight hydration can make removing the gels easier.

11. To detach the gel, place one side of your tweezer tip under the overhanging part of your coverslip. Rest the tweezer on the side of the dish. In one motion, push on the tweezer handle, which should act as a lever to lift your coverslip with the covalently bonded gels from the microscope slide.

TABLE 1 | Polyacrylamide gels recipe.

Standard solution recipe					
Standard solution stiffness (Pa)	**16,315**	**10,014**	**460**	**336**	**253.48**
40% acrylamide (mL)	2.5	3.125	1.25	1.25	1.25
2% bis-acrylamide (mL)	0.604166667	1.666666667	0.666666667	0.5833333	0.5
Water (mL)	1.895833333	0.208333333	3.083333333	3.16666667	3.25
Total volume	5	5	5	5	5
Gel recipe (μL)					
Standard solution volume	300	150	150	150	150
Water	191.75	341.75	341.75	341.75	341.75
Beads	5	5	5	5	5
TEMED	0.75	0.75	0.75	0.75	0.75
10% APS	2.5	2.5	2.5	2.5	2.5
Total volume	500	500	500	500	500
Final acrylamide percentage	12	7.5	3	3	3
Final bis percentage	0.145	0.2	0.08	0.07	0.06

Note: Each color represents a different final percentage of acrylamide. Each stiffness has been confirmed by measuring the indentation of the gel with three-dimensional confocal stacks based on a previously developed approach (Lee et al. 2015). Other stiffnesses can be achieved by changing the recipe based on previous reports in the literature (Yeung et al. 2005).

NOTE: If the gel is detached too slowly, you may observe deformations or patterns on your gel.

NOTE: If the gel has moved during the polymerization process and there is no longer an overhanging edge, a razor blade edge can be slid between the coverslip and the slide to detach them.

12. Once the gels are detached, flip them face up and remove the microscope slide from the dish. Care should be taken to not damage the gel itself.

2.3 | Coat PAAm Hydrogels (Optional, Refer to Figure 3)

NOTE: Individual projects will require different substrate coatings. For a non-adhesive substrate, PAAm hydrogels are biochemically inert and should prevent any nonspecific attachment of proteins. This step is therefore optional. Multiple methods have been developed to modify the surface of the gels with different proteins of interest (Lee et al. 2016; Vignaud et al. 2014; Wolfel et al. 2022; Missirlis et al. 2022; Kumai et al. 2021). We describe a common and easily accessible approach below using Sulfo-SANPAH, a hetero-bifunctional crosslinker that releases Sulfo-N-hydroxy-succinimide (Sulfo-NHS) upon UV-radiation. NHS esters form a stable bond with primary amino groups (—NH2), allowing strong bonds between the hydrogels and proteins used for coating.

Reagents and material needed	Brand	Catalog number
Sulfo-SANPAH	Covachem	NC2028693
Fibronectin	Millipore Sigma	FC010

2.3.1 | Treat Your PAAm Hydrogels With Sulfo-SANPAH

1. Prepare a sample holder by placing a piece of parafilm inside a petri dish or taping it to a piece of cardboard.
2. Place your coverslips with PAAm hydrogels face up on the parafilm, removing any extra water by touching the corner of the coverslip to a dry kimwipe.

   NOTE: This step should be performed quickly, as once the gels are removed from the water they can dry out.
3. Resuspend Sulfo-SANPAH by adding 560 μL of miliQ water to the 40 μL Sulfo-SANPAH aliquot (from Step 1.2).

   NOTE: Keep the Sulfo-SANPAH aliquot on ice and proceed quickly, since it degrades rapidly once exposed to moisture. One aliquot is good for two bottom gels on a 22 × 30 mm coverslip and two top gels on a 12 mm round coverslip.
4. Add 200 μL of the suspension per bottom gel and 100 μL of the suspension per top gel.
5. Place your gels face up with Sulfo-SANPAH in the UV light source. Expose the gels to the UV light for 5 min at maximum strength.

   NOTE: In the UV oven, place a tube rack or any type of box that will act as a pedestal for your samples to bring them at ~2–3″ under the light bulb. The Sulfo-SANPAH should change color from bright red to burgundy once it is activated.

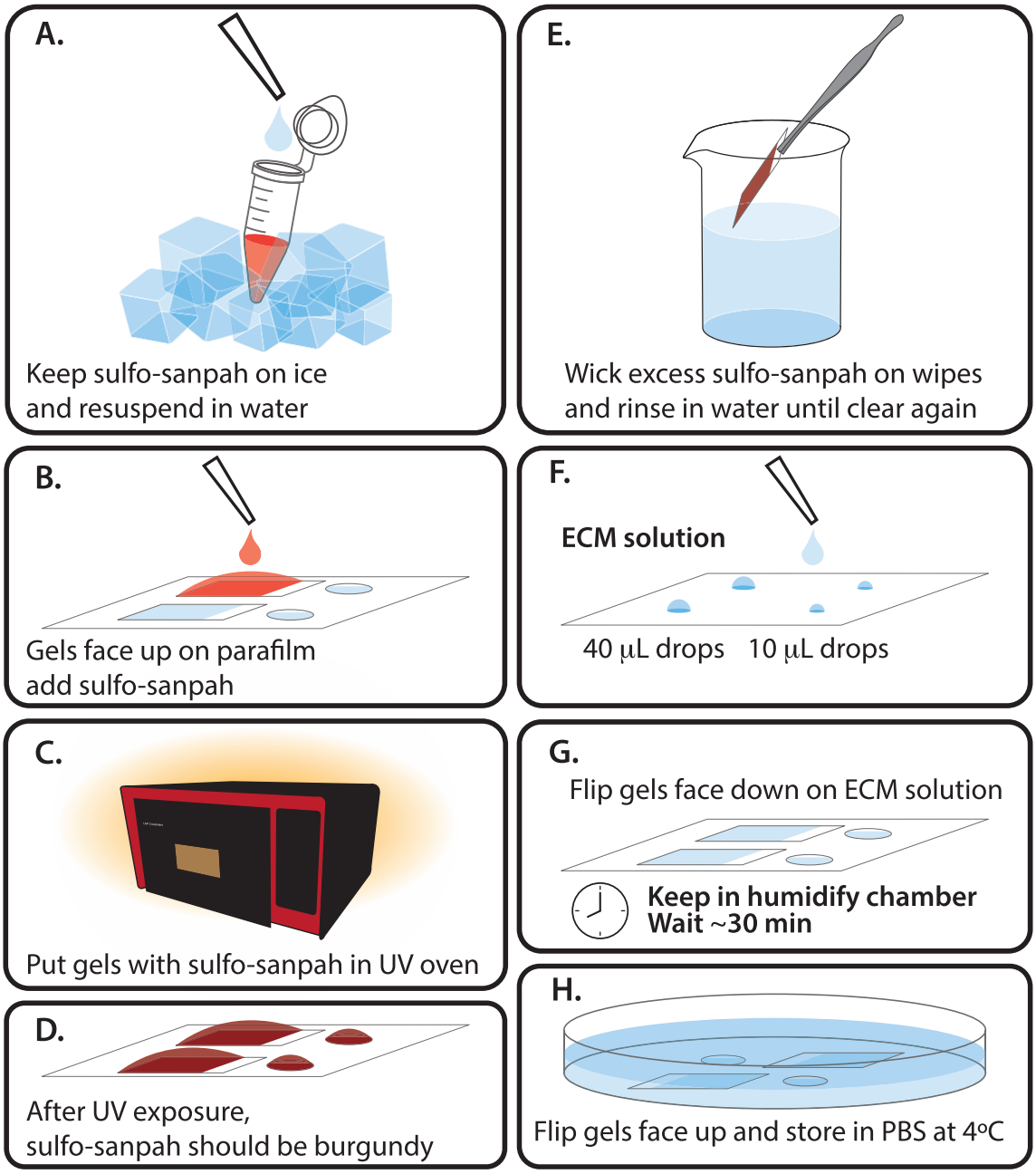

FIGURE 3 | Coat PAAm hydrogels. (A) Take a Sulfo-SANPAH aliquot from the −80°C and keep on ice. Refer to Section 2.1.2 on how to make Sulfo-SANPAH aliquots. Proceed quickly as Sulfo-SANPAH is sensitive to humidity. (A–E) Treat your PAAm hydrogels with Sulfo-SANPAH. Refer to Section 2.3.1 for more detail. (F–H) Coat hydrogels with a substrate of choice. Refer to Section 2.3.2 for more detail.

2.3.2 | Coat Hydrogels With Substrate of Choice

1. Prepare a fresh piece of parafilm to hold the gels in a dish.
2. From your ECM solution, make two 40 µL drops (one for each bottom gel on a 22 × 30 mm coverslip) and two 10 µL drops (one for each top gel on a 12 mm round coverslip) on the parafilm.

   NOTE: For fibronectin, we use a 1 mg/mL concentration. Other ECM proteins may require different dilutions.
3. When the UV treatment is done, lift the coverslips with tweezers, remove the excess Sulfo-SANPAH by touching the side of the coverslip to a kimwipe and wash the coverslips by repeatedly dipping into a large beaker of fresh milliQ water. The reddish stain from Sulfo-SANPAH should be barely noticeable. Remove any excess water by touching the side of the coverslip to a kimwipe.
4. Invert the coverslip and place the gel side down on the ECM solutions. To prevent the gels from drying out, place a damp kimwipe in the lid of your petri dish and close the lid. Incubate for 30 min at room temperature.

   NOTE: It may be necessary to optimize the incubation time, temperature, and concentration for different ECM proteins. For example, collagen will polymerize at room

temperature, and so a uniform coating is best achieved by incubating for longer periods of time at 4°C.

5. Use tweezers to flip the gels face up and rinse multiple times with PBS. Coated gels can be stored in a PBS solution for up to 48 h prior to use.

   NOTE: To help lift the gels without disturbing the gels, you can gently add 1 mL of PBS with a pipet on the side of the coverslips. The PBS should lift the coverslips and make it easier to grab with tweezers.

2.4 | Sample Preparation for Imaging (Refer to Figure 4)

2.4.1 | For Adherent Cells (Figure 4A,B)

1. Place the bottom gel face up in a petri dish and cover with cell media.
2. Plate cells in the dish with the gels.

   NOTE: The number of cells to plate will change depending on the experiment. For example, with our fibroblast cell culture line, plating 50,000 cells in a 6 cm dish the day before the experiment produces a coverslip with single isolated cells that are easy to find but not overlapping.

3. Let the cells attach anywhere from 30 min to overnight according to your experimental needs.

   NOTE: Plating time is experiment specific. Over time, some cells will start to ingest fluorescent beads from the gel, making the TFM analysis challenging.

4. Once the cells are sufficiently attached, place the gel face up in a cell chamber and cover with cell media.

2.4.2 | For Non-Adherent Cells (Figure 4B)

1. Place the bottom gel face up in a cell chamber.
2. Add around 500,000 to 1 million cells in a minimum of 300 μL or a maximum of 2 mL of media.

   NOTE: At least 300 μL is needed to prevent the gel from drying. Make sure the microscope chamber is humidified when using this low of a volume.

3. Allow cells to settle for 10–15 min.

2.4.3 | Confine the Cells

NOTE: This approach can be easily adapted to a variety of imaging chambers (see Figure 5 for examples) and is not dependent on a specific chamber design. We describe below the approach for the chambers we use, the details of which we are happy to share upon request.

1. Place the PDMS pillar on the metal lid (Figure 4C).

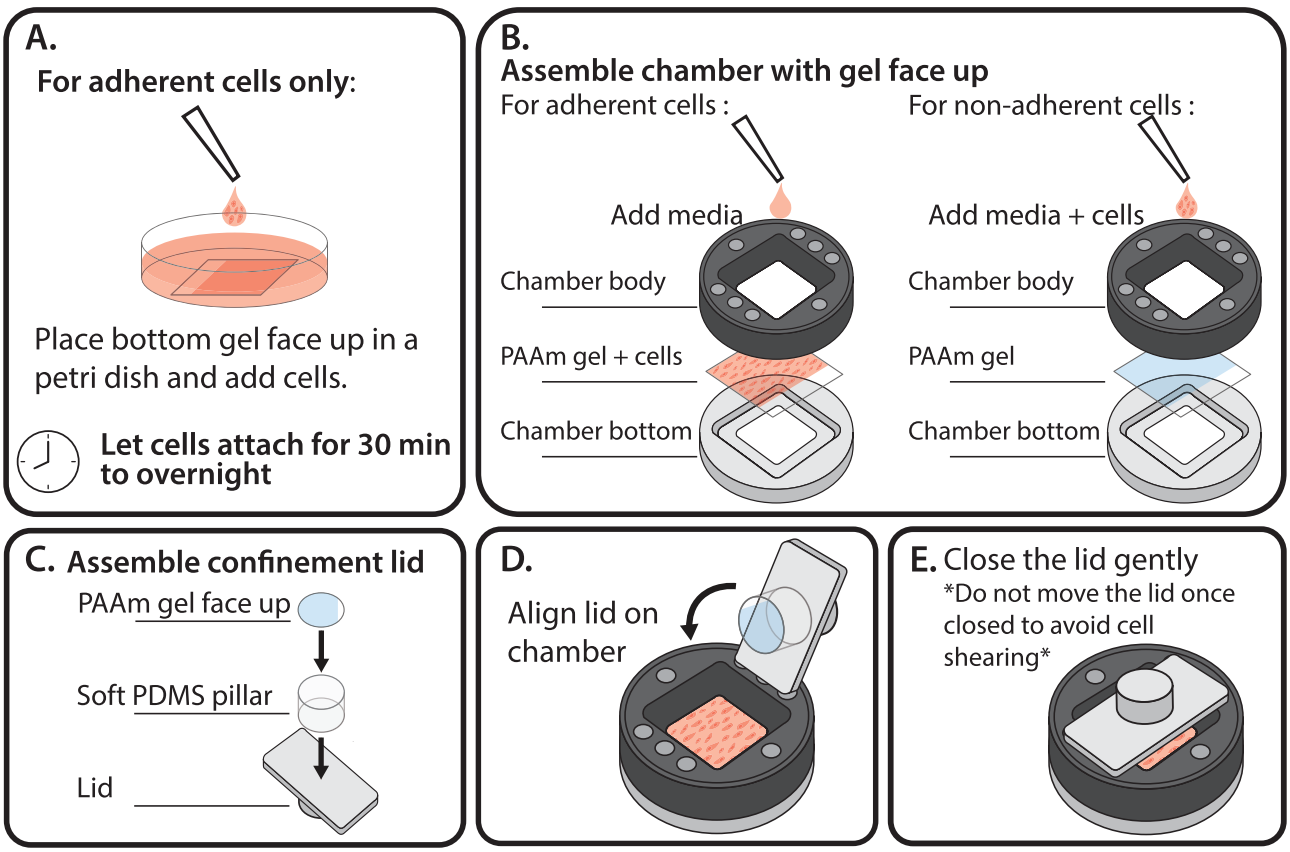

FIGURE 4 | Prepare samples for imaging. (A) Pre-seed adherent cells to give them time to attach. For non-adherent cells, proceed to assemble the chamber (B) and then add the cells. Refer to Sections 2.4.1 and 2.4.2 for more detail. (C–E) Assemble the confinement lid, making sure the gel is facing outward (not toward the pillar), and confine the cells. Refer to Section 2.4.3 for more details. Proceed to Section 2.5 when the sample is ready.

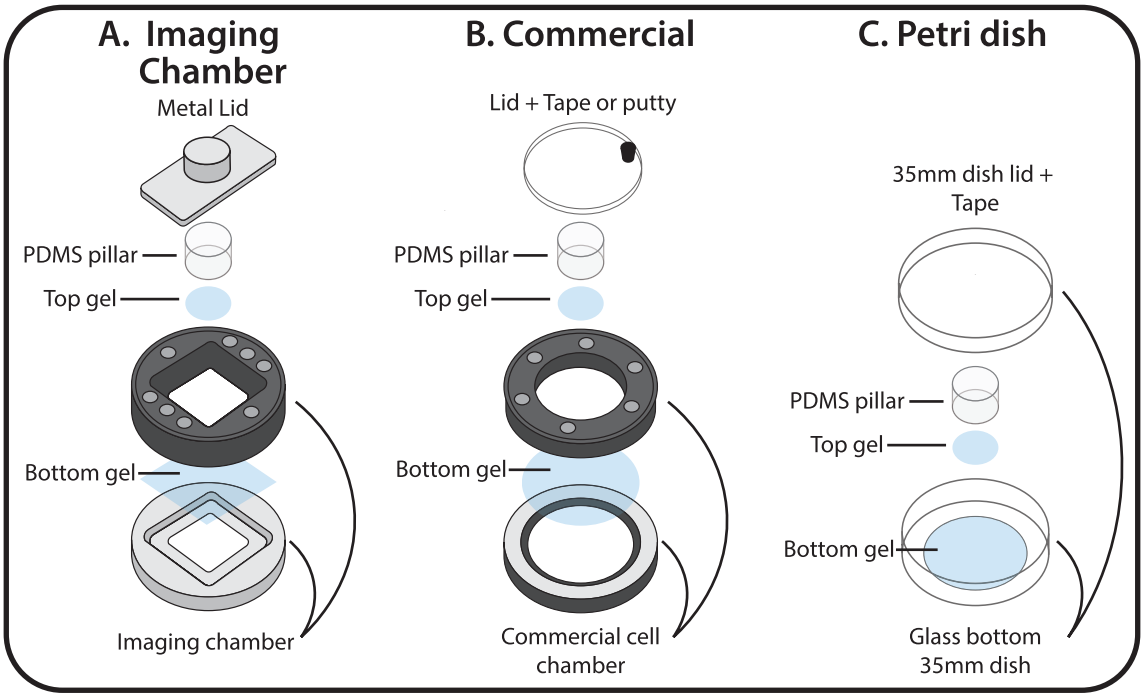

FIGURE 5 | Different possible experimental set-ups. (A) Set up used in this protocol. (B) An example of a commercial magnetic chamber, such as the Chamlide series of chambers offered by Live Cell Instruments (e.g., Product name: CMB, 35 mm Dish Type 1-Well Magnetic Chambers for Round Coverslip; CMS for 18, 20, 22, or 24 coverslips). The included lid can be secured on the magnetic chamber using tape or mounting putty, or could be combined with a small circular piece of magnetic metal which may reduce the chance of shearing the cells. (C) A glass bottom Petri dish is also adaptable to this protocol. When polymerizing the gel, use a water-repellent round coverslip instead of a slide. When coating the gel, a small piece of parafilm can be used to minimize the liquid required to coat the gel. When using tape or putty to secure the lid, be careful not to slide or rotate the lid which can shear the cells.

NOTE: Make sure the pillar is dry and clean. When using drug treatments, separate pillars should be kept for each condition, as PDMS is porous and can absorb the drug.

2. Place the top gel, gel face up, on the PDMS pillar (Figure 4C).
3. Align the lid with the cell chamber (Figure 4D). Gently close the lid on the cells (Figure 4E).

   NOTE: Try to minimize lateral motion to prevent shearing of the cells on the surface between the gels. Closing in one smooth motion can help minimize shearing. For non-adherent cells, most of the cells will get pushed out from between the gels, but if a high number of cells are plated, there should be enough left behind to image.

2.5 | Imaging Confined Cells

NOTE: Depending on the lid of the chamber and the microscope set up, imaging with transmitted light can be challenging as the light source can be obscured. This issue can be alleviated by using a fluorescent probe to visualize the cell.

2.5.1 | Identify the Best Confinement Level

NOTE: Because the central PDMS pillar is pushing on the bottom coverslip, it can cause the bottom coverslip to flex and create uneven levels of confinement (see Figure 6C). This can be adjusted by modifying the height and composition of the PDMS pillar (Figure 6).

1. Find an area where your cells are confined with your desired confinement level. Measure the spacing by focusing on the top of your bottom gel, taking note of your Z location and then focusing on the first plane you detect your top gel and again taking note of your Z location. The difference between both Z positions should be the distance between both gels.

   NOTE: You want your cells to be confined enough that they are touching both gels, but not too confined that the cell is embedded in the gel. If this is the case, you will start to see the shadow of your cell in the gels because the beads are pushed out of the focal plane, which will affect the analysis afterward.

2. Save a plane at the bottom of your cell. This should be near the top of your bottom gel.

   NOTE: if they are not the same, it might mean the coating on your gel is creating that spacing.

3. Save a plane at the top of your cell, which should be near the first plane visible of your top gel.

   NOTE: If you do not see the top gel when you are at the top of the cell, it means your cell is not actually confined.

2.5.2 | Acquire Images to Make a Movie

1. The frequency and the length will vary depending on the experiment. For example, when using highly migratory cells like T cells, imaging will be faster than when using slower cells like fibroblasts.

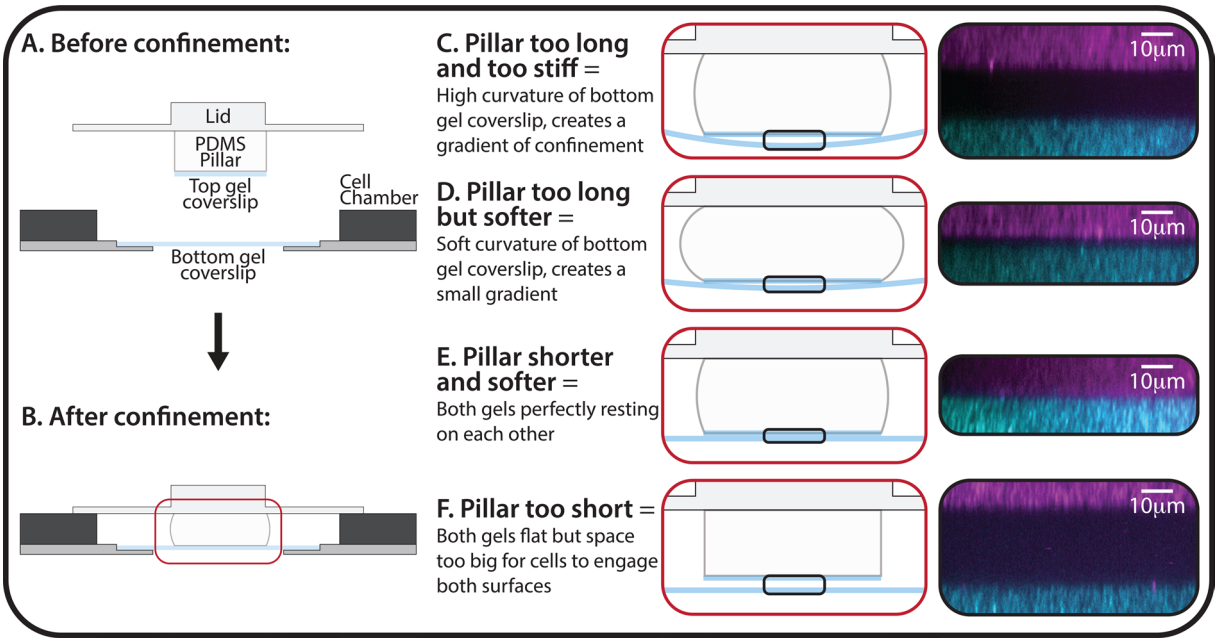

FIGURE 6 | PDMS pillar optimization. (A) Detailed side view of the components of the confinement chamber before (A) and after (B) confinement. (C–F) Importance of PDMS pillar height and stiffness on confinement level. (C) If the pillar is putting too much pressure on the bottom coverslip, it will curve the bottom coverslip, creating a space in the middle and a tight confinement on the edge of the top coverslip. This type of set up can be interesting if you want to have a gradient of confinement or to find the ideal level of confinement for a specific cell model. However, it can impact the directionality of cell migration, as cells might want to migrate toward a less confined area. (D) The pillar is the same size as in (C) but just changing the PDMS ratio from 30:1 to 40:1 made the pillar much softer and reduced the pressure applied to the bottom coverslip, reducing the curvature of the bottom coverslip. (E) The perfect height and stiffness should result in no space between gels and uniform confinement throughout. In this condition, however, the confinement is very sensitive to any faults in the gel (e.g., a fold, a tear, or an uneven gel) that would act as a spacer preventing them from perfectly resting on each other. (F) If the pillar is too short, a space should be observed throughout, even on the edge of the top coverslip. Refer to Sections 2.1.3 and 2.5.1.

NOTE: In ideal levels of confinement, the cells will still generate traction forces that are largely parallel to the surface of the gels that are compatible with traditional TFM methods (Sabass et al. 2008). In cases where the cells are generating significant out-of-plane displacements, short *z*-stacks of the beads may be required to fully capture the gel displacements. In this case the TFM analysis needs to be adjusted to account for the non-planar deformations following previously described methods (Plotnikov et al. 2014) or using alternative computational approaches (Legant et al. 2009; Franck et al. 2011; Hur et al. 2009; Bergert et al. 2016; del Álamo et al. 2013; Apolinar-Fernández et al. 2024).

2.5.3 | Take a Reference Image

NOTE: We need to have a reference image of the gel without cells on it to use as a reference for measuring the bead displacement. In a classical TFM protocol we would simply detach the cells, but this can be more challenging in confinement. The tight spacing makes diffusion slow, and killing the cells can leave behind cell debris that can still deform the gel. The best way to get a reference image is simply to let the cell migrate out of the initial position to get a view of the gel without the cell on it.

1. Wait for your cells to migrate out its initial position, then acquire an image of the gel at both surfaces without your cell present for reference.

2.6 | Analysis

NOTE: This protocol is primarily focused on constructing a setup to measure traction forces in confinement. The analysis of the measurements follows the classical TFM analysis methods that have been previously described (Denisin et al. 2024). We note that multiple references (Huang et al. 2020; Bauer et al. 2021; Schmitt et al. 2024; Han et al. 2015) include detailed step-by-step instructions and software that are freely available for the analysis. The specific code we use is described in Schmitt et al. (2024) and can be downloaded from: https://github.com/OakesLab/TFM. Installation instructions, example data, and a detailed walkthrough are included in the repository. Here we briefly explain the main steps and show a representative result using t cells in Figure 7. Note that each surface in the confined space is treated separately, and thus the analysis needs to be run twice for a given experiment to reveal forces that are on either the top or bottom surface. For more specific technical details of the analysis, readers are urged to look at the above references.

1. Images should be preprocessed as necessary. Adjustments may include cropping large fields of view and photobleaching or flat field correcting images. These steps are not required but can improve signal to noise in the final product.
2. Images of the beads in the gel should be aligned to the reference image. If additional channels are captured during

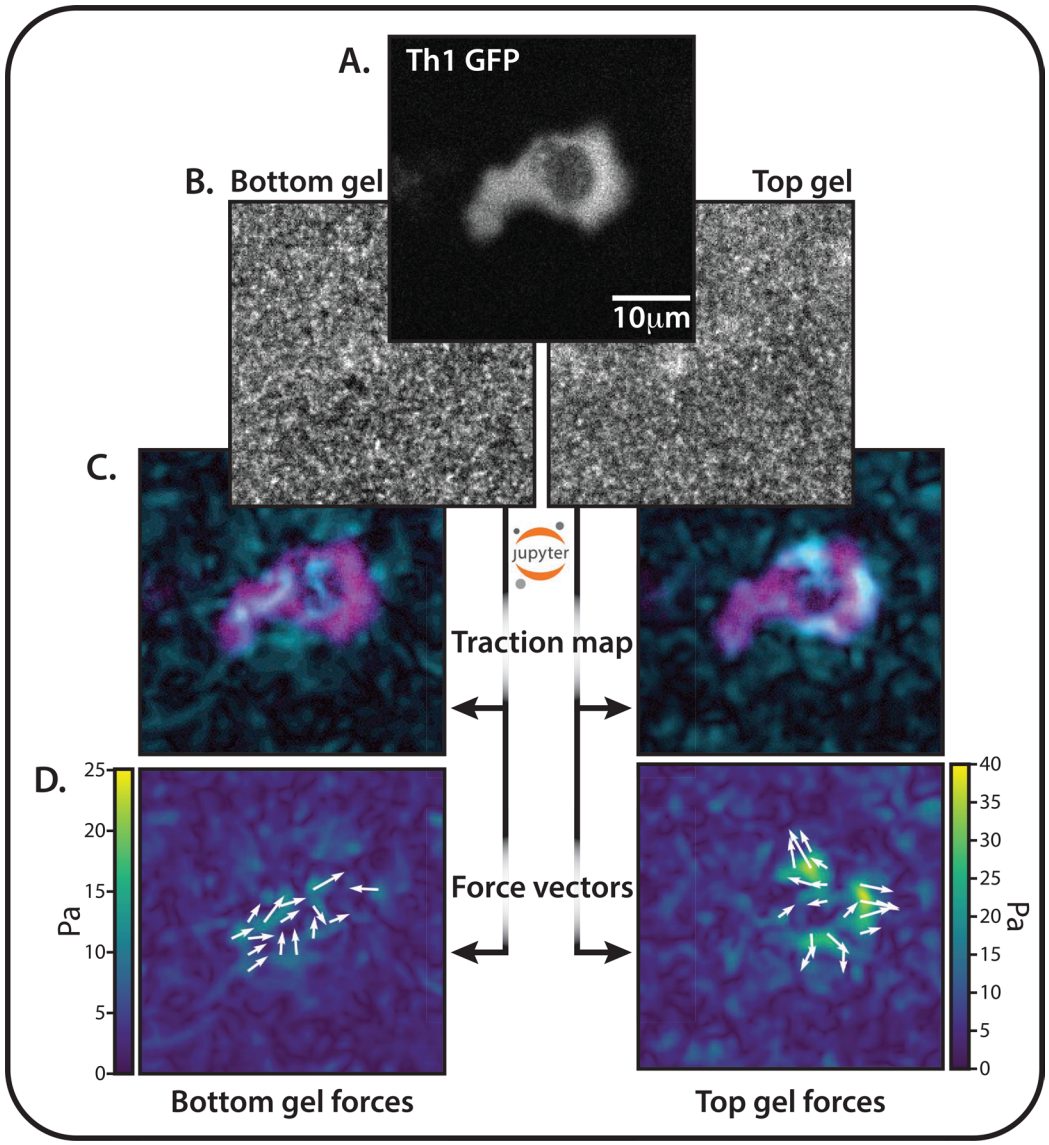

FIGURE 7 | Image analysis. (A) An example image of a helper T cell (Th1) expressing a free GFP fluorophore migrating between two polyacrylamide gels (B). (C) Representation of cell traction localization on the bottom gel (left panel) and the top gel (right panel). (D) The magnitude of the force (represented as Pa in the colorbar) and direction (white arrows) exerted by a Th1 t cell against the bottom gel (left panel) and top gel (right panel). For more detail refer to Section 2.6 from the text.

imaging, they can be registered by applying the same shift as the bead channel.

3. Measure the displacements in the bead images. Displacement calculations can be performed in multiple ways. See ref (Denisin et al. 2024) for a discussion of the different approaches.

4. The calculation of the traction forces is most easily performed in Fourier space (Butler et al. 2002). Here one solves the inverse problem, essentially calculating the forces that would be required to produce the measured displacements.

   NOTE: As an ill-posed problem, a regularization parameter must be chosen. Functionally, the regularization parameter smooths out the resultant traction vectors and suppresses noise (Stricker et al. 2010). There are multiple approaches to calculate this parameter (Sabass et al. 2008; Han et al. 2015; Huang et al. 2019; Apolinar-Fernández et al. 2024; Kandasamy et al. 2025), but none is definitively better than others. In our experience, the simplest way is to empirically test different values paying particular attention to regions where there are no cells, i.e., the background. These background values should be small compared to the relevant signal of real tractions generated by the cell. Once a regularization parameter is chosen, however, it is critical that the same value is used for all experiments. If different values are used, comparisons between data sets may no longer be valid.

5. The remaining analyses are highly specific to the experimental questions asked. Relevant values may include the strain energy (Oakes et al. 2014), contractile work performed, or the average distribution of forces and their direction (Caillier et al. 2024).

3 | Discussion

The approach presented here allows for the confinement of cells between two surfaces of physiological stiffness and can be used to induce migration of weakly adherent cells and to measure traction forces in a confined system. While this technique offers a new adaptation on other confinement methods (Liu et al. 2015), there are caveats that should be considered with its implementation. The system is not a true 3D environment as cells cannot migrate vertically into the gel. It is more accurately described as a 2.5D system, since it consists of two planar surfaces. For true 3D approaches to TFM or approaches that can incorporate out of plane forces in similar 2.5D setups, we direct the reader to other works (Legant et al. 2009; Franck et al. 2011; Hur et al. 2009; Bergert et al. 2016; del Álamo et al. 2013; Song et al. 2020; Mulligan et al. 2019; Toyjanova et al. 2014). The advantage of the presented approach is that it confines cells to a single plane, making it easier to track small and dynamic features, and keeps weakly adherent cells in contact with the substrate. Both features were highly advantageous in our recent work studying T cell migration (Caillier et al. 2024). Using this confinement, we were able to get T cells to migrate robustly on fibronectin coated substrates and still detect adhesion molecules that were not previously visible in 3D environments.

One key element of this approach is finding the right balance between cell confinement levels and gel stiffness. If the cells are not confined enough, they may not interact with the surface long enough to generate adhesion. If they are too confined, however, cells can be embedded in the gel, which can restrict their movement and may cause DNA damage (Shah et al. 2021). If the gels are too stiff, cells can become excessively compressed, and they might not be strong enough to deform the elastic substrate. On the other hand, if the gel is too soft, the cells will embed themselves in the gel, causing deformation out of plane, which can complicate the force reconstruction. The gels therefore need to be in that middle ground where the cells can generate deformations that are linear and planar. These considerations will lead to different needs for each experiment, depending on the context of the experimental question and cell type.

The main benefit of this technique is moving closer to a physiological environment, while retaining all the control of traditional 2D systems. These PAAm gels retain their compatibility with high-resolution imaging, micropatterning, and other covalent modifications of the gel. While we have not explicitly tried, one could also theoretically combine this approach with gels that contain a stiffness gradient (Isomursu et al. 2022). Additionally, the magnitude of confinement can be controlled (see Figure 6), making it possible to image cells in a variety of different positions and even watch as they transition between levels of confinement.

Beyond being more akin to physiological environments, the ability to measure forces in confinement provides an additional benefit, especially for weakly adherent cells. In part, this is because it causes them to maintain more contact with the substrate. Having adhesions on both surfaces can also alter the contractile machinery of the cytoskeleton of strongly adherent cells and reveal insights into how cells adapt their traction stresses in response to complex architectures. This is especially powerful as we consider how competing signals from both the variety of ECM molecules present and the changes in stiffness can influence cell behavior. In particular, this method might prove amenable to measuring non-specific friction forces that are proposed to drive some types of confined migration (Liu et al. 2015; Bergert et al. 2015; Ullo et al. 2024; García-Arcos et al. 2024).

In conclusion, confinement between two flexible substrates can be a powerful tool to study the behavior of cells in more physiological environments that retain a high degree of customizability and are amenable for high-resolution live-cell imaging.

Acknowledgments

We want to thank the Piel lab from Institut Curie for their help teaching us their static confinement technique which provided the basis for our approach. We also want to thank the members of the Fritz-Laylin lab from the University of Massachusetts (UMass), the Velle lab from UMass Dartmouth, and Titus lab from the University of Minnesota for their precious feedback on this method. This work was supported by NIH National Institute of General Medical Sciences (NIGMS) award R01-GM148644 and National Institute of Allergy and Infectious Disease (NIAID) Award P01-AI102851 to P.W.O.

Funding

This work was supported by the National Institute of General Medical Sciences (R01-GM148644) and the National Institute of Allergy and Infectious Diseases (P01-AI102851).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

References

Apolinar-Fernández, A., P. Blázquez-Carmona, R. Ruiz-Mateos, et al. 2024. "Regularization Techniques and Inverse Approaches in 3D Traction Force Microscopy." *International Journal of Mechanical Sciences* 283: 109592.

Bangasser, B. L., G. A. Shamsan, C. E. Chan, et al. 2017. "Shifting the Optimal Stiffness for Cell Migration." *Nature Communications* 8: 15313.

Bauer, A., M. Prechová, L. Fischer, I. Thievessen, M. Gregor, and B. Fabry. 2021. "pyTFM: A Tool for Traction Force and Monolayer Stress Microscopy." *PLoS Computational Biology* 17: e1008364.

Bergert, M., A. Erzberger, R. A. Desai, et al. 2015. "Force Transmission During Adhesion-Independent Migration." *Nature Cell Biology* 17: 524–529.

Bergert, M., T. Lendenmann, M. Zündel, et al. 2016. "Confocal Reference Free Traction Force Microscopy." *Nature Communications* 7: 12814.

Butler, J. P., I. M. Tolić-Nørrelykke, B. Fabry, and J. J. Fredberg. 2002. "Traction Fields, Moments, and Strain Energy That Cells Exert on Their Surroundings." *American Journal of Physiology* 282: 595–605.

Caillier, A., D. Oleksyn, D. J. Fowell, J. Miller, and P. W. Oakes. 2024. "T Cells Use Focal Adhesions to Pull Themselves Through Confined Environments." *Journal of Cell Biology* 223: e202310067.

Charras, G., and E. Sahai. 2014. "Physical Influences of the Extracellular Environment on Cell Migration." *Nature Reviews. Molecular Cell Biology* 15: 813–824.

De Belly, H., E. K. Paluch, and K. J. Chalut. 2022. "Interplay Between Mechanics and Signalling in Regulating Cell Fate." *Nature Reviews. Molecular Cell Biology* 23: 465–480.

del Álamo, J. C., R. Meili, B. Álvarez-González, et al. 2013. "Three-Dimensional Quantification of Cellular Traction Forces and Mechanosensing of Thin Substrata by Fourier Traction Force Microscopy." *PLoS One* 8: e69850.

Dembo, M., and Y.-L. Wang. 1999. Stresses at the Cell-to-Substrate Interface During Locomotion of Fibroblasts.

Denisin, A. K., H. Kim, I. H. Riedel-Kruse, and B. L. Pruitt. 2024. "Field Guide to Traction Force Microscopy." *Cellular and Molecular Bioengineering* 17: 87–106. https://doi.org/10.1007/s12195-024-00801-6.

Devreotes, P., and A. R. Horwitz. 2015. "Signaling Networks That Regulate Cell Migration." *Cold Spring Harbor Perspectives in Biology* 7: a005959.

Engler, A. J., S. Sen, H. L. Sweeney, and D. E. Discher. 2006. "Matrix Elasticity Directs Stem Cell Lineage Specification." *Cell* 126: 677–689.

Feng, M., and F. Nakamura. 2025. "Modulation of Gene Expression by Substrate Stiffness via Ubiquitination of Histone H2B by Ubiquitin-Conjugating Enzyme E2A/B." *ACS Omega* 10: 15799–15809.

Franck, C., S. A. Maskarinec, D. A. Tirrell, and G. Ravichandran. 2011. "Three-Dimensional Traction Force Microscopy: A New Tool for Quantifying Cell-Matrix Interactions." *PLoS One* 6: e17833.

García-Arcos, J. M., J. Ziegler, S. Grigolon, et al. 2024. "Rigidity Percolation and Active Advection Synergize in the Actomyosin Cortex to Drive Amoeboid Cell Motility." *Developmental Cell* 59: 2990–3007.e7.

Gaylo, A., D. C. Schrock, N. R. J. Fernandes, and D. J. Fowell. 2016. "T Cell Interstitial Migration: Motility Cues From the Inflamed Tissue for Micro- and Macro-Positioning." *Frontiers in Immunology* 7: 1–13.

Han, S. J., Y. Oak, A. Groisman, and G. Danuser. 2015. "Traction Microscopy to Identify Force Modulation in Subresolution Adhesions." *Nature Methods* 12: 653–656.

Harris, A. K., P. Wild, and D. Stopak. 1980. "Silicone Rubber Substrata: A New Wrinkle in the Study of Cell Locomotion." *Science (1979)* 208: 177–179.

Huang, Y., G. Gompper, and B. Sabass. 2020. "A Bayesian Traction Force Microscopy Method With Automated Denoising in a User-Friendly Software Package." *Computer Physics Communications* 256: 107313.

Huang, Y., C. Schell, T. B. Huber, et al. 2019. "Traction Force Microscopy With Optimized Regularization and Automated Bayesian Parameter Selection for Comparing Cells." *Scientific Reports* 9: 539.

Hur, S. S., Y. Zhao, Y. S. Li, E. Botvinick, and S. Chien. 2009. "Live Cells Exert 3-Dimensional Traction Forces on Their Substrata." *Cellular and Molecular Bioengineering* 2: 425–436.

Isomursu, A., K. Y. Park, J. Hou, et al. 2022. "Directed Cell Migration Towards Softer Environments." *Nature Materials* 21: 1081–1090.

Janmey, P. A., D. A. Fletcher, and C. A. Reinhart-King. 2020. "Stiffness Sensing by Cells." *Physiological Reviews* 100: 695–724.

Janmey, P. A., and R. T. Miller. 2011. "Mechanisms of Mechanical Signaling in Development and Disease." *Journal of Cell Science* 124: 9–18. https://doi.org/10.1242/jcs.071001.

Kandasamy, A., Y. T. Yeh, R. Serrano, M. Mercola, and J. C. del Alamo. 2025. "Uncertainty-Aware Traction Force Microscopy." *PLoS Computational Biology* 21: e1013079.

Kim, J., and R. C. Hayward. 2012. "Mimicking Dynamic In Vivo Environments With Stimuli-Responsive Materials for Cell Culture." *Trends in Biotechnology* 30: 426–439. https://doi.org/10.1016/j.tibtech.2012.04.003.

Kumai, J., S. Sasagawa, M. Horie, and Y. Yui. 2021. "A Novel Method for Polyacrylamide Gel Preparation Using N-Hydroxysuccinimide-Acrylamide Ester to Study Cell-Extracellular Matrix Mechanical Interactions." *Frontiers in Materials* 8: 637278.

Lee, D., M. M. Rahman, Y. Zhou, and S. Ryu. 2015. "Three-Dimensional Confocal Microscopy Indentation Method for Hydrogel Elasticity Measurement." *Langmuir* 31: 9684–9693.

Lee, J. P., E. Kassianidou, J. I. MacDonald, M. B. Francis, and S. Kumar. 2016. "N-Terminal Specific Conjugation of Extracellular Matrix Proteins to 2-Pyridinecarboxaldehyde Functionalized Polyacrylamide Hydrogels." *Biomaterials* 102: 268–276.

Legant, W. R., A. Pathak, M. T. Yang, et al. 2009. "Microfabricated Tissue Gauges to Measure and Manipulate Forces From 3D Microtissues." *Proceedings of the National Academy of Sciences* 106: 10097–10102.

Liu, N., Y. Shi, J. Li, et al. 2025. "Morphology-Guided Cellular Behavior Modulation With 3D-Printed Engineered ECM." *Cell Biomaterials* 1: 100090.

Liu, Y. J., M. le Berre, F. Lautenschlaeger, et al. 2015. "Confinement and Low Adhesion Induce Fast Amoeboid Migration of Slow Mesenchymal Cells." *Cell* 160: 659–672.

Logue, J. S., A. X. Cartagena-Rivera, M. A. Baird, M. W. Davidson, R. S. Chadwick, and C. M. Waterman. 2015. "Erk Regulation of Actin Capping and Bundling by Eps8 Promotes Cortex Tension and Leader Bleb-Based Migration." *eLife* 4: e08314.

Miller, C. J., and L. A. Davidson. 2013. "The Interplay Between Cell Signalling and Mechanics in Developmental Processes." *Nature Reviews Genetics* 14: 733–744. https://doi.org/10.1038/nrg3513.

Missirlis, D., M. Baños, F. Lussier, and J. P. Spatz. 2022. "Facile and Versatile Method for Micropatterning Poly(Acrylamide) Hydrogels Using Photocleavable Comonomers." *ACS Applied Materials & Interfaces* 14: 3643–3652.

Mulligan, J. A., X. Feng, and S. G. Adie. 2019. "Quantitative Reconstruction of Time-Varying 3D Cell Forces With Traction Force Optical Coherence Microscopy." *Scientific Reports* 9: 4086.

Muncie, J. M., and V. M. Weaver. 2018. "The Physical and Biochemical Properties of the Extracellular Matrix Regulate Cell Fate." *Current Topics in Developmental Biology* 130: 1–37.

Munevar, S., Y.-L. Wang, and M. Dembo. 2001. Traction Force Microscopy of Migrating Normal and H-Ras Transformed 3T3 Fibroblasts.

Oakes, P. W. 2018. "Balancing Forces in Migration." *Current Opinion in Cell Biology* 54: 43–49.

Oakes, P. W., S. Banerjee, M. C. Marchetti, and M. L. Gardel. 2014. "Geometry Regulates Traction Stresses in Adherent Cells." *Biophysical Journal* 107: 825–833.

Oudin, M. J., and V. M. Weaver. 2016. "Physical and Chemical Gradients in the Tumor Microenvironment Regulate Tumor Cell Invasion, Migration, and Metastasis." *Cold Spring Harbor Symposia on Quantitative Biology* 81: 189–205.

Pelham, R. J., and Y.-L. Wang. 1997. "Cell Locomotion and Focal Adhesions Are Regulated by Substrate Flexibility." *Cell Biology* 94: 13661–13665.

Plotnikov, S. V., B. Sabass, U. S. Schwarz, and C. M. Waterman. 2014. "High-Resolution Traction Force Microscopy." *Physiology & Behavior* 176: 367–394.

Sabass, B., M. L. Gardel, C. M. Waterman, and U. S. Schwarz. 2008. "High Resolution Traction Force Microscopy Based on Experimental and Computational Advances." *Biophysical Journal* 94: 207–220.

Saha, K., A. J. Keung, E. F. Irwin, et al. 2008. "Substrate Modulus Directs Neural Stem Cell Behavior." *Biophysical Journal* 95: 4426–4438.

Sala, S., A. Caillier, and P. W. Oakes. 2024. "Principles and Regulation of Mechanosensing." *Journal of Cell Science* 137: jcs261338. https://doi.org/10.1242/jcs.261338.

Schmitt, M. S., J. Colen, S. Sala, et al. 2024. "Machine Learning Interpretable Models of Cell Mechanics From Protein Images." *Cell* 187: 481–494.e24.

Shah, P., C. M. Hobson, S. Cheng, et al. 2021. "Nuclear Deformation Causes DNA Damage by Increasing Replication Stress." *Current Biology* 31: 753–765.e6.

Song, D., L. Dong, M. Gupta, et al. 2020. "Recovery of Tractions Exerted by Single Cells in Three-Dimensional Nonlinear Matrices." *Journal of Biomechanical Engineering* 142: 081012.

Stricker, J., B. Sabass, U. S. Schwarz, and M. L. Gardel. 2010. "Optimization of Traction Force Microscopy for Micron-Sized Focal Adhesions." *Journal of Physics. Condensed Matter* 22: 194104.

Toyjanova, J., E. Bar-Kochba, C. López-Fagundo, J. Reichner, D. Hoffman-Kim, and C. Franck. 2014. "High Resolution, Large Deformation 3D Traction Force Microscopy." *PLoS One* 9: e90976.

Ullo, M. F., A. E. D'Amico, S. B. Lavenus, and J. S. Logue. 2024. "The Amoeboid Migration of Monocytes in Confining Channels Requires the Local Remodeling of the Cortical Actin Cytoskeleton by Cofilin-1." *Scientific Reports* 14: 10241.

Ventura, G., and J. Sedzinski. 2022. "Emerging Concepts on the Mechanical Interplay Between Migrating Cells and Microenvironment In Vivo." *Frontiers in Cell and Developmental Biology* 10: 961460. https://doi.org/10.3389/fcell.2022.961460.

Vignaud, T., H. Ennomani, and M. Théry. 2014. "Polyacrylamide Hydrogel Micropatterning." *Methods in Cell Biology* 120: 93–116.

Wolfel, A., M. Jin, and J. I. Paez. 2022. "Current Strategies for Ligand Bioconjugation to Poly(Acrylamide) Gels for 2D Cell Culture: Balancing Chemo-Selectivity, Biofunctionality, and User-Friendliness." *Frontiers in Chemistry* 10: 1012443. https://doi.org/10.3389/fchem.2022.1012443.

Wolfram, L., C. Gimpel, M. Schwämmle, S. J. Clark, D. Böhringer, and G. Schlunck. 2024. "The Impact of Substrate Stiffness on Morphological, Transcriptional and Functional Aspects in RPE." *Scientific Reports* 14: 7488.

Yamada, K. M., and M. Sixt. 2019. "Mechanisms of 3D Cell Migration." *Nature Reviews. Molecular Cell Biology* 20: 738–752. https://doi.org/10.1038/s41580-019-0172-9.

Yang, Y., K. Wang, X. Gu, and K. W. Leong. 2017. "Biophysical Regulation of Cell Behavior—Cross Talk Between Substrate Stiffness and Nanotopography." *Engineering* 3: 36–54. https://doi.org/10.1016/J.ENG.2017.01.014.

Yeung, T., P. C. Georges, L. A. Flanagan, et al. 2005. "Effects of Substrate Stiffness on Cell Morphology, Cytoskeletal Structure, and Adhesion." *Cell Motility and the Cytoskeleton* 60: 24–34.