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Agarose-based 3D cell confinement assay to study nuclear mechanobiology

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Abstract

Cells in living tissues are exposed to substantial mechanical forces and constraints, imposed by neighboring cells, the extracellular matrix, and external factors. The mechanical forces and physical confinement can drive various cellular responses, including changes in gene expression, cell growth, differentiation, and migration. These responses have important implications in both physiological and pathological processes, such as immune cell migration or cancer metastasis. Previous studies have shown that nuclear deformation induced by 3D confinement promotes cell contractility but can also cause DNA damage and changes in chromatin organization, thereby motivating further studies in nuclear mechanobiology. In this protocol, we present a custom-developed, easy-to-use, robust, and low-cost approach to induce precisely defined physical confinement on cells using agarose pads with micropillars and externally applied weights. We validated the device by confirming nuclear deformation, changes in nuclear area, and cell viability after confinement. The device is suitable for short- and long-term confinement studies and compatible with imaging of both live and fixed samples, thus presenting a versatile approach to study the impact of 3D cell confinement and nuclear deformation on cellular function. This article contains detailed protocols for the fabrication and use of the confinement device, including live cell imaging and labeling of fixed cells for subsequent analysis. These protocols can be amended for specific applications.

Basic Protocol 1: Design and fabrication of the confinement device wafer

Basic Protocol 2: Cell confinement assay

Support Protocol 1: Fixation and staining of cells after confinement

Support protocol 2: Live/dead staining of cells during confinement

Keywords

Agarose; confinement; compression; nucleus; mechanobiology

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CONFLICT OF INTEREST STATEMENT:
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INTRODUCTION:

Within the human body, cells often experience considerable physical confinement due to their 3D microenvironment. For instance, endothelial cells navigate through dense matrices during angiogenesis (Herbert & Stainier, 2011), immune cells migrate through vascular endothelial barriers and tissues during inflammation and wound healing (Friedl & Weigelin, 2008; Luster et al., 2005), and cancer cells penetrate interstitial spaces as small as 1-2 μm in diameter during invasion, intravasation, and extravasation (Weigelin et al., 2012). Moreover, confinement in the tumor microenvironment promotes increased metastatic potential and drug resistance in tumor cells (Shen et al., 2021; Tse et al., 2012). In these processes, cells experience extensive deformations of the cell nucleus (Davidson et al., 2014; Friedl et al., 2011), the largest and stiffest organelle in the cell (Kalukula et al., 2022). Emerging evidence supports the idea that such nuclear deformation can modulate various cellular functions, ranging from cell contractility, cytoskeletal organization, chromatin modifications and gene expression to cell differentiation and viability (Gupta et al., 2012; Hsia et al., 2022; Lomakin et al., 2020; Venturini et al., 2020). The nucleus is thought to play a central role in cellular mechanosensing, i.e., in translating mechanical forces and physical confinement into biological responses (Enyedi & Niethammer, 2016; Kalukula et al., 2022; Kirby & Lammerding, 2018). Nonetheless, many of the molecular mechanisms linking mechanical inputs to biochemical outputs, as well as the mechanisms determining how 3D confinement alters cellular morphology and function of various cell types, remain to be elucidated.

These open questions have motivated the increasing use of either custom-developed or commercially available devices that provide precisely defined vertical or 3D confinement to cells. However, many of these devices are not suitable for long-term cell confinement, which is needed to accurately assess the biological consequences of confinement. For example, many devices use PDMS micropillars or glass coverslips to induce cell confinement (le Berre et al., 2012; Lee et al., 2018; Liu et al., 2015). Because these materials allow only limited media access to the cells, and thus limited nutrient availability, they substantially limit the duration that cells can be confined *in vitro*. Additionally, these devices often require the use of costly and bulky hardware such as vacuum pumps, syringe pumps, or pressurized air to induce confinement. Inconsistent or non-uniform confinement presents further challenges in some existing confinement assays. More recently, some devices have overcome these issues by using agarose pads for confinement, which allows nutrient accessibility for long-term cellular confinement (Prunet et al., 2020, García-Arcos et al., 2023).

Here, we present a simple, easy to use confinement device containing agarose pads with micropillars of a specified height, a 3D printed weight holder, and simple disk weights (Figure 1A, B) to induce 3D confinement of cells to specific heights for up to 24 hours (Figure 1C). Note that 3D confinement is typically associated with both (1) spatial constraint resulting from the confinement and (2) mechanical compression, resulting in deformation of the cell and nucleus. Since the compressive forces may vary over time, whereas the spatial constraint of confinement remains constant once applied to the cells, we refer to these devices as confinement devices, and not compression devices. To fabricate the device, photolithography is used to create a pillar array with SU-8 photoresist on a silicon wafer.

The agarose device is then formed by casting agarose onto the silicon wafer containing the pillar design fabricated to the height of interest. After the agarose device is formed and individual devices are cut out, it is placed onto cells seeded in a glass dish upon which the weight holder and weights can be added directly on top of the agarose device to induce nuclear confinement.

We provide protocols to fabricate the silicon wafer used to form the agarose confinement devices (Basic Protocol 1) and how to successfully induce and measure confinement of cells with the device (Basic Protocol 2). Additionally, we provide two supplemental protocols to demonstrate how to fix cells after confinement for further imaging applications (Support Protocol 1) and how to perform live/dead cell staining and imaging after confinement (Support Protocol 2). This device can effectively induce precisely controlled cell confinement to elucidate how cells respond to 3D confinement and identify underlying mechanisms of mechanotransduction.

BASIC PROTOCOL 1: Design and fabrication of the confinement device wafer

To form the agarose devices with micropillars, agarose is cast onto a mold consisting of a silicon wafer microfabricated to contain the negatives of the desired device features. Since the mold contains features of different heights, it is fabricated using two layers of photoresist, each corresponding to a specific height. The silicon wafer is fabricated using photolithography techniques, in which SU-8 photoresist is spun onto the wafer, exposed with UV light through a photomask containing the design of interest for each layer, and developed to remove the unexposed photoresist. This process is repeated for each layer of photoresist. The final silicon wafer contains two layers of SU-8 photoresist: a lower layer and an upper layer. The lower layer is used to fabricate the micropillar features, with the height of the layer corresponding to the desired height of the cells during confinement, whereas the upper layer serves to help remove the agarose device from the silicon wafer without damaging the micropillars or creating an agarose lip when cutting out the device that protrudes beyond the micropillars. We recommend a micropillar height of 2-5 μm for confinement and a height of 10-15 μm for the control condition, but the exact dimensions will depend on the height of the cell when in unconfined 2D conditions, which varies between cell types and cell culture conditions. In the following, we provide protocols for the fabrication of devices with micropillar heights of either 3 μm (confinement) or 10 μm (unconfined control). One wafer should be fabricated containing a 3 μm lower layer, and a separate wafer should be fabricated containing a 10 μm lower layer. The upper layer contains a 500 μm tall by 3mm wide circular outline of the device to help with removing the agarose from the wafer. This layer will result in a circular undercut groove in the agarose pad to ensure that when cutting out individual agarose devices, the pillar features are not cut, which would prevent solid contact of the pillars with the substrate during confinement. The upper layer is identical for both wafers. The protocol assumes some basic knowledge and experience with photolithography and wafer fabrication. If needed, please consult with available experts or external service providers. All fabrication steps should be performed in a clean room facility with proper safety equipment and precautions.

Materials:

Piranha etch solution* (3:1 sulfuric acid: 30% hydrogen peroxide)

SU-8 2002 (Kayaku Advanced Materials, item no. Y111029 0500L1GL)

SU-8 10 (Kayaku Advanced Materials, item no. Y131259 0500L1GL)

SU-8 2150 (Kayaku Advanced Materials, item no. Y111077 0500L1GL)

Acetone, semiconductor grade* (Transene, product no. UN109)

SU-8 developer* (Kayaku Advanced Materials, item no. Y020100 4000L1PE)

Deionized (DI) water

Isopropyl alcohol (IPA), semiconductor grade* (VWR, cat. no. JT9079-05)

Hydrophobic wafer coating* (FOTS (1*H*,1*H*,2*H*,2*H*-perfluorooctyl trichlorosilane) deposition or Bosch process to perform sulfur hexafluoride (SF_6) etch with octafluorocyclobutane (C_4F_8) deposition).

AutoCAD software (Tanner L-edit version 2020u7.1, KLayout, or equivalent)

GenISys BEAMER software (version 6.3.1)

Chrome Photomask, 5 × 5 × 0.90 inch, quartz substrate (Telic)

Heidelberg DLW 2000 (Heidelberg Instruments)

Hamatech wafer processor

CZ silicon wafer, 4-inch diameter, n-type, 500-550 μm thick* (Nova Wafers)

Oven capable of reaching and maintaining temperature of 90°C

Chemical fume hood compatible with class II photoresist materials

Disposable pipettes* (VWR cat. no. 414004-023)

Resist spin coater

Wafer tweezers (Excelta Corp, model 491-SA-PL)

CleanTips ® Swabs (Texwipe, TX761)

Cleanroom Amplitude ™ Zeta ™ Wipe (Contec, AMZE0001)

Hot plate with adjustable ramp rate (Fairweather TPS66 or equivalent)

Contact aligner (Karl Suss MA6 or equivalent)

Long pass filter for near UV light (PL-360LP from Omega Optical or equivalent)

Rubylith masking film (Ulano, RU3 151800)

Pyrex ® Glass dish, 1200 mL, with dimeter larger than 4-inch wafer (Millipore Sigma, SKU CLS3140150)

Kapton® tape*, 1/8 inch width (ULINE S-14532)

Razor blade* (VWR cat. no. 55411-040)

Profilometer (Tencor P7 profilometer or equivalent)

Oven capable of maintaining temperature of 200°C (YES polyimide bake oven or equivalent)

Molecular vapor deposition tool (MVD 100 or equivalent) or deep silicon etcher capable of running the Bosch Process (Unaxis 770 Deep Silicon Etcher or equivalent)

* Many of these items are general supplies available in clean-room facilities and provided by the facility. We have provided catalog numbers when available, but the specific product available in other clean room facilities may vary. We do not expect this to be an issue. Please consult with your local clean room operators if needed.

Protocol steps with *step annotations:*

Design and generate photomasks, prepare silicon wafers

1. Design the photomask for each layer using the desired features and geometries using an AutoCAD software such as Tanner L-edit (version 2020u7.1) or KLayout. For complex designs, such as our design containing multiple circular features, we recommend performing Boolean subtraction using the GenISys BEAMER software (version 6.3.1). In that case, to design the first layer photomask, the array of individual circles was subtracted from the larger blue circle (Figure 2A). The photomask designs for the device used in our applications, which fits into a 35-mm glass bottom dish, is available on GitHub (https://github.com/Lammerding/Confinement-Device) and can be modified if desired. Each photomask design includes complementary alignment marks to align each layer with the previous one on the wafer during exposure. The first layer photomask will generate alignment marks on the lower layer of photoresist. The second layer mask will contain alignment marks that will be aligned to the wafer containing the first layer alignment marks (Figure 2B, C). After ensuring the tonality of the digital mask design is correct, convert the mask design to a GDSII file. The file will then be converted to a DXF file using a conversion program. This is the file format accepted by the Heidelberg DLW2000 mask writer. Other instruments may use a different file format.

2. For each layer, print the photomask design on a chrome mask using a mask writer, such as Heidelberg DLW2000, following the manufacturer’s instructions.

3. Develop the photomasks and perform a chrome etch using a Hamatech wafer processor. Strip the excess photoresist from the photomasks using a resist hot strip bath and dry the photomasks completely. The final photomasks will be used to expose the photoresist on the silicon wafer.

   The Hamatech wafer processor is a general tool containing a closed and safe environment that can be used with many photomasks and wafer processes. The photomask will first go through a developer process, and then a separate chrome etch process. Many cleanrooms will have standard protocols for these processes.

4. Clean the silicon wafers to be used with a Hamatech wafer processor equipped with a standard Piranha etch solution. The tool will automatically mix the sulfuric acid and hydrogen peroxide solutions, ensuring user safety. The tool performs a series of cleaning and DI water rinsing steps for approximately 8 minutes.

   The Piranha etch solution is used to clean organic material from the wafer and prepare the surface of the wafer. Alternatively, a Nanostrip solution can be used in a general chemistry hood. We recommend following your particular clean room's guidelines. When planning to make multiple identical wafers, for example, to produce multiple confinement devices at once, it is more efficient to fabricate the wafers in parallel. In that case, each wafer is processed as described here.

5. Dehydrate the wafers overnight in a 90°C oven. This is critical before spinning each layer of SU-8 photoresist. Allow the wafer to cool to room temperature before spinning photoresist.

SU-8 fabrication of 3 μm lower layer features (use first wafer for this)

6. Center the wafer on a spin coater, add 3 mL of SU-8 2002 to the center of the wafer, and run the spin process, selecting settings (Table 1) to produce a 3 μm thick SU-8 layer. Remove photoresist from the outer 5 mm perimeter of the wafer and the back of the wafer using a clean room swab soaked with acetone.

   Removing the outer 5 mm of photoresist (the "edgebead") is common practice to ensure the wafer does not get stuck in other tools used (i.e., the deep silicon etcher tool). Additionally, any photoresist on the back of the wafer will come in contact with the surfaces of the hot plates and can cause the wafer to adhere to the hot plate during baking and leave residual photoresist on the hotplates.

7. Bake the wafer for 2.5 minutes on a hot plate pre-heated to 95°C. Remove the wafer from the hotplate and allow the wafer to cool gradually to room temperature.

   If you have access to a cooling block, place the wafer on the cooling block and allow it to cool for approximately 1 minute. Alternatively, you can place the wafer in a wafer carrier, and it will cool in approximately 3-5 minutes.

8. Mount the wafer with the photoresist and the photomask of interest on a contact aligner tool. The set up will vary depending on the contact aligner used, but in general the wafer is placed with the photoresist side up and the photomask mask is placed on top of the wafer with the chrome side of the photomask facing the photoresist. During the exposure (see next step), the light source will expose the photoresist side of the wafer through the photo mask.

9. Expose the photoresist to UV light using a vacuum contact setting. The exact duration will depend on the light source, thickness of photoresist, and the area of photoresist to be exposed. In our protocol, an exposure time of 15 seconds produced the correct sized features for the 3 μm tall SU-8 layer. A UV light long pass filter must be used for SU-8.

   It is necessary to perform an exposure array testing multiple exposure times to determine the correct exposure time. Rubylith can be used to cover portions of the photomask to perform an exposure array on one wafer. Exposure time will depend on the power of the UV source, so it will vary between different contact aligners/steppers and can also change over time. For large features that do not need high resolution such as the pillars in our design, we used ‘hard contact mode’ for alignment, which is less precise than ‘vacuum contact mode’ as described in SUSS MA6 user manual section “1.4.1 Exposure Programs”. After exposure and development, inspect the size of the features on the wafer with a microscope to determine the optimal exposure time.

10. Remove the wafer from contact aligner and bake the exposed wafer on a 95°C hot plate for 3.5 minutes. Remove the wafer from the hotplate and allow the wafer to cool gradually to room temperature on a cooling block for 1 minute.

11. Add the wafer to a glass dish containing SU-8 developer. Gently swirl the dish for 5 seconds and remove the wafer from the developer. Rinse the wafer once with DI water. Repeat 5-second developer and water rinses until there is no residue left on the wafer. For thin layers, repeated short rinses with developer and DI water are helpful to avoid overdeveloping the wafer.

12. Let the wafer dry in a fume hood for at least 4 hours and up to 24 hours. Rinse the wafer once with isopropyl alcohol (IPA) and then DI water. Dry the wafer using a pressurized nitrogen nozzle.

    It is important to use pressurized nitrogen nozzles to dry substrates instead of air because nitrogen is inert, does not contain water, and nitrogen will not react with water on the surface of substrates.

13. Measure the height of the photoresist layer using a Profilometer (e.g., Tencor P7).

14. Dehydrate wafers overnight in a 90°C oven overnight before spinning a second layer of photoresist on the wafer (see step 15 and following).

SU-8 fabrication of 10 μm lower layer features (use second wafer for this)

6. Center the wafer on a spin coater, add 3 mL of SU-8 10 to the center of the wafer, and run the spin process using the settings provided in Table 2 to produce a 10-μm thick photoresist layer. Remove photoresist from the outer 5 mm and back of the wafer using a clean room swab soaked with acetone.

7. Bake the wafer on a hot plate heated to 95°C for 3.5 minutes. Remove the wafer from the hotplate and allow the wafer to cool gradually to room temperature on a cooling block for 1 minute.

8. Mount the wafer containing photoresist and the photomask of interest on a contact aligner tool. The set up will vary depending on the contact aligner used, but in general the photomask mask is placed over the wafer containing photoresist, with the chrome side of the photomask facing the photoresist on the wafer. During the exposure, the light source will expose through the photo mask to the wafer containing photoresist.

9. Expose the photoresist to UV light using a vacuum contact setting. In our protocol, an exposure time of 17.5 seconds produced features in the correct size for the 10 μm tall SU-8 layer. A UV light long pass filter must be used for SU-8.

10. Remove the exposed wafer from the contact aligner and bake the exposed wafer on a 95°C hot plate for 3.5 minutes. Remove the wafer from the hotplate and allow the wafer to cool gradually to room temperature on a cooling block for 1 minute.

11. Add the wafer to a glass dish containing SU-8 developer. Gently swirl the dish for 5 seconds and remove the wafer from the developer. Rinse the wafer once with DI water. Repeat 5 second developer and water rinses until there is no residue on the wafer. For thin layers, repeated short rinses with developer and DI water are helpful to avoid over developing the wafer.

12. Let the wafer dry in a fume hood for at least 4 hours and up to 24 hours. Rinse the wafer once with IPA and then DI water. Dry the wafer using a pressurized nitrogen nozzle.

13. Measure the height of the photoresist layer using a Profilometer (e.g., Tencor P7 Profilometer) to confirm that it matches the desired height of the corresponding features.

14. Dehydrate wafers overnight in a 90°C oven before spinning a second layer of photoresist on the wafer.

SU-8 fabrication of 500 μm upper layer features and final processing (apply to both wafers)

15. Cover alignment marks on the wafer containing lower layer features using a small piece of Kapton® tape. This preserves alignment marks during the upper layer spin process.

16. Center the wafer on a spin coater, pour 5-7 mL of SU-8 2150 on the center of the wafer, and run the spin process. The spin settings for obtaining a 500 μm

layer are provided in Table 3. Let the wafer rest for 10 minutes in the spin coater. Remove the photoresist from the outer 5 mm and back of the wafer using a clean room swab and clean room wipes soaked with acetone.

SU-8 2150 is extremely viscous and must be poured onto the wafer (i.e., it cannot be pipetted). If the spin is uneven, pour more photoresist onto the center of the wafer and spin the wafer again. Letting the wafer rest for 10 minutes after spinning will help create an even thickness. Be careful to hold the wafer level while removing the outer 5 mm photoresist and cleaning the back of the wafer.

17. Place the wafer on a room temperature hotplate and ramp the temperature to 65°C at a rate of 1.5 °C/minute. Hold at 65°C for 30 minutes, and then ramp to 95°C at 1.5°C/minute. Hold the temperature at 95°C for 16-24 hours, turn off the hotplate, and allow the wafer to gradually cool to room temperature on the hot plate for approximately 2 hours.

The pre-exposure bake is usually performed at 95°C for SU-8 photoresist. The longer bake time and more elaborate protocol is needed for the thicker photoresist layer to avoid cracks or delamination due to thermal stress.

18. Remove the Kapton® tape under the upper layer of photoresist covering the alignment marks on the lower layer of photoresist. This can be done by carefully using a razor blade to score the edges of the taped area, followed by tweezers to peel the tape off of the wafer. Use a microscope to confirm the alignment marks are visible on the wafer.

19. Mount the wafer with the photoresist and the photomask of interest on a contact aligner tool. Align the photomask to the alignment marks on the wafer. Expose the photoresist for 3 intervals of 20 seconds each, with 20 seconds of rest in between exposures using a hard contact setting. A UV light long pass filter must be used for SU-8.

Longer exposure times (above 30 seconds) risk SU-8 cracking and defects, so we recommend performing multiple shorter exposures to allow the wafer to cool between multiple exposures.

20. Place the wafer on a room temperature hotplate and ramp the temperature to 95°C at a rate of 1.5 °C/minute. Hold the temperature at 95°C for 5 minutes, turn off the hotplate, and allow the wafer to gradually cool to room temperature on the hot plate for approximately 2 hours.

21. Add the wafer to the bottom of a glass dish containing ~500 mL of SU-8 developer. The amount of developer can vary but should generously cover the entire wafer. Allow the wafer to develop for one hour, replace with fresh developer, and allow the wafer to develop for one additional hour. Rise the wafer with DI water.

If there is still residue on the wafer after rinsing with DI water, place the wafer back into developer for 5-minute periods and perform alternating developer and DI water rinses until there is no longer residue. You should use a microscope

to confirm features on the device do not contain debris from undeveloped photoresist. For the development process, we found that including a rotating stir bar in the developer solution can cause delamination of the photoresist and should be avoided, even if it requires a longer development process.

22. Let the wafer dry in a fume hood for at least 4 hours and up to 24 hours. Rinse the wafer once with IPA and then DI water. Dry the wafer using a pressurized nitrogen nozzle.

A long drying step is recommended to allow any remaining solvents to evaporate. These solvents can otherwise interfere with the next steps of applying the hydrophobic coating to the wafer.

23. Perform a hard-bake on the wafer in an oven by gradually ramping the temperature to 200°C, holding for one hour, and then gradually allowing the wafer to cool to 50°C. Depending on how fast the oven cools it will take approximately 2 hours to reach 50°C. Transfer wafer to a wafer holder and allow the wafers to cool to room temperature for approximately 10 minutes.

Hard baking increases the thermal stability of the photoresist, and removes any leftover solvent in the photoresist that could interfere with future etching processes (i.e. the hydrophobic coating with C_4F_8). Do not open the oven door during the bake while the oven is above 100°C. This will cause the wafers to shatter. When removing the wafers from the oven, place them in an upright wafer holder to cool gradually to room temperature. Do not place the wafers flat directly on a cold metal benchtop.

24. Apply a hydrophobic coating to the wafer. This can be done using either fluoro-octyl trichlorosilane (FOTS) deposition with a Molecular Vapor Deposition (MVD) tool or by running a SF_6 etch and C_4F_8 deposition (Bosch process) in a Deep Silicon Etcher. Measure the contact angle on the silicon wafer using a video contact angle (VCA Optima) system, ensuring a contact angle of ≥ 95°.

Unlike PDMS, agarose can be cast directly from the silicon wafer, without substantial wear or delamination of the wafer. However, if desired, a plastic mold can be generated from a PDMS cast of the silicon wafer to provide an even more permanent mold that can be used to cast the agarose devices. We have previously published a detailed protocol for the fabrication of plastic molds from PDMS casts (Agrawal et al., 2022).

BASIC PROTOCOL 2: Cell confinement assay

This section describes the steps to make the agarose devices, induce confinement of cells using the devices (Figure 3), and confirm cell confinement by measuring nuclear cross-sectional area and nuclear height, which should increase (area) and decrease (height) under confinement. For biological studies, we recommend comparing cells under three different conditions: (1) an unconfined control, (2) confinement with a device sufficient to induce substantial cellular and nuclear deformation (typically 3-5 μm height), and (3) a control with a device that is taller than the unconfined cell height and thus does not induce cellular

deformation but controls for the presence of the agarose pad and the squishing of some cells under the micropillars (typically 10-15 μm height). In our example, we use MDA-MB-231 human breast cancer cells confined to 3 μm height, which is approximately 60% of the original nuclear height of ~5 μm, and a control device with 10-μm tall micropillars (Figure 4A). Different cell types may have different heights and may tolerate different degrees of confinement.

For the experiments, the cells of interest are seeded onto 35-mm glass bottom dishes that have been coated previously with the appropriate extracellular matrix (e.g., collagen or fibronectin), using standard cell culture protocol. After overnight incubation to promote cell adhesion, the cells are imaged on confocal microscope to measure nuclear height prior to confinement. Immediately prior to confinement, the agarose devices are prepared by casting agarose onto the silicon wafer described above in Basic Protocol 1. The individual devices are cut out and soaked in media to reduce media absorption once they are placed onto the dish with the cells. To induce confinement, a 3D-printed weight holder is placed onto the agarose device, followed by the desired amount of weight. For short experiments inducing confinement for 3-6 hours, 10 grams of weight is added to the device. For longer experiments (e.g., 18-24 hours), the amount of weight is decreased to 5 grams to avoid excessive compression of the device over time. Cells can be imaged during the experiment to confirm confinement, e.g., based on the changes in nuclear height and/or cross-section. The nuclear cross-sectional area is expected to increase upon confinement to 3 μm but should not change when cells are subjected to the control device with taller micropillars (Figure 4B). Nuclear height quantification is used to confirm even confinement in cells across the entire device (Figure 4C). This protocol can be applied to a wide variety of cell types, as it is easy to modulate various parameters such as the cell confluency and amount of weight used.

Materials:

Cells of interest (e.g., MDA-MB-231 human breast cancer cells (ATCC HTB-26 ™) genetically modified to express H2B mNeonGreen (Allele Biotechnology) or another nuclear label)

Dulbecco’s Modified Eagle Medium (DMEM) cell culture media supplemented with fetal bovine serum (FBS) and penicillin/streptomycin (see recipe in Reagents and Solutions)

1× Dulbecco’s phosphate-buffered saline (DPBS), no calcium, no magnesium (see recipe in Reagents and Solutions)

Fibronectin (EMD Millipore Corp, cat. no. FC10-5MG) diluted in 1× DPBS to 5 μg/mL or type I collagen (Corning, cat. no. 354236) diluted in 0.02N acetic acid to 0.05 mg/mL

Trypsin-EDTA, 0.25% with phenol red (Gibco/ThermoFisher, cat. no. 25200-072)

Cell media suitable for live cell imaging (e.g., see recipe for Fluorobrite media or use media compatible with specific cell type of interest)

Agarose (SeaKem LE Agarose, Lonza, cat. no. 50004, or comparable)

FITC dextran (Invitrogen, cat. no. D7136 or equivalent)

CO_2 incubator for cell culture (e.g., Thermo Scientific Forma™ Steri-Cycle™)

Water bath set to 37°C

Laminar flow biosafety cabinet (e.g., The Baker Company SterilGARD® Class II Type A2)

35 mm dish with glass bottom 0.17mm thick (e.g., FluoroDish, World Precision Instruments, cat. no. FD35-100)

Cell culture flasks (e.g., 25 cm^2 flask, Corning, cat. no. 430639)

Inverted bright field microscope

15 mL conical tube (e.g., Corning, cat. no. 352096)

Cell culture centrifuge

Cell counter (e.g., Reichert Bright-Line® Hemocytometer or CellDrop™ Automated Cell Counter)

Inverted confocal microscope with a 40× immersion objective, motorized stage, and mounted microscope camera for image acquisition (cells can also be observed with a 10× or 20× non-immersion objective, but high NA objectives are needed for accurate nuclear height measurements)

100 mm polystyrene plastic petri dish (e.g., VWR, cat. no. 25384-342) with bottom removed carefully using an X-Acto knife

17 mm diameter circular metal punch or X-Acto knife

Flat-tipped tweezer (VWR, cat. no. 89259-954)

Kimwipes (Kimtech, cat. no. 34133)

35 mm polystyrene plastic petri dish (e.g., VWR, cat. no. 25384-342)

3D printed weight holder (printed using an Ultimaker S5 3D printer with Ultimaker Tough PLA filament, STL files available on GitHub.

Disk weights ranging from 2g to 10g with slots (e.g., Amazon)

Microscopy software capable of automated capture of Z-stack (e.g., Zeiss Zen Black)

FIJI/ImageJ or equivalent image-processing software

Protocol steps with *step annotations:*

Extracellular matrix coating and cell seeding

1. Culture cells according to methods appropriate for specific cell lines (Phelan & May, 2017; Picot, 2004). Cells should be cultured in a 37°C incubator with 5% CO_2. All culture media should be warmed to 37°C in a water bath. Work should be performed in an approved biosafety cabinet. We have demonstrated that this protocol works consistently with adherent cells, however, due to the ability to modulate experimental parameters, this device should also be suitable for cells cultured in suspension.

2. 18-24 hours before the cell confinement assay, coat the glass bottom dishes with the appropriate extracellular matrix molecules (e.g., 5 μg/mL fibronectin or 0.05 mg/mL type I collagen solution) for 2 hours at 37°C or overnight at 4°C (Agrawal et al., 2022).

   The specific choice of extracellular matrix coating will depend on the cell lines used in the experiments. Fibronectin should be used at a final concentration of 5 μg/mL in 1X DBPS. Incubate glass dishes with fibronectin solution for 2 hours at 37°C. Dilute type 1 collagen in 0.02 N Acetic acid to a final concentration of 0.05 mg/mL. Coat glass dishes with collagen solution and incubate overnight at 4°C. For collagen coatings, it is necessary to wash the dishes with PBS or cell media prior to seeding the cells to avoid acidification of cells and cell media due to the acetic acid present in the collagen solution. Seeding cells on glass will likely change the nuclear and cellular morphology and impact the cellular response to confinement. In our experience, fibronectin provides a more even coating and consistent nuclear height compared to a collagen substrate, but this may depend on the specific conditions and cell lines.

3. Detach cells using standard cell culture methods. For MDA-MB-231 cells in a T-25 flask, aspirate the cell media and rinse flask once with 1× DPBS. Add 500 μL of trypsin-EDTA and incubate for 3-5 min until cells are detached. Check that all cells have detached using an inverted bright field microscope. Add 2 mL culture media to neutralize trypsin and transfer cell suspension to a conical tube. Rinse flask with 2 mL media three times, adding the media to the conical tube and bringing the final volume to 10 mL.

4. Centrifuge detached cells at 300 × g for 5 min to pellet the cells.

5. Aspirate the supernatant and resuspend cell pellet in 5 mL culture media.

6. Count the cells using a hemocytometer or an automated cell counting device.

7. Seed cells onto the coated dishes at a density of 75,000-100,000 cells/dish (corresponding to ca. 37,500 – 50,000 cells/mL in 2 mL of media).

   For optimal confinement, cells should be between 50 to 70% confluence on the day of the experiment.

Preparing cells for confinement and imaging

8. Optionally, for cells that do not contain a genetically encoded fluorescent nuclear label, stain cells with a live cell nuclear/DNA stain (e.g., Hoechst 33342 or SPY555-DNA) prior to imaging.

For live cell imaging, fluorescent labels or dyes that use longer wavelength for excitation (e.g., mNeonGreen, mCherry, mScarlet, SPY555 or SPY650) are preferred as imaging them is less phototoxic compared to fluorophores requiring shorter wavelengths.

9. 18-24 hours after cell plating, replace cell culture media with 500 µL of cell imaging media (e.g., Fluorobrite) and place the 35 mm glass bottom dish on the microscope stage.

Changing the amount of media will affect the efficiency of confinement, as the agarose must displace media to come in contact with the bottom of the dish. If using a dish with a different surface area, the amount of media will need to be adjusted.

10. Acquire multiple fields of Z-stack images using a 40× immersion objective on a confocal microscope to measure the average nuclear height prior to confinement. We found that Z-stacks with 0.5 µm intervals are typically sufficient. The dish can be kept on the microscope while the agarose device and weights are added. Alternatively, the dish can be removed from the microscope until after step 19, and then the dish can be placed back onto the microscope to confirm confinement.

Preparing the agarose confinement device

11. Prepare a 3% agarose solution by adding 1.5 grams of agarose to 50 mL of 1× DPBS. Dissolve the agarose by microwaving the solution in 15 second intervals for approximately one minute. Swirl the solution to ensure that all the agarose is dissolved and then let the solution cool down for 2 minutes.

Typically, a minimum of 50 mL of 3 % agarose is prepared. Tissue culture grade DPBS is essential to maintain the correct pH of the agarose. If air bubbles form during the microwaving step, use a vacuum desiccator to evacuate the bubbles. Usually, a short vacuuming for 10- 30 seconds and the rapidly releasing the vacuum will clear bubbles from the agarose. A video of steps 11-15 is available (Supplemental Video 1).

12. While the agarose is cooling down, prepare the wafer that serves as the mold for the desired confinement height. Rinse the wafer once with DI water and dry with an air nozzle. Place the 100 mm plastic ring on top of the wafer to serve as a mold to cast the melted agarose. This will ensure the thickness of agarose is the same for every casting. The melted agarose should not leak under the plastic ring due to its high viscosity.

13. Carefully poor 15 mL of the 3 % agarose solution on the wafer. Rotate the wafer gently, while keeping the 100 mm plastic ring in place to ensure that the agarose

is evenly spread across the wafer. The height of the agarose layer should be approximately 15 mm. Let the agarose solidify for 10 minutes.

The agarose devices should be freshly made for each experiment, and immediately used after preparing. This will ensure the agarose does not dry out before being placed into the dish with cells.

14. Carefully remove the 100 mm plastic ring without removing agarose by using tweezers to separate the agarose from the plastic ring. Cut out the individual agarose devices using the 17 mm diameter circular metal punch or an X-Acto knife, but do not yet remove the agarose from the silicon wafer.

15. Remove the surrounding excess agarose using flat-tipped tweezers, leaving the individual agarose devices on the wafer.

In our experiments, we do not sterilize the agarose, and we do not see any signs of contamination when culturing cells in the device for up to 24 hours (using media containing antibiotics and using tissue culture grade PBS). Optionally, the agarose devices can be sterilized with UV light, but this should be limited to 10 minutes or less to minimize the risk of the devices drying out before use.

Cell confinement

16. Gently lift up the edge of the agarose device and pass the flat-tip tweezer under the agarose without puncturing the agarose. Place the agarose device in a 35 mm dish containing 2 mL of cell imaging media and soak in the media for 1 minute.

Soaking the agarose is essential to prevent initial absorption of media once the agarose device is placed into the dish with the cells. Flat-tip tweezers are essential for handling of the agarose devices from this point forward. Pointed tweezers can easily puncture or tear the agarose. A video of steps 16-19 is available (Supplemental Video 2).

17. Using the flat-tip tweezers, gently lift the agarose device out of the dish used to soak it in media and place the agarose device on cells in the dish. Wait for ~3 minutes to let the dish with the device equilibrate. At this point, the device should not be compressing the cells yet. Confocal Z-stacks can be acquired after adding the agarose device onto the cells to ensure that no confinement is occurring.

If using 35 mm dishes that contain a circular well with the glass coverslip at the bottom, ensure that the cell culture media (500 μL) that had been added to the dish in step 9 is covering the cells at the bottom of the well, and has not been displaced to the periphery of the 35-mm dish.

18. Spray the weight holder with 70% ethanol to disinfect it and dry with a Kimwipe. Gently add the weight holder to the agarose device and let it sit for 3 minutes. At this point, cells should not be compressed yet, which can be verified by acquiring Z-stacks of the cells, or by assessing nuclear cross-section.

19. Use the tweezers and carefully add the desired amount of weight onto the weight holder. For short term confinement experiments (3-6 hours) we add 10 grams of weight onto the device and confirm that confinement occurs within 30 minutes of adding the weight. For experiments in which cells are confined for 18-24 hours, we add 5 grams of weight, and confirm that confinement occurs within 2 hours of placing the weight onto the device.

    The relatively low weight and long time to achieve full confinement helps to minimize the shear stress associated with displacing the media between the glass surface and the agarose pad. Larger weights will result in faster confinement but may also result in increased fluid shear stress and could lead to excessive compression of the agarose pad, and thus over-confinement. Particularly for longer experiments (>18 hours), we found that although using less weight takes longer to achieve full confinement, it lowers the risk of over-confinement. We anticipate that depending on the cell type, cell density, and stiffness of the cells, the exact amount of weight added will need to be adjusted. Optionally, fluorescently labeled dextran can be added to the cell media to visualize the amount of media between the glass dish and agarose, further confirming confinement (Figure 5A). For overnight experiments, add 0.5 mL of appropriate cell media to the outside of the dish to ensure the cells have adequate nutrient supply.

20. Acquire Z-stacks to measure the height of the nuclei after confinement. We recommend imaging multiple positions to confirm that the confinement height is uniform. Another quick way to confirm that the micropillars of the agarose device are touching the glass dish, and thus the device is achieving the desired confinement, is by looking for areas where the cells are completely squished under the pillars (Figure 5B). In addition, confinement should result in an increased nuclear cross-sectional area in cells located between the pillars.

Image analysis with Fiji/Image J

Quantification of nuclear cross-sectional area to confirm confinement

1. Import Z-stacks of images containing fluorescently labeled nuclei.

    If only performing nuclear cross-sectional area measurements, 3D confocal Z-stacks may not be required, and single sections capturing the mid-plane of the nucleus or wide-field images of the cell/nucleus may be sufficient. If only using single images instead of 3D image stacks, skip forward to step 3.

2. Select *Image* > *Stacks* > *Z project*. You will be prompted to select the projection type and the first and last stack. Keep the “start slice” and “stop slice” as they are and make sure to select “average intensity” for the projection type. Click “OK” to confirm selections.

    If you did not take Z-stacks, you can skip this step.

3. Select *Image > Adjust > Threshold.* A Threshold window will open; change the default filter to ‘Huang’ and select the “dark background” option. Click “Apply” to apply changes.

   If you notice that some nuclei are touching, it may be necessary to apply an additional filter to split them. In that case, select Process > Binary > Watershed.

4. Select *Analyze > Set measurements*. A window will open to check parameters of interest. Ensure that “Area” is checked.

5. Select *Analyze > Analyze Particles*. You will be prompted to set the minimum and maximum sizes of the particles. For our cells, we set the minimum size at 40 μm^2 and select “infinity” for the maximum size. Select “Add to Manager” and “Exclude on edges”. Click “OK” to confirm selections.

6. A region of interest (ROI) Manager window will open and an ROI outlining the nuclei will appear on the thresholded image. In this window, click “Measure”.

7. A new window will open with the measurement results. Results are given in units of μm^2.

8. Compare results between cells in unconfined conditions, confinement, and in the control device. Confinement should result in an increase in nuclear cross-sectional area (Figure 4B).

Quantification of nuclear height to confirm confinement

9. Import Z-stacks of images containing fluorescently labeled nuclei. We use the “3D Objects Counter” plugin to measure nuclear height (Bolte & Cordelières, 2006).

   Nuclear height measurements require confocal Z-stacks acquired with a high resolution, high NA objective to ensure adequate resolution in the Z-direction. We find that images acquired with a 40× water immersion objective (NA = 1.2) or a 63× oil immersion objective (NA = 1.4) work well.

10. Select *Analyze > 3D OC Options.* You will be prompted to set measurements of interest. To measure nuclear height, select “Bounding Box”. Click “OK” to confirm selection.

11. Select *Analyze > 3D Object Counter.* You will be prompted to set a threshold to the image stack. Optionally, you can enter a size filter for objects. Select “Exclude objects on edges” and under “Maps to show” select “Objects”. This will generate a new image stack with numbered objects that are measured. Check the “Statistics” option to export the data with results to a new window in Fiji. When finished, select “OK” to perform the analysis.

12. The window with the results will open. The dimensions of the bounding box are listed as “B-width”, “B-height”, and “B-depth” in units of pixels. In this case, “B-depth” represents the height of the nuclei. Convert the height to micrometers using the image acquisition parameters.

13. Compare results between cells in unconfined conditions, confinement, and in the control device. Confinement should result in a decrease in nuclear height (Figure 4C).

SUPPORT PROTOCOL 1: Fixation and staining of cells after confinement

Cell fixation is a common practice used to preserve cellular structure and morphology for further analysis while reducing the risk of sample degradation. Cell fixation is often necessary for immunofluorescence (IF) analysis of intracellular proteins or for fluorescence labeling using cell impermeable dyes. After fixation, cells are permeabilized to allow intracellular labeling and then incubated in blocking solution to reduce non-specific antibody staining. For immunofluorescence labeling, the fixed sample is then typically incubated with a primary antibody directed against the protein of interest and then, after repeated washing to remove unbound primary antibodies, incubated with a secondary antibody specific to the host species of the primary antibody. In addition, fixed cells can also be labeled with fluorescent stains labeling specific cellular structures, such as DNA using DAPI, or F-actin using fluorescently labeled phalloidin. In this protocol, we provide instructions for the fixation of cells in the confinement devices and for subsequently staining the cells with phalloidin to visualize F-actin (Figure 6). The protocol can be readily adjusted to other applications, and we also include instructions for antibody staining for IF applications.

Materials:

Cell fixation agent (e.g., 4 % paraformaldehyde, see recipe in Reagents and Solutions)

1× DPBS (see recipe in Reagents and Solutions)

0.1% Triton solution (see recipe in Reagents and Solutions)

Blocking solution (3% BSA in PBS, see recipe in Reagents and Solutions)

Phalloidin Alexa Fluor 568 (ThermoFischer, cat. no. A12380)

Fluorescent dye for DNA (e.g. DAPI, Hoechst)

Chemical fume hood

Brightfield microscope

Benchtop rocking platform shaker (VWR, cat. no. 75832-308 or equivalent)

Flat tip tweezer (e.g., VWR, cat. no. 89259-954)

Inverted fluorescence microscope (with applicable fluorescence excitation/emission filters) or inverted confocal microscope with 20× objective or higher with mounted microscope camera for image acquisition (motorized stage optional, but useful)

Microscopy software for image acquisition (e.g., Zeiss ZEN Black, Zeiss ZEN Blue)

Protocol steps with *step annotations*:

1. Follow the confinement protocol described in Basic Protocol 2 and confine cells for the intended period of time. Depending on the application, we recommend inducing confinement for 2-24 hours. Alternatively, instead of imaging cells immediately post-confinement to confirm nuclear height (Basic Protocol 2, step 20), confinement can be confirmed after cell fixation.

2. After confining cells for the desired time, gently aspirate media from the 35 mm glass dish with the cells, without disturbing the agarose device, weight holder and weights.

3. In a chemical fume hood, add 1 mL of fixation agent and wait 1 hour for fixation to occur. For our application, we use pre-warmed 4% paraformaldehyde (PFA) for fixation, which works well for phalloidin and DAPI staining. Other labeling, however, may require alternative fixation methods such as methanol or ethanol-based protocol. The specific fixation method should be selected depending on the cell type used and application of interest (Hobro & Smith, 2017).

4. Carefully remove fixation agent and replace with 1 mL of PBS. Gently lift the weight holder and weight off the agarose device. Using tweezers, carefully peel the agarose device off the fixed cells. Wash cells 2-times with 1.0 mL DPBS for 5 minutes each.

   After the initial fixation, the confined cell morphology should be preserved, even after removing the agarose pad. Sufficient fixation is required to ensure that cells retain their morphology and do not detach from the glass bottom when the agarose device is removed.

5. Examine cells using a brightfield microscope. You should be able see the circular areas where the micropillars were, as the squished cells under the micropillars are typically peeled off together with the agarose device. Cells confined to 3 μm will appear to be flatter and have increased cross-sectional areas compared to cells confined to 10 μm or unconfined cells.

   At this point, samples can be stored in DPBS at 4°C until staining, or you can proceed with the staining protocol. For longer term storage, add sodium azide to the DPBS (0.02% w/v) to prevent microbial contamination. Note that for some sensitive applications (e.g., analyzing protein phosphorylation), cells should be stained and imaged as soon as possible.

6. Permeabilize cells using a 0.1% Triton solution at room temperature for 15 minutes. Wash cells 2-times at room temperature with 1.0 mL PBS for 5 minutes each.

7. Aspirate PBS and add 1 mL of blocking solution to the dish. Incubate for 1 hour on a benchtop rocking platform set to low speed at room temperature.

8. For phalloidin staining: add 250 μL of 1:200 Phalloidin-568 in PBS. Incubate at room temperature rocking for 1 hour. For immunofluorescence labeling: add

250 μL primary antibody solution to dish and incubate at 4°C overnight on a benchtop rocking platform.

For immunofluorescence labeling, primary antibody concentrations typically range from 1:100- 1:500 in PBS containing 3% BSA. The exact concentrations will depend on the antibody and protein of interest and should be optimized for each new target. For some antibodies, incubation at 4° C for a longer period may be preferable. Secondary antibody-only controls should be used to address non-specific binding. For phalloidin staining, skip steps 10 and 11 and resume at step 12. Phalloidin-568 is a fluorescent stain already conjugated to a fluorophore, so no secondary antibody is needed. Phalloidin is also available with many other fluorescence labels.

9. Aspirate antibody or staining solution from samples. Wash with PBS 2-times for 5 minutes each on a rocking platform.

10. For immunofluorescence labeling: incubate samples on benchtop rocking platform for 1 hour with secondary antibody solution compatible with primary antibody used. We recommend a secondary antibody concentration of 1:250 diluted in PBS containing 3% BSA, but the specific concentration will depend on the particular antibodies used.

11. Aspirate solution from samples. Wash with PBS two-times for 5 minutes each on a rocking platform at room temperature.

12. Stain with DAPI at a dilution of 1:500 in PBS for 30 minutes at room temperature on rocking platform. Wash 2-times for 5 minutes each with DPBS and store samples in 0.5 mL DPBS at 4°C.

DAPI is a versatile, robust, and bright nuclear stain that binds to DNA. Alternatively, Hoechst or other DNA intercalating dyes can be used. The concentration of the dye should be optimized for each application.

13. The fixed and stained cells can be temporarily stored at 4°C for further analysis or imaged right away on a microscope. For longer term storage, sodium azide should be added to the DPBS solution (0.02% final concentration). Images of the stained cells can be acquired on a confocal or wide-field fluorescence microscope, depending on the specific application. We recommend acquiring high resolution Z-stacks on a confocal microscope to confirm the confined morphology of the cells.

SUPPORT PROTOCOL 2: Live/dead staining of cells during confinement

Staining living cells, combined with time-lapse microscopy and multi-positions setting, can be used to assess dynamic effects of 3D confinement, and allows the comparison of the same cells pre- and post-confinement, thereby reducing cell-to-cell variability. Additionally, live/dead cell staining is useful to ensure cell viability of cells at the end of various assays. Here, we present a basic protocol for live/dead staining of MDA-MB-231 cells with propidium iodide after 24 hours of 3-μm height confinement, 10-μm height confinement (control), and

in unconfined cells. Our results demonstrate that cells remain viable even under prolonged confinement to 3-μm height, with only cells that were located under the agarose micropillars and thus were squished completely staining positive for propidium iodide (Figure 7). This protocol can also be applied to other live cell stains, as we show that the cell stain diffuses throughout the confinement device within one hour of adding the staining reagent.

Materials:

All materials from Basic Protocol 2

Live/dead cell stain of interest (e.g., propidium iodide, which labels dead cells, used at 1 μg/mL in cell culture media)

Protocol steps:

1. Follow the confinement protocol described in Basic Protocol 2 to apply confinement.

   For live cell imaging of cells starting before confinement is applied, the desired cell stain of interest can already be applied to the cells prior to applying the confinement device. In that case, it is important to avoid moving the dish with the cells when applying the device, or carefully repositioning it after applying the confinement device to capture the same cells again. Note that prolonged incubation of cells in media containing propidium iodide can result in false positive propidium iodide labeling, as even live cells may take up the dye over time. Thus, for lived/dead staining with propidium iodide, we recommend applying the propidium iodide only at the end, as described here.

2. After incubating confined cells for the desired amount of time, gently aspirate media from the edge of the dish, make sure not to move the confinement device.

3. Add 1 mL of cell culture media containing the cell stain of interest to dish, just outside of the confinement device. For our live/dead stain application, we used 1 μg/mL of propidium iodide in Fluorobrite media.

   It is essential to keep the weight holder and weight on the agarose device during the staining. This is to ensure the agarose does not detach from the substrate and shear the cells.

4. Incubate the dishes at 37°C for 1 hour. We found that diffusion of the cell stain across the cells in the device consistently occurs by 1 hour.

   If performing experiments at room temperature (which is suitable for many cell types), it may be necessary to extend the incubation time to ensure that propidium iodide has diffused completely in all areas below the agarose device.

5. Gently aspirate the media from the outside of the device. For live cell imaging, e.g., to quantify the fraction of cells positive for propidium iodide, add 1 mL of cell imaging media to the dish. The cells can then be imagined on a fluorescence microscope or maintained in culture. Alternatively, if using a live-cell stain

compatible with fixation, the cells in the device can be fixed and stained using Support Protocol 1.

REAGENTS AND SOLUTIONS:

Cell culture media (prepare in sterile environment)

- 445.0 mL Dulbecco's Modified Eagle Medium (DMEM) (Gibco/ThermoFisher, cat. no. 11965092)
- 50.0 mL Fetal Bovine Serum (Avantar by VWR, cat. no 89510-186)
- 5.0 mL Penicillin/Streptavidin (Gibco/ ThermoFisher cat. no. 15070-063)

Fluorobrite medium (prepare in sterile environment)

- 42.5 mL Fluorobrite™ Dulbecco's Modified Eagle Medium (Gibco/ ThermoFisher, cat. no. A1896701)
- 1.5 mL 1M HEPES for imaging without 5% CO_2 (Gibco/ThermoFisher cat. no. 15630080)
- 0.5 mL 100× GlutaMAX (Gibco/ThermoFisher cat. no. 35050061)
- 5.0 mL Fetal Bovine Serum (Avantar by VWR, cat. no. 89510-186)
- 0.5 mL Penicillin/Streptavidin (Gibco/ ThermoFisher cat. no. 15070-063)

1× DPBS (tissue culture grade, prepare in sterile environment))

- 5.0 mL 10× DPBS (Gibco/ThermoFisher cat. no. 14-200-075)
- 45.0 mL autoclaved DI water

10× PBS (non-tissue culture grade)

- 1.4 g/L Potassium dihydrogen phosphate (KH_2PO_4)
- 90 g/L Sodium chloride (NaCl)
- 7.9 g/L di-Sodium hydrogen phosphate anhydrous (Na_2HPO_4 $7H_2O$)
- 1.0 L DI water, adjust to pH 7.4

1× PBS (non-tissue culture grade)

- 900 mL DI water
- 100 mL 10× PBS (see above)

4% PFA

- 4.0 g paraformaldehyde (Sigma, SKU 158127-500G)
- 100 mL warm PBS
- Store at 4°C

0.1% Triton

- 0.5 mL Triton X100 stock (Sigma, SKU X100-500ML)
- 49.5 mL 1× PBS (see recipe above)

Blocking solution

- 1.5 g bovine serum albumin (Sigma Aldrich, cat. no. A7906)
- 50 mL 1× PBS
- Vortex for 30 seconds and rock on benchtop rocking platform until BSA dissolves
- Store at 4°C

COMMENTARY:

Background Information:

Researchers have long been studying the effect of mechanical forces and the physical environment on cells, as it is well recognized that these factors can substantially modulate cellular fate and function. This research is now generally categorized as ‘mechanobiology’. In recent years, an increasing interest has fallen on the cell nucleus, and nuclear mechanobiology has gained a spotlight as a promising avenue to elucidate the molecular mechanisms surrounding various diseases and physiological processes, ranging from cancer metastasis to immune cell function and muscle diseases. Although various cell confinement systems have been developed over the past 15 years, many of the systems come with their own challenges and limitations, particularly when applying prolonged confinement. To address these challenges, and after having tested numerous confinement systems in our laboratory, we have developed a robust confinement assay that can be easily tailored to specific applications due to its simple design. While ultimately *in vivo* assays are still needed to fully recapitulate the 3D biological microenvironment of cells, the system presented here provides a cost effective, reliable *in vitro* assay to probe consequences of cell confinement and nuclear deformation with precise control over the degree of confinement. The main advantage of this device is the simple concept of adding weight to the agarose micropillars to induce cell confinement. This device does not require costly external hardware, specially designed microscope stages, or other machined parts. Although the cost and time consideration to fabricate the silicon wafer must be taken into account, this is a relative low one-time costs that should not exceed a few hundred dollars, and that can also be outsourced to commercial or academic service providers if necessary.

Critical Parameters:

Fabrication of the silicon wafer—During the fabrication of the silicon wafer, it is essential to precisely follow the protocol in the critical steps listed below, as small variations can result in highly variable outcomes or non-usable wafers. For the lower layer SU-8 features, baking steps should be timed precisely, while for the upper layer SU-8 features, the pre- and post-exposure bake times are more flexible. It is essential to always let the wafer cool to room temperature after baking before starting the next step. When exposing

the silicon wafer, always take note of the UV source power and adjust the exposure time proportional to the change in lamp power if needed. The hard bake step is critical to ensure that upper layer features do not delaminate after multiple casts of agarose from the silicon wafer. As with any other baking step, let the silicon wafer cool gradually to room temperature.

Cell seeding—Proper cell seeding density is essential to ensure consistent results using the same amount of weight, as a larger number of cells will offer more resistance to confinement. Although more weight can be added if the cells are seeded at a high density to ensure confinement, we found that this approach often leads to over confinement, likely due to the collapse of the micropillars under the weight. Similarly, if the cell density is lower than expected, over confinement can occur if the standard weight is applied. In our experience, we found that dishes with 50-70% cell confluency work best to achieve uniform and robust confinement even for extended periods of time.

Preparing the agarose confinement device—Prior to preparing the agarose devices, rinse the silicon wafer once with DI water to ensure that no dust or other agarose is left on the wafer. Completely dry the wafer before casting the agarose. Since agarose is a hydrogel that contains a large volume fraction of liquid, it is important to dissolve the agarose in tissue culture grade DPBS to ensure that the pH in the cell culture media does not change when the device is applied to the cells. If the agarose is dissolved in PBS outside the range of 7-7.3, the amount of media the agarose device absorbs once placed onto the cells will be affected and can negatively impact the cells and cause cell death. Conversely, dissolving the agarose in water will cause osmotic swelling of the cells once the agarose device is placed into the dish. We highly recommend preparing fresh agarose for each experiment. While handling the agarose devices, it is essential to use flat-tip tweezers and gentle handling to avoid puncturing the agarose. We recommend preparing the agarose devices immediately prior to the confinement assay to ensure that the agarose devices do not dry out.

Cell confinement and imaging—It is essential to soak the agarose device in cell media for 1 minute prior to the assay. Under-soaking the device risks the agarose device absorbing too much media when placed in the dish, which can result in over-confining the cells. After starting the cell confinement, avoid moving the dishes too often, as this can cause the weight holder and weight to slide around and either (1) induce non-uniform confinement if the weight is not distributed evenly, or (2) cause the agarose to slide and shear the cells. When imaging the cells, be sure to avoid saturation of fluorescent markers, as this can affect the height and cross-sectional area measurements of the nuclei.

Cell fixation—It is essential to keep the agarose firmly in contact with the bottom of the dish during fixation by keeping the weight and weight holder in place during incubation. If the weight and weight holder is removed prematurely, the agarose can detach and shear cells off the dish.

Troubleshooting:

Table 4 includes a troubleshooting guide for issues that can occur during the described protocols. We have included probable causes of the issues observed, along with solutions we found helpful to address common problems.

Understanding Results:

The confinement of cells can be confirmed by acquiring Z-stack images and measuring nuclear height (Figure 1C). Confined nuclei typically appear flat in the X-Z view as shown in the representative images, while unconfined nuclei will appear more rounded in the X-Z view. Given the limited resolution in the Z-axis, even when using high NA objectives, we typically allow for 0.5 μm of error when measuring nuclear height (i.e., nuclei confined to 3 μm should measure 2.5-3.5 μm in height), and this could vary further depending on the imaging parameters set. We recommend using the same imaging parameters across experiments to achieve consistent measurements of nuclear height. Cells confined to 3 μm tall micropillars should also exhibit an increase in nuclear cross-sectional area (Figure 4B) as their nuclear height decreases (Figure 4C) when compared to cells confined to 10 μm tall or in unconfined conditions. For the MDA-MB-231 cells used in our example, the nuclei measured to be around 7 μm high on average when grown in unconfined conditions. Thus, confinement to 10-μm height does not affect nuclear height or cross-sectional area compared to unconfined cells, whereas confinement to 3-μm height decreases nuclear height and increases nuclear cross-sectional area. Additionally, we demonstrate that confinement of MDA-MB-231 cells below 5 μm height induces nuclear envelope rupture (Video 1), as previously reported (Denais et al., 2016; Nader et al., 2021; Shah et al., 2021). Consequently, the confinement device can be used to induce nuclear envelope rupture in cells and to study the downstream consequences such as induction of DNA damage and activation of DNA damage response pathways.

For initial validation of achieving uniform confinement across the device, we recommend adding fluorescently labeled dextran to the cell media before applying confinement. When combined with 3D confocal imaging, this will enable to visualize the space occupied by media between the glass bottom/cells and the agarose pad (Figure 5A). This approach is particularly useful to ensure the agarose micropillars are touching the glass dish, and the apical cell surface is in contact with the agarose when applying severe confinement. The contact of the micropillars with the glass dish can also be confirmed without using dextran by looking for circular patches of completely squished cells under the agarose micropillars (Figure 5B). Cells on the edge of the micropillars will be easily distinguished using the orthogonal view as they will show an apparent change in height at the location of the pillar.

Fluorescent labeling or immunofluorescence of cellular structures should demonstrate clear effects in conditions where the vertical confinement is lower than the height of unconfined cells. The exact results will depend on the application. In our example, we stained MDA-MB-231 cells with phalloidin and DAPI to visualize F-actin and DNA, respectively (Figure 6). Because the nuclei are fixed under the device during confinement for immunostaining applications, the nuclear height should not change compared to before fixation, and you should confirm that cells of interest are confined to the desired height. Our propidium iodide

staining shows that cells remain viable even after 24 hours of confinement (Figure 7). If cell viability is reduced substantially during or after confinement, this indicates that cells were likely over-confined or that the pH or osmolarity of the solution changed due to the addition of the device (see Troubleshooting tips).

Time Considerations:

Design and fabrication of confinement device wafer—The design of this photomask takes 2 to 4 hours. More complex designs may take much longer. Writing and developing the photomask will take 3 to 4 hours. Piranha cleaning one silicon wafer takes 5 to 8 minutes, and the wafer must be dehydrated overnight before use. Lower layer feature fabrication (3 μm or 10 μm tall micropillar layer) will take approximately 2 to 4 hours. The 500 μm upper layer takes approximately 36 to 48 hours due to the long bake times. The final hard bake of the silicon wafer and hydrophobic coating will take approximately 4 hours. From start to finish, fabrication of one complete wafer will take about one week for an experienced user. However, troubleshooting and re-optimization of parameters such as the spin recipe, exposure time, bake time, and developer time may require additional time.

Cell confinement and imaging—Coating the 35 mm glass dishes takes 3-5 minutes. Passaging and seeding cells takes approximately 30 minutes. This must be done one day prior to the confinement assay. Preparation of the agarose devices will take 15-20 minutes. For each experiment, it takes approximately 20 minutes from the time of soaking the agarose in media to the time of adding the weight and letting the device reach confinement height after 10 minutes. For live cell imaging experiments, image acquisition time will depend on the parameters chosen. We recommend a confinement time of at least one hour, but this depends on the intended application. Cell confinement can be maintained for 24 hours or longer. For longer confinement periods, we recommend confirming cell viability using appropriate live/dead cell stains, and potentially replacing media every 24 hours.

Immunostaining after confinement—Once the confinement is applied for the desired time, washing with PBS and PFA fixation takes approximately 1 hour. Depending on the intended immunostaining application, staining, and washing times may vary, but we recommend overnight incubation at 4°C for the primary antibody, and 1 hour incubation at room temperature for the secondary antibody. Phalloidin and DAPI staining takes approximately 2 hours to complete after fixation.

Live cell staining during confinement—We found that after the addition of a live/dead cell stain, diffusion under the agarose device can take up to 1 hour to ensure uniform labeling. Optionally, the cells may be washed with media for an additional hour.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT:

The data, tools, and material (or their source) that support the protocol are available from the corresponding author upon reasonable request. Files containing the design for the photomasks and the 3D printed weight holder are available through Github at https://github.com/Lammerding/Confinement-Device

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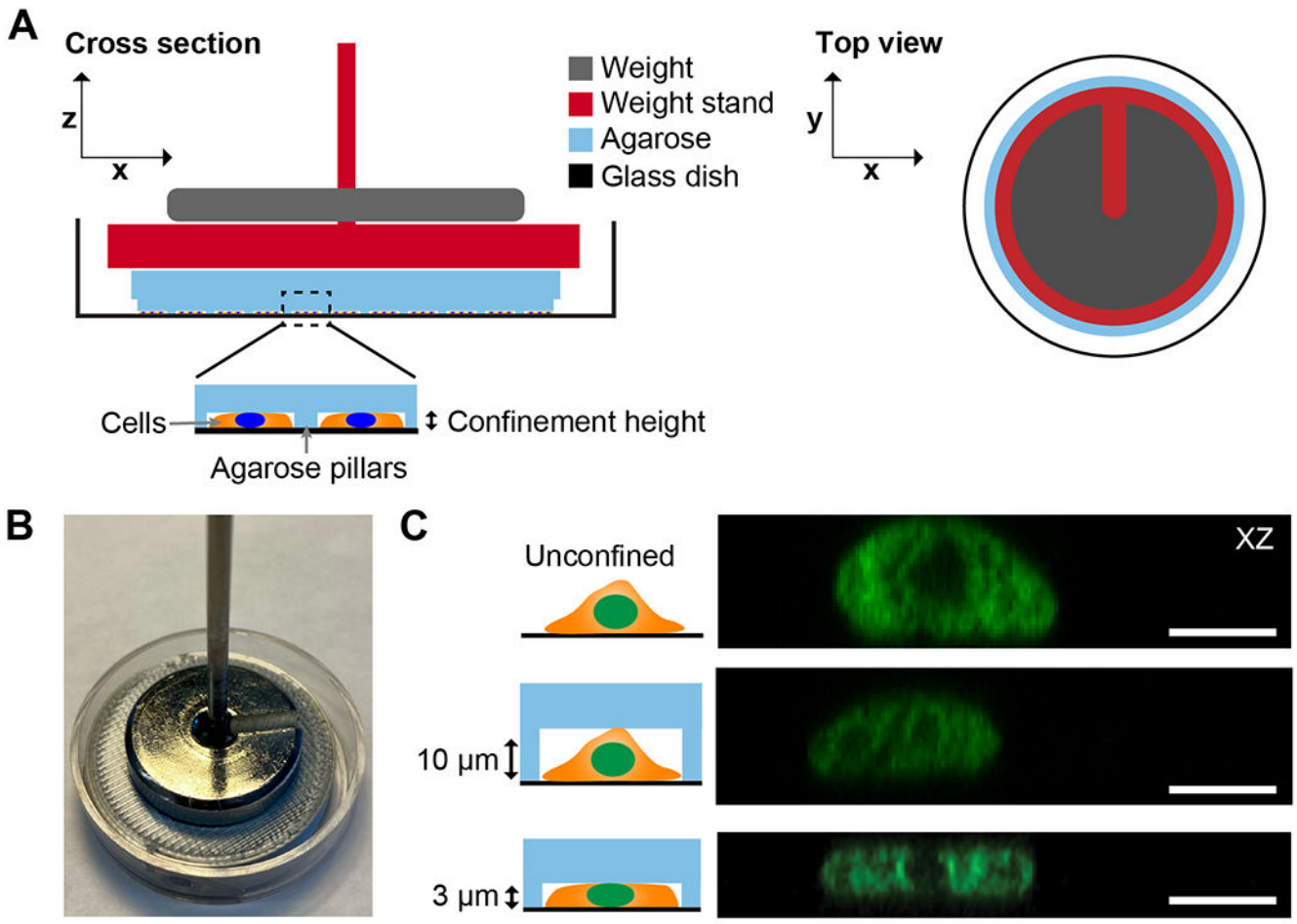

Figure 1. Overview of agarose confinement device.

(**A**) Cross section (left) and top view (right) schematic of the confinement device assembly. Assembly includes a 35 mm glass bottomed dish (black), agarose device (blue), weight holder (red), and weight (gray). (**B**) Photograph of confinement device assembly. (**C**) Representative images of MDA-MB-231 cells with nuclei fluorescently labeled with histone 2B-mNeonGreen under either unconfined, confined to 10 μm, or confined to 3 μm conditions. Images are orthogonal views of Z-stacks. Scale bars = 10 μm.

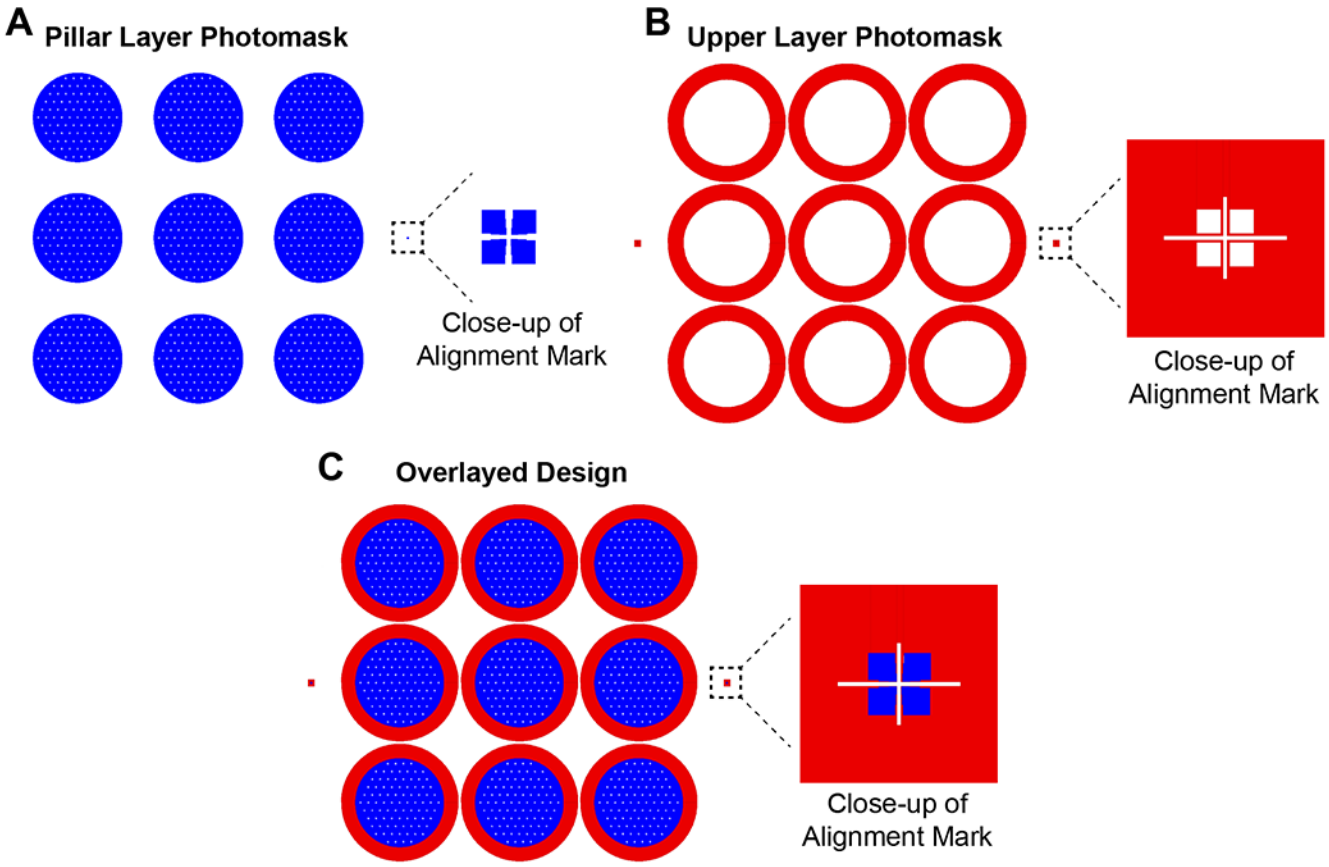

Figure 2. Confinement device photomask design.

(**A**) Lower layer photomask design containing the array of micropillars to induce confinement and alignment marks for aligning the second layer photomask to the first layer of photoresist. (**B**) Upper layer photomask design for the 500 μm tall layer and complementary alignment marks. (**C**) Overlay of the lower- and upper-layer photomask designs. Areas colored in blue and red areas show the pattern that will be exposed to UV-light on the wafer. SU-8 is a negative photoresist, so exposed photoresist will become insoluble to SU-8 developer, thus forming the intended device design.

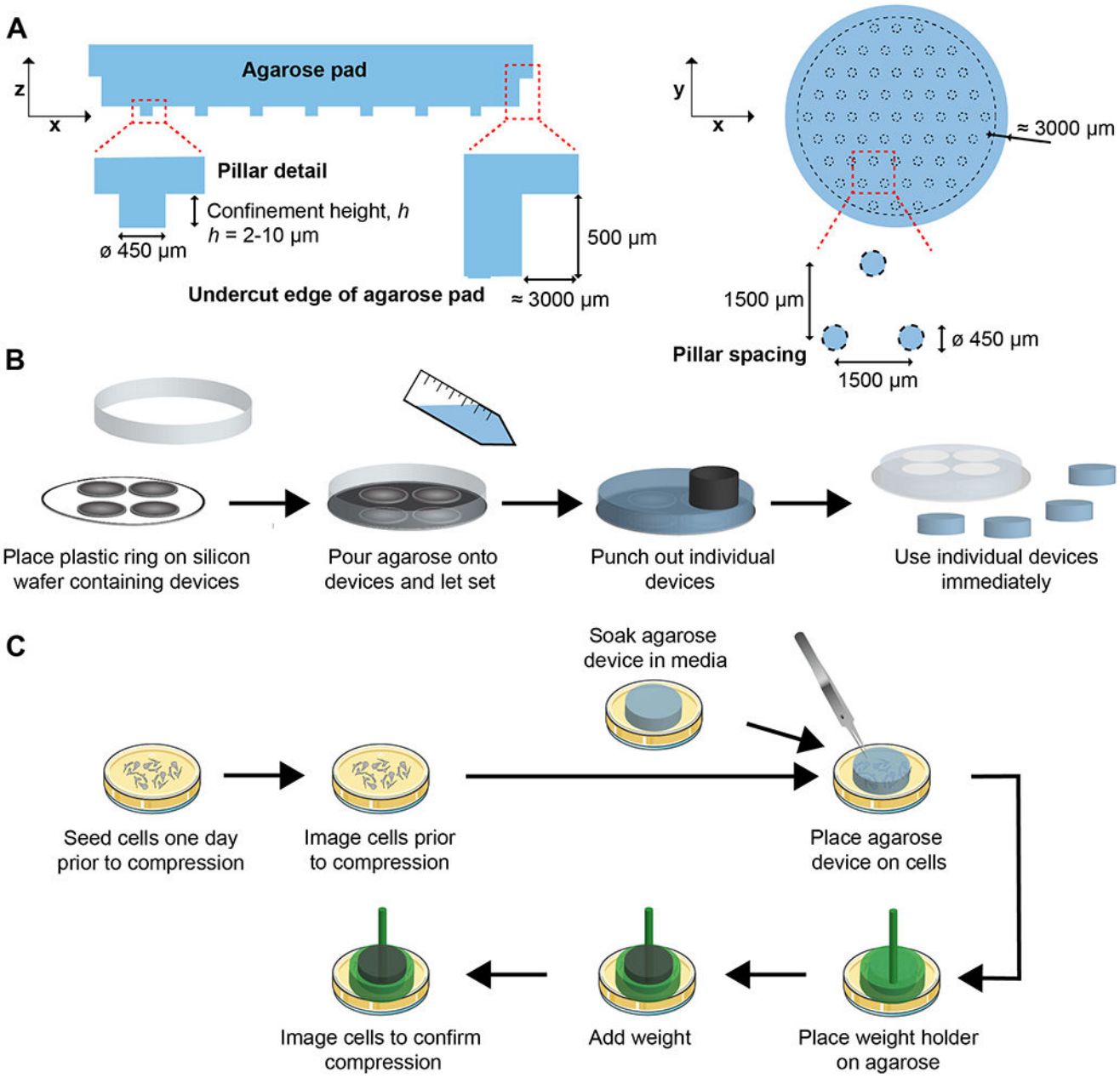

Figure 3. Device design and workflow schematic.

(**A**) Schematic depiction of a single agarose device. The agarose device (light blue) contains an array of micropillars (outlined in black) of a defined height to facilitate confinement. The second layer of SU-8 provides a region to cut out each individual device without cutting through the agarose micropillars. Images are not drawn to scale. (**B**) Preparation of agarose device from silicon wafer. A plastic ring is placed over the silicon wafer and agarose is poured onto the wafer and allowed to set. Individual devices are punched out of the agarose and are ready to be used. For simplification, only 4 devices are shown on one silicon wafer; however, up to 9 devices can be fabricated on one wafer. (**C**) Cell confinement and imaging. Cells are seeded ~24 hours prior to the confinement assay. Cells are imaged

before confinement. Immediately prior to use, the agarose device is soaked in media and then immediately placed onto the dish containing cells. The weight holder is placed on top of the agarose, and the weight is placed onto the holder. Confinement should occur within 30 minutes and should be validated via confocal microscopy. This figure uses icons from Bioicons, an open-source library of science illustrations.

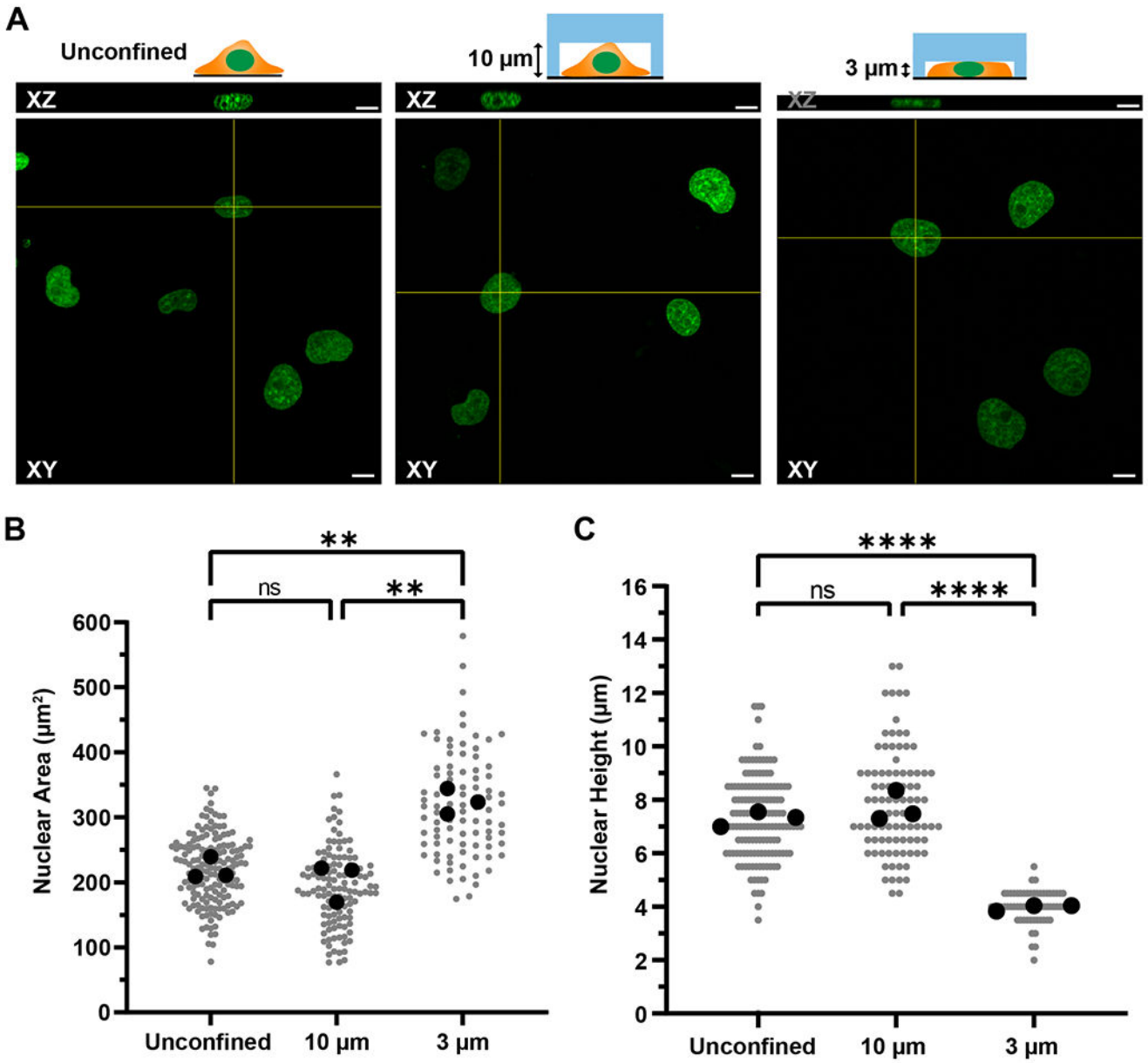

Figure 4. Confinement device validation.

(**A**) Representative images of MDA-MB-231 cells expressing histone 2B-mNeongreen under either unconfined, confined to 10 µm, or confined to 3 µm conditions. Yellow lines indicate orthogonal viewpoint. Scale bars = 10 µm. (**B**) Quantification of nuclear cross-sectional area of unconfined cells, cells confined to 10 µm, and cells confined to 3 µm. (**C**) Quantification of nuclear height of cells from the three conditions. Data is plotted as measurements from individual cells with the mean of each biological replicate indicated by a black dot. n= 65-162 cells per condition from three independent experiments. ** $p < 0.01$, **** $p < 0.0001$ based on one-way ANOVA with multiple comparisons.

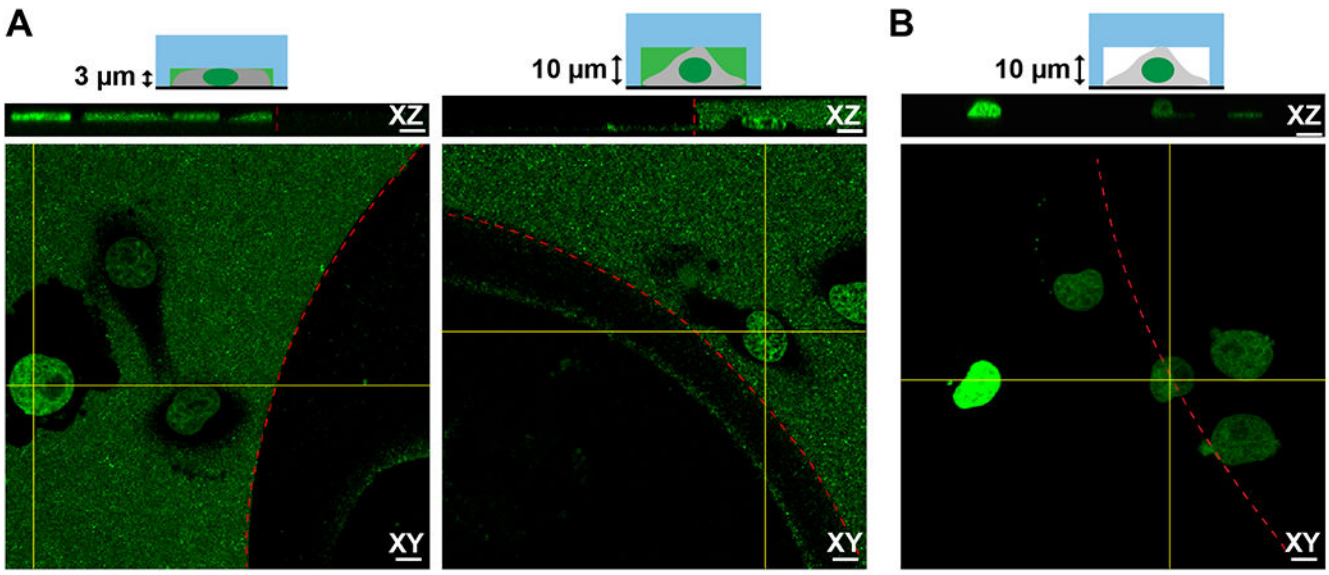

Figure 5. Visualizing agarose micropillars

(**A**) MDA-MB-231 cells expressing Histone 2B-mNeonGreen were confined to either 3 μm or 10 μm height, with FITC-labeled dextran added to the cell media to visualize the space between the agarose, agarose micropillars (red dash line), and the cells and glass dish. Representative images of both the X-Z and X-Y planes are shown. Yellow lines indicate orthogonal viewpoint. (**B**) The agarose pillars (red dash line) can be visualized without dextran in the media to confirm the agarose device is touching the bottom of the dish. Cells under the pillars will be completely squished and should measure a height of less than 2μm. Yellow lines indicate orthogonal viewpoint. Scale bars = 10 μm.

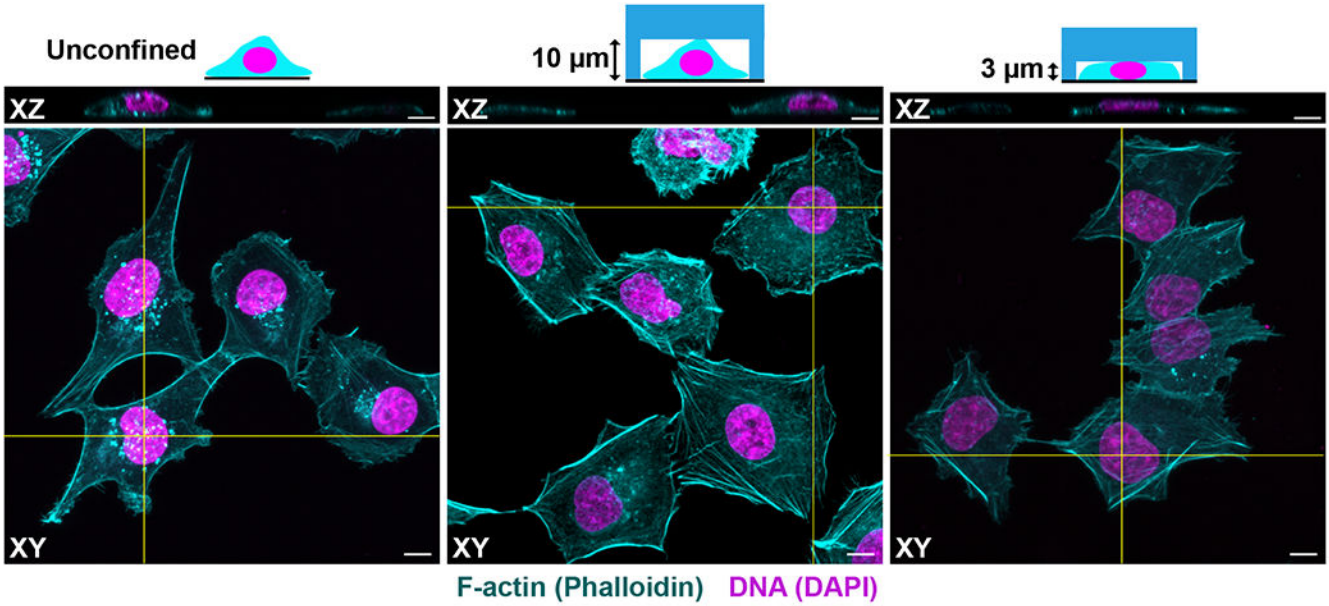

Figure 6. Validation of cell viability and immunofluorescence staining after confinement assay.
MDA-M-231 breast cancer cells were stained with Phalloidin-568 to visualize F-actin (cyan) and DAPI to visualize nuclei (magenta). Representative images of both the X-Z and X-Y planes are shown. Yellow lines indicate orthogonal viewpoint. Scale bars = 10 µm.

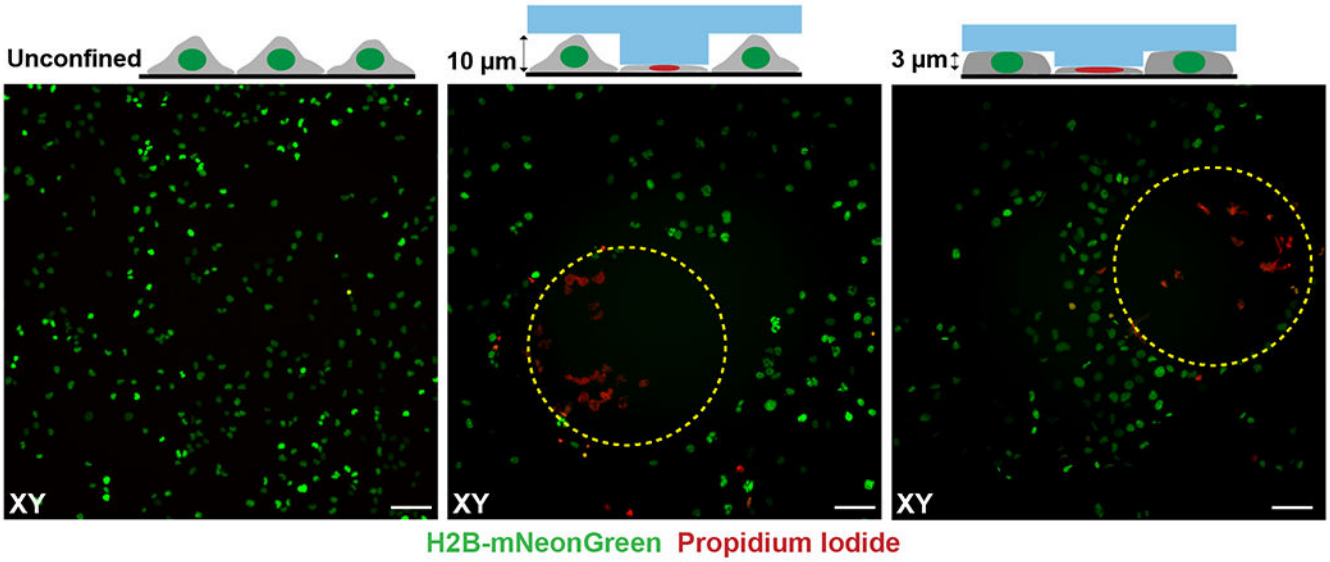

Figure 7. Live/dead staining after 24 hours of confinement.

MDA-MB-231 breast cancer cells expressing Histone 2B-mNeonGreen remain viable after 24 hours in the device under unconfined, 10 μm confinement, and 3 μm confinement conditions. Cells completely squished under the agarose pillars (red) stain positive after incubation with propidium iodide. Scale bars = 100 μm.

Video 1.

MDA-MB-231 cells expressing NLS-GFP and stained with Hoechst 33342 to label DNA experiencing nuclear envelope rupture after confinement to 3 μm tall. Nuclear envelope rupture is detectable by rapid leakage of the NLS-GFP from the nucleus into the cytoplasm. Scale bar = 10 μm.

Table 1:

Spin Curves for 3 μm thick layer with SU-8 2002

Step	RMP	Acceleration /deceleration (rpm/second)	Seconds
Ramp up	500	100	10
Spin	600	100	10
Ramp down	100	100	10

Table 2:

Spin Curves for 10 μm thick layer with SU-8 10

Step	RMP	Acceleration /deceleration (rpm/second)	Seconds
Ramp up	500	100	10
Spin	3300	100	33
Ramp down	100	100	35

Table 3:

Spin Curves for 500 μm thick layer with SU-8 2150

Step	RMP	Acceleration /deceleration (rpm/second)	Seconds
Ramp up	500	100	10
Spin	650	100	60
Ramp down	100	100	15

Table 4.

Troubleshooting guide for cell confinement

Problem	Possible Cause	Solution
Immediate over confinement and cell death after adding the agarose device, before adding weights	The agarose is absorbing the cell medium due to inefficient soaking of agarose device or altered pH of agarose.	Soak the agarose for a longer time or reduce its thickness by using 10 mL of agarose solution instead of 15 mL. Ensure the pH of the PBS used to make the agarose is ~7.1 or the same pH as cell culture media.
No confinement occurs	Cells are too confluent.	Reduce the number of cells when seeding to get a 50% confluency.
	Agarose micropillars were damaged when cutting or handling the device causing displacement of agarose and interfering with the device being able to evenly touch the dish.	Recast agarose and carefully cut out new devices. Be sure to use flat tweezers and do not forcefully clamp down on the agarose device with tweezers.
	Not enough weight was applied.	Add more weight to the device. We recommend adding weight in 5 to 10 gram increments.
	Not enough time has passed for confinement to occur.	Robust confinement should occur within 30 minutes. If this is not the case, incubate cells for 30 more minutes, check nuclear height, and then proceed to try and add additional weight.
Over confinement	Cells are not confluent enough.	Increase the cell density until obtaining 50% to 70% confluency.
	Too much weight is applied.	Reduce the amount of weight added to the device.