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High Throughput Traction Force Microscopy using PDMS Reveals Dose-Dependent Effects of TGF-β on the Epithelial-to-Mesenchymal Transition

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Cover Letter

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February 27, 2019

Dear Dr DSouza:

Please find attached our revised manuscript; the editorial and reviewer comments were constructive and improved the manuscript, and we have addressed all points raised in the text, table, and Figures. These are addressed line by line in the rebuttal letter.

Sincerely yours,

Allen J Ehrlicher

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TITLE:

High Throughput Traction Force Microscopy using PDMS Reveals Dose-Dependent Effects of Transforming Growth Factor-β on the Epithelial-to-Mesenchymal Transition

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KEYWORDS:

PDMS, polydimethylsiloxane\, traction force microscopy, high throughput, contractile force screening, epithelial-to-mesenchymal transition, mechanobiology, cancer

SUMMARY:

We present a high throughput traction force assay fabricated with silicone rubber (PDMS). This novel assay is suitable for studying physical changes in cell contractility during various biological and biomedical processes and diseases. We demonstrate this method's utility by measuring a TGF-β dependent increase in contractility during the epithelial-to-mesenchymal transition.

ABSTRACT:

Cellular contractility is essential in diverse aspects of biology, driving processes that range from motility and division, to tissue contraction and mechanical stability, and represents a core element of multi-cellular animal life. In adherent cells, acto-myosin contraction is seen in traction forces that cells exert on their substrate. Dysregulation of cellular contractility appears in a myriad of pathologies, making contractility a promising target in diverse diagnostic approaches using biophysics as a metric. Moreover, novel therapeutic strategies can be based on correcting the apparent malfunction of cell contractility. These applications; however, require direct quantification of these forces.

We have developed silicone elastomer-based traction force microscopy (TFM) in a parallelized multiwell format. Our use of a silicone rubber, specifically polydimethylsiloxane (PDMS), rather than the commonly employed hydrogel polyacrylamide (PAA) enables us to make robust and inert substrates with indefinite shelf-lives requiring no specialized storage conditions. Unlike pillar-PDMS based approaches that have a modulus in the GPa range, the PDMS used here is very compliant, ranging from approximately 0.4 kPa to 100 kPa. We create a high-throughput platform for TFM by partitioning these large monolithic substrates spatially into biochemically independent wells, creating a multiwell platform for traction force screening that is compatible with existing multiwell systems.

In this manuscript, we use this multiwell traction force system to examine the Epithelial to Mesenchymal Transition (EMT); we induce EMT in NMuMG cells by exposing them to TGF-β, and to quantify the biophysical changes during EMT. We measure the contractility as a function of concentration and duration of TGF-β exposure. Our findings here demonstrate the utility of parallelized TFM in the context of disease biophysics.

INTRODUCTION:

Acto-myosin contractility is an essential element of active cell mechanics, impacting cell behaviors from motility and proliferation to stem cell differentiation. In tissues, contractility drives activity from polar separation in embryogenesis, to airway constriction and cardiac activity. Critically, to generate tension, cells must first adhere to their extracellular environment. In doing so, this contractility generates traction forces on their surroundings. Traction Force Microscopy (TFM) has emerged in a multitude of forms as a way to quantify these forces from diverse cells under different conditions.

The field of TFM has seen an exceptional breadth of innovation and application, and the results have paved the way for new perspectives in biology, which incorporate mechanics and physical forces. Starting with wrinkling silicone substrates[1], researchers have applied various techniques to measure cell traction forces. These approaches have been continuously improved and have now reached a level of resolution on the order of several microns[2]. However, one principal problem has emerged, which is the difficulty in creating substrates of suitably low moduli using the available silicones. To circumvent this problem, polyacrylamide was adopted as a replacement due to the ease of creating substrates on the order of 1–20 kPa[3]. We recently implemented very compliant silicones in TFM[4], allowing us to fabricate the same range of moduli as polyacrylamide, but with the advantages of inert and robust silicone.

TFM approaches have enabled valuable mechano-biological discoveries, however, a persistent shortcoming is their complexity, often restricting their use to researchers in the engineering or physical sciences disciplines. This is due, in large part, to the detailed calibrations and challenging calculations that are required to quantify contractility. Another significant challenge is that TFM methods are largely low-throughput and therefore ill-suited to study many different conditions or populations simultaneously[5]. This has presented a bottleneck, which has hampered transfer of TFM from a specialist biophysics setting into broader biological sciences and pharmacology applications.

We have recently developed a multi-well format TFM plate, which allows researchers to parallelize their TFM measurements for faster quantification of contractility metrics, while exploring the impact of different compounds and also using less reagents[4]. This methodology has broad utility in diverse mechanobiology studies, from evaluating the effects of compounds on cellular activity, to quantifying the contractile changes in differentiation or disease.

One area of biomedical research that will benefit greatly from TFM is the study of how physical cues impact the malignant phenotypes of cancer cells. Metastasis, responsible for 90% of cancer-related deaths, is characterized by cancerous cells leaving their original tumor site and colonizing a secondary site. For cells to migrate through tissue and pass in and out of the vascular system, they must radically change their shapes to squeeze through these physical barriers while generating substantial forces to pull their way along extracellular matrix or move between other cells. These forces are transmitted to the substrate through focal adhesion interactions[2-3], and can be quantified using TFM. While cancers are biochemically exceptionally diverse, with an expanding repertoire of known mutations and protein changes, some common physical changes have been observed; in a variety of cancers, including breast, prostate, and lung cancers, metastatic cells have been shown to exert 2–3 times the traction forces of non-metastatic cells[6-8]. These results suggest that there may be a strong correlation between metastatic progression and the traction forces exerted by cells; however, the detailed time-dependent changes in contractility are difficult to examine.

The epithelial-to-mesenchymal transition (EMT) is a process whereby cells reduce adherens- and tight-junction mediated cell-cell adhesion, becoming more migratory and invasive. In addition to physiological functions that include wound healing and developmental processes, EMT is also a process exploited during metastasis, making it a useful model system to study this process. Using TGF-β, we can induce the EMT in ErbB2-transformed murine mammary epithelial cells (NMuMG-derived)[9] to directly quantify the physical changes during this transformation, and characterize the time and dose-dependent effects of TGF-β on EMT and cell contractility. In this article, we demonstrate the utility of this approach by measuring the changes in contractility during an induced EMT.

PROTOCOL:

NOTE: The following protocol will guide researchers in fabricating and using the multi-well TFM dish shown in **Figure 1**.

1. Preparation of PDMS silicone substrates

1.1. Preparation of PDMS silicone rubber mixture based on a composite mixture of two commercially available kits.

1.1.1. Add Part A and Part B of PDMS kit (e.g., GEL-8100, see the **Table of Materials**) in a 1:1 weight ratio into the 50 mL tube.

NOTE: The mixture is mixed on a rotator at a speed slow enough for the mixture to flow back and forth during revolution to ensure complete mixing.

1.1.2. Add the required amount of curing agent for the desired modulus of the substrate.

NOTE: The amount of curing agent to be added to the mixture depends on the desired modulus of the substrate and may typically range from 0.1% to 1.8%. Refer to **Table 1** and **Figure 3** for a guide to specific crosslinker concentrations and resulting moduli.

1.1.3. Mix the formulation on the rotator for 30–45 min; ensure the rotation is slow enough for thorough mixing.

1.2. Bottom layer: coating PDMS substrates on the glass slide

1.2.1. Place the custom-built chuck illustrated in **Figure 2** on the spin-coater. Clean the glass with ethanol or isopropanol, and dry with a lint-free wipe. Place the glass slide in the chuck and turn on the vacuum to hold the slide in place.

1.2.2. Apply uncured PDMS approximately 1 cm from the edges and work in towards the center. Apply enough (3–4 mL) PDMS to ensure the whole surface will be covered.

NOTE: To ensure that the PDMS is evenly spread on the surface of the glass, a pipette tip may be helpful to spread the PDMS mixture from the center to the edges.

1.2.3. Spin the glass with the PDMS mixture on a spin-coater with the following protocol.

1.2.3.1. To spread the uncured PDMS on the slide, accelerate at 50 rpm/s from 0 to 200 rpm; hold at 200 rpm for 1 min.

1.2.3.2. To achieve a 100 µm thick PDMS layer, accelerate at 50 rpm/s to 300 rpm and hold for 1 min at 300 rpm. Different desired thicknesses other than 100 µm will require specific speed values.

1.2.3.3. To remove, decelerate at 50 rpm/s to 0 rpm. Disable vacuum and remove the coated slide, taking care not to touch the coated surface.

NOTE: It is important to include the acceleration and deceleration steps to ensure a smooth continuous surface.

CAUTION: To ensure that the sample does not fly off the chuck, the custom-made holder should be used to hold the slide, and not rely simply on vacuum and the existing flat chuck. The details and specifications of this holder are given in **Figure 2**.

1.2.4. Place the spin-coated sample in the oven at the manufacturer recommended temperature (100 °C) for 2 h.

NOTE: The surface of the oven where the sample is placed should be solid (i.e., not a wire rack) and level surface to ensure the uniform heating and thickness of the sample. A ceramic or steel plate makes an ideal surface.

1.3. Top bead layer

1.3.1. Add bead solution in the appropriate ratio to the remaining PDMS mixture.

NOTE: This ratio depends on the concentration of the stock bead solution and the desired bead density on the sample. Typical final values are 9.2×10^{11} beads/mL and 0.05–0.2 beads/μm^2, and an excess of beads is generally preferable to an inadequate amount.

1.3.2. Mix the bead solution with the uncured PDMS. This may be accomplished by placing the tube on a rotator for approximately 30 min, vortexing for 1–2 min, or sonication for 30 min. These methods may be combined.

NOTE: In our application, we find that sonication is effective in breaking bead aggregates, and rotation is effective in mixing. Synthesized fiduciary beads may aggregate in storage. Prior to use, they may be resuspended in hexane and sonicated. If there are significant large aggregates, one can filter the bead suspension through a 5 µm syringe filter. This filtration step is optional; it helps to coat the sample with monodispersed beads, but a significant fraction of beads may be lost in the filter.

1.3.3. Take out the slide from the oven, allow to cool to room temperature, and place it on the spin-coater.

1.3.4. Add 3–4 mL of the bead and uncured PDMS mixture onto the surface of the coated sample.

NOTE: The mixture with beads added is less viscous due to the hexane. Make sure not to touch the surface of the substrate as it may damage the already-coated PDMS substrate. Additionally, the bead mixture may initially not wet the surface; take care that the mixture does not immediately flow off the cured PDMS surface.

1.3.5. Spin the sample with the following protocol.

1.3.5.1. To spread the bead and uncured PDMS mixture, accelerate at 100 rpm/s from 0 to 500 rpm; hold at 500 rpm for 1 min.

1.3.5.2. To achieve a thin layer of bead-embedded PDMS (~1 µm), accelerate at 200 rpm/s from 500 to 5000 rpm; hold at 5000 rpm for 10 s.

1.3.5.3. To remove, decelerate at 100 rpm/s to 0 rpm. Disable vacuum and remove coated slide, taking care not to touch the coated surface.

1.3.6. Place the spin-coated sample in the oven at 100 °C for 1 h.

NOTE: Temperatures above 100 °C or durations longer than 1 h can reduce the bead fluorescence. Make sure that the oven temperature is set to 100 °C and not higher.

1.3.7. The protocol can be paused here. To store the sample, cover the surface to avoid dust and light exposure. Make sure that nothing touches the surface. The sample is shelf-stable at room temperature indefinitely.

1.4. Assembling the plate

1.4.1. Add Part A (base) and Part B (curing agent, e.g., Sylgard) of a PDMS elastomer kit in a 1:1 weight ratio into the 50 mL tube.

1.4.2. Mix the mixture on the rotator for 30–45 min.

NOTE: For one plate, mix 5 mL of base with 0.5 mL of curing agent. Up to 1 mL of hexane can be added to reduce the viscosity of the mixture.

1.4.3. Apply the mixture to the bottom of the divider and spread the mixture.

NOTE: The divider should be placed upside-down.

1.4.4. Lay the substrate onto the divider upside-down.

1.4.5. Place the sample upside-down in the oven at 65 °C for 2 h.

1.4.6. Take out samples from the oven and clean the bottom of the glass with 70% ethanol or isopropanol to remove any PDMS residue.

NOTE: The protocol can be paused here. To store the sample, place a lid on the substrate and wrap the device in aluminum foil to avoid exposure to the light. The sample is shelf-stable at room temperature indefinitely. The divider utilized in this method is in a 96-well format; however, researchers may employ other formats (384-well, 2-well, 4-well, 8-well, etc.) depending on desired experiment setups and availability of dividing structures. Some further optimization may be required.

2. Surface functionalization

2.1. Dissolve 80 µL of a Sulfo-SANPAH aliquot in 40 mL of 0.1 M HEPES buffer (pH 7–9).

NOTE: Prepare Sulfo-SANPAH aliquots by dissolving 100 mg of Sulfo-SANPAH powder in 2 mL of sterile dimethyl sulfoxide (DMSO). Prepare 0.1 M HEPES buffer by diluting 50 mL of HEPES in 450 mL of sterile deionized water and filter through a 0.22 µm pore filter.

2.2. Add 200 µL of diluted Sulfo-SANPAH solution to each well of the 96-well plate.

2.3. Expose the plate to UV (300–460nm) light at appropriate distance and duration.

NOTE: After UV exposure, the color of the solution should be darker. UV exposure distance and duration depend on the UV lamp power. In our application, we expose for 10-15 min at a distance of 5 cm.

2.4. Remove the Sulfo-SANPAH solution from the wells and add 200 µL of 5 µg/mL fibronectin solution to each well.

NOTE: Researcher-specified protein can be used for surface coating. Some commonly used proteins are collagen, fibronectin, and laminin. We have found Sulfo-SANPAH to be the most effective method, and plasma cleaning while sometimes employing in PDMS is discouraged as it creates a silicon dioxide layer and visibly damages the surface.

2.5. Incubate the plate at 4 °C overnight.

NOTE: Different incubation methods can be applied depending on the protein used for coating.

2.6. Remove the fibronectin solution and wash each well with phosphate-buffered saline (PBS0 twice.

2.7. Place a lid on the sample.

2.8. Add 200 µL of PBS to each well.

NOTE: The protocol can be paused here. The fibronectin-coated samples can be stored at 4 °C for up to 2 weeks.

3. UV sterilization

3.1. Sterilize the sample under UV light in a biological safety cabinet for 30 min.

NOTE: Longer UV exposure times may negatively impact the bead fluorescence. All subsequent steps must be performed under sterile conditions.

4. Cell culture

4.1. Remove PBS from each well and add 200 µL of cells suspended in culture media to each well.

4.1.1. Plate the cells at the desired cell density. Cell density depends on the desired experiment. For single cell studies, cells should be minimum of 50 µm apart and cells near the edges of the imaging window should be not be included in the TFM measurement. For monolayer cells, the imaging window should have the viewing field covered with a confluent layer of cells.

4.1.2. Prepare the complete growth culture media for NMuMG cells by supplementing DMEM with 5% FBS, 10 mM HEPES, 10 µg/mL insulin, 1% penicillin-streptomycin, 1 mM L-glutamine, and 0.5 µg/mL amphotericin B.

4.1.3. Prepare insulin stock by reconstituting in acidified water (2.5 mL of glacial acetic acid in 130 mL of deionized water) to a concentration of 10 mg/mL. Store the stock solution at 4 °C. Wait until the solution is clear, and then filter through a 0.22 µm pore filter.

4.2. TGF-β addition

4.2.1. To prepare of TGF-β stock, dissolve 2 µg of TGF-β in 100 µL of 10 mM citric acid (pH 3.0) and filter sterilize with 0.22 µm pores. Vortex the tube and aliquot into the desired volumes. Store the aliquots at -80 °C.

4.2.2. Add 1.5 µL of TGF-β stock solution to 10 mL of the complete cell culture media to constitute the cell culture media with the final TGF-β concentration of 3 ng/mL.

NOTE: To make 10 mM pH 3.0 citric acid, dilute the acid in water and adjust pH to 3.0 by adding HCl.

5. Data acquisition

5.1. For each position, acquire at least one image of fiduciary particles and cells. Focus on the bead layer.

NOTE: Pixel size should be optimized based on size of the fiduciary particles and image processing method being used. In this application, the authors use a 10x 0.4 NA objective, and images are acquired with 1024 x 1024 resolution, with 455 nm/pixel. In general, it is helpful to retain a resolution of at least approximately 1–5 pixels per bead; here, beads are polydisperse and have an individual size of 300–500 nm. It is critical that the fluorescent fiduciary beads be in focus for images to be used for TFM calculations. The focus and imaging quality of the beads should be prioritized over imaging the cells themselves. There should be no cross-talk between different channels, particularly any fluorescence not from the fiduciary beads which appears in the imaging spectra of the beads.

5.2. Once all the positions of interest have been recorded, add detachment solution to each well to acquire force-free reference images of the fiduciary particles.

5.2.1. To prepare cell detachment solution, mix an aqueous solution containing 2% TritonX-100, 50 mM sodium azide, and 500 mM potassium hydroxide.

NOTE: The above is provided as an example of an effective detachment solution. Different detachment solutions at researchers' discretion may be used to detach the cells off the substrate surface.

6. Image analysis

6.1. Perform appropriate image analysis as desired.

NOTE: Analysis software was developed in-house. Image analysis may be done with custom-made software or software available online.

7. Bead synthesis

NOTE: The following protocol is based on the synthesis method described by Klein et al.[10].

7.1. Under a fume hood, prepare the three-neck flask with a water-cooled reflux condenser.

CAUTION: Set up a synthesis in a well-ventilated chemical fume hood.

7.2. Add 0.5 mL of PDMS stabilizer and fluorophore to the flask.

7.3. Equip one neck with a rubber septum with a nitrogen inlet needle and an outlet needle and equip the other neck with a rubber septum for adding reagent with a syringe.

7.4. Add 100 mL of anhydrous hexane in 250 mL to the flask and add a small magnetic stir bar.

7.5. Place the flask in the mineral oil bath at 75 °C and purge it with nitrogen gas for 1 h.

7.6. Add 6 mL of methylmethacrylate to a 25 mL round bottom flask.

7.7. Add 0.100 g of 2,2'-azobisisobutyronitrile (AIBN) to the round bottom flask and purge the mixture with nitrogen gas for 1 h.

NOTE: Flush methylamethacrylate through prepacked column to remove inhibitors before use. Add methylamethacrylate and AIBN mixture to the three-neck flask.

7.8. The solution initially becomes cloudy and turns milky. Let the reaction run for 3 h after the solution becomes cloudy.

7.9. After 3 h, place the flask in an ice water bath.

7.10. Vacuum-filter the solution with coarse filter paper.

7.11. Centrifuge the filtrate and re-suspend the particles in hexane.

NOTE: The volume of the hexane to be added depends on the desired concentration of the bead solution. Sonication facilitates re-dispersion of the bead particles in hexane solution. Beads produced by the authors have polydisperse diameters of approximately 300–500nm. Due to the use of cross-correlation pattern tracking to determine displacements, monodisperse beads are not required.

8. Rheology measurement protocol

NOTE: Rheology is not required for every researcher or experiment, but is necessary to quantify the moduli for new formulations of PDMS. In this protocol, we employ a shear rheometer to measure the effects of crosslinker, frequency, and strain on moduli of PDMS samples. Depending on the available tools and expertise, moduli may also be measured using many other mechanical analysis approaches. Additionally, researchers using this protocol may elect to use our published moduli presented in **Table 1**, **Figure 3** and **Figure 4**.

8.1. Use a rheometer with a 25 mm diameter parallel plate geometry. Other geometries may be used.

8.2. Initialize the system and calibrate the device and measuring system (parallel plate, d = 25 mm). After measuring the zero gap, begin loading the PDMS sample.

8.3. As soon as the PDMS elastomer and crosslinking agent have been mixed, pipet the mixture onto the bottom plate of rheometer.

8.3.1. Move the spindle down to completely contact the top of the PDMS sample.

8.3.2. Carefully trim the loaded sample excess from the bottom plate.

8.4. In a strain sweep test, apply increasing strains for each composition with different crosslinking density to ensure the polymer structure remains in the linear viscoelastic regime during all shear measurements.

NOTE: Strain values relevant for cell studies are typically in the range of 0.1–10%. We have found PDMS to be linear up to approximately 100% strain.

8.5. Measure the dynamic shear storage modulus (G’), and loss modulus (G’’) of the PDMS network in a time sweep test with frequency of 1 Hz and oscillatory shear strain of 0.5% at 100 °C.

8.6. To determine the viscoelasticity and time dependency of the final PDMS network, apply a frequency sweep test with frequency ranging 0.1–100 Hz and oscillatory shear strain of 0.5%.

REPRESENTATIVE RESULTS:

Before addition of TGF-β, a confluent monolayer of cells has a cobblestone like shape and is tightly packed. Upon TGF-β treatment, cells become more elongated in morphology, enlarging the cell area and acquiring a more mesenchymal phenotype. Utilizing the multi-well device fabricated with soft PDMS elastomers, the physical properties of cells in a total 17 different conditions were studied. The cells were treated with four different TGF-β concentrations (0.5, 1, 2, and 4 ng/mL) and four different incubation times (12, 24, 48, 96 h), and these results are summarized in **Figure 5**. The cells treated with TGF-β applied larger traction stresses and strain energies than the cells cultured without TGF-β. Cells incubated with TGF-β for 96 h showed the largest traction stresses and strain energies. The cells applied larger stresses and strain energies when treated with higher concentration of TGF-β. The difference in tractions and strain energies were more distinct at longer incubation times.

The surface of the substrate needs to be smooth and uniformly coated with ligands, such as fibronectin or collagen. Scratched surface and/or non-uniform coating of ligands may lead to improper cell attachment, resulting in inaccurate traction measurement. **Figure 6** shows the localized traction stresses due to the non-uniform substrate surface.

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of multi-well plate fabrication. (**A**) A custom-cut glass slide is the starting point. (**B**) The glass slide is coated with a thick (~100 µm layer of PDMS). (**C**) A layer of fiduciary beads (shown in green) are then spin-coated in a ~1 µm thick layer on top of the previous layer. (**D**) The multi-well divider is carefully placed on top of the fiduciary bead layer. (**E**) The complete multi-well plate is assembled and ready for use or storage.

Figure 2: Custom chuck for spin-coater. To prevent the bottom glass (where PDMS is coated) from flying off of the standard spin-coater chuck, a custom-made holder is placed onto the chuck. (**A**) A custom-made holder for the spin-coater chuck. (**B**) Engineering drawing of the holder with all the dimensions.

Figure 3: Substrate stiffness with changing crosslinker concentration (wt%). PDMS mixtures are prepared as described, with the additional crosslinker percentage increasing the elastic modulus until a max of 100 kPa at 1.8 weight percent (wt%) crosslinker. As a guide, the modulus as a function of crosslinker is fit linearly, which can be used by researchers to formulate their own mixture at a particular desired modulus within this range.

Figure 4: Rheology of PDMS elastomer containing 0, 0.15, 0.36 and 1 wt% of crosslink agents at a temperature of 100 °C. Triangle symbols indicate loss modulus (G'') and square symbols indicate storage modulus (G'). (**A**) Oscillatory time sweep at frequency of 1 Hz and shear strain of

0.5% during gelation. (**B**) Oscillatory frequency sweep of PDMS network at shear strain of 0.5%. (**C**) Strain sweep of PDMS network at frequency of 1 Hz. All data points were acquired in triplicate.

Figure 5: Representative results utilizing traction stress device with multiwell format. Monolayer of cells in different conditions were cultured in a multi-well device to measure contractility and strain energy. (**A**) Graph of traction stresses with increasing TGF-β incubation time for different TGF-β concentrations. Traction stresses increased with increasing TGF-β concentrations and incubation time. All data are statistically significant with respect to control (i.e., no TGF-β) except the following: 12 h - 0.5 ng, 12 h - 1 ng, 48 h - 1 ng. (**B**) Graph of strain energy with increasing TGF-β incubation time for different TGF-β concentrations. Strain energies increased with increasing TGF-β concentrations and incubation time. Sample sizes range from n = 7 to n = 15. All data are statistically significant with respect to control (i.e., no TGF- β) except the following: 12 h - 0.5 ng, 12 h – 1 ng, 24 h - 0.5 ng, 24 h – 1 ng, 48 h – 1 ng. Statistical significance was determined using the Kruskal Wallis test, which does not assume a normal distribution.

Figure 6: Representative results from a sub-optimal (A, B) and a satisfactory (C, D) experiment. (**A**) Reflection image of the substrate surface. Undesired contaminants, which can include dust or fibers, or material defects such as scratches are present on the substrate surface. (**B**) Stress map of cell contractility: due to the non-uniform surface, irregular and inaccurate traction stresses are observed around the objects. (**C**) Reflection image of the substrate surface. No apparent contaminants are visible. (**D**) Stress map of cell contractility: no artifact discontinuities are visible.

Table 1: Substrate stiffness with changing crosslinker concentration (wt%). By changing additional crosslinker concentrations, PDMS substrates of the desired Young's moduli are fabricated. PDMS shear moduli were measured with a rheometer and Young's moduli were determined. For each data point, 3 independent preparations were done and the standard deviation is given.

DISCUSSION:

For the success of this method, it is critical to have a uniformly coated sample with a constant thickness of approximately 100 µm. The modulus should be carefully chosen to examine the physical significance of the biological system of interest. When fabricating a top layer, the concentration of the fiducial fluorescent particles should be optimized for accurate analysis of displacement and traction stress. Analyzing isolated single cells requires a denser fiduciary layer than measuring confluent monolayers. Additionally, the surface of the substrate should have stable and uniform coating of adhesion molecules such as collagen, fibronectin, and laminin to ensure proper attachment of the cells to the substrate surface. Particular care must be taken when attaching the multiwell divider; it should be placed without sliding to prevent sealing PDMS glue from being smeared on the culture surface, and the outer edge must be carefully aligned with the glass dimensions to prevent any leaks on the border wells.

To prevent wells from being leaky, an adequate amount of PDMS glue needs to be applied to the well-divider to hold it in place. However, use of excessive amounts will cover cell culture surface. Sufficient volume of PDMS needs to be added during the spin-coating procedure. When curing, the oven and racks need to be level. Inadequate PDMS or an unleveled oven may lead to uneven PDMS thickness.

Bead density needs to be optimized for proper analysis. When the bead density is not adequate, concentration of the beads in fiduciary PDMS mixture needs to be increased. Additionally, an adequate amount of the mixture needs to be added for spin coating. To ensure proper fluorescent signals of the fiduciary bead particles, curing condition should be carefully monitored. Longer times and higher temperatures than the ones specified in this protocol may degrade the fluorescence. Improper protein coating on the surface of the sample may lead to poor cell adhesion. To prevent this, the surface needs to be cleaned and the expiration of reagents such as Sulfo-SANPAH and ligands needs to be checked. UV exposure time and strength should be optimized. Immunofluorescence or other fluorescent protein construct may be tested to ensure uniform ligand coating.

The device fabricated with this method and set of PDMS formulations is only applicable to substrates with Young's moduli ranging from 0.4 kPa to 100 kPa. Other moduli may be possible using alternative formulations, however, the current range spans a large set of physiologically relevant values. While this method is specifically for mutli-well fabrication, it may also be used to prepare individual slides or other slide geometries, and in these instances further user troubleshooting may be necessary. In this embodiment, a relatively thick (1 mm) glass slide is employed, precluding common high numerical aperture (NA) objectives on an inverted microscope. While it is technically feasible to construct these multi-well TFM dishes using thinner glass, the fragility of these in processing and assembly may result in a high rate of breakage, and can run contrary to the higher-throughput approach. Furthermore, surface coating may be investigated further for wider applications of the device.

Utilizing a multiwell format, high-throughput experiments are possible. Multiwell format enabled us to examine the physical changes of NMuMG cells during EMT with TGF-β treatment with combinations of different concentrations and different incubation times in a single dish. Fabrication of traction stress devices with hydrogels such as polyacrylamide gel have several limitations. Dominant ingredient being water, hydrogels are sensitive to salt concentrations (osmolarity), temperature, humidity, and pH changes. Changes in any of these properties can lead to the change in mechanical properties of the material, requiring extra care in using samples and interpreting results. Furthermore, having water, hydrogels are more susceptible to infections, requiring extra cautions. In contrast to water-based hydrogels, silicone-based PDMS is stable and inert. Once fabricated, it can be stored at room temperature indefinitely without requiring any special handling and storing condition.

The impermeable nature of PDMS allows us to fabricate a monolithic substrate, ensuring that all wells are truly identical in substrate thickness and composition, while allowing unique biochemistry to be applied in the media, or different cells to be utilized in each well. A hydrogel-

based surface allows diffusive factors to permeate and travel through it, necessitating adding the hydrogel to wells that are otherwise partitioned to prevent cross-contamination.

This method allows researchers to study cell contractility, a basic and ubiquitous aspect of cell biology, in a high-throughput manner, enabling more efficient and reproducible data acquisition. This helps to translate Traction Force Microscopy into Contractile Force Screening, allowing researchers to truly use contractility as a metric for cell activity, or efficacy of a compound. As a methodology, this has broad applications across biophysical and biomedical sciences, from understanding the physics of cells, to characterizing and testing pharmaceutical compounds against standardized cell types, or perhaps highly specialized individual cells for personalized medicine.

ACKNOWLEDGMENTS:

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DISCLOSURES:

AJE and RK have interest in Live Cell Technologies, a company which fabricates materials described in this article.

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Figure 1

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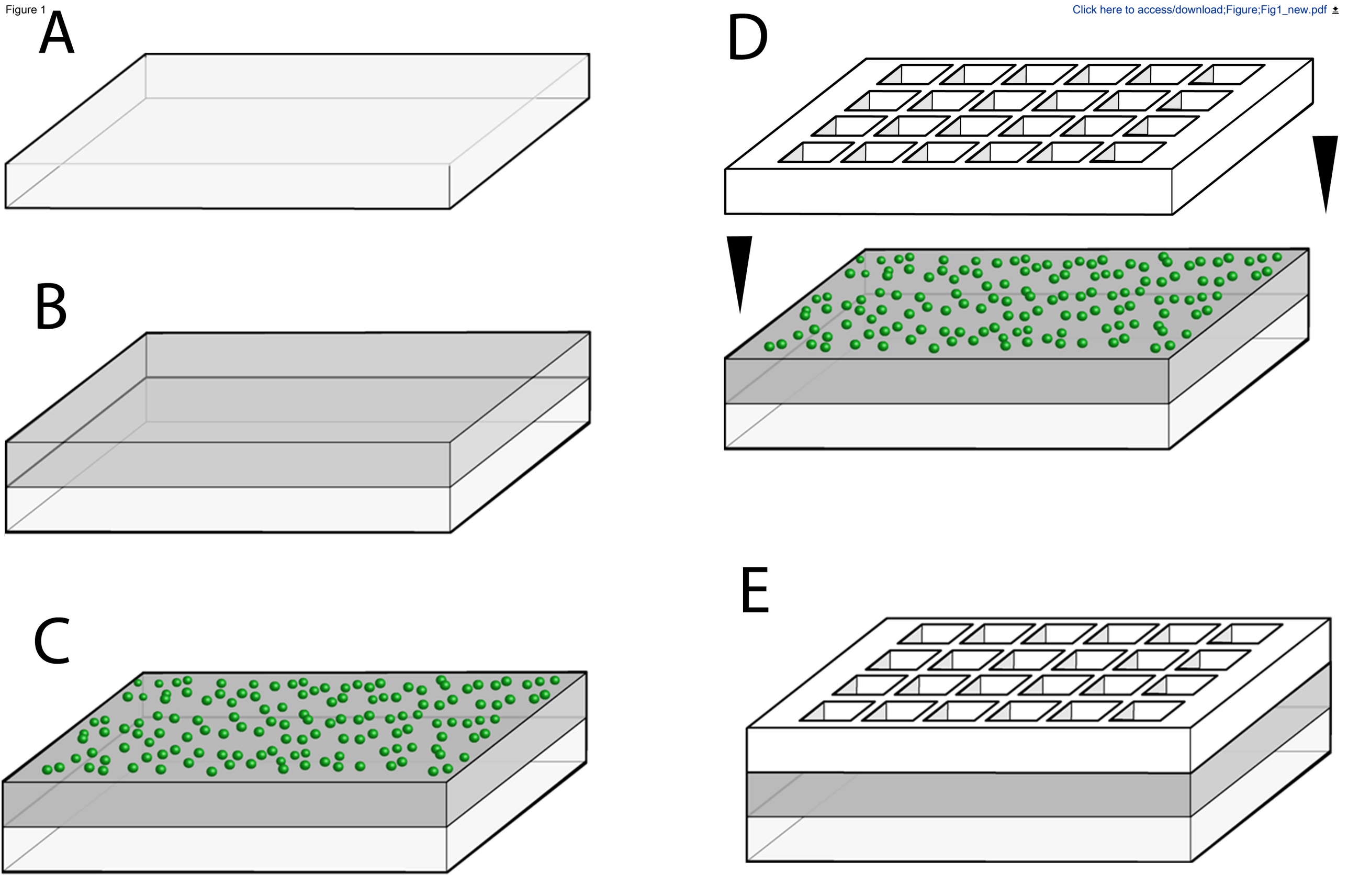

Figure 2

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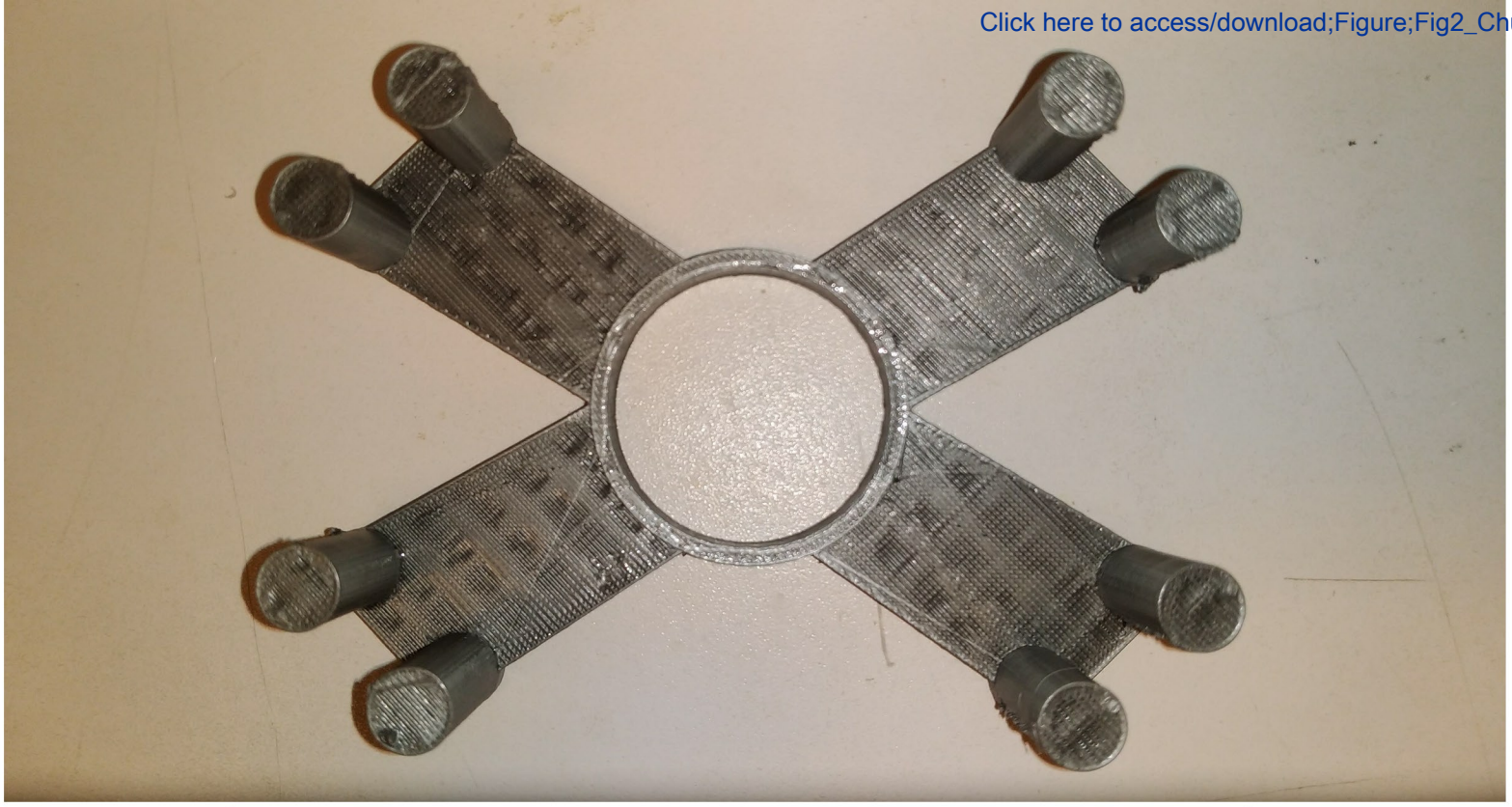
A



2 1

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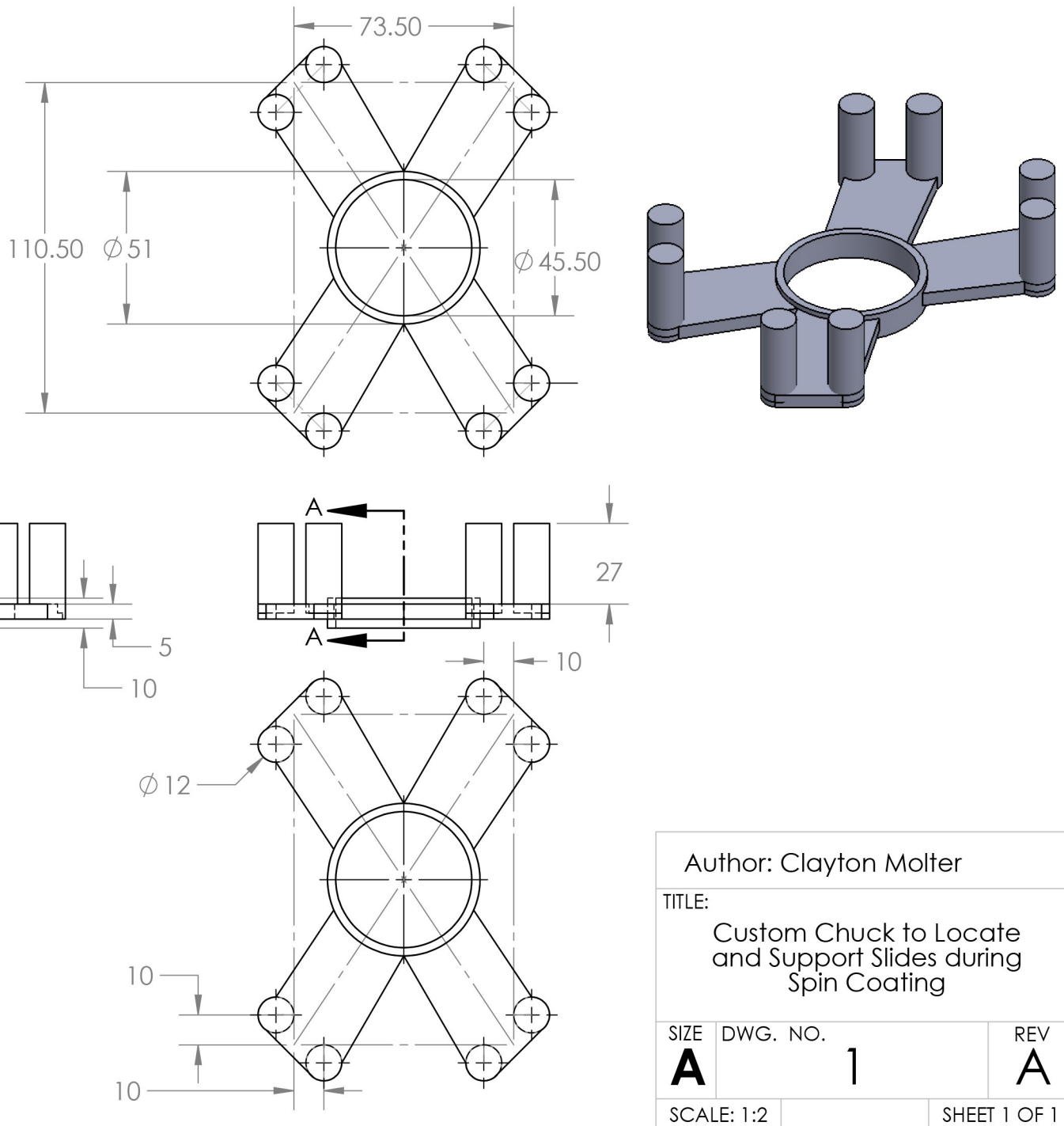

All dimensions are in millimeters (mm)

Author: Clayton Molter		
TITLE: Custom Chuck to Locate and Support Slides during Spin Coating		
SIZE A	DWG. NO. 1	REV A
SCALE: 1:2		SHEET 1 OF 1

Figure 3

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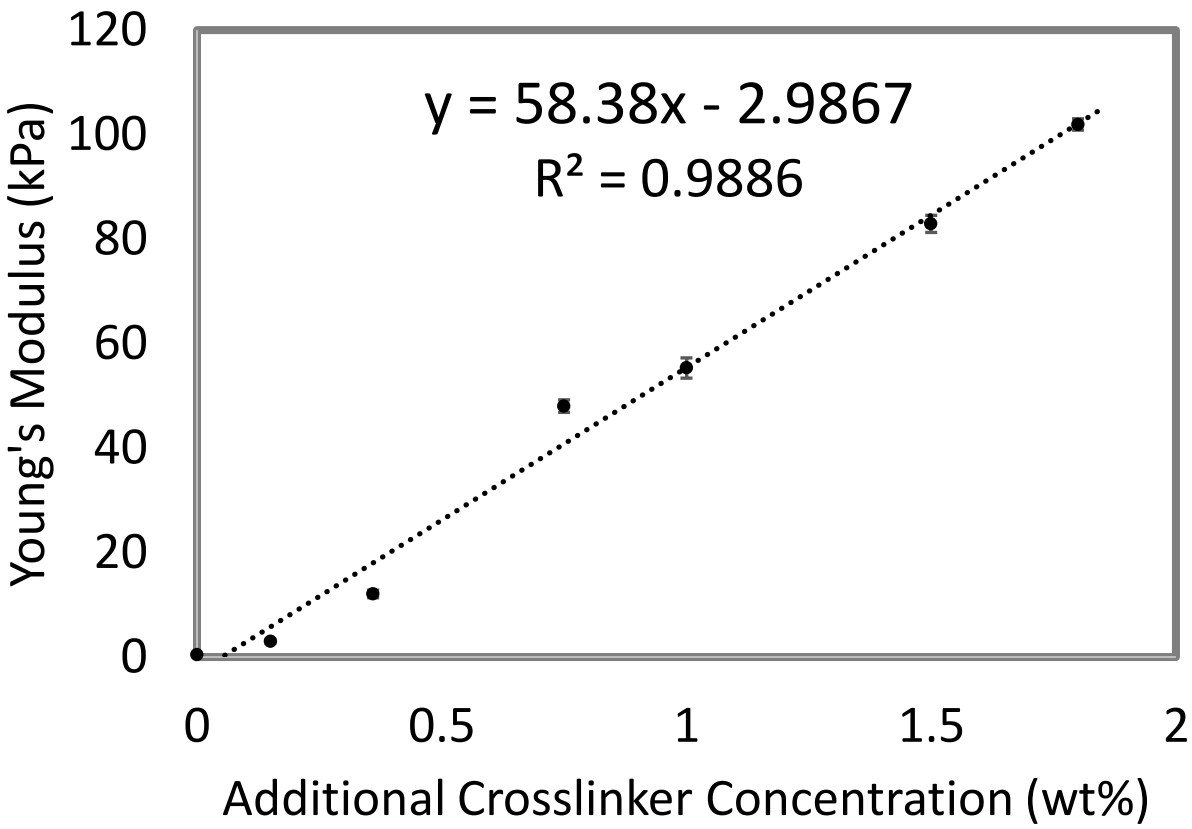

Figure 4

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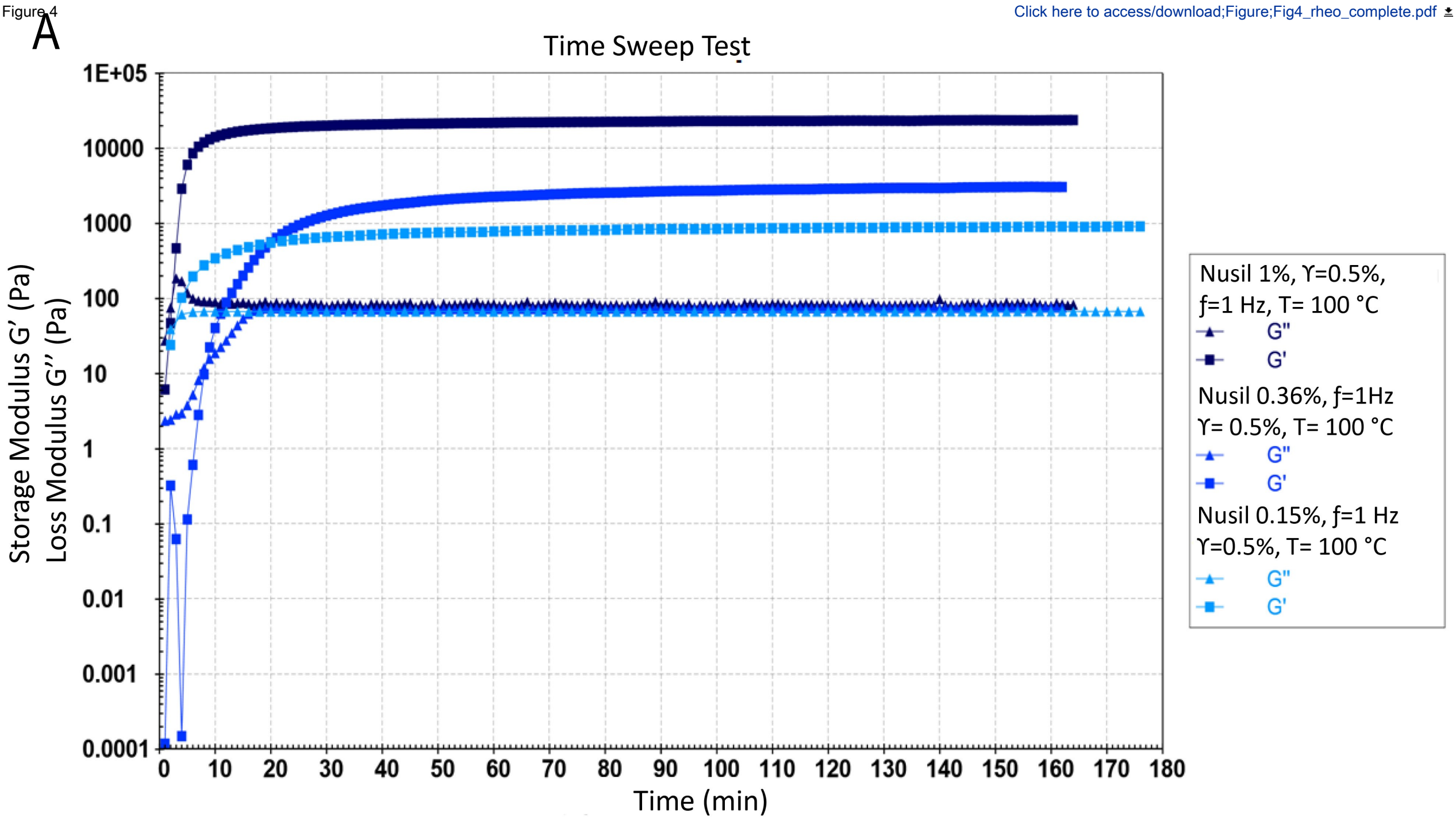

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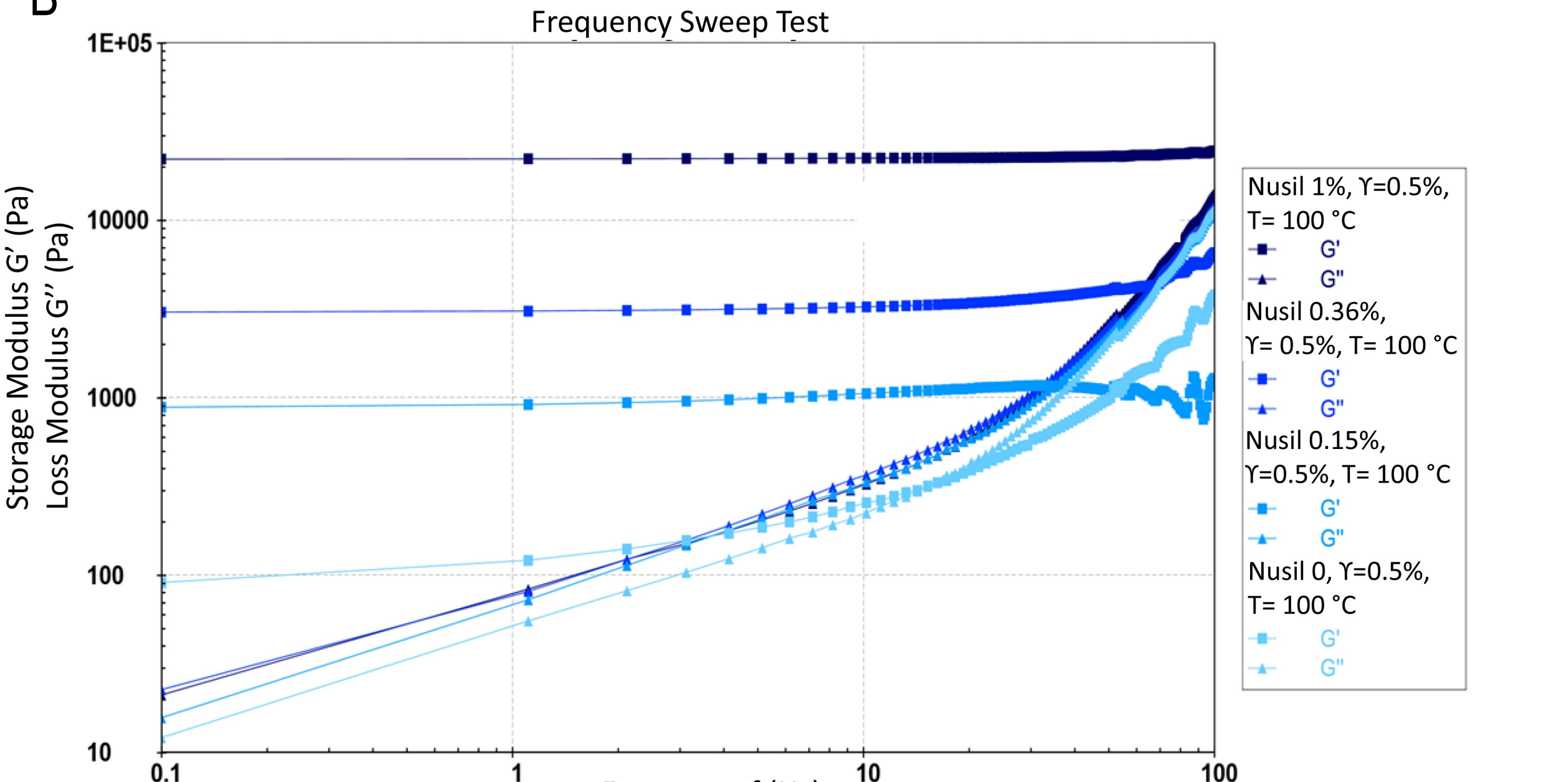

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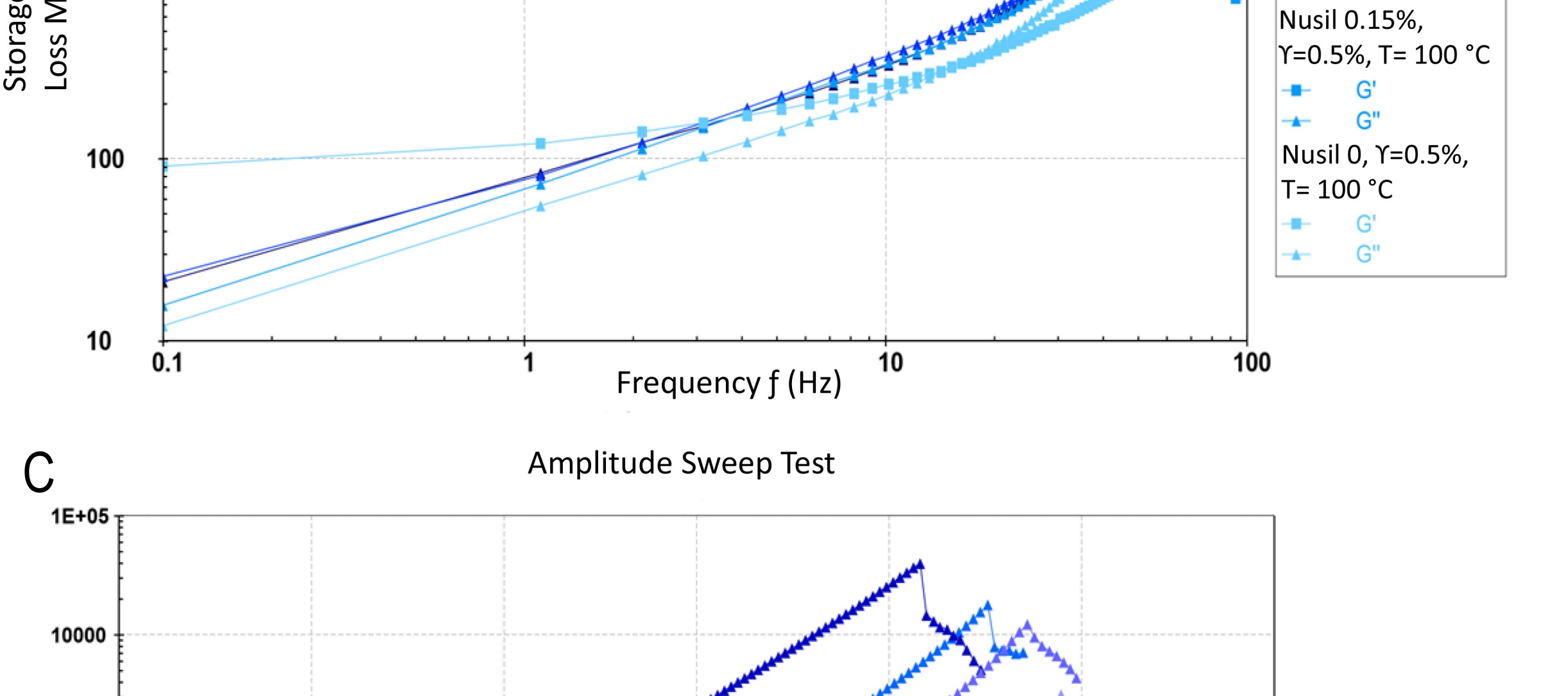

Figure 5

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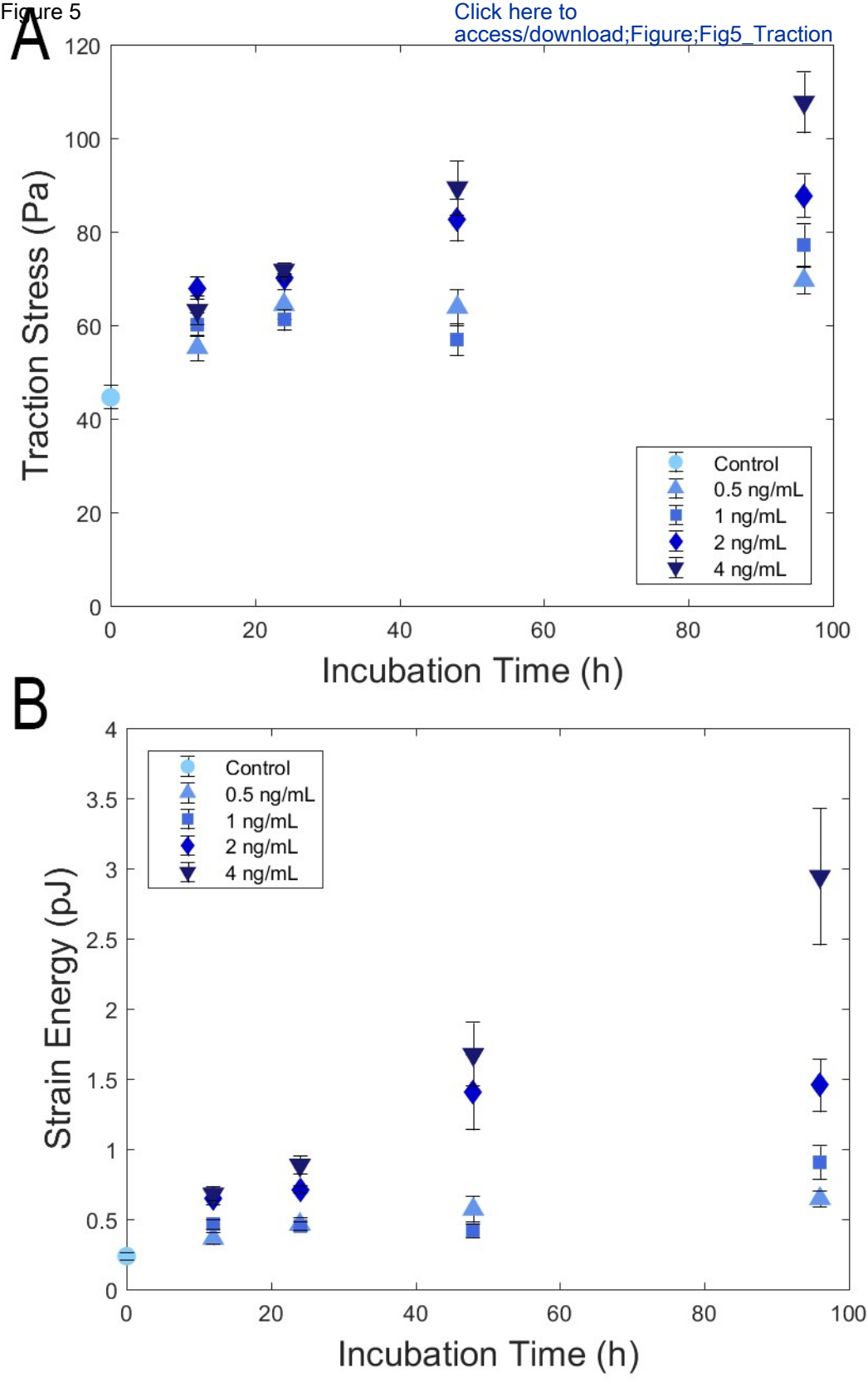

Figure 6 Revised

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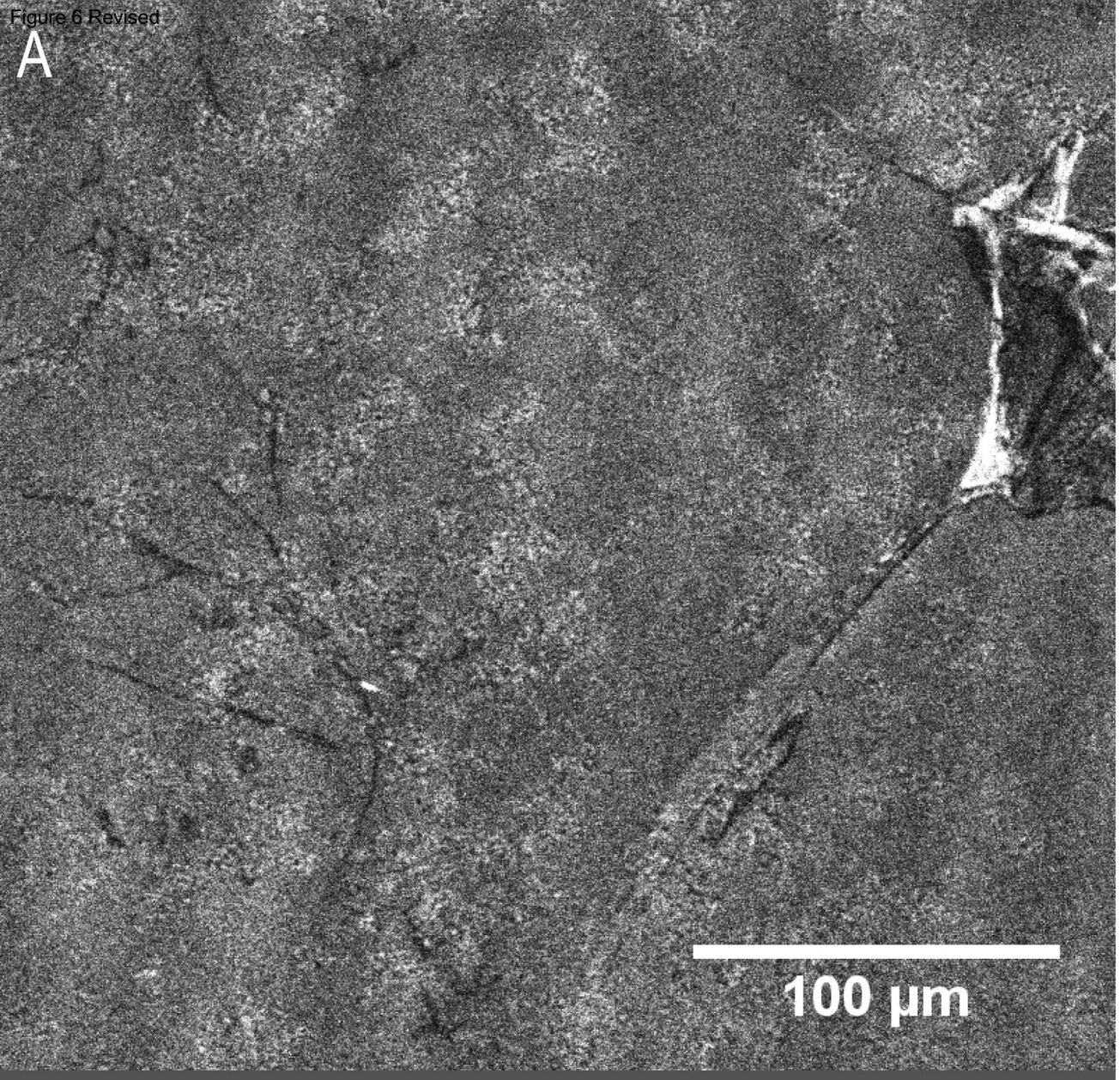

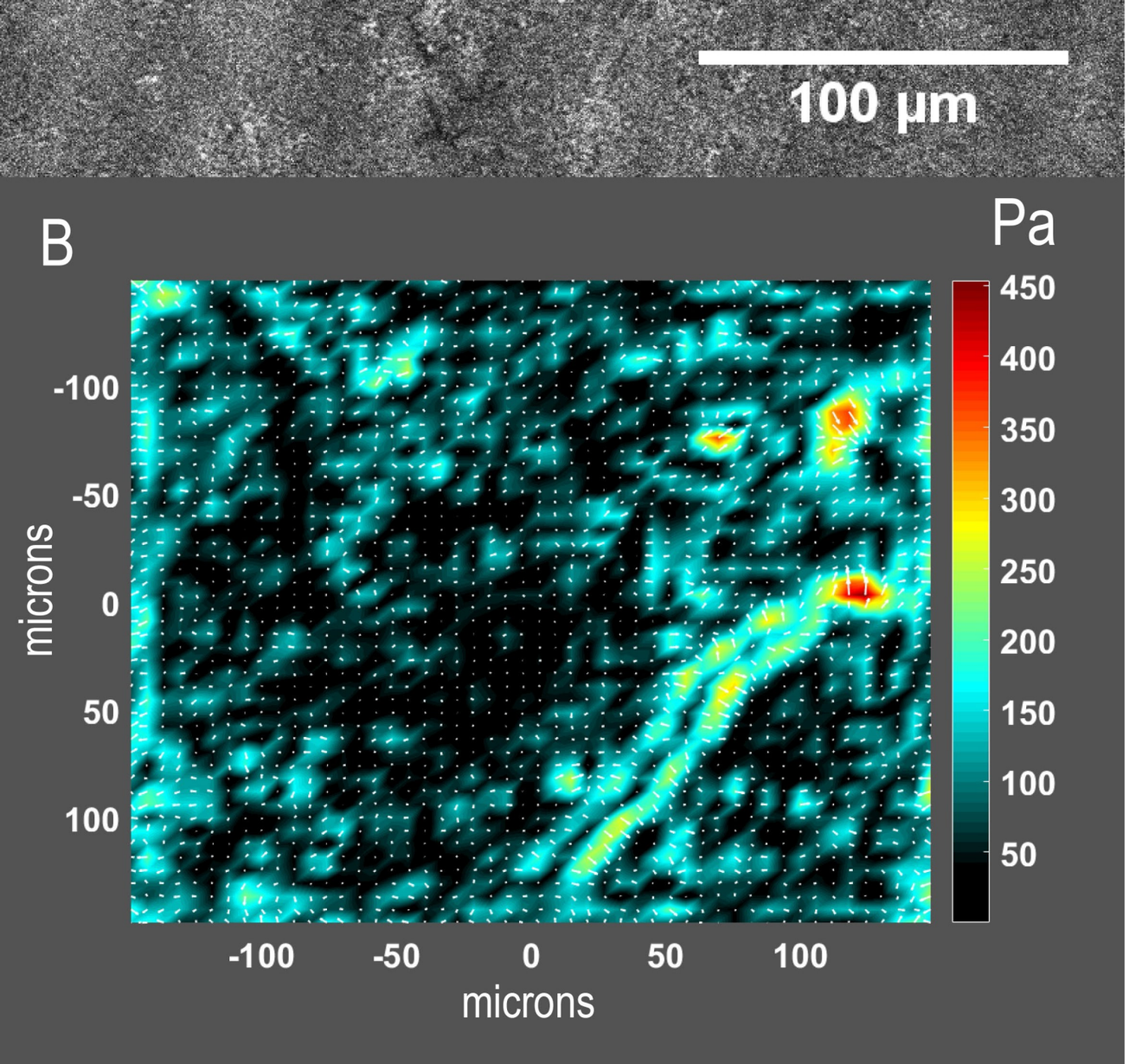

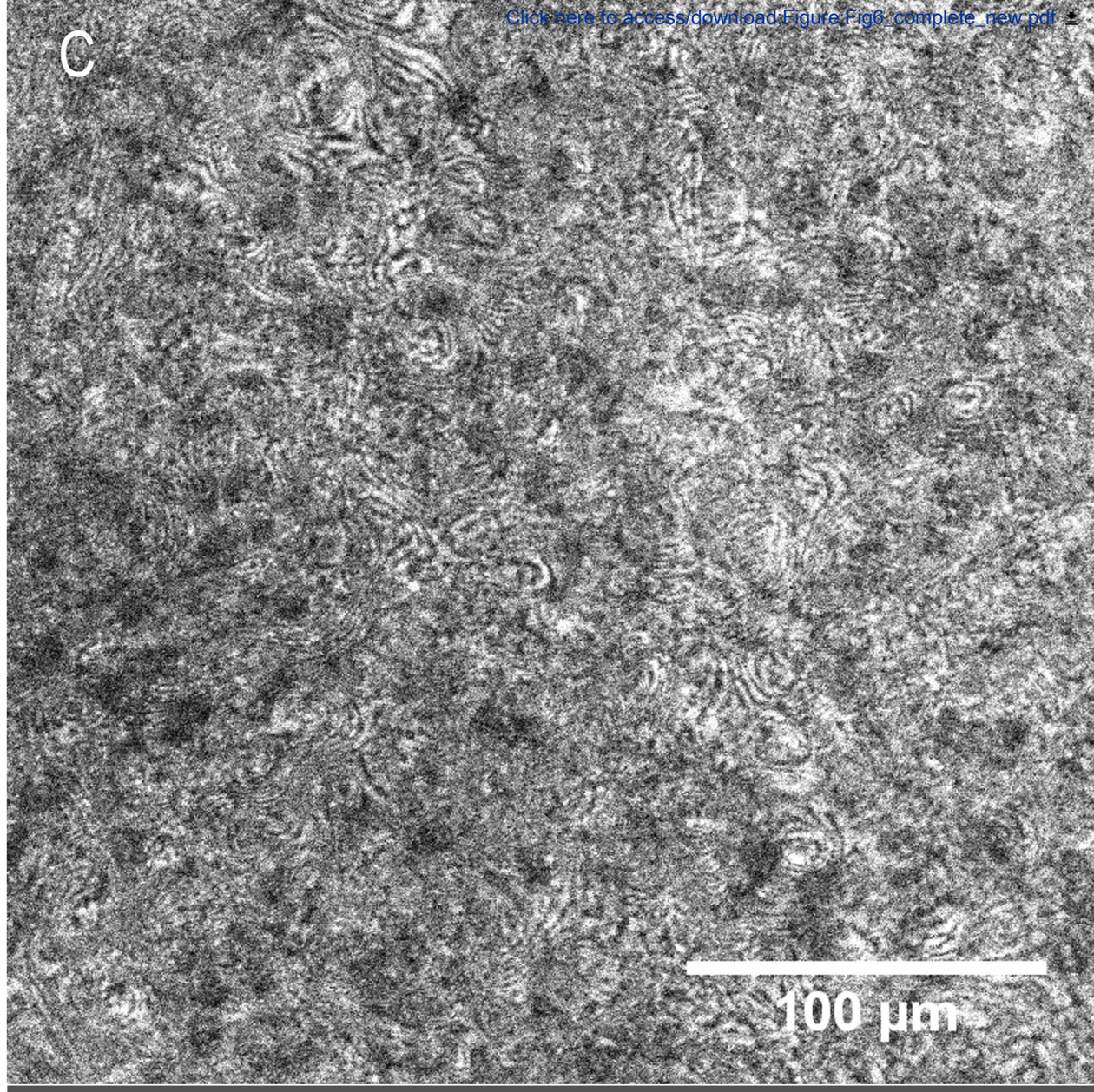

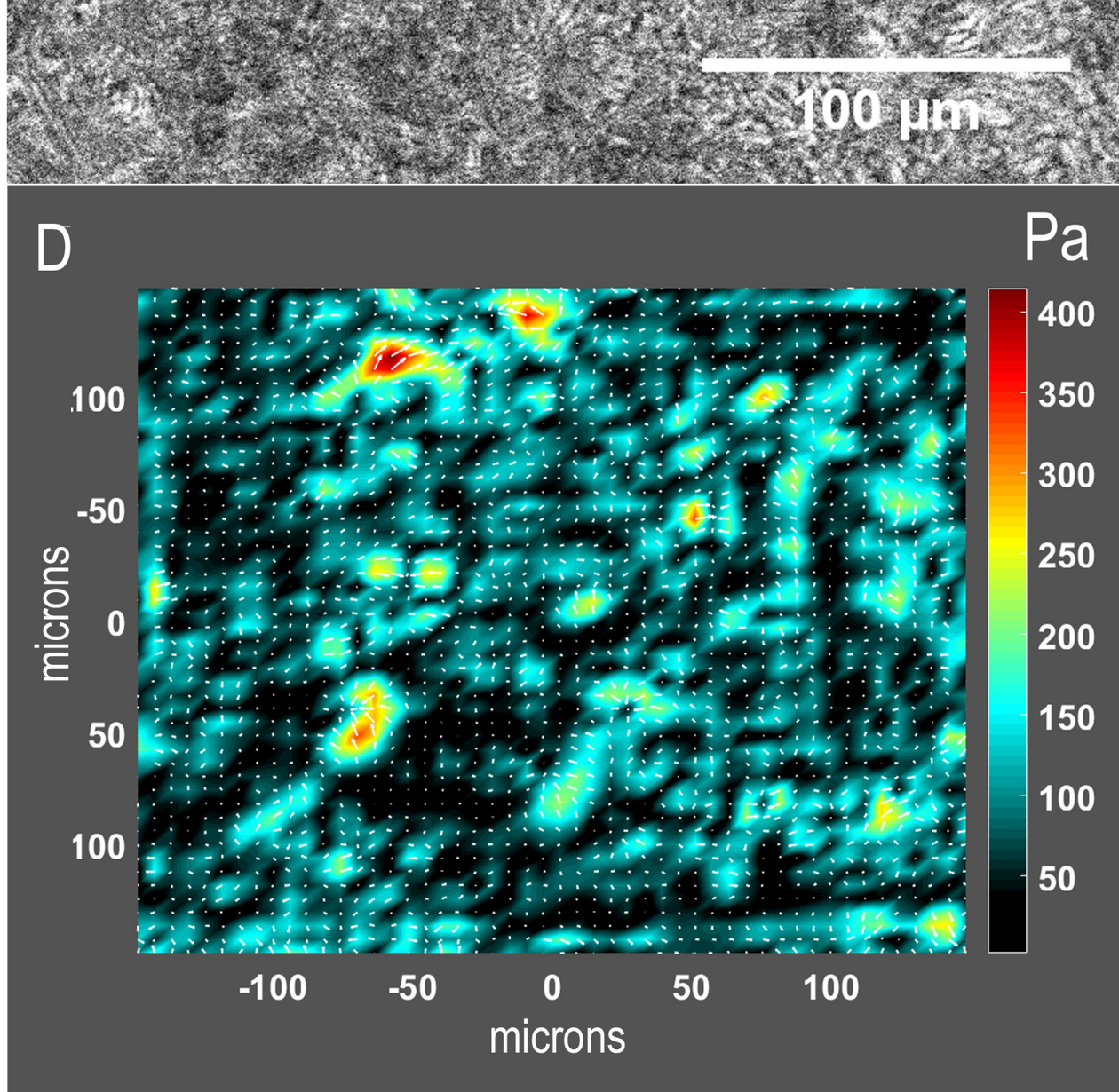

Table 1

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Crosslinker concentration (wt%)	G' (Pa)	Standard Deviation	E (Pa)	Standard Deviation
0	0.135	0.014	0.405	0.043
0.15	1	0.1	3	0.35
0.36	4.027	0.245	12.081	0.73
0.75	16.01	0.49	48.03	1.2
1	18.44	0.989	55.32	1.94
1.5	27.638	0.93	82.91	1.64
1.8	33.986	0.88	101.94	1.088
2	33.36	0.67	100.08	1.1

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
Plate		
GEL-8100	Nusil Technology	GEL-8100
Dow Corning Sylgard 184 Silicone Encapsulant Clear 0.5 kg Kit	Ellsworth Adhesives	184 SIL ELAST KIT 0.5KG
Custom Cut Glass	Hausser Scientific Company	
Target 2TM Nylon Syringe Filter	ThermoFisher Scientific	F2513-4
96-well Stripwell Egg Crate Strip Holder	Corning	2572
Polystyrene Universal Microplate Lid With Corner Notch	Corning	3099
Ethyl alcohol	Greenfield Global	P016EA95
2-Propanol	Sigma-Aldrich	190764
Surface Coating		
Sulfo-SANPAH Crosslinker	Proteochem	c1111-100mg
Fibronectin bovine plasma	Sigma-Aldrich	F1141-1MG
PBS, 1X	Wisent	319-005-CL
DMSO	Sigma-Aldrich	472301
Cell Culture		
DMEM, 1X	Wisent	319-005-CL
FBS (Fetal Bovine Serum)	Wisent	080-150
HEPES	Wisent	330-050-EL
Human Insulin Recombinant	Wisent	511-016-CM
Penicillin-Streptomycin Solution	Wisent	450-201-EL
L-Glutamine solution	Wisent	609-065-EL

Amphotericine B	Wisent	450-105-QL
Recombinant Human TGF-β1	Peprotech	100-21
Acetic acid	Sigma-Aldrich	537020
Cictric acid	Sigma-Aldrich	251275
NMuMG	ATCC	CRL-1636
Sodium azide	Fisher Schientific	AC190385000
Potassium hydroxide	Sigma-Aldrich	221473
TritonX-100	Sigma-Aldrich	X100
Bead Synthesis		
1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)	Sigma-Aldrich	468495-100MG
Methyl methacrylate	Sigma-Aldrich	M55909-500ML
Inhibitor Remover	Sigma-Aldrich	306312-1EA
Methacryloxylpropyl Terminated Polydimethylsiloxane	Gelest	DMS-R31 (25,000g/mol)
2,2'-Azobis(2-methylpropionitrile) (AIBN)	Sigma-Aldrich	441090-25G
Hexane	Sigma-Aldrich	296090-2L
Hexane, mixture of isomers	Sigma-Aldrich	227064-1L
Whatman qualitative filter paper, Grade 1	Sigma-Aldrich	WHA1001055
Equipment		
Laurell WS-650Mz-23NPPB	Laurell Technologies	
UVP Handheld UV Lamp Model UVGL-58	VWR	21474-622
Rheometer	Anton Paar	MCR 302 WESP

Comments/Description
High Purity Dielectric, Soft Silicone Gel kit
curing agent
109.6mm± x 72.8mm± x 1mm thickness
0.95 ACS reagent, ≥99.5%
solution, sterile-filtered, BioReagent, suitable for cell culture
pH 7.4, without calcium and magnesium
4.5g/L glucose, with L-glutamine, sodium pyruvate and phenol red Premium Quality, Endotoxin <1, Hemoglobin <25 1M, free acid USP grade 100 X, sterile filtered for cell culture 200mM solution, sterile filtered for cell culture

250µg/ml, sterile filtered for cell culture

HEK293 Derived
Glacial, ≥99.85%
ACS reagent, ≥99.5%
Mouse Mammary Gland Cell Line
99%, extra pure, ACROS Organics
ACS reagent, ≥85%, pellets
laboratory grade

97%

contains ≤30 ppm MEHQ as inhibitor, 99%
Prepacked column for removing
hydroquinone and monomethyl ether
hydroquinone
Polydimethylsiloxane stabilizer,
25,000g/mol, 1,000 cSt

98%

anhydrous, 95%
anhydrous, ≥99%

circles, diam. 55 mm,

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Author(s): Haruka Yoshie, Newsha Koushki, Clayton Molter, Peter M. Siegel, Ramaswamy Krishnan, Allen J. Ehrlicher

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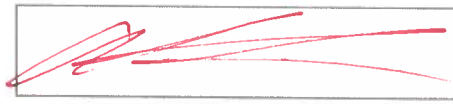
Institution:	McGill University
Article Title:	High Throughput Traction Force Microscopy using PDMS Reveals Dose-Dependent Effects of Transforming Growth Factor-β on the Epithelial-to-Mesenchymal Transition
Signature:	
Date:	Nov 5 2018

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Rebuttal letter.
Page 1 addresses editorial comments, and pages 2 & 3 address reviewer comments.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We have checked the manuscript again and not found any spelling or grammatical errors.

• **Protocol Detail:** Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. Some examples of missing items are listed below. Please double check all your protocol steps and ensure that the manuscript matches the video.

We believe that button clicks and software actions are not constructive for the reader, as these vary substantially between versions and systems, whereas the physical aspects do not. We have added details to acquisition specifics that should allow methodology, platform, and system independence.

1) 5.1: how is the image acquired? Mention magnification, type of microscopy and any relevant settings.

The method of image acquisition is not key, simply that it is fluorescence microscopy, of which confocal microscopy is employed and subset thereof. We have added this in the protocol, and provided details about minimum resolution required.

2) Please ensure that all specific details from the video are mentioned in the manuscript (the manuscript should be able to serve as a standalone article). Examples of missing items: 05:44-06:09, 06:18-07:20, 07:32-07:45, etc.

We have corrected these in the manuscript.

3) Please be consistent with naming between the manuscript text and video (For eg, in section 1.2 you use “silicone” in the manuscript and PDMS in the video.

Silicone is more general term for PDMS, but we have now specified PDMS throughout.

4) 1.3.2: details provided here don’t match the video.

We have corrected these in the manuscript.

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Figure 1 is similar to that published in our previous manuscript (Yoshie et al Biophysical Journal 2018), however, it was newly created by the authors for this JoVE manuscript, and thus is not a reproduction, or modification of a reproduction.

• **Video Comment: at 13:31** - Please remove the 'www.jove.com' from the bottom of the title card, since the video wasn't produced by JoVE.

We have removed this.

Comments from Peer-Reviewers:

Reviewer #1:
The authors present a high throughput traction force assay fabricated with silicone rubber. In the last two decades, the key role of cell mechanics in health and disease (e.g. cancer) has spurred the development of several tools to measure forces at single cell level and at optical resolution. However, such tools generally lack any true high throughput capability and have been mainly used by specialist biophysics lab rather than biology/pharmacology groups. The present contractile force screening assay highly contributes to overcoming these limitations and its power is exemplified by quantifying changes in the mechanical response of epithelial cells after induction of EMT through TGF-β.

We thank the reviewer for their positive appraisal.

The authors are encouraged to address the following points:
*Line 57: "Unlike pillar-PDMS based approaches that have a modulus in the GPa range". It is not clear to which approaches the authors refer to. The common PDMS micropillar/micropost arrays are prepared using 10:1 base:curing agent ratio (e.g. Sylgard 184) that confers a modulus in the MPa range for the bulk array. By properly tuning the geometry (pillar length and diameter), the effective stiffness that cells sense on top of the pillars is in the 10-150 kPa range (e.g. as described in Ghibaudo et al. Soft Matter 2008 - Ladoux group). Therefore the authors are encouraged to clarify their sentence.

We regret the typo of GPa, which should indeed read as MPa; this has been corrected, and we thank the reviewer for pointing this out. We are indeed referring to approaches such as that referenced by the reviewer, where the pillar geometry results in a given stiffness, from a MPa modulus. As any technique has its merits and drawbacks, and the discontinuous nature of pillar-based TFM has both, we are simply differentiating our approach from those to prevent confusion regarding the use of PDMS in TFM, as virtually all PDMS applications in TFM employ Sylgard 184.

**While it is clear from the video, the protocol reads confusing on which PDMS kit is being used. Readers that are mainly familiar with the Sylgard 184 kit (base and curing agent) might get confused when reading Part A and Part B (of GEL-8100?) followed by the addition of curing agent (from Sylgard 184?).*

We agree with the reviewer and had omitted the specifications based on our understanding of JoVE's request to leave out product names, however, these are specified in the list of materials. Nevertheless, to prevent confusion, we have specified these names in the protocol, and that the general approach is a combination of two commercially available kits.

**The glass coverslip is 1 mm thick. This prevents the use of high NA oil immersion objectives for high resolution imaging. The achievable resolution is clearly enough to calculate the force fields, whereas insufficient to resolve subcellular structures such as focal adhesions. In the "Limitations of the method" section, the authors should address whether it is possible to use thinner coverslips (#0, 1, 1.5).*

We have added a comment in the text. While it is not the goal of this protocol to resolve subcellular structures, and so it is somewhat beyond the scope to address this, we have made multi-well plates with coverslips, as it is technically possible, but we do not pursue it as it is problematic in this approach for several reasons, and thus not recommended for a high-throughput general contractility assay for which this is designed. First, the general fragility of coverslips, particularly in a large format, results in a large percent of breakage in fabrication. Second, immersion objectives require immersion fluid, and using this over a large area again is problematic. TFM with PDMS on coverslips is performed by the authors and others, but not employing this current multiwell high-throughput methodology for the above reasons.

**Statistical tests of data in Figure 5 are necessary to support the significant change in the mechanical response as claimed in the manuscript.*

We have quantified statistical significance using the Kruskal Wallis test, which does not assume a normal distribution, and have added this information in Figure 5.

**Figure 6 should contain also a representative image of an optimal experiment.*
We have added this as Figure 6C&D.

**To achieve surface coating, would it be possible to use UV/Ozone treatment instead of Sulfo-SANPAH? Please address this point in the manuscript.*

While we believe that there are likely many methods to functionalize the surface, in this protocol we report sulfo-Sanpah as it has been the most reliable method. Plasma cleaning is discouraged as it creates a silicon dioxide layer and visibly damages the surface. We have added a note to this effect in the text.

Reviewer #2:

Manuscript Summary:

The protocol is very clear and easy to follow with the video and the manuscript. Overall, I think it is very useful protocol to perform high-throughput TFM. I like the strategy presented for making the plates; however, it's unclear how traction stress measurements will be affected by the multi-well plate format. It would be helpful to include information about data acquisition, including limitations in resolution and drift correction. It's unclear how accurate the measurements are, directly comparing single well traction to multi-well plate format might clear this up.

We thank the reviewer for their comments.

Major Concerns:

The paper doesn't include some essential information. Questions below address these points.

1. What is the size range for the synthesized beads that are being used?

2. What is the objective magnification? If air vs oil/water objective will provide any distinct advantage?

3. What is the UV exposure time used for the reported experiment to activate sulfo-sanpah?

4. Table 1 is missing from the document.

5. Will the thickness of the glass plate interfere with image resolution at higher magnifications. Discussion of image resolution seems important.

6. Is the final PDMS coated plate able to be autoclaved? Sterility would be a concern unless the whole process is performed in a sterile environment.

7. Data analysis would be problematic if there is high amount of stage drift between initial acquisition and cell detachment, as each point would have to be corrected for. However, with adequate software this drift should be able to be corrected post acquisition. Some discussion of difficulties in multiwell plate analysis would be helpful.

Author replies:

1. Beads are polydisperse and approximately 300nm- this has been added to the manuscript
2. A 10x air objective is used, this has been added to the manuscript. Most immersion objectives are not suitable due to a) the small working distance offered, requiring the substrate and plate to be thinner increasing fragility and bending, b) the necessity of immersion fluid being applied over a large surface causes problems with dripping. Employing immersion optics and higher magnification could provide up to approximately 3-fold improvements in resolution, but would not result in qualitative changes, and is better suited for lower throughput sub-cellular studies.
3. We have added a note in the text- in our application we have used 10-15 min at a distance of 5 cm. These values may vary depending on effective illumination power.
4. We regret the omission. Thank you- Table 1 has been added.
5. The thickness of the glass does not interfere with 10x objective usage. We have added notes in the manuscript on image resolution and size.
6. The PDMS may be autoclaved, however, this damages the fluorescence of the beads, and catastrophically damages the well-divider employed here. The plate is recommended to be sterilized under biosafety cabinet UV light as described in Section 3.1
7. Dedrift software accommodates for this, provided that the drift distance is not overly large; in our usage, 90 microns seems to be a practical limit. This limit may be increased by imaging a larger field of view, and increasing the search window size.