Journal of Visualized Experiments

Patterning the geometry of human embryonic stem cell colonies on compliant substrates to control tissue-level mechanics
--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60334R1
Full Title:	Patterning the geometry of human embryonic stem cell colonies on compliant substrates to control tissue-level mechanics
Keywords:	Human embryonic stem cells (hESCs); polyacrylamide hydrogels; traction force microscopy (TFM); Photolithography; cell patterning; extracellular matrix; tissue engineering; regenerative medicine
Corresponding Author:	Jonathon M Muncie University of California San Francisco San Francisco, California UNITED STATES
Corresponding Author's Institution:	University of California San Francisco
Corresponding Author E-Mail:	muncie@berkeley.edu
Order of Authors:	Jonathon M Muncie
	Roberto Falcón-Banchs
	Johnathon N. Lakins
	Lydia L. Sohn
	Valerie M. Weaver
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	San Francisco, California, United States of America

1 **TITLE:**
2 Patterning the Geometry of Human Embryonic Stem Cell Colonies on Compliant Substrates to
3 Control Tissue-Level Mechanics
4
5 **AUTHORS AND AFFILIATIONS:**

6 Jonathon M. Muncie[1,2], Roberto Falcón-Banchs[1], Johnathon N. Lakins[2], Lydia L. Sohn[1,3], Valerie
7 M. Weaver[2,4,5,6]
8
9 [1]Graduate Program in Bioengineering, University of California San Francisco and University of
10 California Berkeley, San Francisco, CA, USA
11 [2]Center for Bioengineering and Tissue Regeneration, Department of Surgery, University of
12 California San Francisco, San Francisco, CA, USA
13 [3]Department of Mechanical Engineering, University of California Berkeley, Berkeley, CA, USA
14 [4]Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, University of
15 California San Francisco, San Francisco, CA, USA
16 [5]UCSF Comprehensive Cancer Center, Helen Diller Family Cancer Research Center, University of
17 California San Francisco, San Francisco, CA, USA
18 [6]Department of Anatomy, Department of Bioengineering and Therapeutic Sciences, and
19 Department of Radiation Oncology, University of California San Francisco, San Francisco, CA,
20 USA
21
22 Corresponding Author:
23 Valerie M. Weaver
24 valerie.weaver@ucsf.edu
25
26 Email addresses of Co-authors:
27 Jonathon M. Muncie   muncie@berkeley.edu
28 Roberto Falcón-Banchs   r.falcon@berkeley.edu
29 Johnathon N. Lakins   jon.lakins@ucsf.edu
30 Lydia L. Sohn   sohn@berkeley.edu
31

32 **KEYWORDS:**
33 human embryonic stem cells (hESCs), polyacrylamide hydrogels, traction force microscopy
34 (TFM), photolithography, cell patterning, extracellular matrix (ECM), tissue engineering,
35 regenerative medicine
36
37 **SUMMARY:**

38 Extracellular matrix ligands can be patterned onto polyacrylamide hydrogels to enable the
39 culture of human embryonic stem cells in confined colonies on compliant substrates. This
40 method can be combined with traction force microscopy and biochemical assays to examine
41 the interplay between tissue geometry, cell-generated forces, and fate specification.
42
43 **ABSTRACT:**

44 Human embryonic stem cells demonstrate a unique ability to respond to morphogens in vitro

45 by self-organizing patterns of cell fate specification that correspond to primary germ layer
46 formation during embryogenesis. Thus, these cells represent a powerful tool with which to
47 examine the mechanisms that drive early human development. We have developed a method
48 to culture human embryonic stem cells in confined colonies on compliant substrates that
49 provides control over both the geometry of the colonies and their mechanical environment in
50 order to recapitulate the physical parameters that underlie embryogenesis. The key feature of
51 this method is the ability to generate polyacrylamide hydrogels with defined patterns of
52 extracellular matrix ligand at the surface to promote cell attachment. This is achieved by
53 fabricating stencils with the desired geometric patterns, using these stencils to create patterns
54 of extracellular matrix ligand on glass coverslips, and transferring these patterns to
55 polyacrylamide hydrogels during polymerization. This method is also compatible with traction
56 force microscopy, allowing the user to measure and map the distribution of cell-generated
57 forces within the confined colonies. In combination with standard biochemical assays, these
58 measurements can be used to examine the role mechanical cues play in fate specification and
59 morphogenesis during early human development.
60
61 **INTRODUCTION:**
62 Human embryonic stem cells (hESCs) hold great promise for use in regenerative medicine and
63 tissue engineering applications. The pluripotent nature of these cells gives them the ability to
64 differentiate into any adult cell type. While great strides have been made in directing the fate
65 of hESCs to particular cell types, it has remained very difficult to generate whole tissues or
66 organs *de novo*[1-5]. This is due, in large part, to a limited understanding of the mechanisms that
67 drive the formation of these tissues during human development. In order to fill this gap in
68 knowledge, a number of methods have emerged in recent years to model the early embryo and
69 subsequent stages of development with embryonic stem cells[6-13].
70
71 Shortly after the derivation of the first hESC lines[14], it was demonstrated that embryoid bodies
72 formed from hESCs were capable of spontaneously producing cells of the three primary germ
73 layers[6]. However, due to the inherent lack of control over the size and morphology of embryoid
74 bodies, the organization of germ layers varied significantly and failed to match the organization
75 of the early embryo. More recently, Warmflash et al. developed a method to confine colonies
76 of hESCs on glass substrates via micropatterning, providing control and consistency over the
77 size and geometry of the colonies[8]. In the presence of BMP4, an important morphogen in early
78 development, these confined colonies were capable of self-organizing reproducible patterns of
79 specification to fates representing the primary germ layers. Although this provided a useful
80 model for studying the mechanisms by which primary germ layers are established, the patterns
81 of fate specification did not precisely match the organization and morphogenesis observed
82 during embryogenesis[15]. A more faithful recapitulation of early embryonic development was
83 achieved by embedding hESCs in a three-dimensional extracellular matrix (ECM) of matrigel[11],
84 providing the strongest evidence to date for the ability of hESCs to self-organize and model the
85 early stages of embryogenesis ex vivo. However, this method yields inconsistent results and is
86 thus incompatible with a number of assays that could be used to reveal the underlying
87 mechanisms of self-organization and fate specification.
88

89 Given these existing methods and their respective limitations, we sought to develop a method
90 for reproducibly culturing hESC colonies of defined geometries in conditions that model the
91 extracellular environment of the early embryo. To achieve this, we used polyacrylamide
92 hydrogels of tunable elasticity to control the mechanical properties of the substrate. Using
93 atomic force microscopy on gastrulation-stage chicken embryos, we found that the elasticity of
94 the epiblast ranged from hundreds of pascals to a few kilopascals. Thus, we focused on
95 generating polyacrylamide hydrogels with elasticity in this range to serve as the substrate for
96 hESC colonies. We modified our previous methods for culturing hESCs on polyacrylamide
97 hydrogels[7,9] to provide robust control over the geometry of the colonies. We achieved this by
98 first patterning ECM ligands, namely matrigel, onto glass coverslips through microfabricated
99 stencils, as previously reported[16]. We then designed a novel technique to transfer the
100 patterned ligand to the surface of polyacrylamide hydrogels during polymerization. The method
101 we describe here involves using photolithography to fabricate a silicon wafer with the desired
102 geometric patterns, creating stamps of theses geometric features with polydimethylsiloxane
103 (PDMS), and using these stamps to generate the stencils that ultimately allow patterning of
104 ligand onto the surface of glass coverslips and transfer to polyacrylamide.
105
106 In addition to recapitulating the mechanical environment of the early embryo, confining hESC
107 colonies on polyacrylamide enables the measurement of cell-generated forces with traction
108 force microscopy (TFM), as reported in our previous method[9]. In brief, fluorescent beads can be
109 embedded in the polyacrylamide and used as fiducial markers. Cell-generated forces are
110 calculated by imaging the displacement of these beads after seeding hESCs onto the patterned
111 substrate. Furthermore, the resulting traction force maps can be combined with traditional
112 assays, such as immunostaining, to examine how the distribution of cell-generated forces in
113 confined hESC colonies may regulate or modulate downstream signaling. We expect these
114 methods will reveal that mechanical forces play a critical role in the patterning of cell fate
115 specification during early embryonic development that is currently overlooked.
116
117 **PROTOCOL:**
118 All methods described here pertaining to the use of hESCs have been approved by the Human
119 Gamete, Embryo and Stem Cell Research (GESCR) Committee at the University of California San
120 Francisco.
121
122 **1. Preparation of silicon wafer with geometric features**
123
124 1.1.   Create a photomask with desired geometric features. Use computer-aided drafting
125 software to design the features. For use with negative photoresist, make features opaque and
126 the remainder of the mask transparent.
127
128 NOTE: For patterning onto 18 mm diameter coverslips, group features for each experimental
129 condition into 10 x 10 mm areas to ensure the stencils generated in Step 3 fit onto coverslips.
130
131 1.2.   Spin coat negative photoresist onto a 100 mm silicon wafer. Use the photoresist data
132 sheet to determine the speed and length of spin to generate a film thickness of 100-250 μm.

133

134 NOTE: The film thickness should be adjusted such that the aspect ratio of the width of the
135 patterns to the height of the stencil is as close to 1:1 as possible. However, we recommend a
136 minimum thickness of 100 µm, as anything less produces delicate stencils that easily tear.

137

138 1.3.   Process the photoresist according to the product data sheet. This typically involves a
139 soft bake, UV exposure with photomask in a mask aligner, post exposure bake, development,
140 and hard bake. As an example, the procedure used to process the wafers used in this protocol is
141 outlined in **Table 1.**

142

143 NOTE: Be cautious when handling silicon wafers as they are fragile. Once generated, wafers can
144 be used repeatedly as long as they are not damaged.

145

146 **2. Preparation of coverslips**

147

148 2.1.   **Preparation of acid-washed "top" coverslips**

149

150 2.1.1. Place 18 mm diameter #1 thickness coverslips into a glass Petri dish. The appropriate
151 number of coverslips depends on the size of the dish. For a 100 mm dish, prepare 50-100
152 coverslips at a time. Volume amounts given through the remainder of this section correspond
153 to a 100 mm dish.

154

155 2.1.2. Add 20 mL of 1 M HCl to the Petri dish and gently shake the dish to disperse the
156 coverslips. Ensure all the coverslips are submerged and release air bubbles from between the
157 coverslips. Incubate overnight at room temperature with gentle shaking.

158

159 CAUTION: HCl is acidic and corrosive. Use caution and wear appropriate PPE while working with
160 HCl.

161

162 2.1.3. Decant HCl from the dish. Add 20 mL of ultrapure water and wash with gentle shaking
163 for 10 min. Discard water and repeat for a total of five washes.

164

165 2.1.4. After discarding the final wash, add 20 mL of 100% ethanol to the dish. Using forceps,
166 remove the coverslips individually and arrange between two pieces of filter paper to dry.

167

168 2.1.5. Store dried coverslips in a sealed container. Acid-washed coverslips can be prepared in
169 advance and stored for a month or longer in dust-free conditions.

170

171 2.2.   **Preparation og glutaraldehyde-activated "bottom" coverslips**

172

173 NOTE: Refer to previous methods for additional details[7,9].

174

175 2.2.1. Place 18 mm diameter #1 thickness coverslips into a Petri dish.

176

177 2.2.2. Add 20 mL of 0.2 M HCl to the petri dish and gently shake the dish to disperse the
178 coverslips. Ensure all the coverslips are submerged and release air bubbles from between the
179 coverslips. Incubate overnight at room temperature with gentle shaking.

181 2.2.3. Decant HCl from the dish. Add 20 mL of ultrapure water and wash with gentle shaking
182 for 10 min. Discard water and repeat for a total of five washes.

184 2.2.4. After discarding the final wash, add 20 mL of 0.1 M NaOH and shake gently to disperse
185 and submerge coverslips. Incubate at room temperature with gentle shaking for 1 h.

187 2.2.5. Decant NaOH from the dish. Add 20 mL of ultrapure water and wash with gentle shaking
188 for 10 min. Discard water and repeat for a total of five washes.

190 2.2.6. After discarding the final wash, add 20 mL of a 1:200 dilution of 3-
191 aminopropyltrimethoxysilane in ultrapure water and shake gently to disperse and submerge
192 coverslips. Incubate at room temperature with gentle shaking for 1 h or overnight.

194 CAUTION: 3-aminopropyltrimethoxysilane is flammable and can cause skin irritation. Handle
195 with care, wear appropriate PPE, and discard waste according to local disposal regulations.

197 2.2.7. Decant the diluted 3-aminopropyltrimethoxysilane solution from the dish. Add 20 mL of
198 ultrapure water and wash with gentle shaking for 10 min. Discard water and repeat for a total
199 of at least five washes. It is critical to remove all 3-aminopropyltrimethoxysilane before
200 proceeding.

202 2.2.8. After discarding the final wash, add 20 mL of a 1:140 dilution of 70% glutaraldehyde in
203 phosphate buffered saline (PBS) and shake gently to disperse and submerge coverslips.
204 Incubate at room temperature with gentle shaking for 1 h or overnight.

206 CAUTION: 70% glutaraldehyde is toxic and can cause skin irritation. Handle with care, wear
207 appropriate PPE, and discard waste according to local disposal regulations.

209 2.2.9. Decant the diluted glutaraldehyde solution from the dish. Add 20 mL of ultrapure water
210 and wash with gentle shaking for 10 min. Discard water and repeat for a total of five washes.

212 2.2.10. After discarding the final wash, add 20 mL of 100% ethanol. Using forceps, remove the
213 coverslips individually and arrange between two pieces of filter paper to dry.

215 2.2.11. Store dried coverslips in a sealed container. Glutaraldehyde-activated coverslips can be
216 prepared in advance and stored for up to six months.

218 **3. Generation of stencils for patterning ECM ligand**

220 3.1.   **Generating polydimethylsiloxane (PDMS) intermediate stamps**

3.1.1.   Mix PDMS base with PDMS curing agent at a 10:1 ratio, by weight. Mix thoroughly.

NOTE: For the initial application of PDMS, prepare approximately 100 g of PDMS to fill a 100 mm dish. For subsequent applications, remove PDMS from only the center of the dish where the silicon wafer features are located and prepare 20-30 g of new PDMS.

3.1.2.   Degas the PDMS mixture in a desiccator for 30-60 min or until all air bubbles are removed.

3.1.3.   Place the modified silicon wafer from Step 1 into a plastic 100 mm dish. Slowly and evenly pour the PDMS mixture over the surface of the wafer. Tap the dish on the work surface to release any air bubbles from the surface of the wafer.

3.1.4.   Degas the PDMS mixture poured over the wafer for 10 min or until all air bubbles are removed or have come to the surface of the PDMS.

3.1.5.   Bake the PDMS at 70 °C for 2 h. Allow to cool to room temperature.

3.1.6.   Using a scalpel or box cutter, cut out the section of PDMS from the center of the dish that contains the geometric features.

3.1.7.   Cut the PDMS into roughly 10 x 10 mm squares, each containing the features for a single experimental condition. These are referred to as "stamps" in subsequent steps of the protocol.

3.2.   **Generating flat slabs of PDMS**

3.2.1.   Mix PDMS base with PDMS curing agent at an 8:1 ratio, by weight. Prepare approximately 20 g for each 100 mm dish to be used. Mix thoroughly.

3.2.2.   Degas the PDMS mixture in a desiccator for 30-60 min or until all air bubbles are removed.

3.2.3.   Slowly and evenly pour the PDMS into a clean 100 mm dish. Tap the dish on the work surface to release any air bubbles.

3.2.4.   Bake the PDMS at 70 °C for 2 h. Allow to cool to room temperature.

3.2.5.   Remove the PDMS from the dish. Invert such that the surface of the PDMS that is in contact with the bottom of the dish is facing up.

3.2.6.   For each stamp generated in Step 3.1, cut a 15 x 15 mm square of PDMS. These are referred to as "flat slabs" in subsequent steps of the protocol.

265   3.3.   **Generating stencils**
266
267   3.3.1.   Arrange flat slabs of PDMS on the lid of a 150 mm dish, or other flat surface for easier
268   handling. Ensure sufficient spacing between slabs (e.g., for a 150 mm dish, arrange nine flat
269   slabs with even spacing).
270
271   3.3.2.   Invert each stamp generated in Step 3.1 onto a flat slab of PDMS, such that the features
272   on the stamp are in contact with the flat slab of PDMS. Gently press on the top of the stamp
273   with forceps to ensure even contact.
274
275   3.3.3.   Carefully prop the lid holding PDMS stamp/slab pairs up on the base of the dish, such
276   that the lid rests at an angle. This assists the wicking of the UV-curable polymer in the next step.
277
278   3.3.4 Place a small drop of UV-curable polymer at the top interface of each stamp/slab pair. The
279   polymer will be wicked between the two by surface tension, assisted by gravity.
280
281   NOTE: Many different UV-curable polymers may work in this protocol. We selected Norland
282   Optical Adhesive 74 for the following properties: i) Fast curing upon exposure to UV light, ii)
283   Easy removal from PDMS stamps upon curing, iii) Robust adhesion to glass coverslips with
284   manual pressure.
285
286   3.3.4.   Once the polymer has been completely wicked through, place the lid with stamp/slab
287   pairs flat on the work surface. Place a small drop of UV-curable polymer at each of the
288   remaining three sides of each stamp/slab interface.
289
290   3.3.5.   Using a 200 µL pipette tip, connect the drops of polymer around the edges of the
291   stamp/slab interface. This creates a border that will hold ligand solution in Step 4.
292
293   NOTE: Be careful when working the polymer around the edges of the stamp. If the stamp/slab
294   interface is disrupted, the polymer will wick underneath the features and the stencil will not
295   form properly.
296
297   3.3.6.   Carefully place the stamp/slab pairs into a UV-sterilization box. Use the sterile "Str"
298   power setting and expose for 10 min.
299
300   3.3.7.   Remove the stamp/slab pairs from the UV-sterilization box. Using two pairs of forceps,
301   gently remove the PDMS stamp while holding down the flat slab and stencil that formed from
302   the UV-curable polymer.
303
304   3.3.8.   Using forceps, carefully remove the stencil and invert it such that the surface that is in
305   contact with the flat slab of PDMS is facing up.
306
307   NOTE: The stencils are delicate. Be careful while handing them, especially while initially
308   removing them from the PDMS, so that they do not tear.

309

310 3.3.9. Place the inverted stencils back into a UV-sterilization box. Use the sterile "Str" power
311 setting and expose for 3 min.

312

313 **4. Patterning ECM ligand on coverslips**

314

315 **4.1. Pressing stencils onto acid-washed coverslips**

316

317 4.1.1. Place one acid-washed coverslip for each stencil onto a clean piece of laboratory film.

318

319 4.1.2. Using forceps, carefully place each stencil, flat-side-down, onto an acid-washed
320 coverslip. Place such that features are centered on the coverslip.

321

322 4.1.3. Place a small piece of laboratory film on top of each stencil. Firmly and evenly press
323 down on the stencil to create strong contact between the stencil and the coverslip.

324

325 NOTE: **This is a critical step!** Press firmly and across the entire surface of the stencil. If sufficient
326 contact is not created, the ligand solution will leak between the stencil and the coverslips and
327 the patterning will fail.

328

329 4.1.3.1. OPTIONAL: To confirm sufficient contact between the stencil and the coverslip, pipette
330 100 µL of ultrapure sterile water onto the surface of each stencil and incubate at room
331 temperature. After 1 h, check for leaking. Aspirate water from successfully bonded stencils and
332 proceed.

333

334 **4.2. Plasma cleaning the surface of the stenciled coverslips to increase hydrophilicity**

335

336 4.2.1. Place the coverslips with stencils into a plasma cleaner. Apply high power plasma for 30
337 s.

338

339 **4.3. Preparation of ECM ligand solution**

340

341 NOTE: All subsequent steps should be completed in sterile conditions, if possible.

342

343 4.3.1. Place sterile 100 mM HEPES, 100 mM NaCl, pH 8.0 solution on ice. Once ice cold, add
344 matrigel and collagen to achieve concentrations of 225 µg/mL and 25 µg/mL, respectively.

345

346 4.3.1.1. OPTIONAL: For troubleshooting or for ligand visualization, include a fluorescently
347 tagged ligand in the ligand solution.

348

349 NOTE: Different ECM ligands can be used with this method. For different ligands, additional
350 optimization may be required to determine ideal concentration, incubation time, and
351 incubation temperature. See discussion section for more information.

352

353   4.3.2. Pipette the ligand solution onto the surface of each stencil. For stencils made from 10 x
354   10 mm square stamps, apply 100 µL per stencil.

356   4.3.3. Check for air bubbles in stencil features. If necessary, use fine-tipped forceps or a 2 µL
357   pipette tip to remove air bubbles from features.

359   NOTE: **This is a critical step!** If air bubbles remain trapped at the surface of the coverslip, the
360   ligand will not adsorb to the surface and the resulting pattern will be incomplete.

362   4.3.4. Place the stenciled coverslips with ligand into a dish, wrap with laboratory film, and
363   incubate at 4 °C overnight.

365   **5.   Transfer of ligand to polyacrylamide gel**

367   5.1.   **Removing ligand solution and stencil from each coverslip**

369   5.1.1. Aspirate the ligand solution from the surface of a stencil. Ligand solution can be
370   collected and stored at 4 °C and reused for up to a month.

372   5.1.2. Using forceps, briefly submerge the coverslip with stencil into a dish containing sterile
373   PBS to wash.

375   5.1.3. Briefly submerge the coverslip with stencil into a second dish of sterile PBS to wash.

377   5.1.4. Remove the stencil from the surface of the coverslip. Be careful to not break the
378   coverslip.

380   5.1.5. Briefly submerge the coverslip into a dish containing ultrapure sterile water to remove
381   salts from the PBS washes. Tap the edge of the coverslip to a delicate task wipe to wick away
382   excess water.

384   5.1.6. Dry the coverslip under an inert gas, such as nitrogen. Mark the underside of the
385   coverslip to keep track of orientation from this point forward. Be careful to keep the patterned
386   side facing up.

388   5.1.7. Repeat steps 5.1.1 through 5.1.6 for each stenciled coverslip.

390   5.2.   **Making polyacrylamide hydrogels with patterned coverslips** (refer to previous methods
391   for additional details[7,9]).

393   NOTE: Sterilize all cap holders, tubes, and spacers used to make polyacrylamide gels by washing
394   with 10% bleach overnight, rinsing with water at least five times to remove bleach, and washing
395   briefly in 70% ethanol. Place all pieces on delicate task wipes in a sterile biosafety cabinet to dry
396   before use.

397

398　5.2.1.　Prepare polyacrylamide solution to obtain desired hydrogel elasticity (**Table 2**). Mix
399　together all components except the fluorescent microspheres and the potassium persulfate
400　(PPS).

401

402　NOTE: Prepare 1% potassium persulfate solution fresh each time.

403

404　5.2.2.　Degas the polyacrylamide solution, microspheres, and PPS in separate tubes under
405　vacuum for 30 min.

406

407　5.2.3.　For each patterned coverslip, prepare a glutaraldehyde-activated "bottom" coverslip
408　with a spacer (18 mm outer diameter, 14 mm inner diameter) placed on top.

409

410　5.2.4.　After degassing, briefly sonicate microspheres and add appropriate volume to
411　polyacrylamide solution. Mix by pipetting up and down, being careful not to introduce air
412　bubbles. Briefly sonicate.

413

414　5.2.5.　Add appropriate volume of PPS to the polyacrylamide solution. Pipette up and down to
415　mix the solution, being careful not to introduce air bubbles.

416

417　NOTE: After adding PPS, work rapidly to complete steps 5.2.6 through 5.2.11 before
418　polyacrylamide polymerizes.

419

420　5.2.6.　Pipette 75-150 µL (depending on thickness of spacer) to the center of each
421　glutaraldehyde-activated coverslip.

422

423　5.2.7.　Using forceps, place a patterned coverslip onto each glutaraldehyde-activated coverslip
424　with polyacrylamide and spacer, such that the patterned ligand faces the polyacrylamide
425　solution. If sufficient polyacrylamide solution was added, surface tension will wick the
426　polyacrylamide between the two coverslips and no air bubbles will be present.

427

428　5.2.8.　Pick up each polyacrylamide "sandwich" and carefully touch the side to a delicate task
429　wipe to wick away excess polyacrylamide solution.

430

431　5.2.9.　Carefully place each polyacrylamide "sandwich" into a cap holder with threads that are
432　compatible with 15 mL conical-bottom tubes. The orientation should be such that the bottom
433　of the glutaraldehyde coverslip is in contact with the bottom of the cap holder, and the
434　patterned coverslip is on top.

435

436　5.2.10. Screw a 15 mL conical-bottom tube into each cap holder to hold the polyacrylamide
437　"sandwiches" in place. The tubes should be tight to prevent leaking, but be careful to not
438　overtighten and crack the coverslips.

439

440　5.2.11. Centrifuge the polyacrylamide "sandwiches" in the tubes in swing-buckets at 200 x *g* for

441 10 min at room temperature. Remove the tubes from the centrifuge and place in tube racks to
442 maintain orientation for an additional 50 min to ensure full polymerization. Keep covered with
443 foil to prevent bleaching of fluorescent microspheres.
444
445 NOTE: Centrifugation is critical when using this method to perform TFM. The centrifugal force
446 moves all the microspheres to a single plane, which will ultimately be just below the surface of
447 the hydrogel. This is ideal for imaging microsphere displacements that are used to calculate
448 cell-generated traction stresses. If not performing TFM, microspheres can be left out of the
449 polyacrylamide solution and polymerization can occur at the benchtop without centrifugation.
450
451 5.2.12. Remove polymerized polyacrylamide "sandwiches" from the tubes and submerge in PBS
452 in a 100 mm dish. Wrap in laboratory film and incubate for 3 h at room temperature or
453 overnight at 4 °C.
454
455 5.3.   **Preparation of patterned gels for seeding cells**
456
457 5.3.1. Use a scalpel and forceps to carefully remove the patterned coverslip and the spacer
458 from the polyacrylamide "sandwich" while it remains submerged in PBS. The patterned ligand
459 will have transferred to the polyacrylamide during polymerization and will remain after
460 removing the coverslip. The polyacrylamide gel will remain attached to the glutaraldehyde-
461 activated coverslip.
462
463 NOTE: **This is a critical step!** The polyacrylamide **must** be submerged in PBS while removing the
464 coverslip or the ligand patterns will be destroyed.
465
466 5.3.2. Place each coverslip with patterned polyacrylamide into a cap holder. Place a gasket (18
467 mm outer diameter, 14 mm inner diameter) on top of the coverslip and around the
468 polyacrylamide, where the spacer was located. Screw a sawed-off 15 mm conical-bottom tube
469 into the cap holder, forming a well with the patterned polyacrylamide at the bottom. These
470 assemblies fit into the wells of a standard 12-well plate for easy handling.
471
472 5.3.3. Wash each gel by adding 500 µL of PBS to the well assembly. Incubate for 10 min at
473 room temperature with gentle shaking. Remove PBS and repeat twice for a total of three
474 washes.
475
476 5.3.4. After the final PBS wash, add 500 µL of knockout-DMEM media to gels and incubate at
477 37 °C overnight. Gels can be kept at 37 °C with media for up to 5 days before seeding cells.
478
479 5.3.5. The day before seeding cells, replace knockout-DMEM for complete KSR media and
480 incubate at 37 °C overnight.
481
482 **6. Culturing hESCs on patterned gels**
483
484 NOTE: OPTIONAL: If planning to fix samples for immunostaining after TFM, take images of

485   unstressed microsphere positions prior to seeding cells. In this case, fluorescently tagged ligand
486   should be used in step 4.3.1 so that the eventual locations of cells will be known before seeding
487   and microspheres can be imaged in those regions.
488
489   6.1.   Maintain stock hESC cultures and secondary feeder-free cultures prepared on matrigel-
490   coated plates in conditioned KSR media prior to seeding on patterned polyacrylamide gels as
491   previously described[9].
492
493   NOTE: Generate conditioned KSR media by culturing irradiated mouse embryonic fibroblasts in
494   KSR media supplemented with 4 ng/mL basic fibroblast growth factor (bFGF). Collecting and
495   replace media every 24 h.
496
497   6.2.   Aspirate the media from hESCs. Briefly wash with knockout-DMEM media. Add 0.05%
498   trypsin-EDTA supplemented with 10 µM Y27632 (Rho kinase inhibitor) and incubate at 37 °C for
499   5-10 min.
500
501   6.3.   Add media with serum to inhibit trypsin. Gently aspirate media and pipette over the
502   dish to remove cells. Continue pipetting gently to remove all cells and dissociate cell clusters to
503   single cells.
504
505   6.4.   Collect the cell suspension and centrifuge at 200 x g for 3 min.
506
507   6.5.   Aspirate the supernatant and resuspend the pellet in an equivalent volume of KSR
508   media to wash. Centrifuge at 200 x g for 3 min.
509
510   6.6.   Aspirate the supernatant and resuspend the pellet in conditioned KSR media
511   supplemented with 10 ng/mL bFGF and 10 µM Y27632.
512
513   6.7.   Count the cells with a hemocytometer and adjust the cell suspension to a concentration
514   of 300,000 cells per mL. Pipette 500 µL of the cell suspension onto each patterned gel (150,000
515   cells per gel).
516
517   6.8.   3 h after seeding, use a pipette to carefully aspirate the media from each gel and replace
518   with fresh conditioned KSR media supplemented with 10 ng/mL bFGF and 10 µM Y27632. This
519   removes excess cells that did not adhere to patterned regions of the polyacrylamide.
520
521   NOTE: **This is a critical step!** At this stage cells will be loosely adhered to the patterned ligand.
522   Be very careful when swapping media to not detach the cells. Equal care should be taken with
523   each of the media swaps in the subsequent steps.
524
525   6.9.   24 h after seeding, use a pipette to carefully aspirate the media and exchange for
526   conditioned KSR media supplemented with 10 ng/mL bFGF and 5 µM Y27632.
527
528   6.10.   48 h after seeding, use a pipette to carefully aspirate the media and exchange for

529   conditioned KSR media supplemented with 10 ng/mL bFGF. This removes the Rho kinase
530   inhibitor from the media and experiments can begin the next day, 72 h after seeding.
531
532   **7. Performing TFM**
533
534   7.1.   When desired, perform TFM as previously described[9] under desired experimental
535   conditions.
536
537   7.2.   If unstressed microsphere positions were imaged before seeding cells, fix the cells after
538   taking stressed microsphere positions and perform immunostaining to determine localization of
539   proteins of interest relative to regions of high and low traction forces.
540
541   **REPRESENTATIVE RESULTS:**
542   The main challenge to overcome in attempting to culture hESCs in colonies of controlled
543   geometry on compliant substrates is to generate a homogenous pattern of ECM-ligand on the
544   surface of the substrate. The strategy presented in this method involves first generating the
545   desired pattern on the surface of a glass coverslip and then subsequently transferring that
546   pattern to the surface of a polyacrylamide hydrogel during polymerization of the gel (**Figure
547   1A**). Thus, it is important to ensure the desired pattern is created successfully on the surface of
548   the glass coverslip prior to proceeding to polymerization of the hydrogel and transfer of the
549   pattern (**Figure 1B**). Based on imaging of fluorescent ligand patterns transferred to
550   polyacrylamide, the ligand appears to be present only in a single plane at the surface of the
551   polyacrylamide, though we did not precisely characterize the thickness of this layer. There are
552   two common types of defects observed following pattern generation on the glass coverslip,
553   each with its own source of error. The first is the appearance of fluorescent ligand extending
554   beyond the margins of the desired pattern (**Figure 1C, top**), which results from leaking of the
555   fluorescent ligand solution due to a small tear in the stencil or insufficient sealing of the stencil
556   to the glass coverslip. The second is the appearance of an incomplete pattern (**Figure 1C,
557   bottom**), which is typically due to an air bubble trapped at the coverslip interface that prevents
558   adsorption of the fluorescent ligand.
559
560   The ultimate measure of success for this method is the ability to culture hESCs in desired
561   geometries on the patterned hydrogels (**Figure 2)**. In order to achieve this, hESCs are seeded at
562   a relatively high density (300,000 cells/mL) in the presence of a Rho kinase inhibitor (Y27632)
563   and incubated for 3 h to facilitate adhesion to the patterns of ligand. Media is then replaced to
564   remove non-adhered cells. Over the course of 72 h, the Y27632 is gradually diluted out of the
565   media by a series of media exchanges at 24 and 48 h post-seeding. Typically, the hESCs
566   proliferate to complete the patterned geometries by 48-72 h, such that experiments can begin
567   at 72 h post-seeding, once the Y27632 is completely removed from the media.
568
569   Culturing hESC colonies in confined geometries on polyacrylamide hydrogels permits the
570   measurement of cell-generated traction forces using TFM. These measurements are made by
571   embedding fluorescent microspheres in the hydrogel and imaging the positions of these beads
572   before and after seeding hESCs (**Figure 3A**). The displacement of the beads following cell

573   seeding is a function of cell-generated forces and the elasticity of the hydrogel, thus the images
574   of the bead positions can be used to generate maps of bead displacements and subsequently
575   used to calculate the underlying traction stresses. In circular colonies of hESCs, the largest
576   traction stresses are found near the peripheral edge of the colonies, while the center of the
577   colonies display uniformly low traction stresses (**Figure 3B**). Interestingly, the highest traction
578   stresses are found in clusters near the edge of the colonies, rather than forming a continuous
579   ring of maximal stress. This implies that although colony geometry plays a key role in
580   determining the distribution of traction stresses, more localized regulation and feedback
581   determines the precise locations of maximal stress. Additionally, so long as the image of
582   microsphere positions without adhered cells is taken prior to cell seeding, hESC colonies can be
583   fixed for immunostaining of proteins of interest following traction force measurements. Despite
584   the observed non-uniform distributions of traction stresses, hESCs cultured as patterned circles
585   in maintenance conditions display uniform expression of the pluripotency marker Oct3/4 and
586   cell adhesion molecule E-cadherin (**Figure 3C**).
587
588   The presented method for using stencils to generate patterns of ligand is demonstrably
589   superior to the common technique of microcontact printing for the relatively large geometries
590   used in this method (i.e., for circular colonies 1 mm in diameter as well as triangles and squares
591   of equivalent area). Microcontact printing patterns of ligand at this length scale results in
592   heterogeneous transfer at the edges of patterns, with very little ligand deposited in the central
593   regions of the patterns (**Figure 4A**). This is clearly insufficient for consistently producing hESC
594   colonies of specific geometries. Reducing the cell seeding density also leads to inconsistency in
595   achieving completed colonies. Cells seeded at 200,000 cells/mL, rather than 300,000 cells/mL,
596   are not able to generate sufficient cell-cell contacts to survive the reduced concentration of
597   Y27632 at 24 h post-seeding (**Figure 4B**). It may be possible to generate complete patterns with
598   lower cell densities by extending the period of Y27632 dilution; however, overall it is more
599   efficient to seed at the higher 300,000 cells/mL density. Occasionally, errors in pattern
600   generation that are not detected earlier in the protocol become apparent upon seeding of
601   hESCs. One such error is the leaking of ligand solution underneath the stencil due to poor
602   contact with the coverslip. This ultimately results in ligand being transferred to regions of the
603   polyacrylamide outside the desired geometries, and unconfined growth of hESCs upon seeding
604   **(Figure 4C)**.
605
606   **FIGURE AND TABLE LEGENDS:**
607
608   **Figure 1: Patterning of ECM ligand onto acid-washed coverslips and transfer to**
609   **polyacrylamide hydrogels. (A)** Schematic representation of the protocol for patterning ECM
610   ligand on acid-washed coverslips and transferring the patterned ligand to polyacrylamide
611   hydrogels. **(B)** Immunofluorescent images of patterned ECM ligand on acid-washed coverslips
612   (left) and following transfer to polyacrylamide hydrogel (right). Biotin-tagged matrigel was
613   patterned onto the coverslip and labeled with Alexa Fluor 555 streptavidin prior to transfer to
614   polyacrylamide. Inserts show zoomed-out view of the full patterns generated. **(C)**
615   Representative fluorescent images demonstrating patterning defects of ligand on the glass
616   coverslip. These result from common errors in the protocol, such as leaking of the ligand

617 solution outside the patterned geometry (top, arrow indicates site of leak), and trapping of air
618 bubbles inside the patterned geometries of the stencil upon adding the ligand solution (bottom,
619 arrow indicates site of air bubble). All scale bars = 500 μm.

620

621 **Figure 2: Seeding of hESCs onto patterned polyacrylamide hydrogels.** Representative
622 brightfield images demonstrating successful seeding of hESCs onto polyacrylamide hydrogels
623 with patterned ligand. The timeline at the top indicates the series of media changes used to
624 remove unattached cells and dilute out the Y27632. Note that after initial seeding, cells adhere
625 stochastically to various regions within the patterned ligand and then proliferate to fill the
626 patterned regions over the course of 72 h. Scale bar = 500 μm.

627

628 **Figure 3: Regional localization of traction stresses and immunostaining of confined hESC
629 colonies. (A)** Fluorescent images of microspheres within the polyacrylamide hydrogel before
630 and after seeding hESCs. **(B)** Representative particle image velocimetry (PIV) plot depicting the
631 displacement of microspheres due to traction stresses (left) and corresponding reconstructed
632 traction stresses (right). **(C)** Immunostaining of confined hESC colonies on patterned
633 polyacrylamide hydrogels, demonstrating the ability to compare localization of proteins of
634 interest to traction stresses. All scale bars = 200 μm.

635

636 **Figure 4: Non-ideal results yielded by alternative methods and errors. (A)** Representative
637 fluorescent images of ECM ligand patterned onto acid-washed coverslips via microcontact
638 printing. **(B)** Brightfield images of hESCs that fail to form completed colonies due to insufficient
639 adherence of cells. Large gaps remaining in the colonies at 24 h post-seeding (arrows) are an
640 early indicator of this issue. **(C)** Brightfield images of hESCs that fail to form confined colonies
641 due to errors in patterning that led to presence of ligand outside the desired geometries. All
642 scale bars = 500 μm.

643

644 **Table 1: Example SU8 wafer fabrication protocol.** Silicon wafers used to generate PDMS
645 stamps and subsequent stencils were fabricated using the steps outlined in this table. This
646 protocol was generated using the SU8 3000 data sheet with the aim of creating a film thickness
647 of approximately 100-250 μm.

648

649 **Table 2: Polyacrylamide gel formulations.** Volumes of components for generating
650 polyacrylamide hydrogels of various elastic moduli with fluorescent microspheres for TFM.
651 Volumes can be scaled up or down based on amount of polyacrylamide solution needed. All
652 components except the fluorescent microspheres and PPS are mixed together prior to
653 degassing. PBS = phosphate-buffered saline, TEMED = tetramethylethylenediamine, PPS =
654 potassium persulfate.

655

656 **DISCUSSION:**
657 To simplify a long and detailed protocol, this method consists of three critical stages: 1)
658 generating patterns of ECM ligand on glass coverslips, 2) transferring the patterns to
659 polyacrylamide hydrogels during polymerization of the gel, and 3) seeding hESCs on the
660 patterned hydrogel. There are critical steps that must be considered at each of these three

stages. In order to generate high-fidelity patterns on the glass coverslips, the stencil must be firmly pressed onto the coverslip to prevent leaking of the ligand solution and all air bubbles must be removed after adding ligand solution to the surface of the stencil (**Figure 1C**). If ligand solution does leak through the stencil due to poor contact with the coverslip, ligand will be transferred to the entire surface of the polyacrylamide hydrogel and hESCs will not be confined to the desired colony geometry (**Figure 4C).** The most important step in transferring the patterned ligand to polyacrylamide is gently separating the top coverslip from the hydrogel while all components remain submerged in PBS. If the hydrogel does not remain submerged during separation, the patterns may be completely destroyed. Furthermore, if the separation occurs too rapidly, the surface of the hydrogel may tear or be otherwise damaged. The softer the hydrogel, the more it is at risk for damage during separation. Finally, the user must pipette very carefully when exchanging media during the seeding and culture of hESCs on the patterned hydrogels. The hESCs remain loosely adhered throughout the protocol and the patterned colonies can be easily disrupted by careless or rushed pipetting.

In addition to the critical steps discussed above, there are a number of other steps that may require modification and troubleshooting when adapting this protocol for different applications. While the patterns demonstrated here are on the length scale of hundreds of microns to a millimeter, generating patterned features on silicon wafers with transparency photomasks and negative photoresist allows for feature sizes all the way down to 7-10 μm. Thus, this protocol could be adapted for confining the geometry of single cells or smaller colonies of a few cells on compliant substrates.

Two parameters that will likely require optimization when adapting this protocol for different cell types are the type of ECM ligand used and the concentration of the ligand in solution during adsorption to the glass coverslip through the stencils. A total ligand concentration of 250 μg/mL was sufficient for producing homogenous patterns of matrigel and facilitating attachment of hESCs (**Figure 1B** and **Figure 2**), though it may be possible to achieve similar results with lower concentrations. On the other hand, it may be necessary to further increase the concentration of ligand for cell types that are less adherent or for applications that require shorter incubation times. Increased concentration of ligand may also be required for different ECM ligands, such as fibronectin or collagen, which are less hydrophobic than matrigel and therefore may not adsorb to the hydrogel as strongly. Because the transfer of ligand from coverslip to hydrogel occurs during polymerization of the hydrogel, commonly used techniques for robustly cross-linking the ECM ligand to the hydrogel surface (such as sulfo-SANPAH treatment) are impossible. Using fluorescently-labelled ECM ligands to enable visualization of the patterns at each step of the protocol is extremely helpful when optimizing and troubleshooting these parameters.

Additionally, the protocol for seeding cells may require optimization depending on the cell type and media conditions used. For cells that adhere more rapidly or efficiently, a lower seeding density may be required to prevent cell-cell adhesions that span between patterns and result in aggregates rather than patterned monolayer colonies. For cells that display very poor attachment, a larger seeding density or longer length of time before the initial media swap may be required to facilitate complete formation of confined colonies (**Figure 4B**).

705

706 The key limitation of this method is its technical complexity, which results in relatively low-
707 throughput results compared to similar methods that involve culturing cells on patterned glass
708 substrates[8]. However, this drawback is far outweighed by the physiological relevance achieved
709 by culturing confined hESC colonies on compliant substrates. By effectively recapitulating the
710 mechanical properties of the early embryo, we are able to better model and understand the
711 processes that lead to self-organization of the primary germ layers.

712

713 An additional benefit of confining hESC colonies on polyacrylamide hydrogels is that it enables
714 the use of TFM to examine the link between the organization of an hESC colony, as a model of
715 the early embryo, and the distribution of cell-generated forces that may underlie
716 morphogenesis and cell fate specification. Confining hESC colonies results in distributions of
717 traction stresses that are dependent on colony geometry (**Figure 3B**). Despite the non-
718 uniformity of these traction stresses, cells throughout the colonies remain pluripotent in
719 maintenance conditions (**Figure 3C**). However, we hypothesize that the traction stress
720 distributions may be involved in regulating patterns of cell fate specification by tuning the
721 response to induction cues, such as soluble morphogens. We anticipate that this method will
722 allow us and other groups to better model the early human embryo with hESCs, leading to a
723 more complete understanding of the fundamental processes that underlie human
724 embryogenesis.

725

731

732 **DISCLOSURES:**
733 The authors have nothing to disclose.

734

735 **REFERENCES:**
736 1. Tabar, V., Studer, L. Pluripotent stem cells in regenerative medicine: Challenges and recent
737 progress. *Nature Reviews Genetics*. **15**, 82–92 (2014).
738 2. Avior, Y., Sagi, I., Benvenisty, N. Pluripotent stem cells in disease modelling and drug
739 discovery. *Nature Reviews Molecular Cell Biology*. **17**, 170-182 (2016).
740 3. Trounson, A., DeWitt, N.D. Pluripotent stem cells progressing to the clinic. *Nature Reviews
741 Molecular Cell Biology*. **17**, 194-200 (2016).
742 4. Li, Y., Li, L., Chen, Z.N., Gao, G., Yao, R., Sun, W. Engineering-derived approaches for iPSC
743 preparation, expansion, differentiation and applications. *Biofabrication*. **9** (3), 032001 (2017).
744 5. Stevens, K.R., Murry, C.E. Human Pluripotent Stem Cell-Derived Engineered Tissues: Clinical
745 Considerations. *Cell Stem Cell*. **22** (3), 294-297 (2018).
746 6. Itskovitz-Eldor, J., et al. Differentiation of human embryonic stem cells into embryoid bodies
747 comprising the three embryonic germ layers. *Molecular Medicine*. **6** (2), 88-95 (2000).
748 7. Lakins, J.N., Chin, A.R., Weaver, V.M. Exploring the link between human embryonic stem cell

749 organization and fate using tension-calibrated extracellular matrix functionalized
750 polyacrylamide gels. In: Mace K., Braun K. (eds) *Progenitor Cells. Methods in Molecular Biology*
751 *(Methods and Protocols).* Humana Press, Totowa, NJ. **916**, 317-350 (2012).

752 8. Warmflash, A., Sorre, B., Etoc, F., Siggia, E.D., Brivanlou, A.H. A method to recapitulate early
753 embryonic spatial patterning in human embryonic stem cells. *Nature Methods*. **11** (8), 847–854
754 (2014).

755 9. Przybyla, L., Lakins, J.N., Sunyer, R., Trepat, X., Weaver, V.M. Monitoring developmental force
756 distributions in reconstituted embryonic epithelia. *Methods*. **94**, 101-113 (2016).

757 10. Przybyla, L., Lakins, J.N., Weaver, V.M. Tissue Mechanics Orchestrate Wnt-Dependent
758 Human Embryonic Stem Cell Differentiation. *Cell Stem Cell*. **19** (4), 462–475 (2016).

759 11. Shahbazi, M.N., et al. Self-organization of the human embryo in the absence of maternal
760 tissues. *Nature Cell Biology*. **18**, 700-708 (2016).

761 12. Shao, Y., Taniguchi, K., Townshend, R.F., Miki, T., Gumucio, D.L., Fu, J. A pluripotent stem
762 cell-based model for post-implantation human amniotic sac development. *Nature*
763 *Communications*. **8** (208), 1-15 (2017).

764 13. Simunovic, M., Brivanlou, A.H. Embryoids, organoids and gastruloids: new approaches to
765 understanding embryogenesis. *Development*. **144**, 976-985 (2017).

766 14. Thomson, J.A., et al. Embryonic stem cell lines derived from human blastocysts. *Science.* **282**
767 (5391), 1145-1147 (1998).

768 15. Shahbazi, M.N., Zernicka-Goetz, M. Deconstructing and reconstructing the mouse and
769 human early embryo. *Nature Cell Biology*. **20**, 878-887 (2018).

770 16. Li, Q., et al. Extracellular matrix scaffolding guides lumen elongation by inducing anisotropic
771 intercellular mechanical tension. *Nature Cell Biology*. **18** (3), 311-318 (2016).

A Pattern ECM Ligand | Transfer to Polyacrylamide Hydrogel
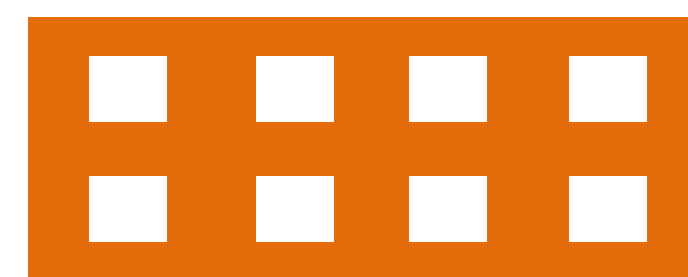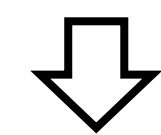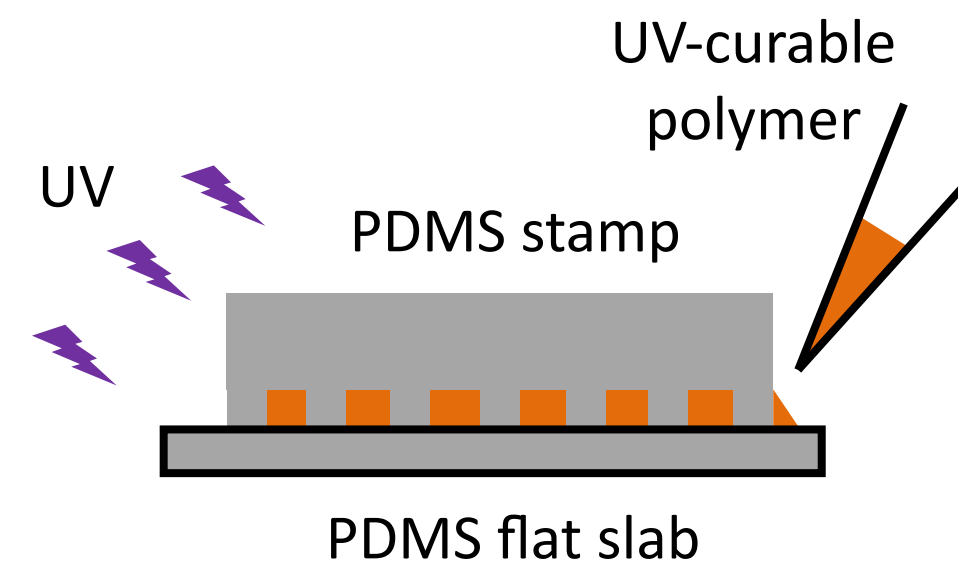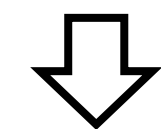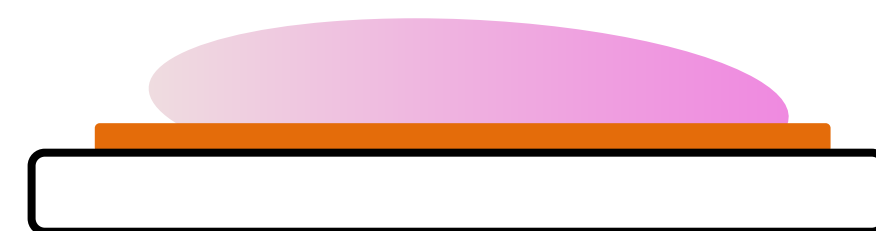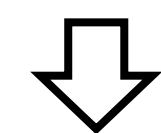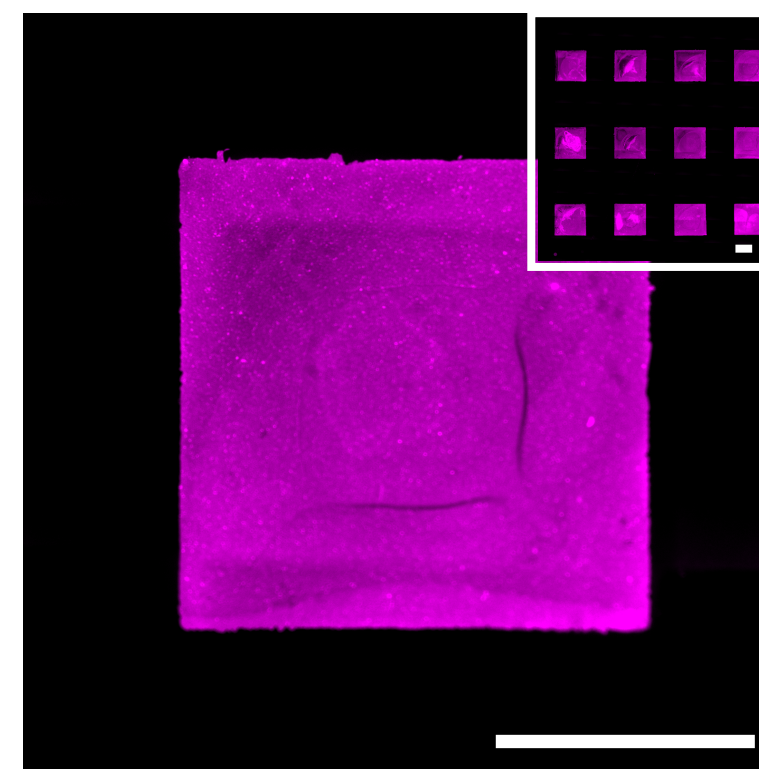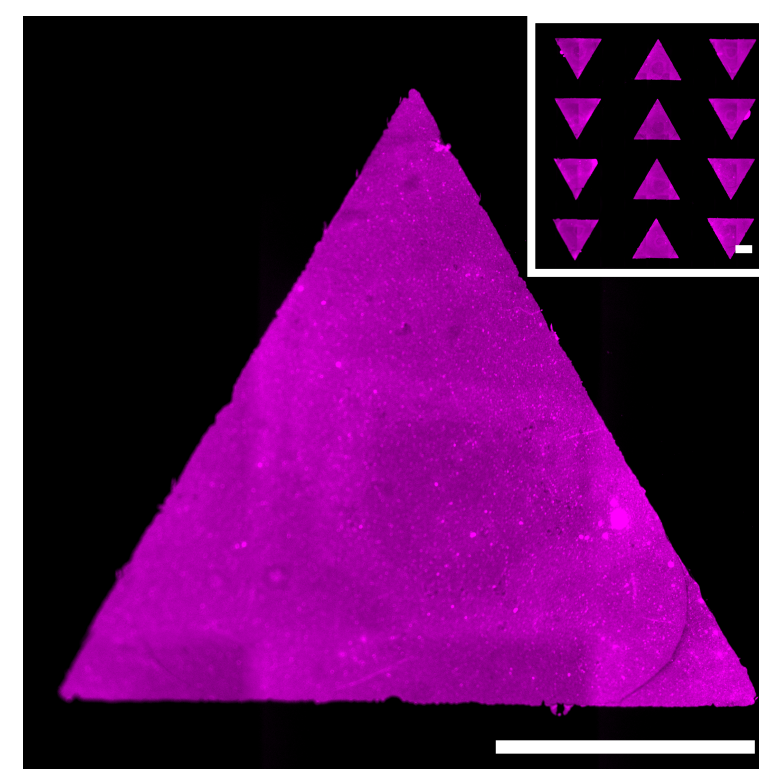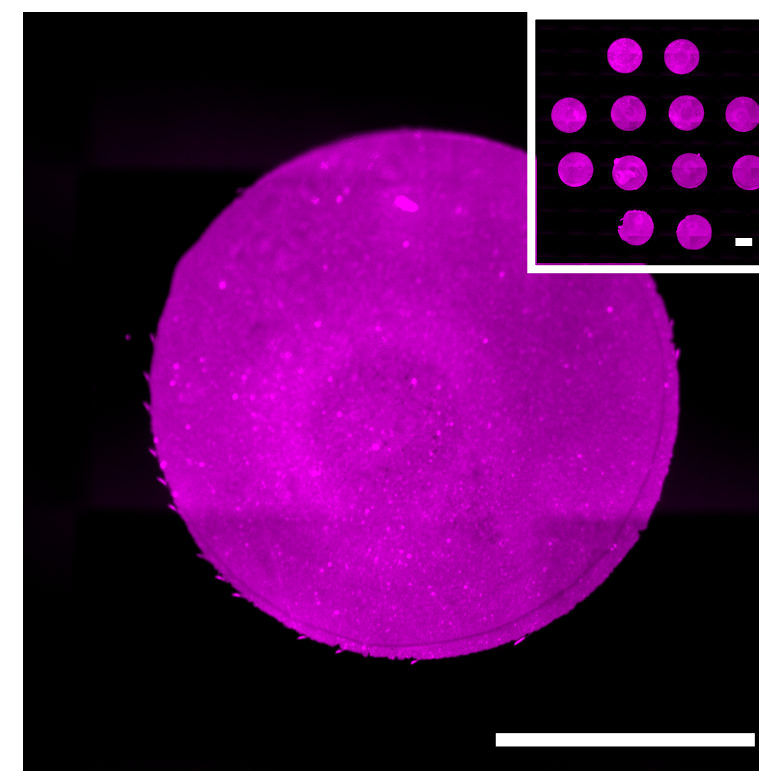
B Patterned Coverslip | Patterned Hydrogel

C Patterning Defects

Figure 2

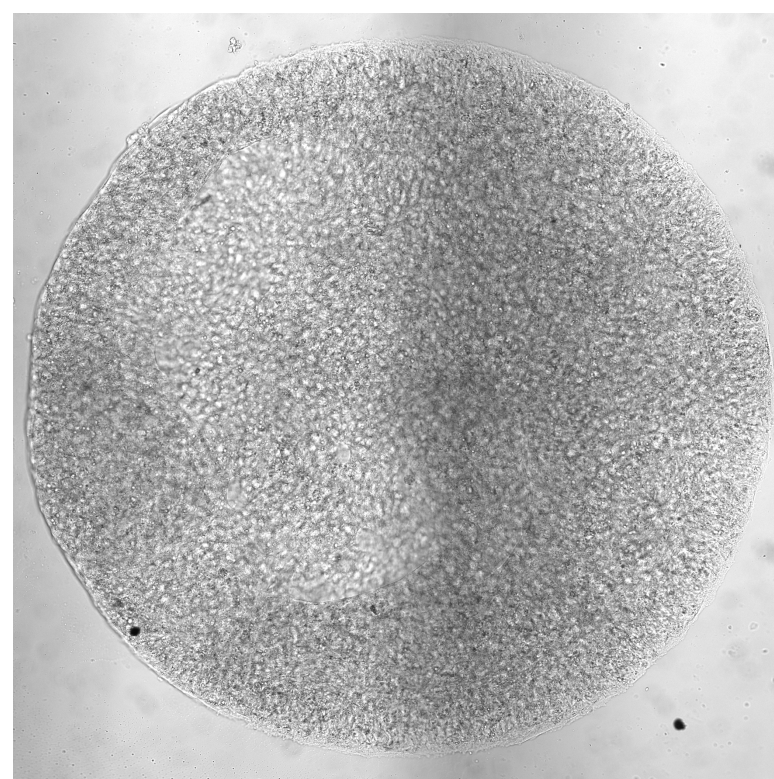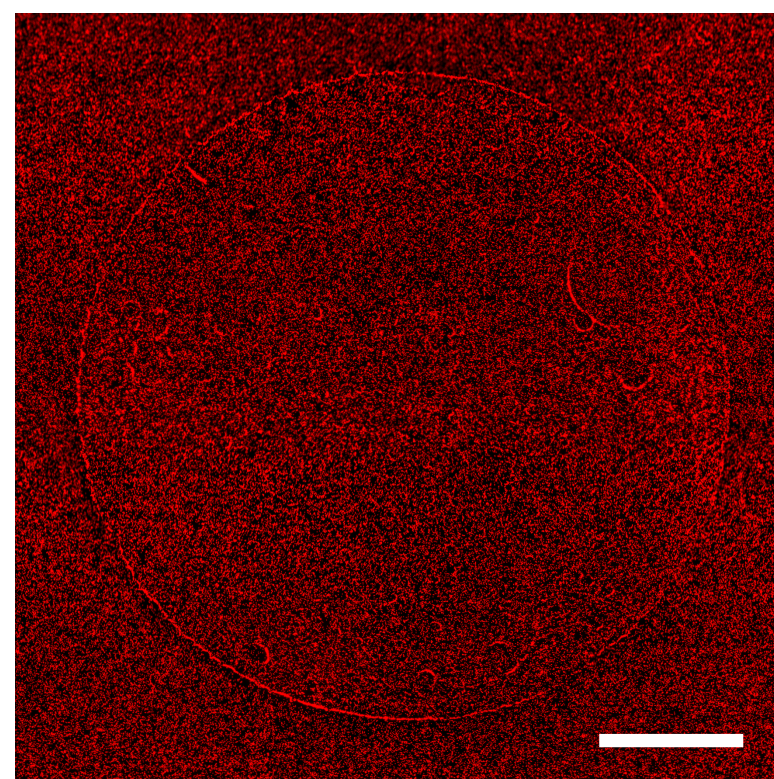| Seed	3 h post-seed	24 h post-seed	48 h post-seed	72 h post-seed
10 µM Y27632	10 µM Y27632	5 µM Y27632	0 µM Y27632	0 µM Y27632

Figure 3
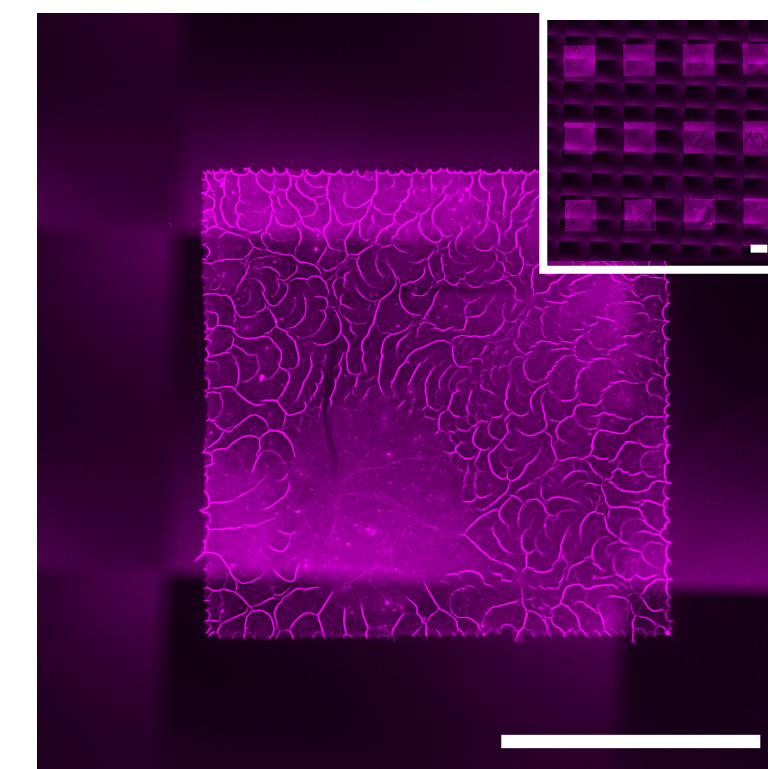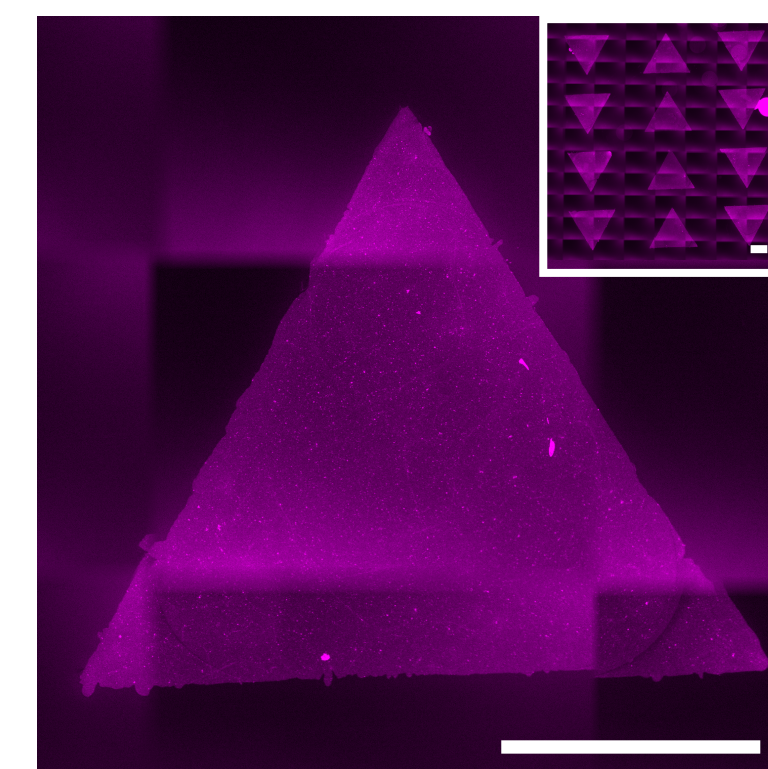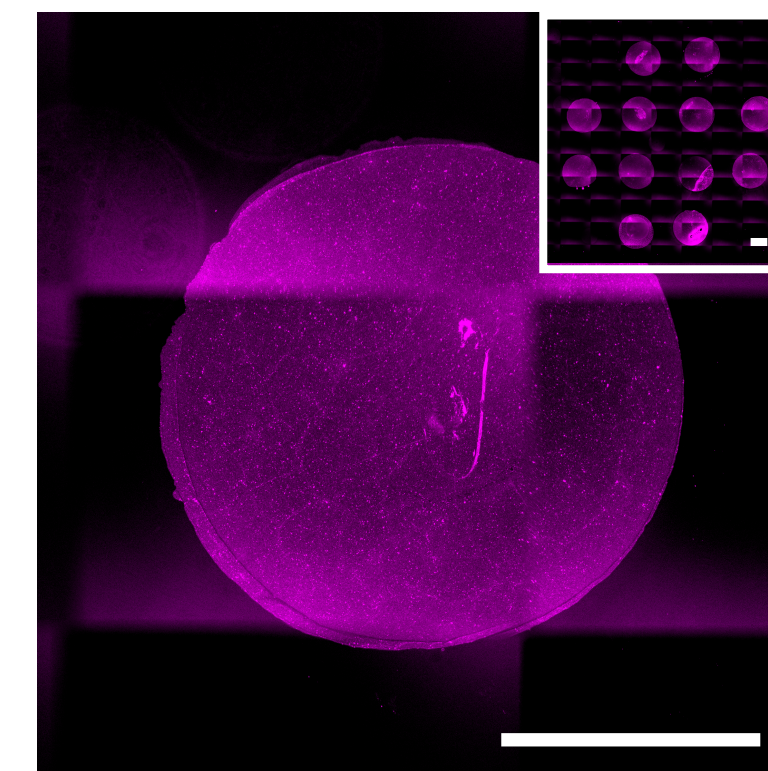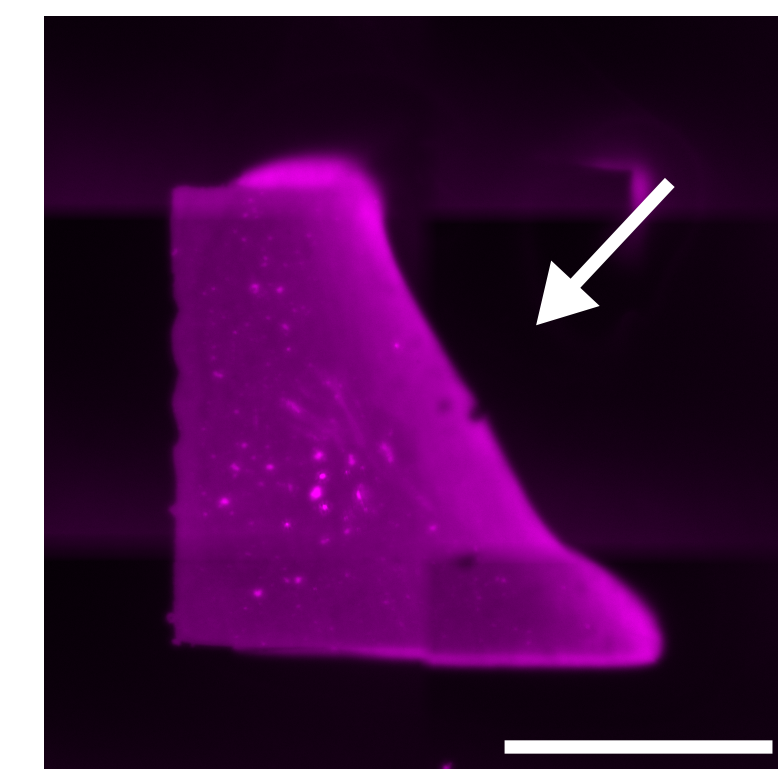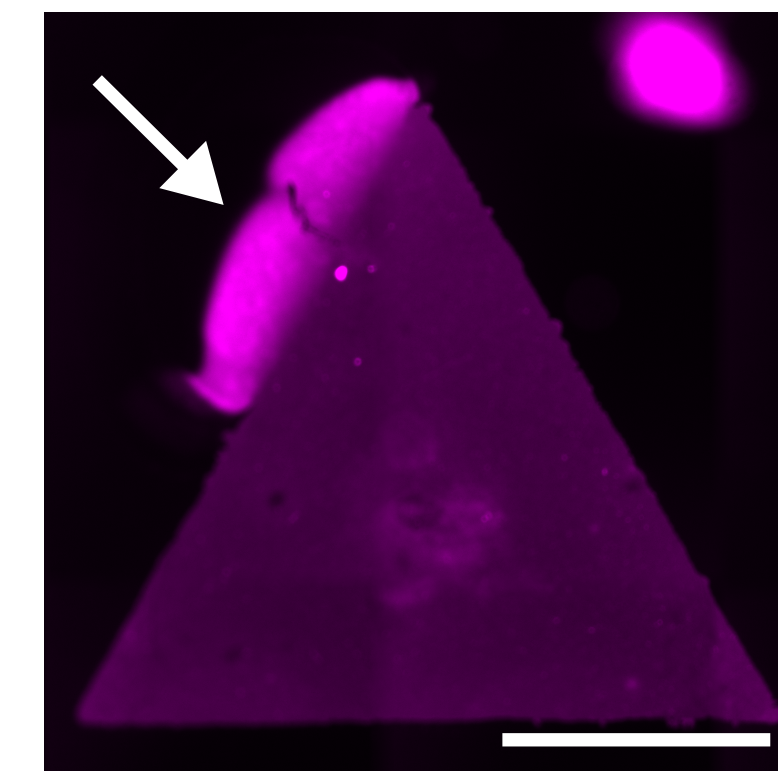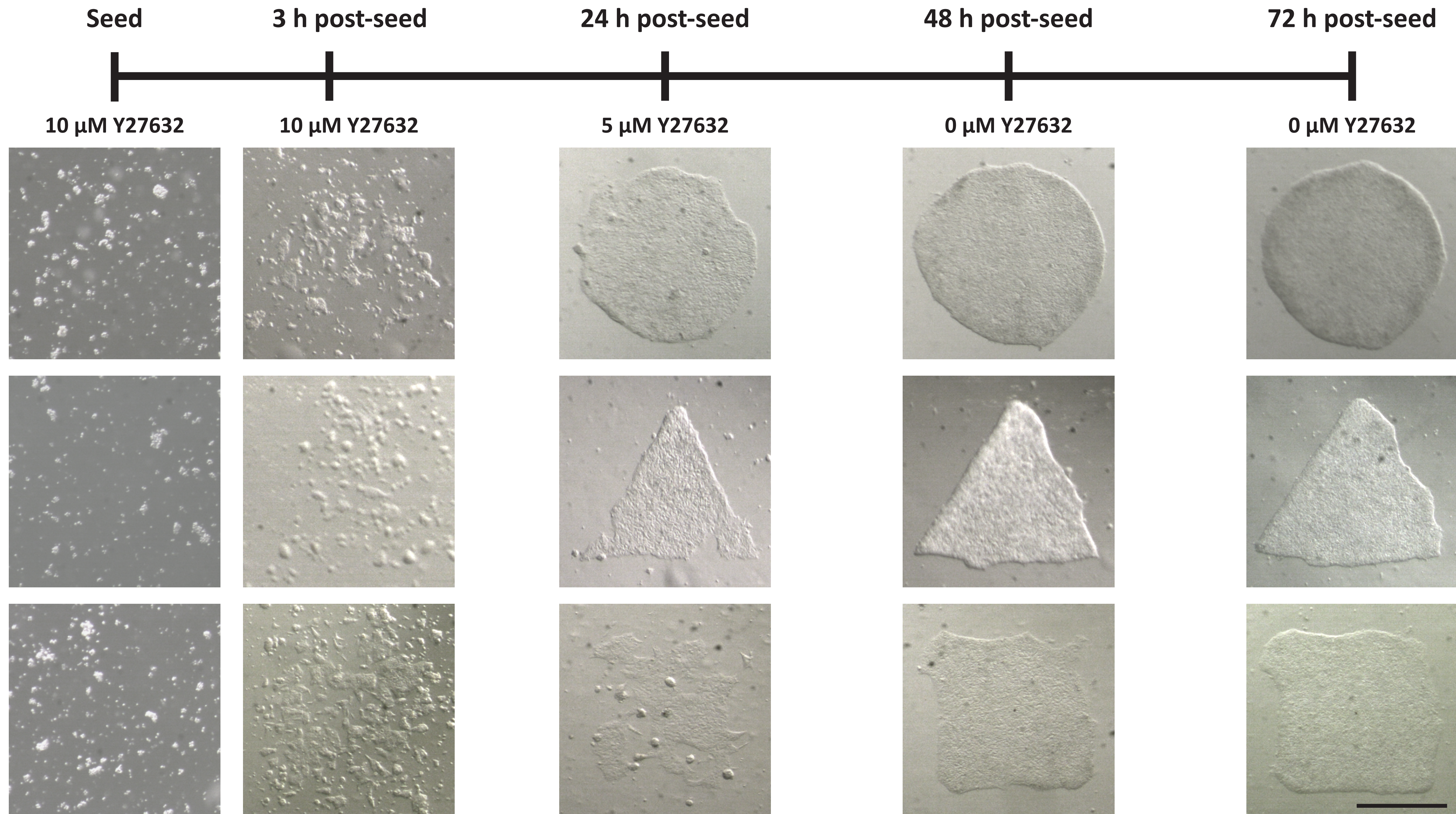
A

Pre-Seeding
Microsphere Positions
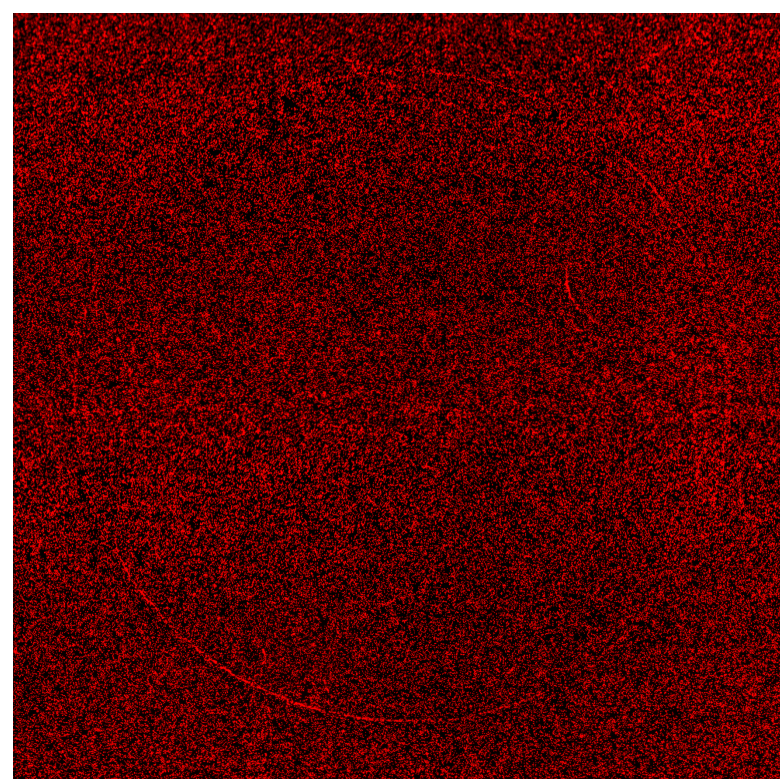
Seeded hESC Colony

Post-Seeding
Microsphere Positions
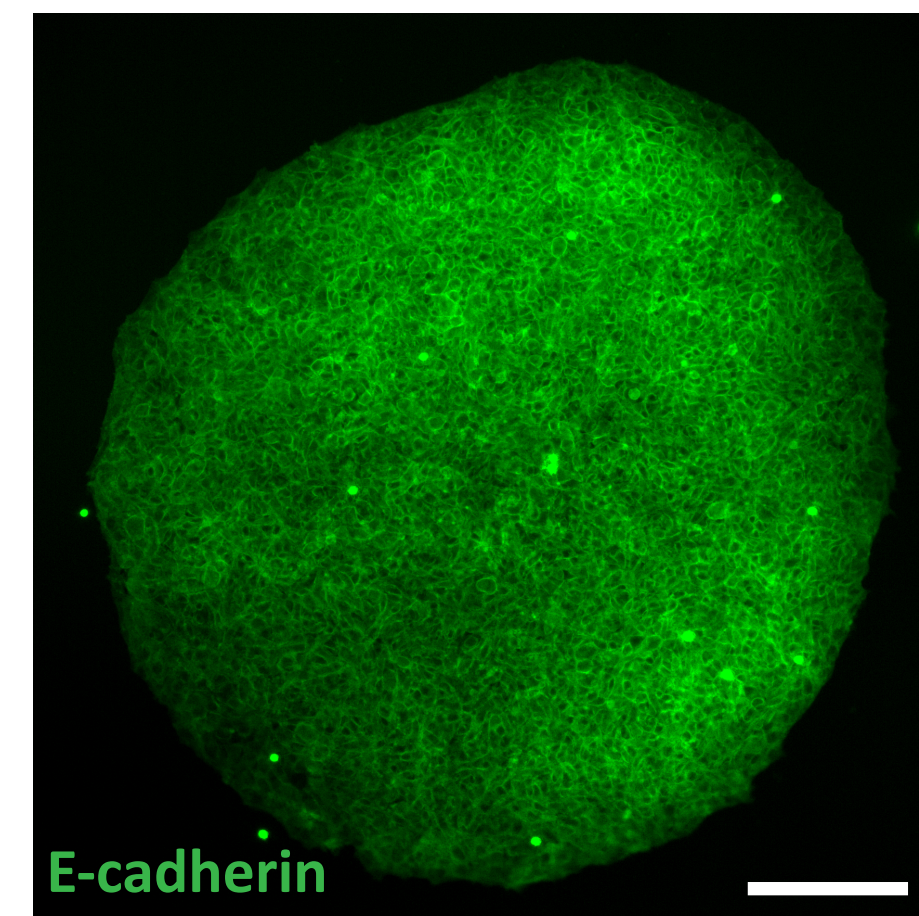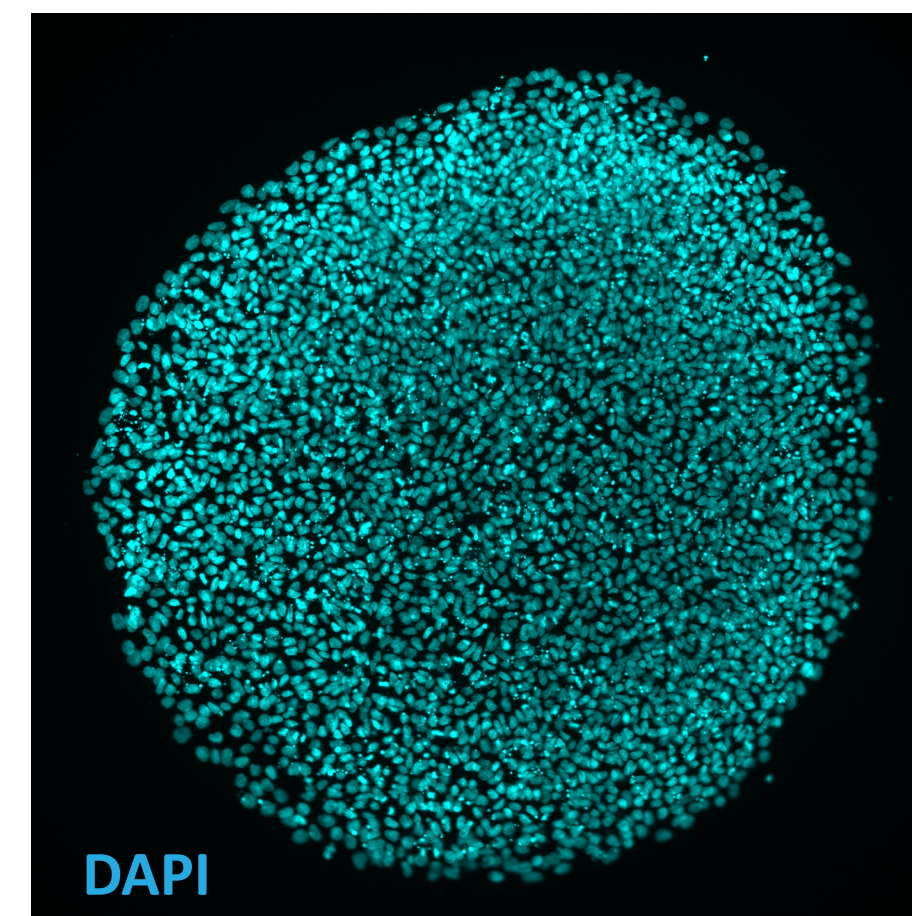
B

Particle Image Velocimetry
(Microsphere Displacements)
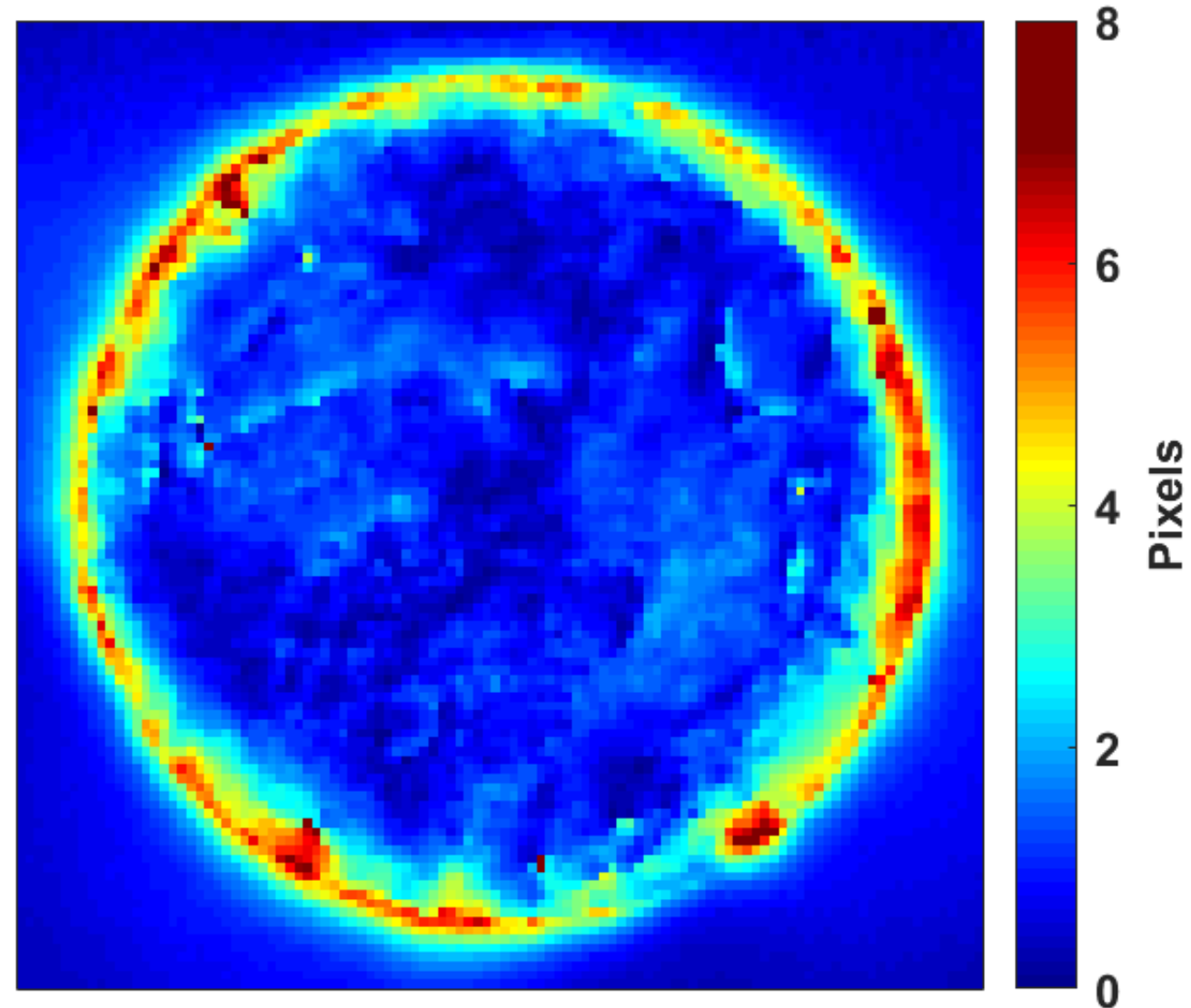
Reconstructed Traction
Stresses

Pixels

Pascals

C

DAPI

Oct 3/4

DAPI

E-cadherin

Figure 4

A

B

Seed	3 h post-seed	24 h post-seed	48 h post-seed	72 h post-seed
10 µM Y27632	10 µM Y27632	5 µM Y27632	0 µM Y27632	0 µM Y27632

C

Table 1
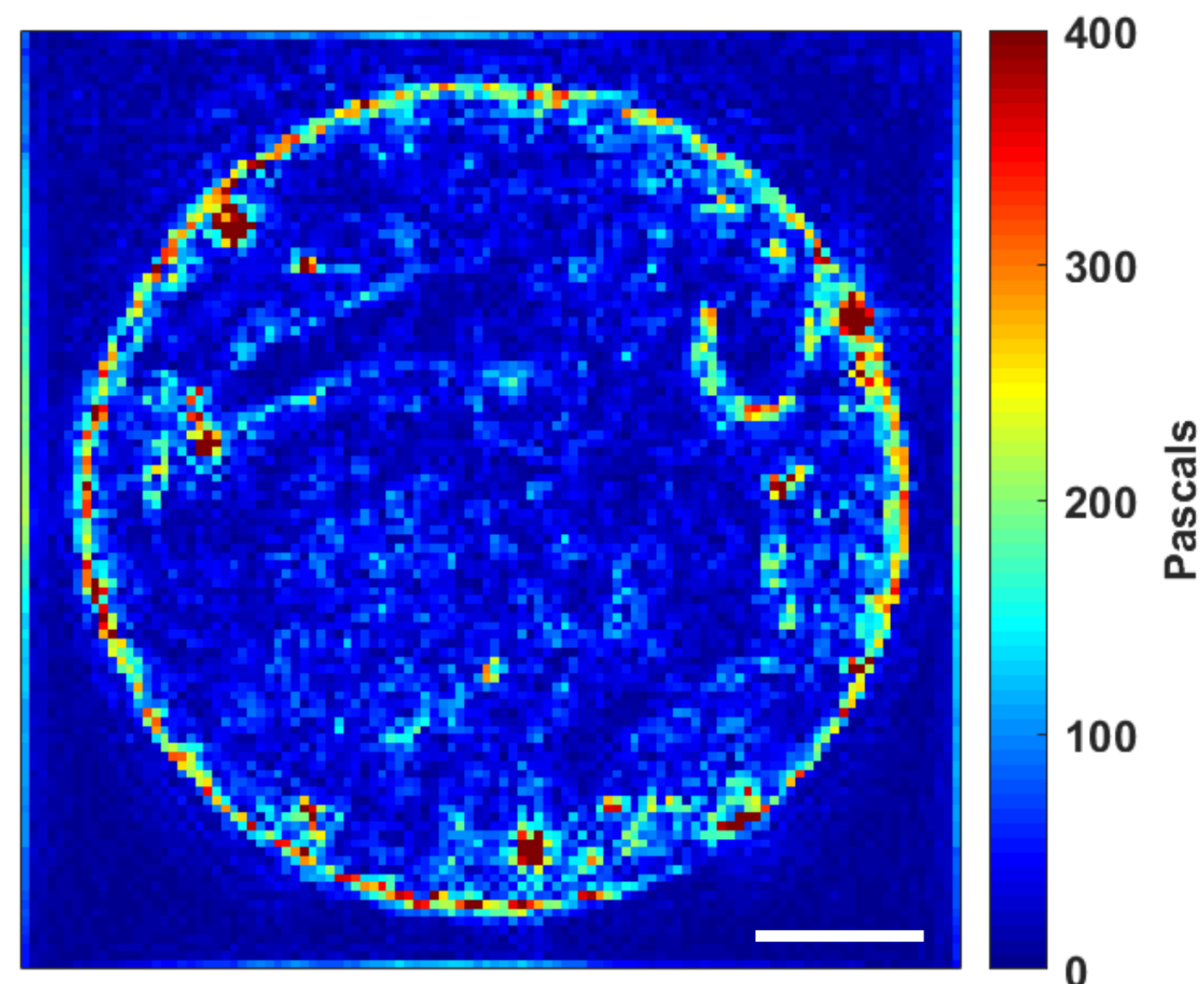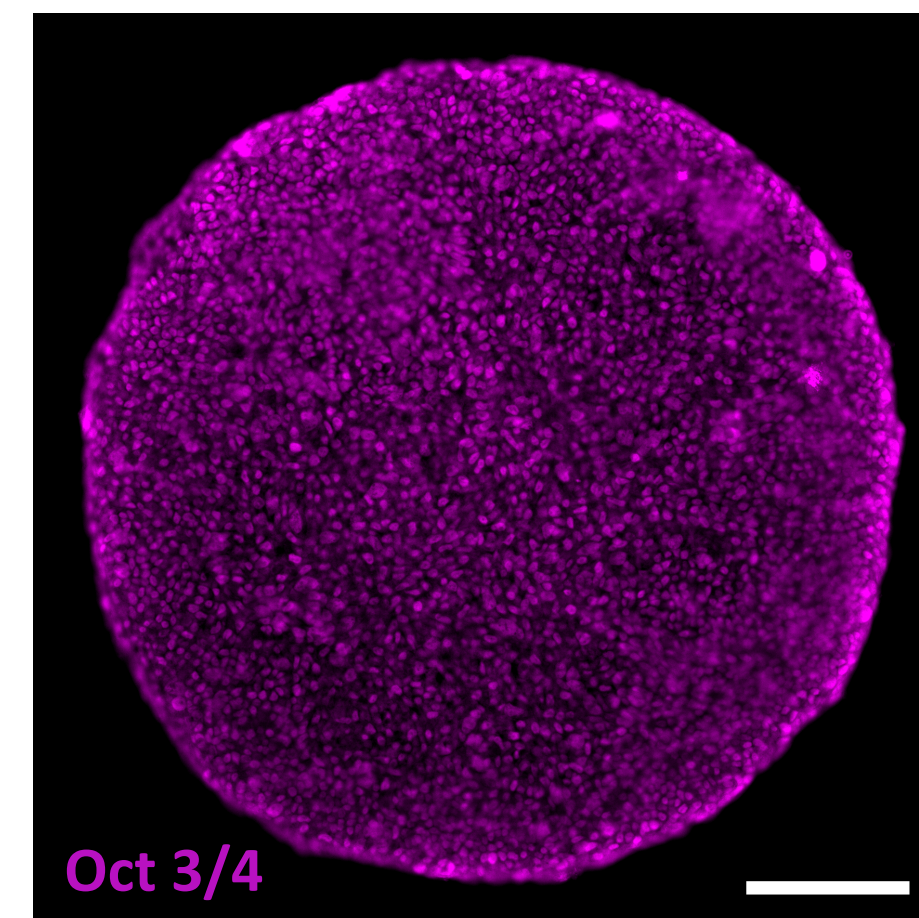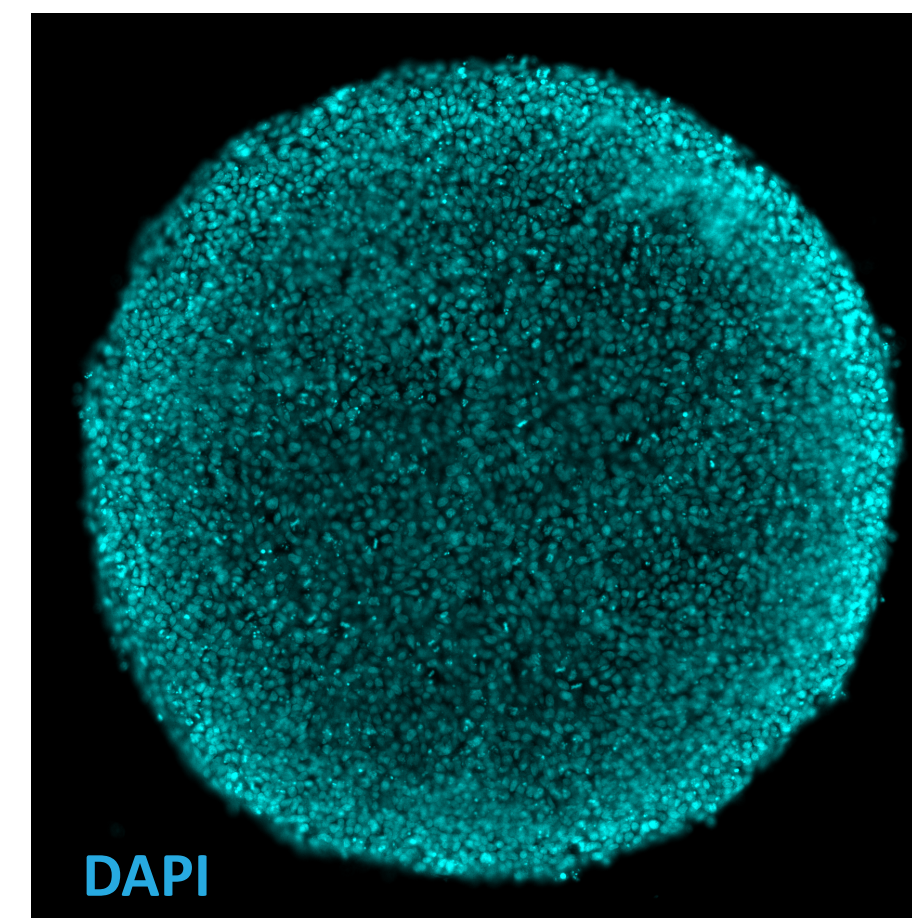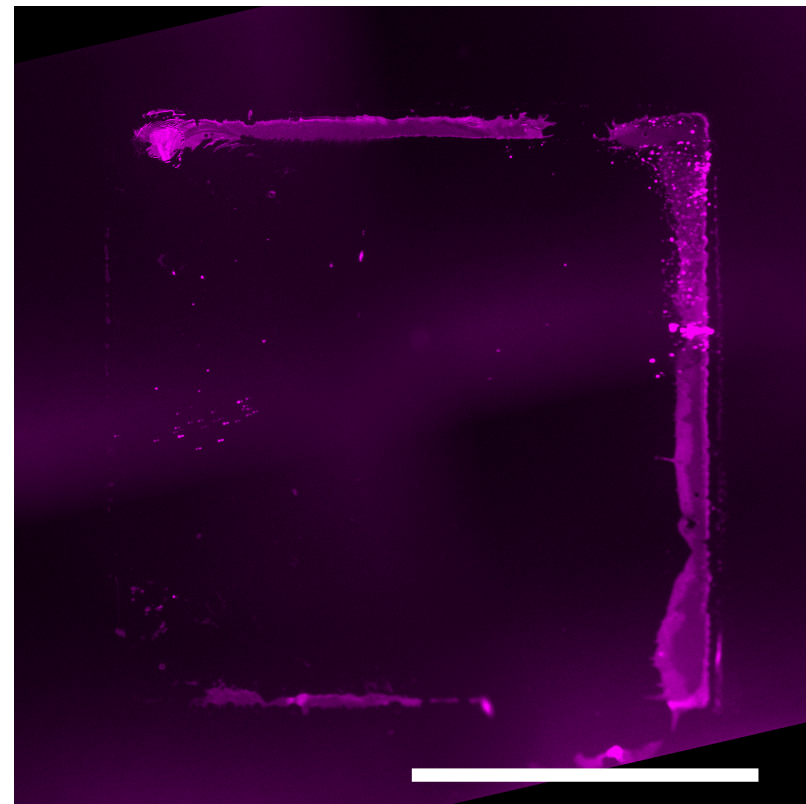
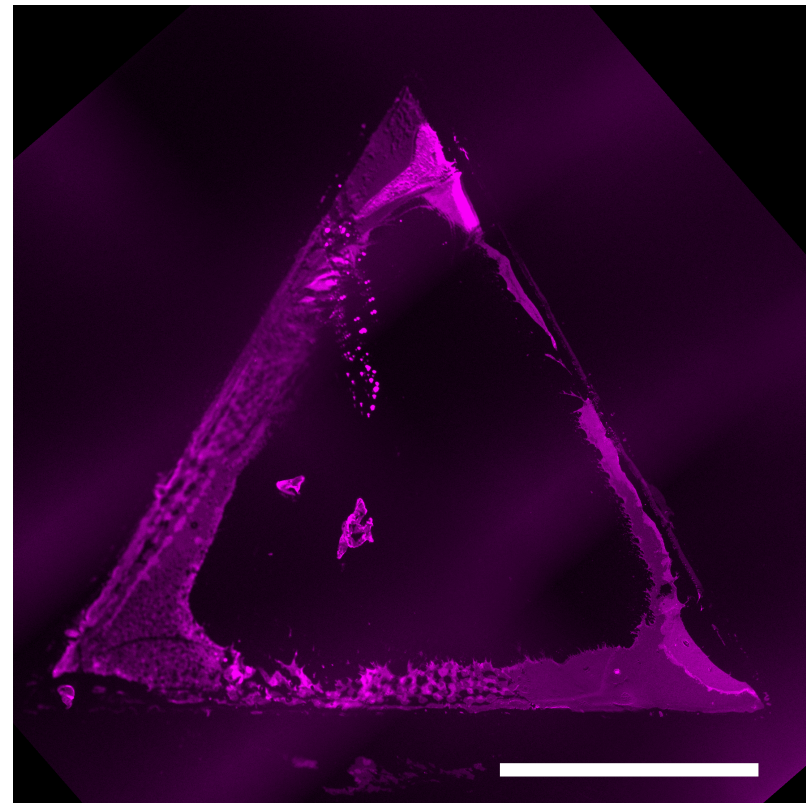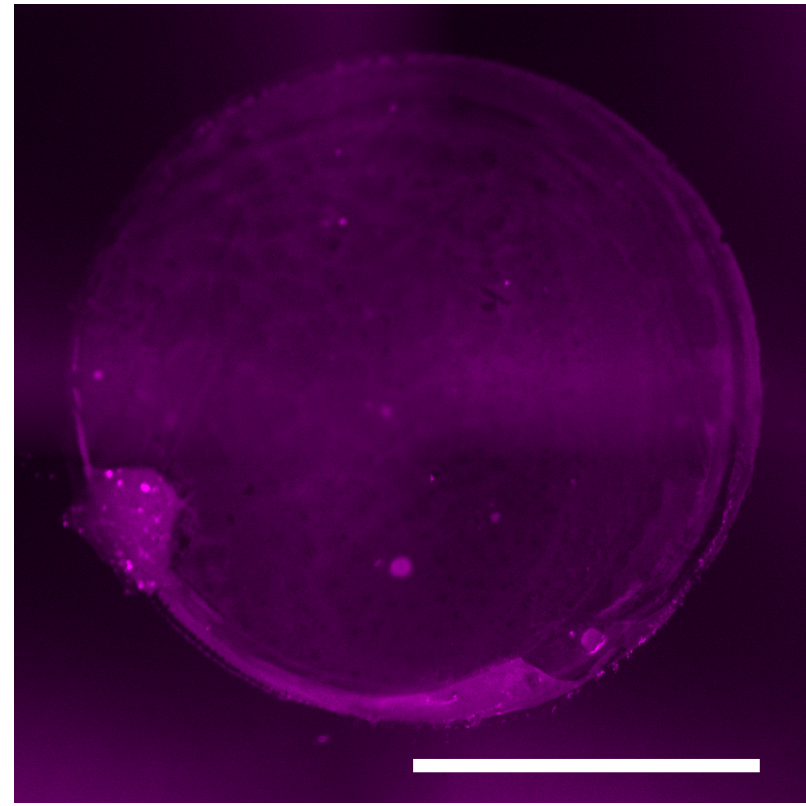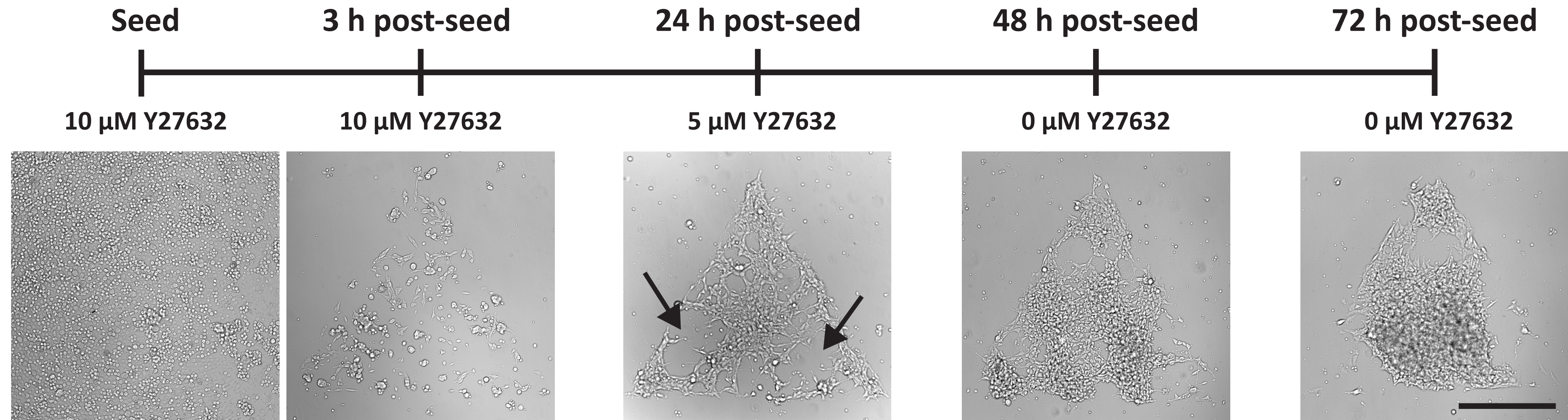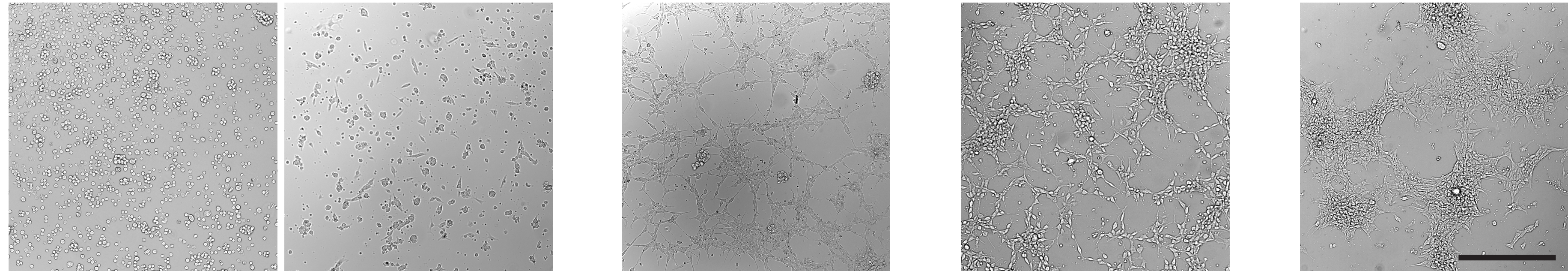| Example SU8 Wafer Fabrication	
1. Spin coat wafer with SU8-3050	Spin at 1,000 RPM for 30 s
2. Soft bake wafer on hot plate	i. 3 min at 65 °C ii. 45 min at 95 °C iii. 3 min at 65 °C iv. Cool to room temperature
3. Expose in mask aligner	i. Align wafer and photomask in mask aligner ii. Expose with energy of 250 mJ/cm^2 ▪ E.g. for lamp with intensity of 11 mW/cm^2, expose for 23 s
4. Post exposure bake on hot plate	i. 1 min at 65 °C ii. 15 min at 95 °C iii. 1 min at 65 °C iv. Cool to room temperature
5. Develop	i. Agitate in SU8 Developer, approx. 5-10 min ii. Check development with isopropyl alcohol (IPA) ▪ If under-developed, IPA rinse will produce a white residue
6. Hard bake on hot plate (optional)	1-2 h at 150 °C

Table 2

Polyacrylamide Gel Formulations									
			Volumes for 1 mL complete solution						
Elastic Modulus (Pa)	Final % acrylamide	Final % Bis-acrylamide	ddH$_2$O (µL)	40% acrylamide (µL)	2% Bis-acrylamide (µL)	10x PBS (µL)	1% TEMED in ddH$_2$O (µL)	Microspheres 0.5% solids in ddH$_2$O (µL)	1% PPS in ddH$_2$O (µL)
1050	3	0.1	565	75	50	100	75	60	75
2700	7.5	0.035	485	187.5	17.5	100	75	60	75
4000	7.5	0.05	477.5	187.5	25	100	75	60	75
6000	7.5	0.07	467.5	187.5	35	100	75	60	75

Table of Materials

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
0.05% Trypsin	Gibco	25300054	
100 mm glass petri dish	Fisher Scientific	08-747B	
100 mm plastic petri dish	Fisher Scientific	FB0875712	
15 mL conical-bottom tubes	Corning	352095	
150 mm plastic petri dish	Fisher Scientific	FB0875714	
18 mm diameter #1 coverslips	Thermo Scientific	18CIR-1	
2% bisacrylamide	Bio-Rad	161-0142	
3-aminopropyltrimethoxysilane	ACROS Organics	313251000	
40% acrylamide	Bio-Rad	161-0140	
Aluminum foil	Fisher Scientific	01-213-100	
Basic fibroblast growth factor	Sigma-Aldrich	F0291	
Bleach	Clorox	N/A	
Centrifuge with swing-buckets	Eppendorf	22623508	Model: 5804 R
Collagen	Corning	354236	
Dessicator	Fisher Scientific	08-642-7	
Ethanol	Fisher Scientific	AC615095000	
Fetal bovine serum	Gibco	16000044	
Fluorescent microspheres	Thermo Scientific	F8821	
Forceps (for coverslips)	Fisher Scientific	16-100-122	
Forceps (for wafers)	Fisher Scientific	17-467-328	
Gel holders	N/A	N/A	Gel holders are custom 3D-printed, CAD drawing available on request
Glutaraldehyde	Fisher Scientific	50-261-94	
HEPES	Thermo Scientific	J16926A1	
Hot plate	Fisher Scientific	HP88854100	
Hydrochloric acid	Fisher Scientific	A144S-500	
Isopropyl alcohol	Fisher Scientific	A416-500	
Kimwipes (delicate task wipes)	Kimberly-Clark Professional	34120	
Knockout serum replacement	Gibco	10828028	
Knockout-DMEM	Gibco	10829018	
Mask aligner (for photolithography)	Karl Suss America, Inc.	Karl Suss MJB3 Mask Aligner	

Matrigel	Corning	354277	
Microscope for traction force	Nikon	N/A	Model: Eclipse TE200 U
Motorized positioning stage	Prior Scientific	N/A	Model: HLD117
Nitrogen gas	Airgas	NI 250	
Norland optical adhesive 74 (UV-curable polymer)	Norland Products	NOA 74	
Oven	Thermo Scientific	PR305225G	
Parafilm (laboratory film)	Fisher Scientific	13-374-12	
PDMS (Sylgard 184)	Fisher Scientific	NC9285739	
Photomask	CAD/Art Services, Inc.	N/A	Photomasks are custom made. CAD drawing for our designs available upon request
Plasma cleaner	Fisher Scientific	NC9332171	
Plastic for gasket	Marian Chicago	HT6135	
Plastic for spacer	TAP Plastics	N/A	Polycarbonate sheet, .01 inch thickness
Potassium chloride (for making PBS)	Fisher Scientific	P217-500	
Potassium phosphate monobasic (for making PBS)	Fisher Scientific	P285-500	
Pottassium persulfate	ACROS Organics	424185000	
Scalpel	Fisher Scientific	14-840-00	
Silicon wafer	Electron Microscopy Sciences	71893-06	Type P, 3 inch, silicon wafers
Sodium chloride (for making PBS)	Fisher Scientific	S271-1	
Sodium hydroxide	Fisher Scientific	S318-100	
Sodium phosphate dibasic dihydrate (for making PBS)	Fisher Scientific	S472-500	
SU8-3050 Photoresist	MicroChem	SU8-3000	
SU8-Developer	MicroChem	Y020100	
TEMED	Bio-Rad	161-0800	
UV-sterilization box	Bio-Rad	N/A	Bio-Rad GS Gene Linker UV Chamber
Y27632 (Rho kinase inhibitor)	StemCell Technologies	72304	

jove
JOURNAL OF
VISUALIZED EXPERIMENTS

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Patterning the geometry of human embryonic stem cell colonies on compliant substrates to control tissue-level mechanics
Author(s):	Jonathon M Muncie, Roberto Falcon-Banchs, Johnathon N. Lakins, Lydia L. .Sohn, Valerie M. Weaver

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

[X] Standard Access   [ ] Open Access

Item 2: Please select one of the following items:

[X] The Author is **NOT** a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

612542.6   For questions, please contact us at submissions@jove.com or +1.617.945.9051.

jove
JOURNAL OF
VISUALIZED EXPERIMENTS

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to
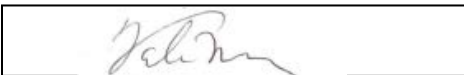
the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Valerie M. Weaver
Department:	Department of Surgery
Institution:	University of California San Francisco
Title:	Professor
Signature:	*[signature]*
Date:	May 23, 2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:
1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

612542.6   For questions, please contact us at submissions@jove.com or +1.617.945.9051.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been thoroughly proofread and checked for spelling and grammatical issues.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

No copyright permission is required since all figures are original.

3. Please use h, min, s for time units.

The manuscript formatting has been modified as requested.

4. Please sort the items in alphabetical order according to the name of material/equipment.

The table of materials has now been sorted in alphabetical order according to the name of the material/equipment.

5. Figure 2: Please use µM instead of uM.

We have updated Figure 2 to use µM instead of uM.

Reviewers' comments:

We thank the reviewers for the constructive comments. We have carefully edited the manuscript and addressed all issues raised by the reviewers. A point by point rebuttal is outlined below. We are confident that our manuscript is now suitable for publication.

Reviewer #1:

Manuscript Summary:

The authors have developed an excellent method that consist in culturing hESC in confined colonies on compliant surfaces which allow then to have control on both the geometry of the culture and the mechanical properties of the substrate. The aim of the method is to recreate a biomimetic environment present during human embryogenesis.

Major Concerns:
No major concerns.

Minor Concerns:
Few typos, just change when appropriated um by µm.

All instances of "uM" have been replaced with "µM".

Reviewer #2:

Manuscript Summary:

This paper reports the method of micro-contact patterning of ECM molecules on PAAm hydrogels with tunable elasticity, which enable to culture hES cells in a confined area of elastic substrate and to apply for traction force microscopy. The detailed protocol for this is useful for many readers.

Major Concerns:

In their method, ECM molecules are contacted to the PAAm surface from the stencil film. In this process, ECM molecules are just adsorbed on the gel surface and not chemically-fixed. Typically, chemical-crosslinkers such as sulfo-SANPAH are used for immobilizing the ECM molecules onto the PAAm surface. They did not describe this typical treatment, which should cause problem on reproducibility of hES cells behaviors. In the case of Matrigel, it works well because Matrigel is hydrophobic and easily strong adsorbed onto substrate surface. But, for other type of ECM molecules such as collagen, fibronectin, their present protocol is rather weak. This problem should be noted.

We agree with the reviewer that this issue constitutes a potential caveat of the method. Because the transfer of ligand from coverslip to hydrogel occurs **during** polymerization of the polyacrylamide, and not after, cross-linking methods such as sulfo-SANPAH treatment are impossible. It may be that other ECM ligands do not adsorb to the polyacrylamide as robustly as matrigel. To address this issue in our revised manuscript we have now incorporated a more thorough description of this caveat in the third paragraph of the discussion section beginning at line 678.

Reviewer #3:

Manuscript Summary:

This manuscript describes a method for generating micropatterned stem cell colonies with defined geometries on hydrogel substrates. This platform supports traction force microscopy and immunostaining-based analysis.

Minor Concerns:

1. The authors indicate a range of photoresist thickness (100-250 microns), which would dictate the features of the PDMS mold and stencil thickness. An expanded commentary on the ideal thickness of the stencil would be helpful to include.

A note has been added to the protocol at line 131 that explains the recommended range of photoresist thickness.

2. A briefly expanded discussion on the UV curable polymer used, and specifically, if there are unique features provided by the Norland 74 material would be beneficial.

We have now added a reference to prior work describing the use of Norland 74 to create patterns of ECM ligand. We have also added a note in the protocol at line 275 that details the properties of Norland 74 that make it ideal for the applications outlined in this methods article.

3. For adding the stencil to the coverslip (step 4.1.3), is manual (by hand) pressure appropriate? Is it possible to check/confirm sufficient contact?

Manual pressure is sufficient for creating a water-tight seal between the stencil and the coverslip. Nevertheless, given the potential that this strategy could contribute to user variability and inconsistency we have now incorporated an optional step to the protocol at line 323 outlining a strategy to confirm sufficient contact is made to create a water-tight seal between the stencil and the coverslip.

4. As currently presented, the polyacrylamide polymerization process during centrifugation is somewhat confusing, although this will likely be more clear in the video representation. Nonetheless, an expanded discussion regarding why this polymerization approach was chosen would be helpful. Could other configurations be used for this gelling process?

We agree that the description needs clarification. The centrifugation process is critical when using this method for traction force microscopy. The centrifugation brings all the microspheres to a single plane just below the top surface of the hydrogel, which is ideal for imaging microsphere displacements that are converted to cell traction stresses. When not performing TFM, centrifugation is unnecessary. We have included this explanation in a note at line 436 of the protocol to clarify any confusion.

5. Is it known how deep into the polyacrylamide the matrigel/collagen is present? Is it transferred solely onto the surface of the polyacrylamide? Also, does the centrifugation process influence the spatial distribution of the fluorescent microspheres used for TFM- is that desired?

We have addressed the purpose of the centrifugation steps above in 4. We have not precisely characterized the thickness of the patterned matrigel/collagen layer that is transferred to the polyacrylamide. Due to the fact that ligand is only transferred at the interface between the coverslip and the polymerizing polyacrylamide, we suggest that the ligand would most likely only be present at the surface of the hydrogel. This is consistent with our imaging of patterned fluorescent ligand, wherein we only observe ligand at the surface of the polyacrylamide. We have now included these details in the representative results at line 543.