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Direct bioprinting of 3D multicellular breast spheroids onto endothelial networks

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1 **TITLE:**
2 **Direct bioprinting of 3D multicellular breast spheroids onto endothelial networks**
3
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16 **KEYWORDS:**
17 Bioprinting, bioinks, 3D in vitro co-culture models, breast spheroids, endothelial cells, cancer
18
19 **SUMMARY:**
20 The goal of this protocol is to directly bioprint breast epithelial cells as multicellular spheroids
21 onto pre-formed endothelial networks to rapidly create 3D breast-endothelial co-culture models
22 which can be used for drug screening studies.
23
24 **ABSTRACT:**
25 Bioprinting is emerging as a promising tool to fabricate 3D human cancer models that better
26 recapitulate critical hallmarks of in vivo tissue architecture. In current layer-by-layer extrusion
27 bioprinting, individual cells are extruded in a bioink together with complex spatial and temporal
28 cues to promote hierarchical tissue self-assembly. However, this biofabrication technique relies
29 on complex interactions among cells, bioinks and biochemical and biophysical cues. Thus, self-
30 assembly may take days or even weeks, may require specific bioinks, and may not always occur
31 when there is more than one cell type involved. We therefore developed a technique to directly
32 bioprint pre-formed 3D breast epithelial spheroids in a variety of bioinks. Bioprinted pre-formed
33 3D breast epithelial spheroids sustained their viability and polarized architecture after printing.
34 We additionally printed the 3D spheroids onto vascular endothelial cell networks to create a co-
35 culture model. Thus, the novel bioprinting technique rapidly creates a more physiologically
36 relevant 3D human breast model at lower cost and with higher flexibility than traditional
37 bioprinting techniques. This versatile bioprinting technique can be extrapolated to create 3D
38 models of other tissues in additional bioinks.
39
40 **INTRODUCTION:**
41 3D in vitro vascularized tumor models are essential tools for mechanistic study of cancer growth
42 and metastasis. For breast cancer in particular, breast epithelial cells cultured in Matrigel
43 organize into polarized spheroids that more closely resemble the in vivo mammary acinus
44 architecture[1-8]. 3D breast epithelial cell culture also impacts cell function, with 3D cultures

45 showing differences in epidermal growth factor (EGF) receptor modulation[8,9]; oncogene function,
46 including ErbB2[10]; growth and apoptosis signaling[11,12]; and chemotherapy resistance[13,14].
47 Vascular endothelial cells similarly respond differently to environmental stimuli in 3D vs.
48 traditional 2D culture[15-18]. However, much of the understanding of vascular endothelial and
49 breast epithelial interactions comes from 2D culture using conditioned medium or Transwell
50 inserts, or 3D models in which the two cell types are physically separated[19-23]. These co-culture
51 models provide limited physiological insight, since both 3D culture and cell-cell contact are critical
52 to vascular endothelial – breast epithelial cell interactions[24-26].
53
54 3D cancer models have been fabricated using a variety of techniques, including hanging drop
55 spheroid formation, bioprinting, magnetic assembly, and culture within hydrogels or on
56 engineered scaffolds[5,27-29]. More recently, 3D tumor models were created with multiple cell
57 types arranged in their respective 3D structures. In one example of a tumor-on-a-chip platform,
58 cancer, endothelial, and stromal cells were mixed into a matrix and then injected into the three
59 central tissue chambers in a polydimethylsiloxane (PDMS) device. The tissue chambers were
60 bordered by two outer channels that represented an artery and venule. After 5-7 days of
61 culture, endothelial cells formed a microvascular network and cancer cells proliferated to form
62 small tumors near the vasculature. This platform was then used to screen drugs and drug
63 combinations[30]. Additional tumor-on-a-chip platforms have been created to study metastasis
64 and cancer types with specific mechanical stimuli (e.g., mechanical strain in the lung)[31,32].
65 However, these platforms generally do not include both vasculature and cancer in their
66 respective 3D structures.
67
68 Biofabrication shows great promise in advancing 3D in vitro vascularized tumor models, since it
69 enables tight spatial control over cell location. Despite growth of bioprinting over the past
70 decade, few studies focus specifically on tumors[33,34]. In one example, 3D printing of HeLa cells in
71 a gelatin/alginate/fibrinogen hydrogel was used to create an in vitro cervical cancer model.
72 Tumor cells were bioprinted as individual cells and then allowed to form spheroids, which
73 showed a higher proliferation rate, increased matrix metalloproteinase expression, and higher
74 chemoresistance than cells in 2D culture[35]. In these studies, as in many others[36,37], dissociated
75 cell suspensions were bioprinted, and then the cell cultures were provided with the required
76 mechanical and biochemical cues to enable the cells to form a 3D structure. However, cellular
77 self-assembly may take days or weeks, may require complex spatial and temporal environmental
78 cues, or may not occur when two cell types are co-cultured. For example, breast epithelial cells
79 induced cell death in endothelial cells in 2D co-culture, and dissociated breast epithelial cells did
80 not form 3D spheroids when bioprinted in alginate/gelatin hydrogels[38]. Dissociated breast
81 epithelial or cancer cells formed spheroids in alginate based bioinks only when entrapped in
82 circular PDMS mold. In other cases, spheroids were formed using suspended droplets in ultra-
83 low attachment circular well plates and then mixed into alginate based bioinks[39,40].
84
85 We now describe an alternative 3D tissue biomanufacturing method in this protocol. Rather than
86 seed dissociated cells and wait for these cells to form the 3D structures, we describe how to
87 create and bioprint 3D tumor spheroids on an vascular tube network to create a tumor co-culture
88 model that can be used almost immediately. Tumor spheroids can be grown in vitro or derived

89 from human tissues (organoids). Similarly, vascular tubes can be grown or can be derived from
90 adipose tissue microvascular fragments. Bioinks can range from biologically inactive alginate to
91 the highly biologically active Matrigel[41]. Since this 3D tumor co-culture model can be created with
92 a variety of cell structures and bioinks, it can incorporate multiple cell types, extracellular
93 matrices, and chemokine gradients[15,42]. While in its current formulation, the endothelial
94 networks cannot be perfused, future iterations could integrate this method with microfluids or -
95 on-chip systems. Bioprinting 3D breast epithelial spheroids onto endothelial networks enables
96 rapid biofabrication of human breast models for drug testing and personalized precision
97 medicine [27].
98
99 **PROTOCOL:**
100
101 **1. Breast Epithelial Cell Growth and Assay Media**
102
103 1.1. MCF10A breast epithelial cells
104
105 NOTE: The non-tumorigenic immortalized breast epithelial cell line is derived from a patient
106 with fibrocystic disease[43]. Cells do not express estrogen receptor.
107
108 1.1.1. To prepare 20 µg/mL epidermal growth factor (EGF), dissolve 100 µg of lyophilized EGF
109 in 500 µL of sterile dH_2O to make 200 µg/mL EGF. Add 500 µL of 200 µg/mL EGF into 4.5 mL of
110 sterile 0.1% BSA in dH_2O to make a 20 µg/mL EGF stock solution. Store prepared 20 µg/mL EGF
111 aliquots at -20 °C for up to 12 months.
112
113 1.1.2. To prepare 500 µg/mL hydrocortisone, dilute 1 mg of hydrocortisone in 1 mL of absolute
114 ethanol (200 proof). Add 1 mL of sterile DMEM F:12 to this mixture to make a 500 µg/mL
115 hydrocortisone stock solution. Store hydrocortisone stock aliquots at -80 °C for up to 12
116 months.
117
118 1.1.3. To prepare 10 µg/mL cholera toxin, dissolve 1 mg of cholera toxin lyophilized powder in
119 1 mL of sterile dH_2O to make a 1 mg/mL cholera toxin stock solution. Store cholera toxin stock
120 aliquots at -80 °C for up to 12 months.
121
122 1.1.4. To prepare Growth Medium, add 500 µL of EGF (20 µg/mL), 500 µL of hydrocortisone
123 (500 µg/mL), 5 µL of cholera toxin (1 mg/mL), 500 µL of bovine insulin (10 mg/mL), 10 mL of
124 penicillin and streptomycin, and 25 mL of horse serum to 500 mL of DMEM F:12 for a final
125 concentration of 20 ng/mL EGF, 500 ng/mL hydrocortisone, 10 ng/mL cholera toxin, 10 ng/mL
126 bovine insulin, 2% v/v penicillin and streptomycin, and 5% v/v horse serum. Antibiotic
127 concentration was increased to 2% to account for decreased sterility in the bioprinting process;
128 however, the antibiotic concentration can be lowered to 1%.
129
130 1.1.5. To prepare Assay Medium, add 500 µL of hydrocortisone (500 µg/mL), 5 µL of cholera
131 toxin (1 mg/mL), 500 µL of bovine insulin (10 mg/mL), 10 mL of penicillin and streptomycin, and
132 25 mL of horse serum to 500 mL of DMEM F:12 for a final concentration of 500 ng/mL

133 hydrocortisone, 10 ng/mL cholera toxin, 10 ng/mL bovine insulin, 2% v/v penicillin and
134 streptomycin, and 5% v/v horse serum.
135
136 1.2. MDA-MB-231
137
138 NOTE: The triple-negative (lacking estrogen receptor, progesterone receptor, and epidermal
139 growth factor receptor 2) breast cancer epithelial cell line is created from pleural effusion of a
140 female patient with metastatic mammary adenocarcinoma[44]. Cells represent an aggressive,
141 invasive, and poorly differentiated breast cancer.
142
143 1.2.1. To prepare Growth and Assay Medium, add 50 mL of fetal bovine serum, 10 mL of
144 penicillin and streptomycin, and 25 mL of horse serum to 500 mL of DMEM for a final
145 concentration 10% v/v fetal bovine serum, 2% v/v penicillin and streptomycin, and 5% v/v horse
146 serum.
147
148 **2. Breast Epithelial Cell Culture**
149
150 2.1. Seed 500,000 MCF10A or MDA-MB-231 cells in a 10 cm (P100) tissue culture dish with
151 10 mL of MCF10A or MDA-MB-231 growth media. MCF10A and MDA-MB-231 cells reach >90%
152 confluence in 48 hours.
153
154 2.2. To **passage breast epithelial cells,** first wash cells with 10 mL of warm PBS.
155
156 2.3. Detach breast epithelial cells by adding 2 mL of 0.05% Trypsin-EDTA to the dish. Place
157 the dish in a 37 °C, 5% CO_2 incubator for 20-25 minutes.
158
159 2.4. Add 5 mL of MCF10A or MDA-MB-231 Growth Medium to the trypsinized cells to
160 neutralize the trypsin. Pipette the cell-media mixture up and down to resuspend cells and break
161 up cell clusters.
162
163 2.5. Add the cell suspension to a 15 mL conical tube and centrifuge at 1,200 x *g* for 3
164 minutes. Carefully aspirate the supernatant and resuspend the cell pellet in 5 mL of MCF10A or
165 MDA-MB-231 Growth Medium.
166
167 2.6. Plate 1 mL of cell suspension in 5 new 10 cm tissue culture dishes along with MCF10A or
168 MDA-MB-231 Growth Medium. Place the dishes in a 37 °C, 5% CO_2 incubator. Cells will be ready
169 to be passaged again in 2-3 days. MCF10A cells can be maintained to passage number 35, after
170 which they show morphological changes. MDA-MB-231 cells can be maintained to passage
171 number 24, after which they show morphological changes.
172
173 **3. Breast Epithelial Spheroid Formation**
174
175 3.1. Freeze 200 µL pipette tips for 30 minutes prior to starting the assay. Keep growth-factor
176 reduced matrix solution (e.g., Matrigel) (10 mg/mL) on ice for the entire process.

177
178 3.2. Slowly pipette 30 µL of ice-cold matrix solution using ice-cold 200 µL pipette tips into
179 each well of an 8-well chamber slide. Start from the sides and drag the pipette tip along the
180 corners, adding a final drop to the center of the well to ensure even coating of each well. Avoid
181 air bubbles during this step to ensure a uniform matrix solution layer. Use new pipette tips for
182 each well.
183
184 3.3. Incubate chamber slides in a 37 °C, 5% CO_2 incubator for 15-20 minutes to polymerize
185 the matrix solution.
186
187 3.4. Resuspend trypsinized MCF10A cells in MCF10A Assay Medium at 200,000 cells/mL or
188 resuspend trypsinized MDA-MB-231 cells in MDA-MB-231 Growth Medium at 200,000 cells/mL.
189
190 3.5. If using MCF10A cells, prepare fresh MCF10A Spheroid Growth Medium by adding 5 µL
191 of EGF (20 µg/mL) and 100 µL of matrix solution to 5 mL of Assay Medium for a final
192 concentration of 2% matrix solution.
193
194 3.6. Remove the chamber slide precoated with matrix solution from the incubator. Add 50
195 µL of MCF10A or MDA-MB-231 cell suspension (10,000 cells) to each well. If using MCF10A
196 cells, add 450 µL of MCF10A Spheroid Growth Medium. If using MDA-MB-231 cells, add 450 µL
197 of MDA-MB-231 Growth Medium premixed with 2% matrix solution (9 µL). Immediate place the
198 chamber slide in a 37 °C, 5% CO_2 incubator.
199
200 3.7. Replace the medium every 4 four days. Carefully pipette 200 µL of old media out from
201 one corner of each well using a 200 µL pipette tip. During this process, tilt the chamber slide at
202 45° to ensure that the spheroids are not disturbed. Add 200 µL of freshly prepared MCF10A
203 Spheroid Growth Medium or MDA-MB-231 Growth Medium previously mixed with 2% matrix
204 solution to each well at the corner in drops to ensure that spheroids remain attached to the
205 matrix layer. Only ~50% of the media is replaced so that all the cytokines produced by the
206 spheroids are not completely depleted.
207
208 NOTE: MCF10A breast epithelial spheroids take up to 8 days to polarize and form hollow
209 centers. MDA-MB-231 breast epithelial spheroids take up to 5 days to form and have no hollow
210 centers.
211
212 **4. Endothelial Cell Network Formation**
213
214 4.1. Human Umbilical Vein Endothelial Cell (HUVEC) Culture
215
216 4.1.1. Add the contents of a single Endothelial Growth Medium-2 kit containing insulin growth
217 factor, fibroblast growth factor, vascular endothelial growth factor, ascorbic acid, epidermal
218 growth factor, heparin and gentamicin-amphotericin B, along with 50 mL of fetal bovine serum,
219 5 mL penicillin-streptomycin and 5 mL of 200 mM glutamine to 500 mL of Endothelial Basal
220 Medium-2 (EBM-2) to create complete Endothelial Growth Medium (EGM-2).

221
222 4.1.2. Seed 500,000 endothelial cells in a 10 cm tissue culture dish in 10 mL of EGM-2. Place
223 cells in a 37 °C, 5% CO_2 incubator until they reach >80% confluency (around 48 hours).
224
225 4.1.3. To passage cells, wash endothelial cells with 10 mL of warm PBS. Detach HUVEC by
226 adding 2 mL of 0.05% Trypsin-EDTA to the 10 cm tissue culture dish. Closely monitor the cells by
227 phase contrast microscopy. Cells are ready when they are balled up but remain attached to the
228 dish.
229
230 4.1.4. Carefully aspirate the trypsin out of the dish. Add 8 mL of EGM-2 to the dish. Wash cells
231 off the dish by pipetting the EGM-2 up and down over the entire dish surface.
232
233 4.1.5. Alternatively, add 5 mL of EGM-2 to the trypsinized cells to neutralize the trypsin.
234 Pipette the cell-media mixture up and down to resuspend cells and break up cell clusters. Add
235 the cell suspension to a 15 mL conical tube and centrifuge at 1,200 x *g* for 3 minutes. Carefully
236 aspirate the supernatant and resuspend the cell pellet in 10 mL of EGM-2.
237
238 4.1.6. Add 1 mL of cell suspension to 9 mL of EGM-2 in a 10 cm tissue culture dish. Place the
239 dishes in a 37 °C, 5% CO_2 incubator. Exchange 2/3 of the medium with fresh EGM-2 every 2
240 days. Cells will be ready to passage in 3-5 days. HUVEC can be maintained up to passage 8 after
241 which they fail to form networks.
242
243 4.2. Endothelial Cell (HUVEC) Network Formation
244
245 4.3. Freeze 200 µL pipette tips for 30 minutes prior to starting the assay. Keep growth-factor
246 reduced matrix solution (10 mg/mL) on ice for the entire process.
247
248 4.4. Slowly pipette 30 µL of ice-cold matrix solution using ice-cold 200 µL pipette tips into
249 each well of an 8-well chamber slide. Start from the sides and drag the pipette tip along the
250 corners, adding a final drop to the center of the well to ensure even coating of each well. Avoid
251 air bubbles during this step to ensure a uniform matrix solution layer. Use new pipette tips for
252 each well.
253
254 4.5. Incubate chamber slides in a 37 °C, 5% CO_2 incubator for 15-20 minutes to polymerize
255 Matrigel.
256
257 4.5.1. Pre-stain a 10 cm dish of HUVEC with 10 µL of red cell tracker (1:1000) for 30 minutes in
258 37 °C, 5% CO_2 incubator.
259
260 4.5.2. Wash endothelial cells with 10 mL of warm PBS. Detach HUVEC by adding 2 mL of 0.05%
261 Trypsin-EDTA to the 10 cm tissue culture dish. Place the dish in a 37 °C, 5% CO_2 incubator for 5
262 minutes to fully detach the cells.
263

4.5.3. Add 5 mL of EGM-2 to the dish to neutralize the trypsin. Transfer the cell suspension to a 25 mL conical tube. Centrifuge HUVEC at 1,200 x *g* for 5 minutes. Aspirate the supernatant and resuspend the cell pellet in 5 mL of serum-free EBM-2.

4.5.4. Count cells using Trypan blue to determine viable cells. Create a 1 x 10^6 cell/mL solution.

4.5.5. Add 100,000 HUVEC to each well with 200 µL of serum free EBM-2. If more cells are added, they will create a surface monolayer rather than a tube network.

4.5.6. Incubate HUVEC in a 37 °C, 5% CO_2 incubator. After 6 hours, image HUVEC networks by phase contrast microscopy. Networks are now ready for coculture with breast spheroids.

5. Bioprinting Breast epithelial spheroids on pre-formed HUVEC Networks

NOTE: A dual nozzle bio-deposition system should be used for the biofabrication process. In this case, the system had three motion arms to allow micron-scale spatial control of material deposition as well as two screw driven motors to deposit bioink from 10 mL syringes. The system should be functionalized with a high efficiency particulate air filtration system as well as UV-sterilization capabilities to maintain a sterile environment during bioprinting. The bioprinter is UV sterilized for an hour before the printing process.

5.1. Create breast epithelial spheroids and HUVEC networks as previously described. Estimate the number of breast epithelial spheroids in each well by counting spheroids in representative phase contrast microscopy images.

5.2. Cut 0.5 cm off the end of a 1000 µL pipette tip. Use the cut pipette tip to carefully pipette all spheroids out of the 8-well chamber slide and into a 50 mL tube. Resuspend spheroids in the selected bioink at 100 spheroids/100 µL.

NOTE: Higher spheroid concentrations may result in spheroid clustering and prevent visualization of interactions between the spheroids and the endothelial networks.

5.3. Pass the spheroid mixture through a 70 µm cell strainer to remove any large or clustered spheroids.

5.4. Load the pooled spheroids into a 10 mL sterile syringe and cap it with a 25-gauge sterile needle. Attach the syringe to the bio-deposition system.

5.5. Aspirate the medium off the HUVEC networks in the 8-well chamber slide.

5.6. Extrude 100 µL of breast epithelial spheroids onto 6 wells of HUVEC networks at a flow rate of 1 mL/min. Maintain 2 wells of HUVEC networks as controls.

307 5.7. Add 400 µL of MCF10A Spheroid Growth Medium if using MCF10A spheroids printed on
308 HUVEC networks or add 400 µL MDA-MB-231 Growth Medium previously mixed with 2% matrix
309 solution if using MDA-MB-231 spheroids printed on HUVEC networks. The respective spheroid
310 growth medium should be used in HUVEC control wells.
311
312 5.8. Incubate co-cultures in a 37 °C, 5% CO_2 incubator for 24 – 96 hours without a media
313 change. Co-cultures will remain viable in the original medium for up to four days.
314
315 **6. Confocal Microscopy**
316
317 6.1. Immunofluorescence and PBS-Glycine Wash Buffer Preparation
318
319 6.1.1. Prepare Immunofluorescence (IF) buffer 10x stock solution by adding 2.5 g of sodium
320 azide, 5 g of bovine serum albumin, 10 mL of Triton X-100, and 2.05 mL of Tween-20 in 500 mL
321 of 10x PBS. Adjust pH to 7.4. Store the stock solution for up to a year at 4 °C to avoid
322 sedimentation.
323
324 6.1.2. Create a working IF buffer by diluting 50 mL of 10x IF buffer in 450 mL of sterile
325 deionized water. Store the working solution at room temperature for up to one week.
326
327 6.1.3. Prepare 10x PBS-Glycine buffer stock solution by adding 37.5 g of glycine to 500 mL of
328 10x PBS. Adjust pH to 7.4. Store the stock solution for up to 6 months at 4 °C to avoid
329 sedimentation.
330
331 6.1.4. Create a working PBS-Glycine solution by diluting 50 mL of 10x PBS-Glycine buffer in 450
332 mL of sterile deionized water. Store the working solution at 4 °C for up to one week.
333
334 6.2. Label Bioprinted Samples and Image by Confocal Microscopy
335
336 6.2.1. Aspirate medium from bioprinted 3D co-cultures and rinse 3 times with warm PBS. Fix
337 bioprinted 3D co-cultures with 4% paraformaldehyde for 1 h at room temperature. Rinse
338 samples 3 times for 20 minutes with 1x PBS-Glycine.
339
340 6.2.2. Block samples with IF buffer mixed with 10% goat serum for 90 minutes (primary block),
341 followed by 40 minutes with IF buffer plus 10% goat serum and Affinipure Fab fragment (1:100,
342 secondary block).
343
344 6.2.3. If using MCF10A spheroids, label samples with a primary antibody for integrin α6 (1:100)
345 in secondary blocking buffer overnight at 4 °C, followed by an Alexa Fluor 488 secondary
346 antibody (1:200) and Hoescht 33342 (1:1000) for 1 h at room temperature protected from light.
347 MCF10A cell lines express high levels of integrin α6, which is essential for showing spheroid
348 polarization and morphology.
349

350 6.2.4. If using MDA-MB-231 spheroids, which express low levels of integrin α6, label samples
351 with Alexa Fluor 488 phalloidin (1:100) and Hoescht 33342 (1:1000) in secondary blocking
352 buffer for 4 hours at room temperature protected from light. Phalloidin enables actin filament
353 visualization so spheroid amorphous and invasive morphology can be assessed.
354
355 6.2.5. Wash samples with 1X PBS-glycine 3 times for 20 minutes.
356
357 6.2.6. Prepare samples for mounting by removing the chamber slide using the manufacturer's
358 tool. Add a small drop of antifade solution to each well. Place a 22 mm x 60 mm coverslip on
359 each chamber slide and carefully seal the edges with clear nail polish.
360
361 6.2.7. Image samples using a confocal microscope as Z stacks of ~10 slices in 5 µm steps. If
362 desired, compress Z planes into a single plane using the Extended Focus command in cell
363 imaging software.
364
365 6.2.8. Quantify spheroid adhesion to endothelial networks using Image J. The number of
366 adhered spheroids can be quantified using the analyze plugin with the appropriate particle size
367 and circularity. Normalize the number of attached spheroids to the image area.
368
369 6.2.9. To ensure reproducibility, quantify the number of adhered spheroids in 4 x 4 tiled
370 images of co-cultures. If the spheroid number is statistically significantly lower than other
371 experiments, the experiment should be repeated.
372
373 **REPRESENTATIVE RESULTS:**
374 Breast epithelial cells should self-organize into 3D spheroids after 5-8 days of culture on matrix
375 solution and in culture medium with 2% matrix solution. Non-tumorigenic MCF10A breast
376 epithelial spheroids should appear round and have a hollow center, with integrin α6 polarized to
377 the outer edge of the spheroid (**Figure 1**, inset shows hollow centers). Highly invasive MDA-MB-
378 231 breast cancer epithelial cells form irregular spheroids. Spheroids should be used when they
379 are around 100 – 300 µm in diameter. When spheroids become too large and get in close
380 proximity, the spheroids will join together to form megaspheroids. In addition, MDA-MB-231
381 breast epithelial spheroids may show cells migrating out of the spheroids if maintained in the
382 Matrigel culture for too long.
383
384 HUVEC should self-organize into tube-like networks after 6-8 hours of sparse, serum-free culture.
385 Samples will have multicellular nodes with connections that are formed of lines of 1-3 cells in
386 parallel. The HUVEC networks can be imaged by phase contrast microscopy or by confocal
387 microscopy if they are labeled with Cell Tracker and Hoescht (**Figure 2**). The ImageJ angiogenesis
388 analyzer can be used to quantify network junctions, segments, and branches. HUVEC networks
389 will die if left in serum-free medium for longer than 16 hours.
390
391 When breast epithelial spheroids are bioprinted onto the HUVEC networks, both spheroids and
392 networks should maintain their original morphology for at least 24 hours. MCF10A breast
393 epithelial spheroids will appear as round objects that adhere directly to the endothelial networks,

394 while MDA-MB-231 breast epithelial spheroids will appear more amorphous yet still attached or
395 in close proximity to the endothelial networks (**Figure 3**). HUVEC networks will be maintained
396 when co-cultured with breast epithelial spheroids. For co-cultures longer than 24 hours, the
397 breast epithelial cells may migrate out of the spheroids and along the endothelial networks. In
398 our experience, this happens earlier in tumorigenic rather than non-tumorigenic breast epithelial
399 cells[38]. We previously demonstrated using bioprinted co-cultures that drug testing can be
400 initiated as early as 2 hours after spheroid bioprinting, for example to test spheroid adhesion
401 onto endothelial networks[45]. We have also shown that 3D breast spheroids are more resistant to
402 anti-cancer drugs like Paclitaxel than when printed as individual cells or in co-culture[41,45]. In the
403 absence of bioprinted spheroids, HUVEC network control wells on their own fail to hold their
404 network morphology and die after 16 h.
405
406 **FIGURE AND TABLE LEGENDS:**
407 **Figure 1: Representative confocal microscopy images of breast epithelial spheroids.** MCF10A
408 spheroids were labeled for integrin α6 (green) and nuclei (blue). Cell phenotype can be confirmed
409 after bioprinting when spheroids appear round with a hollow center (inset) and have integrin α6
410 polarized at the outer edges. MDA-MB-231 spheroids were labeled for actin (green) and nuclei
411 (blue). Cell phenotype can be confirmed when spheroids are irregularly shaped without hollow
412 centers and have cell processes invading into the surrounding matrix. Scale bar = 50 µm.
413
414 **Figure 2: Representative images of HUVEC networks by phase contrast and confocal**
415 **microscopy.** HUVEC networks appear as small multicellular nodes with lines of cells connecting
416 the nodes. For confocal microscopy, cells were labeled with Cell Tracker Red and Hoescht for
417 nuclei (blue). Scale bar = 100 µm.
418
419 **Figure 3: Representative images of breast epithelial spheroids co-cultured with HUVEC**
420 **networks.** MCF10A spheroids, labeled for integrin α6 (green) and nuclei (blue), remain round and
421 appear adhered directly to the endothelial networks. MDA-MB-231 spheroids, labeled for actin
422 (green) and nuclei (blue), appear amorphous yet remain near or on endothelial networks. Co-
423 cultures maintain this morphology for at least 24 hours after bioprinting, after which breast cells
424 may migrate out along the endothelial tubes. Scale bar = 50 µm.
425
426 **DISCUSSION:**
427 This protocol is first of its kind to bioprint spheroids in their 3D architecture for co-culture with
428 endothelial cells also in their 3D architecture. Critical protocol steps include the initial formation
429 of breast epithelial spheroids and HUVEC networks. Extreme caution must be taken in feeding
430 breast epithelial spheroids, as they are easily disrupted from the matrix solution. Similarly, breast
431 epithelial spheroids must be treated with care when they are pipetted off the matrix solution and
432 mixed into the networks. HUVEC networks should not be plated at too high of a density or left
433 for longer than 16 hours, as they will form a monolayer or die, respectively. Finally, all bioprinting
434 should occur in a sterile environment at 37 °C to maximize cell viability.
435
436 Breast epithelial spheroids can be bioprinted in a variety of bioinks besides Matrigel, including
437 alginate and alginate-collagen blends. We demonstrated that spheroids were viable and

438 maintained their morphology when printed in alginate-based bioinks[41]. Thus while we present
439 bioprinting here in matrix solution-based bioink, other less expensive and easier to use bioinks
440 are also possible. We additionally used other breast cancer cell lines, including MCF-7 and
441 genetically modified MCF10A-NeuN cells with similar success[38,41,45]. Alternative means could also
442 be used to create the breast epithelial spheroids. For example, Lee et al. used hydrogel microwell
443 arrays produced using PDMS stamps to create uniformly sized spheroids of controlled size[46].
444 Finally, alternative endothelial cells such as tumor-derived endothelial cells could be used, and
445 rather than forming HUVEC networks, adipose-tissue derived microvessels could be directly
446 bioprinted as 3D vascular structures[47].
447
448 A primary limitation of this method was the challenge in controlling bioprinted spheroid location
449 and number. Spheroids had to be printed with relatively large nozzles and in inviscid fluids to
450 prevent spheroid damage. Rapidly gelling bioinks might better control spheroid location.
451 Spheroids also could not be counted in the bioink, since they were too large for our cell counter.
452 We relied instead on spheroid counts derived from phase contrast images taken prior to pipetting
453 spheroids off the Matrigel surface. Alternative means of forming the spheroids could better
454 control their number and size. We were able to control spheroid number and size with moderate
455 accuracy by using cell strainers and culturing spheroids in the same way each time. A final
456 limitation is that breast epithelial cells migrate out of the spheroids and along the endothelial
457 networks over time. It is possible that alternative bioinks would abrogate this limitation.
458
459 Direct bioprinting of breast epithelial spheroids on pre-formed HUVEC networks enables the
460 creation of a 3D in vitro tumor co-culture model in a short time. Researchers can then rapidly
461 examine interactions between spheroids and vasculature with higher throughput. In the future,
462 breast epithelial spheroids could be bioprinted into perfused vasculatures, which would allow
463 study of flow effects. In addition, tumor-derived organoids and endothelial cells could be
464 bioprinted to enable precision medicine through testing of drug efficacy in a patient-specific
465 model.
466
467 **ACKNOWLEDGMENTS:**
468 This research was funded by NIH 1R01HL140239-01 to AMC. We would like to thank the Cell
469 Imaging Center at Drexel University.
470
471 **DISCLOSURES:**
472 The authors have nothing to disclose.
473
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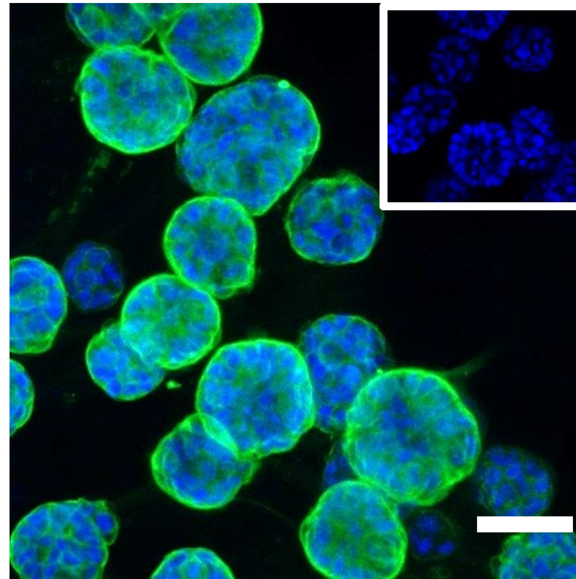

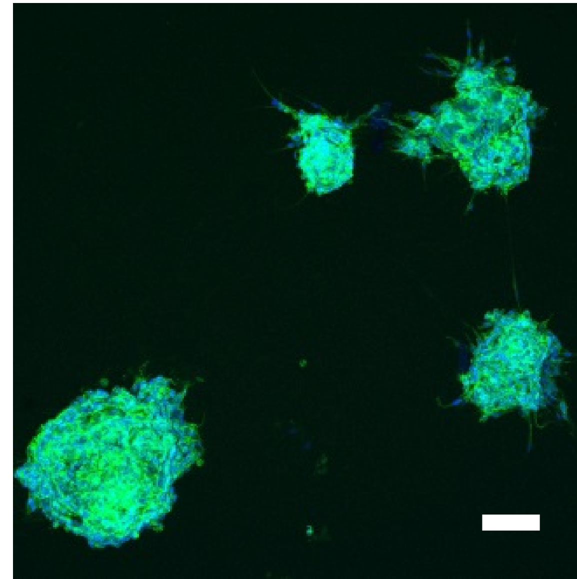

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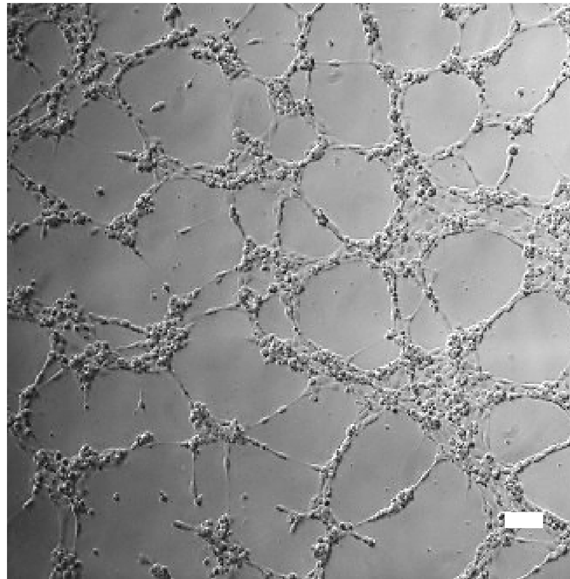

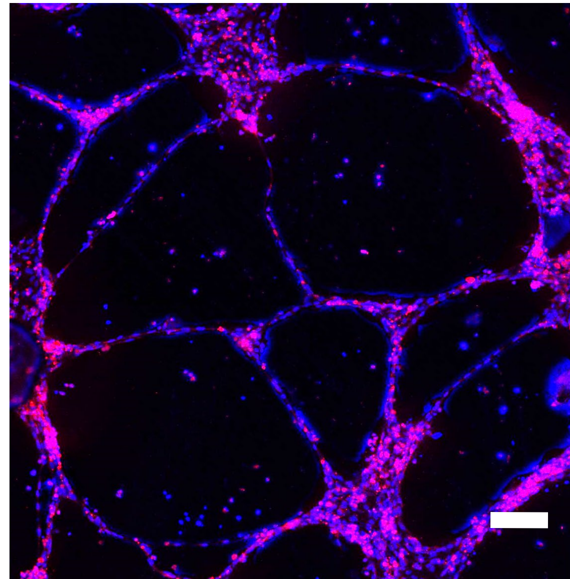

Figure 3

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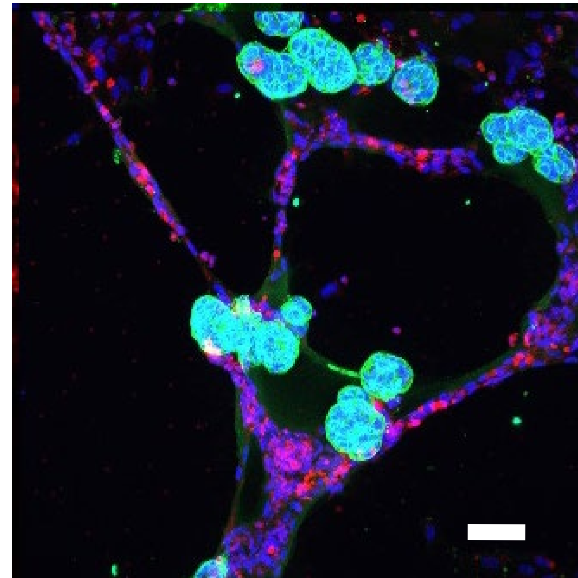

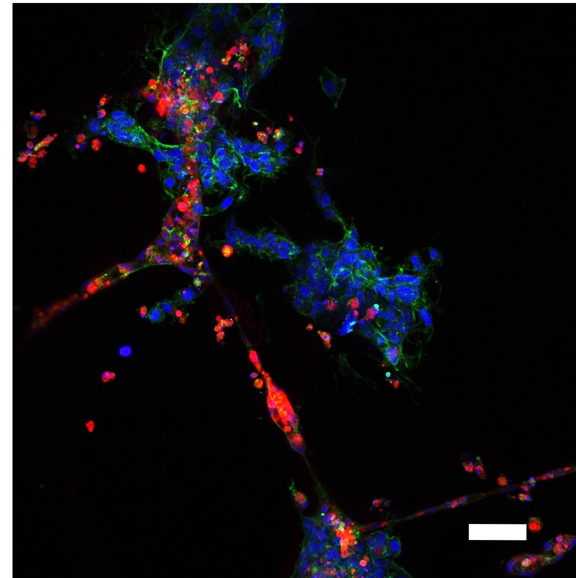

Name of Material/Equipment	Company
37°C incubator, 5% CO2 and 95% humidity	Sanyo
3D Bio printer	custom-made
8-well chamber slides	VWR, Radnor, PA
25-gauge needle	Sigma, St. Louis, MO
Absolute ethanol (200 proof)	Sigma, St.Louis, MO
Affinipure F(ab')2 fragment goat anti-mouse IgG	Jackson ImmunoResearch, West Grove, PA
Alexa Fluor 488 (1:200)	Thermo Fisher, Waltham, MA
Bovine insulin	Sigma, St.Louis, MO
Bovine serum albumin (BSA)	Sigma, St.Louis, MO
Falcon 70 µm Cell Strainer	Corning, Corning, NY
CellTracker™ Red CMTPX Dye	Thermo Fisher, Waltham, MA
Compact Centrifuge	Hermle- Labnet, Edison ,NJ
Cholera Toxin	Sigma, St.Louis, MO
Conical tubes 15 mL	VWR, Radnor, PA
Countess II-FL Cell counter	Thermo Fisher, Waltham, MA
Glass pipettes (10 mL)	VWR, Radnor, PA
DMEM F:12	Thermo Fisher, Waltham, MA
DMEM 1X	VWR, Radnor, PA
Endothelial Basal Medium-2 (EBM-2)	Lonza, Durham, NC
Endothelial Growth Medium-2 (EGM-2)	Lonza, Durham, NC
Alexa Fluor™ 488 Phalloidin	Thermo Fisher, Waltham, MA
Fetal Bovine serum	Cytiva, Logan, UT
Goat serum	Thermo Fisher, Waltham, MA
Glycine	Sigma, St.Louis, MO
Hoescht 33342	Thermo Fisher, Waltham, MA
Horse Serum	Thermo Fisher, Waltham, MA
Hydrocortisone	Sigma, St.Louis, MO
Human Umblical Vein Endothelial cells (HUVECs)	Cell applications, San Diego , CA
Integrin α6	Millipore, Billerica, MA
Live Dead assay	Thermo Fisher, Waltham, MA
LSM 700 Confocal microscope	Zeiss, Thornwood, NY

Matrigel - growth factor reduced 10 mg/ml	VWR, Radnor, PA
MCF10A cells	ATCC
MDA-MB-231 cells	ATCC
Paraformaldehyde	Sigma, St.Louis, MO
Penicillin and streptomycin	Thermo Fisher, Waltham, MA
Phosphate Buffered Saline 1X (PBS)	Thermo Fisher, Waltham, MA
Phosphate buffer saline 10X	Thermo Fisher, Waltham, MA
Prolong gold antifade	Thermo Fisher, Waltham, MA
Recombinant Human Epidermal Growth Factor, EGF	Peprotech, Rocky Hill, NJ
Sodium Azide	Sigma, St.Louis, MO
Sterile syringe (10 mL)	VWR, Radnor, PA
Tissue culture dish (10cm)	VWR, Radnor, PA
Triton X-100	Sigma, St.Louis, MO
Trypan blue 0.4%	Thermo Fisher, Waltham, MA
Trypsin-EDTA 0.05%	Thermo Fisher, Waltham, MA
Tween -20	Thermo Fisher, Waltham, MA
Vascular Endothelial Growth factor ($VEGF_{165}$)	Peprotech, Rocky Hill, NJ
Volocity 6.3 cell imaging software	PerkinElmer, Hopkinton, MA

Catalog Number	Comments/Description
MCO-20AIC	Cell incubation
None	Used for bioprinting
53106-306	for seeding spheroids
Z192406-100EA	bioprinting syringe needle
E7023-500ML	reconsitution of media components
115006020	secondary block - Immunofluorescence
A-11006	Seconday antibody-Immunofluorescence
I-035-0.5ML	MCF10A Media additive
A2153-500G	Blocking agent -Immunofluorescence
352350	Remove large or clustered spheroids
C34552	pre-stain for HUVEC tubes
Z206A	For cell centrifugations
C8052-.5MG	MCF10A Media additive
62406-200	Collecting and resuspending cells
AMQAF1000	counting cells
76184-746	cell resuspension
11320033	MCF10A basal media
10-014-CV	MDA-MB-231 basal media
CC-3156	HUVEC basal media
CC-3162	Accompanied with a Bulletkit (containing growth factors)
	Labelling MDA-MB-231 spheroids
SH30071.03	HUVEC/MDA-MB-231 media additive
16210064	Live and dead cell stain assay for cell viability
G8898-500G	immunofluorescence buffer component
62249	Nuclei stain immunofluorescence
16050130	MCF10A Media additive
H0888-5G	MCF10A Media additive
200-05f	Endothelial cell lines
MAB1378	Immunofluorescence spheroid labelling component
L3224	Live and dead cell stain assay for cell viability
	Used to visualize cells

354230	Spheroid formation
CRL-10317	Breast cell line
HTB-26	Breast cell line
158127-500G	cell fixative
15140122	MCF10A / MDA-MB-231/HUVEC Media additive
7001106	Wash buffer for cells before trypsinization
AM9625	immunofluorescence buffer component
P36934	immunofluorescence mountant medium
AF-100-15	MCF10A/ assay media component
S2002-25G	immunofluorescence buffer component
75846-757	bioprinting process
25382-166	monolayer cell culture
T8787-250ML	immunofluorescence buffer component
15250061	cell counter additive
25300054	cell detachment
85113	immunofluorescence buffer component
100-20	HUVEC tube additive
	Z stack compresser

Rebuttal Letter
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August 24, 2020

Nam Nguyen, Ph.D.
Manager of Review
JOVE – Journal of Visualized Experiments

Original Manuscript: JoVE61791
Authors: Swaminathan and Clyne
Original Title: Direct bioprinting of 3D multicellular spheroids onto endothelial tubes enables rapid biofabrication of human vascularized breast cancer models

Dear,

Thank you for the thorough review of our manuscript # JoVE61791 with the new title "Direct bioprinting of 3D multicellular breast spheroids onto endothelial networks." We appreciate the thoughtful critique. We are pleased that the reviewers find the paper "well-written, with clearly defined steps provided for the protocol section."

We addressed all of the reviewers' comments, as detailed in the following document. Specifically, we clarified the writing, including replacing terms such as vascularized and tubes; added additional references for 3D tissue models; clarified methods including spheroid counting, labeling, and image quantification; and added protocol advantages and disadvantages in the introduction. The revised paper is improved after implementation of the reviewers' suggestions.

Enclosed please find the revised version of our manuscript. Appended below is a detailed response to each comment raised. Thank you for reconsidering our manuscript for publication in the Journal of Visualized Experiments.

Sincerely,

Alisa Morss Clyne, Ph.D.
Associate Professor

RESPONSE TO REVIEWERS

Thank you for your expert and insightful comments on the manuscript. We responded to and acted upon each of your suggestions and concerns. This paper is significantly improved as a result of the review, and we are grateful for the scrutiny and care with which it was previously considered. Below we include our response to each item in detail. We listed each concern raised verbatim and followed with an explanation of how we revised the manuscript through textual emendation as suggested.

Reviewer #1

Concern 1:

Abstract: Line 36: "While bioprinted individual breast epithelial cells formed spheroids only in Matrigel, the pre-formed 3D spheroids sustained their viability, polarized architecture, and function for over a week in inexpensive alginate-based bioinks." Alginate-based bioinks were used by the authors in a different article, not in this manuscript. These claims should be substantiated in the current work with additional data.

Response 1:

Thank you for your suggestion. We removed this sentence from the abstract, which now reads:

> *Abstract, page 1*
> "Bioprinted pre-formed 3D breast epithelial spheroids sustained their viability and polarized architecture after printing."

Concern 2:

Abstract Line 40: "…and used the co-culture model for drug screening studies." No drug screening studies were performed in the current manuscript.

Response 2:

Thank you for pointing this out. We removed the statement from the abstract, which now reads:

> *Abstract, page 1*
> "We additionally printed the 3D spheroids onto vascular endothelial cell networks to create a co-culture model."

Concern 3:

Introduction: Only two examples of 3D models are discussed: tumor-on-a-chip and 3D printing of Hela cells in a gelatin/alginate/fibrinogen hydrogel. A few more could be included.

Response 3:

Thank you for the suggestion. We included several additional references as examples of 3D models.

> *Introduction, page 2*
> "3D cancer models have been fabricated using a variety of techniques, including hanging drop spheroid formation, bioprinting, magnetic assembly, and culture within hydrogels or on engineered scaffolds[5,27-29]."

Concern 4:

Introduction: Line 83: "…dissociated breast epithelial cells did not form 3D spheroids when bioprinted in alginate/gelatin hydrogels": Other researchers have successfully fabricated with different bioinks. Please give more examples to support lines 81 and 82.

Response 4:

We apologize for not clarifying this statement in the original paper. Breast epithelial and cancer cells fail to form spheroids on their own in bioinks like alginate or gelatin. To form spheroids in these bioinks, the

cells must be trapped in synthetic molds or suspended in ultra-low attachment well plates and later collected and mixed in these bioinks. We now clarify this as:

> *Introduction, pages 2-3*
> "Dissociated breast epithelial or cancer cells formed spheroids in alginate based bioinks only when entrapped in circular PDMS mold. In other cases, spheroids were formed using suspended droplets in ultra-low attachment circular well plates and then mixed into alginate based bioinks[39,40]."

Concern 5:
Line 273: Two out of 8 wells were used as controls but have not been discussed. Also, authors have not mentioned what medium was added to the controls.

Response 5:
We are sorry for omitting this information. We now include:

> *Protocol 5.8, page 8*
> "The respective spheroid growth medium should be used in HUVEC control wells."

Concern 6:
Recheck scale bars in all the figures. Why do the spheroids of MCF10A look smaller in Figure 3 when compared to Figure 1 although the scale bars are same/similar?

Response 6:
Thank you for bringing this to our attention. We double checked the scale bars and modified them in Figures 1 and 3.

Concern 7:
Please discuss the time-window available for drug-screening tests post-fabrication of the 3D model; especially when the authors state - "For co-cultures times longer than 24 hours, the breast epithelial cells may migrate out of the spheroids and along the endothelial tubes"

Response 7:
We previously demonstrated using co-cultures that drug testing can be done as early as 2 hours after bioprinting. We now cite this in the paper as:

> *Representative Results, page 10*
> "We previously demonstrated using bioprinted co-cultures that drug testing can be initiated as early as 2 hours after spheroid bioprinting, for example to test spheroid adhesion onto endothelial networks[44]."

Concern 8:
Line 389: "We also demonstrated that breast organoids derived directly from tumors could be bioprinted." - Authors have not shown this in the current study. Please cite appropriate references.

Response 8:
Thank you for bringing this to our attention. These data have not been published. We removed this statement.

Concern 9:
Line 397: "Spheroids also could not be counted in the bioink, since they were too large for our cell counter." How did the authors resuspend spheroids in the selected bioink at 100 spheroids/100 µL (line 266)?

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Response 9:
Thank you for pointing out this ambiguity. Spheroids were counted by in image taken prior to removing the spheroids from the Matrigel. They were then resuspended at 100 spheroids/100 µL using these counts. We now clarify this in the text as:

Discussion, page 11
"Spheroids also could not be counted in the bioink, since they were too large for our cell counter. We relied instead on spheroid counts derived from phase contrast images taken prior to pipetting spheroids off the Matrigel surface."

Concern 10:
Authors mention using cell strainers on line 399 in the discussion, but this was not stated in the protocol.

Response 10:
Thank you for finding this omission. We added a step in the protocol on controlling spheroid size using cell strainers and added cell strainers to the materials list.

Protocol 5.4, page 7
"5.4. Pass the spheroid mixture through a 70 µm cell strainer to remove any large or clustered spheroids."

Concern 11:
Please confirm cell phenotype post-printing using an appropriate assay. Also, how does this model compare with other 3D models in which the two cell types are physically separated?

Response 11:
MCF10A and MDA-MB-231 phenotypes are confirmed morphologically using confocal microscopy. MCF10A spheroids are round with hollow centers, and integrin α6 is polarized to the outer spheroid edge. In contrast, MDA-MB-231 spheroids are irregularly shaped with cell processes invading into the surrounding matrix. We observed these morphological featured both when the breast spheroids were printed individually (Figure 1) and onto the vascular endothelial networks (Figure 2). We now clarify this in the text as:

Figure and Table Legends, page 10
"Figure 1: Representative confocal microscopy images of breast epithelial spheroids. MCF10A spheroids were labeled for integrin α6 (green) and nuclei (blue). Cell phenotype can be confirmed after bioprinting when spheroids appear round with a hollow center (inset) and have integrin α6 polarized at the outer edges. MDA-MB-231 spheroids were labeled for actin (green) and nuclei (blue). Cell phenotype can be confirmed when spheroids are irregularly shaped without hollow centers and have cell processes invading into the surrounding matrix. Scale bar = 100 µm."

Minor Concerns:

Concern 12:
Table of materials: (a) add the company for 3D Bioprinter, (b) the description of "LSM 700 Confocal microscope" says "MCF10A Media additive" which is incorrect, (c) Remove "Live Dead assay" from the list as it was not used in this protocol, (d) Trypan blue was not mentioned in the manuscript text.

Response 12:
Thank you for bringing these to our attention. We added that the 3D Bioprinter was custom-made, updated the confocal microscope description, removed the Live/Dead assay, and added Trypan blue into the cell counting protocol as:

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Protocol 4.5.4, page 6

"4.5.4. Count cells using Trypan blue to determine viable cells. Create a 1 x 10^6 cell/mL solution."

Concern 13:

Line 78: "In these studies, as in many others, ..." Please cite other references.

Response 13:

Thank you for your suggestion. We have included references to support the statement:

Introduction, page 2

"In these studies, as in many others[36,37], dissociated cell suspensions were bioprinted, and then the cell cultures were provided with the required mechanical and biochemical cues to enable the cells to form a 3D structure."

Concern 14:

Line 185: "… MDA-MB-231 cells, add 450 µL MDA-MB-231 Growth Medium along with 2% Matrigel (9 µL)." Please clarify that the Matrigel is mixed together first with medium prior to addition.

Response 14:

Thank you for bringing this to our attention. We amended the protocol to clarify that Matrigel was previously mixed with the medium as:

Protocol 3.7, page 5

"Add 200 µL of freshly prepared MCF10A Spheroid Growth Medium or MDA-MB-231 Growth Medium previously mixed with 2% Matrigel to each well at the corner in drops to ensure that spheroids remain attached to the Matrigel layer."

Concern 15:

Section 3.7: Please clarify why only 200 µL out of the 450 µL medium is replaced every 4 days.

Response 15:

Only ~50% of the media is replaced so that all the cytokines produced by the cells are not completely depleted. We now include this as:

Protocol 3.7, page 5

"Only ~50% of the media is replaced so that all the cytokines produced by the spheroids are not completely depleted."

Concern 16:

In section 4.1, there is no centrifugation step unlike sections 3 and 4.2. Please clarify.

Response 16:

Cells can be passaged either by trypsinizing them only until they ball up and then washing them off the plate with medium, or by trypsinizing them until they float and then centrifuging them. It is easier to passage HUVECs using the first method, while it is easier to passage breast epithelial and cancer cells using the second method since they are more adherent. We added an alternative method for passaging the HUVECs using centrifugation, in case the reader finds that methods to be more straightforward.

Protocol 4.1.5, page 6

"4.1.5. Alternatively, add 5 mL EGM-2 to the trypsinized cells to neutralize the trypsin. Pipette the cell-media mixture up and down to resuspend cells and break up cell clusters. Add the cell suspension to a 15 mL conical tube and centrifuge at 1200 x g for 3 minutes. Carefully aspirate the supernatant and resuspend the cell pellet in 10 mL EGM-2."

Concern 17:

Line 278: "Incubate co-cultures in a 37°C, 5% CO2 incubator for 24 - 96 hours." Please clarify media exchanges during this period.

Response 17:

No media replacement was necessary during the co-culture since media replacements for spheroids were done once every 4 days. We now clarify this as:

> *Protocol 5.9, page 8*
>
> "5.9. Incubate co-cultures in a 37°C, 5% CO2 incubator for 24 – 96 hours without a media change. Co-cultures will remain viable in the original medium for up to four days."

Concern 18:

Sections 1.1.1., 1.1.2. and 1.1.3. - Sub-heading says 20 ng/mL, 500 ng/mL and 10 ng/mL, respectively. However, the associated text says 20 µg/mL, 500 µg/mL and 1 mg/mL. Please recheck the concentrations.

Response 18:

We modified the text accordingly.

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Reviewer #2

Manuscript Summary:
The manuscript by Swathi Swaminathan and Alisa Morss Clyne describes the preparation and co-culture of breast cancer spheroids and HUVECs. The title is a bit misleading as the model is not vascularized in the physiological sense, but rather it is co-cultured with HUVEC tubules that are not perfusable. A simple search in PubMed using the search words "bioprinting spheroids" provides many other publications in which spheroids are bioprinted with endothelial cells, so this is not really new to the field. It is also not really justified that it is necessary to prepare these constructs using bioprinting. It seems like similar results could be obtained simply by pipetting the spheroids onto the HUVECs.

Response:
Thank you for your suggestion. We simplified the title to remove the vascularization and replaced “tubes“ with “networks“ throughout the paper. The reviewer is correct that the spheroids could be pipetted onto the HUVEC networks; however, bioprinting enables improved spatial control of spheroid location.

Concern 1:
Source of the Breast cancer cell lines is not listed. Citation #37 is referenced. Were the cells obtained from those authors? Were the patients consented? Considered human subjects research or de-identified?

Response 1:
Breast epithelial and cancer cells are cells lines obtainable from commercial sources including ATCC. We updated this information in the Table of Materials.

Concern 2:
Method 5.5: 1 mL/min is a flow rate, not a printing speed.

Response 2:
Thank you for pointing this out. We changed “printing speed“ to “flow rate.“

Concern 3:
Line 392 in the Discussion needs a reference cited for "adipose tissue-derived microvessels".

Response 3:
We apologize for this omission. We added a reference for the adipose tissue-derived microvessels.

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Reviewer #3

Major comments:

Comment 1:
It is frequently stated that this method is "suitable for any bioink". It would be helpful to describe within this manuscript a specific example of a bioink used, as stating "resuspend spheroids in the selected bioink" is rather vague and leaves details missing.

Response 1:
Thank you for your suggestion. We now include several examples of bioinks as:

> *Introduction, page 3*
> "Bioinks can range from biologically inactive alginate to the highly biologically active Matrigel[41]"

Comment 2:
The specific advantages and disadvantages of using this system should be outlined in the introduction, to better inform the reader if this system is right for their use.

Response 2:
Thank you for this comment. We now include the system's advantages and disadvantages in the introduction as:

> *Introduction, page 3*
> "Since this 3D tumor co-culture model can be created with a variety of cell structures and bioinks, it can incorporate many additional aspects of the cancer microenvironment, including heterogeneous cell types, varied extracellular matrix composition and mechanical properties, as well as growth factor and cytokine gradients[15,42]. While in its current formulation, the endothelial networks cannot be perfused, future iterations could integrate this method with microfluids or -on-chip systems. Bioprinting 3D breast epithelial spheroids onto endothelial networks enables rapid biofabrication of human breast cancer models for drug testing and personalized precision medicine[27]."

Comment 3:
More representative results, in respect to figures, are necessary to better supplement the protocol. For example, a 3D image should be provided to show the creation of the 3D lumens for the vasculature, as well as an array of images to demonstrate how the co-culture should look over time (i.e. an image at Day 0 of bioprinting, and an image at the end of the assay to demonstrate how the co-culture morphology changes).

Response 3:
We do not show 3D images since the endothelial networks do not consistently contain a lumen and cannot be perfused. We modified the text to change "endothelial tubes" to "endothelial networks" to avoid and confusion. We do not observe any significant morphology change in the co-cultures over the 24 hours that we recommend. After 24 hours, we found that breast cells migrated out of the spheroids along the endothelial tubes. We now clarify this in the manuscript as:

> *Figure and Table Legends, page 10*
> "Figure 3: Representative images of breast epithelial spheroids co-cultured with HUVEC networks. MCF10A spheroids, labeled for integrin α6 (green) and nuclei (blue), remain round and appear adhered directly to the endothelial networks. MDA-MB-231 spheroids, labeled for actin (green) and nuclei (blue), appear amorphous yet remain near or on endothelial networks. Co-cultures maintain this morphology for at least 24 hours after

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bioprinting, after which breast cells may migrate out along the endothelial tubes. Scale bar = 100 µm."

Minor comments:

Comment 1:
There are a couple typos/grammar errors, including in the introduction at line 61: "simultaneous" should be "simultaneously"; and line 151: "placing" should be "place".

Response 1:
Thank you for bringing these to our attention. We have fixed the typos as per your suggestions.

Comment 2:
There are inconsistencies as to how units are referenced, specifically milliliters, where all abbreviations should be "mL", not "ml", like seen at lines 180, 206, 209, 256, and 273.

Response 2:
Thank you for bringing this to our attention. We modified all the units to "mL" maintain consistency.

Comment 3:
All the media used contains 2% penicillin/streptomyocin- what is the reason for this high concentration? Usually, 1% pen/strep is the standard for mammalian cell culture.

Response 3:
In our usual cell culture, we also use media that contains 1% penicillin/streptomyocin. However, during the bioprinting process, the cell suspensions are carried to the bioprinter and extensively handled during the bioprinting process. To reduce the risk of contamination in our bioprinted co-cultures, we increased the antibiotic concentration. We now include this in the paper as

Protocol 1.1.4, page 4

"Antibiotic concentration was increased to 2% to account for decreased sterility in the bioprinting process; however, the antibiotic concentration can be lowered to 1%."

Reviewer #4

Major Concerns

Concern 1:
The title indicates that the authors are biofabricating "vascularized breast cancer models". However, there are two breast cell lines used for their studies, one of which, MCF10A, is a non-tumorigenic breast epithelial cell line, not a breast cancer cell line. Perhaps the authors mean the model biofabricated from MCF10A cells to be used for comparison with the model biofabricated from MDA-MB-231 breast cancer cells. If so, such a rationale is not stated. Rather they refer to both models as "breast cancer models." There is little to no value in a model comprised of one cancer cell line and a model comprised of one "non-tumorigenic" cell line, even for comparative purposes.

Response 1:
We thank the reviewer for this comment. We previously used this technique to bioprint other types of breast cancer cells; however, we did not include those data in this paper. We therefore simplified the title to remove the reference to vascularized breast cancer models. We also changed the text to describe the models as breast-endothelial co-cultures.

Concern 2:
The vascular component of the models is biofabricated from HUVECs, a single endothelial cell line that is not of microvascular origin and thus is not representative of tumor-associated vasculature in vivo. The authors do not provide any evidence that the structures formed by the HUVECs are patent tubes and thus would contribute a perfusable vasculature to the "biofabricated vascularized breast cancer models."

Response 2:
The reviewer is correct about the relevance of HUVECs. Indeed, the HUVECs networks are not perfusable. We therefore modified "endothelial tubes" to "endothelial networks." We also state in the discussion that tumor-derived endothelial cells could be used in this model.

Concern 3:
As stated above, a unique aspect of the biofabricated models herein is their generation from preformed structures. The contention is that these models are physiologically relevant. What is the authors' evidence that in vivo interactions between breast cancer cells and endothelial cells occur between multicellular breast spheroids and preformed endothelial tubes? Will drug screening studies with these models be translatable?

Response 3:
The reviewer raises an interesting point. We modified the paper to state that the 3D model is more physiologically relevant, since we cannot state that the interactions between the breast spheroids and endothelial tubes *in vitro* are replicated *in vivo.* Certainly, we and others have found that 3D cell cultures display different characteristics than 2D cell cultures, and that these characteristics are often more similar to those observed *in vivo.* We also observed that breast cells migrated along endothelial tubes in our other work with this model. This behavior has also been observed *in vivo.* We believe that drug screening studies with this model will be more translatable than those done in 2D culture, especially since in our hands breast epithelial cells and endothelial cells could not be successfully co-cultured in 2D.

Concern 4:
Methodology for confocal imaging is presented but there is nothing on how to interpret the confocal images, quantify them, ensure reproducibility, etc.---all of which would be required for assessing efficacy of drug treatments.

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Response 4:
Thank you for your suggestion. We now included statements on quantifying spheroid number per unit area and reproducibility.

> *Protocol 6.2.8 and 6.2.9, page 9*
> "6.2.8. Quantify spheroid adhesion to endothelial networks using Image J. The number of adhered spheroids can be quantified using the analyze plugin with the appropriate particle size and circularity. Normalize the number of attached spheroids to the image area.
> 6.2.9. To ensure reproducibility, quantify the number of adhered spheroids in 4 x 4 tiled images of co-cultures. If the spheroid number is statistically significantly lower than other experiments, the experiment should be repeated."

Minor Concerns

Concern 1:
Figures 1 and 3 include images of MCF10A and MDA-MB-231 spheroids alone and in co-culture with HUVEC tubes, respectively. In both cases, MCF10A are stained for integrin α6 and nuclei and MDA-MB-231 are stained for actin and nuclei. How can a reader compare these images when the two breast cell lines are not stained for the same proteins? What are the authors trying to show?

Response 1:
We apologize for omitting this important point. We agree that it would be ideal to label each breast cell line for the same proteins, However, MCF10A cell lines express high levels of integrin α6 while MDA-MB-231 cells do not. MCF10A cells are labeled for integrin α6 to show spheroid polarization and morphology, which is essential to phenotype. MDA-MB-231 were labeled with phalloidin to visualize actin filaments so that their amorphous and invasive structure could be seen. We now clarify this in the text as:

> *Protocol 6.2.3 and 6.2.4, pages 8-9*
> "6.2.3. If using MCF10A spheroids, label samples with a primary antibody for integrin α6 (1:100) in secondary blocking buffer overnight at 4°C, followed by an Alexa Fluor 488 secondary antibody (1:200) and Hoescht 33342 (1:1000) for one hour at room temperature protected from light. MCF10A cell lines express high levels of integrin α6, which is essential for showing spheroid polarization and morphology.
> 6.2.4. If using MDA-MB-231 spheroids, which express low levels of integrin α6, label samples with Alexa Fluor 488 phalloidin (1:100) and Hoescht 33342 (1:1000) in secondary blocking buffer for 4 hours at room temperature protected from light. Phalloidin enables actin filament visualization so spheroid amorphous and invasive morphology can be assessed."

Concern 2:
In the Discussion, line 386, the authors describe MCF7 cells as a breast epithelial cell line rather than as a breast cancer cell line.

Response 2:
Thank you for bringing this to our attention. We modified the sentence to describe MCF-7 as breast cancer cell line.

Concern 3:
Lines 389-90, this statement should be referenced.

Response 7:
The line was removed as the data were not previously published.

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Alisa Morss Clyne is currently an Associate Professor in the Fischell Department of Bioengineering at the University of Maryland. Prior to joining the University of Maryland in January 2019, she was an associate professor of Mechanical Engineering at Drexel University in Philadelphia, PA. Dr. Clyne is director of the Vascular Kinetics Laboratory, which investigates integrated mechanical and biochemical interactions among cells and proteins of the cardiovascular system. She is particularly interested in how endothelial cell mechanotransduction changes in a diseased environment, and how fluid shear stress and substrate mechanics affect glucose metabolism and transport. She is a fellow of the American Society of Mechanical Engineers, the American Heart Association, and the American Institute for Medical and Biological Engineering. She is also a member of ASEE, BMES, NAVBO, and SWE. Her teaching focuses on engineering applications in biological systems, and she founded several programs to enhance diversity within engineering.

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Swathi Swaminathan is a Research Scientist in the Department of Biology at Drexel University. Dr. Swaminathan has an MS in Molecular Biology from the University of Leicester, and a PhD in Biological Engineering from the University of Utah. She has extensive experience in tumor spheroid formation and interaction with endothelial cells.