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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)?

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:

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.

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.

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

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.

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.