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TITLE:

Mammary Epithelial and Endothelial Cell Spheroids as a Potential Functional *In Vitro* Model for Breast Cancer Research

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breast cancer, tumor, spheroids, neovascularization, lymphatic vessels, tumor growth, endothelial cells, epithelial cells, cell proliferation.

SUMMARY:

Crosstalk between mammary epithelial cells and endothelial cells importantly contributes to breast cancer progression, tumor growth, and metastasis. In this study, spheroids have been made from breast cancer cells together with vascular and/or lymphatic endothelial cells and demonstrate their applicability as an *in vitro* system for breast cancer research.

ABSTRACT:

Breast cancer is the leading cause of mortality in women. The growth of breast cancer cells and their subsequent metastasis is a key factor for its progression. Although the mechanisms involved in promoting breast cancer growth have been intensively studied using monocultures of breast cancer cells such as MCF-7 cells, the contribution of other cell types, such as vascular and lymphatic endothelial cells that are intimately involved in tumor growth, has not been investigated in depth. Cell-cell interaction plays a key role in tumor growth and progression.

Neoangiogenesis, or the development of vessels, is essential for tumor growth, whereas the lymphatic system serves as a portal for cancer cell migration and subsequent metastasis. Recent studies provide evidence that vascular and lymphatic endothelial cells can significantly influence cancer cell growth. These observations imply a need for developing *in vitro* models that would more realistically reflect breast cancer growth processes *in vivo*. Moreover, restrictions in animal research require the development of *ex vivo* models to elucidate better the mechanisms involved.

This article describes the development of breast cancer spheroids composed of both breast cancer cells (estrogen receptor-positive MCF-7 cells) and vascular and/or lymphatic endothelial cells. The protocol describes a detailed step-by-step approach in creating dual-cell spheroids using two different approaches, hanging drop (gold standard and cheap) and 96-well U-bottom plates (expensive). In-depth instructions are provided for how to delicately pick up the formed spheroids to monitor growth by microscopic sizing and assessing viability using dead and live cell staining. Moreover, procedures to fix the spheroids for sectioning and staining with growth-specific antibodies to differentiate growth patterns in spheroids are delineated. Additionally, details for preparing spheroids with transfected cells and methods to extract RNA for molecular analysis are provided. In conclusion, this article provides in-depth instructions for preparing multi-cell spheroids for breast cancer research.

INTRODUCTION:

The use of animals for experiments has limitations. Animal studies cannot accurately mimic disease progression in humans, and animals and humans do not have identical responses to pathogens. Additionally, restrictions in animal experimentation due to concerns for animal suffering and ethical problems[1,2] increasingly constrain research programs. *In vitro* systems have been widely developed to circumvent the use of animals; moreover, the use of human cells has made *in vitro* models more relevant for the pathophysiological and therapeutic investigation. Conventional monolayer (2D) cell cultures are widely used because they mimic human tissues to some degree. However, 2D monocultures fail to mimic human organs, and 2D monocultures are unable to simulate the complex microenvironment of the original organs and mimic the *in vivo* situation[3–6]. Additionally, in monolayer cell cultures, drug treatments could easily destroy/damage all of the cells. Importantly, some of these limitations can be overcome by switching to multilayer three-dimensional (3D) cell cultures[7,8]. In fact, 3D culture models have been shown to better reflect the cellular structure, layout, and function of cells in primary tissues. These 3D cultures can be formed using a variety of cell lines, similar to a functional organ. Indeed, there are two models of 3D cultures. One model produces spheroids of aggregated cells that form clusters and reorganize them into spheres (scaffold-free models). The second yields organoids, which have a more complex structure and consist of combinations of multiple organ-specific cells, which are considered as a miniature version of organs[9,10]. Due to this, 3D culture systems represent an innovative technology with many biological and clinical applications. Thus, spheroids and organoids have numerous applications for disease modeling and studies related to regenerative medicine, drug screening, and toxicological studies[6,11–15]. Carcinogenic spheroids, derived from 3D technology, recreate the morphology and phenotype of relevant cell types, mimic the *in vivo* tumor microenvironment, and model cell communications and signaling

pathways that are operational during tumor development[16–18]. In addition, to improve cancer biology understanding, tumor spheroids/organoids can also be used to identify a potential patient-specific anti-cancer therapy (personalized) and assess its efficacy, toxicity, and long-term effects[19–22]. Spheroids have opened prominent opportunities to investigate pathophysiology, disease modeling and drug screening because of their ability to preserve cellular and three-dimensional tissue architecture, the ability to mimic the *in vivo* situation, and the cell-cell interactions. However, one must also be aware of the limitations of this system, such as the lack of vascular/systemic component, functional immune or nervous system, and the system represents a reductionist approach as compared to animal models. Indeed, in contrast to animal models, 3D structures provide only an approximation of the biology within a human body. Understanding the limitations of the 3D method may help researchers to design more refined and valid processes for producing spheroids that better represent an organ at a larger scale[23–25].

Cancer is the leading cause of death worldwide, and breast cancer is the most common cancer in women[26–28]. To mimic the complex microenvironment of breast cancer, breast cancer spheroids should be cultured using cells that play a prominent role in breast tumors, i.e., epithelial cells, endothelial cells, fibroblasts, and/or immune cells. Moreover, for a spheroid representing breast cancer, the expression of female hormone receptors (estrogen/progesterone receptors), ability to conserve the patient tumor histological status, and ability to mimic response to therapy should also be considered. Studies have shown that 3D co-culture systems have a cellular organization similar to that of the primary tissue *in vivo*, have the capability to react in real-time to stimuli, and have functional androgen receptors[29–32]. Hence, a similar approach could be useful to mimic a breast tumor *in vitro*. The purpose of the current protocol is to establish a new method of generating breast cancer spheroids. This method utilizes estrogen receptor-positive MCF-7 cells (an immortalized human cell line of epithelial cells) and vascular endothelial cells (HUVECs) or lymphatic endothelial cells (HMVEC-DNeo) to create a model that mimics or closely reflects the interactions between these cells within a tumor. Although MCF-7 (estrogen-responsive) and endothelial cells have been used to develop spheroids in the present study, other cells such as fibroblasts which represent ˜80% of the breast tumor mass, could also be combined in the future to better represent and mimic breast tumor.

There are several methods to form spheroids, such as: 1) the hanging droplet method that employs gravity[33,34]; 2) the magnetic levitation method that uses magnetic nanoparticles exposed to an external magnet[35], and 3) the spheroid microplate method that is performed by seeding cells on low-attachment plates[36,37]. On the basis of the existing methods, which use only one cell type, the present protocol has been optimized using epithelial and endothelial cells to better mimic the growth conditions of breast cancer tumors *in vivo*[38–41]. This method can be easily achieved in the laboratory at a low cost and with minimal equipment requirements. Based on the need/goals of the lab, different approaches were used to form spheroids and gain relevant cellular material from these spheroids. In this context, for DNA, RNA, or protein analysis, the 3D spheroids are produced by co-culturing endothelial and epithelial cells with the hanging drop method. However, for functional studies, for example, to monitor cell growth after short interfering (siRNA) transfection and/or hormone treatment, the spheroids are generated using 96U-bottom plates.

The purpose of this technical protocol is to provide a detailed step-by-step description for 1) forming breast cancer multicellular-type spheroids, 2) preparing samples for histological staining, and 3) collection of cells for extraction of RNA, DNA, and proteins. Both the inexpensive hanging drop method and the more expensive U-bottom plates are used to form spheroids. Here, a protocol for preparing (fixing) spheroids for sectioning and subsequent immunostaining with markers to assess cell proliferation, apoptosis, and distribution of epithelial and endothelial cells within a spheroid is provided. Additionally, this protocol shows a complete step-by-step analysis of histological data using ImageJ software. Interpretation of biological data varies depending on the type of experiment and the antibodies used. Sectioning of fixed spheroids and subsequent staining of the sections was performed by a routine pathology lab (Sophistolab: info@sophistolab.ch)

PROTOCOL:

1. Cell culture

NOTE: Conduct cell handling under sterile conditions.

1.1. Human Umbilical Vein Endothelial Cells (HUVECs) subculture

1.1.1. Coat 75 cm^2 flasks with collagen (5 µg/cm^2) (rat-tail) overnight (ON) at room temperature (RT): or 2–3 h at 37 °C, rinse with water, and allow the flask to dry.

1.1.2. Grow HUVECs in growth medium (EBM-2, Endothelial Basal Medium-2) supplemented with a commercial glutamine supplement (1x = 2 mM), antibiotic-antimycotic solution (AA; 100 µg/mL of streptomycin, 100 µg/mL of penicillin and 0.025 µg/mL of amphotericin B), LSGS (2% v/v FCS, 1 µg/mL of hydrocortisone, 10 ng/mL of human Epidermal Growth Factor, 3 ng/mL of human basic Fibroblast Growth Factor, 10 µg/mL of heparin) and 10% FCS (Fetal Calf Serum) under standard tissue culture conditions (37 °C, 5% CO_2). Change the medium every 2 days until the cells reach 70%–80% confluence.

1.1.3. Wash the sub-confluent cultures with 5 mL of HBSS (without Ca^{2+} and Mg^{2+}) and add 3 mL of trypsin (0.25% diluted in HBSS (without Ca^{2+} and Mg^{2+}).

NOTE: HBSS can also be supplemented with PBS.

1.1.4. Incubate the cells at 37 °C for 2 min (microscopically check whether the cells are detached and rounded up) and stop the detaching reaction by adding 6 mL of 10% FCS medium (DMEM-F12 or EBM-2, 10% FCS).

1.1.5. Centrifuge the cell suspension at 250 x *g* for 5 min at RT and discard the supernatant.

1.1.6. Suspend the cells in growth medium and seed in 75 cm^2 flasks or culture dishes.

1.2. Michigan Cancer Foundation-7 (MCF-7, human breast adenocarcinoma cells) subculture

1.2.1. Grow human breast cancer cell lines in 75 cm^2 flasks in growth medium (DMEM/F12 medium supplemented with a commercial glutamine supplement (1x), antibiotic-antimycotic solution (AA; 100 µg/mL of streptomycin, 100 µg/mL of penicillin and 0.025 µg/mL of amphotericin B), and 10% FCS) under standard tissue culture conditions (37 °C, 5% CO_2). Change the medium every 2–3 days.

1.2.2. Wash the subconfluent cell cultures (70%–80% confluence) with 5 mL of HBSS (without Ca^{2+} and Mg^{2+}) and add 3 mL of trypsin (0.5% diluted in HBSS (without Ca^{2+} and Mg^{2+}) at 37 °C for 5 min (microscopically verify that the cells are detached).

NOTE: HBSS can also be supplemented with PBS.

1.2.3. Add 8 mL of 10% FCS medium to stop the detaching reaction, centrifuge at 250 x *g* for 5 min at RT and discard the supernatant.

1.2.4. Suspend the pellet in growth medium and plate in 75 cm^2 flasks or culture dishes.

2. Spheroid formation

2.1. Plate 5 x 10^5 cells/mL (HUVECs and MCF-7) in a 3.5 cm round dish and culture for 48 h.

2.2. Treat or transfect the plated cells for 24 h or as desired.

2.3. Optional step performed in 96-well U-bottom plate: Wash the cells with 1 mL of HBSS (Ca^{2+} and Mg^{2+}) and stain HUVECs using a blue dye (0.5 µg/mL) and MCF-7 cells using a green dye (0.5 µM) diluted in the respective growth medium and place into a 37 °C and 5% CO_2 incubator for 30–40 min.

2.4. Wash the cells with 1 mL of HBSS (without Ca^{2+} and Mg^{2+}), add 1 mL of enzymatic detachment reagent (0.25% trypsin for HUVEC and 0.5% trypsin for MCF-7) to each round dish using a P1000 pipette, and then incubate for 2–5 min until the cells are detached.

2.5. Collect each cell type separately in a 5 mL round-bottom Polystyrene tube and add 1 mL of 10% FCS medium using a P1000 pipette to stop the enzymatic reaction. Centrifuge the cell suspensions at 250 x *g* for 5 min.

2.6. Carefully aspirate the supernatant and suspend the cells in 1 mL of steroid-free medium (EBM-2, glutamine supplement, antibiotic-antimycotic, 0.4% FCS sf (charcoal-stripped)).

NOTE: Steroid-free medium can be replaced with normal growth medium.

2.7. Use 100 µL of each cell suspension diluted with 10 mL of Diluent II (see **Table of Materials**) to determine the total cell number and calculate the amount of treatment medium (EBM-2, Glutamax, antibiotic-antimycotic, 0.4% FCS sf, vehicle/Treatment) needed to obtain a cell suspension of 5 x 10^3 cells/mL or 3.4 x 10^5 cells/mL per cell type.

2.7.1. Cell count

2.7.1.1. Turn on the machine and flush by pressing the **Function** and **Start** buttons (set-up S3), with Diluent II solution in a cell counter vial.

2.7.1.2. Use set up S4 and press **Start** to measure the blank with fresh Diluent II solution.

2.7.1.3. Change the scintillation vial with 100 µL of cell suspension in 10 mL of Diluent II solution and measure the samples: set-up S4 and press **Start**.

2.7.1.4. Note the number of cells per mL on the digital display.

NOTE: Cell counting can also be performed using other manual or automatic systems: Hemocytometer gridlines, light-scatter cell-counting technology, electrical impedance, imaging-based systems using cell staining, e.g., trypan blue.

2.7.2. 96-well U-bottom plates

2.7.2.1. Prepare 5 mL of each cell suspension (5 x 10^3 cells/mL) and mix them to get a final volume of 10 mL in the ratio of 1:1.

2.7.2.2. Use a manual repeating pipette to pipette out 100 µL of the cell suspension into each well of the 96-well U-bottom plates.

2.7.2.3. Place the plate into an incubator under standard tissue culture conditions and check for spheroids formation after 48 h.

2.7.2.4. Optional step: Take pictures of the spheroids (40x) using a fluorescence stereo microscope.

NOTE: This method generates 96 spheroids (one spheroid/well) after 48 h (area 1–2 pixel^2), and it is usually used for growth studies.

2.7.3. Hanging drop

2.7.3.1. Mix the cell suspensions (3.4 x 10^5 cells/mL) in a 1:1 ratio to get a final volume of 2 mL.

2.7.3.2. Use a P20 pipette to seed the cell mixture in the form of 15 µL drops on the

inverted lid of a 10 cm Petri dish.

2.7.3.3. Invert the lid with the drops and add 5 mL of base medium (EBM-2) at the bottom of the dish to avoid evaporation of the drops.

NOTE: This method generates one spheroid/drop after 48 h. Ensure the drops are sufficiently apart from each other, so that they do not merge while switching the lid. Use more than one 10 cm Petri dish if necessary.

3. Spheroid growth study

3.1. Plate 100 µL of HUVECs and MCF-7 cell mixture (5 x 10^2 cells/well) in 96-well U-bottom plates and place them in the incubator.

3.2. 48 h after plating, check for spheroids formation with an inverted microscope (Bright field). Take pictures (at least six pictures per treatment, 40x) at 96 h of culture.

3.3. Open the pictures using an image processing software and process the image to 300 pixel/in and save the image as a tiff file.

3.4. Download the ImageJ software and open it. Click on the **File** option and go to **Open**.

3.5. Convert the areas of interest to saturated black areas in a uniform manner to have a binary image (black and white). For this, select the **Image** option, choose **Type | 8-bit**. Now, the image is black and white.

3.6. Use the Freehand Selection Tool to draw the border of the spheroids.

3.7. To calculate the area of interest and to separate the object or the foreground pixels from the background pixels, use the threshold function: Select the **Image** option, choose **Adjust** and click on **Threshold**.

3.8. Now a Threshold pop-up window will open. The top bar indicates the minimum threshold value: set the value at zero. The bottom bar indicates the maximum threshold value: move the bar until the area of interest becomes completely red.

NOTE: If the color is not red, select **Red** from the Threshold window.

3.9. Go to **Analyze | Set Measurements** and select **Area** and **Perimeter**.

3.10. To measure the area of interest, select the **Analyze** option and use the **Analyze Particles** tool. In the **Analyze Particles** window, set the **Size** to measure (0.00003-Infinity or 3-Infinity, depending on the size of the spheroids), select **Summarize** and **Display Results**. Then, click on **OK**.

3.11. The results will show up in a chart. Copy the measurements into a spreadsheet to analyze the data.

NOTE: The area is calculated as the number of total pixels; use the Total Area for calculation.

4. Spheroid immunohistochemistry study

4.1. Sample preparation

4.1.1. Plate the cell mixture of HUVECs and MCF-7 (5 x 10^3 cells/well) in 96-well U-bottom plates in HUVEC growth medium for 96 h.

4.1.2. Collect approximately 50 spheroids into a 1.5 mL tube with a cut tip of a P200 µL pipette; allow them to settle to the bottom of the tube by gravity, and then discard the supernatant.

4.1.3. Fix the spheroids with 500 µL of 4% PFA for 1 h, RT.

4.1.4. Boil 2% Nobel Agar solution in PBS using a hot plate with a magnetic stirrer for 3–5 min to dissolve the agarose powder completely and cool down to around 60 °C.

4.1.5. Remove the PFA with a P1000 pipette, wash the spheroids with 500 µL of PBS, and allow them to sediment. Discard the supernatant.

4.1.6. Carefully pipette 600 µL of agarose solution into the 1.5 mL tube with spheroids and place the tube immediately in a centrifuge with a horizontal rotor at 177 x *g* for 2 min.

4.1.7. Add a short string in the middle of the agarose solution to easily remove the plug from the tube.

4.1.8. Solidify agarose plug on ice or at 4 °C. Add 500 µL of PBS into the 1.5 mL tube to avoid drying out of the pellet.

NOTE: The samples are ready for subsequent dehydration, paraffin embedding, sectioning, transfer onto the microscope slides, and IHC staining (collaboration with Sophistolab).

4.2. Image acquisition and processing

4.2.1. Acquire images of spheroid sections using a stereomicroscope.

4.2.2. Save three images for each sample as .jpg files.

4.2.3. Open the ImageJ software. To open the JPEG image, click on **File** and then on **Open**.

4.2.4. Select **Color** and **Color Deconvolution** in the **Image** option.

4.2.5. Select **H DAB** vectors in the Color Deconvolution 1.7 window, and the three images appear.

NOTE: The three images are: **Color 1** represents only the Hematoxylin staining (blue/purple), and **Color 2** represents only the DAB staining (brown). **Color 3** is not needed for the image analysis with two stainings.

4.2.6. Select the **Color 1** window and set the Threshold: go to **Image | Adjust** and click on **Threshold**. The **Threshold** window appears.

4.2.7. Leave the minimum threshold value set at zero and adjust the maximum threshold value to remove the background signal without influencing the true signal. Click on **Apply**.

4.2.7.1. Area measurement

4.2.7.1.1. Click on **Analyze**, choose **Set Measurement**. Select **Area**, **Mean grey value**, and **Min and Max grey value** and click on **OK** to confirm.

4.2.7.1.2. Measure the size of the nucleus using the **Analyze** option, and then select **Measure**.

NOTE: The **Results** window gives the name of the image (Label), size of the image (Area), the average pixel intensity of the IHC image (Mean), and the minimum and maximum gray values (Min, Max). Use the Mean value for calculation.

4.2.7.1.3. Repeat steps 4.2.6–4.7.1.2 for the **Color 2** (and **Color 3** if there is dual staining) window.

4.2.7.2. Cell quantification

4.2.7.2.1. Click on **Process**, choose the option **Binary** and select **Watershed** to separate cells.

4.2.7.2.2. Go to **Analyze** and click on **Analyze Particles**. On the **Analyze Particles** pop-up window, set the size of the cells to exclude unspecific particles. Next, on the **Show** option, click on the drop-down box and select **Outlines**. Then, click on **OK**.

4.2.7.2.3. Repeat steps 4.2.6–4.2.7 and cell quantification steps (section 4.2.7.2) for the **Color 2** (and **Color 3** if there is dual staining) window.

NOTE: Now three windows will appear: 1) Summary results (number of cells counted, total area, average size, % of area and mean), 2) Results (respective to the cells that are counted), and 3)

Drawing window (depicted image of the cells that are counted).

5. Live/dead staining in Spheroids

5.1. Prepare 4 mM stock solutions of Calcein AM in DMSO (stains live cells green) and 2 mM of Ethidium homodimer in DMSO (stains dead cells red) in 1.5 mL tubes.

5.2. Calculate the amount of solution necessary to add 10 µL/well of a mixture of Calcein AM and Ethidium homodimer diluted 1:50 in EBM-2.

5.3. Add the staining mixture to the spheroids and place the plate in the incubator under standard tissue culture conditions for 30–60 min.

5.4. Acquire pictures (40x) using the Fluorescence stereo microscope.

6. Spheroid's protein and RNA isolation

6.1. Prepare cell suspensions at 3.4 x 10^5cells/mL per cell type, mix them at a ratio of 1:1, and seed the cell mixture using a P20 pipette in the form of 15 µL drops on the lid of a 10 cm Petri dish. Invert the lid and place it in the incubator under standard tissue culture conditions for 96 h.

6.2. Use 5–6 mL of PBS to collect spheroids in a 15 mL round-bottom Polystyrene tube using 5 mL sterile polystyrene pipettes.

NOTE: It is also possible to use 15 mL conical tubes.

6.3. Centrifuge the spheroids suspension at 250 x *g* for 5 min, and then carefully aspirate the supernatant.

6.4. Add 500 µL of trypsin (100%) and pipette up and down using a P1000 pipette for 30 s to disaggregate spheroids, achieving a cell suspension.

6.5. Neutralize trypsin with 10% FCS medium, centrifuge the tubes at 250 x *g* for 5 min, and aspirate the supernatant.

6.6. According to the manufacturer's protocol (specific kit for DNA-free RNA isolation), use 300 µL of RNA lysis buffer to lyse the cell pellet.

NOTE: Lysis buffer can also be directly added to the intact spheroids (no trypsin step required) to extract RNA. Add 300 µL of the lysis buffer to the pelleted spheroids, place tubes in ice, and triturate 10 times using a P200 pipette. Subsequently, isolate RNA as above. Spheroid dissociation may be needed if RNA recovery is low due to matrix interference.

6.7. Alternatively, use 70 µL of the Lysis buffer for protein isolation. Homogenize for 2–4 s by

sonication and determine the protein concentration according to the manufacturer's protocol using a BCA Assay Kit.

NOTE: For protein isolation, pelleted spheroids can be lysed directly in lysis buffer followed by sonication. Use 25 µg of total protein to perform western Blot.

REPRESENTATIVE RESULTS:

The spheroids model using epithelial and endothelial co-cultures is required to closely mimic *in vivo* conditions of breast tumors for *in vitro* experiments. The scheme in **Figure 1** depicts the protocol to form spheroids with breast cancer epithelial cells and vascular or lymphatic endothelial cells (**Figure 1**). Each cell type is seeded separately in a 3.5 cm round dish and treated with growth stimulators/inhibitors or transfected with oligonucleotides using Lipofectamine. The confluent monolayers of epithelial or endothelial cells are harvested following trypsinization, washed, and mixed at a 1:1 ratio. The cells are subsequently plated in 96-well U-bottom plates (**Figure 1A**) or on the inverted lid of a 10 cm diameter round culture dish to facilitate hanging drop culture (**Figure 1B**). Soon after seeding, the cells appear as flat, dense sheets of cells at the bottom of each well or drop, and, subsequently, they aggregate with time (24–48 h), thus forming intact spheroids at 48 h. In order to prevent any damage to the loosely formed cell aggregates, the plates must not be disturbed for at least 24 h.

In U-bottom plates, seeding of MCF-7 cells, but not endothelial cells or lymphatic cells, resulted in spheroid formation after 48 h (**Figure 2A,B,** respectively). Moreover, seeding of MCF-7 plus endothelial cells or lymphatic cells at a 1:1 ratio also resulted in spheroid formation (**Figure 2C,D,** respectively). To validate spheroids as a model to study tumor growth, time-dependent changes in spheroid size in response to growth-stimulating solution was measured/quantified and compared with the untreated controls (**Figure 2**). Photomicrographs in **Figure 2E** show time-dependent (24–120 h) sequential growth of a representative MCF-7 spheroid. The line graph in **Figure 2F** depicts the change in the area of MCF-7 spheroids over time when treated with or without a growth stimulator. The spheroid size increased from ~100 µm to ~200 µm over 5 days; moreover, an increased growth rate was observed in spheroids treated with the growth stimulator (**Figure 2F**). Because long-term treatment (168 h) resulted in reduced MCF-7 viability, potentially due to hypoxic conditions in the center of the spheroids (**Figure 3**), 3D structure growth was studied till 96 h. Spheroids formed with MCF-7 cells and HUVECs show a lower number of dead cells at 168 h compared to spheroids formed with MCF-7 alone.

In the initial experiments, only a small growth in spheroids' size was observed after 4 days of culture. It was hypothesized that this might be due to the high number of cells seeded to form spheroids. In the U-bottom culture plates, the growth of spheroids could have been limited by the concave shape of the well. Since growth was dependent on the initial number of cells seeded, different cell numbers were used to form spheroids to test and establish conditions that would be optimal to monitor spheroid growth. As shown in **Figure 4**, the findings suggest that for spheroid growth studies, seeding 500 cells/well to form spheroids was reliable and reproducible for monitoring of spheroids' growth for 4–6 days in response to growth modulatory factors. Spheroids that were most suitable for histological or immune-histological observations were

obtained on day 4, after seeding of 5 x 10^3 cells/well. This same initial concentration was used for cell protein or RNA extractions. Increasing the initial cell concentration by more than 7.5 x 10^3 cells/well did not improve spheroid formation, and the cells did not aggregate properly and assumed irregular structures (**Figure 4**).

In order to evaluate cell composition, proliferation, and apoptosis status, histological sections of spheroids formed with breast cancer cells plus endothelial cells were examined using H&E and Ki67 (dilution 1:300) and Cleaved Caspase-3 (dilution 1:500) antibodies. The process for final sample preparation requires care/attention and precision. **Figure 5A** depicts the workflow for the collection and incorporation of spheroids into agarose for sectioning. Photomicrographs in **Figure 5B–C** demonstrate the difference in MCF-7 spheroid formation in the presence (**Figure 5B**) and absence (**Figure 5C**) of Lipofectamine (0.17% final concentration), a commonly used transfection agent. Bright-field microscopic images of histological sections show a circular, compact structure of a homogenous mixture of two cell types (**Figure 5D–G**). The structure of the spheroids and the shape of their cells showed no abnormalities following transfection with control oligonucleotides in the presence of Lipofectamine (**Figure 5D**). Moreover, cells expressing the proliferative marker, Ki67, were distributed homogeneously in the spheroids; however, a ring of proliferative cells was also observed on the surface of the spheroids (**Figure 5E**). Cleaved Caspase-3 staining showed apoptotic cells after 4 days of culture (**Figure 5F**). Histological sections are useful to evaluate the distribution of epithelial and endothelial cells in spheroids using specific markers. Using CD31 as a specific marker for endothelial cells, it is possible to determine their distribution within the 3D structure. Since all the cells are stained with Hematoxylin (blue staining of the nucleus of the cell), the calculation of CD31 expression could provide the area of the spheroids containing endothelial cells following treatment or transfection (protocol section 4.2.7.1.). Moreover, by performing a dual staining assay, it is possible to have even more information, for example, as shown in **Figure 5G**, CD31 stains endothelial cells (red) and Ki67 stains proliferative cells (brown). Following the protocol section 4.2.7.2, **Figure 5G** revealed that 55% are epithelial cells, 45% are endothelial cells, and 25% of cells are proliferating.

The structure of spheroids may also be studied using immunofluorescence analysis following the labeling of cells with live dyes. Confluent 2D cultures of HUVEC and MCF-7 cells were separately stained with blue (HUVEC) and green (MCF-7) dyes. Subsequently, the stained cells were collected and mixed at a 1:1 ratio and seeded in wells for spheroid formation. Images of fluorescent cells stained were taken immediately after seeding (**Figure 6A**) and after 3 days of culture (**Figure 6B**). With the aim of assessing the percentage of live and dead cells, 4-day old spheroids were stained with calcein-AM (green) and with ethidium homodimer (red) (**Figure 6C**). Spheroids formed with MCF-7 and HUVECs could not be successfully frozen/preserved using the standard protocols for freezing cells (growth medium supplemented with 10% DMSO and 10% FCS) for freezing cells. In frozen and thawed spheroids, rupturing of cell aggregates and increased loss of cell viability were evident. The representative image in **Figure 6D** shows a freshly thawed spheroid stained for dead and live cells. It shows that spheroids are very sensitive to the freezing conditions.

After 5 days of culture in experimental conditions, lysis of around 100 spheroids was necessary

to collect enough protein (~3 μg/mL) or RNA (~60 ng/μL) for analysis. **Figure 7** represents a workflow from spheroids collection (generated with the hanging drops method) to cell lysis for protein extraction to perform western blotting or RNA isolation for future Microarray assays or analysis by RT-PCR. Isolation of RNA/DNA from a heterogeneous cell population in a spheroid for microarray analysis can bring some useful information. However, to identify key cell-specific mechanisms or cell-cell interactions, the spheroid cells can be separated after enzymatic (trypsin or accutase) dissociation, using FACS analysis and RNA from specific cells used for microarray analysis. Moreover, antibiotic-resistant cell lines (e.g., by using EGFP and/or puromycin resistance cell lines) could be used in spheroids to address the role of a specific cell type of cell-cell interaction. Additionally, gene silencing tools can be used to engineer the cells to address specific cell-cell interaction issues.

FIGURE AND TABLE LEGENDS:

Figure 1: Workflow of spheroids formation. MCF-7 and HUVEC or LEC cells in 2D cultures are grown separately and collected after various desired treatments. Spheroids are subsequently generated by direct mixing of cells in (**A**) 96-well U-bottom plates, which promote the formation of 3D structure by cell-to-cell aggregation, or by using (**B**) hanging drop culture that supports spheroids formation via force of gravity.

Figure 2: Spheroid formation and development over time. Images depict the formation of a spheroid by MCF-7 cells (**Panel A**), the lack of spheroid formation by endothelial or lymphatic cells (HUVECs; (**Panel B**) plated at the same density, and spheroid formation by MCF-7 cells plus HUVECs or LECs mixed at a 1:1 ratio (**Panels C,D**). Also depicted is the growth in size of a spheroid over time (24 h, 48 h, 72 h, 96 h, and 120 h). This spheroid was formed from a mixture of MCF-7 plus HUVECs in a 1:1 ratio and seeded at a density of 500 cells/well of a 96-well U-bottom plate (**Panel E**), (scale bar = 200 μm, 40x). Line graph (**Panel F**) shows the time-dependent growth of control (CTR) versus growth-stimulated (Treatment) spheroids. Spheroid's areas were measured and compared between untreated and treated spheroids. Results represent ± SEM, *** $p < 0.001$ compared to the respective control.

Figure 3: **Spheroids long-term culture and viability.** Photomicrograph shows cell viability in spheroids formed with MCF-7 cells alone at 96 h (**Panel A**) and 168 h (**Panel C**), whereas **Panels B** and **D** depict cell viability in spheroids made with MCF-7 plus HUVECs (1:1 ratio) at 96 h (**Panel B**) and 168 h (**Panel D**) (scale bar = 100 μm). Cells were stained with Calcein (red, dead cells) and Ethidium homodimer (green, live cells).

Figure 4: **Cell seeding density and time-dependent growth profile of MCF-7 plus HUVEC spheroid.** HUVECs and MCF-7 cells mixed at a 1:1 ratio were seeded at increasing densities (10^2–10^4 cells/well), and the growth of spheroids was monitored over 24–96 h. Microscopic bright-field images were taken (scale bar = 200 μm, 40x) to assess spheroid growth over time. Cells at a density of 500 cells/well provided optimal size to study spheroid growth.

Figure 5: Spheroid embedding for sectioning and histological staining. Panel A: Cartoon depicting the workflow for collection and incorporation of HUVECs/MCF-7 spheroids into

agarose. After paraffin embedding of the spheroids in an agarose plug and sectioning of paraffin blocks, the samples were used for standard histological staining. **Panels B** and **C** show representative MCF-7 plus HUVEC spheroids treated without and with Lipofectamine 2000, respectively (scale bar = 200 µm, 40x)**. Panels D–G** show representative images of spheroid sections stained with specific markers. **Panel D** shows spheroids stained with hematoxylin-eosin to assess spheroid structure (scale bar = 100 µm). Staining in **Panel E** depicts proliferating cells positively stained with Ki67. **Panel F** shows apoptotic cells in a spheroid positively stained with Cleaved Caspase-3 (scale bar = 50 µm). **Panel G** depicts spheroid sections with dual staining: CD31 (endothelial cells marker) and Ki67 (proliferative marker) (scale bar = 100 µm). The sectioning of spheroids and staining were performed by Sophistolab (info@sophistolab.ch).

Figure 6: Fluorescence images of MCF-7/HUVEC spheroids showing cell distribution and viability. Panels A and **B** depict representative spheroids with HUVECs stained with a blue dye and MCF-7 cells stained with a green dye. The localization of HUVEC and MCF-7 within the spheroid varies immediately after seeding (**A**) and after spheroid formation (**B**) (scale bar = 250 µm). **Panels C–D** show spheroids stained with Calcein/Ethidium Homodimer to identify live (green) and dead cells (red) in the 3D structures in normal culture condition (**C**) and after freezing (**D**) (Scale bar = 100 µm).

Figure 7: Schematic representation of spheroid collection for biochemical analysis. Cartoon showing the scheme to harvest spheroids, separate or release cells, and suspend them in lysis solution for protein analysis or RNA extraction.

DISCUSSION:

Compared to 2D cell cultures, revolutionary 3D spheroid culture technology is a better and more powerful tool to reconstruct an organ's microenvironment, cell-cell interactions, and drug responses *in vitro*. This is the first protocol describing the formation of spheroids from multicellular (epithelial and endothelial) cell lines for breast cancer research. This protocol ensures spheroidal 3D growth of spheroids for up to 5 days, and spheroids can be examined after paraffin embedding, sectioning, and histological staining. Interestingly, the cellular elements within the spheroid still express receptors that promote cell growth and are responsive to proliferative and apoptotic stimuli. The described protocol generates sufficient biological material for protein or RNA/DNA analysis. The above co-culture system, easily achieved in laboratory conditions and at low cost, could be an advantage for future applications, especially in breast cancer therapy and for drug sensitivity testing. In addition, this protocol can be applied to different epithelial and endothelial cell types.

It is extremely difficult to mimic *in vitro* a complex tissue that exists *in vivo*. This is because *in vivo* tissues consist of many interacting components, including nerves, blood vessels, mesenchyme, and immune cells[30,42]. The aim of this protocol is to overcome some of these limitations by using a 3D co-culture system with two different cell types to approach the actual tumor state. Here, spheroids have been formed with epithelial tumor cells in combination with endothelial cells or lymphatic cells to mimic the *in vivo* situation as much as possible, including cell-cell interactions, susceptibility to apoptotic factors released into the intercellular spaces by dying cells, and hypoxic

conditions in the center of the spheroids. This protocol has been optimized for testing of cellular responses to growth factors, signaling factors, and hormones, which rapidly activate signaling cascades and target gene transcription and stimulate protein expression and transport[30,31]. In spheroids, but not *in vivo*, responses of tumor cells to stimuli can be assessed rapidly and frequently.

In the present study, endothelial cells in the spheroids do not seem to form capillary-like structures. Since endothelial cells have been shown to form capillaries when plated at low density and monolayers at high density, it is feasible that lowering the MCF-7 to endothelial cells ratio may result in capillary formation within the spheroids. Alternatively, the addition of specific matrix proteins or matrigel may promote capillary formation. The lack of capillary-like structure in the spheroid does not dilute the advantage of MCF-7 plus endothelial cells versus MCF-7 cells per se, as the factors released by MCF-7 cells could promote endothelial cell proliferation and vice versa; such interactions would remain undetected in MCF-7 only spheroids.

Several studies have shown that 5×10^3 cells/well was the appropriate concentration to seed cells to form spheroids[43–47]; however, in 96-well U-bottom plates, this number of cells limited the coordinated growth of spheroids in experimental conditions. Therefore, in each new study, for each new cell type, it is important to monitor the growth of the 3D system after drug treatment, to determine the effects of the treatment on spheroid size.

The limitation of the current protocol is that the culture of previously formed spheroids cannot be continued after freezing and thawing by the classical DMSO method. In fact, when thawed, most of the cells were dead. Vitrification may prove to be an alternative to keeping the cells alive[48].

Careful attention to technical details is necessary to avoid errors and produce and maintain well-formed spheroids. One challenge is the sample preparation for immunostaining (section 4.1). To facilitate the transfer by pipetting the spheroids into the 1.5 mL tube, it is important to use a P200 tip cut with scissors such that the area of the hole is larger than the spheroids themselves. Otherwise, the transfer could destroy the spheres. Another challenge is how to aspirate the medium after the spheroids have settled to the bottom of a test tube. In this regard, it is better to use a P1000 pipette instead of a vacuum pump to avoid the aspiration of spheroids. An important and delicate step in preparing the spheroids for sectioning is to add the agarose to the pelleted spheroids. The spheroids, once suspended in agarose, must be pelleted quickly to the bottom of the microfuge tube using horizontal centrifugation at room temperature and before the agarose polymerizes.

Overall, the two-cell spheroid made of MCF-7 and endothelial cells provides a viable alternative to investigate cell-cell interactions and mechanisms that drive the growth of either cancer cells or endothelial cells within a tumor. The spheroids could serve as a model to assess the efficacy of therapeutic agents targeting tumor or cancer growth. Importantly, this two-cell model could be further improvised to include other cell types relevant in tumor growth. In this context, fibroblasts that constitute 80% of the breast tumor mass may be included to form an MCF-7,

endothelial cells, and fibroblast spheroid. Moreover, gene silencing and genetically engineered cells could be used to address the role of cell-cell interaction, hormone receptors (estrogen/ progesterone), growth factors, and signaling pathways on tumor/cancer growth as well as to test the efficacy of new anti-cancer molecules.

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DISCLOSURES:

The authors have nothing to disclose.

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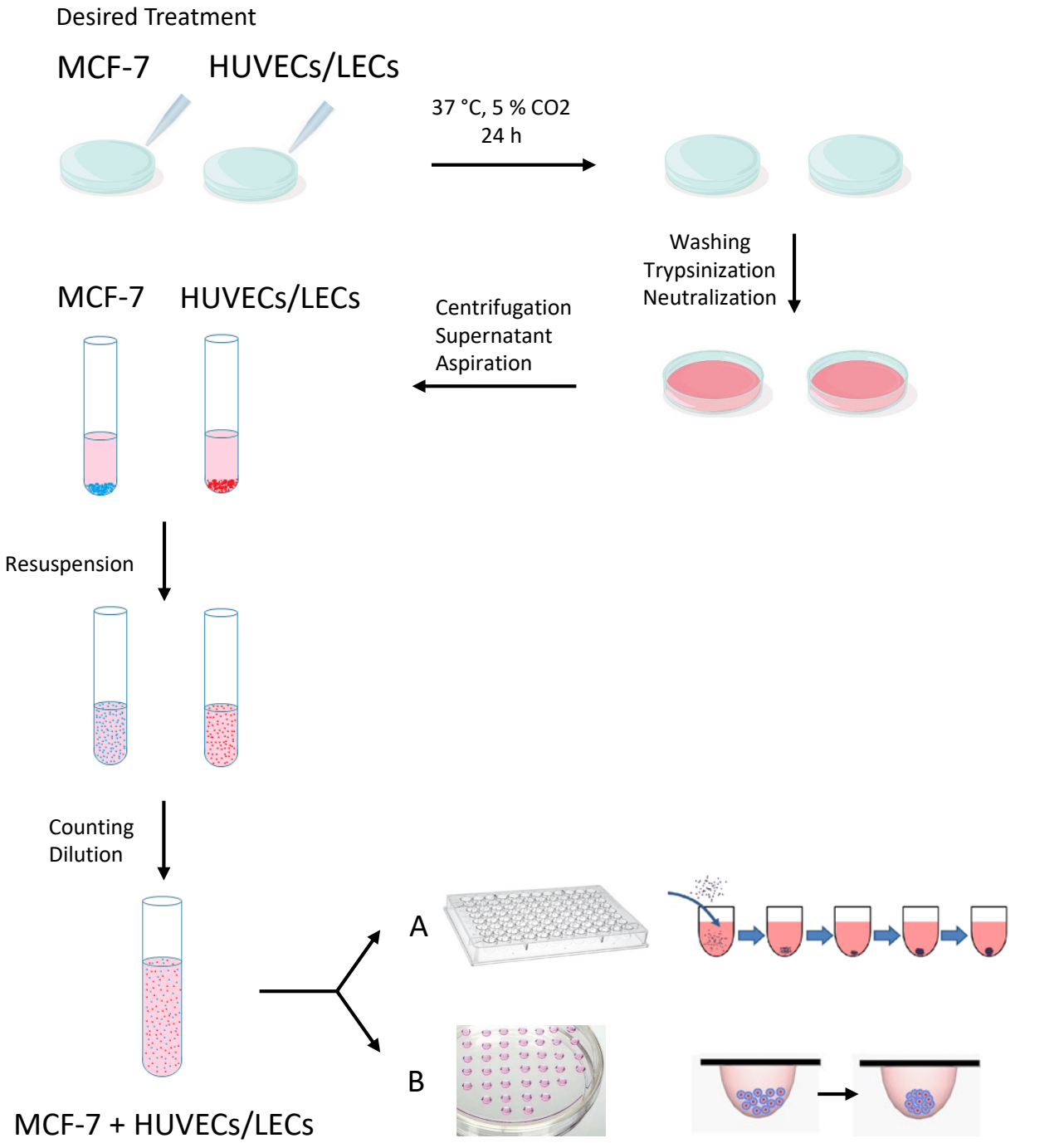
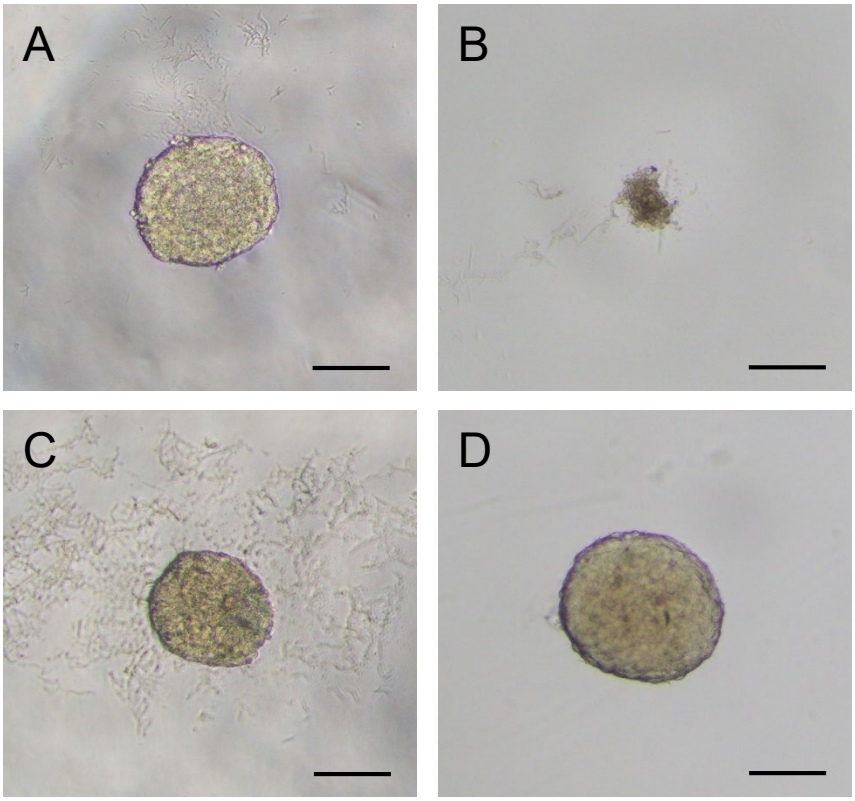
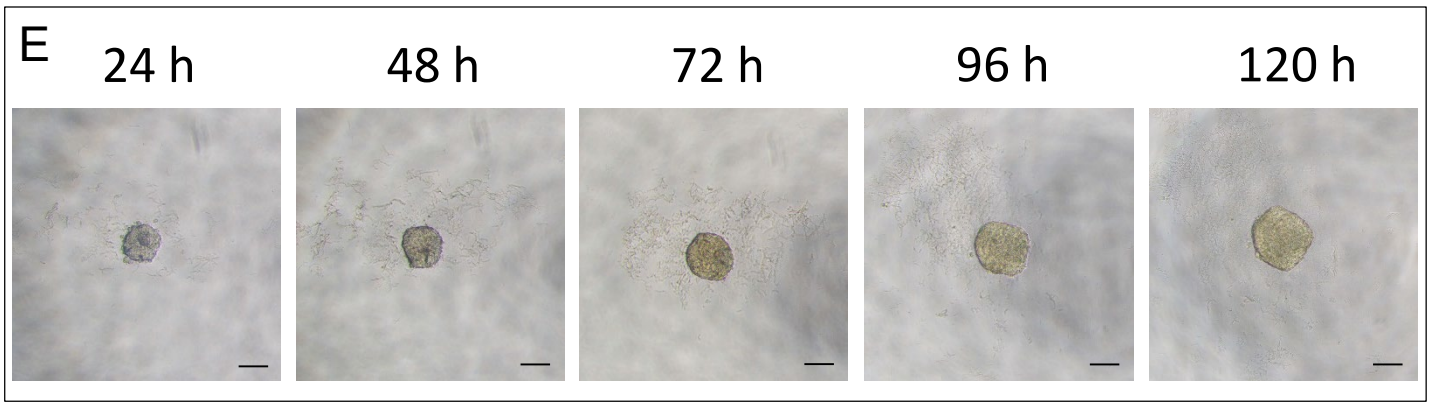
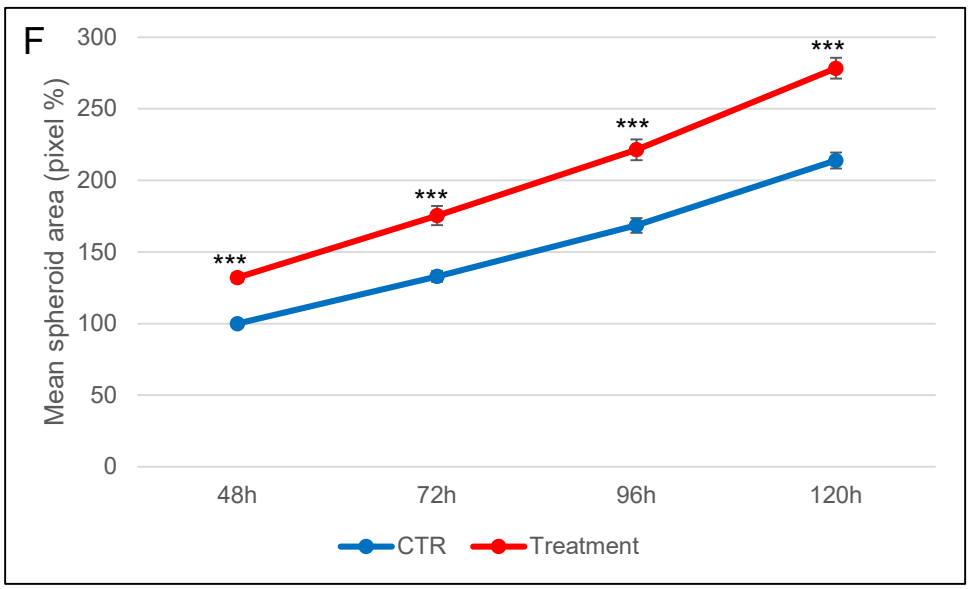


Figure-2







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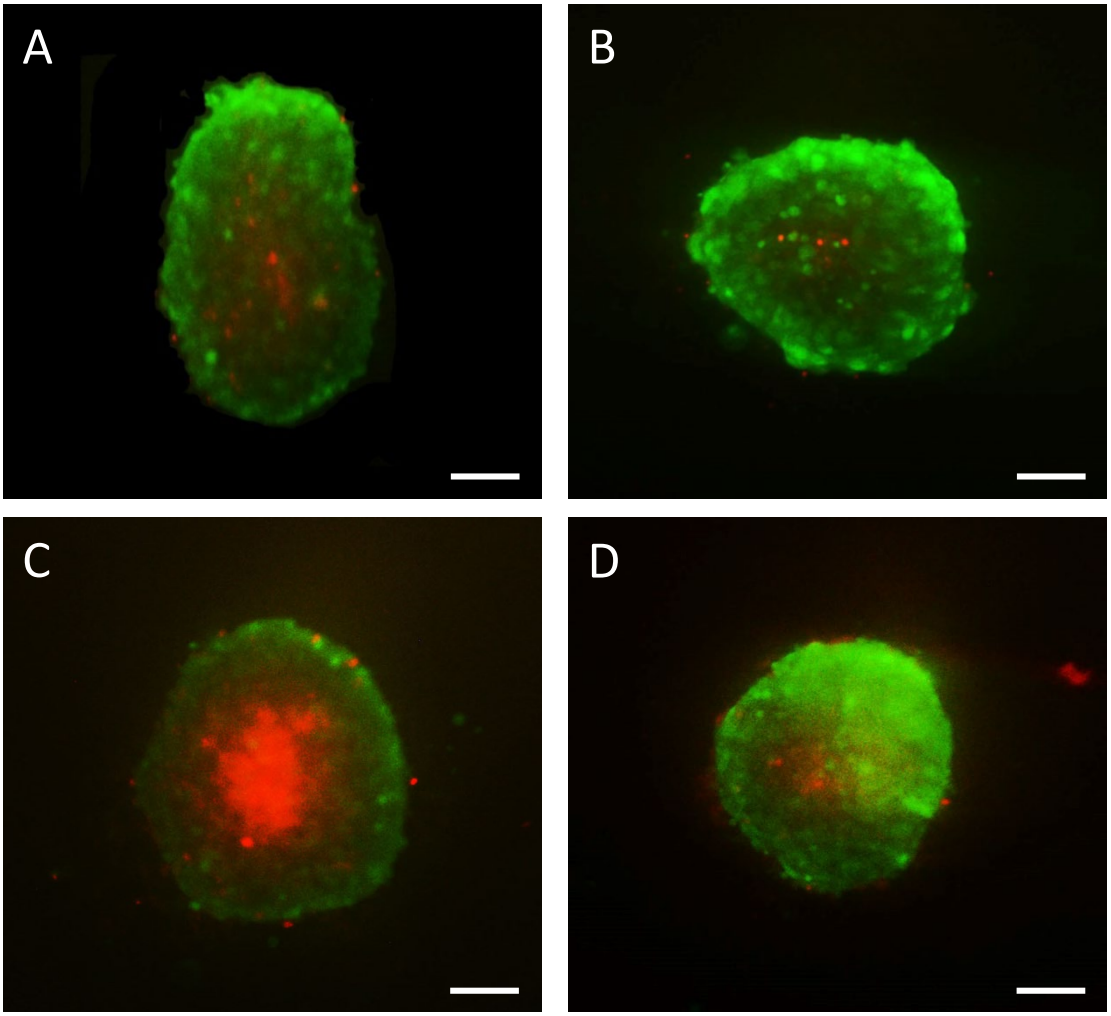

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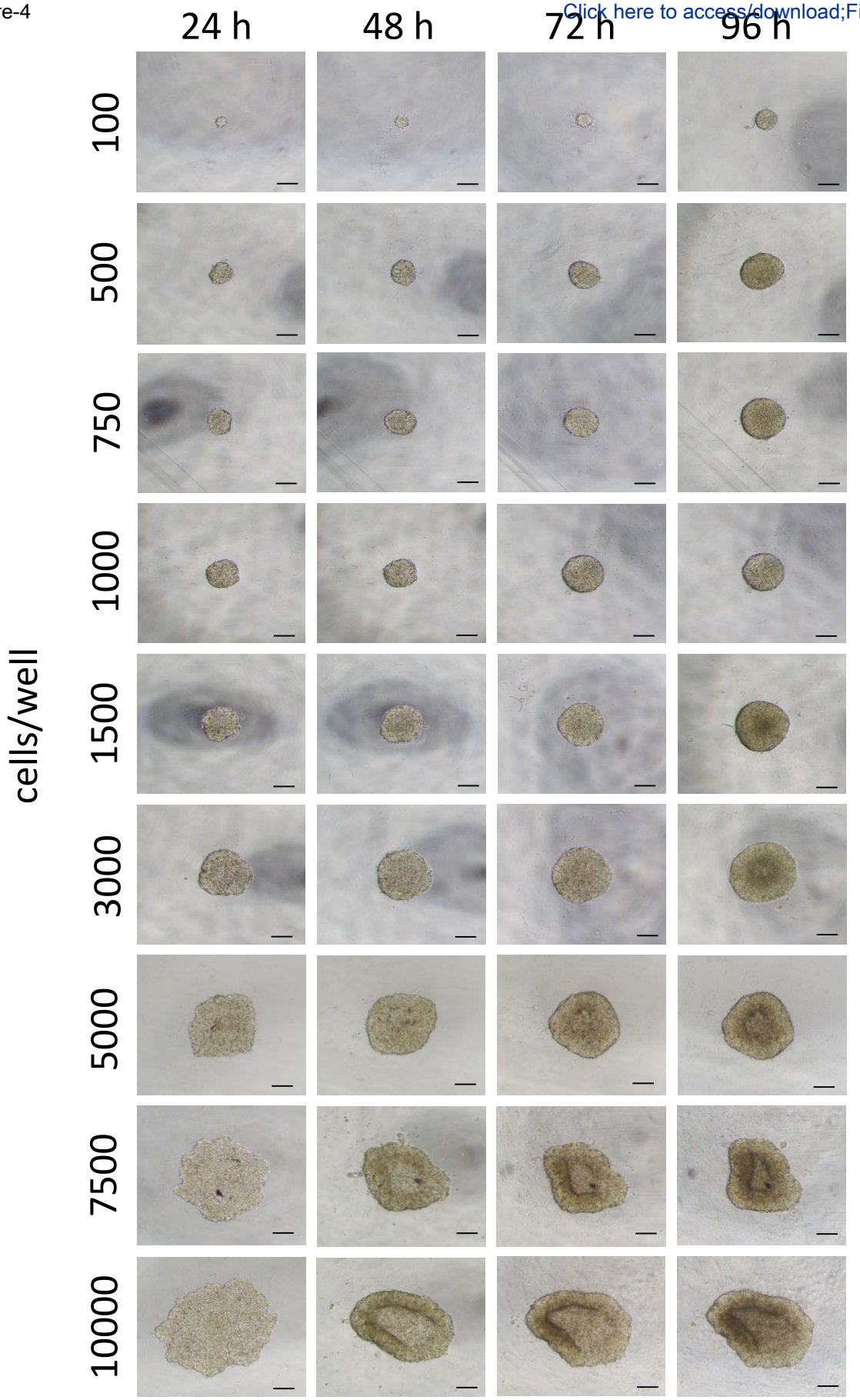

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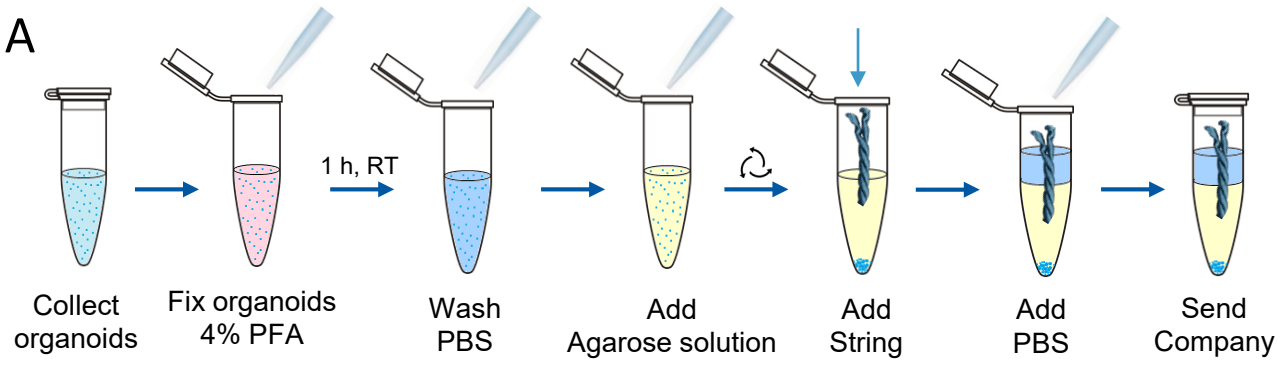

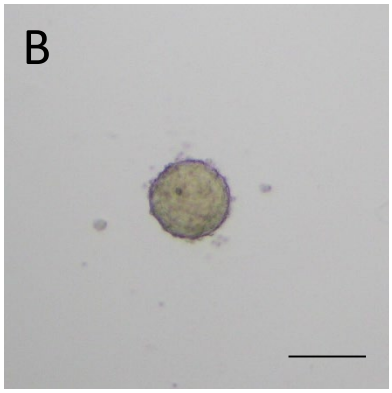

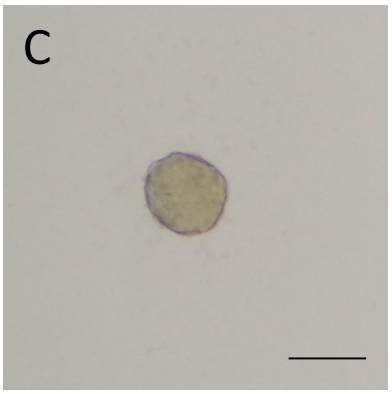

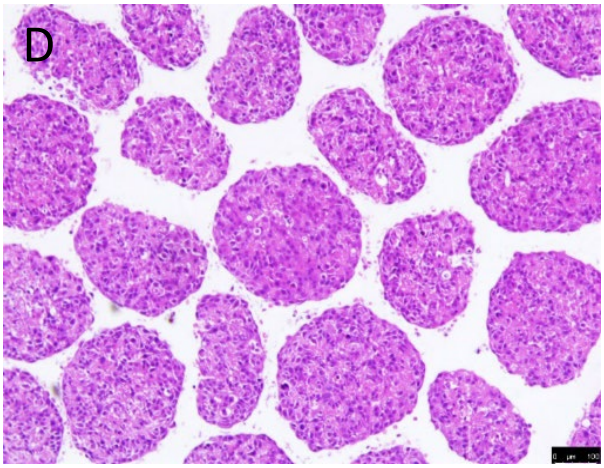

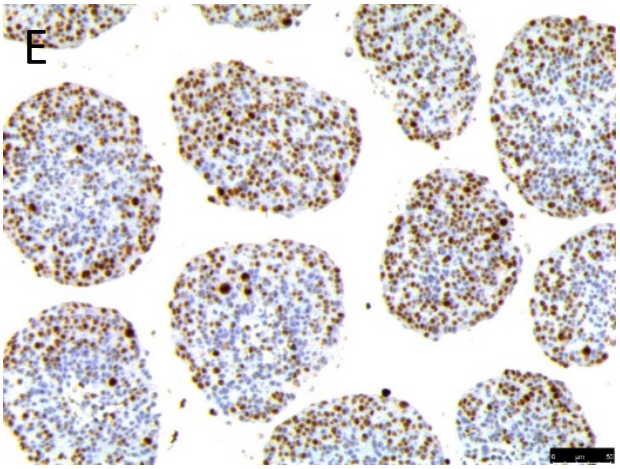

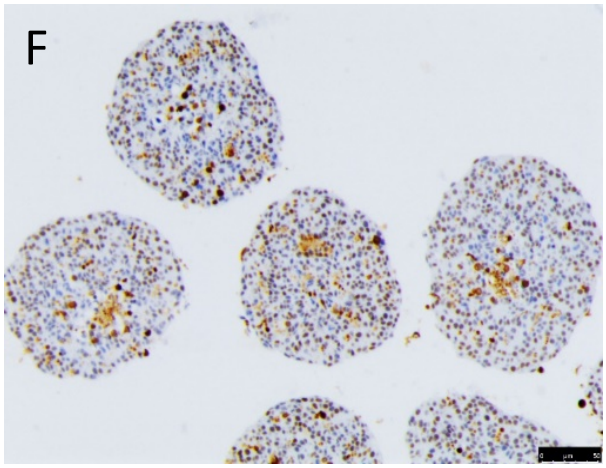

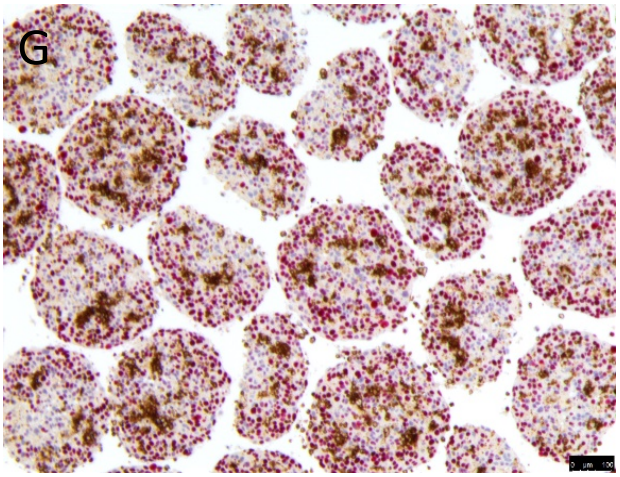

Figure-6

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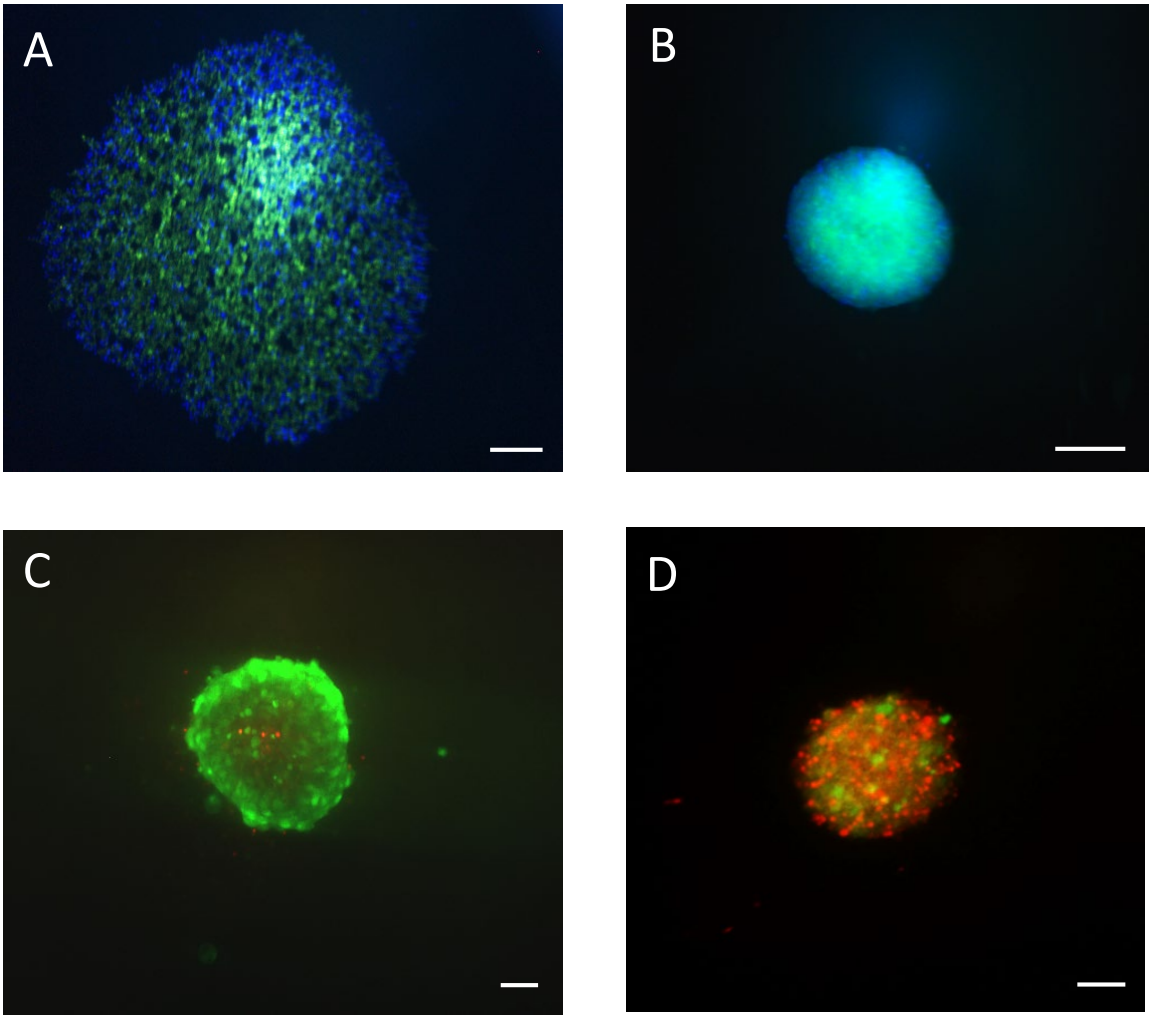

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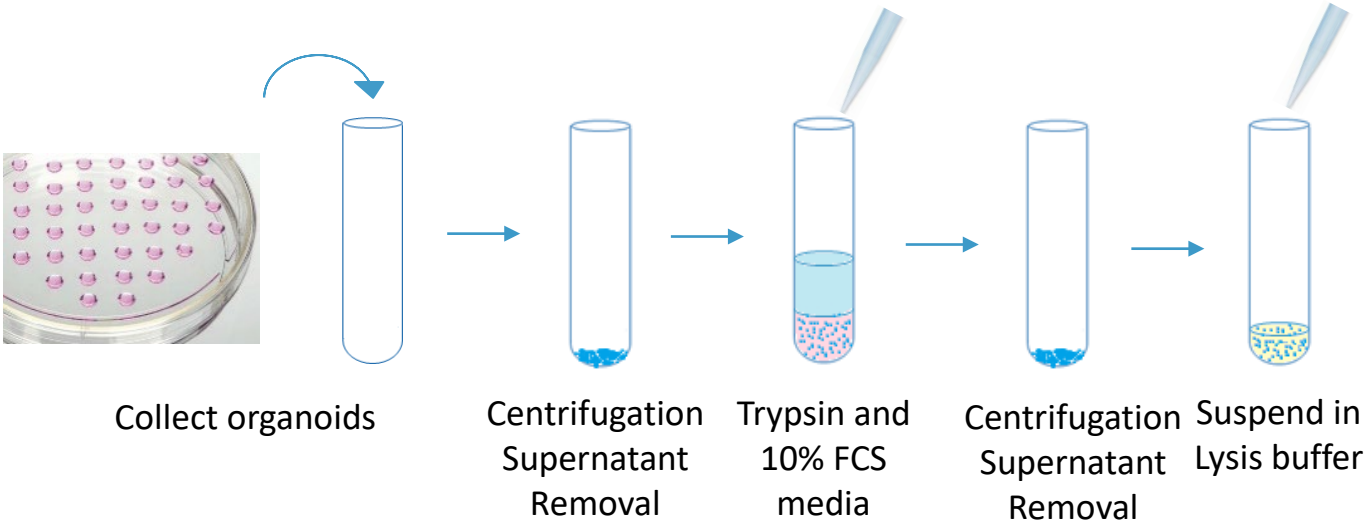

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Ref: *Submission of Revised Manuscript* ***JoVE62940*** *"Mammary Epithelial and Endothelial Cell Spheroids as a Potential Functional In Vitro Model for Breast Cancer Research"*

Dear Dr. Krishnan:

Thank you for giving us the chance to revise and resubmit our manuscript entitled **"Mammary Epithelial and Endothelial Cell Spheroids as a Potential Functional In Vitro Model for Breast Cancer Research"** for consideration for publication in ***JoVE.*** In the revised manuscript, we have fully addressed the issues raised by the editor and the four reviewers (See point by point response to the editorial and reviewers comments).

In the revised manuscript, the sections highlighted with YELLOW indicate material for constructing the Video. Whereas, **underlined** sections of the script indicate the changes made in response to the comments of reviewers and editor. The revised manuscript has been prepared as per the guidelines for publication in ***JoVE.*** All authors have read and approved the submission of the manuscript. It has not been published, nor is being considered for publication elsewhere, in whole or in part, in any language, except as an abstract. We hope that you will find our revisions adequate and are looking forward to your comments and final decision.

Thanking you.

Yours Sincerely

Raghvendra K. Dubey, Ph.D

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Response to Editorial Comments:

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

   Response: *We have gone through the manuscript to make sure there are no spelling or grammar issue.*

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

   Response: *We have made these corrections throughout the manuscript.*

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
   For example: GlutaMAX, PhotoshopCS2, Cell Tracker, Leica, Quick-RNA MiniPrep, Nunclon Sphera, etc.

   Response: *As requested, we have checked the manuscript and removed - trademark symbols, registered symbols, commercial language and company names from the manuscript. Moreover, all commercial products have been sufficiently referenced in the Table of Materials.*

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

   Response: *As requested, we have made the required changes.*

5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc.) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

   Response: *As requested we have made the adequate changes in the protocols to provide more details. (Section 3 lines 246-266, Sections 4.2.7.1. and 4.2.7.2. lines 311-336)*

6. Line 134: Please specify the concentration of collagen used.

   Response: *In the revised manuscript, we now provide the concentration of collagen used (Line 147).*

7. Line 205-206/284-285/294: Please specify the settings (magnification) for taking the images.

   Response: *In the revised manuscript, we provide the settings (magnification) for the images (Lines 221,243, 346, 479, 494, 502).*

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8. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

   Response: *We have formatted and marked the revised manuscript as requested.*

9. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

   Response: *We have reformatted the references in the script to the journal style.*

10. Please do not use the &-sign or the word “and” when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

    Response: *We have reformatted the references in the script to the journal style.*

11. **Figure 1:** Please ensure that the Figure or the figure legends do not contain any commercial term (Nunclon Sphera). Please use generic terms instead.

    Response: *We have checked the figures and figure legends to make sure they do not have any commercial term (Line 470 in Figure1).*

12. Figure2: Please maintain a single space between the number and the unit. Revise the labels in Figure 2D to “24 h”, 48 h”, etc. instead of “24h”, “48h”, etc. Please define the scale bars of Panel A, B, C in the figure legends.

    Response: As *requested, changes have been made in Figure2 and the article: Line 478.*

13. Figure 3: Please define the scale bars and the magnification used to capture images in the figure legends.

    Response: *Since we have added a new Figure to the revised manuscript, the original Figure 3 is now the Figure 4. Also, changes have been made in legends of Figures 2 (Line 480), 4 (Line 495), 5 (Line 503).*

14. Figure 4: Please maintain a unit spacing between the number and the unit (“1 h” instead of “1h”).

    Response: *As requested, we have made the required changes in the Figure.*

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Response to Reviewer #1:

Manuscript Summary:

Use of organoids as a model to mimic and study in-vivo pathophysiological conditions is of considerable relevance. Although single cell based organoids have been widely used, very little is known about two or multi-cell organoids. Here the authors demonstrate, in depth, the feasibility of forming breast cancer cell (MCF-7) organoids together with vascular and/or lymphatic endothelial cells. The authors hypothesize that cell-cell interaction between epithelial and endothelial cells may provide a better model to investigate the mechanisms regulating breast cancer and tumor growth. Importantly, this model could also be useful for testing anti-tumor therapeutic agents.

Overall, this methodology based article provides detailed information on how to culture/form epithelial plus endothelial cell organoids. The procedure provides all the important details and highlights critical steps where one has to pay attention. Monitoring and evaluating growth of an organoid is key to assessing tumor growth over time, and the authors describe this in detail and is simple to follow. Performing immunostaining helps in assessing cell growth, apoptosis and signaling mechanisms, and the authors describe the procedure for fixing the organoids for sectioning. They also provide evidence that transfection of cells using Lipofectamine, a commonly used delivery system, does not interfere with organoid formation and could be used for silencing or other cell transfection based studies.

The paper is well written, easy to follow and the methods described in great detail and with all relevant information to make organoids. I have some minor comments that may further help the article and make it more informative.

Major Concerns:

No

Minor Concerns:

1. Did the authors observe growth of organoids beyond 96 hours? Could the organoids be cultured for a longer time? The authors should provide some information on this issue as some experiments might require long-term treatments.

   Response: *We did conduct experiments to monitor the growth of spheroids for longer term (168 hours). As shown in the new figure 3 of the revised manuscript, MCF and MCF+HUVEC spheroids are more viable at 96 hours, as compared to 168 hours. Moreover, spheroids of MCF-7 cells alone have more dead cells at 168 hours, as compared to MCF-7 + HUVEC spheroids. We have included this data in the revised manuscript (Lines 399-402) and Figure 3 was added (Legend: lines 485-489).*

2. Some comments and helpful pointers on the issue of organoid viability in short term versus longer term cultures should be added.

   Response: *As mentioned in our response to comment 1 (above), we provide new data (Figure 3) showing the viability of cells in spheroids at 96 and 168 hours. We also discuss these changes in the revised article (lines 399-402 and figure 3- Legend in article lines 485-489).*

3. Authors mention the use of Lymphatic endothelial cells to make organoids with MCF-7 cells, but they do not provide any photomicrographs. They should show an organoid formed with MCF-7 + Lymphatic ECs.

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Response: *We thank the reviewer for pointing out this deficiency. In the revised manuscript we now provide a representative photomicrograph of a MCF-7 plus Lymphatic EC spheroid. See Figure 2 panel D and text in lines 391-392 and line 477 (legend to figure 2) of the revised manuscript.*

4. The authors have used live labelling to show presence of endothelial and epithelial cells in an organoid, however, the distribution of epithelial and/or endothelial cells should also be shown in organoid sections.

   Response: *We thank the reviewer for this constructive comment. In the revised manuscript we now show relative distribution of these cells (See figure 5G, legend to figure lines 507-508 and text on lines 429-438)*

5. Are the lymphatic endothelial cells capable of forming organoids alone or similar to HUVECs they form monolayers? A statement in this context would be helpful.

   Response: *The lymphatic endothelial cells behave like HUVECs and form monolayers when in monocultures. They do not form spheroids by themselves, but do so in presence of MCF-7 cells. We added a statement in this context in the text (Line 389).*

6. During co-culturing of MCF-7 together with vascular and/or lymphatic endothelial cells to form organoids, it is of fundamental importance to know the proportion of MCF-7 to the endothelial cells. In other words, MCF-7 are cancer cells and I can imagine that they proliferate much faster than the endothelial cells; Do the authors have an idea how many percent of the organoids cells are MCF-7 and how many percent endothelial cells (e.g., after 96 h co-culturing?). This could probably explain the absent of the vessel structures.

   Response: *We agree and as mentioned in our response to comment 4 (above), we have included a new image (figure 5 panel G) in the revised manuscript, moreover, the calculation of cell % (data shown in (lines 437-438) was performed using the protocol section "Cell quantification 4.7.2.2."*
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7. The isolation of RNA or DNA of a mixture of different cells for performing microarray analysis brings some information's useful for the model. However, to identify some key mechanisms of the effects of the one cell type for the other one (by analyzing the transcriptome), organoid cells should be separated for separate RNA isolation and microarray analysis. This can be done by FACS analysis and by using antibiotics resistance cell lines (e.g., using EGFP and/or puromycin resistance cell lines). This point should be discussed too.

   Response*: We agree and thank the reviewer for this constructive suggestion. In the revised manuscript we discuss these possibilities. (See lines -457 – 464).*

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Response to Reviewer #2:

Manuscript Summary:

The study by Azzarito et al, provides essential and in-depth information on generating multi-cellular organoids combining breast cancer and endothelial cells. The authors propose a method to create a useful cell culture system to study tumor growth in vitro in a system that mimics the real tumor setting. To that regard, taking into account the fact that endothelial cells from both blood vessels and lymphatic system, play a key role in cancer development, they introduce a model system by which cancer cells are mixed with endothelial cells to form tumor organoids with both cell-cell and cell-matrix interactions in all three dimensions.

This is an interesting study which will be quite useful for the study of cancer cell behavior, metastasis and the molecular mechanisms involved using in vitro systems. The paper is well-written and easy to follow, the figures are nicely designed, the aims are clear and the methodology is described in sufficient detail. It is definitely worth publishing as it will greatly enhance cancer research.

Major Concerns:

None

Minor Concerns:

However, there are a few comments that need to be addressed:

1. The authors claim to have used both human umbilical vein (HUVECs) and lymphatic endothelial cells (LECs) to make organoids with MCF-7 cells. However, they provide pictures only of organoids formed with MCF-7 plus HUVECs. It would be useful and interesting to also provide a representative photomicrograph of an organoid formed using MCF-7 plus LECs.

   Response*:* *We agree and as mentioned in our response to reviewer 1, comment 3, in the revised manuscript we have included a photomicrograph of a spheroid composed of MCF-7 plus Lymphatic ECs. See Figure 2 panel D.*

2. The authors show the sections of organoids that have been stained to assess cell growth and apoptosis etc. It would also be more informative if the authors can also include representative sections showing the presence of epithelial cells and endothelial cells within an organoid.

   Response**:** *We agree and as also mentioned in our response to Reviewer 1, we have included a new photomicrograph (figure 5 panel G) in the revised manuscript showing the presence of the two cell types in a spheroid.*

3. For consistency, the authors should use "breast-cancer" throughout the manuscript**.**

   Response: *Since we largely used the term "breast cancer" in the manuscript, for consistency, we have changed the term "breast-cancer" used only in the abstract to "breast cancer".*

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Response to Reviewer #3:

Manuscript Summary:

This is a very well written and presented protocol for the preparation of in vitro organoids suitable for cancer research. The protocol is comprehensive and reads well. I appreciate the limitations and possible troubleshooting advice, since this type of information is usually missing in these types or manuscripts.

Major Concerns:

None

Minor Concerns:

A small section of what experiments can be done with the resulting organoids (exposure to a certain substance, analysis of a specific pathway) would be welcome.

Response: *We thank the reviewer for the helpful suggestion. In the revised manuscript, we elaborate on the potential use of these organoids for experiments to elucidate growth rate in response to mitogens, inhibitors, and autocrine/paracrine factors, study cell-cell interaction in tumour pathophysiology, molecular (microarray) screening of proteins and genes, test development of new anti-cancer agents, and use gene manipulation of specific cells to regulate tumour growth. This is described on lines 461-464 and 582-585 of the revised manuscript.*

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Response to Reviewer #4:

Manuscript Summary:

In the manuscript by Azzarito et al. entitled „Mammary Epithelial and Endothelial Cell Organoids as a Potential Functional Ex vivo Model for Breast Cancer Research" the authors describe a model for production, culture, and agarose embedding of spheroids-aggregates, composed of two cell types: MCF7 breast cancer cells and HUVEC endothelial cells. This manuscript describes a method, which is not novel, but useful to researchers in the breast cancer field who are interested in breast cancer-endothelial cell crosstalk.

Major Concerns:

1. This reviewer has a strong objection against the incorrect use of the terms "organoid" and "ex vivo" in this manuscript, including the misleading title. The cellular structures that the authors produce are not organoids at all. Organoids are cellular structures, which resemble organs in their architecture and function, formed from stem cells or isolated from primary tissue (primary organoids), and cultured in extracellular matrix (ECM), characteristics of which are essential for organoid formation, further development, and function of the organoid. The structures described in this manuscripts are **spheroids**/aggregates formed from cell lines by aggregation of cells by hanging drop method or in aggregation plates. The cells do not organize properly, which also the authors admit in the discussion - that the HUVEC cells do not form capillary-like structures in the spheroids. Therefore, all misuse of the word "organoid" in this manuscript must be corrected.

   Response: *We fully agree and thank the reviewer for correcting us. As correctly pointed out by the reviewer, we have replaced the use of the term "organoid" with "spheroid" throughout the manuscript, including the title.*

2. Another term which is abused in this manuscript is "ex vivo". None of the cells, which are part of the aggregates, come from in vivo - all are established cells lines. Ex vivo can be correctly used only for primary tissues and cells, which have been extracted from in vivo - i.e. fresh patient sample or animal donor organism. Therefore, the "ex vivo" in the title needs to be corrected to "**in vitro**".

   Response: *We fully agree and thank the reviewer for correcting us. As correctly pointed out by the reviewer, we have replaced the use of the term "ex vivo" with "in vitro" throughout the manuscript, including the title.*

Further major concerns include:

3. In the introduction, the authors discuss the limitations and ethical issues of the use of animal models with the focus to highlight the 3D cell cultures. While this is all well, at the same time they shall discuss the limitations of the 3D cell cultures, which do not offer systemic influence and represent a reductionist approach in comparison to the animal models.

   Response: *We agree and as suggested by the reviewer we have added a statement stating the limitations of 3D cell cultures and the fact that they do not replace studies in animal models. A statement in this regard is included on lines 89-95 of the revised manuscript.*

4. Line 93-94: The authors completely ignore the presence of fibroblasts (cancer-associated fibroblasts) in breast tumors, despite they form up to 80% of the tumor mass!

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Response: *We agree with the reviewer. This was an oversight on our part as we were focused on spheroids made form epithelial and endothelial cells. As the reviewer correctly pointed out, we have revised our statement, to correctly state, the presence of fibroblasts in breast tumors. (See lines 100/112 and 581-585 of the revised manuscript).*

5. **Lines 94-97:** the authors describe some of the useful features of the breast 3D cultures, but miss important characteristics such as expression of female hormone receptors (progesterone/estrogen receptors), ability to conserve the patient tumor histological status, faithfully mimic response to therapy etc.

   Response: *We thank the reviewer for pointing out our mistake. As we were using ER-positive cells, we did not think about providing details. As pointed out by the reviewer, we have incorporated the importance of other features like expression of female hormone receptors (progesterone/estrogen receptors), ability to conserve the patient tumor histological status, faithfully mimic response to therapy etc., in the revised text. (See lines 100-103)*

6. The protocol/method section is written quite redundantly. Several parts are unnecessarily repeated. For example, the part "2. Organoid formation" (which should be correctly named Spheroid formation) contains subpart 1 on spheroid formation in U-bottom plates and part 2 on spheroid formation using the hanging drop method. The procedure of cell trypsinization and counting is identical and the difference is only in how much volume of cell suspension is pipetted in the wells of 96-well plate or in drops on the lid. Therefore, these two subsections shall be merged in one with two alternative endings.

   Response: *We fully agree with the reviewers comment. As suggested by the reviewer, we have revised part 2 to avoid redundancy and streamlined the protocol. See lines 181-237 of the Spheroid formation section.*

   Furthermore, the authors should write in this section already, how fast the spheroids form, how big, how many per well/drop etc.

   Response: *We agree and have revised this section. See changes made on Lines 224 and 235 (NOTE sections).*

   Another concern is that the authors should write already in this section on spheroid formation, how to prepare spheroids of cells stained with two different colors - because it is relevant here, during spheroid formation, not after when it is formed - which is relevant for the live/dead staining only in this protocol.

   Response: *We agree and have revised this section to include steps to prepare spheroids using cells stained with live cells tracker dyes. See lines 185-188 (2.3 Optional step) and lines 222-223 (2.7.1.4. Optional step) of the revised manuscript.*

7. Another major concern is the trypsinization of the spheroids for bulk RNA and protein isolation. This is both unnecessary and incorrect - trypsinization changes expression profile of the cells and destroys some of the proteins of the samples. No trypsinization is needed to isolate quality RNA/proteins from spheroids, the authors need to include proper homogenization step instead.

   Response: *We agree with the reviewer. Since this procedure was being used to improve RNA recovery from spheroids grown on matrix, we just continued to use the same approach. Since the trypsin treatment lasts for less than 1 minute and we do not see any adverse effects we continued to use. However, the reviewers point is well taken and we*

have added a note in this section of the revised manuscript stating that pelleted spheroids can be directly lysed by adding lysis buffer and trituration. See lines 365-369 of the revised manuscript.

Minor Concerns:

1. The authors use inconsistently abbreviations, such as FBS/FCS.

   Response: *We agree and thank the reviewer for pointing this mistake. In the revised manuscript we have corrected this error for consistency.*

2. The authors incorrectly use on many occasions "media". "Media" is plural of "medium".

   Response: *We agree and thank the reviewer for correcting us. In the revised manuscript we have corrected the spelling mistake for the term "medium".*

3. Line 135: "…let the collagen polymerize". This is incorrect. The collagen is polymerized by that time already - it polymerizes by 37°C.

   Response: *We agree, and have corrected this error. See changes made on line 149.*

4. The HUVEC medium is missing information on how much LSGS is supplemented.
   Response: *We agree, and have updated this information. See lines 152-154 (HUVEC subculture section 1.1.2.).*

5. Why HBSS is used to wash the plates/dishes, why not PBS (a cheaper option)?

   Response: *We agree with the reviewer and have added a note in the methods that HBSS could be replaced with PBS. See lines 159 / 176 of the revised manuscript.*

6. Why do authors insist on collecting cells (after trypsinization) into round-bottom tubes? Conical 15 ml falcon centrifuge tubes would work just fine, too.

   Response: *We fully agree with the reviewer.*
   Since round-bottom tubes were readily available in the lab, we decided to use them. However,15 ml conical tubes would also work well for this step. This option is stated in a note on lines 356.of the revised manuscript. However, we would like to point out that in practical terms a disadvantage of the conical 15 ml tube is that we can miss some organoids in the very bottom of the conical tube when picking them with a P1000 pipette.

7. Define what "10% FBS medium" is.

   Response: *We are sorry for not clearly defining the medium and in the revised manuscript, we provide this information. See line Line162 of the revised manuscript.*

8. For the spheroid formation, information is missing in what medium the cells should be resuspended after trypsinization - only "medium" is written on lines 170 and 190.

   Response: *We are sorry for this error and provide the missing information in the revised manuscript. See lines 196-198 for the revised manuscript.*

   However, when the cells are stained with CellTrackers, HUVEC medium is specified for the spheroid formation. So which is it and why?

   Response: *MCF-7 cells are stained with CellTracker green CMFDA diluted in MCF-7 growth medium (DMEM/F12 medium supplemented with 1x Glutamax, 1x antibiotic-antimycotic solution and 10% FCS), whereas HUVEC are stained with HOECHST diluted*

in HUVEC growth medium (EBM-2, 1% Glutamax, 1% antibiotic-antimycotic solution, LSGS and 10% FCS). Since the HUVEC require EBM-2 medium and MCF-7 DMEM/F12 we used them accordingly to label the cells. In the revised manuscript, we provide this information for clarity (See lines 186-188).

9. The authors use incorrectly throughout the manuscript "disc" instead of "dish".

   Response: *We thank the reviewer and have corrected this mistake in the revised manuscript*.

10. Lines 142-143: This reviewer believes that the efficiency of trypsinization shall be checked also visually under the microscope and after adding 10% FBS medium, the suspension shall be pipetted several times to ensure single celled suspension.

    Response: *Yes the efficiency of trypsinization is monitored microscopically (See lines 160 and 173).*
    Following, addition of 10% FCS medium to neutralize the trypsin the cells are centrifuged and the pellet is re-suspended in growth medium, single cells monitored and subsequently counted using Coulter counter. Additionally, the step of pipetting several times to ensure single cells in suspension is done after centrifugation of cells when growth medium is added (Step 1.1.6. and 1.2.4.)

11. Line 170: What "medium" shall be used to resuspend the cells? Define!

    Response: *To re-suspend the cells we used – "Steroid free medium" (lines 196-197 step 2.6.); because we were investigating the effect of the growth stimulator. However, the medium we used in our experiments (to assess spheroid growth) can be substituted with normal growth medium (See note line 198).*

12. Lines 171-173: Describe in more detail, how the cells are counted using the Isoton II Diluent and what other, alternative methods can be used if the reader do not have Coulter Z1 Cell Counter.

    Response: *We agree and in the revised manuscript we provide details for cell counting using Isoton II Diluent. Additionally we provide information on alternative methods that can be used, if a Coulter counter is not available. See lines 203-212 of the revised manuscript.*

13. Information on the statistical methods used is missing.

    Response: *We agree and have added this section in the revised manuscript. See line 483 of the revised manuscript.*

14. Figure 2E: It is unclear what the "treatment" of the cells was. While the authors do not have to disclose the exact character of the compound, it is confusing to the reader that in the result section they use the terms "growth stimulator" and "growth hormone" interchangeably. And growth hormone is actually a name of a hormone… So is this really growth hormone they used? This needs to be clarified.

    Response: *We fully agree with the reviewer. To avoid confusion now use the term "growth stimulator". See lines 381 / 397 / 398 of the revised manuscript.*

15. The authors show figure on spheroid destruction by freezing (Figure 6), though this protocol does not include any procedure for spheroid freezing nor proper discussion on how to avoid spheroid damage by freezing. Therefore, this Figure is unnecessary and out of line, no use to the reader. Moreover, the Figure 6B is rather theatrical - the magnification

in the right panel is inefficient and unnecessary to present in this way - the authors should present only the magnified photo.

Response: *We agree with the reviewer's opinion. We have removed all panels of this figure, except the magnified photo, which we have moved to Figure 6 - Panel D. Since we are still working on the freezing protocol, we could only state that the freezing protocol for single cells does not work for spheroids.*

16. Figure 5 legend: It is not clear what is the difference between panels A and B. Please clarify.

Response: *We agree and have rewritten this section for clarity. See revised legend to Figure 6 (originally figure 5). Panel A depicts cells immediately/shortly after seeding, whereas, Panel B depicts stained cells within a spheroid formed 48 hours after seeding.*

17. Language must be improved and several typos need to be corrected.

Response: *We agree and have revised the manuscript to correct all typos as well as spelling and grammatical mistakes.*

We thank the reviewer for the constructive comments and for pointing out critical errors.