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Growth and Characterization of Irradiated Organoids from Mammary Glands

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23 October 2018

Dear Dr. Singh,

We are pleased to submit our manuscript entitled, “Growth and Characterization of Irradiated Organoids from Mammary Glands” for your consideration in *Journal of Visualized Experiments* (JoVE) as a Methods Research Article in the Biomedicine category.

Breast conserving therapy (BCT) is used to treat the majority of triple negative breast cancer (TNBC) patients. In BCT, the tumor is surgically removed, and the surrounding area is exposed to ionizing radiation. Treatment can reduce cancer recurrence in much of the breast cancer population; however, more than one in eight treated TNBC patients experience local recurrence. Studying how radiation may recruit circulating tumor cells will therefore lead to insights about mechanisms contributing to local recurrence for TNBC patients.

There is currently a need to develop models that accurately reproduce host interactions *in vitro* as these models can be viewed in real time with techniques like live cell imaging. Organoids, obtained from digested tissue, are three-dimensional cellular constructs. They retain some functionality of the original organ, and are therefore ideal candidates for *in vivo* mimicking models. In this protocol, we report a method for reproducibly growing organoids from mouse mammary glands. We explored different methods of growing organoids, including plating in various protein matrices and seeding on tissue culture treated plastic; however, we determined that optimal growth resulted from culturing organoids on low-adhesion plates. We then characterized organoid growth and both epithelial marker and F-actin expression. Finally, we showed that organoids could be used for co-culture with macrophages. This presents exciting opportunities to further develop this model, including evaluation of cell-cell interactions between the organoids and other cells, including tumor cells, CD8+ T cells, and adipocytes.

We feel strongly that this work will be of wide interest to the readers of JoVE as it presents a model of irradiated normal mammary tissue that combines biological complexity and accessibility. We believe that this method will contribute to a greater understanding of breast cancer recurrence mechanisms following radiotherapy and may have significant implications for TNBC patients.

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We hope you share our excitement, and we look forward to your response. Please do not hesitate to reach out if you have any questions.

Sincerely,

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TITLE:

Growth and Characterization of Irradiated Organoids from Mammary Glands

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KEYWORDS:

3D organoid culture, mammary gland, normal tissue radiation response, cell-cell interactions, breast cancer, cancer immunology

SUMMARY:

Organoids developed from mouse mammary glands were irradiated and characterized to assess epithelial traits and interactions with immune cells. Irradiated organoids can be used to better evaluate cell-cell interactions that may lead to tumor cell recruitment in irradiated normal tissue.

ABSTRACT:

Organoids derived from the digested tissue are multicellular three-dimensional (3D) constructs that better recapitulate in vivo conditions than cell monolayers. Although they cannot completely model in vivo complexity, they retain some functionality of the original organ. In cancer models, organoids are commonly used to study tumor cell invasion. This protocol aims to develop and characterize organoids from the normal and irradiated mouse mammary gland tissue to evaluate the radiation response in normal tissues. These organoids can be applied to future in vitro cancer studies to evaluate tumor cell interactions with irradiated organoids. Mammary glands were resected, irradiated to 20 Gy and digested in a collagenase VIII solution. Epithelial organoids were separated via centrifugal differentiation, and 3D organoids were developed in 96-well low-adhesion microplates. Organoids expressed the characteristic epithelial marker cytokeratin 14. Macrophage interaction with the organoids was observed in co-culture experiments. This model may be useful for studying tumor-stromal interactions, infiltration of immune cells, and macrophage polarization within an irradiated microenvironment.

INTRODUCTION:

Approximately 60% of the triple negative breast cancer (TNBC) patients choose breast-conserving therapy (BCT) as a form of treatment[1]. In this treatment modality, the tumor containing part of

the breast tissue is removed, and the surrounding normal tissue is exposed to ionizing radiation to kill any residual tumor cells. Treatment reduces recurrence in much of the breast cancer population; however, approximately 13.5% of treated patients with TNBC experience locoregional recurrences[2]. Therefore, studying how radiation may recruit circulating tumor cells (CTCs) will lead to important insights into local recurrence[3,4].

Previous work has shown that radiation of the normal tissue increases recruitment of various cell types[5]. In pre-clinical models of TNBC, irradiation of normal tissue increased macrophage and subsequently tumor cell recruitment to normal tissues[5]. Immune status influenced tumor cell recruitment to irradiated sites, with tumor cell migration observed in immunocompromised subjects. Recapitulating these interactions using organoids derived from mammary glands will allow the observation of cell migration and cell-stromal interactions in real time with microscopy and live cell imaging to determine the role of radiation damage in altering tumor cell behavior.

Mouse mammary organoids have helped elucidate key steps in the development of the mammary gland. A mammary organoid is a multicellular, three dimensional construct of isolated mammary epithelium that is larger than 50 µm[6–10]. Using primary epithelial organoids, Simian et al. evaluated necessary factors for branching in the mammary gland[7]. Shamir et al. discovered that dissemination can occur without an epithelial to mesenchymal transition, providing insight into the metastatic cascade[8]. Methods for generating and characterizing organoids from mammary gland tissue are well established[6,11–13]. However, to our knowledge, methods for growing irradiated organoids from mammary glands have not been reported. A protocol for growing and characterizing irradiated organoids would be a critical step in recapitulating radiation-induced immune and tumor cell recruitment.

In this paper, we report a method for growing and characterizing irradiated mammary epithelial organoids in low adhesion microplates coated with a hydrophilic polymer that supports the formation of spheroids. These organoids were co-cultured with macrophages to examine immune cell infiltration kinetics. This work can be extended to include co-culturing organoids with adipose cells to recapitulate mammary characteristics, breast cancer cells to visualize tumor cell recruitment, and CD8+ T cells to study tumor-immune cell interactions. Previously established protocols may be used to evaluate irradiated organoids. Earlier models co-culturing mammary organoids and immune cells have shed light on mechanisms of metastasis and dissemination. DeNardo et al. found that CD4+ T cell regulation of tumor associated macrophages enhanced a metastatic phenotype of mammary adenocarcinomas[14]. Co-culture models have also been used to elucidate mechanisms of biological development. Plaks et al. clarified the role of CD4+ T cells as down-regulators of mammary organogenesis[15]. However, our group is the first to establish a procedure of visualizing how normal tissue irradiation influences immune cell behavior. Because normal tissue irradiation has been shown to enhance tumor cell recruitment[5], this protocol can be further developed to analyze how tumor cell behavior is altered by irradiation of normal tissue and cells, leading to a greater understanding of cancer recurrence.

PROTOCOL:

Animal studies were performed in accordance with institutional guidelines and protocols approved by the Vanderbilt University Institutional Animal Care and Use Committee.

1. Preparation of mice and cell acquisition (adapted from Nguyen-Ngoc et al.[11])

1.1. Sacrifice athymic Nu/Nu mice (8-10 weeks old) using CO_2 asphyxiation followed by cervical dislocation. Clean the skin using 70% ethanol.

1.2. Resect abdominal and inguinal mammary glands from mice using pre-sterilized scissors and forceps. Remove lymph nodes before resection. Rinse in sterile 1x phosphate buffered saline (PBS) (**Figure 1A**).

1.3. Place it in 15 mL tubes with 10 mL of Dulbecco's Modified Eagle Media/Nutrient Mixture F12 (DMEM/F12) for transport. Samples can be kept overnight at 4 °C or processed immediately. Keep on ice.

1.4. Irradiate samples at 20 Gy using a cesium source (**Figure 1B**).

1.5. 45 min after irradiation, place mammary glands in a 35 mm sterile cell plate and mince with scalpels (**Figure 1C,D**). Mince with approximately 40 strokes until the tissue relaxes and pieces are obtained that are no larger than approximately 1 mm^2 in area.

1.6. Transfer to the collagenase solution in a 50 mL centrifuge tube. Collagenase solution consists of 2 mg/mL collagenase (see **Table of Materials**), 2 mg/mL trypsin, 5% v/v fetal bovine serum (FBS), 5 µg/mL insulin, and 50 µg/mL gentamicin in DMEM/F12 media. Use 10 mL collagenase solution per mouse.

1.7. Place in a water bath at 37 °C, vortexing every 10 min for 30-60 min. Digestion is complete when the collagenase solution is cloudy (**Figure 1E,F**).

1.8. Spin down the digested solution at 450 x *g* for 10 min at room temperature (RT). Three layers will be observed. The supernatant is composed of fat, the middle layer is an aqueous solution, and the bottom is a pellet. The pellet will appear red as it is a mixture of epithelial cells, individual stromal cells, and red blood cells (**Figure 1G**).

1.9. Precoat all pipettes, pipette tips, and centrifuge tubes with bovine serum albumin (BSA) solution prior to contact. BSA solution consists of 2.5 w/v % BSA in Dulbecco's Phosphate Buffered Saline (DPBS). For pre-coating, simply add then remove BSA solution to the inside of the pipette tip and tubes. BSA solution can be reused, although it should be sterile filtered before each experiment.

1.10. For additional recovery, transfer the supernatant to a fresh BSA coated 15 mL tube. Pipette up and down vigorously to disperse fat layer. Centrifuge at 450 x *g* for 10 min at RT.

Aspirate the supernatant, leaving a small amount of media in the tube to avoid aspirating the cell pellet.

1.11. Aspirate the aqueous layer from the tube with the original pellet.

1.12. Add 10 mL of DMEM/F12 to the tube with the original pellet and transfer to the second tube. Pipette vigorously to combine and resuspend the two pellets.

1.13. Centrifuge at 450 x *g* for 10 min at RT. Aspirate the supernatant and add 4 mL of DMEM/F12 to the tube.

1.14. Add 40 µL of deoxyribonuclease (DNase) to the suspension and gently shake by hand for 2-5 min at RT. DNase solution consists of 4 U/mL DNase in DMEM/F12.

1.15. Add 6 mL of DMEM/F12 and pipette thoroughly. Centrifuge the tube at 450 x *g* for 10 min at RT.

1.16. Aspirate supernatant to the 0.5 mL mark. Resuspend in 10 mL of DMEM/F12 and pipette thoroughly.

1.17. Pulse to 450 x *g* and stop 4 s after reaching that speed.

1.18. Repeat steps 1.16-1.17 three more times to purify organoids via centrifugal differentiation. The pellet should now be an off-white color consisting of only epithelial organoids (**Figure 1H**).

NOTE: Organoids can also be filtered using sterile mesh 40 µm filters. After step 1.16, pipette media containing organoids through a filter into a centrifuge tube, and then rinse with 5 to 10 mL of DMEM/F12 media. Flip the filter over a new 50 mL centrifuge tube. Pass 10 mL of DMEM/F12 media through, going the opposite way to rinse off any retentate. The retentate should consist of organoids, and the filtrate should consist mainly of stromal cells, which can be discarded or kept if desired.

2. Determining density and plating organoids

2.1. Resuspend pellet in 10 mL of DMEM/F12. Pipette thoroughly to create a homogenous solution.

2.2. Transfer 50 µL to a 30 mm Petri dish, and view under a phase contrast microscope at 20x. Count the number of organoids with a tally counter.

NOTE: Here pipette tips have been consistently used with a minimal diameter of 457 µm, which is 5-10 times the diameter of the organoids that are seeded. For transferring volumes of 2 mL

or larger (e.g., steps 1.16 and 2.1), use serological pipettes with tip diameters excess of 1,500 µm.

2.3. Calculate the organoid density using the following equation:

$$\frac{\#\ organoids\ in\ 50\mu L}{50\mu L} = \frac{\#\ organoids\ in\ tube}{volume\ of\ tube}\ [=]\ \frac{organoids}{\mu L}.$$

The desired density is 1,000 organoids/mL to simplify further dilution. If the density is too low, centrifuge at 450 x *g* for 5 min and aspirate media. Add media necessary to reach 1,000 organoids/mL, and pipette thoroughly to create a homogenous mixture.

2.3.1. To grow organoids in a protein matrix, seed organoids at a concentration of 1 organoid/µL in collagen type 1 diluted to 87% or in basement membrane extracted from Engelbreth-Holm-Swarm mouse sarcoma. While working with samples, keep on ice.

2.3.2. To freeze organoids, transfer the desired volume to a separate centrifuge tube. Spin down at 450 x *g* for 5 min. Aspirate media, and then add the same volume of 90% FBS/10% DMSO. Resuspend the organoids, and then aliquot into cryotubes. Transfer to -80 ˚C, and then to liquid nitrogen within one week.

2.3.3. To thaw, warm in a 37 ˚C water bath for one min. Centrifuge at 450 x *g* for 5 min, and then aspirate freezing media. Rinse with sterile DPBS, and then centrifuge again. Aspirate DPBS and add organoid media.

2.4. Pipette 50 µL (50 organoids) into each well of the low adhesion plate (**Figure 1I**).

2.5. Add 150 µL of organoid media to bring the total working volume to 200 µL. Organoid media consists of 1% penicillin-streptomycin and 1% insulin-transferrin-selenium (ITS) in DMEM/F12 media.

2.6. Every 2 days replace media carefully.

NOTE: Low adhesion plates are not tissue culture treated; therefore, the cells can be easily detached. Aspirate media slowly by tilting the plate and inserting the pipette tip at the edge of each well. Leave a small amount of media in the bottom of the well. Add new media slowly to avoid applying unnecessary shear forces to organoids.

3. Co-culturing with macrophages

3.1. Maintain GFP or dTomato-labelled RAW 264.7 macrophages in DMEM media supplemented with 10% FBS and 1% penicillin-streptomycin. Seed 1 x 10^4, 5 x 10^4, or 1 x 10^5 cells/mL into organoid media.

3.2. Use live cell phase contrast and fluorescent imaging to monitor macrophage infiltration over time.

4. Immunofluorescence staining of organoids

NOTE: Organoids can be stained in low adhesion wells or can be transferred to chamber slides. To transfer, gently pipette up and down until organoids have detached from plates. Transfer to chamber slides and incubate for 4-8 h to allow organoids to adhere to the plate surface.

4.1. Remove organoid medium from the wells by carefully aspirating. Fix samples with 10% neutral buffered formalin for 15 min at RT.

4.2. Wash 3x 5 min in 1x PBS. If desired, fixed samples can be stored at 4 °C for one week for further staining.

4.3. Permeabilize with 0.1% 4-(1,1,3,3-Tetramethylbutyl) phenyl-polyethylene glycol for 5 min.

NOTE: To stain for F-actin, incubate samples with phalloidin diluted 1:1,000 and 1.67 nM bisbenzimide nuclear dye in 1% PBS/BSA for 1 h at RT. Then, proceed to step 4.8.

4.4. Block with 5% normal goat serum in 0.1% PBS/Polyethylene glycol sorbitan monolaurate (PBST) for 1 h at RT. Wash 3x 5 min with PBS.

4.5. Incubate with Anti-Cytokeratin 14 diluted 1:1,000, E-Cadherin diluted 1:200, or Tight Junction Protein One diluted 1:100 in 1% NGS in PBST for 1 h at RT. Wash 3x 5 min in PBST.

4.6. Incubate with Goat Anti-Rabbit secondary diluted 1:200 with 1% NGS/PBST for 1 h at RT. Cover with foil to avoid light exposure.

4.7. Wash 3x 5 min in PBS. Use the nuclear dye (see **Table of Materials**) to stain nuclei.

4.8. Wash 3x 5 min in PBS. If using chamber slide, mount with a coverslip. Store wrapped in foil at 4 °C for up to 2 weeks.

REPRESENTATIVE RESULTS:

Irradiated epithelial mammary organoids were successfully obtained from mouse mammary glands, processed, and cultured on low-adhesion plates (**Figure 1**). Organoid yield was tested by seeding in different growth environments (**Figure 2A-G**). Seeding cells directly onto tissue culture treated 10 cm cell plates yielded an overgrowth of fibroblast cells. Fibroblasts were identified under phase contrast microscopy in or near the same plane of focus as organoids, and they quickly grew out from plated organoids within a few days. An outgrowth of fibroblasts was also observed when organoids were seeded in basement membrane and collagen protein matrices (**Figure 2E,F**).

A variety of conditions were tested in optimizing irradiated organoid growth (**Figure 2H**). Collagenase types I and VIII from *clostridium histolyticum* were used as the enzyme in the organoid digestion step[11,20]. Organoid yields were significantly higher after digestion with collagenase VIII. This may be due to the purification processes used in producing the enzyme: collagenase type I is partially purified and may cause unnecessary damage to membrane proteins and receptors, leading to poor organoid formation, cell lysis, or over-digestion[20–22]. No significant differences in yield between irradiated and control organoids were observed.

Irradiated organoids could be cultured in low adhesion plates (**Figure 3A-C**) or within basement membrane (**Figure 3D-G**), but the most rapid growth occurred in low adhesion plates (**Figure 3H**). Organoids recapitulated mammary gland characteristics. White arrowheads indicate constructs morphologically similar to ducts and lobes[24,25] (**Figure 3C**), which are critical for the production and transport of milk in the mammary gland[25]. However, further characterization is required to confirm this observation. Growth trends indicated that non-irradiated organoids grew faster than irradiated organoids (**Figure 3H**), most likely due to cell growth arrest resulting from mechanisms of DNA damage repair; however, the trend was not statistically significant[26]. Occasional clumping of low adhesion organoids was observed, and organoids could be cultured up to two weeks before dissociating.

Organoids expressed epithelial characteristics, which were evaluated through immunofluorescence staining of Cytokeratin 14 (K14), E-Cadherin (E-cad), and Tight Junction Protein 1 (ZO-1)[19, 27, 28] (**Figure 4**). Irradiated organoids expressed epithelial markers. K14, a marker of myoepithelium[19], was expressed strongly on the surface of irradiated organoids (**Figure 4A**). Additionally, E-cad and ZO-1 were expressed within cellular junctions of organoids (**Figure 4B, C**). These proteins are essential for proper cell adhesion[27]. After irradiation, organoids continued to retain their epithelial characteristics.

Fluorescent staining of organoids could be visualized within low adhesion plates using fluorescence microscopy (**Figure 5A-D**); however, the clearest visualization was obtained via confocal microscopy (**Figure 5E-F**). Corrected total fluorescence intensity was calculated by subtracting the background and normalizing by organoid area (**Figure 5G**). Growing organoids in the 96-well low adhesion plates also simplified co-culture experiments. When seeded at concentrations typical in the mammary gland, macrophages co-localized with control and irradiated organoids (**Figure 6**)[28,30].

FIGURE AND TABLE LEGENDS:

Figure 1. Method Workflow. (**A**) Mammary glands were resected from mice. The abdominal and inguinal mammary glands were used. (**B**) Mammary glands were irradiated in 50 mL centrifuge tubes containing DMEM/F12 media. (**C**) Mammary glands were transferred to sterile six-well plates and cut with surgical scalpels until minced (**D**). (**E**) Mammary glands were transferred into 50 mL centrifuge tubes containing 5 mL of sterile DMEM/F12 media per gland and digested in a collagenase VIII solution (**F**). (**G**) After being transferred to a 15 mL tube, centrifugal differentiation was utilized to remove stromal cells, single cells, and red blood cells, observed in

a red pellet (white arrow-head) until only white epithelial organoids were obtained (**H**). (**I**). 50 organoids were plated in 200 µL of media in 96-well low adhesion plates and imaged using phase contrast microscopy Scale bar represents 50 µm.

Figure 2. Organoid Plating in 3D Protein Matrices and on Tissue Culture Treated Plastic. Organoids seeded in collagen (**A**) and basement membrane (**B**), imaged after 84 hours of growth. Outgrowth of fibroblasts occurred in matrix plated organoids (**C**, **D**). Phase contrast images of organoids sorted through filtration were obtained 192 hours after seeding. No major differences between the filtrate (**E**) and retentate (**F**) were observed, with both resulting in confluent fibroblast growth. Cells in **E** and **F** were seeded on tissue culture treated plastic. After trypsinizing for 5 min at RT, fibroblasts were removed via aspiration; however, remaining epithelial cells formed a monolayer culture instead of three-dimensional organoids (**G**). Scale bars for **A-G** represent 100 µm. (**H**) Different collagenase types (I (CI) and VIII (CVIII)) and cell processing methods (filtration and centrifugal differentiation (Cent Diff)) were tested, and organoid yield per mammary gland was quantified (n = 2 glands for CI, Filter; 2 glands for CVIII, Filter; 4 glands for CI, Cent Diff, and 12 glands for CVIII, Cent Diff). Statistical significance was determined using a two-tailed, unpaired t-test, ***p<0.0001. Error bars represent standard error.

Figure 3. Representative Organoid Growth. Phase contrast images of irradiated organoid growth in low adhesion plates obtained 20 (**A**), 44 (**B**), and 106 (**C**) hours after seeding. White arrowheads indicate structures that have similar morphology to ducts and lobes. Phase contrast images of irradiated organoid growth in basement membrane obtained 42 (**D**), 66 (**E**), 60 (**F**), and 114 (**G**) hours after seeding. Scale bars represent 50 µm. **H.** Area measurements were obtained in different growth conditions: organoids immediately seeded after digestion and sorting (Irradiated (●), Control (■)), and organoids seeded in basement membrane (Irradiated (○), Control (□)) (n = 3 glands each). Area calculations were made using ImageJ software. Error bars represent standard error.

Figure 4. Epithelial Marker Expression on Irradiated Organoids. Cytokeratin 14 (K14, green), a marker for the basal layer of squamous and non-squamous epithelia, was expressed on irradiated organoids (**A**). E-cadherin (E-Cad), a protein essential for adhesion, was expressed within the junctions between cells in irradiated organoids (**B**). Tight junction protein one (ZO-1) was also expressed within cell junctions of irradiated organoids (**C**). Images were obtained in chamber slides via confocal microscopy. A nucleic acid stain was used to visualize nuclei (blue). All organoids were fixed and imaged after one week of growth. Scale bars are 50 µm.

Figure 5. F-actin expression in organoids. F-actin (red), a microfilament in epithelial cells, was expressed with lower intensity in non-irradiated organoids (**A, C, E**) than in irradiated (**B, D, F**) organoids. A nucleic acid stain was used to visualize nuclei (blue). Images were taken on low adhesion 96-well plates (**A, B**) and 16-well chamber slides (**C, D**). Images were also taken using confocal microscopy (**E, F**). All organoids were fixed and imaged after one week of growth. Scale bars are 50 µm. **G.** Phalloidin fluorescence data from low adhesion plate images were quantified in ImageJ (n = 3 glands). Error bars indicate standard error.

Figure 6. Evaluating cell-cell interactions through macrophage-organoid co-culture. Macrophages (red) infiltrated control (**A**) and irradiated (**B**) organoids. Scale bars represent 50 µm. Average percent area of macrophages in the image field (**C**) was reported at 24 hours of co-culture for control (yellow) and irradiated (orange) organoids (n=3 glands for each sample). Macrophages were seeded at concentrations of 10,000 cells/mL, 50,000 cells/mL, and 100,000 cells/mL, and their infiltration was captured every 30 minutes via live cell fluorescence imaging. All co-culture experiments commenced 7 days after initial organoid seeding. Statistical significance was determined using a two-tailed, unpaired t-test, *p<0.05, ***p<0.0001.

DISCUSSION:

In this protocol, we have developed a method for reproducible growth and characterization of irradiated mammary organoids (**Figure 1**). An irradiation dose of 20 Gy was applied to mirror previous in vivo models of tumor cell recruitment[5]. Irradiation of mammary glands ex vivo prior to organoid formation allowed for isolation of radiation damage effects without a corresponding infiltration of immune cells. The development of an in vitro irradiated normal tissue model enables real time viewing of cellular interactions that may contribute to radiation induced CTC recruitment[11,12].

Closely following steps 1.5-1.18 was critical for maximizing organoid yield. We added thawed aliquots of concentrated collagenase to the digestive solution. Due to the highly viscous nature of the concentrated collagenase aliquot, there can be some variations in amount and therefore in enzymatic activity, so organoid digestion must be closely monitored to avoid over-digestion. It is also important to digest organoids in a 50 mL tube as this allows for an even surface area for digestion. Other studies have used filtration for purifying organoids[19,32]; however, we obtained a much higher yield purifying with centrifugal differentiation (**Figure 2H**). Pre-coating pipettes, pipette tips, and centrifuge tubes with the BSA solution is essential for maximizing yield. Organoids noticeably adhere to uncoated plastic when solution application is neglected.

Great care must be taken to avoid aspirating organoids. This is a risk that occurs when purifying, changing media, and staining for fluorescent markers. Using low adhesion plates for growth allows for easy transfer of organoids and removes the need for organoids to be sectioned in OCT for further staining, a procedure required for basement membrane embedded organoids[11]. In addition to benefits from superior growth, seeding irradiated organoids in low adhesion plates required fewer steps and was less technically challenging than culturing organoids in basement membrane or collagen. However, when staining for markers, it may be helpful to view organoids under a microscope to ensure that accidental aspiration does not occur.

Moreover, there are many considerations that must be accounted for when imaging organoids. Within basement membrane embedded organoids, occasional fibroblast growth may be observed (**Figure 2C,D**). Fibroblast outgrowth in 3D cultured organoids may be caused by organoids making contact with the tissue culture treated surface as adhesion leads to upregulated fibroblast growth factor production in adherent cells[31]. Interestingly, the morphology of these fibroblasts is strikingly similar to pre-adipocytes as both cell types exhibit

spindly, elongated shapes[16]. In further investigation, exposure to insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) may yield cells with an adipogenic lineage, spurring a shift towards a more spherical cellular shape associated with adipocytes[17,18]. We obtained clear images using phase contrast microscopy of free-growing low adhesion (**Figure 3A-C**) and basement membrane embedded (**Figure 3D-G**) organoids. Tracking individual organoid growth in low adhesion plates, however, was difficult due to minimal focal adhesions between the cells and well surface, resulting in organoid movement and occasional aspiration.

Once stained for surface markers, confocal microscopy rendered clearer marker localization (**Figure 5E,F**) than widefield microscopy (**Figure 5A-D**). From fluorescence quantification, trends in phalloidin expression suggest that irradiated organoids expressed increased F-actin relative to the control (**Figure 5G**). Actin cytoskeleton reorganization has been observed in dermal microvascular endothelial cells irradiated at similar dosages[32].

For extended imaging sequences, like time lapse co-culture with immune cells (**Figure 6**), a live cell imaging chamber with humidity and CO_2 control is required[33]. Live cell images taken every 30 minutes revealed that macrophages co-localized with organoids after 24 hours (**Figure 6A,B**), preferentially migrating toward irradiated organoids (**Figure 6C**). Macrophage infiltration into irradiated normal tissue has been observed in vivo, is attributed to chemokine and cytokine gradients, and typically precedes CTC recruitment[5]. Future studies will evaluate classically and alternatively activated macrophage interactions with organoids as polarized macrophage dynamics may play an important role in determining response to radiation[31,32]. Additional analyses will evaluate the consequence of serum starvation and the growth effects of culturing organoids in complete media since these variables may have significant effects on organoid-immune cell interactions. This system can further be adapted for co-culture with other cell types, including CD8+ T cells, stromal cells, adipocytes, and breast cancer cells. Real time observation with techniques like live cell imaging will facilitate the elucidation of potential mechanisms that contribute to CTC recruitment to irradiated normal tissue, which may have significant implications for patients suffering from recurrent TNBC.

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DISCLOSURES:

The authors have nothing to disclose.

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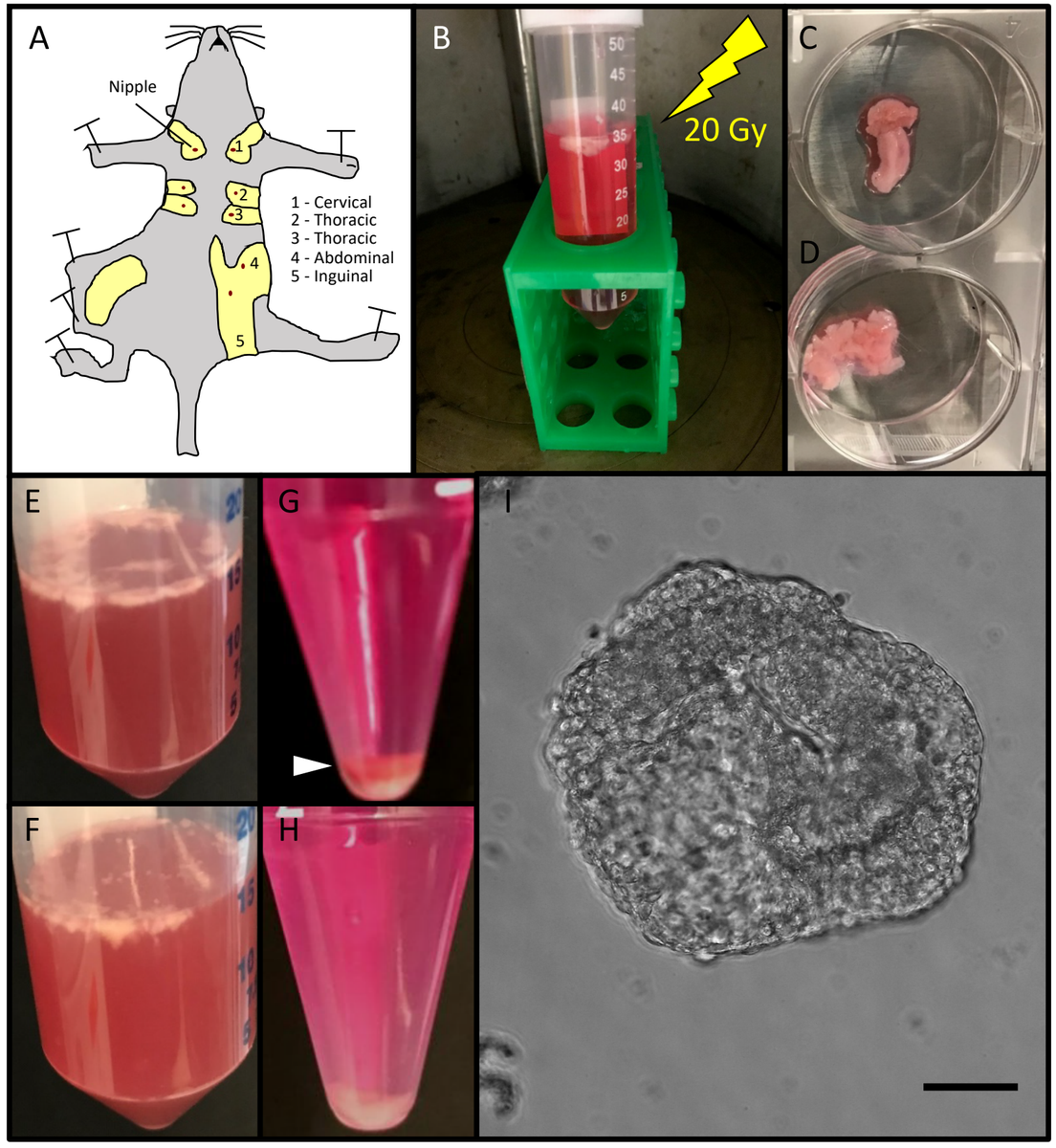

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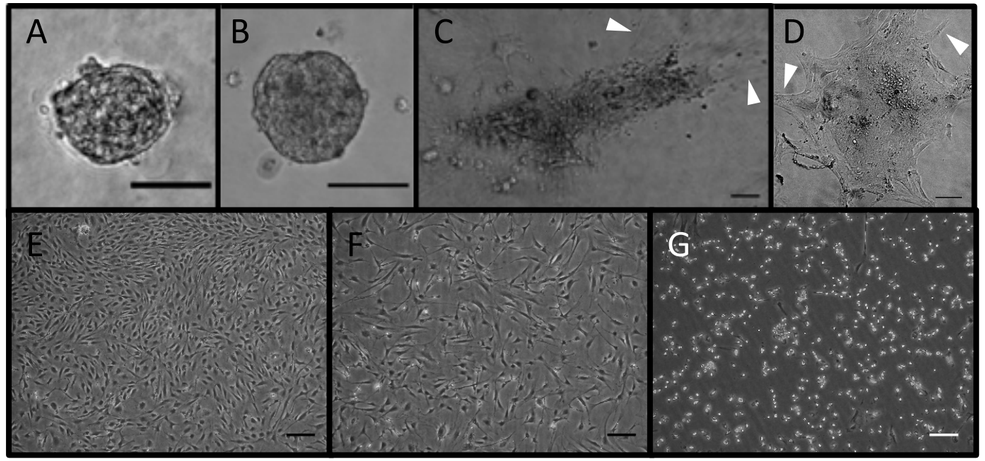

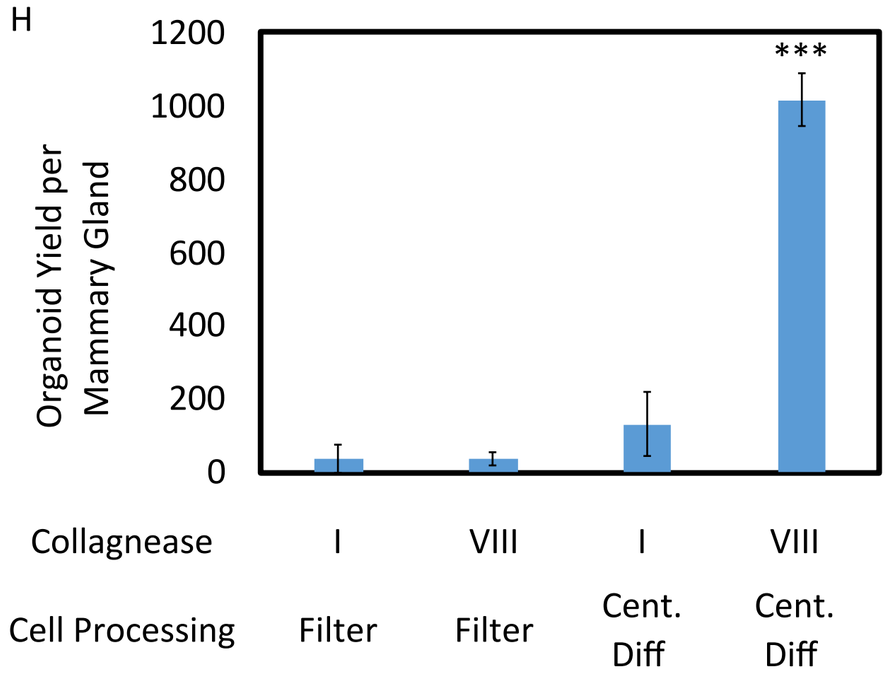

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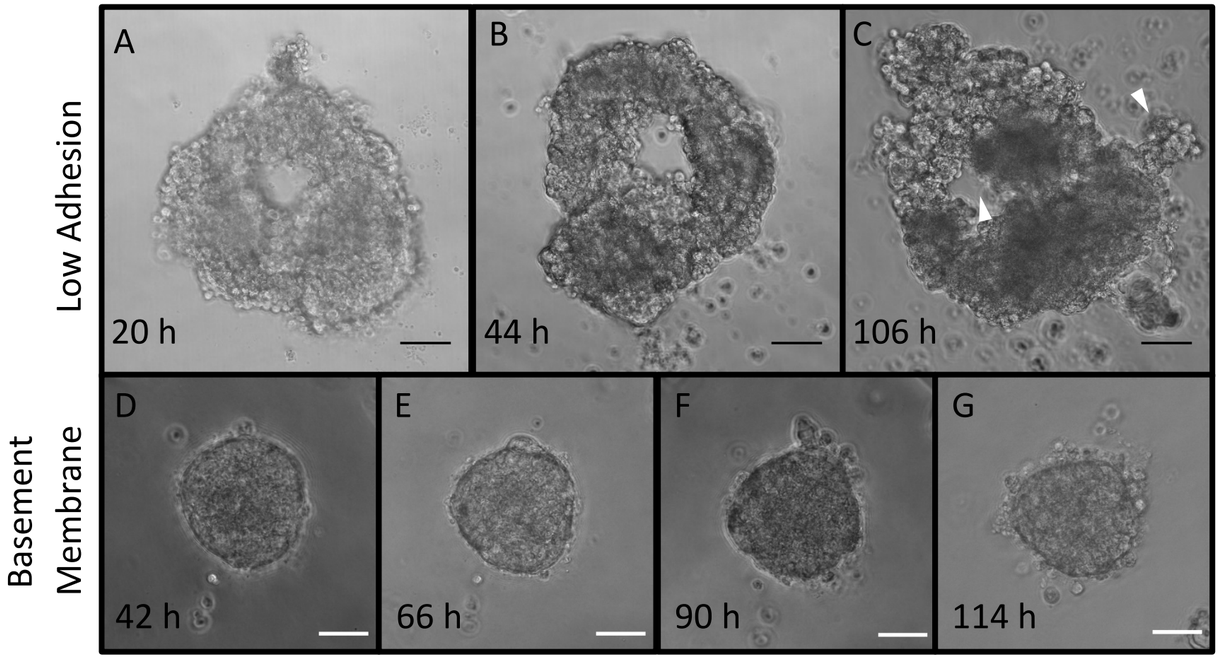

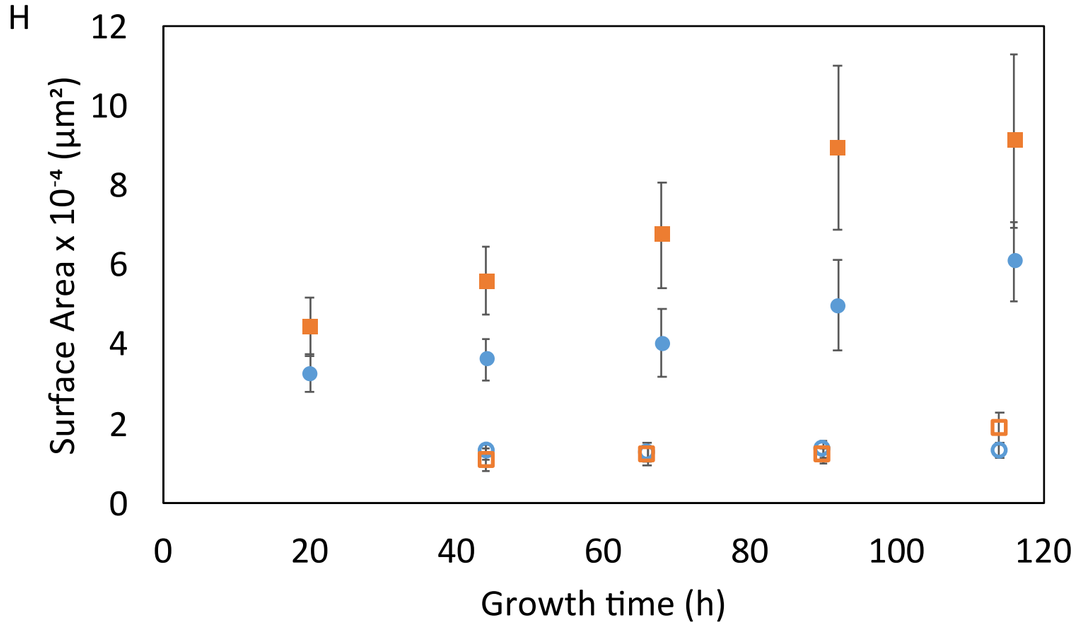

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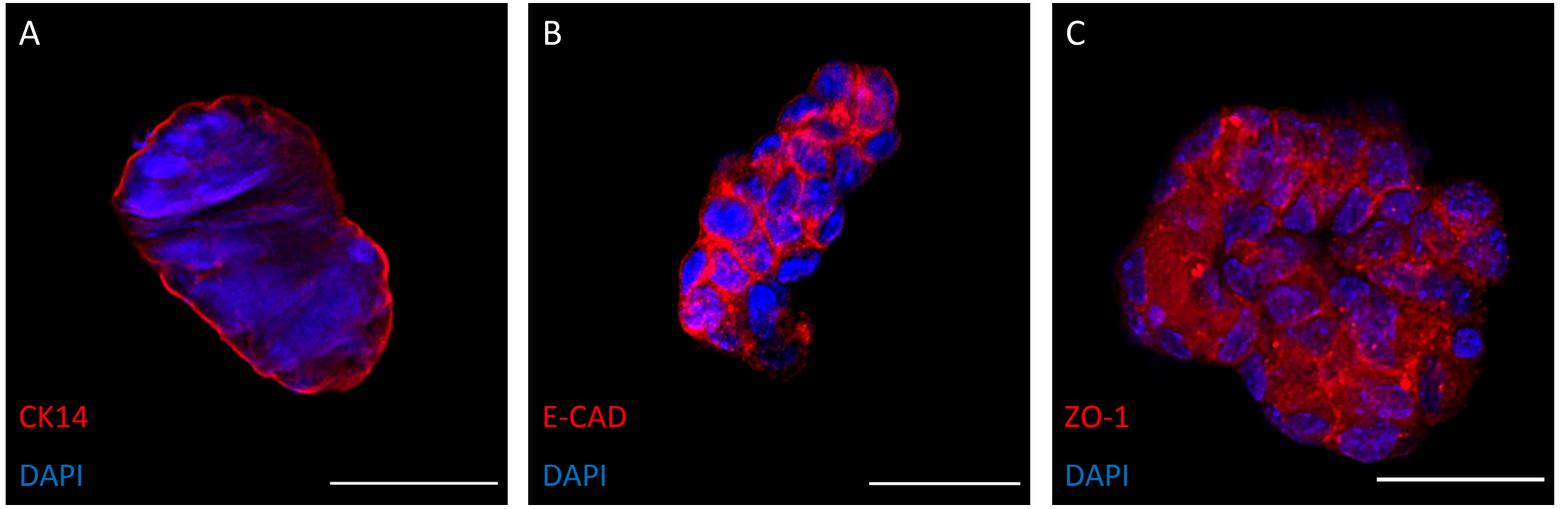

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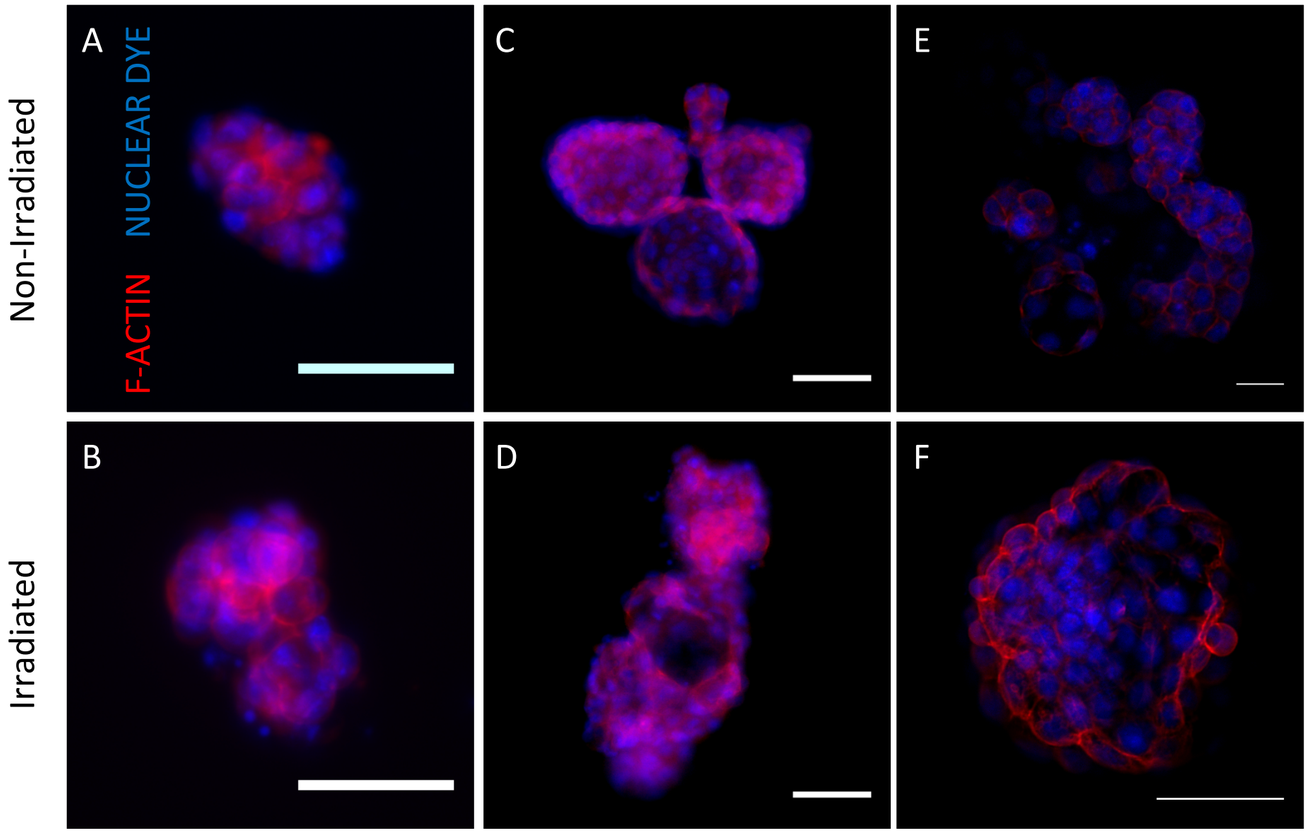

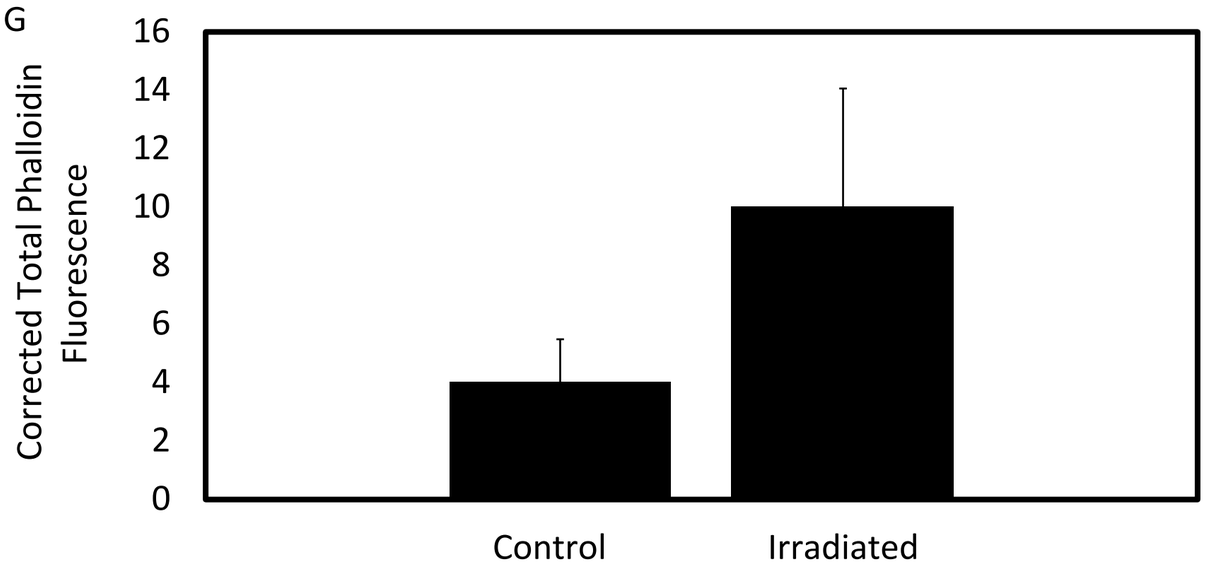

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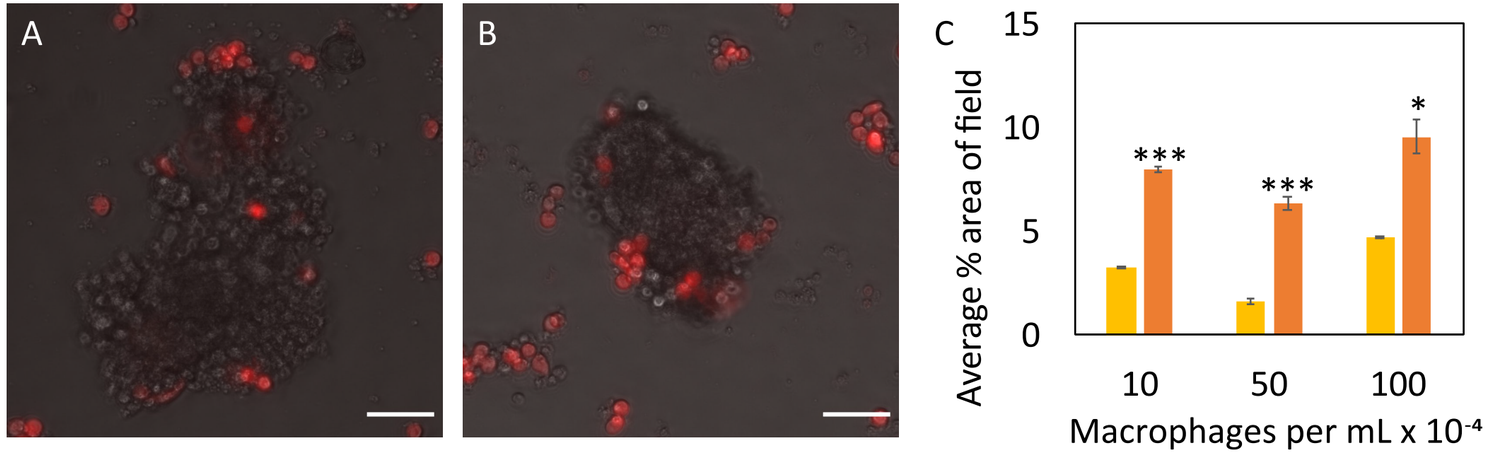

Name of Material/ Equipment	Company	Catalog Number	Lot
10% Neutral Buffered Formalin	VWR	16004-128	
Anti-cytokeratin 14	abcam	ab181595	GR3200524-3
Bovine Serum Albumin	Sigma	A1933-25G	
Collagen Type I	Corning	354236	
Collagenase from Clostridium Histolyticum, Type VIII	Sigma	C2139	
Collagenase I	Gibco	17018029	
DMEM/F12	Thermofisher	11320-033	
DNAse	Roche	10104159001	
DPBS	Fisher	14190250	
E-Cadherin	Cell Signaling	24E10	13
FBS	Sigma	F0926	
Gentamicin	Gibco	15750	
Goat anti-rabbit secondary	abcam	ab150077	GR3203000-1
Goat anti-rabbit secondary	abcam	ab150080	GR3192711-1
Hoechst 33342	Fisher	62249	TG2611041
Insulin (10 mg/mL)	Sigma	I9278	
Insulin-Transferrin-Selenium, 100x	Gibco	51500-056	
Matrigel Basement Membrane (basement membrane extracted from Engelbreth-Holm-Swarm mouse sarcoma)	Corning	356237	
Normal Goat Serum	Vector Laboratories	S-1000	
Nuclon Sphera 96 well plates	Thermo	174927	
PBS	VWR	10128-856	
Pen/strep	Fisher	15140122	
Phalloidin	abcam	ab176757	GR3214582-16
Tight Junction Protein 1	Novus	NBP1-85047	C115428
Triton X-100 (4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol)	Sigma	X100-100ML	
Trypsin	Gibco	27250-018	

Tween-20 (Polyethylene glycol sorbitan monolaurate)	Sigma	P1379-100ML

Comments/Description
green
red

DMEM/F12
composition

Components	Molecular Weight	Concentration (mg/L)	mM
Amino Acids			
Glycine	75	18.75	0.25
L-Alanine	89	4.45	0.049999997
L-Arginine hydrochloride	211	147.5	0.69905216
L-Asparagine-H2O	150	7.5	0.05
L-Aspartic acid	133	6.65	0.05
L-Cysteine hydrochloride-H2O	176	17.56	0.09977272
L-Cystine 2HCl	313	31.29	0.09996805
L-Glutamic Acid	147	7.35	0.05
L-Glutamine	146	365	2.5
L-Histidine hydrochloride-H2O	210	31.48	0.14990476
L-Isoleucine	131	54.47	0.41580153
L-Leucine	131	59.05	0.45076334
L-Lysine hydrochloride	183	91.25	0.4986339
L-Methionine	149	17.24	0.11570469
L-Phenylalanine	165	35.48	0.2150303
L-Proline	115	17.25	0.15
L-Serine	105	26.25	0.25
L-Threonine	119	53.45	0.44915968
L-Tryptophan	204	9.02	0.04421569
L-Tyrosine disodium salt dihydrate	261	55.79	0.21375479
L-Valine	117	52.85	0.4517094
Vitamins			
Biotin	244	0.0035	1.43E-05
Choline chloride	140	8.98	0.06414285
D-Calcium pantothenate	477	2.24	0.004696017
Folic Acid	441	2.65	0.006009071
Niacinamide	122	2.02	0.016557377
Pyridoxine hydrochloride	206	2.013	0.009771844
Riboflavin	376	0.219	5.82E-04
Thiamine hydrochloride	337	2.17	0.006439169
Vitamin B12	1355	0.68	5.02E-04

i-Inositol	180	12.6	0.07
Inorganic Salts			
Calcium Chloride (CaCl2) (anhyd.)	111	116.6	1.0504504
Cupric sulfate (CuSO4-5H2O)	250	0.0013	5.20E-06
Ferric Nitrate (Fe(NO3)3"9H2O)	404	0.05	1.24E-04
Ferric sulfate (FeSO4-7H2O)	278	0.417	0.0015
Magnesium Chloride (anhydrous)	95	28.64	0.30147368
Magnesium Sulfate (MgSO4) (anhyd.)	120	48.84	0.407
Potassium Chloride (KCl)	75	311.8	4.1573334
Sodium Bicarbonate (NaHCO3)	84	2438	29.02381
Sodium Chloride (NaCl)	58	6995.5	120.61207
Sodium Phosphate dibasic (Na2HPO4) anhydrous	142	71.02	0.50014085
Sodium Phosphate monobasic (NaH2PO4-H2O)	138	62.5	0.45289856
Zinc sulfate (ZnSO4-7H2O)	288	0.432	0.0015
Other Components			
D-Glucose (Dextrose)	180	3151	17.505556
Hypoxanthine Na	159	2.39	0.015031448
Linoleic Acid	280	0.042	1.50E-04
Lipoic Acid	206	0.105	5.10E-04
Phenol Red	376.4	8.1	0.021519661
Putrescine 2HCl	161	0.081	5.03E-04
Sodium Pyruvate	110	55	0.5
Thymidine	242	0.365	0.001508265

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15 January 2019

Dear Dr. Singh,

Please find enclosed our revised manuscript entitled, “Growth and Characterization of Irradiated Organoids from Mammary Glands.” We would like to thank the editorial staff and the reviewers for their supportive statements of the article. We appreciate the feedback and have addressed each comment in the attachment. We have also updated our manuscript to reflect the editorial comments.

Thank you for considering our revised manuscript for publication. Please contact us if you have any questions.

Sincerely,

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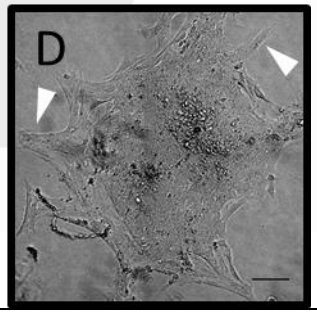
Ms. No. JoVE59293R2
Title: Growth and Characterization of Irradiated Organoids from Mammary Glands

We thank the editor and reviewers for their careful feedback, which has helped us to strengthen our manuscript significantly. We believe that we have addressed their comments and criticisms thoroughly. Below, we respond to the specific issues raised by the reviewers and discuss how we have revised the paper to address these points.

Response to the Editor's Comments

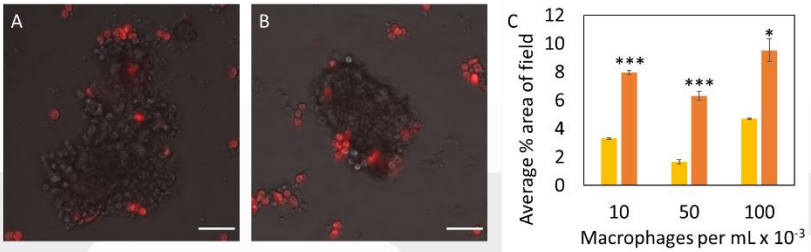
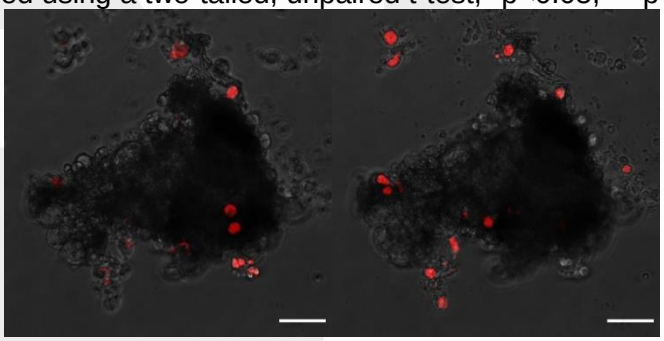
Editor Comments	Response
1. In a tube, petridish, please specify. (step 1.6)	We appreciate the request for clarification and have updated the protocol to read: "Transfer to the collagenase solution in a 50 mL centrifuge tube."
2. Aspirate from the tube? (step 1.11)	We thank you for the comment and have updated the text with the following clarification: "Aspirate the aqueous layer from the tube with original pellet."
3. Question about counting organoids in step 2.2	We appreciate the question and have clarified the procedure as follows: "Transfer 50 µL to a 30 mm Petri dish, and view under a phase contrast microscope at 20x. Count the number of organoids with a tally counter."
4. Question about calculating the organoid density in step 2.3	We appreciate the request for mathematical clarification and have included an equation for calculating density. $\frac{\#\ organoids\ in\ 50\mu L}{50\mu L} = \frac{\#\ organoids\ in\ tube}{volume\ of\ tube}\ [=]\ \frac{organoids}{\mu L}$
5. Please include how to visually identify the fibroblast?	We appreciate the request for clarification. We have updated the second paragraph of representative results to read: "Interestingly, the morphology of these fibroblasts is strikingly similar to pre-adipocytes, as both cell types exhibit spindly, elongated shapes (**Figure 2E, F**)[16]." [16]Gregoire, F.M., *et al, Physiological Reviews,* 1998.
6. How do you look for stromal growth?	We appreciate the question. Stromal cells are easily identifiable under phase contrast microscopy. They will usually be in or near the same plane of view as epithelial cells/organoids and appear within a few days of plating. They grow out from the organoid quickly. We have updated the protocol representative results to read: "Fibroblasts were identified under phase contrast microscopy in or near the same plane of focus as organoids, and they quickly grew out from plated organoids within a few days."
7. Request for citation in 5th paragraph of representative results	We appreciate the comment, and have inserted the following references: LaBarge *et al, JoVE,* 2013; Campbell *et al, Integrative Biology,* 2014;Chanson *et al, PNAS,* 2011.
8. Please refer to the figure panels wherever applicable in the protocol section to bring out clarity.	We appreciate the request for clarification and have inserted references to figure one into the protocol section. 1.2. Resect abdominal and inguinal mammary glands from mice using pre-sterilized scissors and forceps. Remove lymph nodes before resection. Rinse in sterile 1x phosphate buffered saline (PBS) (**Figure 1A**). 1.4. Irradiate samples at 20 Gy using a cesium source (**Figure 1B**). 1.5. 45 min after irradiating, place mammary glands in a 35 mm sterile cell plate and mince with scalpels (**Figure 1C, D**). Mince approximately 40 strokes until the tissue relaxes and pieces are obtained that are no larger than approximately 1 mm^2 in area.

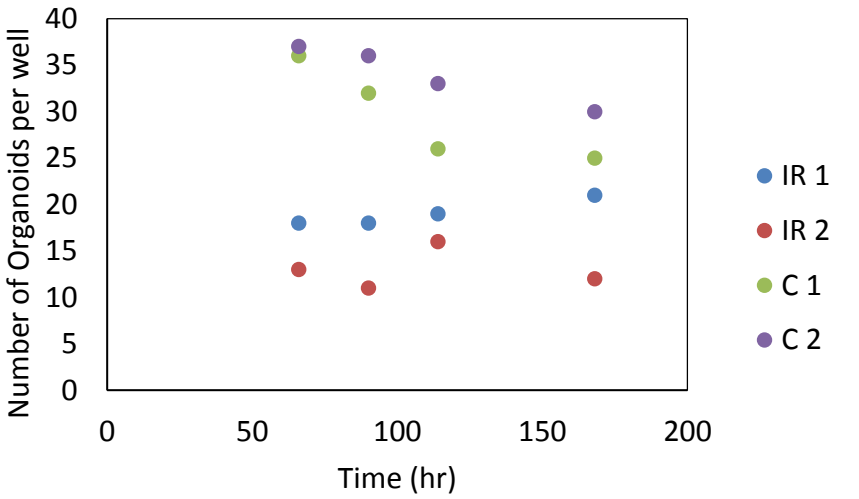
	1.7. Place in water bath at 37 °C, vortexing every 10 min for 30-60 min. Digestion is complete when the collagenase solution is cloudy (**Figure 1E, F**). 1.8. Spin down at 450 x g for 10 min at room temperature. Three layers will be observed. The supernatant is composed of fat, the middle layer is an aqueous solution, and the bottom is a pellet. The pellet will appear red as it is a mixture of epithelial cells, individual stromal cells, and red blood cells (**Figure 1G**). 1.18. Repeat steps 1.16-1.17 three more times to purify organoids via centrifugal differentiation. The pellet should now be an off-white color consisting of only epithelial organoids (**Figure 1H**).
9. What does the yellow arrow show here, please describe?	We appreciate the request for clarification. The yellow lightning bolt is a pictorial representation of ionizing radiation, and we have added a descriptor below the arrow (20 Gy) in the figure to clarify its presence.
10. E and G are 15 mL tube while G and H are 1.5 ml Tube. Please clarify this. Also in the figure caption put the panel number A… and then describe what is being shown. It gets confusing from D onwards	We appreciate the comment. E and F are in 50 mL tubes while G and H are in 15mL tubes. We have updated the caption to read as follows: "**Figure 1. Method Workflow. A.** Mammary glands were resected from mice. The abdominal and inguinal mammary glands were used. **B.** Mammary glands were irradiated in 50 mL centrifuge tubes containing DMEM/F12 media. **C.** Mammary glands were transferred to sterile six-well plates and cut with surgical scalpels until minced (**D**). **E.** Mammary glands were transferred into 50 mL centrifuge tubes containing 5 mL sterile DMEM/F12 media per gland and digested in a collagenase VIII solution (**F**). **G.** After being transferred to a 15 mL tube, centrifugal differentiation was utilized to remove stromal cells, single cells, and red blood cells, observed in a red pellet (white arrow-head) until only white epithelial organoids were obtained (**H**). (**I**). 50 organoids were plated in 200 µL of media in 96-well low adhesion plates and imaged using phase contrast microscopy Scale bar represents 50 µm."
11. Please describe what does scale bar represent in each panel.	We appreciate the request for clarification and have added "Scale bars for **A-G** represent 100 µm" in the caption for Figure 2.
12. In D the cells are not clear.	We appreciate the comment and have replaced the image with one that is more clear in Figure 2D. 
13. In the figure please replace hrs to h. Please remember to leave a single space between number and units.	We thank the editor for the comment and have updated the units as requested.
14. Is this also shown on irradiated organoid?	We appreciate the question. All images shown are irradiated organoids. We have updated the figure caption title to reflect this:

	"**Figure 4. Epithelial Marker Expression on Irradiated Organoids."**
15. Presently the discussion essentially describes the result. Please move the result details to the representative result section. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations: a) Critical steps within the protocol b) Any modifications and troubleshooting of the technique c) Any limitations of the technique d) The significance with respect to existing methods e) Any future applications of the technique	We appreciate the editor's comment. We have moved these discussion points to the representative results sections and have addressed the desired points in the discussion section.

Response to Reviewer #1

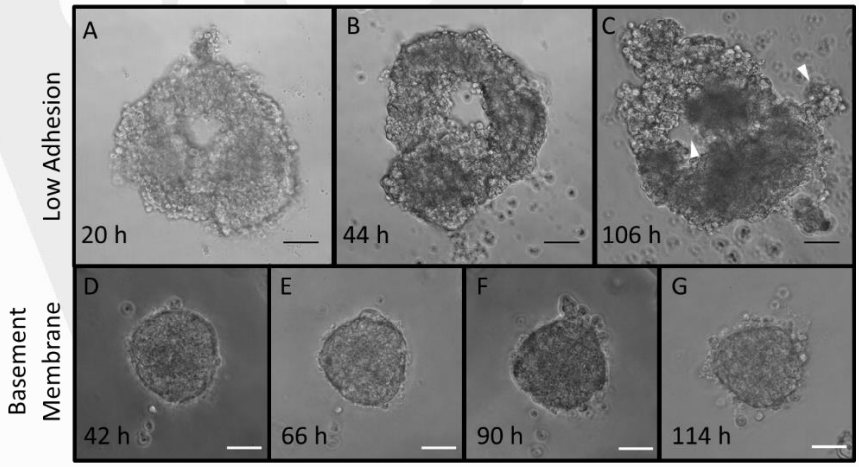
Reviewer 1 Comments	Response
1. The explanation and justification of the radiation treatment and dosing needs more detail. Why are glands treated in a 50mL conical tube as opposed to irradiating the mouse before resection? Why irradiate resected glands as opposed to organoids? Is 20 Gy the appropriate dose in all cases, how was that dose determined?	We thank the reviewer for bringing up this point of clarification. I*n vivo* irradiation is an experiment that we will indeed be performing in the future; however, whole glands were instead treated *ex vivo* to isolate the damage of ionizing radiation on the tissue itself. Additional studies will explore the effect of *in vivo* irradiation as that would allow for a wound healing response and infiltration of multiple cell types. 20 Gy was chosen to mirror previous in vivo models of tumor cell recruitment (Rafat et al., *Cancer Research* 2018). Typically, patients receive ionizing radiation of 60 Gy in fractionated doses of 2 Gy. However, intraoperative and post-operative radiotherapy is also given to human patients at doses consistent with 20 Gy. In addition, previous pre-clinical studies have used a single dose of 20 Gy due to aggressive metastasis of the 4T1 mouse mammary carcinoma model, which makes fractionated dosing infeasible. To this end, we have updated paragraph one of the discussion section to read: “An irradiation dose of 20 Gy was applied to mirror previous *in vivo* models of tumor cell recruitment[5]. Irradiation of mammary glands *ex vivo* prior to organoid formation allowed for isolation of radiation damage effects without a corresponding infiltration of immune cells.” [5]Rafat, M. *et al. Cancer Research,* 2018.
2. When co-culturing macrophages with organoids, are there considerations that need to be taken for the long-term culture of immune cells e.g. treatment with cytokines, effects of serum starvation by switching to organoid media? Will the reproducibility of this protocol be affected by choice of cytokines, source of macrophages (e.g. bone marrow derived vs. cell line), and organoid culture system? If so, the authors should acknowledge deviations could yield very different conclusions about tumor-immune interactions.	We thank the reviewer for this comment. We began our experiments by using non-polarized RAW 264.7 macrophages to determine whether macrophage migration was influenced by the cytokine secretion of the organoids. Future studies will evaluate the effect of both macrophage polarization and serum starvation. We will evaluate the growth effects of culturing organoids in complete media once they have formed. We have also conducted initial macrophage polarization experiments using RAW 264.7 macrophages. We have found macrophages polarized to an M1 phenotype (in DMEM media with 10% FBS, and cytokines LPS and IFN-gamma) retain their polarization when transferred to cytokine-free media and are currently conducting similar experiments with M2 macrophages. We will look more thoroughly at the effects of serum free organoid media on polarization status before doing further co-culture experiments with M1 or M2 macrophages. Following experiments with the RAW 264.7 cell line, we will use primary bone marrow derived macrophages in our studies. We have updated the final paragraph of the discussion section to read: “Additional analyses will evaluate the consequence of serum starvation and the growth effects of culturing organoids in complete media, as these variables may have significant effects on organoid-immune cell interactions.”
3. Additionally the protocol to "monitor macrophage infiltration over time" is unclear. How are infiltration and kinetics measured? At 100,000 macrophages/mL,	We appreciate the reviewer’s concern. We monitored macrophage behavior by using live cell imaging where we quantified the kinetics of macrophage association with organoids over time through fluorescence intensity measurements. This procedure is now clarified, and the results are reported in in a new Figure 6. We conducted co-culture experiments at the previously reported concentration of

identifying specific interactions between macrophages and organoid cells or migration appears difficult. The authors report that macrophages colocalized with organoids within 14 hours - however, the images included in Fig. 5 show a very high local density of macrophages, so it is unclear if they are actively infiltrating/colocalizing with the organoids or just randomly associating due to high density. If this is included in the protocol, additional detail for robust and reproducible measurements of the described phenotype (i.e. infiltration) should be included.	100,000 macs/mL and also at 50,000 and 10,000 macs/mL. Co-localization continues to be observed even at the lowest concentrations, and quantification of fluorescence signal over 24 hours indicates that macrophage migration is enhanced toward irradiated organoids. Macrophages also completely co-localized with both control and irradiated organoids or parts of dissociated organoid tissue by 48 hours. We also obtained Z-stack images to confirm macrophage infiltration into organoids (shown below).  **Figure 6. Evaluating cell-cell interactions through macrophage-organoid co-culture.** Macrophages (red) infiltrated control (**A**) and irradiated (**B**) organoids. Scale bars represent 50 µm. Average percent area of macrophages in the image field (**C**) was reported at 24 hours of co-culture for control (yellow) and irradiated (orange) organoids (n=3 glands for each sample). Macrophages were seeded at concentrations of 10,000 cells/mL, 50,000 cells/mL, and 100,000 cells/mL, and their infiltration was captured every 30 minutes via live cell fluorescence imaging. All co-culture experiments commenced 7 days after initial organoid seeding. Statistical significance was determined using a two-tailed, unpaired t-test, *p<0.05, ***p<0.0001.  Z-stack slices of irradiated organoid after 48 hours of co-culture. A step size of 1.56 µm was used. Scale bars are 50 µm.
4. The authors should acknowledge previous papers that describe co-culture of mammary organoids and immune cells (e.g. DeNardo et al. Cancer Cell 2009).	We thank the reviewer for this comment and have updated the manuscript to include additional relevant studies where organoids and immune cells were co-cultured. We have updated the final paragraph of the introduction to read as follows: “Earlier models co-culturing mammary organoids and immune cells have shed light on mechanisms of metastasis and dissemination. DeNardo et al. found that CD4+ T cell regulation of tumor associated macrophages enhanced a metastatic phenotype of mammary adenocarcinomas[14]. Co-culture models have also been used to elucidate mechanisms of biological development. Plaks et al. clarified the role of CD4+ T cells as down-regulators of mammary organogenesis[15].” [14]DeNardo, D.G. *et al. Cancer Cell,* 2009. [15]Plaks, V. *et al. Developmental Cell,* 2015.
5. For growth measurements of organoids (as in 3D), were area measurements taken	We appreciate the question. Images were taken every 24 hours, and the area was measured from these images. At each time point, a 5x overview of the wells growing the organoids was taken. While

from time lapse images or start and endpoint images? If the latter, how confident are the authors that the organoids did not clump together in the non-adherent Sphera plates between time points?	clumping did occur occasionally, the vast majority of the time organoids did not clump together. This can be seen from representative data attached, where organoids per well were counted over time in irradiated and control wells. Any decrease in organoid count over time is attributed to aspiration when changing media while any increase is attributed to dissociation. We have added a sentence in the fourth paragraph of the discussion to read: “Occasional clumping of low adhesion organoids was observed, and organoids could be cultured up to two weeks before dissociating.” 
6. N (# mice or glands) should be reported for each experiment, unless representative data are used. If the experiment or image is representative, that should be noted in the legend. For experiments where p-values can be calculated, they should be reported. Without statistical support, unqualified statements such as "irradiated organoids grew more slowly than non-irradiated controls" regarding Fig. 3D should be avoided. If p>0.05, the authors should report these as trends, but not draw direct conclusions.	We thank the reviewer for this comment. We have updated the paper to address sample size and statistical significance. This includes updates to the following figure captions: **Figure 2H**: “Different collagenase types (I (CI) and VIII (CVIII)) and cell processing methods (filtration and centrifugal differentiation (Cent Diff)) were tested, and organoid yield per mammary gland was quantified (n = 2 glands for CI, Filter; 2 glands for CVIII, Filter; 4 glands for CI, Cent Diff, and 12 glands for CVIII, Cent Diff). Statistical significance was determined using a two-tailed, unpaired t-test, ***p<0.0001. Error bars represent standard error.” **Figure 3H**: “Area measurements were obtained in different growth conditions: organoids immediately seeded after digestion and sorting (Irradiated (●), Control (■)),and organoids seeded in basement membrane (Irradiated (○), Control (□)) (n = 3 glands each). Area calculations were made using ImageJ software. Error bars represent standard error. Scale bars represent 50 µm.” **Figure 5G**: “Phalloidin fluorescence data from low adhesion plate images were quantified in ImageJ (n = 3 glands).” **Figure 6C**: “Average percent area of macrophages in the image field (**C**) was reported at 24 hours of co-culture for control and irradiated organoids (n=3 glands for each sample)."
7. In Fig 3A-C the white arrows are not described in the figure legend - it appears	We appreciate this comment. We have updated our language to describe what we observed as potential ducts and lobes that cannot be confirmed without further characterization. We have included the

that they are meant to mark "ducts" and "lobes". Without further characterization it is not appropriate to use these specific terms.	following clarification in the representative results section: “White arrowheads indicate constructs morphologically similar to ducts and lobes[23,24] (**Figure 3C**), which are critical for the production and transport of milk in the mammary gland[25]. However, further characterization is required to confirm this observation.” The Figure 3 caption has been updated to read: “White arrowheads indicate structures that have similar morphology to ducts and lobes.” [23]Zhang, L. *et al. Cellular Signaling,* 2016. [24]Sokol, E.S. *et al, Breast Cancer Research,* 2016. [25]Richert, M.M. *et al. Journal of Mammary Gland Biology and Neoplasia.* 2000.

Response to Reviewer #2

Reviewer 2 Comments	Response
1. Figure1 please provide the overall yield of viable organoids from irradiated fatpad compared to non irradiated fatpads to provide an estimate to the viewers to develop any quantitative assays.	We appreciate the reviewer's comment. We did not observe significant differences in yield of viable organoids comparing irradiated to non-irradiated. On average, we obtain 1170 +/- 157 organoids per mammary gland for unirradiated organoids, and 1006 +/- 364 organoids per mammary gland for irradiated organoids. We have updated the third paragraph of the results section to read: "No significant differences in yield between irradiated and control organoids were observed."
2. It is not clear fig 2G was from 3D or 2D plastic grown cells.	We appreciate the reviewer's comment. We have updated the protocol with the following revisions: In the Figure 2 caption: "Cells in Figures E-F were seeded on tissue culture treated plastic." We have also updated the 2nd paragraph of the results section to: "Seeding cells directly onto tissue culture treated 10 cm cell plates yielded an overgrowth of fibroblast cells."
3. Presence of fibroblast is a common factor in organoid preparation especially when they are grown in 2D plastic.	We thank the reviewer for their comment and agree. However, we are interested in evaluating purely epithelial organoids.
4. Mammary organoids undergo branching morphogenesis upon plating on 3d matrix. It is not clear whether irradiated organoids also underwent morphogenesis. More representative images of good quality will guide the viewers on the efficiency of the protocol.	We thank the reviewer for this comment. While it is true that organoids undergo branching morphogenesis upon plating in Matrigel, this requires the addition of growth factors in the media, typically FGF2 (Nguyen-Ngoc *et al., Tissue Morphogenesis: Methods and Protocols,* 2015). We have included additional panels (**Figure 3 A-G**) to better guide the reader on the efficiency of this protocol.  **Figure 3. Representative Organoid Growth.** Phase contrast images of irradiated organoid growth in low adhesion plates obtained 20 (**A**), 44 (**B**), and 106 (**C**) hours after seeding. White arrowheads indicate structures that have similar morphology to ducts and lobes. Phase contrast images of irradiated organoid growth in basement membrane obtained 42 (**D**), 66 (**E**), 60 (**F**), and 114 (**G**) hours after seeding. Scale bars represent 50 μm.
5. The centrifugation speed should be provided in g (rcf)	We appreciate the reviewer's comment. We have updated the protocol, converting all "rpm" values to "x g."

6. Page 2 line 112, presence of red pellet indicates RBC contamination. Pure epithelial cells should be a light brown pellet.	We thank the reviewer for this comment. We have now included additional details about our procedure to indicate where RBCs have been removed following centrifugation. We have updated the manuscript to read: “The pellet will appear red as it is a mixture of epithelial cells, individual stromal cells, and red blood cells (**Figure 1G**)” in step 1.8 following centrifugation, and “The pellet should now be an off-white color consisting of only epithelial organoids (**Figure 1H**)” in step 1.18 following centrifugal differentiation.

Response to Reviewer #3

Reviewer 3 Comments	Response
1. The introduction should describe further how the presented protocol is different from the well established organoid development protocols. In other words, why would these protocols not work for growing organoids from irradiated tissue?	We appreciate the reviewer's comment. This protocol expands upon existing protocols. We show that irradiated organoids can be grown and characterized in a variety of ways. While other protocols may be used for growing organoids from irradiated tissue, we have focused here on describing a working protocol to characterize irradiated organoids as well as how immune cells interact with these organoids. To our knowledge, no other group has developed irradiated organoids to establish how normal tissue damage can alter immune cell behavior as we have done. We have updated the introduction to include the following: "Previously established protocols may be used to evaluate irradiated organoids. Earlier models co-culturing mammary organoids and immune cells have shed light on mechanisms of metastasis and dissemination. DeNardo et al. found that CD4+ T cell regulation of tumor associated macrophages enhanced a metastatic phenotype of mammary adenocarcinomas[14]. Co-culture models have also been used to elucidate mechanisms of biological development. Plaks et al. clarified the role of CD4+ T cells as down-regulators of mammary organogenesis[15]. However, our group is the first to establish a procedure of visualizing how normal tissue irradiation influences immune cell behavior." [14]DeNardo, D.G. *et al. Cancer Cell,* 2009. [15]Plaks, V. *et al. Developmental Cell,* 2015.
2. Does the pipet tips used in steps 1.20, 2.1 and so on, need to be of a particular size to make sure the organoid sizes are maintained? Some of the established organoid protocols use different pipet tip sizes to break apart organoids during passaging, so it seems like the size should be carefully chosen.	We appreciate the reviewer's comment. For measurements and translating of organoids in media of volume 1 mL or smaller, we consistently used pipette tips with a minimal diameter of 457 µm. This is 5-10 times the diameter of the organoids that are seeded. In step 1.20 and 2.1, serological pipettes are used that have tip diameters excess of 1500 µm. In the future, we will explore how pipette tip size can be used to control organoid size. Here, we have clarified the pipette tip sizes used in our protocol to form organoids and specified that the centrifugation step was used to differentiate organoids by size after step 2.2: "Note: We have consistently used pipette tips with a minimal diameter of 457 µm, which is 5-10 times the diameter of the organoids that are seeded. For transferring volumes of 2 mL or larger (e.g. steps 1.16 and 2.1), we used serological pipettes with tip diameters excess of 1500 µm."
3. I am confused about the definition of the organoids in this manuscript. In most protocols, after dissociation of cells from excised tissue and seeding them, usually it takes a while for the cells to grow into organoids. However, step 2.2 directly to refers to counting organoids. What qualifies as a organoid? Please clarify.	We thank the reader for this observation. The term "organoid" has a wide variety of definitions in the literature. We define an epithelial organoid as a 3D construct of isolated mammary epithelium larger than 50 µm, which is supported by other studies (Shamir and Ewald, *Nature Reviews Molecular Cell Biology*, 2014; Simian et al., *Development*, 2001; Shamir et al., *The Journal of Cell Biology*, 2014; Ewald et al., *Developmental Cell*, 2008; Nguyen-Ngoc et al., *Proceedings of the National Academy of Sciences*, 2012). We have updated the manuscript with this clarification, stating in the third paragraph of the introduction: "A mammary organoid is a multicellular, three dimensional construct of isolated mammary epithelium that is larger than 50 µm[6–10]." [6]Shamir, E.R. and Ewald, A.J. *Nature Reviews Molecular Cell Biology,* 2014. [7]Simian, M. *et al.*

	Development (Cambridge, England), 2001. [8]Shamir, E.R. *et al. Journal of Cell Biology,* 2014. [9]Ewald, A.J. *et al. Developmental Cell,* 2008. [10]Nguyen-Ngoc, K.-V. *et al. Proceedings of the National Academy of Sciences,* 2012.
4. Does this protocol yield organoids of homogeneous sizes? How is this ensured?	We thank the reviewer for this request for further detail. After 20 hr of plating, the surface area of organoids exhibit the following area range (μm^2) Irradiated (±Std. dev.): 32776 ± 15990 Control (±Std. dev.): 44403 ± 24320 We ensure that the organoids have similar size between experiments by using the same amount of collagenase solution per trial (10 mL for each mouse, or 5 mL per gland) and by sorting with centrifugal differentiation.
5. How long can the organoids grow in cultures? Can these be passaged to maintain growth?	We thank the reviewer for these questions. The longest that the organoids can be grown is two weeks, after which they begin to dissociate. Passaging is not required, as the organoids do not grow to confluency on the microplates. We have frozen them in 90% FBS and 10% DMSO and found that they grow well after thawing. We have updated the protocol to include directions for freezing and thawing in steps 2.3.2-2.3.3: "Note: To freeze organoids, transfer desired volume to a separate centrifuge tube. Spin down at 450 x g for 5 minutes. Aspirate media, then add the same volume of 90% FBS/10% DMSO. Resuspend the organoids, then aliquot into cryotubes. Transfer to -80˚C, then to liquid nitrogen within one week. To thaw, warm in a 37˚C water bath for one minute. Centrifuge at 450 x g for five minutes, then aspirate freezing media. Rinse with sterile DPBS, then centrifuge again. Aspirate DPBS, and add organoid media." We have also made a note in the fourth paragraph of the discussion that "organoids can be cultured up to two weeks before dissociating."
6. Figure 2 caption, page 5, line 222, the manuscript does not talk about the 'filtration' mentioned here. How does the technique sort organoids? Please include filtration protocol in the main manuscript. Also describe what is expected to be present in the 'filtrate' and 'retentate' mentioned in line 223.	We thank the reviewer for bringing up this point for clarification. The technique sorts organoids from individual cells via differences in size. We have updated the protocol to read as follows after step 1.18: "Note: organoids can also be filtered using sterile mesh 40µm filters. After step 1.16, pipette media containing organoids through a filter into a centrifuge tube, and then rinse with 5 to 10 mL of DMEM/F12 media. Flip the filter over a new 50 mL centrifuge tube. Pass 10 mL of DMEM/F12 media through, going the opposite way to rinse off any retentate. The retentate should consist of organoids, and the filtrate should contain mainly stromal cells, which can be discarded or kept if desired."
7. Line 224, why does trypsinizing allow selective removal of fibroblast? Is there a reason for not including this step in the main protocol?	We thank the reviewer for this remark. We used a partial trypsinization method to remove fibroblasts as described by LaBarge *et al*, *Journal of Visualized Experiments*, 2013. In this protocol, the authors grow organoids on tissue culture treated plastic, and they claim to use differential trypsinization to "promote rapid detachment of fibroblasts from the surface plastic." We did not include this step in the main protocol as we do not culture organoids on tissue culture treated plastic, and we did not observe fibroblast growth on the low adhesion plates.
8. Describe the ' cell processing methods'	We appreciate the reviewer's comment and have updated the caption for Figure 2 to read the following: "(**H**) Different collagenase types (I

referred here. Define 'Cent. Diff.' in figure 2H.	(CI) and VIII (CVIII)) and cell processing methods (filtration and centrifugal differentiation (Cent Diff)) were tested, and organoid yield per MFP was quantified (n = 2 glands for CI Filter; 2 glands for CVIII, Filter; 4 glands for C1, Cent Diff, and 12 glands for CVIII, Cent Diff)."
9. Figure 3 C and Fig 5, what does the white arrowheads indicate?	We thank the reviewer for the requesting clarification. We have updated the Figure 3 caption to read: "White arrowheads indicate structures that have similar morphology to ducts and lobes." A new figure has been added, moving the macrophage results from Figure 5 to Figure 6. We have removed the arrows and instead indicated that the macrophages are red in color.
10. Page 6, line 286, could the authors indicate ' ducts and lobes' formed in the organoid images shown in Fig 3 referred to in the discussion.	We appreciate the comment and have updated the manuscript to include the following clarifications. The Figure 3 caption now reads: "White arrowheads indicate structures that have similar morphology to ducts and lobes." The 4th paragraph of the results section now reads: "White arrowheads indicate constructs morphologically similar to ducts and lobes[23, 24](**Figure 3C**), which are critical for the production and transport of milk in the mammary gland[25]." [23]Zhang, L. *et al. Cellular Signaling,* 2016. [24]Sokol, E.S. *et al, Breast Cancer Research,* 2016. [25]Richert, M.M. *et al. Journal of Mammary Gland Biology and Neoplasia.* 2000.
11. Page 1, line 60, citation is required	We thank the reviewer for the comment and have updated the protocol with a citation. That sentence now reads: "Previous work has shown that radiation of normal tissue increases recruitment of various cell types[5]." [5]Rafat, M. *et al. Cancer Research,* 2018.
12. Page 4, line 184-185, define PBST	We thank the reviewer for the comment and have defined PBST as "0.1% PBS/Polyethylene glycol sorbitan monolaurate"
13. Page 5, line 243, rephrase 'less intensify'	We appreciate the reviewer's comment and have rephrased it to read "with lower intensity."