Journal of Visualized Experiments

Studying Normal Tissue Radiation Effects using Extracellular Matrix Hydrogels

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59304R1
Full Title:	Studying Normal Tissue Radiation Effects using Extracellular Matrix Hydrogels
Keywords:	Breast cancer; radiation; mammary fat pad; extracellular matrix; decellularization; hydrogels
Corresponding Author:	Marjan Rafat Vanderbilt University Nashville, Tennessee UNITED STATES
Corresponding Author's Institution:	Vanderbilt University
Corresponding Author E-Mail:	mrafat@stanford.edu
Order of Authors:	Steven M Alves
	Tian Zhu
	Anastasia Shostak
	Ninna S Rossen
	Marjan Rafat
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Nashville, Tennessee, United States of America

TITLE:

Studying Normal Tissue Radiation Effects using Extracellular Matrix Hydrogels

AUTHORS AND AFFILIATIONS:

Steven M. Alves[1], Tian Zhu[1], Anastasia Shostak[1], Ninna S. Rossen[2], Marjan Rafat[1,3,4]

[1]Department of Chemical and Biomolecular Engineering, Vanderbilt University, Nashville, TN, USA
[2]Department of Radiation Oncology, Stanford University, Stanford, CA, USA
[3]Depattment of Biomedical Engineering, Vanderbilt University, Nashville, TN, USA
[4]Department of Radiation Oncology, Vanderbilt University Medical Center, Nashville, TN, USA

Corresponding Author:

Marjan Rafat (marjan.rafat@vanderbilt.edu)

Email Addresses of Co-authors:

Steven Alves (steven.m.alves@vanderbilt.edu)
Tian Zhu (tian.zhu@vanderbilt.edu)
Anastasia Shostak (anastasia.sho@gmail.com)
Ninna Rossen (nsrossen@stanford.edu)

KEYWORDS:

Breast cancer, radiation, extracellular matrix, decellularization, hydrogels

SUMMARY:

This protocol presents a method for decellularization and subsequent hydrogel formation of murine mammary fat pads following ex vivo irradiation.

ABSTRACT:

Radiation is a therapy for patients with triple negative breast cancer. The effect of radiation on the extracellular matrix (ECM) of healthy breast tissue and its role in local recurrence at the primary tumor site are unknown. Here we present a method for the decellularization, lyophilization, and fabrication of ECM hydrogels derived from murine mammary fat pads. Results are presented on the effectiveness of the decellularization process, and rheological parameters were assessed. GFP- and luciferase-labeled breast cancer cells encapsulated in the hydrogels demonstrated an increase in proliferation in irradiated hydrogels. Finally, phalloidin conjugate staining was employed to visualize cytoskeleton organization of encapsulated tumor cells. Our goal is to present a method for fabricating hydrogels for in vitro study that mimic the in vivo breast tissue environment and its response to radiation in order to study tumor cell behavior.

INTRODUCTION:

Cancer is characterized by excess proliferation of cells that can evade apoptosis and also metastasize to distant sites[1]. Breast cancer is one of the most common forms among females in the US, with an estimated 266,000 new cases and 40,000 deaths in 2018[2]. A particularly

aggressive and difficult to treat subtype is triple negative breast cancer (TNBC), which lacks estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor (HER2). Radiation therapy is commonly used in breast cancer to eliminate residual tumor cells following lumpectomy, but over 13% of TNBC patients still experience recurrence at the primary tumor site[3].

It is known that radiation therapy is effective in mitigating metastasis and recurrence because the combination of lumpectomy and radiation results in the same long-term survival as mastectomy[4]. However, it has recently been shown that radiation treatment is associated with local recurrence to the primary tumor site in immunocompromised settings[5,6]. Also, it is well known that radiation changes the extracellular matrix (ECM) of normal tissue by inducing fibrosis[7]. Therefore, it is important to understand the role of radiation-induced ECM changes in dictating tumor cell behavior.

Decellularized tissues have been used as in vitro models to study disease[8,9]. These decellularized tissues preserve ECM composition and recapitulate the complex in vivo ECM. This decellularized tissue ECM can be further processed and digested to form reconstituted ECM hydrogels that can be used to study cell growth and function[10,11]. For example, injectable hydrogels derived from decellularized human lipoaspirate and from myocardial tissue served as non-invasive methods of tissue engineering, and a hydrogel derived from porcine lung tissue was utilized as an in vitro method of testing mesenchymal stem cell attachment and viability[12-14]. The effect of normal tissue radiation damage on ECM properties, however, has not been investigated.

Hydrogels derived from ECM have the greatest potential for in vitro study of in vivo phenomena. Several other materials have been studied, including collagen, fibrin, and matrigel, but it is difficult to synthetically recapitulate the composition of the ECM[13]. An advantage of using ECM-derived hydrogels is that the ECM contains the necessary proteins and growth factors for a particular tissue[14,15]. Irradiation of normal tissue during lumpectomy causes significant changes to the ECM, and ECM-derived hydrogels can be used to study this effect in vitro. This method could lead to more complex and more accurate in vitro models of disease.

In this study, we subjected murine mammary fat pads (MFPs) to radiation ex vivo. The MFPs were decellularized and made into pre-gel solution. Hydrogels were formed with embedded 4T1 cells, a murine TNBC cell line. The rheological properties of the hydrogel material were examined, and tumor cell dynamics were evaluated within the hydrogels. Hydrogels fabricated from irradiated MFPs enhanced tumor cell proliferation. Future studies will incorporate other cell types to study cell-cell interactions in the context of cancer recurrence following therapy.

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 and ex vivo irradiation of MFPs

1.1. Sacrifice athymic Nu/Nu mice (8–10 weeks) using CO_2 asphyxiation followed by cervical dislocation.

1.2. Clean the skin using 70% ethanol.

1.3. Collect mammary fat pads (MFPs) from sacrificed mice using pre-sterilized scissors and forceps in a 15 mL conical tube containing complete RPMI media (RPMI supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum) (see the **Table of Materials**).

1.4. Irradiate samples to 20 Gy using a cesium source.

1.5. Bring the irradiated MFPs and complete RPMI media into a biosafety cabinet. The media will be dependent on the cell line to be grown in the final hydrogel. Fill 6 cm or 10 cm dishes with enough media to submerge the MFPs. For 6 cm dishes, use 8 mL of media, and for 10 cm dishes, use 20 mL of media.

1.6. Incubate in a 37 °C/5% CO_2 incubator for two days. The length of time in the incubator may be adjusted.

1.7. Rinse tissues in phosphate-buffered saline (PBS), blot excess moisture, and place MFPs into 15 mL conical tubes for storage at -80 °C until decellularization. This freezing step aids the decellularization step and the sample should be frozen even if the next steps are otherwise ready.

2. Decellularization (adapted from references[12,16,17])

NOTE: This procedure was adapted from previously published methods focused on adipose decellularization, which included the sodium deoxycholate ionic detergent rather than sodium dodecyl sulfate to remove DNA efficiently[12,16,17].

2.1. On **day 1**, remove frozen MFPs from -80 °C and thaw at room temperature.

2.2. Once thawed, dry MFPs briefly on a delicate task wipe. Weigh the MFPs using an analytical scale.

2.3. Using a pair of forceps with scissors or a scalpel, divide tissue up into 3 mm x 3 mm x 3 mm samples for study of the intact ECM and the remaining tissue for hydrogel production.

NOTE: The number of samples is dependent on the number of testing methods, e.g. the collection of two samples is described below: one for paraffin embedding (step 2.5) and one for freezing in cryostat embedding medium, if desired (see the **Table of Materials** and step 2.6).

2.4. Weigh the tissues. If embedding in paraffin for sectioning, continue to step 2.5. If freezing in cryostat embedding medium for sectioning, continue to step 2.6.

2.5. In a chemical hood, submerge the tissue in 10% neutral buffered formalin (NBF) (see the **Table of Materials**) for 24 h at 4 °C. Wash 3 times in PBS for 5 min each. Submerge the tissue in 30% sucrose for 48 h at 4 °C.

2.5.1. Weigh the tissue now that this piece has been removed. Continue to step 2.6.

2.6. In a chemical hood, place MFP pieces in a labeled cassette prepped with cryostat embedding medium. Add more cryostat embedding medium to cover the tissue.

2.6.1. Place the cassette into a beaker of 2-methylbutane (see the **Table of Materials**) that is pre-cooled with liquid nitrogen. The beaker should have enough 2-methylbutane to cover the bottom but not enough to submerge the cassette because the cryostat embedding medium should not touch the 2-methylbutane. Let the cassette sit in the 2-methylbutane until the cryostat embedding medium freezes and becomes opaque.

2.6.2. Wrap the cassette(s) in foil, label, and leave at -80 °C until used for sectioning.

NOTE: Tissues placed immediately in cryostat embedding medium were sectioned at 5 µm while tissues incubated in sucrose were sectioned at 30 µm to retain adipocyte morphology.

2.7. Use forceps to manually massage the remaining tissue.

Note: Tissue pieces may also be placed in 10% NBF for 24–48 h, rinsed in PBS, and left in 70% ethanol until embedding in paraffin. Following embedding, 5 µm sections can be used for hematoxylin and eosin (H&E) staining (see section 7 below).

2.8. Place the MFPs in 6 cm dishes with 5 mL 0.02% trypsin/0.05% EDTA solution. Incubate at 37 °C for 1 h. Spray and wipe the dishes with 70% ethanol before placing in the incubator.

2.9. Use 0.7 mm strainers to wash the MFPs with deionized (DI) water by pouring water over the tissue three times. Use forceps to manually massage the tissue in between washes.

2.10. Briefly dry tissue on a delicate task wipe and weigh. Place tissues in a pre-autoclaved beaker containing an appropriately sized stir bar. Cover tissues with 60 mL of 3% *t*-octylphenoxypolyethoxyethanol (see the **Table of Materials**) per 1 g of tissue and stir for 1 h at room temperature. Use a minimum of 20 mL.

2.11. Dump tissue and contents into a strainer. Rinse the beaker with de-ionized (DI) water and pour onto tissues. Repeat two more times. Use forceps to manually massage the tissue in between rinses.

2.12. Briefly dry tissue on a delicate task wipe and weigh. Place tissues and stir bars back in the same beakers, and cover with 60 mL of 4% deoxycholic acid per 1 g of tissue. Stir for 1 h at room temperature. Use a minimum of 20 mL.

2.13. Dump tissue and contents into a mesh strainer. Rinse the beaker with DI water and pour onto tissues. Repeat two more times. Use forceps to manually massage the tissue in between rinses.

2.14. Briefly dry tissue on a delicate task wipe and weigh.

2.15. Place tissues in the same beaker with fresh DI water supplemented with 1% penicillin-streptomycin. Cover tightly with paraffin film. Leave overnight at 4 °C.

2.16. Wash strainers and beakers for use the following day.

2.17. On **day 2**, drain beaker contents into a strainer. Briefly dry tissue on a delicate task wipe and weigh.

2.18. Place MFPs in the same beaker with an appropriately sized stir bar. Cover with 60 mL 4% ethanol/0.1% peracetic acid solution per 1 g of tissue. Use a minimum of 20 mL. Stir for 2 h at room temperature.

2.19. Dump tissue and contents into a 0.7 mm strainer. Use forceps to manually massage the tissue. Place contents back into the beaker. Wash tissue by covering it with 60 mL of 1x PBS per 1 g of tissue. Use a minimum of 20 mL. Stir for 15 min at room temperature. Repeat once.

2.20. Dump tissue and contents into a 0.7 mm strainer. Use forceps to manually massage the tissue. Place contents back into beaker. Wash tissue by covering it with 60 mL DI water per 1 g of tissue. Use a minimum of 20 mL. Stir for 15 min at room temperature. Repeat once.

2.21. Briefly dry tissue on a delicate task wipe and weigh. Dump tissue and contents into a strainer. Use forceps to manually massage the tissue.

2.22. Place contents back into beaker. Cover tissues with 60 mL of 100% n-propanol per 1 g of tissue. Use a minimum of 20 mL. Stir for 1 h at room temperature.

2.23. Briefly dry tissue on a delicate task wipe and weigh. Dump tissue and contents into a 0.7 mm strainer. Use forceps to manually massage the tissue.

2.24. Place contents back into beaker. Wash tissue by covering it with 60 mL of DI water per 1 g of tissue. Use a minimum of 20 mL. Stir for 15 min at room temperature. Repeat three times.

2.25. Dump tissue and contents into a strainer. Repeat steps 2.3–2.6 to collect pieces of tissue for sucrose incubation and freezing in cryostat embedding medium.

2.26. Briefly dry tissue on a delicate task wipe and weigh. Place in a labeled 15 mL tube. Freeze at -80 °C overnight.

3. Lyophilization

3.1. Remove the 15 mL tubes from -80 °C and place on dry ice. Keep samples frozen on dry ice until on the lyophilizer.

3.2. Remove caps. Use a rubber band to attach a delicate task wipe to the top to cover the opening. Put samples on the lyophilizer for at least 2 days.

3.3. Remove samples from the lyophilizer and place tubes on dry ice. Remove delicate task wipes weigh each sample on an analytical scale. Attach caps and place at -80 °C overnight.

4. Milling

4.1. Fill a shallow container with liquid nitrogen. Remove samples from the -80 °C freezer. Weigh each lyophilized MFP.

4.2. Place one sample in the mortar. Use a cryogenic glove to hold the mortar in the liquid nitrogen.

4.3. Use a pestle attached to a handheld drill to mill the sample. Mill in 1 min intervals to check progress and remove gloved hand from liquid nitrogen. Mill for a minimum of 5 min.

4.4. Repeat for all samples. Spray and wipe the mortar and pestle with ethanol between each sample.

4.5. Store powdered samples in 15 mL tubes at -80 °C until ready for use.

NOTE: Samples may be used immediately or stored overnight.

5. Hydrogel formation

5.1. If stored at -80 °C overnight, remove and thaw at room temperature. While thawing, calculate the necessary weight of pepsin (see **Table of Materials**) and volume of hydrochloric acid (HCl) needed for each sample that results in a solution with 1% w/v sample powder and 0.1% w/v pepsin in 0.01 M HCl. Add the pepsin to the HCl to form a pepsin-HCl solution.

5.2. Add sample powder and pepsin-HCl solution to a 15 mL tube. Add a small stir bar, and stir for 48 h.

5.3. Place the tubes on ice for 5 min. Calculate the necessary volume of 10x PBS needed for each sample that results in a solution with a 1x PBS concentration. Add the appropriate volume of 10x PBS to each tube.

5.4. Add 10% v/v 0.1 M NaOH to each solution to reach pH 7.4. Use a pH meter to test individually.

NOTE: Gel solution may be stored at 4 °C for one week.

6. Encapsulating cells in hydrogels

6.1. Using GFP- and luciferase-labeled cells

6.1.1. Using the pH 7.4 gel solution, resuspend pelleted GFP- and luciferase-labeled 4T1 cells to a concentration of 500,000 or 1,000,000 cells/mL of gel solution. Add 16 µL of gel-cell solution to each well of a 16-well chamber slide. Incubate for 30 min at 37 °C.

6.1.2. Add 100 µL of complete RPMI media to each well. Continue incubation for 48 h at 37 °C. The GFP- and luciferase-labeled cells can be visualized using fluorescence microscopy at 0 hours, 24 hours, and 48 hours after gelation.

NOTE: Cell proliferation can be measured for the GFP- and luciferase-labeled 4T1 cells used here by adding (S)-4,5-Dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazolecarboxylic acid potassium salt (see the **Table of Materials**) to the wells for 10 min and performing bioluminescence imaging using a bioluminescence imaging system (see **Table of Materials**). Following bioluminescence imaging, the cytoskeleton can be visualized (see section 10).

6.2. Using unlabeled cells

6.2.1. Using the pH 7.4 gel solution, resuspend pelleted unlabeled 4T1 cells to a concentration of 500,000 or 1,000,000 cells/mL of gel solution. Add 16 µL of gel-cell solution to each well of a 16-well chamber slide and a 96-well plate. Incubate for 30 min at 37 °C.

6.2.2. Add 100 µL of media to each well. Continue incubation for 48 h at 37 °C.

NOTE: Cell viability can be measured (see section 11). The 16-well chamber slide can be used for imaging, and the 96-well plate can be used for quantification.

7. H & E staining

7.1. For formalin-fixed, paraffin-embedded tissue, submerge slides containing 5 µm sections twice in 100% xylene (5–10 min) for de-paraffinization. Rehydrate by submerging in 100%, 95%, 85%, and 70% ethanol for 5 min each followed by DI water for 5 min. Continue to step 7.6.

7.2. For frozen tissue sections, take sections immediately from freezer and submerge them in 10% NBF for 10 min. Wash in 1x PBS three times for 5 min each. Rinse in water for 5 min.

7.3. Stain nuclei with hematoxylin for 3 min. Rinse in running tap water.

7.4. Differentiate by dipping 1–2 times in 0.3% acid alcohol (0.3% HCl in 70% ethanol). Rinse in running tap water for 5 min.

7.5. Add bluing agent for 30 s. Rinse in running tap water for 5 min. Submerge in 95% ethanol for 30 s.

7.6. Incubate with eosin for 90 s at room temperature. Dehydrate in 3 changes of 100% ethanol for 5 min each.

7.7. Submerge twice in 100% xylene for 5 min each. Add distyrene-plasticiser-xylene (DPX0 mounting media to the slide and add a coverslip. Let it cure overnight before imaging.

8. 1-([4-(Xylylazo)xylyl]azo)-2-naphthol staining

8.1. Prepare 1-([4-(Xylylazo)xylyl]azo)-2-naphthol solution.

8.1.1. Add 0.5 g of 1-([4-(Xylylazo)xylyl]azo)-2-naphthol powder to a beaker with 100 mL propylene glycol. Heat to 95–100 °C while stirring for at least 30 min. Prevent the temperature from exceeding 100 °C.

8.1.2. Remove beaker from heat and allow to cool slightly. Pour solution through grade 4 qualitative filter paper to remove any residual particulates.

NOTE: The solution can be stored at room temperature and should be filtered through a 0.45 µm syringe filter immediately before staining.

8.2. Stain frozen tissue sections.

8.2.1. Remove slides from the -80 °C freezer and air dry for 30 min. Pre-cool 10% NBF at -20 °C for 30 min in a Coplin jar. Fix the slides at room temperature for 10 min. Wash in DI water 3 times for 5 min each.

8.2.2. Remove excess water using a delicate task wipe before immersing in propylene glycol 2 times for 5 min each. Remove slides from propylene glycol. Do not rinse.

8.2.3. Place slides into syringe filtered Oil Red O staining solution for 3 h at room temperature.

8.2.4. Differentiate by placing slides in 85% propylene glycol for 5 min. Rinse samples in PBS twice for 5 min each.

8.2.5. Stain samples with hematoxylin for 30 s. Wash in running tap water for 5 min and then place the slides in DI water.

8.2.6. Mount samples using aqueous based mounting medium and allow media to dry overnight before imaging.

9. Rheology

9.1. Bring pre-gel solutions from step 5.4 to the rheometer on ice.

9.2. Attach a 25 mm arm and plate to the rheometer. Open the rheology software on the computer connected to the rheometer.

9.3. Perform rotational mapping and determine the zero gap. Raise the arm when complete.

9.4. Pipette 500 µL of pre-gel on the plate. Lower the arm until the pre-gel solution completely occupies the gap between the plate and the arm. Be conservative because lowering the arm past this point will result in pre-gel solution spilling out of the side.

9.5. Perform a frequency sweep on the pre-gel solution from 0.1–100 Hz after it has remained at 37 °C for 30 min.

9.6. Raise the rheometer arm when complete. Wipe liquid with a delicate task wipe. Rinse with DI water and wipe with a delicate task wipe. Repeat steps 9.4–9.6 with additional samples.

10. Phalloidin conjugate staining of F-actin

10.1. Bring phalloidin conjugate solution to room temperature. Briefly centrifuge at a low speed prior to opening. Aliquot enough solution for the assay, and store the rest at -20 °C.

10.2. Dilute 1000x phalloidin conjugate stock solution to a 1x working solution by adding 1 µL stock solution to 1 mL 1x PBS + 1% bovine serum albumin (BSA). This makes enough for 10 wells (100 µL/well).

NOTE: One may use plain 1x PBS, but 1x PBS + BSA is preferred to prevent phalloidin conjugate from sticking to the tube. Do not store this solution after the assay. 1 µL of blue fluorescent dye (see the **Table of Materials**) working solution may be added to stain for nuclei. Working solution may be made by mixing 1 µL of 20 mM blue fluorescent dye with 11.3 µL 1x PBS.

10.3. Aspirate media from wells (step 6.1). Rinse wells with 1x PBS.

10.4. Add 10% NBF. Incubate wells with 10% NBF for 20 min at room temperature. Remove supernatant. Wash 3 times with PBS for 5 min each time.

10.5. Add 0.1% *t*-Octylphenoxypolyethoxyethanol in PBS for 5 min. Aspirate and wash 3 times with PBS for 5 min each time.

10.6. Add 100 µL of working solution from step 10.2 to each well. Incubate for 1 h at room temperature. Aspirate and wash 3 times with PBS for 5 min each time. Leave in PBS at 4 °C.

10.7. Aspirate and remove wells from the gasket on the chamber slide. Use needle nose tweezers to remove the gasket from the slide. Mount and coverslip samples using aqueous based mounting medium. Allow to cure (5 min).

10.8. Observe cells under a fluorescence microscope at excitation/emission of 590/618 nm.

11. Viability assay

11.1. Rinse the cells with Dulbecco's PBS (DPBS). Add 100 µL of DPBS containing 1 µM calcein AM and 2 µM ethidium homodimer to each well. Incubate for 30 min at room temperature.

11.2. Image the 16-well chamber slide by fluorescence microscopy. Calcein acetoxymethyl (calcein AM) can be observed at excitation/emission = 494/517 nm. Ethidium homodimer can be observed at excitation/emission = 528/645 nm.

11.3. Read the 96-well plate on a plate reader using the same wavelengths in step 11.2.

REPRESENTATIVE RESULTS:

MFPs were decellularized following irradiation using the procedure shown in **Figure 1A**. MFPs pre-decellularization (**Figure 1B**) and post-decellularization (**Figure 1C**) are shown. Decellularization was confirmed using hematoxylin and eosin (H & E) staining, and 1-([4-(Xylylazo)xylyl]azo)-2-naphthol staining was used to evaluate lipid content (**Figure 2**). Rheological properties of the ECM hydrogels were also assessed at 37 °C (**Figure 3**). The storage modulus was higher than the loss modulus for all conditions, demonstrating stable hydrogel formation.

GFP- and luciferase-labeled 4T1 mammary carcinoma cells were encapsulated in the hydrogels. Cell proliferation was examined by fluorescence microscopy, bioluminescence measurements, and viability staining 48 h after encapsulation (**Figure 4**). Irradiated hydrogels showed an increasing trend in tumor cell proliferation. Phalloidin conjugate was used to visualize F-actin in the encapsulated cells (**Figure 5**). This technique can be used to examine cell morphology and cytoskeletal properties.

FIGURE AND TABLE LEGENDS:

Figure 1: Experimental workflow. (**A**) Schematic of hydrogel formation. Digital camera images were taken of MFPs pre- (**B**) and post-decellularization (**C**).

Figure 2: Confirmation of decellularization and de-lipidation in MFPs. Hematoxylin and eosin staining (H & E) of unirradiated MFPs embedded in paraffin and sectioned at 5 µm (**A**) was compared to MFPs frozen in cryostat embedding medium (5 µm sections) before (**B**) and after decellularization (**C**), incubated with sucrose prior to freezing in cryostat embedding medium and

sectioned at 30 µm (**D**). 1-([4-(Xylylazo)xylyl]azo)-2-naphthol staining was done to visualize lipid retention in MFPs frozen in cryostat embedding medium (5 µm sections) before (**E**) and after decellularization (**F**) and incubated with sucrose prior to freezing in cryostat embedding medium and sectioned at 30 µm sections (**G**). Scale bars represent 50 µm. De-cell = decellularization.

Figure 3: Confirmation of hydrogel formation. Rheology was used to determine the storage and loss modulus of control (**A**) and irradiated (**B**) pre-gel solution made from MFPs at 37 °C and 0.5% strain. Error bars show standard deviation.

Figure 4: Tumor cell proliferation in irradiated ECM hydrogels. 4T1 cell proliferation 48 h after inoculation is shown with pre-gel derived from control (**A**) and irradiated (**B**) MFPs. (**C**) Bioluminescence signal from 4T1 cells embedded within control and irradiated hydrogels. Calcein AM stained live cells and ethidium homodimer stained dead cells were evaluated in control (**D**) and irradiated (**E**) hydrogels, and the live/dead ratio was quantified (**F**). Scale bars represent 200 µm. Error bars show standard error.

Figure 5: Cytoskeletal properties in ECM hydrogels. Cells within (**A**) control and (**B**) irradiated ECM hydrogels are stained with phalloidin conjugate to visualize F-actin (red) and blue fluorescent dye to visualize nuclei (blue) in irradiated MFPs. Scale bar represents 100 µm.

Table 1: Tissue weights before and after decellularization. Representative tissue weights for each condition were measured before and after MFP decellularization.

DISCUSSION:

This method of hydrogel formation is largely dependent on the amount of starting tissue. Murine MFPs are small, and the decellularization process results in a significant reduction of material (**Table 1**). The process can be repeated with more MFPs to increase final yield. Milling is another important step that may lead to loss of material. Others have shown success with a cryogenic mill, but this protocol is based on milling via a handheld mortar and electric drill with a pestle attachment[8,17]. This has the advantage of lower capital costs and minimizing material loss but may introduce variability in the final product.

A challenge to confirmation of decellularization and de-lipidation is in freezing adipose tissue in cryostat embedding medium. **Figure 2A** shows H & E staining of an unirradiated MFP embedded and sectioned in paraffin. Distinct nuclei are visible on the edges of adipose cells near junctions with other cells, and adipocyte morphology is well-maintained. **Figure 2B,C,E,F** show MFPs prepared by embedding and freezing MFPs in cryostat embedding medium and sectioning 5 µm slices. This process was unable to retain adipocyte morphology and shape. However, decellularization was confirmed through the loss of nuclei and other traces of DNA (**Figure 2C**), and de-lipidation was visualized with the loss of neutral lipid content staining (**Figure 2F**). Adipocyte morphology was maintained by incubating MFPs in sucrose, embedding and freezing in cryostat embedding medium, and sectioning 30 µm slices (**Figure 2D,G**). While the visualization of H & E staining was difficult with this method (**Figure 2D**), 1-([4-(Xylylazo)xylyl]azo)-2-naphthol staining confirmed the retention of adipocyte morphology

(**Figure 2G**).

We have developed an in vitro hydrogel model that can mimic the in vivo normal tissue microenvironment and its response to radiation damage. ECM hydrogels have been fabricated in similar studies, but the impact of radiation damage on tumor cell behavior has not been assessed[9,12,16-18]. We expect that irradiation of MFPs will alter ECM remodeling and composition, and these compositional changes will be characterized in future studies. We observed an increasing trend in the proliferation of 4T1 cells within irradiated ECM hydrogels using both bioluminescence imaging and viability staining (**Figure 4**). In addition, we used phalloidin conjugate to stain F-actin filaments in encapsulated tumor cells and found a qualitative increase in actin alignment and tumor cell elongation in irradiated ECM hydrogels, which suggests an increase in adhesion strength and invasive capacity (**Figure 5**)[19,20]. Future experiments will explore changes in focal adhesion dynamics and protease-mediated remodeling for the evaluation of cell migration and invasion.

This method was developed using a murine TNBC cell line, but this method may be used as a platform for evaluating the proliferation and invasiveness of other cell types. Future studies may incorporate immune cells to determine their role in response to radiation as well as other forms of tissue damage (e.g., surgery). Although this study evaluated ECM hydrogels from MFPs irradiated ex vivo, additional studies will explore in vivo radiation of MFPs to evaluate the effect of physiological radiation response and infiltrating immune cells on ECM characteristics. We have established a method to fabricate ECM hydrogels from mouse MFPs to study the effect of normal tissue radiation on tumor cell behavior, and this technique may be extended to human tissue for a more relevant hydrogel model. Overall, examining normal tissue damage through ECM hydrogels may lead to insights into the role of ECM changes following radiation therapy in local recurrence.

ACKNOWLEDGMENTS:

The authors thank Dr. Laura L. Bronsart for providing the GFP- and luciferase-4T1 cells, Dr. Edward L. LaGory for advice on 1-([4-(Xylylazo)xylyl]azo)-2-naphthol staining, Dr. Craig L. Duvall for IVIS and lyophilizer use, and Dr. Scott A. Guelcher for rheometer use. This research was financially supported by NIH grant #R00CA201304.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Hanahan, D., Weinberg, R.A. The hallmarks of cancer. *Cell*. **100** (1), 57–70, doi: 10.1007/s00262-010-0968-0 (2000).
2. Siegel, R.L., Miller, K.D., Jemal, A. Cancer statistics, 2018. *CA: A Cancer Journal for Clinicians*. **68** (1), 7–30, doi: 10.3322/caac.21442 (2018).
3. Lowery, A.J., Kell, M.R., Glynn, R.W., Kerin, M.J., Sweeney, K.J. Locoregional recurrence after breast cancer surgery: a systematic review by receptor phenotype. *Breast Cancer Research and Treatment*. **133** (3), 831–841, doi: 10.1007/s10549-011-1891-6 (2012).

4. Miller, K.D. et al. Cancer treatment and survivorship statistics, 2016. *CA: A Cancer Journal for Clinicians*. **66** (4), 271–289, doi: 10.3322/caac.21349 (2016).
5. Vilalta, M., Rafat, M., Giaccia, A.J., Graves, E.E. Recruitment of Circulating Breast Cancer Cells Is Stimulated by Radiotherapy. *Cell Reports*. **8** (2), 402–409, doi: 10.1016/j.celrep.2014.06.011 (2014).
6. Rafat M, Aguilera TA, Vilalta M, Bronsart LL, Soto LA, von Eyben R, Golla MA, Ahrari Y, Melemenidis S, Afghahi A, Jenkins MJ, Kurian AW, Horst KC, Giaccia AJ, G.E. Macrophages Promote Circulating Tumor Cell-Mediated Local Recurrence Following Radiation Therapy in Immunosuppressed Patients. (650), doi: 10.1158/0008-5472.CAN-17-3623 (2018).
7. Haubner, F., Ohmann, E., Pohl, F., Strutz, J., Gassner, H.G. Wound healing after radiation therapy: Review of the literature. *Radiation Oncology*. **7** (1), 1–9, doi: 10.1186/1748-717X-7-162 (2012).
8. Beachley, V.Z. et al. Tissue matrix arrays for high throughput screening and systems analysis of cell function. *Nature Methods*. **12** (12), 1197–1204, doi: 10.1038/nmeth.3619.Tissue (2015).
9. Tian, X. et al. Organ-specific metastases obtained by culturing colorectal cancer cells on tissue-specific decellularized scaffolds. *Nature Biomedical Engineering*. **2** (6), 443–452, doi: 10.1038/s41551-018-0231-0 (2018).
10. Saldin, L.T., Cramer, M.C., Velankar, S.S., White, L.J., Badylak, S.F. Extracellular matrix hydrogels from decellularized tissues: Structure and function. *Acta Biomaterialia*. **49**, 1–15, doi: 10.1016/j.actbio.2016.11.068 (2017).
11. Hinderer, S., Layland, S.L., Schenke-Layland, K. ECM and ECM-like materials — Biomaterials for applications in regenerative medicine and cancer therapy. *Advanced Drug Delivery Reviews*. **97**, 260–269, doi: 10.1016/j.addr.2015.11.019 (2016).
12. Young, D.A., Ibrahim, D.O., Hu, D., Christman, K.L. Injectable hydrogel scaffold from decellularized human lipoaspirate. *Acta Biomaterialia*. **7** (3), 1040–1049, doi: 10.1016/j.actbio.2010.09.035 (2011).
13. Singelyn, J.M., Christman, K.L., Littlefield, R.B., Schup-Magoffin, P.J., DeQuach, J.A., Seif-Naraghi, S.B. Naturally derived myocardial matrix as an injectable scaffold for cardiac tissue engineering. *Biomaterials*. **30** (29), 5409–5416, doi: 10.1016/j.biomaterials.2009.06.045 (2009).
14. Pouliot, R.A. et al. Development and characterization of a naturally derived lung extracellular matrix hydrogel. *Journal of Biomedical Materials Research Part A*. **104** (8), 1922–1935, doi: 10.1002/jbm.a.35726 (2016).
15. Bonvillain, R.W. et al. A Nonhuman Primate Model of Lung Regeneration: Detergent-Mediated Decellularization and Initial In Vitro Recellularization with Mesenchymal Stem Cells. *Tissue Engineering Part A*. **18** (23–24), 2437–2452, doi: 10.1089/ten.tea.2011.0594 (2012).
16. Brown, B.N. et al. Comparison of Three Methods for the Derivation of a Biologic Scaffold Composed of Adipose Tissue Extracellular Matrix. *Tissue Engineering Part C: Methods*. **17** (4), 411–421, doi: 10.1089/ten.tec.2010.0342 (2011).
17. Link, P.A., Pouliot, R.A., Mikhaiel, N.S., Young, B.M., Heise, R.L. Tunable Hydrogels from Pulmonary Extracellular Matrix for 3D Cell Culture. *Journal of Visualized Experiments*. (119), 1–9, doi: 10.3791/55094 (2017).
18. Massensini, A.R. et al. Concentration-dependent rheological properties of ECM hydrogel for intracerebral delivery to a stroke cavity. *Acta Biomaterialia*. **27**, 116–130, doi:

573 10.1016/j.actbio.2015.08.040 (2015).
574 19. Mierke, C.T., Frey, B., Fellner, M., Herrmann, M., Fabry, B. Integrin 5 1 facilitates cancer
575 cell invasion through enhanced contractile forces. *Journal of Cell Science*. **124** (3), 369–383, doi:
576 10.1242/jcs.071985 (2011).
577 20. Ahmadzadeh, H. et al. Modeling the two-way feedback between contractility and matrix
578 realignment reveals a nonlinear mode of cancer cell invasion. *Proceedings of the National*
579 *Academy of Sciences*. **114** (9), E1617–E1626, doi: 10.1073/pnas.1617037114 (2017).
580

Figure 1

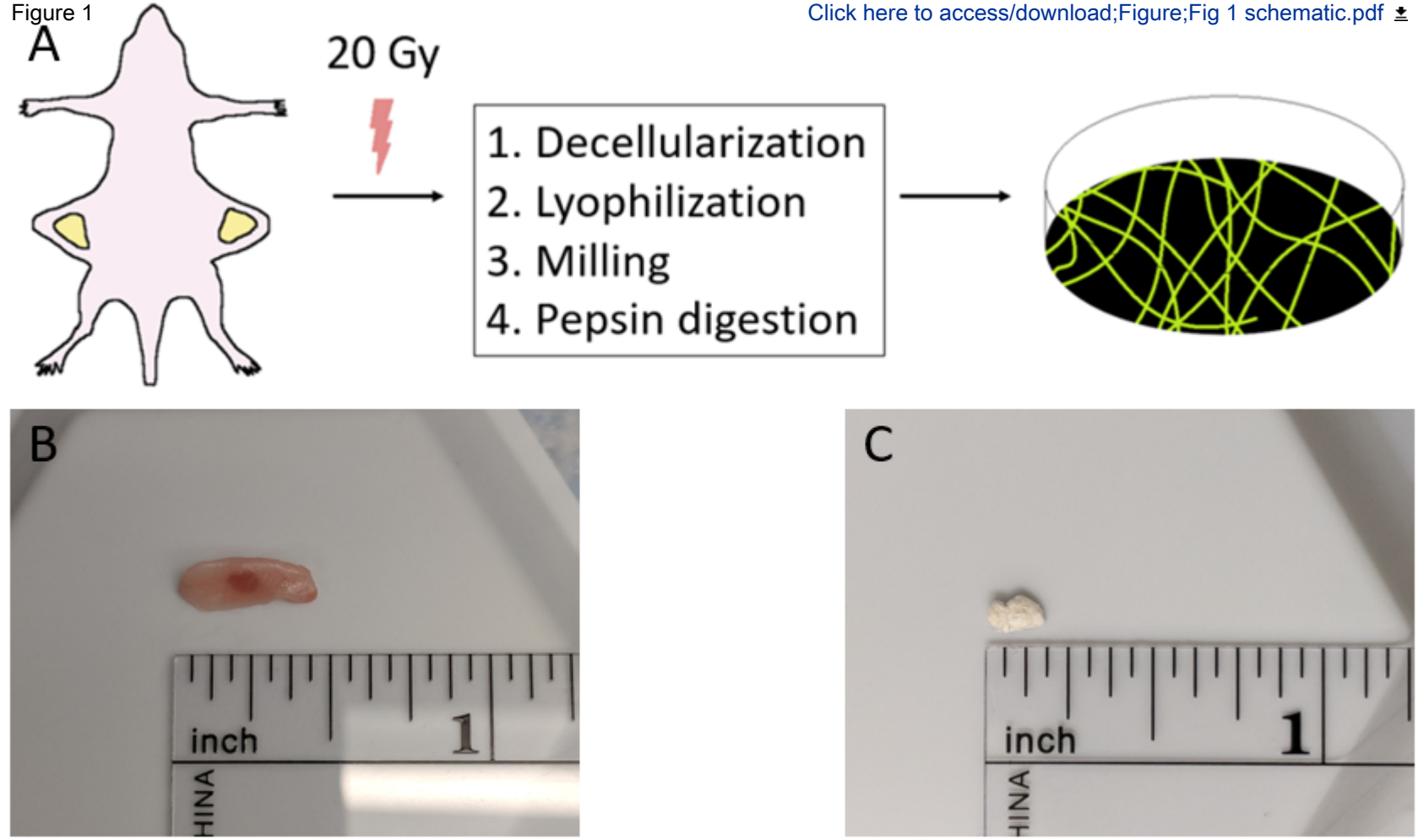


Figure 2

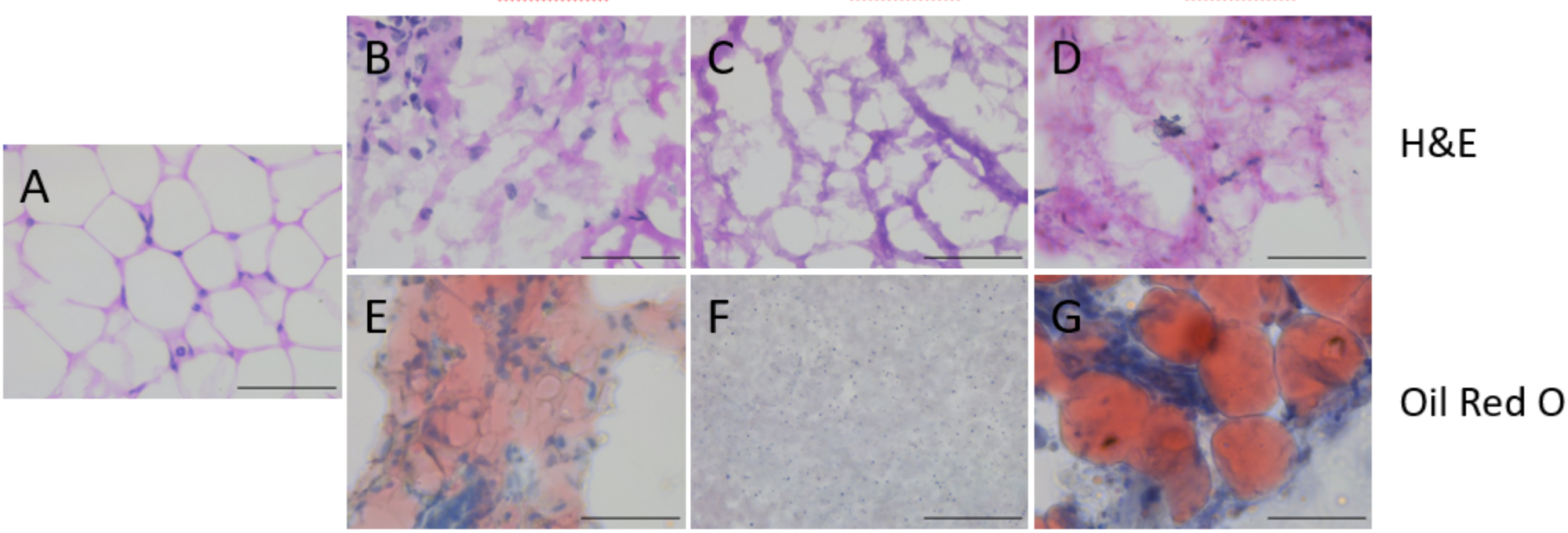


Figure 3

Click here to access/download;Figure;Fig 3 rheology.pdf

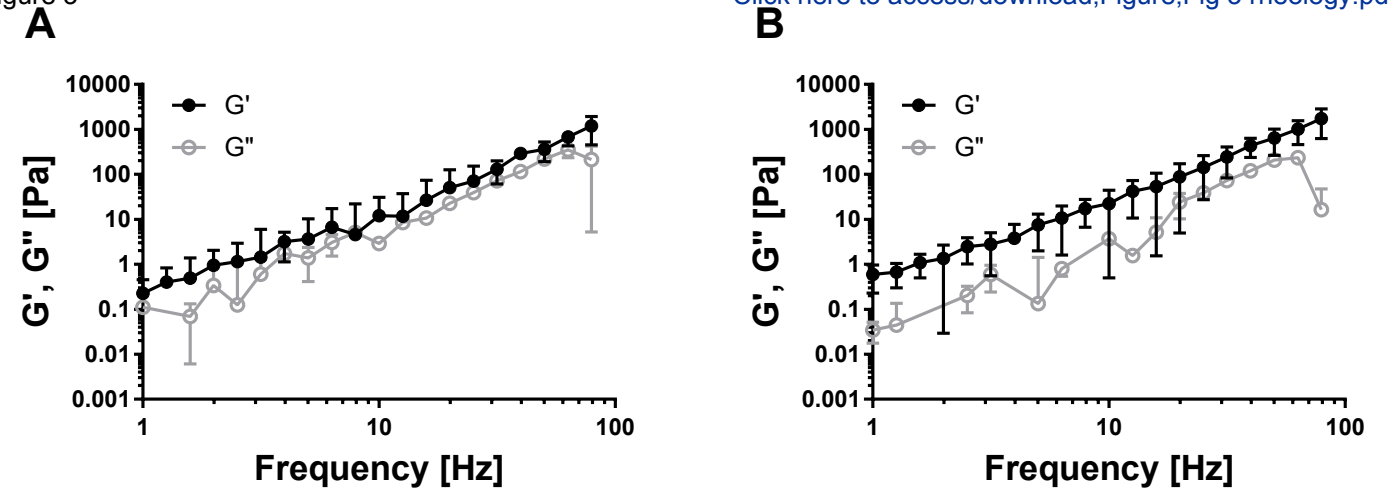
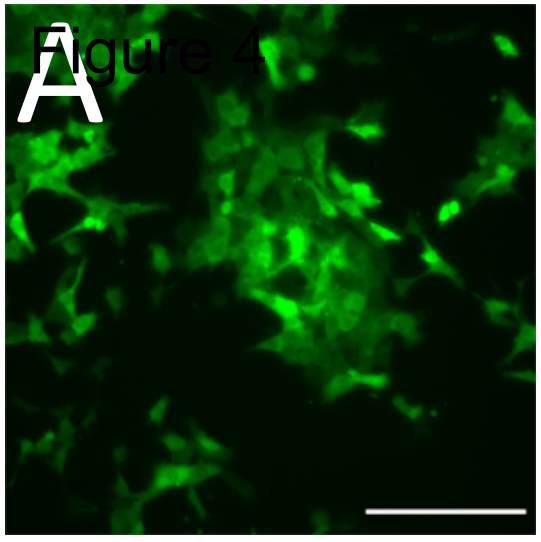
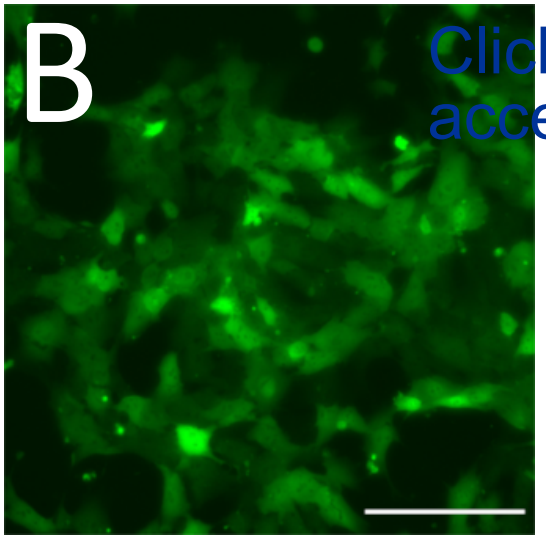
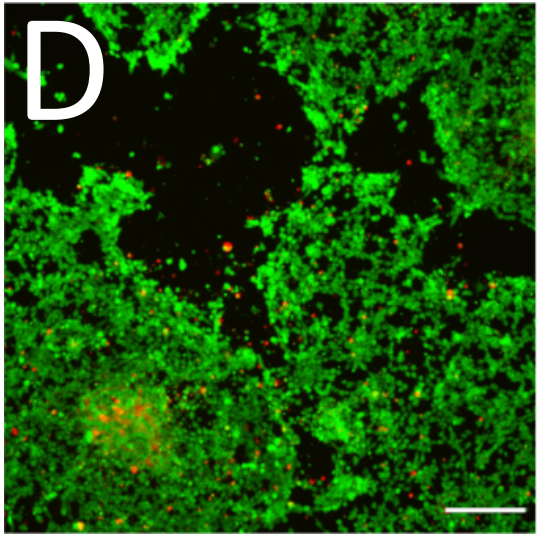
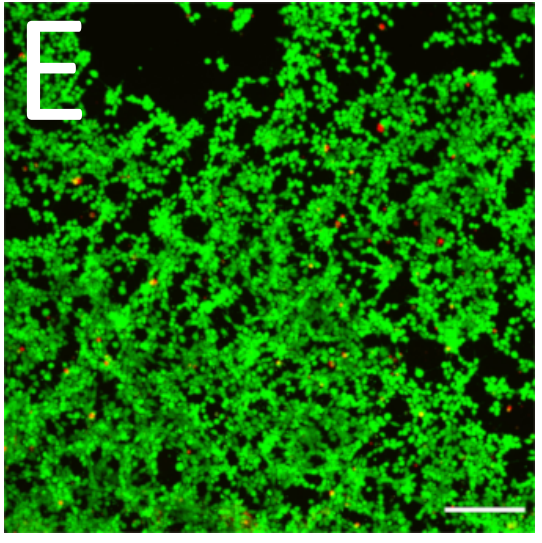


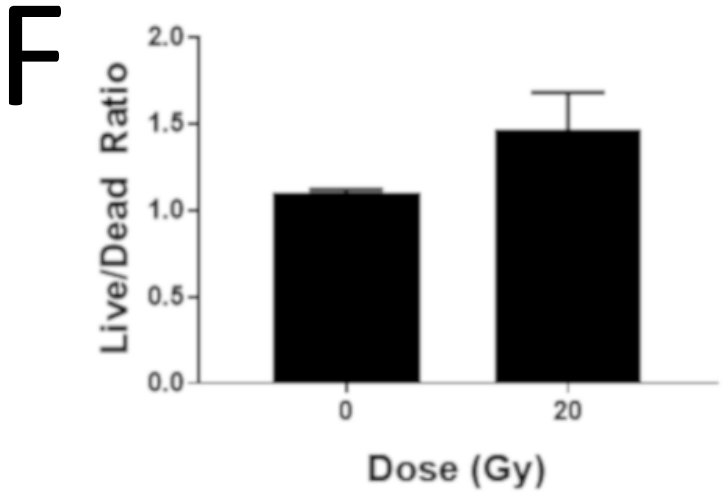
Figure 4











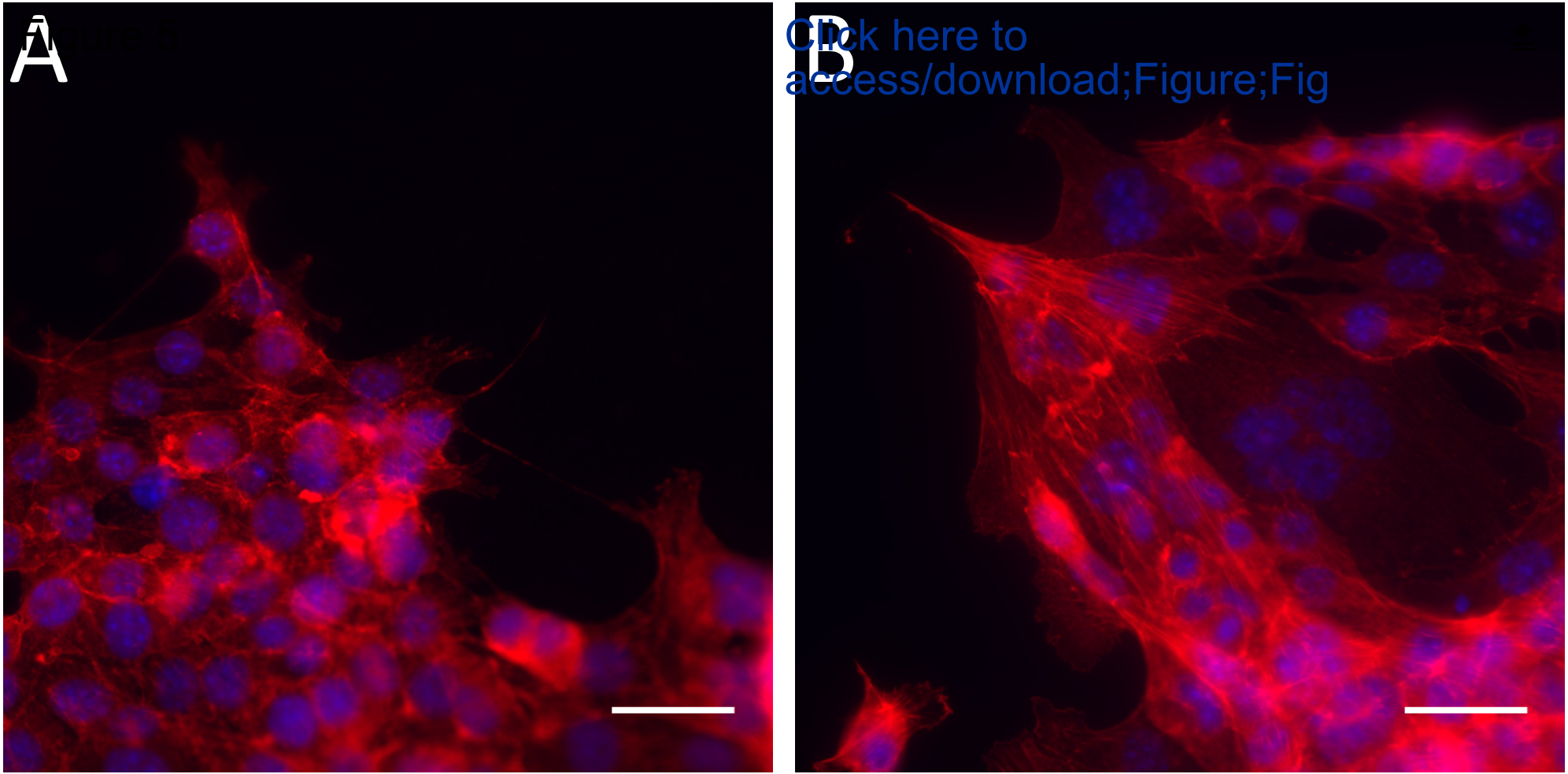


Table 1

Tissue weight (g)	Control (0 Gy)	Irradiated (20 Gy)
Initial MFP weight	0.461	0.457
MFP weight following histology sample removal	0.423	0.416
MFP weight after decellularization	0.025	0.025
Decellularized MFP weight after histology sample removal	0.015	0.016

Table of Materials

Click here to access/download;Table of Materials;2019-02-21 JoVE_Materials resubmission_v1.xlsx

Name of Reagent/ Equipment	Company
10% Neutral Buffered Formalin, Cube with Spigot	VWR
2-methylbutane	Alfa Aesar
AR 2000ex Rheometer	TA Instruments
Bovine Serum Albumin	Sigma-Aldrich
calcein acetoxymethyl (calcein AM)	Molecular Probes, Inc.
D-Luciferin Firefly, potassium salt	Biosynth Chemistry & Biology
DPX Mountant for Histology	Sigma-Aldrich
Dulbecco's phosphate-buffered saline	Gibco
Eosin-Y with Phloxine	Richard-Allan Scientific
ethidium homodimer	Molecular Probes, Inc.
Fetal Bovine Serum	Sigma-Aldrich
Fisher Healthcare Tissue-Plus O.C.T. Compound	Fisher Scientific
Fluoromount-G	SouthernBiotech
FreeZone 4.5	Labconco
Hoechst 33342 Solution (20 mM)	Thermo Scientific
Hydrochloric acid	Sigma-Aldrich
IVIS Lumina III	PerkinElmer
Kimtech Science Kimwipes	Kimberly Clark
n-Propanol (Peroxide-Free/Sequencing), Fisher BioReagents	Fisher Scientific
Oil Red O	Sigma-Aldrich
OPS Diagnostics CryoGrinder	OPS Diagnostics, LLC
PBS (10X), pH 7.4	Quality Biological, Inc.
Penicillin-Streptomycin	Gibco
Pepsin from porcine gastric mucosa	Sigma-Aldrich
Peracetic acid	Sigma-Aldrich
Phalloidin-iFluor 594 Reagent (ab176757)	abcam
Propylene glycol	Sigma-Aldrich
Richard-Allan Scientific Signature Series Bluing Reagent	Richard-Allan Scientific
Richard-Allan Scientific Signature Series Hematoxylin 7211	Richard-Allan Scientific
RPMI Medium 1640	Gibco
Sodium deoxycholate, 98%	Frontier Scientific
Sucrose	Sigma-Aldrich

Triton x-100	Sigma-Aldrich
Trypsin-EDTA (0.25%), phenol red	Gibco
Whatman qualitative filter paper, Grade 4	Whatman
Xylenes (Certified ACS), Fisher Chemical	Fisher Scientific

Catalog Number	Comments/Description
16004-128	-
19387	-
10D4335	rheometer
A1933-25G	-
C1430	-
L-8820	(S)-4,5-Dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazolecarboxylic acid potassium salt
06522-500ML	-
14040133	-
71304	eosin
E1169	ethidium homodimer-1 (EthD-1)
F0926-500ML	-
23-730-571	cryostat embedding medium
0100-01	aqueous based mounting medium
7751020	lyophilizer
62249	blue fluorescent dye
258148-500ML	-
CLS136334	bioluminescence imaging system
	delicate task wipes
BP1130-500	-
O0625-25G	1-([4-(Xylylazo)xylyl]azo)-2-naphthol
CG-08-02	-
119-069-151	Phosphate-buffered saline
15140-122	-
P6887-5G	pepsin
77240-100ML	-
ab176757	phalloidin conjugate
W294004-1KG-K	-
7301L	bluing agent
7211	-
11875-093	-
JK559522	deoxycholic acid
S5016	-

X100-100ML	*t* -Octylphenoxypolyethoxyethanol
25200-056	-
1004-110	grade 4 qualitative filter paper
X5-4	-

jove JOURNAL OF VISUALIZED EXPERIMENTS

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

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Studying Normal Tissue Radiation Effects using Extracellular Matrix Hydrogels

Author(s): Steven M Alves, Tian Zhu, Anastasia Shostak, Nimma S Rossen, Marjan Rafat

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

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

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

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

ARTICLE AND VIDEO LICENSE AGREEMENT

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

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

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

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

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

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

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

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

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

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

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

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

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

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

CORRESPONDING AUTHOR:

Name:	Marjan Rafat
Department:	Chemical and Biomolecular Engineering
Institution:	Vanderbilt University
Article Title:	Studying Normal Tissue Radiation using Extracellular Matrix Hydrogels
Signature:	
Date:	2/22/19

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

VANDERBILT UNIVERSITY
NASHVILLE, TENNESSEE 37235 Chemical & Biomolecular Engineering

Marjan Rafat, PhD
Assistant Professor of Chemical and Biomolecular Engineering
2414 Highland Ave.
ESB 426
Nashville, TN 37212
Phone: (615) 343-3899 • Fax: (615) 343-7951
Email: marjan.rafat@vanderbilt.edu

Nandita Singh, Ph.D.
Senior Science Editor
Journal of Visualized Experiments
Email: nandita.singh@jove.com

11 February 2019

Dear Dr. Singh,

Please find enclosed our revised manuscript entitled, “Studying Normal Tissue Radiation Effects using Extracellular Matrix Hydrogels.” 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,

Marjan Rafat, Ph.D.
Assistant Professor of Chemical and Biomolecular Engineering
Assistant Professor of Biomedical Engineering (Secondary)
Assistant Professor of Radiation Oncology (Secondary)
Faculty Member, Breast Cancer Research Program, Vanderbilt Ingram Cancer Center
Vanderbilt University

Ms. No. JoVE59304
Title: Studying Normal Tissue Radiation Effects using Extracellular Matrix Hydrogels

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 Editors

Editor Comments	Response
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.	Completed.
2. 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 and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Kimwipe, Triton x-100, IVIS Lumina, Whatman, TA Instruments, Fluoromount, etc.	Completed.
3. Please add more details 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. 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. See examples below.	Completed.
4. Please add more details 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. 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. See examples below.	MFPs are collected into a 15 mL conical tube containing RPMI media supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum.
5. 1.6: Please specify the size of the tube used.	15 mL.
6. 2.3: How to divide the tissue?	Now 2.4. It is suggested to use a pair of forceps with scissors or a scalpel.
7. 2.6: Please split into two or three steps so that each step contains only 2-3 actions and a maximum of 4 sentences per step.	Now 2.8. Completed.
8. 2.9: Please specify the mesh size of the strainer.	Size 25 mesh strainers.

9. 7.13: What is the incubation temperature?	H&E slides are incubated with eosin for 90 seconds at room temperature.
10. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.	Completed.
11. Please include single-line spaces between all paragraphs, headings, steps, etc.	Completed.
12. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less 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.	Completed.
13. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.	Completed.
14. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.	Completed.
15. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.	Completed.

Response to Reviewer #1

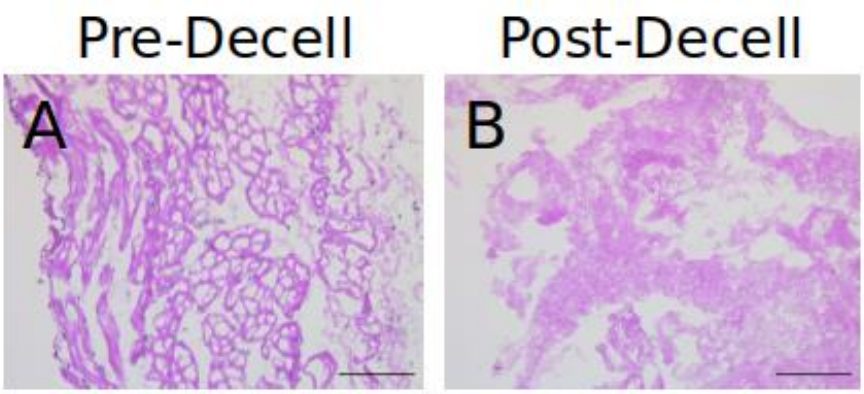
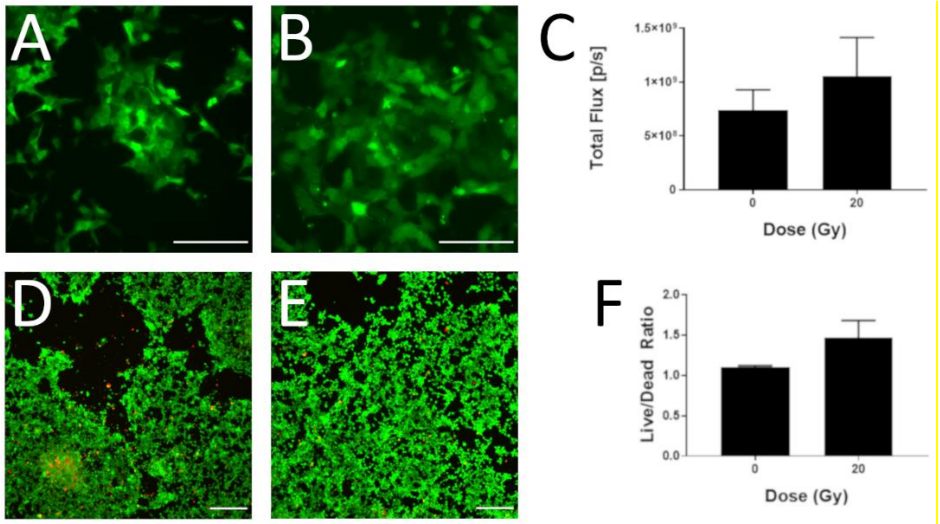
Reviewer 1 Comments	Response
1. There would be benefit to adding minor rationale to some of the steps of the protocol or discussion. For example, what is expected to change in the tissue upon radiation? Further, are there concerns with the irradiated tissue not decellularizing as well under the same conditions as the control tissue? How was this checked?	We thank the reviewer for this comment. It has been well documented that radiation induces fibrosis (Haubner, F. et al. Wound healing after radiation therapy: Review of the literature. *Radiation Oncology*. **7** (1), 1–9, doi: 10.1186/1748-717X-7-162 (2012)), and we expect to see changes to extracellular matrix remodeling. In future studies, we will characterize these changes. For clarification, we have added the following sentence to the discussion: "We expect that irradiation of MFPs will alter ECM remodeling and composition, and these compositional changes will be characterized in future studies." We evaluated decellularization and delipidation by H&E staining and Oil Red O staining, respectively. Figure 2 shows these results in the unirradiated condition, which was representative of irradiated condition as well. Based on the results of these stains, incomplete decellularization was not considered an issue.

Response to Reviewer #2

<table>
<tr><th>Reviewer 2 Comments</th><th>Response</th></tr>
<tr><td>1. The tissues were weighed before and after decellularization, but no data were presented related to the weight in the manuscript. Could the authors give the data related to weight change or the water contents before and after decellularization?</td><td>We thank the reviewer for this suggestion. The data related to weight change is provided in the added Table 1.

<table>
<tr><th>Tissue weight (g)</th><th>Control (0 Gy)</th><th>Irradiated (20 Gy)</th></tr>
<tr><td>Initial MFP weight</td><td>0.461</td><td>0.457</td></tr>
<tr><td>MFP weight following histology sample removal</td><td>0.423</td><td>0.416</td></tr>
<tr><td>MFP weight after decellularization</td><td>0.025</td><td>0.025</td></tr>
<tr><td>Decellularized MFP weight after histology sample removal</td><td>0.015</td><td>0.016</td></tr>
</table>
</td></tr>
<tr><td>2. What is the advantage of the decellularization procedure in this study over the others in the literature employing SDS treatment? Could the authors compare their method to others?</td><td>We thank the reviewer for this comment. While SDS treatment and sodium deoxycholate treatments are comparable in removing cellular material, sodium deoxycholate has been shown to be more effective at removing DNA (Young, D.A. et al. Injectable hydrogel scaffold from decellularized human lipoaspirate. Acta Biomaterialia. 7 (3), 1040–1049, doi: 10.1016/j.actbio.2010.09.035 (2011)). This cited publication studies the effect of decellularization on human lipoasiprate, which is comparable to the adipose in murine mammary fat pads. We have added the following to the protocol for clarification:
“This procedure was adapted from previously published methods focused on adipose decellularization, which included the sodium deoxycholate ionic detergent rather than sodium dodecyl sulfate to remove DNA efficiently [12,16,17].”</td></tr>
<tr><td>3. Do the authors have images with lower magnification to show an overall picture of the tissues after H&E and Oil Red O staining (Figure 2)?</td><td>We thank the reviewer for this suggestion. Sectioning adipose was technically challenging and resulted in few images at lower magnifications due to the inability of OCT to retain tissue morphology. Included here are images of H&E staining at 20x magnification as opposed to 64x shown in Figure 2, which demonstrates again the successful removal of cellular material following decellularization. We unfortunately cannot provide Oil Red O stained slides at lower magnifications due to the aforementioned technical difficulties. We would like to note that the staining shown in Figure 2 is confirmed by other previously published work, and we are confident that we observed de-lipidation: Brown BN et al. Tissue Engineering. Part C, Methods. 2011; 17(4): 411-421 and Choi YS et al. Biochem Biophys Res Commun. 2006; 345(2): 613-637 both show similar Oil Red O staining in adipose tissue.

H&E staining before decellularization (A) and after decellularization (B). Scale bars represent 100 μm.</td></tr>
</table>

	
4. In Figure 4C, bioluminescence data show a significant difference between the cells embedded in hydrogels from radiation-treated and untreated matrices. How do the authors explain such a difference after only one dose of 20 Gy of radiation as compared to multiple doses of 40-50 Gy of radiation applied clinically for radiotherapy? In Figure 4A and B, GFP results also show higher staining in the irradiated gels. However, whether the cells in the control are alive or dead is not clear. Live/dead assay would be of importance to compare the cells in the control and irradiated samples.	We thank the reviewer for these questions. We have updated Figure 4 to include data from additional replicates of the proliferation experiment. In the clinic, patients are given approximately 50 Gy in 28 fractions post-mastectomy or 50 Gy with a 10 Gy boost following lumpectomy. We chose to irradiate to a single dose of 20 Gy as opposed to fractionated doses given the time constraints in our previous *in vivo* study that would influence overall tumor burden. It is well known that fractionating doses results in higher cell survival due to the possibility for repair of double-strand breaks in between fractions that is not possible when giving a single dose (see Hall EJ and Giaccia AJ. *Radiobiology for the Radiologist*. 2012, 7th edition). We therefore used a 20 Gy dose in a single fraction to ensure tissue damage. After multiple experiments, our results continue to show an increasing trend toward higher proliferation using bioluminescence imaging. We have also added live/dead staining analysis in Figure 4 to evaluate whether interaction with ECM hydrogels altered cytotoxicity (see below). We found that there was a trend toward a higher live/dead ratio in the irradiated ECM hydrogels, indicating that the ECM hydrogels are not cytotoxic and that the irradiated ECM hydrogels may promote increased proliferation. We have added the following to the Discussion section to describe this finding: "We observed an increasing trend in the proliferation of 4T1 cells within irradiated ECM hydrogels using both bioluminescence imaging and viability staining (Figure 4)." Scale bars represent 200 μm. 

5. In Fig. 5, the image of the cells on the untreated control is missing. It would be more complete if the authors provide an image for the control. That would also show the cell number on the untreated control and support Fig. 4.	We thank the reviewer for this observation. We have updated Figure 5 to compare the cytoskeleton in cells grown in the control and irradiated ECM hydrogels (see below). We observed that cells grown on irradiated ECM exhibited enhanced actin alignment and tumor cell elongation, which may be indicative of enhanced tumor cell invasion. We have added the following in the Discussion section to describe this finding: "In addition, we used phalloidin conjugate to stain F-actin filaments in encapsulated tumor cells and found a qualitative increase in actin alignment and tumor cell elongation in irradiated ECM hydrogels, which suggests an increase in adhesion strength and invasive capacity (Figure 5) (Mierke, C.T., Frey, B., Fellner, M., Herrmann, M., Fabry, B. Integrin 5 1 facilitates cancer cell invasion through enhanced contractile forces. *Journal of Cell Science*. **124** (3), 369–383, doi: 10.1242/jcs.071985 (2011).; Ahmadzadeh, H. *et al.* Modeling the two-way feedback between contractility and matrix realignment reveals a nonlinear mode of cancer cell invasion. *Proceedings of the National Academy of Sciences*. **114** (9), E1617–E1626, doi: 10.1073/pnas.1617037114 (2017).). Future experiments will explore changes in focal adhesion dynamics and protease-mediated remodeling for the evaluation of cell migration and invasion." Scale bars represent 100 μm.