Journal of Visualized Experiments

Generation of Mosaic Mammary Organoids by Differential Trypsinization

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60742R1
Full Title:	Generation of Mosaic Mammary Organoids by Differential Trypsinization
Section/Category:	JoVE Developmental Biology
Keywords:	mammary; breast; organoid; luminal; basal; myoepithelial; epithelial; 3D culture
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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.	Santa Cruz, California, United States of America

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Dr. Alisha DSouza
Senior Review Editor
JoVE

October 31, 2019

Dear Dr. DSouza,

Thank you for serving as the review editor for our manuscript entitled, *Generation of Mosaic Mammary Organoids by Differential Trypsinization*. We appreciated the time and attention that six reviewers spent in assessing our manuscript and uploaded you will find a point-by-point response to each comment. We also appreciated the editorial review and comments. Please find our point-by-point response below.

Best Regards,

Lindsay Hinck

Editorial comments:
You will find Editorial comments and Peer-Review comments listed below. Please read this entire email before making edits to your manuscript.
NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Examples:
1) 1.1: Is the animal euthanized/anesthetized? Mention all surgical prep steps and anesthesia method. Mention all tools used and describe how sterility is maintained. What is the animal age, strain, sex?

We updated the first step of our protocol to include more details about how the animals are euthanized.

2) 1.1.: please clarify if the lymph nodes are to be included in the rest of the experiment. "Excise"/"extract" may be better words to use here.

We added detailed images to Figure 1 showing the lymph nodes.

3) 1.2,2.10: What is the composition of Digestion Media/ Maintenance Media?

All of the media recipes are listed in the excel speadsheet with recipes.

• **Protocol Highlight:**
1) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
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Thank you, we made the changes to our highlighted selections.

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We included details about our results in the Representative Results section.

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The discussion was rewritten to match the formatting requirements.

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Yes, we changed the use of Matrigel to ECM.

• **Table of Materials:**Please revise the table of the essential supplies, reagents, and equipment. The table

should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as animal strain, media used, etc.

We have made changes to make the tables more consistent.

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We uploaded the Elsevier License that we obtained through RightsLink and cited Figure 2 as suggested above in the legend.

TITLE:

Generation of Mosaic Mammary Organoids by Differential Trypsinization

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KEYWORDS:

mammary, breast, organoid, luminal, basal, myoepithelial, epithelial, 3D culture

SUMMARY:

The mammary gland is a bilayered structure, comprising outer myoepithelial and inner luminal epithelial cells. Presented is a protocol to prepare organoids using differential trypsinization. This efficient method allows researchers to separately manipulate these two cell types to explore questions concerning their roles in mammary gland form and function.

ABSTRACT:

Organoids offer self-organizing, three-dimensional tissue structures that recapitulate physiological processes in the convenience of a dish. The murine mammary gland is composed of two distinct epithelial cell compartments, serving different functions: the outer, contractile myoepithelial compartment and the inner, secretory luminal compartment. Here, we describe a method by which the cells comprising these compartments are isolated and then combined to investigate their individual lineage contributions to mammary gland morphogenesis and differentiation. The method is simple and efficient and does not require sophisticated separation technologies such as fluorescence activated cell sorting. Instead, we harvest and enzymatically digest the tissue, seed the epithelium on adherent tissue culture dishes, and then use differential trypsinization to separate myoepithelial from luminal cells with ~90% purity. The cells are then plated in an extracellular matrix where they organize into bilayered, three-dimensional (3D) organoids that can be differentiated to produce milk after 10 days in culture. To test the effects of genetic mutations, cells can be harvested from wild type or genetically engineered mouse models, or they can be genetically manipulated prior to 3D culture. This technique can be used to generate mosaic organoids that allow investigation of gene function specifically in the luminal or myoepithelial compartment.

INTRODUCTION:

The mammary gland (MG) is a tree-like, tubular epithelial structure embedded within an adipocyte rich stroma. The bilayered ductal epithelium comprises an outer, basal layer of contractile, myoepithelial cells (MyoECs) and an inner layer of luminal, secretory epithelial cells (LECs), encircling a central lumen[1]. During lactation when the outer MyoECs contract to squeeze milk from the inner alveolar LECs, the mammary gland undergoes numerous changes that are under the control of growth factors (e.g., EGF and FGF) and hormones (e.g. progesterone, insulin, and prolactin). These changes cause the differentiation of specialized structures, alveoli, which synthesize and secrete milk during lactation[1]. The mammary epithelia can be experimentally manipulated using techniques in which either epithelial tissue fragments, cells, or even a single basal cell are transplanted into host mammary fat pads, precleared of endogenous mammary parenchyma, and allowed to grow out to reconstitute an entire, functional epithelial tree[2-5]. Transplantation is a powerful technique, but it is time-consuming and impossible if a mutation results in early embryonic lethality (prior to E14) that prevents the rescue of transplantable mammary anlage. Furthermore, investigators frequently wish to research the roles of the two different compartments, which are derived from lineage-restricted progenitor cells. While Cre-lox technology allows differential genetic manipulation of MyoECs and LECs, this is also a time-consuming and expensive undertaking. Thus, since the 1950s, investigators have used in vitro mammary organoids as a relatively easy and efficient way to address questions concerning mammary tissue structure and function[6,7].

In early protocols describing the isolation and culture of primary mammary epithelial cells, investigators found that a basement membrane matrix (BME), composed of a plasma clot and chicken embryo extract, was required for MG fragments grown on a dish[6]. In the following decades, extracellular matrices (ECMs, collagen, and jellylike protein matrix secreted by Engelbreth-Holm-Swarm murine sarcoma cells) were developed to facilitate 3D culture and better mimic the in vivo environment[7-10]. Culturing cells in 3D matrices revealed by multiple criteria (morphology, gene expression, and hormone responsiveness) that such a microenvironment better models in vivo physiological processes[9-12]. Research using primary murine cells identified key growth factors and morphogens necessary for the extended maintenance and differentiation of organoids[13]. These studies have set the stage for the protocol presented here, and for the culture of human breast cells as 3D organoids, which is now a modern clinical tool, allowing for drug discovery and drug testing on patient samples[14]. Overall, organoid culturing highlights the self-organization capacities of primary cells and their contributions to morphogenesis and differentiation.

Presented here is a protocol to culture murine epithelia that can be differentiated into milk-producing acini. A differential trypsinization technique is used to isolate the MyoECs and LECs that comprise the two distinct MG cell compartments. These separated cell fractions can then be genetically manipulated to overexpress or knockdown gene function. Because lineage-intrinsic, self-organization is an innate property of mammary epithelial cells[15-17], recombining these cell fractions allows researchers to generate bilayered, mosaic organoids. We begin by enzymatically digesting the adipose tissue, and then incubating the mammary fragments on a tissue culture

dish for 24 h (**Figure 1**). The tissue fragments settle on polystyrene dishes as bilayered fragments with their in vivo organization: outer myoepithelial layer surrounding inner luminal layers. This cellular organization allows for the isolation of the outer MyoECs by trypsin-EDTA (0.5%) treatment for 3–6 min followed by a second round of trypsin-EDTA (0.5%) treatment that detaches the remaining inner LECs (**Figure 2**). Thus, these cell types with different trypsin sensitivity are isolated and can subsequently be mixed and plated in ECM (**Figure 3**). The cells undergo self-organization to form bilayered spheres, comprising an outer layer of MyoECs surrounding inner LECs. Lumen formation occurs as the cells grow in a medium containing a cocktail of growth factors (see recipes for Growth Medium)[13]. After 5 days, organoids can be differentiated into milk-producing acini by switching to Alveologenesis Medium (see recipes and **Figure 3F**) and incubated for another 5 days. Alternatively, organoids will continue to expand and branch in Growth Medium for at least 10 days. Organoids can be analyzed using immunofluorescence (**Figure 3D–F**) or released from the ECM using a recovery solution (see **Table of Materials**) and analyzed via other methods (e.g., immunoblot, RT-qPCR).

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Santa Cruz.

1. Day 1: Mammary gland digestion

1.1. Prepare to harvest the MGs from mature female mice 10–14 weeks of age.

1.1.1. Perform the harvesting on an open bench under aseptic conditions.

1.1.2. Sterilize all surgical supplies, cork boards, and pins by autoclaving and soaking in 70% alcohol for 20 min prior to surgery.

1.1.3. Anesthetize animals with sodium pentobarbital (2X anesthetic dose of 0.06 mg/g body weight) delivered via intraperitoneal injection with a 0.5 mL insulin syringe.

1.1.4. Monitor the level of anesthesia by pinching the animal's toes to check for a reflex response and commence the protocol only after the animal is fully anesthetized.

1.1.5. Place the animal on its back, pin its appendages to the corkboard, and wipe down its abdomen and chest with ethanol.

1.2. To harvest the #2, 3, 4, and 5 MGs from one mouse (i.e., 8 MGs, **Figure 1A**), identify the midline between the two hind legs and make a small incision (1 cm) on the abdominal skin with sharp scissors, then extend the cut up to the neck[18].

1.3. Follow by making small cuts laterally towards the legs and arms to allow for the release of the skin using a cotton swab. Pull the skin away and stretch it tight before pinning it down on one

side (**Figure 1B**, step 1)[18]. Remove the MGs by cutting under them, and remove the lymph nodes from the #4 glands (**Figure 1B**, steps 2, 3)[18]. Repeat the procedure on the other side of the body.

1.4. Collect the MG tissue in 50 mL of 4 ˚C Dulbecco's Modified Eagle's Media (DMEM)/Nutrient Mixture F12 (F12) supplemented with 5% fetal bovine serum (FBS) and 1X Antibiotic-Antimycotic (Anti-Anti)[18].

1.5. Chop the glands in a 35 mm dish or on a ceramic plate using a razor blade or tissue chopper. Rotate the plate every five manual chops or every round on the tissue chopper until the tissue pieces can fit through a 1 mL micropipette tip with ease (~0.1 mm/fragment, **Figure 1C**).

1.6. Digest the MGs in Digestion Medium (see **Table 1**) for 14 h in a 6 well low adhesion dish at 37 ˚C, 5% CO_2.

2. Day 2: Isolation of mammary epithelial tissue fragments

2.1. After 14 h of digestion, gently mix by pipetting the digested tissue 10X using a 1 mL micropipette to break down any remaining stroma or adipose tissue, ensuring that neither bubbles nor excess mechanical force are generated.

NOTE**:** If there is incomplete digestion after 14 h, this could be due to the accumulation of sheared DNA. In this case, add 1 µL of 1 mg/mL deoxyribonuclease I (DNase I) per 2 mL of Digestion Medium. Incubate for another 30 min at 37 ˚C, 5% CO_2.

2.2. Collect tissue in a 15 mL tube and rinse the well used for digestion with 2–3 mL of tissue culture grade 1X Dulbecco's Phosphate Buffered Saline (DPBS) free of Ca^{2+} and Mg^{2+}. Centrifuge at 600 x *g* for 10 min.

2.3. Evacuate the supernatant containing the lipid layer and medium, and then resuspend the pellet in 5 mL of DPBS and switch to a new 15 mL tube. Centrifuge at 600 x *g* for 10 min.

2.4. During centrifugation place a 70 µm nylon cell strainer in a 50 mL tube and prewet the strainer using 10 mL of 37 ˚C DMEM/F12.

2.5. Resuspend tissue fragments from protocol step 2.4 using 5 mL of DPBS and pass the suspension through a prewet 70 µm nylon cell strainer to remove stromal cells and single cells (**Figure 1D**).

2.6. Collect the tissue fragments on the cell strainer. Rinse 4X with 10 mL of 37 ˚C DMEM/F12 (**Figure 1D**).

CAUTION: Incomplete rinsing will result in cultures contaminated with non-epithelial cells.

2.7. Release the tissue fragments by holding the strainer tab with gloved fingers, inverting the strainer over a 60 mm tissue culture dish and passing 1 mL aliquots of Maintenance Medium (see **Table 1**) through the bottom of the strainer 4X (**Figure 1E**).

2.8. Check the strainer for tissue fragment remnants, which will be visible by the naked eye, and rinse the strainer 1X more with 1 mL of Maintenance Medium if any fragments are still adhering to the strainer. The rinsed tissue fragments should now be free of stromal cells.

2.9. Quickly examine the 60 mm dish containing the MG fragments from protocol step 2.9 under an inverted microscope (4X or 10X objective, **Figure 1F**). A typical preparation of eight MGs yields ~500 fragments. Look for single cells or fat droplets and whether there are contaminating cells.

NOTE: If there are contaminating cells, repeat the filtration step by collecting the medium and fragments from the 60 mm dish using a 5 mL pipette and filtering the fragment again through a fresh 70 µm strainer, repeating protocol steps 2.6, 2.8–2.10.

2.10. Incubate 24 h at 37 ˚C, 5% CO_2, allowing the tissue fragment to adhere and generate bilayered fragments (**Figure 2A**).

NOTE: If the fragments have not settled by 24 h, continue to incubate until adhered. If the fragments do not adhere well, the separation of the cell compartments will not work. If researchers are concerned about adhesion, the tissue culture plates can be treated to promote fragment attachment (e.g., poly-L-lysine).

3. Day 3: Differential trypsinization of myoepithelial and luminal epithelial cells

3.1. To separate MyoECs from LECs, empty the media from the dish, rinse 1x with 1 mL of DPBS and add 1 mL of fresh trypsin-EDTA (0.5%), and carefully monitor the digestion under an inverted microscope (10X or 20X objective, **Figure 2B**, **Figure C**, **Figure F**). The detachment of the outer MyoEC layer will require 3–6 min, depending on trypsin-EDTA (0.5%) strength.

NOTE: Please see **Figure 2** for representative images showing this process. Under brightfield illumination, the MyoECs appear rounded and have a brighter appearance in comparison to the LECs, which remain adhered in the center and appear darker.

3.2. Collect the MyoEC fraction in a 15 mL tube containing 2 mL 10% FBS/DPBS. Without disturbing the LECs, gently rinse the 60 mm dish with 2 mL of DPBS and then dispose of the DPBS (**Figure 2H–I**).

NOTE: The usual recovery for MyoECs is within a range of (3.5×10^6–1.5×10^6) depending on the size of the MGs.

3.3. To remove the LEC fraction, add 1 mL of trypsin-EDTA (0.5%) to the dish again and incubate 7–15 min, monitoring carefully to prevent overdigestion. Quench the trypsin-EDTA (0.5%) on the dish with 2 mL of 10% FBS/DPBS. Collect the LEC fraction in a new 15 mL tube.

NOTE: The usual recovery for LECs is within a range (2×10^6–4.2×10^6) depending on the size of the MGs. Routinely, the purity of both fractions as assayed by immunohistochemistry is ~90%[19] (**Figure 2E**).

3.4. Centrifuge each fraction for 5 min at 300 x *g* to remove the trypsin-EDTA (0.5%). Resuspend the pellet in 250 µL of Maintenance Medium and count each cell population using a hemocytometer or automated cell counter. Place the cells on ice while counting.

NOTE: If genetic manipulation of the cell fractions is desired, primary cells can be grown on low adhesion dishes and infected with lentivirus[20].

4. Day 3: Combining and embedding cell fractions in an extracellular matrix

NOTE: Once the MyoEC and LEC fractions have been collected and counted, they can be combined. The typical MyoEC/LEC ratio is 1:3 (**Figure 3A**)[19]. Different studies can be performed. For example, to perform mosaic studies, fractions can be generated from wild type (WT) and mutant (Mut) mice and combined (MyoEC/LEC: WT/WT; WT/Mut; Mut/WT; Mut/Mut)[21], or fractions can be combined using different ratios of MyoECs/LECs[19].

4.1. Based on cell counts, calculate the number of wells (8 well chamber, see **Table of Materials**) that need to be prepared for 12,000 cells/well (e.g. 3,000 MyoECs:9,000 LECs).

4.2. Establish the base layer for 3D culture by adding 90 µL of 50% ECM (50% ECM/50% DMEM/F12, without phenol red – see the **Table of Materials**) to each well. Ensure there are no bubbles and the wells are coated evenly. To solidify the base layer, incubate the slides at 37 ˚C, 5% CO_2 for 30 min.

Note: The ECM needs to stay ice-cold until this step, otherwise it will polymerize prematurely and lead to uneven base coating and polymerization. Using a base ECM enhances organoid growth in a single plane, which aids image capture. This also obtains faster organoid growth and uses less ECM[22].

4.3. During polymerization, prepare the cell mixes. Pellet the MyoEC and LEC fractions at 300 x *g* for 5 min and resuspend each cell fraction in 10% ECM/90% Growth Medium so each well has 100 µL (see **Table 1** for how to make Growth Medium).

Note: For ease of preparation, replicate wells using the same cell mixes. These can be combined and prepared in one tube (e.g., four wells of the same cell mix can be prepared in 400 µL of 10% ECM/90% Growth Medium).

4.4. Add 100 µL of each cell mix in 10% ECM/90% Growth Medium to each well and allow organoids to settle for 20 min at 37 ˚C, 5% CO_2.

4.5. Once the cells have settled, gently add 100 µL of Growth Medium by gently pipetting down the chamber wall of each well. Incubate the slides at 37 ˚C, 5% CO_2 (see **Figure 3A** for the total composition of each well).

NOTE: The Rho Kinase inhibitor, R-spondin, and Nrg1 are factors that have been identified as important for the long-term culture of organoids grown from both primary murine mammary cells and human breast cancer cells[13,14]. In addition, the stem cell factors B27 and N2 extend the time that organoids can be cultured.

4.6. Image cells every 24 h to track their growth and gently renew the Growth Medium every 2–3 days using 100 µL/well (**Figure 3B–E**).

NOTE: Use extreme care when renewing the medium. Tilt the chamber slides to collect the medium at one corner of the wells. Remove ≤100 µL of the medium and replenish with care to leave the ECM layer undisturbed.

4.7. If researchers are interested in investigating lactation/alveologenesis, switch to Alveologenesis Medium (see **Table 1**) on day 5 and continue to renew the medium every 2–3 days until day 10 (**Figure 3F**) or beyond by passaging using recovery solution (see the **Table of Materials**)[13].

NOTE: At this point, organoids can be isolated from the ECM by incubating at 4 ˚C in 400 µL of 4 ˚C recovery solution (see **Table of Materials** for protocol and reagent info).

5. Day 5 or 10: Fixing and immunostaining organoids

5.1. Remove the medium carefully by gently pipetting off the media (a bulb pipette works best). Rinse each well using 200 µL of 1x Dulbecco's Phosphate Buffered Saline (DPBS, see recipes).

5.2. Fix the organoids using cold (4 ˚C) 4% (w/v) paraformaldehyde (PFA, see recipes) for 10 min at room temperature.

CAUTION: PFA is hazardous. Wear personal protective equipment (lab coat, gloves, and safety glasses). This step should be performed inside a fume hood.

NOTE: The ECM is dissolved by the PFA treatment. Incomplete ECM removal can lead to background staining when the organoids are analyzed by immunofluorescence.

5.3. Remove the 4% PFA and add 200 µL of 0.2% (w/v) glycine/DPBS (see **Table 1**) to each well. Incubate the slides at room temperature for 30 min or 4 ˚C overnight on a rocking surface set to a slow setting.

NOTE: The organoids can be stored for 1–3 days in DPBS at 4 ˚C prior to the next step.

5.4. Permeabilize the organoids using DPBS + 0.25% Triton X-100 (PBST, see **Table 1**) for 10 min at room temperature.

5.5. Block the organoids using 5% donkey serum (DS) in DPBS for 1 h on a rocking surface.

NOTE: This step can be performed overnight at 4 ˚C on a rocking surface.

5.6. Prepare primary antibodies in 1% DS/DPBS. Use 125–200 µL for each well. Perform immunostaining by incubating the organoids in primary antibody overnight at 4 ˚C on a rocking surface.

6. Day 11: Complete immunofluorescence

6.1. Wash each well 2X with 200 µL PBST for 5 min. Add secondary antibody in 1% DS/DPBS using 125–200 µL for each well. Incubate at room temperature on a rocking surface for 45 min.

6.2. Wash each well 2X using 200 µL DPBS per well.

6.3. Stain the nuclei using Hoechst DNA dye in DPBS (1:2,000 in DPBS) for 5 min at room temperature.

6.4. Remove all liquid left on the well by gently suctioning with a vacuum.

6.5. Carefully remove the chambers and gasket, place one drop (~30 µL) of mounting media (see **Table of Materials**) on each well and coverslip, taking care to remove bubbles. Allow the slide to dry in a dark space for 1–2 days. Seal with clear nail polish. Image the organoids on a confocal microscope (**Figure 3E**–**F**).

REPRESENTATIVE RESULTS:

The protocol presented here describes a method for investigating specific lineage contributions of mammary epithelial cells by making use of mosaic organoids. To obtain primary murine cells for organoids, the mammary gland epithelium must first be isolated from the surrounding adipocyte rich stroma (**Figure 1**). This process is described briefly here and is also described in a previously published study[18]. To obtain enough cells, it is recommended that #2, 3, 4, and 5 MGs be removed (**Figure 1A**). An important step key to isolating a pure population of epithelial cells is removal of the lymph nodes from the #4 MGs, which are rich in immune cells that will contaminate the preparation (**Figure 1A**, **B**). The MGs were minced to generate fragments ~0.1 mm in size (**Figure 1B**). The tissue fragments were then enzymatically digested, a process occurring in the presence of collagenase, to release epithelia from stroma, and in the absence of trypsin, to prevent the digestion of proteins such as cadherins that maintain cell-cell contacts. The digested tissue was then centrifuged to remove lipids and filtered through a cell strainer and

washed (**Figure 1C**). Epithelial fragments, adhering to the strainer, were released by inverting the filter and washing the membrane, which transferred the epithelial fragments onto a polystyrene dish (**Figure 1D**). These fragments appeared as small, branched structures (**Figure 1E**).

The purified epithelial fragments were incubated for 24 h. They settled down onto the dish and adhered, forming flat, pancake-like structures with an outer layer of MyoECs encircling inner LECs (**Figure 2A**–**B**). **Figure 2C** shows the edge of such a pancake-like structure from a wild type animal. Trypsin treatment differentially detached the MyoECs, which detached first and appeared as bright, rounded cells that encircled the core of remaining cuboidal LECs (**Figure 2C**, **2F**). The detachment of the MyoECs was carefully monitored using brightfield microscopy and occurred within 3–6 min. Once the MECs were collected, LECs were subsequently detached through a second, longer trypsin treatment of 7–15 min. The time required for cell detachment depends on the trypsin concentration and freshness. The overall purity of the two cell compartments was ~90%, as assayed by counting cells that were KRT14-positive and E-Cadherin (CDH1)-negative in the MyoEC fraction and cells that were KRT14-negative and E-Cadherin-positive in the LEC fraction (**Figure 2D**–**E**)[19]. We discovered that some of the MyoECs were removed from the top of the pancake-like structure as well as from the outer edges. This was observed by using tissue fragments collected from mice labeled with an inducible, fluorescent basal marker (Cytokeratin 14 (KRT14)-CreERT1; R26RYFP/+) and injected with 75 mg/kg tamoxifen 5 days prior to harvest. In **Figure 2F**, G the detachment of MyoECs from around the edges of the pancake structure is readily apparent. This occurred within the first 2 min of trypsin treatment (**Figure 2F**). In addition, YFP-KRT14-positive cells were observed on top of the structure, where they rounded up after trypsin treatment and were removed by the rinse/collection step (**Figure 2G**). The unlabeled core of LECs (**Figure 2H**), which contained few or no YFP-KRT14-positive cells, (**Figure 2I**) subsequently detached in the second round of trypsin treatment.

The MyoEC and LEC fractions were collected, combined, and embedded into 10% ECM plated onto a 50% ECM base. This allowed for better optical resolution of the organoids that grew primarily along the base layer (**Figure 3A**). After 24 h, the cells assembled into aggregated structures that largely lacked a lumen (**Figure 3B**). After 48 h, nascent organoids formed as the central lumens hollowed and appeared as a lighter internal space (**Figure 3C**). After 10 days, the organoids were large, branched structures with well-developed lumens. Mosaic organoids generated from MyoECs harvested from wild type mice and LECs harvested from ACTb-EGFP mice were fixed in situ, immunostained with an antibody against the basal marker alpha-smooth muscle actin (SMA), and stained with the Hoechst DNA stain to show the nuclei. In the figures, the top and section views show different sets of images collected as a Z-stack and reconstructed into a 3D view (**Figure 3D**). The top view reveals the branched morphology of the organoids (**Figure 3E'**). The section view shows the bilayered epithelial structure and open lumen of these organoids (**Figure 3E''**). These organoids can also be differentiated at Day 5 using Alveologenesis Medium and incubated for an additional 5 days (**Figure 3F**). The organoids grew larger, had more branches, and contained milk. Differentiated organoids were generated as described above and immunostained with an antibody directed against the milk marker, whey acidic protein (WAP, **Figure 3F**). WAP is a soluble protein secreted into milk. Much of this liquid was lost when the cells were fixed and immunostained in situ. Therefore, in the top and section views, WAP staining is

visible intracellularly in secreting cells and extracellularly in milk that was trapped at the cell surface during fixation (**Figure 3F**), although in section view a small organoid appears to contain liquid milk (**Figure 3F''** boxed overlay).

FIGURE AND TABLE LEGENDS:

Figure 1: Mammary fragment isolation. (**A**) Labeled schematic of a mouse's 5 MGs with unlabeled, contralateral paired MGs. (**B**) Images of mouse MGs with the #4 MG boxed and magnified to show how to identify the lymph node for removal. (**C**) Image of chopped MGs in a 6 well low adhesion plate with a ruler showing the size of the tissue pieces (~0.1 mm each). (**D**–**E**) Schematic illustrating protocol steps 2.7–2.9. (**D**) MG fragments were filtered through a 70 µm strainer and rinsed 4X. (**E**) The strainer was then inverted over a 60 mm polystyrene tissue culture dish and fragments were released into the dish. (**F**) Image showing the filtered tissue fragments collected on a 60 mm dish that are free of stroma. The arrows point to the smallest fragments that are collected on the 70 µm strainer. Scale bar = 100 µm.

Figure 2: Differential trypsinization. (**A**) Brightfield image showing a tissue fragment adhered on a polystyrene dish, forming a pancake-like structure. (**B**–**C**) The first differential trypsinization step detached MyoECs that are clearly visible as bright, rounded cells after 3 min. (**D**) Immunofluorescent images of MyoECs (bottom) and LECs (top) using the Cytokeratin 14 (KRT14) cell marker for MyoECs, and E-Cadherin (CDH1) cell marker for LECs. (**E**) The expression of KRT14 and CDH1 was used to quantify the yield and purity of the differentially trypsinized cell fractions. (**F**–**I).** Representative phase-contrast and fluorescence (YFP) images of tissue fragments from Cytokeratin 14 (KRT14)-CreERT1; R26RYFP/+ MGs. Mice were injected with 75 mg/kg tamoxifen 5 days prior to harvest. (**F**) Detaching MyoECs (arrows) during the initial trypsin-EDTA (0.5%) 2 min after incubation. (**G**) A sprinkling of KRT14-YFP-MyoECs (arrows) on top of a pancake of unlabeled LECs. (**H**) Brightfield image of LECs after initial trypsinization and MyoEC detachment. (**I**) After MyoEC detachment, KRT14-YFP-MyoECs are no longer visible as shown by the absence of YFP expression. Scale bars = 30 µm (A, brightfield) 100 µm (F, brightfield), 50 µm (G, fluorescence), 100 µm (H, I). Panels A–E of this figure are modified from Macias et al.[19]

Figure 3: Three-dimensional organoid culture. (**A**) Schematic representation of single cells embedded in 10% ECM/90% Growth Medium and grown on a 50% ECM/50% DMEM base layer (protocol step 4.5). (**B**–**C**) Illustrations and phase-contrast images showing the rapid self-organizing capacities of mammary organoids generated from differentially trypsinized and recombined MyoECs and LECs at 24 h (**B**) and 48 h (**C**). Images collected using a digital widefield microscope (**D**) Schematic representation illustrating the top (left) or section (right) views used in E–F to show immunostained organoids. (**E**) Schematic representation of a single well of an 8 well chamber slide containing mammary organoids grown for 5–10 days in Growth Medium. (**E'-E"**) Top view (**E'**) and section view (**E"**) of immunostained organoids at day 10 of growth. MyoECs are marked with smooth actin muscle (SMA) in pseudocolor magenta. The LECs are from ACTb-EGFP mice and are shown in pseudocolor green. Nuclei were stained with Hoechst dye. (**F**) Schematic representation of a single well of an 8 well chamber slide containing mammary organoids grown for 5 days in Growth Medium and 5 days in Alveologenesis Medium. (**F'-F"**). Top

view (**F'**) and section view (**F"**) of immunostained organoids at day 10 of growth. The MyoECs are unmarked. The LECs from ACTb-EGFP mice are shown in pseudocolor green. The milk protein, whey acidic protein (WAP), is shown in pseudocolor yellow in the LECs and coating the inside of the organoids' lumens. Nuclei were stained with Hoechst dye. Images collected using a spinning disk confocal microscope and reconstructed in 3D using Imaris (E', E') or bottom section ~30 slices (F', F"). Scale bars = 100 µm (C), 20 µm (E), 40 µm (F).

DISCUSSION:

Here, a method is presented detailing how researchers can generate 3D organoid cultures using primary MG cells. The difference between this and other protocols is that we detail a method to separate the two, distinct MG cell compartments: the outer basal MyoECs and inner LECs. Our method employs a two-step trypsin-EDTA (0.5%) treatment that we call differential trypsinization[19]. This procedure allows researchers to isolate basal and luminal cells without using sophisticated flow cytometry and thus can be used for studying MGs harvested from a wide variety of mammalian species that may not have the well-characterized biomarkers required for FACS. The ability to segregate the two cell subpopulations enables researchers to genetically modify the isolated cells independently or recombine cells from animals harboring genetic mutations or labels, and thus generate mosaic organoids in 3D culture. A limitation of the current protocol is that the stromal compartment is not included in the culturing conditions. However, new methods are being developed to coculture stromal components with organoids generated from either primary cells or cell lines to better recapitulate in vivo ECM[23-26], and these methods may be adapted to this protocol. In addition, it is important to note that while this protocol achieves a great enrichment of the MyoEC and LEC fractions (~90% purification), the fractions do not represent pure cell lineages.

The success of this protocol relies on a number of key steps. First, it is important to gently but thoroughly digest the MG tissue. Overdigestion of the tissue will lead to cell death and lower recovery of epithelial cells. Incomplete digestion will result in stromal and adipose cell contamination, which will interfere with later analyses (e.g., immunofluorescence, protein analysis, and mRNA measurements). Second, it is important to thoroughly rinse the MG tissue to remove contaminating cells in protocol step 2.8. In protocol step 2.9, the MG tissue fragments are released into a 60 mm dish. Researchers should monitor the released fragments immediately, before they adhere to the dish. If fat droplets or single cells are observed, protocol steps 2.6 and 2.8–2.11 must be repeated. To do this, the medium and tissue fragments are collected from the dish, placed into a new 70 µm strainer, washed 4X with 37 ˚C DMEM/F12 and then released into a new 60 mm dish. Third, it is essential to watch the first trypsin-EDTA (0.5%) incubation closely because the MyoECs can detach within the first 3 min, but they can also adhere for up to 6 min. There have been instances when the trypsin-EDTA (0.5%) was suboptimal, and incubation proceeded for 10 min with successful purification of MyoECs. However, >10 min of trypsinization resulted in the simultaneous collection of MyoECs and LECs. It is also important that the dish remain undisturbed during the first incubation. Otherwise, contamination of the MyoEC fraction with LECs can occur. The reverse is also true; if MyoECs are not completely detached from the dish, they will contaminate the LEC fraction. If researchers are using reporter mice that label MyoECs or LECs exclusively, it is easier to visualize the separation under a fluorescence

microscope (**Figure 2F–I**). Finally, if researchers plan on fixing organoids for immunofluorescence analyses, the pH (7.4) and temperature (4 ˚C) of the 4% PFA is important for successful dissociation of the ECM. If the organoids are collected for other analyses (e.g., protein and mRNA measurements), it is important that the recovery solution be at 4 ˚C. If the ECM is not dissolving, incubation with the recovery solution can be extended by 10 min (i.e., 30 min total incubation). However, longer incubation periods will lead to loss of 3D structure and cell death. The recovery protocol (listed in the **Table of Materials**) specifies the use of wide-bore tips. This is important for maintaining the 3D structure of the organoids as well as the integrity of the cells.

In addition to these four key steps, there are two factors that influence the success of the protocol. First, organoid growth can be limited by genetic mutations that reduce cell proliferation and therefore reduce organoid growth in ECM. If only a few organoids are obtained, the subsequent fixation step frequently results in their loss. To address this, the number of cells embedded within the ECM should be increased while retaining the ratio of MyoECs:LECs (protocol steps 4.1–4.2). Second, once the cells are transferred into an ECM it is important to watch their growth daily and be vigilant about media renewal (every 2–3 days). This protocol specifies phenol red free reagents for better visualization, but the same success and growth is achieved using phenol red positive reagents. The days when medium renewal occurs prior to fixation (protocol step 4.6) should be performed with extreme care to reduce cell loss. The 10% ECM top layer is delicate; therefore washes or medium renewal should be performed by pipetting fluid down the chamber walls to minimize mechanical disturbances.

Differentiation of the organoids into milk-producing acini requires treatment with differentiation supplements: hydrocortisone or dexamethasone, insulin, and prolactin. In this protocol, dexamethasone is recommended. In addition, while prolactin is commercially available, the prolactin used in this protocol was obtained from the National Hormone and Peptide Program. Again, it is very important to leave the organoids undisturbed when changing the Alveologenesis Medium. Differentiation requires a minimum of 5 days. This can be extended another 3–5 days, but the base layer of ECM degrades after 10–12 days. Differentiated organoids are filled with milk and their lumens appear darker.

This is an efficient technique that can be used to address compartment-specific, lineage contributions to mammary epithelial morphogenesis and differentiation. With this technique, researchers can generate mosaic organoids comprising differentially genetically manipulated MyoECs and LECs[21], or MyoECs and LECs obtained from mice harboring different genetic mutations. This allows researchers to better understand the contributions of lineage-specific cell compartments to organ morphogenesis and the acquisition of specialized functions such as milk production.

DISCLOSURES:

The authors have nothing to disclose.

ACKNOWLEDGMENTS:

We thank Ben Abrams for technical assistance and core support from the University of California, Santa Cruz (UCSC) Institute for the Biology of Stem Cells (IBSC). We thank Susan Strome and Bill Saxton for the use of their Solamere Spinning Disk Confocal Microscope. This work was supported in part by grants to UCSC from the Howard Hughes Medical Institute through the James H. Gilliam Fellowships for Advanced Study program (S.R.), from the NIH (NIH GM058903) for the initiative for maximizing student development (H.M.) and from the National Science Foundation for a graduate research fellowship (O.C. DGE 1339067) and by a grant (A18-0370) from the UC-Cancer Research Coordinating Committee (LH).

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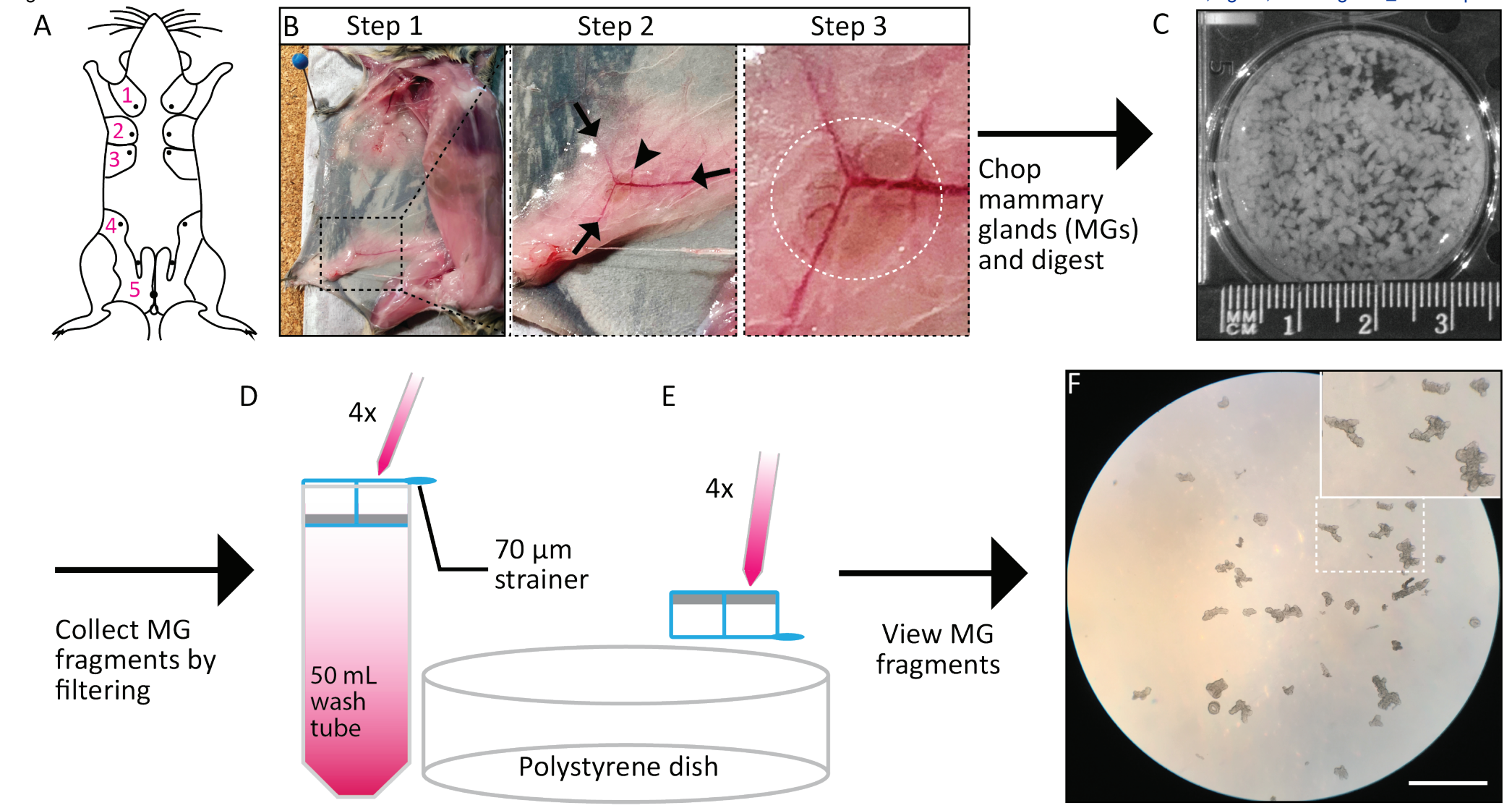
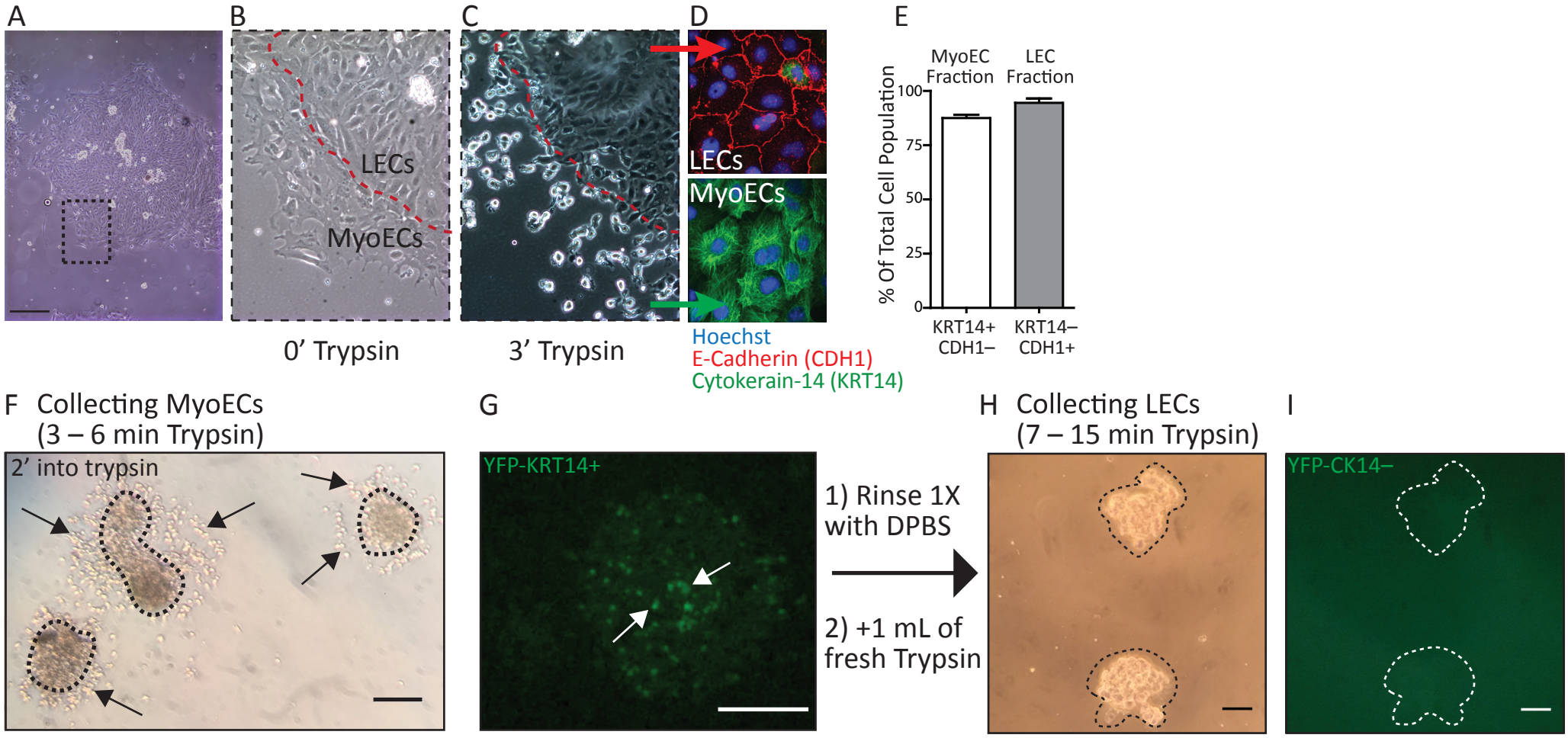
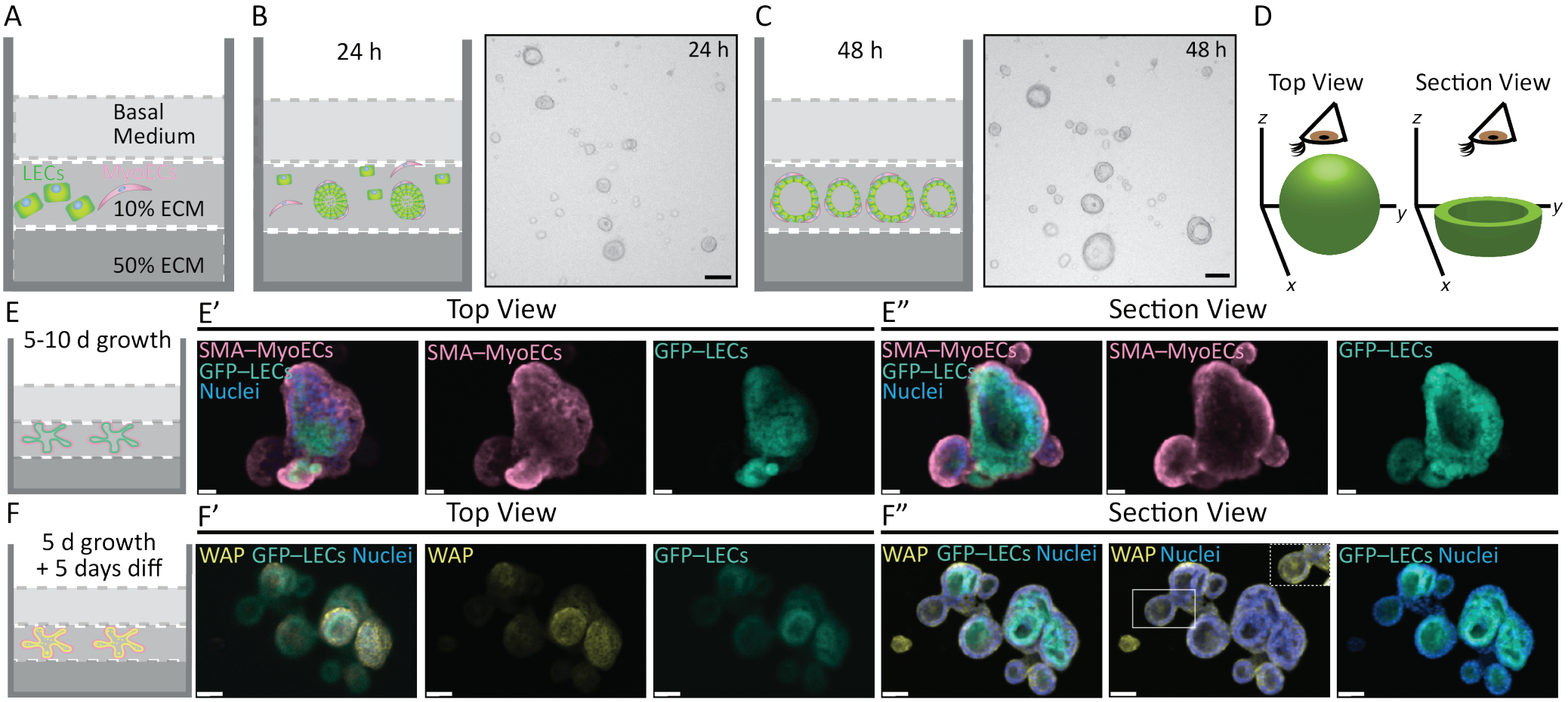
Figure



Figure



Figure



Table

10 mL	**Digestion Medium**	
Amount	*Reagent*	*Notes*
9.45 mL	DMEM/F12	
100 µL	Antibiotic-Antimycotic (100X)	
0.04 g	Class 3 Collagenase	
0.04 g	Class 2 Dispase	
50 µL	Gentamicin	Final Concentration: 500 µg
2.5 mL	Fetal Bovine Serum	Final concentration: 5% (v/v)
Pass through 0.22µm filter		
50 mL	**Maintenance Medium**	
Amount	*Reagent*	
49.47 mL	DMEM/F12	
0.5 mL	Antibiotic-Antimycotic (100X)	
2.5 mL	Fetal Bovine Serum	
25 µL	Insulin	
5 µL	EGF	
10 mL	**Growth Medium**	
Amount	*Reagent*	
9.6455 mL	DMEM/F12, no phenol red	
100 µL	N-2 Supplement (100x)	
200 µL	B27 supplement without vitamin A (50x)	
10 µL	Nrg1	
42.5 µL	R-spondin	
1 µL	Rho inhibitor Y-27632	
1 µL	EGF	
10 mL	**Alveologenesis Medium**	
Amount	*Reagent*	
9.6355 mL	DMEM/F12, no phenol red	
100 µL	N-2 Supplement (100x)	
200 µL	B27 supplement without vitamin A (50x)	
10 µL	Nrg1	
42.5 µL	R-spondin	
1 µL	Rho inhibitor Y-27632	
5 µL	Ovine Pituitary Prolactin	
1 µL	Dexamethasone	
5 µL	Insulin	

1 L	**10X DPBS**
Amount	*Reagent*
80 g	NaCl
2 g	KCl
14.4 g	NaH2PO4
2.4 g	KH2PO4
1 L	di H2O

Fill to 800 mL before adding dry reagents and dissolve. Fill volume to 1 L. Adjust pH to 7.4. A

1 L	**1X DPBS**
Amount	*Reagent*
100 mL	10X PBS
900 mL	di H2O
1 L	**PBST**
Amount	Reagent
100 mL	10X PBS
2.5 mL	Triton X-100
250 mL	**4% Paraformaldehyde**
Amount	*Reagent*
10 g	Paraformaldehyde
200 mL	di H20
25 mL	10X DPBS
50 µL	10 N Sodium Hydroxide

Pass through a 0.45 µm filter to sterilize and assure pH is 7.4

10 mL	**1 % Donkey Serum**
Amount	*Reagent*
100 µL	Sterile Filtered Donkey Serum
9.9 mL	1X DPBS
10 mL	**0.2% Glycine**
Amount	*Reagent*
0.02 g	Glycine
10 mL	1X DPBS

Notes
Final concentration: 5% (v/v)
Final concentration: 250 µg
Final concentration: 500 ng
Notes
Stock: 100µg/mL
Stock: 10 µg/mL
Stock: 10 µM
Stock: 0.1 µg/µL
Notes
Stock: 100µg/mL
Stock: 10 µg/mL
Stock: 10 µM
Final concentration: 1 µg/mL
Final concentration: 5 µg/mL
Final concentration: 5 µg/mL

Notes

utoclave to sterilize.

Notes

Notes

Notes

water must be at 60 ˚C

Notes

Notes

Name of Material/ Equipment
15 ml High-Clarity Polypropylene Conical Tube (BD Falcon)
24 well ultra–low attachment plate (Corning)
35 mm TC-treated Easy-Grip Style Cell Culture Dish (BD Falcon)
50 ml High-Clarity polypropylene conical tube (BD Falcon)
60 mm TC-treated Easy-Grip Style Cell Culture Dish (BD Falcon)
70µM nylon cell strainer (Corning)
Antibiotic-Antimycotic (100X)
B27 supplement without vitamin A (50x)
B6 ACTb-EGFP mice
BD Insulin syringe 0.5 mL
Class 2 Dispase (Roche)
Class 3 Collagenase
Corning Cell Recovery solution
Corning Costar Ultra-Low Attachment 6-well
Dexamethasone
DMEM/F12, no phenol red
DNase (Deoxyribonuclease I)
Donkey anti-Goat 647
Donkey anti-Mouse 647
Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12)
Dulbecco's phosphate-buffered saline (DPBS)
EGF
Fetal Bovine Serum
Fluoromount-G (Southern Biotech)
Gentamicin
Glycine
Goat anti-WAP
Hoechst 33342
Insulin
KCl

KH2PO4
KRT14–CreERtam
Matrigel Growth Factor Reduced (GFR); Phenol Red-Free; 10 mL
MillexGV Filter Unit 0.22µm
Millicell EZ SLIDE 8-well glass, sterile
Mouse anti-SMA
N-2 Supplement (100x)
NaCl
NaH2PO4
Nrg1
Ovine Pituitary Prolactin
Paraformaldahyde
Pentobarbital
R26R-EYFP
Rho inhibitor Y-27632
R-spondin
Sodium Hydroxide
Sterile Filtered Donkey Serum
Sterile Filtered Donkey Serum
Triton X-100
Trypsin EDTA 0.05%

Company	Catalog Number
Fisher Scientific	352096
Fisher Scientific	CLS3473-24EA
Fisher Scientific	353001
Fisher Scientific	352098
Fisher Scientific	353004
Fisher Scientific	08-771-2
Thermo Fisher Scientific	15240062
Thermo Fisher Scientific	12587010
The Jackson Laboratory	003291
Thermo Fisher Scientific	14-826-79
Millipore Sigma	4942078001
Worthington Biochemical	LS004206
Fisher Scientific	354253
Fisher Scientific	CLS3471
Millipore Sigma	D4902-25MG
Thermo Fisher Scientific	11039-021
Worthington Biochemical	LS002007
Thermo Fisher Scientific	A21447
Jackson ImmunoResearch	715-606-150
Thermo Fisher Scientific	11330-057
Thermo Fisher Scientific	14190-250
Fisher Scientific	AF-100-15-100ug
VWR	97068–085
Fisher Scientific	0100-01
Thermo Fisher Scientific	15710064
Fisher Scientific	BP381-5
Santa Cruz Biotech	SC-14832
AnaSpec	AS-83218
Millipore Sigma	I6634-100mg
Fisher Scientific	P217-500

Fisher Scientific	P285-500
The Jackson Laboratory	5107
Fisher Scientific	CB-40230C
Millipore Sigma	SLGV033RS
Millipore Sigma	PEZGS0816
Millipore Sigma	A2547
Thermo Fisher Scientific	17502048
Fisher Scientific	S671-3
Fisher Scientific	S468-500
R&D	5898-NR-050
National Hormone and Peptide Program	
Millipore Sigma	PX0055-3
Millipore Sigma	P3761
The Jackson Laboratory	6148
Tocris	1254
Peprotech	120-38
Fisher Scientific	S318-500
Equitech-Bio Inc.	SD30-0500
Equitech-Bio Inc.	SD30-0500
Millipore Sigma	x100-500ML
Thermo Fisher Scientific	25300-062

Comments/Description
Pen/Strep also works
Follow the guidelines for use – Extraction of Three-Dimensional Structures from Corning Matrigel Matrix
Use at 1:500, Lot: 1608641, stock 2 mg/mL, RRID:AB_2535864
Use at 1:1000, Lot: 140554, stock 1.4 mg/mL
Without Mg2+/Ca2+
100% US Origin, premium grade, Lot: 059B18
Referred to as mounting media in text
Use at 1:250, Lot: J1011, stock 200 µg/mL, RRID:AB_677601
Use 1:2000, stock is 20mM

Lot: 8204010, stock concentration 8.9 mg/mL
These chamber slides are great for gasket removal but other brands can work well (e.g. Lab Tek II).
Use at 1:500, Lot: 128M4881V, stock 5.2 mg/mL, RRID:AB_476701
Purchased from Dr. Parlow at Harbor-UCLA Research and Education Institute
Laboratory grade

We want to thank the Reviewers for their thoughtful comments, which we have used as detailed below to strengthen our manuscript.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
As the authors clearly spell out, the 3 dimensional structures of organoids have been proven to be a biologically relevant way to study mammary epithelial function and crosstalk between epithelial cell subpopulations in vitro. Moreover, the easy application of organoid cultures to the study of human normal tissues and cancer patient samples has emphasized the utility of this culture system. As noted by the authors, many important biological questions can be addressed by genetic manipulation of one or the other epithelial compartment. While FACS based on surface markers is the gold standard for separating these populations, it is not only expensive, but also damages the sorted cells, reducing viability and further perturbing the biology.The straightforward procedure described herein very neatly addresses this need. The title, abstract, and methods are all very clearly written. This is an important contribution.

Major Concerns:
none

Minor Concerns:
1. In the 6th line from the end of the Introduction ("Cells with different genotypes are isolated, mixed together…"), "genotypes" is probably not what is intended here—rather e.g., "sensitivity to trypsization"?

Yes, thank you we changed that sentence to say different trypsin sensitivity.
"Thus, these cell types with differential trypsin sensitivity are isolated, and can subsequently be mixed together and plated in ECM (Figure 3)."

2. "MEC" is used here as an abbreviation for myoepithelial cells. However, many investigators use it more generally used as an abbreviation for "mammary epithelial cells". Could I suggest an alternative abbreviation, such as MyoEC?

Thank you for the suggestion, we have changed all MEC abbreviations to MyoEC.

3. on day 1.2: do the investigators want to specify the approximate size of the fragments to be generated?

We included an image in Figure 1 with a ruler to show fragment size, which is about 0.1mm/fragment. This size fragments are small enough to fit through a p1000 micropipette tip.

Reviewer #2:
Manuscript Summary:
This protocol describes a method for separating myoepithelial epithelial cells (MECs) from

luminal epithelial cells (LECs) from mammary tissue fragments using differential trypsinization. Overall, the paper fits the scope of the journal and provides a rational approach to separating MECs from LECs.

Major Concerns:

1. The impact of this novel technique could be expanded if desired. For example, FACS is limited to well-studied mammals with known biomarkers. This technique could be applied in less studied mammals with unknown biomarkers.

Yes, indeed. Thank you for your suggestion that addresses another significant point to our method. We included this application in our Discussion.

2. It would be helpful to point out the benefit of plating varied concentrations of Matrigel in layers, instead of using only one concentration of Matrigel, as has been described by many other groups.

We adopted the use of varying Matrigel concentration from the on top method used for cell lines {Lee, 2007 #804}. The benefit of this method is that it the organoids settle on the base layer such that they are in a single focal plane and then stabilized by the top layer. We find that this aids the capture of images. Further, this approach decreases the amount of Matrigel required per prep, reducing costs. We also find that it speeds the growth of the organoids. We included this information in the manuscript at Step 4.2:

"Note: The ECM needs to stay ice-cold until this step otherwise it will polymerize prematurely and lead to uneven base coating and polymerization. Using a base ECM enhances organoid growth in a single plane, which aids image capture. We also obtain faster organoid growth and use less ECM (Lee et al. 2007 Nature Methods)."

3. Matrigel comes in different protein concentrations depending on the batch and it is advised by the product's producer to dilute their product to a specific protein concentration. That being said, it appears that diluting in fold changes (e.g. 1:1 Matrigel:DMEM 1x) is common. It would be good to report the protein concentration of the Matrigel batch(es) used in the study, in case others wish to dilute their Matrigel with more stringency.

We agree and have included the information in the notes portion of the materials list.

4. Figure 3B is a schematic that claims to show how organoids rapidly self-organize by 24-48 hours. The argument that organoids rapidly self-organize at 24-48 hours would be better supported with a picture of immunostained organoids at 24-48 rather than a drawing. In the final publication, a video or taking hourly/daily transitional images of the developing organoids would nicely demonstrate how these cells arrange in a 3D-scaffold.

We updated this figure based on these comments. In the revised Figure 3 we show brightfield images representing the 24 h and 48 h time points.

Minor Concerns:
1. Clarify in Figure 3F whether the milk protein staining is seen in the luminal cells or in the lumen. Based on the Figure, the milk protein doesn't seem to be present in the lumen itself but in the surrounding luminal cells.

We agree with your assessment. WAP is soluble protein, secreted into milk and the fact that the organoids are not embedded in paraffin, but instead fixed and immunostained in situ, results in the loss of the milk and therefore WAP. Consequently, what is observed is intracellular WAP in secreting cells and extracellular WAP in milk that is trapped at the cell surfaces at fixation. In the revised manuscript, we have made this clear in the text and we show WAP that has been trapped in the lumen of one small organoid overlayered in the top, right corner of Figure 3F.

In the revised manuscript:

Differentiated organoids were generated as described above and immunostained with an antibody directed against the milk marker, whey acidic protein (WAP) (Figure 3F). WAP is a soluble protein, secreted into milk, and much of this liquid is lost when the cells are fixed and immunostained in situ. Therefore, in the top and section views, WAP staining is visible intracellularly in secreting cells and extracellularly in milk that is trapped at the cell surface during fixation, although in section view a small organoid appears to contain liquid milk (Figure 3D, F'' boxed overlay).

2. There is a repeated instruction on page #4: at the end of 2.4 it says to resuspend the pellet in 5 mL DPBS, and in 2.7 the first sentence repeats this instruction when it is not needed.

We addressed the language between these steps to clearly distinguish the separate resuspension steps. In short, the second resuspension in protocol step 2.7 occurs after the centrifugation that occurs in step 2.6.

In the revised manuscript:

*"2.6. **During the centrifugation time p**lace a 70 µm nylon cell strainer in a 50 mL tube and pre-wet the strainer using 10 mL of 37 ˚C DMEM/F12.*
*2.7. Resuspend tissue fragments **from protocol step 2.5** using 5 mL DPBS and pass the suspension through a 70 µm nylon cell strainer to remove stromal cells and single cells."*

Reviewer #3:
Manuscript Summary:
The paper focuses on a protocol to separate the epithelial compartment from the stroma, further separating the luminal and myoepithelial mammary epithelial cells, then recombining them to form organoids in vitro. The cell separation is based on enzymatic digestions rather

than flow sorting, followed by culture as 3D organoids in Matrigel for further functional assays, such as cellular differentiation followed by immunofluorescence.

COMMENTS

The paper provides an important protocol for the field, as separating the different epithelial compartments can help researchers with several questions, such as the role of certain proteins in specific epithelial compartments. It is easy to follow and provides a detailed step-by-step procedure, along with validation where necessary. The identity of the myoepithelial and luminal compartments were confirmed by immunofluorescence with an antibody directed against cytokeratin 14. Differentiated luminal cells were identified with an antibody directed against when acidic protein. The schematics provided were helpful. Much of the information provided was sufficient, but there can be a few improvements as detailed below.

Major Concerns:

*On page 3, paragraph 2, line 1 it is mentioned that the murine epithelia can be differentiated to produce milk producing acini and in line 9 "alveologenesis media "is mentioned. A short description of lactation in the mammary gland in paragraph 1 will be useful to give insight into what those mean.

Thank you for bringing this to our attention. In the revised manuscript we include a sentence addressing lactation in the first paragraph as suggested.

"During lactation when the outer MyoECs contract to squeeze milk from the inner alveolar luminal cells, the mammary gland undergoes numerous changes that are under the control of growth factors (e.g. EGF and FGF) and hormones (e.g progesterone, prolactin, insulin), which promote the differentiation of specialized structures, alveoli, which synthesize and secrete milk during lactation"

*On page 3, paragraph 2, line 10: a protocol to release cells from Matrigel is alluded to but should be provided.

We added the protocol name in the materials list. Please note that we cannot use the copyrighted name Matrigel within the text so we now refer to these types of basement membranes as extracellular matrix (ECMs).

*Step 2.12: What is the typical percentage of recovery for the tissue fragments and the typical percentage of contaminating cells? How does it impact subsequent organoid formation?

The typical recovery is about 500 fragments per prep (8 MGs). We see very few contaminating cells in our preps. It is key to perform the 4x10 mL washes of the fragments on the strainer to avoid any single cell retention. Carry over of single cells will affect the purity of the myoepithelial cells collected in the first trypsin treatment because they detach quickly and can be from either compartment.

Minor Concerns:
*Step 5.2: Clarification of why 1x DPBS is used sometimes but 1x PBS used the other times.

We apologize for this error and in the revised manuscript clearly state the difference in using DPBS in tissue culture and 1X PBS in fixation steps.

*Step 5.3: An important safety point should be mentioned with respect to the use of PFA, specifically that it should be used in the hood.

Indeed, we added this to the safety note about PFA. Thank you for pointing this out.

*Step 5.7: The concentrations of primary and secondary antibodies should be provided.

We added the concentrations and now this information is available in the notes portion of the materials list.

*Step 6.4: The concentration in ug/ml for Hoechst is more informative and should be added.

We included this information in the notes portion of the materials list as well.

*Figure 3 part (C): details of the microscope used for phase contrast microscopy are needed

We included this information in the figure legend.

*In the Materials List section, the table formatting is not consistent with respect to borders.

Thank you for the comment; we addressed the formatting of the materials list.

*Additionally, in that table for ovine pituitary prolactin, the name of the person/source it was purchased should be mentioned.

The formatting of the materials list affected the visibility of the information about prolactin. We have expanded the borders, so all the information is visible.

*In the recipes for organoids section, the RRIDs for any antibodies should be identified.

Thank you for the suggestion, we added the RRIDs to the notes of the materials list for each of the antibodies used. We did not, however, find an RRID for the donkey anti-mouse 647 secondary antibody we obtained from Jackson ImmunoResearch Labs.

Reviewer #4:
Rubio et al have submitted the article entitled 'Generation of Mosaic Mammary organoids by

Differential Trypsinization' for publication in JoVE. This is a straightforward and well written protocol and should be published with only minor revisions. Furthermore, the figures are clear and the combination of immunofluorescence, bright-field and models makes everything quite clear. This reviewer also appreciates the historical narrative included in the introduction which places the growth of mammary glands in context! Very nice work - and here are my suggestions:

1. Day one: can you provide any more detail regarding the removal of the lymph nodes? Does it need to be done under a dissection scope? What happens if all the tissue isn't removed?
a. Should any of these steps be done in the hood? And at what temp?

The harvesting of the mammary gland can be done at room temperature at a clean bench top using surgical lighting. The lymph nodes are found in mammary gland #4 in the center of 3 deep red lymphatic vessels. We included a figure to clearly show this step of the protocol. Please see revised Figure 1.

2. Day two: what does 'triturate' mean? For the pipetting, should this be a serological pipette? In step 2.8, what volume should be used for rinsing?

Trituration is the name of several different methods used to reduce the particle size of a substance (https://en.m.wikipedia.org/wiki/Trituration)

In the revised manuscript, we removed the word "triturate" and changed the description of this step to be clearer.

"2.1. After 14 h of digestion, gently mix by pipetting the digested tissue 10X using a 1 mL micropipette to break down any remaining stroma or adipose tissue, ensuring that neither bubbles nor excess mechanical force are generated.

Reviewer #5:
Manuscript Summary:
In this manuscript, the authors describe a useful protocol for FACS-free isolation of luminal and basal cells from mammary epithelial organoids. This technique is potentially useful to mammary gland biologists who seek to study the roles of luminal (LECs) and basal/myoepithelial mammary epithelial cells (MECs), manipulate them etc. and do not have access to FACS or prefer not to use FACS. The authors further describe a protocol for MEC+LEC 3D culture and immunostaining.

Major Concerns:
1. Step 1.2: The critical information on how finely the tissue should be chopped is missing.

We added an image of the chopped tissue with a ruler to clearly show the size of the tissue after chopping (Figure 1B).

2. Regarding tissue digestion, the question comes in mind why do authors digest the tissue for long 14 hours (in comparison to other published protocols, including the one by Nguyen-Ngoc that they cite - ref. 20, that use 30 min or so) without the help of any shaker on expensive low-adhesion plates? (The shaker would speed up the digestion significantly and low-adhesion plates would not be needed.) One of the central ideas of the manuscript is that the method is fast and cheaper than others (esp. if it does not need FACS). Also, the digestion enzyme concentration is 2x higher than in other protocols (that use short incubation times), which makes this isolation significantly more expensive than using other protocols.

We have tried a number of different digestion strategies, including shorter protocols that include the use of trypsin {Nguyen-Ngoc, 2015 #1380}{Jarde, 2016 #1186}. Simply put, we find that the use of trypsin, likely due to its actions on proteins such as cadherins, disrupts the outer basal layer such that the structures plated on TC dishes are primarily composed of luminal cells and not bilayered (Steps 3.1-3.3). In our protocol, this intermediate step in tissue culture is required because we differentially trypsinize the outer basal layer of these plated organoids from the inner luminal cells. In contrast, shorter protocols have researchers directly resuspending tissue fragments in Matrigel, which likely stabilizes the tissue fragments, allowing for cells to organize into structures, proliferate and generate bilayered organoids. A consequence of not using trypsin is that our digestion protocol requires longer incubation times and higher concentrations of enzymes. A benefit to our method is that it allows researchers to achieve separation of basal and luminal cells without the use of expensive flow cytometers that are unavailable at some institutions. We appreciate, however, that the digestion reagents are expensive and we have distinguished the cost savings (equipment versus reagents) in the revised manuscript.

3. The expression "stromal, blood, immune and single cells" (used in 2.7 and 2.10) is rather incorrect. Immune cells are stromal cells, too. Immune cells are blood cells, too. And all those listed cell types are usually stromal cells.

We addressed the repetitive language and appreciate the feedback.

4. The term "2D organoids" is not correct. By definition, organoids are 3D structures. On the other hand, the term 2D would suggest that the structures are monolayered, yet, they are still at least bilayered - that is one of the prerequisites of the differential trypsinization protocol. The authors themselves talk about an "outer MEC layer", suggesting there is an inner LEC layer, too, therefore the structures are not 2D. In the "Digestion Media" recipe, the amount of FBS should be in ml, not % (all other ingredients are listed as their weights or volumes to be used, not final concentration). The same for "Maintenance Media".

We apologize for the confusion. In the lab, we refer to the pancake-like, bi-structure that settles down on the TC dish as 2D organoids. We see, however, how this may be confusing to the reader. We have revised our description as follows: *forming flat, pancake-like structures with an outer layer of MyoECs encircling inner LECs (Figure 2A-B).*

5. In step 3.3., the authors talk about the purity of MEC/LEC populations as assayed by IHC. The purity of cell fractions is a critical parameter of the whole protocol, therefore, example protocol to test for lineage markers as well as representative results of such test should be included in the manuscript.

We referenced our 2011 publication that included Supplemental data showing the trypsinization of plated, bi-layered structures with lineage markers and quantification of lineage purity {Macias, 2011 #856}. In the revised manuscript, we re-publish these data in Figure 2.

6. In Fig. 2, it would be useful to include also a photograph of ongoing digestion of the luminal cells (just like shown in 2A for MECs), so that the reader can see what to expect.

Thank you for the suggestion. In the revised manuscript, we include an image of LECs (Figure 2B) and we clearly describe the process in the note under step 3.1.

***"Note**: Please see Figure 2 for representative images showing this process. Under brightfield illumination, the MyoECs appear rounded up and have a brighter appearance in comparison to the LECs, which remain adhered in the center and appear darker."*

7. It would be very useful to the reader to include information on typical yields of MECs and LECs (i.e. average number of cells) per mouse of certain age.

We added this information as a note to that step of the protocol (steps 3.2-3.3).

"Note: The usual recovery for MyoECs is within a range of (3.5e6 – 1.5e6) depending on the size of the MGs."
"Note: The usual recovery for LECs is within a range (2e6 – 4.2e6) depending on the size of the MGs."

8. To refer to the publication by Nguyen-Ngoc (ref. 20) in the Discussion, point 1, as a source of reference images of appropriately digested and overdigested MG tissue is not correct. In this publication the authors used different protocol for tissue digestion. The authors should provide their own pictures of digested and under- or overdigested tissue, resulting from their protocol.

Thank you for the suggestion. We added our own image of chopped and digested tissue in Figure 1C.

9. Are there any morphological features of the MECs and LECs that could be used to

discriminate them and to observe the progress of the trypsinization procedure in a brightfield microscope, when the reader does not have mouse with genetically labelled MECs/LECs? To be able to tell, when the first trypsinization should be stopped, this is absolutely critical for the protocol - how does an unexperienced user do this?

In the revised manuscript, we clearly state these differences to aid the identification of the different cell types during differential trypsinization. We added a note under step 3.1 and expanded Figure 2 to show the process with more detail.

*"**Note**: Please see Figure 2 for representative images showing this process. Under brightfield illumination, the MyoECs appear rounded up and have a brighter appearance in comparison to the LECs, which remain adhered in the center and appear darker."*

10. How sensitive/fail-safe is the differential trypsinization protocol with different trypsin brands?

We have not explored a wide-range of different trypsins. We suggest that researchers closely observe the trypsinizing cells using the morphological parameters described (Step 3.1) and shown (Figure A inset and C) to monitor digestion and determine harvest time.

Minor Concerns:
1. There is incorrect use of the term „media" throughout the manuscript. Media is plural, medium is singular.

Thank you for the grammatical correction. We changed the term media to medium throughout the entire manuscript.

2. "Growth Media", "Alveologenesis Media": "Fill to 10 mL" is confusing. Write the exact volume the user should use.

Thank you for the suggestion; we added exact volumes.

3. There is inconsistency in the instructions for use of hormones, growth factors, inhibitors. Sometimes stock concentration is listed and the volume to add to the medium is listed, at other occasions only final concentration in the medium is listed. The author should make this consistent.

We agree that this was confusing, and we have changed the recipes to use volumes only and added concentration details under the notes' column.

4. The expression "fat and adipose tissue" in the introduction, page 3, is incorrect. Fat tissue is adipose tissue; use one or the latter, not both like this.

We apologize for the repetitive language; we have corrected it and appreciate the feedback.

5. In Figures, there is inconsistent thickness and placement of the scale bar.

We addressed this issue and consistently placed and sized all scale bars.

6. In Figure 3, it would be clearer to the reader, if the colour of cells (MECs vs. LECs) used in the schematic drawing (E) were the same as those shown in the IF images (E'-E''). Similarly, in Figure 3F, the authors indicate milk droplets with yellow colour inside the organoids. However, the WAP protein, detected in the organoids as shown in the pictures F'-F'' in yellow, is only in the cells, there is no accumulation of WAP in the lumen of the organoids (although the authors claim in the Figure legend there is some, none is obvious). The use of the same (yellow) colour could be confusing to the reader. Moreover, the absence of WAP secretion into the lumen is suspicious.

We agree that the colors should be the same, and we changed the illustrations in figure 3 to match the MyoECs in magenta and LECs in green.

We agree with your assessment. WAP is soluble protein, secreted into milk and the fact that the organoids are not embedded in paraffin, but instead fixed and immunostained in situ, results in the loss of the milk and therefore WAP. Consequently, what is observed is intracellular WAP in secreting cells and extracellular WAP in milk that is trapped at the cell surfaces at fixation. In the revised manuscript, we have made this clear in the text and we show WAP that has been trapped in the lumen of one small organoid overlayered in the top, right corner of Figure 3F.

In the revised manuscript:

Differentiated organoids were generated as described above and immunostained with an antibody directed against the milk marker, whey acidic protein (WAP) (Figure 3F). WAP is a soluble protein, secreted into milk, and much of this liquid is lost when the cells are fixed and immunostained in situ. Therefore, in the top and section views, WAP staining is visible intracellularly in secreting cells and extracellularly in milk that is trapped at the cell surface during fixation, although in section view a small organoid appears to contain liquid milk (Figure 3D, F'' boxed overlay).

7. The IF photographs in Fig. 3E'-E'' and 3F'-F'' are of low quality and resolution, should be replaced by better quality ones, both for the manuscript as well as for the future produced video.

Thank you for your attention to this Figure, which we agree was not presented at high enough resolution. We have provided Figure 3 with new images.

8. "NUCLEI" is not an abbreviation; therefore, it should be written in lowercase, i.e. "Nuclei".

We corrected nuclei to lowercase.

9. In the list of materials, there are inconsistencies in references to the producer. For example, Peprotech is sometimes listed independently, on other occasion it looks as if it was a Fisher brand. Similar loose and incorrect references go for products by Corning, Sigma brands etc. Authors need to fix this.

We made all of the vendors consistent with the supplier.

10. There is incomplete information on the source of prolactin: “Purchased from Dr."

Yes, we apologize our Excel formatting affecting the visibility of the source for prolactin. We have modified the column widths to show all the information.

Reviewer #6:
Manuscript Summary:
The authors describe a protocol allowing isolation of distinct epithelial mammary cell types, organoid culture and imaging.
This is a very useful study for the field.

Major Concerns:
NA

Minor Concerns:
1. Please make sure there is consistency between figure 2A (3-6 min trypsin incubation for MECs isolation) and text (2-6 min trypsin incubation).

Thank you, we corrected our typo, please see revised Figure 2A.

2. Please clarify in the text whether Rho Kinase Inhibitor is required for long-term culture or only added for short period of time.

This information is indeed important and we added it to protocol step 4.5.

3. The source of FBS is not provided. There are important variations between different FBS origins and this information is critical for reproducibility. The name of the Prolactin provider is missing (purchased from Dr?).

We added the source of FBS. The formatting of our Excel tables limited the view of the prolactin’s source and we have reformatted the table to clearly show all the information.

4. The paper by Dale's team (Nature Com., 2016) should be discussed in the introduction. This

paper describes the culture conditions (Nrg1/Rspo/Rho kinase inhibitor) that are used in this protocol for mammary organoid establishment and are the basis of the culture conditions used for human breast cancer organoids published in 2018 (reference 13).

This is indeed an important point to be made and we have added the suggested information to our introduction.

In the revised manuscript:

"*Research using primary murine cells identified key growth factors and morphogens necessary for the extended maintenance and differentiation of organoids* [13]*. These studies have set the stage for the protocol presented here, and for the culture of human breast cells as 3D organoids, which is now a modern clinical tool, allowing for drug discovery and drug testing on patient samples* [14]."

5. Please correct on page 4: Incubate another ==> Incubate for another.

Thank you. We corrected our text based on your suggestion.

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