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.