Response to reviewers - Duclos et al. JoVE62365

We are grateful to the editors and the reviewers for their assessment of our work and their comments, which we found to be insightful. We have revised the manuscript to address these issues. We believe that the modifications strengthened the paper. Changes are marked in the revised document.

Below we give point-by-point answers to editorial and reviewers' comments, which are shown in **bold**. Our responses are in regular text with quotes from the edited manuscript shown in *italics*.

Editorial comments:

1.Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. E.g. line 81: "pooled" instead of "pulled", etc.

We thank the editor for this comment. We have now carefully proofread the manuscript and have fixed misspelled words.

2.Please provide an email address for each author.

We draw your attention on the fact that during revision of the manuscript the corresponding author and one of the co-authors accepted new positions. The e-mail address of each co-author is below:

Maela Duclos: maela.duclos@univ-rennes1.fr
Claude Prigent: claude.prigent@crbm.cnrs.fr
Roland Le Borgne: roland.leborgne@univ-rennes1.fr
Vincent Guen: vincent.guen@univ-nantes.fr

We have amended the e-mail address of the corresponding author in the manuscript.

3.Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

We thank the editor for this comment. We have extended the note at the beginning of the protocol by adding the following statement:

> *"Organoids were derived from mouse mammary stem cells. Donor mice were housed and handled in accordance with protocols approved by the Animal Care Committee of the University of Rennes (France)."*

4.Use "s" instead of "sec", "min" instead of "minutes", etc. Add a space between the quantity and its unit, e.g. "4 oC" instead of "4oC", etc.

We have modified abbreviations and units accordingly.

5.Line 168-169: What do you mean by "progressively removing it.."? Please rephrase for clarity.

We apologize if the initial manuscript was somehow misleading regarding this aspect. We have rephrased this part as follows:

> *"Place the glass capillary in the observation chamber. Locate the capillary with the front camera and place the tip of the glass capillary at the upper limit of the detection objective. Select locate sample and set the optimal focus using the tip of the glass capillary in brightfield illumination. Push out the agarose sample slowly from the capillary and locate organoids within the solidified agarose."*

6.Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Include a single line space between successive protocol steps.

We have adjusted the numbering accordingly.

7.Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." E.g. lines 85-95, etc.

We have amended the protocol accordingly.

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

We have highlighted in blue the protocol that identifies the essential steps for the video (3 pages).

9.Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate journal names.

We have used the JoVE EndNote style to format the references in the manuscript. The references appear as described above.

10.Please sort the Materials Table alphabetically by the name of the material.

We have now sorted the Materials Table accordingly.

<u>Reviewers' comments:</u>

Reviewer #1:

Major Concerns:

While the authors present a detailed and well explained protocol which is useful for a diversity of studies, the protocol may be of somewhat limited novelty. Recent papers by van Ineveld et al JoVE, 2020 (doi: 10.3791/60709) and Pleguezuelos-Manzano et al Current Protocols in Immunology;2020 (doi: 10.1002/cpim.106) for instance cover parts of the protocol described here. One novelty: the authors introduce glass capillaries in combination with light sheet microscopy. I would advise that the authors underscore the unique aspects (plus their applicability) of the current protocol. Apart from the novelty, the following recommendations are to be considered to improve the manuscript.

We are grateful to reviewer #1 for his enthusiasm about our work and specifically for highlighting the level of description and clarity of the protocol that we present. We agree with the reviewer that other methods for whole-mount immunofluorescent staining of organoids were recently published. We also note that published methods present protocols for the preparation of organoids for confocal microscopy. We present a protocol for the preparation of organoids for light sheet microscopy. Light sheet microscopy has recently transformed our capabilities to rapidly observe in 3-dimension complex biological structures. We believe that our study will be helpful to scientists interested in using this imaging technique for the analysis of organoids.
Another important distinctive aspect of our manuscript resides in the fact that we specifically describe a protocol for the staining and visualization of primary cilia in organoids. We draw the attention of reviewer 1 on the fact that our paper, if published, will be part of a specific collection titled: Current Advances in Techniques to Study Cilia Structure and Function.
To the best of our knowledge, our manuscript will be the first, if published, which presents a protocol for the immunofluorescence staining of primary cilia in organoids through light sheet microscopy.

We fully agree with the reviewer that we need to clearly state these specificities in the text. We modified the abstract and extended the introduction accordingly and as follows:

Abstract:

> *"Here we present a comprehensive protocol for the immunofluorescence staining of cell lineage and primary cilia markers, in whole-mount mouse mammary organoids, for light sheet microscopy."*

Introduction:

> *"Alternative methods were recently published for the immunofluorescence staining and imaging of organoids through confocal microscopy[15,16]. Our protocol focuses instead on the preparation and imaging of organoids through light sheet microscopy."*

Discussion:

> *"The protocol presented here enables whole-mount immunofluorescence staining and preparation of organoids for light sheet microscopy. Alternative methods for the staining and preparation of organoids for confocal microscopy were recently published[15,16]. The light sheet microscopy technology enables faster imaging of organoids than confocal microscopy and offers the possibility to image organoids at the subcellular scale while keeping the overview of entire 3D structures. Visualization of*

entire structures facilitates the analysis of molecular and cellular mechanisms that occur in a heterogeneous manner in organoids, such as primary ciliogenesis. In the protocol presented here, organoids are embedded in agarose in a glass capillary and can be rotated along the Y-axis in order to facilitate their imaging through the best-suited angle of observation."

These modifications are marked in the revised document.

Major other concerns:

1. While the title is focussed around the study of primary ciliogenesis, the manuscript is aimed at a much broader staining protocol for any intracellular and membrane bound protein in organoids. The title could therefore be taken into a broader context.

We thank the reviewer for his suggestion. However, we believe that we should keep primary cilia in the title for the following reasons: (1) our manuscript, if published, will be part of a specific collection of articles titled: Current Advances in Techniques to Study Cilia Structure and Function, (2) our protocol is optimized for the staining and visualization of primary cilia, which represents one of the specificities of our manuscript (as discussed above).

Minor Concerns:

1. Line 80-81 describes the quantity of organoids needed at the start of the protocol. Other well formats like 48-well and 24-well plates could be added. Another option is to mention the volume of cultures used.

We thank the reviewer for his suggestion, we have now added the number of organoids that we suggest to start with.

2. Line 81: "pulled" is misspelled and should be "pooled"

We thank the reviewer for this comment. We fixed this typing error.

3. Line 143: other (nuclear) stains (such as DAPI) can be mentioned

We agree with the reviewer. We extended this part as follows:

"NOTE: Hoechst (or other nuclear dyes, such as DAPI or DRAQ5) can be added to the buffer with secondary antibodies."

4. Figure 1C: The authors show "DNA" for Hoechst staining. It would be better to mention Hoechst as label instead of DNA

We agree that Hoechst could be shown in the figure. However, we feel that mentioning the fluorophores instead of the targets of the fluorophores on the image may be misleading for the reader. Therefore, we kept the initial labeling. However, we added the information in the figure legend. We thank the reviewer for his suggestion.

5. Figure 1C: Slug is depicted in red and gTub in pink. The distinction between these two colours is difficult. One of the colours could be changed to -for example- white.

We agree with the reviewer and now show the gTub staining in blue.

Reviewer #2:

Manuscript Summary:

This is a very detailed protocol for immunofluorescent staining of organoid and it's full of instructive senses. However, maybe some creative descriptions of this method compared to other protocols should be outstanded. More information should be added and more references are necessary to help researchers get into the professional phrases or techniques in this field.

Major Concerns:

In the introduction part, more explanations about the importance of this method should be described, for example, what the skills are commonly used to deal with the 3D display of organoids, what the disadvantages are these skills and what we need in this field.
In the discussion part, more background information of the issue you solved should be added to let the researchers know why your protocols is advantaged.

We are grateful to reviewer #2 for his enthusiasm about our work and for considering it full of instructive senses. We thank the reviewer for his suggestion to put more emphasis on the novelty of our protocol in comparison to other published methods. This suggestion was also made by reviewer 1. As discussed above, methods for whole-mount immunofluorescence staining and preparation of organoids for confocal microscopy were recently published. Our manuscript presents a protocol for staining and preparation of organoids for light sheet microscopy. Light sheet microscopy has recently transformed our capabilities to rapidly observe in 3-dimension complex biological structures. We believe that our work, if published will be helpful to scientists interested in using this imaging technique for the analysis of organoids, and specifically for the analysis of primary ciliogenesis in organoids.

We have now amended the text in all sections to highlight these aspects (abstract, introduction, discussion) as follows:

Abstract:

> *"Here we present a comprehensive protocol for the immunofluorescence staining of cell lineage and primary cilia markers, in whole-mount mouse mammary organoids, for light sheet microscopy."*

Introduction:

> *"Alternative methods were recently published for the immunofluorescence staining and imaging of organoids through confocal microscopy[15,16]. Our protocol focuses instead on the preparation and imaging of organoids through light sheet microscopy."*

Discussion:

> *"The protocol presented here enables whole-mount immunofluorescence staining and preparation of organoids for light sheet microscopy. Alternative methods for the staining and preparation of organoids for confocal microscopy were recently published[15,16]. The light sheet microscopy technology enables faster imaging of organoids than confocal microscopy and offers the possibility to image organoids at the subcellular scale while keeping the overview of entire 3D structures. Visualization of entire structures facilitates the analysis of molecular and cellular mechanisms that occur in a heterogeneous manner in organoids, such as primary ciliogenesis. In the protocol presented here, organoids are embedded in agarose in a glass capillary and can be rotated along the Y-axis in order to facilitate their imaging through the best-suited angle of observation."*

These modifications are marked in the revised document.