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Three-dimensional imaging of organoids to study primary ciliogenesis during ex vivo organogenesis

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TITLE:

Three-dimensional imaging of organoids to study primary ciliogenesis during *ex vivo* organogenesis

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SUMMARY

Stem cell-derived organoids facilitate the analysis of molecular and cellular processes that regulate stem cell self-renewal and differentiation during organogenesis in mammalian tissues. Here we present a protocol for the analysis of the biology of the primary cilium in mouse mammary organoids.

ABSTRACT:

Organoids are stem cell-derived three-dimensional structures that reproduce *ex vivo* the complex architecture and physiology of organs. Thus, organoids represent useful models to study the mechanisms that control stem cell self-renewal and differentiation in mammals, including primary ciliogenesis and ciliary signaling. Primary ciliogenesis is the dynamic process of assembling the primary cilium, a key cell signaling center that controls stem cell self-renewal and/or differentiation in various tissues. 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. We describe the microscopy imaging method and an image processing technique for the quantitative analysis of primary cilium assembly and length in organoids. This protocol enables a precise analysis of primary cilia in complex three-dimensional structures at the single cell level. This method is applicable for immunofluorescence staining and imaging of primary cilia and ciliary signaling in mammary organoids derived from normal and genetically modified stem cells, from healthy and pathological tissues, to study the biology of the primary cilium in health and disease.

INTRODUCTION

Development of multicellular organisms and the maintenance of homeostasis in their adult tissues reside in a fine-tuned regulation between self-renewal and differentiation of stem cells,

which orchestrate in time and space normal tissue development and regeneration[1]. Subversion of this regulation causes developmental anomalies and cancers[2]. Thus, understanding the molecular and cellular mechanisms that orchestrate stem cell self-renewal and differentiation is of key interest in developmental and cancer biology.

Recent development of *ex vivo* organogenesis methods, in which tissue stem cells generate three-dimensional organoids have transformed our capabilities to study the dynamics of stem cells during mammalian organogenesis and maintenance of tissue homeostasis in a dish[3]. Organoids represent a good alternative to cumbersome genetically modified animal models to study these processes. Protocols for the development of organoids from tissue stem cells of many organs have now been developed[3], including small intestine and colon, stomach, liver, pancreas, prostate, and mammary gland[3]. Additionally, the development of somatic genome-editing techniques in organoid-forming stem cells now enables to quickly interrogate the molecular and cellular mechanisms that control their biology[4,5].

The primary cilium is a microtubule-based structure that is assembled at the surface of stem and/or differentiated cells of various tissues[6]. It is generally non-motile and is assembled as a single structure per cell[7]. Primary ciliogenesis is the dynamic process of assembling the primary cilium[7]. At the cell surface, the cilium acts as a cell signaling platform[8]. Thus, the primary cilium is thought to act as a key regulator of stem cell self-renewal and/or differentiation in many tissues, including the brain[9,10], the mammary gland[4,11], the adipose tissue[12], and the olfactory epithelium[13], among others. Primary ciliogenesis and/or ciliary signaling are dynamically regulated in distinct cell lineages and at different developmental stages[4,13,14], but the underlying mechanisms remain to be largely determined.

Ex vivo organogenesis shows promise for the development of basic knowledge on the molecular and cellular mechanisms that control stem cell biology, including primary ciliogenesis and ciliary signaling. However, it relies on the ability to properly image whole mount organoids at the single cell level and at sub-cellular scales. We recently used a mouse mammary stem cell-derived organoid model to show that primary cilia positively control mouse mammary stem cell organoid-forming capacity[4]. Here we present a comprehensive protocol for the immunofluorescence staining of whole mount mouse mammary organoids (**Figure 1A,B**), which enables the analysis of primary cilia through light sheet microscopy during *ex vivo* organogenesis in three-dimension. Alternative methods were recently published for the immunofluorescence staining and imaging of organoids through confocal microscopy[15,16]. This protocol focuses instead on the preparation and imaging of organoids through light sheet microscopy.

PROTOCOL

NOTE: The protocol below is recommended for the staining of organoids that were grown in 5 wells of a 96 well plate and pooled together (> 100 organoids). 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).

1. Reagents

1.1. To prepare the fixative solution, dilute 125 µL of 16% paraformaldehyde (PFA) aqueous commercial solution in 375 µL of phosphate-buffered saline (PBS) to generate a 4% PFA solution.

CAUTION: Manipulate PFA that is a toxic substance under a chemical hood.

1.2. To prepare the permeabilization buffer, dilute 1.5 µL of Triton X100 in 500 µL of PBS, to produce a 0.3% Triton X100 solution.

1.3. To prepare the blocking buffer, dilute 1.5 µL of Tween-20 and 75 µL of normal goat serum in PBS, to generate a 5% goat serum-0.1% Tween 20 solution.

1.4. To prepare the light sheet mounting medium, dissolve 1 g of ultrapure low melting point agarose in 100 mL of dH20 or PBS at 65 °C. Prepare 1 mL aliquots and store at room temperature.

2. Organoids recovery

2.1. Transfer organoids from the culture wells to a low binding polymer 1.7 mL tube, after pipetting them up and down 3 times in the wells, with an FBS-coated tip for which the end was cut (minimal diameter of the extremity 1.5 mm).

NOTE: The coating prevents organoids from sticking to the tip. The low-binding polymer material enables to reduce the attachment of organoids to the side of the tube during the entire staining procedure.

2.2. Fill the tube with PBS, and spin down at 350 x *g* for 3 min. Remove the supernatant.

3. Fixation, permeabilization and blocking

3.1. Resuspend the organoids in 500 µL of 4% PFA. Incubate for 30 min at room temperature. Spin down at 350 x *g* for 30 s. Remove the supernatant.

NOTE: From this step onwards, resuspend organoids in the different buffers by simply adding the buffers on the pellet of organoids without touching them. Perform all washing steps at room temperature.

3.2. Wash the organoids with 1 mL of PBS for 3 min. Spin down at 350 x *g* for 30 s. Remove the supernatant.

PAUSE: Fixed organoids can be kept in PBS at 4 °C for at least a week.

3.3. Permeabilize the organoids by resuspending them in 500 µL of PBS-Triton X100 0.3% and incubate for 30 min at room temperature. Spin down the organoids at 350 g for 30 s.

3.4. Wash the organoids by resuspending them with 1 mL of PBS and incubate for 3 min. Spin down at 350 x *g* for 30 s. Remove the supernatant. Repeat once.

3.5. Optional: Resuspend the organoids in 500 µL of ice-cold methanol. Incubate at - 20 °C for 10 min. This step may be required for the staining of specific centrosomal markers.

3.6. Optional (if step 5 was performed): Wash the organoids with 1 mL of PBS for 3 min. Spin down at 350 x *g* for 30 s. Remove the supernatant. Repeat once.

3.7. Block non-specific antibody binding sites in organoids by resuspending them with 500 µL of blocking buffer (PBS, 5% goat serum, 0.1% Tween 20). Incubate 1 h and 30 min at room temperature. Spin down at 350 g for 30 s. Remove the supernatant.

3.8. Wash the organoids by resuspending them with 1 mL of PBS and incubate for 3 min. Spin down at 350 x *g* for 30 s. Remove the supernatant.

4. Labelling

4.1. Resuspend the organoids with 200 µL of blocking buffer with diluted primary antibodies and incubate overnight at 4 °C with mild shaking (60 rpm on a horizontal shaker). Place the tubes with a 45° angle with the horizontal plan of the shaker. It will maintain the organoids at the bottom of the tubes in the staining buffer.

4.2. Wash the organoids by resuspending them with 1 mL of PBS and incubate for 5 min. Spin down at 350 x *g* for 30 s. Remove the supernatant. Repeat twice.

NOTE: Some organoids in the last step may stick to the side of the tube, resulting in organoid loss during aspiration of the supernatant after centrifugation, adding 0.2% (w/v) bovine serum albumin (BSA) to the PBS in the washing steps may reduce organoid loss.

4.3. Resuspend the organoids with 200 µL of blocking buffer with secondary antibodies and incubate for 1 h and 30 min with mild shaking (60 rpm on a horizontal shaker). Place the tubes with a 45° angle with the horizontal plan of the shaker. It will maintain the organoids at the bottom of the tubes in the staining buffer.

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

4.4. Wash the organoids by resuspending them with 1 mL of PBS and incubate for 5 min. Spin down at 350 x *g* for 30 s. Remove the supernatant. Repeat twice.

NOTE: Some organoids in the last step may stick to the side of the tube, resulting in organoid loss during aspiration of the supernatant after centrifugation, adding 0.2% (w/v) BSA to the PBS in the

washing steps may reduce organoid loss.

5. Preparation of the agarose sample for imaging

5.1. Melt light sheet mounting medium by incubating it at 65 °C. Once the medium has melted, incubate it at 37 °C for 5 min.

5.2. Resuspend the organoids in 100 µL of mounting medium using a 200 µL tip, with the extremity of the tip cut (minimal size of the extremity: 1.5 mm), by pipetting up and down twice.

5.3. Suck the mounting medium with the organoids in a glass capillary (green capillary, inner diameter: 1.5 mm) using a plunger. Incubate the capillary at room temperature for 5 min for the mounting medium to solidify.

PAUSE: Capillary can be stored in PBS at 4 °C for a week before imaging.

6. Imaging

6.1. Using a light sheet microscope (e.g., ZEISS Lightsheet Z.1), image the organoids with 20x or 40x water immersion objectives.

6.1.1. 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.

6.1.2. Select the 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.

6.1.3. Keep the organoids to be imaged close to the tip of the capillary, to reduce movements of the agarose sample in the PBS. Rotate the capillary to set the optimal sample orientation and define the desired zoom.

6.1.4. Select acquisition mode. Define the channels to image, set proper orientations of the light sheet, set the laser power for each channel (to reduce photobleaching, use low laser power), set the size of the image and the desired illumination side(s). Example of imaging parameters: Image size 1920 x 1920, illumination: dual side.

6.1.5. Define the Z-stack to image by setting the Z-extremities of the stack using the Z-stack module and set the Z-step size to optimal.

6.2. Process the output file using the image processing module of the microscope software to navigate in the sample, obtain a Z-projection and 3D-representation.

6.3. Turn the sample and acquire a new image from a different angle or image other organoids.

6.4. Analyze images with an interactive microscopy image analysis software (e.g., Imaris) enabling (i) the visualization of the sample in 3D (ii) the segmentation of objects (iii) identification and quantitative analysis of objects.

REPRESENTATIVE RESULTS

Ex vivo organogenesis methods are transforming our capabilities to study mammalian tissue development and maintenance of tissue homeostasis in a dish. The analysis of molecular and cellular mechanisms that regulate these processes, including primary ciliogenesis and ciliary signaling, relies on the ability to image organoids in three-dimension.

The protocol described above enables the staining of whole-mount mammary organoids. They arise from mammary stem cell-enriched basal cells that are FACS-purified from adult female mice (**Figure 1A**)[4]. The staining procedure enables the visualization of cell lineage, centrosome, and primary cilium markers in organoids in less than 24 h (**Figure 1B**). The strength of the immunofluorescence staining and imaging procedures that we describe for the visualization of the three-dimensional architecture of organoids is exemplified here through the staining and visualization of integrin α6, also known as CD49f (**Figure 1C**). Additionally, the staining of primary cilia (through the staining of Arl13b, a primary cilium marker), of centrosomes (through a γtubulin staining), and of mammary-stem cell enriched basal cells (through the staining of Slug, an epithelial-mesenchymal transition marker) illustrates the ability to visualize whole-mount organoids at the cellular and sub-cellular scales (**Figure 1C**).

The method described here enables semi-automated segmentation of objects and image analysis (**Figure 1C, Animated Figure 1**). Using an interactive microscopy image analysis software, the total cell number, the number of cells from distinct cell lineages, and the number and size of primary cilia can be quantified with accuracy.

FIGURE LEGEND

Figure 1: Overview of the immunofluorescence staining and imaging procedures for the analysis of mammary organoids. (**A**) Mammary glands from adult C57BL6/J female mice are dissociated and mammary stem cell-enriched basal cells are FACS-purified (CD49f^{high};CD24^{med} phenotype) and plated in three-dimension according to Guen et al., 2017[4]. Mammary organoids ranging from 100-300 µm in size are observed 7-14 days after plating by brightfield microscopy. Scale bar: 100 µm. (**B**) Organoids are recovered, fixed, permeabilized and stained prior to be embedded in agarose in a glass capillary. The procedure takes less than 24 h. Agarose samples containing the organoids are imaged through light sheet microscopy. Imaging of an organoid takes 2-4 min. (**C**) Mammary organoids were stained for the indicated proteins and imaged following the procedure described above. Hoechst was used as a nuclear dye. All scale bars 100 µm.

Animated Figure 1: Three-dimensional analysis of an organoid at the cellular and sub-cellular scales. Slug-expressing basal cells (Slug staining: red), primary cilia (Arl13b staining: green) and centrosomes (gTubulin staining: magenta) were observed in a mouse mammary organoid through post-acquisition image processing using an interactive image analysis software enabling semi-automated segmentation. Hoechst was used as a nuclear dye (blue).

DISCUSSION

The detailed protocol presented here enables the staining and imaging of mouse mammary organoids that grow in semi-solid medium. This protocol is presumably applicable to the staining of organoids mimicking the architecture of various tissues that grow in semi-solid and solid media. For organoids that grow in 100% Matrigel with medium on top, the recovery and fixation steps slightly differ. The culture medium must be removed from the culture well. After a quick PBS wash, the fixative solution (4% PFA) may be directly added in the culture well on the Matrigel-containing organoids. Organoids in Matrigel can be incubated with the fixative solution for 30-60 min at room temperature. Fixed organoids are subsequently transferred to the staining tube after resuspending them in the fixative solution. The fixative solution and the residual Matrigel must be completely removed after centrifugation and prior to the permeabilization step. Organoid permeabilization is a critical step in the protocol. Co-staining of primary cilium and centrosome markers generally requires Triton X-100 and methanol incubation steps. The co-staining of other markers such as cell-cell junction markers or other cytoskeletal proteins may be affected by these permeabilization steps and other strategies may have to be used.

Loss of organoids is an issue during the entire staining procedure. To reduce organoid loss, it is critical to coat the tip that is used to transfer the organoids from the culture well to the staining tube. It is also critical to use a low-binding polymer tube as the staining tube. These measures will prevent organoids from sticking to the tip or the tube during the transferring and washing steps, respectively. Organoids may still be lost during the washing steps after the incubations with antibodies. Adding 0.2% (w/v) BSA to the PBS in the washing steps may reduce organoid sticking to the side of the tube and organoid loss. To ensure enough recovery of organoids for imaging at the end of the procedure, we recommend starting the experiment with more than 100 organoids.

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. Proper embedding of the organoids in agarose for imaging is a critical step of the protocol. In the last PBS wash following the incubation with the secondary antibodies, as much PBS as possible must be removed from the staining tube, without sucking the organoids.

Reducing the amount of PBS in the staining tube prior to organoid resuspension in the mounting medium will reduce dilution of the agarose solution and ensure proper solidification of the agarose sample in the capillary.

Proper positioning of the agarose sample containing the organoids during imaging is another critical step. It is important to keep the organoids to be imaged in the agarose sample out but close to the tip of the glass capillary. The agarose sample far from the glass capillary tends to move in the PBS in the observation chamber. However, the agarose close to the glass capillary remain immobilized for proper imaging of the organoids. If a movement close to the capillary is still affecting proper imaging, cutting the extremity of the agarose sample that is floating in the observation chamber may solve the problem. While the glass capillary may be re-used for multiple samples, we do not recommend the re-use of the plunger. We found that the agarose sample tend to slowly escape from the glass capillary when a plunger is re-used, affecting significantly the possibility to image organoids.

The protocol presented here does not include optical clearing of organoids. We found that organoids can be efficiently imaged up to 100-150 µm in depth using our protocol. Imaging of larger whole organoids may require the acquisition of two images from each side of the same organoid. The images can be stitched during the image processing step. Nevertheless, an optical clearing step, as described by others[15], is presumably compatible with the protocol as well. It must be performed after the labelling step and before agarose-embedding of the samples. While optical clearing is not strictly necessary for organoid imaging using a light sheet microscope, optical clearing is especially valuable when imaging organoids with a confocal microscope. Rotation of samples is not possible with this type of microscope.

Light sheet microscopes enable faster image acquisition than standard confocal microscopes and thus enable the analysis of many organoids in a short period of time. The study of multiple organoids enables robust statistical analysis when quantitative measurements are performed during post-acquisition image processing. Advanced confocal microscopes may, however, offer higher image resolution than light sheet microscopes. Both types of microscopy require time consuming post-acquisition image processing for the analysis of organoids in three-dimension. This limitation should be considered. The output file in light sheet microscopy is very large (3-8 GB per image) and management of large datasets can be an issue. The protocol presented here enables three-dimensional analysis of fixed organoids. The fixation and agarose-embedding steps may of course represent a limitation for some applications that require the analysis of very dynamic events in the same organoid over an extended period of time. Live-imaging of organoids, which express fluorescent reporters, may be more appropriate for such analysis. In this setting, organoids would have to be embedded and imaged in a specific gel and in a medium at the appropriate temperature, which preserve the viability and normal physiology of organoids. The light sheet illumination method that reduces photobleaching and phototoxicity and enables faster imaging of samples, in comparison to standard illumination with a confocal microscope, is especially valuable for live-imaging.

In conclusion, the protocol presented here enables the immunofluorescence staining of whole

mount mammary organoids that arise from normal and genetically modified mouse mammary stem cells with or without diverse pharmacological perturbations. Thus, it allows an in-depth analysis of the molecular and cellular processes, including primary ciliogenesis and ciliary signaling, that regulate stem-cell self-renewal and differentiation during the course of *ex vivo* organogenesis. This protocol may also be applicable to the staining of mammary organoids that arise from transformed cells of cancerous tissues. Therefore, this method should contribute to the development of our knowledge on the biology of the cilium during mammary organoid formation in healthy and pathological conditions. This protocol is presumably applicable to the staining of organoids derived from stem cells of different tissues, with minor modifications. Thus, it may enable the analysis of the biology of the primary cilium in many tissues in health and disease.

ACKNOWLEDGMENTS

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DISCLOSURES

The authors declare no conflict of interest.

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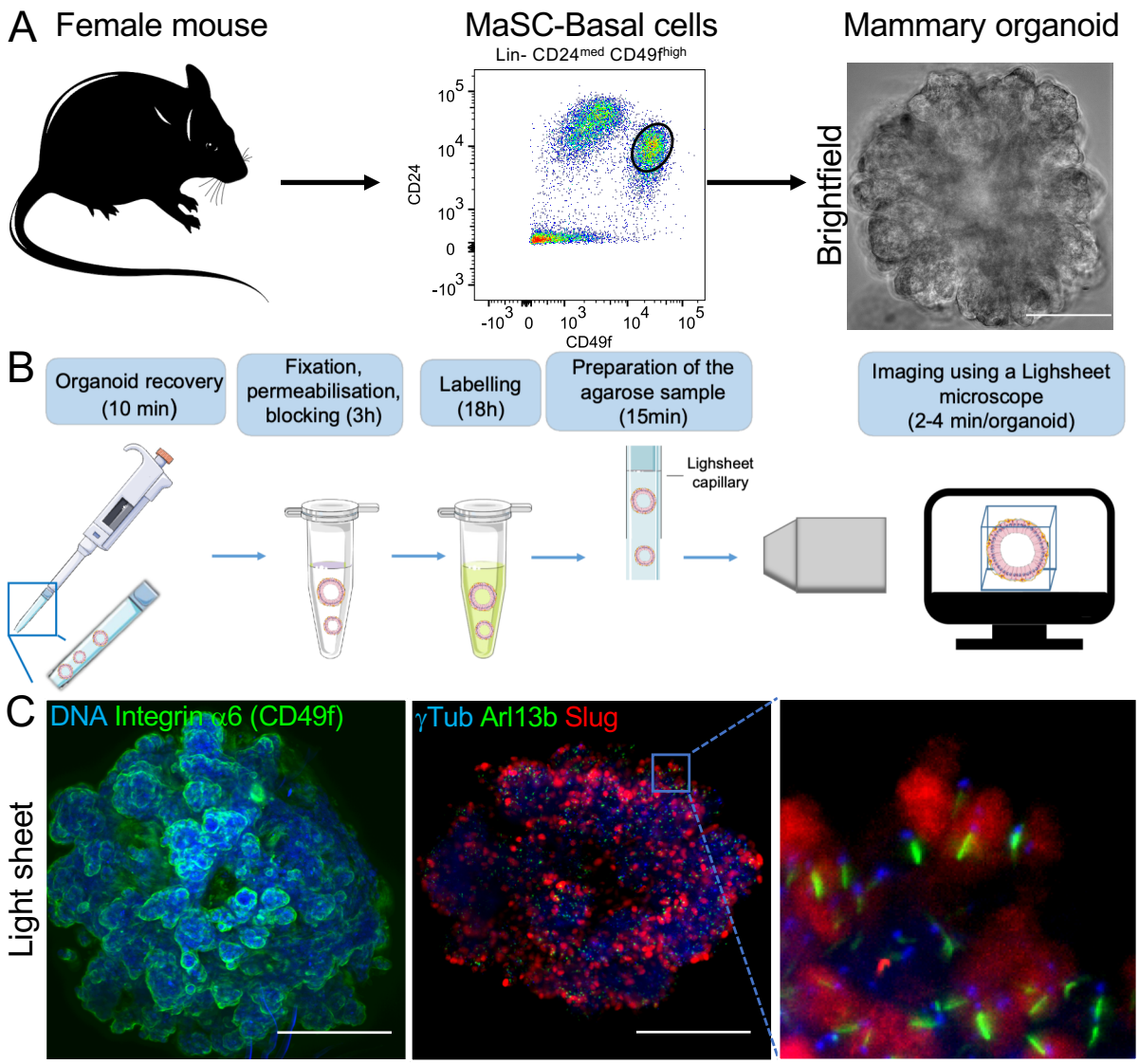

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Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Anti-mouse IgG1 647	Thermo-Fisher	A21240
Anti-mouse IgG2A 488	Thermo-Fisher	A21131
Anti-rabbit 546	Thermo-Fisher	A11035
Arl13b	NeuroMab	73-287
EMS 16% Paraformaldehyde Aqueous Solution, EM Grade	Electron Microscopy Sciences	15710
FBS	Thermo-Fisher	10270106
gtubulin	Sigma-Aldrich	T5326
Hoechst 33342	Thermo-Fisher	62249
Integrin a6	Biolegend	313616
Light Sheet Capillary	Zeiss	701908
Light Sheet plunger	Zeiss	701998
Low binding Microcentrifuge tubes	BioScience	27210
Normal Goat Serum Blocking Solution	Vector labs	S-1000
PBS	Sigma-Aldrich	p3587
Slug	Cell Signaling Technology	9585
Triton-X100	Sigma-Aldrich	T9284
Tween-20	Euromedex	9005-64-5
UltraPure Low Melting Point Agarose	Thermo-Fisher	16520050

Comments/Description

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
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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.