Submission ID #: 61404
Scriptwriter Name: Bridget Colvin
Project Page Link: https://www.jove.com/account/file-uploader?src=18728268

Title: Generation and Quantitative Characterization of Functional and Polarized Biliary Epithelial Cysts

Authors and Affiliations: Latifa Bouzhir*[1], Emilie Gontran*[1], Lorena Loarca[1], Mauricette Collado-Hilly[1], Pascale Dupuis-Williams[1,2]
*These authors contributed equally to the work

[1]Université Paris-Saclay, Inserm U1193, Physiopathogénèse et traitement des maladies du foie
[2]ESPCI Paris – Université PSL

Corresponding Author:
Pascale Dupuis-Williams
pascale.dupuis-williams@universite-paris-saclay.fr

Co-authors:
latifa.bouzhir@universite-paris-saclay.fr
emilie.gontran@universite-paris-saclay.fr
lorena.loarca@universite-paris-saclay.fr
mauricette.collado-hilly@universite-paris-saclay.fr

Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **Y**

If **Yes**, can you record movies/images using your own microscope camera?

Y

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **42**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Pascale Dupuis-Williams**: This is the first protocol for generating biliary cysts, providing a systematic analysis that allows the evaluation of cyst formation, efficiency, and size over time **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Pascale Dupuis-Williams**: This method allows quantitative assessment of epithelial cyst formation and makes it possible to evaluate this process as a function of the type of epithelial cells or hydrogel **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Lorena Loarca**: Since the therapeutic options for biliary disorders are limited, this protocol opens the door for standardizing drug studies, identifying novel therapeutic targets, and understanding disease mechanisms **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.4. **Lorena Loarca**: This simple method allows important questions about the mechanisms of lumen formation within the bioengineering of tubular structures field to be addressed **[1]**.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Introduction of Demonstrator on Camera

1.5. **Pascale Dupuis-Williams**: Demonstrating the cyst generation and cyst imaging procedures will be Latifa Bouzhir, an engineer, and Emilie Gontran, a post-doctoral fellow, both from my laboratory **[1][2]**.

1.5.1. INTERVIEW: Author saying the above

1.5.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera

Protocol

2. Chamber Slide preparation

2.1. Before beginning an experiment, thaw an appropriate hydrogel solution at 4 degrees Celsius **[1]** and pre-cool pipette tips and an 8-well chamber slide at minus 20 degrees Celsius overnight **[2]**.

2.1.1. WIDE: Talent placing solution at 4 °C *Videographer: Difficult step*

2.1.2. Talent placing tips and slide at -20 °C *Videographer: Difficult step*

2.2. The next morning, place the hydrogel and 8-well chamber slide on ice **[1]** and add the appropriate volume of hydrogel to cold normal rat cholangiocyte complete medium to obtain 500 microliters of 40% hydrogel solution **[2-TXT]**.

2.2.1. Talent placing hydrogel and slide on ice *Videographer: Important step*

2.2.2. Talent adding hydrogel to medium, with hydrogel and solution containers visible in frame *Videographer: Important step* **TEXT: See text for all medium and solution preparation details**

2.3. Then use the cold pipette tips to add 50 microliters of hydrogel solution to the center of each well of the chamber slide **[1]** and use a pipette tip to spread the solution over the entire surface of the well bottom as evenly as possible without bubbles **[2]**.

2.3.1. Talent adding hydrogel to well(s), with solution container visible in frame *Videographer: Important step*

2.3.2. Solution being spread over well bottom *Videographer: Important step*

3. Normal Rat Cholangiocyte Preparation

3.1. To prepare the cells for the experiment, while the hydrogel is polymerizing, wash the cells from a 70% confluent normal rat cholangiocyte culture with pre-warmed PBS **[1-TXT]** and incubate the cells with 5 milliliters of fresh pre-warmed PBS for 20 minutes in the cell culture incubator **[2]**.

3.1.1. WIDE: Talent washing culture, with PBS container visible in frame **TEXT: Warm PBS, trypsin-EDTA, and medium in 37 °C water bath use**

3.1.2. Talent adding PBS to flask, with PBS container visible in frame

3.2. At the end of the incubation, replace the PBS with 1 milliliter of pre-warmed trypsin-EDTA **[1]** and return the flask to the cell culture incubator for 5-10 minutes **[2]**.

3.2.1. Talent adding tryspin-EDTA to container, with trypsin-EDTA container visible in frame

3.2.2. Talent placing flask into incubator

3.3. When the cells have detached, neutralize the reaction with 4 milliliters of pre-warmed complete normal rat cholangiocyte medium **[1]** and transfer the cells to a 15-milliliter tube for centrifugation **[2-TXT]**.

3.3.1. Talent adding medium to flask, with medium container visible in frame

3.3.2. Talent adding cells to tube **TEXT: 4 min, 150 x g, RT**

3.4. Then resuspend the pellet in 5 milliliters of pre-warmed medium **[1]** and filter the cells through a 40-micron strainer into a 50-milliliter tube for counting **[2]**.

3.4.1. Shot of pellet if visible, then medium being added to tube, with medium container visible in frame *Videographer: Important step*

3.4.2. Cells being filtered *Videographer: Important step*

4. Cyst Generation

4.1. To generate cysts, add the appropriate volume of hydrogel to cold, complete normal rat cholangiocyte medium to obtain 1600 microliters of an 80% hydrogel solution on ice **[1]** and dilute the cells to a 5 x 10^5 cells/milliliter of cold complete normal rat cholangiocyte medium concentration in 1600 microliters of medium **[2]**.

4.1.1. WIDE: Talent adding hydrogel to medium, with hydrogel and medium containers visible in frame *Videographer: Important/difficult step*

4.1.2. Talent adding cells to medium, with cell container visible in frame *Videographer: Important/difficult step*

4.2. Immediately mix the cell and hydrogel cell solutions **[1]** and add 400 microliters of cells to each well of the hydrogel-coated chamber slide, taking care to avoid bubbles **[2]**.

4.2.1. Talent adding cell solution to hydrogel solution *Videographer: Important step*

4.2.2. Talent adding cells-hydrogel solution to well(s) *Videographer: Important step*

4.3. **Latifa Bouzhir**: To ensure the acquisition of reproducible and significant results, be sure to handle the hydrogel carefully and to thoroughly mix the initial cell-hydrogel solution to obtain a homogenous solution **[1]**.

4.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

4.4. When all of the cells have been seeded, place the slide in the cell culture incubator **[1]**.

4.4.1. Talent placing slide into incubator

4.5. After 2 days in culture, remove 250 microliters of the medium from one corner of each well, taking care not flush out the hydrogel **[1]**, and slowly replace the discarded supernatant with 250 microliters of fresh culture medium **[2-TXT]**.

4.5.1. Medium being aspirated *Videographer: Important step*

4.5.2. Medium being added *Videographer: Important step* **TEXT: Refresh medium every 2 d**

5. Cyst Imaging

5.1. For cyst imaging, on the appropriate day of culture, select the 10x objective on a phase-contrast microscope equipped with image acquisition software **[1-TXT]**, switch on the white lamp **[2]**, and select the brightfield imaging option **[3]**.

5.1.1. WIDE: Talent selecting objective, with open acquisition software on monitor visible in frame **TEXT: *e.g.*, 1, 2, 4, 7, and 10**

5.1.2. Talent switching on lamp

5.1.3. SCREEN: JoVE61404_2.1.2_to_2.1.4: 00:00-00:11

5.2. Select **Play** to switch on the camera and focus on a field of cysts **[1]**.

5.2.1. SCREEN: JoVE61404_2.1.2_to_2.1.4: 00:12-00:30 *Video Editor: please speed up*

5.3. Set the exposure time and open the **Auto Capture Folder** window to allow automatic saving of the images **[1]**.

5.3.1. SCREEN: JoVE61404_2.1.2_to_2.1.4: 00:34-00:54 *Video Editor: please speed up*

5.4. Open the capture Z-series window, reset the default position **[1]**, and use the Z screw to define the top and bottom planes of the Z-stack, adjusting the Z-step depending on the objective and the level of resolution **[2]**.

5.4.1. SCREEN: JoVE61404_2.1.2_to_2.1.4: 01:00-01:12

5.4.2. Added SCREEN: JoVE61404_2.1.2_to_2.1.4: 01:20-03:05 *Video Editor: please speed up*

5.5. Then click **Run now** to launch the acquisition **[1-TXT]**.

5.5.1. SCREEN: JoVE61404_2.1.2_to_2.1.4: 03:06-03:58 *Video Editor: please speed up*
TEXT: Obtain ≥ 3 non-overlapping Z-stacks/well

6. Image Processing

6.1. After imaging, open the z-stack in Fiji **[1]**. To duplicate the stack, click **Image**, **Duplicate**, and **Duplicate stack [2]**.

6.1.1. WIDE: Talent opening z-stack, with monitor visible in frame

6.1.2. SCREEN: 2.2.1_to_2.2.4_t1: 00:00-00:18 *Video Editor: please speed up*

6.2. To create a minimum intensity projection from the duplicated stack, under the **Image** menu, select **Stacks** and **Z project [1]**.

6.2.1. SCREEN: 2.2.1_to_2.2.4_t1: 00:19-00:24

6.3. Select Minimum Intensity for the **Projection type** and click **OK [1]**.

6.3.1. SCREEN: 2.2.1_to_2.2.4_t1: 00:25-00:29

6.4. To subtract the background from the projection, under the **Process** menu select **Subtract Background** and select 500 pixels of rolling ball radius and **light background** to render the cysts more contrasted than the background **[1]**.

6.4.1. SCREEN: 2.2.1_to_2.2.4_t1: 00:30-00:40

7. Cyst Quantification

7.1. To measure the approximate cyst diameter, zoom on the targeted cyst, select the **Straight line** tool **[1]**, and press the T button on the keyboard **[2]**.

7.1.1. WIDE: Talent selecting line tool, with monitor visible in frame

7.1.2. Talent pressing T button

7.2. Draw a line across the diameter of each cyst on the final projection. The next region of interest created for each cyst will be added to the Region of Interest manager **[2]**.

7.2.1. SCREEN: 2.3.1_to_2.3.4_t2: 00:10-00:30

7.3. When all of the cysts have been counted, click on the Z-stack window to select it and click **Show All** to see the counted cysts **[1]**.

7.3.1. SCREEN: 2.3.1_to_2.3.4_t2: 00:32-00:40

7.4. Move the cursor along the Z-stack to check that all of the cysts have been counted in each image, adding new cysts to the region of interest manager as necessary **[1]**.

7.4.1. SCREEN: 2.3.1_to_2.3.4_t2: 00:41-00:50

7.5. When all of the cysts have been counted, select the region of interest set and click **More** and **Save** to save the data **[1]**.

7.5.1. SCREEN: 2.3.1_to_2.3.4_t2: 02:27-02:35

7.6. To determine the size of each cysts, with the regions of interest still selected, click **Measure**. A window listing each cyst and its estimated size will appear **[1]**.

7.6.1. SCREEN: 2.3.1_to_2.3.4_t2: 02:36-02:38

7.7. Then save the data in .csv format **[1]**.

7.7.1. SCREEN: 2.3.1_to_2.3.4_t2: 02:45-02:49

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.2., 2.3., 3.4., 4.1., 4.2., 4.5.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
2.1., 4.2.

Results

8. Results: Representative Cyst Formation, Viability, Functionality, and Polarity Analyses

8.1. As demonstrated, recording the number of cysts **[1]** and their respective sizes over time allows analysis of the evolution of cyst formation and growth **[2]**.

8.1.1. LAB MEDIA: Figures 4A, 4B and 4C *Video Editor: please emphasize Figure 4B*
8.1.2. LAB MEDIA: Figures 4A, 4B and 4C *Video Editor: please emphasize Figure 4C*

8.2. The viability of the starting cell population **[1]** and the 10-day-old cysts can be evaluated by live dead staining **[2]**.

8.2.1. LAB MEDIA: Figure 5A *Video Editor: please emphasize day 0 image/green signal in day 0 image*
8.2.2. LAB MEDIA: Figure 5A *Video Editor: please emphasize day 10 image/green cysts in day 10 image*

8.3. Dead cells represent less than 3% of the cell population at day 10 **[1]** and are mostly located outside of the cysts as isolated cells or as part of small aggregates **[2]**, although some necrotic cell debris accumulation is observed within some large cysts **[3]**.

8.3.1. LAB MEDIA: Figure 5B
8.3.2. LAB MEDIA: Figure 5B *Video Editor: please emphasize red signal outside of green signal in right of image*

8.4. Incubation with fluorescein diacetate and Hoechst (hookst) **[1]** allows assessment of the formation and secretion of fluorescein from the basal to the apical luminal space **[2]**.

8.4.1. LAB MEDIA: Figures 6A and 6B *Video Editor: please sequentially emphasize Hoechst and FDA images in Figure 6A*
8.4.2. LAB MEDIA: Figures 6A and 6B *Video Editor: please emphasize merge image in Figure 6B*

8.5. Notably, the secretion of fluorescein is inhibited by pre-treatment with a multi-drug resistant inhibitor **[1]**, indicating that fluorescent fluorescein accumulation within the lumen is due to secretion through the multi-drug resistant transporter and not leakage from the intercellular space **[2]**.

8.5.1. LAB MEDIA: Figure 6C *Video Editor: please emphasize merge image*

8.5.2. LAB MEDIA: Figure 6C

8.6. Staining for E-cadherin expression reveals that the normal rat cholangiocytes maintain their epithelial phenotype in the hydrogel for at least 10 days **[1]**.

8.6.1. LAB MEDIA: Figure 7B *Video Editor: please emphasize green signal in images*

8.7. When preparing the cysts for immunofluorescence evaluation **[1]**, keeping the bovine serum albumin at 0.1% or less during saturation is key to maintaining cyst integrity, as higher concentrations result in cyst retraction and lumen collapse **[2]**.

8.7.1. LAB MEDIA: Figure 7A

8.7.2. LAB MEDIA: Figure 7A *Video Editor: please emphasize Saturation image*

Conclusion

9. Conclusion Interview Statements

9.1. **Latifa Bouzhir**: Overnight hydrogel thawing, pipette tip and chamber slide pre-cooling, working on ice, and spreading the homogeneous cell-hydrogel mixture uniformly onto the coated chamber slide are all critical for experimental success **[1]**.

9.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (2.1.-2.3., 4.1., 4.2.)

9.2. **Emilie Gontran**: This method can be used to generate cysts from other epithelial cells or hydrogels, allowing comparisons for a better understanding of epithelial polarization **[1]**.

9.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

9.3. **Lorena Loarca**: This quantitative method can be used to test the effects of drugs or genetic mutations on biliary function and organogenesis **[1]**.

9.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera