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Title: Single-Cell Resolution Three-Dimensional Imaging of Intact Organoids

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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**

If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps.

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

If **Yes**, how far apart are the locations? 1 km

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Hendrikus C.R. Ariese**: Our protocol can be used to visualize the structural complexity of organoids, allowing mapping of the identity, distribution, and cell state in these 3D structures at the single cell resolution **[1]**.

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

REQUIRED:

1.2. **Hendrikus C.R. Ariese**: Our quick, three-day protocol is fully optimized for organoids of various origins and uses a straight-forward sample preparation that includes a non-toxic optical clearing step and a silicone-based mounting method **[1]**.

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

OPTIONAL:

1.3. **Ravian L. van Ineveld**: Although our protocol is straightforward, some of the crucial steps, such as the organoid handling and slide preparation, are better explained by visual demonstration than by text **[1]**.

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

Protocol

2. Organoid Recovery

2.1. To recover 100-500-micrometer-diameter organoids grown in basement membrane extract in 24-well plates **[1]**, wash each well to be harvested with PBS without disrupting the 3D matrices **[2]** and place the plate on ice **[3]**.

2.1.1. WIDE: Talent removing plate from incubator

2.1.2. Well being washed, with PBS container visible in frame

2.1.3. Talent placing plate on ice

2.2. Add 1 milliliter of ice-cold recovery solution to each well **[1-TXT]** and place the plate on a horizontal shaker at 4 degrees Celsius for 30-60 minutes **[2-TXT]**.

2.2.1. Talent adding solution to well(s), with solution container visible in frame **TEXT: See text for all solution preparation details**

2.2.2. Talent placing plate onto shaker *Videographer: Difficult step* **TEXT: Increase incubation until BME dissolved as necessary**

2.3. Dip 1-milliliter pipette tips into a 1% BSA (B-S-A) in PBS solution **[1-TXT]** and pipette up and down two times to coat each tip with BSA **[2]**.

2.3.1. Talent dipping tip into BSA-PBS, with PBS-BSA container visible in frame *Videographer: Important step* **TEXT: BSA: bovine serum albumin**

2.3.2. Solution being pipetted *Videographer: Important step*

2.4. Next, add 5 milliliters of 1% PBS-BSA to one 15-milliliter tube per condition **[1]** and invert the tube 2-3 times before discarding the solution to coat the inside of the tube **[2]**.

2.4.1. Talent adding PBS-BSA to tube, with PBS-BSA container visible in frame *Videographer: Important step*

2.4.2. Talent inverting tube *Videographer: Important step*

2.5. To collect the organoids, use a coated tip to gently resuspend the well contents 5-10 times **[1]** and pool all of the organoids from each condition in a single, coated 15-

milliliter tube **[2]**.

2.5.1. Organoid being resuspended
2.5.2. Talent adding organoid to tube

2.6. Rinse each well with 1 milliliter of ice-cold 1% PBS-BSA to ensure that all of the organoids have been collected **[1]** and transfer the washes to the appropriate tubes **[2]**.

2.6.1. Well(s) being rinsed, with PBS-BSA container visible in frame
2.6.2. Talent adding wash to tube

2.7. Bring the final volume in each tube up to 10 milliliters with cold PBS **[1]** and sediment the organoids by centrifugation **[2-TXT]** to obtain a tight pellet without a visible layer of 3D matrix **[3]**.

2.7.1. Talent adding PBS to tube(s), with PBS container visible in frame
2.7.2. Talent adding tube(s) to centrifuge **TEXT: 3 min, 70 x g, 4 °C**
2.7.3. Shot of pellet, then supernatant being removed

3. Fixation and Blocking

3.1. To fix the organoids, use a coated 1-milliliter pipette tip to carefully resuspend each pellet in 1 milliliter of ice-cold paraformaldehyde **[1]**.

3.1.1. WIDE: Talent resuspending pellet, with PFA container visible in frame

3.2. After 45 minutes at 4 degrees Celsius, add 10-milliliters of ice-cold PBS plus Tween-20 to each tube **[1]** and gently mix by inversion **[2-TXT]** before placing the tubes at 4 degrees Celsius for 10 minutes **[3]**.

3.2.1. Talent adding PBT to tube, with PBT container visible in frame
3.2.2. Talent resuspending by pipetting **TEXT: Gently resuspend organoids halfway through fixation time** NOTE: Move this right after 3.1.1, along with the text
3.2.3. Talent placing tube(s) at 4 °C

3.3. To block the organoids, at the end of the incubation, spin down the samples **[1]** and resuspend the pellets in at least 200 microliters of ice-cold organoid washing buffer per well to be plated **[2]**.

3.3.1. Talent adding tube(s) to centrifuge **TEXT: 3 min, 70 x g, 4 °C**
3.3.2. Talent adding OWB to tube(s), with OWB container visible in frame

3.4. Then transfer the organoids to individual wells of a 24-well suspension plate for a 15-

minute incubation at 4 degrees Celsius **[1]**.

3.4.1. Talent adding organoid(s) to well

4. Immunolabeling

4.1. For immunolabeling, add 200 microliters of organoid washing buffer into an empty reference well **[1]** and allow the organoids to settle to the bottom of the plate **[2]**.

4.1.1. WIDE: Talent adding organoid washing buffer to well
4.1.2. Talent checking plate at microscope *Videographer: Important step*

4.2. When the organoids have settled, tilt the plate at a 45-degree angle **[1]** to allow removal of all but the last 200 microliters of wash buffer **[2]**.

4.2.1. Plate being tilted
4.2.2. OWB being removed

4.3. Next, add 200 microliters of organoid washing buffer containing the primary antibodies of interest **[1-TXT]** and place the plate overnight at 4 degrees Celsius with mild rocking and shaking at 40 revolutions per minute **[2]**.

4.3.1. Talent adding antibod(ies) to well, with antibody container(s) visible in frame
TEXT: See text for all Ab suggestion and concentration details
4.3.2. Added shot: Talent placing plate on shaking platform

4.4. The next morning, add 1 milliliter of organoid washing buffer to each well **[1]** and allow the organoids to settle to the bottom of the plate for 3 minutes **[2]**.

4.4.1. Talent adding OWB to well(s), with OWB container visible in frame
4.4.2. Talent setting timer, with plate visible in frame

4.5. When the organoids have settled, remove all but the last 200 microliters from each well **[1]** and wash the organoids with three, 2-hour washes with 1 milliliter of fresh organoid washing buffer and mild rocking and shaking per wash **[2]**.

4.5.1. OWB being removed from well
4.5.2. Talent placing plate onto rocker

4.6. After the third wash, allow the organoids to settle at the bottom of the plate for 3 minutes **[1]** before removing all but the last 200 microliters of organoid washing buffer from each well **[2]**.

4.6.1. Talent setting timer, with plate visible
4.6.2. OWB being removed from well

4.7. Add 200 microliter of organoid washing buffer containing the appropriate secondary antibodies to each well **[1]** for an overnight incubation at 4 degrees Celsius with mild rocking and shaking **[2]**.

4.7.1. Talent adding antibod(ies) to well(s), with antibody container(s) visible in frame
4.7.2. Plate on shaker

4.8. The next morning, wash the organoids with three, 2-hour washes in 1 milliliter of fresh organoid washing buffer per wash as demonstrated **[1]**.

4.8.1. Talent adding OWB to well(s), with OWB container visible in frame

4.9. After the last wash, transfer the organoids into one 1.5-milliliter tube per well **[1]** and collect the organoids by centrifugation **[2-TXT]**.

4.9.1. Talent adding organoids to tube(s)
4.9.2. Talent adding tube(s) to centrifuge **TEXT: 3 min, 70 x g**

5. Optical Clearing

5.1. For optical clearing of the organoids, remove as much wash buffer from each tube as possible without disrupting the organoids **[1]** and use a modified 200-microliter pipette tip to add at least 50 microliters of FUnGI (fungi) to each pellet **[2]**.

5.1.1. WIDE: Talent removing OWB *Videographer: Important step*
5.1.2. FUnGI being added to tube, with cut tip and FUnGI container visible in frame *Videographer: Important step*

5.2. After a 20-minute incubation at room temperature, the organoids can be stored for up to 1 week at 4 degrees Celsius **[1]** or for up to 6 months at minus 20 degrees Celsius **[2]**.

5.2.1. Talent placing tube at 4 °C
5.2.2. Talent placing tube at -20 °C

6. Confocal Imaging Slide Preparation

6.1. To prepare slides for organoid imaging by confocal microscopy, fill a 10-milliliter syringe with a silicone sealant **[1]** and attach a modified 200-microliter pipette tip to the syringe **[2]**.

6.1.1. WIDE: Talent filling syringe with sealant, with sealant container visible in frame
6.1.2. Tip being attached to syringe

6.2. Use the syringe to draw a rectangle of 1- x 2-centimeter in the middle of a slide **[1]** and use a second modified 200-microliter pipette tip to place cleared organoids into the middle of the rectangle **[2]**.

6.2.1. Rectangle being drawn *Videographer: Important step*
6.2.2. Organoids being added to slide *Videographer: Important step*

6.3. To place a coverslip over the organoids, place the left side of the coverslip down first **[1]** before slowly lowering the coverslip from left to right until there is no trapped air **[2-TXT]**.

6.3.1. Left side of coverslip being placed *Videographer: Important step*
6.3.2. Coverslip being lowered onto slide *Videographer: Important step* **TEXT: Optional: Place spacers between slide and coverslip**

6.4. Then gently apply pressure to all of the edges of the coverslip to firmly attach it to the silicone sealant **[1]**.

6.4.1. Pressure being applied

7. Image Acquisition and Processing

7.1. To image the organoids, place the slide onto the stage of a confocal laser scanning microscope **[1]** and select a multi-immersion 25x objective for confocal imaging **[2]**.

7.1.1. WIDE: Talent placing slide onto stage
7.1.2. Talent selecting objective

7.2. Set the microscope to the appropriate acquisition settings **[1]**, selecting a low laser power to reduce photobleaching **[2-TXT]**.

7.2.1. SCREEN: Screenshot_1: 00:00-00:25 *Video Editor: please speed up*
7.2.2. SCREEN: Screenshot_1: 00:26-00:32 **TEXT: <5% low laser power; <10% for weak staining**

7.3. Use the Z-stack mode to define the lower and upper bounds and set the Z-step size to optimal **[1]**.

7.3.1. SCREEN: Screenshot_2: 00:00-00:37 *Video Editor: please speed up*

7.4. When imaging large organoid structures or multiple organoids together, use the tiling mode with a 10% overlap and indicate the area of interest **[1]**.

7.4.1. SCREEN: Screenshot_3: 00:00-00:24 *Video Editor: please speed up*

7.5. When all of the parameters have been set, obtain a 3D-rendered representation of the imaging in the imaging software and optimize the brightness, contrast, and 3D rendering properties **[1]**.

7.5.1. SCREEN: Screenshot_4: 00:13-00:44 *Video Editor: please speed up*

7.6. Then export RGB snapshots of the results as TIFF files **[1]**.

7.6.1. SCREEN: Screenshot_5: 00:03-00:08

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?

2.3., 2.4., 4.2., 5.1., 6.2., 6.3.

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

2.2. The most important aspect is the complete removal of BME. We keep the 24-wells plate and recovery solution ice-cold, while checking the dissolving of BME under a microscope. When not completely dissolved, we increase incubation time.

Results

8. Results: Representative 3D Imaging and Organoid Clearing

8.1. The strength of 3D imaging compared to 2D imaging is illustrated by these images of mouse mammary gland organoids that were generated as demonstrated **[1]**.

8.1.1. LAB MEDIA: Figure 2A

8.2. The central layer of these representative organoids consists of columnar-shaped K8-K18 (K-eight-K-eighteen)-positive luminal cells **[1]** and the outer layer contains elongated K5-postive basal cells **[2]**, recapitulating the morphology of the mammary gland in vivo **[3]**.

8.2.1. LAB MEDIA: Figure 2A *Video Editor: please emphasize blue signal in left and/or right image*

8.2.2. LAB MEDIA: Figure 2A *Video Editor: please emphasize green signal in left and/or right image*

8.3. This polarized organization is challenging to appreciate from a 2D optical section of the same organoid **[1]**.

8.3.1. LAB MEDIA: Figure 2A *Video Editor: please emphasize middle image*

8.4. Another example of a complex structure that is impossible to interpret without 3D information is the network of MRP2 (M-R-P-two)-positive canaliculi that facilitate the collection of the bile fluid of human liver organoids **[1]**.

8.4.1. LAB MEDIA: Figure 2B *Video Editor: please emphasize left image*

8.5. Moreover, the obtained quality and resolution allows for semi-automated segmentation and image analysis **[1]**.

8.5.1. LAB MEDIA: Figure 2B *Video Editor: please emphasize middle image*

8.6. Thus, total cell numbers and the presence of markers can be quantified in specific cellular subtypes in whole organoids **[1]**.

8.6.1. LAB MEDIA: Figure 2B *Video Editor: please emphasize right image*

8.7. By segmenting the nuclei of an entire organoid containing 140 cells **[1]**, 3 cells that display a high positivity for the Ki67 (K-I-sixty-seven) cell cycle marker can be identified **[2]**.

8.7.1. LAB MEDIA: Figure 2C *Video Editor: please emphasize show left image then right image*

8.7.2. LAB MEDIA: Figure 2C graph *Video Editor: please emphasize/encircle top three data points*

8.8. The optical clearing agent FUnGI **[1]** outperforms uncleared **[2]** and fructose-glycerol-clearing in fluorescent signal quality deep within an organoid **[3]**, with FUnGI-cleared organoids demonstrating an overall enhanced fluorescence intensity **[4]** compared to uncleared organoids **[5]**.

8.8.1. LAB MEDIA: Figures 3A and 3B *Video Editor: please emphasize FUnGI images and blue data line*

8.8.2. LAB MEDIA: Figures 3A and 3B *Video Editor: please emphasize No Clearing images and black data line*

8.8.3. LAB MEDIA: Figures 3A and 3B *Video Editor: please emphasize Fructose-glycerol images and red data line*

8.8.4. LAB MEDIA: Figures 3C *Video Editor: please emphasize blue FUnGI data bar*

8.8.5. LAB MEDIA: Figures 3C *Video Editor: please emphasize black No Clearing data bar*

Conclusion

9. Conclusion Interview Statements

9.1. **Ravian L. van Ineveld**: Note that leftover BME in the sample can impair antibody penetration and may result in high background signal and that the clearing step should be skipped when cystic organoids collapse **[1]**.

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

9.2. **Ravian L. van Ineveld**: With slight adaptations to the protocol, the samples can be made compatible with super-resolution confocal, multi-photon, and light sheet microscopy imaging **[1]**.

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