Video Article

Single-Cell Resolution Three-Dimensional Imaging of Intact Organoids

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Abstract

Organoid technology, in vitro 3D culturing of miniature tissue, has opened a new experimental window for cellular processes that govern organ development and function as well as disease. Fluorescence microscopy has played a major role in characterizing their cellular composition in detail and demonstrating their similarity to the tissue they originate from. In this article, we present a comprehensive protocol for high-resolution 3D imaging of whole organoids upon immunofluorescent labeling. This method is widely applicable for imaging of organoids differing in origin, size and shape. Thus far we have applied the method to airway, colon, kidney, and liver organoids derived from healthy human tissue, as well as human breast tumor organoids and mouse mammary gland organoids. We use an optical clearing agent, FUnGI, which enables the acquisition of whole 3D organoids with the opportunity for single-cell quantification of markers. This three-day protocol from organoid harvesting to image analysis is optimized for 3D imaging using confocal microscopy.

Introduction

The advancement of novel culture methods, such as organoid technology, has enabled the culture of organs in a dish¹. Organoids grow into three-dimensional (3D) structures that resemble their tissue of origin as they preserve phenotypic and functional traits. Organoids are now instrumental for addressing fundamental biological questions², modelling diseases including cancer³, and developing personalized treatment strategies^{4,5,6,7}. Since the first protocol for generating organoids derived from intestinal adult stem cells⁸, organoid technology has extended to include a wide range of healthy and cancerous tissues derived from organs including prostate⁹, brain¹⁰, liver^{11,12}, stomach¹³, breast^{14,15}, endometrium¹⁶, salivary gland¹⁷, taste bud¹⁸, pancreas¹⁹, and kidney²⁰.

The development of organoids has concurred with the rise of new volumetric microscopy techniques that can visualize the architecture of whole mount tissue in 3D^{21,22,23,24}. 3D imaging is superior to traditional 2D tissue section imaging in visualizing the complex organization of biological specimens. 3D information proves to be essential for understanding cellular composition, cell shape, cell-fate decisions and cell-cell interactions of intact biological samples. Nondestructive optical sectioning techniques, such as confocal or multi-photon laser scanning microscopy (CLSM and MLSM) and light sheet fluorescence microscopy (LSFM), now enable the combined visualization of both fine details, as well as general tissue architecture, within a single biological specimen. This powerful imaging approach provides the opportunity to study the structural complexity that can be modeled with organoids²⁵ and map the spatial distribution, phenotypic identity and cellular state of all individual cells composing these 3D structures.

Recently we published a detailed protocol for high-resolution 3D imaging of fixed and cleared organoids²⁶. This protocol is specifically designed and optimized for processing delicate organoid structures, as opposed to methodologies for large intact tissues such as DISCO^{27,28}, CUBIC^{29,30,31}, and CLARITY^{32,33}. As such, this method is generally applicable to a wide variety of organoids differing in origin, size and shape and cellular content. Furthermore, as compared to other volumetric imaging protocols that often require considerable time and effort, our protocol is undemanding and can be completed within 3 days. We have applied our 3D imaging protocol to visualize the architecture and cellular composition of newly developed organoid systems derived from various tissues, including human airways³⁴, kidney²⁰, liver¹¹, and human breast cancer organoids¹⁵. In combination with multicolored fluorescent lineage tracing, this method has also been used to reveal the biopotency of basal cells in mouse mammary organoids¹⁴.

Here, we refine the protocol by introducing the nontoxic clearing agent FUnGI³⁵. FUnGI clearing is achieved in a single incubation step, is easier to mount due to its viscosity, and better preserves fluorescence during storage. In addition, we introduce sodium dodecyl sulfate (SDS) to the wash buffer to enhance nuclear stainings as well as a silicone based-mounting method for easy slide preparation prior to microscopy. **Figure 1** provides the graphical overview of the protocol (**Figure 1A**) and examples of 3D-imaged organoids (**Figure 1B–D**). In short, organoids are recovered from their 3D matrix, fixed and immunolabeled, optically cleared, imaged using confocal microscopy and then 3D rendered with visualization software.

Protocol

Use of mouse-derived organoids conformed to regulatory standards and was approved by the Walter and Eliza Hall Institute (WEHI) Animal Ethics Committee. All human organoid samples were retrieved from biobanks through the Hubrecht Organoid Technology (HUB, www.hub4organoids.nl). Authorizations were obtained by the Medical Ethical Committee of UMC Utrecht (METC UMCU) at request of the HUB in order to ensure compliance with the Dutch Medical Research Involving Human Subjects Act and informed consent was obtained from donors when appropriate.

1. Preparation of reagents

- To prepare 4% (w/v) paraformaldehyde (PFA), heat 400 mL of phosphate-buffered saline (PBS) to just under 60 °C in a water bath. Add 20 g of PFA powder and dissolve using a stirrer.
 - Next, add a few drops of 10 M NaOH. Let cool on ice and add a few drops of 10 M HCl to adjust the pH to 7.4. Top up with PBS to 500 mL and aliquot (store at -20 °C for up to 2 months).
NOTE: Do not heat above 60 °C to avoid degradation of the PFA. Preparation time = 4 h.
- To prepare PBS with Tween-20 (PBT) (0.1% v/v), add 1 mL of Tween-20 to 1 L of PBS (store at 4 °C for up to 4 weeks).
NOTE: Preparation time = 10 min.
- To prepare 100 mL of 0.5 M ethylenediaminetetraacetic acid (EDTA), add 18.6 g of EDTA and 2.5 g of NaOH to 80 mL of dH₂O. Adjust the pH to 8 with 1 M NaOH and fill to 100 mL with dH₂O.
- To prepare 500 mL of 1 M Tris, dissolve 60.55 g of Tris with 42 mL of concentrated (36–38%) HCl in 300 mL of dH₂O. Adjust the pH to 8 and fill to 500 mL.
- To prepare organoid washing buffer (OWB), add 1 mL of Triton X-100, 2 mL of 10% (w/v) SDS and 2 g of bovine serum albumin (BSA) to 1 L of PBS (store at 4 °C up to 2 weeks).
NOTE: Preparation time = 10 min.
- To make 220 mL of FUnGI, mix 110 mL of glycerol with 20 mL of dH₂O, 2.2 mL of Tris buffer (1 M, pH 8.0) and 440 µL of EDTA (0.5 M). Add 50 g of fructose and mix at room temperature (RT) in the dark until dissolved. When clear, add 49 g of fructose and mix until dissolved. Then add 33.1 g of urea and mix until dissolved (store at 4 °C in the dark).
NOTE: Do not heat as fructose caramelizes at higher temperatures. FUnGI consists of 50% (v/v) glycerol, 9.4% (v/v) dH₂O, 10.6 mM tris base, 1.1 mM EDTA, 2.5 M fructose and 2.5 M urea. Preparation time = 1 day.
- To prepare PBS-BSA (1% w/v), dissolve 1 g of BSA in 100 mL of PBS (store at 4 °C up to 2 weeks).
NOTE: Preparation time = 10 min.

2. Organoid recovery

NOTE: The following steps apply to organoids grown in basement membrane extract (BME) that were cultured in a 24 well plate with a size of 100–500 µm.

- Aspirate the culture medium and wash 1x with ice-cold PBS. Avoid disrupting the 3D matrix.
- Put the plate on ice and add 1 mL of ice-cold cell recovery solution (**Table of Materials**) to each well. Incubate 30–60 min at 4 °C on a horizontal shaker (40 rpm).
NOTE: The 3D matrix droplets should be completely dissolved.
- Coat a 1 mL pipette tip with BSA by dipping in 1% PBS-BSA and pipetting up and down 2x. This coating will prevent organoids from sticking to the tip. To coat the inner side of a 15 mL conical tube, fill with 5 mL of 1% PBS-BSA, invert 2–3x and discard the PBS-BSA.
NOTE: This coating is necessary for all plastic consumables until fixation (step 3.3).
- Using a coated tip, gently resuspend the content of the well 5–10x and transfer the organoids to coated 15 mL tubes. Organoids from different wells with the same identity can be pooled into the same tube.
- Add 1 mL of ice-cold 1% PBS-BSA to each culture well to rinse and collect all organoids.
- Add cold PBS to 10 mL and spin down for 3 min at 70 x g and 4 °C to obtain a tight pellet without a visible layer of 3D matrix. Carefully remove the supernatant.

3. Fixation and blocking

- Carefully resuspend the organoids in 1 mL of ice-cold PFA using a coated 1 mL tip.
- Fix at 4 °C for 45 min. Gently resuspend the organoids halfway through the fixation time using a coated 1 mL tip to ensure even fixation among all organoids.
- Add 10 mL of ice-cold PBT to the tube, gently mix by inverting the tube, incubate for 10 min and spin down at 70 x g, both at 4 °C.
NOTE: From this step onwards coating of tips is generally not needed as most organoid types do not stick to the tip after fixation. However, some organoids may require coated plastics even after fixation.
- Block the organoids by resuspending the pellet in ice-cold OWB (at least 200 µL of OWB per well) and transfer the organoids to a 24 well suspension plate.
NOTE: Organoids from one large pellet can be split over multiple wells to perform different stainings.
- Incubate at 4 °C for at least 15 min.

4. Immunolabeling

1. Pipette 200 μ L of OWB in an empty well to serve as a reference well.
NOTE: The immunolabeling can also be performed in 48- or 96-wells plates to reduce antibody usage. However, the user should be aware that both staining and washing performance could be reduced due to the smaller volume.
2. Allow the organoids to settle at the bottom of the plate.
NOTE: This can be checked using a stereomicroscope and is made easier by using a dark background.
3. Tilt the plate 45° and remove OWB leaving the organoids in 200 μ L of OWB (use the reference well to estimate 200 μ L).
4. Add 200 μ L of OWB with primary antibodies 2x concentrated (e.g., E-cadherin [1:400] and Ki67 [1:200] for results in **Figure 1**; keratin 5 [1:500], keratin 8/18 [1:200], MRP2 [1:50], and Ki67 [1:200] for results in **Figure 2**) and incubate overnight at 4 °C while mildly rocking/shaking (40 rpm on horizontal shaker).
5. The next day, add 1 mL of OWB.
6. Allow the organoids to settle at the bottom of the plate for 3 min. Remove OWB leaving 200 μ L in the plate. Add 1 mL of OWB and wash 2 h with mild rocking/shaking.
7. Repeat step 4.6 two more times.
8. Allow the organoids to settle at the bottom of the plate for 3 min. Remove OWB leaving 200 μ L in the well.
9. Add 200 μ L of OWB with secondary antibodies, conjugated antibodies and dyes 2x concentrated (e.g., DAPI [1:1000], rat-AF488 [1:500], mouse-AF555 [1:500], phalloidin-AF647 [1:100] for results in **Figure 1**; DAPI [1:1000], rat-AF488 [1:500], rabbit-AF555 [1:500], mouse-AF555 [1:500], phalloidin-AF647 [1:100] for results in **Figure 2**; DAPI [1:1000], phalloidin-AF647 [1:100] for results in **Figure 3**) and incubate overnight at 4 °C while mildly rocking/shaking.
10. The next day, repeat steps 4.5–4.7.
11. Transfer the organoids to a 1.5 mL tube and spin down at 70 x g for 3 min.

5. Optical clearing of organoids

1. Remove as much as possible the OWB by pipetting without disrupting the organoids.
2. Add FUnGI (at least 50 μ L, RT) using a 200 μ L tip with the end cut off and resuspend gently to prevent bubble formation. Incubate at RT for 20 min.
NOTE: Optical clearing by FUnGI may cause minor tissue shrinkage. This will not affect the general morphology of monolayered and multilayered organoids; however, spherical shaped monolayered organoids with large lumens may collapse. The protocol can be paused here and samples can be stored at 4 °C (for at least 1 week) or at -20 °C (for at least 6 months).

6. Slide preparation for confocal imaging

1. Prepare a 10 mL syringe with a silicone sealant (**Table of Materials**). Attach a 200 μ L tip and cut off the end to allow a gentle flow of the viscous silicone after pressing the syringe. Use the syringe to draw a rectangle of 1 cm x 2 cm in the middle of a slide.
2. Cut off the end of a 200 μ L tip and transfer the organoids in FUnGI to the middle of the rectangle.
3. Place a coverslip on top. To minimize trapped air bubbles, place the left side of the coverslip first, then slowly lower the coverslip from left to right until there is no trapped air and then release the coverslip.
NOTE: Spacers that are similar in size to the organoids can be used to prevent them from being damaged.
4. Gently apply pressure on all edges of the coverslip to firmly attach it to the silicone sealant. The slide is now ready for imaging.
NOTE: The protocol can be paused here and the sample can be stored at 4 °C (for at least 1 week) or -20 °C (for at least 6 months).

7. Image acquisition and processing

1. Using a confocal laser scanning microscope (**Table of Materials**), image the slide with a multi-immersion 25x or oil immersion 40x objective for confocal imaging.
 1. Use the following acquisition settings for the 25x objective: scan mode frame, frame size 1024 x 1024, voxel size 332 nm x 332 nm x 1.2 μ m, pixel dwell time <2 μ s, bidirectional scanning, averaging number 1, bit depth 8. To reduce photobleaching, use low laser power (<5% in general, <10% for weak staining).
 2. Use the Z-stack mode to define the lower and upper bounds and set the Z-step size to optimal. When imaging large organoid structures or multiple organoids together, use the tiling mode with 10% overlap and indicate the area of interest.
NOTE: With these settings, the data size for a typical organoid with a diameter of <300 μ m is <1 GB.
2. For tile scan datasets, stitch the imaging files in the software accompanied with the microscope (**Table of Materials**). In the processing section, select **Stitching** as the method, choose **New Output** under parameters and select the file to stitch. Press **Apply** to start stitching.
3. Obtain a 3D rendered representation of the imaging under the **3D View** tab in the imaging software (**Table of Materials**) and subsequently optimize brightness, contrasting and 3D rendering properties. Export RGB snapshots of the results as TIFF files.

Representative Results

Imaging organoids in 3D enables visualization of architecture, cellular composition as well intracellular processes in great detail. The presented technique is undemanding and can presumably be applied to a wide range of organoid systems that are derived from various organs or host species.

The strength of 3D imaging compared to 2D imaging is illustrated by images of mouse mammary gland organoids that were generated using recently published methods¹⁴. The central layer of these organoids consists of columnar-shaped K8/K18-positive luminal cells and the outer

layer contains elongated K5-positive basal cells (**Figure 2A**), which recapitulates the morphology of the mammary gland in vivo. This polarized organization is challenging to appreciate from a 2D optical section of that same organoid (**Figure 2B**, middle panel). Another example of a complex structure that is impossible to interpret without 3D information is the network of MRP2-positive canaliculi that facilitate the collection of the bile fluid of human liver organoids¹¹ (**Figure 2B**). This exemplifies how our method allows visualization of essential structural features of organoids. Moreover, the obtained quality and resolution allows for semi-automated segmentation and image analysis. Thus, total cell numbers and presence of markers can be quantified in specific cellular subtypes in whole organoids. We illustrate this by segmenting the nuclei of an entire organoid containing 140 cells, of which 3 cells display high positivity for the Ki67 cell cycle marker (**Figure 2C**). The DAPI channel is selected as source channel, and segments are generated based on an intensity thresholding step and a sphere diameter of 10 μm . Touching objects are split by region growing from seed points. Lastly, a size filter of 10 voxels is applied to remove small noise induced segments. For every segment representing a nucleus, the mean intensity of the Ki67 channel is then exported for plotting.

We recently developed the optical clearing agent FUnGI³⁵, which we now integrated into this protocol to refine the transparency of the organoids. FUnGI is easy to use, as clearing is readily achieved by a single incubation step after immunofluorescent staining. An added advantage of the agent is its viscosity, which makes it easier for sample handling during slide mounting. Fluorescent samples in FUnGI preserve their fluorescence even when stored for multiple months at $-20\text{ }^{\circ}\text{C}$. We demonstrate that FUnGI outperforms uncleared and fructose-glycerol in fluorescent signal quality deep in the organoid (**Figure 3A,B**), and that FUnGI-cleared organoids have overall enhanced fluorescence intensity compared to uncleared organoids (**Figure 3C**).

In summary, we describe an undemanding, reproducible 3D imaging technique for acquiring volumetric data of immunolabeled organoids. This protocol can be readily used to image a variety of organoids including those of both mouse and human origin, from healthy and disease models. The straightforward sample preparation can be adapted to facilitate confocal, multi-photon and light sheet fluorescent microscopes to obtain cellular to subcellular resolution of entire organoids.

Figure 1: Schematic overview of the high-resolution 3D imaging protocol. Organoids are recovered from their 3D matrix. Fixation and blocking is performed prior to immunolabeling with antibodies and dyes. Optical clearing is achieved in a single step using the FUnGI clearing agent. 3D rendering of images can be performed by using imaging software. **(A)** Schematic overview of the procedure. **(B)** Cleared whole-mount 3D confocal image of a human colonic organoid immunolabeled for F-actin and E-cadherin (E-cad) (25x oil objective). Scale bar = 40 μm . **(C)** Cleared whole-mount 3D confocal image. Scale bar = 20 μm . **(D)** Enlarged optical section of a human colonic organoid immunolabeled for F-actin, E-cadherin (E-cad) and Ki67 (25x oil objective). Scale bar = 5 μm . This figure has been modified from Dekkers et al.²⁶. [Please click here to view a larger version of this figure.](#)

Figure 2: Volumetric imaging visualizes complex 3D architecture. Confocal images representing whole-mount 3D datasets (left panel), 2D optical sections (middle panel) and 3D areas of an enlarged region (right panel). **(A)** An organoid derived from a single basal cell of the mouse mammary gland illustrating the 3D organization of elongated mammary basal cells that surround luminal cells or labeled for K8/18, K5 and F-actin (fructose-glycerol clearing; 25x oil objective). Scale bars represent 55 μm (left panel) and 40 μm (middle and right panels). **(B)** A human fetal liver organoid with a complex 3D network of MRP2-positive canaliculi, labeled for DAPI, MRP2 and F-actin (fructose-glycerol clearing; 40x oil objective). Scale bars represent 25 μm (left panel) and 8 μm (middle and right panels). **(C)** Confocal 3D whole-mount image of a human fetal liver organoid labeled with DAPI and Ki67 (left panel) and a segmented image on the DAPI channel using imaging software (middle panel). Scale bar = 15 μm . Graph plotted representing the Ki67 mean intensity in all the cells (DAPI-segmented) of the entire organoid (140 cells) (right panel). This figure has been modified from Dekkers et al.²⁶. [Please click here to view a larger version of this figure.](#)

Figure 3: Optical clearing of organoids with FUNGI. (A) Representative images of human colonic organoids labeled with F-actin (green) and DAPI (grey) and imaged with no clearing, cleared with fructose-glycerol or cleared with FUNGI (25x oil objective). Left panel: 3D rendering of the organoid. Right panel: optical-section of the organoid at 150 µm depth. For the “no clearing” condition the brightness of the image had to be increased in comparison to the “fructose-glycerol” and “FUNGI” conditions to visualize the organoid. Scale bar = 50 µm. (B) Nonlinear regression fit showing the decrease of DAPI intensity with increasing Z-depth for different optical clearing methods. Values represent intensities of individual cells detected by DAPI segmentation and are normalized to the average DAPI intensity of the first 50 µm of the organoid. To avoid underestimation of the attenuation caused by brighter cells on the deeper edges and budding structures, only the center regions of the organoids were analyzed. (C) Three organoids per condition of similar size and depth towards the coverslip were imaged using identical microscope settings. The full 3D datasets were single cell segmented on DAPI signal for comparison. Bar graph showing average DAPI intensity with different clearing methods on full segmented datasets. Data are depicted as mean ± SD. Values are intensities of >3800 individual cells detected by DAPI segmentation. **** = $p < 0.0001$, Kruskal-Wallis test with two-sided Dunn's multiple comparison post-hoc testing. [Please click here to view a larger version of this figure.](#)

Discussion

Here, we put forward a detailed protocol for 3D imaging of intact organoids with single-cell resolution. To successfully perform this protocol, some critical steps have to be taken. In this section we highlight these steps and provide troubleshooting.

The first critical step is the removal of the 3D matrix. Most organoids are propagated *in vitro* with the use of matrices that mimic the *in vivo* extracellular environment to enhance the formation of well-polarized 3D structures. Fixating and subsequent staining within the 3D matrix is possible, but can be disadvantageous for the penetration of antibodies or can generate high background signal (data not shown). Efficient removal of matrices can be influenced by the type of matrix, the amount and size of the organoids and prolonged culturing. Therefore, optimization may be required for different culture conditions. For organoids cultured in Matrigel or BME, a 30–60 min step in ice-cold cell recovery solution is sufficient to dissolve the matrix without damaging the organoids. In addition, removal of the supporting 3D matrix could result in loss of native structures and disruption of organoid contacts with other cell types, for instance when organoids are co-cultured with fibroblasts or immune cells. Furthermore, optimal organoid fixation is crucial in preserving 3D tissue architecture, protein antigenicity and minimizing autofluorescence. Fixing for 45 min with 4% PFA at 4 °C is normally sufficient for labeling of a wide range of organoids and antigens. However, a longer fixation step, up to 4 h, is typically more appropriate for organoids expressing fluorescent reporter proteins, but will require optimization for different fluorophores. Fixation times shorter than 20 min are insufficient to properly label F-actin using phalloidin probes. Another common issue is the loss of organoids during the protocol. It is therefore important to (i) carefully coat pipet tips and tubes with 1% BSA-PBS as described when handling unfixed organoids to prevent them from sticking to plastics, (ii) use low-adherence or suspension plates to avert the sample from sticking to the plate, and (iii) allow enough time for the organoids to settle at the bottom of the plate before carefully removing buffers. Pipetting viscous FUNGI may introduce bubbles. Handling the cleared sample at RT decreases viscosity and improves ease-of-use, thereby minimizing loss of organoids. While most organoids are easy to handle, cystic organoids with an enlarged lumen have a high tendency to collapse when fixing with 4% PFA or when cleared with FUNGI. This effect can be reduced, but not completely prevented, by using a different fixative (e.g., formalin or PFA-glutaraldehyde). However, this could potentially impact autofluorescence, antibody penetration and epitope availability. When cystic organoids appear folded after clearing, it is advised to skip the clearing step and image by multi-photon microscopy, which is less hampered by light scattering. Lastly, obtaining the entire 3D structure of organoids can be challenging and requires minimal distance between coverslip and organoid. In addition, when organoids have room to move in their mounting agent, this can result in X- and Y-shifts while recording data in Z-depth. Using less silicone sealant during slide preparation can solve suboptimal mounting between coverslip and microscope slide.

However, too little silicone may lead to organoid compression and loss of their inherent 3D structure. FUnGI improves handling for slide mounting and stability of organoids while imaging, due to its higher viscosity.

While this protocol can be used for a broad range of applications to study in depth cellular content and 3D architecture of intact organoids, certain limitations should be considered. This methodology is rather low-throughput and time-consuming. Indeed, users should bear in mind that imaging large intact organoids in 3D requires both tiling and sample acquisition in Z, leading to prolonged acquisition times. Faster imaging could be achieved by using microscope assets, including resonant or spinning disk scanner, or by light sheet microscope technology²⁶. Another consideration is that markers can be heterogeneously expressed between different organoids from the same sample. Therefore, multiple organoids should be acquired to better capture this organoid heterogeneity in culture. Lastly, while the complete wet lab procedure is straightforward, postprocessing of data requires skills in image analysis software for 3D visualization and quantification, as well as statistics for mining all the information present in the dataset.

In the last decade, the field of volume imaging has greatly advanced, due to both the development of a wide range of optical clearing agents and improvements in microscopy and computational technologies^{27,30,31}. While in the past most studies focused on large volume imaging of organs or associated tumors, more recently methods for smaller and more fragile tissues, including organoid structures, have been developed^{36,37,38}. We recently published a simple and fast method for imaging whole-mount organoids of various origin, size and shape at the single cell level for subsequent 3D rendering and image analysis²⁶, which we presented here with some improvements (e.g., FUnGI, silicone mounting) and accompanied by a video protocol. This method is superior to conventional 2D section-based imaging in deciphering complex cell morphology and tissue architecture (**Figure 2**) and easy to implement in laboratories with a confocal microscope. With slight adaptations to the protocol, the samples can be made compatible with, super-resolution confocal, multi-photon as well as light sheet imaging, which makes this protocol widely applicable and provides users with a powerful tool to better comprehend the multidimensional complexity that can be modeled with organoids.

Disclosures

The authors have nothing to disclose.

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