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Single-Cell Resolution Three-Dimensional Imaging of Intact Organoids

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Letter to the editor

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To Benjamin Werth, BS
Science Editor
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Dear Benjamin Werth,

After your kind invitation, we hereby submit our manuscript entitled 'Single-cell resolution three-dimensional imaging of intact organoids' by Ravian L. van Ineveld, Hendrikus C.R. Ariese, Ellen J. Wehrens, Johanna F. Dekkers and Anne C. Rios

If you have any questions, please do not hesitate to contact me.

Sincerely,

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TITLE:

Single-Cell Resolution Three-Dimensional Imaging of Intact Organoids

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KEYWORDS:

organoid, single-cell resolution, 3D imaging, confocal microscopy, immunolabelling, optical clearing

SUMMARY:

The entire 3D structure and cellular content of organoids, as well as their phenotypic resemblance to the original tissue can be captured using the single-cell resolution 3D imaging protocol described here. This protocol can be applied to a wide range of organoids varying in origin, size and shape.

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 dish[1]. Organoids grow into three-dimensional (3D) structures that ressemble their tissue of origin as they preserve phenotypic and functional traits. Organoids are now instrumental for addressing fundamental biological questions[2], modelling diseases including cancer[3], and developing personalized treatment strategies[4–7]. Since the first protocol for generating organoids derived from intestinal adult stem cells[8], organoid technology has extended to include a wide range of healthy and cancerous tissues derived from organs including prostate[9], brain[10], liver[11,12], stomach[13], breast[14,15], endometrium[16], salivary gland[17], taste bud[18], pancreas[19], and kidney[20].

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[21–24]. 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 organoids[25] 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 organoids[26]. 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[29–31], 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 airways[34], kidney[20], liver[11], and human breast cancer organoids[15]. In combination with multicolored fluorescent lineage tracing, this method has also been used to reveal the biopotency of basal cells in mouse mammary organoids[14].

Here, we refine the protocol by introducing the nontoxic clearing agent FUnGI[35]. 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

1.1. 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.

1.1.1. 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.

1.2. 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.

1.3. 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 dH_2O. Adjust the pH to 8 with 1 M NaOH and fill to 100 mL with dH_2O.

1.4. 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 dH_2O. Adjust the pH to 8 and fill to 500 mL.

1.5. 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.

1.6. To make 220 mL of FUnGI, mix 110 mL of glycerol with 20 mL of dH_2O, 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.

1.7. 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 .

2.1. Aspirate the culture medium and wash 1x with ice-cold PBS. Avoid disrupting the 3D matrix.

2.2. 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.

2.3. 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).

2.4. 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.

2.5. Add 1 mL of ice-cold 1% PBS-BSA to each culture well to rinse and collect all organoids.

2.6. 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

3.1. Carefully resuspend the organoids in 1 mL of ice-cold PFA using a coated 1 mL tip.

3.2. 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.

3.3. 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.

3.4. 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.

3.5. Incubate at 4 °C for at least 15 min.

4. Immunolabeling

4.1. Pipette 200 µL 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.

4.2. 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.

4.3. Tilt the plate 45° and remove OWB leaving the organoids in 200 µL of OWB (use the reference well to estimate 200 µL).

4.4. Add 200 µL 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).

4.5. The next day, add 1 mL of OWB.

4.6. Allow the organoids to settle at the bottom of the plate for 3 min. Remove OWB leaving 200 µL in the plate. Add 1 mL of OWB and wash 2 h with mild rocking/shaking.

4.7. Repeat step 4.6 two more times.

4.8. Allow the organoids to settle at the bottom of the plate for 3 min. Remove OWB leaving 200 µL in the well.

4.9. Add 200 µL 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.

4.10. The next day, repeat steps 4.5–4.7.

4.11. Transfer the organoids to a 1.5 mL tube and spin down at 70 x *g* for 3 min.

5. Optical clearing of organoids

5.1. Remove as much as possible the OWB by pipetting without disrupting the organoids.

5.2. Add FUnGI (at least 50 µL, RT) using a 200 µL 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

6.1. Prepare a 10 mL syringe with a silicone sealant (**Table of Materials**). Attach a 200 µL 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.

6.2. Cut off the end of a 200 µL tip and transfer the organoids in FUnGI to the middle of the rectangle.

6.3. 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 simililar in size to the organoids can be used to prevent them from being damaged.

6.4. 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

7.1. 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.

7.1.1. 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 µm, pixel dwell time <2 µs, bidirectional scanning, averaging number 1, bit depth 8. To reduce photobleaching, use low laser power (<5% in general, <10% for weak staining).

7.1.2. 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 µm is <1 GB.

7.2. 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.

7.3. 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 methods[14]. The central layer of these organoids consists of columnar-shaped K8/K18-positive luminal cells and the outer layer contains elongated K5-postive 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 organoids[11] (**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 FUnGI[35], 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 °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 LEGENDS:

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].

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].

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.

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 technology[26]. 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[36–38]. 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 analysis[26], 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.

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DISCLOSURES:

The authors have nothing to disclose.

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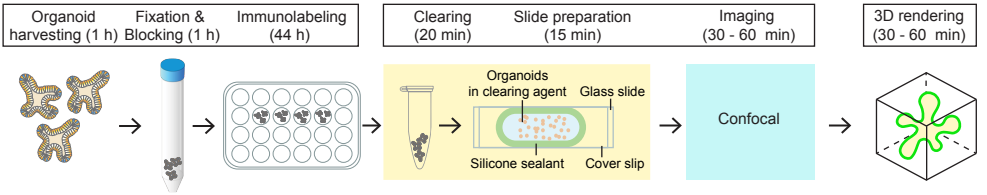
Figure

A

Click here to access/download;Figure;Ineveld,



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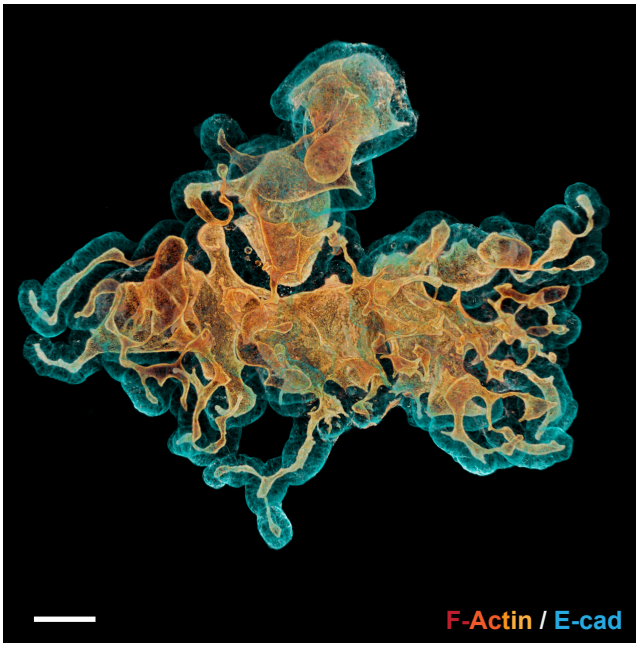

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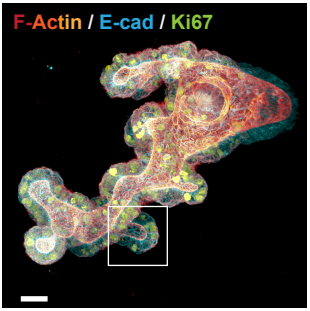

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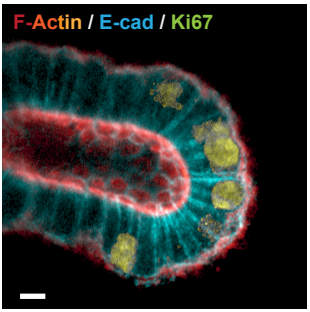
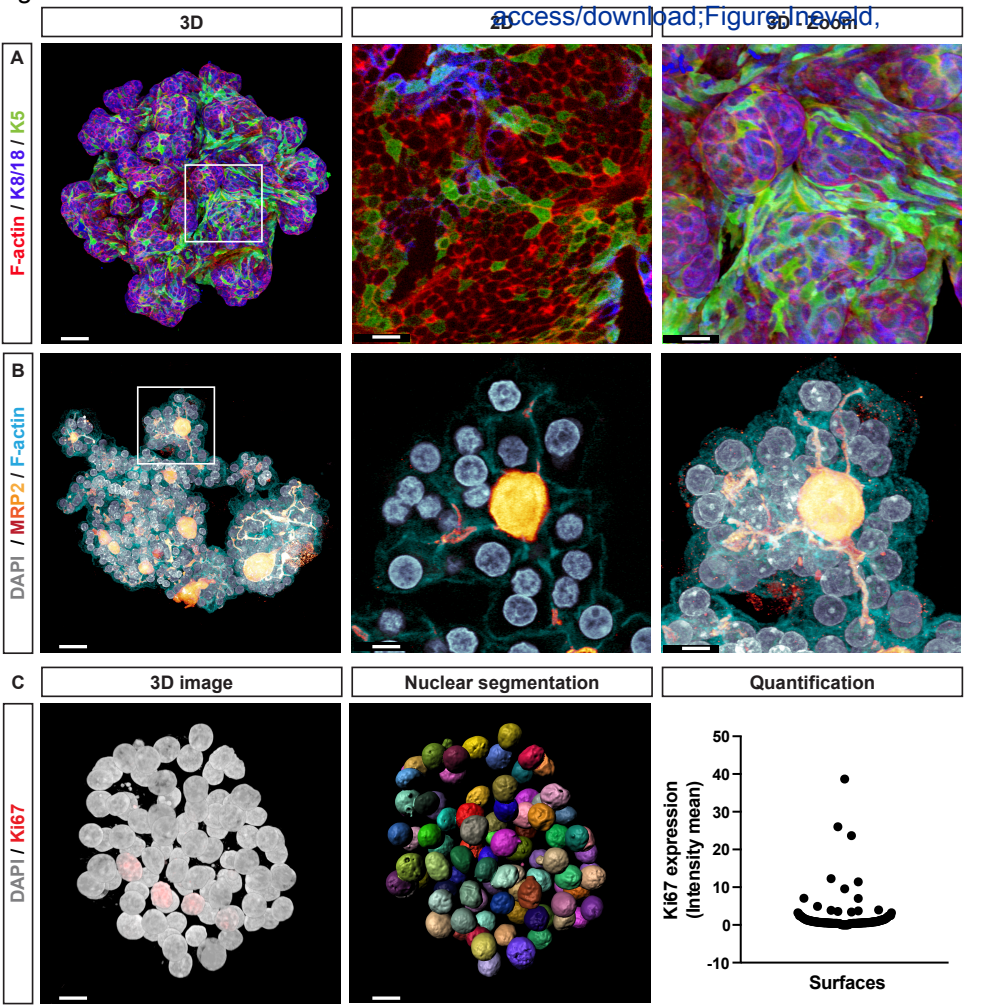
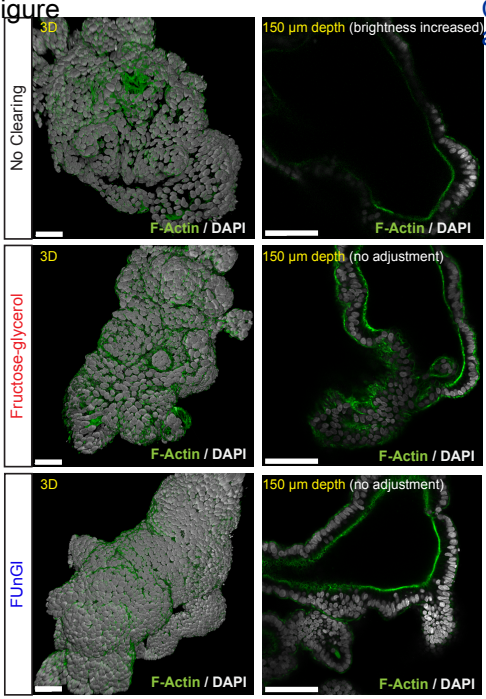
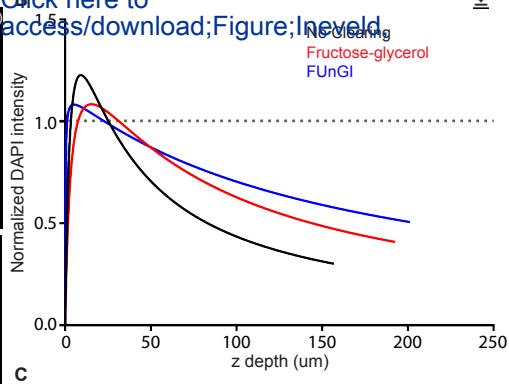


Figure



Figure





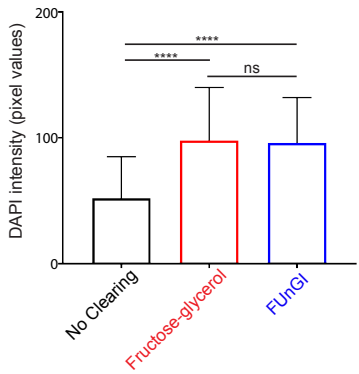


Table of Materials

Click here to access/download;Table of Materials;60709_JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 ml safe-lock centrifuge tubes	Eppendorf	EP0030 120.094	
2 ml safe-lock centrifuge tubes	Eppendorf	EP0030 120.094	
Bovine Serum Albumin (BSA)	Sigma Aldrich	A3059	
Cell recovery solution	Corning	354253	
Confocal microscope	Zeiss	LSM880	
Confocal microscope software ZEN	Zeiss	ZEN black/blue	
Conical tubes 15 ml	Greiner Bio-One	5618-8271	
Coverglass #1.5 24x60mm	Menzel-Glazer	G418-15	
Coverglass #1.5 48x60mm	ProSciTech	G425-4860	
DAPI	ThermoFisher	D3571	dilution 1:1000
Dissection Stereomicroscope	Leica	M205 FA	
Double sided sticky tape 12,7 mm 6,35 m	Scotch 3M		
Dulbecco's Phosphate-bufferd Saline (DPBS)	Gibco	14190144	1x
EDTA	Invitrogen	15576-028	
Focus Clear	CelExplorer	FC-101	
Fructose	Sigma Aldrich	F0127	
Glycerol	Boom	76050771.0500	
Graduated Transfer Pipets	Samco	222-15	
Horizontal shaker	VWR	444-2900	
Hydrochloric acid (HCl)	Ajax Firechem.	265.2.5L-PL	10M stock solution, corrosive
Imaris software	Bitplane		
Microscope slides Superfrost	ThermoFisher	10143352	ground edge, 90 degrees 26 mm~76 mm
Paraformaldehyde	Sigma Aldrich	P6148-500g	Hazardous
Phalloidin Alexa Fluor 647	ThermoFisher	A22287	dilution 1:100-200
E-cadherin	ThermoFisher	13-1900	dilution 1:400
Ki-67	BD Biosciences	B56	dilution 1:200
Keratin 5	BioLegend	905501	dilution 1:500

Keratin 8/18	DSHB (University of Iowa)			dilution 1:200
MRP2	Abcam	ab3373		dilution 1:50
Secondary Rat IgG (H+L) Alexa Fluor 488	ThermoFisher	A-21208		dilution 1:500
Secondary Mouse IgG (H+L) Alexa Fluor 555	ThermoFisher	A-31570		dilution 1:500
Secondary Rabbit IgG (H+L) Alexa Fluor 555	ThermoFisher	A-31572		dilution 1:500
Silicone Sealant	Griffon	S-200		
Sodium Dodecyl Sulfate	ThermoFisher		28312	
Sodium Hydroxide (NaOH) pellets	Merck Millipore	567530		10 M stock solution, corrosive
Suspension cell culture plates	Greiner Bio-One	662102		24-well
Tris	Fisher Scientific	11486631		
Triton X-100	Sigma Aldrich	T8532		Hazardous
Tween-20	Sigma Aldrich	P1379		
UltraPure Low Melting Point (LMP) Agarose	ThermoFisher	16520050		
Urea	Sigma Aldrich	51456		
Workstation	Dell			

Rebuttal to peer review comments

We thank the editorial board for considering our manuscript and addressed the comments below.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please revise lines 65-69, 108-110, 222-223, 254-256, 264-266, 335-343, and 369-371 to avoid textual overlap with previously published work.

We have revised the mentioned sections.

3. Authors and affiliations: Please provide an email address for each author in the manuscript.

These are now added.

4. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

We adapted the text to remove personal pronouns and colloquial phrases.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Zeiss, Leica, Olympus, etc.

We removed company names in the text and referred to the Table of Materials.

6. Please define acronyms/abbreviations (PFA, PBT, SDS, BSA, etc.) upon first use in the main text.

We defined the acronyms

7. 2.1: For how many times are the organoids washed with PBS?

We clarified this by adding 'one time' to step 2.1

8. Line 152: How the coating is done? Is the 1% PBS-BSA removed after coating?

Indeed, the PBS-BSA is removed. We clarified the step as follows:

To coat the inner side of a 15ml conical tube, fill the tube with 5 ml of 1% PBS-BSA, invert 2-3 times and discard the PBS-BSA.

9. 7.1: Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "NOTE".

We moved the second part of the description to a NOTE.

10. 7.2: Please describe how this is done or include a relevant reference.

We added the following description of the stitching process:

7.2 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 method, choose 'New Output' under parameters and select the file to stitch. Press 'Apply' to start stitching.

11. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

We obtained the copyright permission from Springer Nature and included it separately. All figure legends contain the citation.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript describes a protocol for in toto imaging of organoids. This protocol describes step by step the preparation of the sample that do not require the extensive clearing of organoids and tissues above 1 mm in size but still require processing to image them completely.

We thank the reviewer for the feedback and the proposed modifications to further clarify our protocol. We have addressed these in the point by point response below and throughout the revised manuscript.

Major Concerns:

none

Minor Concerns:

1. 1.6 Fructose-glycerol is not used in the protocol and only mentioned in Figure 3, thus, it is not necessary to explain how it is made for this protocol.

We agree with the reviewer and have removed the section from the protocol.

2. 3.3 The authors should emphasize that it needs to be tested if organoids will not stick to the tip after fixation. From our own experience, we know that mouse gastruloids and human early brain organoids do stick to the tip after fixation.

We thank the reviewer for this suggestion. We have now included this in the NOTE.

3. 4 The authors could emphasize that placing the plate on a dark background (e.g. black paper) helps with seeing and not aspirating small organoids.

We agree with the reviewer and included this in step 4.2.

4. 4.3 Performing the immunolabelling in 48- instead of 24-well plates can help in minimizing the antibody volume.

We thank the reviewer for this suggestion and we added a NOTE:

The immunolabeling can be performed in 48- or 96-well 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.

4.11 Is the nuclear staining performed together with the secondary antibody staining or afterwards? This is not specified.

Indeed, nuclear staining by DAPI can be performed together with the secondary antibodies. We clarified this in the text by adding 'conjugated antibodies and dyes' to the sentence.

5. 6.1 + 6.2 It is not clear why a tip with the end cut off depending on the size of the organoid needs to be attached to the syringe if in the next step only the silicone is applied and no organoids are pipetted. This is repeated in 6.3 where it is clear why it is needed.

We clarified this by adding 'to allow a gentle flow of the viscous silicone after pressing the syringe' to the sentence.

6. 6.3 Specify if organoids are transferred with FUnGI.

Adapted accordingly

6.4

Do larger organoids require spacers or does the silicone prevent organoids from being squeezed?

Indeed, the thickness of the silicone prevents this, but an additional spacer may definitely help. Therefore we added a NOTE: Spacers that are similar in size to the organoids can be used to prevent them from being damaged.

Reviewer #2:

Manuscript Summary:

In this manuscript, Ravian L. van Ineveld et al. provides detailed protocol for 3D imaging of intact organoids with single-cell resolution. They describe every step from organoid recovery till image post processing with sufficient detail and present high-quality representation of the anticipated results. Moreover, the Authors provide fair discussion regarding pros and cons of their clearing/imaging technique. Taking the increasing role of organoids in biological research and broad applicability of the presented, easy-to-prepare, non-toxic tissue optical clearing approach into account, I believe this will be an important, useful protocol to follow for new users of this kind of techniques.

We are pleased to hear that the reviewer believes our protocol is of added value to the scientific community. The comments are addressed below and in the revised manuscript.

Major Concerns:

1. The authors discuss, that "while most studies focused on large volume imaging of organs or associated tumors, sample preparation for smaller and more fragile tissues, including organoid structures, were lacking.". This seems to be overestimation of their own work. It should be noted that tissue optical clearing of the organoids was widely presented in the recent work of Masselink et al. Broad applicability of a streamlined ethyl cinnamate-based clearing procedure and applied by the Chen et al. Application of Three-Dimensional Imaging to the Intestinal Crypt Organoids and Biopsied Intestinal Tissues (using FocusClear). Moreover, although these are not organoids but spheroids, I believe that work focused on clearing and imaging of this similar, surely small and fragile tissues, should be mentioned in the manuscript (more information can be found in recent review of Costa et al. Optical clearing methods: An overview of the techniques used for the imaging of 3D spheroids).

We agree with the reviewer. We added the suggested references and toned down the statement as follows:

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.

2. The authors claim that "FunGI cleared organoids have overall enhanced fluorescence intensity compared to uncleared organoids". Although I appreciate that this data was obtained on 3800 cells and FUnGI-cleared specimens can be stored for weeks/months with the fluorescence retained, such statement requires more information about the image acquisition itself, e.g. is it possible that, in the uncleared group, cells selected for quantification were at ~50-60 um depth (or deeper) and thus their lower fluorescent signal might be partially caused by light absorption/obstruction not by an increase in fluorescence intensity of the cleared group? Did you quantified the background signal and subtracted its value from the overall signal of fluorescence intensity?

3 organoids per condition were analyzed in full, meaning all cells in all z-positions were taken along and no cells were specifically selected.

To exemplify this we attached the descriptive statistics of the data of figure 3C for the reviewer.

		uncleared	Glyc-Fruc	FUNGI
1	Number of values	3839	4982	5882
2				
3	Minimum	14.70	16.38	18.98
4	Maximum	127.7	218.3	205.2
5	Range	113.0	202.0	186.2
6				
7	Mean	63.78	97.80	96.35
8	Std. Deviation	20.46	42.02	36.67
9	Std. Error of Mean	0.3301	0.5953	0.4781

For all conditions the cells in the first planes, closest to the coverslip, are always the brightest. However, compared to In uncleared condition, (maximum 127.7) cells in the cleared conditions were always brighter, even at low depth (205.2 for FUnGI), thus supporting the statement that FunGI cleared organoids have an overall enhanced fluorescence intensity compared to uncleared organoids.

To better clarify this, we adapted the description of figure 3C as follows:

(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 plotted in GraphPad Prism 8 showing average DAPI intensity with different clearing methods on full segmented datasets. Data 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).

The background signal in the DAPI channel was very low and therefore not corrected for.

Minor Concerns:

In 1.7, the authors could provide more information regarding approximate time of reagent dissolution (I'm often asked about this by new users, as this helps them plan other steps accordingly and assure that reagents were added in correct order, amount etc.). Is this step performed at room temperature? Did the authors tried to heat the solution to decrease time of dissolution?

We added preparation times to 4% PFA, PBT, OWB, FUnGI and PBS-BSA. Furthermore two notes were added about heating:

NOTE: Do not heat above 60 °C to avoid degradation of the PFA.

NOTE: Do not heat. Fructose caramelizes at higher temperatures.

In 7.2, could you add on more info about: % of the recommended image overlap, the anticipated data size (e.g. for a presented entire organoid containing 140 cells), along with a brief description of stitching protocol that you use with an ImageJ?

We added a NOTE describing general acquisition settings and expected data size with these settings.

We did not use ImageJ for stitching, but we now provide a brief description of stitching using the ZEN software (added to the list of materials) provided with the microscope in step 7.2.

In Name of Material - full list of antibodies used in this study along with the information regarding the concentration used should be provided.

We added the antibodies and their concentrations.

4.3 and 4.11 - please adhere to one style of writing (2x/2 times concentrated).

Adapted accordingly.

Basement Membrane Extract abbreviation was introduced twice.

Adapted accordingly.

Reviewer #3:

Manuscript Summary:

van Ineveld et al. describe a method for extracting 3D organoids from a matrix, then fixing, labeling, clearing, and imaging them at high resolution. The steps outlined are similar to those published recently

in Nature Protocols, but with some improvements. The resulting images of organoids are very impressive and demonstrate the utility of this technique. The recipes for FUnGI and organoid washing buffer are particularly helpful.

We thank the reviewer for the positive feedback and are pleased to hear that the recipes are appreciated. Please find our point-to-point reply below.

Major Concerns:

- More discussion of the settings used to acquire the images would be useful. For example, what Z-stack step size was used to generate the 3D images in the figures? What power settings were used to obtain the immunofluorescence signals without photobleaching? While this depends on the equipment and magnification used, the authors experience in this area would be useful to share. Some of the imaging settings given, such as "speed 7" may only be applicable to the image acquisition software used in the study, which is not stated.

In response to multiple reviewer comments we adapted a NOTE after step 7.1 addressing general acquisition settings.

- A discussion of DAPI and KI67 segmentation or a reference describing how these were done would also be useful. This would be very interesting to most readers of this article.

We added a more detailed description of the segmentation analysis:

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.

Minor Concerns:

Copy editing needed throughout

Step 1.3 - Please recommend how to adjust the pH to 8 in more detail. Also please state what should be used to fill to 100mL, I assume it's dH2O?

Adapted: Set pH to 8 with 1 M NaOH and fill to 100 ml with dH2O.

Step 1.4 - Please clarify what is meant by "36-38% 1:1 with dH2O". What exactly is the percentage referring to?

We adapted the step to using concentrated HCL:
To prepare 500 ml 1 M Tris, dissolve 60,55 g Tris with 42 ml concentrated HCl (36-38%) in 300 ml dH2O. Set pH to 8 and fill to 500 ml.

Step 1.6 - Please explain why this step is needed in this protocol. FUnGI is the new clearing agent recommended by the authors in this article, not the previously published fructose-glycerol. Also, please explain why the refractive index is given.

As indicated by other reviewers, we agree and have removed the section from the protocol.

Step 1.7 - Please be consistent when referring to Tris, as both the 1M solution as well as the powder form are both referred to as Tris or tris base. Perhaps the solution can be referred to as Tris buffer.

Adapted

Step 1.7 - Please explain "Add 99g fructose in two batches". Does this mean add half of the fructose, wait for it to dissolve, then add the other half? It is unclear.

We clarified this as follows:

FUnGI consists of 50% (vol/vol) glycerol, 9.4% (vol/vol) dH2O, 10.6 mM tris base, 1.1 mM EDTA, 2.5 M fructose and 2.5 M urea. To make 220 ml of FUnGI, mix 110 ml glycerol with 20 ml of dH2O, 2.2 ml Tris buffer (1M, pH 8.0) and 440 µl EDTA (0.5 M). Add 50 g fructose and mix at room temperature (RT) in the dark until dissolved. When clear, add 49 g fructose and mix until dissolved. Add 33,1 g of urea and mix until dissolved (store at 4 °C in the dark). Preparation time 1 day.

Step 2.1 - Give the ideal temperature of the PBS for this step.

We added 'ice cold'

Step 2.2 - Please give units for horizontal shaker speed. Currently, only "1.5" is given.

We added '40 rpm'

Step 2.3 - It is unclear what is meant by "resuspending". What is being suspended in this step?

We agree with the reviewer that the term 'resuspending' is confusing and have therefore replaced it with 'pipetting up and down'

Step 4.13 - Please give the amount of time for spinning down.

We added '3 minutes'

Please add Tween-20 and SDS to the materials list.

Adapted accordingly

Reviewer #4:

Rios and coworkers demonstrate their technology for high-resolution imaging of the organoids upon immunostaining, which can work on a wide range of organoids of different origin, e.g., for airway, colon, kidney, and liver organoids as well as human breast tumor organoids and mouse mammary gland organoids. The FUnGI clearing method enables to capture of 3D organoids in full and enables marker quantification on a cell by cell basis.

Overall, a very useful and well-described method. I will suggest that authors proofread the manuscript at least once more before publication. Otherwise, I cannot see any issue with the work.

I recommend the publication.

We are pleased that the reviewer considers our protocol useful and well-described and thank the reviewer for the positive recommendation.

Reviewer #5:

Manuscript Summary:

The manuscript presents the detailed protocol of three dimensional high resolution imaging and image processing of the organoids in vitro. Method has been approved by previous publication. Topic of the manuscript reflects the urgent needs for the appropriate methods of analysis of cell distribution in 3D cellular samples. Unfortunately, described protocol is not suitable for high throughput analysis, but this was not the main aim of the method proposed in the manuscript and thus it does not affect the value of the work.

We agree with the reviewer that a high throughput protocol would be an interesting direction for future research and we are grateful for the proposed editorial changes that we address below and in the revised manuscript.

Major Concerns:

I do not have major concerns for this manuscrit.

Minor Concerns:

I have few comments concerning the text of the manuscript:

Line 58. I would mention also pancreatic and renal organoids here.

Line 108. If exact temperature is required, then the water bath would be preferable versus microwave.

Adapted and clarified as follows:

1.1 To prepare paraformaldehyde (PFA) 4% (wt/vol), heat 400 ml of phosphate-buffered saline (PBS) to just under 60 °C in a water bath. Add 20 g 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). Preparation time 4 hours.

NOTE: Do not heat above 60 °C to avoid degradation of the PFA.

Line 125. Fructose-glycerol clearing solution is mentioned in Figure 3 legend, but it does not appear in the protocol. Should it be rather removed from the section "Preparation of reagents"?

We agree with the reviewer and have removed this section from the protocol.

Line 153. It would be better to write: "step 3.3." here instead of "step 3"

Adapted accordingly.

Line 168. It is not very clear what is the meaning of "resuspend organoids half way".

We clarified the step by changing it to:

“3.2 Fix at 4 °C for 45 min. Gently resuspend the organoids halfway through the fixation time using a coated 1 ml tip to allow for a more even fixation among all organoids.”

Line 243. It would be advisable to use some spacers similar in size to organoids to make the compression more accurate (controllable).

We thank the reviewer for the suggestion. We added a note after step 6.4 about spacers.

Line 388. To prevent the x, y and z shifts of the coverslip we use glue pistol to make a rigid scaffold at the periphery of the coverslip and to fix it to preparate glass.

In our protocol, the coverslip is ‘glued’ to the slide by the silicone, similar to the function of the glue pistol mentioned by the reviewer.

Line 392. Using spacers can help here as well.

We agree and refer again to the note after step 6.4

Reviewer #6:

Manuscript Summary:

The interesting article "Single-cell resolution three-dimensional imaging of intact organoids" clearly describe the workflow from organoids recovery to confocal image acquisition and processing. The Authors implemented the method described in the original paper "High-resolution 3D imaging of fixed and cleared organoids" published in 2019 on Nature methods, adding an improved optical clearing step, and an improved mounting method of the slide using silicon sealant.

The fact that the described method is applicable to different types of organoids and does not require dedicated and special instrumentation both for sample preparation (including optical clearing) and for imaging (standard confocal microscopy seems to be sufficient in most cases) makes this protocol widely usable.

We are pleased that the reviewer appreciated the wide usability of our protocol and are thankful for the comments that we address below and in the revised manuscript.

The major concern is that Figure 3 is not visible. This makes the evaluation of the new clearing protocol impossible. The Authors should add Figure 3 to the article.

Major Concerns:

Row 297: Fig 3 is missing (3A, 3B and 3C). The Authors should add Figure 3.

We apologize for this inconvenience. Together with the editor we will try to find the reason for this to solve the issue for the next submission.

Minor Concerns:

Row 136: substitute 1 gram with 1 g.

Adapted accordingly

Row 251-256: to reproduce images with the same settings with other systems than the Zeiss LSM880, the voxel size used both with the 25x and the 40x would be more useful than the frame size. I would suggest the Authors to include the voxel size in the image acquisition description paragraph. Moreover, I would also suggest to include the numerical aperture of the objective lenses used.

In response to multiple reviewer comments we added a NOTE with general acquisition settings where we incorporated the voxel size.

NOTE: General acquisition settings using the 25x objective are: scan mode frame, frame size 1024x1024, voxel size 332 nm x 332 nm x 1,2 µm, pixel dwell time <2 µs, bidirectional scanning, averaging number 1, bit depth 8. To reduce photobleaching, use low laser power (<5% in general, <10% for weak staining). 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. With these settings the data size for a typical organoid <300 µm in diameter is <1GB.

Row 384: Do the Authors have data or indications about the possible autofluorescence and about the eventually more difficult antibody penetration inside the organoids upon PFA-Glutaraldehyde fixation (2% vol/vol)? Is the 2% vol/vol referred only to Glutaraldehyde (GA) or to PFA-GA? Both concentrations should be given.

As we unfortunately don't have data to support these concerns, we have adapted the text as follows:

This effect can be reduced, but not completely prevented, by using a different fixative, for example Formalin or PFA-Glutaraldehyde. However, this could potentially impact autofluorescence, antibody penetration and epitope availability.

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Dear Nature Permissions department,

I have a specific question regarding a Nature Protocols publication by Dekkers et al. (doi: 10.1038/s41596-019-0160-8 / NP-P180819B). We receive a lot of request from readers regarding our protocol and have now been offered the opportunity to publish an accompanying video protocol through JoVE (Journal of Visual Experiments) to help users perform the protocol steps. Of course the video will be completely new from the Nature Protocols publication, but there might be overlap in the protocol text.

My question is whether it is possible to publish a video protocol regarding the same protocol and, if so, what the next steps would be to adhere with Nature Protocols regulations for this.

Many thanks in advance,

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