Journal of Visualized Experiments

Staining and high-resolution imaging of 3D organoid and spheroid models.

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62280R1
Full Title:	Staining and high-resolution imaging of 3D organoid and spheroid models.
Corresponding Author:	Laura Broutier CRCL: Centre de Recherche en Cancerologie de Lyon FRANCE
Corresponding Author's Institution:	CRCL: Centre de Recherche en Cancerologie de Lyon
Corresponding Author E-Mail:	laura.broutier@lyon.unicancer.fr
Order of Authors:	Alejandro Gonzalez
	Léa Luciana
	Clémentine Le Nevé
	Julie Valantin
	Laura Francols
	Nicolas Gadot
	Christophe Vanbelle
	Laurianne Davignon
	Laura Broutier
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Biology
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	LYON, RHONE-ALPES, FRANCE
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	/

TITLE:

Staining and High-resolution Imaging of Three-dimensional Organoid and Spheroid Models

AUTHORS AND AFFILIATIONS:

Alejandro Lopez Gonzalez[1*], Léa Luciana[1*], Clémentine Le Nevé[2], Julie Valantin[2], Laura Francols[2], Nicolas Gadot[2], Christophe Vanbelle[3], Laurianne Davignon[4], Laura Broutier[1]

[1]Université de Lyon, Université Claude Bernard Lyon 1, INSERM 1052, CNRS 5286, Centre Léon Bérard, Centre de recherche en cancérologie de Lyon (CRCL), Lyon 69373, France

[2]Plateforme Anatomopathologie Recherche, Université de Lyon, Université Claude Bernard Lyon 1, INSERM 1052, CNRS 5286, Centre Léon Bérard, Centre de recherche en cancérologie de Lyon (CRCL), Lyon 69373, France

[3]Plateforme d'Imagerie cellulaire, Université de Lyon, Université Claude Bernard Lyon 1, INSERM 1052, CNRS 5286, Centre Léon Bérard, Centre de recherche en cancérologie de Lyon (CRCL), Lyon 69373, France

[4]PerkinElmer S.A.S., 14 Av. du Québec, Villebon-sur-Yvette 91140, France

*Contributed equally to the work

Emails of co-authors:

Alejandro Lopez Gonzalez	Alejandro.LOPEZGONZALEZ@lyon.unicancer.fr
Léa Luciana	Lea.LUCIANA@lyon.unicancer.fr
Clémentine Le Nevé	Clementine.LENEVE@lyon.unicancer.fr
Julie Valantin	Julie.VALANTIN@lyon.unicancer.fr
Laura Francols	Laura.FRANCOLS@lyon.unicancer.fr
Nicolas Gadot	Nicolas.GADOT@lyon.unicancer.fr
Christophe Vanbelle	Christophe.VANBELLE@lyon.unicancer.fr
Laurianne Davignon	Laurianne.Davignon@PERKINELMER.com

Corresponding author:

Laura Broutier Laura.BROUTIER@lyon.unicancer.fr

KEYWORDS:

3D cell model, spheroid, organoid, staining, imaging, high-resolution

SUMMARY:

Here, we provide detailed, robust, and complementary protocols to perform staining and subcellular resolution imaging of fixed three-dimensional cell culture models ranging from 100 µm to several millimeters, thus enabling the visualization of their morphology, cell-type composition, and interactions.

ABSTRACT:

In vitro three-dimensional (3D) cell culture models, such as organoids and spheroids, are valuable tools for many applications including development and disease modeling, drug discovery, and

regenerative medicine. To fully exploit these models, it is crucial to study them at cellular and subcellular levels. However, characterizing such in vitro 3D cell culture models can be technically challenging and requires specific expertise to perform effective analyses. Here, this paper provides detailed, robust, and complementary protocols to perform staining and subcellular resolution imaging of fixed in vitro 3D cell culture models ranging from 100 µm to several millimeters. These protocols are applicable to a wide variety of organoids and spheroids that differ in their cell-of-origin, morphology, and culture conditions. From 3D structure harvesting to image analysis, these protocols can be completed within 4–5 days. Briefly, 3D structures are collected, fixed, and can then be processed either through paraffin-embedding and histological/immunohistochemical staining, or directly immunolabeled and prepared for optical clearing and 3D reconstruction (200 µm depth) by confocal microscopy.

INTRODUCTION:

Over the past decades, advances in stem cell biology and in vitro 3D culture technologies have heralded a revolution in biology and medicine. Higher complexity cell models in 3D have become very popular as they allow cells to grow and interact with a surrounding extracellular framework, closely recapitulating aspects of living tissues including their architecture, cell organization and interactions, or even diffusion characteristics. As such, 3D cell culture models can provide unique insights into the behavior of cells in developing or diseased tissues in vitro. Organoids and spheroids are both multicellular 3D structures, ranging from several micrometers to millimeters, and are the most prominent in vitro 3D structures. Both may be cultured within a supporting scaffold including (i) hydrogels derived from animals (basement membrane extract, collagen), plants (alginate/agarose), or synthesized from chemicals, or (ii) inert matrices containing pores to promote cell proliferation and growth.

Organoids and spheroids can also develop without the presence of a supporting scaffold by relying on cells to self-assemble into clusters. This relies on different techniques such as the use of non-adhesive materials to inhibit cell attachment, surface tension and gravitational force (e.g., hanging drop techniques), or constant circular rotation of vessels (e.g., spinner culture). In all cases, these techniques facilitate cell-cell and cell-matrix interactions to overcome the limitations of traditional monolayer cell culture[1]. The terms “organoids” and “spheroids” have been used interchangeably in the past, but there are key differences between these two 3D cell culture models. Organoids are in vitro 3D cellular clusters derived from pluripotent stem cells or tissue-specific stem cells, in which cells spontaneously self-organize into progenitors and differentiated cell types and which recapitulate at least some functions of the organ of interest[2]. Spheroids comprise a broader range of multicellular 3D structures formed under non-adherent conditions and can arise from a large diversity of cell types such as immortalized cell lines or primary cells[3]. Hence, inherent to their intrinsic stem cell origins, organoids have a higher propensity for self-assembly, viability, and stability than spheroids.

Nevertheless, in essence, these two models are 3D structures composed of multiple cells, and the techniques developed to study them are thus very similar. For example, powerful imaging approaches at the single-cell resolution level are necessary for probing the cellular complexity of both organoids and spheroids. Here, by summarizing this group’s expertise and that of leaders in

the field of organoids[4], this paper describes detailed procedures to perform two-dimensional (2D) and 3D whole-mount staining, imaging, and analyses of the cellular and subcellular composition and spatial organization of organoids and spheroids ranging from 100 µm to several millimeters. Indeed, this procedure presents two different and complementary types of staining and imaging acquisition to analyze a large variety of sizes and types of in vitro 3D cell culture models. The use of one (3D whole-mount analysis) or the other (2D section analysis) will depend on the model studied and the answers sought. 3D whole-mount analysis by confocal microscopy can, for instance, be applied to visualize cells in 3D culture up to 200 µm in depth, irrespective of the overall size of the 3D structure, whereas the analysis of 2D sections provides insights into samples of any size, albeit at the 2D level. This procedure has been successfully applied across a variety of organoids[4,5] and spheroids derived from human and murine cells, originating from different embryonic germ layers. The overview of the procedure is shown in **Figure 1**. The major stages, the relationships between them, decisive steps, and the expected timing are indicated.

[Place **Figure 1** here]

PROTOCOL:

NOTE: A loss of ≤25% of the initial number of 3D structures should be expected during the steps involving reagent changes and washing in the following procedure. Plan to use a final number of at least ten 3D structures, with a size ranging from 100 to 500 µm, per tested condition to perform qualitative and quantitative image analyses. If necessary, for larger structures, cut the ends of 1 mL pipette tips to avoid breaking the structures. For all steps, if 3D structure sedimentation is too long, cells can be gently spun at 50 × *g* for 5 min at room temperature (RT). Depending on the issue investigated, advantages/disadvantages of such a spinning step should be considered, as centrifugation can compromise the shape of the 3D structures. Avoid spinning at >100 × *g*.

1. Collection and fixation of 3D cell culture models

NOTE: Be careful not to aspirate the 3D structures, which will be only loosely attached to the tube wall.

1.1. Harvesting of 3D cell culture models embedded in a matrix

NOTE: This section describes the recovery of 3D structures grown in drops of a basement membrane extract from murine Engelbreth-Holm-Swarm sarcoma (BME), but may be adapted to other matrices. See the discussion for crucial points regarding ECM.

1.1.1. Remove the culture medium from the wells without disrupting the 3D matrix. Precoat the inside and outside of a 1 mL pipette tip with protein (called **precoated 1 mL tip** hereafter) by dipping the full length of the tip in 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (called **PBS-BSA 0.1% solution** hereafter) and pipetting 1 mL of this solution up and down twice.

NOTE**:** This precoating will prevent the cells from sticking to the tip and minimize any loss.

1.1.2. Precoat the inside of a centrifuge (15 mL) tube with protein (called **precoated centrifuge tube** hereafter) by repeatedly filling with PBS-BSA 0.1% solution and emptying the tube.

NOTE: This will prevent the cells from sticking to the tube and minimize any loss.

1.1.3. Using the precoated 1 mL tip, carefully resuspend the 3D structures of the well using 1 mL of ice-cold 1x PBS, and gently transfer the suspension containing the 3D structures to the precoated centrifuge tube.

1.1.4. Gently add 13 mL of ice-cold 1x PBS, and allow the 3D structures to sediment on ice for at least 10 min.

NOTE: If required, spin for 5 min at 50 × *g* at 4 °C. Avoid spinning >100 × *g*, as this will compromise the shape of the 3D structures.

1.1.5. Remove the supernatant. Using a precoated 1 mL tip, gently resuspend the 3D structures in 1 mL of ice-cold 1x PBS. Repeat steps 1.1.4 to 1.1.5 to obtain a homogenous pellet without any 3D matrix residue.

NOTE: Efficient matrix removal is influenced by the type of matrix, the number, and size of 3D structures and requires optimization for different culture conditions. For 3D structures grown in BME, recovery from the matrix removal typically takes 45–60 min.

1.1.6. Using a precoated 1 mL tip, transfer the 1 mL 1x PBS suspension containing the 3D structures to a precoated 1.5 mL centrifuge tube, and proceed with section 1.3.

1.2. Harvesting floating 3D cell culture models

1.2.1. Using a precoated 1 mL tip, carefully collect and transfer the 3D structures to a precoated 1.5 mL centrifuge tube. Allow the 3D structures to sediment, or spin for 5 min at 50 × *g* at RT.

1.2.2. Remove the supernatant. Using a precoated 1 mL tip, resuspend the 3D structures in 1 mL of 1x PBS. Proceed with section 1.3.

1.3. Fixation of 3D cell culture models

1.3.1. Allow the 3D structures to sediment. Carefully remove the supernatant; under a fume hood, gently resuspend the 3D structures in 1 mL of formalin using a precoated 1 mL tip.

NOTE: Formalin contains formaldehyde, which is hazardous. Manipulate the chemical in a chemical hood. Wear rubber gloves and safety eye goggles.

1.3.2. Incubate the 3D structures for 30 min at RT.

NOTE: A 30 min fixation step with formalin is required for immunostaining of a wide range of 3D structures (varying in size, shape, and origin). However, in general, longer fixation times (>3 h) are better suited to preserve the fluorescence of reporter proteins.

1.3.3. Allow the 3D structures to sediment, or spin for 5 min at 50 × *g* at RT. Gently remove the formalin, and replace it with 1 mL of 1x PBS. Repeat this washing step in 1x PBS twice. Store the samples at 4 °C, and proceed with section 2 or section 3.

NOTE: The protocol can be paused here, and the cells can be maintained at 4 °C for long-term storage (>1 year).

2. 3D whole mount staining, imaging, and analysis of 3D cell culture models

NOTE: As the organoids are loosely attached to the tube wall, handle them gently as all following reagent changes can cause sample loss. Before starting, ensure the availability of the correct controls for staining. Positive and negative controls can be cells, in which the protein of interest is known to be either overexpressed or absent, respectively. Incubate samples without the primary antibody to determine if the observed signal is due to non-specific binding of the secondary antibody. As some cells tend to display high levels of autofluorescence, use controls devoid of secondary antibody to determine if the observed fluorescence is coming from background autofluorescence. Immunolabeling and fluorescent reporter visualization can be combined.

2.1. 3D whole mount staining

2.1.1. Prepare the permeabilization-blocking (PB) solution by supplementing 1x PBS with 0.1%–1% of a non-ionic surfactant (see the **Table of Materials**), 1% dimethylsulfoxide, 1% BSA, and 1% donkey serum (or from the animal in which the secondary antibodies were raised).

NOTE: Carefully optimize the concentration of the non-ionic surfactant depending on the localization of the target: membrane (0–0.5%), cytoplasm (0.5–1%), and nucleus (1%). This solution can be stored at 4 °C for up to 1 month. BSA usually works well for the blocking step, but in case of high background noise, perform an empirical test to obtain the best possible results for a given combination of antibodies.

2.1.2. Transfer the organoids from the 1.5 mL centrifuge tube to a 0.5 mL tube using a precoated 1 mL tip. Let the organoids sediment, gently remove the 1x PBS, and replace it with 0.5 mL of PB solution. Incubate the organoids with gentle horizontal agitation (30–50 rpm) for 1 h at RT.

2.1.3. Let the organoids sediment, gently remove the PB solution, and wash twice in 1 mL PBS-BSA 0.1% for 3 min.

NOTE: Waiting for 3 min allows the structures to sediment at the bottom of the tube.

2.1.4. Gently remove the PBS-BSA 0.1%, and add 250 µL of primary antibody diluted at the appropriate concentration in PB:1x PBS (1:10) solution. To prepare 10 mL of PB:1x PBS (1:10) solution, dilute 1 mL of PB solution in 9 mL of 1x PBS. Incubate for 2–3 days with gentle horizontal agitation (30–50 rpm) at 4 °C.

NOTE: An appropriate antibody incubation time is crucial for a suitable antibody penetration as 3D structures can sometimes reach large sizes.

2.1.5. Let the organoids sediment, and gently remove the primary antibody solution. Wash 5x in PBS-BSA 0.1% for 3 min per wash and then 2x in 1 mL PBS-BSA 0.1% for 15 min per wash with gentle horizontal agitation.

2.1.6. Add 250 µL of secondary antibody diluted at 1:250 in PB:1x PBS (1:10) solution. Incubate for 24 h at 4 °C with gentle horizontal agitation (30–50 rpm). For this step, protect the samples from light.

2.1.7. Add 250 µL of Hoechst 33342 (20 µM stock solution) diluted at 1:1000 in PB:1x PBS (1:10) solution, and incubate for another 2 h at 4 °C with gentle horizontal agitation (30–50 rpm).

2.1.8. Let the organoids sediment, and gently remove the solution containing secondary antibody + Hoechst 33342. Wash the organoids 5x in 1 mL of 1x PBS for 3 min per wash and then 2x in 1 mL of 1x PBS for 15 min per wash with gentle horizontal agitation (30–50 rpm).

NOTE: It is crucial to extensively wash the samples to avoid background noise or loss of signal.

2.1.9. Store the samples in PBS at 4 °C until image acquisition. Proceed with section 2.2.

NOTE: The protocol can be paused here, and the samples can be stored at 4 °C for several months, protected from light.

2.2. Sample preparation for confocal imaging

2.2.1. Using a precoated 1 mL tip, carefully transfer the organoids into 50 µL of the 1x PBS per well in a 96-well black polystyrene microplate. Proceed with step 2.2.3 or section 2.3.

NOTE: At this stage, the sample can be protected from light and stored at 4 °C for many weeks.

2.2.2. Clearing

NOTE: The clearing step is optional and can be used to either immunolabel organoids or to detect endogenous fluorescence. Clearing can cause 3D structure shrinkage, but does not change the general morphology except for spherical mono-layered organoids with large lumens[4]. For these

cystic organoids, skip the clearing step, and perform deep-tissue imaging[6].

2.2.2.1. Prepare 2.5 M glycerol-fructose clearing solution containing 50% v/v glycerol, 11% v/v of distilled water, and 45% w/v fructose by mixing the components on a magnetic stirrer overnight. Store at 4 °C in the dark for up to 1 month.

2.2.2.2. Remove as much 1x PBS as possible without touching the organoids. Add 200 µL of the clearing solution using a 1 mL pipette tip after removing the end, and resuspend gently to prevent the formation of bubbles. Incubate at RT for at least 12 h, and proceed with section 3.

NOTE: As the clearing solution is viscous, small volumes are difficult to handle. To facilitate handling, make sure the solution is at RT, and pipette slowly. For optimal clearing, allow the sample to sediment in the clearing solution for at least 24 h before imaging. If 3D structures are floating at the time of acquisition, perform an optional spin for 10 min at <100 × *g* at RT, or allow more time (one to several days) to let them sediment. The protocol can be paused at this step before proceeding to imaging if it is protected from light and stored at 4 °C (for weeks) or -20 °C (for months).

2.3. Image acquisition and analysis

NOTE: Image sectioning technology will be required to image 3D structures.

2.3.1. Use confocal microscopes, and favor immersion objectives with higher numerical aperture (NA) compared to air. Choose magnification objectives (10x, 20x, 40x) according to the size of 3D structures, image reconstruction (stitching), and solutions used for the analysis.

2.3.2. When selecting the acquisition mode, take into consideration the depth of focus of the objective used to define the step for Z stacking; allow for optimal 3D rendering.

NOTE: Image analysis solutions vary, and the analysis will need to be adjusted to the software used. For instance, this analysis protocol was established on a high-content analysis software (see **Table of Materials** and **Supplementary Figure 1** for details) and provides data on object segmentation, calculation of properties, and cell population selection within a 3D reconstructed object.

3. 2D sectioning, staining, imaging, and analysis of 3D cell culture models

NOTE: 3D cell culture models vary in size. Proceed with section 3.1 or 3.2 for efficient paraffin embedding (**Figure 2**). Allow sufficient time for 3D structure sedimentation before any washes and reagent changes. Be careful not to aspirate the organoids that will be floating at the bottom of the tube. For paraffin embedding, refer to **Figure 2** for guidance.

3.1. Paraffin embedding of large (Ø ≥ 400 µm) 3D cell culture models

3.1.1. On the day before embedding, prewarm two 150 mL flasks filled with paraffin (paraffin baths), a small metal embedding mold per sample, and fine forceps to 65 °C.

3.1.2. Using a precoated 1 mL tip, carefully transfer the organoids in 1x PBS to a flat-bottom glass tube with a polytetrafluoroethylene-lined bottle cap. Let the organoids sediment, carefully remove the 1x PBS, and replace it with 70% ethanol. Incubate for at least 30 min.

3.1.3. Let the organoids sediment, and carefully remove the 70% ethanol. Replace it with 1 mL of ready-to-use eosin Y solution. Flick the tube, and stain for at least 30 min. Carefully remove the eosin solution, and dehydrate the organoids in three successive washes with 1 mL of 100% ethanol for ~30 min each.

NOTE: Ethanol, a flammable and volatile liquid, causes severe eye and respiratory tract irritation. Manipulate it in a fume hood, and wear protective eye goggles.

3.1.4. Carefully remove the 100% ethanol, and under a chemical hood, clear the organoids in 3 successive washes with 1 mL of xylene for ~30 min each.

NOTE: Xylene is a toxic, liquid flammable whose vapors may cause irritation. Manipulate it in a fume hood. Avoid direct contact with skin, and wear rubber gloves and protective eye goggles.

3.1.5. Under a chemical hood, prepare a white microtwin tissue cassette by placing a piece of biopsy pad (previously soaked in xylene) inside one of the compartments of the cassette. Carefully transfer the 3D structures using a precoated 2 mL plastic Pasteur pipette to the biopsy pad. Cover them with another biopsy pad soaked in xylene to prevent the organoids from moving, and close the cassette.

3.1.6. If several samples are processed, place the cassette in a xylene bath to await further processing. Once all samples are transferred into cassettes, place the cassettes in a prewarmed paraffin bath for 30 min at 65 °C. Transfer the cassettes to a fresh prewarmed paraffin bath overnight.

3.1.7. After paraffin impregnation, take a prewarmed embedding mold, and add the heated paraffin to it. Place the biopsy pad containing the 3D structures into the mold, and gently agitate it until all of the organoids drop to the bottom of the mold. Very carefully place the 3D structures at the center of the mold using prewarmed fine forceps. Proceed with section 3.3.

NOTE: Be careful not to disrupt the 3D structures with the forceps; push, but do not pinch them.

3.2. Paraffin embedding of small (Ø ≤ 400 µm) 3D cell culture models

3.2.1. On the day before embedding, prewarm two 150 mL flasks filled with paraffin (paraffin baths), a small metal embedding mold per sample, and fine forceps to 65 °C.

3.2.2. Carefully remove the 1x PBS from the organoid suspension. Gently perform 3 washed in 1 mL of 1x Tris-buffered saline (TBS). Remove as much 1x TBS as possible without touching the organoids.

NOTE: Be careful not to aspirate the sample. If necessary, perform a 5 min spin at 50 x g at RT. Remaining traces of phosphate will interfere with the following steps, notably preventing gel polymerization. Therefore, do not use PBS solutions during any processing step. For this step, a commercial kit, containing cassettes, Reagent #1 (clear fluid), and Reagent #2 (colored fluid), was used to facilitate the paraffin-embedded procedure without potentially loosing tiny fragments (see **Table of Materials**). Follow kit instructions. The cassettes are preassembled with backing papers and board inserts already in place.

3.2.3. Add 2 drops of Reagent #2 into the tube, and mix gently by tapping the tube. Add 2 drops of Reagent #1, and mix again by tapping to make the gel solidify. Using the fine forceps, remove the gel from the tube, and place it in the well of the cassette.

3.2.4. Under the fume hood, dehydrate the sample by placing the cassette in successive baths as follows (use the 150 mL flasks, and use fresh ethanol or xylene for each bath): ethanol 70%, 30 min; ethanol 96%, 30 min; ethanol 100%, three washes, 30 min each; xylene, three washes, 30 min each.

3.2.5. Place the cassettes in a prewarmed paraffin bath for 30 min at 65 °C, and transfer them to a fresh prewarmed paraffin bath overnight. After paraffin impregnation, take a prewarmed embedding mold, and add heated paraffin into it. Open the cassette, carefully dislodge the gel with fine forceps, and place the gel containing the 3D structures onto the center of the embedding mold. Proceed with section 3.3.

3.3. Common steps for paraffin embedding

3.3.1. Gently transfer the mold to a cold area to let the paraffin solidify in a thin layer, which will maintain the 3D structures in the appropriate position. Add a tissue cassette on top of the mold, and add hot paraffin to cover this plastic cassette. Remove the mold once it is completely solidified, and proceed with section 3.4.

NOTE: Paraffin blocks can be stored at room temperature for years.

[Place **Figure 2** here]

3.4. Block sectioning and staining

3.4.1. Cut 4 µm sections using a standard microtome, and perform standard histological and immunohistochemical techniques. Proceed with section 3.5.

NOTE: Specific slides (see **Table of Materials**) were used for a better adhesion of sections. The

slides can be stored at room temperature or at 4 °C for years.

3.5. Image acquisition and analysis

3.5.1. Perform imaging using a digital slide scanner or upright microscope, and analyze data using a platform for fast digital quantitative analysis that reports morphological and multiplexed expression data on a cell-by-cell basis across entire 3D structure sections (see **Supplementary Figure 2** for details).

NOTE: The 20x objective is used routinely by this group.

REPRESENTATIVE RESULTS:

This protocol provides an overview of the critical steps for 2D and 3D whole-mount staining, as well as imaging and quantitative analyses of 3D cell culture models **(Figure 3** and **Figure 4)**. It is applicable to a wide range of 3D cell culture models—from spheroids to organoids from different host species or tissues—and enables the acquisition of accurate and quantitative information on architecture, cell organization, and interactions at cellular and subcellular levels **(Figure 3** and **Figure 4)**. Laboratories may need to optimize 2D histological and immunohistochemical techniques and antibody concentrations according to their own needs.

Both methods yield valuable biological information. 3D whole-mount staining and confocal microscopy provide visual information on cellular composition and spatial position with a field of depth of up to 200 µm **(Figure 3B)**. However, 2D sectioning is convenient for larger 3D structures to reveal detailed cellular morphological traits in the entire section of 3D structures that can be otherwise challenging to observe in situ due to light scattering that compromises resolution in larger samples. Moreover, both techniques can provide quantitative data. Indeed, the resolution obtained allows the application of cellular and subcellular segmentation algorithms for the quantification of the number of cells and the detection of the presence of various cell markers in different cellular subtypes **(Figure 3F** and **Figure 4)**. In summary, the imaging techniques described here are reproducible, simple, and complementary and represent valuable tools for studying cellular heterogeneity.

[Place **Figure 3** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic overview of the procedure. In vitro 3D cell culture models are collected and fixed, then either prepared for 3D whole mount staining (**option a**) or embedded in paraffin for 2D sectioning and staining (**option b**). For 3D whole-mount staining experiments, fixed 3D structures are rehydrated and immunolabeled following the fixation step. An optional optical-clearing step can be performed to improve imaging quality and depth of optical microscopy by reducing light scattering during image processing. Images are captured on an inverted confocal microscope or a confocal high content system and analyzed using the appropriate software. For paraffin embedding, 3D structures are directly processed (**option b.1 for large structures ≥ 400 µm**) or included in a gel (**b.2; small structures ≤ 400 µm**) for dehydration and paraffin embedding.

Paraffin blocks are then cut and stained (histological or immunochemical staining). Images of 2D sections are obtained on a digital slide scanner or an upright microscope and analyzed on an image analysis platform using fast digital quantitative analysis.

Figure 2: Overview of the procedure for paraffin embedding of large and small in vitro 3D cell culture models. (**A**) Standard procedure for paraffin embedding. After fixation and dehydration, 3D structures are stained with eosin to facilitate their visualization (top and bottom left). 3D structures are carefully placed on the biopsy pad (blue) in the cassette using a 2 mL Pasteur pipet (middle). After paraffin impregnation, the 3D structures are gently dropped into the liquid paraffin using forceps and gently agitated in the biopsy pad. Small 3D structures are lost during this step as they cannot be released from the pad (bottom right: failed embedding). Only large 3D structures will be embedded (top right: successful embedding). Arrowheads point to 3D cultures. (**B**) Alternative to the standard paraffin embedding protocol. After having fixed small 3D structures, a commercial kit is used to maintain cells in a gel and facilitate their transfer to the mold after paraffin impregnation (right: successful embedding).

Figure 3: Representative results for 3D whole mount, imaging, and analyses of 3D and 2D optical sections. (**A**) Confocal images of human (h) high-grade glioma spheroid cultured for a week and labeled with Hoechst (blue), Olig2 (yellow), and Actin (red) (20x water objective). For all acquired images, microscope settings were established using a positive control (top), and then the negative control was imaged using identical settings to control the lack of fluorescence in the absence of primary antibody (bottom). (**B**) Orthogonal 3D whole-mount representation of Ki67 staining performed in (h) high-grade glioma spheroid cultured for a week (glycerol-fructose clearing; 20x water objective, confocal). (**C**) Confocal images of (h) high-grade glioma spheroid cultured for a week and labeled with Hoechst (blue), Olig2 (yellow), and Phalloidine-488 (green) (glycerol-fructose clearing; 20x water objective). (**D**) Confocal images of human (h) rhabdomyosarcoma (top) and mouse (m) neural crest cell (bottom) spheroids cultured for a week and labeled with Hoechst (blue), Actin (red), and Ki67 (green), respectively (glycerol-fructose clearing; 20x dry objective). (**E**) Confocal images of (h) high-grade glioma spheroid cultured for a week and labeled with Hoechst (blue) and Ki67 (green) (glycerol-fructose clearing; 40x water objective) (top left). Segmented images on the Hoechst channel and Ki67-positive (+) nuclear regions on the green channel were generated using high-content analysis software (see **Supplementary Figure 1** and **Table of materials**) (bottom). Output given is the percentage of $Ki67^+$ nuclei per segmented 3D structure (top right). Scale bar = 100 µm.

Figure 4: Representative results for imaging and analyses of 2D optical sections. (**A, D**) 2D section images of a 3D cell model (human rhabdomyosarcoma spheroids cultured for a month) obtained with a digital slide scanner and analyzed on a platform for fast digital quantitative analysis. (**A**) H&E staining and detection of cells according to their size. Scale bar = 500 µm. (**B**) Histogram shows percentage of cells < 100 μm^2 and > 100 μm^2 detected using software for fast digital quantitative analysis (left: Halo) or manual counting (right: MC). (**C**) Ki67 staining and detection of cells according to the intensity of their 3,3'-diaminobenzidine (DAB) signal. Negative (blue), weakly positive (yellow), positive (red). Scale bar = 500 µm. (**D**) Histogram shows percentage of Ki67-negative, weakly positive, and positive cells. Abbreviations: H&E =

hematoxylin and eosin; MC = manual counting.

Supplementary Figure 1: Overview of the steps in the imaging analysis software. Analyses are based on the association of **building blocks**. Each building block corresponding to a function—segmentation, calculation, association, output definition—and offers multiple algorithms and variable selections to match the biological sample being imaged. The software provides multiple RMS (Ready Made Solution) analysis protocols that can easily be used and modified. Integrated image analysis protocols can be saved, applied to different datasets, and shared between users. Briefly, the analysis protocol implies sequential object segmentation: spheroid, nuclei and finally, Ki67 pockets (A488). Then, the mean intensity of the Ki67 pockets is calculated to further discriminate the positive events. Finally, nuclei encompassing Ki67 positive pockets are positively selected.

Supplementary Figure 2: Overview of the procedure steps of the quantitative analysis software. Step 1. Upload the files using the **Studies** tab. Files will be opened in the **Image Actions** section. Step 2. Open the **Annotations** tab, then click on **Layer Actions** to design a new layer all around the structure using the **circle** tool of the toolbar. For non-circular structures, the **pen** tool can be used instead. Step 3. The toolbar can be used to design annotations and visualize the quantification with the ✓ tool. Step 4. Open the **Analysis** tab, and select the best conditions for the analysis of the sample (several trials may be necessary here). Step 4.1. Use the **Stain Selection** section to set up the staining condition. In the event of several stains, these can be added and renamed, and the **virtual** color can be modified. The localization detection can be specified—nuclear or cytoplasm staining. Step 4.2. Use the **Cell Detection** section to set up the cell detection. This section will be the most important for the analysis. The **Nuclear Contrast Threshold** section will enable detection of all nuclei. Attention must be paid in case there are multiple population sizes, the software can detect several cells instead of a unique big one. **Nuclear Size** and **Nuclear segmentation aggressiveness** sections can be used to quantify cell size population ranges. Step 5. Description on how to run sample analysis. Follow steps shown in the figure. **Annotation Layer** section will run the setting only on this slide. The quantification can be visualized using the ✓ tool. Repeat steps 4.1–5 until suitable quantification is achieved. Steps 6–6.1. These steps enable you to draw a figure using the software. Step 7. Quantification graphics obtained via software can be saved. Step 8. Data can be exported.

DISCUSSION:

Cell culture is an indispensable tool to uncover fundamental biological mechanisms involved in tissue and organ development, function, regeneration and disruption, and disease. Although monolayered 2D cell culture has predominated, recent research has shifted towards cultures generating 3D structures more reflective of in vivo cellular responses, owing notably to additional spatial organization and cell-cell contacts that influence gene expression and cellular behavior and could thus provide more predictive data[7]. Nevertheless, many challenges remain, including the need for user-friendly staining and imaging techniques for detailed microscopic visualization and evaluation of complex 3D structures at the cellular and subcellular levels. In that context, detailed, robust, and complementary protocols have been provided to perform staining and cellular and subcellular resolution imaging of fixed in vitro 3D cell culture models ranging

from 100 µm to several millimeters in size.

This procedure presents two different strategies to deal with a large variety of sizes and types of in vitro 3D cell culture models. The choice of one (3D whole-mount analysis) or the other (2D sectioning analysis) will depend on the model used and issue investigated. 3D whole-mount analysis by confocal microscopy enables the visualization of cells with a field of depth of up to 200 µm, irrespective of the overall size of the 3D structure, whereas 2D sectioning is applicable to samples of any size, but visualization remains 2D dimensional. Below are some suggestions for troubleshooting and technical considerations. Loss of 3D structures during the workflow is the most common drawback. They can remain adherent to the tips and tubes, which is why precoating tips and tubes with PBS-BSA 0.1% solution is key.

Moreover, it is crucial to let the 3D structures sediment between reagent changes and to perform all pipetting very carefully. As mentioned in the procedure, for all steps, if 3D structure sedimentation is too long, cells can be gently spun at 50 × *g* for 5 min at RT. Depending on the aim of the study, the advantages/disadvantages of such a spinning step should be considered as centrifugation can compromise the shape of the 3D structures. Moreover, care should be taken to preserve this morphology during the fixation step because cystic organoids tend to collapse. Fixing structures under 400 µm in size should prevent structural changes. For optimal immunolabeling, recovery of organoids from their 3D matrices is a crucial step. The 3D matrix can impede adequate antibody penetration or lead to high background staining because of non-specific binding to the matrix.

ECM removal may alter the morphology of the outer segments of organoids (notably in case of small cellular protrusions extending from studied 3D structures) and partially hamper analyses. For such 3D structures, the matrix can be retained throughout the procedure; however, culture conditions should be carefully adapted to grow cells in a minimum amount of matrix to prevent insufficient penetration of solutions and antibodies and to avoid successive washing steps aimed at reducing excessive background noise[6,8]. The optical clearing step described in this protocol in the 3D whole mount staining section is pertinent for the imaging of 3D structures up to 150–200 µm in depth instead of 50–80 µm without clearing. Compared to other clearing methodologies that often requiring several weeks and using toxic clearing agents, a previously published fast and safe clearing step was used in this protocol[4,9].

In addition, this clearing step is reversible, and new antibodies can be added to the initial staining with no loss of resolution or brightness[4]. Nevertheless, depending on the 3D cell culture model studied, a depth of 150–200 µm might not be sufficient to image the 3D structure in an informative way, and this clearing protocol can cause changes in the general morphology of spherical, monolayered organoids with large lumens[4]. Users should carefully design their experiment, and if necessary, optimize the timing of the permeabilization/blocking step (to allow penetration of antibodies and solution), the clearing step (to penetrate deeper than 200 µm, specimens should be totally cleared), and image acquisition. The two most prevalent technologies available in core facilities would be light sheet and confocal microscopy. Users will

need to carefully choose a technology based on the size of their 3D structures and their biological question[10]. However, compared to confocal microscopy, light sheet microscopy resolution obtained for such deep structures remains suboptimal for obtaining subcellular resolution.

Here, a detailed and robust process has been reported that is dedicated to paraffin embedding of single samples. Interestingly, Gabriel et al. recently developed a protocol embedding 3D cell cultures in paraffin with an increased throughput. They used a polydimethylsiloxane (PDMS) mold to confine 96 3D structures in a microarray pattern in one block, providing novel perspectives for studies on 3D tumor models encompassing more groups, time points, treatment conditions, and replicates[11]. However, this method requires extensive skills and machinery, notably for the fabrication of the premold used to created PDMS molds. In summary, this paper describes two different, complementary, and adaptable approaches enabling the acquisition of accurate and quantitative information on architectural and cellular composition of 3D cellular models. Both parameters are crucial for studying biological processes such as intratumoral cellular heterogeneity and its role in resistance to treatments.

ACKNOWLEDGMENTS:

This work was supported by the St Baldrick's Robert J. Arceci Innovation Award #604303.

DISCLOSURES:

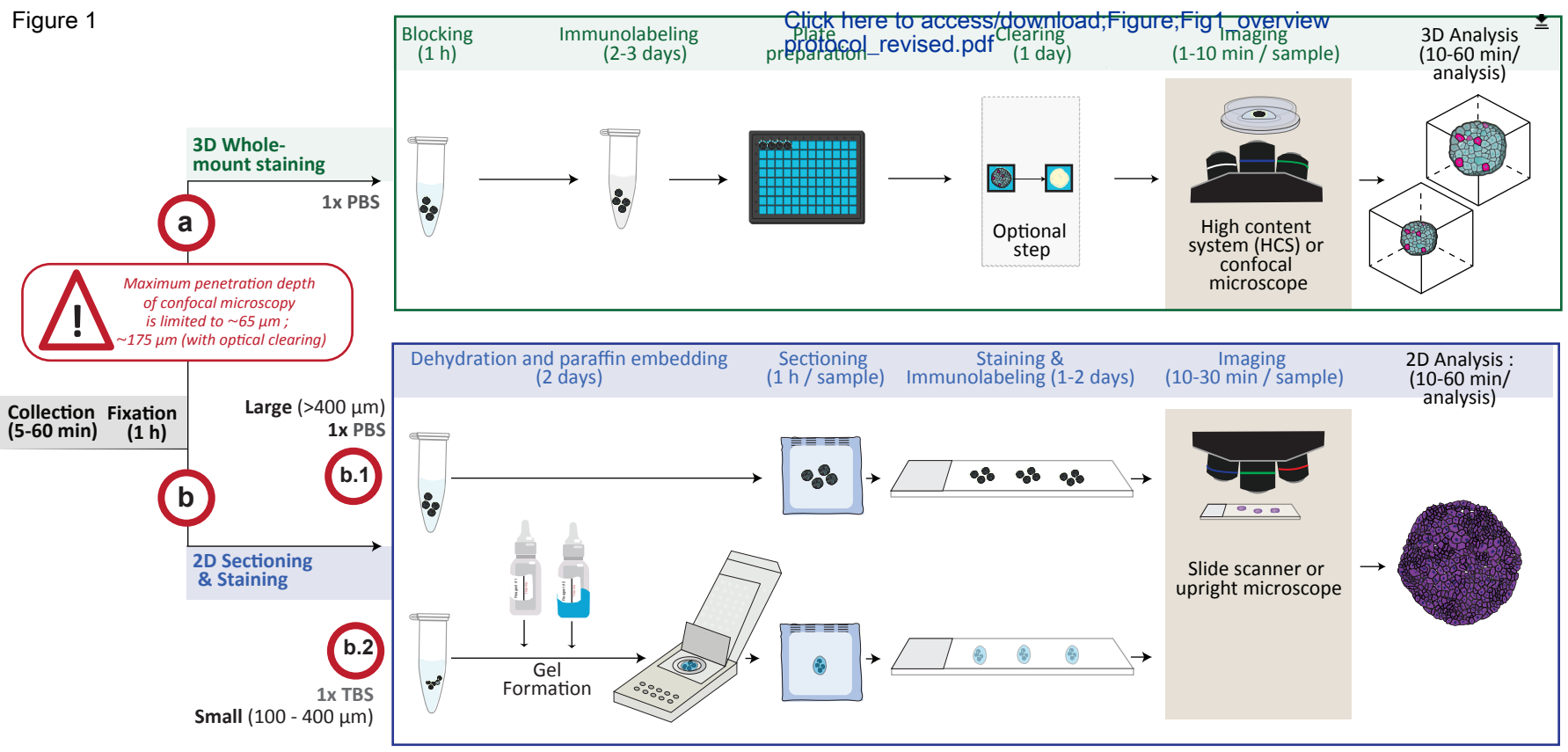
The authors have nothing to disclose.

REFERENCES:

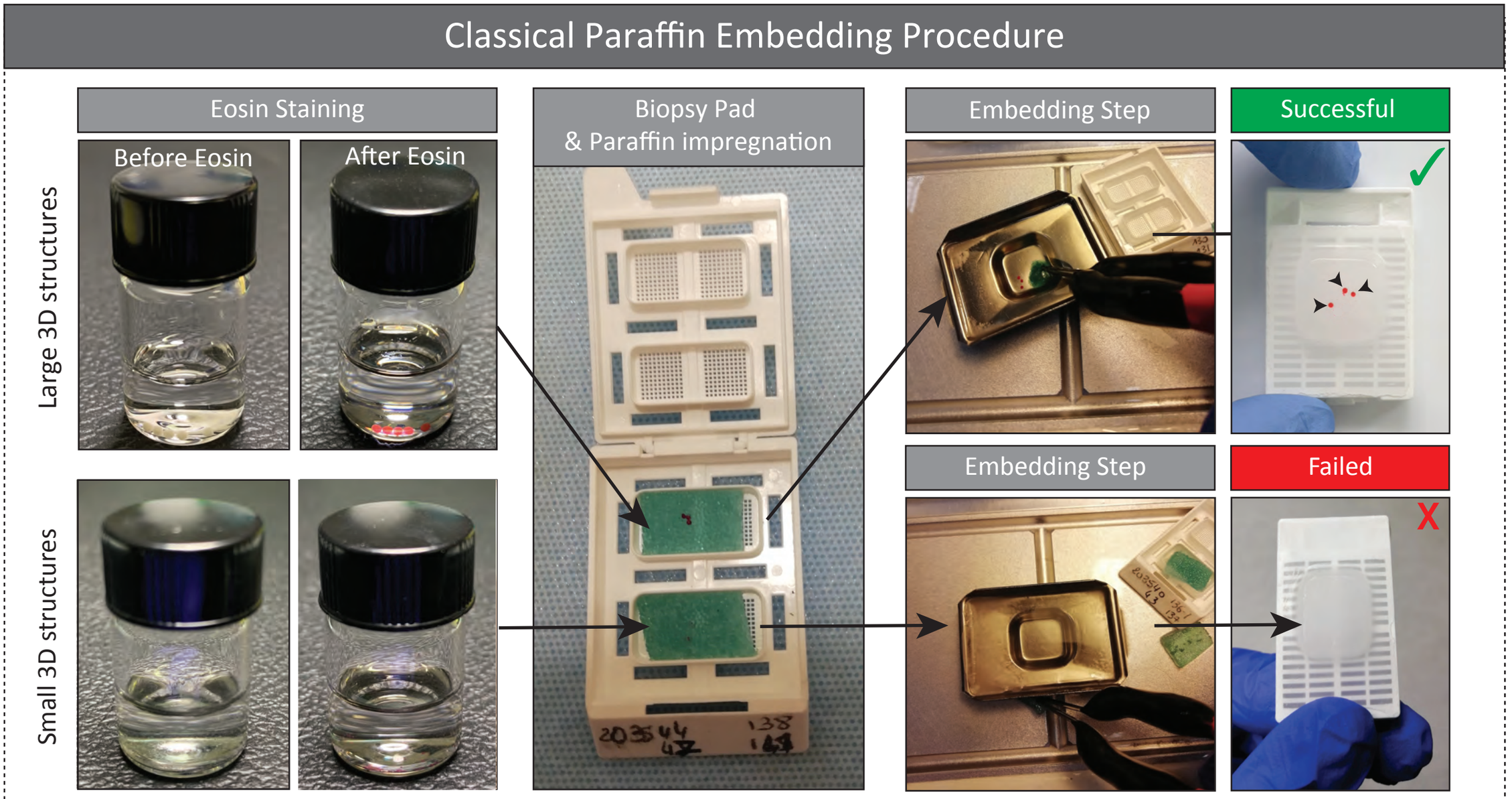
1. Ryu, N. E., Lee, S. H., Park, H. Spheroid culture system methods and applications for mesenchymal stem cells. *Cells*. **8** (12), 1–13 (2019).
2. Bartfeld, S., Clevers, H. Stem cell-derived organoids and their application for medical research and patient treatment. *Journal of Molecular Medicine*. **95** (7), 729–738 (2017).
3. Cui, X., Hartanto, Y., Zhang H. Advances in multicellular spheroids formation. *Journal of the Royal Society, Interface*. **14** (127) doi:10.1098/rsif.2016.0877 (2017).
4. Dekkers, J. F. et al. High-resolution 3D imaging of fixed and cleared organoids. *Nature Protocols*. **14**, 1756–1771 (2019).
5. Broutier, L. et al. Culture and establishment of self-renewing human and mouse adult liver and pancreas 3D organoids and their genetic manipulation. *Nature Protocols*. **11** (9), 1724–1743 (2016).
6. Rezanejad, H., Lock, J. H., Sullivan, B. A., Bonner-Weir, S. Generation of pancreatic ductal organoids and whole-mount immunostaining of intact organoids. *Current Protocols in Cell Biology*. **83** (1), ee82 (2019).
7. Edmondson, R., Broglie, J. J., Adcock, A. F., Yang, L. Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. *Assay and Drug Development Technologies*. **12** (4), 207–218 (2014).
8. McCray, T., Richards, Z., Marsili, J., Prins, G. S., Nonn, L. Handling and assessment of human primary prostate organoid culture. *Journal of Visualized Experiments: JoVE*. (143), 10.3791/59051 (2019).
9. Ueda, H. R. et al. Tissue clearing and its applications in neuroscience. *Nature Reviews:*

Neuroscience. **21** (2), 61-79 (2020).
10. Lazzari, G. et al. Light sheet fluorescence microscopy versus confocal microscopy: in quest of a suitable tool to assess drug and nanomedicine penetration into multicellular tumor spheroids. *European Journal of Pharmaceutics and Biopharmaceutics*. **142**, 195–203 (2019).
11. Gabriel, J., Brennan, D., Elisseeff, J. H., Beachley, V. Microarray embedding/sectioning for parallel analysis of 3D cell spheroids. *Scientific Reports*. **9**, 16287 (2019).

Figure 1



A



B

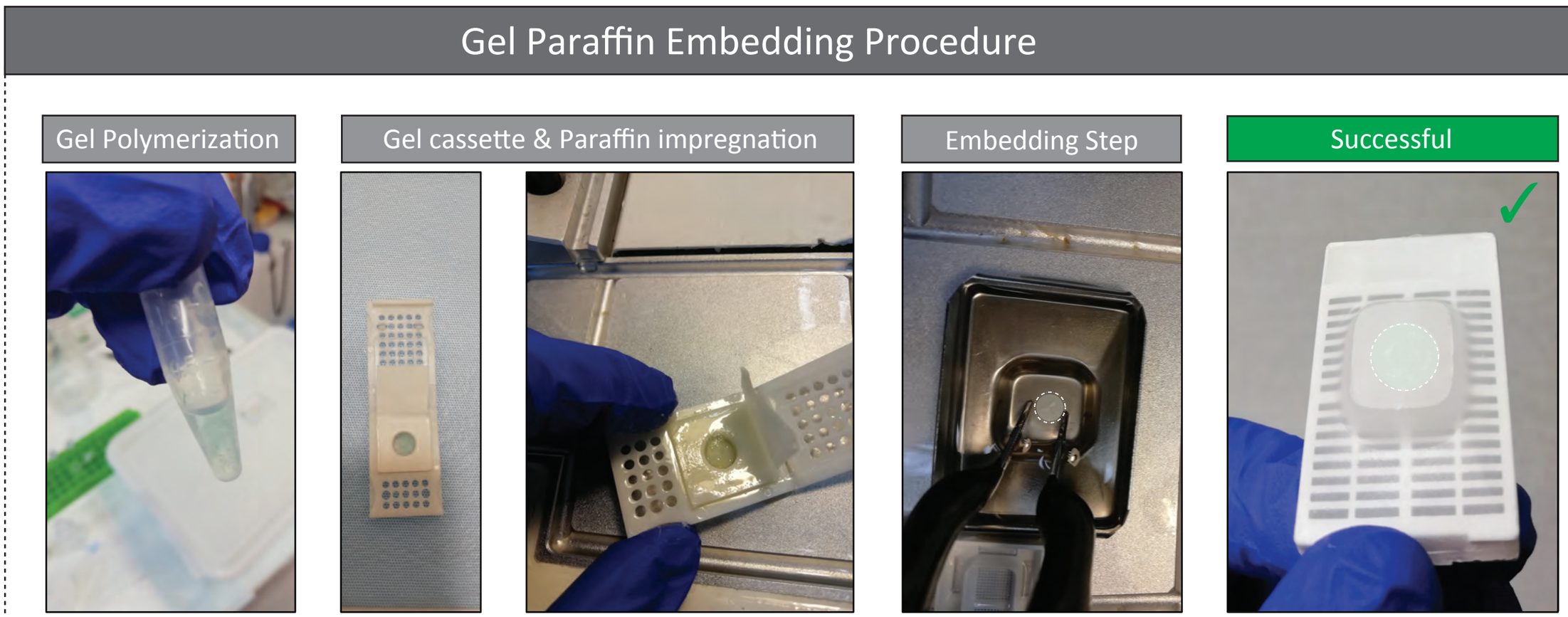


Figure 3

Click here to access/download;Figure;Fig3_Representative results wholemount_revised_comp.pdf

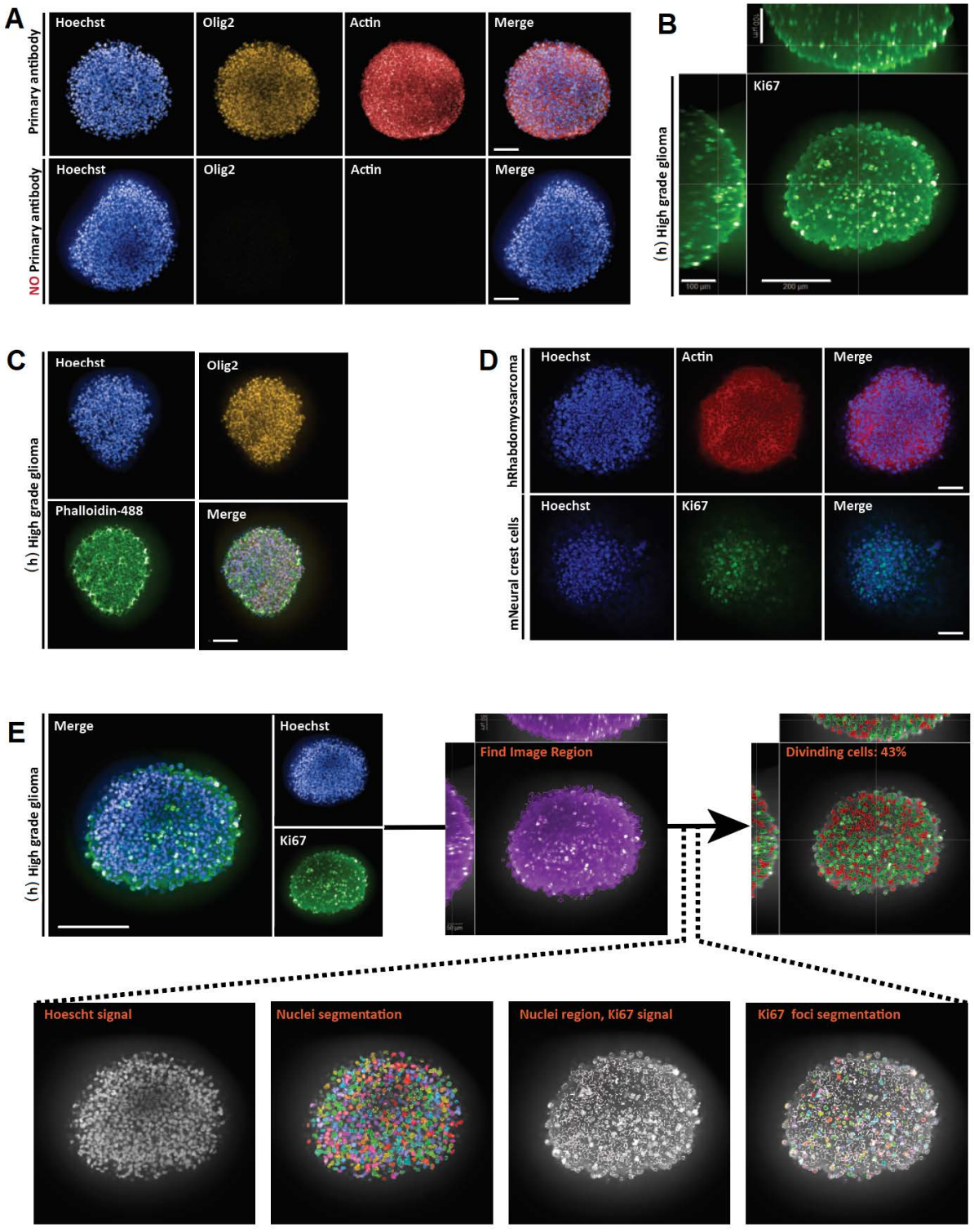
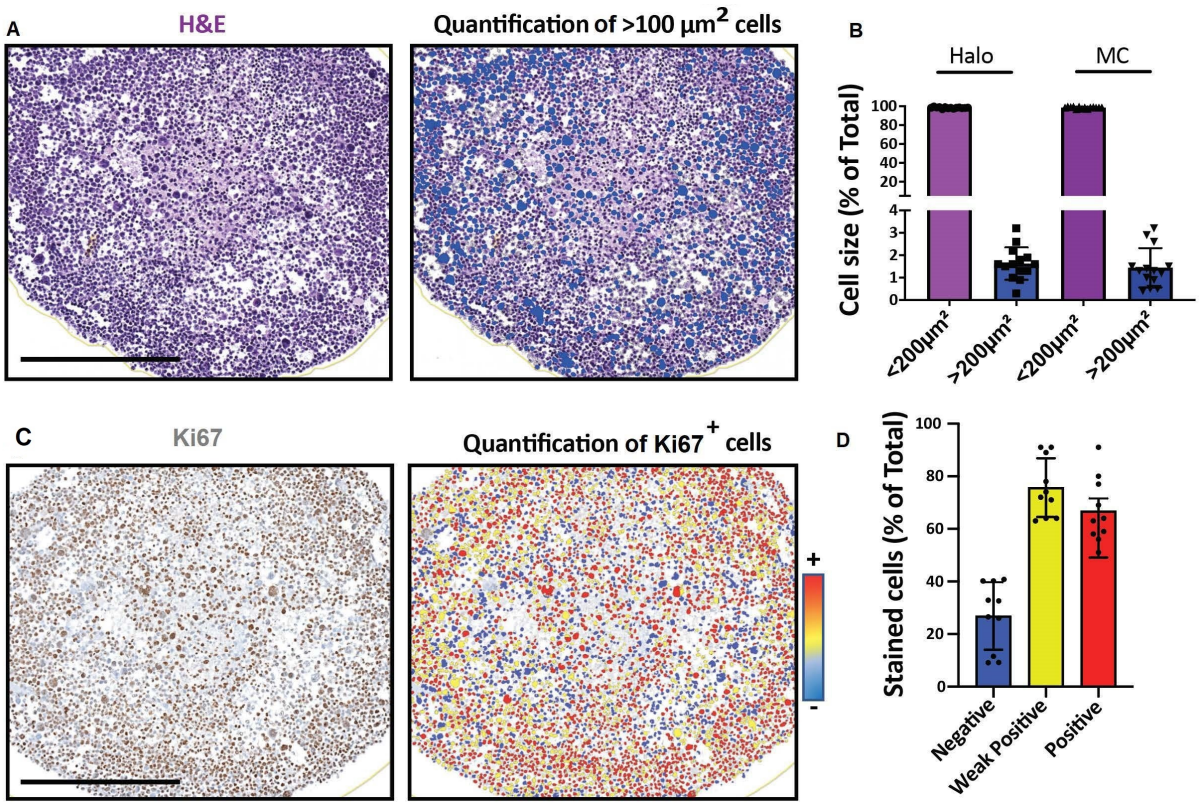


Figure 4

Click here to access/download;Figure;Fig4_Representative results Parrafin_revised_comp.pdf



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Equipment			
Biopsy pad Q Path blue	VWR	720-2254	
Cassettes macrostar III Blc couv. Char. x1500	VWR	720-2233	
Cassette microtwin white	VWR	720-2183	
Chemical hood	Erlab	FI82 5585-06	
Filter tips 1000 µL	Star lab Tip-One	S1122-1730	
Fine forceps	Pyramid innovation	R35002-E	
Flat-bottom glass tubes with PTFE lined 2 mL	Fisher Scientific	11784259	Excellent for environmental samples, pharmaceuticals and diagnostic reagents. PTFE is designed for the ultimate in product safety. PTFE provides totally inert inner seal and surface facing the sample or product.
Glass bottom dish plate 35 mm	Ibedi	2018003	
Horizontal agitation	N-BIOTEK	NB-205	
Incubator prewarmed to 65 °C	Memmert Incubator	LAB129	
Inox molds 15x15	VWR	720-1918	
Microscope Slides Matsunami TOMO-11/90	Roche diagnostics	8082286001	these slides are used for a better adhesion of sections
Microtome	Microm Microtech France	HM340E	
Panoramic scan II	3dhistech	2397612	
Paraffin embedding equipment	Leica	EG1150C	
Plastic pipette Pasteur 2 mL	VWR	612-1681	

Q Path flacon 150mL cape blanc x250	VWR	216-1308	Good for environmental samples, pharmaceuticals and diagnostic reagents. Polypropylene (PP) are rigid, solid, provide excellent stress crack and impact resistance and have a good oil and alcohol barrier and chemical resistance. PE-lined cap is stress crack resistant and offers excellent sealing characteristics.
Set of micropipettors (p200, p1000)	Thermo Scientific	11877351 (20-200) 11887351(p1000)	
OPERA PHENIX	PerkinElmer	HH14000000	
SP5 inverted confocal microscope	Leica	LSM780	
Tissue cassette	VWR	720-0228	
Zeiss Axiomager microscope	Leica	SIP 60549	
Reagent			
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A7030-100G	
Cytoblock (kit)	Thermofisher Scientific	10066588	
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	57648266	CAUTION: toxic and flammable. Vapors may cause irritation. Manipulate in a fume hood. Avoid direct contact with skin. Wear rubber gloves, protective eye goggles.
Eosin aqueous 1%	Sigma-Aldrich	HT110316	
Ethanol 96%	VWR	83804.360	CAUTION: Causes severe eye irritation. Flammable liquid and vapor. Causes respiratory tract irritation. Manipulate in a fume hood. Wear protective eye goggles.
Ethanol 100%	VWR	20821.365	CAUTION: Causes severe eye irritation. Flammable liquid and vapor. Causes respiratory tract irritation. Manipulate in a fume hood. Wear protective eye goggles.

Formalin 4%	Microm Microtech France	F/40877-36	CAUTION: Formalin contains formaldehyde which is hazardous. Manipulate in a fume hood. Avoid direct contact with skin. Wear rubber gloves and protective eye goggles.
Fructose	Sigma-Aldrich	F0127	
Gill hematoxylin type II	Microm Microtech France	F/CP813	
Glycerol	Sigma-Aldrich	G5516	500 mL
Hoechst 33342	Life Technologies	H3570	CAUTION: Suspected of causing genetic defects. Avoid direct contact with skin. Wear rubber gloves and protective eye goggles.
Normal donkey serum	Sigma-Aldrich	D9663	10 mL
Paraffin Wax tek III	Sakura	4511	
Phosphate Buffer Saline (PBS) 1 X	Gibco	14190-094	
Tris-Buffered Saline (TBS) 10X	Microm Microtech France	F/00801	100 mL
Triton X-100	Sigma-Aldrich	T8532	CAUTION: Triton X-100 is hazardous. Avoid contact with skin and eyes.
Xylene	Sigma-Aldrich	534056	CAUTION: Xylene is toxic and flammable. Vapors may cause irritation. Manipulate in a fume hood. Avoid direct contact with skin. Wear rubber gloves, protective eye goggles.
Solutions			
Clearing solution			Glycerol-Fructose clearing solution is 60% (vol/w) glycerol and 2.5 M fructose. To prepare 10 mL of this solution, mix 6 mL of glycerol and 4.5 g of fructose. Complete to 10 mL with dH2O. Use a magnetic stirrer overnight. Refractive index = 1.4688 at room temperature (RT: 19–23 °C). Store at 4 °C in dark for up to 1 month.

PBS-BSA 0,1% solution			To prepare 0,1% (vol/wt) PBS-BSA 0,1% solution, dissolve 500 mg of BSA in 50 mL of PBS-1X (store at 4°C for up to 2 weeks). And dilute 1mL of this solution into 9mL of PBS-1X. This solution can be used to precoat the tip and centrifugation tube.
Permeabilisation-blocking solution (PB solution)			The PBSDT blocking solution is PBS-1X supplemented with 0.1% – 1% Tritonx-100 (depending on the protein localization membrane/nucleus), 1% DMSO, 1% BSA and 1% donkey serum (or from the animal in which the secondary antibodies were raised). This solution can be stored at 4°C for up to 1 month.
PB:PBS-1X (1:10) solution			PB:PBS-1X (1:10) solution is a 10 time diluted PB solution. To prepare 10 mL of this solution dilute 1 mL of PB solution in 9 mL of PBS-1X.
Software			
Halo software	Indicalabs	NM 87114	
Harmony software	PerkinElmer	HH17000010	

Dr. Laura Broutier
« Childhood Cancers and Cell Death - C^3 Team »
Centre de Recherche en Cancérologie de Lyon,
UMR 1052 – CNRS 5286 – CRCL– CLB
laura.broutier@lyon.unicancer.fr

25/01/2021,

RESPONSE TO EDITORIAL AND REVIEWER COMMENTS

Dear Editor,

Thank you very much for considering our manuscript for publication and for your feedback. We thank the reviewers for their constructive and supportive comments and have edited the manuscript to address their concerns. We hereby provide a substantially revised manuscript, now entitled **"Staining and high-resolution imaging of 3D organoid and spheroid models"**, in which we have integrated all of the reviewers' comments.

We hope that with these changes and additions, JoVE will deem our manuscript suitable for publication.

With kind regards,

Dr. Laura Broutier

Editorial comments:

Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.	✓
Please revise the following lines to avoid previously published work: 35-36, 91-93, 103-104, 135-136, 139-140, 149-150, 171-172, 182-183, 189-190, 196-199, 207-208, 245-2456, 282-283, 291-292, 308-313, 343-345, 358-360, 430-433.	✓
JoVE cannot publish manuscripts containing commercial language. 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: e.g., Matrigel, OPERA Phenix, PerkinElmer, Harmony 4.9, Q Path, Cytoblock, Cytoblock Cassettes, Cytoblock Reagent #1, Cytoblock Reagent #2, Thermo Scientific, Panoramic SCAN II, Axio- Imager, Halo, Indica labs, etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.	✓
Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.	✓
Line 111/126/132/141: Please italicize “g” which indicates the centrifugal speed.	✓
Line 352-366/ 367-377: Please ensure that each Figure Legend includes a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.	✓
Please remove the lines 419-422	✓
Please do not use the &-sign or the word “and” when listing authors. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. End the list of authors with a period. Example: Bedford, C. D., Harris, R. N., Howd, R. A., Goff, D. A., Koolpe, G. A. Quaternary salts of 2-[(hydroxyimino)methyl]limidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).	✓
Please remove the commercial names (Harmony, Halo, Panoramic Scan II, Zeiss AxioImager).	✓
Figure 2B: Please remove the commercial name “Cytoblock”.	✓
Figure 3: Please provide the scale bars for all the images. Please define the scale bars either in the image or the Figure Legend.	✓
Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.	✓
Files uploaded to the “Supplemental Files (as requested by JoVE)” section of your Editorial Manager account are only for JoVE’s internal use and will NOT be published with your article. If you would like your files to be available for download with your article, then please move them to the “Supplemental Code Files” section of your Editorial Manager account. However, if these files are only to help the scriptwriter visualize the computational steps, then they can remain in the current section.	✓
14. Supplementary File 1: Please consider removing the commercial name Harmony 4.9	✓
15. Supplementary File 2: Please consider removing the commercial name Halo.	✓

Reviewers' comments:

Reviewer #1: Manuscript Summary: This protocol substantially covers 3D tissue fixation and embedding that can be standardized across many 3D model systems. The authors have included an exceptional level of detail that can be replicated by other researchers. Overall, I find the protocol and manuscript of good quality and of high value to the scientific community. As an organoid researcher myself, the methods within research publications are often too abbreviated to replicate. Thus, this new protocol will likely be highly utilized and cited.

We thank the Reviewer #1 for providing such constructive feedback on our manuscript. We have attempted to address all concerns raised to submit an improved version of our manuscript that we renamed: **Staining and high-resolution imaging of 3D organoid and spheroid models**. Below a point-by-point response to Reviewer #1 questions is provided and the manuscript has been adjusted accordingly. All modifications made to the manuscript are highlighted in red.

Major Concerns:

1) The protocol is general to 3D cultures, which is a strength, yet the introduction is unfocused and goes into too much depth about several types of 3D cultures. This should be revised to be general and provide examples without going into too much detail.

We have revised the Introduction to be more general and provide examples.

2) The figures are a great addition to the manuscript, but the legends should include the cell type and days in culture. This also may provide more focus to the introduction in which they may refer to the examples shown.

We have adjusted the legend and cited the examples in the Introduction.

3) I'm not sure that the inclusion of the Axio imager with Halo software are permitted in JoVE. Also, although acquisition mode settings are included for one instrument, this section is not particularly powerful in reproducibility, as many laboratories utilize different confocal microscopes. Thus, this section may need to be removed, in which case, the title could be revised to something like "Staining and preparation of 3D cell models for high-resolution imaging".

We agree with the Reviewer #1 than we should avoid commercial language. Since imaging is a critical part of the procedure and details on acquisition mode settings and analyses are necessary and helpful for users, we decided to provide broader acquisition mode settings to help users regardless of their tools. Moreover, we kept details of our own acquisition and analysis settings in figure legends and supplementary figures but emphasized in the text that they can be used as helpful examples but should be adapted to the microscope and analysis software used.

4) A video for harvesting 3D cell cultures is only somewhat helpful, but the field would greatly benefit from videos of procedures 2 (whole mount staining) and 3 (paraffin embedding).

We thank the reviewer for this interesting feedback and have changed the highlighted sections accordingly in the manuscript.

5) The following is used interchangeably throughout the text, and is a source of confusion: spheroids, 3D cell cultures, 3D-cells, tumor spheroids, organoids. The authors should clarify the terminology throughout the protocol.

We apologize for the confusion. We have now clarified our terminology in the manuscript. We have carefully defined what we consider in this manuscript as *in vitro* 3D cell culture models, spheroids and organoids. Moreover, to help with manuscript clarity towards the procedure itself we only use the term “3D structures” to refer to *in vitro* 3D cell culture models.

6) The discussion can be more thorough by integrating more than one comparison with previously published protocols to this one for each section of the procedure.

As suggested, we have revised our Discussion to include comparisons of our protocol with other previously published protocols.

Minor Concerns:

7) Areas of odd wording throughout the manuscript

We have carefully edited our manuscript.

8) Add cell type and days in culture to the figure legends

We apologize for this oversight. We have adjusted the legends.

9) Figure 1:
   - Overall clear schematics. The organization could be improved to have clearer zones. Consider splitting this figure into "a" and "b" sections for the separate procedures described above.
   - Change "sectionning" to "sectioning"
   - Consider including "&" to read as "2D Sectioning & Staining"
   - Refer to the different zones in your figure to guide the reader. Example, line 354: "...and then either prepared for immunofluorescence or embedded in paraffin." to "...and then either prepared for 3D whole mount staining (top) or 2D sectioning and staining (bottom)."

We thank the Reviewer#1 for the suggestions. We have adjusted Figure 1 and the corresponding Legend to address all comments.

10) Figure 2:
   - It would be useful to guide the reader through the text by referring to figure parts. Also the legend (or the text) should describe "good embedding" versus "failed embedding." Here the authors should indicate arrowheads point to 3D cultures. This are much larger than the actual organoids and not useful. A higher magnification to see the organoids would help.

We have adjusted Figure 2 and the corresponding Legend to address all comments.

11) Figure 3:
   - In reference to figure, define "Weak positive" in the legend or in the text.

As suggested, we defined "Weak positive" in the legend of the Figure 3.

Reviewer #2: Overall, the aim to provide simple, robust, and reproducible protocols for quantifying cells in 3D environments is very worthwhile, and I applaud the authors for choosing to address this challenge. While I have no doubt that the protocol as described here works well for the cell models used by the authors, I wonder whether they would scale well for larger organoids or different types of organoids. As it stands, the description of the procedure lacks key details and does not adequately address limitations. As such, I think the broad claims of the title and text need to be significantly narrowed down in order to avoid misleading future readers in trying a protocol that has not been vetted for their desired conditions. Also, the authors fail to distinguish the relative advantages and disadvantages of their protocol to a plethora of already published 3D staining, clearing, and quantification protocols. More details in the individual comments below.

We thank the Reviewer #2 for providing constructive feedback on our manuscript. We have attempted to address all concerns raised to submit an improved version of our manuscript that we renamed: **Staining and high-resolution imaging of 3D organoid and spheroid models**. Below a point-by-point response to Reviewer #2 questions is provided and the manuscript has been adjusted accordingly. All modifications made to the manuscript are highlighted in red.

Major Concerns:

1) The authors claim a "Staining and high-resolution imaging of fixed 3D cell models". The field of 3D cell models is quite vast, spanning many different tissue and cell-types and aggregates with small (tens of microns) to very large diameters (several millimeters). Many organoid protocols result in tissues that are larger than one millimeter, and the broad claims in this manuscript appear not to have been vetted with samples larger than a few hundred micrometers. There is a sentence in the discussion mentioning this, but the title and introduction may mislead readers looking for a protocol to fix, stain, and clear organoid models that commonly result in structures in the millimeter range. The authors should either considerably narrow their claims and amend their title and text, or they need to provide data showing the applicability to various 3D spheroid and organoid models spanning at least tissues derived from the three germ layers to account for the heterogeneity of tissue behaviors of different origin during fixing, staining, and clearing.

We apologize for the lack of clarity. Our procedure presents two different and complementary types of staining and imaging acquisition to deal with a large variety of sizes (from 50-100 µm to several millimeters) and types of *in vitro* 3D cell culture models. The choice of one (3D whole-mount analysis) or the other (2D sectioning analysis) will depend on the model used and aim of the study. 3D whole-mount analysis by confocal enables the visualization of cells with a depth of field of 200 µm, irrespective of the overall size of the 3D structure, whereas the 2D sectioning allows the analysis of larger samples reaching several millimeters, but providing only 2D dimensional results. We have now clarified the procedure and the Figure 1 to help users choose the best experimental set-up according to their needs.

We agree with Reviewer #2 and considering the large variety of 3D *in vitro* cell culture models we have limited our procedure to organoids and spheroids, and carefully defined in the introduction what these structures represent. We have amended our title accordingly. Moreover, following Reviewer #2 suggestions, we have included in the revised manuscript references and new staining in Figure 3 to show the validity of our procedure for cells arising from the three germ layers to account for the heterogenous tissues. Please find further details in the following answers.

2) The authors only test their claims using one, maybe two different 3D cell culture models. They also fail to identify what their model system is (or are they two different ones in figure 3?), making it additionally difficult for readers to judge whether this protocol might be of use for their own samples. The authors need to clearly identify what their samples are and how they were generated.

We apologize for this oversight. In the first version of this manuscript, we used one of our models, a primary human rhabdomyosarcoma culture grown in 3D structures in low attachment suspension culture conditions for 15 days. We have adjusted the legend of Figure 3 accordingly. We have now added more models (see below), and have clarified this point.

3) The authors do present their example data without any discernible controls. How can a reader judge whether the technique results in abnormally high background stainings? How do we know whether the technique alters morphology of the markers? The study should include control samples imaged without primary antibodies and treated and acquired with identical settings. These should be presented side-by-side with the fully stained samples. Similarly, to judge fidelity of the segmentation, it would be advantageous to count a few samples by hand and compare results with the automated segmentation. The way things are presented here, it is impossible to judge the quality of the technique employed due to a lack of conditions to compare it to.

For all acquired images, we established settings using a negative control to control the lack of fluorescence when the primary antibody is absent (see Figure 3a) and to validate the specificity of our staining. In the procedure and in the legend of Figure 3, we have now clarified this step, which we agree with Reviewer #2, is a prerequisite for any analysis. Nevertheless, we would prefer not to include all these control images in our Figure 3 to maintain a level of clarity.
Moreover, to provide data supporting our automated segmentation methods, we have now performed manual counting and obtained highly consistent results, though this procedure was 10 times longer than the automated segmentation. We have modified Figure 3 accordingly.

4) The authors use a single nuclear stain (Ki67) to test and present their protocol. This should be considerably widened to include cytoplasmic and membrane-based stains to ascertain whether their techniques also function for these classes of antigens.

We thank the Reviewer #2 for this comment, which has significantly improved our manuscript. In the first version of this manuscript, we decided to present an example using a nuclear marker. To our knowledge, if nuclear staining works, and considering that the penetration of the antibodies is the limiting step here, membrane-based and cytoplasmic staining should follow. However, this protocol was already performed using a variety of markers (cytoplasmic, membrane-based and nuclear) in my previously published work (Broutier et al. Nature medicine, 2017). Please find below a panel with examples of different staining obtained using the described procedure on primary liver cancer organoids.

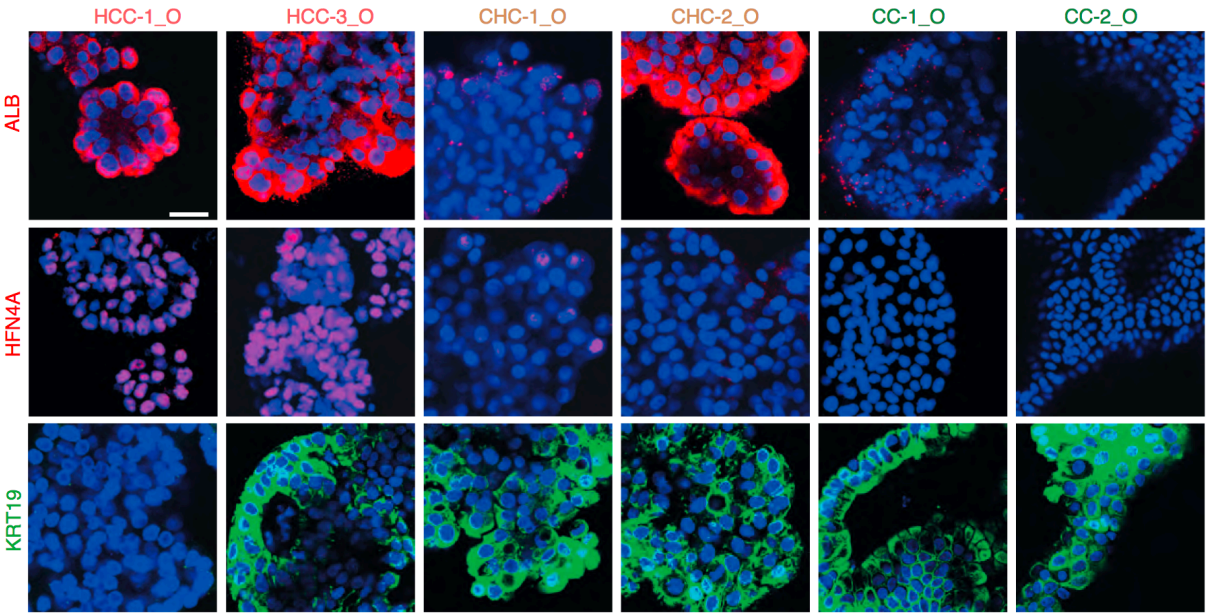


Figure A: IF analysis for the hepatocyte markers ALB and HFN4A (red) and ductal/CC marker KRT19 (green) on tumoroids expanded in culture for at least 3 months. Nuclei were counterstained with Hoechst33342 (blue). Scale bar, 30µm.

We are now mentioning this reference in the introduction to help readers to evaluate the quality of our procedure. Moreover, following Reviewer #2 suggestions, we have now included in Figure 3 cytoplasmic and nuclear staining on 3 different models from different origins (human rhabdomyosarcoma, human glioma, murine neural crest cells). Together with our cited previous work, we consequently applied our procedure to cells arising from the three germ layers to account for the effect on heterogenous tissues of fixing, staining, and clearing.

5) The removal of ECM suggested by the authors amounts to a relatively "blind" mechanical abrasion. These risks damaging the outer structures of organoids, some of which can extend small cellular processes into the ECM. How can the process guarantee not removing the outer layer of morphologies of embedded 3D cellular structures? See below, our combined response to point 5 and 6.
6) Authors caution the readers that "Remaining 3D matrix can hamper proper antibody penetration of the organoid structure or lead to high background staining". This is a big caveat. Removal of ECM may drastically alter morphology and the understanding of the cellular niche in the outer segments of organoids. If this protocol requires removal of ECM to function, then it only provides an incomplete picture of the very cellular niches that 3D cell culture aims to understand. Authors should amend their claims.

As Reviewer #2 noted, the 3D matrix can hamper proper antibody penetration of the organoid structure or lead to high background staining. We thus suggest whenever possible to remove ECM before staining. In many cases, this removal, based on very gentle washes, results in the preservation of the 3D structures of the organoids, as nicely exemplified by *Dekkers et al.* (Nature protocols, 2019). Owing to the wide variety of 3D *in vitro* cell culture models, we agree that ECM removal may alter the morphology of the outer segments of organoids (notably in case of small cellular protrusions) and partially hamper the analysis. To clarify these points, we have now (i) limited our procedure to organoids and spheroids, and carefully defined these structures in the introduction (ii) added a note in the appropriate section to encourage the user to adapt the “Harvesting of 3D cell culture models embedded in a matrix” step according to their systems and needs. Moreover, we have given tips and included a reference to help in the process of keeping the ECM and pursuing with our procedure.

7) The evidence for the ability to quantify in 3D in figure 3 is based on confocal stacks spanning 75 x 0.7 um = 52.5 um. These are very small samples and the side views indicate that the stacks do not even remotely include the rather small spheroids. The authors should quantify whole microtissues to support their claims or describe potential limitations in detail. Ideally, they showcase proper segmentation at the bottom, center, and top of the spheroids using a single image segmentation algorithm.

We have re-acquired a more accurate sample to sustain our claim. We have re-acquired a more accurate sample to sustain our claim. The choice of the step in between each focal plan was carefully adjusted to the specifications of each objective use and its depth of focus. For larger structures, we acquired up to 200µm to facilitate the analysis of larger 3D structures.

8) "Notably, since cystic organoids tend to collapse during fixation, we advise not to let them grow over 400 µm in size to avoid this issue." Most organoids above a certain size will develop necrotic centers due to diffusion-limited nutrient and oxygen supply. As cells die, the center hollows, turning them essentially into cystic organoids. The incompatibility of this protocol with cystic structures prohibits the use of this protocol for a vast group of organoid structures.

We apologize for the lack of clarity. By cystic organoids, we meant monolayered organoids (see answer to comment 18 from Reviewer #2). Hence, we respectfully disagree with Reviewer #2. Indeed, necrotic 3D structures are usually empty in their middle but very rarely (if never) mono-layered. Thus, to our knowledge, they do not collapse during fixation, nor during the clearing step. Moreover, whenever possible we void studying 3D structures suffering massive necrotic events due to diffusion-limited nutrients and oxygen supply, since this will affect the physiology of the studied system. Depending on the question addressed, setting-up smaller 3D structures, to limit necrosis, could drastically improve the culture system and subsequent high-resolution imaging.

Minor Concerns:

9) "Reagent changes and transfer of 3D structures can cause sample loss throughout the following procedure" -- a very useful cautionary note. It would be helpful to be more specific in describing different avenues of sample loss as to sensitize the reader to particular caution during these steps.

We have now included the following **NOTES** throughout the manuscript to emphasize the different avenues of sample loss and sensitize the reader to taking extra care during these steps.

- ***NOTE:*** *Be careful not to aspirate the 3D structures, which will be only loosely attached to the tube wall.*
- ***NOTE:*** *If necessary, cut the top of the tip to avoid breaking the 3D structures.*
- ***NOTE:*** *Tip precoating will prevent the cells from sticking to the tip and minimize any loss of 3D structures.*

10) We recommend using 3D cell culture models with a size ranging from 100 to 500 µm. -This is very small for many organoid models. Neural organoids can grow to 4 mm and larger. Is the protocol capable of dealing with this type of sample? What if any adjustments need to be made? Can you show sample data from larger organoids or at least provide an upper bound of suitable size for your protocol?

We apologize for the lack of clarity. Our procedure presents two different and complementary types of staining and imaging acquisition to deal with a large variety of sizes and types of *in vitro* 3D cell culture models. The choice of one (3D whole-mount analysis) or the other (2D sectioning analysis) will depend on the model used and issue addressed. 3D whole-mount analysis by confocal enables the visualization of cells at a depth of field of up to 200 µm, irrespective of the overall size of the 3D structures, whereas the 2D sectioning method allows the analysis of samples of any size, but results remain 2D dimensional. We have now clarified the procedure and the Figure 1 to help users choose the best experimental set-up according to their needs.

11) Spinning as part of the protocol is surprising, as most 3D cell cultures tend to settle quickly by gravity. Why are potentially compromising centrifugation steps part of the protocol? Could they be omitted to maximize quality of the resulting samples?

This is indeed an optional step for some 3D structures that sink rather slowly (10-30 minutes) and partially. Readers should be able to choose carefully considering the advantages/disadvantages of this optional step according to the aim of their study. We have rephrased the manuscript to emphasize this point.

12) "Prepare the permeabilization-blocking (PB) solution. PB solution is PBS-1X supplemented with 0.1%-1% Triton X-100" -- this is a very wide range. It would be helpful to add a table with specific use scenarios as to maximize utility to the reader.

We have carefully rephrased this sentence to provide the range of Triton X-100 concentration we routinely use depending on the cellular localization.

13) Does a 1h blocking time allow penetration to deeper tissue layers in large samples? How would the authors suggest the reader to optimize this step for their own samples?

To our knowledge, 1h is sufficient to allow adequate penetration into the 3D structures analyzed, for a field of depth of 200 µm. Users working on larger samples and having to do 3D-whole mount staining should optimize blocking time (increase), clearing step (the specimen should be totally cleared) and image acquisition (e.g. light sheet microscopy) for their own samples. However, to our knowledge resolution obtained for such deep structures remains suboptimal, compared to confocal microscopy, for obtaining sub-cellular resolution. This has now been stated in the revised version.

14) Why is the PB solution diluted for the antibody step?

We carefully optimized this protocol. The use of PB 1:10 solution provides better results than pure PB solution or classically used PBS1X and PBS1X-BSA solutions -probably thanks to the presence of a lower concentration of Triton X-100, helping the spread of primary antibodies without damaging them, and BSA and donkey serum helping with lowering non-specific binding. Moreover, we decided to use a PB 1:10 as this was more cost-effective.

15) Washing after primary ab is 5x3 min. Is this enough to remove the primary antibodies from the tissues? If it takes 3-4 days to penetrate into the tissue, why are 5x3 min sufficient for proper removal? Would the protocol not risk the formation of larger primary/secondary antibody complexes in the tissue? Alternatively, could the incubation time with primary ab be reduced?

To our knowledge, this is sufficient to remove the non-bound primary antibodies. Indeed, we never observed the presence of larger primary/secondary antibody complexes in the 200µm sections analyzed. This is also sufficient to prevent background noise.

16) 1:250 is a very high secondary concentration. Most 3D protocols use 1:1000 or 1:2000. Why use them at 10x higher concentration than this?

We carefully optimized this protocol and in our setting this concentration of secondary antibodies gave the most robust and qualitative results for a large variety of antibodies.

17) Hoechst diluted 1:1000 from which stock concentration?

We apologize for this oversight. We have now provided Hoechst stock concentration in the manuscript.

18) Please define the term "mono-layered organoid". It seems like an oxymoron.

Please find below a panel from my work published in Nature medicine in 2017 with an example of non-layered organoid (left) versus more compact multi-layered cancer organoids.

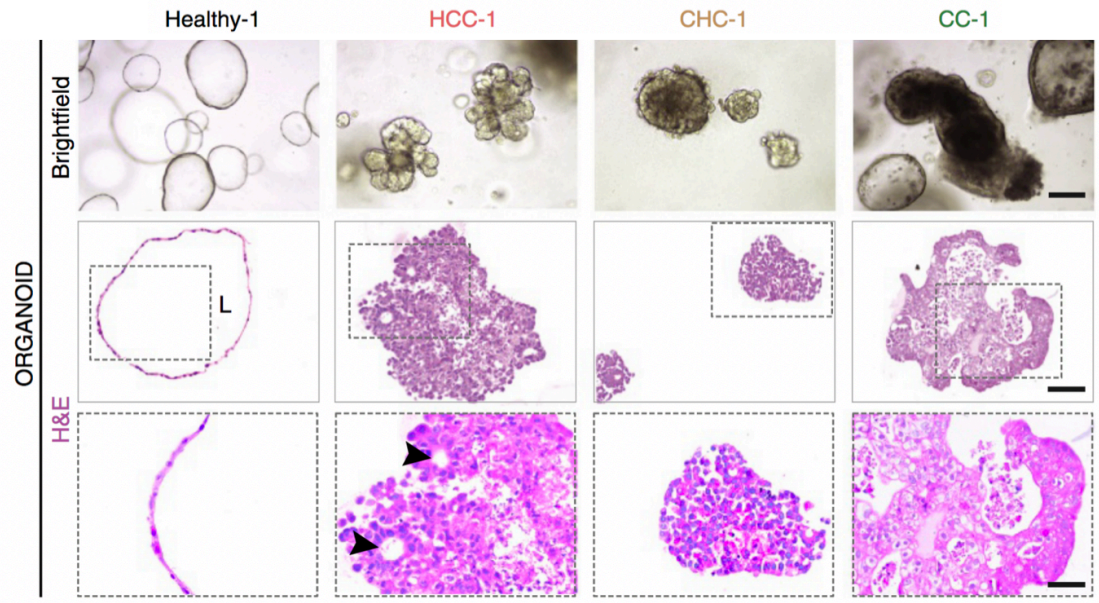


Figure B: Representative brightfield microscopy images (top row) and H&E histological analysis of primary liver cancer organoids (middle and bottom row). Note that, whereas healthy liver-derived organoids (left) grew as a single-layered epithelium of ductal-like cells surrounding a central lumen (L, lumen), tumor-derived organoids (right) formed compact structures. HCC-1 tumoroids, similarly to their parental tissue, exhibit pseudoglandular rosettes (arrowheads), a hallmark of HCC. CC-1 tumoroids present a glandular lumen, similar to the patient's tumor (top row). Scale bars, middle row, 100 µm; top and bottom rows, 50 µm.

19) Reversal of clearing can be very useful. Please provide a brief summary of steps for proper reversal. Incidentally, are the samples damaged by drastic changes in osmolarity? In any case, the authors should include data/images if they include this claim.

We do not have ready-to-publish data to support this claim and did not have time to generate them in the short time of this rebuttal. Consequently, the claim has been removed but mentioned in the discussion with an appropriate reference.

20) "Place the biopsy pad containing the 3D structures into the mold and gently fold it until all of the organoids drop to the bottom of the mold" - I do not understand what is being folded how in this step. Please describe in more detail.

We have carefully rephrased this sentence in our manuscript.

21) Cytoblock kit steps: do these instructions differ from the manufacturer's manual? If so, how?

We did follow the instructions from the manufacturer’s manual. This has now been stated in the revised version.

22) Quote: "Polymerized gel containing 3D structures is carefully handle using forceps for block completion." Please fix sentence.

We thank the reviewer for noticing this mistake. We have carefully rephrased this sentence.

Suppl1

suppl2