# CLEARING ORGANOID PROTOCOL

(High-resolution 3D imaging of fixed and cleared organoids (NATURE PROTOCOLS VOL 14 JUNE 2019 |1756–1771) https://doi.org/10.1038/s41596-019-0160-8

## Fructose-Glycerol Protocol

### Reagent setup

**PFA (4% (wt/vol))**
To prepare 4% (wt/vol) PFA, heat 500 ml of PBS to 60 °C in a microwave. Add 20 g of PFA and dissolve on a stirrer. Add a few drops of 10 M NaOH. Cool on ice and adjust the pH to 7.4 by adding a few drops of 10 M HCl (store at −20 °C for up to 2 months).

**PBT (0.1% (vol/vol))**
To prepare 0.1% (vol/vol) PBS-Tween (PBT), add 1 ml of Tween 20 to 1,000 ml of PBS (store at 4 °C for up to 4 weeks).

**Organoid washing buffer**
To prepare organoid washing buffer (OWB), add 1 ml of Triton X-100 and 2 g of BSA to 1 liter of PBS (store at 4 °C for up to 2 weeks).

**Fructose–glycerol clearing solution**
Fructose–glycerol clearing solution is 60% (vol/vol) glycerol and 2.5 M fructose. To prepare 660 ml of this solution, mix 330 ml of glycerol, 70 ml of $dH_2O$ and 297.2 g of fructose on a magnetic stirrer. Refractive index = 1.4688 at room temperature (RT: 19–23 °C). Store at 4 °C in dark for up to 1 month.

**PBS–BSA (1% (wt/vol))**
To prepare 1% (wt/vol) PBS–BSA, dissolve 1 g of BSA in 100 ml of PBS (store at 4 °C for up to 2 weeks).
*(Esta solução serve para preparar pontas para pipetar os organoides, não utilizámos)*

**Light-sheet embedding solution**
To prepare light-sheet embedding solution, dissolve 0.4 g of LMP agarose in 10 ml of water using a microwave and let it cool to 40 °C on a heat block with a magnetic stirrer. Add 10 ml of fructose–glycerol clearing solution (40 °C) and mix on a heat block with a magnetic stirrer until the solution is clear; keep at 40 °C until use. **CRITICAL** Prepare the solution fresh every time.

## Fixation and blocking ● Timing 1 h

1. Gently resuspend the pellet of organoids in 1 ml of PFA, using a 1-ml tip (precoated with 1% (wt/ vol) PBS–BSA as described in Step 3 – *não preparámos estas pontas*).

2. Incubate at 4 °C for 45 min (halfway through the incubation period, gently resuspend the organoids, using a 1-ml tip precoated with 1% (wt/vol) PBS–BSA). *During fixation, a minimal change in morphology is observed for most mono- or multilayered organoids. However, fixation can cause spherical, monolayered organoids that contain large fluid-filled lumens to 'collapse'. This has been observed for human intestinal samples and may depend on the donor and the quality of the culture medium.*

3. Wash the organoids by filling (até cobrir bem) the eppendorf with cold (4 °C) PBT, gently mix by swirling the tube, incubate for 10 min at 4 °C. *(From this step onward, precoating of tips is not needed*).

   **PAUSE POINT** Organoids can be stored in PBT at 4 °C for 2 d or over the weekend, but, ideally, one should continue with the next steps as soon as possible.

4. For blocking the organoids, resuspend the pellet in cold (4 °C) OWB and transfer (usar ponta amarela cortada) the appropriate amount of organoids per staining to a low-adherence/suspension 24-well plate (use at least 400 µl per well).

   **CRITICAL STEP** Compared to blocking and immunolabeling in tubes or Eppendorf tubes, the 24- well format allows gentle movement of organoids for optimal antibody penetration and washings, and allows organoid visualization at any moment during the procedure using microscopy.

5. Incubate at 4 °C for 15 min.

## Immunolabeling ● Timing 44 h

6. Add 200 µl of OWB to one of the empty wells and use this as a reference well.

7. Make sure that the organoids are settled at the bottom of each well; then tilt the plate to 45° and remove the OWB (200 µl), leaving the organoids in 200 µl of OWB (use the reference well to estimate 200 µl)

8. Add 200 µl of OWB with primary antibodies (2× concentration) to each well and incubate overnight at 4 °C with mild rocking/shaking (60 r.p.m. on a horizontal shaker).

**Washing**

9. Add 1 ml of OWB per well.

10. Wait for 3 min until all organoids are settled at the bottom of each well.

11. Remove 1 ml of OWB, leaving 200 µl in each well.

12. Add 1 ml of OWB per well and incubate for 2 h with mild rocking/shaking.

13. Repeat Steps 10–12 two more times.

14. Wait for 3 min, until all organoids are settled at the bottom of each well.

15. Remove 1 ml of OWB, leaving 200 µl in each well.

16. Add 200 µl of OWB with secondary antibodies (2× concentration) per well and incubate overnight at 4 °C with mild rocking/shaking.

17. Repeat Steps 9–13.

18. Transfer the organoids of each well to a **1.5-ml** (confocal or multiphoton imaging) **or 2-ml** (light-sheet imaging) Eppendorf tube and let the organoids settle at the bottom or spin down (70g, 4 °C, 2 min).

**CRITICAL STEP** Reagent changes and organoid transfers can cause sample loss during fixation and immunolabeling. A loss of ≤10–20% of the initial number of organoids in the culture well (Step 1) is expected. We advise reading 'Troubleshooting' section for Steps 1, 10 and 20 to ensure a minimal loss of sample.

**PAUSE POINT** At any point between Steps 6 and 17, the organoids can be stored in OWB (with or without antibodies) at 4 °C for 2 d or over the weekend without rocking, but, ideally, one should continue with the next steps as soon as possible.

## Sample preparation for imaging

Continue with confocal or multiphoton sample preparation (**option A**) or with light-sheet sample preparation (**option B***). (mesmo sendo para light-sheet seguimos a opção A)*

### (A) Sample preparation for single-photon confocal or multiphoton imaging ● Timing 35 min

I. **Clearing**. Remove as much of the OWB as possible without touching the organoids.

II. Add fructose–glycerol clearing solution (minimum of 50 µl at RT – até cobrir bem) using a 200-µl tip with the end cut off and resuspend gently. Prevent bubble formation.

**CRITICALSTEP** The fructose–glycerol clearing solution has high viscosity. It is therefore difficult to handle small volumes. Make sure to use the fructose–glycerol clearing solution at RT and pipette slowly and carefully.

III. Incubate at RT for 20 min. *Clearing causes organoid shrinkage. This does not change the general morphology of most mono- and multilayered organoids, but it can alter the spherical shape of mono-layered organoids with large lumens.*

**PAUSE POINT/SHIPPING** At this stage, the sample can be stored at 4 °C (for at least 1 week) or −20 °C (for at least 6 months).

IV. *Slide preparation for imaging*. Draw a 1 × 2-cm rectangle in the middle of a slide, using a PAP pen.

V. Place a 1-cm-long piece of sticky tape at both sides of the rectangle. Use 1, 2 or 3 layers, depending on the organoid size.

VI. Cut off the end of a 200-µl tip and use it to place the organoids in the middle of the rectangle. Use 20 µl per layer of tape.

VII. Place a coverslip on top. Place the left side of the coverslip on the left stack of sticky tape and then slowly lower the right side of the coverslip until it touches the right stack of sticky tape; then let go of the coverslip.

VIII. Wait for 1 min to allow the fluid to spread out.

IX. Gently apply pressure to both sides of the coverslip to firmly attach it to the double-sided sticky tape. The slide is now ready for imaging.

**PAUSE POINT** At this stage, the sample can be stored at 4 °C (for at least 1 week) or −20 °C (for at least 6 months).

**(B) Sample preparation for light-sheet microscopy ● Timing 2–16 h**

I. Remove most of the OWB and place the Eppendorf tube on a heat block at 40 °C.

II. Before the pellet dries out, resuspend the pellet in 300 µl of light-sheet embedding solution and put back at 40 °C.

III. While the Eppendorf tube is at 40 °C, use a light-sheet glass capillary to aspirate the mix and let it solidify at 4 °C.

IV. Place the capillary containing the sample in the light-sheet chamber and fill the chamber with fructose–glycerol clearing solution.

V. Push down the agarose-based clearing solution (light-sheet embedding solution) of the capillary to expose the sample for imaging.

**CRITICAL STEP** The quality of light-sheet images is severely affected by the presence of air bubbles or other small objects in the light path, so work cleanly and prevent bubble formation. In addition, allow the sample to settle in the imaging chamber for a couple of hours to overnight at RT before imaging.

**Imaging and image processing**

Proceed with single-photon or multiphoton (**option A**) or light-sheet (**option B**) imaging.

**(A) Single-photon and multiphoton imaging ● Timing 1–2 h**

I. Use a confocal microscope to image the slide, using a multi-immersion 25× or oil immersion 40× objective for single-photon imaging or a 32× water immersion objective for multiphoton imaging. The acquisition mode settings we use most commonly are as follows: scan mode = frame, frame size = 1,024 × 1,024, line step = 2, bidirectional scanning, speed = 7, averaging number = 1, bit depth = 12 and bidirectional scanning. Activate the z-stack mode and define the lower and upper limits. For imaging large organoids or multiple organoids together, activate the tile scan and indicate the borders of the organoids. Of note, other confocal microscopes can be used for similar results (we have tested the protocol with the Leica SP8 and Olympus FV 3000).

II. For tile scan acquisition, stitch the imaging using the ZEN Black software (Imaris, Fiji or ImageJ software can also be used).

III. Use the 3D view tab in the Imaris imaging software to optimize brightness, contrast and 3D-rendering properties for the best representation of the imaging. After corrections, the RGB images can be exported as TIFF files.

**(B) Light-sheet imaging ● Timing 40–90 min**

I. Use a light-sheet microscope to image the samples in the light-sheet embedding solution, using a 20× detection objective (clearing immersion NA = 1.0). The acquisition mode settings we most commonly use are as follows: scan mode = frame, frame size = 1,024 × 1,024, averaging number = 1, bit depth = 8. Activate the z-stack mode (define lower and upper limits) and multi-view mode (×4). Set the light-sheet thickness to ~6 µm and use dual illumination.

II. Use the 3D view tab in the Arivis imaging software to optimize brightness, contrast and 3D-rendering properties for the best representation of the imaging. After corrections, the RGB images can be exported as TIFF files (>300 d.p.i. is recommended).