OmniStain ORGANOIDS PROTOCOL

** perform all steps at 20-25°C (room temperature) unless otherwise specified.

Tissue pretreatment

1. Carefully dissociate the organoid from Matrigel using the organoid recovery solution as specified by the manufacturer.
2. Use a cut Pasteur pipette to suck up and transfer the organoids to a microcentrifuge tube, or, a 96-well plate made of polypropylene.
3. Dehydrate the fixed tissue in 50% v/v Methanol / H_2O x 2, then in 100% Methanol x 3, 10min each.
4. Delipidate the tissue in 2:1 v/v mixture of CH_2Cl_2 / Methanol (delipidation mix) for 1 hour. Mix and add the solutions in a fume hood.
5. Wash the tissue in 100% Methanol x 3, 50% Methanol / H_2O x 2, and PBSN x 2, 10min each.
6. Pre-incubate the tissue in 100µl **1x OmniStain buffer A** at 37°C for 1 hour. Prepare the 1x OmniStain buffer A by mixing **2x OmniStain buffer A** with equal volumes of PBSN.

OmniStaining

7. Prepare the staining solution as follows:

2x OmniStain buffer A	50µl
Primary antibody	1µg each
Secondary antibody Fab fragments, fluorophore-labelled	1µg each
Lectins, fluorophore-labelled (optional)	1µg each
Pre-mixed nuclear stain and OmniStain buffer C *(the nuclear stain and OmniStain buffer C should be pre-mixed in a separate tube)*	1µl
PBSN	to 100µl
Total for each organoid:	100µl

8. Remove and discard the spent 1x OmniStain buffer A in pre-incubation. Add 100µl staining solution to each organoid sample. Incubate at 20-25°C overnight with gentle shaking in the dark.

Washing and Tissue clearing

9. Wash the tissue in 500µl **1x OmniSain buffer B** at 20-25°C for 2 x 1 hour.
   Shake the tube horizontally to ensure adequate washing.
10. Wash the tissue thoroughly with PBSN at 20-25°C for 3 x 15 min.
    Shake the tube horizontally to ensure adequate washing.
11. Dehydrate the tissue as in Step 3 to 100% Methanol.
12. Use a cut Pasteur pipette, carefully suck up and transfer the organoid onto the coverslip of a confocal imaging dish. Remove all the methanol around the organoid, and add a small drop of BABB, DBE or ECi to the organoid and let it incubate without shaking. The organoid will turn transparent within 1 hour and be ready for imaging.

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OmniStain ORGANOIDS PROTOCOL

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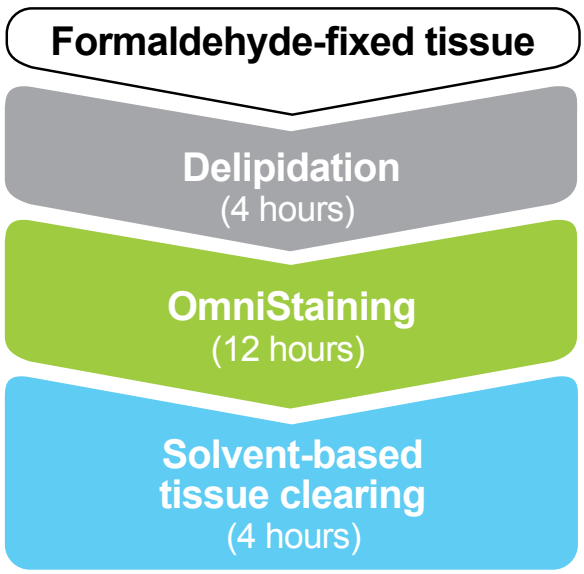
Summary



Materials provided

- 2x OmniStain buffer A
- 1x OmniStain buffer B
- 1x OmniStain buffer C

Materials not provided but required

- 37°C incubator
- Shaker
- Fume hood
- Glass or polypropylene containers / tubes (e.g., microcentrifuge tubes, conical tubes)
- 2.5 µL to 1000 µL adjustable micropipettes with disposable tips
- Deionized water
- PBSN: 1x PBS + 0.5% w/v sodium azide
- Methanol
- Dichloromethane
- Solvent-based clearing solution, either
  - 1:2 v/v mixture of benzyl alcohol / benzyl benzoate (BABB),
  - dibenzyl ether (DBE) or
  - ethyl cinnamate (ECi).
- Primary antibodies
- Fluorescent secondary antibody Fab fragments or nanobodies
- Nuclear stains

Introduction

- OmniStain (also known as INSIHGT) is a **multiplexed 3D immunostaining** method.
- Increases antibody penetration depth and uniformity by **20-30x**, perfect for high-quality tissue clearing.
- The **staining only takes <24 hours for whole organoids <2 mm**, without specialized equipment.
- Works with simple formaldehyde-, glutaraldehyde- and polyepoxide-fixed tissues.
- Fluorophore-conjugated primary antibodies are NOT required. To date, **>360** commercially available primary antibodies have been proven to be compatible.
- OmniStain quenches endogenous fluorescence, so the localization of fluorescent protein must be revealed with their corresponding anti/nanobodies.

Read the paper:
Yau *et al. Nature communications.* 2024. **15**:10888.

Video and resources:
https://illumos.co/omnistain-for-organoid-protocol/

OmniStaining with conventional secondary antibodies (whole IgGs)

***Note: the penetration homogeneity will be suboptimal using the bivalent whole IgG secondary antibodies.*

- Follow the protocol on the opposite page of this leaflet till completing Step 10, but omit secondary Fabs in Step 7.

- you will obtain a tissue stained with primary antibodies.

- Pre-incubate the primary antibody-stained tissue again in 1x OmniStain buffer A as described in Step 6. Then stain the tissue as in Step 7 with fluorescent secondary IgGs, at half the specified amount.

- you can include lectins and nuclear stain in this step.

- Process the fluorescently stained tissue through the rest of the protocol.