OmniStain ORGANOIDS v2

*** Unless stated otherwise, incubate at 20–25 °C with 100-rpm orbital shaking. Use fresh solution for each exchange; keep samples submerged and covered to prevent evaporation.*

****Starting amounts are per organoid (<2 mm); scale for larger or pooled organoids.***

Pretreatment

1. Carefully dissociate the organoids from Matrigel using the organoid recovery solution as specified by the manufacturer.
2. Use a wide-bore pipette to transfer the organoids to a 2 mL microcentrifuge tube, or a 96-well plate made of polypropylene.
3. Dehydrate in 200 µL 50% v/v methanol in H_2O x 2, then in 200 µL 100% methanol x 3, 10 min each.
4. Delipidate in 200 µL 2:1 v/v dichloromethane:methanol mixture for 1 hour in a fume hood.
5. Wash in 200 µL 100% methanol x 3, 50% v/v methanol in H_2O x 2, and PBSN x 2, 10 min each.
6. Pre-incubate in 100 µL **1x OmniStain Organoids v2 buffer A** at 37°C for 1 hour.
   • Prepare the 1x buffer A by mixing equal volumes of **2x OmniStain Organoids v2 buffer A** and PBSN.

OmniStaining v2

7. Prepare the staining solution. Use sufficiently concentrated probe stocks for a final volume of 100 µL.

2x OmniStain Organoids v2 buffer A	50 µL
Species-compatible unconjugated primary antibodies	1 µg each
Matched fluorophore-conjugated secondary Fab fragments	1 µg each
Fluorophore-conjugated lectins and primary antibodies (optional)	1 µg each
PBSN	to 100 µL
Total for each organoid:	100 µL

** DO NOT add nuclear stains to OmniStain Organoids v2 buffer A. Perform nuclear staining as in **Step 9**.*

8. Remove and discard the pre-incubation solution. Add 100 µL staining solution to each organoid. Incubate at 20-25°C overnight (12-18 hours) with gentle shaking in the dark.

Washing and Tissue clearing

9. Prepare 200 µL of 1× OmniStain Organoids v2 Buffer B, optionally containing 1–5 µM nuclear stain at final concentration. Incubate at 20-25°C for 4 hours or overnight (12-18 hours) with gentle shaking.
10. Wash in 200 µL PBSN for 3 x 15 min.
11. Dehydrate in 200 µL 50% v/v methanol in H_2O x 2, then in 200 µL 100% methanol x 3, 10 min each.
12. Transfer the organoid with a wide-bore pipette to a solvent-compatible confocal dish. Remove residual methanol without allowing the organoid to dry; immediately cover with the selected clearing agent and incubate without shaking until transparent (≤1 h).

Have questions? Contact us at sales@illumos.co or visit www.illumos.co

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OmniStain ORGANOIDS v2

Summary

Formaldehyde-fixed organoids

Pretreatment (4 hours)

OmniStaining (~24 hours)

Post-incubation and clearing (6 hours)

Materials provided

- **2x OmniStain Organoids v2 buffer A**
- **1x OmniStain Organoids v2 buffer B**

***** Reagents are NOT interchangeable with OmniStain Organoids or OmniStain IMMUNO***

Materials not provided but required

- PBSN: 1x PBS + 0.02% w/v sodium azide
- Methanol
- Dichloromethane
- Omni CLEAR or solvent-based clearing agents (BABB (1:2 v/v benzyl alcohol:benzyl benzoate), DBE or ECi)
- Antibodies and nuclear stains
  - ** Compatible with DAPI, SYTOX™ Green or SYTOX™ Orange only
- Deionized water
- 37°C shaking incubator
- Fume hood
- 2 mL microcentrifuge tubes, or 96-well polypropylene plates

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

Resources:
https://illumos.co/omnistain-for-organoid-protocol/

Introduction

- OmniStain (also known as INSIHGT) is a **multiplexed 3D immunostaining** method.
- Increases antibody penetration depth and uniformity.
- The **staining takes ~36 hours for whole organoids <2 mm**, without specialized equipment.
- Compatible with formaldehyde-, glutaraldehyde- and polyepoxide-fixed tissues.
- Fluorophore-conjugated primary antibodies are NOT required. **>360** commercially available primary antibodies were validated under specified conditions.
- Methanol quenches fluorescent proteins; use anti-fluorescent protein antibodies to visualize them.

Optional two-stage staining with conventional whole-IgG secondary antibodies

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

- Follow Steps 1–6.
- Prepare the Step 7 staining solution without secondary Fab fragments. If lectins will be added during the secondary stage, omit them during this first stage.
- Incubate as in Step 8, then complete Steps 9-10 without nuclear stain.
- Repeat Step 6 - preincubation **at 20-25°C**.
- Prepare 100 µL secondary staining solution:
  1. 50 µL of 2x OmniStain Organoids v2 Buffer A;
  2. fluorophore-conjugated whole-IgG secondary antibodies;
     - Add 1 µg of whole-IgG secondary antibody per 1 µg of the corresponding primary antibody.
  3. PBSN to a final volume of 100 µL.
- Incubate as described in Step 8.
- Complete Steps 9–12. Add the nuclear dye during this second buffer B incubation, if desired.