OmniStain H&E+IMMUNO PROTOCOL

*** Incubate at 20–25°C unless otherwise specified.*
*** Use fresh solution for each repeat. Keep the specimen fully submerged at all times and agitate on an orbital shaker at 100 rpm, and place the tube horizontally to maximize fluid flow unless otherwise specified.*

PBSN: 1x PBS with 0.02% w/v sodium azide.

Compatible with formalin-fixed tissues. For FFPE tissues, retrieve antigens with **OmniStain REZCUE** *first.*

Important: Plan the eosin, nuclear dye, and antibody-fluorophore panel before beginning the protocol to minimize spectral overlap and channel bleed-through.

Tissue Pretreatment

1. Place the tissue in a 50 mL conical tube.
2. Dehydrate in 25 mL 50% v/v methanol in H_2O 2 x 30 min, then in 25 mL 100% methanol 3 x 30 min.
3. Delipidate in 25 mL 2:1 v/v dichloromethane:methanol mix for 6-18 hours in a fume hood.
4. Wash in 25 mL 100% methanol 3 x 15 min.
5. Wash in 5 mL **OmniStain H&E pre-wash solution** (2 x 30 min), agitate with the tube upright.

H&E-like Staining

6. Prepare 0.4% w/v eosin Y stock solution in water.
7. Prepare a 1 mM or 5 mM nucleic acid dye stock solution for hematoxylin-like staining in DMSO.
   - Compatible dyes include BOBO-1, BOBO-3, POPO-1, TOTO-3, and YOYO-3.
8. Prepare 5 mL of final **OmniStain H&E staining solution** per specimen by adding the following dyes:
   - eosin at 0.004% w/v final concentration (i.e. 1:100 dilution),
   - nucleic acid dye at 5 µM final concentration (i.e. 1:1000 dilution for a 5 mM stock or 1:200 for a 1 mM stock).
9. Stain in 5 mL **OmniStain H&E staining solution** for 6-18 hours with the tube upright.
   - If necessary, add more staining solution with the same dye concentrations to cover the tissue.
   - Protect the tissue from light from this step onwards.

Washing and Pre-incubation

10. Wash in 25 mL 50% v/v methanol in H_2O, 2 x 15 min.
11. Wash in 10 mL **OmniStain H&E post-wash solution** (2 x 30 min) and agitate with the tube upright.
12. Wash sequentially in 25 mL 100% methanol 3 x 30 min, 50% v/v methanol in H_2O 2 x 30 min, and PBSN 2 x 30 min.
13. Pre-incubate in 2 mL **1x OmniStain buffer A** at 37°C with the tube upright for 6-18 hours.
    - Prepare the 1x buffer A by mixing equal volumes of **2x OmniStain buffer A** and PBSN.

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OmniStaining (values were illustrated for 1cm³ tissues or whole adult mouse brains and can be scaled)

14. Discard the preincubation solution and add 1 mL **2x OmniStain buffer A**.

15. Add 10 µg of each primary antibody or lectin per 1 cm³ of tissue or per whole adult mouse brain as a starting amount. Directly fluorophore-conjugated primary antibodies and lectins may be included.
    - Reduce the amount only after validation for the individual probe and specimen type.
    - You can add multiple species-compatible primary antibodies for multiplexed 3D IHC.

16. Top up with PBSN or **2x OmniStain buffer A** to reach a final 1x OmniStain buffer A concentration in the staining solution. To do so, let V (in mL) equal the combined volume added in Step 15:
    - if $V < 1$ mL add $(1 - V)$ mL PBSN.
    - if $V > 1$ mL, add $(V - 1)$ mL **2x OmniStain buffer A**.

17. If necessary, add the minimum volume of **1x OmniStain buffer A** with the same concentration of probes to fully submerge the tissue.

18. Incubate and agitate the tube upright at 20-25°C for at least 4 days in the dark.
    - If penetration is suboptimal, extending the incubation time to up to 14 days may help.

Post-primary incubation

19. Replace with 5 mL **1x OmniStain buffer B** at 20-25°C for 6-18 hours, agitate the tube upright.

20. Wash in 25 mL PBSN (3 x 2 hours), shake the tube horizontally.
    - If all primary antibodies and lectins are directly fluorophore-conjugated, proceed directly to Step 28.

OmniStaining - secondary

21. Pre-incubate in 2 mL **1x OmniStain buffer A** **at 20-25°C** for 6-18 hours while agitating the tube upright.

22. Discard the preincubation solution and add 1 mL **2x OmniStain buffer A**.

23. Add secondary reagents based on the mass of each unconjugated primary antibody in Step 15.
    - Add 1 µg of secondary IgG or Fab fragment per 1 µg of the corresponding primary antibody.
    - Add 0.4 µg of nanobody per 1 µg of the corresponding primary antibody.

24. Top up with PBSN, **2x OmniStain buffer A** or **1x OmniStain buffer A**, using the dilution and coverage rules in Steps 16-17.

25. Incubate and agitate the tube upright at 20-25°C for 12-18 hours in the dark.

Post-secondary incubation and Clearing

26. Replace with 5 mL **1x OmniStain buffer B** at 20-25°C for 6-18 hours, agitate the tube upright.

27. Wash in 25 mL PBSN (3 x 2 hours), shake the tube horizontally.

28. Dehydrate in 25 mL 50% v/v methanol in H_2O 2 x 30 min, then in 25 mL 100% methanol 3 x 30 min.

29. Clear in 20 mL **Omni CLEAR**. Laboratory-prepared BABB consisting of benzyl alcohol:benzyl benzoate at 1:2 v/v, DBE or ECi may be used as alternatives. Replace with 20 mL fresh clearing solution after 3 h and continue for at least 6 additional hours, or until the tissue is optically clear.

**Antibody staining results may depend on antibody lot, specimen, fixation conditions and biological context.