Amamoto Lab, 2022

Protocol: Immunofluorescence

Section Immunofluorescence

1. Take out cryosectioned slides from the -80C freezer and place in a slide container, face-up.
    a. Note: Make sure the slides don't touch the sides of the container as this will cause the liquid to drain to the bottom, quickly drying out the slides.
2. Using a plastic transfer pipette, put ~3 mL of PBS on the slide and incubate for ~1 minute.
3. Drain the PBS and dab onto a paper towel.
4. Repeat 1x with PBS.
5. Dry the slide and the section by keeping the slide at a 45 degree angle.
    a. Note: Drying at this step makes sure that the OCT is completely gone and that the section adheres to the slide. After this step, the section cannot dry until the very end.
6. Re-hydrate the section with ~3 mL of PBS.
7. Remove the PBS and place 500 uL of Donkey Block (see Reagents). Incubate for at least 15 minutes at RT.
8. Remove the Donkey Block and add 500 uL of Primary Antibody, diluted in Donkey Block. Incubate for 2 hours at RT.
    a. Note: For most antibodies, 2 hours at RT is optimal. If this doesn't work, try O/N at 4C or RT. The dilution of the antibody needs to be determined empirically.
9. Wash 3x with PBS.
10. Add 500 uL of Secondary Antibody, diluted in Donkey Block at 1:750. Incubate for 1 hour at RT.
    a. Note: Secondary antibody at 488 nm will have the most autofluorescence. Avoid 405 nm as it is very dim. If using mouse tissue, Anti-mouse antibody will also have fluorescence in the blood vessels. "Red" secondary antibody can be good, but make sure that the emission wavelength matches the microscope specification (546 nm? 595 nm?). 647 nm is very good, but you will not be able to see it by eye.
11. Wash 3x with PBS, 5 minutes each.
12. Dry the slide.
13. Place 5 drops of Fluoromount-G (without DAPI) across the slide, continuous.
14. Place one end of a 24x50mm No. 1.5 coverslip into the Fluoromount-G and press down from one end to the other, making sure that no air bubble gets in.
15. Let the slide dry overnight.

Retina Flatmount Immunofluorescence

1. Take a fixed retina (with the lens) and place in one well of a 24-well plate with PBS.
2. Using a plastic transfer pipette with the end cut off, transfer the retina into the next well with 500 uL of Donkey Block. Incubate on orbital shaker for 15 minutes at RT.
3. Transfer the retina into the next well with 500 uL of Primary Antibody, diluted in Donkey Block. Incubate on orbital shaker for 4 hours at RT.
    a. Note: For some good antibodies, 2 hours is sufficient.
4. Wash 3x with PBS by transferring retinas between wells, incubating 5 minutes each.
5. Transfer the retina into the next well with 500 uL of Secondary Antibody, diluted in Donkey Block. Incubate on orbital shaker for 1 hour at RT.
6. Wash 3x with PBS by transferring retinas between wells, incubating 5 minutes each.
7. Under a microscope, use forceps to remove the lens and the ciliary margin in PBS.
8. Transfer the retina on a No. 1.5 coverslip, photoreceptor side down, with a drop of PBS.
9. With 4 mm spring scissors, make 4 cuts. If the orientation has been marked by a small cut in the retina, make sure to mark the orientation on the coverslip.
10. With the corner of a Kimwipe, slowly touch the PBS drop to suck up the PBS. This will flatten the retina.
11. Using a fine paintbrush, gently flatten out the folded over edges.

12. Dry the retina.
13. Place 5 drops of Fluoromount-G (without DAPI) across a Superfrost Plus slide, continuous.
14. Place one end of the coverslip into the Fluoromount-G and press down from one end to the other, making sure that no air bubble gets in.
    a. Note: If you want to image from the GCL side, instead of placing the coverslip on a slide, place on another coverslip.
15. Let the slide dry overnight.

Reagents

Donkey Block

2 mL Donkey Serum (Jackson Immuno cat. # 017-000-121)
1.5 mL 10% Triton-X
150 mg BSA
Fill to 50 mL with PBS

Store at 4C for up to 6 months