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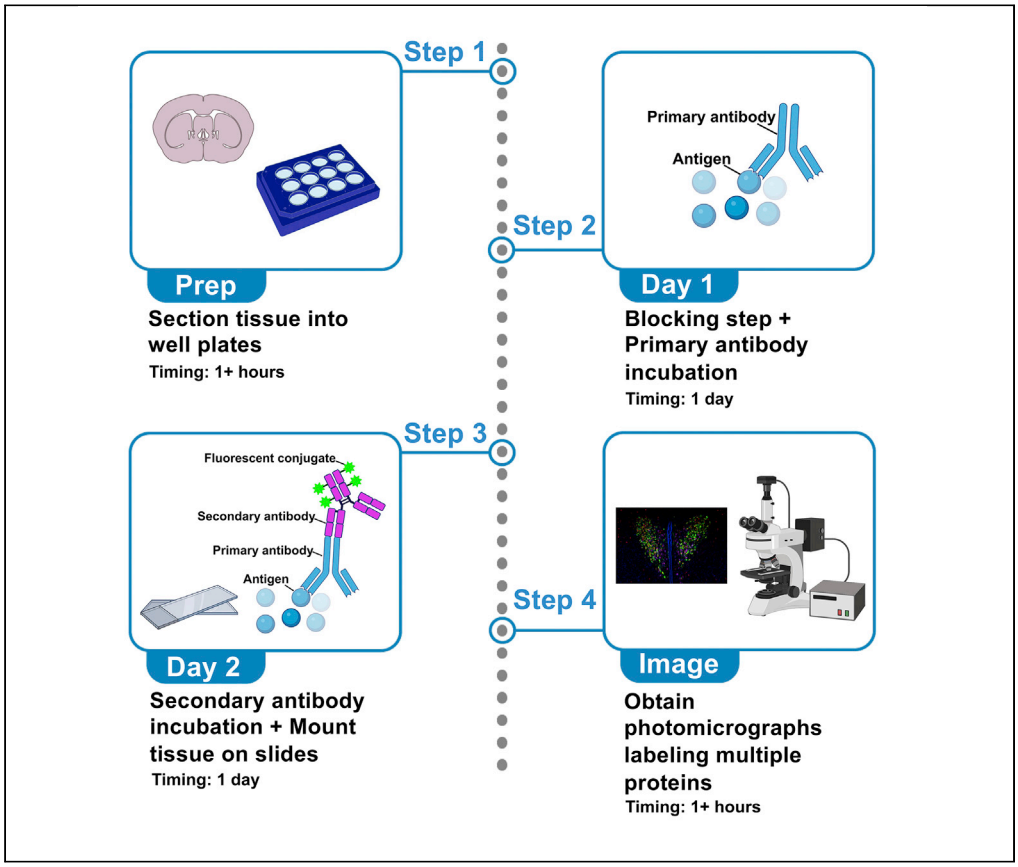
Protocol for multiplex fluorescent immunohistochemistry in free-floating rodent brain tissues



Identifying multiple proteins within the same tissue allows for assessing protein colocalization, is cost effective, and maximizes efficiency. Here, we describe a protocol for multiplex immunolabeling of proteins in free-floating rodent brain sections. As opposed to slide-mounted immunohistochemistry, the free-floating approach results in less tissue loss and greater antibody penetration. Using distinct fluorophores for individual proteins, this protocol allows for visualization of three or more proteins within tissue sections. The protocol can be applied to other tissue types.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Multiplex fluorescent IHC allows for simultaneous visualization of multiple proteins

A protocol for immunolabeling proteins in free-floating rodent brain tissue

The free-floating approach results in less tissue loss and greater antibody penetration

Can be applied to brain tissue of different vertebrates or other tissue types

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Protocol for multiplex fluorescent immunohistochemistry in free-floating rodent brain tissues

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SUMMARY

Identifying multiple proteins within the same tissue allows for assessing protein colocalization, is cost effective, and maximizes efficiency. Here, we describe a protocol for multiplex immunolabeling of proteins in free-floating rodent brain sections. As opposed to slide-mounted immunohistochemistry, the free-floating approach results in less tissue loss and greater antibody penetration. Using distinct fluorophores for individual proteins, this protocol allows for visualization of three or more proteins within tissue sections. The protocol can be applied to other tissue types.

For complete details on the use and execution of this protocol, please refer to Gonzalez Abreu et al. (2022).

BEFORE YOU BEGIN

The protocol below describes the specific steps for validating and using oxytocin, vasopressin, and Fos antibodies in rodent brain tissue. However, we have also used this protocol to label numerous other proteins (i.e., tyrosine hydroxylase, pERK, DOPA decarboxylase) in rodent, bird, amphibian, and fish brain tissue.

Institutional permissions

Obtain institutional permission to perform animal studies and collect tissues under an approved Institutional Animal Care and Use Committee (IACUC) or Institutional Review Board protocol. Our protocol was approved by Emory University's Institutional Animal Care and Use Committee.

Cryosection tissue

Timing: 1 h+

1. This protocol is optimized for brains from animals that were perfused with 4% paraformaldehyde. Section tissue at a thickness of 30 μm or 40 μm using a cryostat or microtome. The protocol below has been conducted successfully on both tissue section thicknesses in adult fish, bird, and rodent brains.
2. If beginning the protocol below within 3 days, tissue sections (3–6 sections per well) can be stored in porcelain 12 well plates with 0.1 M PBS in a humid chamber (sandwich Tupperware with a wet paper towel at the bottom) at 4°C. Otherwise store tissue sections in cryoprotectant in plastic 12 well culture plates with a lid at −20°C for 1 year or −80°C for 10+ years. To conserve freezer space, up to 6 tissue sections can be stored in cryoprotectant in 1.5 mL microcentrifuge tubes.

Validate primary antibodies

Timing: 2 days - 1 week

3. If the primary antibodies have not been used in your species before, characterize your antibodies via western blot to ensure that the primary antibodies bind specifically to the organism's proteins.
4. Conduct specificity controls on the primary antibodies that yielded expected molecular weights from the western analyses.
   a. Preabsorb primary antibodies with 50 μM protein from company in which antibody was obtained or with synthetic peptide from GenScript for 4 h at room temp (20°C–22°C). Proceed with the immunohistochemistry protocol below using the antibody that is being validated. If the antibody is specific, immunoreactive staining should be eliminated in preabsorbed tissue.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-oxytocin (1:250)	Millipore	Catalog# MAB5296; RRID: AB_2157626
Guinea pig anti-vasopressin (1:1000)	BMA Biomedicals/Peninsula Laboratories	Catalog# T-5048.0050; RRID: AB_518680
Rabbit anti-Fos (1:500)	Synaptic Systems	Catalog# 226 003; RRID: AB_2231974
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 680 (1:150)	Thermo Fisher Scientific	Catalog#: A10038; RRID: AB_2534014
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594 (1:300)	Thermo Fisher Scientific	Catalog#: A-21207; RRID: AB_141637
Biological samples		
Adult (PND60-100) male and female Mongolian gerbil (*Meriones unguiculatus*) brain tissue	Charles River Laboratories	https://www.criver.com
Chemicals, peptides, and recombinant proteins		
Normal donkey serum	Jackson ImmunoResearch	Catalog#: 017-000-121
Biotin-SP (long spacer) AffiniPure Donkey Anti-Guinea Pig IgG (H+L)	Jackson ImmunoResearch	Catalog#: 706-065-148
Streptavidin, Alexa Fluor™ 488 Conjugate	Thermo Fisher Scientific	Catalog#: S32354
ProLong™ Gold Antifade Mountant with DAPI	Thermo Fisher Scientific	Catalog#: P36931
Triton™ X-100	Fisher Scientific	Catalog#: BP151-100
PBS, Phosphate Buffered Saline, 10× Solution, Fisher BioReagents™	Fisher Scientific	Catalog#: BP3991
Sucrose	Fisher Scientific	Catalog#: S5-500
Polyvinylpyrrolidone	Fisher Scientific	Catalog#: AAJ61381A1
Ethylene glycol	Fisher Scientific	Catalog#: AAA1159136
Other		
Electron Microscopy Sciences TruBond™ 380 Adhesion Slides	Fisher Scientific	Catalog#: 50-340-33
Fisherbrand™ Premium Cover Glasses	Fisher Scientific	Catalog#: 12-548-5PP
Dark blue/black 12-well porcelain spot plate	SciOptic USA	Catalog#: BPSP-001
Princeton Series 4350 Synthetic Watercolor & Acrylic Brushes 4 Flat Shader	Amazon	N/A
Princeton Select Artiste, Series 3750, Paint Brush for Acrylic, Watercolor and Oil, Flat Shader, 6	Amazon	N/A
Clear nail polish	Amazon	N/A
Rocking Shaker	VWR	Catalog#: 10127-876
Cryostat or microtome	Leica	Catalog#: CM1860
Stereo microscope	Labomed	Catalog#: 4141000
Fluorescent microscope	ZEISS	Axio Imager 2

MATERIALS AND EQUIPMENT

Cryoprotectant		
Reagent	Final concentration	Amount
0.1 M PBS	1×	250 mL
Sucrose	37.5%	150 g
Polyvinylpyrrolidone	1.25%	5 g
Ethylene glycol	37.5%	150 mL
Total	N/A	400 mL
Store at 4°C for 1 year.		

Note: It can take several hours for the polyvinylpyrrolidone to dissolve.

⚠ CRITICAL: Ethylene glycol is harmful if ingested or inhaled and may cause damage to organs through prolonged or repeated exposure. Proper PPE (i.e., gloves, goggles) and engineering controls (i.e., chemical fume hood) should be used when handling these solvents – consult the SDS.

STEP-BY-STEP METHOD DETAILS

Outline of protocol

Here we provide a table outlining the steps of the protocol (Table 1).

Table 1. Step-by-step outline of protocol

Step	Time	Temperature
Rinse × 5	25 min	Room temp (20°C–22°C)
Block	1 h	Room temp
Primary	24 h	4°C
Rinse × 2	1 h	Room temp
Biotin	1 h	Room temp
Rinse × 2	30 min	Room temp
Secondary	2 h	Room temp
Rinse × 2	20 min	Room temp
Mount tissue on microscope slides or store unmounted tissue at 4°C.		

Day 1: Rinse tissue

Timing: 25 min

Prior to beginning immunohistochemistry (IHC), it is important to rinse tissue in 0.1 M PBS, particularly if the tissue has been previously stored in cryoprotectant.

1. Rinse tissue for 5 min in fresh 0.1 M PBS on a shaker.
   a. Repeat 5 times (use a new well plate with fresh 0.1 M PBS for each rinse).

Note: See Methods video S1 for a demonstration of transferring tissue between wells using a paint brush. Using a paint brush for transferring tissue is more affordable than purchasing mesh well inserts (i.e., Netwell). We have also found that fragile tissue (i.e., neonatal brains, poorly perfused brains) gets caught or stuck in mesh well inserts, yet we find little to no tissue damage occurs when using a paint brush to transfer tissue.

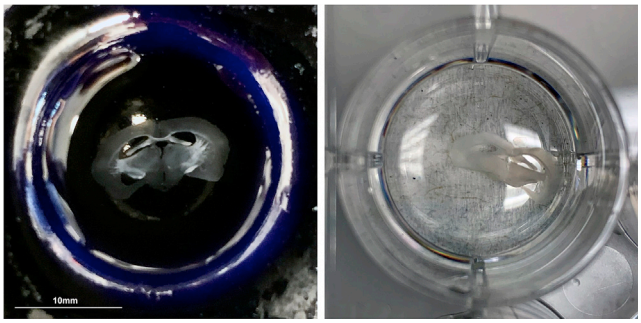

Figure 1. Well plate options

Left: a coronal rodent brain section in a porcelain spot plate. Right: a coronal rodent brain section in a plastic cell culture plate. We prefer porcelain spot plates for ease of visualizing tissue and the concave shape of the wells reduces the amount of solution needed and results in less tissue damage when transferring tissue from well to well. Scale bar represents 10 mm.

Optional: To better observe tissue, we prefer using dark colored porcelain spot plates, instead of clear plastic culture plates (Figure 1). Additionally, the concave shape of the porcelain spot plate wells results in the need for less solution to completely cover tissue and is thus cost effective for antibody use.

Day 1: Calculations

Timing: 5 min

For this protocol, all calculations will be made for 12 wells of tissue, each well containing 5 tissue sections, each with 40 μm thickness, and 500 μL solution.

2. Number of tissue sections per well:
   a. Depending on brain size and tissue quality, 3–6 tissue sections can be placed in a single well. If the tissue is poorly perfused and is 'sticky,' place fewer sections per well.
3. Amount of solution per well:
   a. The total amount of solution per well can range from 350–500 μL. Scale the solution based on the number of tissue sections, such that all sections in each well are completely submerged.
4. For this protocol, a 12 well plate needs 6 mL (500 μL ×12) of solution per step (i.e., Primary, Biotin, and Secondary steps).

Day 1: Block non-specific sites

Timing: 1 h

Antibodies may weakly bind nonspecifically to random proteins, thus creating noise in fluorescent signal. To reduce background staining, tissue is incubated in a blocking buffer prior to primary antibody incubation to block non-specific sites that the primary and/or secondary antibodies may bind to. The blocking buffer is made with serum from the species your secondary antibodies were raised in. For example, if you are using a donkey anti-mouse secondary antibody, you will make a blocking buffer with normal donkey serum.

5. Make blocking buffer:
   a. Make a 1× PBS solution with 10% normal donkey serum and 0.3% Triton X-100 in a glass beaker.
6. Pipet 500 μL blocking buffer into each of 12 wells in a new well plate.
7. Transfer tissue from the final PBS rinse above into the well plate containing blocking buffer.

8. Place well plate in a humid chamber and incubate at room temperature for 1 h.

Blocking buffer		
Reagent	Final concentration	Amount
Normal donkey serum	10%	600 μL
Triton X-100	0.3%	18 μL
0.1 M PBS	1×	5,382 μL
Total	**N/A**	**6 mL**
Can be made 1 day ahead and stored at 4°C.		

Note: Triton X-100 is very viscous. To avoid the Triton X-100 sticking in a clump to the bottom of a glass beaker when making the blocking buffer, mix the donkey serum in 0.1 M PBS and have a stir bar actively spinning in the solution on a stir plate. Add the Triton X-100 via a pipet directly into the solution and allow it to completely dissolve for at least 2 min. Some bubbling may occur.

Optional: We preferentially use Normal Donkey Serum, however, Bovine Serum Albumin is commonly used with success.

Day 1: Make diluent for primary, biotin, and secondary steps

Timing: 20 min

While the tissue incubates in the blocking buffer, make the diluent that will serve as the base for mixing antibodies in subsequent steps. The diluent helps to stabilize the antibodies, promotes diffusion into the tissue, and minimizes non-specific binding.

9. Make enough diluent for each step (i.e., in this example, 6 mL is needed per step). There will always be a primary and secondary step, whereas a third biotinylation step is optional.
10. Make diluent:
    a. Make a 1× PBS solution with 5% normal donkey serum and 0.3% Triton X-100 in a glass beaker.

Diluent		
Reagent	Final concentration	Amount
Normal donkey serum	5%	900 μL
Triton X-100	0.3%	54 μL
0.1 M PBS	1×	17,046 μL
Total	**N/A**	**18 mL**
Store at 4°C for 1 week.		

Note: See note above about Triton X-100.

Day 1: Primary antibody incubation

Timing: 24 h

There are two types of immunofluorescence assays – direct and indirect. Direct immunofluorescence uses only one fluorophore-conjugated antibody directed against the antigen of interest. Indirect immunofluorescence uses an unconjugated primary antibody that binds to the target of interest

and a secondary antibody containing a fluorescent marker that is directed against the primary antibody. This protocol uses indirect immunofluorescence, and thus there are primary and secondary antibody incubation steps. Specifically, we use a primary anti-oxytocin antibody raised in a mouse, a primary anti-vasopressin antibody raised in a guinea pig, and a primary anti-Fos antibody raised in a rabbit.

11. Primary antibody concentrations:
    a. Mouse anti-oxytocin: 1:250.
    b. Guinea pig anti-vasopressin: 1:1,000.
    c. Rabbit anti-Fos: 1:500.
12. Make primary antibody solution:
    a. Mix primary antibodies into diluent.
13. Pipet 500 μL primary antibody solution into each of 12 wells in a new well plate.
14. Transfer tissue directly from the blocking buffer into the well plate containing the primary antibody solution.
15. Place well plate in a humid chamber and incubate at 4°C for 24 h.

Diluent		
Reagent	Final concentration	Amount
Mouse anti-oxytocin	1:250	24 μL
Guinea pig anti-vasopressin	1:1,000	6 μL
Rabbit anti-Fos	1:500	12 μL
0.1 M PBS	1×	5,958 μL
Total	N/A	6 mL
Can be made 1 day ahead and stored at 4°C.		

Note: To determine antibody concentrations for the primary antibody incubation step, one can peruse the literature to identify concentrations used by other labs. If validating a new antibody (or the concentration used by other labs does not work in your tissue), we recommend conducting a pilot with the following concentrations: 1:100, 1:250, 1:500, 1:1,000. Each concentration can be run in a single well of tissue that contains 4–5 tissue sections (i.e., the pilot would use 4 wells of tissue total). Depending on results from the pilot, concentrations can be scaled up or down.

Optional: For the antibodies listed here, we have conducted primary antibody incubations for 24 h and for 48 h and have found no statistical difference in cell labeling. Thus, after testing your specific primary antibodies, a 48 h incubation is potentially an option.

Day 2: Rinse tissue

Timing: 1 h

Rinsing the tissue after the primary antibody incubation removes unbound antibody as well as antibody that has partially bound to non-specific sites.

16. Rinse tissue in fresh 0.1 M PBS on a shaker.
    a. Rinse tissue for 30 min in fresh 0.1 M PBS on a shaker.
    b. Repeat tissue rinsing, step 16 a.

Day 2: Biotin incubation

Timing: 1 h

Antibodies that do not exhibit strong expression can be enhanced by increasing the number of fluorophores bound to the tissue. Signal amplification can be achieved by applying a biotin step. In this protocol, the guinea pig anti-vasopressin antibody from BMA Biomedicals/Peninsula Laboratories requires biotinylation for sufficient visualization in brain tissue. Thus, we use a donkey anti-guinea pig biotin, which will target and bind to the previously bound guinea pig anti-vasopressin antibody.

17. Biotin concentration:
    a. Donkey anti-guinea pig biotin conjugated to streptavidin: 1:125.
18. Make biotin solution:
    a. Mix biotin into diluent.
19. Pipet 500 μL primary antibody solution into each of 12 wells in a new well plate.
20. Transfer rinsed tissues into a new well plate containing the biotin solution.
21. Place well plate in a humid chamber and incubate at room temperature for 1 h.

Diluent		
Reagent	Final concentration	Amount
Donkey anti-guinea pig biotin	1:125	48 μL
0.1 M PBS	1×	5,952 μL
Total	N/A	6 mL

Can be made 1 day ahead and stored at 4°C.

Day 2: Rinse tissue

Timing: 30 min

Rinsing the tissue after the biotin incubation removes unbound biotin.

22. Rinse tissue in fresh 0.1 M PBS on a shaker.
    a. Rinse tissue for 15 min in fresh 0.1 M PBS on a shaker.
    b. Repeat tissue rinsing, step 22 a.

Day 2: Secondary antibody incubation

Timing: 2 h

Because primary antibodies and biotin complex do not contain fluorescent markers, a secondary antibody incubation containing fluorophores is necessary to visualize antibody binding. We use a donkey anti-mouse antibody conjugated with the Alexa Fluor 680 dye to bind to and visualize the mouse anti-oxytocin antibody to visualize oxytocin cells in the far red (not visible to the naked eye). A donkey anti-rabbit antibody conjugated with the Alexa Fluor 594 antibody is used to bind to and visualize the rabbit anti-Fos antibody to visualize Fos cells in red. Finally, because there is an extraordinary affinity of avidin for biotin (Chivers et al., 2011), a streptavidin with the Alexa Fluor 488 conjugate is used to bind to and visualize the anti-guinea pig biotin, which previously bound to the guinea pig anti-vasopressin primary antibody; vasopressin will thus be visualized in green.

23. Secondary antibody concentrations:
    a. Donkey anti-mouse 680: 1:150.
    b. Donkey anti-rabbit 594: 1:300.
    c. Streptavidin 488: 1:300.
24. Make secondary antibody solution:
    a. Mix secondary antibodies into diluent at the concentrations suggested in step 23.

25. Pipet 500 μL primary antibody solution into each of 12 wells in a new well plate.
26. Transfer tissue from the rinse into the well plate containing the secondary antibody solution.
27. Place well plate in a humid chamber in the dark (i.e., a cupboard) at room temperature for 2 h.

Diluent		
Reagent	Final concentration	Amount
Donkey anti-mouse 680	1:150	40 μL
Donkey anti-rabbit 594	1:300	20 μL
Streptavidin 488	1:300	20 μL
0.1 M PBS	1×	5,920 μL
Total	N/A	6 mL

Can be made 1 day ahead and stored at 4°C (in the dark; wrapped in foil).

Optional: We have used Alexa Fluor conjugated secondary antibodies in 350, 488, 594, 647, and 680 nano meter (nm) wavelengths for primary antibodies generated in mouse, guinea pig, rabbit, and goat with great success.

Note: Regardless of the primary antibody, we use the same concentrations for Alexa Fluor secondary antibodies based on the fluorophore. For example, here we used a donkey anti-rabbit 594 antibody at a concentration of 1:300 to label Fos. A donkey anti-mouse 594 antibody to label oxytocin would also have a concentration of 1:300.

⚠ CRITICAL: Keep secondary antibody solution covered from light. The beaker of solution can be wrapped in foil. The well plates with solution should be covered with a box lid. Although fluorescent antibodies should be protected from light, we have found that Alexa Fluor antibodies can withstand periods of direct light without affecting signal. Therefore, it is not necessary to conduct immunohistochemistry with the lights off.

Day 2: Rinse tissue

Timing: 20 min

Rinsing the tissue after the secondary antibody incubation removes unbound antibody, thus improving the signal-to-noise ratio (SNR).

28. Rinse tissue in fresh 0.1 M PBS on a shaker. Cover well plate with a box lid to protect from light.
    a. Rinse tissue for 10 min in fresh 0.1 M PBS on a shaker. Cover well plate with a box lid to protect from light.
    b. Repeat tissue rinsing, step 28a.
29. At this stage, tissues can be directly mounted to microscope slides or stored in 0.1 M PBS solution in a well plate inside a humid chamber at 4°C for up to 1 week.

Mount tissue on microscope slides

Timing: 30+ min

We mount stained tissue onto subbed slides to enhance adhesion of the tissue to the slide. Non-subbed slides can be subbed by applying a gelatin-coating. We use Trubond 380 Adhesion slides that come pre-subbed and are ready for use. Once tissue sections are mounted to slides, slides are then coverslipped and allowed to dry for 24 h. Slides are then sealed and are ready for imaging.

30. Mount tissue on slides:
    a. Distribute tissue across 0.1 M PBS-filled well plates such that there is a single tissue section per well.
    b. Using a paint brush held parallel to the well plate, gently slide the brush underneath the tissue.
        i. Slowly lift the paint brush such that the lateral sides of the tissue each fold around the paint brush.
        ii. Holding the paint brush parallel to the slide, press the right side of the paint brush to the slide and slowly roll the brush clockwise.

    Note: The tissue section should roll off the paint brush and onto the slide.

        iii. Use a 0.1 M PBS-saturated paint brush to gently move the tissue so that it lies completely flat on the slide.

    Note: We refer to this as the 'pancake method' because the paint brush acts as a spatula to scoop under the tissue, and then the tissue is flipped onto the slide. See Methods video S2 for a demonstration.

    c. As an alternative to step 30b, a paint brush can be used to push the tissue to the side of a well of 0.1 M PBS.
        i. Scrunch the tissue onto the paint brush using a sweeping motion as the brush sweeps along the side of the well to bring the tissue out.
        ii. Use a second paint brush, saturated in 0.1 M PBS, to scoot the tissue off the first paint brush onto the slide.
        iii. Use the brushes to unfurl the tissue on the slide.

    Note: If using this method, ensure the tissue is well perfused so that it can withstand scrunching/folding and gentle tugging with paint brushes when flattening the tissue on the slide. We also recommend using sufficient amounts of 0.1 M PBS for this method. See Methods video S3 for a demonstration.

31. Coverslip slides:
    a. Allow tissue sections to dry.

    Note: All tissue sections on a slide must partially dry in order to fully adhere to the slide. Tissue sections begin to appear crystalline and sparkly when they are partially dried (see Figure 2).

    ⚠ CRITICAL: Ensure that all individual tissue sections reach the crystalline stage once prior to moving to the next step.

    b. Rewet tissue by gently dabbing 0.1 M PBS onto the tissue section with a paint brush.

    Note: This aids in evenly spreading the mounting media across the slide.

    c. Pipet ~3 drops of the mounting medium (we use Prolong Gold with DAPI) evenly across the slide.
    d. Gently place the left side of a coverslip onto the left side of the slide and use forceps to slowly lower the right side of the coverslip onto the slide. See Methods video S4.

    Note: Once the coverslip is on the slide, it should not be removed because the tissue will unadhere from the slide and likely tear. If tissue sections substantially slide and overlap one

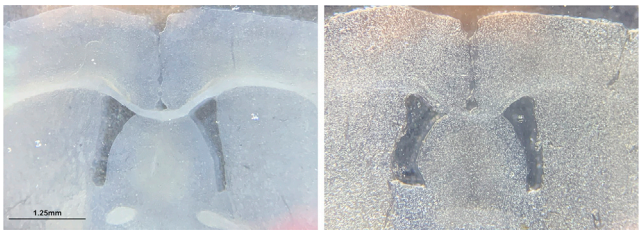

Figure 2. Tissue drying stages prior to coverslipping microscope slides

Left: An example of a tissue section on a slide that is too wet and has not yet sufficiently dried for coverslipping. Right: An example of a tissue section that has dried sufficiently to adhere to the slide and appears crystalline; this tissue section is ready for coverslipping. Scale bar represents 1.25 mm.

another, the entire slide, including the coverslip, can be immediately placed into a dish of 0.1 M PBS. With the slide submerged, gently slide the coverslip off; all tissue will detach and float in the dish. The mounting process can then be started anew.

e. Place coverslipped slides on a paper towel under a box lid to dry for 24 h.

32. Seal coverslipped slides:
    a. After slides have been coverslipped for 24 h, apply clear nail polish around the edges of the coverslip to seal the gap between the coverslip and the slide.
33. Slides are ready for imaging and can be stored at room temperature in light-blocking slide boxes. We have successfully stored slides in boxes at room temperature for 15+ years. Note that some antibodies may lose their fluorescence over time.

Note: For smaller brains (vole, mouse, finch), we prefer using the Princeton flat shader 4 paint brush. For larger brains (i.e., gerbil and rat), we prefer the Princeton flat shader 6 paint brush.

Optional: There are numerous methods for mounting free-floating tissue sections onto slides. An additional alternative is to have a small dish of 0.1 M PBS with tissue sections floating in it. The slide is then held mostly parallel to the water and slightly submerged; the tissue is then scooted onto the slide with a paint brush.

⚠ CRITICAL: We prefer to mount tissue using desktop stereo microscopes (e.g., Labomed 4141000 Model Luxeo 2S Binocular Stereo Microscope, 10×/30× Magnification). It is critical to ensure that no tissue is folded over onto itself or overlaps with other sections of tissue; tissue overlap will result in difficulties with region-specific imaging.

EXPECTED OUTCOMES

This protocol should result in tissue sections that, when imaged with a fluorescent microscope with appropriate filter cubes, will generate data for 3+ proteins within tissue sections (see Figure 3). This protocol will allow for the quantification of total cell-numbers and target proteins and of colocalization of multiple proteins.

LIMITATIONS

This protocol is ideal for brain tissue from birds or rodents because of brain size, but can also be successfully applied to smaller brains such as those from amphibians and fish. Smaller brain sections can be difficult to mount with a paint brush, but more importantly can be difficult to order along an anterior-poster axis. Additionally, the mounting procedures described here require patience and delicate hand motor movements. We recommend practicing before attempting the procedure with experimental tissue.

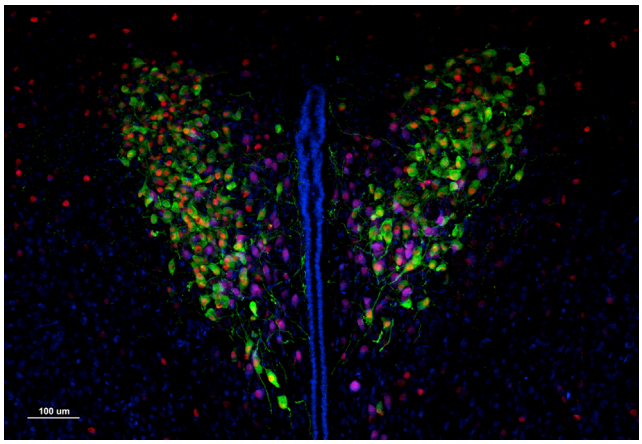

Figure 3. Multi-fluorescent immunohistochemical labeling of brain tissue

An example of rodent brain tissue that has been immunohistochemically stained using the protocol described above. Vasopressin (green), oxytocin (purple), Fos (red), and DAPI nuclear stain (blue) in the paraventricular nucleus of the hypothalamus. Scale bar represents 100 um.

TROUBLESHOOTING

Problem 1

I see too much fluorescent signal.

If your tissue shows too much binding, this may be the result of too much antibody and non-specific binding (see validate primary antibodies under before you begin).

Potential solution

Revalidate your antibody by running it at lower concentrations (i.e., 1:10, 1:100, 1:500, 1:1000). Western blots and/or preabsorption controls should also be performed to confirm the antibody targets your protein of interest in your tissue.

Problem 2

I see no expression.

If you see no fluorescent expression of cells, then you need to determine if your antibodies don't work or if there was an accidental error that occurred during the protocol (steps 11–15).

Potential solution

To confirm that your antibody works, run it at higher concentrations (i.e., 1:10, 1:100, 1:500, 1:1000). An antigen retrieval step (i.e., enzymatic or heat-mediated) can also be conducted prior to the blocking step to unmask antigenic sites to enhance antibody binding.

Problem 3

I see no colocalization.

Sometimes antibodies may compete with one another and block binding at similar sites (steps 1–15).

Potential solution

Running sequential IHC can often mitigate antibody competition. To do this, repeat the Blocking and Primary Incubation steps for each individual antibody, with rinses in between, prior to proceeding to the Biotin and/or Secondary Incubation steps. Each primary antibody will still need a 24–48 h incubation period, and thus sequential IHC takes several days longer than non-sequential IHC.

Problem 4

Fluorescent signal is not consistent across tissue sections.

If some tissue sections show fluorescent signal and other section do not, or if half of a tissue section shows signal and the other half does not or shows dim signal, this may be the result of insufficient antibody penetration of tissue (step 2).

Potential solution

Having fewer tissue sections per well can help avoid tissue sections sticking to one another, thus allowing for sufficient antibody penetration of all tissue.

Problem 5

I see staining for one protein in multiple fluorescent channels.

If you see cellular signal across multiple channels (i.e., the same cells, and identical expression pattern, are visible in red and green), this may mean that one primary antibody cross-reacted with another primary antibody or that your primary antibody is non-specific (steps 1–33).

Potential solution

To rule out that your antibody is non-specifically binding, re-run IHC with just the one primary antibody in question; it should fluoresce only in one channel if it is specific to its antigen. Alternatively, sometimes primary antibodies may "stick" to one another. To avoid this, you can run sequential IHC (see solution to problem 3 above).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Aubrey M. Kelly (aubrey.kelly@emory.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze [datasets/code].

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101672.

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AUTHOR CONTRIBUTIONS

A.M.K. wrote the protocol. B.A.F. and K.J.W. assisted with image and video acquisition and edited the protocol.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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