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Main protocol for immunohistochemistry and histology (light microscopy)

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Abbreviations

dH_2O, bi-distilled water

PBS, phosphate buffered saline

PFA, paraformaldehyde

RT, room temperature

Contents:

Part I Immunohistochemistry

I. Overview

Successful immunohistochemistry depends on several factors, which may be summarized as follows:

- Well-characterized antibodies, with high specificity and affinity
- Preservation of antigenicity
- Preservation of adequate cell structure
- Absence of barrier likely to prevent penetration of antibodies into tissue
- Avoiding undesired nonspecific cross-reactivity (background staining)

A unique, optimal tissue processing for all antigens does not exist. Therefore, for each new antibody, an evaluation of the most effective method of tissue processing is needed.

1.1 Characterization of primary antibodies

– Immunohistochemistry is fraught with false negative and false positive results. Failure to detect an antigen in a particular cellular compartment, cell population, or brain region does not allow any conclusion about the presence or absence of the corresponding protein!
– Antibodies typically have a threshold of detection below which they produce no specific signal
– The balance between specific and non-specific staining is difficult to determine. Using antibodies too concentrated increases the chances of non-specific labeling; applying them in tissue containing very little or no epitope can result in non-specific labeling patterns that are not seen when the epitope is abundant!
– Prior knowledge about the nature and expected distribution of the antigen is often necessary to determine whether a given staining pattern is specific or not.

Whenever possible, the following criteria should be applied when evaluating the specificity of an antibody:

1. The distribution of the staining should correspond to the distribution of the mRNA of the corresponding protein
2. The antibody should reveal in Western blots a protein band of the expected size. Affinity-purified antibodies should show only epitope-related bands (including degradation products, etc).
3. The staining should disappear upon competition with the peptide antigen (this test does not rule out that the antibody might recognize one or several additional or unrelated epitopes).
4. Two antibodies raised against different parts of the protein should give similar staining patterns.
5. The specific staining pattern should be absent in a knockout (in which the gene sequence coding for the epitope has been deleted); however, it is not rare that the specific staining seen in wildtype tissue is replaced by a different, non-specific pattern in knockout tissue!

1.2 Characterization of secondary antibodies

– Secondary antibodies are (mostly) IgG or IgG fragments raised against immunoglobulins of the host species in which primary antibodies were raised. Their species-specificity is variable and should always be verified in double-staining experiments.
– Most companies provide secondary antibodies that have been affinity-purified sequentially to remove all cross-reacting antibodies against the most commonly used species. However, the affinity of these antibodies tends to be lower.
– Secondary antibodies from Jackson Immunoresearch raised in donkey are purified for species cross-reactivity (but not those raised in goat).
– Staining specificity is verified by omission of the primary antibodies, keeping all other reagents.

Trouble-shooting with secondary antibodies

– Blocking unspecific binding of secondary antibodies is required for all immunohistochemistry experiments. Dry milk powder is very efficacious, but difficult to use with free floating sections. The best alternative is excess normal serum from the species in which the secondary antibodies were raised.
– Secondary antibodies are used at fairly high concentration, increasing the risk of non-specific binding to brain tissue sections.
– In addition, anti-mouse IgGs are potentially a problem when applied onto mouse tissue. The biggest problems arise when the tissue was lesioned (experimentally or during the fixation), in young or in aged mice, and in weakly fixed tissue. Sometimes, the only way to circumvent the problem is to use another primary antibody (rat monoclonal onto mouse tissue, or any polyclonal antibody).
– Anti-mouse IgGs also produce high background staining when applied onto neonatal rat tissue; the problem can be solved by application of excess normal rat serum during the primary antibody incubation. Similarly, excess mouse normal serum reduces background of rat monoclonal antibodies in mouse tissue.

II. Tissue preparation and sectioning

Different methods for tissue fixation and sectioning are used depending on the antibodies used and expected results. The strength of fixation has a major influence on immunoreactivity and detection of proteins in specific subcellular compartments, such as postsynaptic densities or cell nucleus. Antigen retrieval following fixation represents offers multiple possibilities to enhance immunoreactivity. In addition, working with weakly fixed tissue allows overcoming poor detection of postsynaptic proteins, but often comes at the cost of tissue preservation.

More recently, the possibility to combine biochemical procedures, requiring fresh tissue, and immunohistochemistry, performed on fixed tissue, has arisen, using a perfusion that keeps tissue alive until used for biochemistry or being fixed by immersion for immunostaining. This method is herewith called “ACSF perfusion”. In addition, three classical methods are described for tissue preparation and sectioning.

Staining of tissue sections is then common to all protocols and described in the following chapters.

2.1 Overview of methods

- M1: Standard protocol, with perfusion-fixation and staining of free-floating sections cut with a sliding microtome, vibratome, or cryostat. The strength of fixation can be adjusted with appropriate fixatives.

- ACSF-perfusion: Protocol for combining immunohistochemistry with biochemistry on tissue from the same animal, and for optimal detection of synaptic proteins. Mice are perfused with ACSF under conditions keeping the tissue alive; tissue blocks of interest are prepared and postfixed for 60 min to 3 hours for immunohistochemistry (depending on the target antigen), followed by staining of free-floating sections cut with a sliding microtome, vibratome, or cryostat. Tissue for biochemistry can be processed immediately or snap-frozen.

- M2: Antigen retrieval methods applied to fixed tissue (either prior of after cutting sections)

- M3: Protocol for fresh- frozen tissue cut in the cryostat, following by quick fixation by immersion in various fixatives

For primary cell cultures (see Part II)

2.2 Standard protocol (M1)

2.2.1 Perfusion, postfixation

The strength of fixation can be varied by adjusting the time of postfixation, reducing the concentration of PFA (2%), or changing the pH of the buffer. A weaker fixation often (but not always) results in better immunostaining, but the sections are more fragile and difficult to handle during the staining procedure. Overnight postfixation is deleterious to most antibodies!

1. Prepare the solutions for perfusion (PBS, fixative at 4 ºC).
2. Organize the equipment and surgical instruments in the perfusion hood.
3. Weigh the animal and anesthetize it with an overdose of pentobarbital (nembutal, >50 mg/kg; i.p.). Young animals (<P3) are anesthetized by hypothermia on ice.
4. Perfuse transcardially with PBS followed by fixative solution as follows (see table "perfusion parameters"):
   - Pin the deeply anesthetized animal on a cork board
   - Open the thorax rapidly to expose the heart and major vessels. Hold the heart with a forceps, cut off the tip of the heart with sharp scissors and insert a blunted canula (Ø 1mm for mice; 3 mm for rats) through the left ventricle into the ascending aorta (follow the axis of the heart)
   - Open the right atrium with a sharp hook and start the peristaltic pump

   Caution: a good perfusion depends critically on the speed of the surgery. The pump should be started with 1-2 min after opening the thorax; avoid any unnecessary procedure to be as fast and precise as possible

   - Let the PBS flush the blood away; the liver should turn beige-yellow
   - Once the fixation is started, clamp the forceps holding the heart (that will fix the canula inside the heart).
   - Pour cold (4°C) fixative into the glass cylinder before it is completely empty, avoiding air bubbles in the tube (without stopping the pump).

Perfusion Parameters (light microscopy)

Animal	Age	Flow mL/min	Vol. PBS 22°C	Vol. Fix 4°C	Postfix4°C Time	vol.
Mouse	Adult	20	10 mL	60 mL	3-6 h	30 mL
Mouse	P7	10	½ tube	20 mL	48 h	20 mL
Mouse	P0	5	fine tube	10 mL	72 h	10 mL
Rat	Adult (> 120g)	50	100 mL	450 mL	2-6h	50 mL
Rat	P7	20	tube	50 mL	48 h	20 mL
Rat	P0	10	1/3 tube	20 mL	72 h	10 mL

Note: Neonates (mice) are perfused best with a butterfly needled connected to a 10 mL syringe.

5. After perfusion, rapidly dissect the tissue of interest (avoid damage to the brain or spinal cord by removing first the dura mater).
6. Postfix in the same fixative at 4°C for several hours (see table Perfusion Parameters).
7. Rinse the brain in PBS prior to either cryoprotection or cutting on the vibratome, or antigen retrieval, depending on your experiment.

2.2.2 Cryoprotection

Protects the tissue to prevent ice crystal damage during freezing; after postfixation, rinse the brain tissue with PBS and transfer to one of these cryoprotectant solutions:

Sucrose at 4°C

30 % in PBS for 24-72 h in PBS (until block sinks); for large brains (rat, primates), it is better to start with a lower concentration (10% overnight).

For long-term storage, tissue blocks or entire brain can be frozen with powdered dry ice, wrapped tightly in aluminum foil and stored in air-tight containers at -80°C.

10% DMSO in PBS at 4°C

This procedure allows a fast cryoprotection, but not suited for extreme cold (dry ice, -80°C)

Adult rat brain	at least 3h; until maximal 15 h
P0 rat brain	2h
Mouse brain	2h

Thereafter, the brain can be frozen on the stage of the sliding microtome (see below)

2.2.3 Sectioning

Fixed tissue sections can be cut without embedding using three procedures:
– Frozen blocks with a sliding microtome
– Frozen blocks with a cryostat
– Non-frozen blocks with a vibratome (no cryoprotection required)

Sections can be processed fresh-floating (preferred) or mounted onto coated glass slides.

Sections from fresh-frozen tissue are cut with a cryostat and mounted onto coated glass slides. Thereafter, they can be fixed by immersion (see Chapter 2.4.2).

Sliding Microtome

The brain tissue needs to be perfusion-fixed, cryoprotected, and frozen at about -30ºC – -40ºC, using the freezing stage of the microtome (or a metal plate cooled with dry ice). It is recommended to use “one-way” blades (but they can be reused….).

1. Start the freezing stage
2. Mount the blade into the blade holder and adjust it onto the microtome
3. Set section thickness to 40 µm (default) or more
4. Block the tissue in the desired orientation (using a razor blade)
5. Place a drop of tissue mounting fluid on the freezing stage and wait until it is almost completely frozen
6. Place the fixed, cryoprotected brain in desired orientation at the surface of the mounting fluid
7. Wait until the block is completely frozen
8. Start sectioning

9. Pick sections one by one with a wet brush and collect them in a multi-well plastic box filled with ice-cold PBS (place the box on ice, using a dark background)
10. Collect sections row by row; we use boxes containing 3 rows of 12 wells; later on, one can use 1:12; 1:6; 1:4; 1:3, etc. series of sections by taking them from 1, 2, 3, 4, etc equally spaced columns
11. Store the sections at 4° < 24h in PBS
12. For long-term storage (-20°) transfer the sections in antifreeze solution (in slide storage boxes, closed hermetically with parafilm

Cryostat

After cryoprotection in sucrose,

1. Freeze the tissue with powdered dry ice
2. Place the blocks to be cut into the cryostat chamber in advance to allow for the temperature to equilibrate
3. Set the chamber temperature to -20°C and the knife temperature to -18°C; adjust the knife temperature (±2°C) if the sections do not cut well. Do not use the antiroll plate.
4. Mount the cork carrying the frozen tissue block onto the metal specimen holder using tissue mounting fluid
5. Start sectioning at 40 µm
6. Collect sections with a wet brush and transfer them in ice-cold PBS (see Sliding microtome)
7. Store the sections at 4° < 24h in PBS
8. For long-term storage (-20°) transfer the sections in antifreeze solution
9. Keep the cryostat chamber clean….discard the one-way blade wipe the chamber with acetone after each session

Vibratome (fresh or fixed tissue)

1. The day before, prepare ice cubes of PBS in the freezer
2. Insert the one-way blade
3. Block the tissue in the desired orientation and mount it on the holder with cyanoacrylate glue
4. Immerse the block under ice-cold PBS and add PBS ice-cubes
5. Set the advance speed at a low (minimal value) and vibration amplitude at a high value
6. Start cutting at the desired thickness and collect sections in PBS

Comment: Soft tissue (young animal, spinal cord, etc) can be stabilized with Agar blocks

Proceed with immunohistochemistry

2.3 ACSF perfusion (for mice)

1. Prepare fixative (4% PFA in 0.15 M Na-phosphate buffer, pH 7.4) and ACSF from stock solution and cool to 0° on ice
2. Anesthetize the mouse with sodium pentobarbital (Nembutal) and perfuse intra-cardially (as described in section 2.2.1) with 15-20 mL ice-cold, oxygenated ACSF at a flow rate of 10-15 mL/min (perfusion time, <90 sec)
3. **Immediately** decapitate the mouse and place the head on ice. Carefully, but rapidly, dissect out the brain on ice (use a metal block placed on the ice as support).
4. Cut the block of interest (e.g., cut the brain sagitally into two halves, or isolate with transverse cuts the blocks containing the olfactory bulb/prefrontal cortex, hippocampal formation, brainstem/cerebellum), transfer them in ice-cold fixative and store in the refrigerator for the time desired (see "Fixation time" below). Note: do not post-fix the entire brain, because the penetration of fixative is insufficient!!!
5. Rinse the brain with ice-cold PBS and transfer to ice-cold 30% sucrose solution in PBS for cryoprotection until the tissue sinks to the bottom of the tube (at least overnight; see section 2.2.2).
6. Freeze and cut with a sliding-microtome or a cryostat as described in section 2.2.3; collect free-floating sections in PBS (store in anti-freeze if desired) and proceed with immunohistochemistry as usual.

Fixation time:
Minimum required: 60 minutes

- Time-window for postsynaptic molecules ($GABA_A$ receptors, gephyrin, neuroligin2, PSD-95): 60 – 150 min
- Time-window for presynaptic markers (VGAT, vGluT1, dopamine-β-hydroxylase): 90 – 240 min
- Time-window for soluble proteins (eGFP, parvalbumin, serotonin): 90 – 240 min
- Time-window for structural or transmembrane proteins (APP, glial markers, myelin-binding protein: 150 – 360 min

2.4 Antigen-retrieval methods (M2)

Antigen-retrieval is performed after perfusion-fixation or after sectioning (see standard protocol) to enhance detection of fixation-sensitive antigens.

2.4.1 Antigen-retrieval by microwave irradiation (prior to sectioning)

This procedure performed in acidic buffer results in a marked reduction of background, but is only suitable for epitopes that are not destroyed by heating. Each candidate antibody has to be tested separately.

1. After postfixation, rinse brain tissue well with PBS and incubate overnight at RT in 0.1 M Na-citrate buffer (pH 4.5), using a shaker

2. Block the brain in the desired orientation and place it in a Becher containing 80 mL fresh Na-citrate buffer (volume to be calibrated to boil within 60 sec.)
3. Put in microwave oven and heat at about 650 W for the following time:

rat brain	2-3 min
rat spinal cord	90 sec
mouse brain	90 sec
mouse spinal cord	45-60 sec
neonatal tissue (P0 – P7)	30 sec

4. Let the buffer cool down for about 5 min
5. Rinse the brain in PBS
6. Proceed with cryoprotection and sectioning (see Standard protocol)

2.4.2 Antigen-retrieval for tissue sections (e.g., anti-Ki-67 or anti-PCNA antibodies)

Free floating sections

1. Fill a container with 1 L water.
2. Fill Falcon tube with 50 mL Na-citrate buffer pH 6, 10 mM, add tissue sections, don't close the lid (or make a hole)
3. Put Falcon tube in a water-filled Erlenmeyer to hold position in the container
4. Microwave heating at 800 W, heat up the tissue sections twice for 7 min each time (→ open the door of the microwave each time to cool down for 1 min)
5. Allow to cool down, rinse sections in PBS (or Tris buffer) and proceed with staining

Mounted sections (better results)

→ *Use superfrost slides (gelatin may melt).*

1. Prepare water container, 1 L
2. Put the slides with mounted sections into 50 mL Falcon tubes filled with citrate buffer pH 6, 10 mM; do not close the lid
3. Place Falcon tubes in a water-filled Becher to hold position in the water bowl
4. Microwave heating at 800 W, heat up the tissue sections twice for 7 min each time (→ open the door of the microwave each time to cool down for 1 min)
5. Allow to cool down, rinse sections in PBS (or Tris buffer) and proceed with staining

2.4.3 Tissue denaturation prior to BrdU immunohistochemistry

To visualize BrdU immunoreactivity, DNA has to be denatured to reveal the epitope.

1. If sections are in antifreeze solution, rinse them 2 x 5 min in PBS in 12-well culture plates
2. Incubate the sections in 50% formamide/50% 2XSSC solution, pH 7.4 (0.3 M NaCl/0.03 M sodium citrate) at 65°C for 2 h on a shaker
3. Rinse with 2XSSC, pH 7.4 for 10 min at RT
4. Incubate in 2 N HCl for 30 min at 37°C
5. Rinse 10 min in 0.1 M borate buffer pH 8.5 at RT
6. Wash 3x5 min in Tris-Triton
7. Proceed with immunohistochemistry

2.4.4 Antigen-retrieval by pepsin digestion

This procedure is used for free-floating sections from perfusion-fixed tissue and was originally described by Watanabe et al, 1998, EJN 10, 478-487. It markedly improves the detection of membrane-bound synaptic proteins.

Pepsin (Dako, S3002) is stored as 20x stock in 0.2 M HCl at -20ºC

1. Preheat the oven at 37°C (at least one hour before)
2. If sections are in antifreeze solution, rinse them 2 x 5 min in PBS in 12-well culture plates at RT
3. Make fresh 0.2M HCl solution (in dH_2O) from a 1 M or 10 M HCl stock and pre-warm it in the oven
4. Preheat metal plates and 9-well glass plates at 37°C in the oven
5. Pipet 500 mL/well 0.2 M HCl solution in 9-well glass plates, transfer the sections and keep warm at 37°C for 10 min
6. Dilute pepsin stock 1:20 (0.15 mg/mL) in warm 0.2M HCl
7. Take the metal plate (with the 9-well plates on it) out of the oven and replace the HCl with the pepsin solution; return to the oven and incubate for 10 min at 37ºC
8. Wash sections 5 min in PBAS and in Tris-saline buffer 2x 10 min at RT

Proceed with immunohistochemistry

Comments: Works poorly with tissue from juvenile animals; the method can be modified on demand (e.g., a longer digestion can be achieved on mounted sections); the effect of pepsin is very superficial (a few μm) and these sections need to be analyzed by confocal microscopy.

2.5 Fresh frozen tissue (M3)

This procedure involves the preparation of cryostat sections from fresh-frozen tissue, followed by mild fixation prior to immunostaining.

Rat and mouse brain tissue can be frozen with powdered dry ice. For larger brains, use isopentane cooled at -40°C with liquid nitrogen (control the temperature).

1. Decapitate the animal (anesthetized with isoflurane or methophane, if needed) and quickly remove the brain out of the skull (work on ice)
2. Prepare tissue blocks depending on the orientation of the sections to be cut later
3. Place them of a piece of cork (2x2 cm, thickness 2-3 mm) on a drop of PBS or saline; cover the brain or the mounted tissue block with powdered dry ice
4. Once frozen, pack them in aluminum foil (airtight), labeled with colored tape.
5. Transfer into Falcon tube (50 mL) or other air-tight container and store at -80°C

2.5.1 Cryostat (fresh tissue)

1. Place the frozen blocks to be cut into the cryostat chamber in advance to allow for the temperature to equilibrate
2. Set the chamber temperature to -20°C and the knife temperature to -20°C; adjust the knife temperature (±2°C) if the sections do not cut well
3. Mount the cork carrying the frozen tissue block onto the metal specimen holder using tissue mounting fluid
4. Carefully position the antiroll plate
5. Start sectioning at 12-14 µm
6. Collect sections on gelatin-coated (or superfrost) glass slides; two options are possible: keep slides at RT (outside the cryostat) and melt the section directly onto the slide or place the slices a few minutes prior to use into the cryostat; place the section onto the cold slide, and melt it in place by locally warming the glass from below with your finger.
7. Place the glass slides at RT for a short while (30 sec – 2 min) to allow the sections to adhere well and store the slides inside the cryostat until cutting is finished
8. Store the slides until use at -80ºC
9. Keep the cryostat chamber clean….discard the one-way blade wipe the chamber with acetone after each session

2.5.2 Immersion-fixation of fresh tissue sections

The choice of fixative and duration of fixation depend on the age of the tissue and the purpose of the staining:

– Routine immunohistochemistry requires 20-30 min immersion-fixation in ice-cold fixative (4% PFA in 0.1 M Na-phosphate buffer)
– Detection of sensitive antigens can be achieved by short fixation (45 sec) in 0.1% PFA in 0.1 M Na-phosphate buffer under microwave irradiation
– Alternatively, and with tissue from juvenile animals, sections can be fixed for 30-60 sec. in cold (-20ºC) methanol

1. Thaw slides stored at -20ºC or -80ºC (for a few weeks)
2. Fix them as described above
3. For light fixation with microwaves, place 3 slides in a Petri-dish (ø 14,5 cm) containing 50 mL fixative, transfer into the microwave oven and irradiate for 30-45 sec at 500 W
4. Transfer slides immediately in PBS (100 mL glass container)
5. Wash three times for 10 min in PBS; the slides should never dry
6. Proceed with immunohistochemistry (see chapter IV)

Proceed with immunohistochemistry

III. Immunohistochemical staining protocols

– Use Tris-Triton (pH 7.4) for washing and diluting reagents; the addition of Triton X-100 serves to reduce superficial tension, avoiding damaging free-floating sections during washing steps.
– Tris can be replaced with PBS for immunofluorescence (but not for immunoperoxidase).
– In most cases, preincubation in buffer containing blocking serum is not necessary and can be skipped.
– Fluorochromes should not be exposed to bright light but the procedure can be performed under normal room illumination.

3.1 Immunoperoxidase staining (ABC method)

Elite ABC kit, Vector Laboratories

1. If sections are stored in antifreeze solution, rinse them in Tris-Triton in 12-well culture plates
2. Calculate the amount of primary antibodies required (and serial dilutions if necessary): 300 – 500 µl/well; 1 well can hold up to 4-5 sections
3. Prepare primary antibody solutions in Tris-Triton pH 7.4 containing 2% normal goat serum and 0.2%Triton X-100. Mix well (vortex briefly)
4. Pipet primary antibody solution into 9-well glass plates labeled with colored tape
5. Transfer sections with a wet brush; rinse the brush when changing antibody
6. Incubate sections overnight at 4°C with continuous agitation **(100 rpm)** in a moist chamber
7. Wash sections 3x10 min in Tris-Triton in 12-well cell culture plates
8. In the mean time, prepare biotinylated secondary antibody solution (1:300 in Tris-Triton containing 2% normal serum) and pipet into the 9-well glass plates
9. Incubate sections under agitation for 30 min at RT in secondary antibody; longer incubation increase background staining
10. At least 30 min prior to use, prepare the Avidin-Peroxidase-Complex (ABC) solution (Vectastain Elite kit standard): mix 1% reagent A and 1% reagent B in Tris-Triton
11. Wash sections 3x10 min in Tris-Triton
12. Pipet the ABC complex solution into the 9-well glass plates and incubate sections for 30 min at RT under agitation
13. Wash 3x10 min in Tris-Triton
14. Prepare Tris-Triton pH 7.7 and make DAB solution
    (The final DAB solution contains 0.5 mg DAB/mL Tris buffer (or 0.05%) and 0.01% H_2O_2)
    Dissolve 1 vial DAB (500 µL; frozen at –80°C, 50 mg/mL dH_2O) in 50 mL Tris-Triton (pH7.7); 50 mL are enough for one 12-well plate

Caution: DAB is light-sensitive (cover with alufoil and carcinogenic; handle only in a hood and wear gloves)

15. Split the DAB solution into two halves and pipet 2 mL/well into a 12-well plate
16. Preincubate sections for 5 min in DAB solution (to allow for diffusion into the tissue before starting the enzymatic reaction)

17. The reaction is catalyzed with H_2O_2. Just before use, dilute 150 µL of a 35% H_2O_2 solution in 5 mL dH_2O (1% solution). Add 20 µL of this mixture per mL of remaining DAB solution. Note: if you work with large DAB volumes, H_2O_2 can be dissolved directly in the DAB solution at a final concentration of 0.01%
18. Start the reaction by adding 2 mL DAB-H_2O_2 solution to each well. Use a timer!
19. When sections have the appropriate color, stop the reaction by transferring them rapidly into ice-cold PBS
20. Transfer immediately in a second wash for 10 min
21. Wash the sections twice more in PBS
22. Mount the sections on gelatinized glass slides (fill a large Petri dish with dH_2O containing 10-20% PBS; hold the slides on two corners and dip them in the water; place the sections in the desired orientation with a brush)
23. Let sections air-dry overnight prior to dehydration and coverslipping; label slides with a pencil.

Caution: H_2O_2 is not stable; the bottle has to be tightly sealed and kept at 4ºC. It should be replaced after a few months

Comment: good agitation (at least 100 rpm) is crucial to ensure uniform exposure to antibodies and uniform washes

3.1.1 Decontamination of DAB

Immerse all glass- and plastic ware in contact with DAB in water containing about 10% bleach (Javel; 14% Na-hypochloride) under the hood. The **following** day, inactivate the bleach with Na-thiosulfate (about 100 g per 5 l; stir). When all bubbles are gone, discard in the sink. Thoroughly rinse the glass- and plasticware and put it to wash.

3.1.2 Mounting and coverslip DAB-sections

Place the slides with the dried sections in a slide holder and dehydrate the sections in increasing ethanol concentration (70%, 70%, 96%, 96%, 100%, 100%, 100%) 5 min each, clear 4 times in xylene (5 min each) and coveslip with Eukitt (do not let dry and avoid air bubbles). Caution: Work in the hood and wear gloves! Xylene is toxic by inhalation and contact with skin!

- Check if ethanol and xylene solutions are clean
- For each series of sections, change xylene cuvette by discarding (in special container) the cuvette #1 and making a fresh cuvette #4
- Do not store Eukitt in the hood to avoid excessive evaporation
- If the Eukitt is too thick, dilute with xylene

3.2 Immunofluorescence (free-floating sections)

1. If sections are stored in antifreeze solution, rinse them in Tris-Triton or PBS (10 min/wash in 12-well cell culture plates) using a wet brush to transfer the sections
2. Decide whether to use regular (non-affinity purified for cross-reactivity) secondary antibodies raised in goat, or “multiple labeling” affinity purified secondary antibodies raised in donkey
3. Prepare primary antibody solution in Tris-Triton containing 2% normal serum (from the species in which the secondary antibody is raised) and 0.2% Triton X-100

4. Incubate sections in 9-well glass plates (300-500 µl/well) in a moist chamber with continuous agitation **(100 rpm)** overnight at 4°C
5. Wash sections in Tris-Triton, 3x10 min (using 12-well cell culture plates)
6. Dilute the secondary antibody in Tris-Triton containing 2% normal serum (see table "Secondary antibodies" for details)
7. Incubate sections in 9-well glass plates under agitation in secondary antibody diluted in Tris-Triton/2% normal serum for 30 min at RT
8. Wash 3x10 min in Tris-Triton
9. Mount sections on gelatin-coated slides and let them air dry
10. Coverslip with Dako fluorescence mounting medium (50 µl per slide); let it polymerize at least overnight prior to microscopy. Use this mounting medium only if the sections are important and need to be stored for a long time
11. Alternatively, coverslip with buffered glycerol (1:1 in 0.1 M Na-bicarbonate buffer, pH 9.2) or with DAKO Dapi
12. Store at 4°C in a closed slide carton

3.3 Immunofluorescence with sections mounted on glass slides

1. Draw with a Pap-pen a closed circle around the sections to prevent spilling antibody solutions
2. Lay wet paper towels in a slide box to make a moist chamber and place horizontal slide holder inside
3. When working with weakly fixed tissue, pre-incubate the sections with Tris-Triton (or PBS) containing 4% normal serum for 1 hour at RT
4. Prepare primary antibody solution in Tris-Triton containing 4% normal serum and 0.2% Triton (300 µl per slide)
5. Take slides out of PBS, weep them summarily with a Kleenex tissue, place them horizontally in the moist chamber and pipet primary antibody solution over the slides. Be careful not letting the sections dry out at any time
6. Incubate overnight at 4°C with slow rotation
7. Wash slides 3x10 min in Tris-Triton
8. Pipet secondary antibody solution (in Tris-Triton + 4% normal serum) over sections and incubate in moist chamber 1 h at RT
9. Wash slides 3x10min in PBS
10. Dip slides briefly in dH_2O to remove excess of salt and let air dry
11. Coverslip with Dako mounting medium or glycerol, as above
12. Store at 4°C in a closed slide carton

3.4 Multiple fluorescence staining

Proceed as above, incubating sections in a mixture of primary antibodies raised in different species (in Tris-saline buffer containing 2% normal serum from the species in which secondary antibodies were raised and 0.2% Triton X-100).

Secondary antibodies (e.g. anti-guinea pig, anti-rabbit etc.) must all be raised in the same species (goat, donkey). This condition is absolutely necessary for successful multiple labeling.

Table: Overview of fluorescent secondary antibodies

Fluorochrome	Adsorption λ	Emission λ	Color	Dilution
Alexa 405	405	450	Blue	1:1000
Alexa 488	488	520	Green	1:1000 – 1:2000
Alexa 594**	594	620	Red	1:1000
Alexa 647	647	700	Far red	1:1000
Cy3	568	600	Orange-red	1:500 – 1:1000
Cy5	640	700	Far red	1:300 – 1:500
Dye-light 488	488	520	Green	1:500 – 1:1000
Dye-light 594**	594	620	Red	1:500 – 1:1000
Dye-light 649	649	700	Far red	1:500

+ For blue emission (quadruple labeling)
**Alternative to Cy3, or well suited for quadruple labeling (with linear unmixing)

3.5 Direct immunofluorescence

In this case, the primary antibody is directly coupled to a fluorochrome (e.g., Cy3-conjugated rabbit anti-GFAP antibody). It can readily be combined with other antibodies in double or triple immunofluorescence staining experiments.

1. After completion of the double immunofluorescence staining (with Alexa488 and Cy5 fluorochromes), wash the sections 3x15 min in Tris-Triton
2. Pre-incubate in 50% normal serum* in Tris-Triton for 3 hours
3. Incubate overnight at room temperature with the conjugated primary antibody (10 μg/mL in Tris buffer, pH 7.4, containing 2% BSA and 10% normal serum*)
4. Wash 3x10 min in Tris-triton and mount the sections on glass slides
5. Coverslipp as usual

*The normal serum is from the species in which the primary antibody was raised.

IV. Reagents and Solutions

4.1 Buffers

4.1.1 PBS

Stock I (base)
42.6 g Na_2HPO_4 anhydr.
238.4 g NaCl
3000.0 mL dH_2O

Stock II (acid)
13.8 g NaH_2PO_4 monohydr.
79.5 g NaCl
1000.0 mL dH_2O

Store at RT

Working solution:
dH_20 1800 mL
stock II 35 mL
stock I 165 mL
adjust pH to 7.4

4.1.2 Tris-saline

Stock Trizma base 10x

Tris (0.5 M)	60.6 g/l
NaCl (1.5 M)	87.7 g/l

Store at RT

Working solutions:

Tris-saline pH 7.4
dilute stock 1:10
adjust pH with HCl to 7.4

Tris-Triton pH 7.4
dilute stock 1:10
adjust pH with HCl to 7.4
add 0.05% Triton X-100

Tris-Triton pH 7.7
dilute stock 1:10
adjust pH with HCl to 7.7
add 0.05% Triton X-100

4.1.3 Na-phosphate buffer stock (0.4 M)

Na_2HPO_4 anhydr.	46.0 g
NaH_2PO_4 monohydr.	10.5 g
dH_2O	1000.0 mL

Store at 4°C

mix well by stirring

before use: warm to RT
stir to dissolve crystals

4.1.4 Na-citrate buffer (for antigen retrieval)

Stock

Na_2HPO_4 dihydrate (0.2M)	35.6g/L
Citric acid (0.1M)	21.0g/L

Store at RT

Working solution:
take about 50% of final volume citric acid and add Na-phosphate buffer until pH reaches 4.5 (or 6)

4.1.5 Artificial cerebrospinal fluid (ACSF)

Composition (in mM): NaCl 125, KCl 2.5, $CaCl_2$ 2.5, $MgCl_2$ 2, $NaHCO_3$ 26, NaH_2PO_4 1.25, glucose 25; pH 7.4

Stock 10X		*for 250 mL*
NaCl	73.05 g/L	18.26 g
$NaHCO_3$	21.84 g/L	5.46 g
NaH_2PO_4 1xH_2O	1.73 g/L	0.43 g
KCl	1.86 g/L	0.47 g
dH_2O	up to 1 L	
Store at 4°C up to 2-3 weeks		

Working solution (1X; 100 mL):
90 mL of dH_2O
0.55 g glucose (25 mM)
370 µL $CaCl_2$(1xH_2O) (1 M stock; 2.5 mM final concentration)
200 µL $MgCl_2$(6H_2O)(from 1M stock)
10 mL of 10X ACSF
Adjust pH to 7.4 as necessary

Make up fresh on day of experiment

4.1.6 Pepsin (for antigen retrieval)

Stock solution (20X): Make 0.2 M HCl solution (from 1-10 M HCl with dH_2O). Dissolve pepsin (Dako, S3002) (3 mg/mL) in 0.2 M HCl; aliquot (500 µL) and store frozen at -20ºC (except one vial that is kept in the refrigerator).
Working solution: Dissolve stock solution in 0.2 M HCl in dH_2O to a final concentration of 0.15 mg/mL

4.1.7 Buffered glycerol for mounting immunofluorescence sections

Prepare a 1:1 mixture of glycerol and Na-bicarbonate buffer, 0.1 M, pH 9.2.
Store at RT

4.2 Cryoprotectants

4.2.1 10% DMSO in PBS (%v/v) (make fresh)

4.2.2 10%, 30% Sucrose in PBS (%w/v) (make fresh)

4.2.3 Antifreeze solution (Long-term storage of free-floating sections at -20°C)

Mix the following ingredients in this order (can be stored at 4°C)

Na-phosphate buffer 50 mM, pH 7.4	500 mL (or 62.5 mL 0.4 M Na-phosphate buffer and 437.5 mL dH_2O)
Glucose	150 g
Ethylene glycol	300 mL
Sodium azide	200 mg (caution: toxic !)

4.3 Fixatives

Fixatives are to be prepared on the day of use. PFA is not stable in solution

4.3.1 Fixative for perfusion (4% PFA in neutral buffer)

Composition : 4% PFA in 0.15 M Na-phosphate buffer and 15% (± 1.95 g/L) saturated picric acid; pH 7.4

Note: Picric acid improves penetration of primary antibodies, but is not absolutely required. It is not compatible with some tracers and histological markers (e.g. DiI). Problems can also occur after antigen-retrieval and microwaving. Only use the purest quality!

Volume of fixative required: As a rule of thumb, twice the animal body weight (500 mL/adult rat; 60-80 mL per adult mouse); see chapter II.1 for details

Caution: work in a ventilated hood; picric acid is explosive when dry; always keep as saturated solution in dH_2O

For 1 liter:

- Dissolve 40 g PFA in ~200 mL dH_2O; add 20 drops of NaOH (8 N); warm up to max. 60°C while stirring continuously until entirely dissolved
- Add 375 mL 0.4 M Na-phosphate buffer, 150 mL saturated picric acid solution, and dH_2O to make up 1000 mL
- Filtrate the solution, cool it to RT, adjust the pH to 7.4, and put the solution on ice

4.3.2 Fixative for fresh-frozen sections (0.5% PFA)

for 200 mL:

Dissolve 1 g PFA in 125 mL dH_2O by stirring at max 60°C, add 4 drops NaOH (8 N) and let the solution clear. Add 75 mL 0.4 M Na-phosphate buffer, cool to RT, and adjust pH to 7.4.

4.3.3 Fixative for cell culture (4% PFA)

for 200 mL:

Dissolve 8 g paraformaldehyde in 125 mL dH_2O with 4 drops NaOH (8N) by stirring at max. 60°C and let the solution clear. Add 75 mL 0.4 M phosphate buffer, cool to RT, and adjust the pH to 7,4.

Note: this solution can be aliquoted, stored at -20ºC, and thawed briefly before use

4.4 Detergents

4.4.1 Triton X-100 (Stock solution)

10% Triton X-100 (%v/v) in dH_2O. Dissolve by stirring slowly.
Store at 4 ºC for 2-3 months

4.5 Glass-slide coating (Gelatin-Chromalun)

1. Fill slide holders with slides (19/holder)
2. Wash the slides overnight in Extran MA 01, diluted 1:100 in tap water
3. Wash the slides in running tap water for 2-4 h
4. Add 5 g gelatin in 1 liter dH_2O at 55°C. Stir until dissolved
5. Add 0.5 g $CrKO_8S_2$ .12 H_2O
6. Filter the solution while hot
7. Coat the slides by dipping them for 30 sec in the solution (RT); work carefully to achieve a uniform thickness
8. Blot the edge of the slides, dry them in a hot oven (70-80°C), at least 48 h
9. Put them back in the cardboard boxes, labeled accordingly.

Part II Staining of cell cultures

1) Antibody staining of living cells on glass coverslips and methanol fixation

This protocol describes double immunofluorescence staining for a cell surface and an intracellular epitope

- Live staining is recommended for membrane proteins with an extracellular epitope.
- Live staining at 37°C requires adjustment of pH of incubation buffer (buffer A). Note that some membrane proteins become rapidly internalized at 37°C, which can affect the staining pattern!
- To avoid internalization, cells can be stained very briefly at 4°C with a high concentration of antibodies
- Methanol fixation does also permeabilize the membrane, which allows the antibodies to penetrate into the cell

Staining protocol

1. Warm up buffer A to RT
2. Prepare a wet Kleenex with a Parafilm on top in a large glass Petri dish and label for different samples
3. Dilute 1° antibody in buffer A and pipet 120 µL of antibody solution on the Parafilm
4. Rinse the cells growing on glass coverslips in 12-well plates with buffer A and place them up-site down on the antibody drops.
5. Incubate on a shaker (very slow rotation) for 90 min.
6. Wash 3x10 min with buffer A in a 12-well plate
7. Fix the cells for 10 min with methanol at –20°C (critical)
8. Rinse 3 times with PBS
9. Incubate cells for 90 minutes with the second 1° antibody diluted in PBS containing 10% normal serum
10. Wash 3x10 min with PBS
11. Incubate cells for 30 minutes with the mixture of 2° antibody diluted in PBS containing 10% normal serum (Cy3: 1:500; Alexa488: 1:1000; Cy5: 1:200)
12. Wash 3 times 3 min with PBS
13. Let cells dry and cover them up-site down with DAKO mounting medium or with glycerol/$NaHCO_3$ (1:1; pH 9.2)

Buffer A (modified from Archibald et al. 1998):

Substance	Company	MW	Stock	Amount	Final conc.
$CaCl_2$ x $2H_2O$	Fluka, Microselect	147.02	2 M	1 mL	2 mM
$MgCl_2$	Sigma, Cat. No. 104-20	95.21	4.9 M	408 µl	2 mM
Glycin	Merck, p.a.	75.07	10 mM	100 µl	1 µM
TTX*	Latoxan, Valence, F	319.3	1 mM	500 µl	500 nM
Glucose	Fluka, for microbial.	198.17	-	5.945 g	30 mM
HEPES	Fluka, microselect	238.31	-	5.958 g	25 mM
KCl	Merck, p.a.	74.55	500 mM	10 mL	5 mM
NaCl	Fluka, puriss p.a.	58.44	-	6.954 g	119 mM
H_2O bidest.	-	18.02	-	ad 1000 mL	-

* TTX stock, 1 mM: Dissolve 1 mg TTX in 3.1 mL 0.1 N HCl and make 500 µL aliquots (1 mg TTX = 3.132 µmol). Caution: very toxic!! Discard all waste separately!

2) Antibody staining of living cells on glass coverslips and PFA fixation

- See remarks for live staining with methanol fixation!
- PFA solution should always be freshly prepared or stored frozen at –20°C
- PFA fixation partially permeabilizes the plasma membrane. For optimal staining of intracellular proteins, fully permeabilize with Triton X-100. Conversely, permeabilization can be reduced by adding 4% sucrose to the PFA solution.

Staining protocol

1. Proceed as above until fixation
2. Fix cells for 10 to 15 min with 4% PFA at RT
3. Rinse 3 times with PBS
4. If necessary, permeabilize 4 to 5 min with 0.2% Triton X-100 solution in PBS with 10% NGS at RT (do not wash after permeabilization)
5. Proceed as above for the second primary antibody and the secondary antibodies

PFA-solution 4%, 50 mL

1. Warm up 0.4M phosphate buffer
2. Dissolve 2.0 g paraformaldehyde in ca. 20 mL dH_2O (max. 60°C), adding few drops of NaOH and let cool down
3. Add 18.75 mL of 0.4 M phosphate buffer
4. Add water dH_2O up to 50 mL. Adjust pH to 7.4 (only when solution is at RT!)

3) SYTOX green staining for cell nuclei

SYTOX Green nucleic acid stain is a green-fluorescent nuclear and chromosome counterstain (binds to DNA) that is not cell permeant, making it a useful indicator of dead cells within a population. The dye is well excited by the 488 nm argon-ion laser line commonly used with confocal microscopes and flow cytometers. Spectrum: absorption 504 nm; emission 523 nm

Molecular Probes, Cat. No. S-7020, 5 mM solution in DMSO

Staining protocol

1. Rinse cells with Tris buffer
2. Fix cells with methanol, 10 min at –20°C
3. Wash 3 times with Tris buffer
4. Incubate cells with 100nM SYTOX green solution in Tris buffer at RT during 30 min
5. Wash 3 times 10 min with Tris buffer
6. Let dry and cover

Note: Sytox Green staining can also be added to the secondary antibody solution when performing an immunofluorescence staining, as described above.

4) Staining of F-actin with phallotoxins

- Phallotoxins (from the deadly mushroom *amanita phalloides*) bind competitively to F-actin in the nanomolar range
- At elevated pH they loose their affinity for F-actin
- Phallotoxins are unable to bind to monomeric G-actin. They shift the monomer/polymer equilibrium toward the polymer and stabilize F-actin
- **Staining does not work after methanol fixation!**

Molecular probes: Alexa fluor 488 phalloidin (A-12379), rhodamine phalloidin (R-415)

Protocol

1. Perform immunofluorescence in live cells if required
2. Fix cells with 4% PFA at RT for 15 min
3. Rinse 3 times with PBS
4. Permeabilize with 0.2% Triton X-100 in PBS with 10% NGS for 5 min at RT
5. Do not rinse again and add directly the solution containing the phallotoxin (1:20, for both colors) and the secondary antibodies in PBS with 10% NGS for 1 hour.
6. Wash 3x10 min with PBS
7. Let dry and cover with embedding medium

5) DiD labeling of the plasma membrane

– Vybrant™ DiI/O/D cell-labeling solution is a dye delivery solution that can be added directly to normal culture media to uniformLy label suspended or attached culture cells.
– The standard protocol of Molecular Probes suggests live staining in medium. The same protocol can also be used in living cells with buffer A (see antibody staining in living cells)
– Does not work optimally in already fixed cells (live staining recommended)

Further information and general protocol:
VybrantTM DiD, Molecular Probes, V-22887;
http://probes.invitrogen.com/media/pis/mp22885.pdf

Labeling protocol

1. Rinse cells with buffer A
2. Dilute vibrant DiD solution 1:200 in buffer A
3. Proceed as described in the live cell labeling protocol. Incubate cells for approximately 20 min in the vibrant DiD solution
4. Wash 3 times 5 to 10 min with buffer A
5. Fix cells with methanol
6. Proceed for further antibody staining if desired…

Part III Histological Stains

1) Nissl staining

Note: This protocol is not suited for tissue processed with antigen-retrieval (use protocol 2)

Mount sections on gelatin-coated glass slides and let dry overnight.
A few slides can be handled one by one in 100 mL glass containers. Otherwise, use slide holders (in 250 mL containers).

Dip them successively in the following solutions:

dH_2O	5 min
Cresyl violet solution (filter prior use)	5 min
dH_2O	30 sec
96% ethanol and 0.5% acetic acid	1-5 min until the desired coloration is obtained (check in the microscope)
Isopropanol	5 min
Isopropanol:Xylol (1:2)	5 min
Xylol	4 x 2 min

Coverslip in Eukitt

2) Nissl staining protocol for human tissue

Mount sections on gelatin-coated glass slides and let dry overnight.

Dip them successively in the following solutions:

Xylol	2 x 20 min
100 % ethanol	20 min
Chloroform	60 min
100% ethanol	10 min
96% ethanol	10 min
70% ethanol	5 min
50% ethanol	5 min
dH_2O	5 min
Cresyl violet solution (filter prior use)	2 min
dH_2O	30 sec
70% ethanol + 0.1% acetic acid	30 sec
96% ethanol + 0.1% acetic acid	30 sec
96% ethanol	5 min
100% ethanol	2 x 5 min
Xylol	4 x 5 min

Coverslip in Eukitt

3) Preparation of Cresyl violet

Dissolve 1 g Cresyl violet acetate (Fluka 61123) in 100 mL dH_2O containing 0.25 mL glacial acetic acid; stir for at least 1 hour; dilute 1:1 with dH_2O. Filter before use.

4) Fluorojade B staining

Fluoro-Jade B, is an anionic fluorescein derivative useful for detecting neurons undergoing degeneration in tissue sections.

1. Prepare sections from perfusion-fixed rodent brain
2. Mount them on SuperFrost® Plus Gold slides and them then air-dry overnight
3. Immerse the slides in 100% ethanol solution for 3 min
4. Rinse for 3 min in 70% ethanol and 2 min in dH_2O
5. Transfer the slides to a solution of 0.06% potassium permanganate in dH_2O for 15 min on a shaker
6. Rinse for 2 min in dH_2O
7. Prepare staining solution from a 0.01% Fluoro-Jade B stock solution (10 mg dye powder in 100 mL dH_2O*). To make up 100 mL of staining solution, mix 10 mL stock solution with 90 mL 0.1% glacial acetic acid. This results in a final dye concentration of 0.001%.
8. Incubate slides for 30 min in the staining solution
9. Rinse 3x1 min in dH_2O
10. Excess water is removed by briefly (about 15 s) draining the slides vertically on a paper towel.
11. The slides are air-dried, cleared by immersion in xylene for at least 1 min and coverslipping with DPX (a non-aqueous non-fluorescent plastic mounting media). Do not coverslip with Dako® mounting medium or buffered glycerol!
12. Store the slides at 4° C in the dark.

*Store at 4ºC up to 2-3 months

Note: Depending on the number of degenerating cells and tissue specimen, different potassium permanganate/Fluoro-Jade B incubation time/concentration should be tested to ensure optimal signal/noise ratio.