A method for triple fluorescence labeling with *Vicia villosa* agglutinin, an anti-parvalbumin antibody and an anti-G-protein-coupled receptor antibody

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Accepted 2 March 1998

Abstract

The aim of the original study [S.B. Bausch, C. Chavkin, *Vicia villosa* agglutinin labels a subset of neurons coexpressing both the mu opioid receptor and parvalbumin in the developing rat subiculum, Dev. Brain Res., 97, 1996, 169–177] [3] was to develop a method for identifying a subset of mu opioid receptor-expressing interneurons in the rat subiculum for electrophysiological studies. Previous studies had shown that a subset of parvalbumin-positive neurons in the rat subiculum could be labeled with the lectin, *Vicia villosa* agglutinin (VVA) [C.T. Drake, K.A. Mulligan, T.L. Wimpey, A. Hendrickson, C. Chavkin, Characterization of *Vicia villosa* agglutinin-labeled GABAergic neurons in the hippocampal formation and in acutely dissociated hippocampus, Brain Res., 554, 1991, 176–185] [11], and that mu opioid receptor immunoreactivity (IR) and parvalbumin-IR were colocalized in a subset of neurons in the hippocampus and dentate gyrus [S.B. Bausch, C. Chavkin, Colocalization of mu and delta opioid receptors with GABA, parvalbumin and a G-protein-coupled inwardly rectifying potassium channel in the rodent brain, Analgesia, 1, 1995, 282–285] [2]. We hypothesized that a subset of mu opioid receptor-expressing neurons in the subiculum also would express the calcium binding protein, parvalbumin, and could be labeled with VVA. Labeling of live neurons with VVA [11] then could be used to identify these neurons. This protocol was designed to triple-label neurons expressing the mu opioid receptor, parvalbumin and the carbohydrate group, N-acetylgalactosamine. Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation, J. Immunol. Methods, 78, 1985, 143–153 [25,29]. VVA labeling and immunocytochemistry with an affinity-purified anti-mu opioid receptor antibody [S.B. Bausch, T.A. Patterson, M.U. Ehrengruber, H.A. Lester, N. Davidson, C. Chavkin, Colocalization of mu opioid receptors with GIRK1 potassium channels in rat brain: an immunocytochemical study, Recept. Channels, 3, 1995, 221–241] [4] and an anti-parvalbumin antibody [M.R. Celio, W. Baier, L. Scharer, P.A. de Viragh, C. Gerday, Monoclonal antibodies directed against the calcium binding protein parvalbumin, Cell Calcium, 9, 1988, 81–86] [8] were used to accomplish this goal. Immunofluorescence was used as the detection method; visualization was accomplished with three fluorophores with different excitation/emission spectra and a one laser confocal microscope. This protocol can be modified easily to triple-label neurons for other carbohydrate groups and proteins. © 1998 Elsevier Science B.V. All rights reserved.

Themes: Cell biology

Topics: Staining, tracing, and imaging techniques

Keywords: Confocal microscopy; Lectin; Immunofluorescence; Mu opioid receptor; N-acetylgalactosamine; Perineuronal net

1. Type of research

Anatomy, Immunocytochemistry, Neurobiology.

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PII S1385-299X(98)00011-7
2. Time required

- Perfusion of tissue with fixative solution and cryoprotection
  (A)–(C) Anesthetize and perfuse rat; remove brain ∼ 30 min
  (D) Postfix: 2 h
  (E) Cryoprotect: 24–72 h
- Section tissue ∼ 30 min
- Immunofluorescent labeling
  Day 1: (A)–(E) ∼ 5 h
  Day 2: Continued incubation
  Day 3: (F)–(J) ∼ 5.5 h
- Mount tissue onto slides
  (A) Mounting: dependent upon number of wells of tissue ∼ 10 min/well
  (B) Drying: overnight
- Coverslip slides
  (A) Coverslipping ∼ 10 min
  (B) ‘Drying’ of mounting media: overnight
  (C) Sealing ∼ 10 min
  (D) Drying of sealant ∼ 1 h

* Indicates incubation or drying only—no bench time.

3. Materials

3.1. Animals

Male Sprague–Dawley rats (5 weeks) were obtained from Bantin and Kingman (Belleview, WA) and housed in the University of Washington vivarium. All treatment of animals was according to National Institutes of Health and institutional guidelines.

3.2. Special equipment

- Adjustable speed shaker
- 37°C oven
- 4°C cold room or refrigerator with internal power supply outlet (for shaker)
- Biorad MRC 600 confocal microscope equipped with two photomultiplier tubes and one Kr/Ar laser capable of excitation wavelengths for fluorescein (488Å), rhodamine (568Å) and Cy5 (647Å). Wavelengths in parentheses indicate the Kr/Ar laser lines. Emission filter blocks designed for use with multiple fluorescent labels, specifically: T1, triple dichroic; GR2, 560LP dichroic with 605 emission filter for channel 1 and 522 emission filter for channel 2; FR2, 640LP dichroic with 680 emission filter for channel 1 and 598/40 emission filter for channel 2
- Computer equipped with COMOS 6.03 software

3.3. Chemicals, reagents and solutions

Chemicals
- Bovine serum albumin
- Gelatin
- Glutaraldehyde
- KCl
- N-acetylgalactosamine
- NaCl
- Nail polish
- Parafomaldehyde
- Pentobarbital
- Periodic acid
- Sodium phosphate dibasic
- Sodium phosphate monobasic
Sucrose
• Tris base
• Tris–HCl
• Triton X-100

All chemicals, except nail polish and pentobarbital, were obtained from Sigma. Nail polish was purchased from a local drug store and pentobarbital was supplied through the University of Washington Medical Center Pharmacy.

**Reagents**

- Affinity-purified Cy5™-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) (Cy5 is a trademark owned by Biological Detection Systems, Pittsburgh, PA)
- Affinity-purified fluorescein-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch)
- Affinity-purified anti-peptide rabbit anti-mu opioid receptor antibody (MT-2, #2148 [4]; not commercially available)
- Lissamine rhodamine–conjugated streptavidin (Jackson ImmunoResearch)
- Monoclonal mouse anti-parvalbumin antibody (clone #PA-235 [8]; Sigma)
- Normal goat serum (Vector)
- Vectashield mounting media (Vector)
- *Vicia villosa* agglutinin (VVA)–biotin (Vector & EY Laboratories)

**Solutions**

Make all solutions fresh (< 1 week) for each staining series; subbed slides, 0.4 M sodium phosphate buffer and 1 M Tris buffer stock solutions may be stored longer.

- 0.4 M Sodium phosphate buffer (PB) [pH 7.4] (per liter)

  10.5 g Sodium phosphate monobasic
  45.9 g Sodium phosphate dibasic

  Heat to dissolve; let cool to room temperature (RT). Adjust pH to 7.4 with 10 M NaOH. Store at RT.

- 1 M Tris buffer (TB) [pH 7.4] (per liter)

  132.2 g Tris–HCl
  1.94 g Tris base

  Adjust pH to 7.4 with 10 M NaOH. Store at 4°C.

- PBS = PB containing 0.15 M NaCl and 2.7 mM KCl [pH 7.4]
- TBS = TB containing 0.15 M NaCl and 2.7 mM KCl [pH 7.4]

  Per liter

<table>
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<th>Final concentration</th>
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<tbody>
<tr>
<td>8.77 g NaCl</td>
<td>0.15 M NaCl</td>
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<tr>
<td>0.2 g KCl</td>
<td>2.7 mM KCl</td>
</tr>
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- Fixative: 2% paraformaldehyde in 0.1 M PB containing 0.1% glutaraldehyde (per liter)

  20 g Paraformaldehyde/1 of 0.1 M PB
  4 ml of 25% glutaraldehyde

  Mix paraformaldehyde in 0.1 M PB; heat to dissolve (do not exceed 65°C); let cool to RT; filter. Store at 4°C for no longer than 24 h before use; if stored, warm to RT before use. Add glutaraldehyde immediately prior to use.

- Subbed slides

  Per 456 ml

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
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<tbody>
<tr>
<td>6 g gelatin</td>
<td>1.3% gelatin</td>
</tr>
<tr>
<td>288 ml water</td>
<td></td>
</tr>
<tr>
<td>120 ml 95% ethanol</td>
<td>25% ethanol</td>
</tr>
<tr>
<td>20 ml of fresh 2% CrKSO₄</td>
<td>0.09% CrKSO₄</td>
</tr>
<tr>
<td>28 ml glacial acetic acid</td>
<td>0.06% glacial acetic acid</td>
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  Mix gelatin and water; heat to 50°C to dissolve; cool to RT. Add ethanol, CrKSO₄ and glacial acetic acid. Dip clean slides in subbing solution for 1 min; allow to dry overnight.

- VVA–biotin diluent: 0.01 M PBS containing 0.5% gelatin

  Per 25 ml

<table>
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<tbody>
<tr>
<td>25 ml 0.01 M PBS [pH 7.4]</td>
<td>0.01 M PBS</td>
</tr>
<tr>
<td>0.5 g gelatin</td>
<td>2% gelatin</td>
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</table>
Heat to dissolve gelatin; cool to RT before use.

- **General block:** 0.1 M TBS with 2% gelatin and 10% normal goat serum

<table>
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<th>Per 25 ml</th>
<th>Final concentration</th>
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<tbody>
<tr>
<td>22.5 ml 0.1 M TBS [pH 7.4]</td>
<td>0.1 M TBS</td>
</tr>
<tr>
<td>0.5 g gelatin</td>
<td>2% gelatin</td>
</tr>
<tr>
<td>2.5 ml normal goat serum</td>
<td>10% normal goat serum</td>
</tr>
</tbody>
</table>

  Mix TBS and gelatin; heat to dissolve gelatin. Cool to RT before adding normal goat serum.

  - **Antibody diluent:** 0.1 M TBS containing 0.1% bovine serum albumin (BSA), 10% normal goat serum and 0.1% Triton X-100

    | Per 50 ml | Final concentration |
    |-----------|---------------------|
    | 45 ml 0.1 M TBS [pH 7.4] | 0.1 M TBS          |
    | 50 mg BSA                  | 0.1% BSA           |
    | 5 ml normal goat serum     | 10% normal goat serum |
    | 50 μl Triton X-100         | 0.1% Triton X-100  |

  - **Streptavidin diluent:** 0.1 M TBS containing 0.1% Triton X-100

    | Per 25 ml | Final concentration |
    |-----------|---------------------|
    | 25 ml 0.1 M TBS [pH 7.4] | 0.1 M TBS          |
    | 25 μl Triton X-100         | 0.1% Triton X-100  |

4. Detailed procedure

4.1. **Perfusion of tissue with fixative solution and cryoprotection**

(A) Deeply anesthetize rat with 50 mg/kg i.p. pentobarbital.
(B) Perfuse rat intracardially with fixative for 10 min.
(C) Remove brain from skull.
(D) Postfix brain in fixative for 2 h.
(E) Cryoprotect brain by sinking (24–72 h) in 30% sucrose in 0.1 M PB.

4.2. **Section tissue**

(A) Cut brains into 40-μm sections using a freezing sliding microtome.
(B) Place sections into 0.1 M PB.
(C) Sort free-floating sections into individual staining wells (6–10 sections/well depending upon sizes of wells and sections).

4.3. **Immunofluorescence labeling**

All steps at RT unless otherwise stated. All steps at RT and 4°C should be performed on a shaker at medium speed.

(A) Rinse sections in 0.01 M PB 5 min
    Rinse sections in 0.01 M PBS 3 × 5 min

(B) Incubate sections in VVA–biotin diluted in VVA–biotin diluent to 35 μg/ml (Vector) or 50–75 μg/ml (EY Laboratories) 2 h

(C) Rinse sections in 0.01 M PBS 3 × 5 min
    Rinse sections in 0.1 M TBS 6 × 5 min

(D) Incubate sections in General block 1 h at 37°C

(E) Incubate sections in primary antibodies diluted in antibody diluent:
    mouse anti-parvalbumin antibody diluted 1:2000 1 h at RT then
    rabbit anti-G-protein receptor antibody diluted accordingly 36 h at 4°C

(F) Rinse sections with 0.1 M TBS containing 0.1% Triton X-100 10 × 6 min
4.4. Mount sections onto slides

(A) Mount sections onto slides.
(B) Dry overnight.

4.5. Coverslip slides

(A) Coverslip slides with Vectashield mounting media.
(B) Allow mounting media to ‘dry’ overnight (will not completely dry).
(C) Seal coverslips to slides by placing a line of nail polish along all edges of the coverslip.
(D) Allow nail polish to dry for at least 1 h before viewing sections at high magnification or cleaning.

4.6. View sections / collect images with Biorad MRC 600 confocal microscope with COMOS 6.01 software

(A) View/collect images for fluorophores simultaneously from triple-labeled tissue to determine colocalization.
(1) Collect images simultaneously for lissamine rhodamine and fluorescein.
   (a) Emission filter blocks
      T1 into channel 1
      GR2 into channel 2
   (b) Excitation lines
      488/568 $\lambda$
   (c) Image output is
      lissamine rhodamine for channel 1
      fluorescein for channel 2

   (2) Collect images simultaneously for Cy5 and lissamine rhodamine.
      (a) Emission filter blocks
         T1 into channel 1
         FR2 into channel 2
      (b) Excitation lines
         All lines
      (c) Image output is
         Cy5 for channel 1
         lissamine rhodamine for channel 2

   (B) View/collect images for fluorophores individually from triple-labeled tissue to control for ‘bleed-through’, false positives/negatives (Use the same confocal settings as used in (A)).
   (1) Collect images individually for fluorescein.
      (a) Emission filter blocks
         T1 into channel 1
         GR2 into channel 2
      (b) Excitation lines
         488 $\lambda$
      (c) Image output is
         ‘bleed-through’ of signal for channel 1
         fluorescein for channel 2

   (2) Collect images individually for lissamine rhodamine.
      (a) Emission filter blocks
         T1 into channel 1
         GR2 into channel 2
      (b) Excitation lines
         568 $\lambda$
      (c) Image output is
         lissamine rhodamine for channel 1
         ‘bleed-through’ of signal for channel 2

   (3) Collect images individually for lissamine rhodamine.
      (a) Emission filter blocks
         T1 into channel 1
         FR2 into channel 2
(b) Excitation lines 568 Å
(c) Image output is ‘bleed-through’ of signal for channel 1
lissamine rhodamine for channel 2

No specific signal should be seen in ‘bleed-through’ channels. Furthermore, images for each fluorophore should be virtually identical in (A) and (B).

(C) View/collect images for fluorophores individually from single-labeled tissue to control for ‘bleed-through’, false positives/negatives (Use the same confocal settings as used in (A), (B)).

(1) Collect images for fluorescein.
(a) Emission filter blocks T1 into channel 1
GR2 into channel 2
(b) Excitation lines 488 Å (or 488/568 Å)
(c) Image output is ‘bleed-through’ of signal for channel 1
fluorescein for channel 2

(2) Collect images for lissamine rhodamine.
(a) Emission filter blocks T1 into channel 1
GR2 into channel 2
(b) Excitation lines 568 Å (or 488/568 Å)
(c) Image output is lissamine rhodamine for channel 1
‘bleed-through’ of signal for channel 2

(3) Collect images for lissamine rhodamine.
(a) Emission filter blocks T1 into channel 1
FR2 into channel 2
(b) Excitation lines 568 Å (or All lines)
(c) Image output is lissamine rhodamine for channel 1
‘bleed-through’ of signal for channel 2

(4) Collect images for Cy5™.
(a) Emission filter blocks T1 into channel 1
FR2 into channel 2
(b) Excitation lines All lines
(c) Image output is Cy5™ for channel 1
‘bleed-through’ of signal for channel 2

No specific signal should be seen in ‘bleed-through’ channels. Furthermore, single and triple-labeled tissue should show labeling of similar numbers and types of structures for each fluorophore.

Fig. 1. Schematic of the reactions used in the triple fluorescence labeling protocol.
5. Results

This protocol visualized mu opioid receptor-IR with fluorescein, parvalbumin-IR with Cy5™ and VVA-labeling with lissamine rhodamine (Fig. 1; schematic of labeling). These fluorophores were chosen for maximum separation of excitation/emission spectra (Fig. 2) with the available Kr/Ar laser equipped confocal system (see Section 3.2).

The results obtained using the protocol have been described in detail previously [3] and one example is shown in Fig. 3. Labeling for the mu opioid receptor, parvalbumin and VVA individually, was found scattered throughout the rat subiculum.

![Excitation and emission spectra](image)

Fig. 2. Excitation (A) and emission (B) spectra of different fluorophore conjugated, affinity-purified antibodies. This figure illustrates only the relative shape and position of each fluorophore in the peak region of its excitation and emission following conjugation to antibodies. Quantitative comparisons should not be made since peak heights have been normalized. All spectra were obtained with an M-Series spectrofluorometer system from Photon Technology International. Abbreviations: Cy5, Indodicarbocyanine; FITC, Fluorescein Isothiocyanate; LRSC, Lissamine Rhodamine Sulfonyl Chloride (lissamine rhodamine) (Figure and legend kindly provided by Dr. William Stegeman at Jackson ImmunoResearch).
Fig. 3. Triple-labeling for the mu opioid receptor, parvalbumin and N-acetylgalactosamine in the rat subiculum. This sagittal rat brain section was labeled with affinity-purified MT-2 (#2148) antibody, anti-parvalbumin antibody and VVA using the described protocol. Mu opioid receptor-IR was visualized with fluorescein green; parvalbumin-IR was visualized with Cy5™ (purple); VVA-labeling was visualized with lissamine rhodamine (red). Colocalization of mu opioid receptor-IR with parvalbumin-IR appears light blue; colocalization of parvalbumin-IR with VVA appears pink; colocalization of mu opioid receptor-IR with parvalbumin-IR and VVA appears white. Image was obtained using a confocal microscope and is a reconstruction of eight optical sections. Control experiments established the specificity of labeling (data not shown). Omission of VVA–biotin and all primary antibodies yielded no labeling. Omission of each primary antibody or VVA–biotin separately showed labeling only for the primary antibody or VVA–biotin included. Omission of each secondary antibody or lissamine rhodamine–streptavidin yielded labeling only for the appropriate primary antibodies or VVA–biotin, respectively. Preadsorption of MT-2 (#2148) with 30 μM MT-2 peptide blocked MT-2 (#2148) labeling. Preadsorption of VVA–biotin with 0.1 M N-acetylgalactosamine and pretreatment of tissue with 44 mM periodic acid blocked all VVA labeling. Scale bar = 25 μm.

Mu opioid receptor-IR was seen in neuronal somata and proximal dendrites. Parvalbumin-IR was observed in neuronal somata, dendrites and fibers. VVA-labeling was found in lattice-like structures surrounding neurons (perineuronal nets). Approximately 30% of all parvalbumin-positive neurons were triple-labeled with mu opioid receptor-IR, parvalbumin-IR
and VVA whereas, on average, approximately 37% of all VVA-positive neurons were triple-labeled. Triple-labeled neurons were found scattered throughout the subiculum, but most of these neurons were found deep in the pyramidal cell layer.

6. Discussion

The principal conclusion is that the described triple-labeling protocol can be used to colocalize the mu opioid receptor, parvalbumin and N-acetylgalactosamine. This protocol can be altered easily for detection of other antigens and carbohydrate groups and is likely to be useful for many neuroscientific applications.

6.1. Colocalization of VVA, parvalbumin-IR and mu opioid receptor-IR

6.1.1. Perineuronal nets

VVA is just one of many lectin and antibody markers that can be used to label perineuronal nets (for reviews, see Refs. [6,7]). Perineuronal nets are described as a fine mesh covering which surrounds the soma and proximal dendrites of a subset of neurons in the brain. The function of perineuronal nets is speculative; proposed functions include: (1) stabilizing synapses, (2) concentrating growth factors, (3) mediating interactions between glia and neurons, (4) physically blocking formation of new synapses, and (5) linking the extracellular matrix with the cytoskeleton.

6.1.2. Parvalbumin

Parvalbumin is a soluble calcium binding protein that is proposed to play a role in the relaxation of muscles (for review, see Ref. [14]). The function of parvalbumin in the brain is unknown, but it has been proposed to act as a calcium binding protein, which subsequently could modulate specialized calcium-dependent processes and neuronal excitability. Parvalbumin is localized to a defined population of neurons in the brain and its expression has been proposed to depend on the physiological properties of the neuron i.e., fast firing neurons with a high metabolic rate (for a review, see Ref. [1]).

6.1.3. Mu opioid receptors

The mu opioid receptor is one of three opioid receptor types shown to exist based upon results from radioligand binding assays, bioassays and electrophysiological experiments (for a review, see Ref. [20]). Opioid receptors activate pertussis toxin-sensitive G-proteins and couple to a variety of second messenger systems and ion channels (for review, see Ref. [23]). In the rat subiculum, mu opioid receptors anatomically colocalize with [4,12] and functionally couple to [12,28] either an inwardly rectifying or a voltage-gated delayed-rectifier potassium channel in distinct and separate populations of neurons.

6.1.4. Colocalization

Although others had shown that a subset of parvalbumin-positive neurons could be labeled with the lectin, VVA [11,15,17–19,22], we were the first to describe the colocalization of mu opioid receptor-IR with parvalbumin-IR and VVA labeling [3]. The functional significance of finding mu opioid receptors, parvalbumin and perineuronal nets in the same neuron remains speculative. However, VVA-labeling of live tissue [11] may prove useful for identifying a defined subset of neurons in which mu opioid receptors couple to either the inward rectifier or the voltage-gated delayed-rectifier potassium channel.

6.2. Protocol

6.2.1. Choice of fluorophores

As stated in the results, fluorescein, lissamine rhodamine and Cy5™ were chosen as fluorophores for maximum separation of excitation/emission spectra (Fig. 2) with the available Kr/Ar laser equipped confocal system (see Section 3.2). Other fluorophores may be substituted for increased signal or for better separation of fluorescent signals with the user’s microscopy system. In regard to fluorophores used in this protocol, Cy5™ is advantageous because of lower tissue autofluorescence in the far red region. However, disadvantages of using Cy5™ include: (1) lower resolution due to the use of longer wavelength light; and (2) lower sensitivity of human vision to light above 650 nm (emission maximum of Cy5™ is 670 nm). Therefore, Cy5™ cannot be visualized with conventional epifluorescence microscopes and is most commonly visualized with electronic fluorescence microscopes equipped with a far red detector [10]. Also of notable concern is the rapid photobleaching of fluorescein. Cy2™ has been proposed to be a superior green fluorescence-emitting fluorophore compared to fluorescein because of lower photobleaching and increased fluorescence intensity (Ref. [13] but see Ref. [5]). (Cy2™ and Cy5™ are trademarks owned by Biological Detection Systems, Pittsburgh, PA).
6.2.2. False negatives

All negative colocalization results should be interpreted with great caution. The immunofluorescence method used in this protocol is less sensitive than methods using amplification (i.e., immunoenzymatic reactions) for detection of low levels of antigen. Thus, low levels of antigen may be present, but may not be detected using this method. Furthermore, competition of reagents [21] and quenching of fluorophores by other fluorophores [9] have been reported and also may yield false negative results. Appropriate controls to rule out false negative results include: (1) comparison of single- and triple-labeled tissue to determine if fewer or different immunoreactive structures are observed in the triple- compared to single-labeled tissue, and (2) single-labeling of sequential serial sections.

6.2.3. False positives

Positive colocalization results also should be interpreted with caution until appropriate control experiments are performed to rule out false positive results. False positives can be caused by: (1) nonspecific autofluorescence of compounds intrinsic to the tissue; (2) nonspecific filter combinations for a defined set of fluorophores; (3) nonspecific primary antibody interactions; (4) nonspecific secondary antibody interactions [26] and (5) nonspecific lectin interactions. Autofluorescence can be detected by viewing unlabeled tissue under the same conditions used to view fluorescently labeled tissue. In some instances, autofluorescence can be decreased by treating the tissue with sodium borohydride or sodium metaperiodate (see Ref. [26]).

False positive results due to nonspecific filter combinations can be minimized by choosing fluorophores which show good separation of excitation/emission spectra. Additionally, filters should be chosen that block the signal from undesired fluorophores while allowing good visualization of the desired signal. It should be noted, however, that total separation is difficult to achieve when visualizing multiple fluorophores simultaneously, especially if one fluorescent signal is particularly bright. Therefore, the fluorophores can be viewed simultaneously to determine colocalization, but each fluorophore should be viewed separately to verify the source of the fluorescent signal. Filters are specific if no signal is observed in the ‘bleed-through’ channels (see B,C in Section 4.6). If ‘bleed-through’ is observed, then: (1) more restrictive barrier filters, (2) more restrictive excitation filters, or (3) different fluorophores should be used.

False positive results due to nonspecific antibody or lectin interactions can be caused by: (1) nonspecific interactions or cross-reactivity between the primary or secondary antibodies/lectins and the tissue; (2) cross-reactivity between the primary antibodies; (3) cross-reactivity between the secondary antibodies, and (4) cross-reactivity between secondary antibodies and the wrong primary antibodies. Appropriate controls to rule out false positive results due to nonspecific antibody or lectin interactions include: (1) omission of all primary antibodies; (2) sequential omission of each primary or secondary antibody; (3) omission of the lectin; (4) preadsorption of the primary antibody with its immunogen; (5) preadsorption of the lectin with its carbohydrate binding partner, and (6) (for lectins) oxidation of the carbohydrate binding partner (see Section 8). For a more detailed account of the causes and controls for false positive results, readers are directed to an excellent review by Wessendorf [26].

6.2.4. Alteration of the protocol

If altering this protocol for use with other antigen/carbohydrate group combinations: (1) the most abundant IR or labeling should be visualized with Cy5 since Cy5 is not as readily detectable as other fluorophores, and (2) particular attention should be given to the species of animals used to generate the primary and secondary antibodies. In general, each primary antibody should be raised in a different animal species while secondary antibodies should be raised in the same species to minimize unwanted cross-reactivity between antibodies. For further guidance on modifications, readers are directed to several excellent Refs. [16,24,26,27] on multiple fluorescent labeling and confocal microscopy.

7. Troubleshooting

The following is a list of potential solutions for problems that were encountered specifically during the development of this protocol.

7.1. High background VVA-labeling

(1) According to product literature, high background labeling may result if lectins are diluted in serum-containing solutions. Therefore, if high background lectin-labeling is a problem, the lectin binding step should precede any incubations utilizing serum in the diluent or can be used following sufficient rinsing to remove serum constituents from the tissue. (2) According to product literature, high background labeling also may result if streptavidin is diluted in serum-containing solutions. Therefore, if high background streptavidin-labeling is a problem, do not combine incubation of the conjugated
streptavidin with the prior antibody incubations because the antibody diluent contains serum. Thorough rinsing of the tissue following incubations with serum-containing diluents also will help alleviate this potential problem. (3) Low background fluorescence was seen for lissamine rhodamine–conjugated streptavidin in the absence of VVA–biotin using the described protocol. However, if high background fluorescence for lissamine rhodamine–conjugated streptavidin persists after omission of VVA–biotin, BSA (0.05%–0.5%) may be added to the streptavidin diluent to decrease nonspecific binding.

7.2. No or low VVA-labeling

Treatment of tissue with (1) sodium borohydride (used to decrease the background fluorescence of tissue perfused with glutaraldehyde containing fixatives), or (2) methanol (used to delipidate tissue) may decrease VVA-labeling below the detection limits of this protocol in regions exhibiting low levels of VVA-labeling. (3) The binding of many lectins, including VVA, is sensitive to the concentration of salt in the diluent.

8. Support protocols

The specificity of labeling should be checked for each series of experiments. Specificity controls always should be done in parallel with the normal protocol, which serves as a positive control for labeling. The following is a list of specificity control protocols.

8.1. No primary antibodies and no VVA control (Reagent control)

Sections are incubated under the same conditions listed in the described protocol, but in diluent alone in Steps 3B (no VVA) and 3E (no antibodies). Omission of VVA–biotin and all primary antibodies should yield no labeling.

8.2. No primary antibody or no VVA control (Control for cross-reactivity of the secondary antibodies or streptavidin)

Again, sections are incubated under the same conditions listed in the described protocol, but in diluent alone in Steps 3B (no VVA) or 3E (no antibody; omit each antibody separately). Omission of each primary antibody or VVA–biotin separately should show labeling only for the primary antibody or VVA–biotin included.

8.3. Antibody preadsorption control (Control for the specificity of the antibody for its immunogen)

Antibody is preincubated with the immunogen, both diluted in antibody diluent, overnight on the shaker at 4°C or for at least 2 h on the shaker at RT, then used as described in Step 3E of the protocol. Preadsorption of the antibody with the immunogen should block labeling for the adsorbed antibody; preadsorption of the antibody with a nonspecific protein or peptide should not block labeling.

8.4. VVA preadsorption control (Control for the specificity of VVA for N-acetylgalactosamine)

VVA–biotin is preincubated with 0.1 M N-acetylgalactosamine (the carbohydrate group that binds VVA [25]) in VVA diluent overnight on the shaker at 4°C or for at least 2 h on the shaker at RT, then used as described in Step 3B of the protocol. Preadsorption of VVA–biotin with N-acetylgalactosamine should block all VVA labeling.

8.5. VVA oxidation control (Control for the specificity of VVA for N-acetylgalactosamine)

Sections are treated with 44 mM periodic acid (in distilled water) (oxidizes terminal carbohydrate groups [29]) for 10–15 min at RT just prior to Step 3A; then proceed with the normal protocol. Pretreatment of tissue with periodic acid should block all VVA labeling.

9. Quick procedure

(1) Perfusion of tissue with fixative solution and cryoprotection.
(2) Section tissue.
3. Immunofluorescence labeling. All steps at RT unless otherwise stated. All steps at RT and 4°C should be performed on a shaker at medium speed.

(A) Rinse sections in 0.01 M PB 5 min
Rinse sections in 0.01 M PBS 3 × 5 min

(B) Incubate sections in VVA–biotin diluted in VVA–biotin diluent 2 h

(C) Rinse sections in 0.01 M PBS 3 × 5 min
Rinse sections in 0.1 M TBS 6 × 5 min

(D) Incubate sections in general block 1 h at 37°C

(E) Incubate sections in primary antibodies diluted in antibody diluent 1 h at RT then 36 h at 4°C

(F) Rinse sections with 0.1 M TBS containing 0.1% Triton X-100 10 × 6 min

(G) Incubate sections in fluorescently-labeled secondary antibodies diluted in antibody diluent 1 h at 37°C

(H) Rinse sections with 0.1 M TBS containing 0.1% Triton X-100 10 × 6 min

(I) Incubate sections in fluorescently-labeled streptavidin in streptavidin diluent 1 h

(J) Rinse sections with 0.1 M TBS 8 × 5 min
Rinse section with 0.1 M TB 15 min

4. Mount sections onto slides.

5. Coverslip slides.


10. Essential literature references

Refs. [1,3,7,16,25–27,29].

Acknowledgements

I wish to thank Dr. Charles Chavkin for support, Dr. Ruth Westenbroek and Ms. Paulette Brunner for helpful discussions and Dr. William Stegeman for providing the excitation/emission spectra. Work was performed in the lab of Dr. Charles Chavkin and the W.M. Keck Center for Advanced Studies of Neuronal Signaling at the University of Washington.

References


