NeuroVue® Filter Squares For Neuronal Tract Tracing in Fixed Tissue

Overview of Labeling Strategy (see page 2 for more detailed instructions)

- Fix tissue in 4% buffered formaldehyde.
- Initiate labeling by inserting NeuroVue micro-strip(s) into nerve tract(s) to be traced. The highly lipophilic NeuroVue dyes transfer from the micro-strip into nerve cell membranes and diffuse along the lipid bilayer in both directions from the insertion site (anterograde and retrograde labeling).
- Incubate tissue in 4% phosphate buffered formaldehyde at 37°C.
- Monitor the progress of dye diffusion using light microscopy and/or fluorescence microscopy.
- When dye(s) have reached the region(s) to be studied, remove NeuroVue micro-strip(s) and prepare whole mounts or tissue sections for fluorescence imaging.

Advantages of NeuroVue® Technology

- Different fibers can be traced in the same specimen by using fluorescent NeuroVue dyes that excite and emit in the green, orange, red and/or far red.
- Use of dye-coated filters allows more precise positioning than is possible with crystals or oils, avoids tissue damage caused by high pressure microinjection, and provides sharp high resolution images of both afferent and efferent fibers arising at the point of filter insertion (Fritzsch et al., Brain Res Bul 66:249-258, 2005).
- Use of NeuroVue dyes reduces the complexity of labeling procedures because these dyes have been selected to have similar diffusion rates, allowing simultaneous or near-simultaneous application of different colors in most cases. (Fritzsch et al., Brain Res Bul, 66:249-258, 2005).

Equipment and Supplies Needed

<table>
<thead>
<tr>
<th>Item</th>
<th>Micro-strip Preparation</th>
<th>Specimen Fixation</th>
<th>Labeling/Diffusion Monitoring</th>
<th>Tissue Mounting</th>
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<td>Glycerol (or other mounting medium free of organic solvents)</td>
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<tr>
<td>Microscope slides and coverslips</td>
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* Prepared from ultrapure formaldehyde solution (Polysciences, Cat. #04018) or equivalent or reagent grade paraformaldehyde powder or equivalent.

NeuroVue® Filter Squares For Neuronal Tract Tracing in Fixed Tissue

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* Prepared from ultrapure formaldehyde solution (Polysciences, Cat. #04018) or equivalent or reagent grade paraformaldehyde powder or equivalent.
**Procedures**

The examples below, kindly provided by Drs. Bernd Fritzsch and Lucy Feng (Creighton University), illustrate applications of NeuroVue® dyes for tracing various sensory nerves of the head and ear to and from the brain. For additional examples in a variety of developmental systems, see the NeuroVue® bibliography.

1. **Preparation of NeuroVue® Filter Micro-strips**

   a) Put on a clean pair of protective gloves.

   b) Agitate scissors and forceps in alcohol to remove any dye residues. Air dry completely before use.

   **Note:** To avoid cross-contamination among dyes, it is preferable to use a separate set of instruments for each dye being handled in Steps 1, 3 and 4.

   c) Using a dissecting microscope, hold the NeuroVue dye coated filter with forceps and use microscissors to cut into triangular pieces approximately 0.3 - 1 mm on a side (Figure 1).

   **Note:** Shape and size of pieces can be adjusted depending upon the size of the tissue specimen to be labeled and the number of colors to be used simultaneously.

   d) After cutting micro-strips, agitate scissors and forceps in alcohol to remove any dye residues, then air dry completely.

2. **Preparation of Tissue Specimens for Labeling with NeuroVue® Micro-strips**

   a) Put on a pair of protective gloves.

   b) Perfusion fix embryos with 4-10% formaldehyde in 0.1M phosphate buffer (pH 7.4).

   **Note:** In order to minimize background tissue fluorescence caused by autofluorescent impurities in the fixative it is important that fixative be prepared using ultrapure methanol free formaldehyde (e.g. Polysciences, Cat. #04018) or paraformaldehyde.

   c) Store fixed specimen at 4°C in 4% neutral buffered formaldehyde for at least 3 days (or up to 1 year).

   **Note:** Prolonged fixation (>1 month) results in greater tissue crosslinking and slower dye diffusion.

   d) Rinse forceps to be used for manipulation with alcohol to remove any dye residues.

   e) For brain, remove and pin down the head. If needed, dissect to expose application site (see Fritzsch Jackson Lab Presentation 2005 for examples of “inside-out” labeling of the inner ear). For more rigid tissues, make incision(s) as needed to allow insertion of filter micro-strip(s) and note a landmark to find the cut later.

   **Note:** To avoid spread of dye through unwanted neuronal profiles through peripheral anastomoses, cut nerve(s) for which labeling is not desired. For example, cut the facial nerve root to avoid cross labeling of the trigeminal nerve, and vice versa.

---

**Figure 1. Preparation of NeuroVue Red micro-strips for use in tissue labeling.**
Using a dissecting microscope, microscissors (at left) are used to cut small triangles from a 1cm x 1cm coated filter square (Cat. #24835). Scale bar = 2 mm (~25X magnification).
3. Insertion of NeuroVue® Micro-strips in Tissue to Initiate Labeling

a) Put on a pair of protective gloves.

b) Agitate forceps in alcohol before and after insertion of each micro-strip. Air dry completely before use.

Note: To avoid cross-contamination, use a separate forceps for each different NeuroVue dye.

Note: It is important to avoid residual alcohol on tools used to handle the NeuroVue filters or micro-strips, especially in Steps 1 and 3. Contact with alcohol (or other organic solvents) can potentially alter the standardized dye concentration coated on the filter. This in turn may cause increased variability in diffusion times from sample to sample or study to study.

c) Pick up NeuroVue micro-strip with forceps and insert in tissue site.

d) Insert additional NeuroVue micro-strips as needed (Figure 2).

Note: In soft tissue, such as brain, the filter can be directly inserted with the sharp (tip) end first.

Note: In more rigid tissues, re-locate the landmark(s) noted during tissue preparation (Step 3) and insert the micro-strip, using its tip to assist in finding the incision.

Note: Do NOT use a dissecting needle to push filter into tissue. This can cause inaccurate placement and also inadvertent labeling of non-target fibers.

e) Place tissue with inserted micro-strip(s) in a closed jar with 4% buffered formaldehyde and incubate at 22°C – 37°C, monitoring periodically as described in Step 4 below, until neuronal profiles of interest are filled.

Note: Increasing incubation temperature decreases structural stability of fixed tissue but decreases diffusion times required for filling of neuronal profiles.

4. Monitoring Dye Diffusion

a) Estimation of diffusion times needed.

Diffusion times must generally be determined empirically for each laboratory’s neuronal profile(s) of interest since they depend on a number of variables:

i) Distance over which neuronal profile(s) are to be traced (Table 1).

ii) Dye concentration (standardized for each NeuroVue dye)

iii) Incubation temperature (increasing temperature decreases diffusion time but may reduce structural preservation; temperatures substantially above 37°C may reduce dye stability)

iv) Extent of tissue cross linking (higher fixative concentration and/or longer times in fixative will increase diffusion times).

Table 1. Approximate Diffusion Times for Filling of Murine Ear Projections to and from the Brain

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>Time @ 37°C</th>
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<tr>
<td>Embryonic day 11.5 (E11.5)</td>
<td>24 – 36h</td>
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<tr>
<td>Embryonic day 13.5 (E13.5)</td>
<td>48 – 60h</td>
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<tr>
<td>Embryonic day 16.5 (E16.5)</td>
<td>72 – 80h</td>
</tr>
<tr>
<td>Newborn (P0)</td>
<td>80 – 96h</td>
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</table>

Figure 2. Placement of NeuroVue® microstrips for multicolor neurotracing. Lateral view of murine head (embryonic day 12.5); with micro-strips placed to obtain central projections of NeuroVue Red labeled trigeminal nerve, NeuroVue Maroon labeled facial nerve and NeuroVue Jade labeled glossopharyngeal nerve. The eye is visible as a brown spot at left (anterior). Same magnification as in Figure 1 (~25X).

Figure 3. Monitoring diffusion distance using NeuroVue dye absorbance. After incubation for 36 h at 37°C, diffusion in all directions from the point of micro-strip insertion is readily visualized using a dissecting microscope (same specimen as in Figure 2; magnification ~25X).

b) Checking Diffusion Distance.
   i) Put on a pair of protective gloves.
   ii) Use a dissecting microscope to observe how far the dye front has traveled from the point of micro-strip insertion. All of the NeuroVue dyes absorb strongly in the visible: NeuroVue Jade appears yellow/orange, NeuroVue Red appears magenta/red and NeuroVue Maroon appears cyan/blue (see Figure 3).

Note: Fluorescence detection of NeuroVue dyes is much more sensitive than detection using absorbance. Thus, use of absorbance for monitoring (Figure 3) will underestimate the true extent of neuronal profile filling. If dye front extends to the final target region when observed using a dissecting microscope, diffusion time may have been too long.

Note: Dye concentrations high enough to give visible color may also cause absorption quenching of emitted fluorescence.

Note: Like other membrane dyes used for neurotracing, the NeuroVue dyes will also label muscle fibers or any other macroscopic structures surrounded by continuous lipid bilayers (Figure 4). Care must therefore be taken in Step 3 to insert the micro-strip in a location that maximizes labeling of target neurons and minimizes labeling of non-target structures.

5. Preparation of Labeled Tissue For Imaging

Note: For the following procedures, wear protective gloves. Agitate tools used for tissue manipulation in alcohol and air dry between steps to avoid dye cross contamination during the dissection process.

a) Whole mounts or surface preparations.
   (Figure 5) below illustrates the major steps involved in preparing and imaging whole brain mounts for the type of specimen shown in Figure 2. Use of such preparations in combination with confocal microscopy is the preferred method for imaging patterns of innervation in brain or peripheral structures such as ear, cochlea, etc. (see Figure 6).

   Step 1 Remove NeuroVue micro-strips.
   Step 2 Begin removal of midbrain by cutting through midbrain flexure.
   Step 3 Complete removal of midbrain by cutting between cerebellum and midbrain.
   Step 4 Make dorsal cut through choroid plexus of the hindbrain and cerebellum.
   Step 5 Make a lateral cut to remove ear and all cranial nerves.
   Final Result Ventral view of isolated brain, showing labeled nerve tracts.

Figure 5. Dissection of NeuroVue labeled murine brain (E12.5). Multicolor neurotracing was initiated by placement of NeuroVue Maroon, NeuroVue Red and NeuroVue Jade micro-strips as shown in Figure 2. After incubation in 4% formaldehyde in PBS for 36h at 37°C, the brain was prepared for mounting and confocal imaging as shown (magnification ~25X).

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b) Serial sections
Use of such preparations is essential when studying larger specimens (e.g., older stages of brain development) and provides additional information for complex peripheral structures such as the ear. Embedding in 15% gelatin, post-fixation for at least 48 h in 4-10% buffered formaldehyde, preparatio of serial vibratome sections and mounting in glycerol (Gurung & Fritsch, J Comp Neurol 479:309-327, 2004) is the preferred method of tissue preparation when whole mounts or surface mounts do not allow adequate visualization of tissue regions of interest.

Note: The following precautions have been found to minimize transcellular dye spreading and maximize ability to maintain good resolution of labeled fibers when preparing serial sections from gelatinembedded tissue:

- Prepare sections in chilled (4°C) buffer and keep them in chilled buffer until just before mounting.
- Image quality may be reduced if sections are mounted in glycerol and held at room temperature for longer than 1 hour before viewing. Therefore delays between mounting and imaging should be minimized by mounting and coverslipping only a limited number of sections (8-12) in 100% glycerol at any one time, and viewing or imaging them as soon as practical.
- If sections must be held in glycerol for longer than 1 hour, store at 4°C. Some image degradation will be seen in cut profiles at the tissue surface but overall image quality will be good even after overnight storage at 4°C.

Note: Frozen sections are less optimal due to greater dye leakage during the sectioning process (von Bartheld et al., J. Histochem Cytochem 38:725-733, 1990; Köbbert et al., Progr Neurobiol 62: 327-351, 2000).

Note: As with other membrane dyes used for neurotracing, paraffin embedding or use of organic solvents should be avoided when using the NeuroVue dyes. Such solvents extract membrane lipids and associated dyes, reducing or eliminating ability to detect labeled nerve fibers.

6. Imaging of Whole Mounts or Tissue Sections
As of September 2006, NeuroVue dyes are available that enable 3-5 color neuronal profiling in combination with commonly used genetic markers. (Table 2) briefly summarizes the spectral properties of the commercially available NeuroVue dyes. Full excitation and emission spectra, appropriate laser lines and filter combinations for confocal imaging, and suggested excitation-emission filter sets for epifluorescence imaging of each of these dye may be found in the product data sheets.

Which dyes are optimal depends on the instrument configuration available and the length of time required for diffusion from point of filter insertion to the region of interest (Table 3). For some studies with tissue which exhibits high levels of green/yellow autofluorescence background, it is recommended that the green channel be reserved for genetic markers and the longer wavelength channels for the NeuroVue dyes.

Table 2 - Spectral Characteristics of NeuroVue® Dyes

<table>
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<th>Product Number</th>
<th>Product Name</th>
<th>Excitation Maximum</th>
<th>Emission Maximum</th>
<th>Ext. coeff. (M⁻¹cm⁻¹)</th>
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<tr>
<td>24834</td>
<td>NeuroVue Maroon</td>
<td>647 nm</td>
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<td>NeuroVue Red</td>
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<td>NeuroVue Burgundy</td>
<td>683 nm</td>
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Table 3 - Selection of NeuroVue® Combinations for Multicolor Neurotracing

<table>
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<th>Type of Study</th>
<th>Dye Combination</th>
<th>Instrument Requirement</th>
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<tr>
<td>3 color neurotracing</td>
<td>NeuroVue® Jade, NeuroVue® Red, NeuroVue® Maroon</td>
<td>For standard epifluorescence microscopes and confocal systems having 488nm, 568nm and 633/647nm excitation</td>
</tr>
<tr>
<td>3 color neurotracing</td>
<td>NeuroVue® Jade, NeuroVue® Orange, NeuroVue® Maroon</td>
<td>For standard systems with 543nm excitation instead of 568nm</td>
</tr>
<tr>
<td>4 color neurotracing</td>
<td>NeuroVue® Orange, NeuroVue® Red, NeuroVue® Maroon NeuroVue® Burgundy</td>
<td>For standard systems with spectral detection. Color unmixing required for Orange/Red and Maroon/Burgundy combinations.</td>
</tr>
<tr>
<td>5 color neurotracing</td>
<td>NeuroVue® Jade, NeuroVue® Orange, NeuroVue® Red, NeuroVue® Maroon, NeuroVue® Burgundy</td>
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As shown in the examples below, the NeuroVue dyes can be used for neuroanatomical tracing at many levels of resolution, in combination with one another or with genetic markers (e.g., eGFP) specific to neuronal subtypes of interest.

**Figure 6** illustrates confocal imaging of facial and cranial nerve tracts using a whole brain mount.

Whole brain mount in glycerol (labeled as shown in Figure 2 and prepared as shown in Figure 5). Confocal images A and B at right were collected from regions indicated by boxes A and B, respectively.

Images were collected on a BioRad Radiance 2000 confocal system with 488 nm, 568 nm, 637 nm laser excitation; dichroic mirrors at 500 nm and 650 nm, and 515/40 nm BP; 600/40 nm BP; and 660 nm LP for NeuroVue Green, Red and Maroon, respectively.

**Figure 6 Whole brain mount (left) and central projections (panels A and B) in E12.5 murine embryo.** Vagus, trigeminal and facial / VIII / glossopharyngeal nerves were labeled with NeuroVue Jade, NeuroVue Red and NeuroVue Maroon, respectively.

**Figure 7** illustrates confocal imaging of innervation in the murine inner ear using a surface preparation of the cochlea. Note that even very thin Type II inner hair cells (IHC), which are normally quite difficult to visualize, are clearly visible at the single fiber level (right panel).

Specimen #1 (left panel; scale bar = 2 mm) was labeled by insertion of NeuroVue Red in the modiolus (see Figure 8), resulting in strong labeling of all cochlear nerve fibers. For Specimen #2 (center and right panels), no labeling was done in the modiolus but micro-strips of NeuroVue Red or NeuroVue Maroon were inserted apically at the locations indicated schematically in the left panel (red and green rectangles, respectively). This allowed labeling of much smaller numbers of fibers, allowing clear visualization of individual type II afferent fibers (red) and branches of efferent fibers (green) to IHC and OHC, both at intermediate power (center panel; scale bar = 100µm) and high power (right panel; scale bar =10µm). Surface cochlear preparations from 1 week old (P7) mice were labeled as described; all dye incubations were carried out in 4% formaldehyde in PBS for 48h at 37°C.
Figure 8 illustrates confocal imaging of the interactions between radial fibers in the murine inner ear and supporting cells in the organ of Corti, again using a surface preparation of the cochlea.

![Organ of Corti, Radial fibers, Spiral ganglion, Modiolus](image1)

**Low power (scale bar =100µm)**

**High power (scale bar =10µm)**

Figure 8. Combined labeling with eGFP (green pseudocolor) and NeuroVue Red (red pseudocolor) allows high resolution imaging of nerve fibers and transgenically labeled cells in the inner ear. (Morris et al., Brain Res 1091:186-199 2006).

**Left panel:** Z-axis collapse of confocally imaged NeuroVue Red labeled fibers (red pseudocolor) overlaid with a single optical section of PLPeGFP labeled cells (green pseudocolor). Note that both the Schwann cells, which surround the spiral ganglion cells, and supporting cells in the organ of Corti are PLP-eGFP positive, and therefore appear yellow in this collapsed image. **Right Panel:** Individual NeuroVue Red labeled fibers are readily seen in the apex as they grow out between the eGFP-labeled supporting cells.

Figure 9 illustrates the use of two color neurotracing to optically dissect anatomically overlapping fibers.

![Figure 9](image2)

Figure 9. Double labeling with NeuroVue Maroon (A, green pseudocolor) inserted in the utricle and NeuroVue Red (B, red pseudocolor) inserted in the cochlea allows visualization of overlapping cell and fiber distribution (C) in the brainstem of E18.5 mouse hindbrain.

After incubation for 96h at 37°C and embedding in 15% gelatin, a 100 µm thick vibratome section was cut, mounted in glycerol, and imaged using the Zeiss LSM 510 Meta confocal system, with laser excitation at 543 nm or 633 nm and emission at 565 - 615 nm or 650 - 710 nm for NeuroVue Red or NeuroVue Maroon, respectively. Each dye allowed imaging to the single cell or fiber level, with no overlap in emissions between the two channels (panels A and B). Optically combining both channels (panel C) shows that vestibular nucleus afferent fibers were labeled only by NeuroVue Maroon and cochlear nucleus afferent fibers only by NeuroVue Red, whereas overlapping fibers of both types (yellow pseudocolor) are found in the cochlear-vestibular efferent bundle. Scale bar = 100 µm.
For further examples of how the NeuroVue dyes have been used to trace neuronal interactions in a wide variety of systems, see the NeuroVue Bibliography below.

**References Cited**


**NeuroVue® Bibliography**


de Caprona MD, Beisel KW, Nichols DH, Fritzsch B. 2004. Partial behavioral compensation is revealed in balance taskd mutant mice lacking otocina. Brain Res Bull 64:289-301. Both NeuroVue Maroon (previously PTIR271) and NeuroVue Red (previously PTIR278) were used in Figure 8 (B. Fritzsch, personal communication).

Fritzsch B. 2003. Development of inner ear afferent connections: forming primary neurons and connecting them to the developing sensory epithelia. Brain Res Bull 60:423-433. NeuroVue Maroon (previously PTIR271) and NeuroVue Green (previously PTIR281) were used in Figure 4a, 4c, and 4d; NeuroVue Maroon and PKH26 were used for cover image (B. Fritzsch, personal communication).

Fritzsch, B, Tessorollo L, Coppola E, Reichardt LF. 2004. Neurotrophins in the ear: their roles in sensory neuron survival and fiber guidance. Prog Brain Res 146:265-278. NeuroVue Maroon (formerly PTIR271) and DiI were used in Figure 2 (B. Fritzsch, personal communication).


Fritzsch B, Jackson Lab Presentation, 2005: http://www.biomedsci.creighton.edu/facilities/nccb/media/Jackson_lab_presentation.ppt NeuroVue Maroon (formerly PTIR271); NeuroVue Red (previously PTIR278); NeuroVue Maroon (previously PTIR278).


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<td>24835</td>
<td>NeuroVue® Red Filter Square For Neuronal Tract Tracing</td>
<td>1 filter</td>
</tr>
<tr>
<td>24836</td>
<td>NeuroVue® Orange Filter Square For Neuronal Tract Tracing</td>
<td>1 filter</td>
</tr>
<tr>
<td>24837</td>
<td>NeuroVue® Jade Filter Square For Neuronal Tract Tracing</td>
<td>1 filter</td>
</tr>
<tr>
<td>24838</td>
<td>NeuroVue® Burgundy Filter Square For Neuronal Tract Tracing</td>
<td>1 filter</td>
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</table>

Ordering Information

<table>
<thead>
<tr>
<th>Cat. #</th>
<th>Description</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>24839</td>
<td>Vannas scissors, super fine</td>
<td>1 pair</td>
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<tr>
<td>19636</td>
<td>PolyFreeze (Frozen Mounting Medium) 6x120ml</td>
<td>1 Case</td>
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<tr>
<td>18606</td>
<td>Aqua-Poly/Mount</td>
<td>20ml, 100ml, 5x20ml</td>
</tr>
</tbody>
</table>

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