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Protocol NT001:
Neuronal Tract Tracing in Fixed Tissue Using NeuroVue® Dye-Coated Filters

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 (*http://www.mtarget.com/product/neurovue.aspx*).
- Neuronal connections can be studied in embryos lacking receptors needed for neuronal identification as well as in juveniles and adults (*Gurung & Fritzsch, J Comp Neurol 479:309-327, 2004; Morris et al., Brain Res 1091:186-199, 2006; Hsieh & Cramer, J Comp Neurol 497:589-599, 2006*).
- 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 Bull 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

Item	Micro-strip Preparation	Specimen Fixation	Labeling/ Diffusion Monitoring	Tissue Mounting
NeuroVue Filter square (1cm x 1cm)	X			
Protective gloves	X	X	X	X
Microscissors*	X			
Forceps	X	X	X	X
95% ethyl alcohol	X	X		
Dissecting microscope	X		X	
4% formaldehyde#; pH 7.4 in phosphate buffered saline (PBS)		X	X	
Refrigerator or cold room (4 °C.)		X		
NeuroVue Micro-strips (see Step 1, page 2)			X	
Screw-cap vials for specimen storage/incubation		X	X	
37 °C incubator or oven			X	
15% gelatin in PBS (or other non-paraffin embedding material)				X
Glycerol (or other mounting medium free of organic solvents)				X
Microscope slides and coverslips				X
Fluorescence microscope with confocal and/or epi-illumination)				

* World Precision Instruments #500086, (http://store.wpiinc.com) or equivalent

\# Prepared from ultrapure formaldehyde solution (e.g., Polysciences, #04018, www.polysciences.com/shop/ or equivalent) or reagent grade paraformaldenyde powder (e.g., Sigma P-6148, www.sigmaaldrich.com or equivalent).

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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 below.

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.

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.

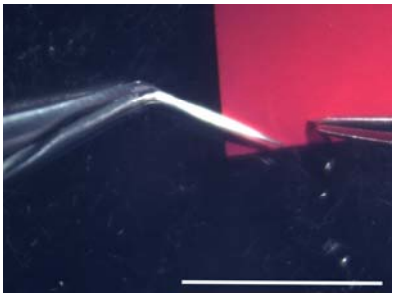


Figure 1. Preparation of NeuroVue Red micro-strips for use in tissue labeling. Using a dissecting microscope, microscissors (at left) are used to cut small triangles from a 1cm x 1 cm coated filter square (Product #FS-1002). Scale bar = 2 mm (~25X magnification).

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;** to view details, enlarge image electronically).

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 catalog #04018) or paraformaldehyde.

c) Store fixed specimen at 4^{o}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.

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.*

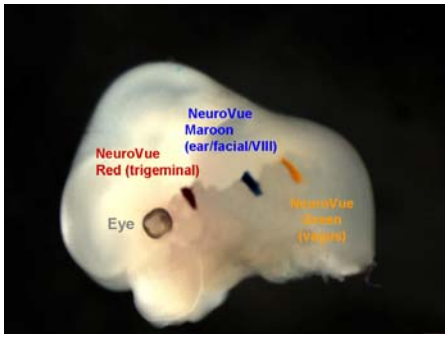


Figure 2. Placement of NeuroVue micro-strips for multicolor neurotracing. Lateral view of murine head (embryonic day 12.5); with micro-strips placed to obtain central projections of NeuroVue Red labeled trigeminal nerve, NeuroVue Maroon labeled facial nerve and NeuroVue Green labeled glossopharyngeal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

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

Developmental Stage	Time @ 37°C.
Embryonic day 11.5 (E11.5)	24 – 36h
Embryonic day 13.5 (E13.5)	48 – 60h
Embryonic day 16.5 (E16.5)	72 – 80h
Newborn (P0)	80 – 96h

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 Green/ Emerald/Jade appear yellow/orange, NeuroVue Red appears magenta/red and NeuroVue Maroon appears cyan/blue (**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.

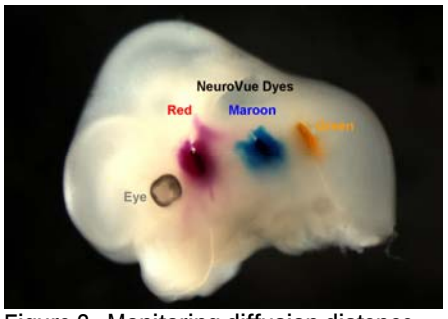


Figure 3. Monitoring diffusion distance using NeuroVue dye absorbance. After incubation for 36 h at 37°C, diffusion in all directions from the point of micro-strip insertion is readily visualized using a dissecting microscope (same specimen as in Figure 2; magnification ~25X).

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.*

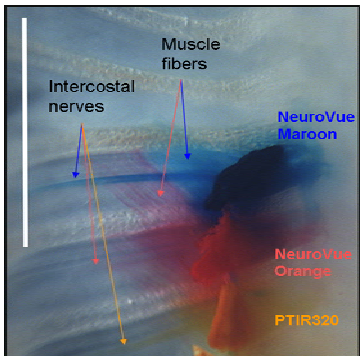


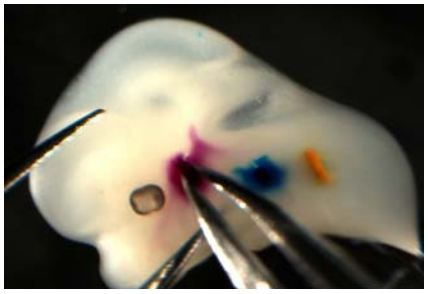
Figure 4. Monitoring proper placement of NeuroVue micro-strips. Correct insertion in murine spinal cord labels only intercostal nerves after incubation for 10 days at 37°C (not shown). Suboptimal insertion can also label intercostal muscle fibers to varying extents (here, strong labeling for NeuroVue Orange, moderate for NeuroVue Maroon). Scale bar = 2 mm.

5. Preparation of Labeled Tissue For Imaging

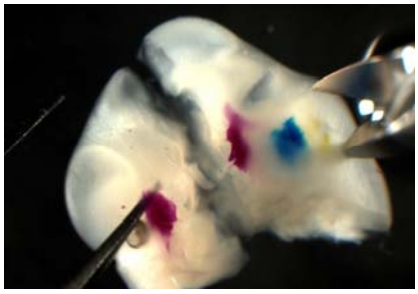
Note: For the following procedures, wear protective gloves. Agitate tools used for tissue manipulation in alcohol and air dry between steps to avoid dye cross contamination during the dissection process.

a) Whole mounts or surface preparations

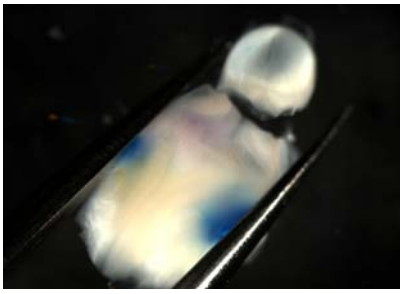
Figure 5 below illustrates the major steps involved in preparing and imaging whole brain mounts for the type of specimen shown in Figure 2. Use of such preparations in combination with confocal microscopy is the preferred method for imaging patterns of innervation in brain or peripheral structures such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



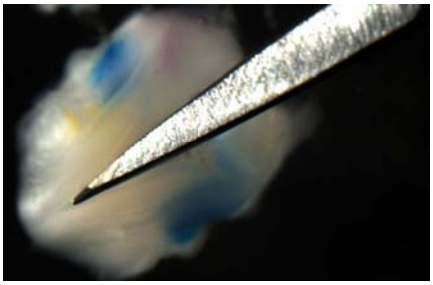
Step 1. Remove NeuroVue micro-strips.



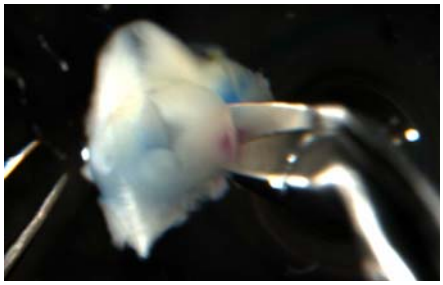
Step 2. Begin removal of midbrain by cutting through midbrain flexure.



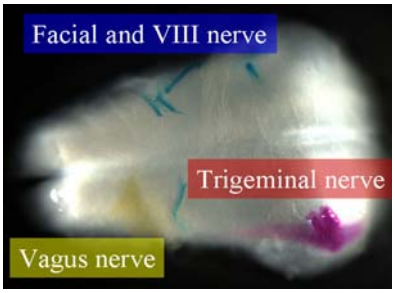
Step 3. Complete removal of midbrain by cutting between cerebellum and midbrain.



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 Green 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).

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, preparation

of serial vibratome sections and mounting in glycerol (*Gurung & Fritzsch, 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 gelatin-embedded 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 January 2008, 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 individual 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.

*Note: **Due their very long red fluorescence emissions, most people cannot see NeuroVue Maroon or NeuroVue Burgundy emissions by eye.** Detection by camera will be more sensitive than with the unaided eye.*

Table 2. Spectral Characteristics of NeuroVue® Dyes

Product Number	*Product Name*	*Excitation maximum*	*Emission Maximum*	*Ext. coeff. ($M^{-1}cm^{-1}$)*
FS-1001	NeuroVue Maroon	647 nm	667 nm	222,368
FS-1002	NeuroVue Red	567 nm	588 nm	120,000
FS-1003	NeuroVue Orange	550 nm	570 nm	139,800
FS1005	NeuroVue Burgundy	683 nm	707 nm	192,832
FS-1006	NeuroVue Jade	478 nm	508 nm	87,850

Table 3. Selection of NeuroVue® Combinations for Multicolor Neurotracing

Type of Study	Dye Combination	Instrument requirement
3 color neurotracing Medium term (≤ 5 days)	NeuroVue Jade NeuroVueRed, NeuroVue Maroon	For standard epifluorescence microscopes and confocal systems having 488nm, 568nm and 633/647nm excitation
3 color neurotracing Medium term (≤ 5 days)	NeuroVue Jade, NeuroVue Orange NeuroVue Maroon	For standard systems with 543nm excitation instead of 568nm
4 color neurotracing Long term studies (3-4 wk)[1]	NeuroVue Orange, NeuroVue Red NeuroVue Maroon NeuroVue Burgundy	For systems with spectral detection. Color unmixing required for Orange/Red and Maroon/Burgundy combinations
5 color neurotracing Medium term (≤5 days)	NeuroVue Jade NeuroVue Orange, NeuroVue Red NeuroVue Maroon NeuroVue Burgundy	

[1] Compatible with green fluorescent genetic markers such as eGFP or photoactivated X-Gal reaction product BCI [*Matei, Brain Res Bull 70:33-43, 2006*])

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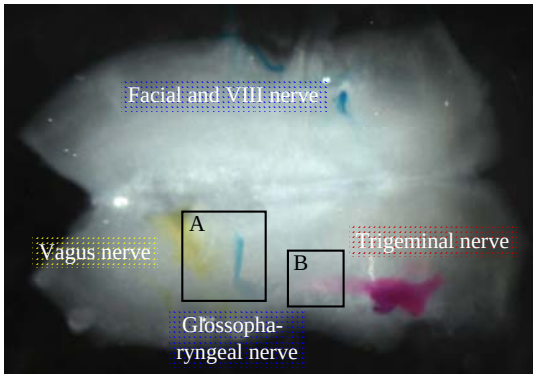
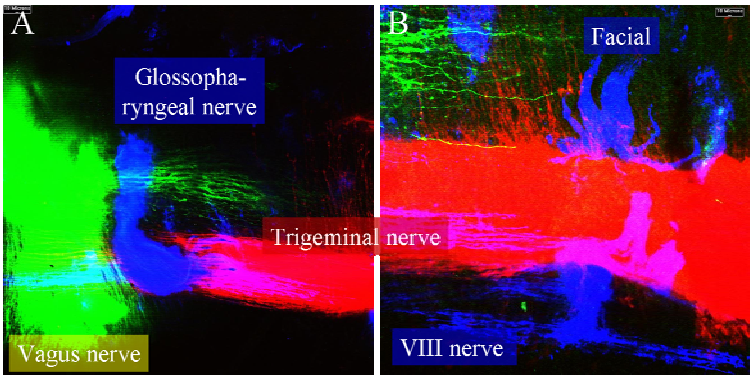
Note: New NeuroVue family members are also in development at MTTI. If you have special needs that are not met by the products listed in Table 2, please contact us via phone, fax or e-mail to inquire.

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.



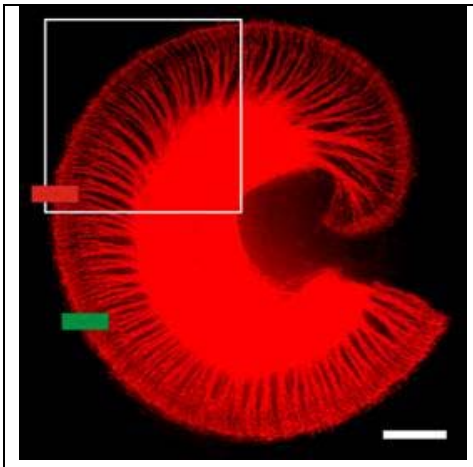
Whole brain mount in glycerol (labeled as shown in Figure 2 and prepared as shown in Figure 5). Confocal images A and B at right were collected from regions indicated by boxes A and B, respectively

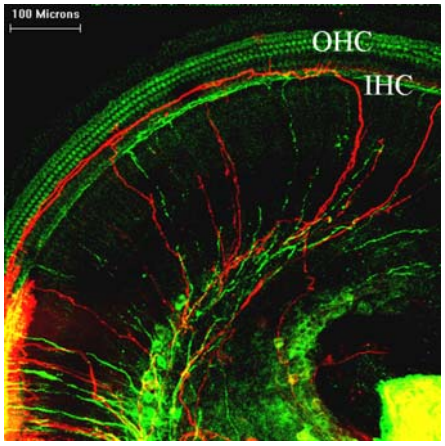


Images were collected on a BioRad Radiance 2000 confocal system with 488 nm, 568 nm, 637 nm laser excitation; dichroic mirrors at 500 nm and 650 nm, and 515/40 nmBP; 600/40 nm BP ; and 660 nm LP for NeuroVue Green, Red and Maroon, respectively.

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 Green, 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).





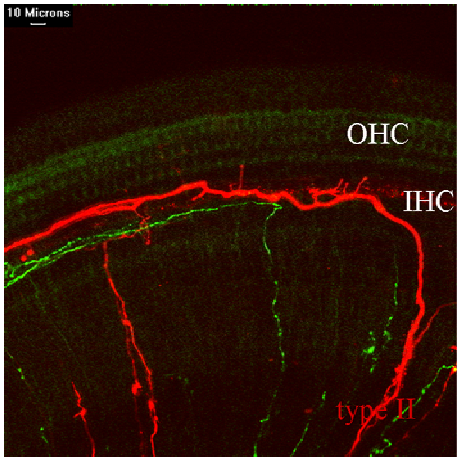
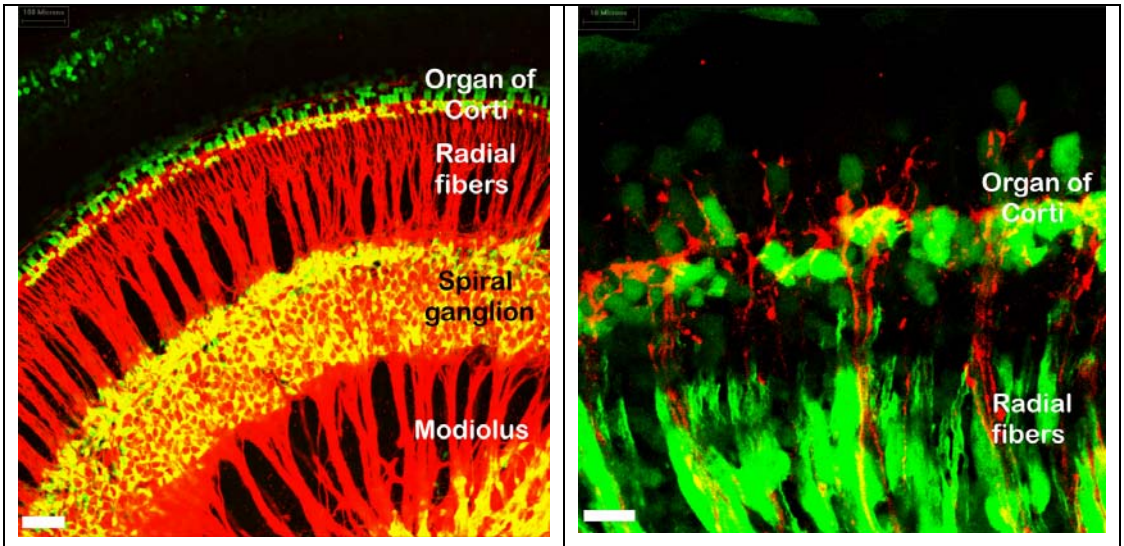


Figure 7. Low, medium and high resolution imaging of afferent and efferent fibers to outer and inner hair cells (OHC, IHC) in murine inner ear after dual labeling with NeuroVue Red (red pseudocolor) and NeuroVue Maroon (green pseudocolor). Specimen #1 (left panel; scale bar = 2 mm) was labeled by insertion 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 = 100 μm) and high power (right panel; scale bar =10 μm). 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.



Low power (scale bar =100 μm) | High power (scale bar =10 μm)

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 PLP-eGFP 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.

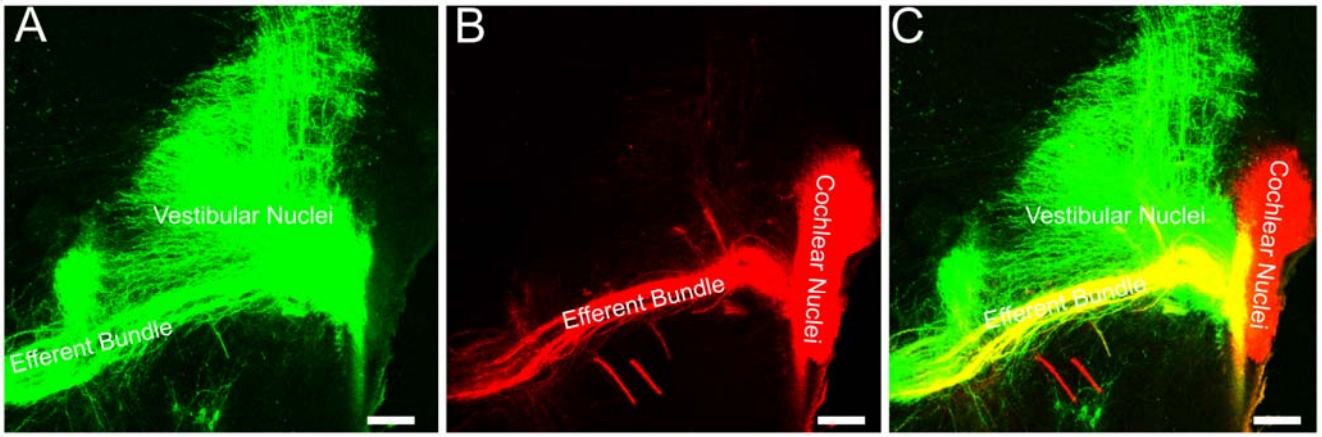


Figure 9. Double labeling with NeuroVue Maroon (A, green pseudocolor) inserted in the utricle and NeuroVue Red (B, red pseudocolor) inserted in the cochlea allows visualization of overlapping cell and fiber distribution (C) in the brainstem of E18.5 mouse hindbrain. After incubation for 96h at 37°C and 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, watch for emerging publications on our website (www.mtarget.com), and send us yours to add to the list!

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