Photo- and bio-physical characterization of novel violet and near-infrared lipophilic fluorophores for neuronal tracing

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Summary

Lipophilic fluorescent dyes have been used to trace neuronal connections because of their ability to diffuse laterally within nerve cell membranes. Given the hundreds to thousands of connections that a typical neuron makes with its neighbours, a diffusion-matched set of spectrally distinct dyes is desirable. To extend a set of these dyes to obtain six independent labels, we have characterized the properties of novel violet and near-infrared candidates. By combining two-photon and confocal microscopy all of these candidates can be imaged using a single Titanium Sapphire laser. Here we present measurements of the two-photon action cross-sections and diffusion properties of the dyes, using either the relative diffusion distance or fluorescence recovery after photobleaching techniques, and demonstrate six-colour neuronal tracing within the spinal cord and brain tissue.

Introduction

Neurons are cells with highly branched processes of variable length (micrometres to metres) that form an interwoven network of processes and contacts. Since the discovery of the Golgi technique, labelling single cells has been the hallmark of neuroanatomy, currently revived with the invention of ‘brainbow’ (Livet et al., 2007). Despite the superior visibility of these whole label single cell approaches, the time consuming breeding of transgenic mice in addition with the ‘brainbow’ reporter system makes these techniques of limited use for many rare combinations of mutant mice currently being generated using sophisticated combinations of genetic engineering (Tian et al., 2006). In addition, although breathtaking in their clarity, single cell labelling does not provide the often needed information about the projection patterns of distinct subsets of neurons to establish, for example, topology of cochlear projections to the cochlear nuclei or distribution of all motoneurons to a given muscle. These limitations had in the past led to the decline of the Golgi technique and the rise of tracing techniques such as horseradish peroxidase, dextran amines and lipophilic dyes (Godement et al., 1987; Fritzsch, 1993; Glover, 1995; Fritzsch et al., 2005). In particular, fluorescence imaging of lipophilic dyes with the potential to combine three distinct colours to analyse the topology of connections has been the technique of choice for embryonic analysis in many vertebrates. In fact, the use of lipophilic dye diffusion in embryonic tissue has proven to be essential for the analysis of the hundreds of mutant mice that have early lethality. However, given the task to decipher millions of connections in trillions of neurons in a given mouse, the three colour system we previously developed (Jensen-Smith et al., 2007) and multicolour systems developed by others (Livet et al., 2007) fall short of the needed multicolour resolution.

Previously, a trio of NeuroVue® (NV) dyes was developed with well-separated green, red and far red fluorescence emission that permitted easy and fully segregated triple labelling (Jensen-Smith et al., 2007) and multicolour systems developed by others (Livet et al., 2007) fall short of the needed multicolour resolution.

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filter sets that allow, at present, six-colour images to be produced through combinations of single- and two-photon excitation. To determine how well the candidate neurotracers performed in comparison to the existing probes, we have characterized the diffusive transport of these compounds in the peripheral nerves of fixed murine spinal cord tissue. Finally, we demonstrate six-colour neuroimaging in the brain and intercostal preparations.

Materials and methods

Lipophilic dyes

NeuroVue Red (NVR), Maroon (NVM), Orange (NVO), Jade (NVJ), Burgundy (NVB), PTIR326, PTIR327, PTIR301, PTIR334, PTIR336 and MTTI129 were provided as 1 mM solutions in ethanol and also as dye coated filters (at concentrations of 11–18 nmol mm\(^{-2}\)) by Molecular Targeting Technologies, Inc. (West Chester, PA, U.S.A.) holding proprietary rights to novel fluorescence-based technologies from PTI Research, Inc (Exton, PA). The chemical structures of the fluorescent head groups of the candidate dyes are shown in Fig. 1.

\[
\begin{align*}
\text{PTIR334} & \quad \text{PTIR336} \\
\text{PTIR326} & \quad \text{PTIR327} \\
\text{PTIR301 and MTTI129} & 
\end{align*}
\]

Fig. 1. Chemical structures of the head groups developed for candidate NV dyes. The substituent X, varies for each candidate dye. The asymmetric hydrocarbon chains R and R1 were the same for all candidate dyes and had a total of 36 carbons except for MTTI129, which has symmetrical hydrocarbon chains and a total of 32 carbons. All structures were confirmed by NMR and mass spectroscopy. Structures of the commercialized NV dyes are not shown, but these dyes can be obtained from Molecular Targeting Technologies, Inc.

Preparation of tissue

Mice used for this study were E12.5, E18.5 or P0 when they were euthanized. The mothers were injected with Avertin intraperitoneally for the purpose of anesthetizing the embryos, and the newborn were directly anesthetized with Avertin. The embryos were perfused immediately following cervical dislocation of the mother. Both newborns and embryos were perfused with 4% paraformaldehyde (PFA) in the left ventricle using a peristaltic pump with glass capillaries glass capillaries or 27 gauge needles. After perfusing for 1 min, the dead mice were placed in 4% PFA and stored at 4°C from 1 week to 9 months. All breeding and euthanasia were conducted using procedures approved by the Creighton University IACUC (protocol #0630.1) and University of Iowa ACURF (approval #86198). All mice used for the studies discussed here were carcasses from wild-type mice generated in the breeding of various mutant lines and thus did not result in any specific breeding or euthanasia.

Intercostal nerve labelling

The intercostal nerves were prepared by completely removing all internal organs and tissue in the thoracic and abdominal cavities. A small incision was made with microscissors using an anterior approach through two adjacent ribs and intercostal muscles. The incision was parallel to and near the spinal cord, running the length of two to three ribs. A rectangular piece of dye labelled filter strip was cut to size and placed into the incision and held in place by the cut ribs to keep the label adjacent to the intercostal nerves. After insertion of the filter paper, the preparation was placed into 4% PFA and incubated at 36–40°C for 24, 48, 64.5, 72 or 96 h. Because nerves are the only continuous lipid layers, they will be preferentially filled much like a wick.

Dissection and mounting

After the appropriate incubation time all tissue posterior to the injected intercostal spaces was dissected away including skin, superficial and deep back muscles. The labelled intercostal nerves were removed along with one unlabelled intercostal space to be used as a control. The preparation was then mounted on a glass slide with glycerol and a glass cover slip placed on top. Generally the slide was imaged immediately following dissection, but if this could not be done it was kept at 4°C until it was imaged. With this approach the preparation was stable for approximately 24 h.

Two-photon cross-section measurements

Absolute two-photon action cross-sections of commercial and candidate NV fluorescence probes were determined through a comparison to fluorescein, a well-known standard (Xu &
Webb, 1996). Lucifer Yellow was measured in parallel with the NV probes, and was used as an internal control (Xu et al., 1996). Each dye was prepared in either ethanol or water, and placed in a sealed quartz cuvette (Starna Cells, Atascadero, CA, U.S.A.). The cuvette was placed at the focus of a 10×/0.25 NA microscope objective, and the sample was excited at wavelengths ranging from 720–1000 nm using the modelocked pulse train of a femtosecond Titanium Sapphire (Ti:S) laser (Chameleon XR, Coherent, Inc., Santa Clara, CA, U.S.A.) (Fig. 2). The excitation beam was directed into the objective using a 700 nm short pass dichroic (700 DCSP, Chroma Technology Corp., Rockingham, VT, U.S.A.) The resulting fluorescence was collected using the same objective, passed back through the dichroic and was filtered using a BG22 blue-glass filter before being detected by a photomultiplier tube (PMT) (Hamamatsu Corp., Bridgewater, NJ, U.S.A.). Photons arriving at the PMT were counted using a SR400 (Stanford Research Systems, Inc., Sunnyvale, CA, U.S.A.). The cuvette was placed at the focus of a 10×/0.25 NA microscope objective, and the sample was excited at wavelengths ranging from 720–1000 nm using the modelocked pulse train of a femtosecond Titanium Sapphire (Ti:S) laser (Chameleon XR, Coherent, Inc., Santa Clara, CA, U.S.A.) (Fig. 2). The excitation beam was directed into the objective using a 700 nm short pass dichroic (700 DCSP, Chroma Technology Corp., Rockingham, VT, U.S.A.) The resulting fluorescence was collected using the same objective, passed back through the dichroic and was filtered using a BG22 blue-glass filter before being detected by a photomultiplier tube (PMT) (Hamamatsu Corp., Bridgewater, NJ, U.S.A.). Photons arriving at the PMT were counted using a SR400 (Stanford Research Systems, Inc., Sunnyvale, CA, U.S.A.). Photons arriving at the PMT were counted using a SR400 (Stanford Research Systems, Inc., Sunnyvale, CA, U.S.A.) photon counter controlled by a LabView® program, which was also used to simultaneously measure the average excitation laser power on a laser power meter. For each dye, the mean count rate of three trials was determined as a function of excitation laser power, adjusted by attenuation with a neutral density filter wheel.

To eliminate the need for accurate spatio-temporal measurements of the excitation pulse train, we employed a known two-photon standard and ratiometric analysis. The action cross section of the unknown dye (U), relative to the known (K) was determined from Eq. 1.

\[
(\eta \sigma)_U = \eta_K \sigma_K \left( \phi_K \phi_U \right) \left( n_K n_U \right) \left( C_K C_U \right) \left( D_K D_U \right),
\]

where \( \eta \) is the fluorescence quantum efficiency, \( \sigma \) is the two-photon excitation cross-section, measured in Goppert–Mayers (GM, 1 GM = 10⁻⁵⁰ cm² s), \( \phi \) is the fluorescence detection efficiency, \( C \) is the dye concentration, \( n \) is the index of refraction of the solvent at the excitation wavelength, and \( D \) is the quadratic rate of increase of fluorescence with laser power. The quadratic coefficients were obtained by fitting the detected fluorescence intensity to a polynomial of the average laser power using the Levenberg–Marquardt algorithm. Generally, the data were well fit by a single quadratic term, although for some dyes and at some wavelengths, additional linear, cubic and quartic terms were justified according to an F-test. The concentrations were measured spectrophotometrically, using known literature values of the molar extinction coefficients. Because the fluorescence emission spectrum of each dye varied considerably, the fluorescence detection efficiency (ratio of the number of detected and emitted fluorescence photons) had to be measured for each dye. This was calculated from Eq. 2

\[
\varphi_{\text{dye}} = \frac{\int F(\lambda) \varphi(\lambda) d\lambda}{\int F(\lambda) d\lambda \int \varphi(\lambda) d\lambda},
\]

where \( F(\lambda) \) is the fluorescence emission spectrum of the dye molecule and \( \varphi \) is the detection efficiency of the system. The detection efficiency was calculated from the ratio of the detected photon count rate to the incident photon count rate when a stable, tunable, narrowband (10 nm) beam of light was passed through the collection optics (10 nm) beam of light was passed through the collection optics in Fig. 2. To produce this light source, emission from an air-cooled quartz-tungsten-halogen lamp was passed through a home-built Czerny–Turner monochromator, fibre-optic coupled and then collimated for delivery.

Confocal single- and multiphoton imaging

All of the samples were imaged on a Zeiss LSM 510 META microscope at the Creighton University Integrated Biological Imaging Facility (CU-IBIF) or with a Leica TCS SP5 at the University of Iowa, Department of Biology Roy J. Carver Center for Imaging. Three objectives were used during the course of data acquisition: the Plan Neofluar 40× oil 1.3 NA DIC, Plan Neo 20× 0.75 NA DIC and the Plan Apo 10× 0.4 NA. The wavelength, excitation mechanism and the filter settings that were used to image each dye are listed in Tables 1–4. Images of each dye in a multiply labelled sample were acquired sequentially with optimized excitation wavelength and emission filtering.

Using the z-stack setting of the Zeiss LSM or Leica confocal software, images of the labelled nerves were acquired at several heights throughout the axial thickness. This collection of images was then collapsed to a two-dimensional projection of the labelled nerve. In order to construct a complete composite of each labelled nerve, from the incision site to a point in the background (past the attenuated fluorescent signal), it was often necessary to acquire several z-stack projections; these projections were later stitched together in software.

Confocal images were also taken with the Leica TCS SP5 system using acousto-optical filters to tune the emission bandpass. The Leica system was also used for multicolour unmixing. Briefly, individual emission profiles were generated for each of the six dyes within the tissue and the overlapping
Table 1. Summary of peak one- and two-photon excitation wavelengths and emission wavelengths of NV dyes and candidates. Uncertainties in the least significant digits are given in parentheses.

<table>
<thead>
<tr>
<th>Dye</th>
<th>1-Photon exc. Wavelength peak (nm)</th>
<th>ε [M−1 cm−1]</th>
<th>2-Photon exc. wavelength peak (nm)</th>
<th>ησ (2)</th>
<th>[GM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lucifer Yellow</td>
<td>428</td>
<td>12,000</td>
<td>480</td>
<td>0.94(6)</td>
<td>12,000</td>
</tr>
<tr>
<td>334</td>
<td>440</td>
<td>51,600</td>
<td>550</td>
<td>0.183(4)</td>
<td>51,600</td>
</tr>
<tr>
<td>336</td>
<td>412</td>
<td>20,100</td>
<td>570</td>
<td>0.056(7)</td>
<td>20,100</td>
</tr>
<tr>
<td>NVJ</td>
<td>480</td>
<td>85,525</td>
<td>720</td>
<td>2.0(2)</td>
<td>85,525</td>
</tr>
<tr>
<td>NVO</td>
<td>550</td>
<td>139,800</td>
<td>760</td>
<td>258(30)</td>
<td>139,800</td>
</tr>
<tr>
<td>NVR</td>
<td>570</td>
<td>113,450</td>
<td>1040</td>
<td>28.3(6)</td>
<td>113,450</td>
</tr>
<tr>
<td>NVM</td>
<td>674</td>
<td>216,700</td>
<td>14.6(1.2)</td>
<td>468(52)</td>
<td>216,700</td>
</tr>
<tr>
<td>NVB</td>
<td>685</td>
<td>199,200</td>
<td></td>
<td></td>
<td>199,200</td>
</tr>
<tr>
<td>301</td>
<td>772</td>
<td>199,300</td>
<td></td>
<td></td>
<td>199,300</td>
</tr>
</tbody>
</table>

*Not measured.

Table 2. Imaging filter sets used for diffusion measurements of NV dyes and candidates in fixed spinal cord preparations.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Excitation mechanism</th>
<th>λ_ex (nm)</th>
<th>Emission bandpass (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>334</td>
<td>Two-photon</td>
<td>860</td>
<td>480–520</td>
</tr>
<tr>
<td>336</td>
<td>Two-photon</td>
<td>900</td>
<td>480–520</td>
</tr>
<tr>
<td>327</td>
<td>Single-photon</td>
<td>458</td>
<td>480–520</td>
</tr>
<tr>
<td>326</td>
<td>Single-photon</td>
<td>488</td>
<td>500–550</td>
</tr>
<tr>
<td>NVJ</td>
<td>Single-photon</td>
<td>488</td>
<td>500–550</td>
</tr>
<tr>
<td>NVR</td>
<td>Single-photon</td>
<td>543</td>
<td>565–615</td>
</tr>
<tr>
<td>NVM</td>
<td>Single-photon</td>
<td>633</td>
<td>650–710</td>
</tr>
<tr>
<td>301</td>
<td>Single-photon</td>
<td>720</td>
<td>755–815</td>
</tr>
</tbody>
</table>

Table 3. Imaging filter sets used for six-colour imaging of NV dyes and candidates (Fig. 10).

<table>
<thead>
<tr>
<th>Dye</th>
<th>Excitation mechanism</th>
<th>λ_ex (nm)</th>
<th>Emission bandpass (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>334</td>
<td>Two-photon</td>
<td>820</td>
<td>430–480</td>
</tr>
<tr>
<td>NVJ</td>
<td>Two-photon</td>
<td>940</td>
<td>500–540</td>
</tr>
<tr>
<td>NVO</td>
<td>Two-photon</td>
<td>720</td>
<td>535–590</td>
</tr>
<tr>
<td>NVR</td>
<td>Two-photon</td>
<td>820</td>
<td>650–710</td>
</tr>
<tr>
<td>NVM</td>
<td>Single-photon</td>
<td>633</td>
<td>650–710</td>
</tr>
<tr>
<td>301</td>
<td>Single-photon</td>
<td>720</td>
<td>755–815</td>
</tr>
</tbody>
</table>

Table 4. Imaging filter sets used for six-colour imaging of NV dyes and candidates (Figs 11 and 12).

<table>
<thead>
<tr>
<th>Dye</th>
<th>Excitation mechanism</th>
<th>λ_ex (nm)</th>
<th>Emission bandpass (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>334</td>
<td>Single-photon</td>
<td>458</td>
<td>465–485</td>
</tr>
<tr>
<td>NVJ</td>
<td>Single-photon</td>
<td>488</td>
<td>520–550</td>
</tr>
<tr>
<td>NVR</td>
<td>Two-photon</td>
<td>800</td>
<td>565–605</td>
</tr>
<tr>
<td>NVO</td>
<td>Single-photon</td>
<td>543</td>
<td>550–570</td>
</tr>
<tr>
<td>NVM</td>
<td>Single-photon</td>
<td>633</td>
<td>646–675</td>
</tr>
<tr>
<td>NVB</td>
<td>Single-photon</td>
<td>633</td>
<td>725–745</td>
</tr>
</tbody>
</table>

spectra in the composite tissue profile were subsequently subtracted to isolate the fluorescence signature of each dye.

Brain and embryo nerve labelling

Forebrains of juvenile and adult mice were used to extend the peripheral nerve study. These brains, like all other tissue used in this study, were from mice euthanized for other purposes. To demonstrate the ease of handling of the established protocol and to show the usefulness of six-colour labelling in a single brain, we implanted six NV dyes into three cranial nerves on either side of an E12.5 wild-type mouse embryo that had been perfused as part of a litter of timed mouse breeding and kept in 4% PFA. Small pieces of dye coated filters were cut and placed in different locations to label distinct cranial nerves: NVO was placed posterior to the left eye to label the trigeminal nerve; NVB was placed anterior to the right ear labelling the facial nerve and a portion of the vestibulocochlear nerve; PTIR334 was placed ventral to the ear at the internal carotid artery to label the glossopharyngal and vagus nerves; NVM was placed in the right posterior-inferior orbit to label the maxillary division of the trigeminal nerve; NVJ was placed just posterior to the lower right jaw to label the mandibular division of the trigeminal nerve; NVR was placed anterior to the right inner ear to label the facial nerve. The preparation was then placed into 4% PFA at 60°C for 24 h. After that time images of the preparation were taken with a Leica M205 FA microscope using a Leica DFC 420 camera, conventional epifluorescence and Leica Application Suite software. The hindbrain was then removed, placed dorsal side up and imaged with conventional epifluorescence as before. This allowed the visualization of cranial nerve nuclei that were labelled with lipophilic dye. The hindbrain was then mounted on a slide with glycerol and a cover slip was placed on top. It was then imaged on a Leica TCS SP5 confocal microscope using the settings in Table 4 allowing for complete discrimination of all six dyes.

Distance measurements

For a consistent measure of the diffusion distance, the labelled nerve tract was measured from the incision point to a point on the fibre where the fluorescence intensity had decayed to a value of five standard deviations above the value of the background. The pixel values and the length of the labelled nerve were established with the plot profile tool found in Origin (OriginLab, Northampton, MA, U.S.A.). From the three-dimensional diffusion equation, allowing for anomalous diffusion, we expect a mean-squared diffusion length of

\[
\langle \Delta X^2 \rangle = 6D(t)t = \Gamma t^\alpha,
\]
where $\Gamma$ is the transport coefficient and $\alpha$ is the time-scaling exponent. Taking the log of both sides linearizes this equation:

$$\log \Delta X_{\text{RMS}} = 0.5 \log \Gamma + 0.5\alpha \log t. \quad (4)$$

So by plotting the distance that each dye diffuses for several time points on a log–log scale, the time-scaling exponent and the transport coefficient are obtained from the slope and intercept of the best-fit line. The value of the diffusion coefficient is then established from

$$D(t) = \frac{1}{6} \Gamma t^{\alpha-1}. \quad (5)$$

Note that Fickian diffusion, with a constant diffusion coefficient, is a special case of the more general anomalous diffusion (Künz & Lavallée, 2004). There are several possible explanations for a departure from normal Fickian diffusion, including diffusion within a complex geometry, and both spatial and temporal changes in the diffusion coefficient (Weiss et al., 2003; Künz & Lavallée, 2004; Sanabria et al., 2007). The case of $\alpha < 1$ is anomalous sub-diffusion, where the diffusion rate is less than that expected by Fick’s law. This has proven to be the case in many biological contexts, including the movement of lipids and proteins within membranes (Saxton, 1993, 1994; Goins et al., 2008). The case of $\alpha > 1$, anomalous super-diffusion, generally occurs when the dimensionality of diffusion is restricted or in the case of directed transport. All of this can be anticipated when considering diffusion of lipophilic tracer molecules over long distances in the heterogeneous environment of the peripheral nerve.

**Fluorescence recovery after photobleaching (FRAP)**

Measurements were made with the Region of Interest (ROI) and Time Series feature of the Zeiss LSM confocal software. Briefly, a small circular region of interest (either 4.4 or 8.8 μm in diameter) was selected on the peripheral nerve. Regions within 100 μm of the dye filter insertion point (proximal) and approximately 500 μm further away (distal) were studied to determine if the diffusion coefficient depended on location. The initial intensity of each region was collected in five scans prior to bleaching. The recovery sequence was recorded through a series of one hundred scans, on the average, separated by 1 s intervals. Assuming the general case of $b$-photon photobleaching with $p$-photon excitation by the probe beam (where $b$ and $p$ are integers, either 1 for one-photon excitation, or 2 for two-photon excitation), we followed a combination of the approaches of Brown et al. (1999) and Feder et al. (1996), but extended the treatment to the case of a uniformly bleached disk (rather than a Gaussian spot) to match the experimental method. In this case, the time-dependent fluorescence recovery signal is

$$F_{b,p}(t) = F_0 f_{nr} + (1 - f_{nr}) F^0$$

$$= \left[ 1 + (t/\tau_1)^\beta \sqrt{2p(t/\tau_2)\gamma} \right] \times \left[ \sum_{m=0}^{\infty} A_m^2 ((\tau_1/t)\gamma) \times \sum_{l=1}^{\infty} (-\beta)^{l-1} \left( \frac{1}{1 + 2lt(\tau_3)^\gamma} \right)^{-1/2} \left( 1 + 2lt(\tau_3)^\gamma \right)^{-1/2} \right], \quad (6)$$

with

$$A_m(x) = 1 - e^{-x} \left( \sum_{n=0}^{m} \frac{x^{m-n}}{(m-n)!} \right).$$

In Eq. 6, the three characteristic recovery times depend on the diffusion parameters,

$$\tau_1 = \left( \frac{R^2}{4\Gamma} \right)^{1/\alpha}, \quad \tau_2 = \left( \frac{\omega_z^2}{4\Gamma} \right)^{1/\alpha}, \quad \text{and} \quad \tau_3 = \left( \frac{\omega_z^2}{4\Gamma} \right)^{1/\alpha}. \quad (7)$$

$F^0$ is the fluorescence prior to photobleaching, $F_0$ is the fluorescence of the bleached spot immediately following photobleaching, $\beta$ is the bleach depth, $f_{nr}$ is the immobile fraction, $\omega_z$ is the $1/e^2$ axial radius of the bleaching beam and $\omega_{zp}$ is the $1/e^2$ axial radius of the probe beam. For NVR and NVM, two-photon photobleaching and monitoring with the same excitation wavelength was used, so $b = p = 2$, $\omega_z = \omega_{zp}$ and $\tau_1 = \tau_2$.

A non-linear least-squares fit to the recovery curve was used to optimize the values of $\alpha, \beta, \tau_1$ and $\tau_2$. The axial radius of the beam was calculated using the standard assumptions and verified through photobleaching measurements in solid fluorescent samples. The non-recoverable fraction was introduced as a fitting parameter only when statistically justified by an $F$-test (Bevington & Robinson, 2002). The values of the transport coefficient and diffusion coefficient were calculated from the best-fit parameters. With this information, the diffusion coefficient was calculated from Eqs 5 and 7.

**Results**

**Two-photon excitation of NV dyes**

Figures 3 and 4 show the measured one- and two-photon excitation spectra for the commercially available and candidate NV dyes obtained in pure ethanol. The peak wavelengths for each dye are also summarized in Table 1. PTIR334, 336 and 301 represent new candidates that extend the fluorescence emission spectrum to the violet and near-infrared, respectively. Nevertheless, given the broad fluorescence emission spectrum of molecular dyes, there remains considerable overlap between both violet candidates and NVJ, though PTIR301 is clearly differentiated from NVB.
The single-photon excitation peak of PTIR301 is beyond that of the conventional HeNe laser, but can easily be generated by the tunable Ti:S laser that is available with the Zeiss and Leica platforms. Similarly, the violet candidates are not well positioned to commonly available single-photon laser sources, so we investigated the possibility of exciting these using two-photon excitation. Figure 4 compares the two-photon action cross-section spectra of all of the NV dyes. All could be efficiently multiphoton excited over the tuning range of the Ti:S laser. But given the long wavelength absorption of NVB, two-photon excitation was only reasonable beyond 900 nm, and pure multiphoton excitation of PTIR301 was not possible.

The violet candidates (PTIR334 and 336) had similar multiphoton excitation cross-sections, with peak values ranging from 0.05–0.20 GM at wavelengths approximately double that of their single photon excitation peaks. These candidates are the least bright of the series, but comparable with NVJ and certainly of reasonable brightness in comparison with other biologically relevant fluorophores (Zipfel et al., 2003). NVR and NVO appear to be very bright two-photon fluorophores with peak cross-sections of 28.3 ± 0.6 and 258 ± 30 GM, respectively, which compare favourably to bright fluorophores such as fluorescein and rhodamine. Somewhat surprising was the fact that the peak excitations for both of these occurred at wavelengths below 800 nm, blue-shifted with respect to their single-photon excitation peaks.

Knowledge of the two-photon excitation spectra makes it possible to plan multicolour imaging protocols. For example, 850 nm would be a good choice for a single wavelength to simultaneously excite the NV violet candidates, along with NVJ, NVO and NVR. Similarly 980 nm is ideal for the simultaneous two-photon excitation of NVJ, NVR, NVM, NVO and NVB. To selectively excite NVR without interference from NVO, 800 nm would be an ideal wavelength, although an appropriate narrowband emission filter is also required to effectively distinguish these dyes. Furthermore, because slight spectral differences may occur when the dyes are used in cells and tissue instead of pure ethanol, some final tissue-specific optimization of the imaging protocol may still be required. Nevertheless, six and possibly seven colour dye sets are possible using the commercial and candidate NV dyes by using a combination of multiphoton and confocal single-photon excitation in multilaser sequential imaging. The additional labels will significantly enhance neurotracing of several distinct nerves within the same brain to better elucidate both the topology and connections made within multiple neuronal projections.

Multiphoton excitation often provides unanticipated advantages over conventional single photon excitation. For example, Matei et al. (2006) demonstrated photoactivation of non-fluorescent dyes. Multiphoton excitation can also enable imaging strategies that allow similar fluorophores to be more easily distinguished from one another. For example, Fig. 5(A) compares the one- and two-photon action...
Fig. 5. Comparison of single- and two-photon excitation strategies to improve the imaging of NVO and NVR in the same sample. The one-photon (dots, right and bottom axes) and two-photon (solid, left and top axes) action spectra for NVO and NVR are plotted in (A). Vertical arrows indicate maximum...
cross-section spectra for NVO and NVR. To reduce the cross talk resulting from NVO fluorescence being recorded in the NVR emission channel, the excitation wavelength should be chosen to maximize the ratio of NVR to NVO absorption. With single photon excitation (bottom and right axes), the maximum ratio is approximately 100 when exciting the red edge of the NVR absorption band in the range of 590–600 nm. Two-photon excitation (left and top axes) improves simultaneous imaging of NVR and NVO by increasing this excitation ratio to approximately 700. This occurs at 800 nm, a wavelength that is easily accessible to the Ti:S laser and near the peak two-photon excitation wavelength for NVR. By contrast, single-photon excitation of NVR with 590–600 nm light reduces its brightness by 5–10-fold, and the typical gas lasers used by most commercial confocal microscopes do not emit in this range.

This is readily demonstrated in brain tissue that has been labelled with both NVO and NVR. Figure 5(B)–(D) was obtained using single photon excitation of both probes at 543 nm, the closest available match to the optimum wavelength range of 590–600 nm. Narrowband excitation filtering from 550–570 nm effectively excludes NVR from the NVO channel, but NVO fluorescence in the 590–620 nm wavelength range of the NVR filter is considerable. As a result, the red channel is truly a mixture of NVR and NVO, and there is no spatial discrimination of the dyes. Two-photon excitation at 760 nm also shows considerable mixing of NVR and NVO throughout the image (Fig. 5E–G). By contrast, using 890 nm two-photon excitation to preferentially excite NVO (Fig. 5H), and 800 nm to preferentially excite NVR (Fig. 5I) reveals that the NVR signal is actually localized towards the top of the sample, with NVO distributed throughout (Fig. 5). This was corroborated by spectral imaging and linear unmixing.

The influence of the fluorescent head group on diffusion

Although spectral separation is important, for neurotracing studies it is equally important that the candidate dyes diffuse at similar rates as the other dyes in the series. Vastly different diffusion rates would necessitate a staged set of injections of different dyes for equal diffusion, a more cumbersome approach compared to simultaneous implantation. In principle, the diffusion rate will depend on interactions between the local environment and both the head group and the hydrocarbon tails. Previous studies of NVM, NVO, NVR, PTIR326 and PTIR327 showed that all of these dyes diffuse at similar rates in murine spinal cord with incubation times ranging from 24 to 96 h (Fritzsch et al., 2005; Jensen-Smith et al., 2007). Nevertheless, the substantial variation in the head groups of the new candidate dyes could affect their relative diffusion rates. If this is the case, it may be possible to tune the hydrocarbon chain length to optimize the diffusion rate. Therefore, we tested the hypothesis that the configuration of the fluorescent head group impacts the rate of diffusion.

Comparison of singly labelled samples

In one study, individual NV tracers were introduced to peripheral nerve preparations through a dye-coated nylon filter that was applied to the base of the intercostal nerve, proximal to the spinal cord. After allowing 24–128 h of incubation at 37 °C, the effective diffusion distance was measured. The data for NVM, NVR, PTIR301, PTIR334 and PTIR336 are presented in Fig. 6. Generally the distances range from nearly 1 mm, at 24 h, to nearly 2 mm, at 96 h of incubation. After 24 h, PTIR301 reached a distance similar to that of PTIR336 and NVR and it diffused further than PTIR334 and NVM (P < 0.005). At later times, however, PTIR301 lags behind the other probes. By contrast, although PTIR334 began with the shortest diffusion distance at 24 h, by 96 h it reached a distance common to PTIR336, NVM and NVR. PTIR336 and NVR had similar diffusion distances at each incubation period. Finally, the diffusion distance of NVM fell below both that of PTIR336 (P < 0.05) and NVR (P < 0.005) after 24 and 48 h (P < 0.005) of incubation.

As expected from Eq. 4, the diffusion distances exhibit power-law scaling with the incubation period. Although PTIR336 and NVR have similar slopes, the slope of NVM is slightly steeper. PTIR334 with the steepest slope and 301 with the shallowest slope, clearly behave differently than the other probes in the set. The time-scaling exponent of PTIR301 is only 0.48, clearly sub-diffusive and significantly smaller than ratios in NVR:NVO excitation of 100 for single-photon excitation and 700 for two-photon excitation. A 200-μm-thick coronal section of a newborn mouse forebrain received a sub-cortical injection of NVO (pseudocolour green) and NVR (pseudocolour red) (B–J). Dye-soaked filters were removed prior to imaging, therefore leaving dark voids in the labelled tissue. The image panels directly compare 543 nm, single photon excitation (B, C), 760 nm two-photon excitation (E, F) and a combination of 890 nm (H) and 800 nm (I) two-photon excitation. Emission bandpasses were 550–570 nm (pseudocolour green; B, E, H) and 580–610 nm (pseudocolour red; C, F, I). The right column shows the two-channel merge (D, G, J). The spectral overlap of the two dyes provides a false impression of spatial overlap (B–G). However, both dyes can be completely segregated using a two-photon excitation at 800 nm (H–J). Scale bar: 100 μm.
any of the other probes ($P < 0.005$). At the other extreme, PTIR334 had a time-scaling exponent of 1.6, clearly super-diffusive and significantly greater than the rest ($P < 0.005$).

In the middle PTIR336, NVM and NVR were all slightly super-diffusive, and generally well matched, though PTIR336 is significantly different from NVM ($P < 0.005$).

Figure 6(C) compares the values of the effective transport coefficient for all of these dyes, derived from the intercepts of the best-fit lines in Fig. 6(A). All values of the transport coefficient are significantly different for all of the probes tested, and it is clear that the values of $\Gamma$ are inversely correlated with $\alpha$: PTIR301 has the greatest value of $\Gamma$, exceeding that of the other dyes by several orders of magnitude. Together, the results in Fig. 6(B) and (C) can be used to determine the time-dependent diffusion coefficient from Eq. 4. This result is shown in Fig. 6(D).

The effective diffusion coefficients of all dyes were in the range of 0.75 to 2.1 $\mu$m$^2$s$^{-1}$ for all time points tested. Dyes that exhibited anomalous super-diffusion have a diffusion coefficient that increases with time, whereas sub-diffusive behaviour has a continuously decreasing diffusion coefficient. Initially, PTIR334 begins with the smallest diffusion coefficient, significantly less than NVM, PTIR336 and PTIR301 ($P < 0.005$), due to its extremely low transport coefficient. By contrast, although sub-diffusive, PTIR301 nevertheless has a diffusion coefficient that is very well matched with all of the other dyes after 24 h, with the exception of PTIR334. However, after 48 h, the diffusion coefficient of PTIR301 decreases significantly relative to all of the other dyes. By 96 h, all of the dyes are well matched except for PTIR301 ($P < 0.005$).

Comparison of samples with two sets of fluorophores

Because these dyes are meant to be used together for multicolour neurotracing, we next examined whether or not dye-dye interactions could influence the diffusive transport of these dyes. In this study, intercostal nerves were labelled with a pair of spectrally distinct fluorescent molecules. NVR served as a common co-label to NVM, PTIR326 and NVJ. The diffusion distances of both labels were tracked over a period of up to 96 h.

Figure 7(A) depicts the diffusion distance of each independent co-label for several incubation periods. After 24 and 48 h, all co-labels travelled similar distances. However, by 96 h, NVM has diffused significantly further than PTIR326 ($P < 0.05$) and NVJ ($P < 0.005$). Between the 48 and the 96 h incubation periods, the diffusion distance of NVJ did not change significantly, leading to a poor power-law fit. At
96 h, the diffusion distance of NVJ is smaller than PTIR326 and NVM ($P < 0.005$).

Figure 7(B) compares the time-scaling exponent for each independent co-label. The time-scaling exponents for these dyes lie within the same range as those values that were reported in the single-label study. NVM was used in both the single- and double-label studies, and the time-scaling exponent did not change significantly with the addition of the NVR co-label. NVJ and PTIR326 were both sub-diffusive, similar to PTIR301 in the single-label study. All of the transport coefficients of the co-labels were significantly different and anti-correlated with the time-scaling exponent (Fig. 7C). Although there are significant differences between dyes when comparing these two parameters, when taken together there is very little difference between the diffusion coefficients over the periods of incubation studied (Fig. 7D). Furthermore, these diffusion coefficients are all numerically similar, or even greater than, the diffusion coefficients measured in the single-label study. NVM, for example saw a 3-fold increase in the transport coefficient and a slight reduction in the time-scaling exponent, resulting in a nearly 2-fold increased diffusion coefficient when co-labelled with NVR. Although this experiment cannot say anything about the underlying mechanism, it is clear that some dye interaction is occurring, yet (in the case of NVM) this is not necessarily resulting in a slower diffusion rate. To determine whether the difference in diffusion rates is due to just the increase in total dye concentration, or whether it depends on the specific pairings of dye molecules, we investigated the diffusive transport of NVR as a common co-label paired with other PTIR dyes. These results are summarized in Fig. 8.

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After 24 h, the diffusion distance of NVR is the same regardless of co-label, but the diffusion distances begin to diverge after this point. NVR diffuses more slowly when paired with NVJ than it does when paired with NVM. The results are clearly seen as changes in the time-scaling exponent (Fig. 8B). Recall that as a single label, NVR was somewhat super-diffusive with a time-scaling exponent of 1.12 ± 0.06. When paired with NVJ, this value drops nearly 2-fold ($P < 0.005$). But when paired with NVM the reduction is insignificant. For all dyes studied, the time-scaling coefficient was smaller when paired with another dye than when used as a single label. It is also important to consider the effect on the transport coefficient (Fig. 8C). Regardless of the dye it is paired with, the transport coefficient is significantly larger when a co-label is present, than alone. As a result, the diffusion coefficient
for NVR is initially greater than it would be if it is unpaired (Fig. 8D). But the diffusion coefficient either decreases or increases less quickly with time than was seen when used alone due to the reduction in the time-scaling exponent. Although these observations are statistically significant, the overall change in the diffusion rate is relatively minor, with an effective diffusion coefficient of approximately 2 μm² s⁻¹ when used in a single- or double-label study.

**FRAP measurements of the diffusion coefficients of select dyes**

Although time–distance measurements have practical value for evaluating the relative diffusion distance of a probe in tissue, there are also several disadvantages with these measurements. Because the fluorescence intensity of the end of the profile is chosen pragmatically to be the farthest point that can definitely be discerned over background, the distance measured is expected to exceed the mathematical diffusion distance. This in turn leads to a systematic over estimation of the diffusion distance. This in turn leads to a systematic over estimation of the diffusion coefficient. Furthermore, given the long time periods required to observe diffusion over macroscopic distances, the measurements can be affected by many external factors. Thermal instability, for example, may be seen as a decrease in the rate of diffusion with time, complicating the interpretation of the anomalous diffusion exponent. It is also complicated by the inherent heterogeneity of the tissue sample occurring over several millimetres of distance.

To address these issues, we have also performed FRAP measurements to make point diffusion measurements. In these measurements, NV probes were introduced to the peripheral nerve of the spinal cord with a 12 h, 37°C incubation. The fluorescence recovery following photobleaching of small regions proximal and distal to the nylon filter insertion point was monitored for a few minutes. Sample FRAP recovery curves for NVM and NVR are shown in Fig. 9(A). Although the depth of the fluorescence bleach depends on the bleaching protocol, the time course of the recovery depends on the transport coefficient, anomalous diffusion coefficient and the shape and geometry of the photobleached region. These parameters were determined by fitting the data with Eq. 6 (Fig. 9A). Figure 9(B) shows the time-scaling exponent from at least eight measurements of each dye made at each location. There was a systematic, yet slight increase in the time-scaling factors measured at distal locations as compared to proximal locations for each probe. But both NVM and NVR had similar time-scaling exponents. Furthermore, there is no significant difference between the distal values of these parameters as measured by FRAP or the time–distance method (Fig. 6B). By contrast, the transport coefficients of the two probes are significantly different, regardless of the position...
Fig. 9. FRAP recovery of NVM and NVR in individually labelled peripheral nerve fibres. Measurements were made proximal (p, solid symbols and bars) or distal (d, open symbols and hatched bars) to the nylon filter insertion point. Panel (A) shows the normalized fluorescence recovery curves following the bleaching of a circular spot. The time-scaling exponent (B) transport coefficient (C) and projected diffusion coefficients (D), are obtained by fitting the recovery curve to Eq. 6, using Eqs 5 and 7. The average and standard error of the mean from at least eight measurements for each dye and location are shown.

of the measurement. As a result, the diffusion coefficients of NVM and NVR, calculated 3 min after bleaching, are significantly different. Although it was anticipated that the diffusion coefficients would be smaller, the large disparity in the diffusion coefficients as measured by FRAP was surprising.

Simultaneous six-colour neurotracing

To demonstrate multicolour neurotracing, fixed spinal cord (Fig. 10, Zeiss 510 LSM) and brain tissue (Figs 11 and 12, Leica TCS SP5) was stained with four commercially available NV dyes, PTIR334 and either PTIR301 (Fig. 10) or NVB (Figs 11 and 12). To efficiently excite all six dyes while minimizing fluorescence cross talk, a combination of single- and two-photon excitation was employed in each case. Figure 10 shows the intercostal nerves of a single mouse labelled with these six dyes. The two peripheral halves were mounted next to each other (jagged black line in the middle) to show the six-colour labelling and the level of segregation. Each inserted filter strip spanned two to three intercostal nerves which project as coloured lines to the left and right. In this example, any apparent differences in diffusion distances may relate to proper contact of the inserted filter strip with the intercostal nerve. This is required for dye uptake from the filter into the lipid bilayer. The excitation wavelengths, mechanisms, and filter sets used to record the contribution from each dye are provided in Table 3. The clear spectral separation of all six probes is clearly evident by the unique labelling patterns of each dye.

The same clear spectral separation is also apparent in brain tissue. Figure 11 shows a 1-mm-thick coronal section of the forebrain of an adult mouse. Small strips of filter were implanted and the tissue was incubated in 4% PFA for 72 h at 36°C. Removal of the filter strips prior to imaging produced holes near the centre of the dye distribution. This image was obtained with the Leica TCS SP5 microscope, which has acousto-optic filters that enable precisely tuned narrowband detection of fluorescence emission. This allowed for confocal imaging of several of the dyes with excitation wavelengths closer (~10 nm) to the detection bandpass (Table 4).

Finally, to demonstrate the reliability of this approach we prepared a single E12.5 mouse embryo for six-colour labelling (Fig. 12). Three (out of 12) cranial nerves on either side were distinctly and separately labelled, permitting co-localization of various nerve components contributing to different cranial nerves or branches of a single cranial nerve with unprecedented clarity (Fig. 12). Using six-colour labelling routinely can reduce the number of mutant embryos while
increasing accuracy of findings through imaging in register with each other.

Discussion

One disadvantage of carbocyan dyes is their broad excitation and emission spectra, in the range of 80–100 nm. As a consequence only a few dyes can be imaged with non-overlapping signals within the visible spectrum. Multiphoton excitation of the NV dyes opens up new possibilities for multicolour neurotracing. Figure 4 demonstrates how probes with single-photon absorption bands from 400–700 nm can all be multiphoton excited with a tunable near-infrared Ti:S laser. This both reduces the number of lasers that must be used for multicolour neurotracing, while providing improved penetration and reduced photo-toxicity of near-infrared light (Zipfel et al., 2003). All of the NV dyes tested can be efficiently two-photon excited, but NVO, NVR and NVB are particularly bright with peak two-photon action cross-sections similar to that of fluorescein or rhodamine. Choosing a wavelength of approximately 900 nm permits simultaneous excitation of all of the candidate dyes. And although the action cross-section for NVM is approximately 1000 times greater than the violet candidates at that wavelength, given the reduced quantum efficiency of many standard photomultiplier tubes in the near-infrared (near-IR) compared to the violet, in practice the detected fluorescence from equivalent concentrations are fairly similar. When imaging on microscopes lacking a 405 nm violet diode laser, multiphoton excitation of the violet candidates is particularly useful. Although it is possible to multiphoton excite the entire NV line at a single wavelength, the overlapping emission spectra of some of the dyes would necessitate spectral unmixing. This is especially true of the violet candidates and NVJ, so expanding into the UV remains a priority.

For neuroimaging in samples labelled with the far-red–near-IR NV probes (NVM, NVB, 301) along with the violet-red probes, multitrack acquisition using a combination of confocal and multiphoton excitation provides an advantage. For example, to produce a single multitrack image of the PTIR 334, NVJ, NVR, NVO, NVM and PTIR 301, we combined six single-channel images taken sequentially (Fig. 10). As
Fig. 11. Six-colour image of a NV labelled 1-mm-thick coronal section of the forebrain of an adult mouse showing the segregation of six colours in adjacent filter injections. Small strips of filter were implanted and the tissue was incubated in 4% PFA for 72 h at 36°C. The filter strips were removed (leaving dark voids in the centre of each dye distribution) and the section was mounted flat in glycerol for immediate imaging with the Leica TCS SP5 microscope using the excitation and emission strategy detailed in Table 4. For clarity, the insets show the independent detection channels associated with each NV probe to verify that there was no bleed through between the dyes used for this image. Scale bars: 200 μm.

shown in Fig. 4. 820 nm light efficiently excites PTIR334, NVJ and NVR, while avoiding excitation of the remaining dyes and capitalizing on the fact that the two-photon cross-section of NVO is one-fifth of PTIR334 at 820 nm. NVJ was easily separated from PTIR334 by using 940 nm excitation, where NVJ has a substantially higher cross-section than PTIR334. Because the two-photon cross-section of PTIR334 never exceeds that of NVJ, excitation at 820 nm and a narrowband emission filter was used for the image of PTIR334 without contamination by NVJ. Unfortunately, this resulted in autofluorescence also being visible in the PTIR334 image. Still, the peripheral nerve is clearly visible. The Ti:S laser was then tuned down to 720 nm for confocal imaging of PTIR301 and two-photon imaging of NVO. Finally, confocal imaging of NVM was accomplished use the 633 nm HeNe laser to complete the six-colour image using the Zeiss LSM 510 microscope.

Although this sequential imaging strategy can effectively distinguish the individual signals from a large number of overlapping fluorophores without spectral unmixing, the time resolution is limited to several seconds. Current commercial microscopes do not readily accommodate simultaneous imaging of more than a few fluorophores. Nevertheless, this limitation can be overcome in a customized imaging platform.

Two further examples of six-colour imaging in brain tissue, obtained with the Leica TCS SP5 microscope are shown in Figs 11 and 12. For these images, dye filters were placed in the motor cortex and allowed to diffuse for 3 days at 36°C. Again, a combination of confocal and multiphoton excitation was used to optimally separate the signal of the six NV probes (Table 4). In this case, single photon 458 nm excitation of PTIR334 was used in combination with precisely tuned narrowband acousto-optic tunable filters to minimize the autofluorescence background. As in Fig. 10, optimal separation of NVR and NVO was achieved using two-photon excitation at 820 nm.

Higher magnification images show that all of these dyes compare favourably to other widely used lipophilic tracers in their ability to completely fill single neurons with a profile touched by the injected dyes. To our knowledge, this is the first time that six lipophilic dyes have been used to simultaneously label neuronal profiles in the same tissue with equal effectiveness. This achievement enables new multiple labelling studies. For example, cranial nerves III–VIII could be simultaneously labelled to show how a given mutation might result in the aberration of a subset of the nerves in the same tissue. This will dramatically reduce the need to generate multiple mutant embryos that would otherwise be needed if imaging was limited to one, or at best two, populations of neurons. Even within a given terminal field, multiple colours would help to distinguish adjacent projections. For example adjacent hairs in the whisker pads of the mouse are known to project to discrete areas in the trigeminal sensory complex. Labelling six adjacent whisker hairs, each with a different coloured dye, would allow for simultaneous imaging of all of those projections. This would reduce the total number of mice needed and enhance the validity of the conclusions by allowing for intra-tissue comparisons rather than inter-tissue comparisons. Figure 12 shows the result of a single injection of six dyes into three cranial nerves of a 12-day-old mouse embryo bilaterally, clearly demonstrating the ease of handling, reliability and detailed imaging quality of these dyes.
Fig. 12. Six-colour image of NV labelled cranial nerve distribution in hindbrain. (A) Side of E12.5 mouse showing dye injections of NVO (red), NVB (non-fluorescent), and PTIR334 (green) labelling trigeminal (V), facial (VII) and glossopharyngeal/vagal (IX/X), respectively. Dorsal is up and rostral to the left. (B) Opposite side of same mouse in (A) showing dye injections NVM (blue), NVJ (green), NVR (red) labelling maxillary branch of trigeminal (V2), mandibular branch of trigeminal (V3) and facial nerves (VII), respectively. Dorsal is up and rostral is to the right. (C) Hindbrain of same mouse in A and B flat-mounted and imaged using conventional epifluorescence to visualize the central projections of nerves labelled. With conventional Texas red (566 nm) fluorescein (488 nm) and Cy5 (625 nm) filter cubes, NVO and NVR cannot be distinguished, PTIR334 and NVJ cannot be distinguished, and NVB cannot be imaged. (D) Confocal images of same hindbrain shown in (C); NVO, (yellow), NVB (cyan), PTIR334 (blue), NVM (magenta), NVJ (green) and NVR (red). (E) Individual nerve fibres can be distinguished and all six dyes can be separated using the settings from Table 4. (F) Higher power image of...
Transport coefficients and time-scaling exponents obtained from time–distance measurements are useful parameters to help organize molecular probes into diffusion-matched groups. Although the measured diffusion distances of two probes may agree at a given time point, if the time-scaling exponents are substantially different, then the probes will not be well matched at other times. Here we find that PTIR 336, NVR and NVM are well matched in their time-scaling exponents and transport coefficients, and consequently, their effective diffusion coefficients. Although our measurements were made only over a 96 h period, it can be assumed that they will be well matched for longer periods of time. By contrast, PTIR 301 and PTIR 334 have significantly different diffusion parameters than the others, and may be less useful in multicolour neurotracing studies.

For multicolour neurotracing, addition of a second label results in a decrease in the time-scaling coefficient, suggesting that dye aggregation may decrease diffusion rates in these studies. This is countered by a greater transport coefficient, resulting in very similar diffusion distances measured at the 24 h time point. This was also seen when the concentration of a single label (NVR) was increased. One possible interpretation is that increased dye concentration on the nylon filter leads to a more rapid initial desorption as the dye enters the tissue, followed by a slower diffusion rate within tissue. Additional studies would be needed to confirm this hypothesis.

Although distance measurements are perhaps the most useful measure of probe transport for neurotracing studies, an accurate measurement of the diffusion distance in tissue is difficult. For this reason, relative diffusion distances of two probes are often compared. The distance measured in this study is the distance at which the probe fluorescence has dropped to 5% above the background noise level. Although somewhat arbitrary, this serves as a uniform criterion where the signal is unambiguously distinguished from background. The distance can be expected to exceed the mathematical diffusion distance, which for a point source would be found when the fluorescence decreases to 13.5% of the maximum value. Furthermore, we are systematically underestimating the actual length of the labelled nerve by measuring the two-dimensional projection of a nerve that extends into three-dimensions. Based on the average thickness of an image stack, the systematic error associated with this length discrepancy is estimated to be less than 3%. Despite these qualifications, this measure of the diffusion distance serves as a quantitatively useful measure for comparing across a class of molecular probes.

This is supported by the general agreement in the diffusion properties determined using either distance measurements or FRAP. When averaging distal and proximal sites, the FRAP time-scaling exponents of NVM and NVR (Fig. 9B) are similar to those obtained using the distance measurements (Fig. 6B). The diffusion coefficients calculated from the FRAP recovery (Fig. 9D) are also qualitatively similar to those determined by the time–distance technique, though FRAP reveals a greater difference between NVM and NVR. It is important to recognize that FRAP measures the diffusion coefficient from the fluorescence recovery over just a few minutes, a completely different timescale than the 24–96 h required by the time–distance technique. For a homogeneous sample exhibiting normal Fickian diffusion, we would expect quantitative agreement between the two techniques. But this is clearly not the case for the more complex spinal cord sample. Nevertheless, the qualitative agreement suggests that FRAP can also be used in place of the more physiologically relevant, but laborious, time–distance measurements. FRAP has the added advantage of determining a well-defined absolute diffusion coefficient (albeit on short time and length scales), whereas the time–distance measurements are limited to relative comparisons (over large time and length scales).

Given the heterogeneous structure of the spinal cord sample, it is not surprising that different diffusion parameters were obtained at points proximal and distal to the filter insertion point. There are several possible reasons for this. First, we were expecting that the increased probe concentration proximal to the insertion point may lead to the formation of dye aggregates with a corresponding decrease in the diffusion rate. In fact, we found a significant decrease in the diffusion of NVM at proximal points when compared with distal points. But this was not the case with NVR, where diffusion was slightly enhanced nearer the insertion point. At this point it is not clear why these two probes behave differently, but the smaller probed volume and greater time resolution of the FRAP measurements is revealing a greater variation in the transport of the dye than the time–distance measurements.

NVO, NVR, NVM, NVJ, NVB, PTIR 326, PTIR 301, PTIR 334 and PTIR 336 all have the same long chain hydrocarbon tail configuration (two asymmetric chains, 36 carbons total), so the variations in the diffusion rates must be attributed to the chemical modifications of the head groups. With the reduced diffusion rate of PTIR 301 at the longer time points, characteristic of anomalous sub-diffusion, we have produced additional near IR candidates, modifying both the head group and the structure of the hydrocarbon tails to

NVO, NVB and PTIR 334 dyes. Individual nerve fibres can be seen along with individual cell bodies. (G) Higher power image of NVM, NVJ and NVR dyes. Again individual neurons can be distinguished and individual cell bodies can be seen. (C–G) Rostral is to the right. Arrows point to individual trigeminal mesencephalic cell bodies. FBM, facial branchial motor nucleus; SS, superior salivatory nucleus; Vm, trigeminal motor nucleus; ST, solitary tract. Scale bars: (A–D) 1 mm; (E–G) 100 μm.
increase performance. One in particular, MTTI129, displays very promising long range diffusion, as shown in Fig. 13. With absorption and emission wavelengths of 785 nm and 805 nm, respectively, this molecule is an excellent spectral complement to the NV probes. Its symmetrical hydrocarbon chain configuration (32 carbons total) contributes to a super-diffusive time-scaling exponent of 2.07 in peripheral spinal cord tissue. This is a dramatic improvement over PTIR301, with more than a 2-fold increase in diffusion distance by 96 h, and still diffusion-matched with the NV probes between 48 and 96 h. We are currently investigating how the hydrocarbon chains lengths might be further tuned in conjunction with the head group to optimize the diffusion rates of this and similar candidates for improved matching in long distance applications.

In summary, we report here for the first time the two-photon cross-section data of lipophilic dyes widely used for neuronal tracing and demonstrate that our newly generated dyes expand the repertoire to six dyes that can be fully spectrally segregated using defined excitation and emission settings. We also demonstrate that long-term diffusion measured along nerves are comparable with FRAP data. Finally, we demonstrate in a few samples how these multicoloured dyes can be used to enhance simultaneous tracing of multiple nerves or connections within the same brain. This technique will provide a better intra-tissue comparison of normal and aberrant projections and reduce the number of animals that will need to be analysed for a given study.

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References


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