Supplementary Materials for

Prototype Nerve-Specific Near-Infrared Fluorophores

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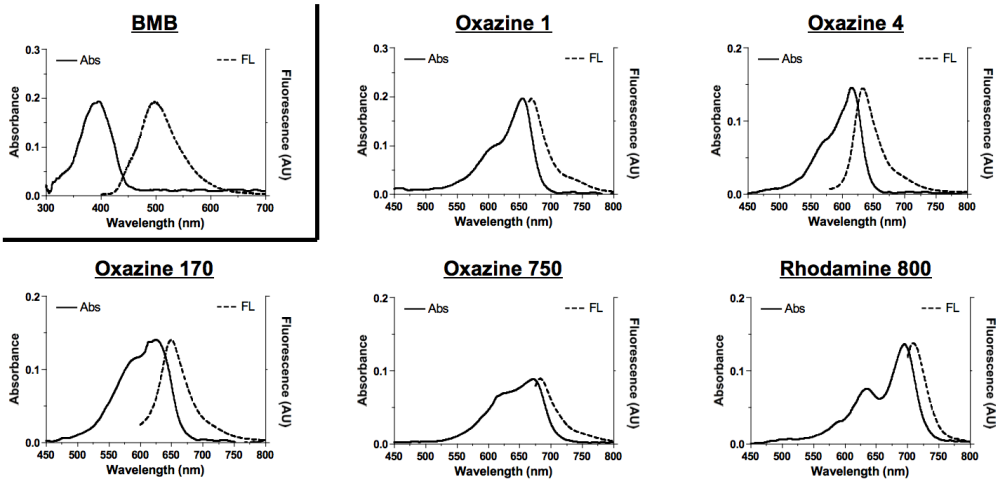
SUPPLEMENTARY METHODS

Optical Property Measurements: All optical measurements were performed at 37°C in phosphate-buffered saline (PBS), pH 7.4, 100% fetal bovine serum (FBS) buffered with 50 mM HEPES, pH 7.4, methanol (MeOH), or dimethyl sulfoxide (DMSO). Absorbance and fluorescence emission spectra of the series of NIR fluorophores were measured using fiber optic HR2000 absorbance (200–1100 nm) and USB2000FL fluorescence (350–1000 nm) spectrometers (Ocean Optics, Dunedin, FL). Excitation light was provided by a 532 nm green laser pointer (Opcom Inc., Xiamen, China) set to 5 mW and coupled through a 300 μm core diameter, NA 0.22 fiber (Fiberguide Industries, Stirling, NJ). *In silico* calculation of the partition coefficient (logD at pH 7.4) was calculated using Marvin and J Chem calculator plugins (ChemAxon, Budapest, Hungary).

In Vivo **Nerve-Specificity Screening:** Sprague-Dawley (SD) male rats weighing 250-300 g were purchased from Charles River Laboratories (Wilmington, MA). BMB, Oxazine 1, Oxazine 4, Oxazine 170, Oxazine 750, and Rhodamine 800 were initially screened to investigate nerve specificity in rats. For the initial screening, we administered a relatively high dose (2 mg/kg; 1 μmol) of each fluorophore intravenously. 4 h after injection, brachial plexus (BP) and sciatic nerve (SN) were imaged using the intraoperative FLARE™ system for *in vivo* nerve-specific fluorophores screening.

A.



B.

Optical Properties of Oxazine 4 in Various Solvents.

Solvent	Extinction Coefficient ($M^{-1}cm^{-1}$)	Peak Absorbance (nm)	Peak Emission (nm)	Stokes Shift (nm)
PBS	143,000	616	634	17
FBS	143,000	616	635	17
MeOH	182,000	611	631	20
DMSO	147,000	621	640	19

DMSO = dimethyl sulfoxide; FBS = fetal bovine serum supplemented with 50 mM HEPES, pH 7.4; MeOH = methanol; PBS = phosphate buffered saline, pH 7.4.

Figure S1. **A**) Optical Properties of Nerve-Targeting Fluorophores. Absorbance and fluorescence spectra were obtained at a concentration of 1 µM in 100% FBS supplemented with 50 mM HEPES, pH 7.4. **B**) Optical properties of Oxazine 4 were measured in PBS (pH 7.4), FBS (pH 7.4), MeOH, and DMSO.

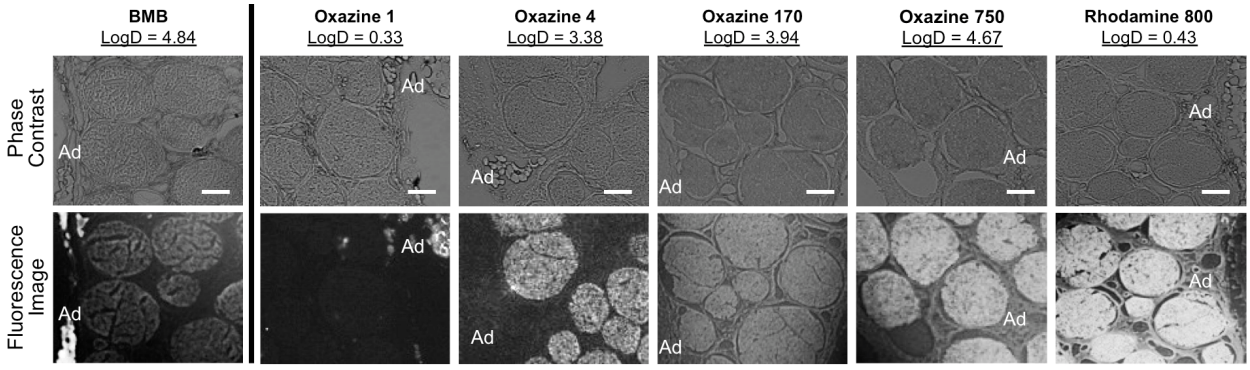


Figure S2. *Ex Vivo* Screening Assay of Fluorophores for Nerve-Specificity. Nerve-specific fluorescence intensity was determined using staining of pig sciatic nerve cut in cross section and incubated with 100 µM of each fluorophore. Ad = adipose tissue. Scale bars = 100 µm. The partition coefficient (logD at pH 7.4) was calculated using JChem calculator plugins.

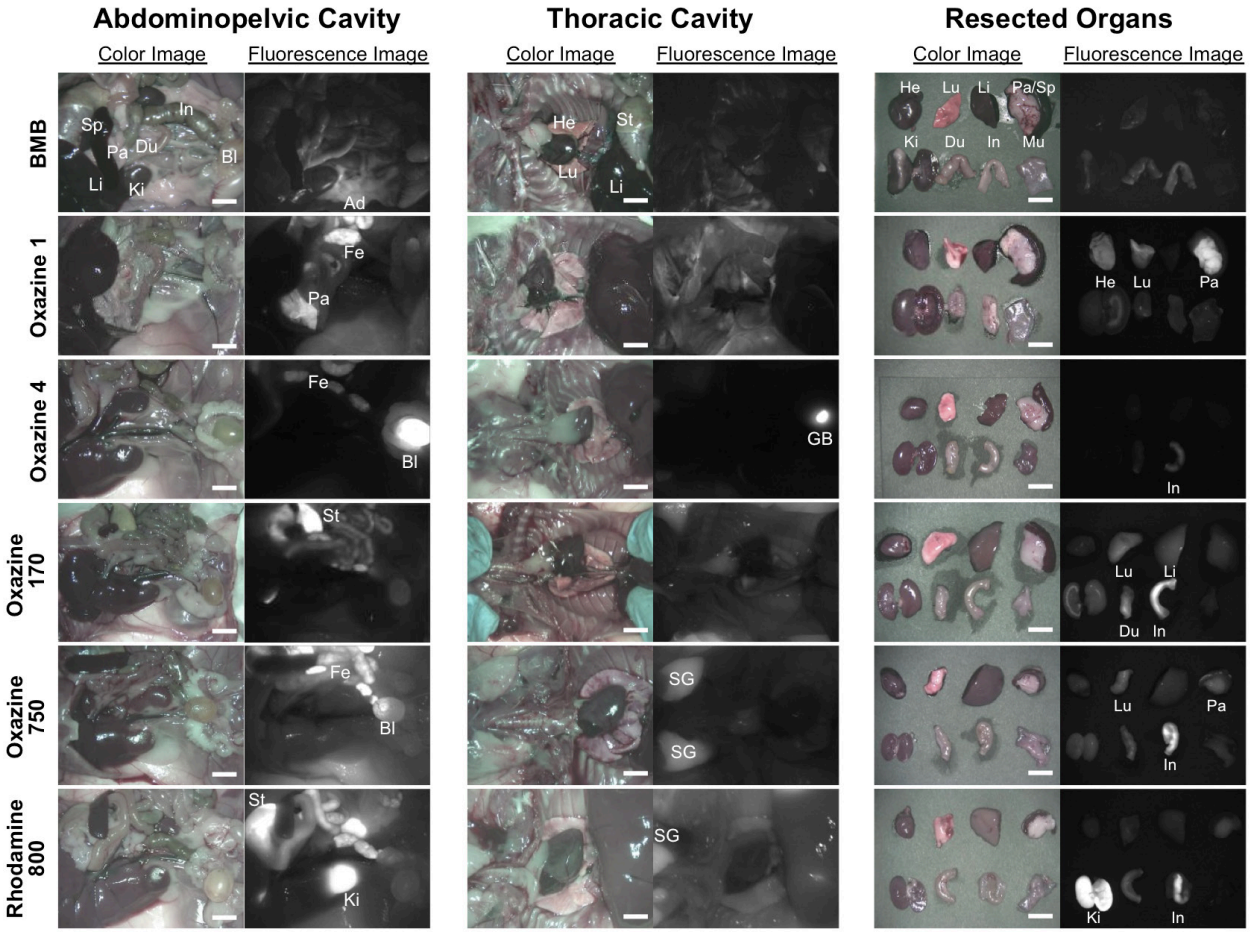


Figure S3. *In Vivo* Biodistribution and Clearance of Nerve-Specific Fluorophores. All fluorophores used in *ex vivo* screening were tested into SD rats for *in vivo* tracking biodistribution 4 h post-injection. Among nerve highlighting fluorophores (BMB, Oxazine 4, and Oxazine 170), Oxazine 4 showed higher nerve-specific fluorescence signal and lower background (muscle and adipose tissue) fluorescence signal than Oxazine 170 and BMB. Abbreviations used are: Ad, adipose; Bl, bladder; Du, duodenum; Fe, feces; GB; gallbladder; He, Heart; In, intestine; Ki, kidneys; Li, liver; Lu, lungs; Mu, muscle; Pa, pancreas; Sp, spleen; St, stomach; and SG, salivary gland. Scale bars = 1 cm. All NIR fluorescence images have identical exposure and normalizations.

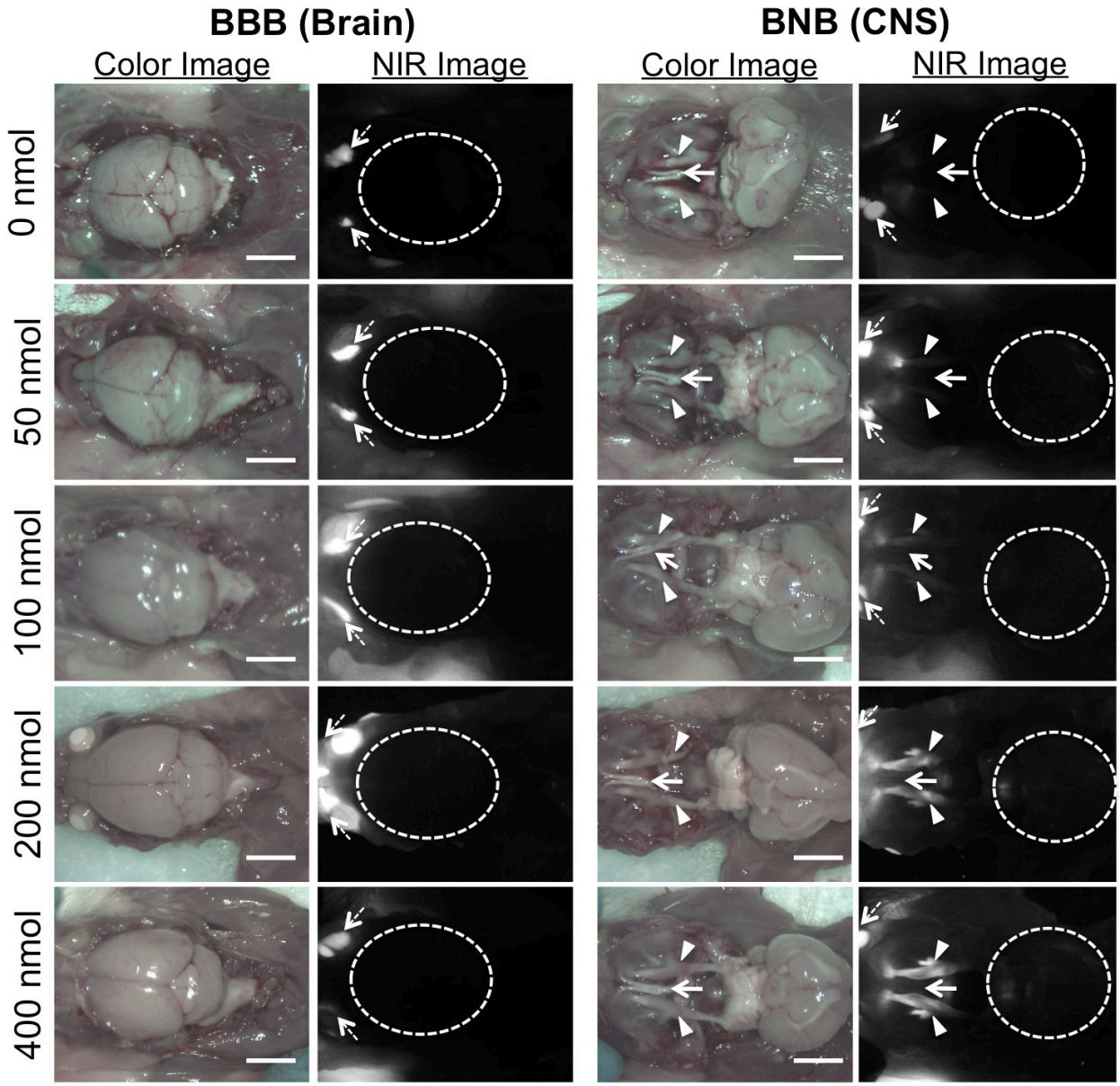


Figure S4. *In Vivo* Dose-Dependent Brain and Central Nerve Uptake of Oxazine 4 in Mice. 0, 50, 100, 200, and 400 nmol of Oxazine 4 were injected intravenously into CD-1 mice, and BBB (brain-blood barrier) and BNB (blood-nerve-barrier) uptake at CNS (central nervous system) was imaged at 4 h post-injection. Dotted arrows indicate Harderian glands (autofluorescence from porphyrins) and dotted circles indicate intact brain shown from the top. Arrowheads indicate trigeminal ganglia (peripheral nerves) and solid arrows indicate optic nerves (central nerves). Images are representative of N = 5 independent experiments. Scale bars = 0.5 cm. All NIR fluorescence images have identical exposure times (500 msec) and normalizations.

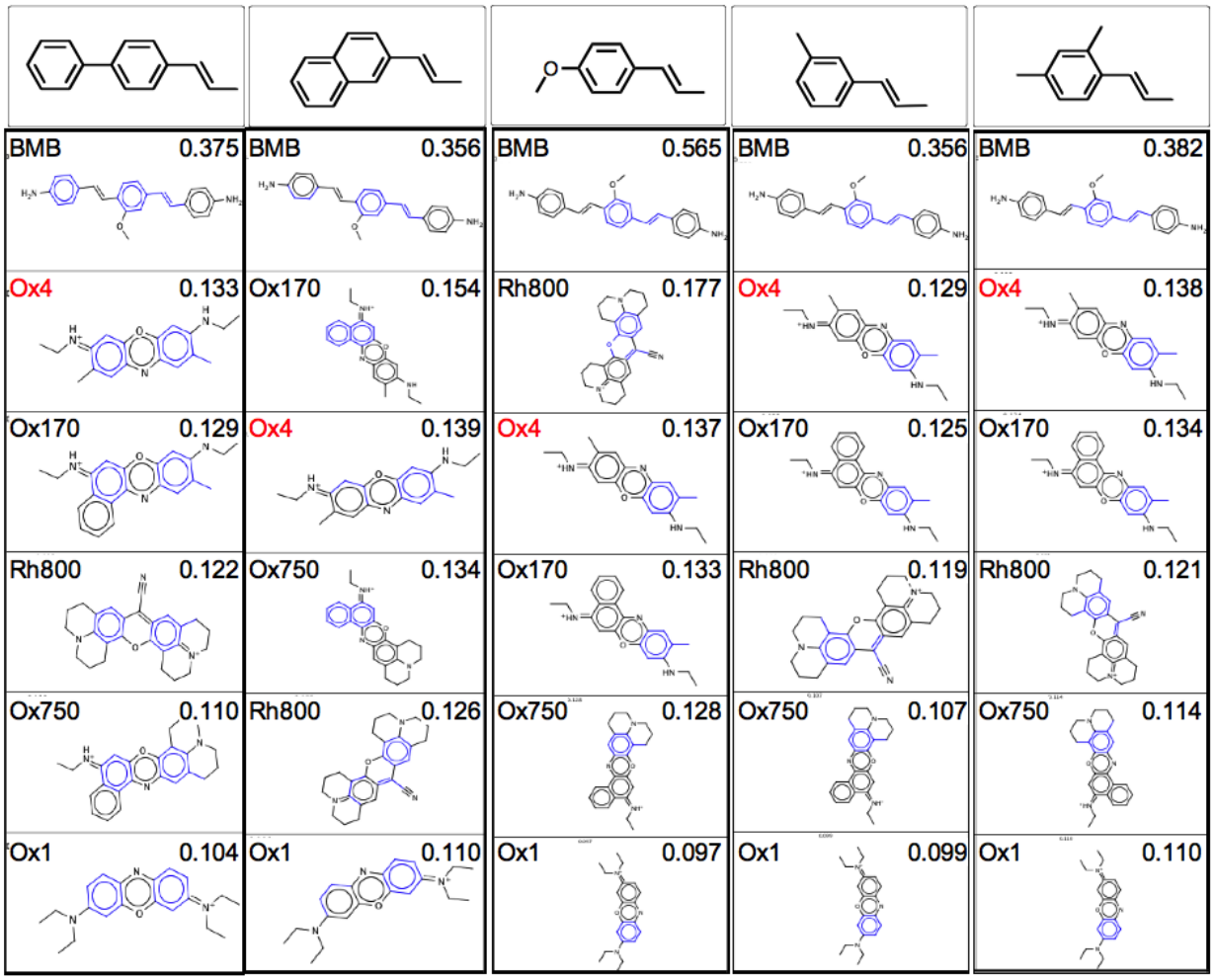


Figure S5. Chemical Similarity Calculated by Comparing Fluorophores to Positive Nerve Binding Fingerprints Proposed by Gibbs et al. (*PLoS One* 2013; 8, e73493). Based on the QSAR screening and fingerprinting, Oxazine 4 fits with the previously proposed basic requirement of a balanced para-configuration of the core. In addition, Oxazine 4 shows the highest similarity with the five highest ranked fingerprints except for BMB. Although Oxazine 170 and Rhodamine 800 show relatively high similarities, the former is rather lipophilic (logD at pH 7.4 = 3.94) and the latter is rather hydrophilic (logD at pH 7.4 = 0.43), which resulted in high accumulation in adipose tissue or low targeting affinity to the nerve, respectively.