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Effects of laser polarization on responses of the fluorescent Ca^{2+} indicator X-Rhod-1 in neurons and myelin

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Abstract. Laser-scanning optical microscopes generally do not control the polarization of the exciting laser field. We show that laser polarization and imaging mode (confocal versus two photon) exert a profound influence on the ability to detect Ca^{2+} changes in both cultured neurons and living myelin. With two-photon excitation, increasing ellipticity resulted in a $\approx 50\%$ reduction in resting X-Rhod-1 fluorescence in homogeneous bulk solution, cell cytoplasm, and myelin. In contrast, varying the angle of a linearly polarized laser field only had appreciable effects on dyes that partitioned into myelin in an ordered manner. During injury-induced Ca^{2+} increases, larger ellipticities resulted in a significantly greater injury-induced signal increase in neurons, and particularly in myelin. Indeed, the traditional method of measuring Ca^{2+} changes using one-photon confocal mode with linearly polarized continuous wave laser illumination produced no appreciable X-Rhod-1 signal increase in ischemic myelin, compared to a robust $\approx 50\%$ fluorescence increase with two-photon excitation and optimized ellipticity with the identical injury paradigm. This underscores the differences in one- versus two-photon excitation and, in particular, the under-appreciated effects of laser polarization on the behavior of certain Ca^{2+} reporters, which may lead to substantial underestimates of the real Ca^{2+} fluctuations in various cellular compartments. © 2017 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.NPh.4.2.025002]

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1 Introduction

Fluorescence methods in the life sciences such as flow cytometry, fluorescence resonance energy transfer, two-photon excited fluorescence, fluorescence correlation spectroscopy, or super-resolution microscopy^{1–7} have revolutionized our ability to interrogate cells and tissues. One powerful technique involves the use of fluorophores or genetically encoded proteins that are sensitive to ions such as Ca^{2+} and H^+ , allowing measurements of Ca^{2+} and pH in live cells and organelles in real time. A typical application utilizes microscopic imaging of a sample by irradiation of a fluorescent reporter with light from a lamp in wide-field or by a focused laser spot in a point scanning instrument. The latter can make use of single-photon (e.g., confocal continuous wave) or multiphoton (usually two-photon) excitation to achieve optical sectioning. While some fluorophores allow ratio-metric measurements (either by exciting at two wavelengths such as with fura-2 or measuring emission at two wavelengths as with indo-1),^{8,9} most applications involve a semiquantitative measurement of fluorescence intensity to track relative changes in ionic concentration. Although the effects of varying illumination intensity and wavelength on responses of indicators are well understood,^{10,11} influences of polarization of exciting laser light have not been well studied.¹² Here, we report unexpectedly large differences in the fluorescence response of the Ca^{2+} indicator X-Rhod-1 as a function of polarization of the exciting laser light and significant differences depending on whether one- or two-photon excitation is used.

X-Rhod-1 is a chemically engineered Ca^{2+} indicator based on tetramethylrhodamine developed by Molecular Probes modeled on Roger Tsien's Rhod-2 dye;¹³ its longer excitation (580 nm) and emission (600 nm) wavelengths offer the advantage of avoiding excitation of endogenous cell fluorescence that may interfere with measured Ca^{2+} responses. X-Rhod-1 has been used for imaging mitochondria¹⁴ and cell cytosol of astrocytes,¹⁵ cultured neuroblastoma cells,¹⁶ cardiac monolayers,¹⁷ retinal glia cells,¹⁸ liver cell lines,¹⁹ sensory neurons,²⁰ oligodendrocytes, and myelin.^{21–25} Rhodamine dyes are also known to be photostable, have high absorption coefficients, and can be used in parallel with shorter wavelength dyes and fluorescent proteins.²⁶

Accurate quantification of Ca^{2+} in living cells or organelles provides important information for understanding physiological cell signaling pathways, as well as mechanisms of cellular and organellar injury. Overall functional integrity of the nervous system depends critically on health of glia, neurons, and axonal connections, both myelinated and unmyelinated. As in most cell types, Ca^{2+} overload appears to be the final common pathway of injury; therefore, reliable measurement of Ca^{2+} fluctuations is critical. During the course of our investigation of injury mechanisms of myelinated tracts of the central nervous system,²³ we observed that the state of polarization of the exciting laser field exerted a profound effect on the ability of X-Rhod-1 to reliably report intracellular Ca^{2+} changes. Here, we describe a technique that allows precise control of the polarization of the exciting light at the sample and report the significant effects that

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changes in polarization, in concert with the type of excitation (one- versus two-photon), exert on the Ca^{2+} -dependent fluorescent responses of this indicator.

2 Polarization-Controlled Ca^{2+} Imaging in Multiphoton and Confocal Laser-Scanning Systems

Two-photon fluorescence images were collected using a Nikon D-Eclipse-C1 confocal microscope (Nikon Instruments Inc. Melville, New York), custom modified for multiphoton imaging.²⁷ Samples were excited with 925-nm light generated by a Ti:sapphire laser (Tsunami; Spectra-Physics, Irvine, California). Emitted fluorescence was collected with a 525 ± 25 -nm bandpass and 590-nm long-pass filters (Chroma Technologies, Bellows Falls, Vermont), together with 735-nm primary (FF735-DiO2, Semrock, Rochester, New York) and 585-nm secondary (FF585-DiO1, Semrock, Rochester, New York) dichroics. Detection was performed by two photomultiplier tubes (Hamamatsu R5929; Hamamatsu Corporation, Bridgewater, New Jersey). Samples were placed on the stage of a Nikon E800 upright microscope and imaged with a water-immersion dipping objective (60×1.0 NA, Fluor, Nikon, Japan).

Two-photon fluorescence images were also acquired using a Bergamo II Rotating microscope (ThorLabs, Inc. Newton, New Jersey) using the galvo-galvo scan path. Samples were excited in two-photon mode at 925 nm using a Ti:Sapphire Chameleon Ultra II (Coherent, Santa Clara). Emitted fluorescence was collected using bandpass filters: FF03-525/50 (green), FF01-607/

70 (red), and FF705-DiO1LP primary dichroic and FF562-DiO3LP secondary dichroic (Semrock, Rochester, New York). Two-photon images were collected using a water-dipping N20X-PFH-20X Olympus XLUMPLFLN Objective, 1.00 NA (Olympus Scientific Solutions Americas Inc., Waltham, Massachusetts). Samples (X-Rhod-1 in a dish, cultured hippocampal neurons in a Petri dish, or fully myelinated rat optic nerves in an imaging chamber) were placed on a custom-made imaging stage.

For multiphoton imaging, the polarization state of the input laser beam at the sample (angle, ellipticity, and direction: clockwise versus counter-clockwise) and the power were controlled using a series of motorized waveplates placed in the laser beam prior to entering the microscope (Fig. 1). The power of the beam delivered to the microscope was controlled by a half-waveplate (WPLH05M-4500, Thorlabs, Newton, New Jersey) and a broadband polarizing beam splitter cube (PBS102, Thorlabs) that allowed only horizontally polarized light to pass. The resulting power at the sample plane was measured by a power meter (Thorlabs PM100D, Newton, New Jersey) with a S130C Si-photodiode detector. To control the polarization state of the laser beam, a broadband zero-order half-waveplate (WPLH05M-4500, Thorlabs, Newton, New Jersey) and a quarter-waveplate (WPLQ05M-4500, Thorlabs, Newton, New Jersey) controlled the angle and ellipticity of the beam, respectively. All three waveplates were placed in motorized precision rotation mounts (PRM1Z8, Thorlabs, Newton, New Jersey) and connected to T-cube DC Servo Controllers (TDC001, Thorlabs, Newton, New Jersey) to provide automated rotation of the plates. The positioning of the waveplates was monitored by custom

Fig. 1 Schematic representation of the polarization microscopy setup: A pulsed laser beam with $\lambda = 925$ nm is power controlled by rotating the polarization via a half-waveplate ($\lambda/2$) and then splitting the rotated laser beam into orthogonal s and p components via a PBS. The p-component of the beam next travels through a half- and quarter-waveplate ($\lambda/2$, $\lambda/4$) that manipulates the polarization state of the beam. The system compensates for polarization and power changes due to the optical path using motorized rotation mounts to adjust both half-waveplates and the single-quarter waveplate. The polarization is calibrated by measuring the polarization state and average power at the sample. A polarimeter measured the polarization state of the beam at the sample while a power meter measured the power of the beam at the sample. The power and polarization information were fed to custom-designed LabView software that manipulated the position of the motorized waveplates to achieve the desired polarization state at the sample while maintaining a constant optical power. The resulting waveplate positions were entered into a lookup table, allowing each state and power level to be recalled during imaging of a sample. The lookup tables were calibrated and maintained by additional LabView software, allowing the polarization and power stability to be confirmed before each imaging session.

LabView software (National Instruments, Austin, Texas) and the advanced positioning technology framework (Thorlabs, Newton, New Jersey). Polarization (ellipticity, angle θ) and beam power were measured at the sample (under the objective lens of the microscope) with a polarimeter (Meadowlark Optics D3000, Frederick, Colorado) and power meter. Calibration for output power and polarization was performed before each experiment using additional custom LabView software. The combined software package interfaced with the polarimeter, power meter, and the waveplate rotation servos and was synchronized with the two-photon microscope imaging system via a transistor–transistor logic triggering pulse. This permitted the software to automatically adjust the polarization and laser power at the sample plane before triggering acquisition of an image. The pulse was received in Nikon EZC1 software (on the C1) or ThorImage LS (on the Bergamo 2), triggering acquisition of an image when the correct power and polarization state were achieved.

Confocal images of fluorescent samples (X-Rhod-1 in HEPES buffer, cultured hippocampal neurons, myelin in optic nerve; Appendix B) were obtained using a commercial confocal system (Nikon D-Eclipse C, Nikon Instruments Inc., Melville). For excitation, the 488-nm line of an Argon laser (Spectra Physics Lasers, Mountain View, California) and the 594-nm line of a HeNe laser (JDS Uniphase, Manteca, California) were used. Fluorescence emission was separated using Omega Optical 630 ± 15 nm and 540 ± 30 nm filters (Chroma Technologies, Brattleboro, Vermont).

To change the ellipticity of the 594-nm laser line in the confocal C1-Nikon imaging system from 0.2 to linear, a linear polarizer ($\varnothing$ 12.5 mm SM05-Mounted Linear Polarizer, 500- to 720 nm-LPVISB050-MP, Thorlabs, Newton, New Jersey) was inserted into the laser path in the scan head of the microscope. The resulting ellipticity and power were measured to confirm the state at the sample. The insertion of the polarizer resulted in a slight power loss at 0 ellipticity versus 0.2 ellipticity, which was compensated for via a neutral density filter positioned in the laser path for the 0.2 case to maintain comparable power levels.

3 Results and Discussions

We examined the effects of various polarization states of exciting light on the Ca^{2+} -dependent fluorescence of X-Rhod-1. The tri-potassium salt of this indicator was dissolved to a concentration of 40 μM in HEPES buffer at pH 7.4 with saturating 2 mM Ca^{2+} , ensuring maximal fluorescence. Pulsed laser light with linear polarization (ellipticity = 0) at the sample was used for two-photon excitation of the dye solution while the polarization angle θ was varied from -90 deg to $+90$ deg. As expected, varying θ had little effect on fluorescence intensity of this dye, expected to be randomly oriented in bulk solution [Fig. 2(a)]; in contrast, gradually increasing ellipticity while maintaining constant power at the sample resulted in a substantial drop in intensity [Fig. 2(b)], but only with two-photon excitation [with one-photon excitation, ellipticity exerted a negligible effect, Fig. 2(c)]. This is in agreement with previous reports.^{28,29}

We then investigated dynamic changes in X-Rhod-1 emission as a function of $[\text{Ca}^{2+}]$ and the influence of polarization on the ability of this dye to report Ca^{2+} fluctuations in living cells. Cultured hippocampal neurons bath-loaded with X-Rhod-1-AM and imaged in two-photon mode at rest exhibited similar behavior to the homogeneous dye solution [Fig. 3(a)–3(c)],

confirming that dye molecules are also randomly oriented in the neuronal cytoplasm. However, the Ca^{2+} -dependent increase of X-Rhod-1 emission in anoxic neurons was strongly dependent on ellipticity, in both one-photon confocal mode and even more so with two-photon excitation [Fig. 3(d)]; for the same paradigm, the percent fluorescence rise doubled as polarization was changed from linear to 0.4 ellipticity. Although the absolute intensity diminished at greater ellipticity [Fig. 3(c)], the anoxic Ca^{2+} -dependent rise was significantly augmented [Fig. 3(d)].

The myelin sheath is a compact regular wrap of numerous layers of lipid rich membrane produced by myelinating glia to electrically insulate axons³⁰ and it exhibits important Ca^{2+} fluctuations within its thin cytosolic spiral in response to physiological and pathological stimuli.^{23,25} We, therefore, studied the effects of varying polarization on the fluorescence of dyes partitioned into this highly anisotropic compartment [Fig. 4(a)]. Given the small features of myelin, with fluid-filled spaces on the order of only several nanometers in thickness,³¹ it is likely that any fluorescent molecules partitioned into this structure would assume an ordered orientation and, therefore, would be potentially influenced by polarization states of exciting light. We first measured the behavior of the green Ca^{2+} -independent lipophilic probe DiOC6(3), whose acyl chains facilitate its partitioning into lipid-rich membrane leaflets, leading to a large rise in quantum yield within the hydrophobic environment of a cell membrane or myelin³² [Fig. 4(a)]. In contrast to dyes in bulk solution and neuronal cytoplasm, varying θ while maintaining linear polarization induced a substantial decrease in fluorescence intensity as θ was varied from its optimal orientation of 0 deg to ± 90 deg [Fig. 4(b)], suggesting a high degree of order of the DiOC6(3) molecules. We have previously shown that the acetoxymethyl ester of X-Rhod-1 loads into the major dense line

Fig. 2 Effects of laser polarization on fluorescence intensity of X-Rhod-1 in bulk solution. (a) Linearly polarized laser light at various angles caused insignificant differences in measured intensity (two-photon excitation). (b) In contrast, increasingly elliptical polarization resulted in a significant reduction in intensity, which was independent of left or right circular directions for this nonchiral molecule (two-photon excitation; orange). (c) Increasing elliptical polarization (while maintaining constant power at the sample) had a significant effect on fluorescence with a substantial decrease in intensity, but only during two-photon excitation ($13\% \pm 0.9\%$ decrease, $P = 1.2 \times 10^{-7}$ compared to linear polarization). In contrast, changing ellipticity during one-photon excitation (blue) had a negligible effect ($3\% \pm 1\%$ increase, $P = 0.03$).

Fig. 3 Effect of polarization on Ca^{2+} -dependent X-Rhod-1 fluorescence in cultured neurons. (a) Example of micrographs of an X-Rhod-1-loaded neuron at different ellipticities recorded using two photon imaging. (b) and (c) As in bulk solution (Fig. 2), varying θ while maintaining linear polarization had little effect on fluorescence intensity, whereas increasing ellipticity substantially reduced emission with multiphoton excitation. (d) Neurons exposed to chemical anoxia underwent a substantial increase in cytosolic Ca^{2+} levels reported by an increase in X-Rhod-1 fluorescence. This increase varied substantially depending on the degree of ellipticity, which was most pronounced with two-photon (orange) than with one-photon excitation. Numbers within bars represent number of ROIs analyzed (neurons)/number of independent experiments. Bars are mean $\pm$ SEM. Scale bar in (a) is 5 μm .

of myelin, the thin cytosolic spiral that is in continuity with the cytosol of the parent glial cell.²² The effect of varying θ on X-Rhod-1 signal from myelin was similar but less pronounced ($-29\% \pm 5\%$ change at $\theta = 0^\circ \pm 90^\circ$) than with DiOC6(3) ($-55\% \pm 11\%$ change at $\theta = 0^\circ \pm 90^\circ$) [Fig. 4(b)], suggesting that the Ca^{2+} reporter probe was also ordered within myelin, but to a lesser degree than the highly lipophilic DiOC6(3). Interestingly, varying ellipticity while maintaining θ at optimal values resulted in a pattern similar to that in bulk solution or homogeneous neuronal cytoplasm [$\approx 40\%$ decrease at ellipticities ± 0.6 ; Fig. 4(c)].

Energy deprivation in the form of anoxia or ischemia is known to increase myelinic Ca^{2+} levels.^{23,33} Given the ordered nature of dye molecules within this structure, we examined whether excitation mode and/or polarization would influence the magnitude of measured Ca^{2+} -dependent X-Rhod-1 fluorescence rise after 30 min of chemical ischemia [Figs. 4(d) and 4(e)], as it

did in neuronal cytoplasm. Surprisingly, one-photon excitation using confocal mode caused an insignificant increase in signal even after 30 min of profound chemical ischemia when polarization was maintained linear (ellipticity = 0) at the sample [$2\% \pm 2\%$ increase, $P = 0.07$ versus time-matched control; Fig. 4(d)]. Only when ellipticity was increased to 0.2 did the same paradigm result in a modest but significant rise in Ca^{2+} -dependent fluorescence ($12\% \pm 2\%$ increase after 30 min). In contrast, two-photon excitation induced substantially larger fluorescence changes: at 0 ellipticity (linear polarization), 30 min of ischemia induced a $12\% \pm 1\%$ fluorescence rise, which increased further to $\approx 50\%$ at ellipticity ± 0.2 [Fig. 4(e)]. Interestingly, in myelin an optimum was reached at ellipticity 0.2 with the Ca^{2+} -dependent rise diminishing at ellipticity 0.4 compared to 0.2. This is in contrast to the disordered indicator in neuronal cytoplasm where the rise in Ca^{2+} signal continued to increase at ellipticities greater than 0.2 [Fig. 3 (d)]. As in other paradigms, left versus right rotation of polarization

Fig. 4 Effect of polarization on X-Rhod-1 behavior in highly anisotropic myelin. (a) In contrast to bulk solution and cell cytoplasm, dyes [DiOC6(3) (green) and X-Rhod-1 (red)] partitioned into myelin in a highly ordered manner as evidenced by a substantial reduction of intensity as linearly polarized exciting light is rotated from the optimal 0 deg to 90 deg. Fluorescence from the larger glial process (*) where dyes are unordered was not heavily influenced by polarization. (b) Graphical representation of fluorescence from myelin as a function of polarization angle. (c) Effects of ellipticity were similar to those in solution and cell cytoplasm [two-photon excitation in (a)–(c)]. (d) Ca^{2+} -dependent X-Rhod-1 signal from myelin after 30 min of chemical ischemia excited by one-photon absorption (confocal); a detectable increase was only observed at higher ellipticity. With two-photon excitation (e), the signal increase was greater than with confocal and exhibited an optimum at ellipticity ≈ 0.2 . aCSF: time-matched measurements without ischemia. Signal from the chemically related Ca^{2+} -insensitive dye 5(6)-ROX exhibited no signal change after 30 min of ischemia. (d) and (e) Numbers above/below /within bars represent number of ROIs analyzed (myelin)/number of optic nerves studied. Bars are mean $\pm$ SEM Scale bar in (a) is 5 μm .

at ellipticities > 0 showed no differences. The chemically related Ca^{2+} -insensitive fluorophore 5(6)-ROX showed no changes in fluorescence with chemical ischemia, excluding other changes in ischemic myelin as potential causes for the signal increase of X-Rhod-1 [Fig. 4(e)].

The effects of elliptical polarization on the dynamic changes in X-Rhod-1 fluorescence induced by increases in Ca^{2+} was unexpected and has significant implications on estimates of Ca^{2+} changes in various experimental paradigms. The effect is particularly pronounced in myelin, where dye molecules, especially those partitioned into the sheath such as X-Rhod-1, assume an ordered arrangement as they intercalate into myelin leaflets. The differences are particularly pronounced when comparing linear polarization using one-photon excitation (confocal), where seemingly no Ca^{2+} change was observed, versus two-photon excitation at the optimized ellipticity of 0.2, where a 50% fluorescence increase was measured in otherwise exactly the same experimental paradigm (Fig. 4).

One explanation likely involves a gradual disordering of myelin as ischemia proceeds,^{21,23} which would tend to reduce

fluorescence below baseline as a larger proportion of molecules are free to rotate from optimum orientation. Combined with a Ca^{2+} rise (which increases X-Rhod-1 fluorescence), the net effect may be either no change or an increase in signal depending on which phenomenon predominates. In addition, the $\cos^2(\theta)$ versus $\cos^4(\theta)$ relationship of photon absorption for one- versus two-photon excitation (respectively), where θ is the angle between the polarization of the exciting laser field and the dipole moment of the dye molecule,³⁴ will impart additional important differences between the two excitation modes of X-Rhod-1. Ellipticizing the exciting field sacrifices some initial signal [Fig. 4(c)] but provides an orthogonal component that will contribute to the excitation of rotated dye molecules in increasingly disordered myelin, resulting in an optimal net fluorescence rise at modest ellipticities, as was observed.

This photophysical phenomenon is the likely explanation for a recent study suggesting no myelinic Ca^{2+} increase reported by X-Rhod-1 in response to activation of *N*-methyl-D-aspartate receptors;²¹ these authors used one-photon excitation in confocal mode, and, although the polarization state of their exciting

light was not reported, as Appendix E-Table 1 shows, most modern confocal systems maintain near-linear polarization at the sample (≈ 0 ellipticity), precisely the characteristics that result in a very weak fluorescence increase from the myelin compartment as shown in Fig. 4. In contrast, our work with myelin Ca^{2+} imaging was conducted with two-photon excitation and with ellipticity deliberately optimized at ≈ 0.2 to maximize the Ca^{2+} -dependent signal changes reported by X-Rhod-1 from this compartment.²⁵

In the case of signal reduction for two photon elliptic states versus pure linear polarization, molecular transition rules may impact the probability of two-photon absorption events. The net angular momentum change for electronic transitions in most fluorophores is zero, requiring the net angular momentum of the two-photon absorption event to be zero. This is achieved when one photon is polarized clockwise and the other counterclockwise (pure linear polarization), leading to a cancellation of angular momentum and optimal two-photon absorption.^{35,36} As a result, increasingly elliptical polarization, with an increasing mismatch between probabilities of clockwise versus counterclockwise photon pairs, will result in less efficient two-photon excitation and a reduction in fluorescence as we observed (Figs. 2 and 3).^{35,36}

Our results underscore the important effects of the polarization state of exciting laser light on the dynamic changes of certain Ca^{2+} reporters such as X-Rhod-1. In particular, linearly polarized one-photon excitation may yield significant underestimates of the degree of Ca^{2+} increase, which becomes more important as reporter molecules become ordered, as in the confined spaces of anisotropic structures such as the highly regular myelin sheath of nerve fibers.

Appendix A: Hippocampal Cell Culture and Optic Nerve Preparations

Experimental animal protocols were approved by the University of Calgary Animal Care Committee and followed the Canadian Council on Animal Care guidelines.

Primary cultures of hippocampal neurons were prepared from C57B/L6 mouse embryos at day 18 (E18).³⁷ Pregnant female mice were deeply anesthetized with 80% CO_2 /20% O_2 and euthanized by cervical dislocation. The intact uterus was removed via C-section and placed on ice in a Petri dish containing dissection buffer: Hanks' balanced salt solution without Ca^{2+} / Mg^{2+} (Thermo Fisher 14170-112) supplemented with 10 mM HEPES (ThermoFisher 15360-080), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (Thermo Fisher 10378-018). Embryos were detached from the uterus and placed in a Petri dish containing the same ice-cold dissection buffer, and the brains were dissected out. Next, brains were cut along the midline and hippocampi were carefully cut from the cortex. Harvested hippocampi were minced and then mechanically dissociated in 0.05% Trypsin/EDTA (Thermo Fisher 25300-054) at 37°C for 15 to 18 min with vigorous shaking every 3 min. To terminate trypsinization, hippocampi were neutralized in Neurobasal medium (Thermo Fisher 21103-049) supplemented with 20% heat-inactivated goat serum (Thermo Fisher 16210-064) followed by 30 strokes of trituration using a 10-ml serological pipette (VWR 89130-898). Then, the tissue suspension was spun down at 80 g for 5 min at room temperature and the supernatant discarded. The pellet was resuspended evenly in neurobasal medium supplemented with 2% B-27 supplement

(Thermo Fisher 17504-044), 2 mM GlutaMAX (Thermo Fisher 35050-061), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (Thermo Fisher 10378-018) by 30 strokes of trituration in a 5-ml serological pipette (VWR 89130-886) before being filtered through a 70- μm cell strainer (VWR CA21008-952). Resuspended cells were plated onto poly-ornithine and fibronectin-coated glass bottomed imaging dishes and placed in a 5% CO_2 37°C incubator. Culture media was half changed every 72 h. Cells were grown for 10 to 14 days in the incubator before use.

To prepare the optic nerve, adult Long Evans rats (Charles Rivers Laboratories, Montreal, Canada) (200 to 250 g) were deeply anesthetized with 80% CO_2 /20% O_2 and decapitated, and nerves were immediately excised on ice in 0.5 mM Ca^{2+} -CSF buffer containing (in mM) NaCl 126, NaHCO_3 26, KCl 3, NaH_2PO_4 1.25, MgSO_4 2, CaCl_2 0.5, and D-glucose 10; and oxygenated by carbogen 95% O_2 /5% CO_2 , pH 7.4.

Appendix B: Dye-Loading of Cells and Tissues

Cultured hippocampal neurons were loaded with 2 μM of the membrane-permeable Ca^{2+} indicator X-Rhod-1 (as the acetoxymethyl ester; Thermo Fisher Scientific, Waltham, Massachusetts, X14210) in cell culture media for 1 h at 37°C, and then rinsed and incubated for 30 min with the same culture media to allow de-esterification of the dye. For imaging, neurons were bathed in aCSF containing (in mM) 148 NaCl, 3 KCl, 3 CaCl_2 , 1 MgCl_2 , 10 HEPES, 8 glucose, 0.5 μM CuSO_4 , and pH 7.4. Chemical anoxia was induced by the addition of 2 mM sodium azide.

Myelinic Ca^{2+} fluctuations were measured as previously described.²² Adult rat optic nerves were incubated at room temperature in 0.5 mM Ca^{2+} -aCSF buffer for 2 h with the Ca^{2+} indicator X-Rhod-1 acetoxymethyl ester (40 μM) or with the 5(6)-ROX [5-(and-6)-carboxyl-X-rhodamine] (AS-81110, Anaspec, Fremont, California), an X-Rhod-1 analog that lacks the Ca^{2+} indicator moiety, together with the green lipophilic dye DiOC6(3) (1 μM) (3,3'-dihexyloxacarbocyanine iodide; Thermo Fisher Scientific-D273) to clearly outline myelin sheaths [Fig. 4 (a)]. Nerves were transferred to aCSF at 35°C for 30 min before imaging to allow de-esterification and washout of excess dye. Experiments were performed in aCSF buffer containing (in mM) NaCl 126, NaHCO_3 26, KCl 3, NaH_2PO_4 1.25, MgSO_4 2, CaCl_2 2, and D-glucose 10; and bubbled with 95% O_2 /5% CO_2 , pH 7.4 at 36°C. Chemical ischemia was induced by equimolar replacement of glucose with sucrose and the addition of the mitochondrial inhibitor sodium azide (2 mM).

X-Rhod-1 trisodium salt, 40 μM (Thermo Fisher Scientific, Waltham, Massachusetts, X14209), was dissolved in HEPES buffer (in mM) 10 mM HEPES, 150 mM NaCl, 2 mM CaCl_2 , and pH 7.4.

Appendix C: Image Acquisition and Processing

For imaging, optic nerves were placed in a chamber (RC-27LD, Harvard Apparatus, St. Laurent, Quebec, Canada) and were immobilized under a net made of Lycra. Nerves were continuously perfused by a peristaltic pump (Gilson Minipuls 3, Mandel, Guelph, Ontario, Canada) and bath temperature maintained at 36°C (TC 324B, Warner Instruments, LLC, Hamden, Connecticut). All solutions were aerated with a 95% O_2 /5%

Table 1 Polarization measurements obtained from five commercial laser scanning microscopes. Polarization states of the laser beam at the sample were measured as described for different objective lenses and wavelengths. For the majority of confocal and multiphoton microscopes, the polarization state was highly linear (ellipticity < 0.1) although the exact degree of ellipticity/linearity varied with the different combinations of objectives and microscopes.

Microscope	Objective	λ (nm)	V (μ W)	H (μ W)	Ellipticity	Direction
Nikon C1Si	40 $\times$ 1.3 NA	405	0.657	0.024	0.037	V
		488	2.77	0.127	0.046	V
		638	0.007	0.208	0.033	H
	20 $\times$ 0.75 NA	405	6.07	0.311	0.051	V
		488	14.08	0.52	0.037	V
		638	0.038	0.77	0.049	H
	10 $\times$ 0.45 NA	405	29.5	3.72	0.13	V
		488	35.6	1.048	0.03	V
		638	0.039	2.47	0.02	H
	Nikon A1	405	8.58	0.559	0.06	V
		488	1.143	0.045	0.04	V
		561	8.24	0.534	0.06	V
		640	8.56	0.645	0.07	V
Zeiss LSM880	20 $\times$ 0.8 NA	405	12.02	3.47	0.29	V
		488	8.12	1.62	0.20	V
		561	11.08	2.38	0.21	V
		640	11.04	2.54	0.23	V
	63 $\times$ 1.4 NA	405	8.44	315	0.03	H
		488	58.2	236	0.25	H
		561	14.37	1180	0.01	H
		633	4.82	318	0.01	H
	40 $\times$ 1.3 NA	405	2.84	41.8	0.07	H
		488	7.99	28.5	0.28	H
		561	8.76	148.2	0.06	H
		633	2.41	35.9	0.07	H
Olympus FV1000 confocal	20 $\times$ 0.75 NA	488	35.2	20.1	0.57	V
		559	132.7	59.8	0.45	V
		635	54.5	20.5	0.38	V
	40 $\times$ 1.3 NA	488	11.03	6.37	0.58	V

Table 1 (Continued).

Microscope	Objective	λ (nm)	V (μ W)	H (μ W)	Ellipticity	Direction
Olympus FV1000 multiphoton	20 $\times$ 1.0 NA	559	32.2	9.78	0.30	V
		635	13.35	2.45	0.18	V
		710	3250	816	0.25	V
	40 $\times$ 0.8 NA	850	2130	210	0.1	V
		950	2950	243	0.08	V
		710	371	91.2	0.25	V
		850	204	24.9	0.12	V
		950	224	70.2	0.31	V

Note: V, power measured with the polarizer oriented vertically relative to the stage.

H, power measured with the polarizer oriented horizontally relative to the stage.

λ , wavelength of the laser in nm.

Ellipticity, the highest measured power divided by the lowest measured power of H and V.

Direction, indication of which of H or V had the highest intensity thus indicating the predominant direction of polarization relative to the microscope stage.

CO₂ gas mixture. A Petri dish containing X-Rhod-1 in a solution of cultured neurons was placed on the microscope stage and immobilized. Images of X-Rhod-1 or 5,6 ROX and DiOC6(3) stained myelin in optic nerve were recorded every 2 min for 45 min in two-photon imaging mode and every 15 min for 45 min in confocal mode to avoid photobleaching. Images of cultured neurons stained with X-Rhod-1 were recorded every 5 s for 10 min.

Images of bulk X-Rhod-1 or 5,6 ROX in solution, cells, and myelin were imported into ImageTrak software (written by P.K. S.)³⁸ for visualization and analysis. Entire cells or myelin regions (2 to 3 μ m long and $\sim$ 0.5 μ m wide) were randomly selected for analysis after 3 min of anoxia for neurons or 30 min of ischemia for myelin, times at which Ca²⁺ increases plateaued and were maximal. Three to seventeen experiments were replicated for each polarization state. Changes induced by treatments are reported as the percent fluorescence change with respect to baseline intensity ($\Delta F/F_0$) for each region of interest (ROI).

Appendix D: Statistics

Data are presented as mean $\pm$ SEM. N represents the number of optic nerves or dishes in each group, and n indicates the number of ROIs for myelin in optic nerve or hippocampal neurons. First, the homogeneity of variance was assessed using Levene and Bartlett's tests. Statistical significance was determined using ANOVA with the nonparametric multiple comparison; Dunn–Holland–Wolfe test or Wilcoxon rank test were used as appropriate, unless otherwise noted. Igor software was used for statistical analysis (WaveMetrics, Lake Oswego, Oregon).

Fig. 5 Dichroics exhibited only minor effects on detection of polarized fluorescence. Fluorescent green and red bulk plastic slides excited with linearly polarized 925 nm fs laser light. Linearly polarized fluorescence was recorded while the angle of polarization at the sample varied between -90 deg to $+90$ deg at 15 deg increments. (a) Fluorescence intensity versus angle for the green slide using both primary and secondary dichroics by less than 5%. (b) The red slide with excitation light passing through the primary dichroic only showed less than 8% change in fluorescence intensity (black) and less than 3% for the combination of primary + secondary dichroics. Taken together, these results show that while the optical train imparts a very minor effect on detected fluorescence intensity when passing/reflecting polarized emission (mainly due to dichroic elements), the large effects observed from biological samples (Figs. 3 and 4) were real and not artifacts induced by microscope optics.

Appendix E: Measurement of the Polarization State at the Sample for Five Commercial Laser-Scanning Microscopes

After observing the substantial impact of polarization state, the question was raised regarding the default polarization state from “typical” confocal microscopes. To get a sense of the polarization state of the laser at the sample, we performed measurements on several commercial confocal and multiphoton laser-scanning microscopes for different lenses and excitation wavelengths (Table 1).

We performed measurements of the polarization state at the sample on five commercial laser-scanning microscopes for different objectives, wavelengths, and imaging modes (confocal versus two photon) (Table 1).

Polarization of the laser beam at the sample was measured using a power meter (Thorlabs PM100D, Newton, New Jersey) with Si Photodiode detector (Thorlabs, S130C) and a rotating linear polarizer (Thorlabs, LPVISA050). The power of the beam with the polarizer oriented vertically (V) or horizontally (H) relative to the microscope stage was measured and reported in μW . The ellipticity was calculated by dividing the minimum versus maximum power, with the dominant direction (V or H) noted based on which direction recorded the maximum value. Linear states have an ellipticity value <0.1 ($V \gg H$ or $H \gg V$), while a perfectly circular state has an ellipticity of 1.0 ($V = H$).

For the majority of confocal or multiphoton microscopes studied, the polarization state was highly linear (ellipticity <0.1) with the exception of the Olympus FV 1000 Confocal microscope that exhibited ellipticities between 0.2 and 0.58 depending on the laser wavelength. A Zeiss LSM880 also deviated somewhat from linear with an ellipticity of ≈ 0.25 at 488 nm. Based on our observations, the polarization state is dependent on a combination of the microscope and the objective lens. However for some systems, one factor or the other tended to dominate: for the Nikon C1Si the polarization state at the sample was linear and was independent of the objective or wavelength. In contrast, for the Nikon A1, the polarization depended on the objective, with the $10\times/0.45$ NA objective exhibiting elliptical polarization ≈ 0.2 , whereas a higher performance $25\times/1.1$ NA objective resulted in ellipticity ≤ 0.07 across a

wide wavelength range. The Zeiss microscope exhibited linear polarization regardless of objective, except at 488 nm where ellipticity was ≈ 0.2 , suggesting the laser itself was somewhat ellipticized. The Olympus Confocal microscope showed elliptical polarization that was slightly dependent on the wavelength of the laser beam. For the Olympus multiphoton system, the polarization state was not dependent on the lens; however, it was dependent on wavelength, implying a wavelength-dependent birefringence in the system. The variability in polarization states at the sample for different systems and optics may significantly affect detection of the fluorescence intensity changes in some biological samples.

Appendix F: The Role of the Dichroic in the Excitation and Emission Path

Angled dichroic filters may impact the polarization of the excitation or emission light within the microscope. To examine the influence of the dichroic on the emission path, we used a fluorescent green and red plastic slide standard (Chroma). The solid plastic of the slides prevents the contained fluorescent dipoles from rotating in the excited state, so the orientation of the emitted light is the same as the incident light. Green and red slides were used to examine whether the difference in the emission wavelength influenced the system response to polarization. Two-photon excited images of the slides were recorded with linear polarization from -90 deg to $+90$ deg as shown in Fig. 5.

Disclosures

The authors have no financial or potential conflicts of interest.

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