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Near infrared two-photon excitation cross-sections of voltage-sensitive dyes

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Abstract

Microscopy based on voltage-sensitive dyes has proven effective for revealing spatio-temporal patterns of neuronal activity in vivo and in vitro. Two-photon microscopy using voltage-sensitive dyes offers the possibility of wide-field visualization of membrane potential on sub-cellular length scales, hundreds of microns below the tissue surface. Very little information is available, however, about the utility of voltage-sensitive dyes for two-photon imaging purposes. Here we report on measurements of two-photon fluorescence excitation cross-sections for nine voltage-sensitive dyes in a solvent, octanol, intended to simulate the membrane environment. Ultrashort light pulses from a Ti:sapphire laser were used for excitation from 790 to 960 nm, and fluorescein dye was used as a calibration standard. Overall, dyes RH795, RH421, RH414, di-8-ANEPPS, and di-8-ANEPPDHQ had the largest two-photon excitation cross-sections ($\sim 15 \times 10^{-50}$ cm^4 s photon^{-1}) in this wavelength region and are therefore potentially useful for two-photon microscopy. Interestingly, di-8-ANEPPDHQ, a chimera constructed from the potentiometric dyes RH795 and di-8-ANEPPS, exhibited larger cross-sections than either of its constituents.

Keywords: Voltage-sensitive dyes; Potentiometric dyes; Two-photon microscopy; Cross-sections; Styryl dyes; Nonlinear optical properties; Chimeric dyes

1. Introduction

Functional imaging of neuronal activity in three dimensions offers new possibilities for understanding brain physiology. Since the functional architecture of the brain is three-dimensional, one goal of neuroimaging is to accurately resolve neuronal activity in three-dimensions with high spatial- and temporal-resolution. The work of Llinas and others, for example, has shown that large-scale aspects of brain function can originate from electrical properties of individual neurons (Llinas, 1988; Llinas et al., 1998), underscoring the need for cellular and sub-cellular scale imaging. Measurements of membrane potential in small neurons and their processes, however, are extremely difficult using traditional electrode techniques. Measurements of macroscopic tissue volumes are similarly difficult, requiring large impractical electrode arrays.

The use of voltage-sensitive (potentiometric) dyes as molecular voltmeters (Cohen and Salzberg, 1978; Salzberg, 1983) is currently the only optical technique enabling direct measurements of neuronal membrane potential. The sensitivity of this methodology ranges from ∼10 mV scales, relevant for subthreshold membrane potential dynamics, to the ∼100 mV scales associated with action potentials. Voltage-sensitive dyes have proven to be effective for measuring electrical activity in neurons in vitro (Contreras and Llinas, 2001; Grinvald et al., 1983; Salzberg et al., 1973, 1977; Yuste et al., 1997) and in vivo (Grinvald et al., 1994; Orbach and Cohen, 1983; Petersen et al., 2003). To date, several studies have visualized voltage-sensitive dye responses in three-dimensions using one-photon fluorescence in vivo (Kleinfeld and Delaney, 1996; Petersen et al., 2003), and recently gradient-index (GRIN) lens optics and computational optical sectioning techniques have been used to achieve

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high-speed three-dimensional microscopy with voltage-sensitive dyes in near surface tissues (Fisher et al., 2004). However, in all these studies optical sectioning is inherently limited by one-photon fluorescence microscopy techniques; i.e. the images are sensitive to fluorescence from the entire depth of focus and do not explicitly reject out-of-focus light.

Two-photon laser scanning microscopy (Denk et al., 1990) enables true three-dimensional imaging because of its intrinsic optical sectioning properties. Additionally, high contrast images can be obtained from deeper within biological tissues compared to confocal microscopy (Centonze and White, 1998). Since its inception, two-photon laser scanning microscopy has found wide-spread applicability throughout the field of neuroscience, both in vitro (Mainen et al., 1999; Yuste et al., 1997) and in vivo (Helmchen et al., 2001; Stosiek et al., 2003; Svoboda et al., 1999; Yoder and Kleinfeld, 2002). Calcium Green, GFP, and other fluorophores with absorption peaks in the blue are among the most widely used dyes for in vivo studies, in part because their peak absorption wavelength can be reached by two photons within the tuning range of most commercially available pulsed laser sources. Calcium-indicators yield large signals following two-photon excitation and can reveal rapid intracellular Ca^{2+} concentration changes dependent upon action potentials (Svoboda et al., 1997), but they most certainly do not provide a direct measurement of electrical activity.

Second harmonic generation (SHG) imaging (Hellwarth and Christensen, 1974; Sheppard et al., 1977) also provides inherent optical sectioning, and has recently been used in conjunction with voltage-sensitive dyes (Campagnola et al., 1999; Dombeck et al., 2004; Millard et al., 2003) to produce exceptional images of activity in cultured neurons. However, because SHG is a parametric nonlinear process, the resulting second harmonic wave travels predominantly in the same direction as the incident light (Mertz and Moreaux, 2001) and yields signal in the reflectance direction only after backscattering. By contrast, even at the scale of a single fluorophore, fluorescence emission following two-photon absorption of polarized light by membrane-bound voltage-sensitive dyes leads to a symmetric dipole radiation distribution (Lakowicz, 1999). In sub-surface tissue imaging conditions, where the excitation volume is small compared to the imaging depth, photons are generally assumed to be emitted isotropically (Oheim et al., 2001). These factors make two-photon microscopy preferable to SHG for backward detection, a critical criterion for in vivo imaging.

Albota, Xu and Webb have measured two-photon excitation cross-sections for a variety of biologically relevant molecular fluorophores (Albota et al., 1998; Xu, 2000; Xu and Webb, 1996). Very little information, however, exists on the suitability of voltage-sensitive dyes for two-photon imaging purposes (Hess and Webb, 1998). To this end, we have analyzed the two-photon spectral properties of some of the most common voltage-sensitive dyes, including recent novel dyes di-8-ANEPPDHQ (Obaid et al., 2004) and RH1692 (Shoham et al., 1999). We also included in our study Nile Blue A, which is a lipophilic membrane-permeant potentiometric dye (Beeler et al., 1981; Cohen et al., 1974; Vergara et al., 1978). Using a ratiometric method (Albota et al., 1998) with fluorescein as a reference, two-photon excitation cross-sections were obtained for nine voltage-sensitive dyes at incident wavelengths ranging from 790 to 960 nm. We used octanol as a solvent to approximate the environment of these dyes when bound to membrane (Sims et al., 1974) and we identified dyes with comparatively high cross-sections in this spectral region. Our investigation of the novel napthylstyryl-pyridinium "chimera" dye di-8-ANEPPDHQ, which is a combination of an RH795 (Grinvald et al., 1994) quarternary ammonium head group and a di-8-ANEPPS (Bedlack et al., 1992) chromophore, revealed that its two-photon excitation cross-section in this spectral region was larger than that of either of its constituent components.

2. Materials and methods

2.1. Background theory

In two-photon absorption, an atom or molecule simultaneously absorbs two photons and makes a transition from its ground state to an excited state. Assuming no linear (i.e. one-photon) absorption, the two-photon absorption of light propagating through an optically thin sample of C molecules per unit volume (cm^{-3}) is characterized by the following differential equation for the input light intensity I:

$$\frac{dI}{dz} = -\beta I^2. \tag{1}$$

Here I is the source intensity ($erg\,cm^{-2}\,s^{-1}$), and β is the two-photon absorption coefficient. The two-photon absorption coefficient, β, can also be expressed in terms of the two-photon cross-section, Σ, i.e.

$$\beta = 2C\frac{\Sigma}{\hbar\omega}, \tag{2}$$

where ω is the angular frequency of the incident light field; the factor of 2 arises because two photons are absorbed, and Σ has cgs units of $cm^4\,s$. In Eq. (2), $\hbar\omega$ is the energy per photon ($erg\,photon^{-1}$) at the excitation wavelength. The informal unit for Σ is the Goeppert-Mayer (GM), where $1\,GM = 10^{-50}\,cm^4\,s\,photon^{-1}$. Σ is described as a "cross-section" in order to establish a two-photon analog to the linear (one-photon) absorption cross-section, which has true units of area. The extra factor of cm^2 is due to the extra factor of I in Eq. (1).

The two-photon absorption cross-section, in turn, can be defined in terms of the material third-order susceptibility, $\chi^{(3)}$, i.e.

$$\Sigma = \frac{4\pi^2\hbar\omega^2\,\mathrm{Im}\,\chi^{(3)}}{C\eta^2c^2}. \tag{3}$$