Sensitivity of Second Harmonic Generation from Styryl Dyes to Trans-Membrane Potential

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Running Title: SHG Imaging of Membrane Potential

Keywords: non-linear optical imaging, electrophysiology, two-photon excitation fluorescence

Supplemental Material

TABLE OF MIRROR AND FILTER COMBINATIONS FOR SHG DETECTION

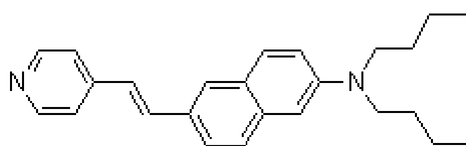
wavelengths	mirror, M in Figure 2	filters, F_{SHG} in Figure 2
830!– 870!nm	CVI Laser TLM1-425-45-UNP-0737 reflecting 425!± 50!nm	Chroma Technology D425/40 with CVI Laser SPF-450 passing 425!± 10!nm
890 and 910!nm	CVI Laser TLM1-450-45-UNP-0737 reflecting 450!± 50!nm	Chroma Technology D460/50 with Oriel Instruments 57530 passing 450!± 10!nm
930!– 970!nm	CVI Laser TLM1-475-45-UNP-0737 reflecting 475!± 50!nm	Chroma Technology D470/40 with 475!± 50!nm band-pass passing 470!± 15!nm

TABLE OF DICHROIC AND FILTER COMBINATIONS FOR 2PF DETECTION

wavelengths	dichroic, D in Figure 2	filters, F_{2PF} in Figure 2
830!– 910!nm	560!nm long-pass	CVI Laser LWP-450 with 3!mm BG!39
910!– 970!nm	Chroma Technology 770DCXR	CVI Laser SWP-700 with 3!mm BG!39

SYNTHESIS OF CHIRAL DYES DI-4-ANEPMRF AND DI-4-ANEPMPOH

The chiral reagents for the syntheses were purchased from Sigma Chemical and stored refrigerated prior to use. Fast atom bombardment (FAB) mass spectral analyses were obtained from the Midwest Center for Mass Spectrometry with partial support by the National Science Foundation, Biology Division (Grant No. DIR9017262).

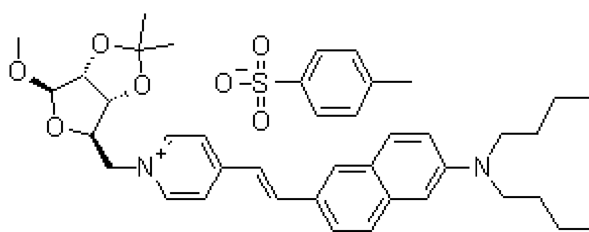


starting compound

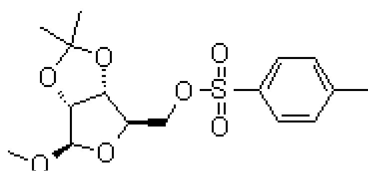
The starting compound β-(2-(di-*n*-butylamino)-6-napthyl)-4-vinyl-pyridine was prepared from 6-bromo-2-(di-*n*-butylamino)-naphthalene by the palladium-catalysed Heck coupling procedure described by Hassner et al. (Hassner et al. 1984).

The dye 1-(2-methyl-3-hydroxypropyl)-4-(2-(di-*n*-butylamino)-6-naphthyl)-vinyl)-pyridinium bromide or di-4-ANEPMPOH was prepared as follows. A solution of 358!mg (1!mmol) of the starting compound and 306!mg (2.0!mmol) (S)-(+)-3-bromo-2-methylpropanol in 3!ml dry acetonitrile was heated at reflux in a nitrogen atmosphere while stirring for a period of 24 hours. After cooling to ambient temperature, the solvent was removed under reduced pressure on a rotary evaporator and the dark-red semi-crystalline residue stirred in 40!ml ether and refrigerated overnight. The resulting brick-red solid was separated by filtration, rinsed with ether and dried in

air. The crude dye was further purified by chromatography on a silica gel column. Unreacted starting compound was eluted first with chloroform and ethylacetate and the dye eluted as a dark red oil with portions of methanol. This gave 450!mg of the dye, which was further recrystallized from methanol-ether as a dark brown hygroscopic solid. The peak absorption wavelength in ethanol was 502!nm, and the peak emission wavelength in ethanol was 709!nm. Thin layer chromatography (silica gel, one part methanol to four parts chloroform) showed one homogeneous rose-coloured fluorescent spot with Rf 0.193. FAB mass spectral analysis showed M^+ ion at 431.3.



intermediate compound



toluene sulfonic acid ester

The intermediate compound 1-(methyl-2,3-*o*-isopropylidene-5-β-D-ribofuranosyl)-4-(β-(2-(di-*n*-butylamino)-6-naphthyl)-vinyl)-pyridinium-*p*-toluenesulfonate was prepared as follows. A solution of 330!mg (0.92!mmol) starting compound and 330!mg (0.92!mmol) methyl-2,3-*o*-isopropylidene-5-*o*-(*p*-tolylsulfonyl)-β-D-ribofuranoside in 3!ml anhydrous *N*,*N*-dimethylformamide was stirred magnetically and heated by means of an oil bath at 110!to 120 C under a nitrogen atmosphere for a

period of 38 hours. The DMF solvent was removed under reduced pressure at 60 C via water aspirator then vacuum pump at 2!mm Hg, and the dark-red viscous residue taken up in chloroform and charged to a column of silica gel-60. Unreacted naphthyl-vinyl-pyridine starting compound was eluted with chloroform then ethyl acetate, and the intermediate compound was eluted with a 1:1 mixture of methanol and chloroform to give 580!mg of a dark-red viscous oil.

The dye 1-(methyl-5-β-D-ribofuranosyl)-4-(β-(2-(di-*n*-butylamino)-6-naphthyl)-vinyl)-pyridinium chloride or di-4-ANEPMRF was prepared as follows. A solution of the intermediate compound and 5!ml de-ionised water containing 3!ml concentrated hydrochloric acid in 15!ml methanol was stirred and heated at reflux for a period of 1.5 hours. After standing at room temperature overnight, the methanol solvent was removed under reduced pressure on a rotary evaporator and the residual aqueous mixture neutralized to pH 8–10 by gradual addition of aqueous sodium carbonate with stirring and cooling in an ice bath. The resultant aqueous dye mixture was extracted into chloroform and the combined extracts washed with saturated brine solution, dried over $MgSO_4$ and concentrated on a rotary evaporator to leave the crude dye as a red-brown resin. The peak absorption wavelength in ethanol was 502!nm, and the peak emission wavelength in ethanol was 712!nm. Thin layer chromatography (C18RPS, EtOH) showed one homogeneous rose-coloured fluorescent spot just off the origin and the absence of any unhydrolyzed intermediate compound. FAB mass spectral analysis showed M^+ at 505.3.

ESTIMATE OF 2PF VERSUS SHG PHOTON RATE

In our use of the Fluoview system, we select imaging conditions such that the membrane pixel values (recorded as 12-bit numbers) are comparable for both SHG and 2PF channels. With a software gain of 10!x on the SHG channel, we set the Pockels cell for the minimum power into the scanner such that we still obtain a reasonable SHG image. In order to obtain a similarly bright 2PF image, we find that a PMT voltage of ~600!V is necessary. Given that the images are comparable, we may use the known characteristics of the detectors to estimate the ratio of the photon rates on the two channels.

Hamamatsu's documentation for the H7421-40 photon-counting head (SHG detector) gives a rate of ~6!x!10^5!counts s^{-1} /!pW at 450!nm. The head produces 5!V TTL pulses with a pulse width of 30!ns. We assume that each pulse going into the analogue input electronics of the Fluoview contributes to an average signal collected over the dwell time for each pixel (~5!µs), such that the analogue equivalent of the head's TTL output is 5!V x 30!ns x 6!x 10^5!s^{-1} /!pW = 0.09!V /!pW. The filters on the SHG channel transmit 60% at 450!nm so, including the 10!x software gain, the effective SHG signal as a function of emitted SHG is ~5!x 10^{11}!V /!W.

Hamamatsu's documentation for the R3896 photo-multiplier tube (2PF detector) gives a radiant sensitivity of ~80!mA /!W at 600!nm and a gain of ~2!x 10^5 for a PMT voltage of 600!V. The Fluoview amplifier board has an I-to-V gain of 570!kΩ, so the voltage output is 570!kΩ x 2!x 10^5 x 80!mA /!W = ~9!x 10^9!V /!W. The filters on the 2PF channel transmit ~50% between 430!nm and 615!nm, which range covers ~40% of the fluorescence emission spectrum, so the effective 2PF

signal as a function of emitted 2PF is ~2!x 10^{9}!V /!W. For the same signal on both channels, the ratio of 2PF power to SHG power is therefore 5!x 10^{11} /!2!x 10^{9} = 250. However, SHG photons at 450!nm have ~1.4!x the energy of 2PF photons at 640!nm, so the ratio of 2PF photon rate to SHG photon rate is ~360. For comparison, Moreaux et al. (Moreaux et al. 2000) calculate that the SHG cross-section for a single molecule is four orders of magnitude smaller than the 2PF cross-section, but note that the coherence of SHG can make SHG and 2PF powers more comparable with a sufficient density of dye.

IMAGE PROCESSING

We performed image processing for the display of images using code written in Perl (http://www.perl.com/) and using the Netpbm package (http://netpbm.sourceforge.net/), running under Mac OS X (Apple Computer, Inc., Cupertino CA). The frames were first nudged, at most by a few pixels, to correct for any slight drift in the position of the cell during imaging. With the frames aligned, a mask corresponding to the cell membrane was generated. Within each frame, the mask was applied to select the bright membrane pixels, and a nearest-neighbour mean filter was applied, with the filter constrained to use only pixels within the mask. Just as the total intensity values, $I\,(t_{i})$, were normalised as described in Materials and Methods, so were the intensity values of each pixel normalised across the frames by a running average interpolated from the values of that pixel in the frames corresponding to the 0!mV reference voltage. We use Wayne Rasband’s ImageJ (http://rsb.info.nih.gov/ij/) to display the images using a “Fire” lookup table.

DISCUSSION OF POTASSIUM-INDUCED DEPOLARISATION EXPERIMENTS

Peleg et al. (Peleg et al. 1999) used undifferentiated P19 neuronal cells to investigate the enhancement of SHG from di-4-ANEPRMF by gold nanoparticles conjugated to antibodies for membrane proteins. The excitation wavelength was again 1064!nm. They depolarised the cells by changing the extracellular potassium concentration from ~5mM to ~45mM, resulting in a reduction in signal by 59%. Calculating a change in TMP of ~50!mV for the depolarisation, and assuming that this represents almost complete depolarisation, this corresponds to a voltage-sensitivity of ~290% / 100!mV using our definition of sensitivity in terms of the signal change relative to the 0!mV intensity.

Campagnola et al. (Campagnola et al. 1999) used an earlier version of the microscope described in this paper to measure SHG and 2PF from L1210 (mouse leukæmia) cells stained with di-8-ANEPRMF and excited at 880!nm. They changed the extracellular potassium concentration from ~5!mM to ~135!mM, giving a ~25!mV depolarisation as determined by TMRE. Since ~135!mM potassium will completely depolarise the cell (and in fact damage it beyond recovery), we may assume that the TMP at ~5!mM was ~-25!mV. Using both imaging and cuvette-based spectroscopy, the SHG:2PF signal ratio was found to be reduced by ~54% which, converting to response expressed as a signal change relative to the 0!mV intensity, corresponds to a voltage-sensitivity of ~470% / 100!mV!

One possibility for non-voltage-dependent effects on SHG involves the quadratic dependence of SHG signal on concentration. Changes in dye concentration resulting from changes to the cells could result in significant changes in SHG. For instance, a decrease in concentration of 36% would

produce the same reduction in SHG signal of 59% reported by Peleg et al. (Peleg et al. 1999). In the case of the 54% reduction in SHG:2PF signal ratio reported by Campagnola et al. (Campagnola et al. 1999), the reduction in dye concentration would have had to be 54% as well: SHG being quadratic in concentration and 2PF being linear, the SHG:2PF signal ratio is linear in concentration. (At a maximum staining level of ~5!µM, self-quenching of 2PF would not have been an issue.) However, such significant changes in dye concentration can be reasonably ruled out in the latter case, because Campagnola et al. report that the 2PF signal increased significantly at the same time that the SHG signal decreased. A redistribution of dye molecules between the membrane leaflets would also result in a reduction in SHG signal — particularly for non-chiral dye molecules — while not affecting 2PF signal. (In our voltage-clamping experiments, the simultaneous measurement of 2PF and SHG provides a control for changes in dye concentration. Depending on the dichroic used, either we find 2PF sensitivities of the opposite sign to the SHG sensitivities or we find no apparent changes in 2PF with voltage.)

More generally, we may consider three sources of voltage-sensitivity. (1) In some dyes, there is an orientational response of the dye to TMP, equivalent to electric field induced second harmonic generation or EFISH (Butcher and Cotter 1990). (2) Some dyes, such as TMRE, redistribute between the intracellular and the extracellular media (Ehrenberg et al. 1988) or between the cell membrane and the extracellular medium as a function of TMP (Loew 1999). Such dye redistributions typically require tens of seconds for equilibration. (3) The ideal mechanism for voltage-sensitivity is directly electrochromic. We have previously demonstrated that the reorientation and redistribution mechanisms do not pertain to the fast responses of styryl dyes in 1PF experiments on HLB apparatus (Loew et al. 1979; Loew and Simpson 1981; Fluhler et al. 1985). The agreement of the HLB measurementss and our voltage-clamping measurements of SHG

responses to TMP suggests that the SHG voltage-sensitivities reported here are electrochromic. In contrast, the potassium-induced depolarisation experiments (Campagnola et al. 1999; Peleg et al. 1999) make gross changes to the extracellular medium that affect the dye–membrane system, perhaps driving dye into or out of the cell, and hence affecting SHG beyond straightforward electrochromism. For instance, potassium-induced depolarisation is not generally reversible — the 135!mM potassium used by Campagnola et al. damages cells beyond recovery — and the potassium-induced depolarisation experiments do not attempt repolarisation to verify that SHG signals return to their original levels. In contrast, voltage-clamping permits us to repeatedly cycle TMP between the reference voltage and a test voltage. By returning to the test voltage many times during the course of imaging, we verify that the response is both reversible and robust.

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