

Action spectra of electrochromic voltage-sensitive dyes in an intact excitable tissue

Joseph Foley
Martin Muschol

University of South Florida
Department of Physics
Tampa, Florida 33620

Abstract. Voltage-sensitive dyes (VSDs) provide a spatially resolved optical read-out of electrical signals in excitable tissues. Several common fluorescent VSDs display electrochromic shifts of their emission spectra, making them suitable candidates for ratiometric measurements of transmembrane voltages. These advantages of VSDs are tempered by tissue-specific shifts to their fluorescence emission. In addition, the optimal electrochromic dye response occurs in wavelength bands distinct from the dye's maximal resting emission. This "action spectrum" can undergo tissue-specific shifts as well. We have developed a technique for *in situ* measurements of the action spectra of VSDs in intact excitable tissues. Fluorescence emission spectra of VSDs during action-potential depolarization were obtained within a single sweep of a spectrophotometer equipped with a charge-coupled device (CCD) array detector. To resolve the subtle electrochromic shifts in voltage-induced dye emission, fluorescence emission spectra measured right before and during field-induced action-potential depolarization were averaged over about 100 trials. Removing white-noise contributions from the spectrometer's CCD detector/amplifier via low-pass filtering in Fourier space, the action spectra of all dyes could be readily determined. © 2008 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3013326]

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1 Introduction

Voltage-sensitive dyes (VSDs) are powerful optical indicators of electrical activity in tissues that are either inaccessible to electrode recordings (e.g., neuronal axons or dendrites)¹⁻³ or that require multisite recordings in order to unravel the complex spatio-temporal patterns of electrical activity (e.g., neuronal assemblies, neuroendocrine tissue, and heart and muscle physiology).⁴⁻⁷ Since their discovery over 30 years ago,^{8,9} fast-response dyes have been shown to provide linear optical (absorption or fluorescence emission) responses with response times sufficient for faithful optical recordings of action-potentials from excitable tissues.^{1,10-12} Many of the commonly used fluorescent styryl VSDs are electrochromic, i.e., their fluorescence spectra undergo a spectral shift in response to changes in transmembrane voltage.¹³ Electrochromic dyes with shifts in their fluorescence emission are the only practical choices for ratiometric optical recordings of brief voltage transients in excitable tissues. Ratiometric optical recordings eliminate artifacts of optical signals due to dye bleaching or nonvoltage-related changes arising from motion in contractile tissue.⁶ They also hold the promise of calibration of optical responses against transmembrane voltage.^{14,15}

Widespread use of voltage-sensitive dyes has been hampered by several practical limitations, among them the small magnitude of the optical responses and the risk of phototoxic damage to the preparation. While detection of small fractional changes in optical properties improves with increased dye staining and illumination intensity, the risk of phototoxic damage is reduced by minimizing staining and light exposure. Due to these conflicting requirements on optical recordings, optimizing optical recording parameters for the specific dye/tissue combination used in a given experiment is important.

Among the important optical parameters relevant for optimizing optical voltage recordings are the specific fluorescence excitation and emission characteristics of the VSD.¹⁶⁻²⁰ Unfortunately, the staining and fluorescence characteristics of fluorescent VSDs tend to vary significantly from preparation to preparation. Fluorescence emission spectra of voltage-sensitive dyes, for example, can undergo significant shifts of their spectral emission, depending on their immediate environment.²¹ Staining characteristics can even vary for different membranes within the same cell. Confocal imaging of di-8-ANEPPS fluorescence in human embryonic kidney (HEK) cells indicated that emission of internalized dye molecules was significantly blue-shifted when compared to its counterparts bound to the plasma membrane.²²

Address all correspondence to Martin Muschol University of South Florida, Dept. of Physics, 4202 E. Fowler Ave-Tampa, FL 33620-5700; Tel: 813-974-2564; Fax: 813-974-5813; E-mail: mmuschol@cas.usf.edu

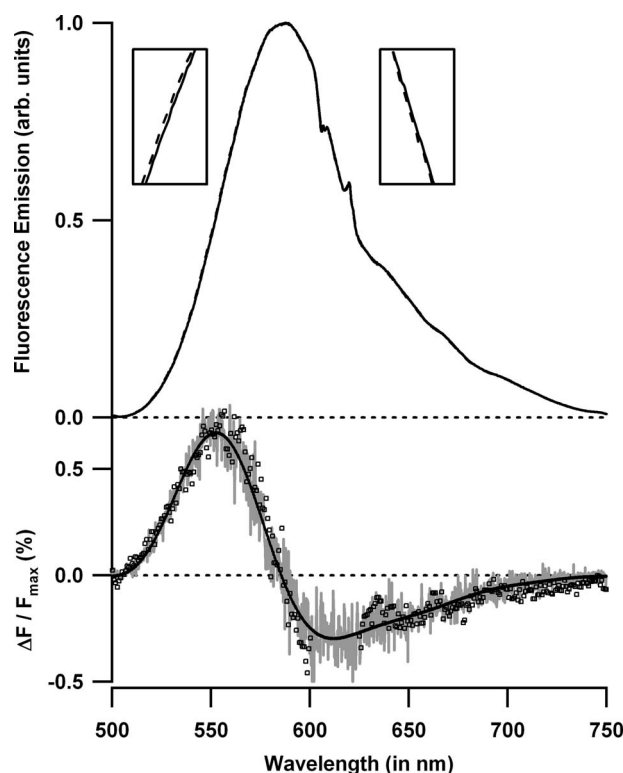


Fig. 1 In situ fluorescence emission and action spectrum of di-4-ANEPPDHQ. (Top) Superposition of the fluorescence emission spectrum of the electrochromic dye di-4-ANEPPDHQ measured in the stained mouse neurohypophysis, either at rest (solid line) or during action-potential depolarization (dashed line). (Inset): Enlarged version of the two spectra indicating the slight and asymmetric blue-shift induced by depolarization. (Bottom) The difference or action spectrum $\Delta F/F_{\max} = (F_{\text{AP}} - F_0)/F_{\max}$ for di-4-ANEPPDHQ obtained from the difference of 100 recordings (noisy gray trace) and after low-pass Fourier filtering (solid line). Here, F_{AP} and F_0 refer to the fluorescence emission measured during action-potential stimulation and at rest, respectively. F_{\max} is the maximum in the resting fluorescence. Superimposed on the measured action spectra is the local derivative $dF_0/d\lambda$ of the resting fluorescence (hollow squares), rescaled to match the peak value in $\Delta F/F_{\max}$. $dF_0/d\lambda$ values near the two spectrometer-induced kinks in the fluorescence spectra were excessively noisy and were removed from the figure (gap between 600 and 630 nm). The agreement between $\Delta F/F_{\max}$ and $dF_0/d\lambda$ indicates that changes in transmembrane voltage uniformly shift fluorescence emission, consistent with the behavior expected for electrochromic dyes.²⁰

While ratiometric optical recordings of voltage signals offer distinct advantages, the assessment of tissue-specific shifts in the voltage responses of electrochromic dyes is complicated by three factors. First, the optimal wavelength region for optical recording of voltage responses with electrochromic dyes is distinct from the peak in their resting fluorescence. As shown in Fig. 1, the voltage-induced spectral shift in fluorescence emission implies that potentiometric changes in dye fluorescence are largest at wavelengths on either side of the peak in their resting fluorescence (in fact, the action spectrum crosses zero near the resting peak of dye fluorescence). It is the measurement of the “action spectrum” of VSD responses that is required for optimizing optical recording parameters. However, the blue shifts of VSDs induced by typical membrane depolarizations (100 mV or less) tend to be very small

(again, see Fig. 1). Hence, resolution of these weak spectral shifts require long integration times and good control over transmembrane potential.^{16,19,20} Yet, optical measurement of voltage signals and, therefore, measurements of VSD action spectra are most useful in preparations which are not accessible to recordings with glass electrodes and thus in convenient control of transmembrane voltage.

To address these problems, we have developed an approach for *in situ* measurements of VSD action spectra by resolving the small spectral shifts induced during transient action-potential depolarization. We performed all our measurements in the mammalian neurohypophysis. The mammalian neurohypophysis is a good example of an intact, excitable preparation consisting mostly of hypothalamic axons and their secretory swellings, glial cells known as pituicytes, and dense vascularization. Magnocellular neurons in the hypothalamus project their axons into the neurohypophysis, where they release arginine vasopressin and oxytocin into the circulation. Field stimulation of the axons passing through the infundibular stalk generates action potentials invading the entire posterior pituitary. Optical signals recorded from the neurohypophysis during trains of action potentials include intrinsic scattering changes,²³ voltage responses recorded with potentiometric dyes,^{3,24,25} intraterminal calcium changes,²⁶ and intrinsic fluorescence changes.²⁷ The small physical dimensions of axons and secretory varicosities, the difficulty of controlling their voltage with electrodes, and the high optical density of neurohypophysial tissue make recordings of dye spectra from individual axons and terminals very challenging. To overcome these limitations, we have measured the action spectra of electrochromic VSDs by averaging the optical signals over the entire tissue and by selectively and synchronously depolarizing neurohypophysial axons using action potentials. We employed a CCD-based spectrophotometer for rapid and triggered acquisition of complete dye spectra immediately preceding and during the brief action potential depolarization of the neurohypophysis. Using averaging over multiple *in situ* recordings and Fourier filtering of the resulting spectra, we have determined the action spectra of the five different fluorescent VSDs.

2 Materials and Methods

2.1 Tissue Preparation

Typically, 30- to 60-day-old NIH Swiss female mice were euthanized and their pituitary glands were harvested following Institutional Animal Care and Use Committee approved procedures. The neurohypophysis along with a portion of the infundibular stalk and the surrounding pars intermedia were separated from the anterior pituitary. The intact neurohypophysis was mounted in a custom-designed optical recording chamber and perfused with oxygenated (95% O₂, 5% CO₂) physiological saline (154 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl₂, 1 mM MgCl₂, 20 mM N-2-hydroxyethyl perazine-N-2-eteanesulfonic acid, 10 mM glucose; pH 7.4). The infundibular stalk was placed between two platinum-iridium (90/10%) field electrodes, and action potentials were initiated by brief (500 μ s) bipolar shocks, supplied by an SIU-102 stimulus-isolation unit (Warner Instruments, LLC; Hamden, CT). The health of the neurohypophysis following dissection was assessed by measuring the intrinsic optical sig-

nal associated with excitation-secretion coupling in the unstained preparation.²³ All measurements were performed at room temperature (24 °C).

2.2 Staining with VSDs

The action spectra (i.e., the changes in fluorescence dye emission induced by membrane depolarization) of five different styryl-based voltage-sensitive dyes were determined: di-8-ANEPPS, JPW 1114 (di-2-ANEPEQ), RH 414, FM1-43, and the recently developed di-4-ANEPPDHQ.²⁸ All dyes were obtained from Invitrogen (Carlsbad, CA). Stock solutions of most dyes were prepared by dissolving the dye into a (90/10%) mixture of dimethyl sulfoxide and Pluoronic F-127 to a final dye concentration of 5–10 mM. FM1-43 was dissolved directly in distilled water instead. For staining of the preparation, 3–5 μL of dye stock was added to 1 mL of physiological saline. The preparations were typically stained for 45 min, with di-8-ANEPPS requiring 80–90 min incubation periods. Prior to fluorescence measurements, the dye solutions were washed out with physiological saline for 15 min. Figure 5 compares the relative intensity of resting fluorescence emission (integrated over the total peak area) of all dyes after their respective staining period.

2.3 Optical Setup and Spectral Recordings of Fluorescence Emission

All fluorescence measurements were performed on an upright BX61WI microscope (Olympus America; Center Valley, PA) using a 20×0.5 NA water-immersion objective. A 470 nm high-power LED (MBLED; Thorlabs, Newton, NJ), driven at 75–600 mA with a stabilized constant-current LED controller (Mightex; Pleasanton, CA), was used as excitation light source.²⁹ The emission from the LED was bandpass limited with a 482/35 nm filter (Semrock; Rochester, NY). To record the broadband fluorescence emission from the dye, we replaced the bandpass emission filter of our fluorescein isothiocyanate (FITC) filter cube (Semrock; Rochester, NY) with a 515 nm long-pass filter (Schott glass OG 515 lp). To minimize dye bleaching and possible phototoxic damage to the preparation, LED illumination was limited to a total of 0.6 s prior to and during electrical stimulation.

To capture the brief and transient changes in fluorescence dye emission during action-potential stimulation, we used an AvaSpec 2048-USB2 CCD-array spectrophotometer (Avantes USA; Broomfield, CO). The spectrophotometer was fitted with a 600 lines/mm grating and a 2048 pixel CCD with a detection range of 267 to 819 nm, resulting in a nominal resolution of 2.1 nm. On the CCD detector, however, the spectral data are sampled every 0.27 nm and with 16 bit resolution of intensity changes. A built-in order-sorting filter reduces contamination of the spectra from higher-order diffraction peaks. To preserve the small voltage-induced shifts in fluorescence emission, the default smoothing algorithm in the acquisition software had to be disabled. Light output from the camera port on the BX61WI microscope was focused with 10×0.25 NA objective onto the 600 μm fiber-optic cable input to the spectrophotometer. Synchronization between the electrical stimulation of the neurohypophysis with spectral re-

cordings of dye emission was provided by an STG1004 stimulus generator (Multichannel Systems, Reutlingen, Germany).

Measurements of changes to the emission spectra of voltage-sensitive dyes during action-potential depolarization are complicated by two factors. First, the spectral shifts in fluorescence dye emission are quite small (see Fig. 1). In addition, membrane depolarization induced by action potentials is short and transient. To address these limitations, we typically summed one hundred individual measurements of emission spectra for resting fluorescence and depolarization-induced spectral changes measured from a single preparation. During action-potential depolarization, spectra of dye fluorescence emission were recorded in a single 8-ms sweep of the spectrophotometer. Resting spectra of all voltage-sensitive dyes were recorded 120 ms prior to generating an action potential. Minimizing the spacing between spectra recorded during rest and during stimulation and reducing illumination intensity avoided distortion of the action spectra due to dye bleaching. Stimulation at frequencies above 1 Hz induces plastic changes in the excitable responses of the neurohypophysis.³ Hence, consecutive spectral recordings were spaced at least 4 s apart. The total number of stimuli within a given stimulus train was limited to 20, and successive stimulus trains were separated by 15 min rest periods.

2.4 Data Processing

All data were analyzed using Igor Pro 5.02 software (WaveMetrics; Lake Oswego, OR). The emission spectra recorded during rest were subtracted from those taken during stimulation and averaged over 100–200 trials. We used the following procedure to resolve the small voltage-induced wavelength shifts in fluorescence emission. For small spectral shifts, $\Delta F/\Delta\lambda \approx dF_0/d\lambda$ and, therefore, $\Delta\lambda \approx \Delta F/(dF_0/d\lambda)$. Here, ΔF is the experimentally measured change in fluorescence emission, and $dF_0/d\lambda$ is the local slope of the resting fluorescence emission F_0 at a given wavelength λ . Therefore, plots of $\Delta F/(dF_0/d\lambda)$ over the entire emission range can be used to estimate $\Delta\lambda$. The above considerations also indicate that even very small wavelength shifts $\Delta\lambda$ result in significant changes in fluorescence emission ΔF wherever the fluorescence emission changes rapidly with λ (see insert in Fig. 1). Therefore, high sensitivity to small fluorescence changes permits us to resolve wavelength shifts $\Delta\lambda$ close to the spectral sampling rate of the CCD array (0.27 nm) and well below the nominal 2.1 nm resolution of the spectrophotometer.

The raw difference spectra were still rather noisy due to significant noise from the uncooled CCD array detector and current-to-voltage converters. We used Fourier analysis to filter out the high-frequency components of this white noise. A typical power spectrum obtained via fast Fourier transform (FFT) of the raw difference spectra is shown in Fig. 2. The first 10–15 components of the power spectrum at low spatial frequencies rise by several orders of magnitude above the uniform background of white noise dominating high spatial frequencies. It is these low-frequency components of the Fourier spectrum that represent the portions of the action spectra slowly varying with wavelength. Hence, we low-pass filtered the spectra by zeroing out all Fourier components below five times the root-mean-square amplitude of the white noise or

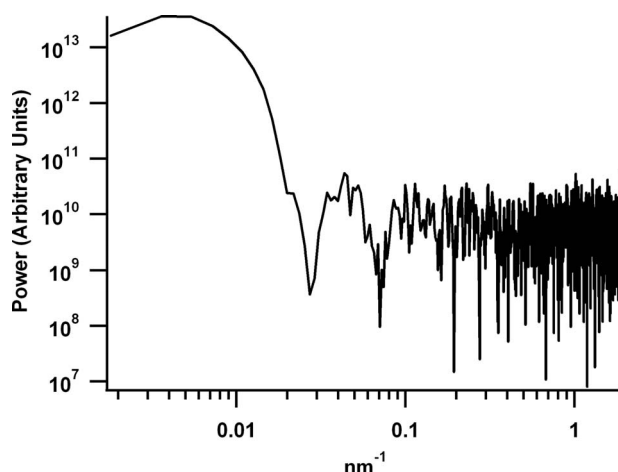


Fig. 2 Fourier analysis of the action spectrum for di-4-ANEPPDHQ. Power spectrum for the action spectrum of di-4-ANEPPDHQ shown in Fig. 1. Note the large amplitudes at low spatial frequencies, followed by white noise at higher frequencies. By zeroing out the high-frequency noise components, the S/N ratio of the action spectra could be significantly improved (see Materials and Methods section).

beyond spatial frequencies of 0.1 nm^{-1} or higher. Filtered action spectra were obtained via inverse FFT of the filtered Fourier spectra.

2.5 Ratiometric Camera Recordings

We tested whether we could obtain ratiometric dye signals using RH414, a dye with a weak action spectrum (i.e., small voltage-induced spectral shifts). Changes in RH414 fluorescence were induced by a single action potential invading the neurohypophysis. Fluorescence changes were recorded using a fast 128×128 pixel CCD camera cooled to -60°C (Ixon DV860BV, Andor; Northern Ireland). To resolve fast voltage transients, spatial resolution was reduced by 4×4 binning, thereby increasing the frame rate to 1613 FPS. A 470-nm LED was used for dye excitation. To catch either one of the two peaks in the action spectrum of RH414, fluorescence emission was recorded using a standard FITC filter cube with either a 536/40-nm bandpass or a 610-nm long-pass emission filter. The tissue averaged fluorescence in either emission band was normalized to the fractional change from the resting fluorescence just before stimulation. Typical optical recordings of action potentials obtained in consecutive recordings from either peak in the action spectrum are shown in Fig. 6.

3 Results

3.1 In Situ Measurements of Action Spectra

We have measured the resting fluorescence emission spectra and action spectra of four electrochromic dyes: di-8-ANEPPS, di-4-ANEPPDHQ, di-2-ANEPEQ (JPW 1114), and RH 414. We have also included the voltage-sensitive dye FM1-43 for comparison and control. In contrast to the spectral shifts exhibited by the former, voltage changes predominately modulate the amplitude of FM1-43 fluorescence emission.

Figure 1 displays the resting fluorescence-emission spectrum of di-4-ANEPPDHQ in the neurohypophysis averaged over 100 8-ms single-sweep recordings of a CCD-array spec-

trophotometer. The resting emission spectra are somewhat distorted by contributions from higher-order diffraction off the diffraction grating (e.g., the two spikes between 600 and 650 nm). Because we are mostly interested in the voltage-induced changes to the resting emission spectra, we did not attempt to correct for these distortions to the resting spectra. Superimposed on the resting spectrum (solid line) is the emission spectrum recorded during action-potential-induced depolarization of the neurohypophysis, also averaged over 100 recordings. As indicated by the inserts in Fig. 1, the emission spectrum of di-4-ANEPPDHQ is slightly blue-shifted during membrane depolarization. We estimated that the average wavelength shift $\Delta\lambda$ induced during action-potential depolarization was slightly less than 0.4 nm (for details, see Materials and Methods). The action spectrum of the dye obtained from one single-sweep recording was too noisy to warrant analysis. To overcome this noise limitation, measurements of the resting and voltage-shifted emission spectra were repeated 100 times and the action spectrum was calculated from their difference. The resulting action spectrum is shown as noisy gray trace in Fig. 1.

3.2 Low-Pass Filtering of Action Spectra

As shown in Fig. 1, the overall shape of the action spectrum is clearly discernable after the summation of 100 difference spectra. Yet, the signal-to-noise (S/N) ratio is rather disappointing. Closer inspection of the difference spectra reveals that much of the noise seems to arise from broadband white noise, while most of the features of the difference spectrum are slowly varying with wavelength. This is confirmed by calculating the spectral power distribution of the action spectra (Fig. 2). There are several large peaks in the power spectrum at low spatial frequencies, with a background of weaker but uniform white-noise components present at all spatial frequencies. We low-pass filtered the summed action spectra by eliminating all high-frequency Fourier components while preserving the low-frequency components (see Materials and Methods for details). This yielded the filtered action spectrum for di-4-ANEPPDHQ shown as a solid line in Fig. 1.

To ascertain whether low-pass filtering might alter the shape of the difference spectra, we performed the following test. A single emission spectrum of di-4-ANEPPDHQ was recorded at a very low LED intensity (LED current set to 1 mA) and 2 ms integration time of the spectrophotometer (Fig. 3(a)). This artificially noisy resting spectrum was Fourier-analyzed as described in the previous paragraph (Fig. 3(b)) and transformed back into real space. The filtered spectrum was plotted together with the rescaled sum of 100 emission spectra for the same dye recorded at normal illumination (Fig. 3(c)). Although the count rate of the summed resting spectra was over 10^4 times that for the single, low-intensity measurement, low-pass filtering essentially reproduced the emission spectrum recorded at normal illumination intensities.

Action spectra for all five VSDs investigated are presented in Fig. 4. The action spectra of the recently developed dye di-4-ANEPPDHQ yielded larger responses in fluorescence emission with transmembrane voltage than di-8-ANEPPS, JPW 1114, or RH414. More importantly, di-4-ANEPPDHQ much more efficiently stained the preparation (see Fig. 5) and, therefore, significantly improved the S/N ratio of the weak

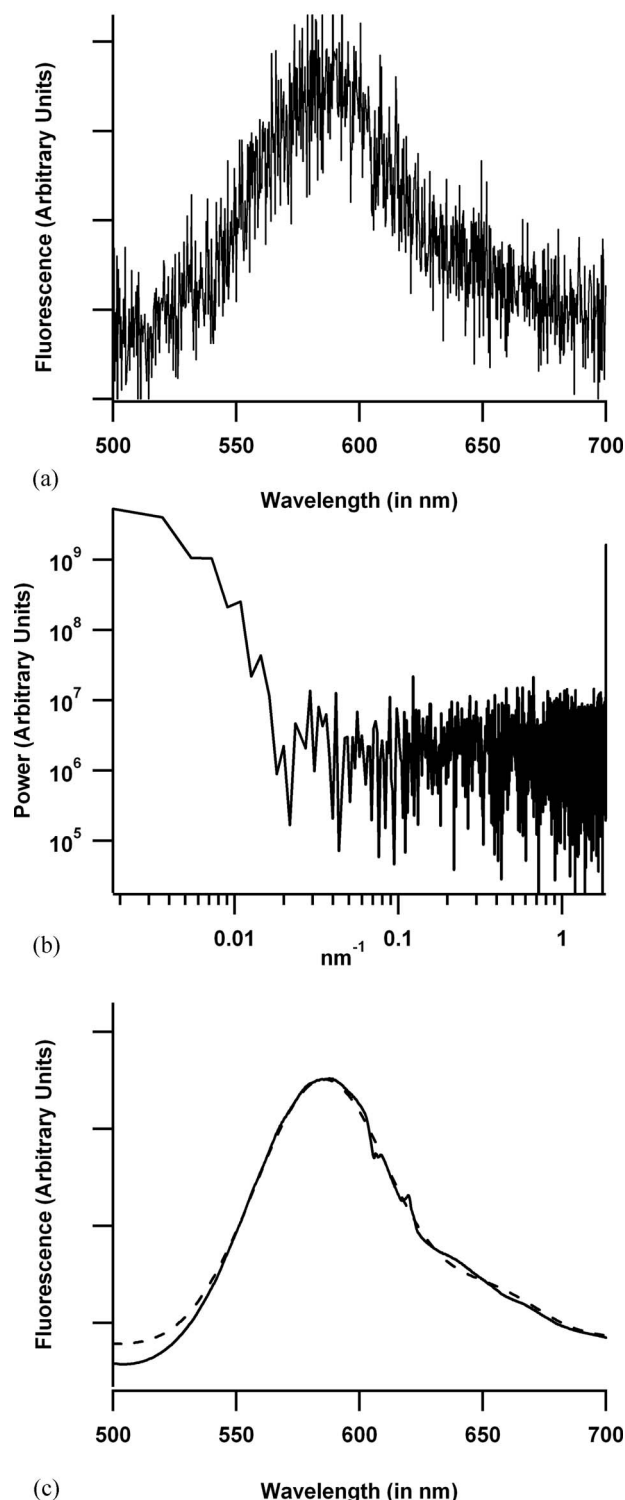


Fig. 3 Testing the effects of Fourier filtering on fluorescence spectra. (a) A single emission spectrum of di-4-ANEPPDHQ was recorded at very low illumination and short integration time. (b) The power spectrum of this artificially noisy emission spectrum shows strong low-frequency components and a base level of high-frequency noise. (c) After low-pass filtering (for details, see Materials and Methods), the filtered emission spectrum (dashed line) was superimposed onto the sum of 100 emission spectra taken at much higher illumination intensity and with much longer integration times (solid line).

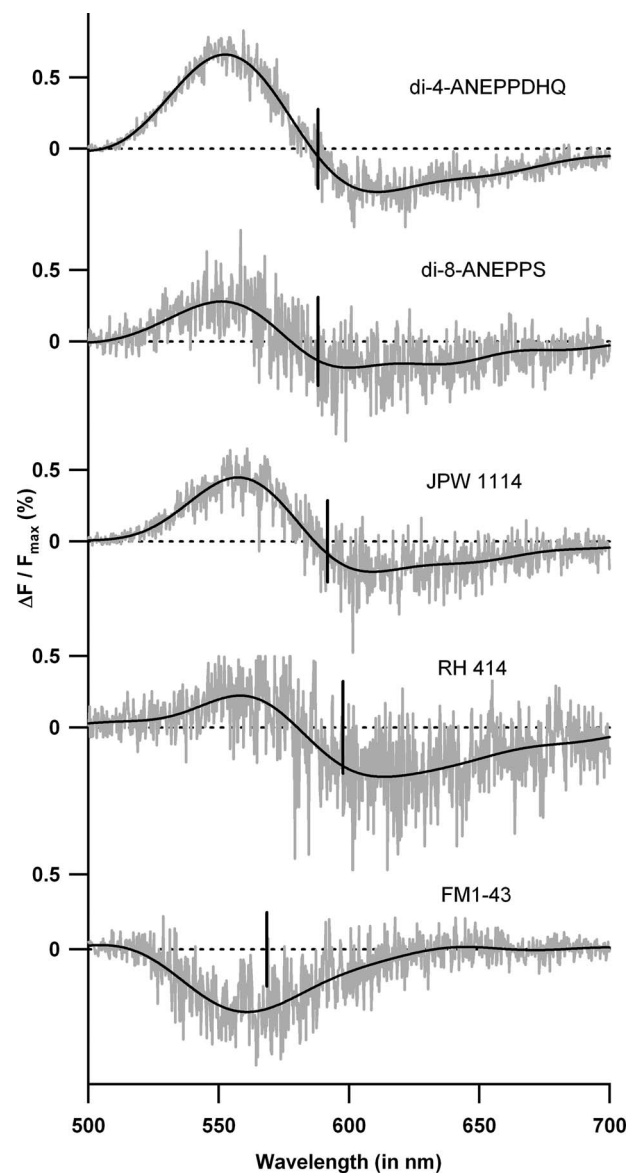


Fig. 4 Action spectra for various VSDs. Action spectra $\Delta F/F_{\max}$ for five VSDs obtained from the mouse neurohypophysis during action-potential depolarization. For each dye, the raw difference spectrum is shown in light gray, with the Fourier filtered spectrum superimposed as a solid line. All spectra are plotted to the same scale. The vertical lines indicate the location of the peak in the resting fluorescence emission. The action spectra of di-4-ANEPPDHQ, JPW 1114, RH 414, and FM1-43 were measured from the average of 100 spectra, while 200 spectra were averaged for di-8-ANEPPS.

fluorescence changes ΔF . The maximal voltage response for all electrochromic dyes occurs in wavelength bands on either side of the fluorescence emission peaks. Not surprisingly, the action spectra of all amino-naphthylethenylpyridinium-based dyes display similar features, with the largest peak of their action spectra on the short wavelength side of their fluorescence emission. The action spectrum of RH 414, in contrast, develops its maximal response on the long-wavelength shoulder of its resting fluorescence emission. For optical recordings of electrical activity within a single emission band, recording on the favorable emission shoulder clearly improves the opti-

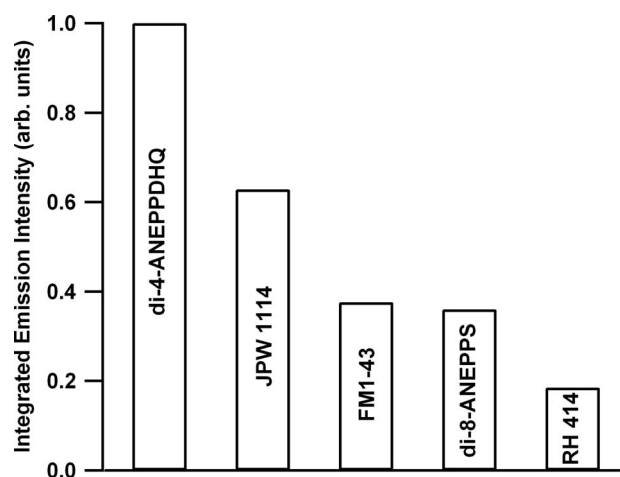


Fig. 5 Staining efficacy of various VSDs in the mammalian neurohypophysis. Relative intensity of the integrated fluorescence emission of various VSDs after staining of the mammalian neurohypophysis. The preparations were incubated with 20 μ M of dye for 45 min, and the dye was washed out prior to measurement. Di-8-ANEPPS was diluted to 40 μ M and was incubated for 90 min instead.

cal S/N. The asymmetry in the peak amplitude of the action spectra on either side of the isosbestic point represents an additional challenge to ratiometric optical recordings of voltage transients. Response amplitudes of voltage-sensitive dyes are intrinsically small. In addition, voltage-induced fluorescence signals in complex tissue only arise from a fraction of stained tissue. Hence, the asymmetric peak amplitudes of the action spectra reduces S/N levels during ratiometric measurements of voltage signals, further highlighting the benefit of *in situ* measurements of action spectra.

3.3 Ratiometric Measurements of Transmembrane Voltage

Ratiometric measurements of fluorescence changes allow us to discriminate against contributions to the optical transients not related to voltage signals, including dye bleaching or dye internalization. Based on the action spectra measured above, we decided to perform some preliminary ratiometric measurements of voltage transients in the neurohypophysis. With the exception of FM1-43, all of the dyes in this study can be used for ratiometric measurements of transmembrane voltage. For this preparation, di-4-ANEPPDHQ clearly provides the most robust fluorescence signal. However, as shown in Fig. 6, even with the relatively weak peaks in the action spectrum of RH 414, ratiometric measurements are possible. Optical recordings of the population action potential were obtained with 482/35 nm excitation and fluorescence emission recorded with either a 536/40 nm bandpass filter (upper trace) or a 610 nm long-pass filter (lower trace). The emission signals at the two opposite peaks of the action spectrum were recorded in consecutive trials using a fast CCD camera acquiring 1613 frames/s. The fast transient dominant in either emission band is related to the population action potential depolarizing neurohypophysial axons and their secretory swellings. In addition, a slow after-depolarization following the action-potential transient is present in both recordings. These ratiometric recordings indicate that this slow depolarization is

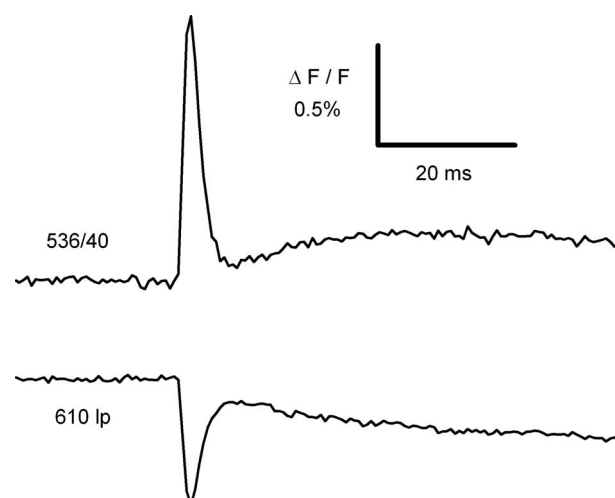


Fig. 6 Ratiometric recordings of neurohypophysial action potential. Fluorescence emission changes recorded with a fast CCD camera (1613 frames/s) during action-potential depolarization of a mouse neurohypophysis stained with RH 414 (20 μ M for 40 min) using 482/35 nm excitation. The upper trace shows optical voltage signal recorded with a 536/40 nm bandpass filter, while the lower curve shows the signal from the same preparation obtained with a 610 nm long-pass filter.

dominated by trans-membrane voltage changes, supporting our previous contention that it arises from glial depolarization following electrical activity.³

4 Summary and Discussion

We have determined the action spectra of several electrochromic voltage-sensitive dyes in an intact tissue during brief action-potential depolarizations. These action spectra were measured using a standard fiber-optically coupled CCD-array spectrophotometer. Because of the intrinsic noise and limited sensitivity of these commercial detectors and our requirement to resolve the spectral shifts induced by action-potential stimulation, fluorescence emission had to be summed over approximately 100 trials. Such summation, however, did suppress the noise level sufficiently to resolve the small difference spectra of resting versus stimulated fluorescence emission. Fourier filtering further improved the S/N ratios of the summed action spectra significantly.

The action spectra of all styryl dyes, together with a marker indicating the peaks of their resting fluorescence, are displayed in Fig. 4. Aside from significant amplitude-difference variations in the net shift induced during action-potential stimulation, the action spectra of the four styryl dyes recorded from the neurohypophysis have similar shapes. More importantly, the action spectra reveal that the voltage-induced fluorescence changes ΔF on opposite sides of the dye emission peaks (indicated by vertical reference lines in Fig. 4) are rather asymmetric. This asymmetry is related to the asymmetric shape of the fluorescence emission spectra themselves. For a small and uniform wavelength shift $\Delta\lambda$, the local change in fluorescence emission ΔF should be directly proportional to the local slope ($dF_0/d\lambda$) of the resting fluorescence emission (see Materials and Methods and Ref. 20). Figure 1 shows the superposition of the measured action spectrum $\Delta F/F_{\max}$ and

the local derivative ($dF_0/d\lambda$) of the resting emission of di-4-ANEPPDHQ. The two data sets are identical to within the noise, indicating that the asymmetry of the action spectra indeed arises from the asymmetric shape of the dye emission spectrum. Furthermore, the agreement between ΔF and ($dF_0/d\lambda$) confirms that the voltage-induced wavelength shift $\Delta\lambda$ in fluorescence dye emission is independent of wavelength, consistent with the behavior of electrochromic dyes.²⁰ Several of the other dyes in Fig. 4 show apparent offsets between the peak in fluorescence resting emission and the zero-crossing of the action spectra. These offsets are likely due to the significantly lower S/N ratios for the action spectra and the corresponding uncertainties in determining the crossover wavelength.

The asymmetry in the amplitude of voltage-induced dye response ΔF on opposite sides of the resting fluorescence poses a challenge when trying to record ratiometric voltage signals. As shown in Fig. 6, the fractional fluorescence change $\Delta F/F_0$ induced by single action potentials on either side of the zero-crossing of the action spectrum are quite different. Optimization of the emission bands used for ratiometric recordings is therefore particularly important. For optimal performance in a given setup, the spectral response characteristics of the optical train and the noise characteristics of the fluorescence excitation and detection system will need to be considered as well.

Overall, our measurement approach provides a convenient and cost-effective means for measuring the action spectra of voltage-sensitive dyes *in situ*, in the preparation of interest, and with the same microscope setup used for fluorescence recordings of electrical activity. We presume that this approach could be extended to calibrate activity-induced changes to the emission spectrum against the absolute-voltage excursions during slow or fast changes in transmembrane voltage.

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