J Membrane Biol (2013) 246:679–688
DOI 10.1007/s00232-013-9584-1

Evaluation of Voltage-Sensitive Fluorescence Dyes for Monitoring Neuronal Activity in the Embryonic Central Nervous System

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Received: 28 June 2013 / Accepted: 4 August 2013 / Published online: 22 August 2013
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Abstract Using an optical imaging technique with voltage-sensitive dyes (VSDs), we investigated the functional organization and architecture of the central nervous system (CNS) during embryogenesis. In the embryonic nervous system, a merocyanine-rhodanine dye, NK2761, has proved to be the most useful absorption dye for detecting neuronal activity because of its high signal-to-noise ratio (*S*/*N*), low toxicity and small dye bleaching. In the present study, we evaluated the suitability of fluorescence VSDs for optical recording in the embryonic CNS. We screened eight styryl (hemicyanine) dyes in isolated brainstem–spinal cord preparations from 7-day-old chick embryos. Measurements of voltage-related optical signals were made using a multiple-site optical recording system. The signal size, *S*/*N*, photobleaching, effects of perfusion and recovery of neural responses after staining were compared. We also evaluated optical responses with various magnifications. Although the *S*/*N* was lower than with the absorption dye, clear optical responses were detected with several fluorescence dyes, including di-2-ANEPEQ, di-4-ANEPPS, di-3-ANEPPDHQ, di-4-AN(F)EPPTEA, di-2-AN(F)EPPTEA and di-2-ANEPPTEA. Di-2-ANEPEQ showed the largest *S*/*N*, whereas its photobleaching was faster and the recovery of neural responses after staining was slower. Di-4-ANEPPS and di-3-ANEPPDHQ also exhibited a large *S*/*N* but required a relatively long time for recovery of neural activity. Di-4-AN(F)EPPTEA, di-2-AN(F)EPPTEA and di-2-ANEPPTEA showed smaller *S*/*N*s than di-2-ANEPEQ, di-4-ANEPPS and di-3-ANEPPDHQ; but the recovery of neural responses after staining was faster. This study demonstrates the potential utility of these styryl dyes in optical monitoring of voltage changes in the embryonic CNS.

Keywords Optical recording · Voltage-sensitive dye · Embryo · Nervous system · Fluorescence · Screening

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Introduction

Optical recording with voltage-sensitive dyes (VSDs) is a powerful tool with which to monitor transmembrane voltage changes simultaneously in many excitable cells (Grinvald et al. 1977; Salzberg et al. 1977). The combination of a VSD with a multichannel device, such as a photodiode array or complementary metal oxide semiconductor (CMOS) sensor, has made it possible to investigate the spatiotemporal dynamics of electrical activity in the neural and cardiac systems (for reviews, see Cohen and Salzberg 1978; Salzberg 1983; Grinvald et al. 1988; Loew 1988; Kamino 1991; Ebner and Chen 1995; Baker et al. 2005; Canepari and Zecevic 2010).

We have applied optical techniques to the embryonic nervous system and established the feasibility of using VSD recording to analyze neuronal activity in the peripheral and central nervous systems (CNSs) (for reviews, see Momose-Sato et al. 2001, 2002; Glover et al. 2008; Momose-Sato and Sato 2011). The physiological approach to the embryonic nervous system has been greatly advanced by the optical technique because conventional electrophysiological measurements are technically difficult or impossible in the early developing CNS, in which the cells are small and fragile. In our previous studies, we used a merocyanine-rhodanine dye, NK2761, to monitor neuronal activity at the population level because this VSD is an optimal absorption dye (a dye used in the absorption mode in an optical voltage recording experiment) for the embryonic nervous system due to its large response, large signal to noise ratio (*S*/*N*), low phototoxicity and small dye bleaching (Momose-Sato et al. 1995).

Recently, newer optical methods, such as confocal or multiphoton microscopy, have been developed to investigate physiological events at the cellular level. Using these methods together with a fluorescence VSD (a dye used in the fluorescence mode in an optical voltage recording experiment), some pioneer studies have succeeded in monitoring transmembrane voltage changes in neural and cardiac systems (Saggau et al. 1998; Fisher et al. 2008; Acker et al. 2011; Yan et al. 2012). Although these techniques are potentially useful in the embryo, it is not yet known, even by standard microscopic measurement, whether fluorescence probes have enough sensitivity to monitor embryonic neuronal activity and which dyes are most suitable for the embryonic nervous system.

In the present study, we examined the performance of eight styryl (hemicyanine) VSDs, including four commonly used probes and three recently synthesized dyes, to evaluate the utility of fluorescence dyes for monitoring electrical activity in the embryonic CNS. Preliminary results have appeared previously in abstract form (Sato et al. 2011).

Materials and Methods

Preparations

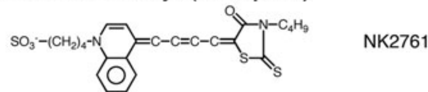
Experiments were carried out in accordance with the guidelines of the US National Institutes of Health and Komazawa Women's University for the care and use of laboratory animals. All efforts were made to minimize the number of animals used and their suffering. All of the experiments were performed at Komazawa Women's University. Fertilized eggs of white leghorn chickens (Saitama Experimental Animals Supply, Saitama, Japan) were incubated for 7 days (Hamburger-Hamilton stages 30–31; Hamburger and Hamilton 1951) in a forced-draft incubator (type P-008; Showa Incubator, Urawa, Japan) at 37.5 °C and 60 % humidity and turned once each hour. The embryo was decapitated, and the brainstem–spinal cord preparation was dissected by transecting the spinal cord at the level of the lower cervical segment. The preparation was kept in a physiological solution, which contained (in mM) NaCl, 138; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 0.5; glucose, 10; and Tris–HCl buffer (pH 7.3), 10. The solution was equilibrated with oxygen.

Staining with VSDs

For optical recording, the meningeal tissue surrounding the brain was carefully removed in a bathing solution under a dissection microscope. In this study, we tested eight fluorescence styryl (hemicyanine) dyes: RH414, RH795, di-4-ANEPPS, di-2-ANEPEQ, di-3-ANEPPDHQ, di-2-AN(F)EPPTEA, di-4-AN(F)EPPTEA and di-2-ANEPPTEA (Fig. 1). The meaning of the short-coded nomenclature, such as di-4-ANEPPS, is explained in Yan et al. (2012). RH414 and RH795 were acquired from Molecular Probes (Eugene, OR), and di-4-ANEPPS, di-2-ANEPEQ and di-3-ANEPPDHQ were from Life Technologies (Carlsbad, CA). Di-2-AN(F)EPPTEA, di-4-AN(F)EPPTEA and di-2-ANEPPTEA were newly synthesized at the R. D. Berlin Center for Cell Analysis and Modeling, University of Connecticut Health Center, and were kind gifts of Prof. Leslie Loew. The fluorescence dyes were dissolved in a small amount of ethanol and stored at −20 °C. The preparation was stained by incubating it for 20 min in a solution containing 0.04 mg/ml of each dye. In photobleaching experiments, however, the dye concentration was 0.1 mg/ml. In the measurements using NK2761 (Hayashibara Biochemical Laboratories/Kankoh-Shikiso Kenkyusho, Okayama, Japan) (Kamino et al. 1981, 1989; Salzberg et al. 1983), the dye was freshly dissolved in dimethylsulfoxide (DMSO) and then diluted with the physiological solution to a concentration of 0.2 mg/ml (20-min staining). The final concentration of ethanol and DMSO in the staining solution was <0.1 %.

In 7-day-old chick embryos, the thickness of the medulla along the ventrodorsal axis is about 1,000 μm. The immature cellular-interstitial structure of the embryonic tissue allowed the dye to diffuse well from the surface and into deeper regions. After staining, the preparation was attached to the silicone bottom of a recording chamber with the ventral side facing up by pinning it with tungsten wires. The preparation was continuously perfused with the bathing solution at 1–2 ml/min at 26–28 °C.

Merocyanine-Rhodanine Dye (absorption)



Styryl Dyes (fluorescence)

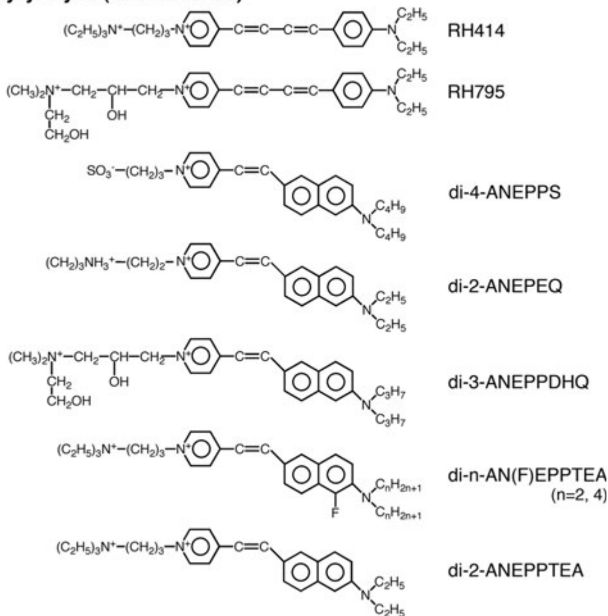


Fig. 1 Chemical structures of the fluorescence VSDs screened in this study, together with the structure of a merocyanine-rhodanine absorption dye, NK2761

Electrical Stimulation for Inducing the Evoked Depolarization Wave

The widely propagating correlated wave, termed the "depolarization wave" (Momose-Sato and Sato 2013a, b), was evoked by applying a single square current pulse to the cervical spinal cord using a concentric bipolar tungsten electrode (TOG204-006; Unique Medical, Tokyo, Japan) at 100–200 μA/1 ms, which induced the maximum response.

Optical Recording

The methods used for multiple-site optical recording of electrical activity have been described in detail elsewhere (Salzberg et al. 1977; Wu and Cohen 1993; Momose-Sato et al. 2001; Canepari and Zecevic 2010). For absorption measurements, light from a 300-W tungsten-halogen lamp (type JC-24 V/300 W; Kondo Philips, Tokyo, Japan) driven by a DC power supply (PAD35-20L; Kikusui Electronic, Yokohama, Japan) was collimated, rendered quasi-monochromatic with a heat filter and an interference filter with a transmission maximum at 703 ± 12 nm (Asahi Spectra, Tokyo, Japan) and focused onto the preparation. For epifluorescence measurements, light from a 300-W tungsten-halogen lamp of the same type as used in the absorption measurements was collimated, rendered quasi-monochromatic with a heat filter and an excitation filter (510–560 nm) and then reflected off a 575-nm dichroic mirror. The fluorescence emission from preparations was transmitted first through the dichroic mirror and then through a 590-nm long-pass emission filter.

An objective (Plan Apo 4× [0.2 NA, numerical aperture], Plan Apo 10× [0.45 NA] or Fluor 20× [0.50 NA]) and a photographic (magnification 1.67×) eyepiece projected a real image of the preparation onto a hexagonally arranged photodiode-fiber optic camera with 464 photodiodes (H-469II; WuTech Instruments, Gaithersburg, MD) mounted on an upright microscope (Eclipse E800; Nikon, Tokyo, Japan). Using objectives of 4×, 10× and 20× with an eyepiece of 1.67×, each detector collected light from a round area having a diameter of 115, 46 and 23 μm, respectively. The output of each detector was amplified individually, digitalized and stored on a computer (NeuroPlex; RedShirtImaging, Fairfield, CT). The feedback resistance of the first-stage amplifier was 20 MΩ (for absorption measurements) or 1 GΩ (for fluorescence measurements), and the second-stage amplifier gain was set to 200×. The amplified outputs were high pass-filtered with a 2.2-s time constant and low pass-filtered with a corner frequency of 1,000 Hz. The amplified signals were acquired at a sampling rate of 0.941 ms per frame using 464 detectors with a 12-bit A/D converter (DAP-3200e/214; Microstar, Bellevue, WA). The optical signals are presented as fractional changes (changes in transmitted or fluorescence light intensity normalized to resting light intensity: $\Delta I/I$ or $\Delta F/F$). During optical recording, perfusion was briefly stopped to minimize motion artifacts. The incident light was turned off using an electronic shutter except during the recording period. VSD signals were analyzed with the computer program NeuroPlex (RedShirtImaging), which runs under IDL (Research Systems, Boulder, CO). To reduce high-frequency noise, detected signals were processed off-line with a low-pass filter (binomial 30×).

Results

Optical Recording of the Depolarization Wave with Absorption/Fluorescence Dyes

In chick, rat and mouse embryos, widely propagating correlated activity, the depolarization wave, is observed during a specific period of development (for reviews, see Momose-Sato and Sato 2013a, b). This activity occurs spontaneously but is also induced by electrical stimulation of the sensory nerve or the brain. In the present dye screening, we evaluated optical signals related to the

evoked depolarization wave because (1) the signal is large and stable and (2) it is easy to make preparations and to induce the signal.

Figure 2a illustrates an example of multiple-site optical recording of the depolarization wave in a 7-day-old embryonic chick brainstem–spinal cord preparation stained with a merocyanine-rhodanine dye (an absorption dye), NK2761. The signals were evoked by direct electrical stimulation applied to the upper cervical cord (lower inset), and the recording was made using a 464-element photodiode array in a single sweep. In Fig. 2b, optical signals recorded from four different sites (a–d in Fig. 2a) are enlarged. The polarity of the optical signals was dependent upon the wavelengths of incident light (data not shown), indicating that the detected signals were indeed dye-absorption changes related to the membrane potential and do not correspond to changes in light scattering related to mechanical or other factors. Using NK2761, the depolarization wave was clearly detected in the same manner as we reported previously (Momose-Sato et al. 2003).

Figure 3a shows an example of multiple-site optical recording of the depolarization wave in a preparation stained with a styryl dye (a fluorescence dye), di-2-ANEPEQ. Optical signals detected in different sites (a–d in Fig. 3a) are enlarged in Fig. 3b. The recording conditions were the same as those in Fig. 2, except for the difference between absorption and fluorescence. Compared with the recording of NK2761 (Fig. 2), the *S*/*N* was smaller with di-2-ANEPEQ because of marked noise. Nonetheless, the waveform and distribution pattern of the optical signals were similar between the two recordings. These similarities indicate that neuronal activity in the embryonic brain is detectable with a fluorescence VSD in a manner reminiscent of that with the absorption dye.

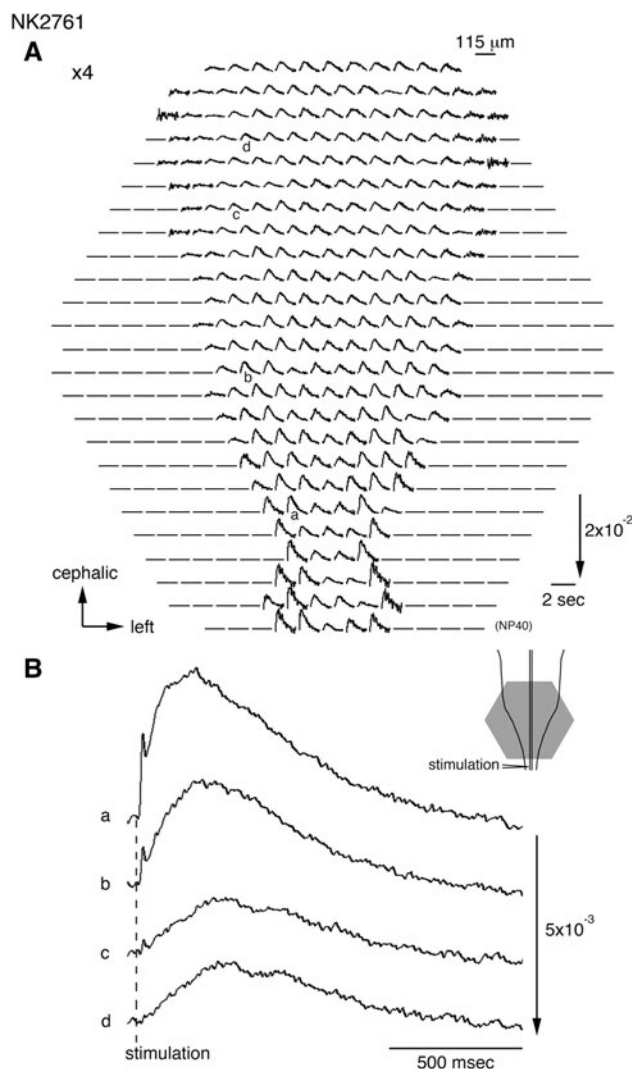


Fig. 2 **a** Multiple-site optical recording of neural responses to upper spinal cord stimulation in a 7-day-old embryonic chick brainstem–spinal cord preparation. The preparation was stained with a merocyanine-rhodanine absorption dye, NK2761. The optical signals evoked by electrical stimulation (200 µA/1 ms) were recorded simultaneously from 464 contiguous regions of the preparation with a magnification of ×4 (an objective) ×1.67 (an eyepiece). The direction of the *arrow* in the *lower right* indicates an increase in transmitted light intensity (a decrease in dye absorption), and the length of the *arrow* represents the stated value of the fractional change (the change in light intensity divided by DC-background intensity). Electrical stimulation elicited a propagating depolarization wave in the spinal cord and the brainstem. An illustration of the preparation is shown in the *lower right*, in which the detected area is marked with a *gray hexagon*. The recording was made in a single sweep. **b** Enlarged traces of the optical signals detected from four regions indicated by *a*–*d* in **a**

Comparison of the Performance of Fluorescence VSDs

Next, we examined the performance of the eight fluorescence VSDs shown in Fig. 1.

Signal Size and S/N

The signal size and *S*/*N* provide good indications of which dyes are likely to be useful for monitoring membrane potential (Cohen et al. 1974; Ross et al. 1977; Gupta et al. 1981; for review, see Cohen and Salzberg 1978). In Table 1, we compared the maximum signal size (maximum $\Delta F/F$, $\Delta I/I$) and *S*/*N* of the optical signals obtained from some preparations (magnification 4× and 1.67×). With the tested fluorescence dyes, the maximum signal sizes were larger, while *S*/*N*s were 10–20 times smaller than those with an absorption dye, NK2761. Among the fluorescence dyes tested, di-4-ANEPPS, di-2-ANEPEQ, di-4-AN(F)EPPTEA, di-2-AN(F)EPPTEA and di-2-ANEPPTEA exhibited the largest signals. With regard to the *S*/*N*, di-2-ANEPEQ showed the best performance, followed by di-3-ANEPPDHQ and then di-4-ANEPPS. When RH414 and RH795 were applied, we obtained very small or undetectable signals.

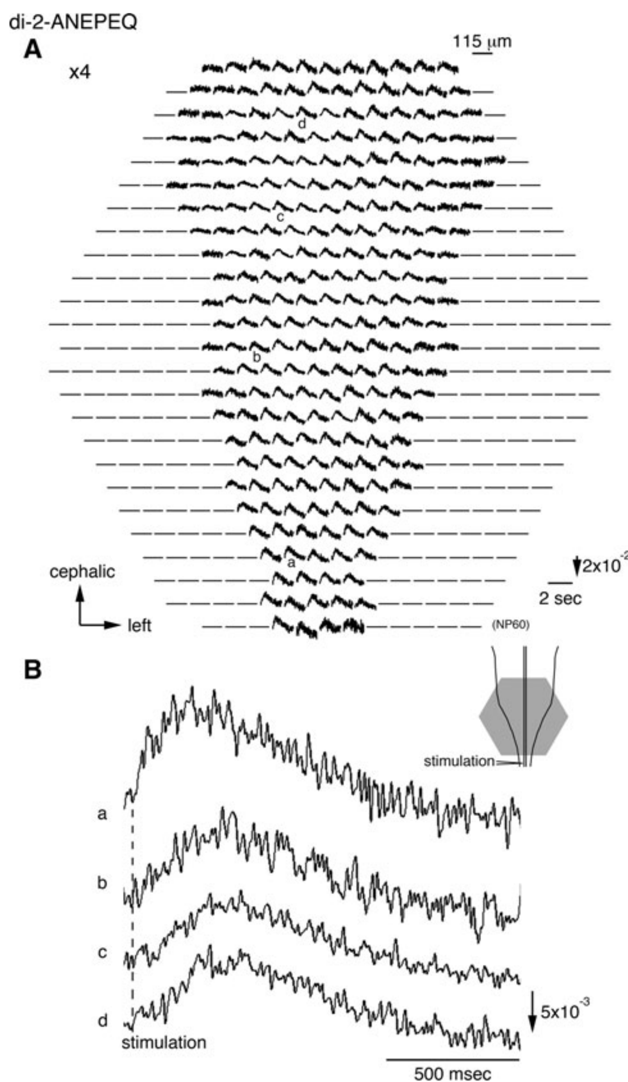


Fig. 3 **a** Multiple-site optical recording of neural responses detected with a styryl fluorescence dye, di-2-ANEPEQ. The direction of the *arrow* in the *lower right* indicates an increase in fluorescence intensity, and the length of the *arrow* represents the stated value of the fractional change. Other experimental conditions were the same as those in Fig. 2. The fractional change was about five times larger than that in Fig. 2, but the *S*/*N* was lower because of larger noise. **b** Enlarged traces of the optical signals detected from four regions indicated by *a*–*d* in **a**

High-Magnification Recording

For optical analysis of neural responses at the cellular level, it is necessary to detect optical responses at high magnifications. In Fig. 4, we compared optical responses recorded with 4×, 10× and 20× objectives for several dyes. As shown in Fig. 4a, although the *S*/*N* was much smaller than with the absorption dye (also see Fig. 2 and Table 1), neural responses were clearly discernable for every fluorescence dye at every magnification. In Table 2, the maximum *S*/*N* evaluated for each dye is summarized. In Fig. 4b, we made spatiotemporal color-coded representations of the optical responses recorded with di-2-ANEPEQ using the 20× objective. This figure, together with the data shown in Table 2, indicates that the quality of fluorescence signals is high enough to resolve neural responses at higher magnifications.

Photobleaching

Exposure of stained preparations to illumination with light causes photobleaching. The less a dye bleaches, the longer the time that stable recording is possible. In Fig. 5, the time courses of photobleaching are compared among the dyes. The abscissa is illumination time, and the ordinate is normalized background light intensity monitored without any stimulation under continuous illumination. As shown in Fig. 5a, the time courses of photobleaching differed among the dyes. For example, di-4-ANEPPS showed almost no change in the background fluorescence, while the fluorescence intensity of di-2-ANEPEQ was reduced to 75 % with 700 s of continuous illumination. Among the newly synthesized dyes (Fig. 5b), di-4-AN(F)EPPTEA showed the least photobleaching, like di-4-ANEPPS. Di-2-AN(F)EPPTEA and di-2-ANEPPTEA exhibited modest photobleaching.

Effects of Perfusion

Perfusion with the physiological solution is considered to cause detachment of dye bound to the cell membrane, which results in a reduction in signal amplitude (Momose-Sato et al. 1999). In Fig. 6, we compared the effects of perfusion among several dyes. In this experiment, incident light was turned off except during the measuring period (about 5 s/h). Normalized fluorescence intensity is plotted against time of perfusion (1 ml/min, lower abscissa), together with total time of illumination (upper abscissa). For di-2-AN(F)EPPTEA, di-2-ANEPPTEA and di-2-ANEPEQ, the fluorescence intensity gradually decreased with perfusion but the relative intensity was > 75 % even after 5 h, suggesting that the effect was not serious (also see Fig. 7). Unexpectedly, for di-4-ANEPPS, di-4-AN(F)EPPTEA and di-3-ANEPPDHQ, the fluorescence intensity increased with time. The rate of increase in fluorescence intensity was in the following order: di-4-ANEPPS > di-4-AN(F)EPPTEA > di-3-ANEPPDHQ. This result might be related to the chemical structure of the dyes, but the underlying mechanism is not understood at present (see “Discussion” section).

Recovery After Staining

Some VSDs are toxic to living cells, which causes a transient reduction in neural responses during staining. In Fig. 7, we examined the time course of recovery in optical

Table 1 Signal size and signal-to-noise ratio

Dye	Preparation reference	Objective 4×	
		Maximum $\Delta F/F$ ($\times 10^{-2}$)	*S/N*
RH414	NP12	0.17	1.1
	NP13	0.18	1.1
	NP17	n.d.	
RH795	NP14	0.40	1.4
	NP20	0.42	1.2
Di-4-ANEPPS	NP15	1.6	1.8
	NP16	1.5	2.1
	NP25	1.7	2.5
	NP59	1.1	2.8
Di-2-ANEPEQ	NP23	1.4	4.3
	NP57	1.7	4.4
	NP60	1.3	4.0
Di-3-ANEPPDHQ	NP30	0.70	2.7
	NP58	0.81	3.0
	NP61	0.75	2.7
Di-4-AN(F)EPPTEA	NP50	1.5	1.5
	NP51	1.2	1.5
	NP71	1.1	1.5
	NP72	1.2	2.5
	NP77	1.3	1.5
Di-2-AN(F)EPPTEA	NP67	1.1	1.4
	NP68	1.1	1.5
	NP73	1.5	1.5
	NP75	1.5	1.8
Di-2-ANEPPTEA	NP63	1.2	1.2
	NP66	1.1	1.1
	NP69	1.4	1.1
	NP74	1.5	1.8
	NP84	1.0	1.3
Dye	**Preparation reference**	**Maximum $\Delta I/I$ ($\times 10^{-2}$)**	***S/N***
NK2761	NP11	0.35	24
	NP32	0.33	22
	NP38	0.34	23
	NP40	0.36	26

The signal size and signal-to-noise ratio were evaluated for the largest signal obtained in each preparation. The signal size and signal-to-noise ratio with NK2761 were also compared. Recordings were made with a magnification of ×4 and ×1.67

$\Delta F/F$ fractional change in fluorescence intensity, $\Delta I/I$ fractional change in transmitted light intensity, *S/N* signal-to-noise ratio, *n.d.* not detectable (*S/N* < 1.0)

responses after staining. To exclude the effects of damage caused by dissection, optical signals were evaluated from 1 h after staining. In this experiment, the incident light was turned off except during the measuring period (about 10 s/measurement), so the effects of photobleaching seemed to be negligibly small.

In Fig. 7, the waveforms of the optical signal differed somewhat between 1 and 3 h for several dyes, including di-4-ANEPPS, di-3-ANEPPDHQ and di-2-ANEPEQ, while those of di-4-AN(F)EPPTEA and di-2-ANEPPTEA were relatively stable for 1–3 h. In Fig. 8, we plot the amplitude and duration of the optical signals against time. The amplitude and duration were normalized to the values at 1 h after staining. The graphs show that for di-2-AN(F)EPPTEA, di-4-AN(F)EPPTEA and di-2-ANEPPTEA the optical signal fully recovered within 1 h after staining, whereas for di-2-ANEPPS, di-3-ANEPPDHQ and di-4-ANEPPS the recovery took longer.

Discussion

In the present study, we screened several styryl VSDs with an emphasis on performance in the embryonic CNS. The tested dyes have been used in various other preparations to monitor transmembrane voltage changes. RH414, RH795, di-4-ANEPPS and di-2-ANEPEQ are commercially available dyes, which are frequently used for monitoring neuronal activity in the adult/postnatal nervous system (Grinvald et al. 1984, 1988; Loew et al. 1992; Zecevic 1996; Onimaru and Homma 2003). Di-3-ANEPPDHQ is a naphthylstyryl-pyridinium potentiometric dye, which is a chimera of RH795 and di-3-ANEPPS. This dye is also commercially available and useful for monitoring neuronal activity in the enteric nervous system (Obaid et al. 2004). Di-2-AN(F)EPPTEA and di-4-AN(F)EPPTEA are recently synthesized fluorinated VSDs, and di-2-ANEPPTEA is a nonfluorinated analog of di-2-AN(F)EPPTEA. Di-2-AN(F)EPPTEA and di-4-AN(F)EPPTEA have been shown to exhibit large optical signals in random-access, two-photon imaging and in multiwavelength imaging (Acker et al. 2011; Yan et al. 2012).

The ideal VSD is highly sensitive to transmembrane voltage changes and has little or no pharmacological or phototoxic effects (Cohen and Salzberg 1978). In addition, it is required that photobleaching is small and that long-term stable recording is possible. To apply a VSD to a new preparation, dye screening is crucial for obtaining successful results because the characteristics of the dye, such as the signal size, *S/N* and toxicity, vary depending on the species and tissues (Ross and Reichardt 1979; Senseman and Salzberg 1980; Grinvald et al. 1988). Evaluation of the dye is especially critical in the embryonic nervous system since embryonic neurons are easily damaged because of the fragility and immaturity of the cells. Here, we discuss the relative utility of different fluorescence dyes for monitoring neuronal activity in the embryonic CNS, focusing on the signal size, *S/N*, photobleaching, effects of perfusion and

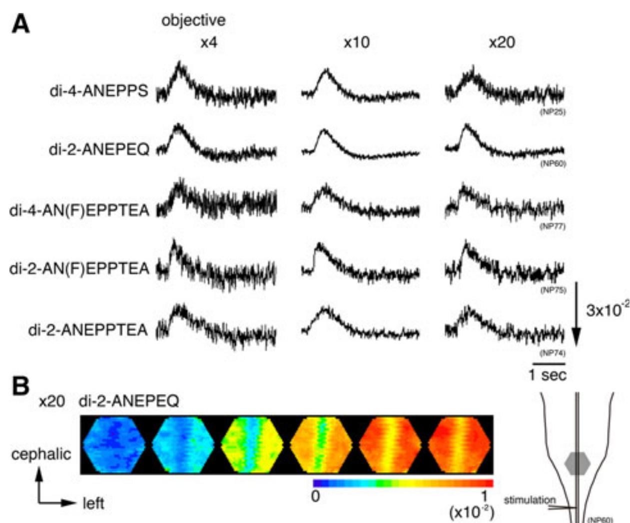


Fig. 4 **a** Optical signals at various microscopic magnifications. Optical recordings were made with an eyepiece (×1.67) and three objectives (×4, ×10 and ×20) for five fluorescence dyes and an absorption dye. **b** An example of color-coded representations of the optical signal related to the depolarization wave. The preparation was stained with di-2-ANEPEQ, and the optical signals were detected with a magnification of ×20 and ×1.67. An illustration of the preparations is shown on the *right*, and the detected area is marked with a *gray hexagon*. The frame interval was 50 ms. The stimulation was applied at the first frame

Table 2 Maximum signal to noise ratio (*S*/*N*) obtained with the objectives of 4×, 10× and 20×

Dye	Objective (maximum *S*/*N*)		
	4×	10×	20×
Di-4-ANEPPS	2.8	6.0	2.4
Di-2-ANEPEQ	4.4	9.0	7.2
Di-4-AN(F)EPPTEA	2.5	3.6	2.0
Di-2-AN(F)EPPTEA	1.8	3.6	2.3
Di-2-ANEPPTEA	1.8	3.8	2.0

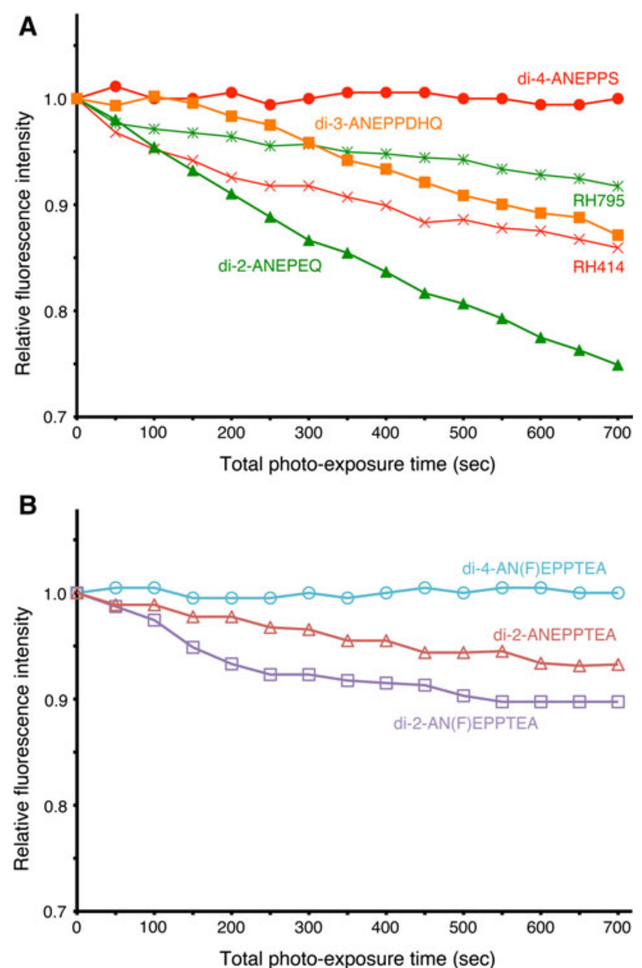


Fig. 5 Effects of illumination on the fluorescence background intensity of the dye. The ordinate represents the relative fluorescence intensity (DC-background light intensity at each time divided by that at time 0), and the abscissa is time of continuous illumination. **a** Results of five fluorescence dyes. **b** results of three recently synthesized dyes

chemical toxicity. It should be emphasized, however, that the relative merits of different fluorescence dyes are different for different preparations (e.g., enteric neurons of the guinea pig [Obaid et al. 2004]).

Signal Size and *S*/*N*

In the present study, the tested fluorescence dyes, except for RH414 and RH795, exhibited large enough signals to be recorded in single sweeps. In the embryonic nervous system, RH414 and RH795 showed very small or undetectable signals, although it has been reported that these dyes are useful in other preparations (Grinvald et al. 1984, 1988; Obaid et al. 2004). Differences in the performance of the VSD between embryonic and adult tissues have also been observed in absorption dyes, such as an oxonol dye, NK3041 (RH155), which provides large absorption signals in adult hippocampal slices, while it gives small signals in the embryonic CNS (Momose-Sato et al. 1995, 1999).

In the embryonic CNS, the synapses are functionally immature and easy to fatigue, even with 0.1 Hz repetitive stimulation (Sato et al. 1999; Mochida et al. 2001; Momose-Sato et al. 2001). Thus, it is usually necessary to record neural responses in single sweeps. In the present study, although the *S*/*N* of the fluorescence dyes was smaller than that of NK2761, it was sufficient for the analysis without averaging, even at high magnifications (Fig. 4; Table 2). Using the 20× objective with the 1.67× eyepiece, each detector collects light from a round area with a diameter of 23 μm, suggesting that cellular-level analysis might be possible.

Photobleaching

For all of the tested fluorescence dyes except di-2-ANEPEQ, the change in background fluorescence intensity was <15 % after 700 s of continuous illumination. This result suggests that the effect of photobleaching is not serious in experiments in which the incident light is turned off between recordings. The largest change in the background light intensity was observed for di-2-ANEPEQ, which might be problematic when long-term recording is performed.

Washout by Perfusion

In our previous study of absorption dye screening, we reported that background light intensity changed with perfusion, which is possibly caused by detachment of dye molecules from the cell membrane (Momose-Sato et al. 1999) or by internalization of the dye molecules. In the present study, we observed that fluorescence intensity slightly decreased with perfusion for di-2-AN(F)EPPTEA, di-2-ANEPPTEA and di-2-ANEPEQ. Although speculative, a similar mechanism as suggested for the absorption dye might underlie the present result. Surprisingly, for di-4-ANEPPS, di-4-AN(F)EPPTEA and di-3-ANEPPDHQ, the fluorescence intensity gradually increased with time in the order of di-4-ANEPPS > di-4-AN(F)EPPTEA > di-3-ANEPPDHQ. The styryl dye is anchored to the membrane with a pair of alkyl groups on the amino end, and the longer chain alkyl groups bind more tightly to the membrane (Yan et al. 2012). But the dyes with longer alkyl chains are less soluble in aqueous solution, may associate with the walls of the chamber and may take longer to equilibrate through the tissue and bind to the neuron membranes. Since only the membrane-bound form of the dyes is fluorescent, this may explain the increase in fluorescence for the dye molecules with longer alkyl chains.

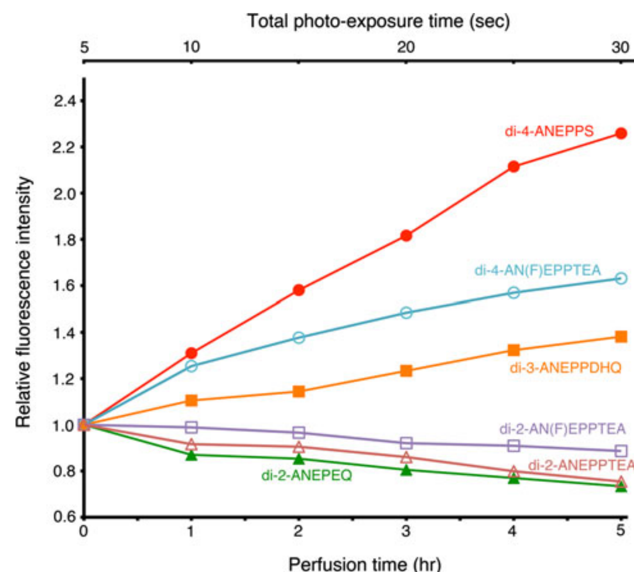


Fig. 6 Effects of perfusion on the fluorescence background intensity of the dye. The incident light was turned off except during the measuring period. The ordinate represents the relative fluorescence intensity (DC-background light intensity at each time divided by that at time 0). The lower abscissa is time after staining (perfusion rate 1 ml/min), and the upper abscissa is the total illumination time

Toxicity

In the embryonic CNS, optical signals for di-2-AN(F)EPPTEA, di-4-AN(F)EPPTEA and di-2-ANEPPTEA recovered within 1 h after staining. However, di-4-ANEPPS, di-3-ANEPPDHQ and di-2-ANEPPS required a longer time for recovery. These results suggest that the former dyes are less toxic than the latter ones in the embryonic CNS.

In the present study, it has been shown that some of the fluorescence VSDs are potentially useful for monitoring neuronal activity in the embryonic CNS. Among the dyes tested, di-2-ANEPEQ exhibited the largest *S*/*N*. However, its photobleaching was faster, and the recovery of neural activity after staining was slower. This dye seems to be useful for short-term recording, when enough time is allowed for recovery. Di-4-ANEPPS and di-3-ANEPPDHQ also showed large *S*/*N*s, together with less photobleaching. However, these dyes also required a relatively long time for the recovery of neural activity after staining. Furthermore, the background fluorescence intensity changed unstably with time, the mechanism of which is unknown. The newly synthesized styryl dyes di-4-AN(F)EPPTEA,

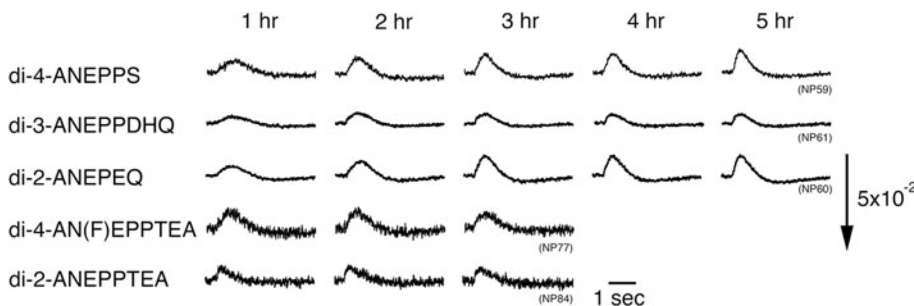


Fig. 7 Time-dependent changes in optical signals after staining. Preparations were stained with five fluorescence dyes. Optical signals related to the depolarization wave were detected with a magnification of ×10 and ×1.67 in single sweeps at 1, 2, 3, 4 and 5 h after staining

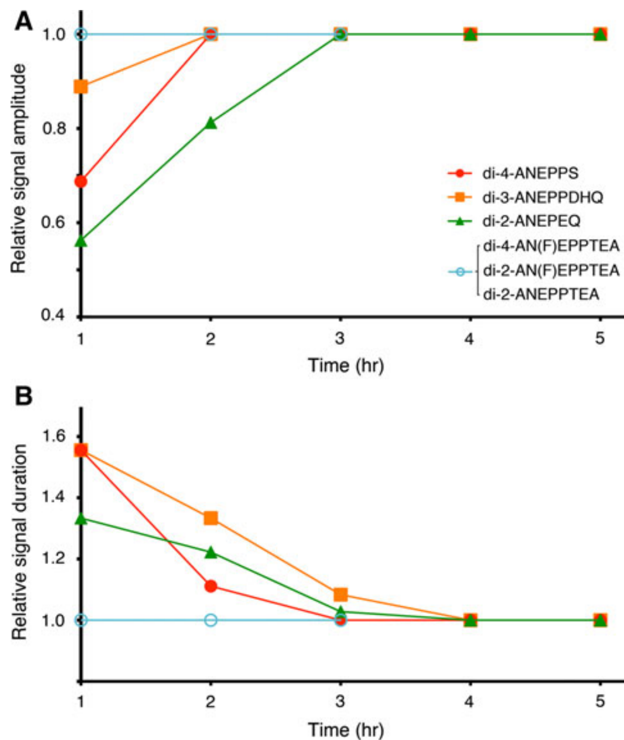


Fig. 8 Time-dependent changes in optical signals after staining. Amplitudes (**a**) and durations (**b**) of the optical signal normalized to the values at 1 h after staining are plotted against time. The signal duration was measured at 50 % of the maximum signal amplitude

di-2-AN(F)EPPTEA and di-2-ANEPPTEA exhibited smaller *S*/*N*s than di-2-ANEPEQ; but their photobleaching was slower, and the recovery after staining was faster. These dyes appear to be suitable for long-term recording or recording in which only a short time is available for recovery after staining.

The early embryonic brain has a histologically loose structure with immature neurons/glia and undifferentiated connective tissue. In addition, the brain is small and thin. The high translucency of the embryonic brain gives large *S*/*N* in absorption measurements; thus, absorption dyes, rather than fluorescence dyes, have usually been used as molecular voltmeters in embryonic preparations. Indeed, in the 7-day-old chick brainstem, the *S*/*N* of the fluorescence signal was inferior to that of the absorption signal, even with di-2-ANEPEQ, which showed the best performance in the present study. Nevertheless, the translucency of the tissue becomes low as development proceeds, and fluorescence dyes might be a better choice when optical recording is performed at the late embryonic stage or using postnatal/posthatching animals. Also, fluorescence dyes are far superior when used in an epi-illumination configuration (i.e., whole brain).

In general, the *S*/*N* in fluorescence is degraded by nonspecific binding of the dye to extraneous material, as in the case of brain slices. However, in situations where the dye is bound only to the cell membrane and there is only one cell in the light path (e.g., tissue-cultured neurons), the *S*/*N* in fluorescence can be much larger than that in absorption (Wu and Cohen 1993). Considering this, it is possible that the fluorescence dyes tested in the present study give better performance in optical recording at the single-cell level. Continuing efforts are being made to develop and screen dyes with larger *S*/*N*s, which may ultimately lead to improvements in optical analyses of the developmental progress of the CNS.

Acknowledgments This research was supported by Grants from the Monbu-Kagaku-sho of Japan, the Human Frontier Science Program (Grant RGP0027/2009) and the US National Institutes of Health (grant R01EB001963). S. H. M. was supported by the Opto-Medical Institute as a postdoctoral fellowship.

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