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New near-infrared optical probes of cardiac electrical activity

Aryvydas Matiukas,1,3 Bogdan G. Mitrea,1 Arkady M. Pertsov,1 Joseph P. Wuskell,2 Mei-de Wei,2 James Watras,2 Andrew C. Millard,2 and Leslie M. Loew2

1Department of Pharmacology, State University of New York Upstate Medical University, Syracuse, New York; 2Department of Cell Biology, Center for Cell Analysis and Modeling, University of Connecticut Health Center, Farmington, Connecticut; and 3Department of Physics, Kaunas University of Technology, Kaunas, Lithuania

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VOLTAGE-SENSITIVE DYES were designed either to assess average membrane potentials in cell population measurements obtained with a spectrofluorometer or to determine spatial patterns of voltage distributions associated with tissues, individual cells, or organelles. The two most common fields of application are neurology (10, 11, 31, 43) and cardiology (12, 28, 33, 40). These dyes allowed unique experiments [such as measuring the membrane potentials across the inner membranes of individual mitochondria within a single living cell (19)] that could not be accomplished with conventional electrical measurements obtained using microelectrode or patch clamping techniques.

Voltage-sensitive dyes in combination with advanced optical methods have had an especially important impact on the study of patterns of electrical activity in cardiac tissue (18). The styryl dye di-4-ANEPPS proved to be a particularly effective voltage probe for cardiac electrophysiology, providing submillisecond temporal resolution, high fluorescence, stable staining, low photobleaching, and low toxicity (34).

Use of di-4-ANEPPS and high-speed video cameras or photodiode arrays yielded high spatial and temporal resolution recordings of electrical activity in the subsurface layers of cardiac tissue. It permitted visualization of such complex structures as spiral waves (6, 12). Although di-4-ANEPPS is now the primary probe of choice for many laboratories, it has an important limitation in that it allows optical potentials from only a few hundred micrometers of subsurface layer (9).

There is intense interest to extend these investigations to spiral and scroll waves in millimeter-thick layers or across whole ventricular wall (thickness up to ~10 mm), with the final goal being to reconstruct three-dimensional scroll waves. This requires new voltage-sensitive dyes that exhibit excitation/emission peaks at longer wavelengths to provide lower light scattering, and less background fluorescence from endogenous chromophores in cardiac tissue. An additional issue is to shift the dye excitation spectrum away from the blood absorption peak. The Grinvald laboratory has synthesized a series of dyes with an oxonol chromophore that have absorbance maxima at ~630 nm; these have proven useful in recording neuronal activity (36). New optical methods such as transillumination, narrow illuminating beam scanning, and transmitted light recording all promise to improve deeper layer imaging (7, 17) provided that effective dyes can be identified. Our goal was to investigate new long-wavelength styryl dyes (41) because of the proven ability of this chromophore class to be particularly useful for cardiac preparations.

Styryl dyes such as di-4-ANEPPS have large Stokes shifts (i.e., the difference between the excitation and emission wavelengths) that make them particularly convenient for microscopy, because a large Stokes shift eases the exclusion of scattered and reflected light and the filtering away of background autofluorescence. Another favorable feature of the styryl dyes is their high fluorescence quantum efficiency when bound to membranes but negligible fluorescence in aqueous solution; thus only stained cells contribute to the fluorescence signal even if the experimental protocol does not permit washing away the staining solution. The existing repertoire of styryl potentiometric dyes has varying solubility, lipid avidity, tissue penetrability, and ionic charge that allows them to be customized for specific types of experimental demands (1, 2, 29, 39, 42). However, one limitation of the currently available set of styryl dyes is that their range of absorbance spectra is limited...
to the blue-green region of the spectrum, with the longest wavelength dye extending only to ~520 nm. Longer wavelength dyes would permit the design of experiments with even lower autofluorescence and values away from the absorbance of many biological chromophores such as NADH and hemoglobin. Also, because light scattering decreases with wavelength in the range of 600–1,000 nm (26, 38), longer wavelength dyes would permit deeper light penetration into intact tissue for both cardiac tissue slices and in vivo preparations. This report describes and characterizes three new styryl dyes with absorption and emission peaks that occur at wavelengths >150 nm longer than those of di-4-ANEPPS. We have examined these dyes in detail for their ability to report electrical activity in cardiac tissue from three animal models: rat, guinea pig, and pig. Evaluation of these dyes alongside di-4-ANEPPS indicates that this new set of styryl dyes provides comparable sensitivities to membrane potential and can be chosen to optimize properties such as dye penetration and persistence.

**METHODS**

Dye spectra and sensitivity measurement in model membranes. Absorbance and emission spectra in ethanol and model membranes (25) were measured as described previously (41). Voltage-dependent spectra in model membranes were measured using a voltage-clamped hemispherical lipid bilayer (HBLB) apparatus (14, 22) modified for near-infrared fluorescence detection (41). In this experiment, light from a tungsten lamp is sent through a monochromator and then focused onto the bottom of the HLB, stained with dye from the external aqueous bathing solution. The monochromator is then scanned over the wavelength range of interest while a train of ±50-mV voltage steps are applied to the membrane at a frequency of 40 Hz, and the modulation of the detected light signal is measured with a lock-in amplifier. The transmitted light signal is collected at 180° from the incident light. Fluorescence is collected at 90° via fiber optic light guide (22, 41) through cutoff filters at 715, 780, and 780 nm for the dyes JPW3067, JPWS034, and JPWS020, respectively.

Emission spectra in stained rat heart tissue were measured using an Ocean Optics spectrometer, as specified. Adult rats were euthanized by carbon dioxide asphyxiation, and then the heart was excised and placed in ice-cold Hanks’ buffered saline solution (HBSS). The left ventricular myocardium was divided into four sections and incubated in ice-cold HBSS with or without the specified styryl dye (at a final concentration of 100 μM). After a 20-min incubation in the specified styryl dye solution (on ice), the emission spectra of the dye in the ventricular myocardium were obtained (as described below). For these emission spectra, the myocardial pieces were excited with a 30-W helium-neon laser at 633 nm (providing 80 μW at the specimen), using a 650-nm long-pass dichroic mirror and a 650-nm long-pass emission filter. Emission signals were acquired for 200 ms and were corrected for the nonideal transmission of both the emission filter and the dichroic mirror (by normalizing to their transmission spectra). Comparable emission spectra (though much higher levels of fluorescence) were obtained in right ventricular myocardium after injection of 100 μM JPWS034 (in HBSS) into a coronary vessel.

Cardiac tissue preparation and staining methods. All experimental protocols conformed to the Guide for the Care and Use of Laboratory Animals [DHAEW Publication No. (NIH) 85-23, Revised 1996, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205]. For rats (Sprague-Dawley, 300–400 g, female, n = 30) and guinea pigs (n = 6), animals were injected with heparin (550 U/100 g) and euthanized with pentobarbital sodium (1 ml/100 g for rats and 0.75 ml/100 g for guinea pigs), after which the heart was immediately excised and placed in ice-cold cardioplegia solution (CPS) composed of (in mM) 280 glucose, 13.44 KCl, 12.6 NaHCO3, and 34 mannitol (15). After removal of extraneous tissues, the aorta was cannulated and Langendorff perfusion was started with a standard oxygenated Tyrode solution [composed of (in mM) 130 NaCl, 24 NaHCO3, 1.2 NaH2PO4, 1.0 MgCl2, 5.6 glucose, 4.0 KCl, and 1.8 CaCl2, buffered to a pH of 7.4] at 80 mmHg and 36°C (3). Young pigs (15–20 kg, n = 14) were heparinized (500 IU iv) and subsequently anesthetized with pentobarbital sodium (35 mg/kg iv). The heart was rapidly removed and Langendorff perfused with cold (4°C) CPS (15). The right free ventricular wall was quickly excised, and the right coronary artery was cannulated. Nonperfused tissue was removed, leaving a preparation of typically 5 × 5 cm and a thickness of 8 mm. The preparation was stretched on a plastic frame and perfused with a standard oxygenated Tyrode solution at 80 mmHg and 36°C. Whole cannulated heart (rat or guinea pig) or stretched pig tissue was put into a special transparent chamber and superfused with the same solution at a rate of 30–40 ml/min. Perfusion and superfusion temperatures were continuously monitored and kept at 36 ± 1°C by using two sets of glass heating coils and heated-refrigerated circulators. Electrodes were saturated to whole hearts or inserted into pig tissue to monitor ECG. All preparations were continuously paced at frequencies of 5, 3.3, and 2 Hz for rat, guinea pig, and pig tissue, respectively.

After a 30-min stabilization period during which the perfusion flow and ECG were monitored, the excitation-contraction uncoupler diacetyl monoxide (DAM) was added to the Tyrode solution (15 mM) to stop contractions of cardiac tissue. After a 20-min equilibration, the preparation was stained by injecting voltage-sensitive dye solution into the perfusion flow.

Solid dyes were dissolved in DMSO to make 10–50 mM stock solution that was kept frozen. The dye solution was made by dissolving the required amount of the stock solution into 1 ml of Ringer solution [composed of (in mM) 140 NaCl, 5.6 KCl, 1.0 MgCl2, 5.0 HEPES, 10.0 glucose, 2.0 NaH2PO4, and 2.2 CaCl2] with 0.1–0.2% Pluronic F-127 (final concentration) to facilitate loading. Various dye doses (1–1,000 nmol) were administered, and optimal doses and concentrations were found by trial and error. We found 400, 250, and 150 μM to be optimal concentrations, and 200, 200, and 100 nmol per 1 g of tissue to be optimal doses, of the dyes JPW3067, JPWS034, and JPWS020, respectively (we used only 10% of these doses in the case of pig preparations because of much higher dye consumption). However, it should be noted that optimal doses depend on many factors such as perfusion system, staining method, and protocol, and the best values should be determined for each experimental setup and protocol.

The dye loading (perfusion) was found best to be performed at temperatures of 36 ± 1°C, because at higher temperatures low dye doses often evoked significant arrhythmias (ventricular tachycardia or fibrillation) immediately or a few minutes after dye injection. The normal excitation wave propagation in cardiac tissue was verified by injecting a small amount of di-4-ANEPPS (3–10 nmol) at both the beginning and end of each experiment (using appropriate excitation/emission filters to prevent cross talk between the dyes, as described below).

Optical action potential recording. The optical setup for testing the new dyes was similar to the optical mapping setup described previously (5, 16). A collimated beam provided by a 250-W tungsten halogen lamp uniformly illuminated the epicardial surface of the preparation. The light was heat filtered and then passed through the excitation filter. For excitation of di-4-ANEPPS, light was filtered through 520 ± 40-nm band-pass filters, and for the new styryl dyes, through 650 ± 20-nm band-pass filters. Optical action potentials (OP) were recorded with a cooled fast charge-coupled device (CCD) camera (Little Jo; SciMeasure) with a Computar H1212FI lens (focal length 12 mm, 1:1.2 aperture ratio, diameter 28 mm; CBC), located at ~100 mm from the sample. Lenses of both the light source and the camera were adjusted to illuminate and image an area 25 mm in diameter at the center of the preparation; hence, spatial resolution in the images was 0.31 mm/pixel. The fluorescent light emitted by the voltage-sensitive dye was isolated from excitation by using a 640 ±
50-nm band-pass filter for di-4-ANEPPS and long-pass filters for styryl dyes with 50% transmission at 720 nm (for JPW3067 and JPW5020) and 850 nm (for JPW5034) (filters for the new dyes were determined experimentally). The camera was located either on the same side of the preparation for epifluorescence mode recording or at the opposite side for transillumination mode recording. To reduce cardiac tissue motion artifacts, we used DAM as described above; in addition, whole rat or guinea pig hearts were gently pressed against stretched nylon mesh (on a single side opposite both the light source and camera so as not to affect the amount of collected fluorescence). These measures in most cases completely eliminated motion artifacts.

The video images (80 × 80 pixels, 14 bits) were acquired at 500–2,000 frames/s, and the background autofluorescence (endogenous fluorescence recorded under the same conditions before dye injection) was subtracted from each frame to obtain the voltage-dependent optical signal. To allow temporal alignment and subsequent averaging of successive paced action potentials, we recorded the trigger for the pacing stimulus as a single pixel in the movie frames. The alignment error was no more than one-half frame (~0.5 ms). We used ensemble averaging to improve signal-to-noise ratio (i.e., averaging the OP signals from 10–20 sequential recordings). To further reduce noise, we then low-pass filtered the OP signal in both time (with a 3- to 7-point triangular window, depending on the camera frame rate) and spatial domains (with a pyramidal 5 × 5 kernel). The effective spatial and temporal resolution were 1.76 –3.33 ms and 0.78 mm, respectively. Dye loading and washout dynamics were assessed from averaged sets of OP recordings (3–5 specimens). To facilitate comparison, we normalized fluorescence and OP signals to their peak values (defined as the difference between maximum and minimum values) in some figures.

For the dye loading dynamics, the time to reach peak values of background fluorescence (membrane voltage-independent component of the dye molecule fluorescence), OP, and relative fluorescence were determined by simple visual inspection of the dynamic plots. For the dye washout dynamics, analogous half-times of the same parameters were determined.

**RESULTS**

New dyes structures, spectra, and sensitivity in model membranes. We tested and characterized three new voltage-sensitive dyes: JPW3067, JPW5034, and JPW5020. The structures of these dyes are shown in Fig. 1. All three dyes have the same chromophore, but they differ by the length of hydrocarbon chains (41). Molecular weights are 659.59, 743.74, and 855.95, respectively. For comparison, the structure of di-4-ANEPPS is included at the bottom of Fig. 1.

To successfully apply these new dyes, one has to know the excitation and fluorescence wavelengths. They can be chosen from absorbance and emission spectra shown in Fig. 2. Absorbance spectra were measured in ethanol (Fig. 2A) and in a model membrane made of multilamellar lipid vesicles (MLV) composed of egg phosphatidylcholine (Fig. 2B). The absorbance spectra reveal several maxima that have variable amplitudes under different pH or solvent conditions and may represent different configurational isomers. The longest wavelength peak in MLV is ~200 nm to the red compared with that of the widely used voltage-sensitive dye di-4-ANEPPS. When the three different JPW dyes are compared, the long-wavelength absorbance peaks range from 670 to 705 nm in ethanol and from 630 to 646 nm in MLV. However, efficient (>50% of maximum absorbance) excitation can be achieved over a much broader range, from 510 to 800 nm, allowing one to optimize excitation for the dye in different tissues, including blood-perfused tissue. Figure 2, C–E, shows the emission spectra in
ethanol, MLV, and electrically inactive rat heart tissue (we did not observe any time dependence of the spectra), respectively. When excited at 633–650 nm, all three JPW styryl dyes emit over the range of 750–900 nm in MLV (Fig. 2D) or with a peak at 750–780 nm in rat myocardium (Fig. 2E). In ethanol, the emission spectra are shifted to higher wavelengths (Fig. 2C). Thus the new voltage-sensitive dyes emit fluorescence light in the near-infrared region (NIR). The emission spectra of the new dyes in MLV and muscle have maxima 150–170 nm to the red compared with di-4-ANEPPS.

Figure 3 shows the wavelength dependence of the relative transmitted light (A) and fluorescence (B) responses for the new dyes in a voltage-clamped HLB (i.e., $\Delta T/T$ and $\Delta F/F$ vs. wavelength for 100-mV voltage steps) for the new dyes. The biphasic response results from a voltage-dependent shift of the absorbance or emission spectrum to shorter wavelength with depolarization. Thus for a depolarizing voltage step (+100 mV), the absorbance increases on the low-wavelength side of the absorbance maximum, decreases on the high-wavelength side, and crosses zero near the absorbance maximum. Fluorescence is being excited by absorption and exhibits the same trend. Because transmittance is the opposite of absorbance, relative transmission shows an opposite trend (decreasing on the low-wavelength side of the absorption maximum and increasing on the high-wavelength side). Relative fluorescence is two orders of magnitude higher, because very strong illumination background is eliminated by the fluorescence collection filter. This should be kept in mind when designing dye applications (unless working with very thick tissues that absorb almost all incident light). The biphasic shape of $\Delta T/T$ and $\Delta F/F$
curves is consistent with an electrochromic mechanism for the new dyes, as has been suggested for the older styryl dyes. This mechanism provides submillisecond response time and very linear response to the membrane potential (22).

Amplitude of OPs in rat, guinea pig, and pig. Figure 4A shows unprocessed sample images of the fluorescence in rat tissue stained with the new styryl dyes and di-4-ANEPPS, as well. The “+” shape marker shows the pixel on the image from which an unprocessed time course for the optical signal is shown as a shaded trace in Fig. 4B. Negative polarity of raw action potentials means that the voltage-sensitive fluorescence was collected on the red (longer wavelength) wing of the dye emission spectrum. Thick black trace in Fig. 4B shows processed optical signals (except inversion). Processed signals were used for all further measurement results.

Figure 5 shows processed and inverted OPs in various cardiac tissues stained with the new styryl dyes (for Figs. 5–9, curves represent means of 3–5 specimens). OPs for di-4-ANEPPS also are provided for comparison. Figure 5A shows OP in rat heart. JPW3067 exhibits an OP amplitude comparable to that of di-4-ANEPPS. JPW5020 displays a smaller fluorescence change but has other useful features such as very slow washout (discussed below). JPW5034 has both a medium fluorescence response and washout time. However, it should be noted that a much higher dye concentration (up to 40 times compared with di-4-ANEPPS) has to be applied for the new styryl dyes to work effectively. The rat heart was stained with optimal concentrations of 400, 250, and 150 μM of the dyes JPW3067, JPW5034, and JPW5020, respectively (di-4-ANEPPS was applied at 10 μM). On the other hand, applicable dye concentration is limited by the toxic effect of the dye. We observed toxic effects of the new styryl dyes on the cardiac tissue when concentrations exceeding 500 μM were applied. Longer upstroke in the OP recorded by the new dyes is related to deeper sampling of the cardiac tissue because of deeper penetration of red excitation light (16).

Figure 5B shows OP in guinea pig heart. All dye concentrations are identical to those for a rat. Guinea pig myocardium generally provides a higher OP. In this tissue, 500 μM JPW3067 provides an even higher fluorescence change than 10 μM di-4-ANEPPS. JPW5034 (250 μM) provides fluorescence sensitivity similar to that of 10 μM di-4-ANEPPS, whereas the fluorescence of 150 μM JPW5020 is lower.

Figure 5C shows fluorescence OP in coronary-perfused, isolated, right ventricular pig tissue. Again, as measured by...
relative fluorescence $\Delta F/F$, JPW3067 outperforms di-4-ANEPPS, JPW5034 provides similar performance, and JPW5020 provides a lower response. Figure 6 provides transillumination OP in pig heart. We can clearly see that in epifluorescence mode, the new dyes performed comparably or worse than di-4-ANEPPS. In transillumination mode, where much deeper layers contribute to the OP signal (7), the most efficient styryl dye JPW3067 definitely outperformed di-4-ANEPPS.

Table 1 summarizes the maximal fluorescence efficacies (expressed as $\Delta F/F$) for the new styryl dyes in different cardiac tissues, and Table 2 provides the maximal OP signal-to-noise ratio for the same dyes and tissues. Efficacy for di-4-ANEPPS also is provided for comparison in the same setup. Generally, JPW3067 has the highest efficacy among the new dyes, being comparable or higher than that of di-4-ANEPPS [the quantum efficiency of the new dyes has not been measured, but on the basis of other similar styryl dyes, we assume it to be on the order of 0.3 (14)]. JPW5034 has medium efficacy, whereas JPW5020 has the lowest efficacy. Regarding tissue-specific efficacy, efficacies in rat and guinea pig are similar. The efficacies for the pig tissue are slightly lower; this is probably related to lower staining level, because the weight of pig tissue was typically 15–20 times higher. This finding also shows that much less dye is required per 1 g of pig tissue to obtain the same OP.

The data from Table 2 show that, in most cases, the new styryl dyes provided better signal-to-noise ratio. This is usually important for the optical mapping of cells or cell cultures. In addition, in transillumination mode in pig, the two best dyes (JPW3067 and JPW5034) provide much higher S/N ratio than di-4-ANEPPS.

Dye staining and washout in rat, guinea pig, and pig. Figure 7 shows the new dye loading and washout dynamics in rat (photobleaching was not observed in our experiments, see DISCUSSION). Figure 7 shows the dynamics for the normalized OP amplitude (A) and the normalized fluorescence (B). Figure 7A also shows $Y$ error bars to indicate the level of variation of the dynamics ($Y$ error bars for any curve in Figs. 7–9 do not exceed 20% during dye-loading phase and 50% during washout; curves represent means of 3–5 specimens). Figure 7C shows the dynamics for the relative fluorescence, $\Delta F/F$. The dynamics of the dye di-4-ANEPPS also are shown for comparison. JPW3067 provides good relative fluorescence and also rapidly equilibrates with the tissue: it both loads and washes out in $\sim 50$ s. The remaining background fluorescence level is lower than 10%. Depending on the application, this either allows multiple staining with good $\Delta F/F$ and low accumulated toxicity or may require continuous dye administration to maintain a constant level of OP. JPW5034 has a loading and washout profile similar to that of di-4-ANEPPS but with a longer loading phase and a slightly higher relative fluorescence level.
washout rate similar to that of di-4-ANEPPS. The remaining background fluorescence level is ~40%, indicating some internalization of the dye. JPW5020 is a long-lasting dye that loads a little slower and washes out very slowly, allowing one to record OP in rat twice as long as di-4-ANEPPS (i.e., 30 min). A second staining of the heart with JPW5020 did not extend the recording of OPs, probably because of internalization of the dye (as evidenced by a high background fluorescence).

The loading and washout dynamics of the styryl dyes in myocardium from guinea pig and pig are shown in Figs. 8 and 9, respectively. Data in Figs. 8 and 9 are presented in a manner similar to the data in Fig. 7. The dynamics progressively slow

![Fig. 7. Dye loading and washout dynamics in rat. Dynamics are shown for the normalized optical action potential (OP) amplitude (A), normalized fluorescence (B), and ΔF/F (C). The dynamics of the dye di-4-ANEPPS (Di-4) are shown for comparison. Error bars in A indicate the level of data variation.](image1)

![Fig. 8. Dye loading and washout dynamics in guinea pig. Dynamics are shown for the normalized optical action potential (OP) amplitude (A), normalized fluorescence (B), and ΔF/F (C).](image2)
down from rat to guinea pig to pig. The difference in the dye washout dynamics is even more pronounced between guinea pig and pig (note the longer time scale in Fig. 9).

Tables 3, 4, and 5 summarize the loading and washout times (in s) for rat, guinea pig, and pig, respectively. The 100\% loading time indicates when the maximum value of a parameter is reached after dye injection, and the washout time shows the time interval (starting from the loading time) required for the parameter to drop to 50\% of its maximum value (50\% washout time). An inset in the Fig. 9C illustrates the definition of these times. The times are provided for three parameters: OP amplitude, background fluorescence level, and relative fluorescence, because their values generally differ significantly ($P < 0.05$).

The time values of most parameters progressively increase from rat to guinea pig to pig.

The general trend for the new dyes is that JPW3067 loads and washes out the fastest, whereas JPW5034 and JPW5020 show progressively longer loading and washout kinetics. Also, there was a trend in the loading and washout of the dyes in the studied species. In rat, this happens the fastest; in guinea pig, at a medium rate; and in pig, the slowest.

### DISCUSSION

In this report, we have described and characterized three new NIR styryl dyes with absorption and emission peaks that occur at wavelengths $\sim 150$ nm longer than those of di-4-ANEPPS. We examined these dyes in detail for their ability to report electrical activity in cardiac tissue from three animal models: rat, guinea pig, and pig. We did not test the new dyes on rabbits, but we point out, however, that extensive literature on optical mapping utilizing styryl voltage-sensitive dyes in various species including rats, guinea pigs, pigs, and rabbits does not show significant interspecies difference in the signal amplitudes (38–41). In this study, the measured efficacy range of 6.9–9.1\% for di-4-ANEPPS well agrees with the value range of 4–15\% obtained by other researchers (4, 23, 34). On the basis of this agreement, we expect the new dyes also perform efficiently in rabbits. Our study shows that one may

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**Table 3. Loading and washout times of the new styryl dyes and di-4-ANEPPS in rat**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>JPW3067</th>
<th>JPW5020</th>
<th>JPW5034</th>
<th>Di-4-ANEPPS</th>
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<tbody>
<tr>
<td>100% Fl* loading</td>
<td>47±7</td>
<td>540±51</td>
<td>275±35</td>
<td>292±39</td>
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<tr>
<td>100% OP loading</td>
<td>45±3</td>
<td>540±50</td>
<td>225±21</td>
<td>332±41</td>
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<tr>
<td>100% ΔF/F loading</td>
<td>45±2</td>
<td>540±51</td>
<td>225±22</td>
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<tr>
<td>50% Fl* washout</td>
<td>83±15</td>
<td>3050±340</td>
<td>1755±295</td>
<td>870±147</td>
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<tr>
<td>50% OP washout</td>
<td>45±3</td>
<td>990±125</td>
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<td>70±10</td>
<td>1110±195</td>
<td>475±64</td>
<td>1900±145</td>
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</table>

Values are means ± SD (in seconds). Fl*, background fluorescence level; OP, optical action potential.

---

**Table 4. Loading and washout times of the new styryl dyes and di-4-ANEPPS in guinea pig**

<table>
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<tr>
<th>Parameter</th>
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<th>JPW5020</th>
<th>JPW5034</th>
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<tr>
<td>100% Fl* loading</td>
<td>45±7</td>
<td>1,050±75</td>
<td>250±27</td>
<td>315±49</td>
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<tr>
<td>100% OP loading</td>
<td>45±7</td>
<td>600±59</td>
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<tr>
<td>100% ΔF/F loading</td>
<td>45±7</td>
<td>630±62</td>
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<tr>
<td>50% Fl* washout</td>
<td>65±8</td>
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<td>1,350±123</td>
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<tr>
<td>50% OP washout</td>
<td>48±6</td>
<td>925±119</td>
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<td>600±117</td>
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<tr>
<td>50% ΔF/F washout</td>
<td>120±10</td>
<td>1,400±223</td>
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Values are means ± SD (in seconds). †Extrapolated value.
Table 5. Loading and washout times of the new styryl dyes and di-4-ANEPPS in pig

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<th>Parameter</th>
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<th>JPW5034</th>
<th>Di-4-ANEPPS</th>
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<tr>
<td>100% Fl⁺ loading</td>
<td>60±9</td>
<td>390±41</td>
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<td>100% OP loading</td>
<td>60±8</td>
<td>510±48</td>
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<td>100% ΔF/F loading</td>
<td>60±4</td>
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<td>340±18</td>
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</tbody>
</table>

Values are means ± SD (in seconds).

expect only longer loading and washout times, with values somewhere in between these for guinea pig and pig.

The new dyes were found to perform efficiently in various cardiac tissues and to provide OP comparable to those of di-4-ANEPPS. The two most successful dyes, JPW3067 and JPW5034, provide 40–120% of the relative fluorescence response of di-4-ANEPPS. In thin HLB layers, the new dyes provide lower relative fluorescence (5%) than di-4-ANEPPS (9%) (20, 35) so that their higher efficacy in cardiac tissues is related to the probing of deeper layers (8). In transillumination mode (when an excitation source and an emission detector are at the opposite sides of tissue) for thick cardiac tissues (~10 mm), the relative fluorescence change of the new dyes also approached or even exceeded that of di-4-ANEPPS. Optimization of filters for these dyes yet to be performed could yield further improvement in their fluorescent efficacies.

It should be noted that raw optical signals are quite noisy. To obtain good quality, it is necessary to apply standard noise reduction procedures such as accumulation of the periodic optical signal and spatiotemporal filtering (compare traces in Fig. 4B). We usually accumulated 10–20 sequential OPs. If very detailed dynamics are not of primary interest, it is possible to accumulate an even larger number of OPs. It is well known that the noise level decreases as the square root of the number of accumulated samples. Spatiotemporal filtering allows further noise reduction by filtering out CCD camera noise and possible vibrations of the tissue surface in the superfusion flow.

The new NIR dyes provide advantages in the choice of excitation methods and/or light sources. Especially when working with a large area and/or thick cardiac tissues, one needs a quite powerful light source capable of providing excitation at the levels of 100 mW/cm². When using a halogen light source with an excitation filter, shifting the central filter wavelength from 520 to 650 nm allows a severalfold reduction of supplied power to the halogen bulb. Alternatively, one can use less dye or record at higher speed and still have good OP. The possible use of inexpensive excitation sources such as red LED, red laser diodes, and helium-neon lasers (at 633 nm) also represents potential advantages. We found that all of these sources produce high dye-excitation efficiency.

Another advantage of the new NIR dyes is lower endogenous absorption for both excitation and emission light in cardiac tissue. Most of the endogenous fluorescence was blocked by emission filters; subtraction of the autofluorescence background corrected the true background fluorescence only ~10%. According to the optical properties of cardiac tissue (8, 32, 37), shifting the wavelength 150–200 nm to the red reduces the absorption coefficient several times. Our measurements show that for 10-mm-thick cardiac tissue (bloodless), this means about a 30-fold increase of excitation light in deep layers. Also, because scattering decreases with increasing wavelength (26, 38), that would allow better depth resolution of the recorded OP. Finally, the new NIR dyes have both excitation and emission spectra that are the farthest to the red from the blood absorption maximum (~580 nm) among all known voltage-sensitive dyes. This allows the possibility of using them in blood-perfused tissues.

We investigated in detail the dynamics of dye loading, washout, and internalization. This little-studied characteristic of the dyes may be important for long-term experiments (a half hour or longer) or large area (volume) monitoring of electrical activity in cardiac tissue. In individual cell or cell culture applications, internalization of an electrochromic dye is the primary pathway for degradation of the OP, but in Langendorff type or other constantly perfused preparations, dye washout also may play an important role. We did not observe any photobleaching: the dynamics of the fluorescent signal are the same under permanent or intermittent (several seconds each minute) excitation.

Comparison of Figs. 7–9 shows that the dynamics of background fluorescence, OP amplitude, and relative fluorescence variation are slightly different. These three parameters are related to the concentration of dye molecules unbound to excitable cells or internalized dye within excitable cells, the concentration of molecules in membrane of electrically active cells, and the efficacy of the voltage-sensitive fluorescence. The latter can be degraded not only because of the dye washout but also because of internalization of the dye. When dye molecules migrate through the membrane and attach to its inner leaflet, chromophore within them is pointed in the opposite direction and undergoes opposite spectrum shifts at the same potential change. Such molecules still fluoresce, but the voltage-sensitive signal reverses phase to partially cancel the detected fluorescence response; this is especially undesirable.

Although there were generally no significant differences between the loading times measured using the three parameters, the washout times generally increase in the order OP < background fluorescence < relative potentiometric fluorescence change (ΔF/F). This would be the behavior expected if dye internalization was the main factor degrading the efficacy of the potentiometric probes over time.

Analysis of the data in Tables 3–5 shows that the values of all three parameters are lowest for the dye JPW3067 and gradually increase for JPW5034 and JPW5020. This is related to the gradually increasing molecular weight (and also size) as well as the length of the hydrocarbon chains of the dye molecules. Longer hydrocarbon chains make more hydrophobic dyes that bind to a cell membrane more tightly and internalize more slowly, according to systematic studies with the ANEP dye series (14, 24). During washout, the OP amplitude decreases faster than the total fluorescence, suggesting that some dye molecules are being internalized. The very fast loading and washout of JPW3067 can be quite an advantage in specific applications.

Considering the species-specific dynamics (compare Figs. 7–9), we noticed that the dye loading and washout progressively slows down from rat to guinea pig to pig. Although metabolic rate follows a similar trend (being highest in the rat
and lowest in the pig), it remains to be determined whether metabolic rate influences the washout dynamics of these dyes. Disadvantages and limitations of the new dyes are 1) some internalization and washout that limit the experiment length or require continuously controlled dye injection, and 2) toxicity of some dyes, especially taking into account that, to obtain OP transients simultaneously from the intact heart. Am J Physiol Heart Circ Physiol 280: H2053–H2060, 2001.


