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Near Infrared Voltage Sensitive Fluorescent Dyes Optimized for Optical Mapping in Blood-Perfused Myocardium

Arvydas Matiukas, PhD^{*,†}, Bogdan G. Mitrea, BS^{*}, Maochun Qin, MD, Arkady M. Pertsov, PhD^{*}, Alexander G. Shvedko, PhD^{**}, Mark D. Warren, PhD^{**}, Alexey V. Zaitsev, PhD^{**}, Joseph P. Wuskell, PhD^{***}, Mei-de Wei, PhD^{***}, James Watras, PhD^{***}, and Leslie M. Loew, PhD^{***}

^{*}Department of Pharmacology, State University of New York, Upstate Medical University, 750 E Adams Street, Syracuse, NY13210

^{**}Nora Eccles Harrison CVRTI, University of Utah, 95 S 2000 East, Salt Lake City, UT 84112-5000

^{***}Department of Cell Biology, Center for Cell Analysis and Modeling, University of Connecticut Health Center, Farmington, CT 06030

[†]Department of Physics, Kaunas University of Technology, Studentu 50, Kaunas, LT51368, Lithuania

Abstract

Background—Styryl voltage-sensitive dyes (e.g. di-4-ANEPPS) have been successfully used for optical mapping in cardiac cells and tissues. However, their utility for probing electrical activity deep inside the myocardial wall and in blood-perfused myocardium has been limited because of light scattering and high absorption by endogenous chromophores and hemoglobin at blue-green excitation wavelengths.

Objectives—The purpose of this study was to characterize two new styryl dyes Di-4-ANBDQPQ (JPW-6003) and Di-4-ANBDQBS (JPW-6033) optimized for blood-perfused tissue and intramural optical mapping.

Methods—Voltage-dependent spectra were recorded in a model lipid bilayer. Optical mapping experiments were conducted in 4 species (mouse, rat, guinea pig, and pig). Hearts were Langendorff-perfused using Tyrode's solution and blood (pig). Dyes were loaded via bolus injection into perfusate. Transillumination experiments were conducted in isolated coronary-perfused pig right ventricular wall preparations.

Results—The optimal excitation wavelength in cardiac tissues (650nm) was >70nm beyond the absorption maximum of hemoglobin. Voltage-sensitivity of both dyes was ~10–20%. Signal decay half-life due to dye internalization was 80–210 minutes, which is 5–7 times slower than for di-4-ANEPPS. In transillumination mode, $\Delta F/F$ was as high as 20%. In blood-perfused tissues, $\Delta F/F$ reached 5.5% (1.8 times higher than for di-4-ANEPPS).

Conclusion—We have synthesized and characterized two new NIR dyes with excitation/emission wavelengths shifted >100nm to the red. They provide both high voltage-sensitivity, and 5–7 times slower internalization rate compared to conventional dyes. The dyes are optimized for deeper tissue

Address for correspondence: Arkady Pertsov, PhD, Dept. Pharmacology, SUNY Upstate Medical University, 750 E Adams Street, Syracuse, NY 13210; Telephone: 315-464 7986; Fax: 315-464 8014; E-mail: pertsova@upstate.edu.

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probing and optical mapping of blood-perfused tissue, and can also be used for conventional applications.

Keywords

voltage-sensitive dye; optical mapping; internalization; blood-perfusion

INTRODUCTION

Optical mapping and its key element – voltage-sensitive dyes, have had an especially important impact on the study of patterns of electrical activity in cardiac tissue¹ and cultured cardiac myocytes^{2,3} leading to improved understanding of cardiac arrhythmias. Rapid progress in laser technology, as well as increased sensitivity, dynamic range, and frame rate of recording devices, has significantly extended the range of potential applications of optical mapping. The most recent developments include intramural optical mapping utilizing diffusive optical tomographic approaches⁴, endoscopic mapping⁵, and optical imaging in intact, blood-perfused hearts⁶. Successful development of these technologies will likely reduce the barrier between basic and clinical applications of optical mapping.

The emerging applications created the need for new near-infrared (NIR) voltage sensitive dyes. Until recently, the most common were dyes with blue-green excitation (di-4-ANEPPS). However, in the presence of blood, which strongly absorbs green light, even conventional surface recordings are difficult to obtain⁷, requiring extremely high illumination intensities. Strong absorption and scattering of green excitation light is also a significant obstacle for deep tissue imaging. Transillumination signals are several orders of magnitude smaller compared to surface fluorescence⁸, though the development of NIR voltage-sensitive dyes with the excitation spectrum shifted to the red beyond the absorption maxima of hemoglobin and other endogenous chromophores should significantly improve both transillumination and fluorescence signals in blood-perfused tissues. It is well known that the shift from 520 nm to the red by 130 nm increases tissue transmittance by more than an order of magnitude⁹. Reduced absorption in the red part of the spectrum is accompanied by a decrease in light scattering, since light scattering is inversely related to wavelength⁹. Both of these factors should contribute to an improvement of optical signals.

There have been ongoing efforts to develop dyes with red excitation and NIR fluorescence^{10,11,12}. Despite noticeable progress, neither of the new dyes has all of the necessary properties to fully satisfy the requirements of the new applications. Although PGH dyes¹¹ have high voltage sensitivity in the red part of the spectrum, they are difficult to load. Dye JPW-3067¹², on the other hand, has a high sensitivity and is easy to load, but rapidly washes out, which makes it difficult to use.

In the present study, two new NIR dyes are introduced, which combine high voltage-sensitivity, easy loading, and slow washout. The dyes show superior characteristics not only in blood-perfused hearts and transillumination experiments, but also in conventional optical mapping applications. Reduced photobleaching (important for tissue culture experiments where light intensity is very high) and slow internalization may make these new dyes a useful tool in conventional applications as well.

METHODS

Dye synthesis, spectra and sensitivity measurement in model membranes

The dye synthesis was done as described earlier¹⁰. Two alternate strategies were employed for the key chromophore assembly step: aldol condensation or Heck coupling. Dye absorbance

and emission spectra in ethanol were measured as described previously¹⁰. Voltage-dependent spectra in model membranes were measured by a voltage-clamped hemispherical lipid bilayer (HLB) apparatus¹³ modified for near infrared fluorescence detection¹⁰. In this experiment, light from a tungsten lamp was sent through a monochromator and then focused onto the bottom of the HLB, which was stained with dye from the external aqueous bathing solution. The monochromator was then scanned over the wavelength range of interest, while a train of ± 50 mV voltage steps was applied to the membrane at a frequency of 40 Hz and the modulation of the detected light signal was measured with a lock-in amplifier. The transmitted light signal was collected at 180° from the incident light. Fluorescence was collected at 90° via fiber optic light guide¹⁰ through a > 715 nm long-pass filter.

Cardiac tissue preparation, perfusion, and staining methods

The dye testing experiments were performed on Langendorff-perfused hearts of mice (n=8), rats (n=14), guinea pigs (n=9), and pigs (n=16). The species were chosen based on the most common optical mapping applications. Transillumination experiments were performed on pig isolated coronary-perfused left and right ventricular free wall preparations (n=12). We used C57BL/6 mice (20–25g), Sprague Dawley rats (300–400g), guinea pigs (400–500g), and pigs (20–25 kg). Blood-perfused experiments (n=9) were conducted on Langendorff pig hearts.

All experimental protocols conformed to the *Guide for the Care and Use of Laboratory Animals* and were approved by the Committee for the Humane Use of Animal of the SUNY Upstate Medical University. The heart isolation and perfusion procedures were identical to those described previously¹². All preparations were continuously paced at the frequency 6.7Hz, 5Hz, 3.3Hz and 2Hz for mouse, rat, guinea pig, and pig, respectively. After a 20–30 min stabilization verified by a vigorous contraction and normal sinus rhythm, the excitation-contraction uncoupler diacetyl-monoxime (DAM) was added to the perfusate (15 mmol/l) to stop contractions. Whole mouse, rat, or guinea pig hearts were additionally gently pressed against stretched nylon mesh (on a single side opposite to both light source and camera so as not to affect the amount of collected fluorescence). These measures, in most cases, completely eliminated motion artifacts.

Blood-perfused Langendorff preparation of pig heart was implemented as described earlier⁶. Briefly, approximately 1.5 liters of modified Tyrode's solution (37°C, composition in mmol/L: Na⁺, 157; K⁺, 4.7; Ca²⁺, 1.5; Mg²⁺, 0.7, H₂PO₄⁻ 0.5, Cl⁻ 137.6, HCO₃⁻ 28.0, glucose 11.0, dextran 4% and insulin 10U) was infused in an external jugular vein, and blood-Tyrode's mixture was collected from a carotid artery. The mixture was oxygenated (CO₂ 5%/O₂ 95%), heated (37°C) and filtered using a Liliput hollow fiber oxygenator (COBE Cardiovascular, Arvada, Co). After cannulating the aorta, collector tubes were inserted into the right and left ventricles to collect all the blood coming out of the coronary sinus and Thebesian veins. To ensure that all the outflow of perfused blood is collected for recirculation, all the heart orifices were meticulously sutured so that the heart chambers were completely sealed. Subsequently, the heart was placed in a warm bath with heated water-jacketed transparent glass wall and was superfused with an oxygenated, heated Tyrode's solution.

All dyes were injected directly into the aortic cannula during uninterrupted aortic perfusion. The new dyes and di-4-ANEPPS were delivered in Tyrode's solution at concentrations of 40 μ M and 10 μ M, respectively. It is important that the rate of injection is sufficiently slow so that the flow rate during injection does not increase by more than 10%. Faster dye injection may lead to preparation damage and arrhythmias. The perfusion rate was continuously monitored using a flow meter (Cole Parmer, Vernon Hills, IL). The injection continued until the staining level (as determined from measuring background fluorescence) reached the optimum (close to the level of saturation of the camera). Typically the staining procedure did

not last longer than 10 min. In blood perfused pig hearts, dye injection time was close to 1 min because of high perfusion flow.

To load JPW-6003, Pluronic F-127 was added to the bolus, achieving a final concentration of 0.2–0.5%. Otherwise no signal was observed.

Stock solutions: JPW-6003 (18.8 mg/ml of pure ethanol); Pluronic F-127 (2g in 10ml DMSO); JPW-6033 (16.7 mg/ml of pure ethanol); di-4-ANEPPS (5 mg in 1 ml DMSO).

Dose for different species were 50 nmol/g of heart tissue in pigs and 100 nmol/g in all other species. These doses were derived empirically from experiments (see above).

Action potential recording

The optical setup was described previously^{12,14}. Blood-perfused pig heart imaging was done as described by Zaitsev et al.⁶. For illumination, a tungsten halogen lamp was used (400 W for blood-perfused hearts; 250 W for all other conditions/tissues). In a majority of experiments, optical action potentials (OAP) were recorded with a cooled fast 14 bit CCD camera (Little Jo by SciMeasure) with a Computar H1212FI lens (focal length 12 mm, F 1.2; CBC Corp; or model HG0608FCS: focal length 6mm, F 0.8 in the case of mouse heart), located at about 100 mm (20 mm for mouse heart) from the sample. Acquisition rates were 500–2000 Hz at 80×80 pixels resolution.

Optical recordings in blood-perfused hearts were obtained using an externally cooled 12 bit CCD camera (Dalsa CA-D1-0128T, Ontario, Canada) equipped with a Computar objective lens (HG1208FCS-HSP, focal length 12 mm, F 0.8). Images were acquired at the frame rate of 400–800 Hz and 64×64 pixels resolution.

Excitation of the JPW dyes was done using a 650±20 nm band-pass filter; whereas, fluorescence was recorded using a 720 nm long-pass filter. In control experiments with di-4-ANEPPS, 520±40 nm and 640±50 nm band-pass filters were used for excitation and fluorescence, respectively.

In experiments with small hearts (mouse, rat, guinea pig), the field of view of the camera was adjusted to fit the whole ventricles. In pig experiments, the field of view was fixed at 25×25 mm². In transillumination experiments, the epicardial surface was illuminated, while fluorescence was recorded from the endocardium.

Electrical recordings of conduction velocity were done using monophasic electrode array and techniques similar to those described by Horner et al.¹⁵. The signals were amplified by bioamplifiers (ISO DAM-8A, World Precision Instruments) and digitized at 10 kHz by 16-bit data acquisition card (PCI-6229, National Instruments) in the data acquisition computer (P4, 2.8 GHz).

Data processing

The acquired images were processed offline to improve their quality. 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. Ensemble averaging was used to improve signal to noise ratio (i.e., averaging the OAP signals from 10–20 sequential recordings). To allow temporal alignment of successive paced action potentials, the trigger for the pacing stimulus was recorded as a single pixel in the movie frames. The alignment error was no more than one-half frame (~0.5 ms). To further reduce noise, the OAP signal was low-pass filtered in both time (with 3–7 point triangular window, depending on the camera frame rate) and spatial domain (with pyramidal 5×5 kernel).

Dye loading and washout dynamics were assessed from averaged sets of OAP and voltage independent background fluorescence recordings. To facilitate comparison, fluorescence and OAPs were normalized to their peak values. Action potential duration (APD) was measured at 50% repolarization as an average of 35×35 pixel (~10×10 mm) area.

Statistical analysis

Data are presented as mean±SD. A non-parametric Mann-Whitney U-test was used for the statistical comparison between the parameters of the new dyes and di-4-ANEPPS. $P < 0.05$ was considered significant. To provide a better comparison, some of the kinetics curves had their washout portion fitted with a linear function and extrapolated; to estimate half-lives curves were extrapolated to a 50% decrease (Origin software).

RESULTS

Dye spectra and voltage-sensitivity

In the present study, two new voltage sensitive dyes were synthesized and characterized: JPW-6003 (Di-4-ANBDQPQ) and JPW-6033 (Di-4-ANBDQBS). The structures of these dyes are shown in Figure 1. Both dyes have the same chromophore, but have different polar groups attached to the quinolinium nitrogen: propyltrimethylammonium and butylsulfonate for JPW-6003 and JPW-6033, respectively. Molecular weights are 695.63, and 570.73, respectively. Using a quaternary ammonium in JPW-6003 (Di-4-ANBDQPQ) instead of the sulfonate polar group in JPW-6033 (Di-4-ANBDQBS) was intended to improve water solubility and to reduce the rate of flipping into the cytoplasmic face of the cell membrane, since JPW-6003 is a doubly charged cation whereas JPW-6033 is a neutral zwitterion. For comparison, the structure of di-4-ANEPPS is included at the bottom of the Figure 1. In order to shift the spectral range of the new dyes to the red, the length of the linker region was increased from a single double bond in di-4-ANEPPS to a dienyl linkage in the new compounds; additionally, the new probes have a quinolinium group to increase the overall size of the pi system compared to the pyridinium moiety in the ANEP dyes. These changes shifted both the absorbance and emission spectra of the new dyes to longer wavelengths compared to di-4-ANEPPS.

Figure 2 shows spectral characteristics of the new dyes in ethanol (panel A). The absorbance spectra in ethanol reveal single maxima, indicating a high level of the dye purity and absence of different configurational isomers. The absorption maxima for JPW-6003 occurred at 603nm. For JPW-6033, peak absorption is shifted by 40 nm to shorter wavelengths, but is 2–3 times higher than JPW-6003. Due to the broad width of the peaks, in practical applications both dyes can be efficiently excited over the wavelength range 500 – 700 nm, allowing one to optimize excitation of the dye in different tissues, including blood-perfused tissue. Panel B shows the dye emission spectra in ethanol. When excited at 560 nm, both dyes emit over the range 700–900 nm.

Spectral responses of the new dyes to changes in membrane potential (action spectra) are given in panels C and D. They show transmission $\Delta T/T$ and fluorescence $\Delta F/F$ changes at various wavelength caused by 100mV voltage steps in a voltage clamped hemispherical lipid bilayer. Fluorescence was collected at >715 nm, while the excitation wavelength varied from 500 nm to 650 nm. The spectra are sometimes referred to as differential (obtained by subtraction of spectra obtained at membrane voltages of –50 and +50 mV).

Similar to older styryl dyes, both $\Delta T/T$ and $\Delta F/F$ curves have a biphasic shape, which is consistent with an electrochromic mechanism¹⁶. (In the provided measurement range above 500 nm mostly the high-wavelength sensitivity is observed). The biphasic response results

from a voltage-dependent shift of the absorbance or emission spectrum to a shorter wavelength with depolarization¹². The JPW-6003 dye shows the maximal voltage sensitivity $\Delta F/F = -20\%$ value when excited at 618 nm. The maximum for JPW-6033 is shifted by approximately 15 nm to the red and is lower ($\Delta F/F = -15\%$). Compared to other voltage-sensitive dyes, the values of $\Delta F/F$ for both dyes are relatively large. In fact, $\Delta F/F = 20\%$ is the highest absolute value for voltage-sensitive dye ever reported in a hemispherical lipid bilayer (HLB).

In cardiac tissue, the optimal excitation wavelengths were found to be longer than in HLB (~650 nm versus 618 nm). The maximal amplitude of the optical action potential was observed at excitation wavelengths around 650 nm, whereas in HLB at such wavelengths, we observed a significant drop in $\Delta F/F$. White and black squares in Figure 2D show the average values of $\Delta F/F$ for optical action potentials at 650 nm obtained in pig using JPW-6003 and JPW-6033 (see next section for more detail).

The positive (short wavelength) voltage-sensitivity of the new dyes in cardiac tissue was more pronounced than in HLB. At 520 nm (emission collected at 610–670 nm), $\Delta F/F$ was as high as 2%–5% in the heart (squares in the top left corner, Figure 2D). These findings are consistent with the overall shift of the action spectrum in the heart tissue to the red compared to HLB. The presence of significant positive voltage-sensitivity is important for ratiometric applications.

Optical action potentials in cardiac tissue

To assess performance of the new dyes we conducted epicardial optical mapping experiments in Tyrode's and blood-perfused Langendorff heart preparations of various species as well as transillumination experiments in isolated perfused preparations of pig left and right ventricular wall. Figure 3, Panel A shows representative epicardial optical action potentials (OAP) in Tyrode's perfused mouse, rat, guinea pig, and pig hearts 15 minutes after dye injection. One can see characteristic long upstrokes, which is the manifestation of deep subsurface sampling by red excitation light^{12,17}. In all species, the maximum relative fluorescence (voltage-sensitivity $\Delta F/F$) is $> 10\%$, which puts JPW-6003 and JPW-6033 in line with the best blue-green voltage-sensitive dyes including the gold standard di-4-ANEPPS. Table 1 summarizes the maximal voltage-sensitivities $\Delta F/F$ for the new styryl dyes and di-4-ANEPPS in different Tyrode's-perfused cardiac tissues. In pig and rat, $\Delta F/F$ is significantly higher ($p < 0.01$) for the new NIR dyes than di-4-ANEPPS. This is also true for $\Delta F/F$ averaged across all studied species (see the last row in Table 1). The difference between JPW-6003 and JPW-6033 was not significant.

In blood perfused pig heart, the new dyes could be used at a low intensity of illumination. At 1 mW/mm^2 they yielded OAP amplitude higher than di-4-ANEPPS at 10 mW/mm^2 illumination. Figure 3, Panel B shows action potential recordings obtained using JPW-dyes and di-4-ANEPPS in a Langendorff perfused pig heart about 50 minutes after dye injection. The corresponding $\Delta F/F$ were 5.5% and 5.4% for JPW-6003 and JPW-6033 which is almost two times higher than those observed for di-4-ANEPPS. Lower values of $\Delta F/F$ for blood-perfused than in Tyrode's preparations is a result of the significant binding of dyes to blood, which increases voltage-independent component of fluorescence (see next section).

The new dyes also significantly outperformed di-4-ANEPPS in transillumination mode. Figure 3, Panel C shows transillumination signals in pig RV. The results were even more dramatic in thicker LV preparations. We were able to record excellent transillumination signals in 10–12 mm thick preparations, in which no di-4-ANEPPS signal could be recorded.

Table 2 summarizes the maximal voltage-sensitivities $\Delta F/F$ for the new styryl dyes and di-4-ANEPPS in pig for blood-perfusion and transillumination experiments. In both cases, the new dyes showed a higher $\Delta F/F$ than di-4-ANEPPS ($p < 0.024$).

Kinetics of dye loading and washout

In Tyrode's-perfused preparations, the loading times are quite uniform across species, and typically do not exceed 5–10 minutes after the end of dye injection. In small animals, the rate of injection has to be small to avoid side effects such as arrhythmia and AV-blockade. This makes the duration of dye injection an essential component of the loading time. In this regard, however, the new dyes are not significantly different from the majority of voltage sensitive dyes including di-4-ANEPPS.

The most remarkable feature of the new dyes is their extremely slow washout kinetics and persistence of voltage-sensitive signal. Figure 4 shows the time dependence of normalized total fluorescence and its voltage-dependent component in rat preparation. One can see that over the course of the experiment, >150 minute changes in total fluorescence F were less than 17% for both dyes which suggest a very slow washout and an insignificant photobleaching. This contrasts with di-4-ANEPPS, whose fluorescence drops by 50% within first 15 min (see panel A). The decay of voltage sensitive signal $\Delta F/F$ (see panel B) is also very slow for JPW-6003 and JPW-6033. The half-life $\tau_{1/2}(\Delta F/F)$ is 161 min for JPW-6003, and 119 min for JPW-6033. These are 4–5 times greater than the half-life for di-4-ANEPPS (32 min).

Figure 5 shows washout kinetics in Tyrode's-perfused pig preparation. The drop in total fluorescence is less than 9% in 2 hours (panel A), whereas the half-lives of voltage-sensitive fluorescence ($\tau_{1/2}(\Delta F/F)$; panel B) are 210 min (JPW-6003) and 165 min (JPW-6033). This is the slowest kinetics among all studied species (Table 3).

The half-lives of total $\tau_{1/2}(F)$ and voltage-sensitive fluorescence $\tau_{1/2}(\Delta F/F)$ for mouse, rat, guinea pig, and pig are shown in Table 3. There is a tendency for these parameters to increase from mouse to larger species, yet they are all 3–11 times higher than for di-4-ANEPPS. The increased half-lives represent a major advantage of the new dyes, which enables experiments of significantly longer duration.

Kinetics of loading and washout in blood-perfused hearts

Direct injection of dye in blood produces kinetics that are quite different from those observed in Tyrode's perfused preparations (see Fig. 6A). The $\Delta F/F$ reaches the level of ~5% very rapidly (within 2 min after the dye injection) and remains constant over the duration of the experiment (115 minutes). The total fluorescence increases very slowly and reaches maximum about 1 hour after injection and remains relatively constant for the next hour. Consequently, maximal optical action potential amplitudes (which equal the product $\Delta F/F * F$) are reached approximately one hour after the end of dye injection. A possible mechanism of such kinetics is a strong binding of dye to blood with subsequent slow redistribution between blood and cardiac myocytes' membrane. Replacement of blood by Tyrode's solution during the stationary stage (see Fig. 6B) reduces overall fluorescence F and increases $\Delta F/F$, which supports this hypothesis (see discussion for more detail).

The loading of dyes can be accelerated if dye injections are performed while the heart is perfused with Tyrode's, and the switch to blood perfusion occurs only after the total fluorescence reached the maximum. Figure 7, panels A and B shows kinetics of total fluorescence F , and voltage-sensitive fluorescence $\Delta F/F$, respectively, for such a loading protocol when dyes were injected into aorta during perfusion with Tyrode's solution. Maximal amplitude of both F and $\Delta F/F$ were reached within 15 minutes. After the switch to blood

perfusion (indicated by thick lines above the time scale), the total fluorescence intensity F dropped ~50% within 5–10 min, whereas $\Delta F/F$ decreased more gradually. Both F and $\Delta F/F$ decreased to a steady-state level within 40–50 minutes. The initial, fast drop in F and $\Delta F/F$ after switching to blood perfusion is a result of hemoglobin absorption of excitation and emission light, whereas the subsequent, slower decline in F and $\Delta F/F$ is caused by the redistribution of dye between blood and cardiac myocytes' membrane. Using this loading protocol, $\Delta F/F$ signals reached 12% during the first 5 min after switching to blood perfusion. Rapid changes in F and $\Delta F/F$, however, may not always be convenient.

Effect on electrophysiological characteristics

The JPW dyes do not manifest apparent acute toxic effects in the range of concentrations required to achieve maximal optical signals. Figure 8 shows a volume-conducted ECG (panel A), conduction velocity (CV; panel B), and action potential duration (APD50; panel C) as a function of time after dye injection, in various experimental models (blood-perfused pig heart, guinea pig, and rat, respectively). There were no apparent changes in these electrophysiological parameters after dye injection.

DISCUSSION

In this paper, we introduced two new styryl dyes JPW-6003 and JPW-6033 with a near infrared spectral range. Both dyes can be efficiently excited at 650 nm and manifest strong voltage-sensitive fluorescence signals at >720 nm, which constitutes an approximately 100 nm shift to the red compared to conventional dyes with blue-green excitation. The most commonly used styryl dye di-4-ANEPPS is usually excited at 520 nm and its fluorescence recorded at 640 nm¹². The significance of the development of new dyes is that they enable optical mapping in the NIR part of the spectrum, where both blood and cardiac tissue are significantly more transparent. NIR optical mapping will be particularly beneficial for blood-perfused preparations and 3D imaging, paving the way towards clinical applications including endocardial optical mapping which is currently limited to Tyrode's perfused cavities⁵.

The usefulness of the voltage-sensitive dyes is determined by several factors, which include: voltage-sensitivity $\Delta F/F$, efficiency of loading (as many dyes have difficulty penetrating capillaries to reach cardiac myocytes), the resistance to photobleaching, and kinetics of washout and internalization. The kinetics of washout and internalization determine the rate of decay of the optical signal. In all categories, both JPW dyes performed exceptionally well.

Voltage-sensitivity

The voltage-sensitivity of the new dyes, tested in 4 different species and various experimental conditions, was either comparable, or superior to the best voltage-sensitive dyes used for optical mapping of electrical excitation in the heart. In Tyrode's perfused ventricular wall preparations, $\Delta F/F$ was in the range of 10–18% for both JPW-6003 and JPW-6033. This is 50–100% greater than $\Delta F/F$ reported for di-4-ANEPPS, which is considered a “gold standard” of optical mapping, and is comparable to what has been achieved for Ca^{2+} dyes¹⁸. As expected, this dye performed very efficiently in transillumination mode (when an excitation source and an emission detector are on opposite sides of tissue). In this mode, both JPW-6003 and JPW-6033 showed $\Delta F/F$ as high as 20%. The fact that the excitation wavelength (~650nm) of the new dyes is well separated from the absorption maximum of blood (~580 nm) conveys a major advantage in blood-perfused preparations. In blood-perfused Langendorff pig hearts, the new JPW dyes yielded almost double the $\Delta F/F$ (compared to Di-4-ANEPPS), while requiring only one tenth the intensity of illumination.

Some of the previously reported NIR dyes also have relatively high voltage-sensitivity. For example, $\Delta F/F$ for JPW-3067¹² and PGH¹¹ dyes was reported to be as high as 9–18%. However, rapid washout of JPW-3067 dye and lack of reliable loading protocols at normal pH for PGH dyes makes them difficult to use. The new dyes do not have the limitations discussed above. They can both maintain high fluorescence levels for several hours (see Table 3) versus several minutes in case of JPW-3067¹². They can also be readily loaded (JPW-6003) using FDA-approved Pluronic F-127¹¹ or without any vehicle (JPW-6033).

Internalization and washout

One of the important features of the new dyes is the significantly longer retention time in the tissue and the significantly slower decay time of the voltage-dependent optical signal thus allowing significantly longer experimental recordings. The decay of voltage-sensitive signal at a faster rate than the background fluorescence is usually attributed to dye internalization. The internalization rate for JPW-6003 provides the longest experiment time (in cardiac tissue) to date among known voltage-sensitive dyes (namely, up to 4hrs, which is several times longer than with di-4-ANEPPS). Substitution in JPW-6033 of the quaternary ammonium by butylsulfonate (as in PGH dyes¹¹) increased the rate of dye washout and internalization, yet it was still significantly slower than with di-4-ANEPPS.

Washout and internalization rates were species-specific. The dynamics of dye internalization progressively slowed down (Table 3) from mouse to larger species. Metabolic rate follows a similar trend (being highest in the mouse and lowest in the pig), though it remains to be determined if metabolic rate influence the washout dynamics of these dyes.

Peculiarities of dye loading and washout in blood-perfused tissue

The kinetics of dye loading in blood-perfused hearts was quite different from those perfused with Tyrode's solution. Loading the dye in blood resulted in a very slow increase in total fluorescence for about 40 minutes (Fig. 6A), whereas loading the dye in Tyrode's solution resulted in maximum fluorescence within 10–15 min (Fig. 4–5). These differences in kinetics may reflect binding of JPW dyes to blood, with subsequent slow redistribution between blood and membranes of cardiac myocytes.

As a further test of this hypothesis, hearts were loaded with JPW-6003 while being perfused with blood, then after reaching maximal fluorescence and $\Delta F/F$, the perfusion media was switched to Tyrode's solution. Total fluorescence dropped quickly after switching to the Tyrode's solution, whereas $\Delta F/F$ increased. Both responses could be explained by some of the dye binding to the blood, with the switch to Tyrode's solution resulting in a decrease in "background" or "non-voltage sensitive" fluorescence in the blood, and hence a greater proportion of the fluorescence originating from the cardiac membranes.

If, on the other hand, the heart is loaded with JPW-6003 while being perfused with Tyrode's solution, and then after reaching maximal fluorescence and $\Delta F/F$, the perfusion media is changed to blood, then both total fluorescence and $\Delta F/F$ decrease (Fig. 7). In this case, the dye is initially bound to cardiac myocytes and is subsequently taken up by blood, which causes both a drop in total fluorescence and in $\Delta F/F$. Note that the equilibration time (40 min) and the steady-state value of $\Delta F/F$ (5–6%) after switching from Tyrode's solution to blood (Fig 7B) is comparable to that obtained when the dye is loaded directly into blood perfused heart (Fig. 6A).

The initial rapid drop in fluorescence immediately after replacement of Tyrode with blood in Fig 7A occurred before the drop in $\Delta F/F$ (Fig 7B). The initial drop in total fluorescence cannot be caused by dye redistribution between cardiac myocytes and blood, since redistribution

would lead to a significant reduction of $\Delta F/F$. Instead, the initial rapid drop in fluorescence may reflect a purely optical effect, with blood acting as an optical filter, reducing the intensity of illumination.

Another interesting finding that deserves discussion is the relatively constant $\Delta F/F$ when dye is delivered directly into blood (see Fig 6A). A similar effect is observed in experiments when blood perfusion was turned on after dye loading (see Figure 7B). Indeed, after 40 min of equilibration $\Delta F/F$ reaches a level of 5–6% and practically does not change for up to another 100 min. This contrasts with the more rapid decay of voltage-sensitive signal in Tyrode's perfused preparations (see Fig 4–5, Table 3), and raises the possibility that decreased $\Delta F/F$ in Tyrode's perfused preparation is not caused exclusively by dye internalization, but may also reflect to some extent a run-down of the preparation. That is, blood-perfused preparations may have a slower run-down and thus slower decay of the signal. Another possible explanation is that blood can serve as a buffer that supplies additional dye to the membrane of cardiac myocytes as dye gets internalized. Additional experiments are needed to provide further details on the mechanism(s) underlying dye loading in blood-perfused tissue.

Using new dyes in conventional applications

Although JPW-6033 and JPW-6003 were designed to increase the depth of imaging of electrical activity in tissues, and to facilitate imaging during blood perfusion, these dyes are also useful in conventional epicardial mapping applications. Specifically, these dyes have greater $\Delta F/F$ and slower internalization rate than other available voltage-sensitive dyes, plus the dyes can be readily loaded. Moreover, the red shift is likely to be a useful feature in combination with recently reported uncoupler blebbistatin, which is inactivated by blue-green light¹⁹. These dyes may as well be useful for optical mapping of monolayers and individual cardiac myocytes, given the ability of these dyes to respond to low levels of illumination (1 mW/mm²) and high $\Delta F/F$. Monolayers and individual cardiac myocytes usually require high intensities of illumination, which often results in photobleaching²⁰.

Due to an increased depth penetration in thick, Tyrode's-perfused tissues, optical recordings obtained using the NIR dyes contain significant contributions from deep tissue layers. In order to resolve activation patterns in a single layer, special deconvolution algorithms are needed (see for example²¹). The interpretation of conventional epicardial maps obtained without taking into account depth effects will require extreme caution. It is worth noting that contributions from deep layers is also an issue, although to a lesser extent, with conventional green-blue dyes (i.e., calcium-sensitive and voltage-sensitive dyes^{22,23,24}). For the applications that require the thinnest probing layer possible, there is a choice of di-4-ANEPPS excited by blue light which, among commonly used techniques, will provide the thinnest signal collection layer.

The new dyes can show both positive and negative fluorescence responses (see Figure 2D), and thus can be used in ratiometric applications²⁵.

Limitations of the study

The selection of excitation and emission wavelengths for new dyes was based on spectral measurements in a lipid bilayer. The spectral characteristics of the dyes, however, could be different in myocardial tissues^{11,12}. In the present study, no attempt was made to adjust for possible differences in spectral properties of the JPW dyes. For convenience, identical filters were used for both dyes. Although excellent results were achieved with the indicated filter selection, optimizing the excitation and emission filters could improve the results further. One of the best ways of such optimization would be through the measurement of voltage dependence of excitation and emission spectra in myocardial tissue.

It should be noted that doses of the dyes that were used in the present study were optimized for maximal signal-to-noise ratio in a specific experimental setting. Using brighter light sources and more sensitive sensors in the NIR range of the spectrum may require less dye. The opposite is also true, in that using less powerful light sources, or detectors with lower sensitivity in the NIR spectral range would require a higher dose.

High doses of voltage-sensitive dyes may affect ion channels via influencing membrane fluidity and dipole potential of lipid bilayer, as well as by electrostatic interaction with channel proteins. In isolated cardiac myocytes, voltage-sensitive dyes can prolong action potential duration²⁶ (APD90; di-8-ANEPPS) and induce early afterdepolarizations^{26,27} (di-4-ANEPPS). In the whole heart, di-4-ANEPPS and RH421 has been reported to change perfusion pressure, to reduce sinus rate, and to cause AV nodal block^{28,29}.

The dye doses used in the present study did not affect electrophysiological characteristics of myocardial tissue (see Fig. 8). However, further study is required to determine how much the dose can be increased without causing adverse effects. In the early stage of this project, doses of 200 nmol/g (which is 2 times higher than those indicated in the methods section) had no adverse effects.

Conclusion

Two new NIR dyes with excitation/emission wavelengths shifted >100nm to the red were synthesized and shown to provide both high voltage-sensitivity, and 5–7 times slower internalization rate compared to conventional dyes. The dyes are optimized for deeper tissue probing and optical mapping of blood-perfused tissue, but can also be used for conventional applications.

These improvements enable new experiments related to deeper tissue probing, mapping of blood-perfused tissue, and more efficient double-dye (Ca/V_m) optical mapping.

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References

1. Jalife J. Ventricular fibrillation: mechanisms of initiation maintenance. *Annu Rev Physiol* 2000;62:25–50. [PubMed: 10845083]
2. Rohr S, Salzberg BM. Multiple site optical recording of transmembrane voltage (MSORTV) in patterned growth heart cell cultures: assessing electrical behavior, with microsecond resolution, on a cellular and subcellular scale. *Biophys J* 1994;67:1301–15. [PubMed: 7811945]
3. Fast V, Kleber A. Microscopic conduction in cultured strands of neonatal rat heart cells measured with voltage-sensitive dyes. *Circ Res* 1993;73:914–25. [PubMed: 8403261]
4. Khait VD, Bernus O, Mironov SF, Pertsov AM. Method for the three-dimensional localization of intramyocardial excitation centers using optical imaging. *J Biomed Opt* 2006;11:34007. [PubMed: 16822057]
5. Kalifa J, Klos M, Zlochiver S, Mironov S, Tanaka K, Ulahannan N, Yamazaki M, Jalife J, Berenfeld O. Endoscopic fluorescence mapping of the left atrium: A novel experimental approach for high resolution endocardial mapping in the intact heart. *Heart Rhythm*. 2007;10.1016/j.hrthm.2007.03.009Epub 2007 March 16

6. Zaitsev AV, Guha PK, Sarmast F, Kolli A, Berenfeld O, Pertsov AM, de Groot JR, Coronel R, Jalife J. Wavebreak formation during ventricular fibrillation in the isolated, regionally ischemic pig heart. *Circ Res* 2003;92:546–53. [PubMed: 12600877]
7. Huizar JF, Warren MD, Shvedko AG, Kalifa J, Moreno J, Mironov SF, Jalife J, Zaitsev AV. Three Distinct Phases of VF during Global Ischemia in the Isolated Blood-Perfused Pig Heart. *Am J Physiol Heart Circ Physiol*. 2007 Jun 1;[Epub ahead of print]
8. Baxter WT, Mironov SF, Zaitsev AV, Jalife J, Pertsov AM. Visualizing excitation waves inside cardiac muscle using transillumination. *Biophys J* 2001;80:516–30. [PubMed: 11159422]
9. Van Gemert, MJC.; Welch, AJ.; Jacques, SL.; Cheong, QF.; Star, WM. Light distribution, optical properties, and cardiovascular tissues. In: Abela, GS., editor. *Lasers in cardiovascular medicine and surgery: Fundamentals and techniques*. Norwell, MA: Kluwer Academic Publishers; 1990.
10. Wuskell JP, Boudreau D, Wei MD, Jin L, Engl R, Chebolu R, Bullen A, Hoffacker KD, Kerimo J, Cohen LB, Zochowski MR, Loew LM. Synthesis, spectra, delivery potentiometric responses of new styryl dyes with extended spectral ranges. *J Neurosci Meth* 2006;151:200–15.
11. Salama G, Choi B-R, Azour G, Lavasani M, Tumbe V, Salzberg BM, Patrick MJ, Ernst LA, Waggoner AS. Properties of new, long-wavelength, voltage-sensitive dyes in the heart. *J Membrane Biol* 2005;208:125–40. [PubMed: 16645742]
12. Matiukas A, Mitrea BG, Pertsov AM, Wuskell JP, Wei M, Watras J, Millard AC, Loew LM. New near-infrared optical probes of cardiac electrical activity. *Am J Physiol Heart Circ Physiol* 2006;290:H2633–43. [PubMed: 16399869]
13. Fluhler E, Burnham VG, Loew LM. Spectra, Membrane Binding Potentiometric Responses of New Charge Shift Probes. *Biochemistry* 1985;24:5749–55. [PubMed: 4084490]
14. Hyatt CJ, Mironov SF, Vetter FJ, Zemlin CW, Pertsov AM. Optical Action Potential Upstroke Morphology Reveals Near-Surface Transmural Propagation Direction. *Circ Res* 2005;97:277–84. [PubMed: 15994436]
15. Horner SM, Vespalcova Z, Lab MJ. Electrode for recording direction of activation, conduction velocity, and monophasic action potential of myocardium. *Am J Physiol Heart Circ Physiol* 1997;272:H1917–27.
16. Loew LM, Scully S, Simpson L, Waggoner AS. Evidence for a Charge-Shift Electrochromic Mechanism in a Probe of Membrane Potential. *Nature* 1979;281:497–9. [PubMed: 492309]
17. Ding L, Splinter R, Knisley SB. Quantifying spatial localization of optical mapping using Monte Carlo simulations. *IEEE Trans Biomed Eng* 2001;48:1098–1107. [PubMed: 11585033]
18. Takahashi A, Camacho P, Lechleiter JD, Herman B. Measurement of intracellular calcium. *Physiol Rev* 1999;79:1089–125. [PubMed: 10508230]
19. Fedorov VV, Lozinsky IT, Sosunov EA, Anyukhovskiy EP, Rosen MR, Balke CW, Efimov IR. Application of blebbistatin as an excitation-contraction uncoupler for electrophysiologic study of rat and rabbit hearts. *Heart Rhythm* 2007;4:619–26. [PubMed: 17467631]
20. Cheng DK, Tung LEA, Sobie EA. Nonuniform responses of transmembrane potential during electric field stimulation of single cardiac cells. *Am J Physiol* 1999;277:H351–62. [PubMed: 10409215]
21. Gibson AP, Hebden JC, Arridge SR. Recent advances in diffuse optical imaging. *Phys Med Biol* 2005;50:R1–R43. [PubMed: 15773619]
22. Hyatt CJ, Mironov SF, Wellner M, Berenfeld O, Popp AK, Weitz DA, Jalife J, Pertsov AM. Synthesis of voltage-sensitive fluorescence signals from three-dimensional myocardial activation patterns. *Biophys J* 2003;85:2673–83. [PubMed: 14507730]
23. Bishop MJ, Rodriguez B, Trayanova N, Gavaghan DJ. Inference of intramural wavefront orientation from optical recordings in realistic whole-heart models. *Biophys J* 2006;91:3957–8. [PubMed: 16935956]
24. Janks DL, Roth BJ. Averaging over depth during optical mapping of unipolar stimulation. *IEEE Trans Biomed Eng* 2002;49:1051–4. [PubMed: 12214878]
25. Knisley SB, Justice RK, Kong W, Johnson PL. Ratiometry of transmembrane voltage-sensitive fluorescent dye emission in hearts. *Am J Physiol Heart Circ Physiol* 2000;279:H1421–33. [PubMed: 10993810]

26. Hardy ME, Lawrence CL, Standen NB, Rodrigo GC. Can optical recordings of membrane potential be used to screen for drug-induced action potential prolongation in single cardiac myocytes? *J Pharmacol Toxicol Methods* 2006;54:173–82. [PubMed: 16632384]
27. Schaffer P, Ahammer H, Muller W, Koidl B, Windisch H. Di-4-ANEPPS causes photodynamic damage to isolated cardiomyocytes. *Pflugers Arch* 1994;426:548–51. [PubMed: 8052525]
28. Cheng Y, Van Wagoner DR, Mazgalev TN, Tchou PJ, Efimov IR. Voltage-sensitive dye RH421 increases contractility of cardiac muscle. *Can J Physiol Pharmacol* 1998;76:1146–50. [PubMed: 10326837]
29. Nygren A, Kondo C, Clark RB, Giles WR. Voltage-sensitive dye mapping in Langendorff-perfused rat hearts. *Am J Physiol Heart Circ Physiol* 2003;284:H892–902. [PubMed: 12424095]

Glossary of abbreviations

NIR	near infrared
HLB	hemispherical lipid bilayer
CPS	cardioplegia solution
DAM	diacetyl-monoxime
OAP	optical action potential(s)
APD	action potential duration

di-4-ANBDQPQ (JPW-6003)

di-4-ANBDQBS (JPW-6033)

di-4-ANNEPS

Figure 1.

Structure of the new NIR styryl dyes JPW-6003 (Di-4-ANBDQPQ), and JPW-6033 (Di-4-ANBDQBS). Structure of di-4-ANNEPPS is shown for comparison. Grey square shows the chromophores, and the white dashed square is a linker.

Figure 2.

Spectral characteristics of JPW-6003 and JPW-6033. *A, B.* Dye absorbance and emission spectra in ethanol. *C, D.* Voltage-sensitive transmission $\Delta T/T$ and fluorescence excitation $\Delta F/F$ spectra in the hemispherical lipid bilayer for 100 mV voltage steps. The fluorescence was collected through a >715 nm long-pass filter. For comparison, the voltage-sensitivity ($\Delta F/F$) in pig heart derived from optical action potentials at 520 and 650 nm are shown (black squares for JPW-6003, white squares for JPW-6033).

Figure 3.

Optical action potentials (OAP) recorded in different experimental models using JPW-6003 and JPW-6033. Gray and black traces show raw and processed recordings, respectively. A.: Epifluorescence recordings in Tyrode's-perfused preparations of pig, guinea pig, rat, and mouse. B.: Epifluorescence recordings in blood-perfused Langendorff pig (Recordings were made from LV). Right OAP was recorded using di-4-ANEPPS. C.: Transillumination recordings in Tyrode's-perfused pig (RV).

Figure 4.

Washout kinetics of JPW-6003 and JPW-6033 in rat (Tyrode's perfusion). Panels A and B show the time dependence of the total (F) and voltage-sensitive fluorescence ($\Delta F/F$), respectively. Zero on the time scale corresponds to the maxima. Vertical lines show the times corresponding to 50% drop from the maximum value. Labels next to the lines show the half-life. The kinetics of the dye di-4-ANEPPS is shown for comparison. All curves are averages of 3–5 experiments, and error bars show the level of data variation.

Figure 5.

Dye washout kinetics in pig (Tyrode's perfusion). Notations are the same as in Figure 4. To obtain the half-life, the curves were extrapolated (dotted lines).

Figure 6.

Loading and washout kinetics of JPW-6003 in blood-perfused pig heart (direct injection into blood flow). *A.*: Time dependence of total F and voltage-sensitive fluorescence $\Delta F/F$. $t=0$ corresponds to the end of dye injection. F is normalized to maximum. *B.*: Responses of F and $\Delta F/F$ to a switch from blood- to Tyrode's-perfusion. F is normalized to the stationary value. The switch was performed after 120 minutes of blood-perfusion (during stationary stage). Dashed lines show extrapolation of the blood-perfusion curves. All curves with error bars are averages of 3–5 experiments. Thick black and grey lines near time axis indicate switching between blood- and Tyrode's-perfusion, respectively.

Figure 7.

Dye loading and washout kinetics in blood-perfused pig heart with accelerated (Tyrode's) loading. $t=0$ corresponds to the end of dye injection. The switch from Tyrode's- to blood-perfusion at 14 minutes after dye injection. *A.*: The kinetics of total fluorescence F (normalized to maximum value). *B.*: Voltage-sensitive fluorescence $\Delta F/F$. Notations are the same as in Figure 6.

Figure 8.

Stability of electrophysiological characteristics after injection of JPW-6003 and JPW-6033.

A.: Volume-conducted ECG recorded in an isolated blood-perfused porcine heart before and 30 min after injection of 8.7 μmol JPW-6033 (50 nmol/g tissue). B.: Time dependence of conduction velocity (CV) in guinea pig during and after injection of 220 nmol JPW-6033 (100 nmol/g tissue). Bottom plot shows dose vs. time. C.: Time dependence of action potential duration (APD) in rat after injection of 160 nmol JPW-6003 (100 nmol/g tissue). Plot shows data from 3 experiments (different symbols). The trend (solid line) shows slight increase (0.8 ms/hr).

Table 1

Voltage sensitivity $\Delta F/F$ of JPW-6003 and JPW-6033 in different cardiac tissues (Tyrode's-perfusion, epifluorescence).

Animal	Dye		
	JPW-6003	JPW-6033	di-4-ANEPPS
Pig (n = 16)	15.2±1.1 [*] (n = 5)	15.0±1.1 [*] (n = 5)	8.9±1.4 (n = 6)
Rat (n = 14)	11.1±1.3 [*] (n = 4)	13.9±1.2 [*] (n = 4)	6.8±0.6 (n = 6)
Guinea pig (n = 9)	11.3±1.0 (n = 3)	12.5±1.4 (n = 3)	8.2±1.0 (n = 3)
Mouse (n = 8)	9.1±1.1 (n = 4)	10.5±1.6 (n = 4)	-
All species (n = 47)	11.9±2.6 [*] (n = 16)	13.1±2.2 [*] (n = 16)	7.9±1.4 (n = 15)

Values are mean±SD. Relative fluorescence $\Delta F/F$ is expressed in percent. Last column shows data for di-4-ANEPPS.

*
p<0.01 vs di-4-ANEPPS

Table 2

Voltage sensitivity $\Delta F/F$ of JPW-6003 and JPW-6033 for blood-perfusion and transillumination experiments (pig).

Modality	Dye		
	JPW-6003	JPW-6033	di-4-ANEPPS
Blood-perfusion (direct loading) (n = 9)	5.5±1.1 ^{*+} (n = 3)	5.4±1.0 ^{*+} (n = 3)	3.1±0.5 (n = 3)
Transillumination (Tyrode's-perfusion) (n = 12)	20.0±1.3 [*] (n = 3)	20.7±1.4 [*] (n = 3)	8.0±1.1 (n = 6)

Values are mean±SD. Relative fluorescence $\Delta F/F$ is expressed in percent. Last column shows data for di-4-ANEPPS.

^{*} p<0.024 vs di-4-ANEPPS

⁺ Data on JPW dyes were combined into one group

Table 3
Half-lives of total and voltage-sensitive fluorescence of JPW-6003 (Tyrode's-perfusion, epifluorescence).

Animal	Dye/Parameter					
	JPW-6003			JPW-6033		
	$\tau_{1/2}(F)$	$\tau_{1/2}(\Delta F/F)$	$\tau_{1/2}(F)$	$\tau_{1/2}(\Delta F/F)$	$\tau_{1/2}(F)$	$\tau_{1/2}(\Delta F/F)$
Pig (n = 16)	>160	210±22 [§]	>160	165±17 [§]	17±3	67±3
Rat (n = 14)	>170	161±11 [*]	>160	119±15 [*]	15±2	32±2
Guinea pig (n = 9)	>180	149±16	>160	131±15	23±2	22±2
Mouse (n = 8)	>120	105±13	>90	80±10	-	-

Values are means ± SD (in minutes). $\tau_{1/2}(F)$ - half-life (washout time) for normalized total fluorescence. $\tau_{1/2}(\Delta F/F)$ – half-life of relative voltage-sensitive fluorescence. Last column shows data for di-4-ANEPPS. The n values for individual groups are the same as in Table 1.

^{*} p<0.01 vs di-4-ANEPPS
[§] extrapolated