Ratiometry of transmembrane voltage-sensitive fluorescent dye emission in hearts

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which should affect both red and green signals, may be lessened.

Also motion artifacts may be lessened, which was described for transmembrane voltage changes in arterioles and heart cell cultures and calcium signals from hearts (2, 4, 23). Motion is a limitation of optical mapping of transmembrane potentials in the heart, requiring mechanical stabilization, pharmacological electro-mechanical uncoupling, or use of temporal derivatives of the optical signal to detect repolarization of the action potential (6, 8, 9, 12, 13).

This study considers ratiometry of epicardial di-4-ANEPPS emission in isolated rabbit hearts with excitation by a laser beam. Ratiometry was performed for action potentials and resting membrane potential changes produced by potassium infusion. We measured emission in two optical bands and, in separate experiments, 16 bands using a spectrophotometer to determine the impact of the selection of emission wavelengths used for the numerator and denominator.

METHODS

Heart preparation. Hearts (n = 11) were removed from pentobarbital-anesthetized New Zealand White rabbits in accordance with Institutional Animal Care and Use Committee guidelines. Hearts were initially perfused with a solution containing (in mmol/l) 129 NaCl, 5.4 KCl, 1.8 CaCl2, 1.1 MgCl2, 26 NaHCO3, 1 Na2HPO4, 11 glucose, and 0.04 g/l cellular potassium, we used potassium bolus injections or the perfusate heater.

One of the hearts fibrillated during pacing at 150 ms. Orders of perfusion with and without diacetyl monoxime and of the potassium signal was recorded simultaneously with optical signals.

Ratiometric fluorescence emission measurements. Hearts were stained with di-4-ANEPPS dissolved in ethanol that was gradually injected into the perfusion tubing (total 0.2–0.3 ml in 15 min). The heart gently contacted a transparent plate through which excitation and emission light passed. In some experiments the plate contained a 1-cm diameter hole to allow positioning of an intracellular microelectrode near the optical recording site. Two optical recording systems were used with different hearts (Fig. 1). In both systems, a 488-nm argon laser excited the di-4-ANEPPS. This wavelength is near the excitation peak or crossover wavelength (480 nm in bilayer), which minimizes transmembrane voltage dependence of absorption (5, 10). Thus the fractional change of red fluorescence is expected to be less than that found when a longer excitation wavelength is used (5). With a two-multiplier tube system, the laser scanned 63 spots in a 1-cm2 region of epicardium. Red fluorescence was collected with a photomultiplier tube that had its photocathode covered with a long-pass glass filter (>610 nm), whereas green fluorescence was collected with another photomultiplier tube covered with an interference filter (half-height bandwidth of 534–546 nm). The photomultiplier tubes were positioned side by side and sufficiently far from the heart so that the optical path from the heart was essentially the same for both tubes (maximum angular difference <3° and incident fluorescence was approximately perpendicular to the filter surface.

Before recordings, high-voltages (i.e., gains) for each photomultiplier tube were adjusted during laser excitation to produce identical DC fluorescence signals. The relative flux density of fluorescence in the two wavelength bands was determined by moving both optical filters to the opposite photomultiplier tube without changing the high voltages. Before moving, flux densities were inversely proportional to photomultiplier gains. After moving, the photomultiplier with larger gain received the fluorescence wavelength band with larger flux density, and hence the ratio of signals represented the square of the ratio of flux densities. This method ignores differences in photocathode sensitivity at different wavelengths and maximum filter transmittances. The ratio was compared with theoretical ratios calculated from areas under a gaussian curve with a peak at 620 nm and standard deviation of 62 nm that was fit to a di-4-ANEPPS emission spectrum with previously described methods (17).

In preliminary studies, the ratio of the two photomultiplier tube signals during action potentials was determined online with an analog circuit (MPY634BM, Burr-Brown, Tucson, AZ). For experiments included in results, the system hardware was altered to digitize signals from both of the photomultiplier tubes alternately at a rate of 500 Hz per tube. Changes in the ratio were then calculated from digitized data.

In some hearts, a spectrofluorometer system was used that contained a spectograph (77400, Oriel, Stratford, CT) and a steady perfusion of hyperkalemic solution to alter the resting membrane potential (18). In preliminary tests, the delay from potassium bolus injection to the solution change at the aortic cannula was determined with blue dye and with measurement of transmittance of laser light through the cannula. Also elevated potassium concentration in effluent from hearts after bolus injection was verified with an electrolyte analyzer. In some experiments, a custom-fabricated aortic cannula containing a potassium ion-selective electrode (TIPK, World Precision Instruments, Sarasota, FL), reference, and ground electrodes was used to indicate timing of the potassium change just above the left ventricle. The potassium signal was recorded simultaneously with optical signals.

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16-element photodiode array (PDB-C216, Photonic Detectors, Simi Valley, CA) as described (17). Fluorescence emission in 15 or 16 adjacent bands of wavelengths from just above the laser wavelength of 488 nm to 817 or 838 nm was digitized at a sampling rate of 1,000 Hz.

An intracellular glass microelectrode filled with 3 M KCl was positioned near the optical recording sites in six hearts to measure transmembrane potentials. The microelectrode signal was recorded simultaneously with optical signals on a digitizing oscilloscope (Norland 3001A, Atkinson, WI) or a separate channel of each of the optical recording systems. All recordings in this study were DC coupled.

Data analysis. Fluorescence signals and ratios were calculated and graphed with a computer (Sparcstation 20, Sun Microsystems, Mountain View, CA) using PV Wave or Matlab. So that differences after versus before action potential phase-zero depolarization or potassium infusion represent all available data from the two-photomultiplier tube system, measurements were performed on averaged recordings for all laser spots. Recordings were not temporally smoothed. Changes in fluorescence and ratios produced by phase-zero depolarizations or potassium-induced depolarizations were determined from the mean values in 6-ms segments before and after depolarization. The RMS noise (i.e., positive square root of the average of the squares of the deviations of the fluorescence samples about their mean) was determined for 6-ms segments before depolarization. Comparisons of action potential duration measured with the microelectrode versus that measured ratiometrically were performed with a single laser spot near the microelectrode. The green signal (wavelengths of 540 ± 6 nm), the red signal (wavelengths >610 nm), and their ratio are shown. The red signal contains an inverted action potential, i.e., downward deflection corresponding to the phase-zero depolarization and upward deflection corresponding to the gradual repolarization of the action potential. The green signal contains an upright action potential. These recordings indicate a shift toward shorter emission wavelengths when the membrane depolarizes.

In all 16 recordings from ventricular epicardium of 5 rabbit hearts using the two-photomultiplier tube system with solution potassium concentration of 5.4 mM, the fractional fluorescence change (ΔF/F) during the action potential phase-zero depolarization was 2.1 ± 0.8% for green fluorescence and -3.8 ± 0.5% for red fluorescence. The fractional change of the ratio of the green fluorescence to the red fluorescence (ΔRatio/Ratio) was 6.1 ± 1.1%. Magnitudes of fractional changes were significantly larger for the ratio versus either of the individual fluorescence signals (P < 0.001, paired 2-tailed t-test).

Figure 3 shows an example of the ratio of the green and red signals during potassium infusion and the simultaneous recording from a potassium ion-sensitive electrode located in the aortic cannula ~1 cm above the heart. Data were recorded without pacing the heart. Baseline drift in the ratio, which was not negligible, has been canceled with linear subtraction. Resting membrane potential decreased and the heart became quiescent when potassium concentration was raised. The action potential amplitude and duration also decreased during the first 20 s of hyperkalemia. Recovery
occurred after returning to the initial potassium concentration. In a different heart that was paced continuously to prevent the ventricles from becoming quiescent, again the emission ratio after subtraction of drift followed depolarization and recovery of the resting membrane potential.

The estimated emission flux density ratio in the green collection band to that in the red collection band for the two-photomultiplier tube system was $0.083 \pm 0.036 (P = 0.001, \text{value vs. 1, two-tailed } t\text{-test, } n = 3)$. This is comparable to theoretical ratios calculated from areas under a curve fit to the emission spectrum of the dye (METHODS) of 0.1 or 0.05 for the green bands of $540 \pm 10$ nm or $540 \pm 5$ nm, respectively, to the red band of $>610$ nm.

Spectrofluorometric measurements. To gain insight into the role of the emission wavelengths chosen for ratiometric measurements, the emitted fluorescence was measured with a spectrograph (77400, Oriel, Stratford, CT). Discrete wavelength bands having a width of 21.9 nm were measured for wavelengths just above the laser light wavelength of 488 to 817 or 838 nm (17). Of six hearts with no diacetyl monoxime that were used to test the ability to lessen motion artifacts with ratiometry, two were endocardially prefrozen and four were not prefrozen.

Figures 4 and 5 show examples of the motion artifacts and their reduction by emission ratiometry in endocardially prefrozen hearts. In Fig. 4, simultaneous recordings from two diodes that collected green and red emission wavelengths and their ratio are shown. The green signal (Fig. 4A) and red signal (Fig. 4B) exhibited a rapid increase and decrease, respectively, that corresponded to phase-zero depolarization. The rapid changes were followed by motion artifacts that obscured repolarization in signals from both diodes. The ratio of the green signal to the red signal (Fig. 4C) canceled the artifact and revealed the action potential repolarization.

Ratiometry also lessened effects of motion when the heart was not endocardially prefrozen. In the example shown in Fig. 6, the phase-zero depolarization expressed as a fraction of the peak-to-peak deflection during a cardiac cycle was much larger for the ratio signal compared with either the green (Fig. 6A) or red (Fig. 6B) signal.

The ability of ratiometry to lessen motion artifacts was quantitatively evaluated by measuring the ratio of the magnitude of change in the signal during phase-zero depolarization to the total peak-to-peak change during the cardiac cycle ($\Phi_0/p$). This analysis was performed on the first recording obtained in each heart without diacetyl monoxime. The $\Phi_0/p$ will have a maximum possible range of 0–1, will have a small value when motion causes the signal to exceed the upper or lower values that occur at the beginning and
end of the phase-zero depolarization, and will have a larger value when the effects of motion are reduced. Wavelength bands were 510–576 nm and 598–751 nm for numerator and denominator.

In the two hearts that were endocardially prefrozen and received no diacetyl monoxime, the Ph₀/p₂p was 0.386 ± 0.104 for the numerator, 0.700 ± 0.311 for the denominator, and 0.941 ± 0.083 for the ratio (P < 0.02 for ratio vs. numerator, paired 2-tailed t-test). In the four hearts that were not endocardially prefrozen and received no diacetyl monoxime, the Ph₀/p₂p was 0.273 ± 0.099 for the numerator, 0.351 ± 0.164 for the denominator, and 0.785 ± 0.144 for the ratio (P < 0.02 for ratio vs. denominator or numerator, paired 2-tailed t-test).

Effects of photobleaching, i.e., a slow decrease in emission, were evident in long-duration recordings obtained with the two-photomultiplier tube system and...
with the spectrofluorometer system as seen in Fig. 5. Results from both systems indicated that the ratiometry decreased drift but did not fully eliminate it. This may occur if the fractional decrease in emission due to drift is different for different wavelengths. In all seven trials with the spectrofluorometer in two endocardially prefrozen hearts that did not receive diacetyl monoxime, the emission at wavelengths of 532–554 nm (green) decreased $53 \pm 18\%$ in 120 s, whereas the emission at wavelengths of 663–685 (red) decreased $41 \pm 16\%$ ($P = 0.011$, for red vs. green, paired 2-tailed $t$-test). The ratio of the two emission signals (green/red) decreased only $18 \pm 13\%$ ($P < 0.015$ for value vs. decrease in red or green emission, paired 2-tailed $t$-test).

Fig. 5. Reduction of photobleaching-induced drift and motion artifact by emission ratiometry with spectrofluorometer system. The ordinates have been normalized to the value at the beginning of the recording. Drift in the green signal (A) was $-17\%$, drift in the red signal (B) was $-12\%$, and drift in the ratio of green to red signals (C) was $-5\%$. Motion artifact was also reduced by ratiometry. Perfusing solution did not contain diacetyl monoxime. The heart was endocardially prefrozen.

Fig. 6. Reduction of motion artifact by emission ratiometry with spectrofluorometer system in intact heart. For the green and red signals (A and B), magnitudes of deflections during phase-zero depolarization were a small fraction of the peak-to-peak magnitude of deflection during the cardiac cycle. For the ratio (C), magnitude of phase-zero depolarization was essentially the same as the peak-to-peak magnitude. Perfusing solution did not contain diacetyl monoxime. The heart was not endocardially prefrozen.
The signals obtained with ratiometry may depend on the choice of emission wavelengths that are collected. To understand this, we examined the emission spectrum and its change during an action potential or potassium bolus. Figure 7 shows the results for an action potential. The diastolic emission spectrum is shown (Fig. 7A). Also, the changes in emission (emission after the phase-zero depolarization minus emission before it) for each diode are shown as a fraction of both the diastolic emission (Fig. 7B) and RMS noise [i.e., signal-to-noise ratio (signal/noise), Fig. 7C]. The emission of green light increased while emission of red light decreased, which is consistent with action potentials in previous figures.

Figures 8 and 9 show the effects of altering numerator and denominator wavelengths on the fractional change and signal/noise obtained with ratiometry of an action potential. The optimal fractional changes and signal/noise occurred when wavelengths near 525–550 nm and 650–700 nm were used for the numerator and denominator, respectively. The highest fractional changes and signal/noise were larger for the ratiometric measurements than for individual diodes shown in Fig. 7 (e.g., fractional change of 8% for ratio vs. 6% for diodes, and signal/noise of 60 for ratio vs. 35 for diodes).

Changes in emission for individual diodes and ratios during the potassium-induced change in resting membrane potential (i.e., emission after injection of a potassium bolus minus emission before injection) were also examined. To correct for emission drift by photobleaching, each signal from the diode after injection was multiplied by a factor that represented the drift for that diode measured at a different laser spot and in a different trial with no potassium bolus. The wavelengths of emission used in the numerator and denominator that gave the largest fractional changes and signal/noise during potassium infusion were similar to those found during the action potential shown in Figs. 7–9. Also, the highest fractional changes and signal/

![Fig. 7. Fluorescence emission spectrum and spectral response of di-4-ANEPPS during an action potential in rabbit heart recorded with spectrotetrofluorometer system. Relative intensities of emitted fluorescence at various wavelengths during diastole (F, A), fractional change in intensities during action potential phase-zero depolarization (ΔF/F, B) and signal-to-noise ratio (signal/noise) (ΔF/RMS noise, C) are shown as functions of emission wavelength. Each measurement is from a single photodiode that collected light in an optical bandwidth of 21.9 nm. The wavelength of maximum emission was near 640 nm. Emission of green light (e.g., 520–550 nm) increased during phase-zero depolarization, whereas emission of red light (e.g., 630–700 nm) decreased. Signal/noise was small at wavelengths near 600 nm. Perfusing solution did not contain diacetyl monoxime. Heart was endocardially prefrozen.](http://ajpheart.physiology.org/DownloadedFrom)
noise during potassium infusion were larger for the ratiometric measurements than for individual diodes.

As a further test of the ability of ratiometry to reduce fluorescence changes that are unrelated to the transmembrane potential, the coefficients of variation (i.e., SD/mean) (22) were determined for all seven trials in the two hearts that were endocardially prefrozen and did not receive diacetyl monoxime. Measurements were performed with the spectrofluorometer system for the 10-s segment of each recording beginning ~1 s after the onset of laser illumination. This segment was selected because it contained pronounced drift in green and red signals due to onset of laser illumination, and it was after any effects of vibrations in the apparatus caused by movement of the laser shutter. The hearts were in sinus rhythm so that recordings contained diastolic intervals and action potentials. The coefficient of variation represents all of the variation in fluorescence, including effects of drift, motion and transmembrane potential changes. However, because we found

![Diagram](attachment:image.png)

**Fig. 8.** Fractional change in ratiometric measurement (ΔRatio/Ratio) during the same action potential as in Fig. 7. Results for ratios of all pairs of diodes are shown. Each diode collected light in an optical bandwidth of 21.9 nm. Large fractional changes occurred with ratios of signals having wavelengths of ~525 nm and 600–750 nm. Fractional changes were larger than those found with nonratiometric measurements (Fig. 7B). Perfusing solution did not contain diacetyl monoxime. The heart was endocardially prefrozen.

![Diagram](attachment:image.png)

**Fig. 9.** Signal/noise in ratiometric measurement (ΔRatio/RMS noise) during the same action potential as in Fig. 8. Results for ratios of pairs of diodes are shown. (Signal/noise of ratiometric measurements on principal diagonal is undefined.) Large signal/noise occurred with ratios of signals having wavelengths of ~550 nm and 650–700 nm. Signal/noise was larger than that found with nonratiometric measurements (see Fig. 7C). Perfusing solution did not contain diacetyl monoxime. The heart was endocardially prefrozen.
that fractional changes due to transmembrane potential were larger for the ratio, a decrease in the coefficient of variation for the ratio would indicate reduction of fluorescence changes that are unrelated to the transmembrane potential. Emissions at wavelengths just above the laser wavelength of 488–575 nm (numerator) and 598–838 nm (denominator) and their ratio were determined. These ranges contain the optimal wavelengths indicated in the preceding analysis. The coefficient of variation was 0.06 ± 0.03 (range of 0.01–0.1) for the numerator, 0.05 ± 0.03 (range of 0.01–0.1) for the denominator, and 0.02 ± 0.01 (range of 0.004–0.035) for their ratio ($P = 0.019$ for ratio vs. numerator or denominator, paired two-tailed t-test). Thus variability was reduced for the ratio even though the ratio had increased fractional fluorescence changes for action potentials and potassium-induced depolarization.

**Ratiometric measurement of action potential repolarization.** The ability of the ratio to indicate action potential repolarization was tested in six hearts with simultaneous microelectrode and optical recordings during perfusion with warm solution containing 15 or 20 mM diacetyl monoxime. Because multiple trials were performed at some laser spots, the durations of recordings were shortened to 2–4 s to lessen photobleaching. Figure 10 shows recordings of the ratio and transmembrane voltage during pacing at intervals of 150–1,250 ms in one heart. Ratio and microelectrode signals indicate similar action potential contours and durations for a given pacing interval. Even though activation time at the laser recording spot differed from that at the microelectrode tip by $3.8 \pm 1$ ms as expected due to their spatial separation, the plots of ratio versus transmembrane voltage indicate a linear

![Fig. 10. Ratiometric and microelectrode recordings plotted vs. time and vs. each other from one heart. Each row shows simultaneous recordings during pacing at intervals of 150 ms (A), 250 ms (B), 500 ms (C), and 1,250 ms (D).](http://ajpheart.physiology.org/)
relationship between the two signals during most of the action potential duration.

The combined results shown in Fig. 11 indicate agreement of action potential duration at 75% repolarization (APD$_{75}$) measured ratiometrically with that measured from microelectrode signals. Two-way analysis of variance of APD$_{75}$ in the six hearts during warm perfusion indicated $P = 0.003$ for an effect of the pacing interval and $P = 0.847$ for an effect of the recording method (i.e., microelectrode vs. ratiometry).

In the three hearts that were paced at rates of 150, 250, 500, and 1,250 ms and did not fibrillate, APD$_{75}$ measured ratiometrically (wavelength bands of 510–576 and 598–751 nm for the numerator and denominator) and with microelectrodes, respectively, was 99 ± 6 and 98 ± 9 ms during pacing at 150 ms, 116 ± 9 and 124 ± 4 ms during pacing at 250 ms, 144 ± 15 and 145 ± 14 ms during pacing at 500 ms, and 153 ± 33 and 155 ± 33 ms during pacing at 1,250 ms ($P > 0.1$ for ratiometric measurement vs. microelectrode measurement at same pacing interval, $P \leq 0.05$ for ratiometric or microelectrode measurements at ≥500 ms vs. those at 150 ms, paired 2-tail $t$-tests).

The effect of a potassium bolus on the action potential duration was recorded with the two-photomultiplier tube system in two hearts perfused with warm solution containing 15 mM diacetyl monoxime. The APD$_{75}$ measured ratiometrically at a single laser spot or with microelectrodes changed from 159 ± 25 ms to 102 ± 8 ms when potassium was introduced ($P = 0.003$, paired 2-tailed $t$-test, $n = 4$ hearts × recording methods). Two-way analysis of variance of the APD$_{75}$ indicated $P = 0.022$ for an effect of the potassium and $P = 0.65$ for an effect of the recording method (i.e., microelectrode vs. ratiometry).

Calibration of ratiometric measurements. The calibration factor between the ratio and the transmembrane voltage during perfusion with solution containing normal potassium was determined in the six hearts in which microelectrode recordings were obtained (of which 2 were studied with the 2-photomultiplier tube system and 4 were studied with the spectrofluorometer). For spectrofluorometric measurements, emission wavelength bands of 510–576 nm and 598–751 nm were used for the numerator and denominator, respectively, which were wavelengths that produced large fractional changes and low noise in the ratio described. When all recordings were included, the calibration slope depended on the order of the recording in each heart ($P = 0.03$, analysis of variance, $n = 30$). A linear regression of the calibration slope versus the order of the recording indicated that the calibration slope decreased at an average rate of 0.0058 per recording. This decrease may be due to dye internalization or photo-bleaching (14). To estimate the calibration slope when these effects are minimized, only a single ratiometric recording from each heart was included. The criterion for selection was that the recording had the largest $\Delta$Ratio/Ratio found in the heart, which consistently occurred for recordings obtained within 20 min after staining the heart with 4,4-ANEPPS.

In all six hearts, transmembrane voltages before and after phase-zero depolarization were $-85 \pm 6$ mV and $18 \pm 7$ mV ($P > 0.6$ for hearts studied with photomultiplier tubes versus hearts studied with spectrofluorometer, nonpaired 2-tail $t$-test). In the two hearts studied with photomultiplier tubes for which gains were initially set to produce a ratio of unity, ratios before and after phase-zero depolarization were $0.98 \pm 0.03$ and $1.05 \pm 0.02$, and the calibration slope was $(0.071 \pm 0.017)/100$ mV. In the four hearts studied with the spectrofluorometer, ratios before and after phase-zero depolarization were $0.21 \pm 0.03$ and $0.23 \pm 0.04$, and the calibration slope was $(0.017 \pm 0.002)/100$ mV.

Reduction of effects of laser noise. The ability of emission ratiometry to reduce common mode noise due to fluctuations of the excitation light intensity was studied by disabling a regulator in the laser power supply that controlled laser light intensity. Under this condition, the peak-to-peak magnitude of periodic laser intensity fluctuations at a rate of 60 Hz was 1.7% of the average laser intensity. For green and red emission signals, 60-Hz fluctuations were approximately half as large as the deflections produced during the phase-zero depolarization, as shown in Fig. 12, A and B. However, for the ratio of these signals (Fig. 12C), any 60-Hz fluctuations were much smaller than the phase-zero depolarization.

DISCUSSION

Emission ratiometry for transmembrane voltage measurements in hearts. The results indicate that emission ratiometry reduces drift and effects of epicardial motion, which is indicated in individual recordings (Figs. 4–6 and 12), in the measurements of drift over a 2-min period and in the decreased coefficient of variation. The ratiometry allows optical recordings of car-
Diastolic repolarization without requiring electromechanical uncoupling drugs. This is a potentially important advantage because the drugs can have undesired effects in myocardium (3, 16, 25, 26). Also ratiometry reduces drift, which affects long-duration recordings. Whereas slow changes in transmembrane potential that occur over a longer time period than that studied here may still be obscured by drift, our results show that ratiometry can reveal changes in transmembrane potential that occur during a potassium bolus.

Photobleaching-induced drift was only partly canceled by ratiometry as evident from drift in the ratio of green light to red light (Fig. 5). The spectrofluorometric results indicated that there was a greater decrease in recorded green emission compared with red. This is not expected if photobleaching only destroys fluorophore molecules, in which case the fractional decrease in emission should be the same for all fluorescence wavelengths. A greater decrease in green emission may result from a different emission spectrum of the remaining fluorophore, possibly due to the environment of the fluorophore, or from a phosphorescence emission at the longer wavelengths (11, 24). Also, dye internalization may be enhanced with intense laser illumination. If the emission spectrum and photobleaching decay rates differ in different membranes within the cells, this would produce drift of the ratio during photobleaching when dye has internalized, as proposed by Kao, WY, Davis C, Kim YI, and Beach JM (unpublished observations). Also a change in the membrane environment, if it occurs intracellularly, may alter spectral properties of internalized dye because of changes in the membrane dipole potential (14).

The emission ratiometry has potential advantages for studies in which changes in absorption due to chemical effects unrelated to transmembrane voltage may interfere with transmembrane voltage measurements. The ratio is expected to reject effects of changes in absorption because they are common to both green and red emissions. The finding that ratiometry detected similar transmembrane voltage-dependent shifts of emission wavelengths for the cardiac action potential and hyperkalemia indicates that any absorption changes due to potassium were rejected.

Regarding the possibility of improved calibration with ratiometry, Bullen and Saggau (5) found that slopes of ratio versus transmembrane voltage were approximately constant among cells; however, variable offset existed in the ratio that required subtraction. They also found that when offset was subtracted, slopes indicated a conversion factor between transmembrane voltage and ratio of 0.015/100 mV, which is similar to our mean slope of 0.017/100 mV found with the spectrofluorometer. The calibration slope may depend on detector sensitivities and optical bandwidths. Whereas gains and bandwidths were constant among all diodes in our spectrofluorometer, they were different in our photomultiplier tubes, which produced a mean slope of 0.071/100 mV when the gains were adjusted to produce an initial ratio of unity. Had our spectrofluorometer diode gains been adjusted to produce an initial ratio of unity (which would require increasing the gains of diodes sensing green light by a factor of ~4.5), then a slope of 0.077/100 mV can be calculated, which is close to the value obtained with our two photomultiplier tubes. Despite similarities in
our mean slopes obtained with the spectrofluorometer compared with that reported in neurons by Bullen and Saggau, the variation of slopes among hearts was larger than the variation among neurons. This may be due to nonspecific fluorescence in the heart in which various types of tissue are present, as discussed by Bullen and Saggau.

The signal/noise obtained ratiometrically (Fig. 9) is optimal at numerator wavelengths slightly longer than those that give the largest fractional ratio change. This may be due to larger fluorescence emission at the longer numerator wavelengths (Fig. 7), which increases DC level and lessens effects of shot noise. In systems that use two detectors for which the optical bandwidths of numerator and denominator signals can be selected a priori, the shot noise should decrease when a wider optical bandwidth is used. This is expected to increase signal/noise if the additional wavelengths add to the change in ratio. For the denominator, wavelengths will add to the signal in a wide band of approximately >600 nm (Fig. 8). For the numerator, the widest possible band is bounded by the excitation wavelength and ~580 nm, above which wavelengths add no fractional change in the ratio. However, a narrower numerator band gives a larger fractional change and signal/noise by excluding wavelengths that have a small change (Figs. 8 and 9). Also, neither band needs to be wide if there is sufficient light intensity to overcome shot noise, because the largest signal/noise was obtained with narrow numerator and denominator bands near 550 and 680 nm. Another issue impacting on the choice of bands is that nonspecific fluorescence from surrounding cellular structures or dye bound in nonspecific orientation may be rejected when optical bandwidth is small (5).

Motion artifacts in optical action potential recordings may have multiple causes that include possible changes in light transport properties (reflectance, scattering, or absorption of light) at the excitation and emission wavelengths due to stretching of the tissue and translational movement of the tissue when the light transport properties or dye distribution among voltage-sensing and nonspecific environments are heterogeneous. Ratiometry may eliminate distortion of the action potential due to changes in light transport properties provided that identical changes occur for the two emission wavelength bands used for the numerator and denominator. Even when ratiometry eliminates visible distortion in the optically recorded action potential, translational movement would cause different phases of an action potential to be recorded from different groups of cells. This limitation is potentially important in cases of heterogeneous repolarization and resting potential. In these cases, ratiometry may be advantageous with endocardially prefrozen or mechanically stabilized hearts that already have motion lessened by contact with adjacent underlying necrotic tissue or external stabilizers.

Laser scanner method for ratiometry. Recording systems in which spatial resolution of the recording is determined by a narrow excitation laser beam have potential advantages for emission ratiometry in hearts as well as microscopy (5, 7). The fact that localization of a recording is controlled by the excitation beam ensures that both red and green emission signals are colocal. For systems in which broad-field excitation is used and spatial resolution is determined by locations of detectors in image planes, colocal ratiometric recordings depend on exact alignment of detectors (23). Effects of laser intensity fluctuations, which have been a limitation of laser scanner systems and required additional methods for cancellation (15, 19), are reduced by emission ratiometry because the effect is common to numerator and denominator signals (Fig. 12). Also, the use of a narrow laser excitation bandwidth (~10⁻⁵ nm) allows short emission wavelengths (e.g., 500 nm, Fig. 8) to be included in the numerator if necessary.

The emission spectrum in hearts. The emission spectrum and its change during depolarization in hearts are qualitatively similar to previous measurements of di-4-ANEPPS emission in other preparations. For Retzius cells from the leech, Fromherz and Lambacher (11) found an emission peak at 604 nm and emission crossover at 575 nm for excitation at 482 nm with a positive change of ~6% in emission at 533 nm and a negative change of 5% at 636 nm for 100 mV of depolarization. For hemispherical bilayers, Fluhler et al. (10) found an emission peak at 640 nm and emission crossover at 620 nm with a positive change of 7.6% at 540 nm and a negative change of ~2.2% at 680 nm for 100 mV of depolarization. Our emission peak (Fig. 7) is similar to that of Fluhler et al. (10), whereas our emission crossover for the action potential is similar to that of Fromherz and Lambacher (11). Both of these reports agree with our findings in hearts in which positive changes in green emission and negative changes in red emission occur during membrane depolarization.

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