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Single-detector Simultaneous Optical Mapping of V_m and $[Ca^{2+}]_i$ in Cardiac Monolayers

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Abstract

Simultaneous mapping of transmembrane voltage (V_m) and intracellular Ca^{2+} concentration (Ca_i) has been used for studies of normal and abnormal impulse propagation in cardiac tissues. Existing dual mapping systems typically utilize one excitation and two emission bandwidths, requiring two photodetectors with precise pixel registration. In this study we describe a novel, single-detector mapping system that utilizes two excitation and one emission bandwidth for the simultaneous recording of action potentials and calcium transients in monolayers of neonatal rat cardiomyocytes. Cells stained with the Ca^{2+} -sensitive dye X-Rhod-1 and the voltage-sensitive dye Di-4-ANEPPS were illuminated by a programmable, multicolor LED matrix. Blue and green LED pulses were flashed in opposite phase at a rate of 488.3Hz using a custom-built dual bandpass excitation filter that transmitted blue (482 ± 6 nm) and green (577 ± 31 nm) light. A long-pass emission filter (>605 nm) and a 504-channel photodiode array were used to record combined signals from cardiomyocytes. Green excitation yielded Ca_i transients without significant crosstalk from V_m . Crosstalk present in V_m signals obtained with blue excitation was removed by subtracting an appropriately scaled version of the Ca_i transient. This method was applied to study delay between onsets of action potentials and Ca_i transients in anisotropic cardiac monolayers.

Keywords

dual mapping; voltage/calcium delay; cardiac monolayer; photodiode array

Introduction

Over the last 30 years, progress in basic arrhythmia research has been largely facilitated by the use of techniques to optically map spatiotemporal changes in cardiac transmembrane voltage (V_m) and intracellular Ca^{2+} concentration (Ca_i) (1, 2). Changes in the cardiac V_m regulate the flow of Ca^{2+} ions through voltage-sensitive channels, and by altering Ca_i , indirectly affect force generation through a process of excitation-contraction coupling (3). Changes in cardiac Ca_i on the other hand, modulate the activity of different membrane ion channels (e.g. L-type Ca^{2+} channel) and transporters (e.g. Na^+ - Ca^{2+} exchanger) and thus influence the shape of the cardiac action potential. This complex bidirectional coupling between Ca_i and V_m dynamics is known to govern normal cardiac function and pacemaking (4-6), but if disrupted or altered, can also precipitate different forms of cardiac arrhythmias (7-11). Hence, simultaneous optical mapping of V_m and Ca_i (dual mapping) from the same area of the tissue, while technically challenging, is of utmost value when studying electrical phenomena in the healthy and diseased heart.

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Dual optical mapping of V_m and Ca_i can be performed using a single excitation source and two separate detectors that simultaneously record the resulting fluorescent signals at two distinct emission bands (1, 2). A major drawback of this approach lies with the fact that the corresponding pixels in the two detectors (and their imaging optics) must be precisely aligned so as to gather fluorescence from the exact same tissue region. The use of smaller pixel size in modern high-resolution detectors and/or magnification optics for studies of microscopic electrical events further complicate the spatial registration process. Alternative methods for simultaneous detection of multiple fluorophores involve alternating application of multiple excitation wavelengths and recording of the resulting fluorescence signals by a single detector (12-14). Although single-detector, multiple excitation wavelength techniques are inherently non-simultaneous, the method may be considered simultaneous if the switch of the excitation wavelengths and sampling of emission for different fluorophores is rapid with respect to the rate of change in the measured physiological parameters.

Due to the difficulty in rapidly switching excitation wavelengths with traditional filament and arc lamp sources, most previous cardiac dual mapping studies have used a single excitation source and two recording detectors. In 2000, Fast and Ideker developed a system for simultaneous dual mapping in neonatal rat cardiac monolayers using RH-237 and Fluo-3 AM by employing a microscope, two photodiode arrays (PDAs), and two dichroic mirrors to separate excitation and emission wavelengths within the same optical path (15). In the same year, Choi and Salama used a tungsten excitation source, a dichroic mirror, and two PDAs with corresponding emission filters to simultaneously record RH-237 and Rhod-2 signals in whole guinea pig hearts (16). Fast's group went on to test various derivatives of Fluo and Rhod dyes (17-19). Later, other groups adopted a similar strategy, with Laurita et al. using two PDAs with Di-4-ANEPPS and Indo-1 dyes in guinea pig and dog heart preparations (20, 21), and Omichi et al. using a single laser band to excite RH-237 and Rhod-2 and two CCD cameras to record fluorescence signals in pig hearts (22). These and various other studies (8, 23-28) have been complicated by the use of two detectors (CCD, CMOS or PDA) and the corresponding need to carefully co-register the two recorded images on a pixel-by-pixel basis, using custom-made algorithms.

Recently, high power LEDs have been employed as excitation sources in a number of optical mapping studies in both cardiac monolayers and intact tissues (12, 14, 23, 29-32). The use of light emitting diodes (LEDs) offers several advantages compared to filament and arc lamp sources, including low price and power consumption, relatively narrow excitation band, intrinsically low noise, and importantly, rapid switch-on and -off times allowing fast multiplexing of different excitation wavelengths. In this study, we utilized the advantages of LED technology to develop a novel dual optical mapping approach where a multicolored matrix of LEDs was used to rapidly alternate between blue and green illumination to excite the voltage sensitive dye Di-4-ANEPPS and the calcium indicator X-Rhod-1. The red fluorescent signal emitted from both dyes was recorded with a single PDA, temporally separated into blue and green channels, and then processed to remove optical crosstalk, yielding simultaneously recorded V_m and Ca_i signals. This approach was applied to study the temporal relationships between V_m and Ca_i in isotropic and anisotropic monolayers of neonatal rat cardiac myocytes.

Materials and Methods

All animals were treated according to protocols approved by the Duke University Institutional Animal Care and Use Committee.

Cardiac cell culture

Neonatal rat ventricular monolayers were prepared similar to previously described methods (33, 34), and were used for optical mapping studies on culture day 5-7.

LED illumination system

The high power Lamina Ceramics BL-3000 RGB+ LED array (Lamina Ceramics, Inc., Westampton, NJ) was selected as the excitation source. The BL-3000 consists of 39 individual 6-element LED arrays (with 2 LEDs for green, blue, and red color) arranged in a rectangular matrix (schematic shown in Fig. 1). The use of the excitation source with many small singlecolor LEDs densely interweaved in a large matrix enabled us to apply spatially uniform and virtually identical illumination patterns for each of the LED colors. Intensity of the illumination was further improved by adding a focusing lens array (60° Wide Flood Optic, Lamina) in front of the LED matrix. A small heat sink (Lamina HS-4100-0345) was attached to the back of the matrix to dissipate the heat generated by the LEDs. For this study, only blue and green output of the tri-color BL-3000 were used to excite voltage- and Ca_i -sensitive fluorophores, respectively. The operation of the LED matrix was controlled by a manually programmable, high power LED driver (PP600, Gardasoft Vision Limited, Cambridge, UK) which was externally triggered and synchronized with the data acquisition and electrical stimulation using two A/D boards (DAP 5400a and DAP 840, Microstar Labs) controlled by a custom-designed LabVIEW program (National Instruments Corporation, Austin, TX) running on Windows XP (Supplementary Fig. S1). The LED matrix was operated in either a constant or pulsed illumination mode with blue, green, or blue and green lights combined. The details of the hardware and software used for the control of LED illumination and data acquisition are provided in the Supporting Material.

Custom design of a dual bandpass excitation filter

Dual mapping of V_m and Ca_i in cardiac monolayers was performed by co-staining the cells with the voltage sensitive dye Di-4-ANEPPS and the calcium indicator dye X-Rhod-1 (Invitrogen, Corp., Carlsbad, CA). When unfiltered, either blue (484 ± 11 nm) or green (542 ± 14 nm) LED illumination simultaneously excited both dyes (Fig. 2A), leading to a significant optical crosstalk between V_m and Ca_i signals during dual mapping (not shown). We therefore custom-designed a dual bandpass optical filter with a “blue band” of 482 ± 6 nm and a “green band” of 577 ± 31 nm (Chroma Technology Corp., Bellows Falls, VT), which were selected to improve separation between the two LED excitation bandwidths and to avoid exciting Di-4-ANEPPS during green LED illumination. Moreover, the blue band in the filter was designed to be narrow in order to minimize a relatively high baseline fluorescence level during blue illumination (F_{blue}) caused by Di-4-ANEPPS, while simultaneously increasing the fractional changes in fluorescence ($\Delta F/F$ ratio) during recorded action potentials (35). Furthermore, the upper cut-off wavelength (608 nm) of the green band in the filter was chosen to be slightly higher than the ~ 605 nm cut-on wavelength of the long-pass emission filter (Fig. 2B). This small overlap in the excitation and emission bands served to enhance the baseline fluorescence level during green illumination (F_{green}). By reducing the difference between F_{green} and F_{blue} , this enabled better utilization of the full dynamic range of the detector during dual mapping (additional detail provided in Supporting Material). The long-pass emission filter consisted of a 0.1 mm thick glass coverslip spin-coated with five layers of photoresist (PSCRed, Brewer Science, Inc., St. Louis, MO) that was sandwiched between the recording chamber and fiber optic bundle (36, 37).

Optical mapping of impulse propagation

Cardiac monolayer cultures were optically mapped in contact fluorescence imaging mode (37) using a single detector consisting of a bundle of 504 optical fibers (750 μm in diameter arranged in a hexagonal pattern with a 10 mm side length) connected to an array of photodiodes (RedShirt Imaging LLC, Decatur, GA) (37, 38). The photodiode array (PDA) was used in the maximum gain configuration that also includes low pass filters with a fixed cut-off frequency of ~ 950 Hz. The PDA also contains a resettable, high pass filter (>0.5 Hz) that enables the use of the full dynamic range of the PDA in recording of the ΔF signal.

For single fluorophore recordings, monolayers were incubated either with the 15 μM solution of Di-4-ANEPPS for 5 min at room temperature, or with the 1.5 μM solution of X-Rhod-1 for 30 minutes at 37°C followed by a 30 min incubation in fresh media at 37°C. Stained cultures were placed in a heated recording chamber and perfused with Tyrode's solution. For dual mapping, cultures were first stained with X-Rhod-1, placed in the heated chamber, and recordings from blue and green constant LED illumination were saved for later use in optical crosstalk removal. The chamber was then cooled, and the cultures were stained with Di-4-ANEPPS. After the dye was washed out for 5 minutes and the temperature was restored to 37°C, bipolar point stimuli ($1.2\times$ threshold, 10 ms duration) were applied at a 2 Hz pacing rate using an XYZ-micropositioned platinum point electrode. The pacing rate was increased every minute in steps of 0.5 Hz, and V_m and Ca_i signals were recorded at the end of each step.

Removal of optical crosstalk between V_m and Ca_i signals

Since any fluorophore has a non-zero response to all excitation wavelengths, multi-fluorophore recordings will always contain some degree of optical crosstalk. Since the custom dual bandpass filter was designed to avoid Di-4-ANEPPS excitation during green LED illumination, the separation algorithm for V_m and Ca_i signals was only performed for the blue illumination channel. Removal of the crosstalk was performed for each recording site by subtracting a scaled version of the Ca_i signal obtained during green pulsed illumination (dF_{green}) from the simultaneously recorded mixed V_m and Ca_i signal obtained during blue pulsed illumination ($dF_{\text{blue}}^{\text{uncorr}}$) (Fig. 3 and Supplementary Fig. S2). The scaling factor was determined during initial staining of the culture with X-Rhod-1 by finding the ratio of calcium transient amplitudes in response to constant blue ($\Delta F_{\text{green}}^{\text{xrhod}}$) vs. constant green ($\Delta F_{\text{blue}}^{\text{xrhod}}$) illumination. This scaling factor ($\Delta F_{\text{blue}}^{\text{xrhod}} / \Delta F_{\text{green}}^{\text{xrhod}}$) remained constant over multiple recordings (Supplementary Fig. S3). The blue channel signal recorded after addition of Di-4-ANEPPS was corrected to obtain V_m using the following equation:

$$\begin{aligned} V_m = dF_{\text{blue}}^{\text{corr}} &= dF_{\text{blue}}^{\text{uncorr}} - dF_{\text{green}} \cdot \Delta F_{\text{blue}}^{\text{xrhod}} / \Delta F_{\text{green}}^{\text{xrhod}} \\ &= dF_{\text{blue}}^{\text{uncorr}} - \text{Ca}_i \cdot \Delta F_{\text{blue}}^{\text{xrhod}} / \Delta F_{\text{green}}^{\text{xrhod}} \end{aligned}$$

The green channel signal represented Ca_i and was only inverted to display calcium transients with positive amplitude. Since calculation of the scaling factor and crosstalk removal was performed individually for each recording site in the fiberoptic bundle, it was critical that the position of the coverslip remained fixed during all recordings and dye loadings. A custom-made polydimethylsiloxane (PDMS) ring was therefore placed around the perimeter of the coverslip in order to secure its position within the mapping chamber and prevent its translation or rotation during the experiments.

Data analysis

Following acquisition and crosstalk removal, obtained V_m and Ca_i signals were detrended by subtracting a best-fit 3rd order polynomial to remove baseline drift. Signals were then filtered using a 12-point moving median filter followed by a 31-point Savitsky-Golay filter (31). Activation times used to calculate conduction velocities (CVs (39)) and construct isochrones maps were determined as the times of maximum positive slopes of action potentials and calcium transients. Action potential duration (APD) and Ca_i transient duration (Ca_iD) were determined by taking the difference between the activation time and time of 80% repolarization. Average V_m - Ca_i activation delay for each recording was calculated as the difference between the activation times of Ca_i transient and action potential in select recording sites in the monolayer. In isotropic cultures, all parameters (CV, APD, Ca_iD , and V_m - Ca_i delay) were obtained by averaging values from all recording sites excluding those within 1.5 mm of the pacing site. In anisotropic cultures, longitudinal and transverse parameters were determined at the long and short axes of elliptical isochrones, excluding recording sites within 1.5 mm of the pacing site. The signal to noise (S/N) ratios for raw V_m and Ca_i signals were calculated during 2 Hz pacing by dividing ΔF amplitude by the root-mean-square (RMS) noise and averaging over all recording sites. The RMS noise level was calculated from linearly detrended signal during the 150 ms interval immediately preceding depolarization. To determine percentage amplitudes for electrical crosstalk, RMS noise was subtracted from the signal amplitude, and the difference was divided by the peak signal amplitude.

Statistics

Data were expressed as mean $\pm$ SE. Differences between longitudinal and transverse measurements were analyzed using a paired t-test. V_m - Ca_i delay vs. pacing rate data were fit with linear regression and a positive trend was assigned if the slope parameter was significantly greater than zero. Data were considered statistically significant when $p < 0.05$.

Results

Evaluation of optical crosstalk during constant green and blue LED excitation

While the use of a dual bandpass excitation filter limited the spectral ranges of blue and green LED emissions, it was expected that a single LED color would still excite both X-Rhod-1 and Di-4-ANEPPS because of their overlapping absorption spectra (Fig. 2). To quantify the amount of optical crosstalk between the two dyes we applied either blue or green constant LED illumination through the dual bandpass filter to excite isotropic cardiac monolayers stained with either X-Rhod-1 or Di-4-ANEPPS (Fig. 4A-D). Optical crosstalk, expressed as the percent of undesired signal amplitude relative to desired signal amplitude (e.g., panel 4D vs. inverted panel 4B and inverted 4A vs. 4C) was $3.5 \pm 0.9\%$ and $34.5 \pm 14.5\%$ during green and blue excitation, respectively (evaluated in $n=8$ Di-4-ANEPPS stained monolayers vs. $n=13$ X-Rhod-1 stained monolayers, respectively). The relatively small crosstalk present during green excitation validated the choice of the dual bandpass filter and was considered negligible (16, 20, 28). Similar amounts of crosstalk were also evident in cardiac monolayers stained with both X-Rhod-1 and Di-4-ANEPPS in which constant blue illumination produced a combination of V_m and Ca_i signals (Fig. 4E), while constant green illumination resulted in a virtually pure (inverted) Ca_i signal (Fig. 4F).

Choice of LED flashing frequency

The RedShirt Imaging PDA has a built-in low-pass filter with a cut-off frequency of ~ 950 Hz yielding a transient exponential response to a step LED pulse (Fig. 5A). During dual mapping, light levels alternate between the total fluorescence response to blue excitation

($F_{blue} + \Delta F_{blue}$) and the total response to green excitation ($F_{green} + \Delta F_{green}$). Unless the ΔF response during an LED pulse decays to a smaller value than the noise floor (approximately ± 5 mV) before the sampling takes place during the following pulse, there will be unwanted “electrical crosstalk” between V_m and Ca_i signals upon separation. The proper sampling time was determined experimentally in cultures stained only with Di-4-ANEPPS and illuminated by pulsed blue LED light (with an equal turn-on and turn-off phase), by finding the minimum sample time that allowed the ΔF_{blue} to decay below noise level during the turn-off phase of the excitation (Fig. 5). As the signal was sampled later in the turn-off phase, the electric crosstalk in the green channel gave false action potential traces of decreasing amplitude (Fig. 5C-E). Specifically, when the signal was sampled from 0 ms to 0.3071, 0.6141, and 0.9216 ms in the turn-off period, the amplitude of action potential due to electrical crosstalk decreased from $\sim 100\%$ to 55.1%, 15.2%, and 2.6% of the amplitude recorded 0.9216 ms into the turn-on period (Fig. 5F). If the LED flashing period was increased and signal was sampled at times greater than 0.9216 ms after the onset of the on and off phases, no improvement in ΔF_{blue} occurred and the electrical crosstalk remained below the noise level (Fig. 5F). Thus, the maximal LED flashing rate that yielded negligible electrical crosstalk was $1/(2 \times 0.9216 \text{ ms}) \approx 542.5 \text{ Hz}$. To account for the discreteness of two A/D board timings, temporal resolution of the LED driver (see supplemental material), and potential variability in timings, an extra sample (102.4 μs) was added which increased the flashing period per color to $2 \times (0.9216 + 0.1024) = 2.048 \text{ ms}$, yielding a final sampling rate for V_m and Ca_i signals of $\sim 488.3 \text{ Hz}$.

Dual mapping of V_m and Ca_i in isotropic monolayers

To validate the choice of LED flashing frequency and the post-recording procedure to separate V_m and Ca_i signals, isotropic monolayers were stained with both X-Rhod-1 and Di-4-ANEPPS and mapped in flashing mode with either the green or blue LED illumination alone (Fig. 6A), or alternating green and blue illumination (Fig. 6B). Similar to the application of continuous light (Fig. 4), applying pulsed green illumination yielded only Ca_i signal in the green channel and no signal in the blue channel (during the off phase of green illumination) (Fig. 6A, left), while applying pulsed blue illumination yielded mixed V_m and Ca_i signal in the blue channel and no signal in the green channel (Fig. 6A, right). When both blue and green LEDs were pulsed 180 degrees out of phase, the signal separation procedure yielded Ca_i signal in the green channel and mixed V_m and Ca_i signals in the blue channel (Fig. 6B, left). By applying the crosstalk correction algorithm, the scaled Ca_i component was effectively removed from the V_m signal in the blue channel (Fig. 6B, right). The average S/N ratios of the raw corrected V_m and Ca_i signals were 21.1 ± 2.4 and 21.5 ± 1.8 , respectively ($n=5$ monolayers). The V_m and Ca_i isochrones constructed from the filtered signals showed that the calcium transients uniformly trailed behind action potential propagation (Fig. 6D). For each experiment, specific values of driving currents for blue and green LED illumination were adjusted empirically at the onset of dual mapping in order to maximize S/N ratios for V_m and Ca_i while limiting the difference between total fluorescence intensities ($F + \Delta F$) of X-Rhod-1 and Di-4-ANEPPS and avoiding saturation of the PDA (additional details are provided in Supporting Materials).

To further verify accurate separation of V_m and Ca_i signals during dual mapping, 10 μM verapamil in standard (with 1.8 mM Ca^{2+}) or Ca^{2+} -free Tyrode's solution was superfused over an isotropic cardiac monolayer. Compared to recordings in drug-free standard Tyrode's solution (Fig. 7A), propagated action potentials in the presence of verapamil exhibited a significantly increased duration and partially attenuated Ca^{2+} transients (Fig. 7B), while upon the removal of extracellular Ca^{2+} , as expected, Ca^{2+} transients were virtually abolished (Fig. 7C).

Action potential and Ca_i transient mapping in anisotropic cardiac monolayers

Simultaneous mapping of V_m and Ca_i was performed in anisotropic cardiac monolayers during point pacing to assess how delay between action potential and Ca_i transient changes with pacing rate and direction of impulse propagation (longitudinal vs. transverse). As expected, calcium transients and action potentials activated in an elliptical pattern around the pacing site (Fig. 8A) with calcium transient activation following action potential activation for all pacing rates (Fig. 8B). No significant difference was found when conduction velocities were calculated using V_m vs. Ca_i data. As the pacing rate was increased from 2 Hz to 6 Hz, the conduction velocity decreased from 21.2 ± 0.7 cm/s to 16.7 ± 2.4 cm/s in the longitudinal direction and from 10.9 ± 1.1 cm/s to 7.9 ± 1.8 cm/s in the transverse direction (Fig. 8C). Durations of action potential (APD) and Ca_i transient (Ca_iD) were not statistically different for longitudinal vs. transverse propagation (Fig. 8D). As pacing rate increased from 2 Hz to 6 Hz, Ca_iD was decreased from 313.6 ± 52.3 ms to 127.0 ± 2.9 ms, while APD remained approximately unchanged and averaged over all pacing rates to 129.3 ± 21.5 ms. Finally, with increase in pacing rate from 2 to 6 Hz, V_m - Ca_i delay increased with linear regression slope of 0.49 ± 0.11 ms/Hz in the longitudinal and 0.45 ± 0.09 ms/Hz in the transverse direction (Fig. 8E). In addition, at a pacing rate of 2 Hz, the V_m - Ca_i delay was significantly longer in the transverse than longitudinal direction ($p = 0.0198$, $n = 3$ monolayers).

Discussion

This study describes a novel method for simultaneous macroscopic mapping of V_m and Ca_i signals in the neonatal rat cardiomyocyte monolayers using a single fluorescence emission filter and PDA detector. Filtered blue and green LED lights were pulsed in an alternating fashion to separately excite two fluorophores, Di-4-ANEPPS and X-Rhod-1. Custom design of a dual bandpass excitation filter, careful synchronization of the LED illumination and data acquisition via LabVIEW software, and specific experimental procedures during staining with X-Rhod-1 followed by Di-4-ANEPPS combined with a post-processing algorithm in MATLAB, were developed to separate V_m and Ca_i signals and minimize their electrical and optical crosstalk to $<3.5\%$. Relatively high sampling rate and signal-to-noise ratio of the separated signals allowed us to study how the delay between action potential and calcium transient activation is affected by the excitation rate and direction of impulse propagation in anisotropic cardiac monolayers. From these dual mapping studies, we found that calcium transients in monolayers of neonatal rat myocytes always activated after action potentials with a delay that was increased for higher pacing rates and was larger for transverse vs. longitudinal propagation.

Choice of System Components

We combined the BL-3000 LED matrix, Lamina lens array, and high power PP600 LED driver (all available from commercial vendors) to create a pulsed excitation source capable of generating bright and uniform illumination field over a large recording area. The application of sub-maximum driving currents in this setup enabled dual mapping of V_m and Ca_i with relatively high S/N ratios (~ 21), despite intrinsically low light levels generated by a cardiac monolayer. Using the same illumination setup for dual mapping of whole hearts would be expected to yield signals with significantly higher S/N ratios. Furthermore, the use of the LED matrix with a large number of interweaved blue and green LEDs positioned in close proximity to each other and directly above the monolayer, enabled the generation of spatially uniform and virtually identical blue and green illumination patterns, otherwise difficult to accomplish if blue and green excitation sources were spatially separated. Since existing imaging detectors and acquisition boards do not allow independent control of gain

in individual pixels/channels, spatially uniform blue and green illumination are necessary to ensure uniformly high S/N ratios and no saturation in different recording sites.

In this study, we recorded V_m using Di-4-ANEPPS, a high S/N ratio voltage-sensitive dye commonly utilized in studies of cardiac action potential propagation (1, 27). On the other hand, although non-ratiometric imaging of Ca_i is often performed using Rhod-2 dye (1, 27), we decided to instead use X-Rhod-1, which compared to Rhod-2 has excitation and emission spectra shifted toward longer wavelengths. With these shifts, the excitation spectra of X-Rhod-1 (but not Rhod-2) and Di-4-ANEPPS were sufficiently separated to enable use of a custom-made dual bandpass excitation filter to obtain pure Ca_i signal during green flash excitation without the need for post-processing. Furthermore, the shifted emission spectrum of the X-Rhod-1 enabled the use of a long-pass red emission filter (PSCRed-coated coverslip) to record both V_m and Ca_i signals. However, since Ca_i indicators typically have a broad excitation spectrum, and show a large fractional fluorescence compared to V_m indicators (such as Di-4-ANEPPS), significant cross-talk between Ca_i and V_m during blue flash excitation was unavoidable. As the relative change in the X-Rhod-1 signal during blue vs. green excitation was stable over time, it was possible to simply subtract a scaled version of the Ca_i signal from the blue channel to successfully extract V_m with minimal remaining crosstalk. This extraction process (i.e., calculation of the scaling factor and subtraction), was individually performed for each of the 504 recording sites to minimize potential artifacts due to spatial non-uniformities in the blue vs. green excitation, dye loading, or thickness of the emission filter.

Comparison with other single detector multi-parameteric optical mapping methods

Two recently published studies have described optical mapping methods that utilized pulsed LED excitation and a single detector to ratiometrically record a single or two electrophysiological parameters. An elegant study by Lee et al. utilized 8 separate LED sources with 4 different colors and an EMCCD camera (Cascade-128+, Photometrics) to ratiometrically record V_m and Ca_i in Langendorff-perfused rat hearts stained with Di-4-ANBDQPQ and Fura-2 (14). Custom-designed electronic circuitry was used to synchronize LED illumination with the frame acquisition rate of the camera. Unlike our methodology, this mapping system was not designed to address the potential issue of optical cross-talk between the two fluorophores and relied on the use of a specific combination of dyes (of which Di-4-ANBDQPQ is not commercially available) with widely separated excitation as well as emission spectra. Two distinct emission spectra required the design of a custom dual bandpass emission filter to enable recording of V_m and Ca_i . The 4 distinct color LEDs used for excitation were not spatially co-registered as in our study, potentially leading to unequal illumination patterns. Furthermore, this method also required the use of a camera capable of outputting a frame-exposure signal. The 465 Hz sampling rate used to map V_m and Ca_i was comparable to the 488.3 Hz sampling rate in our study. S/N ratios were significantly higher, likely because recordings in the intact heart preparations derive signals from not only one, but multiple cardiac layers beneath the surface (24, 40, 41).

Another study by Bachtel et al. used two separate LED sources and a similar EMCCD camera to ratiometrically record V_m and remove motion artifacts from recorded signals in Langendorff-perfused pig hearts stained with Di-4-ANEPPS (12). Similar to the study by Lee et al., the two LED sources used were not spatially co-registered, but used a single dye to circumvent the need for removal of optical cross-talk, which is likely to emerge in multi-dye preparations. Their sampling rate of 375 Hz and S/N ratio of 5.8 were significantly lower than those obtained in our study.

Restitution of CV, APD, Ca_iD, and V_m-Ca_i delay

Dual optical mapping in 5-10 μm thick cardiac monolayers offers an important advantage in that the origin of the recorded signals is known with high confidence. In contrast, results obtained using multiple excitation bands in thick tissue preparations such as the whole hearts, ventricular wedges, or nodal tissues must be interpreted with caution because the interrogation depth of light is wavelength dependent (24, 42, 43). In our study, combining the monolayer preparation and the use of a single detector for dual mapping ensured that both recorded signals (V_m and Ca_i) originated from the same sites within the tissue culture. This allowed us to unambiguously derive how the delay between V_m and Ca_i activation changes as a function of the activation rate and direction of wave propagation. As expected, we found that both V_m and Ca_i activation patterns had elliptical shapes in anisotropic monolayers, CV restitution relationships derived from either V_m or Ca_i signals were similar, while Ca_iD restitution showed stronger rate dependence relative to that of the APD restitution. The average V_m - Ca_i delay of 9.6 ± 0.5 ms measured between the action potential and Ca_i transient upstrokes during basic pacing was in excellent agreement with those reported for human ventricular wedge preparations (44) and guinea-pig epicardium (16), but higher than those previously reported in neonatal rat ventricular myocyte (NRVM) cultures (15). The V_m - Ca_i delay increased for higher pacing rates, similar to findings by Choi and Salama who attributed this increase to an inadequate recovery of Ryanodine receptors and/or Ca^{2+} currents at shorter diastolic intervals (16). One minute-long rapid excitation in our study (vs. S1-S2 protocol by Choi and Salama) may have also yielded a rise in the resting Ca_i , causing a decrease in the Ca^{2+} gradient and ion flow across the sarcolemma, and consequently, a delay in the Ca^{2+} -induced Ca^{2+} release from the sarcoplasmic reticulum.

Current Limitations

In its current configuration, the described methodology yields relatively high S/N ratios (~ 21) for unfiltered V_m and Ca_i signals which, combined with a moderate level of temporal filtering, allow for the generation of clear activation isochrone maps as well as APD and Ca_iD measurements. Still, a substantial portion of the PDA dynamic range is used to accommodate the difference between base fluorescence levels of the two dyes, while the S/N ratio is limited by the amount of blue light (exciting Di-4-ANEPPS) that can be delivered without saturating the PDA. One potential strategy to overcome this limitation would be to add a second PP600 driver to flash the red LEDs from the BL-3000 matrix in synchrony with the green LEDs. The amplitude of the red illumination could be then fine-tuned to minimize the difference between the blue and green channel baselines, thus allowing the delivery of brighter blue light. This would result in increases in both the dynamic range and S/N ratio of the recorded signals.

Presently, the sampling rate during dual mapping is limited to ~ 488 Hz which is sufficient for tracking of V_m spatiotemporal dynamics at low (45) but not high (35) magnifications. However, this is a technical limitation of the RedShirt Imaging PDA, rather than a theoretical limitation of the method. The use of tunable filters (made with switched capacitors) and broad-band amplifiers would allow us to increase the sampling rate and accurately capture fast dynamic events at the microscopic scale (46). Finally, the versatility of the methodology could be further improved by the use of a high numerical aperture lens to separate the detector from the recording chamber, which would allow the use of other combinations of dyes and/or (single or multiband) emission filters.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Schematic of the dual mapping system. The LED matrix (BL-3000, Lamina) contained 39 individual LED elements, each made of 6 LEDs (2 red, 2 green, and 2 blue). Light from the LED matrix was focused by a lens array and filtered through a custom-made dual bandpass filter. The matrix was used to illuminate from above the cardiac monolayer, which was placed in a perfused heated chamber. The PDA detector (RedShirt Imaging) was used to record from below the monolayer in a trans-illumination (contact fluorescence imaging) mode with a red emission filter (PSCRed stained coverslip), which was sandwiched between the PDA and chamber.

FIGURE 2.

Filter and dye spectra for dual mapping. A) Illumination spectra. Dotted lines show absorption spectra for Di-4-ANEPPS (blue) and X-Rhod-1 (green), while solid lines show emission spectra for blue (blue) and green (green) LED sources, and transmission spectrum for the dual bandpass filter (black). B) Emission spectra of Di-4-ANEPPS, X-Rhod-1, and the PSCRed long-pass emission filter. The spectra of the LEDs and the emission filter were measured using an Ocean Optics USB2000 spectrophotometer. Spectra for the dual bandpass filter were obtained from Chroma Technology. Emission spectra for Di-4-ANEPPS and X-Rhod-1 from Invitrogen were measured using excitation wavelengths of 497 nm and 580 nm, respectively.

Experimental Protocol: **Analysis Protocol:** **FIGURE 3.**

Experimental and data analysis algorithm for dual mapping. The experimental procedure involves a first step where recordings were performed using only X-Rhod-1 dye followed by the second step when Di-4-ANEPPS was added. Data obtained from both steps are combined in post-processing analysis to separate V_m and Ca_i signals.

FIGURE 4.

Optical crosstalk between X-Rhod-1 and Di-4-ANEPPS. A monolayer stained only with X-Rhod-1 displayed Ca_i transients in response to both A) blue and B) green constant LED illumination. A monolayer stained only with Di-4-ANEPPS displayed action potentials in response to C) blue LED illumination, but not D) green LED illumination. E) A monolayer stained with both dyes displayed a combined V_m and Ca_i response during E) blue LED illumination and only Ca_i transients during F) green LED illumination.

FIGURE 5.

Determination of maximum LED flashing rate. A) In response to pulsed LED illumination (blue), the PDA generates a low-pass filtering response (black). Open squares represent times when signal is sampled by the A/D board (every 102.4 μ s). B) V_m signal obtained in a Di-4-ANEPPS stained monolayer by sampling 0.9216 ms after blue LED turn-on. C-D) Due to the low-pass filtering response of the PDA, green channel signals sampled C) 0.512 ms and D) 0.7168 ms after blue LED turn-off show “electrical crosstalk” in the form of a residual V_m signal. E) 0.9216 ms after the blue LED turn-off, the electrical crosstalk has decayed to a negligible amount. F) The dependence of the level of electrical crosstalk in the green channel on the time used to sample blue channel after the LED turn-off.

FIGURE 6.

Validation of LED flashing rate and crosstalk removal during dual mapping in isotropic cardiac monolayers. A) Flashing the green or blue LED alone in dual-stained monolayers yielded signals only in the green or blue channel, respectively. B) Flashing both the green and blue LEDs in the opposite phase produced a mixed V_m and Ca_i signal in the blue channel and pure Ca_i signal in the green channel, similar to those shown in A. After applying the algorithm for optical crosstalk removal, the blue channel displays only V_m signal. C) V_m (blue) and Ca_i (green) isochrone maps created from the separated signals. Times (in ms) apply to both V_m and Ca_i isochrone lines. The pulse sign indicates the location of a bipolar pacing electrode.

A Normal Tyrode's Solution

B Normal Tyrode's + 10 μ M verapamil

C 0 Ca^{2+} Tyrode's + 10 μ M verapamil

FIGURE 7.

Effect of application of verapamil and 0 mM extracellular Ca^{2+} on the V_m and Ca_i signals in isotropic cardiac monolayers. A) Representative V_m and Ca_i traces from a monolayer perfused with normal Tyrode's solution. B-C) V_m and Ca_i traces from the same optical channel after the addition of 10 μ M verapamil (B) or 10 μ M verapamil and removal of extracellular Ca^{2+} (C) from the perfused solution. Note that the application of 10 μ M verapamil with 0 mM extracellular Ca^{2+} , but not 10 μ M verapamil alone, eliminated Ca_i transients in cardiomyocytes.

FIGURE 8.

Dual mapping of V_m and Ca_i in anisotropic cardiac monolayers. A) Isochrone maps of action potential (blue) and Ca_i transient (green) activation during point-pacing from the center of the monolayer (pulse sign) at 2, 4, and 6 Hz. B) Representative V_m and Ca_i traces from a single channel shown for the three pacing rates. C-E) Effects of pacing rate on conduction velocity, action potential and Ca_i transient duration, and activation delay between action potential and Ca_i transient (V_m - Ca_i delay).