Optical Recording of Single Cardiomyocyte Transmembrane Potential in Langendorff-Perfused Mouse Hearts

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Abstract

Spatial heterogeneity of action potential properties has been related to cardiac arrhythmogenesis. In this study we used laser scanning confocal microscopy in conjunction with the fast potentiometric dye ANNINE-6 to monitor changes in cardiomyocyte transmembrane potentials in Langendorff-perfused mouse hearts on a subcellular scale. Line-scan images from up to three neighboring cardiomyocytes were obtained during continuous electrical stimulation at 3 Hz. Fluorescence changes for each cardiomyocyte along the scan line were resolved from the corresponding line-scan image. Peak changes in fluorescence intensity during an action potential exceeded 20%. Signal-to-noise ratio of the optical signal was >20. Action potential durations were not significantly different between adjacent cardio-myocytes under our conditions. We conclude that this imaging technique can be used to investigate cell-to-cell repolarization heterogeneity in the intact heart.

1. Introduction

Optical recordings of cardiac electrical activity using fast voltage-sensitive dyes (VSDs) have been widely applied to study the activation and repolarization of the heart.[1-4] These optical mapping studies revealed different patterns of electrical heterogeneity, including base-to-apex[2] or endo-to-epicardial[3] action potential duration gradients at the macroscopic level and such spatial heterogeneity of action potential properties has been related to arrhythmogenesis in the heart.[1-3] Usually custom-made optical mapping systems based on photodiode array or charge-coupled device cameras[2,3] are used to record action potentials simultaneously from multiple sites. Given the nature of the illumination of the excitation light and the light-tissue interactions, each element of the camera takes fluorescent signals from many layers of cardiomyocytes.[2,3,5] On the other hand, recent studies suggested that measurement of action potentials with cellular and/or subcellular resolution in the intact heart would provide valuable insights into arrhythmogenesis.[6,7] Whether similar repolarization gradients occur on the cellular scale in the intact heart has remained uncertain. To resolve signals with high spatial resolution in 3-dimensional space, laser scanning microscopy (LSM), which has superior depth penetration and resolution in thick tissue, has been used for functional imaging.[8] Unfortunately, LSM introduces at least three serious technical difficulties for recording the transmembrane potentials. First, only fluorescent photons from a tiny volume (~μm^3) are collected, requiring much higher concentration of the dye molecules and density of excitation photons at the focus. The high energy of the excitation subsequently results in severe photobleaching of dye molecules and photodamage to the tissue. Second, the traditional voltage sensitive dyes such as di-4-ANEPPS possess low voltage sensitivity, typically <10% per 100-mV of membrane potential change.[9, 10] Such low sensitivity yields optical signals with low signal-to-noise ratios (SNR) since the noise level in LSM imaging is significantly elevated by the shot-noise of the photo-multiplier tube (PMT), the speckles from the coherent excitation laser, and the light-tissue interaction in scattering tissues. Third, the image is built point-by-point in LSM, posing constrains in temporal resolution.

Accordingly, we used the line-scan mode of a laser scanning confocal microscope (LSCM) in conjunction with the novel fast potentiometric dye ANNINE-6, which has reportedly a markedly higher voltage sensitivity than traditional dyes,[10] to record membrane potentials of single cardiomyocyte within the intact heart with high spatiotemporal resolution. Signal processing algorithms were developed to improve SNR.

2. Methods

Isolated Heart Preparation. Adult mouse hearts were excised and an 18-G cannula was inserted into the ascending aorta for retrograde perfusion (60cm H_2O) with oxygenated (100% O_2) Tyrode's solution containing (mM) 133.5 NaCl, 4 KCl, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, 2 $CaCl_2$, 10 HEPES, and 11 glucose (pH 7.4, adjusted with

10N NaOH). The Tyrode's solution was filtered through a 0.22µm microfilter to remove particles. For loading the heart with ANNINE-6,[10] the dye was first dissolved in 20% (w/v) Pluronic-F 127 in DMSO at 0.42 mM. Aliquots of this stock were diluted 1:100 in 10 mL Tyrode's solution which was then used for continuous retrograde perfusion. Finally, the heart was perfused with 30 mL dye-free Tyrode's solution to wash out unbound dye molecules. Staining and washout were performed in a water bath at 36°C.

Confocal Line-scan Imaging of Cardiomyocyte Action Potentials. All imaging experiments were carried out on an upright Zeiss LSM 510 META NLO microscope at room temperature (19-21°C). The heart was placed in a custom-made chamber in which a pair of platinum electrodes is embedded. Electrical stimuli (3 Hz, 2-ms pulses) were delivered to the right ventricle of the heart through the embedded electrodes. A volume-conducted electrogram of the heart was continuously monitored between an electrode near the base, and another close to the apex. A National Instruments multi-functional card AI-16XE-50 was programmed to generate the pacing pulse train and record the electrogram in real time.

Illumination of ANNINE-6 was provided by the 488-nm line of an Argon laser and fluorescence was collected in the >560 nm range. A frame image of the ventricular tissue about 30 µm beneath the epicardium was taken first and a line along a segment of the sarcolemma was selected for high-frequency line scanning. The length of the line ranged from a few microns for subcellular recordings to 80 µm for multiple-cell recording. The nominal lateral resolution was 0.1 to 0.6 µm, depending on the digital zoom. The highest actual lateral resolution was about 0.3 µm, which is defined by the numerical aperture (NA) of the objective (NA=1.2) and the wavelength of the excitation laser (λ=488 nm). To achieve high spatial resolution in 3-dimensional space, the confocal detector aperture was set to 250 µm, providing a nominal axial resolution of 3 µm. The line-scan mode of the microscope was used to achieve high temporal resolution. The same line was repeatedly scanned at a rate of 1.042 kHz for 8 to 10 seconds and the intensity of each line was stacked sequentially to generate the line scan images. Each scan line encompassed 128 pixels. Fluorescence intensity was digitized at 12-bit resolution and the data was stored on the hard disk for off-line analysis.

To completely suppress motion during the rapid line scan imaging, the heart was electromechanically dissociated by supplementing the Tyrode's solution with 50 µM cytochalasin-D and 1 µM ryanodine.

Data Processing. The line-scan images were exported as 12-bit TIFF images and were subsequently processed with routines written in Matlab. Details of data processing are described in Section 3.2.

3. Results

ANNINE-6 Selectively Stains the Sarcolemma. Figure 1A is a frame image of a 230×230 µm^2 area from the anterior left ventricle of a mouse heart stained with ANNINE-6. The VSD stains only the outer leaf of the lipid bilayer of the cell membrane as indicated by the lack of nuclear membrane staining. Various sarcolemmal structures can be readily identified, including lateral membrane (side-to-side), intercalated disc (end-to-end) and transverse tubules (t-tubules) of cardiomyocyte outer membrane. Endothelial cells lining the capillary lumen are heavily stained since the dye is delivered through the perfusate.

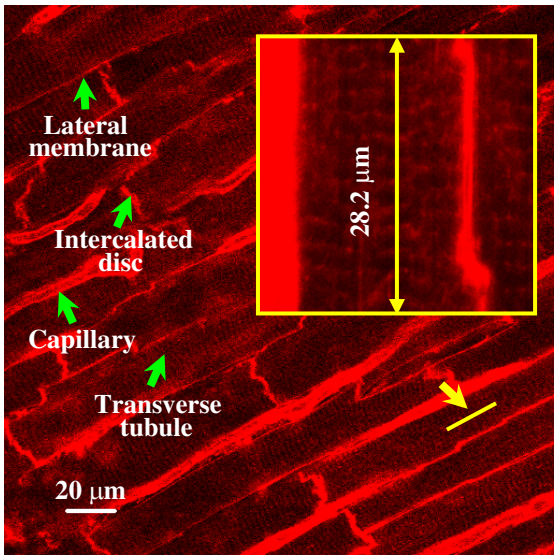

Figure 1. Frame image from the anterior left ventricle of a mouse heart stained with ANNINE-6. Green arrows denote various cardiomyocyte membrane structures and a capillary in the intact mouse heart. Inset: zoom-in frame of the region of interest whose center is indicated by the yellow line. Repeated line-scan is performed along the yellow line across the t-tubules.

Line Scan Images and Action Potentials. Line-scan images were acquired from various sarcolemmal structures, including t-tubules, side-to-side and end-to-end junctions. Figure 2 shows a typical line-scan image acquired across multiple t-tubules (see the yellow line in Figure 1). ANNINE-6 has been shown previously to exhibit a decrease in fluorescence emission upon membrane depolarization.[10] In Figure 2, periodic electrical stimulation resulted in periodic decreases in dye fluorescence along the scan line (see arrows in Figure 2). The image also exhibited a more gradual decrease in dye fluorescence, which most likely reflects photobleaching.

To resolve the action potential associated-optical signal, we averaged the pixels along the line and plotted the time course of the spatially averaged signal, as shown in Figure 3.

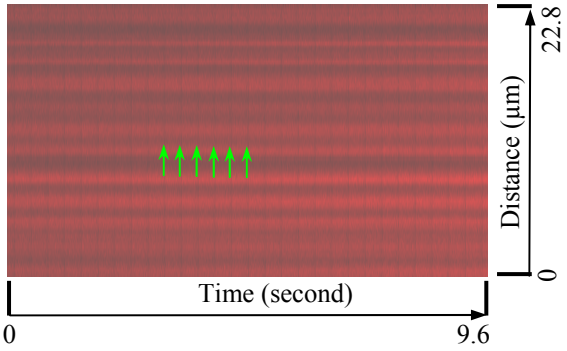

Figure 2. A typical line-scan image scanned along the yellow line in Figure 1. Green arrows mark the periodical decreases in ANNINE-6 fluorescence induced by action potential-associated membrane depolarization (vertical dark bands).

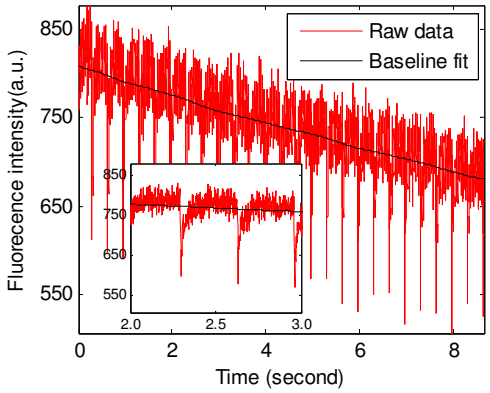

Figure 3. Time course of spatially averaged signal of line-scan image in Figure 2. The notches are action potential-associated decreases in fluorescence. Inset: zoom in of 1-second spatially averaged signal.

Figure 2 shows both the action potential-associated fluorescence decrease and the photobleaching-induced decline in baseline fluorescence. To compensate for the effect of photobleaching, a filtering-based estimation algorithm was developed, utilizing a combination of a moving averaging filter and a low-pass Butterworth filter with a cut-off frequency of 3 Hz. This baseline estimator has been tested with >300 scans and is robust in fitting the baselines. We then normalized the change in dye fluorescence (ΔF) against its baseline (F). Figure 4 shows a 4-second period of the normalized signal (ΔF/F, blue trace). Peak changes in fluorescence intensity during an action potential were about 25% in this case. To improve the SNR of the signal, we first constructed an objective trace of the action potential based on the ensemble average and subsequently subtracted the objective trace from the ΔF/F signal. The power spectrum of the residual suggested that the residual has the characteristics of white noise. An optimal filtering strategy was applied to the normalized signal ΔF/F. The result is shown in Figure 4 (red trace). The SNR is defined as the ratio of peak ΔF/F to the standard deviation of the baseline ΔF/F, which was calculated from the 50-ms segment preceding each action potential. A SNR of 50 was achieved in this case.

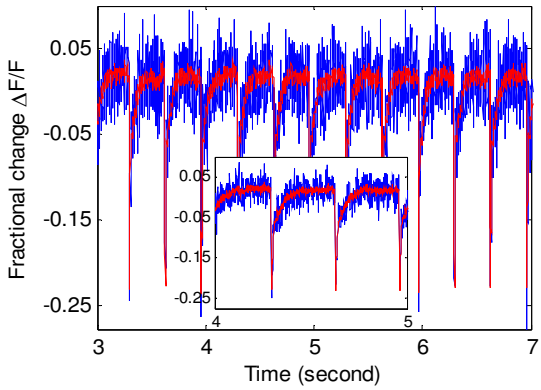

Figure 4. Time course of spatially averaged, normalized fluorescence signal ΔF/F. Data from the line-scan image in Figure 2 was used. The blue trace was the ΔF/F following photobleaching correction and normalization. The red trace was the result after optimal filtering, whose SNR was significantly improved. Inset: 1-second of ΔF/F before (blue) and after (red) optimal filtering.

We also performed the microelectrode impalement of the same preparation and recorded the transmembrane action potentials. The resolved optical signal followed the contours of action potentials recorded with microelectrodes (not shown).

Signals recorded from end-to-end and side-to-side junctions as well as those recorded across multiple t-tubules within a single cardiomyocyte had similar time courses. The peak fractional fluorescence ΔF/F was -21.7 ± 3.0% (mean ± SD, n=80). Action potential durations derived from the optical recordings were 19.5±7.2 ms and 4.6±1.6 ms for 70% and 30% repolarization, respectively.

Simultaneous Imaging of Action Potentials from Multiple Neighboring Cardiomyocytes.

One of the desirable features of the optical approach is the ability to record signals simultaneously from multiple sites. Action potentials were recorded from 2~3 neighboring cells in ANNINE-6 stained hearts. Figure 5A shows a representative recording site and the line-scan image of 3 neighboring cardiomyocytes. Action potentials of each myocyte were resolved from the corresponding

portion of the line-scan image (shown in panel A, cell junctions were not included), as shown in Figure 5.B. No significant differences were observed between the optical signals obtained from the three cells. Other simultaneous multiple-cell recordings gave identical results.

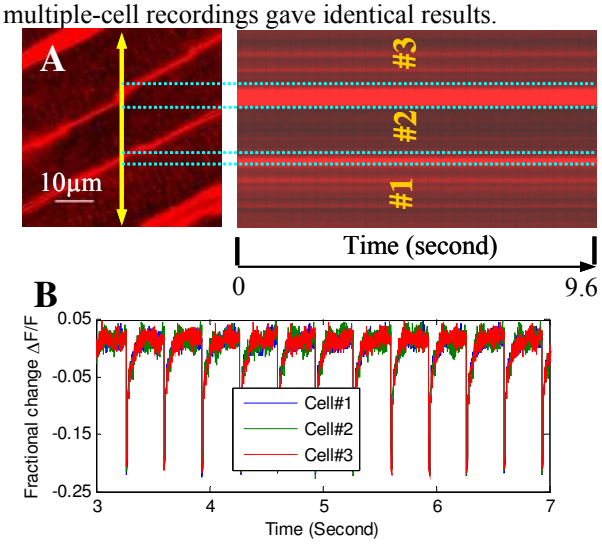

Figure 5. Simultaneous optical recordings of action potentials from 3 neighboring cardiomyocytes. (A) Frame mode image and line-scan image along the yellow line. (B) Resolved action potentials from each of the 3 cells.

4. Discussion and conclusions

In this proof-of-concept study, we demonstrated, for the first time, the ability to directly record changes in cardiomyocyte transmembrane voltage with subcellular resolution in an intact heart. This resulted from a combination of multiple factors, including the high voltage sensitivity of the novel VSD ANNINE-6, the superior spatial resolution of LSCM, and a set of signal processing algorithms to improve SNR. The ability to record action potentials with subcellular resolution opens up new perspectives for investigating repolarization heterogeneity and heterocellular electrical communication in intact heart.

We achieved a sampling rate of 1,042 Hz by using the line-scan mode of the imaging system. This rate is sufficient to faithfully track action potential repolarization. However, it is insufficient to study the fast depolarization during phase 0 of the cardiac action potential. Such temporal constraints are due to the commercially available LSCM. The response time of ANNINE-6, which is in the range of nanosecond, does not constitute a limiting factor.

The pharmacological agents applied to eliminate motion artefacts interfere with the cardiac electro-physiology. The action potential durations derived in this study therefore should be interpreted in the context of continuous presence of cytochalasin-D and ryanodine. Other techniques for excitation-contraction uncoupling are under investigation.

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