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Optical Mapping of Intra-Sarcoplasmic Reticulum Ca^{2+} and Transmembrane Potential in the Langendorff-perfused Rabbit Heart

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Abstract

Sarcoplasmic reticulum (SR) Ca^{2+} handling plays a key role in normal excitation-contraction coupling and aberrant SR Ca^{2+} handling is known to play a significant role in certain types of arrhythmia. Because arrhythmias are spatially distinct, emergent phenomena, they must be investigated at the tissue level. However, methods for directly probing SR Ca^{2+} in the intact heart remain limited. This article describes the protocol for dual optical mapping of transmembrane potential (V_m) and free intra-SR [Ca^{2+}] ([Ca^{2+}]$_{SR}$) in the Langendorff-perfused rabbit heart. This approach takes advantage of the low-affinity Ca^{2+} indicator Fluo-5N, which has minimal fluorescence in the cytosol where intracellular [Ca^{2+}] ([Ca^{2+}]$_i$) is relatively low but exhibits significant fluorescence in the SR lumen where [Ca^{2+}]$_{SR}$ is in the millimolar range. In addition to revealing SR Ca^{2+} characteristics spatially across the epicardial surface of the heart, this approach has the distinct advantage of simultaneous monitoring of V_m, allowing for investigations into the bidirectional relationship between V_m and SR Ca^{2+} and the role of SR Ca^{2+} in arrhythmogenic phenomena.

Video Link

The video component of this article can be found at http://www.jove.com/video/53166/

Introduction

Dual optical mapping of intracellular Ca^{2+} and transmembrane potential (V_m) in the intact Langendorff-perfused heart has become a mainstay of investigations in cardiac electrophysiology, including mechanisms of arrhythmia and excitation-contraction coupling[1-4]. This approach has provided unprecedented knowledge into normal and abnormal electrophysiology and, importantly, into the bidirectional relationship between V_m and intracellular Ca^{2+}. However, optical mapping of intracellular Ca^{2+} with high-affinity fluorescent indicators (such as Rhod-2 and Fluo-4) only reports on bulk changes in intracellular Ca^{2+} and is unable to distinguish whether these changes are due to transmembrane Ca^{2+} flux, release and reuptake into intracellular stores, or in most instances, some combination of both. Furthermore, high-affinity Ca^{2+} indicators have slow on-off kinetics and may not accurately report rapid changes in Ca^{2+} concentration[5].

Each action potential triggers a rise in intracellular Ca^{2+}, known as the intracellular Ca^{2+} transient (CaT). In the mammalian heart, approximately 70 – 90% of the total CaT is due to release of Ca^{2+} from the sarcoplasmic reticulum (SR) via opening of ryanodine receptors (RyRs)[6]. Within the SR, approximately half of the total Ca^{2+} is bound to calsequestrin (CSQ) and other intra-SR buffers[7], which play an important role in SR Ca^{2+} homeostasis[8,9]. The amount of free SR Ca^{2+} dictates the driving force for SR Ca^{2+} release as well as gating of RyR, and therefore has a significant impact on the intracellular CaT. Furthermore, alterations in SR Ca^{2+} release or reuptake can, in turn, impact V_m via the electrogenic Na^+-Ca^{2+} exchange, which may have arrhythmogenic consequences. Therefore, in addition to the CaT, monitoring of free SR Ca^{2+} can provide important insights into contractile and electrophysiological dysfunction.

Over the past several years, investigators have made significant advances in the monitoring of SR Ca^{2+} in isolated cardiac myocytes and from a single location on the intact heart. One such method requires rapid pulses of caffeine to open RyRs and the SR Ca^{2+} content is then inferred or calculated from the immediate rise in intracellular Ca^{2+}[10]. Another intriguing approach uses low-affinity Ca^{2+} indicators, such as Fluo-5N[11] or Mag-Fluo4[12], which bind to free SR Ca^{2+}. These indicators have dissociation constants (K_d) in the range of 10 – 400 μM and therefore exhibit minimal fluorescence in the cytosol compared to the SR lumen, where the Ca^{2+} concentration ([Ca^{2+}]$_{SR}$) is in the millimolar range. Using low-affinity Ca^{2+} indicators, several aspects of SR Ca^{2+} cycling have been investigated at the level of the isolated myocyte, including fractional SR Ca^{2+} release and the mechanisms of Ca^{2+} alternans[13,14]. However, in order to fully understand the heterogeneous nature of SR Ca^{2+} cycling in the intact heart and the role of SR Ca^{2+} in spatially distinct arrhythmic phenomena, methods for imaging SR Ca^{2+} across the epicardial surface of the intact heart are required[15].

This article describes methodology for dual optical mapping of free SR Ca^{2+} and V_m in the intact Langendorff-perfused rabbit heart with the low-affinity Ca^{2+} indicator Fluo-5N. In addition to revealing SR Ca^{2+} characteristics spatially across the epicardial surface of the heart, this approach has the advantage of simultaneous monitoring of V_m, allowing for investigations into the bidirectional relationship between V_m and SR Ca^{2+}.

Protocol

All procedures involving animals were approved by the Animal Care and Use Committee of the University of California, Davis, and adhered to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

1. Preparation

1. Prepare two concentrated (25X) stocks of modified Tyrode's solution in advance and store at 4 °C: (1) Stock I (in mM: NaCl 3,205, $CaCl_2$ 32.5, KCl 117.5, NaH_2PO_4 29.75, $MgCl_2$ 26.25) and (2) Stock II (in mM: $NaHCO_3$ 500).
    1. Freshly prepare 2 L Tyrode's solution of the following composition (in mM): NaCl 128.2, $CaCl_2$ 1.3, KCl 4.7, $MgCl_2$ 1.05, NaH_2PO_4 1.19, $NaHCO_3$ 20 and glucose 11.1 by combining 1840 ml of deionized (DI) water, 80 ml Stock I, 80 ml of Stock II, and 4 g of glucose. Do not directly mix Stock I and Stock II; add them to 1,840 ml DI to avoid precipitation.

2. Prepare stock solution of the excitation-contraction uncoupler blebbistatin at 10 mg/ml in anhydrous di-methylsulfoxide (DMSO) in advance and store the stock solution at 4 °C.
3. Prepare stock solutions of fluorescent dyes: (1) Voltage-sensitive dye RH237 (1 mg/ml in DMSO) and (2) Low-affinity calcium indicator Fluo-5N AM (2 mg/ml in DMSO) according to manufacturer's recommendations. Use the preparations immediately to avoid decomposition with subsequent loss of loading capacity.
4. Prime the recirculating Langendorff perfusion system with oxygenated (95% O_2, 5% CO_2) Tyrode's solution. Adjust the flow of O_2/CO_2 to keep the pH of the Tyrode's solution at 7.4 ± 0.05. Use an in-line nylon woven net filter (pore size: 11 µm) to continuously filter the perfusate.
    1. Prime the perfusion system at RT, as subsequent loading of Fluo-5N AM is performed at RT. For Fluo-5N AM loading, use a small volume of recirculating perfusate (~ 150 ml). After dye loading, use a larger volume of perfusate (1 - 2 L, see **Figure 1C**).

5. Turn on data acquisition system and prepare for continuous monitoring of ECG and perfusion pressure. Prepare pressure-monitoring system: Connect a pressure transducer to the perfusion line and monitor the perfusion pressure with a transbridge amplifier. Adjust the baseline perfusion pressure to 0 mmHg when the heart is not attached to the perfusion system.
6. Align the optical mapping cameras to ensure spatial alignment between the V_m and SR Ca^{2+} signals.
    1. First, focus the cameras by placing a ruler or an object with text into the perfusion dish. Turn the mapping cameras into a live view mode and adjust the focus until the text is crisp and clear. Acquire a single frame image from each camera.
    2. Overlay these images and adjust the transparency of the top image so that both images are visible. The text in the images should overlap exactly. If not perfectly aligned, adjust the angle of the dichroic mirror or the position of each camera until the two images are aligned.

2. Harvesting, Perfusion, and Dye-loading of Rabbit Heart

1. Secure the rabbit in an approved rabbit restrainer. Deeply anesthetize via an intravenous injection (marginal ear vein) of sodium pentobarbital (50 mg/kg) and heparin (1,000 IU).
    1. When the rabbit displays lack of nociceptive reflexes, make a midline skin incision to expose the sternum and ribs. Cut through the sternum with blunt-tip surgical scissors from the xyphoid to the manubrium. Take care not to damage the heart while opening the sternum. Spread the ribs to expose the heart.
    2. Quickly excise the entire heart-lung block by rapidly cutting all vessels and connective tissue. Immediately place the heart-lung block into ice-cold Tyrode's solution.

2. Locate the aorta for retrograde perfusion. Cut the ascending aorta just proximal to the three branches of the aortic arch. Cannulate the aorta to an 8 G cannula connected to the perfusion system. Use a piece of USP 0 silk suture to secure the aorta onto the cannula.
3. Carefully dissect the trachea, lungs, and epicardial fat from the heart. With a sharp forceps and dissection scissors, locate the mitral valve and carefully damage or remove one leaflet to prevent solution congestion in the left ventricle.
4. Submerge the heart in the glass-jacketed perfusion chamber horizontally with the anterior surface of the heart facing up for imaging. Secure the cannula to a piece of Sylgard with U-shaped pins onto the silicon bottom of the dish to prevent movement of the heart during mapping (**Figure 1D**). If desired, insert a small insect pin at the apex of the heart for stabilization.
5. Position electrocardiogram (ECG) electrodes in the bath on the right and left side of the heart. Fully submerge the electrodes in the bath and ensure they are not in contact with the heart surface to provide a volume-conducted ECG analogous to a Lead I configuration. Verify a normal Lead I ECG morphology. Adjust the flow (25 - 35 ml/min) to ensure that the aortic pressure is maintained at 60 - 70 mmHg.
6. Turn off the room lights and add 0.3 - 0.6 ml of blebbistatin stock solution (step 1.2) to the perfusate (final concentration 10 - 20 µM).
    1. Turn off room lights to avoid photoinactivation of blebbistatin. If necessary, a small spotlight or headlamp can be used to provide task lighting.
    Note: Blebbistatin is a myosin ATPase inhibitor and an excitation-contraction uncoupler. Because Fluo-5N loading requires extended (60 min) RT (hypothermia) conditions (see steps 2.8 – 2.10), cardiac energy production may be compromised. Therefore, addition of blebbistatin into the perfusate prior to dye loading may reduce the energy demand[16] and eliminate motion artifacts during subsequent optical recordings[17].

7. When contraction of the heart has ceased (10 - 15 min, verified on the aortic pressure recording), switch to the small-volume recirculating perfusion system (see step 1.4.1).

8. Prepare and load Fluo-5N AM loading solution: Add 0.25 ml of Fluo-5N AM stock solution (step 1.3) to 0.25 ml warm 20% Pluronic F127. Add 0.5 ml warm Tyrode's solution to the mixture, mix well, and add to the small-volume recirculating perfusion system.
9. Continuously monitor perfusion pressure and ECG and adjust the flow if necessary. Dye loading takes approximately 1 hr at RT. 45 min after loading has begun, turn on the circulating water bath to begin warming the perfusion system and perfusate to 37 °C.
10. After 60 min of Fluo-5N AM loading, switch the perfusion to the larger volume recirculating perfusion system (see step 1.4).
11. Dilute 50 µl of voltage sensitive dye RH237 stock solution (step 1.3) in 1 ml of warm Tyrode's solution and add slowly (over ~ 5 min) into an injection port proximal to the cannula.

3. Optical Mapping

1. During the final moments of dye loading, position the heart and focus the optical mapping cameras to ensure the appropriate field of view for the experiment.
2. If desired, place a bipolar pacing electrode on the epicardial surface of the heart for pacing.
3. Place a plastic or glass cover slip on the surface of the perfusion chamber to reduce motion artifact on the liquid surface that may be present due to recirculation of the perfusate.
   Note: As an alternative to a cover slip, use a clean plastic 50 mm Petri dish cover.
4. Focus the light guides to uniformly illuminate the surface of the heart with excitation light. Use blue light emitting diode (LED) light sources and further filter the light with a 475 - 495 nm bandpass filter (**Figure 1A**).
5. Collect emitted fluorescence with a macroscope and two complementary metal-oxide-semiconductor (CMOS) optical mapping cameras. Split the emitted light with a dichroic mirror at 545 nm.
   1. Long-pass filter the longer wavelength moiety, containing the V_m signal, at 700 nm. Band-pass filter the shorter wavelength moiety, containing the SR Ca^{2+} signal, from 502 - 534 nm (**Figure 1A**). Set the frequency of optical data acquisition to 0.5 - 1 kHz.
6. For each optical recording, first initiate the desired pacing protocol, turn on the excitation light, and collect 1 - 4 sec of data. If desired, synchronize the pacing protocols and data acquisition.
   Note: It is not possible to re-load the Fluo-5N AM, therefore the signal-to-noise ratio (SNR) will decrease throughout the experiment due to dye leakage from the SR. Limit experimental protocol to 1 - 2 hr to ensure high SNR values.
7. Following the experiment, wash the perfusion system in the sequence of DI water, 70% reagent alcohol, and again with DI water.
8. Filter each dataset with a spatial Gaussian filter (radius 3 pixels) using commercially available software packages according to the manufacturer's protocol.
9. If necessary, spatially align the V_m and SR Ca^{2+} data sets. As in Step 1.6, overlay images from each camera and verify that the anatomical structures of the heart (*i.e.*, coronary vasculature, edges of the atria or ventricles) or other items in the field of view (*i.e.,* cannula, pacing electrode) exactly overlap. If not, spatial alignment of the datasets is necessary.
10. Shift the top transparent image in the x- and y-direction until exact overlap is achieved, making note of the number of pixels the image must be shifted in each direction. The entire dataset corresponding to the top image (either V_m or SR Ca^{2+}) must then be shifted by the determined number of pixels in each direction to assure precise alignment between the V_m and SR Ca^{2+} datasets.

Representative Results

Figure 1A shows a schematic diagram of the optical configuration for dual V_m and SR Ca^{2+} mapping. With this setup, there is complete spectral separation of the V_m and SR Ca^{2+} signals (**Figure 1B**). A diagram of the dual-loop perfusion system used for Fluo-5N dye loading is illustrated in **Figure 1C**. **Figure 1D** shows the horizontal orientation of the heart in the perfusion dish. Representative V_m and SR Ca^{2+} optical traces during continuous pacing from different locations on the epicardial surface of the heart are shown in **Figure 2A**. The optical field of view with this setup is 31 mm x 31 mm and the orientation of the heart within the field of view is shown in **Figure 2B**. Activation maps are also shown in **Figure 2B** and are constructed by selecting the activation time of each pixel and plotting this value on an isochronal map (note the typical delay of ~ 8 - 10 msec between the V_m and SR Ca^{2+} activation maps). Importantly, key action potential (AP) properties such as AP rise time (from 10% to 90% of the amplitude [Trise]), and AP duration at 80% repolarization (APD_{80}), are not statistically different between hearts loaded with RH237 and Fluo-5N compared to those loaded with RH237 and Rhod-2 (Trise: 14.9 msec ± 1.9 msec vs. 14.2 msec ± 1.2 msec and APD_{80}: 155.3 msec ± 5.8 msec *vs.* 156.9 msec ± 5.7 msec for Fluo-5N and Rhod-2, respectively; p >0.05 for both; n = 8/group), indicating minimal effect of Fluo-5N or dye loading on the AP. **Figure 2C** demonstrates the ability to observe and quantify relative changes in diastolic SR Ca^{2+} load and systolic SR Ca^{2+} release amplitudes at various pacing cycle lengths. Only relative changes in these parameters can be quantified, as absolute calibration ($[Ca^{2+}]_{SR}$) of the signal in the intact heart is not possible.

This approach is particularly useful for characterizing and quantifying pathological SR Ca^{2+} handling, as is shown in **Figure 3**. Following application of the β-adrenergic receptor agonist isoproterenol (100 nM), clear diastolic Ca^{2+} leak from the SR is observed (red arrows, **Figure 3A**). If the leak is large enough, Ca^{2+}-mediated triggered activity may occur (**Figure 3B**).

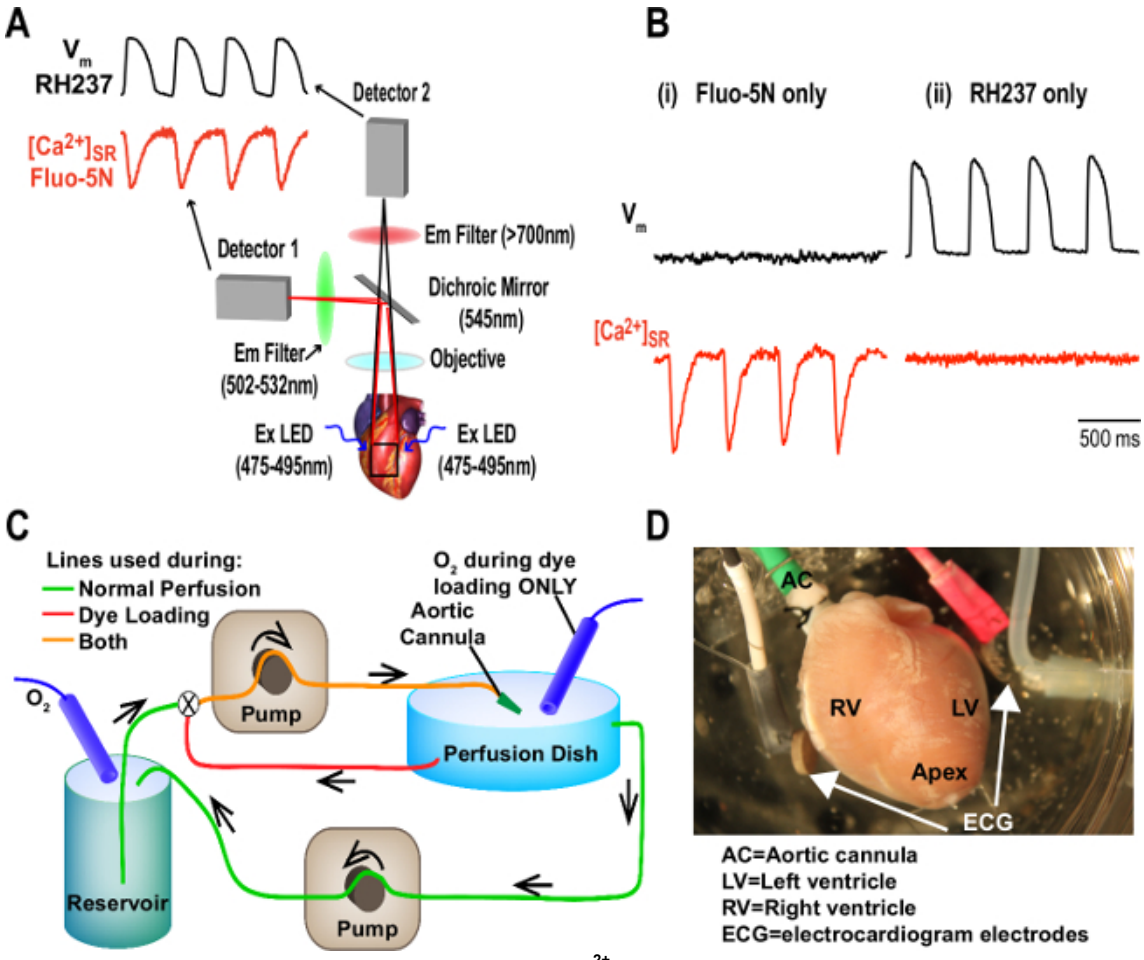

Figure 1. System Setup for Dual Mapping of V_m and SR Ca^{2+}. **(A)** Diagram of the optical setup, including necessary filters and dichroic mirror for proper spectral separation. **(B)** Imaging of hearts loaded with either Fluo-5N only (i) or RH237 only (ii) indicates complete spectral separation of the V_m and SR Ca^{2+} fluorescent signals. Ex: excitation, Em: emission. **(C)** Diagram of the dual-loop perfusion system for normal perfusion and Fluo-5N dye loading. **(D)** Image of a cannulated heart and ECG electrodes in the perfusion dish. Panels A and B reproduced with permission from Wang *et al.*[15]. Please click here to view a larger version of this figure.

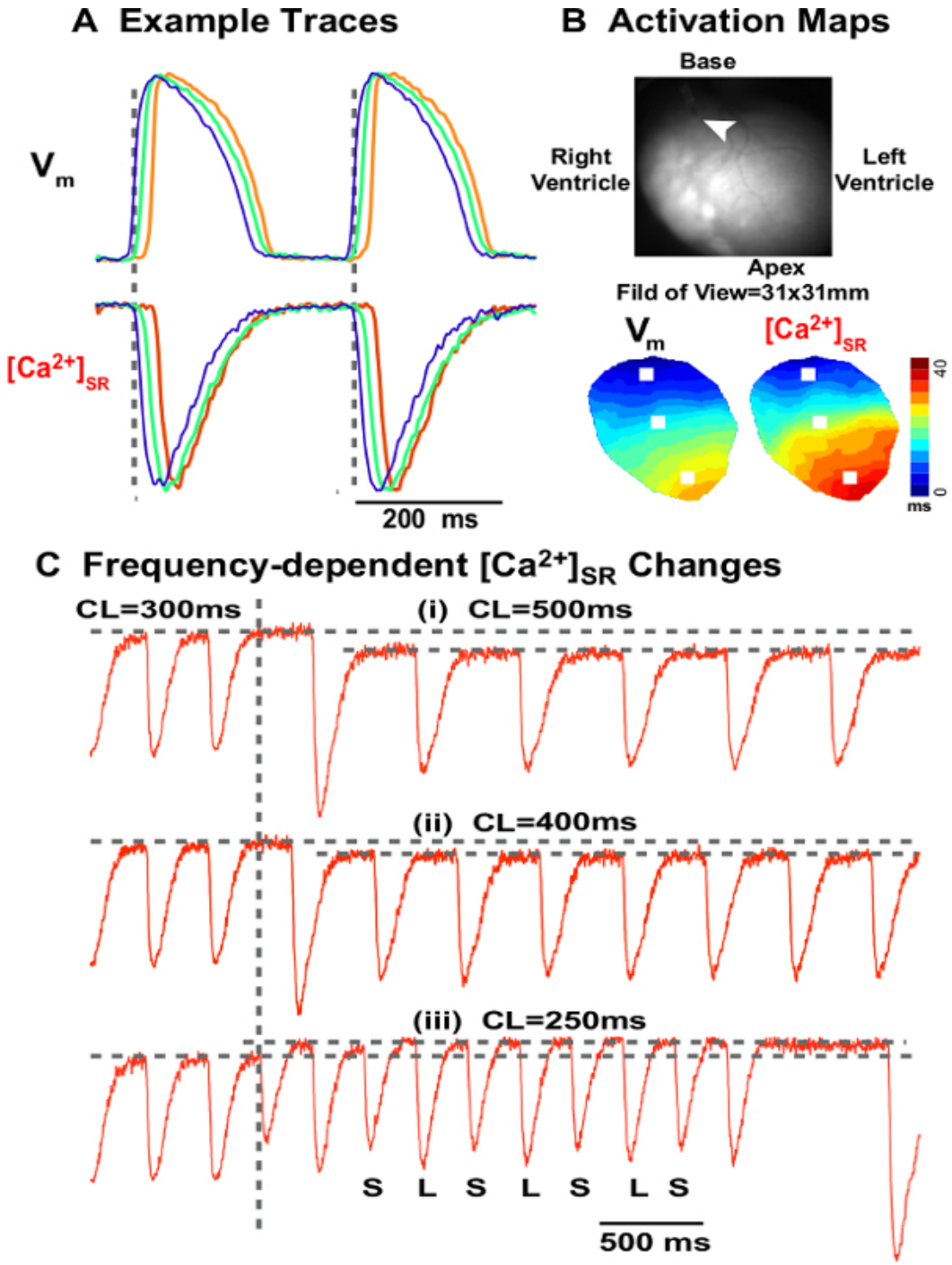

Figure 2. V_m and SR Ca^{2+} Optical Traces during Continuous and Non-continuous Pacing. **(A)** Representative V_m and SR Ca^{2+} optical traces during continuous pacing at a cycle length of 300 msec. Signals are shown from the individual locations indicated in **(B)**. Note the delay of ~ 8 - 10 msec between the V_m and SR Ca^{2+} activation times as is typical for normal excitation-contraction coupling. (B) Image of a heart within the mapping field of view (white arrowhead indicates pacing electrodes) and activation maps of V_m and SR Ca^{2+}. **(C)** SR Ca^{2+} optical traces demonstrating changes in diastolic SR Ca^{2+} load and the amplitude of SR Ca^{2+} release following abrupt changes in pacing rate. All traces start with pacing at CL = 300 msec. Pacing rate is then abruptly changed to CL = 500 msec (i), CL = 400 msec (ii), or CL = 250 msec (iii). When longer CLs are initiated (i - ii), a larger SR Ca^{2+} release first occurs (due to the longer diastolic interval and recovery of RyRs) and the diastolic SR Ca^{2+} load slightly decreases to a new steady-state (horizontal dashed line). When a shorter CL is initiated, however, a smaller SR Ca^{2+} release amplitude occurs (due to the shorter diastolic interval and incomplete recovery of RyRs) and diastolic SR Ca^{2+} load increases to a new steady-state (horizontal dashed line). Alternation of SR Ca^{2+} release amplitude also occurs at CL = 250 msec. S: small release amplitude, L: large release amplitude. (CL: cycle length) Please click here to view a larger version of this figure.

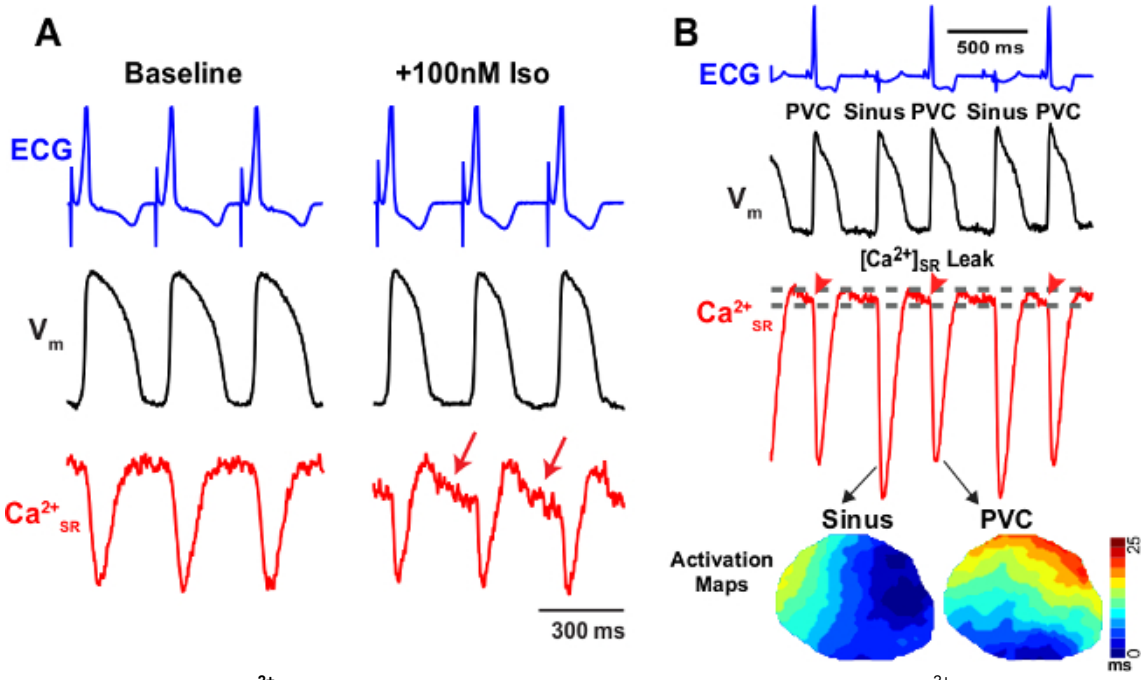

Figure 3. Diastolic SR Ca^{2+} Leak and Subsequent Triggered Activity. (A) ECG, V_m, and SR Ca^{2+} traces during baseline (left) and following treatment with 100 nM isoproterenol (Iso, right). With Iso, significant diastolic SR Ca^{2+} leak is observed (red arrows). SR Ca^{2+} signal amplitudes have been normalized before and after Iso. The sino-atrial node was ablated to allow for pacing CL = 300 msec in both cases. **(B)** If SR Ca^{2+} leak is large enough, it may trigger focal activity (premature ventricular complexes, PVCs). In this example, PVCs are interrupting the normal sinus rhythm. Please click here to view a larger version of this figure.

Discussion

The keys to successful Fluo-5N dye loading are the small-volume recirculating perfusion setup, which allows for a high Fluo-5N concentration without the need for large amounts of dye, the length of loading time (1 hr), and performing the loading at RT. If loading is performed at physiological temperatures, cellular enzymatic activity quickly cleaves the –AM tag when the dye crosses the cell membrane, trapping the dye molecules in the cytosol and not allowing them to cross the SR membrane. At RT, however, enzymatic activity is slowed and a sufficient amount of dye can cross both the cell membrane and SR membrane before the –AM tag is cleaved, trapping the dye in the SR.

Even with optimal loading conditions, however, signal amplitudes and fractional fluorescence changes (ΔF/F) with this approach will be lower than with traditional optical mapping of V_m and intracellular Ca^{2+} with high-affinity dyes. For example, with the optical setup used here that consists of LED excitation light, macroscopic objectives, large filters and dichroic mirror (5 cm x 5 cm), and CMOS cameras, fractional fluorescence changes (ΔF/F) are on the order of 1 - 2% for the Fluo-5N signal and 2 - 3% for the RH237 signal[15]. The fractional fluorescence will, of course, depend on the specific optical configuration, light source, and cameras of a particular setup, but even under optimal conditions the Fluo-5N signal is significantly lower than what is typically observed for recordings of intracellular Ca^{2+} with high-affinity dyes. For example, on the same setup, ΔF/F is on the order of 20 - 30% for Rhod-2 when green (545 nm) excitation light is used[18]. The green excitation light is also closer to the excitation peak of RH237, thus the ΔF/F of RH237 is also larger in this case, around 4 - 5%. Thus, for those labs already performing dual optical mapping of V_m and intracellular Ca^{2+}, care should be taken to optimize all components of the optical setup to ensure maximal ΔF/F for the Fluo-5N signal.

With this approach, investigators can precisely probe SR Ca^{2+} dynamics and its impact on V_m in ways that are not possible with existing approaches. For example, quantitative measurements of SR Ca^{2+} leak (as in **Figure 3A**) and the precise time constant of SR Ca^{2+} recovery (tau –a measure of SR Ca^{2+}-ATPase [SERCA] activity) can be performed. Using existing approaches for optical mapping of intracellular Ca^{2+} with high-affinity dyes, these measurements may be 'contaminated' with transmembrane Ca^{2+} flux (*i.e.*, contributions from L-type Ca^{2+} channels and the Na^{+}-Ca^{2+} exchanger). Furthermore, high-affinity Ca^{2+} indicators have inherently slower kinetics than low-affinity indicators[5], thus Fluo-5N signals are expected to report on precise SR Ca^{2+} movements with high fidelity and essentially no time delay between actual Ca^{2+} changes and corresponding changes in fluorescence.

The ability to investigate the detailed mechanisms by which SR Ca^{2+} release and reuptake contribute to arrhythmogenic phenomena is another advantage of this approach. For example, using this methodology, we recently reported how refractoriness of SR Ca^{2+} release contributes to the onset of Ca^{2+} alternans and that during ventricular fibrillation, SR Ca^{2+} release remains nearly continuously refractory[15]. This was the first report of SR Ca^{2+} dynamics during fibrillation and highlights the key advantage of SR Ca^{2+} imaging in the intact heart: spatially distinct and complex phenomena such as spatially discordant alternans and fibrillation can be studied. Unfortunately, this type of information cannot be gleaned from isolated cells, where fibrillation is not possible, or from methods that only record from a single location on the intact heart[12].

Furthermore, simultaneous optical mapping of V_m and free SR Ca^{2+} in the intact heart may provide a novel tool for assessing abnormal Ca^{2+} handling in pathological conditions, including heart failure. The abnormalities in SR Ca^{2+} regulation, including activation and termination of SR Ca^{2+} release, diastolic SR Ca^{2+} leak, and SR Ca^{2+} uptake - all major steps in Ca^{2+} cycling and all potentially altered in heart failure - can be

directly studied. In addition, this methodology could also be a useful tool for assessing the effects of novel therapeutic interventions, such as RyR stabilization[19] and SERCA modulation[20].

This approach is not without limitations, however, including the low ΔF/F as discussed above and the inability to precisely calibrate fluorescence values to exact $[Ca^{2+}]_{SR}$ concentrations. Unfortunately, variability in dye loading, non-uniform excitation and emission light due to the curved surface of the heart, and photo-bleaching and dye leak from the SR throughout the course of the experiment make it extremely difficult to calibrate Fluo-5N signals to exact $[Ca^{2+}]_{SR}$ in the intact heart. Previous *in vitro* studies in physiological buffer solutions and permeabilized isolated myocytes have reported such calibrations and have indicated that changes in Fluo-5N fluorescence are proportional to changes in $[Ca^{2+}]_{SR}$ and the fluorescence is not expected to saturate at physiological $[Ca^{2+}]_{SR}$ levels[11]. Thus, investigators should choose the method of Ca^{2+} mapping (low- or high-affinity dye) based on their specific experimental objectives and physiological investigations.

Disclosures

The authors declare that they have no competing financial interests.

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