Part 1
Electrophysiology

1 Measurement of calcium transient *ex vivo*

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Introduction

Intracellular calcium is a key regulatory signal for cardiomyocyte contraction and has become increasingly recognized as an important mechanism of electrical instability in the heart (arrhythmias) [1]. Measuring intracellular calcium in heart tissue is based on established techniques for measuring intracellular calcium levels in isolated myocytes using fluorescent indicators [2]. However, in tissue preparations additional factors need to be accounted for. Nonetheless, the advantages are: measurements can be performed while cardiomyocytes are in their native setting; it is possible to account for regional heterogeneities of cellular function as they exist normally and in disease conditions; and calcium-mediated arrhythmias can be investigated. The present chapter focuses on measuring intracellular calcium with high temporal and spatial resolution from intact heart preparations.

Preparation and fluorescent indicator loading

Measuring intracellular calcium in heart preparations is most effective when performed *ex vivo*, to provide efficient fluorescent indicator delivery, light excitation and emission collection. Efficient and uniform delivery of the fluorescent indicator is key; accordingly, perfusion in whole hearts (e.g. Langendorff) will provide the best result in this regard. Perfusion of tissue samples (e.g. left ventricular wedge preparations [3], isolated atria [4]) is also possible but careful attention must be paid to the location of the perfusion bed relative to the imaging field of view. Loading of calcium fluorescent indicators by superfusion is difficult in large (i.e. thick-walled) preparations when diffusion to deeper cell layers is limited; however, superfusion of smaller (i.e. thin-walled) preparations, such as the isolated zebrafish heart, is feasible [5].

In general, most calcium-sensitive fluorescent indicators that have been used in isolated myocytes can also be used in *ex vivo* tissue preparations. However, in tissue the cell permanent or acetoxymethyl (AM) ester form of the fluorescent indicator should be considered. AM indicators, being uncharged and lipophilic, readily penetrate the cell membrane. Once inside the cell, esterases liberate the charged fluorescent indicator by hydrolysis, which is then less likely to cross the cell membrane. Many calcium fluorescent indicators are available in the AM form and can also be used in *ex vivo* preparations, such as Indo-1 [6,7], Rhod-2 [8], Fluo-4 [9], and Fura-2 [5]. The choice of fluorescent indicator depends on several factors, the main being the wavelengths at which peak excitation and emission occur. With some fluorescent indicators, emission (Indo-1) or excitation (Fura-2) occurs at distinct wavelengths depending on whether calcium is bound or not, which can be used to aid calibration (see section Calibration in this chapter). The affinity

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of the fluorescent indicator for calcium (i.e. K_d) must also be taken into account. Typically, K_d is chosen within the range of expected calcium concentration to enable sufficient sensitivity without saturation. Unlike voltage-sensitive fluorescent indicators, special attention should be given to the concentration of the calcium fluorescent indicator used. If the concentration used is too large (>5 μM) intracellular calcium levels could be unintentionally reduced by the buffering capacity of the indicator.

Several co-agents can be administered with the fluorescent indicator to improve loading and fluorescent measurements *ex vivo*. Pluronic F-127 is a detergent that can aid the dispersion of the lipophilic AM form of fluorescent indicators. In addition, when inside the cell the indicator can be actively removed from the cytosol by anion transporters, which can quickly reduce signal intensity. Probenecid can be used to block this transporter; however, its effects on cellular physiology should also be weighed. Finally, it is very difficult to accurately image fluorescence from contracting heart tissue, thus, inhibiting contraction is essential. It is possible to mechanically restrain the preparation or use ratiometric techniques [10] to reduce some motion artifact, but currently the most common and effective method is to use pharmacological agents that maintain electrical excitation and calcium release yet inhibit mechanical contraction, such as 2,3-butanedione monoxime, cytochalasin-D, and blebbistatin. However, these agents should be used with caution because they can influence calcium regulation and electrophysiological function [11,12].

Optical setup

Shown in Figure 1.1 (Panel A) is an example of an optical mapping system in its simplest configuration to measure intracellular calcium from the Langendorff perfused heart using Indo-1 [7]. One of the advantages of using optical mapping techniques to measure cellular function is that a wide range of preparation size (i.e. field of view) can be accommodated. However, there are several limitations that should be considered. Measuring fluorescence at the level of the whole heart is best achieved using macroscopic objectives (e.g. standard photography lens). In Figure 1.1 (Panel A), two separate camera lenses are optically aligned to face each other in a tandem lens configuration, which significantly improves light collection. Optical magnification is determined by the focal length ratio of the back ("B") lens to the front lens ("F"). For example, an 85-mm front lens with a 105-mm back lens yields a magnification of 1.24×. High numerical aperture lenses are optimized for maximal light collection and are preferred. Macroscopic systems based on standard photography lenses are suitable for imaging fields (i.e. preparations) that range from a few mm (mouse) to a few cm (canine isolated atrial preparations). Larger preparations would require significant demagnification because total detector size is typically not much larger than 1–2 cm. However, standard photography lenses are not optimized for significant demagnification and may produce a fading out of the image at the periphery, known as vignetting. For preparations <1 mm^2 (e.g. embryonic hearts), a standard fluorescent microscope may be better suited.

Measuring fluorescence *ex vivo* requires a light source for fluorescence excitation, a light detector for fluorescence emission, and a judicious selection of optical filters to optimize light transmission. The light source and optical filters are determined by the fluorescent indicator chosen. For example, shown in Figure 1.1 (Panel A) is UV excitation light (Hg lamp, 365 nm) for Indo-1. In contrast, Fluo-4 is excited with 488 nm light; thus, a quartz tungsten halogen (QTH) lamp that produces light in the visible range will suffice. Light sources with broad output spectra require excitation filters (Ex Filter, Figure 1.1) to pass only wavelengths that maximally excite the fluorescent indicator and do not overlap with the emission wavelength. Filter specifications such as bandwidth, peak transmission, and blocking will all determine the amount of light that excites the fluorescent indicator and will, thus, impact signal fidelity. Modern LED technology can provide an excellent alternative to Hg and QTH light sources given their low cost, low noise, and high power output [13]. Moreover, the wide availability of quasimonochromatic LEDs may obviate the need for an excitation filter.

Light collected from the preparation includes excitation light that reflects off the preparation as well as fluorescence emission. The amount of excitation light is much larger than fluoresced light,

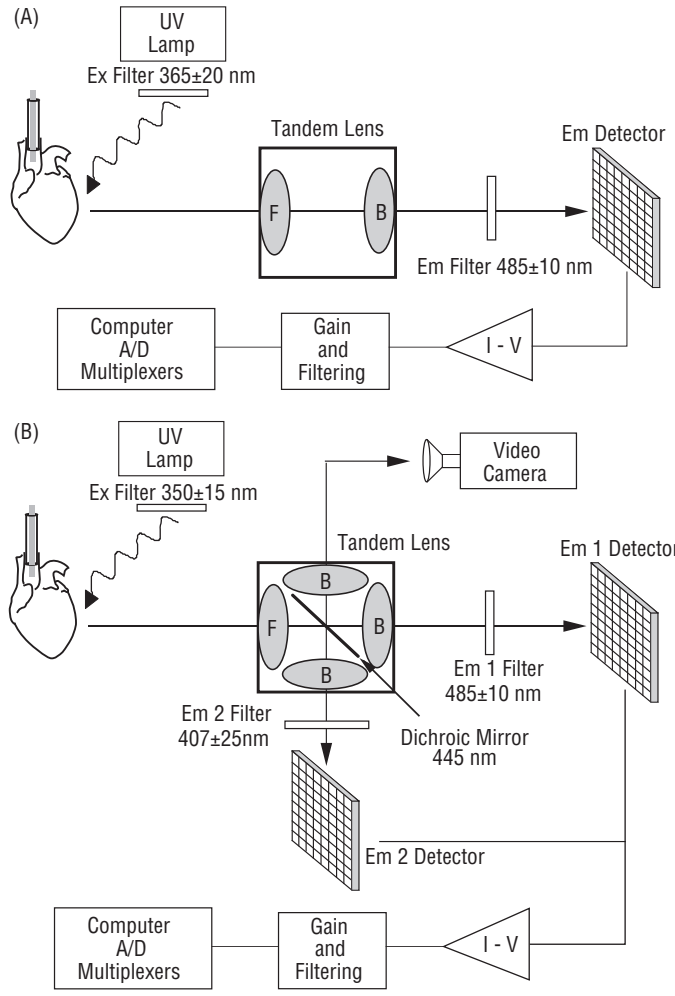

Figure 1.1 System diagram for measuring un-calibrated (A) or calibrated (B) intracellular calcium from intact heart preparations. (A) shows excitation light from an Hg arc lamp directed to the preparation. Fluorescence is collected by a tandem lens assembly consisting of a front (F) and back (B) camera lens. Fluoresced light is allowed to pass through an emission filter (Em Filter) to a detector array (Em Detector). Signals corresponding to each pixel channel are amplified, filtered, and digitized for analysis. (B) shows filtered excitation light (350 nm) from an Hg arc lamp specifically for dual wavelength emission. Fluorescence is collected by a tandem lens assembly consisting of four camera lenses (1 front, 3 back). A dichroic mirror (445 nm) passes fluorescence of longer wavelengths to an emission filter (Em 1 Filter) and detector array (Em 1 Detector) and reflects fluorescence of shorter wavelengths to a second emission filter (Em 2) and detector array (Em 2 Detector). To view the preparation, the dichroic mirror is rotated clockwise by exactly 90° to reflect an image of the preparation to a standard video camera.

requiring the need for an emission filter (Em Filter, Figure 1.1 Panel A) to block excitation light from reaching the detector (Em Detector, Figure 1.1 Panel A). The bandwidth, center wavelength, and maximum transmittance of the emission filter must be taken into account to maximize the amount of fluorescence passed and excitation light blocked.

A variety of detector technologies with sufficient sensitivity and speed (e.g. frame rate) are capable of accurately measuring fluorescence from *ex vivo* preparations. Photomultiplier tubes have sufficient fidelity; however, being a single detector they can only measure fluorescence from multiple sites if the preparation is scanned site-by-site in a sequential

manner with excitation light (e.g. laser) [14]. On the other hand, detector arrays, such as photodiode arrays (PDA), charge-coupled devices (CCD), and complementary metal–oxide–semiconductor (CMOS) sensors can capture fluorescence from hundreds to thousands of sites simultaneously when excitation light illuminates the entire imaging field [15]. These detector arrays are based on similar technology, except for their output format. The PDA output is a parallel stream of voltage signals corresponding to each recording location where each signal needs to be, conditioned (amplified and filtered), multiplexed, and digitized (Figure 1.1). This offers flexibility in signal conditioning and sampling speed in hardware, but with the added cost of a specialized design. CCD and CMOS sensors on the other hand, output a single serial stream of data that is digitized and multiplexed "on chip". This limits signal conditioning to mostly software methods, but offers the advantage of being "plug and play". Currently, the speed and signal fidelity of these arrays are similar; however, CCD and CMOS arrays offer spatial resolutions that can be orders of magnitude higher (more pixels) than PDAs.

Calcium recordings from an intact heart using currently available detectors should not required further signal processing (e.g. filtering) to achieve acceptable signal fidelity (Figure 1.3A). However, in some situations the signal source may be very small (e.g. embryonic hearts, monolayers), which may result in the appearance of excessive noise. Typically, this noise is high frequency and can be filtered out in the time and/or space domain. In the time domain, the signal from each "pixel" can be low-pass filtered in software using standard digital signal processing techniques. In the space domain, signals from neighboring pixels can be averaged together (i.e. binned) in either hardware or software. The choice of filter characteristics for calcium transients may be different than that for action potentials [16]. For example, action potentials have a much faster rise time than calcium transients. However, action potentials are naturally "smoothed" in space due to electrotonic forces (i.e. space constant), but calcium transients are not.

Confocal microscopy can be used to image intracellular calcium from heart tissue [9,17]. The field of view is limited to several cells; however, measured fluorescence only arises from cells within the confocal plane. As such, subcellular events within single cells (calcium sparks, waves) can be resolved, as well as heterogeneities in calcium cycling between neighboring cells. Confocal imaging, however, requires exquisite control of motion, and depth of penetration is very limited depending on the technology used (e.g. single or two photon technology). Finally, the first reported measurements of intracellular calcium *ex vivo* were achieved by attaching a fiber-optic cable to the epicardial surface of a beating rabbit heart [18]. The advantage of such a system is that the fiber-optic cable can be sutured to the heart surface so it can move with the beating heart, thus minimizing motion artifact.

Calibration

Intracellular free calcium concentration and its change during the heartbeat have very important physiological meaning. However, the fluorescence intensity measured can depend on factors unrelated to calcium levels such as the intensity of excitation light, optical transmission, and fluorescent indicator washout. Thus, to quantify intracellular calcium concentration, the fluorescence signal needs to be calibrated to absolute calcium levels. Standard procedures have been developed for isolated cells; however *ex vivo* methods are much more complex due to spatial heterogeneities and limited control of the cellular environment.

A fluorescent indicator that exhibits a dual spectral response or a shifting of excitation wavelength with free calcium concentration is ideal for calibration. For example, fluorescence from Indo-1 will increase with increasing intracellular calcium when measured at ~407 nm but will decrease with increasing calcium when measured at ~485 nm. By calculating the ratio of fluorescence measured at each wavelength it is possible to normalize for factors that influence fluorescence that are unrelated to calcium levels (e.g. excitation light intensity, fluorescent indicator washout). Shown in Figure 1.1 (Panel B) is an example of an optical mapping system designed to measure the fluorescence ratio of Indo-1. This setup is similar to that shown in Panel A, except with several important changes. First, fluorescence emission of Indo-1 at both peak wavelengths is best achieved by ~350 nm excitation.

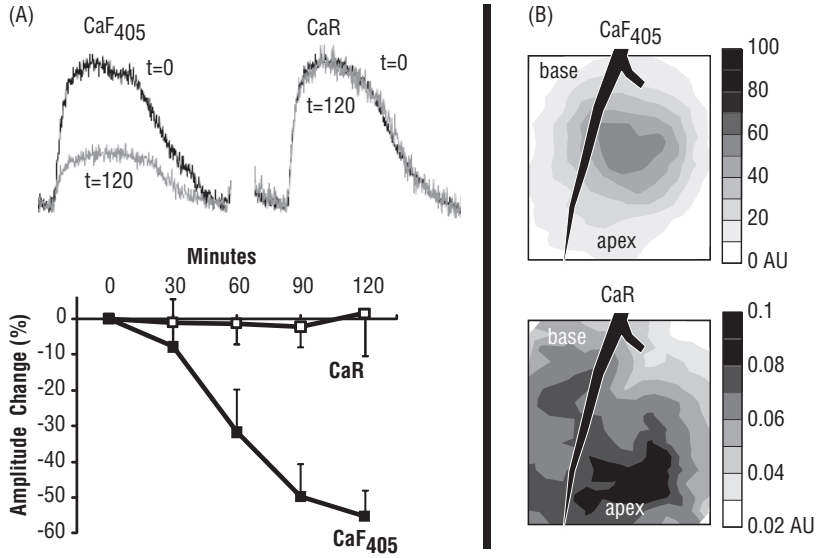

Figure 1.2 (A) shows calcium fluorescence (CaF_{405}) and ratio (CaR, background corrected CaF_{405}/CaF_{485}) transients from the same site over a 2-h period. As demonstrated in the traces (top) and graph (bottom), CaF_{405} significantly decreased, but CaR did not (error bars show SD). (B) shows contour maps of CaF_{405} and CaR amplitude measured across the epicardial surface of the intact heart preparation. CaF_{405} exhibited a circular "bull's-eye" pattern similar to the pattern of excitation light (not shown). In contrast, CaR reflects the physiologic heterogeneity of calcium transient amplitude that is independent of excitation light intensity and heterogeneity.

Measuring fluorescence simultaneously at two separate wavelengths requires two detectors. Therefore, fluorescence emission corresponding to each peak wavelength needs to be split into two light paths. A dichroic mirror placed between the tandem lenses passes light of longer wavelengths (>445 nm) and reflects light of shorter wavelengths. Light directed to each detector is then band-pass filtered to optimally detect emission at each peak wavelength (Em 1 Filter, Em 2 Filter). It is important that both detectors (Em 1 Detector, Em 2 Detector) are in perfect alignment to ensure a one-to-one correspondence (i.e. registration) between the field of view and both detectors. Finally, before the fluorescent indicator is loaded, background fluorescence intensity at each wavelength is measured and then subtracted from all subsequent recordings (i.e. background corrected) after the fluorescent indicator is loaded.

Shown in Figure 1.2 are examples of how the ratio of Indo-1 fluorescence can correct for changes that occur over time due to dye washout (Figure 1.2A) and heterogeneities of excitation light and indicator loading (Figure 1.2B). Calcium transient amplitudes measured *ex vivo* from Indo-1 fluorescence directly (CaF) can significantly decrease by more than 50% over 2 hours. However, the background corrected ratio of Indo-1 fluorescence (CaR) remains unchanged over the same period. Spatial heterogeneities of excitation light and indicator can also significantly influence the calcium transient amplitude measured *ex vivo*. Shown in Figure 1.2B is a contour map of calcium transient amplitude measured across the epicardial surface of the guinea pig heart as measured directly by fluorescence (CaF, top) or the ratio of fluorescence (CaR, bottom). CaF reveals a bull's-eye pattern that mostly depends on the pattern of excitation light directed to the heart. In contrast, CaR reveals a pattern that is dependent on physiological heterogeneities (largest amplitude at apex) that remains the same even if excitation light is reduced or moved to a different position. However, CaR alone cannot account for the nonlinear response of fluorescence to calcium that occurs at concentrations far from K_d (e.g. saturation).

Calibrating the ratio (CaR) to calcium levels *ex vivo* can be performed as it is done in isolated cells using:

$$[Ca^{2+}]_i = K_d S_2 \left\{ \frac{R - Rmin}{Rmax - R} \right\}$$

where R is CaR (as described here) and S_2 is the ratio of fluorescence at the longer peak wavelength (485 nm) measured in absence of calcium (minimum) and in the presence of saturating calcium (maximum). Calibration constants (e.g. K_d) can be borrowed from that used in isolated cells; however, this may not be the same in tissue and there are several factors that complicate the calibration procedure [19]. In addition, R_{min} and R_{max}, the ratios that correspond to maximum and minimum calcium respectively, can be difficult to measure. For example, the reagents that are used to minimize (zero) intracellular calcium (e.g. ionophores) can only be used at the end of an experiment, and these can change the shape of (e.g. shrink) the preparation making it difficult to measure R_{min} at the same recording sites where calcium transients were measured during an experiment. Nevertheless, accurate calibration *ex vivo* is possible [6,7].

Analysis and interpretation

The signal morphology of intracellular calcium (e.g. calcium transient) measured *ex vivo* is similar to that measured from isolated cells *in vitro*. Normally, calcium levels are low at rest (diastole, ~100–200 nM), but several milliseconds after electrical excitation (action potential upstroke) calcium levels quickly increase to levels close to 1000 nM (systole), corresponding to the release of calcium from the sarcoplasmic reticulum (SR) by the ryanodine receptor (RyR). Following systole, calcium levels return to diastolic levels due to, mostly, the uptake of calcium back into the SR by SR calcium ATPase and extrusion of calcium from the cell by the sodium–calcium exchanger. To quantify the rate of calcium removal from the cytosol, the decay phase can be fit to an exponential function, or the duration of the calcium transient can be measured at a predefined amplitude percentage (e.g. duration at 90% amplitude). Unlike calcium measured from single cells *in vitro*, calcium measured *ex vivo* typically originates from an aggregate of cells. Thus, it is possible that if significant heterogeneities exist between cells or single cell events occur (e.g. calcium sparks and waves), the measured response may be significantly different than measured from an individual cell.

One of the advantages of measuring calcium *ex vivo* is that regional heterogeneities can be determined and linked to normal and abnormal physiology. For example, we have previously shown that the decay phase of the calcium transient is significantly slower near the endocardium compared to the epicardium [3]. Shown in Figure 1.3A is an example of calcium transients measured from the endocardium (ENDO) and epicardium (EPI) of the intact canine LV wedge preparation. The decay phase of the calcium transient, when fit to a single exponential, reveals a shorter time constant (135 ms) near the EPI compared to the ENDO (250 ms). These heterogeneities have been linked to the occurrence of cardiac alternans [3] and triggered arrhythmias [20]. In addition, we have also shown in the guinea pig heart that calcium transient amplitude is larger near the apex of the heart compared to the base (Figure 1.2B), which is consistent with a stronger contraction near the apex compared to the base.

Abnormal calcium regulation has become increasingly recognized as an important mechanism of arrhythmia. Accordingly, a very important advantage of measuring calcium *ex vivo* is that calcium-mediated arrhythmias can be fully investigated. For example, arrhythmias can be caused by spontaneous release of calcium from internal stores. Such events are well characterized as calcium sparks and waves in isolated cells [21]. These manifest as a membrane depolarization that, if large enough, can initiate multiple extra beats (extrasystoles) [4,22]. Shown in Figure 1.3B is an example of spontaneous calcium release measured *ex vivo* in a rat heart with myocardial infarction. Immediately following four paced calcium transients, stimulation is halted and activity is measured as spontaneous calcium release (arrows) and full calcium transients (asterisks). Similar studies in canine demonstrate the focal nature

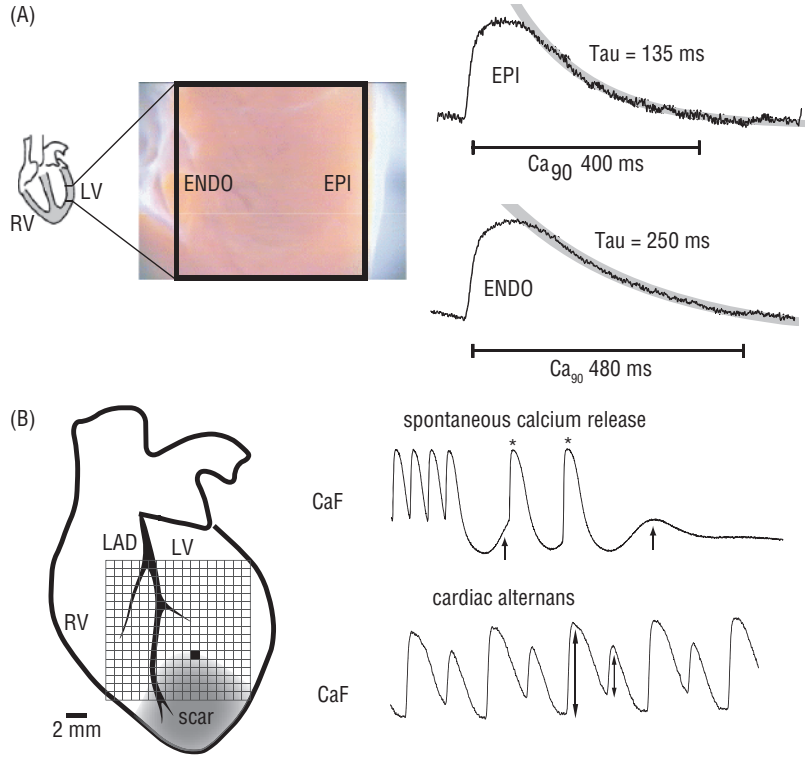

Figure 1.3 (A) shows an image of the LV canine wedge preparation with the mapping field superimposed. Calcium transients (right) reveal that the return of intracellular calcium to diastolic levels is slower at the endocardium (ENDO) compared to the epicardium (EPI), as indicated by the exponential fit (i.e. bold gray line) to the decay phase of the calcium transient and by the calcium transient duration (i.e. CaF_{90}). (B) shows examples of calcium-mediated arrhythmia substrates present in an intact rat heart with myocardial infarction. Calcium transient recordings (CaF) from the mapping field (grid) show spontaneous calcium release (arrows) associated with un-stimulated beats (asterisks), and alternans of calcium transient amplitude (double arrows).

of spontaneous calcium release in tissue [20] (Video clip 1.1). However, CaF recordings and in particular spontaneous calcium release measured *ex vivo* (what we call an m-SCR) must be interpreted with caution. For example, it is not clear if m-SCR activity represents calcium waves and/or calcium sparks. Abnormal calcium regulation has also been linked to reentrant arrhythmias. For example, our laboratory [23] and others [24] have linked calcium transient alternans, a beat-to-beat fluctuation of calcium transient amplitude, to repolarization alternans, an important mechanism of sudden cardiac death [25]. Shown in Figure 1.3B is an example of calcium transient alternans measured *ex vivo* in a rat heart with myocardial infarction. The arrows indicate alternation in the calcium transient amplitude on a beat-by-beat basis.

Set-by-step procedure

For details of the procedure see Katra *et al.* [7].

1. Optical setup (for Indo-1 AM):
   - **a.** excitation 365 nm, or 350 nm for ratio;
   - **b.** emission 485 nm, or 485 nm + 405 nm with 445 nm dichroic mirror for ratio.
2. Prepare tissue sample and perfuse/ superfuse (e.g. Langendorff).
3. Monitor ECG, and perfusion pressure and flow.
4. Confirm preparation viability and stability (~10 minutes).

5. Position preparation within mapping field and adjust focus.
6. For ratio, record background fluorescence in the absence of calcium indicator. At this point, the preparation position must be maintained (or accurately restored later) for all subsequent recordings.
7. Administer calcium-sensitive dye (~30-45 minutes) prepared in perfusion/ superfusion solution at ~5 µM final concentration, initially dissolved in a 0.5 mL solution of DMSO and Pluronic (Molecular Probes, Inc. Eugene, OR) 20% wt/vol.
8. For ratio, a 20-minute washout period to remove unhydrolyzed dye.
9. Administer mechanical–electrical uncoupler (e.g. blebbistatin) continuously.
10. Confirm pacing electrode placement and threshold strength.
11. Perform experimental protocol.

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