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A technical review of optical mapping of intracellular calcium within myocardial tissue

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1Department of Biomedical Engineering, The George Washington University, Washington, District of Columbia; 2Université de Bordeaux, Centre de Recherche Cardio-Thoracique de Bordeaux U1045, Bordeaux, France; 3Institut National de la Santé et de la Recherche Médicale, Centre de Recherche Cardio-Thoracique de Bordeaux U1045, Bordeaux, France; and 4L’Institut de Rythmologie et Modélisation Cardiaque LIRYC, Université de Bordeaux, Bordeaux, France

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Jaimes R 3rd, Walton RD, Pasdois P, Bernus O, Efimov IR, Kay MW. A technical review of optical mapping of intracellular calcium within myocardial tissue. Am J Physiol Heart Circ Physiol 310: H1388–H1401, 2016. First published March 25, 2016; doi:10.1152/ajpheart.00665.2015.—Optical mapping of Ca2+-sensitive fluorescence probes has become an extremely useful approach and been adopted by many cardiovascular research laboratories to study a spectrum of myocardial physiology and disease conditions. Optical mapping data are often displayed as detailed pseudocolor images, providing unique insight for interpreting mechanisms of ectopic activity, action potential and Ca2+ transient alternans, tachycardia, and fibrillation. Ca2+-sensitive fluorescent probes and optical mapping systems continue to evolve in the ongoing effort to improve therapies that ease the growing worldwide burden of cardiovascular disease. In this technical review we provide an updated overview of conventional approaches for optical mapping of Ca2+ within intact myocardium. In doing so, a brief history of Ca2+ probes is provided, and nonratiometric and ratiometric Ca2+ probes are discussed, including probes for imaging sarcolemmal calcium cycling and SR Ca2+ release and SR Ca2+ resequestration (55). Optical mapping is often used to image the fluorescence of a Ca2+ probe administered to myocardial tissue to measure spatial changes in the kinetics and amplitude of Ca2+ transients (52, 70, 72, 106). Additional insights into the mechanisms of EC coupling are provided when optical mapping of a Ca2+ probe is combined with other modes of optical mapping, such as imaging of a potentiometric dye to measure action potentials (20), and is particularly useful for studying pathological mechanisms, including arrhythmogenesis, within living myocardial tissue (32, 49, 78).

There are excellent reviews of Ca2+ cycling in cardiac cells (10, 48, 117) and Ca2+ imaging of single cardiac myocytes (13). Salama and Hwang have also provided a detailed review of Ca2+ optical mapping that focuses on dual mapping of action potentials and Ca2+ transients (82). The objective of the present review is to provide an updated overview of conventional approaches for optical mapping of Ca2+ within intact myocardium. In doing so, a brief history of Ca2+ probes is provided, and nonratiometric and ratiometric Ca2+ probes are discussed, including probes for imaging SR Ca2+ and probes compatible with potentiometric dyes for dual optical mapping. Typical measurements derived from optical Ca2+ signals are explained, and the analytics used to compute them are presented. Last, recent studies using Ca2+ optical mapping to study arrhythmias, heart failure, and metabolic perturbations are summarized.

Calcium (Ca2+) is the primary ion associated with the activation and modulation of contraction in cardiac myocytes (10). Intracellular calcium (Ca2+) measurements from myocardial tissue are critical for understanding the physiology of excitation-contraction (EC) coupling and mechanisms of disease. High-fidelity Ca2+-sensitive fluorescent probes provide for high temporal resolution measurements of Ca2+ transients, which are generated by processes associated with a sudden increase in cytosolic Ca2+ and subsequent reduction of cytosolic Ca2+. For the most part, the Ca2+ transient represents sarcoplasmic reticulum (SR) Ca2+ release and SR Ca2+ resequestration (55). Optical mapping is often used to image the fluorescence of a Ca2+ probe administered to myocardial tissue to measure spatial changes in the kinetics and amplitude of Ca2+ transients (52, 70, 72, 106). Additional insights into the mechanisms of EC coupling are provided when optical mapping of a Ca2+ probe is combined with other modes of optical mapping, such as imaging of a potentiometric dye to measure action potentials (20), and is particularly useful for studying pathological mechanisms, including arrhythmogenesis, within living myocardial tissue (32, 49, 78).

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**Ca^{2+} Fluorescence Probes**

A variety of light-emitting compounds have been used to image intracellular Ca^{2+} within myocardial tissue. In recent work, these include ratiometric and nonratiometric probes, genetically expressed probes, and low-affinity Ca^{2+} probes to image SR Ca^{2+} (Table 1). Aequorin was one of the first light-emitting compounds used to study Ca^{2+} in muscle cells (12, 25, 79). It was isolated from the jellyfish Aequorea victoria and is bioluminescent blue when bound to Ca^{2+}, as Shimomura et al. reported in 1963 (86). Aequorin is a photoprotein that emits light by an intramolecular reaction and does not require optical excitation, minimizing complications of cellular autofluorescence. It does not localize to intracellular compartments but also does not pass freely across the sarcolemma for intracellular measurements (25). Other limitations are that it emits less than one photon per molecule, and its light-emitting component (coelenterazine) is irreversibly consumed to produce light, requiring that it be added to the media to maintain luminescence (67).

In the 1980s Tsien developed fluorescent probes based on tetracarboxylate chelators for Ca^{2+} that could enter cells by passing through the sarcolemma and not be consumed when emitting light (103), two properties required for imaging Ca^{2+} from myocardial tissue. Quin 2, a highly selective Ca^{2+} indicator derived from fluorescent quinoline (100, 101), was one of these early probes. Esterification (explained below) enabled quin 2 to enter and remain within cells. Its fluorescence (339 nm excitation, 492 nm emission) increased fivefold between quin 2 to enter and remain within cells. Its fluorescence (339 nm excitation, 492 nm emission) increased fivefold between

The drawbacks of the Ca^{2+}-binding and -unbinding kinetics of a probe to Ca^{2+} concentration changes. Kd should be carefully considered when selecting a Ca^{2+} probe to minimize the effect of Ca^{2+}-binding and -unbinding kinetics on accurate measurements of changes in Ca^{2+}. Rhod 2 has a Kd of 570–710 nM, a range that provides for acceptable measurements of Ca^{2+} transients for typical animal species used to study cardiac physiology (43). Kd values for popular Ca^{2+} probes with their excitation/emission peaks and quantum yields are listed in Table 1. Recent work by Kong and Fast (43)

### Table 1. Properties of common Ca^{2+} probes used to study cardiac physiology

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Excitation, nm</th>
<th>Emission, nm</th>
<th>Quantum Yield</th>
<th>Kd, nM*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ratiometric</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fura 2</td>
<td>362 (330–393)</td>
<td>335 (311–358)</td>
<td>512 (476–590)</td>
<td>505 (467–559)</td>
</tr>
<tr>
<td>Fura 4F</td>
<td>362 (330–393)</td>
<td>335 (311–358)</td>
<td>512 (476–590)</td>
<td>505 (467–559)</td>
</tr>
<tr>
<td>Indo 1</td>
<td>349 (315–372)</td>
<td>331 (303–354)</td>
<td>485 (441–538)</td>
<td>410 (375–488)</td>
</tr>
<tr>
<td><strong>Nonratiometric</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhod 2</td>
<td>553 (535–568)</td>
<td>576 (561–601)</td>
<td>516 (502–541)</td>
<td>0.102 (65)</td>
</tr>
<tr>
<td>Fluo 4</td>
<td>194 (178–307)</td>
<td>516</td>
<td>516</td>
<td>9.700</td>
</tr>
<tr>
<td>Fluo 2MA</td>
<td>194</td>
<td>516</td>
<td>516</td>
<td>6.700</td>
</tr>
<tr>
<td>Fluo 4F</td>
<td>194</td>
<td>516</td>
<td>516</td>
<td>6.700</td>
</tr>
<tr>
<td>Fluo 2LA</td>
<td>194</td>
<td>516</td>
<td>516</td>
<td>6.700</td>
</tr>
<tr>
<td><strong>Genetic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCaMP2</td>
<td>485–487</td>
<td>508</td>
<td>0.53 (3); 0.93 (96)</td>
<td>545 ± 32 (3)</td>
</tr>
<tr>
<td>GCaMP3</td>
<td>497</td>
<td>500–550</td>
<td>0.65 (3)</td>
<td>405 ± 9 (3)</td>
</tr>
<tr>
<td><strong>SR Ca^{2+}</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mag-fura 2</td>
<td>362 (330–393)</td>
<td>335 (311–358)</td>
<td>512 (476–590)</td>
<td>505 (467–559)</td>
</tr>
<tr>
<td>Mag-fluo 4</td>
<td>494</td>
<td>516</td>
<td>22,000</td>
<td></td>
</tr>
<tr>
<td>Fluo 5N</td>
<td>494</td>
<td>516</td>
<td>90,000</td>
<td></td>
</tr>
</tbody>
</table>

Wavelengths at the spectral peaks of excitation and emission are provided followed by the half-maximum bandwidth in parentheses, when available. *Dissociation constants (Kd) are listed as a range: the lower value is dye in solution and the higher value is the dye in a cell.
provided an experimental survey of probes with a range of $K_d$ and demonstrated that if a probe’s Ca$^{2+}$/H$^{11001}$ affinity is too high ($K_d$ > 570 nM) then the CaD transient duration could be misrepresented. For example, for long pacing cycle lengths in the swine left ventricle, CaD measured at a 50% level of recovery was almost 81% longer with high-affinity probes (fluor 4 and fluor 2MA) than with low-affinity probes (fluor 4FF and fluor 2LA) (43). Very-low-affinity probes with extremely high $K_d$ ($K_d$ > 22–90 M), such as fluor 5N (75, 84, 87) and ratiometric mag-fura 2 (33), have been used to study SR Ca$^{2+}$/H$^{11001}$ in isolated cardiac myocytes. SR Ca$^{2+}$/H$^{11001}$ has also been optically mapped in myocardial tissue using mag-fluo 4 (45, 104) and fluor 5N (111). In recent studies, fluor 5N was optically mapped simultaneously with the potentiometric probe RH-237 to study SR Ca$^{2+}$/H$^{11001}$ loading during action potential alternans and ventricular fibrillation (111).

**Ca$^{2+}$/H$^{11001}$ probe esterification.** Many early experiments studied cellular Ca$^{2+}$/H$^{11001}$ dynamics in isolated cells using either aequorin or the free acid form of a synthetic Ca$^{2+}$/H$^{11001}$ probe. These agents were administered to the cytosol using microinjection (e.g., Ref. 16) or disruption of the sarcolemma (e.g., Ref. 25). Of course, imaging Ca$^{2+}$/H$^{11001}$ within myocardial tissue requires a much more efficient approach for cytosolic delivery of the probe. Mechanical dissociation after low-Ca$^{2+}$/H$^{11001}$ enzymatic treatment can deliver indo 1 to a large number of isolated myocytes (88), but Ca$^{2+}$/H$^{11001}$ probe esterification was critical for enabling Ca$^{2+}$/H$^{11001}$ imaging within myocardial tissue.

Probes with AM esters (Fig. 1, B and C) are denoted with an “AM,” as in rhod 2-AM. The ester neutralizes the charge of the probe to promote its passage across the sarcolemmal membrane. Charge neutralization of a probe comes at a price: the probe can no longer be dissolved in water. Because of this, esterified probes are often dissolved in small volumes of dimethyl sulfoxide and stored in aliquots at $-20^\circ$C. Before an experiment, the aliquot is thawed and mixed 1:1 with 20% pluronic F-127 (50). Sonication will improve probe dissolution. The dissolved probe mixture is then diluted in a small volume of perfusate media. Camphor oil can also be used for dispersing the probe within the solution. The solution is then administered to the coronary vasculature, either by slow injection or by recirculating perfusate containing the probe. Once in the cytosol, intrinsic esterases cleave the AM ester, trapping the probe in the cell. Although a majority of the probe will be trapped in the cytosol, some will be extruded by an adenosine-binding cassette protein called multidrug resistant glycoprotein (63). Probe efflux by this glycoprotein is partially inhibited by probenecid, with minimal effect on Ca$^{2+}$/H$^{11001}$ transients, as shown in macrophages (107, 108). The activity of the

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**Table 2. Properties of common potentiometric probes used to study myocardial electrophysiology**

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>DI-4-ANBDQBS</td>
<td>563 [EtOH]</td>
<td>500–700</td>
<td>820</td>
<td>700–900</td>
<td>15</td>
<td>62</td>
</tr>
</tbody>
</table>

EtOH, ethanol.

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Fig. 1. Generalized diagrams of typical Ca$^{2+}$/H$^{11001}$-sensitive fluorescent probes. A: the general structure of a probe includes a fluorophore for fluorescence, a chelator for binding Ca$^{2+}$/H$^{11001}$, and an optional conjugation to allow the probe to cross the cell membrane. The basic structures of rhod 2-acetoxyethyl ester (AM) (B), fura 2-AM (C), and bis-fura 2 (D) are shown. BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid.
glycoprotein is temperature-dependent so probe efflux is reduced by performing experiments at lower temperatures, such as 32°C (63). As explained by Sollott and colleagues (88), limitations of probe esterification include noncytosolic probe compartmentalization, incomplete hydrolysis of the ester, and potential alterations of the spectral properties of the probe. Genetically encoded Ca\(^{2+}\) probes (discussed below), such as GCaMP, address many of these limitations (97).

**Probe localization.** Ca\(^{2+}\) probe intracellular localization, organelle and lysosome retention, and intracellular origin of the fluorescence signal can vary with experimental conditions. This is apparent when examining published reports that use rhod 2-AM, such as those of Brandes and Bers (14) and Del Nido et al. (72). Using myocytes from rat heart trabeculae, Brandes and Bers selectively permeabilized the sarcomemlal and mitochondrial membranes. They found that 55% of rhod 2 fluorescence originated from mitochondria and 45% originated from the cytosol (14). In contrast, Del Nido et al. loaded isolated guinea pig myocytes with rhod 2-AM and treated the cells with FCCP to permeabilize the mitochondrial membrane. They observed no significant change in the distribution of rhod 2, indicating that rhod 2 was primarily localized to the cytosol. Furthermore, digitonin was administered, and extensive rhod 2 fluorescence was lost, substantiating that fluorescence was primarily of cytosolic origin (72). Other studies used a cold/ warm protocol to load rhod 2-AM in the mitochondria of isolated myocytes to measure beat-to-beat changes in mitochondrial Ca\(^{2+}\) (99). Substantial uptake of rhod 2 and fluo 3 by lysosomes/endosomes was also noted in those studies. Overall, this previous work underscores careful consideration of Ca\(^{2+}\) probe compartmentalization and how it could be influenced by probe concentration and loading conditions, especially duration and temperature.

**Quantum yield.** Quantum yield (QY) is an important characteristic of a fluorescence probe and is defined as the probability that an absorbed photon will result in an emitted photon. Higher QY is advantageous because less excitation light and less sensitive imaging equipment are required to achieve acceptable signal quality. However, the QY of a probe and its excitation spectrum are both important to consider when imaging myocardial tissue. For example, rhod 2 (a nonratiometric probe) has a QY of 0.102, and fura 2 (a ratiometric probe) has a Ca\(^{2+}\)-bound QY of 0.49 (Table 1). It is usually easier to obtain high-quality rhod 2 signals from the myocardium than fura 2 signals because peak excitation for rhod 2 is in the visible (green) range. Peak excitation for fura 2 is in the ultraviolet range, meaning that it may be more difficult to energize an adequate amount of fura 2. This is because the intensity of UV light sources is usually low, UV light does not penetrate the myocardium as deep as longer wavelengths, and the intensities of UV light required for imaging may damage myocardial tissue.

**Genetically encoded probes.** Recent developments in the expression of genetically encoded Ca\(^{2+}\) probes have overcome the limitations of intracellular loading associated with exogenous Ca\(^{2+}\) probes (46, 116). Genetically encoded probes often provide adequate and stable optical Ca\(^{2+}\) transients for cells throughout the myocardium. The first genetically encoded Ca\(^{2+}\) probe was based on the green fluorescent protein and calmodulin (67). A variant (GCaMP) with higher signal-to-noise ratio and a \(K_d\) of 235 nM was reported several years later (71). GCaMP was subsequently improved for in vivo expression in rodents to produce GCaMP2 and was optically mapped to study atrioventricular conduction in anesthetized mice (96). Those studies found that GCaMP2 fluorescence was 45% slower than the fluorescence of rhod 2, indicating the slower fluorescence transition kinetics of GCaMP2. The most recent version, GCaMP3, has increased baseline fluorescence and dynamic range (both by 3-fold) as well as 1.3-fold higher affinity for Ca\(^{2+}\) (97).

Transgenic human embryonic stem cell-derived cardiomyocytes that stably express GCaMP3 have been used in myocardial grafts (85). These cells were grafted within the infarct of guinea pig hearts, and GCaMP3 fluorescence was optically mapped to provide Ca\(^{2+}\) transients originating exclusively from the graft. In conjunction with the ECG, the graft transients were used to show graft-host coupling in ex vivo perfused heart studies (85). A caveat of these studies was that the fluorescence of blebbistatin interfered with that of GCaMP3 due to overlap of their blue-green emission bands. 2,3-Butanedione monoxime, a less-specific action-myosin ATPase inhibitor, was used in lieu of blebbistatin for some experiments (85). Improved GCaMP variants GCaMP6 and GCaMP7 have been recently introduced but not yet implemented for optical mapping of intracellular Ca\(^{2+}\) (3, 18).

**Ratiometry.** Ca\(^{2+}\) fluorescence that is optically mapped from myocardial tissue will vary with location and time. Variations are caused by uneven distribution of the probe within the tissue, heterogeneous tissue structure, nonuniform excitation light intensity, and probe photobleaching and washout (exocytosis). These variations are usually removed by scaling the signal at each pixel to have the same range, as is commonly done for rhod 2 signals, but this scaling also makes it impossible to measure changes in diastolic Ca\(^{2+}\) level and Ca\(^{2+}\) transient amplitudes.

Ratiometry is an approach where the fluorescence ratio at each pixel is computed using the values at two wavelengths. This cancels fluorescence variability common to both wavelengths to provide a signal that is proportional to Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). Ratiometry requires a Ca\(^{2+}\) probe that undergoes a shift in the emission or excitation spectra when bound to Ca\(^{2+}\). For example, indo 1 is an emission ratiometric probe because the peak of its emission spectrum shifts to shorter wavelengths as Ca\(^{2+}\) concentration increases (35). Fura 2 is an excitation ratiometric probe because its excitation peak shifts to shorter wavelengths with increases in Ca\(^{2+}\) concentration (35). Ratiometric probes have an isosbestic wavelength, below which fluorescence increases with Ca\(^{2+}\) and above which fluorescence decreases. The fluorescence ratio of wavelengths below and above the isosbestic point cancels common variability while amplifying the Ca\(^{2+}\) signal.

Microscope systems are used to image ratiometric Ca\(^{2+}\) probes within cells to study cytosolic and organelle Ca\(^{2+}\) concentrations (90). Both excitation ratiometry (59, 61) and emission ratiometry (6, 22, 95) are used in microscopy studies. Ratiometric imaging is not commonly used in myocardial optical mapping studies because of the additional required illumination wavelengths (for excitation ratiometry) or imaging instrumentation (for emission ratiometry). When imaging myocardium, additional instrumenta-
tion, if available, is often used to image an additional signal, such as that of a potentiometric probe (36), as discussed below. The additional spectral bandwidth required to perform emission or excitation ratiometry may also overlap with that of other probes or endogenous compounds, such as myoglobin or NAD(P)H (31, 47, 106). Furthermore, most ratiometric Ca$^{2+}$ probes are excited by UV light (Table 1), and, as discussed above, providing adequate UV illumination to myocardial tissue to image the fluorescence of a Ca$^{2+}$ probe over a large area could be challenging.

Even so, an early myocardial imaging study used emission ratiometry (indo 1) to measure changes in Ca$^{2+}$ at discrete epicardial locations during ischemia in perfused rabbit hearts (54). Since then, ratiometric imaging of indo 1 was used to study the progression of Ca$^{2+}$ activation across the epicardial surface of perfused rat hearts (23). Recent work has used excitation ratiometry (fura 2) to study changes in diastolic and systolic Ca$^{2+}$ during ischemia-reperfusion injury (106). Although optical mapping of ratiometric probes is not common, this could change as inexpensive high-power light-emitting diode (LED) light sources, inexpensive high-speed cameras, and new ratiometric Ca$^{2+}$ probes are developed. For example, fura 4F is a newer ratiometric probe (118) that has a $K_d$ similar to that of rhod 2 (Table 1). Even though fura 4F has not yet been used in optical mapping experiments, sophisticated multi-parameter optical mapping systems are being developed for ratiometric imaging of both high- and low-affinity Ca$^{2+}$ probes (36, 56). Future studies of myocardial metabolism, where changes in Ca$^{2+}$, mitochondrial redox state, and mitochondrial membrane potential are measured (2, 39, 91, 98), would certainly benefit from accurate measurements of relative changes in Ca$^{2+}$ using ratiometry.

Quantification of nonratiometric signals. The nonratiometric probe rhod 2 is optically compatible with RH-237, a popular potentiometric probe, so it is used in many studies that simultaneously map Ca$^{2+}$ transients and transmembrane potential (20, 69, 70), as discussed below. Rhod 2 signals are often scaled, as explained above, but the fluorescence could be calibrated to accurately measure [Ca$^{2+}$], within myocardial tissue (20). A calibration approach was developed by Del Nido and colleagues for perfused rabbit hearts (72) and then used to study arrhythmia mechanisms in subsequent work (7, 19, 20). Measurements of true [Ca$^{2+}$] using calibrated rhod 2 fluorescence provide valuable physiological insight but also require long-term dye stability, which can be challenging to maintain. Furthermore, calibration of rhod 2 fluorescence depends on the optical absorbance of the dye within the tissue, and that absorbance may differ between species as well as between normal and disease states. Prior experiments are therefore required to determine the characteristics of rhod 2 in each type of tissue. Experiments are also prolonged by additional protocols to measure the full range of [Ca$^{2+}$], for calibration of the fluorescence signal. Thus, rhod 2 fluorescence calibration is typically only used for experiments that require measurements of true [Ca$^{2+}$].

Even so, it is not possible to estimate relative changes in Ca$^{2+}$ transient amplitude in myocardial tissue. We tested this using simultaneous mapping of rhod 2-AM and the potentiometric dye RH-237 in rat hearts before and after administering the β-adrenergic agonist isoprenaline (1 µM). Hearts were paced at 7 Hz, and fluorescence signals were imaged using a dual optical mapping system. RH-237 photobleaching and washout were identified as slow reductions in optical action potential amplitude (Fig. 2A). However, rhod 2 fluorescence, and the associated Ca$^{2+}$ transient amplitudes, was stable for >20 min (Fig. 2B). Ca$^{2+}$ transients recorded immediately before, and 2 min after, administering isoprenaline were compared, revealing a significant increase in Ca$^{2+}$ transient amplitude after isoprenaline (Fig. 2C). The higher transient amplitude was not the result of drifting background fluorescence (Fig. 2D) but was entirely attributed to a change in fluorescence due to Ca$^{2+}$ (Fig. 2E).

Resolving such amplitude differences by comparing DF/F0 suggests that factors influencing background fluorescence such as distribution of fibrosis, inhomogeneous dye loading, and nonuniformity of illumination could, in certain situations, have little impact on the detection of physiological changes in Ca$^{2+}$ transient amplitude.

Potentiometric Probes

Ca$^{2+}$ transients and optical action potentials can be measured simultaneously when tissue is stained with both a Ca$^{2+}$ fluorescence probe and a potentiometric probe. Potentiometric probes are lipid-soluble compounds that embed with high affinity within lipid bilayers. When embedded within the sarcolemma, the emission spectrum of a potentiometric probe shifts with changes in transmembrane potential ($V_m$) to provide a fluorescence signal that represents the action potential. Table 2 lists several potentiometric probes that are commonly used in optical mapping studies of myocardial tissue.

RH-237 and DI-4-ANEPPS are potentiometric probes that are excited by wavelengths in the blue-green band. Fluorescence emission amplitudes ($\Delta F/F$) lie within the range of 5–20% for most potentiometric probes (Table 2). RH-237 and DI-4-ANEPPS exhibit rapid washout and internalization kinetics, requiring frequent reloading of the probe throughout an experiment. Unlike Ca$^{2+}$ fluorescence probes, potentiometric probes do not gain direct access to organelle membranes. Cellular internalization of the probe occurs, but, once internalized, the probe will no longer respond to changes in membrane potential. Although this increases myocardial background fluorescence and reduces $\Delta F/F$, the assessment of optical action potential kinetics is usually unaffected.

PGH-I and DI-4-ANBDQBS are potentiometric probes with peak excitation within the red band and emission in the near infrared band. Such probes are advantageous for imaging deeper within the myocardium (>4 mm) due to reduced absorption and scattering by endogenous chromophores within the excitation and emission bands of the probes (110). Another advantage is that near-infrared probes enable optical mapping of blood-perfused myocardium (62), avoiding the high absorbance of blood in the shorter blue-green band. PGH-I has far red-shifted excitation and emission spectra and provides large signal amplitudes, but it can be somewhat difficult to load in myocardial tissue. Administering PGH-I in a bolus of slightly acidic perfusate (pH 6.0) provides for optimal tissue loading (81). DI-4-ANBDQBS, the more recently developed styryl dye, can be loaded without an acidic bolus and has similar excitation and emission spectra as PGH-I and low phototoxicity and low washout rate (62).
Optical Mapping $\text{Ca}^{2+}$ Fluorescence from Myocardial Tissue

The same general principles for imaging $\text{Ca}^{2+}$ from myocytes in solution apply to imaging myocytes in situ, where the differences include the optics, detectors, and frame rates used to image large areas of the myocardium (Table 3). One important difference is that $\text{Ca}^{2+}$ transients from myocardial tissue are acquired from a volume of tissue, containing a multitude of myocytes, while $\text{Ca}^{2+}$ transients from suspended myocytes are usually acquired from a single cell, typically using confocal microscopy. Furthermore, myocardial contraction introduces motion artifact in optically mapped $\text{Ca}^{2+}$ signals while signals from cellular studies are less affected by contraction.

Conventional optical mapping systems image myocardial tissue using charge-coupled device (CCD) or complementary metal-oxide semiconductor (CMOS) cameras (21, 69). High-power LED light sources, which are cheaper and have a wider range of wavelengths than lasers, provide illumination. Some LEDs have a wide spectral band that may interfere with other fluorescent probes. This is remedied using an excitation filter to shift the spectral peak and narrow the bandwidth. Emitted light is often filtered at a half-width of at least 40 nm at the probe’s peak emission wavelength to ensure that adequate light reaches the imaging unit.

Simultaneous imaging of $\text{Ca}^{2+}$ and $V_m$. The time of events and intervals between $\text{Ca}^{2+}$ transients and action potentials can be measured by acquiring $\text{Ca}^{2+}$ and $V_m$ signals from the same myocardial sites. Such colocated signals provide valuable insight into excitation contraction coupling and arrhythmia mechanisms. The most desirable and commonly used approach is to couple probes that share the same excitation band and have sufficiently distinct emission bands. Fluorescence signals can then be separated into different detectors using dichroic mirrors and emission filters. With this approach, separate detectors image the fluorescence of each probe to maximize the spatiotemporal resolution of the data. Detectors must be precisely aligned to ensure that $\text{Ca}^{2+}$ and $V_m$ signals are indeed acquired from the same myocardial sites. In early studies, two photodiode arrays were carefully aligned using a six-step manual procedure (19, 20). In contrast to photodiode arrays, CCD and CMOS cameras can be aligned more easily because these detectors provide images. Images of reference grids placed in the field of view are used to align cameras either semi-manually (73) or automatically using custom software and postexperiment image processing (37).

Simultaneous imaging of more than one probe also requires careful analysis of spectral content and filtering to minimize signal cross talk. Johnson et al. demonstrated significant cross talk when DI-4-ANEPPS and fluo 3/fluo 4 were used simultaneously (40). Other probe combinations minimize cross talk and maximize signal-to-noise ratios (Table 4). For example, in guinea pig hearts, rhod 2 and RH-237 were shown to be compatible (20) and are commonly used together (17, 49, 50, 60, 70). Systems to simultaneously image indo 1 and DI-4-ANEPPS have been constructed (52, 53), and simultaneous imaging of rhod 2 and DI-4-ANB-DQPO has recently been reported (57).
**Examples of important aspects to consider when imaging Ca\(^{2+}\)**

**Table 3.**

<table>
<thead>
<tr>
<th>Probe compartmentalization</th>
<th>Isolated Cardiomyocytes and Monolayers</th>
<th>Myocardium Cytosolic Ca(^{2+}) Imaging</th>
<th>Myocardium SR Ca(^{2+}) Imaging</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Compartmentalization is condition-dependent. Mitochondria retain rhod 2 (14, 99) and indo 1 (89). Fluo 3 localizes to lysosomes (99). Fluo 5N (84) and mag-fura 2 (33) are retained in the SR after cytosolic washout.</td>
<td>Rhod 2 compartmentalization depends upon experimental conditions. Although rhod 2 may localize to the mitochondria (14), it is often used for myocardium cytosolic Ca(^{2+}) imaging (49, 70, 72) and is compatible with other modes of fluorescence imaging (58).</td>
<td>Mag-fluo 4 (45, 104) and fluo 5N (111) are low-affinity dyes that have been used to image SR Ca(^{2+}). For SR localization, cytosolic washout at 37°C follows long-term loading at room temperature (45, 111).</td>
</tr>
</tbody>
</table>

| Probe dissociation constants | Ca\(^{2+}\) transients from isolated cells and monolayers are typically imaged with high-affinity probes such as fluo 4 (95), which may prolong CaD (26, 27). | Fluo 4 is a higher affinity probe than rhod 2, and causes artifacts in the Ca\(^{2+}\) transient, as shown for swine LV at long cycle lengths (43). | Very-low-affinity Ca\(^{2+}\) dyes (K\(_d\) >22 μM) are typically used to image SR Ca\(^{2+}\). |

| Measurements derived from Ca\(^{2+}\) transients | Transient amplitudes are typically measured (76). Ratiometry provides Ca\(^{2+}\) concentration and kinetic measurements (22, 90). | Ca\(^{2+}\) kinetics usually measured (39, 52, 70) but not amplitudes. Ratiometry of fura 2 (106, 121), indo 1 (15, 52, 83), or fura red (119) has been used to measure amplitudes. | Nonratiometric imaging provides kinetic measurements and short-term relative assessments of SR Ca\(^{2+}\) release amplitudes (45, 104, 111). |

| Imaging approach | Confocal imaging at rates usually >16 frames/s for monolayers (1, 4). Photomultipliers can provide 1,000 samples/s for isolated cell measurements (92). Photodiode array imaging >500 samples/s (118) and CCD imaging >100 frames/s (1, 11). | Photodiode array systems (20, 52) and CCD/CMOS camera systems (49, 58, 85) are used to image myocardial Ca\(^{2+}\) fluorescence. Frame rates are typically >250 frames/s, but most conventional mapping systems use cameras that image >1,000 frames/s (49). | SR Ca\(^{2+}\) (fluor 5N fluorescence) has been imaged from the epicardium of perfused rabbit hearts at frame rates between 500 and 1,000 frames/s using CMOS cameras (111). |

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**CaD,** intracellular calcium transient duration; LV, left ventricular.

**Acquisition rates.** Frame rates used to image Ca\(^{2+}\) probe fluorescence should provide adequate reconstruction of the Ca\(^{2+}\) transient. The Nyquist sampling theorem specifies the minimum sample rate to be two times that of the highest frequency content in the signal. SR Ca\(^{2+}\) release is the fastest part of the transient, and the time to peak (TTP) of a Ca\(^{2+}\) transient has been measured to be 20–30 ms for cells paced at 2 Hz (38) and ~15 ms in rat hearts paced at 5.5 Hz (39). Frame rates as low as 80 frames/s have been used to optically map Ca\(^{2+}\) transients (85). Most conventional optical mapping systems consist of either CCD or CMOS cameras, either of which can acquire full frames at rates of at least 500 frames/s. Rates over 1,000 frames/s are optimal to provide enough samples to accurately measure the rate of SR Ca\(^{2+}\) release and transient amplitudes. The upper limit of useful camera frame rates is ~4,000, the point where signal-to-noise ratio becomes low. Such high-speed imaging was used to measure changes in the TTP Ca\(^{2+}\) at 4,000 frames/s with a photodiode array (20).

**Motion artifact.** Elimination of the motion artifact requires suppression of contraction using electromechanical uncoupling agents (7, 28, 77) or, less frequently, mechanical constraint (112). Blebbistatin is a popular uncoupling agent for optical mapping, especially when imaging Ca\(^{2+}\) because it specifically inhibits the myosin ATPase without altering sarclellial ion currents or SR Ca\(^{2+}\) kinetics (28). However, myocyte ATP utilization is severely reduced by electromechanical uncoupling, potentially altering the time course of physiological changes, especially mitochondrial function, during experimental perturbations (47, 105, 114). Motion tracking algorithms (41, 80, 115) and ratiometry (42, 44, 93) are sometimes effective in removing motion artifact from optical action potentials, but there has been less work in the application of motion tracking algorithms to reduce artifact in optically mapped Ca\(^{2+}\) transients. In theory, the same motion tracking algorithms could be used for Ca\(^{2+}\) signals, but publications demonstrating this are presently unknown to us. Ca\(^{2+}\) transients from single sites can be measured in contracting hearts without motion sup-

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**Table 4.** Selected studies that used Ca\(^{2+}\) fluorescence probes with other exogenous or endogenous fluorescent compounds

<table>
<thead>
<tr>
<th>Probes</th>
<th>Description</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhod 2 and RH-237</td>
<td>Ca(^{2+}) and (V_m)</td>
<td>20, 49, 70</td>
</tr>
<tr>
<td>GCaMP3 and RH-237</td>
<td>Ca(^{2+}) and (V_m)</td>
<td>85</td>
</tr>
<tr>
<td>Fluo 5N and RH-237</td>
<td>Ca(^{2+}) and (V_m)</td>
<td>111</td>
</tr>
<tr>
<td>Rhod 2 and di-4-ANBDQPOQ</td>
<td>Ca(^{2+}) and (V_m)</td>
<td>57</td>
</tr>
<tr>
<td>Indo 1 and di-4-ANEPPS</td>
<td>Ratio-Ca(^{2+}) and (V_m)</td>
<td>52, 53</td>
</tr>
<tr>
<td>Fura 4F and di-8-ANEPPS</td>
<td>Ratio-Ca(^{2+}) and (V_m)</td>
<td>36</td>
</tr>
<tr>
<td>Fura 2 and FAD(^{+})</td>
<td>Ratio-Ca(^{2+}) and endogenous FAD(^{+})</td>
<td>106</td>
</tr>
</tbody>
</table>

\(V_m\), transmembrane potential; FAD\(^{+}\), flavin adenine dinucleotide; ratio-Ca\(^{2+}\), ratiometric Ca\(^{2+}\) measurement.

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pression by coupling a fiber optic light guide to the myocardium (29, 64). This provides high temporal resolution Ca\textsuperscript{2+} signals, but high spatial resolution Ca\textsuperscript{2+} imaging is not possible.

**Analysis of Ca\textsuperscript{2+} Signals**

A handful of physiological measurements are derived from Ca\textsuperscript{2+} probe fluorescence using a variety of analyses. The most common measurements used to study Ca\textsuperscript{2+} cycling (Table 5), and methods to compute them, are presented in this section.

**Preprocessing.** Ca\textsuperscript{2+} signals, especially those from nonratiometric probes, are averaged, filtered to reduce noise, and normalized before analysis. Spatial smoothing, either by pixel averaging within a defined radius or by convolution with a geometric kernel, is common in Ca\textsuperscript{2+} optical mapping. Ca\textsuperscript{2+} signals at each pixel are sometimes ensemble-averaged and/or low pass filtered. Infinite impulse response filtering, as suggested for \( V_m \) signals (51), is also appropriate for Ca\textsuperscript{2+} signals. In fact, spatiotemporal filtering (66) and wavelet analyses (5, 120) that have been optimized for \( V_m \) signals could also substantially improve the signal-to-noise ratio of Ca\textsuperscript{2+} imaging data. Two-dimensional wavelet processing of Ca\textsuperscript{2+} images, similar to that demonstrated for \( V_m \) images (120), shows promise for selective removal of noise while maintaining local image features. This would be advantageous for locating sites of early SR Ca\textsuperscript{2+} release with precision. In any preprocessing approach, signal distortion should be checked to ensure that any changes in Ca\textsuperscript{2+} transient morphology, especially the fast upstroke phase, would not alter interpretation of the mapping data.

**Cytosolic Ca\textsuperscript{2+} transient analysis.** The two main phases of the Ca\textsuperscript{2+} transient (upstroke and extrusion phases) are usually analyzed separately to quantify the activity of exchangers [Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX)], pumps [SR Ca\textsuperscript{2+}-ATPase (SERCA) and sarcoplasmic Ca\textsuperscript{2+}-ATPase], and the SR Ca\textsuperscript{2+} release channels [ryanodine receptors (RyRs)]. The first step is to identify the onset of the rise in cytosolic Ca\textsuperscript{2+} (\( t_0 \)), the beginning of the upstroke. The maximum first derivative could be used as a marker for \( t_0 \) (20), as done for sarcolemmal depolarization (24). An alternative is to detect when fluorescence first exceeds 10% of the amplitude above baseline (20, 53). The maximum of the second derivative has also been used (39), and it provides a value closer to the onset of the upstroke (Fig. 3A). The maximum first derivative of the upstroke is often interpreted as the fastest speed of SR Ca\textsuperscript{2+} release, an important measure of RyR activity and couplon (9) function. This point usually lies halfway between \( t_0 \) (if measured using the maximum second derivative) and the transient peak.

The Ca\textsuperscript{4+} transient TTP is another important assessment of RyR activity and couplon function, requiring accurate detection of the true transient peak. Signal smoothing and low imaging frame rates introduce errors in measuring the true transient peak. This issue is diminished by measuring the duration of the transient upstroke from 10% above baseline to 90% of the peak, a value known as rise time (53, 70). While the TTP is always longer than rise time, the two values are altered pari passu, as shown in isolated heart studies (63). Signal smoothing and spatial integration may also alter rise time if they significantly alter the upstroke phase of the transient.

Ca\textsuperscript{D} is a Ca\textsuperscript{2+} analog of action potential duration (APD) and is a ubiquitous Ca\textsuperscript{2+} transient measurement. Even so, there is little consensus on how best to compute Ca\textsuperscript{D}, as is also true for APD. Ca\textsuperscript{D} is the difference between the time at a specified level of cytosolic Ca\textsuperscript{2+} extrusion and \( t_0 \). Levels of 30, 80, and 90% of extrusion have been used for Ca\textsuperscript{D}, denoted as Ca\textsuperscript{D}30, Ca\textsuperscript{D}80, Ca\textsuperscript{D}90, respectively. The issue is that the percent of cytosolic Ca\textsuperscript{2+} removal used to measure Ca\textsuperscript{D} in a particular study depends upon the intracellular processes that are affected in an experiment. For example, Ca\textsuperscript{D}30 was measured in studies of pyruvate dehydrogenase activation (39) while Ca\textsuperscript{D}80 was measured in studies of adrenergic signaling in human heart failure (49). Furthermore, the approach used to identify \( t_0 \) could alter Ca\textsuperscript{D}; the first derivative approach will shorten Ca\textsuperscript{D} while the second derivative approach will lengthen it. We suggest using the second derivative approach because the complete Ca\textsuperscript{2+} upstroke phase will be represented in the measurement of Ca\textsuperscript{D} (82).

**Table 5. Typical measurements derived from Ca\textsuperscript{2+} fluorescence signals and the general physiological insight they provide**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Physiological Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time-to-peak (TTP)</td>
<td>The time from initiation of Ca\textsuperscript{2+} departure (( t_0 )) to peak fluorescence</td>
<td>RyR Ca\textsuperscript{2+} release kinetics</td>
</tr>
<tr>
<td>Rise time</td>
<td>Duration from 10% to 90% of Ca\textsuperscript{2+} upstroke</td>
<td>RyR Ca\textsuperscript{2+} release kinetics</td>
</tr>
<tr>
<td>Maximum departure velocity</td>
<td>The maximum first derivative (( dF/dt )) of the Ca\textsuperscript{2+} upstroke</td>
<td>RyR Ca\textsuperscript{2+} release kinetics</td>
</tr>
<tr>
<td>Ca\textsuperscript{D}50, Ca\textsuperscript{D}80, Ca\textsuperscript{D}90</td>
<td>The duration from ( t_0 ) to 50, 80, or 90% of cytosolic Ca\textsuperscript{2+} extrusion</td>
<td>Duration of cytosolic Ca\textsuperscript{2+} extrusion</td>
</tr>
<tr>
<td>( T_{50} )</td>
<td>The duration from peak to 50% of cytosolic Ca\textsuperscript{2+} extrusion</td>
<td>Early extrusion phase, fast Ca\textsuperscript{2+} extrusion, emphasis on SERCA activity (49)</td>
</tr>
<tr>
<td>Decay time constant (( \tau_{\text{fall}} ))</td>
<td>The time required for cytosolic Ca\textsuperscript{2+} extrusion to reach 1-( e ) (( \sim 63.2% )) of baseline</td>
<td>Late Ca\textsuperscript{2+} extrusion phase, slow Ca\textsuperscript{2+} extrusion, emphasis on NCX activity (49)</td>
</tr>
<tr>
<td>Ca\textsuperscript{D}30/Ca\textsuperscript{D}80</td>
<td>Ratio of early Ca\textsuperscript{2+} extrusion phase to late Ca\textsuperscript{2+} extrusion phase</td>
<td>Balance of SERCA and NCX activity (49)</td>
</tr>
<tr>
<td>( V_m/Ca\textsuperscript{2+} ) phase chirality</td>
<td>The chirality of the phase plot of the normalized Ca\textsuperscript{2+} signal vs. the normalized ( V_m ) signal</td>
<td>Counterclockwise chirality indicates normal ( V_m/Ca\textsuperscript{2+} ) coupling, and clockwise chirality indicates abnormal ( V_m/Ca\textsuperscript{2+} ) coupling (70)</td>
</tr>
<tr>
<td>Ca\textsuperscript{D}-APD</td>
<td>Difference between Ca\textsuperscript{2+} transient duration and optical action potential duration</td>
<td>Increasing difference between Ca\textsuperscript{D} and APD may lead to DAD (49)</td>
</tr>
</tbody>
</table>

RyR, ryanodine receptor; SERCA, sarco(endoplasmin reticulum Ca\textsuperscript{2+}-ATPase; NCX, sodium-calcium exchanger; DAD, delayed afterdepolarization.
Fig. 3. Ca\(^{2+}\) transient analysis and features used for measuring Ca\(^{2+}\) kinetics. A: Ca\(^{2+}\) transient measured from a perfused rat heart via rhod 2 fluorescence [F(t)]. The first and second derivative of fluorescence (dF/dt and d\(^2\)F/dt\(^2\)) are shown for the Ca\(^{2+}\) transient upstroke phase. A monoexponential function was fitted to the late Ca\(^{2+}\) extrusion phase (solid line) from 30% of Ca\(^{2+}\) extrusion to baseline. CaD80 is the time from \(t_0\) to 80% of Ca\(^{2+}\) extrusion, corresponding to the marker at 20% above baseline. Time-to-peak (TTP) is the time from \(t_0\) to the peak of the Ca\(^{2+}\) transient. B: example of a monoexponential fitted to the late Ca\(^{2+}\) extrusion phase. The residuals of the fit indicate that the main features of the late extrusion phase were fitted with low error. C: example of a biexponential fitted to the same Ca\(^{2+}\) transient shown in B. Residuals of the fit indicate fitting error within the late upstroke phase and the early extrusion phase. \(\tau_{\text{Fall}},\) time constant of cytosolic Ca\(^{2+}\) extrusion; \(\tau_{\text{Rise}},\) upstroke time constant.

Optical mapping in guinea pig hearts revealed the effect of activation cycle length on TTP and CaD (20). CaD shortens with cycle length, an outcome linked to APD restitution, which is based on the slow recovery of sarcolemmal L-type Ca\(^{2+}\) channels (78). Reductions in cycle length reduce L-type Ca\(^{2+}\) current, which shortens the plateau phase of the action potential, reducing SR Ca\(^{2+}\) release, resulting in shorter CaD (20). This “CaD restitution” has implications on the TTP. TTP decreases as pacing rate increases, sometimes only within a certain dynamic range. Because L-type Ca\(^{2+}\) channels are still recovering by the next beat during very fast pacing rates (basic cycle length <190 ms in guinea pigs), the TTP can no longer shorten. This effect has only been found in the base, not the apex, of guinea pig hearts (20).

**Time constant of cytosolic Ca\(^{2+}\) extrusion.** The time constant of cytosolic Ca\(^{2+}\) extrusion (\(\tau_{\text{Fall}}\)) is a value that quantifies the rate of fluorescence reduction after the Ca\(^{2+}\) transient upstroke (Fig. 3). This time constant represents the activity of pumps and exchangers that remove Ca\(^{2+}\) from the cytosol. The activity of NCX and SERCA both significantly influence \(\tau_{\text{Fall}},\) but one or the other may have a greater influence on the rate of extrusion at different intervals. For example, in human myocardium, the NCX is thought to dominate the late phase of cytosolic Ca\(^{2+}\) extrusion (49). The extrusion phase of the cytosolic Ca\(^{2+}\) transient is often modeled as a single decaying exponential (Eq. 1) (8)

\[
F(t) = Ae^{-\frac{t}{\tau_{\text{Fall}}}} + B
\]

and \(\tau_{\text{Fall}}\) is measured as shown in Fig. 3B. The transient is often rounded for a short duration after the peak and, depending upon species, may have a shape that is dramatically different from the rest of the extrusion phase. This introduces errors when fitting a single decaying exponential. In this case, a biexponential function could be fitted to the entire Ca\(^{2+}\) transient (Eq. 2). This provides two time constants; one that includes the upstroke (\(\tau_{\text{Rise}}\)) and one for the extrusion phase (\(\tau_{\text{Fall}}\)) (74).

\[
F(t) = A(e^{-\frac{t}{\tau_{\text{Fall}}}} - e^{-\frac{t}{\tau_{\text{Rise}}}}) + B
\]

\(\tau_{\text{Fall}}\) computed using Eq. 2, as shown in Fig. 3C, often differs slightly from \(\tau_{\text{Fall}}\) computed using Eq. 1. An accurate way to fit the late phase of Ca\(^{2+}\) extrusion is to use Eq. 1 and restrict the fit to fluorescence values between 70% of the transient peak and the diastolic baseline (52).

Although \(\tau_{\text{Fall}}\) is a time constant, it is not independent of transient amplitude, since there is a nonlinear relationship between the two parameters (8). Maximum departure velocity is also dependent upon amplitude, underscoring the importance of maintaining continuity of illumination and camera position, especially when using a nonratiometric probe. Nonratiometric signals were usually normalized to a set range to minimize the...
effect of amplitude on kinetic measurements such as \( \tau_{\text{Fall}} \) and departure velocity.

Investigations of Physiology and Disease

Optical mapping of \( \text{Ca}^{2+} \)-sensitive fluorescent probes and quantitative analysis of \( \text{Ca}^{2+} \) transients have been used to study a spectrum of myocardial physiology and disease conditions. The data are often displayed as a pseudocolor map, providing detailed spatial information for interpreting mechanisms of ectopic activity, alternans, tachycardia, and fibrillation. Recent work using \( \text{Ca}^{2+} \) optical mapping to study heart failure, fatal arrhythmias, and metabolic perturbations is briefly discussed below.

**Human heart failure.** Increased cardiac \( \beta \)-adrenergic activity is a common cause of arrhythmia and sudden death in heart failure patients. Recent dual optical mapping studies (RH-237 and rhod 2 AM) in human left ventricle wedge preparations have provided new insights into the response of donor and end-stage failing hearts to \( \beta_1 \)- and \( \beta_2 \)-adrenergic stimulation (49). Careful analysis of CaD80 and APD (Fig. 4) revealed that failing hearts were desensitized to \( \beta_1 \) stimulation, but \( \beta_2 \) stimulation facilitated delayed afterdepolarizations (DADs) and premature ventricular contractions (PVCs). The DADs were caused by an increase in the time difference between CaD80 and APD \( \Delta(CaD-APD) \), as shown in Fig. 4A. This value indicates a vulnerable window of elevated diastolic \( \text{Ca}^{2+} \) that motivates inward NCX current. Maps of \( \Delta(CaD-APD) \) identified transmural heterogeneities (Fig. 4B), a primary mechanism of PVCs (Fig. 4C). Such \( \text{Ca}^{2+} \) optical mapping studies of human myocardium are providing novel insights to improve heart failure therapies, such as the utility of blockers that specifically target \( \beta_2 \) receptors.

\( \text{Ca}^{2+} \)-mediated sarcolemma depolarization. Dual imaging of RH-237 and rhod 2-AM in healthy rabbit hearts has also provided insights into the initiation of spontaneous sarcolemma depolarization after local \( \beta \)-adrenergic receptor activation (Fig. 5) (70). In those studies, subepicardial injections of norepinephrine caused PVCs that exhibited abnormal delays between sarcolemmal depolarization and the \( \text{Ca}^{2+} \) transient upstroke (Fig. 5, C and D). The delays were analyzed using phase plots that were constructed by plotting normalized rhod 2 fluorescence vs. normalized RH-237 fluorescence (19). During PVCs, the chirality of the phase plot was reversed (Fig. 5E), indicating depolarization was mediated by local and synchronous release of SR \( \text{Ca}^{2+} \) during diastole. The imaging data of these studies provided the first quantitative confirmation in perfused hearts of PVCs caused by SR \( \text{Ca}^{2+} \) release during local adrenergic activity.

\( \text{Ca}^{2+} \) dynamics during fibrillation. Ventricular fibrillation is often preceded by ventricular tachycardia. The transition can be caused by elevated diastolic \( \text{Ca}^{2+} \) that leads to \( \text{Ca}^{2+} \) transient alternans and then APD alternans that ultimately destabilize the tachycardia (109, 113). Simultaneous imaging studies (RH-237 and rhod 2-AM) of swine right ventricles further demonstrated that, during fibrillation, \( \text{Ca}^{2+} \) transients and sarcolemmal potential could become decoupled, without...
distinct relationships between fluorescence maps and each process having significantly different fundamental rates (73). Later, excised blood-perfused swine hearts were studied using simultaneous imaging to determine the relationship between conduction block and Ca\textsuperscript{2+} cycling during fibrillation (112). Little difference in the fundamental rates of transmembrane potential and Ca\textsuperscript{2+} transients was observed, and in most of the area that was imaged 80% of all transient upstrokes occurred during the initial 25% of sarcomemal depolarization, indicating substantial coupling. However, decoupled Ca\textsuperscript{2+} transients and sarcomemal potentials were observed, but exclusively near sites of conduction block, leading to the conclusion that decoupling was a consequence rather than a cause of block (112). Overall, these previous studies demonstrate the unique insights into complex spatiotemporal activity that can be developed using simultaneous imaging of a potentiometric and Ca\textsuperscript{2+}-sensitive probe.

\textit{Ca}_{i}\textsuperscript{2+} dynamics and metabolism.} In metabolic studies, changes in Ca\textsuperscript{2+} kinetics that alter contractile function in perfused hearts are measured using Ca\textsuperscript{2+} fluorescence probes (14, 39, 98). For example, paired optical mapping studies that measured developed pressure, NADH fluorescence, and Ca\textsuperscript{2+} transients in perfused rat hearts provided mechanistic insights into the inotropic effects of pyruvate and dichloroacetate, two compounds that promote full glucose oxidation in myocytes (39). TTP and CaD30 were reduced by pyruvate and dichloroacetate, indicating increased RyR activity and increased SERCA activity. However, CaD80 remained unchanged due to significant lengthening of \( r_{\text{Fut}} \), possibly secondary to increased SR Ca\textsuperscript{2+} load. These studies are an example of increased ventricular contractility without companion measurements of Ca\textsuperscript{2+} transient amplitude because of the use of a nonratiometric probe (rhod 2) (39). Optical mapping of ratiometric Ca\textsuperscript{2+} probes would be an especially useful approach in these types of experiments.

\textbf{Summary}

Since the early work of Fabiato in 1985 (25), fluorescence imaging of Ca\textsuperscript{2+}-sensitive fluorescence probes has become an extremely useful approach in optical mapping studies of myocardial tissue and adopted by many cardiovascular research laboratories. Future developments may provide probes with greater quantum yield, enhanced genetically encoded probes that are faster and brighter, and multimode optical mapping systems for imaging ratiometric probes simultaneously with other fluorescent compounds, as well as new developments that are unforeseen. The technology continues to evolve in the ongoing effort to improve therapies that ease the growing worldwide burden of cardiovascular disease.

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\textbf{DISCLOSURES}

No conflicts of interest, financial or otherwise, are declared by the authors.

\textbf{AUTHOR CONTRIBUTIONS}

R.J., R.D.W., P.L.C.P., O.B., and M.W.K. conception and design of research; R.J., R.D.W., P.L.C.P., and O.B. performed experiments; R.J., R.D.W.,
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