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CHAPTER 2

Ca^{2+}-Sensitive Fluorescent Dyes and Intracellular Ca^{2+} Imaging

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Imaging Ca^{2+}-sensitive fluorescent indicators provides a common approach for studying Ca^{2+} signals in many contexts. Fluorescent indicators are particularly useful for measuring acute Ca^{2+} changes in a relatively noninvasive manner. The availability of indicators that can be targeted to specific cellular domains, coupled with variations in affinity, brightness or spectral characteristics, provides tools for exploring spatially and temporally diverse Ca^{2+} signals, and moreover, multiplexing the readout of Ca^{2+} with other cellular functions. This article aims to give the novice experimentalist some insight into the considerations and potential pitfalls that impinge on the use of fluorescent Ca^{2+} indicators.

INTRODUCTION

A multitude of Ca^{2+} signals, varying from nanoscopic, subcellular domains to whole-cell Ca^{2+} waves and lasting for microseconds to hours, are used by different types of cells (Berridge 2006; Bootman et al. 2001). The characteristics of these cellular Ca^{2+} signals depend on the expression of tissue-specific Ca^{2+} transport systems (Berridge et al. 2000). The diversity of cellular Ca^{2+} signaling means that there is no single technique that can be used to monitor Ca^{2+} changes in all situations. However, fluorescent Ca^{2+} indicators and imaging provide the most versatile and widely used method for analyzing cellular Ca^{2+} responses. Since their introduction by Tsien and colleagues (Tsien et al. 1982), fluorescent Ca^{2+} indicators have underpinned the investigation of Ca^{2+} signaling in a host of different experimental settings. Using appropriate technology and suitable indicators, it is possible to monitor Ca^{2+} signals spanning from subcellular to multicellular, at high speed or time lapse, within living cells.

This chapter describes practical approaches for using fluorescent indicators to monitor intracellular Ca^{2+} concentration. These indicators include fluorescent protein reporters such as pericams (Nagai et al. 2001), cameleons (Truong et al. 2007), modified yellow cameleons (YCs), (Nagai et al. 2004), and camgaroos (Griesbeck et al. 2001). However, our emphasis will be on measuring Ca^{2+} signals with synthetic indicators such Fura-2, Indol-1, and Fluo-4 (Grynkiewicz et al. 1985; Minta et al. 1989; Tsien 1980). The procedures described can be applied to many imaging modalities, including wide-field, confocal, and total internal reflection (TIRF) microscopy. However, the experimental details can vary depending on the cell type, imaging system, and characteristics of the Ca^{2+} signals being studied.

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For further background information regarding the process of fluorescence and imaging hardware related to the separation of excitation and emitted light with dichroic mirrors and optical filters, the reader is directed to excellent online tutorials http://www.olympus.co.uk/microscopy and https://www.micro-shop.zeiss.com. For further information related to indicator types and their specifications, the reader is referred to http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook.html or http://www.teflabs.com/ion-indicators-0/).

CHOOSING AN APPROPRIATE Ca^{2+} INDICATOR

There is a wide variety of Ca^{2+} indicators available, with excitation and emission spectra ranging from ultraviolet (UV) to the far red, in addition to differences in Ca^{2+} affinity, basal fluorescence, and cell permeability. Consequently, the choice of a Ca^{2+} indicator to be used in an experiment must be carefully evaluated in terms of the Ca^{2+} signals to be measured, the importance of quantitative or qualitative data, the imaging hardware available, and the potential problems that might be encountered.

Factors Determining Fluorescence Intensity

The central concept underlying the use of Ca^{2+} indicators is that an acute increase or decrease in their fluorescence intensity reflects a change in Ca^{2+} concentration. However, it is very important to appreciate that fluorescence intensity is also a function of several other factors. These include indicator concentration, excitation path length, and cytosolic localization. If the cellular indicator concentration alters during an experiment (assuming fluorescence per molecule remains the same), then fluorescence intensity will change too, irrespective of the Ca^{2+} concentration. Typical causes of changes in indicator concentration are photobleaching, indicator compartmentalization, or indicator extrusion. Changes in fluorescence intensity will also accompany variations in cell thickness, for example, caused by dynamic movement, even though the cell indicator concentration is uniform. Finally, local conditions such as viscosity, pH, etc. can alter Ca^{2+} indicator properties. Variations in these conditions may be encountered between indicators located in the cytosol or organelles such as nuclei (see later), mitochondria, or vesicular structures. Thus, possible artifactual changes of fluorescence intensity must be considered carefully to faithfully monitor Ca^{2+} signals.

Dual-Wavelength Ca^{2+} Indicators

Ca^{2+}-sensitive fluorescent indicators can be broadly divided into ratiometric (dual-wavelength) or single-wavelength indicators based on their response to a Ca^{2+} elevation. Ratiometric indicators are the most useful for quantitative measurements of Ca^{2+} concentration, whereas single-wavelength indicators are more commonly used for qualitative data, indicating relative changes in Ca^{2+} (Grynkiewicz et al. 1985; Minta et al. 1989; Tsien 1980). However, ratiometric indicators can equally be used for qualitative data. One disadvantage of dual-wavelength indicators, compared to single-wavelength indicators, is that their dynamic range (difference in intensities between Ca^{2+}-free and Ca^{2+}-bound indicator) is often smaller, thus making it harder to detect modest Ca^{2+} changes.

When Ca^{2+} binds to a ratiometric indicator, it changes the optimum excitation or emission wavelength of the indicator. For example, Fura-2 is a dual excitation ratiometric indicator; the Ca^{2+}-free form of Fura-2 has a peak excitation wavelength of ~380 nm, whereas the peak excitation wavelength for Ca^{2+}-bound Fura-2 is ~340 nm. An elevation of Ca^{2+} concentration induces an increase in Fura-2 emission fluorescence when the indicator is excited at 340 nm, with a corresponding decrease in fluorescence at 380-nm excitation. Conversely, Indo-1 is a dual emission ratiometric indicator; the Ca^{2+}-free and Ca^{2+}-bound forms of Indo-1 have peak emissions of ~405 nm and ~475 nm, respectively. Indo-1 is typically excited with ~340 nm light. An elevation in Ca^{2+} concentration induces an increase and decrease in Indo-1 emission at wavelengths 405 nm and 475 nm, respectively.

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Ratiometric indicators also display an excitation or emission wavelength at which the indicator appears insensitive to Ca^{2+}. This is known as the "isosbestic point." This Ca^{2+}-insensitive wavelength can be used to measure the indicator concentration. With Fura-2, the isosbestic wavelength (∼360 nm excitation) can also be used to measure changes in other metal ions, such as Mn^{2+}. The binding of Mn^{2+} to Fura-2 quenches its fluorescence. Consequently, at the isosbestic point, a decrease in Fura-2 emission fluorescence indicates an increase in Mn^{2+}. This is useful because Mn^{2+} can be used as a Ca^{2+} surrogate to monitor store-operated Ca^{2+} entry; Mn^{2+} entering the cell in place of Ca^{2+} will be detected by decreased Fura-2 fluorescence. Despite the quenching by Mn^{2+}, Fura-2 can still be used to report Ca^{2+} in a quantitative, ratiometric manner. This combined application of monitoring Ca^{2+} concentration and Ca^{2+} entry illustrates the considerable advantage of dual-wavelength indicators.

Although Fura-2 has the advantage of delivering quantitative data, the need to alternately excite the indicator with 340 nm and 380 nm light can slow image acquisition. Fortunately, present-day filter changers, or monochromators, can rapidly switch excitation wavelengths making Fura-2 a plausible candidate (assuming equally fast detectors) for high-speed (e.g., 100 Hz) imaging (see http://www.pti-nj.com/RatioMaster/RatioMaster.html). Even if slow data sampling speeds are required, it is best to acquire ratiometric image pairs in quick succession, and then pause. Otherwise, the Ca^{2+} concentration could change considerably in the time between image capture, and the ratiometric output is not a true reflection of the Ca^{2+} concentration. For long-term experiments where a slow sampling rate is sufficient, we sequentially capture images using 340-nm and 380-nm excitation (200 msec per image, 50 msec delay between images for wavelength change), then shutter the excitation light until the next sampling point is due. In this way, we can monitor Ca^{2+} using Fura-2 for >30 min with little bleaching.

Because Indo-1 only requires excitation at one wavelength, rapid monitoring of Ca^{2+} concentration can be obtained with dual emission detectors (the 410-nm and 475-nm emitted light can be separated with a single dichroic mirror). We use Indo-1 and twin photomultipliers to monitor Ca^{2+} in cardiac myocytes with millisecond resolution (Proven et al. 2006). For similar reasons, Indo-1 is commonly used for flow cytometry. A disadvantage of Indo-1 is that it has a lower dynamic range (change in fluorescence between the Ca^{2+}-free and Ca^{2+}-bound form) than Fura-2.

Although very commonly used, both Fura-2 and Indo-1 are excited by UV light, which has potential drawbacks. Aside from its biological effects (see below), UV light is generally poorly transmitted by most objectives and other optical components such as light guides. However, dual-wavelength indicators that absorb visible light are available. These include ratio pericam (Shimozono et al. 2002) and Fura Red (Kurebayashi et al. 1993; Lohr 2003). Fura Red also has the advantage of being sufficiently red-shifted in its emission that it can be used easily in combination with green fluorescent protein (GFP) without the need for spectral cross-talk correction. Alternatively, the combined use of two single-wavelength reporters, such as Fluo-4 and Fura Red, offers the possibility of dual-emission ratiometric confocal measurements using visible light excitation (Lipp and Niggli 1993; Thomas et al. 2000).

Single-Wavelength Ca^{2+} Indicators

Single-wavelength indicators display a change in their fluorescence emission when Ca^{2+} signals occur. In contrast to dual-wavelength indicators, there is not a sufficient shift in either excitation and/or emission wavelengths on Ca^{2+} binding to allow ratiometric measurements. A key issue with single-wavelength indicators is that variations in fluorescence emission may not reflect differences in Ca^{2+} concentration. For example, we find that neighboring clonal cells (e.g., HeLa cells) within a single field of view do not load with equivalent amounts of Ca^{2+} indicator. Monitoring a single-wavelength indicator, such as Fluo-4, therefore yields adjacent cells that have obviously different fluorescence intensities. Without calibrating the fluorescence within each individual cell, it would be impossible to tell whether the different fluorescent intensities solely reflect disparity in Ca^{2+} indicator loading, or perhaps variation of their Ca^{2+} concentration. For this reason, single-wavelength indicators are most useful for measuring relative changes in Ca^{2+} concentration.

Although single-wavelength measurements are not ideal when quantitative data are required, a practical way of correcting for uneven indicator concentration is to express the fluorescence signal

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relative to its starting signal. This is often denoted F/F_0 (or $\Delta F/F_0$ if the change in fluorescence is used). F is the intensity of fluorescence emission recorded as the experiment runs. F_0 is the fluorescence intensity at the start of the experiment. This simple approach normalizes differences of indicator concentration between cells, and provides a plausible method for comparing data between experiments. However, it does not compensate for changes in indicator concentration caused by bleaching, extrusion, or compartmentalization that may occur during the experiment (see section on photodamage).

Affinity of Indicator for Ca^{2+}

A key consideration for indicator selection is the range of Ca^{2+} concentrations to be measured during an experiment. Ideally, the dissociation constant (K_d) of the indicator for Ca^{2+} is near to the midpoint of the expected Ca^{2+} range. The relationship between indicator fluorescence and Ca^{2+} concentration is linear around the K_d. Outside this range, large changes in Ca^{2+} concentration can be represented by only small changes in fluorescence. Low-affinity indicators are suitable for large Ca^{2+} signals, but may barely resolve small Ca^{2+} changes. Conversely, high-affinity Ca^{2+} indicators are suitable for relatively small changes in Ca^{2+} concentration, but may become saturated (and therefore not report accurately) by substantial Ca^{2+} changes. In some situations, the combination of low- and high-affinity indicators may be necessary to follow cellular Ca^{2+} responses accurately.

Imaging Hardware

Imaging fluorescent Ca^{2+} indicators within live cells can be readily achieved using a fairly modest setup. A basic wide-field system will comprise of a research-grade microscope (typically inverted), an intense broad-spectrum light source, appropriate mirrors and filters to separate excitation and emission light, a suitable objective lens, and a camera. Many manufacturers offer complete wide-field systems, but it is also possible to build such a system using components from a variety of manufacturers and control it using open source software such as Micro-Manager (http://valelab.ucsf.edu/~MM/MMwiki/). The choice of specific components will depend on the application and budget. However, maximizing the efficiency of light transmission by using the right objective (see below), high efficiency filters, and a sensitive camera will reduce the amount of excitation light required for an acceptable signal. This will increase the rate at which images can be acquired and/or reduce the risk of photodamage (see below).

Mercury arc lamps have traditionally been used as the light source for wide-field imaging systems because they provide a broad spectrum of light from which specific excitation wavelengths can be filtered. However, mercury arc lamps have a short life span (typically 200–400 h), show a non-uniform spectrum (though this can be an advantage if a peak excitation wavelength is paired with an appropriate Ca^{2+} indicator), do not tolerate rapid on/off cycles, require regular alignment, and have the potential to cause hazardous explosion. There are now many alternatives including metal halide lamps, light-emitting diodes (LEDs), and hybrid solid-state illuminators. Again, application and budget will dictate the best option, although there are currently no 340-nm LEDs available (required for Indo-1 and ratiometric Fura-2 imaging). The range of cameras available for fluorescence microscopy has also increased rapidly over recent years, but for general Ca^{2+} imaging applications, cameras based on sCMOS sensors (http://www.scmos.com/) offer the best combination of low noise, high dynamic range, high sensitivity, high frame rates, and wide field of view. Expensive EM-CCD cameras (generally considered to be the most sensitive type of camera) are only required for very low light imaging/single-molecule detection or for use with spinning disk confocal imaging systems (see below).

Wide-field imaging has the advantages of simplicity and sensitivity, but is compromised by out-of-focus light that reduces resolution. Selective illumination or rejection of out-of-focus light can overcome these problems, but requires a more complex (and thus expensive) imaging system. TIRF microscopy is an example of a technique utilizing selective illumination. The evanescent wave created by TIRF illumination excites fluorophore molecules within ~150 nm of the glass coverslip.

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This technique can be used to visualize cellular structures or Ca^{2+} signals that occur close to the plasma membrane, and has been particularly useful for studies of Ca^{2+} influx (Luik et al. 2008). Selective illumination deeper within a cell requires an alternative technique such as confocal microscopy. The basic concepts underlying confocal microscopy are the focusing of intense light at a particular plane within a sample, combined with a pinhole in the emission light path to ensure that only light arising from the illuminated plane is used to form an image. Confocal microscopy therefore allows "optical sectioning" of a sample. The depth of the optical section is determined by physical and hardware parameters. For example, opening up the pinhole diameter will increase the depth of the optical section. Removing the pinhole altogether will approximate a wide-field imaging system. A standard point-scanning confocal microscope will use galvanometer-mounted mirrors to scan a spot of high-intensity laser light across a sample in a raster scan, with the emitted fluorescence detected using a photomultiplier tube (PMT). The image is constructed pixel-by-pixel as the scan proceeds across the sample.

Although confocal microscopy can generate crisp, high-resolution images, there are a number of factors to consider in its application for live cell Ca^{2+} imaging. For example, the intensity of laser light and pixel dwell time (i.e., the speed of the scan) required to generate an image with sufficient signal to noise can induce photodamage (see below). This is typically seen as membrane blebbing, cell rounding, and retraction of adherent cells away from the imaged area. One option is to open up the confocal pinhole to allow more fluorescence signal to reach the PMT and reduce the scanning laser power. The resulting image will become blurred by out-of-focus light, and, with a large pinhole setting will look more like a wide-field image, somewhat obviating the use of a confocal system. Using a confocal system that has fast scanning capability, such as a spinning disk confocal, resonant scanning confocal, or an acousto-optical deflector (AOD) confocal can significantly reduce photodamage by reducing the pixel dwell times. These systems can capture images at hundreds of frames per second so they are ideally suited to imaging rapid intracellular events such as Ca^{2+} puffs and Ca^{2+} sparks.

Cell Damage and Indicator Tolerance

Prolonged irradiation of cells with intense light can result in cell damage. Because UV light has more energy than that in the visible spectrum, selecting a Ca^{2+} indicator that is excited by green or red light may reduce this problem. However, strong illumination at any wavelength of light may be sufficient to elicit a cellular response. For example, we found that excitation of rhodamine-based indicators (e.g., Rhod-2) in HeLa cells provoked Ca^{2+} signals via the production of reactive oxygen species (ROS; see below). The impact of excitation light should be examined by running control experiments with the same illumination intensity and duration, but without any other stimulus. If the indicator/cell combination is stable for the duration of a control recording then it is suitable for examining evoked Ca^{2+} responses. A further consideration is the impact of the indicator on the viability of the cells. The most widely used method for introducing Ca^{2+} indicators into cells is as a membrane-permeant acetoxymethyl ester, but this is not entirely benign. The liberation of the Ca^{2+}-sensitive indicator yields formaldehyde and acetic acid as by-products. Although there are few studies examining the effect of Ca^{2+} indicators on cell activity, it has been shown that indicator loading can reduce cellular ATP content. A further consideration is the buffering of Ca^{2+} by the indicators. Excessive loading of cells with indicator can severely reduce, or even abolish, the Ca^{2+} signals under observation (Richardson and Taylor 1993; Alonso et al. 2003).

Photobleaching and Photodamage

Photobleaching of a fluorophore results in irreversible loss of fluorescence and is a major impediment to many experimental regimes, especially those that involve rapid image acquisition or long duration. The main cause of photobleaching is the reaction of indicators with oxygen while in their excited state, thereby generating a nonfluorescent molecule. Different indicators have unique propensities to photobleach. For example, we observed that Fluo-4 showed a slower rate of bleaching than Fluo-3 in the

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same environment (Thomas et al. 2000). Those indicators that are more prone to bleaching generally undergo fewer rounds of excitation–emission before they lose their ability to fluoresce.

The continuous loss of indicator during an experiment can compromise the detection of Ca^{2+} signals, and the calibration of Ca^{2+} concentration (Becker and Fay 1987; Scheenen et al. 1996). Intense illumination of Ca^{2+} indicators can also give rise to the production of ROS, which can themselves trigger Ca^{2+} signals. This response recapitulates the events involved in photodynamic therapy where intense light is delivered to tumor cells containing a photosensitive compound (Hong et al. 2009). Cell-permeant ROS scavengers (e.g., ascorbate and Trolox) have been successfully used to reduce photobleaching (Scheenen et al. 1996). In some cases, the effect of photobleaching may be simply mathematically corrected because it follows a predictable exponential decay (Thomas et al. 2000). The simplest approach is to minimize photobleaching through optimization of experimental parameters, reducing the image acquisition rate or lowering the intensity of excitation light.

Indicator Leakage

Some cell types rapidly extrude or compartmentalize Ca^{2+} indicators. This is particularly evident with experiments performed at 37°C (Malgaroli et al. 1987). Indicators that are specific for a cellular compartment can accumulate in other places if conditions allow. For example, we routinely use Fluo-4 to monitor cytosolic Ca^{2+}. Under our standard conditions for loading Fluo-4AM (see Protocol 1: Loading Fluorescent Ca^{2+} Indicators into Living Cells [Bootman et al. 2013a]), we observe an even cytosolic accumulation of the indicator. However, with extended loading times, Fluo-4 will begin to accumulate in mitochondria.

If excessive indicator leakage or sequestration occurs, it is usually feasible to conduct experiments at a lower temperature. Although Ca^{2+} signaling systems are in part enzymatic and may be affected by temperature, cellular Ca^{2+} signaling systems can generally be faithfully observed below 37°C. Alternatively, a leakage-resistant Ca^{2+} indicator or a pharmacological block of multispecific organic anion transporters can be used (Di Virgilio et al. 1988). A few Ca^{2+} indicators have been designed to be more leakage resistant as a result of their zwitterionic nature. The most popular is Fura-PE3 (also called fura LeakRes; http://www.teflabs.com), which is essentially a leakage-resistant form of Fura-2 (Vorndran et al. 1995). Leakage-resistant Ca^{2+} indicators can be formed by conjugation to dextran, a large biologically inert molecule. Dextran-conjugated Ca^{2+} indicators do not leak, or become compartmentalized, under conditions in which other indicators are adversely transported. However, because they are designed not to be membrane-permeant, dextran-conjugated Ca^{2+} indicators need to be introduced into cells by microinjection or diffusion from a patch pipette.

IMAGING PARAMETERS

Because there are many different imaging approaches for monitoring fluorescent Ca^{2+} indicators, it is not possible to describe all potential experimental procedures. However, there are a number of key factors to consider in the setup of an experiment (such as choice of objective lens and image acquisition configuration) to ensure optimal image acquisition (see Protocol 1: Loading Fluorescent Ca^{2+} Indicators into Living Cells [Bootman et al. 2013a]).

The Objective Lens and Pixel Size

Imaging cellular Ca^{2+} signals usually requires a trade-off between factors such as image resolution, rate of image acquisition, and excitation intensity. It could be desirable to obtain high-resolution images at high speed with strong illumination. However, this is usually only possible for short duration experiments. A key determinant of the optical performance of any microscope is the objective lens. In particular, the maximum spatial resolution of a microscope is dependent on the numerical aperture (NA) of the objective lens. For fluorescence microscopy, the light-gathering power of an objective is proportional to the fourth power of the NA, and inversely proportional to the square of the

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TABLE 1. Comparison of the optical performance of common microscope objectives used for live cell microscopy.

Magnification	Numerical aperture	Brightness (normalized to 10× objective)	Spatial resolution (µm)
10×	0.45	1.0	0.75
20×	0.75	1.9	0.45
40×	1.3	4.4	0.26
60×	1.4	2.6	0.24
100×	1.4	0.9	0.24

Brightness values are normalized to the 10×, 0.45 NA objective. Spatial resolution is based on 550-nm light.

magnification. This means high-NA (but low-magnification) lenses are desirable to maximize the light gathering power of the system. NA and magnification are often proportional to each other. Table 1 lists features of typical commercially available objectives. It should be stressed that the actual achievable NA is often less than that stated, and that mismatch of the refractive indices of the lens, immersion fluid, and sample can lead to aberration and deterioration of the image. Consideration should be given to the use of water- or oil-immersion objectives depending on the specimen and depth of focus required (for a discussion, see http://www.microscopyu.com/articles/optics/waterimmersio-nobjectives.html). In particular, a water-immersion objective may be ideal if the experiment involves imaging cells in aqueous solution. Although it may be tempting to reach for the highest magnification objective, it may be better to use a lower power lens. Another way to ensure good light capture when using a high NA objective is to use a relay lens between the microscope and camera.

High-NA (1.4) objectives are ideal for monitoring subcellular Ca^{2+} signals, for example in confocal microscopy. But if the particular experiment is to sample global Ca^{2+} signals within individual cells in a field of view, then a lower magnification, lesser NA objective could be appropriate. To monitor Ca^{2+} oscillations in single cells, we routinely use 40× (NA 1.3) or 20x (NA 0.75) objectives. Although these lenses do not afford the best spatial discrimination, they have larger fields of view, and allow many cells to be simultaneously monitored. With a high-NA objective, the spatial resolution of the objective lens would be far greater than is experimentally required to detect global Ca^{2+} oscillations.

Another consideration with regard to the objective lens is how well it transmits UV light. As mentioned earlier, some of the most widely used Ca^{2+} indicators, such as Indo-1 and Fura-2 families, require UV excitation. Glass transmits UV poorly and specific UV-optimized objectives are necessary for excitation of these indicators. UV-compatible objectives are usually designated "Fluor," "Fluorite," "Fluar," etc. However, UV objectives are often not well corrected for spatial aberration, which can be problematic, especially toward the edges of the field of view. Phase-contrast objectives contain a light-attenuating phase-plate and should be avoided.

For single point-scanning confocal microscopes, the pixel size within an image is determined by factors such as the objective magnification, software zoom, and image frame size settings. For camera-based systems, pixel size is determined by the objective/system magnification, camera pixel size, and software "binning" settings. Most camera imaging systems allow the user to define the level of pixel binning from 1 × 1 (full resolution) to 4 × 4, or greater. Pixel binning essentially allows the fluorescence of a group of neighboring pixels to be summed, or averaged, and for those pixels to then be regarded as a single pixel. This in effect changes the image pixel size, and the same effect can be achieved with a point-scanning confocal by adjustment of the image "frame size." Although there is a loss of spatial resolution, fluorescent images become much brighter when pixel binning is used and discrimination from background can be improved. This may allow the excitation intensity to be decreased.

Bit Depth

Whether the detectors in an imaging system are PMTs or cameras, consideration has to be given to their setup. Almost all systems have controls to alter the sensitivity of the detectors to the incoming

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photons of light. These controls must be carefully set to use an appropriate amount of the available bit depth when capturing images.

The consideration of bit depth is important because it determines how well a system will be able discriminate Ca^{2+} signals of different amplitudes. Bit depth indicates the number of arbitrary levels (or gray levels) that a system can use to quantify the range of light intensities within an image. With 1-bit recording, all pixels that form an image could have two gray levels (black or white). Whereas, with 8-bit recording all pixels can have 256 gray levels (2^8). Therefore, an 8-bit image has better resolution for small changes in intensity. A bit depth of <8 bits was a significant problem with older imaging systems where strong signals could be easily saturated (see below), or small signals missed. By contrast, modern-day systems generally have at least 8 bits of image depth. Moreover, many cameras or PMTs are capable of 12- (4,096 gray levels) or 16-bit (65,536 gray levels) resolution. Although 12- and 16-bit images allow for greater discrimination between gray levels, this extra resolution increases memory requirements for image storage, and may not be necessary.

Detector Settings: Gain

A principal method for ensuring the appropriate use of the available bit depth is the adjustment of "gain" for the camera/PMT (sometimes denoted as "contrast"). This control regulates the amplification of the signal by the detector. Gain control is particularly important in Ca^{2+} imaging because it needs to be adjusted before an experiment is performed (Fig. 1). If the gain setting is too high, the signal may saturate the available bit depth and the peak of a Ca^{2+} signal will be lost. Alternatively, if the

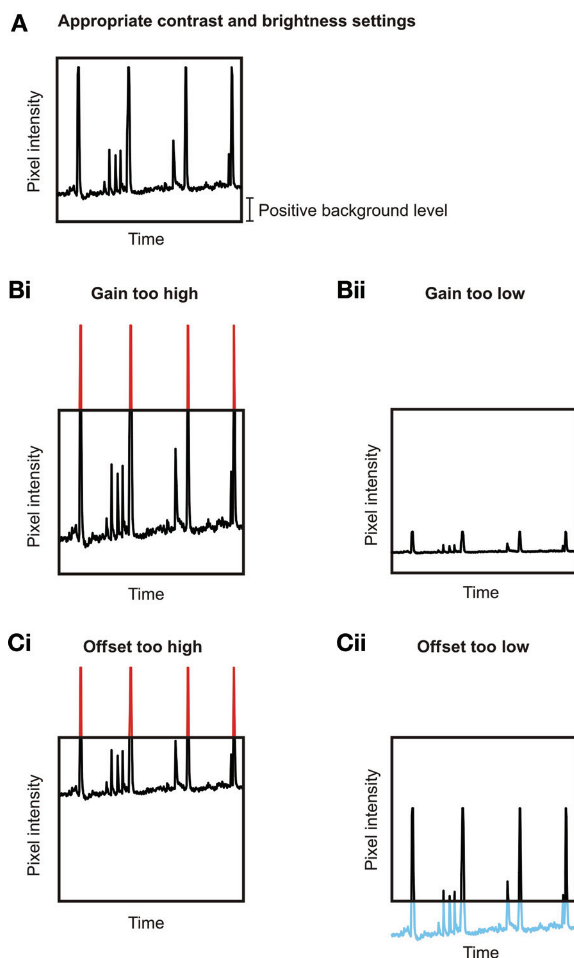

FIGURE 1. Effects of incorrect adjustment of gain and offset during Ca^{2+} imaging. Panel *A* depicts a real Ca^{2+} response recorded from a Fluo-4-loaded cardiac myocyte. It is evident that there are Ca^{2+} transients with different amplitudes. The large amplitude signals were Ca^{2+} waves, and the smaller events were Ca^{2+} sparks. Both types of event are clearly visible as the full bit depth of the imaging system was used for the recording. In Panel *B*, the same Ca^{2+} trace is presented, but now showing the effect of too high gain (*Bi*) or too low gain (*Bii*). In the former situation, the small Ca^{2+} signals have better detection, but the large amplitude responses saturate the detector (the red portions of the trace show parts of the signal that are saturating). With too low contrast, the large Ca^{2+} signals are still visible, but the small Ca^{2+} signals minimized. Panel *C* illustrates the effects of incorrect offset on the Ca^{2+} signal. Essentially, the *top* (*Ci*; offset too high) or *bottom* (*Cii*; offset too low) parts of the recording are missed (the blue portions of the trace show parts of the signal that are not recorded).

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gain setting is too low, a signal will only occupy a fraction of the bit depth and a Ca^{2+} rise will be poorly resolved. The necessary insight into the likely properties of a Ca^{2+} rise can only be achieved with some preliminary experimentation to determine the ideal gain settings. Ideally, the gain should be moderated so that Ca^{2+} signals use a broad portion of the bit depth of the system without saturation. This is critical in experiments where different amplitude Ca^{2+} signals may occur in the same recording, as shown in Figure 1.

Two important considerations that must be taken into account during the adjustment of gain are noise and autofluorescence. Almost all systems introduce random noise into the detected image because of electronic fluctuations. Autofluorescence results from sources within a biological preparation that are intrinsically fluorescent at the excitation wavelength used and is usually most prevalent with UV excitation and blue-green emission, that is, the channels used by Fura-2 and Indo-1. In practical terms, noise and autofluorescence only marginally influence most Ca^{2+} measurements because Ca^{2+} indicators generally give much greater signals. However, noise and autofluorescence can be a problem when cells are loaded poorly with fluorescent indicators. Increasing the gain to amplify a weak signal will also enhance both the noise and autofluorescence.

Detector Settings: Offset

The detectors in imaging systems have an “offset” control (sometimes “black level” or “brightness”). The offset determines the base intensity at which a camera or PMT detects a signal—presuming any signal in the detector below this level represents background signal. Decreasing the offset will darken images and nonfluorescent background areas will appear black. It is tempting to adjust the offset to produce a black background because this yields images with seemingly no noise. However, if the offset setting is too low, some real fluorescence emission will be lost and thereby invalidate the estimation of Ca^{2+} concentration (Fig. 1). To facilitate selection of appropriate offset and gain setting, most imaging systems use a look-up table (LUT) that alerts the user (by color codes) to the intensity of the pixels that are saturated, or that are zero intensity. Ideally the image should not contain many pixels flagged as zero, indicating that the background is low, but not below zero.

It is important to differentiate between the effect of adjusting camera/PMT offset and gain and altering the display settings for the visualization of images. With many imaging systems, it is possible to select various LUTs, adjust the “gray levels to view,” or alter the display contrast and brightness settings. These controls can dramatically alter the visualization of images, but they only affect the way that data is displayed and do not change the recorded information.

DATA PROCESSING AND CALIBRATION

The method of data analysis depends on the type of data collected (e.g., single- versus dual-wavelength Ca^{2+} indicator) and the required output (e.g., image sequence, plot of fluorescence intensity from a region of interest [ROI], or a calibrated Ca^{2+} measurement). Typically, data analysis proceeds by the removal of background signals (noise, autofluorescence) and the normalization or calibration of the true indicator fluorescence, followed by relevant data presentation.

Image Storage

With photometry or the simple extraction of fluorescence intensity values from groups of cells, the resulting ASCII or text data files are not large. However, imaging experiments have the potential to generate significant volumes of data. Consequently, a viable approach to image storage must be initially established. If possible, the best practice is to store original raw image files because they can be re-analyzed if necessary. However, image acquisition and storage can be reasonably demanding; an 8-bit camera capturing images of 512×512 pixels every 5 sec for 10 min (i.e., 120 images) will generate a file of $\sim$32 megabytes. This value will swell dramatically if the image format was larger, the bit-depth extended, or the number of images captured increased. Plausible storage options are raid

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drives with very large capacity. Portable data stores are CD-ROMs, DVDs BluRay disks, or portable hard drives. Disks and hard drives are not infallible, so multiple data copies are recommended. Given that terabyte memory devices are relatively cheap, the storage of data is not the headache that it once was. However, some consideration of the temporal/spatial resolution that is appropriate for the data needed could reduce long-term storage issues.

Background Removal

The subtraction of background signal is an important step because it has a significant effect on the apparent change in fluorescence. This is easily shown in the following simple example. Suppose the background pixel intensity equals 50 (arbitrary units), while the pixel intensity within a cell at rest equals 100. After stimulation, the pixel intensity in the cell increases to 250. Without background subtraction, the response would appear to be a 2.5-fold change in fluorescence (i.e., $250 \div 100 = 2.5$). However, with background subtraction, the response is a 4-fold change in fluorescence (i.e., $[250 - 50] \div [100 - 50] = 4$). This may not be critical if an experiment is simply designed to identify whether a response occurred or not. However, without background subtraction, it is not possible to accurately convert fluorescence into Ca^{2+} concentration or compare results between experiments using different imaging system settings. The dramatic effect of not having appropriate background subtraction is illustrated in Figure 2, which illustrates different quantitative output from the same experimental data set with or without background removal.

There are several ways in which background data can be obtained. For example, a "background image" without fluorescent cells can be captured at the start of an experiment and subtracted from all the subsequent experiment images. Alternatively, an averaged intensity value can be derived from a region of background pixels (excluded from fluorescent cells). This background intensity value can be subtracted from all the pixels in an image sequence, or simply from numerical data obtained by analyzing the fluorescence of cellular regions over time.

Uneven Illumination

If quantitative data are required, care should be taken to ensure even illumination of the field of view. This is particularly important when performing ratiometric measurements with two different excitation wavelengths; if the illumination is not uniform at each wavelength, the resulting ratio value will vary with respect to image location. Ideally, the illumination should be checked at the beginning of an experiment, and, if necessary, lamps or other parts of the imaging system should be appropriately adjusted. Because of the variation in the spectral output of the arc in mercury and xenon bulbs, it is frequently not possible to adjust a system to give even illumination at all wavelengths. In this case, an alternative approach can be used. The best way to correct for uneven illumination is to acquire images during a calibration procedure. For example, with Fura-2 it would be appropriate to obtain images of a uniform layer of the Ca^{2+}-saturated indicator at 340-nm excitation (i.e., without cells), and images of the Ca^{2+}-free indicator at 380-nm excitation. A uniform layer of Fura-2 ($\sim$50 µM) can be imaged using a thin chamber constructed from two coverslips separated by a thin film of plastic wrap (see Protocol 2: Converting Fluorescence Data into Ca^{2+} Concentration [Bootman et al. 2013b]). In fact, this chamber with indicator should ideally be used before experimentation to adjust for uniform illumination. These images should be stored as a reference (denoted here as "field reference"). The data images captured during an experiment should be divided on a pixel-by-pixel basis by the appropriate field reference image and multiplied by the mean intensity of the data image. This approach corrects for non-uniform excitation without changing the average fluorescence intensity of the data image. These corrected images can then be used for ratiometric measurements. Our experience suggests that this method works best if there are experimental and field reference images that have similar intensity ranges.

An additional adjustment that is required for ratiometric imaging is that the excitation intensity at each wavelength is adjusted to match the resultant fluorescence to the bit-depth of the system. With Fura-2, the maximal fluorescence intensity at 340 nm is approximately four times greater than at

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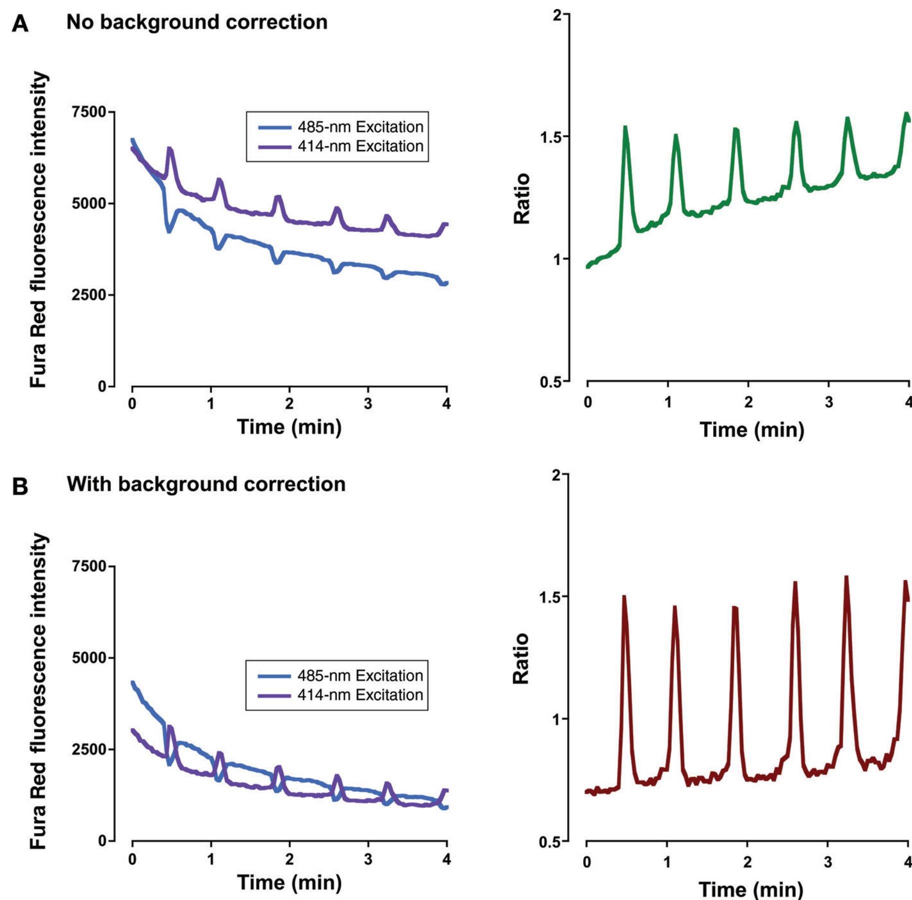

FIGURE 2. The effect of background correction on Ca^{2+} measurement using fluorescent indicators. The figure shows an experiment in which a Fura-Red-loaded HeLa cell was stimulated with histamine to evoke Ca^{2+} oscillations. Panel *A* depicts the ratiometric measurement of fluorescence without background subtraction. The data appear to show Ca^{2+} oscillations superimposed on an elevating baseline. Panel *B* shows the same data, but after subtraction of background from each image using the rolling ball procedure. The baseline Ca^{2+} value is now not drifting upward and the peaks of the Ca^{2+} oscillations are larger. The cells were loaded with Fura Red by incubation with 1 μM Fura Red AM for 30 min, followed by 45 min de-esterification. The Fura Red was alternately excited at two wavelengths (D414/30 and HQ485/15 filters) to monitor Ca^{2+}-bound and Ca^{2+}-free indicator (emission was sampled using a 650/50 filter). Images were acquired with a 40×, 1.3-NA objective. An image pair (i.e., images at both excitation wavelengths) was collected every 2 sec. After 1 min of basal recording 10 μM histamine was added to elicit Ca^{2+} oscillations.

380 nm if the illumination intensity is equal. Because the output of 340-nm and 380-nm light by the excitation light source varies and the transmission of 340-nm light by the microscope optics is considerably less than that of 380 nm, it is unlikely that in practice the excitation intensities at 340 nm and 380 nm are equal. However, by using the thin chamber with indicator dye, the maximal fluorescence intensities can be adjusted to be similar by the combination of neutral density filters with the excitation bandpass filters.

A more problematic situation can arise when an experiment has been performed, the illumination is found to be uneven, and it is not possible to obtain additional images. In this situation, post-hoc image manipulation can be attempted as a last resort. One method for reducing the effect of uneven illumination when attempting background removal is the “rolling ball” subtraction method. This can be an effective tool to remove background on a pixel-by-pixel basis, albeit requiring user input and computer time. Because this is an ImageJ application (see section on ImageJ below), it will work on all standard image formats. An example of the utility of background correction using the rolling ball method is shown in Figure 3.

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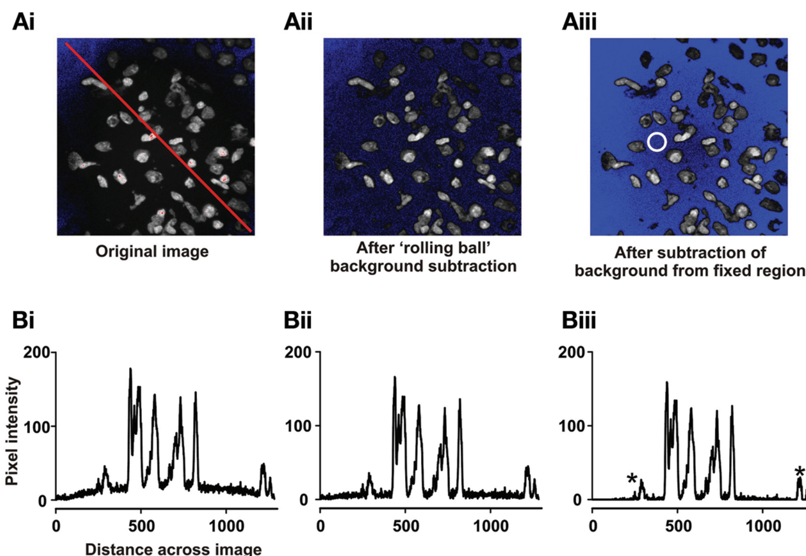

FIGURE 3. Rolling ball method for uneven background correction. The figure shows the effect of a simple method for correcting uneven background illumination. Panel *Ai* depicts an original image of Fura-Red-loaded cells. The background is less intense at the sides of image. Panel *Aii* shows the same image as *Ai*, but following rolling ball background subtraction. Panel *Aiii* illustrates that removing a fixed background value (sampled from the region within the white circle) does not resolve the background gradient. Panels *Bi*–*Biii* shows profiles of fluorescence intensity across the area marked by the red line in *Ai*. The background gradient is evident in *Bi*, but less so in *Bii* following rolling ball background subtraction. Subtracting a fixed background value removes most of gradient, but impacts on the amplitude of the real fluorescence signals. If the fixed background region is chosen from an area of high intensity, then some real fluorescence signal is lost. This is evident from the reduced amplitudes of the peaks in *Biii* (e.g., peaks marked with *).

Calibration of Fluorescence into Ca^{2+} Concentration

The calibration procedure is similar for dual- and single-wavelength indicators, and involves measuring the fluorescence intensity in Ca^{2+}-free or Ca^{2+}-saturating conditions at the appropriate excitation or emission wavelengths (see Protocol 2: Converting Fluorescence Data into Ca^{2+} Concentration [Bootman et al. 2013b]). The mathematical conversion of the fluorescence emission to Ca^{2+} concentrations requires that the affinity K_d of the indicator is known. This is usually empirically determined using absolute standards.

Once the background is subtracted, the images can be ratioed between appropriate wavelength images ($F_{\lambda1}/F_{\lambda2}$) if a dual-wavelength indicator was used. For example, with Fura-2, data is often presented as a 340/380 ratio; the intensity of emitted light recorded with 340 nm excitation divided by the intensity of emitted light recorded with 380 nm excitation. Alternatively, if a single wavelength indicator was used, the later images are ratioed with the initial image (F/F_0 or $\Delta F/F_0$) as described above. After this processes, it is possible to extract the change in fluorescence ratio with respect to time in user-defined ROIs (usually cells, or areas within cells) from the image sequence. Wavelength ratios can be converted to Ca^{2+} concentrations with additional calibration data.

An issue that has plagued Ca^{2+} literature for many years is the discrepant response of Ca^{2+} indicators in the nucleus and cytosol. It is important to realize that the K_d of indicators for Ca^{2+} and their fluorescent properties are different in the cytosol and nucleus. We find that most Ca^{2+} indicators have a greater resting fluorescence, and larger relative response, in the nucleus compared to their signal in the cytosol (Thomas et al. 2000). Careful calibration of the responses, using distinct values for affinity, etc., shows that the Ca^{2+} concentration is equivalent in both compartments. Because the nucleus occupies a large proportion of the cell volume and fluorescent indicators are much brighter in the nucleus, most cellular Ca^{2+} recordings will be dominated by the response of the nucleus. This is particularly true for wide-field imaging, where it is generally not possible to distinguish the cytosolic and nuclear boundary. By contrast, confocal microscopy provides

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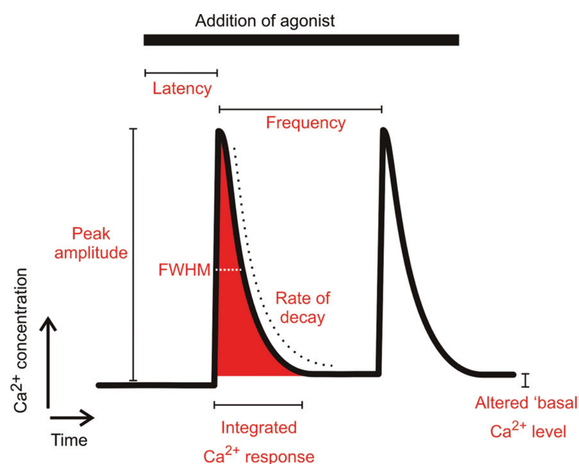

FIGURE 4. Suggested parameters for measuring cellular Ca^{2+} signals. The cartoon depicts an idealized oscillatory Ca^{2+} signal evoked by application of an agonist, and several suggested parameters that can be used to monitor the response. FWHM, full-width at half-maximal amplitude.

the spatial resolution so that Ca^{2+} within the cytosol and nucleus can be distinguished. It is plausible that cells generate real differences between nuclear and cytosolic Ca^{2+} responses. Indeed, there is a growing literature that nuclear Ca^{2+} signals may be discrete from the cytosol (Bootman et al. 2009). However, this cannot be assumed. In many cases nuclear and cytosolic Ca^{2+} concentration are in equilibrium, with the nuclear envelope simply introducing a kinetic delay in the movement of Ca^{2+} between compartments.

Data Presentation

The presentation of Ca^{2+} responses is very much determined by the message that is being conveyed. In the majority of situations, showing traces of evoked Ca^{2+} responses and associated statistical analysis is sufficient. We generally analyze multiple parameters to characterize cellular responses to stimulation (Fig. 4). Calculating the proportion of cells showing a detectable Ca^{2+} signal is useful, as it gives a measure of the threshold and efficacy of a stimulus. In addition, peak Ca^{2+} signal amplitude, latency, area integrated Ca^{2+} signal, and frequency of Ca^{2+} transients can be used to provide concentration-response relationships for different stimuli. The parameters most appropriate depend on the cell type and nature of the experiment. For example, some cell types display all-or-none responses where the amplitude of their Ca^{2+} signals is not affected by the stimulus concentration, whereas other cell types have smoothly graded peak Ca^{2+} signals (Bootman et al. 1994). For the former cell type, peak Ca^{2+} signal or an integrated Ca^{2+} signal has little use, but the proportion of responding cells could be used to describe the response to the stimulus.

SAMPLE MOUNTING, PERFUSION, AND SOLUTION EXCHANGES

If at all possible, samples should be prepared on optically optimum substrates. Most high-NA objectives are designed for use with glass coverslips, typically with a refractive index of 1.52 and 170 µm thick. Most adherent cells can be cultured directly on glass. Nonadherent cells can often be immobilized by coating the surface of the coverslip with synthetic substrates (e.g., poly-D-lysine) or extracellular matrix proteins (e.g., collagen).

Suitable coverslips are available in a range of diameters. We routinely use 16- or 22-mm diameter #1.5 coverslips. Larger coverslips provide an increased area over which an objective can be scanned. However, we have found that coverslips in excess of 22 mm are less rigid and can cause focal drift during experiments. Furthermore, 16- or 22-mm coverslips fit well into 35-mm culture dishes and six-well plates. For use, the coverslips can be placed in custom-made chambers (an example is shown in Fig. 5) or commercially resourced chambers (e.g., Atto chamber, Harvard Apparatus, Bioptechs).

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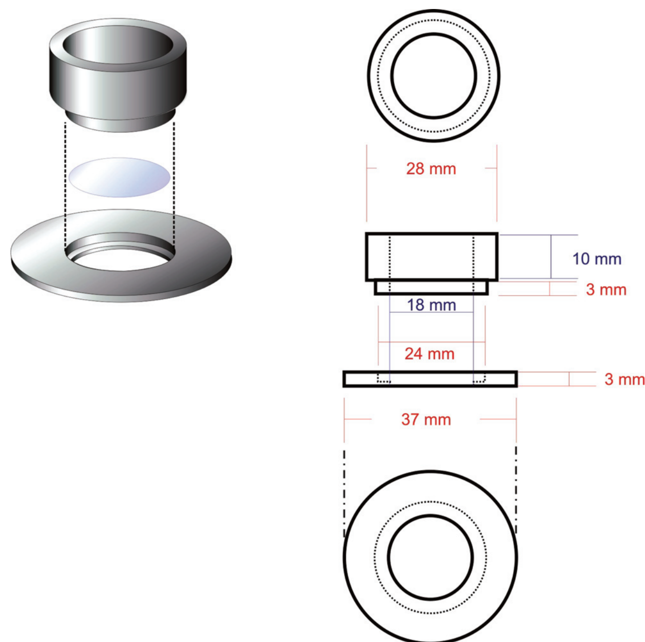

FIGURE 5. Customized design for an imaging chamber. The figure depicts the dimensions of a typical imaging chamber designed to use 22-mm diameter coverslips. The outer dimensions of the chamber can be altered to fit particular microscope stages.

Transferring a coverslip to the imaging chamber is best done with fine-tipped tweezers, but care needs to be taken not to crack the coverslip. It is advisable to seed more coverslips with cells than are required for an experiment, so that there are sufficient replacements. It is advisable to mount a coverslip within the chamber before indicator loading—this minimizes the frustration and lost time if a coverslip is dropped or cracked. Chambers can be made water tight using inert silicone grease. Care should be taken not to let the grease come into contact with the cells because it will kill them. The bottom of the glass coverslip should be wiped with a tissue when the chamber is constructed. Otherwise there will be a residual carry-over of culture medium. When the water evaporates, the salts within the medium will be left behind and form an obvious layer on the glass surface. This can hinder image resolution.

Characterization of Ca^{2+} signals typically requires the addition/removal of an agonist/antagonist. Media exchanges often cause experimental issues, such as focal drift or detachment of cells. The simplest method for media exchange is bolus addition of a solution using a large-bore pipette. An aspiration system should be used concurrently to maintain a constant volume in the chamber. We generally use a bent and blunt 16-gauge needle positioned in one side of the chamber (Fig. 6). As with all sharp items, care should be taken when handling the needle. The height of the aspiration system is at the user's discretion. Typically, we allow 0.5–1 mL volume in the chamber. However, smaller volumes would be suitable if expensive reagents are being used. A key factor is that the cells should not dry out in the chamber or experience dramatic mechanical effects from solution changes. Sophisticated, multichannel perfusion systems, allowing fast exchange and rapid alternation of bath solutions are commercially available (e.g., http://www.wpiinc.com/index.php/vmchk/MPS-2.html,

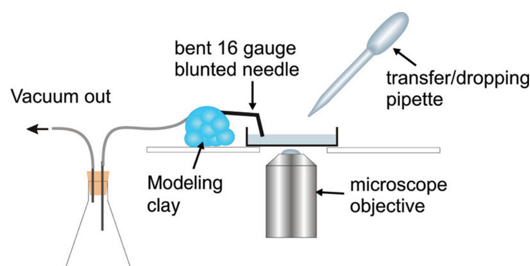

FIGURE 6. Simple superfusion setup for solution change during Ca^{2+} imaging experiments. The figure depicts the typical superfusion system that we use for routine imaging experiments with adherent cells. Most cell types tolerate bolus addition of agonists without problem. The speed of fluid addition should be moderated if cells detach, or mechanically activated responses are seen.

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http://www.biosciencetools.com/catalog/perfusionmini.htm). In addition to allowing fast solution exchange, such systems can be automated to allow precise repetition of experiments while freeing the user.

ANALYZING IMAGING DATA WITH IMAGEJ

ImageJ is an open-source software application from the National Institutes of Health for scientific image processing and analysis. It is free to download and, because it is a Java-based system, ImageJ will run on Windows, Mac, and Linux workstations (http://rsbweb.nih.gov/ij/). ImageJ is fairly intuitive and a great deal of helpful hints can be found on-line. Below, we discuss a few of the more typical ImageJ applications that are relevant to Ca^{2+} imaging.

Importing Data

Imaging data is acquired as a time series and saved as either a multi-file format or a single-file format. In the multi-file format, each file corresponds to one field of view, one channel, and one time point. In the single-file format, all of the images, channels, and time points are saved as a single file. This single file can be a multi-page TIFF file (also known as a "TIFFstack") or proprietary file format from the acquisition software vendor. Each image in a multi-page image stack is often referred to as a "slice."

ImageJ can open multi-page TIFF stacks via the *File* > *Open* menu command. Multi-file image series (one image per file) can be opened with the *File* > *Import* > *Image Sequence* command. There are several options for the ordering of these files and the user must ensure the files are imported in the correct time sequence.

The advantage of the proprietary file format is that meta-data can be stored directly with the image. This typically includes pixel size, acquisition parameters, and channel information. The disadvantage is that ImageJ does not support many proprietary file formats directly. Installation of the Bio-Formats ImageJ plugin (http://loci.wisc.edu/software/bio-formats) from the LOCI group in Wisconsin (http://loci.wisc.edu/) solves this problem so that ImageJ can seamlessly open most proprietary formats along with the image metadata via the *File* > *Open* command.

Background Subtraction

The first step after importing an image stack would be to perform a background subtraction. As described above, the ideal situation would be to simply subtract the intensity of a noncellular region. However, if the illumination is not even across the field of view, a suitable region often cannot be found. If a field reference image (described in the Uneven Illumination section above) was obtained then the experimental images may be corrected as suggested earlier. In the situation where no field reference is available, images within a stack may be corrected using the rolling ball method.

The menu command *Process* > *Background Subtraction* will open a dialog for the user to set some parameters for a rolling ball background subtraction. As described above, the rolling ball background subtraction is a way to correct for uneven background. One way to visualize the rolling process is to think of the image as topography, where bright areas (i.e., cells) are "high ground" and dark areas are "low ground." Now invert the topography so that it is upside down, and the bright areas become "potholes" in the ground. If you roll a large ball across the inverted topography then the lowest point of the ball corresponds to the ground level *so long as the ball does not fall into the holes*. The ground level at each point is then subtracted to create a new background corrected image. The most important option to select for the rolling ball background subtraction is the ball radius, so that it finds the background and the ball does not fall into the potholes. It is best to err on the side of being too large—50 pixels is often a good starting point. The user should ensure the cells centers are not being erased.

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Analyze Intensity Over Time

The polyline selection tool from the main ImageJ tool window (third from the left) is the simplest way to select individual cells to analyze. The selected region is referred to as an ROI. Once the cell is selected, the intensity over time can be quantified by the menu command *Image > Stacks > Plot Z-axis profile*. The image stack is in effect a three-dimensional (3D) data set: X, Y, and T. ImageJ presumes 3D data sets to be *XYZ*, which is somewhat confusing at first, but does not change the analysis. The intensity profile plot should appear along with the numerical intensity values as a table, which can be copied to a spreadsheet.

Analyzing one cell is useful for a quick inspection of data, but is inefficient when analyzing many cells per image. This can be expedited by drawing around each cell and adding the ROI to the ROI manager. The ROI manager can be opened via the menu command *Analyze > Tools > ROI Manager*. Once a cell is selected, it can be added to the list of ROIs in the ROI Manager via the *Add* button on the ROI manager dialog or more simply with the "T" key on the keyboard. When all the cells are selected (selections can be made visible by the "Show All" checkbox in the ROI manager dialog), they can be analyzed via the *More >> MultiMeasure* button. Select *Measure all slices* and *One row per slice*. A table with the intensity over time for each cell will appear in the Results window.

SUMMARY

Imaging cells loaded with fluorescent indicators is a powerful method for measuring cellular Ca^{2+} concentration with good spatial and temporal resolution. The experimental procedures for using Ca^{2+} indicators are relatively easy and usually work robustly for primary cells and cell lines. The considerable brightness of fluorescent Ca^{2+} indicators typically gives signals with excellent signal-to-noise. However, despite their ease of use, there are numerous issues that require consideration to ensure optimal quantitative data output and minimal cell invasiveness.

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REFERENCES

Alonso MT, Chamero P, Villalobos C, Garcia-Sancho J. 2003. Fura-2 antagonizes calcium-induced calcium release. *Cell Calcium* **33:** 27–35.

Becker PL, Fay FS. 1987. Photobleaching of fura-2 and its effect on determination of calcium concentrations. *Am J Physiol* **253:** C613–C618.

Berridge MJ. 2006. Calcium microdomains: organization and function. *Cell Calcium* **40:** 405–412.

Berridge MJ, Lipp P, Bootman MD. 2000. The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* **1:** 11–21.

Bootman MD, Cheek TR, Moreton RB, Bennett DL, Berridge MJ. 1994. Smoothly graded Ca^{2+} release from inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores. *J Biol Chem* **269:** 24783–24791.

Bootman MD, Lipp P, Berridge MJ. 2001. The organization and functions of local Ca^{2+} signals. *J Cell Sci* **114:** 2213–2222.

Bootman MD, Fearnley C, Smyrnias I, MacDonald F, Roderick HL. 2009. An update on nuclear calcium signaling. *J Cell Sci* **122:** 2337–2350.

Bootman MD, Rietdorf K, Collins T, Walker S, Sanderson M. 2013a. Loading fluorescent Ca^{2+} indicators into living cells. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot072801.

Bootman MD, Rietdorf K, Collins T, Walker S, Sanderson M. 2013b. Converting fluorescence data into Ca^{2+} concentration. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot072827.

Di Virgilio F, Fasolato C, Steinberg TH. 1988. Inhibitors of membrane transport system for organic anions block fura-2 excretion from PC12 and N2A cells. *Biochem J* **256:** 959–963.

Griesbeck O, Baird GS, Campbell RE, Zacharias DA, Tsien RY. 2001. Reducing the environmental sensitivity of yellow fluorescent protein. Mechanism and applications. *J Biol Chem* **276:** 29188–29194.

Grynkiewicz G, Poenie M, Tsien RY. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260:** 3440–3450.

Hong X, Jiang F, Kalkanis SN, Zhang ZG, Zhang X, Zheng X, Jiang H, Chopp M. 2009. Intracellular free calcium mediates glioma cell detachment and cytotoxicity after photodynamic therapy. *Lasers Med Sci* **24:** 777–786.

Kurebayashi N, Harkins AB, Baylor SM. 1993. Use of fura red as an intracellular calcium indicator in frog skeletal muscle fibers. *Biophys J* **64:** 1934–1960.

Lipp P, Niggli E. 1993. Ratiometric confocal Ca^{2+}-measurements with visible wavelength indicators in isolated cardiac myocytes. *Cell Calcium* **14:** 359–372.

Lohr C. 2003. Monitoring neuronal calcium signaling using a new method for ratiometric confocal calcium imaging. *Cell Calcium* **34:** 295–303.

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Luik RM, Wang B, Prakriya M, Wu MM, Lewis RS. 2008. Oligomerization of STIM1 couples ER calcium depletion to CRAC channel activation. *Nature* **454:** 538–542.

Malgaroli A, Milani D, Meldolesi J, Pozzan T. 1987. Fura-2 measurement of cytosolic free Ca^{2+} in monolayers and suspensions of various types of animal cells. *J Cell Biol* **105:** 2145–2155.

Minta A, Kao JP, Tsien RY. 1989. Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J Biol Chem* **264:** 8171–8178.

Nagai T, Sawano A, Park ES, Miyawaki A. 2001. Circularly permuted green fluorescent proteins engineered to sense Ca^{2+}. *Proc Natl Acad Sci* **98:** 3197–3202.

Nagai T, Yamada S, Tominaga T, Ichikawa M, Miyawaki A. 2004. Expanded dynamic range of fluorescent indicators for Ca^{2+} by circularly permuted yellow fluorescent proteins. *Proc Natl Acad Sci* **101:** 10554–10559.

Proven A, Roderick HL, Conway SJ, Berridge MJ, Horton JK, Capper SJ, Bootman MD. 2006. Inositol 1,4,5-trisphosphate supports the arrhythmogenic action of endothelin-1 on ventricular cardiac myocytes. *J Cell Sci* **119:** 3363–3375.

Richardson A, Taylor CW. 1993. Effects of Ca^{2+} chelators on purified inositol 1,4,5-trisphosphate (InsP3) receptors and InsP3-stimulated Ca^{2+} mobilization. *J Biol Chem* **268:** 11528–11533.

Scheenen WJ, Makings LR, Gross LR, Pozzan T, Tsien RY. 1996. Photodegradation of indo-1 and its effect on apparent Ca^{2+} concentrations. *Chem Biol* **3:** 765–774.

Shimozono S, Fukano T, Nagai T, Kirino Y, Mizuno H, Miyawaki A. 2002. Confocal imaging of subcellular Ca^{2+} concentrations using a dual-excitation ratiometric indicator based on green fluorescent protein. *Sci STKE* **2002:** l4.

Thomas D, Tovey SC, Collins TJ, Bootman MD, Berridge MJ, Lipp P. 2000. A comparison of fluorescent Ca^{2+} indicator properties and their use in measuring elementary and global Ca^{2+} signals. *Cell Calcium* **28:** 213–223.

Truong K, Sawano A, Miyawaki A, Ikura M. 2007. Calcium indicators based on calmodulin fluorescent protein fusions. *Methods Mol Biol* **352:** 71–82.

Tsien RY. 1980. New calcium indicators and buffers with high selectivity against magnesium and protons: Design, synthesis, and properties of prototype structures. *Biochemistry* **19:** 2396–2404.

Tsien RY, Pozzan T, Rink TJ. 1982. T-cell mitogens cause early changes in cytoplasmic free Ca^{2+} and membrane potential in lymphocytes. *Nature* **295:** 68–71.

Vorndran C, Minta A, Poenie M. 1995. New fluorescent calcium indicators designed for cytosolic retention or measuring calcium near membranes. *Biophys J* **69:** 2112–2124.

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Protocol 1

Loading Fluorescent Ca^{2+} Indicators into Living Cells

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Small-molecule fluorescent Ca^{2+} reporters are the most widely used tools in the field of Ca^{2+} signaling. The excellent spatial and temporal resolution afforded by fluorescent reporters has driven the understanding of Ca^{2+} as a messenger in many different cell types. In many situations, the cellular loading and monitoring of fluorescent Ca^{2+} indicators is quite trivial. However, there are numerous pitfalls that require consideration to ensure that optimal data are recorded. Fluorescent Ca^{2+} indicators have carboxylic acid groups for binding of Ca^{2+}. Because these "free-acid" forms of the indicators are hydrophilic they cannot readily cross cell membranes and need to be introduced into cells using techniques such as microinjection, pinocytosis, or diffusion from a patch pipette. However, the most convenient and widely used method for loading indicators into cells is as hydrophobic compounds in which the carboxylic acid groups are esterified (commonly as acetoxymethyl [AM] or acetate esters). The ester versions of the indicators permeate the plasma membrane. The Ca^{2+}-sensitive, free-acid form of the indicator is liberated following hydrolysis of the ester groups by intracellular esterases.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

Reagents

Buffered salt solution (see Step 2)

Ca^{2+} indicator of choice (in ester form)

Ca^{2+} indicators are generally purchased as powders that need to be dissolved before use (see Step 1).

Cells grown on circular glass coverslips (16 or 22 mm, depending on the experiment)

Alternatively, cells can be grown free in suspension. Before imaging, they must be allowed to settle and attach to the glass, so that they are not washed away with solution changes.

Pluronic F-127 (20% [w/v]) in dimethyl sulfoxide (DMSO)

Pluronic F-127 dissolved in DMSO is available commercially and we recommend buying this reagent. To prepare the reagent in the laboratory, dissolve pluronic F-127 in an appropriate volume of dry DMSO to give a final concentration of 20% (w/v). Stir the mixture until the pluronic F-127 is dissolved. This procedure may

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require warming. Do not allow the temperature to exceed 40°C. Noxious fumes will be produced, so use a properly ventilated cabinet. The mixture is stable for ~6 mo at room temperature.

Equipment

Imaging system

Sonicator or vortex mixer

METHOD

Loading Cells with the Ester Form of a Ca^{2+} Indicator

Because Ca^{2+} indicators are light-sensitive, perform as many steps as possible in reduced light.

1. Prepare a suitable, high-concentration stock of Ca^{2+} indicator (e.g., 1 mM) by dissolving the powder in an appropriate volume of 20% pluronic F-127 in DMSO. Vortex or sonicate as necessary for full dissolution. This can be stored in the dark at –20°C for up to several weeks.

   DMSO serves as a solvent and is required to maintain stability during storage. Pluronic acid acts as a detergent to help keep the indicator in solution when it is diluted to the working concentration in saline (in Step 2). Pluronic acid has been shown to enhance Ca^{2+} indicator loading. It works by preventing the amphipathic Ca^{2+} indicator molecules from forming micelles.

2. Before application to cells, dilute the stock solution of Ca^{2+} indicator to an appropriate concentration (e.g., 2 µM) using an appropriate buffered salt solution (e.g., Ringer's solution or cell culture medium). Vortex or sonicate the diluted indicator solution to prevent formation of micelles, and thereby enhance loading.

   The concentration of Ca^{2+} indicator ester required for loading will depend on the cell type, and usually has to be established through trial and error. Some cell types appear to load and hydrolyse the indicator ester rapidly, whereas others are more difficult to load, or are able to extrude the indicator. For most of the cell types we study, we prepare a loading solution containing 2 µM Ca^{2+} indicator ester by addition of 4 µL of the 1 mM stock solution to 2 mL of the experimental salt solution. Although 2 µM is appropriate for loading many cell types, occasionally much higher indicator concentrations are required (e.g., >10 µM).

   We typically dilute the Ca^{2+} indicator stock with the same HEPES-buffered salt solution that the cells will be incubated in during subsequent experiments. However, Ca^{2+} indicators can be loaded into cells maintained in serum-replete culture medium.

3. If appropriate, wash cells free of serum before applying the Ca^{2+} indicator loading solution.

   The presence or absence of serum during Ca^{2+} indicator loading and subsequent experiments must be rigorously considered. Constituents of serum can sequester indicators, thereby reducing their free concentration and inhibiting loading. For acute experiments, the continual presence of serum is often not required. However, the absence of serum can lead to the rapid induction of autophagy, which could be a confounding cellular response.

4. Add a sufficient volume of the Ca^{2+} indicator loading solution to cover the cells entirely. (Our coverslips are placed in a water-tight chamber with the cells facing upward. Typically we add 0.5–2 mL of the loading solution to the cells within the chamber.) Then incubate the cells in a dark place for an appropriate length of time (e.g., 30 min) to allow the Ca^{2+} indicator ester to permeate the cells and be hydrolysed by intracellular esterases to the Ca^{2+}-sensitive free-acid form (Tsien 1981).

   The duration of the incubation is dependent on the cell type, and should be determined through trial and error. For most of the cell types that we study, a period of 30 min is sufficient. Once the concentration of the Ca^{2+} indicator loading solution and the loading time are established, they should be rigorously adhered to. Changing either parameter will alter the amount of Ca^{2+} indicator within the cells, and could lead to disparate responses between experiments.

   The temperature at which Ca^{2+} indicator loading takes place also needs to be carefully considered. We find that indicators generally load into cells more consistently at room temperature (i.e., 18°C–22°C). At higher temperatures, some cell types can extrude indicators, or sequester them into organelles. If

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experiments are intended to be performed at temperatures >22°C, it is plausible to perform the Ca^{2+} indicator loading at room temperature and then gently warm the cells.

See Troubleshooting.

5. When the prescribed incubation time with the Ca^{2+} indicator loading solution has expired, wash the cells a couple of times with indicator-free saline solution. To allow complete de-esterification of the indicator, leave the cells for an additional 20–30 min before use. During this period of time, gradually adjust the cells to the required experimental temperature (e.g., 37°C) if necessary.

Setting Up a Ca^{2+} Imaging Experiment

Fluorescence indicators allow Ca^{2+} changes to be monitored with high spatial and temporal resolution in living cells. There are turn-key systems available from multiple manufacturers, in addition to numerous home-made fluorescence imaging systems. It is therefore not possible to write a general method that will apply to every imaging device. However, the following are typical steps, and potential pitfalls, that need to be considered in the design of an experiment to image cells loaded with a fluorescent Ca^{2+} indicator.

6. Turn on the imaging system before the experiment with sufficient time for the electronics to synchronize, and for the light sources to have stable emission.

   The latter is particularly important with arc lamps and gas lasers; light-emitting diodes and solid-state lasers are virtually instantaneous.

7. Mount the coverslip-bearing Ca^{2+} indicator-loaded cells into a chamber suitable for imaging and reagent superfusion (if this has not yet been done). (This chamber should be stably installed on the stage of the microscope connected to the imaging system.)

8. Set the imaging system's controls (light intensity, gain, and offset) so that the cells are well-illuminated and visible, but will not be irradiated to the extent that indicator bleaching or cell damage occurs. In addition, ensure that the background pixels (in non-cellular regions) have a positive value (sometimes called a positive offset).

   Most imaging systems have a "range check" look-up table that encodes pixel intensity into color. This can be used to make sure the background pixels have a positive value. The system gain is a key parameter in determining how much of the bit depth of the system will be used in the recording of a signal. Ideally, a large proportion of the bit depth should be used, but care needs to be taken to ensure that the light output during the experiment does not saturate the system. Of course, the amplitude of a Ca^{2+} signal cannot be known before it has occurred, so setting the gain requires some considered guesswork, or a trial experiment.

9. Set the imaging parameters (e.g., experiment duration, image capture frequency, number of wavelengths).

10. After the cells are appropriately loaded with a Ca^{2+} indicator, the imaging system is primed with appropriate settings, and reagent solutions are ready to apply to the cells, initiate the experiment.

    Ideally the imaging system will allow a real-time readout of the fluorescence intensity of the cells within a field of view. This allows the user to follow the progression of an experiment, and to terminate data collection if cells detach or if the focus drifts. Generally, cells can be individually outlined and independently monitored during an experiment. See Troubleshooting.

TROUBLESHOOTING

Problem (Step 4): Cells do not load with Ca^{2+} indicator.

Solution: For reasons that are not always understood, some cell types do not load well with the ester forms of Ca^{2+} indicators. Alternative methods for Ca^{2+} indicator loading (including microinjection, transient cell permeabilization, or pinocytic uptake of extracellular indicator) can be applied. These methods allow the introduction of the free-acid (Ca^{2+}-sensitive) forms of indicators. Alternatively, cell transfection methods can be used to introduce genetically encoded Ca^{2+} indicators.

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Problem (Step 4): Cells load with Ca^{2+} indicator, but it rapidly leaks out.

Solution: A plausible strategy to prevent leakage of Ca^{2+} indicators is to block MDR-mediated anion transport. The compound used most often for this purpose is sulfinpyrazone, which is a uricosuric medication commonly used in the treatment of gout. We have used sulfinpyrazone in situations where the loading of fluorescent Ca^{2+} indicators (or subsequent experiments) needs to be performed above room temperature (Bootman et al. 1992). Sulfinpyrazone is highly lipophilic and will therefore need to be dissolved in a nonpolar solvent such as DMSO. Since the AM forms of indicator are also reconstituted in DMSO (see above), care must be taken not to exceed deleterious levels of nonpolar solvent. We typically use final sulfinpyrazone and DMSO concentrations of 100 µM and 0.1%, respectively. An alternative to sulfinpyrazone is sulfobromophthalein, which has been shown to help Ca^{2+} indicator retention in lung slices (Perez and Sanderson 2005). A further alternative is probenecid, but this appears to be effective at higher concentrations (e.g., 1 mM) than sulfinpyrazone. To date, we are unaware of any effects of sulfinpyrazone or sulfobromophthalein on Ca^{2+} signal transduction. However, as with all drugs, they should be used with caution.

Problem (Steps 4, 10): The loading and/or responsiveness of the Ca^{2+} indicator deteriorates over time, even when stored frozen.

Solution: In practice, the usefulness of an indicator declines as soon as it is reconstituted in pluronic acid/DMSO. Our empirical observations suggest that deterioration rate depends on the indicator type. For example, we find Rhod-2 to be rapidly labile (within weeks), whereas Fura-2 is relatively stable (useable for several weeks). Some indicators appear to load into cells and are brightly fluorescent even though they do not faithfully report Ca^{2+}changes. If the cellular loading and/or responsiveness of a Ca^{2+} indicator declines, the stock solution should be discarded and a fresh batch of indicator prepared. The key is to only make up small quantities of the Ca^{2+} indicator and to discard it as soon as its performance declines.

RELATED INFORMATION

For a detailed discussion of practical considerations and potential problems regarding the use of fluorescent Ca^{2+} indicators, see Introduction: Ca^{2+}-Sensitive Fluorescent Dyes and Intracellular Ca^{2+} Imaging (Bootman et al. 2013).

REFERENCES

Bootman MD, Taylor CW, Berridge MJ. 1992. The thiol reagent, thimerosal, evokes Ca^{2+} spikes in HeLa cells by sensitizing the inositol 1,4,5-trisphosphate receptor. *J Biol Chem* **267:** 25113–25119.

Bootman MD, Rietdorf K, Collins T, Walker S, Sanderson M. 2013. Ca^{2+}-sensitive fluorescent dyes and intracellular Ca^{2+} imaging. *Cold Spring Harb Protoc* doi: 10.1101/pdb.top066050.

Perez JF, Sanderson MJ. 2005. The frequency of calcium oscillations induced by 5-HT, ACH, and KCl determine the contraction of smooth muscle cells of intrapulmonary bronchioles. *J Gen Physiol* **125:** 535–553.

Tsien RY. 1981. A non-disruptive technique for loading calcium buffers and indicators into cells. *Nature* **290:** 527–528.

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Protocol 2

Converting Fluorescence Data into Ca^{2+} Concentration

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In many situations, fluorescent Ca^{2+} reporters are used to simply indicate that a change of Ca^{2+} concentration has occurred. Monitoring the emission from a Ca^{2+}-sensitive indicator can be sufficient to tell whether a signal has arisen, and what its kinetic/spatial parameters were. The emission from an indicator does not have a linear relationship to the Ca^{2+} concentration within a cell; rather, the relationship between fluorescence emission and Ca^{2+} concentration is described by a logistic function. Simply recording fluorescence emission, therefore, provides a relative indication of the magnitude of a Ca^{2+} signal that should not be used for generating mean amplitude data. However, with a little consideration and effort, the fluorescence output can be calibrated to yield actual Ca^{2+} concentration.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

Reagents

Buffered salt solution (see Step 1)

Ca^{2+} ionophore of choice (see Step 4)

$CaCl_2$

Cells grown on a glass coverslip and loaded with Ca^{2+} indicator (see Protocol 1: Loading Fluorescent Ca^{2+} Indicators into Living Cells [Bootman et al. 2013a])

Calibration data can be obtained from cells that have been used for an experiment, or from a fresh batch of control cells. The advantage of using cells from a prior experiment is that calibration data can be obtained for each individual cell. The disadvantage of this approach is that the Ca^{2+} indicator may have been significantly bleached during the prior experiment, in which case using fresh cells would be ideal.

EGTA

Weak alkali for dissolving EGTA

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Equipment

Imaging system (see Introduction: Ca^{2+}-Sensitive Fluorescent Dyes and Intracellular Ca^{2+} Imaging [Bootman et al. 2013b])

pH meter or paper

METHOD

Prepare Calibration Solutions

Essentially, calibration of fluorescence emission from a Ca^{2+} indicator involves obtaining maximal (Ca^{2+}-saturated) and minimal (Ca^{2+}-free) readings. For this, it is necessary to prepare two calibration solutions made from the same stock solution.

1. Make the stock solution using the recipe employed for the experimental extracellular salt solution, but without adding Ca^{2+}.

   Typically, ~200 mL of this stock solution is sufficient.

2. Split the stock solution, without added Ca^{2+}, into two equal volumes.
   i. To one volume, add Ca^{2+} (e.g., $CaCl_2$) so that its final Ca^{2+} concentration is 4 mM.

      This solution can be used to determine fluorescence from Ca^{2+}-saturated indicator.

   ii. To the other volume, add EGTA so that its final concentration is 2 mM.

      Note that EGTA is difficult to dissolve in neutral solutions, but can be rapidly dissolved in alkali. This solution can be used to determine fluorescence from Ca^{2+}-free indicator.

      These solutions can be kept frozen or refrigerated, and used again, to provide economical use of salts, etc. However, the solutions should be discarded if there are any signs of precipitation or bacterial growth.

3. Check the pH of the calibration solutions and correct, if necessary.

   EGTA is a strong acid and can readily alter the pH of solutions it is dissolved in.

4. Add the Ca^{2+} ionophore to a small volume (e.g., 10 mL) of both the Ca^{2+}-saturated and Ca^{2+}-free calibration solutions.

   Equilibration of intracellular and extracellular Ca^{2+} is achieved using a Ca^{2+} ionophore such as ionomycin or A23187. 4-Bromo-A23184 may be a better choice if the Ca^{2+} indicator is excited in the ultraviolet range because it is non-fluorescent.

Obtain Minimum and Maximum Fluorescence Data

The minimum and maximum fluorescence signals must be determined with the same settings (contrast, brightness, etc.) as the experimental cells being studied. In practice, it is easiest to obtain the minimal (Ca^{2+}-free) reading first.

5. Superfuse the cells with the ionophore/2 mM EGTA calibration solution. (The exact volume required depends on the chamber in which the cells are situated. Generally, several flushes with 1–2 mL volumes is sufficient.)

   The ionophore will allow the rapid equilibration of intracellular and extracellular Ca^{2+}. The presence of EGTA ensures that any Ca^{2+} leaking out of the cell is sequestered. In this way, the Ca^{2+} concentration within the cell attains a low level.

6. Obtain the Ca^{2+}-free reading.

   Depending on the cell type and the concentration of ionophore, the steady-state Ca^{2+}-free fluorescence signal should be obtained in ~10 min. This is typically denoted F_{min} or R_{min} for single-wavelength or dual-wavelength Ca^{2+} indicators, respectively. See Troubleshooting.

7. After determining the minimal (Ca^{2+}-free) fluorescence signal, superfuse the cells with the ionophore/4 mM Ca^{2+} calibration solution. (Again, several flushes with 1–2-mL volumes may be required.)

   The Ca^{2+} present in this solution will cause a rapid increase in cellular Ca^{2+} concentration, such that the Ca^{2+} indicator is eventually saturated.

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8. Obtain the Ca^{2+}-saturated reading.

   The Ca^{2+}-saturated condition typically reaches steady state more rapidly than the Ca^{2+}-free situation. The Ca^{2+}-saturated fluorescence signal is denoted F_{max} or R_{max} for single-wavelength or dual-wavelength Ca^{2+} indicators, respectively. See Troubleshooting.

9. Obtain background readings of pixel intensity from non-cellular areas. Subtract the average background pixel intensity from the minimal and maximal fluorescence data.

   Complications can arise in the determination of background pixel intensity if the field of view is not illuminated evenly. See Troubleshooting.

Determine Ca^{2+} Concentration

10. Use the minimal and maximal calibration data (background subtracted) and the following equations to convert the fluorescence emission signal obtained during an experiment into Ca^{2+} concentration.

    - For a single-wavelength indicator:

    $$[\mathrm{Ca}^{2+}] = K_\mathrm{d}\left(\frac{F - F_\mathrm{min}}{F_\mathrm{max} - F}\right),$$

    where F is the background-corrected fluorescence intensity recorded from cells during the experiment, F_{min} is the Ca^{2+}-free indicator fluorescence (determined in the ionophore/2 mM EGTA solution), F_{max} is the Ca^{2+}-saturated indicator fluorescence (determined in the ionophore/4 mM Ca^{2+} solution), and K_d is the affinity of the indicator for Ca^{2+}.

    - For a dual-wavelength indicator:

    $$[\mathrm{Ca}^{2+}] = K_\mathrm{d}\left(\frac{R - R_\mathrm{min}}{R_\mathrm{max} - R}\right)\left(\frac{F_\mathrm{max},\ \lambda_2}{F_\mathrm{min},\ \lambda_2}\right),$$

    where R is the ratio value of the background-corrected fluorescence recorded from cells during the experiment, R_{min} is the fluorescence ratio from Ca^{2+}-free indicator, R_{max} is the fluorescence ratio from Ca^{2+}-saturated indicator, and K_d is the affinity of the indicator for Ca^{2+}. The (F_{max}, λ_2/F_{min}, λ_2) factor (often denoted as β) is the ratio F_{max}/F_{min} at the wavelength of the Ca^{2+}-free form of the indicator, λ_2 (e.g., 380 nm in the case of Fura-2).

    These calculations require knowledge of the Ca^{2+} indicator's K_d. The K_d values of Ca^{2+} indicators can be obtained from the vendors, the published literature, or can be determined in situ (Thomas et al. 2000). Empirical determination of the K_d in the same cell type as that being studied will generally provide the most accurate calibration. In many cases, the K_d values provided by Ca^{2+} indicator vendors have been determined in simple solutions that do not resemble the cellular milieu. Many factors, such as ionic strength, pH, and temperature can affect the affinity of an indicator for Ca^{2+} (Uto et al. 1991). Of these, temperature is plausibly the most critical. The same temperature should be used for experimentation and calibration.

TROUBLESHOOTING

Problem: There is Ca^{2+} indicator loss during the experiment or the cells look poor at the end of the experiment.
Solution: Obtain calibration data using a fresh batch of cells loaded with Ca^{2+} indicator and examined under the same experimental settings.

Problem (Steps 6 and 8): There is difficulty in obtaining stable fluorescence in cells treated with ionophore.
Solution: Perform an in vitro calibration using the procedure described below. This will not take into account the effect of cytoplasmic compartments on the K_d of the indicator.

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Problem (Step 9): Illumination is uneven, and there are no obvious regions from which background signal can be sampled.

Solution: The illumination pattern needs to be imaged in a cell-free situation. One approach to do this is to image a thin layer of Ca^{2+} indicator (in both Ca^{2+}-free and Ca^{2+}-saturated conditions). Press plastic wrap down on a glass coverslip until it appears tightly adhered to the surface. Cut a central channel in the wrap by drawing a scalpel blade (two parallel cuts) across the coverslip. Remove the plastic wrap in the channel by scratching and lifting the film. Place a second coverslip over the channel. Thus, the channel forms a chamber with a height of the plastic wrap. Draw the Ca^{2+} indicator solution into the channel by capillary action. Solutions can easily be exchanged by adding a new solution at one end of the chamber and filter paper to the other end (to suck the fluid out). A 50-μM Ca^{2+} indicator solution is usually sufficient. Record a background image (no Ca^{2+} indicator; saline solution only) and subtract it from the reference image before use. The "field reference" images can be used to correct uneven illumination. In addition, the chamber can be easily used to obtain λ_1/λ_2 images for R_{max}, R_{min}, and background images for subtraction at each wavelength to perform an in vitro calibration. The $F_{min}\lambda_2$ and $F_{max}\lambda_2$ images are part of the image data set collected for R_{max} and R_{min}.

RELATED INFORMATION

For a detailed discussion of practical considerations and potential problems regarding the use of fluorescent Ca^{2+} indicators, see Introduction: Ca^{2+}-Sensitive Fluorescent Dyes and Intracellular Ca^{2+} Imaging (Bootman et al. 2013b).

REFERENCES

Bootman MD, Rietdorf K, Collins T, Walker S, Sanderson M. 2013a. Loading fluorescent Ca^{2+} indicators into living cells. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot072801.

Bootman MD, Rietdorf K, Collins T, Walker S, Sanderson M. 2013b. Ca^{2+}-sensitive fluorescent dyes and intracellular Ca^{2+} imaging. *Cold Spring Harb Protoc* doi: 10.1101/pdb.top066050.

Thomas D, Tovey SC, Collins TJ, Bootman MD, Berridge MJ, Lipp P. 2000. A comparison of fluorescent Ca^{2+} indicator properties and their use in measuring elementary and global Ca^{2+} signals. *Cell Calcium* **28:** 213–223.

Uto A, Arai H, Ogawa Y. 1991. Reassessment of Fura-2 and the ratio method for determination of intracellular Ca^{2+} concentrations. *Cell Calcium* **12:** 29–37.

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