Topic Introduction

Ca²⁺-Sensitive Fluorescent Dyes and Intracellular Ca²⁺ Imaging

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Imaging Ca²⁺-sensitive fluorescent indicators provides a common approach for studying Ca²⁺ signals in many contexts. Fluorescent indicators are particularly useful for measuring acute Ca²⁺ 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 Ca²⁺ signals, and moreover, multiplexing the readout of Ca²⁺ 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 Ca²⁺ indicators.

INTRODUCTION

A multitude of Ca²⁺ signals, varying from nanoscopic, subcellular domains to whole-cell Ca²⁺ 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 Ca²⁺ signals depend on the expression of tissue-specific Ca²⁺ transport systems (Berridge et al. 2000). The diversity of cellular Ca²⁺ signaling means that there is no single technique that can be used to monitor Ca²⁺ changes in all situations. However, fluorescent Ca²⁺ indicators and imaging provide the most versatile and widely used method for analyzing cellular Ca²⁺ responses. Since their introduction by Tsien and colleagues (Tsien et al. 1982), fluorescent Ca²⁺ indicators have underpinned the investigation of Ca²⁺ signaling in a host of different experimental settings. Using appropriate technology and suitable indicators, it is possible to monitor Ca²⁺ 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 Ca²⁺ 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 camgaros (Griesbeck et al. 2001). However, our emphasis will be on measuring Ca²⁺ signals with synthetic indicators such as 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 Ca²⁺ 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.

Ratiometric indicators also display an excitation or emission wavelength at which the indicator appears insensitive to Ca²⁺. This is known as the “isosbestic point.” This Ca²⁺-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 Mn²⁺. The binding of Mn²⁺ to Fura-2 quenches its fluorescence. Consequently, at the isosbestic point, a decrease in Fura-2 emission fluorescence indicates an increase in Mn²⁺. This is useful because Mn²⁺ can be used as a Ca²⁺ surrogate to monitor store-operated Ca²⁺ entry; Mn²⁺ entering the cell in place of Ca²⁺ will be detected by decreased Fura-2 fluorescence. Despite the quenching by Mn²⁺, Fura-2 can still be used to report Ca²⁺ in a quantitative, ratiometric manner. This combined application of monitoring Ca²⁺ concentration and Ca²⁺ 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 Ca²⁺ concentration could change considerably in the time between image capture, and the ratiometric output is not a true reflection of the Ca²⁺ 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 Ca²⁺ using Fura-2 for >30 min with little bleaching.

Because Indo-1 only requires excitation at one wavelength, rapid monitoring of Ca²⁺ 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 Ca²⁺ 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 Ca²⁺-free and Ca²⁺-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 Ca²⁺ Indicators

Single-wavelength indicators display a change in their fluorescence emission when Ca²⁺ signals occur. In contrast to dual-wavelength indicators, there is not a sufficient shift in either excitation and/or emission wavelengths on Ca²⁺ binding to allow ratiometric measurements. A key issue with single-wavelength indicators is that variations in fluorescence emission may not reflect differences in Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ indicator loading, or perhaps variation of their Ca²⁺ concentration. For this reason, single-wavelength indicators are most useful for measuring relative changes in Ca²⁺ 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

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.

This technique can be used to visualize cellular structures or Ca²⁺ signals that occur close to the plasma membrane, and has been particularly useful for studies of Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ puffs and Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ responses. A further consideration is the impact of the indicator on the viability of the cells. The most widely used method for introducing Ca²⁺ indicators into cells is as a membrane-permeant acetoxymethyl ester, but this is not entirely benign. The liberation of the Ca²⁺-sensitive indicator yields formaldehyde and acetic acid as by-products. Although there are few studies examining the effect of Ca²⁺ indicators on cell activity, it has been shown that indicator loading can reduce cellular ATP content. A further consideration is the buffering of Ca²⁺ by the indicators. Excessive loading of cells with indicator can severely reduce, or even abolish, the Ca²⁺ 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

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 **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 **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

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/waterimmersionobjectives.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 Ca²⁺ signals, for example in confocal microscopy. But if the particular experiment is to sample global Ca²⁺ signals within individual cells in a field of view, then a lower magnification, lesser NA objective could be appropriate. To monitor Ca²⁺ oscillations in single cells, we routinely use 40× (NA 1.3) or 20× (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 Ca²⁺ 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 Ca²⁺ 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

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 to 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).

gain setting is too low, a signal will only occupy a fraction of the bit depth and a Ca²⁺ rise will be poorly resolved. The necessary insight into the likely properties of a Ca²⁺ rise can only be achieved with some preliminary experimentation to determine the ideal gain settings. Ideally, the gain should be moderated so that Ca²⁺ signals use a broad portion of the bit depth of the system without saturation. This is critical in experiments where different amplitude Ca²⁺ 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 Ca²⁺ measurements because Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ indicator) and the required output (e.g., image sequence, plot of fluorescence intensity from a region of interest [ROI], or a calibrated Ca²⁺ 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 ~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

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 ($\sim 50 \mu\text{m}$) can be imaged using a thin chamber constructed from two coverslips separated by a thin film of plastic wrap (see **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

FIGURE 2. The effect of background correction on Ca²⁺ measurement using fluorescent indicators. The figure shows an experiment in which a Fura-Red-loaded HeLa cell was stimulated with histamine to evoke Ca²⁺ oscillations. Panel A depicts the ratiometric measurement of fluorescence without background subtraction. The data appear to show Ca²⁺ 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 Ca²⁺ value is now not drifting upward and the peaks of the Ca²⁺ oscillations are larger. The cells were loaded with Fura Red by incubation with 1 μ M 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 Ca²⁺-bound and Ca²⁺-free indicator (emission was sampled using a 650/50 filter). Images were acquired with a 40 $\times$, 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 μ M histamine was added to elicit Ca²⁺ 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.

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 **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 ($F_{\lambda 1}/F_{\lambda 2}$) 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

FIGURE 4. Suggested parameters for measuring cellular Ca²⁺ signals. The cartoon depicts an idealized oscillatory Ca²⁺ 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 Ca²⁺ within the cytosol and nucleus can be distinguished. It is plausible that cells generate real differences between nuclear and cytosolic Ca²⁺ responses. Indeed, there is a growing literature that nuclear Ca²⁺ signals may be discrete from the cytosol (Bootman et al. 2009). However, this cannot be assumed. In many cases nuclear and cytosolic Ca²⁺ concentration are in equilibrium, with the nuclear envelope simply introducing a kinetic delay in the movement of Ca²⁺ between compartments.

Data Presentation

The presentation of Ca²⁺ responses is very much determined by the message that is being conveyed. In the majority of situations, showing traces of evoked Ca²⁺ 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 Ca²⁺ signal is useful, as it gives a measure of the threshold and efficacy of a stimulus. In addition, peak Ca²⁺ signal amplitude, latency, area integrated Ca²⁺ signal, and frequency of Ca²⁺ 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 Ca²⁺ signals is not affected by the stimulus concentration, whereas other cell types have smoothly graded peak Ca²⁺ signals (Bootman et al. 1994). For the former cell type, peak Ca²⁺ signal or an integrated Ca²⁺ 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).

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.

<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 Ca²⁺ 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.

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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