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Protocol

Loading Fluorescent Ca²⁺ Indicators into Living Cells

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Small-molecule fluorescent Ca²⁺ reporters are the most widely used tools in the field of Ca²⁺ signaling. The excellent spatial and temporal resolution afforded by fluorescent reporters has driven the understanding of Ca²⁺ as a messenger in many different cell types. In many situations, the cellular loading and monitoring of fluorescent Ca²⁺ indicators is quite trivial. However, there are numerous pitfalls that require consideration to ensure that optimal data are recorded. Fluorescent Ca²⁺ indicators have carboxylic acid groups for binding of Ca²⁺. 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 Ca²⁺-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)

Ca²⁺ indicator of choice (in ester form)

Ca²⁺ 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 Ca²⁺ Indicator

Because Ca²⁺ indicators are light-sensitive, perform as many steps as possible in reduced light.

1. Prepare a suitable, high-concentration stock of Ca²⁺ 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 Ca²⁺ indicator loading. It works by preventing the amphipathic Ca²⁺ indicator molecules from forming micelles.

2. Before application to cells, dilute the stock solution of Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ indicator stock with the same HEPES-buffered salt solution that the cells will be incubated in during subsequent experiments. However, Ca²⁺ indicators can be loaded into cells maintained in serum-replete culture medium.

3. If appropriate, wash cells free of serum before applying the Ca²⁺ indicator loading solution.

The presence or absence of serum during Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ indicator ester to permeate the cells and be hydrolysed by intracellular esterases to the Ca²⁺-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 Ca²⁺ indicator loading solution and the loading time are established, they should be rigorously adhered to. Changing either parameter will alter the amount of Ca²⁺ indicator within the cells, and could lead to disparate responses between experiments.

The temperature at which Ca²⁺ 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

experiments are intended to be performed at temperatures >22°C, it is plausible to perform the Ca²⁺ indicator loading at room temperature and then gently warm the cells.

See Troubleshooting.

5. When the prescribed incubation time with the Ca²⁺ 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 Ca²⁺ Imaging Experiment

Fluorescence indicators allow Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ indicator.

Solution: For reasons that are not always understood, some cell types do not load well with the ester forms of Ca²⁺ indicators. Alternative methods for Ca²⁺ indicator loading (including microinjection, transient cell permeabilization, or pinocytotic uptake of extracellular indicator) can be applied. These methods allow the introduction of the free-acid (Ca²⁺-sensitive) forms of indicators. Alternatively, cell transfection methods can be used to introduce genetically encoded Ca²⁺ indicators.

Problem (Step 4): Cells load with Ca²⁺ indicator, but it rapidly leaks out.

Solution: A plausible strategy to prevent leakage of Ca²⁺ indicators is to block MDR-mediated anion transport. The compound used most often for this purpose is sulfinpyrazone, which is a uricostatic medication commonly used in the treatment of gout. We have used sulfinpyrazone in situations where the loading of fluorescent Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ signal transduction. However, as with all drugs, they should be used with caution.

Problem (Steps 4, 10): The loading and/or responsiveness of the Ca²⁺ 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 Ca²⁺ changes. If the cellular loading and/or responsiveness of a Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ indicators, see **Ca²⁺-Sensitive Fluorescent Dyes and Intracellular Ca²⁺ Imaging** (Bootman et al. 2013).

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