Chapter 27

Imaging Changes in Cytoplasmic Calcium Using the Yellow Cameleon 3.6 Biosensor and Confocal Microscopy

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Abstract

Changes in the concentration of cytoplasmic calcium, $[Ca^{2+}]_{cyt}$ are central regulators in many cellular signal transduction pathways including many lipid-mediated regulatory networks. Given this central role that $[Ca^{2+}]$ has during plant growth, monitoring spatial and temporal $[Ca^{2+}]$ dynamics can reveal a critical component of cellular physiology. Here, we describe the measurement of $[Ca^{2+}]_{cyt}$ in *Arabidopsis* root cells using plants expressing Yellow Cameleon 3.6 (YC 3.6). YC3.6 is a Ca^{2+}-sensitive biosensor where the intensity of its fluorescence resonance energy transfer (FRET) signal changes as the Ca^{2+} level within the cell rises and falls. The FRET from this calcium reporter can be visualized using confocal microscopy and the resultant images converted to a quantitative map of the levels of Ca^{2+} using an approach called ratio analysis.

Key words Yellow Cameleon 3.6, Calcium, Ratio imaging, Fluorescence resonance energy transfer

1 Introduction

Plants generally maintain cytoplasmic $[Ca^{2+}]$ at very low levels (<200 nM), by sequestration of calcium into subcellular compartments or by its removal from the cell via transporters on the plasma membrane. Signaling events originating either during normal development or during a response to an environmental stimulus can trigger an increase in $[Ca^{2+}]$ that then acts as a second messenger, regulating subsequent cellular responses (1, 2). Such signal-related changes in Ca^{2+} have been inferred to play roles in a host of lipid-based signaling networks, making direct measurement of Ca^{2+} signals an important goal in understanding plant lipid-based signaling systems.

The dynamics of $[Ca^{2+}]_{cyt}$ have been successfully observed in plants using a number of small molecule, calcium-sensitive, fluorescent probes, including calcium green, fura-2, and indo-1 (3). However, use of these dyes has important limitations related

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to difficulties in introducing them into plant cells and ensuring that once inside, they remain in the cytosol (3). The fluorescent protein-based [Ca^{2+}] sensors of the Yellow Cameleon (YC) family obviate these problems because they can be stably expressed in the plant cells of interest and stay localized to the cytoplasm.

The Yellow Cameleons are calcium sensors based on fluorescence resonance energy transfer (FRET) between two fluorescent proteins: cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP); these fluorophores are connected by a calcium sensing domain that includes calmodulin and the M13 calmodulin-binding domain of myosin light chain kinase (Fig. 1a; (4, 5)). The original YC 2.1 version of this sensor has been successfully used in plants (6, 7) but has now been superseded by YC 3.6, where the YFP is replaced by a circularly permutated form called Venus, yielding a protein with a greatly improved brightness and dynamic range (8). It is important to note that there are now many versions of Yellow Cameleon which have shifted Kd's for Ca^{2+} and a range of other Ca^{2+} reporters designed around fusing GFP to other Ca^{2+}-responsive proteins (e.g., (9, 10)); however, currently most of the GFP reporter-based Ca^{2+} imaging in plants is being made using YC 3.6.

The imaging and analysis of YC3.6 expressing plants is relatively straight-forward and readily accomplished with the confocal

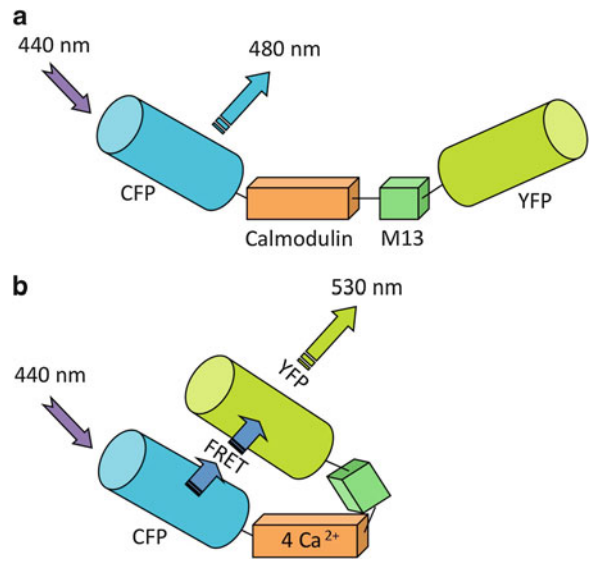

Fig. 1 Structure of the YC 3.6 calcium indicator. (**a**) Domains of YC 3.6 include cyan fluorescent protein (CFP), calmodulin (a calcium binding protein), M13 (the calmodulin-binding domain of myosin light chain kinase), and Yellow Fluorescent Protein (YFP). (**b**) Conformation of the YC 3.6 protein in the presence of high calcium. Calmodulin binds calcium, then this activated calmodulin is able to bind M13. This allows the YFP and CFP to more closely interact such that FRET is able to occur. FRET results in less CFP emission and more YFP emission from the YC 3.6 protein

microscopes available in most university imaging facilities. At low resting [Ca^{2+}], YC 3.6 shows a low FRET signal; however, upon an increase in [Ca^{2+}] the calmodulin domain becomes activated by calcium and binds to the calmodulin-binding M13 region of the sensor. Therefore, at high [Ca^{2+}] the sensor changes conformation, bringing the two fluorescent proteins into closer proximity/more optimal orientation thus facilitating an increase in the FRET signal and a concomitant decrease in CFP signal (Fig. 1b). Analysis of the fluorescence from both CFP and FRET enables accurate measurement of the [Ca^{2+}]. In practice, this is performed by calculating the ratio of FRET signal/CFP signal as the value of this ratio corrects for many potential optical artifacts in such fluorescence measurements, such as changes due to differences in reporter concentration or photobleaching (3).

In the method described below, we will focus on the measurement of [Ca^{2+}] in *Arabidopsis* roots using the confocal microscope for YC 3.6 data collection. Most confocal microscopes have: (1) an emission line from the argon ion laser suitable for CFP/FRET excitation, (2) filters able to separate the CFP and FRET fluorescence, (3) two channels capable of collecting the CFP and FRET signals simultaneously, and (4) detectors that allow quantitative analysis of the resultant fluorescent signals, making them highly suited to YC3.6 imaging.

2 Materials

2.1 Plant Material

Imaging is greatly facilitated by the use of plants stably expressing the fluorescent protein-based sensor YC 3.6 (*see* **Note 1**).

1. Choose a promoter to drive YC 3.6 expression that is appropriate for the cell type studied. For example, the CaMV35S promoter gives strong YC 3.6 expression in the epidermis, cortex, and root cap. Other regions of the plant may require a cell-specific promoter (e.g., Lat52 rather than CaMV35S for pollen, ref. (11)).
2. When developing YC 3.6 expressing lines, screen transformants for fluorescence to ensure usable signal for microscopy. In addition, have untransformed plants on hand which have been grown and handled identically as the experimental material, to provide an essential control for autofluorescence (*see* **Note 2**).

2.2 Sample Preparation

Handle plants as little as possible prior to imaging to minimize cytosolic calcium changes due to mechanical stress. Additionally, ensure that the sample will not dry out or otherwise become stressed during imaging. For cytosolic calcium studies in *Arabidopsis* seedling roots, grow plants on a cover glass coated in phytagel

(an optically clear agar substitute available from Sigma-Aldrich). Suitable cover glass sizes range from 40 to 60 mm, dependent on the type of microscope stage available and/or sample chamber used during imaging.

1. Autoclave cover glasses by packing vertically into a beaker, alternating cover glass with a piece of filter paper to prevent each glass form sticking to its neighbor. Once autoclaved, work in the sterile hood and use tweezers to place each cover glass in a sterile petri dish.
2. Add 0.5 % (w/v) phytagel to growth medium and autoclave. Allow to cool until the bottle can be handled with a bare hand. Warm liquid phytagel solution can be pipetted onto the cover glass in the petri plate, forming a "pillow" shape 1–2 mm thick, then allowed to cool and solidify (*see* **Note 3**).
3. After cooling, surface-sterilized seed (2 min, 70 % ethanol) can be planted in the phytagel using a sterile spatula. Poke the seed through the gel such that it is resting on the cover glass itself. Plant 2–4 seeds per cover glass, then seal the plate with parafilm or gas-permeable tape to minimize contamination and to prevent drying (Fig. 2a).

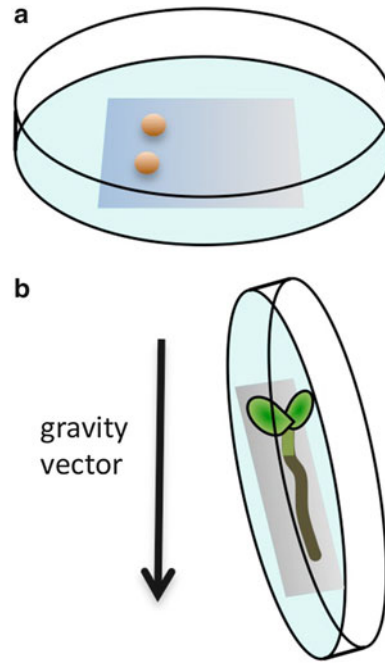

Fig. 2 Preparation of the cover glass and growth of Arabidopsis roots for microscopy. (**a**) Petri plate with sterile cover glass; seeds are planted into the "pillow" of phytagel on the cover glass. (**b**) Cartoon of a vertically grown 7-day-old Arabidopsis plant with its root growing down along the cover glass through the phytagel media

4. Germinate in growth chamber with the plate flat for one day. On day 2, set the plate on edge such that it is angled back slightly from the vertical, so that the roots grow down along the cover glass (Fig. 2b). *Arabidopsis* roots that are 4–10 days old are usable for microscopy when grown this way (*see* **Note 4**).

3 Methods

3.1 Configuration of the Confocal Microscope

1. For successful FRET, YC 3.6 requires CFP excitation. Commonly used laser lines for CFP excitation are the 458 nm line of the argon-ion laser (generally installed on most confocal microscopes) or the 405 nm emission of a diode laser.
2. Use as little excitation energy as possible to minimize photobleaching and potential damage to the sample. Excess excitation energy has the potential to alter the cellular physiology under study (*see* **Note 5**).
3. Select the correct primary dichroic mirror to separate the excitation energy from the fluorescence signal. Generally, the primary dichroic mirror for confocal microscopy matches the laser line selected (e.g., 458 or 405 nm; see Fig. 3).
4. Select the range of YC 3.6 fluorescence wavelengths collected in each channel. For example, a usable range would be 460–505 nm for the CFP channel and 525–535 nm for the FRET channel (Fig. 3; *see* **Note 6**).

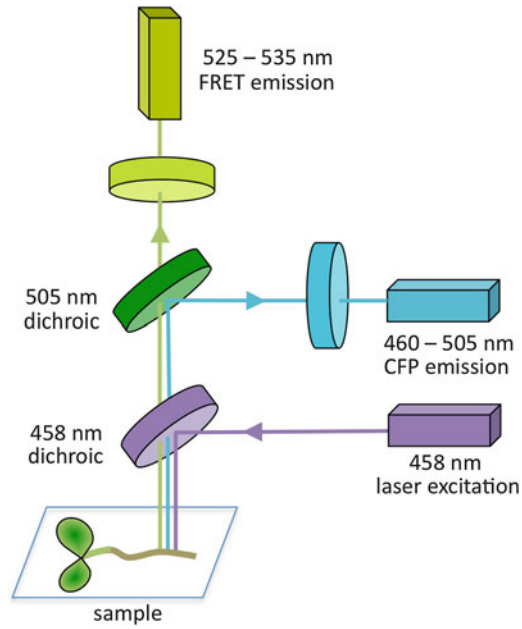

Fig. 3 Configuration of the confocal microscope for imaging the YC 3.6 calcium indicator

5. Some confocal microscopes have the option of using another channel for a transmission (bright field) image. If this is available, enable the transmission channel for the purpose of focusing during the time course.
6. Pseudocolor the image from the CFP channel to green-scale (where increasing intensity of signal is represented as an increasing green brightness), and the image from the FRET channel to red-scale (*see* **Note 7**).

3.2 Imaging Parameters of the Confocal Microscope

For ion imaging using the confocal microscope, generally some sacrifice in spatial resolution is needed to achieve the temporal resolution desired, because the higher the spatial resolution, the longer the microscope takes to acquire each image. If too much time is taken obtaining each image or too long elapses between frames of a time course, important changes in $[Ca^{2+}]$ will be missed (*see* **Note 8**).

1. With the sample on the stage of the microscope, select the proper objective and focus on the cells of interest. Confirm that the sample is fluorescent.
2. Select a pixel resolution that is compatible with fast scan times. Smaller images will allow faster scan times and a shorter interval between each image in the time course.
3. Likewise, select a fast scan speed that will enable rapid image acquisition.
4. Reduce image averaging (usually applied to reduce noise in the image by averaging the signal in multiple frames) or remove completely; this will also allow collection of more frames in a given time period.
5. The confocal pinhole diameter for both the CFP and the FRET detection channels should be adjusted to give the same optical section thickness (*see* **Note 9**).
6. The confocal pinhole diameter should be at the physical optimum of 1 Airy unit or slightly larger (to capture a greater Z section of the sample). Reducing the pinhole below one Airy unit will reduce signal with little gain in resolution.
7. Detector gain should be carefully adjusted for the CFP and FRET detection channels such that there are no pixels that are saturated in intensity in the image (*see* **Note 10**).
8. Confirm that at resting calcium, the detector gain for the FRET channel is set such that the signal is low, allowing for increases in fluorescence from FRET during a $[Ca^{2+}]$ increase (*see* **Note 11**).
9. Some confocal microscope detectors will have an offset that adjusts the output of the low end of fluorescence intensities (i.e., filters out low signals to improve the contrast of the image). For quantitative imaging, set the offset to 0 for both the CFP and FRET channels.

3.3 Data Collection

Once configuration and scanning parameters are established, it is important that these settings be reused for every sample. Changes in laser strength, wavelengths collected, scan time, pinhole diameter, or gain can alter the measured FRET/CFP ratio independently of $[Ca^{2+}]$ changes. Therefore, all settings must be the same in order to compare results between samples and between data collection sessions (*see* **Note 12**).

1. With the sample on the stage of the microscope, select the proper objective and focus on the cells of interest. Confirm using epifluorescence that the sample is fluorescent.
2. Load the previously saved YC 3.6 configuration and scanning parameters.
3. Begin imaging with the scanning laser and perform a continual scan to focus on the desired optical section. Crop to zoom into the region of interest and rotate if necessary.
4. Set up the time series interval and number of frames to collect in the time course (*see* **Note 13**).
5. Start the time course. If possible, view the signal from each channel separately in addition to the overlay in order to monitor any calcium changes.
6. Collect ~30 frames for a baseline prior to addition of any treatments.
7. After ~30 frames, apply treatment (*see* **Note 14**).
8. If available, observe the transmission image during the time course to confirm that the sample stays in focus.
9. Follow any $[Ca^{2+}]$ changes in real time by observing the overlay of the FRET and CFP channels.
10. When the $[Ca^{2+}]$ response is finished, collect ~30 more frames at the end of your time course then save the image stack.
11. In addition to the experimental material, perform the same experiment on a number of samples that are not transformed with YC 3.6 using the saved settings to *see* the amount and distribution of autofluorescence (*see* **Note 15**).

3.4 Ratio Analysis

A number of confocal systems have software to process the CFP and FRET image pairs into a ratio image. Alternatively, it is possible to analyze the data using an image-processing program such as ImageJ (NIH) or a commercial package such as MetaFlour (Molecular Devices) or iVision (BioVision Technologies).

1. Confocal data is usually collected with pixel intensity values ranging from 0 to 255 (8 bit) or from 0 to 4,095 (12 bit). Export the images to a file type able to contain this data depth that is usable by the image analysis program, e.g., TIFF (RGB full color). Export without using compression or scaling in order to preserve the raw intensity values.

2. Export such that the CFP image and FRET image are each contained in their own color channel (red, green or blue) in the TIFF for every frame in the time course as this easily keeps the paired CFP and FRET images together (*see* **Note 16**).
3. In the image analysis program, open the TIFF and separate the CFP and FRET color channels into individual image windows containing the raw data.
4. Quantify the amount of background signal in both the CFP and FRET raw images by measuring an average intensity in an area away from the sample (*see* **Note 17**).
5. Subtract out the average CFP background signal from every pixel in the CFP image, then repeat for the FRET image with the FRET background value. Typically, image analysis programs have a "pixel arithmetic" type of command able to do this arithmetic on each pixel of the image.
6. Generate a ratio image by dividing the FRET image by the CFP image. Again there will generally be a command within the image analysis language for this purpose (*see* **Note 18**).
7. The resulting ratio image will contain extreme values generated by arithmetic performed on noise and/or areas of low signal in the raw images; defining the significant regions of the image to eliminate these extreme values will give a more robust analysis (*see* **Note 19**).
8. It is now possible to determine an average ratio of the sample region of interest. Repeat the analysis for each image in your time course to generate data for a graph showing relative changes in $[Ca^{2+}]$ over time.
9. It is often desirable to pseudocolor a ratio image to more easily *see* differences in ratios, and therefore differences in $[Ca^{2+}]$. This can be performed from a "pseudocolor" command within the image analysis software or by generating a scaled image which allows a more user-controlled mapping of color to ratio value (*see* **Note 20**).

3.5 Calibration of YC 3.6

Often YC 3.6 $[Ca^{2+}]$ data is presented as a change in ratio over time; however, it is possible to approximate an absolute $[Ca^{2+}]$ by calibration of the YC 3.6 ratio to known $[Ca^{2+}]$. Two approaches exist for calibration, in vivo and in vitro. In theory, the in vivo calibration is more accurate because any impact of the cytoplasmic milieu on YC 3.6 fluorescence is included, although in practice the permeabilization/buffering approach required for in vivo calibration has proven extremely difficult to apply to intact plants (*see* **Note 21**). Therefore, most analyses calibrate to an in vitro standard. An in vitro calibration can be performed by ratio analysis of the fluorescence from purified recombinant YC 3.6 in buffer solutions of known free Ca^{2+} levels.

It is also possible to use the published K_d for YC 3.6 (250 nM, ref. (8)) to estimate $[Ca^{2+}]$ according to the equation: $[Ca^{2+}] = (K_d[R - R_{min}]/[R_{max} - R])(F_{min}/F_{max})$, where R is the measured ratio value, R_{max} and R_{min} represent the maximum and minimum ratio values, and F_{min} and F_{max} represent the maximum and minimum FRET fluorescence obtainable from the sample, respectively (12). This approach will provide a rough estimate of the cytoplasmic Ca^{2+} level.

1. To obtain R_{min}, treat the plant with 20 μM of the Ca^{2+} ionophore Br-A23187 and 5 mM EGTA and record the minimum ratio obtained.
2. Replace the EGTA solution with 5 mM Ca^{2+} and record the maximum Ca^{2+} level obtained [R_{max}]. Alternatively, alcohol with 1 M Ca^{2+} has been used to drive Ca^{2+} levels to R_{max} (*see* **Note 22**).

4 Notes

1. Stably transformed lines for a range of Arabidopsis mutants can be readily generated via either crossing to available CaMV35S driven YC3.6 expressing lines (e.g., ref. (13)) or generated de novo via *Agrobacterium* dipping (ref. (14)).
2. Relying only on PCR screening or screening for antibiotic resistance can yield transformed lines that are too dim or show non-uniform expression and so are unsuitable for subsequent microscopy.
3. Make medium fresh each time, do not reheat solidified phytagel solution. Keep plates in the sterile hood with the lid closed to minimize drying of the gel while cooling. Prepared plates can be stacked and stored at 4 °C in a plastic sleeve until use.
4. For experiments requiring addition of a test solution, cut a window in the gel a couple of mm ahead of the root a few hours in advance of imaging and fill the window with sterile media lacking phytagel. Allow the root to grow into the window. Add subsequent treatments to the liquid in this window.
5. For example, too much excitation energy while monitoring the tip-focussed calcium gradient in a growing root hair can slow tip growth and dissipate the $[Ca^{2+}]$ gradient. Yet the cell remains viable (i.e., cytoplasmic streaming continues) and morphologically the disruption of growth is highly cryptic.
6. When selecting the range of wavelengths, keep in mind that bleed-through of the CFP signal into the FRET channel can be a problem and can be tested for by imaging plants expressing only CFP with the imaging parameters used for YC3.6.

7. When viewed in an overlay, changes in the signal intensity from each channel (and thus changes in [Ca^{2+}]) will be easily visualized in real time, with increases in $[Ca^{2+}]_{cyt}$ appearing as increased red coloration in the image.
8. Another potential problem to avoid is scanning an individual frame so slowly (e.g., tens of seconds) that the physiological state of the region is different at the start of the scan than it is at the end of the scan. Typically for Ca^{2+} imaging, image acquisition of around 1 s and up to 5 s between images will capture most of the fast Ca^{2+} kinetics seen in plants to date.
9. This is not necessary if both channels share the same pinhole.
10. Saturating pixels do not report the true signal intensity (i.e., that area could in fact be much brighter), therefore images containing saturated pixels are not amenable to quantification and analysis.
11. For example, if intensity values range from 0 (no signal) to 255 (saturating), then adjust the gain for the FRET channel so that most pixels in the region of interest have an intensity of 100–150.
12. When collecting data for quantitative analysis, use the confocal microscope after it has been on for at least an hour to ensure that room temperature is stable and all components are warmed up. Additionally, circadian rhythm has been shown to have an effect on cytosolic [Ca^{2+}] responses in plants (15, 16), therefore, conducting YC 3.6 imaging at the same time of day may yield more reproducible results.
13. Confirm that the interval between frames is longer than the time is takes to scan a single frame.
14. For example, cold shock alters cytoplasmic [Ca^{2+}] quickly in most plant cells and is an easy treatment to administer by pipetting 100 μl ice-cold buffer onto the root during imaging. This is a useful control to test the imaging parameters to be used.
15. If there is significant autofluorescence in the plant material (not uncommon), it may not be possible to use YC 3.6 for calcium measurements. Some confocal microscopes have software to perform "linear unmixing," a mathematical calculation performed on the image based on reference spectra with the goal of separating autofluorescence from fluorescent protein signal. However, because it infers the pixel intensity values, linear unmixing cannot be used in conjunction with quantitative ratio analysis without caution.
16. Take care to note which color channel of the TIFF corresponds to each fluorescence channel of the confocal microscope data.

17. Alternatively, measure the average intensity in an image taken with identical configuration and scanning parameters in a region on the microscope slide away from your sample. Either of these background signal measurements will include detector dark noise level in addition to anything non-sample in the optical path that may generate a signal.
18. To generate a ratio image with fractional values (e.g., the calculated ratios from YC 3.6 can cover the range from 0.3 to 2.3) it may be necessary to change the data type of your raw FRET and CFP images to floating point (the raw images are generally in "byte" or "short integer" formats that cannot record fractional numbers whereas "floating point" format can).
19. To define the significant regions of your image, many image analysis programs will be able to generate a thresholded mask that excludes low-fluorescence and/or noisy areas outside the region of interest. Use the raw image that is less bright, e.g., the CFP image, to generate this mask.
20. To convert the fractional ratio image data into a form usable to display in byte-form pseudocolor, first multiply each pixel in the ratio image by a scaling factor to bring the intensity values into byte range (0–255). For example, if your ratios range from 0.3 to 2.3 then multiplying by 100 will give values from 30 to 230. Once the ratio image is in byte format, then apply the color look-up table (CLUT) from within the image processing package; a rainbow CLUT is usually effective at showing differences and the accepted convention is to map low ratio values to the blue end of the spectrum and high ratios to the red.
21. In vivo calibration involves adding Ca^{2+} buffers to the medium to set a known extracellular Ca^{2+} level and permeabilizing the plasma membrane with ionophore (such as A23187 or ionomycin) or detergent so that internal and external Ca^{2+} levels are the same.
22. It is often very difficult to obtain R_{min} and R_{max} from tissues deep within the plant, likely due to difficulties in access for the Ca^{2+} buffer and ionophore to cells below the epidermis. 1 M $CaCl_2$ plus 10 % ethanol has been used as an alternative strategy to elevate Ca^{2+} throughout the plant (17).

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