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Calcium Indicators Based on Calmodulin–Fluorescent Protein Fusions

Kevin Truong, Asako Sawano, Atsushi Miyawaki, and Mitsuhiko Ikura

Summary

Calmodulin (CaM) is an ubiquitous protein involved in Ca^{2+}-mediated signal transduction. On Ca^{2+} influx, CaM acquires a strong affinity to various cellular proteins with one or more CaM recognition sequences, resulting in the onset or termination of Ca^{2+}-regulated cascades. Through nuclear magnetic resonance and crystallographic structural studies of these Ca^{2+}–CaM complexes, we have gained a deep understanding of CaM target recognition mechanisms. One immediate application is the creation of protein-based Ca^{2+} sensors using CaM complexes and green fluorescent proteins, previously named "chameleon." The major advantage of chameleons is that they can be expressed in single cells and targeted to the specific organelles or tissues to measure localized Ca^{2+} changes. This chapter describes the methods involved in cloning chameleons, characterizing their biochemical and biophysical properties, and imaging them in single cells using a digital fluorescence microscope.

Key Words: Fluorescence resonance energy transfer; calmodulin; green fluorescent protein; chameleon; calcium signaling.

1. Introduction

Because Ca^{2+} concentration ($[Ca^{2+}]$)changes are involved in many cellular processes, such as cell development, differentiation, and apoptosis, the study of these changes within their spatial and temporal context can provide valuable insights into their biological significance. Synthetic dyes (such as Fura and Indo) and aequorin are common tools for studying $[Ca^{2}]$ changes, however, most synthetic dyes leak rapidly from cells, and aequorin has a weak bioluminescence and is not ratiometric ***(1)***. In contrast, protein-based Ca^{2+} sensors based on Ca^{2+}–calmodulin (CaM) complexes and green fluorescent protein (GFP), previously named chameleons, overcome both previous limitations (*see* **Note 1**). In this chapter, we describe the methods involved in cloning chameleons, characterizing

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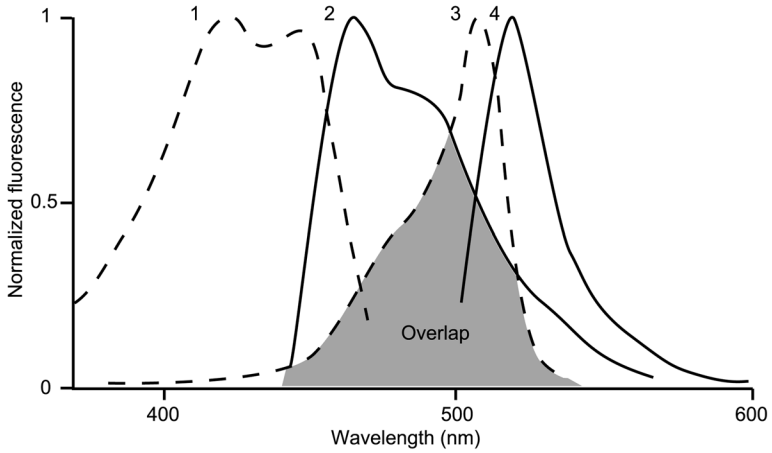

Fig. 1. Emission (solid) and excitation (dotted) spectrums of CFP (labeled 1 and 2) and YFP (labeled 3 and 4). The overlap between the CFP emission spectrum and YFP excitation spectrum allows FRET to occur between these fluorophores.

their biochemical properties, and imaging them in single cells using a digital fluorescence microscope. Before we can describe the methods, it is necessary to understand the theory of fluorescence resonance energy transfer (FRET; *see* **Subheading 1.1.**) and the general design of chameleons (*see* **Subheading 1.2.**).

1.1. Concept of FRET

FRET is the transfer of energy from the donor to acceptor fluorophore that can occur if the donor's emission spectrum strongly overlaps with the acceptor's excitation spectrum. This transfer of energy occurs at distances less than 80 Å and is most efficient when the fluorophores are closest and in a parallel orientation ***(2,3)***. In the design of chameleons, GFP mutants are used as fluorophore partners: cyan fluorescent protein (CFP) as donor and yellow fluorescent protein (YFP) as acceptor (*see* **Fig. 1**; **ref. *4***). Intermolecular FRET can be used to detect protein–protein interactions if both fluorophores are fused to different interacting partners, whereas intramolecular FRET can be used to detect protein cleavage and conformational changes if both fluorophores are fused to the same protein ***(3)***.

Fig. 1

1.2. General Design of Chameleons

Using the concept of intramolecular FRET, a chameleon consists of a tandem fusion of N- and C-terminal domains of CaM (N-CaM and C-CaM, respectively) and CaM recognition sequences (CRS), sandwiched between CFP and YFP ***(5–7)***. Depending on the binding orientation of the CRS, it can either be placed N-terminal to CaM, C-terminal to CaM, or in between N-CaM

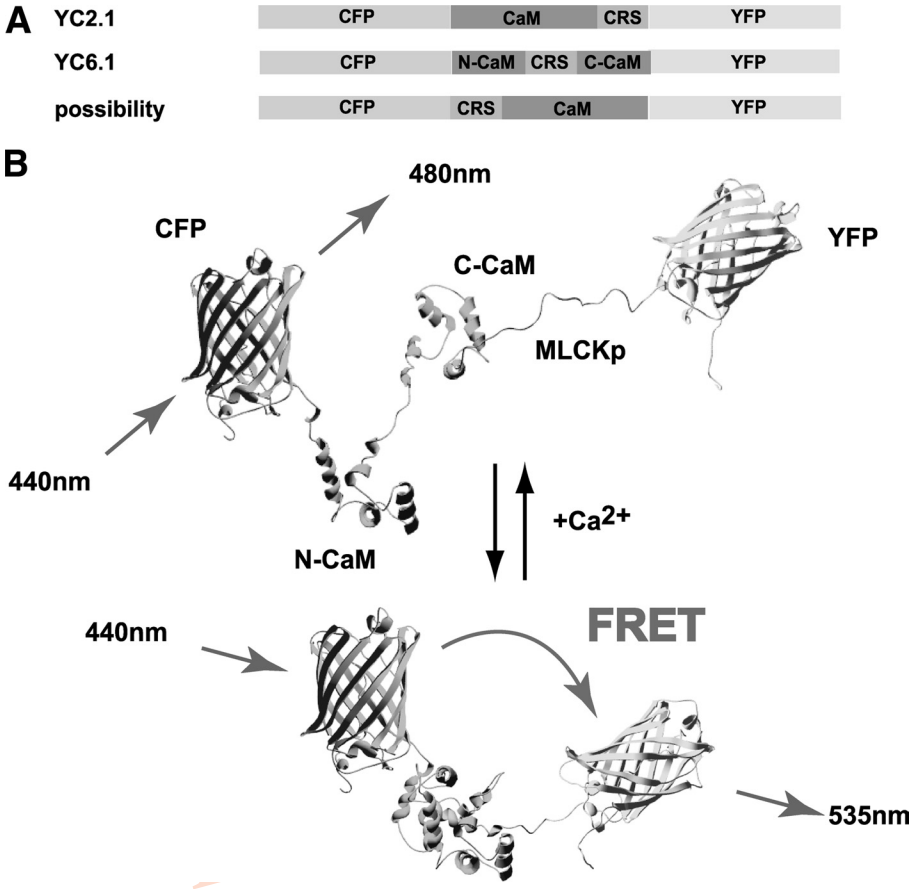

Fig. 2. **(A)** The possibilities for fusing the CRS to CaM. **(B)** A model of the possible the conformational change of YC2.1. Before the Ca^{2+} influx, the relative position of the fluorophores is far apart and, therefore, there is minimal FRET. After the Ca^{2+} influx, the fluorophores are brought closer by the Ca^{2+} CaM–CRS complex, resulting in an increased FRET.

and C-CaM (*see* **Fig. 2A** and **Note 2**). After a Ca^{2+} influx, CaM binds the CRS, causing a conformational change that brings CFP and YFP closer, for an increased FRET efficiency (*see* **Fig. 2B**). Therefore, a change in FRET efficiency is directly correlated to a change in $[Ca^{2+}]$. A number of yellow chameleons (YC), which use the CFP–YFP FRET partners, have been created that have varying Ca^{2+} binding affinities, subcellular localizations, and dynamic ranges. The methods presented in this chapter apply to all published YCs for Ca^{2+} imaging, including: YC2.1, based on the structure of CaM bound to the myosin light chain kinase CRS peptide (MLCKp) *(7)*; YC3.1, a mutant with a single E104Q

mutation to CaM that lowers Ca^{2+} affinity ***(7)***; YC2nu, targeted to the nucleus ***(6)***; YC6.1, based on the structure of CaM bound to CaM-dependent kinase kinase CRS peptide (CKKp) that improves dynamic range ***(5)***.

AU: Please spell out PRSETB, if appropriate.

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2. Materials

2.1. Cloning of Chameleons

1. PRSETB bacterial expression plasmid (Invitrogen): His-tag for Ni-nitrilotriacetic acid (NTA) agarose protein purification; ampicillin resistance gene for selection; and isopropyl-β-D-thio-galactopyranoside (IPTG) for inducible protein expression.
2. pcDNA3 mammalian transient expression plasmid (Invitrogen): cytomegalovirus promoter and ampicillin resistance gene for bacterial selection.

2.2. Biochemical and Biophysical Characterization

1. Luria-Bertani (LB) medium: 10 g tryptone, 5 g yeast extract, and 5 g NaCl; add water to a final volume of 1 L.
2. IPTG.
3. Phenylmethylsulfonyl fluoride.
4. Ni-NTA agarose (Qiagen).
5. *Escherichia coli* strain BL21 (DE3).
6. Sonicator.
7. EGTA buffer: 100 m*M* KCl; 50 m*M* HEPES pH 7.4; and 10 m*M* EGTA.
8. $CaC1_2$ buffer: 100 m*M* KCl; 50 m*M* HEPES, pH 7.4; 10 m*M* EGTA; and 10 m*M* $CaCl_2$.
9. Spectrofluorophotometer (Shimadzu).

2.3. Imaging With Chameleons

1. Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS).
2. Hanks' Balanced Salts Solution (HBSS) with Ca^{2+} (Gibco).
3. 37°C CO_2 incubator.
4. HeLa cell strain.
5. GeneJuice (Novagen).
6. Olympus IX70 inverted epifluorescence microscope.
7. Olympus Xenon lamp.
8. MicroMax 1300YHS charge-coupled device (CCD) camera and Sutter Lambda 10-2 filter changers controlled by Metafluor 4.5r2 software (Universal Imaging).
9. CFP–YFP FRET filter set (*see* **Note 3**) (Omega Optical): 440AF21 excitation filter (CFP excitation), 455DRLP dichroic mirror, 480AF30 emission filter (CFP emission), and 535AF26 emission filter (YFP emission).
10. Neutral density (ND) filter set (Omega Optical).
11. UApo ×40 oil Iris/340 objective (Olympus).
12. U-MNIBA band pass mirror cube unit (Olympus).

13. Histamine, ionomycin, EGTA, and BAPTA-AM (Sigma).
14. 35-mm-diameter glass-bottom dishes (Maltek).

3. Methods

The methods described below outline the cloning of chameleons for expression in bacterial and mammalian cells (*see* **Subheading 3.1.**), the biochemical and biophysical characterization of chameleons (*see* **Subheading 3.2.**), and the use of chameleons in FRET in vivo Ca^{2+} imaging (*see* **Subheading 3.3.**).

3.1. Cloning of Chameleons

Using standard recombinant DNA methods ***(8)***, chameleons are assembled modularly using five different components: CFP, YFP, N-CaM, C-CaM, and CRS. CFP and YFP plasmids are available from Clontech; CaM from Dr. Ikura; CRS from polymerase chain reaction oligonucleotide synthesis. The DNA manipulations to construct a chameleon are not described here in detail because there is no single strategy that applies to all possibilities (*see* **Note 4**). After the construction of a chameleon, the fusion fragment is cloned into the PRSETB plasmid for sufficient protein expression needed to perform biochemical and biophysical characterization. For transient mammalian expression of chameleons used in in vivo Ca^{2+}-imaging experiments, we cloned into the pcDNA3 plasmid (*see* **Note 5**).

3.2. Biochemical and Biophysical Characterization

Before the chameleon can be used in in vivo Ca^{2+} imaging, it is purified (*see* **Subheading 3.2.1.** and **Note 6**), and in vitro experiments with a fluorometer are performed to determine its dynamic range (*see* **Subheading 3.2.2.**) and Ca^{2+}-binding curve (*see* **Subheading 3.2.3.**).

3.2.1. Purification of Chameleons From Bacterial Cells

1. Transform BL21 (DE3) cells with the bacterial plasmid using standard molecular biology methods ***(8)***.
2. Plate the cells on LB plates containing ampicillin and incubate overnight at 37°C.
3. Select a single colony and grow in 100 mL of LB medium containing 100 µ*M* ampicillin at 37°C.
4. At an optical density at 600 nm of 0.7, induce with 0.5 m*M* IPTG for 3 h.
5. Centrifuge the cells for 30 min at 3000*g*.
6. Resuspend the cell pellet in 10 mL of 50 m*M* HEPES, pH 7.4; 10% glycerol; 100 m*M* KCl; 1 m*M* $CaCl_2$; and 1 m*M* phenylmethylsulfonyl fluoride.
7. Sonicate with the 0.5-in. horn at maximum power, 10% duty cycle, four times, for 4 min each. Cool the solution for 5 min between sonications.
8. Centrifuge the cell debris for 20 min at 30,000*g*.
9. Gently mix the supernatant with 1 mL of slurry Ni-NTA agarose for 30 min at 4°C.

10. Wash the column with 10 mL of 50 m*M* HEPES, pH 7.4; 100 m*M* KCl; and 5 m*M* imidazole.
11. Elute with 1 mL of 50 m*M* HEPES, 100 m*M* KCl, and 100 m*M* imidazole. It is unnecessary to cleave the His-Tag because it does not interfere with the fluorescent properties.
12. Dialyze the sample with 2 L of 50 m*M* HEPES, pH 7.4, and 100 m*M* KCl at 4°C.

3.2.2. Fluorescence Spectral Analysis

A change in FRET efficiency (or change in $[Ca^{2+}]$, in the case of chameleons) is often observed by a change in the emission ratio (R is the peak emission intensity of the acceptor divided by the peak emission intensity of the donor). The dynamic range of a Ca^{2+} indicator is defined as the division of the maximum ratio, R_{max}, by the minimum ratio, R_{min}. A chameleon with a larger dynamic range is a more effective in vivo Ca^{2+} indicator.

1. Dilute the sample in 50 m*M* HEPES, pH 7.4; 100 m*M* KCl; and 20 µ*M* EGTA into a 1-mL cuvet. Any dilution factor is acceptable as long as the emission signal remains detectable. Record the fluorescence emission spectrum from 450 to 570 nm at 433 nm excitation.
2. Record the fluorescence emission spectrum of the blank sample.
3. Subtract the spectrum in **step 2** (background) from **step 1** to find the spectra of the chameleon in the absence of Ca^{2+}. Determine R_{min} from this spectrum.
4. Repeat **steps 1** to **3** in the presence of 1 m*M* $CaCl_2$ to find the spectra of the chameleon in the presence of Ca^{2+}. Determine R_{max} from this spectrum.
5. **Figure 3** shows the fluorescence spectrum of YC6.1 ***(5)*** before and after Ca^{2+}, with an R_{min} and an R_{max} of 1.1 and 2.4, respectively.

Fig. 3

3.2.3. Ca^{2+}-Binding Properties

The Ca^{2+}-binding curve is used to assess the effective range of $[Ca^{2+}]$ that the Ca^{2+} indicator can measure. Ca^{2+}/EDTA and Ca^{2+}/EGTA buffer are used as standards because at low $[Ca^{2+}]$, Ca^{2+} contaminants can significantly distort the free $[Ca^{2+}]$ levels (*see* **Note 7**).

1. In a 1-mL cuvet, dilute the sample in the EGTA buffer. Record the fluorescence emission spectrum from 450 to 570 nm at 433 nm excitation. Determine the emission ratio.
2. To obtain the Ca^{2+}-binding curve, add successive fractions of the $CaC1_2$ solution to the sample and determine the emission ratio. Because the experiment is performed at 20°C with the EGTA and $CaCl_2$ buffers described in this chapter, the free calcium can be calculated by solving the quadratic equation (for different conditions, please consult **ref. *9***):

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$$([Ca^{2+}]_{free})^2 + \{(10{,}000{,}060.5 - [Ca^{2+}]_{total}) \times [Ca^{2+}]_{free}\} - (60.5 \times [Ca^{2+}]_{total}) = 0$$

3. To produce the Ca^{2+}-binding curve, plot the free$[Ca^{2+}]$ vs the change in the emission ratio. **Figure 4** shows the Ca^{2+}-binding curve of YC6.1.

Fig. 4

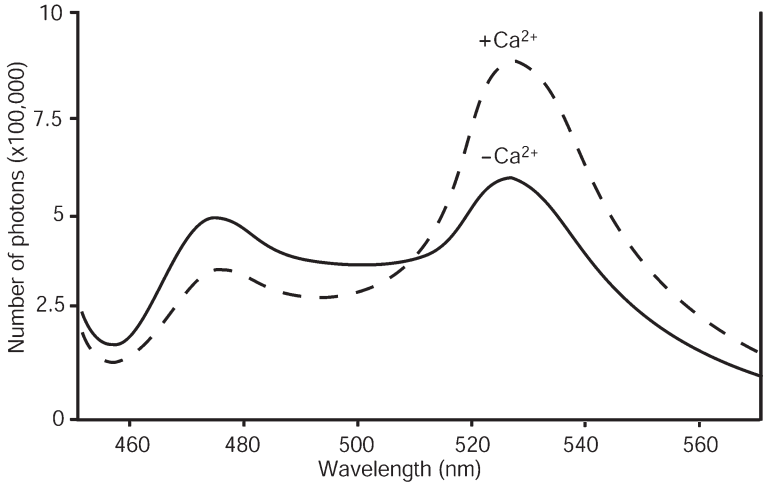

Fig. 3. Example fluorescence spectrum of YC6.1.

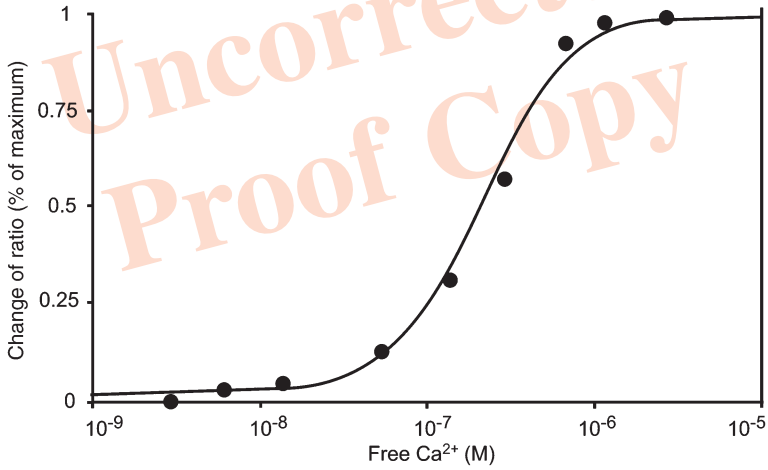

Fig. 4. Example Ca^{2+}-binding curve of YC6.1.

3.3. Ca^{2+} Imaging With Chameleons

The following section describes a simple Ca^{2+}-imaging experiment using HeLa cells; however, with minor modifications, the method can be applied to other adherent cells and physiological contexts. In this section, we describe the preparation of the cell culture (*see* **Subheading 3.3.1.**) and the acquisition of data (*see* **Subheading 3.3.2.**).

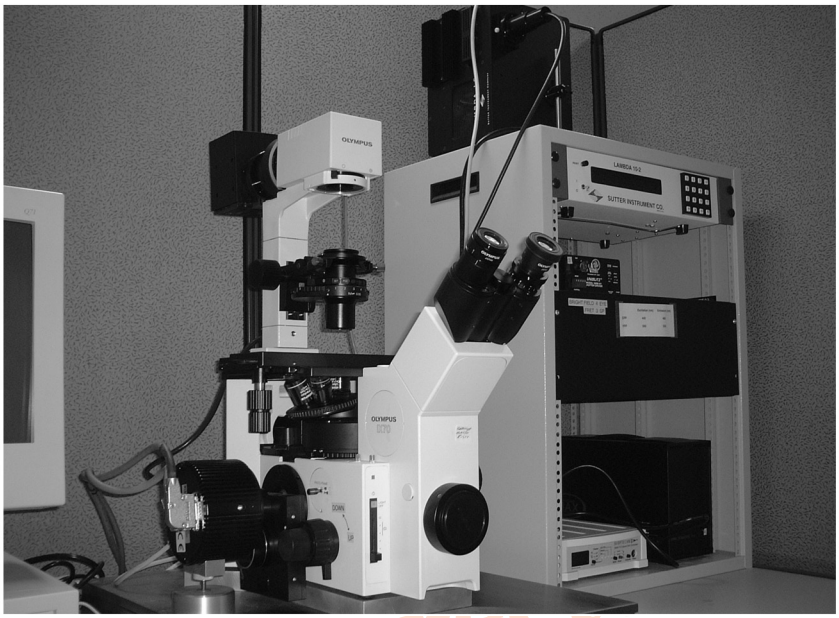

Fig. 5. Digital fluorescence microscope facilities.

3.3.1. Preparation of the Cell Culture

1. Plate HeLa cells on 35-mm-diameter glass-bottom dishes with DMEM–10% FBS media.
2. Incubate the cells at 37°C (5% CO_2) until cells are 50 to 80% confluent.
3. Transfect cells with the mammalian expression plasmid containing your chameleon, using GeneJuice (Novagen).
4. Remove the transfection mixture after 6 h and replace with 1.5 mL of DMEM–10% FBS media.
5. Incubate the cells at 37°C (5% CO_2) for 24 h. The cells are ready to perform the Ca^{2+}-imaging experiment.

3.3.2. Data Acquisition

The experiment should be performed in a dark room to reduce background light. **Figure 5** shows an image of our microscopy facilities.

Fig. 5

1. Turn on the Xenon lamp, microscope, filter changers, and computer.
2. Remove the growth media from the culture dish.
3. Wash with 1 mL of HBSS (+$CaCl_2$).
4. Add 1 mL of fresh HBSS (+$CaCl_2$).
5. Put the cells on the stage of microscope.
6. Start the MetaFluor software. The MetaFluor software controls the shutters, filter exchangers, and camera during data acquisition.

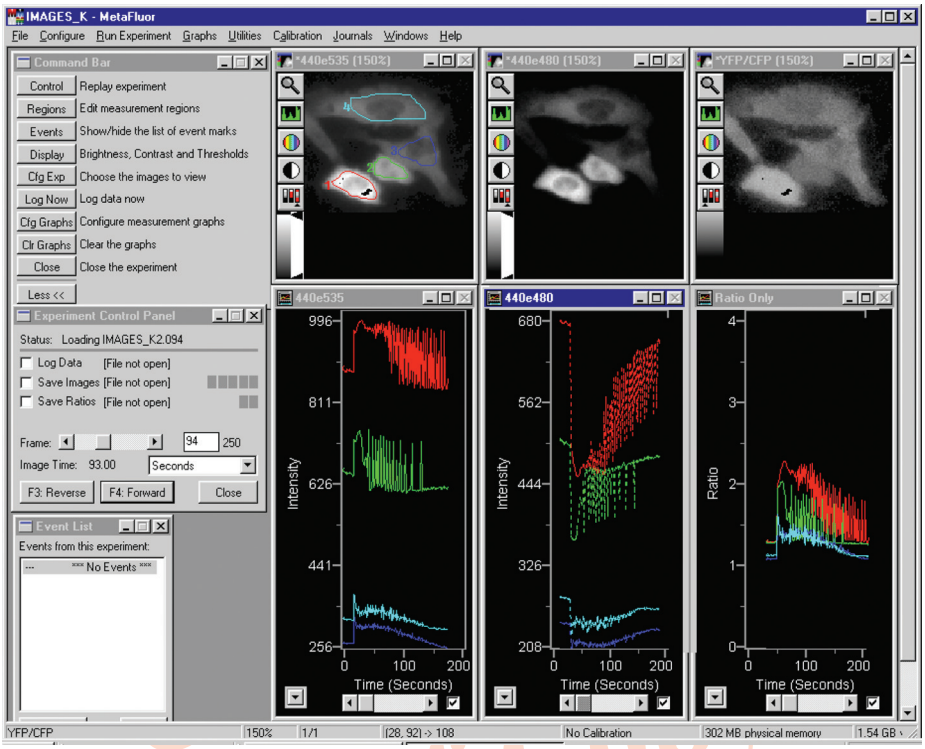

Fig. 6. Screen shot of the MetaFluor software. The regions of observation are highlighted in the 440e535 panel. The 440e535 panel and graph plot the change in YFP fluorescence as a result of CFP excitation; the 440e480 panel and graph plot the change in CFP fluorescence as a result of CFP excitation; the YFP/CFP panel and graph is the ratio of 440e535 and 440e480 panels and graphs. In this experiment, the graphs display a Ca^{2+} oscillation as a result of histamine stimulation.

7. To find cells expressing the chameleon construct, screen for YFP fluorescence using the eyepiece. Turn the filter turret to the U-WNIBA band-pass mirror cube and use the ND filter in the range of 0.1 to 10% depending on the level of fluorescence emission. The U-WNIBA mirror cube is ideal for quickly finding cells with YFP fluorescence using the eyepiece, and the 10% ND filter is used to reduce photobleaching.
8. Using the ×40 oil objective, center the microscope viewing area on a cell that has a healthy morphology and displays a strong cytosolic fluorescence. **Figure 6** shows a panel with the typical fluorescence distribution of a healthy HeLa cell.
9. Acquire single images on the computer screen using MetaFluor, while adjusting the focus until you have the sharpest image.
10. Turn the filter turret to the CFP–YFP FRET mirror cube.
11. To monitor the peak emissions of CFP and YFP as a result of CFP peak excitation over time, set the data acquisition conditions as follows: time interval, every 10 s;

and exposure time, 200 ms for CFP and YFP. MetaFluor will display the emission ratio over time.

12. Draw a region of interest on the field of view of the CCD. Usually, this region will outline the cell of interest. Then, start data acquisition. The fluorescence emission intensities of the CFP and YFP, together with their emission ratios in the region of interest, will be monitored over time (*see* **Note 8**).
13. When the emission ratio reaches a steady state, add 50 µL of 2 m*M* histamine to the culture dish for a final concentration of approx 100 µ*M*. Be careful not to move the culture dish in this process. The histamine binds to cell receptors on the plasma membrane that initiate a signaling cascade, resulting in the release of Ca^{2+} from the endoplasmic reticulum through the inositol-1,4,5-triphosphate receptor ***(10,11)***. This should cause a conformational change in the chameleon that can be observed by a rise in emission intensity of YFP and a decline in CFP intensity. Therefore, the emission ratio should increase. The emission ratio should return to steady-state levels when the effect of the histamine wanes. **Figure 6** shows a screen shot of MetaFluor software as it is collecting data.
14. To correlate the emission ratio to the cytosolic [Ca^{2+}], it is necessary to determine R_{min} and R_{max} so that emission ratios can be mapped to the Ca^{2+}-binding curve. Add 50 µL of 20 µ*M* ionomycin for a final concentration of approx 1 µ*M*. Ionomycin opens pores on the plasma membrane to allow permeability to Ca^{2+} ions. Because the medium is saturated with $CaCl_2$, the ratio will rise to R_{max}. To determine R_{min}, add 50 µL of 100 m*M* EGTA and 600 µ*M* BAPTA-AM for final concentrations of approx 5 m*M* and approx 30 µ*M*, respectively. The emission ratio should drop to R_{min}.
15. Stop the data acquisition.

4. Notes

1. Chameleons are not without their own limitations. In general, they have a lower signal-to-noise ratio than other techniques mentioned, but this ratio can be improved by the careful design of a new construct ***(5)*** or by finding optimal experimental conditions, as described in the text.
2. The best place to insert the CRS depends on the arrangement of the two CaM domains with respect to the CRS in the three-dimensional structure. If the N terminus of the CRS in the CaM-CRS complex is within 20 Å of the C-terminus of CaM, a CaM–CRS fusion would be effective ***(6)***. However, if the both N- and C-termini are within 10 Å of the hinge of CaM, a (N-CaM)–CRS–(C-CaM) fusion would be most effective ***(5)***.
3. If you are only interested in using CFP and YFP in your applications, the CFP–YFP FRET filter set will satisfy your needs. However, if you are using dsRFP ***(12,13)*** for FRET, you will need the following dichoric mirrors and filters from Omega Optical or equivalents: 450-520-590TBDR for the dichoric mirror; 440DF21 for CFP excitation; 510DF23 for YFP excitation; 575DF26 for RFP excitation; 480AF30 for CFP emission; 535AF26 for YFP emission; 600ALP for RFP emission. This filter set will allow you to excite or acquire emission from CFP, YFP, and RFP individually, however, with a tradeoff in efficiency.

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4. In our chameleon constructs, we truncated the last 11 C-terminal amino acids of CFP (the minimal region to form GFP) to reduce the relative tumbling of the fluorophores. Additionally, glycinyl–glycine linkers were introduced between CaM and CRS fusion points to increase conformational freedom for the formation of the Ca^{2+} CaM–CRS complex.
5. When amplifying the chameleon DNA by polymerase chain reaction for insertion into PRSETB or pcDNA3, remember that the 5′ and 3′ primers for the chameleon will have internal primer sites because CFP and YFP are only different by a few point mutations. It is necessary to purify the DNA fragment of the correct size from the agarose gel.
6. If your particular chameleon degrades or is insoluble in bacterial cells, it is possible to perform the characterization using mammalian cell lysate. For harvesting, cells should be grown in 10-cm-diameter culture dishes. Twenty-four hours after transfection, cells are scraped off the culture dish and lysed in a hypotonic lysis buffer (50 m*M* HEPES, pH 7.4; 100 m*M* KCl; 5 m*M* $MgCl_2$; and 0.5% Triton X-100). Then, the mixture is centrifuged to remove cell debris. Next, the supernatant is dialyzed in 2 L of buffer (50 m*M* HEPES, pH 7.4, and 100 m*M* KCl). Finally, the sample can be used for characterization as described in **Subheadings 3.2.2.** and **3.2.3.**
7. The accuracy of the Ca^{2+}-binding curve depends on the accurate preparation of the Ca^{2+}/EGTA buffers ***(9)***.
8. The emission intensities will decrease during the course of the experiment because of photobleaching; however, the emission ratio should remain constant if there is no change in FRET. To reduce photobleaching, decrease exposure time and excitation light intensity. Binning is summing the signal from multiple pixels on the CCD camera, so that less light is required, while maintaining a good signal-to-noise ratio.

Acknowledgments

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