Protocol

Imaging Intracellular Free Ca^{2+} Concentration Using Yellow Cameleons

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Green fluorescent protein (GFP)-based fluorescent indicators for Ca^{2+} offer significant promise for monitoring Ca^{2+} in previously unexplored organisms, tissues, and submicroscopic environments because they are genetically encoded, function without cofactors, can be targeted to any intracellular location, and are bright enough for single-cell imaging. These probes use simple GFP variants, circularly permuted GFP (cpGFP), in which the amino and carboxyl portions have been interchanged and reconnected by short spacers between the original termini, or pairs of GFP variants that permit fluorescence resonance energy transfer (FRET). Yellow cameleons (YCs) use FRET between cyan- and yellow-emitting variants of *Aequorea* GFP (cyan fluorescent protein [CFP] and yellow fluorescent protein [YFP], respectively). YCs are composed of a linear combination of CFP, calmodulin (CaM), a glycylglycine linker, the CaM-binding peptide of myosin light-chain kinase (M13), and YFP. Binding of Ca^{2+} to the CaM moiety of the YC initiates an intramolecular interaction between the CaM and the M13 domains, causing the chimeric protein to shift from an extended conformation to a more compact one, thereby increasing the efficiency of FRET from CFP to YFP. This technique is amenable to emission ratioing, which is more quantitative than single-wavelength monitoring. YC3.60 was engineered to enhance its performance as a fluorescent Ca^{2+} indicator and here we describe the use of thisameleon to image rapid changes in intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) within HeLa cells. FRET imaging is performed using a laser-scanning confocal microscope.

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

Ca^{2+} -free medium

To HBSS that is nominally Ca^{2+} free, add 1 μM ionomycin, 1 mM EGTA, and 5 mM MgCl_2 .

Dulbecco's modified Eagle medium (DMEM)

Hanks' balanced salt solution (HBSS) containing 1.26 mM CaCl_2

Heat-inactivated fetal bovine serum (FBS)

HeLa cells

Ionomycin

Mammalian expression vector containing the YC3.60 gene (pcDNA3/YC3.60)

Transfection reagents such as Superfect (QIAGEN) and Lipofectamine 2000 (Invitrogen)

Adapted from *Imaging: A Laboratory Manual* (ed. Yuste). CSHL Press, Cold Spring Harbor, NY, USA, 2011.

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Equipment

3CCD color camera (ORCA-3CCD Ashura from Hamamatsu Photonics; C7780-22)

For fast and simultaneous acquisition of cpVenus173 and CFP images from HeLa cells expressing YC3.60, this camera, which comprises three charge-coupled device (CCD) chips (RGB: red, green, and blue) and a prism, can be used. The cpVenus173 and CFP images are captured by the G and B chips, respectively. In addition, to improve spatial resolution along the z-axis, a spinning-disk unit (e.g., CSU21 from Yokogawa Electric Corporation) can be placed in front of the camera.

Diode-pumped solid-state laser (430 nm) (Melles Griot)

Glass-bottomed dishes (35 mm) (Matsunami Glass Ind., Ltd., D111300)

Image acquisition software to control the Ashura 3CCD camera (e.g., AQUACOSMOS [Hamamatsu Photonics])

Image processing software to make pseudocolor ratio images (e.g., AQUACOSMOS [Hamamatsu Photonics] or MetaMorph [Molecular Devices])

Incubator for cell culture (set at 37°C)

Inverted microscope IX-71 (Olympus) equipped with the best chromatically corrected objective UPLSAPO 60× 1.35 numerical aperture (NA) (Olympus)

METHOD

Transfection

1. Plate HeLa cells on coverslips in a Petri dish and allow them to grow in DMEM containing 10% heat-inactivated FBS for 12 h.
2. Transfect the cells in the dish with 1 µg of cDNA (pcDNA3/YC3.60) using Superfect or Lipofectamine 2000.

Imaging

3. Between 1 and 3 d posttransfection, image the HeLa cells on an inverted microscope. Expose the cells to reagents at room temperature in HBSS containing 1.26 mM CaCl_2 .
4. Choose moderately bright cells in which the fluorescence is uniformly distributed in the cytosolic compartment but excluded from the nucleus, as would be expected for a 74-kDa protein without targeting signals.
5. Define several variables for fast image acquisition (stream mode). These include intensity of the laser, scanning speed, binning, and sensitivity of the camera.

In our experience, the intensity of the laser should be attenuated greatly using neutral-density filters for Ca^{2+} imaging on a timescale of subseconds. Photobleaching does not happen with a laser that is sufficiently attenuated.

6. Observe fluorescence signals from CFP and cp173Venus by the blue and green channels of the 3CCD camera, respectively.
7. Set 4 × 4 binning for high-sensitive fluorescence detection at video rate observation.
8. Adjust the rotational speed of the spinning disk. To remove the artificial streak raster pattern, synchronize spinning with the frame CCD readout.
9. Acquire video rate confocal FRET images (31.5-msec accumulation time/frame) during stimulation such as an application of histamine.
10. At the end of an experiment, convert the fluorescence signal into $[\text{Ca}^{2+}]_c$. Obtain $R_{\max}$ and $R_{\min}$ values as follows.
 - i. Saturate intracellular YC3.60 with Ca^{2+} by increasing the extracellular $[\text{Ca}^{2+}]$ to 10–20 mM in the presence of 1–5 µM ionomycin. Wait until the fluorescence intensity reaches a plateau.
 - ii. Deplete the indicator of Ca^{2+} by washing the cells with Ca^{2+} -free medium.

- iii. The in situ calibration for $[Ca^{2+}]$ uses the equation

$$[Ca^{2+}] = K'_d[(R - R_{min})/(R_{max} - R)]^{1/n},$$

where K'_d is the apparent dissociation constant corresponding to the Ca^{2+} concentration at which R is midway between R_{max} and R_{min} and n is the Hill coefficient. YC3.60 shows a monophasic Ca^{2+} response curve ($K'_d = 0.25 \mu M$; $n = 1.7$).

11. Take an image without laser illumination to make a background image.

Background-corrected images for CFP and cpVenus173 were used to make pseudocolor ratio images.

DISCUSSION

Structure, Properties, and Uses of YCs

The prototype cameleon was generated in 1997 (Miyawaki et al. 1997) and since then several versions have been engineered to improve performance as a fluorescent Ca^{2+} indicator. Version YC3.1 is less sensitive to acidic pH than the prototype (Miyawaki et al. 1999). Version YC3.12 contains Venus (Nagai et al. 2002), a bright YFP variant that matures rapidly in mammalian cells at 37°C and thereby allows the immediate detection of $[Ca^{2+}]_i$ transients after gene introduction in freshly prepared brain slices. To increase the dynamic range of the Ca^{2+} response, FRET and circular-permutation techniques have been combined. A large Ca^{2+} -dependent change in the relative orientation and distance between CFP and YFP has been achieved using cpYFPs (Nagai et al. 2004) in which the amino and carboxyl portions have been interchanged and reconnected by a short spacer between the original termini (Baird et al. 1999). Such circular permutation has been conducted on Venus, and new termini have been introduced into surface-exposed loop regions of the β -can. One of the variants, cp173Venus, has a new amino terminus at Asp-173, which is far removed at the opposite end of the β -can from the original amino terminus, Met1 (Fig. 1A). YC3.60 was generated by replacing Venus in YC3.12 with cp173Venus (Fig. 1B). Compared with YC3.12, YC3.60 is equally bright but shows a five- to sixfold larger dynamic range (Fig. 1C). Thus, YC3.60 gives a greatly enhanced signal-to-noise ratio (SNR), thereby enabling Ca^{2+} imaging experiments that were not previously possible. A confocal image of YC3.60-expressing HeLa cells is shown in Figure 1D. A series of ratio images in pseudocolor acquired at video rate (displayed at 16.7 Hz) shows how the increase in $[Ca^{2+}]_c$ (cytosolic free Ca^{2+} concentration) appeared and propagated within the individual cells after stimulation with histamine.

It has been pointed out that the dynamic range of YCs is damped down in neuronal cell types that have a large amount of CaM and CaM-associated proteins. Thus, the interface between the CaM and the M13 peptide has been redesigned to generate highly specific protein/peptide pairs that are not perturbed by endogenous CaM or CaM-binding proteins (Palmer et al. 2004). A mutant CaM/peptide pair, D1, was cloned between CFP and YFP to yield a re-engineered YC. The D1-containing YC is indifferent to a large excess of CaM. Moreover, it displays a low- Ca^{2+} affinity with a K_d value of 60 μM and has proven to be an ideal probe for measuring the Ca^{2+} concentration in the endoplasmic reticulum. The binding interface of CaM and M13 was further reengineered by computationally designing complementary bumps and holes (Palmer et al. 2006). Another attempt to eliminate the sensitivity of genetically encoded Ca^{2+} indicators to endogenous CaM and CaM-binding proteins involved the use of troponin C proteins from skeletal and cardiac muscles (Heim and Griesbeck 2004). Because troponin C is specifically expressed in muscle, these novel probes should not be perturbed by endogenous proteins when expressed in nonmuscle tissues. Generation of transgenic mouse lines expressing variants of the troponin C-based Ca^{2+} indicators, such as CerTN-L15 (Heim et al. 2007) and TN-XXL (Mank et al. 2008), allowed the investigators to perform reliable in vivo two-photon Ca^{2+} imaging of neurons.

Because of their emission of ratiometric responses, YCs are the best choice for observing Ca^{2+} dynamics in motile animals. Calcium transients in neurons have been observed in the process of sensation of gentle touch (Suzuki et al. 2003) or temperature (Clark et al. 2007) of *Caenorhabditis*

FIGURE 1. Development and performance of YC3.60. (A) The three-dimensional structure of GFP with the positions of the original (Met1) and the new (Asp173) amino termini indicated. (B) Domain structures of YC3.12 and YC3.60. (CaM) *Xenopus* calmodulin; (E104Q) mutation of the conserved bidentate glutamate (E104) at position 12 of the third Ca^{2+} -binding loop to glutamine. (C) Emission spectra of YC3.12 (left) and YC3.60 (right) (excitation at 435 nm) at zero (blue line) and saturated Ca^{2+} (red line). (D) A series of confocal pseudocolored ratio images showing propagation of $[\text{Ca}^{2+}]_c$ in HeLa cells that expressed YC3.60.

elegans and during escape behaviors of zebrafish (Higashijima et al. 2003). The ratiometric approach using YCs is also of great advantage for quantitative Ca^{2+} measurements. A version of YC (Synapcam) was localized to the postsynaptic terminals at the *Drosophila* larval neuromuscular junction to selectively and quantitatively measure Ca^{2+} influx through glutamate receptors with single-impulse and single-bouton resolutions. The Ca^{2+} influx signals (i.e., the transmission strength) varied along the axonal branches with a gradient of weak, at the proximal boutons, to strong, at the distal ones. More recently, quantitative in vivo imaging using YC3.60 revealed elevated $[\text{Ca}^{2+}]_i$ in neurites near A β plaques (Kuchibhotla et al. 2008).

Although FRET-based indicators for Ca^{2+} have been developed, our understanding of the structure–photochemistry relationships of GFP has enabled the development of Ca^{2+} probes based on a single GFP variant. cpGFPs, in which the amino and carboxyl portions had been interchanged around position 145 and reconnected by short spacers between the original termini (Baird et al. 1999), were fused to CaM and its target peptide, M13. Chimeric proteins G-CaMP (Nakai et al. 2001) and pericam (Nakai et al. 2001) are fluorescent, and their spectral properties change reversibly with Ca^{2+} concentration, probably caused by the interaction between CaM and M13, leading to an alteration of the environment surrounding the chromophore. G-CaMP is a single-wavelength intensity-modulating probe for Ca^{2+} , which is now widely used. The probe has been iteratively improved in terms of brightness, response linearity, photostability, and calcium affinity. The most updated version (G-CaMP3) allows for imaging neural activity in multiple model animals, such as worms, flies, and mice (Tian et al. 2009). Three types of pericam have been obtained by mutating several amino acids adjacent to the chromophore. Of these, flash pericam becomes bright with Ca^{2+} -like G-CaMP, whereas inverse pericam dims. On the other hand, ratiometric pericam has an excitation wavelength that changes in a Ca^{2+} -dependent manner, thereby enabling dual excitation ratiometric Ca^{2+} imaging. Ratiometric pericam permits quantitative Ca^{2+} measurement by minimizing the effects of several artifacts that are unrelated to changes in $[\text{Ca}^{2+}]_i$. The molecular structures of the genetic calcium indicators are shown in Figure 2.

Practical Considerations in Experiments with HeLa Cells

pH

pH-related artifacts were not an issue in the experiments that used HeLa cells because agonist-induced $[\text{Ca}^{2+}]_c$ mobilization did not cause any intracellular pH changes detectable by the pH indicator, BCECF (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein) (data not shown). Correspondingly, comparisons of YC2 with YC2.1 and YC3 with YC3.1 showed no major differences in the reported $[\text{Ca}^{2+}]_c$ attributable to the difference in pH sensitivity. On the other hand, both YC2 and YC3 expressed in dissociated hippocampal neurons were perturbed by acidification following depolariza-

FIGURE 2. Schematics of two genetically encodable indicators for Ca^{2+} . (A) Yellow cameleon. (B) G-CaMP or pericam. (Modified, with permission, from Tsien 2000.)

tion or glutamate stimulation. This problem was solved by using YC2.1 or YC3.1 (Miyawaki et al. 1999). YC2.12, YC3.12, and YC3.60 also give reliable Ca^{2+} responses over a physiological range of pH (Nagai et al. 2004).

Concentration of YCs in Cells

The estimation of cameleon concentration in cells is essential for quantifying the trade-off between optical detectability and Ca^{2+} buffering. It is important to consider the ability of various concentrations of YC3.60 to buffer Ca^{2+} transients in HeLa cells. During a 0.1-mM histamine challenge, sharp $[\text{Ca}^{2+}]_c$ transients followed by $[\text{Ca}^{2+}]_c$ oscillations can be observed in cells expressing $<200 \mu\text{M}$ YC3.60. In contrast, $[\text{Ca}^{2+}]_c$ recovers slowly to the baseline over a period of several hundred seconds, and oscillations are never observed when transfected HeLa cells expressed $>300 \mu\text{M}$ YC3.60. Cameleons at cytosolic concentrations below $<20 \mu\text{M}$ are too dim to give favorable SNRs with our current instruments. This limitation cannot be overcome by increasing the intensity of illumination because of YFP photochromism (see below).

Photochromism of YFP (cpVenus173)

If YFP is excited too strongly, its fluorescence will be reduced. This apparent bleaching is actually photochromism because the fluorescence recovers, to some extent, spontaneously and can be further restored by ultraviolet illumination (Dickson et al. 1997). Intense excitation of YCs also causes photochromism of the YFP moiety, which results in a decrease in the yellow:cyano emission ratio independent of Ca^{2+} change. The extent of photochromism is dependent on excitation power, NA of objective, and exposure time. Therefore, it is necessary to optimize these factors for each cell sample to minimize photochromism while still preserving a high SNR. Because the photochromism is partially reversible, the sampling interval is another factor that should be considered. Illumination at frequent intervals sometimes leads to a decrease in the resting ratio values of YCs. A better solution is to bin pixels at the cost of spatial resolution. The increased SNR permits the decrease in intensity of the excitation light with a neutral-density filter and the observation of $[\text{Ca}^{2+}]_i$ oscillations without significant photochromism of the indicators.

Microscopy

The dynamic measurement of $[\text{Ca}^{2+}]_i$ in space and time is crucial for understanding many important cellular functions. Although common conventional confocal microscopes use a single laser beam to scan a specimen, the Yokogawa Electric Corporation confocal scanning unit (CSU) scans the field of view with approximately 1000 laser beams by means of the microlens-enhanced Nipkow-disk scanning technology. CSU's multibeam scanning technology requires low-light intensity per unit area, thereby significantly reducing photobleaching and phototoxicity in live cells. The most advanced model of the CSU series is the CSU-X1. With an additional camera port, the CSU-X1 makes it easy to image two different emission ranges simultaneously. This is probably best for the fast confocal observation of YC3.60 or indicators based on FRET between CFP and YFP. The 430-nm laser described in this article is no longer available and can be replaced with common solid-state lasers emitting at $\sim 440 \text{ nm}$.

Another option for fast confocal imaging is the LSM 5 *LIVE* (Zeiss). With the use of a coherent line excitation light and a line CCD, this system illuminates 512 pixels along a line in the x direction and scans this line in the y direction with a scan mirror for up to 2048 pixels. This results in the ideal combination of long pixel dwell time and short frame-acquisition time. Unlike the CSU, confocal aperture can be freely adjusted in this system. In addition, scanning speed need not be synchronized with frame CCD readout. Figure 3 shows histamine-induced $[\text{Ca}^{2+}]_c$ transients in HeLa cells imaged with the LSM 5 *LIVE* system. The updated system, LSM 7 *LIVE*, is currently available.

Neural activity can be analyzed in dissociated neuronal cultures, slice cultures, acute slices, and intact animals. Gene transfer techniques, including liposome-mediated transfection, various viral

FIGURE 3. Histamine-induced $[Ca^{2+}]_c$ transients in HeLa cells imaged with LSM 5 *LIVE* system equipped with a 440-nm (laser diode) laser line and an objective lens (Plan-Apochromat 63 $\times$, 1.4 NA, oil). Images were acquired without averaging at 30 Hz. The pinhole size was set to 28 μ m ($\sim$ 2 Airy disk units) for both channels. Scale bar, 10 μ m.

vectors, electroporation, and the gene gun, have shown significant progress in recent years. Because there is a trend toward the understanding of brain functions in a physiological context, the ultimate approach is to construct transgenic lines, to which two-photon excitation microscopy is applied. Although two-photon excitation microscopy has been applied to samples loaded with multiple fluorophores for multicolor or FRET imaging, the excitation of one fluorophore relative to the other fluorophores cannot be controlled well in the conventional system that uses a narrowband laser. Recently, a new technology that enables phase modulation of ultrabroadband laser pulses has been reported (Isobe et al. 2009). Versatile control of two-photon excitation of CFP and YFP was achieved with their ratio (CFP/YFP) being varied 100-fold. Selective excitation of CFP over YFP was indeed appreciated in an experiment using YC.

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