In Vivo Calcium Imaging of Brain Activity in *Drosophila* by Transgenic Cameleon Expression

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Abstract

Various genetically encoded fluorescent sensors that monitor changes in intracellular calcium concentration have been developed over the last few years. The ability to target these calcium indicators to cells and structures of interest makes them valuable tools for diverse applications and gives them distinct advantages over conventional fluorescent dyes in transgenically tractable organisms. In particular, the cameleon calcium sensors have been used successfully in a number of applications. For example, we use cameleon-2.1 to monitor in vivo brain activity in Drosophila. However, using cameleons to image intracellular calcium concentration changes in vivo is still evolving and is by no means a standard technique. Experimental details and “tricks” for dealing with equipment, techniques, and data evaluation are still restricted to a few laboratories. In this protocol for calcium imaging in Drosophila brain using cameleon-2.1, we provide guidelines to the basic principles of this novel technique in Drosophila neuroscience and, more generally, to the broad field of signal transduction research.

Introduction

Calcium imaging has become a standard technique in diverse areas of biology in general, and neuroscience in particular, reflecting the many cellular roles and actions of this ion as a second messenger and as a carrier of charge. Calcium can stimulate biochemical cascades and changes in gene transcription, mediate synaptic potentials, and trigger vesicle release. In neurons, changes in intracellular calcium concentration, which depend on processes such as presynaptic, postsynaptic, and somatic calcium influx, as well as intracellular calcium release, can be a consequence or a cause of neuronal depolarization and, therefore, represent a valuable indicator of neuronal activity. Calcium imaging using synthetic calcium-sensitive dyes, such as Fura-2 or Calcium Green, has been used for a wide range of studies, from cellular processes in vitro to complex networks in living animals. However, those conventional fluorophores have a major drawback: Their distribution within a cell or a tissue relies on diffusion and reflects both the ability of the dye to diffuse and the density of the tissue. The advent of genetically encoded fluorescent sensors [for a review, see (1)], which allow targeting of the sensor to neurons of interest or to subcellular structures within cells, is a major step forward.

Most genetically encoded fluorescent sensors contain variants of green fluorescent protein (GFP) as their molecular core. Two strategies to construct calcium-sensitive fluorescent sensors have been described. The first class of sensors, which includes the cameleons (2, 3), contains two GFP variants that differ in their excitation and emission wavelengths, usually enhanced cyan (ECFP) and enhanced yellow (EYFP) fluorescent protein. These two fluorescent proteins are fused to the calcium-binding region of calmodulin and a calmodulin target peptide to make them responsive to calcium (Fig. 1) (2-6). Upon calcium binding, a conformational shift in the protein occurs, which increases fluorescence resonance energy transfer (FRET) from ECFP to EYFP. When cameleon is excited, it emits light at 485 nm, thereby exciting EYFP, which emits light at 535 nm. An increase in intracellular calcium concentration can be detected by a simultaneous shift in the emission intensities of both fluorophores: ECFP emission decreases, whereas EYFP emission increases (Fig. 1). Thus, the ratio of EYFP emission to ECFP emission reflects the extent by which intracellular calcium concentration changes. The second class of sensors relies on so-called circularly permuted GFP variants (7-9). In these sensors, the original NH2- and COOH-termini have been fused and a “crack” close to the chromophore region has been introduced, providing new NH2- and COOH-termini that are fused to a calcium-binding region of calmodulin and, in some cases, a calmodulin...
target peptide (8, 9). These sensors show very low fluorescence in the unbound state, but increase emission intensity considerably after binding calcium. For the in vivo imaging experiments described here, we prefer FRET-based sensors for two reasons. First, the relatively high background fluorescence allows us to easily detect labeled structures and analyze their morphology in addition to monitoring changes in intracellular calcium concentration. Second, the ratiometric nature of the fluorescence measurements substantially reduces changes in fluorescence resulting from movement artifacts, a serious concern when working with living animals.

*Drosophila* is an ideal model organism in which to make use of these genetically encoded sensors. First, the germ lines of fruit flies can easily be manipulated. Second, using the Gal4-UAS technique (10, 11), the sensor can be targeted to any brain region of interest for which a Gal4 driver line exists. In this two-part approach, one fly strain carries the cameleon DNA under the control of an upstream activator sequence (UAS), so that the gene is silent in the absence of the transcription factor Gal4. A second fly strain expressing Gal4 in a cell type-specific manner is mated to the UAS strain, resulting in progeny expressing cameleon in a transcriptional pattern reflecting the expression pattern of the Gal4 line (Fig. 2). Third, advanced genetic tools available in *Drosophila*, which can be used to manipulate and analyze brain function, can potentially be combined with the optical imaging approach. Fourth, the *Drosophila* brain is small enough to allow simultaneous imaging of large parts of its circuitry.

We used genetically encoded calcium sensors to establish a method to visualize neuronal activity evoked by external stimuli in the brains of living fruit flies (12). The stimuli we used (odorants) and the brain regions we analyzed (neurons within the fly’s olfactory pathway) were determined by our particular research interests. However, the technique can readily be adapted to other areas in the wide realm of *Drosophila* neurobiology. Cameleon has been used successfully to analyze synaptic transmission at the *Drosophila* neuromuscular junction (13) and can certainly be adapted to other *Drosophila* preparations, such as embryos and cell cultures. Thus, despite our focus on our own specific experiments and experiences in this protocol, we wish not only to provide a guideline for using this technology in *Drosophila* olfaction research, but also to elucidate the basic principles of cameleon imaging in *Drosophila*. 

**Protocol**

UAS construct: GAL4 lines: UAS/GAL4:

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**Fig. 2.** Cell-type specific expression of Cameleon 2.1 using the Gal4-UAS system (10). The fly line UAS-Cameleon 2.1 (14) is crossed to any cell type–specific Gal4 line, such as one expressing Gal4 in olfactory projection neurons or in the mushroom bodies, leading to restricted cameleon expression in the F1 generation.
## Materials

Dental glue (Protemp II, ESPE, Seefeld, Germany)

*Note: Other types of glue that are harmless to the fly may be used.*

## Fly Strains

UAS-Cameleon 2.1/82 [homozygous second chromosome insertion (14), available at the Bloomington Stock Center at Indiana University, Bloomington, IN 47405 (http://fly.bio.indiana.edu)]

Gal4 lines of interest, such as the GH 146 line, which expresses Gal4 in a large subset of olfactory projection neurons connecting the antennal lobe with the mushroom body and the lateral protocerebral lobe (15). (Many Gal4 lines are available at the Bloomington Stock Center.)

## Preparation Chamber

2 cm by 2 cm by 2 cm acrylic block

2 cm by 2 cm plastic coverslip with a 5-mm hole punched into the center

Transparent polypropylene plastic sheet

*Note: An experimental chamber is constructed from an acrylic block, a plastic coverslip, and a very thin transparent plastic sheet that can easily be cut with a razor blade (see details under Instructions, below). A notch cut across the top of the acrylic block allows the fixed fly to be placed on the block, and channels drilled into the sides intersect with this notch and allow odorants to be applied from the front (Fig. 3).*

## Dissection

Blade holders (Fine Science Tools, Foster City, CA #10053-13)

Breakable razor blades (Fine Science Tools, #10050-00)

Forceps (Dumont #5) (Fine Science Tools, #11252-20)

Minutien pins (Fine Science Tools, #26002-10)

## Stimulation

Aquarium pump

Electronic solenoid valve (The Lee Company, Essex, CT)

Mineral oil

Odorants

1 ml plastic pipette tip

Teflon tubing
**Equipment**

The calcium imaging setup consists of five parts: an upright microscope, a light source, a charge-coupled device (CCD) camera, a beam-splitter device, and computer hardware and software to control data acquisition (see Fig. 4 for setup). Several companies (for instance, Visitron Systems, Puchheim, Germany; TILL Photonics, Gräfelfing, Germany; and Improvision, Lexington, MA) provide complete calcium imaging system setups.

**Fig. 3.** In vivo preparation of *Drosophila* for optical imaging of the brain. For detailed explanation, see text. CX, calyx; iACT, inner antennocerebral tract; LPL, lateral protocerebral lobe.
**Protocol**

**Microscope**

Upright microscope, such as the Zeiss Axioscope 2FS or Olympus BX 50

*Note: We found that a fixed stage was very convenient for our specific application.*

40× to 63× water immersion objective with a long working distance and a numerical aperture between 0.8 and 1, such as the Zeiss Achromplan or Olympus LumPlanFl

**Illumination**

Polychromator equipped with a 75 to 100 W xenon arc lamp, such as the TILL monochromator system (TILL Photonics, Gräfelfing, Germany) or the Visichrome high-speed polychromator system (Visitron, Puchheim, Germany)

*Note: If the system is used only for cameleon-based imaging, a xenon or mercury arc lamp equipped with a 440-nm band pass filter should be sufficient. It is important to equip the light source with a fast shutter to allow accurate illumination times. The optimal excitation wavelength for this application is 436 nm ± 5 nm bandwidth.*

**CCD Camera**

Fast, high-resolution CCD camera, such as CoolSnap HQ (Roper Scientific, Tucson, AZ), Orca ER (Hamamatsu, Bridgewater, NJ), or Till Photonics SVGA (Gräfelfing, Germany).

*Note: Critical parameters for the choice of a CCD camera include the readout speed (10 to 20 Hz), the chip size (at least 640 by 480 pixels), flexible binning, and quantum efficiency achieved by cooling.*

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**Fig. 4.** Cameleon imaging setup. The prepared fly is placed under the microscope using a water immersion objective. Illumination from an arc lamp and a monochromator at 436 nm evokes a fluorescent emission that is transmitted by a 455- to 470-nm dichroic long pass filter (DCLP). The beam-splitter device separates EYFP from ECFP emission with a 505- to 515-nm DCLP and narrows the emissions with bandpass filters (BP) of 535/30 nm and 485/40 nm, respectively. Images of both emitted wavelengths are projected side by side onto a single CCD camera chip. A computer controls the illumination, the CCD camera, and the stimulus application (in this case, odorant stimulation).
Filter Settings and Beam-Splitter Device

- 455- to 470-nm long-pass (LP) dichroic mirror
- 505- to 515-nm LP dichroic mirror
- 535/30-nm emission filter
- 485/40-nm emission filter

Note: A cameleon 2-filter set is available at Chroma, Brattleboro, VT, part #71007.

Beam-splitter device (Optical Insights, Santa Fe, NM)

Note: Two independent CCD cameras can be used (12) to detect the two wavelengths of emitted light. However, alignment of the two images and accurate, synchronized control of the image acquisition is relatively complicated. A beam-splitter device permits accurate projection of the two images onto two halves of a single CCD chip.

Computer Control of the CCD Camera and Data Acquisition

Software package, such as MetaFluor and MetaMorph (Universal Imaging, Downingtown, PA), TILLVision (TILL Photonics, Gräfelfing, Germany), or Velocity (Improvision, Lexington, MA).

Note: The software controls the light source, the CCD camera, and application of the stimulus. In addition, it is necessary to be able to follow fluorescence intensity changes at given regions within the acquired image for both wavelengths online.

Recipes

Recipe 1: Drosophila Ringer’s Solution (16)

NaCl 130 mM
KCl 5 mM
MgCl₂ 2 mM
CaCl₂ 2 mM
Sucrose 36 mM
Hepes-NaOH, pH 7.3 5 mM

Prepare in 100 ml of distilled, deionized water and store as aliquots at −20°C.

Instructions

Experimental Preparation

1. Using the Gal4-UAS technique (10, 11) flies selectively expressing cameleon in defined subsets of cells can be generated by crossing the appropriate Gal4 line to the transgenic UAS-cameleon strain (Fig. 2). We have crossed the UAS-Cameleon 2.1/82 line with the GH 146 line, which expresses Gal4 in a large subset of olfactory projection neurons connecting the antennal lobe with the mushroom body and the lateral protocerebral lobe (15). The expression level of cameleon can be increased by creating flies homozygous both for Gal4 and the UAS-cameleon insert.

Note: For a detailed description of the Gal4-UAS techniques, see (11).

2. Cool the fly in an empty vial on ice for no longer than 10 min.

Note: Cooling for more than 10 min can produce long-lasting effects on the nervous system. Immobilizing the flies using CO₂ is not recommended because of possible deleterious side effects on the nervous tissue.
3. Glue a thin transparent sheet of plastic to the top of a plastic coverslip in which a hole has been stamped (Fig. 3, step 1).

4. Turn the coverslip over so that the transparent sheet is on the bottom and the opening in the coverslip faces upward.

5. Place a minuten pin into the hole on the transparency and apply a drop of dental glue to it (Fig. 3, step 2).

6. Glue the fly over the minuten pin belly-up, being careful to avoid damaging the antennae (Fig. 3, step 3). The fly is placed this way so that the minuten pin sits between head and thorax; this stretches the head in a position that keeps the antennae away from the glue.

7. Cut the legs and immobilize the abdomen by placing a tiny drop of glue on the abdominal tip.

8. After the glue has dried, turn the coverslip over and place it on an acrylic preparation chamber so that the fly rests in the groove cut into the top of the acrylic block (Fig. 3, step 4).

9. Place a drop (about 300 µl) of *Drosophila* Ringer’s solution (Recipe 1) over the head (Fig. 3, step 5).

10. Use a splinter of a breakable razorblade in a blade holder to cut a hole through the transparency and the head capsule and expose the brain (Fig. 3, step 6). Avoid bleaching by using red light illumination in the room and on the dissection microscope.

11. Carefully remove the trachea and the antennal hearts using a fine forceps. It is important to be extremely careful to avoid damaging the exposed brain.

12. Place the preparation chamber containing the fly under the imaging microscope using a water immersion objective.

13. Focus on the brain using red light illumination (greater than 600 nm). EYFP fluorescence is visible with illumination at 436 nm and a yellow emission filter (Fig. 3, step 7). When focusing on the structures of interest, keep the illumination time as short as possible to avoid photobleaching.

14. To stimulate flies with an odorant, use an aquarium pump to produce a constant air stream. Use an electronic valve and teflon tubing to guide the air stream through filters or vials containing an odorous compound or solvent (usually mineral oil), respectively. The air stream is then guided through a pipette tip placed in a channel drilled through the side of the acrylic block to a point about 5 mm distant from the fly’s antennae and maxillary palps, the chemosensory organs.

*Note: It is very important to avoid any mechanosensory artifacts, such as might be caused by an air pressure decrease, and to avoid any contamination of the control airflow with odorants.*

**Data Acquisition**

1. Adjust the illumination in both the EYFP and ECFP channels to obtain fluorescence intensity values in a reasonable detection range (above ~1000). Reasonable fluorescence intensities can be achieved by adjusting the illumination time (~20 to 200 ms) or by adjusting the binning on the CCD chip. Prolongation of the illumination time enhances the fluorescence intensity at the cost of temporal resolution; increasing the bin size enhances the fluorescence intensity at the cost of spatial resolution.

2. Choose regions of interest (ROIs) within which to evaluate calcium changes online (Fig. 5A).

3. Acquire as many images as necessary, but as few as possible, to determine the baseline before the stimulus and the onset and duration of the evoked calcium influx. It is important to obtain images before, during, and after stimulation. Keep the illumination time as short as possible. The frame rate depends on the signal intensity and the background fluorescence. In our experience, frame rates from 3 to 10 Hz are reasonable, but this will depend on the particular application and the cameleon expression level.

**Data Evaluation**

Changes in calcium concentration can be detected as changes in the fluorescence of ECFP and EYFP in opposite directions. Shifts in fluorescence intensity caused by bleaching or artifacts, on the other hand, such as movement artifacts due to muscle contractions or hemolymph pumping, are characterized by parallel changes in EYFP and ECFP emission. For a preparation in which motion cannot be eliminated, verifying the opposite direction of the shifts in ECFP and EYFP fluorescence is imperative. As shown in Fig. 5B, EYFP emission in the ROI increases and ECFP emission decreases in response to an odorant stimulus. It is also clear, however, that there are parallel shifts in ECFP and EYFP fluorescence before the stimulus. These parallel shifts are due to motion. Therefore, it is often necessary to average several experiments and perform a statistical analysis to eliminate such artifactual responses and discriminate between signal and noise.
1. To obtain normalized values expressed as percentage of fluorescence intensity change, one can calculate a \( \Delta F/F (\%) \) value, where \( F \) is a baseline value before stimulation and \( \Delta F \) refers to the change in fluorescence from baseline (Fig. 5B). Note: We often use the average of three images before onset of stimulus to define the baseline value of \( F \). However, more sophisticated measurements of the baseline (for instance, measurements that take bleaching of the probe into account) may be applied.

2. The ratio of the two fluorescent signals is calculated as \( F (\text{EYFP}) \) divided by \( F (\text{ECFP}) \) for every time point. This value represents the relative magnitude of the FRET signal intensity. To normalize this value, a \( \Delta R/R (\%) \) value can be calculated, where \( R \) is defined as the average of an appropriate number of ratio values before stimulus onset, and \( \Delta R \) is the deviation from \( R \) (Fig. 5B).
3. Correct the values for bleaching. Applying a single or higher order exponential fit to the raw intensities of EYFP or ECFP can approximately correct for bleaching. Alternatively, a linear correction for bleaching can be applied to the ratio (EYFP/ECFP) signals as a function of time.

Note: Bleaching is a highly nonlinear process, particularly with a probe such as cameleon, which consists of two fluorophores that optically interact with each other. Whereas we find that presentation of raw data is useful as far as indicating the actual measurement, quantitative analyses of calcium influx may require correction for bleaching.

Troubleshooting

When setting up the system, we recommend applying KCl (Fig. 6) or a calcium ionophore as an initial test to determine whether the preparation and signal detection are optimized.

Fig. 6. KCl-induced calcium signal in the lateral protocerebral lobe. (A) EYFP fluorescence showing the lateral protocerebral lobe (LPL) with a region of interest (ROI) depicted. (B) The change in ratio (EYFP/ECFP) fluorescence as a function of time as \( \Delta R/R(\%) \) acquired with a frequency of 3 frames per s is shown. The green bar indicates the injection of 5 \( \mu l \) of 1 M KCl into the drop of Drosophila Ringer’s solution above the brain (~300 \( \mu l \)). The trace shows a rapid decrease immediately after injection, due to contraction of the tissue, and a subsequent increase in intracellular calcium concentration due to depolarization. The three numbers indicate the time points for the images shown in (C). The pseudocolor coded frames show the intensity of \( \Delta R/R(\%) \) at the different time points 1 (frame 98), 2 (frame 114), and 3 (frame 250), clearly demonstrating the calcium increase. (D) Screenshots and color scale for Movies 1 through 3 (http://stke.sciencemag.org/cgi/content/full/sigtrans;2003/174/pl6/DC1), which show the time course of the contraction and the subsequent transient increase in calcium concentration. Movie 1 shows the pseudocolor coded ratio (EYFP/ECFP). In Movie 2 the ratio (EYFP/ECFP) is grayscaled and above a threshold pseudocolor coded, indicating the calcium signal above baseline. Movie 3 consists of an average of the grayscaled EYFP images and the ratio (EYFP/ECFP) pseudocolor coded above the baseline.
In each experiment, a ROI that cannot respond to calcium, for instance one from a region that is not part of the cameleon-expressing tissue, should always be evaluated as a control. In the experiment shown in Fig. 6B, cells were depolarized by injecting 5 µl of 1 M KCl (to about a 10-mM final concentration) into the Drosophila Ringer’s solution surrounding the brain. The ratio trace shows a rapid decrease after the KCl application that does not reflect a change in intracellular calcium concentration but a contraction caused by the KCl-evoked depolarization. As can be seen in Movie 1, Movie 2, and Movie 3, the whole brain tissue moves reversibly out of focus, resulting in an unequal decrease in EYFP and ECFP fluorescence. This illustrates the need to evaluate every shift in the ratio trace by analyzing the morphology of the tissue and the time courses of the original fluorescence values for EYFP and ECFP. The calcium influx due to the depolarization is reflected by a subsequent increase in the ratio of EYFP to ECFP.

A continuous flow of oxygenated Drosophila Ringer’s solution prolongs the lifetime of the tissue (17).

Related Techniques

Other calcium sensors, such as camgaroo (7) or G-CamP (9), have also been used in Drosophila with great success (18, 19). These other genetically encoded calcium sensors show a very strong signal-to-noise ratio; they have the disadvantage, however, of showing low background fluorescence, and they cannot be used for ratiometric imaging. The choice of which sensor is best for a particular experiment depends on the application. For experiments in which motion cannot be excluded, such as in whole animal preparations, the ratiometric cameleon sensor is obviously advantageous. For cell culture experiments or tissue preparations, circularly permuted sensors might be the better choice because of their higher signal-to-noise ratio.

Notes and Remarks

Data Presentation

Calcium imaging data can be presented as traces plotting the change in fluorescence as a function of time. It is important to examine the original traces of DF/F (Fig. 5), because R or DR/R values can give false signals that actually derive from movement artifacts.

The change in fluorescence in response to a stimulus can be presented as a series of frames. Such a multidimensional dynamic signal can best be displayed using pseudocolor coded images. By adjusting the color range to cover the range of observed ΔR/R values (color column), the increase in signal can easily be visualized (Fig. 6C). Alternatively, a movie clip can be created to show the dynamic change in fluorescence emission (Fig. 6D). In Movie 1 (http://stke.sciencemag.org/cgi/content/full/sigtrans;2003/174/p6/DC1), the pseudocolor coded ratio EYFP/ECFP is shown as in C, indicating only roughly the morphological structures. Movie 2 (http://stke.sciencemag.org/cgi/content/full/sigtrans;2003/174/p6/DC1) shows the same data, but in a different color scaling. The ratio EYFP/ECFP is grayscale coded, showing the morphology of the tissue. The increase in the ratio is visualized by applying a threshold above R at which the scale is changed to a pseudocolor presentation with a very narrow color range (color column). Similar results are obtained by calculating an average of the grayscale EYFP emissions, again indicating the morphology and superimposing the above-threshold pseudocolor coded ratio signal, indicating the increase in intracellular calcium concentration [Movie 3 (http://stke.sciencemag.org/cgi/content/full/sigtrans;2003/174/p6/DC1)].

Image processing, filtering and averaging can all be used posthoc to eliminate noise and enhance calcium signals.

References

17. C. G. Galizia, personal communication.
20. We are grateful to R. Tsien and A. Miyawaki for granting the use of the cameleon construct and to G. Heimbeck and R. Stocker for providing the line GH 146. We also thank E. Buchner, C. Schuster, D. Reiff, and G. Galizia for support and help. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 554/B2 and Fl 821/1-1).