Chapter 2

Analysis of Calcium Signals in Steering Neuronal Growth Cones In Vitro

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Abstract

Calcium imaging allows us to measure the spatial and temporal changes in intracellular calcium concentration in living cells. Localized calcium elevation often functions as the polarizing signal during guided migration including axon guidance. In this chapter, we describe a protocol to quantitatively monitor the spatiotemporal dynamics of calcium signals in neuronal growth cones in the presence of an extracellular concentration gradient of axon guidance cue.

Key words Growth cone, Axon guidance, Ca^{2+} imaging, Turning assay, Chemotaxis

1 Introduction

Calcium imaging is a widely used technique in laboratories across almost all fields of studies. Indicators for calcium ions (Ca^{2+}) change their fluorescence properties upon Ca^{2+} binding. Therefore, recording the changes in fluorescent intensity of the indicator allows us to quantify changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]$). There are two different types of Ca^{2+} indicators: Chemical indicators and genetically encoded indicators. The first generation of synthetic chemical indicators was UV-excitable and ratiometric indicators. They require either dual-wavelength excitation or dual-emission monitoring [1, 2]. Subsequently, to overcome the disadvantages of UV-excitation, i.e., cytotoxicity and generation of autofluorescence from intracellular constitutions, visible light-excitable indicators have been synthesized [3, 4]. Today there are many commercially available indicators with different excitation and emission spectra that cover a broad range of Ca^{2+} concentrations. As for genetically encoded indicators, modern-day versions are derived from photoproteins isolated from organisms like the luminescent jelly fish [5]. The recent development of genetic engineering technology has produced a number of gene-encoded

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indicators that facilitate the generation of knock-in cell lines and animals, and the selective labeling of specific cell types for in vivo and ex vivo imaging [6, 7]. In dissociated cell culture systems, chemical indicators are, however, better choices because their acetoxymethyl (AM)-conjugated form can easily be loaded into the cytosol and generally provide higher signal to noise ratio. Because growth cones are highly motile, their shape changes during the recording period even if the recording takes only a few minutes. Confounding factors such as changes in thickness can be canceled out by using dual-emission ratiometric imaging. Fura-Red is a dual-excitation indicator. Its peak absorbance shifts from 473 nm to 436 nm upon Ca^{2+} binding and excitation at either wave length results in the emission of red light (longer than 550 nm, [8]). Therefore, as $[Ca^{2+}]$ increases, the intensity of emitted light excited at 473 nm decreases. In combination with another blue light-excitable Ca^{2+} indicator, one can perform dual-emission ratiometric imaging with visible light excitation. There are many reports using this system for monitoring Ca^{2+} dynamics in growth cones [9, 10]. In our laboratory, we employ Fura-Red and Oregon green 488 BAPTA (OGB)-1 for dual-emission ratiometric imaging [11].

The first in vitro chemotaxis assay of neuronal growth cones was reported in 1979, in which a perfusion system was used to generate a stable gradient of nerve growth factor (NGF). In that study, NGF was ejected continuously from a micropipette so that NGF distributes as a gradient along the medium flow [12]. A decade later, Mu-ming Poo's group developed a simpler method for generating a stable gradient of guidance cues using repetitive pulses, rather than the continuous ejection method [13]. Because the duration of a single pulse was short, the distribution of the molecule from each ejecting pulse should ideally be Gaussian, i.e., the distribution of the guidance cue is radial and decays exponentially from the tip of the micropipette. Therefore, a stable gradient can be generated without a perfusion system. This method in combination with optical imaging techniques is now widely used to assess chemotaxis and its associated intracellular signaling pathways [11, 14, 15].

In this chapter, we describe the method for quantitative Ca^{2+} imaging in a growth cone exposed to an NGF gradient.

2 Materials

2.1 Culture Media and Reagents

1. Poly-D-lysine (PDL, Sigma) is dissolved in sterile water and filtrated with 0.45 μm filter to make 10 % (w/v) stock solution. The stock solution, stored at −80 °C, is further diluted to make 0.01 % (w/v) solution. The diluted stock is stored at 4 °C and ready for use.

2. Natural mouse laminin (Invitrogen).
3. Goat anti-human Fc (Jackson) for L1-Fc coating of culture dishes.
4. L1-Fc chimeric proteins that consist of the whole extracellular domain of L1 and the Fc region of human immunoglobulin G is prepared as described [16].
5. RPMI 1640 (Invitrogen) supplemented with 10 % fetal bovine serum (JRH Biosciences) and 20 ng/ml NGF (Promega). Antibiotic-antimycotic, containing penicillin G (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml) (Invitrogen) is also added into the medium.
6. Leibovitz L-15 medium (Invitrogen) supplemented with 20 ng/ml NGF, N2 (Invitrogen), and 750 μg/ml bovine serum albumin (Invitrogen).
7. OGB-1-AM and Fura-Red-AM (Invitrogen) are dissolved in dimethyl sulfoxide (Sigma) to make stock solutions (both 1 mM) and put into an ultrasonic bath for 10 min. Then they are filtrated through 0.22 μm filter (Merck Millipore) by centrifuging at 9,100 × *g*. Store at –20 °C.
8. Cremophor EL (Nacalai Tesque) is dissolved in sterile water to make 10 % stock solution. Store at 4 °C for up to 1 month.
9. Dextran, Alexa Fluor 488 (10,000 MW, Invitrogen) is dissolved in phosphate-buffered saline (PBS, Invitrogen) to make 1 mM stock solution (*see* **Note 1**). Store at –20 °C.

2.2 Animals

Fertilized chicken eggs are obtained from local supplier and incubated at 37.7 °C until embryonic day 9 or 10.

2.3 Apparatus for the Generation of a Microscopic Gradient

1. Borosilicate glass tubings with filament (outer diameter: 1.0 mm, inner diameter: 0.5 mm, length: 10 cm, Sutter Instruments) are pulled using a Flaming/Brown micropipette puller (model P-97; Sutter Instruments) to make micropipettes with 1 μm tip.
2. A motor-drive manipulator (MM-89, Narishige) and a three-axis oil hydraulic micromanipulator (MMO-203, Narishige) for coarse and fine control of the micropipette, respectively. Those manipulators must be fixed rigidly to the microscope via an adaptor.
3. A pneumatic picopump (PV-820, World Precision Instruments) with a holder for micropipettes with 1.0 mm outer diameter (World Precision Instruments).
4. An electric stimulator (SEN-3301, Nihon Kohden) for controlling the picopump.
5. Nitrogen gas cylinder with pressure regulator.

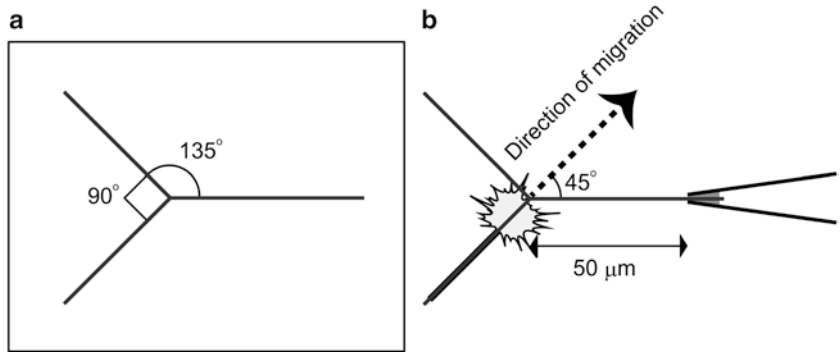

Fig. 1 "Y-sheet" for aligning the micropipette and the growth cone. (**a**) Draw this on a transparent sheet to be attached onto the PC monitor. (**b**) By aligning an axon shaft with either of the two orthogonal lines and positioning the micropipette along the horizontal line, the gradient of the guidance cue of interest can be applied to the growth cone at 45° to its direction of migration

6. The "Y sheet": To facilitate the experiment by showing where the micropipette tip should be positioned with respect to the growth cone (Fig. 1). Affix the "Y sheet" on the monitor of the PC with sticky tape.

2.4 Equipment for Ca^{2+} Imaging

1. An inverted microscope for epifluorescent imaging (IX-81, Olympus) with 20× (UPlanFL N, NA 0.5, Olympus) and 100× (UPlanSApo, NA 1.40, Olympus) objectives. The microscope must be covered by an acrylic box and have a heater in order to maintain the temperature at 37 °C inside the box.
2. A CCD camera (ImagEM, Hamamatsu Photonics).
3. An emission light splitter (Dual View, Roper scientific) with a 565 nm dichroic mirror (DM), and 527/20 nm and 610 nm long pass emission filters (Fig. 2. *See also* **Notes 2** and **3**).
4. Optical filters. In addition to the filters in an emission splitter, an excitation filter (492/18 nm), and a DM (505 nm) are set in the filter wheel with electronic shutter (Ludl Electronic Products) and the microscope, respectively (Fig. 2).
5. A PC with the image acquisition software, MetaMorph (Molecular Devices), that comes with the "split view" function.

2.5 Data Analysis

Software for data quantification, scientific graphing, and statistical analysis: MATLAB 2012a (Math Works) and GraphPad Prism 4.0 (GraphPad Software).

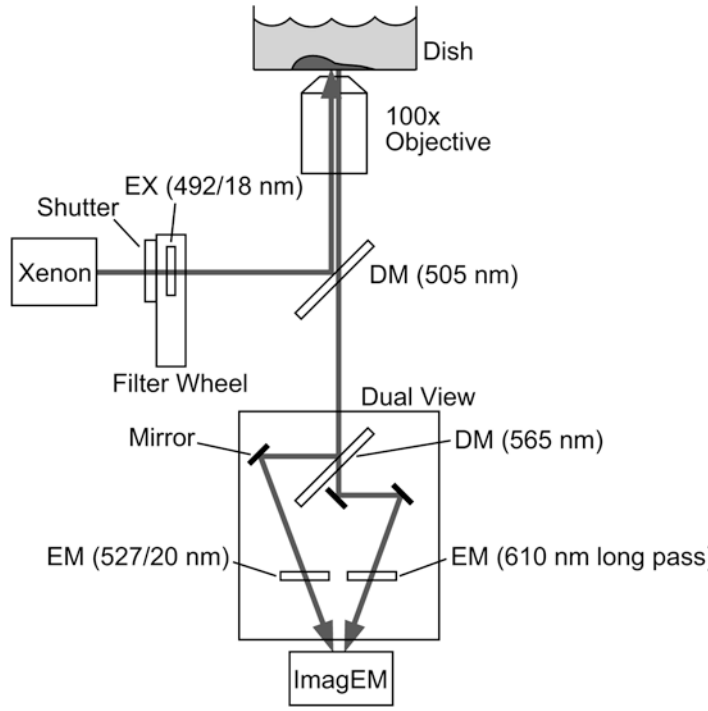

Fig. 2 Schematic drawing of the microscope setup for dual-emission imaging. *EX* excitation filter, *DM* dichroic mirror, *EM* emission filter

3 Methods

3.1 Check for Gradient Generation

1. Switch on the heater inside the acrylic box. Humidify the setup by hanging a moistened paper towel with its bottom tip dipped in a beaker containing distilled water. Wait at least 1 h for the temperature and humidity to stabilize.
2. Mount a glass-based dish containing pre-warmed 2 ml Leibovitz L-15 medium on the microscope.
3. Fill a micropipette with ~2 μl Alexa Fluor 488-dextran solution (1 mM), and connect it to the picopump holder. Then mount the holder onto the micromanipulator.
4. With 20× objective, set the micropipette at 50 μm from the center of the field of view (*see* **Note 4**).
5. Perform imaging as follows: For now, bypass the Dual View unit for full view imaging. Take pictures at 3–5 s intervals with the ImagEM (binning set at 1×1) over a time period of 10 min. Within the 10 min, take 30 images as background, then start Alexa ejection (2 Hz, 20 ms duration, 4 psi) (*see* **Note 5**).

3.2 Dish Coating

1. Put 1 ml PDL solution (0.01 %) onto a glass-based dish (bottom cover slip diameter: 27 mm, IWAKI). Incubate at 37 °C for 1 h (up to overnight).
2. Wash the dish twice with sterile water.
3. Put laminin solution (~50 ng/cm^2) on the dish, and incubate at 37 °C for 1 h; or alternatively,
   (a) Put goat anti-human Fc solution (~4 μg/cm^2) on and incubate at 37 °C for 1 h (up to overnight).
   (b) Wash the dish three times with PBS.
   (c) Put 0.5 ml L1-Fc solution on and incubate at 37 °C for 1 h.
4. Rinse with serum-containing RPMI 1640.

3.3 Cell Culture

1. Dissect dosal root ganglia (DRG) from the lumbrosacral region of an embryonic day 9 or 10 chick into an eppendorf tube containing 1 ml Leibovitz L-15 medium.
2. Spin down at 9,100 × *g* for 1 min at 4 °C.
3. Discard supernatant and resuspend in 100 μl trypsin–EDTA (0.05 %, Invitrogen).
4. Incubate at 37 °C for 18 min.
5. Spin down at 9,100 × *g* for 1 min at 4 °C.
6. Discard supernatant and wash in 1 ml Leibovitz L-15 medium.
7. Spin down at 9,100 × *g* for 1 min at 4 °C.
8. Discard supernatant and resuspend in 30 μl Leibovitz L-15 medium.
9. Gently triturate with the 2–20 μl pipette (Eppendorf) without introducing bubbles until a single cell suspension is obtained (~70 strokes usually).
10. Spin down at 9,100 × *g* for 1 min at 4 °C.
11. Discard supernatant and resuspend in 500 μl pre-warmed RPMI1640 medium supplemented with NGF and FBS.
12. Plate cells (~1.4×10^4 cells/dish) onto the glass-based dish coated with either laminin or L1, and incubate in a humidified atmosphere of 95 % air/5 % CO_2 at 37 °C for at least 2 h before loading Ca^{2+} indicators.

3.4 Loading of Ca^{2+} Indicators

All media used here are pre-warmed at 37 °C.

1. Prepare Ca^{2+} indicator solution: Dissolve 2 μl OGB-1 (2 μM final concentration), 2 μl Fura-Red (2 μM final concentration), 0.2 μl Cremophor EL stock solutions in 1 ml Leibovitz L-15 medium. Vortex to mix well.
2. Gently rinse the cell culture with Leibovitz L-15 medium.

3. Incubate cells with the Ca^{2+} indicator solution at 37 °C for 30 min.
4. Gently rinse the culture with Leibovitz L-15 medium.
5. Incubate with 2 ml Leibovitz L-15 medium supplemented with NGF, N2, and BSA at 37 °C for at least 30 min before mounting onto the microscope.

3.5 Ca^{2+} Imaging in the Growth Cone Exposed to an NGF Gradient

Warm up and humidify the environment inside the acrylic box covering the microscope as described above (*see* Subheading 3.1, **step 1**). Wait at least 1 h before starting recordings, allowing the temperature and humidity to stabilize (*see* **Note 6**).

1. Put a drop of immersion oil onto the 100× objective, and mount the culture dish on top.
2. Fill the micropipette with ~2 μl NGF (100 μg/ml), and connect it to the picopump holder controlled by the micromanipulator.
3. After locating the growth cone for imaging, switch the objective from 100× to 20×.
4. Carefully rotate the cell culture dish to adjust the axon angle using the "Y sheet" (Fig. 1, *see also* **Note 7**).
5. Lower the micropipette to the center of the field of view (*see* **Note 8**).
6. Lift the micropipette out of the culture medium in order to minimize dilution due to backpressure.
7. Switch the objective back to 100× (*see* **Note 9**).
8. Lower the micropipette back down to the original position, and then move it to 50 μm from the growth cone (*see* **Note 10**).
9. Perform imaging as follows: Move the Dual View unit back into the emitted light path. Record over a period of 10 min, taking pictures at 3–5-s intervals with the ImagEM (binning set at 4×4). Within the 10 min of recording, the first 30 images are background recording before NGF ejection. Then start the NGF ejection (2 Hz, 20 ms duration, 4 psi).

3.6 Data Analysis

Custom-made programs written with MATLAB are used for all procedures unless otherwise stated.

1. Creating the ratiometric image: Split the recorded image into OGB-1 and Fura-Red channels with the "split view" function of MetaMorph (those two images must be aligned perfectly in X–Y space). After subtracting the background from each image, divide the OGB-1 image by the Fura-Red image. Create a mask for extracting the growth cone region as follows (*see* **Note 11**): Make two new images by applying Gaussian filters with different kurtosis to the Fura-Red image, and

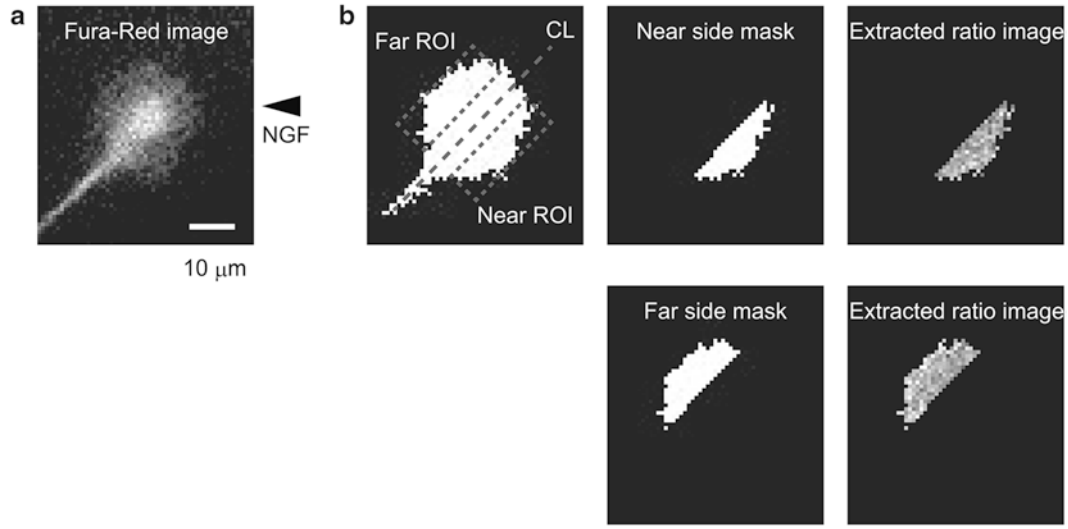

Fig. 3 Extracting the near and far side images of the growth cone for the analysis of asymmetry in Ca^{2+} signals. (**a**) A Fura-Red image of a growth cone loaded with both OGB-1 and Fura-Red. The NGF-containing micropipette was placed on its right side. (**b**) The mask image (*left panel*) is created based on the Fura-Red image (**a**). The center line (CL) and the near and far ROIs are determined as described in the text (*see* Subheading 3.6, **step 2**). By applying the near and far side masks (*middle panels*) over the ratiometric images, the near and far sides of the growth cone are extracted (*right panels*), to be used for further analysis

subtract the more blurred image from the other. Further subtract the resultant image from the original (unprocessed) image. Finally, binarize the last image.

2. Determining the near and far regions of interest (ROIs) (Fig. 3): Draw a 5 μm line along the axon shaft from the point where the shaft connects with the growth cone, start and end points of the line are points "1" and "2". Find the centroid of the growth cone in three consecutive images (points "3," "4," and "5"). A center line for dividing the growth cone into halves is found by taking the linear regression across points 1 to 5. The side facing the NGF source is defined as near side and the opposite as far side. Each growth cone half is further divided into three equal sections along the width (which is perpendicular to the center line). The rectangle enclosing the lateral two-thirds on the near side or far side of the growth cone is defined as the "Near ROI" or "Far ROI," respectively.
3. Calculating the relative changes in $[Ca^{2+}]$ on the near and far sides: R is relative fluorescence between OGB-1 ($F_{\text{OGB-1}}$) and Fura-Red ($F_{\text{Fura-Red}}$).

$$R = \frac{F_{\text{OGB-1}}}{F_{\text{Fura-Red}}}$$

R_0 is the baseline R, taken as the mean of 20 consecutive R before NGF ejection. Calculate R/R_0 at each time point over the period of Ca^{2+} imaging (10 min) on the near and far sides.

4. [Optional] Calculate the near–far asymmetry: Divide the R/R_0 of near side by R/R_0 of far side.
5. Performing statistical analyses with Prism: Calculate the mean R/R_0 during the period from 1 min after the start of NGF ejection till the end of recording for each growth cone. This is because usually the extracellular gradient of NGF takes about a minute to form. Compare R/R_0 on the near side versus the far side using paired *t*-test to show whether the applied gradient causes any asymmetric changes in $[Ca^{2+}]$. Depending on the experiment, it may be necessary to compare near–far asymmetry under different experimental conditions, for example, in the presence versus absence of a pharmacological inhibitor in the bath medium. In this case, use unpaired *t*-test or an appropriate multiple comparison test if there are three or more conditions, to assess the effect of drug action on the Ca^{2+} asymmetry.

4 Notes

1. Because the distribution of the ejected molecule theoretically varies depending on the molecular weight, a fluorescent molecule of similar molecular weight as the guidance cue of interest should be used for checking the gradient.
2. OGB-1 and Fura-Red images are simultaneously projected onto the CCD camera, and this image has to be divided into two separate channels to get the ratiometric image for further analysis. Because misalignment causes a critical error in ratiometric imaging (the R value), OGB-1 and Fura-Red images must be aligned carefully by following the alignment protocol provided by the manufacturer.
3. For accurate calculation of the relative changes in $[Ca^{2+}]$, background intensity should be subtracted from the experimental images. To obtain the background intensity, take five images under the same condition as the experimental images, e.g., the filters, illumination intensity, and exposure time, in an empty area of the culture dish.
4. Hints on finding the tip of the micropipette: Reduce the area of the transmitted light as much as possible using the field stop, to generate a very thin column of light. Adjust the position of the micropipette so that the tip falls within the narrowed beam light; lift up the objective, and observe through the eyepiece to find the pipette tip before the tip touches the bottom of the dish; by moving the pipette back and front, you

should be able to find the tip easily; the tip would be most easily found with a phase contrast rather than a differential interference contrast objective; however, even if a differential interference contrast objective is used, raising the condenser will be helpful for finding the tip easily.

5. The distribution is ideally radial. If the distribution skews too much, try: Changing the pressure intensity and/or pulse duration, changing the heater position, changing the position, and/or number of moistened paper towels.
6. The lower limit of humidity for maintaining cells at healthy level is, by our group's experience, 40 %. This can be achieved by directing the heated air flow toward the moistened paper towel.
7. Observe cells through the eyepiece but not through a CCD camera, otherwise you may easily lose the target growth cone when rotating the dish.
8. Remember the *z*-axis location of the micropipette on the micromanipulator so as not to break the tip of the micropipette in later steps.
9. Two types of oils are available for Olympus objectives. Of those two, we use Type-F immersion oil, because it is more viscous than the other. Higher viscosity should minimize spilling of the oil over the 100× objective when switching to the 20× objective.
10. With the 100× objective, Dual View in place, and ImagEM camera, the pipette tip will be outside the field of view before reaching 50 μm apart from the growth cone, thus the distance between the growth cone and the micropipette tip should be measured using the scale on the micromanipulator.
11. Rapid changes in growth cone shape cause confounding factors. Those factors must be eliminated from the analysis, because they cause artifactual increases or decreases in $[Ca^{2+}]$. The factor derived from changes in thickness (*z*-axis) can be canceled out by utilizing ratiometric imaging ($F_{\text{OGB-1}}/F_{\textit{Fure-Red}}$), and the factor due to changes in shape (on the *xy*-plane) can be excluded by extracting the growth cone area from the image with an appropriate mask.

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