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Bioluminescence imaging of neuronal network dynamics using aequorin-based calcium sensors

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Running Head: Bioluminescence imaging of neuronal networks

ABSTRACT

Optogenetic calcium sensors enable the imaging in real-time of the activities of single or multiple neurons in brain slices and *in vivo*. Bioluminescent probes engineered from the natural calcium sensor aequorin do not require illumination, are virtually devoid of background signal, and exhibit large dynamic range and low cytotoxicity. These probes are thus well-suited for long-duration, whole-field recordings of multiple neurons simultaneously. Here we describe a protocol for monitoring and analyzing the dynamics of neuronal ensembles using whole-field bioluminescence imaging of an aequorin-based sensor in brain slice.

Keywords: Bioluminescence, Genetically Encoded Biosensors, GFP-Aequorin, Calcium Imaging, Neuron, Neuronal Ensemble, Viral Transfer, Coelenterazine (CTZ)

1. INTRODUCTION

Optogenetic Ca^{2+} sensors allow the imaging of transient intracellular Ca^{2+} increases associated with neuronal activities using fluorescence (FL) or bioluminescence (BL) microscopy [1,2]. Unlike FL sensors, imaging using BL sensors does not require optical sectioning and scanning (typically acquired at 5~10 full frame/s), hence avoiding a significant loss of information when imaging activities of neuronal ensembles. BL sensors are based on luciferases, which are enzymes that catalyze light emission from the reaction with small organic compounds called luciferins [3]. BL imaging thus does not require illumination, hence its low invasiveness and absence of phototoxicity, but critically depends on luciferin availability.

Aequorin, isolated from jellyfish *Aequorea* species, has been used for decades as BL indicator of Ca^{2+} physiology in numerous cell types and biological species [4]. It is a stable reaction intermediate formed with the luciferase apoaequorin and the luciferin coelenterazine (CTZ), which emits blue light upon Ca^{2+} binding [5,3]. The rapid kinetics of Ca^{2+} binding to and unbinding from aequorin makes it a suitable indicator of rapid Ca^{2+} transients [6]. Aequorin belongs to the family of photoproteins, which contain three EF-hand Ca^{2+} binding sites and share high structural and functional homology [5,3]. The properties of photoproteins differ from those of other luciferase/luciferin reactions [3] with twofold implications for BL imaging experiments using aequorin-based sensors. First, the apoaequorin-CTZ complex forms slowly, whereas the BL reaction occurs as a rapid flash whose intensity increases with Ca^{2+} concentration, and proceeds to completion in the continuous presence of Ca^{2+} [6-9]. Aequorin is thus generally reconstituted prior to imaging, through incubation of cells or tissue for at least 30 min [10-12] with CTZ concentrations (5~10 μM) below cytotoxicity threshold (~20 μM , refs [13,14]). *In vivo*, the BL of aequorin-based sensors is detectable 15 min after intravenous injection of CTZ and reaches a plateau after 45 min [15]. These procedures enable the imaging in tissue slices and *in vivo* for several hours without recharging aequorin [10,15-17]. In contrast, other luciferases have much faster turnover rates, yielding high BL intensities that allow imaging at higher spatiotemporal resolution than achieved with aequorin-based sensors (see e.g. [18,19] for Nano-lantern-based Ca^{2+} sensors). However, the requirement of these other luciferases for the continuous presence of luciferin (20~40 μM CTZ for Nano-lantern-based Ca^{2+} sensors, [18,19]) is an important drawback, notably because of luciferin toxicity and instability [13,14]. Second, the aequorin complex is stable below Ca^{2+} binding threshold, and aequorin BL intensity varies by several orders of magnitude in the [0.1 μM ~ 1 mM] Ca^{2+} concentration range [20]. As a result, the BL of aequorin-based sensors has far lower background signal and has higher dynamic range [12,17] than sensors based on other luciferases (see e.g. [18,19]).

Aequorin-based BL imaging is thus endowed with high contrast, allowing the detection of discrete events on whole-field signals, and the acquisition of whole field imaging data in a very compact format through the encoding of only active pixel coordinates (see below; and [12,17]).

The GFP-aequorin (GA) is a FL and BL fusion protein, with improved light output and stability of aequorin, which enables Ca^{2+} imaging from single cells to whole animals of diverse species [21,12,22-24,15-17]. Bioluminescence resonance energy transfer (BRET) from aequorin to GFP [25] shifts GA light emission to the green, further shift occurring upon fusion of aequorin with yellow or red FL proteins [26,21,27]. GA minimally alters cellular processes, and behaves as a low-affinity, supralinear indicator of intracellular Ca^{2+} transients [12,24,15,17]. It has been targeted to various cell types using conditional alleles in transgenic animals or through viral transfer, and addressed to different subcellular compartments [10,22,24,15,16].

In the present chapter, we provide unique protocols used in our laboratory to perform whole-field BL imaging of neuronal ensemble activities in brain slices expressing cytosolic GA after transduction with a recombinant Sindbis pseudovirus [17]. The description of slice preparation and viral transduction, reported elsewhere [28,29], has been kept to the minimum; whereas sections dealing with BL data acquisition, handling and analysis are detailed.

2. MATERIALS

2.1 Expression vector and animals

1. Recombinant Sindbis pseudovirions encoding GA (Sin-GA; see **Notes 1** and **2**)
2. Rats or mice (see **Note 3**)

2.2 Materials for the preparation of acute slices

1. 10× artificial cerebrospinal fluid (ACSF): 1260 mM NaCl, 25 mM KCl, 12.5 mM NaH_2PO_4 , 260 mM NaHCO_3 . Weigh 146.1 g NaCl, 3.73 g KCl, 3 g anhydrous NaH_2PO_4 , 43.7 g NaHCO_3 , and dissolve in 2 L deionized H_2O . Store at 4°C for up to two weeks.
2. ACSF: 126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4 , 2 mM CaCl_2 , 1 mM MgCl_2 , 26 mM NaHCO_3 , 20 mM D-glucose and 5 mM Na pyruvate. Add in this order, 750 mL H_2O , glucose, 100 mL 10× ACSF, 2

mL CaCl₂ 1 M, 1 mL MgCl₂ 1 M and 0.55 g Na pyruvate. Adjust to 1 L and filter on 0.22 µm membrane. This solution is prepared daily and saturated with carbogen (95% O₂, 5% CO₂) for 20 min before use.

3. Slicing solution: ACSF, supplemented with 1 mM kynurenic acid (Sigma, cat. no. K3375), and bubbled with carbogen. To help dissolve kynurenic acid, sonicate for 5~10 min in an ultrasound bath.
4. Dissection tools: Scissors for initial dissection, long thin scissors for removing skin, short scissors for cutting skull, short blunt forceps for removing skull, rounded spatula for removing brain, scalpel and blade for dividing brain, rounded spatula for transferring brain halves.
5. Small parts: Adhesive cyanoacrylate (e.g. Loctite 406), Stainless steel blade (Campden Instruments Ltd., Part N. 752/1/SS) or razor blade (Gillette blue extra), plastic Petri dish (diameter 35 mm), Pasteur pipette for manipulating brain slices.
6. Anesthetics: ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight). In a 5 mL tube, add 125 µL Rampun® 0.2% (xylazine), 250 µL Imalgene® 1000 (ketamine) and 2.125 mL NaCl 0.9%. Use this solution at 100 µL per 10 g body weight. Store this solution at 4°C

2.3 Materials for overnight transduction of acute brain slices with Sin-GA

1. Slice culture medium: Mix 250 mL Minimal essential medium (MEM; Invitrogen-Life technologies, cat. no. 32360026), 250 mL 1× Hanks balanced salt solution (HBSS; Invitrogen-Life technologies, cat. no. 24020091), 5 mL Penicillin–streptomycin (P: 10,000 U/mL, S: 10,000 mg/mL), and 2.75 g D-glucose. Filter-sterilize (Steritop, Millipore), aliquot in 50 mL tube, and store at 4°C for up to two months.
2. Millicell culture plate inserts (Millipore, cat. no. PICMORG) (*see Note 4*).
3. Sin-GA. Recombinant pseudovirions are stored at -80°C (*see Note 5*).
4. Native CTZ free base (Prolume, Cat# 303 NF-CTZ-FB), stock solution at 1.25 mM in ethanol (*see Note 6*).

2.4 Instrumentation

1. Laminar flow cabinet and a 5% CO₂ incubator maintained at 35 °C
2. Vibrating slicer (e.g. VT1000S or VT1200; Leica Microsystems)

3. Incubation chamber: a submerged chamber ensuring sufficient oxygenation of the tissue during the recovery period. Usually made of a small plastic cylinder ending in a nylon net.
 4. Grid of nylon threads glued to a U-shaped platinum frame to maintain the slice at the bottom of the recording chamber.
 5. Imaging setup (Figure 1, see **Note 7**):
 - 5.1 Upright microscope (BX51WI Olympus) equipped with water immersion objectives
 - 5.2 Intensified CCD video camera (ICCD225; 768×576 pixels; Photek, St Leonards on Sea, UK) controlled by the data acquisition software IFS32 (Photek)
 - 5.3 Mercury lamp (Olympus) or LED (Thorlabs) for GFP fluorescence excitation
 - 5.4 GFP filter set (e.g. Chroma technology, cat no 41018, or Semrock, cat no GFP-30LP-B-000)
 - 5.5 Recording chamber and a perfusion system
 - 5.6 Homemade dark box housing the setup to avoid light noise.
-
6. Electrical extracellular stimulation (optional, see **Note 8**):
 - 6.1 Micromanipulator (e.g. Mini 25 3Axes, Luigs and Neuman, Germany)
 - 6.2 Concentric bipolar electrode (e.g. CBBSE75, FHC)
 - 6.3 Constant current stimulation unit (e.g. DS2A, Digitimer, UK).
-

3. METHODS

3.1 Preparation of acute slices from neocortex and transduction with Sin-GA

We use 13~17-day-old rats and mice to prepare slices for overnight transduction with Sindbis virus. For a more detailed protocol, see [28].

1. Place a 250 mL bottle of slicing solution in the freezer until ice appears.
2. Transfer the bottle containing the half-frozen slicing solution in a bucket of ice-water mix.
3. Bubble the slicing solution with carbogen for 20 min.
4. Put ice-water mix around the slicing chamber to keep it cold throughout the slicing procedure.
5. Anesthetize the animal with ketamine/xylazine, kill by decapitation, and then rapidly submerge the head in a Petri dish containing ice-cold, oxygenated cutting solution.
6. Cut the scalp and push it aside. Using small scissors, cut the skull from the vertebral foramen to the frontal lobes, and then make four lateral cuts from midline, two anterior and two posterior. Open the skull from midline with blunt forceps.

7. For parasagittal sections, using a scalpel blade, make lateral cuts between olfactory bulb and frontal cortex, and between cerebellum and occipital cortex, then rostro-caudal between the two hemispheres.
8. Extract brain halves from the skull with a spatula, and glue onto a thin film of cyanoacrylate spread on the cooled slicing stage as shown in Figure 2 (*see Note 9*).
9. Transfer and screw the stage with the block of tissue in the slicing chamber, and submerge with ice cold slicing solution continuously bubbled with carbogen at low rate.
10. Cut 300 μm slices (250 μm for mice) with blade fast vibration ($\sim 60\text{--}70$ Hz, amplitude is 0.8 mm), and slow progression through tissue ($0.1\text{--}0.2$ mm s^{-1}).
11. Once the blade has moved through cortex and hippocampus, release the slice by cutting with a thin folded needle and restart sectioning.
12. Using an inverted Pasteur pipette broken at the narrow tip and mounted on a rubber bulb, transfer the slices into the incubation chamber and let them recover in slicing solution for 30 min at room temperature.
13. During recovery, add 1.2 mL of slice culture medium in a 35 mm Petri dish, place a Millicell insert in the dish (remove any bubble from under the insert), and place the dish in a 5% CO_2 incubator.
14. Transfer slices onto the insert with inverted Pasteur pipette and remove excess slicing solution to leave a thin layer of fluid above the slice.
15. Slices are transduced by adding 5 μL of Sin-GA viral solution onto the slice and incubating overnight at 35 $^\circ\text{C}$ in a standard incubator with humidified 5% CO_2 atmosphere.
16. Thirty min after Sin-GA application, add CTZ (final concentration: 10 μM) to the slice culture medium for reconstitution of the aequorin moiety (*see Note 10*).

3.2 Bioluminescence imaging

1. The next day, pour some carbogen-saturated ACSF onto the insert with inverted Pasteur pipette, gently detach the slices using a thin paintbrush, and transfer into an incubation chamber filled with carbogen-saturated ACSF.
2. Let slices equilibrate for at least 1 h at room temperature in the dark (*see Note 11*).
3. Transfer one slice into the recording chamber perfused at 1~2 mL/min with carbogen-saturated ACSF.

4. Stop inflow and suction, gently put the slice at the bottom of the chamber, place a grid onto the slice, and resume perfusion. Recordings are typically performed 15~24 h after viral transduction, either at room temperature or at 32~34°C (*see Note 12*).
5. Using a low magnification objective (e.g. 10×), scan the slice under FL microscopy to locate a region with the desired expression pattern.
6. Optionally, position the tip of the stimulation electrode on the top of the slice (*see Note 13*).
7. Change the objective if necessary and using the low gain acquisition mode of the camera, take a FL picture of GA expression in the imaging field.
8. When investigating the spatial properties of ensemble responses, set the slice in the recording chamber so that X and Y axis have a biological relevance.
9. For the cortex, orient the slice so that the cortical surface (the pia) is parallel to the X-axis (horizontal axis of the field of view). Thus, the dorso-ventral axis of the cortex is parallel to the Y axis.
10. Remove the GFP filter cube from the light path, turn off any unnecessary source of light in the room, and close the dark box. Switch on the high gain mode of the camera and start BL recording (*see Note 14*).
11. Save the file, switch back to the low gain mode, and take a new FL picture of the imaging field in order to detect possible drift of the slice during the recording.
12. Optionally, for post-hoc histological analyses, transfer the slice into a 24 well plate containing 4% paraformaldehyde in PBS and incubate overnight at 4°C. The next day, replace fixative by PBS.

3.3 Data acquisition and handling

1. Preliminary recordings have to be performed to evaluate which acquisition mode of the camera is the most suitable, depending on the spatial and temporal light density emitted by the sample. The Photek camera we use can run under two acquisition modes, “photon counting” or “binary slice” (*see Note 15*).
2. Select the acquisition mode of the camera, which is most appropriate for the imaging experiment, and start recording (*see Note 16*).

3.4 Data analysis

1. Check compatibility between data acquisition and analysis softwares (see **Note 17**).
2. Define the type and number of ROI you wish to analyze. The first part of the analysis is to extract light signal of ROI from the data file. You can define ROI in a systematic way such as an array of identical small ROI (e.g. square) covering the whole field of view. In reference [17], we used an 18 x 24 array of square ROI, each ROI corresponding to a 60 x 60 μm square on the slice (whole imaging field with 10 \times objective: 1.4 mm x 1 mm, Figure 3). For the next steps, we will illustrate different types of data analysis based on arrays of ROI. Alternatively, you can define ROI using predefined shapes (circle, square, polygon, etc.) or draw them manually (see **Note 18**).
3. Extract light signal of each ROI from the data file. At this stage, it is convenient to store the intensity versus time signals in a single table (Figure 3). It is important to be able to map each signal to each corresponding ROI.
4. In order to manipulate easily these data, import this table in a software that allows to manipulate column/row, perform mathematical operations and generate images and graphs (e.g. Matlab, Igor, R, Origin).
5. Convert the intensity versus time table in image [e.g. with the function "*image()*" in Matlab]. This representation, called rasterplot, provides an easy way to visualize the spatiotemporal dynamics of BL signals in the whole field (Figure 3). Time is represented in one dimension, space (i.e. ROIs) in the other dimension, and light intensity is color-coded. Spatial and temporal correlations can thus be identified (see **Note 19**).
6. Optionally, perform other kinds of analysis from the intensity vs. time table to compare light responses of different ROI over time. For example, calculating a cross-correlation table provides a quantitative tool to analyze similarity between the activities of different ROI. Alternatively, using unsupervised clustering (see [17]) and plotting a hierarchical tree allows sorting and grouping together ROI that exhibit similar activity patterns.

4. Notes

1. GA has been expressed using diverse viral vectors or in transgenic mice lines [10,15]. We use Sindbis vectors to transduce acute brain slices because they allow fast expression (within 6~12 h) of transgenes at high level, selectively into neurons [31,12,32,33,17]. Our GA sequence (Genbank accession number: EF212028) is similar to that originally reported [21], except for a few aminoacid substitutions in the GFP moiety, and full codon optimization of the apoaequorin sequence for better

expression in mammalian cells [12]. The GA coding sequence is inserted in the pSinRep5 vector [32] downstream the subgenomic promoter. Detailed protocols for the production of Sindbis recombinant pseudovirions can be found elsewhere [34,29].

2. Recombinant Sindbis viruses are classified as a Biosafety Level-2 (BL-2) agent by the NIH Recombinant DNA Advisory Committee. Recommended precautions include standard microbiological practices, laboratory coats, inactivation of all infectious waste, limited access to working areas, protective gloves, posted biohazard signs, class I or II biological safety cabinets, and proper training of personnel involved in manipulation. Sindbis viruses can be inactivated by organic solvents, bleach, or autoclaving.

3. We use Wistar rats or mice with C57/Bl6 genetic background. All animal experiments must be performed in accordance with the guidelines from relevant authorities.

4. Millicell inserts fit into 35 mm Petri dishes and are suitable for the handling of the slices because of their low walls.

5. We typically use recombinant Sindbis viral stocks with titers ranging $10^7 \sim 10^8$ transducing units/mL, which is enough for slice transduction or for *in vivo* injection.

6. Store CTZ stock solution at -80°C . Caution: manipulate CTZ in the dark as it is photo-sensitive.

7. We use $10\times$ (N.A. = 0.3) and $60\times$ (N.A. = 0.9) water immersion objectives for light acquisition. Following visualization of GFP fluorescence, GFP filters are removed from light path during BL recordings.

8. The stimulus wave form is provided by a TTL signal either by the software pClamp through a digidata A/D converter (Axon Instruments) or by a signal generator (Accupulse, World Precision Instrument).

9. We use a homemade slicing stage with a 10° slope that takes cortical surface curvature into account (see Figure 2).

10. Overnight incubation with CTZ starting from the time of viral transduction, results in visible enhancement of GA fluorescence as compared to 3 h incubation after overnight expression, presumably because aequorin is more stable than apoaequorin *in cellulo* [34]. The reconstituted GA pool is sufficient to record continuously for over 1.5 h without recharging with CTZ [17].

11. This step is necessary to avoid drift along the z-axis during recording.

12. Given that the Sindbis virus does not diffuse deeply into the slice ($< 150\ \mu\text{m}$), it is important to place the transduced side of the slice upward for taking the FL picture and performing BL imaging.

13. The stimulating electrode should be positioned just above, but not penetrate the slice as it may damage tissue.

14. At the end of the recording, it may be necessary to evoke large intracellular Ca^{2+} increase for calibration between slices. This can be achieved by applying glutamate 100~500 μM .

15. The Photek camera we use can run under two acquisition modes, “photon counting” or “binary slice”. It is important that the end user is aware of these two acquisition modes, because they determine how the light signal is stored in the data file. The Photek camera relies on the intensification mechanism that is implemented in the majority of intensified cameras. A photon enters the camera and hits a photocathode. An electron is emitted, which is then drawn towards the microchannel plate (MCP) by an electric field. The MCP is a thin disc (about 1 mm thick), which is a honeycomb of glass channels typically 6~10 μm each, with a resistive coating. A high potential is applied across the MCP, enabling the electron to accelerate along one of the channels in the disc. When the electron has sufficient energy, it generates secondary electrons from the channel walls. These electrons in turn undergo acceleration which results in a cloud of electrons exiting the MCP. Gains in excess of 10,000 can readily be achieved. A phosphor screen then back converts electrons in photons. This beam of photons is then guided through a fiber optic bundle to a CCD camera. Thus, a single photon entering the camera appears on the CCD screen as a little halo covering several pixels. In the “binary slice” mode, the coordinates all the pixels forming the halo are stored. In the “photon counting” mode, the pixels forming the halo are replaced by a single pixel whose coordinates are the centroid of the halo. Thus only the coordinate of the centroid is stored in the data file. These calculations are performed for each frame. The spatial resolution in the photon counting mode is slightly better than with the binary slice mode. However, if the amount of light emitted by the sample is high enough, two or more photons emitted by the same cell and during the same time frame can be detected as a single event in the photon counting mode. Indeed, they will be amplified concomitantly and can appear as a single (though larger) halo on the CCD chip. In this case, replacing the halo by a single event leads to a loss of quantitative information. Thus, preliminary recordings have to be performed to evaluate which detection mode is the most suitable, depending on the spatial and temporal light density emitted by the sample.

16. While the low level of light emission is overcome by the use of intensified CCD camera, the small number of emitted photons allows a compact storage of data. Indeed, the IFS32 Photek software compresses data using a run-length encoding (RLE) strategy that keeps only the X-Y coordinate of a photon hit on the CCD sensor and the number of adjacent hits on the same pixel row. Consequently, BL recordings generate files several thousand-fold smaller than fluorescence (FL) imaging. For

comparison, the 90 minutes BL recording we reported in reference [17] generated a 3.5 Mo data file, whereas an experiment of same duration, acquisition frequency (25 frame/s), pixel number (576 x 768), 8 bits encoding, would generate a 58 Go image file.

17. Some simple measurements on a limited number of ROI can be performed using the Photek acquisition program IFS32. However, if a large number of ROI need to be analyzed, or if more complex calculations are necessary, data need to be transferred to a distinct analysis software. Therefore, we developed a program to convert property files generated by IFS32 into TIFF movies that can be opened in any image analysis software. In general, you may have to contact your camera provider to know how data are stored and compressed in the data file.

18. You can use the FL image to find the border of your cells. Using thresholding, you generate a binary image which then is used as a mask to filter/extract the light signal of interest. For example, if you perform recordings on neurons with well-defined edges, your mask will comprise both the soma and dendrites. Using segmentation, it is then possible to fragment the ROI in several small ROI covering the whole neuron. Thus, it is possible to read simultaneously the light signal of distinct cell compartments.

19. When data of a raster plot are summed along the time dimension, it enables comparing the light emission of the different ROI during the whole recording. Conversely, when data are summed along the ROI axis, it gives the light signal integrated over the whole field of recording versus time.

FIGURE LEGENDS

Figure 1. Schematic representation of a setup for BL recordings. Prior to imaging, samples are visualized by GFP epifluorescence using GFP filter set. Filters reside in a filter wheel (not shown), thus they can be removed from the light path during BL acquisition. FL image is acquired with the low gain mode of the ICCD camera. The BL data comprise the pixel coordinates (X,Y) of each light event together with the time information as the frame number.

Figure 2. 10° slicing stage. We use a homemade slicing stage to compensate for brain surface curvature. The rostro caudal axis is parallel to the blade edge and the dorsal part of the brain (i.e. the cortex) is closest to the blade.

Figure 3. General procedure for bioluminescence imaging data analysis.

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