Chapter 17

Bioluminescence Imaging of Calcium Oscillations Inside Intracellular Organelles

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Abstract

Ca^{2+} oscillations inside intracellular organelles are important for regulation of functions such as gene expression at the nucleus, respiration at mitochondria or protein processing at the endoplasmic reticulum. Targeted aequorins are excellent calcium probes for subcellular analysis, but single-cell imaging has proven difficult because of low light yield. Here we describe a procedure that combines virus-based expression of targeted aequorins with photon-counting imaging. This methodology allows real-time resolution of changes of cytosolic, mitochondrial or nuclear Ca^{2+} signals at the single-cell level.

Key words: Bioluminescence imaging, aequorin, herpes simplex virus, calcium oscillations, mitochondria, nucleus, endoplasmic reticulum, anterior pituitary, pancreatic islets of Langerhans, β cells.

1. Introduction

Genetically encoded probes, such as aequorins (1), can be targeted to specific subcellular locations (2–4) in response to blue light. AEQ offers several advantages for measurements of subcellular Ca^{2+} signals in living cells. Unlike fluorescent probes, aequorin does not require cell radiation for exciting light emission. In addition, it has a larger gain and a wider dynamic range (5). Manipulation of the Ca^{2+} -binding sites together with the use of different synthetic coelenterazines permits to cover a Ca^{2+} concentration range from 10^{-8} to 5×10^{-3} M (1, 6, 7). However, the low amount of light emitted has made difficult the use of this methodology for studies at the single-cell level, which are often required for adequate analysis of subcellular Ca^{2+} oscillations. Here we combine the superb selectivity of targeted aequorin, the high expression

induced by a viral vector and the high sensitivity provided by a photon-counting camera to resolve subcellular changes in $[Ca^{2+}]$ at the single-cell level.

The herpes simplex virus (HSV) based gene transfer system has unique features that make it suitable for use in mammalian cells (8). It promotes high expression of the transduced gene and is suitable for post-mitotic expression “in vivo” or in primary cultures, two conditions that are closer to the physiological condition than the cell lines models. On the other hand, photon-counting imaging of bioluminescence extends sensitivity to the single-cell level. Calibration of the photoluminescent signal into $[Ca^{2+}]$ requires computation of the fraction of the total luminescence that is emitted at each instant. For this reason, every experiment must be finished by cell lysis in the presence of excess Ca^{2+} to release the residual luminescence. We describe here protocols that can be applied to studies with cell lines (GH3 pituitary cells), primary cultures (mouse anterior pituitary cells) or “ex vivo” tissues (pancreatic islets of Langerhans), but the procedures should work efficiently with other cells and tissues.

2. Materials

2.1. Cell Preparation, Culture and Expression of Aequorins

1. Solutions for cell dissociation: Trypsin, type III; Collagenase, type 4 (Worthington, LS004186); Hank's Balanced Salt Solution. Collagenase working solution: 0.2% collagenase in HBSS.
2. Culture media and additives: Dulbecco's Modified Eagle's Medium (DMEM); foetal bovine serum (FBS); penicillin/streptomycin; L-glutamine. Supplemented RPMI medium: RPMI 1640 with 2.5% FBS, 15% horse serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin.
3. Solution containing 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (Trypsin/EDTA)
4. Poly-L-lysine-coated coverslips can be obtained commercially. Alternatively, they can be easily prepared by introducing 12-mm diameter glass sterilized coverslips into a 0.01 mg/mL solution of poly-L-lysine. Treat for 15 min and then wash the coverslips twice with water and air-dry for 30–60 min under sterile conditions.
5. Multi-dish 4 wells for 12-mm glass coverslips
6. Coelenterazine and coelenterazine n (Molecular Probes, Invitrogen) are dissolved in methanol at a concentration of 200 μ M, aliquoted in 30 μ L portions in microcentrifuge

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tubes in ice and gassed briefly with nitrogen before closing the tubes. Wrap with aluminium foil and store at -80°C for up to 6 months.

2.2. Bioluminescence Imaging

1. HEPES-buffered saline (HBS): 145 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 10 mM glucose, 10 mM sodium-HEPES, pH 7.4.
2. Krebs-Ringer bicarbonate-(KRB): 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgCl_2 , 25 mM NaHCO_3 , 3 or 10 mM glucose; gassed with 95% O_2 –5% CO_2 to maintain pH at 7.4.
3. Permeabilizing solution: Digitonin dissolved at 100 μM in 10 mM CaCl_2 . The stock solution of digitonin can be prepared at 10 mM in DMSO.
4. Home-made wood lightproof box to cover the microscope (about $100 \times 100 \times 100$ cm). They are also available from commercial sources (see for example, Hamamatsu Photonics).
5. Zeiss Axiovert S100 TV inverted microscope equipped with a Zeiss Fluor 40 $\times$, 1.3 NA oil objective equipped with a Xenon XBO75 fluorescence excitation lamp and filters for FITC (Excitation, 490 nm, Emission, >520 nm).
6. Hamamatsu VIM photon counting ICCD camera (C2400-35) mounted in the bottom port of the microscope. Argus Image Processor and M4314 image intensifier controller. All the above hardware is handled by the Hamamatsu *Aquacosmos* 2.0 software.
7. Cell perfusion system for living cells mounted in a PH-3 thermostated platform for open 12-mm glass coverslips (Warner Instruments) using an 8-lines gravity-driven perfusion system equipped with pinch valves (VC-8 valve controller). Solutions are heated using a SH-27B inline heating system. All these components may be obtained from Warner Instruments.

3. Methods

3.1. Cell Preparation, Culture and Expression of Aequorins

3.1.1. Cell Preparation and Culture

1. Rat pituitary GH₃ cells (ATCC, CCL-82.1) are cultured in supplemented RPMI medium. The cells are grown in 75 cm^2 flasks at 37°C under atmosphere of 95% air/5% CO_2 . Cells are trypsinized once a week with 0.25% trypsin-EDTA and subcultured at 1:5 dilution. Duplication

time is about 48 h. For the experiments, the cells are seeded on the 12-mm diameter polylysine-coated coverslips at $3\text{--}10 \cdot 10^4$ cells/coverslip.

2. Anterior pituitary (AP) cells are obtained from 12-week-old Wistar rats. The animals are killed by cervical dislocation and pituitaries are quickly removed. The neurointermediate lobe is removed and the AP gland is chopped into little pieces with small dissecting scissors. Cell disaggregation is induced by incubation with 1 mg/mL trypsin in HBSS for 30 min at 37°C with gentle shaking. Every 5 min, the cell suspension is passed five times through the tip of a silanized fire-polished Pasteur pipette to help cell dissociation. The cells are then sedimented by centrifugation (5 min at 200g) and washed twice with HBSS. Monodispersed cells are finally plated on poly-L-lysine-coated coverslips ($5\text{--}15 \cdot 10^4$ cells/coverslip) and cultured in DMEM supplemented with 10% FBS, antibiotics and glutamine.
3. Islets of Langerhans are obtained from 12-week-old Balb/c mice. The mice are killed by cervical dislocation, the pancreatic duct is cannulated with a 27–30 G needle and 1–2 ml of collagenase solution is introduced checking the size increase of the pancreas. The organ is then removed and placed inside a tube containing 1 mL of collagenase solution. After a 5–15 min incubation at 37°C with strong shaking (this time may change considerably with different collagenase lots), the tissue is passed repeatedly through the tip of a plastic Pasteur pipette to complete dissociation. At this time 10 mL of ice cold HBBS containing 1% FBS is added. The tissue is allowed to sediment and washed again with cold HBBS/FBS. The tissue is then transferred to a Petri dish under a stereoscopic microscope and the islets (about 100/mouse) are fished with the tip of an automatic pipette. Once isolated, islets are plated at the centre of 12-mm poly-lysine-treated coverslips (4–8 islets), allowed to attach undisturbed for 30–60 min and cultured for 24 h in DMEM medium containing 5.6 mM glucose and supplemented with 10% FBS and antibiotics.

3.1.2. Expression of Aequorin by Infection with HSV-1-Derived Amplicon Vectors

Amplicon vectors derived from herpes simplex virus type 1 (HSV-1) produce high protein expression in a very wide range of host cells, both dividing and non-dividing (*see Note 1*). The schematic diagram of a prototype aequorin amplicon is depicted in **Fig. 17.1**. The detailed protocol of how to prepare and titer HSV amplicon vectors can be found in another chapter of this book (9).

1. To facilitate cell adhesion, seed about $3 \cdot 10^5$ GH₃ or AP cells on 12-mm diameter poly-L-lysine-coated glass coverslips and place the coverslips in four-well plates containing 0.5 mL of

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Fig. 17.1. Schematic diagram of a prototype aequorin amplicon. The transcriptional unit contains the immediately early (IE) 4/5 promoter, the corresponding targeted GFP–aequorin chimeric gene and a polyadenylation signal. The two genetic elements from HSV –1, the *ori_s* and the HSV packaging sequences, allow replication and packaging of the amplicon. The prokaryotic sequences contain a bacterial origin of replication and an ampicillin selection marker that allow propagation and amplification in *Escherichia coli*. The detailed protocol of how to prepare and titrate HSV amplicon vectors can be found in another chapter of this series (9). Details on targeting sequences and alternative red fluorescent proteins can be searched in Refs. (4 and 14). This figure was reproduced with permission from Ref. (14). Copyright Elsevier (1998).

the culture medium per well. A good attachment is essential, as cells must be perfused with different solutions along the experiment.

2. After an hour to ensure attachment to the glass surface, the cells are infected with an amplicon vector expressing an aequorin targeted to a given subcellular compartment. For this purpose, 5–20 μL of virus stock are added directly into the cell culture medium (*see Note 2*). The multiplicity of infection (MOI) is 1–3. In the present protocol we use amplicon vectors expressing the fusion protein GFP–aequorin targeted to the cytosol, nucleus or mitochondria (*see Note 3*).
3. The islets of Langerhans are allowed to attach to the coverslip for 30–60 min and then $1\text{--}3 \cdot 10^3$ infectious virus particles per islet are added.
4. In all the cases, expression time is usually 12–24 h. A 48-h expression period, tested in a few experiments, was found satisfactory as well.

3.1.3. Reconstitution of Apoaequorin

1. At 12 and 24 h post-infection, check the expression of the aequorin by monitoring the GFP fluorescence under the microscope. The range of positive (green) cells should be 20–40%.
2. Aequorin has to be reconstituted with coelenterazine in order to allow Ca^{2+} -dependent light emission. Different aequorin–coelenterazine combinations may be used to achieve different Ca^{2+} affinities, depending on the range of the $[\text{Ca}^{2+}]$ changes expected (**Fig. 17.2**). The combination of native aequorin and coelenterazine (AEQ1) is suitable for the 10^{-7} – $3 \cdot 10^{-6}$ range. Using coelenterazine n with native aequorin (AEQ2) extends the upper range to $2 \cdot 10^{-5}$. Finally, the combination of mutated aequorin and coelenterazine n (AEQ3) is suitable for the range between $3 \cdot 10^{-6}$ and 10^{-3} M (*see Note 4*).

Fig. 17.2. Calibration curves of different aequorin systems. The three aequorin (AEQ) systems shown, correspond to: wild-type aequorin with either native coelenterazine (AEQ1) or coelenterazine n (AEQ2) or mutated low Ca^{2+} affinity aequorin and coelenterazine n (AEQ3) (6). The function used to relate Ca^{2+} and photoluminescence (expressed as fraction of the total counts emitted at every instant, L/L_{TOTAL}) was: $[\text{Ca}^{2+}]$ (in M) = $[R + (R \times KTR) - 1] / [KR - (R \times KR)]$, where $R = [L / (L_{\text{max}} \cdot \lambda)]^{1/n}$, using the values shown in Table 17.1 for the constants. This figure was redrawn with permission from ref. 5. Copyright Elsevier (2006).

3. For reconstitution, the coverslips containing the cells expressing the apoaequorins (targeted to different organelles and either native or mutated) are transferred to a 4-well plate containing 0.20 ml of HBS. Then, 1 μL of coelenterazine (either native or n, from a 200 μM stock) is added and gently mixed. Finally, the plate is incubated for 1–2 h at room temperature in the dark.

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4. Islets of Langerhans are reconstituted in a similar fashion except that they are initially not attached to coverslips. Instead, they are handled with the tip of an automatic pipette and placed in the coelenterazine suspension by hand. The incubation medium for the islets is KRB containing 3 mM glucose and gassed with 95% O₂/5% CO₂.

3.2. Bioluminescence Measurements

3.2.1. Bioluminescence Imaging of Subcellular Ca²⁺

1. Coverslips containing infected cells or islets (*see Note 5*) are reconstituted with coelenterazine. Then, they are placed in the stage of the microscope into a perfusion chamber thermostated to 37°C and perfused continuously with pre-warmed HBS (for GH3 or AP cells) or KRB medium gassed with 5% CO₂ (for pancreatic islets) at a rate of 5 mL/mL.
2. The cells are examined for GFP fluorescence using the FITC filters and an adequate microscopic field is chosen. A GFP fluorescence image is captured with the help of the Hamamatsu C2400-35 ICCD camera with the sensitivity set to a minimum (0).
3. After turning off the excitation lamp, a bright field image of the same cells is captured using the same camera.
4. Microscope light is turned off and the dark box doors closed for complete darkness (*see Note 6*).
5. Sensitivity of the intensifier is set to maximum (10) and photonic emission images are captured with the Hamamatsu VIM photon-counting camera handled with an Argus-20 image processor and integrated every 10-s periods. Total counts per region of interest may range between 2.10³ and 2.10⁵ for different cells. Background photonic emissions in regions of interest of similar size in non-expressing cells is typically about (mean±S.D.) 1±1 count per second (cps) per typical cell area (about 2000 pixels).
6. Cells are subjected to the different test treatments using the perfusion system. Three examples of the outcome are shown in **Fig. 17.3**.
7. Before ending the experiment, it is necessary to perfuse the cells with permeabilizing solution. Digitonin releases all the remaining aequorin counts, which must be added up in order to estimate the total photonic emissions, a value required for calibration in Ca²⁺ concentrations. Emission of all residual counts may take 2–5 min from the time of lysis. The experiment is finished once aequorin photonic emissions cease.
8. The entire sequence of bioluminescence images is stored in the computer together with the bright field and fluorescence images captured before photon counting.

Fig. 17.3. Examples of Ca^{2+} oscillations inside organelles measured with aequorin. **(A)** The trace represents the spontaneous oscillations of mitochondrial Ca^{2+} in a single anterior pituitary cell expressing mitochondria-targeted aequorin. The images corresponding to one single oscillation are shown on *top*. Photonic emissions, coded in pseudocolor, have been

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3.3. Image Analysis and Quantification

1. Regions of interest (ROIs) are selected with the help of the Aquacosmos software by drawing circles around infected cells according to the fluorescence and bright field images captured at the beginning of the experiment (*see Note 7*).
2. The same ROIs are pasted on every image of the bioluminescence sequence. Total photonic emissions in every ROI are computed with the Aquacosmos software to obtain the luminescence emission value (L) for each cell at each point in time. A few ROIs are drawn in regions devoid of cells to compute background luminescence.
3. All the photonic emissions in the bioluminescence images, including those obtained after digitonin permeabilization, are added up using the Aquacosmos software to obtain a bioluminescence image containing all the photonic emissions. The size of the ROI is adjusted to the area in which photons are emitted from each individual cells, which is usually somewhat larger than the size of the cell. Cells with overlapping of photonic emissions are excluded from the analysis.
4. The luminescence values for every ROI at each time value (L) are computed and exported to a worksheet. Background luminescence is subtracted from each L value. For every ROI, the total luminescence, $\text{Total}(L)$, is computed by adding up the values of all the images. Then, the following values are computed for every time point (t):
 - L : Luminescence emission at time t
 - $\text{Sum}(L) = \sum L$ values from $t = 0$ to t
 - L_{TOTAL} : Total luminescence remaining at time $t = \text{Total}(L) - \text{Sum}(L)$
 - % Remaining luminescence: $100 \times L_{\text{TOTAL}} / \text{Total}(L)$
 - L / L_{TOTAL}
 - $[\text{Ca}^{2+}]$ using the algorithms described in **Table 17.1**.

Fig 17.3. (continued) superimposed to the bright field image. Time sequence goes from *left to right* and from *top to bottom*. Interval between images, 10 s. The size of each image box is $10 \times 10 \mu\text{m}$. Unpublished results by C. Villalobos, L. Nuñez, P. Chamero, M.T. Alonso and J. García-Sancho. **(B)** Glucose-induced mitochondrial Ca^{2+} oscillations in nine single pancreatic β cells within a Langerhans' islet expressing mitochondria-targeted aequorin. The *upper panels* show, from *left to right*, brightfield, GFP fluorescence and pseudocolor-coded aequorin bioluminescence images (integration of the luminescence emission during the whole experiment; scale at *right*). The size of each image box is $200 \times 200 \mu\text{m}$. This figure was reproduced with permission from Ref. (13). Copyright Elsevier (2008). **(C)** Nuclear Ca^{2+} oscillations in four individual cells of a pancreatic islet expressing with nucleus-targeted aequorin and stimulated with high (11 mM) glucose. The stimulation period (bar) was 5 min. *Left*: pseudocolor-coded aequorin bioluminescence image (integration of the luminescence emission during the whole experiment). This figure was reproduced with permission from ref. 5. Copyright Elsevier (2006).

Table 17.1**Values of the constants for calibration of several aequorin–coelenterazine combinations**

Condition	K_R	K_{TR}	n	λ
AEQ1: AEQwt + COEL wt	4.81×10^7	601	2.3	1
AEQ2: AEQwt + COEL n	2.23×10^7	348	2.0	0.129
AEQ3: AEQmut + COEL n	8.47×10^7	156.6×10^3	1.204	0.138

All the values were obtained at 37°C. Calculations were performed using the following formula:

$$[Ca^{2+}] \text{ (in M)} = [R + (R \cdot K_{TR}) + 1] / (K_R - (R \cdot K_R)) \text{ where } R = [L / (L_{TOTAL} \cdot \lambda)]^{(1/n)},$$

where L is the luminescence emitted at the time of measurement and L_{TOTAL} is the addition of the counts present in the tissue at that time, estimated by integrating all the counts from the time of measurement until the release of all the residual luminescence by lysis at the end of the experiment. For more details, see Refs. (5 and 7).

4. Notes

1. Apart from GH3 and AP cells (10–13) and pancreatic islets, this methodology has been successfully applied to express AEQ in HeLa, HEK293 and NIH3T3 cell lines, and in primary cultures of adrenal chromaffin cells, cerebellar granule, sympathetic and dorsal root ganglion neurons, and rat cardiomyocytes and chicken embryonic heart cells (6, 11, 14–16).
2. The virus stocks have titers of $0.2\text{--}1 \times 10^7$ and are stored at -80°C in 50–100 μL aliquots. Virus particles are thermolabile and should be thawed immediately before adding them to the cells, and kept on ice during manipulations. GH₃ and AP cells are spherical and adhere to the glass very rapidly; other cells such as HeLa or HEK293 cells need longer times to become properly attached so that it may be better to wait till next day for infection.
3. The use of the fusion protein GFP–aequorin has many advantages over the native aequorin. It allows direct visualization of the expressed protein under the fluorescence microscope, the protein is more stable, and it gives a higher light yield (17). The original GFP–aequorin fusion gene was obtained from Dr. Brûlet (17) and modified by PCR to fuse it in frame to the corresponding targeted sequences. The nuclear GFP–aequorin was obtained by fusing the *Xenopus* nuclear nucleoplasmin gene; the cytosolic GFP–aequorin, by fusing the luciferase gene; and the mitochondrial aequorin by fusing the first 31 amino acids

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of the human COX VIII subunit. The low Ca^{2+} -affinity mitochondrial GFP-aequorin was generated by swapping the EcoRV-EcoRI fragment of the aequorin moiety by the mutated region of the low Ca^{2+} affinity endoplasmic reticulum aequorin, as previously described (14). All of these fusions were cloned in the multicloning site of the pHSVpuc plasmid. Recently, similar fusion proteins with mRFP have been described (4).

4. Ca^{2+} reaches very different concentrations in different cellular compartments. In cytosol and in nucleus $[\text{Ca}^{2+}]$ is about 10^{-7} M at rest and can increase 10 times or more during cell activation. In the endoplasmic reticulum $[\text{Ca}^{2+}]$ is high at rest (near 10^{-3} M) and decreases during cell activation. In mitochondria $[\text{Ca}^{2+}]$ is low at rest (about 10^{-7} M) but can increase to μM and even near mM levels during cell activation (6).
5. The islets are placed over the glass bottom (coated with 0.1 mg/mL poly-L-lysine) of the perfusion chamber. Small islets are a better choice because they can be accommodated inside the microscopic field and they usually stick better to the coverslip. The flow is stopped at this stage in order to avoid dragging away the islets. After 2–3 min, the islets stick to the glass bottom and perfusion can be started, first at low rate and then increased progressively to reach about 5 mL/min.
6. Photon counting cameras are extremely sensitive to any contaminating light. Accordingly, any light source inside the dark box must be turned off during photon counting. Putative light sources, such as leads of different operators, even if they are outside the dark box, must be turned off. Some motorized microscopes have internal leads that produce a light leak to the camera. This is the case of Zeiss Axiovert 200, for example. Check this disturbance by turning off the microscope during measurements.
7. Calibration of the recorded signals is dependent on the total amount of photonic emissions, including those released after cell permeabilization. If cell density is excessive or the amount of light emitted by some individual cells is too high, overlap between photonic emissions from different cells may happen, and this could influence the calculations of Ca^{2+} concentrations. This can be avoided by optimizing expression efficiency and/or by plating the cells at a proper density.

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