Protocol

Using Targeted Variants of Aequorin to Measure Ca^{2+} Levels in Intracellular Organelles

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Aequorin is a Ca^{2+} -sensitive photoprotein isolated from the jellyfish *Aequorea victoria*. It is an ideal probe for measuring Ca^{2+} concentration ($[\text{Ca}^{2+}]$) in intracellular organelles because it can be modified to include specific targeting sequences. On the binding of Ca^{2+} to three high-affinity sites in aequorin, an irreversible reaction occurs in which the prosthetic group coelenterazine is released and a photon is emitted. This protocol presents procedures for expressing, targeting, and reconstituting aequorin in intact and permeabilized mammalian cells and describes how to use this photoprotein to measure intracellular $[\text{Ca}^{2+}]$ in various subcellular compartments.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

ATP
Bovine serum albumin (BSA)
Bradikynin
 CaCl_2
Carbachol
Coelenterazine or coelenterazine-*n*
 Prepare a 0.5 M stock solution in pure methanol and store aliquots at -80°C .
Cyclopiazonic acid
Digitonin
EGTA
Histamine
Intracellular buffer <R>
Ionomycin
Krebs-Ringer modified buffer (KRB) <R>
Norepinephrine

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Plasmids (e.g., cytAEQ, nuAEQ, cyt/nuAEQ, srAEQ, mtAEQwt, mtAEQmut, mtAEQ2mut, pmAEQ, erAEQ, goAEQ, vampAEQ, perox AEQ, cxAEQ)

Information on how to make these plasmids can be found in Brini et al. (1993, 1994, 1995, 1997), de la Fuente et al. (2012), Lasorsa et al. (2008), Montero et al. (1995), Marsault et al. (1997), Martin et al. (1998), Mitchell et al. (2001), Pinton et al. (1998), Rizzuto et al. (1992, 1998).

Equipment

Apparatus for measuring light production from aequorin (custom-built by Elettrofor; see Fig. 1)

Cell culture plates (24 wells)

Coverslips (glass; 13 mm)

Data recording and analysis system

The acquisition device is a Hamamatsu photon-counting head device (low noise; H7360-01) that is connected to a PC through a counting unit (Hamamatsu C8855-01). Luminescence recording is carried out with software provided by Hamamatsu. Data from each experiment are exported to a CSV file. Luminescence calibration is carried out using a custom-developed macro-enabled Excel spreadsheet, which is available from the authors on request.

FIGURE 1. Apparatus for measuring light production from aequorin. The equipment consists of three parts. (A) A thermostatically controlled chamber with a perfusion system (set at 37°C) is located on top of a hollow cylinder. A coverslip seeded with cells is placed in the chamber and perfused with a continuous flow of KRB in which all the reagents are added. A peristaltic pump is essential to obtain rapid equilibration of the perfusing medium inside the chamber. (B) A photomultiplier tube with a built-in amplifier/discriminator that is able to capture the light emitted during the reaction is placed in close proximity (1 mm) to the top of the hollow cylinder that contains the perfusion chamber. This system is located in a dark box to reduce external light influence and is connected to (C) a data recording and analysis system that is able to convert the luminescent data into $[\text{Ca}^{2+}]$. The graph of luminescence output is displayed on a computer screen during the experiments and the program converts the data into $[\text{Ca}^{2+}]$ values using mathematical algorithm based on the Ca^{2+} response curve of aequorin at fixed conditions of temperature, pH, and $[\text{Mg}^{2+}]$. (D) The system as it is used during the experiments. The thermostatically controlled chamber is located within the luminometer and the coverslip with cells (enlarged in the left square and seen from above) is perfused with the experimental solutions.

Perfusion chamber
Peristaltic pump
Photomultiplier tube (with built-in amplifier/discriminator)
Thermostat
Tubing (Tygon, black, 1.5-mm diameter)

METHOD

The protocol below describes techniques for using aequorin to measure intracellular $[Ca^{2+}]$ in a variety of subcellular compartments (Experiments 1–5). In each experiment, careful regulation of temperature (in a thermostatically controlled perfusion chamber) and pH (which affects the stability of aequorin) is essential for correct calibration of the probe. Steps 1–3 are instructions common to each experiment. Experiments 1–4 are performed with intact cells and Experiment 5 is performed with permeabilized cells.

Transfection

1. Perform transfection using a method appropriate for the cell type of interest. For commonly used cell lines (e.g., HeLa and COS cells), introduce the plasmid codifying recombinant aequorin using calcium phosphate or liposome-based transfection protocols. For cells in primary culture or cells difficult to transfect, try other approaches such as electroporation or viral transduction (Kendall et al. 1996; Alonso et al. 1998). It is also possible to generate stable cell lines (Button and Brownstein 1993).

Reconstitution of Functional Photoprotein

Transfected aequorin is only the polypeptide portion of the photoprotein and to reconstitute the functional holoprotein, cells must be incubated with the prosthetic group coelenterazine. Coelenterazine is highly permeant across biological membranes and we have never encountered a mammalian cell type or intracellular organelle in which coelenterazine entry is limiting. However, we have found that it takes at least 1–2 h to incorporate an amount of coelenterazine that will form sufficient functional photoprotein for accurate luminescence measurements.

2. Transfer the cells from complete growth medium to KRB. For investigations of subcellular compartments with low $[Ca^{2+}]$, incubate the cells with 5 μM coelenterazine for at least 1–2 h. For investigations of subcellular compartments with high $[Ca^{2+}]$, either reduce the $[Ca^{2+}]$ by incubating with the ionophore ionomycin (0.8–1 μM) before reconstitution and/or use the less sensitive analog, coelenterazine-*n*.

*Coelenterazine is hydrophobic and incubation of cells in KRB rather than complete growth medium prevents loss of available prosthetic group by passive absorption to hydrophobic patches of serum proteins. However, transfer to KRB can be detrimental to some primary cells, in which case keep the cells in complete medium and add a higher concentration of coelenterazine. Coelenterazine-*n* is a less sensitive analog of coelenterazine, which can be used to reduce the rate of aequorin luminescence emission and is useful in investigations of cellular compartments endowed with high $[Ca^{2+}]$ (e.g., intracellular stores such as the endoplasmic reticulum [ER] and sarcoplasmic reticulum).*

Calibration

Raw luminescent signals are transformed into $[Ca^{2+}]$ values using an algorithm based on the relationship between $[Ca^{2+}]$ and the ratio between L and L_{max} (Allen and Blinks 1978). In aequorin experiments, L is the light intensity recorded under physiological conditions (counts/sec) and L_{max} is the total light emission when all the aequorin in the cells is suddenly exposed to a saturating Ca^{2+} concentration (Fig. 2).

3. To utilize the Allen and Blinks algorithm, measure L_{max} at the end of every experiment. Perfuse the cells with a lysis solution (i.e., a hypotonic medium containing 10 mM $CaCl_2$ and 100 μM digitonin, or similar detergent).

This procedure discharges all the aequorin that has not been consumed during the experiment. See Troubleshooting.

Experiment 1: Measurement of Cytosolic Ca^{2+}

4. Seed the cells of interest on to 13-mm glass coverslips placed in the wells of a 24-well plate. Transfect the cells with the cytAEQ construct (see Step 1). Reconstitute the holoprotein by incubating the cells with coelenterazine for ~ 2 h (see Step 2).

FIGURE 2. Calibration curve showing the relationship between $[\text{Ca}^{2+}]$ (pCa^{2+}) and the logarithm of L/L_{max} , where L is the instant rate of light emission and L_{max} is the maximal value of light emission measured in saturated conditions. These data were obtained experimentally by mixing a solution containing cytoplasmic aequorin with solutions containing different $[\text{Ca}^{2+}]$, prepared to give defined $\log[\text{Ca}^{2+}]$ values in the final solution. Then, both the light emission obtained immediately after mixing the solutions and the total number of light counts in the sample were measured to obtain L/L_{max} ratios. The relationship between light emission and $[\text{Ca}^{2+}]$ is linear only in the central part of the curve. Different types of aequorin have different $[\text{Ca}^{2+}]$ response curves based on the analog of coelenterazine used. Use these curves to choose the appropriate aequorin to measure a range of $[\text{Ca}^{2+}]$.

- Place a coverslip seeded with cells in the thermostatically controlled chamber of the light-measuring apparatus. Perfuse the cells with KRB using a peristaltic pump. Start the luminometer recording. Determine the background signal in the absence of agonist.

Under basal conditions, variations in $[\text{Ca}^{2+}]$ are very small ($0.1\text{--}0.5\ \mu\text{M}$) and it is impossible to detect differences in light emission.

- Add an appropriate agonist to the perfusion buffer and experiment to determine the lowest concentration of agonist that will induce a maximal Ca^{2+} -release response (e.g., $100\ \mu\text{M}$ histamine, $100\ \mu\text{M}$ ATP, $0.5\ \text{mM}$ carbachol, $100\ \mu\text{M}$ norepinephrine, or $100\ \text{nM}$ bradykinin). Observe the agonist-induced rise in $[\text{Ca}^{2+}]$, which reaches a peak and then declines (Fig. 3A).

In our studies, mostly carried out in nonexcitable cells, we commonly employ histamine, ATP, and bradykinin, which are agonists that act via G proteins to generate diacylglycerol and inositol 1,4,5-trisphosphate (IP_3), thereby stimulating release of Ca^{2+} from the ER. These agonists induce a transient increase in $[\text{Ca}^{2+}]$. For a homogeneous response, it is important to give cells a maximal stimulus so it is necessary to challenge cells with different concentrations of agonist to find the lowest dose that has the maximal response. See Troubleshooting.

- When the $[\text{Ca}^{2+}]$ has returned to the background level, estimate the total content of aequorin by discharging the remaining pool. Perfuse the cells with a hypotonic, Ca^{2+} -rich solution ($10\ \text{mM}$ CaCl_2 , $100\ \mu\text{M}$ digitonin in H_2O), which induces the release of aequorin into a Ca^{2+} -rich environment. Measure L_{max} (see Step 3).

See Troubleshooting.

Experiment 2: Measurement of Capacitative Ca^{2+} Influx

- Seed the cells of interest on to 13-mm glass coverslips placed in the wells of a 24-well plate. Transfect the cells with the cytAEQ construct (see Step 1). Reconstitute the holoprotein by incubating the cells with coelenterazine for $\sim 2\ \text{h}$ (see Step 2). Place a coverslip seeded with cells in the thermostatically controlled chamber of the light-measuring apparatus.
- Perfuse the cells with KRB that is not supplemented with Ca^{2+} and contains $100\ \mu\text{M}$ EGTA. Replace this buffer with KRB that is not supplemented with Ca^{2+} and contains $100\ \mu\text{M}$ EGTA and $20\ \mu\text{M}$ cyclopiazonic acid. Perfuse for 3 min and follow the change in $[\text{Ca}^{2+}]$.

During this period the ER Ca^{2+} stores are emptied and a slow increase in cytoplasmic Ca^{2+} is observed. The cytoplasmic $[\text{Ca}^{2+}]$ correlates directly with the amount of Ca^{2+} released from the ER. Cyclopiazonic acid is a highly selective, reversible inhibitor of Ca^{2+} -ATPase in the intracellular Ca^{2+} storage sites (SERCA). Alternative methods for emptying the ER are to use an irreversible inhibitor of SERCA (e.g., thapsigargin) or to stimulate inositol 1,4,5-trisphosphate receptors with an IP_3 agonist.

FIGURE 3. Measurement of $[Ca^{2+}]$ in different cellular compartments in HeLa cells. When agonists are added to the cells in perfusion buffer, a transient increase in $[Ca^{2+}]$ is recorded. The traces show (A) the cytosolic $[Ca^{2+}]$ increase after agonist stimulation, (B) mitochondrial Ca^{2+} uptake after agonist stimulation, and (C) ER Ca^{2+} uptake and release after agonist stimulation. It is also possible to perfuse a fixed $[Ca^{2+}]$ to trigger mitochondrial Ca^{2+} uptake in permeabilized cells (D). For the details of experimental protocols, see the text.

10. Evaluate Ca^{2+} influx through store-operated channels in the plasma membrane by perfusing the cells with KRB containing 2 mM Ca^{2+} and 20 μ M cyclopiazonic acid.

See Troubleshooting.

Experiment 3: Measurement of Mitochondrial Ca^{2+} Uptake

11. Seed the cells of interest on to 13-mm glass coverslips placed in the wells of a 24-well plate. Transfect the cells with a mtAEQ construct (see Step 1).

The choice of construct depends on the $[Ca^{2+}]$ range predicted. Use the mtAEQwt construct if the maximum level of Ca^{2+} is expected to be 10 μ M and use the mtAEQmut construct if the maximum level of Ca^{2+} is expected to be 200 μ M.

12. Reconstitute the holoprotein by incubating the cells with coelenterazine for ~ 2 h (see Step 2).
13. Place a coverslip seeded with cells in the thermostatically controlled chamber of the light-measuring apparatus. Perfuse the cells with KRB using a peristaltic pump. Start the luminometer recording. Determine the background signal in the absence of agonist.
14. Add an appropriate agonist to the perfusion buffer and experiment to determine the lowest concentration of agonist that will induce a maximal Ca^{2+} -release response. Observe the agonist-induced rise in $[Ca^{2+}]$, which reaches a peak and then declines (Fig. 3B).

See Troubleshooting.

15. When the $[Ca^{2+}]$ has returned to the background level, estimate the total content of aequorin by discharging the remaining pool. Perfuse the cells with a hypotonic, Ca^{2+} -rich solution (10 mM $CaCl_2$, 100 μ M digitonin in H_2O), which induces the release of aequorin into a Ca^{2+} -rich environment. Measure L_{max} (see Step 3).

See Troubleshooting.

Experiment 4: Measurement of Ca^{2+} in the ER and Its Release

16. Transfect cells of interest with the erAEQ construct (see Step 1). Seed the transfected cells on to 13-mm glass coverslips placed in the wells of a 24-well plate.
17. Incubate the cells in KRB supplemented with 600 μM EGTA and 0.8–1 μM ionomycin. Reconstitute the holoprotein by incubating the cells with coelenterazine-*n* for ~ 1 h (see Step 2).
*The $[\text{Ca}^{2+}]$ in the ER is very high (200–500 μM) and so it is necessary to empty the store before reconstituting with the low-affinity analog coelenterazine-*n* (see Step 2).*
18. Remove the ionophore by washing the cells three times with KRB containing 2% BSA and 2 mM EGTA.
19. Place a coverslip seeded with cells in the thermostatically controlled chamber of the light-measuring apparatus. To maintain a low $[\text{Ca}^{2+}]$ in the ER store, perfuse the cells with KRB containing 100 μM EGTA. Wash the cells with KRB containing 2% BSA and 2 mM EGTA for 200 sec.
20. Perfuse the cells with KRB containing a specific $[\text{Ca}^{2+}]$ (e.g., 1 mM CaCl_2) and observe the increase in $[\text{Ca}^{2+}]$ until a plateau is reached that represents the resting $[\text{Ca}^{2+}]$ in the ER (Fig. 3C).
21. Add an appropriate agonist (e.g., histamine) and follow the kinetics of Ca^{2+} release from the ER (Fig. 3C).
See Troubleshooting.
22. When the $[\text{Ca}^{2+}]$ has returned to the background level, estimate the total content of aequorin by discharging the remaining pool. Perfuse the cells with a hypotonic, Ca^{2+} -rich solution (10 mM CaCl_2 , 100 μM digitonin in H_2O), which induces the release of aequorin into a Ca^{2+} -rich environment. Measure L_{max} (see Step 3).
See Troubleshooting.

Experiment 5: Measurement of Mitochondrial Ca^{2+} Uptake in Permeabilized Cells

In this experiment, mitochondrial Ca^{2+} uptake independently to ER Ca^{2+} release is evaluated and microdomains of high $[\text{Ca}^{2+}]$ can be detected close to the mitochondrial Ca^{2+} channels.

23. Seed the cells of interest on to 13-mm glass coverslips placed in the wells of a 24-well plate. Transfect the cells with the mtAEQ construct (see Step 1). Reconstitute the holoprotein by incubating the cells with coelenterazine for 2 h (see Step 2). Permeabilize transfected cells by perfusing with intracellular buffer containing 20 μM digitonin for 60 sec. Wash with intracellular buffer for 60 sec.
24. Perfuse the permeabilized cells with intracellular buffer containing 0.4–2 μM CaCl_2 . Observe the slow increase of Ca^{2+} in the mitochondria (Fig. 3D).
See Troubleshooting.

TROUBLESHOOTING

Problem (Steps 6, 10, 14, 20, and 24): Luminescence signals are low.

Solution: Low luminescence could be the result of low transfection efficiency, which is dependent on the quality of the transfection solution and the purity of DNA plasmid preparation. In most cases, transfection has to be optimized by trial and error. All aequorins are provided with an HA1 tag and the rate of transfection can be determined by performing an immunocytochemistry analysis using the HA1 epitope tag antibody. It is also possible that low luminescence is caused by some difficulty with the reconstitution of functional photoprotein. Usually coelenterazine is cell membrane permeant, but with some cell types it may be better to use a serum-free medium to increase the efficiency of coelenterazine uptake and reconstitution of holoprotein. When investigating intracellular compartments with high $[\text{Ca}^{2+}]$, it may be helpful to use plastic instruments to reduce Ca^{2+} contamination.

Problem (Steps 3, 7, 15, and 22): Calibration values are unreliable.

Solution: Calibration values may be affected by the steepness of the Ca^{2+} -response curve of aequorin. In compartments with nonhomogeneous $[\text{Ca}^{2+}]$, only a small fraction of the protein pool may be exposed to a massive light discharge and this can significantly increase the calibrated mean value. Also, low aequorin counts may occur as a result of high aequorin consumption. This happens particularly in compartments with high $[\text{Ca}^{2+}]$, where most aequorin is consumed after Ca^{2+} binding and this limits the amount of free aequorin in Ca^{2+} -saturating exposition. This problem may be overcome by the use of low-affinity aequorin variants or prosthetic groups and the emptying of the compartments before beginning the experiment. Calibration values may also be affected by variability in the transfection efficiency. Cells must be kept under strictly reproducible conditions, which can be achieved by using the same clones, DNA, and method of transfection. In any case, a different amount of aequorin should only change the quality of the signal and not the value of the calibrated Ca^{2+} signal (Brini 2008).

DISCUSSION

Aequorin is a powerful Ca^{2+} probe because it can be fused with targeting sequences so it localizes specifically to particular subcellular compartments (see **The Use of Aequorin and Its Variants for Ca^{2+} Measurements** [Granatiero et al. 2014]). In contrast, dyes like Fura-2 can measure $[\text{Ca}^{2+}]$ only in the cytosol. Moreover, aequorin probes can be modified to measure $[\text{Ca}^{2+}]$ in compartments like the ER that contain high levels of Ca^{2+} . With a photomultiplier-associated computer, it is easy to convert luminescence data into $[\text{Ca}^{2+}]$. Some limitations of this technique include the potential for inaccurate measurements in organelles with heterogeneous $[\text{Ca}^{2+}]$ and when the amount of light emitted is low. It can also be difficult to optimize the timing for reconstitution of active photoprotein. Nevertheless, aequorin is a highly effective tool for measuring $[\text{Ca}^{2+}]$ in living cells.

RECIPES

Intracellular Buffer

Reagent	Final concentration
KCl	130 mM
NaCl	100 mM
K_2PO_4	20 mM
HEPES	200 mM
Succinic acid	50 mM
Malic acid	10 mM
Pyruvate	10 mM
MgCl_2	10 mM

Adjust the pH to 7.0 with KOH.

Krebs-Ringer Modified Buffer (KRB)

Reagent	Final concentration
NaCl	135 mM
KCl	5 mM
MgSO_4	1 mM
K_2HPO_4	0.4 mM
Glucose	5.5 mM
HEPES	20 mM

Adjust the pH to 7.4. Most experiments are performed in buffer supplemented with 1 mM CaCl_2 .

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