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Ca^{2+} Measurements in Mammalian Cells with Aequorin-based Probes

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[Abstract] Aequorin is a Ca^{2+} sensitive photoprotein suitable to measure intracellular Ca^{2+} transients in mammalian cells. Thanks to recombinant cDNAs expression, aequorin can be specifically targeted to various subcellular compartments, thus allowing an accurate measurement of Ca^{2+} uptake and release of different intracellular organelles. Here, we describe how to use this probe to measure cytosolic Ca^{2+} levels and mitochondrial Ca^{2+} uptake in mammalian cells.
Keywords: Ca^{2+}, Aequorin, Probes, Luminescence

[Background] Aequorin is a 21 kDa photoprotein isolated from jellyfish *Aequorea Victoria* that emits blue light in the presence of Ca^{2+}. In its active form the photoprotein includes an apoprotein and a covalently bound prosthetic group, called coelenterazine. The apoprotein contains four helix-loop-helix 'EF-hand' domains, three of which are Ca^{2+}-binding sites. These domains confer to the protein a particular globular structure forming the hydrophobic core cavity that accommodates the coelenterazine. When Ca^{2+} ions bind to the three high affinity EF-hand sites, coelenterazine is irreversibly oxidized to coelenteramide, with a concomitant release of CO_2 and emission of light (Head *et al.*, 2000).

Aequorin began to be widely used when the cDNA encoding the photoprotein was cloned, thus opening the way to recombinant expression. In particular, recombinant aequorin can be expressed not only in the cytoplasm, but also in single intracellular compartments by including specific targeting sequences in the engineered cDNAs (Hartl *et al.*, 1989). To expand the range of Ca^{2+} sensitivity that can be monitored, point mutations in the EF-hand motives that lower the affinity for Ca^{2+} have been introduced (Granatiero *et al.*, 2014a and 2014b). Reconstitution of an active recombinant aequorin in living cells is obtained by simple addition of coelenterazine into the medium. Coelenterazine is highly hydrophobic and permeates cell membranes of various cell types. Different coelenterazine analogues have been synthetized and are now commercially available.

Materials and Reagents

1. Round glass coverslips 12 mm (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 1014355112NR15) - Sterile upon autoclave cycle
2. 24-well plate (Corning, Costar®, catalog number: 3524)
3. MDA-MB-231 cell line
4. MDA-MB-468 cell line
5. BT-549 cell line

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6. Aequorin-expressing plasmids (Brini, 2008)
7. Gelatin
8. Collagen
9. Coelenterazine 0.5 mM stock solution, in methanol (IS Chemical Technology, catalog number: I14-2266) - Aliquot in 50 µl aliquots. Store at -80 °C. Save it from light
10. Agonist (*e.g.*, ATP, Histamine, Bradykinin, Caffeine, Carbachol, Glutamate)
    Note: In this protocol example 100 µM ATP is used.
11. Milli-Q water
12. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S9888)
13. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P5405)
14. Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) (Sigma-Aldrich, catalog number: M2670)
15. Potassium dihydrogen phosphate (KH_2PO_4) (Sigma-Aldrich, catalog number: NIST200B)
16. Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) (Sigma-Aldrich, catalog number: M2773)
17. HEPES (Sigma-Aldrich, catalog number: H3375)
18. Sodium hydroxide (NaOH) (Sigma-Aldrich, catalog number: 71687)
19. Calcium chloride ($CaCl_2$) (Sigma-Aldrich, catalog number: 449709)
20. Glucose (Sigma-Aldrich, catalog number: G8270)
21. Digitonin (Sigma-Aldrich, catalog number: D5628 or D141)
22. Krebs-Ringer modified buffer (KRB) (see Recipes)
23. Digitonin lysis solution (see Recipes)

Equipment

1. Windows-based computer
2. Perfusion chamber (Elettrofor)
3. Low noise photomultiplier (Hamamatsu Photonics K. K., model: H7360-01)
4. Peristaltic pump (Gilson's MINIPULS® 3)
5. Water bath (temperature-controlled)
6. Photon-counting unit (Hamamatsu Photonics K. K., model: C8855-01)

Note: Equipment is assembled as depicted in Figure 1.

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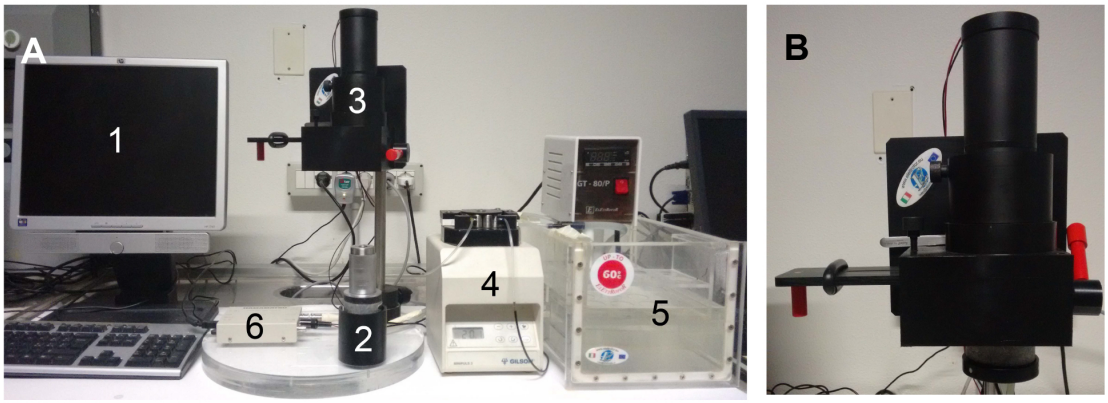

Figure 1. Equipment. A. Fully equipped aequorinometer is composed of: (1) computer, (2) perfusion chamber, (3) photomultiplier, (4) peristaltic pump, (5) water bath, (6) photon-counting unit. B. During the experiment, the perfusion chamber is placed in close proximity to the photomultiplier, protected from light.

Procedure

A. Day 1

Plate cells on 12 mm round glass coverslips, in a 24-well plate at 30-50% confluence (about 70,000 cells/well) and let them grow in their specific medium (Figure 2). Pre-treatment of coverslips with gelatin, collagen or poly-lysine to increase cell adherence is not mandatory, but could be recommended for specific cell types.

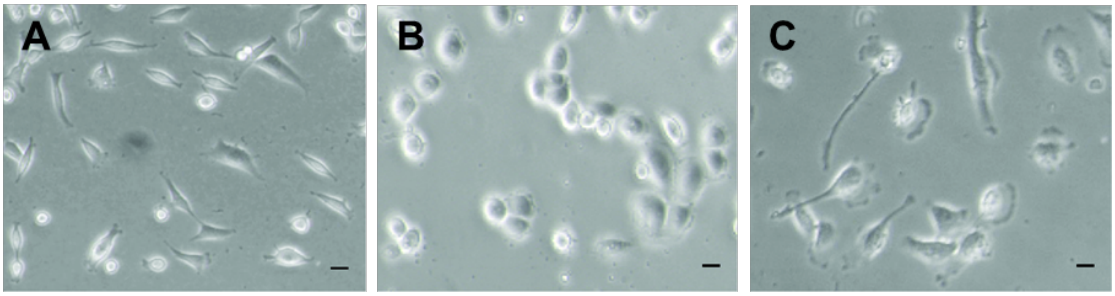

Figure 2. Different mammalian cell lines. MDA-MB-231 (A), MDA-MB-468 (B) and BT-549 (C) breast cancer cell lines at around 40% confluence. Scale bars = 10 µm.

B. Day 2

Transfect cells with the proper aequorin-expressing plasmids, according to the expected free [Ca^{2+}] present in the subcellular compartment (Brini *et al.*, 1995; Brini, 2008). Choose the best transfection/infection protocol depending on the targeted cell type. In this example protocol, Aequorin-wt and mitochondrial mutated aequorin (Asp119Ala) are used.

C. Day 3

1. 24 h after transfection remove medium from the cell plate.

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2. Wash cells with KRB solution once.
3. Incubate cells at 37 °C for 90 min with 5 µM coelenterazine in 200 µl KRB solution.
4. Transfer a coverslip containing the transfected cells to the perfusion chamber. Fix the perfusion chamber in close proximity to the photomultiplier (2-3 mm distance) (Figure 3).
5. Continuously perfuse cells with KRB, at 37 °C in a water bath. Agonists and other drugs should be added to the same solution.
6. Switch on the photomultiplier and start recording the light emission, ideally with a time span of 1 sec (the output of the amplifier-discriminator is captured by the photon-counting unit connected to a Windows-based computer).
7. As soon as the background values are stable, stimulate cells with an appropriate agonist concentration, in order to induce Ca^{2+} release from the endoplasmic reticulum (ER).
8. Follow light emission until suppression of Ca^{2+} signals.
9. Terminate the experiment by lysing cells with the digitonin solution, thus discharging the remaining aequorin pool.
10. Proceed with data analysis and calibration, as described below.

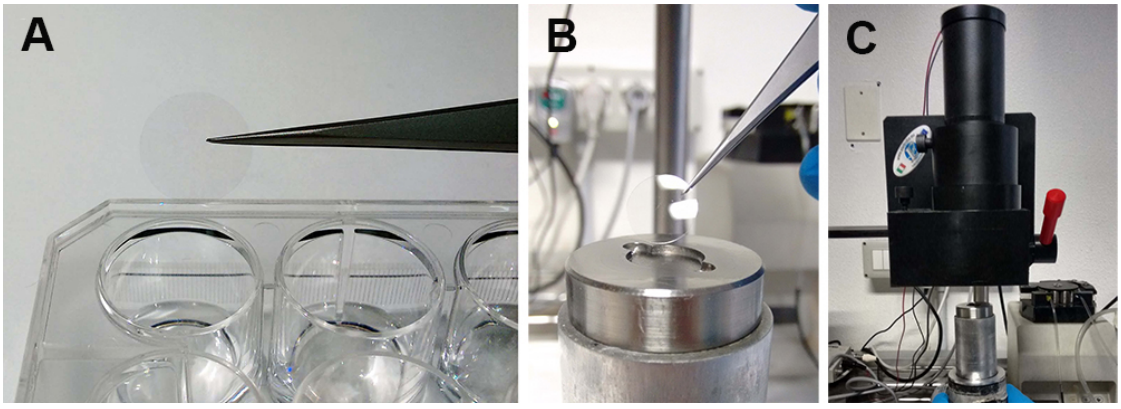

Figure 3. Experimental procedure. The coverslip coated with transfected cells is moved from the plate (A) to the perfusion chamber (B). The perfusion chamber is then placed near the photomultiplier (C).

Data analysis

In order to convert the luminescence signal in $[Ca^{2+}]$, an algorithm has been developed. Three variables are required:

1. The total amount of luminescence that each sample would emit in presence of saturating $[Ca^{2+}]$. In order to calculate this parameter, at the end of each experiment cells are lysed with a digitonin-containing solution (as reported in the Procedure section), and the luminescence emitted by the residual aequorin is measured. Since the total amount of luminescence is directly dependent on the amount of aequorin, this parameter takes into account differences in transfection efficiency.

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2. L_{max}, *i.e.*, the luminescence that would be emitted at a certain time point if all the aequorin had been suddenly discharged. Since aequorin is being constantly consumed, L_{max} progressively decreases during the experiment. At each time point, L_{max} is calculated by subtracting the luminescence recorded before that point from the total light output of the whole experiment.
3. L, *i.e.*, the rate of photon emission at any instant during the experiment.
   The $[Ca^{2+}]$ at each time point is a function of L/L_{max} (Figure 4). The presence of three Ca^{2+} binding sites in the aequorin molecule is responsible for the steep relationship between photon emission rate and free $[Ca^{2+}]$. The rate of luminescence is independent of $[Ca^{2+}]$ at very high (> 10^{-4} M) and very low $[Ca^{2+}]$ (< 10^{-7} M). However, it is possible to expand the range of $[Ca^{2+}]$ that can be monitored by choosing the proper recombinant aequorin construct (Figure 5).
   Data should be plotted as average of $Ca^{2+}{}_{max}$ peaks from replicates of each condition. At least 6 replicates per condition are recommended. In addition, a representative Ca^{2+} trace for each group of samples should be shown.

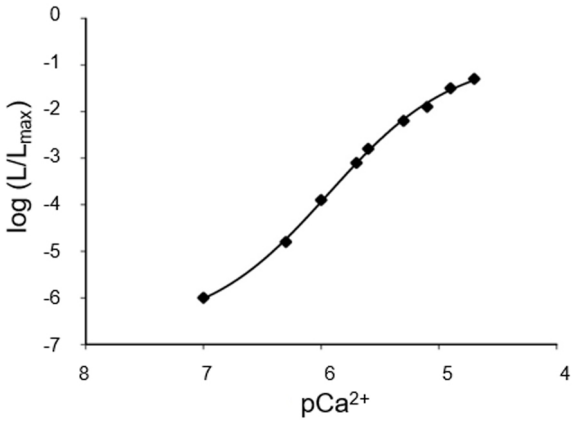

Figure 4. Representative Ca^{2+} calibration curve. At the end of the experiment, after cell lysis, the total amount of aequorin can be estimated and L/L_{max} can be calculated for each data point, thus allowing the conversion of light emitted into free Ca^{2+} concentration values. N = 6.

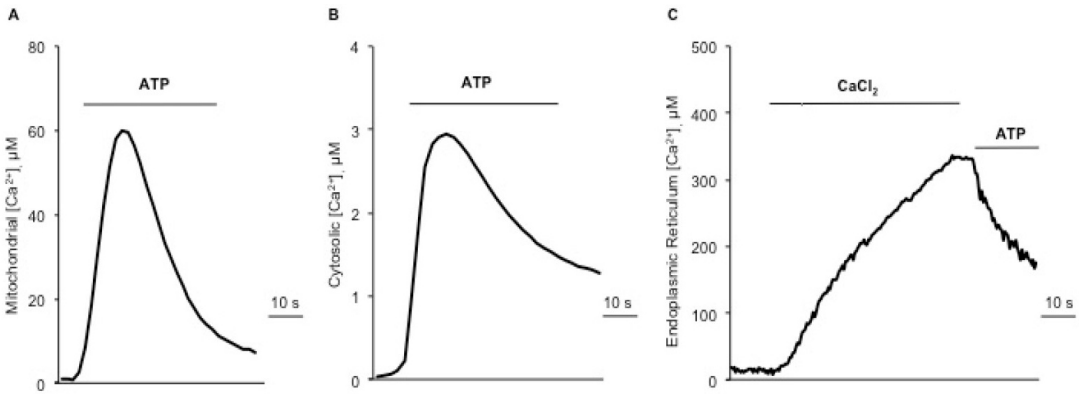

Figure 5. Representative traces of intracellular Ca^{2+} measurements. A. Mitochondrial Ca^{2+} uptake upon 100 µM ATP stimulation, using a mutated isoform (Asp119Ala) of aequorin. B.

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Cytosolic Ca^{2+} transients upon ATP stimulation, using the wild-type aequorin. C. ER Ca^{2+} uptake monitored by adding 1 mM $CaCl_2$ to a Ca^{2+}-free KRB, and subsequent Ca^{2+} release induced by agonist stimulation (ATP).

Notes

1. Although aequorin luminescence is influenced neither by K^+ nor Mg^{2+} (which are the most abundant cations in the intracellular environment and thus the most likely source of interference in physiological experiments) both ions are competitive inhibitors of Ca^{2+}-activated luminescence. pH also affects aequorin luminescence at values below 7. For these reasons, experiments with aequorin need to be done in well-controlled conditions of pH and ionic concentrations, notably of Mg^{2+}.
2. To stimulate Ca^{2+} release from ER, the appropriate agonist should be selected for each cell type (*e.g.*, ATP, Histamine, Bradykinin, Caffeine, Carbachol, Glutamate). The choice depends on cell-type specific expression of the G protein-coupled plasma membrane receptors.

Recipes

1. Krebs-Ringer modified buffer (KRB)
   135 mM NaCl
   5 mM KCl
   1 mM $MgCl_2 \cdot 6H_2O$
   0.4 mM KH_2PO_4
   1 mM $MgSO_4 \cdot 7H_2O$
   20 mM HEPES
   Adjust the pH to 7.4 with NaOH
   Store at 4 °C
   Add 1 mM $CaCl_2$ and 5.5 mM glucose, fresh before use
2. Digitonin lysis solution
   100 µM digitonin
   10 mM $CaCl_2$
   Store at 4 °C for maximum 4 days

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