Measuring [Ca^{2+}] in the endoplasmic reticulum with aequorin

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Summary The photoprotein aequorin was the first probe used to measure specifically the [Ca^{2+}] inside the lumen of the endoplasmic reticulum ([Ca^{2+}]_{ER}) of intact cells and it provides values for the steady-state [Ca^{2+}]_{ER}, around 500 μM, that closely match those obtained now by other procedures. Aequorin-based methods to measure [Ca^{2+}]_{ER} offer several advantages: (i) targeting of the probe is extremely precise; (ii) the use of low Ca^{2+}-affinity aequorin allows covering a large dynamic range of [Ca^{2+}], from 10^{-5} to 10^{-3} M; (iii) aequorin is nearly insensitive to changes in Mg^{2+} or pH, has a high signal-to-noise ratio and calibration of the results in [Ca^{2+}] is made straightforward using a simple algorithm; and (iv) the equipment required for luminescence measurements in cell populations is simple and low-cost. On the negative side, this technique has also some disadvantages: (i) the relatively low amount of emitted light makes difficult performing single-cell imaging studies; (ii) reconstitution of aequorin with coelenterazine requires previous complete depletion of Ca^{2+} of the ER for 1–2 h, a maneuver that may result in deleterious effects in some cells; (iii) because of the high rate of aequorin consumption at steady-state [Ca^{2+}]_{ER}, only relatively brief experiments can be performed; and (iv) expression of ER-targeted aequorin requires previous transfection or infection to introduce the appropriate DNA construct, or alternatively the use of stable cell clones. Choosing aequorin or other techniques to measure [Ca^{2+}]_{ER} will depend of the correct balance between these properties in a particular problem.

HISTORICAL BACKGROUND
Although the ER has been known to be the main intracellular Ca^{2+} store for nearly 20 years, monitoring the free [Ca^{2+}] in the ER ([Ca^{2+}]_{ER}) has proven to be a difficult task. The first studies using low-affinity fluorescent indicators required cell permeabilization to release the cytosolic dye and could not avoid compartmentalization of the dye into other organelles [1–3]. Apart of that, selectivity of these probes over Mg^{2+} is poor and calibration was highly uncertain. On the other side, use of aequorin to measure [Ca^{2+}]_{ER} has required solving two difficult technical problems: getting specific targeting of the probe into the ER and modifying the Ca^{2+}-affinity of the probe to reach the adequate [Ca^{2+}] range.

The problem of targeting Targeting a protein to stay in the ER requires not only adding a N-terminal presequence able to signal translocation into the ER, but also a second signal designed to make the protein stay in the ER, avoiding progression through the secretory pathway. The first approach to targeting aequorin to the ER used the known Lys-Asp-Glu-Leu (KDEL) motif appended to the C-terminal of aequorin to retain the protein in the ER [4,5]. However, this modification caused a spontaneous and Ca^{2+} independent degradation of the protein [6,7]. In addition, the luminescence signal provided quite low [Ca^{2+}]_{ER} values (1–5 μM) [4], hardly consistent with the role of the ER as a Ca^{2+}-store. These findings can be now explained on the basis of the high Ca^{2+}-affinity of the native aequorin used and the presence of a small low-Ca^{2+} compartment in the ER (see below). In order to avoid these problems, a different procedure was designed to retain the chimeric photoprotein in the ER. Immunoglobulin heavy chains are translocated into the ER and then bind to the chaperone BiP through its CHI region until the light chain arrives. Thus, in cells
that do not produce light chains, a chimeric polypeptide containing the CH1 region is expected to be retained into the ER [8]. That was the case. A chimeric aequorin containing a portion of the immunoglobulin heavy chain gene including leader, VDJ and CH1 domains, fused to the N-terminal of aequorin (Fig. 1), was correctly targeted and retained into the ER [9]. Electron microscopy using aequorin-tagged immunogold labeling confirmed that ER-targeting was specific and that the photoprotein was absent in other structures of the secretory pathway such as the Golgi complex [10].

**The problem of the Ca\(^{2+}\)-affinity**

The immunoglobulin-aequorin chimera solved the problem of targeting, but that was not enough to measure [Ca\(^{2+}\)]\(_{Er}\) because of the high Ca\(^{2+}\)-affinity of native aequorin, which makes it unable to measure [Ca\(^{2+}\)] \(_{Er}\) values above 5 \(\mu\)M. Fig. 2 shows calibration curves of a series of aequorins. \(LU_{\text{max}}\) values correspond to the ratio among the luminescence emitted per second at every [Ca\(^{2+}\)] and the total luminescence than remains to be emitted by the sample. Therefore, a value of 0 for log \(LU_{\text{max}}\) corresponds to a situation in which all the aequorin molecules emit their light in one second, and a value of –1 means that 10% of the aequorin molecules are emitting their light every second. Taking into account that emission of light by aequorin is irreversible (every aequorin molecule emits light only once during the experiment), stable measurements of [Ca\(^{2+}\)] \(_{Er}\) when log \(LU_{\text{max}}\) is ≥ –1 are not possible. This means that native aequorin (AEQ1 in Fig. 2) can only measure [Ca\(^{2+}\)] values up to about 5 \(\mu\)M. To extend the measuring range, a point mutation was introduced (Asp119 → Ala) that was known to reduce about 10-fold the Ca\(^{2+}\)-affinity of aequorin [11]. This effectively increased the maximum [Ca\(^{2+}\)] values that could be measured up to around 100 \(\mu\)M (AEQ2, Fig. 2), but that was still not enough to measure steady-state [Ca\(^{2+}\)]\(_{Er}\) values.

On the first place, emission of luminescence requires previous reconstitution with the cofactor coelenterazine, but hardly any reconstitution of native or mutated aequorin could be obtained in intact cells with the ER full of Ca\(^{2+}\). Thus, to avoid consumption of aequorin during reconstitution, the ER was depleted of Ca\(^{2+}\) before reconstitution. Then, when Ca\(^{2+}\) was reintroduced in the extracellular medium, large peaks of luminescence were obtained (no matter if native or mutated aequorin was used) that consumed more than 90% of the aequorin in the first minute [9,10]. Calibrated into [Ca\(^{2+}\)], these peaks corresponded to a [Ca\(^{2+}\)] of about 5 \(\mu\)M using native aequorin and around 100 \(\mu\)M using mutated aequorin. The height of these peaks matched precisely the Ca\(^{2+}\)-saturation level of each type of aequorin and these data clearly suggested that the real steady-state [Ca\(^{2+}\)]\(_{Er}\) was higher than 100 \(\mu\)M.

An initial alternative to obtain data on the dynamics of [Ca\(^{2+}\)] in the ER using these probes was to use Sr\(^{2+}\) as a Ca\(^{2+}\) surrogate. Sr\(^{2+}\) behaves similarly to Ca\(^{2+}\) with respect to Ca\(^{2+}\) pumps and most Ca\(^{2+}\) channels, but its affinity for aequorin is 100-fold smaller. This approach allowed measuring steady-state [Sr\(^{2+}\)] values of 1–2 mM and dynamic movements of this cation under different conditions.
conditions, e.g. a fast $\text{Sr}^{2+}$ release after cell stimulation with agonists producing inositol 1,4,5-trisphosphate or a slow one after treatment with ER-$\text{Ca}^{2+}$-ATPase blockers [9,10]. But the real steady-state [$\text{Ca}^{2+}$]$_{\text{ER}}$ was still unclear.

To measure it, it was necessary to reduce further the affinity of aequorin for $\text{Ca}^{2+}$. That was achieved indirectly by using a semisynthetic coelenterazine, named coelenterazine $n$ [12], that reduces the rate of emission of luminescence by aequorin. The calibration curve of mutated aequorin reconstituted with coelenterazine $n$ (AEQ3, Fig. 2) shows that the range of [$\text{Ca}^{2+}$] that can be measured with this probe go from 10–20 M to about 1 mM. In addition, working at room temperature (22 °C) reduces still further the rate of luminescence, allowing to measure millimolar levels of $\text{Ca}^{2+}$ for a longer period of time.

Using this approach, we could finally obtain measurements of [$\text{Ca}^{2+}$]$_{\text{ER}}$ dynamics in intact HeLa cells [13,14]. Afterwards, this new probe has been also applied to the measure of [$\text{Ca}^{2+}$] in the sarcoplasmic reticulum of skeletal muscle myotubes, by expressing a closely related aequorin chimera made by fusing the resident sarcoplasmic reticulum protein calsequestrin with mutated aequorin [15]. In addition, we have also used the same low-$\text{Ca}^{2+}$ affinity aequorin, targeted to mitochondria instead of ER, to measure large [$\text{Ca}^{2+}$]$_{\text{mit}}$ transients in mitochondria after stimulation of chromaffin cells [16].

The subsequent application of this technique to a wide range of cells was only possible by using viral methods to express the aequorin chimera [17]. Fig. 1 shows the plasmid of herpes simplex virus type 1 used to clone ER-targeted aequorin and generate viruses able to express the construct. Thanks to this technique, it was possible to express ER-targeted aequorin in a variety of cell lines (NIH3T3, PC12, GH3) and primary cultures (cerebellar granule cells, anterior pituitary cells and chromaffin cells), obtaining dynamic measurements of [$\text{Ca}^{2+}$]$_{\text{ER}}$ in these cells [17,18].

**METHODOLOGY**

Fig. 3 outlines a typical experiment, which we can describe as a series of steps.

**Cell seed**

Cells should be seeded at 50% confluency onto glass or plastic coverslips. A good attachment is essential, as cells have to be perfused with different solutions during the experiment. To facilitate attachment in some cell kinds, we treat previously the coverslips with poly-L-lysine. It is important to take into account that reconstitution of aequorin requires prior depletion of $\text{Ca}^{2+}$ of the ER (see below) by incubation in EGTA-containing medium for 1–2h. Some cells may tend to detach from glass during this period. Poly-L-lysine covered coverslips may be obtained commercially. Alternatively, they may be easily prepared by introducing autoclaved coverslips in a filtered sterile 0.01 mg/ml poly-L-lysine solution for 5 min, washing them 2 min in sterile water and finally air drying them completely under sterile conditions.

**Expression of ER-targeted mutated aequorin**

This step may be skipped if the cells come from a cell clone expressing ER-targeted mutated aequorin (ERmutAEQ). For example, we have stable HeLa cell clones that express this construct [9]. To measure [$\text{Ca}^{2+}$]$_{\text{ER}}$ in other cell types, the ERmutAEQ plasmid should be expressed in the desired cell culture. This can be achieved either by transfection or infection methods. We have used successfully Ca-phosphate and transfection reagents such as Lipofectamine (Gibco Labs, Grand Island, NY) and FuGene (Hoffmann-La Roche, Basel, Switzerland) to express this plasmid in cell lines such as HeLa and CHO. However, these methods are not efficient to express the construct in many other cell types, particularly primary cultures. To solve this problem, we have used defective herpes simplex virus (HSV1) carrying the gene, and this method has allowed to obtain a good expression in a series of cell lines and primary culture cells [17,18]. Adequate expression is
usually obtained after 12 h using virus-based methods, and 18–24 h using transfection methods.

**Ca**\(^{2+}\)-depletion of the ER

Even using mutated aequorin and coelenterazine n, reconstitution of aequorin is impossible if the ER remains full of \(\text{Ca}^{2+}\). It is, therefore, necessary to deplete completely the ER of \(\text{Ca}^{2+}\) for 1–2 h. This is achieved by incubating the cells in medium containing EGTA and a reversible inhibitor of the ER-\(\text{Ca}^{2+}\)ATPase, such as 2,5-di-tert-butybenzohydroquinone (BHQ) or ciclopenta-zonic acid. These compounds produce a rapid \(\text{Ca}^{2+}\)-depletion of the ER (half-time 1–2 min) through some still unknown leak pathways. \(\text{Ca}^{2+}\) released from the ER is then extruded out of the cell by the plasma membrane \(\text{Ca}^{2+}\) pump. Briefly, the glass coverslip containing the cells that express ERmutAEQ is transferred to a well (of a 24-well plate) containing 1 ml of extracellular medium (NaCl, 145 mM; KCl, 5 mM; MgCl\(_2\), 1 mM; glucose, 10 mM; Hepes, 10 mM, pH 74) containing 0.5 mM EGTA. After washing once with the same medium, 0.4 ml of depletion solution (extracellular medium supplemented with 0.5 mM EGTA and 10 \(\mu\)M BHQ) is added to the well and left for 10 min at room temperature. This time is enough to empty almost completely the ER of \(\text{Ca}^{2+}\).

**Reconstitution with coelenterazine n**

Reconstitution of aequorin with coelenterazine n (Molecular Probes Europe BV, Leiden, The Netherlands) is much slower than with wild-type coelenterazine (the half-time in vitro is 5 h compared to 22 min for the wild-type [12]). This means that longer times of reconstitution will give an almost linear increase in the total luminescence output. A very important factor is also to keep low the temperature of reconstitution. Increasing the temperature to 37 \(^\circ\)C during reconstitution decreases by near one order of magnitude the total luminescence of the sample, perhaps because of increased aequorin consumption. The usual procedure is as follows: depletion solution is removed and 0.2 ml of the same solution is added to the well. Then, 1 \(\mu\)l of coelenterazine n (stock prepared 200 \(\mu\)M in methanol) is added and gently mixed. The plate should be then left in the dark (coelenterazine is light-sensitive) at room temperature for 1–2 h.

**Luminescence experiment**

After reconstitution, the glass coverslip is transferred to the cell chamber of a home-made luminometer. Cells are then initially perfused for 5 min with extracellular medium containing 0.5 mM EGTA in order to wash completely BHQ and release the inhibition of the ER-\(\text{Ca}^{2+}\)-ATPase. Then, the usual experiment starts by perfusing extracellular medium containing 1 mM \(\text{Ca}^{2+}\) to refill the ER. Once \([\text{Ca}^{2+}]_{\text{ER}}\) reaches the steady-state, different kinds of experiments may be performed (see below). It is important to know also that every experiment has to end with a final step of cell lysis, in order to allow calibration of the luminescence into \([\text{Ca}^{2+}]\) values (see below). The equipment for luminescence measurements in cell populations is composed of several components that can be easily assembled. Briefly, we use a Electron Tubes (Ruislip, U.K.) photomultiplier 9789A refrigerated at 4 \(^\circ\)C inside a cool box. The 10 mm photocathode is placed in almost direct contact with the thermostatted perfusion chamber (Fig. 3), and covers most of the surface of the glass coverslip. A system of eight electrovalves allows perfusing different solutions, placed in a nearby bath, that flow by gravity during the experiment. The photomultiplier is usually set at 1–1.2 kV, and the output goes via an amplifier-discriminator AD2 up to a counter/timer PCB CT1 card in the computer, both from Electron Tubes. Luminescence data are stored every 50 ms, averaged every second and transformed into \([\text{Ca}^{2+}]\) values through a home-made software (see below).

**Calibration of luminescence data**

In order to calibrate the data obtained in terms of \([\text{Ca}^{2+}]_{\text{ER}}\), we need to know the total amount of luminescence that can be emitted by the sample. For that, at the end of every experiment we use a pure lysis solution (10 mM CaCl\(_2\) and 100 \(\mu\)M digitonin in water). Cell permeabilization in the presence of excess \(\text{Ca}^{2+}\) rapidly releases the luminescence from intracellular stores to allow calibration of the luminescence measurements in cell populations. The luminescence values have no meaning in terms of \([\text{Ca}^{2+}]\) until they are transformed into ratios \(L/L_{\text{max}}\). The final calibrated results are therefore independent of the total amount of reconstituted aequorin in the sample. The other hand, as aequorin is being progressively consumed, the value of \(L_{\text{max}}\) (the total remaining luminescence of the sample) decreases continuously along the experiments. Because of this, the behavior of aequorin luminescence is quite different to that of \(\text{Ca}^{2+}\)-sensitive fluorescent dyes.
For example, a constant level of luminescence (L) corresponds to a continuous increase in \( \frac{U}{U_{\text{max}}} \) ratio and thus, to a continuous increase in \([Ca^{2+}]\). Vice-versa, to get a constant level of \([Ca^{2+}]\), we need the \( \frac{U}{U_{\text{max}}} \) ratio to become constant, and for this we need the luminescence to be decreasing exponentially.

Crude records of luminescence are stored in computer files that contain data sampled every 50 ms. To transform these data in \([Ca^{2+}]\) files that contain data sampled every 50 ms. To transform

\[ \text{ICR} = \frac{L}{L_{\text{max}} \times \lambda} \]

This algorithm was derived from a mathematical model proposed originally to explain from a molecular point of view the \([Ca^{2+}]\)-dependence of aequorin luminescence [20], but it can be used as a simple mathematical transformation independent of the model. The values for the parameters of the algorithm that should be used to calculate \([Ca^{2+}]\) in experiments using EdmanAEQ reconstituted with coelenterazine n are:

At 22°C: \( K_{\text{E}} = 5 \times 10^2 \), \( K_{\text{R}} = 1.597 \times 10^3 \), \( n = 1.271 \), \( \lambda = 0.02512 \).

At 37°C: \( K_{\text{E}} = 8.47 \times 10^3 \), \( K_{\text{R}} = 1.656 \times 10^5 \), \( n = 1.203 \), \( \lambda = 0.138 \).

Regarding the significance of the parameters in the model [20], it is worth indicating that \( n \) was the number of \( Ca^{2+} \)-binding sites in the model, and has a value of near 3 in native aequorin. The smaller value of \( n \) observed here is mainly due to the mutation of one of the \( Ca^{2+} \)-binding sites, and corresponds with a decrease in the slope of the calibration curve (compare the slope of the AEQ1 curve with those of AEQ2 or AEQ3 ones in Fig. 2). The parameter \( \lambda \) is the rate constant for aequorin consumption at saturating \([Ca^{2+}]\). This parameter was not included in the original description of the algorithm [19], because the maximum rate constant of native aequorin reconstituted with wild-type coelenterazine is 1.0 s\(^{-1}\). Reconstitution with coelenterazine n reduces considerably the maximum rate constant, and this allows recording high \([Ca^{2+}]\) values with smaller aequorin consumption, particularly at 22°C.
This 1–2% of aequorin contained in a low-Ca⁡²⁺ environment has a dramatic influence in the calibration. In the lower left panel, curve A shows the [Ca⁡²⁺] values obtained by applying directly the algorithm to the experimental data. Addition of Ca⁡²⁺ induces an increase in [Ca⁡²⁺]Lmax that rises smoothly until near 500 μM and then decreases slowly to reach again very low [Ca⁡²⁺]Lmax values within few minutes. To understand why the [Ca⁡²⁺]Lmax decreases instead of reaching a stable steady-state, we should note first that the decrease in [Ca⁡²⁺]Lmax starts when less than 20% of aequorin remains able to emit light (dotted line). At that moment, the 1–2% of aequorin present in a low-Ca⁡²⁺ compartment starts to become quantitatively important. In fact, a few minutes later, this low-Ca⁡²⁺ compartment becomes dominant when most of aequorin has been consumed, and the apparent [Ca⁡²⁺]Lmax returns to very low values. The artifact introduced by this small compartment can be overcome by assuming that aequorin in that compartment does not emit a significant amount of light during the initial part of the experiment. In that case, we can reduce the total amount of aequorin in the algorithm by taking out the 1–2% of aequorin of the low-Ca⁡²⁺ compartment. The exact percentage of this compartment can be estimated from the amount of aequorin luminescence released by lysing the cells at the end of the experiment, when luminescence has returned to near-background levels. The result of this re-calculation of the same data, reducing the value of Lmax by 2%, is shown in curve B. We can see that this small change in the value of the total luminescence hardly affects the initial increase in [Ca⁡²⁺]Lmax, but makes disappear the subsequent decrease. Now [Ca⁡²⁺]Lmax stabilizes at around 500 μM, although the rapid consumption of aequorin allows monitoring this high [Ca⁡²⁺]Lmax value only for few minutes.

Similar results are obtained when this protocol is carried out at 22°C, except for the fact that steady-state [Ca⁡²⁺]Lmax values can be measured for a longer time. Addition of Ca⁡²⁺ induced a much broader peak of luminescence that ended in a long tail (upper right panel). At the end of that period, cell lysis released a small amount of light, usually slightly higher than that obtained in the experiments at 37°C. This is probably due to the overlap of the long tail, that is, the incomplete consumption of the high-Ca⁡²⁺ compartment. Again here, the calibrated [Ca⁡²⁺]Lmax data show a smooth increase followed by stabilization at about 500 μM, and then slow decrease due to the low-Ca⁡²⁺ compartment (curve A). The decrease in [Ca⁡²⁺]Lmax starts also here when only 20% of aequorin remains active in the sample (dotted line, right panel). Recalculation of the data reducing again Lmax in 2% produced curve B, that shows a stable steady-state that allows performing different kinds of experimental maneuvers for more than 10 minutes. This simple experiment constitutes a good example of the ability of aequorin to detect heterogeneities in [Ca⁡²⁺]L in this case, fast aequorin consumption in high [Ca⁡²⁺] areas allowed detecting a small compartment (2% of total) with much lower [Ca⁡²⁺]. This compartment, whose structural nature is unknown, may have been responsible of the first measurements of [Ca⁡²⁺]Lmax with ER-targeted aequorin in the range 1–5 μM [4]. Similar values were also obtained when we expressed our ER-targeted construct containing native aequorin in HeLa cells [10]. On the other hand, this kind of experiments also constitute evidence that most of the ER (98% in HeLa cells) has a nearly homogeneous free [Ca⁡²⁺] around 500 μM. The presence of compartments with grossly different free [Ca⁡²⁺] would have been easily detectable by the presence of different rates of consumption along the refilling process.

Comparing the data obtained at both temperatures, the main reason for the slowest aequorin consumption at 22°C is that the maximum rate of aequorin consumption (λ) decreases about five-fold at 22°C compared with that at 37°C (see also Fig. 2). This increases the time that [Ca⁡²⁺]Lmax can be recorded at steady-state at 22°C, even though the steady-state [Ca⁡²⁺]Lmax level is the same at both temperatures. As a consequence, while at 37°C steady-state [Ca⁡²⁺]Lmax values can be monitored for only 2–3 min, at 22°C the measuring time at the same [Ca⁡²⁺]Lmax values becomes 15–20 min. Solving the problem of fast consumption at 37°C would require introducing a new mutation in the aequorin molecule to reduce further its Ca⁡²⁺ affinity.

As mentioned above, the technique can be used with many cell types, even primary cultures, provided that the preparation is highly pure and that expression of the EBmnTAcrQ gene becomes possible. The purity of the preparation is very important in primary cultures because measurements in cell populations cannot separate the signal coming from different cell types, and the intensity of the expression may also be different in different cell types. We have measured [Ca⁡²⁺]Lmax in some preparations that can be obtained highly pure, such as chromaffin cells and cerebellar granule cells. Fig. 5 shows a typical experiment performed in chromaffin cells (see also [18]).

The upper panel shows the record of luminescence and the lower one the calibrated [Ca⁡²⁺]Lmax values. Addition of extracellular Ca⁡²⁺ starts refilling of the ER, reaching levels close to 500 μM (lower panel). Then, a series of additions of 5 mM caffeine were made. Each caffeine addition induced a rapid but partial decrease of [Ca⁡²⁺]Lmax, followed by recovery to the same concentrations. This maneuver was repeated many times, and [Ca⁡²⁺]Lmax always recovered to steady-state values. This repetitive recovering is extremely important as a test that aequorin consumption does not affect significantly the [Ca⁡²⁺]Lmax values obtained in these cells. Finally, in the last addition, 50 mM caffeine was perfused, producing a fast and full emptying of the ER. It is very interesting to compare the crude luminescence

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Fig. 5 Effect of caffeine on ER-targeted aequorin luminescence and calibrated $[\text{Ca}^{2+}]_{\text{ER}}$. Bovine chromaffin cells were infected with pHSVerAEQ to express ER-targeted mutated aequorin. After reconstitution with coelenterazine n, refilling of the ER was started by perfusing extracellular medium containing 1 mM $\text{Ca}^{2+}$ as indicated. Then, several consecutive stimulations with 5 or 50 mM caffeine were performed as indicated. The upper panel shows the crude luminescence record, and the lower one the calibrated $[\text{Ca}^{2+}]_{\text{ER}}$ values. Temperature was 22°C.

Data with the calibrated values to understand the calibration of aequorin. First, it is evident that a steady-state luminescence is not indicative of a steady-state $[\text{Ca}^{2+}]_{\text{ER}}$. For example, after the addition of $\text{Ca}^{2+}$ to the extracellular medium, luminescence reached a steady-state before the first addition of caffeine (upper panel), but calibrated $[\text{Ca}^{2+}]_{\text{ER}}$ was still going up. Similarly, $[\text{Ca}^{2+}]_{\text{ER}}$ recovered to similar values after each stimulation, but luminescence decreased continuously along the experiment. The technique allows also performing experiments with permeabilized cells (Fig. 6). The protocol here is identical to that described above until the glass coverslip is placed in the luminometer and BHQ is washed. At that point, instead of perfusing extracellular medium containing 1 mM $\text{Ca}^{2+}$, we perfuse intracellular medium containing 0.5 mM EGTA and 100 $\mu$M digitonin for 1 min as indicated. Then, the ER was refilled by perfusion of intracellular medium containing 100$nM$ $\text{Ca}^{2+}$ (buffered with EGTA). Once the steady-state $[\text{Ca}^{2+}]_{\text{ER}}$ had been reached, either 0.1 or 2 $\mu$M InsP$_3$ were added as indicated. Temperature was 22°C.

![Diagram of ER and aequorin](image)

intracellular medium containing 100$nM$ $\text{Ca}^{2+}$ (buffered with EGTA) is perfused to refill the ER. Fig. 6 shows that the ER is refilled at a similar rate that in intact cells (Fig. 4) and reaches similar steady-state $[\text{Ca}^{2+}]_{\text{ER}}$ levels. At that point, we can test the effect of an intracellular $\text{Ca}^{2+}$ mobilizer such as inositol 1,4,5-trisphosphate (InsP$_3$). The figure shows the effect of a maximal concentration of InsP$_3$ (2 $\mu$M) and a submaximal one (0.1 $\mu$M). These experiments on permeabilized cells may be particularly useful when we want to measure precisely the response of the ER to a certain non-permeable intracellular agent.

**ADVANTAGES AND DISADVANTAGES OF THE TECHNIQUE**

In this section, we will try to dissect the main pros and cons of aequorin-based methods of measuring $[\text{Ca}^{2+}]_{\text{ER}}$ dynamics. On the positive side, we can mention the following points:

1. **Selectivity of targeting.** It has been shown both by immunofluorescence [9] and by electron microscopy [10] that the probe is correctly sent and retained in the ER. This is an important advantage with respect to fluorescent dyes, that cannot be targeted specifically.

2. **Large dynamic range and high signal-to-noise ratio.** The luminescence of aequorin can change by a factor of several orders of magnitude after the increase in $[\text{Ca}^{2+}]_{\text{ER}}$, and every type of aequorin is able to follow $[\text{Ca}^{2+}]_{\text{ER}}$ changes within a span of nearly two orders of magnitude of $[\text{Ca}^{2+}]_{\text{ER}}$. In addition, the availability of different kinds of aequorins (Fig. 2) with different $\text{Ca}^{2+}$-affinities allows exploring a wide range of $[\text{Ca}^{2+}]_{\text{ER}}$ from $10^{-7}$ to $10^{-3}$ M.
3. **Straightforward calibration.** As shown above, calibration of luminescence into [Ca\(^{2+}\)] values requires only to know the total luminescence of the sample. This is easily measured by lysing the cells in Ca\(^{2+}\)-containing medium at the end of the experiment. After that, calibration in [Ca\(^{2+}\)] is obtained immediately by applying the algorithm described above through a computer program. The possible presence and size of a low-Ca\(^{2+}\) compartment should be explored and corrected as described above, if possible. On the other hand, aequorin is hardly sensitive to pH in the physiological range, and Mg\(^{2+}\) is unable to trigger luminescence.

4. **Low-cost equipment.** The equipment necessary to perform the luminescence measurements is not commercial but can be easily assembled from a series of low-cost components. Similarly, the software to transform luminescence into [Ca\(^{2+}\)] can be freely obtained from our laboratory.

In the other side of the balance, this methodology has also some important drawbacks that should be considered:

1. **Small amount of light.** The amount of emitted light is relatively small, and this makes difficult performing single-cell imaging studies. Recently, however, it has been shown that the use of a high-sensitivity camera combined with the high gene expression provided by viruses, allows making single-cell studies of mitochondrial or nuclear [Ca\(^{2+}\)] using targeted aequorin [21,22]. Nevertheless, the application of this technique to the measure of [Ca\(^{2+}\)] is more problematic, because of the different pattern of [Ca\(^{2+}\)] changes in the ER compared with mitochondria, nucleus or cytosol. In the ER, the steady-state [Ca\(^{2+}\)]

2. **Depletion of Ca\(^{2+}\) of the ER.** As mentioned above, reconstitution of aequorin with coelenterazine requires depleting the ER of Ca\(^{2+}\) for 1–2h. The question here is whether or not this prolonged depletion alters somehow the cell [Ca\(^{2+}\)]

3. **High rate of consumption.** The length of the experiments is considerably limited by the rapid consumption of aequorin that starts immediately after reintroduction of Ca\(^{2+}\). Fig. 4 shows that, at 37°C, nearly 90% of aequorin has been already consumed when the steady-state is reached. This means that there is hardly any time left to test the effects of agonists or other possible experimental maneuvers. This is why we perform most of our [Ca\(^{2+}\)] experiments at 22°C. At this temperature, the steady-state is reached when aequorin consumption is still around 60% (Fig. 4), and reasonable measurements can be still obtained during 10–20 more minutes (Fig. 5). The problem of time-limitation may be solved by reducing further the Ca\(^{2+}\)-affinity of aequorin, perhaps by introducing a new mutation in any of the Ca\(^{2+}\)-binding sites, although this modification will probably reduce considerably the light output in steady-state.

4. **DNA expression method required.** Transfection or infection methods are required to express the ER-targeted aequorin in the cell type of interest. The efficiency of these methods is variable and depends on the cell type. In addition, expression of the protein requires at least
interference from Mg2+

signal-to-noise ratio, and calibration is complicated due to

specificity of targeting, have a smaller dynamic range and

ER-targeted aequorin and other methodologies. Fluores-

cent dyes, for example, are much better to do single-cell

imaging and have no problems of consumption, but lack

specificity of targeting, have a smaller dynamic range and

signal-to-noise ratio, and calibration is complicated due to

interference from Mg2+, pH or just non-specific distribu-

tion of the dye. Therefore, advantages and disadvantages

are exactly opposite to those of aequorin, so that measure-

ments with both techniques may be strongly reinforcing.

CONCLUSION

Aequorin-based methods to measure [Ca2+] combine a high specificity of targeting with a large dynamic range of [Ca2+] measurement from micromolar to millimolar lev-

eels and also a high signal-to-noise ratio. In contrast, they

also have severe limitations particularly in terms of intensity

of light emission and rapid consumption of the probe. The

balance between pros and cons will determine in which

kind of applications it may be more adequate. It may be

also useful to combine measurements of [Ca2+] with ER-targeted aequorin and other methodologies. Fluores-
cent dyes, for example, are much better to do single-cell

imaging and have no problems of consumption, but lack

specificity of targeting, have a smaller dynamic range and

signal-to-noise ratio, and calibration is complicated due to

interference from Mg2+, pH or just non-specific distribu-
tion of the dye. Therefore, advantages and disadvantages

are exactly opposite to those of aequorin, so that measure-

ments with both techniques may be strongly reinforcing.

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