Subcellular calcium measurements in mammalian cells using jellyfish photoprotein aequorin-based probes

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The jellyfish *Aequorea victoria* produces a 22-kDa protein named aequorin that has had an important role in the study of calcium (Ca^{2+}) signaling. Aequorin reacts with Ca^{2+} via oxidation of the prosthetic group, coelenterazine, which results in emission of light. This signal can be detected by using a special luminescence reader (called aequorinometer) or luminescence plate readers. Here we describe the main characteristics of aequorin as a Ca^{2+} probe and how to measure Ca^{2+} in different intracellular compartments of animal cells (cytosol, different mitochondrial districts, nucleus, endoplasmic reticulum (ER), Golgi apparatus, peroxisomes and subplasma-membrane cytosol), ranging from single-well analyses to high-throughput screening by transfecting animal cells using DNA vectors carrying recombinant aequorin chimeras. The use of aequorin mutants and modified versions of coelenterazione increases the range of calcium concentrations that can be recorded. Cell culture and transfection takes ~3 d. An experiment including signal calibration and the subsequent analyses will take ~1 d.

INTRODUCTION

In all cells, the intracellular calcium concentration ($[Ca^{2+}]$) is strictly controlled by complex interactions among different channels, pumps and transporters[1,2]. Finely tuned changes in $[Ca^{2+}]$ modulate a variety of intracellular functions, and disruption of Ca^{2+} handling leads to various pathological conditions[3–5].

The medusa *Aequorea victoria* produces a 22-kDa protein named aequorin that has had a major role in the study of Ca^{2+} signaling[6]. Upon binding of Ca^{2+} to three high-affinity sites, aequorin undergoes an irreversible reaction in which a photon is emitted. In its active form, the protein includes a prosthetic group (coelenterazine) that is oxidized and released in the Ca^{2+}-triggered reaction[7,8]. Thus, the expression of aequorin cDNA yields the polypeptide, to which the prosthetic group must be added. Coelenterazine is highly hydrophobic and, when added to the culture medium of aequorin-expressing cells, will freely permeate through the cell membrane. Once inside the cell, coelenterazine spontaneously binds to aequorin, generating the active probe. This procedure is generally termed 'reconstitution' and will be described in detail below.

There is a relationship between the fractional rate of consumption (i.e., L/L_{max}, where L_{max} is the maximal rate of discharge at saturating Ca^{2+} concentrations) and $[Ca^{2+}]$ (ref. 9). Owing to cooperation between the three binding sites, light emission is proportional to the second to third power of $[Ca^{2+}]$; this property on the one hand accounts for the excellent signal-to-noise ratio of aequorin and on the other hand may subtantially affect the measurements.

Given that the probe (differently from fluorescent indicators) is gradually consumed throughout the experiment, the signal tends to decrease, and the conversion into $[Ca^{2+}]$ concentration can be obtained only at the end of the experiment, when, after cell lysis, the total aequorin content is estimated and L/L_{max} can be back calculated for each data point.

At present, several tools are available for intracellular $[Ca^{2+}]$ measurements (**Table 1**). Here we will discuss advantages and limitations of the aequorin-based approach to help researchers who choose aequorin from among other Ca^{2+} probes.

TABLE 1 | Summary of the most common techniques for intracellular Ca^{2+} detection.

Probe	Origin	Detection technique	Ref.
Aequorin	Genetically encoded	Luminometry	15
Berovin	Genetically encoded	Luminometry	22
Obelin	Genetically encoded	Luminometry	23
Cameleon	Genetically encoded	FRET microscopy	24
Troponin C biosensor	Genetically encoded	FRET microscopy	25
Camgaroo	Genetically encoded	Fluorescence microscopy	26
Ratiometric Pericam	Genetically encoded	Ratiometric fluorescence microscopy	27
GEM-GECO1	Genetically encoded	Ratiometric fluorescence microscopy	28
Calcium Green-1	Synthetic	Fluorescence microscopy	29
Fluo-3, Fluo-4	Synthetic	Fluorescence microscopy	29
Fura-2, Indo-1	Synthetic	Ratiometric fluorescence microscopy	30

TABLE 2 | Description of the compartment-specific aequorin chimeras available.

Intracellular localization	Acronym	Targeting strategy
Cytosol	CytAEQ	No targeting sequence is added to aequorin; the sequence of aequorin was modified only by adding the epitope tag HA1 (ref. 15)
Nucleus	NuAEQ	A fragment of rat glucocorticoid receptor, lacking the hormone-binding domain and the nuclear localization signal are fused with the HA1-tagged aequorin[31]
	MtAEQwt	Mitochondrial pre-sequence of subunit VIII of cytocrome *c* oxidase (COX) is fused to the HA1-tagged aequorin, for measurements of $[Ca^{2+}]$ up to 10–15 μM (ref. 32)
Mitochondrial matrix	mtAEQmut	The mutated version of mtAEQwt. Because of the cooperativity between the three Ca^{2+}-binding sites of aequorin, the point mutation (Asp119Ala)[13] that affects the second EF-hand domain, produces a mutated aequorin, which can be used to measure $[Ca^{2+}]$ in the range of 10–500 μM (ref. 33)
	mtAEQmut28,119	Double-mutated form (Asp119Ala and Asn28Leu) of mtAEQwt, which can be used to measure $[Ca^{2+}]$ in the millimolar range for long periods of time, without problems derived from aequorin consumption[14]
Mitochondrial intermembrane space	MimsAEQ	HA1-tagged aequorin is fused (sequence in frame) with glycerol phosphate dehydrogenase, an integral protein of the inner mitochondrial membrane, with a large C-terminal tail protruding on the outer side of the membrane, i.e., in the mitochondrial intermembrane space[34]
Plasma membrane	pmAEQ	The targeting of aequorin to the subplasmalemmal space was based on the construction of a fusion protein including the HA1-tagged aequorin and SNAP-25, a protein that is synthesized on free ribosomes and recruited to the inner surface of the plasma membrane after the palmitoylation of specific cysteine residues[35]
Endoplasmic reticulum	erAEQmut	The encoded polypeptide includes the leader sequence (L), the VDJ and CH1 domains of an Igg2b heavy chain (HC) and the HA1-tagged aequorin at the C-terminus. In this chimera, retention in the ER depends on the presence of the CH1 domain at the N terminus of aequorin. This domain is known to interact with the luminal ER protein BiP, thus causing the retention of the Igg2b HC in the lumen. In the absence of the immunoglobulin light chain, the polypeptide is retained in this compartment[36]
Sarcoplasmic reticulum	srAEQmut	Calsequestrin (CSQ), a resident protein of the sarcoplasmic reticulum, is fused to HA1-tagged aequorin. This chimera is used to measure $[Ca^{2+}]$ in the sarcoplasmic reticulum, the specialized muscle compartment involved in the regulation of Ca^{2+} homeostasis[37]
Golgi apparatus	goAEQmut	Fusion of the HA1-tagged aequorin and the transmembrane portion of sialyltransferase, a resident protein of the Golgi lumen[18]
Secretory vescicles	vampAEQmut	Mutated AEQ (AEQmut; Asp119Ala) is fused to the vesicle-associated membrane protein (vamp)2/synaptobrevin (a vesicle-specific SNARE with a single transmembrane-spanning region) allowing intravesicular $[Ca^{2+}]$ to be monitored[38]
Peroxisomes	peroxAEQ	HA1-tagged wild-type and Asp119Ala mutant aequorins were fused with a peroxisomal targeting sequence[17]

Major advantages of using aequorin

- **Selective intracellular distribution.** The main reason for the renewed interest in using aequorin is that, being a protein, it can be engineered to induce its specific localization to a cell region of interest[10,11]. Although wild-type aequorin is exclusively cytosolic (cytAEQ), the addition of specific targeting sequences permits selective localization of the photoprotein, resulting in recombinant aequorin chimeras for different intracellular compartments: nucleus (nuAEQ), mitochondria (mtAEQ and mimsAEQ), subplasma-membrane cytosol (pmAEQ), endoplasmic/sarcoplasmic reticulum (erAEQ/srAEQ), Golgi apparatus (goAEQ), secretory vesicles (vampAEQ) and peroxisomes (peroxAEQ). Details of the construction of the different chimeras are described in **Table 2**. In all cases, the correct intracellular localization of the different probe has been verified through immunofluorescence localization with antibodies against the aequorin protein or the epitope added to its sequence and using specific markers for the different intracellular compartments.

Box 1 | Extending dynamic range of aequorin with the use of new coelenterazine derivatives

In addition to native coelenterazine, other derivates have been produced: these coelenterazines are designed as *cp*, *f*, *h*, *hcp*, *n* and *i*. These variants conserve the characteristics required to be used to reconstitute the aequorin complex both *in vivo* and *in vitro*, and the reconstituted photoprotein exhibits markedly changed luminescence quantum yield and response speed to Ca^{2+} binding. Coelenterazine *n* and *i* display reduced light emission, as low as 3% of the native coelenterazine, and a slower response to Ca^{2+} binding[12].

Development of coelenterazine with reduced light emission was necessary for proper increase of the dynamic range in the plethora of aequorin-based Ca^{2+} assays. Different intracellular compartments display $[Ca^{2+}]$ above the dynamic range of native aequorin or even the low-affinity aequorin mutant Asp119Ala. Introduction of coelenterazine *n* and *i* allowed the reduction of aequorin affinity, to an affinity that was low enough to perform measurements in very high $[Ca^{2+}]$, such as in the ER, Golgi apparatus or mitochondria (**Table 2**).

On the opposite extreme, it has been demonstrated that aequorins reconstituted with coelenterazine derivates *cp*, *f*, *hcp* or *h* display relative intensities that are higher (10–20 times more potent), as compared with apoaequorin containing the native coelenterazine. Of crucial interest is the *h* variant, as it has been shown that aequorin prepared with this derivate was more sensitive (compared with aequorin reconstituted with native coelenterazine), thus delivering a new tool for measuring small changes in Ca^{2+} concentrations[39].

Furthermore, the increase of emitted light made the new coelenterazine *h* suitable for assays that suffer in sensitivity or that require small populations of cells, thus allowing the development of a series of assays for luminescence high-throughput screening.

It should be taken into consideration that until now no calibration curve has been developed for the combined use of native aequorin with the high-sensitivity coelenterazine derivatives (*cp*, *f*, *hcp* and *h*); therefore, absolute $[Ca^{2+}]$ (with the mean of molar values) is impossible to determine and it is possible to describe only relative concentrations (often expressed in arbitrary luminescence units).

- **Wide dynamic range.** Native aequorin and its mutants (**Table 2**), in association with different prosthetic groups, are well suited for measuring $[Ca^{2+}]$ from as low as 0.1 μM and up into the millimolar range. Numerous chemical modifications of the prosthetic group have been made, which modify, in different ways, the Ca-triggered reaction of the photoprotein[12]. Among these, particularly useful have been those that result in lower light emission at high $[Ca^{2+}]$ (especially the *n* form; see **Box 1**). In parallel, single point mutations have been inserted in aequorin cDNAs to reduce its affinity and to allow measurements at higher Ca^{2+} concentrations (**Table 2**)[13,14]. Combinations of mutant aequorins and modified coelenterazine have been used to further increase the dynamic range, allowing measurements in compartments with low $[Ca^{2+}]$ (i.e., cytoplasm or mitochondria in certain cell types[15,16]), high $[Ca^{2+}]$ (i.e., peroxisomes or mitochondria of other cell types[14,17]) or very high $[Ca^{2+}]$ (i.e., Golgi or ER[14,18,19]).
- **High signal-to-noise ratio.** Mammalian cells are not endowed with chemiluminescent proteins, and thus the background of aequorin measurement is very low. Moreover, the steep relationship between the increases in light emission and Ca^{2+} concentration accounts for the very large luminescence peaks observed upon stimulation of cells (>1,000–10,000-fold over background can be detected with cytosolic and mitochondrial aequorin, respectively). Because of the excellent signal-to-noise ratio, reliable aequorin measurements can be obtained with moderate levels of expression of the probe.
- **Low Ca^{2+}-buffering effect.** Aequorin displays an extremely low buffering effect on intracellular Ca^{2+}, negligible if compared with that of fluorescent Ca^{2+} indicators[20]. Thus, although in principle, all Ca^{2+} probes perturb Ca^{2+} homeostasis because they bind Ca^{2+} and thus act as Ca^{2+} buffers, this effect is much less relevant for aequorin than for trappable fluorescent dyes. As an example, Fura-2 measurements in the presence or absence of aequorin display the same cytoplasmic Ca^{2+} levels; on the contrary, aequorin measurements show a strong reduction in cytoplasmic $[Ca^{2+}]$ if Fura-2 is added compared with vehicle[15].

Major disadvantages of using aequorin

- **Low light emission by the photoprotein.** In contrast to fluorescent dyes (where up to 10^4 photons can be emitted by a single molecule before photobleaching occurs), only one photon can be emitted by an aequorin molecule. This means that only a small fraction of the total aequorin pool emits its photon every second: out of the 10^4–10^5 molecules per cell of a typical aequorin transfection, light emission will vary from 0 to 1,000 photons at most.
- **Overestimation of the average rise in cells (or compartments) with inhomogeneous behavior.** Because of the steep Ca^{2+} response curve of aequorin, if the probe is distributed between a high-Ca^{2+} and a low-Ca^{2+} domain, the former will undergo a much larger discharge. The total signal will be calibrated as 'average' $[Ca^{2+}]$ increase, which will be severely biased by the region with high Ca^{2+}.
- **Cells must be amenable to transfection.** The obvious requirement of this approach is that the cell type being studied must be amenable to transfection (**Box 2**).

Experimental design

The proposed protocol allows measurement of Ca^{2+} signaling in a wide variety of cell types in different subcellular compartments. Owing to the variety of $[Ca^{2+}]$ in different subcellular compartments, different combinations of aequorin and coelenterazine, with modified sensitivity, have been developed.

- A general overview of the protocol could be summarized in a few points (represented in **Fig. 1**): cell transfection, aequorin reconstitution, light recording during Ca^{2+} homeostasis perturbation and light recording during cell lysis induction (with consequent measurement of the total aequorin amount).

The first and fundamental issue to be defined is the choice of readout. Different aequorin chimeras have been proposed, depending on the intracellular localization of the $[Ca^{2+}]$ to be investigated (**Table 2**). An appropriate aequorin-coelenterazine combination is also required. A general guideline could be as follows: native aequorin with native coelenterazine for $[Ca^{2+}]$ from 0.1 μM to 10 μM; aequorin(Asp119Ala) with native coelenterazine

Box 2 | Cell preparation and transfection ● TIMING 3 h

We have used a wide variety of cell types (e.g., HeLa, HEK 293T, CHO, COS-7, SH-SY5Y, A7r5, PC12, MEF as well as primary cultures of neurons and skeletal muscle myotubes), with good results in terms of aequorin expression using the appropriate transfection methods. It is not the purpose of this paper to describe all possible transfection procedures, the choice of which depends mainly on the cell type being studied. The Ca^{2+} phosphate procedure is by far the simplest and least expensive and has been used successfully to transfect a number of cell lines, including HeLa, HEK 293T, L929, L, COS-7, A7r5, PC12 cells and myotubes. Other transfection procedures have also been employed, such as liposomes, polyethylenimine and electroporation; viral constructs for some aequorins are also available[40–42].

1. *Cell growth*. Seed cells onto circular glass coverslips (diameter, 13 mm) for single-sample luminescence measurements. For plate-reader analysis, seed cells directly into the preferred multiwell type; avoid the use of coverslips. Allow cells to grow until ~50% confluence; optimize plating densities for each specific cell line.

■ **PAUSE POINT** After seeding cells, wait at least 24 h.

2. *Transfection*. When the cells have reached 40–60% confluence, transfect them with 1–2 μg of vector encoding aequorin per coverslip, depending on the desired transfection method (usually 1 μg cm^{-2} for liposomes or polyethylenimine and 2 μg cm^{-2} for Ca^{2+} phosphate).

Choose the appropriate transfection reagent and optimized protocol for the particular cell line being used. Alternatively, create a stable cell line using standard protocols[15].

■ **PAUSE POINT** After transfection, wait 36–48 h.

for $[Ca^{2+}]$ from 10 μM to 100 μM; and aequorin(Asp119Ala) with coelenterazine *n* for $[Ca^{2+}]$ from 100 μM to 1,000 μM. Once the proper combination has been chosen, the researcher has to set up its proper expression protocol. Several transfection methods have been successfully used to drive expression of aequorin.

The central event in the measurement protocol is the perturbation of Ca^{2+} resting conditions, with the generation of changes in intracellular $[Ca^{2+}]$. The perturbing agent has to be chosen considering two different concepts: first, the source of Ca^{2+} (intracellular stores such as the ER or the extracellular medium); and second, the cell type, because receptors capable of activating Ca^{2+} waves could differ in type and expression, depending on the cell model. Examples of the most commonly used Ca^{2+}-perturbing agents used for aequorin assays are listed in **Table 3**.

To optimize the assay, several controls should be performed. A positive control for probe expression is provided by cell lysis, which is done as the final phase of each procedure. Light emission induced upon cell lysis is directly proportional to the whole amount of aequorin expression. A useful negative control is to perform the preferred reconstitution and recording

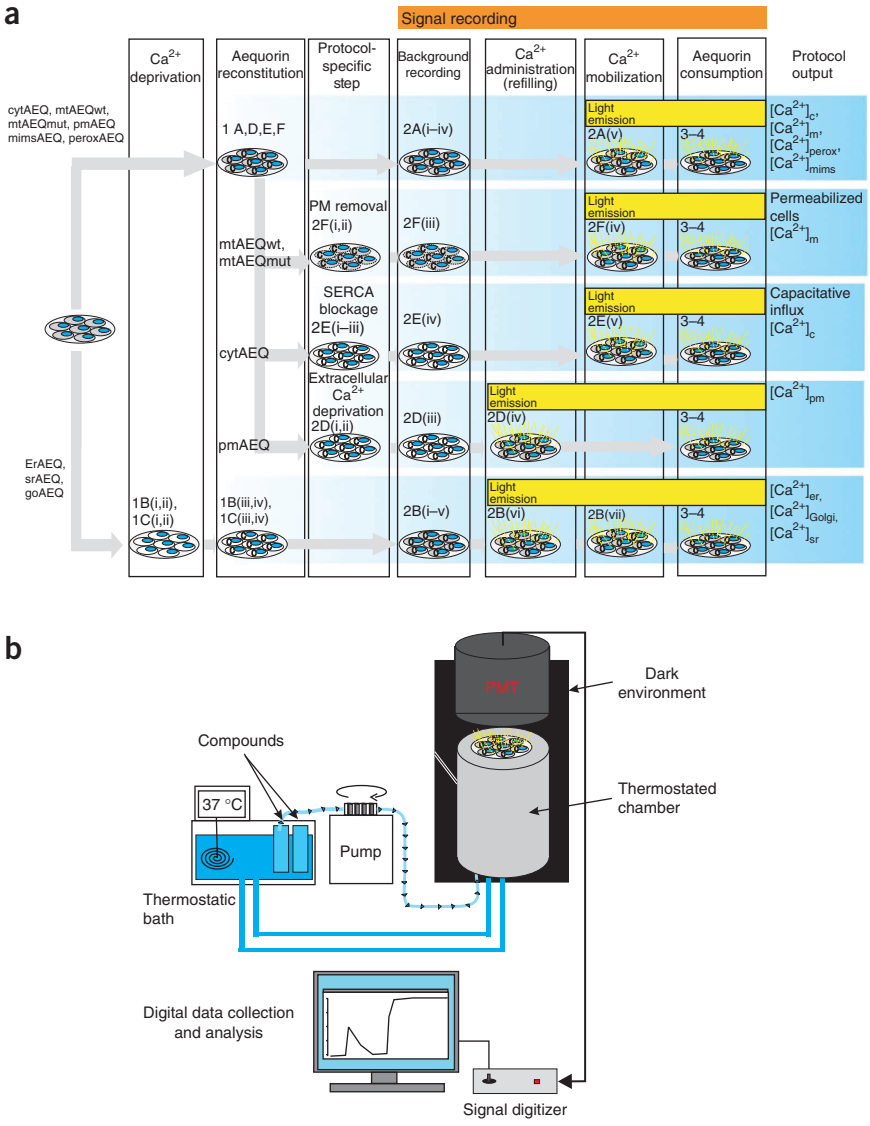

Figure 1 | Setup of an aequorin experiment. (**a**) Diagram of principal events necessary to perform a Ca^{2+} signaling event with aequorin-expressing cells. Forks indicate different actions to be performed, depending on the aequorin type. Steps of the protocol are indicated. Cytoplasmic $[Ca^{2+}] = [Ca^{2+}]_c$; mitochondrial matrix $[Ca^{2+}] = [Ca^{2+}]_m$; peroxisomal $[Ca^{2+}] = [Ca^{2+}]_p$; mitochondrial intermembrane space $[Ca^{2+}] = [Ca^{2+}]_{mims}$; subplasmalemmal $[Ca^{2+}] = [Ca^{2+}]_{pm}$; endoplasmic reticulum luminal $[Ca^{2+}] = [Ca^{2+}]_{er}$; Golgi lumen $[Ca^{2+}] = [Ca^{2+}]_{Golgi}$; sarcoplasmic reticulum luminal $[Ca^{2+}] = [Ca^{2+}]_{sr}$. (**b**) Schematic of the typical organization of a custom-made aequorin recording system for whole-cell population measurements.

TABLE 3 | Examples of Ca^{2+}-perturbing agents.

Agent	Concentration	Pathway	Sensitive cell type
ATP	100 μM	GPCR activation, IP3-dependent ER $[Ca^{2+}]$ ($[Ca^{2+}]_{er}$) release through the IP3R	HeLa, HEK, mouse embryonic fibroblasts, COS7, SKBR3, H1299, A549, DU145, MCF7, NB4, MDA468, IMR90, XTC1, RH36
Bradykinin	100 μM	GPCR activation, IP3-dependent $[Ca^{2+}]_{er}$ release	Human fibroblasts
Caffeine	20 mM	$[Ca^{2+}]_{er}$ release through the Ryanodine receptor	H2C9
Carbachol	500 μM	GPCR activation, IP3-dependent $[Ca^{2+}]_{er}$ release	HCT116, SH SY-5Y, primary rat oligodendrocytes, primary rat or mouse astrocytes
Glutamate	500 μM	Synthetic	Primary rat or mouse neurons, primary rat oligodendrocytes, primary rat or mouse astrocytes
Histamine	100 μM	GPCR activation, IP3-dependent $[Ca^{2+}]_{er}$ release	HeLa, H1299, human fibroblasts
Kainate	100 μM	Modulators of neuronal NMDAR, AMPAR and VGCC	Primary rat oligodendrocytes, primary rat or mouse astrocytes
NMDA	200 μM	Modulators of neuronal NMDAR, AMPAR and VGCC	Primary rat oligodendrocytes, primary rat or mouse astrocytes
Potassium	50 mM	Modulator of VGCC	All excitable cells

procedure in nontransfected cells. Further, the buffering effect of aequorin could be easily verified by performing Fura-2 experiments in cells expressing reconstituted aequorin and in cells expressing empty vector as described by Brini *et al.*[15].

Finally, if new compounds need to be used, it is important to verify whether the compounds themselves condition the aequorin activity. To check this aspect, we recommend the following control experiment. Reconstituted aequorin must be

Box 3 | Cell population analysis with plate reader ● TIMING 3–6 h

The low complexity of protocols for cytoplasmic and mitochondrial $[Ca^{2+}]$ and $[Ca^{2+}]$ measurements with aequorin allows easy automation of the process and development of assays for plate readers. This, in addition to development of high-sensitivity coelenterazines (**Box 1**), permits the development of high-throughput screening. One of these platforms (called Aequoscreen) is based on the expression of apoaequorin in cells that also express the superfamily of G protein–coupled receptors. This cellular aequorin–based functional assay was adapted for high-throughput screening, and it has been reported to have good reproducibility, high signal and high throughput (100,000 compounds per day)[43]. Another platform based on aequorin is the LumiLux (PerkinElmer), which in 2008 was also validated for calcium measurements assays by high-throughput screening. This instrument is reported to allow ultrahigh-throughput cellular imaging (up to 1,536-well microplates)[44].

The final goal of these 'aequorin-based luminescence platforms' is to generate superior-quality screening data of calcium measurements, with automated image acquisition and data analysis, allowing explorative screening and the identification of new drugs by screening tens of thousands of molecules. Beyond drug discovery, the high-content technology could provide useful tools for learning where and when a subset of proteins act or for identifying genes involved in several mechanisms, for example, cell division, cancer and neurodegeneration.

Plate readers based on the aequorin assay could in any case be used in the range of medium to low throughput for exploring Ca^{2+} signals in suspended cell populations, using wild-type coelenterazine.

Here we describe a quick and easy protocol for an aequorin assay with plate readers.

Additional materials

Ca^{2+}-mobilizing solution, 3× (3× CMS). Dissolve the Ca^{2+}-perturbing agent in modified KRB; different cell types require different agents. The most common agents are listed in **Table 3**. Freshly prepare the solution, and do not store it.

Lysis solution, 4×, 0.4% Triton X-100. Add 0.4 ml of Triton X-100 and 1 ml of 1 M $CaCl_2$ to 100 ml of Milli-Q water. This solution can be stored at 4 °C for 1 month.

Luminescence plate reader. Different plate readers are available on the market. These are usually designed to allow maximum sensitivity. Nonetheless, the reading protocol should be optimized for aequorin[45]. In particular, no emission filters should be placed between the sample and the photomultiplier. Reading has to occur from the bottom of the plate, to allow reading during injection of stimuli. Liquid injection in the well has to be optimized to allow both a rapid diffusion of the stimulus, while avoiding cell detachment (two injectors are required; examples include VITOR3 or Microbeta Jet from PerkinElmer). Readers with multiple reading positions are available and should be chosen to avoid having cell samples remain in a non-optimized environment for too long; alternatively, readers with a temperature-controlled environment are also available.

(continued)

Box 3 | (continued)

1. Remove cells from incubator and rinse them twice with modified KRB.
2. After rinsing, place cells in 500 µl of modified KRB per well (24-well plates) or 200 µl of modified KRB per well (96-well plates).
3. Add 5 µM 100× native coelenterazine solution, and incubate the cells for 1.5–2 h.

▲ **CRITICAL STEP** Coelenterazine is very sensitive to light when not bound to aequorin. Avoid light exposure by handling it in a low-light environment and wrapping each aliquot in aluminum foil.

? TROUBLESHOOTING

4. Remove cells from incubator, and rinse them twice with modified KRB. Place the plate in a luminescence-sensitive plate reader equipped with two different liquid injectors.

▲ **CRITICAL STEP** Wash the cells carefully in order to not detach the cells.

5. Start acquisition of Ca^{2+} kinetics. Set up reading time not higher than 1,000 ms, and record signal for at least 5 s to measure baseline.
6. Add 3× CMS to activate Ca^{2+} pathways. Set administration of 250 ml of 3× CMS for 24-well plates and 100 ml for 96-well plates. Record for at least 60 s.

? TROUBLESHOOTING

7. Add 4× lysis solution. Set administration of 250 ml of 4× lysis solution for 24-well plates and 100 ml for 96-well plates. Record for at least 60 s.

? TROUBLESHOOTING

8. Repeat from step 5 for each well (depending on the number of detectors of the plate reader).
9. Repeat from step 3 for each plate.
10. Export results in an Excel-compatible format for offline calibration of signal.

extracted from cells in the absence of Ca^{2+} and in the presence or absence of the compound to be tested. Finally, Ca^{2+} must be added, and the luminescence must be monitored. If the same amount of light is emitted in both conditions, then the compound should not affect aequorin activity and could be safely used.

For adherent cells, we provide several protocols for aequorin measurements through the use of perfusion chamber–based single-tube luminometers. Furthermore, we provide a protocol based on automated plate reader luminometers, which allows for measurements of suspended cells, also in high-throughput assays (**Box 3** and **Fig. 2**). Given the choice to use plate readers, a few concepts must be considered during the experimental setup. For plate reader–based assays, we recommend avoiding the use of erAEQ, srAEQ and goAEQ because of the higher complexity of the measurement protocol. Conversely, cytoplasmic or mitochondrial aequorin displays a substantially less complexity, making them suitable for automation. In our experience, plate-reader luminometers display a reduced sensitivity when compared with single-tube photomultipliers, especially when 96-well plates are used. In this case, if the cell system chosen has a low-expression yield, we suggest the use of high-sensitivity coelenterazine derivatives (**Box 1**) rather than wild-type coelenterazine. It should be noted, however, that for most of these coelenterazine derivatives a calibration curve is still not available (**Box 1**).

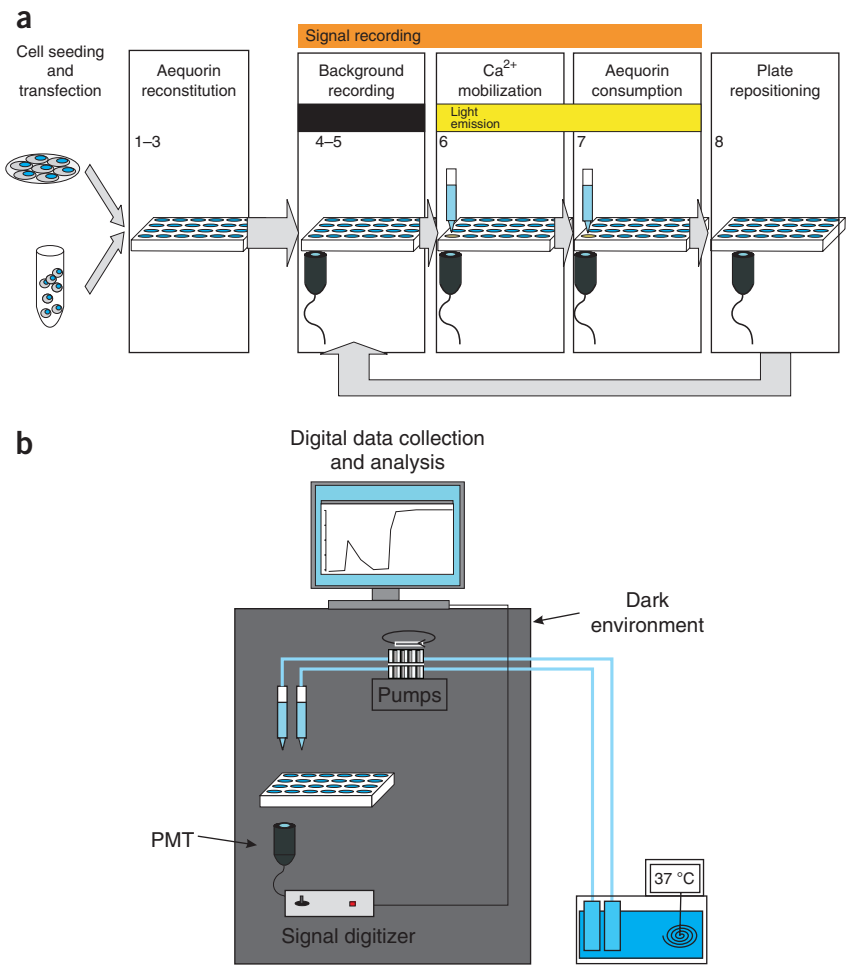

Figure 2 | Setup of the aequorin plate-reader assay. (**a**) Diagram of principal events necessary to perform a Ca^{2+}-signaling event with aequorin-expressing cells. Numerals refer to steps in the protocol. (**b**) Schematic of the typical organization of a plate reader for the aequorin assay.

MATERIALS

REAGENTS

- Coelenterazine, native or *n* (Sigma-Aldrich)
- NaCl (Sigma-Aldrich)
- Potassium chloride (Sigma-Aldrich)
- Potassium phosphate monobasic (Sigma-Aldrich)
- Magnesium sulfate (Sigma-Aldrich)
- $CaCl_2$, 1 M (Fluka)
- Glucose (Sigma-Aldrich)
- Magnesium chloride hexahydrate (Sigma-Aldrich)
- HEPES (Sigma-Aldrich)
- Ionomycin calcium salt (Sigma-Aldrich)
- EGTA (Sigma-Aldrich)
- *N*-(hydroxyethyl)-ethylenediaminetriacetic acid (HEDTA; Sigma-Aldrich)
- *tert*-Butylhydroquinone (tBHQ; Sigma-Aldrich)
- Caffeine (Sigma-Aldrich)
- BSA (Sigma-Aldrich)
- Triton X-100 (Sigma-Aldrich)
- Digitonin (Sigma-Aldrich)
- Ca^{2+}-perturbing agents (**Table 3**)
- Milli-Q water (Millipore)
- NaOH (Sigma-Aldrich)
- DMSO (Sigma-Aldrich)
- HCl (Sigma-Aldrich)
- KH_2PO_4 (Sigma-Aldrich)
- HCl (Sigma-Aldrich)
- Sodium succinate (Sigma-Aldrich)
- KOH (Sigma-Aldrich)

EQUIPMENT

- Parafilm
- Microcentrifuge tubes, 0.5-ml
- Glass coverslip, 13-mm diameter, thickness between 0.16–0.19 mm (Thermo Scientific)
- Custom-made luminescence reader (aequorinometer) or any analog single photomultiplier tube (PMT)
- Peristaltic pump
- Temperature-controlled bath
- Windows-based PC (a custom-made setup could be implemented also on Mac or Linux-based workstations)
- Photon-counting head (Hamamatsu) or equivalent from a different company (only for a custom-made luminescence reader)
- Digitizer counting unit (Hamamatsu) or equivalent from a different company (only for a custom-made luminescence reader)

REAGENT SETUP

Krebs Ringer buffer (KRB) (135 mM NaCl, 5 mM KCl, 0.4 mM KH_2PO_4, 1 mM $MgSO_4$, 20 mM HEPES and 5.5 mM glucose (pH 7.4)) Prepare KRB by dissolving 7.89 g of NaCl, 0.373 g of KCl, 0.054 g of KH_2PO_4, 0.247 g of $MgSO_4$, 4.76 g of HEPES and 1 g of glucose in less than 1,000 ml of Milli-Q water, adjust the pH of the solution to 7.4 with NaOH and then make up the final volume to 1 liter. This solution can be stored at 4 °C for 3 d.

Modified KRB Prepare modified KRB by adding 0.5 ml of 1 M $CaCl_2$ to 500 ml of KRB. This solution can be stored at 4 °C for 3 d. ▲ CRITICAL This solution should be prepared in a polypropylene cylinder; glass containers could bind Ca^{2+}, giving rise to experimental imprecisions.

Coelenterazine solution, 100× Although coelenterazine is relatively stable when stored undissolved at −80 °C, it is extremely labile once dissolved. Coelenterazine is usually dissolved at 0.5 mM in pure methanol as a 100× stock solution kept at −20 °C for short periods (few days) or −80 °C for longer periods. We recommend that the supply of coelenterazine solution be split into small aliquots (~50 µl) and kept protected from light. ▲ CRITICAL Methanol evaporates extremely fast, so we recommend sealing the aliquots with Parafilm and keeping them on ice during handling.

Ca^{2+}-mobilizing solution (CMS) Dissolve the Ca^{2+}-perturbing agent in modified KRB; different cell types require different agents. The most common agents are listed in **Table 3**. Freshly prepare the CMS, and do not store it.

Ionomycin, 1 mM stock solution Prepare 1 mM ionomycin stock solution by dissolving 1 mg of ionomycin in 1.34 ml of DMSO and divide the stock solution into aliquots. This solution can be stored at −20 °C for 6 months. ▲ CRITICAL Avoid multiple freeze-thaw cycles.

tBHQ, 100 mM solution Prepare 100 mM tBHQ solution by dissolving 166 mg of tBHQ in 10 ml of DMSO, and divide the solution into aliquots. This solution can be stored at −20 °C for 6 months.

Lysis solution, 0.1% Triton X-100 Prepare lysis solution by adding 0.1 ml of Triton X-100 and 1 ml of 1 M $CaCl_2$ to 100 ml of Milli-Q water. This solution can be stored at 4 °C for up to 1 month.

EGTA, 0.5 M stock solution Prepare EGTA stock solution by dissolving 19.01 g of EGTA in 100 ml of Milli-Q water. Use NaOH to help dissolving the solution, and then adjust the pH of the solution with HCl to 7.4. This solution can be stored at room temperature (22–30 °C) for 2 months.

Ca-free buffer (CFB) Prepare Ca-free buffer by adding 0.06 ml of EGTA stock solution to 300 ml of KRB (required only for erAEQ, goAEQ, srAEQ and pmAEQ). Freshly prepare the buffer, and do not store it.

BSA, 2% (wt/vol) Prepare 2% (wt/vol) BSA by dissolving 4 g of BSA in 200 ml of CFB. Freshly prepare BSA, and do not store it.

Intracellular buffer stock solution, (10× IB) (1.3 M KCl, 100 mM NaCl, 5 mM KH_2PO_4, 10 mM $MgSO_4$, 50 mM sodium succinate and 200 mM HEPES) Combine 96.87 g of KCl, 5.84 g of NaCl, 0.68 g of KH_2PO_4, 2.46 g of $MgSO_4$, 5.9 g of succinic acid and 47.66 g of HEPES in 1,000 ml of Milli-Q water. Adjust the pH of the solution to 7.0 with KOH. This solution can be stored at 4 °C for up to 1 month.

KEGTA, 0.5 M stock solution Prepare 0.5 M KEGTA stock solution by dissolving 19.01 g of EGTA in 100 ml of Milli-Q water. Use KOH to help dissolving the EGTA, and then adjust the pH of the solution with HCl to 7.0. This solution can be stored at room temperature for 2 months.

HEDTA, 0.5 M stock solution Prepare 0.5 M HEDTA stock solution by dissolving 73.06 g of EDTA in 500 ml of Milli-Q water, and then adjust the pH of the solution to 8 with HCl. This solution can be stored at room temperature for up to 2 months.

Modified IB, 130 mM KCl, 10 mM NaCl, 0.5 mM KH_2PO_4, 1 mM $MgSO_4$, 5 mM sodium succinate, 20 mM HEPES, 2 mM EGTA, 2 mM EDTA, 5.5 mM glucose, 1 mM pyruvic acid and 3 mM $MgCl_2$ Prepare modified IB by dissolving 1 g of glucose, 0.0886 g of pyruvic acid and 0.609 g of $MgCl_2$ in 100 ml of 10× IB. Add 4 ml of 0.5 M KEGTA and 4 ml of 0.5 M EDTA to it. Adjust the volume to 800 ml with Milli-Q water and adjust solution pH to 7 with KOH. Finally, adjust the final volume to 1,000 ml with Milli-Q water. Freshly prepare the solution, and do not store it.

ATP intracellular buffer (AIB) Prepare AIB by dissolving 0.275 g of ATP in 500 ml of modified IB. Adjust the pH of the buffer to 7. Freshly prepare the buffer, and do not store it. ▲ CRITICAL After the addition of $CaCl_2$, check the pH (should be pH 7.0). ▲ CRITICAL Store this solution on ice and use only a small amount (~30 ml) at 37 °C during the experiment, to avoid ATP degradation.

Permeabilization IB, 25 µM digitonin Prepare permeabilization IB by dissolving 3 mg of digitonin in 100 ml of IB. Freshly prepare the solution, and do not store it.

Cell preparation and transfection See **Box 2** for details on preparation of cells and transfection of vectors encoding aequorin and aequorin mutants.

EQUIPMENT SETUP

Luminescence reader: for cell population analysis See **Box 4** for details on luminescence reader (aequorinometer).

Box 4 | Aequorinometer description

Photons emitted during the experimental procedure can be recorded and analyzed using relatively inexpensive equipment; we refer interested readers to refs. 9 and 46–49 for excellent detailed technical descriptions.

In brief, in this system the perfusion chamber, on top of a hollow cylinder, temperature-controlled by a water jacket, is continuously perfused with buffer via a peristaltic pump. The coverslip with cells is placed a few millimeters from the surface of a low-noise PMT. The photon counts are then transferred to a computer for further analysis (**Fig. 1**).

Components list:

- Photon-counting head H7360-01 (Hamamatsu Photonics)
- Perfusion chamber (Elettrofor)
- Elettrofor Termostitc group GT-80/P (Elettrofor)
- Photon counter C8855-01 (Hamamatsu Photonics)
- Peristaltic pump

The aequorinometer is composed of an upper part containing a low-noise photomultiplier inserted into a dark box housing a manual shutter (to protect it from light exposure) and a removable temperature-controlled chamber to hold the sample. During aequorin experiments, the shutter is opened, and the perfusion chamber (13 mm diameter, 2 mm height, volume 265 µl) is placed in close proximity (<3 mm) of the photomultiplier. The perfusion chamber can house a 13-mm coverslip with adherent cells and is sealed on the top with a coverslip using a thin layer of silicon. Cells are continuously perfused with temperature-controlled medium (water bath, GT-80/P) at 37 °C. A peristaltic pump allows liquid perfusion. The output of the amplifier discriminators is captured by the photon counter C8855-01 connected via a USB cable to a workstation and recorded by the sample software (Hamamatsu Photonics) for storage and further analysis.

PROCEDURE

Reconstitution ● TIMING ~60–120 min

1| Depending on intracellular localization, aequorin will be exposed to different intracellular [Ca^{2+}]. As the functional aequorin-coelenterazine probe is irreversibly destroyed by Ca^{2+} ions, as reported before, different reconstitution procedures must be followed depending on the aequorin chimera used and the type of experiment (see **Fig. 1a** to decide which option to use).

In particular, when the recombinant protein is located in a compartment that has high [Ca^{2+}], reconstitution with coelenterazine is highly inefficient because of competition between consumption and reconstitution. To obtain reliable quantitative estimates of [Ca^{2+}] in the lumen of organelles with high [Ca^{2+}], the [Ca^{2+}] needs to be decreased during both the reconstitution of aequorin with coelenterazine and the subsequent initial phase of perfusion in the luminometer (**Table 4**).

(A) Measurement of [Ca^{2+}] in low-[Ca^{2+}] compartments (cytAEQ, nuAEQ, mtAEQ, mtAEQmut, mimsAEQ and peroxAEQ)

(i) Remove cells from an incubator, and rinse them twice with 1 ml of modified KRB to remove residual medium.

(ii) Add 0.2 ml of modified KRB to the coverslip and 2 µl of 100× native coelenterazine.
▲ **CRITICAL STEP** Coelenterazine is very sensitive to light when not bound to aequorin. Avoid light exposure by handling it in a low-light environment and wrapping each aliquot in aluminum foil.

(iii) Incubate the cells for 1.5–2 h at 37 °C in a 5% CO_2 atmosphere for complete equilibration of coelenterazine.
? TROUBLESHOOTING

(B) Measurement of [Ca^{2+}] in high-[Ca^{2+}] compartments: intraluminal [Ca^{2+}], using erAEQ or goAEQ

(i) Wash the cells with KRB supplemented with 200 µl of 600 µM EGTA to remove extracellular Ca^{2+}.

(ii) Remove the buffer, add 0.2 ml of CFB supplemented with 1 µl of 1 mM ionomycin stock solution, and wait for 2 min for complete equilibration of ionomycin and depletion of intraluminal Ca^{2+}.

(iii) Add 2 µl of 100× coelenterazine *n* solution.
▲ **CRITICAL STEP** Coelenterazine is very sensitive to light when not bound to aequorin. Avoid light exposure by handling it in a low-light environment and wrapping each aliquot in aluminum foil.

(iv) Incubate the cells at 4 °C for 45 min for complete equilibration of coelenterazine.

TABLE 4 | Comparison of Ca^{2+} deprivation methods depending on investigated readout.

Assay	Ca^{2+} deprivation method
Capacitative influx	Extracellular Ca^{2+}-free buffer supplemented with 100 µM EGTA and 10 µM tBHQ
$[Ca^{2+}]_{pm}$	Extracellular Ca^{2+}-free buffer supplemented with 100 µM EGTA
$[Ca^{2+}]_{ER}$, $[Ca^{2+}]_{Golgi}$	Extracellular Ca^{2+}-free buffer supplemented with 100 µM EGTA, 1 mM ionomycin
$[Ca^{2+}]_{sr}$	Extracellular buffer supplemented with 3 mM EGTA, 10 µM caffeine and 30 µM tBHQ

(v) After incubation, wash the cells three times with 1 ml of 2% BSA to remove excess ionomycin.
(vi) Maintain the cells in CFB, and transfer them to the perfusion chamber.
? TROUBLESHOOTING

(C) Measurement of [Ca^{2+}] in high-[Ca^{2+}] compartments: intraluminal [Ca^{2+}], using srAEQ

(i) Wash the myotubes for 2 min with 1 ml of KRB containing 3 mM EGTA, 10 mM caffeine and 30 μM tBHQ, to allow depletion of intraluminal Ca^{2+}.
(ii) Remove the buffer, and add 0.2 ml of CFB.
(iii) Add 2 μl of 100× coelenterazine *n* solution.
▲ CRITICAL STEP Coelenterazine is very sensitive to light when not bound to aequorin. Avoid light exposure by handling it in a low-light environment and wrapping each aliquot in aluminum foil.
(iv) Incubate the cells at 25 °C for 1 h for complete equilibration of coelenterazine.
(v) Wash the cell extensively (three times) with 2% BSA to remove excess tBHQ.
(vi) Maintain the cells in CFB, and transfer them to the perfusion chamber.
? TROUBLESHOOTING

(D) Measurement of subplasmalemmal [Ca^{2+}], using pmAEQ

(i) Perform the steps as described in Step 1A.

(E) Measurements of capacitative Ca^{2+} influx (cytAEQ and mtAEQ)

(i) Perform the steps as described in Step 1A.
(ii) Wash the cells with 1 ml of CFB.
(iii) Incubate the cells with 1 ml of CFB supplemented with the sarcoplasmic/endoplasmic Ca^{2+} ATPase (SERCA) blocker, tBHQ (10 μM), for 10 min.

(F) Mitochondrial [Ca^{2+}] measurements in permeabilized cells (mtAEQ and mtAEQmut)

(i) Perform the steps as described in Step 1A.

Signal recording ● TIMING ~3–10 min

! CAUTION Avoid exposing the PMT to a direct light source; keep it off or keep the shutter closed (if present) when not in active use.
▲ CRITICAL Before and after each experiment, the plastic tubes must be extensively washed with Milli-Q water. Ensure that both the tubes and the perfusion chamber contain appropriate buffer when the coverslip is placed inside.
▲ CRITICAL No air bubbles should be present in the perfusion chamber, and it must be carefully sealed to avoid buffer leakage.
▲ CRITICAL All the systems must be at 37 °C before starting the experiment to ensure proper calibration.
▲ CRITICAL After each experiment, remove the 13-mm coverslip from the chamber, reseal the chamber and wash the entire the system extensively by perfusing it with Milli-Q water, thus avoiding spurious signals in the subsequent experiment.
▲ CRITICAL Stop the perfusion before changing each medium to avoid generating air bubbles.

2| As in the reconstitution step, this phase must also be adapted to the aequorin chimera and the intracellular compartment being studied. Choose from options A to F (**Fig. 1a**).

(A) Measurement of [Ca^{2+}] in low-[Ca^{2+}] compartments (cytAEQ, nuAEQ, mtAEQ, mtAEQmut, mimsAEQ and peroxAEQ)

(i) Place the 13-mm coverslip inside the temperature-controlled (37 °C) perfusion chamber.
(ii) Start perfusing the cell monolayer with modified KRB (pH 7.4, at 37 °C) using a peristaltic pump (standard flow rate: 2.5 ml min^{-1}).
(iii) Continue to perfuse the cells constantly, place the chamber in the dark box in close proximity (2–3 mm) to the surface of the low-noise photomultiplier tube, and start recording.
(iv) To measure the resting Ca^{2+}, start recording the basal c.p.s. (counts per second) luminescence value for at least 30 s to obtain a stable signal, and then note the lower value as background.
? TROUBLESHOOTING
(v) To activate Ca^{2+} pathways, pause perfusion and change from modified KRB to CMS. Resume the perfusion and raise its rate from 2.5 to 5 ml min^{-1} to allow rapid equilibration of the compounds in the perfusion chamber. After addition of agonist, light emission (c.p.s. values) rapidly increases, reaches a peak and then starts to decline (at this point, lower the perfusion rate to the original value). In the case of mtAEQ or mtAEQmut, the c.p.s. value will return to almost basal values within 2 min, whereas with cytAEQ, nuAEQ and mimsAEQ there is a sustained increase of the c.p.s. value above normal basal levels throughout the stimulation period.
? TROUBLESHOOTING

(B) Measurement of [Ca^{2+}] in high-[Ca^{2+}] compartments: intraluminal [Ca^{2+}], using erAEQ, goAEQ

(i) Place the 13-mm coverslip inside the temperature-controlled (37 °C) perfusion chamber.

(ii) Start to perfuse the cells with CFB constantly, and place the chamber in the dark box in close proximity (2–3 mm) to the surface of the low-noise photomultiplier tube and start recording.

(iii) Perfuse the cells with CFB for 30 s to ensure depletion of intraluminal Ca^{2+}.

(iv) Afterward perfuse the cells with 2% BSA for 3 min to wash and eliminate excess ionomycin or tBHQ.

(v) Perfuse the cells with CFB for at least 30 s to obtain a stable signal, and note down the lower value as background.

? TROUBLESHOOTING

(vi) Reload the stored Ca^{2+} by perfusing cells with modified KRB. Ca^{2+} reloading will appear as a large peak. Steady state in intraluminal [Ca^{2+}] is usually reached around half of the decreasing phase of c.p.s. values.

▲ **CRITICAL STEP** The Ca^{2+}-rich buffer should be perfused slowly.

? TROUBLESHOOTING

(vii) To activate the Ca^{2+} pathways, pause perfusion and change from modified KRB to CMS. After addition of agonist, light emission (c.p.s. values) rapidly decreases.

▲ **CRITICAL STEP** Agonist perfusion must be carried out in a slow and regular manner, to maintain unaltered the different kinetics of Ca^{2+} release from Ca^{2+} stores.

? TROUBLESHOOTING

(C) Measurement of [Ca^{2+}] in high-[Ca^{2+}]compartments: intraluminal [Ca^{2+}], using srAEQ

(i) Perform the procedure as described in Step 2B.

(D) Measurement of subplasmalemmal [Ca^{2+}], using pmAEQ

(i) Place the 13-mm coverslip inside the temperature-controlled (37 °C) perfusion chamber.

(ii) Start to perfuse the cells with CFB, and place the chamber in the dark box in close proximity (2–3 mm) to the surface of the low-noise photomultiplier tube and start recording.

(iii) Perfuse with CFB for at least 60 s to obtain a stable signal, and note the lower value as background.

? TROUBLESHOOTING

(iv) Reload the stored Ca^{2+} by perfusing with modified KRB. This will cause light emission and consequent rapid increase in c.p.s. values. Once the peak is reached, it will start to decline.

▲ **CRITICAL STEP** Ca^{2+}-rich buffer should be perfused slowly.

? TROUBLESHOOTING

(E) Measurements of capacitative Ca^{2+} influx (cytAEQ)

(i) Place the 13-mm coverslip inside the temperature-controlled (37 °C) perfusion chamber.

(ii) Start to perfuse the cells with CFB, and place the chamber in the dark box in close proximity (2–3 mm) to the surface of the low-noise photomultiplier tube.

(iii) Perfuse with CFB for at least 60 s to obtain a stable signal, and note the lower value as background.

? TROUBLESHOOTING

(iv) To evoke capacitative Ca^{2+} entry, replace CFB with KRB supplemented with 2 mM $CaCl_2$, in the continuous presence of 30 μM tBHQ. This evokes a second, larger increase in $[Ca^{2+}]_c$. Follow the increase in c.p.s. value until a stable plateau value is reached and then the decline to background values (>100 s).

? TROUBLESHOOTING

(F) Mitochondrial [Ca^{2+}] measurements in permeabilized cells (mtAEQ, mtAEQmut)

(i) Place the 13-mm coverslip inside the temperature-controlled (37 °C) perfusion chamber.

(ii) Start the perfusion with modified IB for 60 s to allow complete equilibration of the new ion concentrations.

(iii) Change from modified IB to permeabilization IB, and continue perfusion for 60 s; this leads to the selective permeabilization of the plasma membrane and to the washout of cytosolic Ca^{2+}.

(iv) Change the medium to AIB, and perfuse the cells for 60 s. Note the lower c.p.s. value as background.

? TROUBLESHOOTING

(v) To trigger mitochondrial Ca^{2+} uptake, change the medium to AIB containing the desired free Ca^{2+} concentration ($[Ca^{2+}]_{free}$), calculated with the Chelator software package[21] (**Table 5**). Follow the increase in c.p.s. value until a stable plateau value is reached (>100 s).

? TROUBLESHOOTING

TABLE 5 | Final [Ca^{2+}] necessary to obtain the desired $[Ca^{2+}]_{free}$ in IB buffer containing Ca^{2+}-chelating compounds.

$[Ca^{2+}]_{total}$ (mM)	$[Ca^{2+}]_{free}$ (μM)
1.47	1
1.76	1.5
1.97	2
2.13	2.5
2.27	3
2.37	3.5
2.47	4

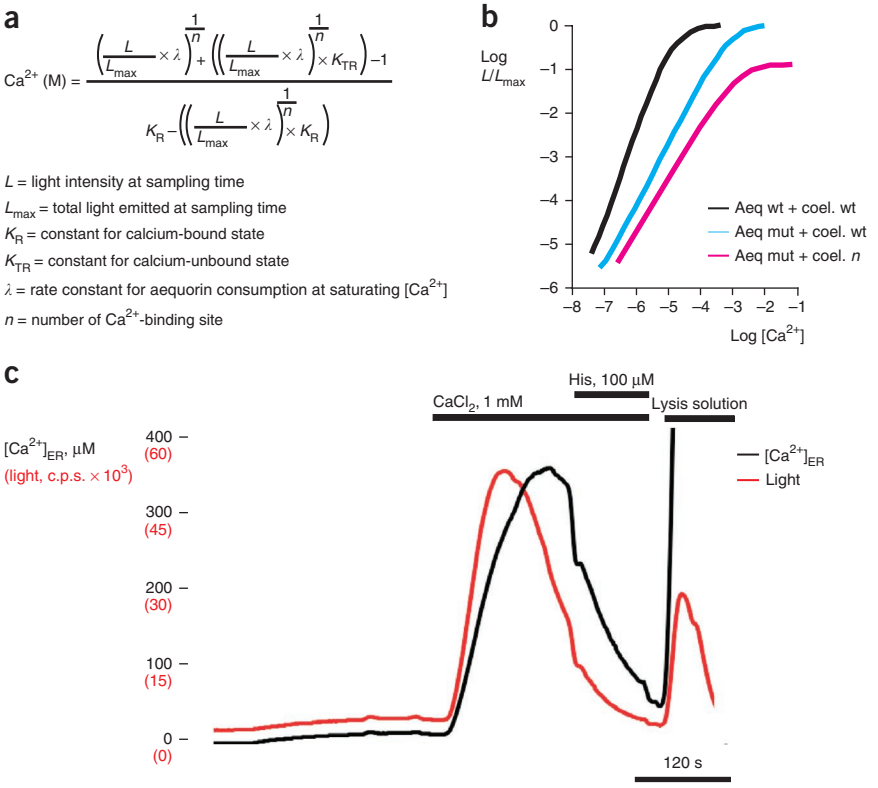

Figure 3 | Aequorin calibration into $[Ca^{2+}]$ values. (**a**) Equation for typical Ca^{2+} calibration starting from luminescence signal. (**b**) Calibration curves of aequorin setup described in this protocol: native aequorin (Aeq wt) reconstituted with wild-type coelenterazine (coel. wt), mutated aequorin (Aeq mut) reconstituted with wild-type coelenterazine wt and mutated aequorin reconstituted with coelenterazine *n* (coel. *n*). (**c**) Representative luminescence and Ca^{2+} kinetics of erAEQ in HeLa cells, displaying the marked change in curve shape induced by calibration.

Total aequorin consumption

● TIMING ~2 min

3| Wait until there is no further change in the c.p.s. value for ~20–30 s. Pause perfusion, and change the medium to lysis solution. Resume the perfusion. A large increase in luminescence (c.p.s. value) will be observed.

4| To accurately determine the total aequorin content, wait until the c.p.s. value returns to the basal level recorded at the beginning of the experiment; it might take up to 2 min, depending on transfection efficiency and cell number. Stop the analysis system and the perfusion.

? TROUBLESHOOTING

Signal calibration and data analysis ● TIMING ~5–10 min

5| Export results in a spreadsheet-compatible format for offline signal calibration.

6| Open exported files using a spreadsheet software (i.e., Microsoft Office Excel, Lotus SmartSuite Lotus 123, LibreOffice Calc, etc.), and arrange each recording in a single column.

7| Subtract the background value noted at the beginning of the signal-recording phase from all the values recorded.

8| Transform the background-corrected values into $[Ca^{2+}]$ using the equation shown in **Figure 3** and calibration constants listed in **Table 6**.

TABLE 6 | Aequorin calibration values.

Aequorin and oelenterazine	KR	KTR	*N*	Λ	Ref.
Wild type + native	7,230,000	120	2.99	1	15
Mutant Asp119Ala + native	22,770,000	22,008	1.43	1	13
Mutant Asp119Ala + *n*	84,700,000	165,600	1.2038	0.138	19

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 7**.

TABLE 7 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1A(iii), 1B(vi), 1C(vi), **Box 3** (step 3)	Cells detach from coverslip	Low adhesion of cells	Prepare a selective coating (depending on cell type) or reconstitute in DMEM supplemented with 1% FBS
2A(iv), 2B(v), 2D(iii), 2E(iii), 2F(iv)	High noise in basal recording (>150–200 c.p.s.)	An air bubble is inside perfusion chamber or a drop of solution is between perfusion chamber and PMT	Stop recording and perfusion. Remove air bubbles and clean the top of perfusion chamber
	Progressive reduction of basal recording	Cells are detaching	Reduce the speed of the pump or reduce the cell number
2B(vi)	Very weak signal increase	Depletion of intraluminal Ca^{2+} could result in cell toxicity	Instead of ionomycin, use the SERCA inhibitor tBHQ (at 40 μM)
2B(vi), 2E(iv)	Very weak signal increase	Incomplete ionomycin or tBHQ washing out	Increase time of washing with 2% BSA
2B(vii)	No changes in curve slope during agonist induced Ca^{2+} release	Degraded agonist or improper agonist choice	Prepare fresh Ca^{2+}-mobilizing agent
2A(v), 2B(vi), 2D(iv), 2F(v), **Box 3** (step 6)	No signal detection	Degraded Ca^{2+}-mobilizing agent or improper Ca^{2+}-mobilizing agent choice	Prepare fresh Ca^{2+}-mobilizing agent
4, **Box 3** (step 7)	Low transfection yield with mimsAEQ	High levels of mimsAEQ expression can induce cell toxicity	Reduce the quantity of DNA used for transfection
	No signal or low signal detection	Low expression yield or low amount of cells	Increase cell amount or change transfection method
	Maximum c.p.s. during lysis agent exposure is lower than during agonist exposure	Cell detachment during perfusion	Reduce amount of cells in the seeding phase. Prepare a selective coating (depending on cell type) or reconstitute in DMEM supplemented with 1% FBS

● TIMING

Step 1: 90–120 min for options A, D, E and F; 50 min for options B and C
Step 2: 1.5–2 min for options A, D and E; 3–4 min for option F; 5–8 min for options B and C (for a single sample)
Steps 3 and 4: 2 min (for a single sample)
Steps 5–8: 5–10 min (for a single sample)
Box 2, step 1: 30–60 min (depending on cell type); step 2: 30 min–2 h (depending on the transfection method)
Box 3, steps 1–3: 90–120 min
Box 3, steps 4–7: 2–3 min
Box 3, step 8: 50–80 min for 24-well plates; 200–240 min for 96-well plates

ANTICIPATED RESULTS

As described in the PROCEDURE, at the end of each experiment, the cells are lysed by perfusing them with a hyposmotic medium containing 10 mM $CaCl_2$ and a detergent (Triton X-100) to discharge all the aequorin that was not consumed during the experiment; this provides an estimation of the total aequorin content, which must be known to convert the luminescence data into $[Ca^{2+}]$ values.

To transform luminescence values into $[Ca^{2+}]$ values, we used essentially the method described by Allen and Blinks[9], which relies on the relationship between $[Ca^{2+}]$ and L/L_{max}: L is the light intensity at a given moment and L_{max} is that which would have been recorded if, at that same moment, all the aequorin present in the cell had been suddenly exposed to a saturating $[Ca^{2+}]$.

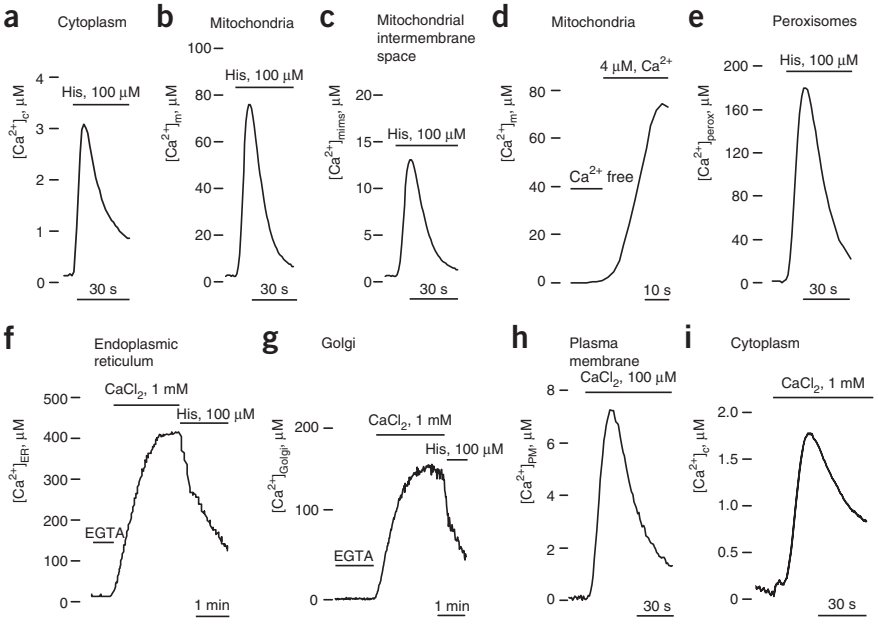

Figure 4 | Measurements of Ca^{2+} in different cellular compartments. (**a–i**) Representative Ca^{2+} kinetics measured with aequorin in cytoplasm (**a**), mitochondria (**b**), mitochondrial intermembrane space (**c**), mitochondria from permeabilized cells (**d**), peroxisomes (**e**), endoplasmic reticulum (**f**), Golgi apparatus (**g**), subplasmalemma (**h**) and cytoplasm during capacitative influx stimulation (**i**) in HeLa cells.

The rate constant of aequorin consumption at saturating $[Ca^{2+}]$ is 1.0 s^{-1}, and a good estimate of L_{max} can be obtained by estimating the total aequorin content by discharging all the aequorin content at the end of the experiment (by adding an excess of Ca^{2+} and detergents) and collecting the total amount of aequorin light output.

As aequorin is consumed continuously, the value of L_{max} is not constant during the experiment. The value of L_{max} to be used for calculating $[Ca^{2+}]$ at every point along the experiment should be calculated as the total light output in the whole experiment minus the light output recorded before that point. The relationship between the ratio L/L_{max} and $[Ca^{2+}]$ has been modeled mathematically; all calibration curves using native aequorin, mutant aequorins and different forms of coelenterazine have been comprehensively represented by the group of Alvarez[14] and summarized in **Figure 3** and **Table 6**.

Various physiological parameters may affect the Ca^{2+} sensitivity of aequorin, such as the $[Mg^{2+}]$ (which decreases the sensitivity of aequorin and thus must be known in order to obtain an accurate $[Ca^{2+}]$ estimate in the cell and/or compartment of interest), the ionic strength and pH (although the effect of this parameter is very modest in the physiological range, i.e., 6.6–7.4). For physiological conditions of pH, temperature and ionic strength, a second to third power relationship between the rate of consumption and the $-\log[Ca^{2+}]$ (pCa^{2+}) can be observed in the pCa^{2+} range of 5–7; the presence of three Ca^{2+}-binding sites in the aequorin molecule is responsible for the high degree of cooperativeness and for the steep relationship between photon-emission rate and $[Ca^{2+}]$.

In **Figure 4**, we report all calibrated aequorin traces discussed above; Ca^{2+} values of distinct subcellular compartments are typical of HeLa cells. Different cell lines have different intraorganelle $[Ca^{2+}]$ (i.e., the mitochondrial Ca^{2+} response in HeLa cells is ~100 μM, whereas in HEK 293 cells it is ~3 μM).

When an experiment is properly set up, Ca^{2+} mobilization induces a large signal increase above background. Such an increase is shaped as a peak in cytoplasm, mitochondria, subplasmalemma and peroxisomes, which is rapidly extinguished in ~60 s. In compartments with high $[Ca^{2+}]$ (endoplasmic/sarcoplasmic reticulum and Golgi apparatus), when the $[Ca^{2+}]$ in the perfusion medium is switched to 1 mM $CaCl_2$, the $[Ca^{2+}]$ in the lumen of the two compartments gradually increases, reaching a plateau generally in 120–180 s. This plateau is stable for some minutes, during which it can be challenged with agonists (e.g., histamine) to induce Ca^{2+} release from intracellular Ca^{2+} stores.

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