Invited Review

Fluorescent Protein–photoprotein Fusions and Their Applications in Calcium Imaging†

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ABSTRACT

Calcium-activated photoproteins, such as aequorin, have been used as luminescent Ca2+ indicators since 1967. After the cloning of aequorin in 1985, microinjection was substituted by its heterologous expression, which opened the way for a widespread use. Molecular fusion of green fluorescent protein (GFP) to aequorin recapitulated the nonradiative energy transfer process that occurs in the jellyfish Aequorea victoria, from which these two proteins were obtained, resulting in an increase of light emission and a shift to longer wavelength. The abundance and location of the chimera are seen by fluorescence, whereas its luminescence reports Ca2+ levels. GFP–aequorin is broadly used in an increasing number of studies, from organelles and cells to intact organisms. By fusing other fluorescent proteins to aequorin, the available luminescence color palette has been expanded for multiplexing assays and for in vivo measurements. In this report, we will attempt to review the various photoproteins available, their reported fusions with fluorescent proteins and their biological applications to image Ca2+ dynamics in organelles, cells, tissue explants and in live organisms.

INTRODUCTION

Bioluminescence is increasingly considered as an alternative to fluorescence for several imaging and nonimaging biological applications, mainly due to its simplicity, cost-effectiveness, sensitivity and potential for high throughput screening in small volumes. As there is no need for excitation, the background is negligible and signal-to-noise is higher than in fluorescence, but photon emission is scarce. Bioluminescent reporters are used to study a myriad of biological processes, such as gene expression, protein–protein interactions and physiological regulatory elements (e.g. Ca2+, cAMP).

Calcium-activated photoproteins are bioluminescent proteins that emit photons upon Ca2+ binding (1). They consist of an apoprotein and coelenterazine, a substrate oxidized by the protein and kept tightly bound to the apoprotein as a hydroperoxide derivative. The most commonly used is aequorin, derived from the hydroid Aequorea victoria (2). It was the first indicator used to record Ca2+ activity by microinjection in single barnacle muscle fibers (3). Since its cloning in 1985, it has been used as a gene-encoded Ca2+ indicator (GECI) (4) in cells, as an alternative to the widely used synthetic fluorescent probes. In the photocytes of A. victoria, the green fluorescent protein (GFP) serves as an antenna for nonradiative energy transfer (5) from aequorin, shifting its emission from blue to green. It has been found that the molecular fusion of GFP and aequorin mimics this process and increases aequorin stability and quantum yield within mammalian cells (6). In addition, the chimera is a bifunctional probe, its fluorescence facilitating visualization and its luminescence being proportional to Ca2+ levels. In the first part of this review, we summarize what is known of aequorin and other Ca2+ photoproteins more recently studied, pointing out their feasibility for use in cells. We then discuss the strategies that have been used for making chimeric fusions of various fluorescent proteins with photoproteins (FP–photoproteins), aimed to displace their emission toward longer wavelengths. Further tuning for particular applications has been achieved, on the one hand, using synthetic coelenterazines and, on the other hand, by introducing certain amino acid mutations in the photoprotein sequences. We end this section by providing some tips to guide the user between the numerous possible combinations of aequorin-based probes, mutations and chemically modified coelenterazines, for typical biological purposes. In the second part of this report, we review the biological studies in which FP–photoproteins have been employed in Ca2+ studies from the subcellular level to single cells, tissue slice preparations and intact organisms, either by transient expression or by the generation of transgenic lines.

CALCIUM-ACTIVATED PHOTOPROTEINS

There are many types of bioluminescent systems currently known. In contrast with luciferases, photoproteins are defined as the bioluminescent proteins capable of emitting light in proportion to the amount of the protein luminesced and not to the
amount of the luciferin substrate (1). In photoproteins, there is a fast catalytic reaction producing the light, whereas the release of the oxidized coelenterazine and binding of fresh substrate to the apoprotein (the regeneration step) are relatively slow. The triggering factors of light emission in these photoproteins are diverse: Ca\(^{2+}\), Fe\(^{2+}\), H\(_2\)O\(_2\), ATP, Mg\(^{2+}\), etc. (7). We will refer here only to the Ca\(^{2+}\)-regulated photoproteins, outlining their main characteristics and amenability for use as reporters fused to fluorescent proteins. The most studied Ca\(^{2+}\)-sensitive photoproteins are those present in the phylum Cnidaria, class Hydrozoa, namely aequorin, obelin, and clytin, of which almost exclusively aequorin has been used in biological applications (Table 1). They have a relative molecular mass of about 21 kDa and use the same substrate, coelenterazine, tightly, but noncovalently bound to the protein structure. The apoproteins of aequorin, obelin, clytin and mitrocomin are closely related and show high amino acid sequence identity (Table 1). Their Ca\(^{2+}\)-binding sites are particularly conserved and differ from other Ca\(^{2+}\)-binding proteins by their high content of cysteine, tryptophan, histidine, proline and tyrosine residues (8). This suggested that these residues play a role in light emission (9).

**Aequorin**

It is, so far, the best-known photoprotein and has been widely used in a variety of applications. Aequorin was isolated and purified from the light organs of the jellyfish *A. victoria* (2,10). The cloning and expression of aequorin cDNA in *Escherichia coli* were accomplished by two groups (11–13). Aequorin is comprised of 189 amino acid residues, with an essential C-terminal proline. Aequorin contains a noncovalently bound hydroperoxy-substituted coelenterazine as the functional light-emitting chromophore. The crystal structure of aequorin (14) shows its globular form with four “EF-hand” domains, of which I, III and IV can bind Ca\(^{2+}\) (15). Aequorin’s conformation is more compact than that of apoaequorin; the substrate coelenterazine is located in the center of the protein, shielded from the solvent in a 600 Å \(^2\) pocket, which is ample enough to fit chemically modified coelenterazines.

When aequorin reacts with at least two and up to three Ca\(^{2+}\) ions, a conformational transition takes place, triggering cyclization of the peroxyde of coelenterazine into the corresponding dioxetanone, which instantly decomposes, producing CO\(_2\) and the excited state of coelenteramide. When the latter falls back to the ground state, blue light is emitted in a broad band with a peak at 465 nm (15–18).

One microgram of aequorin emits 4–5 × 10\(^{12}\) photons and has a quantum yield of 0.16 (19,20). The luminescence reaction of aequorin in the presence of an excess of Ca\(^{2+}\) has a half-time constant of 6–20 ms for the rise and 0.4–0.8 s for the decay (1). Spent apoaequorin can be reconstituted into aequorin by incubation with excess coelenterazine in the presence of oxygen, 2-mercaptoethanol (or DTT) and EDTA. The regeneration, at 4°C, is usually 50% complete within 30 min. Several kinds of cations other than Ca\(^{2+}\) can trigger the luminescence reaction of aequorin, for example La\(^{3+}\), Sr\(^{2+}\), Pb\(^{2+}\) and Cd\(^{2+}\) (21). In testing biological samples, however, aequorin emission is considered to be highly specific to Ca\(^{2+}\), because the occurrence of a significant amount of those metal ions is unlikely. Even if the EF-hands I and III of aequorin bind Mg\(^{2+}\) with lower affinity than Ca\(^{2+}\), this does not trigger the luminescence reaction (22), although it decreases aequorin Ca\(^{2+}\) sensitivity (23).

**Obelin**

Obelin from *Obelia longissima* has been cloned and sequenced in 1995 (24). Its recombinant form was prepared and investigated as Ca\(^{2+}\) indicator in 2000 (25). Obelin from hydroid polyp *Obelia geniculata* was partly described in 1974 (26), and its recombinant form was prepared and investigated by Markova et al. in 2002 (27). The isolated obelin emits blue light (475 nm peak) when Ca\(^{2+}\) is added. Although the chemical mechanism of obelin luminescence is considered to be identical to that of aequorin (27), the onset of emission after binding Ca\(^{2+}\) is rather fast (3 ms) which would make it useful for experiments requiring high temporal resolution (28). As a Ca\(^{2+}\)-indicator, obelin is suitable between 10\(^{-6.5}\) and 10\(^{-3.5}\) mol L\(^{-1}\), whereas aequorin is better used from 10\(^{-7.5}\) to 10\(^{-4.5}\) mol L\(^{-1}\) (29,30). In contrast with aequorin, the bioluminescence of obelin was conserved upon addition of a C-terminal Tyr residue and even whole protein fusions (31).

**Clytin (phialidin)**

Clytin was cloned and sequenced from the jellyfish *Clytia gregaria*, and it shows 62% amino acid sequence homology to aequorin (32). Several isoatypes are known: recombinant clytin-I showed lower Ca\(^{2+}\) sensitivity than aequorin (33); thus, it is suited for measuring higher Ca\(^{2+}\) concentrations. In contrast, clytin-II showed higher initial luminescence intensity than clytin-I and aequorin (34,35). As much as three distinct photoproteins and four GFPs have been recently identified and cloned from *Clytia hemisphaerica* (36). Interestingly, they show differential expression at larval, polyp and medusa stages, which suggests that each fulfills a different role in the organism. Furthermore, clytin-2 and GFP-2 are coexpressed in eggs and imported to mitochondria. It is interesting to speculate why these proteins have acquired their mitochondrial targeting signals independently during evolution. A reason might be that high clytin and GFP protein concentration in mitochondria favor efficient energy transfer *in vivo* (36) (reviewed later); moreover, Ca\(^{2+}\) transients in mitochondria reach higher concentrations than in the cytosol, which would also boost the light response. Clytin properties have been improved by random mutagenesis (37).

**Mitrocomin (halistaurin)**

Mitrocomin was cloned by Fagan et al. (9) and showed 68% and 61% homology to aequorin and clytin-I, respectively. Curiously, its two C-terminal residues are Pro-Tyr, the latter being dispensable. Recently, five isoforms of mitrocomin have been cloned from a single organism which are likely products of two allelic genes differing in only one residue (64R or 64K) (38).

**Berovin and mnemiopsin**

These photoproteins, which appear in organisms of the phylum *Ctenophora*, have been characterized and found to be strongly inactivated by light. Berovin from *Berocr abyssicola* (39) and mnemiopsin from *Mnemiopsis leidyi* (40) were recently cloned. Although berovin sequence identity with aequorin is as low as 28%, three canonical EF-hand Ca\(^{2+}\)-binding sites were discovered, and it was successfully expressed in mammalian cells in culture. Recently, the structure of apoberovin with bound Mg\(^{2+}\) has been determined (41). Mg\(^{2+}\) was found on each functional
Table 1. Ca\(^{2+}\)-regulated photoproteins.

| Phylum       | Organism*            | Photoprotein         | Amino acid identity to Aeq\(^{†}\) | CLZ | Emission maxima (nm) | Ca\(^{2+}\) detection limit | Sensitivity to Mg\(^{2+}\) | Luminescence rate of rise and decay | Observed properties                                                                                   | Refs‡ |
|--------------|----------------------|----------------------|-----------------------------------|-----|----------------------|----------------------------|--------------------------|----------------------------------|---------------------------------------------------------------------------------------|-------|
| Cnidaria     | *Aequorea victoria*  | Aequorin             | 100% (11)                         | Native | 465                  | >Sensitive than Obelin     | Sensitive (due mostly to EF hand 3) | Faster rise and decay than aequorin | Optimal for cytoplasm                                                            | (23,29,42) |
|              | *Obelia geniculata*  | Obelin               | 64% (27)                          | Native | 495                  | Relatively insensitive     | Faster rise and decay than aequorin |                                    | Best for high Ca\(^{2+}\) cell compartments (mitochondria, plasma membrane, peroxisomes or Golgi). Obelins about 10-fold lower luminescence activity in stably expressing CHO cells | (42)  |
|              | *Obelia longissima*  | Obelin               | 66% (24)                          | Native | 485                  | Relatively insensitive     | Faster rise and decay than aequorin |                                    | Optimal for cytoplasm                                                            | (24,42) |
|              |                      | hcp                  |                                   |       |                      | >Sensitivity than Obelin-native; similar to f-Aeq and hcp-Aeq | Insensitive | 3- and 11-fold higher rate than obelin and aequorin with native CLZ | Excellent for fast intracellular Ca\(^{2+}\) transients                                         |       |
|              | *Clytia gregaria*    | Clytin-I and II (phialdin) | 62% (32)                          | Native | 474                  | CL-II shows 4.5-fold higher initial luminescence intensity than CL-I | Insensitive |                                    | High Ca\(^{2+}\) cell compartments (mitochondria, plasma membrane, peroxisomes or Golgi). It worked well in mitochondria in CHO cells | (32,33,42,70) |
| Mitrocoma    | *cellularia*         | Mitrocomin           | 68% (9)                           | Native | 470                  |                                    |                                    |        | Very weak luminescence; poor conversion of apo-mitrocomin into active photoprotein (poor CLZ charging) | (9,42)  |

(continued)
EF loop and, in accordance with this, Mg\(^2+\) at physiological concentrations had an effect on Ca\(^{2+}\) sensitivity, kinetics, thermostability and charging with coelenterazine.

Malikova et al. (42) have reported a comparison of the properties of recombinant aequorin from A. victoria, obelins from O. geniculata and O. longissima, clytin from C. gregaria and mitrocomin from Mitrocoma cellularia in the same in vitro conditions and in live mammalian cells in culture. In spite of their high degree of homology, they differed in the Ca\(^{2+}\) concentration detection limit, Mg\(^{2+}\) sensitivity and rate of luminescence rise. Aequorin was the most sensitive to Ca\(^{2+}\) (K\(_{D}\) = 85 nM with 1 mM Mg\(^{2+}\) present) and clytin the least sensitive (K\(_{D}\) = 500 nM), whereas the obelins and mitrocomin had intermediate Ca\(^{2+}\) affinities. The Ca\(^{2+}\)-independent luminescence (the spontaneous discharge) was lowest with clytin and obelin from O. geniculata, a useful property for in vivo applications.

In stopped flow kinetics experiments, obelin showed the fastest rate of luminescence rise upon mixing with Ca\(^{2+}\), whereas aequorin had the lowest rate constant. The luminescence decay was much slower than the rise, and obelins decayed much faster than the other photoproteins. Aequorin was the most sensitive to Mg\(^{2+}\), an important fact for live cell use because the cytoplasmic Mg\(^{2+}\) levels are in the range of millimolar. In CHO cells expressing mitrocomin, the luminescence signal was just above detection limit owing to its inefficient charging with coelenterazine. In contrast, aequorin and obelin from O. longissima were well suited for measurement of cytosolic Ca\(^{2+}\) signals triggered by receptor stimulation, whereas clytin and obelin from O. geniculata were found to be more appropriate for measurements in compartments with higher Ca\(^{2+}\) concentration (Table 1).

**ENGINEERING FP–PHOTOPROTEIN SENSORS FOR CALCIUM BASED ON BRET**

In vitro bioluminescent assays for Ca\(^{2+}\) are often straightforward and involve studying the cell response to stimulatory conditions, whereas in vivo Ca\(^{2+}\) imaging presents greater challenges. It is well known that red/infrared light is less absorbed and less scattered by animal tissues, owing to the absorption spectra of hemoglobin, melanin and water. Thus, tissues have the so-called optical window, between 600 and 900 nm, in which they absorb and scatter light minimally (43). This significantly narrows down the choice of reporters that can be used in vivo or in tissue slice preparations, as most of them emit blue or green light. Moreover, light penetration depth is limited to between 1 and 2 cm. Although alternatives to luminescence emitting in that optical window exist, such as near-infrared quantum dots and carbon nanotubes, these present potential risk of toxicity to living specimens or damage to the experimenter from accidental inhalation (in case of carbon nanotubes) (44).

The application of photoproteins for Ca\(^{2+}\) imaging in vivo is limited by their blue emission and the lack of diversity in luminescence hue compared to the ample palette of fluorescent proteins (45). Thus, expanding the color emission of Ca\(^{2+}\) photoproteins would be advantageous, not only for in vivo imaging in deep tissues, but also for multiplexing analytical screens. Different photoprotein emission hues have been obtained by mutagenesis, using synthetic coelenterazines and by fusing the photoprotein to fluorescent proteins (which act as BRET acceptors). The latter approach has been shown to produce the largest
Resonance energy transfer and key factors affecting it

Resonance energy transfer (RET), initially described in 1946 by Theodor Förster (46), occurs between two chromophores in close proximity when the emission spectrum of one (the donor) overlaps with the excitation spectrum of the second (the acceptor). Following donor excitation, part of the relaxation energy is transferred to the acceptor in a nonradiative manner through dipole–dipole coupling. The acceptor, in turn, emits light at its characteristic emission band (47). When the donor molecule is fluorescent, the process is known as fluorescence resonance energy transfer (FRET). In the case of a bioluminescent donor molecule (light emission triggered by a biochemical reaction), it is termed bioluminescence resonance energy transfer (BRET). The efficiency of RET between two chromophores depends on several factors. Most importantly, RET occurs when the donor chromophore is within a few nanometers of the acceptor (RET efficiency is inversely proportional to the sixth power of this distance). $R_0$ is the distance resulting in 50% energy transfer efficiency and is characteristic of a given RET pair. For FP and Ca$^{2+}$ photoproteins, typical $R_0$ values range between 40 and 80 Å and large $R_0$ are preferred (48,49). $R_0$ depends on two tunable factors, the spectral overlap between donor and acceptor, and the donor emission quantum yield in the absence of the acceptor. In turn, the relative orientation of the donor and acceptor dipoles also affects $R_0$, but it is rather difficult to control experimentally. The extinction coefficient of the acceptor (its capacity of energy absorbance) is a factor directly related to the overlap integral and, therefore, to RET efficiency.

From natural BRET to designed FP–photoprotein fusions

In addition to the Ca$^{2+}$-sensitive photoproteins we have reviewed in the previous section, the bioluminescence systems of the hydrozoans Aequorea, Clytia and Obelia comprise a cognate GFP. In Aequorea, although the in vitro bioluminescence of the purified and reconstituted aequorin upon contact with Ca$^{2+}$ is blue (peak emission ≈465 nm), that of the intact jellyfish is green and coincides with the fluorescence of A. victoria GFP (avGFP) (1,2,50,51). In Obelia, live specimens also emit green bioluminescence (508 nm peak). The mechanism of Förster theory was invoked to explain these spectral shifts. The GFP was proposed to act as RET acceptor “from an excited product of the calcium-activated photoprotein” (52) and was found to increase the quantum efficiency of light emission. Similar to aequorin/avGFP, a pair clytin/GFP has been discovered and isolated from various species of the jellyfish Clytia and characterized in terms of spectral emission, quantum yield and energy transfer efficiency (36,53,54).

BRET efficiency depends upon several factors (discussed above), yet in its natural milieu (e.g. photocytes), the concentration of the donor photoprotein and the acceptor GFP, ranging from micromolar to millimolar, seems to be a key element (55).

Throughout in vitro studies, a noteworthy observation was made regarding the protein concentrations required to have energy transfer from a photoprotein to GFP. For clytin, the bioluminescence emission was shifted to the green at micromolar concentrations of Clytia GFP (cgGFP), but for aequorin, almost 100 times larger amounts were required (54,56). The intermolecular distances at micromolar concentrations are above those required for BRET; therefore, it was suggested that energy transfer between photoprotein and GFP requires a protein–protein interaction. However, this interaction must be transient as techniques such as analytical ultracentrifugation, size-exclusion chromatography or fluorescence anisotropy have failed to demonstrate an association.

Moreover, no structure of a photoprotein:GFP complex has ever been solved by crystallography or NMR. However, evidence for a complex was obtained with the more sensitive NMR chemical shift perturbation mapping, upon titration of $^{15}$N-1H labeled clytin with unlabeled cgGFP, or vice versa. With these data, the residues and interaction surfaces of clytin and cgGFP were defined, located on the 3D structures (which were known) and the overall topology of a complex was computer-modeled (55,56). The distance between fluorophores was 45 Å, compatible with efficient BRET. The $K_D$ of the interaction was estimated to be 0.9 mmol L$^{-1}$, so this structure probably represents a pre-oriented, low affinity clytin:cgGFP complex. It was proposed that, upon Ca$^{2+}$ binding, clytin adopts a conformation with several orders of magnitude enhanced affinity for cgGFP; then, a very transient complex is formed, in which chemiexcitation of clytin and subsequent energy transfer to cgGFP occur. In other studies, no stable complex of Ca$^{2+}$ discharged clytin with cgGFP has been detected, suggesting that such BRET complex must indeed be a short-lived intermediate in the clytin bioluminescence process (54,57).

Genetically fused photoprotein–fluorescent protein pairs. The energy transfer process occurring naturally in the photocyes of A. victoria between aequorin and avGFP has been reproduced at lower concentrations by bringing them closer to each other, that is by coadsorption onto DEAE-cellulose, by interaction of their fusion partners or by linking them molecularly. This would allow to achieve the small donor-acceptor distances and/or favorable chromophore dipole orientations required for BRET. In 1999, Xu et al. were the first to generate chimeric proteins based on BRET as a sensing tool, by genetically fusing the C-terminal ends of Renilla luciferase and GFP with two interacting circadian clock proteins (58). One year later, Brulet et al. concatenated avGFP and aequorin, which resulted in a bright green-emitting Ca$^{2+}$ sensor named GA, with a 40 nm redshift (6) (Fig. 1). This reporter provided unique advantages like bifunctionality (as the chimeric protein location and its abundance could be monitored by fluorescence, whereas its luminescence reported Ca$^{2+}$), considerably improved overall protein stability within mammalian cells compared to aequorin alone and, probably, also increased quantum yield (6).

A study by Gorokhovatsky et al. provided a mechanism to explain why BRET was so efficient in these fusions (59). Using a variant of avGFP (60), which conserves the 395 nm absorbance peak of wt GFP, fused to aequorin, they demonstrated a Ca$^{2+}$-induced redshift of GFP absorption and fluorescence excitation. In the same study, the fusion of avGFP and obelin (each coming from a different organism) did not show such a Ca$^{2+}$-dependent spectral shift. The authors suggested that an specific interaction between avGFP and aequorin exists, promoting the best chromophore orientation, distance and spectral overlap for efficient BRET. When other avGFP variants are used, lacking the two absorption peaks of the wt protein (6), such weak interaction likely remains, although the avGFP spectral shift is no longer present.
The Ca²⁺-triggered bioluminescence emission spectra were normalized (RLU, relative light units). GA (GFP-aequorin), CitA (Citrine-aequorin), Redquorin (linker-optimized tdTomato-aequorin). The emission profiles of the photoproteins obelin and Clytin (not shown) overlap closely with that of aequorin. The table indicates the Full Width at Half-Maximum of the chimeras (FWHM), the emission maxima of the major peaks, and the counts above 600 nm relative to the total emitted light (expressed in %).

In a follow-up study by Brület’s group, the authors attempted to further displace aequorin emission to longer wavelengths. They reported for the first-time fusions of aequorin with a yellow FP (Venus) and a red FP (mRFP1) (61). The chimeras were functional when expressed in mammalian cells and Venus-aequorin (VA) displayed strong BRET and a 58 nm redshift. This was shown to substantially improve light transmission through the skin and the thoracic cage of mice. Regarding mRFP1-aequorin (RA), its emission spectrum coincided with aequorin luminescence, but it showed a small peak at 600 nm due to energy transfer. Furthermore, RA could be detected with higher sensitivity than GA or VA when a sample of the photoprotein was placed subcranially in a mouse, leading the way to further improvements. Shortly after, Manjarrés et al. reported a chimeric protein composed of another red FP (mRFP1.2) with aequorin. In combination with GA, it was used to record simultaneously and independently the changes in Ca²⁺ concentrations in two subcellular compartments of the same cells (62). The chimeras were targeted to the cytosol, nucleus, mitochondria and the endoplasmic reticulum (ER); aequorin was mutated to decrease its Ca²⁺ affinity in the chimera directed to the ER (ermutGA). Ca²⁺ signals exhibited different time course and regulation in the cytosol/nucleus, in mitochondria, and in the ER. As the efficiency of BRET between mRFP1.2 and aequorin was low, spectral unmixing was used to separate the red and green emissions originating from the organelle combinations. The ability to image Ca²⁺ dynamics in cells using well-separated hues in a synchronous manner was certainly a major progress. However, for in vivo imaging, further red-shifted variants with more efficient BRET were needed to avoid light attenuation from tissue absorbance, as has been discussed.

In the search for a better red acceptor, aequorin was paired with various FP with orange and red emission. The tandem dimer tdTomato (two concatenated copies of the dimerizing RFP TagRFP) showed about 50% energy transfer efficiency in the fusion tdTomato-aequorin (tdTA), with a bimodal spectrum (48). The pair tdTomato and aequorin showed two to four-fold larger spectral overlap integral than other red FPs examined with aequorin; in addition, the RET critical distance R₀ was the largest for tdTA/aequorin (44.7 Å) of all studied combinations (48). Luminescence from tdTA was able to pass animal tissues, as Ca²⁺ signals were imaged through the abdominal wall in mice injected with the purified chimera. Further optimization of tdTA (by shortening the linker, the C-terminus of tdTomato and the N-terminus of aequorin) led to the generation of redquorin, characterized by high BRET efficiency and a major emission peak at 582 nm (63) (Fig. 1). The emission of redquorin was transmitted through blood with less absorption than the previous chimeras. In addition, redquorin allowed recording Ca²⁺ transients associated with muscle contractions in developing zebrafish embryos. In summary, in tuning the luminescence spectra of Ca²⁺-sensitive photoproteins, the approach of fusing them with fluorescent proteins (BRET-based chimeras) has produced so far the largest emission shifts.

It is interesting to note that BRET was very efficient when fusing GFP, citrus or Venus to aequorin (all derived from Aequorea), almost regardless of the linker composition (6,59,61,62,64), but very hard to obtain when the acceptor (mOrange, TagRFP, mRFP1, mRFP1.2, tdTomato) was derived from other organisms (i.e. Discosoma) (48,61,62). Likewise, in the avGFP-obelin fusion mentioned earlier, no evidence of a transient interaction was found (59). It appears that, when the photoprotein and GFP have coevolved, there is an interaction favoring BRET, as it was also found in vitro in the Clytia system (55,56), whereas the use of photoproteins and FPs from distantly related organisms weakens energy transfer. Even though such interaction in these BRET systems is weak and transient, it is still naturally specific and not interchangeable.

It is worth mentioning that gene fusions to aequorin are limited to its N-terminal end, as deletion or modification of the C-terminal proline abolished luminescence (65). Interestingly, obelin was found to tolerate additions to its C-terminus (31). This little exploited fact will allow making FP or other C-terminal fusions on obelin.

Total light output, stability, affinity and emission kinetics of FP–photoprotein fusions. In the first molecular fusions of aequorin with GFP (GA), the fusion proteins showed better Ca²⁺-triggered bioluminescence activity than aequorin alone (6). Experimentally, the light-emitting capacity of extracts obtained from cells transfected with the chimeras increased from 19 to 65 times, a result attributed largely to greater protein stability. The half-life of apoaequorin in the cells was relatively short, 20 min, whereas GFP is known to be a very robust protein and it helps to stabilize aequorin in the fusion. GA in vitro also showed higher sensitivity to Ca²⁺ compared to aequorin alone. More recently, we reported the same observation (63). In the Renilla luciferase-GFP system, it has long been proven that energy transfer to GFP was responsible for a 3.1-fold increase in quantum yield (5). In GA, there was probably also an enhancement of quantum yield. The overall improvement in total luminescent activity of GA fusions allowed for the first time to image Ca²⁺ in single cells with an intensified CCD, with a reasonable time resolution (1 Hz) (6).
The rate of bioluminescence decay is another key feature in the Ca\(^{2+}\)-sensitive photoproteins. It is commonly observed that the decay rate increases with Ca\(^{2+}\) concentration. Some reports hint that the change in such emission decay is related to the structural rigidity of the photoprotein (66,67). In fact, the conjugation of quantum dots with aequorin increased its conformational rigidity, resulting in longer emission decay (68). On the one hand, mutation studies in aequorin showed that there is a relationship between sensitivity to Ca\(^{2+}\) and reaction kinetics (66). On the other hand, bright mutants with similar decay rates showed very different EC\(_{50}\) for Ca\(^{2+}\) and, conversely, some slow decay aequorin mutants displayed unchange\(d\) Ca\(^{2+}\) affinity. In the latter case, it was suggested that a structural link transducing Ca\(^{2+}\) binding to bioluminescence was disrupted. In a study by Drobac et al. (69), the authors compared aequorin and obelin fused to GFP. The fusions acted quite similarly as Ca\(^{2+}\) sensors in live cells, but GFP-obelin showed faster rise and decay kinetics in vitro.

Photoproteins with chemically attached fluorophores. So far, we have discussed how fluorescent proteins fused to photoproteins cause a significant and valuable bioluminescence spectral shift to longer wavelengths. The important precedent for this was reported by Shimomura and Shimomura back in 1985 by covalently attaching fluorescein to aequorin (70,71). The product emitted yellowish light (\(\lambda_{\text{max}} = \text{520 nm}\)) by intramolecular energy transfer from the native chromophore of aequorin to the fluorescein. Using the same BRET principle, other fluorophores (IaNBD and Lucifer Yellow) were attached to unique sites in aequorin, mutated into cysteines. Such labeling induced the appearance of an extra yellow peak around 530 nm (72). More recently, aequorin has been conjugated to quantum-dot particles (CdSe/ZnS), resulting in limited energy transfer with the presence of an emission peak around 540 nm. In addition, a four-fold increase in the decay time was observed, which was attributed to increased conformational rigidity (68). Further studies are needed in order to increase the BRET efficiency from aequorin to these conjugated fluorophores, for example using aequorin mutants to find the most suitable orientation/distance configuration for label attachment. Chemically modified aequorins are suited for in vitro studies, but would have to be delivered to the cell interior for live cell applications.

A GFPuv-aequorin fusion protein (GAP) is a dual mode, fluorescent or bioluminescent Ca\(^{2+}\) indicator. This novel class of GECI (73) deserves a special comment. They are molecular fusions of a particular GFP mutant (GFPuv) (60) and aequorin, able to function either as a luminescent or fluorescent biosensors for Ca\(^{2+}\), depending on whether apoaequorin is reconstituted with coelenterazine or not. To date, most GECIs use as a Ca\(^{2+}\) sensor moiety an endogenous Ca\(^{2+}\)-binding protein (such as calmodulin or troponin C). In some systems, this has been found to cause interference with signaling cascades in the cells expressing the chimeras (4). GAP probes, like other FP–photoprotein fusions, circumvent this issue, as both modules derive from the jellyfish and are absent in mammalian cells. *Aequorea* wild-type GFP, in contrast with GFP from *Renilla* or other organisms, has the particularity of having two absorbance peaks at 395 nm and 475 nm, corresponding to the protonated and anionic hydroxyl on Tyr66, respectively. GFP mutants carrying mutation S65T (like EGFP) have only the long wavelength peak (51). However, the GFPuv used in GAP sensors conserves the two excitation peaks of wild-type GFP. Binding of Ca\(^{2+}\) to the aequorin moiety in GAP caused an excitation spectral shift of GFPuv (73). This property was used to construct an indicator which changes the ratio of 470/403 nm excitation in a Ca\(^{2+}\)-dependent manner. Moreover, GAP was molecularly targeted to accumulate in the cytoplasm, nucleus and various organelles. GAP variants (GAP2, GAP3), with lowered Ca\(^{2+}\) affinities, were developed by mutagenesis to match the higher Ca\(^{2+}\) levels present in mitochondria, Golgi apparatus and the ER (74,75).

**Tuning FP-aequorin properties for biological applications:**

**use of synthetic coelenterazines**

In 1974, a hypothetical compound, later called coelenterazine, was proposed as the light-emitting chromophore of aequorin (16), and soon after, it was isolated and synthesized (76). Later, it was found that aequorin contains coelenterazine-2-hydroperoxide and that the peroxide group is linked to various residues in the protein by hydrogen bonds (14).

Since 1988, Shimomura *et al.* have reported synthetic coelenterazine analogues with different functional group modifications (71,77–79). When apoaequorin is regenerated with a coelenterazine analogue, the resulting active product is called a semisynthetic aequorin. A total of about fifty kinds of semisynthetic aequorins were prepared. Some of their features (Ca\(^{2+}\) affinity, luminescence rise and decay kinetics, emission spectra, regeneration time, total luminescence capacity and cell permeability) are considerably different from those of native aequorin (18,71,78,80,81).

Modification of the structure of coelenterazine results in the following functional consequences. The group on C2 of native coelenterazine is hydroxyphenyl and must be aromatic. Replacement of the original phenolic OH with H (coelenterazine-h) or F (coelenterazine-f) increases the Ca\(^{2+}\) sensitivity and the relative intensity of the semisynthetic aequorin produced (h- or f-aequorin, respectively). However, C2 substituents containing the heavy elements Cl, Br and I, that is p-bromobenzyl or p-iodobenzyl groups, or a bulky p-naphthylmethyl group (coelenterazine-n) decrease the Ca\(^{2+}\) sensitivity and the reaction rates, probably by steric hindrance and interference with the conformational changes of aequorin following Ca\(^{2+}\) binding (1,82).

The group on C8 of coelenterazine must be larger than a methyl group, and no aromaticity is needed at this position. For instance, substitution of the original phenyl group with a cyclopentyl group (coelenterazine-cp and part of coelenterazine-hcp modification) increases aequorin sensitivity to Ca\(^{2+}\) and the rate of the luminescence reaction, shortening the rise and decay times, without significant decrease in total light emission. This is because, unlike the aromatic phenyl group, the aliphatic cyclopentyl group does not form hydrophobic interactions with the surrounding amino acid residues, therefore promoting the light-emitting reaction (82).

Some coelenterazine analogues have been shown as an alternative approach to shifting aequorin and obelin emissions to longer wavelengths (up to 500 nm and 524 nm, respectively), although their luminescence capacity was low compared to native coelenterazine (83–85).

**Tuning of FP-aequorin properties for biological applications:**

**mutagenesis on aequorin**

A diversity of aequorin derivatives have been produced by random and site-directed mutagenesis. Table 2 summarizes several mutations of amino acid residues that have been described to
table 2. Summary of the effects of common amino acid residue mutations on aequorin properties.

| Category            | Aequorin mutants          | Reported effects                                                                 | Ref  |
|---------------------|---------------------------|----------------------------------------------------------------------------------|------|
| **Spectral shift**  | W86F                      | Emission blue-shift (10 nm; 455 nm peak vs 465 nm for WT)                         | (86) |
| Mutant S; E53C      |                           | Emission red-shift (9 nm; 480 nm peak vs 471 nm for WT)                           | (100)|
| **Ca2+ affinity**   | Y82F                      | Emission red-shift (32 nm; 501 nm peak vs 469 nm for WT)                          | (87) |
| D119A, D117G        |                           | Low Ca2+ affinity (Kd of 260 µM and 109 µM vs 1 µM for WT)                       | (89) |
| N28L, N28N, N28H    |                           | Low Ca2+ affinity (one log shift to the right vs WT)                              | (90) |
| Mutant AM20†        |                           | Low Ca2+ sensitivity (half-log shift to the right vs WT)                         | (88) |
| Mutations in GAP1 and GAP2‡ |                     | Low Ca2+ affinity, GAP1 one log decrease vs GAP; GAP2 2.8 log decrease vs GAP | (74,75) |
| **Emission decay kinetics** | Q168R                    | Slightly lower affinity (Kd of 6.8 µM vs 2.6 µM for WT)                          | (66) |
| K30E; A123D; N26D   |                           | Slightly higher affinity (Kd < 2.6 µM)                                            | (66) |
| N26D; D117G         |                           | Slower decay half-life (1.1 and 28.6 s, resp., vs 0.7 s for WT)                   | (66) |
| Y82F                |                           | Slower decay half-life (2.9 s vs 0.4 s for WT)                                    | (108)|
| Mutant AM20; V25K; V25R |                     | Slower decay half-life (14.1, 7.9 and 5.3 s, resp., vs 1.3 s for WT), related to decreased Ca2+ affinity | (88) |
| V25A; V25I; H27N    |                           | Possible higher decay kinetics, inferred by comparison with obelin                | (97) |
| Y82R; W86Y; H58F; A69W; H16F |                     | Substantial loss of luminescence                                                  | (94,100) |
| **Regeneration & luminescence activity** | C145S; C152S; C180S; M71C; S5C; G29R | Poor luminescence activity, regeneration-related                                  | (92,152,160) |
| H169Q; Y184F        |                           | Poor luminescence activity (23% of WT), regeneration-related                     | (95) |
| Mutant S           |                           | Significantly higher luminescence activity vs WT                                  | (92) |
| **Stability**       | Mutant S; E53C            | Higher luminescence activity vs WT per mg of protein                              | (100) |
| P189 deletion/substitution |                     | Loss of luminescence, protein structure stability-related                          | (96) |
| FI49S               |                           | Decreased level of thermostability                                               | (97) |
| Mutant S           |                           | Poor level of thermostability                                                    | (93) |
| Q168R; L170I       |                           | Enhanced thermostability at 37°C                                                  | (97) |
| S32T/E156V         |                           | Enhanced thermostability at 37°C                                                  | (95) |
| S32T/E156V/Q168R/L170I |                     | Highest thermostability at 37°C                                                   | (93) |

*For each category, only mutations highlighted as important by the authors or by other reports are presented. †Mutant S is aequorin with mutations C145S, C152S and C180S. ‡Mutant AM20 is an aequorin variant where the complete Ca2+-binding region of EF-hand I is replaced by that from EF-hand III. §GAP1 is a GFP-aequorin fusion whose aequorin carries the substitutions D117A, D119A and D163A. Mutations in GAP2 are D24N and D119A.

alternative different aequorin properties, and their positions are highlighted on the 2D scheme of aequorin in Fig. 2. A thorough description of these mutations is beyond the scope of this review, so we strived to present only the most critical residues. We distinguish five categories according to the end effects of these mutations on aequorin, namely spectral variation, Ca2+ affinity, decay kinetics, luminescence activity and protein stability. The X-ray crystal structure of aequorin (14) described a hydrophobic pocket surrounded by separate residues that stabilize the coelenterazine-binding H169, showed an increase in aequorin lifetime in cells, an exceptional thermostability, but a decreased Ca2+ affinity (97). Recently, random and rational mutagenesis allowed the creation of the thermostable double mutant, S327E156V and, in particular, the most thermostable mutant to date, with a quadruple mutation S327E156V/Q168R/L170I (93).

Although aequorin structure has been the most extensively studied by mutagenesis, several reports by Vysotski and collaborators have also shown striking structure–function effects in obelin (87,95,98,99). In 2008, Rowe et al. managed to produce aequorin and obelin variants with new emission colors and altered decay kinetics, by combining mutations with synthetic coelenterazines (100). An alternative approach to mutagenesis is to construct hybrid chimeras combining fragments of different photoproteins. For instance, a hybrid version of obelin and clycin, suitable for high throughput screening, was developed and showed increased signal output when expressed in mitochondria (101). Similarly, the Ca2+-sensitive domain of EF-hand I of aequorin was replaced with that of other Ca2+-binding proteins (clycin, mitrocomin, Renilla luciferin-binding protein and calmodulin). The chimeric mutants allowed the determination of the contribution of EF-hand I to aequorin Ca2+ sensitivity and decay kinetics (88).
Development of aequorin variants with altered properties using non-natural amino acids. An alternative way of changing the amino acid composition in aequorin was the strategic incorporation of non-natural amino acids (102). In their study, Rowe and colleagues have incorporated a noncanonical phenylalanine in place of Y82, where they reported up to 40 nm redshift in light emission. In 2016, the same group proposed the modification of the hydrophobic pocket surrounding the coelenterazine, specially the Tyr82–His16–Trp86 triad, by replacing the residues Tyr82 and Trp86 with four different phenylalanine-derived non-natural amino acids, AminoPhe, BromoPhe, IodoPhe and MethoxyPhe. The resulting variants were then complexed with different synthetic coelenterazine analogues to produce combinations with yellow light emission (peak at 526 nm) and long decay kinetics (half-life up to one minute) (103).

A guide to choosing aequorin-based Ca\(^{2+}\) indicators to match a biological application

As we have seen, others and we have generated aequorin-based Ca\(^{2+}\) sensors with a palette of emission hues (blue, green, yellow and red), whose Ca\(^{2+}\) affinities were also different. Redquorin was similar to aequorin in terms of Ca\(^{2+}\) sensitivity, whereas GA and CitA showed higher Ca\(^{2+}\) affinity (63). Regarding the choice of emission color, it depends on the application (Table 3). For multicolor imaging, well-separated hues are required; for in vivo work in animal tissues, red color would be advantageous, as described earlier. The properties afforded by mutagenesis and using the synthetic coelenterazines, reviewed in the previous paragraphs, can be combined with FP–photoprotein fusions to yield an enormous flexibility.

In the case of imaging high Ca\(^{2+}\) containing organelles, the prevailing Ca\(^{2+}\) concentration can vary from several micromolar to millimolar levels during physiological stimulation, depending on the cell type and the organelle (90,91,104,105). In reports of mitochondrial and ER Ca\(^{2+}\), coelenterazine-\(i\) is usually combined with aequorin mutants or aequorin-based probes of low Ca\(^{2+}\) affinity, carrying some of the mutations N28L, D117A, D119A, D163A (73,74,89–91,105–107).

Further combinations of rational site-directed mutations with coelenterazine analogues rendered a series of aequorins whose characteristics varied significantly (108). For instance, a 74 nm shift in the emission peak was observed between aequorin mutant Y82F combined with coelenterazine-\(i\) (519 nm peak emission), and aequorin mutant W86F paired with coelenterazine-\(hcp\) (445 nm). Such combinations highlight the flexibility of aequorin
when it comes to spectral tuning for multiplexing applications (for more combinations, consult (100,108)). In addition to spectral separation, time-resolved bioluminescence has also been achieved between semisynthetic aequorins with different coelenterazine analogues that showed different decay kinetics (108,109). Discrimination of native and f-aequorin was done using two separate time intervals, from 0–6 s and from 6–25 s, to resolve two different cardiovascular analyte responses in vitro. When two aequorin variants are coexpressed in cells, it is not possible to regenerate each one with a different coelenterazine analogue. In this case, time-resolved measurements can only be performed by choosing mutations conferring different decay kinetics (see Table 3, time-resolved multiplexing). In another study, redquorin was reconstituted with coelenterazine-hcp or –f to monitor spontaneous activity in single cultured cells and fast Ca²⁺ transients in zebrafish embryos, respectively, owing to their fast response kinetics (63). Use of coelenterazine-hcp or –cp, resulting in high Ca²⁺ affinity aequorin, might prevent prolonged Ca²⁺ imaging. It may be necessary to combine them with aequorin mutants of reduced affinity. Of the many possible variations of FP-photoproteins and coelenterazines, we summarize some combinations better suited for particular biological applications (Table 3).

**APPLICATIONS OF FP-PHOTOPROTEIN FUSIONS**

The addition of FP-aequorin fusions to the arsenal of aequorin probes has facilitated imaging Ca²⁺ fluxes in cell populations and in intact organisms. FP-aequorins have also allowed measurements of Ca²⁺ in single cells, in the cytoplasm and in cell compartments (48,110). To this goal has also contributed the use of modern detectors, like electron-multiplying charge-coupled devices (EMCCD), which are superseding the more difficult to use image-photon detectors.

### Table 3. Proposed combinations of Ca²⁺ photoprotein-based probes for different applications.

| Target application           | Proposed starting combinations | Observations                                                                 |
|-----------------------------|--------------------------------|-------------------------------------------------------------------------------|
| Cytosol/Nucleus             | GA; CitA; VA | Native h; f | Fusions with higher Ca²⁺ sensitivity than aequorin (start with native CLZ). Fusion with Ca²⁺ sensitivity similar to aequorin (start with a CLZ analogue to enhance light output). |
| High Ca²⁺ microdomains or organelles | GA; CitA; VA | Native h; f | Combinations allow good S/N ratio, with moderate rate of aequorin consumption. If prolonged measurements are desired, consider reducing affinity using mutagenesis (e.g. D117G). |
| Ca²⁺ release points         | GA; CitA; VA | Native h; f | Combinations allow good S/N with prolonged Ca²⁺ measurements and slow consumption rate. |
| Mitoch/ER/Golgi             | GA; CitA; VA | Native i; n | Combinations allow good S/N with low exposure times (CLZ provides fast emission kinetics and increased Ca²⁺ affinity). |
| Fast acquisition            | GA; CitA; VA | Native i; n | Combinations allow good S/N and fast kinetics with residues at COOH-terminus (CLZ provides fast emission kinetics and increased Ca²⁺ affinity). |
| Spontaneous Ca²⁺ release    | GA; CitA; VA | Native i; n | Combinations allow good S/N with prolonged Ca²⁺ measurements and slow consumption rate. |
| Multiplexing                | GA + Redquorin | Native f | Dual-color mode detection (green/red). |
| Spectrally-resolved         | Aequorin + CitA | Native f | Combinations allow good S/N with prolonged Ca²⁺ measurements and slow consumption rate. |
| Time-resolved               | Aequorin + CitA | Native f | Dual-color mode detection (green/red). |
| In vivo                     | Redquorin | Native f | Dual-color mode detection (green/red). |

**Aequorin fusions:** GA (GFP-aequorin), CitA (Citrine-aequorin), VA (Venus-aequorin), Redquorin (63), GO (GFP-obelin). Note that VA could substitute for CitA, GO has faster kinetics than GA (69). Coelenterazine (CLZ) analogues were used to increase light output for some applications. Ca²⁺ affinity: i < n < native < h < cp < f < hcp (1).

**Uses of FP–Ca²⁺ photoprotein fusions in cultured or dissociated cells**

Whole-cell signaling assay systems and high throughput screening. Aequorin and other bioluminescent probes have been extensively used to construct reporter systems of various biochemical activities, that is signal transduction, gene expression, switching on (or off) a promoter, drug discovery in medium or high throughput screening assays, or biosensing of compounds of environmental importance (111,112). Plasma membrane receptors are leading pharmacological targets. In the first steps of drug screening for a particular receptor, its functional activation or inhibition can be confirmed by measuring cytosolic Ca²⁺ rises within cells that express the receptor. In the case of GPCRs, this model is usually assisted by the exogenous co-expression of a promiscuous G-protein subunit. These types of assays are easy to
perform and permit scalability and automation, commonly desired features in medium and high throughput experiments.

Several studies use GFP-aequorin as a reporter of activation of Ca^{2+}-signaling pathways. An investigation on low affinity phospholipid agonists of the G-protein-coupled receptor GPR63 attested to the higher sensitivity of GFP-aequorin compared to other indicators of intracellular Ca^{2+} (113). When using the fluorescent indicator fluo-4 in an imaging plate reader, cells had to be transfected with the receptor GPR63 and a chimeric Gafl (q/f) in order to detect the Ca^{2+} signals. In contrast, overexpression of a promiscuous G-protein was not needed when using the BRET reporter GFP-aequorin for screening. In another functional activation assay, mammalian CHO cells and GA were employed to identify the molecular features of pelagic/benthic transition induced by environmental signals of marine annelids (114). GA from Brület has also been used in several cell lines (mainly CHO-K1 and HEK293) in the initial screening of inhibitors (115) or selective agonists (116), and in the de-orphanization of receptors (117). In a pancreatic cell line, the fusion was stably expressed to mechanistically analyze signaling pathways (118). In cultured embryonic neurons, GA was used to confirm functional activation of membrane receptors (119).

Transient receptor potential vanilloid type 1 (TRPV1) are plasma membrane Ca^{2+} channels involved in nociception. A low Ca^{2+} affinity mutated GFP-aequorin (ermutGA) was used to study the ectopic but functional TRPV1 channels expressed in the ER of dorsal root ganglion neurons (120). In a study by Ayoub et al. in 2015, Ayoub et al. studied the cytosolic Ca^{2+} dynamics of stimulated HEK293 that express FSH or LH receptors using GFP-aequorin (121). The authors cleverly combined various BRET and FRET assays to investigate the activation and regulation of FSH and LH receptors in real time, in living cells.

A bioassay was developed to screen for functional antibodies against muscarinic acetylcholine receptor M3 in sera from patients with Sjogren’s syndrome (122). Rises in Ca^{2+} were detected in response to carbachol by a GFP-Aequorin fusion expressed in CHO cells, and antibodies in patients’ sera inhibited the activation of the receptor. The luminescence assay was found to be more reliable than existing bioassays. GA was also expressed in keratinocytes (HaCaT cells) to detect cell responses to auto-IgGs from patients of the autoimmune blistering disease, pemphigus vulgaris (123).

Another fruitful application of FRET or BRET has been in detection of protease activities, by constructing fusion proteins consisting of a fluorescent or luminescent protein (energy transfer donor) followed by a protease recognition sequence, and a different fluorescent protein (acceptor). In the intact chimeras, there is energy transfer between donor and acceptor but, upon cleavage by the protease, the increased donor-acceptor distance disrupts FRET/BRET (124). Following this principle, an assay system based on GFP-aequorin was constructed to measure alpha-thrombin or caspase-3 activities in vitro and in live cells (64). GFP and aequorin were linked by peptides of 6 or 18 amino acid residues containing the protease recognition sites. A decrease in BRET was indicative of protease activation in a time and dose-dependent form. This study was the second to appear showing that the molecular fusion of GFP and aequorin resulted in highly efficient BRET, after Baubet et al. (6). These assays are sensitive enough to be used in high throughput screening and drug discovery, and are displacing widely used radiolabeled assays (125).

**Ca^{2+} studies in subcellular compartments.** Aequorin was the first GECI whose expression was targeted to subcellular compartments (89) to investigate Ca^{2+} dynamics in organelles like mitochondria, the ER or the Golgi apparatus. One example of a related use of GFP-aequorin was reported by Calvo-Rodriguez et al. (126,127). The authors employed cultures of hippocampal neurons to study the influence of aging on cytosolic and mitochondrial Ca^{2+} responses to excitotoxic stimuli, by combining fura-2 to measure cytosolic Ca^{2+} and low affinity mitochondrially targeted GFP-aequorin (mitoGA) to track mitochondrial matrix Ca^{2+}. This procedure would allow testing drugs interfering with neuron cell death in neurodegeneration and ischemia. MitoGA was also used in Ca^{2+} mobilization assays in a study of the activation of GPCR MrgX receptor by peptides (128).

GFP-aequorin fusions have been also employed to probe Ca^{2+} dynamics in nonmammalian systems, like in fission yeast (129). It was pointed out that the use of GFP-aequorin extended the recording time from 10 to 240 min, compared with using aequorin alone. A recent report on the expression of various clytins in Clytia hemisphaerica has identified a naturally occurring clytin isoform expressed in egg mitochondria (36). Whether this protein offers any advantage for use as a tool in mitochondrial studies will have to be determined.

**Applications of GAP indicators as fluorescent or bioluminescent calcium probes.** As mentioned before, GAP probes are molecular fusions of GFPuv and aequorin, able to function either as a luminescent or fluorescent biosensors for Ca^{2+} (73).

In a study using GAP as a luminescent probe, the low Ca^{2+} affinity variant GAP1 was combined with coelenterazine-n to measure Ca^{2+} in the ER and Golgi apparatus (74). This combination had eight-fold reduced decay rate and 10-fold lower affinity than GAP. Rapid aequorin consumption in high Ca^{2+} environments was prevented, allowing measurements in the ER for extended periods in several cell types.

In a report in 2016, GAP was further modified to measure resting and stimulation levels of Ca^{2+} in the ER (75). GAP probes were used here as a fluorescent reporter (there was no reconstitution with coelenterazines). Transgenic mice and flies expressing GAP2 and GAP3 were generated, and Ca^{2+} was measured in the ER of various tissues from these mice (Langerhans islets, hippocampal slices) and in muscle of living flies during electrical stimulation. Further reports using these transgenic models are expected with excitement. These biosensors, orthogonal to endogenous signaling, have produced so far results compatible with current knowledge in various physiological paradigms and thus seem robust enough to be used in cells, tissues and in vivo.

**Biological applications of red-emitting aequorins.** By far, GFP-aequorin, pioneered by Brület et al. (6), is the most widely used FP-Ca^{2+} photoprotein fusion. Their work prompted others and us to develop yellow- and red-shifted FP-aequorin fusions, as has been discussed (48,61–63). The red chimera tdTAl was well expressed in mammalian cells, reporting agonist-induced cytosolic Ca^{2+} oscillations at the single cell level (48). It was also able to report Ca^{2+} transients after K+ depolarization of cultured mouse cortical neurons. Furthermore, the progression of the Ca^{2+} wave was spatially resolved in neuronal processes and the cell body. Although tdTAl emission was bimodal, with similar peaks at 465 and 581 nm, sufficient red light was emitted to cross the skin and muscle of a mouse (recombinant protein was deposited
in muscle or in the peritoneal cavity). Redquorin is an improved variant of tdTA, sensitive enough to detect spontaneous Ca^{2+} oscillations in single cells (63). With as much as 41% of total emission above 600 nm (cf. Fig. 1), emitted light was detected across a blood sample (hemoglobin is the main absorber below 600 nm in animal tissues). Redquorin remains the aequorin-based probe with the longest emission described so far, without important changes in Ca^{2+} affinity, luminescence activity or emission kinetics compared to aequorin.

**Uses of FP–Ca^{2+} photoprotein fusions in brain slices and tissue explants**

In the applications in cultured neurons and brain slices, Ca^{2+} detected with the photoproteins acts as a proxy for neural activity. The papers reviewed investigate neural networks and circuitry, cell-to-cell relationships and communication. The use of brain or other tissue slices permits studying complex biological processes in preparations retaining the tissue architecture and many of its connections, although they usually have a relatively short lifetime after extraction from the animal. Furthermore, luminescence reporters occupy a technological niche in the domain of photosensitive tissues. In mammalian cells, they are used for studies of specific activity and wiring in the retina.

Rogers et al. targeted GA expression to report Ca^{2+} changes associated with electrical activity in subcellular domains of single neurons and for visualizing activity in tissue slices (130). The chimeras were directed to the mitochondrial matrix and to the ER using appropriate peptides, to synaptic vesicles by fusion to synaptotagmin I (SynGA), and to the postsynaptic density (fusion of GFP-aequorin with PSD-95, PSDGA). All these proteins or peptides were placed at the N-terminus of GFP-aequorin and low affinity versions were made by introducing mutation D119A in the aequorin moiety (89). The Ca^{2+} concentration within the domain where the SynGA probe was localized was higher than in the cytosol, in agreement with other studies demonstrating localized high Ca^{2+} microdomains in excitable cells. SynGA displayed a constant rate of light emission, whereas the probe localized in the postsynaptic density (PSDGA) showed random, nonsynchronized Ca^{2+} transients, behaving like a synaptic event detector. Long-term recordings of up to 13 h of continuous acquisition were achieved in brain cortical slices and in retinal explants, allowing the analysis of multicellular ensembles. In the work of Agulhon et al., a virus-delivered GA reported spontaneous networking activity in groups of Müller cells in mouse retina explants (131).

Droba et al. reported a quantitative comparison of fusions of GFP with the photoproteins aequorin, obelin, and aequorin with point mutation N26D (which conferred increased Ca^{2+} affinity of the first EF hand) (69) (Table 2). The probes, expressed in layer V pyramidal neurons in cortical brain slices by neurotranspc viral transduction, allowed detection of Ca^{2+} signals elicited by electrical stimulation. This is a thorough study showing functionality of these probes in an exquisitely sensitive preparation. Ca^{2+}-dependent bioluminescence was detectable under conditions where Ca^{2+} concentration transients reached the micromolar range (about 5 action potentials per depolarizing step). Above that Ca^{2+} level, the responses rose exponentially, consistent with the slope of 2.5 of the Ca^{2+} sensitivity curves of aequorin (132) and obelin. The physiological levels of Mg^{2+} in neurons likely contributed to decrease the Ca^{2+} sensitivity, compared to the

**FP–photoproteins expressed in whole organisms for in vivo imaging**

GECIs show their full potential when used for imaging whole organisms. Compared to fluorescent probes, bioluminescent Ca^{2+} indicators offer high sensitivity and signal-to-noise, and lack of phototoxicity. In addition, FP–photoprotein probes show no interaction with endogenous signaling proteins and low Ca^{2+} buffering effects.

**Mouse.** Rogers and cols. were able to image Ca^{2+} in intact behaving animals with a transgenic mouse that ubiquitously expresses GA targeted to the mitochondrial matrix (mtGA) (137). Therefore, this model allowed detection of subcellular-scale events (i.e. mitochondrial Ca^{2+}) while imaging at the organism scale, as mitochondria could not be individually resolved. The system, which permitted the detection of Ca^{2+} activity in freely moving newborn mice, was particularly adequate for behavioral studies, like sleep/wake cycles, providing good temporal resolution. Also, this study confirmed that Ca^{2+} is taken up by mitochondria during skeletal muscle contractions. Apoaequorin reconstitution in muscle was complete and stable from about half an hour after coelenterazine injection, and signals could be detected for up to two hours in contracting hindlimbs of adult mice. In a subsequent study, the same mouse model (mtGA) was used to validate a device for simultaneous...
measurement of luminescence and infrared light in whole body imaging (138). The system was based on an intensified CCD-photon-counting camera plus a second CCD recording infrared light to monitor animal position and movement. Ca²⁺ signals from electrically stimulated hindlimbs were recorded together with near-infrared video of the animal at 25 frames per second.

**Zebrafish.** Correlation of brain activity with locomotion has also been studied in transgenic zebrafish expressing GA (139). With the aim of noninvasively monitoring freely swimming larvae, bioluminescence was recorded with a nonimaging device, sacrificing the spatial resolution for the sake of temporal resolution. In this transgenic fish, only a small population of neurons ( hypocretin-positive neurons in the hypothalamus) expressed GA. As in the case of the organellar Ca²⁺ described above, the genetic targeting ensures that the signal originates in the cells of interest, as these cannot be spatially resolved. In this study, neuronluminescence (i.e. Ca²⁺ peaks resulting from bursts of firing neurons) was quantitatively correlated with movements of the larvae, which were simultaneously recorded by an infrared CCD camera in darkness, or by a stroboscopic illumination device to mimic natural light-dark cycles.

Muscle development in zebrafish has been extensively studied by Miller and collaborators in several articles using a transgenic fish expressing aequorin under an alpha-actin promoter (140), under normal and dystrophic conditions (141). Their work has untangled some of the essential molecules involved in Ca²⁺ signaling responsible for slow muscle differentiation (140–142). In these papers, the authors used aequorin rather than GFP-aequorin fusions.

In our recent work, zebrafish embryos were used to test the ability of reduorin to record Ca²⁺ dynamics in living organisms (63). Reduorin mRNA was microinjected into one-cell embryos; bioluminescence, mostly coming from the trunk (muscle), was imaged continuously with an EM-CCD camera for more than 12 h, including the whole segmentation period. Reduorin reconstituted with coelenterazine-fl was detectable at exposure times as short as 50 ms, resolving single twitches of embryos at 30 h post fertilization.

**Drosophila.** Transgenic *Drosophila* lines have been generated to express GA specifically either in the mushroom bodies or in ellipsoid body ring neurons of flies (143). Whole-brain bioluminescence imaging allowed registration of Ca²⁺ responses derived from spontaneous activity, K⁺-depolarization, cholinergic stimulation and trans-synaptic activation of deep cells within the brain. Unexpectedly, delayed (15 min) Ca²⁺ responses in mushroom body lobes after nicotine and acetylcholine stimulation were observed. Pharmacological intervention revealed that this delayed activity was regulated by intracellular Ca²⁺ stores. Although the possible oscillatory nature of this signal could not be resolved with this technique, this approach allowed monitoring a broad range of response timings. The role of CAMP and its effectors in nicotine-triggered Ca²⁺ responses was later investigated with the same transgenic fly model (144).

A similar transgenic system was utilized to target GA expression to olfactory receptor neurons of flies, to explore the complexity of Ca²⁺ responses to odor stimuli of different durations and their role in sensory adaptation (145). In this work, two components or phases in odor-evoked Ca²⁺ signals in olfactory receptor neurons were distinguished. Each component, mathematically deconvolved, had a different contribution of Ca²⁺ from intracellular stores and Ca²⁺ entry. In a subsequent study (146), odor-evoked signals were detected downstream in the olfactory pathway, in the mushroom bodies, confirming the strength of GA bioluminescence for functional brain mapping in vivo. Long-term, overnight recordings of spontaneous brain activity with high temporal resolution (<10 ms) were also achieved in the mushroom bodies, in glial cells or in all neurons (using a pan-neuronal promoter).

**Arabidopsis and other organisms.** The use of aequorin is very popular in plants (147,148). Bioluminescence is not affected by the strong tissue autofluorescence, because there is no need for excitation light. This is also beneficial when performing studies of photochemical properties in the mesophyll and other photosensitive tissues. Aequorin is also pH-insensitive, which is really convenient in plant cells, where signaling pathways include proton extrusion and changes of cytosolic pH.

In a methodological paper, Xiong and cols. reported the use of GA for Ca²⁺ imaging in plant tissues (149). To our knowledge, this is the first report on the use of a chimeric FP–protein in plants. A cooled-CCD camera was sufficient to detect weak darkness-induced Ca²⁺ signals and Ca²⁺ responses occurring in the leaves when the roots were challenged with 200 mM NaCl, causing osmotic stress. The authors compared the performance of aequorin and GA in intact plants and showed that GA behaved significantly better. GA allowed imaging the propagation of slow Ca²⁺ waves at the leaves with good spatial and temporal resolution, enough to determine the speed of the signal through the leaf veins.

In protozoa studies, GA was expressed in purified gametocytes of *Plasmodium berghei* to study the role of PKG in Ca²⁺-driven gametocyte activation upon xanthurenic acid stimulation (150). Although in the literature there are some studies with Ca²⁺ photoproteins in fungi, to our knowledge, none uses FP–photoproteins.

**CONCLUDING REMARKS**

The studies we have reviewed demonstrate the versatility of photoprotein-based Ca²⁺ indicators. Various colors (from blue to red) are now available using the BRET fusions. Other properties, like Ca²⁺ sensitivity, thermostability, total light capacity or emission kinetics, can be further adjusted for particular uses by mutagenesis, and by choosing one of an ever increasing collection of synthetic coelenterazines (85). Opposite characteristics may be required for different applications, that is, whereas for GPCR ligand screening, slow decay mutants are preferred (101,108), probes with fast kinetics are needed for recording Ca²⁺ transients in subcellular locations in neurons (69,151) or in studies of muscle contraction.

In addition to the extensively used synthetic Ca²⁺ indicators, a large number of fluorescent and luminescent probes compose the GECI toolbox (4), each with its own advantages and drawbacks. Ca²⁺-sensitive photoproteins are ideally suited for continuous long-term registration in minimally invasive studies (63,146). As drawbacks, exposure times longer than in fluorescence are usually needed because of their low photon yield. This reduces temporal resolution, which may be required to track fast Ca²⁺ dynamics on behaving animals. The FP–photoprotein chimeras have alleviated this shortcoming, because of increased protein
stability in cells, raised quantum yield and luminescence capacity, and because animal tissues are more transparent to the red-shifted emission variants. A further disadvantage used to be the complexity of working with photon-counting detectors. New detectors such as EM-CCDs have shown improvements and the recent market production of multipixel photon counters, also known as silicon photomultipliers, is expected to enable lower-level light detection and even photon counting with a semiconductor device.

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