

THE THEODOR BÜCHER LECTURE

Investigating signal transduction with genetically encoded fluorescent probes**Delivered on 22 October 2002 at the 28th FEBS Meeting in Istanbul****Tullio Pozzan^{1,2}, Marco Mongillo² and Rüdiger Rudolf¹**¹*Department of Biomedical Sciences, CNR Institute of Neurosciences, University of Padua, Italy;* ²*Venetian Institute for Molecular Medicine, Padua, Italy*

Ca²⁺ and cAMP are ubiquitous second messengers in eukaryotes and control numerous physiological responses ranging from fertilization to cell death induction. To distinguish between these different responses, their subtle regulation in time, space and amplitude is needed. Therefore, the characterization of the signalling process requires measurement of second messengers with tools of precise localization, high dynamic range and as little disturbance of cell physiology as possible. Recently, fluorescent proteins of marine jellyfish have given rise to a set of genetically encoded biosensors

which fulfil these criteria and which have already led to important new insights into the subcellular handling of Ca²⁺ and cAMP. The use of these probes in combination with new microscopical methods such as two-photon microscopy now enables researchers to study second messenger signalling in intact tissues. In this review, the genetically encoded measurement probes and their origin are briefly introduced and some recent insights into the spatio-temporal complexity of both Ca²⁺ and cAMP signalling obtained with these tools are discussed.

Second messengers and the encoding paradigm

Cells are structures of immense spatio-temporal complexity. Myriads of different proteins, fatty acids, carbohydrates, and other organic and inorganic compounds are being produced, degraded or transported at each moment. To enhance their reliability and efficiency, these processes are organized in a highly complex and dynamic system of subcellular compartments. These compartments not only include membrane-enclosed organelles such as the endoplasmic reticulum (ER), mitochondria, etc., but are also generated through the discrete localization of the signalling processes within the apparently homogeneous cytoplasmic environment. In addition, cells are continuously bombarded by signals released by closely located or even distant cells. To translate these extracellular signals into intracellular responses, all eukaryotic cells utilize complex transducing machineries. Such machineries are assembled with transmembrane receptors that sense external stimuli and a series of intracellular relay proteins, the sequential activation of which leads to the

modulation of the levels of a few compounds named second messengers. These are typically very small molecules such as inositol 1,4,5-triphosphate (IP3), Ca²⁺ or cyclic nucleotides. Given that numerous extracellular signals modulate the level of the same intracellular second messenger a problem of specificity obviously arises. Specificity of the signalling through second messengers is achieved by a complex encoding of (a) the localization of second messenger molecule release (compartmentalization); (b) the number of molecules released (amplitude); and (c) its temporal release pattern (frequency). In this way, a plethora of input signals, which may come from hormones, neurotransmitters, cell surface molecules, growth factors or other signalling molecules, is computed intracellularly to give rise to very specific cellular responses, ranging from fertilization to induction of cell death, and including a vast amount of physiological processes such as secretion, contraction, growth and proliferation, to name only a few. Obviously, to exert all these processes the signalling must be finely tuned in a highly dynamic manner. Thus, to study second messenger signalling it is crucial to measure their distribution *in vivo* and under physiological conditions. Furthermore, as signal compartmentalization plays an important role, one ideally would need to analyse levels of second messengers simultaneously, in different subcellular compartments and with a high spatial and temporal resolution. No such ideal system is presently available, but the new tools that have been introduced in the last few years have enormously expanded our possibility to monitor the dynamics of intracellular events in the living cell. In the following paragraphs we will briefly discuss the characteristics of a few genetically engineered probes generated to monitor second messenger levels in living cells, and discuss some of the newest information on cell physiology that has been obtained with these new tools.

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Abbreviations: AEQ, aequorin; cAMP, adenosine cyclic-3',5'-monophosphate; cGMP, guanosine cyclic-3',5'-monophosphate; ER, endoplasmic reticulum; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; IP3, inositol 1,4,5-triphosphate; PKA, protein kinase A.

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***Aequorea victoria*, a jellyfish, sheds light on cell signalling**

Bioluminescence, i.e. the active emission of visible light by living cells, occurs in a wide variety of organisms ranging from bacteria to insects and vertebrates [1]. Of special interest for the current review is the bioluminescence of some coelenterates, in particular that of the medusa *Aequorea victoria*. Shimomura *et al.* sampled these medusae at the marine station of Friday Harbor and described a squeezeate obtained from the organism which luminesced in a Ca^{2+} -induceable manner [2]. Interestingly, the purified protein solution did not emit green light, as does the living medusa or the fresh squeezeate, but emits blue light [2]. Shimomura and coworkers concluded from these experiments that the blue light emitted by the photoactive Ca^{2+} -dependent protein, which they called aequorin (AEQ), would either be filtered or taken up and converted into green light by a second, acceptor protein. This in turn would be sterically very close to AEQ in the medusa, but far away in the squeezeate, and therefore incapable of photon uptake and transformation. Hence AEQ would directly emit the light in its original wavelength, which is blue. Subsequent research strengthened this idea and it was proposed that in the medusa AEQ transmits its energy in a radiation-less manner to the acceptor, a process called fluorescence resonance energy transfer (FRET) [3]. In the case of aequorin, the energy results not from the absorbance of a photon, but from the oxidation of the coenzyme, coelenterazine, and thus from a Ca^{2+} -induced conformational change of the apoprotein. The acceptor protein has been called Green Fluorescent Protein (GFP) [3,4]. In fact, with AEQ, GFP and the wide distribution of the knowledge of FRET, *Aequorea* has revolutionized the world of cell biology and second messenger signalling research. While GFP is the great star of our time (see below), AEQ has a much longer history in laboratory research.

Aequorin, a highly versatile Ca^{2+} indicator

Shortly after the description of its Ca^{2+} -dependent luminescence, microinjected purified AEQ protein was used for intracellular Ca^{2+} measurements [5] and for years it has been one of the tools preferred by physiologists interested in Ca^{2+} handling [6,7]. After its sequencing [8], AEQ could also be genetically expressed in target cells, and thereby previous major difficulties with AEQ protein purification and microinjection could be overcome. A major breakthrough in the use of AEQ came, however, from the possibility of targeting the recombinant protein to different intracellular locations with high selectivity. Because chimeric AEQ also retained its Ca^{2+} -dependent luminescence capacity, it made it possible for the first time to measure Ca^{2+} levels specifically in different cellular organelles such as the ER [9–11], mitochondria [12–15], Golgi [16,17], etc. The use of this technique not only refined our understanding of the cellular Ca^{2+} handling but also led to the discovery of new, unexpected phenomena (see below).

Without the specific targeting of AEQ, it would have been impossible to obtain these insights, but we soon realized that AEQ had a few major drawbacks as a biosensor: (a) the necessity to supply its coenzyme,

coelenterazine, without which the protein does not emit light; (b) the consumption of the probe upon Ca^{2+} binding; and (c) the low amount of light emitted (less than one photon per molecule) [18,19]. In addition, AEQ luminescence can only be triggered by the binding of divalent cations (Ca^{2+} and Sr^{2+} in particular) and cannot be turned into a sensor for other cellular parameters. As to the first problem, coelenterazine is peroxidized upon binding of Ca^{2+} to AEQ [20], with the energy produced resulting in the release of photons of blue color [20], and the irreversible destruction of the chemiluminescent protein. If new coelenterazine is provided, the AEQ molecules can be reconstituted into new fully functional proteins [20], but this process is relatively slow (taking from minutes to hours) and this property has been rarely used for practical applications. The weak luminescence of AEQ [20] limits its utilization in microscopy at the single-cell level, and its use as a probe to obtain single-cell or suborganelle dynamics of the Ca^{2+} level has major limitations in terms of spatial and temporal resolution, at least at the present stage of development of the measuring apparatuses (and see below). Despite the above-mentioned limitations, chimeric targeted AEQs represent a milestone in the development of protein biosensors. From the methodological point of view the chimeric aequorins are in fact the first example of molecularly engineered protein probes and have opened the way to the more recent GFP-based indicators. Conceptually, as far as signalling via Ca^{2+} is concerned, chimeric AEQs have provided a lot of novel information. Particularly relevant appears for example the contribution of AEQ for the understanding of Ca^{2+} handling by mitochondria. Through the use of mitochondrial AEQ it was observed that physiological stimulation with Ca^{2+} -mobilizing agents induced increases of the Ca^{2+} level in mitochondria much larger than those reached in the bulk cytoplasm [12]. This was particularly puzzling because the Ca^{2+} -uptake mechanism was known to have a very low affinity for Ca^{2+} (reviewed in [21]). This observation could be explained only by a close, synapse-like interaction between ER Ca^{2+} -release- and mitochondrial Ca^{2+} -uptake-sites (reviewed in [14,22]). In this case, the local Ca^{2+} concentration would be expected to be much higher than in the surrounding cytoplasm, such that it could be sensed and taken up by the mitochondrial Ca^{2+} uniporter (see model depicted in Fig. 1). Later morphological studies confirmed this idea [13] and meanwhile, a close interaction between mitochondria and ER has been shown for a number of cell systems (reviewed in [23]). Notably, this close association finds its rationale in the regulation of energy metabolism [24]. As such, one major target of the Ca^{2+} spikes released by the ER and sensed by mitochondria are the Ca^{2+} -sensitive mitochondrial dehydrogenases [24,25], which modulate the ATP synthase to produce ATP [26,27].

GFP and its mutants – a colorful story

The natural partner of AEQ in *Aequorea victoria* is GFP (Table 1). Although its discovery [2] and purification [3] occurred only a few years after those of AEQ, its use in biological experiments began only 30 years later. After the publication of the GFP cDNA sequence [28], the seminal paper that made the use of GFP so widespread in the scientific community is that of Chalfie and coworkers in

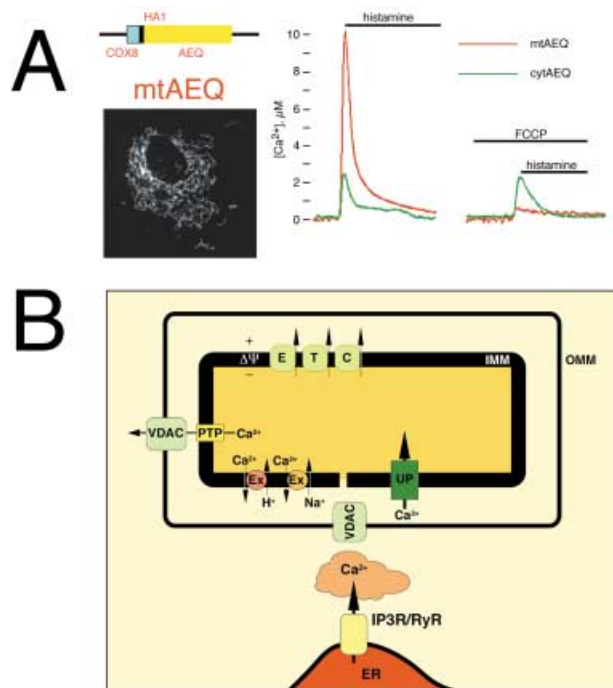


Fig. 1. Mitochondrial targeting of aequorin reveals a tight connection between ER and mitochondria. (A) The schematic representation of the mtAEQ cDNA construct is presented on the upper left. Cox8 is the targeting sequence derived from subunit 8 of cytochrome *c* oxidase and HA1 is the epitope tag derived from hemagglutinin. Below the scheme, the image of a HeLa cell transfected with mtAEQ and revealed by an anti-HA1 Ig is presented. On the upper right are presented typical kinetics of the Ca^{2+} changes elicited by histamine in the cytoplasm (green trace) or the mitochondrial matrix (red trace) in HeLa cells expressing cytosolic or mitochondrial aequorin. The uncoupler FCCP (2 mM) was added where indicated. Note that this completely prevented the increase in $[Ca^{2+}]_{i}$ in the mitochondrial matrix while leaving the cytoplasmic response practically unaffected. (B) Scheme illustrating the close functional relationship between ER and mitochondria. OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; ER, endoplasmic reticulum; UP, Ca^{2+} uniporter; PTP, permeability transition pore; VDAC, voltage dependent anion carrier; ETC, electron transfer chain. $\Delta\Psi$, mitochondrial membrane potential; IP3R, IP3 receptor; RyR, ryanodine receptor. For further information, see text and original references [12,13].

1994, where it was demonstrated that heterologously expressed GFP in *C. elegans* maintained its fluorescent properties [29]. At the beginning, rumours spread that GFP was not fluorescent when expressed in mammals (possibly due to the low rate of folding at 37 °C), but it soon became clear not only that good levels of fluorescence can be obtained in the cytoplasm of these cells, but also that the protein can fold sufficiently well at 37 °C to fluoresce even within organelles ([30], reviewed in [31,32]). GFP was thus directed to diverse subcellular compartments (reviewed in [31,32]) and this allowed in many cases for the first time, and in a very elegant, noninvasive manner, the *in vivo* study of organelle structure (reviewed in [33]), protein trafficking in the cytoplasm ([34], reviewed in [35]) and the intermembrane system (reviewed in [36]), morphology of cells and subcellular structures (reviewed in [37] and [38–41], respectively) or the ligand-induced movement of receptor-subunits (reviewed in [42]). The success of GFP in the scientific community is demonstrated by the impressive increase over the last few years in the number of papers published using this tool (Fig. 2). Surprisingly, despite being 30 kDa in size, GFP behaves rather neutrally, and in most cases the subcellular localization of membrane or soluble proteins fused to GFP was indistinguishable from that of the wild type construct. Furthermore, through the use of tissue-specific promoters it has been possible to visualize gene expression profiles and cell movement in living organisms (reviewed in [43]). To overcome the slow maturation, the relatively weak fluorescence and the unfavourable excitation wavelength of 395 nm of wild type GFP1 (Table 1), a series of mutants have been created. One of the first major improvements in these directions included the S65T mutation [44] which renders GFP considerably brighter, shortens its maturation time and shifts the major excitation wavelength from 395 nm to about 490 nm (Table 1) [44]. Interestingly, concerning the folding to its fluorescent form, this mutant still exhibits a substantial temperature-sensitivity, i.e. it folds much better at temperatures below 37 °C [45]. This temperature-dependent folding could be exploited in the unique environment of the secretory pathway for a pulse/chase-like analysis of post-Golgi vesicular trafficking in the secretory pathway [46–48].

The other key development in the GFP story was the introduction of mutants with different spectral properties.

Table 1. Major GFP mutants and their origin.

Name	Species	Based on	Principal mutations	Ex. max. (nm)	Em. max. (nm)	Ref.
Wild type GFP	<i>A. victoria</i>			395, 475	509	[2,3,28,29]
S65T		wild type GFP	S65T	495	508	[44]
EGFP		GFPmut1	S65T, F64L	488	507	[95]
ECFP		EGFP	S65T, F64L, A146I, M153T, V163A	433, 453	475, 501	[95]
EYFP		GFP-10	S65G, S72A, T203Y	515	528	[50]
Venus		EYFP	S65G, S72A, T203Y, F46L, F64L, M153T, V163A, S175G	515	528	[54]
Citrine		EYFP	S65G, S72A, T203, V68L, Q69M	516	529	[53]
Kaede	<i>T. geoffroyi</i>			380, 508, 572, 533, 508	518, 582	[60]
DsRed	<i>Discosoma</i> sp.			558	583	[58]

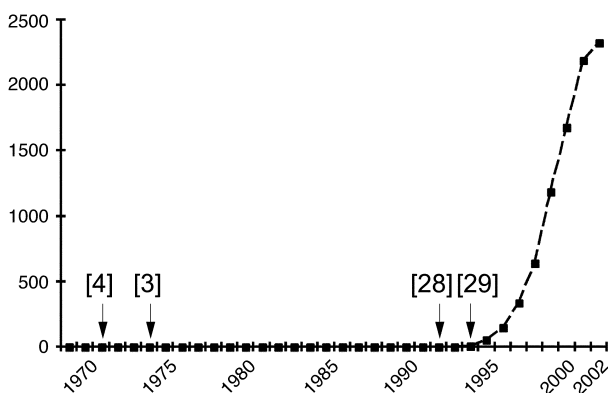


Fig. 2. Use of green fluorescent protein (GFP) in scientific research. The graph shows the number of publications per year found in Entrez browser using the search item 'green fluorescent protein'. Numbers over data points indicate key references.

While the first mutants have been generated by random mutagenesis and selection, in the following years the rational design of many more GFP mutants was greatly facilitated by the determination of the crystal structure of the protein [49,50]. This revealed that GFP exhibits a can-like structure which is made up of 11 β -strands (β -can), closed to the exterior at both ends, and which encloses the central tripeptidic chromophore [49,50]. Starting from this information, mutations affecting the chromophore, its flanking amino acids and the general structure of the protein have been created to obtain a large number of mutants with altered spectral characteristics and folding properties (Table 1) [31,32]. A major improvement was the introduction of mutation F64L in combination with an adaptation of the sequence to the mammalian codon usage, which not only improved the expression rate and the fluorescence intensity per molecule, but also greatly diminished the temperature-sensitivity of GFP [45]. This mutation combined with S65T is called enhanced GFP (EGFP) and served as basis for many later mutants. Of these, a blue-shifted, cyan fluorescent protein (ECFP) is of prime interest because together with a red-shifted, yellow fluorescent protein (EYFP) it is able to undergo FRET pairing, and this fact has already been extensively exploited to construct FRET-based biosensors for, e.g. Ca^{2+} , cyclic nucleotides, and activity of enzymes (see below, reviewed in [32,51,52]).

Further improvements in the basic features of GFP and of its mutants that have found large practical applicability include reduction in the pH- and Cl^- -sensitivity (Citrine and Venus) [53,54] (Table 1) and increased speed of folding (Citrine and Venus) [54]. The most recent among the various mutants of *Aequorea* GFP is a photoactivatable GFP (PA-GFP) [55], which exploits a characteristic of wild type GFP, namely its photoisomerization. Photoisomerization occurs when a chromophore can attain different fluorescent states according to its electric charge. In the case of PA-GFP, which is based on a codon-optimized version of wild type GFP and contains the mutation T203H [55], intense illumination of the protein with light of wavelength about 400 nm leads to photoisomerization of the chromophore predominantly to the anionic form [55]. This in turn increases its fluorescence when illuminated at 488 nm by about 50–

100-fold [55]. By this means, a portion of PA-GFP expressed in a specific subcellular compartment can be lit by photoactivation and then followed in a pulse/chase-like manner.

Already in the early days of bioluminescence research, GFPs were reported in cnidarian species other than *Aequorea* ([56], reviewed in [31,32]). In recent years, it has become evident that many cnidaria such as corals contain GFP-like proteins. Interestingly, in these animals living more or less close to the water surface, their function is most probably to protect the sensitive photosynthetic systems of the endosymbiotic algae, the zooxanthellae, from the strong incident sunlight [57]. Meanwhile the number of sequenced GFPs and GFP-like proteins, all exhibiting the peculiar β -can protein structure, has risen to 27 [57], which is helping to clarify the still partially obscure cnidarian relationships [57]. Apart from the interesting phylogenetic analyses, the ongoing evolutionary and ecophysiological GFP research also gives deeper insights into the factors influencing GFP fluorescence, and has generated new, important tools for cell biology such as a red fluorescent protein, DsRed, from the coral *Discosoma* sp. [58] (Table 1). Interestingly, until now even extensive mutagenetic work on the GFP from *Aequorea* was not able to produce a mutant fluorescing in the red. Such a marker, in turn, would greatly facilitate multiple labelling together with the already available mutants (Table 1). Therefore, the publication of DsRed [58] was highly welcome and the initial problem of its obligate tetramerization has recently been overcome by genetic engineering [59]. Under serendipitous circumstances, a second highly interesting fluorescent protein tool has been produced only very recently in a Japanese laboratory [60]. In the search for new fluorescent proteins with interesting spectral characteristics, the researchers happened to leave two aliquots of a preparation of a green fluorescing protein from the coral *Trachyphyllia geoffroyi* on the laboratory bench (Table 1). One of the aliquots was covered, and the other exposed to sunlight. The following day, the exposed sample had changed its major absorption peak from 508 nm to 572 nm and had turned red [60]. In a systematic investigation, the capacity of this protein to undergo photoconversion, i.e. a light-induced colour-switch, has been reproduced and the protein has been named Kaede (Japanese for maple leaf) [60]. It was shown that photoconversion of Kaede is irreversible and efficient upon illumination with UV light of about 365 nm wavelength [60]. Currently, the tetrameric structure of Kaede is still hampering its widespread use [60], but molecular engineering will almost certainly amend this problem. In summary, the previous and ongoing research work, including crystal-structure inferred positional mutagenesis, development of specific experimental protocols and discovery of new fluorescent proteins, have been and are still furnishing researchers with a highly potent colour box allowing them to view organisms, cells and organelles simultaneously in different colours.

GFP-based biosensors

The availability of spectrally different and highly stable variants of GFP has triggered the production of molecularly engineered biosensors capable of monitoring biochemical events taking place inside cells or organelles in terms of quantifiable changes of fluorescence. Three classes of

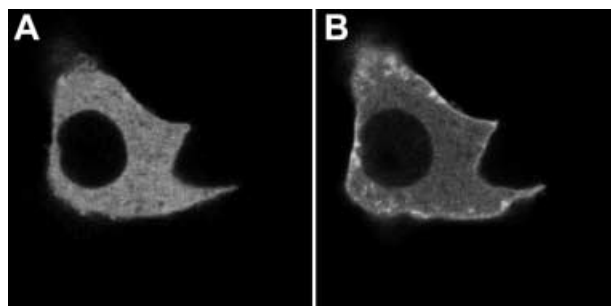


Fig. 3. Effect of carbachol on PKC β localization in MIN6 pancreatic cells. Cells were transfected with a construct where EGFP was fused at the N-terminus of PKC β . Shown is the PKC β distribution at rest (left panel) and at the peak of the response (right panel). For a more detailed description see [34]. Pictures were kindly provided by P. Pinton (Department of Experimental and Diagnostic Medicine, Section of General Pathology, University of Ferrara, Italy).

biosensors have been generated thus far: (a) GFP fusion proteins that change their subcellular distribution upon modification at the level of an intracellular second messenger. Based on this principle, sensors for diacylglycerol, Ca²⁺ and IP₃ have been introduced and characterized (e.g. Fig. 3). (b) A second class makes use of a single GFP molecule that is genetically modified to be directly sensitive to the fluctuations of an intracellular parameter, e.g. pH or Ca²⁺. (c) A third class of biosensors exploits fluorescence FRET as a way of making GFPs biochemically sensitive to different intracellular events, e.g. Ca²⁺, cGMP or cAMP or protein phosphorylation; due to lack of space in the following paragraphs we will concentrate primarily on the biosensors for Ca²⁺ and cAMP that take advantage of FRET, though some of the recently developed single GFP probes will be also mentioned.

FRET is a quantum-mechanical event occurring when two fluorophores are placed in close proximity (< 100 Å) and the emission spectrum of one fluorophore, acting as a donor, overlaps the excitation spectrum of the second fluorophore, that acts as an acceptor. Under these circumstances, part of the energy of the excited state of the donor is transferred to the acceptor, which emits light at its own wavelength [61]. By exciting the donor while simultaneously recording and calculating the ratio of the donor and acceptor emissions, one can therefore estimate FRET changes in a way suitable for most cellular imaging with both conventional and confocal systems. The first GFP mutants used as an appropriate FRET couple were BFP and GFP [44,62,63], which were then replaced in most applications by the brighter and more stable CFP and YFP [52,63].

The common framework of FRET-based biosensors is therefore based on a sequence that will respond to the changes in some intracellular parameter with a conformational change that in turn changes the mutual distance between the two GFP partners [52]. FRET-based biosensors thus report a biochemical event in terms of reciprocal distance between the GFP partners, without perturbing either of the GFPs [52]. Although this phenomenon gives rise to a highly selective readout for chromophore vicinity, FRET-based probes are necessarily large fusion products. To partially overcome this hindrance, a conceptually

different class of bioprobes has been developed whose mechanism relies on the capability of a messenger-induced conformational change to directly modify the photochemical properties of a single GFP.

Ca²⁺ biosensors

As we mentioned above, one of the limitations of AEQ is its low luminescence, which hinders the use of this method in most single-cell experiments. Only in very large cells, such as oocytes [64] or muscle fibres [5] that can be microinjected with large amount of proteins, was the signal strong enough to allow a good time resolution of the [Ca²⁺] change. All other attempts to image an AEQ signal in small mammalian cells need prolonged time integration (> 1 s) and have a limited spatial resolution [65,66]. Conversely, fluorescent Ca²⁺ dyes are very bright and allow single-cell imaging at fast sampling rate, though their selective targeting to subcellular compartments is generally unsatisfactory. To gain insights into the encoding mechanism of Ca²⁺ signals, tools are therefore required that are suitable for single-cell imaging and combine high spatial and temporal resolution. An additional need, to further dissect Ca²⁺-transduction pathways, is the possibility of measuring the concentration of the ion within the different cellular compartments. Genetically encoded Ca²⁺ sensors based on GFP have been generated to fulfil these requirements. By fusing them to organelle-specific targeting sequences, such probes can be used to assess local variations of Ca²⁺ with a high sensitivity and a specificity unattainable with image analysis, thereby combining the targetability of recombinant proteins (pioneered by the chimeric AEQs) with the brightness of fluorescent dyes.

Two families of FRET-based Ca²⁺ probes have been almost contemporarily introduced [67] by the groups of Tsien [63] and of Persechini [68] but only the former have then found a wide applicability in cell biology. These probes, also known as 'Cameleons' (Table 2) [63], have been engineered by exploiting Ca²⁺-sensitive conformational changes of a tandem fusion product sandwiched between two flanking GFP mutants [63]. The Ca²⁺-sensing sequence has been designed by fusing the Ca²⁺-binding domain of calmodulin to its target peptide M13 [63]. Two of the natural properties of calmodulin have been exploited, namely its Ca²⁺ sensitivity and the high affinity of the Ca²⁺-bound form for its substrate, M13. When Ca²⁺ binds calmodulin, the latter wraps M13, thereby engaging CFP and YFP in FRET [63]. Further genetic engineering has provided cameleon variants targeted at the nucleus [63], ER [63], mitochondrial matrix [69], the cytoplasmic side of vesicular membranes [70] and the plasma membrane [70,71]. Cameleons can also be tuned to sense different Ca²⁺ concentrations, by mutating amino acids within the Ca²⁺-binding domain or by substituting M13 with a different target peptide for calmodulin. Given that the physiological Ca²⁺ range is highly dissimilar in diverse cellular compartments [72], such capability is crucial to guarantee the value of the probe [63,69].

Genetic targeting also provides specificity at a molecular level to the measurements in any one compartment. Comparative experiments with cameleons fused to caveolar and non-caveolar plasma membrane domains, showed that

Table 2. GFP-based sensors of second messengers.

	Fluorescence source	Excitation λ (nm)	Emission λ (nm)	Subcellular targeting	Transgenes	Ref.
Calcium						
Cameleon	CFP/YFP CFP/Venus CFP/Citrine	430	480/535	cytosol ER mitochondria golgi nucleus plasma membrane caveolae secretory granules	<i>Drosophila</i> <i>C. elegans</i>	[63,80,96]
Ratiometric pericam	cpYFP	415/490	525	cytosol ER mitochondria nucleus subplasmalemmal space		[77]
Camgaroo	YFP		525	cytosol		[76,97]
Cyclic nucleotides						
cAMP sensor	CFP/YFP	430	480/535	RII RII del		[87,88]
Cygnets	CFP/YFP	430	480/535			[89]
Protein kinases activity						
AKAR	CFP/Citrine	430	480/535			[79]
Tyrosine kinase	CFP/Citrine	430	480/535			[91]
Phocus	CFP/YFP	430	480/535			[98]

caveolae may be a compartment involved in the regulation of store-operated Ca^{2+} entry [71]. Such an observation would be very difficult to obtain with other tools, as it requires a spatial resolution higher than the maximum currently achievable with fluorescence imaging.

Importantly, cameleons are also suitable for ratiometric imaging with two-photon excitation [73–75], which enables detection of Ca^{2+} -signalling processes in thick tissues and living organisms. It is therefore expected that relevant new aspects of Ca^{2+} regulation will soon be uncovered in experimental conditions much closer to the physiological ones.

As mentioned above, one of the drawbacks of cameleons (and more generally, of all FRET-based indicators) is their big molecular size. To partially solve this hassle, smaller probes have been designed by exploiting other biochemical properties of GFPs. The unravelling of the relationship between structure and function of GFPs has provided a crucial observation. Despite the highly ordered structure of GFPs, the product of a complex and precise processing pathway (reviewed in [31,32]), the protein tolerates the insertion of sequences at certain locations surprisingly well [76]. In fact, insertion of calmodulin in place of Y145 of a YFP results in camgaroos (Table 2), whose fluorescence is modified by Ca^{2+} binding [76]. In other words, the Ca^{2+} -dependent conformational change of the calmodulin graft hosted in the pouch (hence the name camgaroo) of YFP makes the latter sevenfold brighter.

More recently, the circularly permuted variant of GFP (cpGFP) has been identified as the preferred fluorescent reporter for constructing probes based on a single protein [76] (Table 2). cpGFP is a GFP in which the amino and

carboxy termini are linked together by a flexible spacer, and new amino and carboxy termini can be created along the original sequence. A biochemically sensitive sequence can therefore be easily inserted into the new amino and carboxy termini. Because the β -barrel structure of GFP bears the chromophore in its centre, it would be expected that such modification would be irreconcilable with the preservation of fluorescence. The outcome of such engineering is instead a fully fluorescent GFP that shows enhanced sensitivity to alterations of the environment surrounding the chromophore [76], making it well suited to serve as the basis for a sensor. Ca^{2+} probes known as pericams have been generated, by inserting the yellow circularly permuted variant of GFP (cpEYFP) between CaM and its target peptide M13 [77]. By introducing subtle mutations in the amino acids close to the chromophore, three variants of pericams have been obtained that display different Ca^{2+} -dependent behaviours. Flash pericam becomes brighter [77], inverse pericam dims [77] whereas ratiometric pericam shifts its excitation spectrum upon binding Ca^{2+} [77]. An assortment of targeted pericams is now available that can be used to probe Ca^{2+} in various subcellular compartments, e.g. the nucleus [77], mitochondria [77] and the subplasma membrane region [34]. For example, employment of these probes in spontaneously beating cardiac myocytes from neonatal rats has revealed unambiguously that mitochondria sense cytoplasmic Ca^{2+} -rises in a beat-to-beat fashion [15], while microdomains of high Ca^{2+} in the subplasma membrane cytoplasmic rim has been monitored in pancreatic β -cells stimulated with glucose [34]. The circularly permuted variant of GFP has been exploited also by Nakai *et al.* to develop a different Ca^{2+} probe based on a single GFP (G-CaMP) [78]. A limit of such a sensor is the requirement

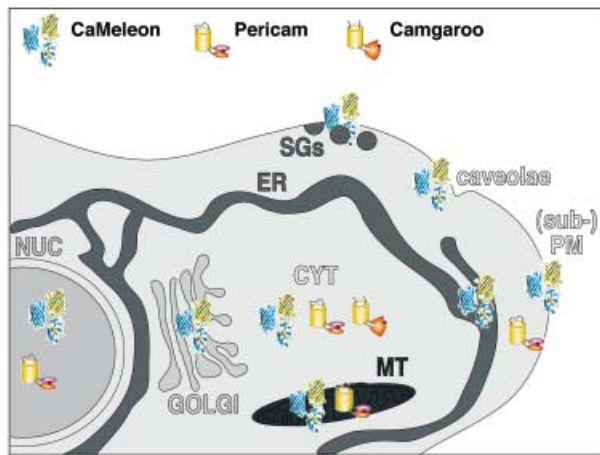


Fig. 4. Targeting of GFP-based calcium sensors. CYT, cytoplasm; ER, endoplasmic reticulum; MT, mitochondria; NUC, nucleus; PM, plasma membrane; SGs, secretory granules. For explanations and references see text.

of folding at temperatures below 37 °C, but its advantage resides in the high signal-to-noise ratio [78].

Although all the tools reviewed here allow interesting experimental approaches in Ca^{2+} signalling, some restrictions still linger. The major of these concerns is that all biosensors utilize calmodulin as the conformationally sensitive domain [78,79]; as a high expression level is unavoidably required to achieve a sufficient fluorescent signal, buffering of intracellular Ca^{2+} and following interference of the exogenous calmodulin with Ca^{2+} homeostasis can be predicted [80]. Such limitation is also witnessed by the difficulty encountered in obtaining transgenic animals expressing these Ca^{2+} biosensors. Despite the potential interest, the only transgene animals at present are *Drosophila* [81–84] and *C. elegans* [85], and in both cases inducible or tissue-specific expression is mandatory. Figure 4 presents a cartoon depicting the available GFP-based probes for monitoring Ca^{2+} at the subcellular level.

At present, a single comprehensive tool to investigate Ca^{2+} dynamics does not exist. It should be also stressed here that the GFP-based Ca^{2+} indicators should always be considered as an alternative to the classical fluorescent dyes such as fura-2, etc. The latter are still preferable in a number of conditions, due to their strong signal and easiness to load in cells. One should therefore choose carefully among the methods available the one that best fulfils one's experimental needs.

Cyclic nucleotide sensors

Although most of the elements of cyclic nucleotide-mediated signalling pathways have been studied extensively, much is still to be uncovered on the dynamics of these ubiquitous second messengers within cells. Indirect evidence indicates that the spatial and temporal modulation of cAMP and cGMP concentrations encode the output signal in a finely regulated manner. The characterization of this aspect of signalling requires methods of direct investigation suitable for living cell experiments, and such tools have been unavailable until recently. As a

consequence, the conception of biosensors for cyclic nucleotides, that could be used to measure cAMP and cGMP selectively and with high spatial and temporal resolution, has been a primary concern for the investigators of this field of signal transduction. To develop such a methodology, researchers have made use of 'physiological' resources, by using cyclic AMP- or GMP-dependent protein kinases as natural sensors for cAMP and cGMP, and FRET phenomenon between GFPs as a reporter.

cAMP-dependent protein kinase (PKA) occurs as a holotetramer of two cAMP-sensing regulatory subunits and two catalytic subunits. Binding of cAMP to the regulatory subunits elicits a conformational change that causes the catalytic subunit to dissociate from the complex. The first FRET-based cAMP biosensor (that used chemically labelled regulatory and catalytic subunits of PKA) was introduced in 1990 by Tsien's group, but the technical difficulties in its use have severely limited its widespread use [86]. The more recent probes, on the other hand, are based on molecular engineering. In particular cyan and yellow mutants of GFP have been fused to regulatory and catalytic subunits, respectively, so that cAMP-induced dissociation between the two is detected as FRET changes (Table 2). By using this probe, we have shown that microdomains of high cAMP are generated in cardiac myocytes when a β -adrenergic stimulus is applied [87,88]. Most notably, because the regulatory subunit of such a probe maintains affinity for endogenous PKA-binding proteins, the sensor is already targeted to physiologically relevant compartments in the cell. By generating a variant of the sensor lacking the PKA-anchoring protein (AKAP) binding domain, we have shown how targeting is essential for the kinase to sense hormone-induced rises in cAMP [88].

A probe for cGMP named 'Cygnet' has been generated on the same basis [89] (Table 2). A protein kinase G sandwiched between CFP and YFP responds to elevation in cGMP concentration with a conformational change that is sufficient to abolish FRET between the two fluorophores. The validity of this approach has been confirmed in cell lines as well as Purkinje neurons, in which responses to cGMP-raising agents have been successfully recorded [89].

A new class of FRET-based probes, which serve as protein kinase activity reporters, has recently seen the light. The first such sensor was designed by Nagai *et al.* [90] but this approach has been exploited mainly in Tsien's laboratory [52]. The novelty of these latter biosensors resides on the modularity of the design strategy [79,91]. The common framework on which this family of sensors is based is made of a consensus substrate sequence for the kinase of interest, fused to a flexible linker and a phosphorylated substrate domain between two flanking GFPs [79,91]. The conformational change induced by the phosphorylation of the substrate brings the partner GFPs in close contact thereby permitting FRET generation [79,91]. On this module, by selecting appropriate consensus sequences, reporters of serine/threonine kinases A, B and C have been designed, as well as reporters of Abl and Src tyrosine kinases [52,79,91] (Table 2). By using the reporter for kinase A activity, Zhang *et al.* have shown that colocalization of a substrate and PKA can modulate its susceptibility to phosphorylation by the kinase, emphasizing the essential role of PKA-anchoring in signal transduction [79].

Finally, FRET between GFP chimeras can also be exploited to study protein–protein interactions, that can serve as readouts of the signalling pathways (e.g. [92]). Compared to biochemical or biophysical methods, such fluorescent protein-based methodology offers the unique possibility of visualizing the interaction of partner proteins with nanometer resolution within their cellular environment (e.g. [93]). There is no prototypical example of this experimental strategy, but rather a wide collection of data elegantly collected thus far, and such use of FRET in combination with recent microscopy techniques will surely uncover novel mechanisms of regulation of the diverse cell functions [94].

In conclusion, over the last 10 years the armoury for studying the dynamics of different aspects of cell physiology has increased tremendously. At present we can follow in a living cell the kinetic changes of many second messengers, the redistribution of proteins, the dynamic organization of organelles, the traffic of proteins, etc. The prototypes of these biosensors have been the chimeric aequorins that have unravelled novel aspects of Ca^{2+} signalling and have introduced the concept of targeted probes. The discovery of GFP and its mutants, and the molecular engineering of a number of constructs from them, has made possible the dissection at the single-cell level of many aspects not only of cell signalling processes, but also of general phenomena of cell biology. This review is by no means comprehensive and has been mostly concentrated on problems that have concerned the laboratory of the authors. We apologise to those colleagues whose important contributions have not been quoted.

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