1
Probing Calcium Ions with Biosensors

JUNICHI NAKAI[1,2] AND MASAMICHI OHKURA[1,3]

[1]Department of Information Physiology, National Institute for Physiological Sciences, Myodaiji, Okazaki, 444-8585, Japan, [2]Laboratory of Neuronal Circuit Dynamics, Brain Science Institute, RIKEN, 2-1 Hirosawa,Wako-shi, 351-0198, Japan and [3]Department of Pharmacology, Kyushu University of Health and Welfare, Yoshinocho, Nobeoka-shi, 882-8508, Japan

Introduction

It is well known that calcium ions (Ca^{2+}) play important roles in cellular functions, such as muscle contraction, secretion, and controlling enzyme activities and membrane potentials. Hence, scientists have measured intracellular Ca^{2+} concentrations ([Ca^{2+}]) in cells. To date, several methods have been developed in order to measure [Ca^{2+}], which include ion-selective electrodes, Ca^{2+} indicator dyes, and biosensors. Amongst them, it is now most popular to use Ca^{2+} indicator dyes such as fura-2, which were developed in the early 1980s (Tsien *et al.*, 1982), because application of acetoxy methyl forms of Ca^{2+} indicator dyes makes loading of the dyes to cells much easier, and because Ca^{2+} imaging is made possible by using Ca^{2+} indicator dyes. The development of Ca^{2+} indicator dyes is indeed one of the big steps in biology. However, these Ca^{2+} indicator dyes occasionally cannot satisfy some particular scientific needs. For example, it is not easy to target Ca^{2+} indicator dyes to specific intracellular organelles, or conversely, unexpected accumulations of the dyes to cells and organelles are occasionally observed. In addition, Ca^{2+} indicator dyes can be extruded from cells. Finally, cell-type specific loadings of Ca^{2+} indicator dyes are not possible, except for some special cases.

Since green fluorescent protein (GFP) was cloned from the jellyfish *Aequorea victoria* (Prasher *et al.*, 1992), it has been applied intensively in the biological field as a marker or a reporter protein. In addition, it is now possible to make several different types of Ca^{2+} probes (biosensors) containing GFP. Because these Ca^{2+} probes are genetically encoded, it becomes possible to express the probes in specific cells or

*To whom correspondence may be addressed (jnakai@brain.riken.go.jp)

Abbreviations: [Ca^{2+}], Ca^{2+} concentration; cpGFP, circularly permutated GFP; CRET, chemiluminescence resonance energy transfer; ER, endoplasmic reticulum; FRET, fluorescent resonance energy transfer; GFP, green fluorescent protein; HEK 293 cell, human embryonic kidney 293 cell; K_d, apparent molar dissociation constant; RFP, red fluorescent protein; YC, yellow cameleon.

Biotechnology and Genetic Engineering Reviews – Vol. 20, December 2003
0264–8725/03/20/3–21 $20.00 + $0.00 © Intercept Ltd. PO Box 716, Andover, Hampshire, SP10 1YG, UK

Table 1.1. Categories of genetically encoded Ca^{2+} probes

1.	**Chemiluminescence group**	
	Chemiluminescence probe	aequorin
	CRET probe	GFP–aequorin fusion protein
2.	**Fluorescence group**	
	FRET probe	cameleon, FIP
	Single-chromophore probe	camgaroo, G-CaMP, pericam

specific organelles. Moreover, it is also possible to generate transgenic animals expressing Ca^{2+} biosensors.

In this review, we will discuss the recent advances in genetically encoded Ca^{2+} probes, as well as the advantages versus the disadvantages of each probe.

Chemiluminescence and fluorescence Ca^{2+} probes

Several kinds of genetically encoded Ca^{2+} probes are currently available. They can be categorized into two groups (*Table 1.1*). The first group utilizes chemiluminescence. This type of probe is based on aequorin, which is a Ca^{2+} binding protein capable of generating light by itself. Therefore, probes of this group require no excitation light source for measurements. Within the group, the chemiluminescence probes, including aequorin and its mutants, and the chemiluminescence resonance energy transfer (CRET) probes, including GFP–aequorin fusion proteins, can be found.

The second group utilizes fluorescence of GFP, so that the excitation light source is essential. So far, all probes within this group use calmodulin, which is a Ca^{2+} binding protein, as a Ca^{2+} sensor. This group can be subdivided into two types: the fluorescent resonance energy transfer (FRET) probes, and the single-chromophore probes. The FRET-type probes, that include cameleons and FIPs, were developed first, followed by the development of the single-chromophore probes, in which camgaroos, G-CaMP, and pericams are included. In the following sections, we will describe the characteristics of each Ca^{2+} probe.

AEQUORIN

Aequorin, which is a chemiluminescence probe in itself, was the first indicator used to monitor intracellular Ca^{2+} changes within cells (Ridgway and Ashley, 1967). Since then, aequorin has been studied extensively, and many applications have been reported. Aequorin, which is isolated from the jellyfish *Aequorea*, is composed of a 21 kDa apoprotein (apoaequorin) and a prosthetic group, coelenterazine (*Figure 1.1*). Apoaequorin consists of 189 amino-acid residues, and has three Ca^{2+} binding sites ('EF hands'). The cDNA of apoaequorin was cloned more than 10 years after aequorin was first used in a biological field (Inouye *et al.*, 1985). Both apoaequorin and coelenterazine are essential for function as a Ca^{2+}-sensing photoprotein.

Aequorin can be introduced into cells by either microinjection of aequorin protein (Miller *et al.*, 1994), or by its expression from cDNAs . Here, we will focus on the cDNA expression method. Introduction of cDNA can be achieved by conventional methods, such as transfection. When the cDNA is expressed in the cells, apoaequorin is synthesized rapidly in the cytosol. Aequorin can be reconstituted in the cells from apoaequorin, oxygen, and coelenterazine, which is added extracellularly. Coelente-

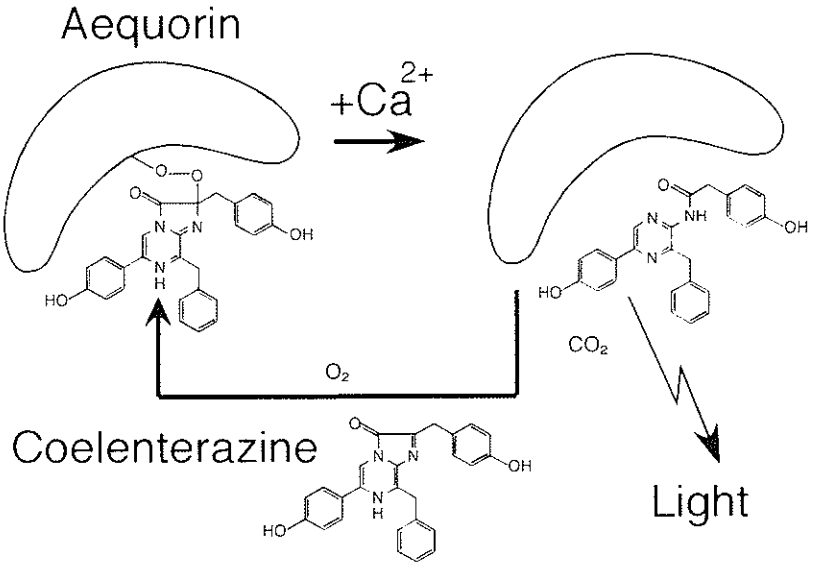

Figure 1.1. Schematic mechanism of acquorin. Aequorin can be reconstituted in cells from apoaequorin, the prosthetic group coelenterazine, and O_2. When two of the three Ca^{2+} binding sites (EF hands) are occupied, covalently bound coelenterazine, that is the chromophore, is dissociated from apoaequorin and emits blue light. The reaction is irreversible, and a single aequorin molecule emits a single photon only once because bound coelenterazine is consumed by the reaction with Ca^{2+}.

razine incorporated into cells binds covalently to apoaequorin via a peroxide linker, and is situated in the binding cavity located at the centre of the apoaequorin molecule (Head *et al.*, 2000). When two of the three Ca^{2+} binding sites are occupied (Tsuji *et al.*, 1986; Shimomura, 1995), aequorin is decomposed into apoaequorin, an active form of coelenterazine and CO_2 (*Figure 1.1*). The active form of coelenterazine subsequently emits blue light, of broad spectrum, with an emission peak at approximately 460 nm. Although pH has less effect on the luminescence, temperature, ionic strength, and Mg^{2+} have some effects on the luminescence (Blinks *et al.*, 1978).

The stability of aequorin in cells depends on the presence of coelenterazine, such that apoaequorin expressed in the cytosol is not stable, with a half-life of approximately 20 min (Badminton *et al.*, 1995). Reconstitution of aequorin with coelenterazine extends its half-life, with no significant decrease in stability observed over 6 hours. Fusion of other molecules with aequorin also extends its half-life. For example, luciferase–apoaequorin and nuclear-targeted apoaequorin (nucleoplasmin–apoaequorin) have longer half-lives than apoaequorin of 80–90 minutes and 60 minutes, respectively (Badminton *et al.*, 1995).

Recombinant aequorin can be targeted to intracellular organelles by fusion of a signal sequence at the N-terminus of the aequorin protein. Aequorins targeted to the nucleus, endoplasmic reticulum (ER), Golgi, plasma membrane, mitochondrial matrix, mitochondrial intermembrane space, and mitochondrial outermembrane are currently available (Rizzuto *et al.*, 1992, 1994; Brandenburger *et al.*, 1999; Brini *et al.*, 1999). For measurement of high $[Ca^{2+}]$ in ER or mitochondria, an aequorin which had lower

Ca^{2+} sensitivity (aequorin-119, apparent dissociation constant K_d 260 μM, which was obtained from the calibration curve relating free Ca^{2+} to light emission) was constructed by introduction of a point mutation at a Ca^{2+} binding site (D119A) (Kendall *et al.*, 1992). In addition, the Ca^{2+} sensitivity of aequorin can be decreased by using Sr^{2+}, which is known as the surrogate cation (Montero *et al.*, 1995), or by reconstitution with a modified coelenterazine analogue (Barrero *et al.*, 1997).

Advantages of aequorin

1) Aequorin has a wide dynamic range (10^{-7}–10^{-1} M). Therefore, it can respond to large Ca^{2+} rises without saturation. 2) Aequorin has a steep Ca^{2+} response curve, such that aequorin emits 10^5 times more photons when the [Ca^{2+}] changes from 10^{-7} M to 10^{-1} M. 3) Aequorin has a low Ca^{2+} buffering capacity. In general, binding of Ca^{2+} to a Ca^{2+} probe (in this case, aequorin) possibly interferes with subsequent intracellular processes. However, aequorin hardly interferes with the processes due to the large K_d for Ca^{2+} (13 μM) (Kendall *et al.*, 1992). 4) Low background signal can be obtained from aequorin. Because autoluminescence, in contrast to autofluorescence, is absent in most organisms, together with the steepness of the Ca^{2+} response curve, small Ca^{2+} changes can be detected easily. 5) Aequorin is not toxic. It does not interact with other intracellular molecules. 6) There is no photodamage, because an excitation light source is not required.

GFP–AEQUORIN FUSION PROTEINS

GFP–aequorin fusion proteins (Baubet *et al.*, 2000), which belong to the CRET probes, were constructed (*Figure 1.2*) in order to improve the low quantum yield of aequorin. By fusion of GFP to the N-terminus of aequorin, the energy released from aequorin upon Ca^{2+} binding was radiationlessly transferred from the chromophore (coelenterazine) to GFP (CRET), and the emission peak shifted from 460 nm (aequorin alone) to 509 nm. The emission peak also became sharper with the bandwidth (between low and high wavelengths at 50% values of the maximum emission), decreasing from 108.3 ± 20.1 nm to 56.0 ± 3.3 nm (pG5A construct). For targeting to the synaptic terminal, the full-length synaptotagmin I (SG5A), or its transmembrane part (tSG5A), was fused to the N-terminus of the GFP–aequorin fusion protein. SG5A was unexpectedly expressed, not only at the synaptic terminal, but also in the plasma membrane. On the other hand, tSG5A was trapped in the Golgi system.

Advantages of GFP–aequorin fusion proteins

The same advantages described above for aequorin also apply to the GFP–aequorin fusion proteins. In addition, there are three further advantages. The first is that the GFP–aequorin fusion proteins emit much more light than aequorin: use of GFP–aequorin fusion proteins will therefore facilitate the reduction of exposure times, and thus achieve higher spatial resolution. The second advantage is that the expression patterns can be followed by GFP fluorescence, which can be detected easily by conventional fluorescent microscopes. The third advantage is that GFP–apoaequorin

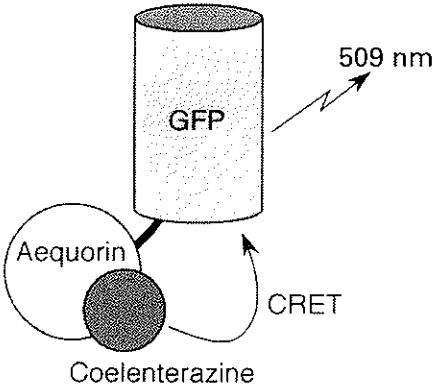

Figure 1.2. Schematic mechanism of GFP–aequorin fusion protein. In this protein, GFP is fused to the N-terminus of aequorin. This fusion protein also needs reconstitution with coelenterazine. When Ca^{2+} binds to aequorin, energy released from coelenterazine is transferred to GFP (CRET), resulting in the emission of green, rather than blue, fluorescence. The reaction is also irreversible. The signal of this probe is much larger than that from aequorin.

is suggested to have a longer half-life in the cytosol than apoaequorin alone. Although Ca^{2+} sensitivity, half-life of the protein in the cytosol and the amplification rate in light-emitting activity of the GFP–aequorin fusion proteins compared with aequorin alone have not been analysed in detail, there is a possibility that the GFP–aequorin fusion proteins replace aequorin.

Disadvantages of aequorin and GFP–aequorin fusion proteins

1) Aequorin and the GFP–aequorin fusion proteins require reconstitution with coelenterazine. Although the reconstitution of normal aequorin with normal coelenterazine *in vitro* takes 22 minutes for 50% regeneration (Shimomura *et al.*, 1993), *in vivo* this procedure takes at least 1 hour. In addition, ER- or mitochondria-targeted aequorin needs additional treatment with ionophores, or other agents, to reduce the local $[Ca^{2+}]$. Moreover, the difficulty of reconstitution becomes a serious limitation for *in vivo* application. 2) Aequorin emits low-intensity light, because of the fact that one aequorin molecule emits only one photon. In contrast, fluorescent probes can emit many more photons from a single molecule, due to the ability of the fluorescent probes to be re-excited. In addition, the quantum yield of aequorin is only 0.23 (Shimomura and Johnson, 1969, 1970), and contrasts with the value for GFP (0.72). This is not a limitation for cell population study, but is for single cell study. The GFP–aequorin fusion proteins will reduce this limitation. 3) Aequorin is consumed by the reaction with Ca^{2+}. With native aequorin, most of the photoprotein is consumed when $[Ca^{2+}]$ rises above a micromolar level for more than a few seconds. Therefore, the chemiluminescence probes are not ideal for long-term recording.

CAMELEONS

In 1997, Miyawaki and colleagues developed Ca^{2+} probes called cameleons (Miyawaki

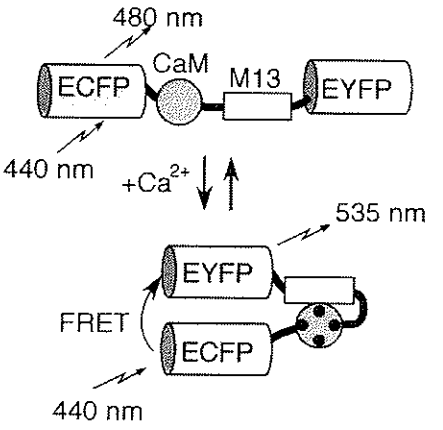

Figure 1.3. Schematic mechanism of yellow cameleon (YC). Cameleons belong to the FRET probes and are composed of a donor GFP (ECFP for YC) and an acceptor GFP (EYFP for YC) connected with calmodulin (CaM) and the M13 peptide. Without Ca^{2+}, the donor GFP absorbs 440 nm light and emits 480 nm blue light. When Ca^{2+} binds to calmodulin, the Ca^{2+}–calmodulin complex subsequently binds to the M13 peptide, resulting in a shortening of the distance between the two GFPs. Because FRET efficiency is proportional to $1/r^6$, where r is the inter-chromophore distance, shortening of the inter-chromophore distance increases FRET efficiency, so that fluorescence at 535 nm increases, and fluorescence at 480 nm decreases. These processes are reversible. When Ca^{2+} dissociates from calmodulin, the fluorescence signal at 535 nm decreases, and the fluorescence at 480 nm increases.

et al., 1997), which use FRET technology. Cameleons have two GFP variants with different colours; for example, one is a donor GFP, which emits blue light (CFP), and the other is an acceptor GFP, which is excited by the blue light and emits yellow light (YFP). An important thing about the GFP pair is that the emission wavelength of the donor GFP should overlap with the excitation wavelength of the acceptor GFP, so that FRET takes place. These GFPs are connected to each other with calmodulin and the M13 peptide of myosin light chain kinase to make a single molecule (*Figure 1.3*). When Ca^{2+} binds to calmodulin, Ca^{2+}–calmodulin subsequently binds to the M13 peptide, which has a binding capability to calmodulin. This interaction changes the distance and geometry between the two GFP variants, resulting in increase of the FRET efficiency, so that the energy acquired by the donor GFP (CFP for yellow cameleon, YC) is not emitted as blue fluorescence, but instead it is transferred efficiently to the acceptor GFP (YFP for YC) to emit yellow fluorescence. These processes are reversible; therefore, when Ca^{2+} is released from calmodulin, the change of the distance and geometry between two GFP variants decreases the FRET efficiency, resulting in an increase of the blue fluorescence and a corresponding decrease of the yellow fluorescence. Hence, this probe forms a single excitation-dual emission probe as indo-1, and ratiometric analysis is possible. The original cameleon has a biphasic Ca^{2+} binding curve with K_ds of 80 nM and 2 μM: it can therefore cover a wide range of Ca^{2+} changes. Two mutations of the Ca^{2+} binding sites in calmodulin – E31Q or E104Q – reduce Ca^{2+} sensitivities of cameleons, such that the K_d of cameleon 3, possessing the mutation E104Q, is 4.4 μM, and the K_ds of cameleon 4, having the mutation E31Q, are 83 nM and 700 μM. By using these cameleons having different K_ds, the measurement of $[Ca^{2+}]$ in ER and mitochondria becomes possible.

After the original report of cameleons, several improvements were carried out. One of the improvements concerned the pH sensitivity of the original cameleons. Because fluorescence of many GFP mutants is pH sensitive in such a way that the fluorescence becomes brighter at higher pH, pH changes also affect the emission ratio of cameleon: yellow cameleon (YC) is particularly pH sensitive (with a pK_a of 6.9 and a Hill coefficient of 1.0). This pH sensitivity was improved in YC2.1 (Miyawaki *et al.*, 1999), in which the pH-sensitive enhanced YFP (EYFP) was replaced by an EYFP mutant having a reduced pH sensitivity (pK_a 6.1, Hill coefficient 0.9). Although this EYFP mutant (EYFP V68L/Q69K) improved the pH sensitivity of YC, it became poorly fluorescent at 37°C. To improve the folding efficiency of EYFP V68L/Q69K, YC2.12 (Nagai *et al.*, 2002) was constructed where EYFP V68L/Q69K was replaced with a fast-maturation mutant of EYFP known as '*Venus*'. The relative fluorescence intensity at 37°C of *Venus* was about 30 times brighter than that of EYFP. Using *Venus*, Ca^{2+} signals from cameleon 2.12 were recorded from brain slices incubated for only 4 hours after introduction of the cDNA by a particle gun. In addition, *Venus* revealed not only fast maturation, but also lower sensitivities to Cl^- (K_d for $Cl^- > 10^4$ mM) and pH (pK_a 6.0). To improve the pH sensitivity of YC, another EYFP mutant *Citrine* (pK_a 5.7, see the camgaroos section below) was also used for the construction of YC2.3 and YC3.3: these were more resistant to both pH and Cl^- than YC2.1 (Griesbeck *et al.*, 2001).

Increasing the dynamic range of the fluorescence emission ratio represents another important improvement of the Ca^{2+} probe. By using a fragment from calmodulin-dependent kinase instead of the M13 peptide, the dynamic range of fluorescence emission ratio of YC6.1 (Truong *et al.*, 2001) becomes *c.* twice that of YC2.1 (at least *in vitro*).

In addition, '*Split YC2.1*' is another cameleon, which also has a large dynamic range. *Split YC2.1* is composed of two proteins, ECFP–calmodulin and M13–EYFP (Miyawaki *et al.*, 1999). Without Ca^{2+}, these two molecules dissociate completely. With Ca^{2+}, ECFP–calmodulin binds to M13–EYFP, resulting in an increase of the FRET efficiency. The dynamic range of *Split YC2.1* is ~4 *in vitro*; however, it is reduced to ~2.5 *in vivo*, because of interactions with native calmodulin and the M13 peptide. Although distribution of cameleon was homogeneous in the cytosol, M13–EYFP and ECFP–calmodulin of *Split YC2.1* distributed unevenly, and migration of ECFP–calmodulin was observed when [Ca^{2+}] increased.

Although both the BFP–GFP and the CFP–YFP pairs were first used for the construction of cameleons, thus far it has been the ECFP–EYFP combination (for YC) which has been the most extensively studied, since it is this pair which emits bright fluorescence with the longer wavelength. Hence, several donor–acceptor GFP combinations for YC are currently available (*Table 1.2*). Using the same strategy, red cameleons were constructed (Mizuno *et al.*, 2001), after red fluorescent proteins (RFPs) had been cloned (Matz *et al.*, 1999). In red cameleons, *DsRed*, which is an RFP, is used as an acceptor and EYFP V68L/Q69K (YRC2), ECFP (CRC2) or Sapphire (SapRC2) is used as a donor. Although these cameleons have K_ds for Ca^{2+} of 0.2–0.4 μM due to the intact calmodulin, SapRC2 seems to be more useful than the other two cameleons. Because *DsRed* has a broad absorption spectrum, excitation light at wavelengths of approximately 440 nm (for CRC2) or 488 nm (for YRC2) both excited *DsRed* to some extent. By contrast, light at approximately 400 nm, which

Table 1.2. Characteristics of genetically encoded Ca^{2+} probes

Probe	Probe name	GFP used	Excitation and emission wavelength (nm)	K_d and (parentheses) Hill coefficient values for Ca^{2+}	References
Aequorin	Aequorin	–	–; 460	13 μM	Kendall, 1992
	aequorin-119	–	–; 460	260 μM	Kendall, 1992
GFP–aequorin	G5A	EGFP	–; 509	13 μM[a]	Baubet, 2000
Cameleon	cameleon 1	BFP-GFP[b]	370; 440/510	70 nM (1.8), 11 μM (1.0)	Miyawaki, 1997
	cameleon 2	EBFP-EGFP	370; 440/510	70 nM (1.8), 11 μM (1.0)	Miyawaki, 1997
	cameleon 3	EBFP-EGFP	370; 440/510	4.4 μM (0.76)	Miyawaki, 1997
	cameleon 4	EBFP-EGFP	370; 440/510	83 nM (1.5), 700 μM (0.87)	Miyawaki, 1997
	YC2	ECFP-EYFP	440; 480/535	70 nM (1.8), 11 μM (1.0)	Miyawaki, 1997
	YC2.1	ECFP-EYFP	440; 480/535	100 nM (1.8), 4.3 μM (0.6)	Miyawaki, 1999
	YC3.1	ECFP-EYFP	440; 480/535	1.5 μM (1.1)	Miyawaki, 1999
	YC2.12	ECFP-Venus	440; 480/535	100 nM (1.8), 4.3 μM (0.6)[c]	Nagai, 2002
	YC2.3	ECFP-*Citrine*	440; 476/528	100 nM (1.8), 4.3 μM (0.6)[c]	Griesbeck, 2001
	YC3.3	ECFP-*Citrine*	440; 476/528	1.5 μM (1.1)[d]	Griesbeck, 2001
	YC6.1	ECFP-EYFP	440; 480/535	110 nM	Truong, 2001
	YRC2	EYFP-RFP	480; 535/565	0.2–0.4 μM	Mizuno, 2001
	CRC2	ECFP-RFP	440; 480/565	0.2–0.4 μM	Mizuno, 2001
	SapRC2	Sapphire-RFP	400; 510/565	0.2–0.4 μM	Mizuno, 2001
FIP	FIP-CB_{SM}	BGFP-RGFP	380; 440/505	1.5 μM (3.9)	Romoser, 1997
	FIP-CA_3	BGFP-RGFP	380; 440/505	100 nM (1.8)	Persechini, 1997
	FIP-CA_9	BGFP-RGFP	380; 440/505	280 nM (2.1)	Persechini, 1997
Camgaroo	camgaroo-1	EYFP[b]	490; 515	7 μM	Baird, 1999
	camgaroo-2	*Citrine*	516; 529	5.3 μM (1.2)	Griesbeck, 2001
G-CaMP	G-CaMP	EGFP[b]	488; 509	235 nM (3.3)	Nakai, 2001
Pericam	flash-pericam	EYFP[b]	490; 514	0.7 μM (0.7)	Nagai, 2001
	inverse-pericam	EYFP	490; 514	0.2 μM (1.0)	Nagai, 2001
	ratiometric-pericam	EYFP	418/494; 514	1.7 μM (1.1)	Nagai, 2001

[a] K_d was estimated from aequorin.
[b] These probes are poorly expressed at 37°C.
[c] K_d and Hill coefficient were estimated from YC2.1.
[d] K_d and Hill coefficient were estimated from YC3.1.

most effectively excites Sapphire, does not excite *DsRed*. Moreover, SapRC2, which uses a Sapphire–*DsRed* pair, is pH resistant, because both Sapphire (pK_a 5.5) and *DsRed* are pH resistant (Miyawaki and Tsien, 2000).

FIPS

FIPs are another type of FRET probe. These probes also use two GFP variants, which have different colours. FIPs are excited at 380 nm light, and fluorescence is observed at 440 nm and 505 nm. In contrast to cameleon, there is only the M13 peptide connected between the two GFP variants (*Figure 1.4a,b*). One of the probes, FIP-CB_{SM} (*Figure 1.4a*), does not have calmodulin (Romoser *et al.*, 1997). Hence, it uses native calmodulin in the cytosol. On the other hand, FIP-CA_3 and FIP-CA_9 (*Figure 1.4b*) have a modified calmodulin at the C-termini of the probes (Persechini *et al.*, 1997). Whether or not they have calmodulin in their molecules, they use calmodulin–M13 interaction for detection of Ca^{2+}. When calmodulin binds to the M13 peptide, the distance between two GFP molecules becomes longer so that the FRET efficiency

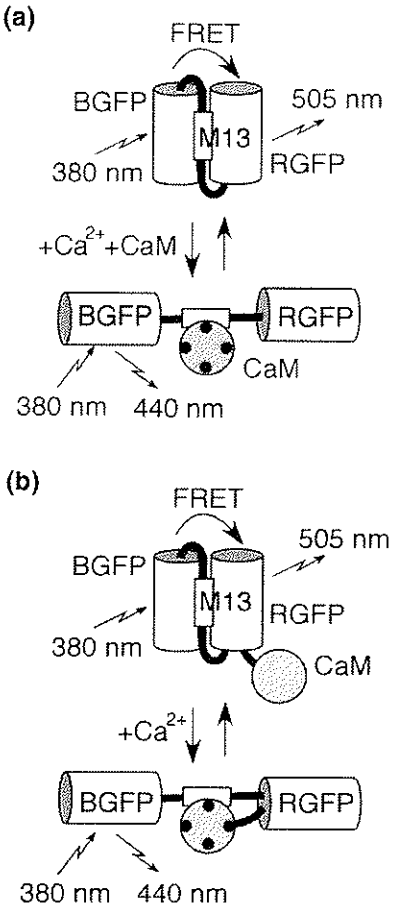

Figure 1.4. Schematic mechanisms of FIPs. FIPs are another FRET probe. Like cameleons, they also have two GFP variants with different colours (BGFP and RGFP), and use the interaction between the M13 peptide and calmodulin for Ca^{2+} detection. Two GFPs are connected with only the M13 peptide. There are two types of FIPs that have been constructed. One of the probes (FIP-CB_{SM}) does not include calmodulin, so that it interacts with native calmodulin inside cells (a). By contrast, the other probes (FIP-AC_3 and FIP-CA_9) have a modified calmodulin molecule at the C-termini of the probes (b). Without Ca^{2+}, FIPs are excited by 380 nm light and emit 505 nm light due to FRET. Whether calmodulin is included in the probes or not, binding of Ca^{2+} to calmodulin makes the Ca^{2+}–calmodulin complex interact with the M13 peptide, resulting in an increase of the distance between two GFPs. This decreases FRET efficiency, and FIPs emit 440 nm light. These processes are reversible.

decreases, resulting in decrease of the fluorescence ratio F_{509}/F_{440}. FIP-CA_3 has a K_d for Ca^{2+} of 100 nM and FIP-CA_9, in which point mutations have been introduced at a Ca^{2+} binding site, has K_d of 280 nM. From stopped-flow experiments, the dissociation kinetics of FIPs have been shown to be biphasic, and the k_{off} values for FIP-CA_3 and FIP-CA_9 (saturated by Ca^{2+}) have been shown to be 0.63 and 7.6 s^{-1} and 1.4 and 34.4 s^{-1}, respectively. Association kinetics are also biphasic: the rate-limiting fluorescent transitions appear to be much slower than 50 s^{-1} (Persechini *et al.*, 1997). The pH

dependence of FIP-CA_3 and FIP-CA_9 was tested over the pH range from 6.5 to 7.4. Fluorescence ratio changes induced by changing pH are mostly observed at low Ca^{2+} concentrations. Although pH affects the fluorescence ratios of the probes, the K_ds for Ca^{2+} are not significantly affected (Persechini *et al.*, 1997). The Cl^- sensitivity of FIPs has not yet been reported.

Advantages of cameleons and FIPs

1) The fluorescence is much brighter than that from single-chromophore Ca^{2+} probes. 2) Ratiometric measurement allows us to estimate [Ca^{2+}] more accurately than single-wavelength measurement.

Disadvantages of cameleons and FIPs

1) Fluorescent ratio changes of the FRET probes are relatively smaller than those for the single-chromophore Ca^{2+} probes. However, new probes such as YC6.1 have large fluorescence ratio changes. 2) FRET probes use two GFPs (for example, CFP and YFP). By contrast, the single-chromophore probes use only one GFP. Therefore, more probes can be used in cells simultaneously by using the single-chromophore approach. 3) Some of the probes have pH and/or Cl^- sensitivities.

CAMGAROOS

Camgaroo-1 is the first Ca^{2+} probe to be developed which is composed of a single chromophore (YFP). GFP has a chromophore surrounded by 11 β strands which create a β-can structure. Instead of connecting calmodulin to the N- or C-terminus of YFP, in camgaroo-1, calmodulin is inserted into the amino-acid sequence that resides in the β-can structure of EYFP (*Figure 1.5*). The mechanism of this type of Ca^{2+} probe is as follows: the binding of Ca^{2+} to calmodulin changes the conformation of the β-can structure of EYFP, which results in a shift from the non-fluorescent to the fluorescent chromophore form. Hence, camgaroo-1 is a single-wavelength Ca^{2+} probe, which is excited by light at approximately 490 nm, and which emits fluorescence at approximately 530 nm. The most attractive feature of this probe is the size of the maximum fluorescence enhancement (~7-fold), which is much larger than that of FRET-type Ca^{2+} probes (only ~2–4-fold). Because camgaroo-1 does not have a calmodulin-binding peptide, such as the M13 peptide, in the molecule, the apparent molar dissociation constant K_d for Ca^{2+} is rather mild (7 μM), and its Hill coefficient is 1.6. Interestingly, pH dependence of camgaroo-1 is different from that of the parental EYFP V68L/Q69K. Its pK_a is shifted from 6.1 to 8.9, or 10.1 in the presence or absence of Ca^{2+}, respectively.

There is no doubt that the development of camgaroo-1 represented a big step for making biosensors. Unfortunately, camgaroo-1 (Baird *et al.*, 1999), which uses EYFP V68L/Q69K, is poorly expressed at 37°C, and cannot be targeted to organelles such as mitochondria. In addition, circularly permutated EYFP V68L/Q69K becomes fluorescent only at 20°C or below. To improve on these problems, random mutations of camgaroo-1 were performed, and camgaroo-2 (Griesbeck *et al.*, 2001) was found, with a K_d of 5.3 μM, a Hill coefficient of 1.24, and a fluorescent

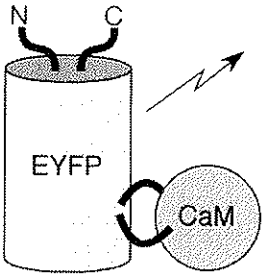

Figure 1.5. Schematic model of camgaroos. Camgaroos are single-chromophore probes based on EYFP, and incorporate calmodulin, which is connected to the outer shell of EYFP (β-can) instead of the N- or C-terminus of EYFP. When Ca^{2+} bind to calmodulin, a conformation change of Ca^{2+}–calmodulin induces a subsequent conformation change of YFP, resulting in fluorescence increase. These processes are reversible.

enhancement of ~7-fold. *Citrine*, which was a YFP variant found in camgaroo-2, had one additional mutation (Q69M) into EYFP V68L/Q69K. With this mutation, excitation and emission peaks of *Citrine* did not change much (516 nm and 529 nm, respectively, which are comparable to those of other YFPs). Instead, *Citrine*, and circularly permutated *Citrine*, became fluorescent at 37°C. The merits of *Citrine* are not only the expression at 37°C, but also proper targeting of camgaroo-2 to organelles such as mitochondrial matrix. In addition, the pK_a of *Citrine* was shifted to the left (5.7), and *Citrine* became less halide sensitive.

Advantages of camgaroos

1) Camgaroos produce large fluorescent changes. 2) Camgaroo-2 is stable at 37°C. 3) Camgaroo-2 is less pH sensitive. 4) Camgaroo-2 is Cl^- resistant.

Disadvantage of camgaroos

Ca^{2+} sensitivities are mild (between ~5.3 and 7.0 μM). Therefore, they are not ideal for measurement of sub-micromolar range concentrations of Ca^{2+}.

G-CAMP

G-CaMP (Nakai *et al.*, 2001) is another single-chromophore Ca^{2+} probe. This was the first Ca^{2+} probe in which a single, circularly permutated enhanced GFP (cpEGFP) was used. In this probe, calmodulin and the M13 peptide from myosin light chain kinase are connected to the new N- and the C-terminal ends of cpEGFP (*Figure 1.6*). Circular permutation of GFP does not change the fluorescence spectra of GFP. So, G-CaMP has excitation and emission peaks at 488 nm and 509 nm, respectively. The Ca^{2+}-sensing mechanism of G-CaMP is similar to those of camgaroos, except that it uses the M13 peptide. Without Ca^{2+}, calmodulin and the M13 peptide are dissociated from each other, and the chromophore emits a weak fluorescence. When Ca^{2+} ions bind to calmodulin, Ca^{2+}–calmodulin produces conformation changes of the β-can structure of cpEGFP, which make the chromophore more fluorescent by a factor of up to 4.5

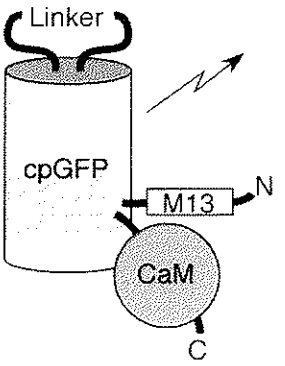

Figure 1.6. Schematic model of G-CaMP and pericam. G-CaMP and pericam are single-chromophore probes, which use circularly permutated GFPs (cpGFPs). The major difference of G-CaMP and pericam is the colour. G-CaMP is based on EGFP, whereas pericam is based on EYFP. In cpGFPs, the original N- and C-termini are connected with a short linker sequence. Both probes have the M13 peptide and calmodulin at the newly-made N- and C-termini of the cpGFPs. Binding of Ca^{2+} to calmodulin makes the Ca^{2+}–calmodulin complex interact with the M13 peptide. This interaction changes the conformation of cpGFPs, resulting in changes of fluorescence intensity. These processes are reversible.

times. Hence, G-CaMP is a Ca^{2+} probe for single-wavelength measurement. Ca^{2+} sensitivity of G-CaMP is high enough for measurement of $[Ca^{2+}]$ (K_d for Ca^{2+} is 235 nM). Because the Ca^{2+} response curve of G-CaMP is steep (Hill coefficient is 3.3), large fluorescent changes are expected at $[Ca^{2+}]$ corresponding to the K_d. Although G-CaMP does not have Cl^- sensitivity, G-CaMP is pH sensitive; its pK_a values are 7.1 and 8.1 with and without Ca^{2+}, respectively. Like EGFP, the fluorescence of G-CaMP becomes brighter at high pH. In addition to pH sensitivity, fluorescence of G-CaMP is temperature sensitive, and it is hardly fluorescent at 37°C. It becomes fluorescent at lower temperature, such as ~28–30°C. When the incubation temperature is lowered from 37 to 28°C, G-CaMP gradually becomes fluorescent. Once G-CaMP becomes fluorescent at lower temperature, it maintains its fluorescence even at 37°C for at least 24 hours.

Like other genetically encoded Ca^{2+} probes, targeted G-CaMPs have also been constructed. Thus far, G-CaMPs targeted to the plasma membrane and nucleus have become available. An example of the plasma-membrane-targeted G-CaMP expressed in a human embryonic kidney (HEK) 293 cell is shown in *Figure 1.7*. The plasma-membrane-targeted G-CaMP was specifically expressed to the plasma membrane (*Figure 1.7a*), and it responded to the application of carbachol, which induced a Ca^{2+} increase in the HEK 293 cell (*Figure 1.7b*). Under the conditions close to the plasma membrane, where calcium ion channels are expressed, fast $[Ca^{2+}]$ changes can take place. To assess whether the probe can respond to rapid $[Ca^{2+}]$ change, the reaction kinetics of G-CaMP was measured. The time constants for the association of Ca^{2+} were dependent on $[Ca^{2+}]$: they were found to be 2 ms at 1 μM $[Ca^{2+}]$ and ~200 ms at 200 nM $[Ca^{2+}]$. The time constant for dissociation of Ca^{2+} was ~200 ms, and was found to be independent of $[Ca^{2+}]$.

Recently, an improved version of G-CaMP, called G-CaMP1.6, has been developed (Ohkura *et al.*, manuscript in preparation). This new probe includes mutations for stabilizing protein folding, so that G-CaMP1.6 becomes approximately 40 times

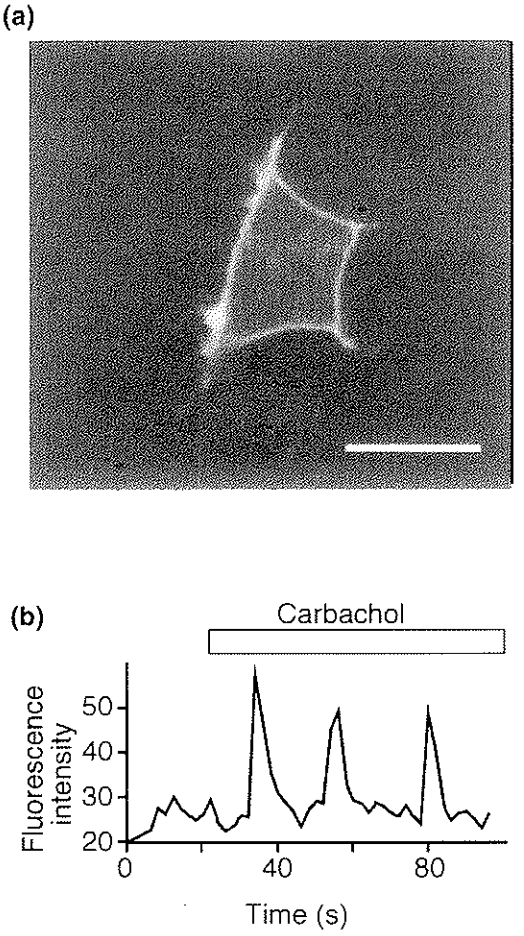

Figure 1.7. Expression of the plasma-membrane-targeted G-CaMP in a human embryonic kidney (HEK) 293 cell. Plasma-membrane-targeted G-CaMP was constructed by connecting the targeting signal from neuromodulin to the N-terminus of G-CaMP. (a) The probe was specifically expressed to the plasma membrane. This image was taken by using a confocal microscope. Bar 4 μm. (b) Fluorescence intensity changes of the probe were recorded upon bath application of 100 μM carbachol.

brighter than the original signal from G-CaMP. Although, unfortunately, G-CaMP1.6 is hardly fluorescent at 37°C, it rapidly becomes fluorescent at lower temperature, such that fluorescence can be recognized within 10 minutes at room temperature. Moreover, another new Ca^{2+} probe, which is based on CFP (therefore, it is called C-CaMP), was recently developed (*Figure 1.8*) (Ohkura *et al.*, manuscript in preparation). It is fluorescent at 37°C, and emits blue light.

Advantages of G-CaMP

1) High sensitivity to Ca^{2+} and a steep Ca^{2+} response curve represent the major advantages of this probe. 2) Fluorescent changes are large. 3) G-CaMP is Cl^- resistant.

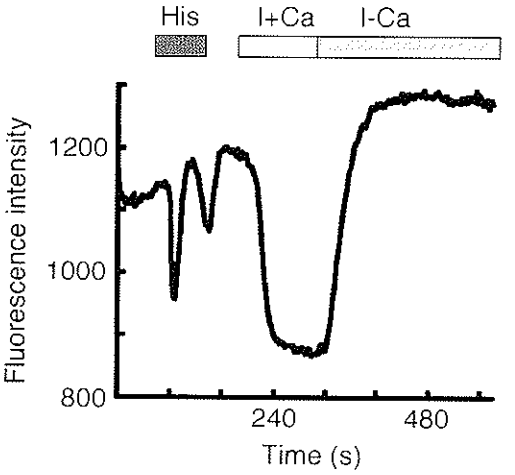

Figure 1.8. Fluorescence responses recorded from a HeLa cell expressing C-CaMP, which is a Ca^{2+} probe based on a single CFP. Like inverse-pericam, C-CaMP is the inverse type Ca^{2+} probe, such that the fluorescence signal becomes weaker upon Ca^{2+} binding. This probe was stable at 37°C. 100 μM histamine (His) and 5 μM ionomycin (I), with and without 2 mM Ca^{2+}, were applied in the bath.

Disadvantages of G-CaMP

1) Fluorescence of G-CaMP is temperature sensitive. 2) G-CaMP is pH sensitive. This pH sensitivity is slightly improved in G-CaMP1.6. 3) The original G-CaMP emits low fluorescence. This was improved in G-CaMP1.6.

PERICAMS

Pericam (Nagai *et al.*, 2001) is also the single-chromophore Ca^{2+} probe which is based on YFP. The structure of pericam is almost the same as that of G-CaMP, which uses calmodulin and the M13 peptide (*Figure 1.6*). Therefore, the molecular mechanism for Ca^{2+} detection is almost the same as that of G-CaMP. The major difference in these probes is the colour of GFP. Pericam is based on the YFP variant, EYFP V68L/Q69K, while G-CaMP is based on EGFP. Three types of pericam have been developed; these are flash-pericam, inverse-pericam, and ratiometric-pericam. Flash-pericam, of which excitation and emission peaks are around 490 nm and 514 nm, respectively, becomes brighter upon Ca^{2+} binding (K_d for Ca^{2+} 0.7 μM, Hill coefficient 0.7). By contrast, inverse-pericam, which is excited at 490~503 nm light and emits 515 nm fluorescence, becomes dimmer upon Ca^{2+} binding (K_d for Ca^{2+} 1.7 μM, Hill coefficient 1.1). Both flash-pericam and inverse-pericam are Ca^{2+} probes for single-wavelength-measurement. Whilst ratiometric-pericam (K_d for Ca^{2+} 0.2 μM, Hill coefficient 1.0) is unique, such that it shifts the excitation peak from 415 nm to 495 nm upon Ca^{2+} binding without a shift of its emission peak (at ~515 nm), so that the ratiometric-pericam is a dual excitation ratiometric indicator. The maximum ratio change values for these probes are the largest of all the single-chromophore probes (~7–10-fold). Although inverse-pericam and ratiometric-pericam are stable at 37°C, flash-pericam

is stable at 28–30°C. Like other single-chromophore probes, these pericams are pH sensitive. Interestingly, the pH dependence of flash- and inverse-pericam are biphasic. At high pH (>9–10), the fluorescent intensities of Ca^{2+}-bound flash- and inverse-pericam become dimmer, suggesting the collapse of the β-can structure. Slight Cl^- sensitivities are also expected for pericams, since EYFP V68L/Q69K, used for the construction of pericams, has been reported to have a slight Cl^- sensitivity (Griesbeck *et al.*, 2001). So far, ratiometric-pericams targeted to the nucleus and mitochondrial matrix are available. Since ratiometric-pericam is a ratiometric probe and has a large maximum fluorescence enhancement, it will be a very useful tool for Ca^{2+} measurement.

Advantages of pericams

1) Fluorescence changes are large. 2) Ratiometric-pericam allows us to estimate $[Ca^{2+}]$ more accurately. 3) The cells expressing inverse-pericam are easily identified due to the bright fluorescence in the resting condition.

Disadvantages of pericams

1) Pericams are pH sensitive. 2) Flash-pericam has temperature sensitivity and is only poorly fluorescent at 37°C.

Common disadvantages of the fluorescence-group probes

1) It is possible that the functional domain such as calmodulin interacts with intracellular molecules, and interferes with physiological functions. 2) Association and dissociation kinetics of the fluorescence-group biosensors are not as fast as those of Ca^{2+} indicator dyes. For example, the reaction kinetics of fura-2 yield a k_{on} of 6.02×10^8 $M^{-1}s^{-1}$ and a k_{off} of 96.7 s^{-1} (Kao and Tsien, 1988) and the reaction kinetics of fluo-3 yield a $k_{on} > 10^9$ $M^{-1}s^{-1}$ and a k_{off} between 550 and 200 s^{-1} (Eberhard and Erne, 1989). By contrast, the k_{on} and k_{off} values for cameleon have been reported to be 2.4×10^6 $M^{-1}s^{-1}$ and 12.9 s^{-1}, respectively. The rate constants for association of FIP-CA_3 and FIP-CA_0 have been reported to be 151 and 1.3 s^{-1} and 189 and 1.8 s^{-1}, respectively, at 150μM $CaCl_2$. The time constant for association of G-CaMP is 2 ms (at 1 μM) to >200 ms (at <200 nM), depending on $[Ca^{2+}]$. The time constant for dissociation of G-CaMP is ~200 ms. 3) If they are expressed at high concentration, they may have some toxicity as a Ca^{2+} buffer. 4) The excitation light source is essential.

Choice of Ca^{2+} biosensors

Table 1.3 summarizes the advantages versus the disadvantages of the genetically encoded Ca^{2+} probes. Probes within the chemiluminescence group have a very wide dynamic range, as well as a high Ca^{2+} sensitivity. Therefore, they are good for measurement of both small and large Ca^{2+} changes. In addition, there is almost no photodamage, and almost no interaction with other molecules. Because incorporation of coelenterazine into cells is more efficient at the tissue surface, the surface culture system is ideal for these kinds of probes. On the other hand, probes within the

Table 1.3. Advantages and disadvantages of genetically encoded Ca^{2+} probes

Category	Probe type	Advantages	Disadvantages
Chemiluminescence group	Chemiluminescence and CRET probes	1) Wide dynamic range (10^{-7}–10^{-4} M) 2) Steep Ca^{2+} response curve 3) Low background signal 4) No requirement for an excitation light source 5) No photodamage 6) There is no interaction between probes and intracellular molecules	1) Need reconstitution with coelenterazine. The probes targeted to ER and mitochondria need additional treatment for reconstitution 2) Weak signal due to a low quantum yield 3) Not ideal for long-time recording due to the consumption of aequorin
Fluorescence group	FRET probes	1) Bright fluorescence 2) Ratiometric measurement enables us more accurate estimation of $[Ca^{2+}]$	1) Fluorescent changes are relatively small 2) Need two GFPs 3) Some probes are pH and/or Cl^- sensitive
	Single-chromophore probes	1) Big fluorescence changes	1) pH and/or Cl^- sensitive 2) Some probes are poorly fluorescent at 37°C

fluorescence group are good for long time-lapse measurements and organelle-specific measurements, such as in ER or mitochondria. They are also ideal for *in vivo* experiments. These probes have different Ca^{2+} sensitivities (as represented by the K_d and Hill coefficient). Therefore, choice depends on the particular experimental condition ($[Ca^{2+}]$ and the size of the response). Phototoxicity by the excitation light, and interaction with other molecules, may sometimes become a problem with these types of probe.

Application of this technology

The most remarkable advantage of the genetically encoded Ca^{2+} probes is the availability of tissue-specific and cell-type-specific expression of the probes. Introducing Ca^{2+} probe cDNA into cells or animals can be performed by standard methods such as transfection, microinjection, electroporation, virus infection, and particle gun.

In addition, the genetically encoded Ca^{2+} probes can be expressed in specific organelles. *Table 1.4* summarizes organelle-targeted probes which are currently available. With the targeting sequence to the organelles, they can be expressed in the nucleus, ER, mitochondria, Golgi, and plasma membrane. For such a purpose, Ca^{2+} indicator dyes such as fura-2 are not ideal. Ca^{2+} indicator dyes usually exist in the cytosol, because the esterase activity, which cleaves acetoxy methyl group from the indicators, is mainly in the cytosol.

Conclusion

In this review, we have described recent advances of the genetically encoded Ca^{2+} probes. These probes are categorized into two groups; chemiluminescence probes, and fluorescence probes. The choice of the probes depends on the experimental

Table 1.4. Targeted Ca^{2+} probes

Probe	Target
aequorin	cytosol, nucleus, ER, plasma membrane, Golgi, mitochondria (matrix, outer membrane, intermembrane space)
GFP–aequorin	cytosol, synaptic terminal (plasma membrane)
cameleon	cytosol, nucleus, ER, mitochondria (matrix)
FIP	cytosol
camgaroo	cytosol, mitochondria (matrix)
G-CaMP	cytosol, nucleus, plasma membrane
pericam	cytosol, nucleus, mitochondria (matrix)

situation. The main advantage of these probes is the cell-type-specific and organelle-specific expression. Therefore, the probes have great potential for transgenic systems.

There are still several remaining drawbacks, such as reconstitution with coelenterazine, weak signal, temperature sensitivity, pH and Cl^- sensitivities, or slow reaction kinetics. It is expected that some of these drawbacks of the current probes will be improved shortly.

In addition to GFP and RFP, many other fluorescent proteins have been cloned from marine organisms. Using these new fluorescent proteins, new Ca^{2+} probes will be constructed. With those probes, which have different colours, multi-colour recording with the Ca^{2+} probe and other biosensors, such as the cAMP probe, will become possible in the near future.

Acknowledgement

We thank Ms. Yoshitomo for the artwork associated with this review.

References

BADMINTON, M.N., SALA-NEWBY, G.B., KENDALL, J.M. AND CAMPBELL, A.K. (1995). Differences in stability of recombinant apoaequorin within subcellular compartments. *Biochemical and Biophysical Research Communications* **217**, 950–957.

BAIRD, G.S., ZACHARIAS, D.A. AND TSIEN, R.Y. (1999). Circular permutation and receptor insertion within green fluorescent proteins. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 11241–11246.

BARRERO, M.J., MONTERO, M. AND ALVAREZ, J. (1997). Dynamics of $[Ca^{2+}]$ in the endoplasmic reticulum and cytoplasm of intact HeLa cells: a comparative study. *Journal of Biological Chemistry* **272**, 27694–27699.

BAUBET, V., MOUELLIC, H.L., CAMPBELL, A.K., LUCAS-MEUNIER, E., FOSSIER, P. AND BRÛLET, P. (2000). Chimeric green fluorescent protein-aequorin as bioluminescent Ca^{2+} reporters at the single-cell level. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 7260–7265.

BLINKS, J.R., MATTINGLY, P.H., JEWELL, B.R., VAN LEEUWEN, M., HARRER, G.C. AND ALLEN, D.G. (1978). Practical aspects of the use of aequorin as a calcium indicator: assay, preparation, microinjection and interpretation of signals. *Methods in Enzymology* **57**, 292–328.

BRANDENBURGER, Y., ARRIGHI, J.F., ROSSIER, M.F., MATURANA, A., VALLOTTON, M.B. AND CAPPONI, A.M. (1999). Measurement of perimitochondrial Ca^{2+} concentration in bovine adrenal glomerulosa cells with aequorin targeted to the outer mitochondrial membrane. *Biochemical Journal* **341**, 745–753.

BRINI, M., PINTON, P., POZZAN, T. AND RIZZUTO, R. (1999). Targeted recombinant aequorins: Tools for monitoring [Ca^{2+}] in the various compartments of a living cell. *Microscopy Research and Technique* **46**, 380–389.

EBERHARD, M. AND ERNE, P. (1989). Kinetics of calcium binding to fluo-3 determined by stopped-flow fluorescence. *Biochemical and Biophysical Research Communications* **163**, 309–314.

GRIESBECK, O., BAIRD, G.S., CAMPBELL, R.E., ZACHARIAS, D.A. AND TSIEN, R.Y. (2001). Reducing the environmental sensitivity of yellow fluorescent protein. Mechanism and applications. *Journal of Biological Chemistry* **276**, 29188–29194.

HEAD, J.F., INOUYE, S., TERANISHI, K. AND SHIMOMURA, O. (2000). The crystal structure of the photoprotein aequorin at 2.3 Å resolution. *Nature* **405**, 372–376.

INOUYE, S., NOGUCHI, M., SAKAKI, Y. *ET AL.* (1985). Cloning and sequence analysis of cDNA for the luminescent protein aequorin. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 3154–3158.

KAO, J.P. AND TSIEN, R.Y. (1988). Ca^{2+} binding kinetics of fura-2 and azo-1 from temperature-jump relaxation measurements. *Biophysical Journal* **53**, 635–639.

KENDALL, J.M., SALA-NEWBY, G., GHALAUT, V., DORMER, R.L. AND CAMPBELL, A.K. (1992). Engineering the Ca^{2+}-activated photoprotein aequorin with reduced affinity for calcium. *Biochemical and Biophysical Research Communications* **187**, 1091–1097.

MATZ, M.V., FRADKOV, A.F., LABAS, Y.A. *ET AL.* (1999). Fluorescent proteins from non-bioluminescent Anthozoa species. *Nature Biotechnology* **17**, 969–973.

MILLER, A.L., KARPLUS, E. AND JAFFE, L.F. (1994). Imaging $[Ca^{2+}]_i$ with aequorin using a photon imaging detector. In: *Methods in cell biology, volume 40: A practical guide to the study of calcium in living cells*. Ed. R. Nuccitelli, pp 305–338. San Diego, USA: Academic Press.

MIYAWAKI, A. AND TSIEN, R.Y. (2000). Monitoring protein conformations and interactions by fluorescence resonance energy transfer between mutants of green fluorescent protein. *Methods in Enzymology* **327**, 472–500.

MIYAWAKI, A., LLOPIS, J., HEIM, R. *ET AL.* (1997). Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* **388**, 882–887.

MIYAWAKI, A., GRIESBECK, O., HEIM, R. AND TSIEN, R.Y. (1999). Dynamic and quantitative Ca^{2+} measurements using improved cameleons. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 2135–2140.

MIZUNO, H., SAWANO, A., ELI, P., HAMA, H. AND MIYAWAKI, A. (2001). Red fluorescent protein from *Discosoma* as a fusion tag and a partner for fluorescence resonance energy transfer. *Biochemistry* **40**, 2502–2510.

MONTERO, M., BRINI, M., MARSAULT, R. *ET AL.* (1995). Monitoring dynamic changes in free Ca^{2+} concentration in the endoplasmic reticulum of intact cells. *EMBO Journal* **14**, 5467–5475.

NAGAI, T., SAWANO, A., PARK, E.S. AND MIYAWAKI, A. (2001). Circularly permuted green fluorescent proteins engineered to sense Ca^{2+}. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 3197–3202.

NAGAI, T., IBATA, K., PARK, E.S., KUBOTA, M., MIKOSHIBA, K. AND MIYAWAKI, A. (2002). A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nature Biotechnology* **20**, 87–90.

NAKAI, J., OHKURA, M. AND IMOTO, K. (2001). A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein. *Nature Biotechnology* **19**, 137–141.

PERSECHINI, A., LYNCH, J.A. AND ROMOSER, V.A. (1997). Novel fluorescent indicator proteins for monitoring free intracellular Ca^{2+}. *Cell Calcium* **22**, 209–216.

PRASHER, D.C., ECKENRODE, V.K., WARD, W.W., PRENDERGAST, F.G. AND CORMIER, M.J. (1992). Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* **111**, 229–233.

RIDGWAY, E.B. AND ASHLEY, C.C. (1967). Calcium transients in single muscle fibers. *Biochemical and Biophysical Research Communications* **29**, 229–234.

RIZZUTO, R., SIMPSON, A.W., BRINI, M. AND POZZAN, T. (1992). Rapid changes of mitochondrial Ca^{2+} revealed by specifically targeted recombinant aequorin. *Nature* **358**, 325–327.

RIZZUTO, R., BRINI, M. AND POZZAN, T. (1994). Targeting recombinant aequorin to specific

intracellular organelles. In: *Methods in cell biology, volume 40: A practical guide to the study of calcium in living cells*. Ed. R. Nuccitelli, pp 339–358. San Diego, USA: Academic Press.

ROMOSER, V.A., HINKLE, P.M. AND PERSECHINI, A. (1997). Detection in living cells of Ca^{2+}-dependent changes in the fluorescence emission of an indicator composed of two green fluorescent protein variants linked by a calmodulin-binding sequence. A new class of fluorescent indicators. *Journal of Biological Chemistry* **272**, 13270–13274.

SHIMOMURA, O. (1995). Luminescence of aequorin is triggered by the binding of two calcium ions. *Biochemical and Biophysical Research Communications* **211**, 359–363.

SHIMOMURA, O. AND JOHNSON, F.H. (1969). Properties of the bioluminescent protein aequorin. *Biochemistry* **8**, 3991–3997.

SHIMOMURA, O. AND JOHNSON, F.H. (1970). Calcium binding, quantum yield, and emitting molecule in aequorin bioluminescence. *Nature* **227**, 1356–1357.

SHIMOMURA, O., KISHI, Y. AND INOUYE, S. (1993). The relative rate of aequorin regeneration from apoaequorin and coelenterazine analogues. *Biochemical Journal* **296**, 549–551.

TRUONG, K., SAWANO, A., MIZUNO, H. *ET AL.* (2001). FRET-based *in vivo* Ca^{2+} imaging by a new calmodulin-GFP fusion molecule. *Nature Structural Biology* **8**, 1069–1073.

TSIEN, R.Y., POZZAN, T. AND RINK, T.J. (1982). T cell mitogens cause early changes in cytoplasmic free Ca^{2+} and membrane potential in lymphocytes. *Nature* **295**, 68–71.

TSUJI, F.I., INOUYE, S., GOTO, T. AND SAKAKI, Y. (1986). Site-specific mutagenesis of the calcium-binding photoprotein aequorin. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 8107–8111.