Published in final edited form as:

Methods Cell Biol. 2010 ; 99: 153–182. doi:10.1016/B978-0-12-374841-6.00006-2.

Genetically-encoded probes for measurement of intracellular calcium

Michael Whitaker

Institute of Cell and Molecular Biosciences, Medical School, Newcastle University, Framlington Place, Newcastle upon Tyne, NE2 4HH

Abstract

Small, fluorescent, calcium-sensing molecules have been enormously useful in mapping intracellular calcium signals in time and space, as chapters in this volume attest. Despite their widespread adoption and utility, they suffer some disadvantages. Genetically-encoded calcium sensors that can be expressed inside cells by transfection or transgenesis are desirable. The last ten years have been marked by a rapid evolution in the laboratory of genetically encoded calcium sensors into two families both figuratively and literally, resulting in distinct configurations of fluorescent proteins and their attendant calcium sensor modules. Here, I describe the design logic and performance of this abundant collection of sensors and describe their use and performance in vitro and in vivo. Genetically-encoded calcium sensors have proved valuable in the measurement of calcium concentration in cellular organelles, for the most part in single cells in vitro. Their success as quantitative calcium sensors in tissues in vitro and in vivo is qualified, but they have proved valuable in imaging the pattern of calcium signals within tissues in whole animals. Some branches of the calcium sensor evolutionary tree continue to evolve rapidly and the steady progress in optimising sensor parameters leads to the certain hope that these drawbacks will eventually be overcome by further genetic engineering.

1. INTRODUCTION

Small, fluorescent, calcium-sensing molecules have been enormously useful in mapping intracellular calcium signals in time and space, as chapters in this volume attest. Despite their widespread adoption and utility, they suffer some disadvantages.

All low molecular mass fluorescent cytoplasmic calcium sensors are highly-charged molecules, so cross the cell's plasma membrane very poorly. They are placed into the cytoplasm by microinjection using fine-tipped micropipette or a patch clamp pipette in whole cell mode. This limits their utility. Cell permeant fluorescent calcium sensors can be made by masking the charged carboxylic groups by forming acetoxymethyl (AM) esters. Once inside the cell, the ester bonds are cleaved, trapping the sensor in the cell. It is straightforward to bathe cells in culture with the aposensor at low concentration and these AM esters have been very widely used. One major drawback of the method is that the calcium sensor finds itself not only in the cytoplasm, but also in intracellular compartments such as the endoplasmic reticulum (ER) (Silver et al., 1992). Calcium concentrations are higher in the ER than in the cytoplasm, so this leads to a significant unwanted fluorescence signal from sensor in the ER that makes interpretation of the true cytoplasmic concentration changes difficult. It is also very challenging to use low molecular mass fluorescent calcium sensors in whole animals.

For these reasons, genetically-encoded calcium sensors that can be expressed inside cells by transfection or transgenesis are desirable. One such sensor is aequorin, a calcium-sensing protein found in the jellyfish *Aequoria victoria*. Originally, aequorin was isolated as a protein from jellyfish and placed inside cells by microinjection (Baker, 1978; Gilkey et al., 1978). More recently, a construct encoding recombinant aequorin has been used to express the aequorin apoprotein in cells directly [see chapter by Miller in this volume]. Aequorin is a luminescent molecule and at the concentrations used inside cells emits relatively few photons when compared to fluorescent molecules at appropriate excitation intensities (Varadi and Rutter Guy, 2002b). However, proteins that are fluorescent at the visible wavelengths best suited to fluorescence imaging are relatively rare. As it happens, *Aequoria victoria* also expresses a fluorescent protein, green fluorescent protein (GFP), and it is the work that has produced the variously-coloured versions of GFP that has increased our knowledge of this fluorophore and led to recombinant fluorescent calcium sensors (Tsien Roger).

The first recombinant fluorescent calcium sensors were described by Tsien and by Persechini in 1997 (Miyawaki et al., 1997; Persechini et al., 1997; Romoser et al., 1997). They were based on a concatenation of a recombinant calcium binding domain with GFP-derived fluorescent protein pairs. This approach has bred a family of these cameleon indicators, so called because they are based on a long tongue-like interaction between calmodulin (CaM) and a binding peptide and change colour (Miyawaki et al., 1997). Later, when it was realised that the GFP beta-can structure lent itself to circular permutation without loss of function (Baird et al., 1999), insertion of a calcium binding domain within the GFP (Baird et al., 1999) or concatenated to new N- or C-terminals (Nakai et al., 2001) led to a second family of calcium sensors based on the fluorescence of a single GFP-derived molecule.

The last ten years have been marked by a rapid evolution in the laboratory of these two families and their relatives, both figuratively and literally, as random mutagenesis and clonal selection in bacteria has on occasion been used to optimise the properties of the sensors (Griesbeck et al., 2001). This rapid diversification has generated continuing improvements in the performance of the sensors, but has also generated a plethora of choice. Reviews have been written to track progress in the field (Barth Alison, 2007; Demarex and Frieden, 2003; Garaschuk et al., 2006; Griesbeck, 2004; Hires et al., 2008; Kotlikoff Michael, 2007; Mank and Griesbeck, 2008; Miyawaki, 2003a; Miyawaki, 2003b; Miyawaki, 2005; Pozzan and Rudolf, 2009; Solovyova and Verkhratsky, 2002; Zacharias et al., 2000). Most of the new variants have first been tested by their makers in living cells as proof of principle rather than to answer substantial questions in biology. I shall first set out the evolution of this growing tribe of genetically-encoded calcium sensing probes, dealing with the three broad families in turn and then describe their application and utility in various biological settings.

2. GENETICALLY-ENCODED SENSORS

2.1 The cameleon family

2.1.1 Origins—The family founders were described in three papers that followed rapidly in succession in 1997. Their conception was aided by previous work in which GFP had been altered by directed mutagenesis to produce different coloured variants with altered excitation and emission spectra (Heim et al., 1995). As an aside, these differently coloured variants are sometimes referred to collectively as GFPs, though they are not green. Persechini's group described a construct (FIP-CB_{sm}) in which a red-shifted excitation variant of GFP (RSGFP (Delagrave et al., 1995), hereafter GFP) and blue fluorescent protein (BFP) are linked by a sequence that includes 17 amino acids from the calmodulin binding domain of avian myosin light chain kinase (MLCK). This novel protein indirectly senses calcium concentrations

inside cells, as when calcium increases, endogenous calmodulin becomes activated and binds to the MLCK calcium binding domain. This in turn alters the disposition of the attached GFPs and leads to changes in Förster resonance energy transfer (FRET) between the blue and green proteins (Romoser et al., 1997).

Förster resonance energy transfer is the phenomenon on which the cameleon sensor family relies. It occurs between closely apposed fluorophores that have overlapping emission and excitation spectra (Jares-Erijman Elizabeth and Jovin Thomas, 2003). In this example, the emission spectrum of BFP overlaps with the excitation spectrum of GFP. The extent of FRET depends on the degree of overlap between the two spectra, the orientation of the fluorescence dipoles and crucially, the distance between them. There is a very steep sixth power relationship with distance, so the energy transfer is very sensitive to distance between fluorophores over the range 1-10 nm (Jares-Erijman Elizabeth and Jovin Thomas, 2003). Calmodulin binds to the helical MLCK sequence by wrapping its two lobes around it (Ikura et al., 1992). In FIP-CB_{sm}, the steric bulk of the calmodulin molecule when it binds to the MLCK peptide linker forces the BFP and RFP further apart and reduces FRET (Romoser et al., 1997). FRET can be measured in a variety of ways (Jares-Erijman Elizabeth and Jovin Thomas, 2003; Visser et al.), but conceptually the simplest method is to excite the **donor** fluorophore, here BFP and measure the emission of both the donor and the **acceptor**, here GFP. FRET takes place by non-radiative energy transfer, so high levels of FRET transfer energy from BFP to GFP, reducing BFP emission at around 440 nm and increasing GFP emission at 510 nm. Calmodulin binding reduces FRET, increasing emission at 440 nm and reducing emission at 510 nm. These changes can be expressed as a ratio of emission at the two wavelengths, a value independent of the concentration of the protein. In HEK-239 cells expressing FIP-CB_{sm}, ratio changes (F_{510} / F_{440}) of around 3-4 fold could be observed after raising free intracellular calcium concentration with the calcium ionophore ionomycin (Romoser et al., 1997).

FIP-CB_{sm} relied on endogenous calmodulin to generate a calcium-sensitive FRET signal between GFPs. Tsien's construct concatenated *Xenopus laevis* calmodulin and an MLCK calmodulin binding peptide, M13 (Ikura et al., 1992), together between BFP and GFP and also in an analogous construct between two other GFP variants, enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP). In this concatenated configuration, binding of calcium to calmodulin causes it to loop back towards the M13 peptide (the cameleon's tongue) as it binds, reducing the distance between the two GFP variants and enhancing FRET (Miyawaki et al., 1997). This study beautifully exemplifies the power of the cameleon concept linked to selective mutagenesis: the original BFP/GFP construct (cameleon-1) worked well in vitro, but did not express sufficiently in mammalian cells; the enhanced variant with mammalian codon usage (EBFP/EGFP –cameleon-2) showed much improved expression, but the best expression, brightness and signal-to-noise data were seen with enhanced cyan and yellow variants of GFP (ECFP/EYFP – yellow cameleon-2). These benefits came however at the expense of a lower FRET change between calcium bound and unbound forms (1.5 vs 1.8 when expressed as a ratio of emission wavelengths) and a greater pH sensitivity. Mutagenesis can also be applied to the calcium binding affinity of the calmodulin moiety: Calmodulin has two classes of calcium binding sites and site-directed mutations in either high (K'_d 70 nM) or low affinity sites (K_d 11 μ M) give rise to constructs in which high affinity sites are suppressed to give a monotonic binding curve (K'_d 4.4 μ M: cameleon-3) or low affinity sites are altered to give an enhanced range over four orders of magnitude of calcium concentration (K'_d s of 83 nM and 700 μ M:cameleon-4). The third dimension of modification adds signal tags to the constructs. Nuclear localisation tags gave cameleon-2nu and ER localisation tags produced yellow cameleon-3er (K'_d 4.4 μ M) and cameleon-4er (K'_d s of 83 nM and 700 μ M).

Tsien's seminal paper also exemplifies some challenges in the approach: on the one hand the complexities of permutation and combination of mutant variants and their concomitant properties and on the other, the relatively low magnitude of FRET modulation by calcium over a very wide range of concentrations. The subsequent proliferation of family members results from attempts to improve brightness and dynamic range, but at the expense of adding to the combinatorial complexity.

Persechini's second sensor design also concatenated GFPs, MLCK peptide and calmodulin, though in different order. A calmodulin whose EF hand calcium binding sites had been reversed in order (CN-CaM) was added to the FIP-CB_{sm} C-terminal to BFP to make FIP-CA (Persechini et al., 1997). This produced a sensor with a monotonic FRET response and an apparent (K'_d of 100 nM). Variants with lower affinities for calcium were obtained by mutating the MLCK calmodulin binding peptide sequence, rather than the calmodulin calcium binding sites. As with FIP-CB_{sm}, calmodulin binding reduced FRET, the ratio (now expressed as F_{440} / F_{510}) increasing approximately 1.7 fold over the calcium dynamic range. The interaction was markedly pH sensitive in the range 6.5-7.5. This configuration of calmodulin and calmodulin binding peptide did not lead to later variants and appears to have been an evolutionary dead end.

Theameleon family of calcium sensors is shown in figure 1.

2.1.2 Evolution—The EYFP in yellow cameleon –2 and –3 show an apparent pK_a of 6.9, so contain a significant proportion of the protonated species at physiological pH (Miyawaki et al., 1999). The protonated species does not participate in FRET (Habuchi et al., 2002). As pH can vary by several tenths of a pH unit when cells are stimulated; changes in pH would be read as a changes in calcium ion concentration. Two adjacent point mutations in EYFP (V68L and Q69K) lower the pK_a to 6.1, markedly reducing the pH sensitivity in the physiological range (Miyawaki et al., 1999). Replacing EYFP with EYFP-V68L/Q69K abolished pH sensitivity above pH 6.9 (Miyawaki et al., 1999). This substitution produces yellow cameleon-2.1 (YC2.1; K'_d s for calcium: 100 nM and 4.3 μ M) and yellow cameleon-3.1 (YC3.1; K'_d for calcium: 1.5 μ M) with around a 2-fold difference in 528/476 nm emission ratios in calcium-free and calcium-saturating conditions. Recalling that the calcium dependent signal from FIP-CB_{sm} relied on binding of endogenous calmodulin, an obvious concern would be that YCs would be perturbed by such interactions and also perhaps themselves perturb downstream calcium signalling pathways. In fact, EC_{50} s for YC2.1 and YC3.1 stimulation of calmodulin-dependent phosphodiesterase were 2-3 order of magnitude greater than for calmodulin and the sensors were unperturbed by addition of 3 μ M calmodulin. Of course, the YC constructs will buffer calcium inside cells. This was tested by studying the calcium oscillations induced in HeLa cells induced by addition of histamine. At a YC3.1 concentration of 150 μ M, calcium oscillations were evident whereas at concentrations greater than 300 μ M, oscillations were not seen, though the overall magnitude of the response was little altered. The loss of oscillations suggests calcium buffering. Below around 20 μ M, the fluorescent signal was too faint to give acceptable signal to noise ratios (Miyawaki et al., 1999). Thus, working YC concentrations in the range 40-150 μ M do not substantially perturb calcium-dependent signalling mechanisms.

Yellow fluorescent proteins, besides being sensitive to pH, are more prone than GFP to photobleaching and to quenching by biological anions such as chloride. Because YFPs show such utility as one of the partners in the CFP/YFP FRET couple, this defect is worth fixing. Mutagenesis by error-prone PCR and expression in *E. coli* uncovered a mutation to methionine in residue 69 that was much more resistant to chloride quenching than EYFP-V68L/Q69K, twice as resistant to photobleaching, with a pK_a of 5.7 rather than 6.1 and of comparable spectral properties including brightness (Griesbeck et al., 2001). This YFP is

known as citrine, and substituted for EYFP-V68L/Q69K as the FRET acceptor produced the cameleons YC2.3 and YC3.3. These two cameleons express well at 37°C, show a ratio change of around 1.5 to calcium over their dynamic range and are pH insensitive down to around pH 6.5. To demonstrate the utility of YC3.3 in an acidic compartment, it was targeted to the Golgi using an 81 residue N terminal construct from human galactosyl transferase type II. The sensor was saturated when expressed in the Golgi, suggesting high resting levels of free calcium concentration in this cellular compartment (Griesbeck et al., 2001).

The CFP/Citrine couple was also used in an ER-targeted sensor, Cameleon D1ER. Here the rationale was to design a sensor based on the M13/CaM binding pair that would be insensitive to interaction with endogenous calmodulin (Palmer Amy et al., 2004), as had been reported (Hasan Mazahir et al., 2004; Heim and Griesbeck, 2004). The M13 and CaM were co-mutated to provide a binding pair that would not interact strongly with endogenous calmodulin. Cameleon D1ER has a very wide range of calcium sensitivity with K_d s of 0.81 and 60 μ M, appropriate for ER calcium sensing, and was successfully used in HeLa cells to monitor cytoplasmic and ER calcium simultaneously in conjunction with Fura2 (Palmer Amy et al., 2004).

The GFP family of proteins is remarkable in possessing a visible wavelength fluorophore that is formed through an oxidation reaction involving adjacent amino acids (Tsien, 1998). Fluorescence develops relatively slowly when the protein is expressed in cells, the process of what is known as maturation taking tens of minutes to hours; maturation is also temperature dependent, oxidation to form the fluorophore being the rate limiting step. Another potential difficulty with FRET-based probes using the CFP/YFP partners is that maturation of YFP is substantially slower than that of CFP, particularly at mammalian body temperatures (Miyawaki et al., 1999), a very important consideration especially for expression in transgenic mammals. If the YFP partner of the FRET couple matures more slowly than the CFP partner, then the sensors dynamic range is compromised, as mature CFP in a sensor that contains immature YFP will contribute to the 476 nm emission in the absence of 528 nm emission from the same construct, so that the overall population 528/476 emission ratio will be depressed as a function of the proportion of disparately-matured sensor constructs (as illustrated by the behaviour of YC6.1 discussed below (Evanko Daniel and Haydon Philip, 2005)). The F46L mutation in YFP greatly accelerates oxidation to the mature fluorophore and four additional point mutations contributed to create a construct that matured two orders of magnitude faster than EYFP from a urea-denatured state (Nagai et al., 2002; Rekas et al., 2002); because of its resulting brightness, this YFP construct was given the name Venus. Venus also has a low pK_a (6.0) and low sensitivity to chloride, comparable to Citrine in these respects (Griesbeck et al., 2001), though it lacks Citrine's improved resistance to photobleaching. Substitution of Venus for EYFP-V68L/Q69K resulted in a new rapidly maturing yellow cameleon (YC2.12). Bright YC2.12 fluorescence was seen to develop rapidly after ballistic transfection of Purkinje cells in cerebellar slices, though the fold ratio change after depolarisation suggests that its dynamic range was not much altered from earlier family members (Nagai et al., 2002).

The challenge of improving dynamic range was addressed systematically by altering the orientation of the YFP fluorescence dipole relative to the CFP dipole (Jares-Erijman Elizabeth and Jovin Thomas, 2003) to maximise FRET (Nagai et al., 2004). Changes in orientation were achieved by circular permutation (see below, section 2.2.1) of the Venus construct. The YC3.12-based construct with EYFP-V68L/Q69K substituted by circularly-permuted Venus with a new N-terminal at Asp-173 (termed YC3.60) showed the largest increase in fluorescence emission ratio dynamic range between calcium free and calcium bound forms in vitro: around 6.6 fold compared to 2.1 fold for YC3.12. This large

improvement in dynamic range was verified by expression of each the two sensors in HeLa cells and challenge with ATP to raise cytoplasmic free calcium levels (Nagai et al., 2004). This study also illustrates the important point that altering the properties and conformation of the FRET partners at the N- and C-terminals of the sensor can also alter the apparent calcium activation characteristics of the calmodulin-M13 inner pair as measured by FRET. YC3.60 showed a monotonic increase with calcium concentration, as would be expected from a construct based on the monotonically-increasing cameleon-3 (Miyawaki et al., 1997), but the apparent dissociation constant for YC3.60 is 0.25 μM , compared to 4.4 μM for cameleon-3. YC2.60, based on cameleon-2, has a single high affinity K'_d of 40 nM, compared to the two K'_d s of 70 nM and 11 μM of cameleon-2 (Miyawaki et al., 1997). YC4.6 has K'_d s of 58nM and 14.4 μM , compared to K'_d s of 83 nM and 700 μM in cameleon-4. The YCX.60 series of cameleons show the rapid maturation and low pH and chloride sensitivities of their Venus forbears, YCX.12, and are the best performing native M13-based cameleons to date.

Both Citrine and the circularly permuted Venus (cpv) of the YCX.60 series were used as alternative acceptors in a further series of cameleons based on Cameleon D1ER (Palmer Amy et al., 2006). Computational design of novel M13 and CaM-based binding pairs led to Cameleons D2, D3 and D4 and D2cpv, D3cpv and D4cpv, offering a wide range of calcium affinities, good sensor dynamic range (the cpv series comparable to YC3.60) and insensitivity to endogenous calmodulin. This cameleon set showed good performance in reporting cytoplasmic and mitochondrial calcium concentrations in HeLa cells and periplasmalemmal calcium concentrations in hippocampal neurone when localised with the appropriate targeting sequences.

ECFP/EYFP-based cameleons require excitation at near-UV wavelengths. It would be convenient to have FRET-based calcium sensors that can be excited at visible wavelengths. One possibly solution is to use a FRET couple in which GFP is paired with a red fluorescent protein. GFP-like red fluorescent proteins are found in corals (Baird et al., 2000; Miyawaki et al., 2003b). However, they are less tractable than GFP and its variants as they oligomerise, mature very slowly via a green-emitting intermediate and in general show low extinction coefficients and quantum yield (Miyawaki et al., 2003b). A GFP/RFP cameleon has been developed using a DsRed variant – a tandem dimer mutant (Yang et al., 2005). The maturation rate is tens of hours and the emission ration change is less than 1.2 fold when cells expressing the sensor are challenged with ionomycin (Yang et al., 2005).

2.1.3 Changing the sensor mechanism 1—Solution NMR showed that the calmodulin binding peptide of calmodulin-dependent kinase kinase (CKKp) has a different relation to the two lobes of calmodulin than M13 peptide (Truong et al., 2001). The structural modelling suggested that the peptide might be concatenated in a recombinant construct *between* the N- and C-terminal lobes of calmodulin. Calculations suggested that if ECFP and EYFP- V68L/Q69K were attached to the N- and C-terminals of the split calmodulin, then the distance between the fluorophores when calcium was bound and the calmodulin interacting with its binding peptide might be less than 40Å, rather than the 50-60Å in M13-based YC2.1. Given the sixth power dependency of FRET on distance between fluorescent dipoles (Jares-Erijman Elizabeth and Jovin Thomas, 2003), this approach promised an improvement of the dynamic range of the ratio of fluorescence emission. The splitting of the N- and C-domains of calmodulin in this construct (termed YC6.1) led to a monotonic calcium binding curve with a K'_d of 110 nM, in some respects more suited to measurement of smaller changes in intracellular free calcium concentration. While in the event, YC6.1 showed a more modest fold emission ratio change than predicted (2.1 vs 1.4 for YC2.1 in parallel experiments), the twofold change was expressed over a

narrower range of calcium concentrations (0.05-1 μM) in the physiologically relevant cytoplasmic range.

YC6.1 of course suffers from the pH and chloride sensitivity and the slow maturation of its EYFP-V68L/Q69K fluorophore that we discussed above. Replacing EYFP-V68L/Q69K with Venus (Evanko Daniel and Haydon Philip, 2005) gives the sensor VC6.1 (Venus cameleon 6.1: the nomenclature is confusing and unhelpful, given that the Venus CaM-M13 cameleons are known as YC2.12 and YC3.12). VC6.1 shows a emission ratio change of around 2.1-fold between zero and saturating calcium concentrations. Thus, as with substitution with Venus for EYFP-V68L/Q69K to produce YC2.12 from YC2.1, dynamic range is not much altered, while improvements in maturation and pH and chloride sensitivity are obtained. It would be logical to develop a YC6 sensor that contains the circularly permuted Venus used in YC2.6 and YC3.6 (Nagai et al., 2004); this would be predicted to much improve the ratio dynamic range.

Small improvements in dynamic range for YC6.1 and VC6.1 can be obtained by excluding from analysis cells that express a low resting YFP/CFP ratio (Evanko Daniel and Haydon Philip, 2005): the authors very reasonable suggest that this screens out cells in which the YFP partner is less-mature relative to its CFP pair.

2.1.4 Changing the sensor mechanism 2—One potential disadvantage of calmodulin-based sensors is that calmodulin is a near-ubiquitous protein with many binding partners. It is possible that calmodulin-based sensors may suffer interference from binding partners when expressed in the cytoplasm or other cellular compartments. While there is no direct evidence to support this conjecture, it is nonetheless true that performance in vivo does not always mirror the sensor properties demonstrated in vitro (Hasan Mazahir et al., 2004; Heim and Griesbeck, 2004). With this potential pitfall in mind, a sensor has been developed based on troponin C, a calcium binding protein and close homologue of calmodulin that is however expressed only in muscle. The approach was to concatenate TnC with CFP and Citrine (Heim and Griesbeck, 2004). While developing these CFP-TnC-Citrine sensors, a variant strategy was pursued to concatenate TNI, a TnC binding partner, alongside TnC by analogy with the M13 binding partner of calmodulin in the classical cameleons; this was unsuccessful. The constructs showing the greatest change in FRET between calcium-free and calcium bound forms contained a chicken skeletal muscle TnC with an N-terminal 14 residue truncation, TN-L15, and a human cardiac TnC, TN-humTnC. TN-L15 showed a 140% change and TN-humTnC a 120% change, measured in the absence of magnesium ion. At physiological (1 mM) magnesium concentrations, the dynamic ranges were 100% and 70% respectively. Apparent dissociation constants were 470 nM for TN-L15 and 1.2 μM for TN-humTnC. The TnC EF hand calcium binding sites in TN-L15 were mutated to give K_d 's of 300 nM and 29 μM . The pH sensitivities were similar to the other CFP/citrine-based sensors, with a reduction in dynamic range below pH 6.8 and little effect in the physiological range pH 6.8-7.3. Calcium off rates were similar to or slightly faster than that of YC2.3 (Heim and Griesbeck, 2004). The TN-L15 sensor was targeted to the plasma membrane using GAP43, Ras or Synaptobrevin. In direct comparison with YC2.1 and YC 3.3, it showed markedly greater sensitivity and no diminution of dynamic range.

Mutations to EF hands III and IV and substitution of Citrine with a circularly permuted variant, Citrine-cp174 produced a TnC-based sensor that showed no magnesium dependence, a 4 fold dynamic range and a K_d of 2.5 μM – TN-XL. TN-XL has a very fast off rate with a dominant component with a time constant of 142 ms (Mank et al., 2006). Expressed in *Drosophila* under a UAS/Gal4 neuronal promoter, it showed response times to calcium signals at the neuromuscular junction significantly faster than other sensors - YC2.0, YC3.3, Inverse Pericam, G-CaMP1.3 and G-CaMP1.6. Further mutagenesis and

rearrangement of the TnC domain gave a higher affinity variant, modestly named TN-XXL, that was capable of long term monitoring of individual neuronal responses in flies and mice (Mank et al., 2008).

2.2 Camgaroos

2.2.1 Circular permutation of EYFP—Remarkably perhaps, the beta-can that surrounds the cyclised and oxidised fluorophore is amenable to circular permutation, by which is meant the insertion of a peptide linker between N- and C-terminals of the protein and the creation of a new N- and C-terminal pair elsewhere in the sequence, in the loops that connect the component beta-sheets and in the beta sheets themselves (Baird et al., 1999). As we have seen, circular permutation of Venus led to YC2.60 and YC3.60, the two cameleons with the largest emission ratio dynamic range (Nagai et al., 2004). The discovery that N- and C-terminal of EYFP could be rearranged prompted the discovery that a calcium sensor could be fashioned by insertion of calmodulin within EYFP itself. *Xenopus* calmodulin was inserted between residues 144 and 146 of each of ECFP, EYFP and EGFP. Each of these constructs was a calcium sensor, with the EYFP insertion giving the largest calcium response. In calcium-free conditions, the construct absorbs predominantly at 400 nm, while in calcium-saturating conditions, the dominant absorption peak is at 490 nm. The 400 nm absorption is due to the protonated form of EYFP and the 490 nm absorption to the unprotonated form. As discussed above in section 2.1.2, in EYFP the protonated species is not fluorescent (Habuchi et al., 2002), so the excitation spectrum shows a single peak at 490 nm and both the excitation and emission spectra are strongly dependent on calcium concentration, with around an eight-fold increase in emission intensity at saturating calcium concentrations. Calcium binding was monotonic with an apparent dissociation constant of 7 μ M. Calcium binding clearly shifts the proportion of protonated and unprotonated forms at constant pH, so the pKa's for the two forms are different: 10.1 and 8.9 respectively. Continuing the whimsical tradition, this calcium sensor is termed Camgaroo-1, because it is yellowish, carries a smaller companion (the calmodulin) in a pouch, can bounce high in signal and may spawn improved progeny (Baird et al., 1999). The increase in fluorescence intensity after addition of histamine to Camgaroo-1 expressing HeLa cells was a modest 40% and the characteristic calcium spiking activity was almost invisible, so the sensor is not quite as bouncy as its name implies when sensing cytoplasmic free calcium; however addition of ionomycin caused an overall 7 fold increase in fluorescence. The modest increase observed in response to histamine is almost certainly due to the 7 μ M K'_d , high relative to the calcium increase from around 100 nM to 1 μ M expected when histamine is added to HeLa cells.

Camgaroo-1 does not fold well at 37°C and could not be targeted to intracellular organelles, for example mitochondria (Baird et al., 1999). In an attempt to live up to another of its attributes, the possibility that it may spawn improved progeny, Camgaroo-1 was subjected to error-prone PCR mutagenesis (Griesbeck et al., 2001); selection of the brightest clone after expression in *E. coli* revealed a point mutation of residue 69 to methionine. This new sensor, Camgaroo-2, had very similar calcium binding properties and fluorescence dynamic range as Camgaroo-1, but expressed far more brightly in HeLa cells grown at 37°C. The response to histamine at around a 5% fluorescence increase was lower even than for Camgaroo-1, but targeting to mitochondria using the targeting sequence of sub-unit VIII of cytochrome *c* oxidase was demonstrated. Mitochondrial calcium increases that raised the resting fluorescence signal by about 70% were demonstrated in response to histamine and subsequent addition of ionomycin gave an overall 1.5-fold increase in fluorescence signal (Griesbeck et al., 2001), lower than that observed with cytoplasmic Camgaroo-2, perhaps because the resting mitochondrial calcium concentration is higher than that of the cytoplasm.

Using a similar camgaroo-like strategy, the EF hand calcium binding site was introduced into EGFP between residues 144-145, 157-158 or 172-173 (Zou et al., 2007). These Ca-G family sensors had extinction coefficients and quantum yields comparable to EGFP. They operate in the ratiometric mode and with excitation at 398 nm and 490 nm showed a sensor dynamic range of 1.8 at a 510 nm emission wavelength. Comprising a single EF hand binding site, the apparent dissociation constants are in the millimolar range (0.4-2 mM) and are therefore suitable only for monitoring high calcium environments such as the ER. They are markedly pH sensitive, with a pKa of around 7.5. Expressed in the ER of HeLa and BHK-21 cells, they showed modest ratio changes in response to agonists (Zou et al., 2007).

2.3 Pericam G-CaMP family

2.3.1 Pericams—In pursuit of the idea that the clefts introduced into the beta can structure by circular permutation might make the fluorophore more accessible to solution protons and so susceptible to structural changes brought about by reorientation of concatenated peptides, Miyawaki's group developed the pericam series of sensors (Nagai et al., 2001). Circular permutation of EYFP-V68L/Q69K to give an EYFP with Y145 as the N-terminal and N144 as the C-terminal (cpEYFP) produced an EYFP variant that could be concatenated with M13 and calmodulin (bearing the E104Q mutation that conferred a monophasic calcium binding curve (Miyawaki et al., 1997)). The construct with calmodulin at the N-terminal (CaM-cpEYFP-M13) showed no calcium-dependent properties, confirming the finding reported for a cpGFP variant (Nakai et al., 2001), but the opposite concatenation (M13-cpEYFP-CaM) gave a construct that showed three fold brighter 520 nm fluorescence in high calcium media compared to calcium-free media when excited at 485 nm. This construct was given the name pericam (from a circularly *permuted* YFP and *CaM* – calmodulin). Pericam was the prototype from which three pericams with enhanced features were developed. Flash pericam has three additional point mutations that confer an eightfold increase in 520 nm fluorescence on calcium binding. Flash Pericam is a single wavelength, non-ratiometric indicator with a K'_d of 0.7 μ M. Knowing that substitution of phenylalanine at residue 203 in YFP conferred fluorescence on the protonated form, this mutation was introduced into Flash Pericam. The result, Ratiometric Pericam, was a sensor whose emission ratio at 520 nm when excited at 494 nm or 415 nm changes ten fold between calcium free and calcium saturating conditions with a K'_d of 1.7 μ M; this excitation ratio sensor is functionally analogous to fura-2 [REF]. Further semirandom mutagenesis of Ratiometric Pericam gave a single wavelength construct whose fluorescence intensity at 513-515 nm *decreased* on calcium binding – Inverse Pericam (K'_d ; 0.2 μ M). Two advantages of Inverse Pericam are that it is bright and has excitation/emission characteristics similar to fluorescein; the latter advantage it shares with Flash Pericam: these two YFP based indicators are functionally equivalent to the Fluo-3 and Fluo-4 single wavelength calcium sensors [REF]. Expression in HeLa cells showed that Ratiometric Pericam and Inverse Pericam expressed significantly better at 37°C than did Flash Pericam. Ratiometric Pericam gave a 2.5 fold increase in excitation ratio emission after addition of histamine, while Flash and Inverse Pericams offer a ~100% increase and decrease in signal respectively with the same agonist. As might be expected from our earlier discussion of the camgaroos, the calcium-free and calcium bound forms of all three pericams showed different pKa's and all three have pH sensitive emissions in the physiological pH range. Miyawaki showed a proof of principle that the excitation ratio-based Ratiometric Pericam can be used in the context of confocal imaging (Shimozono et al., 2002); recent confocal microscopes based on acousto-optical filters offer turnkey solutions to excitation ratiometric imaging.

2.3.2 GCaMPs—Single wavelength non-ratiometric sensors that use the same sensor strategy as pericams but are based on circularly permuted GFP rather than EYFP were developed at almost the same time as the pericams, their development preceding the

pericams' by a matter of months (Nakai et al., 2001). Both the CaM-cpGFP-M13 and M13-GFP-CaM concatenations were tested: only the latter showed significant calcium-sensing properties. Twenty-six variants of the M13-N149cpGFPC144-CaM concatenate were tested and the variant that showed the greatest fluorescence increase in HEK-239 cells after ATP addition was termed G-CaMP (presumably for Green Fluorescent-calmodulin protein). In HEK-239 cells, G-CaMP gave a 1.5-fold increase in fluorescence in response to ATP and a 4 fold increase in response to ionomycin. G-CaMP has very similar fluorescence parameters to Flash Pericam, with an excitation maximum at 489 nm, an emission maximum at 509 nm and a 4.5 fold increase in fluorescence on calcium binding (cf 8 fold for Flash Pericam). The apparent dissociation curve was monotonic, with a K'_d of 0.24 μ M. As with the camgaroos and pericams and for the same reasons, the sensor signal is strongly pH dependent in the physiological range. The association time constant for calcium binding was strongly calcium dependent and varied from 250 ms at low calcium concentration to 2.5 ms at higher concentrations; the dissociation time constant was 200 ms. G-CaMP expresses poorly at 37°C. G-CaMP-expressing smooth muscle showed a response to rapid depolarisation of around 50%, with a time course comparable to that previously measured with Fluo-3. Carbachol addition gave a 2.5 fold increase. pH was monitored in these experiments and did not change (Nakai et al., 2001).

This first GCaMP family member, later designated GCaMP1, had very weak fluorescence when expressed at physiological temperatures compared to GFP itself. This was addressed by introducing two mutations V163A and S175G that were known to improve the temperature-dependent maturation of GFP to give a variant known as G-CaMP1.6 (Ohkura et al., 2005); this increased brightness about 40 fold. However, these modifications did not lead to adequate maturation above 30°C. The G-CaMP construct was subjected to error-prone PCR mutagenesis and the clones fluorescing most brightly at 37°C were selected (Tallini Yvonne et al., 2006). The two new mutations in the brightest clone were identified (D180Y and V93I), but it also turned out that the RSET leader sequence that had been added to facilitate purification of the expressed protein was essential for thermal stability at 37°C. This construct, GCaMP2, is around 200 times brighter than G-CaMP1 at 37°C (with an extinction coefficient at 487 nm of 19,000 and a quantum yield of 0.93 with emission at 508 nm) and shows the same 4-5 fold increase in fluorescence a saturating calcium concentrations when compared to calcium free conditions. Though not reported, it should be assumed that this sensor remains pH-sensitive. GCaMP2 was expressed using tissue-specific promoters in transgenic animals and calcium transients were detected in granule cells in cerebellar slices (Diez-Garcia et al., 2005) and in isolated heart in vitro and in adult and embryonic heart in vivo (Tallini Yvonne et al., 2006). Some insight into the sensor mechanism of GCaMP2 is afforded by its crystal structure (Akerboom et al., 2009; Wang et al., 2008).

Even so, in HEK293 cells, GCaMP2 fluorescence is still 100 fold lower than GFP itself (Tian et al., 2009). HEK293 cell medium-throughput screening assays were used to identify brighter GCaMP2 mutants; attention was also paid to improving the sensitivity to small calcium changes through mutations of the CaM EF hands and of the M13/CaM interaction domains. The upshot was GCaMP3, with a dynamic range of 12, due to a 2 fold decrease in calcium-free fluorescence and a 1.5 fold increase in calcium-saturated fluorescence relative to GCaMP2, and a K'_d of 0.66 μ M (Tian et al., 2009),

2.3.3. Case 12 and 16—The Case (presumably *Calcium sensor*) constructs were developed by analysing the linker sequences between M13 and cpEYFP/GFP and cpEYFP/GFP and calmodulin and the three key residues 148 145 and 203 in the pericams and GCaMPs (Souslova Ekaterina et al., 2007). Based on this analysis, constructs were made containing the G-CaMP linker sequences and the cpEYFP derived from Ratiometric

Pericam. Nine point mutants were made with alterations in both the linker sequences and in the three key residues within cpEYFP. As expected, combinations of Asp148 and Phe203 produced ratiometric indicators akin to Ratiometric Pericam, while Asn or Glu at residue 148 combined with Phe203 had a single excitation peak at 490 nm. The Glu148/Thr145 and Glu148/S145 variants showed a 14.5 fold increase in 490 nm fluorescence between calcium-free and calcium-bound forms. The E148/S145 variant of these pericam-G-CaMP hybrids was optimised for folding at 37°C using error-prone PCR, resulting in a variant with a 12 fold dynamic range named Case12. Substituting Thr for Ser at the 145 position of Case12 gave Case16, with a 16.5 fold dynamic range. The apparent dissociation constant for both Case12 and Case 16 was 1 μ M. Like the pericams and G-CaMP sensors, the calcium-bound forms of Case 12 and Case 16 (pKa 7.2) – and thus their fluorescence – are affected by any changes in pH within the physiological range.

3. APPLICATIONS OF GENETICALLY-ENCODED SENSORS

3.1 Targeting to subcellular locations

Low molecular weight fluorescent calcium sensors do make their way to intracellular compartments (Silver et al., 1992) and can be used to measure calcium there, but they are difficult to target precisely (Varadi and Rutter Guy, 2002b). One of the two major advantages of genetically-encoded calcium sensors is that chimeric constructs and signalling tags can target them specifically to subcellular locations. Methods to achieve some of these specific localisations had already been developed for GFP itself and for the calcium sensor aequorin (De Giorgi et al., 1996). The ability to target cameleons YC-3er and YC-4er was demonstrated in the study in which cameleons were first described (Miyawaki et al., 1997).

3.1.1 Endoplasmic reticulum—ER calcium concentrations have been measured using low molecular weight calcium sensors and with aequorin (Solovyova and Verkhatsky, 2002), but it seems fair to say that theameleon based sensors (YC-3er and YC-4er) have given the best estimates of ER calcium concentration and turnover (Foyouzi-Youssefi et al., 2000; Graves Thomas and Hinkle Patricia, 2003a; Graves Thomas and Hinkle Patricia, 2003b; Varadi and Rutter Guy, 2002a; Yu and Hinkle, 2000). In summary,ameleon based indicators have presented a picture of the ER as an organelle with resting calcium concentrations in the range 250–600 μ M and a very active calcium turnover that depends very heavily on the activity of the SERCA ATPase (Demaurex and Frieden, 2003). Transgenic YC3.3er has been engineered to give tissue specific expression in mouse pancreatic beta cells (Hara et al., 2004). The interpretation of calcium changes in the ER measured byameleon indicators is tempered by the finding that pH changes within the organelle may interfere with estimates of dynamic calcium concentration (Varadi and Rutter Guy, 2004). Improved sensors for ER calcium are now available (Palmer Amy et al., 2004; Zou et al., 2007).

3.1.2 Mitochondria—Mitochondrial targeting of recombinant aequorin was achieved using the N-terminal presequence of subunit VIII of cytochrome oxidase (Rizzuto et al., 1992). The same targeting strategy was used to locate ratiometric pericam within mitochondria (Robert et al., 2001) and to show that the pericam tracked beat to beat calcium changes in cardiomyocytes, just as did aequorin.ameleon probes targeted to mitochondria were effective only at very low expression levels (Arnaudeau et al., 2001). In a comparison of mitochondrially-targetedameleon (mtYC2), camgaroo-2 and Ratiometric Pericam (Nagai et al., 2001) in HeLa cells, it was found that Ratiometric Pericam was the most reliable and faithful of sensors (Filippin et al., 2003). Mislocalisation and poor expression of the mitochondrially-targeted YC2 sensor could be improved by inserting a tandem repeat of the subunit VIII presequence as the targeting sequence (2mtYC2) (Filippin et al., 2005).

2mtYC2 was used successfully to demonstrate calcium handling by skeletal muscle mitochondria during contraction. (Rudolf et al., 2004). Insertion of a tandem targeting repeat was an ineffective strategy for the preferred Citrine or Venus variants (Filippin et al., 2005), but in contrast, the D2cpv, D3cpv, and D4cpv cameleons (Palmer Amy et al., 2006) functioned well as mitochondrial calcium sensors when targeted with the cytochrome oxidase tandem repeat (Palmer Amy et al., 2006). These constructs are now the recommended genetically encoded mitochondrial calcium sensors. An recent overview of calcium sensor approaches in mitochondria is available (Pozzan and Rudolf, 2009).

3.1.3 Peroxisome—Cameleon D3cpv was furnished with a modified peroxisome localisation sequence (D3cpv-KVK-SKL) to monitor calcium concentrations in this organelle in HeLa cells in response to agonists or depolarisation (Drago et al., 2008).

Golgi: The Citrine cameleon YC3.3 has been expressed in the Golgi using an 81 residue N-terminal sequence from human galactosyl transferase type II (Griesbeck et al., 2001); it was saturated, offering no useful information but that the Golgi has a very high resting calcium concentration.

3.1.4 Plasma membrane—Sub-plasmalemmal calcium concentrations may differ from those in bulk cytoplasm. Localised calcium concentrations around secretory vesicles were shown to be higher than those in cytoplasm by using a phogrin chimera to target YC2 to secretory vesicle membrane (Emmanouilidou et al., 1999). A number of targeting strategies have proved successful in localising sensors to the plasma membrane. The cpVenus cameleon YC3.60 has been targeted using a Ki-Ras chimera (Nagai et al., 2004). The TN-L15 sensor localised to the plasma membrane as GAP43, Ras or synaptobrevin chimeras (Heim and Griesbeck, 2004). Localisation can also be achieved with a myristoyl/palmitoyl N-terminal tag (Zacharias David et al., 2002), an approach that was used with the cameleon D series (Palmer Amy et al., 2006). A chimera of GCaMP2 and synaptotagmin (SyGGCamp2) has been used to monitor synaptic calcium signals, in this case in vivo in zebrafish (Dreosti et al., 2009).

3.2 Tissue-specific expression

The other major advantage of genetically-encoded calcium sensors is tissue-specific expression in intact organisms.

3.2.1 YC2.1—The first transgenic tissue specific expression of genetically-encoded calcium sensors was demonstrated in plants. YC2.1 was expressed in *Arabidopsis* guard cells of the leaf, first using a CaMV promoter (Allen et al., 1999) and then a guard cell specific *det* promoter (Allen et al., 2000), demonstrating that aspects of the calcium signalling response in guard cells were under differential genetic control. YC3.1 was used in transgenic *Arabidopsis* plants to visualise calcium signals in the pollen grain (Iwano et al., 2004).

YC2.1 was expressed transgenically in *C. elegans* pharyngeal muscle under the control of the pharyngeal-specific *myo-2* promoter (Kerr et al., 2000) and tracked calcium changes during pharyngeal pumping; YC3.1 tracked temporal changes more faithfully than YC2.1, being the faster sensor, but YC2.1 tracked calcium changes to basal level more faithfully than YC3.1, as might be expected from its lower K_d . Expression of YC2.12 in *C. elegans* touch neurons under the control of the *mec-4* promoter identified a role for specific ion channels in the touch response (Suzuki et al., 2003).

The UAS/Gal4 tissue specific expression system was used to express YC2.1 in a subset of the antennal lobe projection neurones of *Drosophila* in order to study odorant responses in the antennal lobe and mushroom body calyx *in vivo* (Diegelmann et al., 2002; Fiala et al., 2002). Odorant-specific patterns of neuronal excitation were seen in both the antennal lobe and the calyx. In the former, the EYFP/ECFP emission ratio changes were 1.23 % $\pm$ 0.23 % (mean and sem) and in the latter 0.6% $\pm$ 0.06%. In the antennal lobe, the changes in sensor signal were observed in spatially-restricted regions of around 10-30 μ m diameter, the size of individual glomeruli. These very small changes were nonetheless reproducible, with distinct and reproducible patterns of activity from fly to fly associated with different odorants.

The same UAS/Gal 4 technology was used to express YC2 in neurones of larval *Drosophila* (Reiff Dierk et al., 2002) to the evolution of calcium signalling in presynaptic terminals innervating larval muscle. A 28% emission ratio change was measured *in vivo* during spike train stimulation of the neuromuscular junction and signals of this magnitude could be resolved in single synaptic boutons; there were no detectable differences in nmj physiology between wild type and transgenic larvae. This study illustrates the point that targeted expression of genetically-encoded sensors in individual neurones is for some applications superior to the use of low molecular weight synthetic calcium indicators, as the specificity of expression more than compensates for the loss of brightness.

In a similarly mature use of YC2.1 sensor technology coupled to UAS/Gal4 transgenic expression, neuronal calcium measurement coupled with electrophysiology was used to identify thermosensory neurones in the larval nervous system *in vivo* (Liu et al., 2003). 10-50% changes in emission ratio were associated with heating and cooling. A functional map of thermosensory neurones was generated and it was found that neurones with different temperature responses were anatomically segregated.

YC2.1 was also used in zebrafish to record the behaviour of Rohon-Beard (RB) neurones during the fish's escape response (Higashijima et al., 2003). This careful study started with transient expression of the YC2.1 transgene in the RB neurones to show proof of principle before generating transgenic lines in which the calcium signals in the RB neurons could be correlated with the escape response in conscious fish.

3.2.2 YC3.3er—YC3.3er (the citrine-based sensor) was expressed in the beta cells of transgenic mice under the control of the mouse Insulin 1 promoter (Hara et al., 2004). The sensor signal could be detected in isolated pancreatic islets and addition of thapsigargin or carbachol gave the expected decrease in the 535/485 emission ratio.

3.2.3 Camgaroos and Inverse Pericam—UAS/Gal 4 expression was used to create transgenic *Drosophila* that expressed camgaroos-1 and -2 in the mushroom bodies of adult brain (Yu et al., 2003). Dissected fly brains were used. Camgaroo-2 fluorescence in the mushroom bodies was much more intense than that of camgaroo-1, but the camgaroo-1 emission ratio signal on potassium depolarisation was more than double that of camgaroo-2 (38% vs 14% in the mushroom body lobe and 83% vs 28% in the mushroom body itself). It was shown that these increases were not due to changes in pH. Application of the putative mushroom body transmitter, acetylcholine, cause ratio changes of a few percent. In this setting, camgaroo-2, although brighter, showed substantially lower ratio changes than camgaroo-1; it also underwent significantly faster photobleaching.

Inverse pericam is an intensity-coded sensor that decreases its fluorescence as calcium increases. Addition of DsRed2 to the C-terminal of inverse pericam produces a ratiometric indicator whose 615 nm / 510 nm emission ration increases as calcium increases. This

indicator (DsRed2-referenced inverse pericam: DRIP) requires dual excitation and dual emission optics (Shimozono et al., 2004). The DsRed2 fluorescence is a passive, calcium-independent signal that is proportional to the concentration of the sensor and helps control for alterations in overall fluorescence intensity due for example to movement artefacts. DRIP was expressed transgenically in worms under the control of the myo 2 promoter that is specific for pharyngeal muscle. Ratio changes of 30-40% were measured in worms undergoing fast pharyngeal pumping.

After screening six sensors (flash pericam, inverse pericam, G-CaMP, camgaroo-2, YC2.12 and YC 3.12) for calcium sensitivity in stably-transfected fibroblast cell lines, the two with the greatest dynamic range (inverse pericam: -40% and camgaroo-2: +170%), together with YC3.12 that gave inconclusive results in the fibroblast expression screen but is optimised for expression at 37°C, were used to generate transgenic mice under the control of the TET expression system (Hasan Mazahir et al., 2004); the TET system allows tissue-specific expression by crossing the TET mice with mice expressing the TET transactivator under tissue specific control. TET sensor mice were crossed with a line expressing the transactivator under the control of the alpha-calmodulin/calcium dependent kinase II (α CamKII) promoter. All mice developed normally. Five highly expressing lines were obtained out of 36 transgenic lines: two YC3.12, two camgaroo-2 and one inverse pericam. Expression patterns in brain slices and excised retina were analysed by two-photon microscopy. They appeared to be mosaic, not mapping directly to the known patterns of α CamKII expression. Neocortical expression could also be imaged through the thinned skull in anaesthetised mice. Two photon fluorescence recovery after photobleaching suggested that as much as half the fluorescence signal was immobile and this together with punctuate staining patterns suggested that this immobile sensor fraction might be due to interaction between the M13 and CaM moieties of the sensors and their normal cellular targets. Cellular and synaptic stimulation of pyramidal neurones in cortical slices using sharp and patch microelectrodes gave 5-10% increases in 535 nm fluorescence using wide field imaging and around 20-100% for camgaroo-2 and -30% for inverse pericam using two photon imaging. In the retina, a ganglion cell subset was strongly labelled in YC3.1-expressing mice, but no light-evoked responses were detected. In camgaroo-2 expressing lines, bleaching occurred in the retina too quickly for measurements to be made. In one inverse pericam-expressing mouse, seven of 12 ganglion cells tested showed a transient decrease in fluorescence attributable to a calcium increase in response to light. Sensors were imaged in the olfactory bulb in vivo using wide field microscopy. Camgaroo-2 expressing mice showed a 1-3% increase in response to odours, while inverse pericam gave ~8% decrease. Each distinct odour evoked a unique pattern of activity, similar odours evoking similar patterns.

This thoughtful study established four main facts: that around half of the transgenically-expressed cameleon family sensor was immobile, reducing sensitivity and making quantitation of the calcium signals impossible; that nonetheless, it was possible to observe patterns of neuronal activity; that YC3.12 was not an effective transgenic sensor. The study also reports unpublished experiments in which transgenic mice expressing YC3.0 under the control of a β -actin promoter gave only 1-2% ratio changes during wide field imaging in cerebellar slices. The high proportion of immobile sensor in transgenic animals remains for the moment inexplicable – it was not seen in the stably transfected fibroblast lines.

3.2.4 GCaMP—G-CaMP (Nakai et al., 2001) was expressed in mice under the control of a smooth muscle myosin heavy chain promoter and was expressed in vascular and non-vascular smooth muscle (Ji et al., 2004). The signatures of inotropic (ion channel) and metabotropic (InsP₃-mediated) post-synaptic signalling could be distinguished in single excised smooth muscle cells.

In a set of experiments strikingly parallel to those with YC2.1 (Diegelmann et al., 2002; Fiala et al., 2002), but using two photon imaging, G-CaMP was expressed in a subset of projection neurones in *Drosophila* antennal lobe to demonstrate that different odorants activated specific patterns of glomeruli (Wang Jing et al., 2003). Individual glomeruli are differentially sensitive to a given odorant and more are recruited as the odorant concentration is increased. Increases of fluorescence of up to 50% (at 525 nm) were measured in responsive glomeruli.

Transgenic expression of GCaMP2 has been achieved in mouse heart (Tallini Yvonne et al., 2006). The TET system was used: the GCaMP2 sequence was placed downstream of a weakened α myosin heavy chain promoter (α MHC) and seven tetO enhancer sequences to permit suppression of gene expression using doxycycline. These mice were crossed with others with a hemizygous α MHC-tetracycline transactivator allele. The doubly transgenic mice expressed GCaMP2 only in the heart. Doxycycline suppression of the transgene was essential, as mice constitutively expressing GCaMP2 from birth showed markedly enlarged hearts, a phenotype comparable to that seen in mice overexpressing calmodulin. This phenotype was avoided entirely by administering doxycycline in utero and until 13-15 weeks post partum. Subsequent removal of doxycycline for up to six weeks led to no detectable cardiomegaly. Robust GCaMP signals were present four weeks after doxycycline removal.

Striking wide field fluorescence images of cardiac calcium transients in whole mouse heart beating at up to 300 beats/min were obtained anaesthetised, ventilated open-chested mice, the first to be recorded under wholly physiological conditions with the heart under normal load. As expected sympathetic stimulation with isoproterenol markedly increased the calcium signal and also increased end diastolic calcium concentration. Signal to noise ratios were good and it was possible to record very clean signals from a single pixel of the 100×100 pixel imaging array (tens of microns). Using a photodiode array in isolated perfused heart, signals from a membrane potential sensitive dye and from GCaMP2 were acquired simultaneously. Association and dissociation kinetics of calcium were rapid ($\tau=14$ ms and 75 ms respectively) and unaltered in vivo. Comparison with a fast calcium dye Rhod2 nonetheless showed that the rise and decay times of the GCaMP2 signal in beating heart was around 45% slower, but with a three times greater dynamic range. Calcium sparks could not be observed in isolated ventricular myocytes expressing GCaMP2. GCaMP2 imaging in open-chested embryos from embryonic day 10 allowed the analysis of the development of the atrio-ventricular node conduction pathway.

GCaMP2 fused to synaptotagmin localises to synaptic boutons. It reports the location of synapses in zebrafish in vivo and shows a linear response over a wide range of action potential frequencies (Dreosti et al., 2009). It can report both spiking frequencies in optic tectum; it also reports activity in the graded synapses of retinal bipolar cells. GCaMP2 has also been used to map functional connections in the *C. elegans* nervous system (Guo et al., 2009). Connections can be mapped grossly, but the sensor's signals are too weak to distinguish direct from indirect connections.

3.2.5 TN-L15, TN-XL and TN-XXL—A cerulean version of TN-L15, cerTN-L15, was used to create a transgenic mouse line that expressed the sensor widely in brain, especially in the neocortex and hippocampus (Heim et al., 2007). Calcium changes resulting from 2-3 action potentials could be resolved and calcium responses in spiny dendrites of pyramidal cells could be detected after puffing on glutamate, an excitatory neurotransmitter (Garaschuk et al., 2007; Heim et al., 2007). TN-XL was expressed using the UAS/Gal4 tissue specific expression system in *Drosophila* neuromuscular junction (Mank et al., 2006). Its rapid off-rate for calcium made it significantly better at tracking calcium changes than its

counterparts. TN-XXL showed improved sensitivity and long term stability in sensing calcium signals from fly neurones; in mice, tuning curves for orientation specific neurones in visual cortex could be monitored repeatedly over timescales of days or weeks (Mank et al., 2008).

3.2.6 Comparison of the performance of genetically encoded calcium sensors

—Though progress in the field has been periodically reviewed (Barth Alison, 2007; Garaschuk et al., 2007; Griesbeck, 2004; Mank and Griesbeck, 2008), few studies have systematically compared the performance of different genetically encoded calcium sensors, except to demonstrate the superiority of a novel sensor over its predecessors. I have discussed above (section 2.2.3) the systematic comparisons of camgaroo-1 and camgaroo-2 when expressed in *Drosophila* mushroom bodies (Yu et al., 2003) and of inverse pericam, camgaroo-2 and YC3.1 when expressed in mouse brain (Hasan Mazahir et al., 2004).

The performance of GCaMP, inverse pericam and camgaroo-2 was compared with that of the low molecular weight synthetic indicators X-Rhod-5F and Fluo4-FF in apical dendrites of pyramidal cells in hippocampal brain slices from 6-7 day old rats transfected using a biolistic approach and maintained at room temperature (Pologruto et al., 2004). Images were obtained using two photon microscopy. Action potentials were triggered using current injection into the cell body. Under these conditions, X-Rhod-5F and Fluo4-FF could detect calcium changes (signal twice that of noise) in the dendrite due to voltage dependent calcium channel activation after single action potentials while with the same criterion GCaMP required 5 action potentials, camgaroo-2, 33, and inverse pericam over 20. For comparison, the dynamic ranges ($\Delta F/F$) for the three sensors under these conditions in vitro was 1.8, ~2 and -0.25, so the sensitivity of camgaroo-2 was poor despite its larger dynamic range. Power spectrum analysis was used to analyse the fluorescence response during action potential trains at 20 Hz. Most of the power in the frequency analysis of X-Rhod-5F and Fluo4-FF fluorescence was at the fundamental frequency, 20 Hz, indicating that the fluorescence signal tracked each action potential. For the genetically encoded sensors, no clear peak was observed at 20 Hz, indicating that the sensors were too slow to track individual action potentials at this stimulation frequency.

It was possible to measure calcium activation curves in situ for the three sensors and thus their apparent dissociation constants by simultaneously measuring calcium concentration using a calibrated X-Rhod-5F signal and the fluorescence signal from the sensor at various levels of stimulation. For inverse pericam (K'_d 0.9 μ M) and camgaroo-2 (K'_d 8 μ M), these were comparable to those previously reported in vitro; however, GCaMP showed a K'_d (1.7 μ M) almost an order of magnitude greater than that previously reported in vitro (Nakai et al., 2001). Because the calcium concentration profile of dendritic action potentials is well characterised (Pologruto et al., 2004), there seems little doubt that the calcium dissociation characteristics of GCaMP vary markedly in vitro and in vivo. FRAP studies in dendrites showed that the all three sensors were mobile, with mobilities comparable to GFP itself. This result is quite firmly at odds with that reported in mouse brain (Hasan Mazahir et al., 2004) and discussed above (section 2.2.3). It may be that dendrites, being relatively free of organelles, mirror better the behaviour of the sensors in cytoplasm than cell bodies; it should be noted that punctuate staining was reported in mouse brain (Hasan Mazahir et al., 2004). It should also be borne in mind that though the observations on mouse brain slices were carried out at room temperature, as were these experiments in rat brain slices, in the mouse study the sensors had been expressed at body temperature, whereas the biolistically-transfected rat brain slices were maintained throughout at room temperature.

These data, as the authors point out (Pologruto et al., 2004), demonstrate that the genetically-encoded sensors are better-suited to measuring summated neuronal responses

after multiple stimuli, not single action potentials, consistent with their reported use to monitor patterns of neuronal activity (Fiala et al., 2002; Hasan Mazahir et al., 2004; Wang Jing et al., 2003); as it happens these three studies all described odorant-specific patterns of neuronal signalling.

As an addendum to the study, Svoboda's group also provided in vitro solution X-ray scattering evidence that showed that the calcium-dependent fluorescent signal of GCaMP, as theorized, depends on a coupled structural change in which calcium binding to CaM is closely linked to binding of CaM to M13; in contrast, the calcium dependent fluorescence signal in camgaroo-2 is solely due to binding to CaM, the M13 peptide paradoxically playing no part in the sensor response (Pologruto et al., 2004).

A second comparative study was undertaken at the *Drosophila* larva neuromuscular junction (Reiff Dierk et al., 2005), using an approach previously reported (Reiff Dierk et al., 2002). The responses of ten sensors from the three families to 40 and 80 Hz stimulation of the synaptic bouton were compared. Camgaros-1 and -2 and flash pericam did not sense calcium changes in the bouton. YC2.0, 2.3, 3.3, TN-L15, inverse pericam and GCaMP1.3 and 1.6 all showed adequate responses (around 5% on average at 40 Hz and 10-15% at 80 Hz) to pulse train stimuli, but none exhibited dynamic ranges anywhere near comparable to those measured in vitro (Reiff Dierk et al., 2005). None was comparable in performance in this system when compared to the later-developed TN-XL (Mank et al., 2006). In an echo of the work in rat brain slices, the performance of YC3.3, TN-L15, GCaMP1.6, GCaMP2, YC2.60, YC3.60, cameleon D3 and TN-XL were compared one with another and calibrated against a low molecular weight indicator, Oregon-Green-BAPTA-1 (Hendel et al., 2008). The latter four sensors were around two fold more responsive than their earlier counterparts. None of the sensors were seen to detect single action potentials, though YC3.60 and cameleon D3 could detect two action potentials in succession. None showed the fast temporal response of the low molecular weight indicator. A theoretical framework in which to consider the pros and cons of calcium sensors has been adumbrated (Hires et al., 2008).

GCaMP1.6 and GCaMP2 were compared in pyramidal cells dendrites in mammalian brain slices transfected ballistically or by electroporation (Mao et al., 2008) under conditions that allowed comparison with first generation sensors (Pologruto et al., 2004). Their performance was not significantly better than GCaMP, even when localised using membrane and cytoskeletal targeting chimeras (Mao et al., 2008). GCaMP3, however, showed substantial gains in sensitivity and discrimination (Tian et al., 2009): overall, the signal to noise ratio was much improved and responses in dendrites to single action potentials could be reliably detected. Direct comparison with TN-XXL and cameleon D3 showed that, although brighter, the two FRET sensors gave smaller fluorescence changes and less favourable signal to noise ratios. GCaMP3 was also more photostable. After either adenoviral transfection or in utero electroporation, calcium responses in pyramidal neurones could be observed in awake, behaving mice (Tian et al., 2009). Parallel electrical recordings showed that detectable calcium responses were associated with three or more action potentials. Calcium responses were also readily observed in the glomeruli of *Drosophila* antennal lobe and in sensory neurones of *C. elegans*, altogether a methodological tour de force (Tian et al., 2009).

4. USE OF GENETICALLY ENCODED CALCIUM SENSORS

For single cell applications, wide-field fluorescence imaging, spinning disk or confocal microscopy are appropriate methods. Dual excitation laser scanning confocal imaging is achievable (Shimozono et al., 2002). For whole animal applications, particularly in intact brain or brain slices two photon microscopy is recommended, as it reduces tissue damage

and offers improved imaging within tissue (see chapter by Godfrey Smith in this volume and (Fan et al., 1999))

Expression of sensors in cells and tissues, as we have seen, can be achieved by transfection and transgenesis. One advantage of transgenic approaches is that expression can be confined to a specific tissue type, an advantage even if it is excised for imaging. Random expression in a subset of cells can more simply be achieved by using biolistic transfection of excised tissue.

Ratiometric sensors (in this context the FRET-based sensors, ratiometric pericam and DRIP) offer the advantage that the quantitative signal is in theory independent of variations in sensor distribution and concentration within cells (Silver et al., 1992). This allows reliable calibration of the signal in terms of calcium concentration (see chapter by Patton and Bers in this volume). Non-ratiometric sensors (eg GCaMP3) are adequate for determining changes in calcium concentration, for example when measuring overall spatial and temporal patterns of calcium signalling. Even in these circumstances, caution should be exercised in case the responses are non-linear, especially at low calcium concentration, so that a subset of smaller signals is overlooked (Reiff Dierk et al., 2005).

In general, genetically-encoded calcium sensors are not available commercially, though Invitrogen offers YC3.60 (<http://probes.invitrogen.com/media/pis/mp36207.pdf>). Some can be obtained for non-commercial use from their creators (<http://www.tsienlab.ucsd.edu/> and <http://cfds.brain.riken.jp/>). Or you can make your own using the handbook (Miyawaki et al., 2003a; Miyawaki et al., 2005).

5. CONCLUSIONS

Genetically-encoded calcium sensors have proved valuable in the measurement of calcium concentration in cellular organelles, for the most part in single cells in vitro. Their success as sensors in tissues in vitro and in vivo is qualified. They have also proved valuable in imaging the pattern of calcium signals within tissues, particularly in the poikilotherms, *C. elegans*, *Drosophila* and zebrafish. In homeotherms the record is largely disappointing, even when tissue is excised and monitored at room temperature (Pologruto et al., 2004). A striking exception is the use of GCaMP2 to image calcium signalling patterns in mouse heart (Tallini Yvonne et al., 2006). For the most part, sensors are still not capable of sensing individual calcium events in single cells when these cells are part of tissue, though single cell responses can be monitored in disaggregated cells (Kotlikoff Michael, 2007). Some branches of the calcium sensor evolutionary tree continue to evolve rapidly and the steady progress in optimising sensor parameters leads to the certain hope that these drawbacks will eventually be overcome by further genetic engineering.

Acknowledgments

I thank Jill McKenna for help with the manuscript. Our work is supported by grants from the Wellcome Trust.

REFERENCES

- Akerboom J, Rivera Jonathan DV, Guilbe Maria MR, Malave Elisa CA, Hernandez Hector H, Tian L, Hires SA, Marvin Jonathan S, Looger Loren L, Schreier Eric R. Crystal structures of the GCaMP calcium sensor reveal the mechanism of fluorescence signal change and aid rational design. *Journal of biological chemistry*. 2009; 284:6455–64. [PubMed: 19098007]
- Allen GJ, Chu SP, Schumacher K, Shimazaki CT, Vafeados D, Kemper A, Hawke SD, Tallman G, Tsien RY, Harper JF, Chory J, Schroeder JI. Alteration of stimulus-specific guard cell calcium

- oscillations and stomatal closing in *Arabidopsis det3* mutant. *Science* (New York, N.Y.). 2000; 289:2338–42.
- Allen GJ, Kwak JM, Chu SP, Llopis J, Tsien RY, Harper JF, Schroeder JI, Evans C, Oh St U ML. Cameleon calcium indicator reports cytoplasmic calcium dynamics in *Arabidopsis* guard cells. *Plant journal - for cell and molecular biology*. 1999; 19:735–47. [PubMed: 10571859]
- Arnaudeau S, Kelley WL, Walsh JV, Demaurex N. Mitochondria recycle Ca^{2+} to the endoplasmic reticulum and prevent the depletion of neighboring endoplasmic reticulum regions. *Journal of biological chemistry*. 2001; 276:29430–9. [PubMed: 11358971]
- Baird GS, Zacharias DA, Tsien RY. Circular permutation and receptor insertion within green fluorescent proteins. *Proceedings of the National Academy of Sciences of the United States of America*. 1999; 96:11241–6. [PubMed: 10500161]
- Baird GS, Zacharias DA, Tsien RY. Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97:11984–9. [PubMed: 11050229]
- Baker PF. The regulation of intracellular calcium in giant axons of *Loligo* and *Myxicola*. *Annals of the New York Academy of Sciences*. 1978; 307:250–68. [PubMed: 30377]
- Barth Alison L. Visualizing circuits and systems using transgenic reporters of neural activity. *Current opinion in neurobiology*. 2007; 17:567–71. [PubMed: 18036810]
- De Giorgi F, Brini M, Bastianutto C, Marsault R, Montero M, Pizzo P, Rossi R, Rizzuto R. Targeting aequorin and green fluorescent protein to intracellular organelles. *Gene*. 1996; 173:113–7. [PubMed: 8707049]
- Delagrè S, Hawtin RE, Silva CM, Yang MM, youvan DC. Red-Shifted Excitation Mutants of the Green Fluorescent Protein. *Nature Biotechnology*. 1995; 13:151–154.
- Demaurex N, Frieden M. Measurements of the free luminal ER Ca^{2+} concentration with targeted “cameleon” fluorescent proteins. *Cell calcium*. 2003; 34:109–19. [PubMed: 12810053]
- Diegelmann S, Fiala A, Leibold C, Spall T, Buchner E. Transgenic flies expressing the fluorescence calcium sensor Cameleon 2.1 under UAS control. *Genesis* (New York, N.Y. - 2000). 2002; 34:95–8.
- Diez-Garcia J, Matsushita S, Mutoh H, Nakai J, Ohkura M, Yokoyama J, Dimitrov D, Knopfel T. Activation of cerebellar parallel fibers monitored in transgenic mice expressing a fluorescent Ca^{2+} indicator protein. *European journal of neuroscience*. 2005; 22:627–35. [PubMed: 16101744]
- Drago I, Giacomello M, Pizzo P, Pozzan T. Calcium dynamics in the peroxisomal lumen of living cells. *Journal of biological chemistry*. 2008; 283:14384–90. [PubMed: 18362140]
- Dreosti E, Odermatt B, Dorostkar Mario M, Lagnado L. A genetically encoded reporter of synaptic activity in vivo. *Nature methods*. 2009; 6:883–9. [PubMed: 19898484]
- Emmanouilidou E, Teschemacher AG, Pouli AE, Nicholls LI, Seward EP, Rutter GA. Imaging Ca^{2+} concentration changes at the secretory vesicle surface with a recombinant targeted cameleon. *Current biology - CB*. 1999; 9:915–8. [PubMed: 10469598]
- Evanko Daniel S, Haydon Philip G. Elimination of environmental sensitivity in a cameleon FRET-based calcium sensor via replacement of the acceptor with Venus. *Cell calcium*. 2005; 37:341–8. [PubMed: 15755495]
- Fan GY, Fujisaki H, Miyawaki A, Tsay RK, Tsien RY, Ellisman MH. Video-rate scanning two-photon excitation fluorescence microscopy and ratio imaging with cameleons. *Biophysical journal*. 1999; 76:2412–20. [PubMed: 10233058]
- Fiala A, Spall T, Diegelmann S, Eisermann B, Sachse S, Devaud J-M, Buchner E, Galizia CG. Genetically expressed cameleon in *Drosophila melanogaster* is used to visualize olfactory information in projection neurons. *Current biology - CB*. 2002; 12:1877–84. [PubMed: 12419190]
- Filippin L, Abad Maria C, Gastaldello S, Magalhaes Paulo J, Sandona D, Pozzan T. Improved strategies for the delivery of GFP-based Ca^{2+} sensors into the mitochondrial matrix. *Cell calcium*. 2005; 37:129–36. [PubMed: 15589993]
- Filippin L, Magalhaes Paulo J, Di Benedetto G, Colella M, Pozzan T. Stable interactions between mitochondria and endoplasmic reticulum allow rapid accumulation of calcium in a subpopulation of mitochondria. *Journal of biological chemistry*. 2003; 278:39224–34. [PubMed: 12874292]

- Foyouzi-Youssefi R, Arnaudeau S, Borner C, Kelley WL, Tschopp J, Lew DP, Demaurex N, Krause KH. Bcl-2 decreases the free Ca²⁺ concentration within the endoplasmic reticulum. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97:5723–8. [PubMed: 10823933]
- Garaschuk O, Griesbeck O, Konnerth A. Troponin C-based biosensors: a new family of genetically encoded indicators for *in vivo* calcium imaging. *Cell calcium*. 2007; 42:351–361. [PubMed: 17451806]
- Garaschuk O, Milos R-I, Grienberger C, Marandi N, Adelsberger H, Konnerth A. Optical monitoring of brain function in vivo: from neurons to networks. *Pflügers Archiv - European journal of physiology*. 2006; 453:385–96. [PubMed: 17047983]
- Gilkey JC, Jaffe LF, Ridgway EB, Reynolds GT. A free calcium wave traverses the activating egg of the medaka, *Oryzias latipes*. *Journal of cell biology*. 1978; 76:448–66. [PubMed: 10605450]
- Graves Thomas K, Hinkle Patricia M. Ca(2+)-induced Ca(2+) release in the pancreatic beta-cell: direct evidence of endoplasmic reticulum Ca(2+) release. *Endocrinology*. 2003a; 144:3565–74. [PubMed: 12865339]
- Graves Thomas K, Hinkle Patricia M. Endoplasmic reticulum calcium storage and release in cells expressing misfolded growth hormone. *Growth hormone & IGF research - official journal of the Growth Hormone Research Society and the International IGF Research Society*. 2003b; 13:36–43.
- Griesbeck O. Fluorescent proteins as sensors for cellular functions. *Current opinion in neurobiology*. 2004; 14:636–41. [PubMed: 15464898]
- Griesbeck O, Baird GS, Campbell RE, Zacharias DA, Tsien RY. Reducing the environmental sensitivity of yellow fluorescent protein. Mechanism and applications. *Journal of biological chemistry*. 2001; 276:29188–94. [PubMed: 11387331]
- Guo ZV, Hart AC, Ramanathan S. Optical interrogation of neural circuits in *Caenorhabditis elegans*. *Nature methods*. 2009; 6:891–896. [PubMed: 19898486]
- Habuchi S, Cotlet M, Hofkens J, Dirix G, Michiels J, Vanderleyden J, Subramaniam V, De Schryver Frans C. Resonance energy transfer in a calcium concentration-dependentameleon protein. *Biophysical journal*. 2002; 83:3499–506. [PubMed: 12496116]
- Hara M, Bindokas V, Lopez James P, Kaihara K, Landa Luis R, Harbeck M, Roe Michael W. Imaging endoplasmic reticulum calcium with a fluorescent biosensor in transgenic mice. *American journal of physiology*. 2004; 287:C932–8. [PubMed: 15163621]
- Hasan Mazahir T, Friedrich Rainer W, Euler T, Larkum Matthew E, Giese G, Both M, Duebel J, Waters J, Bujard H, Griesbeck O, Tsien Roger Y, Nagai T, Miyawaki A, Denk W. Functional fluorescent Ca²⁺ indicator proteins in transgenic mice under TET control. *PLoS biology*. 2004; 2:e163. [PubMed: 15208716]
- Heim N, Garaschuk O, Friedrich Michael W, Mank M, Milos R-I, Kovalchuk Y, Konnerth A, Griesbeck O. Improved calcium imaging in transgenic mice expressing a troponin-C based biosensor. *Nature methods*. 2007; 4:127–129. [PubMed: 17259991]
- Heim N, Griesbeck O. Genetically encoded indicators of cellular calcium dynamics based on troponin C and green fluorescent protein. *Journal of biological chemistry*. 2004; 279:14280–6. [PubMed: 14742421]
- Heim R, Cubitt AB, Tsien RY. Improved green fluorescence. *Nature*. 1995; 373:663–664. [PubMed: 7854443]
- Hendel T, Mank M, Schnell B, Griesbeck O, Borst A, Reiff Dierk F. Fluorescence changes of genetic calcium indicators and OGB-1 correlated with neural activity and calcium in vivo and in vitro. *Journal of neuroscience - the official journal of the Society for Neuroscience*. 2008; 28:7399–411.
- Higashijima, S.-i.; Masino Mark, A.; Mandel, G.; Fetcho Joseph, R. Imaging neuronal activity during zebrafish behavior with a genetically encoded calcium indicator. *Journal of neurophysiology*. 2003; 90:3986–97. [PubMed: 12930818]
- Hires SA, Tian L, Looger Loren L. Reporting neural activity with genetically encoded calcium indicators. *Brain cell biology*. 2008; 36:69–86. [PubMed: 18941901]
- Ikura M, Clore GM, Gronenborn AM, Zhu G, Klee CB, Bax A. Solution structure of a calmodulin-target peptide complex by multidimensional NMR. *Science (New York, N.Y.)*. 1992; 256:632–8.

- Iwano M, Shiba H, Miwa T, Che F-S, Takayama S, Nagai T, Miyawaki A, Isogai A. Ca^{2+} dynamics in a pollen grain and papilla cell during pollination of *Arabidopsis*. *Plant physiology*. 2004; 136:3562–71. [PubMed: 15489279]
- Jares-Erijman Elizabeth A, Jovin Thomas M. FRET imaging. *Nature biotechnology*. 2003; 21:1387–95.
- Ji G, Feldman Morris E, Deng K-Y, Greene Kai S, Wilson J, Lee Jane C, Johnston Robyn C, Rishniw M, Tallini Y, Zhang J, Wier Winthrop G, Blaustein Mordecai P, Xin H-B, Nakai J, Kotlikoff Michael I. Ca^{2+} -sensing transgenic mice: postsynaptic signaling in smooth muscle. *Journal of biological chemistry*. 2004; 279:21461–8. [PubMed: 14990564]
- Kerr R, Lev-Ram V, Baird G, Vincent P, Tsien RY, Schafer WR. Optical imaging of calcium transients in neurons and pharyngeal muscle of *C. elegans*. *Neuron*. 2000; 26:583–94. [PubMed: 10896155]
- Kotlikoff Michael I. Genetically encoded Ca^{2+} indicators: using genetics and molecular design to understand complex physiology. *Journal of physiology*. 2007; 578:55–67. [PubMed: 17038427]
- Liu L, Yermolaieva O, Johnson Wayne A, Abboud Francois M, Welsh Michael J. Identification and function of thermosensory neurons in *Drosophila* larvae. *Nature neuroscience*. 2003; 6:267–73.
- Mank M, Griesbeck O. Genetically encoded calcium indicators. *Chemical Reviews*. 2008; 108:1550–1564. [PubMed: 18447377]
- Mank M, Reiff Dierk F, Heim N, Friedrich Michael W, Borst A, Griesbeck O. A FRET-based calcium biosensor with fast signal kinetics and high fluorescence change. *Biophysical journal*. 2006; 90:1790–6. [PubMed: 16339891]
- Mank M, Santos AF, Drenth S, Mersic-Flogel TD, Hofer SB, Stein V, Hendel T, Reiff Dierk F, Levelt C, Borst A, Bohoeffer T, Hübener M, Griesbeck O. A genetically encoded calcium indicator for chronic *in vivo* two-photon imaging. *Nature Methods*. 2008; 5:805–811. [PubMed: 19160515]
- Mao T, O'Connor Daniel H, Scheuss V, Nakai J, Svoboda K. Characterization and subcellular targeting of GCaMP-type genetically-encoded calcium indicators. *PLoS one*. 2008; 3:e1796. [PubMed: 18350138]
- Miyawaki A. Fluorescence imaging of physiological activity in complex systems using GFP-based probes. *Current opinion in neurobiology*. 2003a; 13:591–6. [PubMed: 14630223]
- Miyawaki A. Visualization of the spatial and temporal dynamics of intracellular signaling. *Developmental cell*. 2003b; 4:295–305. [PubMed: 12636912]
- Miyawaki A. Innovations in the imaging of brain functions using fluorescent proteins. *Neuron*. 2005; 48:189–99. [PubMed: 16242400]
- Miyawaki A, Griesbeck O, Heim R, Tsien RY. Dynamic and quantitative Ca^{2+} measurements using improved cameleons. *Proceedings of the National Academy of Sciences of the United States of America*. 1999; 96:2135–40. [PubMed: 10051607]
- Miyawaki A, Llopi J, Heim R, McCaffery JM, Adams JA, Ikura M, Tsien RY. Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature*. 1997; 388:882–7. [PubMed: 9278050]
- Miyawaki A, Nagai T, Mizuno H. Mechanisms of protein fluorophore formation and engineering. *Current opinion in chemical biology*. 2003a; 7:557–62. [PubMed: 14580558]
- Miyawaki A, Nagai T, Mizuno H. Engineering fluorescent proteins. *Advances in biochemical engineering/biotechnology*. 2005; 95:1–15. [PubMed: 16080263]
- Miyawaki A, Sawano A, Kogure T. Lighting up cells: labelling proteins with fluorophores. *Nature cell biology*. 2003b; (Suppl):S1–7.
- Nagai T, Ibata K, Park Eun S, Kubota M, Mikoshiba K, Miyawaki A. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nature biotechnology*. 2002; 20:87–90.
- Nagai T, Sawano A, Park ES, Miyawaki A. Circularly permuted green fluorescent proteins engineered to sense Ca^{2+} . *Proceedings of the National Academy of Sciences of the United States of America*. 2001; 98:3197–202. [PubMed: 11248055]
- Nagai T, Yamada S, Tominaga T, Ichikawa M, Miyawaki A. Expanded dynamic range of fluorescent indicators for Ca^{2+} by circularly permuted yellow fluorescent proteins. *Proceedings of the*

- National Academy of Sciences of the United States of America. 2004; 101:10554–9. [PubMed: 15247428]
- Nakai J, Ohkura M, Imoto K. A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein. *Nature biotechnology*. 2001; 19:137–41.
- Ohkura M, Matsuzaki M, Kasai H, Imoto K, Nakai J. Genetically encoded bright Ca^{2+} probe applicable for dynamic Ca^{2+} imaging of dendritic spines. *Analytical chemistry*. 2005; 77:5861–9. [PubMed: 16159115]
- Palmer Amy E, Giacomello M, Kortemme T, Hires SA, Lev-Ram V, Baker D, Tsien Roger Y. Ca^{2+} indicators based on computationally redesigned calmodulin-peptide pairs. *Chemistry & biology*. 2006; 13:521–30. [PubMed: 16720273]
- Palmer Amy E, Jin C, Reed John C, Tsien Roger Y. Bcl-2-mediated alterations in endoplasmic reticulum Ca^{2+} analyzed with an improved genetically encoded fluorescent sensor. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:17404–9. [PubMed: 15585581]
- Persechini A, Lynch JA, Romoser VA. Novel fluorescent indicator proteins for monitoring free intracellular Ca^{2+} Cell calcium. 1997; 22:209–16. [PubMed: 9330791]
- Pologruto TA, Yasuda R, Svoboda K. Monitoring neural activity and $[\text{Ca}^{2+}]$ with genetically encoded Ca^{2+} indicators. *Journal of neuroscience - the official journal of the Society for Neuroscience*. 2004; 24:9572–9579.
- Pozzan T, Rudolf R. Measurements of mitochondrial calcium in vivo. *Biochimica et biophysica acta*. 2009; 1787:1317–23. [PubMed: 19100709]
- Reiff Dierk F, Ihring A, Guerrero G, Isacoff Ehud Y, Joesch M, Nakai J, Borst A. In vivo performance of genetically encoded indicators of neural activity in flies. *Journal of neuroscience - the official journal of the Society for Neuroscience*. 2005; 25:4766–78.
- Reiff Dierk F, Thiel Philippe R, Schuster Christoph M. Differential regulation of active zone density during long-term strengthening of *Drosophila* neuromuscular junctions. *Journal of neuroscience - the official journal of the Society for Neuroscience*. 2002; 22:9399–409.
- Rekas A, Alattia J-R, Nagai T, Miyawaki A, Ikura M. Crystal structure of venus, a yellow fluorescent protein with improved maturation and reduced environmental sensitivity. *Journal of biological chemistry*. 2002; 277:50573–8. [PubMed: 12370172]
- Rizzuto R, Simpson AWM, Brini M, Pozzan T. Rapid changes of mitochondrial Ca^{2+} revealed by specifically targeted recombinant aequorin. *Nature*. 1992; 358:325–327. [PubMed: 1322496]
- Robert V, Gurlini P, Tosello V, Nagai T, Miyawaki A, Di Lisa F, Pozzan T. Beat-to-beat oscillations of mitochondrial $[\text{Ca}^{2+}]$ in cardiac cells. *EMBO journal*. 2001; 20:4998–5007. [PubMed: 11532963]
- Romoser VA, Hinkle PM, Persechini A. 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*. 1997; 272:13270–4. [PubMed: 9148946]
- Rudolf R, Mongillo M, Magalhaes Paulo J, Pozzan T. In vivo monitoring of Ca^{2+} uptake into mitochondria of mouse skeletal muscle during contraction. *Journal of cell biology*. 2004; 166:527–36. [PubMed: 15314066]
- Shimozono S, Fukano T, Kimura KD, Mori I, Kirino Y, Miyawaki A. Slow Ca^{2+} dynamics in pharyngeal muscles in *Caenorhabditis elegans* during fast pumping. *EMBO Reports*. 2004; 5:521–526. [PubMed: 15088067]
- Shimozono S, Fukano T, Nagai T, Kirino Y, Mizuno H, Miyawaki A. Confocal imaging of subcellular Ca^{2+} concentrations using a dual-excitation ratiometric indicator based on green fluorescent protein. *Science's STKE - signal transduction knowledge environment*. 2002; 2002:pl4. [PubMed: 11917155]
- Silver RA, Whitaker M, Bolsover SR. Intracellular ion imaging using fluorescent dyes: artefacts and limits to resolution. *Pflugers Archiv - European journal of physiology*. 1992; 420:595–602. [PubMed: 1614836]

- Solovyova N, Verkhatsky A. Monitoring of free calcium in the neuronal endoplasmic reticulum: an overview of modern approaches. *Journal of neuroscience methods*. 2002; 122:1–12. [PubMed: 12535760]
- Souslova Ekaterina A, Belousov Vsevolod V, Lock John G, Stromblad S, Kasparov S, Bolshakov Alexey P, Pinelis Vsevolod G, Labas Yulii A, Lukyanov S, Mayr Lorenz M, Chudakov Dmitriy M. Single fluorescent protein-based Ca²⁺ sensors with increased dynamic range. *BMC biotechnology*. 2007; 7:37. [PubMed: 17603870]
- Suzuki H, Kerr R, Bianchi L, Frokjaer-Jensen C, Slone D, Xue J, Gerstbrein B, Driscoll M, Schafer William R. In vivo imaging of *C. elegans* mechanosensory neurons demonstrates a specific role for the MEC-4 channel in the process of gentle touch sensation. *Neuron*. 2003; 39:1005–17. [PubMed: 12971899]
- Tallini Yvonne N, Ohkura M, Choi B-R, Ji G, Imoto K, Doran R, Lee J, Plan P, Wilson J, Xin H-B, Sanbe A, Gulick J, Mathai J, Robbins J, Salama G, Nakai J, Kotlikoff Michael I. Imaging cellular signals in the heart in vivo: Cardiac expression of the high-signal Ca²⁺ indicator GCaMP2. *Proceedings of the National Academy of Sciences of the United States of America*. 2006; 103:4753–8. [PubMed: 16537386]
- Tian L, Hires SA, Mao T, Huber D, Chiappe ME, Chalasani Sreekanth H, Petreanu L, Akerboom J, McKinney Sean A, Schreiter Eric R, Bargmann Cornelia I, Jayaraman V, Svoboda K, Looger Loren L. Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. *Nature methods*. 2009; 6:875–81. [PubMed: 19898485]
- Truong K, Sawano A, Mizuno H, Hama H, Tong KI, Mal TK, Miyawaki A, Ikura M. FRET-based in vivo Ca²⁺ imaging by a new calmodulin-GFP fusion molecule. *Nature structural biology*. 2001; 8:1069–73.
- Tsien Roger Y. The 2009 Lindau Nobel Laureate Meeting: Roger Y. Tsien, Chemistry 2008. *Journal of visualized experiments - JoVE*.
- Tsien RY. The green fluorescent protein. *Annual Reviews of Biochemistry*. 1998; 67:509–544.
- Varadi A, Rutter Guy A. Dynamic imaging of endoplasmic reticulum Ca²⁺ concentration in insulin-secreting MIN6 Cells using recombinant targeted cameleons: roles of sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA)-2 and ryanodine receptors. *Diabetes*. 2002a; 51(Suppl 1):S190–201. [PubMed: 11815480]
- Varadi A, Rutter Guy A. Green fluorescent protein calcium biosensors. *Calcium imaging with GFP cameleons. Methods in molecular biology (Clifton, N.J.)*. 2002b; 183:255–64.
- Varadi A, Rutter Guy A. Ca²⁺-induced Ca²⁺ release in pancreatic islet beta-cells: critical evaluation of the use of endoplasmic reticulum-targeted “cameleons”. *Endocrinology*. 2004; 145:4540–9. [PubMed: 15217981]
- Visser AJWG, Liptonok SP, Visser NV, van Hoek A, Birch DJS, Brochon JC, Borst JW. Time-resolved FRET fluorescence spectroscopy of visible fluorescent protein pairs. *European biophysics journal - EBJ*. 39:241–53. [PubMed: 19693494]
- Wang Jing W, Wong Allan M, Flores J, Vossall Leslie B, Axel R. Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. *Cell*. 2003; 112:271–82. [PubMed: 12553914]
- Wang Q, Shui B, Kotlikoff Michael I, Sondermann H. Structural basis for calcium sensing by GCaMP2. *Structure (London, England - 1993)*. 2008; 16:1817–27.
- Yang X, Xu P, Xu T. A new pair for inter- and intra-molecular FRET measurement. *Biochemical and biophysical research communications*. 2005; 330:914–20. [PubMed: 15809083]
- Yu D, Baird Geoffrey S, Tsien Roger Y, Davis Ronald L. Detection of calcium transients in *Drosophila* mushroom body neurons with camgaroo reporters. *Journal of neuroscience - the official journal of the Society for Neuroscience*. 2003; 23:64–72.
- Yu R, Hinkle PM. Rapid turnover of calcium in the endoplasmic reticulum during signaling. *Studies withameleon calcium indicators. Journal of biological chemistry*. 2000; 275:23648–53. [PubMed: 10811650]
- Zacharias DA, Baird GS, Tsien RY. Recent advances in technology for measuring and manipulating cell signals. *Current opinion in neurobiology*. 2000; 10:416–21. [PubMed: 10851181]

- Zacharias David A, Violin Jonathan D, Newton Alexandra C, Tsien Roger Y. Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science (New York, N.Y.)*. 2002; 296:913–6.
- Zou J, Hofer Aldebaran M, Lurtz Monica M, Gadda G, Ellis April L, Chen N, Huang Y, Holder A, Ye Y, Louis Charles F, Welshhans K, Rehder V, Yang Jenny J. Developing sensors for real-time measurement of high Ca^{2+} concentrations. *Biochemistry*. 2007; 46:12275–88. [PubMed: 17924653]

Figure 1. Schematic depiction of the different classes of genetically encoded calcium sensors. EYFP and EGFP variants for individual sensors are shown to the right, as are the identities of the red-emitting sensors.