Fluorescent tags of protein function in living cells

Michael Whitaker

Summary
A cell's biochemistry is now known to be the biochemistry of molecular machines, that is, protein complexes that are assembled and dismantled in particular locations within the cell as needed. One important element in our understanding has been the ability to begin to see where proteins are in cells and what they are doing as they go about their business. Accordingly, there is now a strong impetus to discover new ways of looking at the workings of proteins in living cells. Although the use of fluorescent tags to track individual proteins in cells has a long history, the availability of laser-based confocal microscopes and the imaginative exploitation of the green fluorescent protein from jellyfish have provided new tools of great diversity and utility. It is now possible to watch a protein bind its substrate or its partners in real time and with submicron resolution within a single cell. The importance of processes of self-organisation represented by protein folding on the one hand and subcellular organelles on the other are well recognised. Self-organisation at the intermediate level of multimeric protein complexes is now open to inspection. ***BioEssays*** **22:180–187, 2000.**

Introduction

What you get is what you see

Fluorescence methods are now commonplace in cell biology. Whether used as an enzyme-linked assay, in DNA sequencers, or in confocal microscopy, the major advantage of the approach is that a fluorescent molecule is effectively self-luminous, a light shining in the darkness. Just as our eyes pick out a distant candle flame on a dark night (we can detect about 5 photons in a brief flash[1]), so photometric detectors such as photomultipliers and charge-coupled device (CCD) cameras can be designed to work at the single photon level. With this degree of sensitivity, fluorescence from a single molecule can be detected, amounting to around 500 photons a second.[2] A single molecule generates a point source of light, so that the spatial precision in imaging the source of single molecule fluorescence is at nanometre length scales, well below the half-micron resolution of multipoint imaging in the light microscope.[3] A second advantage is linearity: double the molecules give double the signal, the basis of all fluorescence quantitation. A third is colour, or more properly the emission spectrum of the molecule. This not only allows one fluorescent tag to be distinguished from another within the cell, but is also the basis for approaches that exploit fluorescence resonance energy transfer (FRET) or the sensitivity of a fluorophore to its environment, as we shall see later.

The simplest application of fluorescent tagging of proteins is to attach a fluorophore to a protein to find out where it goes.

Painting proteins

Entomologists study the foraging behaviour of individual ants by applying different coloured paint spots to them as they leave the nest. One of the earliest instances of fluorescent tagging was less sophisticated, more the equivalent of spraying all ants indiscriminately with a paint spray gun. Fluorescent molecules are generally covalently linked to proteins by reaction with side chain amino or sulphydryl groups.[4] If intact cells are treated with reactive fluorescent tags, many membrane proteins become labelled on their extracellular domains. The aim of these sorts of experiments was straightforward, namely to determine which parts of the protein faced the outside world,[5] but the technique was then adapted to measure protein diffusion constants in the plasma membrane using fluorescence recovery after photobleaching (FRAP). An intense illumination will irreversibly bleach most fluorophores; they will no longer be fluorescent.[6,7] A locally applied spot of intense illumination to a labelled plasma membrane produces a dark patch. By measuring the rate at which a fluorescence signal reappears in the patch as unbleached tagged proteins diffuse into it, the protein diffusion constant can be determined.

Achieving the precision of the entomologist is more exacting. Proteins must be isolated in quantity, purified, tagged, and then microinjected into cells. It is not, therefore, surprising that the first proteins to be studied were tubulin (Fig. 1) and actin, which are abundant proteins that can be isolated with relative ease from brain and muscle. The effort was worthwhile. The cytoskeleton was revealed to be a highly dynamic structure with a rapid turnover rate.[8] The

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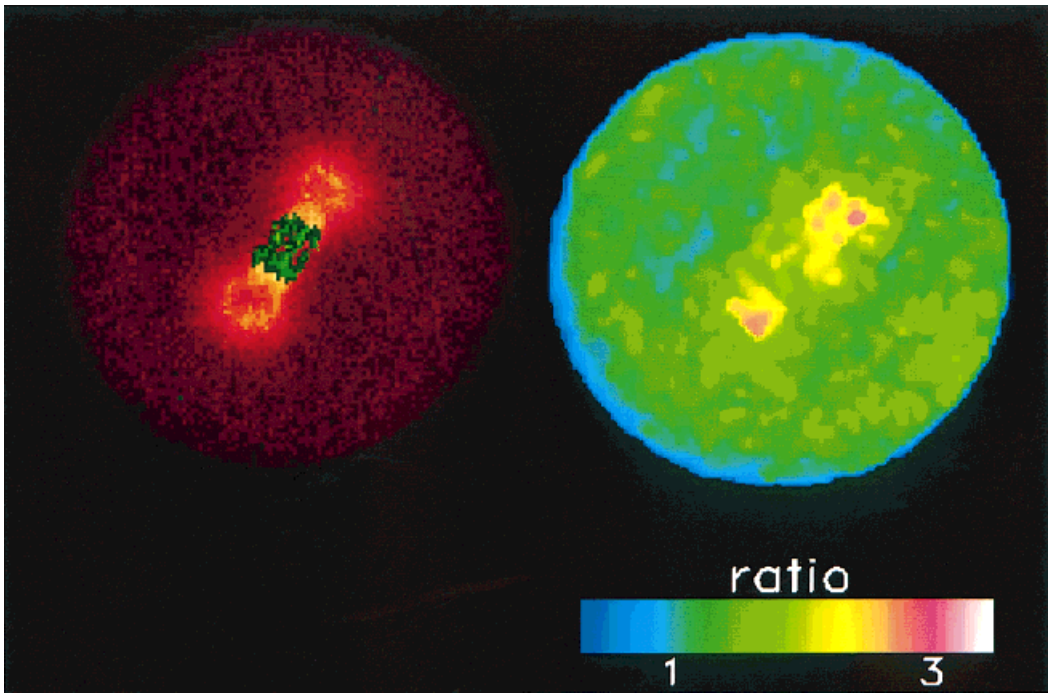

Figure 1. Localization and activation of proteins during mitosis. The sea urchin embryo on the left has been microinjected with rhodamine-tagged tubulin and chromatin stained using Hoechst 33342. The image shows the chromosomes and the tubulin of the mitotic spindle during early anaphase.[62] The embryo on the right is at a similar stage of the mitosis. It has been microinjected with a mixture of TA- and fluorescein-calmodulin. The ratio image of the two signals shows activation of calmodulin at the spindle poles.[17] Images were obtained using a confocal microscope. The embryo is 100 μm across.

movement of tubulin dimers into and along microtubules in the mitotic spindle was seen by using FRAP to follow a cohort of bleached rhodamine-tagged dimers.[9] Better resolution was achieved by turning FRAP on its head and designing a carboxyfluorescein-based fluorophore (C2CF) that *acquired* fluorescence on intense illumination: illumination of the mitotic spindle with a slit of intense light generated a bright band of activated fluorophore that moved polewards.[10]

Actin, α-actinin, vinculin, fibronectin, and tropomyosin were tagged and localized. The approach was given the name fluorescent analog cytochemistry,[11] a neat tag in itself that has, oddly, not caught on. To this list of cytoskeletal proteins we can add calmodulin, again a stable protein present at high concentrations in cells and readily prepared from brain.[12] Another advantage of calmodulin is the good reactivity of the amino group on lysine at position 75 (lys75) with the label, compared to other amino groups, illustrating the point that luck is needed when labelling native proteins: They must by chance contain suitable amino acids for labelling at positions that do not interfere with the protein's function.

Watching painted proteins at work

The above examples show that one way to watch proteins at work is to watch where they go. The simple tagging approach has been extended to less abundant proteins. Protein kinase C translocation to the plasma membrane on stimulation has been imaged using cy3-labelled microinjected protein,[13] as has differential localization of phosphoinositide transfer protein (PITP) isoforms using cy3- and cy5-labelled α and βvariants.[14] PITPα is found in the nucleus and cytoplasm, while PITPβ is localized solely to perinuclear membrane. Perhaps more exciting is the possibility of watching what proteins are doing when they get there, by visualising the conformation change that occurs when proteins are activated.

The excitation and emission spectra of fluorescent molecules all vary to some extent with their environment. This can be a nuisance if it interferes with and complicates the interpretation of straightforward localization experiments. For these a fluorophore with minimal sensitivity to environment is preferred. It can be an advantage to be exploited if the cause of the environmental change can be inferred.

Fluorescent tags that alter their intensity when the environment changes are easiest to measure. Calmodulin undergoes a large conformational change when it binds calcium,[15] altering the environment of appropriate tagged fluorophores. When the TA fluorophore is conjugated to lys75 of calmodulin, its fluorescence increases about 10-fold as calmodulin binds calcium and wraps around its target.[15,16] This alone is useful for determining the kinetics of calcium-calmodulin-substrate interactions in the test tube,[16] but within the cell, the signal is ambiguous, since the intensity in the image reports concentration (localization) as well as activation and substrate binding. One way around this problem is to microinject two calmodulins, one tagged with TA, the other with fluorescein. The fluorescein calmodulin reports concentration alone, so dividing the TA-calmodulin signal by the signal provided by fluorescein calmodulin gives a quotient that represents calmodulin activation. The method shows activation of calmodulin in the nucleus[17] around the nucleus and at the spindle poles (Fig. 1) during mitosis[18] with a temporal resolution of a few seconds. Millisecond time resolution[15] is feasible for these and other tagged proteins.

Environment also affects the polarisation of the fluorescence emission. One advantage of the approach is that polarisation (anisotropy) is an intensive variable largely independent of dye concentration. Fluorescence anisotropy measurements of tagged calmodulin have provided images of calmodulin activation in migrating fibroblasts[19,20] and have yielded information about the orientation of myosin heads during muscle contraction.[21] Concentration-independence of the signal is also the major advantage of time-resolved fluorescence methods. Quantum yield or brightness is not the only thing that alters when the environment of a fluorophore changes; there is a corresponding change in the lifetime of the excited state. Lifetime measurements in a test tube in the fluorimeter are relatively straightforward and have been used, for example, to demonstrate the interaction of protein kinase C with its substrates,[22] using quenching of the trytophan fluorescence of PKC by pyranyl derivatives of phosphoinositides and diacylglycerol. Polarization fluorescence microscopy and FLIM are harder to implement than simple intensity measurements. However, it is very likely that imaging fluorescence lifetimes will become more straightforward in the near future and that fluorescence lifetime imaging microscopy (FLIM) will eventually be available as a routine tool,[23] much as confocal microscopy already is.

It is obvious that signals that are independent of protein concentration are preferable when looking at protein activation in living cells. Fluorescence resonance energy transfer (FRET) provides a concentration-independent signal without the complexities of anisotropy measurements and FLIM, but does so at the cost of labelling a single protein molecule (or a pair) with two fluorophores.

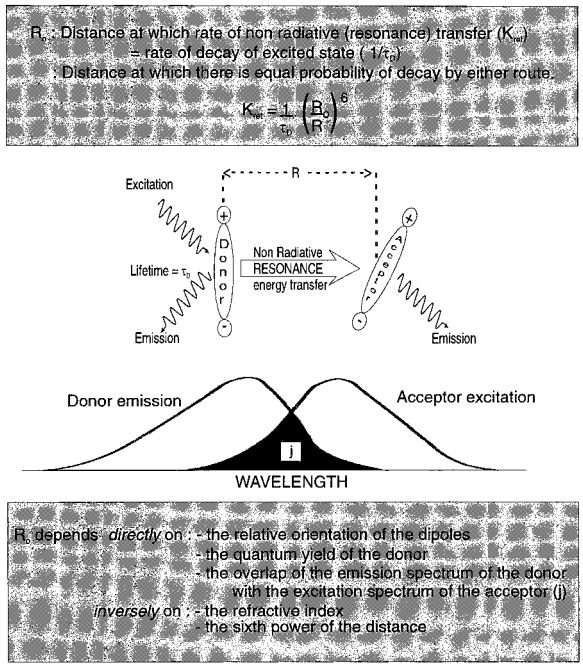

Figure 2. Fluorescence resonance energy transfer occurs via non-radiative energy transfer between fluorescent dipoles.

FRET (Fig. 2) occurs when two fluorophores are close together. The effect falls off with the sixth power of the distance between them.[24] If the distance between two protein domains alters as the protein is activated, then FRET can be used to sense the conformational change. The design of FRET probes requires an additional level of sophistication and relies on a good knowledge of the domain structure and sequence of a protein. An example is again calmodulin, whose cysteine-less sequence permitted the introduction of two novel cysteines by site-directed mutagenesis on the two arms of the molecule. As calmodulin wraps around its target,[15] its two arms come together, enabling FRET between the differently tagged arms.[25] This approach is a model example of the rational design of tagged proteins. It will have become clear that relying on conveniently placed and reactive native side chains is a hit-and-miss affair that depends largely on luck for success.

FRET is also perfect for imaging the interaction between two proteins or a protein and its substrate. Changes in the conformation of the myosin light chain/myosin heavy chain oligomer on phosphorylation of the heavy chain have been imaged with FRET between fluorescein-labelled light chain and rhodamine-tagged heavy chain, providing images of heavy chain phosphorylation patterns in migrating cells.[26] A more intricate fluorescent protein biosensor[27] that mea-

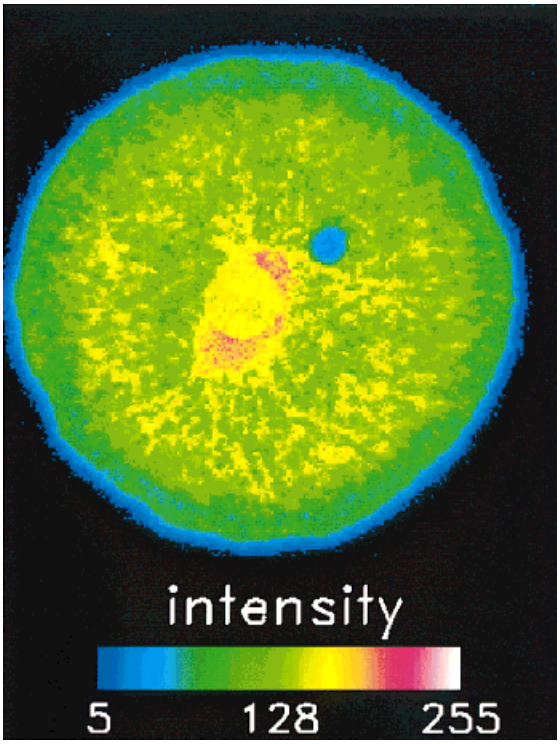

Figure 3. Localization of a protein domain. The sea urchin embryo has been microinjected with a GFP-tagged SH2 domain from PLC 1. The presumption is that the domain localization to the nucleus and perinuclear region just before mitosis, as shown in this confocal micrograph, represents the localization of the parent PLC 1 (Joanne Shearer, Mark Larman, and Michael Whitaker).

sures cytoplasmic cAMP concentration has been developed by Tsien.[28,29] It exploits the fact that the regulatory and catalytic subunits of cyclic AMP-dependent kinase dissociate when the concentration of cAMP increases; the degree of FRET between the tagged subunits is a monotonic function of cytoplasmic cAMP.

Polarization is lost during FRET; FRET can occur between like fluorophores. These photochemical properties are the basis of an ingenious demonstration that a GPI-anchored protein is confined to GPI/sphingolipid/cholesterol microdomains in the cell membrane.[30] A fluorescent folate analogue was used to determine the density-dependent behaviour of the folate receptor. The wild-type GPI-anchored receptor gave a signal whose pixel anisotropy was independent of pixel intensity, indicating that the intensity of the signal was the sum of sub pixel-size microdomains within each of which FRET (and so the distance between receptors) was constant. The control was a chimeric transmembrane anchored folate receptor that was predicted to distribute randomly in the membrane and indeed showed an increase in anisotropy with decrease in pixel density, as theory predicts for a non-confined receptor.

Interior decoration

There is a certain laboriousness in the approaches we have discussed so far. Proteins to be tagged must be obtained in quantity by isolation or recombinant expression, then tagged with fluorescence using covalent linking reactions and finally placed in the cell cytoplasm, usually by microinjection. An elegant way of painting a single recombinant protein *inside* a cell has been devised, based on the reaction of arsenicals with thiols.[31] The recombinant protein contains an alpha-helical motif with well-placed thiol groups that avidly bind an organoarsenical fluorescein derivative. Labelling is carried out in the presence of a vicinal dithiol reagent to protect other cell proteins from attack by the arsenical. This is a very promising approach to tagging a single fluorophore to a single protein. It can only supply one colour, but could be used in conjunction with other labelling approaches to produce polychrome tags for FRET or ratio imaging.

Painting proteins in natural colours

A new and elegant approach to protein tagging has been developed based on the green fluorescent protein from the jellyfish *Aequoria victoria*.[32]

Green fluorescent protein (GFP) is used by the jellyfish to alter the emission wavelengths of its luminescent protein aequorin by FRET from blue to green.[33] The striking feature of GFP is that its fluorescence is due to the internal interaction between amino acids within the protein.[34] Thus, GFP is intrinsically fluorescent and will fold and fluoresce wherever it is translated. This is itself an enormous advantage, as a chimeric protein-GFP construct, once made, is available to all the repertoires of recombinant protein expression. A slightly bizarre, perhaps tasteless, but informative and scientifically useful example is the GFP mouse, all of whose cells express the fluorescent protein[35] and which glows faintly green when illuminated by near UV light. The mouse usefully demonstrates that readily detectable levels of GFP are well tolerated by cells. Moreover, the function of GFP-tagged proteins is preserved, it seems; the relatively small (27 kDa) GFP is usually added to the C-terminal of the protein to be tagged and does not seem to get too much in the way.[36,37]

Another advantage of GFP is that it lends itself to modification by site-directed mutagenesis. The fluorescence properties have been improved in this way, initially to make a brighter (higher quantum yield) variant with a red-shifted emission spectrum.[38] These modifications enhanced its early use as a gene expression tag and lineage tracer.[39,40] Other site-directed mutants show improved thermosensitiv-

A Glossary of Terms

Fluorescence occurs when light absorbed by part of a molecule (the fluorophore) is re-emitted at a longer wavelength. Absorption of light generates an excited state of the fluorophore, so the absorption spectrum of the fluorophore is equivalent to its excitation spectrum. The excited state loses some energy before re-emitting light, generating an emission spectrum that is red-shifted by an amount known as the Stokes shift.

Anisotropy is common in fluorophores and means simply that the fluorophore is sensitive to the plane of polarization of the exciting light. If the fluorophore is tethered and so less free to move, the anisotropy will be greater. Changes in the fluorophore's freedom of movement can be measured using polarized light.

Fluorescence lifetime is the time the fluorophore spends in the excited state. Lifetime is a statistical property and is exponentially distributed, with direct analogy to the lifetimes of radioactive nuclei. It is defined by a half-life, or more usually by an exponential decay constant, τ.

Fluorescence lifetime imaging (FLIM) provides an image of lifetimes across a specimen, rather than the more usual intensity map. The usual method exploits the fact that the lifetime determines the phase lag in modulation of light emitted in response to sinusoidal modulation of the exciting light. The method is straightforward, but is presently used only by imaging specialists.

Fluorescence resonance energy transfer (FRET) occurs when two suitable fluorophores are in close proximity. Dipole effects lead to the transfer of energy from the excited state of one fluorophore to the other, provided the emission spectrum of the donor overlaps significantly with the excitation spectrum of the acceptor (see Figure 2). FRET can be imaged using conventional intensity ratio methods or FLIM, as resonance energy transfer shortens the apparent fluorescent life time of the donor.

Photobleaching is an inevitable consequence of excitation of fluorescent molecules, as there is a small probability that the energy absorbed will cause a permanent chemical change in the molecule each time the molecule absorbs light. For this reason, exciting light intensities in fluorescence imaging are as low as possible, consistent with a detectable signal.

Fluorescence recovery after photobleaching (FRAP) is used to measure the diffusion times of populations of fluorescent molecules. High intensity light is deliberately used to photobleach all the fluorophores in a given area. Subsequent increase (recovery) of fluorescence in this region is monitored with normal levels of excitation light and represents diffusion of unbleach molecules into the area of interest.

Fluorescent Tags Mentioned

TA (2,4-chloro-[6-(4-(N,N′-diethylaminophenyl)-1,3,5-triazine])
An environmentally-sensitive fluorophore used, for example, to sense conformational changes in calmodulin. Ex: 365nm. Em: 410nm.

Fluorescein
A workhorse fluorophore, usually used as 5- or 6-carboxyfluorescein. Ex: 492nm Em: 515nm.

Cy3, Cy3.5, Cy5
Proprietary longer-wavelength fluorophores marketed by Amersham BioSciences. Cy3 Ex: 550nm Em: 570nm. Cy3.5 Ex: 581nm Em: 596nm. Cy5 Ex: 649nm Em: 670nm.

Green fluorescent protein (GFP)
A jellyfish protein whose fluorophore arises from internal isomerization of elements of its amino acid sequence. EGFP variants with increased brightness and altered excitation and emission spectra have been produced by site-directed mutagenesis. EBFP (Blue) Ex: 370nm Em: 440nm. ECFP (Cyan) Ex: 440nm Em: 480nm. EGFP (Green) Ex: 440nm Em: 510nm. EYFP (Yellow) Ex: 480nm Em: 535.

ity.[41] Equally useful, different coloured variants have been engineered, with yellow emission spectra[42] and both blue- and red-shifted excitation spectra.[43]

The colour variants of the fluorescent protein add to the palette and permit multiple fluorescent tagging in a single cell, but perhaps more useful even than this, they open the door to FRET[44] and so to the analysis of protein-protein interaction.

Not just where proteins go . . .

The advantage of GFP chimeras compared to chemical fluorescent tagging for finding out where proteins are inside living cells is the ease with which they may be assembled and introduced into the cytoplasm. It is now fairly straightforward to localise recombinant cellular proteins and indeed to use GFP-tagged proteins as markers of the morphology of entire intracellular compartments.[36,37,39,40,45–49] GFP chimeras have begun to prove their worth in functional studies. A β-adrenergic receptor chimera has been shown to be fully-functional biochemically when expressed in cells.[50] The construct was used to show receptor internalisation after adrenergic stimulation and its plasma membrane diffusion coefficient was determined using FRAP. A protein kinase C β chimera has been used to demonstrate a transient episode of PKC localization to the plasma membrane after stimulation of Gq-linked agonists, but not Gs- or Gi-linked stimulation.[51] The study also found evidence of asymmetric cross desensitisation between angiotensin and endothelin receptors. A third paper in a similar vein used an arrestin chimera to visualise the arrestin-binding kinetics to 16 G protein-linked receptor subtypes to define the time course of receptor desensitisation and internalisation.[52]

These experiments are more a proof-of-concept than the demonstration of novel, unforeseen results, but they very beautifully illustrate the likely impact of such molecular chimeras in the signal transduction field.

A more striking demonstration of the value of GFP chimeras in developing entirely new approaches is the tagging of protein domains to demonstrate their interactions with other cellular components. The cysteine-rich domain of protein kinase Cs binds diacylglycerol. The translocation of the cysteine-rich domain/GFP chimera to the cell membrane can be seen both as a demonstration of where native PKC is translocated on activation and as an indicator for imaging the sites of diacylglycerol production in cells.[53] Translocation to the plasma membrane is observed after stimulation of both G protein- and tyrosine kinase-linked receptors. Differential localization to the nuclear membrane is seen after treatment with phorbol dibutyrate, but not with phorbol myristate acetate (PMA), while the mobility of the domain in the plasma membrane (determined by FRAP) is much reduced after treatment with PMA, but not with DiC_8, a soluble diacylglycerol. These results point to some unexpected complexities in PKC signalling. A second example is the use of the plekstrin homology (PH) domain from PLCδ1.[54] The PH domain binds $PtdInsP_2$ and $InsP_3$. It has been used to follow relative concentration changes in plasma membrane $PtdInsP_2$ and plasma membrane $InsP_3$ during signalling events involving calcium oscillations.[53,55]

. . . But what they do when they get there

As we have already seen, FRET between fluorophores tagged to two domains of the same protein or to two components of a protein oligomer can report the conformational changes associated with protein activation and the interaction of protein oligomers. GFP-based fluorophores offer opportunities for FRET-based assays of activation and oligomerisation, too.

One inventive approach uses a GFP-tagged protein kinase C in association with a phosphothreonine antibody that binds only to the active, autophosphorylated form of PKC.[56] FRET between the GFP and the Cy3.5-tagged phosphothreonine antibody was monitored using FLIM in living cells that were expressing the GFP chimera and that had been microinjected with the antibody. FRET (that is, activation of PKC) was observed to increase first with a punctate distribution at the plasma membrane and later in the perinuclear region after stimulation with PMA. One slight disadvantage of using phosphopeptide antibodies may be, as in this example, that inactivation of the kinase by phosphatases is slowed by the antibody binding.

FRET between GFP variants has also been used successfully to measure protein activation. Active calmodulin can be assayed by using a construct that consists of a complimentary GFP FRET pair separated by a peptide linker corresponding to the calmodulin binding domain of myosin light chain kinase (MLCK).[57] When this activation indicator is expressed in living cells, there is a reduction in FRET between the GFP as activated calmodulin binds and forces the two GFPs further apart. Maximal reduction *in vitro* was determined to be 65%, with a 5- to 6-fold increase in the 440/505 emission ratio.

Activated calmodulin reflects cytoplasmic calcium concentrations, but does not exactly mirror the spatial and temporal pattern of calcium increases.[18] A more faithful calcium indicator was designed by tethering a calmodulin variant C-terminal to the second GFP[58] of the GFP-MLCK-peptide-GFP construct. The tethered calmodulin much more readily interacts with its handcuffed partner and does not therefore interact or bind with its cellular substrates. The calcium indicator shows a maximal 1.7-fold change in the 440/505 emission ratio on calcium binding, corresponding to a 30% reduction in FRET.

A parallel approach with a construct in which the calmodulin and the MLCK peptide are adjacent and flanked on either side by the GFP FRET pair resulted in an indicator whose FRET signal increases as calmodulin binds calcium and so binds to the MLCK peptide, bringing the GFPs closer together.[59] The maximal emission ratio change on calcium binding is comparable to the GFP-MLCKpeptide-GFP-calmodulin variant described above. The GFP-calmodulin-MLCKpeptide-GFP constructs have been called cameleons because they readily change colour and retract and extend a long tongue.[59] Other members of the cameleon family contain modifications to calmodulin calcium affinity and targetting sequences that permit measurement of calcium concentrations within cell organelles such as the endoplasmic reticulum and nucleus.[59]

A colourful future

Cells work by assembling individual functional protein units into molecular machines whose purpose is to perform a particular task at a particular location.[60] A very clear and useful analogy has been drawn between the cell and a large industrial factory where the product of one machine is used as a regulator of or material for another machine.[61] One way to operate a factory is to accumulate the products of one machine in a stockpile, for use in the next. Stockpiles reduce the risk of running out of intermediate product, but are wasteful of resource; most modern manufacturing operates on the just-in-time principle where stocks are kept to a minimum. It seems that cells are just-in-time manufacturers: By concentrating functional protein units into multifunctional molecular machines it is possible to eliminate diffusion as a source of delay and run the machines at a rate limited by their own intrinsic turnover rates.[61]

Molecular machines are assembled by interactions between specific binding domains in proteins; assembly and

function are modified by protein activation. The new protein probes of domain binding and protein function can give us a vivid picture of the assembly and activation of molecular machines that will complement and enhance the existing approaches through structural biology and mutagenesis.

Acknowledgments

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