

CONSTRUCTING AND EXPLOITING THE FLUORESCENT PROTEIN PAINTBOX

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by

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MOTIVATION

My first exposure to visibly fluorescent proteins (FPs) was near the end of my time as a faculty member at the University of California, Berkeley. Prof. Alexander Glazer, a friend and colleague there, was the world's expert on phycobiliproteins, the brilliantly colored and intensely fluorescent proteins that serve as light-harvesting antennae for the photosynthetic apparatus of blue-green algae or cyanobacteria. One day, probably around 1987–88, Glazer told me that his lab had cloned the gene for one of the phycobiliproteins. Furthermore, he said, the apoprotein produced from this gene became fluorescent when mixed with its chromophore, a small molecule cofactor that could be extracted from dried cyanobacteria under conditions that cleaved its bond to the phycobiliprotein. I remember becoming very excited about the prospect that an arbitrary protein could be fluorescently tagged *in situ* by genetically fusing it to the phycobiliprotein, then administering the chromophore, which I hoped would be able to cross membranes and get inside cells. Unfortunately, Glazer's lab then found out that the spontaneous reaction between the apoprotein and the chromophore produced the "wrong" product, whose fluorescence was red-shifted and five-fold lower than that of the native phycobiliprotein¹⁻³. An enzyme from the cyanobacteria was required to insert the chromophore correctly into the apoprotein. This enzyme was a heterodimer of two gene products, so at least three cyanobacterial genes would have to be introduced into any other organism, not counting any gene products needed to synthesize the chromophore⁴.

Meanwhile fluorescence imaging of the second messenger cAMP (cyclic adenosine 3',5'- monophosphate) had become one of my main research goals by 1988. I reasoned that the best way to create a fluorescent sensor to detect cAMP with the necessary affinity and selectivity inside cells would be to hijack a natural cAMP-binding protein. After much consideration of the various candidates known at the time, I chose cAMP-dependent protein kinase, now more commonly abbreviated PKA. PKA contains two types of

subunits, regulatory and catalytic. In the absence of cAMP, the regulatory subunits tightly bind and inhibit the catalytic subunits. When cAMP becomes available, it binds to the regulatory subunits, which then let go of the catalytic subunits, which in turn start transferring phosphate groups from ATP onto selected proteins⁵⁻⁷. But how could activation of PKA by cAMP be made directly visible inside a single living cell? From my graduate student days I had been fascinated by a biophysical phenomenon called fluorescence resonance energy transfer (FRET), in which one excited dye molecule can transfer its energy to a close neighbor, much as a football or basketball player can pass the ball to a teammate with diminishing probability of success the greater the distance between the players. If we could attach one type of dye molecule to the regulatory subunits and the other type of dye molecule to the catalytic subunits, FRET would be possible in intact PKA, because the subunits are in intimate contact. But once cAMP had broken up the PKA complex and allowed the subunits to drift apart, FRET would be disrupted and a change in fluorescence color should be observable.

But to get these experiments to work, we needed abundant supplies of PKA subunits and lots of advice on how to handle them, especially because we had very little experience with protein biochemistry. I contacted Susan Taylor, who had become one of the world's leading experts on PKA and was producing relatively large quantities of recombinant PKA subunits in order to solve their crystal structure^{8,9} (Figure 1). The Taylor lab kindly sent shipment after shipment of proteins on wet or dry ice from UCSD to Berkeley for us to try to label with dyes, but the dyes either refused to stick, or messed up the subunits to the point where they would no longer respond to cAMP. The wish to facilitate this collaboration was an important part of the reason that my lab moved from Berkeley to UCSD in 1989. Eventually, after a year of working side by side, Dr. Stephen Adams in my lab and Ying Ji Buechler and Wolfgang Dostmann in the Taylor lab devised a reproducible procedure to combine fluorescein-labeled catalytic subunits with rhodamine-labeled regulatory subunits to produce FRET-based sensors for cAMP^{10,11}. Over the next few years, we used these protein complexes to study several interesting problems in cAMP signaling¹²⁻¹⁴. For example, we collaborated with Eric Kandel's lab to demonstrate spatial gradients of cAMP within individual sea slug (*Aplysia californica*) neurons undergoing training procedures in a model of synaptic plasticity¹⁵.

cAMP decreases FRET between PKA subunits labeled with fluorescein and rhodamine

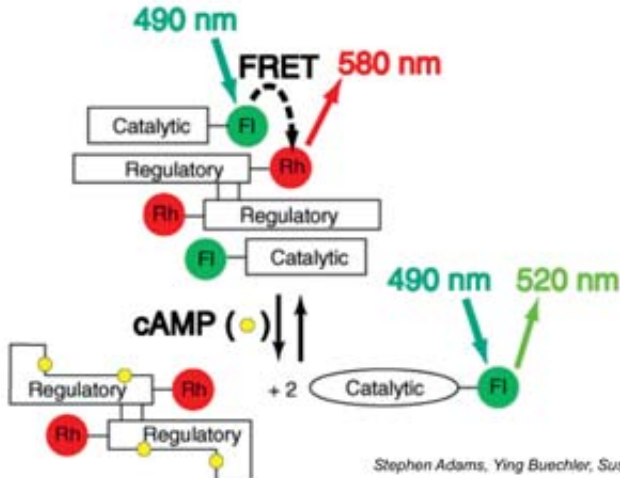


Figure 1. Schematic cartoon showing how cAMP-induced dissociation of regulatory from catalytic subunits of protein kinase A (PKA) can be reported by loss of FRET from fluorescein to rhodamine labels.

Although the cAMP sensor was moderately successful, the general approach would have been very difficult to extend to other proteins because it required high level expression and purification of soluble proteins or subunits, controlled attachment of two different dyes *in vitro* to distinct domains or subunits without destroying the function of the protein, repurification, and microinjection back into living cells. Such cells had to be large and robust enough to tolerate poking with a hollow glass needle, and the experimenter had to be patient and dexterous, unlike me. All of the above obstacles could be circumvented if we had genes encoding two fluorescent proteins of the appropriate colors. These genes could be fused to the genes for the protein(s) of interest. One would still have to get the fusion genes into the cell(s) to be studied, but standard methodology has been worked out for most cells of interest. Introducing genes into cells (transfection) is generally much easier than introducing proteins, because each cell needs only one or a few copies of DNA (compared to billions of molecules of protein), the cell has plenty of time to recover from any membrane damage, and one can selectively propagate those cells that have successfully assimilated the DNA. Once in the cells, these genes would hopefully make composite proteins *in situ* that would both fluoresce and preserve native biological function.

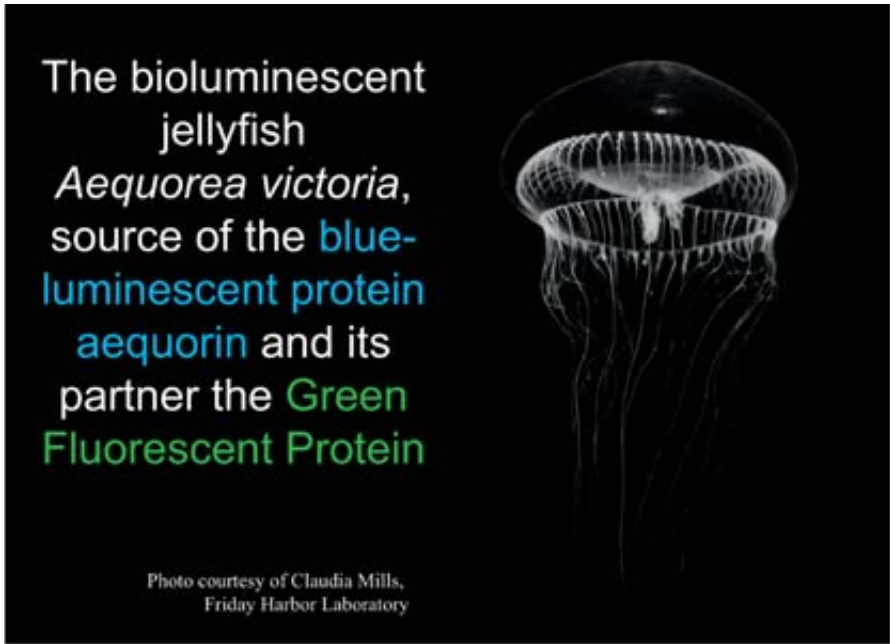


Figure 2. The jellyfish *Aequorea victoria* or *Aequorea aequorea* from which aequorin and Green Fluorescent Protein were isolated. Photo courtesy of Dr. Claudia E. Mills, Friday Harbor Laboratories.

Knowing that Prof. Glazer's lab was still struggling with the phycobiliprotein approach, I sought simpler alternative fluorescent proteins. I remembered that certain jellyfish (Figure 2) contained a green fluorescent protein (GFP), because it was an annoying contaminant to be carefully separated away from aequorin, the jellyfish protein that some of our competitors used to measure calcium signals inside cells¹⁶. One day in April or May 1992, I typed "green fluorescent protein" into Medline, the National Library of Medicine's computerized literature searching tool that had recently become accessible from UCSD. To my astonishment, up popped a citation to a just-published paper by Douglas Prasher and collaborators¹⁷, reporting the cloning of the gene for GFP and the likelihood that the chromophore was an integral part of the protein rather than being an external cofactor. I rushed to the library to photocopy the paper (Figure 3), which conveniently listed Prasher's telephone number at the Woods Hole Oceanographic Institution. I phoned Prasher in May 1992 and was surprised to hear that he did not intend to work on GFP any more. En route to the full-length gene, he had first obtained a ~70% length gene and tried to express it into bacteria, but no fluorescence appeared. Because of funding difficulties and a change in career direction, he was willing to pass the baton to us, provided that we promised to include him as a coauthor if we succeeded. I agreed to this very reasonable request, so he promised to send us a sample of the DNA encoding GFP, and some frozen jellyfish tissue in which we might hunt for the enzyme(s) that we both feared would be necessary to synthesize the chromophore within GFP.

However, I was not immediately ready to receive these samples, because no one in the lab was experienced in molecular genetics. Stephen Adams had learned much protein biochemistry during the collaboration with Susan Taylor, but her lab had already laid the molecular biological foundation^{8,9} before we got involved. So I waited in 1992 for someone with molecular genetics experience to join my lab. That person was Roger Heim, who was just completing his Ph.D. in Switzerland on cloning Ca²⁺ pumps^{18,19}. Heim came strongly recommended by his mentor Prof. Ernesto Carafoli, who said that his lab had an internal joke that if Heim could not get an experiment to work, nobody could. Ironically, Heim wanted a diversion from molecular biology and therefore applied to become a postdoctoral fellow in my lab to learn and use Ca²⁺ imaging, but he accepted my suggestion that he also see if he could make anything useful with Prasher's GFP gene. He arrived at UCSD in late September 1992.

Prasher *et al.* (1992) clone GFP

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Primary structure of the *Aequorea victoria* green-fluorescent protein

(Bioluminescence; Cnidaria; aequorin; energy transfer; chromophore; cloning)

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SUMMARY

Many cnidarians utilize green-fluorescent proteins (GFPs) as energy-transfer acceptors in bioluminescence. GFPs fluoresce *in vivo* upon receiving energy from either a luciferase-oxyluciferin excited-state complex or a Ca²⁺-activated photoprotein. These highly fluorescent proteins are unique due to the chemical nature of their chromophore, which is comprised of modified amino acid (aa) residues within the polypeptide. This report describes the cloning and sequencing of both cDNA and genomic clones of GFP from the cnidarian, *Aequorea victoria*. The *gfp10* cDNA encodes a 238-aa-residue polypeptide with a calculated *M_r* of 26888. Comparison of *A. victoria* GFP genomic clones shows three different restriction enzyme patterns which suggests that at least three different genes are present in the *A. victoria* population at Friday Harbor, Washington. The *gfp* gene encoded by the ΔGFP2 genomic clone is comprised of at least three exons spread over 2.6 kb. The nucleotide sequences of the cDNA and the gene will aid in the elucidation of structure-function relationships in this unique class of proteins.

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Figure 3. Title, abstract, and acknowledgments from the paper describing the cloning of the gene encoding Green Fluorescent Protein and the structure of GFP's chromophore¹⁷.

INITIAL EXPERIMENTS

When I re-contacted Prasher in early October 1992 to say that with Heim's arrival we were ready to receive the samples, Prasher informed me that in the months since my first phone call, Martin Chalfie at Columbia had requested and already received the gene. Shortly thereafter, I met Chalfie at a Society for Neuroscience meeting and heard from him that the gene could make bacteria (*E. coli*) fluorescent, implying no other jellyfish-specific components

were required, which was a great relief. Heim therefore concentrated his efforts on getting GFP to work in yeast (*S. cerevisiae*), partly to avoid wasteful duplication of Chalfie's efforts, partly because we were getting advice and borrowing equipment and reagents from my nearest neighbor at UCSD, Scott Emr, an eminent molecular/cell biologist specializing in yeast. With the help of Chalfie's advice to amplify only the protein-coding region of Prasher's cDNA and thus discard the flanking upstream and downstream stretches of DNA, Heim succeeded in making some yeast cells fluorescent, though there was a huge variation in the brightness of individual cells in the population. Heim and I showed Emr the cells under the microscope and asked if he could suggest any biological question in yeast for which GFP could help supply the answer. Emr was excited to see Heim's results and he indicated GFP may be used to track cargo protein movements through the secretory pathway of yeast. A postdoc in the Emr lab made GFP fusion constructs to a secreted protein and a lysosomal protein in yeast. Unfortunately, the GFP fluorescence signals were very weak and highly variable from cell to cell. This confirmed that wild-type GFP was too unreliable, so Emr's lab put GFP aside until it could be sufficiently improved.

My original hope for fluorescent proteins was to fuse a different color to each of the two types of subunits of PKA, so we obviously needed a second color. Because the chromophore of GFP was mostly constructed from a tyrosine at position 66, I asked Heim to mutate this amino acid to tryptophan, the other aromatic amino acid most conducive to absorbance and fluorescence. This alteration practically destroyed the fluorescence. After the failure of my naïve rational idea, Heim decided to mutate GFP randomly. To our delight, he soon found a blue-fluorescing mutant (Figure 4, upper right quadrant), which upon sequencing proved surprisingly to contain a histidine at position 66²⁰. Later we discovered that tryptophan at 66 gives an even more useful and bleach-resistant cyan fluorescent protein (CFP), intermediate between blue and green, but only if additional mutations carve out extra room inside the protein to accommodate the bulky tryptophan (Figure 4, lower right quadrant). Even histidine at 66 benefits from its own set of compensatory mutations²¹.

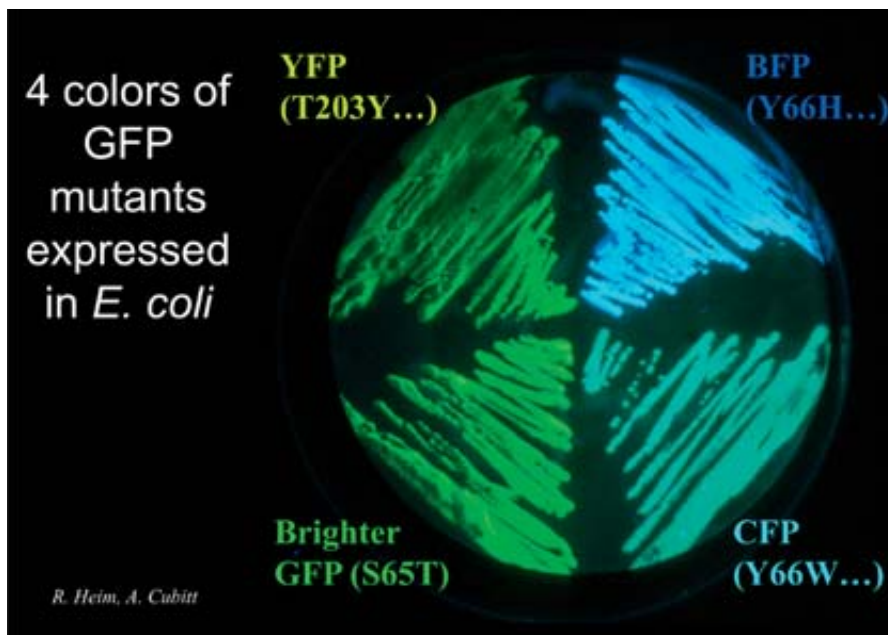


Figure 4. Fluorescence from streaks of bacteria expressing (clockwise from upper right) improved blue, cyan, green, and yellow fluorescent proteins. Each streak is labeled with the mutation most responsible for its spectral alteration.

It still bothered me that we did not understand how the chromophore (technically, a *p*-hydroxybenzylideneimidazolidinone) inside GFP was spontaneously formed from serine 65, tyrosine 66, and glycine 67^{17,22}. There seemed to be no biochemical precedent for such a post-translational modification, which not only formed a new heterocyclic ring but also dehydrogenated the α - β single bond of tyrosine to a double bond. Dehydrogenations either evolve hydrogen gas (H_2), which I thought most unlikely in this case, or require an oxidant to carry away the two hydrogen atoms. The only oxidant we could directly control within the cells was atmospheric O_2 . Heim therefore grew GFP-expressing bacteria under strictly anaerobic conditions and was pleasantly surprised to find that the protein was made but not yet fluorescent. Upon re-exposure to air, that protein became green fluorescent over a few hours²⁰. We were therefore lucky that the requisite oxidant, O_2 , is available in all organisms except obligate anaerobes, yet can be readily eliminated to demonstrate its necessity. This discovery allowed us to propose a plausible mechanism for chromophore formation (Figure 5). An important corollary is that two hydrogens + O_2 gives H_2O_2 = hydrogen peroxide, a potentially toxic byproduct. I noticed this from having to balance chemical equations in high school. Surprisingly few researchers recognized this simple consequence of the conservation of matter, perhaps because detailed mass balance is de-emphasized in most chemistry courses from university level onwards. Much later the predicted generation of one molecule of H_2O_2 per molecule of mature GFP was experimentally confirmed²³.

GFP chromophore formation and its analogy to Asn-Gly hydrolysis

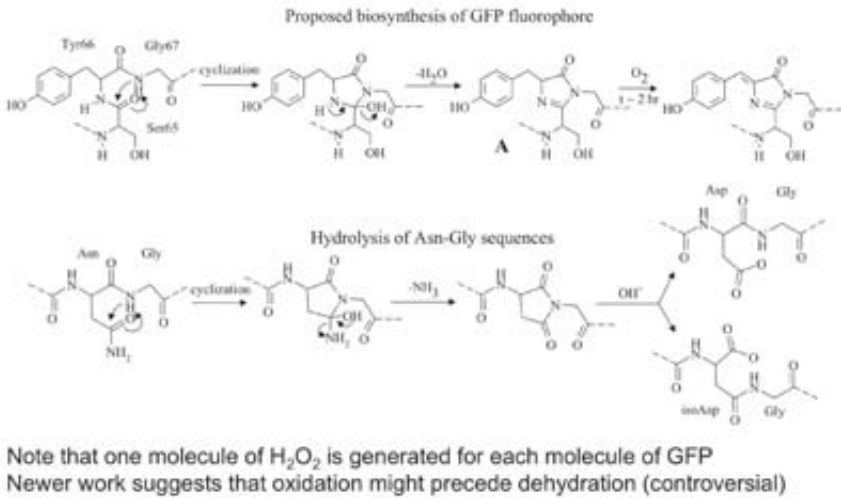


Figure 5. Initial proposal for mechanism of formation of the GFP chromophore⁷⁴. The initial cyclization is analogous to the known tendency of Asn-Gly sequences to cyclize⁷⁵. Recently it has been proposed that oxidation precedes dehydration²³.

We still had one other big problem before we could use FRET from the blue mutant (“BFP”) to GFP to sense protein conformational changes or protein-protein interactions. Ideally, GFP should be excitable only by the same blue wavelengths as BFP emits. Then irradiation with ultraviolet (UV) light to selectively excite BFP would either give blue emission in the absence of FRET, or transfer the energy to GFP to glow green. However, the original GFP was more strongly excited by UV than by blue, so that GFP was a very poor acceptor of FRET from BFP. Why did GFP have two excitation peaks, one big one in the UV and a much smaller one in the blue? I hypothesized that the major UV peak was due to the chromophore structure as guessed by Shimomura²⁴ and Prasher^{17,22}, whereas the minor blue peak was due to a small fraction of the chromophores undergoing dehydration of serine 65 to a dehydroalanine. Such dehydration would create an extra double bond in conjugation with the rest of the chromophore and perhaps explain a shift to longer wavelengths. To test this hypothesis, Heim mutated serine 65 to alanine or cysteine, which I thought would respectively prevent or promote the formation of the extra double bond, eliminating or accentuating the blue peak. Once again I was wrong: both mutations eliminated the UV peak and amplified the desired blue peak. Heim then tried replacing serine 65 by threonine. Even though threonine is the amino acid most closely resembling serine, differing only by a CH_2 group, the unwanted UV peak disappeared completely, the blue peak became 5–6 fold higher, and it even shifted ~10 nm to longer wavelengths (Figure 6)²⁵. We therefore suggested this mutant, “S65T”, as a general improvement on wild-type GFP (Figure 4, lower left

quadrant). This mutation greatly improved GFP's performance in yeast²⁶ and thus began to address Emr's difficulties. As an initial proof of principle²¹ that FRET between GFP mutants could report biochemical signals, Heim genetically fused BFP to S65T GFP via a floppy peptide linker that could be proteolyzed, e.g. by trypsin. FRET was effective in the chimeric protein before enzyme exposure, but was disrupted after cleavage of the linker as expected (Figure 7A). Fortunately the FPs themselves were quite resistant to proteases. Other GFP mutants with spectra like S65T and their use as FRET acceptors from BFP were independently developed by Youvan's group^{27,28}.

Mutations of Ser65 improve excitation spectra

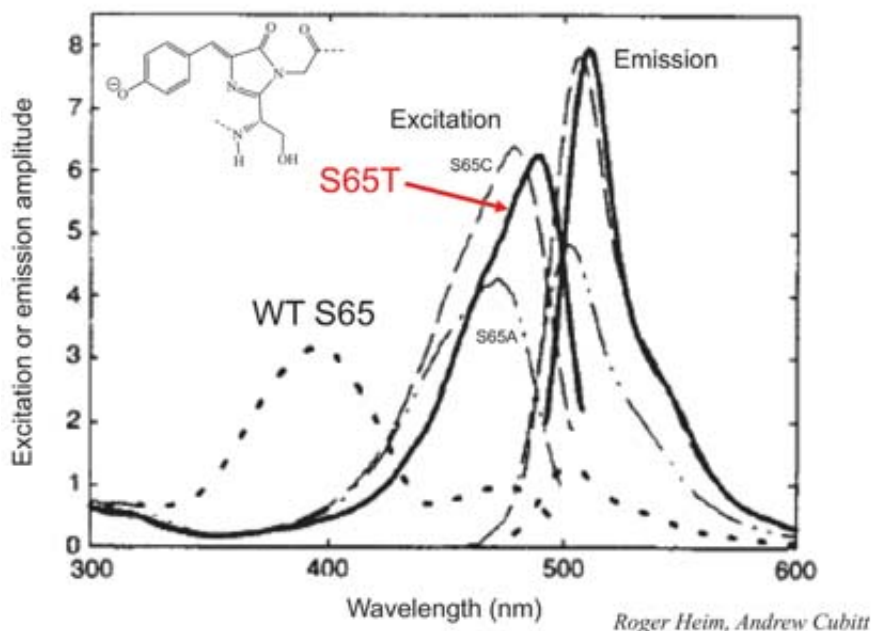


Figure 6. Excitation and emission spectra of wild-type (WT) GFP and several mutants of Ser 65²⁵. The inset shows the structure of the wild-type chromophore.

In a random screen for optimal mutations of residues 55 to 74, Cormack *et al.*²⁹ confirmed the value of S65T and added another mutation, F64L, which permits folding at warmer temperatures. The resulting double mutant, “enhanced GFP”, was aggressively marketed by Clontech, a molecular biology supply company, and became the basis for most subsequent applications of GFP, even when these two mutations are not explicitly acknowledged. Improvements like this helped repay our early debt to the Emr lab, which has exploited the brighter GFPs for numerous studies of protein sorting in the yeast secretory and endocytic pathways.

Examples of genetically encoded FRET sensors

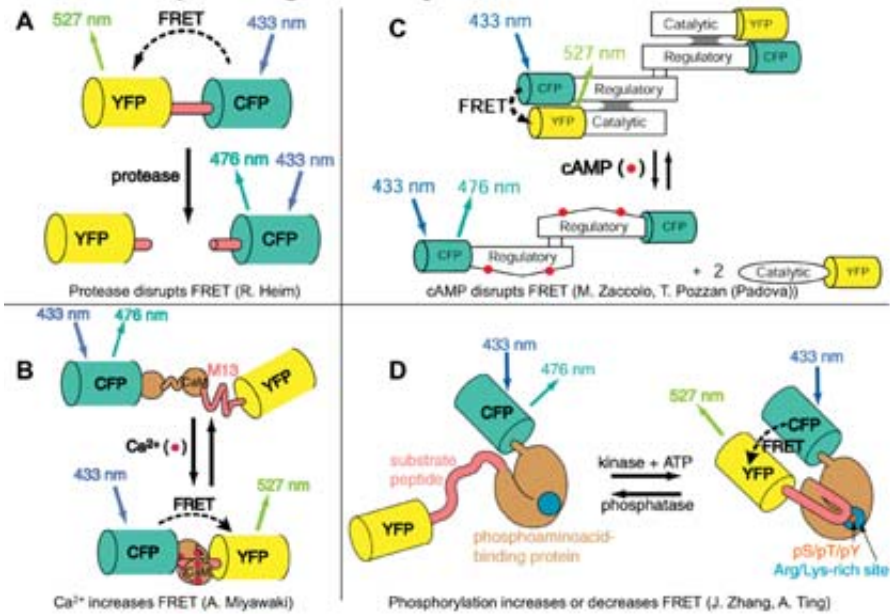


Figure 7. Schematic cartoons of genetically encoded fluorescent indicators based on FRET from CFP to YFP, to report A) protease activity, B) Ca²⁺ concentrations, C) cAMP concentrations, and D) the balance between protein kinase and phosphatase activity.

The above improvements were all made without any three-dimensional structural information, which clearly would greatly facilitate further engineering. My group had no experience with x-ray crystallography, and tentative approaches to local structural biologists found no takers. GFP had already been crystallized³⁰ well before the gene was cloned, and I had heard that several other groups had entered the competition to solve the structure of the wild-type protein. When Jim Remington at the University of Oregon e-mailed me in May 1995 to get an expression vector for GFP, I suggested that he solve the S65T structure, because that ought to be publishable even if another group got the wild-type structure first. Within a few months, Dr. Mats Ormö in Remington's lab had solved the crystal structure (Figure 8), using selenomethionine substitution for phasing. The protein was an almost perfect cylinder, 2.4 nm in diameter by 4.0 nm long, composed of eleven beta-strands surrounding a helix running up the central axis, into which the chromophore was inserted. The chromophore was deeply buried at the center of the protein, explaining how it could be shielded from solvent and rigidified to make it fluorescent. (Once the protein is denatured, the exposed chromophore completely loses its fluorescence.) We could also rationalize why the chromophore had to be formed spontaneously, because no enzyme could reach through the completely encapsulating shell formed by the rest of GFP. Remington noticed a cavity next to the chromophore and suggested that it could accommodate an aromatic ring in a π -stacking relationship, which might shift the fluorescence wavelengths. To introduce

this ring, Andrew Cubitt, a new postdoc in my lab, mutated Thr 203 to various aromatic amino acids, followed by re-annealing. Indeed, both excitation and emission maxima increased about 20 nm, producing a noticeably more yellowish fluorescent protein, hence dubbed YFP (Figure 4, upper left quadrant). These shifts made YFP a good FRET acceptor from CFP. We were glad to have the CFP/YFP pair to replace our previous BFP/GFP combination, because BFP was too easy to bleach and required potentially injurious UV excitation.

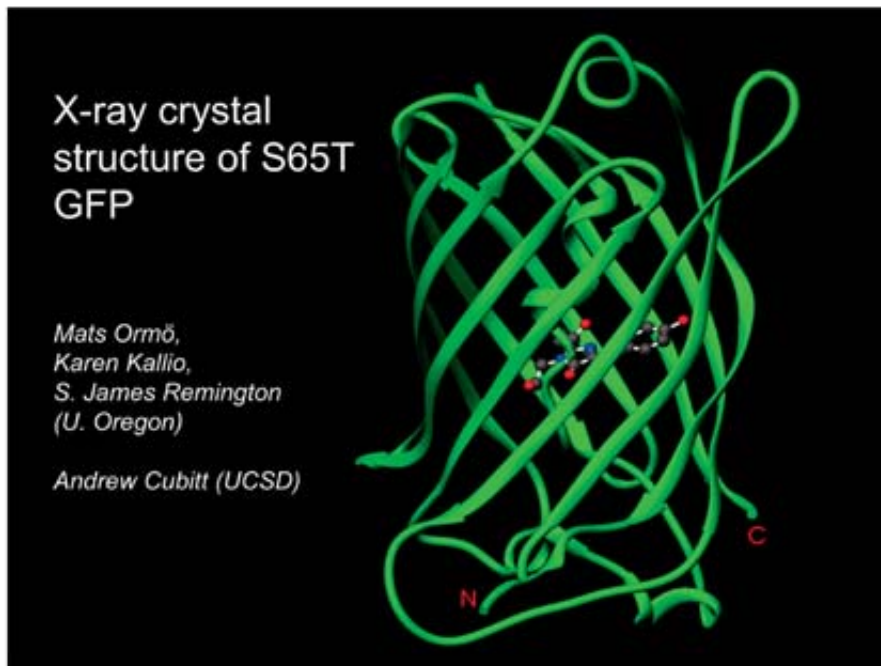


Figure 8. X-ray crystal structure of the S65T mutant of GFP³¹. Alpha helices and beta strands are shown as ribbons, connecting segments as tubes, and the chromophore in ball-and-stick representation. N- and C-terminii are marked.

We felt this novel crystal structure deserved a high-profile publication, because it explained so many features of GFP and enabled immediate rational improvement, so we submitted the work to *Science*. However, the referees were not impressed. One of them acknowledged that the structure was competently determined, but was not convinced that GFP was of sufficient importance or significance. The other reviewer voiced disappointment that our paper failed to answer the really important question about GFP, namely what is the native biological function of GFP within the jellyfish. We felt it unreasonable that a crystal structure should be expected to answer an ecological question, so I appealed to the Editor, who then sent the manuscript to a third reviewer. Many weeks elapsed without a response. Then one of the groups working on the structure of wild-type GFP announced to the Internet newsgroup on fluorescent proteins that they had solved the structure, which

would soon appear in *Nature Biotechnology*. I forwarded this announcement to *Science*, which accepted our paper the next day without the long-awaited third review. Fortunately, the two papers^{31,32}, published within about a week of each other, were in good agreement on the major features of the structure, except that wild-type GFP was dimeric in Yang & Phillips' crystals whereas S65T GFP was monomeric in Ormö and Remington's crystals grown under different conditions. From these structures and a more detailed study by Brejc *et al.*³³, it became evident that the UV and blue excitation peaks in wild-type GFP arose from the neutral and anionic forms of the chromophore respectively, and that mutation of serine 65 to threonine re-oriented the side chain hydroxyl enough to alter the hydrogen bonding network controlling the ionization of the chromophore.

WATCHING INTRACELLULAR BIOCHEMISTRY

Our first attempt to use FRET to measure real intracellular signals was launched by Atsushi Miyawaki, who came to my lab from Tokyo, where he had helped clone and characterize the receptor for inositol 1,4,5-trisphosphate (InsP₃)^{34,35}. I very much wanted to image this second messenger, which is crucial for releasing Ca²⁺ from intracellular organelles such as the endoplasmic reticulum. Miyawaki and I hoped to accomplish this by attaching donor and acceptor FPs to opposite ends of the cytosolic domain of the InsP₃ receptor, which was supposed to undergo a conformational change upon binding InsP₃. However, none of Miyawaki's constructs showed any significant change in FRET in response to InsP₃, and we realized we hardly even knew where InsP₃ bound within its receptor. With the invaluable help of Dr. Mitsuhiko Ikura in Toronto, Miyawaki decided to practice on an engineered receptor of known structure³⁶, a fusion of calmodulin (CaM) with its target peptide, "M13", from skeletal muscle myosin light chain kinase. CaM and M13 are relatively unstructured in the absence of Ca²⁺, but when free Ca²⁺ concentrations increase, Ca²⁺ binds to CaM, causing it to wrap around M13 in a way that is reminiscent of a hamburger bun wrapping around a frankfurter (Figure 7B). My enthusiasm for this diversion was limited at first, because my initial academic success had been in building and exploiting organic synthetic indicators for Ca²⁺, so I did not feel that we needed more Ca²⁺ reporters *per se*. But I conceded that this digression would be a good rehearsal, so Miyawaki put BFP (later replaced by CFP) at the N-terminus of CaM, while he attached S65T GFP (later replaced by YFP) at the C-terminus of M13. This quadripartite fusion (see Figure 7B for schematic structure) indeed responded to Ca²⁺ with a FRET increase. Miyawaki dubbed these chimeric proteins "cameleons", spelled without an "h" to reflect their origin from CaM, yet alluding to their ability to change colors in response to Ca²⁺ and to change that response following seemingly minor mutations^{37,38}. Nevertheless, genetic encodability gives cameleons some big complementary advantages: applicability to any cell or organism into which the DNA can be introduced, long-term continuous production of the indicator, precise targeting to spe-

cific cell types within a complex tissue, precise targeting to subcellular locations, and susceptibility to improvement by rational or random mutation. For these reasons, cameleons and their relatives have become the most popular means to image activity of identified neurons within intact nervous systems. A visually spectacular demonstration of dynamic Ca^{2+} signals seen with an improved cameleon is the transient Ca^{2+} rise seen within each cleavage furrow in the early development of a zebrafish embryo (Figure 9).

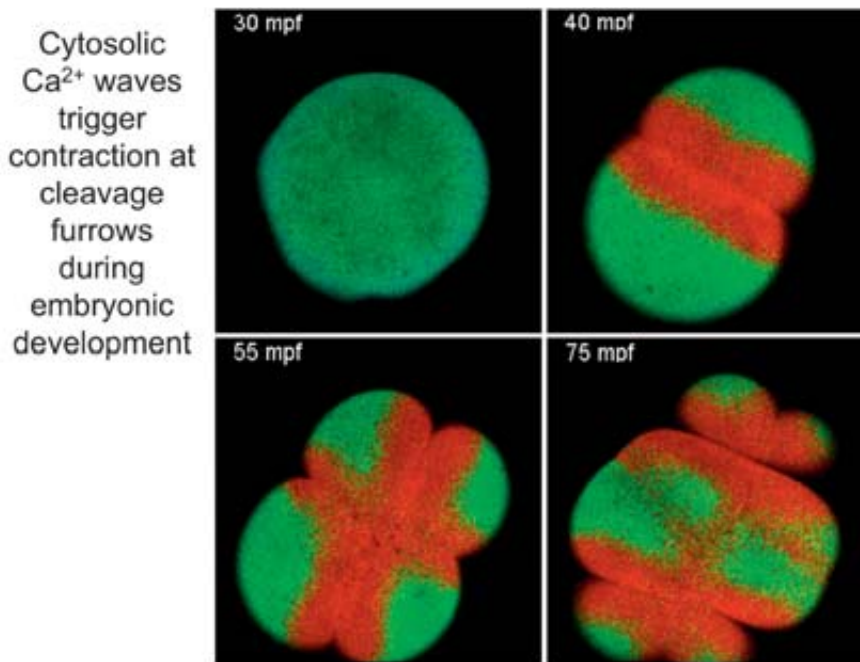


Figure 9. Transgenic zebrafish embryo expressing yellow cameleon 3.60⁷⁶. Single confocal z-plane, imaged every 5 sec (“mpf” = minutes post fertilization). Images selected from a video kindly provided by Hide Mizuno & Atsushi Miyawaki, RIKEN, Japan.

What about labeling PKA, my original motivation for starting work on GFP? Eventually this worked as well (schematic structure in Figure 7C), due mainly to Manuela Zaccolo in Tullio Pozzan’s lab in collaboration with Charles Cho in my lab³⁹. Ironically, PKA proved one of the more difficult proteins to tag with FPs, because an inordinate amount of trial and error was necessary to find linkers that allowed fusion of the FPs without disrupting the ability of the subunits to respond to cAMP. Eventually these molecules allowed Zaccolo and Pozzan to directly image the long hypothesized subcellular compartmentation of cAMP in cardiac muscle cells stimulated by an adrenaline analog⁴⁰. We and others also developed a more general approach to visualize the dynamics of protein kinases, both those phosphorylating serines and threonines^{41–46} as well as those acting on tyrosines^{47–49}. The design principle (Figure 7D) is a variation on the cameleons, in which M13 is replaced by a peptide substrate (pink) for the kinase of interest, and CaM is replaced by a protein domain (brown) that binds phosphorylated Ser, Thr, or Tyr as appro-

priate. Examples of such domains are 14-3-3 (for pSer or pThr), FHA1 (for pThr), and SH2 (for pTyr). Once the kinase phosphorylates the target Ser, Thr, or Tyr, that amino acid is intramolecularly complexed by the phospho-amino acid binding domain, changing the distance or orientation between the donor and acceptor FPs. An example of dynamic imaging of kinase activity is in Figure 10.

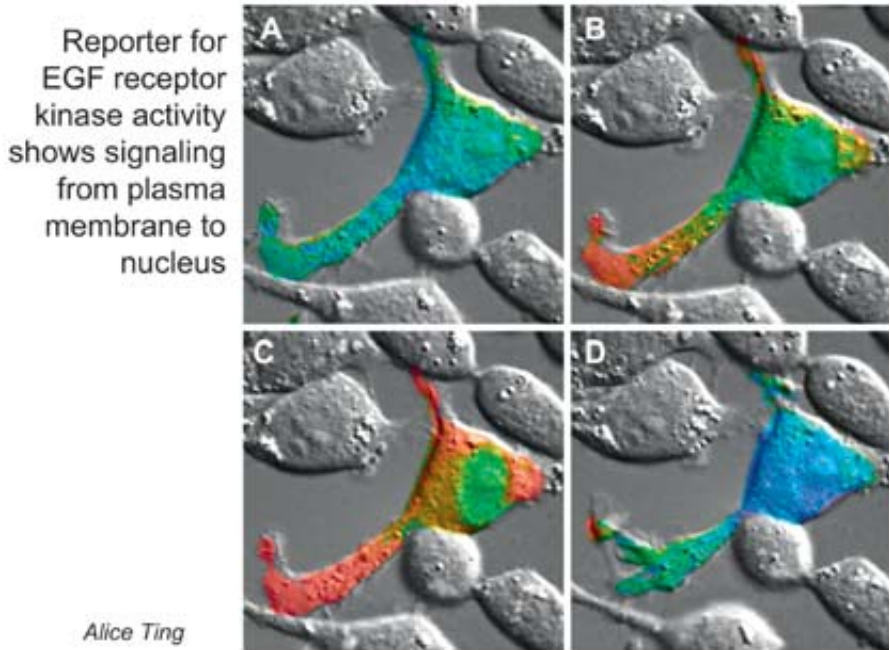


Figure 10. A fibroblast transfected with a reporter⁴⁷ for the tyrosine kinase activity of the receptor for epidermal growth factor (EGF). The extent of FRET, indicating the extent of phosphorylation of the reporter, is shown in pseudocolors ranging from blue (negligible phosphorylation) to red (maximal phosphorylation). These colors are superimposed on differential interference contrast images to show cellular morphology. The colored cell was the only one transfected within this field of view. At rest the indicator showed negligible phosphorylation (A), but upon stimulation with EGF, phosphorylation (shown by warmer pseudocolors) started at the periphery of the cell (B) and spread towards the nucleus (C). After EGF was removed, the tide of phosphorylation receded back towards the most peripheral tip at the far left end of the cell (D).

The DsRed structure drawn using *E. coli* expressing DsRed as “ink”

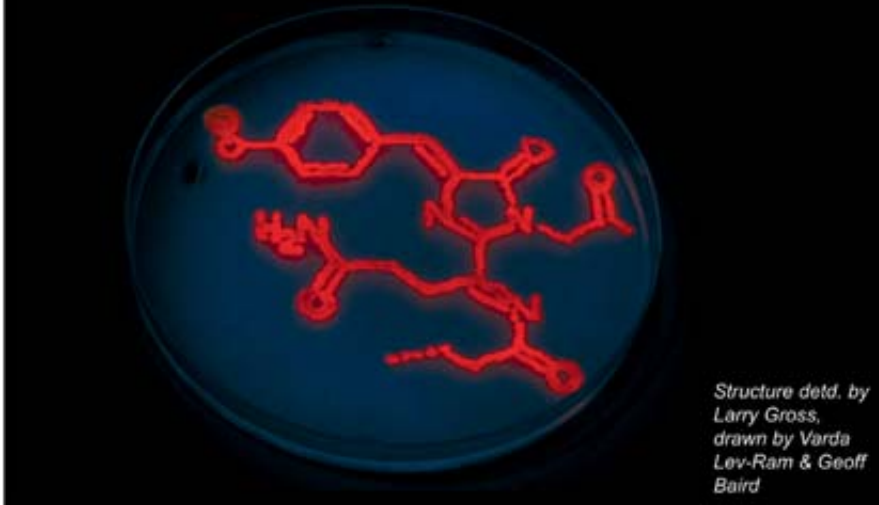


Figure 11. Chromophore structure⁵² of DsRed, drawn on a Petri dish using bacteria expressing the protein and showing its beautiful red fluorescence.

The final major expansion in the palette of FPs originated in the revelation that they are not just accessory proteins in bioluminescent cnidaria but also are responsible for many of the colors of nonbioluminescent corals. In a breakthrough discovery, Matz *et al.* isolated a gene encoding a red fluorescent protein (“DsRed”) from a coral in a Moscow aquarium⁵⁰. The rapid commercial availability of this gene enabled us to discover that DsRed is an obligate tetramer⁵¹ and that its chromophore (Figure 11) is initially the same as that of GFP but then undergoes another dehydrogenation to generate an unprecedented acylimine, which is stable only when buried inside the intact protein⁵². Theoretical calculations by Dr. Kim Baldridge at UCSD verified that extension of the chromophore by two double bonds could account for the red shift in excitation and emission spectra⁵². The tetrameric stoichiometry and chromophore structure were soon confirmed by independent X-ray crystal structures^{53,54}. The very tight mutual binding of the four subunits severely hindered use of DsRed as a fusion tag, because any protein fusion had to become at least tetrameric. If the fusion partner engaged in any protein-protein interactions of its own, massive aggregation and visible precipitation often resulted. Nevertheless, Robert Campbell succeeded over many cycles of directed evolution in generating a monomeric red FP (RFP), which made protein fusions much more reliable⁵⁵. He, Nathan Shaner, and Lei Wang then engineered an extensive palette of monomeric FPs whose emission maxima covered the rest of the visible spectrum out to 648 nm (Figure 12)^{56,57}. Other groups have also developed a multitude of FPs with complementary desirable

phenotypes, including amazing and highly useful photoswitching capabilities⁵⁸, but space does not permit an extensive review here.

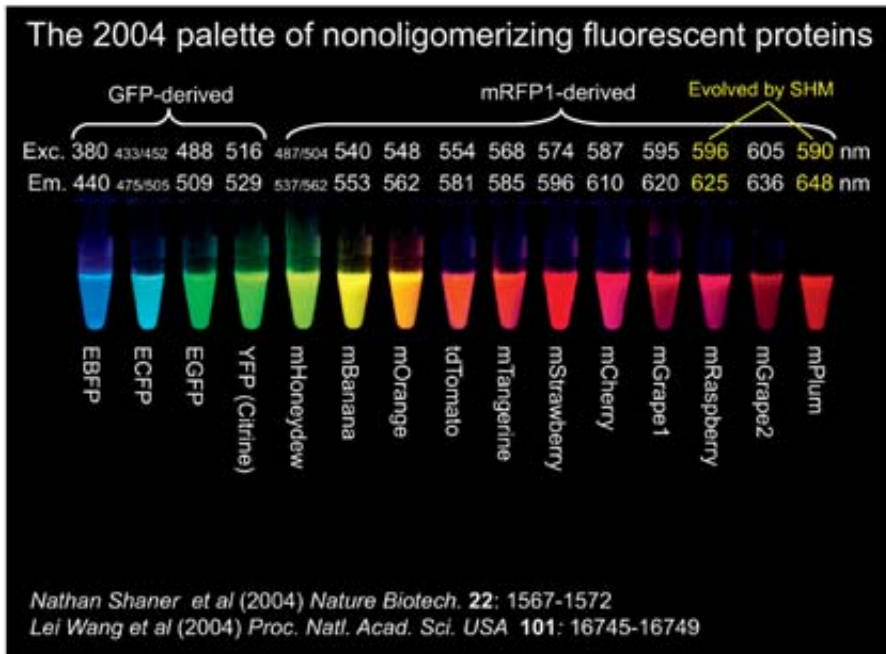


Figure 12. Monomeric or tandem dimeric fluorescent proteins derived from *Aequorea* GFP or *Discosoma* RFP, expressed in bacteria and purified. This photo is a time exposure of fluorescences excited at different wavelengths and viewed through different cutoff filters.

EXAMPLES OF MEDICAL AND EDUCATIONAL SIGNIFICANCE

One of the most common requests I get is to explain why GFP and related FPs are significant, preferably in lay terms. My usual answer has been that their genetically encoded fluorescent colors can make many key biochemical processes directly visible inside living cells and organisms. Using standard molecular biological tricks to connect host cell genes to FP genes, we can watch when and where those host cell genes get switched on and off, when the protein products are born, where they travel, with what other proteins they interact, and how long they survive. Even when we cannot directly tag the signals of interest, e.g. Ca^{2+} , cAMP, protease or kinase activities, we can often engineer FP-based indicators as in Figure 7 to image those signals indirectly. These generalized explanations seem too abstract for many people, who would prefer more specific applications of relevance to major medical problems. I therefore mention a few rather arbitrarily chosen examples:

A vaccine against AIDS that would eradicate the disease from already-infected patients would be extremely desirable. Tagging of the causative virus with GFP and high-resolution time-lapse microscopy of infected T cells contacting naïve cells shows that the virus can pass directly from one cell to another through specialized transient adhesions dubbed virological synapses

(Figure 13). If the virus can really spread between host cells without even transiently exposing itself to the extracellular milieu where antibodies could neutralize it, then the task of any post-infection vaccine will be much harder.

GFP-tagged HIV can be transmitted by cell-cell contact

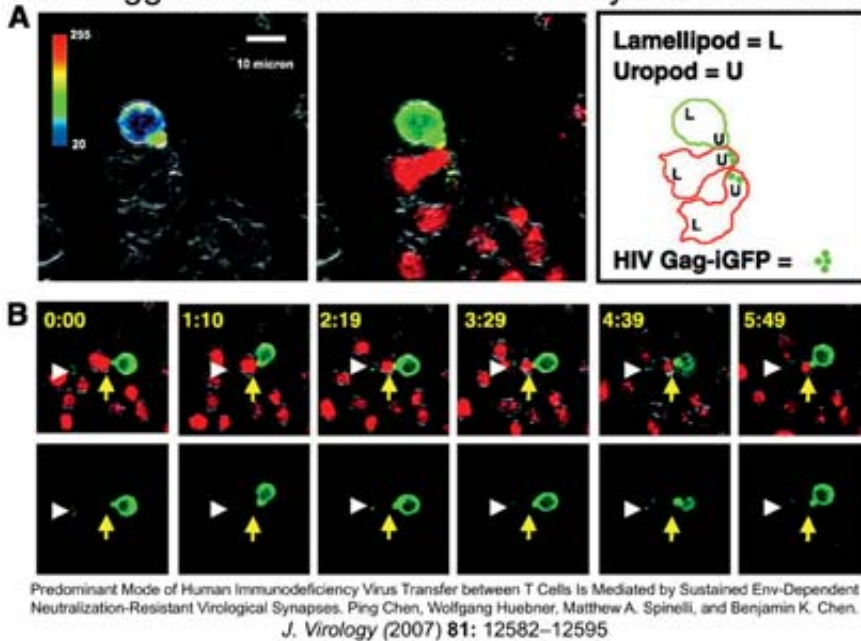


Figure 13. GFP labeling of human immunodeficiency virus (HIV) shows that the virus can spread from cell to cell through virological “synapses” without exposure to antibody neutralization. Reprinted with permission from Figure 6A,B of Chen *et al.*⁷⁷.

There is much interest in the hypothesis that some of the pathology in Alzheimer’s disease results from chronic elevations in cytosolic Ca^{2+} in the affected neurons⁵⁹, but the evidence has come from neurons in culture rather than intact animals. The genetic encodability of cameleons has now enabled imaging of Ca^{2+} in the brains of normal mice vs. transgenic mice with a model for Alzheimer’s disease⁶⁰. In normal animals, free Ca^{2+} in neurons at rest is tightly regulated around 80 nM, whereas in the disease models, about 20% of the dendrites show much higher Ca^{2+} centered around 400 nM, particularly in the neighborhood of Alzheimer’s plaques (Figure 14). This observation provides direct evidence *in vivo* for dysregulation of neuronal Ca^{2+} during Alzheimer’s disease.

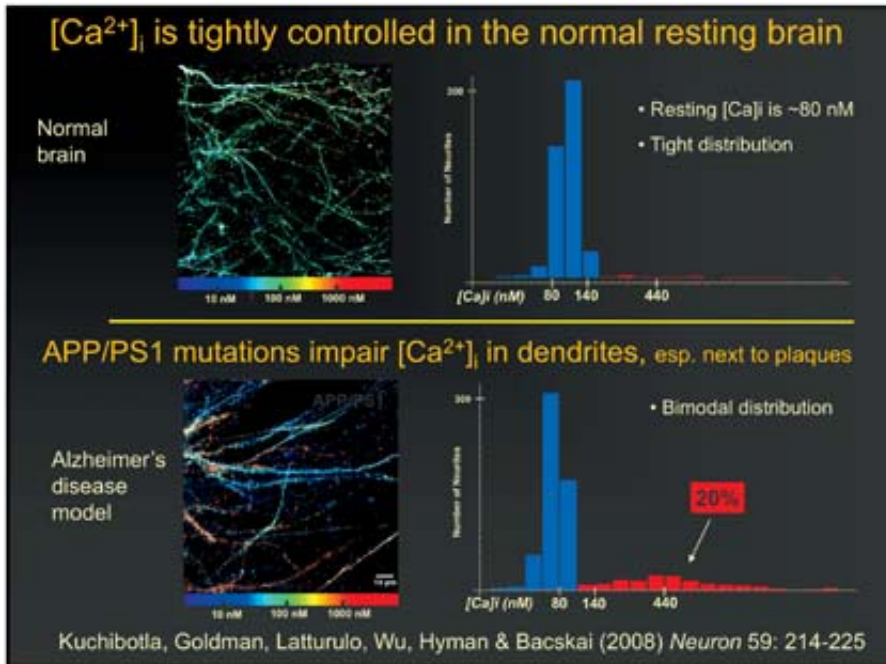


Figure 14. Two-photon imaging of yellow cameleon 3.60⁷⁶ in brains of transgenic mice, without or with APP1/presenilin mutations to generate plaques modeling Alzheimer's disease. Low to high cytosolic $[Ca^{2+}]_i$ level are shown in blue to red pseudocolors respectively. In the disease model, 20% of neurons show a distinctly higher cytosolic $[Ca^{2+}]_i$, especially in the vicinity of disease plaques. Figure donated by Dr. Brian Bacskai and related to Kuchibhotla *et al.*⁶⁰.

An example of the use of FPs in drug discovery comes from efforts to find small molecules that inhibit the aggregation of the Alzheimer's disease peptide A β into β -amyloid. When A β is fused to the N-terminus of GFP, the aggregation of A β is fast enough to prevent the GFP from folding and becoming fluorescent. Candidate drugs that prevent aggregation of A β allow GFP fluorescence to develop, constituting a simple high-throughput way to screen combinatorial libraries (Figure 15)⁶¹.

A High-Throughput Screen for Compounds That Inhibit Aggregation of the Alzheimer's Peptide

Kim Woojin, Kim Yunkyoung, Min Jaeki, Kim Dong Jin, Chang Young-Tae* and Michael H. Hecht (2006) *ACS Chem. Biol.* 1: 461–469

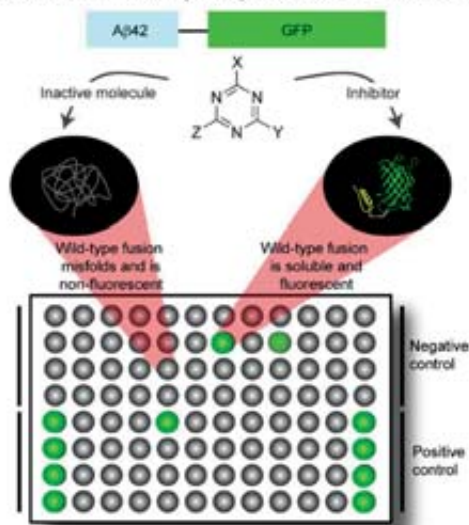


Figure 1. Fluorescence-based screen using the A β 42-GFP fusion. In the absence of inhibition, the A β 42 portion of the fusion aggregates rapidly and causes the entire A β 42-GFP fusion to misfold and aggregate (left). Therefore, no fluorescence is observed. However, inhibition of A β 42 aggregation enables GFP to form its native green fluorescent structure (right). (The green part of the ribbon diagram shows the structure of GFP; the yellow part is merely a schematic representation of a nonaggregated form of A β 42.) The triazine scaffold is shown at the center of the figure. Combinatorial diversity was introduced at sites marked X, Y, and Z. A 96-well plate is shown at the bottom of the figure. Compounds were added to each well, followed by *E. coli* cells expressing the A β 42-GFP fusion.

Figure 15. High-throughput screening of combinatorial drugs to find inhibitors of aggregation of A β , the Alzheimer's disease peptide. Inhibition of aggregation allows an A β 42-GFP fusion to become fluorescent. Reprinted with permission from Figure 1 of Kim *et al.*⁶¹.

Cell cycle indicator using YFP and mCherry

Green = in mitosis; Red = interphase

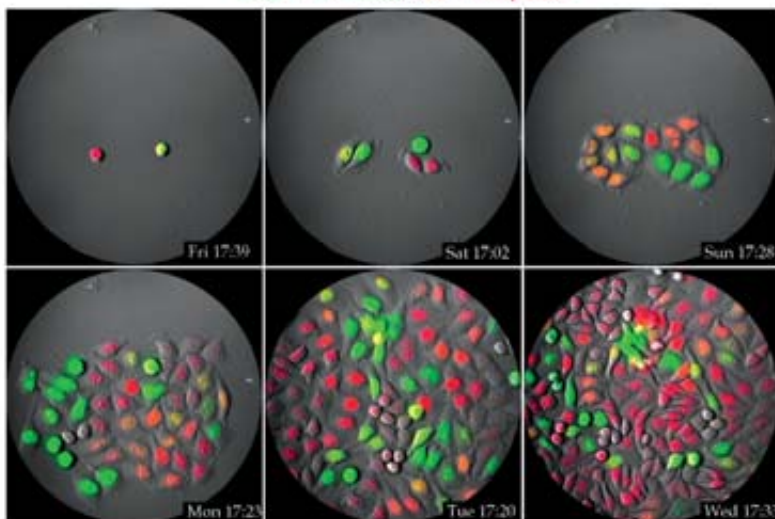


Figure 16. Frames chosen from a time-lapse video of HEK293 cells growing and dividing in tissue culture while transfected with a two-color reporter of cell cycle progression. Green-fluorescing cells are actively undergoing mitosis, whereas red-fluorescing cells are in interphase⁶². Video courtesy of Asako Sawano & Atsushi Miyawaki, RIKEN, Japan.

A final example comes from indicators of the mitotic cell cycle. Certain protein domains tell cells to accumulate those proteins at particular phases of the cell cycle and to destroy them at other phases. If these domains are grafted onto FPs of different colors, the cells can be made to fluoresce one color (say green) while they are actively dividing and another color (say red) while resting⁶². In cell culture, these cells display spectacular oscillations like an aerial view of asynchronized traffic lights (Figure 16). If tagged cells from a benign tumor line (e.g. NMuMG) are transplanted into an animal, the tumor initially looks yellow at low magnification (Figure 17) because it contains a mixture of green- and red-fluorescing cells. Over several days, the tumor shifts to all red, telling us that the cells have stopped dividing, which is why the tumor is benign. If tagged cells from a malignant tumor line (e.g. HeLa) are similarly transplanted, the tumor persists in its yellow color. Higher magnification views show the cell cycle phase pattern of the tumor cells around blood vessels. Since the pattern depends on several factors, including the maturity of vessels and the degree of necrosis in the surrounding tissues, this experiment provides a visually powerful lesson on the importance of new blood vessel formation in permitting cancer cells to proliferate.

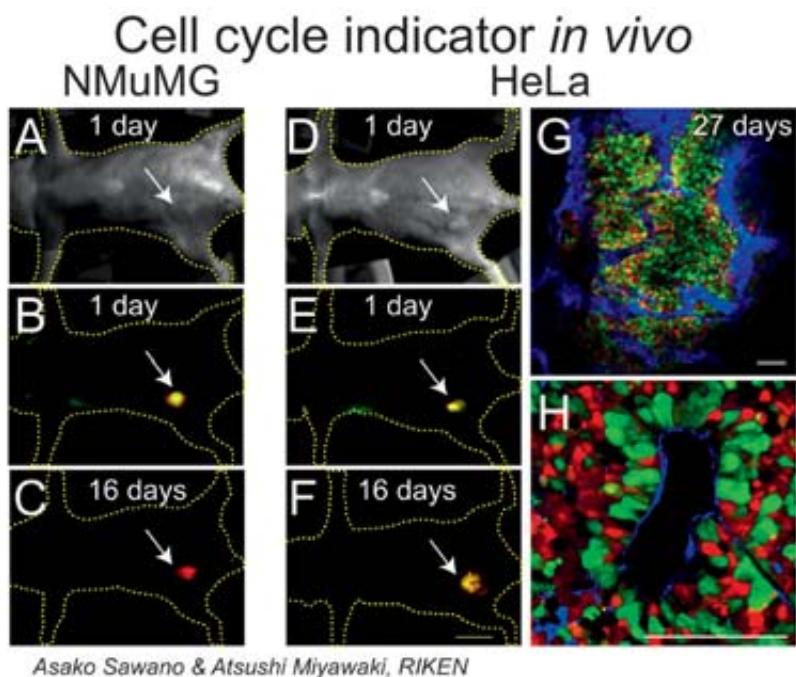


Figure 17. Reporter of cell-cycle progression applied to benign (NMuMG) and malignant (HeLa) tumor implants *in vivo*. Figure 3A–H from Sakaue-Sawano *et al.*⁶², reprinted with permission.

Multicolored FPs are helpful in education as well as research. Simple experiments with FPs enable secondary school students (Figure 18) to see with their own eyes how DNA can transform cells, when mutations have occurred, how proteins behave biochemically, and so on. After Jeremy Babendure

completed a Ph.D. in my lab, he began a very successful program (biobridge.ucsd.edu) to make experiments like these accessible to high school science classes, initially starting close to UCSD but now spreading wherever teachers and students are interested.



Figure 18. High school science classes using fluorescent proteins as laboratory exercises as part of the BioBridge program (biobridge.ucsd.edu).

TRANSCENDING THE LIMITATIONS OF EXISTING FPs

FPs have obvious major limitations:

1. All FPs with structures homologous to jellyfish GFP and coral RFP, i.e. cylinders comprising 11 β -strands, contain >200 amino acids. Occasionally this size proves too large and perturbative when fused to the protein of interest⁶³⁻⁶⁵; also the time required for FPs to fold and form their chromophores is sometimes too slow^{66,67}. In such cases FPs can be replaced by much shorter sequences, e.g. the 12 residue peptide FLNCCPGCCMEP⁶⁸, which bind dyes containing two appropriately placed arsenic atoms that link to the four cysteine sulfhydryls.
2. The O₂ requirement for chromophore maturation prevents applications in organisms that cannot tolerate even transient exposure to any O₂. Aside from the tetracysteine/biarsenical system just mentioned, flavin-binding proteins offer another potential solution⁶⁹.
3. For imaging of transfected cells and organs inside live mammals, excitation and emission wavelengths > 600 nm would be very advantageous because hemoglobin and other pigments tremendously attenuate shorter

wavelengths. Unfortunately, it has been very difficult to find or evolve coral FPs that retain high brightness with excitation wavelengths > 600 nm. As mentioned at the outset, some phycobiliproteins from blue-green algae have such long wavelengths and high brightness. More recently, plant phytochromes have been mutated into long-wavelength FPs dubbed phytofluors^{70,71}. However, none of these has been successfully expressed in mammalian cells, perhaps because the pigment cofactors are foreign. Recently, Xiaokun Shu in my lab discovered that bacterial phytochromes can also be mutated into FPs with excitation maxima in the far red, e.g. 684 nm, and emissions >700 nm⁷² (Figure 19). The crucial advantage of bacterial phytochromes is that they spontaneously incorporate their cofactor, biliverdin, the initial product of heme catabolism in all aerobic organisms including humans. Thus we now have infrared FPs that work in intact animals such as mice and flies, which I find particularly satisfying given my early unsuccessful interest in exploiting phycobiliproteins.

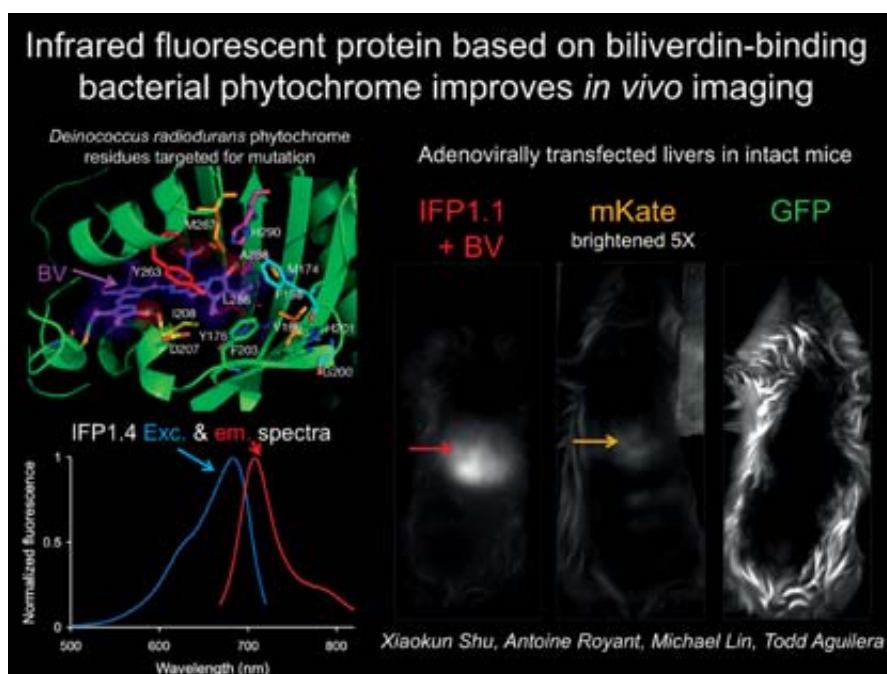


Figure 19. An infrared-fluorescing protein (IFP) can be evolved from a phytochrome from *Deinoxoccus radiodurans*. Upper left: crystal structure of the wild-type phytochrome (PDB: 1ztu)⁷⁸ with its chromophore, biliverdin, in purple, and residues targeted for mutagenesis labeled. Lower left: Normalized excitation (blue) and emission (red) spectra of IFP1.4. Right: Liver fluorescence of intact living mice injected with adenoviruses encoding IFP1.1, mKate (an RFP previously advocated for *in vivo* imaging⁷⁹), or an enhanced GFP. Biliverdin (250 nmol) was injected intravenously 1 hr before obtaining the IFP image. The mKate image was 5-fold brightened in software to render it visible. Belly fur was removed with depilatory cream but the skin was left unbroken. Arrows point to the liver. Note that the GFP images are dominated by autofluorescence, rendering the livers invisible. The much greater visibility in the highly pigmented liver of IFP compared to previous FPs demonstrates the value of very long excitation and emission wavelengths for *in vivo* fluorescence imaging.

4. The crucial advantage of FPs, their genetic encodability, is of no or very limited value in human patients, because human gene therapy would have to become safe and effective, and introduction of an FP gene would have to benefit the patient directly. Molecular imaging in human patients must still rely on synthetic molecules. Furthermore, humans are even thicker and more opaque than mice, so whole-body scanning of patients requires nonoptical techniques such as X-ray computed tomography, positron emission tomography, and magnetic resonance (MR) imaging. Therefore we have been developing activatable cell penetrating peptides (ACPPs), which are polycationic cell penetrating peptides (CPPs) whose cellular uptake is minimized by a polyanionic inhibitory domain and then restored upon proteolysis of the peptide linker connecting the polyanionic and polycationic domains. Local activity of proteases able to cut the linker causes amplified retention in tissues and uptake into cells⁷³. Tumor uptake of ACPPs is up to 4 fold higher with a matrix metalloproteinase substrate (PLGLAG) as the linker than with a negative control composed of D-amino acids (Figure 20). Conjugation of ACPPs to macromolecular carriers such as dendrimers prolongs pharmacokinetics and increases delivery of payload (Cy5 or Gd-DOTA or both in the same molecule) to tumor for far-red or MR imaging. The dual labeled probe with Cy5 and Gd-DOTA enables whole body MR imaging followed by fluorescence-guided surgery. Thrombin-cleavable ACPPs accumulate in atherosclerotic plaques and experimental stroke models, so vascular pathologies can also be imaged. Because the ability of ACPPs to deliver various cargoes with enzymatic amplification to protease-expressing tissues *in vivo* offers clinical potential, this is now the dominant effort in my lab.

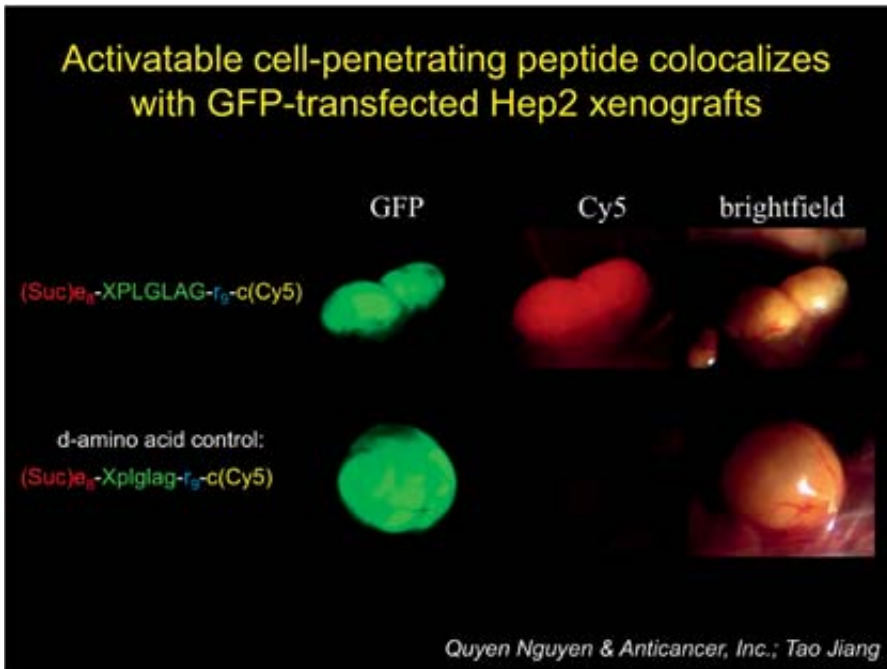


Figure 20. Activatable cell penetrating peptides (ACPPs) localize to tumors *in vivo*. Hep2 (human laryngeal carcinoma) tumor cells stably transfected with EGFP were xenografted into nude mice. 3 weeks after implanting 5×10^6 cells subcutaneously, the tumor had reached ~ 5 mm diameter. Top images: 6 hrs after systemic injection of an ACPP, 10 nmol succinyl-e₈-XPLGLAG-r₉c(Cy5), the skin was removed over the tumor, and GFP fluorescence, Cy5 fluorescence, and reflected light images were acquired from the anesthetized animal. X denotes 6-aminohexanoyl, upper case letters represent L-amino acids, lower case letters are D-amino acids. PLGLAG is a sequence cleavable by matrix metalloproteinases-2 and -9⁷³. Bottom panel: an analogous experiment with a matching negative control, succinyl-e₈-Xplglag-r₉c(Cy5), in which the protease cleavage site has been changed to D-amino acids, rendering it protease-resistant. The lack of Cy5 signal in the tumor indicates that retention depends on cleavage by proteases.

ACKNOWLEDGMENTS

A list of the collaborators most relevant to the work described here is in Figure 21. Senior honorees often point out that the experiments were done by younger colleagues in their lab, but I have to go further and confess that I could not have done the experiments with my own hands because I lack the training in the relevant methods, especially the molecular biology. So I am particularly indebted to these key people as well as many others too numerous to list here. I am also grateful to the Howard Hughes Medical Institute, which has supported me from the time I moved to UCSD, as well as to the National Institutes of Health for many years of funding. Our recent transition to cancer research was supported by the Breast Cancer Research Program of the Department of Defense.

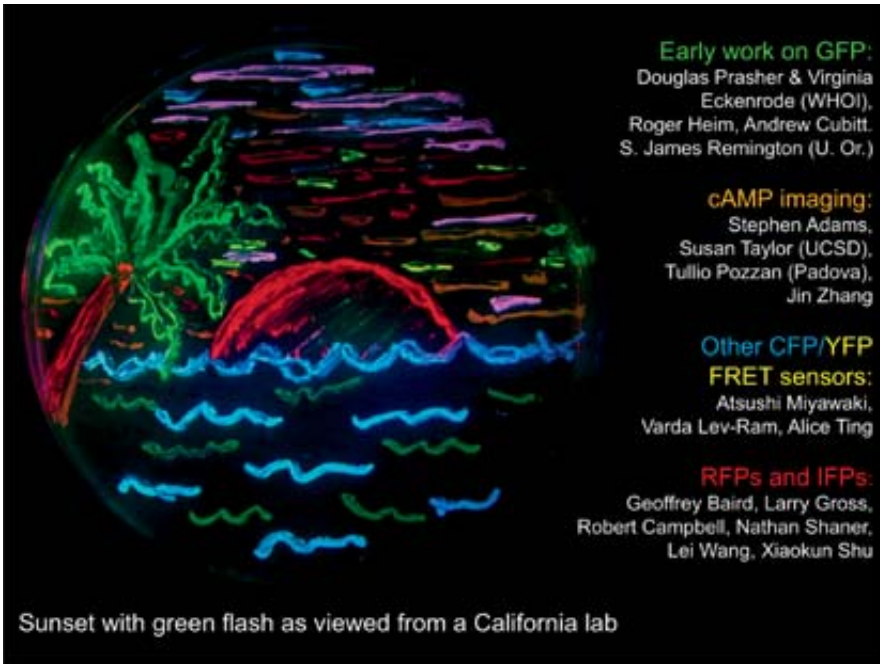


Figure 21. Key collaborators for the work described in this lecture, and a glowing California sunset in a Petri dish, drawn with multiple colors of bacteria expressing fluorescent proteins.

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