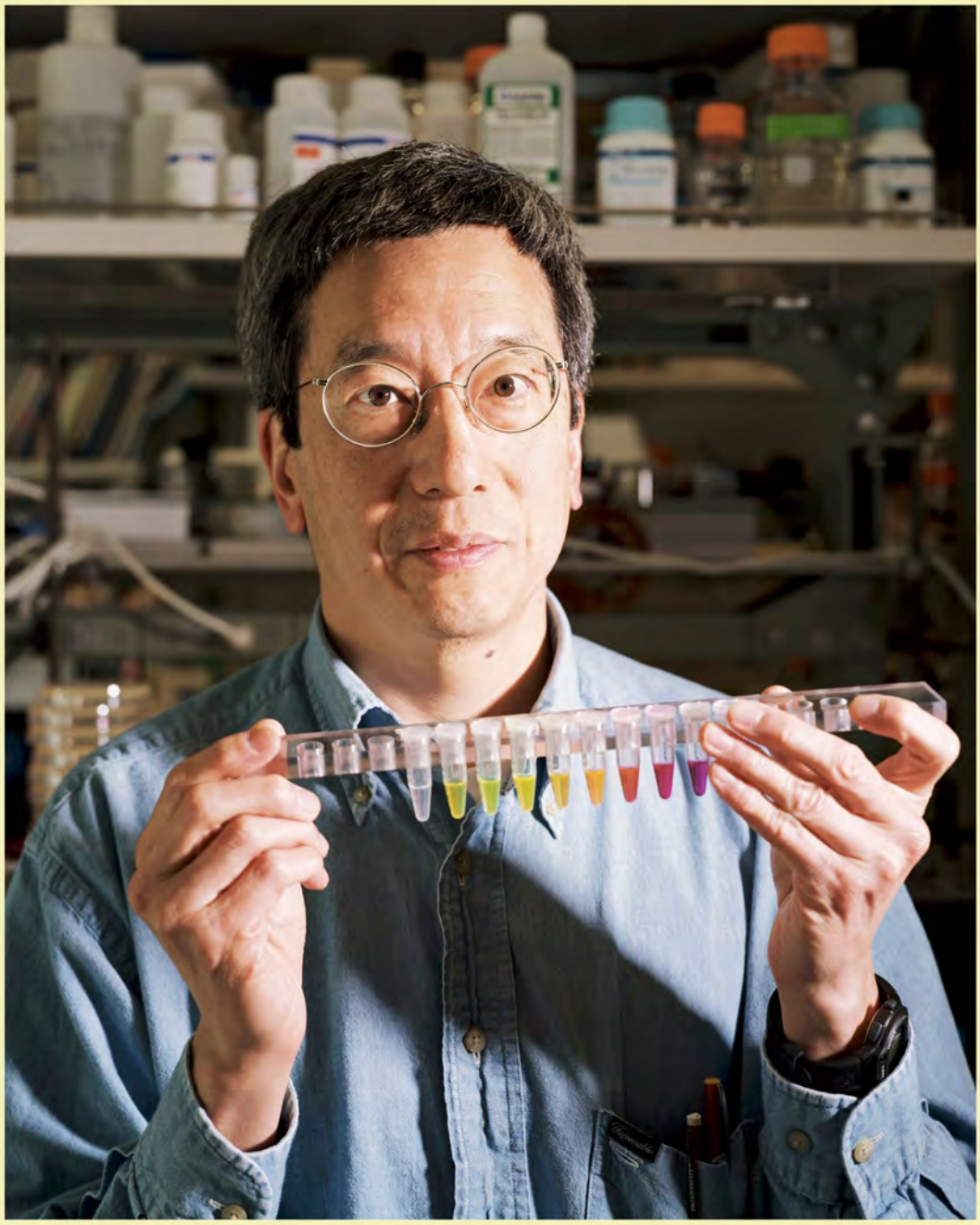

Roger Tsien

The life of our Nobel Prize-winning classmate

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Roger's life story

Written by Roger,
published on
the Nobel website

The Nobel Prize in Chemistry 2008
Osamu Shimomura, Martin Chalfie, Roger Y. Tsien

Roger Y. Tsien - Biographical

Q: What do elementary school pupils and Nobel Laureates have in common?
A: They both have to write autobiographical essays on command.

Ancestors and family

My father, Hsue Chu Tsien (1915–1997), came from the "scholar-gentry" class in Hangzhou, China, where "Tsien" (now more commonly transliterated as Qian) is quite a common surname. Apparently in 907 A.D., Qian Liu, my paternal ancestor 34 generations ago, established a kingdom around Hangzhou and fostered its growth through many civil engineering projects. This fiefdom prospered peacefully under the rule of Qian Liu and his successors until 978, when they surrendered to the Sung dynasty to avoid bloodshed*****. I had thought that descent from Qian Liu was an obscure secret of our family, but this factlet somehow found its way onto Wikipedia through no fault of mine. Furthermore, this genealogy is hardly much of a distinction given that everyone in principle has 2^{34} ancestors from 34 generations ago. 2^{34} (about 17 billion) vastly exceeds the earth's population in the 10^{th} century, so practically everyone, at least from that part of China, probably has Qian Liu as an ancestor, even if not so strictly through the Y chromosome. By far the most famous Tsien in modern times is Hsue Shen Tsien or Qian Xuesen, the aeronautical engineer who was deported from the U.S. during the McCarthy era and then became father of the ballistic missile program of the People's Republic[1]. He and my father were first cousins. Several other Chinese-American bioscientists, including Robert Tjian, now President of the Howard Hughes Medical Institute, and Shu Chien, a prominent bioengineer at UCSD, also have the same Chinese surname as mine and are likewise descended from Qian Liu, so we are distant relatives.

Dad too was excited by flight and airplanes, which were the cutting-edge technology of his day. In the 1930s he won a national scholarship (Tsinghua) to study in America. He went to MIT's mechanical engineering department, where he obtained a Master's degree for research on aircraft engines, including a proposal to boost the thrust during takeoff by injecting water into the exhaust to become steam. Before he could pursue any further studies in America, he had to return to China to serve in the Nationalist (Kuomingtang) Air Force. One of his best friends and fellow engineers, Yao Tzu Li, had an attractive and intelligent sister, Yi Ying Li, who had trained as a nurse at Peking Union Medical College, the most prestigious of Chinese medical institutions. My father courted her eagerly by letters even before they had ever met in person. When they finally did meet, she found him socially awkward

and overly impressed with his own academic prowess[2]. Despite her lack of romantic feelings for him, she agreed to marry him, perhaps because she doubted her own prospects in wartime China. Their first son, Yongyou, was born in March 1945. Soon thereafter, Dad was ordered to go to the U.S. as a liaison officer to try to extract more military aid for the Chinese Air Force. He had to travel over the Himalayas to India and then by ship, zigzagging to avoid enemy submarines, so he did not arrive in the U.S. until the day that Japan's surrender was announced[2]. His mission was therefore futile, but he knew that China would be racked by postwar civil war. Somehow he used contacts in the Defense Department to arrange for Mom and Yongyou to come to the U.S. Such permission was not trivial, because the Chinese Exclusion Act forbidding immigration from China to the U.S. had been repealed only in 1943, at which time the national quota was set at just 105 immigrants per year and thousands were ahead on the waiting list.

After Mom and Yongyou arrived in America in January 1947, life was quite a struggle because Dad could not find a professional job as an aircraft engineer. Such employment at the major firms required a security clearance, which a Chinese citizen could not get. So he started a tiny export-import business in New York City and later an engineering consultancy firm in Westchester County, which yielded enough to live on but not to become prosperous. Nevertheless their next son, Yonglo or Louis, was born in October 1949. Around then, Yongyou started school and needed to pick an American name. He wanted to be "Dick", so the school officials explained to my parents that this was a nickname for "Richard". "Yongyou" was somehow transliterated as "Winyu" to become Richard's middle name in English.

According to Mom, she always planned to have three children, though this statement came many years after the fact. After two sons, even Dad was looking forward to a girl[2], but in February 1952 they got me instead. Dad picked my Chinese name, Yongjian (transliterated Yonchien to become my middle name in English), but Dick insisted that my American name should be Roger. My mother later told me this was because Dick had a playmate at the time named Roger. Much later, perhaps when I was in college, I quizzed Dick about this mysterious namesake. Dick confessed that he actually named me after Roy Rogers, the famous cowboy actor. I mention all this to clarify the origins of the similarity between the names "Richard W. Tsien" and "Roger Y. Tsien", which has continually confused many scientists and their secretaries even up to now. I don't know why my parents chose two different transliterations for "Yong", but if they had not, Richard and I would be completely indistinguishable ("Tsien RY") in bibliographical databases.

Growing up: Home chemistry experiments

One of my earliest memories, probably from age 3 or 4, is of building a sand path at the beach across a strip of coarse pebbles that hurt my feet to cross. I loved to draw maps of imaginary cities with freeways vaulting over or tunneling under the surface streets. Perhaps these were the first signs of my future obsessions with bridge-building and activity-mapping. Some time in elementary school my parents bought a Gilbert chemistry set, but I didn't find it very interesting because the experiments seemed so tame. Then I discovered a book in the school library that had much better experiments and illustrations. Regrettably, I cannot now remember the book's name or author, though I hand-copied many sketches of its experiments into a notebook dated around 1960, now deposited in the Nobel Museum. Two experiments I remember best: 1) silica gardens, in which crystals of metal salts (e.g. $CoCl_2$, $NiSO_4$, $CuSO_4$) dropped into a solution of sodium silicate would develop bright magenta, green, or blue gelatinous coatings from which vertically rising dendrites would sprout; 2) preparation of a strongly alkaline

(0.5M NaOH or KOH) aqueous solution of dilute (~ 0.5 mM) potassium permanganate, which colored the liquid an intense purple. As this solution passed through a folded cone of filter paper, its color changed to a beautiful green, reflecting reduction of MnO_4^- to MnO_4^{2-}, presumably by the cellulose. In November 2008, I reproduced this surprisingly little-known demonstration for Swedish television and Nobel Media as an example of what got me interested in chemistry. Both experiments reflect an early and long-lasting obsession with pretty colors.

Figure 1. Our family in 1960, just before moving to Livingston. From left: Richard (15), Louis (11), H.C. (my father), me (8), Yi Ying (my mother).

In 1959, Dad closed his consulting firm and started working for RCA's vacuum tube division in Harrison, NJ. Mom and Dad looked for a town with affordable homes, within convenient commuting distance, and with good public schools for the three of us. A photo from around then is Figure 1. They chose a new housing development in Livingston, NJ, but the developer refused to sell to us, saying that they could not permit Livingston to become a Chinatown, nor could they afford the likelihood that other customers would refuse to buy houses next to a Chinese family. My parents appealed to the Governor of New Jersey, Robert Meyner. His office sent a letter to the developers warning them that racial discrimination was illegal. Finally a compromise was reached: the developers sold us a house completely surrounded by houses that had already been sold. The problem for us kids was that Livingston has lots of rocks in its soil, left from the glaciers. My parents were determined to have a respectable American-style grassy lawn, which required removal of the rocks. We had to cart away not only our own stones but many from our neighbors, who had used the unoccupied leftover lot as a dumping ground, or so we believed.

The many weeds in the lawn revealed a deep personality difference: Dad, as an impatient mechanical engineer, liked the instant solution of digging them up one by one from close enough to extirpate all the roots. I was an occasionally asthmatic hay fever sufferer, deeply afraid of pollen, so I advocated a chemical approach, sprinkling herbicide on the weeds from a safe distance. We tried my way once. The weeds slowly turned brown but eventually recovered. Dad declared the experiment a failure and went back to hand weeding. I still think about this result in relation to our current research on cancer therapy.

In 1960, RCA closed its vacuum tube division, presumably because semiconductors were replacing tubes, so Dad moved to Esso (later renamed Exxon) Research and Engineering. Esso provided much better projects and pay, so he stayed until his retirement in 1983. I believe some of the chemicals and glassware that enabled me to do the more interesting chemistry experiments were diverted from the company stockroom. Other supplies could be bought by mail order in those days with a parent's signature. Over the next 5 or 6 years I gradually did many of the classic experiments of inorganic chemistry in the basement of our house: preparing and burning H_2 gas, preparing O_2 and burning steel wool in it, preparing NH_3 in a flask and watching it suck water up as a fountain inside the flask. I distilled HF from $CaF_2 + H_2SO_4$ in plastic apparatus and was delighted to see its ability to etch glass. I electrolyzed molten NaOH using a step-down transformer and rectifier from a model train set, the nickel crucible as cathode, and a carbon rod salvaged from a dead flashlight battery as anode. I managed to get a few granules of very impure metallic sodium, which gave off a satisfying hiss when dropped into water. Pyrotechnics were naturally of great interest: I made and ignited gunpowder, ammonium dichromate volcanoes, and even a spectacular thermite reaction with powdered aluminum and chromium oxide. My most ambitious attempt was a multistep sequence aimed at synthesizing aspirin, for which I needed acetic anhydride, which had to be made from acetyl chloride, for which I needed phosphorus trichloride, for which I needed to burn red phosphorus in a stream of chlorine gas. I tried to do this reaction sequence in flasks with rubber stoppers (Figure 2), because I had no glassware with ground glass joints. The corrosive chemicals largely chewed up the rubber, so I did not get beyond acetyl chloride. Because I had no fume hood, I did the more dangerous experiments outdoors on a picnic table on the backyard patio. Looking back, I am appalled at how dangerous all this was for an unsupervised boy of 8 to 15, but it was also very good training in how to improvise equipment, plan and execute experiments, interpret confusing results, and decide how to do things better. These experiments made me confident enough that when I had to earn my first merit badge as a Boy Scout and was advised to pick something really easy, I chose Chemistry. Tougher merit badges like Hiking, with its requirement for a twenty-mile hike in one day, I got later.

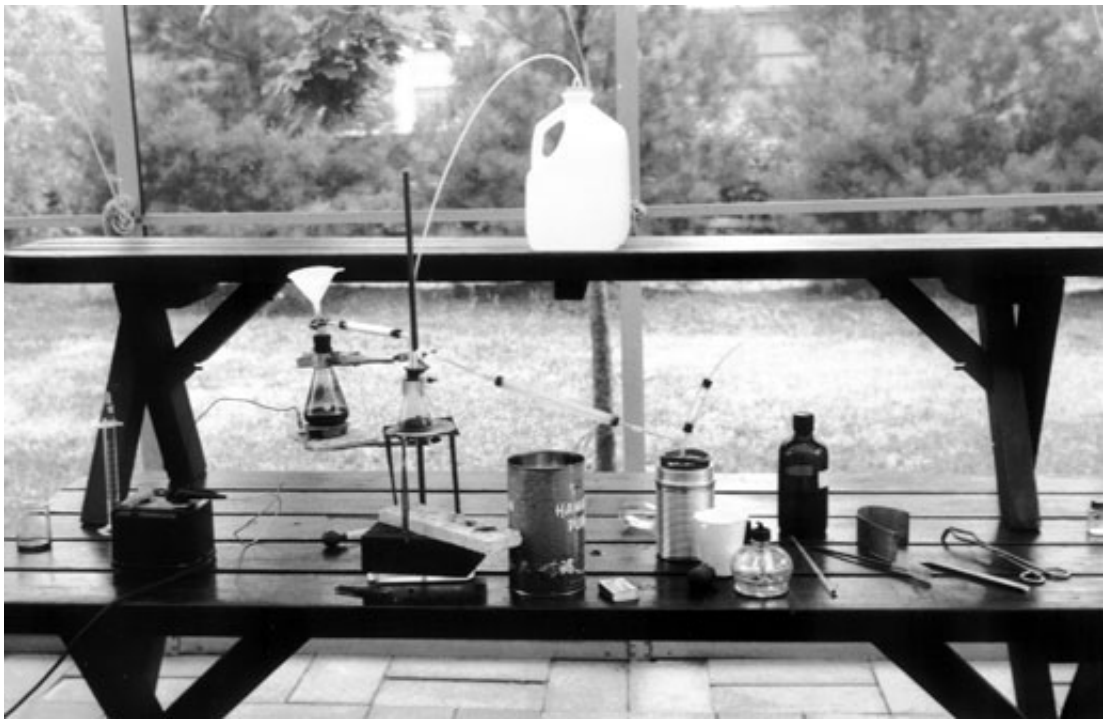

Figure 2. Setup for preparing Cl_2 and reacting it with red phosphorus, sometime in 1966 1967, in our screened backyard patio. The leftmost flask contained $KMnO_4$ to react with aqueous HCl added through the funnel controlled by a pinch clamp. The Cl_2 was dried by passage through $CaCl_2$ then directed onto P_4 in the flask on the ring stand. Because no running water was available, the water to cool the PCl_3 condenser was siphoned from the recycled milk jug and deposited into the waste can labeled "Hawaiian Punch". The receiver for PCl_3 was immersed in ice in the thermos bottle. The alcohol lamp allowed auxiliary heating for the phosphorus. Note rubber stoppers everywhere.

Elementary school to high school;
Westinghouse science talent search

School was usually bearable but frequently boring. I really looked forward to days in winter when heavy snow would close school, so that I could spend the day sledding. I was terrible at ball games at school, such as football, soccer, basketball, and softball, because I was small, nonathletic, and two years younger than my classmates at an age when this makes a huge difference. But I was popular enough in high school to be elected student council treasurer by an overwhelming majority.

Mom tried hard to teach us Chinese after school, but as I got older I found these lessons increasingly tedious. I well understood spoken Chinese at a child's level (e.g. the Chinese for "Tidy your room!" is permanently etched into my brain) but was reluctant to speak it myself, due to the wish (all too common among children of immigrants) to distance myself from my parents' accents and intense pride in their ethnicity and traditions. Likewise they despaired over my refusal (like a "foreign devil") to eat most Chinese food, especially the most authentic dishes with the strongest tastes or smells, or prepared from exotic creatures.

My first exposure to a research environment was in a National Science Foundation-sponsored summer research program at Ohio University in 1967, where I was assigned to work in the laboratory of Prof. Robert Kline on the ambident coordination of thiocyanate (SCN^-). The Pearson theory of hard and soft ligands and metals was new and fashionable at the time, so Prof. Kline wanted me to find out if thiocyanate could simultaneously bind with its "soft" sulfur to a soft metal and its "hard" nitrogen to a hard metal, e.g. PhHg–SCN–Cr(III). He hoped that the

infrared absorbances of thiocyanate would tell us whether such bridging was taking place. I prepared a lot of amorphous precipitates of rather ill-defined composition and measured their infrared spectra. In the winter of 1967, my senior year at Livingston High School, I entered the Westinghouse Science Talent Search, the nationwide "science fair" competition. (This annual event still exists, though sponsorship was taken over by Intel in 1998.) For lack of any alternatives, I wrote up my Ohio University project, trying my best to draw some conclusions from a mess of dubious data. Prof. Kline largely disowned those conclusions, pointing out that my preparations had not given correct carbon, hydrogen, and nitrogen microanalyses. The 40 finalists were summoned to Washington DC in March 1968 for interviews and a public poster session. I remember being envious of my fellow finalists, who were much more adult and sophisticated. Also their projects and exhibits seemed much more exciting and explainable than mine. I felt intimidated by the senior judge, **Glenn Seaborg**, partly because of his commanding height, partly because he was chairman of the U.S. Atomic Energy Commission, partly because of his 1951 Nobel Prize for work in inorganic chemistry. The awards ceremony was very tense for us because the ten scholarship winners were announced in reverse order, forcing everyone to hope their name was called but as late as possible. I am still mystified how I won first prize despite the unsoundness of my project, and I retain a dislike for scientific competitions. Dad did his bit to keep me grounded: when I phoned home, his first comment was that it was a good thing I now had a $10,000 scholarship, because he had recently lost that amount on the stock market. One of the most satisfying compliments I received was that the developer who had not wanted to sell a house to Mom and Dad in 1960 now used my photo in one of their advertisements as evidence of the quality of the local school system.

Harvard

In April 1968 I had to choose between four colleges: Columbia, MIT, Caltech, and Harvard. Dad vetoed Columbia because of the student unrest that spring, and I did not mind because I wanted to get further away from New Jersey. I rejected MIT because Dick and Louis had both gone there and I was tired of being compared to them. The small size of Caltech's undergraduate class sounded attractive, but I finally decided against Caltech because **Richard Feynman** was no longer teaching introductory physics and because the music department was tiny and of negligible fame compared to Harvard's. Indeed Harvard did turn out to be a salutary experience on the whole. Friendships with classmates were crucial in helping me grow up. The student protests of spring 1969 and 1970 provided my first exposures to cannabis, police brutality, and participatory politics. The diversity of courses let me sample art history, visual design, economics, Colonial history, constitutional law, psychology, both music theory and chamber music performance, etc. Ironically, the worst courses were those intended to lead Harvard's elite chemistry majors into research careers. These required courses were so distasteful I abandoned chemistry. Looking for alternatives, I dabbled in molecular biology (taught by **Walter Gilbert**, who later shared a Nobel Prize for DNA sequencing), oceanography, relativistic quantum mechanics, and astrophysics. But what I finally chose was neurobiology, partly because the relationship between brain and mind seemed philosophically the most important problem in science, partly because **David Hubel**, John Nicholls, and **Torsten Wiesel** ran a course charismatically proselytizing undergraduates to become neuroscientists. Hubel and Wiesel were still doing the research on visual cortex that eventually won them the 1981 Nobel Prize in Medicine or Physiology. I asked Prof. Hubel if I could do a summer internship in their lab, but he told me they had no space for undergraduates and suggested that I apply to Nelson Kiang at the Massachusetts Eye and Ear Infirmary instead. In summer 1971, Kiang gave me intensive tutorials in auditory neurophysiology and an interesting project analyzing spike trains from the cochlear nucleus. I am still plugging away at neurobiological problems almost four decades later.

Cambridge

When I asked Hubel and Kiang for advice on where to apply to graduate school in neuroscience, their only point of agreement was that the top places were Cambridge, MA and Cambridge, UK. I felt it was time to leave Cambridge, MA to broaden my horizons, so I applied for a Marshall Scholarship to go to the other Cambridge. In early 1972, while still a senior at Harvard, I learned my application was successful, and that my Ph.D. supervisor would be a Dr. R. H. Adrian, whom I had never heard of. I phoned my brother Dick, who had just become an Assistant Professor at Yale after finishing his D. Phil. from Oxford on cardiac electrophysiology. Dick informed me that R. H. Adrian was one of Britain's most eminent skeletal muscle electrophysiologists, and son of **E. D. Adrian**, a Nobel Laureate in neurophysiology. Moreover R. H. Adrian had been the external examiner on Dick's D. Phil. degree. "But muscle is a backwater," I exclaimed. "I want to work on the brain." Dick assured me that Richard Adrian was a true British gentleman, who would let me work on a topic of my own choosing. So I decided to wait and see. After a summer intensively studying music at Fontainebleau, near Paris, I arrived in Cambridge in October 1972. At my first lunch in Churchill College, an aristocratic-looking don sat down opposite me and asked if I was Roger Tsien. I immediately realized he was Richard Adrian, because only someone who knew a member of my family could pronounce our surname correctly, as he just had. Within the first few minutes of our conversation, he asked "Is it true you think muscle is a backwater?" I had to admit the accuracy of the quotation. (I later found out that Dick had mischievously teased Adrian about this at a conference they had both attended that summer.) Adrian looked a bit pained at my confession, but immediately said that he would not object whenever I wanted to transfer to one of the real neurophysiologists in the department.

Thus began my Ph.D. training. I never did switch to another official supervisor, because I soon realized I did not enjoy doing conventional electrophysiology of the central nervous system. The traditional thesis project, basically following the paradigm so successfully employed by Hubel and Wiesel, was to drop an extracellular microelectrode into the brain of an anesthetized animal and record the activity of individual neurons while providing sensory stimuli. After several hundred such recordings, one could classify the different response patterns and write up a thesis and several publications. To me this seemed too much like ice fishing, i.e. cutting a hole in the ice covering a lake, dropping a fishing line into the opaque water beneath, and patiently waiting for a bite. The brain derives its power from trillions of neurons working in parallel, so I wanted to see lots of neurons simultaneously signaling to each other and processing information. Ideally one would stain the neurons with a dye that would visibly light up or change color whenever and wherever a neuron fired an action potential. A few commercially available dyes had indeed been found that responded to neuronal action potentials, but the optical responses were extremely tiny, e.g. a 10^{-4} or 10^{-5} change in fluorescence.

They were detectable only if thousands of action potentials driven by the investigator were averaged under highly simplified conditions[3]. Many orders of magnitude improvement would be necessary to detect endogenous signals in a complex brain. I rashly decided in winter 1972 that I would try to design and synthesize new dyes for the specific purpose of imaging neuronal activity. One strategy was to target the dye to the vicinity of sodium channels, which were believed to undergo large conformational changes as they generated action potentials. Another strategy was to create "electrochromic dyes" with large changes in dipole moment between ground and excited state, so that a change in neuronal membrane potential could shift the peak wavelengths of absorbance or fluorescence[4]. In either case I would have to learn organic synthesis, which I had hated in those Harvard chemistry courses and which nobody in the Physiological Laboratory could teach me. Fortunately, Dr. Ian Baxter,

a junior faculty member in the Chemistry Department and a friend of a friend of Richard Adrian's, was intrigued by my idea for targeting sodium channels and agreed to supervise me unofficially. Baxter had no other students and had the time, kindness, and patience to look over my shoulder several times a day and show me the necessary techniques. I found to my own surprise that I could do and enjoy organic synthesis once it was for a biological purpose of my own choosing. I remained hooked on this type of research even though the molecule I synthesized proved incapable of binding sodium channels, even though Baxter soon left to become a careers counselor in the north of England, and even after other generations of my synthetic voltage sensors proved inferior to those found by other labs screening large numbers of commercially available dyes and their close analogs[5].

My first glimmer of success required shifting to another biological target. Action potentials almost always generate large increases in intracellular calcium to exert any biological effect such as the release of neurotransmitters to excite or inhibit the next neuron in the pathway. In 1975 there was great excitement over the discovery that arsenazo III, a dye invented to measure heavy metals in nuclear waste, could also be used to monitor calcium in giant axons from squid neurons, though the signals from this dye were very small and somewhat ambiguous[6]. I felt that designing a dye to measure Ca^{2+} should be a far easier problem than designing a dye to track fast changes in neuronal membrane potential. Hundreds of dyes were already known in the chemical literature to respond to Ca^{2+}, e.g. for determination of water hardness. The real problem was that inside cells, the free Mg^{2+} concentration is about four orders of magnitude higher than that of Ca^{2+}, so that an intracellular Ca^{2+} indicator needs yet higher selectivity for Ca^{2+} over its sister ion Mg^{2+}. No chemist had yet recognized the biological need for such a selective indicator. A colorless buffer called EGTA was the only synthetic molecule known to have the necessary Ca^{2+}:Mg^{2+} selectivity[7], but it had never been made into any sort of dye molecule. By doodling on paper and playing with molecular models, I saw a way to make EGTA into a very rudimentary dye molecule. I started on this brand new project without telling Richard Adrian, because any prudent supervisor would have told me I should be bringing older projects to closure rather than starting radically new ones. Fortunately, within a few weeks I managed to make a small, impure sample of the target molecule (much later given the acronym "BAPTA") and found that it had the expected optical response to Ca^{2+} combined with high Ca^{2+}:Mg^{2+} selectivity[8]. After many more years and discoveries, better dyes descended from BAPTA[**] became the most popular way of seeing endogenous intracellular Ca^{2+} signals, screening for ligands and receptors linked to Ca^{2+} signaling, and imaging neuronal activity microscopically.[10]

After my Ph.D., I stayed in Cambridge as a postdoctoral Research Fellow at Gonville & Caius College. My change in focus towards Ca^{2+} signaling led me into collaboration with Dr. Timothy Rink, a new faculty member in the Physiological Laboratory, because Tim wanted to make Ca^{2+}-selective electrodes from materials sent from Switzerland[11]. The directions for assembly were in German, which Tim could not read. I had learned to read chemistry papers in German, so I translated the instructions. Our collaboration started with these Ca^{2+}-selective electrodes and continued with the biological testing and exploitation of my fluorescent indicators for Ca^{2+}. Even more importantly, Tim and his wife Norma invited me to their Christmas party in 1976, where I first met their sister-in-law, Wendy. Soon I was spending every weekend visiting Wendy at her house in North London. When Tim and Norma found out several months later, they were quite astonished at the effectiveness of their entirely unintentional matchmaking. Wendy (Figures 3–4) is still the love of my life.

Berkeley

My fellowship at Gonville & Caius College was to end in late 1981, so in 1979–1980 I started looking for an independent position. Because of Wendy's residence in London, I applied to the National Institute of Medical Research in Mill Hill, but was rejected without an interview. This was not a good time to search for a research job in Britain, because of the austerity program of the new Thatcher administration. It was time to return to the U.S., yet I had almost no contacts and few publications. Almost all my applications were unsuccessful. Biological departments considered me a chemist, while chemistry departments rejected me as a biologist. Nowadays the application of chemistry to solve biological problems is a very fashionable subdiscipline dubbed "chemical biology", but in 1980 the only venue for such interdisciplinary efforts was in the pharmaceutical industry. Even there, individual scientists were typically either chemists or biologists, not both simultaneously.

Figure 3. Wendy with our dog, Kiri, in 2004.

Luck intervened. The Department of Physiology-Anatomy, University of California, Berkeley, had a vacant assistant professorship, for which the chair of the search committee was Terry Machen, whom I had gotten to know while he was on sabbatical in Cambridge. Also Berkeley had two faculty members, Richard Steinhardt and Robert Zucker, who were interested in Ca^{2+} signaling. These connections enabled me to get an interview at Berkeley. Fortunately, the fluorescent indicators for Ca^{2+} had finally progressed enough to enable the first direct measurements of cytosolic Ca^{2+} in lymphocytes, including the elevation due to mitogenic stimulation[12,13]. Now one could investigate Ca^{2+} signals in populations of small mammalian cells, whereas previous techniques required

single cells large and robust enough to withstand microinjection. This prospect, together with the fact that my Ph.D. was in Physiology, convinced the Department to offer me the Assistant Professorship, which I accepted before I found out that Berkeley was suffering a financial crisis. The startup package to get my laboratory going in early 1982 was cut to just a few thousand dollars, and each item had to be justified as a replacement for obsolete instructional equipment. For example, to get me a UV lamp for viewing thin layer chromatography plates, an old microscope illuminator from the teaching lab had to be junked. More importantly, the Department had no resources to provide a fume hood, which I needed to continue synthesizing the Ca^{2+} indicators. Prof. Robert Macey, whose lab was next to mine, kindly donated an old fume hood including its irreplaceable ductwork extending to the roof of the building. For the remainder of my seven years at Berkeley, all our synthetic reactions took place in this single wooden fume hood, less than 4 feet wide, with wire netting embedded in the glass of the front window. The entire lab stank from chemicals in unvented storage cabinets, and became lachrymatory when reactions using excess ethyl bromoacetate had to be worked up outside the hood. I mention these austerities only to remind young scientists that some good research can be accomplished without lavish facilities and startup funds.

Despite these troubles, my time at Berkeley was scientifically quite productive, including collaborations with Machen[14], Steinhardt[15], Zucker[16], and others. I recruited Drs. Grzegorz Grynkiewicz and Akwasi Minta, who synthesized much improved Ca^{2+} indicators (fura-2, indo-1, fluo-3)[17,18] and a Na^{+} indicator (SBFI)[19], all of which are still in use today. After the budget crisis eased, the Berkeley administration helped me buy a primitive image processor, which I painfully programmed[20] to calculate images of the ratio of fluorescences at two alternating excitation wavelengths. Such real-time ratioing revealed Ca^{2+}, Na^{+}, and pH signals[14] inside single living cells, often with unprecedented spatiotemporal resolution.

Moving to UCSD

However, I began to worry about being trapped in a career of imaging inorganic ions. I wanted to explore signals transmitted through more complex biochemicals such as cAMP (cyclic 3',5-adenosine monophosphate) and the wider, more fashionable world of macromolecular interactions. As my bargaining power grew, I also came to want a lab with enough fume hoods, vented storage cabinets, and small darkrooms for fluorescence microscopy to support my unusual combination of chemistry and biology, as well as a joint appointment in a Chemistry department and support from the Howard Hughes Medical Institute. None of these were possible in Berkeley, so in 1989 we moved south to the University of California, San Diego, where we still are. UCSD satisfied the above desires and was much younger, roomier, faster-growing, and less tradition-bound than Berkeley, which I felt more than compensated for its lesser fame. The highlights of the science started at UCSD are recounted in my **Nobel lecture**.

Figure 4. Wendy and I, dressed up for the Nobel Ceremony.

Conclusions

Writing this autobiography has reminded me how my career has been shaped by a strange mixture of chance and fateful predisposition. The use of chemistry to build biologically useful molecules is a form of engineering, so I did not escape the tradition set up by my father, uncles, and brothers. However, I avoided the mechanical, aeronautical, electrical, and computer specialties they chose, probably because like many youngest siblings[21], I had to seek a distinct niche. But if I had not found Ian Baxter to re-instill my enjoyment of chemistry, perhaps I would have chosen yet another direction. My interest in imaging with multiple glowing colors also reflects visual interests from early childhood, which I have been lucky enough to align with a professional career. From a strictly biological point of view, our contributions have mainly been in the development of techniques. Man-made techniques do have a habit of becoming obsolete, whereas basic discoveries about how nature works should last forever. But truly fundamental insights such as those of Darwin or **Watson & Crick** are rare and often subject to intense competition, whereas development of successful techniques to address important problems allows lesser mortals to exert a widespread beneficial impact for at least a few years. Moreover, the same engineering approach is what creates new therapeutic strategies to alleviate disease, not just tools for our fellow researchers.

* The benevolent reign of these kings is commemorated in at least two immaculately maintained shrines, one in Lin'an, a medium-sized city in Zhejiang Province, the other constructed in 2002 on prime real estate on the famous West Lake at the center of Hangzhou. My mother, my wife, and I visited both shrines in 2004. My mother interpreted the prominence of these shrines as an attempt by the current Chinese regime to advertise a historical precedent for reunification with Taiwan.

** The invention of a generalizable structure that sensed Ca^{2+} with unprecedented selectivity was duly reported to the National Research Development Corporation, as required for work funded by the UK Science Research Council. Initially NRDC was enthusiastic enough to file a patent application, 42927/78, but the administrators soon decided that measuring intracellular Ca^{2+} was of negligible commercial value. They felt that the only possible use for biological Ca^{2+} measurements was in clinical assays in blood serum, an application with completely different performance criteria, so they abandoned the patent application. In principle I could have taken over the patent costs out of my own pocket, but the NRDC's estimate of the fees equaled about 20 years of a postdoctoral salary, so I did not try. Eventually, follow-up patent applications by the University of California covering narrower variations in molecular structure proved quite lucrative. A much more important example of the NRDC's conservatism[9] was their failure to patent **Milstein and Köhler**'s monoclonal antibodies, another Cambridge invention of the mid-1970's.

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From ***Les Prix Nobel***. *The Nobel Prizes 2008*, Editor Karl Grandin, [Nobel Foundation], Stockholm, 2009

This autobiography/biography was written at the time of the award and later published in the book series ***Les Prix Nobel/ Nobel Lectures/The Nobel Prizes***. The information is sometimes updated with an addendum submitted by the Laureate.

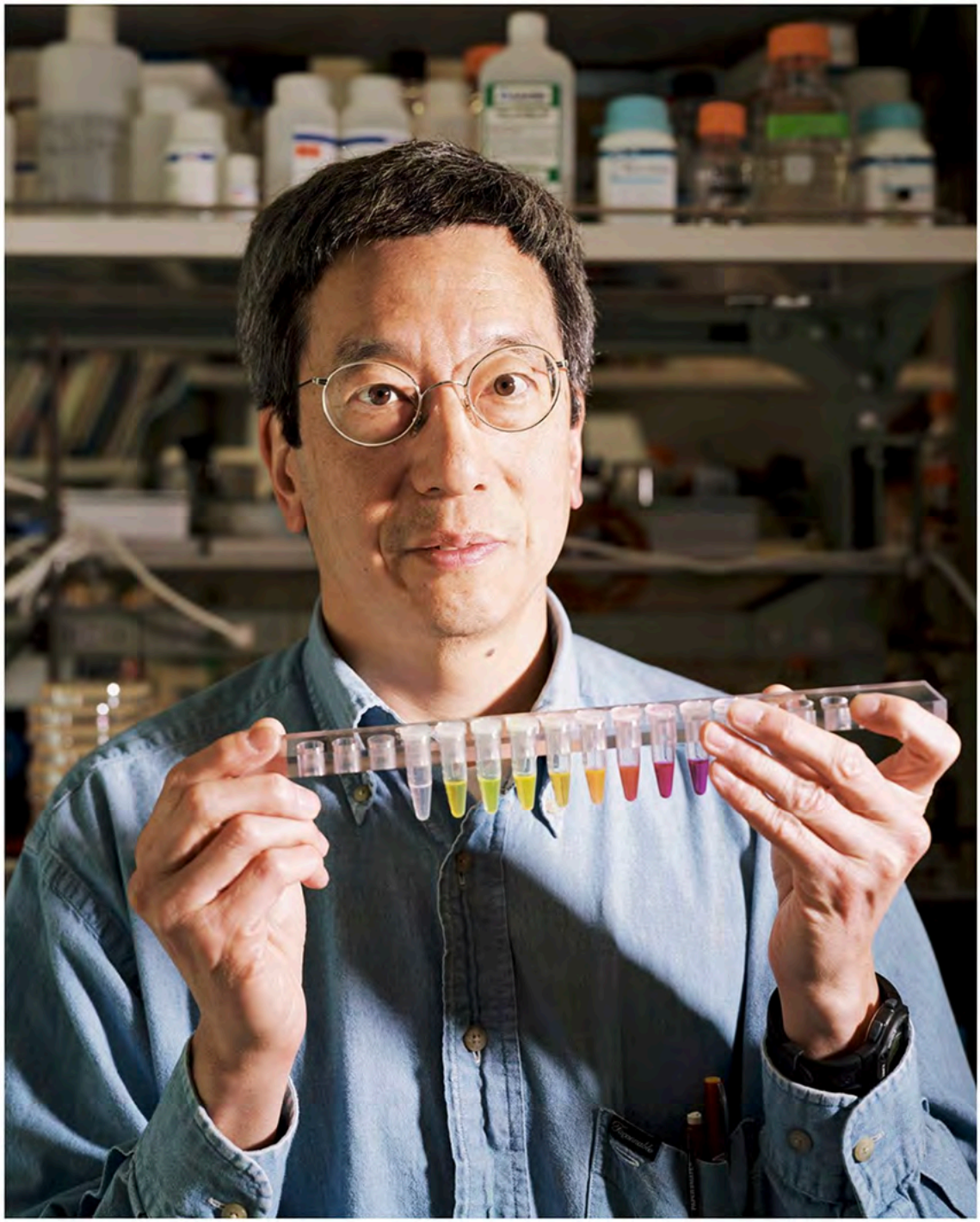
Roger Tsien died on 24 August 2016.

New York Times Obituary

August 24, 2016

The New York Times

Roger Y. Tsien, Nobel Winner for Use of Glowing Proteins, Dies at 64



Roger Y. Tsien shared the 2008 Nobel Prize in Chemistry after making it simpler to follow the dance of molecules within cells. Joe Toreno/Howard Hughes Medical Institute

By Kenneth Chang

Sept. 4, 2016

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Roger Y. Tsien, who won a Nobel Prize in Chemistry for creating a rainbow of fluorescent proteins that could light up the dance of molecules within cells, died on Aug. 24 in Eugene, Ore. He was 64.

His death was announced by the University of California, San Diego, where he was a professor of chemistry and biochemistry. A university spokesman said that he did not have information about the cause, but that Dr. Tsien, who was visiting Eugene, had suffered a "medical event" while bicycling.

While other scientists made the initial discoveries of a green fluorescent protein from jellyfish, Dr. Tsien (pronounced chen) was the one who transformed it into the ubiquitous tool used by biologists today.

After another scientist, Douglas C. Prasher, provided a copy of the gene that encodes the fluorescent protein, Dr. Tsien set out to make a better version.

"Roger, in his brilliant ingenuity, figured it should be possible to play with it," Charles S. Zuker, a former colleague who is now at Columbia, said in an interview. "He would do the simplest, most clever experiments to get at some of the most fundamental questions in contemporary biology."

The original protein glowed green when ultraviolet or blue light was shined on it. Dr. Tsien and the other members of his laboratory mutated the gene so that the proteins glowed brighter under blue light, which made them easier for biologists to use. (Ultraviolet light damages living cells.)

When biologists seek to track the comings and goings of a particular protein in a cell, they first identify the gene that produces it. They then splice the genetic instructions for the green fluorescent protein into the gene. The result is that the protein they want to track is tagged with a

fluorescent snippet, a beacon easily visible under a microscope — as when a car's headlights are turned on at night.

Dr. Tsien's laboratory then created a version that glowed blue instead of green. Other colors followed. That enabled the tracking of multiple molecules inside living cells.

"It created a new universe of biology," Dr. Zuker said.
In 2008, Dr. Tsien shared the chemistry Nobel with Osamu Shimomura of the Marine Biological Laboratory in Woods Hole, Mass., and Boston University School of Medicine, who discovered the jellyfish protein, and Martin Chalfie, a professor of biological sciences at Columbia, the first to insert the green fluorescent protein gene into another organism.

Dr. Tsien wondered why he had been honored and not Dr. Prasher, who had shared the gene with both Dr. Chalfie and Dr. Tsien.

"I think it must have been an agonizing decision for the Nobel committee, and they could easily have given the prize to Doug instead of me," Dr. Tsien told Chemistry World magazine.

Dr. Prasher had by then dropped out of science and was driving a courtesy van for a Toyota dealership in Huntsville, Ala., after budget cuts cost him his job for a NASA subcontractor. But Dr. Chalfie and Dr. Tsien invited him to attend the Nobel ceremony in Stockholm and paid for his trip.

In 2012, Dr. Tsien hired Dr. Prasher to work in his laboratory. Dr. Prasher left last year.

Roger Yonchien Tsien was born on Feb. 1, 1952, in New York City, the third of three sons to Hsue Chu Tsien, a mechanical engineer, and Yi Ying, who was trained as a nurse. In an autobiographical essay on the Nobel Prize website, Dr. Tsien recalled that his parents bought him a chemistry set when he was in elementary school, but that he "didn't find it very interesting because the experiments seemed so tame."

When he was 15, he worked at a summer research program at Ohio University. That turned into a project that won the top prize in the Westinghouse Science Talent Search competition.

He entered Harvard at 16 and graduated with a degree in chemistry and physics in 1972. He earned a doctorate in physiology from the University of Cambridge in England in 1977.

After positions as a postdoctoral researcher at Cambridge and a professor at the University of California, Berkeley, Dr. Tsien moved to the University of California, San Diego, in 1989.

Building on research from his doctorate, he had developed a way to measure levels of calcium in cells, which was a marker for the level of activity in neurons. The markers changed shape in the presence of calcium ions and then would change color.

“It’s typical of Roger,” said Stephen Adams, a scientist who worked in Dr. Tsien’s lab. “He took something that was well known in another field and applied it to neurobiology with a few chemical modifications.” Dr. Adams recalled that Dr. Tsien wanted to track other biological markers as well. Dr. Tsien and his collaborators extracted the proteins they wanted to track, attached dye molecules and injected them back into cells.

It worked, but it was laborious.

Dr. Tsien heard about the green fluorescent protein and found Dr. Prasher’s paper on the gene. Dr. Tsien and Dr. Chalfie independently contacted Dr. Prasher at about the same time.

“He made it a much more powerful molecule for people to do their experiments with,” Dr. Chalfie said of Dr. Tsien’s work.

Dr. Tsien, with Dr. Zuker, founded two companies in the 1990s. The Aurora Biosciences Corporation commercialized drug discovery tools

using fluorescent markers, and Senomyx looked for ways to modulate taste receptors to reduce the amount of sugar in foods without affecting taste.

Dr. Tsien is survived by his wife, Wendy; two brothers, Richard and Louis; and a stepson, Max Rink.
After winning the Nobel, Dr. Tsien shifted his research again, although his work continued to be colorful. One project was a fluorescent molecule that would light up in neon green only in cancer tumors, telling surgeons exactly what to cut out and what to leave untouched. Another stains nerve cells, again to help surgeons avoid cutting them by mistake. The tumor indicators are in initial clinical trials; the nerve stain is being tested in animals.

“We hope the combination of these molecules will significantly improve patient outcome,” said Dr. Quyen T. Nguyen, a professor of head and neck surgery at the University of California, San Diego, School of Medicine, who was collaborating with Dr. Tsien.

Nobel Lecture

December 8, 2008

CONSTRUCTING AND EXPLOITING THE FLUORESCENT PROTEIN PAINTBOX

Nobel Lecture, December 8, 2008

by

Roger Y. Tsien

Howard Hughes Medical Institute, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0647, USA.

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 phycobiliprotein[1–3]. 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 chromophore[4].

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 proteins[5–7]. 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 signaling[12–14]. 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 plasticity[15].

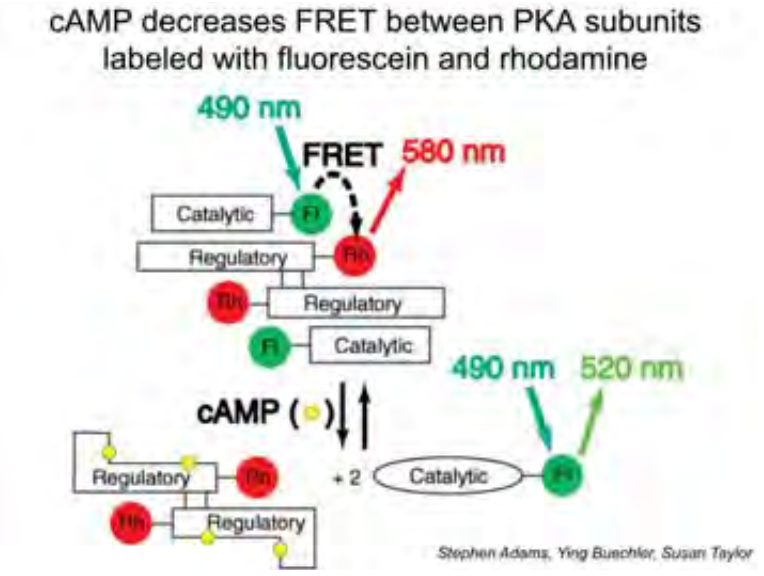


Figure 1. Schematic cartoon showing how cAMP-induced dissociation of regulatory from catalytic subunits of protein kinase A (PKA) can be reported by loss of FRET from fluorescein to rhodamine labels.

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.

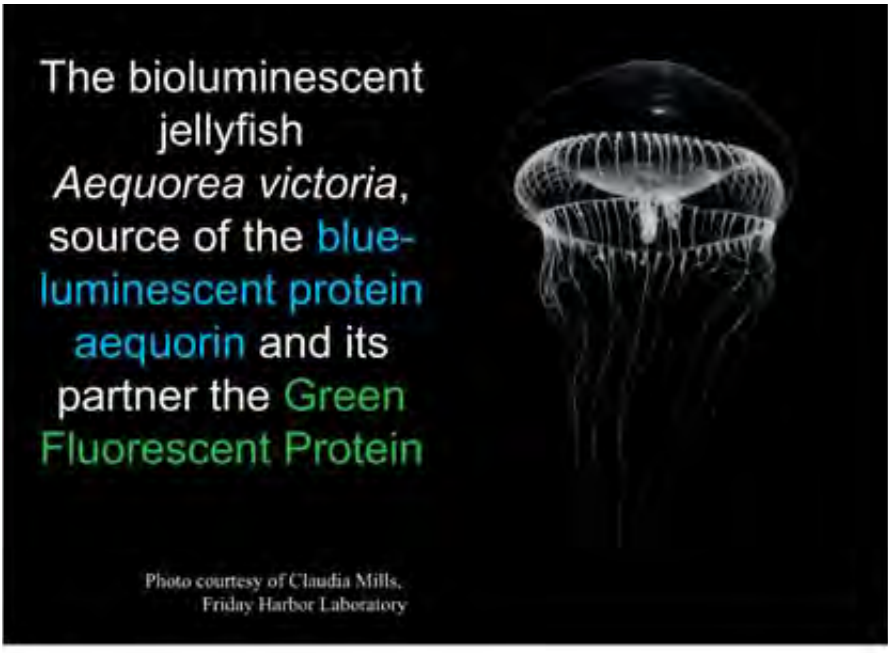
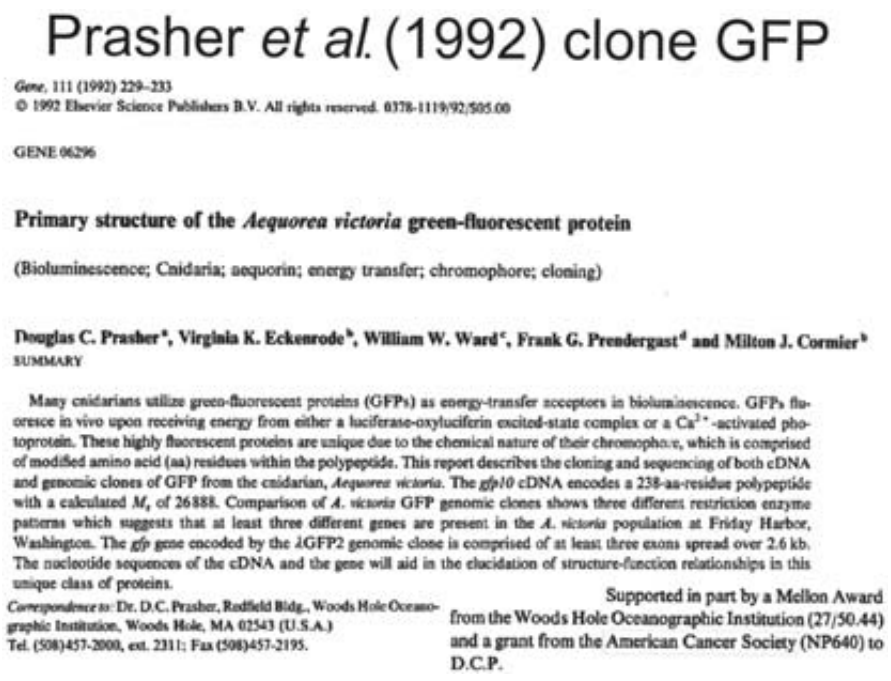


Figure 2. The jellyfish *Aequorea victoria* or *Aequorea aequorea* from which aequorin and Green Fluorescent Protein were isolated. Photo courtesy of Dr. Claudia E. Mills, Friday Harbor Laboratories.

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 cells[16]. 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 collaborators[17], 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 Ca^{2+} 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 Ca^{2+} 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

Gene, 111 (1992) 229–233
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GENE 06296

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

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

Douglas C. Prasher[a], Virginia K. Eckenrode[b], William W. Ward[c], Frank G. Prendergast[d] and Milton J. Cormier[b]

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 Ca^{2+}-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 26 888. 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 λGFP2 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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Tel. (508)457-2000, ext. 2311; Fax (508)457-2195.

Supported in part by a Mellon Award from the Woods Hole Oceanographic Institution (27/50.44) and a grant from the American Cancer Society (NP640) to D.C.P.

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 chromophore[17].

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 66[20]. 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 mutations[21].

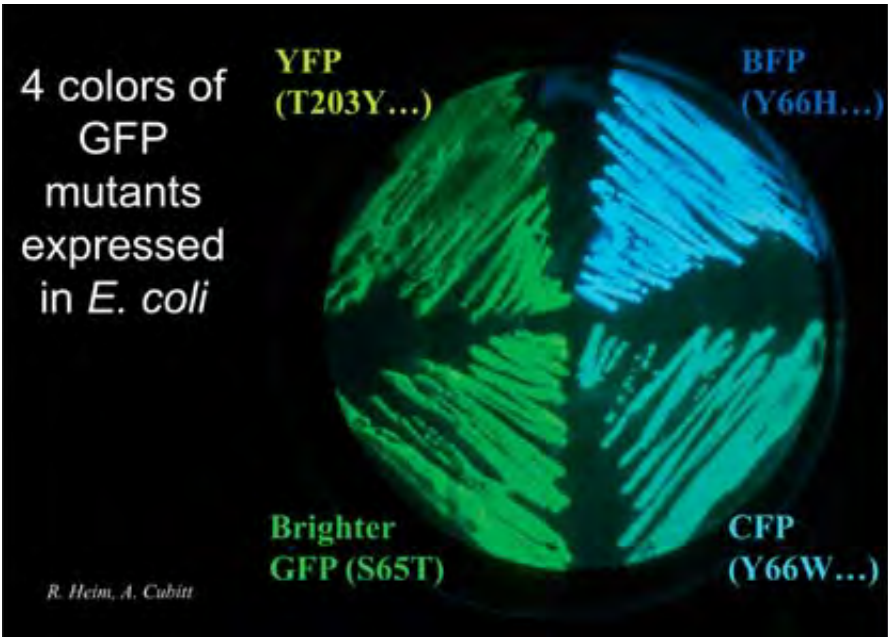

Figure 4. Fluorescence from streaks of bacteria expressing (clockwise from upper right) improved blue, cyan, green, and yellow fluorescent proteins. Each streak is labeled with the mutation most responsible for its spectral alteration.

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 hours[20]. 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 confirmed[23].

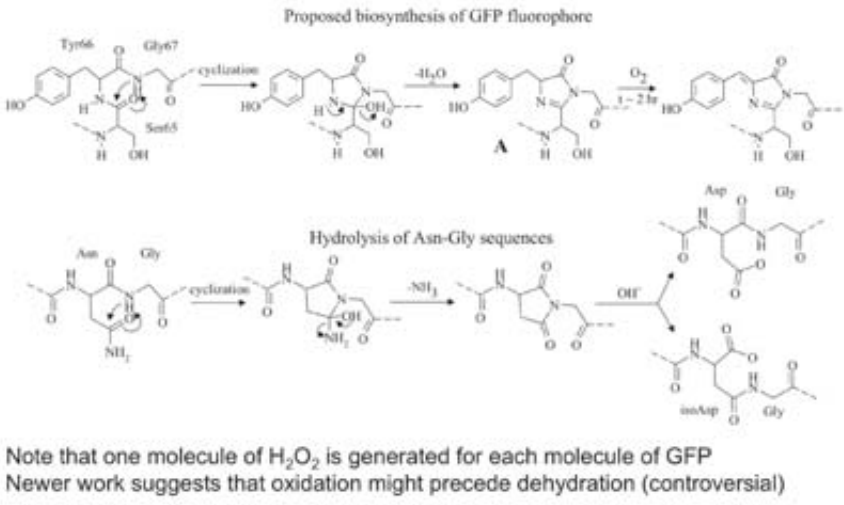

Figure 5. Initial proposal for mechanism of formation of the GFP chromophore[74]. The initial cyclization is analogous to the known tendency of Asn-Gly sequences to cyclize[75]. Recently it has been proposed that oxidation precedes dehydration[23].

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 Shimomura[24] 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 yeast[26] and thus began to address Emr's difficulties. As an initial proof of principle[21] 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].

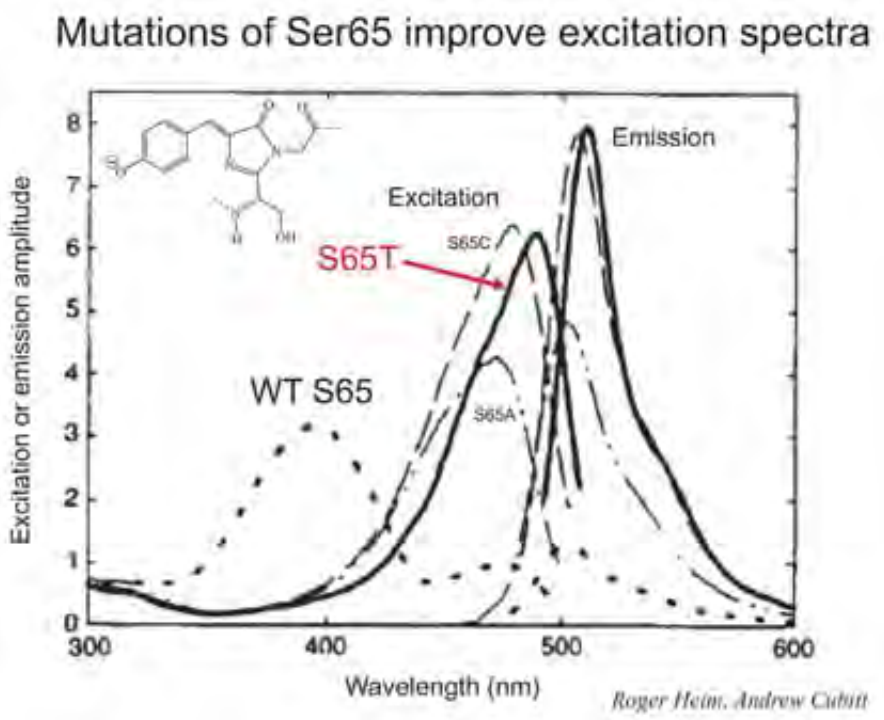

Figure 6. Excitation and emission spectra of wild-type (WT) GFP and several mutants of Ser 65[25]. The inset shows the structure of the wild-type chromophore.

In a random screen for optimal mutations of residues 55 to74, 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.

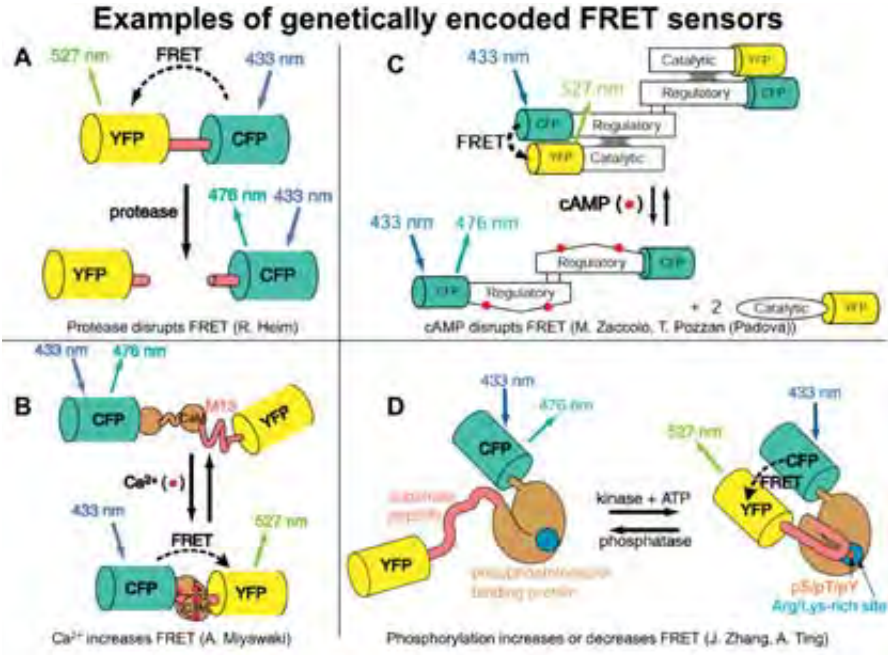

Figure 7. Schematic cartoons of genetically encoded fluorescent indicators based on FRET from CFP to YFP, to report A) protease activity, B) Ca^{2+} concentrations, C) cAMP concentrations, and D) the balance between protein kinase and phosphatase activity.

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 crystallized[30] 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.

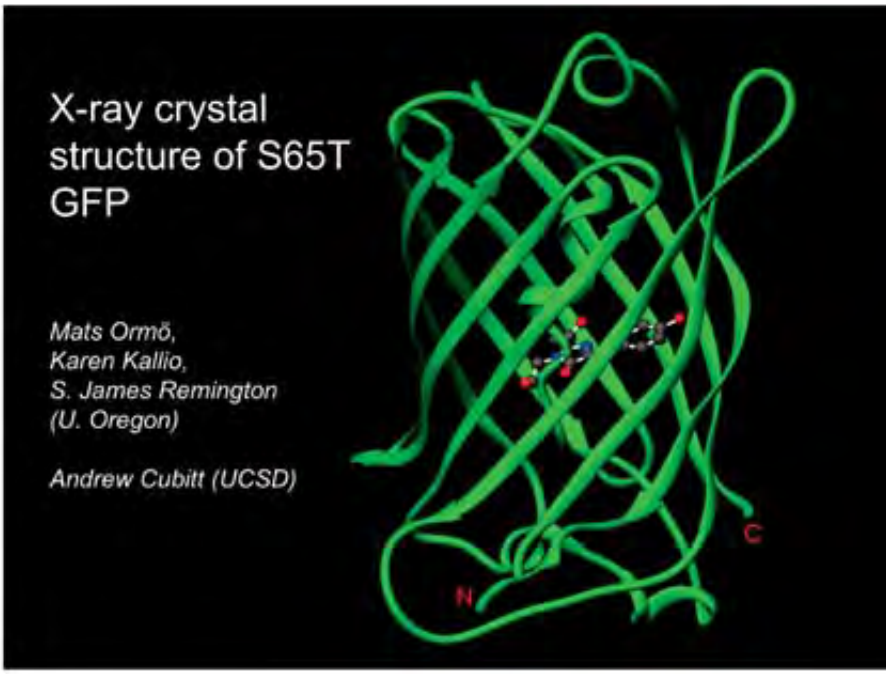

Figure 8. X-ray crystal structure of the S65T mutant of GFP[31]. Alpha helices and beta strands are shown as ribbons, connecting segments as tubes, and the chromophore in ball-and-stick representation. N- and C-terminii are marked.

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 ($InsP_3$)[34,35]. I very much wanted to image this second messenger, which is crucial for releasing Ca^{2+} 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 $InsP_3$ receptor, which was supposed to undergo a conformational change upon binding $InsP_3$. However, none of Miyawaki's constructs showed any significant change in FRET in response to $InsP_3$, and we realized we hardly even knew where $InsP_3$ bound within its receptor. With the invaluable help of Dr. Mitsuhiko Ikura in Toronto, Miyawaki decided to practice on an engineered receptor of known structure[36], 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 Ca^{2+}, but when free Ca^{2+} concentrations increase, Ca^{2+} 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 Ca^{2+}, so I did not feel that we needed more Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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).

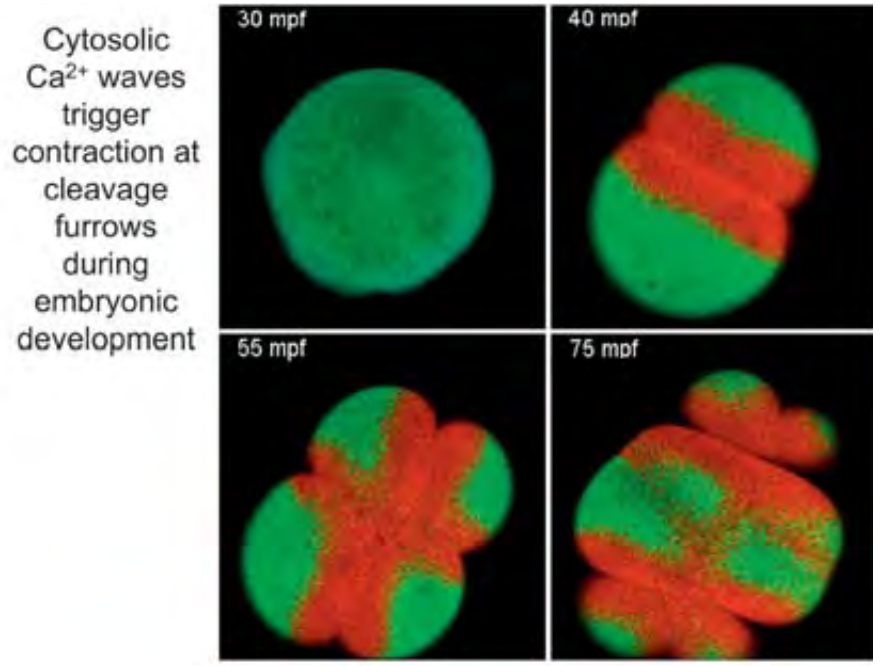

Figure 9. Transgenic zebrafish embryo expressing yellow cameleon 3.60[76]. Single confocal z-plane, imaged every 5 sec ("mpf" = minutes post fertilization). Images selected from a video kindly provided by Hide Mizuno & Atsushi Miyawaki, RIKEN, Japan.

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 lab[39]. 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 analog[40]. 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 phosphoamino 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.

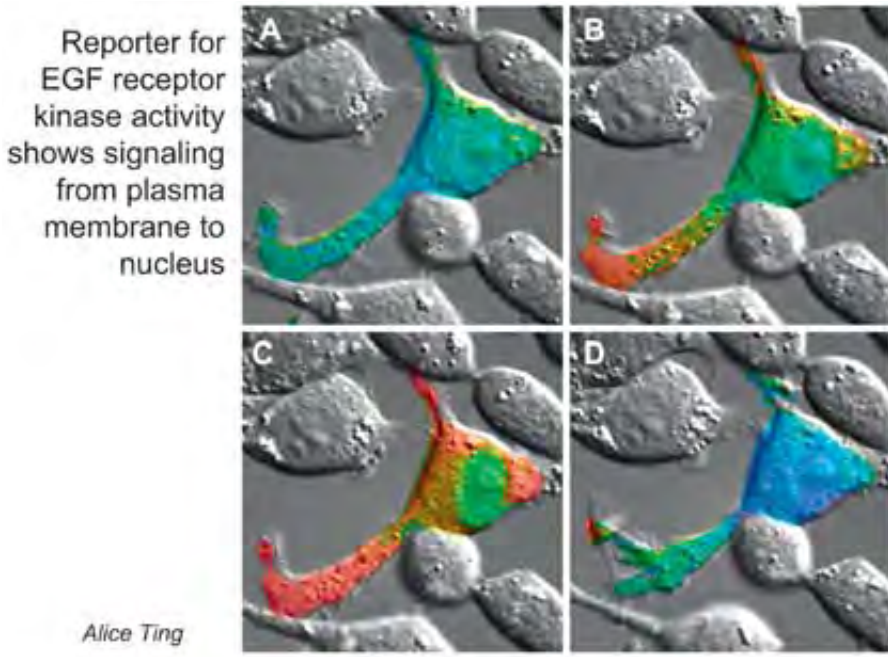

Figure 10. A fibroblast transfected with a reporter[47] for the tyrosine kinase activity of the receptor for epidermal growth factor (EGF). The extent of FRET, indicating the extent of phosphorylation of the reporter, is shown in pseudocolors ranging from blue (negligible phosphorylation) to red (maximal 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).

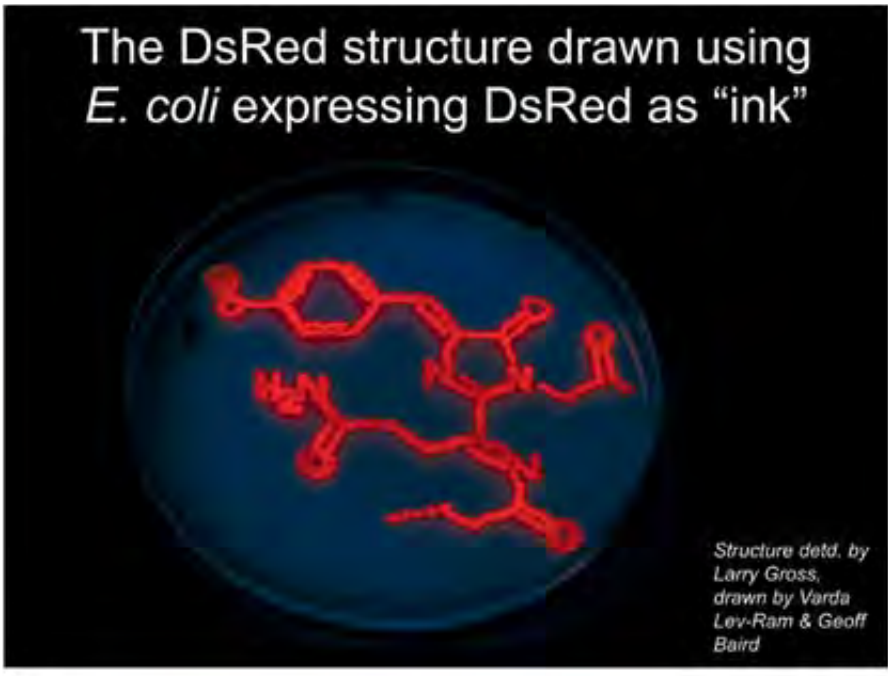

Figure 11. Chromophore structure[52] of DsRed, drawn on a Petri dish using bacteria expressing the protein and showing its beautiful red fluorescence.

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 aquarium[50]. The rapid commercial availability of this gene enabled us to discover that DsRed is an obligate tetramer[51] 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 protein[52]. 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 spectra[52]. 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 reliable[55]. 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 capabilities[58], but space does not permit an extensive review here.

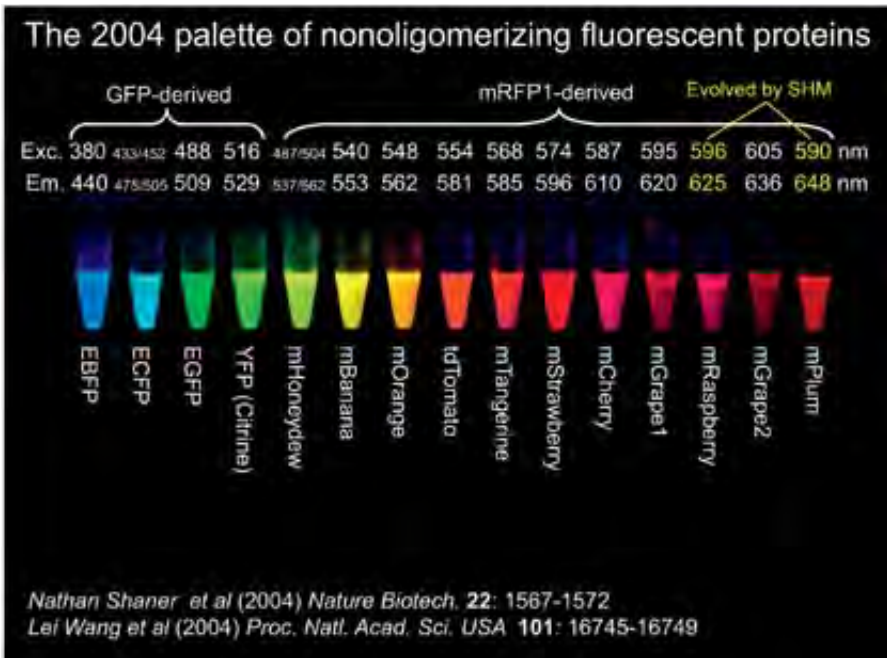

Figure 12. Monomeric or tandem dimeric fluorescent proteins derived from *Aequorea* GFP or *Discosoma* RFP, expressed in bacteria and purified. This photo is a time exposure of fluorescences excited at different wavelengths and viewed through different cutoff filters.

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.

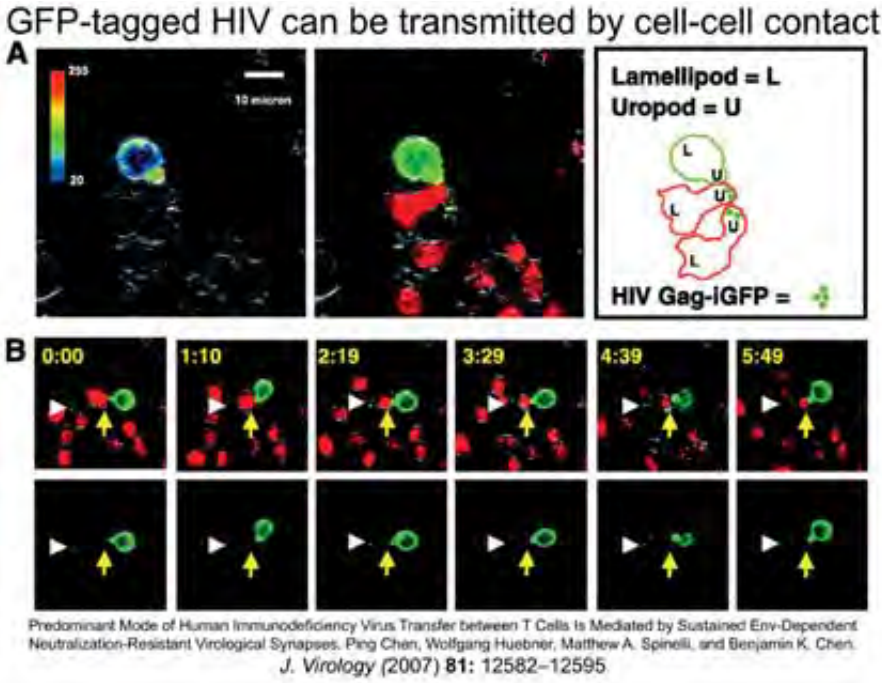

Figure 13. GFP labeling of human immunodeficiency virus (HIV) shows that the virus can spread from cell to cell through virological "synapses" without exposure to antibody neutralization. Reprinted with permission from Figure 6A,B of Chen *et al.*[77].

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 neurons[59], 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 disease[60]. 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.

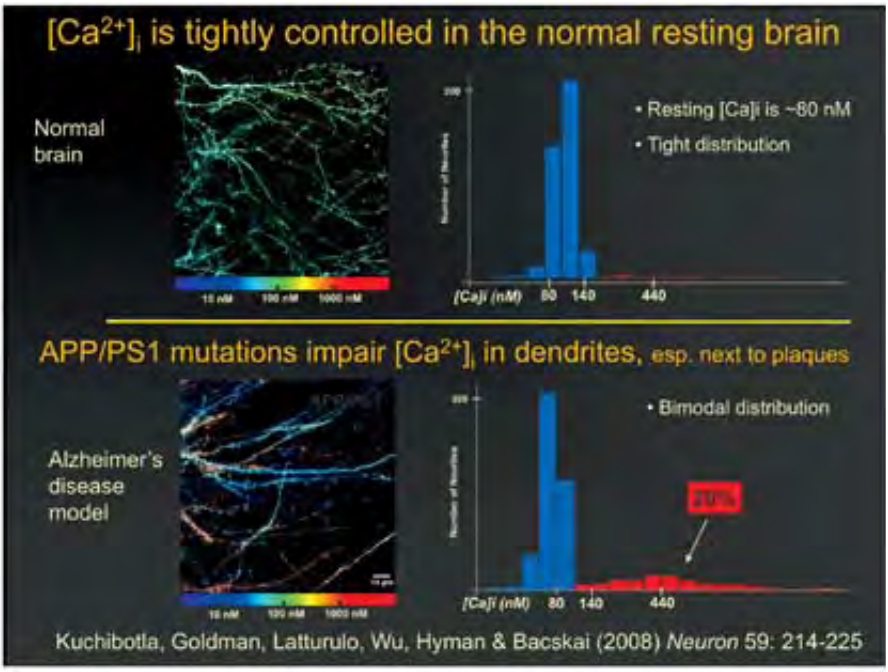

Figure 14. Two-photon imaging of yellow cameleon 3.60[76] in brains of transgenic mice, without or with APP1/presenilin mutations to generate plaques modeling Alzheimer's disease. Low to high cytosolic [Ca^{2+}] level are shown in blue to red pseudocolors respectively. In the disease model, 20% of neurons show a distinctly higher cytosolic [Ca^{2+}], 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 Aβ into β-amyloid. When Aβ is fused to the N-terminus of GFP, the aggregation of Aβ is fast enough to prevent the GFP from folding and becoming fluorescent. Candidate drugs that prevent aggregation of Aβ allow GFP fluorescence to develop, constituting a simple high-throughput way to screen combinatorial libraries (Figure 15)[61].

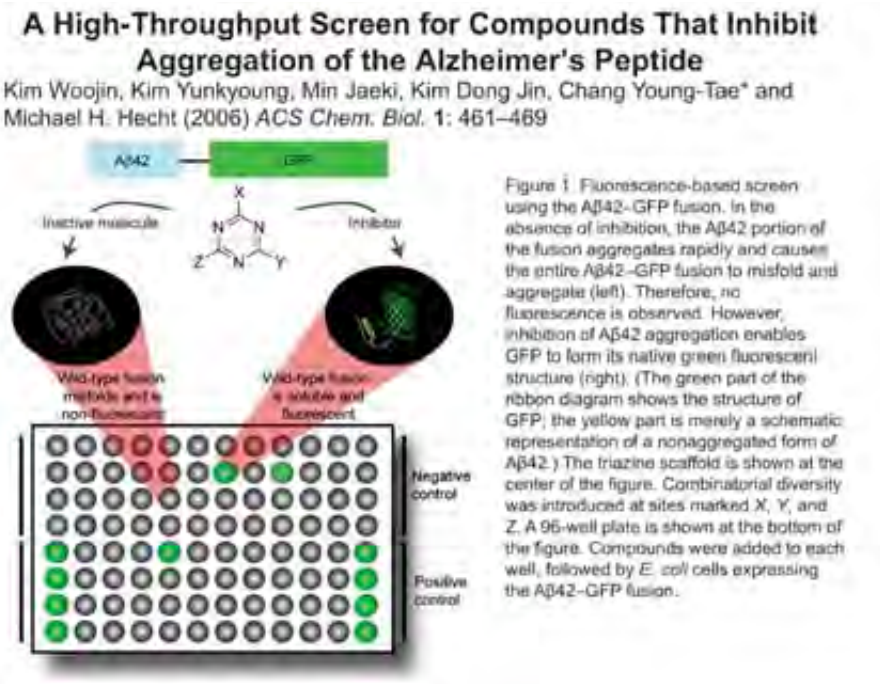

Figure 15. High-throughput screening of combinatorial drugs to find inhibitors of aggregation of Aβ, the Alzheimer's disease peptide. Inhibition of aggregation allows an Aβ42-GFP fusion to become fluorescent. Reprinted with permission from Figure 1 of Kim *et al.*[61].

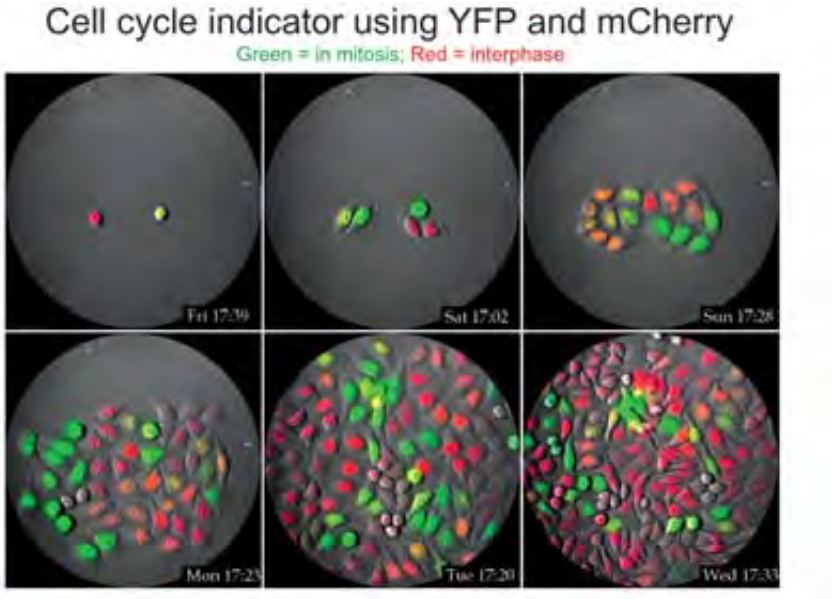

Figure 16. Frames chosen from a time-lapse video of HEK293 cells growing and dividing in tissue culture while transfected with a two-color reporter of cell cycle progression. Green-fluorescing cells are actively undergoing mitosis, whereas red-fluorescing cells are in interphase[62]. Video courtesy of 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 resting[62]. 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.

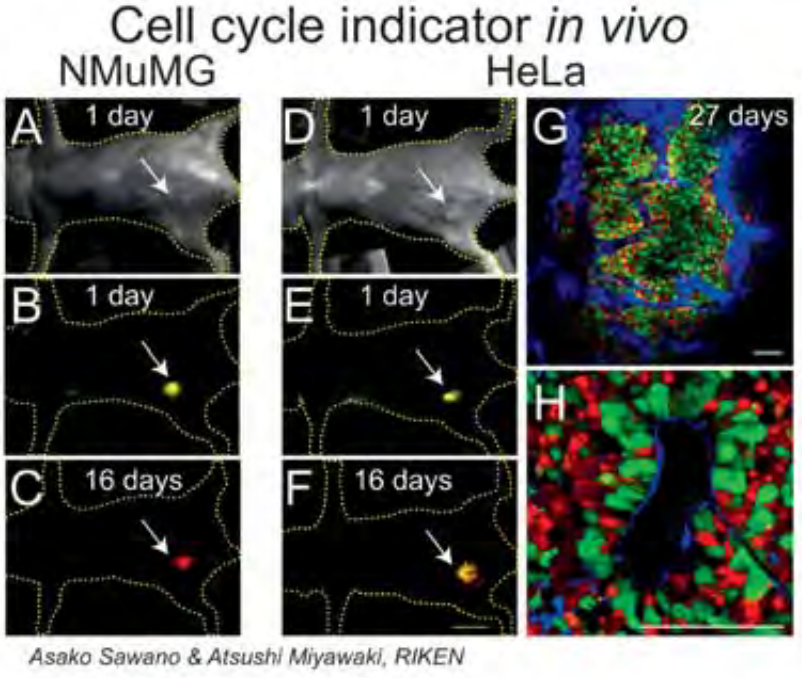

Figure 17. Reporter of cell-cycle progression applied to benign (NMuMG) and malignant (HeLa) tumor implants *in vivo*. Figure 3A–H from Sakaue-Sawano *et al.*[62], reprinted with permission.

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 interest[63–65]; 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 FLNCCPGCCMEP[68], which bind dyes containing two appropriately placed arsenic atoms that link to the four cysteine sulfhydryls.
2. The O_2 requirement for chromophore maturation prevents applications in organisms that cannot tolerate even transient exposure to any O_2. Aside from the tetracysteine/biarsenical system just mentioned, flavin-binding proteins offer another potential solution[69].
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 nm[72] (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.

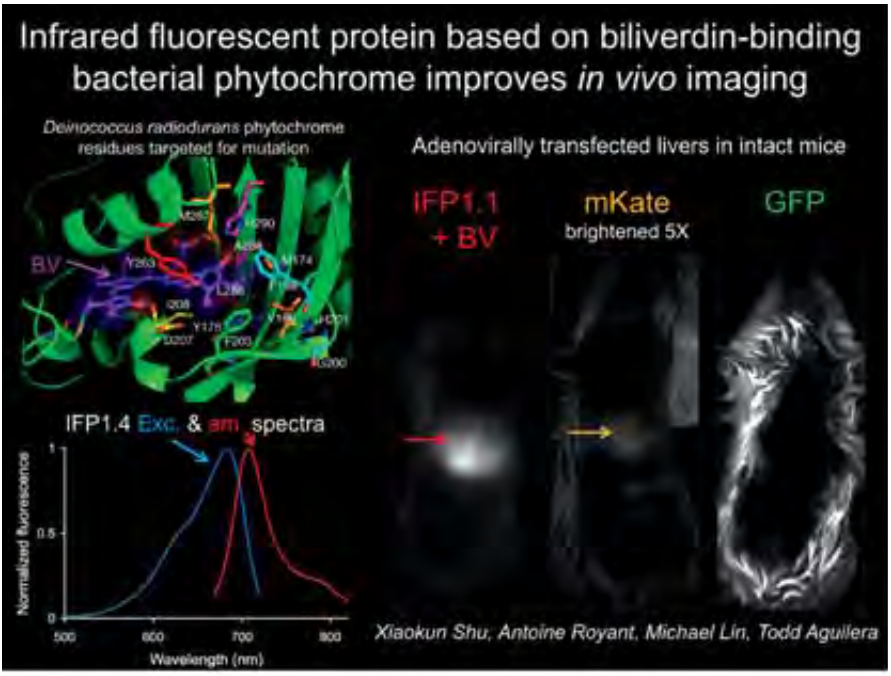

Figure 19. An infrared-fluorescing protein (IFP) can be evolved from a phytochrome from *Deinococcus radiodurans*. Upper left: crystal structure of the wild-type phytochrome (PDB: 1ztu)[78] with its chromophore, biliverdin, in purple, and residues targeted for mutagenesis labeled. Lower left: Normalized 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* imaging[79]), 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 cells[73]. 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.

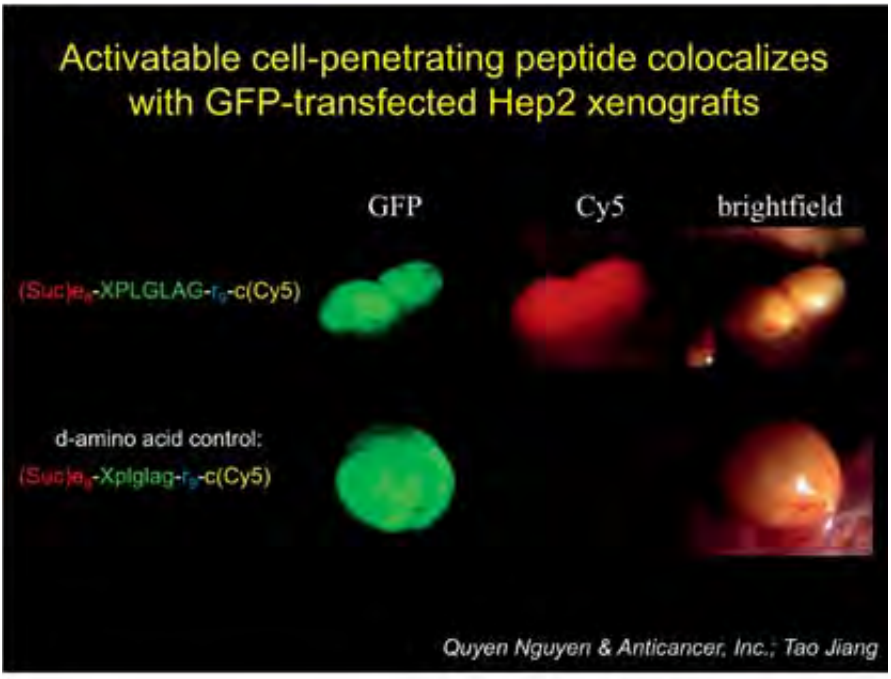

Figure 20. Activatable cell penetrating peptides (ACPPs) localize to tumors *in vivo*. Hep2 (human laryngeal carcinoma) tumor cells stably transfected with EGFP were xenografted into nude mice. 3 weeks after implanting $5x10^6$ cells subcutaneously, the tumor had reached ~ 5 mm diameter. Top images: 6 hrs after systemic injection of an ACPP, 10 nmol succinyl-e_8-XPLGLAG- r_9c(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 -9[73]. Bottom panel: an analogous experiment with a matching negative control, succinyl-e_8-Xplglag-r_9c(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.

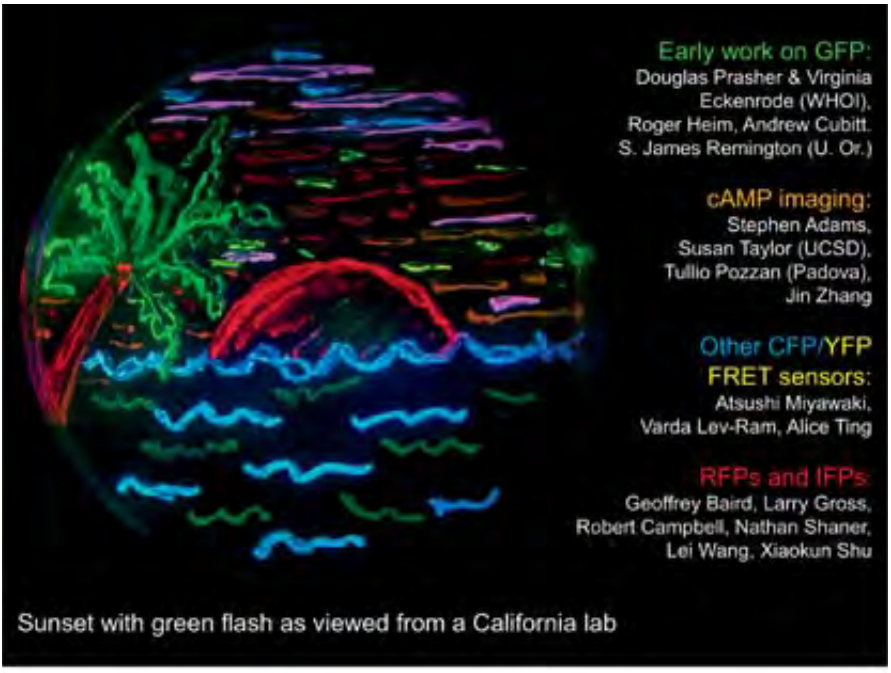

Figure 21. Key collaborators for the work described in this lecture, and a glowing California sunset in a Petri dish, drawn with multiple colors of bacteria expressing fluorescent proteins.

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