BREEDING MOLECULES TO SPY ON CELLS

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In some ways, the cells in our bodies are a bit like social communities. The proteins and other molecules inside the cell interact with each other rather like the people of a medium-sized town. I say medium-sized because the sequencing of the human genome in 2001 indicated that we contain only about 25,000–35,000 genes (International Human Genome Sequencing Consortium, 2001), which specify roughly the same number of protein types. Many people were surprised that the number was so finite and that *Homo sapiens* did not have significantly more genes than other species. A genome sequence is at best a sort of telephone directory or census list of the names of all the citizens: valuable reference information, but not something one would read from cover to cover for fun. It also gives little hint as to how the inhabitants actually live. Like town dwellers, individual protein molecules in a cell are born, get modified or "educated," travel around, and cooperate or compete with each other for partnerships. Some proteins emigrate from the cell. A few have the job of killing other proteins. Eventually all the proteins will die at varying ages, and their components will be recycled.

Suppose you were an anthropologist from a distant continent, or an alien from outer space. You want to understand how a particular town or other community functions, but you yourself cannot fit inside it, so what would you do? You might well train some appropriately sized explorers or journalists and infiltrate them over the city walls, to spy on events and report back to you. Even better spies might be natives who are born naturally into the society but who nevertheless faithfully keep you informed of their whereabouts, their education, their partnerships, or the prevailing economic climate. You might develop some radio collars or spy cameras to clamp onto particular named individuals and send an alarm

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whenever that individual comes within close contact of another tagged inhabitant. Finally, after you had learned all you could by passive observation and recording, you might want to try some perturbations to see how your subjects react. For example, you could suddenly dump in a lot of money by remote control and test how the society reacts. Or you could explode the radio collars to assassinate particular individuals and see which social activities continue vs. collapse. At this gory prospect, perhaps my metaphor is becoming too tortured, but the point is that for each of the forms of espionage described above, we have developed probe molecules that perform somewhat analogous tasks inside living cells.

We communicate with our probes or spies not with radios but by optical microscopy, which has been the leading technology for observing live cells ever since the pioneering discoveries of Antoni van Leeuwenhoek (Bradbury, 1967). Initially he and his successors looked at unstained cells, but the lack of contrast in most tissues soon led to the introduction of dyes empirically chosen for their ability to stain various cell structures more or less selectively. Usually these dyes would not cross intact cell membranes. Moreover, the dyes had to be applied at high concentrations because they bound their intracellular targets with low affinity and could be detected only by absorbance, that is, their ability to block certain colors of light from passing through. To block a significant fraction of an intense light beam, one needs lots of dye molecules, so many that they would interfere with life itself. Therefore, in the nineteenth century biological microscopy and histology became largely the study of dead, fixed, stained tissues, which inevitably became a rather static discipline.

The rebirth of light microscopy began in the 1930s with two developments. One was the introduction of fluorescently labeled antibodies by Coons et al. (1942), which provided the first means to find trace amounts of specific proteins in cells. Antibodies still are the best and most general way to recognize any chosen endogenous protein with high affinity and selectivity. Fluorescence consists of the ability of some dyes not just to absorb light but to re-emit light of a longer wavelength. Only a small percentage of light-absorbing substances have this ability—most just turn the light into heat. If we look through a microscope with color filters that only let through the re-emitted redder wavelengths, then we can see even very low levels of fluorescent glow because the unstained background gives off no light and shows up as black. Therefore fluorescent molecules can be detected with much greater sensitivity than nonfluorescent dyes, and this

is why we still rely so heavily on fluorescence. Unfortunately, immunofluorescence was and still is largely restricted to dead, fixed, sliced-up tissue, because antibodies cannot cross cell membranes. Meanwhile, people began to start looking at live cells again after Zernicke invented phase contrast microscopy in the 1930s, a clever physics technique to image small variations in the speed of light through different parts of cells (Bradbury, 1967). Unfortunately, phase contrast has no ability to pick out particular types of molecules. So if I can briefly return to my metaphor of cells as towns, immunofluorescence means that we can freeze all the inhabitants and localize a few specific individuals as corpses; phase contrast lets us see nondestructively where large crowds are massing but not the identities of the participants.

Our first significant success with biochemical reporters was a series of buffers and indicators for calcium ions (Ca^{2+}), which are crucially important messengers inside cells (for a review see Tsien, 1999). Fluctuations in the concentration of free Ca^{2+} control the contraction of all forms of muscle, the activation of many enzymes, early steps in our immune responses, and most types of rapid secretion. Perhaps the most important class of secretory responses are at the chemical synapses in the nervous system, where brief and localized puffs of intracellular Ca^{2+} not only trigger acute release of neurotransmitters but also activate biochemical cascades, resulting in memory storage via longer-term changes in synapses.

The widespread importance of intracellular Ca^{2+} has encouraged strenuous efforts to measure and control it (Tsien, 1999), but there are some major difficulties: 1) >99% of the total Ca^{2+} in the cell is bound or sequestered, whereas biochemical reactions are driven by the <1% that is free at any time. (A crude analogy might be an economy where most of the total value is locked up in fixed assets, yet buying and selling rely on a much smaller amount of circulating cash.) Destroying cells to measure the total amount of Ca^{2+} in them by traditional analytical techniques is rarely informative. 2) Healthy cells have very little free Ca^{2+} in them, typically 10^{-7} to 10^{-6} M, but they contain plenty of similar divalent cations such as ~10^{-3} M Mg^{2+}, so any reporter molecules must give a big signal only when they see Ca^{2+}. (To continue this economic analogy, our spies must be able to distinguish real currency from counterfeits or other pieces of paper that are much more abundant.) 3) Cells are bathed in saline solutions containing much more free Ca^{2+} than present inside, so any damage to the cell membrane tends to cause a massive influx of Ca^{2+}, which per-

turbs or even kills the cell. Thus the spies must be infiltrated into the cells without making holes in their walls.

One of our popular solutions to these problems is "fura-2" (Grynkiewicz et al., 1985), which binds Ca^{2+} very specifically because the binding pocket is just the right size for Ca^{2+} and is too big for Mg^{2+} (Tsien, 1980). Ca^{2+} twists the $N(CH_2COO^-)_2$ group on the benzofuran and decouples the nitrogen lone pair electrons from the chromophore, decreasing the effective length of the chromophore and shifting the fluorescence excitation spectra to shorter wavelengths. The ratio of fluorescences obtained with 340- to 350-nm vs. 380- to 385-nm excitation reports the free Ca^{2+}. However, the multiple negative charges necessary to bind the Ca^{2+} prevent the molecules from crossing membranes easily. Fortunately, these negative charges can be masked by special blocking groups called acetoxymethyl esters, which allow our cloaked indicators to slip through the cell membrane without any damage to the latter. What makes acetoxymethyl esters special is that they are easily cut off by enzymes present in mammalian cells, thus regenerating the negative charges and trapping the indicators in the cell (Tsien, 1981). Many thousands of studies have been published using such indicators to measure Ca^{2+} dynamics under many physiological and pharmacological conditions.

We built comparable-sized molecules to measure other intracellular signals such as sodium (Minta and Tsien, 1989) and proton (Rink et al., 1982) concentrations, gene expression (Zlokarnik et al., 1998), and membrane potential (Gonzalez and Tsien, 1997; Gonzalez and Tsien, 1995; Rink et al., 1980). For example, the membrane potential probes are based on an unusual type of dye, called an oxonol, that is negatively charged yet highly hydrophobic. It sits either at the extracellular or the intracellular border of the cell's outer membrane and can rapidly hop back and forth between those two locations. When the inside of the cell is at a negative potential, the oxonol is repelled to the extracellular face, whereas when the inside of the cell is positively charged, the oxonol is attracted to the intracellular face of the membrane. Meanwhile, a different dye, a coumarin, is stably anchored at just the extracellular border of the same membrane. When the coumarin and oxonol are on the same (extracellular) side of the membrane, they can get close enough to interact by a quantum-mechanical phenomenon known as fluorescence resonance energy transfer (FRET), in which the oxonol steals excited-state energy away from the coumarin. The result is that when the membrane poten-

tial is inside negative, the coumarin is prevented from glowing blue and the oxonol instead emits red. When the potential is inside positive, the separation between the oxonol and the coumarin reduces FRET and favors blue emission without red. I thought up this basic mechanism as a graduate student in 1975 but was unable to get it to work then. Much later, a postdoctoral fellow named Jesús Gonzalez joined my lab and made it a reality (Gonzalez and Tsien, 1997; Gonzalez and Tsien, 1995). Its most important use is in the pharmaceutical industry in high-throughput screens for drugs that activate or inhibit ion channels and other transporters that affect membrane potentials. It is also proving helpful for basic neurobiological research on small networks of neurons. For example, Tim Cacciatore and Adam Taylor, graduate students working with Profs. William Kristan and David Kleinfeld at UCSD, have used these probes to analyze the connectivity of neurons that modulate swimming in leech ganglia (Cacciatore et al., 1999).

In the mid-1990s we started to put most of our effort into developing genetically encoded macromolecular indicators, which are usually administered not as ready-made proteins but as genes, which tell the cell to make the indicators to our specifications. Thus these spies are native-born citizens of the cell or organism, even though they are partially derived from foreign ancestors. The most important forebear is the green fluorescent protein (Tsien, 1998) from a Pacific Northwest jellyfish, *Aequorea victoria.* When the jellyfish is disturbed, it emits a greenish glow by activating a bioluminescent protein called aequorin, which transfers its energy to the green fluorescent protein (GFP). GFP was discovered as a protein by Osamu Shimomura (Shimomura et al., 1962), the gene was cloned by Douglas Prasher (Prasher et al., 1992), and that gene was first expressed outside the jellyfish by Martin Chalfie's and Frederick Tsuji's labs (Chalfie et al., 1994; Inouye and Tsuji, 1994). Currently it remains mysterious how the glow is useful to the jellyfish and why it evolved two participant proteins for this task rather than just one. Nevertheless, GFP has become tremendously useful in many areas of molecular and cell biology because it provided the first means by which a simple gene could give rise to bright visible fluorescence. Whenever scientists want to make a cellular protein fluorescent, their first thought nowadays is to fuse the gene for their favorite protein to the gene for GFP, then put this composite gene back into the cell or organism of interest. If all goes well, the hybrid generates a chimeric protein in which the host component goes about its normal

business while the attached GFP fluoresces and reports the presence and position of the pair (Tsien, 1998).

Dr. Roger Heim in my lab started working in 1992 on the GFP gene provided by Prasher (Prasher et al., 1992). We very much wanted to create mutants with brighter fluorescence, because the original GFP was dim, fickle, and spectrally impure. We also wanted different colors to enable FRET, which inherently requires a pair of colors. Heim developed the first mutants in which the spectrum of GFP was simplified and enhanced (Heim et al., 1995); one of these mutations, right next to the chromophore, is at the heart of the optimized GFPs now routinely used around the world. Heim also created blue- and cyan-emitting mutants, BFPs and CFPs, respectively (Heim et al., 1994; Heim and Tsien, 1996). Later we helped Prof. Jim Remington's group solve the X-ray crystallographic structure of GFP, which immediately suggested a way to push the emission to somewhat longer wavelengths (Ormö et al., 1996). Dr. Andrew Cubitt made the appropriate mutants, whose yellowish appearance led to the name of YFPs. Currently, the best pair for FRET consists of the cyan and yellow mutants, CFP and YFP, respectively.

An early and still exemplary use of CFP and YFP to monitor important intracellular biochemistry came from Dr. Atsushi Miyawaki, who made a single composite protein (Miyawaki et al., 1999; Miyawaki et al., 1997; Miyawaki and Tsien, 2000) out of four linked modules: CFP, a famous calcium-binding protein called calmodulin, a calmodulin-binding peptide called M13, and finally YFP. When Ca^{2+} concentrations are low, calmodulin does not interact with M13. In this state, FRET from the CFP to the YFP is relatively inefficient. When Ca^{2+} concentrations are high, Ca^{2+} binds to calmodulin, which induces calmodulin to wrap around the neighboring M13 peptide, which increases FRET from the CFP to the YFP, which decreases the amount of cyan emission while increasing the strength of yellow emission. Dr. Miyawaki dubbed these chimeric creatures "cameleons," because the key Ca^{2+} sensor is calmodulin, whose standard abbreviation is CaM. The CaM and M13 are like the body and extensible tongue of a real chameleon, which is famous for retracting its tongue into its body and changing color. Because cameleons are entirely genetically encoded, they can be targeted to specific subcellular locations by using the known postal systems that cells normally use to address proteins to given subcellular locations. Such molecular-scale targeting allows genetically encoded indicators to have much finer spatial

specificity than could be obtained solely from the imaging resolution of an optical microscope. Genetic targeting also works at a more macroscopic level to place the indicators in practically any tissue or organ of an intact animal. For example, we have prepared transgenic mice in which almost all tissues express cameleons. The mice are healthy, continue expressing cameleons generation after generation, and are essentially indistinguishable from normal mice except for a slight fluorescence, as shown in Fig. 4.1A. Figure 4.1B and C show a heart muscle cell from such a mouse, at two moments of its spontaneous beating rhythm, low and high Ca^{2+}, respectively. The yellow-to-cyan ratios of cameleon fluorescence, reflecting the dynamic calcium levels in the muscle cell, are translated into computer-generated colors ranging from blue to red to represent low to high Ca^{2+} levels, respectively. Waves of red sweep through the cell in synchrony with local twitching of the muscle. The real benefit from such mice will come from interbreeding them with mice that have specific genetic abnormalities relevant to human diseases. Progeny that contain both the abnormality and the cameleon will help reveal the involvement of intracellular Ca^{2+} in the disease state.

We have employed somewhat similar four-part fusions (Ting et al., 2001; Zhang et al., 2001) to reveal the activity of protein kinases, which are enzymes that add phosphate groups to other proteins. Such phosphate groups are the most important class of dynamic modifications that proteins can undergo. The fusions consist of CFP, a phosphoaminoacid binding domain, a peptide tuned to be a specific substrate for the kinase of interest, and finally YFP. If kinase activity is high, it phosphorylates the peptide, which internally binds to the phosphoaminoacid binding domain, which changes the mutual orientation or proximity of the CFP and YFP, which alters the efficiency of FRET. If the activity of the kinase declines relative to that of phosphatases, which are the enzymes that cut phosphate groups off proteins, then all the steps including the final color change are reversed. These reporters are like model pupils that constantly broadcast the efficacy of specific teachers within the civic educational system. These particular images represent just two of the many kinase reporters that we and some other groups (Kurokawa et al., 2001; Sato et al., 2002; Violin et al., 2003) have developed.

So far all the applications I have described of fluorescent proteins have been based on mutants of the green fluorescent protein from jellyfish. However, Russian workers recently discovered (Matz et al., 1999) that

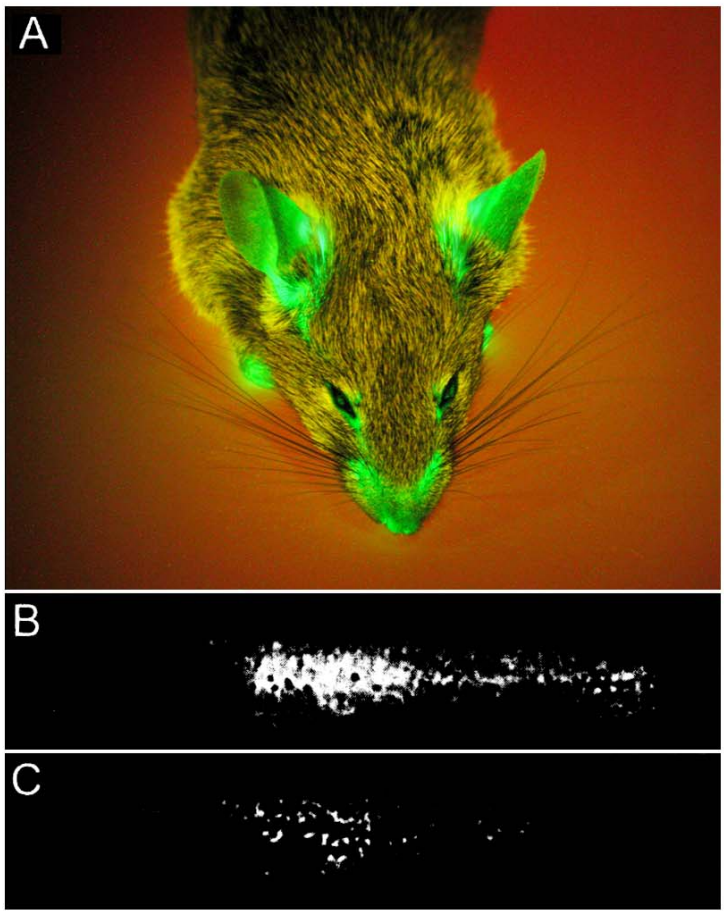

Fig. 4.1. A genetically encoded Ca^{2+} indicator, yellow cameleon 3.0 (Miyawaki et al., 1997), in transgenic mice and cardiac myocytes from such mice. A. External view of a mouse expressing yellow cameleon 3.0 under the control of a beta-actin promoter. Although the fluorescence is most visible where the darkly pigmented fur is thinnest, nearly all the tissues express the fluorescent indicator protein. The mouse was generated by Atsushi Miyawaki, Qing Xiong, and Varda Lev-Ram, and photographed by Paul Steinbach. B and C. Two frames from a movie showing spontaneous Ca^{2+} transients and contractures in a cardiac myocyte from such a mouse. Pseudocolors indicate the ratios of yellow to cyan emissions, which reflect local Ca^{2+} concentrations. Low Ca^{2+} = blue to green, intermediate Ca^{2+} = yellow, high Ca^{2+} = red. The myocytes were prepared by Randa Hilal-Dandan in Prof. Larry Brunton's laboratory.

nonbioluminescent corals contain a wide variety of fluorescent proteins somewhat homologous to GFP. Some of these coral proteins fluoresce at much longer wavelengths, including red. Red fluorescent proteins would be very valuable to provide additional contrast and FRET partners for the blue, cyan, green, and yellow-green colors already available from GFP variants. Also, red wavelengths are more distinct from tissue autofluores-

cence, penetrate tissue better, and are therefore superior for in vivo imaging. Using mass spectroscopy, we discovered the structure of the chromophore in red fluorescent proteins (Gross et al., 2000). The chromophore is almost identical to that of GFP but contains one extra double bond, which is quantitatively sufficient by quantum-mechanical calculations to explain the ~80-nm red shift of the spectra (Gross et al., 2000). Unfortunately, the wild-type coral proteins insist on forming tightly bound gangs of four (Baird et al., 2000; Wall et al., 2000; Yarbrough et al., 2001), which has greatly hindered biological application because anything else fused to the coral protein is usually forced to tetramerize as well. Intensive efforts in my lab to re-engineer the protein so that it remains monomeric were eventually successful, although no less than 33 mutations were required (Campbell et al., 2002). This monomeric red fluorescent protein should make possible many new applications of genetically encoded tags and reporters.

Despite the wonderful feats that jellyfish and coral fluorescent proteins make possible, they do have important limitations. They are at least 220 amino acids in size, so that they are often bigger than the host protein to which they are fused. Their only basic function is fluorescence, whereas artificial small molecule probes encompass a much wider range of useful properties. Ideally, one would like to combine the genetic targetability of fluorescent proteins with the versatility and small size of organically synthesized dyes. A solution to this challenge is to tag the protein of interest with a very small peptide domain, whose essential core is just two cysteines, two spacer amino acids (optimally proline-glycine), then two more cysteines (Adams et al., 2002; Griffin et al., 2000; Griffin et al., 1998). This motif can be labeled in living intact cells with membrane-permeant dyes that carry two arsenic atoms along one edge. Each pair of cysteines binds to one of the arsenics, probably causing the peptide to bend into a hairpin conformation. We have synthesized a variety of biarsenical dyes that can fit into the same peptide domain, but the most useful are a green-emitting fluorescein-based dye called FlAsH (for fluorescein-based arsenical hairpin binder) and a red-emitting resorufin-based analog called ReAsH (for resorufin-based arsenical hairpin binder). FlAsH and ReAsH are both able to cross mammalian cell membranes. They are delivered prebound to an antidote, 1,2-ethanedithiol, that minimizes toxicity by protecting endogenous thiols from the arsenics. The antidote also keeps the dyes relatively nonfluorescent until they find their target

tetracysteine domains. A single tetracysteine site can bind either FlAsH or ReAsH, but not both at the same time. Once bound, the dyes remain stably attached for days under normal conditions.

Our lead application has been a collaboration (Gaietta et al., 2002) with the laboratory of Mark Ellisman (UCSD) to label connexin-43, a major constituent of gap junctions. By sequentially applying FlAsH, removing excess free dye, and then applying ReAsH a few hours later, we can stain older connexin-43 molecules green and newer molecules red, all in live cells. Within each gap junction, the young red molecules show up as a concentric ring around a central core of old green connexins. The longer the time interval between removal of FlAsH and addition of ReAsH, the greater the percentage of old green-stained proteins that have been replaced by red-stained youngsters. This result shows that new molecules are added at the periphery while old ones are drained away at the center. Furthermore, ReAsH can be visualized by electron microscopy through a process called photoconversion. Photoconversion is achieved by vigorously illuminating ReAsH in the presence of pure oxygen and a chemical called diaminobenzidine. The excited state of ReAsH can convert ordinary oxygen into a short-lived and very reactive form called singlet oxygen, which immediately oxidizes the diaminobenzidine into a localized and fine-grained polymer, which is readily marked with osmium tetroxide. The result is that wherever ReAsH is sufficiently abundant, an electron-dense deposit becomes visible in the electron microscope. This process is genetically targeted, catalytic, and amplifying. Aside from the genetically tagged protein, it uses only small, diffusible molecules (ReAsH, O_2, diaminobenzidine, osmium tetroxide), which easily penetrate well-fixed tissue, whereas in traditional immunoelectron microscopy, good fixation destroys the antigens and prevents the antibodies from diffusing into the specimen. This allows one to see first the gap junctions by fluorescence in live cells and then the same gap junctions by low-magnification electron microscopy after fixation and photoconversion. Upon increasing the magnification, the two juxtaposed plasma membranes densely riveted by connexins can be resolved. By comparison, a high-quality immunoelectron micrograph shows much sparser staining, reflecting the low probabiliy of antigen staining and lack of amplification in previous methods.

ReAsH undergoes photoconversion far more efficiently than FlAsH. By using the pulsed application of FlAsH and ReAsH and photoconverting

only the latter, one can distinguish new and old copies of protein at electron microscopic spatial resolution. This enabled us to confirm by electron microscopy that new copies of connexins congregate at the periphery of the gap junction. In images not shown here, we have also shown that newly synthesized connexin-43 bound for the surface membrane is transported in vesicles of 100- to 150-nm diameter, whereas old connexin-43 molecules are disposed of in horseshoe-shaped fragments of double membranes and then fuzzy lysosomal aggregates (Gaietta et al., 2002).

The red biarsenical dye has one other trick in its repertoire. Dr. Oded Tour has found that strong illumination generates reactive oxygen species that can locally and acutely inactivate the protein to which the dye is attached. Only a few seconds of strong illumination suffice to inactivate connexin-43 and L-type voltage-activated Ca^{2+} channels. Thus a short genetically encoded motif containing four cysteines can direct photochemical inactivation of proteins (Jay and Sakurai, 1999) with higher spatial and temporal resolution than possible with conventional genetic deletions, antisense oligonucleotides, or small interfering RNAs. Returning to our opening analogy, ReAsH is like a radio collar that can either report the spatial location of its wearer or explode on command to incapacitate that same host.

How can these methods for visualizing intracellular biochemistry and signaling events help improve human health? Aside from the longer-term benefits of increasing our fundamental knowledge of how cells work, there are more immediate pharmaceutical applications. As a result of huge efforts in gene identification and cloning, culminating in the sequencing of the human genome, we have a basic compilation of the proteins that make up our bodies and that are potential targets for drug therapies. Even if only 10–20% of the proteins prove to be suitable targets, that constitutes many thousands. Meanwhile, new methods in organic chemistry, particularly combinatorial synthesis, are making millions of druglike candidate molecules relatively available, but only in very small quantities initially. Although structure-based rational drug design is very elegant in principle, it currently only works in special cases and to limited extents, whereas the most robust strategy for finding new drug leads remains high-throughput screening, that is, testing huge numbers of compounds for biological activity in physiologically appropriate assays. Mammalian cells are particularly suitable platforms for building such assays because we are mammals and because cells, unlike isolated macromolecules, are complex

enough to model most fundamental biological functions. Cells can be mutagenized, selected for optimal performance, grown in large quantities, and distributed into aliquots, which would not be possible or ethical with whole animals. Fluorescence measurements of membrane potential, protein processing and conformation, and gene expression are among the most versatile ways to build functional assays in live cells. Because these assays work on single cells without killing them, one can rapidly select for the few cells that give the biggest responses to known stimuli and then let those cells multiply into a large, homogeneous population (Whitney et al., 1998). This population is then used to test thousands to millions of candidate drugs (Finney, 1998; Gonzalez et al., 1999; Mere et al., 1999), each candidate in a separate well of a plate with thousands of such tiny wells. The small volume of each well, 1 to 2 μl, minimizes the amount of test compound required and is nicely compatible with fluorescence readings but prevents assays based on radioactivity, aspiration, or filtration. Many of the fluorescence technologies developed in our lab and mentioned in this lecture were commercialized by Aurora Biosciences Corporation, a biotechnology start-up company that I helped start in 1994 and that was eventually acquired by Vertex Pharmaceuticals in 2001.

Here are some general conclusions that I would draw from thirty years of trying to engineer molecules to report cellular events:

- Deliberate design and synthesis of molecules (both small and macro) can have a significant impact on cell biology and drug discovery.
- Cell biology, many aspects of chemistry, and some physics/instrumentation must be closely integrated.
- Small teams of 1–2 postdocs/students in an academic lab of 3–15 can make basic progress in 0.5–5 yr (huge teams not required).
- Translation to medical/pharmacological treatments does require industrial participation.
- Most major biochemical signals can now or will soon be visualized in live cells.
- Cells are highly individualistic; spatial organization (microscopic and submicroscopic) and temporal patterning are all-important.

The type of research that we have done is often dismissed as technology development, inferior to pure biology. However, in many cases a relatively modest investment in developing powerful and general methods can solve a wide range of longstanding biological questions or enable

translation of biological knowledge into practical clinical advances. One is then reminded of the old aphorism: If you give a hungry man a fish, you feed him for a day; if you teach him how to fish, you feed him for a lifetime. That is why we enjoy devising new lures and nets. Fortunately, the sea of potential knowledge is the one ocean that is in no danger of becoming overfished.

In all my research, I have been fortunate to enjoy the collaboration of a great many scientists in my lab and around the world. I only had time to mention a tiny part of our work with small molecule indicators and some more recent results on genetically encoded indicators, whereas I had to omit discussion of caged compounds (Adams and Tsien, 1993; Furuta et al., 1999), permeant esters (Li et al., 1998), and injectable protein indicators (Adams et al., 1993; Bacskai et al., 1993; Hempel et al., 1996) because of time and space limitations.

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