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FLUORESCENT PROTEIN BIOSENSORS: Measurement of Molecular Dynamics in Living Cells

Kenneth A. Giuliano and Penny L. Post*

Center for Light Microscope Imaging and Biotechnology and Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213 and *University of Pittsburgh School of Medicine, Department of Neurological Surgery, Suite B-400, Presbyterian University Hospital, Pittsburgh, Pennsylvania 15213-2582

Klaus M. Hahn

Scripps Research Institute, Department of Neuropharmacology IMM 6, La Jolla, California 92037

D. Lansing Taylor

Center for Light Microscope Imaging and Biotechnology and Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213

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ABSTRACT

A new generation of reagents that report on specific molecular events in living cells, called fluorescent protein biosensors, has evolved from in vitro fluorescence spectroscopy and fluorescent analogue cytochemistry. Creative designs of fluorescent protein biosensors to measure the molecular dynamics of macromolecules, metabolites, and ions in single cells emerge from the integrative use of contemporary synthetic organic chemistry, biochemistry, and molecular biology. Future advances in fluorescent probe design, computer-driven optical instrumentation, and software will allow us to engineer endogenous cellular components that localize and function as reporters of their activities, thus moving molecular measurement beyond the single cell to living tissues and the whole organism.

PERSPECTIVES AND OVERVIEW

A powerful set of tools based on the interactions of proteins with ligands such as other proteins and macromolecules, metabolites, and ions are being used to explore the molecular basis of life functions. We can now genetically modify the structure and function of specific proteins, overexpress or completely knock out selected proteins, or incorporate foreign proteins into cells, tissues, organs, and organisms. High-resolution spectroscopic tools such as X-ray crystallography and nuclear magnetic resonance (NMR) coupled to molecular modeling allow us to define the relationship between the structure and function of proteins in vitro.

An important challenge in biology is to extend our knowledge of protein structure and function in vitro to the level of molecular interactions in living cells because all life functions originate at the cellular level (76, 114, 118). This extension has a strong foundation in the fields of molecular biology, biochemistry, and fluorescence spectroscopy. The investigation of the molecular basis of cellular function in cell-free systems has led to the purification and characterization of numerous proteins. Using this classical in vitro approach, investigators have

amassed data concerning the structure of proteins, how they respond to regulatory signals, and how they associate with other proteins, all in the hope of extrapolating this information to uncover how proteins function in the living cell (61). While the information gained from dilute-solution studies performed in vitro is needed to help interpret the results from living systems, a great challenge is to understand the nonideal chemistry that occurs in the concentrated-fluid and solid phases known to comprise the cell environment (76). Therefore, the goal of the cell biologist is to complete the continuum of information from the high-resolution studies on purified cellular proteins in vitro to the temporal-spatial molecular interrelationships in living cells, tissues, organs, and organisms.

Investigating the molecular dynamics of living systems from cells to organisms is difficult as it requires techniques that minimally perturb normal physiological activities. Historically, biologists have used electromagnetic radiation to study the chosen living biological sample. The earliest use of light microscopes and X-ray scattering demonstrated that this approach was valuable (133). Today, we can explore the temporal-spatial dynamics within living samples with light (29, 114), NMR (31, 35, 54), and a growing list of scanning probe methods based on a variety of physical and chemical properties of the sample (10, 133). Each of these methods has advantages and disadvantages based on type and extent of perturbation, sensitivity, and spatial and temporal resolution, as well as specificity. We believe that future studies will take advantage of more than one of these modes of analysis to simultaneously dissect a multitude of molecular processes in living samples.

This review focuses on the use of labeled proteins, called fluorescent protein biosensors, to explore functional living samples. These biosensors report the functional activity of intracellular proteins through an alteration in the fluorescence spectroscopic properties of the attached probe that reflects some conformational change, reversible ligand binding, or covalent modification.

Proteins are well suited to act as intracellular sensors because their activities are believed to mediate all the chemical reactions in cells (61). However, the approaches discussed here for fluorescence can also be used with chemiluminescence (18), NMR (31, 54), and electron spin resonance (105). The use of fluorescent protein biosensors as sensitive and specific sensors of chemical and molecular changes in living cells has its roots in classical solution spectroscopic studies and fluorescent analogue cytochemistry (118). Fluorescent analogue cytochemistry creates fluorescently labeled, functional protein analogues that have been used to define the distribution and dynamics of molecular processes in

living cells (63, 112, 117, 118, 127). Fluorescent protein biosensors have been the natural outgrowth of fluorescent analogue cytochemistry and are a component of this approach (43, 118).

Here, we discuss the theory behind and design of fluorescent protein biosensors and point out chemical and fluorescence-based methodologies that we believe will lead to the newest members of this growing family of biosensors. We begin by dissecting protein activities into their molecular components and discuss how these activities form the basis of the design of fluorescent protein biosensors. We then describe how fluorescence-based reagents are coupled to methodologies designed to detect and analyze their information-rich fluorescence signals. We follow with sections concerning the design and construction of fluorescent protein biosensors, keeping in mind their use in the unique environment of the living cell. Finally, we discuss the major issues in the future of fluorescent protein biosensors.

APPROACHES TO SENSING PROTEIN ACTIVITY IN LIVING CELLS

The activity of intracellular proteins encompasses many sorts of chemical interactions that include the binding and release of ligands, assembly and disassembly of macromolecular structures, the interaction with membranes or organelles, and the catalytic conversion of specific substrates into metabolic or macromolecular products (see 23 for overview). The activity of a protein is defined by the environmental changes that occur either internally or on its surface. This is true whether the protein is an enzyme with catalytic activity or a cellular structural component that must interact with other macromolecules or organelles. Molecular associations that change protein conformation can dramatically alter protein structure, or they can act locally, causing only subtle environmental changes (34). In both cases, the effects on protein activity may be profound. For example, phosphorylation of a single serine residue on the light chain located in the head region of smooth muscle myosin II converts a compact 10S particle with little ATPase activity into an extended 6S, fully active enzyme (120). In contrast, phosphorylation of a single serine residue in the active site of isocitrate dehydrogenase causes no long-range conformational change in the free enzyme, yet it inhibits enzymatic activity by preventing the enzyme from interacting with its negatively charged substrate (52). Regardless of the extent of reversible conformational changes, proteins stabilize these changes by dynamically rearranging molecular dipoles, hydrogen bonds, and ionic and hydrophobic interactions. Many of these activity-

dependent molecular rearrangements can be sensed by fluorescence-based reagents and thus form the basis of fluorescent protein biosensor design.

Fluorescent Analogues of Proteins

Fluorescent analogue cytochemistry involves the preparation of functional, fluorescent analogues of proteins used to define the dynamic distribution and activity of these proteins in living cells (117, 127). Three classes of fluorescent chromophores (probes) in proteins are potentially useful in engineering fluorescent analogues: intrinsic, coenzyme, and extrinsic (107).

Intrinsic probes consist of the aromatic side chains of tyrosine, phenylalanine, and tryptophan residues, whereas coenzyme probes are comprised of molecules such as flavin-adenine dinucleotide. Fluorescence spectroscopic investigations of intrinsic and coenzyme probes have revealed important structural information and interactions of selected proteins (60, 132). Unfortunately, intrinsic and coenzyme-based probes have two major limitations: The location of the probe is rarely in the optimal region of the protein to sense key environmental changes, and the excitation spectra are very broad and generally do not permit selective investigation of proteins containing these fluorophores in complex mixtures that include living cells.

Weber (129) introduced the approach of extrinsic fluorescent labeling of proteins. The incorporation of extrinsic fluorescent probes at a specific location in a protein allows for site selection as well as spectral selection. The idea of extrinsic labeling was extended by the discovery of Weber & Laurence (134) that a variety of polycyclic aromatic compounds that were nonfluorescent in aqueous media became fluorescent upon binding serum albumin. A series of classic papers by Weber and his colleagues led the way in the use of fluorescence spectroscopy as a tool to study the structure and dynamics of proteins and other macromolecules (24, 93, 107, 133). A wide range of reactive fluorescent dyes is now available that can be used to target specific sites, exhibit environmental sensitivities, and fit into specific spectral regions (45, 125).

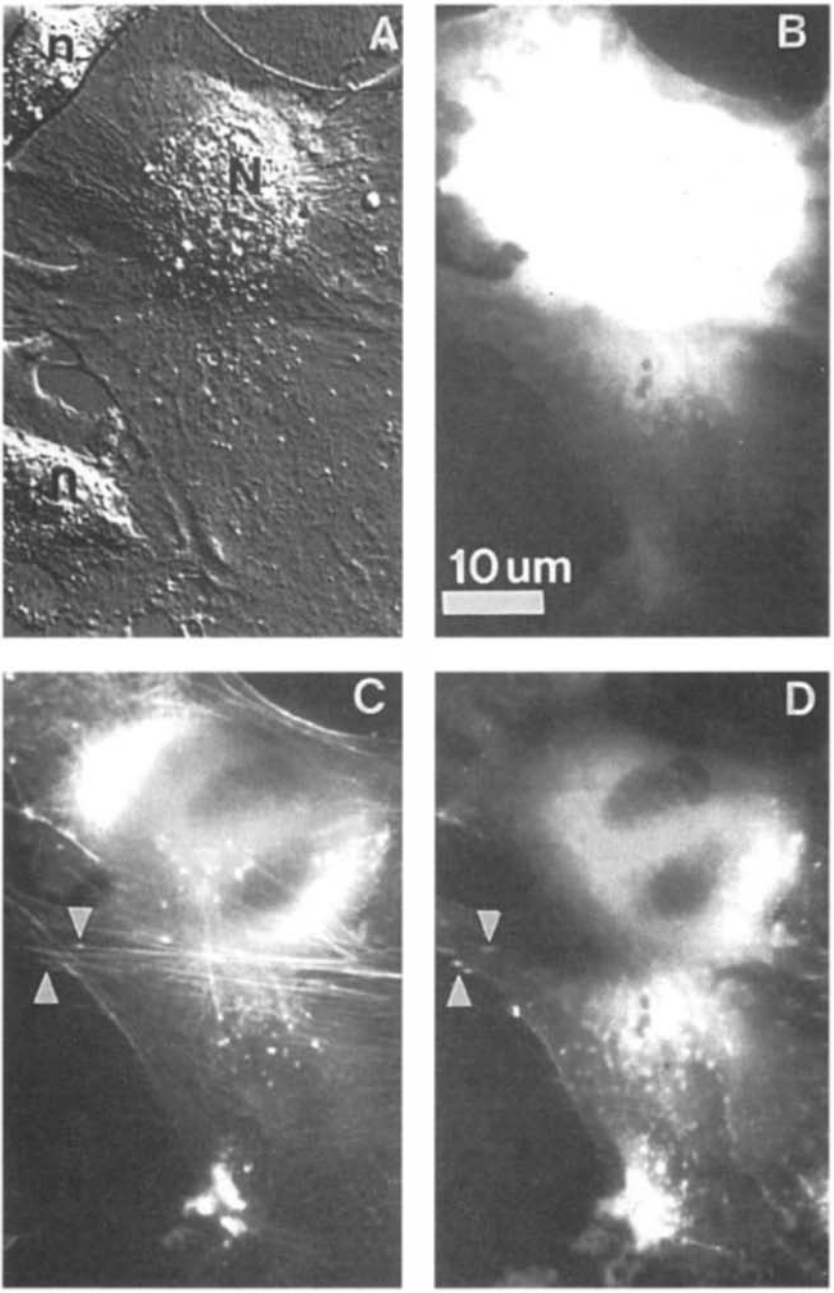
To prepare fluorescent protein analogues, a protein of interest is isolated, covalently modified with a reactive fluorescent dye, and characterized in vitro to determine if the covalent modification somehow affected the original activity (102, 112, 118, 127). Although optimization of the labeling process to produce bright, biologically relevant fluorescent analogues varies with each protein, many analogues have already been prepared and characterized based on general guidelines (63, 102, 128). The characterized fluorescent protein analogue is then introduced

into living cells (85), where the fluorescence signal demonstrates the distribution of the analogue in time and space.

Fluorescent protein analogues have been most often used to describe the dynamics of the actin-based cytoskeleton in living cells. For example, to measure the assembly and disassembly of actin (38) and myosin II (59), they have also been used to localize several accessory proteins (64, 84, 87, 119). Fluorescent analogues have now been prepared for numerous structural proteins (39, 88, 99), enzymes (89, 90), and other proteins and peptides (75, 83). When coupled with fluorescence photobleaching recovery methods, they can be used to measure the physical characteristics of the surrounding cytoplasm (59, 64, 74, 111, 128). Similar experiments can be performed using photoactivation of fluorescence that exhibits a higher signal-to-noise ratio (101).

Fluorescent analogue cytochemistry will continue to guide us in our analysis of the dynamics of living cells. Figure 1 demonstrates the use of multiple probes over a spectral range to map the dynamics of multiple proteins. The living human glioblastoma cell shown here was microinjected with three distinctly labeled components: dextran, to map the accessible cytoplasmic volume of the cell; actin, which incorporates into the dynamic actin cytoskeleton; and vinculin, to map the dynamics of focal adhesions. Figure 1*a* is a video-enhanced differential interference contrast image that depicts glioblastoma morphology and organelle structure. The actin analogue shown here is a fluorescent protein biosensor in that it belongs to a new generation of fluorescent actin analogues that interact optimally with endogenous actin-binding proteins and that therefore report accurately the dynamics of actin assembly in living cells (38). Combining fluorescent analogues and fluorescent protein biosensors in the same cells will give us a powerful set of cell biological reagents. In addition, the fluorescent protein biosensors

Figure 1 Multiparameter fluorescence analogue cytochemistry. Living human glioblastoma cells (SNB-19) were microinjected with three distinctly labeled fluorescent analogues and observed with video-enhanced differential interference contrast microscopy (*A*) or fluorescence microscopy (*B–D*) using the multimode microscope (29, 37). The nuclei of three cells in the field of view are denoted in *A* with the letters n and N. The cell marked with N was microinjected with fluorescent analogues that included: (*B*) an M_r 10,000 dextran (38), (*C*) nonmuscle actin (38), and (*D*) smooth muscle vinculin (KA Giuliano, in preparation). The arrowheads in *C* and *D* denote the colocalization of stress fibers containing actin and vinculin fluorescent analogues. Using a time series of fluorescence ratio images, data from this type of experiment can be used to measure a temporal and spatial map of actin assembly (38) or the dynamics of focal contact formation and dissolution.



described below can simultaneously be used as fluorescent protein analogues in the same experiment, thus yielding a wealth of information from a single reagent.

Fluorescent Protein Biosensors

Proteins express activity through their interaction with various ligands, from ions and metabolites to macromolecules and organelles. To induce activity, some proteins require an interaction with metal ions (e.g. Ca^{2+}, Mg^{2+}, Fe^{2+}, etc) or metabolites (e.g. cAMP, phosphoinositides, and diacylglycerol). Small ligands can directly induce protein activity, as in iron ions binding to myoglobin, diacylglycerol binding to protein kinase C, or a small ligand interacting with one protein that induces complex formation with other proteins. The reversible binding of ligands, interaction with other macromolecules, and posttranslational modifications are activities for which fluorescent protein biosensors have been designed.

Enzyme activity represents an interesting target for intracellular analysis. Fluorogenic substrates for several enzymes are commercially available that have the potential for widespread use in measuring enzyme activity in living cells (46). Moreover, enzyme-based, fluorescent protein biosensors report the modulation of catalytic activity in living cells (2, 91). Therefore, the activity of an enzyme in a living cell can be mapped using fluorescent substrates and to quantify conformational changes of the same enzyme resulting from the interaction with modulators of activity, such as metabolites or other macromolecules.

Intracellular proteins also interact with a myriad of other macromolecules including other proteins, DNA, RNA, complex carbohydrates, and lipids in the plasma membrane and organelles. Fluorescent protein biosensors can be designed to measure the conformational changes that accompany these interactions, specifically in three general classes of activity-dependent conformational changes: spatial rearrangements of the peptide backbone that alter the distance between specific amino acid residues; activity-dependent changes in the exposure of certain amino acid residues to solvent; and changes in the hydrodynamic radius of a protein (e.g. macromolecular shape changes or macromolecular complex formation). The distinctions between these general classes of protein interactions are not clear-cut, and overlap between them is most likely the rule rather than the exception. For instance, a change in the spatial distance between specific amino acid residues within a protein will most likely be accompanied by a change in protein shape or exposure of a previously hidden region of the protein to solvent, or both. Therefore, one must carefully interpret the signals derived from fluores-

cent protein biosensors because these reagents can report a combination of environmental changes, especially in the milieu of living cytoplasm.

Fluorescence Reagents and Spectroscopic Methodologies

Just as we generally classified protein activity–dependent conformational changes, we can also loosely group fluorescent reagents and spectroscopic methodologies used to construct and observe fluorescent protein biosensors in living cells. Three types of fluorescence spectroscopy can be used in conjunction with fluorescence ratio imaging microscopy, a major tool in quantifying fluorescence (1, 14, 40). The following ratio imaging methods are independent of cytoplasmic path length, accessible volume, and local concentration: (*a*) Fluorescence resonance energy transfer (FRET) and fluorescence quenching techniques are sensitive to intra- and intermolecular distances and can therefore sense relatively small changes in protein conformation; (*b*) solvent-sensitive fluorescent reagents can detect intracellular changes in protein-ligand binding; and (*c*) fluorescence anisotropy imaging microscopy relies on the modulation of fluorescence polarization to report macromolecular complex formation or alterations in macromolecular shape.

MECHANISMS OF ACTION AND APPLICATIONS OF FLUORESCENT PROTEIN BIOSENSORS

Solvent Sensitivity

BACKGROUND Molecules that surround fluorophores in solution can exert powerful and useful effects on fluorescence intensities, excitation and emission spectra, and lifetimes. Besides interacting with solvent, dye molecules covalently attached to proteins interact with many other molecules in their immediate environment, including protein amino acid side chains, lipids, nucleic acids, carbohydrates, metabolites, and ions. The diverse mechanisms underlying solvent effects can be divided into general and specific solvent effects (66, 73). General solvent effects result from the interaction of the dipole moment of the dye with the electromagnetic field produced by surrounding solvent molecules, whereas specific solvent effects constitute a diverse class of distinct chemical interactions between a dye and one or more surrounding solvent molecules. Solvent sensitivity manifests itself as an alteration of fluorescence wavelength, intensity (67), or both, changes that are readily detectable in living cells with fluorescence ratio imaging (14).

The sensitivity of fluorescence to its environment has been used to

great advantage in the molecular dissection of biological processes. Solvent-sensitive fluorophores have been harnessed in vitro to study protein structure and ligand binding, membrane structure and dynamics, changes in free ion concentration, and many other biological interactions (66). Solvent-sensitive dyes were originally used to probe the hydrophobic microenvironments of macromolecules (106, 134). These free dyes were also used to measure the equilibria of macromolecular interactions (79) but have since been augmented with protein-reactive solvent-sensitive fluorescent dyes (45). Covalently modified proteins have, for example, been used to measure the binding of Ca^{2+} to calmodulin (44, 80, 137) and parvalbumin (92), the binding of calmodulin to target proteins (86), and the assembly of actin subunits into microfilaments (26, 62). Fluorescently labeled proteins with the ability to provide information about the environment surrounding the fluorophore in dilute solution are the predecessors of the present generation of solvent-sensitive fluorescent protein biosensors.

KEY EXAMPLES OF BIOSENSORS The use of solvent-sensitive fluorophores has recently been extended to the design of protein-based biosensors for studying biochemistry in vitro and in individual, living cells. For example, Hahn et al (44) developed merocyanine fluorophores with long-wavelength, solvent-sensitive fluorescence characteristics specifically for use in live cells. One such fluorophore, modified with side chains that cause it to bind specifically to the calcium-bound conformation of calmodulin, is covalently attached to calmodulin. When calcium binds to the derivatized protein, the dye moves from an aqueous to a hydrophobic environment, with consequent changes in fluorescence (44). This calmodulin analogue is used as an indicator of calmodulin activation in individual living cells, revealing the kinetics and spatial distribution of calmodulin activity during serum stimulation and wound healing (42).

The strengths of biochemistry and molecular biology must sometimes be combined to produce a solvent-sensitive fluorescent protein biosensor. Specific labeling of Cys108 of the regulatory light chain of myosin II with solvent-sensitive dyes was not successful in producing a fluorescent protein biosensor of myosin II phosphorylation (91). Therefore, site-directed mutagenesis was used to introduce a single cysteine-labeling site next to a phosphorylation site on the protein. Myosin light chain kinase activity caused a measurable change in the fluorescence spectrum of labeled myosin II in dilute solution and in isolated muscle fibrils (91).

A new fluorescent protein biosensor for the measurement of submicromolar inorganic phosphate anions in solution represents a new class of ion indicator that has potential for use in living cells. Brune et al (16) developed a genetically engineered phosphate-binding protein to measure the kinetic release of inorganic phosphate from enzymes such as phosphatases. The protein-bound coumarin-based dye exhibits a 5.2-fold increase in fluorescence with a 10-nm peak-wavelength shift in the presence of saturating inorganic phosphate. The authors used the probe to measure the rate of inorganic phosphate release from solutions of actomyosin subfragment 1 during ATPase activity. The design of this in vitro fluorescent probe is echoed in the solvent sensitivity–based fluorescent protein biosensors described below.

Kleinfeld and coworkers used a similar approach to produce fluorescent indicators for the concentration of specific fatty acids. An intestinal fatty acid–binding protein, I-FABP, was labeled with the solvent-sensitive dye acrylodan (94). Fluorescent changes in this indicator are used to monitor concentrations of the fatty acid during activation of living cytotoxic T lymphocytes (4).

CAVEATS OFTHE PRESENT METHODOLOGY Solvent-sensitive fluorescent dyes have found widespread use in the analysis of protein chemistry in dilute solution studies. To study actin assembly in vitro, Kouyama & Mihashi (62) used covalently bound pyrene to measure the assembly of globular actin molecules into actin microfilaments, while Detmers et al (26) produced a similar reagent by reacting 7-chloro-4-nitrobenzeno-2-oxa-1,3 diazole (NBD)–Cl with filamentous actin. Pyrene-actin has since become the reagent of choice for dissecting the assembly and disassembly of actin in vitro because of its large change in fluorescence intensity upon polymerization (22). The limitations of pyrene-actin and other protein-based fluorescent reagents as fluorescent protein biosensors are twofold: the lack of an actin assembly–specific spectral change and the incompatibility of the fluorophore with live cell studies. As originally reported, globular pyrene-actin exhibits a 25-fold increase in fluorescence intensity upon assembly into filamentous actin (62). In the same report, the authors found that the binding of subfragment 1 of myosin II to filamentous pyrene-actin reduced the fluorescence intensity to a level near that of globular pyrene-actin. The fluorescence signal emanating from the pyrene-actin–subfragment 1 complex therefore appeared to be globular actin–like, although actin filaments still predominated. Furthermore, the site of actin modification by pyrene, Cys374, resides in a region of the molecule where several actin-binding proteins

interact (56). This modification may either prevent other proteins from interacting with actin (38, 81) or cause unknown spectral changes in the fluorescence spectrum of pyrene-actin if it were to be used in a living cytoplasm.

The photophysical characteristics of the most popular solvent-sensitive fluorophores used to describe dilute-solution protein chemistry preclude their use in living cells (see "Construction of Fluorescent Protein Biosensors," below), whereas the commercially available fluorophores most compatible with live cells show little solvent sensitivity (45). Overcoming this paradox of practicality will require new strategies in both protein and fluorophore engineering (see "Future," below). Genetic engineering of proteins to direct fluorophore labeling and the development of fluorescence reagents appropriate for use in living cells could make newer versions of solvent-sensitive fluorescent protein biosensors more sensitive and specific (21, 28, 91).

Fluorescence Quenching

BACKGROUND Several processes fall under the general heading of quenching, including energy transfer (see "FRET," below), complex formation, collisional quenching, and excited-state reactions (11, 93, 133). These processes are usually divided into viscosity-dependent dynamic quenching (11) and viscosity-independent static quenching (93). Dynamic quenching results from the interaction of fluorophores in the excited state and affects only the emission spectrum of the fluorophore, whereas static quenching results from the interaction of a fluorophore in the ground state with other molecules and often results in alterations in the absorption spectrum.

The exposure to solvent of small molecules bound to proteins has been assayed using fluorescence quenching induced by solvent perturbation (50). The extent of fluorescence quenching is considered an excellent indicator of this exposure, because fluorescence is quenched by a variety of ions and other molecules (71). For example, Vaughan & Weber (124) used 1-pyrenebutyric acid as a probe attached to proteins together with fluorescence lifetime measurements to determine the quenching by oxygen. The quenching efficiency was taken as a measure of the accessibility of the 1-pyrenebutyric acid probe to O_2. The analysis showed that hydrophobic ligands are buried within the structure of bovine serum albumin. These results suggest that 1-pyrenebutyric acid could be used to measure oxygen concentration under physiological conditions. Based on this work, a fluorescence quenching technique was developed to determine the local oxygen concentration in living liver cells (58). Benson et al (7) extended this approach using video

microscopy and measured the Stern-Volmer quenching constants at different locations within liver cells. The quenching constants were calculated for each pixel by taking the ratio of the fluorescence intensity under nitrogen to the fluorescence intensity for all other pO_2 levels. The spatial variation in the quenching constants was interpreted as variations in the solubility coefficient of oxygen in different locations within the liver cells.

KEY EXAMPLES OF BIOSENSORS Chemiluminescence quenching, which is mechanistically related to fluorescence quenching, has been used to measure protein phosphorylation in living cells. Sala-Newby & Campbell (98) used genetic engineering to develop a luciferase-based reagent to measure protein-kinase activity in a population of living cells. Because quenching of luciferase chemiluminescence is mediated by phosphorylation of the reagent by protein kinase, it formed the basis of the assay.

A quenching-based fluorescent peptide biosensor for calpain protease activity in live hepatocytes was recently described (97). Proteolysis of the intracellularly trapped peptide results in the release of a deprotonated and therefore unquenched coumarin, which is then quantified with video fluorescence microscopy. Calpain protease activity increases concomitantly with intracellular free calcium ion concentration, an observation consistent with the proposed role this protease plays in calcium-mediated signal transduction.

Fluorescence resonance energy transfer (FRET) is a valuable tool for creating quench-based biosensors because of its ability to measure the quenching of the donor fluorescence and the appearance of sensitized fluorescence from the acceptor probe (see "FRET," below). The sensitized fluorescence permits a donor-to-acceptor fluorescence ratio that normalizes the cell signal for variable path length, accessible volume, and local concentration. Alternatively, fluorescence lifetime imaging can be used to map the changes in fluorescence lifetime that accompany fluorescence quenching (68, 123). Because this latter technique provides a spatial map of the lifetime of a particular fluorophore, it does not require that the fluorescent protein biosensor exhibit activity-dependent shifts in light absorption or emission. This technology is also compatible with many presently available intracellular fluorescent probes and promises to spur the development of new fluorescence reagents (68).

The following in vitro study suggests some interesting ways to design biosensors for use in living cells. Tropomyosin is a coiled coil of two parallel α-helical polypeptide chains that functions as a component of

the calcium regulation of skeletal and cardiac muscle. Tropomyosin was specifically labeled at Cys190 with *N*-(1-pyrene)-maleimide. Two structural states of tropomyosin were identified: an excimer-forming state and a nonexcimer state. The excimer was identified by the appearance of a wavelength band at 480 nm, which was long compared with the structured fluorescence emission bands at 383 nm and 403 nm. Steric considerations indicated that chain separation of tropomyosin was required for the pyrenes to form an excimer (41). Conformational changes or assembly-disassembly dynamics of various protein subunits could be studied using reversible excimer formation measured either by a ratio of excimer emission to fluorescence emission or by fluorescence-lifetime imaging.

CAVEATS OF THE PRESENT METHODOLOGY The major limitation to the use of quenching-based fluorescent protein biosensors is the specificity of intracellular quenching. Predicting nonspecific fluorescence quenching in the complex compartments of cytoplasm is problematic because several molecules may participate unpredictably. As described above, oxygen is an efficient quencher, yet its removal may lead to abnormal physiological conditions that would be difficult to predict or quantify. The ability of a constitutive intracellular molecule, pyruvate, to quench fluorescence is predictable enough to form the basis of an in vitro assay for pyruvate reactions (3). Consequently, simple fluorescence-quenching measurements in living cells are difficult to interpret. The release of intracellular quenching may be no easier to decipher. The coumarin-fluorophore–based reagent described above that induces fluorescence as a result of calpain action is also subject to endogenous molecules that may requench its fluorescence. Moreover, coumarin fluorescence is pH sensitive, so a change in intracellular pH concomitant with calpain activation would bias the results. By coupling fluorescence quenching to energy transfer to another fluorophore, or to the formation of eximers, one could take advantage of the benefits of fluorescence ratio imaging microscopy, thus enhancing the specificity of the method. Furthermore, development of new dyes whose fluorescence is chemically protected from oxygen quenching will also improve the specificity of the next generation of quench-based fluorescent protein biosensors.

Fluorescence Resonance Energy Transfer (FRET)

BACKGROUND Protein activities that involve either significant rearrangement of intramolecular domains or reversible association with a ligand can potentially be sensed in living cells with FRET methodology. The theory of FRET was first described by Förster in 1948 and has

been experimentally verified (33, 70, 108, 109). Upon excitation of a donor fluorophore, absorbed energy is transferred in a nonradiative manner to a proximal acceptor fluorophore. Depending on several parameters, this transfer may quench the donor fluorescence and enhance the acceptor fluorescence (30, 48, 66, 136). Most importantly for biological systems, the rate of transfer varies as the inverse sixth power of the distance between donor and acceptor, making FRET extremely sensitive to changes in molecular distances over the range of approximately 1–7 nm. Advances in digital image acquisition and image processing have allowed steady-state and lifetime-dependent (48, 55, 68) FRET measurements to be made with the light microscope. Using this type of microscopy, decreases in the fluorescence intensity and lifetime of the donor caused by specific quenching by the acceptor are temporally and spatially mapped in living cells.

Marsh & Lowey (82) also used FRET to define the interaction between specific sites on the light and heavy chains of the catalytic head region of skeletal muscle myosin II. Huang et al (51) labeled pairs of 30S ribosomal proteins with naphthalene-based and fluorescein dyes and reconstituted 30S particles in vitro. The proximity of 20 ribosomal proteins in the particles was measured and found to agree with previous chemical cross-linking results. Thus, FRET methodology can potentially be used to measure association reactions such as those required for cytoskeletal assembly within living cells (116).

KEY EXAMPLES OF BIOSENSORS FRET-based fluorescent protein biosensors have been used in living cells to measure protein dynamics at the plasma membrane (49), within intracellular compartments (27, 122), and in the cytoplasm during signal transduction (2). To study lectin receptor topography and mobility in single cells during myoblast fusion, Herman & Fernandez (49) incubated chick muscle cells with a mixture of concanavalin A molecules labeled with pyrene (donor) and fluorescein (acceptor). The authors observed a dramatic reorganization of concanavalin A receptors during myogenesis, suggesting a temporal correlation between changes in membrane fluidity and the onset of myoblast fusion. Young et al (138) coupled fluorescence photobleaching and FRET in living cells to describe the differential membrane-binding effects of concanavalin A and succinyl concanavalin A. Uster & Pagano (122) also used a fluorescently labeled lectin as a fluorescence donor but employed a fluorescent analogue of phosphatidylcholine as an acceptor to measure lectin and lipid sorting in living fibroblasts. Those endocytic cellular compartments containing both lectin and lipid exhibited FRET, whereas those compartments subject to sorting contained

only acceptor fluorescent lipids. Adams et al (2) labeled the catalytic and regulatory subunits of cAMP-dependent kinase with an energy-transfer pair that allowed FRET to occur in the holoenzyme complex. However, upon binding of cAMP to the regulatory subunit, the holoenzyme dissociates and FRET is lost as the enzyme becomes active. This fluorescent protein biosensor (1) has been used in several types of living cells to measure changes in intracellular cAMP concentration with high temporal and spatial resolution (2, 100).

Post et al have designed a biosensor of myosin II regulatory light chain phosphorylation that employs energy transfer between fluorescein-labeled regulatory light chains and rhodamine-labeled myosin heavy and essential light chains (PL Post, RL DeBiasio & DL Taylor, in preparation). Phosphorylation of the regulatory light chain produces a change in the energy transfer efficiency. The biosensor has been used in living cells under different conditions to map the spatial distribution of myosin II. An advantage of this method is that both dyes of the energy-transfer pair are on one protein, which eliminates problems of diffusion of donor and acceptor upon mixing with endogenous unlabeled proteins.

CAVEATS OF THE PRESENT METHODOLOGY Several requirements restrict the design of FRET-based fluorescent protein biosensors for use in vivo. First, donor and acceptor molecules must have sufficient spectral overlap and be optimally oriented within 1–7 nm of each other. Specific protein labeling with two different reactive fluorophores to satisfy these conditions is problematic. Donor and acceptor macromolecules are usually labeled separately and recombined before use in living cells (1, 122). Generally applicable methods for labeling specific amino acid residues will facilitate the future construction of FRET-based fluorescent protein biosensors (see "Construction of Fluorescent Biosensors," below).

Second, dilution with endogenous unlabeled proteins is theoretically a major obstacle to the construction of multisubunit FRET-based fluorescent protein biosensors (115). Once separated, acceptor and donor protein subunits are not likely to interact with each other again in a cytoplasm crowded with endogenous unlabeled protein subunits. However, Adams et al (2) found that their FRET-based optical probe for cAMP is reversible in living cells and suggest that exogenous levels of microinjected probe are sufficiently greater than those of endogenous kinase to statistically favor recombination of labeled subunits. Furthermore, Adams et al (1) discuss the possibility that this excess of exogenous holoenzyme may also perturb the cAMP-response pathway. To

avoid the delivery of supraphysiological doses of FRET-based optical probes, Adams et al (1) suggest a chemically tethered version of the biosensor to facilitate recombination of the labeled subunits in living cells. In contrast, the observed reversibility of the multisubunit fluorescent protein biosensor might also be attributed to the structure of the surrounding cytoplasm.

Fluorescence Anisotropy

BACKGROUND Encoded within the rotational Brownian motion of intracellular proteins is information about their size and shape, interactions with other cellular components, and the viscosity of the surrounding cytoplasm (130). Measurement of this rotational diffusion in living cells would yield temporal and spatial maps of the interaction of proteins with ions, metabolites, and other macromolecular structures that would affect the rotational diffusion of the protein. The rotational diffusion coefficient of a protein in solution is directly related to temperature but inversely related to both the first power of the viscosity of the surrounding medium and the cube of the hydrodynamic radius of the protein (40). Inversely related to the rotational diffusion coefficient of a protein is its rotational correlation time, usually in the range of nanoseconds to tens of nanoseconds (66). If a dye with a fluorescence lifetime on the order of the rotational correlation time of a protein is immobilized on that protein, then significant randomization of its emission dipole occurs during the excited state. This randomization is measured by exciting the dye with plane-polarized light and measuring the fluorescence emission through a variable-angle polarizer. Altering the steady-state polarization of a labeled protein therefore provides a sensitive measure of changes in its hydrodynamic radius; these changes may result from protein peptide chain rearrangements or the interaction of the protein with other cellular components.

Fluorescence polarization has emerged as a primary technique for the measurement of the rotational diffusion of biomolecules in dilute solution, and as such, has been extensively reviewed (9, 17, 65, 103, 130). The technique has been used to measure the rotational diffusion of proteins for more than 40 years (129). Although the aromatic amino acids intrinsic to the proteins can be used as fluorescence polarization probes (131), extrinsic fluorophores are the most useful because they can be selected to have fluorescence lifetimes that closely match the rotational correlation times of proteins (20, 129). For example, fluorescence polarization of extrinsically labeled proteins has been used to measure protein molar volumes and their interaction with small and macromolecular ligands (66).

KEY EXAMPLES OF BIOSENSORS Recent efforts have applied fluorescence polarization methods to the measurement of specific components or processes of living cells. For example, organic molecules have been used to probe the structure of cytoplasm (57) and membrane dynamics (9) while small proteins have been used to describe cellular signaling pathways (19, 40).

Fluorescence anisotropy imaging microscopy (FAIM), which is related linearly to solution-based fluorescence polarization techniques, is a simple yet powerful mode of light microscopy that is easily added to existing light microscope–based imaging systems (40). For example, FAIM was performed on fluorescein-labeled calmodulin (M_r 16,790) that was microinjected into migrating and growth factor–stimulated fibroblasts (40). FAIM produced temporal and spatial maps showing gradients of calmodulin activation that correlate well with the time course of the elevation and decline of free–calcium ion concentration and with the previously known dynamics of the actin-cytoskeleton in these cells (36, 42). In another example, temporal anisotropy measurements were made of the interaction of fluorescein-labeled epidermal growth factor (M_r ~6000) with its cellular receptor on living cells (19). Upon binding of the growth factor to its receptor on the cell, a rapid quenching of growth factor fluorescence was detected and attributed to changes in its rotational diffusion. The rapid fluorescence decay during growth factor stimulation was used to determine the rate constants for the interaction of the fluorescent growth factor with its receptor on the cell surface.

CAVEATS OF THE PRESENT METHODOLOGY The limitations of FAIM parallel those of solution-based fluorescence polarization methods and include interferences from light scattering, fluorophore photobleaching, and fluorescence-energy transfer. Methods for dealing with many of these limitations have been described (9, 40), but the photophysics and photochemistry of commonly used protein-reactive fluorophores have thus far hindered full application of FAIM. For fluorescence-polarization techniques to indicate changes in the hydrodynamic radius of proteins or protein complexes, the fluorescence lifetime of the dye used to label the protein of interest must be of the same order as the protein's expected rotational correlation time (40). Prevalent protein-reactive fluorophores able to measure changes in the rotational correlation time changes of medium to large proteins ($M_r > 20,000$) have molar extinction coefficients and excitation wavelengths that are unfavorable for live cell imaging (40). For example, solutions of globular actin labeled with naphthalene-based dyes that have fluorescence lifetimes of about

10 ns show little change in fluorescence anisotropy when induced to undergo self-assembly to a highly structured filamentous state (77, 121, 130). Nevertheless, actin labeled with the triplet probe erythrosin-5-iodoacetamide, which has a phosphorescence lifetime of >100 μs, shows a significant increase in delayed luminescence anisotropy upon polymerization to filamentous actin (77). Unfortunately, experiments using erythrosin-labeled actin are performed under nonphysiological anoxic conditions in vitro to prevent quenching of the phosphorescence, thus limiting the usefulness of delayed luminescence anisotropy in living cells. Clearly, fluorescent and phosphorescent dyes with physical characteristics more amenable to FAIM need to be explored to extend the applicability of the method to a wider range of proteins.

Future enhancements in the design of the instrumentation and fluorescent protein biosensors used for fluorescence anisotropy microscopy will make the technique more flexible and sensitive. Time-resolved or phase-modulation measurements of anisotropy yield more information about the degree of fluorophore rotation and the environment surrounding the fluorophore than steady-state polarization experiments (66). Time-resolved microscopic instrumentation has already been developed to explore intracellular chemistry (32, 69, 123). The recent development of fluorescence lifetime-resolved two-photon microscopy could be a major breakthrough in time-lapse measurements of living systems (123).

CONSTRUCTION OF FLUORESCENT PROTEIN BIOSENSORS

The central challenge in production of fluorescent protein biosensors is site-selective attachment of dyes to proteins. To date, most protein analogues have been made with environmentally insensitive dyes to simplify quantification of dye distribution within cells. Production of such adducts requires only that the dye be attached at a position where biological activity would be minimally perturbed. In the most widely used method, semirandom labeling, the attachment site depends on the relative reactivities of native amino acid side chains and on the reactive group of the dye.

Although many valuable protein adducts have been produced by means of semirandom labeling, this method largely fails in the generation of more sophisticated analogues that have environmentally sensitive dyes precisely placed to respond to protein activity. A few such probes have been produced using more specific methods, as described below. The future of probe development lies in presently available

methods for labeling specific protein amino acid residues, including genetic engineering (91), enzyme-mediated protein labeling (110), and C-terminus labeling (96); in promising new technologies that enable incorporation of nonnative residues, including fluorescently labeled amino acids, during protein synthesis (28); and in the preparation of chimera proteins using the green fluorescent protein or its derviatives as a molecularly targeted fluorophore (21).

To date, all methods use reactive groups on dyes that produce some selectivity for specific amino acid side chains. Dyes with a wide range of reactive groups are now commercially available. Their chemistry and the selection of reaction conditions to maximize selectivity for particular amino acids have been extensively reviewed (15, 45). Although many amino acid side chains can be derivatized or turned into useful labeling sites through chemical or enzymatic modification (47, 78), dyes are usually attached to cysteine or lysine because the nucleophilic side chains of these amino acids are most readily labeled in the presence of other residues. Selectivity for cysteine vs lysine is usually influenced by the pH of the reaction mixture, as cysteine remains in a reactive, deprotonated form at a pH where protonated lysine residues are unreactive. Selection of one lysine over another can be effected by manipulating pH because local protein environments strongly influence the pK_a of individual residues. Finally, ligands that alter protein conformation can be used to alter the relative reactivity of different residues.

When a dye is attached near a ligand-binding site (i.e. to report ligand binding), an affinity-labeling approach can be used to direct the dye to the desired site. Hahn et al (44) produced an indicator of calmodulin conformational change by attaching a dye to the protein where it would bind a hydrophobic pocket found only in the protein's calcium-bound form. A dye was synthesized containing side chains conferring affinity for the pocket. This binding affinity was used to direct the reactive dye to the desired site. A slowly reacting group was used on the dye, so that the dye bound first to the pocket and then covalently attached to a nearby residue. The dye has a strong affinity for the pocket, and its solvent-sensitive fluorescence is affected whenever it moves into the pocket during calcium binding.

Jackson & Puett (53) used a different affinity-labeling tactic to attach a spin label near the active site on calmodulin. This approach may be used to place reporter dyes where they will interact with bound ligands without blocking their binding sites. An inhibitor of calmodulin phosphodiesterase activation was attached to a spin label through a cleavable bridge. The reactive residue for covalent attachment was on the spin label. During labeling, the ligand sat in the binding pocket, directing the reactive group and spin label to a nearby site. The labeled cal-

modulin could not activate phosphodiesterase until cleavage of the inhibitor. Cleavage led to an active calmodulin with a spin label covalently attached near the active site.

Affinity labeling has also been used to attach a reactive residue at a desired site for later selective derivatization with dye. Bock (12, 13) synthesized a reactive derivative of a peptide that bound to the active site of thrombin. The peptide contained a blocked sulfhydryl function that was released after covalent attachment of the peptide. The sulfhydryl group was then specifically labeled with fluorescent dyes.

An interesting hybrid of intrinsic and extrinsic labeling emerged recently that uses the tools of genetic engineering to increase the power of fluorescence. This method could allow the insertion of a sequence of the *Aequorea victoria* green fluorescent protein and related proteins into target proteins, thus creating a genetically engineered, fluorescent, chimeric protein (21). Another possible technique would be to use a small portion of the the green fluorescent protein sequence containing the hexapeptide chromophore to label specific domains of proteins.

New technologies offer the promise that dyes can be incorporated during in vitro translation and chemical synthesis of proteins, thus overcoming the need to selectively derivatize a protein site. tRNAs can now be charged with unnatural amino acids and incorporated at precise positions in a protein during in vitro translation (6, 28). The many unnatural amino acids introduced by this method include a residue labeled with the fluorescent dye NBD (28). To produce novel protein probes for microinjection in cells, these methods still require optimization. They suffer primarily from the need for extensive synthesis of reagents and from the small scales on which proteins can be produced. New methods for linking synthetic peptides offer a promising alternative to labeling intact proteins. Large-scale synthesis of proteins using automated peptide synthesis has already been reported (95). Adaptation of this chemistry to accommodate peptides bearing dyes should be possible. The coupling of our ability to routinely engineer dyes into specific positions within a protein using biochemical, genetic, and synthetic methods with our increasing knowledge of protein structure should lead to production of many new analogues probing a wide range of protein functions.

DESIGN CONSIDERATIONS OF FLUORESCENT PROTEIN BIOSENSORS UNIQUE TO IN VIVO USES

Although issues relating to the site-specific labeling of proteins dominate the design of fluorescent protein biosensors (see previous section),

the use of these reagents in the unique environment of living cells bears some consideration. Fluorophores with properties appropriate for a particular spectroscopic methodology must also exhibit properties favorable for use in live cells (63, 83, 102, 112, 118).

Fluorophore Considerations

For reviews of the fluorophore properties needed for live-cell studies, see Waggoner (126) and Simon & Taylor (102). Here, we summarize the key points:

BRIGHTNESS Fluorophores should retain a high extinction coefficient and high quantum yields when conjugated to a protein to maximize intracellular detection. Unfortunately, many of the commercially available, environmentally sensitive fluorescent dyes, such as those based on naphthalene or pyrene, have fairly low extinction coefficients when compared with the environmentally insensitive xanthenes (45). Nevertheless, new classes of environmentally sensitive fluorescent dyes have been synthesized to construct solvent-sensitive fluorescent protein biosensors (44).

WAVELENGTH OF EXCITATION Optimally, dyes for live cell investigations should absorb light at $\lambda > 500$ nm. Excitation of fluorophores at $\lambda < 500$ nm produces cellular autofluorescence (5, 8). Ultraviolet and near-ultraviolet radiation cause cellular photodamage from the byproducts of irradiation of media components (104) as well as induce fluorescence from endogenous components in the cells and from optical components of the microscope.

PHOTOSTABILITY If a fluorescent protein biosensor is to undergo repeated excitation during a live-cell experiment, then the photostability of the fluorescent protein biosensor becomes one of the most important experimental parameters. Excitation of many dyes in the presence of oxygen may irreversibly photobleach them (102), lead to oxygen-mediated quenching (see "Fluorescence Quenching," above), or generate toxic photoproducts. To help minimize these events, free oxygen can be removed, but the photophysical and physiological effects of such perturbations must also be assessed. Clearly, new approaches are necessary to engineer bright, photostable, and nonphototoxic fluorophores. In addition, new approaches to excitation, such as two-photon excitation, show great promise (25).

SPECIFICITY OF FLUORESCENCE CHANGE The specificity of a biosensor's spectroscopic change must be well characterized before in vivo data

can be interpreted. Concurrent environmental changes may cause spectroscopic changes in the fluorophore (see "Solvent Sensitivity," above). In addition, interactions of the dye with aromatic amino acids present in the cell, other dye molecules, or molecular oxygen can cause unexpected quenching of the fluorophore (see "Fluorescence Quenching," above). Experimental results can be misleading unless these effects are identified first with dilute solution studies.

Protein Considerations

A main consideration in the production of most fluorescent protein biosensors using traditional labeling schemes is the purification of enough protein to synthesize the reagent. Because protein purification from the experimental cells or tissue of interest can be difficult, a protein is often purified from a closely related species or a recombinant expression system.

The protein of interest is usually introduced into living cells and delivered to its proper subcellular location. It must be soluble in a physiological buffer so that it can be either microinjected or bulk loaded into cells (85). Taylor et al (113) have used immunoelectron microscopy to demonstrate molecular incorporation of microinjected fluorescein actin into the stress fibers of living cells. Other proteins, such as organellar proteins, may require attachment of an intracellular targeting signal. The use of chimeras produced between target proteins and the green fluorescent protein (21) or its technical derivative should simplify the methodology dramatically.

The complexity of engineering physiologically relevant fluorescent protein biosensors appears to be daunting at first. Nevertheless, the experience gained in refining fluorophores, genetically manipulating proteins, and performing light microscope–based fluorescence spectroscopy will help focus resources toward developing a straightforward and generally applicable methodology for fluorescent protein biosensor design and use.

FUTURE

Fluorescent analogues of proteins should have an important impact on defining the chemical and molecular dynamics responsible for cell and tissue functions. Advances are expected in four areas: (*a*) the use of molecular biological methods to fluorescently label proteins site specifically; (*b*) the development of new classes of extrinsic luminescent probes designed for wider ranges of excitation and emission spectra, fluorescence lifetimes, and two-photon excitation; (*c*) the development of methods to encapsulate fluorescent probes to minimize photobleach-

ing; and (*d*) the evolution of user-friendly, yet powerful instrumentation to measure temporal-spatial dynamics in living cells and tissues.

The use of the green fluorescent protein and derivative fluorescent peptides should make the labeling of proteins with a fluorescent marker as simple as making any protein chimera (21). The potential revolutionary impact of using molecular biology to label proteins will be realized if a minimum-length peptide can be incorporated into target proteins in one or more sites. In fact, the green fluorescent protein fluorophore could be used as the probe for fluorescent protein biosensors if the peptide sequence can be incorporated into target proteins near binding and active sites. Similarly, the incorporation of fluorescent analogues of amino acids into specific sites during protein synthesis could create a new class of exciting fluorescent protein biosensors (6, 28; see also 85a, this volume).

The new labeling tools based on molecular biology will complement extrinsic dye options rather than make them obsolete. As these tools simplify labeling, we expect more investigators to prepare fluorescent analogues and fluorescent protein biosensors. However, new extrinsic dyes will be needed to span the full range of the useful spectrum (~360–1000 nm), to create luminescent probes with a wide range of lifetimes for use in measuring the anisotropy of proteins, and to optimize two-photon excitation (25).

The photostability of the luminescent probes will continue to be a major challenge. The real value of this technology is based on the ability to measure changes in cells and tissues during life functions. The present fluorescent probes are at least a factor of 10 less photostable than required for many experiments over time. Therefore, creative methods for protecting the fluorophores from destruction will be needed. Finally, the instrumentation will need to continue to evolve toward increased power and user-friendliness, but decreased cost and complexity.

Although we view fluorescent protein biosensors as some of the most potentially powerful tools for dissecting cellular interrelationships at the molecular level, they are clearly intermediates in the evolution of modern biological discovery. Lewis & Lewis (72), using transmitted light microscopy to observe some of the first cultured mammalian cells, deduced some of the intracellular relationships that we can now molecularly dissect with fluorescent protein biosensors. We are witnessing the development of completely synthetic fluorescent probes for a wide range of physiological parameters, an expansion in the number and sophistication of fluorescent protein biosensors, and molecular techniques to prepare fluorescent analogues of proteins. By engineering

endogenous components that constitutively localize and function as reporters of their own activities, we will move beyond the single cell and be able to measure and manipulate molecular dynamics in living tissues and whole organisms.

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