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Reagents to Measure and Manipulate Cell Functions

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Summary

Reagents that are used as part of a discovery platform for the measurement and manipulation of cell functions are at the heart of single and multiplexed high content screening assays. Measurement reagents include physiological indicators, immunoreagents, fluorescent analogs of macromolecules, positional biosensors, and fluorescent protein biosensors. Recent developments in reagents that manipulate specific cell functions including small inhibitory RNAs, caged peptides, proteins, and RNAs, and gene switches complement measurement reagents, especially when both classes of reagents are used in the same living cells. The use measurement and manipulation reagents in multiplexed high content screening assays promises to enable a systems cell biology approach to drug discovery and biomedical research.

Key Words: Biosensors; cell-based assays; compound screening; fluorescent probes; photochemistry; RNAi.

1. Introduction

The cell is the first level of biological organization that exhibits life. A major challenge in the postgenomic era is to fully understand the functional dynamics of living cells. Detailed cellular knowledge is essential for understanding the normal development and function of organisms, the engineering of therapeutics for disease and the creation of novel diagnostics tools. With the human genome mostly in hand and strong activity in identifying and characterizing the proteome, there is a new wave of effort for mapping all the regulatory pathways in a cell, sorting out the mechanisms of regulation, and defining their roles in cell functions. This requires an understanding of the activities of organelles, metabolites, protein–protein interactions, protein modification, protein translocation, conformational changes, lipids, nucleic acids, and carbohydrates, as well as feedback, feedforward mechanisms. Activity is intense in the pharmaceutical industry because these regulatory pathways are obvious targets for drug discovery and a systems biology knowledge base is desired. There are many approaches in use for trying to figure out how cells work. This chapter will focus on the cellular reagent tools that fluorescence detection technologies offer for measurement, as well as some of the technologies that can be used for manipulations.

Fluorescent and luminescent labels have significantly replaced radioactive isotopes for screening of drug candidates and basic biomedical research. Many of these assays still require isolation of cell membranes or intracellular components that can be used to quantify binding of luminescent ligands. In recent years, intact cell-based assays have grown in importance. Although many cell-based assays require simple quantification of the amount or location of labeled macromolecules in cells, many other fluorescent cell-based assays are very sophisticated and require measurement of energy transfer changes, total internal reflectance excitation, confocal,

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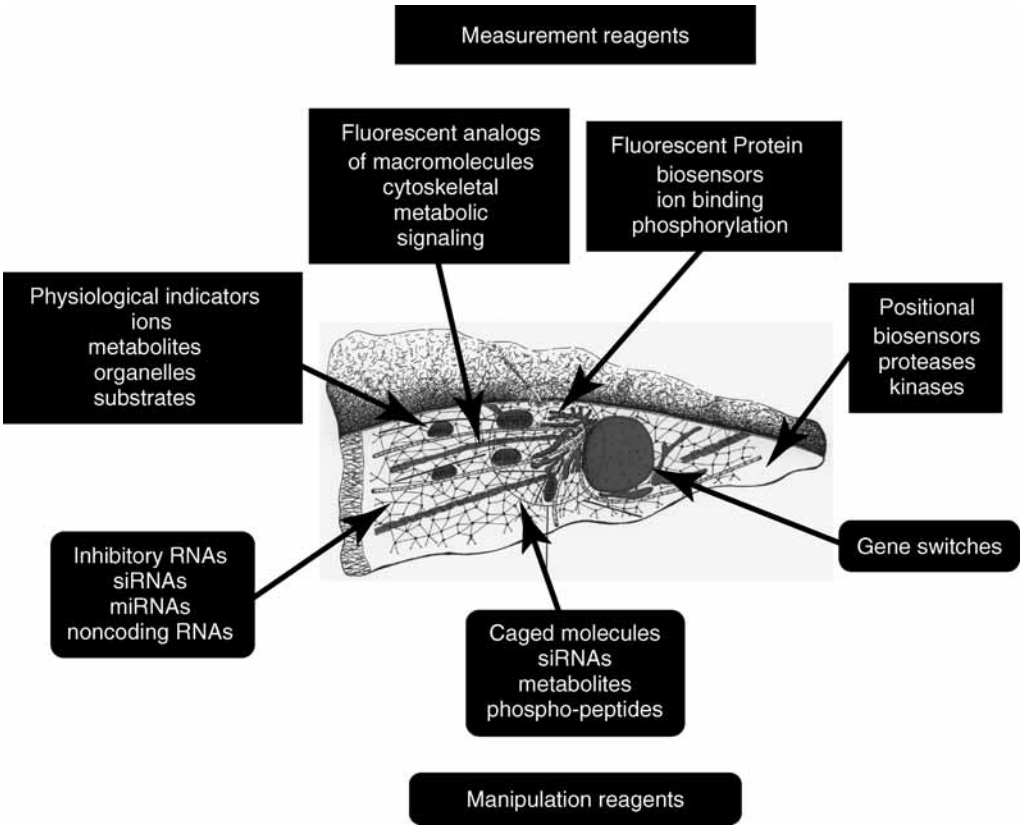

Fig. 1. Reagents to measure and manipulate cell functions. A large and growing repertoire of reagents is being used to measure temporal and spatial processes in living cells that involve ions, metabolites, macromolecules, and organelles. Moreover, new reagents continue to be developed that manipulate specific processes in living cells. A systems cell biology approach combines multiple measurement and manipulation reagents into multiplexed assays that can be used to build new cellular knowledge.

two-photon, or fluorescence correlation spectroscopy. The application of these methods has been widely reviewed *(1–8)*.

Our emphasis here is the evolution and latest developments for assays with fixed and living cells. Reagents that are used to measure and manipulate one or more specific cellular processes in the same living cells have become important tools in a systems cell biology approach to drug discovery and biomedical research (**Fig. 1**). Besides discussing the principles and applications of the fluorescent probes to measure and reagents to manipulate living cells, we will also point out technologies that remain weak and need improvement. This sets the stage for contemplation about what the future might hold.

2. The Evolution of Fluorescent Labels and Probes in Biomedical Research and HCS

More than a half century has passed as the intrinsic fluorescence of proteins and artificially attached fluorescent groups were initially used to measure the conformational dynamics associated with protein activity in vitro; early reviews have covered these accomplishments *(9,10)*. It is from these pioneering studies that a wide range of protein as well as small molecule-based physiological indicator fluorescent reagents evolved to measure cellular processes in living cells. The major developments in fluorescent labels and probes for cell-based assays are briefly

Table 1
Evolution of Reagents for the Measurement of Cell Functions

Year	Fluorescent reagent(s)	Cell function	References
1953	Intrinsic protein fluorescence and artificially attached fluorescent labels	Conformation dynamics associated with protein activity	*9,154,155*
1962	Fluorescently labeled immunoreagents	Antigen localization in tissue sections and tissue culture preparations	*10,19*
1970	Fluorescent membrane analogs	Conformational changes in biological membranes	*156*
1974	Small molecule probes of cellular physiology: fluorescent potential sensitive probes	Changes in cell and organelle membrane potentials	*69,70,157*
1978	FRET-based probes	Protein conformational changes associated with activity	*158–160*
1980	Fluorescent analogs of intracellular proteins	Structural and functional dynamics of the cytoskeleton and other constituents	*56,76,161,162*
1982	Multicolor immunoreagents for flow cytometry	Two-color analysis of lymphocytes with a single color laser	*20*
1982	Fluorescent probes of intracellular ion concentration	Intracelluar free calcium, magnesium, and pH	*163–165*
1987	Five-parameter labeling for live cell kinetic multimode microscopy	Nuclei, mitochondria, endosomes, actin-cytoskeleton, and cell volume	*166*
1989	Introduction of bright water-soluble fluorescent labeling reagents	Multicolor analysis of cellular constituents	*21–24*
1990	Fluorescent protein biosensors	Temporal and spatial measurements of calmodulin activity in living cells	*74,80*
1994	Applications of autofluorescent proteins as tags	Temporal and spatial measurements of endogenously synthesized fluorescent protein analogs in living cells	*102,167,168*
1997	Multiplexed HCS	High content measurements of multiple apoptosis events and kinetic analysis of glucocorticoid receptor translocation after drug treatments	*13*

summarized in **Table 1** and further discussed below. It is essential to understand that fluorescent reagents coevolved with advances in optical detection systems, including microscopy. Semiautomated microscopes with powerful software, multicolor filter sets, and environment controls have been essential for progress *(11,12)*. The development of high content screening (HCS) allowed the application of a high throughput approach to cell biology *(13)*. Reagents and imaging systems are interdependent and progress is optimized by a systems approach to design.

2.1. There are Essentially Three Groups of Fluorescent Reagents

First, covalent labels that allow localization and quantification of biomolecules. This class includes organic dyes with reactive groups. Fluorescein and rhodamine were developed early and became long-time standards, but the cyanine dye labels and then the Alexa dyes brought significant improvements in water solubility, spectral selection, photostability, and brightness. New labels seem to be reported monthly. The fluorescent proteins, such as green fluorescent protein (GFP), and other genetic labeling techniques like FlAsH (*see* **Table 2** and Chapters 14–16)

would also have to be in this category because covalent bonds are formed between the fluorescent species and the labeled protein.

Second, noncovalent labeling reagents include membrane-associating dyes, like diI, and the DNA content probes like DAPI, Hoechst, and the intercalating dyes like propidium idodide. Their specificity depends on a noncovalent but high affinity for the target site. Nevertheless, these have found many applications and some are very widely used in cell biology, drug discovery assays, and diagnostics. Third, the fluorescent indicator dyes are designed to be sensitive to some local environment property such as pH, calcium level, membrane potential, protein conformation, or molecular proximity. These are often much more complicated in their use. They must be delivered to the cytoplasm, membrane, or specific compartment within the cell. Sometimes the indicator dye can also be intentionally localized to a target region of the cell by covalent labeling or by designing a specific noncovalent affinity for the site.

We will elaborate on the uses of these dyes in cell based assays below, but it is worth a brief discussion of the important properties a fluorescent dye must have to have utility in such assays. The chemists who create new fluorescent probes must be intimately aware of following principles and properties in order to synthesize useful probes ***(11)***.

First, fluorescence brightness that results from high absorbancy and high quantum yield. Molar extinction coefficients above 80,000 are characteristic of the best fluorescent labels. There are many mechanisms by which a molecule might have low quantum yield and as a result most light absorbing dyes are not fluorescent. However, most useful labels have quantum yields greater than 0.1 and most are in the 0.3 to 0.8 range (one is the maximum possible). The quantum yields of many longer wavelength fluorophores are substantially lower than those of the best dyes in the visible and near UV region of the spectrum, but during detection this is compensated by reduced background interference at longer wavelengths.

Second, photodegradation (or photobleaching). Currently available fluorophores are all subject to photodegradation. This susceptibility increases at longer absorption wavelengths. Photobleaching is one of the most serious limitations of conventional fluorescent probes in quantitative imaging systems. High efficiency (numerical aperture) collection objectives and high sensitivity cameras in imaging systems are essential so that fluorescence signals can be acquired quickly before bleaching affects quantification. Photobleaching is more pronounced for dyes having longer wavelengths.

Third, chemical stability. On the whole, ultraviolet (UV) and visible dyes (coumarins, xanthenes, and cyanines) are relatively stable to acidic and basic conditions and to a range of redox environments. This simplifies the use of these fluorescent probes under many experimental conditions. These fluorophores can be incorporated by synthetic modification into useful reagents such as amidites, nucleotides, lipid analogs, protein analogs, drug analogs, and so on. However, the chemical stability of longer wavelength emitting fluorophores is lower. Instability of fluorophores in storage, stock solutions in room light or during sample handling can limit the utility and must be controlled.

Fourth, phototoxicity. Dye-sensitized phototoxicity to cells and tissues remains a significant problem in some assays, in which cells are under continuous illumination for more than a few seconds. For example, when forming image stacks either by stepping through a specimen followed by deconvolution or by confocal methods (single or multiphoton), or excessive illumination in live cell HCS studies, toxicity is frequently observed as indicated by the inability of cells to initiate or complete mitosis, membrane blebbing, and nuclear degradation. Production of singlet oxygen and its products is the main cause of phototoxicity. Care must be exercised in using the minimal dose of irradiation to generate the data.

Fifth, nonspecific binding. The more hydrophobic fluorophores are notorious for nonspecifically sticking to plastics and nonspecific cellular structures. Much progress has been made with the cyanine and Alexa dyes that contain sulfonic acid groups on the rings that reduce this complication.

Sixth, perturbation of the reaction to be analyzed. Attachment of an organic dye to a ligand, protein or nucleic acid adds a molecular weight of 500–1000 and bulk 0.5–1.2 nm long. A fluorescent protein has a molecular weight of 70–100 times the mass of an organic label that could produce significant steric interference with function and target binding. In either case, it has been emphasized that functional analysis of the labeled protein or other analog of cellular constituents must be defined before attempting physiological interpretations of results ***(14–17)***. This has not been done as a rule, especially with the advent of rapidly produced analogs of proteins using fluorescent protein gene-target protein gene fusions. Related problems can also occur when fluorescent probes are used at high concentrations, in which they can impact the function of the organelles or constituents that they attach to in the cell. Fluorescent probes must be used at minimally perturbing concentrations. This might preclude the use of some probes in specific cells. With knowledge of these probe requirements and limitations we can move to a discussion of application of the fluorophores for labeling and use as physiological indicators.

3. Fluorescent Reagents for Fixed End Point HCS

3.1. Fluorescent Immunoreagents Have Become Important Tools for Fixed End Point HCS

Sixty-five years ago, an amino-reactive anthracene derivative was used to prepare the original antibody conjugate ***(18)***. These authors found that β-anthryl-carbamido derivative of antipneumococcus III antibody retained its original immunological properties although labeling type-III pneumococci specifically fluorescent in UV light. As these early studies, fluorescein and rhodamine, whose fluorescence spectra were removed from the UV region, in which fixed human tissues exhibit substantial autofluorescence, became the most widely used labeling reagents to create the fluorescent antibodies that allowed microscope visualization of macromolecules in fixed cells ***(10,19)***. In the early 1980s, the advent of monoclonal antibody technology led to the appearance of a wide range of monospecific antibodies and with them interest in developing new colors of fluorescent labels for multicolor detection (originally by flow cytometry) and localization (with microscopes) of different proteins in the same sample. The development of phycobiliprotein reagents in 1982 allowed for the first two-color analysis of lymphocytes with a single laser flow cytometers ***(20)***.

In the late 1980s a range of multicolor cyanine dye reagents ***(21–23)*** (**Table 1**) with excellent water solubility and labeling properties became available and soon after that Molecular Probes Inc. (Eugene, OR) evolved alternative multicolor fluorescent labels called the Alexa dyes ***(24,25)***. Although not yet perfect, the CyDyes and Alexa dyes do well in most of the properties that are essential for a good labeling reagent as described earlier, plus relative insensitivity to molecular environment, availability in multiple colors, and excitation wavelengths compatible with available lasers and white light sources ***(26)***. However, there is room for improvement. Although the brightness of many fluorophores is within a factor of two to three of the theoretical limit for visible absorbing single chromophore organic molecules, they have application-dependent limitations. For example, when detecting more that four or five fluorescent dyes in a sample, the investigator must be concerned about the breadth of the absorption and emission peaks of the dyes that lead to spread of the fluorescence signal of each dye into the detection channels for the other dyes (*see* Chapter 17). Quantum dot labeling reagents have much narrower emission peaks and might help with this problem, but quantum dot reagents offer different challenges (*see* **Subheading 3.2.1.**).

There are now many hundreds of direct and secondary antibodies commercially available that are optimally labeled either directly or indirectly with the fluorescent dyes. They are integral components of staining kits for research and diagnostic applications in flow and image cytometry. They are also now extensively used with nucleic acid probes for chromosome analysis, gene expression and SNP analysis and DNA sequencing. Fluorescent labels are truly the heart of a multibillion-dollar industry based on fluorescence detection.

Antibodies that detect specific protein posttranslational modifications have been in use for about 15 yr *(27)*, and are finding increased use in both multiparameter flow cytometry and multiplexed HCS. For example, the use of fluorescent antiphospho-peptide antibodies to sort out protein phosphorylation and dephosphorylation is an important area, in which drug targets are likely to be found. Nolan and his colleagues have demonstrated the power of flow cytometry with these reagents for sorting out kinase-phosphatase regulated pathways in cancer *(28)* and blood cells *(29)*. This is an exciting area for the application of HCS to correlate phosphorylation status with other cellular activities.

Flow cytometry is another fluorescence readout technology that has the strength of rapid analysis of total cellular fluorescence in heterogeneous populations. This technology combines nicely with the multicolor fluorescent labels and physiological indicators that have been developed. During the past few years, several research groups have employed custom-built flow cytometers with the capacity to quantify as many as 12 fluorescent signals from individual cells. Roederer and his colleagues *(30)* have shown how this multicolor cytometric technology is substantially advancing measurement capabilities and aiding new experimental designs. They are using this technology, for example, to provide a completely new view of the immune system that encompasses a far greater heterogeneity of cell type and function than previously imagined. This capability is being combined with rapid sampling technologies such as those being developed by Larry Sklar at the University of New Mexico *(31)*. A commercial version of this innovative approach called The HyperCyt® is a high-throughput flow cytometry platform that interfaces a flow cytometer and microplate autosampler. Quantitative measurements have been demonstrated in end-point assays at rates of 20–40 samples/min over a four-decade range of fluorescence intensity *(32)*.

3.2. Additional Fluorescent Reagents for Use Mainly in Fixed End-Point HCS Assays

Clearly, immunoreagents have become and will long remain the major reagents for fixed end-point HCS assays. Nonetheless, other types of reagents also exhibit the specificity and sensitivity required to perform HCS assays. These include additional immunoreagents like single chain antibodies, and other labeling approaches such as quantum dots, aptamers, fluorescence signal amplification reagents, molecular beacons, and the highly "avid" noncovalent complex formed between the vitamin biotin and the protein avidin or its analogs (*see* **ref.** *33* for review of histochemical uses of biotin–avidin). Although these reagents have yet to find widespread use in HCS, we envision that the growth in fixed end-point, multiplexed HCS assay development will require these and other new reagent technologies.

3.2.1. Quantum Dots

Quantum dots, semiconductor nanocrystals, have found many applications in imaging, labeling, and sensing. The favorable characteristics of quantum dots, which include size tunable light emission, bright fluorescence signals, resistance against photobleaching, and simultaneous excitation of multiple fluorescence colors have been widely reviewed (*see* **refs.** *34–37* and Chapter 17). Some applications of quantum dots that hold promise for use in HCS include:

1. Encoding cells with mixtures of quantum dots such that individual responses within mixed cell populations can be deconvoluted *(37,38)*.
2. A cell motility assay, in which locomoting cells engulf quantum dots spread on a substrate leaving a fluorescence free area that acts as a record of their migration *(37)*.
3. A *fluorescence resonance energy transfer* (FRET)-based assay of protein activity, in which quantum dots act as energy donors *(35)*.
4. Multiplexed assays, in which five colors of quantum dots are used to label nuclear, cytoskeletal, mitochondrial, and cytoplasmic constituents in the same cells *(35)*. However, the large size of the quantum dots requires less crosslinking of the cytoplasm during fixation, to allow penetration of the quantum dot reagents. Undoubtedly, the limitations of quantum dots and similar, but distinct classes of silica-based

(39,40) and gold-based fluorescence tags *(41)* are being addressed and the future prospects for these unique labeling reagents in HCS are encouraging.

3.2.2. Aptamers and Molecular Beacons

The term aptamer has generally come to define short, single stranded oligonucleotides that fold into distinct three-dimensional structures capable of binding their targets with high affinity and specificity *(42)*. On the other hand, large libraries of peptides with the same properties have also been termed aptamers *(43)*, but the discussion here is focused on nucleic acid aptamers. An aptamer database designed to contain comprehensive sequence information on nucleic acid aptamers and their binding targets is publicly available at http://aptamer.icmb.utexas.edu *(44)*. Aptamers are also considered to be an emerging class of therapeutics and comparisons of their properties with those of antibody-based drugs have been made *(45)*. It is the favorable characteristics of aptamers that we believe will make them important reagents for HCS in the future. These characteristics have been recently reviewed *(45)* and include:

1. Binding affinities in low nanomolar to picomolar range.
2. Candidate selection is a chemical process carried out in vitro and can therefore target any protein.
3. Uniform activity regardless of batch synthesis.
4. A wide variety of chemical modifications for diverse functions is available.
5. Aptamers have a virtually unlimited shelf life.

In addition to their potential use as replacements or complements to existing immunoreagents, aptamers are also being developed as sensors of the activity of cellular constituents. Modular allosteric fluorescent sensors with and without covalently attached fluorophores have been constructed to report the binding of metabolites such as ATP, theophylline, and flavin mononucleotide *(46,47)* as well as protein activity *(42)*.

Molecular beacons and molecular beacon aptamers are a class of DNA probes that act as switches. Target binding induces conformational changes that usually result in the loss of fluorescence quenching *(48)*. Molecular beacons have been used to monitor gene expression in living systems and are being developed as sensors of protein activity *(48)* and DNA–protein interactions *(49,50)*.

3.2.3. Catalyzed Reporter Enzyme Deposition

Catalyzed reporter deposition (CARD), which has also come to be known as tyramide signal amplification is a powerful technique used to detect antigens that are not detectable by ordinary immunochemistry *(51)*. In the immunofluorescence-based version of CARD, a horseradish peroxidase conjugated immunoreagent catalyzes the deposition of a fluorescently labeled phenolic compound in the immediate area of the enzyme *(52)*. The enzymatic amplification obtained by the system results in a one to two orders of magnitude increase in the fluorescence signal *(51,52)*. New modifications to the original procedure have increased its sensitivity (i.e., *see* **ref.** *53*). CARD has also found application in flow cytometry *(54)* and has even been combined with quantum dots for sensitive and photostable immunofluorescence detection *(55)*. Thus, CARD has the potential to greatly increase the number of targets that can be detected by HCS through amplification of the signal strength of low copy number cellular constituents.

4. Fluorescent Reagents For Live Cell and Kinetic HCS Analysis

Measurement of the structure and dynamics of living cells is a great challenge in the era of systems biology. Below we will discuss the types of probes, how they are delivered into cells, and how the fluorescence readout is accomplishes with imaging systems. We will then give examples of applications in HCS.

4.1. Physiological Indicator Dyes

Much of the early research as been reviewed extensively *(11,56–63)*. Some of the earliest uses of probes with living cells are listed in **Table 1**. Britton Chance was a pioneer in the measurement

of fluorescence from living cells. He was able, for example, to detect NADH fluorescence oscillations from mitochondria in intact cells as early as 1964 *(64)*. Another of the early measurements of an important property of living cells was carried out by Tasaki who used an extrinsic probe, anilinonapthalene sulfonate, to detect faint fluorescence changes that occur when stained squid axons conducted an action potential *(65)*. Significant improvements were obtained in a collaboration of a chemist, Alan Waggoner, and a physiologist, Larry Cohen, and his collaborators *(66–68)*. The strategy was to screen a large number of commercially available dyes, synthetically vary the structures of the best candidates to produce improvements, discover the molecular mechanisms of voltage sensitivity of the best of these dyes, and, finally, use knowledge of the molecular mechanisms to create better dyes *(69–71)*. Eventually, probes that showed large signal changes in small cells during single action potentials were obtained *(72)*.

These early studies on membrane potential sensitive fluorescent probes opened the way to designing, developing and applying fluorescent indicators for a wide range of cellular properties ranging from organelle function, pH, free Ca^{2+} ion concentration, Na^{+}, K^{+}, and more. This area has been extensively reviewed *(73–75)*. The "Molecular Probes"—Invitrogen website and Handbook describe many valuable fluorescent probes, their properties and their referenced work (*see* http://probes.invitrogen.com as well as Chapter 17).

4.2. Fluorescent Analog Cytochemistry

Fluorescent analog cytochemistry (initially called molecular cytochemistry) was developed to define the dynamic distribution and activity of specific proteins in living cells *(14,76)*. The name "fluorescent analog" was chosen for this method, as any modification of the protein would yield an analog, not a native protein. However, the technologies and methods were developed to prepare analogs that maintained a majority of the biochemical and molecular properties and activities exhibited by the native molecules. The original methods were quite involved and included purifying a targeted protein, covalently labeling the protein with a reactive fluorescent probe and then characterizing the "analog" in vitro to determine if the covalent modification altered the native activity of the protein (e.g., enzyme activity, ligand binding, binding affinities with other proteins, and so on). Subsequently, the fluorescent analogs were loaded into cells, usually by microinjection and then analyzed by a variety of fluorescence microscopic methods *(14,15)*. These early studies led to the application of this method to a wide range of intracellular proteins *(11,77)*. Some fundamental rules concerning labeling approaches, characterization of functionality and limits on the concentrations of analogs in cells were developed *(16,17,56)*.

A variety of quantitative fluorescence microscope methods evolved in parallel with the development of fluorescent analogs of proteins and other macromolecules with the goal of making quantitative measurements of cell activities using spectroscopic methods and imaging technology *(8,77,78)* One of the key methods was ratio imaging, originally demonstrated as a quantitative method to measure cytoplasmic pH, but also shown as a critical method to normalize the variable pathlengths in cells *(17,79)*, and to measure either spectral changes from environmental changes or energy transfer in proteins engineered as protein-based biosensors (*[80–83]*; *see also* **Subheading 4.4.**).

Cells are not optimal "cuvets" for fluorescence quantitation. Using the average attached and spread mammalian cell in culture, as an example, the pathlength through the peripheral regions of the cell is usually much less than that through the nuclear region. In addition, the presence of membrane-bound organelles can exclude fluorescent analogs, thus decreasing the "accessible volume" in some cellular regions. Therefore, a simple measurement of fluorescence intensity cannot be directly interpreted as a relative local concentration of the fluorescent analog. Two color ratios in which one channel of fluorescence is the fluorescent analog and the second channel of fluorescence is a soluble control molecule such as a crosslinked, nonfunctional analog or a dextran or ficoll, can be applied *(84)*. The image of the fluorescent analog is divided by the

image of the soluble control molecule to yield a "normalized" map of the relative concentration of the analog. This approach identified local cellular domains of concentration and activity in cells ***(82,85)***. Many unnormalized fluorescence images from cells have led to misinterpretations of the biology. This issue will become critical as more investigators use live cell dynamic HCS as a profiling tool.

The preparation and utilization of these first generation fluorescent analogs was very difficult and time-consuming. Experimental preparations could take more than a week to complete with major efforts in protein purification, labeling, and characterization, even before applying the analogs to cells. In addition, the use of biochemical methods to label the proteins required the loading of the labeled proteins into the living cells. A combination of physical microinjection and mechanical shock loading methods were the main approaches, both being time-consuming. A simpler and more elegant approach was required to label and incorporate specific proteins so that the method would grow beyond the use by a relatively small number of biophysically oriented cell biologists. However, these early methods opened the door to quantitative analyses of protein functions in cells, using the cell itself as a "living microcuvet."

4.2.1. Recent Applications of Fluorescent Analogs Relevant to HCS

Translocations between the cytoplasmic and nuclear compartments provide robust HCS assay measurements and have been used as the basis of several HCS assays. For example, fluorescent analogs of the transcription factor NF-κB and its regulatory kinase Iκ-Bα were used to obtain kinetic live cell measurements of cytoplasm-nuclear translocation oscillations that were also correlated with measurements of gene expression mediated by NF-κB ***(86)***. In another high-content assay based on cytoplasm-nuclear translocation, nuclear export inhibitor and kinase inhibitor panels were screened using primary and secondary translocation assays ***(87)***. The primary screen was used to assay for inhibitors of the activation-dependent nuclear export of the p38 kinase substrate mitogen-activated protein kinase (MAPK) activated protein kinase 2 (MK2) using a GFP-MK2 fusion protein as an analog. The hits from the primary screen were categorized in secondary assays either as direct inhibitors of MK2 nuclear transport or as inhibitors of upstream p38 MAPK pathway activities (e.g., nuclear export assays or other signaling proteins and in vitro kinase activity assays).

4.2.2. Methods for Loading Macromolecules Into Living Cells for HCS

Although fluorescent analogs and fluorescent protein biosensors produced in vitro for measurements in living cells have played a major role in understanding the temporal and spatial orchestration of cellular processes, loading protein-based biosensors into cells on a large enough scale for HCS has been problematic. For many academic researchers, cell-loading techniques such as microinjection have provided enough capacity for routine investigations. Most protein loading techniques rely on the reversible perturbation of the plasma membrane ***(88,89)*** and the mechanism of this type of cell injury and subsequent repair have been well defined ***(90)***. Progress has been made in scaling up physical perturbation methods for cell loading. For example, electroporation mediated loading of mammalian cells attached to substrates have been described (i.e., *see* **ref.** ***91***). Several updated electroporation products are now available commercially. Microfabrication of microneedle arrays have been used to simultaneously microinject large populations of cells and even local regions of tissue ***(92,93)***. Finally, optoinjection, which uses light energy to reversibly perturb cellular membranes, is a promising new technology that has the potential for large-scale bulk loading of living cells ***(94)***.

In addition to plasma membrane perturbation, other approaches to large-scale cell loading have been developed. Examples include delivery systems taking advantage of the endocytic pathway ***(95,96)*** as well as direct translocation of specific proteins across the plasma membrane ***(97)***. However, the clinical need for methods capable of delivering macromolecules to cells and tissues has led to the development of peptide-based transduction systems. These methods have

been extensively reviewed *(98–100)* and are mainly based on cationic peptides fused to cargo macromolecules to enable cargo uptake by the cells. Cargo molecules traverse the Golgi and endoplasmic reticulum before being released into the cytoplasm *(101)*. Thus, with progress being made on several technical fronts, we believe that bulk-loading methodology on the scale necessary to support HCS campaigns will soon become a reality. These developments will enable the integration of several classes of in vitro prepared fluorescent analogs and fluorescent protein biosensors into multiplexed HCS assays.

4.2.3. Fluorescent Protein Gene Fusions With Targeted Genes (GFP-Based Fluorescent Analogs)

A simpler and more elegant approach to labeling proteins was made possible by the discovery that the gene for the GFP from the jelly fish Aequoria could be fused to a specific gene in a cell, producing a fluorescent fusion protein *(102)*. Tsien and his colleagues subsequently optimized the GFP through mutagenesis, including making multiple colors *(103)*. This led to an explosion of the production of fluorescent analogs, as the development of this method coincided with the rapid identification of genes *(104,105)* (*see also* Chapter 12 *[106]*).

4.2.4. Fluorescent Analogs Labeled at Specific Sites Using Fluorescent Probe Capture in Living Cells

The production of fluorescent analogs through ectopic expression of autofluorescent fusion proteins is simple, specific, and sensitive, but it has its limitations *(107)*. Several new approaches for labeling fluorescent protein biosensors through the capture of fluorescent probes, which are often added exogenously and taken up by cells in which they are specifically bound the endogenously expressed proteins, have been described and extensively reviewed *(59,108–110)*. These probes fall into four classes *(107)*:

1. Intein-based labels.
2. Proteins that are specifically modified with fluorescent probes by a separate protein.
3. Proteins that reversibly and specifically bind fluorescent probes.
4. Proteins that irreversibly and specifically bind fluorescent probes through covalent bond formation.

A summary of several methods for labeling cellular proteins at specific sites using fluorescent probe capture is presented in **Table 2**. Two of the now commercially available reagents, Lumio Tags and Halo Tags are described and demonstrated in detail elsewhere in this volume (*see* Chapters 14 and 15). The number and breadth of these new labeling approaches means that fluorescent analogs and fluorescent protein biosensors can potentially be multiplexed with other fluorescent probes in the same cells to provide new systems cell biology information.

4.3. Positional Biosensors

Positional biosensors are fluorescent protein biosensors that report on the regulation of intracellular molecular processes through their artificial (engineered) intracellular localization *(111)*. Because HCS platforms are able to measure the translocation of molecules between cellular compartments, positional biosensors have great potential as reagents in both fixed endpoint and live cell kinetic HCS assays. Many proteins naturally exhibit changes in the distribution from one cellular compartment to another because their localization within the cell often determines their functional activity (e.g., transcription factors, β-arrestin, and so on). Fluorescent analogs of these proteins are not considered "positional biosensors." Using an HCS application that quantifies fluorescent aggregates within the cytoplasm of living cells, a library of compounds was screened for specific GPCR agonists with GFP-based sensors of receptor aggregation within stimulated cells *(112)*. Similarly, the aggregation state of a sensor made up of a fusion protein containing the catalytic subunit of protein kinase (PKA) and GFP

Table 2
Methods to Label Cellular Proteins at Specific Sites Using Fluorescent Probe Capture

Method	Mechanism of fluorescent probe capture	References
Formation of covalent bonds		
"FlAsH" or "Lumio" tags	Biarsenical-tetracysteine covalent complex formation	***169****; see also* Chapter 15
"Halo" tags	Engineered hydrolase enzyme that covalently traps a fluorescently labeled substrate after hydrolysis of carbon-halogen bond	Chapter 14
"SNAP" tags	Autoalkylation of O^6-alkylguanine-DNA alkyltransferase (AGT) with fluorescently labeled substrates	***170***
Biotinylation	Biotin-ligase-mediated transfer of labeled biotin analogs to membrane-bound extracellular acceptor peptides	***171***
"Cystope" tagging	Sulfhydryl reactive probes that react with a cysteine residue genetically fused to the C-terminus of target proteins	***172***
Ligation to modified sugars in glycoproteins	Staudinger ligation of fluorescent phosphines with azido-modified sugars on the extracellular face of membrane glycoproteins	***173,174***
Noncovalent ligand binding		
Enzyme substrate binding	Dihydrofolate reductase from *E. coli* binding of fluorescent trimethoprim analogs	***175***
Puromycin conjugation	Labeling of newly synthesized proteins with fluorescent puromycin analogs	***176***

was used to quantify changes in intracellular cAMP concentration in stimulated cells ***(113,114)***.

Positional biosensors are designed and genetically engineered to respond to specific molecular events and to report these activities through translocations between predetermined intracellular compartments using target signals engineered into the biosensors. Hence, this class of biosensor uses the normal targeting sequences, but engineers them into proteins that would not normally translocate. Several positional biosensors are synthetically engineered using modular designs to optimize the specificity and sensitivity with which cellular process measurements can be incorporated into multiplexed HCS assays ***(111)***. A critical component common to all synthetic positional biosensors is the reporting domain, a fluorescent probe whose localization is quantified with HCS. The reporting domain can be an autofluorescent protein such as GFP or it can be labeled with most any of the biosensor labeling methods described here. Positional biosensors also contain a dominant localization domain that maintains the biosensor in a particular cellular compartment until it is modified by a specific biological activity such as protease or kinase activity. Once modified, the dominant localization domain no longer has the capability to maintain the biosensor in a specific cellular compartment. The biosensor is therefore free to diffuse into other compartments or to be possibly actively translocated into a particular compartment by a secondary localization domain that becomes dominant after the biosensor has been modified. The third component of the synthetic positional biosensor is the sensing domain. This domain confers specificity to the positional biosensor. It is the modification of the sensing domain by a specific biological activity that leads to the eventual translocation of the biosensor in to a different compartment.

Positional biosensors of protease activity have been produced and used in HCS assays ***(111,115)***. These biosensors, which were used to detect cytoplasmic protease activities, contained a specific protease cleavage site as their specificity domain. Flanking the specificity domain were localization domains that caused the biosensors to be predominately partitioned in

the cytoplasm until an active protease cleaved the specificity domain, which led to a net translocation of the reporter domain, GFP in this case, into the nucleus. Thus, changes in the cytoplasm-nucleus distribution ratio of the reporter domain provided and HCS readout of protease activity in living cells.

In addition to protease activity, positional biosensor has been used to make HCS measurements of kinase activity in cells *(111)*. A biosensor of PKA was designed to have GFP as the reporter domain and localization domains were chosen to ensure the cycling of the biosensor between the nucleus and cytoplasm. In this example, a dominant nuclear localization signal caused the biosensor to be predominately partitioned in the nucleus in unstimulated cells. The specificity domain of the biosensor contained the protein-binding domain of the cAMP response-element binding (CREB) protein that provided a specific binding site for activated PKA. This binding event hindered the activity of the dominant nuclear localization signal resulting in the predominant repartitioning of the biosensor in the cytoplasm, a translocation easily measured with HCS.

Positional biosensors have the potential to provide high quality measurements of several specific cellular activities in both fixed end point and kinetic modes of HCS. Furthermore, positional biosensors can be incorporated into multiplexed HCS assays to make correlated measurements within the same cells. In a recent example, positional biosensors were designed by fusing compartmental localization domains to GFP to measure agonist-mediated inositol 1,4,5-trisphosphate, diacylglycerol, and protein kinase c signaling in the same cells along with kinetic free calcium measurements *(116)*. Positional biosensors exhibit large (approx > 0.6) Z' factors and should play an important role in HCS. Cellumen, Inc. (Pittsburgh, PA) is developing families of positional biosensors.

4.4. Fluorescent Protein Biosensors of Temporal and Spatial Biological Activities

When converted to sensors of cellular processes, proteins have the potential to report not only the dynamic distribution of specific reactions, but also provide information on reaction kinetics, protein interactions, and post-translational modifications. Thus, in addition to positional biosensors, other classes of fluorescent protein biosensors have been developed to sense and report biological activities through the environmental changes that occur either internally or on their surface, including binding to other proteins *(57,58,73,74,117,118)*. The original fluorescent protein biosensor produced in vitro was used to measure the reversible activation of the calcium-binding protein calmodulin in living cells *(80,119)*. The biosensor, based on an environmentally sensitive merocyanine dye covalently linked to calmodulin before introduction into cells, was used to make temporal and spatial measurements of calcium and calmodulin activation maps in living cells undergoing stimulation and locomotion *(119)*. This biosensor was soon followed by fluorescent protein biosensors of myosin II phosphorylation *(82,83,120)* and cAMP dynamics *(121)*. Nevertheless, there has been recent progress in the development of new fluorescent protein biosensors produced in vitro as well as fluorescent protein biosensors based on autofluorescent proteins such as GFP.

4.4.1. Fluorescent Protein Biosensors Produced In Vitro

The original class of fluorescent protein biosensors were constructed outside of living cells using synthetic fluorescent probes for labeling followed by loading into living cells, in which temporal and spatial measurements were made (reviewed in **ref. *74***). Recently, a synthetic biosensor of intracellular Cdc42 activation was developed and its performance was compared to a FRET-based endogenously produced biosensor *(122,123)*. Instead of FRET, the new biosensor relied on a solvent-sensitive fluorescent merocyanine dye to report changes in the interaction of the WASP protein with activated Cdc42. Addition of GFP to the biosensor for ratio imaging based pathlength correction enabled spatially resolved measurements to be made in all compartments of the cell.

In another example, mRNA molecules encoding a mutant glutamate receptor with an engineered reactive cysteine were microinjected in to oocytes *(124)*. After protein synthesis off of the microinjected mRNA was allowed to occur, the newly synthesized transmembrane glutamate receptors were labeled in a site-specific manner by reaction of the oocytes with a cysteine-reactive Alexa 546 fluorescent dye. Once labeling was complete, changes in the total fluorescence signal emanating from the biosensor was used to measure the kinetic conformational changes in the receptor on glutamate binding. Thus, these two recent examples of the many synthetic fluorescent protein biosensors produced over the last several years indicate how important it will be to rapidly solve issues of bulk loading of fluorescent protein biosensors into large populations of target cells for large scale HCS.

4.4.2. FRET-Based Fluorescent Protein Biosensors Constructed With Pairs of Autofluorescent Proteins

In the class of fluorescent protein biosensors of autofluorescent proteins, those that use FRET as the basis of their sensing and reporting capabilities are the most common *(125)*. These biosensors generally contain two complementary autofluorescent proteins whose FRET efficiency depends on the conformational state of the biosensor within living cells. In a study in which the quantification of FRET efficiency was carefully measured, it was reported that an optimal pair of autofluorescent proteins for FRET-based biosensors was the cyan fluorescent protein (CFP) coupled with a variant of the yellow fluorescent protein (YFP) call citrine *(126)*. The authors found that the CFP-citrine pair had a FRET efficiency twice that of a CFP-YFP pair at neutral pH. However, results from the same study point out at least two caveats to be considered when incorporating FRET-based fluorescent protein biosensors into demanding cell-based assays: (1) the optimal protein pair had a maximal FRET efficiency of 40%; and (2) fluorescent protein biosensors containing linked autofluorescent proteins exhibit some measure of FRET at all times owing to the relatively close proximity of the two fluorophores. Thus, one must consider the photophysical characteristics of FRET-based fluorescent protein biosensors that might limit the FRET assay response window. Nevertheless, FRET-based fluorescent protein biosensors have been designed to measure a wide range of biological activities and recent developments merit discussion.

A FRET-based biosensor was designed to measure changes in cAMP concentration in living cells *(127)*. The cAMP binding protein Epac was used as the basis of the endogenously expressed biosensor. The Epac domain, sandwiched between CFP and YFP, exhibited a conformational change on cAMP binding that induced a measurable change in the CFP/YFP fluorescence ratio. The biosensor was used to measure the rapid disappearance of cAMP in aldosterone-producing adrenal cells resulting from the activation of the phosphodiesterase PDE2. A similar approach was used to build a biosensor of histone H3 phosphorylation *(128)*. In this case, CFP and YFP coding sequences flanked the histone H3 phosphorylation site peptide and a phosphoserine-binding domain from the 14-3-3 protein. On phosphorylation of histone H3, which reversibly occurs during the early phases of mitosis, the biosensor exhibited a 15–25% change in the YFP/CFP emission ratio.

Several FRET-based biosensors have recently been used to study the dynamics of the cytoskeleton in living cells. In one example, a CFP-Citrine FRET pair was fused to the N- and C-terminal of the myosin II regulatory light chain *(129)*. When expressed in primary vascular smooth muscle cells, the biosensor was used to report the temporal and spatial kinetics of the calcium induced changes in myosin II phosphorylation that were associated with smooth muscle contraction. A 10% change in the CFP-Citrine fluorescence ratio was observed in cells stimulated with 80 m*M* potassium ion solution. In another example, the temporal and spatial regulation of neuronal Wiskott-Aldrich syndrome protein activity was measured in living cells using a CFP-YFP based FRET biosensor *(130)*. The authors showed that neuronal Wiskott-Aldrich syndrome protein was activated in filopodia and therefore played a role in regulating the cytoskeletal dynamics involved in

membrane ruffling. Similarly, a CFP-YFP based FRET biosensor was used to determine that the Rho GTPases Rac1 and Cdc42 were differentially localized during NGF-induced neurite outgrowth *(131)*. A major challenge for FRET-based biosensor use in HCS is the relatively low Z' factor that is generally possible resulting from the small changes measured.

4.4.3. Fluorescent Protein Biosensors Based on a Single Species of Autofluorescent Protein

Autofluorescent proteins such as GFP have been engineered to detect and report biological activities through changes in their spectral properties *(111,118,132)*. In one recent application, EYFP was first engineered to have a fluorophore whose fluorescence signal was pH dependent *(133)*. Further modification of the protein through fusion with an amino acid sequence encoding a mitochondrial intermembrane targeting signal was used to direct the biosensor to a particular cellular compartment. The biosensor shows promise in providing temporal measurements of pH in the intermembrane space, which is key to the regulation of cellular energy metabolism, after activation of cell surface receptors or during the onset of apoptosis. Another fluorescent protein biosensor based on a single autofluorescent protein involved engineering a pair of redox sensitive cysteine residues into a GFP molecule at a site, in which their oxidation state would reversibly alter the protonation state, and therefore the fluorescence intensity of the protein-based fluorophore *(134)*. The signals from the biosensor, which were measured in living cells in a fluorescence ratio mode to correct for pathlength artifacts, were used to measure redox changes including during superoxide bursts in macrophage cells, hyper- and hypoxic-conditions, and in response to peroxide stimulating agents such as epidermal growth factor and lysophosphatidic acid.

4.4.4. Other Applications of Fluorescent Biosensors and Analogs

Recent applications of fluorescent biosensors and analogs involve the innovative use of cell-based reagents. In one case, insulin mediated translocation of an EGFP-Akt1 kinase fusion protein to the plasma membrane was measured by adding a fluorescence quencher to the cellular bathing solution *(135)*. When the EGFP-Akt1 analog translocated to the membrane, it was in close enough proximity to the extracellular quenching molecules to reduce the total fluorescence intensity of the entire population of cells expressing the biosensor, thus providing information on a cellular translocation, which could be measured in a high-throughput screening mode. In a second example, a biosensor of the phosphorylation dynamics of the EGF receptor was designed to report the translocation from the cytoplasm to the plasma membrane as a change in fluorescence intensity *(136)*. The biosensor contained the EGF receptor fused to both ECFP and EYFP with a phosphotyrosine-binding domain positioned between the ECFP and the EYFP. When the EGF receptor translocated to the membrane, in which the phosphotyrosine domain was phosphorylated, a conformational change in the biosensor occurred and induced fluorescence energy transfer between the ECFP and EYFP, providing a measurable fluorescence signal change.

In a final example, the EGF-induced nucleocytoplasmic shuttling of the stress kinase ERK was measured using a GFP derived from coral that had unusual photochromic properties *(137)*. The fluorescence intensity of the coral GFP-ERK fusion protein could be reversibly highlighted (photoactivated) or erased (photobleached) depending on the wavelength of light used to irradiate the cells. Irradiating only part of the cells allowed investigators to measure the relatively fast nucleocytoplasmic shuttling of the kinase on cell activation.

4.4.5. Future Potential of Protein-Based Biosensors

Technologies for the creation of new fluorescent protein biosensors plus the development of new applications for existing sensors, many of which will involve multiplexing, will ensure that this class of biosensors will continue to grow in both number and complexity. One of the most promising developments in the technology are new systems cell biology approaches that will be addressed with multiplexed live cell kinetic assays that incorporate two or more biosensors within the same cells.

5. Reagents to Combine Cellular Manipulation With HCS Assay Measurements

Reagents that manipulate the temporal and spatial regulation of specific cellular processes are essential tools for the use of HCS platforms as an approach to systems biology. Earlier reviews described several types of reagents for cellular manipulation ***(57,58,138)***, but new technologies are also having a large impact. Recently, the use of photoremovable protecting groups have been used to "cage" phosphopeptides ***(139)*** and phosphoproteins ***(140)***. It was shown that caged phosphopeptides, which were designed to target the 14-3-3 proteins involved in cell cycle regulation, could displace endogenous proteins from the complexes with 14-3-3 in living cells when irradiated with light. The uncaging of the phosphopeptides caused premature cell cycle entry, release of G1 cells from interphase arrest and loss of the S-phase checkpoint after DNA damage, accompanied by high levels of cell death ***(139)***.

The use of RNA inhibition to modulate expression levels of key proteins in cells has escalated in recent years (***[141,142]***; *see also* Chapter 18). Furthermore, the activity of small inhibitory RNAs (siRNAs) can be controlled in time and space within living cells using photoremovable protecting groups (*see* Chapter 19).

Manipulation of gene expression in living cells also involves the upregulation of RNAs that encode proteins or other gene products including siRNAs and other noncoding RNAs. Systems based on tetracycline-based ***(143)*** activation of gene regulatory proteins have been used to modulate protein and siRNA levels in cells ***(144,145)***. Thus, the coupling of RNA inhibition and gene switching reagents with HCS is an ideal combination for the manipulation and measurement the myriad integrated molecular processes that comprise living cells ***(146–149)***. Recently, Cellumen, Inc. has created some switched molecules using an improved ecdysone receptor technology.

A discussion on reagents for the manipulation of cellular processes would not be complete without mention of the enormous collection of drugs that posses a multitude of biological activities. Strategies for drug discovery often include compound designs that modulate the activity of specific targets whether the compounds are synthetic ***(150)*** or derived from natural products ***(151)***. It is specificity of action that makes many therapeutic drugs valuable as cellular manipulation reagents. For example, panels of drugs have been used to build phenotypic profiles of cellular responses using them as perturbagens ***(152)*** or in combination with other drugs ***(153)*** or in combination with other manipulation reagents such as siRNAs ***(146)***.

6. Prospectus

Reagents to measure and manipulate cell functions are the foundation of cell-based high-throughput as well as high content assays. There are many areas in which technological advances in reagent development would significantly benefit drug discovery and biomedical research. New reagents with which to construct labels and probes are needed. Particularly useful would be low molecular weight, probably organic, dyes with narrow and limited excitation and emission spectra assembled into protective "sleeves." This would enable the development of multiplexed assays with much less crosstalk and would be extremely valuable for constructing FRET pairs to go along with multiplexed measurements. Photostable fluorophores in the near IR would extend the range of multiplexed reagents and would allow reduction of the interference from autofluorescence that is because of native cellular chromophores or to fluorescent candidate drugs. Probes with emission lifetimes longer than autofluorescent fluorophores but short enough for rapid emission photon acquisition would be valuable for reducing background fluorescence by time resolve imaging. The 10–100 ns time-scale for emission would be especially attractive. Dyes with larger two-photon absorption cross-sections, in addition to the properties above, would be useful for research in which optical sectioning is required.

The upcoming decades would greatly benefit from new ways to create fluorescent protein biosensors, and, therefore, the creation of systems biology knowledge, for all the regulatory

events that take place in the networks and pathways of living cells. Development of these reagents and new technologies to simultaneously load multiple reagents into the same cells will require a lot of creativity.

We are in need of instrumentation advances to rapidly and cleanly readout the signals from fluorescent probes. More efficient photon capture, brighter excitation at many wavelengths, further improvements in optical filters to separate the excitation and crosstalk of the "new fluorophores" that will hopefully be developed (*see* Chapters 1 and 4).

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