The Fluorescent Toolbox for Assessing Protein Location and Function

Ben N. G. Giepmans, *et al.*

Science **312**, 217 (2006);
DOI: 10.1126/science.1124618

mass spectrometry are just emerging (*33*). It can be anticipated that biological hypotheses will generate lists of proteins that need to be characterized and quantified in a particular study. Such lists of proteins can then be submitted to the database to produce the minimal set of peptides required to test the hypothesis. This set of peptides can then be measured by targeted methods, including MRM (Fig. 2D). The directed nature of this approach allows the mass spectrometer to focus on a nonredundant set of targets and therefore leads to a substantial gain in throughput and sensitivity. By adding calibrated, isotopically labeled reference peptides, precise quantitative information can be obtained.

Such strategies are best implemented on mass spectrometers with Q-Q-LIT geometry related to the triple quadrupole instrument that has been used for decades to quantify small molecules drugs and their metabolites in serum. The same type of protocols can be applied to proteomics studies.

As a variant of this approach, Smith developed the concept of using accurate mass tags to identify peptides by matching accurately measured peptide masses with those calculated for peptides present in a database (*34*), thus obviating the need to sequence each peptide in each sample. With the rapid increase in accessible data from prior proteomic experiments and the development of mass spectrometer control software that supports large inclusion lists for targeted analyses, the use of the hypothesis-driven strategies can be expected to increase.

Outlook and Conclusion

Protein analysis and, more specifically, proteomics have driven the development of mass spectrometry for the past decade. Technological advances have translated into major improvements in mass accuracy, resolving power, LOD, and accuracy of quantification and new experimental strategies aimed at the routine and comprehensive analysis of whole proteomes. New mass-spectrometric strategies to analyze intact proteins, protein complexes, and low-redundancy target workflows are emerging. Although these mass spectrometry technologies have been driven by protein research; once developed, they will equally effect the analysis of other types of biomolecules, including metabolites, lipids, and carbohydrates. It can therefore be anticipated that the use of mass spectrometry in the life sciences will become even more prevalent and diversified.

References and Notes

1. N. L. Anderson, N. G. Anderson, *Mol. Cell. Proteomics* **1**, 845 (2002).
2. J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong, C. M. Whitehouse, *Science* **246**, 64 (1989).
3. M. Karas, F. Hillenkamp, *Anal. Chem.* **60**, 2299 (1988).
4. R. Aebersold, D. R. Goodlet, *Chem. Rev.* **101**, 269 (2001).
5. R. Aebersold, R. M. Mann, *Nature* **422**, 198 (2003).
6. Mass accuracy and resolving power are two key characteristics of a mass spectrometer. Resolving power characterizes the ability to separate two ions that are close in mass (and it is defined by the ratio mass/mass difference). Mass accuracy is expressed either in absolute terms (difference between the observed and calculated values) or in relative terms expressed in ppm.
7. K. F. Medzihradszky *et al.*, *Anal. Chem.* **72**, 552 (2000).
8. R. G. Cooks, G. L. Glish, S. A. McLuckey, R. E. Kaiser, *Chem. Eng. News* **69**, 26 (1991).
9. In data (or information)-dependent acquisition, the data system will use information collected on the flight in full scan mode (MS1) to trigger the subsequent MS/MS experiments.
10. J. V. Olsen, M. Mann, *Proc. Natl. Acad. Sci. U.S.A.* **101**, 13417 (2004).
11. J. W. Hager, *Rapid Commun. Mass. Spectrom.* **16**, 512 (2002).
12. M. W. Senko, C. L. Henderickson, M. R. Emmett, S. D. Shi, A. G. Marshall, *J. Am. Soc. Mass Spectrom.* **8**, 970 (1997).
13. M. Hardmanand, A. Makarov, *Anal. Chem.* **75**, 1699 (2003).
14. Q. Hu *et al.*, *J. Mass Spectrom.* **40**, 430 (2005).
15. M. J. Huddleston, M. F. Bean, S. A. Carr, *Anal. Biochem.* **239**, 180 (1996).
16. J. C. Le Blanc *et al.*, *Proteomics* **3**, 859 (2003).
17. M. J. Huddleston, R. J. Annan, M. F. Bean, S. A. Carr, *Anal. Chem.* **65**, 877 (1993).
18. R. A. Zubarev, N. L. Kelleher, F. W. McLafferty, *J. Am. Chem. Soc.* **120**, 3265 (1998).
19. J. E. P. Syka, J. J. Coon, M. J. Schroeder, J. Shabanowitz, D. F. Hunt, *Proc. Natl. Acad. Sci. U.S.A.* **101**, 9528 (2004).
20. N. L. Kelleher, *Anal. Chem.* **76**, 197A (2004).
21. M. A. Baldwin, *Mol. Cell. Proteomics* **3**, 1 (2004).
22. S. Carr *et al.*, *Mol. Cell. Proteomics* **3**, 531 (2004).
23. A. Keller, A. I. Nesvizhskii, E. Kolker, R. Aebersold, *Anal. Chem.* **74**, 5383 (2002).
24. H. Wang *et al.*, *Mol. Cell. Proteomics* **4**, 618 (2005).
25. S. Hu *et al.*, *Anal. Chem.* **76**, 4044 (2004).
26. S. P. Gigy *et al.*, *Nat. Biotechnol.* **17**, 994 (1999).
27. H. Zhou *et al.*, *Nat. Biotechnol.* **19**, 375 (2001).
28. S. Ficarro *et al.*, *Nat. Biotechnol.* **20**, 301 (2002).
29. H. Zhang, X. J. Li, D. B. Martin, R. Aebersold, *Nat. Biotechnol.* **21**, 660 (2003).
30. P. Kearney, P. Thibault, *J. Bioinform. Comput. Biol.* **1**, 183 (2003).
31. X. J. Li *et al.*, *Anal. Chem.* **76**, 3856 (2004).
32. B. Kuster, M. Schirle, P. Mallick, R. Aebersold, *Nat. Rev. Mol. Cell Biol.* **6**, 577 (2005).
33. F. Desiere *et al.*, *Genome Biol.* **6**, R9 (2004).
34. R. D. Smith, G. A. Anderson, M. S. Lipton, *Proteomics* **2**, 513 (2002).
35. S. E. Ong *et al.*, *Mol. Cell. Proteomics* **1**, 376 (2002).
36. X. Yao, A. Freas, J. Ramirez, P. A. Demirev, C. Fenselau, *Anal. Chem.* **73**, 2836 (2001).
37. A. Thompson *et al.*, *Anal. Chem.* **75**, 1895 (2003).
38. P. L. Ross *et al.*, *Mol. Cell. Proteomics* **3**, 1154 (2004).

39. We gratefully acknowledge funding with federal funds from the National Heart, Lung, and Blood Institute of the NIH under contract no. N01-HV-28179.

10.1126/science.1124619

REVIEW

The Fluorescent Toolbox for Assessing Protein Location and Function

Ben N. G. Giepmans,[1,2] Stephen R. Adams,[2] Mark H. Ellisman,[1] Roger Y. Tsien[2,3]*

Advances in molecular biology, organic chemistry, and materials science have recently created several new classes of fluorescent probes for imaging in cell biology. Here we review the characteristic benefits and limitations of fluorescent probes to study proteins. The focus is on protein detection in live versus fixed cells: determination of protein expression, localization, activity state, and the possibility for combination of fluorescent light microscopy with electron microscopy. Small organic fluorescent dyes, nanocrystals ("quantum dots"), autofluorescent proteins, small genetic encoded tags that can be complexed with fluorochromes, and combinations of these probes are highlighted.

Fluorescence has long been used to visualize cell biology at many levels, from molecules to complete organisms. Originally, fluorescence was mainly observed from small organic dyes attached by means of antibodies to the protein of interest. However, antibody targeting of intracellular proteins normally requires cell fixation and permeabilization. Later, fluorophores could directly recognize organelles, nucleic acids, and certain important ions in living cells. In the past decade, fluorescent proteins have enabled noninvasive imaging in living cells and organisms of reporter gene expression, protein trafficking, and many dynamic biochemical signals. Hybrid systems in which small organic fluorophores are genetically targeted are filling other useful niches including determination of protein age, correlative electron-microscopic localization, and rapid photoinactivation of selected proteins. Meanwhile, semiconductor nanocrystals have been developed with higher brightness and photostability than previous fluorophores, but their targeting currently remains challenging. This review will discuss recent developments in fluores-

[1]National Center for Microscopy and Imaging Research, Center for Research in Biological Systems, Department of Neurosciences; [2]Department of Pharmacology; [3]Department of Chemistry and Biochemistry and Howard Hughes Medical Institute, University of California, San Diego, La Jolla, CA 92093, USA.

*To whom correspondence should be addressed. E-mail: rtsien@ucsd.edu

cent probes and techniques to determine protein expression, activity, and function.

Fluorophores

Small organic dyes. Small organic fluorophores (<1 kD) for covalent labeling of macromolecules have undergone industrial optimization of wavelength range, brightness (extinction coefficient for absorbance × quantum yield of fluorescence), photostability, and reduction in self-quenching. Molecular strategies have included extension of double-bond conjugation, rigidification through extra rings, and decoration with electron-withdrawing or obligatorily charged substituents such as fluorines or sulfonates. Hundreds of such dyes are commercially available (*1*), and further progress is likely to be incremental. Because these dyes lack specificity for any particular protein, most applications use antibodies (Fig. 1, A to C) in fixed and permeabilized cells.

Fluorescent proteins. The first fluorescent proteins to become useful in cell biology were phycobiliproteins, photosynthetic antenna pigments extracted from cyanobacteria (*2*). Each macromolecule contains multiple bilin chromophores encapsulated in a matrix evolved to minimize quenching, making phycobiliproteins up to two orders of magnitude brighter than small organic fluorophores. However, their size (200 kD) limits diffusion, so their application has been mainly in antibody conjugates for surface labeling in flow cytometry and enzyme-linked immunosorbent assay (ELISA) [reviewed in (*3*)]. They could become much more widely useful if genetically expressed in situ, but the problem is that bilin chromophores have to be supplied and inserted into the apoproteins. Fortunately, much progress has been made (*4*, *5*).

A revolution in cell biological imaging has resulted from the discovery (*6*), gene cloning (*7*), and heterologous expression of the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* [reviewed in (*8*)]. Expression of GFP alone or in most genetic fusions with other proteins results in visible fluorescence (Fig. 1, C and D) without requiring any cofactors other than O_2, because the chromophore is generated by spontaneous cyclization and oxidation of three amino acids buried at the heart of the 2.4- by 4-nm beta barrel. GFP is just one member of a large family of homologous fluorescent proteins (FPs) (*9*, *10*), mostly from marine coelenterates, with different colors from variations in chromophore covalent structure and noncovalent environment (*11*). Laboratory mutagenesis has further diversified FPs' spectra, increased their brightness and folding efficiencies (*12*), and decreased oligomerization [reviewed in (*11*)]. Mutation can either increase the photostability for standard fluorescence observation or conversely generate FPs that are photoswitchable from dark to bright or from one color to another [reviewed in (*13*)]. Such photoswitching can be reversible or irreversible and is useful for monitoring protein diffusion, trafficking, and age. Although FPs generate stoichiometric amounts of H_2O_2 during chromophore formation (*8*), they seem to generate relatively little reactive oxygen species (ROS) during illumination, which is not

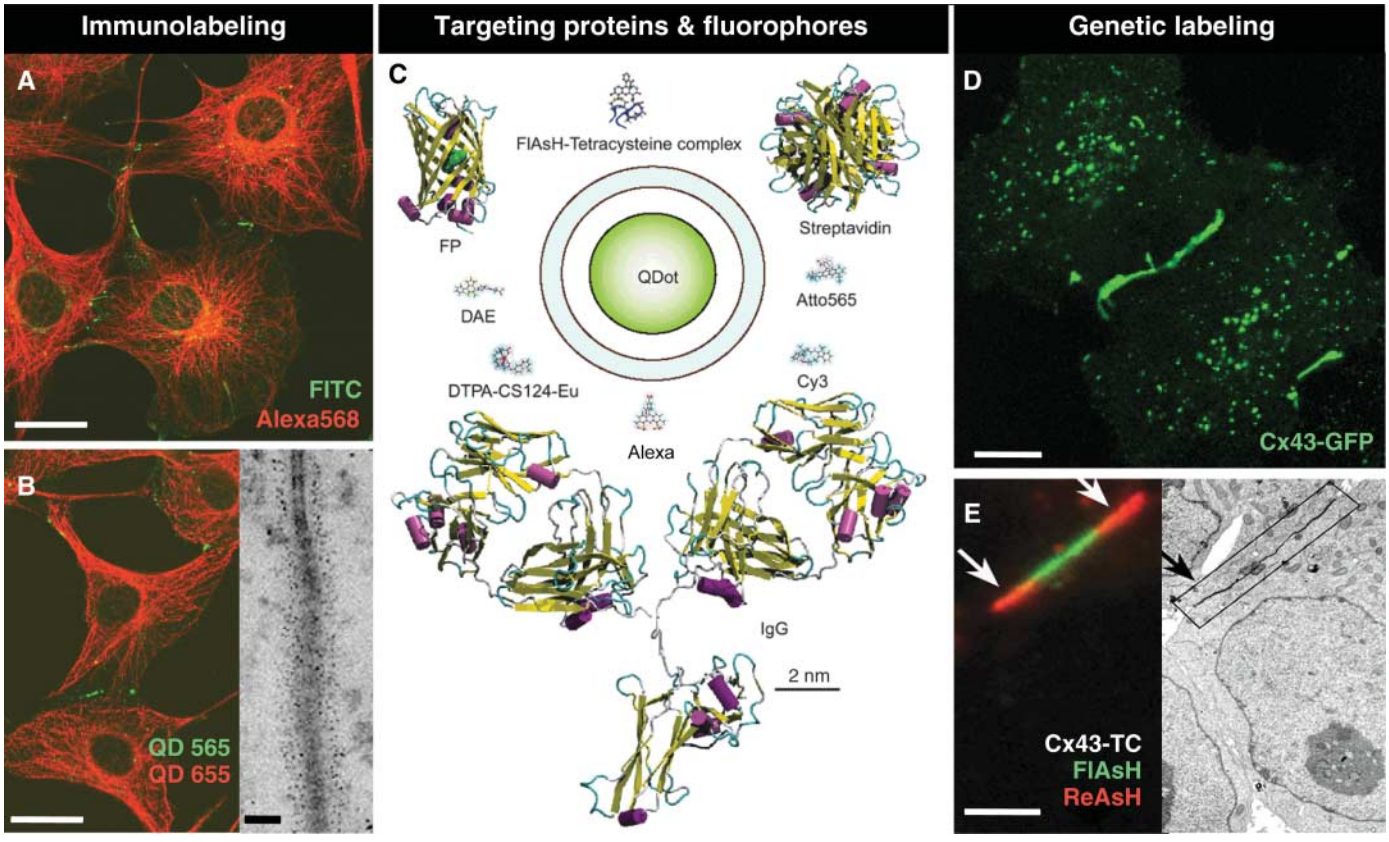

Fig. 1. Characteristics and applications of types of fluorophores in protein detection. Applications of different targeting methods and types of fluorophores are illustrated on connexin43 (green) and α-tubulin (red) in fibroblasts (**A** and **B**) and connexin43 in HeLa cells (**D** and **E**) as indicated. The structures of different types of targeting proteins and fluorophores (**C**) are shown to scale. [Scale bar is 2 nm; reproduced from (*58*).] Endogenous proteins are labeled using primary antibodies followed by secondary antibodies conjugated to small organic dyes (A) or Fab fragments attached to QDs (B), which are also detected at the EM level; (B) (right), QD 565 at connexin43–based gap junction. Genetically encoded intrinsic FPs (D) or tetracysteine tags labeled with biarsenicals (E) rely on ectopic expression. Tetracysteines on connexin43 were pulse-labeled with FlAsH (green) and subsequently ReAsH (red), thus distinguishing old from new connexins, respectively. ReAsH is also visualized in EM using photooxidation (E) (right). [Reproduced from (*37*).] Scale bars in (A), (B), and (D), 20 μm (LM); (B), 50 nm (EM); (C), 2 nm; (E), 2 μm.

surprising given their evolution within organisms exposed to sunlight. Nevertheless, it has been possible to mutate FPs to generate ROS for photodestruction of cells (*14*). The fluorescence of FPs is normally rather insensitive to their biochemical environment except for quenching by acid pH or denaturation, but they have been engineered for enhanced pH sensitivity or responsiveness to metal or halide ions and thiol-disulfide redox potentials [reviewed in (*10*, *15*)].

Quantum dots. Quantum dots (QDs) are inorganic nanocrystals that fluoresce at sharp and discrete wavelengths depending on their size, have high extinction coefficients (10 to 100 times those of small fluorophores and FPs), and have good quantum yields. QDs typically contain a CdSe or CdTe core and ZnS shell (Fig. 1C). Their absorbance extends from short wavelengths up to just below the emission wavelength, so that a single excitation wavelength readily excites QDs of multiple emission maxima. Crucial for biological applications was the development of coatings that make QDs water soluble, prevent quenching by water, and allow conjugation to protein-targeting molecules such as antibodies and streptavidin (Fig. 1B) (*16*–*20*). The large size of QDs conjugated to biomolecules (~10 to 30 nm) prevents efficient traversal of intact membranes, which restricts their use to permeabilized cells or extracellular or endocytosed proteins. The photostability of QDs allows repeated imaging of single molecules, and their size and electron-density permits correlated electron microscopy (EM) (*21*).

Gold and silver nanodots formed within hydrophilic dendrimers are also highly fluorescent and wavelength tunable (*22*). Their derivatization and application in cell biology is eagerly awaited.

Techniques to Tag Proteins

Immunolabeling. Immunolabeling and other techniques for tagging proteins are summarized in Table 1. The most widespread technique to detect endogenous proteins using fluorescence is labeling with a primary antibody followed by amplification with a secondary antibody conjugated to small organic dyes, a phycobiliprotein, or a QD (Fig. 1. A to C). Alternatively, primary antibodies can be directly conjugated to fluorophores or to biotin, which is then detected using streptavidin. Direct conjugation is especially useful when injecting antibodies into live cells or to increase spectral diversity when analyzing multiple proteins. When high-quality antibodies to the target protein are not available, the target can be recombinantly expressed with an epitope tag, although it is no longer completely endogenous. Of course, the accuracy of protein recognition depends on the specificity of the primary antibodies, so this should be validated using parallel methods. Disadvantages of immunofluorescence are that it is usually restricted to permeabilized cells or extracellular or endocytosed proteins, and the multivalency of these probes might lead to oligomerization of target proteins on live cells. In standard immunolabeling, the size of the fluorophore-targeting complex typically exceeds 200 kD and might interfere with multiprotein recognition in protein complexes.

Genetic tagging. A key advantage of genetically encoded FPs is that they can be covalently fused to the protein of interest, so that targeting is precise (Fig. 1, C and D). Transfection and transgenic techniques often make exogenous DNA easier than dyes to deliver to cells or organisms. Limitations include the need for ectopic expression, the significant size of FPs, the possibility that the fusion may interfere with the function of the protein of interest, and the restriction to fluorescence as the only useful property. Therefore, several hybrid systems have been described by which small molecules can be covalently targeted to genetically specified proteins inside or on the surface of living cells, either by spontaneous attachment or enzymatic ligation [reviewed in (*23*–*25*)]. Most of these techniques are too new to have had much cell biological application. The most developed is the tetracysteine-biarsenical system (*26*), which requires modification of the target protein by a 12-residue peptide sequence that includes four cysteines, which binds membrane-permeable biarsenical molecules, notably the green and red dyes "FlAsH" and "ReAsH" (*27*), with picomolar affinity (Fig. 1, C and E). Small dithiol antidotes are coadministered to minimize binding and toxicity to endogenous proteins. The tetracysteine motif has undergone multiple rounds of improvement to increase its affinity for the biarsenical dyes, enabling lower dye concentrations and higher antidote concentrations to reduce background staining (*28*). Examples where protein function is less perturbed by the small tetracysteine-biarsenical combination than by FP fusions include tubulin in yeast (*29*), coupling of receptors to heterotrimeric guanine nucleotide–binding proteins (G proteins) (*30*), translocation to the nucleus (*31*), and type III secretion of pathogenic proteins from bacteria into eukaryotic cells (*32*). Tetracysteine-biarsenicals also enable manipulations not readily possible with FPs, such as affinity purification (*27*), fluorophore-assisted light inactivation (*33*, *34*), cotranslational detection of protein synthesis (*35*, *36*), pulse-chase labeling (*37*, *38*), and correlative EM localization (*37*). However, the biarsenical dyes give higher background fluorescence and poorer contrast than FPs, have not yet been demonstrated in intact transgenic animals, require the cysteines to be reduced during labeling, and do not permit two different proteins in the same compartment to be simultaneously labeled with different colors. The tetracysteine sequence occasionally provides a palmitoylation site, although such modification can sometimes be

Table 1. Applications of fluorophores in protein detection. Applicability ranges from most optimal (++) to generally not applicable (–), and (+/–) indicates applicable in some cases.

Fluorophores for examination of	Small organic dyes (antibody-targeted)	Quantum dots (antibody-targeted)	Fluorescent proteins	Genetic tags with small dyes
Endogenous proteins	++	+	–	–
Clinical specimens	++	+	–	–
Animals	Ex vivo	Ex vivo	Transgene live	Transgene ex vivo
Primary tissues	++	+	Transgene/virus	Transgene/virus
Live cells in culture	Surface	Surface	++	+
Multiple proteins at once	++	++	++	–
Dynamic interactions	+/–	+/–	++	Combination with FP
Turnover/synthesis	–	–	+	+
Activation state	Phospho-specific	Phospho-specific	FRET sensors	Combination with FP
CALI	+	–	+	++
EM	+/–	++	+/–	+
Protein microarrays	++	+	–	–
In gel fluorescence	–	–	+	+
Western blot	–	+	–	–
Major advantages	Diversity of properties	Bright and photostable	Live cells and specificity	Live cells and small size
Major limitations	Targeting in live cells	Targeting and penetration	Ectopic expression	Ectopic expression, background staining
Improvements expected	Generic conjugated primary antibodies	Smaller, diversity of properties	Better properties, generic sensors	New applications

prevented by a preceding epitope tag (*28*). Genetic tags have unique applications, but other methods should be used in parallel to decide whether tagging or ectopic expression levels are perturbing protein function or localization.

Studying Protein Expression and Localization in Primary Cells and Fixed Tissues

Expression and activity profiling with flow cytometry. To study endogenous proteins, immunofluorescence approaches are usually most suitable. Fluorophore-conjugated antibodies specific for phosphoproteins conjugated to fluorophores enable visualization of the activation state of endogenous proteins and are crucial in single-cell profiling of the activity of multiple cytoplasmic proteins simultaneously using fluorescent flow cytometry (*39*). Single-cell profiling can delineate signaling networks in cells (*40*) and might be extendable to clinical drug testing on patients' blood cells in ex vivo experiments. For cytoplasmic proteins, small dyes are currently preferable to QDs because of the better penetration in fixed and mildly permeabilized cells (Table 1). However, the brightness of QDs improves the detection limit, and the different spectral characteristics aid in multilabel separation. Using small dyes, phycobiliproteins, and QDs, 17 fluorophores have been detected simultaneously in a flow cytometer (*41*).

Protein localization at light and EM levels. The best information on localization of proteins in the context of organelles and other subcellular structures is achieved by EM. Most naked fluorophores upon illumination generate some singlet oxygen, which not only tends to bleach the fluorophore itself but can locally oxidize diaminobenzidine (DAB) to an osmiophilic polymer identifiable by EM. This process of "photoconversion" was first shown with Lucifer Yellow (*42*) and later improved by eosin immunostaining (*43*). Recently, tetracysteine-tagged proteins labeled with the biarsenical dye ReAsH were shown to photoconvert DAB in protocols that allow stringent fixation and optimal preservation of ultrastructure (Figs. 1E and 2H). Moreover, the technique allows pulse-chase distinction between young and old copies of a given protein at the EM level (Fig. 1E) (*37*). GFP has been claimed to photoconvert DAB (*44*), albeit with much lower efficiency than ReAsH.

Photoconversion works best for proteins concentrated in subcellular structures. To detect multiple or more diffuse proteins, QDs are a promising tool for protein detection at the light microscope (LM) and EM levels (Fig. 1B). The electron-dense core and the size of QDs make them visible at the EM level, and this visibility can be enhanced by silver staining (*45*). Correlated LM and EM was shown by postsectioning labeling of a nuclear protein with QDs (*46*). Recently a straightforward application to label multiple endogenous proteins simultaneously for correlated LM-EM in cells and tissues using immunolabeling with QDs has been reported (*21*). QDs permit quick LM prescreening of labeling efficiency and areas of interest before EM examination. Because of the different sizes of QDs, three different proteins can be easily discriminated (*21*). Because every QD that fluoresces should be visible in EM, all fluores-

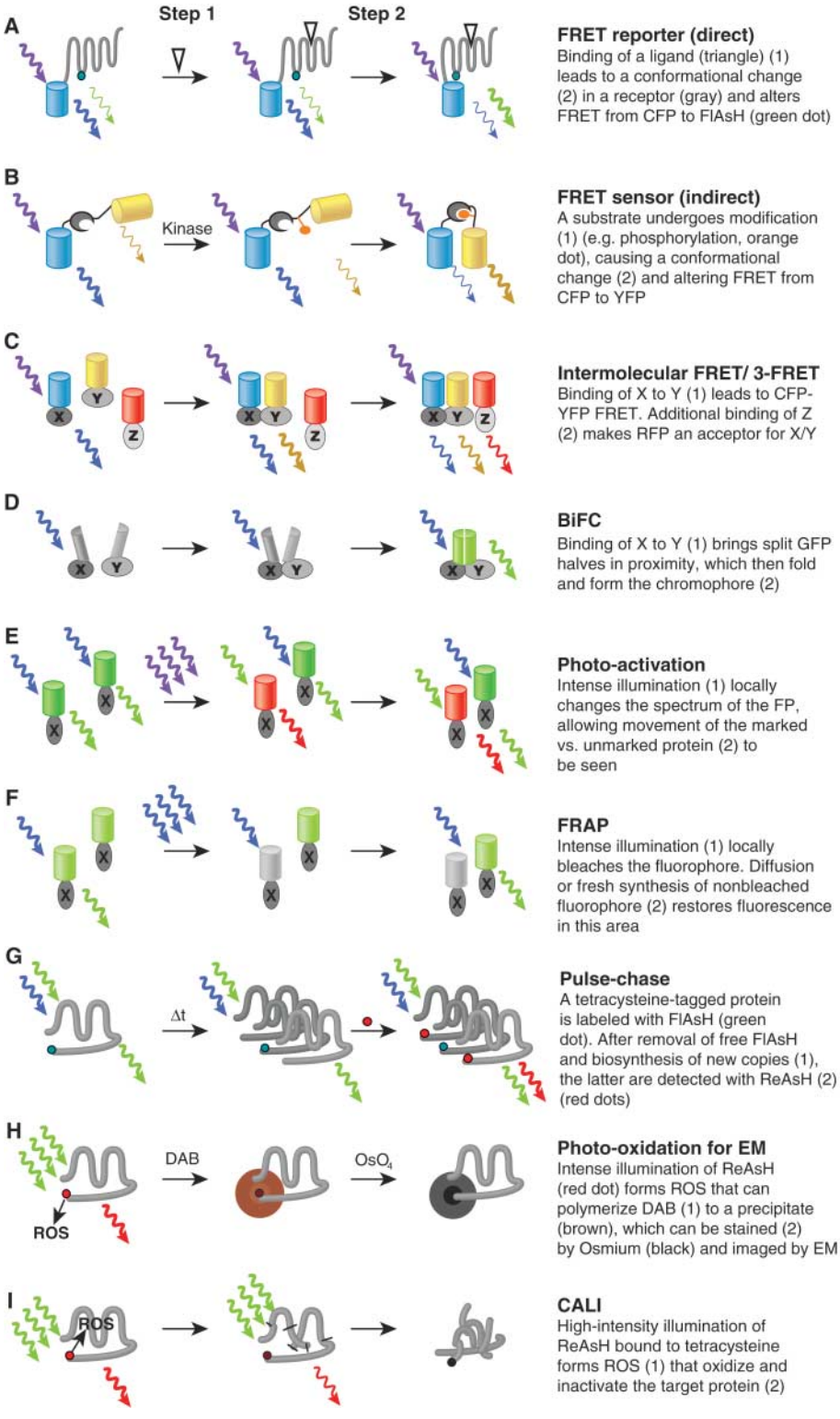

Fig. 2. Advanced fluorescent applications with genetic tags. (**A** to **I**) Principles of advanced techniques are depicted and explained. Barrels represent cyan, green, yellow, and red fluorescent proteins. X, Y, and Z represent target proteins. Light intensity is indicated by the thickness of the waves. See text for further details.

Excitation (nm):	800 (2 photon)	488	432	568	637
Emission (nm):	410-490	500-530	555-565	580-620	>660
Fluorophore:	Hoechst	GFP	QD565	ReAsH	Cy5
Targeting:	direct affinity	genetic	immuno	genetic	immuno
Target:	DNA	α-tubulin	giantin	β-actin	Cytochrome c
Structure:	nuclei	microtubules	golgi	stress fibers	mitochondria

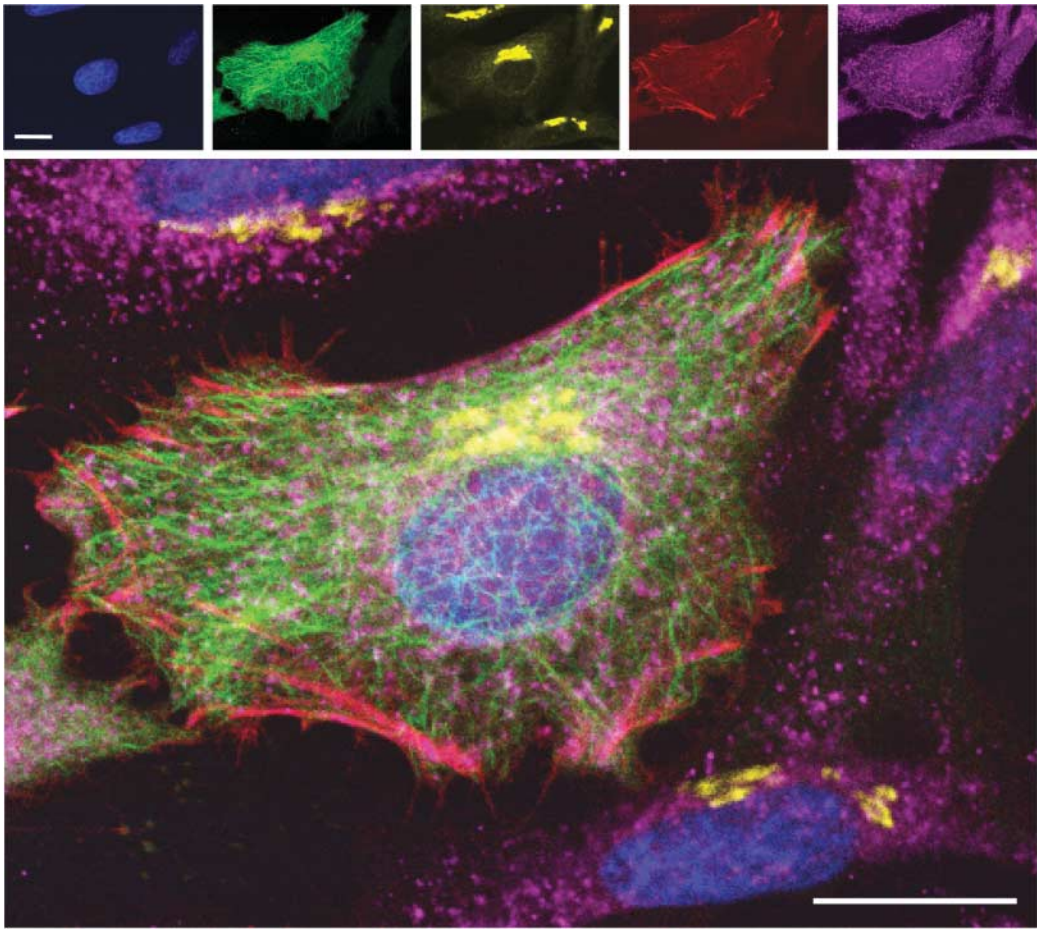

Fig. 3. Parallel application of targeting methods and fluorophores. HeLa cells transfected with GFP–α-tubulin and tetracysteine–β-actin were stained with ReAsH. After fixation, cells were immunolabeled for the Golgi matrix protein giantin with QDs and for the mitochondrial enzyme cytochrome c with Cy5 as indicated. DNA was stained with Hoechst 33342. Images were acquired from Z planes that best represent each structure using excitation and emission wavelengths as indicated. Individual channels are false-colored (middle) and merged (bottom). Scale bars, 20 μm.

cent studies using QDs could, in principle, be followed up at the EM, including nonantibody techniques such as using biotin ligase to tag cell surface proteins for detection by streptavidin-conjugated QDs (*47*). Because preembedding immunolabeling requires permeabilization and does not allow direct stringent fixation, the ultrastructure is less well preserved than when using tetracysteine-based correlated microscopy.

Protein Dynamics in Live Cells

Protein diffusion and trafficking. When a labeled protein undergoes major net redistribution, direct imaging can see this motion. Such translocation can be deliberately engineered as a readout for protein activation or second messengers, e.g., translocation of FP-tagged pleckstrin homology domains to monitor accumulation of polyphosphoinositides in the plasma membrane [reviewed in (*15*, *48*)]. But even at steady state, proteins are typically undergoing diffusion or intercompartmental exchange. Three general approaches for measuring such normally invisible fluxes are single-particle tracking, correlation spectroscopy, and photomarking methods. In single-particle tracking, the individual molecules, aggregates, or organelles must be both bright and sparse enough to be tracked from one video frame to the next, preferably by automated software. For example, low concentrations (<0.5%) of fluorescent-conjugated actin or tubulin form fluorescent speckles in filamentous actin or microtubules, respectively. The assembly, flow, and turnover within the macromolecular structure is reflected by the dynamic movement of the fluorescent speckles [reviewed in (*49*)]. Fluorescence correlation spectroscopy statistically analyzes the intensity fluctuations resulting from migration of fluorescent objects into and out of a small volume at the focus of a laser (*50*–*52*). Extensions include image correlation spectroscopy, which measures changes in fluorescence from microscopic images and thus allows more global mapping of interactions and dynamics of labeled constituents in living cells (*53*, *54*). Photochemical sensitivity of the fluorophore is undesirable in the above techniques, whereas it is essential for photomarking, another set of techniques to image protein dynamics. Various FPs (*10*, *11*, *13*) can be destroyed (Fig. 2F), dequenched, or changed in color, as by photoactivation (Fig. 2E) in a subcellular area with the use of intense local illumination (*55*). The subsequent fate of the marked molecules can then be imaged directly. For active transport, the size of the fluorescent tag is usually not very important, but for passive diffusion, small tags are much less likely to cause perturbations.

Single QDs can be imaged repeatedly because of their brightness and resistance to photobleaching, but the difficulty in targeting cytoplasmic proteins in live cells is a major limitation. For cell surface proteins, however, QDs are advancing single-molecule live-cell imaging. Glycine receptor diffusion was followed by immunotargeted streptavidin-QDs (*45*). More than a thousand high-quality images could be acquired from endogenous receptors in living neurons, which revealed that glycine receptors diffuse with different rates in extrasynaptic, perisynaptic,

and synaptic domains. Lidke and co-workers recently obtained new insight into epidermal growth factor (EGF) signaling using a combination of many of the probes and techniques reviewed here. They found that EGF-conjugated QDs specifically colocalize with an ErbB1-GFP receptor chimera and activate the receptor, as determined by Cy5-conjugated antibodies against activated ErbB1. The complex was internalized by a clathrin-dependent process, as shown by colocalization of Alexa-labeled transferrin (*56*). Retrograde transport of single EGF-QD bound to the receptor was found to depend on receptor oligomerization, as shown using spectrally distinct EGF-QDs. Fluorescence recovery after photobleaching (FRAP) of GFP-actin revealed that retrograde receptor transport and actin flow was at the same rate. Receptors did not internalize on filopodia, but at the cell body, as was found by specific quenching of extracellular EGF-QDs-streptavidin by using impermeable biotin-Alexa. Thus, filopodia might serve as antennae and could regulate transport of activated receptors to the cell body (*57*).

Conformational changes. One of the most general ways to detect dynamic conformational changes with spatiotemporal resolution is to sandwich the protein domain between two fluorophores (most commonly cyan and yellow FPs, CFP and YFP, respectively) and monitor fluorescence resonance energy transfer (FRET) [reviewed in (*15*, *58*, *59*)] (Fig. 2, A to C). A typical FRET-based sensor is shown in Fig. 2B. FRET efficiency depends both on the distance and the orientation between donor and acceptor. FRET responses to internal conformational changes most often result from reorientation rather than distance changes, because circular permutation of one of the FPs (*60*) or small changes in linker length alter the FRET response much more drastically than can be explained by any alteration in the distance between the FPs. The protein linking the FPs can itself be engineered to change conformation in response to important biochemical signals. These sensors can be targeted to specific subcellular compartments when fused to an appropriate localization signal. In this way, FRET-based indicators have been developed to measure several ions, cyclic nucleotides, metabolites, neurotransmitters, the balance between protein kinase and phosphatase activities, and activities of proteases, small G proteins, and histone acetylases (*15*, *58*, *59*).

Protein-protein interactions. FRET can also detect dynamic protein-protein interaction in live cells of ectopically expressed FP-tagged proteins (Fig. 2C), provided that the FPs get within 6 to 8 nm of each other. Recently, the possibility of using three fluorescent proteins to study higher order complexes has been addressed by adding a monomeric red fluorescent protein (mRFP) to the CFP/YFP pair. In trimeric complexes, CFP is the FRET donor for YFP; subsequently, YFP can act as a FRET donor for mRFP (Fig. 2C). 3-FRET has been shown in multiprotein complexes (*61*) and in protein trimerization (*62*). Further optimization of a higher wavelength FRET pair, as well as spectral deconvolution, might improve the 3-FRET technique.

FPs split at appropriate sites can fold and reconstitute the chromophore when the two halves are fused to interacting partners (*63*), a two-hybrid system termed bimolecular fluorescence complementation (BiFC) (Fig. 2D). Self-assembling fragments of GFP have also been reported (*64*), in which the two fragments only have to exist in the same compartment to generate fluorescence, without requiring splinting by other protein-protein interactions. BiFC can be used to study gene expression of at least two promotors, as has been demonstrated in *Caenorhabditis elegans* (*65*). BiFC has a high signal-to-background ratio, because it creates new fluorescence rather than modulating existing fluorescence. Multiple protein-protein interactions can be studied in parallel using spectrally distinct split FPs (*65*, *66*). However, BiFC is slow (hours to days) and irreversible, and the geometrical and affinity requirements for the protein-protein interaction have not yet been characterized.

In some cases, simple colocalization can be sufficient to indicate protein-protein interaction. For example, if the regulatory subunit of protein kinase A is targeted to the plasma membrane and a fluorescently tagged catalytic subunit is coexpressed, the latter is dragged to the plasma membrane but released when elevation of adenosine 3′,5′-monophosphate (cAMP) dissociates the subunits (*31*). Two-color fluorescence correlation spectroscopy can also show when partners with differently colored tags diffuse in pairs and are therefore likely to be interacting (*51*).

Protein synthesis and turnover. The determination of protein turnover requires discriminating old from new copies of the protein, typically by irreversibly labeling with one color all copies made up to a certain time. Optionally, proteins made later can be labeled with a different color after a delay. Endogenous, extracellularly exposed epitopes or receptors can be tagged with high-affinity ligands or antibodies conjugated to a fluorophore. Tetracysteine motifs can be labeled with one biarsenical, followed with another biarsenical, to study synthesis of assembled protein structures (*37*) (Figs. 1E and 2G) or to determine the subcellular site of protein translation (*38*). Chimeric proteins tagged with photomarkable FPs can be photoactivated or bleached to highlight proteins synthesized before (*13*) (Fig. 2E) or after (Fig. 2F) illumination. Some FP mutants irreversibly misfold above a critical threshold temperature, so a temperature shift can also serve as the triggering event (*67*). Yet others spontaneously change color from green to red as they complete their chromophore maturation, so that no external stimulus is required (*68*). In this case, the time resolution is modest because the color change in a population of molecules takes place over hours.

Manipulation of protein activity using chromophore-activated light inactivation. The ability of illuminated fluorophores to generate ROS, especially singlet oxygen (see EM section), can be used to inactivate the protein of interest (Fig. 2I) with much better spatiotemporal control than possible with genetic knockouts or RNA interference. This technique, chromophore-activated light inactivation (CALI), was introduced using the dye malachite green immunotargeted to the protein of interest (*69*). To avoid the difficulties of delivering dye-conjugated antibodies into living cells, the use of genetic tags for CALI has been explored. GFP (*70*), tetracysteine-bound biarsenical dyes FlAsH (*33*) and ReAsH (*34*), and a fluorescein conjugate targeted to the FK506-binding protein–12 FKBP12(F36V) (*71*) have been used to specifically destroy ectopically expressed proteins in living cells, with an order of efficiency ReAsH > FlAsH ~ fluorescein ≫ malachite green ~ GFP (*33*, *34*). Nonspecific toxic effects can be further minimized by multiphoton excitation at longer, less damaging wavelengths (*72*). "KillerRed," recently identified in a screen for phototoxic FPs (*14*), is a promising prototype of a wholly genetically encoded sensitizer for CALI and photoconversion, but it is an obligate dimer and its current CALI efficiency is less than that of ReAsH (*14*).

Seeing endogenous enzyme activity. Antibody recognition and genetic fusion are essentially stoichiometric, i.e., each target associates with one or, at most, a small number of fluorophores. Fluorogenic substrates (*1*) for endogenous enzymes offer much greater amplification and detect whether the enzyme is in an activated versus latent or inhibited state. The latter distinction is particularly important for proteases, because for many proteases only a small fraction of the total pool is active. Because of the great importance of proteases in infectious diseases, apoptosis, inflammation, tumor development, and metastasis, several approaches have recently been developed for imaging protease activity in intact animals (*73*), including disruption of FRET or other fluorescence quenching on multiply labeled substrates (*74*), and activation of cationic cell penetrating peptides to carry fluorophores onto and into cells (*75*).

Increasing spatial resolution and depth penetration. Because so many molecular and cell biological phenomena operate over distances

from one to a few hundred nanometers, much effort has been devoted to increasing the spatial resolution of fluorescence microscopy beyond the limits (~200 nm) set by classical diffraction theory. When an emitter is known to be a single point, its position can be determined with subnanometer accuracy if enough photons can be collected. Such measurements have been particularly valuable in deciphering step sizes of motor proteins and processive enzymes in vitro (*52*, *76*). A recent extension to live cells revealed that the step size of GFP-labeled peroxisomes along microtubules is ~8 nm (*77*). For more complex objects of unknown size and shape, several optically innovative approaches such as structured illumination, coherent observation with diametrically opposed objectives, and stimulated emission depletion are showing great promise (*78*–*80*).

For high-resolution imaging in scattering tissue at depths up to ~1 mm, multiphoton excitation with pulsed infrared is the method of choice because scattering is weakest in the infrared and because all of the collected emission photons must have originated from the illumination focus, even if they have suffered scattering (*81*, *82*). Fluorescence imaging at even greater depths is possible using novel serial reconstruction techniques in fixed tissue (*83*) or by tomography in live tissues, albeit with reduced resolution (*84*, *85*).

Further Considerations and Concluding Remarks

The power of fluorescence imaging is expanding rapidly because of synergistic advancements in fluorescent probes, targeting strategies, instrumentation, and data analysis, enabling high-throughput screens, single-molecule detection, multiprotein and live-cell imaging. The ecological diversity of natural FPs, further expanded by deliberate engineering by rational or randomly combinatorial mutagenesis, is producing an amazing range of useful phenotypes, although no one mutant combines all desirable properties. Reporters for many key metabolites, regulatory enzymes, and biochemical processes have already been developed, with no obvious limit in sight. Although genetic tagging is an unprecedented tool in live-cell protein imaging, other methods should be used in parallel (Figs. 1 and 3) to address whether tags perturb endogenous protein function. These include fluorophores conjugated to well-characterized antibodies, often specific for given posttranslational modifications, as well as genetic knock-in experiments to test whether the modified protein is an adequate in vivo replacement for its endogenous parent. Conversely, immunofluorescence labeling of FP-tagged target proteins tests the specificity of primary antibodies. Immunodetection using bright and stable QDs lowers the detection limit, increases the feasibility of multiprotein detection, and can be applied in correlated EM studies and in vitro assays that currently rely on enzymatic activity, such as Western blots and ELISA (Table 1). But better targeting and penetration will be required for QDs to reach their fullest potential.

Fluorescence detection will also be increasingly applied in clinical assays like protein-activity profiling in patient cells and biopsies, and to study effects of drugs on cell signaling in individual patients. Fluorescence assays are well suited for high-throughput drug screening both in biochemical assays such as protein microarrays and in functional assays in live cells or animals (*86*). The unique combination of high spatial and temporal resolution, nondestructive compatibility with living cells and organisms, and molecular specificity insure that fluorescence techniques will remain central in the analysis of protein networks and systems biology.

References and Notes

1. R. P. Haugland, *The Handbook—A Guide to Fluorescent Probes and Labeling Technologies* (Molecular Probes, Eugene, OR, 10th ed., 2005).
2. A. N. Glazer, *J. Biol. Chem.* **264**, 1 (1989).
3. A. Waggoner, *Curr. Opin. Chem. Biol.* **10**, 62 (2006).
4. A. J. Fischer, J. C. Lagarias, *Proc. Natl. Acad. Sci. U.S.A.* **101**, 17334 (2004).
5. A. J. Tooley, A. N. Glazer, *J. Bacteriol.* **184**, 4666 (2002).
6. O. Shimomura, F. H. Johnson, Y. Saiga, *J. Cell. Comp. Physiol.* **59**, 223 (1962).
7. D. C. Prasher, V. K. Eckenrode, W. W. Ward, F. G. Prendergast, M. J. Cormier, *Gene* **111**, 229 (1992).
8. R. Y. Tsien, *Annu. Rev. Biochem.* **67**, 509 (1998).
9. M. V. Matz, Y. A. Labas, J. Ugalde, *Methods Biochem. Anal.* **47**, 139 (2006).
10. D. M. Chudakov, S. Lukyanov, K. A. Lukyanov, *Trends Biotechnol.* **23**, 605 (2005).
11. N. C. Shaner, P. A. Steinbach, R. Y. Tsien, *Nat. Methods* **2**, 905 (2005).
12. J. D. Pedelacq, S. Cabantous, T. Tran, T. C. Terwilliger, G. S. Waldo, *Nat. Biotechnol.* **24**, 79 (2006).
13. K. A. Lukyanov, D. M. Chudakov, S. Lukyanov, V. V. Verkhusha, *Nat. Rev. Mol. Cell Biol.* **6**, 885 (2005).
14. M. E. Bulina *et al.*, *Nat. Biotechnol.* **24**, 95 (2006).
15. J. Zhang, R. E. Campbell, A. Y. Ting, R. Y. Tsien, *Nat. Rev. Mol. Cell Biol.* **3**, 906 (2002).
16. X. Michalet *et al.*, *Science* **307**, 538 (2005).
17. J. K. Jaiswal, S. M. Simon, *Trends Cell Biol.* **14**, 497 (2004).
18. X. Gao *et al.*, *Curr. Opin. Biotechnol.* **16**, 63 (2005).
19. A. P. Alivisatos, W. Gu, C. Larabell, *Annu. Rev. Biomed. Eng.* **7**, 55 (2005).
20. F. Pinaud *et al.*, *Biomaterials* **27**, 1679 (2006).
21. B. N. Giepmans, T. J. Deerinck, B. L. Smarr, Y. Z. Jones, M. H. Ellisman, *Nat. Methods* **2**, 743 (2005).
22. T. H. Lee, J. I. Gonzalez, J. Zheng, R. M. Dickson, *Acc. Chem. Res.* **38**, 534 (2005).
23. T. Gronemeyer, G. Godin, K. Johnsson, *Curr. Opin. Biotechnol.* **16**, 453 (2005).
24. L. W. Miller, V. W. Cornish, *Curr. Opin. Chem. Biol.* **9**, 56 (2005).
25. I. Chen, A. Y. Ting, *Curr. Opin. Biotechnol.* **16**, 35 (2005).
26. B. A. Griffin, S. R. Adams, R. Y. Tsien, *Science* **281**, 269 (1998).
27. S. R. Adams *et al.*, *J. Am. Chem. Soc.* **124**, 6063 (2002).
28. B. R. Martin, B. N. Giepmans, S. R. Adams, R. Y. Tsien, *Nat. Biotechnol.* **23**, 1308 (2005).
29. M. Andresen, R. Schmitz-Salue, S. Jakobs, *Mol. Biol. Cell* **15**, 5616 (2004).
30. C. Hoffmann *et al.*, *Nat. Methods* **2**, 171 (2005).
31. O. Dyachok, Y. Isakov, J. Sagetorp, A. Tengholm, *Nature* **439**, 349 (2006).
32. J. Enninga, J. Mounier, P. Sansonetti, G. Tran Van Nhieu, *Nat. Methods* **2**, 959 (2005).
33. K. W. Marek, G. W. Davis, *Neuron* **36**, 805 (2002).
34. O. Tour, R. M. Meijer, D. A. Zacharias, S. R. Adams, R. Y. Tsien, *Nat. Biotechnol.* **21**, 1505 (2003).
35. M. C. Rice, M. Bruner, K. Czymmek, E. B. Kmiec, *Mol. Microbiol.* **40**, 857 (2001).
36. M. C. Rice, K. Czymmek, E. B. Kmiec, *Nat. Biotechnol.* **19**, 321 (2001).
37. G. Gaietta *et al.*, *Science* **296**, 503 (2002).
38. W. Ju *et al.*, *Nat. Neurosci.* **7**, 244 (2004).
39. E. A. Danna, G. P. Nolan, *Curr. Opin. Chem. Biol.* **10**, 20 (2006).
40. K. Sachs, O. Perez, D. Pe'er, D. A. Lauffenburger, G. P. Nolan, *Science* **308**, 523 (2005).
41. S. P. Perfetto, P. K. Chattopadhyay, M. Roederer, *Nat. Rev. Immunol.* **4**, 648 (2004).
42. A. R. Maranto, *Science* **217**, 953 (1982).
43. T. J. Deerinck *et al.*, *J. Cell Biol.* **126**, 901 (1994).
44. M. Grabenbauer *et al.*, *Nat. Methods* **2**, 857 (2005).
45. M. Dahan *et al.*, *Science* **302**, 442 (2003).
46. R. Nisman, G. Dellaire, Y. Ren, R. Li, D. P. Bazett-Jones, *J. Histochem. Cytochem.* **52**, 13 (2004).
47. M. Howarth, K. Takao, Y. Hayashi, A. Y. Ting, *Proc. Natl. Acad. Sci. U.S.A.* **102**, 7583 (2005).
48. N. A. O'Rourke, T. Meyer, G. Chandy, *Curr. Opin. Chem. Biol.* **9**, 82 (2005).
49. C. M. Waterman-Storer, G. Danuser, *Curr. Biol.* **12**, R633 (2002).
50. S. T. Hess, S. Huang, A. A. Heikal, W. W. Webb, *Biochemistry* **41**, 697 (2002).
51. E. Haustein, P. Schwille, *Curr. Opin. Struct. Biol.* **14**, 531 (2004).
52. S. Weiss, *Science* **283**, 1676 (1999).
53. P. W. Wiseman *et al.*, *J. Cell Sci.* **117**, 5521 (2004).
54. P. W. Wiseman, J. A. Squier, M. H. Ellisman, K. R. Wilson, *J. Microsc.* **200**, 14 (2000).
55. T. H. Ward, J. Lippincott-Schwartz, *Methods Biochem. Anal.* **47**, 305 (2006).
56. D. S. Lidke *et al.*, *Nat. Biotechnol.* **22**, 198 (2004).
57. D. S. Lidke, K. A. Lidke, B. Rieger, T. M. Jovin, D. J. Arndt-Jovin, *J. Cell Biol.* **170**, 619 (2005).
58. E. A. Jares-Erijman, T. M. Jovin, *Nat. Biotechnol.* **21**, 1387 (2003).
59. H. Wallrabe, A. Periasamy, *Curr. Opin. Biotechnol.* **16**, 19 (2005).
60. T. Nagai, S. Yamada, T. Tominaga, M. Ichikawa, A. Miyawaki, *Proc. Natl. Acad. Sci. U.S.A.* **101**, 10554 (2004).
61. E. Galperin, V. V. Verkhusha, A. Sorkin, *Nat. Methods* **1**, 209 (2004).
62. L. He, X. Wu, J. Simone, D. Hewgill, P. E. Lipsky, *Nucleic Acids Res.* **33**, e61 (2005).
63. T. J. Magliery, L. Regan, *Methods Biochem. Anal.* **47**, 391 (2006).
64. S. Cabantous, T. C. Terwilliger, G. S. Waldo, *Nat. Biotechnol.* **23**, 102 (2005).
65. S. Zhang, C. Ma, M. Chalfie, *Cell* **119**, 137 (2004).
66. C. D. Hu, T. K. Kerppola, *Nat. Biotechnol.* **21**, 539 (2003).
67. C. Kaether, H. H. Gerdes, *FEBS Lett.* **369**, 267 (1995).
68. A. Terskikh *et al.*, *Science* **290**, 1585 (2000).
69. D. G. Jay, T. Sakurai, *Biochim. Biophys. Acta* **1424**, M39 (1999).
70. Z. Rajfur, P. Roy, C. Otey, L. Romer, K. Jacobson, *Nat. Cell Biol.* **4**, 286 (2002).
71. K. M. Marks, P. D. Braun, G. P. Nolan, *Proc. Natl. Acad. Sci. U.S.A.* **101**, 9982 (2004).
72. T. Tanabe *et al.*, *Nat. Methods* **2**, 503 (2005).
73. K. Shah, R. Weissleder, *NeuroRx* **2**, 215 (2005).
74. D. Stockholm *et al.*, *J. Mol. Biol.* **346**, 215 (2005).
75. T. Jiang *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **101**, 17867 (2004).
76. A. Yildiz *et al.*, *Science* **300**, 2061 (2003).
77. C. Kural *et al.*, *Science* **308**, 1469 (2005).

78. M. Hofmann, C. Eggeling, S. Jakobs, S. W. Hell, *Proc. Natl. Acad. Sci. U.S.A.* **102**, 17565 (2005).
79. A. Egner, S. W. Hell, *Trends Cell Biol.* **15**, 207 (2005).
80. M. G. Gustafsson, *Proc. Natl. Acad. Sci. U.S.A.* **102**, 13081 (2005).
81. W. R. Zipfel, R. M. Williams, W. W. Webb, *Nat. Biotechnol.* **21**, 1369 (2003).
82. F. Helmchen, W. Denk, *Nat. Methods* **2**, 932 (2005).
83. P. S. Tsai *et al.*, *Neuron* **39**, 27 (2003).
84. P. J. Keller, F. Pampaloni, E. H. Stelzer, *Curr. Opin. Cell Biol.* **18**, 117 (2006).
85. V. Ntziachristos, J. Ripoll, L. V. Wang, R. Weissleder, *Nat. Biotechnol.* **23**, 313 (2005).
86. P. A. Clemons, *Curr. Opin. Chem. Biol.* **8**, 334 (2004).

87. We apologize that many original research papers could not be cited because of space limitations. Work by the authors directly related to this review is supported by NIH RR04050, NS27177, GM 72033, and by the Howard Hughes Medical Institute.

10.1126/science.1124618

REVIEW

New Tools Provide New Insights in NMR Studies of Protein Dynamics

Anthony Mittermaier[1] and Lewis E. Kay[2]

There is growing evidence that structural flexibility plays a central role in the function of protein molecules. Many of the experimental data come from nuclear magnetic resonance (NMR) spectroscopy, a technique that allows internal motions to be probed with exquisite time and spatial resolution. Recent methodological advancements in NMR have extended our ability to characterize protein dynamics and promise to shed new light on the mechanisms by which these molecules function. Here, we present a brief overview of some of the new methods, together with applications that illustrate the level of detail at which protein motions can now be observed.

NMR spectroscopy is an experimental tool developed over half a century ago by physicists who were interested in exploring fundamental properties of matter. They could have hardly imagined the wide utility of their creation. One such example is in the area of structural biology, where since the early 1970s the technique has been used to study the interplay between biomolecular structure, dynamics, and function. An early experiment by Wagner and Wüthrich (*1*) foreshadowed the importance of NMR in protein science. In this seminal contribution, the authors studied the positions and intensities of peaks in one-dimensional (1D) ^{1}H NMR spectra of aromatic residues in a small globular protein as a function of temperature and found compelling evidence for rotation of bulky aromatic side chains within the hydrophobic core. This showed that proteins were in fact dynamic over a spectrum of time scales and complemented the beautiful and static pictures of protein structure that were already emerging from x-ray diffraction. We now know that there is an intimate relation between dynamics and molecular function. For example, protein dynamics contribute to the thermodynamic stability of functional states and play an important role in catalysis, where conformational rearrangements can juxtapose key catalytic residues; in ligand binding, which often involves the entry of molecules into areas that would normally be occluded; in molecular recognition processes, which are often fine-tuned by disorder-to-order transitions; and in allostery, where coupled structural fluctuations can transmit information between distant sites in a protein. NMR spectroscopy is uniquely suited to study many of these dynamic processes, because site-specific information can be obtained for motions that span many time scales, from rapid bond librations (picoseconds) to events that take seconds (*2*, *3*). Here, we review a number of recent advances in solution NMR spectroscopy that have substantially extended our ability to measure protein motions and that promise to improve our understanding of protein dynamics and their relation to biological activity.

[1]Department of Chemistry, McGill University, Montreal, Quebec H3A 2K6, Canada. E-mail: anthony.mittermaier@mcgill.ca [2]Department of Medical Genetics, Department of Biochemistry, and Department of Chemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada. E-mail: kay@pound.med.utoronto.ca

Investigating Micro- to Millisecond Time Scale Dynamics

Many biochemical events occur on the microsecond to millisecond time scale, and it is of considerable interest to characterize the conformational transitions that are involved in such processes. However, intermediates are often formed only transiently and are populated at levels that are not amenable to traditional structural approaches. Figure 1 illustrates the situation schematically for the case of a protein that can exist in two distinct conformational states, $A \underset{k_{BA}}{\overset{k_{AB}}{\rightleftharpoons}} B$, with one state substantially more populated than the other. Because the frequency of the magnetic energy absorbed by each nucleus depends on its chemical environment, a given probe will likely have distinct chemical shifts in each conformation, separated by $\Delta\nu$ (Fig. 1A). If the exchange rate, $k_{ex} = k_{AB} + k_{BA}$, between conformers is very much less than $2\pi\Delta\nu$, then separate peaks may be observed for a single site in each of the conformations, so long as the population of the minor species is on the order of a few percent. However, for systems where k_{ex} is not much smaller than $2\pi\Delta\nu$, peaks derived from the weakly populated conformer (excited state) are most often not observed, because the transient nature of this state leads to substantial peak broadening. As a result, a spectrum is obtained (Fig. 1B) where for each probe a peak is observed only from the more populated state, slightly shifted from its position in the absence of a slow exchange limit. How, then, does one obtain information about the excited state when it is essentially invisible in NMR spectra?

One way is to use an experimental approach, based on an idea from Erwin Hahn in the 1950s, called a spin echo. The basic phenomenon can be explained as follows. Imagine that a group of runners, composed of both slow and fast individ-

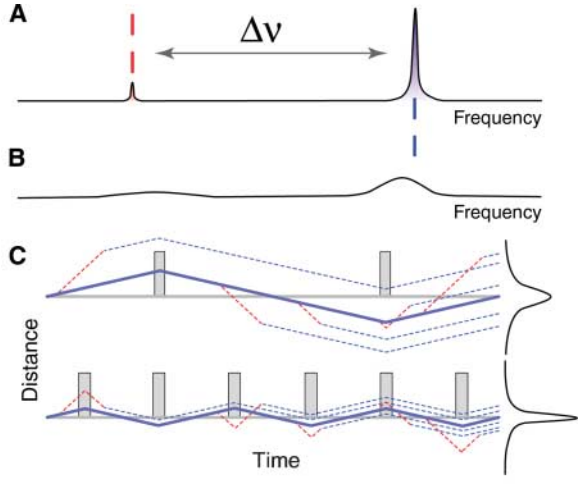

Fig. 1. Spectra from a single protein site undergoing (**A**) slow conformational exchange, $k_{ex} \ll 2\pi\Delta\nu$, and (**B**) intermediate conformational exchange, $k_{ex} \approx 2\pi\Delta\nu$. (**C**) Schematic representation of signal dephasing during CPMG pulse trains based on the analogy to the runners described in the text, where the *y* axis plots the distance of the runners from the starting position. A blue or red line indicates a spin in the major or minor state, respectively. Dashed lines correspond to spins experiencing at least one conformational transition, whereas the solid lines correspond to no transitions.