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REVIEW

Fluorescent Histochemical Techniques for Analysis of Intracellular Signaling

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SUMMARY Intracellular signaling relies on the orchestrated cooperation of signaling proteins and modules, their intracellular localization, and membrane trafficking. Recently, a repertoire of fluorescence-based techniques, which significantly increases our potential for detailed studies of the involved mechanisms, has been introduced. Microscopic techniques with increased resolution have been combined with improved techniques for detection of signaling proteins. Transfections of fluorescently tagged proteins have allowed in vivo microscopy of their trafficking and interactions with other proteins and intracellular structures. We present an overview of general signaling principles and a description of techniques based on fluorescent microscopy suited for studies of signaling mechanisms.
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extended resolution
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During the inception of the postgenomic era, protein interactions and regulatory mechanisms have been emphasized in cell biology and biomedical research. This also includes investigations of cell signaling. Diverse applications of fluorescence microscopy have earned increasing interest for such studies. Multistaining techniques with new fluorochromes, confocal microscopy, transfections with probes expressing fluorescently labeled proteins, and in vivo microscopy have all contributed to the increasing merits of fluorescence microscopy. In particular, these techniques have contributed to the notion that intracellular signaling is not the result of disordered chemical reactions of freely diffusible components but rather is spatially highly regulated. Transduction of signals is highly dependent on protein interactions, subcellular localization, and trafficking of signaling modules. Specific signaling emanates from intracellular organelles and structures, such as plasma membrane rafts, endosomes, and mitochondria. Currently, a painstaking mapping of the functions, interplay, and crosstalk among the diverse proteins and structures participating in cell regulation, and how these control specific cell functions, is taking place. Here we discuss applications of fluorescence microscopy for studies of intracellular signaling.

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Concepts of Signal Transduction

Intracellular signaling is initiated by activation of receptors in the cytoplasm and the plasma membrane. This initiates cascades of protein interactions, ultimately leading to altered activities of transcription factors in the nucleus. This review focuses on four families of plasma membrane receptors to discuss fluorescence microscopy methods in signal transduction studies: G-protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs), cytokine receptors (CRs), and the immunoreceptor (IR) family. Despite striking differences in receptor structure and function, the different transmembrane receptor families share several downstream signal transducers. Often, each receptor type can initiate several signaling chains, and different receptors can use the same or similar chains. There is ubiquitous crosstalk among the different signaling pathways, and signaling along one chain often modifies transduction along other chains. Our current knowledge of these signaling chains has been examined in several contemporary reviews (Schlessinger 2000; Gadina et al. 2001; Latour and Veillette 2001; Pierce et al. 2001; and references therein). Some of the general signaling pathways emanating from the different receptor types are depicted in Figure 1.

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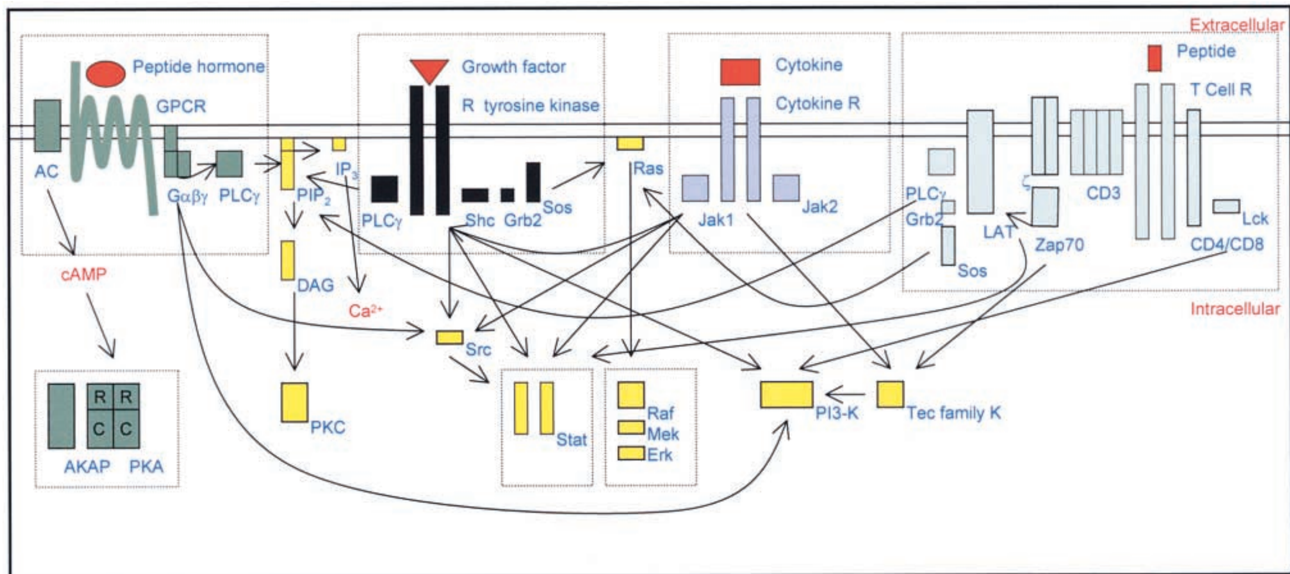

Figure 1 Overview of some important signaling modules involved in signal transduction from plasma membrane receptors. Dotted boxes indicate individual signaling modules. AC, adenylyl cyclase; R, receptor; K, kinase.

After ligand binding, plasma membrane receptors transmit signals through chains of signal transducers. Receptor or adaptor protein kinases become activated, and protein phosphorylations create docking sites for downstream transducers containing SH2 or PTB domains. Other pathways rely on activation of enzymes that produce second messengers such as cAMP, inositol-1,4,5-trisphosphate (IP_3), and diacylglycerol. Not only receptors but also many of their downstream effectors are located in or close to the plasma membrane. GPCRs are situated together with heterotrimeric G-proteins, the effector enzyme adenylyl cyclase or phospholipase C (PLC), whereas adaptor proteins such as Shc and Src and small G-proteins such as Ras are found in the vicinity of RTKs. Cytokine receptors are without intrinsic kinase activities but are complexed with tyrosine kinases of the Jak family. The immunoreceptors, T-cell receptor, B-cell receptor, and Fc-receptors, are multimeric complexes containing ligand-binding subunits, co-receptors, and chains involved in signal transduction. Signals from plasma membrane receptors are transmitted to cytoplasmic modules, often via G-protein-mediated mechanisms. Some of the most studied cytoplasmic modules are the MAP kinase module consisting of an MAP kinase kinase kinase, an MAP kinase kinase, and an MAP kinase. Examples include the Raf, MEK1, and Erk1/2 modules. There are strong indications that these modules are physically integrated by specific scaffolding proteins, as has been demonstrated for JIP-1 integrating the MLK, MKK7, and JNK modules (Whitmarsh and Davis 1998). The specific locations of signaling modules are often critical for signal speed and specificity (Pawson and Nash 2000). Modules containing different subtypes of Type II cAMP-regulated protein kinase (PKA) tetramers are attached to the plasma membrane, endosomes, mitochondria, and nucleus by distinct A kinase anchoring proteins (AKAPs) (Feliciello et al. 2001). Ultimately, signaling from plasma membrane receptors regulates nuclear transcription factors through kinase activation. Erk, JAK, and the catalytic unit of PKA all translocate to the nucleus, where they activate specific transcription factors in response to receptor activation. There is growing evidence that nuclear import and accumulation of these enzymes are regulated processes, adding to the complexity of intracellular regulation (Cyert 2001).

The significance of subcellular localization and activation-induced relocation of signaling proteins and modules for signaling efficiency and specificity has recently been realized. Plasma membrane receptors are probably not floating randomly in the lipid bilayer, as previously hypothesized by the Singer–Nicolson fluid mosaic model, but are distributed in specific regions that differ in their composition of lipids and structural proteins (Singer and Nicolson 1972; Simons and Ikonen 1997). Plasma membrane microdomains, or rafts, are very small clusters of glycosphingolipids, cholesterol, and lipids with saturated acyl chains (Brown and London 1998; Rietveld and Simons 1998). The redistribution of stimulated T-cell receptors and B-cell receptors to these structures appears to be required for rapid and efficient receptor-mediated signal transduction. (Xavier et al. 1998; Cheng et al. 1999,2001). Another specialized plasma membrane structure are the caveolae: small cell surface invagina-

tions implicated in endocytosis, lipid traffic, and signal transduction (Fielding 2001). Heterotrimeric Gα, PDGF receptor, Rap1, and PKCα are examples from a growing list of signaling proteins that have been found enriched in caveolae (Anderson 1998). After ligand binding and activation, most plasma membrane receptors are internalized. The activated receptors, often in complex with downstream signal transducers, are delivered to the endocytic pathway. They are either transferred to lysosomes for degradation or recycled to the plasma membrane. Receptor endocytosis was previously perceived simply as signal attenuation, but accumulating evidence has revealed that receptors continue to signal during endocytosis. The finding that tyrosine kinase activity for the EGF and insulin receptor was maintained after endocytosis (Cohen and Fava 1985; Kay et al. 1986; Khan et al. 1986) initiated studies of signal transduction from other endocytosed plasma membrane receptors. It has become clear that GPCR, insulin-like growth factor-1 receptor, and receptors for platelet-derived growth factor and nerve growth factor all are able to transduce signals after internalization (Lammers et al. 1989; Kapeller et al. 1993; Ehlers et al. 1995; Daaka et al. 1998). In some cases it has been demonstrated that signaling from the same activated receptor differs depending on its intracellular localization (Skarpen et al. 1998). Signaling from several RTKs and GPCRs is significantly altered when ligand-induced internalization is inhibited in dynamin- and β-arrestin-defective cells (Ceresa and Schmid 2000). It was, for example, recently proposed that endosomes are a site from which the NGF receptor induces the prolonged activation of Rap1 and MAPK (Wu et al. 2001). Nuclear import and export of signaling proteins represent another arena of signal regulation in the nuclear membrane. After mitogenic stimulation, ERK is phosphorylated and translocates from the cytoplasm to the nucleus (Lenormand et al. 1993). This can be observed by immunofluorescence staining for ERK1/2 a few minutes after stimulation of EGFRs (Skarpen et al. 2000). The cytoskeleton has also been implicated in intracellular signaling (Bahler 1996). The endoplasmic reticulum and mitochondria participate in Ca^{2+}-mediated signaling, and the mitochondrion is also a main regulator of apoptosis (Duchen 2000; Ferry and Kroemer 2001; Petersen et al. 2001).

Therefore, different principles can be identified for maintenance of signaling specificity, efficiency, and modulation. The phenotype of expressed receptors, signal transducers, regulating proteins, and transcription factors determines which pathways are available in a particular cell. Signaling proteins possesses several protein–protein (e.g., SH2 and SH3) and protein–phospholipid (e.g., FYVE) domains for specific docking along signaling cascades of pre-formed and activation-induced complexes. Scaffolding several signaling components into functional signal modules is another way of maintaining signaling specificity and efficiency. Finally, the location of signaling modules in subcellular organelles or specialized organelle regions and their activation-induced routing along membrane compartments modify the cellular effects. These principles prompt the requirement for accurate techniques to determine intracellular distribution, activation-induced translocations, and co-localization of signaling proteins in their native and activated state.

Detection of Signaling Molecules

Probes for Signal Proteins

Since the discovery of oncogenes, antibodies have been used for detection of their products, oncoproteins (Krueger et al. 1980; Anderson et al. 1982). It was later realized that the majority of oncogenes were mutated forms of genes coding for cell regulatory proteins and in particular for signal transduction proteins including growth factors, receptors, intracellular signal transducers, and transcription factors. Immunofluorescence techniques have been broadly used to demonstrate which cells or tumors express them and to reveal their intracellular localization as an indication of their function (Ward et al. 1986; Perrot–Applanat et al. 1992; Huitfeldt et al. 1996; Fonseca and Brown 1997). Initially, primarily monoclonal antibodies were implemented because they could be made highly specific to specific proteins or epitopes through strict selection procedures. Later, polyclonal antisera to short amino-acid sequences have become increasingly employed (Nigg et al. 1982a; Bulinski 1986; Gullick 1994). These can easily be produced based on the cDNA structure of a gene and therefore require neither cellular expression nor purification of the protein. However, the use of amino acid sequence- or epitope-specific polyclonal or monoclonal antibodies may lead to unexpected crossreactivity with unrelated proteins containing a similar sequence or epitope (Nigg et al. 1982b). It is therefore highly important to perform control experiments to ensure antibody specificity. Absorption with the peptide or protein of interest cannot control for all unwanted crossreactivity because other proteins may contain a similar epitope or peptide sequence. Western immunoblotting analysis can ensure that the correct protein is identified. As a control for immunofluorescence, the full Western blotting membrane should be displayed to document lack of reactivity at all but the correct molecular weight. Because Western blotting, but not immunofluorescence staining, involves protein denaturation and reduction, this technique is not a perfect control (Burry 2000).

Recently, antibodies to activated forms of signaling proteins have become available. Initially, antibodies to

phosphorylated tyrosine were utilized (Nairn et al. 1982; Comoglio et al. 1984). Tyrosine phosphorylations of many signaling proteins induce activation both by creating binding sites for downstream transducers with SH2 or PTB domains and by activating intrinsic kinase domains by alteration of the tertiary protein structure (Hubbard and Till 2000). Used in immunofluorescence, anti-phosphotyrosine antibodies cannot discriminate among different activated proteins. It was therefore a significant improvement when antibodies to phosphorylated synthetic peptides were introduced. These antibodies recognize phosphorylated tyrosine, serine, or threonine in the context of the surrounding peptide sequence. Thus, they are specific not only for certain signaling proteins but also for distinct phosphorylation sites within the same protein (Bangalore et al. 1992). Such antibodies have been used to demonstrate that the EGF receptor (EGFR) remained activated after internalization and during endocytosis (Carpentier et al. 1987), and to detect activation of the MAP kinases Erk1/2 (Yung et al. 1997). A wide range of antibodies to phosphorylated receptors, signal transducers, and transcription factors are now commercially available. However, it should be remembered that the phosphorylated sequences of different proteins might be quite similar, making crossreactivity a possibility. An antiserum raised against a synthetic phosphotyrosine-containing peptide of c-Neu also recognized the activated EGFR (Bangalore et al. 1992). In addition, the sensitivities of many of the phospho-specific antibodies are not high enough to produce reliable immunofluorescence staining. A different approach to detect phosphorylation of distinct SH2- and PTB-binding domains of EGFR was used by Emlet et al. (1997). A GST fusion protein containing the two SH2 domains of PLC-γ1, which docks to autophosphorylated EGFR, was conjugated to Cy5 and used as a probe for fluorescence microscopy. Antibodies have also been made to other activation-induced conformation alterations (Campos–Gonzalez and Glenney 1991). An antiserum to a specific non-phosphorylated 16-mer long sequence of human β-type PDGF-receptor was shown to react only with the activated receptor (Bishayee et al. 1988). Interestingly, the antiserum also recognized the activated EGFR, although the presumptive epitope containing the two flanking three-peptide sequences in this case was 169 amino acids apart. Thus, a conformational change induced by phosphorylation was implied to alter the tertiary structure of the receptor so that these sequences became closely located (Bishayee 2000). Other examples of conformational changes recognizable by antibodies include the cleaved or activated caspase enzymes (Srinivasan et al. 1998). In Western blots these are easily detected because of their shift in molecular weight. Antibodies that specifically detect the activated forms have been raised using synthetic peptides designed to match the N-terminal sequence of the cleaved product. The newly formed amino group at the N-terminus appears to constitute an essential part of the epitope (Saido et al. 1992).

Tagging of Transfected Gene Products

A different approach to study the functions of signal transducing proteins has been to transfect cells or animals with probes containing the corresponding genes. This can be the full-length gene, the wild-type gene, or the gene containing deletions or mutations in specific domains for functional studies (Scherer and Davis 1979; Barton et al. 1990). To identify these gene products both microscopically and for biochemical studies, they are often ligated to a reporter gene coding for a protein normally not found in the cell under study. The genes for several non-mammalian enzymes or peptides have been used as such tags, including genes for peroxidase, β-galactosidase (lacZ), luciferase (luc), β-lactamase (bla), bacterial GST, hemaglutinin (HA), and the FLAG peptide sequence (Spergel et al. 2001). These tags can be detected enzymatically or with appropriate antibodies and can therefore be used to study the localization of the transfected gene products microscopically.

Recently, genes encoding fluorescent proteins were introduced as tags for transfected proteins (Chalfie et al. 1994). These are well suited for fluorescence microscopy and allow in vivo microscopy of protein trafficking (Tsien 1998). Initially, the green fluorescent protein (GFP) from the luminescent jellyfish *Aequorea victoria* was used (Chalfie et al. 1994). GFP has been successfully expressed both in bacteria and eukaryotic cells and is widely used for dual-color imaging and real-time membrane trafficking studies of living cells (Rizzuto et al. 1995; Lippincott–Schwartz et al. 2001). There is now available a wide variety of GFP mutants that show increased fluorescence (e.g., EGFP), are more photostable, or display different excitation and emission properties and emit other colors such as cyan, blue, or yellow (Heim and Tsien 1996). A red fluorescent protein (DsRed) isolated from sea anemone has also recently been introduced (Matz et al. 1999). The availability of fluorescent tags with overlapping fluorescence allows fluorescence resonance energy transfer (FRET) imaging (see below). Recently a mutated red fluorescent protein (E5) that changes its fluorescence from green to red over time was introduced (Terskikh et al. 2000). This form of "fluorescent timer" can be used to analyze the "history" of gene expression and to monitor both activation and downregulation of gene expression.

A prerequisite for the use of a reporter gene is to establish that it does not interfere with the localization or function of the protein. The size of GFP (238 amino acids) suggests that it can interfere with the normal

functions of a fused protein. Whereas an EGFR construct fused to GFP was shown to function normally (Carter and Sorkin 1998), others have reported unwanted side effects of GFP fusions. For example, a cytochrome c–GFP chimera was not released from the mitochondria after Bax induction as the wild-type protein would have been (Roucou et al. 2000). There are alternatives to GFP for fluorescence protein tagging in living cells. A recombinant protein composed of six amino acids with four cysteines in an α-helical conformation has been designed for genetic incorporation into proteins of interest (Griffin et al. 1998). In living cells the recombinant protein was specifically detected by extracellular administration of the fluorescent membrane-permeant ligand 4′,5′-*bis*(1,3,2-dithioarsolan-2-yl)fluorescein. Farinas and Verkman (1999) have recently described a similar receptor-mediated fluorophore-targeting approach using single-chain antibodies. Expressed constructs of a single-chain antibody and a specific protein were labeled with cell-permeable synthesized conjugates of a hapten (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one) and different fluorescent probes. The method was used to localize specific proteins residing in the Golgi, the endoplasmic reticulum (ER), and the plasma membrane.

It should be noted that transfected genes are ectopically expressed, often at high levels. The localization and functions of the proteins may therefore be corrupted. It has been shown that overexpression of the putative scaffold protein KSR inhibits Ras signal transduction, although it was identified as a mediator of Ras signaling by genetic means (Cacace et al. 1999). Therefore, ectopic expression of scaffold proteins may severely alter the signaling properties of signal protein modules (Burack and Shaw 2000).

Detection of Second Messengers

In some cases, intracellular signaling can be detected because a signaling intermediate binds to and thus alters the fluorescent properties of a fluorochrome. In particular, such techniques have been used to study Ca^{2+} fluxes in cells after growth signals or other stimuli. Ca^{2+} signaling depends on Ca^{2+} influx from outside the cell or from intracellular stores in the ER. Voltage-operated Ca^{2+} channels and receptor-operated Ca^{2+} channels regulate influx over the plasma membrane, whereas IP_3 binding to its receptor releases Ca^{2+} from the ER (Berridge et al. 1998). Ratiometric Ca^{2+} probes (indo 1, fura 2) change excitation and/or emission maxima when bound to Ca^{2+}, whereas non-ratiometric probes (fluo 3, calcium green class, rhod 2) increase emission intensities when bound to Ca^{2+} (Takahashi et al. 1999). A limitation of most Ca^{2+} probes is their inability to penetrate cell membranes and their need to be microinjected. Ca^{2+} fluxes are studied in vivo with an inverted microscope equipped either with a digitized video camera or with a confocal scanning unit. The latter offers higher resolution but has a more limited number of excitation lines and poor temporal resolution.

Recently it has been realized that reactive oxygen species (ROS) (e.g., O^{-}_2, H_2O_2, OH^-) are signaling intermediates (Finkel 1998). Rac1-regulated NADPH-oxidase appears to be the main enzyme system involved in ROS production induced by growth factors such as EGF and NGF. Inhibition of this ROS production attenuates many of the responses to growth factors, such as proliferation and differentiation. Intracellular ROS can be detected by loading the cells with 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA), which becomes fluorescent on reaction with oxidants. This compound diffuses into the cells and intracellular esterases hydrolyze its acetate groups. The resulting 2′,7′-dichlorofluorescein then reacts with intracellular oxidants, resulting in the observed fluorescence (Bass et al. 1983). As an example, intracellular ROS production in response to EGF stimulation has been demonstrated in PC12 cells by confocal microscopy with this method (Mills et al. 1998). It has been demonstrated that dihydrofluorescein diacetate may be a superior probe for confocal microscopy (Hempel et al. 1999).

Demonstration of Gene Activation

Several techniques have been applied to detect gene activation, the end point in signaling cascades emanating from the plasma membrane, at a cellular level. For example, immunocytochemistry (ICC) has been used to detect c-fos protein induction in specific cholinergic and non-cholinergic neurons of rat basal forebrain after ventricular instillation of NGF (Gibbs and Martynowski 1997). Similarly, in situ hybridization (ISH) techniques have been used to detect mRNA induction of c-fos and other genes after growth factor stimulation of hippocampal neurons (Ip et al. 1993). Non-radioactive ISH techniques for detection of mRNA have been described elsewhere (Hougaard et al. 1997). Activation of transfected genes tagged with a reporter gene can also be studied by fluorescence microscopy (Kyung et al. 2001). The DNA-binding capability of activated transcription factors can also be detected using labeled DNA sequences representing their response elements (Wang et al. 1992; Koji et al. 1994). Such methods have been named Southwestern histochemistry or in situ DNA–protein binding.

High-resolution Localization of Signaling Proteins

Multicolor Overlaying

The increasing versatility of fluorescence microscopy for signaling studies relies partly on the ability to de-

tect and compare several independent probes in the same cell sample or tissue section. Multicolor staining techniques can be used to identify cell populations expressing a particular signal transducer, to define which subcellular organelles contain a signal transducer, and to determine if different signal transducers co-localize and may therefore interact. Such techniques have been used to show that pre-neoplastic liver lesions and unaltered hepatocytes contain similar levels of EGFR, whereas bile duct cells and putative stem cells contain considerable lower levels (Huitfeldt et al. 1996). Several internalized ligand-activated receptors have been found co-localized with their downstream signal transducers, suggesting that they remain signaling-competent (Ceresa and Schmid 2000). Two-color immunofluorescence microscopy was important to demonstrate the location of internalized EGFR together with Shc, Grb2, and Cbl in early and late endosomes (Oksvold et al. 2000,2001). Multicolor techniques have also been applied to double-transfected cells using GFP variants with different fluorescence spectra (Ellenberg et al. 1999).

Immunological multistaining recquires crossover controls to ensure absence of immunological crossreactions (Brandtzaeg et al. 1997). In addition, the combination of fluorochromes requires careful consideration of epifluorescence filters, laser excitation lines, and fluorochrome specifications. Since the finding by Marrack (1934) that covalent addition of dye molecules to antibodies did not affect their ability to bind antigen, a diversity of fluorochromes have been introduced. In addition to the classical fluorescein isothiocyanate (FITC)- and rhodamine-derived fluorochromes, several new fluorochromes have been introduced as more intense or longer-wavelength alternatives. These include the rhodamine Red-X and Texas Red-X fluorochromes and the cyanogen family of fluorochromes (Cy2, Cy3, Cy5, Cy5.5, and Cy7) (Mujumdar et al. 1993; Lefevre et al. 1996). Recently, Alexa dyes with a wide variety in their emission and excitation maxima have become available. These generally show high intensities and are more resistant to fading (Panchuk–Voloshina et al. 1999). When fluorochromes are chosen, different aspects should be considered: the excitation maximum should match the lamp or laser, resistance to photobleaching is important when strong or prolonged excitation is needed, overlapping emissions can perturb multistaining, and the fluorochrome size may impede staining penetration. Although Texas Red and Alexa 594 are excellent choices for a krypton laser, Cy3 or RRX may prove more suitable for an argon laser. When two or more fluorochromes are observed or recorded simultaneously, bleed-through may become a problem if the emissions of the two fluorochromes are not well separated. If FITC emission is strong, its emission may be detected as red with a monochrome camera or photomultiplier tuned to detect weak rhodamine emission. Sequential detection of fluorochromes will often solve this problem. In addition, multistaining techniques require highly specific secondary immunoglobulins and strict controls to rule out crossreactivity. In practice, when fluorochrome reagents are purchased as conjugates with antisera to immunoglobulins, the fluorescence is highly dependent on the antiserum quality. It may be difficult to find combinations of fluorochrome reagents and antisera for multicolor staining that are appropriate for the light source, show minimal crossreactivity, and have high fluorescence efficiency.

Analysis of multistained cells requires accurate overlaying of the images representing each fluorochrome. Often it is also necessary to quantify fluorescence intensities, the degree of fluorochrome co-localization, or the intensity ratios of two or more fluorochromes. Images captured by video or digital cameras or by confocal laser scan microscopes are well suited for computerized image analysis for such purposes (Huitfeldt et al. 1991). Although software for specific microscopic systems or analyses are available, flexible and complete general-purpose software for fluorescence analysis would be welcomed.

Markers of Intracellular Organelles and Structures

For functional characterization of signaling mechanisms, it is necessary to precisely define the intracellular localization of the participating signaling transducers. It was early realized that oncogene protein functions relied on their intracellular localization (Weinberg 1985). Since then, it has become clear that intracellular signaling is not the result of freely occurring chemical reactions among soluble reactants, initiated at the plasma membrane and diffusing to the nucleus. These reactions are spatially highly regulated, often located on specific intracellular organelles or structures, and organized in modules containing subsets of signaling transducers (Leof 2000), thereby obtaining signaling efficiency and specificity. At the same time, this organization contributes to signaling diversity and modulation. The precise intracellular localization of many signal transducers is still not well characterized, which raises controversies about their functions (Prior et al. 2001; White and Anderson 2001). A primary signaling organelle is the plasma membrane. In polarized cells, different signaling functions may be attributed to the basolateral or apical cell membranes (Khurana et al. 1996; Saunders et al. 1998; Muallem and Wilkie 1999). At present the role of specialized plasma membrane regions, so-called cholesterol-enriched rafts, in signaling is under exploration (Simons and Toomre 2000). In addition, compartments of the

endocytic pathway have been implicated in intracellular signaling (McPherson et al. 2001). These examples illustrate the need for specific markers of different subcellular organelles and structures. Some fluorescent or fluorochrome-labeled chemicals show high affinity towards specific organelles (e.g., mitochondria staining with MitoTracker) or alter fluorescence properties based on pH (e.g., LysoTracker in the endocytic pathway) or redox status (RedoxSensor Red). Antibodies recognizing unique organelle proteins or lipids have also been widely used for such purposes. Whereas the list of available markers is long, the current nomenclature and precise localization of subcellular markers is often confusingly described in the literature. An overview of suitable markers for subcellular organelles and structures involved in signal transduction is given in Table 1. In many cases one can also use antibodies to proteins that anchor signaling proteins or modules to specific organelles. Good examples of such are the AKAP proteins, which anchor different cAMP-dependent protein kinases to specific subcellular organelles (Feliciello et al. 2001). It is our impression that many of these markers are more extensively distributed in the cells than their original characterizations indicate. It is important to have in mind certain criteria for subcellular markers. First, the distribution of the markers should not change due to external or internal stimuli. We have found that the plasma membrane marker PMCA redistributes along the endocytic pathway after growth stimulation (MPO, ES, and HSH, personal observations). Second, the membrane markers should be expressed at levels detectable with ICC and Western immunoblotting analysis, preferable in most cell types. We have, for example, not been able to obtain reliable immunofluorescence detection of Rab proteins as markers of intracellular organelles in untransfected cells.

It is now also possible to identify specific organelles in living cells transfected with organelle-targeted GFP (Pizzo et al. 1995). Several organelles can be distinguished in the same cell using combinations of fluorescence-shifted GFP mutants (Rizzuto et al. 1996). Probes for labeling of plasma membranes, endosomes, mitochondria, and nuclei are now commercially available. In combination with GFP-tagged signal transducers, such probes will become increasingly useful for signal transduction studies of cultured cells.

Microscopic Resolution and Optical Sectioning

Confocal microscopy offers increased horizontal and, in particular, vertical resolution compared to conventional fluorescence microscopy (White et al. 1987). This has been advantageous for intracellular localization in subcellular structures. The theoretical resolution limit of conventional light microscopy, known as the “diffraction resolution barrier,” is half the wavelength of light, which means about 0.2–0.4 μm for visible light. Whereas protein sizes are in the 1–10-nm range, mitochondria are about 0.3–1 μm wide, endosomes about 0.2 μm, and plasma membrane rafts are believed to be smaller than 50 nm. Compared to conventional microscopy, confocal microscopy improves resolution by a factor of 1.4 (2 if deconvolution is applied). Therefore, this improvement is significant for studies of intracellular organelles. In practice, confocal fluorescence resolution is highly dependent on the light intensity. At low intensities the pinhole must be

Table 1 Organelles/structures involved in intracellular signaling and markers for their individual detection

Organelle/structure	Marker	Examples of signaling proteins	References[a]
Plasma membrane	PMCA	EGFR, GPCR, CR	1, 2, 3, 4
Plasma membrane raft	DiI, filipin	BCR, TCR	5, 6, 7
Caveolae	Caveolin	PKCα, PLCγ, TrkA, Src, Ras	8, 9
Clathrin-coated pit	Clathrin H-chain	EGFR, GPCR, CR	10, 2, 3
Primary vesicle	Clathrin H-chain	EGFR, GPCR, CR	10, 2, 3
Early endosome	EEA1, Rab5	EGFR, Shc, Grb2, ERK1/2	11, 12, 13, 14
Recycling endosome	Transferrin, Rab4	?	15, 16
Multivesicular body	pAnnexin I	EGFR, MAPK ERK	17, 18, 14
Late endosome	CD63, M6PR, Rab7	EGFR, ERK, MP1	19, 20, 12, 14
Lysosome	LAMP-1	?	21
Caveosome	Caveolin-1	?	22
Endoplasmic reticulum	Calnexin, calreticulin	Bcl-2	23, 24
Mitochondrion	hsp60	NGFI-B, Bax, Bcl-2	25, 26, 24
Centrosome	Centrin, γ-tubulin	PKAIIα, Phos. diesterase 4D	27, 28, 29
Microtubuli	α-Tubulin	Rac, JNK, MAPK	30
Nucleus	LAP2	NFk-B, MAPK	31, 32

[a]1, Garcia and Strehler 1999; 2, Schlessinger 2000; 3, Pierce et al. 2000; 4, Gadina et al. 2001; 5, Simons and Toomre 2000; 6, Xavier et al. 1998; 7, Cheng et al. 1999; 8, Okamoto et al. 1998; 9, Anderson 1998; 10, Schmid 1997; 11, Mu et al. 1995; 12, Chavrier et al. 1990; 13, Di Guglielmo et al. 1994; 14, Oksvold et al. 2001; 15, Willingham et al. 1984; 16, Daro et al. 1996; 17, Futter et al. 1993; 18, Felder et al. 1990; 19, Kobayashi et al. 2000; 20, Geuze et al. 1988; 21, Carlsson and Fukuda 1992; 22, Pelkmans et al. 2001; 23, Wada et al. 1991; 24, Krajewski et al. 1993; 25, Craig et al. 1993; 26, Ferry and Kroemer 2001; 27, Salisbury JL 1995; 28, Feliciello et al. 2001; 29, Tasken et al. 2001; 30, Gundersen and Cook 1999; 31, Furukawa et al. 1995; 32, Cyert 2001.

increased, which reduces the resolution and increases the thickness of optical sections. Low intensities also reduce the signal-to-noise ratio. Under such conditions, a conventional fluorescence microscope equipped with a digital camera may provide better images. Although the vertical resolution of confocal microscopy is much poorer than the horizontal resolution, it makes optical sectioning through individual cells possible. Therefore, the interior of cells or nuclei can be visualized unaffected by fluorescence from surrounding plasma membrane or cytoplasm. This feature has been exploited in a number of signaling studies. However, the uneven shape of cultured cells easily obscures the location of plasma membrane proteins, particularly when they are enriched in specific regions. EGFR and caveolin-1, a structural and signal protein-integrating protein found in caveolae, may easily be perceived as located in intracellular vesicles when viewed by conventional fluorescence microscopy or in a horizontal optical section by confocal microscopy (Figure 2). Three-dimensional reconstruction reveals that both EGFR and caveolin-1 are enriched in structures at the cell surface (Figure 2). Immunoelectron microscopy confirms the plasma membrane location of caveolin-1 in aggregates of invaginated plasma membrane. For detailed studies of the intracellular localizations of caveolar proteins, immunoelectron microscopy is highly recommended (Simons and Toomre 2000).

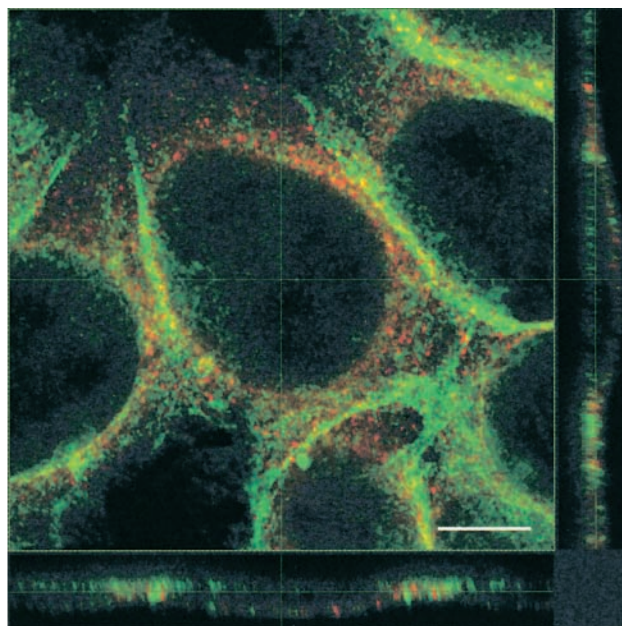

Figure 2 Three-dimensional reconstruction of HeLa cells stained by two-color immunofluorescence for EGFR (green) and caveolin-1 (red). A horizontal (xy) section leaves the impression that EGFR was located both along the plasma plasma membrane and in cytoplasmic organelles, whereas caveolin was enriched in cytoplasmic organelles. Vertical sections obtained from 3D reconstructions (yz right and xz, bottom) demonstrated that EGFR was predominantly located in the plasma membrane, whereas the distribution of caveolin was limited to the plasma membrane facing the culture dish. Serum starved cells were fixed in 4% paraformaldehyde and exposed to 0.2% Triton-X-100. Immunostaining with sheep anti-EGFR and rabbit anti-caveolin was detected with Cy2-conjugated donkey anti-sheep and RRX-conjugated anti-rabbit immunoglobulins, respectively. Confocal images were obtained with a ×100, NA 1.25 objective in a Leica TCS SP confocal microscope equipped with TCS NT software. Thirty optical sections were used for 3D reconstruction. Bar = 10 μm.

Different techniques have been developed to extend the resolution of fluorescence microscopy. Images from confocal microscopy and digital fluorescence microscopy can be digitally deconvoluted (Wang 1998). When a series of images at different depths are compared, out-of-focus fluorescence can be removed using the point-spread function of the microscope optics. Such techniques have been used to demonstrate endocytosis of EGFR (Benveniste et al. 1989; Carter and Sorkin 1998). In addition, optical techniques to improve resolution have been developed, rendering a resolution of 0.1 μm or better possible (reviewed by Gustafsson 1999). Recently, a promising technique obtaining a significant increase in optical resolution was described by Klar et al. (2000). The process, termed point-spread function engineering by stimulated-emission depletion (PSFE by STED) improves confocal sectioning approximately fivefold. This is achieved by quenching excited fluorochromes along the edge of the focal spot through stimulated emission, reducing the spot size. The axial resolution can also be improved by the coherent use of two opposing lenses. Interference of the excitation or emission light (or both) is utilized in different techniques, termed 4Pi confocal microscopy, or image interference microscopy (I2M) when applied to widefield microscopy (Hell et al. 1997; Schrader et al. 1998; Gustafsson 1999). Axial resolution better than 100 nm has been reported with these techniques. These methods have recently been demonstrated in the imaging of membrane-stained living bacteria, in which a fourfold increased axial resolution was achieved (Bahlmann et al. 2001). These techniques have until now not been commonly available, and their potential for signaling studies remains unexplored. With evanescent wave microscopy (also called total internal reflection fluorescence microscopy) it has been possible to demonstrate in vivo binding of single EGF molecules to the plasma membrane of cultured A431 cells (Sako et al. 2000). By this method, it was shown that a complex of one EGF molecule and an EGFR dimer was formed, followed by the arrest of a second EGF molecule and receptor phosphorylation. In another study, this technique was used to demonstrate plasma membrane translocation of a GFP-labeled pleckstrin homology domain of Akt induced by PDGF (Haugh et al. 2000). This technique is based on the fact that light hitting a

less dense medium beyond a certain angle is reflected (Toomre and Manstein 2001). Some of the light, however, slightly penetrates the less dense medium. The intensity of this light falls off exponentially from the density interface, and an approximately 70-nm layer is selectively illuminated. The change in light density is obtained at the "basal" dish–cell interface, and at the "apical" cell–culture medium interface, making fluorescence microscopy close to the plasma membrane possible at high resolution.

Dynamics of Protein Interactions and Trafficking

In Vivo and Two-photon Microscopy

By in vivo fluorescence microscopy, the movement of signaling molecules and their allocations to cellular organelles can be studied in cultured cells, or in some cases in cultured organ slices (Rizzuto et al. 1998). Depending on the kinetics of the biological event being studied, such experiments can be performed at video rate, or as time-lapse microscopy with sequentially captured images. In addition to the sensitivity and resolution considerations, image acquisition speed needs concern. Fast-moving objects require short, possible video rate image capture, and this may deteriorate sensitivity and resolution. In addition, the fluorochrome fading characteristics must be related to exposure times and excitation intensities. In particular, such methods have been applied to Ca^{2+} measurements and in investigations of ligand–receptor interactions and internalization. For example, fluorochrome-labeled EGF binding to EGFR and consequent complex endocytosis was demonstrated by such means (Schlessinger et al. 1978; Benveniste et al. 1989; Hopkins et al. 1990). Lately, in vivo protein trafficking has also been investigated with GFP-tagged transfected probes (Royston and Sorkin 1998). Both inverted and upright microscopes have been used, the latter with water-immersion objectives. In both cases temperature and pH must be controlled, preferably in an incubator.

In vivo microscopy of thick multicellular preparations, such as brain slices and embryos, has gained from the development of two-photon microscopy (Denk et al. 1990; Piston 1999; Konig 2000). In this technique, excitation arises from the simultaneous absorption of two photons. A fluorophore that normally absorbs ultraviolet light (~350 nm) can also be excited by two red photons (~700 nm) if they reach the fluorophore at the same time. To obtain this, the photon density must be approximately a million times what is required for one-photon absorption. Pulsed lasers are used to obtain this. The peak laser power is very high but the average power is only slightly higher than in confocal microscopy. Because two-photon excitation is obtained only at the focal point of the microscope, photobleaching and cell damage are minimized, which is crucial for in vivo microscopy. The sample penetration is also increased, often two- to threefold what is obtainable with confocal microscopy. The resolution of two-photon microscopy may be somewhat poorer than that of confocal microscopy because longer excitation wavelengths are used. In particular, Ca^{2+} signaling has been studied with this technique, but GFP-tagged AMPA receptor distribution in living neurons has also been investigated with this method (Koester et al. 1999; Shi et al. 1999).

Measurements of Protein Mobility In Vivo

Fluorescence recovery after photobleaching (FRAP) was introduced 30 years ago as a technique to monitor the mobility and dynamics of fluorescent proteins in living cells. With the introduction of GFP its usefulness has increased, and FRAP is now widely used in studies of intracellular signaling (reviewed by Reits and Neefjes 2001). In this technique, fluorescent-tagged proteins are irreversibly photobleached in a small part of the cell with maximal laser intensity. The re-distribution of non-bleached fluorescent molecules to the photobleached region is then visualized in the microscope. This method has several advantages: it is fast, suitable for most confocal microscopes, and easy to establish. A common FRAP application is to study the mobile fraction of a protein, i.e., fraction that is free to diffuse into the bleached region during a time interval. For example, Mochizuki and co-workers (2001) used FRAP to visualize turnover of Ras and Rap1 at the neurite. In this work, yellow-emitting mutant GFP conjugated to Ras and Rap1 was photobleached in the neurites of differentiated PC12 cells and the fluorescence intensity was recovered within 240 sec. In another study, FRAP was used to examine the intranuclear dynamics of fluorescent estrogen receptor-α (Stenoien et al. 2001). With this technique, it was demonstrated that the estrogen receptor exhibits differential mobility depending on several factors that are linked to its transcription function.

Whereas FRAP measures recovery of fluorescence, a technique called fluorescence loss in photobleaching (FLIP) characterizes the reduction of fluorescence in cellular regions when a small region is photobleached. With this technique, Lillemeier et al. (2001) introduced a random walk model for movement of STAT1 from the plasma membrane to the nucleus after stimulation with interferon. In this study, FLIP and FRAP analysis of a GFP–STAT1 fusion protein were combined. When a specified area (approximately 20 μm^2) of the cytoplasm or the nucleus was scanned with maximal laser power, FLIP was clearly observed in the whole cytoplasm and nucleus, respectively.

Detection of Protein Binding in Intracellular Organelles

Very low levels of a fluorescently labeled molecule can be measured, and its binding to other structures determined, by fluorescence correlation spectroscopy (Eigen and Rigler 1994; Maiti et al. 1997; Lippincott–Schwartz et al. 2001). Applied to confocal or multiphoton microscopy, the method has been termed fluorescence correlation microscopy (FCM) (Brock and Jovin 1998). Very small sample volumes (~1 femtoliter or less) can be measured in living cells, allowing studies of plasma membrane regions and intracellular organelles. FCM measures the fluctuations in emission when fluorescently labeled molecules diffuse in and out of a defined volume. These fluctuations reflect the average number of labeled molecules in the volume, as well as the characteristic diffusion time of each molecule across the defined volume. Binding of a labeled molecule with another (unlabeled) molecule will slow down the diffusion and is readily detectable. This technique has just recently been adapted to microscopy and its feasibility is still not fully explored. However, it was used to characterize tagged EGFR diffusion in different cellular structures (Brock et al. 1999). In addition, FCM detected binding of proinsulin C-peptide to human cell membranes and characterized the binding of Raf-1 to activated Ras (Rigler et al. 1999; Trier et al. 1999).

Molecule Interactions by Fluorescence Resonance Energy Transfer (FRET)

Protein interactions, either among components of a signaling cascade or among signaling and scaffolding proteins, are essential for signal transduction. Demonstrations of such interactions are therefore useful verifications of signal activation or other regulatory mechanisms. Traditionally, co-immunoprecipitation analyses have been used to detect such protein interactions. These assays are often difficult to perform because signaling complexes easily break up during protein extraction from cells. Furthermore, proteins may co-precipitate on the basis of their presence in the same membrane and not because of direct interactions. Similarly, induction of signaling protein co-localization can be monitored by fluorescence multi-staining techniques. For example, EGFR and Shc co-localize in early endosomes after EGF stimulation. The resolution limit of conventional microscopy is not good enough to prove direct molecular interaction by co-localization. Fluorescence resonance energy transfer (FRET) is a method that can detect protein co-localization in the 5-nm range and thus can provide a much better basis for proving direct interactions. The technique is based on the fact that when two overlapping fluorochromes are closely located, excitation of the lower-wavelength fluorochrome induces energy transfer to the higher-wavelength fluorochrome (Clegg 1995). When closely located, excitation of FITC by a 488-nm light source will not lead to the normal emission with a 520–540 nm peak but rather energy transfer to a closely situated rhodamine fluorochrome, which then will emit light in the 560–600-nm range. Thus, the emission is shifted from green to red light. This effect is highly dependent on the distance between the fluorochromes and is negligible when they are more than ~5 nm apart. Energy can also be transferred to a closely situated non-fluorescent molecule, and this can be detected as attenuation of emission. Energy transfer also protects against fading, which can be exploited to detect protein interaction. FRET and related techniques can be adapted to microscopic examinations and have been particularly useful for studies of cultured cells doubly transfected with genes tagged to GFP mutants of overlapping fluorescence. The significance of these methods is their potential to directly detect protein interactions in living cells. Recently, visualization of receptor-mediated activation of G-proteins by monitoring FRET between α- and β-subunits fused to cyan and yellow fluorescent proteins was described in living *Dictyostelium discoideum* cells (Janetopoulos et al. 2001). Mochizuki and co-workers (2001) demonstrated the use of a "Ras and interacting protein chimeric unit" to compare interaction between Ras/Rap1 and Raf with FRET. Binding of GTP-Ras to Raf resulted in an intramolecular conformational change that brought CFP close to YFP, which resulted in FRET. A fluorescent indicator for tyrosine phosphorylation of the insulin receptor substrate-1 (IRS-1) based on FRET has also been reported (Sato et al. 1999). The FRET pair was a synthetic phosphopeptide from the tyrosine phosphorylation domain of IRS-1 and a targed SH2 protein, labeled with fluorescein and tetramethyl rhodamine, respectively. FRET techniques have also been used to detect signaling from internalized plasma membrane receptors. Sorkin (2001) recently used FRET measurements to study the localization of signaling complexes of EGFR(CFP)–Shc(YFP) and Shc(YFP)–Grb2(CFP) in endosomes of EGF-stimulated cells. Although endocytic EGFR–Grb2/Shc complexes have been described earlier, this work exemplifies the incorporation of new more accurate methods for studying signal transduction from receptor complexes in endosomes. FRET is not limited to interaction studies between proteins, but can also be used to monitor physiological effects within a specific protein. Fusion constructs of GFP variants separated by a sequence that changes conformation after modification have been used to detect kinase activity (Nagai et al. 2000), protease activity (Xu et al. 1998), and Ca^{2+} release (Miyawaki et al. 1997). FRET microscopy tech-

niques have been limited to combinations of cyan/yellow and blue/green fluorescent proteins because of their appropriate spectral separation. Recently, Harpur and co-workers (2001) introduced a FRET method that was independent of spectral separation of the GFPs. This technique takes advantage of the fluorescence lifetime of the combined donor/acceptor emission by fluorescence lifetime imaging microscopy. Thus, a combination of EYFP and EGFP can be monitored for FRET. The major benefit is that EYFP and EGFP are brighter than the other GFP mutants.

Conclusions and Perspectives

During the post-genomic era, emphasis is turning from the identification of genes and proteins towards characterization of their functions. Multicolor fluorescence techniques have proved highly versatile for such studies. In particular, this is due to the significance of accurate intracellular localization and co-localization of signal transducers with other proteins. Recent publications have presented the prospect of increasing the confocal microscopic resolution even beyond the resolution limit of light. At present, the intracellular localization of signaling proteins has much to gain from a better supply and characterization of markers for intracellular organelles and subregions. The development of user-friendly, general-purpose image analysis programs integrating image overlaying, co-localization analysis, fluorometry, 3D reconstruction, image ratioing, and time-lapse analysis will further this progress. Furthermore, the inherent possibility of crossreactions, which in many cases is difficult to control for, underscores the need to supplement fluorescence microscopy with other techniques including Western blotting, co-immunoprecipitation, subcellular fractionation techniques, and immunoelectron microscopy.

Particularly for studies of transfected, wild-type, and mutated signal proteins, fluorescence microscopy has become indispensable for characterization of specific molecular mechanisms. In vivo fluorescence, in which trafficking of signaling proteins can be examined in living cells under controlled conditions and after specific stimuli, is receiving increasing attention. Such techniques are particularly suited for FRET analysis for direct demonstration of protein interactions. It should be kept in mind, however, that the effects of ectopically expressed proteins do not necessarily reflect physiological processes. Overexpression of a protein may titer out binding proteins, as observed for AKAP proteins, or may lead to non-physiological intracellular localization (Burack and Shaw 2000).

The prospect of "imaging biochemistry inside cells" has been introduced (Wouters et al. 2001). We are on the verge of the possibility to directly visualize chemical interactions in cellular organelles in vivo. Close collaborations between genetic engineers and microscopists are crucial to achieve the maximal advantage of these techniques.

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