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Technical Advancement

Recent Advances in Fluorescent Labeling Techniques for Fluorescence Microscopy

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Tremendous progress in recent computer-controlled systems for fluorescence and laser-confocal microscopy has provided us with powerful tools to visualize and analyze molecular events in the cells. Various fluorescent staining and labeling techniques have also been developed to be used with these powerful instruments. Fluorescent proteins such as green fluorescent protein (GFP) allow us to directly label particular proteins of interest in living cells. This technique has been extended over a large area of cell biology, and a variety of fluorescent protein-derived techniques have been developed to visualize the functions and conditions of the molecules within living cells. In this review, we summarize the techniques for fluorescent staining and labeling for recent fluorescence microscopy.

Key words: fluorescence microscopy, fluorescent labeling, immunofluorescent staining, fluorescent dyes, fluorescent proteins

I. Introduction

Since biology entered the new post-genome era, the function of each gene product in the cell has attracted researchers' interests. The development of techniques of fluorescent protein (FP) labeling and recent computer-controlled systems for fluorescence and laser-confocal microscopes has also boosted our eagerness to see directly the behavior of particular proteins of interest in living cells [17, 26]. New techniques for fluorescent labeling of molecules have been devised depending on such requirements to see directly into the molecules in living cells. Live-cell imaging techniques make it possible to see the molecular basis of a variety of functions in the cell [12, 20]. Originally, fluorescence microscopy itself was a powerful technique to visualize the location of particular molecules in the cell [30, 31]. The combination of new techniques for fluorescence labeling and staining with advanced fluorescence microscopy has provided us with new tools to analyze the dynamic behavior of cellular molecules.

Before the emergence of GFP-labeling technique, the advantage of fluorescence microscopy was the detailed mapping of the molecules in cells and tissues by multiple fluorescent staining. After the advent of GFP-labeling, fluorescence microscopes including laser-confocal microscopes have been actively involved in elucidating the molecular functions in the cell as follows: 1) live-cell imaging for the molecular dynamics by FP-fusion probes [12]; 2) the analyses of the activity or status of signal transduction by visualizing the binding of proteins with other molecules or their folding in itself by fluorescence resonance energy transfer (FRET) microscopy [9, 18] or fluorescence correlation spectroscopy [19]; 3) the analyses of movement and trafficking of particular proteins by fluorescence recovery after photobleaching (FRAP) techniques [35]; 4) the analyses of turnover of proteins by employing molecular tags that specifically bind the particular membrane-permeable dyes [4]. In this review, we summarize the techniques for fluorescent staining and labeling for recent fluorescence microscopy.

II. Overview

The techniques of fluorescent labeling of molecules are divided broadly into two categories: the conventional fluorescent staining and the molecular tagging by introducing the genes of FPs or specific binding motifs for fluorochromes by genetic engineering. Conventional fluorescent staining includes the immunostaining using the fluorochrome-labeled specific antibodies and chemical staining

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using the fluorochrome-labeled chemical reagents that specifically react with the target molecules in the cell. The techniques for molecular labeling with FPs by genetic engineering do not need the staining procedure for microscopy, hence we can easily see the behavior of molecules of interest in living cells. All the other labeling techniques require the procedures of fluorescent staining prior to microscopic examination. Moreover, immunofluorescence staining usually requires fixation procedures and is not suitable for live-cell imaging.

III. Fluorescent Staining Techniques Using the Fluorescent-labeled Molecular Probes to Detect the Particular Biomolecules, Structures, or Molecular Events in the Cell

Immunofluorescent staining

Immunofluorescence microscopy is a versatile procedure and is able to detect any biomolecules in the cell so far as the specific antibodies are provided in advance [16]. To stain intracellular biomolecules, permeabilization of plasma membrane or thin-sectioning of samples is required for the easy access of antibody molecules to the target molecules in the cell. The technique generally requires the fixation procedure of tissues and cells prior to the staining, hence is unsuitable for live-imaging. Specific antibodies for the particular molecules are the key issues in this method. One can either purchase them from manufacturers, order to have them made, or make them by oneself. Two methods, i.e., direct and indirect immunofluorescent staining have been widely used. In the direct-method, the fluorochrome-labeled antibodies are used as probes. The indirect method, on the other hand, uses non-labeled antibodies for the particular biomolecules as the primary antibodies. The fluorochrome-labeled secondary antibodies are used for fluorescent

Table 1. *Fluorochromes for immunofluorescence microscopy*

Fluorochromes	excitation (nm)	emission (nm)
AMCA	347	445
Alexa Fluor 350	345	440
Alexa Fluor 488	488	520
Cy2	492	510
FITC	496	518
Bodipy-FL	503	511
TRITC	544	572
Cy3	550	570
LRSC	572	590
Rhodamine Red-X	570	590
Texas Red	596	620
Cy5	650	670
Alexa Fluor 647	650	668

AMCA (aminomethylcoumarin acetic acid), Cy2 (cyanine), FITC (fluorescein isothiocyanate), TRITC (tetramethylrhodamine isothiocyanate), Cy3 (indocarbocyanine), LRSC (lissamine rhodamine sulfonyl chloride), Cy5 (indodicarbocyanine).

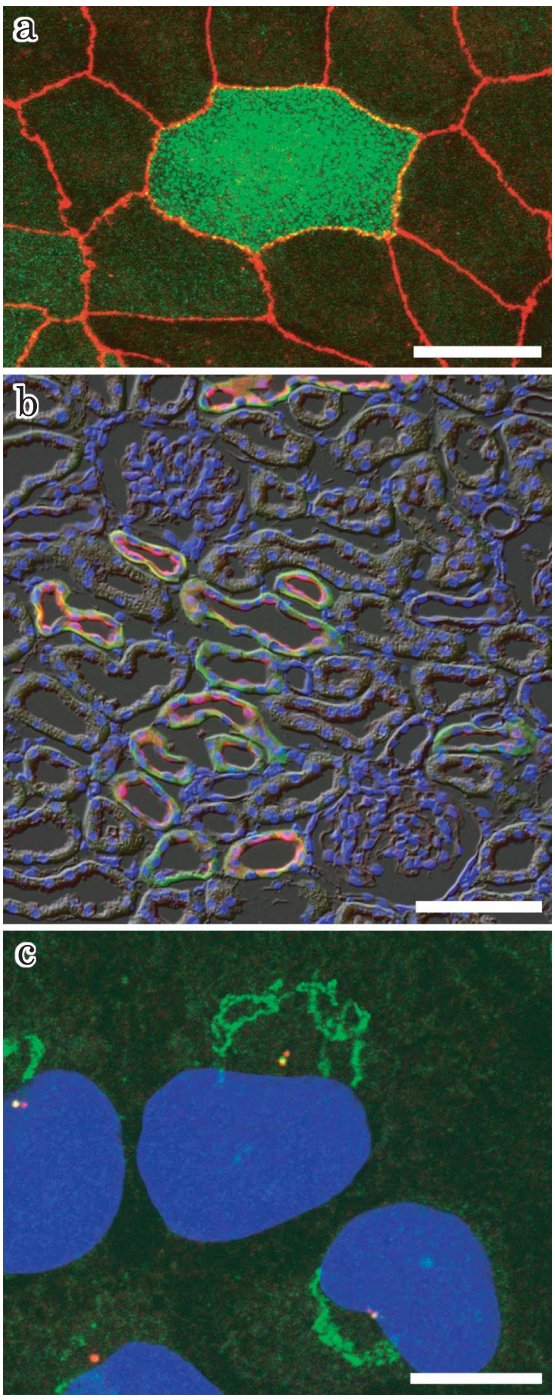

Fig. 1. **a.** Laser confocal image for SGLT1 and ZO-1 of confluent MDCK cells. Green signal indicates the GFP fluorescence of SGLT1-GFP, and red signal indicates the immunofluorescent labeling of ZO-1. Bar=10 μm. Reproduced and modified from [29]. **b.** Immunofluorescence image of rat kidney with anti-aquaporin 2 antibody (red), anti-aquaporin 3 antibody (green), and DAPI (blue). Fluorescence image was merged with corresponding differential interference-contrast (DIC) image. Bar=100 μm. **c.** Immunofluorescent staining image of HSC-4 cells with γ-tubulin antibody (red), Golgi 58K (green), and TO-PRO-3 (blue). The Golgi apparatus is labeled by Golgi 58K. Golgi 58K is also localized to one (arrow) of two dots of the centrosome [8]. Bar=10 μm.

labeling of the primary antibodies. Since the various fluorochrome-labeled secondary antibodies are commercially provided (Table 1), one can choose the proper antibodies by considering the properties of fluorochromes and the animal species of the primary antibodies. The direct method is simple and easy as long as the fluorochrome-labeled antibodies are available. Zenon method is a modification of the direct method, and has a wide range of applications especially for multi-color labeling [21]. As to sensitivity, the indirect method is better than the direct method. In the indirect method, multi-color-staining can be carried out by the combination of secondary antibodies coupled with different fluorochromes (Fig. 1) [15, 29, 32]. Special caution should be paid to the possible cross-reactivity of antibodies to the unintended molecules in the tissue cells [14].

Fluorescence in situ hybridization (FISH)

Antisense single-stranded DNAs for the particular genes or sequences are labeled with proper fluorochromes and used as the molecular probes. The fluorescent-labeled probes hybridize with the complementary RNA or DNA molecules in the cells and are used to detect the particular gene expression in the cells or the number of copies and their localization of particular genes or sequences in chromosomes or nuclei.

Chemical fluorescent probes

Cellular structures such as nucleus, plasma membrane, and cytoskeletons can be visualized with chemical reagents that bind to the biomolecules specific to particular structures. When the regents have fluorescent chromophores or are coupled with fluorochromes, the cellular structures can be seen by fluorescence microscopy. Various fluorescent probes for staining particular cellular structures are commercially available as shown in Table 2. You can choose suitable ones for your experiments depending on the character of the probes. You can also choose fluorochromes in a variety of aspects such as wavelength (excitation and emission), photobleaching speed, and permeability in the cellular membrane. In the case of nuclear staining, the specificity of fluorescent probes to DNA is important because most of the DNA-binding fluorescent probes also bind RNAs (Fig. 2, Table 3) [13, 27, 28]. You have to choose the DNA-specific dyes, or otherwise, RNase pretreatment is required. Phalloidin is a toxic substance from *Amanita phalloides*, commonly known as the death cap poisonous mushroom. Fluorochrome-coupled phalloidin specifically decorates F-actin in the cell. Chemical fluorescent probes are easy to use and are best suited for counter-staining. Cell permeable fluorescent dyes such as Hoechst dyes are also useful for live-cell imaging.

Table 2. *Chemical fluorescent probes for counter-staining*

	target	Ex/Em (nm)	cell permeability
FITC-phalloidin	actin fiber	496/516	impermeant
Rhodamine-phalloidin	actin fiber	554/573	impermeant
MitoTrackers	mitochondria	*	permeant
Rhodamine 123	mitochondria	507/529	permeant
$DiOC_6$	ER+mitochondria	484/501	permeant
ER-Trackers	ER	374/575	permeant
LysoTrackers	lysosome	**	permeant
NBD C_6-ceramide	Golgi apparatus	466/536	permeant

* There are five color-variants of MitoTrackers of green (490/516), orange (551/576), red (578/599), far red (581/644), and deep red (644/665).

** There are four color-variants of LysoTrackers of blue (373/422), green (504/511), yellow (465/535), and red (577/590).

Calcium indicators

Fluorescent probes that exhibit a spectral response upon Ca^{2+} binding have enabled us to directly visualize changes of intracellular Ca^{2+} concentrations in living cells by

Table 3. *Chemical fluorescent dyes for nuclear staining*

	Ex/Em (nm)	DNA	RNA	cell permeablity
DAPI	358/461	+++	−	semi-permeant
Hoechst 33258	352/461	+++	−	permeant
BO-PRO-1	462/509	+	++	impermeant
YO-PRO-1	491/509	+++	+	impermeant
SYBR Green I	494/512	++	−	impermeant
PicoGreen	502/523	+	−	impermeant
SYTOX Green	504/523	+++	−	impermeant
TO-PRO-1	515/531	+	+	impermeant
POPO-3	534/570	+++	+++	impermeant
PI	535/617	+++	++	impermeant
YO-PRO-3	612/631	+++	±	impermeant
TO-TO-3	642/660	++	+++	impermeant
TO-PRO-3	642/661	+++	±	impermeant

DAPI (4',6-diamidino-2-phenylindole), PI (propidium iodide).
Reproduced and modified from [13].

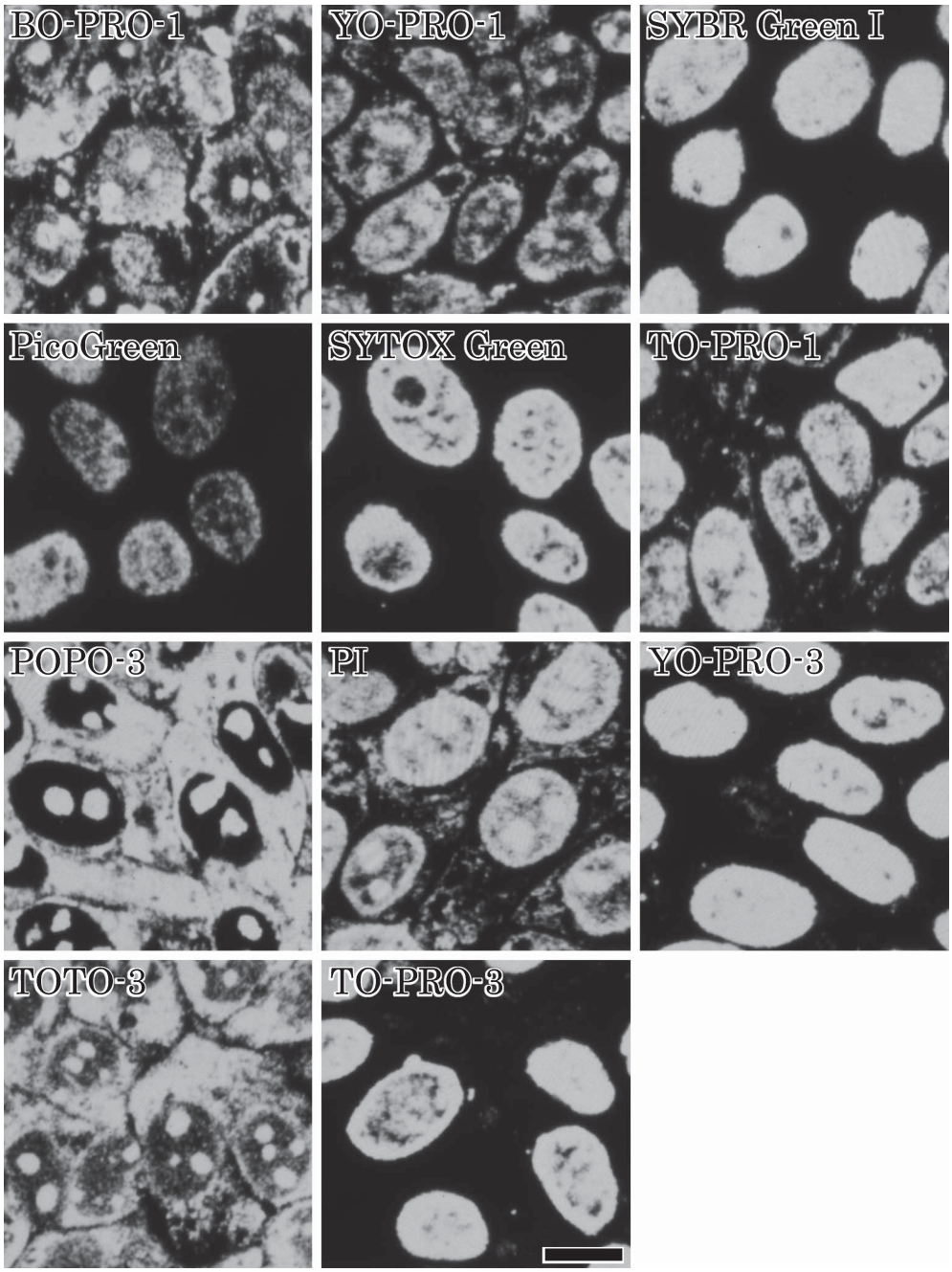

Fig. 2. Laser confocal images of MDCK cells with various fluorescent dyes for nucleic acid. Bar=10 μm. Reproduced and modified from [13].

fluorescence microscopy [33]. Most of calcium indicators are derivatives of the Ca^{2+} chelators such as EGTA, APTRA and BAPTA. Acetoxymethyl esters of the calcium indicators are permeable to cellular membranes and passively taken up into the cytoplasm of living cells, where they are cleaved to cell-impermeant products by intracellular esterases.

IV. Direct Fluorescent Labeling of Particular Gene Products by Genetically Engineered Molecular Tags

Particular gene products can be labeled directly by genetic engineering with the molecular tags such as FPs. They can also be tagged with certain amino acid sequences that specifically bind to the particular cell-permeable fluorescent dyes. FP-fusion gene products are detected after the transformation of the cells. In the case of non-fluorescent molecular tags, incubation with specific fluorescent dyes

and subsequent washing procedure are needed prior to observation.

FP-fusion proteins

FPs such as GFP have made it possible to see not only the localization *per se* but also the dynamics of the labeled molecules in the cell, allowing the development of new ways to analyze the functional behavior of proteins of interest in living cells [5, 11, 29]. This technique is also applicable to the functional motifs or domains of proteins as noted in the accompanying sections. Labeling with FPs is carried out by genetic engineering including recombinant DNA technology. Many FPs have been identified and engineered as shown in Table 4. We can choose suitable ones for the specific experiments by taking into account the wavelength (excitation and emission), the quantum-yield that correlates to the signal intensity, and the degree of polymerization (monomer, dimer or tetramer) [25]. For example, monomeric FPs are suitable for labeling when one wants to see the tracking of biomolecules. Although dimeric and tetrameric FPs emit relatively strong signals, multimeric FPs have a tendency to affect the behavior of biomolecules by the artificial clumping of labeled molecules. The behavior of FP-labeled molecules thus is sometimes different from that of endogenous ones. Moreover, tagging with monomeric FPs cannot completely exclude the possibility of adverse effects on the behavior and/or localization of fusion proteins. The localization and behavior of the non-labeled endogenous proteins should be checked by immunofluorescent staining to avoid possible artifacts.

Molecular tags for specific cell-permeable fluorescent dyes

Just like FP-tagging, tagging with proper amino acid sequences that specifically bind to the particular cell-permeable fluorescent dyes by genetic engineering is also useful to see the behavior and localization of particular molecules in living cells. Two types of molecular tag systems for specific cell-permeable fluorescent dyes are commercially available (Table 5). Tetracysteine tag (TC-Tag: formerly Lumio tag) consists of the six-amino acid sequence of -Cys-Cys-Pro-Gly-Cys-Cys- and specifically binds to the cell permeable labeling reagents of FlAsH-EDT_2 and ReAsH-EDT_2. FlAsH-EDT_2 and ReAsH-EDT_2 labeling reagents become strongly fluorescent (green and red, respectively) only upon binding to the TC-Tag, allowing specific detection of tagged proteins. In the case of TC-Tag, however, the procedures of staining and washing prior to the observation are necessary. Although the labeling-reagent for the TC-Tag itself is not fluorescent, the analogues that are present in the serum bind to the reagent and become fluorescent and disturb the examination of specific labeling, making the washing procedure necessary. Pretreatment of culture medium with a non-fluorescent and cell-impermeable reagent that binds to such endogenous analogues could eliminate the necessity of washing procedures.

The HaloTag protein, a genetically engineered derivative of a hydrolase, is also used as a molecular tag. Since the HaloTag protein is large as FPs (297 amino acid residues), utmost care should be taken as to possible artifacts just like the FP tagging. Five types of Halo-ligands of cell-permeable fluorescent dyes are available as shown Table 5.

Unlike the FPs, the tags themselves do not have their own fluorescence in either case. Elaborate experiments such as pulse-chase labeling using a single fluorescent dye or time-sequential pulse-labeling by different-colored fluorescent dyes for the same tag are possible. These pulse-labeling techniques have enabled us to analyze the turnover of a particular protein or the directivity of protein distribution [4].

Table 4. *Fluorescent proteins [2, 7, 20, 22]*

	Ex/Em (nm)	molar absorptivity	quantum yield	polymerization
EBFP	380/440	29,000	0.31	monomer
ECFP	433/475	32,500	0.40	monomer
Midoriishi-Cyan1	472/495	27,250	0.90	dimer
AmCyan1	458/489	40,000	0.24	tetramer
EGFP	488/509	56,000	0.60	monomer
Azami-Green	492/505	72,300	0.67	tetramer
mAzami-Green1	492/505	55,000	0.74	monomer
ZsGreen1	493/505	35,600	0.63	tetramer
EYFP	514/527	83,400	0.61	monomer
Venus	515/528	92,200	0.57	monomer
ZsYellow	529/539	20,200	0.42	tetramer
Kusabira-Orange1	548/561	73,700	0.45	dimer
mKusabira-Orange1	548/559	51,600	0.60	monomer
DsRed2	563/582	43,800	0.55	tetramer
mRFP1	584/607	44,000	0.25	monomer
HcRed1	588/618	20,000	0.02	dimer

Intensities of FPs are in proportion to the product of molar absorptivity and quantum yield.

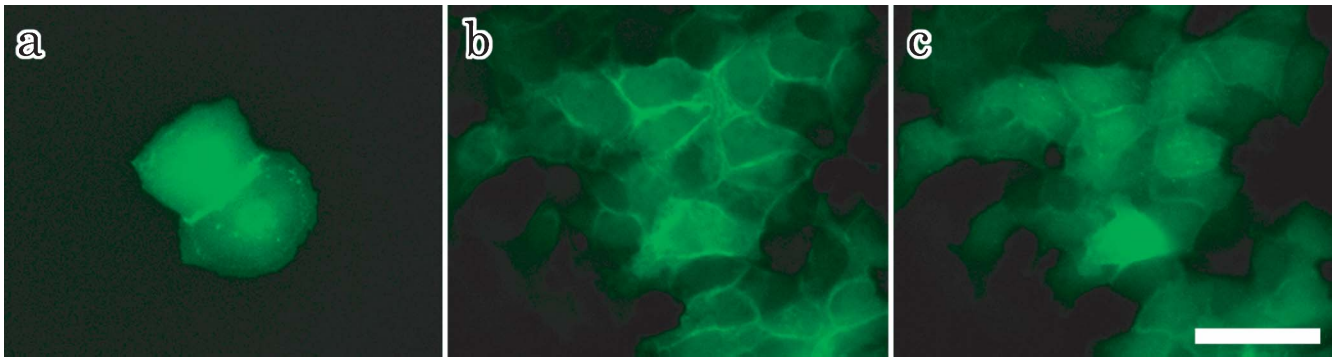

Fig. 3. GFP-tagged AKT-PH domain expressed in the MDCK cells. **a.** The GFP-AKT-PH accumulates at the cell-contact area between the freshly cultured MDCK cells. **b.** The probe also accumulates at the cell-contact area in the densely plated MDCK cells. **c.** The signals of GFP-AKT-PH are internalized from the cell-contact area depending on the completion of epithelial sheet. Panels of **b** and **c** are the same view area of time-lapse microscopy. Panel **c** was captured 16 hr after capturing panel **b**. Bar=40 μm.

Table 5. *Specific dyes for molecular tags [1, 6, 10]*

	target tag	Ex/Em (nm)
FlAsH-EDT_2	TC-Tag	508/528
ReAsH-EDT_2	TC-Tag	593/608
HaloTag Coumarin Ligand	HaloTag	353/434
HaloTag Oregon Green Ligand	HaloTag	496/516
HaloTag diAcFAM Ligand	HaloTag	494/526
HaloTag Alexa Fluor 488 Ligand	HaloTag	494/517
HaloTag TMR Ligand	HaloTag	555/585

Table 6. *Functional motifs and domains used to visualize cellular structures and molecular functions*

Domain or motif	target molecules or cellular structure
Nuclear localization signal of SV40 large T-antigen [23]	nuclei
ER targeting sequence of calreticulin [24]	ER
Mitochondrial targeting sequence of cytochrome c oxidase [36]	mitochondria
N-terminal domain of β-1,4-galactosyltransferase [34]	Golgi apparatus
Farnesylation signal of c-Ha-Ras	plasma membrane
PH domain of AKT [34]	PtdIns(3,4,5)P_3
PH domain of PLC [34]	PtdIns(4,5)P_2
C1 domain of PKC [3]	diacylglycerol

Functional motifs and domains of proteins to visualize cellular structures and molecular functions

A variety of functional motifs and domains of proteins have been known and can be used to design probes to visualize particular cellular structures such as the plasma membrane, mitochondria, cell nucleus, Golgi apparatus, and endoplasmic reticulum by FP-tagging (Table 6). Visualization of the particular cellular structures is useful for the purpose of counter-staining of living cells. Lipid-second-messengers such as phosphatidylinositol (PtdIns) derivatives can also be visualized by the probes of FP-tagged functional motifs or domains in living cells (Table 6). For example, the pleckstrin-homology domain (PH domain) of AKT (protein kinase B) specifically binds to phosphatidylinositol-(3,4,5)-trisphosphate (PtdIns(3,4,5)P_3) that is the product of PI3-kinase. The FP-tagged AKT-PH probe can then detect the active-site for PI3-kinase in living cells (Fig. 3). Since the second messengers are transiently formed and localized at the specific sites in cells during the course of signal transduction, live-cell imaging by FP-tagged probes is a powerful tool for the study of signal transduction mechanisms.

V. Prospects for Fluorescent Labeling

Tagging by FPs has provided us with a great means to analyze the molecular behavior and functions of living cells. Tagging with FPs, however, sometimes has an adverse effect on the behavior of tagged molecules by altering their original molecular properties. Such effects can be minimized by the use of small-sized tags such as TC-Tag that consists of only 6 amino acids. Combined use of such labeling procedures with conventional immunofluorescent methods will remain powerful tools in examining the cellular functions.

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