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Live-cell reporters for fluorescence imaging

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Advances in the development of new fluorescent reporters and imaging techniques have revolutionized our ability to directly visualize biological processes in living systems. Real-time analysis of protein localization, dynamics, and interactions has been made possible by site-specific protein labeling with custom designed probes. This review outlines some of the most recent advances in the design and application of live-cell imaging probes, with a particular focus on SNAP-tag technology. Specific examples illustrating applications in superresolution and single-molecule imaging, protein trafficking and recycling, and protein–protein interactions are presented.

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Introduction

Fluorescence microscopy is a powerful tool for studying the dynamics and localization of proteins in living cells. Proteins are routinely imaged by fusion to auto-fluorescent proteins or to protein/peptide tags that can be site-specifically labeled with synthetic fluorophores. The success of any strategy for live-cell imaging lies in the ability to specifically confer the desired optical properties to the protein of interest, thereby providing means to visualize and interrogate the protein in its native environment. Tag-based approaches utilize specific recognition sequences to recruit chemical probes for *in situ* labeling of the target protein. The binding of the probe to the protein tag occurs either through a tag-mediated self-labeling reaction or is assisted by an auxiliary enzyme. A key advantage of site-specific labeling over imaging approaches on the basis of auto-fluorescence proteins is the ability to use chemistry to modulate the biophysical properties of a given synthetic fluorophore to the needs and constraints imposed by the experiment. Moreover, the high spatial and temporal resolution, molecular specificity, and nondestructive compatibility with living systems make site-specific labeling suitable for a broad range of applications from *in vivo* imaging to drug discovery processes. Various peptide and protein fusion tags have been developed that permit the study of proteins in live cells and organisms, including self-labeling systems such as Tetracysteine-tag, SNAP-tag [1,2], CLIP-tag [3], HaloTag [4], TMP-tag [5] and BL-tag [6], and the enzyme-mediated systems such as the ones on the basis of phosphopantetheinyl transferases (AcpS and Sfp) [7], sortase (SrtA) [8], and lipoic acid ligase (LplA) [9]. In this review, I will focus on the development and recent applications of live-cell fluorescence imaging reporters for one particular site-specific labeling technique, SNAP-tag. For further information on other site-specific labeling approaches as well as on the use of auto-fluorescent proteins, the reader is encouraged to consult some excellent reviews published in recent years [10,11••,12].

The SNAP-tag is an engineered mutant of the human repair protein O^6-alkylguanine-DNA alkyltransferase (hAGT) which reacts with O^6-benzylguanine (BG) [1,2] or O^6-benzyl-4-chloropyrimidine (CP) [13] substrates modified with a synthetic label (e.g., fluorophores, quantum dots, affinity ligands, and gold nanoparticles). The label is covalently attached to the fusion tag in a well-defined mechanism, predictable stoichiometry, and rapid kinetics (Figure 1a) [14]. Selective labeling of intracellular or membrane proteins can be achieved by the appropriate selection of cell-permeable or cell-impermeable substrates, respectively. The SNAP-tag protein labeling system is distinguished from auto-fluorescence protein-based approaches by the wealth of different reporter probes it makes available for imaging (e.g., fluorophores, quantum dots, affinity ligands, gold nanoparticles, etc.) as well as the easy synthesis of custom probes for new applications, such as fluorogenic probes [15,16], photoactivatable fluorophores [17–19], as well as fluorescent sensors for metal ions [20,21] and cell metabolites [22–24]. Additionally, the ability to noninvasively label proteins in any compartment of the cell, including the nucleus, cytoplasm, and at both faces of the plasma membrane, significantly expands the applications into a wide variety of experimental settings in cell biology [25]. SNAP-tag has been successfully utilized in many cellular and *in vivo* imaging studies, including analysis of protein function [26], protein half-life [27], protein trafficking and recycling [28], protein–protein interactions [29], and protein–drug interactions [30,31]. Its high labeling specificity allied with the superior optical properties of synthetic fluorophores has greatly increased our ability to study proteins in living systems. Here I will discuss the design of new fluorescent reporters and present examples of recent applications, such as those for pulse-chase

experiments, multi-color protein imaging, superresolution microscopy, and tracking of single protein molecules in live-cell experiments.

Design of new live-cell reporters for fluorescence imaging

Fluorogenic reporters

Fluorogenic reporters allow for direct monitoring of fluorescence signals with high sensitivity and spatial resolution by minimizing the fluorescence background caused by unreacted or nonspecifically bound probes. Fluorogenic ('dark' or 'quenched') probes exhibit nearly no basal fluorescence, but generate an intense fluorescence response after they have bound to their intended target, thereby resulting in much higher signal-to-noise ratios than conventional fluorophores. We and others have developed SNAP-tag fluorogenic substrates to enable real-time imaging of dynamic cellular processes, such as protein expression, localization, trafficking and degradation [15,16]. These substrates consist of a benzylguanine scaffold bearing a fluorophore attached at the para position of the benzylic ring and a dark quencher attached at the C-8 position of the guanine ring (this position was shown to be the only one amenable for chemical modification without significant deterioration of probe reactivity towards SNAP-tag) (Figure 1b). The fluorescence emission of the reporter fluorophore is intramolecularly quenched through a combination of Förster-type resonance energy transfer (FRET) and photo-induced electron transfer (PET) [16]. Upon reaction with the tagged protein, the quencher group is dissociated leading to up to 50-fold increase in the relative fluorescence intensity of the fluorophore. By turning 'on' the fluorescence signal only at the target protein, fluorogenic reporters meet the critical need for systematic 'no-wash' before fluorescence imaging measurements (Figure 1c). The usefulness of fluorogenic reporters to continually monitor the spatiotemporal dynamics of the EGF receptor (EGFR) during cell migration, without the need for washing the cells, was demonstrated by Urano and co-workers [15].

Of particular interest is the development of near-infrared fluorogenic reporters, which could virtually eliminate background fluorescence in live-animal or deep tissue analysis, and therefore, circumvent probe clearance issues for real-time imaging. In attempt to achieve this goal, SNAP-tag fluorogenic substrates carrying a near-infrared emitting fluorophore (IRDye 800CW) and a non-fluorescent broad range quencher (IRDye QC-1) were synthesized; however, these probes failed to detect xenograph tumors expressing SNAP-tag fused to the ADRβ2 adrenergic receptor, presumably due to the reduced reaction rate of these substrates [32]. More recently, Johnsson and co-workers have introduced a novel class of cell-permeable near-infrared fluorescent reporters on the basis of a silicon–rhodamine (SiR) fluorophore which permits the imaging of proteins in living cells without washing steps [33••]. The fluorogenic character of the probe stems from an equilibrium between its fluorescent zwitterionic and non-fluorescent spirolactone forms (Figure 2a). Coupling of the SiR probe to the protein tag favors the formation of the fluorescent zwitterion, whereas the aggregation of unreacted probe and nonspecific binding to hydrophobic structures favors the formation of the non-fluorescent spirolactone, thereby resulting in minimal background staining in live-cell experiments. The high sensitivity of the SiR fluorophore at a wavelength that exhibits low cellular autofluorescence and phototoxicity has enabled the labeling of SNAP-tag in cortical neurons in rat-brain sections. Furthermore, the excellent brightness and photostability of this probe were confirmed in long-term imaging experiment in which cells that expressed SNAP-tag fusions were grown in the presence of the probe and imaged continuously over 48 h. The utility of SiR for live-cell superresolution microscopy has also been demonstrated [33••].

Photoactivatable fluorescent reporters

Dynamic measurements often require spatial tracking of a molecule or an entire organelle within a cell. The tools of choice for these purposes are photoactivatable or photoswitchable fluorescent reporters. These probes are sensitive to photoinduced conversions, such that their optical properties are modified upon irradiation with light of a specific wavelength. Photoactivatable probes show fluorescence quantum yield enhancement, whereas photoswitchable probes exhibit emission wavelength switching. The possibility to actively control the fluorescence emission at a time in a diffraction-limited region allow for the acquisition of superresolution images for nanoscale visualization. Photoinduced fluorescent proteins have often been used for live-cell superresolution microscopy because of their ability of being genetically targeted; however, they provide 10-fold fewer photons before photobleaching than good small-molecule emitters [34]. Single-molecule-based imaging by means of photoactivatable fluorescent proteins, particularly in the green region of the optical spectrum, suffers from high background and low contrast ratios. As site-specific labeling technologies, such as the SNAP-tag, allow the spatial targeting of synthetic fluorophores to specific subcellular locations, they can be advantageously employed for visualizing, monitoring, and quantifying dynamic molecular events in living cells with high spatial and temporal resolution.

SNAP-tag photoinduced substrates have been generated by a so-called 'caging' process in which a fluorophore of choice is converted into a non-fluorescent state by the incorporation of a photocleavable group, for example 4,5-dimethoxy-2-nitrobenzyl (DMNB) (Figure 2b) [35]. Fluorophore uncaging and consequent fluorescence