

Chemical tags: inspiration for advanced imaging techniques

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This review summarizes recent applications of chemical tags in conjunction with advanced bio-imaging techniques including single-molecule fluorescence, spatiotemporally resolved ensemble microscopy techniques, and imaging modalities beyond fluorescence. We aim to illustrate the unique advantages of chemical tags in facilitating contemporary microscopy to address biological problems that are difficult or near impossible to approach otherwise. We hope our review will inspire more innovative applications enabled by the mingling of these two growing fields.

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Introduction

Advances in microscopy have tremendously expanded our knowledge of biological processes at the microscopic level. The achievements therein are the result of close collaborations between physicists/engineers who build the imaging instruments and chemists/biochemists who design the corresponding probe molecules. One classic example representing this trend is the use of GFP to visualize specific proteins within living organisms by fluorescence microscopy [1]. Recent developments in more advanced imaging schemes (e.g. single-molecule fluorescence imaging, fluorescence lifetime imaging, triplet-state lifetime imaging, luminescence imaging, vibrational absorption imaging or magnetic resonance imaging) have emerged as next-generation tools to unravel complex biological processes in space and time from particular vantage points. In contrast to genetically encodable fluorescent proteins, the probes for these advanced imaging modalities, however, generally lack biocompatible targeting strategies to specific biomolecules. Since proteins are the most diversified functional biomolecules, protein-specific targeting capability, if achievable, will tremendously enrich the applications of corresponding imaging methods.

Chemical tags have emerged as a new generation protein labeling strategy compatible with live cells. Chemical tags are composed of a defined polypeptide sequence that is fused to a protein of interest, and which can be subsequently modified with a chemical reagent, such as an appropriately derivatized fluorescent dye. The first chemical tag, FAsH, was invented in 1998 by the Tsien lab [2]. Since then, several commonly used chemical tags have been developed, including self-labeling FAsH/ReAsH [3], SNAP/CLIP tag [4,5], TMP-tag [6], HaloTag [7], β -lactamase tag [8q] and enzyme-mediated labeling methods based on lipoic acid ligase [9]. Methodologically, chemists have used a variety of strategies to engineer and optimize chemical tags, including directed evolution [10], proximity-induced reactivity [11] and pro-drug loading [9,12]. With efforts from many research groups, chemical tags have reached a relatively mature stage, and the question has shifted from ‘How to label’ to ‘What to label with’, as discussed in several recent review articles [13[•],14[•],15]. In our opinion, the most powerful feature of chemical tags, compared to the classic fluorescent proteins, is the rendered chemical diversity in the label/reporter moiety. We discuss in this present review how this rendered chemical diversity perfectly matches the demand of protein-specific imaging for a variety of advanced imaging methods.

In this review, we discuss the selected works that use chemical tags in combination with bio-imaging schemes beyond traditional fluorescence, such as wide-field or confocal microscopy. Reminiscent to the revolutionizing role of GFP to fluorescence microscopy, we highlight the bridging role of chemical tags that renders targeted protein specificity in modern advanced microscopies. And we also demonstrate the advantage of chemical tags in obtaining new and valuable information that would be difficult to collect otherwise (Table 1).

Chemical tag-enabled imaging techniques based on single-molecule fluorescence

Single-molecule fluorescence imaging techniques have brought considerable excitement to biological research. These techniques enable characterization of biomolecules on the individual level, providing complementary data to that obtained from ensemble experiments. Because it only detects one molecule, the single-molecule fluorescence assay is technically demanding and requires high-photon-output fluorophores. A typical fluorescent protein molecule can emit roughly 4×10^5 photons before photobleaching [16], while the best organic dye molecules have a typical photon output on the order of 10^6 to 10^8 [17]. Therefore, dye molecules conjugated with chemical tags provide high

Table 1

Implementations of chemical tags in advanced microscopy discussed in this review

Imaging technique	Detected signal	Representative tag/probe	Significance	Ref.
PALM; STORM	Precise location of single-molecule fluorophores	Halo/azido DCDHF; TMP/Atto655	Reconstructed super-resolution images	[21,23, 26–28,29*]
Single-molecule tracking	Precise single-molecule trajectories	SNAP/Dy547; Halo/Alexa488	Dynamic information of target protein	[30–32]
CosMos	Colocalization of multi-color single-molecule fluorescence	TMP/Cy5; SNAP/DY549	Mechanistic analysis of macromolecular machines	[33**]
STED	Peripherally depleted fluorescence	Halo/Atto655; SNAP/SiR	Super-resolution imaging	[29*,37–40]
FLIM	Fluorescence lifetime	TMP/Cy3	Indicative of protein-specific micro-environment	[42*]
Triplet imaging	Triplet-state lifetime	SNAP/TMR	Indicative of cellular oxygen consumption	[43]
TR-FRET/LRET	Time-resolved luminescence resonance energy transfer	SNAP/K (europium cryptate); TMP/Lumi4 (terbium chelate)	Background-free detection of FRET for protein–protein interaction	[44,45]
OLID-FRET	Modulated donor fluorescence due to acceptor photoswitching	SNAP/NitroBIPS; SNAP/Cy3–NISO	Sensitive detection of FRET	[48,49]
EM	Electron beam	FIAsH–ReAsH	Protein-specific EM contrast	[3]
Infrared near-field microscopy	Infrared absorption	ACP/Alexa 488	Photobleaching-free	[51]
PET	Gamma ray from positron emission	Halo/ ⁶⁴ Cu NOTA	<i>In Vivo</i> imaging of whole animal	[52]
MRI	Nuclear magnetic resonance	Halo/2CHTgD (gadolinium chelate)	Increased MRI sensitivity with protein-specificity	[53*]

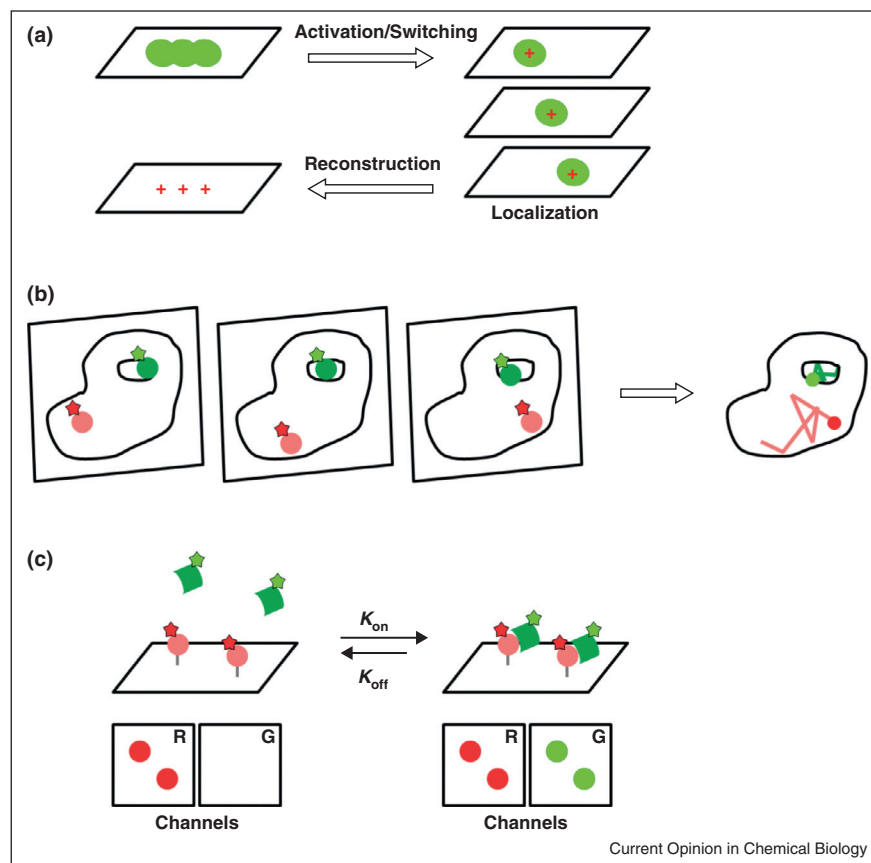
photon budgets, more precise localization, longer observation time and a higher signal-to-noise ratio. These merits make chemical tags excellent tools for the study of proteins by single-molecule fluorescence.

Single-molecule fluorescence detection enables the reconstruction of sub-wavelength resolution images by two fundamentally similar approaches: PALM (photoactivation localization microscopy) [18,19] and STORM (stochastic optical reconstruction microscopy) (Figure 1a) [20]. In PALM, target proteins are labeled with photoactivatable fluorophores, which are then photo-activated sparsely and repeatedly, allowing the record of a collection of single-molecule resolved images. The fluorophores are finally localized to a precise location using software and the super-resolution image is generated. Compared to the photoactivatable fluorescent proteins, chemical tags allow more accurate localization due to the larger number of detected photons. From a chemical point of view, chemical tag-based labeling methods provide diverse photo-chemical strategies toward dye photoactivation. In 2010, the Moerner group demonstrated the first example of live bacteria PALM imaging of a labeled target protein using the chemical tag, HaloTag/azido DCDHF conjugate [21]. Azido DCDHF has an extraordinary high quantum yield of photoactivation under UV exposure [22]. Therefore, a low-intensity UV light source can be used, reducing the UV-induced damage to living cells. More recently, the Johnson group utilized a caged rhodamine derivative as an

alternative probe for PALM imaging in conjunction with the SNAP-tag [23]. Similar to PALM, STORM takes advantage of the reversible photoswitching of fluorescent dyes. It has recently been shown that photoswitching is a rather universal process for a wide spectrum of organic dyes, especially rhodamines, cyanines and oxazines [24,25]. Live cell dSTORM (direct STORM) imaging of labeled intracellular protein H2B was demonstrated using a TMP–Atto655 conjugate, taking advantage of the photoswitching behavior observed in the presence of cellular oxygen and reductants [26]. Several alternative chemical tag/dye combinations have been successfully applied to live cell dSTORM [27,28], with a noteworthy example being the newly developed SNAP-tag/NIR fluorophore silicon–rhodamine [29*]. With the growing availability of PALM/STORM microscope and chemical tag-dye conjugates, we expect super-resolution imaging to become a routine protocol for live cell studies in the near future.

The high-photon output of synthetic fluorophores could further enable prolonged sub-resolution tracking of single proteins with high temporal-resolution inside live cells (Figure 1b). Appelhans *et al.* observed single-molecule diffusion behavior of mitochondrial proteins using HaloTag-rhodamine labels [30]. Benke *et al.* later reported dual-color single-molecule tracking of cellular proteins using multiple chemical tags [31]. In pursuit of brighter and more photostable material, Liu *et al.* reported a targeting strategy of quantum dots that combines both

Figure 1



Imaging strategies based on single-molecule fluorescence of labeled target proteins. **(a)** PALM/STORM image is reconstructed based on precise localization of a stack of images rendered by stochastic photoactivation/photoswitching on single-molecule level. **(b)** Single-molecule tracking is indicative of spatial-temporal dynamics of different proteins in live cell. **(c)** Colocalization of multi-color single-molecule fluorescence reveals kinetic information of complex biochemical machineries.

the lipoic acid ligase and the HaloTag. This stepwise strategy enables the tracking of membrane targets at the single-molecule level and is considered a promising new method for tracking membrane-bound receptors in neurons [32].

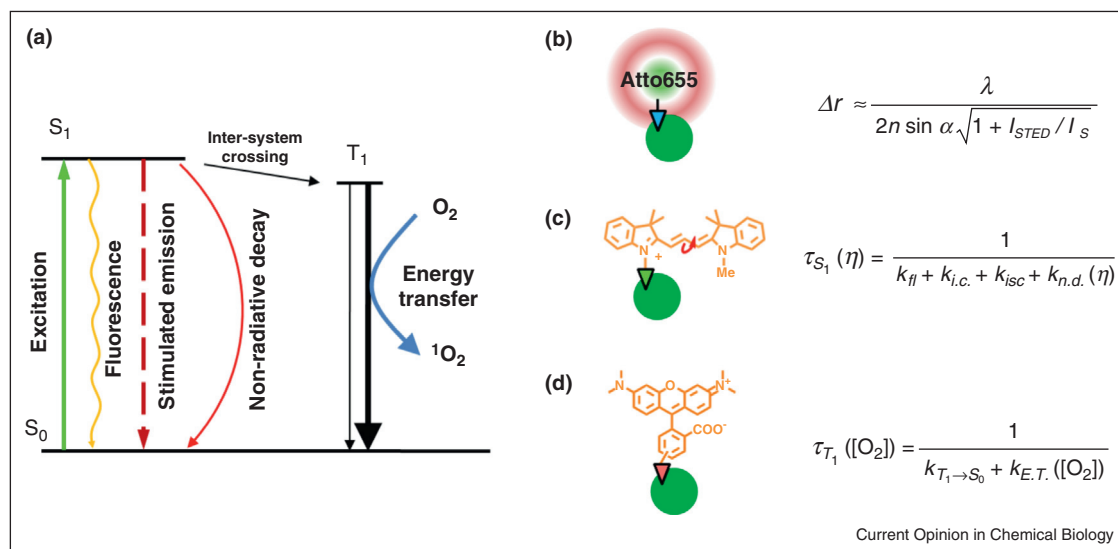
The high photo-stability of organic dyes allows the observation of a single protein molecule over an extended period of time. The biochemical interactions within this time period can be seen under the same microscope if the interaction partners are labeled with other fluorescent markers with different colors, characterized as a colocalization event of multi-color single-molecule fluorescence (Figure 1c). This method, dubbed CoSMoS (colocalization single-molecule spectroscopy), is especially useful for the study of protein complexes which are difficult to reconstitute *in vitro*, as each component of the complex could be fluorescently labeled directly in cell extracts using chemical tags. Hoskins et al. have deciphered the dynamic assembly process of spliceosomes, which are mega-Dalton protein-RNA complexes for mRNA maturation, using

CosMos with the TMP-tag and the SNAP-tag as labeling methods for individual protein components. This work highlights the orthogonality between different chemical tags, enabling their simultaneous use for multi-color imaging [33•]. In addition to probing biochemical interactions, single-molecule approaches are also useful for detecting low efficiency protein modifications due to its superior detection sensitivity. For example, Yang and Zhang demonstrated single-molecule measurements of simultaneous SUMOylation (small ubiquitin-like modifier) using the SNAP/CLIP tags, with a sensitivity ~ 100 fold greater than immunoblotting assays [34]. These examples are pioneering yet promising attempts to use chemical tags and single-molecule imaging to decipher protein functions and modifications.

Spatiotemporally resolved ensemble microscopy techniques

STED microscopy is the first demonstrated super-resolution technique for far-field fluorescence imaging. It uses a high-power doughnut-shaped stimulated emission

Figure 2



Principles of selected spatiotemporally resolved imaging techniques. **(a)** Energy diagram of a typical fluorophore. **(b)** HaloTag–Atto655 is used in STED microscopy to break the diffraction limit. Resolution of STED microscopy is a function of STED beam intensity. **(c)** TMP–Cy3 is used with FLIM microscopy to sense local micro-environment in live cells. Cy3 S_1 state lifetime is a function of local viscosity. **(d)** SNAP–TMR is able to sense oxygen concentration using triplet imaging microscopy. TMR T_1 state lifetime is a function of local oxygen concentration.

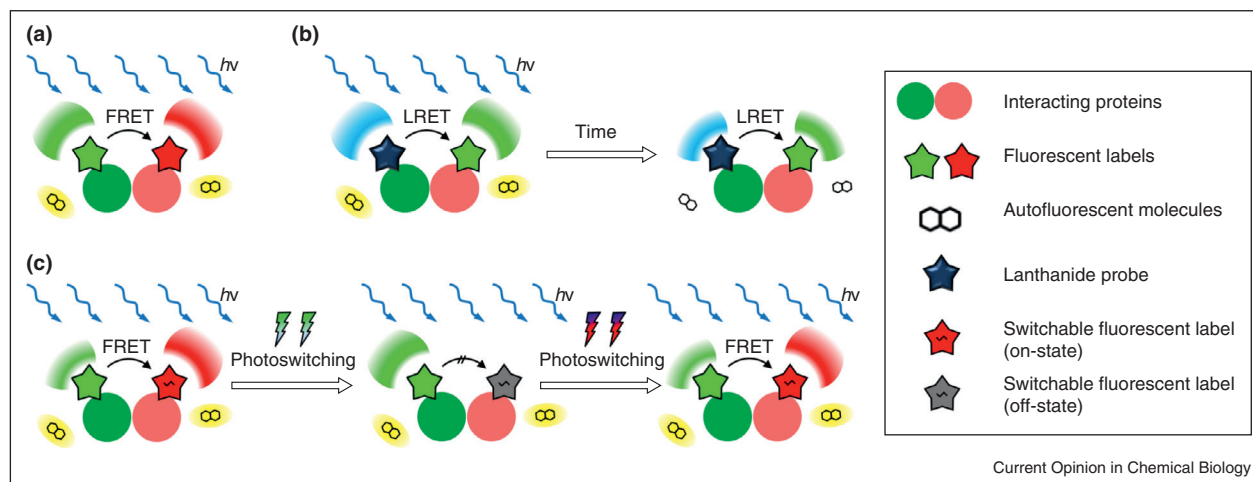
beam to deplete the peripheral fluorescence of the focal point, therefore narrows the effective point spread function to improve spatial resolution [35,36]. In principle, stimulated emission depletion can be applied to all fluorophores (Figure 2a,b), but photostable dyes are preferable in practice since a high-intensity depletion beam is used. To date, live cell STED imaging has been demonstrated with several chemical tag/fluorophore conjugates [29,37–39]. Multi-color STED imaging with orthogonal chemical tags was also demonstrated [40].

Besides implementations in super-resolution imaging, chemical tags have also advanced time-resolved fluorescence techniques. Fluorescence lifetime imaging microscopy (FLIM) is a powerful time-resolved fluorescence technique that characterizes the singlet excited state of fluorophores [41]. The fluorescence lifetime of the singlet excited state, defined as the time delay between the absorption of a photon and the emission event, is highly sensitive to the surrounding micro-environments. It can be measured in frequency-domain by modulating the excitation source sinusoidally at high frequency (>10 MHz) and recording the phase delay of the fluorescence signal. Gatzogiannis *et al.* have developed a protein micro-environment sensor based on fluorescence lifetime measurements of a TMP–Cy3 probe. Cy3 has a prolonged fluorescence lifetime in a high viscosity environment due to the lowered efficiency of its non-radiative isomerization pathway (Figure 2a,c). Using TMP-tag, Cy3 is targeted to the nucleus and cell

membrane, and heterogeneity inside the cell nucleus is visualized using FLIM [42]. This work highlights the greater environmental accessibility of chemical tag-labeled organic dyes over barreled fluorescent proteins as biophysical probes. Using varying pulsed excitation, the triplet-state lifetime of fluorophores can also be measured by utilizing the fact that triplet-state build-up is a function of excitation pulse width. Geissbuehler *et al.* developed a wide-field imaging-fitting protocol to measure the triplet-state lifetime of tetramethylrhodamine (TMR) conjugated to a cytosol protein with SNAP-tag. Because oxygen induces triplet-state lifetime changes via an energy transfer process (Figure 2a,d), this approach enables fast mapping of cellular oxygen concentration during muscle cell contraction [43].

Time-resolved fluorescence is especially useful in FRET-based methods to study protein–protein interactions (Figure 3a). Time-resolved FRET relies on a lanthanide-based donor which has a long-lived (on the order of ms) luminescence. Therefore, donor and acceptor emissions can be collected following pulsed excitations, minimizing the detection of cross-excitation of the acceptor and autofluorescence (Figure 3b). Maurel *et al.* designed a donor–acceptor pair, europium cryptate-d2, to GPCRs using SNAP-tag to study their interaction and oligomerization on the cell surface [44]. Rajapakse *et al.* have reported a TMP–Lumi4 probe for studying cytosolic protein–protein interaction by luminescence resonance energy transfer (LRET) between a terbium

Figure 3



Improving FRET using novel detection methods in combination with labeled fluorophores. **(a)** Traditional FRET suffers from high background resulting from autofluorescence as well as spectrum bleed-through. **(b)** Using long-lived lanthanide probes as donors, emission can be detected shortly after the removal of the excitation light, minimizing the autofluorescence and cross-excitation. **(c)** Using OLID-FRET, the absorption spectrum of photoswitchable acceptor is directly modulated with light and FRET signal is detected from the modulated donor fluorescence.

complex and GFP [45]. Recently, TMP-lanthanide probes with improved cell permeability were reported [46]. With higher signal-to-noise ratio, time-resolved FRET is gaining increasing attention as a promising detection strategy of protein–protein interactions [47].

Conceptually different from time-resolved FRET, OLID (optical lock-in detection)-FRET microscopy was developed as an alternative method to improve the detection accuracy of the FRET signal. In OLID-FRET, a photoswitchable fluorophore that can be reversibly photo-switched by light is used as the FRET acceptor. Donor fluorescence with and without sensitizing the acceptor was measured repeatedly in the same cell, allowing unambiguous resolution of the FRET signal (Figure 3c). Mao *et al.* reported a live cell OLID-FRET system using GFP as the donor and a photoswitchable NitroBIPS, conjugated to GFP via SNAP-tag, as the acceptor. This method enhances the sensitivity of FRET down to 1% FRET efficiency [48]. Recently Cy3/NISO was demonstrated to be a suitable OLID-FRET pair and can be used to label membrane proteins via the SNAP-tag [49].

Beyond fluorescence contrast

Although fluorescence is considered one of the most sensitive optical detection methods, it has several drawbacks including limited optical resolution, poor penetration depth and inevitable fluorophore photobleaching. Development of non-fluorescence-based imaging methods could complement fluorescence imaging in these regards. However, often times there are few genetically encodable protein tags for these non-fluorescence methods. We aim to demonstrate the growing interest of

using chemical tags in non-fluorescence-based imaging methods with targeted protein-specificity.

In pursuit of superior resolution beyond optical microscopy, electron microscopy (EM) was developed based on the fact that electron beams have orders of magnitude shorter wavelengths compared to that of visible light. By using the electron beam as the illumination source, EM can resolve sub-cellular structures down to 1 nm. The contrast of EM is usually rendered by staining with osmium tetroxide, which intrinsically lacks protein specificity. Gaietta *et al.* implemented ReAsH as a contrast reagent for electron microscopy by taking advantage of its photo-catalytic effect of diaminobenzidine oxidation toward an osmophilic polymeric product. Cooperation of fluorescence and electron microscopy of FIAH/ReAsH labeled connexin43 revealed the transportation and incorporation processes of connexin43 into existing gap junctions [3]. This work is considered the benchmark for using chemical tags to achieve a resolution beyond optical microscopy.

As a non-bleaching alternative to traditional fluorescence approaches, infrared-based microscopy is being explored for potential imaging applications. Infrared (IR) absorption does not subject molecules to irreversible damage. The IR bands of biomolecules, however, are often superimposed with each other and hard to distinguish. Generosi *et al.* observed that Alexa488 molecules had a specific IR absorption band which minimally overlapped with cellular IR absorption. Alexa488 was labeled to glutamate receptors on neurons with the ACP-tag [50] and live cell images were recorded using infrared scanning near-field

microscopy [51]. This work presents an interesting application of chemical tags towards photobleaching-free non-fluorescence optical imaging.

To achieve deeper penetration for live animal imaging, positron emission tomography (PET) is one of the most commonly used techniques. In PET, isotope probes are localized based on the emitted γ -rays, which are generated from the annihilation event between a positron and an electron. Hong *et al.* recently reported a HaloTag- ^{64}Cu NOTA probe for PET imaging in live animals. Tumor cells expressing HaloTag protein could be detected in live mice using injected ^{64}Cu NOTA probe [52]. Another widely used method for live animal imaging is magnetic resonance imaging (MRI), which uses penetrative magnetic field to magnetize and probe selected atomic nuclei and reconstruct images. Recently, a protein-targetable MRI contrast reagent based on HaloTag-gadolinium chelate (2CHTGD) was developed and characterized *in vitro* [53^{*}]. While further *in vivo* applications of the chemical tag-targeted MRI probe are still being evaluated, this work, along with the PET imaging approach discussed above, exemplify the potential of chemical tags in promoting protein-specific imaging in live animals.

Concluding Remarks

By introducing diverse reporting moieties specifically to their target proteins, chemical tags have unforeseeable potential in promoting novel techniques towards various biological problems. Stimulating to each other, the co-evolution of chemical tagging and imaging strategies is becoming a fruitful source of innovation for the toolbox of biological research.

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