Protein modification for single molecule fluorescence microscopy

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Single molecule methods have emerged as a powerful new tool for exploring biological phenomena. We provide a brief overview of the scope of current experiments and assess the limitations of both fluorescent labels and the means to achieve protein modification for single molecule microscopy.

Why single molecule fluorescence?

Over the last decade, techniques capable of resolving the fluorescence from individual molecules have become a powerful tool for probing the biological world. Such methods have improved our understanding of a wide range of biological phenomena, including: the dynamic changes in catalytic rate present in many enzymes,[1–3] the mechanism of translocation of motor proteins,[4,5] telomerase assembly,[6] RNA polymerase translocation,[7] the activation and regulation of individual genes,[8,9] infection from a single virus,[10] the transport of molecules through the nucleopore complex,[11] and the clustering and movement of receptors in the cell membrane.[12–14]

The success of these experiments relies on two key advantages of single molecule methods: first, by resolving individual molecules, the distribution of a molecular property can be measured without ensemble averaging. This enables the observation of subpopulations and intermediates that would not be measurable in a corresponding bulk experiment. Second, temporal changes in the behaviour of individual molecules can be resolved. As kinetics can be measured without the need to synchronize the entire population, pathways and rare intermediates can be probed at equilibrium.

Single molecule methods have been the subject of considerable review.[15–22] We instead focus on some of the current limitations of single molecule methods, and the role of chemistry in overcoming these limits.

In order to achieve single molecule detection, we must be able to detect the emitted light from a single molecule in the presence of background noise. Most single molecule methods achieve this by restricting the volume from which light is collected. Through volume restriction, the background light is reduced while the signal from the fluorescent molecule remains constant. Although different techniques vary in the methods used to achieve this restriction, all methods have an upper limit on fluorophore concentration of approximately one nanomolar. Consider the constraints this places on a bimolecular reaction such as the turnover of a fluorogenic substrate by an enzyme: Michaelis constants for many enzymes are much higher than nanomolar,[23,24] and although it is possible to study such reactions at low substrate concentrations, this places a serious limitation on the time required to observe a statistically significant number of events. Recent single molecule methods exploiting photo-activation[25] can excite a small number of fluorophores allowing single molecule detection at higher concentrations. However, it is important to realise that the concentration of photoactivated molecules at any one instant is still low, and hence there are still significant limitations on the range of biological processes that can be probed. For example,

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it would still be difficult to study weak bimolecular interactions between two fluorescently-labelled species using photoactivation. In the future, methods capable of enhanced volume reduction may enable single molecule methods to operate over a wider range of fluorophore concentrations.[23,26]

The second major obstacle, and the focus of this article, lies in the limitations of the fluorescent labels themselves, both in their photophysical properties and in the means to attach a label without perturbing protein behaviour.

Fluorophore properties

In general, the requirements for single molecule fluorophores are common with other optical imaging techniques such as confocal microscopy.[27]

Brightness

For SMF detection we must discriminate a weak fluorescent emission from background noise. Hence, for a constant background signal, the problem is one of the rate and number of photons emitted by a molecule. In designing new fluorescent labels, one must therefore attempt to maximise the rate of photon emission whilst minimising the potential for photo-induced chemical changes that might lead to a molecule becoming non-fluorescent.

Maximising the rate of photon emission from a fluorophore can be achieved either through high quantum yields, or high absorption cross sections. A good experimental measure of the suitability of a fluorophore is the molecular brightness that encompasses these two requirements.[28,29] An experimental comparison of the brightness of a synthetic fluorophore and intrinsically fluorescent protein is shown in Fig. 1.

The need for high brightness is further compounded by the transmission characteristics of microscope optics and the wavelength range of detectors. In practice, our selection of fluorophore is restricted to the visible spectrum. Table 1 summarises the properties of several fluorophores typically used for SMF.

For many synthetic fluorophores, we can approach the theoretical limit for the maximum rate of photon emission from a particular molecule.[30] Much recent effort has thus focused on attempts to enhance fluorophore photostability.

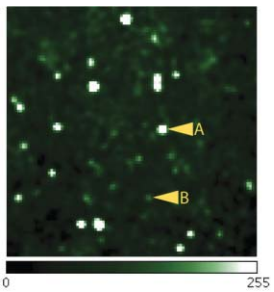

Fig. 1 200 ms 300 × 300 μm Total internal reflection fluorescence image of an Alexa488/eGFP mixture giving an indication of difference in brightness. Suggested Alexa488 (A) and eGFP (B) molecules are marked.

Photostability

Photostability can be divided into two categories: (1) photoblinking, the intermittent changes in fluorophore intensity due to triplet state population. (2) Photobleaching, the irreversible loss of fluorescence in a molecule due to changes in its structure following a light-induced chemical reaction. For an individual fluorophore, these processes are observed as stepwise changes in fluorescence intensity (Fig. 2). Photoblinking on the measurement timescale can prevent accurate single-molecule measurement. This is in contrast to conventional confocal microscopy, where such blinking could

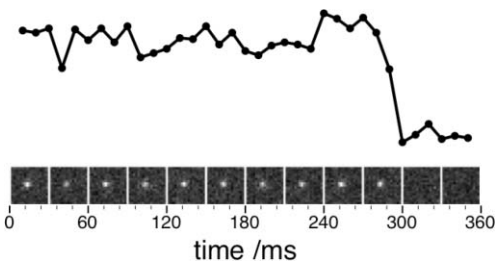

Fig. 2 Photobleaching of a single Cy3b-labelled biomolecule is observed as a single stepwise event. (Top) TIRF microscopy of spot intensity, (bottom) CCD images.

Table 1 Properties of typical single molecule fluorophores[a]

	Excitation maximum/nm	Emission maximum/nm	Quantum Yield	Absorption cross section/$M^{-1}cm^{-1}$	Approximate photobleaching rate[a]/s^{-1}	Reference
Fluorescein	494	521	0.95	76 000	5×10^{-2}	[18]
R6G	488	530	0.95	116 000	6.5×10^{-2}	[19,20]
TMR	540	565	0.38	95 000	2.2×10^{-2}	[19,21]
Cy3b	558	572	0.67	130 000	3.0×10^{-2}	[22]
Cy5	620	649	0.27	250 000	0.8×10^{-2}	
AlexaFluor488	494	517	0.92	73 000	4.9×10^{-3}	[23]
eGFP	489	508	0.60	55 000	3.3×10^{-2}	[24]

[a] Experimental conditions vary in the cited references. Direct comparison between different fluorophores is of limited use; this is especially true of photobleaching rate, which is highly dependent on the excitation intensity.

be tolerated. Stepwise changes in fluorescence intensity are not always unwelcome; for example, counting photobleaching steps can be used to determine the stoichiometry of macromolecular complexes.[31,32]

The primary mechanism for photobleaching is photo-oxidation of a fluorophore by molecular oxygen. Singlet oxygen is created by reaction of triplet oxygen with a fluorophore excited to a triplet state during photoexcitation. This singlet oxygen can then react with the fluorophore and cause photobleaching. Photo-ionization of excited dye molecules provides an alternative mechanism to produce reactive species capable of causing photobleaching, and this second pathway may even dominate in the case of multi-photon excitation.[33]

Several attempts have been made to improve the photostability of the current generation of fluorophores, including the rigidification of cyanine dyes,[34] the use of larger near-infrared terrylene dimide dyes,[35] and the encapsulation of fluorophores within rotaxanes[36] or supramolecular hosts such as cucurbituril.[37,38] Improved stability may also be achieved through the use of different laser pulse schemes designed to suppress population of fluorophore triplet states.[27,39]

Another simple indirect method to limit photobleaching is to reduce the effect of dissolved oxygen. This can be achieved by either suppressing triplet-state excitation of the fluorophore, or by the removal of oxygen from the system using degassing and/or oxygen scavenging. The effectiveness of many such photo-protective agents has been tested recently.[40,41]

Semiconductor nanoparticles[42] and nanodiamonds[43] provide two other alternatives for single molecule imaging. Quantum dots are approximately 10 times brighter and 100 times more resistant to photobleaching than their chemical dye counterparts. However, these advantages must be taken in the context of the increased size of such a label. In addition to label size, significant photoblinking also limits the usefulness of quantum dots for many single molecule experiments.[44]

Fluorescent reporters

Beyond the requirements for photostability and brightness, many experiments require further properties from the fluorophore. For example, changes in Förster resonance energy transfer (FRET), fluorescence quenching, and electron-transfer have all been used to probe changes in biomolecule conformation.[15,45,46] Given suitably robust fluorophores, many properties routinely measured in bulk can be probed at the single molecule level, including pH,[47] voltage,[48] and ion concentration.[49] Recent techniques exploiting photoactivation of fluorophores have created the demand for new molecules capable of efficient photoswitching between dark and bright states.[25]

Protein modification techniques

The requirements for labelling applied to single molecule experiments are similar to those for conventional bulk approaches. However, highly-specific labelling methodologies are required for successful orthogonal labelling, which is often important for single molecule measurements. This importance is due to the ability of single molecule methods to reveal the dynamics associated with protein conformational changes. The independent labelling of multiple sites with different fluorophores, either within the same protein of interest (POI), or on multiple different POIs will open up new directions in the study of protein conformational fluctuations, or intermolecular protein–protein interactions using, for example, FRET or fluorescence cross correlation spectroscopy.

Recent research at the interface of chemistry and biochemistry has greatly diversified the tools available for protein modification with fluorescent labels (Fig. 3 and Table 2). Such techniques may

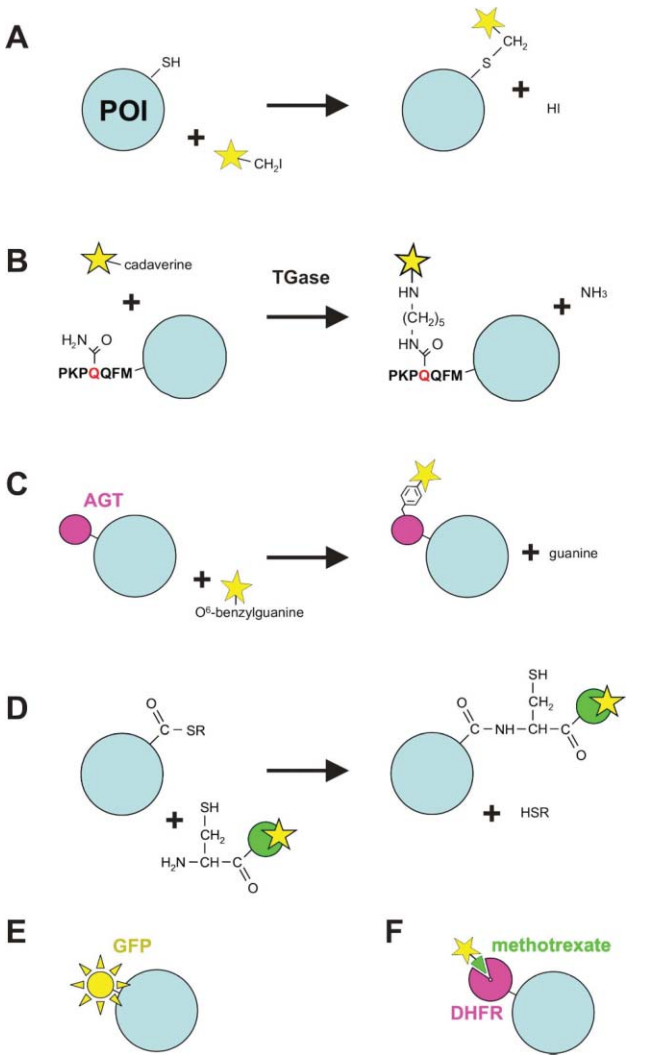

Fig. 3 Approaches for the fluorescent modification of proteins. A generalised fluorophore is shown as a yellow star throughout, and the protein-of-interest (POI) is a blue circle. See text for further details of the methods shown. **A. Direct chemical labelling**. The example given is the labelling of a free thiol with an iodoacetamide conjugate. **B. Labelling of peptide/protein** tags illustrated by the modification of a Q-tag motif with a fluorophore–cadaverine conjugate catalysed by transglutaminase (TGase). **C. Auto-labelling of fusion proteins**. In this example, a POI–AGT fusion is auto-catalytically labelled using a fluorescent benzylguanine derivative. **D. Protein ligation techniques.** In this expressed-enzyme ligation method, the POI is produced with a C-terminal thioester, allowing chemical ligation with a fluorescently labelled peptide/protein containing an N-terminal cysteine residue. **E. Fusion to intrinsically fluorescent proteins**, such as GFP, provides a simple means for *in vivo* labelling. **F. Fluorescent ligand binding.** A POI–DHFR fusion is shown binding to fluorescently labelled methotrexate as an example.

Table 2 Summary of protein modification techniques

Approach	Examples	Pros	Cons
Direct chemical labelling.	Labelling at cysteine residues.	• Very small modification.	• Only suitable for labelling of highly purified protein for use *in vitro.*
	Labelling at lysine residues.	• Exceptionally wide range of fluorophores available.	• High-resolution structural information desirable.
	Labelling of non-natural amino acids.	• Simple and robust labelling methodology.	• Not appropriate as a general method: every POI will behave differently.
		• Precise positioning of fluorophore.	• (New techniques for non-natural amino acid incorporation may help alleviate these problems.)
Fusion to intrinsically fluorescent proteins.	Fluorescent proteins and their derivatives.	• Appropriate as a general method.	• Rather large tag with the potential to oligomerize or degrade.
		• Simple *in vivo* labelling.	• Broad excitation and emission spectra.
		• Well-developed technology.	• Limited opportunity to improve photostability of fluorophore.
Labelling of peptide/protein tags.	Peptide carrier protein domains-Sfp phosphopantetheinyl transferase Q-tag-transglutaminase FlAsH and ReAsH.	• Appropriate as a general method.	• Range of fluorophores limited.
	Biotin carboxyl carrier protein/AviTag–BirA. CVIA motif–protein farnesyltransferase.		• (See text for method-specific drawbacks.)
Auto-labelling of fusion proteins.	SnapTag.	• Appropriate as a general method.	• Rather large tag.
	HaloTag.	• Cell permeable substrates allow *in vivo* labelling.	• Range of fluorophores limited.
Protein ligation techniques.	Native chemical ligation.	• Appropriate as a general method.	• Intracellular imaging not possible.
	Expressed protein ligation.	• Streamlined purification and labelling process (EPL).	
	Expressed enzyme ligation.	• Applicable to a wide range of fluorophores.	
	Sortase-mediated ligation.		
Fluorescent ligand binding.	Ni:NTA labelling of his tag HisZiFiT.	• Reversible binding may circumvent photobleaching problem.	• Generally a rather large tag size.
	Dihydrofolate reductase-methotrexate FKBP12–SLF′. Immunolabelling.	• Potential for *in vivo* labelling (SLF′).	• (See text for method-specific drawbacks.)

be classified as direct, if amino acids intrinsic to the POI are modified, or indirect, if protein or peptide tags are attached to the POI. Indirect labelling may be achieved using an intrinsically fluorescent tag, or *via* subsequent modification of a tag with a fluorescent probe. They may also be differentiated on the basis of whether the modification is achieved *via* covalent attachment of the fluorophore or simply *via* reversible binding.

Direct chemical labelling

A well-established technique for *in vitro* modification of purified proteins involves reaction with cysteine residues. The relative infrequency of cysteine residues in proteins may allow specific, stoichiometric labelling with small molecules. The robust reaction of solvent-accessible thiols with maleimide–fluorophore conjugates is specific (in the sense that it occurs mainly at cysteine residues) and occurs rapidly under "protein-friendly" conditions in biological buffers of moderate pH and temperature. Iodoacetamide-conjugates are also widely used to modify thiols and benefit from being less sensitive to the presence of reducing agents such as DTT and TCEP, which are often employed before labelling to ensure that cysteines are in the reduced form.[50] If the POI contains multiple cysteine residues then labelling is likely to result in a non-homogenous preparation and/or reduced activity. This problem may be circumvented by site-directed mutagenesis of the POI to replace the native cysteine residues. If tolerated, this "Cys-light" derivative can be used as a blank canvas for the re-introduction of surface-accessible cysteines. Clearly, this design process is made far easier by the availability of high-resolution structural information for the POI.

Chemical modification of lysine ε-amino groups can be achieved with amine-reactive conjugates (commonly succinimidyl esters). Unfortunately, the high frequency of lysine residues generally results in multiple labelling, and subsequent detrimental effects on protein activity. However, the relatively low pK_a value of the N-terminal α-amino group allows moderately specific modification by reaction with succinimidyl ester conjugates at near-neutral pH. For example, this technique was successfully applied to label the bacterial RecA protein and to observe the assembly and disassembly of RecA nucleoprotein filaments on single DNA molecules.[51]

An important drawback of labelling natural amino acids is that the reaction is not specific with respect to other proteins. Consequently, these techniques are applied to highly purified

proteins and are not useful for *in vivo* labelling. Orthogonal labelling at two cysteine residues with different fluorophores is also difficult. It may be possible to chromatographically separate the differentially labelled products, or to exploit two cysteine residues with dramatically different reactivities. New strategies for independent labelling of cysteine residues include the reversible protection of thiols by metal-chelation or redox control mechanisms.[52]

To overcome the limitations imposed by the 20 commonly occurring amino acids, the Schultz lab has pioneered co-translational protein modification methods in which unnatural amino acids are incorporated into proteins *in vivo* (for review and methodology see Wang *et al.*[53]). These techniques exploit modified aminoacyl tRNA synthetases that charge tRNAs with unnatural amino acids to recognise nonsense, quadruplet or (potentially) degenerate codons. This expands the available genetic code allowing site-directed mutagenesis with over 30 novel amino acids, including the fluorescent dansyl-alanine.[54] Several of these amino acids possess orthogonal chemical reactivities, including keto, azide and thioester groups, which can be used to modify the POI with fluorophore conjugates. For example, *p*-acetyl-L-phenylalanine was incorporated into a Z domain protein and modified with fluorescein hydrazide.[55] Unnatural amino acids have also been incorporated into proteins using modified *in vitro* translation techniques.[56–58] These co-translational methods are potentially powerful as they incorporate the advantages of conventional amino acid labelling with small molecules (precise positioning and small tag size) with the added benefits of higher specificity.

Fusion to intrinsically fluorescent proteins

Using recombinant DNA technology, the POI may be fused to a fluorescent protein (FP) at either the N- or C-terminus. The jellyfish green fluorescent protein (GFP) was first heterologously expressed as a fluorescent marker in 1994.[59,60] Since then, the widespread use of FP-fusions in cell imaging has fuelled the development of many GFP variants and the isolation of novel classes of FP with improved or novel properties with respect to excitation/emission spectra, brightness, photostability and a reduced propensity to oligomerize (for reviews see Zhang et al;[61] Shaner *et al.*[62]). A major advantage of this approach is the potential to image the POI *in vivo* without the need to develop cell permeable small molecule dyes. Single molecule imaging requires controlled low-level expression, which in turn alleviates the potential complexities associated with oligomerisation of FPs. There are now many examples of single molecule imaging using POI–GFP fusions in both live cells[63] and for *in vitro* single molecule detection.[64,65] Disadvantages of FPs include their relatively large size (~30 kDa), which may inhibit POI activity, their reduced brightness in comparison to chemical labels, their tendency to oligomerize, and their potential for *in vivo* degradation. Moreover, for FRET, dual-colour or multi-colour work, FPs are generally outperformed by small molecule dyes, which display narrower excitation and emission spectra.

Labelling of protein/peptide tags

Several methods employ relatively small protein tags that can be fused to the POI and enzymatically-labelled with moderate to high specificity. Peptide carrier protein domains (~10 kDa) allow modification with phosphopantetheinyl-conjugates *via* Sfp phosphopantetheinyl transferase.[66,67] Much smaller are the Q-tag peptide motifs targeted by transglutaminase (TGase): a protein crosslinking agent that catalyses amide bond formation between glutamine and lysine residues. The guinea pig TGase is promiscuous, such that the lysine substrate can be substituted with a variety of primary amine donors, including cadaverine-conjugates.[68,69] The specificity is moderate and probably not sufficient for unique labelling of the POI in a complex protein mixture. Nevertheless, a two-step TGase-based modification was used to label proteins on the surface of live cells.[48] Notably, orthogonal labelling of chymotrypsin inhibitor 2 with AlexaFluor647–cadaverine and AlexaFluor488–maleimide for single molecule FRET analysis was also demonstrated.[70] The Tsien lab has developed the biarsenical–tetracysteine system for labelling a short motif (CCPGCC) with a membrane permeable dye.[71] These tetra-cysteine tags, which form reversible chemical bonds with the fluorescent biarsenical compounds FlAsH and ReAsH, have shown some promise for imaging in live cells. However, important drawbacks include arsenical toxicity, the redox sensitivity of the binding reaction, and the non-specific labelling of other cysteine rich motifs.

Fluorescent labelling of the POI can also be achieved in two steps if a biotin moiety is first attached to the target. Biotin carboxyl carrier protein (BCCP) fusions are modified at a conserved lysine with biotin by *E. coli* biotin ligase (BirA) both *in vivo* and *in vitro.* However, BCCP is undesirably large and this led to the development of a 14mer biotin acceptor peptide that is efficiently modified by BirA.[72] This technology is commercially available under the name AviTag (Avidity). The biotin-labelled POI can subsequently be imaged using fluorophore–streptavidin conjugates, but the streptavidin is large and exists as a tetramer that can bind four biotin molecules. In a modification of this approach, it was demonstrated that a ketone isostere of biotin also served as a substrate for BirA. This allows subsequent reaction with a hydrazide or hydroxylamine for which many fluorescent derivatives are available.[73] The use of biotin–acceptor peptide technology was extended further by the isolation of an orthogonal BirA–biotin acceptor peptide pair from yeast. Use of the *E. coli* and yeast systems together allows independent labelling of two different acceptor peptides for dual-colour or FRET studies.[74] It is important to note that two-step methods involving biotin labelling may be of particular interest to the single molecule biologist: the biotin–avidin interaction can also be used for immobilisation and purification of the POI, giving the biotin tag triple functionality. A second example of two-step labelling employs protein farnesyltransferase (PFTase) to catalyse the covalent attachment of farnesyl molecules to the small tetrapeptide motif CVIA. Proteins containing this sequence at their C-terminus can be alkyne-functionalised using modified PFTase substrates and subsequently modified with reporter groups using azide conjugates.[75]

Auto-labelling of fusion proteins

These methods 'hijack' natural enzymatic mechanisms for covalent auto-modification of fusion constructs. For example, the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (hAGT) is able to modify itself with an alkyl group from its natural substrate O^6-alkylguanine-DNA, but also tolerates O^6-benzylguanine and

a variety of fluorescent derivatives as substrates.[76] A potential difficulty of this method, commercially available under the name SnapTag (Covalys), is non-specific labelling of endogenous AGT. The HaloTag method (Promega) exploits auto-labelling of a mutant halogen dehalogenase with chloro-alkane derivatives. This system benefits from high specificity, as halogen dehalogenase activity is not present in *E. coli* or mammalian cells. Both methods provide the opportunity to label *in vivo* as the fluorescent substrates are cell-permeable. However, a general drawback of auto-labelling methods is that the tag must be relatively large to maintain its catalytic integrity.

Protein ligation techniques

Protein and peptide tags may be attached to proteins using native chemical ligation.[77] Proteins produced with N-terminal cysteines can be modified with synthetic, fluorescent peptides containing C-terminal thioesters or with fluorophore–thioester conjugates, which are easily derived from commercially available succinimidyl esters.[78] Nature has harnessed a similar chemistry in the form of inteins: self-splicing protein elements. Excision of genetically-modified inteins from expressed proteins can generate C-terminal thioesters or N-terminal cysteines, which can be ligated to small synthetic peptides or other proteins in a technique called expressed protein ligation.[79–81] This technology was recently exploited to create a general method for C-terminal fluorescence labelling.[82] Expressed enzyme ligation techniques resemble expressed protein ligation, but make use of a V8 protease to ligate N-terminal serines to C-terminal thioesters.[83] Sortase-mediated ligation is a further enzyme-based ligation method that exploits the ability of sortases to attach proteins containing a short C-terminal "sorting signal" to the cell surface of bacteria.[84] They are able to both cleave a POI-sorting signal fusion and attach it to the N-terminal amino group of pentaglycine peptides. Sortase-based methods were recently used to image proteins on the surface of live cells.[85]

Fluorescent ligand binding by protein tags

One potential advantage of reversible binding of fluorophores, as opposed to covalent labelling, is that the POI can be repeatedly re-labelled to circumvent the photobleaching problems that are a critical limiting factor in single molecule investigations. In one recent example, single molecule imaging of histidine-tagged proteins was achieved on the surface of live cells using Ni:NTA–Atto647.[86] Although the histidine-tag is small and compatible with many existing recombinant DNA technologies, the use of a nickel-based label may cause toxicity in cells. A zinc-based alternative, HisZiFiT, was developed for histidine-tag labelling with fluorescein.[87] The tight binding of dihydrofolate reductase to methotrexate-conjugates, which are readily cell-permeable, has also been exploited for labelling.[88] Likewise, an FKBP12 mutant protein binds very tightly to the synthetic ligand SLF′. Conjugates of SLF′ to fluorescein are cell permeable and non-toxic, and have been used to label FKBP12–POI fusions in mammalian cells.[89]

A final way in which to exploit binding for labelling is to harness the specificity of antibody–antigen interactions. For example, one may fuse the POI to an antigen for which a fluorescent antibody is available. This was applied to monitor the translocation of a GST–Rad54 fusion along a single DNA molecule using a FITC-labelled anti-GST antibody.[90] In another method, the POI is fused to a single chain anti-fluorescein antibody to allow labelling with cell-permeable hapten–fluorophore conjugates.[91] An obvious drawback of such techniques is the large size of the antibody.

Conclusions

This array of new strategies for protein modification presents many opportunities for new single molecule measurements. This is particularly true for single molecule FRET, where the targeting of two labels to particular sites on a biomolecule is required. Although dual-labelling is possible *in vitro* for purified proteins, a robust means to introduce specific orthogonal functionality *in vivo* would greatly expand the utility of single molecule methods. In contrast to the vast number of new labelling techniques, many problems still remain for the fluorescent label itself. In particular, photobleaching remains a major challenge. New, more stable fluorophores would greatly expand the timescale of single molecule experiments and allow a wider range of biological phenomena to be explored.

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