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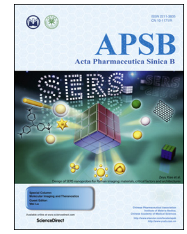

REVIEW

Fluorogen-activating proteins: beyond classical fluorescent proteins

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KEY WORDS

Fluorogen activating proteins;
Fluorogens;
Genetically encoded sensors;
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Abstract Fluorescence imaging is a powerful technique for the real-time noninvasive monitoring of protein dynamics. Recently, fluorogen activating proteins (FAPs)/fluorogen probes for protein imaging were developed. Unlike the traditional fluorescent proteins (FPs), FAPs do not fluoresce unless bound to their specific small-molecule fluorogens. When using FAPs/fluorogen probes, a washing step is not required for the removal of free probes from the cells, thus allowing rapid and specific detection of proteins in living cells with high signal-to-noise ratio. Furthermore, with different fluorogens, living cell multi-color proteins labeling system was developed. In this review, we describe about the discovery of FAPs, the design strategy of FAP fluorogens, the application of the FAP technology and the advances of FAP technology in protein labeling systems.

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1. Introduction

Fluorescence imaging is one of the most powerful techniques to observe biomolecules in real-time with high spatial and temporal resolution, which reveals the fundamental insights into the production, localization, trafficking, and biological functions of biomolecules in living systems[1–4]. As the biological objects are poorly fluorescent, fluorescent probes including fluorescent proteins and organic fluorescent dyes are essential molecular tools for bio-imaging[5,6]. Among them, a diverse set of genetically encodable fluorescent biosensors have been designed to probe dynamic cellular events. These sensors that generally involve the incorporation of a fluorescent tag into a protein or a selected protein domain, have enabled researchers to track various components of intracellular signaling networks in real time within the native cellular environment. In the past several decades, two approaches have been developed to construct genetically encodable biosensors for live cell studies (Fig. 1): 1) fluorescent protein-based reporters: chimeric genetic fusions of fluorescent proteins (*e.g.*, GFP and its variants) with a protein (or RNA) domain[7]; 2) fluorogen-based reporters: a genetically encodable tag binds a fluorogenic ligand (endogenously present or exogenously applied) and activates its fluorescence. As the fluorogenic chromophore is non-fluorescent by its own and becomes strongly fluorescent only upon binding its target, unspecific fluorescence background in cells remains minimal even in the presence of an excess of dye, thus ensuring high imaging contrast[8]. Labeling with fluorogenic probes can be covalent, relying on chemical or enzymatic reaction, or non-covalent, relying on binding equilibrium. In the past 20 years, great efforts have been dedicated to exploring covalence-based self-labeling tags, such as the commercially available SNAP-tag[9–11], CLIP-tag[12] and Halo Tag[13–15]. Parallel to the development of covalent fluorogenic protein labeling strategies, methods based on the non-covalent interaction between a protein tag and a fluorogenic dye have emerged[16,17]. Unlike the covalent labeling strategies, non-covalent labeling can be very fast since no covalent bond has to be created. Moreover, systems based on reversible non-covalent binding could provide an additional degree of control as fluorescence could also be switched off by washing away the fluorogenic ligand, given that the off-rate is fast enough. In this review, we describe the discovery of one of the non-covalence-based fluorogenic probes based on fluorogen activating proteins (FAPs), the design strategy of FAP fluorogens, the application of the FAP technology and the advances of FAP technology in protein labeling systems.

1.1. The discovery of the FAPs

FAP technology was first introduced in 2008 by Szent-Gyorgyi et al.[18] by using single-chain antibodies as genetically encodable FAP. FAPs binding modified thiazole orange (TO) and malachite green (MG) were first generated by screening a yeast surface-displayed library of human single-chain antibodies (scFvs) using fluorescence-activating cell sorting (FACS). Eight unique FAPs were isolated from the library, among which six proteins specifically activate modified MG. scFvs are engineered proteins composed of Immunoglobulin (IgG) variable heavy (V_H) and variable light (V_L) domains tethered together *via* a short flexible peptide linker, which retain the wide range of antigen recognition capabilities of the full-length antibodies, and are also conformable to be used as recombinant tags in diverse fusion proteins[19].

The FAP technology is a fluorogenic tagging approach that utilizes molecular recognition to directly activate the fluorescence of otherwise nonfluorescent small-molecule dyes (fluorogens). Selected FAPs bind TO and MG with nanomolar affinity and increase their respective green and red fluorescence by as much as thousands of fold. The fluorescence enhancement results from FAPs constraining the rapid rotation around a single bond within the chromophore (Fig. 2)[6]. The non-covalent interactions between the fluorogens and FAPs are like those of ligands and their receptors, mainly including van der Waals forces, π-effects and hydrogen bonds. Molecular recognition capabilities are largely determined by these loops of FAPs, termed complementarity determining regions (CDRs), which undergo somatic hypermutation during the immune response to generate specific high affinity binding to the antigen (Fig. 3)[20,21]. FAPs represent a new class of fluorogen-based reporters, which provide a fluorescent tool for imaging fusion protein's location and abundance in time and space. FAP-fluorogen imaging system offers a number of distinct advantages in bio-applications: 1) unbound dye remains nonfluorescent in solution, allowing for the simple addition of dyes to the cellular media without any need for fixation or washout, a property that will enable imaging in more complicated tissue environments and live-cell imaging; 2) fluorogen binding to most FAPs occurs within seconds of addition, and can be carried out in a near physiological buffer or medium of choice. The interaction between the fluorogen and FAP is highly specific, with some FAP clones exhibiting subnanomolar affinity; 3) since FAPs are small in size (<30 kDa), they are easy to genetically engineer. The FAP technology thus allows specific fluorescent labeling of fusion proteins of interest in both living or chemically fixed cells; 4) the possibility offered to completely control the concentration of fluorogens paves the way for on-demand applications wherein

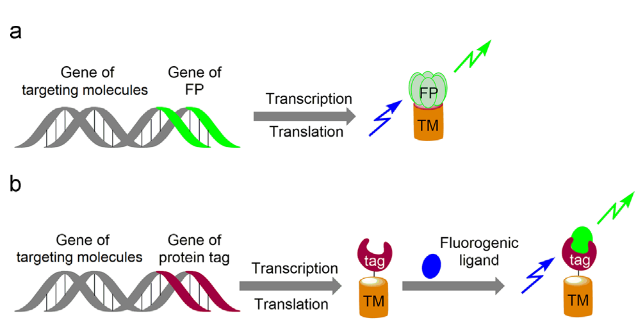

Figure 1 (a) Fluorescent protein-based reporters and (b) fluorogen-based reporters for fluorescence imaging, TM: targeting molecules.

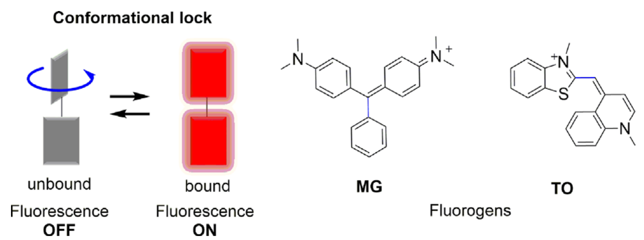

Figure 2 Concept and examples of the fluorogen-based reporters operating by intramolecular rotation. Adapted with permission from Ref. 6. Copyright (2017) American Chemical Society.

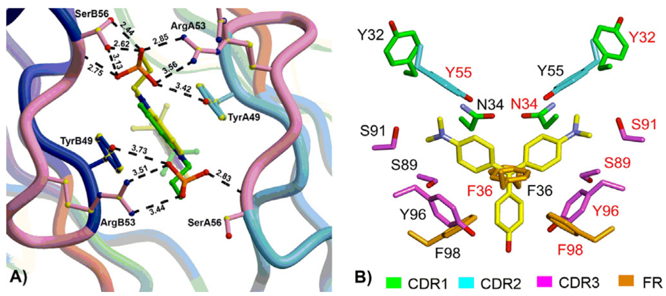

Figure 3 (A) The interactions between the DIR sulfonate and M8V_L show the two orientations of the bound DIR in the crystal structure (green and yellow), and the A (light blue) and B (blue) V_L chains. CDR2 is colored pink. Ser^{B56} and Arg^{A53} also sample two alternate conformations. One sulfonate (top) has more interactions with the protein than the alternate (bottom) sulfonate. Adapted with permission from Ref. 20. Copyright (2012) American Chemical Society. (B) MG interactions with L5*. Spatial distribution of amino acid side chains that contact MG. Shown are contacting side chains from both V_L A (black letters) and V_L B (red letters) that comprise a set of pairwise symmetrical locations and orientations. Adapted with permission from Ref. 21.

fluorescence is desired only at a specific time or at a given density as exemplified with the FAPs; 5) fluorescence visualization can be spatially controlled by the appropriate choice of the membrane permeable and impermeable fluorogens, enabling one to selectively observe FAP fusion proteins inside cells, on the cell surface, or within trafficking vesicles; 6) variations of the fluorogens have been shown to produce a variety of distinct spectral and sensing properties for a given FAP, which is very useful in a variety of multicolor experiments. To sum up, the FAP-fluorogen system is a versatile, effective fluorogenic labeling strategy.

1.2. The variations of the FAPs

For many of the scFvs, both V_H and V_L domains are essential for dye binding and fluorescence, however, the analysis of other scFvs revealed that either V_H or the V_L domain alone is sufficient to cause the fluorogenic dye activation[19,22] (Table 1). The existence of FAPs comprised of only of V_H or V_L domains that activate MG, such as L5, H6 and H8 FAPs, have already been demonstrated by Szent-Gyorgyi et al.[18]. In order to discover more desirable FAPs with appropriate and superior properties, such as high quantum yield and binding affinity, scFvs-based FAPs have been reconstructed and researched. For instance, the L5* FAP is a V_L domain that binds MG to activate intense fluorescence, which is a leucine-to-serine point mutant (Ser89) obtained by directed evolution of L5[21]. L5* binds to MG to form a bright fluorescent complex that improves on the quantum yield of the original L5 FAP by about 5-fold (quantum yield = 0.24). Another improved version of L5, the dL5** FAP (quantum yield = 0.20), is a synthetic dimer of a light chain with a disulfide forming pair of cysteines in each monomer. When expressed as a monomer putatively assembled into ternary complex with MG dye, the dL5** FAP confers tighter binding while maintaining increased brightness. The V_H domain alone FAP, dH6.2 FAP, is a synthetic

Table 1 Properties of FAPs and fluorogens. Fluorogens are derivatives of MG or TO, FAPs are comprised of hypervariable heavy (H) and light (L) chains joined by a flexible linker. All information is taken from Refs. 18 and 21.

FAP	Fluorogen	Size (kDa)	Excitation λ_{max} (nm)	Emission λ_{max} (nm)	Quantum yield	Fluorescence enhancement
L5-MG	MG-2p	11.5	640	668	0.048	4100
H6-MG	MG-2p	14.4	635	656	0.25	18,000
H8-MG	MG-2p	13.6	626	646		
HL4-MG	MG-2p	26.1	629	649	0.16	15,700
HL1.0.1-TO1	TO1-2p	25.9	509	530	0.47	2600
L5*-MG	MG-2p	14.2	634	667	0.24	

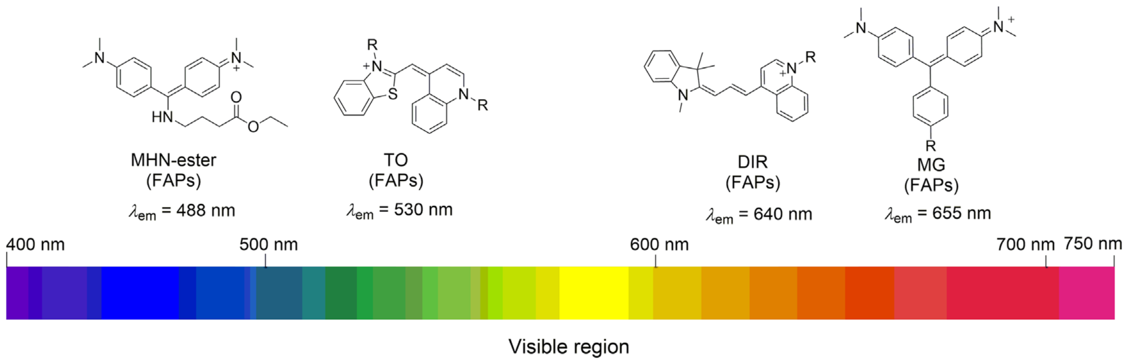

Figure 4 Fluorogens utilized for the development of FAP labeling methods. The maximal emission wavelengths of the fluorogens bound to their cognate FAPs are given.

dimer derived from a heavy chain with the second cysteine changed to alanine in each monomer[23]. This kind of FAP was normally bright but considerably less photostable across multiple compartments, which has proven useful for super-resolution imaging[22].

In addition, these FAPs contained internal disulfide bonds, which restricted their use to non-reducing environments such as the cell surface and secretory apparatus, since these FAPs may not fold properly in the reducing environment of the cytosol. The engineering of disulfide-free FAPs, like p13-CW FAP, a classic heavy-light scFv (HL4) with the second cysteine in each domain changed to an alanine, improved labeling in the cytoplasm and various other reducing subcellular compartments[24,25]. Furthermore, selection of scFvs against other fluorogens successfully extended the chromatic palette of FAPs[26,27]. Of particular interest, some scFv promiscuously activate various dimethylindole red (DIR) analogs, providing access to wavelengths ranging from the blue to the near infrared (NIR, 650–900 nm)[26]. There are also promiscuous FAPs that can bind more than one fluorogen, with alternate excitation and emission wavelengths and varying affinity constants for fluorogen binding[21]. Further improvements in brightness would result in better sensitivity and lower phototoxicity under typical imaging conditions.

2. The fluorogens of the FAP technology

MG, TO and DIR are classic FAP fluorogens (Fig. 4). In aqueous buffer these fluorogens exhibit strong absorbance maxima at 607 nm (MG), 504 nm (TO) and 610 nm (DIR), but exhibit extremely low levels of fluorescence. When bound to their cognate

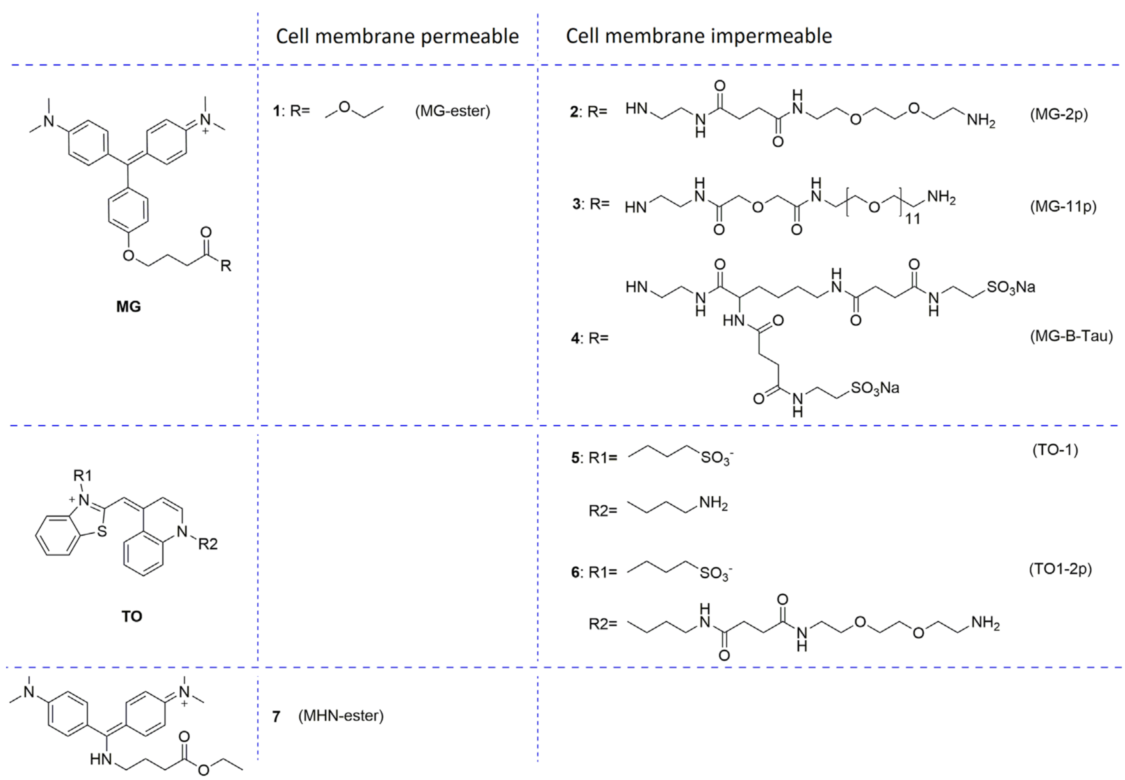

Figure 5 Structures of cell membrane permeable and impermeable fluorogens.

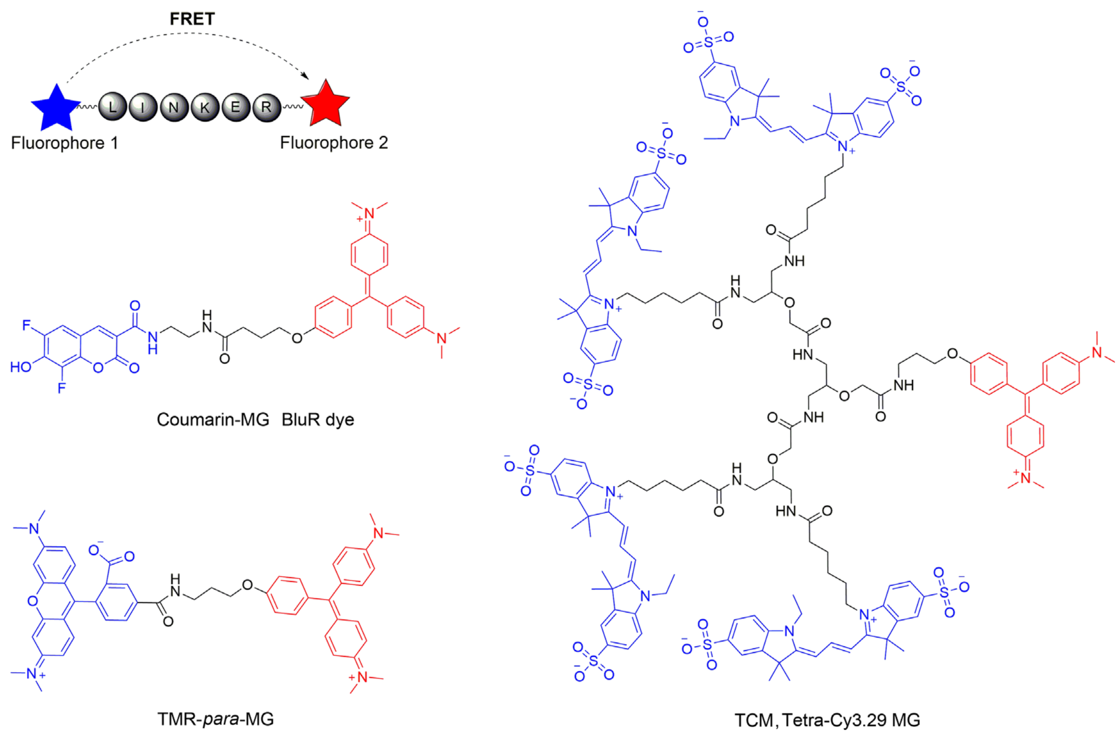

Figure 6 Structures of FRET based MG fluorogens with large pseudo-Stokes shifts. Donor groups are depicted in blue, and acceptor groups in red.

FAPs, the fluorogens exhibit bathochromic excitation maxima that are well matched to lasers (MG and DIR, 633 nm; TO1, 488 or 514 nm) generally used in flow cytometry and microscopy.

Along with the progress of genetically encodable FAPs to specifically label a protein-of-interest within the cellular milieu, various fluorogens of FAPs based on MG, TO or DIR were explored for tracking cellular proteins and other signaling molecules within their endogenous environment, providing unprecedented insights into the dynamic regulation of signaling networks in living cells.

2.1. Cell membrane permeable and impermeable fluorogens

Cell surface proteins tagged with FAP are readily detected with cell membrane impermeable fluorogens, whereas proteins within the secretory compartments in the cell only could be stained with membrane permeable fluorogens. In order to increase or decrease cell membrane permeability, fluorogen **1–6** were developed based on MG or TO (Fig. 5). With the intention of shorting the emission wavelength to blue, fluorogen **7** (MHN-ester) was explored[28]. MG-ester[18,29] and MHN-ester (fluorogen **1** and **7**) showed good cell permeability. On the other hand, sulfonated groups, polyethelyene glycol groups and amino groups were used to reduce the cell membrane permeability, resulting in cell impermeable sulfonated fluorogens TO1, TO1–2p, polyethelyene glycol modified fluorogens MG-2p, MG-11p and sulfonated fluorogens MG-B-Tau[30], respectively.

2.2. Fluorogens with large pseudo-Stokes shifts

Large Stokes shift is desirable in fluorescent labeling applications of dyes, as it reduces self-quenching effects and interference from excitation source[31]. Usually, fluorogen MG exhibited small Stokes shifts ($\Delta\lambda = 20$ nm), great efforts have been dedicated to increase the wavelength length difference between the excitation and emission and three series of Förster resonance energy transfer (FRET)-based MG fluorogens with large pseudo-Stokes shifts have been developed (Fig. 6)[32–34]. Very recently, we have rationally designed and synthesized a series of novel 3-indole-Malachite Green-based FAP fluorogens[35]. The important features of this class of FAP fluorogens are the efficient internal charge transfer resulting in significant fluorescence enhancements, remarkable large "pseudo-Stokes" shifts, low toxicity to cells, as well as very fast onset in response to FAP in both live mammalian cells and bacterial cells (Fig. 7). They have the potential to be an alternative to FRET-based MG fluorogens with large pseudo-Stokes shifts in multiplexing applications with FAP imaging.

2.3. In vivo FAP fluorogens

Cellular and tissue imaging in the near-infrared (NIR) wavelengths between 650 and 900 nm is advantageous for *in vivo* because of the low absorption of biological molecules in this region[36,37]. In the past decade, significant advances have been made in the design of molecular probes for *in vivo* imaging. Two series of NIR FAP fluoromodules have been developed by modification of TO[26] or MG[38] *via* conjugating methine groups (Fig. 8), among which, MG modified fluorogens have been successfully used for the detection of protein–protein interactions *in vivo*.

3. The application of the FAP technology

3.1. Protein locations visualization

FAP-based fluoromodules have been successfully and widely utilized in selectively visualization of protein location, internalization and trafficking in mammalian and yeast cells since 2008. As

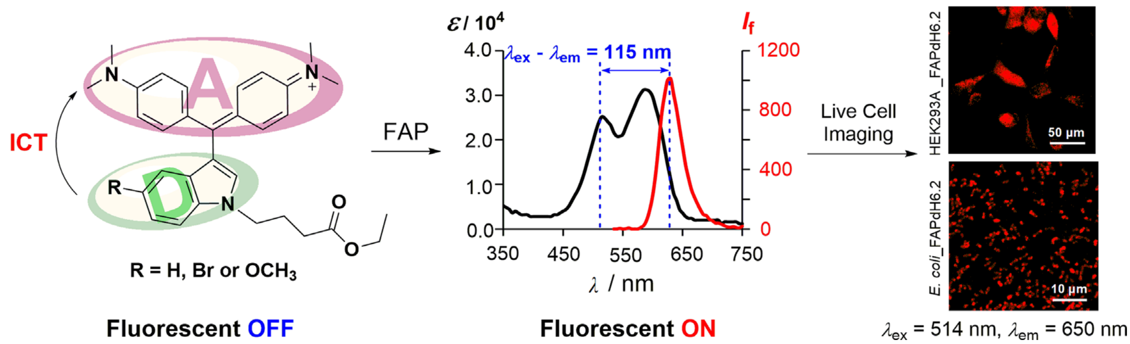

Figure 7 3-Indole Malachite Green based FAP fluorogens and their applications in live mammalian cells and bacterial cells imaging. Adapted with permission from Ref. 35. Copyright (2017) American Chemical Society.

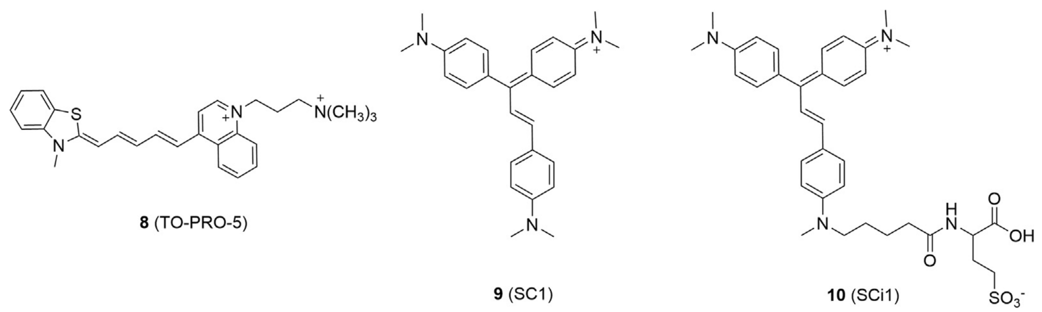

Figure 8 Structures of NIR FAP fluorogens.

discussed above, FAP technology has multiple advantages in biological imaging: 1) experimental flexibility—fluorescence is generated only upon addition of the fluorogen; 2) fast responses—FAPs can be visualized few seconds or minutes after addition of the fluorogen; 3) high spatial labeling discrimination—by playing with the cell-permeability of the fluorogen, fusion proteins in a given cell location can be selectively observed. For instance, comprehensive studies of plasma membrane G-protein coupled receptors (GPCRs), especially for β2-adrenergic receptor (β2-AR), expression, location, trafficking and quantification, as well as other membrane proteins, have been investigated by genetically encoding FAP tags in targeting molecules and imaging with modified TO or MG fluorogens[29,39–42]. Meanwhile, FAP-fluorogen technology was shown to be also suitable for labeling intracellular/cytosolic targets with the evolution of fluorogens. Based on FAPs and membrane permeable fluorogen MG-ester, Bruchez et al.[25] presented a new labeling technology for cytoplasmic compartments, which is no-wash, far-red, highly fluorogenic, photostable, and nonphototoxic and functions in all organelles. Later, a two-color, Green-Inside Red-Outside (GIRO) (Fig. 9), compartment selective FAP-based approach that generates distinct signals from surface and internal proteins in live cells for simultaneous detection is also demonstrated by Bruchez's group[28]. Lately, the same group established a three-color labeling approach, allowing excitation-dependent visualization of extracellular, intracellular, and total protein pools in the same cells by using one fluorogenic tag that combines with distinct dyes to affect different spectral characteristics[34].

3.2. *Drug discovery platform*

The FAP-based fluorescence detection and quantification approach also provides a platform for high-throughput screening of receptor proteins[29]. The most successful application is the discovery of novel cystic fibrosis transmembrane conductance regulator (CFTR) F508del correctors, using FAP tagging method in the trafficking studies of CFTR[43–45]. In addition, FAPs have an enormous potential for use in flow cytometry cell surface-based assays because fluorescence can be limited to proteins that are or have recently been resident on the surface membrane. Wu et al.[46] developed a platform combining FAP technology with high-throughput flow cytometry to detect real-time protein trafficking to and from the plasma membrane in living cells. The hybrid platform allows drug discovery for trafficking receptors such as GPCRs, and has been validated using the β2-AR system. They later expanded the hybrid system to a new type of biosensor, which provides the opportunity to study multiple trafficking proteins in the same cell[47]. Recently, Jarvik et al.[48] developed a novel approach based on FAPs and tethered fluorogen for visualizing regions of close apposition between the surfaces of living cells, which has the potential to provide a real-time readout of the proximity status of the membranes of the two cells. More recently, we and Brönstrup group first applied MG/FAP to study translocation efficiencies of molecular scaffolds designed to transport cargos in bacteria, which provided a general method for investigating the translocation capability of compounds across the membrane of bacterial cells (Fig. 10)[49].

3.3. *Super-resolution imaging and single molecule tracking*

In 2014, the Nobel Prize in Chemistry recognized super resolution imaging, a breakthrough in optical microscopy that enables researchers to visualize and investigate biological processes at the individual molecule level inside living cells. Fluorogen-based reporters were furthermore shown to open great prospects for super-resolution microscopy and single molecule tracking. Highly

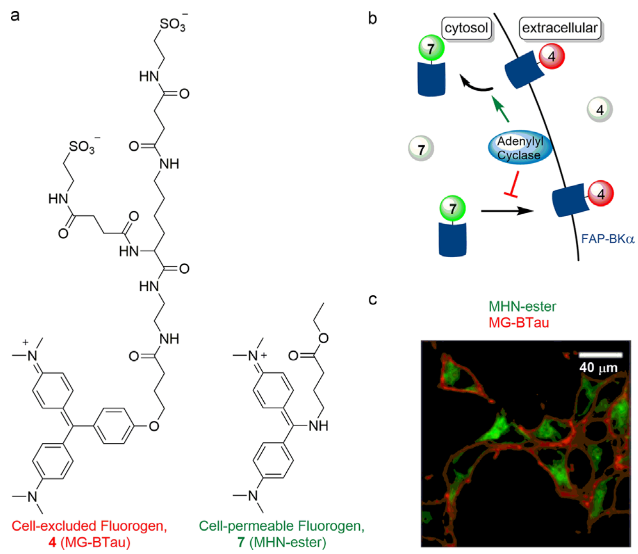

Figure 9 An example for membrane protein and intracellular protein visualization with cell permeable and impermeable fluorogens. (a) The chemical structure of the cell excluded MG-BTau (**4**), and cell permeable MHN-ester (**7**). (b) Schematized paradigm for GIRO labeling. (c) Live cells labeled with MG-BTau (red) and MHN-Ester (green) to label surface and internal protein in FAP-BK*α* expressing live cells. Adapted with permission from Ref. 28. Copyright (2015) American Chemical Society.

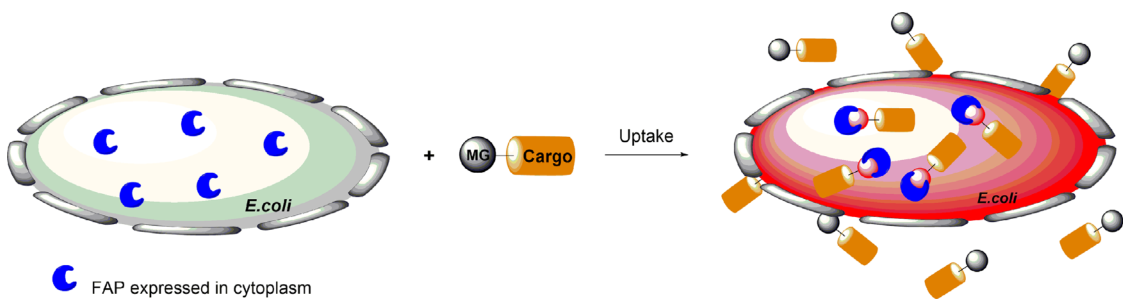

Figure 10 Principle illustrating the application of the FAP system for uptake visualization of MG–carrier conjugates in *E. coli*. Adapted with permission from Ref. 49. Copyright (2017) Wiley-VCH.

photostable far-red MG-based FAPs fluoromodules were reported to be well suitable for live cell imaging with stimulated emission depletion (STED) nanoscopy in mammalian cells and bacteria[23,50]. The ability to control the labeling density of the FAPs by adjusting the fluorogen concentration in the milieu was used to obtain sparse labeling distribution of densely genetically tagged proteins for single molecule localization imaging in living cells, further demonstrating the interest of such probes for super-resolution microscopy[22]. This system is advantageous over traditional approaches[51,52], because it does not require special imaging buffers or photo-activation or photo-switching with a second laser line. The ability to label a subset of proteins independently of the expression level also enabled to track individual receptors in living cells[53], confirming the usefulness of FAPs for single molecule studies.

3.4. Functional biomaterials and biosensors

FAPs proved to be beneficial for the design of functional biomaterials and biosensors, as well. For instance, combined with FAP, a genetically encoded pH sensor was developed by coupling modified TO with a pH sensitive Cy5 analog. It was successfully used to track surface proteins through endocytosis, which undergoes a significant change in FRET efficiency in response to environmental pH change (Fig. 11)[54]. Saunders et al.[55] developed a membrane materials in which dL5 FAP and AEAEAKAK, an amphiphilic peptide, are combined to form a solid-phase fluorescence detection platform. It is envisioned that dL5 FAP membranes can be established in diseased locales to monitor infiltration and migration of inflammatory cells marked with antibodies conjugated to MG. In combination with polymeric materials, targeted biosensor have been developed to distinct subcellular structures within living cells[56]. More recently, Meng group[57] reported an injectable film by which antibodies can be localized *in vivo*. Their system builds upon a bifunctional polypeptide consisting of a FAP and a *β*-fibrillizing peptide (*β*FP). The FAP domain generates fluorescence that reflects IgG binding sites conferred by protein A/G (pAG) conjugated with the fluorogen MG. When injected into the subcutaneous space of mouse footpads, film-embedded IgG were retained locally, with distribution through the lymphatics impeded. Further, a targetable near-infrared photosensitizer (TAP)-FAP that has been described by Bruchez et al.[58], which allows researchers to study protein inactivation, targeted-damage introduction and cellular ablation with unprecedented precision (Fig. 12).

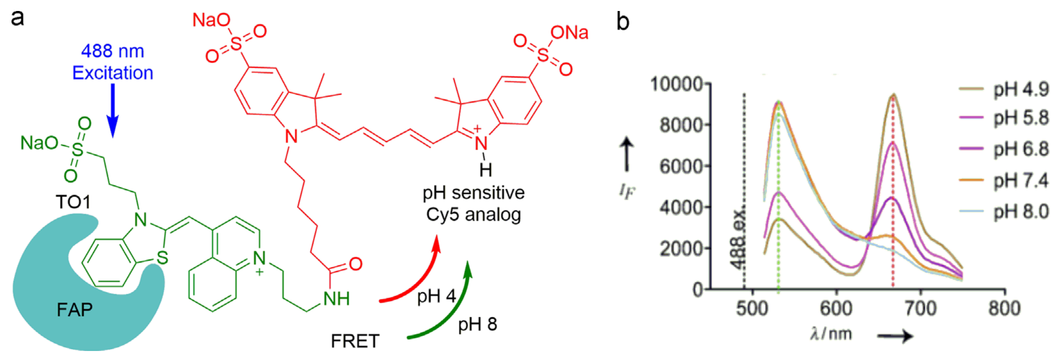

Figure 11 Illustration of the FRET-based pH biosensor. (a) The chemical structure of the FRET-based dye. A derivative of TO is linked to the pH-dependent Cy5 analogue. (b) The fluorescence emission spectra of the FAP-bound dye in citrate/phosphate buffer at various pH values. Upon excitation at $\lambda=488$ nm, energy transfer from the donor (TO) to the acceptor (Cy5 analogue) depends on the pH value. Adapted with permission from Ref. 54. Copyright (2012) Wiley-VCH.

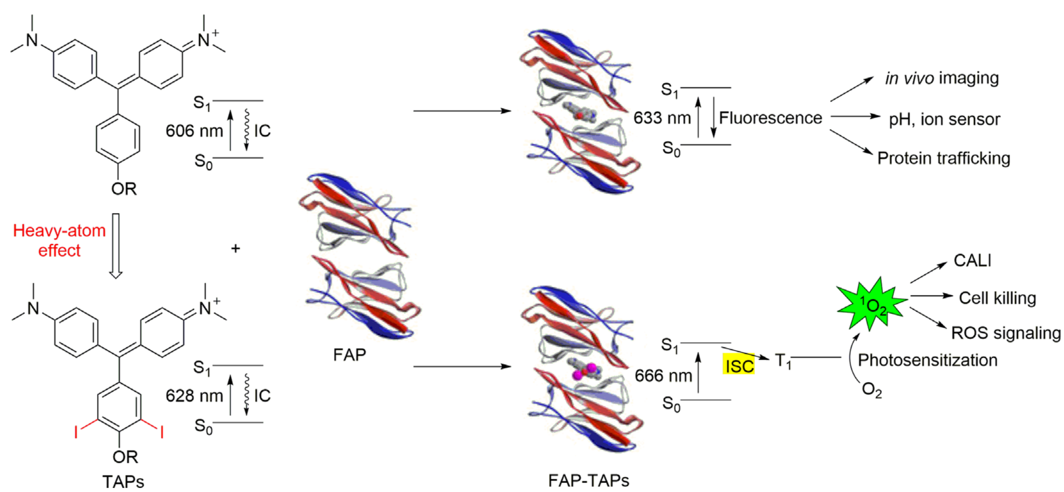

Figure 12 Mechanism of ROS generation by the targetable NIR photosensitizer, FAP-TAPs. IC, internal conversion by molecule's free rotation; ISC, intersystem crossing. Reprinted with permission from Ref. 58. Copyright (2016) Nature Publishing Group.

3.5. *In vivo imaging*

With the development and maturity of FAP technology, its applications have begun to expand into imaging of living animals. Pena et al.[59] first described the combined use of novel genetically targeted probes and high resolution optical imaging technologies to explore mitochondrial metabolism, ROS generation and function/dysfunction in the context of the living zebrafish. Remarkably, Bruchez and co-workers[60] developed a new tumor-targeting probe, affiFAP, containing a protein that specifically binds EGFR (affibody) and dL5** FAP. Fluorescence activation was achieved through either systemic (affiFAPs were pre-complexed with fluorogens prior to injection) or topical (affiFAPs were pre-targeted to the tumor site) administration of fluorogen. The latter approach was expected to minimize any undesired non-specific probe fluorescence and to demonstrate a possible no-wash platform for surgical guidance. They extended the application of affiFAP to *in vivo* imaging of a xenografted human EGFR-enriched tumor model in mice (Fig. 13), and establish its utility as a pretargeted fluorogen activating reagent, which is promising to be used in clinical settings to molecularly define tumor margins.

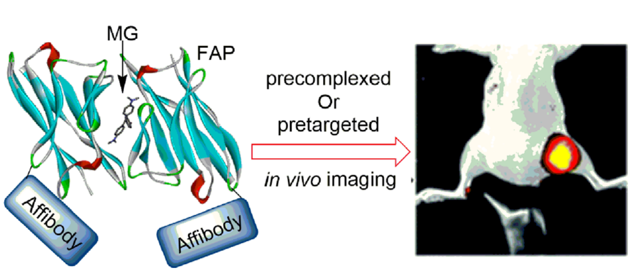

Figure 13 Affibody-fused FAP for targeted *in vivo* tumor imaging. Affibody is a protein that specifically binds EGFR. Adapted with permission from Ref. 60. Copyright (2017) Royal Society of Chemistry.

4. Conclusion and perspectives

As summarized in this review, fluorogenic labeling is a general concept for imaging biomolecules with high contrast in living systems, with great potential for pushing the limit of biological imaging. In this review, we have discussed in detail about the discovery, development and application of the FAP technology as a novel effective fluorogenic labeling method. Clearly, the FAP-fluorogen system displays a few unprecedented attributes, such as a small size, no-wash, high signal-to-noise ratio, and tunable spectral properties, which make them interesting alternatives to classical fluorescent proteins and open great prospects for advanced imaging, such as super-resolution microscopies. In this two-component system, both the FAP and the fluorogen can be tuned in order to obtain the desired properties. For example, fluorogens can be designed to display improved spectral properties, brightness, and photostability. Meanwhile, through random mutagenesis and directed evolution, FAPs can be selected to accommodate a large repertoire of fluorogens. With further efforts, we expect that the FAP-fluorogen system will be useful for addressing exciting unexplored biological questions and will greatly advance our understanding of human disease and normal cell function. However, in all cases introduced above, the FAP was expressed from a recombinant gene that encoded a protein fusion between the FAP and the protein of interest. This approach results in two significant setbacks[61]: (1) time and labor regarding quality control and generation of each recombinant protein, and (2) artificial protein expression from a nonnative promoter, typically altering protein regulation and abundance in the cell. Therefore, careful upstream studies of their toxicity and their influence on cellular processes is still required. At present, our group is conducting an in-depth study on this issue. Moreover, whether the FAP approach is feasible with smaller antibody fragments such as nanobodies remains to be investigated.

Acknowledgments

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