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Fluorescence-Activating and Absorption-Shifting Tags for Advanced Imaging and Biosensing

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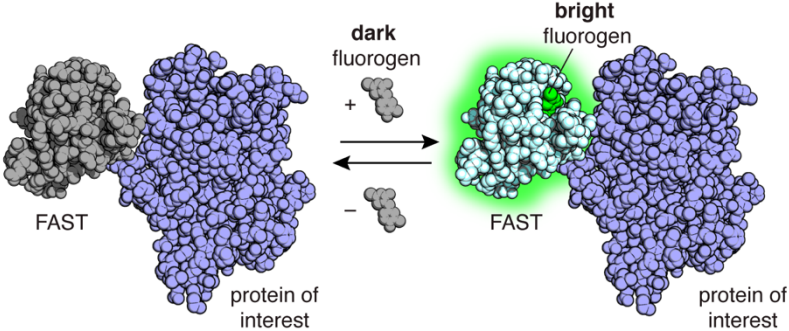
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Conspectus

Fluorescent labels and biosensors play central roles in biological and medical research. Targeted to specific biomolecules or cells, they allow non-invasive imaging of the machinery that govern cells and organisms in real time. Recently, chemogenetic reporters made of organic dyes specifically anchored to genetic tags have challenged the paradigm of fully genetically encoded fluorescent proteins. Combining the advantage of synthetic fluorophores with the targeting selectivity of genetically encoded tags, these chemogenetic reporters open new exciting prospects for studying cell biochemistry and biology. In this Account, we present the growing toolbox of Fluorescence-Activating and absorption-Shifting Tags (FASTs), small monomeric proteins of 14 kDa (125 amino acids residues) that can be used as markers to monitor gene expression and protein localization in live cells and organisms. Engineered by directed protein evolution from the Photoactive Yellow Protein (PYP) from the bacterium *Halorhodospira halophila*, prototypical FAST binds and stabilizes the fluorescent state of live-cell compatible hydroxybenzylidene rhodanine chromophores. This class of chromophores are normally dark when free in solution or in cells because they dissipate light energy through non-radiative processes. The protein cavity of FAST allows the stabilization of the deprotonated state of the chromophore and blocks the chromophore into a planar conformation, which leads to highly fluorescent protein-chromophore assemblies. The use of such fluorogenic dyes (also called fluorogens) enables the imaging of FAST fusion proteins in cells with high contrast without the need to remove unbound ligands through separate washing steps. Fluorogens with various spectral properties exist nowadays allowing investigators to adjust the spectral properties of FAST to their experimental conditions. Molecular engineering allowed furthermore to generate membrane-impermeant fluorogens for the selective labeling of cell-surface proteins. Over the years, we generated a collection of FAST variants with expanded spectral properties or fluorogen selectivity using a concerted strategy involving molecular engineering and directed protein evolution. Moreover, protein engineering allowed us to adapt FASTs for the design of fluorescent biosensors. Circular permutation enabled the generation of FAST variants with increased conformational flexibility for the design of biosensors in which fluorogen binding is

conditioned to the recognition of a given analyte. Bisection of FASTs into two complementary fragments allowed us furthermore to create split variants with reversible complementation that allow the detection and imaging of dynamic protein-protein interactions. We provide here a general overview of the current state of development of these different systems and their applications for advanced live cell imaging and biosensing, and discuss potential future directions.



Key references

Plamont, M.-A.; Billon-Denis, E.; Maurin, S.; Gauron, C.; Pimenta, F. M.; Specht, C. G.; Shi, J.; Querard, J.; Pan, B.; Rossignol, J.; Moncoq, K.; Morellet, N.; Volovitch, M.; Lescop, E.; Chen, Y.; Triller, A.; Vrizz, S.; Le Saux, T.; Jullien, L.; Gautier, A. Small Fluorescence-Activating and Absorption-Shifting Tag for Tunable Protein Imaging in Vivo. *Proc Natl Acad Sci USA* **2016**, *113*, 497–502.

This paper is the seminal paper presenting the development of canonical FAST through engineering of the photoactive yellow protein (PYP) by directed protein evolution, and its applications as reporter of gene expression and protein localization in bacteria, yeast, mammalian cells and in zebrafish embryo.

Benaissa, H.; Ounoughi, K.; Aujard, I.; Fischer, E.; Goïame, R.; Nguyen, J.; Tebo, A. G.; Li, C.; Le Saux, T.; Bertolin, G.; Tramier, M.; Danglot, L.; Pietrancosta, N.; Morin, X.; Jullien, L.; Gautier, A. Engineering of a Fluorescent Chemogenetic Reporter with Tunable Color for Advanced Live-Cell Imaging. *Nat Commun* **2021**, *12*, 6989.

This paper presents the evolution of FAST into pFAST a promiscuous variant able to bind a collection of fluorogenic chromophores covering most of the visible spectrum, and its application for advanced cellular imaging such as super-resolution microscopy and FLIM-FRET.

Tebo, A. G.; Gautier, A. A Split Fluorescent Reporter with Rapid and Reversible Complementation. *Nat Commun* **2019**, *10*, 2822.

This paper presents the development of splitFAST, a biosensor of protein-protein interaction generated through bisection of FAST into two complementary fragments that can complement when in close proximity.

Introduction

Fluorescence microscopy has become the method of choice for characterizing fluorescently labeled cellular structures and biomolecules with high spatial and temporal resolution in living cells and organisms. A large collection of fluorescent probes and biosensors has been developed and is nowadays available in the toolbox of cell biologists. Among them, fluorescent proteins such as the green fluorescent protein (GFP) and its derivatives play a central role, as they enable to label virtually any proteins in live cells and organisms through simple and efficient genetic fusion techniques¹. The discovery and development of fluorescent proteins with various spectral properties have been essential for pushing the limits of multicolor imaging and designing various types of biosensors.

The hegemony of fluorescent proteins has been recently challenged by the development of genetic tags that allow the selective anchoring of synthetic fluorophores, whose spectral properties, brightness and photostability can be tuned using modern chemistry. In such chemogenetic reporters, fluorogenic chromophores (also called fluorogens), whose fluorescence is activated upon target binding, are usually preferred to ordinary 'always-on' fluorophores because they allow imaging with high contrast in cells without the need for separated washing steps to remove unbound dyes. Molecular rotors such as Malachite Green and Thiazole Orange can for instance be activated by interaction with genetically encoded fluorogen-activating proteins (FAPs) based on single-chain variable fragments². These fluorogens are non-fluorescent when free because rotational freedom allows the dissipation of light energy via non-radiative pathways, but they can strongly fluoresce when locked into a planar conformation in the complementary cavity of FAPs. Another illustrative example is rhodamine-based chromophores that can be fluorogenic through tuning of the environment-sensitive equilibrium between the non-fluorescent spirocyclic, and the fluorescent quinoid form³⁻⁵. These fluorogenic chromophores were elegantly used for the fluorogenic labeling of proteins fused to the self-labeling proteins SNAP-tag⁶ and Halo-tag⁷.

Our laboratory recently expanded the fluorescence toolbox with Fluorescence-Activating and absorption-Shifting Tags (FASTs), small monomeric proteins of 14 kDa (125 amino acids residues) capable of stabilizing the fluorescent state of various live-cell

compatible fluorogens that are otherwise dark when free in solution or in cells. FASTs can be used as alternatives to fluorescent proteins or self-labeling tags to monitor gene expression and protein localization in live cells and organisms (Figure 1a). We provide here a general overview of the current state of their development and applications for advanced imaging and biosensing in live cells and organisms, and discuss potential future directions.

Prototypical FAST

Prototypical FAST binds and stabilizes the fluorescent anionic state of 4-hydroxybenzylidene rhodanine (HBR) derivatives⁸ (Figure 1). HBR derivatives are push-pull chromophores composed of an electron-donating phenol conjugated to an electron-withdrawing rhodanine heterocycle. These dyes are essentially non-fluorescent in solution because of ultrafast radiationless decay (Figure 1b). When bound to FAST, they strongly fluoresce because the protein environment prevents large amplitude motion suppressing internal conversion and locks the chromophore in an emissive planar conformation (Figure 1b). The fluorogenic behavior of HBR derivatives is similar to that of the 4-hydroxybenzylidene-imidazolinone (HBI) chromophore found in GFP. Outside the protein environment, HBI and its derivatives are essentially non-fluorescent. Theoretical studies have suggested that the ultrafast excited-state decay proceeds along two alternative pathways corresponding to rotation around one of the methine bridge bonds allowing internal conversion through conical intersections⁹⁻¹¹. The fluorescence quenching of HBR derivatives in solution is believed to result from similar deactivation pathways.

To create prototypical FAST, we engineered the blue light photosensor Photoactive Yellow Protein (PYP) implicated in the photophobic behavior of the bacterium *Halorhodospira halophila* (Figure 2a). PYP senses light via trans-cis isomerization of the 4-hydroxycinnamoyl prosthetic group covalently attached to the residue Cys69 through a thioester bond. The residue Glu46 presents in the low dielectric protein interior is protonated and hydrogen-bonded to the chromophore phenolate oxygen, which stabilizes its negative charge and gives PYP its yellow color in solution. As the biosynthetic

enzymes responsible for maturation of PYP are absent in most organisms, we replaced the 4-hydroxycinnamoyl group with HBR derivatives, which share structural similarities.

To identify protein variants able to non-covalently bind HBR derivatives and stabilize their anionic fluorescent state, we developed a general directed evolution strategy⁸ (Figure 2b). Directed evolution can enable to identify proteins with improved affinity and brightness. After mutagenizing Cys69 to alanine, we created a combinatorial library of variants through randomization of the loop 94-101 (that gates the chromophore binding pocket) using saturation mutagenesis. As yeast surface display can allow high-throughput evaluation and selection of fluorescence properties using fluorescence-activating cell sorting (FACS), the constructed library was expressed in yeast so that every cell displays many copies of one variant sequence. Iterative rounds of sorting allowed the identification of a protein variant, initially called Y-FAST and renamed FAST, able to form tight and bright green-yellow fluorescent assembly with 4-hydroxy-3-methylbenzylidene rhodanine (HMBR) (with 480/540 nm absorption/emission peaks).

The assembly between FAST and HMBR exhibits red-shifted absorption compared to HMBR in solution at physiological pH in agreement with a shift of the protonation/deprotonation equilibrium from a protonated state (in solution) to a deprotonated anionic state (in FAST) (Figure 1c). As fluorescence brightness is the convolution of the fluorescence quantum yield and the molar absorptivity at the excitation wavelength, the red-shift in absorption upon binding participates to the strong and selective fluorogenic activation (Figure 1c). It was originally proposed that the chromophore adopts in FAST a quasi-planar conformation with an orientation of the phenolic ring that enables Glu46 to stabilize the anionic phenolate state (Figure 2a), explaining both the high fluorescence quantum yield and the red-shifted absorption. This mode of binding was recently confirmed independently by the determination of the structure of FAST by nuclear magnetic resonance¹² and the in silico modeling of FAST variants bound to various fluorogens¹³. The unique fluorogenicity mechanism involving both quantum yield increase through conformational locking and absorption red-shift through protonation enables to minimize fluorescence background that could result from potential off-target interactions.

Molecular engineering studies showed that varying the substituents on the aromatic ring of the fluorogen could generate various spectral properties in the green to red region¹⁴ (Figure 3). This set of fluorogens enabled to adapt the fluorescence properties of FAST to the experimental settings without the need for changing the reporter tag, offering experimental versatility not encountered with conventional fluorescent proteins. In particular, we discovered that introduction of two dimethoxy groups in ortho position of the phenol (HBR-3,5DOM) significantly red-shifts absorption and emission, generating a bright orange-red fluorescent assembly with 520/600 nm absorption/emission peaks. This unique property allows one to easily change the color of FAST from green/yellow to orange/red by a simple change of fluorogen. Beyond providing an elegant way to tune the spectral properties of FAST, this pair of fluorogens could be used to switch color dynamically in cells. Because fluorogen binds FAST in a reversible and dynamic fashion, color can be rapidly changed from green to red (or red to green) by exchanging fluorogens. We showed that measuring the degree of anti-correlation of the green and red fluorescence signals upon such dynamic color switching was an effective method to quantitatively extract the signal of FAST in spectrally crowded cells expressing both green and red fluorescent proteins¹⁴. This proof-of-principle experiment opened exciting prospects to overcome spectral crowding and push the frontiers of multiplexed imaging.

Chromophore engineering also allowed the development of fluorogenic chromophores suitable for the selective labeling of cell-surface proteins¹⁵ (Figure 3). Introduction of carboxymethyl group on the rhodanine head enabled to generate HBR analogs that are negatively charged at physiological pH, allowing to reduce their ability to cross the plasma membrane of mammalian cells¹⁵. These chromophores with reduced cellular uptake retained their ability to form fluorescent, albeit dimmer, assembly with FAST, allowing the selective labeling of FAST-tagged surface proteins without labeling intracellular proteins present in the secretory pathway. This property allowed the localization of a membrane protein by a simple fluorescence measurement, without microscopy or image analysis¹⁵. This work opened perspectives for the study of secretion, transport and recycling of membrane proteins, as well as for the development of new

methods for screening therapeutic molecules capable of repairing defective protein trafficking.

Properties and applications of FAST

FAST can be used as marker to monitor gene expression and protein localization in live cells and organisms. FAST was shown to be functional in various cell types (e.g. mammalian cells^{6,12-14}, neurons⁸), microorganisms (e.g. bacteria^{8,16-23} and yeast²⁴) and multicellular organisms (e.g. zebrafish embryo⁸). The low cytotoxicity of the fluorogens at the concentrations used for labeling and the low phototoxicity of the FAST:fluorogen assemblies allow for long-term imaging in various systems. In addition, the small size (125 amino acid residues, i.e. 14 kDa), reduced genetic footprint (375 bp), and full monomericity of FAST should make it less perturbing when fused to proteins than fluorescent proteins, whose general use can be limited by their larger size (25-30 kDa) and tendency to oligomerize.

Submicromolar fluorogen binding affinity enables to reach high labeling selectivity in cells. In presence of fluorogen concentrations ensuring full labeling, labeling is extremely rapid and completed in a few hundred milliseconds. Consequently, labeling kinetics in cells or organisms is almost only limited by fluorogen cell uptake and diffusion. In practice, incubation with the fluorogen for few tens of seconds is sufficient for full labeling in cultured mammalian cells. For multicellular organisms such as zebrafish embryo, full labeling is reached in few tens of minutes⁸. At steady state (i.e. when cells are fully loaded with fluorogen), fluorescence maturation is quasi-instantaneous as it is only limited by fluorogen binding. This property distinguishes FAST from GFP-like fluorescent proteins that are notoriously limited by their rather long (10 minutes up to several hours) chromophore maturation and from self-labeling tags such as HaloTag and SNAP-tag that have a longer labeling time. This feature opens new opportunities for monitoring dynamic processes such as protein synthesis in real-time. Note that, moreover, because fluorogen binding is non-covalent, protein degradation results in an immediate loss of fluorescence, providing new ways to monitor protein turnover in real-time. The reversibility of the labeling is a unique trait that distinguishes FAST from self-labeling tags such as HaloTag⁷, SNAP-tag⁶ or CLIP-tag²⁵. As fluorogens bind FAST non-covalently, labeling can be

reversible, allowing one to switch off FAST at will by fluorogen washout^{8,14}. This feature should make FAST an ideal reporter for the monitoring of transgenesis or genome editing, as fluorescence could be activated only transiently for screening modified cells or organisms when needed. In addition, non-covalent fluorogen binding results in continuous fluorogen exchange at steady state. This continuous fluorogen renewal provides good photostability, as photodamaged fluorogen can be replaced by new ones²⁶. However, photobleaching can still occur. Prolonged and/or intense illumination leads to photooxidation of the FAST protein²⁶, as observed for regular fluorescent proteins. Protein photodamage alters very likely fluorogen binding, leading ultimately to photobleaching²⁶.

FAST was shown to offer an attractive alternative to fluorescent proteins for studying protein function in anaerobic conditions. While fluorescent proteins require molecular oxygen for chromophore maturation, FAST is fluorescent no matter the concentration of molecular oxygen. FAST was shown (i) to outperform fluorescent proteins to monitor the formation of bacterial biofilms where the distributions of molecular oxygen can be highly variable^{16,22}, and (ii) to be strongly fluorescent in strict anaerobes such as *Clostridium* organisms^{17,19}. FAST was used to study promoter activity in *Clostridium acetobutylicum*, a strict solventogenic anaerobe, and allowed the tagging of a previously uncharacterized *Clostridium* cell divisome protein, ZapA¹⁷. FAST and HaloTag⁷ were recently combined to demonstrate that *Clostridium ljungdahlii* and *Clostridium acetobutylicum* can exchange protein material in syntrophic co-culture²⁷. Before that work, only flavin mononucleotide-based fluorescent proteins (FbFPs) were shown to be somewhat functional in anaerobes²⁸, but their use was limited by fairly low brightness²⁹. This work has opened the door for applications in other anaerobes. FAST was used for instance to monitor the levels of proteins involved in (i) the production of the biocommodities butanol and acetone from methanol in *Eubacterium limosum*²³, and (ii) the autotrophic production of lactate from dihydrogen and carbon dioxide in *Acetobacterium woodii*³⁰. More recently, FAST was shown to allow the monitoring of protein dynamics in live cells of *Methanococcus maripaludis*³¹, opening great prospects for future studies focused on the metabolism and physiology of methanogenic archaea. Finally, because of its molecular oxygen-independent fluorescence, FAST was shown to be usable to study the vitality of bacteria transplanted to the gut microbiota of mice³².

Besides offering previously unobtainable options for studying protein function in anaerobes, FAST provides also new options for studying bacterial protein localization as it remains functional after secretion by Gram-positive and Gram-negative bacteria²¹. Real-time imaging of bacterial virulence factors or adhesion proteins has been hampered for a long time by the limited number of fluorescent tools suitable for tagging secreted proteins. FAST was used to tag bacterial effectors that could be secreted by Sec-dependent secretion systems in Gram-positive *Listeria monocytogenes* or by a type III secretion system in the Gram-negative pathogen *Shigella flexneri*²⁰. FAST tagging enabled to monitor infection dynamics in epithelial cells exposed to the human pathogen *Listeria monocytogenes* (*Lm*) and revealed active proliferation in entry vacuoles²⁰. We anticipate that this property is general and that FAST should allow the visualization of protein secretion in various bacteria and other microorganisms.

The growing family of FAST reporters

In addition to the original FAST, an improved FAST (iFAST) variant was developed by replacing Val107 with isoleucine in order to increase the conformational locking of the chromophore in the binding site³³. Compared to FAST, iFAST is brighter and binds fluorogens with higher affinity. iFAST allowed the generation of a tandem iFAST (td-iFAST) that forms green and red fluorescent reporters 1.6-fold and 2-fold brighter than EGFP and mCherry, respectively, while having a comparable size. This brighter variant can be used as genetic tag for characterizing low-expressing proteins.

Recently, new variants and fluorogenic ligands were developed to add new colors to the FAST family (Figure 4). Molecular engineering of the fluorogenic ligand followed by cycles of affinity/brightness improvement by yeast display allowed us to further tune spectral properties. Addition of an extra double bond between the phenol and the rhodanine moieties of the fluorogen allowed to further red-shift the absorption and emission properties. Elongating the π -electron conjugation diminishes the energy gap between the high occupied molecular orbital (HOMO) and the low unoccupied molecular orbital (LUMO), red-shifting the absorption and emission properties. Yeast display screening allowed the isolation of a FAST variant able to form bright far-red fluorescent assembly with (4-hydroxy-3-methoxyphenyl)allylidene rhodanine (HPAR-3OM)³⁴. With

555/670 nm absorption-emission peaks, far-red (fr)FAST expands the fluorescence toolkit with new properties and possibilities. The use of far-red fluorescent tags is particularly important when working with multicellular organisms, due to the lower autofluorescence and the better light penetration in tissues at longer wavelengths. frFAST was shown to be an efficient far-red fluorescent tag for imaging proteins at various stages of zebrafish embryogenesis. Efficient labeling and detection could be achieved in tissue of internal organs such as the myocardium in beating heart in live zebrafish larvae. Finally, the far-red emission of frFAST allowed multicolor imaging in cells and in chicken embryo tissues.

Very recently, a FAST variant, named pFAST, with highly tunable spectral and optical properties was engineered by a coordinated strategy involving directed evolution and fluorogen engineering¹³. pFAST is a promiscuous variant capable of recognizing a palette of chromophores with various electronic properties (Figure 4). The bimolecular assemblies obtained show optical properties spanning the visible spectrum from blue to red. This extensive alteration of the absorption and emission wavelengths was achieved by replacing the rhodanine found in prototypical HBR derivatives by isosteric heterocycles with various electron-withdrawing abilities. Fluorescence screening strategy based on yeast display and FACS allowed us to isolate a variant showing good-to-excellent binding/spectroscopic properties with the entire set of fluorogenic compounds. The ability to tune the spectral properties of pFAST through simple molecular modulation allows investigators to optimize spectral orthogonality for multicolor imaging. This work has opened the door for new ways to optimize energy transfer efficiency in Förster resonance energy transfer (FRET) biosensors. The intrinsic property of FAST variants to be imaged with and without fluorogen allows measurements with or without acceptor. The use of pFAST as FRET acceptor allowed with a single genetic construct to easily optimize FRET efficiency by testing various fluorogens for identifying those that give optimal energy transfer. This optimization could be done in single cells by sequential addition of the fluorogens, allowing to compare biosensors in the exact same cellular context. During this work, it was discovered that pFAST can assemble with the 4-hydroxy-3-methylbenzylidene isorhodanine (HBIR-3M) into a very tight non-fluorescent bimolecular assembly that absorbs in the green region. This discovery opened new opportunities for

the design of dark acceptors for FRET measurements based on fluorescence lifetime imaging microscopy (FLIM) because pFAST labeled with a dark chromophore does not fluoresce and can thus modulate the fluorescence lifetime of a donor without blocking an imaging channel. HBIR-3M was found to be well suited also for other applications. The possibility to replace bright chromophores with a dark one by direct competition for the binding site was shown to allow the switching off pFAST fluorescence in cells and in multicellular organisms with high time resolution. Beside a higher chromophore binding promiscuity, pFAST displays improved properties with the membrane-permeant and membrane-impermeant HBR analogs originally designed for prototypical FAST, making it today the FAST variant of choice for imaging applications. pFAST binds most HBR analogs with higher affinity and usually forms assemblies with increased brightness, enabling more efficient labeling in live cells, including neurons, and in multicellular organisms. Moreover, these assemblies exhibit superior photostability, allowing longer or more power-demanding imaging experiments. The high photostability allowed the imaging of cellular structures below the diffraction limit using stimulated emission depletion (STED) nanoscopy, whose broad use is currently limited by the low numbers of fluorophores displaying compatible spectral properties and photostability. The labeling of pFAST with HBR-3,5DOM allowed STED imaging of membranes, microtubules, and actin filaments with sub-diffraction resolution in live cells and neurons.

In addition to these developments, directed evolution allowed us to create variants with orthogonal fluorogen selectivity³⁵, further expanding the possibilities offered by the FAST toolbox. As HMBR and HBR-3,5DOM provide respectively green and red fluorescence, yeast display evolution was used to isolate variants of FAST with orthogonal recognition capabilities, and thus orthogonal spectral properties (Figure 4). Sorting in presence of both fluorogens allowed us to isolate variants forming fluorescent assemblies preferentially with either fluorogen. The resulting greenFAST and redFAST selectively recognize HMBR and HBR-3,5DOM, and form bimolecular assemblies with 480 nm / 540 nm and 560 nm / 600 nm absorption/emission peaks. GreenFAST binds HMBR 100-fold tighter than HBR-3,5DOM, while redFAST binds HBR-3,5DOM 10-fold tighter than HMBR, ensuring orthogonality. This set of spectrally orthogonal tags was shown to be well suited for two-color fluorescence microscopy in live cells and

multicellular organisms. In particular, greenFAST and redFAST allowed the creation of a two-color cell cycle indicator based on the Fluorescent Ubiquitination-based Cell Cycle Indicator (FUCCI) design. FUCCI enables to delineate cell cycle phases via the use of a red fluorescent protein and a green fluorescent protein fused to the N-terminal domains of Cdt1 and geminin, two cell cycle regulators whose levels show biphasic cycling during the cell cycle³⁶. Although very powerful, FUCCI could not be used to analyze very short cell cycles, such as those found in early fish or amphibian embryos, because of the time limitations imposed by the slow maturation of GFP-like fluorescent proteins. As greenFAST and redFAST are instantaneously fluorescent provided that the fluorogens are present, and as their fluorescence disappears as soon as they are degraded, they appeared well suited for the design of a FUCCI-like cell cycle indicator. Fusions of greenFAST and redFAST to the N-terminal domains of zebrafish Cdt1 and geminin enabled to delineate all phase transitions in cell cycles at the mid-blastula transition in zebrafish embryogenesis. The quasi-instantaneous fluorescence maturation of greenFAST and redFAST allowed detailed analysis of cell cycles as short as 15 min and as early as 3.5 hours post-fertilization. Moreover, proliferation patterns and asynchronous division could be observed as early as 256-cell stage. This behavior had been previously described only in fixed embryo³⁷ or in live embryos analyzed by label-free non-linear microscopy³⁸, but these techniques did not give access to the individual phases of the cell cycle. GreenFAST and redFAST were recently used as fluorescent reporters for investigating the coculture dynamics of metabolically engineered *Clostridium saccharoperbutylacetonicum* strains under anaerobic conditions³⁹.

FAST-based sensors

Topological variants of FAST were developed to design fluorescent chemogenetic biosensors (Figure 5). The possibility to condition fluorogen binding, and thus fluorescence, to a given recognition event or cellular signal enabled the development of sensors for various applications in biological research. Circularly permuted (cp)FAST variants were created through relocation of the N- and C-termini at position 115 and 114 (Figure 5a,b)⁴⁰. Relocating the N- and C-termini enabled to retain the same (or similar) three-dimensional structure (and thus function) while positioning the N- and C-termini

close in space. Such circular permutations can have various applications. One is to be used to tag protein through insertion at a permissive insertion site when N- or C-terminal fusion are not possible. Another one is for the creation of biosensors. Circular permutation often increases conformational flexibility enabling the design of conformational protein switches. Flanking cpFAST variants with the Ca^{2+} -binding protein Calmodulin and the M13 peptide (successfully used in the past for the design of the GCaMP series) allowed us to create a Ca^{2+} -responsive system where binding of Ca^{2+} increases the affinity for the fluorogen⁴⁰. The ability to condition fluorogen binding, and thus fluorescence, to Ca^{2+} binding allowed the design of fluorescent sensors for the monitoring of Ca^{2+} in live cells. The ability to modulate the spectral properties of FAST through fluorogen change allowed us to modulate the spectral properties of this sensor from green to red without the need for protein reengineering.

FAST also allowed the creation of a split fluorescent complementation system, named splitFAST, for the monitoring of dynamic protein-protein interactions in live cells⁴¹ (Figure 5c,d). Because interactions between proteins play an essential role in most cellular processes, methods to identify interacting partners and characterize how they interact in space and time are essential. SplitFAST was created through bisection of FAST in its last loop between Ser 114 and Gly 115. The resulting N-terminal fragment and the C-terminal fragment show modest affinity in presence of fluorogen. However, when fused to two interacting proteins, these two complementary fragments can assemble into a functional reporter, enabling visualization of the interaction. SplitFAST allowed imaging of known interactions in various cellular localizations. Interestingly, as fluorogen binding is rapid, non-covalent and reversible, splitFAST displays rapid and reversible complementation, enabling the monitoring of not only protein complex assembly but also protein complex disassembly. The rapid and reversible complementation is a unique trait that distinguishes splitFAST from split fluorescent proteins that exhibit slow and irreversible complementation because of their chromophore maturation. This work showed also that splitFAST could be well suited for the design of fluorescent biosensors. Fusion of the two complementary split fragments to Calmodulin and M13 allowed to monitor Calcium waves with high temporal resolution in mammalian cells. An apoptosis sensor could be generated by conditioning splitFAST

complementation to the activity of the caspase-3. Recently, the design of splitFAST could be extended to other FAST variants. Functional split reporters could be generated through bisection of frFAST, greenFAST and redFAST at position 114-115^{34,35}. Noteworthy, greenFAST and redFAST share a common C-terminal fragment after bisection, which allowed the detection of two interactions sharing a common partner³⁵.

Perspectives

The chemogenetic nature of FASTs opens various options for engineering new original and innovative tools. Recently, the group of Mikhail Baranov has shown that FAST can bind and stabilize the fluorescent states of other dyes derived from the benzylidene imidazolone found in GFP⁴²⁻⁴⁴, opening new prospects for spectral tuning. Future engineering efforts should aim at increasing fluorescence brightness through increase of the fluorescence quantum yield and shaping of the absorption bands, and at pushing the spectral properties to the near infrared region for enabling imaging deeper into tissues.

Alteration of the chemical structure of HBR analogs could also be used to alter the fate of the excited state, in particular by promoting intersystem crossing. As relaxation from the triplet state to the ground state can excite triplet oxygen into singlet oxygen, such compounds could be used to generate photosensitizers able to generate singlet oxygen or other reactive oxygen species (ROS) locally upon light excitation. Such genetically encoded fluorescent photosensitizers could be used for (i) selective inactivation of proteins or ablation of cells for functional studies⁴⁵⁻⁴⁷, or (ii) local polymerization of diaminobenzidine into an osmiophilic product detectable by electron microscopy for applications in correlative light and electron microscopy^{47,48}.

Innovation could also arise from protein engineering. Recently, the group of Mikhail Baranov showed that the size of FAST could be reduced to 98 amino acid residues through truncation of the N-terminal domain¹². Although the resulting nanoFAST lost its ability to bind canonical FAST fluorogens, nanoFAST is able to bind and stabilize the fluorescent state of 4-hydroxy-2,5-dimethoxybenzylidene rhodanine. This work opens very exciting prospects for the design of protein tags with minimal size. Protein engineering could also allow the generation of FAST variants able to form fluorescent assemblies displaying various fluorescence lifetimes for applications in multiplexed

imaging. We previously serendipitously discovered that, although FAST and greenFAST both form green fluorescent assembly with HMBR with superimposable spectral properties, these two tags exhibit different fluorescence lifetimes that can be used to distinguish FAST and greenFAST fusions by fluorescence lifetime imaging³⁵. Protein engineering could enable the identification of a repertoire of FAST variants able to form fluorescent assemblies displaying various fluorescence lifetime imaging when labeled with a single fluorogen for fluorescence lifetime multiplexing, as recently elegantly shown using HaloTag variants that enable the fluorescence lifetime multiplexing of several cellular targets in one spectral channel⁴⁹. Protein engineering should also lead to innovations in the design of optical sensors. Optical protein sensors play central roles in biological research. Incorporation of sensing proteins units within FAST variants and their topological derivatives could generate biosensors that recognize various analytes with high selectivity. Efficient and large conformational coupling could allow the design of analyte responsive optical protein sensors through allosteric-like modulation of fluorogen binding.

Future research direction should also include the optimization of FAST:fluorogen pairs for super-resolution microscopy techniques. Previous studies showed that FAST has features suitable for such techniques. Since investigators can control fluorogen concentrations at will, it is possible to label only a subset of proteins independently of their expression level. This property was shown to be useful to improve resolution using super-resolution imaging by radial fluctuations (SRRF)⁵⁰ and super-resolution optical fluctuation imaging (SOFI)³⁵, which are sensitive to fluorophore density, or by single-molecule localization microscopy (SMLM)⁵¹, which necessitates the stochastic generation of sparse subsets of emitters over time. The recent demonstration that pFAST was suitable for STED microscopy in live cells and neurons should stimulate further developments in super-resolution imaging¹³. Future engineering efforts should focus on increasing photostability, improving single molecule photon budget, and tuning/engineering blinking properties for applications in SMLM.

Finally, the methodology developed to create fluorescent reporters with new spectral properties could be applied to identify genetically encoded probes for other imaging modalities such as photoacoustic imaging. Photoacoustic imaging recently

appears as a promising alternative to optical imaging combining deep-penetrating excitation light with acoustic detection^{52,53}. Photoacoustic signal can be generated by light absorbers able to dissipate light energy non-radiatively. Local heat release results in a thermoelastic expansion that produces a change in pressure. By using pulsed excitation, it is possible to generate fluctuating pressure waves, which propagates as detectable acoustic waves. Ideal photoacoustic probes (i) absorb near-infrared light, and (ii) have high excitation coefficients and low fluorescence quantum yield to maximize heat release. Weakly fluorescent FAST-like systems absorbing far-red and near-infrared light could maximize photoacoustic effect and be efficient genetically encoded photoacoustic probes. The chromogenic effect (absorption red-shift) undergone by the chromophore upon binding could ensure high selectivity as the free chromophore would not absorb the excitatory light.

In conclusion, FAST and its variants are attractive alternatives to fluorescent proteins and self-labeling proteins. They provide new options for the imaging of proteins in living cells and organisms, and open exciting prospects for biosensor design, multiplex imaging, and high-resolution imaging. The design and use of FASTs are still in their infancy, and we expect that the coming years will see demonstrations of their full potentials and further exciting and unpredictable advances.

Bibliographical information

Arnaud Gautier is Professor of Chemistry at Sorbonne University in Paris, where he develops chemogenetic methods and probes for imaging and controlling biomolecules and cellular functions. He studied chemistry at the École Normale Supérieure de Lyon, where he received a PhD in chemistry in 2005. In 2006, he joined the group of Kai Johnsson at the École Polytechnique Fédérale of Lausanne where he developed methods for the selective labeling of proteins with synthetic probes in living cells. In 2009, he joined the group of Jason W. Chin at the MRC laboratory of Molecular Biology in Cambridge, where he worked on the expansion of the genetic code of mammalian cells. Arnaud Gautier was Assistant Professor at the École Normale Supérieure in Paris from 2010 to 2019, before joining Sorbonne University in 2019 as Full Professor.

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Notes

A.G. is cofounder and holds equity in Twinkle Bioscience/The Twinkle Factory, a company commercializing the FAST technology.

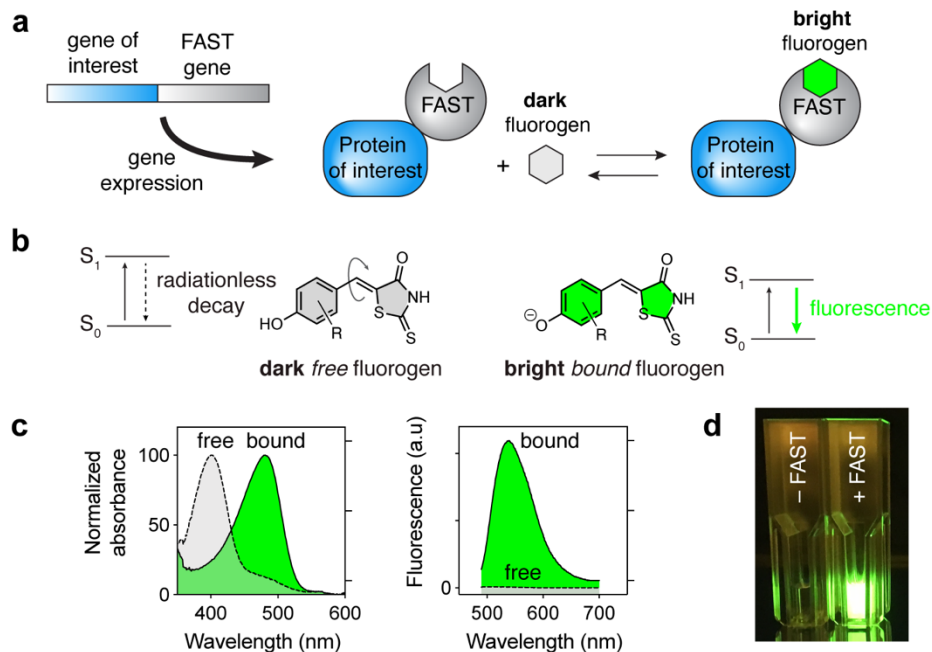


Figure 1. Fluorescence-activating and absorption shifting tag (FAST). **a** Prototypical FAST is a small monomeric protein of 14 kDa that can be used to monitor gene expression and protein localization in live cells and organisms. **b,c** FAST binds and stabilizes the fluorescent state of hydroxybenzylidene rhodanine chromophores that are otherwise dark when free in solution or in cells. The protein cavity of FAST allows the stabilization of the deprotonated state of the chromophore, leading to a red-shift in absorption, and blocks the chromophore into a planar conformation, which leads to highly fluorescent protein-chromophore assemblies. **d** Fluorescence activation undergone by the fluorogenic chromophore HMBR upon binding to recombinant FAST.

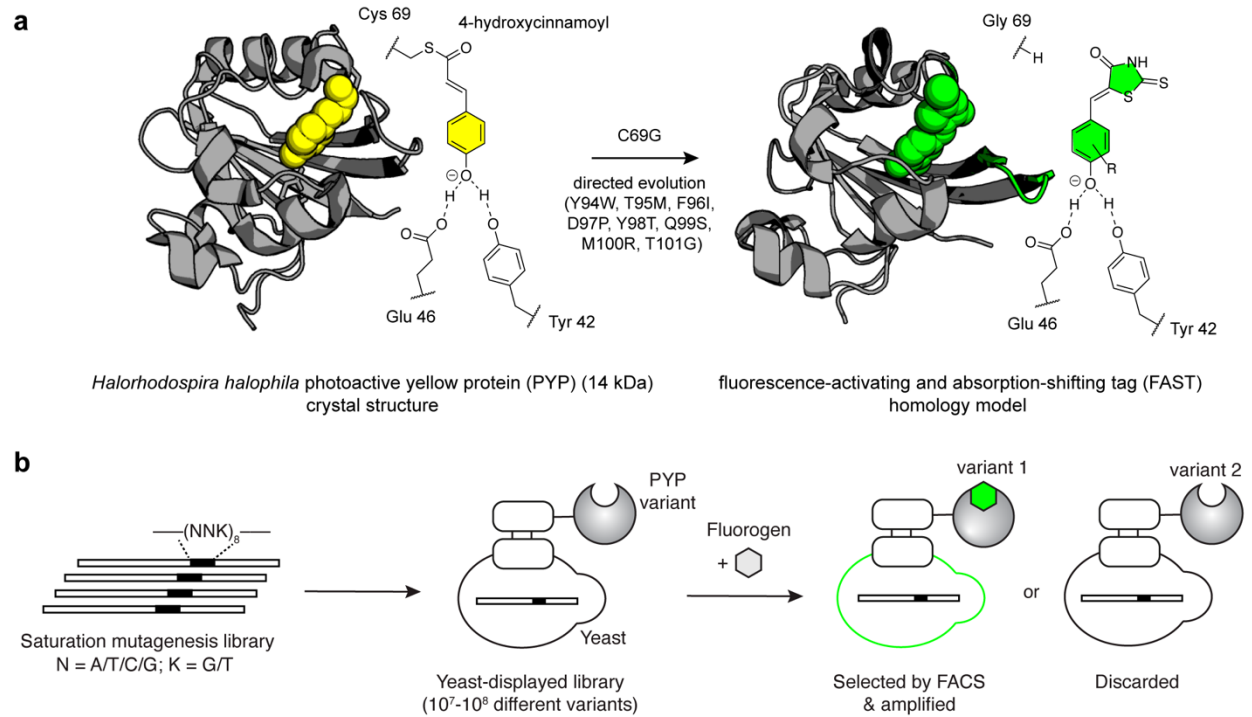


Figure 2. Engineering of prototypical FAST. **a** FAST was engineered from the *Halorhodospira halophila* photoactive yellow protein (PYP) by site-directed mutagenesis and directed evolution. **b** Yeast display enabled to isolate a variant forming bright fluorescent assembly with hydroxybenzylidene rhodanine (HBR) analogs.

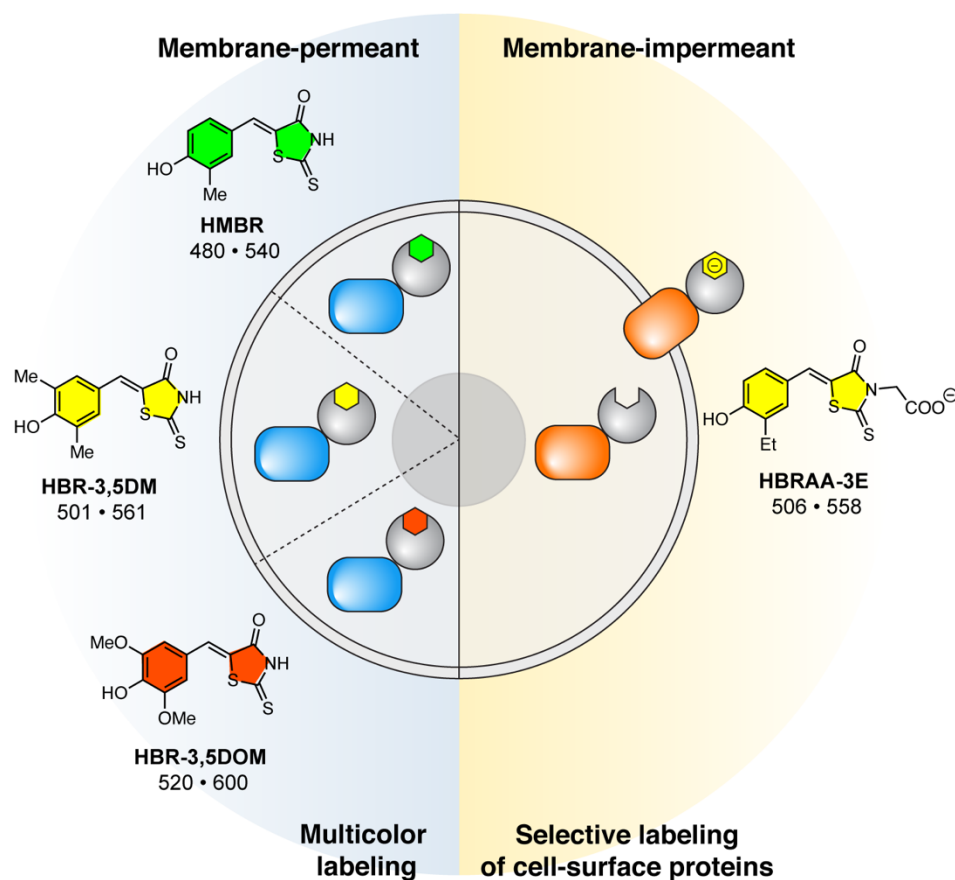


Figure 3. Expansion of FAST properties through molecular engineering. a Varying the substituents on the aromatic ring of HBR analogs enables to tune the spectral properties of the FAST:fluorogen assembly. The wavelengths of maximum absorbance and emission are given. **b** Introduction of carboxymethyl group on the rhodanine head enabled to generate HBR analogs that are negatively charged at physiological pH, allowing to reduce their ability to cross the plasma membrane of mammalian cells.

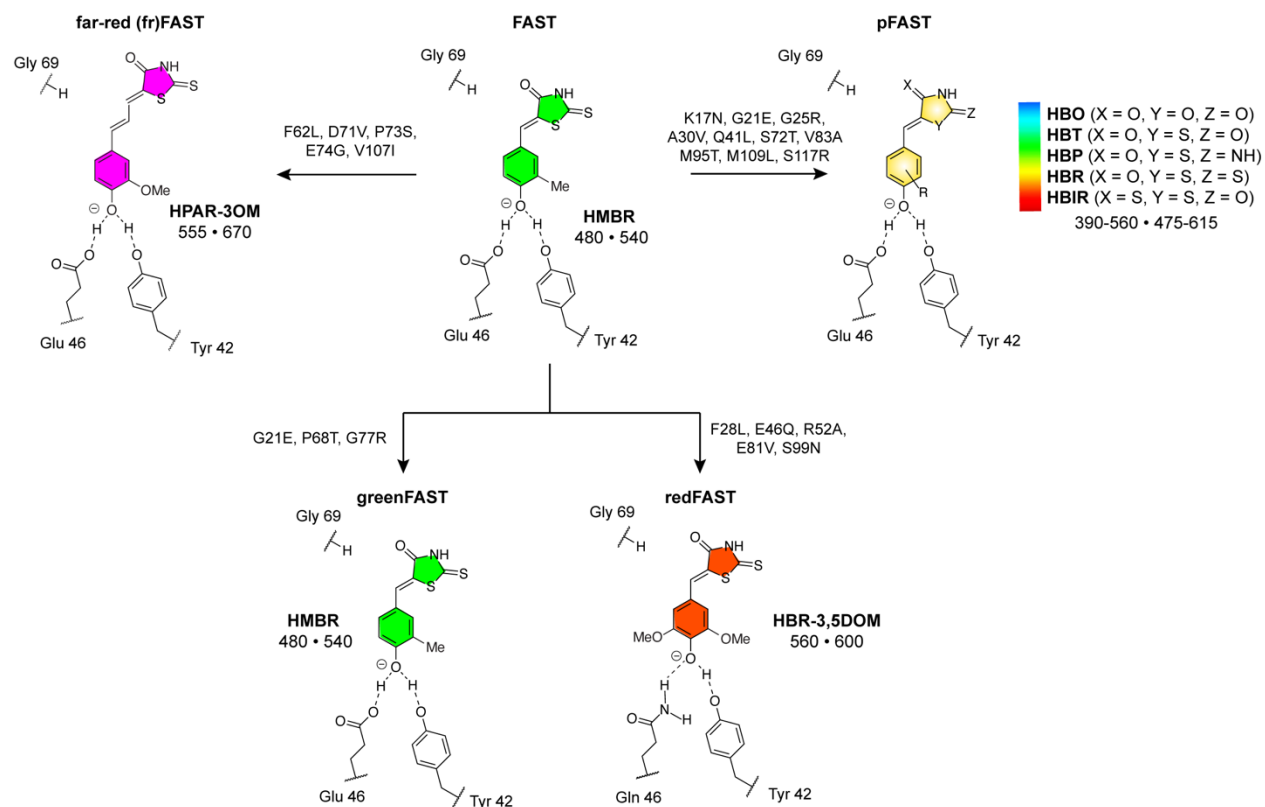


Figure 4. FAST family tree. A concerted strategy of molecular engineering and directed evolution allowed the generation of (i) far-red(fr) FAST forming far-red fluorescent assembly with HPAR-3OM, (ii) a promiscuous variant (pFAST) able to form tunable fluorescent assemblies with a collection of fluorogens with spectral properties covering most of the visible spectrum, and (iii) orthogonal variants, greenFAST and redFAST, forming selectively green and red fluorescent assemblies with HMBR and HBR-3,5DOM, respectively, providing spectrally orthogonal reporters.

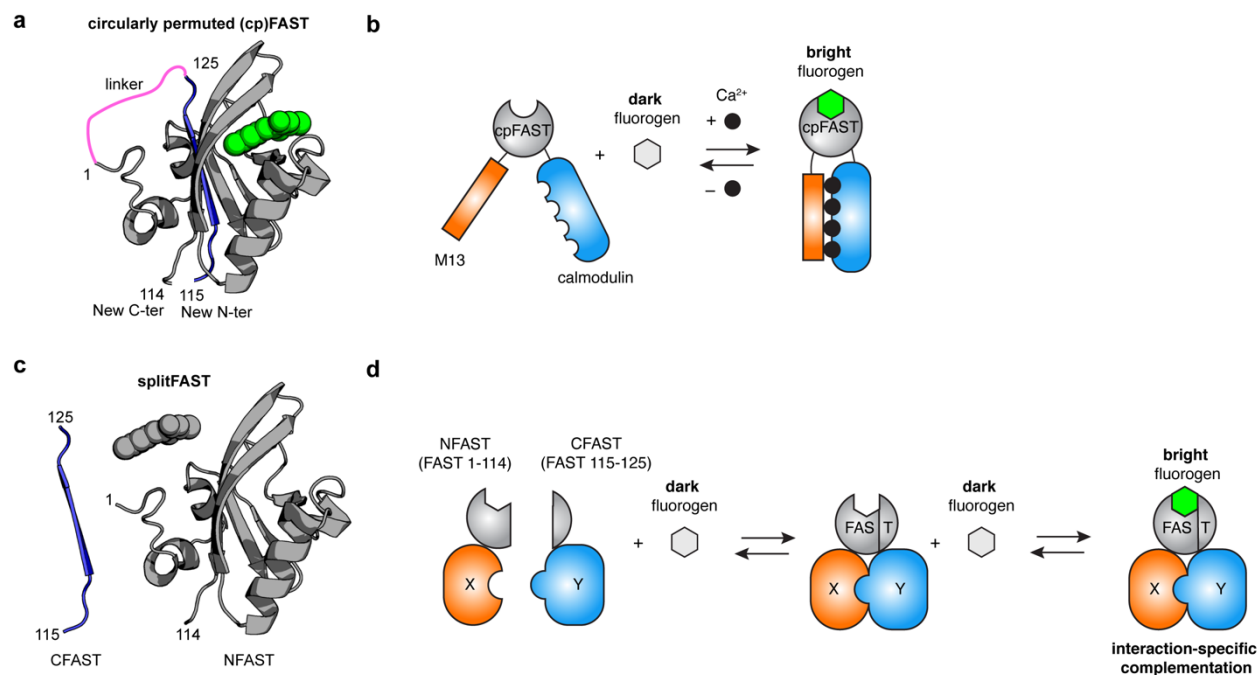


Figure 5. Topological FAST variants for the design of biosensors. **a** Relocation of the N- and C-termini at position 115 and 114 allowed the generation of circularly permuted (cp)FAST variants that can be used to (i) tag protein through insertion at a permissive insertion site or (ii) create biosensors. **b** The conformational flexibility of cpFAST enabled the creation of Ca²⁺ sensors through fusion to calmodulin and the M13 peptide, two domains that interact together in presence of Ca²⁺. A conformational coupling enables to condition fluorogen binding, and thus fluorescence, to Ca²⁺ recognition. **c,d** Bisection of FAST in its last loop between Ser 114 and Gly 115 allowed the creation of a split fluorescent complementation system, named splitFAST, enabling the visualization of protein-protein interactions.

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