Methods and Applications in Fluorescence

TOPICAL REVIEW

Fluorescence protein complementation in microscopy: applications beyond detecting bi-molecular interactions

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Abstract

Conventional fragments of fluorescent proteins used in bimolecular fluorescence complementation technique (BiFC), form light-emitting species only when they are kept in close proximity by interacting proteins of interest. By contrast, certain fluorescent protein fragments complement spontaneously, namely those corresponding to the 1st to 10th beta-strands (GFP1-10) and the 11th beta-strand of superfolder GFP (GFP11). They were designed as folding reporters for high throughput expression and structure biology. Besides, for light microscopy, self-associating fluorescent protein fragments constitute a valuable and sometimes unique tool. The GFP11 tag is very advantageous when a full-length fluorescent protein cannot be fused to a protein of interest, namely for live imaging of certain pathogens. Self-associating GFP fragments enable live labelling of specific synapses, visualization of proteins topology and their exposure to particular subcellular compartments. Present review aims to attract attention of scientific community to these tools and to inspire their further development and applications.

1. Introduction

Conventional bimolecular fluorescence complementation (BiFC) technique has been developed for detection of protein-protein interactions in situ. In BiFC, the two fragments of a fluorescent protein are fused to the two proteins of interest; these fragments fold together, enabling fluorophore ‘maturation’, only when they are brought into close proximity by complex formation between the proteins of interest (first published in [1]). However, another type of fluorescent protein complementation turned out to be useful for completely different applications in microscopy.

The history of self-associating fluorescent protein fragments started with the works of Waldo group on the development of GFP-based protein folding reporters to detect expression of soluble protein constructs [2–4]. A mutant capable to fold very efficiently, the superfolder GFP, was designed [5]. Further, it was found that two fragments of the superfolder GFP, GFP1-10 (containing 1th to 10th beta-strands of beta-barrel) and GFP11 (11th beta-strand) (figure 1) assemble spontaneously, without the need to be fused to interacting proteins, yielding a highly fluorescent species [6–8]. This property makes GFP1-10/GFP11 complementation useless to detect interactions by conventional BiFC technique, but provides a sensitive way to detect a protein tagged with GFP11 by adding the GFP1-10 fragment (relevant glossary is in table 1). GFP1-10/GFP11 system was originally invented to screen for soluble protein constructs [6]; however, it turned out to be very useful to visualize proteins of interest by live microscopy. Most common schemes of GFP1-10/GFP11 based microscopy applications are represented on figure 2. GFP11 tag is particularly advantageous when a full-length fluorescent protein tag cannot be fused to a protein of interest, and when other short peptide tags (e.g. the tetracysteine tag requiring chemical labelling) are not suitable.

Since pioneer application of GFP1-10/GFP11 system in microscopy in 2007 [10], many diverse applications have been reported. The present review is devoted to the basics, practically important features, applications and perspectives of self-associating fluorescent proteins in microscopy. Neither conventional BiFC, nor applications of split fluorescent proteins in vitro are covered. The goal of the current review is to
attract attention of scientific community to this promising tools and to stimulate development of their novel applications in imaging.

2. Properties of self-associating fragments of superfolder GFP

‘Classical’ fragments of the fluorescent proteins used in BiFC constitute roughly the ‘halves’ of the protein: truncation positions are after the 155th or the 173th aminoacid residue. Successful folding and subsequent fluorophore maturation requires that the fragments are kept in proximity for quite a long time ($t_{1/2} = 60$ s) by an interaction between the proteins which they are tagged to [1, 11]. In the absence of interacting partners, co-expressed fragments of fluorescent proteins do not yield a fluorescent species (ibid), which is a prerequisite of BiFC technique. In contrast, the non-symmetrically split fragments of superfolder GFP (GFP1-10 and GFP11) associate spontaneously [6]. GFP1-10 is apparently non-fluorescent (ibid); it contains more random coils than the reassembled GFP, according to the circular dichroism spectra of the purified proteins [12]. It was concluded that GFP1-10

![Figure 1. Schematic representation of the splitting the superfolder GFP into self-assembling GFP1-10 and GFP11 fragments. Based on data from [5, 6]. The GFP1-10, GFP11, and the superfolder GFP ‘tail’ (which is truncated in GFP11) are shown in blue, green, and light grey respectively. Arrows, cylinders and lines represent alpha-helices, beta-strands and linker sequences, respectively. Numbers indicate aminoacid residues (numbering of superfolder GFP). The split position is marked with lightening. The chromophore is shown as orange spark. Adapted with permission from Springer Nature: Nature Biotechnology [5]. Copyright (2006).](image-url)

| Table 1. Glossary. |
|-------------------|
| **Term**          | **Definition**                                                                 |
| GFP1-10 (detector fragment) | Polypeptide comprising residues 1 to 215 (1th to 10th beta strands) of the superfolder GFP |
| GFP11 (GFP S11, S11)       | Peptide which corresponds to residues 216 to 230 (the 11th beta strand) of the superfolder GFP |
| GFP$_{complement}$ (complemented split GFP) | Complex formed by fragments of superfolder green fluorescent protein |
| Self-association (self-assembly, self-complementation) of a split fluorescent protein | Spontaneous binding of the two independently synthesized fragments of a fluorescent protein which diffuse in the same compartment, but are not necessarily brought to proximity by other interacting entities. It is commonly implied that self-association leads to formation of a fluorescent chromophore |
alone lacks the structure needed for chromophore formation, while GFP11 binding induces the necessary structural constraints. The reassembled GFP has apparently the same spectral characteristics as the ‘single polypeptide’ superfolder GFP; it is sufficiently stable to be observed by electrospray time of flight mass spectroscopy under mild conditions (ibid). This suggests that for microscopy applications, the reassembled GFP can be considered as a conventional, ‘single-polypeptide chain’-FP tag. One observation contradicts this: photobleaching of complemented GFP was slower than photobleaching of equivalent single-polypeptide chain GFP, which was explained by dissociation of the bleached GFP1-10 and binding of the ‘fresh’ non-bleached GFP1-10 fragments to the same tag [13]. However, no experimental proof of that was reported. When GFP1-10/GFP11 complementation had already been used in microscopy, it was reported that in vitro refolded GFP1-10 is unable to bind GFP11 in the darkness; only upon illumination with visible light, refolded GFP1-10 acquires capacity to bind GFP11 [14]. The authors demonstrated that the chromophore in refolded GFP1-10 is in trans-conformation; isomerization towards the cis-conformation requires illumination with visible light, and only the cis-conformation is capable to bind GFP11 (ibid). The latter was supposed to possess some secondary structure in the absence of GFP11, according to in vitro observations of its circular dichroism spectra, and non-negligible fluorescence 1/3 of that of the complete GFP [14]. The need for illumination to convert trans-GFP1-10 to the cis-form capable to bind GFP11 suggests an opportunity to control the GFP1-10/GFP11 complementation in space and time in the cells. That reaction might occur in the cells being induced by ambient light and/or the excitation light of the microscope. However, significant fluorescence of the cells expressing GFP1-10 alone has never been reported nor observed by us. No study of the effect of illumination on in cellulo GFP1-10/GFP11 complementation has been reported so far, which is not a surprise, considering that culturing cells in darkness is complicated and that it is hardly feasible to acquire fluorescent image without affecting trans-cis isomerization of GFP1-10 by excitation light. The apparent discrepancy may be explained by the following. The above-described in vitro experiments on GFP1-10 isomerization and interaction with GFP11 [14] used GFP1-10 fragment obtained by cutting the GFP11 fragment off the ‘single-chain’ superfolder GFP, where the chromophore had matured in the microenvironment of intact GFP barrel; meanwhile, GFP1-10 expressed in the cells in the absence of GFP11 hardly constitutes an equivalent microenvironment for chromophore maturation. Thus, in vitro studies provide quantitative information on the

Figure 2. Principle of the use of GFP1-10/GFP11 complementation in microscopy and summary of the strategies to deliver GFP1-10 and GFP11-tagged protein of interest (POI) into a compartment of interest (which may refer to cytoplasm, other intra-cellular compartments or to extra-cellular space, as in the case of GRASP [9]).
photophysics of the reactions of GFP complementation, but they do not necessary describe the processes which occur in cellulo.

3. Spontaneous GFP assembly meets microscopy

What advantages can spontaneous complementation of GFP1-10 and GFP11 fragments offer to microscopy? First, development of fluorescence upon adding GFP1-10 to the cells containing a GFP11-tagged protein provides an easy way for specific staining of the latter. In a study co-authored by the inventors of self-assembling GFP [15], purified GFP1-10 was added as a 'reagent' to fixed and permeabilized cells expressing GFP11-tagged protein, and the assay was proposed as an alternative to immunostaining protocols. Indeed, when one needs to stain a tagged protein of interest, then the single-step, 'wash-free' staining of GFP11 has obvious advantages over immunofluorescence staining protocols for e.g., Flag- or HA-tags. Most important, GFP1-10 is a genetically encoded 'reagent', which provides important advantages in comparison to any conventional labelling, including live-cell staining methods, such as biarsenical labelling of the tetracysteine tag [16], because the chemical labelling and washing steps may be perturbing or unfeasible for living samples. GFP1-10 can be continuously expressed in cells; it binds GFP11 spontaneously and without any 'adaptor' moieties, which enables one to visualize protein of interest without any labelling step and in virtually intact cells, like with conventional fluorescent protein tags. But, in contrast to them, GFP11 is small. This is an important advantage if a full-length FP tag perturbs maturation or trafficking of the protein of interest (which was likely the case for coxsackievirus B3 [17], see below), or if the object has limited coding capacity (the case for influenza virus [18]). Further, spontaneous complementation still requires that the two fragments are present in the same compartment, so targeting GFP1-10 to a particular compartment permits to specifically highlight GFP11-tagged proteins only in that compartment. In addition, cell lines stably expressing GFP1-10 were designed [18], enabling reproducible and convenient visualization of GFP11 tag. Thus, such accessories and the ‘behavior’ of GFP1-10/GFP11 system in the cells make it a convenient tool for microscopy. In the subsequent chapters, these applications of self-assembling GFP fragments will be described.

4. False positives and false negatives. How to avoid?

Like in any technique, the experimenter should take into account possibility of false-positive and false-negative readouts while using GFP1-10/GFP11 system. The probability of false signals depends on a specific application of the system and the design of experiment.

GFP1-10/GFP11 complementation is not intended to detect binary interactions. So, there is no problem of ‘false-positive’ signal due to accidental collisions of the GFP fragments. The goal is to detect presence of protein(s) of interest in a compartment or cell of interest, so any collision between GFP1-10 and GFP11 may produce only ‘true’ signal. The only theoretically possible source of false positive signal would be fluorescence of GFP1-10 alone or GFP11 alone. However, this putative signal has never been reported to our best knowledge. On the contrary, in the works were ‘GFP1-10-only’ and/or ‘GFP11-only’ control samples were mentioned, their fluorescence was never detectable with microscope settings used to image GFPcomp samples, for example [19–21]; in our experiments, if any fluorescence existed, it was indistinguishable from weak cellular autofluorescence (e.g. figure 1(B) in [22]).

‘False-negative’ readout (absence of fluorescence despite of the presence of both GFP1-10 and GFP11 in the same compartment) is not as unlikely as the false-positives discussed above. The probability of overlooking a true signal obviously decreases with increasing sensitivity of microscope detectors. Most important, risk of missing the signal depends on the timeline of experiment: how soon observation is performed after GFP1-10 and GFP11 occurred in the same compartment. One should keep in mind that complementation is not an instant event. Upon mixing GFP1-10 and GFP11-tagged protein in vitro (final concentrations 68 and 127 μM), detectable fluorescence appeared within 30 min; the authors proposed a two-step model comprising the bi-molecular complementation step (half-time of 34 min) and the monomolecular chromophore formation step (half time of 167 min). However, these kinetics measurements were performed at 25°C, so we expect that in mammalian cells at 37°C, GFPcomp chromophore forms much faster. Direct measurements of the kinetics of GFPcomp formation in live cells (this could be feasible by photocoupling of GFP1-10 or GFP11) have never been reported. Nevertheless, certain hints on that can be obtained from time-lapse imaging of GFP1-10 expressing cells upon infection with a bacterium [23] or with a virus [18] encoding GFP11-tagged protein. In Salmonella infection experiments, fluorescence of complemented GFP developed within 2 h of the GFP11-tagged protein translocation [23]. In our experiments, fluorescence intensity grew quickly from the 2nd hour post-infection [18], which is very fast considering that the delay included trafficking of viral genome to the nucleus and initiation of its expression. In another study, GFP signal emerged 4 h after triggering apoptosis in the cells containing GFP1-10 and a GFP11-tagged apoptosis reporter [21]. In these experiments, like in all time-lapse observations of GFP complementation, it was not possible to detect GFP1-10
and GFP11 before they formed GFP$_{\text{comp}}$ fluorophore, so absence of fluorescence might be interpreted either as ‘the fragments are not synthesized/translated yet’ or as ‘the fragments are there but have not complemented yet’. To our best knowledge, ‘true’ false-negative readout (when GFP1-10 and GFP11 tags are present in the same compartment, but no fluorescence appear) have never been reported in single time point experiments, which permitted enough time for chromophore formation. We have neither observed ‘false GFP-negative’ cells when we infected (at high multiplicity of infection) the cells stably expressing GFP1-10 with influenza virus encoding a GFP11-tagged protein [18, 24]. Thus, for steady-state experiments, to avoid overlooking or under-estimation of complemented GFP signal, one has to check that the signal intensity reached a plateau. In the dynamic experiments (e.g. time course of translocation of a GFP11-tagged protein to a compartment containing GFP1-10), a possible delay between translocation and appearance of fluorescence should be kept in mind, and change of GFP$_{\text{comp}}$ fluorescence over time should not be simplistically interpreted as a change of GFP11-tagged protein concentration.

5. ‘Folding-sensitive’ imaging of proteins of interest

Since self-associating GFP fragments have been designed to detect proper folding of proteins of interest, the most straightforward application of this system in microscopy was to detect protein aggregation in the cell. The most well-known folding-related disease is Alzheimer’s syndrome; so, the first in situ (more precisely, in cellulo) application of GFP1-10/GFP11 complementation was quantification of tau protein aggregation [10]. The principle there was very similar to the original in vitro use of self-assembling GFP fragments by Waldo group: soluble GFP11-tagged tau protein became fluorescent thanks to GFP1-10/GFP11 complementation, while in tau protein aggregates, GFP11 was ‘buried’ and hence inaccessible to GFP1-10, so no fluorescence developed. Surprisingly, this paper was not followed shortly by other groups, and has not been extensively cited (only 39 citations by August 2018). Only several years later, the 2nd report on application of GFP1-10/GFP11 system to study protein aggregation in situ appeared: the same approach was used to visualize the wild type and mutants of soluble alpha-synuclein in the cells, and to distinguish the soluble form from non-fluorescent alpha-synuclein aggregates [25]. Besides microscopy, flow cytometry was used in that work to quantify the populations of cells according to their content of soluble alpha-synuclein. In another elegant study, GFP11 did not serve as a tag of a protein of interest. Instead, GFP11-dependent folding of GFP1-10 itself was used to characterize an unconventional secretion pathway for misfolded proteins (the misfolding-associated protein secretion) [26]. GFP1-10 expressed alone served as a model unfolded protein, while complemented split-GFP (GFP1-10 co-expressed with GFP11) was folded protein. For instance, the authors demonstrated that GFP1-10 was efficiently secreted via the secretion pathway of interest, while the folded split-GFP was not.

Obviously, GFP1-10/GFP11 complementation can do much more than distinguishing between soluble and aggregated states of a protein in cell. That pioneer application in microscopy paved the way to a range of other ones, expanding far away from screening for soluble protein constructs for which split GFP was invented [6, 27].

6. Visualizing proximity between organelles and cells

If GFP1-10 and GFP11 fragments are targeted to specific compartments or structures of interest, then spontaneous complementation of the two fragments may be used to visualize proximity of these structures. For instance, organelle contact sites in live cells were visualized this way [28]. Punctate signals of complemented GFP were observed when GFP1-10 and GFP11 fragments were tethered to any pairs of the organelles among the lipid droplets, vacuole peroxisomes, mitochondria and endoplasmatic reticulum (ER), suggesting that these organelles form contact sites. The authors could not definitely exclude the possibility that the contacts between organelles were artifacts created by irreversible GFP1-10 to GFP11 binding. However, it was unlikely the case, since complemented GFP signals of ER-mitochondria contacts mostly co-localized to ERMES, the known structure which tethers ER to mitochondria. This indicates that assembly of GFP occurs at the existing contacts between the organelles.

A particularly important field of application of GFP1-10/GFP11 complementation is neurobiology, namely neuron connectivity studies. Mapping synapses within brain tissue is a very important but very challenging task. Synaptic clefts are too thin to be directly visualized by light microscopy, while electron microscopy needs ultra-thin slices where 3D structures can hardly be preserved. An elegant solution for this problem, based on GFP1-10/GFP11 complementation, was proposed by Feinberg and co-authors: the technique called ‘GFP reconstitution across synaptic partners’ (GRASP) [9, 29]. In GRASP, the two self-associating fragments of GFP are fused to targeting sequences which ensure their trafficking and appropriate localization on the pre- and post-synaptic membranes of the neurons of interest. Fluorescent GFP is reconstituted only if a cell expressing GFP1-10 forms a synapse with a cell expressing GFP11-tagged protein. For a recent example, GRASP enabled the
authors to show that synaptic partner recognition (SPR) between the PHB sensory neurons and the AVA interneurons needs the receptor protein tyrosine phosphatase (RPTP) dcr-I [30]. On the basis of split-GFP system, a protocol called ‘SynView’ was developed and used to demonstrate that neu republican-1beta and neuroligin-1 and neuroligin-2 (synaptic cell-adhesion molecules that are needed for synapse specification and function) form a trans-synaptic complex [31, 32]. Importantly, on the basis of crystal structures, the authors carefully selected the positions to insert GFP1-10 and GFP11 tags into neurexinn1/beta and neuroligin, such that: (1) they did not perturb functions of these proteins; (2) GFP1-10 and GFP11 tags became close to each other only upon binding between the proteins of interest, thus avoiding false-positive complexes driven by GFP1-10/GFP11 complementation (ibid).

7. Visualizing protein subcellular localization and topology

Important information to understand function of virtually any protein is its subcellular localization and, in case of a (trans)-membrane protein, its topology (namely exposure of the C- and N-termini to particular compartments). Resolution of light microscopy is typically insufficient to address these questions. Fluorescence Resonance Energy Transfer (FRET) technique enables to detect 5–10 nm proximity between structures of interest, but FRET signal depends primarily on the distance between the two labels, regardless to the presence of a membrane separating them. Moreover, FRET cannot detect if a protein is exposed to a given compartment. By contrast, spontaneous superfolder GFP complementation only requires that the both fragments are in the same compartment, but it does not require continuous nanometer-scale proximity between the labelled species during signal acquisition. Thus the most popular imaging application of GFP1-10 / GFP11 system became probing protein subcellular localization and topology, because this type of information is highly demanded, alternative techniques to address the question are limited, and split-GFP based approach is straightforward. Below, several examples will be described.

In budding yeast S. cerevisiae, Split-GFP system has been used to clarify the localization of Kar5p, a highly conserved transmembrane protein essential for fusion of two haploid nuclei which occurs during mating process [33]. Kar5p localizes adjacent to the spindle pole body (SPB), mediates nuclear envelope fusion, and recruits another important factor Prm3p to SPB. Kar5p has been tagged with GFP1-10, while GFP11 was fused to either ScST (a protein which resides in outer nuclear membrane and ER), either to Psu1p (which is exclusively nucleoplasmic). Formation of GFP signal upon co-expression of Kar5p-GFP1-10 with either of the above-mentioned GFP11-tagged constructs enabled authors to demonstrate the presence of Kar5p in both inner and outer nuclear envelope. By a similar strategy, using GFP1-10 versions targeted to endoplasmic reticulum (ER) lumen and to the cytoplasm, and vaccinia virus protein L2 protein constructs with GFP11 tag at the appropriate termini, Hyun et al demonstrated exposure of the N- and C-termini of L2 to the cytoplasmic and luminal sides of ER, respectively [34]. Orientation of NADH type II dehydrogenase (NDH2) isoforms in mitochondrial inner membrane in T. gondii was determined by split-GFP system as well [35]. The NADH binding sites of these enzymes in various species can either be exposed to the interembrane space where they oxidize cytosolic NADH, or to the matrix where they oxidize NADH in that compartment. In the cited report, GFP1-10 was targeted to mitochondrial matrix via fusion to the matrix protein succinyl-CoA synthetase, while GFP11 was fused to either isoform I or to isoform II of NDH2 present in T. gondii. Development of GFP fluorescence in mitochondria was observed, suggesting that the both isoforms of T. gondii are exposed to mitochondrial matrix. Interestingly, NDH2 isoform I initially showed no mitochondrial fluorescence; however the authors found that it occurred because its C-terminal part contained an amphipathic helix which favored immersion of the C-terminus into membrane lipid bilayer, thus hindering GFP11 accessibility to mitochondrial matrix-targeted GFP1-10. When the amphipathic helix was removed, the complemented GFP signal appeared, indicating exposure of the isoform I to mitochondrial matrix. Another study of sub-mitochondrial localization concerned wild type and a mutant tau protein, which were tagged with GFP11, while GFP1-10 constructs were targeted to mitochondrial matrix, outer mitochondrial membrane or inter-membrane space (IMS) [36]. This permitted authors to detect tau protein in the IMS and outer mitochondrial membrane, but not in the matrix.

A split-GFP-based assay has been developed to quantify peroxisomal subpopulation of alanine-glyoxylate aminotransferase (AGT), an enzyme which is normally localized in peroxisomes, while aberrant accumulation in mitochondria occurs for a G170R mutant related to hyperoxaluria type I [19]. GFP1-10 was targeted to peroxisomes by fusion to peroxisome localization signal, while AGT and its mutant were tagged with GFP11. Only the AGT-GFP11 molecules transported to peroxisomes became fluorescent, while AGT aberrantly transported to mitochondria and/or misfolded remained dark. This assay called Glow-AGT enabled the authors to visualize AGT in peroxisomes by fluorescence microscopy, as well as to quantify the population of cells possessing peroxisomal AGT by flow cytometry, and thus to quantitatively estimate the capacity of certain small molecules to restore normal peroxisomal localization of a mutant AGT.
Spontaneous GFP-10/GFP11 complementation is particularly useful to determine protein topology and subcellular localization in protozoans, some of which have very complicated subcellular organization. For instance, Chromalveolates, such as a diatom *Phaeodactylum tricornutum* contain the secondary symbiotic plastid engulfed by peri-plastide membrane. Orientation of pTE3 protein which is integral to that membrane, has been determined [37]. GFP-10 was targeted to the ER or the peri-plastidal compartment via specific marker protein fusion; GFP11 was fused to the C-terminus of pTE3P. The authors demonstrate that the C-terminus of pTE3P, which contains the catalytic RING finger domain, is exposed to the periplastidal side of the membrane (ibid.). Furthermore, split GFP was used to study *Apicomplexa*, a group of parasitic protozoans characterized by rather small size and a complicated life cycle, part of which occurs inside mammalian host cells. For example, exposure of PFE55, an exported protein of *Plasmodium falciparum*, to erythrocyte cytosol has been detected using GFP-10 targeted to that compartment [38]. In another study, GFP11 has been fused to the C-terminus of *Tg*Tic20 protein of *Toxoplasma gondii*, while GFP-10 has been targeted to the stroma of apicoplast, a unique multi-membranous organelle of this group. Development of fluorescence in that compartment enabled authors to demonstrate exposure of the *Tg*Tic20 C-terminus [39].

An assay for efficiency of cytoplasmic delivery of a cargo by CPP (cell-penetrating peptides) has been developed on the basis of GFP-10/GFP11 complementation [40]. The problem was that many of the cell-penetrating peptides are retained in endosomes and thus fail to deliver their cargos to cytosol, which is essential for biological effects. By conventional techniques, it was difficult to distinguish this retention in endosomes from cytosolic localization. In the proposed assay called ‘Split-complementation Endosomal Escape’, GFP-10 was expressed in the cytosol of cells, while the GFP11 protein fragment is fused to a moiety containing the cell penetrating peptide. Only if the CPP-GFP11 construct is released to cytoplasm, fluorescence signal develops. The authors claim ‘minimal background’ and suitability of the assay for high-throughput. The same approach has been used to distinguish the cytosolic pool of antibody TMab4 fused to GFP11 fragment from the pool trapped in the endocytic vesicles, using a cell line expressing GFP-10 [41].

Similarly to protein sub-cellular localization, GFP-10/GFP11 complementation constitutes an excellent tool to detect fusion between cells. For instance, Kodaka and co-authors visualized myofusion [42, 43]. Two myoblast cell lines C2C12 were established: one expressing GFP-10 (C2C12-GFP-10) and another one expressing GFP11 fused to blasticidin S resistance gene product (C2C12-BSR-GFP11). GFP fluorescence only occurred when the two co-cultured cell lines started to differentiate and to fuse, yielding multi-nuclear myotubes where GFP-10 and GFP11 fragments could form the GFP fluorophore. Even more powerful approach has been used to monitor membrane fusion between the effector cells expressing viral Env protein and the target cells expressing its receptors [44]: each type of cells expressed one of the two inactive fragments of the reporter dual split protein (DSP) which the authors had previously developed [45]. Thus, fusion of the Env-containing effector cells and target cells was detected by restoration of the both activities of DSP: GFP fluorescence and luciferase activity.

8. Visualization of pathogen-encoded proteins in infected cells. Size matters.

Spontaneous GFP-10/GFP11 complementation is particularly useful for *in situ* visualization of proteins of interest encoded by various pathogens (figure 3). Expression of the two fragments of split-GFP by the host and the pathogen enables a straightforward opportunity to detect a pathogen-encoded protein only when it is present in those host cells of interest which expresses GFP-10 fragment. Furthermore, many viruses have limited coding capacity, which makes it difficult to insert a full-length FP gene into their genomes. Meanwhile, GFP11 coding sequence may be easier to insert; a small tag is less likely to compromise pathogen functions. A small tag alternative to GFP11 is the tetracysteine tag [16], which can be specifically labelled in live cells with biarsenical reagents FlAsH or ReAsH, yielding a fluorescent label. However, FlAsH/ReAsH labelling has significant drawbacks for imaging infection process: (1) chemical labeling step may perturb the cells; (2) tetracysteine tag permits to label only the tagged molecules which existed when the reagent is added. Meanwhile, GFP-10 "reagent" is permanently present in the cell and may react with newly synthesized tagged protein molecules, making them visible (Comparison of chemical labelling versus GFP11 tag is illustrated on figure 4). Kinetics of the formation of split GFP fluorophore (we mean both translation and post-translational chemical reactions yielding the fluorophore) is its another important advantage over conventional FPs, besides small size. This process takes some time for any fluorescent protein, causing a time delay between the actual synthesis of a FP-tagged protein of interest and the moment when it becomes fluorescent. This does not cause any problem for observations of ‘steady-state’ in the cell. However, expression of pathogen-encoded proteins during infection cycle may change very quickly, so quick formation of the fluorophore can be critical. GFP-10/GFP11 complementation enables to shorten the fluorophore maturation: if GFP-10 is encoded by the host, then experiment can be arranged such that GFP-10 is expressed before the infection event, so the
rate of fluorescence signal development is limited by the synthesis or by the delivery to the cell of the GFP11-tagged pathogen molecule. The latter is illustrated by the first report on split-GFP application in pathogens [23]. GFP1-10 was expressed in the host cells, while GFP11 was fused to SteA or SteC - the effector proteins of Salmonella enterica which are injected into the host cell via type III secretion system. Upon translocation, GFP11 tag complemented with GFP1-10, yielding fluorescent labelling of cytoplasmic subpopulation of the bacterial effector protein. Intracellular dynamics of PipB2 was characterized. This study was only feasible thanks to a self-assembling fluorescent protein complementation. With a conventional FP tag, it would not be possible to discriminate between the intra-bacterial subpopulation of effector proteins; moreover, full-length fluorescent protein tag would perturb the translocation of the effector proteins. The same approach was used in other studies of spatio-temporal localization of bacterial effector proteins. Park et al fused GFP11 tag to Pseudomonas syringae effector proteins AvrB and AvrRps4 delivered via type III secretion system, and generated a number of plant host transgenic lines expressing GFP1-10 targeted to various subcellular compartments [46]. Wang and co-authors tagged with GFP11 a range of chlamydial effector proteins encoded by the bacterium, while the host cells stably expressed GFP1-10 [47]. Interestingly, CT733, one of GFP11-tagged chlamydial proteins, did not penetrate the host cell and thus did not become fluorescent in live samples; nevertheless authors managed to visualize it via adding of purified GFP1-10 as a ‘stain’, upon fixation and permeabilization. This illustrates versatility of GFP11 which can serve not only as a smart live FP-tag, but also as an affinity tag for ‘IF-like’ staining. Split-GFP approach enabled Li et al for the first time to directly visualize trafficking of VirE2 (a bacterial virulence protein of Agrobacterium tumefaciens) within the recipient plant cells [48]. The authors created VirE2-GFP11 fusion construct by inserting GFP11 coding sequence into permissive site of VirE2 gene, which was expressed in A. tumefaciens. GFP1-10 was expressed in recipient cells (plant and yeast). Comparison of motions of VirE2-GFP-containing particles within natural recipient (plant cells) and non-natural recipient (yeast cells) enabled to authors to demonstrate the importance of active trafficking for the efficiency of genetic transformation of the host. Translocation of A. tumefaciens VirE2 protein to yeast cells was visualized by split-GFP approach as well in another study [49].
Small size and rather fast development of fluorescence upon GFP1-10 binding makes GFP11 tag even more advantageous for live imaging of viral infections. Namely, with conventional FPs, it was impossible to characterize time-dependent changes of composition and localization of influenza virus-encoded proteins in real infection cycle, because full-length FP tags led to genetic instability and/or severely impaired the virus functions, for instance, packaging of the viral genomic RNAs containing FP coding sequences [50]. To circumvent the problem, we developed a recombinant influenza A virus encoding the GFP11 tag fused to viral RNA polymerase PB2 subunit. PB2 bearing the complemented split GFP was incorporated into the progeny viral ribonucleoproteins which were efficiently packaged into the virions preserving their infectivity. Further, cell lines stably expressing the ‘detector’ fragment were designed [18], enabling reproducible and convenient visualization of any protein tagged with GFP11. The designed virus enabled us to characterize interactions and trafficking of influenza polymerase and ribonucleoprotein throughout the infection cycle [18, 22, 24]. Another example is a study of an enterovirus coxsackievirus B3 (CVB3) by van der Schaar and co-authors [17]. In the host cell, enteroviruses generate the replication organelles (ROs) where they replicate their genome. No virus-encoded construct had been reported for live-cell imaging of the RO. Attempts to generate enteroviruses that encode full-length FP-tagged, membrane-anchored viral proteins to label ROs were unsuccessful, presumably due to impairment by full-length FPs of a function of RO-anchored viral proteins or perturbing their liberation from polyprotein. To overcome this limitation, the authors inserted GFP11 into the 3A protein of CVB3 and demonstrated that the tag did not perturb any function of 3A nor compromises the virus. Further, they monitored the development of ROs in the host cells expressing GFP1-10 upon infection with the engineered CVB3 encoding GFP11-tagged 3A. In addition, another CVB3 was designed, encoding both 3A-GFP11 and GFP1-10 [17]. This highlights a way to avoid the need for host cells expressing GFP1-10, thus greatly extending flexibility of a researcher in the choice of hosts, which can be genetically unmodified cells, tissues or even whole animals.

9. Nevertheless, detecting protein-protein interactions!

Despite that complementation between GFP1-10 and GFP11 fragments occur spontaneously and is therefore unsuitable to detect interactions, a smart way to apply this system for interaction analysis has been found. Superfolder GFP was splitted into three fragments: GFP1-9 (beta-strands 1 to 9), GFP10 (beta-strand 10) and GFP11; the two latter are fused to the two putatively interacting partners. Only if they interact, complete chromophore forms and fluorescence develops [51]. The tripartite split-GFP...
combines the advantages of a small GFP11 tag and the capacity of ‘classical’ BiFC to detect complex formation. For the first time, tripartite split-GFP was used in a study of receptor for advanced glycation end products (RAGE) demonstrating that RAGE dimerization occurs in ER [52]. A series of applications followed that pioneering work. Interaction between p52 and TTDA subunits of the transcription factor II H (TFIIH) were detected, and dynamics of the complexes was characterized in live cells [53]. The tri-partite split-GFP revealed interactions between ubiquitination enzymes MuRF1 and E2, and localization of the complexes in live human cells was determined by microscopy [54]. The tri-partite split-GFP has also been demonstrated to work for detecting protein-protein interactions in plants [55]. State-of the art use of the tripartite split GFP is represented by the work of Koraichi et al [56]: the authors not only developed a sensor for GTPase—effector interaction that was suitable for high-content screening to evaluate modulators of small GTPase activation, but also designed a ‘reporter’ cell line expressing GFP1-9 and the single chain nanobody which bound the complemented GFP and amplified its fluorescence signal.

10. Synopsis and perspectives

There are still many low-hanging fruits in the field of imaging applications of self-assembling fluorescent proteins. Being invented in the high-throughput expression field, these tools seem to be under-appreciated by cell biologists and microscopists. Spontaneous fluorescent protein complementation provides various opportunities, beyond just a smart labelling.

Diversity of known fluorescent proteins suggests that fragments of various proteins may self-assemble in various combinations and yield fluorescent entities. For instance, GFP11 containing E222Q mutation (superfolder GFP numbering) binds GFP1-10 and yields a chromophore with significantly higher ratio of the 460 nm absorbance band to the 400 nm band, in comparison to the reassembled GFP containing E222 [12]. Even more interesting results were obtained on another pair of self-associating fragments of superfolder GFP: the 10th beta-strand of superfolder GFP (GFP10) and the circularly permuted mutant containing the ‘rest’ of superfolder GFP, referred to as ‘split-GFP’ (the dark sfGFP lacking the 10th beta-strand) [57]. split-GFP yielded a green fluorophore upon assembly with GFP10, while it yielded a yellow fluorophore when bound to the ‘GFP10’ bearing T203Y mutation which converts full-length GFP to YFP. This provides an opportunity to detect two (and in principle, more) proteins of interest tagged with different color versions of GFP10, in the presence of the same ‘detector’ fragment. Even more interesting, irradiation with 405 nm light facilitated dissociation of GFP10 from split-GFP in vitro (in the darkness, complex was essentially irreversible, dissociation constant (Kd) of 78.7 ± 13.8 pM). This occurred under non-denaturing conditions, suggesting that in cells, light-induced dissociation may also be achieved and potentially used for optogenetics or similar molecular tools.

‘Promiscuous complementation’ of the opposite type, when the same 11th beta-strand binds to different detector fragments, has been reported as well: GFP11 binding to CFP1-10 and to YFP1-10 ‘detector’ fragments yielded fluorophores of the respective colors [13]. This enables other interesting applications: for example, if CFP1-10 is targeted to one compartment, while YFP1-10 is directed to another one, then subpopulations of a GFP11-tagged protein of interest resided in these compartments can be independently tracked, for example, after a treatment perturbing an ‘initial’ steady-state.

Possibility of simultaneous observations of several split fluorescent protein-tagged proteins of interest would be very useful for microscopy. Therefore, attempts are made to extend the color palette of self-associating split fluorescent proteins. A self-associating far-red fluorescent protein mPlum was designed [58]. In contrast to split superfolder GFP where the C-terminal 11th beta-strand is split from the first ten beta-strands, for split mPlum, the efficient complementation occurs between the N-terminal beta strand (bearing E16V mutation) and the larger fragment which contains beta-strands from 2 to 11. The split mPlum system was used for imaging of soluble alpha-synuclein protein in mammalian cells (ibid). However, no further use of split mPlum has ever been reported; lack of interest to that tool can be explained by mediocre brightness of mPlum fluorophore [59]. Recently self-associating split superfolder Cherry (sfCherry) has been designed, consisting of sfCherry1-10 detector fragment (beta strands 1 to 10) and the 18-aminoacid peptide sfCherry11 [13]. The authors report that fluorescence intensity of the complemented sfCherry is substantially lower than that of full-length, ‘non-split’ sfCherry, presumably due to less efficient self-complementation between these fragments. Further optimization of the split protein may improve the situation.

Fusion of several fluorescent protein fragments to a single protein of interest is another promising direction. Like in the case of conventional FPs, tandem arrangement of GFP11-like tags enables one to increase brightness several-fold and to reduce photo-bleaching rate. 4x-GFP11, 7x-GFP11 and 4xsfCherry11 tags were designed and characterized [13]. Combinations of tags having different colors (e.g. GFP11 + the short fragment of split mPlum) would enable to label a protein of interest with an additional hue, distinguishable from both GFP alone and mPlum alone, thus extending the palette for multi-color imaging.
The tripartite split GFP [51] combines advantages of self-assembling split fluorescent proteins and those of conventional BiFC, thus opening new opportunities for detecting bimolecular interactions. Other tripartite split-fluorescent protein tools will likely be developed in the near future.

Interestingly, it has been reported that the truncated circularly permuted GFP lacking the 10th beta-strand can bind an oligohistidine tag in the groove vacated by the native strand, yielding a fluorescent GFP [60]. Moreover, exposure to light triggered dissociation of the peptide. These findings provide hints for design of ‘non-canonical’ and light-controllable complementing partners of split GFP, and highlight new possible applications for split-GFP, such as optogenetics. By analogy to GFP1-10 + GFP11 complementation, self-assembling fragments of several enzymatic tags have been designed, namely Renilla luciferase and HaloTag [45]. Self-assembling HaloTag seems to be particularly promising, since it should combine advantages of the split GFP system, and the advantages of self-labeling tags, namely the possibility to label one tag with virtually any organic dye of the researcher’s choice. Particular dyes with optimal physico-chemical properties are critical for a range of applications, namely, chromophore-assisted light inactivation (CALI), single molecule localization microscopy (SMLM) and stimulated emission depletion (STED); thus self-assembling split-HaloTag would greatly facilitate super-resolution imaging for nearly all objects which are currently labelled with GFP1-10/GFP11 system, e.g. it would be possible to image synapses beyond diffraction limit by GRASP technique. In addition, a split-SNAP-tag was designed [61]; although the reported SNAP-tag fragments assemble only when they are brought to proximity by interacting partners (like in classical BiFC), this gives the hope that self-assembling split-SNAP-tag fragments may be designed in the future.

Highly specific and nearly irreversible GFP1-10/GFP11 binding may serve as a platform for introducing various labels to a tagged protein of interest. As a simple example, we designed mCherry-GFP1-10 construct which yielded a two-color mCherry-GFPcomp chimera used as a positive control for FRET microscopy [24]. Going further in this direction, Ishikawa et al attached luciferase fragment to GFP11-labelled protein of interest [45]. The authors developed an interesting and powerful strategy: combination of several self-assembling pairs in a single reporter system, namely, self-assembling split GFP and a self-assembling enzymatic reporter. This has been realized in the dual-functional split-reporter protein (DSP) [45]. Each part of DSP is a chimera composed of a fragment of GFP and a fragment of Renilla luciferase; upon self-complementation, DSP recovers both the luciferase activity and GFP fluorescence. Purpose of making DSP is not only to combine ‘imaging-friendly’ fluorescence signal with ‘quantification-friendly’ enzymatic activity. The authors reported that self-assembly of DSP was mostly driven by fragments of GFP, while self-assembly of Renilla fragments was inefficient by itself. Moreover, using split GFP as a self-association module, Ishikawa et al designed a new methodology called ‘GFP-scanning’ to facilitate generation of self-association-capable split proteins; GFP-scanning was reported to be in particular efficient in identifying the optimal split points of the reporter proteins. One would expect discovery of numerous new split reporters thanks to this platform.

In addition, in cellulo applications of self-associating split fluorescent proteins are not restricted to microscopy. Cells containing complemented GFP can be analysed by flow cytometry (e.g. [25]), which would be more frequently used upon expansion of color palette of self-assembling fluorescent proteins.

Thus, self-complementing fluorescent (and non-fluorescent) protein tags constitute an already powerful, but still incomplete toolbox which has various opportunities for development. Deep understanding of fluorescent protein photophysics, combined with the productivity of modern high-throughput technologies, would permit to design novel more powerful and smarter molecular tools. The most common cell biologist’s approach ‘One protein—one tag’ becomes obsolete. Various combinations of self-complementing (and/or multi-functional) tags should provide even more opportunities to quantify and visualize diverse processes occurring in the living cell.

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