Novel uses of fluorescent proteins

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Abstract

The field of genetically encoded fluorescent probes is developing rapidly. New chromophore structures were characterized in proteins of Green Fluorescent Protein (GFP) family. A number of red fluorescent sensors, e.g., for pH, Ca^{2+} and H_2O_2 , were engineered for multiparameter imaging. Progress in development of microscopy hardware and software together with specially designed FPs pushed super-resolution fluorescence microscopy towards fast live-cell imaging. Deeper understanding of FPs structure and photophysics led to further development of imaging techniques. In addition to commonly used GFP-like proteins, unrelated types of FPs on the base of flavin-, bilirubin- or biliverdin-binding domains were designed. Their distinct biochemical and photophysical properties opened previously unexplored niches of FP uses such as labeling under anaerobic conditions, deep tissue imaging and even patients' blood analysis.

Introduction

In the broad instrumentarium of modern biology, fluorescent proteins occupy an important and unique niche enabling direct observation of molecular processes in live systems [1].

This technology began from a single known member, green fluorescent protein GFP from jellyfish *Aequorea victoria*. In 1990s, a number of its improved and color-shifted variants from blue to yellow were created. Since the discovery of GFP-like proteins in corals in 1999, great efforts were directed towards characterization of natural diversity of this protein family. As a result, GFP-like proteins were found not only in coelenterates, but also in combjellies, crustaceans, and lower chordates - lancelets [1]. Proteins of diverse spectral, biochemical and biophysical properties were found in nature leading to development of a multitude of FP-based techniques.

The interest in novel natural GFP-like proteins has diminished by now. The last notable finding in this field was a discovery of multidomain FPs in Hydrozoa in 2012 [2]. In these FPs, two or four GFP-like domains are repeated within the same polypeptide chain. Curiously, a linker between these domains contains amino acid sequence VAMPRIVET that means “hello to you” in Russian. This “best regards” from Nature should encourage scientists to further study biological functions and evolution diversity of this amazing protein family.

The focus of the GFP-like proteins research in the last few years has shifted towards deeper understanding of structure, photophysics and photochemistry of existing FPs and development of advanced imaging methods. An important new direction is engineering of FPs unrelated to GFP.

Applications of FPs are published in thousands of papers every year. In this short review, we focus only on a few topics, which in our opinion are of a special interest.

From novel fluorescent proteins to novel applications

In contrast to other natural proteinaceous pigments, which carry protein-bound cofactors, proteins of GFP family form chromophore by self-catalyzed posttranslational modifications of their own internal amino acids [1]. Different chromophores can be formed within the protein β -barrel (220-240 amino acids), and the list of known chromophore structures continues to expand (Fig. 1a). For example, an unusual linkage of GFP-type chromophore with the hydroxyl of a nearby tyrosine was recently revealed in red laRFP from a lancelet [3]. Also, in engineered green WasCFP a possibility to deprotonate the tryptophan-based CFP-type chromophore was demonstrated [4]. WasCFP possesses a record high fluorescence lifetime that is advantageous for multiparameter fluorescence lifetime imaging (FLIM) and fluorescence resonance energy transfer (FRET). Potentially, these new types of chromophores can facilitate further expansion of spectral diversity of GFP-like proteins.

Two-color labeling with regular FPs is a useful approach for quantitative imaging. For example, labeling of nascent mRNA with green and red FPs fused to specific RNA-binding domains was used to quantify kinetics of transcription of a single gene in live cells [5]. Also, artificial minigene constructs allow to use FP's green-to-red fluorescence ratio as a measure of activity of

alternative splicing, mRNA translation or nonsense-mediated mRNA decay at single cell level [6–9]. Such analysis in some cases revealed strong heterogeneity within seemingly homogenous cell populations [5,7,9] - phenomena that cannot be discovered by classical methods.

In addition to the GFP-like proteins, unrelated types of fluorescent proteins were designed during the last several years. These “genetically encoded” FPs are based on protein domains that specifically bind to ubiquitously present chromophores. The first representatives of such FPs were flavin mononucleotide (FMN)-based fluorescent proteins (FbFPs) developed in 2007 [10].

In FbFPs, light-oxygen-voltage-sensing (LOV) domains (about 100 amino acids) from bacterial or plant proteins are mutated to make the non-covalently bound FMN green fluorescent [10,11] (Fig. 1b). The main advantages of FbFPs over GFP-like proteins are fast oxygen-independent maturation, which opens a whole area of applications under anaerobic conditions, and a smaller size, which was demonstrated to be crucial in some models. At the same time, compared to GFPs, FbFPs possess much lower fluorescence brightness because of low extinction coefficient.

An FbFP named miniSOG (Singlet Oxygen Generator) was designed to produce high level of reactive oxygen species (ROS) upon blue light illumination [12]. MiniSOG was found to be an efficient genetically encoded tag for electron microscopy. For live models, miniSOG and GFP-like protein KillerRed [13] (the latter produces radical-based ROS under green-orange light) can be used as genetically encoded photosensitizers. It is a unique tool that enables very precise (in space and time) light-induced killing of specific cell populations [13–17], inactivation of target proteins [13,18*,19], and damaging genomic DNA [16,20]. Moreover, a new approach to study protein arrangement in large complexes was developed [21**]. This method named singlet oxygen triplet energy transfer (STET) evaluates distance between proteins using bleaching of infrared fluorescent protein IFP1.4 (fused to one target protein) by singlet oxygen produced by miniSOG (fused to another target protein). In contrast to commonly used FRET, which is efficient at short distances only (<10 nm), STET makes it possible to determine long-range (several tens of nanometres) inter-protein relationships. A recent appearance of an FbFP variant with enhanced level of singlet oxygen production [22] instils confidence in further development of phototoxic FP-based optogenetic techniques.

Another type of green FPs was discovered in Japanese eel muscles recently [23**]. This 139-amino acid protein named UnaG belongs to the fatty acid binding protein (FABP) family. UnaG binds bilirubin non-covalently with a subnanomolar affinity and activates its fluorescence (Fig. 1c). Due to high affinity and specificity of bilirubin binding, UnaG can be used as a fluorescent sensor for the easy and fast measurement of bilirubin in blood of patients - the first case of suitability of a fluorescent protein for clinics [23**].

Engineered bacterial phytochromes represent the third type of non-GFP-like FPs [24]. These extensively mutated ~315 amino acids long domains carry covalently bound biliverdin that fluoresces in near-infrared (Fig. 1d). This spectral region (excitation-emission maxima at 640-700 and 670-720 nm, respectively) is unattainable for other types of FPs. The main drawback of bacteriophytochrome-based infrared FPs (IFPs) is low fluorescence quantum yield. Another

problem of IFPs is insufficient amounts of biliverdin in some tissues [25]. This problem can be solved by co-expression of heme oxygenase, which produces biliverdin from heme [25].

In spite of low molecular brightness, IFPs far outperform GFP-like proteins in whole body imaging in mice, where near-infrared light penetrates tissues much deeper compared to shorter-wavelength light [26,27*]. The use of a reversibly photoswitchable variants (PAiRFP1 and PAiRFP2) enables strong enhancement of signal-to-noise ratio by controlled optical modulation of the signal in *in vivo* imaging, where high autofluorescence represents a significant problem [28].

High extinction coefficient and low fluorescence quantum yield of IFPs are advantageous for photoacoustic tomography (PAT) [29]. PAT is a hybrid imaging technique, which is based on detection of ultrasound waves resulted from thermoelastic expansion of pigmented objects upon illumination with a short pulse of intense light. It was demonstrated that iRFP670 and iRFP720 [26] can be used for multiparameter PAT of deep tumors in mice, enabling clear spectral separation from each other and from blood hemoglobin [30*].

Genetically encoded fluorescent biosensors go red

One of the most powerful uses of GFP-like proteins is constructing genetically encoded fluorescent sensors for various analytes and protein activities [1]. Among different sensors' designs, perhaps the most promising one is based on circularly permuted FPs linked to the domain(s) that change conformation upon interaction with the molecule of interest. Green fluorescent proteins originated from *Aequorea victoria* GFP undergo circular permutation relatively easy. This resulted in many green fluorescent single-fluorophore sensors of superior performance for intracellular detection of Ca^{2+} [31], NAD^+/NADH [32], ATP/ADP [33], H_2O_2 [34], etc. Sensors of other colors are in high demand for multiparameter imaging.

Recently, red fluorescent biosensors were developed. First, a number of red fluorescent pH sensors appeared [35–37] (Fig. 2a). With pHRed [35], quantitative imaging of pH changes is possible due to the ratiometric response. The probe has two excitation peaks at 440 and 585 nm, and a single emission peak at 610 nm. $\text{p}K_a$ of pHRed is 6.6 making it more suitable for pH measurements in neutral and slightly acidic compartments. But the sensor was used also to quantify mitochondrial pH changes. Later, two other red pH reporters appeared, pHTomato [36] and pHuji [37]. Both are intensimetric and used mostly to monitor endocytosis and exocytosis events. pHuji is based on mApple template and outperforms pHTomato due to significantly higher dynamic range.

In 2011, a palette of GECOs—genetically encoded Ca^{2+} probes was reported [38], including the red one based on circularly permuted mApple FP (Fig. 2b). Later, other red fluorescent Ca^{2+} reporters appeared, namely RCaMP [39], R-CaMP1.07 [40], ratiometric REX-GECO1 [41], and low-affinity LAR-GECO for Ca^{2+} imaging in mitochondria and ER [42].

Whereas all existing green and red Ca^{2+} sensors detect Ca^{2+} transients, there is a need in a kind of "memory" indicator that would "remember" its Ca^{2+} -bound state. This would allow whole-brain functional circuits labeling and analysis. Foscue et al. described a green-to-red photoconvertible Ca^{2+} sensor CaMPARI whose photoswitching efficiency strongly increases in the presence of

Ca^{2+} [43**] (Fig. 2c). Thus, upon violet illumination only those neurons undergoing activity-dependent Ca^{2+} elevation will turn red. The utility of the probe was demonstrated in a number of animal models ranging from fly to mouse.

cpmApple, a fluorophore of R-GECO1, was used to build HyPerRed, a genetically encoded red fluorescent H_2O_2 probe [44*]. HyPerRed enabled a first multiparameter imaging of mitochondrial H_2O_2 in parallel with glutathione redox state and pH in the mitochondrial matrix and in the cytoplasm imaged with green fluorescent biosensors, and also to monitor H_2O_2 dynamics in the mitochondria and in the cytoplasm of the same cells combining red and green probes for H_2O_2 .

Most recently, a new design type for genetically encoded biosensors was suggested [45*]. It is based on green and red dimerization-dependent FPs that are fluorescent in the dimeric form and non-fluorescent as monomers. A number of biosensors, e.g., for caspase activity (Fig. 2c) and Ca^{2+} , were built implementing an interchange of a non-fluorescent monomer between red and green dimers. Despite some limitations, such that non-quantitative readout, slow response and relatively low dynamic range, the dimerization-dependent biosensors provide a new useful way of probe design.

Novel tags and approaches for super-resolution fluorescence microscopy

The application of photoactivatable FPs (PAFPs) in two major types of super-resolution fluorescence microscopy – PALM (photoactivated localization microscopy) and RESOLFT (reversible saturatable optical fluorescence transition) was recently reviewed in Ref [46].

Despite the progress in methods and image reconstruction algorithms, we are still very far from the ultimate goal - prolonged live-cell super-resolution imaging at high temporal resolution. It should come as no surprise, that the certain single-molecule properties of fluorescent proteins (such as blinking behavior, emission rate, photoswitching rate, and the total photon budget) are essential for implementation of super-resolution techniques in living cells or tissues. For years, however, improvements of FPs was guided solely by ensemble properties, such as spectra, brightness, and photostability. Recently, the development of FPs placed emphasis on the specific requirements of super-resolution techniques. Recent works show comparison of single-molecule behaviour of multiple PAFP in fixed [47] and living cells [48].

In PALM and other similar techniques, super-resolution information is derived from localization of individual fluorophores, visible on image series as a result of controllable or stochastic single-molecule switching. The requirement for spatial and temporal separation of fluorescence bursts significantly limits acquisition speed. An improvement in temporal resolution can be achieved by tuning of localization algorithms to novel sCMOS cameras with high frame rates [49]. Also, the progress in algorithms for more densely labelled samples brings the timescale of PALM closer to actual cellular dynamics [50]. Recently published mMaple2 and mMaple3 [51] were designed specifically to meet the requirements of PALM: high photon numbers per burst, optimal on-off switching rate ratio, reduced oligomerization tendency. The unavoidable intrinsic blinking of fluorescent proteins complicates single-molecule counting. A novel stochastic approach allows for extraction of accurate stoichiometry information from PALM data [52*].

Robust blinking of fluorophores can be advantageous, as in super-resolution optical fluctuation imaging (SOFI), a promising algorithm for densely labelled samples [53]. Recent extension of this method promises higher (up to 5-fold) resolution improvement and extraction of additional useful information from the fluctuations [54], such as molecular brightness. SOFI has been shown to work with PAFP [55,56] even in 3D [57**] in living cells. An improved reversibly switchable green FP Skyran-S was recently designed to provide robust fluctuations and low photofatigue for SOFI [58].

In RESOLFT, reversible photoswitching of fluorescent probes is utilized in coordinate-based system with structured nonlinear light beams. An elegant combination of RESOLFT and time-domain microscopy, dubbed $\square$ -RESOLFT, allowed for dual-channel super-resolution imaging in living cells of spectrally similar rsEGFP and Dronpa with distinct fluorescent lifetimes and switching kinetics [59]. PAFPs for RESOLFT should also exhibit high switching rate and very low photofatigue over multiple cycles of photoconversion. The improvement of this property has already attracted researchers [60].

Correlative light and electron microscopy (CLEM) impose additional requirements on FPs. Most PAFPs lost fluorescence or the ability to photoactivation under conditions necessary for optimal ultrastructure fixation for EM. Recently published EosFP variants survive heavy OsO_4 fixation, enabling better correlative PALM and electron microscopy [61].

All of these techniques are rapidly improving towards implementation *in vivo*. One recent example is the observation of presence of caveolae membrane domains in a living zebrafish embryo [62].

Identification and utilization of the long-lived dark states in fluorescent proteins

All FPs have fluorescence quantum yields lower than unity, indicating the presence of non-radiative processes competing with fluorescence. In some cases, such non-radiative processes may involve the formation of kinetically trapped dark states (DS) of various origins. The formation and utilization of such DS are largely unexplored, and this section will discuss few recent achievements in this area.

Dickson et al. proposed the utilization of DS using the principles of modulated spectroscopy [62]. The simplified Jablonski diagram of the suggested approach is shown in Figure 3a. A direct depopulation of transient DS by coillumination of the sample with secondary laser was suggested. This will lead to repopulation of the chromophore emitting singlet excited state enhancing its fluorescence. Thus the crucial requirement for optically enhanced fluorescence is to have a relatively long lived and the bathochromically shifted optically reversible dark state. One of obvious advantages of this method is that photon energy of the secondary light source ($h\nu_3$) is less than that of the primary $S_0 \rightarrow S_1$ excitation ($h\nu_1$) and the fluorescence ($h\nu_2$). Turning this coillumination on and off at a specific frequency dynamically modulates collected fluorescence without generating additional background. This method, initially developed and utilized for metal nanodots and organic dyes, has been recently applied to FPs. Some blue (omBFP, the variants of mKalama1), green (AcGFP), red, and other FPs were susceptible to secondary pulsed excitation [64–66]. One impressive example includes the fluorescence

recovery of the green FP in the presence of highly fluorescent, but unmodulatable another green FP (Figure 3b). Another example demonstrates a dramatic improvement of signal-to-noise ratio for the blue FP (Figure 3c).

Using the broadband transient absorption (TA) spectroscopy spanning the time scale from picoseconds to seconds, a hidden photoinduced reactivity of various FPs and the nature of the DS can be revealed. Figure 3d shows the TA spectra of selected modulatable FPs in comparison to their DS action spectra. In case of bright blue-light emitting protein omBFP, the DS transient was associated with the anionic species, which are formed as a result of isomerization-coupled internal conversion and deprotonation [67,68]. The unpublished DS action spectrum of mDsRed had a maximum around 710 nm (see Fig. 5.4 in Ref. [66]), which nicely fits the TA spectra of various RFPs [69*]. Based on the results of QM/MM calculations this transient was assigned to the unusual open-shell dianionic chromophore (dianion-radical) formed via photoreduction. The results raise the question of whether photoreduction occurs in other FPs and if the corresponding electron-attached states are common gateway states for photobleaching and blinking. The nature of the DS in AcGFP is unclear at the moment.

We believe that previously unexplored arena of FP DS holds great promise for future understanding, development, and utilization of FPs.

Conclusions

At first glance, applications of fluorescent proteins (FPs) seem to be so well-established that nothing really new can appear in this field, but recent works convince of the opposite. Directed molecular evolution is extensively used to generate GFP-like proteins with new chromophores, unusual spectra, extended fluorescence lifetime, enhanced photostability, improved parameters of photoconversion, *etc.* Another important growing point is construction of new biosensors, in particular red fluorescent ones. The use of sensors of distinct colors makes it possible to simultaneously observe various analytes and activities within the same cell.

GFP-unrelated FPs based on protein domains bound to natural intracellular cofactors (FMN, bilirubin or biliverdin) are of a special interest. These FPs are seemingly “genetically encoded” since there is no need to introduce exogenous dyes into the cells. Non-GFP-like FPs possess unique properties such as oxygen-independent maturation, near infrared fluorescence, or efficient ROS production, which enable novel areas of applications where GFP-like proteins are inefficient.

Novel improved FPs are envisioned for further improvement of quantitative multiparameter imaging of live systems at different scales, from single molecules to whole organisms.

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Figure legends

Figure 1. Structures of fluorescent proteins of different types. 3D protein structures are shown to the same scale in cartoon representation (rainbow colors from blue at N-termini to red at C-termini). Chromophore groups are shown in spacefill representation. Images were created using PyMol (DeLano Scientific) from PDB files 2Y0G, 4EET, 4I3B, and 1ZTU for (a-d), respectively. In chemical structures of chromophores the moieties responsible for fluorescence are highlighted by corresponding colors. **(a)** GFP-like proteins. Main types of chromophores formed by autocatalytic modifications of internal amino acids are shown. **(b)** FMN-binding fluorescent proteins (PpFbFP, iLOV, miniSOG, *etc.*). **(c)** Bilirubin-binding protein UnaG. **(d)** Biliverdin-binding fluorescent proteins (IFP1.4, iRFP, IFP2.0, *etc.*).

Figure 2. Design of FP-based sensors. Selected sensor types discussed in the review are shown. **(a)** Single FP-based pH sensors, which increase red fluorescence at higher pH. **(b)** Calcium sensors based on circularly permuted red FPs fused to M13 peptide and calmodulin (CaM), which increase red fluorescence in Ca^{2+} -bound state. **(c)** Ca^{2+} sensor CaMPARI. Its green-to-red photoswitching under violet light illumination occurs efficiently only from the Ca^{2+} -bound state. **(d)** Sensor for caspase-3 activity based on dimerization-dependent green and red FPs, which become brightly fluorescent only upon binding with a non-fluorescent monomer (grey).

Figure 3. Dark states in action. **(a)** Jablonski diagram illustrating the reverse intersystem crossing between bright and dark states of a fluorophore. Note that this scheme works for FPs and other systems which depopulates DS and repopulates S_0 . For some other systems the direct repopulation of S_1 is possible. **(b)** Selective fluorescence recovery of mitochondria-targeted AcGFP in the presence of high nuclear-targeted EGFP fluorescence for live NIH 3T3 cells: left image - raw fluorescence of AcGFP-labeled mitochondria and EGFP excited at 476 nm; right image - demodulated AcGFP fluorescence upon coexcitation with 561 nm 300 Hz laser. (Reproduced with permission from ref [65] which include all experimental details. Copyright 2012 American Chemical Society). **(c)** Live-cell demodulation of mitochondria-targeted modBFP/H148K. Upon 405 nm illumination, blue fluorescence was collected from modBFP/H148K-mito mixed with high background emission. Coillumination at 514.5 nm modulated at 2 Hz (secondary illumination only within the white circle) recovered only the modBFP/H148K-mito signal on a greatly reduced background (lower circle). (Reproduced with permission from ref [63]. Copyright 2013 American Chemical Society. See the original reference for the experimental details). **(d)** Optical properties of the modulatable FPs. Normalized absorption (blue lines) and emission (green lines) spectra, as well as transient absorption spectra (magenta lines) recorded at 4 ms after laser excitation. DS action cross section spectra scanned by secondary laser wavelength of various FPs (red squares). modBFP was excited at 372 nm, AcGFP at 476 nm. TA of DsRed is presented.

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First example of broadband TA spectroscopy in FPs. Identification of the DS using QM/MM methods.

Figure 2

(a)

(b)

(c)

(d)

Figure 3

Multiparameter functional imaging
Anaerobic cell labeling
ROS-optogenetics
Deep tissue imaging
Photoacoustic tomography
Super-resolution microscopy