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Fluorescent Proteins: Shine on, You Crazy Diamond

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ABSTRACT: In this Perspective we discuss recent trends in the development and applications of fluorescent proteins. We start by providing a historical and structural perspective of their spectroscopic and structural aspects and describe how these properties have made fluorescent proteins essential as 'smart labels' for biosensing and advanced fluorescence imaging. We show that the strong link between the spectroscopic properties and protein structure and properties is a necessary element in these developments and that this dependence makes the proteins excellent model systems for a variety of fields. We pay particular attention to emerging or future research opportunities and unsolved questions.

Two decades have passed since the gene for green fluorescent protein (GFP) was cloned from the jellyfish *Aequorea victoria*,[1] and 18 years since the first demonstrations of its expression and maturation in other organisms.[2] These discoveries set the stage for an extraordinary success story that rapidly propelled these proteins to the forefront of the modern life sciences.[3−7] The key to this success is the protein's ability to attain fluorescence after expression without the need for cofactors or chaperones. Simply put, introducing an appropriate DNA fragment encoding a fluorescent protein into nearly any organism will lead to the appearance of fluorescence, meaning that the emission is contained with the coding gene. Even more exciting is that this gene can be conveniently manipulated using standard molecular biology tools, allowing the creation of recombinant proteins, fusion constructs, or even transgenic organisms. In this way complete fluorescent labeling of nearly arbitrary target proteins in living organisms can be realized, including intact multicellular animals that would otherwise be impossible to label using nongenetically encoded fluorophores.

Because it is a protein, the primary sequence of a FP, and therefore its structure and properties, can be readily manipulated using the available tools for DNA mutagenesis. Depending on the extent to which the desired changes can be accomplished rationally, or must be accomplished through trial-and-error, these manipulations can be performed in a completely deterministic, site-directed random, or completely random fashion. While the development of useful FPs requires that these techniques are performed in a careful and highly demanding scientific context, the lab manipulations themselves have become so routine that they can be performed by undergraduate students, and in fact make ideal introductory molecular biology experiments since immediate feedback is available simply by observing the presence or absence, and color of the fluorescence of the produced protein. Starting from the original *A. victoria* GFP (hereafter denoted avGFP), it was found that the spectral properties of the protein could be changed dramatically by introducing just one or a few mutations,[3] showing that the fluorescence of these labels very delicately depends on the protein structure. However, some developments proved elusive: despite years of effort, the performance of avGFP-based mutants could not be extended toward red emission and help had to come from nature in the form of the discovery of red-fluorescent FPs in corals.[8] This development was particularly desirable not only because a wider 'color palette' allows more labels to be used simultaneously but also because light scattering and background absorption and emission are all strongly reduced at longer wavelengths, allowing more in depth imaging into the tissues of multicellular organisms with less distortions and better contrast. The main result of these developments has been the generation of a range of brightly fluorescent and biologically tolerated labels that cover the entire visible spectrum.

On the whole, these developments have been so successful that the wide availability of variants with subtly different properties, sometimes with rather whimsical names, has led to a broad range of options for any experiment. Occasionally this can be confusing for "end users" of fluorescent proteins who wish to find and employ the optimal label for their application, and with that has come a strong need for quantification of their performance. In most experiments, the main parameters of interest are typically the spectral performance (excitation and emission wavelength, brightness, photostability) and biological tolerance of the label. However, the complexity of biological systems, including the labels themselves, means that the performance of a particular label is a multifaceted question that requires the consideration of other properties, such as the time required to reach fluorescence after expression (the maturation time), the sensitivity of the label to external factors such as pH, its tendency for oligomerization, fusion tolerance, or cytotoxicity. A major complication is that these aspects are much harder to quantify exactly. Fortunately, several resources and reviews provide assistance in choosing suitable fluorescent proteins (http://zeiss-campus.magnet.fsu.edu/articles/probes/index.html; http://zeiss-campus.magnet.fsu.edu/articles/probes/index.html; http://www.olympusconfocal.com/applications/fpcolorpalette.html)[5,9,10].

The broad palette of available fluorescent proteins also begs the question as to what extent opportunities for important improvements in performance remain. Is there still room at the top or has all the low hanging fruit (and perhaps the next couple of levels up) been picked? The answer to this question is mixed: Increasingly indications are emerging that the current labels are converging on fundamental limitations in terms of brightness and emission wavelength, suggesting that the days of

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explosive gains in FP performance may be numbered. However, in this perspective we argue that there remains plenty of room for discovery, invention, and engineering, but that the field is also adapting to changing contexts. First we briefly describe some of the defining aspects of fluorescent proteins.

Fluorescent Proteins: Diversity in Similarity and Similarity in Diversity. Fluorescent proteins or nonfluorescent homologues have been found in a wide range of aquatic organisms, including jellyfish, corals, or crustaceans and appear to trace back to a single, ancient, ancestor gene.[5,11,12] This ancestor is thought to have arisen early in the evolution of metazoa (multicellular organisms) and has diverged to encompass two superfamilies that share the "GFP-fold" structure. The first family constitutes the G2F domains that occur in multidomain proteins found in the extracellular matrix and are found in a wide range of multicellular organisms. These domains are involved in protein binding and do not form chromophores but are highly structurally similar to FPs. An example is the G2 domain of nidogen, a basement membrane protein present in many organisms including mammals.[13] In contrast, the distribution of the chromophore-forming FPs among the Metazoa is rather more sparse. At present fluorescent proteins and chromoproteins (FPs with a very low fluorescence quantum yield) have been discovered in marine life belonging to a variety of different phyla, including lancelets (Chordata), copepods (Arthropoda), corals and sea anemones (Anthozoa), hydrozoa, and comb jellies (Ctenophora).[5]

This conservation along divergent parts of the tree of life demonstrates that FPs play one or more roles that are essential to the host organisms, though this purpose remains unclear. Likewise, it remains unclear whether fluorescence emission is important to the host organism or whether it is more of an accidental byproduct. Evolution may well have repurposed FPs for different tasks in different organisms, though the present lack of FP-like proteins in freshwater or terrestrial organisms suggests that these are related to the marine environment. Some of the proposed functionality focuses on the protein's fluorescence property, including communication (with peers, to attract prey, or to dazzle predators),[5] or conversion of part of the sunlight spectrum to wavelengths more suitable for photosynthesis.[14] Others focus more on the protein's ability to absorb light, as a way to achieve coloration,[15] as photoprotection from intensive sunlight,[12,16] as photoreceptors,[12] or even as photochemical agents in the host metabolism.[17,18] Finally, apparently light-independent functions, such as antioxidant properties, have also been proposed.[19,20]

For all their diversity, all fluorescent proteins known to date consist of a single polypeptide chain of about 230 amino acids in length and an approximate molar weight of 25 to 30 kDa, though a natural organism expressing multiple FP domains in a single protein was recently reported.[21] Despite showing low sequence similarity across templates, the three-dimensional structures of all fluorescent proteins known to date are remarkably similar, consisting of an 11-stranded β-barrel structure (Figure 1). A single distorted helix spirals down the center of the barrel and contains three amino acids that cyclize to form the chromophore. These amino acids, located at positions 65−67 (avGFP numbering) always correspond to an XZG tripeptide, where X is variable, Z is an aromatic amino acid (tyrosine in naturally occurring proteins), and G denotes glycine, which is strictly conserved and is required to achieve

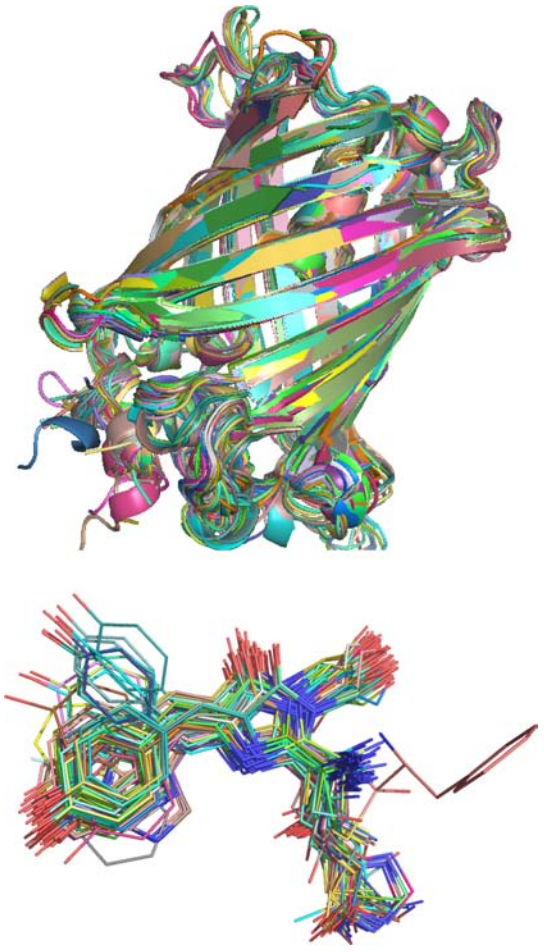

Figure 1. (a) Alignment of 202 crystal structures of different fluorescent proteins extracted from the protein data bank (PDB). (b) Detail of (a) showing the alignment of the chromophores. Note that the majority of the chromophores are in the cis state.

fluorescence. The formation of the chromophore is autocatalytic in the sense that it does not require external scaffolds or cofactors, though a carefully structured chromophore environment, and particularly certain strongly conserved residues, such as arginine-96 are required.[22−25]

Fusions to both the N and C termini are readily tolerated and generally do not impede fluorescence emission, though in some cases poor folding of the fused protein can negatively affect or even prevent the folding and maturation of the FP,[26] and likewise the FP can aid or impede the folding of the fused target.[27] In cases where the target protein is intolerant of N- or C-terminal fusions, insertions of the FP can occasionally be used.[28] However, attempts to reduce the size of the label by creating truncated or otherwise shortened FPs have met with little success and show that much or all of the GFP structure is required for chromophore formation and fluorescence.[29]

The structure of the naturally occurring green chromophore was first determined over 30 years ago[30] (Figure 2). The majority of fluorescent proteins have a chromophore in the cis conformation, though exceptions have been encountered.[31] The exact mechanism of the chromophore maturation has been thoroughly investigated and is fairly well-understood for the formation of the naturally occurring green chromophore (Figure 2). Starting from the three amino acids that will eventually form the chromophore, nucleophilic attack of the

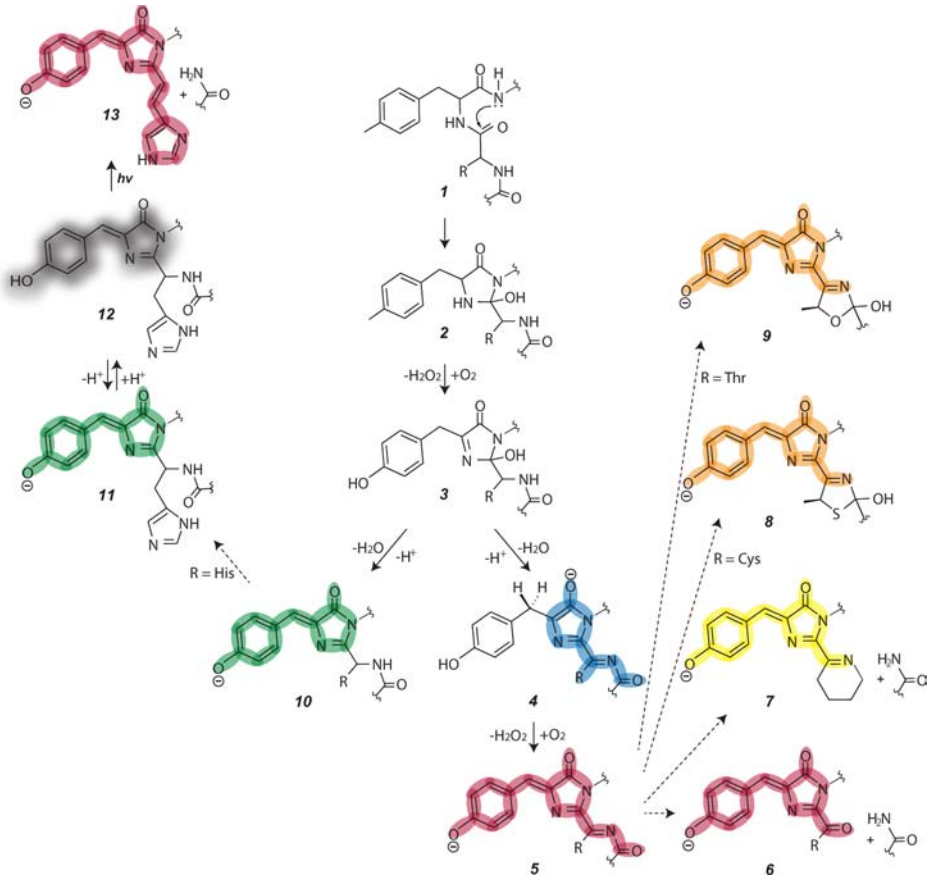

Figure 2. Proposed maturation mechanisms for a range of chromophores. Starting from the chromophore tripeptide (**1**), the abundantly occurring green chromophore (**10**) is formed in a number of steps. In selected photoconvertible fluorescent proteins containing a histidine residue in the tripeptide (discussed below), this structure can convert to a red emitting state (**9**) by irradiation with UV light. In other cases the green chromophore (**4**) matures via the blue-emitting intermediate (**5**) to the DsRed-like chromophore (**6**). Through chromophore and environment mutagenesis, a range of different chromophores can be generated (**10**–**13**). Adapted from ref 39 with permission from Elsevier.

Gly67 amide nitrogen on the carbonyl carbon of Ser65 results in the formation of a heterocyclic structure, followed by oxidation by molecular oxygen, and dehydration[23,32−37] (Figure 2). The oxidation is the rate-limiting step,[35] and oxygen is the only external reagent that is consumed by the maturation process. Depending on the particular FP, this process generally takes minutes to hours to complete. The formation of other types of chromophores is somewhat less understood, and particularly the formation of red chromophores has met with considerable debate,[34,38,39] though it is known that most red fluorescent proteins mature along a common DsRed-like pathway. Conceptually, all red chromophores known to date consist of the green FP chromophore combined with additional double bonds that extend the conjugated system (Figure 2). The chemistry involved in these processes is complex, and several competing mechanisms have been proposed.[34,38,40−42] For example, it was long thought that the red fluorescence of DsRed arose via a green fluorescent intermediate,[43] but evidence is increasingly emerging that maturation occurs via an unusual blue intermediate, though other red fluorescent proteins do mature via a green chromophore intermediate.[38] The blue-to-red conversion can be blocked from occurring, resulting in a blue FP with a novel chromophore structure.[44] Often the maturation of red fluorescence is fraught with pitfalls, in the sense that a significant fraction of proteins fail to achieve red fluorescence and become trapped in, e.g., a green-emissive state via a competing pathway (Figure 2), leading to significant experimental complexity.[45]

A range of different chromophore structures have been discovered (Figure 2), though the GFP-like green chromophore and dsRed-like red chromophore are the most abundant, both in terms of research uses as well as the number of variants that have been extracted from marine species. Other chromophores have been artificially created by mutating the template proteins provided by nature, e.g., through the introduction of different aromatic amino acids to replace the central tyrosine in the chromophore tripeptide, leading to UV, cyan-, and blue-emitting fluorescent proteins.[3]

Having said all that, one of the most fascinating aspects of fluorescent proteins is that the covalent structure of the chromophore structure determines only part of the spectroscopic properties. Located at the center of the barrel, the chromophore engages in a wide range of interactions with surrounding amino acids and possibly highly ordered water molecules observed in the crystal structures, forming an intrinsic part of an interaction network that encompasses the entire protein. Single mutations in nonchromophore amino acids can strongly influence the spectroscopic properties of the protein, shifting its absorption and/or emission spectrum or even rendering it nonfluorescent.[3] This tightly coupled network also imparts a very favorable stability: As proteins go, FPs are remarkably resistant to heat- or chemically induced denaturation, or even proteinases.[46]

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The effect of the chromophore environment on the fluorescence emission has been extensively studied by creating synthetic analogues of the chromophore or using short digestion fragments.[47,48] The simplest of these is 4′-hydroxybenzilidene-2,3-dimethylimidazolinone (HBDI, Figure 3). At room temperature and in aqueous solution, HBDI is very

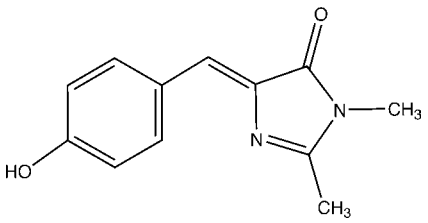

Figure 3. Structure of HBDI (compare with structure **10** in Figure 2).

weakly fluorescent with a fluorescence quantum yield of about 10^{-4}, compared to avGFP's fluorescence quantum yield of about 0.8.[49] Ultrafast spectroscopy has attributed this low quantum yield to highly efficient subpicosecond internal conversion that occurs in a wide range of solvents,[50−53] causing deactivation of the excited state on a time scale that is too short for fluorescence to be competitive (by comparison, most GFP display excited-state lifetimes of about 3 ns). Further study revealed that this process is only weakly dependent on viscosity, suggesting that no large structural rearrangements are involved in the process,[50,53] and is essentially barrierless.[54] HDBI does become fluorescent at very low temperatures and especially below the glass transition of the solvent.[49,51,55] While the exact mechanism associated with this fast internal conversion is still under debate, the current consensus appears to favor rotation or flexibility along one of the bonds associated with the methylene bridge.[47,48] Evidently, to obtain the remarkable fluorescence quantum yields observed in FPs, the chromophore environment in FPs is very good at preventing this internal conversion from occurring. However, a simple packing argument, in which the immediate chromophore environment simply prevents the internal conversion through steric hindrance, is not sufficient to explain this effect,[47] and other factors, such as hydrogen bonding[56] and charge[57] or stacking interactions,[58] must be included.

Much like HBDI,[59] the chromophore in fluorescent proteins is known to exist in differing protonation states depending on the pH of the solution.[3,60] Most ensemble spectroscopy can be explained in terms of the protonation state of the phenolic oxygen of the chromophore: a deprotonated anionic state, which is fluorescent and absorbs at about 470 nm in avGFP, and a protonated neutral state that absorbs at about 390 nm in avGFP but emits only very dim fluorescence at about 450 nm.[61] An analogous observation (but at different wavelengths) can be made for all known fluorescent proteins. Except in very rare cases,[62] only the anionic state is fluorescent, showing that the chromophore environment is specifically tuned to prevent fast internal conversion in this state but not in others. However, in avGFP excitation of the neutral form at 395 nm is unusual in that it rapidly leads to bright green fluorescence emission that is similar to that of the anionic state. The mechanism involved for this transition was discovered to be ultrafast excited-state proton transfer (ESPT)[61] via an internal proton wire.[63] ESPT does not occur in all fluorescent proteins, though when it does it can be exploited to create labels that display very large apparent Stokes shifts.[64,65] In a nutshell, then, we have uncovered most of the mechanisms commonly relied upon to explain FP excited-state dynamics: changes in protonation, induced by the chromophore or protein environment, or possibly ESPT, and structural isomerization ("cis/trans") associated with the same, or a very similar, mechanism as that responsible for the fast internal conversion of isolated HBDI. While sufficient for a conceptual level, detailed investigations have shown that the actual picture is rather more complex.[66]

While the immediate chromophore environment is the natural place to look when studying FP photophysics or photochemistry, the spectroscopic properties are sensitive to the conformation of the protein as a whole. For example, in trying to reconstruct the evolution of red FPs from green ancestors, it was found that 12 mutations were necessary to obtain red emission, though some of the mutations were not in proximity of the chromophore and only affected the overall fold of the protein.[67] Likewise, a light-induced change in the protonation state and configuration of a fluorescent protein chromophore were found to profoundly affect the conformational dynamics of large segments of the protein[68] (Figure 8), indicating that the chromophore and protein matrix must be considered as a whole. In effect the spectroscopic properties of the protein become dependent not only on the potential energy surfaces of the chromophore but also on the surfaces associated with practically the entire protein.

This complexity and the associated dynamics are reflected in the excited-state dynamics: a single fluorescent protein, irradiated with uniform and constant excitation light, does not emit fluorescence uniformly but rather displays a rich dynamic behavior with nonfluorescent intermittencies ('blinking') on time scales ranging from microseconds to seconds.[69−73] The detailed mechanistic picture of these transitions between different states is often unclear, though as we previously mentioned most explanations associate these with different protonation states of the chromophore[74] and the occurrence of ESPT or cis/trans isomerization.[75]

The size of the protein also presents it with a large 'surface area' with which to engage in interactions with other proteins of cellular constituents. Fortunately, experience has shown that in most use cases FPs remain largely inert, though this also depends on the particulars of the FP in question, and care must always be taken.[76] Perhaps the most common interaction in many FPs is a tendency to oligomerize, which is particularly pronounced in FPs derived from corals, though this tendency can be suppressed using dedicated engineering.[77,78]

FPs as Passive Reporters. The vast majority of current FP applications rely on their use as passive reporters, meaning that the FP is expected to emit bright fluorescence while essentially not interacting with the system under study. From a purely spectroscopic viewpoint, the spectroscopic properties of fluorescent proteins are markedly inferior to those of optimized organic fluorophores, such as rhodamine or carbocyanine derivatives, in terms of brightness and particularly resistance to photodestruction.[79] Reagents or buffers that enhance the brightness and photostability of organic dyes[80] often have little effect on fluorescent proteins (M. Sauer, personal communication). Were it not for their all-redeeming property of genetically encoded fluorescence, the complexity and limited photostability of FPs would probably make them less attractive than organic dyes in most 'classical' imaging experiments.

The ease with which mutations in the protein structure can be introduced does suggest plenty of room for improvement,

though in the green and yellow region of the spectrum many of these gains have been more evolutionary than revolutionary. For example, EGFP continues to be an FP of choice in many applications due to its attractive combination of fast and quantitative maturation, brightness, photostability, and tolerance toward fusions, well over a decade after its discovery.[81] This suggests that green fluorescent proteins, and possibly cyan and yellow proteins as well, are close to realizing their full potential.

Figure 4 plots the fluorescence brightness of a number of popular or otherwise interesting fluorescent proteins as a function of their wavelength of maximal emission. This figure highlights two major trends: greenish fluorescent proteins tend to achieve the most favorable fluorescence brightness, with performance lagging toward either end of the visible spectrum, but also that there is a fairly large spread in brightness among the labels. The latter reflects that many different properties govern the attractiveness of a particular label for a given experiment, and optimization of one parameter (e.g., brightness) can negatively affect the performance of another (e.g., photostability). Therefore the brightest fluorescent protein is not necessarily the best choice for a particular experiment. Given the widespread availability of well-performing cyan, green, and yellow FPs, the most promising opportunities in terms of optimization potential probably come from the fluorescent proteins at the edge of the visual spectrum, which are less well characterized. However, as Figure 4 shows, it is probable that the spectroscopic performance of these variants is fundamentally limited compared to that of the green variants, and the choice of emission wavelength is an important factor for experiments that require, e.g., high sensitivity.

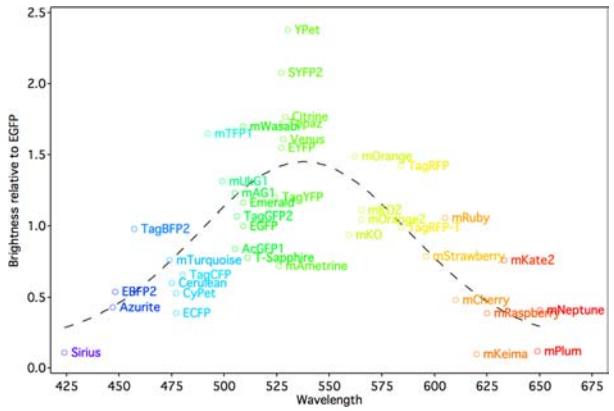

Figure 4. Reported fluorescence brightness (extinction coefficient times fluorescence quantum yield) of a number of popular or unique fluorescent proteins as a function of emission wavelength (data as reported in refs 10 and 44). The dashed line serves as a guide to highlight the general trend. The coloring of the tags approximates the appearance of the corresponding emission wavelength to the naked eye. The brightness of some fluorophores has been altered by very small amounts for clearer visual presentation.

An ongoing challenge, and probably one of most prominent driving forces in FP development, is the creation of variants that emit red or far-red fluorescence, though it is an open question to what extent fundamental limitations will ultimately limit the performance achievable within the fluorescent protein scaffold. The available red FPs tend to display much larger degrees of cellular toxicity and lower tolerances for protein fusion[82] while also suffering from slower or even incomplete chromophore maturation.[38] Importantly, a significant fraction of even well-known red fluorescent proteins fails to achieve any red fluorescence whatsoever: In a study combining fluorescence correlation spectroscopy and pulsed-interleaved excitation on samples consisting of two FPs fused together,[83] the authors found that essentially all EGFP fragments become fluorescent, but only 40% of mCherry and 22% of mRFP fragments reach the fully matured red state. Obviously this is an area in which further improvement is urgently needed. Given the strong dependence of red FP fluorescence on the entire protein structure, evident from the difficulty in monomerizing these FPs without compromising spectroscopic performance,[77] balancing association tendencies or cellular toxicity with favorable spectroscopic properties presents a formidable challenge.

The effect of fluorescent protein complexity is nicely revealed in a discussion of their photostability or resistance to the complete loss of fluorescence through photodestruction. The exact mechanisms associated with FP photodestruction remain poorly understood. Increasing evidence points to the direct involvement of intermediate nonfluorescent (blinking) species, formed from the singlet excited state, as being the major pathways for photodestruction.[79,84] In some cases, however, permanent loss of fluorescence through photodestruction can be confused with processes that cause a shift in the emission spectrum or a reversible loss of fluorescence, such as decarboxylation,[85,86] oxidative reddening,[87] or reversible photochromism.[73,88] A main complication in developing more photostable fluorescent proteins is the sensitivity of this parameter to the environment and observation conditions of the protein. Observing a given fluorescent protein in different fusion constructs, different cellular compartments or environments or cell states can all alter the absolute kinetics and even relative kinetics of photodestruction.[5] The photostability also depends strongly on the manner of observation: The relative stability of different FPs can change dramatically depending on whether the observation is performed using confocal imaging, widefield imaging, or another type of observation. An example is TagRFP-T, which was shown to be more photostable than its ancestor TagRFP in whole-colony screening,[89] but is actually surpassed by TagRFP at higher excitation intensities.[90] Similarly, mRaspberry is more photostable than mPlum in confocal microscopy but less stable in wide field microscopy.[91] This dependence of the photodestruction on the imaging conditions is not surprising given that this process is thought to occur from transiently formed intermediate states. As a result, the best approach in selecting a photostable fluorescent protein is to make use of the literature only for general guidance and to compare a number of well-performing variants under the actual experimental conditions.

These findings are somewhat emblematic for the challenges faced in FP development. While it is straightforward to combine DNA mutagenesis with high-throughput screening to achieve directed evolution, a choice has to be made regarding which parameter will be optimized, be it fluorescence brightness,[81] photostability,[89] folding/maturation,[27] photochromism,[92,93] or even the excited-state lifetime.[94] While this will result in improvements of that specific parameter, other parameters will likely deteriorate if no further action is taken. Also, the results strongly depend on the manner in which the screening was performed, and different results will be obtained under different imaging or targeting conditions. Since this complexity and sensitivity is an intrinsic part of the fluorescent

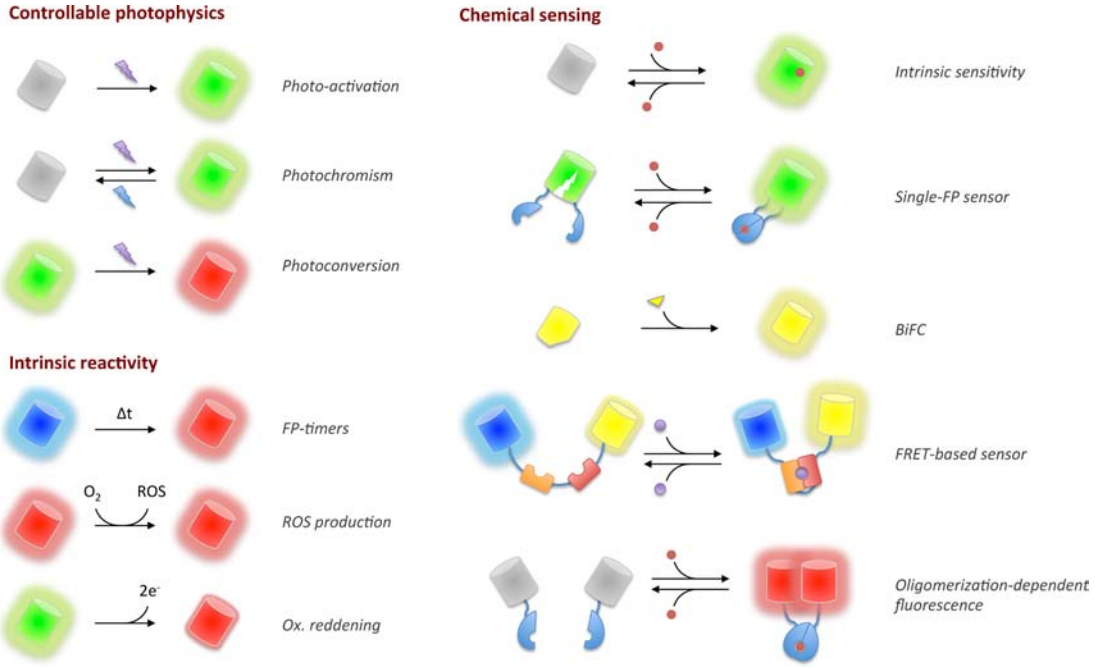

Figure 5. Fluorescent proteins as smart labels: schematic overview of the different types of functionality discussed in the text.

proteins, the likely way forward is to perform high-throughput screening in conditions that approximate actual usage as close as possible, while standardizing the assays used to tabulate their performance.

From Passive Reporters to Smart Labels. One of the most exciting directions in FP development distinguishes itself from these developments by not aiming to suppress the spectroscopic and structural complexity of these labels, but rather by exploiting this complexity to achieve enhanced imaging capabilities. Compared to classical experiments, these strategies do not use the labels as passive reporters but turn them into "smart" fluorophores. For the sake of this perspective, we will somewhat arbitrarily divide these fluorophores into those that are "chemically smart", meaning that they exploit the complexity and easy modifiability of fluorescent proteins to obtain sensitivity to certain chemical signatures (well-defined compounds, environments, or chemical reactions) and those that are "photophysically smart", meaning that their photophysics deviate away from the 'passive reporter' standard, in a way that can be exploited in imaging. The former class effectively reports on its environment, while the latter allows imaging with an enhanced spatial resolution or increased spatiotemporal discrimination between adjacent labels.

Chemically Smart Fluorescent Proteins. Chemically smart fluorescent proteins display a change in emission in the presence of certain compounds, cellular components, or chemical reactivity. In a biological context, a more typical name would be "biosensors".[10,95−97] The first FP-based biosensors were readily available by using the intrinsic sensitivity of the proteins to, e.g., pH[98−101] or halogen ions,[102] though the response or selectivity can often be improved further by introducing mutations.[103] More challenging is introducing sensitivity to environmental factors that the fluorescent protein does not display an innate sensitivity to. In this case, one can create recombinant proteins consisting of one more domains sensitive to the parameter in question and one or more fluorescent proteins, in such a way that the presence or absence of the target causes a structural change in the sensing domains. This change can then be linked to the fluorescence by designing the construct so that the structural rearrangement is transmitted to the FP barrel and the chromophore environment (Figure 5). In some cases the modifications can be very limited, such as introducing only a few strategically placed cysteines to obtain a redox potential sensor based on a disulfide-dependent conformational change.[104,105] In other cases the modification amounts to the insertion or grafting of entire peptide domains onto the FP, such as M13 and Calmodulin domains, to obtain a sensor for Ca^{2+}.[106,107]

Biosensors need not be limited to only a single FP moiety. An alternative strategy is to make use of Förster energy transfer (FRET) between two fluorescent proteins, by creating a construct in which a donor and acceptor fluorescent protein are fused together with one or more binding motifs, such that FRET will be increased or decreased upon occurrence of the target parameter (Figure 5).[108] Since FRET depends on the relative orientation and distance between the fluorophores, changes in either or both of these parameters can be used to provide a signal contrast. In principle, the donor and acceptor need not be part of the same protein but can be expressed as separate constructs with compatible sensing domains, that associate on occurrence of the parameter of interest. In these cases one gains the advantage of a lower background ('off') level, but the temporal response is typically slower and problems can arise due to uneven expression or different total concentrations of the donor and acceptor fragments.[10] If possible, intramolecular FRET is typically preferable.

FPs are uniquely positioned for use as biosensors simply by virtue of being proteins. Since much of the cellular machinery consists of proteins, the odds of finding naturally occurring binding or substrate motifs for biologically relevant targets or reactions are good, and the affinities of these substrates are also likely to be in the appropriate dynamic range for biologically relevant information. Creating these fusion constructs can be

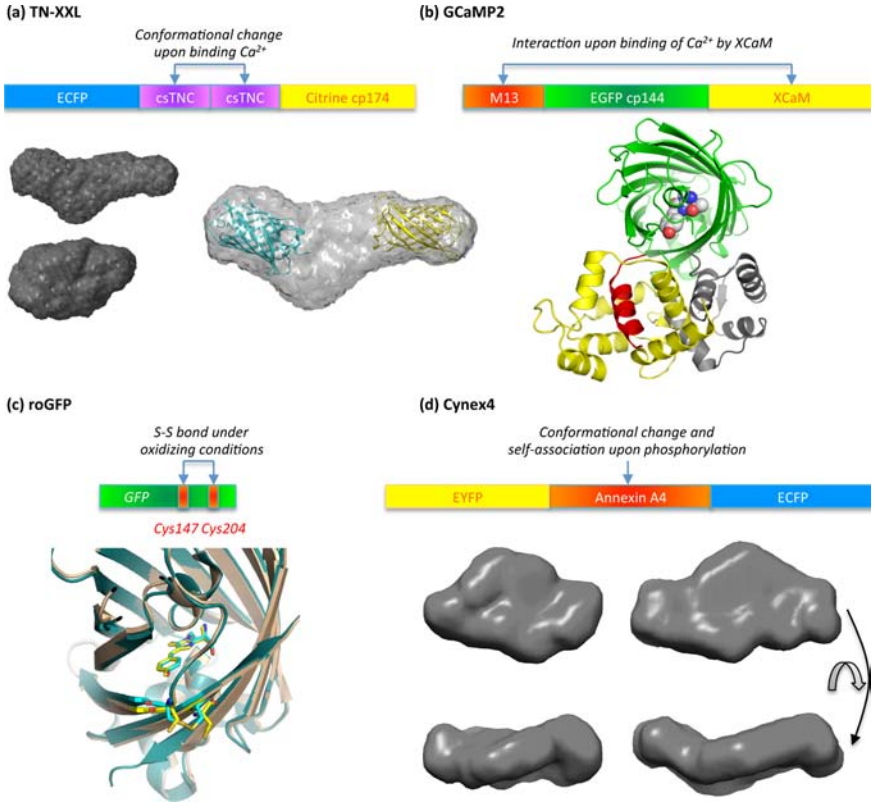

Figure 6. Structural rearrangements observed for selected biosensors using SAXS or crystallography. A schematic overview of each construct is shown using the colored bars. (a) Molecular shape of TN-XXL in the calcium-free and calcium-bound states, reconstructed from SAXS data, and a manual docking of the FP moieties in the Ca-free state. (b) Crystal structures of GCaMP2 in the calcium-bound and calcium-free state (PDB ID 3EKJ and 3EK4). The FP moiety is shown in green, M13 and CaM are shown in red and yellow in the Ca-bound state, and in gray in the Ca-free state. For added clarity the chromophore atoms are shown as spheres. (c) Crystal structures of roGFP1-R7 in a reducing (cyan/teal) and oxidizing (yellow/beige) environment (PDB ID 2AH8 and 2AHA). The chromophore and cystein 147 and 204 residues are shown using stick representation. (d) Molecular shape of Cynex4 reconstructed from SAXS data, and of a Cynex4-T226D mutant which mimics the activated sensor. Panels showing SAXS data in figures (a) and (d) reproduced from refs 227 and 138 with permission from Elsevier.

achieved using undergraduate-level molecular biology techniques, though the process is often labor intensive. In cases where the binding motif or substrate is based on a template naturally present in the organism, the excess of these motifs compared to endogenous levels can introduce distortions in the reported dynamics. A possible solution is to introduce mutations that destroy additional functionalities that are not critical for the sensing mechanism[109] or to use a binding motif that is not naturally present in the organism.[110] The modularity of this concept also allows considerable freedom in the parameter that is probed: a highly interesting class of sensors does not report on the presence or absence of certain molecules, but rather reports on the presence of a given chemical activity, such as protein phosphorylation.[111−117] By moving beyond the sensing of local concentrations, into the sensing of local reactivity, an entirely new class of problems can be addressed. Instead of asking, "what is present at this location?", we can now ask questions as to "what is happening at this location?".

In practical use, the main difference between single-FP and FRET-based sensors is that the former is usually intensiometric, transmitting signals as changes in fluorescence brightness, while the latter is usually ratiometric, meaning that signals are transmitted as a change in ratio of two quantities, traditionally the signal of the donor and that of the acceptor (though many other ways to quantify FRET exist). Ratiometric measurements typically allow more exact quantification, though the absolute contrast is often higher for single-FP based sensors.[118,119] The boundary between these types is not absolute: some single-FP sensors can be used in a ratiometric way[100,105,107,120−125] or can be exploited as a switchable acceptor in a FRET pair, resulting in FRET-based readout.[126,127] Likewise, FRET pairs can be constructed using a nonfluorescent (chromoprotein) acceptor, resulting in intensiometric readout especially suitable for fluorescence lifetime imaging (FLIM).[128,129] One potential disadvantage of the FRET-based approach is that the presence of two, differently colored FPs means that more of the visual spectrum is used by a single probe, complicating the simultaneous use of multiple probes or labels.

An important asset for the development of FP-based biosensors is the availability of structural variants or 'functionalized versions' of fluorescent proteins. For example, a highly useful type of structural modification is the creation of circular permutations, structural variants in which the original termini of the polypeptide backbone have been bridged using a peptide linker, and new termini have been created at a different position.[106,130] While a large number of circular permutations are theoretically possible, the vast majority result in non-fluorescent proteins, and successful new termini are usually, but not always, located in loop regions between β-sheet segments on the protein barrel.[106,130,131] Since the termini are in different positions, any peptide fusions will result a different geometry with respect to the chromophore, allowing the sensitivity of the

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fluorescence to conformation to be enhanced.[106] Also, the relative orientations of the donor and acceptor FP in a FRET sensor can be improved, resulting in probes with a higher contrast.[132]

In principle, the mechanisms of FP-based sensing can be extended to any event that results in conformational changes or the association/dissociation of proteins or fragments. From a protein engineering perspective, a highly appealing aspect of these sensors is the rather modular way in which they are constructed. By and large, the sensors currently available can be divided into a number of fixed template designs (Figure 5), suggesting that arbitrary sensors can be constructed by straightforward swapping of the binding domains and/or fluorescent proteins in a modular fashion. However, this appearance is somewhat misleading since the creation of these sensors is very much a trial-and-error undertaking, involving the repetitive creation of large amounts of candidates that are then screened at various levels of detail. In part this is because little direct experimental data is available on the exact structural mechanisms through which these probes operate. For example, no crystal structures are available for FRET-based probes, and only a limited number are available for probes consisting of a single FP. To some extent, a mechanistic picture can be approximated by making use of the crystal structures of the individual components, possibly combined with computational modeling,[133,134] though the large sizes of these proteins preclude rigorous and large-scale computational analysis. That said, a limited amount of direct structural information has been obtained for selected constructs and provides illustrative pictures of these mechanisms in action.

Figure 6 shows small-angle X-ray scattering (SAXS) and X-ray crystallography data acquired on two single-FP and two FRET-based probes. Compared to X-ray crystallography, SAXS is capable of working in solution, side stepping crystallization entirely, but is limited to providing information on the overall molecular shape only. To our knowledge, SAXS data are currently available for just two FRET probes, both of which we will briefly describe here. Figure 6a shows SAXS data acquired on the TN-XXL sensor for Ca^{2+},[135] which is a fairly representative FRET sensor in that it consists of the typical FP−binding domain−FP structure, where the binding domain consists of two repeats of troponin-C subunits. While TN-XXL has been carefully optimized to a very high level of optical and biochemical performance,[136] the SAXS data provide the first direct picture of the dramatic change in molecular structure that occurs when the TN-XXL sensor binds Ca^{2+}. While care must be taken in interpreting these results, the overall compaction of the structure shows that a significant change in distance between the donor and acceptor FP is an important part of the contrast mechanism. Interestingly, the 'off' conformation indicates that the FP moieties are not only spaced apart but also adopt relative orientations that are unfavorable for FRET, indicating that not only the distance between the FPs but also their relative orientation forms an essential part of the contrast mechanism. In this way the structural data directly supports the high optical performance of TN-XXL.

Figure 6c shows SAXS data for another sensor, Cynex4, which provides information on the regulation of Annexin proteins and is sensitive to Ca^{2+} and phosphorylation.[137,138] Unable to obtain phosphorylated Cynex4 in high quantities, the authors instead sought to mimic the structure of the activated sensor via the creation of an 'always on' T266D mutant.[138] Compared to TN-XXL, the data reveal much subtler changes in molecular shape, consistent with the lower FRET performance (compared to TN-XXL) associated with this construct. However, its *in vivo* structural mechanism is more difficult to extract from this picture since the contrast mechanism involves not only conformational change of the Annexin domain but also association of multiple copies of the protein.[137]

Figure 6b displays crystallography data for the 'off' and 'on' states of an intensiometric Ca^{2+} sensor, GCaMP2.[139−141] The operating mechanism of GCaMP2 relies on the fusion of M13 and calmodulin (CaM) domains to the N and C terminus of a circularly permuted EGFP. Upon binding of Ca^{2+}, CaM wraps around the M13 peptide, causing a dramatic increase in fluorescence. The crystallographic data provide an unprecedented structural insight into the mechanism of this probe: in the absence of calcium, an opening in the protein barrel allows solvent access to the chromophore, efficiently preventing fluorescence emission. Upon binding of Ca^{2+}, the CaM−M13 pair rearranges to block solvent access, restoring the protein's fluorescence ability.

Finally, Figure 6d shows crystallography data obtained on roGFP, a family of sensors for redox potential.[105,142] In roGFP, an elevated redox potential results in the formation of a disulfide bond between cysteine residues engineered into the protein. Formation of this bond causes a change in the ratio of the 400−490 nm excitation peaks (both of which will emit green fluorescence due to ESPT), allowing this probe to be used in an excitation-ratiometric way. Note the relative subtlety of the overall structural distortion in roGFP, once again highlighting the sensitivity of the fluorescence emission to the protein structure as well as the largely similar position with respect to the chromophore at which both GCaMP2 and roGFP affect the FP moiety.

In addition to shedding light on the detailed mechanisms of existing probes, the availability of detailed structural data can be used to generate improved sensors. However, in practice very little feedback is usually available on why a particular construct displays poor performance, or what steps should be taken to improve it.[143] This complexity is not just due to the inherent structural complexity of FPs but also arises from the many steps that are involved in expressing the protein, such as translation and folding, and the general complexity of the cellular environment. As a result, sensor optimization remains a laborious undertaking that often provides sensors with rather limited performance (i.e., contrast). This limited performance is not fundamental; the available Ca^{2+} sensors have been heavily optimized over the years and achieve impressive FRET or intensity contrasts when exposed to differing concentrations of Ca^{2+}.[118,119,132,133,135] However, very few other sensors have been so rigorously optimized.

What is it that makes a certain sensor perform better than others or makes certain fluorescent proteins work better in some constructs but not others? By and large, we do not know. Predicting the behavior of a given construct or gaining insight into the reasons for its failure is still an extreme challenge. Particularly in the case of FRET-based sensors, some of the performance limitations are undoubtedly due to limitations in the fluorescent proteins themselves, such as incomplete maturation.[83,144,145] In the case of single-FP sensors, much of the complexity also derives from having to walk a fine line between too much distortion of the FP structure, rendering it entirely nonfluorescent or too little, resulting in low contrast.

Gaining further insight into why some sensor constructs display limited performance, or accelerating the laborious

process of sensor creation, is one of the biggest open challenges in the field. Given the complexity of the sensors and their working environment, the way forward in addressing this question is most likely a combination of additional structural or computational characterization, combined with the design and implementation of an experimental strategy that allows the slow creation of the constructs to be parallelized using high-throughput combinatorial cloning followed by equally high-throughput screening of the created constructs. Typically the method of choice in performing high-throughput screening in FP development is to evaluate the fluorophore properties in bacteria or another fast growing and easy to maintain organism, allowing for easy selection and DNA purification. However, in the case of biosensors, this is more complicated since the relevant pathways may not be present in 'simpler' organisms, or the construct may rely on expression features (glycosylation, etc.) not present in these organisms. In some cases eukaryotic or even human cell lines will be required, while in other cases 'intermediate' model systems, such as yeast, may provide the answer. Further insight into the sensor performance will likely have to come from combining the resulting data set on what works and what does not with advanced techniques, such as computational data mining and modeling. While several important and innovative steps in this direction have been taken,[119,146−148] the field is still packed with challenges.

The structural variability and tolerance of fluorescent proteins have also resulted in several innovative schemes for interaction sensing. It turns out that some fluorescent proteins can be chopped into two fragments, each of which can be expressed independently. When the two pieces meet (for instance, because each piece is fused to fusion partners that interact with each other), a virtually intact fluorophore assembles and becomes fluorescent, a process known as biomolecular fluorescence complementation (BiFC).[149] If sufficient care is taken to take into account suppression of spontaneous assembly of the two fragments, any fluorescence that appears is a sign of the interaction of the fusion partners (Figure 5). Recent research has also revealed that it is possible to replace virtually any part of a fluorescent protein with an artificially created peptide post-translationally, indicating that this self-assembly mechanism is highly robust and can be used to introduce functionalities not achievable via traditional means.[150] Yet another way to probe interactions relies on the use of fluorescent proteins that are nonfluorescent when expressed in isolation but become fluorescent when two FPs meet and dimerize (Figure 5). Known as oligomerization-dependent fluorescence,[151] this technique is conceptually similar to BiFC but differs in that the association can be reversible, though at the current stage the obtained contrast is lower.

Photophysically Smart Fluorescent Proteins. The "photophysically smart" probes display excited-state properties that can be advantageously used in imaging and that is intrinsic to the probe in the sense that it is (or should be) largely independent of the environment. The two areas in which these properties are commonly used are dynamic labeling[152] and far-field fluorescence microscopy with a diffraction-unlimited resolution.[153−155] We will focus on diffraction-unlimited imaging in this paper.

The key to diffraction-unlimited imaging is to have a fluorophore with the light-inducible ability to not emit fluorescence even though it is experiencing a nonzero excitation intensity[154] (this is a simplified view but it will serve to understand the concepts involved). In effect, fluorescence dynamics are used to decouple emission from excitation, such that the photophysical complexities of the fluorophore are no longer avoided but rather become active partners in the imaging.[154,156]

Of course, not all fluorescence dynamics are intrinsically useful: blinking dynamics that are essentially completely stochastic cannot be controlled and are therefore of limited usefulness. One of the most exciting developments in fluorescence imaging has been the discovery of fluorescent proteins that allow light-induced control of the fluorescence emission, effectively allowing these fluorophores to act a tiny light switches. Surprisingly, a range of different ways to control the fluorescence has been discovered (Figure 5) and has become known under a variety of names. Unfortunately, this nomenclature is not uniform, and the different terms are often assigned different meanings. In this work we will use the nomenclature shown in Figure 5.

An important first class of smart FPs displays a light-induced, reversible, and thermally stable transition between a fluorescent and nonfluorescent state. In many proteins this is an 'on to off' type switching, where irradiation with the same wavelength that induces fluorescence emission also induces off-switching of the fluorescence, while irradiation with UV light leads to recovery of the fluorescence. First reported on individual molecules of an avGFP mutant,[69] the ability appears to be present in a wide range of other proteins as well,[89,157−159] though it can be masked by fast thermal recovery to the fluorescent state or confused with photobleaching. However, a few proteins stand out as having taken this behavior to the extreme, combining a very high contrast with good reversibility and high thermal stability. Examples include Dronpa,[88] mTFP0.7,[160] rsTagRFP,[161] rsEGFP,[93] or mGeos.[162] Interestingly, asFP595[163] displays opposite behavior, in that irradiation at the fluorescence excitation wavelength causes an increase in fluorescence, while UV irradiation suppresses it. Both types of behavior are possible within a single scaffold; a conversion between the two by mutagenesis has been demonstrated for Dronpa and a mutant protein, Padron.[164] A potential downside to these probes is that they form 'two-band' systems, in the sense that both fluorescence excitation and on- or off-switching are caused via irradiation at the same wavelength, making it impossible to trigger one without also triggering the other. In an example of the versatility of fluorescent proteins, a three-band photochromic FP, Dreiklang, was recently reported and offers a solution to this issue.[165]

What mechanism is responsible for the photochromism seen in these fluorescent proteins? The Dronpa photochromism is perhaps the best studied,[68,71,166−175] and two events have been put forward as responsible for the photochromism: a change in chromophore protonation, based on ensemble absorption and emission spectroscopy, and cis/trans isomerization of the chromophore, based on crystallography data, though both events are likely linked to one another.[176] In a highly interesting experiment, Mizuno et al. used NMR spectroscopy to compare the structural differences between the fluorescent and non-fluorescent state and found that a major part of the protein and the chromophore are fairly rigid in the fluorescent state but experience large structural flexibility upon off-switching (Figure 8).[68,177] This suggests that, with some probability upon absorbing an excitation photon, the chromophore 'breaks free' from its tethers in the environment, and as a result part of the β barrel becomes flexible in turn. The involvement of major

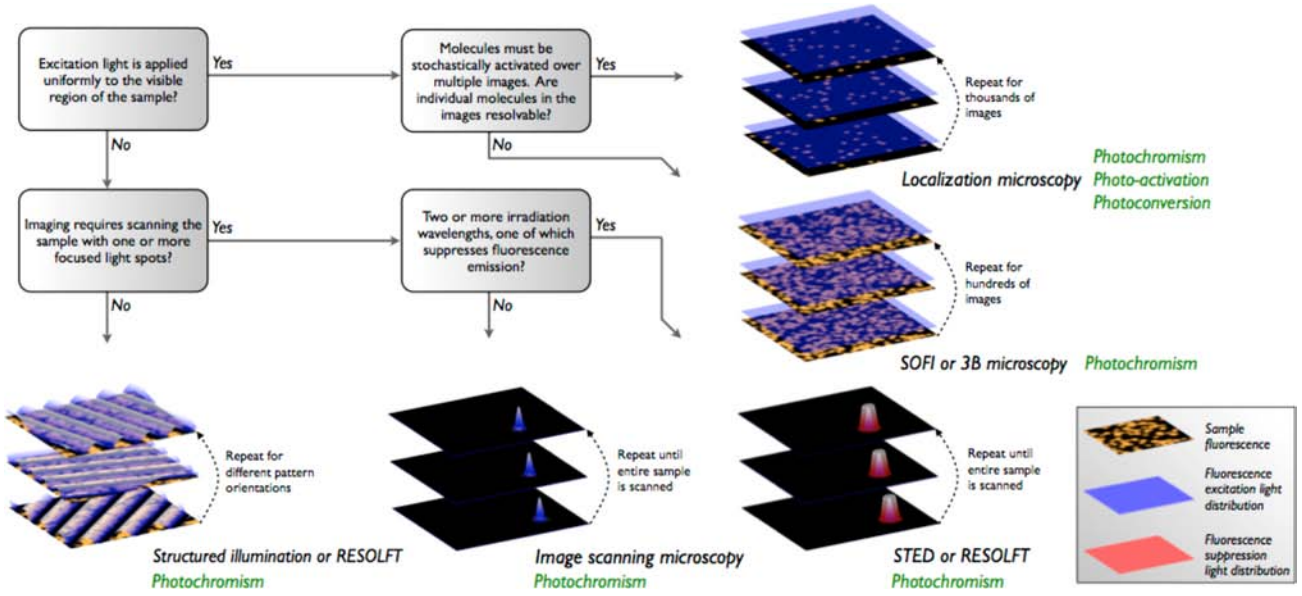

Figure 7. Simplified overview of different types of diffraction-unlimited fluorescence imaging. The flowchart, starting from the top left, serves to identify major differentiators between the techniques. The images show a selection of the consecutive steps involved in the image acquisition. The green text indicates the type of smart FP that can be used for each technique, though these are not exclusive. References for and a short explanation of each of these techniques are given in the main text.

parts of the protein suggests that the comparative lack of well-performing photochromic proteins, but surprising pervasiveness of short-lived photochromism in many fluorescent proteins, may not be due only to the immediate environment of the chromophore but can also have its origins in the ability of the chromophore interaction partners to find sufficiently stable alternative configurations by rewiring the interaction network of other parts of the protein.

In contrast to this are the fluorescent proteins that display irreversible light-induced changes in fluorescence. In general these divide into two types: FPs that display 'off' to 'on' conversion, and FPs that convert from shorter to longer wavelength emission (typically green to red). In all cases known thus far, this transition requires irradiation with UV to blue light. Examples of 'off' to 'on' conversion include PA-GFP,[178] PS-CFP[179] and PS-CFP2, and PAmCherry.[180] Example proteins that undergo color conversion include Kaede,[181] EosFP[182] and its monomeric and tandem variants, Dendra2,[183] mKikGR,[78] and PSmOrange.[184,185] However, in some applications, such as localization microscopy (discussed below) or the counting of individual fluorophores based on activation, care must be taken to take into account long-lived blinking events occurring in the converted fluorescent state,[186] that could otherwise be confused with the activation of new emitters. It would be highly interesting to have quantitative estimates of the fraction of fluorophores that reach, e.g., the green fluorescent state, and that convert from this state to the red state, since absorption spectra recorded on purified FPs irradiated with UV often reveal significant absorption bands associated with the green chromophore even after prolonged irradiation, suggesting that many of the fluorophores never reach the red state.

While it is known that irradiation with UV light affects the maturation of DsRed-like chromophores,[38] the defining elements of these FPs is that maturation is conditional upon irradiation with light. These probes are thought to divide in two classes based on the mechanism responsible for this chromophore formation. The light-induced maturation of PA-GFP, PS-CFP, and PAmCherry is thought to proceed via oxidative decarboxylation of Glu222 (using avGFP numbering), shifting the protonation equilibrium of the chromophore to the fluorescent anionic state.[179,180,187] The second group, known as Kaede-like fluorescent proteins, contain a histidine residue as the first residue of the chromophore tripeptide. In these proteins the conversion process starts from the protonated form of the green chromophore[188] and results in the formation of the red form via cleavage of the peptide backbone (Figure 2),[189,190] for which a number of different mechanisms have been proposed.[189,191−193]

Interestingly, fluorescent proteins that combine different behaviors into a single fluorophore have also been discovered,[194,195] demonstrating that these different types of fluorescence dynamics need not be exclusive.

How are these properties used to provide a superior spatial resolution? Figure 7 provides a simplified overview of some of the different diffraction-unlimited techniques that make use of these smart fluorophores. Broadly speaking, these techniques divide into two approaches. The first approach combines spatial patterning of the irradiation light with the fluorophore photophysics or photochemistry to create deterministic spatial distributions that are sharper than diffraction allows (this is once again a somewhat simplified view). Examples of this approach include stimulated emission depletion microscopy (STED),[196,197] reversible, optically linear fluorescence transitions microscopy (RESOLFT),[197−199] nonlinear structured illumination (NSIM),[200,201] and nonlinear image scanning microscopy (ISM).[202] In STED or RESOLFT imaging, the patterned light is used to suppress the fluorescence emission using, e.g., stimulated emission (STED) or photochromism (RESOLFT), such that emission is confined to regions with spatial dimensions that are smaller than the diffraction limit.[203] The irradiation patterns can be carefully tuned through appropriate optical engineering, but it is typical to use a "donut mode"[204] if the sample is scanned point-by-point or a

sinusoidal pattern if an entire region of the sample is imaged simultaneously[205] (Figure 7). While the patterns themselves are diffraction-limited, a theoretically unlimited resolution is possible because the fluorescence suppression is a saturable process.[197]

Remarkably, the 'inverse' of this process, where spatially patterned irradiation is used not to suppress but to excite fluorescence, can be exploited to achieve a theoretically unlimited resolution by relying on saturation of fluorescence emission or on photochromism. This is the approach taken by NSIM and nonlinear ISM, depending on whether the sample is scanned in a point-by-point fashion, or an entire field-of-view is acquired simultaneously. However, a consequence of these techniques is that the data must be processed using a computer before images can be constructed.

The second approach uses the fluorescence dynamics of the sample so that multiple images can be acquired from the same (stationary) sample, with each image offering a different and therefore complementary view on the sample. A straightforward way to achieve such imaging is to make use of reversible photochromism, since individual molecules activate stochastically throughout the measurement and therefore different combinations of fluorophores are active in any given image. These complementary images can then be processed to obtain a single image containing super-resolution information. If the imaging conditions allow the emission spots of individual molecules to be clearly resolved, then very high imaging resolutions can be achieved by fitting these spots to a model of the point-spread function to determine the location of the emitters. These powerful techniques are known as photoactivation localization (PALM) microscopy[206,207] or stochastic optical reconstruction microscopy (STORM).[208] If resolving individual emitters is not possible due to low signal-to-noise or high numbers of active molecules or not desirable (e.g., when faster imaging is needed), (photochromic) stochastic fluctuation imaging (SOFI) or Bayesian localization microscopy (3B microscopy) can be used instead, though at a lower spatial resolution.[209−211] However, all of these techniques require that the data are processed using a computer before meaningful images can be generated.

The precise control over the fluorescence emission afforded by photochromism can also be used to enhance the imaging in other ways. For example, periodic modulation of the intensity of the on- or off-switching illumination can be used to achieve higher signal-to-background levels by using lock-in amplification[212] or to obtain higher FRET imaging accuracies using photochromic FRET.[161,213,214]

While only a comparatively limited range of fluorescent proteins displaying these behaviors with good performance are known, they are at the state of the art in imaging. Given the requirement for these smart labels as active partners in the imaging[154] and the very high degree to which their photophysics determine the obtainable performance,[92,93,170,199,210] one of the biggest questions in the field is to what extent these behaviors can be rationally tuned to changing experimental requirements. We anticipate that in advanced (e.g., diffraction-unlimited) imaging, changing the properties of the fluorophore will one day be as desirable as changing, say, the power of the excitation laser or the exposure time of the camera. Is it possible to rationally create photophysically smart fluorophores with tailored excited-state properties? Several developments have hinted that the answer to this question will ultimately turn out to be 'yes',[195,215,216] though insights gained on one protein have often been difficult to generalize to other fluorescent proteins. For now, (semi-) random mutagenesis followed by high-throughput screening continues to be the technique of choice.[92,93,161,180,217−220] A realization of this challenging prospect will open new perspectives in advanced fluorescence imaging.

Fluorescent Proteins As Chemical Actors. Research efforts have also focused on mapping the intrinsic chemical reactivity of 'native' fluorescent proteins and/or the extent to which these labels are capable of modifying their environment. A prime example of FP chemistry is the maturation of the chromophore itself. Usually the main aim in optimizing FPs is to accelerate this process as much as possible, but an interesting class of fluorescent proteins are the so-called fluorescent timers,[221,222] which mature from a blue or green emitting state to a red emitting state over the course of a few hours. Since the kinetics of this conversion can be determined *in vitro* and verified *in vivo*, the ratio of the two emission bands provides an estimate of how long ago the labels were expressed. Additional control over the lifespan of these proteins can potentially be achieved by attaching a degradation motif.[223]

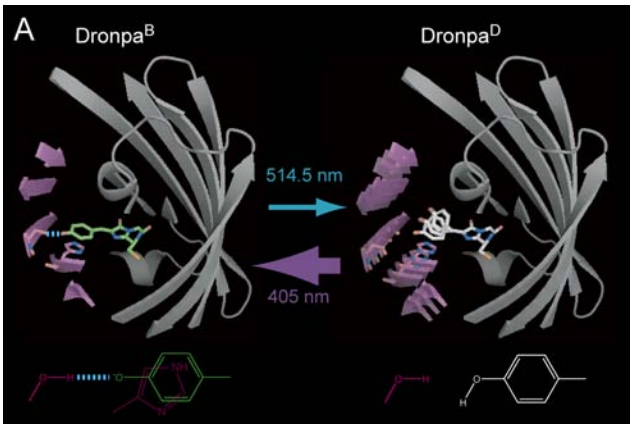

Figure 8. NMR measurements on the Dronpa photoswitching: In the fluorescent state (left), the chromophore (green) and adjacent β strands (magenta) are structurally inflexible, showing up as well-defined resonances in the NMR spectrum. Upon switching to the nonfluorescent state, the mutual stabilization is lost, and NMR resonances disappear due to peak broadening, indicating high structural flexibility. The bottom panels show a schematic overview of the chromophore stabilization via a hydrogen bond to Ser142 and stacking interactions with His193. Reproduced with permission from ref 68. Copyright 2008 National Academy of Sciences, U.S.A.

Another application of FPs has centered on the production of reactive oxygen species (ROS) that occurs upon repeated excitation of the chromophore, though not to the same degree in all fluorescent proteins.[224] By virtue of their highly reactive nature, these species induce cellular damage or even cell death when produced in sufficiently high quantities. Special 'killer' FPs with optimized ROS production are now available,[225] though the quantum yield of ROS production is still well below that of some organic dyes.

Finally we also note the particularly surprising property that some green fluorescent proteins can act as light-induced electron donors in the presence of suitable oxidants,[18] including biologically relevant compounds such as FAD or NAD^+. Upon oxidation, the GFP permanently converts to a dimly red fluorescent state, by which this process has become known as

'oxidative reddening'. Interestingly, this process was shown to be one of the main contributors to GFP photobleaching, and removing oxidants from the medium was shown to be effective in enhancing the photostability.[87] Whether this process plays a relevant part in the biological function of FPs is not clear at present.[226] Still, this finding does make one wonder to what extent this functionality could be used in practice and whether other functionality could be embedded by building on this mechanism.

CONCLUSIONS AND OUTLOOK

Why did we choose to refer to fluorescent proteins as 'crazy diamonds' in the title of this work? For starters, these proteins truly are tiny treasures if one considers the extraordinary knowledge and capability that has become accessible through their discovery. But their dynamics and properties are also mindboggling in their own rights. Frankly, we think it is fascinating, exciting, and awe-inspiring to consider just what range of functionality can be packed into proteins that all end up looking pretty much the same in the crystal structures (Figure 1). Clearly, two decades ago one would have been hard pressed to imagine the range of behaviors and functionalities available in the fluorescent proteins of today. And the success of these proteins does not stand in isolation, since they have turned out to be enablers for a wide variety of techniques, such as diffraction-unlimited fluorescence microscopy.

These developments also mark one of the major success stories in protein engineering. Starting from an initially limited number of templates, the FP family has expanded to include a rainbow of colors and a wide range of photophysical behaviors as well as becoming integral scaffolding elements for biosensors. Apart from the discovery of new template proteins in nature, the tool of choice in these achievements has been, and still is, (semi-) random mutagenesis. This random approach is considerably facilitated by the ease with which the fenotype of each construct can be determined simply by looking at the color and fluorescence brightness associated with, e.g., a bacterial colony. However, it is now firmly recognized that the FP behavior can change dramatically depending on the environment, and one of the main challenges in continuing this work will be to come up with screening metrics that are more tailored to typical applications.

The continued reliance on randomization in FP development also shows that a clear understanding of the structure−function relationship of fluorescent proteins remains elusive. This should come as no surprise given the complex nature of the protein, though major strides have been and continue to be made. However, the ultimate goal of rationally predicting the photophysical and photochemical behaviors of arbitrary FPs is still firmly out of reach. The realization of such a model would not only be important for the FP field itself but also would be applicable to a range of much broader fields, such as the fundamental study of protein dynamics and chemistry (folding, maturation, etc.). Fluorescent proteins are probably some of the best model systems in this regard due to the tight coupling between fluorescence and protein structure and the ease with which this parameter can be observed. These developments are also highly relevant to fields that do not deal with proteins exclusively, such as photophysics and photochemistry. By themselves the chromophores contained in the proteins are not very complex, but rather derive their fascinating properties from the tight interactions with the well-defined structure of the protein as a whole. Fluorescent proteins present some of the most well-defined and easily modifiable systems known to date for the study of light-induced processes in thoroughly anisotropic and dynamic environments.

Meanwhile the subfields of FP-based sensor development continue to expand rapidly, allowing complex questions to be addressed *in vivo* with unprecedented power. By allowing the sensing of nonprotein targets and of chemical reactivities, these approaches enable complementary assays to 'standard' FP labeling and allow drastically more of the picture of life to be revealed. And all of this has been done in just two decades. It has been an amazing ride, and we are no where near the finish line.

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Notes

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