Chemistry & Biology
Crosstalk

Fluorescent Revelations

Carsten Schultz[1,*]
[1]European Molecular Biology Laboratory, Cell Biology and Biophysical Instrumentation Unit, and Biophysics Unit, Meyerhofstr. 1, 69117 Heidelberg, Germany
*Correspondence: schultz@embl.de
DOI 10.1016/j.chembiol.2009.02.001

A fluorescent protein from jellyfish changed the way life science research is performed today. Its discovery, the first expression in an animal, the determination of its structure, the details of the mechanism behind the fluorescence, and diversification of the fluorescent properties has made green fluorescent protein a unique tool in the biological sciences, and the scientists that made key contributions to these developments were awarded the 2008 Nobel Prize in Chemistry.

Introduction

The 2008 Nobel Prize in Chemistry was awarded to three scientists for work that represents a story of discovery, innovation through engineering, and the application of a true gift that nature has provided to science. The award was given for the discovery and isolation of the green fluorescent protein (GFP) from *Aequorea aequorea* (now commonly called *Aequorea victoria*) to the pharmacist and chemist Osamu Shimomura; for the transfer of the GFP gene into the genome of another animal and demonstration of its use as a tag to visualize biological processes to the biologist Martin Chalfie; and for elucidating the chemical mechanism of fluorophore formation, which made the engineering of GFP into a set of essential GFP variants possible, to the chemist and physicist Roger Y. Tsien.

GFP: From The Sea to the Bench

In the early 1960s, seeking to explain the source and molecular mechanism of bioluminescence in the aquatic world, Shimomura focused on a species of jellyfish, *Aequorea victoria,* which produced green bioluminescence from its photogenic cells. Initially, Shimomura and his coworker isolated a blue light-emitting protein they named *aequorin*, which was found to emit blue light in a Ca^{2+}-dependent manner. Subsequently, they discovered that the typical green fluorescence of the jellyfish was generated by another protein and suggested that energy transfer between aequorin to this other protein leads to green light emission. While only a footnote in the original article on aequorin (Shimomura et al., 1962), the source of the green fluorescence, GFP, was quickly isolated, and some of its physicochemical properties were characterized (Johnson et al., 1962; Shimomura and Johnson, 1969). Later, the size of the protein and the basic structure of the fluorophore, a *p*-hydroxybenzylidene-imidazoline (Figure 1A) responsible for the green emission, was determined (Shimomura, 1979).

It took almost two decades to finally resolve the mystery of this unusual fluorophore formation. Initial steps toward untangling the enigma of GFP and *p*-hydroxybenzylidene-imidazoline formation were made by Douglas Prasher during his postdoctoral time at the University of North Carolina and later in Woods Hole Oceanographic Institution in the 1980s. Prasher sequenced the GFP gene from *Aequorea aequorea* and cloned it into bacteria for the first time (Prasher et al., 1992). However, unable to achieve GFP fluorescence in bacteria, Prasher's funding ran out, and he left Woods Hole. Perhaps this could have been the end of the GFP story, had it not been for the willingness of Prasher to share GFP clones and Chalfie's and Tsien's interest in pursuing further GFP studies.

Marty Chalfie and coworkers were more successful in producing a fluorescent product in bacteria as well as in entire worms (*Caenorhabditis elegans*). Additionally, they demonstrated that the formation of the GFP fluorophore did not require any special cofactors or other proteins (Chalfie et al., 1994) and suggested that it is most likely formed through an autocatalytic process initiated by blue light or through engagement of cellular components present across species. Importantly, GFP could not only be expressed in any organism, but by cloning it as a fusion to a protein of interest, researchers were suddenly able to track expression and localization of tagged proteins inside a living cell or an entire organism without the need to use any exogenous factors or manipulation (Wang and Hazelrigg, 1994). Chalfie's results thus made it evident that GFP could be used as a genetically encoded tag for monitoring complex dynamic cellular processes.

GFP through a Mechanistic Lens

While Shimomura continued to focus on bioluminescent proteins and Chalfie was most interested in the developmental biology of *C. elegans*, the third laureate, Roger Tsien, was interested in better understanding the molecular mechanism of GFP fluorescence and extending the range of GFP variants to be applied to study of biological function.

In the early 1990s, Shimomura's structure of the fluorofore was confirmed and the precursor for *p*-hydroxybenzylidene-imidazoline formation was identified to correspond to GFP residues Ser^{65}-Tyr^{66}-Gly^{67} (Cody et al., 1993). Next, Roger Heim in Roger Tsien's laboratory found that residues 65-67 were sufficient to spontaneously form the fluorophore in the presence of oxygen and proposed a mechanism for fluorophore maturation based on the nucleophilic attack of the Gly^{67} amido group on the Ser^{65} carbonyl group, resulting in the formation of a cyclic intermediate that subsequently oxidized at the Tyr66 moiety in the presence of oxygen to form the conjugated fluorophore (Figure 1B.) (Heim et al., 1994).

A direct result from the successful expression of GFP in bacteria was the ability to produce sufficient amounts of the purified protein for X-ray structure analysis (Ormo et al., 1996; Yang et al., 1996). The GFP structure provided additional clues to further explain the

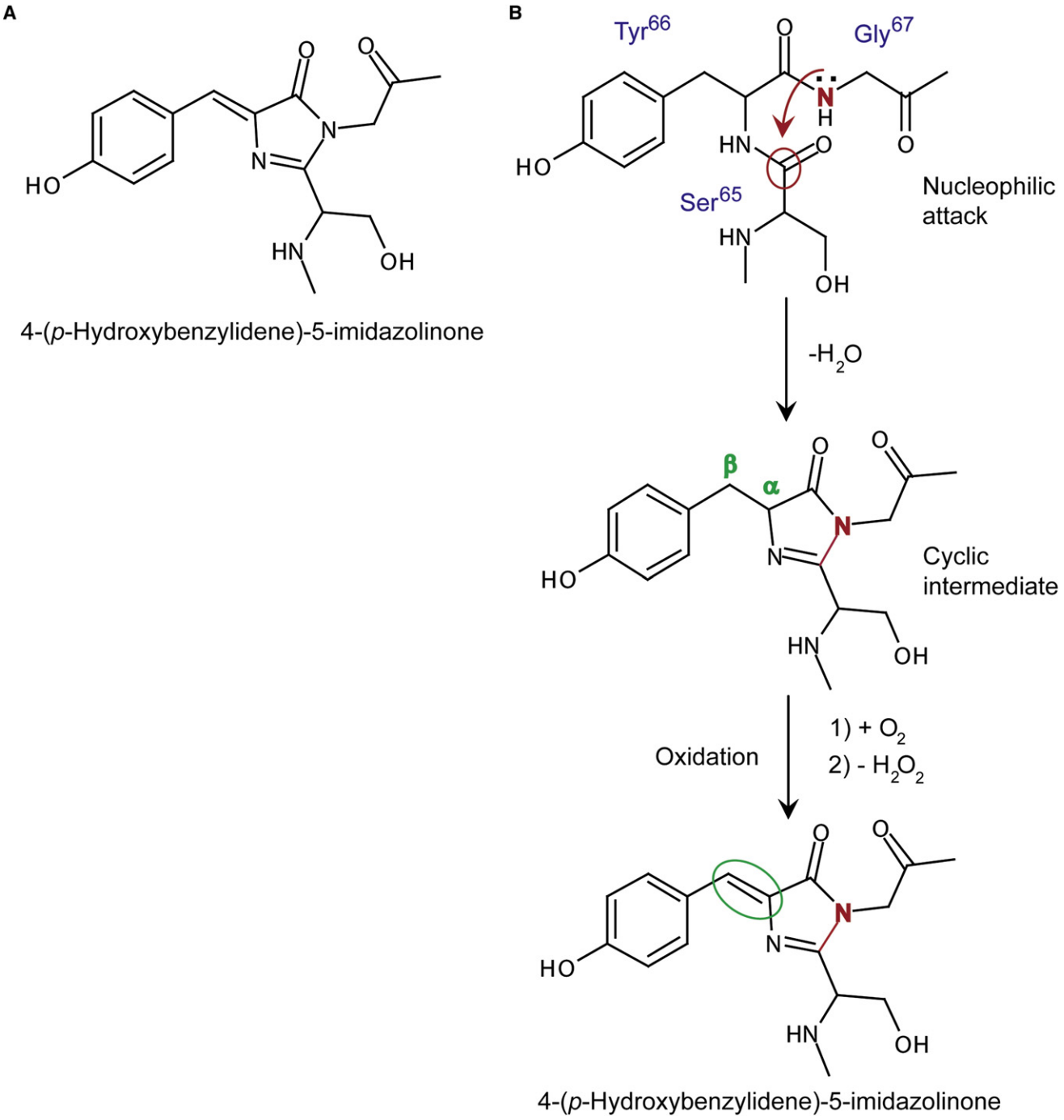

Figure 1. GFP Chromophore and its Mechanism of Formation
(A) The chromophore of GFP is formed by the amino acids Gly65, Tyr66, and Ser67.
(B) Maturation of the chromophore involves an intramolecular cyclization followed by oxidation of the tyrosine side chain by oxygen. The latter is the only cofactor required for the chromophore synthesis, an essential feature for the general applicability of GFP.

remarkable intramolecular transformation that led to the formation of *p*-hydroxybenzylidene-imidazoline. GFP folded into an 11-stranded β-barrel, with the fluorophore located in its center (Figure 2), where it is fully protected from the bulk solvent. Elucidation of the precise structure rapidly enabled the development of mutants with different properties, the basis of today's fluorescent protein toolbox.

Discovering the Fluorescent Protein Rainbow

In addition to being mechanistically intriguing, GFP provided a tool to tackle many of the scientific problems Tsien had encountered in

his previous research. Tsien, who was already renowned for the preparation and application of an extended series of ratiometric ion-sensitive indicators and a number of sophisticated reporter molecules based on fluorescence resonance energy transfer (FRET), often faced a reoccurring obstacle: the probes were not membrane-permeant. Although this could be solved by prodrug approaches for small molecules, protein- or peptide-based reporters needed to be applied by invasive techniques such as microinjection (Adams et al., 1991), potentially jeopardizing the experimental outcome. Thus, use of GFP could overcome these problems, because GFP-fusion proteins were made by the cell or organism of interest itself. For Tsien, real-time imaging experiments were his bread and butter, because he had revolutionized cellular imaging with the invention of small molecule fluorescent ion indicators, such as the calcium chelator Fura-2 (Grynkiewicz et al., 1985).

In order to generate genetically encoded FRET probes suitable for ratiometric measurements, it was necessary to employ more than one fluorophore. This and the ability to track several proteins simultaneously in living cells were crucial for the future application of fluorescent proteins. Therefore, Tsien generated variants of fluorescent proteins by random mutagenesis of GFP. The S65T variant was one of the first mutants to be described. It maintained green fluorescence but exhibited overall improved spectral properties (Heim et al., 1995). The first successful new proteins, such as blue fluorescent protein and cyan fluorescent protein, were emitting at shorter wavelengths (Heim et al., 1994; Heim and Tsien, 1996). After the crystal structure became available in 1996, red-shifted proteins such as yellow fluorescent protein followed (Heim and Tsien, 1996). In combination with the many variants based on fluorescent Anthozoa proteins, pioneered by the group of Konstantin and Sergej Lukyanov in Moscow, the entire visible spectra is now covered (Matz et al., 2002; Shaner et al., 2004; Shaner et al., 2008). Systematic engineering, merging features from Anthazoa and *Aequorea* proteins and tricks such as circular permutation provided a large number of fluorescent proteins with an array of colors including the "fruit" series (mCherry, mHoneydew, mBanana, mOrange, mStrawberry, mTangarine described in Shaner et al., 2004), improved brightness, better folding at 37°C, photostability, and environmental insensitivity toward pH and anions (Shaner et al., 2005). Through constant improvement by many groups worldwide, often by introducing dozens of mutations, the best fluorescent protein variants available to researchers today have better physical and photochemical properties than most small molecule fluorescent probes. Finally, by using two genetically encoded fluorophores in one reporter construct, Tsien's laboratory pioneered the use of GFP-based FRET probes to measure intracellular calcium (Miyawaki et al., 1999) or phosphorylation levels (Zhang et al., 2001). The wealth of innovations introduced by Tsien undoubtedly helped to elevate GFP to its current popularity.

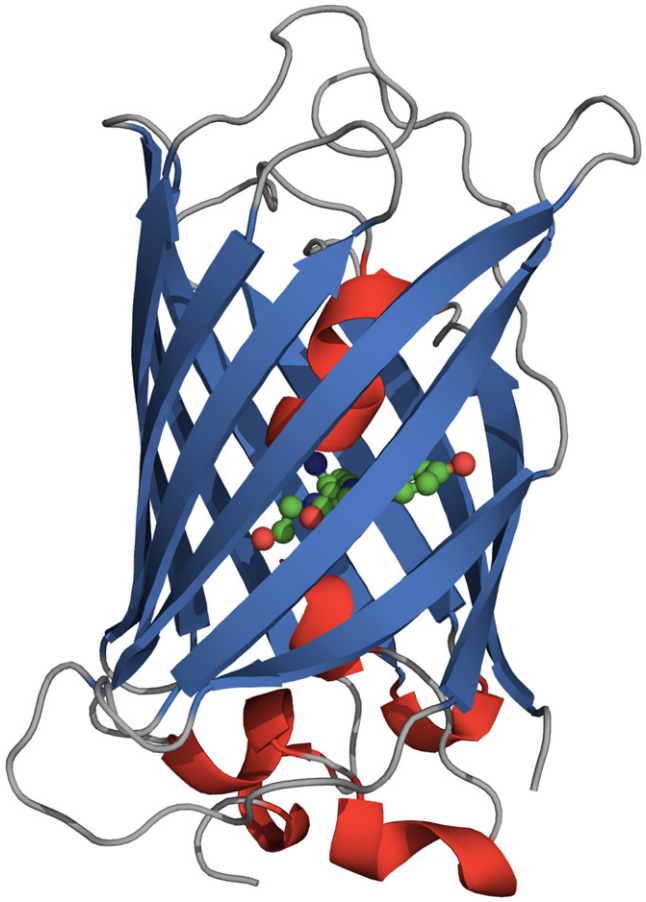

Figure 2. The 27-kDa β-Barrel of GFP Shields the Chromophore from the Bulk of Water Molecules
The image was prepared using Pymol (http://www.pymol.org).

GFP Tricks of the Trade

The beauty of working with GFP is its simple application and, in most cases, the lack of interference with the properties of the molecule to which it is fused. Even small peptides fused to GFP will still bind to an interacting protein in living cells (Piljic and Schultz, 2008). In fact, the protein's tendency to dimerize with neighboring fluorescent proteins is often considered the largest caveat (Zacharias, 2002). Therefore, intrinsically monomeric versions of almost all fluorescent proteins are now known. The general use of GFP also stems from the fact that it allows both the observation of a relatively small number of fusion proteins in cells and, at the same time, the expression of millions of copies per cell without negative impact on cell viability (Chalfie et al., 1994). High expression is necessary to follow cells in living organisms, especially when the organisms are larger and not perfectly transparent. Initially, GFP was used to track protein localization and monitor gene expression by producing GFP fused to the protein of interest or by placing GFP under the control of a promoter of interest. However, while expression of proteins at the level of cell batches can be monitored via western blotting, translocation events in culture dishes or single cells require a method with spatial resolution. Only microscopy is able to provide these data, and GFP has made real-time imaging in living cells a common technique.

Fluorescent proteins are not solely inert tags enabling us to visualize molecules and follow their movements. Engineering of GFP based on structural information has made them photo-switchable (Chudakov et al., 2003; Gurskaya et al., 2006; Lukyanov et al., 2005; Patterson and Lippincott-Schwartz, 2002). This means that

upon illumination with a certain wavelength, the fluorescence of the protein will either appear or switch colors. These features are remarkably useful for monitoring the complex timing of cellular events—for example, when protein mobility in living cells needs to be monitored (Gurskaya et al., 2006). When earlier expressed proteins need to be distinguished from more recently synthesized ones, so-called "fluorescent timer proteins" are employed (Subach et al., 2009). An example of the power of fluorescent proteins as a genetically encoded photosensitizer is Killer red (Bulina et al., 2006), a red fluorescent protein that produces reactive oxygen species (ROS) when excited by light. In general, fluorophores often produce ROS, and fluorescent proteins are no exception in this respect. Though normally without impact on cell viability, in some cases the amount of ROS produced by a fluorescent protein can be so high that molecules in the immediate environment can be structurally and/or functionally damaged. Of course, prolonged ROS production would be cell-toxic.

For studying protein-protein interactions by a method called bimolecular fluorescence complementation (BiFC), GFP was cut in half (Kerppola, 2006). Both halves are nonfluorescent, sufficiently stable, and maintain enough of the three-dimensional structure to permit their fusion in solution thereby generating the fluorescent GFP. When two interacting proteins are labeled, each with one of the halves, their physical interaction permits the formation of the fluorescent protein and is hence immediately trackable.

The applications of the various fluorescent proteins summarized above focused on the investigation of proteins and their function. For investigation of biologically relevant small molecules, fluorescent derivatives are either difficult to bring into cells or, if passive cell entry is possible, fluorophores often obscure the function of the molecule of interest. For instance, lipids will mostly behave according to the fluorophore attached instead of following its headgroup or fatty acid composition (Neef & Schultz, 2009). GFP and its relatives help with this dilemma enormously. By labeling a lipid binding domain with GFP, the recognition of a lipid in a particular membrane is possible allowing estimates regarding lipid distribution and enzymatic turnover in the natural environment (Teruel and Meyer, 2000; van der Wal et al., 2001). This concept is not limited to lipids, although their distinct location often permits good spatial separation.

For many small molecules and ions inside or outside cells GFP-based reporter molecules have been prepared including sugars, amino acids, and xenobiotics (Fehr et al., 2005). Many of these reporter molecules are based on FRET. The fluorescent protein of choice is usually connected to a sensor unit reporting the abundance of analyte and a second chromo- or fluorophore, most often a second fluorescent protein with suitable spectral properties. One of the most common FRET pairs is cyan fluorescent protein and yellow fluorescent protein, but others such as mOrange and mCherry or GFP and red fluorescent protein have also been used (van der Krogt et al., 2008). Other GFP-based FRET reporter constructs monitor intracellular ion levels or enzyme activities such as phosphorylation and dephosphorylation in cells. One of the earliest of the latter kind, for protein kinase A phosphorylation, was also prepared by the Tsien lab (Zhang et al., 2001).

Bright Outlook for the Future

With translocation probes and FRET reporters, we are now able to look at dynamic processes and to estimate mass changes of biologically important compounds in living cells and sometimes in living organisms. These experiments will lay the groundwork for systems biology and the challenge of mathematically modeling an intact cell. In order to accomplish this in a way that will enable us to make biochemical predictions from these models, they need to be thoroughly validated. This requires detailed quantitative analysis of molecule numbers and enzymatic activity levels with good spatial and temporal resolution. The above-mentioned reporters will be instrumental for providing the required data through combination with quantitative microscopy. To date, very few of these challenging studies have been performed. One option for counting molecules requires the formation of a reference particle—for example, the expression of a viral capsid with a precise number of protein copies per capsid delivering a particle with a precise number of fluorescent proteins attached (Rabut et al., 2004). Another option is measuring single molecules in a defined subcellular volume of the cell by fluorescence correlation spectroscopy. Again, fluorescent proteins will be indispensible for following protein diffusion and molecular interactions.

In the future, the search for brighter, more photostable fluorescent proteins will continue. Fluorescent proteins from other organisms will be added and the incorporation of artificial amino acids will provide new means of engineering the optical properties in a more focused way. For in vivo applications, the generation of genetically encoded dyes in the near infrared part of the spectrum will be of particular importance. On the application side, we need to learn much more about the behavior of fluorescent proteins in certain environments. For instance, the outcome of preparing genetically encoded FRET sensors with two fluorescent proteins is still a lottery. Only when we follow the conformational changes that lead to the structural change in fluorophore orientation and/or distance (e.g., by NMR) will we be able to design ratiometric fluorescent probes with adapted sensitivities. Even to date, we can state that bringing GFP from the jellyfish to laboratory benches worldwide has changed the way we perform experiments and look at events in living cells and organisms. For that, we say: "Thank you!"

REFERENCES

Adams, S.R., Harootunian, A.T., Buechler, Y.J., Taylor, S.S., and Tsien, R.Y. (1991). Fluorescence ratio imaging of cyclic-AMP in single cells. Nature *349*, 694–697.

Bulina, M.E., Chudakov, D.M., Britanova, O.V., Yanushevich, Y.G., Staroverov, D.B., Chepurnykh, T.V., Merzlyak, E.M., Shkrob, M.A., Lukyanov, S., and Lukyanov, K.A. (2006). A genetically encoded photosensitizer. Nat. Biotechnol. *24*, 95–99.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. (1994). Green fluorescent protein as a marker for gene expression. Science *263*, 802–805.

Chudakov, D.M., Belousov, V.V., Zaraisky, A.G., Novoselov, V.V., Staroverov, D.B., Zorov, D.B., Lukyanov, S., and Lukyanov, K.A. (2003). Kindling fluorescent proteins for precise in vivo photolabeling. Nat. Biotechnol. *21*, 191–194.

Cody, C.W., Prasher, D.C., Westler, W.M., Prendergast, F.G., and Ward, W.W. (1993). Chemical structure of the hexapeptide chromophore of the *Aequorea* green-fluorescent protein. Biochemistry *32*, 1212–1218.

Fehr, M., Okumoto, S., Deuschle, K., Lager, I., Looger, L.L., Persson, J., Kozhukh, L., Lalonde,

S., and Frommer, W.B. (2005). Development and use of fluorescent nanosensors for metabolite imaging in living cells. Biochem. Soc. Trans. *33*, 287–290.

Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985). A new generation of Ca2+ indicators with greatly improved fluorescence properties. J. Biol. Chem. *260*, 3440–3450.

Gurskaya, N.G., Verkhusha, V.V., Shcheglov, A.S., Staroverov, D.B., Chepurnykh, T.V., Fradkov, A.F., Lukyanov, S., and Lukyanov, K.A. (2006). Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light. Nat. Biotechnol. *24*, 461–465.

Heim, R., and Tsien, R.Y. (1996). Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. Curr. Biol. *6*, 178–182.

Heim, R., Prasher, D.C., and Tsien, R.Y. (1994). Wavelength mutations and posttranslational autoxidation of green fluorescent protein. Proc. Natl. Acad. Sci. USA *91*, 12501–12504.

Heim, R., Cubitt, A.B., and Tsien, R.Y. (1995). Improved green fluorescence. Nature *373*, 663–664.

Johnson, F.H., Shimomura, O., Saiga, Y., Gershman, L.C., Reynolds, G.T., and Waters, J.R. (1962). Quantum efficiency of *Cypridina* luminescence, with a note on that of *Aequorea*. J. Cell. Comp. Physiol. *60*, 85–103.

Kerppola, T.K. (2006). Visualization of molecular interactions by fluorescence complementation. Nat. Rev. Mol. Cell Biol. *7*, 449–456.

Lukyanov, K.A., Chudakov, D.M., Lukyanov, S., and Verkhusha, V.V. (2005). Photoactivatable fluorescent proteins. Nat. Rev. Mol. Cell Biol. *6*, 885–891.

Matz, M.V., Lukyanov, K.A., and Lukyanov, S.A. (2002). Family of the green fluorescent protein: journey to the end of the rainbow. Bioessays *24*, 953–959.

Miyawaki, A., Griesbeck, O., Heim, R., and Tsien, R.Y. (1999). Dynamic and quantitative Ca^{2+} measurements using improved cameleons. Proc. Natl. Acad. Sci. USA *96*, 2135–2140.

Neef, A.B., and Schultz, C. (2009). Selective fluorescent labeling of lipids in living cells. Angew. Chem. Int. Ed. Engl. *48*, 1498–1500.

Ormo, M., Cubitt, A.B., Kallio, K., Gross, L.A., Tsien, R.Y., and Remington, S.J. (1996). Crystal structure of the *Aequorea victoria* green fluorescent protein. Science *273*, 1392–1395.

Patterson, G.H., and Lippincott-Schwartz, J. (2002). A photoactivatable GFP for selective photolabeling of proteins and cells. Science *297*, 1873–1877.

Piljic, A., and Schultz, C. (2008). Analysis of protein complex hierarchy in living cells. ACS Chem. Biol. *3*, 749–755.

Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., and Cormier, M.J. (1992). Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene *111*, 229–233.

Rabut, G., Doye, V., and Ellenberg, J. (2004). Mapping the dynamic organization of the nuclear pore complex inside single living cells. Nat. Cell Biol. *6*, 1114–1127.

Shaner, N.C., Campbell, R.E., Steinbach, P.A., Giepmans, B.N., Palmer, A.E., and Tsien, R.Y. (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma sp*. red fluorescent protein. Nat. Biotechnol. *22*, 1567–1572.

Shaner, N.C., Steinbach, P.A., and Tsien, R.Y. (2005). A guide to choosing fluorescent proteins. Nat. Methods *2*, 905–909.

Shaner, N.C., Lin, M.Z., McKeown, M.R., Steinbach, P.A., Hazelwood, K.L., Davidson, M.W., and Tsien, R.Y. (2008). Improving the photostability of bright monomeric orange and red fluorescent proteins. Nat. Methods *5*, 545–551.

Shimomura, O. (1979). Structure of the chromophore of *Aequorea* green fluorescent protein. FEBS Lett. *104*, 220–222.

Shimomura, O., and Johnson, F.H. (1969). Properties of the bioluminescent protein Aequorin. Biochemistry *8*, 3991–3997.

Shimomura, O., Johnson, F.H., and Saiga, Y. (1962). Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. J. Cell. Comp. Physiol. *59*, 223–239.

Subach, F.V., Subach, O.M., Gundorov, I.S., Morozova, K.S., Piatkevich, K.D., Cuervo, A.M., and Verkhusha, V.V. (2009). Monomeric fluorescent timers that change color from blue to red report on cellular trafficking. Nat. Chem. Biol. *5*, 118–126.

Teruel, M.N., and Meyer, T. (2000). Translocation and reversible localization of signaling proteins: a dynamic future for signal transduction. Cell *103*, 181–184.

van der Krogt, G.N.M., Ogink, J., Ponsioen, B., and Jalink, K. (2008). A comparison of donor-acceptor pairs for genetically encoded FRET sensors: application to the Epac cAMP sensor as an example. PLoS ONE *3*, e1916.

van der Wal, J., Habets, R., Varnai, P., Balla, T., and Jalink, K. (2001). Monitoring agonist-induced phospholipase C activation in live cells by fluorescence resonance energy transfer. J. Biol. Chem. *276*, 15337–15344.

Wang, S., and Hazelrigg, T. (1994). Implications for Bcd messenger-RNA localization from spatial-distribution of Exu protein in Drosophila oogenesis. Nature *369*, 400–403.

Yang, F., Moss, L.G., and Phillips, G.N. (1996). The molecular structure of green fluorescent protein. Nat. Biotechnol. *14*, 1246–1251.

Zacharias, D.A. (2002). Sticky caveats in an otherwise glowing report: oligomerizing fluorescent proteins and their use in cell biology. Sci. STKE PE23.

Zhang, J., Ma, Y.L., Taylor, S.S., and Tsien, R.Y. (2001). Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering. Proc. Natl. Acad. Sci. USA *98*, 14997–15002.