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The Green Fluorescent Protein: A Key Tool to Study Chemical Processes in Living Cells**

*G. Ulrich Nienhaus**

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Our knowledge of the molecular basis of life has grown enormously over the past decades. Whole-genome sequencing has revealed the blueprints of the biomolecular constituents, proteins and nucleic acids, and of living organisms. Tens of thousands of protein structures have been determined by crystallography and nuclear magnetic resonance spectroscopy, and functionally important dynamics have been explored in great detail for many proteins by using a wide variety of biochemical and biophysical techniques. However, the function of proteins cannot be understood merely on the basis of in-vitro studies on isolated molecules. Within the cell, proteins interact with many other molecules, localize in different cellular compartments, and associate with the extended cytoskeletal and membranous structures of the cell. Moreover, they may undergo modifications and translocate within the cell by diffusion or active transport as part of their function. Therefore, many life-science researchers have turned their attention to the investigation of biomolecular interactions and the resulting functional phenomena within the living cell, tissue or organism, as they proceed in both space and time.

For these studies, fluorescence-based optical microscopy is the method of choice because 1) it is a minimally invasive technique that allows processes in living cells and tissues to be observed over extended periods of time; 2) it is exceedingly sensitive (down to the single-molecule level); and 3) novel microscope designs offer image resolution substantially better than the Abbe (diffraction) limit. The key advantage of fluorescence microscopy is, however, that 4) particular functional processes can be focused on by specifically attaching fluorophores to molecules (or other structures such as membranes, organelles or cells) of interest.

The 2008 Nobel Prize in Chemistry was awarded to Osamu Shimomura, Martin Chalfie, and Roger Y. Tsien "for the discovery and development of the green fluorescent protein, GFP". GFP and the related fluorescent proteins (FPs) of the GFP family have proved to be exceedingly powerful and versatile tools for the specific fluorescence labeling of proteins in cells. Tens of thousands of papers have been published based on GFP fluorescence; this small protein has truly revolutionized the life sciences. The GFP polypeptide chain consists of 238 amino acids; it folds into a rigid, 11-stranded β barrel with a central helix running along its axis (Figure 1). A fluorescent chromophore, which forms autocatalytically from a tripeptide (for GFP: Ser65-Tyr66-Gly67), is held in the middle of the helix, essentially in the center of the can-shaped protein. The GFP gene can be introduced into the cellular DNA so that the cell synthesizes the protein. Moreover, the GFP gene can be fused to the gene of a protein that the researcher wants to study. The fusion protein is thus the protein of interest with an added GFP domain (Figure 2), which becomes visible by its fluorescence when exciting it with light of a suitable wavelength (Figure 3). This strategy has been widely employed to monitor a multitude of cellular processes, including gene expression, protein localization, translocation and degradation, and many biomolecular interactions. Consequently, GFP and its cousins have become standard tools for thousands of researchers around the globe.

Remarkably, it took several decades from the discovery of GFP to its development into one of the most powerful imaging tools. Many researchers have contributed to this work, and Shimomura, Chalfie, and Tsien were rightfully selected as Nobel Laureates because their names are associated with three decisive steps in GFP development. In 1962, Osamu Shimomura isolated the protein from tissue extracts of the jellyfish *Aequorea victoria* in his pursuit of understanding the bioluminescence of this organism.[1] He purified the protein, characterized its optical properties, and, in 1979, identified the chemical nature of the fluorophore.[2] Cloning the gene of GFP by Prasher and co-workers in 1992[3] laid the basis for the second stage of GFP research, which started with the recombinant expression of GFP in *Escherichia coli* and *Caenorhabditis elegans* by Martin Chalfie and collaborators.[4] Whereas it was earlier assumed that special enzymes were required to convert the polypeptide chain into the functional fluorescent protein, this seminal work proved that no other component from *Aequorea victoria* is needed. Because the fluorophore forms spontaneously, GFP can be expressed and utilized as a genetically encoded fluorescence marker in essentially all organisms. In 1996, the three-dimensional

[*] G. U. Nienhaus
Institute of Biophysics, University of Ulm
89069 Ulm (Germany)
and
Department of Physics, University of Illinois at Urbana-Champaign
Urbana, IL 61801 (USA)
E-mail: uli@uiuc.edu

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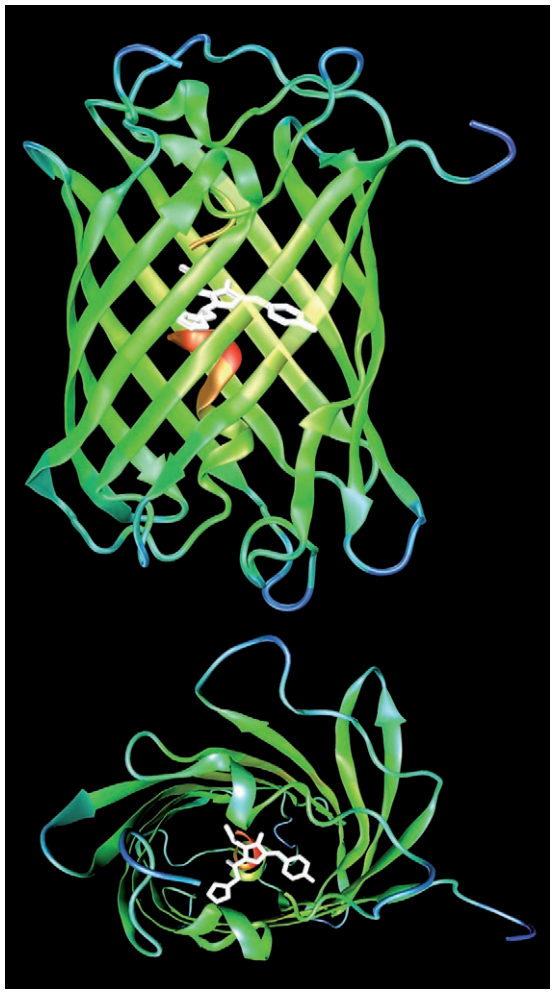

Figure 1. Cartoon representations (side and top view) showing the characteristic polypeptide fold of a protein from the GFP family (EosFP).[13] The chromophore (white) is included as a stick model.

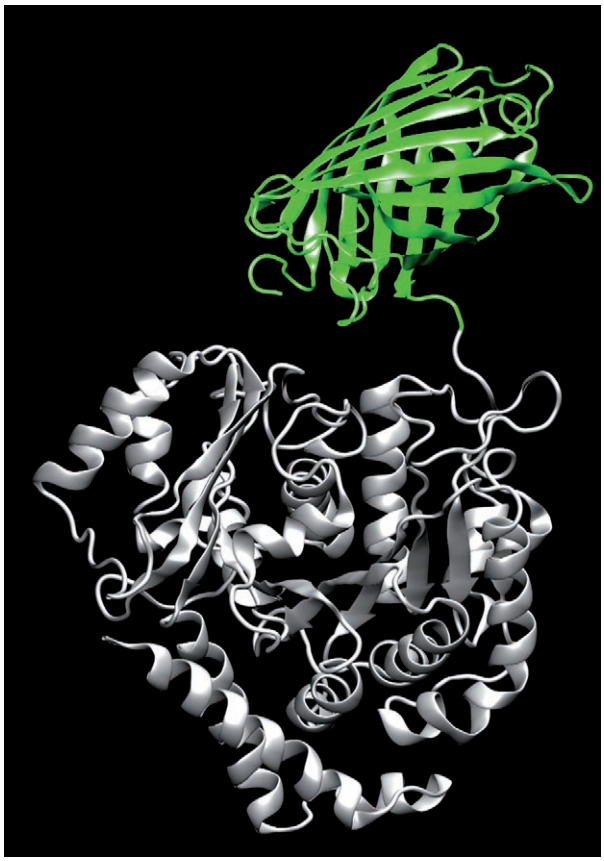

Figure 2. Schematic depiction of a fusion protein, consisting of a GFP domain (green) connected to another protein (white).

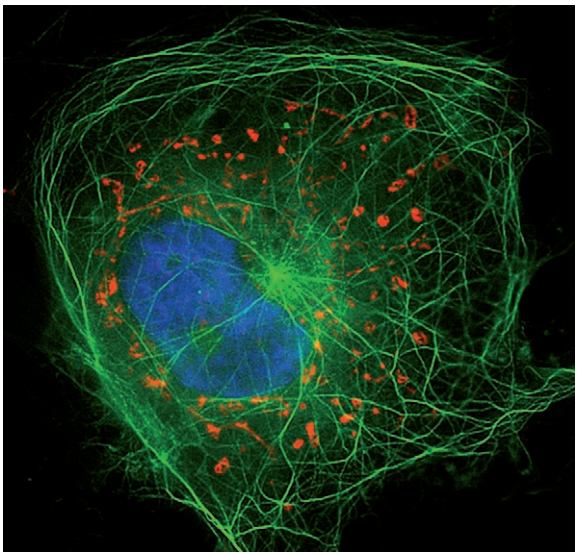

Figure 3. Three-color image of a cultured cell. Tubulin fibers were marked green by a GFP-labeled tubulin-binding protein; mitochondria were labeled by a red FP fused to a mitochondrial localization signal. The nucleus was stained blue with DAPI, an organic dye. Reprinted from Ref. [19].

structure of GFP was solved by the groups of Remington (PDB: 1EMA) and Phillips, Jr. (PDB: 1GFL) using X-ray crystallography.[5,6] Tsien and co-workers were able to show that the strongly fluorescent chromophore, 4-(*p*-hydroxybenzylidene)-5-imidazolinone, forms autocatalytically from the tripeptide Ser-Tyr-Gly, requiring nothing else but dioxygen.[7] A large variety of GFP and GFP-like protein mutants with new and improved properties as fluorescence tags were produced in his laboratory by protein engineering. Variants in novel hues, blue (BFP), cyan (CFP), and yellow (YFP), were created, enabling multicolor applications, Förster resonance energy transfer (FRET)-based studies of protein–protein interaction, and the development of FRET-based biosensors, for example in the measurement of intracellular calcium ion levels.

In the late 1990s, it was quite a surprise when proteins of the GFP family were discovered in non-bioluminescent anthozoa by Wiedenmann[8] and Lukyanov and co-workers.[9] Since then, many different GFP-like proteins have been characterized, some showing entirely novel properties. Importantly, the long-sought orange and red FPs were finally identified. These are highly desirable for live-cell and tissue imaging applications because of the reduced cellular autofluorescence and scattering in the red spectral range. Furthermore, they extend the color palette for multicolor labeling or FRET experiments.

In recent years, so-called "photoactivatable", or "optical highlighter" FPs have emerged as powerful new tools for cellular imaging.[10] Upon irradiation with light of specific wavelengths, these FPs either switch between a fluorescent and non-fluorescent state (photoswitching),[11,12] or they change their fluorescence emission from one wavelength to another (photoconversion).[13,14] By focused illumination of specific regions in a living cell, subsets of proteins can be highlighted, and their subsequent dynamics followed in space and time. Another most exciting application is in the area of superresolution optical microscopy, which is also known as "nanoscopy".[15–17] Image resolution way below the Abbe (diffraction) limit is achieved by collecting fluorescence photons from individual molecules (or ensembles that are confined in space to a region much smaller than the wavelength of light). These spots can be localized with a precision down to about 10 nm. Stunning superresolution images have recently been obtained based on photoactivatable FPs.[16,17]

At the moment, FP technology is being vigorously advanced by laboratories all over the world, aiming to develop monomeric, rapidly maturing, bright, and photostable FP variants, to extend the color palette further to the red and to optimize the behavior of photoactivatable FPs.[18] Swift progress is expected by rational protein engineering, based on our continuously improving knowledge of structure–function relations in FPs, and random mutagenesis combined with sophisticated screening procedures.

We are currently at the beginning of an era in which chemical interactions are observed within living cells. The Nobel Prize 2008 in Chemistry celebrates three scientists who have established GFP as a tool central to this endeavor. With further advances in FP technology and rapid improvements in quantitative optical imaging, the mysteries of living cells and organisms may finally be unraveled.

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