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FEATURE ARTICLE

Discovery and development of the green fluorescent protein, GFP: the 2008 Nobel Prize

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The 2008 Nobel Prize in Chemistry was particularly important for the scientific community working in the analytical and bioanalytical fields. It was awarded for the plethora of work and information gathered after almost 50 years of research in an area that started with the study of bioluminescence as a natural phenomenon in living marine organisms. What began as a relatively narrow field of research, and almost a marine biologist's curiosity, turned into an unexpectedly fruitful new area of science with far-reaching discoveries beyond understanding bioluminescence in marine species.

Thanks to this pioneering work, nowadays we have powerful tools to study and quantify molecular events at the nanoscale in complex biological systems. The use of green fluorescence protein (GFP) has enabled visualization—sometimes for the first time—in intact animals and single cells.

The 2008 Nobel Prize in Chemistry was awarded jointly to three scientists, namely, *Osamu Shimomura* of the Marine Biological Laboratory in Woods Hole, MA, USA, and of Boston University Medical School, Boston, MA, USA, *Martin Chalfie*, Columbia University, New York, NY, USA, and *Roger Y. Tsien*, Howard Hughes Medical Institute, University of California San Diego, La Jolla, CA, USA, for "*the discovery and development of the green fluorescent protein, GFP.*"

The Nobel Prize press release stated*: "Glowing proteins – a guiding star for biochemistry*," truly reflecting the essence of the discovery. In that regard, each laureate had made a specific and seminal contribution to the field: Osamu Shimomura first isolated GFP from the jellyfish *Aequorea victoria* and discovered that this protein glows bright green under ultraviolet light. Martin Chalfie demonstrated the use of GFP as a luminescent reporter gene. In one of his pioneering experiments, he made six individual cells fluoresce by expressing GFP in the transparent roundworm *C. elegans*. Roger Y. Tsien contributed to a better understanding of the mechanism of GFP fluorescence. He also created genetically modified variants of the wild-type GFP extending the fluorescence emission colors to a wide range of emission wavelengths, spanning through the visible spectrum. The implications of this work are enormous and the new GFP variants are extraordinary tools that enable several biochemical processes to be followed at the same time by employing emission spectra resolution approaches.

The question that some may pose is: *Why are these studies centered around a marine organism's protein so important? Why has the scientific community benefited so widely from these discoveries?*

The main reason was that the discovery of GFP and its physical and chemical properties enabled basic photochemistry and photophysics to be translated from conventional small organic fluorescent molecules to larger and more complex macromolecules such as proteins. GFP combines the properties of a conventional chromophore/fluorophore with the added bonus of being able to be used as a reporter molecule in recombinant DNA-based technologies. For example, a fluorescence analytical signal can be obtained from the GFP fused to other proteins capable of specifically recognizing a target analyte. It can be used as a reporter gene for the development of luminescence cell-based assays, cellular biosensors, fluorescence resonance energy

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transfer (FRET), and bioluminescence resonance energy transfer (BRET) methods. Molecular biology has been successfully used to improve fluorescence efficiency and, particularly, to alter the wavelength of emission of GFP by changing the amino acid sequence of the protein through site-directed mutagenesis studies. In this manner, a battery of rainbow-emitting GFP variants was obtained that could be divided into seven different classes based on their distinct chromophores. The number of scientific publications in which GFP variants have been used has increased exponentially in recent years, reaching an average of 2500 papers per year, many of them in bioanalytical chemistry.

Three main periods can be distinguished that cover the history of GFP.

1955–1985 Discovery and Pioneering Work

In 1955, Davenport and Nicol [1] reported that the jellyfish *Aequorea victoria* is bioluminescent, i.e., it produces light with the help of chemical reactions that provide the energy for photon emission, and it emits green light.

This photon-emitting protein was GFP, later discovered by Shimomura et al. in the early 1960s [2] as a companion protein to aequorin, the bioluminescent protein from *Aequorea victoria*.

> "...During purification steps they cut off the edges of the jelly fish and pressed them through a filter to get what they called a 'squeezate' ... when he poured it into the sink, it flashed brightly. He realised that there was seawater in the sink and that it was calcium ions in the seawater that had caused the chemical reaction. Strangely enough, the flash of light was not green like the edges of the jellyfish. It was blue." [3]

In a living jellyfish, GFP fluorescence occurs only when aequorin interacts with Ca^{2+} ions, inducing a change in the conformation of the protein, which in turn causes a chemical reaction in which the chromophore goes through an excitation state and relaxes back to the ground state emitting light with a blue glow. Some of the energy released during the relaxation process of aequorin can be transferred to GFP in a radiationless manner, shifting the blue color of emission towards green.

The main scientific contribution of Osamu Shimomura was the study and understanding of the bioluminescence mechanism of this jellyfish, as he had accomplished previously with other marine organisms. This is a typical and representative example of how basic research can lead to an unexpected scientific revolution. Morin and Hastings [4] found the same type of bioluminescent phenomenon in the related coelenterates *Obelia* (a hydroid) and *Renilla* (a sea pansy), and they were the first to suggest *radiationless energy transfer* as an in-vivo mechanism for GFP's excitation and emission.

Morise et al. [5] purified and crystallized GFP, and demonstrated that aequorin could efficiently transfer its luminescence energy to GFP. In 1970, Shimomura [6] proposed that the chromophore was a 4-(*p*-hydroxybenzylidene)imidazolidin-5-one attached to the peptide backbone through the 1 and 2 positions of the ring.

1985–1995 GFP as a tool for the Biosciences

The idea of using GFP as a tool for the development molecular biology-based bioanalytical methods began much later. Martin Chalfie, who learned about the existence of GFP for the first time in 1988 at a seminar about bioluminescent organisms at Columbia University in New York, realized that GFP would be a fantastic tool for mapping cells by connecting its gene with those encoding for other proteins. In 1992, Douglas Prasher cloned and identified the nucleotide sequence of wild-type GFP [7] and in 1994, Martin Chalfie expressed its coding sequence in *E. coli* and *C. elegans* [8]. One month later, Frederick Tsuji's laboratory independently reported the expression of the recombinant protein [9].

1995–2008 Modern Applications

The wild-type GFP had several drawbacks and limitations for use as a robust analytical tool, for example dual-peaked excitation spectra, pH and chloride sensitivity, poor fluorescence quantum yield, poor photostability, and folding at 37 °C. Elucidation of the GFP crystal structure was a milestone that enabled study of the *chromophore* and neighboring residues, which were later modified by directed mutagenesis to produce the wide variety of GFP derivatives in use today. In 1995, Roger Tsien engineered the wild-type GFP, obtaining mutants with different emission wavelengths and improved thermostability [10]. Later, enhanced GFP (EGFP) characterized by an extinction coefficient (ε) of 55,000 $mol^{-1}L\ cm^{-1}$ and by a fluorescence quantum yield of 0.60 was prepared. Other blue mutants (EBFP, EBFP2), cyan fluorescent proteins (ECFP, Cerulean, CyPet), and yellow fluorescent protein derivatives (YFP, Citrine, Venus, YPet) were also constructed and characterized. One color, however, that Tsien could not produce with GFP was red. Red light penetrates biological tissues more easily and is, therefore, especially useful for researchers who study body cells and organs.

Mikhail Matz and Sergei Lukyanov searched for GFP-like proteins in fluorescent corals and discovered six more proteins, one red, one blue, and the others green [11]. The desired red protein, DsRED, was unfortunately larger and

heavier than GFP. Tsien's research group solved this problem by redesigning DsRED so that the protein is now stable and fluoresces as a single amino acid chain that can easily be connected to other proteins by genetic fusion [12]. From this smaller protein, Tsien also developed proteins with mouth-watering names like mPlum, mCherry, mStrawberry, mOrange, and mCitrine, depending on the color that they glow. Several other researchers and companies have also added new colors to this growing palette. Today, 46 years after Shimomura first wrote about the discovery of the green fluorescent protein, there is a kaleidoscope of GFP-like proteins that shine throughout the colors of the rainbow.

One of the main exciting applications of GFP and its variants is their use as fluorescence reporter donor and acceptor pairs in fluorescence resonance energy transfer, FRET. In his Nobel Laureate lecture, Roger Tsien stated:

> "... I had been fascinated by a biophysical phenomenon called FRET in which one excited dye molecule can transfer its energy to a close neighbor, much as a football or basketball player can pass the ball to a teammate with diminishing probability of success the greater the distance between the players".

By appropriately selecting a fluorescent donor and an acceptor it is possible to study functional protein–protein interactions and receptor dimerization, by detecting the switch in fluorescence emission to a higher wavelength because of the resonance energy transfer process. Bioluminescence resonance energy transfer, BRET, was also the direct result of the GFP discovery, because the jellyfish luminescence machinery is derived from the natural resonance energy-transfer process occurring in the presence of Ca^{2+} from the blue-emitting photoprotein aequorin to GFP. Many different applications of BRET involve other bioluminescent donors, for example *Renilla* or *Gaussia* luciferases, and many GFP variants (Fig. 1) [13].

The GFP discovery paved the way to molecular luminescence imaging in which GFP-labeled cells can be visualized in intact whole experimental animals, for example mice or rats, enabling precise localization in different target organs. Several animal models have been established using xenograft cancer models to study cancer growth and metastasis and the effect of drugs.

Fluorescence imaging is an important tool complementary to positron emission tomography (PET) or other optical-based imaging technologies. The use of red-shifted GFP mutants improves the sensitivity and resolution, because red light penetrates biological fluids and tissues more deeply than green light [14].

Recently, a transgenic line of monkeys carrying a gene encoding for GFP fully integrated into their genome has been created [15]. This work expanded the application of the photoprotein to non-human primates, and set the stage for the development of new animal models of human disease. Although the use of GFP to label cells and to obtain transgenic fluorescent mice contributed in a significant manner to advances in life sciences, these models are still quite different in terms of genotypes and phenotypes from humans. The "glowing" transgenic monkeys render new opportunities for pathophysiological studies and for the design of new therapies, particularly in the neurosciences, where small animal models do not fully reflect human physiology. On the other hand, concern related to the use of genetically modified organisms as models for medical research is a serious ethical aspect, and the balance between benefit and risk factors for humanity must be continuously monitored, regulated, and publicly discussed.

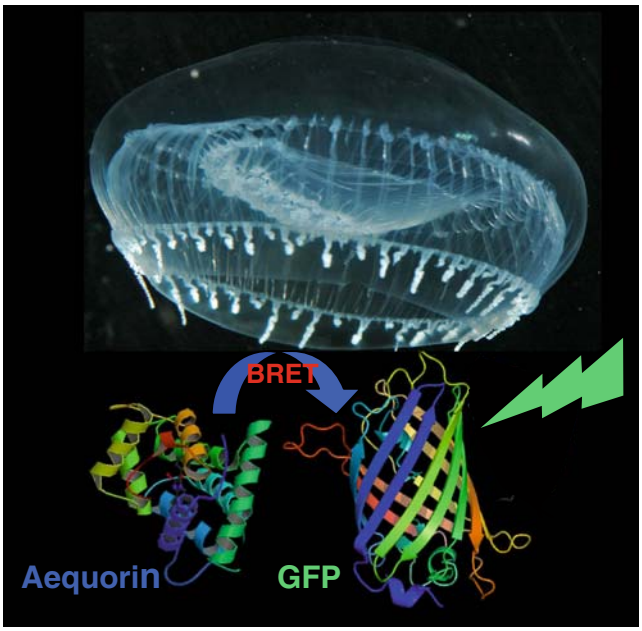

Fig. 1 The mechanism of GFP fluorescence in the jellyfish Aequorea involves two proteins. An influx of Ca^{2+} causes the first protein, aequorin, to become excited and transfer energy to the second protein, GFP, which loses the energy by emitting a photon of green light. Aequorin does not transfer its energy by fluorescence in vivo but rather by a quantum chemical (Förster type energy transfer) process (BRET). (Photo courtesy of Aldo Roda, University Bologna, Italy)

In conclusion, we have discussed the discovery, modification, and applications of GFP performed by the three 2008 Nobel Laureates in Chemistry. While we have learned much about GFP in recent decades, there is still one mystery that has still not been fully disclosed and that is: *Why does the jellyfish Aequorea victoria shine?* Many organisms living in the sea use light from bio/fluorescent proteins to confuse their enemies, to attract food, or to tempt a partner. But, is this the real purpose of aequorin? We leave it to readers to find out or, if they prefer, to remain

with us for a follow through of this fascinating story of light-emitting marine organisms on a future occasion.

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