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The Colorful Journey of Green Fluorescent Protein

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The 100th Nobel Prize in Chemistry was recently awarded to three distinguished scientists, Osamu Shimomura, Martin Chalfie, and Roger Y. Tsien, "for the discovery and development of the green fluorescent protein, GFP." To commemorate this occasion, I have been given the opportunity to review some of the critical moments in the long, yet colorful, journey of this inspirational molecule from nature. In so doing, it is impossible not to reflect upon the contributions of these critical minds that have made this journey possible.

CRITICAL MOMENTS

Discovery of GFP. Though the story of GFP began millions of years ago when the jellyfish *Aequorea aequorea* (also commonly referred to as *Aequorea victoria* and *Aequorea forskalea*) successfully evolved a fluorescent protein species with the uncanny ability to convert the excited blue energy of the bioluminescent protein aequorin into the green light observed in nature, its journey to "Nature's Scientific Contributors Hall of Fame" started much more recently, in 1961. At that time, Osamu Shimomura was studying the bioluminescence of *Aequorea aequorea*, trying to identify the substance that makes the jellyfish glow. To accomplish this task, Shimomura and the late Prof. Frank Johnson collected hundreds of thousands of jellyfish specimens and subjected them to various isolation strategies. Finally, after many months of intense research, the light-emitting substance, a protein later named aequorin, was purified and its biochemical properties were measured. As Shimomura demonstrated, aequorin becomes luminescent in the presence of Ca^{2+}, a feature that serves as the basis for its later development as a Ca^{2+} sensor. Much to Shimomura's surprise, the light emitted from purified aequorin (or from the jellyfish extract) was clearly blue, contrary to his expectation of green light similar to that emitted by the live jellyfish. During the purification of aequorin, however, Shimomura also noticed the existence of a green protein in the jellyfish extract. This observation, which represents the initial discovery of GFP, was included as a footnote in a paper by Shimomura and Johnson (*1*) describing the purification and characterization of aequorin: "A protein giving solutions that look slightly greenish in sunlight though only yellowish under tungsten lights, and exhibiting a very bright, greenish fluorescence in the ultraviolet of a Mineralite, has also been isolated from the squeezates."

Cloning and Expression of GFP. This protein, which was later named green fluorescent protein by Morin and Hastings (*2*), remained relatively quiet for another 30 years before it stirred a huge wave of interest within the biological community. At that time, Martin Chalfie was studying gene expression in *Caenorhabditis elegans*. Specifically, he was interested in understanding the mechanosensation of the worm and was attracted to the idea of using fluorescent

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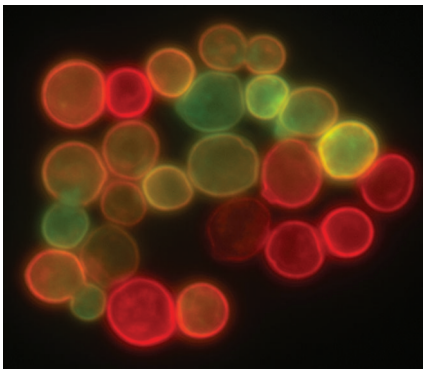

Figure 1. Yeast cell membrane visualized by some membrane proteins fused with RFP and GFP fluorescent markers. Imposition of light from both of markers results in yellow color. Credit: Copyright 2008, Masur. Accessed at http://commons.wikimedia.org/wiki/File:Yeast_membrane_proteins.jpg (February 2, 2009).

markers to visualize gene expression and protein localization. With a GFP cDNA clone obtained from Douglas Prasher, who successfully cloned GFP and published his results in 1992 (*3*), a rotation student in the Chalfie laboratory expressed GFP in bacteria and demonstrated that the expressors were indeed fluorescent. Soon thereafter, *C. elegans* expressing GFP and thereby glowing in green were also generated (*4*). These experiments demonstrated, for the first time, that GFP does not require additional cofactors or specific jellyfish converting enzymes to gain fluorescence and therefore can be used as a fluorescent marker for tracking gene expression and protein localization in other organisms. This critical moment in the history of GFP marked its transition from a curious fluorescent substance to a powerful imaging tool.

Development of GFP. The wild-type GFP, though shown to fluoresce in several organisms, is not a gem without flaws. In particular, its complex excitation spectrum and poor folding efficiency significantly limited its application in mammalian systems. In fact, GFP's meteoric rise as a molecular imaging tool took place after Roger Tsien stepped onto the stage. Tsien's contributions, which have led to many shining and colorful moments in GFP's career, include the development of GFP variants with much improved bio- and photophysical properties (*5*, *6*), as well as additional color variants with distinct emissions ranging from blue and cyan to yellow (*7*, *8*). These advancements not only have cemented GFP's status as the marker of choice for tracking gene expression and protein localization in intact cells and organisms but more importantly have opened new vistas in various biological applications, which collectively have led to the GFP revolution as we now know.

CRITICAL MINDS

In 1945, the year an atomic bomb was dropped on the city of Nagasaki, Japan, **Osamu Shimomura** was a 16-year-old high school student living not far from the bomb's epicenter. Although educational opportunities were extremely limited in post-World War II Japan, his determination to earn an education carried him to great academic success. His lifetime scientific interest in biolumiscence began as a graduate student at Nagoya University, where he was assigned the challenging task of purifying and crystalizing luciferin from *Cypridina*, a molecule that was notoriously unstable under normal conditions. After numerous attempts, Shimomura finally succeeded in identifying key conditions that allowed the crystalization of this substance. This work earned him an opportunity to work with Johnson at Princeton Unversity on the identification of the bioluminescent species in jellyfish.

This task turned out to be even more challenging than the one he tackled during his graduate studies. For one, only minute amounts of the luminescent substance could be obtained from a single jellyfish. Therefore, in order to collect enough specimen to isolate and characterize the substance at the biochemical level, in addition to laboratory scientists, Shimomura and Johnson needed to become excellent (jelly)

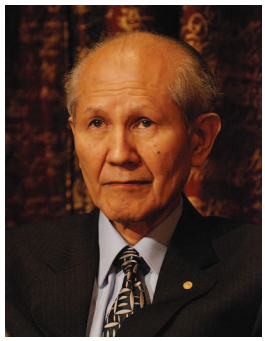

Figure 2. Osamu Shimomura, Nobel Prize Laureate for Chemistry 2008, at a press conference at the Swedish Academy of Science in Stockholm. Credit: Copyright 2008, Prolineserver (talk). Accessed at http://commons.wikimedia.org/wiki/File:Osamu_Shimomura-press_conference_Dec_06th,_2008-2.jpg (February 2, 2009).

fishermen. Every morning during the summer of 1961, the Shimomura and Johnson families would go to Friday Harbor, WA, to collect jellyfish. Shimomura estimates that 50,000 jellyfish were needed to produce a milligram of the luminescent species under study and that, in total, over 850,000 *Aequorea* specimens were collected during 19 years of research. Nonetheless, despite various attempts, Shimomura and Johnson failed to isolate luciferin-like substances from jellyfish, even though the prevailing hypothesis at that time held that all bioluminescence results from reactions of luciferin-like molecules. In the face of these apparent failings, Shimomura decided to desert the previous hypothesis and to extract the bioluminescent species, whatever it may be. During the course of these studies, he realized that the bioluminescence may, in fact, involve a protein. Moreover, he discovered that pH conditions, which could be used to reversibly inhibit the emission of light, could be used to facilitate the extraction of the light-emitting substance. The purification strategies that he devised eventually led to the isolation of aequorin and, unexpectedly, the discovery of GFP. Clearly, an open mind fueled by dedication and commitment

served as a crucial element in the discovery of GFP.

Martin Chalfie was a college student at Harvard when he first became interested in biochemistry. However, his interest was somewhat dampened by one summer of failed experiments after his junior year. Following graduation, he worked at a variety of temporary jobs but was eventually lured back to biological research as a graduate student at Harvard. For the most part, Chalfie's scientific interest has been focused on the mechanisms of mechanosensation in *C. elegans*, a poweful genetic model system that itself is in Nature's Scientific Contributors Hall of Fame. In fact, it was the transparency of this organism that originally inspired Chalfie to visualize biological processes in live worms using light. This was during the late 1980s, when Chalfie, while studying gene expression in *C. elegans*, was becoming increasingly dissatisfied with the static images of gene expression and protein localization patterns generated by antibody and β-galactosidase staining. Having realized the limitations of these commonly used techniques, Chalfie was on the look-out for new tools that would allow him to visualize these biological phenomena in a more dynamic manner. As the old adage goes, "Opportunity favors the prepared mind." Upon hearing about GFP at a seminar, Chalfie was immediately struck by the notion that GFP could be the tool that he had been searching for. By Chalfie's estimation, GFP had several favorable properties as an imaging tool: (i) Because GFP is fluorescent, it is well-suited for experiments in living animals. Indeed, observing fluorescence is relatively noninvasive and can be done using live cells, tissues, and organisms. (ii) GFP appeared to be a fairly small protein without enzymatic activities. This suggested that GFP could function as a relatively inert fusion tag. However, at that point, it was not clear whether GFP could become fluorescent in other species. In fact, the prevailing view was that the formation of the

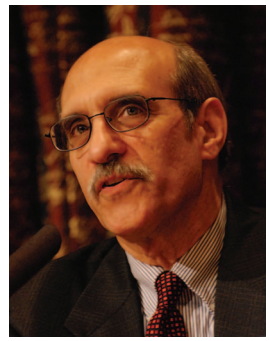

Figure 3. Martin Chalfie, Nobel Prize Laureate for Chemistry 2008, at a press conference at the Swedish Academy of Science in Stockholm. Credit: Copyright 2008, Prolineserver (talk). Accessed at http://commons.wikimedia.org/wiki/File:Martin_Chalfie-press_conference_Dec_07th,_2008-4.jpg (February 2, 2009).

GFP chromophore likely required the help of some converting enzymes from the jellyfish. Driven by the dream of seeing the worm glowing in green, Chalfie carried out the pioneering work to bring GFP to life inside bacteria and worms and showed, indeed, that no other jellyfish proteins are needed to generate fluorescent GFP molecules.

Coming from a family known for having produced a number of top engineers, **Roger Tsien**, who calls his own work molecular engineering, once said, "I'm doomed by heredity to do this kind of work." Tsien showed very early interests in science, particularly in chemistry. When he was 8 years old, he embarked upon some dangerous, but pretty exciting, chemistry experiments that he had found in a book from the school library, in the basement of the family house. While in college, Tsien developed a strong interest in neurobiology. However, he eventually became a chemist as he realized that chemistry is the source of the molecular tools needed to solve the biological problems that intrigued him. That essentially is the theme of his scentific endeavor: to build molecules to probe biological phenomena.

The first set of molecular tools that Tsien created were motivated by his desire to probe neurobiology. These tools consisted of a series of fluorescent dyes designed to track the movement and dynamics of Ca^{2+}, a small-molecule second messenger that provides a direct measure of neuronal activity as well as playing a critical role in numerous other physiological processes. To that point, the best way to measure intracellular Ca^{2+} levels was to microinject aequorin and image its Ca^{2+}-dependent bioluminscence, which was a tedious and difficult procedure. Using synthetic chemistry, Tsien developed a series of fluorescent Ca^{2+} chelators whose fluorescence properties change upon binding Ca^{2+}. Since these dyes are charged molecules that do not readily traverse the cell membrane on their own, Tsien further devised a means of introducing these intracellular indicators into cells by masking the charges with groups that can be removed by intracellular enzymes. Fura-2, one of such Ca^{2+} dyes developed more than 20 years ago, is still the most widely used Ca^{2+} probe. In addition to developing Ca^{2+} probes and similar dyes designed to measure the dynamics of other intracellular ions, pH fluctuations, and the voltage across cell membranes, Tsien has also devised strategies to photochemically release or absorb messenger substances such as Ca^{2+}, $InsP_3$,

Figure 4. Roger Tsien, Nobel Prize Laureate for Chemistry 2008, at a press conference at the Swedish Academy of Science in Stockholm. Credit: Copyright 2008, Prolineserver (talk). Accessed at http://commons.wikimedia.org/wiki/File:Roger_Tsien-press_conference_Dec_07th,_2008-2.jpg (February 2, 2009).

and nitric oxide, among others. Together, these molecular tools have greatly impacted our understanding about both the regulation and functional roles of these biologically important messenger molecules inside cells.

However, it was the study of cyclic AMP (cAMP), another important second messenger molecule, that led Tsien to his first encounter with GFP. To better understand how cAMP regulates a plethora of cellular processes with high specificity, Tsien, in collaboration with Susan Taylor, had devised a fluorescent sensor of cAMP to visualize its intracellular dynamics. This sensor was based on the main effector molecule of cAMP, protein kinase A (PKA). The regulatory and catalytic subunits of PKA were labeled with two organic fluorophores, fluoroscein and rhodamine, respectively, which undergo fluorescence resonance energy transfer (FRET) when they are in close proximity. Binding of cAMP to the regulatory subunit leads to dissociation of the catalytic subunit from the regulatory subunit, thereby disrupting FRET. Though this sensor can be used in live cells, it requires a series of technically challenging steps, including protein purification, fluorescence labeling, and microinjection. Considering the technical challenges and limitations in applying this technique to probe other cellular events, Tsien had tremendous interest in finding new ways of labeling genetically designated proteins in living cells, ideally with at least two colors. Given the circumstances, Tsien's encounter with GFP was both serendipitous and inevitable. One day in 1992, a medline search of "green fluorescent protein" popped up Prasher's paper, and an immediate phone call brought GFP into the hands of a molecular engineer. However, to make a genetically encoded cAMP sensor similar to the FRET-based indicator that he and Taylor had developed, two color variants with favorable properties were needed. In order to make GFP change color, he started with in-depth studies to gain insights into the critical steps of the formation of GFP chromophore, *p*-hydroxybenzylidene-imidazolinone, which was correctly proposed by Shimomura (*9*). Using both random mutagenesis and structure-based engineering strategies, GFP variants with improved properties as well as several spectrally distinct GFP color variants were created in the Tsien laboratory. These improvements have not only made it possible to generate a genetically encoded cAMP sensor but have also enabled the development of a plethora of fluorescent biosensors—by Tsien himself and others—that are designed to illuminate various cellular events that are normally invisible, including protease activation, Ca^{2+} dynamics, kinase activities, and redox changes, to name a few. In fact, the general principles behind the designs of these biosensors are applicable to essentially any biochemical event to enable its visualization in living cells.

Behind this pioneering work, there is a vision to identify the biological problems to be solved. This visionary perspective, combined with enormous insights drawing from knowledge in various disciplines such as chemistry, biology, physics, and instrumentation, has enabled the identification of the most efficient path for solving the problem at hand. I have had the privilege of working with Roger Tsien to apply some of the general principles used for engineering genetically encoded biosensors to visualize a highly dynamic regulatory mechanism that controls numerous cellular processes, namely, protein phosphorylation. The general method we developed has now been successfully applied to illuminate the spatiotemproal regulation of many kinases. I have also had many opportunities to witness his passion for science and love of colors. In fact, during my time there, he still tried to find time to carry out chemical syntheses, which usually happened during Christmas. As a proof for the latter, many colorful illustrations were created in the Tsien Laboratory using bacteria expressing fluorescent proteins or purified fluorescent proteins. In my opinion, his love of colors may be best reflected in his desire to give a changing color to all the invisible biochemical events in living cells.

GFP has come a long way through the years, from a humble beginning to a glorious stand in Nature's Scientific Contributors Hall of Fame. This "molecular flashlight" not only contributed to a large number of insights about the inner workings of living cells but now provides a rainbow of joyful colors that fluoresce in culture dishes, Eppendorf tubes, and even in the hands of high school students to motivate great minds of the future. Indeed, with many more breakthroughs based on this remarkable molecule still on the horizon, it is certain that the colorful journey of GFP will continue.

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