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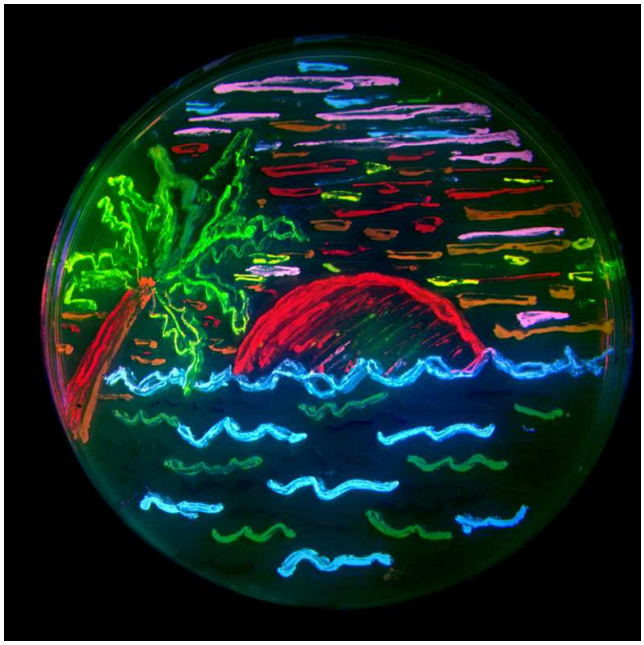
Fisica delle nanostrutture



The Green Fluorescent Protein

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Cover: Sunset with green flash as viewed from a California lab. Artwork with bacteria expressing various colors of fluorescent protein. Further information on page 11, fig. 5.

1 Nanostructures for biology

The demand for smaller and smaller microelectronic chips is one of the driving force for the research in material sciences all over the world. The scaling down of the devices requires both theoretical and experimental tools to face the new phenomena occurring when the typical observed length scales are of the same order of magnitude of the typical length scales of that material. It is necessary to take into consideration the carrier mean free path (it determines if the transport properties are described with a ballistic or diffusive regime), the phase-breaking length (it determines the occurrence of interference phenomena) and the thermal De Broglie wavelength (it determines the quantization of the kinetic energy).

The first integrated transistor length was about ten centimeters in 1958; the Moore's law predicted a doubling of the number of components on a square inch of silicon every 18 months. Nowadays the channel length of the commercial devices is 40 nm. It allows the production of very performant devices. The comparison between the length of the devices and the length of the natural objects can help to estabilish new links between the inorganic and the organic world (see fig. 1). While the first transistor was comparable to an human hand, the actual dimensions of the devices are comparable to those of viruses or proteins. By continuing the scaling down, it is expected to reach the dimensions of DNA or of the atoms. So the biological and the nanostructures world are closer than one can image and very intriguing properties can be discovered by integrating biological samples in solid state research.

One of the topic still under investigation is the capability of some proteins to emit light under optical excitation, and the possibility to use them for in-vivo cellular imaging. Among them, the Green Fluorescent

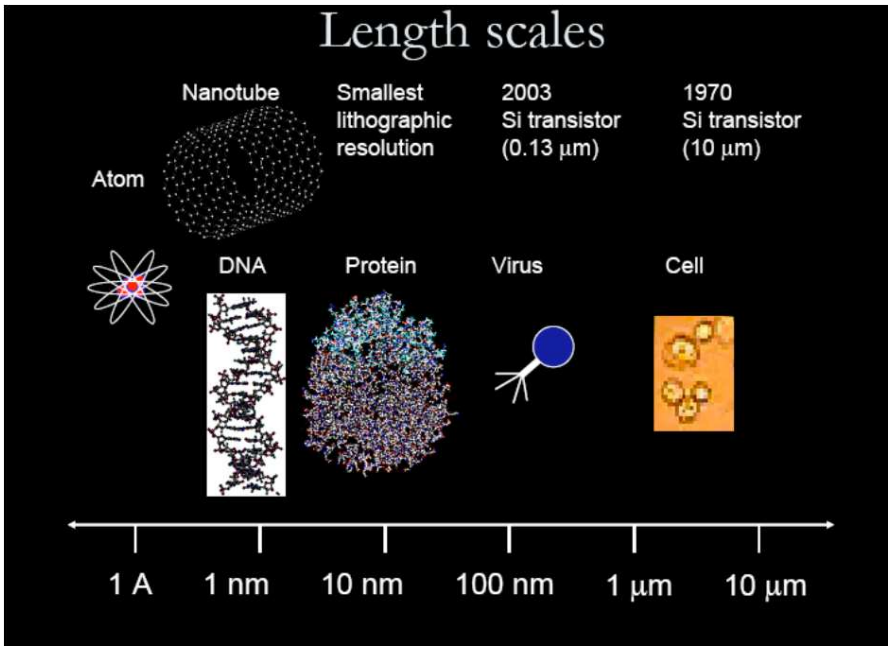

Figure 1: Comparison between the typical dimension of natural object and the dimension of the current silicon-based technology.

Protein GFP received great attention from the scientific community.

2 Glowing proteins - a guiding star for biochemistry

Martin Chalfie, Osamu Shimomura and Roger Y. Tsien (figure 2) were awarded the 2008 Nobel Prize in Chemistry on 8 October 2008 for the discovery and characterization of the green fluorescent protein GFP, for first expressing GFP in fluorescent form in important model organisms and for the development of GFP and its homologues to a universal set of genetic tags for protein localisation, protein movement and protein interactions in the cells of all types of organisms [1–3]. Herein I report an extract from the Presentation Speech by Professor Mans Ehrenberg

Figure 2: Left: Osamu Shimomura; centre: Martin Chalfie; right: Roger Y. Tsien. They were awarded the 2008 Nobel Prize in Chemistry for the discovery and development of the green fluorescent protein, GFP.

[4]:

> [...] The discovery and development of the green fluorescent protein (GFP) have radically changed the scientific agenda. Improved variants of GFP and GFP-like proteins in synergy with high-resolution microscopes, computational techniques and powerful theoretical approaches are currently fuelling a scientific revolution focused on quantitative analyses of complex biological systems. The gradual appearance of a world of hitherto unseen structures and dynamic principles is now impacting virtually all aspects of biological, medical and pharmaceutical research.
>
> The story of GFP has three acts: the discovery of GFP, the expression of fluorescing GFP in key model organisms and the development of GFP-like proteins into a universal set of genetic tags.
>
> The first act began in Japan fifty years ago, when Osamu Shimomura studied the self-luminous small crustacean ostracod

Cypridina. His successful work brought him to the US, where he collaborated with Professor Frank Johnson in the study of the green self-luminescence of the jellyfish Aequorea victoria, peacefully swimming in the waters of the Pacific Ocean outside Friday Harbor, Washington State. In 1961 Shimomura made the surprising discovery that the protein aequorin, responsible for the self-luminescence of *A. victoria*, emits blue and not green light. Fortunately, he also discovered the green fluorescent protein now known as GFP, and was able to eventually explain that the green light from the jellyfish was due to electronic excitation of GFP by radiation-less transfer of blue self-luminescence from aequorin, followed by emission of green fluorescence from GFP. Thanks to Professor Shimomura's discovery, GFP with its remarkable optical properties was pulled out from its hiding place in the Pacific and made available for scientific scrutiny.

In the beginning of the second act, hardly anyone believed that expression of GFP in organisms other than *A. victoria* would lead to a fluorescent protein. It was generally assumed that formation of its chromophore would require enzymes specific to *A. victoria*, but there was one GFP believer named Martin Chalfie who had a different view. His research focused on the nervous system of the small roundworm Caenorhabditis elegans. He was filled with enthusiasm for the experiments that would become possible if only one could express fluorescent GFP in *C. elegans*. Using a GFP clone provided by researcher Douglas Prasher, he demonstrated in 1993 and 1994 that brightly fluorescent GFP was expressed in both *E. coli* and *C. elegans*. Professor Chalfie's results not only

> showed the power of experiments over scientific prejudice but also made it clear to many that GFP was destined to become a universal genetic marker.
> The third act of the GFP story began in 1994, when Roger Tsien explained how the chromophore of GFP can form spontaneously in the presence of oxygen and engineered a GFP variant with blue fluorescence, demonstrating that point mutations in the primary structure of GFP can modulate its fluorescence emission spectrum. Since then, Tsien has provided many engineered variants of GFP. They fluoresce throughout the whole visible spectrum, have enhanced photostability and brightness as well as greatly reduced time for their chromophore to mature into its fluorescent state after protein folding. GFP has revolutionised the biological sciences, thanks to the creative engineering of continuously improved forms of GFP and GFP-like proteins carried out by Professor Tsien.

The decision to award people working in this topic helps us to evaluate the enormous impact on the scientific community of the studies about the GFP. Moreover, the number of papers published in the last years on this topics (see figure 3) has quickly increased up to about 3,500 thus indicating the growing interest of the scientists on the properties of this protein. In just three years, the GFP from the jellyfish *Aequorea victoria* has vaulted from obscurity to become one of the most widely studied and exploited proteins in biochemistry and cell biology [5, 6].

GFP amazing ability to generate an highly visible, efficiently emitting internal fluorophore is both intrinsically fascinating and tremendously valuable. High-resolution crystal structures of GFP offer unprecedented opportunities to understand and manipulate the relation between protein

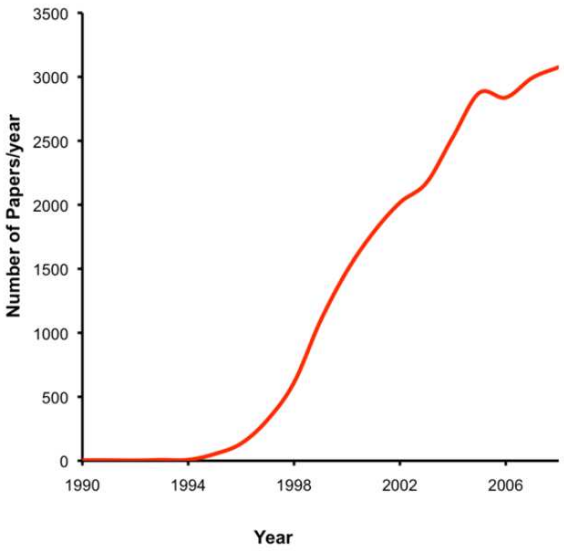

Figure 3: Number of scientific papers published about the GFP as a function of the year.

structure and spectroscopic function. GFP has become well established as a marker of gene expression and protein targeting in intact cells and organisms. Mutagenesis and engineering of GFP into chimeric proteins are opening new vistas in physiological indicators, biosensors, and photochemical memories [7].

3 Discovery and Major Milestones

Bioluminescence was written about as early as the first century AD by Pliny the Elder, who noticed the bright light eminating from some jellyfish. The presence of a fluorescent component in the bioluminescent organs of Aequorea victoria jellyfish (*phylum Cnidaria*, class *Hydrozoa*) was noted by Davenport and Nicol in 1955 [8], but it was Osamu Shimomura who was the first to realize that this fluorophore was actually

a protein [9]. In a footnote to his paper describing the isolation of the bioluminescent protein aequorin from Aequorea, Shimomura wrote:

> 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 [a handheld ultraviolet lamp], has also been isolated from squeezates.

Squeezate refers to the solution that resulted from the squeezing of the excised bioluminescent tissues of the jellyfish through a cotton bag. This description of the appearance of GFP solutions is still accurate. Soon after, Shimomura reported the fluorescence emission spectrum of this protein, which peaked at 508 nm [10]. They noted that the green bioluminescence of living *Aequorea tissue* also peaked near this wavelength, whereas the chemiluminescence of pure aequorin was blue and peaked near 470 nm, which was close to one of the excitation peaks of GFP. Therefore the GFP converted the blue emission of aequorin to the green glow of the intact cells and animals. In 1971 Morin & Hastings [11] found the same color shift in the related *coelenterates Obelia* (a hydroid) and *Renilla* (a sea pansy) and were the first to suggest radiation-less energy transfer as the mechanism for exciting coelenterate GFPs in vivo. The nature of the chromophore itself remained a mystery until 1979 when Shimomura correctly determined the chemical structure of the chromophore [12]. Achieving this breakthrough necessitated the laborious harvest of 100 mg of the naturally occurring green fluorescent protein from Aequorea.

However, the GFP utility as a tool for molecular biologists did not begin to be realized until 1992 when Douglas Prasher reported the cloning and nucleotide sequence of wtGFP [13]. The funding for this project had

run out, so Prasher sent cDNA samples to several labs. The lab of Martin Chalfie expressed the coding sequence of wtGFP, with the first few amino acids deleted, in heterologous cells of *E. coli* and *C. elegans*, publishing the results in Science in 1994 [14] (see figure 4). Remarkably, the

Figure 4: Cover of the volume 263, issue 5148 of the journal *Science* dedicated to the paper of M. Chalfie et al. entitled *Green fluorescent protein as a marker for gene expression* [14]

GFP molecule folded and was fluorescent at room temperature, without the need for exogenous cofactors specific to the jellyfish. This is the first dramatic demonstrations that the gene was self-sufficient to undergo the

post-translational modifications necessary for chromophore formation. It is the so called "first GFP revolution" because it is demonstrated that GFP chromophore formation does not require host factors. The biological research community, armed with the arsenal of powerful molecular biology techniques developed during the preceding two decades, was quick to recognize the unique utility of a genetically-encoded fluorophore as a marker of gene expression and protein localization. Accordingly, by November 1995 there were at least 36 additional examples of applications of recombinant *Aequorea* GFP and in the decade that followed the number of publications per year with *GFP* or *fluorescent protein* as a keyword rose at a rate of 500-600 publications per year per year. In hindsight, the cloning of the gene and the first demonstrations of recombinant expression in non-jellyfish organisms marks a clearly discernible turning point in the history of fluorescent protein research.

The growing popularity of *Aequorea* GFP, and the demand for additional variants that fluoresced at wavelengths other than green, prompted researchers to begin the search for homologues in other marine organisms. This effort came to fruition in late 1999 when a team of researchers from the Russian Academy of Science reported that reef *Anthozoa* contain fluorescent proteins with hues ranging from cyan to red [15]. As an example, in figure 5 a close-up of the adult red-cyan morph of the stony coral and an artwork with bacteria expressing various colors of fluorescent protein are shown. The inspiration behind this breakthrough discovery is credited to the evolutionary biologist Yulii A. Labas who had prompted Mikhail V. Matz, then a graduate student in the lab of Sergey A. Lukyanov, to attempt to clone *Aequorea* GFP homologues from the brightly colored tentacle tips of a sea anemone and several other Anthozoan organisms. A red fluorescent protein (commonly known as DsRed) from *Discosoma sp.* mushroom anemone served as the focal point of much of the initial

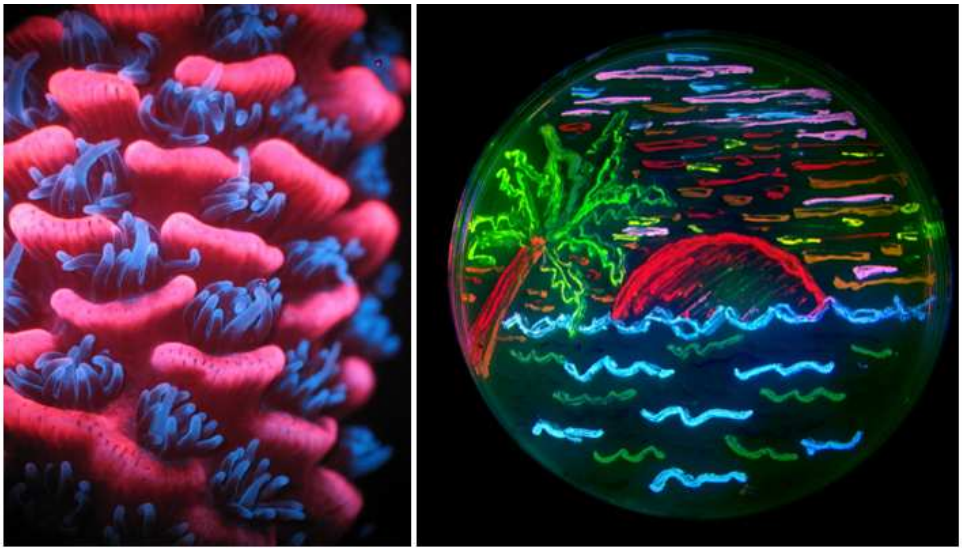

Figure 5: Left: A close-up of the adult red-cyan morph of the stony coral *Acropora millepora*. The red fluorescence is attributable to *amilRFP* and the cyan fluorescence is attributable to *amilCFP*. Right: Sunset with green flash as viewed from a California lab. Artwork with bacteria expressing various colors of fluorescent protein.

excitement and just one year after the initial report, papers describing its chromophore structure, obligate tetrameric structure, and x-ray crystal structure began to appear in the literature. Figure 6 shows the fluorescence excitation and emission spectra of wild-type *Aequorea* GFP and of mutant the DsRed. These reports revealed that Discosoma red fluorescent protein was very similar to Aequorea green fluorescent protein in terms of its overall fold and chromophore-formation chemistry. The key difference between the red and green proteins is that Discosoma red fluorescent protein undergoes an additional fourth step in the chromophore maturation pathway [16].

It is now generally understood that many of the bright and varied colors of reef coral are due to fluorescent proteins and their nonfluorescent homologues. Indeed, 4-years prior to the groundbreaking paper from

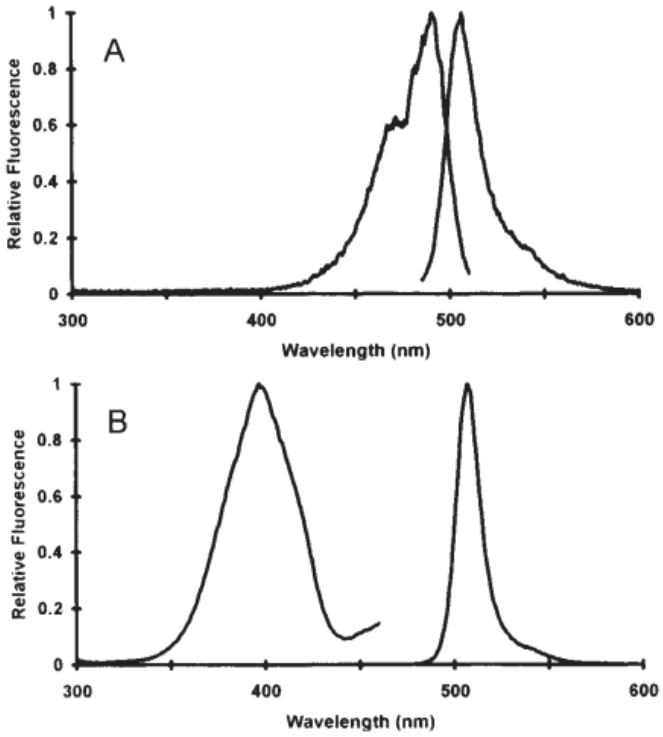

Figure 6: Top: Fluorescence excitation and emission spectra of wild-type *Aequorea* GFP. Bottom: Fluorescence excitation and emission spectra of the DsRed. Note that the excitation spectrum of RSGFP4 is red-shifted almost 100 nm relative to wild-type GFP.

Lukyanov, the pink pigment of the Anthozoan coral *Pocillopora damicornis* had been isolated and shown to be a proteinaceous pigment, as opposed to a non-protein pigment, which the authors dubbed pocilloporin. The authors did not, at that time, recognize that the protein was indeed a member of the GFP-like superfamily. This revelation was reported sometime after the Lukyanov paper, when the gene encoding pocilloporin was sequenced and shown to encode a protein with 19.6% identity with Aequorea green fluorescent protein. It is now apparent that, in terms of extinction coefficient and quantum yield, naturally occurring

fluorescent proteins lie on a broad continuum. Pocilloporin (often referred to as a nonfluorescent chromoprotein) can be conceptualized as a fluorescent protein that has a quantum yield of effectively zero and thus lies at one extreme of the continuum.

These GFPs seem to be partners with chemiluminescent proteins and to control the color of the emission in vivo. Despite interesting speculations, it remains unclear why these coelenterates glow, why green emission should be ecologically so superior to the blue of the primary emitters, and why the animals synthesize a separate GFP rather than mutate the chemiluminescent protein to shift its wavelengths. The discover of such a classes of luminescent proteines belonging to the same superfamily lead to the so called second GFP revolution. Unfortunately, Aequorea GFP genes are the only GFP genes that have been cloned. Several other bioluminescent species also have emission-shifting accessory proteins, but so far the chromophores all seem to be external cofactors which diminish their attractiveness as biotechnological tags and probes.

4 Molecular structure

X-ray crystal structures revealed that the GFP has a unique overall fold comprised of an 11-stranded β-sheet wrapped into a cylinderical β-barrel protein that is 42 $\mathring{A}$ in height and 24 $\mathring{A}$ in diameter. Small sections of α-helix also form caps on the ends of the cylinders. This type of protein fold has been given the blithe and befitting name of a β-can due to its resemblance to a soup can in terms of shape and proportions [17]. The chromophore - a modified tyrosine sidechain and cyclized protein backbone - is located near the center of the protein, attached to a helical segment of the protein that threads through the center of the β-can along its long axis (see figure 7). The regularity of the β-can of GFP

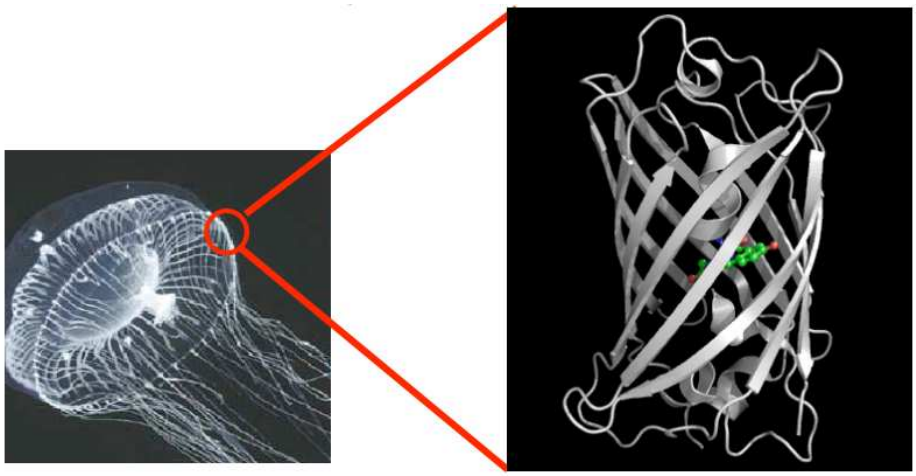

Figure 7: Picture of the GFP structure.

is quite remarkable. The eleven strands of the sheet form an almost seamless symmetrical structure, the only irregularities being between two of the strands. In fact, the structure is so regular that water molecules on the outside of the can also form stripes around the surface of the cylinder. The tightly constructed β barrel would appear to serve the role of protecting the fluorophore well, providing overall stability and resistance to unfolding caused by heat and denaturants [18].

Analysis of a hexapeptide derived by proteolysis of purified GFP has led to the prediction that the fluorophore originates from an internal serine 65, tyrosine 66, glycine 67 tripeptide (Ser-Tyr-Gly) sequence which is post-translationally modified to a 4-(p-hydroxybenzylidene)-imidazolidin-5-one structure. The spontaneous formation of the Aequorea green fluorescent protein chromophore within the folded β-can protein structure must necessarily involve at least three key steps: cyclization of the main chain, loss of a molecule of water (dehydration), and oxidation with molecular oxygen. The exact order and mechanism

of these steps is a matter of ongoing investigation. An early, and still generally accepted, proposed mechanism is shown in figure 8. Once pro-

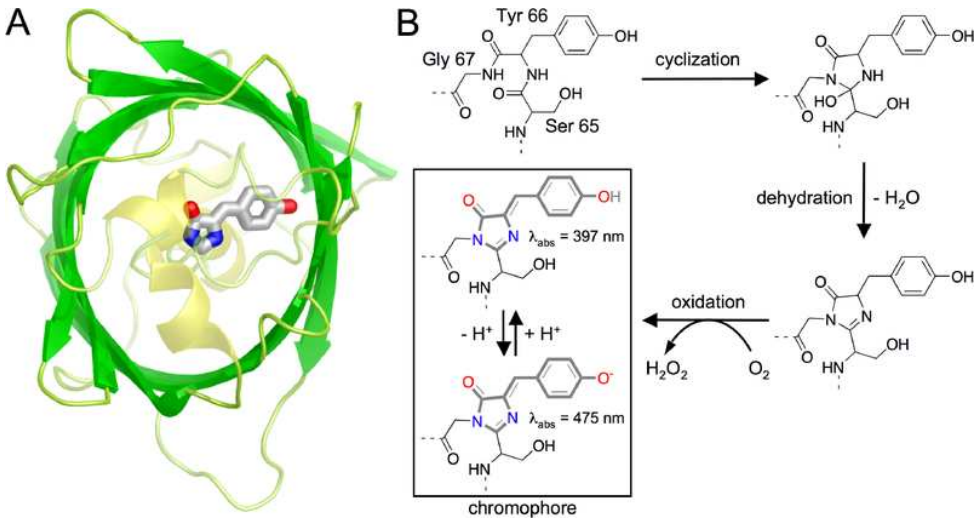

Figure 8: Left: The molecular structure of Aequorea green fluorescent protein as viewed from the top. The chromophore is shown in stick representation where gray represents carbon, red represents oxygen, and blue represents nitrogen. Helices closest to the viewer are rendered transparent. Right: A proposed mechanism for the series of post-translational modifications that converts the serine 65, tyrosine 66, glycine 67 tripeptide sequence into the fluorescent chromophore.

duced, however, GFP is quite thermostable.

Numerous subsequent structures of fluorescent protein homologues from other organisms have revealed that all fluorescent proteins share the β-can fold and thus belong to a protein superfamily defined by similarity to the Aequorea green fluorescent protein archetype. Mutations in regions of the sequence adjacent to the fluorophorc, that is, in the range of positions 65-67, have been systematically explored in order to engeneer the structure to control the emission properties of the protein. Actually some of these attempts produced significant wavelength shifts, most suffer a loss of fluorescence intensity.

5 Absorbance and fluorescent properties

The currently known GFP variants may be divided into seven classes based on the distinctive component of their chromophores: class 1, wild-type mixture of neutral phenol and anionic phenolate; class 2, phenolate anion; class 3, neutral phenol; class 4, phenolate anion with stackedπ-electron system; class 5, indole; class 6, imidazole; and class 7, phenyl [7]. Each class has a distinct set of excitation and emission wavelengths. Structures of the resulting chromophores of the six major classes of GFP-mutant are shown in figure 9, together with typical fluorescence spectra. The denatured wild-type protein absorbs maximally at 384 nm at neutral or acidic pH and at 448 nm at alkaline pH, with a pKa of 8.1. This rough similarity to the absorbance and excitation maxima of the intact protein was a primary motivation for assigning the 395- and 470-nm excitation peaks of the latter to the neutral and anionic chromophores. Denatured GFPs or small proteolytic fragments carrying the chromophore are essentially totally non fluorescent, presumably because the chromophore is unprotected from quenching by jostling water dipoles, paramagnetic oxygen molecules, or cis-trans isomerization. The slight difference in absorbance wavelengths between denatured and intact proteins is not unreasonable for the structured environment of the latter. Theoretical calculations of the energy levels of the chromophore in vacuo have led to the proposal that the imidazolinonering nitrogen adjacent to the hydroxybenzylidene must be protonated. However, the large effects on the chromophore of buried water molecules and the microenvironment supplied by the protein would seem to provide a chemically more plausible explanation.

One of the most promising new techniques in high-resolution fluorescence microscopy is two-photon excitation, in which two infrared photons

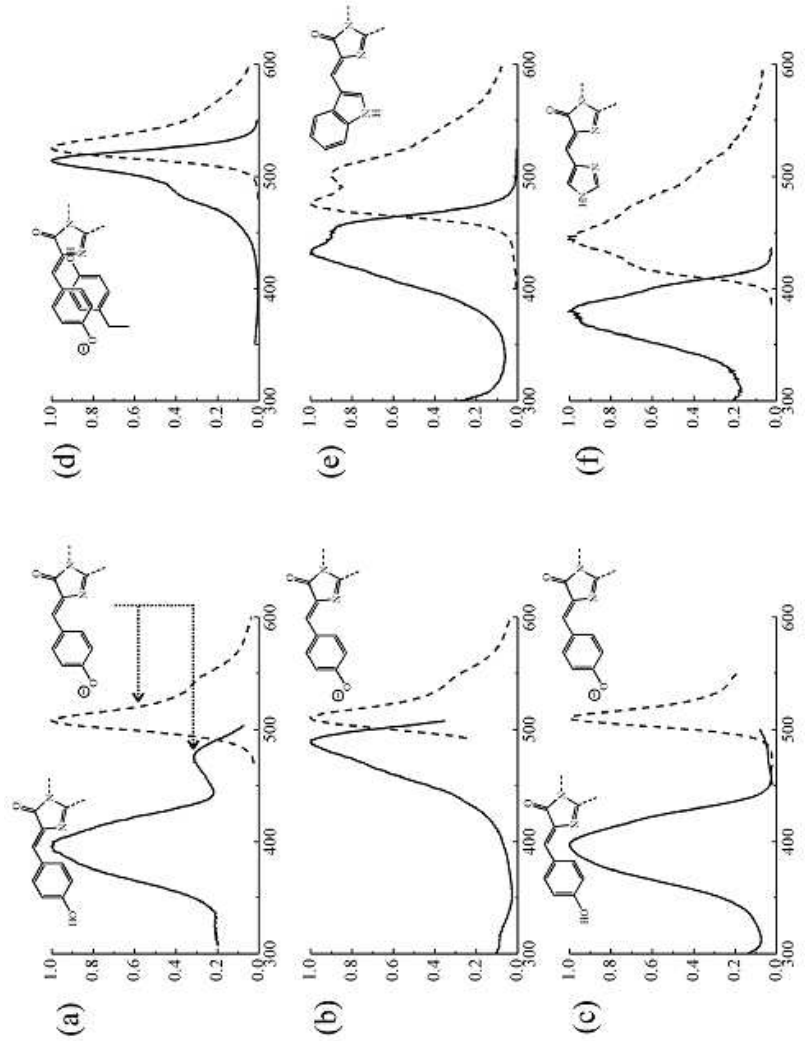

Figure 9: Fluorescence excitation and emission spectra (solid and dashed lines, respectively) for typical members of the six major classes of GFPmutants, together with the chromophore structures believed to be responsible for the spectra. Spectra have been normalized to a maximum amplitude of 1. The actual GFPs depicted are (a) wild-type, (b) Emerald, (c) H9-40, (d) Topaz, (e) W1B, and (f) P4-3. [7]

hit a fluorophore within a few femtoseconds of each other and sum their energy to simulate a single photon of half wavelength, that is, ultraviolet to blue. Such coincidence of infrared photons requires extremely high fluxes and therefore occurs to a significant extent only at the focus of a microscope objective of high numerical aperture, illuminated by a pulsed laser. Because other regions of the specimen are effectively not excited, they neither emit fluorescence nor are subject to photobleaching or photodynamic damage. As in confocal microscopy, the image is built up by scanning the focus point in a raster, but unlike confocal microscopy, out-of-focus planes are protected from bleaching, which is a tremendous advantage for two-photon excitation. GFPs are quite good fluorophores for two-photon excitation [19]. Wild-type GFP is readily excited with 780- to 800-nm pulses, which are in the optimal output range for commercial mode-locked titanium-sapphire lasers. However, the photoisomerization proceeds just as with 390- to 400-nm single-photon excitation (55). Class 3 (neutral phenol) GFP mutants have not been tried but should be better because they disfavor photoisomerization. S65T, the prototypic class 2 GFP mutant, is optimally excited near 910 nm and has a slightly higher two-photon cross-section than the wild type. Two-photon excitation is also effective on class 6 (imidazole) blue mutants (43) as well as class 5 (indole) cyan mutants.

As noted above, wild-type GFP at high pH loses absorbance and excitation amplitude at 395 nm and gains amplitude at 470 nm. Such pH values, though mechanistically revealing, are almost never encountered in biology. Wild-type GFP is also quenched by acidic pH values with an apparent pKa near 4.5. Several of the mutants with enhanced spectral properties at pH 7 are actually more acid sensitive than is the wild type; thus EGFP is 50% quenched at pH 5.5. pKa as high as 6.8 are found in some of the class 4 mutants with Thr203 replaced by an aromatic

residue. The mechanistic explanation for these relatively high pKa is not entirely clear. Loss of the Thr203 hydroxyl would indeed be expected to destabilize the phenolate form of the chromophore. However, the effect of acid is to quench the fluorescence altogether rather than simply shift it toward the short wavelengths expected of a protonated chromophore. The sensitivity of some GFPs to mildly acidic pH values carries both advantages and disadvantages. Such GFPs could be quenched to a major extent in acidic organelles such as lysosomes, endosomes, and Golgi compartments. The pH sensitivity of some GFPs can also be put to good use to measure organellar pH by targeting appropriate GFPs to those locations.

Higher GFP concentrations amplify the main excitation peak at 395 nm at the expense of the subsidiary peak at 470 nm. Because the 395- and 470-nm peaks are believed to result from neutral and anionic fluorophores, respectively, aggregation probably inhibits ionization of the fluorophores. Increasing temperature from 15 to 65 °C modestly decreases the 395-nmand increases the 470-nm excitation peak of mature wild-type GFP. Yet higher temperatures cause denaturation, with 50% of fluorescence lost at 78 °C. As already mentioned, much more modest temperature increases from 20 to 37 °C can profoundly decrease maturation efficiency of GFPs lacking mutations to improve folding.

6 Photobleaching

GFPs have a variety of remarkable abilities to undergo photochemical transformations, which enables visualization of the diffusion or trafficking of GFP-tagged proteins. A defined zone within a cell or tissue is momentarily exposed to very bright illumination, which initiates the photochemistry. The subsequent fate of the photoconverted protein is

imaged over time.

In 1997 two different papers demonstrated by performing single molecule imaging some interesting properties about the fluorescence dynamics of the GFP molecule. The first one [20] observed under costant illumination at 488 nm the on/off switching and blinking of single GFP molecules (see figure 10) The second one [21] demonstrated that single-molecule emis-

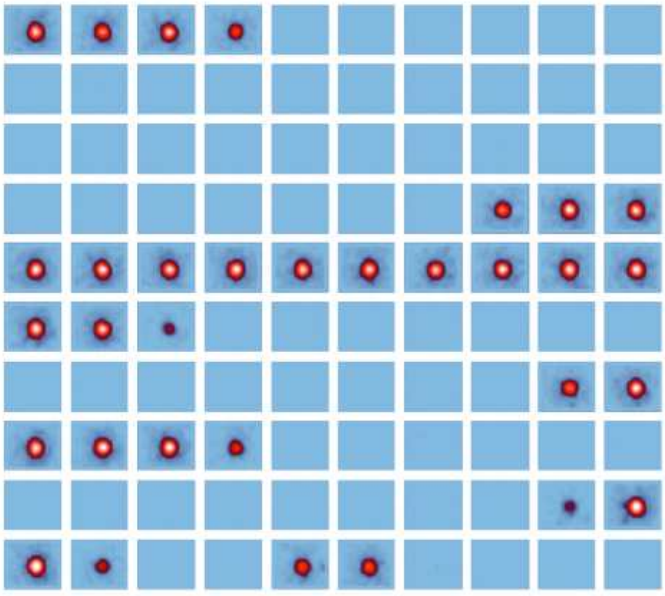

Figure 10: Luminescence of a single GFP molecule as a funcion of time under costant illumination at 488 nm: the on/off switching and blinking of single GFP molecules is evident. [20]

sion of GFP exhibits fast reversible turning on and off events (blinking) and ultimate switching off (photobleaching) (see figure 11).

At least four distinct types of semipermanent photochemical transformation have been reported from one or more GFPs: (a) simple irreversible photobleaching, (b) conversion from a 395- to 475-nm excitation maximum, (c) loss of 488-nm-excited fluorescence, reversible by illumination at 406 nm, and (d) generation of rhodamine-like orange or red

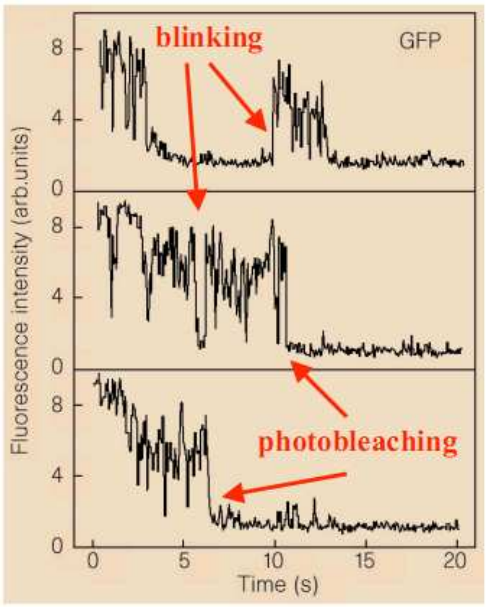

Figure 11: Fluorescence intensity of GFP molecule as a function of time: GFP exhibits fast reversible turning on and off events (blinking) and ultimate switching off (photobleaching). [21]

fluorescence upon illumination at 488 nm under strictly anaerobic conditions.

Photobleaching is the simplest and most universal behavior of fluorophores. Most GFPs are relatively resistant to photobleaching, perhaps because the fluorophore is well shielded from chemical reactants such as O_2. The bleach rate of the prototypic class 2 GFP, the S65T mutant, was reported to be relatively indifferent to equilibration with 0-100% oxygen or addition of quenchers of triplet states, singlet oxygen, and radicals. Nevertheless, with sufficient laser power, photobleaching is easily observed and exploited for measurements of fluorescence recovery. The class 6 mutants (BFPs) are generally more photosensitive than classes 1-5. Cell-permeant antioxidants may be helpful in protecting such GFPs from bleaching.

The shifting to a longer-wavelength excitation peak is characteristic of wild-type and other class 1 GFPs. As discussed previously, the mechanism is probably a light-driven proton transfer from the neutral chromophore to the carboxylate of Glu222, yielding an anionic chromophore and a protonated Glu222. This UV-induced enhancement of the blue excitation peak has been exploited to measure lateral diffusion of GFP-tagged proteins. Because the proton transfer is mediated by Thr203 and Ser205, mutation of those residues might be a promising way to enhance this photochromic effect. Indeed, UV irradiation of the double mutant T203S, S205T increases the amplitude of its long-wavelength excitation peak 11.8-fold, whereas wild-type GFP under the same conditions increases by at most 3.6-fold.

The opposite behavior, a shift from a longer to a shorter excitation wavelength, seems to occur in class 4 mutants. Upon intense laser llumination and observation of the fluorescence from single molecules immobilized in a polyacrylamide gel, such mutants both blink reversibly on a time scale of seconds and switch the fluorescence off over tens of seconds. However, the apparent bleaching can be reversed by illumination at short wavelengths such as 406 nm. Probably the chromophore, which is normally mostly anionic, can eventually be driven into a protonated state with an excitation maximum near 405 nm, where upon it appears non fluorescent and bleached to the probe laser at 488 nm. However, excitation of the protonated state then restores the normal anionic state. Such cycling can be repeated many times with apparently no fatigue, so that it potentially represents a basis for an optical memory at the single molecule level. It might also be particularly advantageous for multiple determinations of diffusion or trafficking on the same region of interest.

A variety of GFPs, including wild-type, S65T, and EGFP, undergo a remarkable photoconversion to a red fluorescent species under rigor-

ously anaerobic conditions, for example, in microorganisms that have exhausted the oxygen in the medium, or in the presence of oxygen scavengers such as glucose plus glucose oxidase and catalase. The nature of this species emitting at 600 nm remains to be clarified. This effect has been used to measure the diffusibility of GFP in live bacteria. One complication is that the red emission develops with an exponential time constant of about 0.7 s after the illuminating flash.

Photobleaching of GFP has been reported to be slow [22], certainly much slower than fluorescein under similar conditions. For istance, continuous observation in a confocal microscope for 20 min only reduced GFP intensity to one-half its original value. Contrary to early reports, the photobleaching rate of wild-type GFP is close to the same whether it is excited in the UV or the blue, but the decrease in UV-excited fluorescence appears more rapid because of photoisomerization. Wild-type GFP has two absorption peak, 395 and 475 nm, which are thought to be related via rotation about a bond (isomerization) within the chromophore structure. The isomerization can be induced by irradiation of wild-type GFP with either 395- and 490-nm light and the kinetics of this photoinduced reaction have recently been measured. The photoisomerization affects the brightness of wild-type GFP during visualization. The photoisomerization can also be used to photoactivate wild-type GFP. That is, the fluorescence properties of GFP change after irradiation. When wild-type GFP is irradiated with either UV (395 nm) or blue light (488 nm), photoisomerization occurs and causes an increase in the 475 nm peak and a decrease in the 395 nm peak. In this manner, UV pre-exposure can be used to increase the blue excitation brightness of wild type GFP.

7 Applications

The most popular applications of fluorescent proteins involve exploiting them for imaging of the localization and dynamics of specific organelles or recombinant proteins in live cells. Through the use of fluorescence microscopy, the morphology, dynamics, and distribution of the organelle can be imaged as a function of time. The procedure for imaging of a fusion between a fluorescent protein and a specific protein-of-interest (in order to gain insight into its localization and dynamics) is identical. The availability of a broad selection of colors of fluorescent protein has provided researchers with the means to image the localization of multiple organelles and/or proteins-of-interest, simultaneously.

The availability of a broad selection of colors of fluorescent protein has also enabled researchers to develop methods to probe whether two proteins are within a distance of less than 10 nm of each other. The observation of colocalization for two different proteins-of-interest fused to different colors of fluorescent protein is insufficient to address this question, since the theoretical resolution limit of conventional optical imaging is several hundred nanometers. To obtain information on the proximity of two proteins at better than 10 nm resolution, investigators exploit the phenomenon of Förster resonance energy transfer (FRET)[1]. Accordingly, by expressing the donor fluorescent protein as a fusion with one protein-of-interest and the acceptor fluorescent protein as a fusion with a second protein-of-interest, the distance between the two proteins-of-

[1]FRET is the distance- and orientation-dependent radiationless transfer of excitation energy from a donor fluorophore to an acceptor chromophore. The greater the extent of the spectral overlap between the donor emission and the acceptor excitation, the more efficient the energy transfer is for a particular FRET pair of fluorescent proteins. Since spectral overlap is constant, and orientations are assumed to be random, the efficiency of FRET is generally a good indicator of distance between the donor and acceptor fluorescent protein.

interest can be inferred from the FRET efficiency measured using live cell fluorescence microsopy. In contrast to intermolecular FRET for the investigation of protein-protein interactions, intramolecular FRET between two fluorescent proteins fused in the same polypeptide chain can be used to investigate small molecule dynamics and enzyme activities in a live cell. These intramolecular FRET constructs are often referred to as reporters or biosensors of biochemical activities. One of the most well known examples of such reporters are the *Cameleons* which enable imaging of intracellular calcium ion concentrations.

Variants derived from Aequorea green fluorescent protein have proven to be fairly tolerant of a variety of dramatic structural manipulations including the genetic insertion of a second protein, circular permutation, and even splitting into two polypeptide chains that are competent to fold into a functional fluorescent protein when brought into close proximity. In certain cases, fluorescent protein chimeras can be used as single fluorescent protein-based (as opposed FRET-based) biosensors. These biosensors are designed such that the binding of the second protein to its ligand, or the ligand-dependent interaction of the attached proteins and/or peptides, results in a change in the protein environment (and thus the fluorescence properties) of the chromophore. Single fluorescent protein-based biosensors have been successfully used for the imaging of localized calcium ion and hydrogen peroxide concentrations.

Illumination can induce conversions between chromophore stereoisomers or photochemical reactions of the chromophore in certain fluorescent proteins. Researchers have exploited these illumination-dependent changes in spectral properties to create optical highlighters that can be turned *on*, and sometimes *off*, by illumination at specific wavelengths. These tools are often referred to as photoactivatable, photoconvertable, or photoswitchable fluorescent proteins, depending on the particular mech-

anism by which the illumination-dependent change occurs. These optical highlighter fluorescent proteins have enabled numerous new applications including imaging of sub-populations of cells during organism development, imaging of fast protein dynamics, and imaging with sub-diffraction limit resolution.

While the creation of fluorescent cells and tissues has now become a relatively mundane practice for many practicing scientists, the creation of a transgenic animal expressing visible amounts of a fluorescent protein is still considered exotic and worthy of media attention. Some of the most highly publicized examples from recent years include: fluorescent zebrafish commercially available from GloFish, a green fluorescent pig, and red fluorescent cats. Some examples are shown in figure 12. Perhaps the most dramatic and technically sophisticated example of creating a fluorescent transgenic animal is the recently reported *Brainbow* mouse in which each cell of a living mouse's brain was colored one of about 90 different colors. Not all examples of transgenic organisms receive so much attention, and numerous researchers are using fluorescent proteins in organisms ranging from plants to mice for the study of the basic biological processes associated with normal and diseased cells. Fluorescent mice have been particularly useful for the study of cancer cells in living animals.

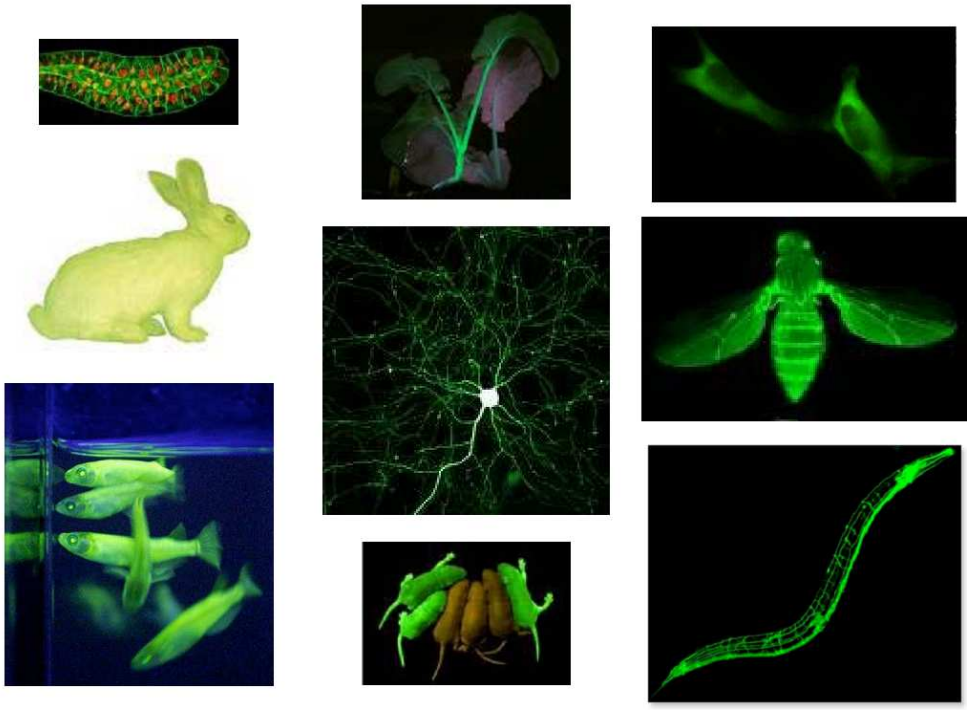

Figure 12: Some examples of the creation of a transgenic animal expressing visible amounts of a fluorescent protein.

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