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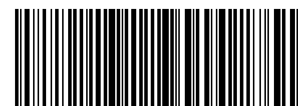

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2009 Green Fluorescent Protein issue

Reviewing the latest developments in the science of green fluorescent protein

Guest Editors Dr Sophie Jackson and Professor Jeremy Sanders

All authors contributed to this issue in honour of the 2008 Nobel Prize winners in Chemistry, Professors Osamu Shimomura, Martin Chalfie and Roger Y. Tsien

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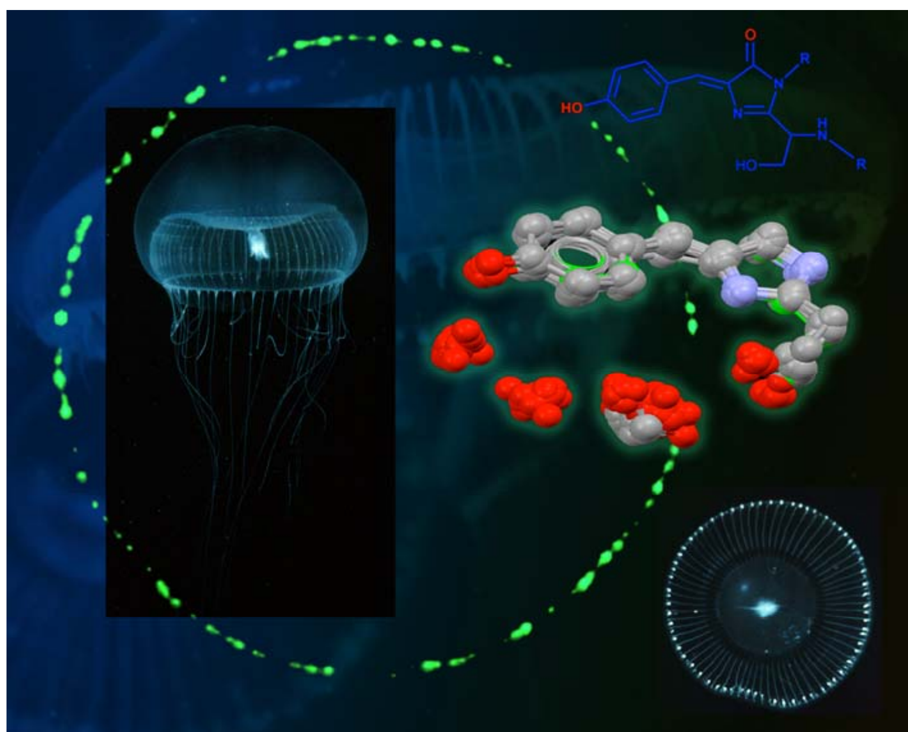

GFP: from jellyfish to the Nobel prize and beyond†

Marc Zimmer

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On December 10, 2008 Osamu Shimomura, Martin Chalfie and Roger Tsien were awarded the Nobel Prize in Chemistry for "the discovery and development of the green fluorescent protein, GFP". The path taken by this jellyfish protein to become one of the most useful tools in modern science and medicine is described. Osamu Shimomura painstakingly isolated GFP from hundreds of thousands of jellyfish, characterized the chromophore and elucidated the mechanism of Aequorean bioluminescence. Martin Chalfie expressed the protein in *E. coli* and *C. elegans*, and Roger Tsien developed a palette of fluorescent proteins that could be used in a myriad of applications.

1. From jellyfish to the Nobel prize

"I decided to find out who the schnook was that won this year's prize. So I opened up my laptop and found out I was the schnook." That was how Marty Chalfie described his discovery that he had been awarded the Nobel Prize in Chemistry for 2008. He shared the award with Roger Tsien and Osamu Shimomura "for the discovery and development of the green fluorescent protein, GFP." This year's award is particularly interesting as it recognizes the basic research that Osamu Shimomura did in order to understand the photophysics involved in Aequorean bioluminescence (a field of research that would probably not be funded under current funding criteria) and the work of Chalfie and Tsien that took an interesting but esoteric protein and made it one of the most useful tools in modern biology and medicine. It is my hope that by the end of this highlight the reader will realize that Shimomura, Chalfie and Tsien are no schnooks and that the GFP Nobel award was richly deserved.

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† Part of a themed issue on the topic of green fluorescent protein (GFP) in honour of the 2008 Nobel Prize winners in Chemistry, Professors Osamu Shimomura, Martin Chalfie and Roger Y. Tsien.

In August 1960 **Osamu Shimomura** left Japan with a Fulbright Fellowship to work in the laboratory of Prof. Frank Johnson at Princeton University. His project was to elucidate the mechanism of bioluminescence of the jellyfish *Aequorea aequorea* (also known as *Aequorea victoria*). The jellyfish were found in the Northeastern Pacific so every summer from 1961 to the eighties Shimomura and his family would make the 5000 km drive from Princeton, New Jersey to the University of Washington's Friday Harbor laboratory. Jellyfish were abundant and could be scooped up from a pier using large shallow nets. Each jellyfish has a couple hundred photoorgans located on the edge of its umbrella, when stimulated they give off green light, see Fig. 1.

Prior to Shimomura's jellyfish work, all known bioluminescent organisms, such as *Cypridina hilgedorfii* studied by Shimomura in Japan[2] and the firefly,[3] used a luciferin/luciferase system to produce light. Shimomura and Johnson discovered that *Aequorea victoria* was different. Two proteins were involved in *Aequorea* bioluminescence—a calcium binding protein and a green fluorescent protein. In their first summer at Friday Harbor Shimomura and Johnson caught over 10 000 jellyfish from which they isolated 1 mg of the luminescent calcium binding protein which they named aequorin.[4] In a 1962 paper devoted to the extraction, purification and properties of aequorin,[4] the fluorescent protein was described as "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." The green fluorescence of the *Aequorea* light organs had been described before,[5] but this was the first

Marc Zimmer at the Nobel Award Ceremony

Marc Zimmer uses computational methods to examine the chromophore formation and photophysics of fluorescent proteins. He has been a faculty member at Connecticut College since 1990. Douglas Prasher (see this highlight) and Bruce Branchini (a firefly luciferase chemist at Connecticut College) introduced him to GFP. Marc wrote Glowing Genes *the first book to be published about GFP and is responsible for the upkeep of "The GFP Site" at http://gfp.conncoll.edu.*

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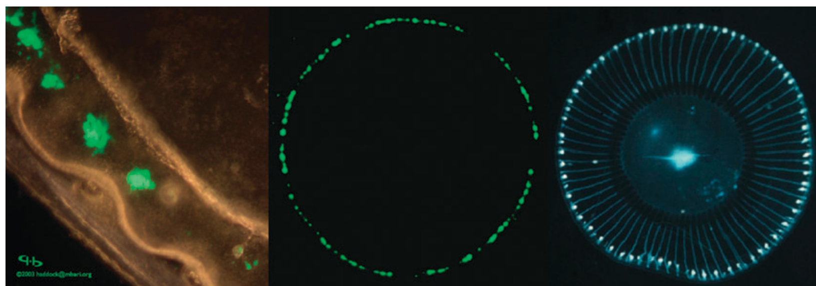

Fig. 1 *Aequorea victoria* photo organs (left), the whole jellyfish in the dark (middle) and under visible light (right). (Photocredits: Steve Haddock and his bioluminescence web page,[1] Monterey Bay Aquarium Research Institute (left image). Osamu Shimomura (right and middle images)).

time it was shown that the green substance responsible for the fluorescence was a protein.

Over the next 20 years in order to isolate enough of the jellyfish proteins Shimomura caught hundreds of thousands of jellyfish. They were plentiful at Friday Harbor "a constant stream of floating jellyfish passed along the side of the lab dock every morning and evening, riding with the current caused by the tide. Sometimes they were extremely abundant, covering the surface of the water."[6] Once caught Shimomura used a homemade jellyfish slicer to cut-off the part of the jellyfish umbrella that contained the photoorgans. When the rings of twenty to thirty jellyfish were squeezed through a rayon gauze, a faintly luminescent liquid called squeezate was obtained. In the squeezate aequorin gives off blue light upon binding calcium, however in the jellyfish radiationless (Förster-type) energy transfer occurs and the fluorescent protein absorbs the blue light emitted by aequorin (λ_{max} = 470 nm) and fluoresces green (λ_{max} = 509 nm).[7,8] Hence it was named green fluorescent protein (GFP),[9] eqn (1).

It was easier to isolate aequorin than GFP, therefore Shimomura concentrated most of his research effort on studying aequorin[6,10,11] and he thinks that his best work was done in this area, but it is his research on GFP, the protein associated with aequorin in *Aequorea victoria*, that garnered him the Nobel prize. By 1971 Shimomura and his co-workers had collected enough GFP to start analyzing it. In 1974 he described the purification and crystallization of GFP, as well as the intermolecular energy transfer between aequorin and GFP in the jellyfish. This Förster-type energy transfer also occurs when aequorin and GFP are co-absorbed on a Sephadex column, eqn (1).[8]

Aequorea GFP and the GFP found in the sea pansy *Renilla*[12] were the only fluorescent proteins known at the time. One of Shimomura's most important contributions to the field was to determine the structure of the chromophore in GFP. He denatured GFP and digested it with papain. Only one of the fragments obtained absorbed above 300 nm and had a similar absorption spectrum to GFP. Although it did not fluoresce it was assumed that this was the chromophore. Acid hydrolysis, UV and mass spectroscopy as well as synthesis of model compounds were used to determine a structure for the chromophore shown in Fig. 2.[13] Since then the structure of the chromophore proposed by Shimomura has been confirmed.[14–16]

Shimomura's research was basic research at its best. He spent more than twenty years elucidating the photophysics of *Aequorea* bioluminescence. Shimomura never foresaw the multitude of uses for GFP and although he was intrigued by the potential uses of aequorin as a calcium monitor that never drove his research.[17] In today's funding climate it is unlikely that his research would have been funded. Fortunately he found funding and laid the foundation of the 2008 Nobel Prize in Chemistry.

During the late seventies Milt Cormier's laboratory isolated and characterized the proteins involved in the bioluminescence observed in the sea pansy, *Renilla*. There are many similarities between *Renilla* and *Aequorea*, both have a green fluorescent protein that is excited by radiationless energy transfer from a neighboring blue luminescent protein and both their GFPs have similar but not identical chromophores.[12] Therefore it is not surprising that the Cormier group examined the bioluminescence of both organisms. Isolation, purification and characterization of these proteins was painstaking work since thousands of animals were needed to obtain the few milligrams required to do the characterization. Advances in cloning promised to solve these problems. Bill Ward, a postdoc in Cormier's lab, took the first step by sequencing *Aequorea* aequorin and GFP, then another postdoc in the lab, Doug Prasher, cloned *Aequorea* aequorin.[18] In Cormier's lab Prasher also started to clone *Aequorea* GFP. He successfully cloned the GFP gene from the lab's *Aequorea* cDNA library, but upon sequencing the gene found that it only represented 70% of the full-length gene.[19] At that time Prasher moved from the Cormier lab and got a position at Woods Hole Oceanographic Institute.

In the Cormier group the main incentive for the cloning of aequorin and GFP was the production of larger amounts of the proteins,[19] but no one had considered using it as a genetically encoded fluorophore. While at Woods Hole, Douglas Prasher worked on using aequorin as a genetically incorporated calcium sensor and was the first to get the idea that GFP could be used in imaging. After collecting more jellyfish at Friday Harbor, Prasher sequenced and cloned GFP.[20] Fig. 3 lists the DNA

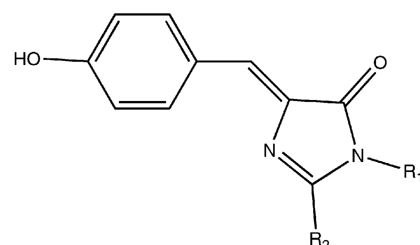

Fig. 2 Structure of the chromophore of *Aequorea* GFP.[13]

$$\text{Aequorin} + \text{Ca}^{2+} \xrightarrow{\text{Blue Light}} \text{GFP} \xrightarrow{\text{Green Light}} \qquad (1)$$

Eqn 1 Upon binding calcium aequorin emits blue light that undergoes radiationless energy transfer to green fluorescent protein, which emits the green light shown in Fig. 1.

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tacacacgaataaaagataaacaaag atg agt aaa gga gaa gaa ctt ttc act
                            M   S   K   G   E   E   L   F   T
gga gtt gtc cca att ctt gtt gaa tta gat ggc gat gtt aat ggg
 G   V   V   P   I   L   V   E   L   D   G   D   V   N   G
caa aaa ttc tct gtc agt gga gag ggt gaa ggt gat gca aca tac
 Q   K   F   S   V   S   G   E   G   E   G   D   A   T   Y
gga aaa ctt acc ctt aaa ttt att tgc act act ggg aag cta cct
 G   K   L   T   L   K   F   I   C   T   T   G   K   L   P
gtt cca tgg cca aca ctt gtc act act ttc tct tat ggt gtt caa
 V   P   W   P   T   L   V   T   T   F   S   Y   G   V   Q
tgc ttt tca aga tac cca gat cat atg aaa cag cat gac ttt ttc
 C   F   S   R   Y   P   D   H   M   K   Q   H   D   F   F
aag agt gcc atg ccc gaa ggt tat gta cag gaa aga act ata ttt
 K   S   A   M   P   E   G   Y   V   Q   E   R   T   I   F
tac aaa gat gac ggg aac tac aag aca cgt gct gaa gtc aag ttt
 Y   K   D   D   G   N   Y   K   T   R   A   E   V   K   F
gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt att gat
 E   G   D   T   L   V   N   R   I   E   L   K   G   I   D
ttt aaa gaa gat gga aac att ctt gga cac aaa atg gaa tac aac
 F   K   E   D   G   N   I   L   G   H   K   M   E   Y   N
tat aac tca cat aat gta tac atc atg gca gac aaa cca aag aat
 Y   N   S   H   N   V   Y   I   M   A   D   K   Q   K   N
gga atc aaa gtt aac ttc aaa att aga cac aac att caa gat gga
 G   I   K   V   N   F   K   I   R   H   N   I   K   D   G
agc gtt caa tta gca gac cat tat caa caa aat act cca att ggc
 S   V   Q   L   A   D   H   Y   Q   Q   N   T   P   I   G
gat ggc cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa
 D   G   P   V   L   L   P   D   N   H   Y   L   S   T   Q
tct gcc ctt tcc aaa gat ccc aac gaa aag aga gat cac atg atc
 S   A   L   S   K   D   P   N   E   K   R   D   H   M   I
ctt Ctt gag ttt gta aca gct gct ggg att aca cat ggc atg gat
 L   L   E   F   V   T   A   A   G   I   T   H   G   M   D
gaa cta tac aaa taa atgtccagac ttccaattaga cactaaagtg
 E   L   Y   K
tccgaacaat tactaaaatc tcagggttcc tggttaaatt caggctgaga
tattatttat atatttatag attcattaaa attgtatgaa taatttattg
atgttattga tagaggttat tttcttatta aacaggctac ttggagagta
ttcttaattc tatattaatt acaatttgat ttgacttgct caaa
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Fig. 3 Nucleotide and amino acid sequence of GFP cDNA as reported by Prasher.[20] The chromophore forming amino acids are bold and underlined. Additional nucleotides preceding and following the GFP gene in the Prasher vector are italicized.

and protein sequence. The resultant cloned GFP was not fluorescent and Prasher concluded, "These results will enable us to construct an expression vector for the preparation of non-fluorescent apoGFP."[20] This view that GFP would not be the genetically encoded fluorophore envisioned by Prasher when he started cloning GFP was reinforced in a follow-up paper on the structure of the chromophore in GFP in which Bill Ward wrote, "The post-translational events required for chromophore formation are not yet understood. It is very unlikely that the chromophore forms spontaneously, but its formation probably requires enzymatic machinery."[14] Unable to find more funding for his GFP work and not confident that GFP would function as a tracer molecule Doug Prasher focused on his other research projects.

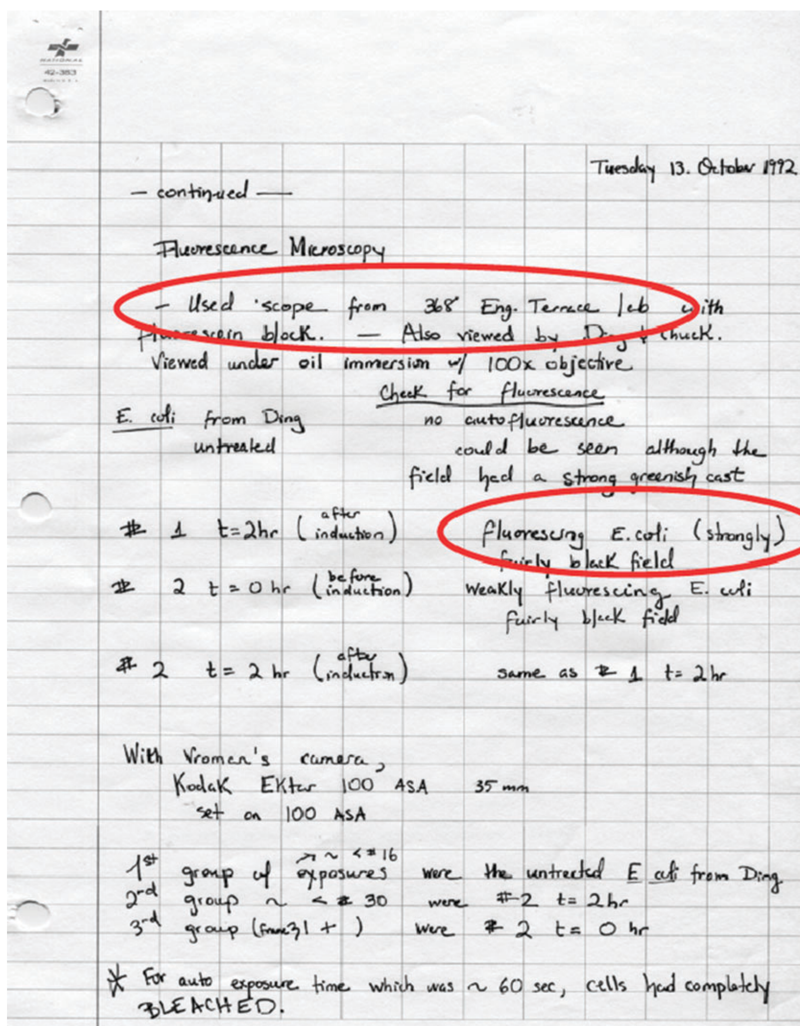
Marty Chalfie's road to the Nobel Chemistry Prize started in High School, where he was friends with Bob Horvitz. After an undergraduate degree with marginal chemistry grades, a year of high school chemistry teaching and a PhD in physiology from Harvard University in 1972, it was the advice of the same Bob Horvitz that convinced Chalfie to do his postdoctoral studies in the laboratory of Sydney Brenner. There he worked on *Caenorhabditis elegans* with Brenner, Horvitz and Sulston, who would go on to be awarded the 2002 Nobel Prize in Medicine. *C. elegans* is see-through and so it was that Chalfie first had the idea of using fluorescent proteins to see when protein expression occurred in *C. elegans*. The idea came to him just a little after noon on Tuesday, April 25th, 1989. Paul Brehm, then at Tufts, was giving a noon seminar to the neurobiology groups at Columbia University. In the talk he described Shimomura's work and the role of GFP in emission of green light by *Aequorea victoria*, eqn (1). At that point of the seminar Chalfie stopped paying attention and started dreaming about using the mechanosensor promoters he was studying in *C. elegans* to promote expression of the fluorescent GFP. After the talk he spent a few days trying to find out whether someone had cloned GFP. He heard about Prasher's work and contacted him. They discovered they had similar ideas and agreed to collaborate once Prasher had succeeded in cloning the GFP cDNA.

A few years later after having cloned (non-fluorescent) GFP Prasher tried contacting Chalfie, but was unsuccessful as Chalfie was on sabbatical at the University of Utah. That would have been the end of Chalfie's involvement in the GFP story, however in September 1992 Chalfie got a rotation student with fluorescence microscopy experience. The GFP idea popped up again as it would be a great rotation project, so he decided to see if Prasher had cloned GFP. Chalfie found the GFP cloning paper in *Gene*, called Prasher to re-establish the collaboration and 6 days later was sent the GFP gene. At this point Chalfie had two options, he could cut the GFP gene out of the vector sent by Prasher using the same restriction enzymes Prasher had used, giving him a GFP gene with some additional nucleotides before and after

the GFP gene, see Fig. 3, or he could use PCR to amplify the GFP coding gene. Fortunately he chose the latter route for it was the DNA that preceded the GFP gene, Fig. 3, that prevent the correct folding of GFP and its subsequent autocatalytic chromophore formation.[21] One month after receiving the GFP cDNA the rotation student, Ghia Euskirchen, succeeded in creating green fluorescent *E. coli*, see Fig. 4. There were two reasons she was successful. Firstly, she used only the GFP coding region, and secondly she had experience with and had access to fluorescence microscopes, which allowed her to distinguish between the inherent green autofluorescence of the bacteria and GFP fluorescence. This was a major breakthrough. GFP autocatalytically formed its own chromophore. It didn't need any other enzymes to become fluorescent, which presumably meant that fluorescent GFP could be expressed in all living organisms. Indeed Chalfie was soon able to use known promoters to express GFP in the touch neurons of *C. elegans*.[22]

Bill Ward, who had worked with Cormier and Prasher, joined the collaboration and showed that the absorption and emission properties of GFP were identical in *E. coli* and in the native jellyfish GFP.[22] Tulle Hazelrigg, who happens to be married to Martin Chalfie and is an excellent scientist independent of Chalfie, was responsible for the next important contribution to the GFP field. She made the first GFP fusion protein and proved that it could functionally replace the original protein thereby showing where in the cell the protein resided.[23]

Douglas Prasher had two requests for his GFP gene, both Martin Chalfie and **Roger Tsien** had conceived of using GFP as a genetically encoded tracer molecule. Tsien wanted to follow cAMP in live cells and also collaborated with Prasher. In fact Roger Tsien's request for the gene preceded Chalfie's request and he had the gene before Chalfie. However Roger Tsien's lab was a chemistry lab and he didn't have a molecular biologist who could work with GFP DNA. He had to wait for a post-doc with the appropriate experience to arrive in his lab before he could try using GFP as a fusion tag. By the time Roger Heim, the post-doc, arrived in the Tsien lab, Euskirchen and Chalfie had already expressed fluorescent GFP in *E. coli*. This did not deter Tsien who has largely been responsible for maturing the fluorescent protein (FP) field and for developing a palette of user friendly FPs.


Tuesday 13. October 1992

— continued —

Fluorescence Microscopy

– Used 'scope from 368' Eng. Terrace lab with [illegible] block. — Also viewed by [illegible] Chuck.
Viewed under oil immersion w/ 100x objective

Check for fluorescence

E. coli from Ding untreated — no autofluorescence could be seen although the field had a strong greenish cast

1 t=2hr (after induction) — fluorescing E. coli (strongly) fairly black field

2 t = 0 hr (before induction) — Weakly fluorescing E. coli fairly black field

2 t= 2 hr (after induction) — same as # 1 t= 2 hr

With Vromen's camera,
Kodak Ektar 100 ASA 35 mm
set on 100 ASA

1st group of exposures ~ < # 16 were the untreated E coli from Ding
2nd group ~ < # 30 were #2 t= 2 hr
3rd group (from 31 +) were # 2 t= 0 hr

* For auto exposure time which was ~ 60 sec, cells had completely BLEACHED.

Fig. 4 Ghia Euskirchen's lab notebook for October 13, 1992.

In the same year that Chalfie reported[22] the expression of GFP in *E. coli* and *C. elegans* Tsien reported that the autocatalytic chromophore formation in GFP was oxygen dependent and proposed the biosynthetic pathway for chromophore formation shown in Fig. 5.[24] He also described the creation of the first wavelength mutation of GFP and proposed the possibility of utilizing fluorescence energy transfer (FRET) measurements between GFP and its mutants, such as the newly created blue fluorescent protein (BFP = a Y66H GFP mutant).[24]

Wild-type GFP has some deficiencies; one of them being the fact that it has two excitation peaks due to the neutral and anionic forms of the chromophore shown in Fig. 5. Tsien found that the S65T GFP mutant has only one excitation peak, a six-fold increased brightness and a four-fold increase in the rate of oxidation of chromophore. It is the basis of the most commonly used FP, enhanced green fluorescent protein (EGFP).[26,27]

Diffraction quality crystals of GFP were grown by Ward[28] long before it was used as a fluorescent tracer molecule, but it was only in 1996 that the crystal structure of GFP was solved and then it was solved simultaneously by the Phillips[16] (wild-type GFP) and Tsien/Remington[15] (enhanced GFP) groups. GFP has an 11-stranded β-barrel with an α-helix running through the β-barrel. The chromophore is located in the center of the barrel and is protected from bulk

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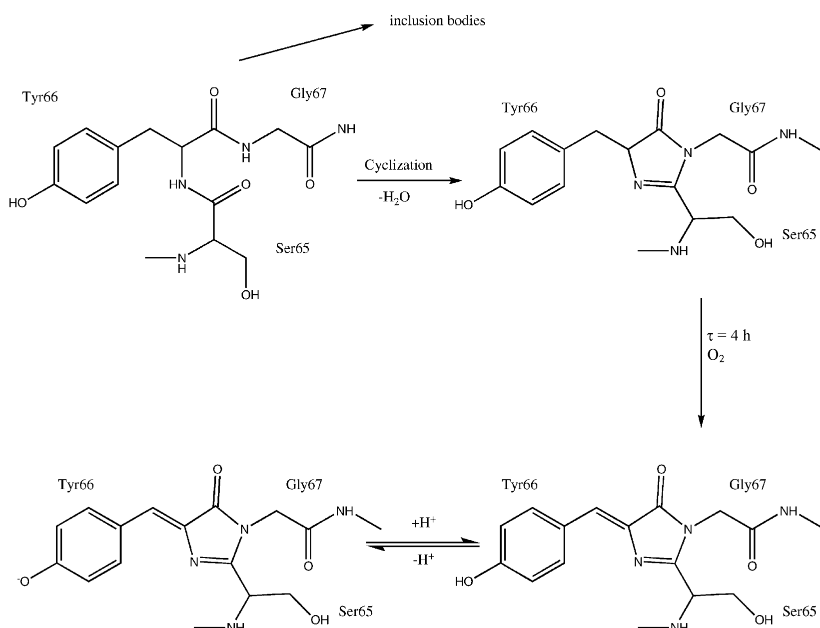

Fig. 5 Proposed scheme for the formation of the GFP chromophore. The upper two forms of GFP are non-fluorescent. Oxidation of Tyr66 is required to form two fluorescent states. The neutral form is excited at 395 nm while the anionic form is excited at 475 nm.[24,25]

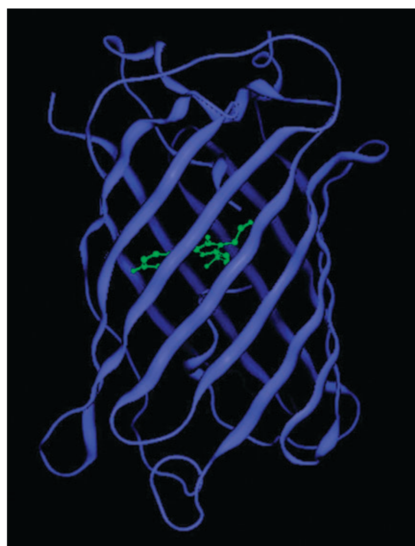

Fig. 6 Crystal structure of GFP. The chromophore is shown in green and is located in the center of the β-barrel. Coordinates obtained from the PDB (1GFL).

solvent, see Fig. 6. The barrel has a diameter of about 24 Å and a height of 42 Å.

The crystal structures revealed several polar residues and water molecules that comprise a hydrogen bonding network around the chromophore. Fig. 7 shows all the short-range interactions between the chromophore and the surrounding protein in S65T GFP.[15]

In the first rationally designed mutant based on the crystal structure of GFP-S65T Tsien and co-workers decided to mutate T203 into a tyrosine so that it could π stack with the phenolic group in the chromophore.[15] The resultant yellow fluorescent protein, YFP, is red-shifted by 16 nm relative to GFP-S65T and does indeed have a π stacking interaction between the chromophore and Tyr203.[29]

The green, blue, cyan and yellow fluorescent proteins developed by the late 90's were the start of a color palette of FPs but a very important color, red, was still missing. A large search for red FPs was initiated. Groups all over the world tried mutating GFP to form a red GFP mutant. This strategy was not very successful and we would have to wait until 2008 before a red mutant of *Aequorea victoria* GFP was created.[30] Other groups took to oceans to look for red bioluminescent organisms. They were no more successful. It took a conceptual shift to find red fluorescent proteins. Lukyanov and Labas made the breakthrough.[31] Thinking that aequorin and GFP might have evolved separately and that fluorescent proteins did not necessarily have to be associated with other chemiluminescent proteins, they decided to look for organisms that were red fluorescent but were not bioluminescent. In aquarium shops in Moscow they found corals containing the first "red" fluorescent protein, DsRed. Since Lukyanov found DsRed in 1999, over 150 distinct fluorescent or colored GFP-like proteins have been reported. In fact the majority of GFP containing organisms are non-bioluminescent.[32] These FPs can be divided into seven groups according to their color and chromophore structure,[33] see Fig. 8.

DsRed was not ideal for imaging work, it is more orange than red, tetrameric, slow to mature and goes through an intermediate green state before the red fluorescent form is obtained. Using mass spectroscopy, theoretical calculations and other methods, Tsien showed that the DsRed chromophore was formed by an additional oxidation which extended the conjugation of the GFP chromophore as shown in Fig. 8C.[34] The red chromophore structure was later confirmed by two crystal structures.[35,36]

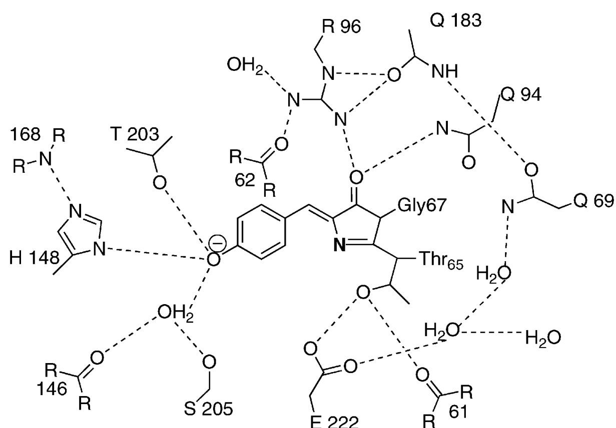

Fig. 7 Schematic diagram of the interactions between the chromophore and its surroundings in the S65T mutant.[15] Possible hydrogen bonds are drawn as dashed lines.

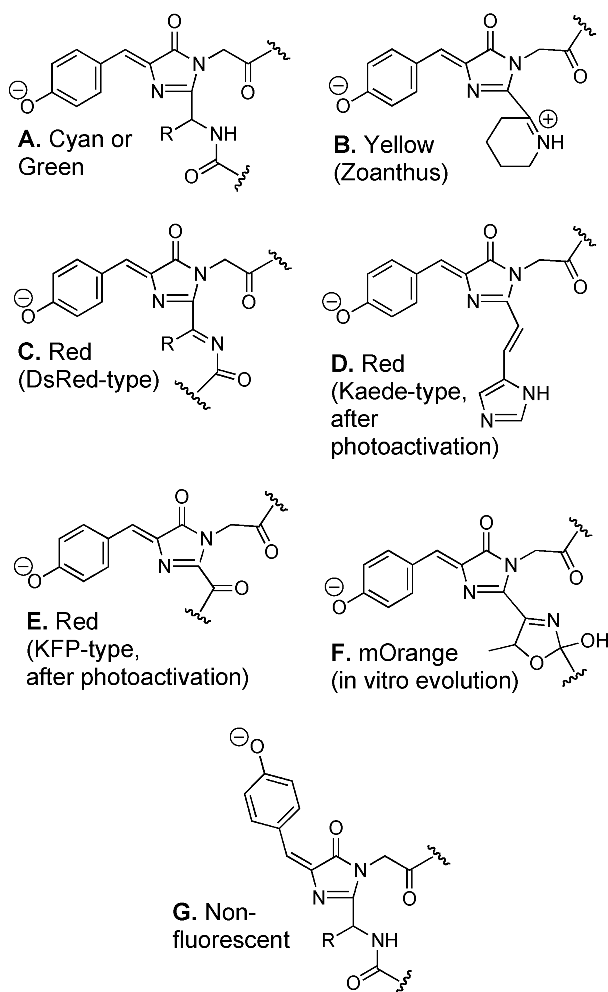

Fig. 8 Chemical diversity of chromophores generated in GFP-like proteins.

It would take 33 mutations to DsRed to create the first monomeric red FP (mRFP1).[37] However the Tsien group was not happy with mRFP1 as it photobleaches quickly and has a significantly reduced fluorescence, they therefore continued to search for more FPs. In 2004 Tsien introduced the mFruits;[38,39] a palette of FPs was rapidly being created, see Fig. 9.

A number of groups have randomly mutated fluorescent proteins and screened for brightness or specific wavelengths.

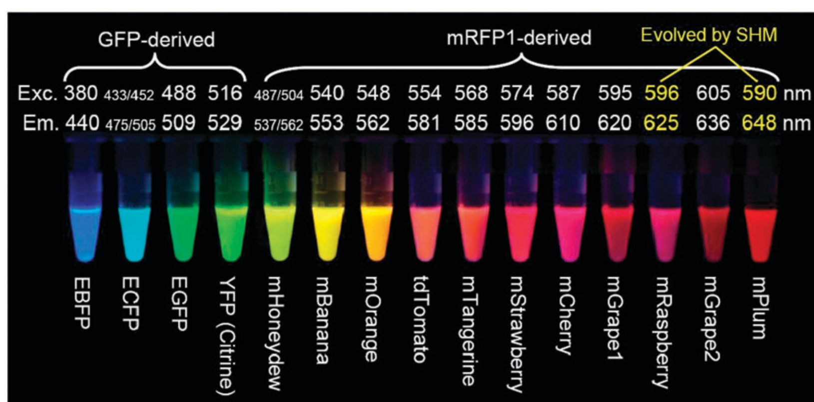

Fig. 9 mFruit FPs derived from mRFP1[39] and by somatic hypermutation (SHM).[38] E stands for enhanced versions of GFP, m are monomeric proteins and tdTomato is a head-to-tail dimer. (Image from Roger Y. Tsien Nobel Lecture 8 December 2008).

In a fairly recent *Nature Methods* article Tsien *et al.* describe how they improved the photostability of bright monomeric orange and red fluorescent proteins by screening for enhanced photostability.[40]

Initially it was Tsien's desire to create a fluorescent sensor for cAMP that got him involved in fluorescent protein research. It is therefore not surprising that over the past 10 years Tsien has also created a number of genetically encoded FRET sensors,[41] such as a calcium,[42,43] protease,[44] phosphorylation[45] and of course a cAMP sensor.[46,47]

2. Beyond

A literature search for papers with "green fluorescent protein" in the title, abstract or keywords found one paper published in 1990, 1441 in 1999 and 4210 in 2008.‡ Two books for the non-scientist have been written about GFP,[17,48] fluorescent proteins have appeared in numerous art exhibits, and a Google search reveals more than 150 000 GFP images. There is clearly a lot of interesting and important GFP research that is beyond the direct jellyfish ⇒ Osamu Shimomura ⇒ Martin Chalfie ⇒ Roger Tsien ⇒ Nobel Prize linage. Many of the most important developments will be highlighted in other reviews in this issue of *Chemical Society Reviews*. I will use Fig. 10 to introduce some GFP research that is beyond the work that was rewarded in 2008's Nobel Chemistry Prize and to discuss some future directions fluorescent protein research might take.

2.1 Spectral diversity and quantum yield

The beach scene in Fig. 10 shows some of the mFruit colors available since 2004.[38,39] There is a continuous effort to find new and brighter colored FPs. The spectral diversity of the fluorescent proteins is obtained by slight variations in the structure of the imidazolinone-based-chromophore (see Fig. 8) and the interactions of these chromophores with the protein environment. The different chromophores are responsible for coarse

‡ Basic Scopus (Elsevier B.V.) search for "green fluorescent protein" in article title, abstract and keywords in all subject areas.

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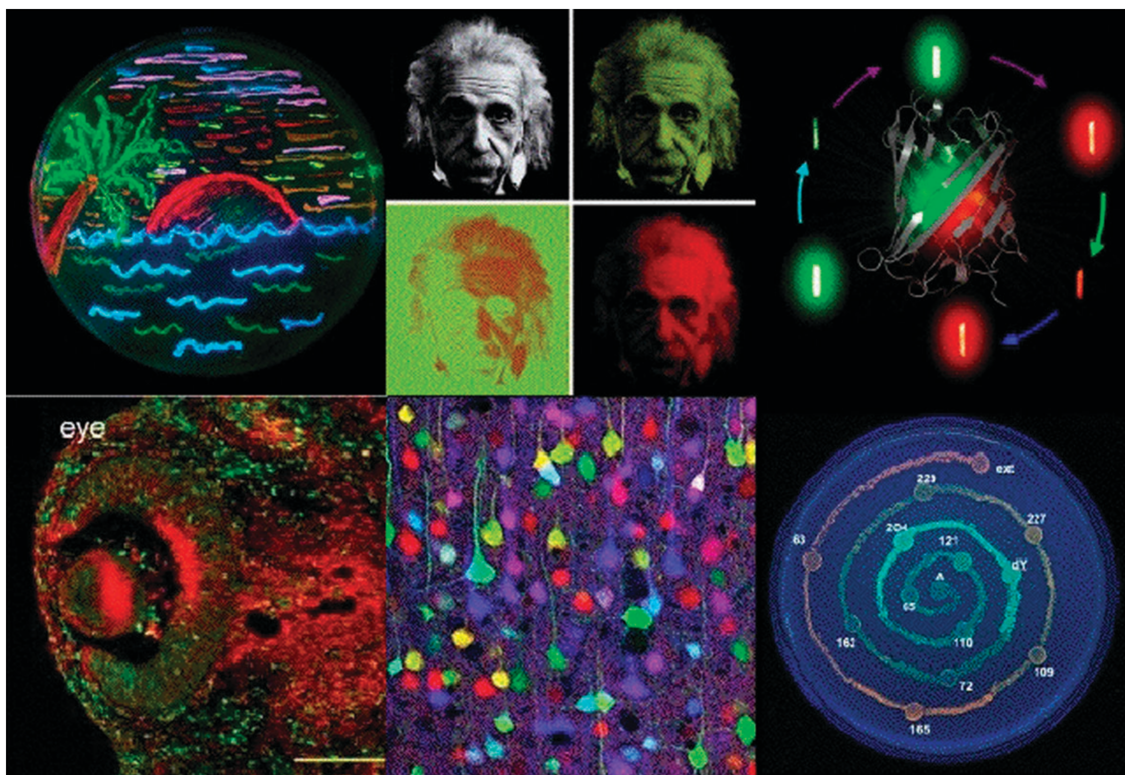

Fig. 10 Clockwise from top left corner. Agar plate of bacterial colonies expressing mFruit fluorescent proteins (*R. Tsien*). To celebrate the 125th birthday of Albert Einstein, a photograph of the scientist was covered with a EosFP tagged polymer coating, which was excited to produce a green fluorescent Einstein and then photoconverted to the red fluorescent version (*J. Wiedenmann*). The X-ray structure of IrisFP colored green and red, surrounded by photographs of IrisFP crystals in its different forms (switched on/off, green/red), recorded in the fluorescence mode (*V. Adam*). Visual appearance of bacteria expressing mutant proteins that retrace the green to red transition within the phylogenetic tree of colors from corals of the family Faviida (*M. Matz*). Brainbow confocal image of cerebral cortex (*Confocal image by Tamily Weissman. Mouse by Jean Livet and Ryan Draft*). Fucci (fluorescent, ubiquitination-based cell cycle indicator) modified cells are yellow at the start of replication, switch to green during S phase and to red during G1. Here they are used to visualize cell cycle progression in mouse eye development (*A. Miyawaki*).

spectral adjustments, while the fine wavelength shifts are accomplished by changing amino acids adjacent to the chromophore. A number of groups are trying to generate fluorescent proteins with new colors by using structural insights into spectral tuning. The field has recently been reviewed by Martynov.[49] Several computational groups have also been examining FPs with an aim of understanding their spectral properties and quantum yields.[50–56]

There is a need for FPs that fluoresce in the far red and infra-red range, therefore existing FPs are continually being mutated and new red FPs, such as mKate,[57,58] mRuby[59] and R10-3,[60] are being created. The brightness of the fluorescent proteins is related to the amount of conformational freedom available to the chromophore within the protein matrix.[61–64] This knowledge has been used to create brighter DsRed mutants.[65]

The palette of mutated FPs is also continually enhanced by the addition of new FPs found in nature. The majority of these FPs have been found in corals,[32,66,67] however FPs have also been found in copepods[68] and even in amphioxus (lower chordates that look like eyeless fishes).[69]

2.2 Optical highlighters

"Facing the Light" was first displayed at the "125 years of Albert Einstein" exhibition of the University of Ulm, which commemorated the 125th birthday of Albert Einstein. Jörg Wiedenmann and Franz Oswald created the images by taking a black and white image of Albert Einstein (top left Einstein in Fig. 10) covering it with a nitrocellulose membrane that had EosFP immobilized on it (top right Einstein). EosFP, named after the goddess of dawn, is a photoconvertible fluorescent protein. Initially it is green fluorescent (bottom left Einstein) but irradiation with UV light (390 ± 30 nm) induces cleavage between the amide nitrogen and the α-carbon atom in the histidine adjacent to the chromophore resulting in a red fluorescent form (bottom right Einstein).

EosFP is an excellent example of a group of FPs that have been found and created that change their emission upon irradiation. They are known as optical highlighters. For convenience they have been classified into three groups: photoactivatable, photoconvertible and photoswitchable FPs.[70]

Photoactivatable FPs are dark and are irreversibly activated by irradiation. For example irradiation of PA-GFP[71] with intense violet light results in a 100-fold increase in green fluorescence. It is presumed that the violet light causes the decarboxylation of Glu222, which aids in the formation of the anionic fluorescent form of the chromophore, see Fig. 11A.

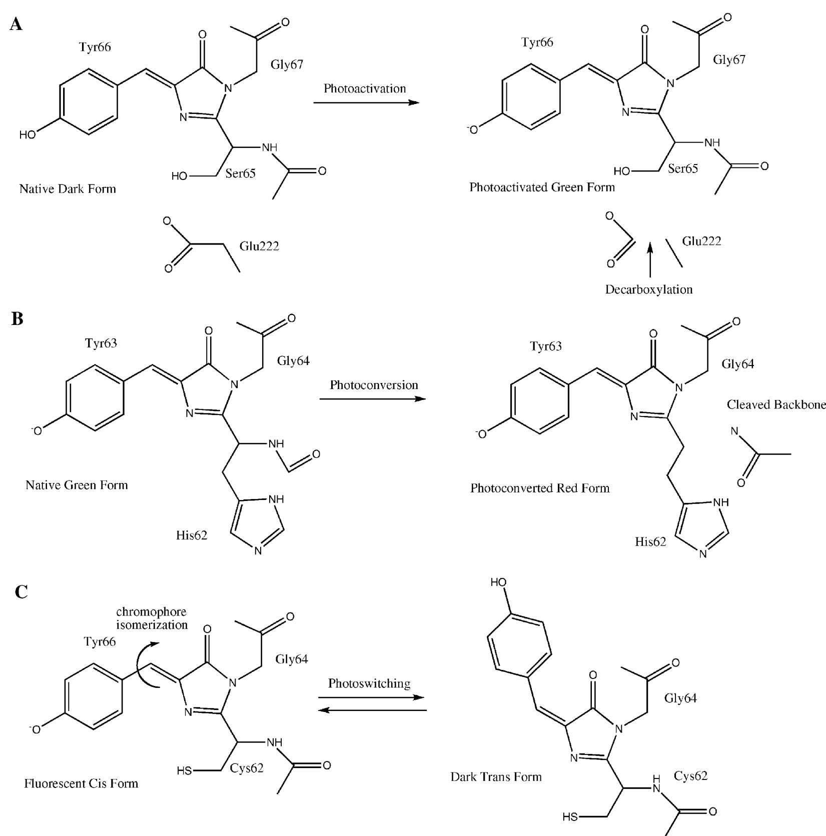

Fig. 11 Photoactivatable (A), photoconvertible (B) and photoswitchable (C) highlighter proteins. See text for more detailed description.

Photoconvertible FPs such as EosFP,[72] Kaede,[73] and Dendra2[74] can be irreversibly converted from a green fluorescent form to a red fluorescent form by violet or ultraviolet irradiation. The photoconversion is presumably associated with a cleavage occurring between the amide nitrogen and the alpha carbon of His62 that is followed by oxidation of the His62 sidechain, see Fig. 11B.

Finally there are photoswitchable FPs which are dark and are reversibly activated by irradiation. It is presumed that photoswitchable FPs such as Dronpa,[75,76] mTFP0.7[77] and KFP[33,78] switch between the dark E (or *trans*) state and the fluorescent Z (or *cis*) state, see Fig. 11C.

Optical highlighters are sure to be an area of much research in the post GFP Nobel prize era. The driving force in this area is the need for more genetically encoded photoactivatable and photoswitchable fluorescent proteins that can be used in the newly developed super-resolution microscopy techniques—FPALM (fluorescence photoactivated localization microscopy),[79] PALM (photoactivated localization microscopy),[80] iPALM (interferometric photoactivated localization microscopy)[81] and STORM (stochastic optical reconstruction microscopy).[82] In late 2008 a mutant of the photoconvertible EosFP was reported. "Like its parent protein EosFP, IrisFP also photoconverts irreversibly to a red-emitting state under violet light because of an extension of the conjugated pi-cloud of the chromophore, accompanied by a cleavage of the polypeptide back-bone. The red form of IrisFP exhibits a second reversible photo-switching process, which may also involve *cis–trans* isomerization of the chromophore."[83] More recently another EosFP mutant, mEos2, was reported, which has a much lower aggregation tendency than EosFP.[84] In the same issue of *Nature Methods* a series of photoactivatable mCherry mutants, named PAmCherry proteins, were reported.[85]

2.3 Evolution and function

More than 15 000 papers have been published that use fluorescent proteins or have studied them, and yet we do not know the function of the fluorescent proteins. Understanding the evolution of fluorescent proteins may one day lead to more knowledge about its function. Did the original FP ancestors have a function that had nothing to do with fluorescence? Quiet possibly since a number of GFP-like proteins are non-fluorescent chromoproteins. Most interesting amongst these non-fluorescent proteins is nidogen, a protein found in basement membranes of animals, including humans. Although it does not contain a central chromophore, the overlap between the G2 nidogen domain and GFP barrels is extremely close (rms deviation of 2.5 Å for a superimposition of all 195 Ca atoms).[86]

An evolutionary analysis has shown that GFP and nidogen belong to the same superfamily;[68] its function is unknown. Parsimony analysis[68] and ancestral reconstruction experiments[32,87] suggest that all but one of the non-green colors arose from an ancestor with a canonical green chromophore. The exception is the yellow protein from *Zoanthus* sp., which is likely to have evolved from a DsRed-like red ancestor.[32] The phylogeny provides an excellent scaffold for identifying the key color-converting sequence changes. The bottom right image in Fig. 10 shows the visual appearance of bacteria expressing mutant proteins that retrace the green-to-red transition from the ancestral green protein to the least evolved red ancestor within the coral family Faviida. A minimum of 12 mutations are required to fully recapitulate the present-day red fluorescence from the ancestral green protein.

2.4 Applications

The 2008 Nobel Prize in Chemistry was awarded to Shimomura, Chalfie and Tsien for their GFP research because GFP has developed into a tremendously useful molecule with applications in many areas of science and medicine. Therefore a highlight of this type should at least mention some GFP applications. Unfortunately it is impossible to review all the applications of fluorescent proteins, I will just mention two of my favorites.

Never before have brains been as beautiful as those shown in the bottom center image in Fig. 10. They belong to

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transgenic mice with fluorescent multicolored neurons created by a genetic strategy that randomly mixes green, cyan and yellow fluorescent proteins in individual neurons, thereby creating a palette of ninety distinctive hues and colors.[88] Using a brainbow of colors, researchers will now be able to map the neural circuits of the brain.

Cell growth occurs through an ordered sequence of events—the cell cycle, which consists of four distinct phases, G1, S, G2 and M phase. Fucci (fluorescent, ubiquitination-based cell cycle indicator) allows cell cycle researchers to visualize cell cycle progression.[89] The Fucci modified cells are yellow at the start of replication, switch to green during S phase and to red during G1. To demonstrate the utility of Fucci, a Fucci mouse was created. The bottom left image in Fig. 10 shows the equilibrium between cell differentiation and cell proliferation that occurs during the development of a mouse eye.

Hopefully these two examples demonstrate some of the utility, beauty and versatility of fluorescent protein-based-techniques, and give the reader a hint that the development and the associated chemical understanding of FPs has just begun.

3. Conclusion

The 2008 Nobel Prize in Chemistry rewards both basic research as well as applied research. While Shimomura's primary interest in GFP was its role in *Aequorea* bioluminescence, Tsien was interested in its practical applications. Hence he has developed brighter, faster maturing, more photostable fluorescent proteins covering a spectrum of colors and has incorporated them into *in vivo* sensors. I hope that this award will remind those in charge of funding research that basic research can open the doors to very useful and often unexpected discoveries. On the other hand I hope that the award will also silence the purists who do not value applied research. Roger Tsien should not have to finish his Nobel speech with the following justification: "Some people have at times criticized us for mainly working on techniques. I would like to draw their attention to an old Chinese proverb that says that if you give a man a fish you feed him for one day, if you teach him how to fish you feed him for a lifetime. That's why we enjoy devising fishing tackle and nets to scoop from the ocean of knowledge."

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