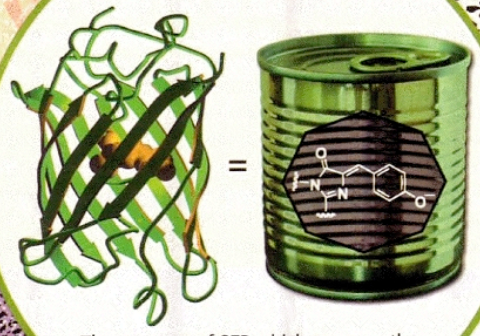
By Hui-wang Ai & Robert E. Campbell

IMAGING

More than just pretty COLOURS:

THE GROWING IMPACT OF FLUORESCENT PROTEINS IN THE LIFE SCIENCES

Figure 1



The structure of GFP, which serves as the archetype for all FPs, is often referred to as a β-can.

How is it that a jellyfish sparked a revolution in biotechnology? The year was 1962 when a Princeton University researcher by the name of Osamu Shimomura reported on the purification and characterization of the protein responsible for the bioluminescence of Aequorea jellyfish. Shimomura had painstakingly harvested many thousands of jellyfish, cut off its bioluminescent organs with scissors and squeezed the proteins from the tissue wrapped in a handkerchief: a procedure that produced a solution known aptly as 'squeezate'. From this squeezate Shimomura isolated the bioluminescent protein aequorin; a tremendous accomplishment in its own right. In a footnote within the manuscript describing this work, he mentions the presence of another curious protein in squeezate that was not bioluminescent but rather fluorescent.[1]

'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 squeezates'

Shimomura could not have anticipated that with this second protein, known as Aequorea Green Fluorescent Protein (GFP), would emerge in the mid 1990s as a powerful tool for life science research. Today GFP is used in thousands of labs around the world as a non-obtrusive genetically encoded marker that enables an untold number of otherwise unimaginable applications.[2] Some of the more common applications include: tracking and imaging fusion proteins in live cells; fluorescence imaging in transgenic tissue and animals; sensing of intracellular enzyme activities; and imaging of transplanted tumours in rodent models of cancer.

Not only is GFP one of the most utilitarian proteins known to science, it is also one of the most aesthetically pleasing. From the macroscopic perspective, solutions of GFP have a pleasant greenish hue as was accurately described by Shimomura. However, it is at the molecular level that GFP is most fetching (Figure 1). GFP is composed of 238 amino acids arranged in an 11-stranded -sheet that is wrapped into an attractive pseudosymmetric cylinder. Given that this structure is somewhat squat, it is often referred to as a β-can.

Located near the centre of the can is the heart of GFP: the visible wavelength fluorophore that makes this protein so unique. Unlike other proteins, such as hemoglobin, that are coloured due to the presence of a bound non-protein chromophore, GFP is self sufficient to generate its intrinsic chromophore from its own polypeptide sequence. That is, the protein requires no other jellyfish-specific proteins or factors for it to undergo the post-translational modifications (a process referred to as maturation) leading to formation of the mature chromophore.

In the presence of molecular oxygen, expression of the GFP gene in a variety of organisms ranging from bacteria to plants to mammals, will result in the formation of green fluorescing cells and/or tissues. It is this functional autonomy that makes GFP so exceptionally useful as a tool in life science research.

One might expect that there is relatively little scientific advance to be made by simply expressing GFP in various cell types and thereby rendering them green fluorescent. Indeed, this is not exactly the type of application that has driven the explosive growth in the popularity of GFP for life science research. The single most important and most common application of GFP is in the study of fusion-protein localization, dynamics and function in live cells. Investigators interested in the biological role of a certain protein can fuse the gene encoding that protein to the gene encoding GFP.

This fusion is done in such a way the protein product will be expressed as a single polypeptide, creating a covalent link between the protein of interest and the GFP. A plasmid containing the chimeric gene under control of a suitable promoter is then used to transfect mammalian cells. The transfected cells integrate the DNA into their own chromosomes and express the chimeric gene, as they would any other gene in their genome, to produce the protein of interest fused to the fluorescent label that is GFP.

In the ideal situation, the protein of interest dictates the localization and dynamics of the fusion protein and the GFP tag is a non-perturbing bystander. Through the use of time-lapse fluorescence microscopy or one of a growing number of variations on this technique, one can create movies that reveal the intracellular spatiotemporal dynamics of the protein of interest. To confirm that the GFP is not disrupting the normal localization of the protein of interest, researchers often stain the cells with labeled antibodies against the endogenous protein of interest and compare the results to that obtained with the GFP tag. Needless to say, the major advantage of the GFP based approach relative to traditional immunohistochemistry is that it enables researchers to investigate protein dynamics in living cells and tissue and is not limited to static localization of proteins in dead and fixed tissue.

By itself, GFP was a wonderful gift from Nature to the scientific community. However, in several regards the wild-type protein is sub-optimal with respect to the quite 'unnatural' applications in which it is typically used. For example, the wild-type protein has evolved to fold and undergo the chromophore-forming reaction most efficiently in the cool temperatures of the ocean waters. When transplanted to tissue culture or a living organism it is often expected to perform these same tasks at a balmy 37 °C; a process which it could accomplish only inefficiently. Fortunately, GFP is remarkably amenable to protein engineering by genetic modification and this process can be used for the discovery of variants with improved properties. Accordingly, one of the first and most significant improvements in the properties of GFP was the engineering of variants that matured much more efficiently at 37 °C.

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Another drawback of GFP is that it was originally available in just the one fluorescent colour that Aequorea had generously provided. However, this limitation was also soon overcome through the efforts of protein engineers, most notably Professor Roger Y. Tsien of the University of California San Diego, who was primarily responsible for the development of blue, cyan, enhanced green, and yellow versions of folding enhanced GFP.[2] This small palette of colour variants, known as BFP, CFP, EGFP, and YFP, respectively (the four leftmost samples in Figure 2), greatly expanded the range of possible applications of the growing family of fluorescent protein (FP) variants. In particular, the availability of a selection of colours enabled simultaneous imaging of multiple different fusion proteins in live cells (Figure 3A) to identify protein pairs that did, or did not, colocalize to specific intracellular compartments. This selection of colours also enabled researchers to begin to ask whether two proteins were physically interacting (or at least within a distance of less than 10 nm of each other).

Such questions cannot be answered by the observation of colocalization since the theoretical resolution limit of conventional optical imaging with visible light is limited to several hundred nanometers. To obtain information on the proximity of two proteins in live cells and with better than 10 nm resolution, investigators exploit the strange phenomenon of Förster (a.k.a. fluorescence) resonance energy transfer (FRET). Although the physical basis of FRET requires a good deal of theory to explain properly, it manifests itself in quite simple and practical manner.

If two fluorophores are close enough together in space, the higher energy fluorophore passes its excited state energy to the lower energy fluorophore that will then emit a photon at its characteristic fluorescence wavelength. In the case of GFP variants, the CFP (higher energy donor) and YFP (lower energy acceptor) pair is widely used for FRET-based detection of protein-protein proximity's and interactions in live cells. By exciting CFP with blue light and imaging of the ratio of yellow to cyan fluorescence with band pass filters, FRET efficiency between protein scan be easily and quantitatively measured using widely available epifluorescence microscopy equipment.

The progress described in the preceding paragraphs describes the state-of-the-art in FP technology circa 1999: developments since then have continued at a breathtaking pace! For example, in late 1999 it was reported, by Professor Sergey A. Lukyanov of the Russian Academy of Science, that coral is a source of homologues of GFP with hues ranging from cyan all the way to red.[3] Despite the best efforts of protein engineers,

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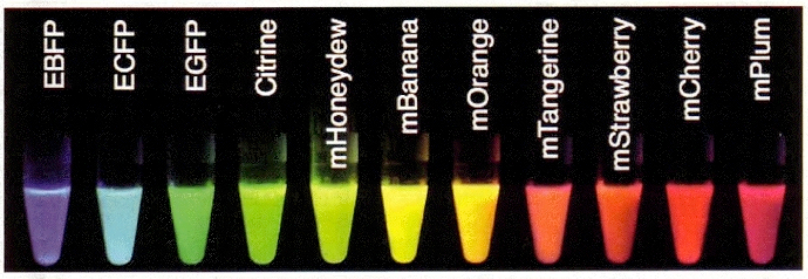


The 'palette' of monomeric FPs for use in live cell imaging (Credit: Nathan Shaner, Paul Steinbach, and Roger Y. Tsien).

variants with only a modest red-shift (i.e. YFP) had been engineered from Aequorea GFP. Clearly the coral had figured out how to do something that scientists had not!

It is now generally understood that the bright and varied colours of reef coral are due to GFP homologues. As was originally done with the Aequorea GFP, coral FPs have now been subjected to extensive amounts of engineering for brighter fluorescence and shifted hues. One particular coral FP, the red fluorescent protein (RFP) from Discosoma coral, has been a particularly fruitful source of improved variants. As with most coral FPs, the wild-type Discosoma RFP is an obligate tetramer.

For some applications the tetrameric structure is not a detriment: the cell shown in Figure 3A is expressing a tetrameric Discosoma RFP that is genetically targeted and correctly localized to the mitochondria. However, there are many other examples in which fusion to a tetrameric RFP drastically perturbs the localization of the protein of interest. Fortunately this obstacle was eventually overcome by the use of extensive protein engineering to convert the tetrameric Discosoma RFP to a monomeric RFP known as mRFP1. Further directed evolution of mRFP1 with selection for brighter and wavelength-shifted variants eventually produced the mFruit series of monomeric yellow, orange, and red FPs (the 7 rightmost samples in Figure 2).[4]

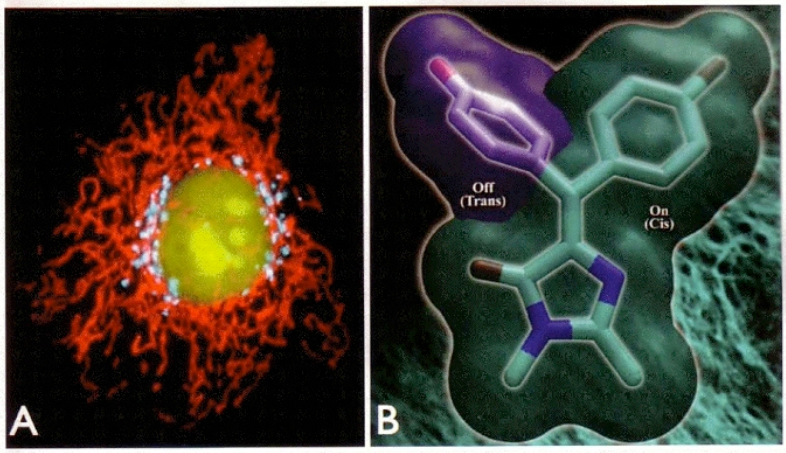
Plasmid DNA containing the genes for each of the mFruit variants is now commercially available from Clontech Laboratories, Inc. of Mountain View, California. A variety of other commercial vendors are now offering engineered coral FPs of a wide variety of colours including, but not limited to: Evrogen of Moscow, Russia; MBL International of Woburn, Massachusetts; and Allele Biotechnology of San Diego, California. Most of the original Aequorea-derived colours are now available from Invitrogen of Carlsbad, California, though many of more recently reported and further improved versions are typically distributed by the lab in which they were developed or by a non-profit plasmid repository such as Addgene Inc. of Cambridge, Massachusetts.

The engineering of various colours of monomeric FPs have been but one of many recent highlights in the fast moving field of FP research. For example, major effort within the FP field has been invested in the engineering of biosensors that change its fluorescence intensity, or fluorescence colour, in response to an external stimulus. One approach to the development of such biosensors is to exploit FRET, and sandwich a 'sensing' domain (that is, a region of protein that undergoes a conformational change when bound to a particular target molecule or when post-translationally modified by an enzyme) between donor and acceptor FPs.

Some notable examples of such biosensors include ones that detect Ca2+, kinase activity, and histone methylation. Another recent highlight has been the development of photoactivable FPs that transition from a non-fluorescent to a fluorescent state when irradiated with a specific wavelength of light, and photoconvertable FPs that transition from one colour to another when irradiated with a specific wavelength of light. By being able to 'turn on' or otherwise manipulate the properties of a subpopulation of FP molecules with light, researchers are gaining exciting new insight into intracellular protein dynamics. In the case of FP variant developed by the authors, atomic structures of the FP in both the 'on' and 'off' state have revealed that a photoinduced cis-trans isomerization of the chromophore is the molecular basis for this intriguing photoswitching phenomenon (Figure 3B).[5]

The field of FP engineering and applications has obviously come a long way since Shimomura's first observation of a greenish solution derived from jellyfish squeezate. Researchers now have at their disposal a set of genetically encoded fluorophores that enable cells, tissues, and even whole animals to be investigated with a level of detail and subtlety that would otherwise be unimaginable. Some of the remaining challenges in this field include the development of extremely photostable FPs suitable for single molecule imaging applications, near-infrared FPs for deep tissue imaging, multiple FRET pairs, and imaging modalities that break the diffraction barrier. Today, in the lab of the author and numerous other labs around the world, researchers are working hard to address these challenges and help ensure that the future of FP-based imaging is a bright one indeed.

Figure 3



A. Fluorescence image of a HeLa cell expressing YFP targeted to the nucleus, CFP targeted to the Golgi apparatus, and Discosoma RFP targeted to the mitochondria (Credit: Michael Davidson). B. The chromophore of a FP in both the 'fluorescence on' and 'fluorescence off' states (Credit: REC and MD).

References:

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Robert E. Campbell is an assistant professor in the Department of Chemistry at the University of Alberta and currently holds a Canada Research Chair in Bioanalytical Chemistry. His research interests lie in the area of protein engineering, with specific emphasis on the development of new fluorescent proteins and sensors for use in live cell imaging.

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