1. The fluorescence process.

1.1 introduction

Fluorescence is the result of a three-stage process that occurs in certain molecules (generally polyaromatic hydrocarbons or heterocycles) called **fluorophores or fluorescent dyes**. A **fluorescent probe** is a fluorophore designed to **respond to a specific stimulus** or **to localize within a specific region of a biological specimen**. The process responsible for the fluorescence of fluorescent probes and other fluorophores is illustrated by the simple electronic-state diagram (Jablonski diagram) shown in **Figure 1**.

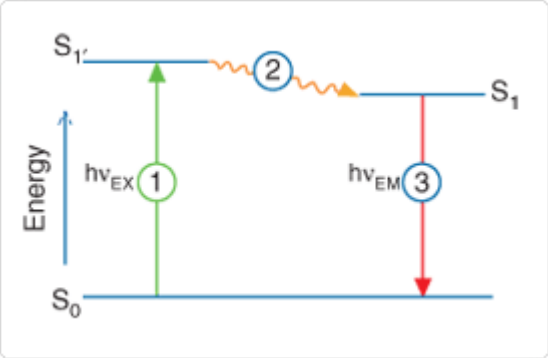


Figure 1. Jablonski diagram illustrating the processes involved in the creation of an excited electronic singlet state by optical absorption and subsequent emission of fluorescence. The labeled stages 1, 2 and 3 are explained in the adjoining text.

Stage 1: Excitation

A photon of energy $h\nu_{EX}$ is supplied by an external source such as an incandescent lamp or a laser and absorbed by the fluorophore, creating an excited electronic singlet state (S_1'). This process distinguishes fluorescence from chemiluminescence, in which the excited state is populated by a chemical reaction.

Stage 2: Excited-State Lifetime

The excited state exists for a finite time (typically 1–10 nanoseconds). During this time, the fluorophore undergoes conformational changes and is also subject to a multitude of possible interactions with its molecular environment. These processes have two important consequences. First, **the energy of S_1' is partially dissipated**, yielding a relaxed singlet excited state (S_1) from which fluorescence emission originates. Second**, not all the molecules initially excited by absorption (Stage 1) return to the ground state (S_0) by fluorescence emission**. Other processes such as collisional quenching, fluorescence resonance energy transfer (FRET) and intersystem crossing (see below) may also depopulate S_1. The fluorescence **quantum yield**, which is the ratio of the number of fluorescence photons emitted (Stage 3) to the number of photons absorbed (Stage 1), is a measure of the relative extent to which these processes occur.

Stage 3: Fluorescence Emission

A photon of energy $h\nu_{EM}$ is emitted, returning the fluorophore to its ground state S_0. Due to energy dissipation during the excited-state lifetime, the energy of this photon is lower, and therefore of longer wavelength, than the excitation photon $h\nu_{EX}$. The difference in energy or wavelength represented by

(hv_{EX} – hv_{EM}) is called the **Stokes shift.** The Stokes shift is fundamental to the sensitivity of fluorescence techniques because it allows emission photons to be detected against a low background, isolated from excitation photons. In contrast, absorption spectrophotometry requires measurement of transmitted light relative to high incident light levels at the same wavelength.

1.2 Fluorescence Spectra

The entire fluorescence process is cyclical. Unless the fluorophore is irreversibly destroyed in the excited state (an important phenomenon known as **photobleaching,** see below), the same fluorophore can be repeatedly excited and detected. The fact that a **single fluorophore can generate many thousands of detectable photons** is fundamental to the **high sensitivity** of fluorescence detection techniques.

For polyatomic molecules in solution, the discrete electronic transitions represented by hv_{EX} and hv_{EM} in Figure 1 are replaced by rather broad energy spectra called the **fluorescence excitation spectrum** and **fluorescence emission spectrum**, respectively. The bandwidths of these spectra are parameters of particular importance for applications in which two or more different fluorophores are simultaneously detected. Under the same conditions, the fluorescence emission spectrum is independent of the excitation wavelength, due to the partial dissipation of excitation energy during the excited-state lifetime, as illustrated in Figure 1. The emission intensity is proportional to the amplitude of the fluorescence excitation spectrum at the excitation wavelength (Figure 2).

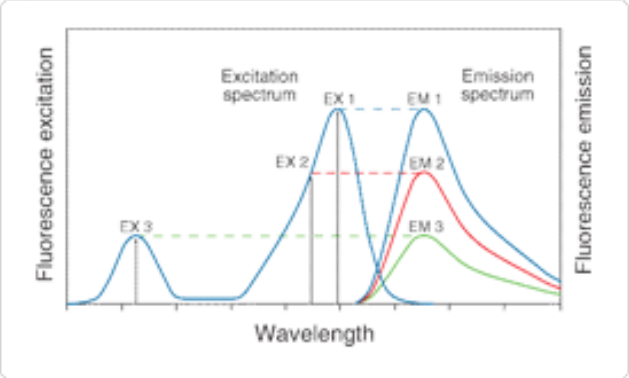


Figure 2. Excitation of a fluorophore at three different wavelengths (EX 1, EX 2, EX 3) does not change the emission profile but **does produce variations in fluorescence emission intensity** (EM 1, EM 2, EM 3) that correspond to the amplitude of the excitation spectrum.

1.3 Fluorescence Detection

Fluorescence Instrumentation

Four essential elements of fluorescence detection systems can be identified::

1) an excitation source, 2) a fluorophore, **3) wavelength filters** to isolate emission photons from excitation photons, **4) a detector** that registers emission photons and produces a recordable output, usually as an electrical signal or a photographic image. Regardless of the application, compatibility of these four elements is essential for optimizing fluorescence detection.

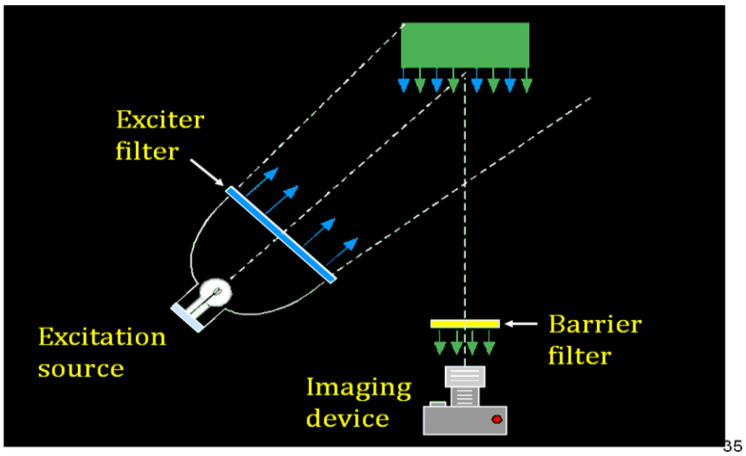


Fig 3 Fluoresce detection system

Fluorescence instruments are primarily of four types, each providing distinctly different information:

- **Spectrofluorometers and microplate readers** measure the *average* properties of bulk (µL to mL) samples.
- **Fluorescence microscopes** resolve fluorescence as a function of spatial coordinates in two or three dimensions for microscopic objects (less than ~0.1 mm diameter).
- **Fluorescence scanners**, including microarray readers, resolve fluorescence as a function of spatial coordinates in two dimensions for macroscopic objects such as electrophoresis gels, blots and chromatograms.
- **Flow cytometers** measure fluorescence per cell in a flowing stream, allowing subpopulations within a large sample to be identified and quantitated.

Other types of instrumentation that use fluorescence detection include capillary electrophoresis apparatus, DNA sequencers and microfluidic devices. Each type of instrument produces different measurement artifacts and makes different demands on the fluorescent probe. For example, although photobleaching is often a significant problem in fluorescence microscopy, it is not a major impediment in flow cytometry or DNA sequencers because the dwell time of individual cells or DNA molecules in the excitation beam is short.

1.4 Multicolor Labeling Experiments

A multicolor labeling experiment entails the deliberate introduction of two or more probes to simultaneously monitor different biochemical functions. This technique has major applications in flow cytometry, DNA sequencing, fluorescence *in situ* hybridization and fluorescence microscopy.

Signal isolation and data analysis are facilitated by maximizing the spectral separation of the multiple emissions. Consequently, fluorophores with narrow spectral bandwidths, such as Alexa Fluor dyes are particularly useful in multicolor applications. An ideal combination of dyes for multicolor labeling would exhibit strong absorption at a coincident excitation wavelength and well-separated emission spectra. Unfortunately, it is not easy to find single dyes with the requisite combination of a large extinction coefficient for absorption and a large Stokes shift.

1.5 Photobleaching

Under high-intensity illumination conditions, the **irreversible destruction** or **photobleaching** of the excited fluorophore becomes the primary factor limiting fluorescence detectability. The most effective remedy for photobleaching is to **maximize detection sensitivity**, which allows the excitation intensity

to be reduced. Detection sensitivity is enhanced by low-light detection devices such as CCD cameras, as well as by high–numerical aperture objectives and the widest bandpass emission filters compatible with satisfactory signal isolation. Alternatively, a less photolabile fluorophore may be substituted in the experiment. Alexa Fluor 488 dye is an important fluorescein substitute that provides significantly greater photostability than fluorescein (Figure 4), yet is compatible with standard fluorescein optical filters.

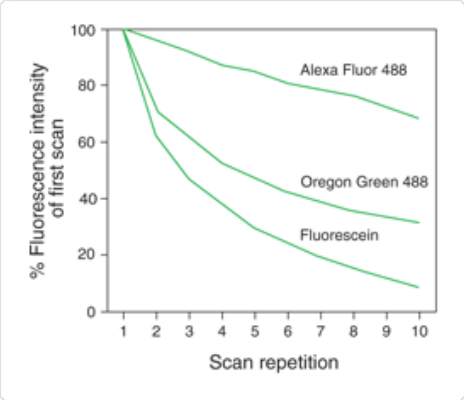


Figure 4 Photobleaching resistance of the green-fluorescent Alexa Fluor 488, Oregon Green 488 and fluorescein dyes, as determined by laser-scanning cytometry.

1.6 Comparing Different Dyes

Fluorophores currently used as fluorescent probes offer sufficient permutations of wavelength range, Stokes shift and spectral bandwidth to meet requirements imposed by instrumentation (e.g., 488 nm excitation), while allowing flexibility in the design of multicolor labeling experiments. The fluorescence output of a given dye depends on the efficiency with which it absorbs and emits photons, and its ability to undergo repeated excitation/emission cycles. Absorption and emission efficiencies are most usefully quantified in terms of the **molar extinction coefficient (ε)** for absorption and the **quantum yield (QY)** for fluorescence. Both are constants under specific environmental conditions. QY is a measure of the total photon emission over the entire fluorescence spectral profile. Fluorescence intensity per dye molecule is proportional to the product of ε and QY.

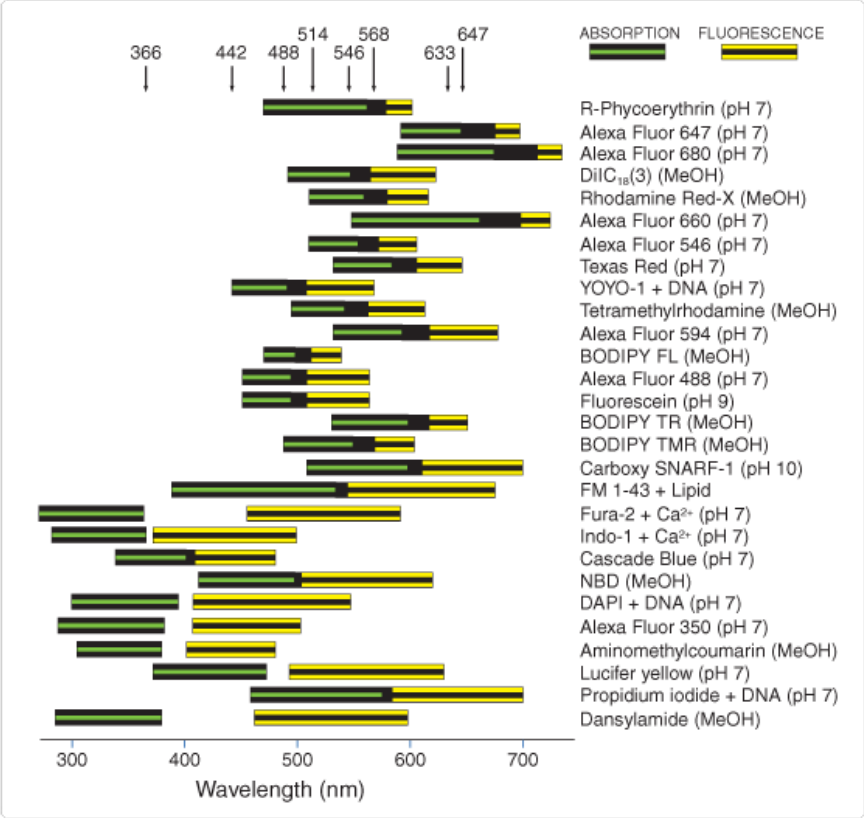


Figure 5. Absorption and fluorescence spectral ranges for 28 fluorophores of current practical importance. The range encompasses only those values of the absorbance or the fluorescence emission that are >25% of the maximum value. Fluorophores are arranged vertically in rank order of the maximum molar extinction coefficient (εmax), in either methanol or aqueous buffer as specified. Some important excitation source lines are indicated on the upper horizontal axis.

2. Applications of luminescence in cell biology

2.1. Green Fluorescence Protein

Please have a look at the following web-site for more info

http://zeiss-campus.magnet.fsu.edu/articles/probes/fpintroduction.html

An Introduction to GFP

GFP stands for **green fluorescent protein** (the official name for the molecule) and is, imaginatively, a protein that **fluoresces green in the presence of UV light**. It has found its use in all areas of cellular biology and advances have reached the point where it is the focus of works of art, such as a pet rabbit called Alba, whose fur glowed green under UV light. In November 2008, three men, Osamu Shimomura, Martin Chalfie and Roger Tsien, were awarded the Nobel Prize in chemistry, "for the discovery and development of green fluorescent protein".

So what makes this such a versatile and important molecule?

History of GFP

Osamu Shimomura is the starting point for GFP. As a young boy, he was only miles from the atomic bomb that landed on Nagasaki, close enough to be temporarily blinded by the explosion. In 1960, Osamu Shimomura moved from Japan to work at Princeton University and here he worked in a research group studying the jellyfish *Aequorea victoria*. The project he was working on involved bioluminescence, the edges of the skirt, or umbrella, of this species of jellyfish emitting a green light, figure 6. So, along with two colleagues from the university he went to Puget Sound, a series of waterways extending into northern Washington State from the Pacific Ocean. The three scientists collected roughly 10,000 specimens of the jellyfish, all with hand nets to avoid catching anything else, from which they removed a 5mm-thick ring from around the skirt, responsible for the glow observed. Somewhat gruesomely, these rings were crushed to remove the liquid constituents and forced through cheese-cloth to separate the wanted material from the solid that made up the jellyfish.

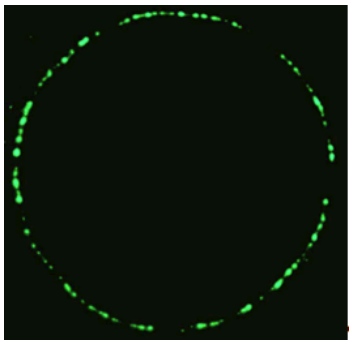


Figure 6. The ring of fluorescence seen in the jellyfish *A. victoria*.

The crushed rings, or "squeezate" as it was referred to in the original paper, was purified to produce just over 5 mg of glowing substance, though this proved not be the green light observed, but possessed a blue glow instead, originating from a protein named *Aequorin*. In the purification process another, discarded, protein was found that did posses this green fluorescence, but was practically ignored as it only emitted the distinctive green light when irradiated with blue or UV light. In 1962 the results of the work was published, with only a minor mention of the fluorescing green protein. Realizing the potential of the green fluorescing protein, Shimomura shifted his focus, and over the next 17 years continued to work on the jellyfish. In this time, over 850,000 of the organisms were captured and killed. At the end of this, Shimomura had finally deciphered the structure of the fluorescing portion of the molecule.

Research into GFP slowed considerably until in **1992** the **gene for GFP was eventually coded by Douglas Prasher**, genes and DNA, in general, being the blueprints from which proteins are synthesized. This was a significant breakthrough and led to a race to be the first research teams to successfully express the gene in and organisms. The first person to manage this was **Martin Chalfie** and in 1994 published the results of this work. Chalfie succeeded in inserting the gene for GFP into the bacteria *E. coli*, which then fluoresce with a green light in the presence of UV radiation. Since then, GFP has been used for thousands of different applications, and improvements have been made to the properties of the protein by mutations in the gene, creating brighter variants, as well as multiple different colours. As well as this**, GFP and GFP variants have been found in dozens of different marine species**, from sea anemones to sea pansies, which produce their own light, to corals that

possess no bioluminescence, but have the ability to fluoresce. And the natural varieties do not just stop at green, with almost all imaginable colours having been found.

What is the structure of GFP and how does it work?

The structure of GFP is built up in the same way as any protein and as such has multiple levels of structure, as well as multiple methods of chemical interaction.

- The base or primary structure of **GFP is a chain of 238 amino acids** weighing roughly 27,000 atomic mass units (**27 kDa**), with **only about 4 of the amino-acids directly producing any fluorescence effect**.
- The secondary structure is **a series of helices and pleated sheets,** caused by hydrogen-bonding within the chain, while the tertiary structure **is a barrel made from 11 of the sheets**, capped with the helices. At the centre of this lies the **chromophore,** a short chain of altered amino-acids **responsible for the light emission**. The barrel structure keeps the chromophore away from solvents, making **GFP capable of fluorescing under almost any conditions**, being able to fluoresce nearly to the point at which the protein is denatured by things such as heat and pH. Figure 7 shows the structure of GFP, the cylinder being ~42 Å long and ~24 Å in diameter.

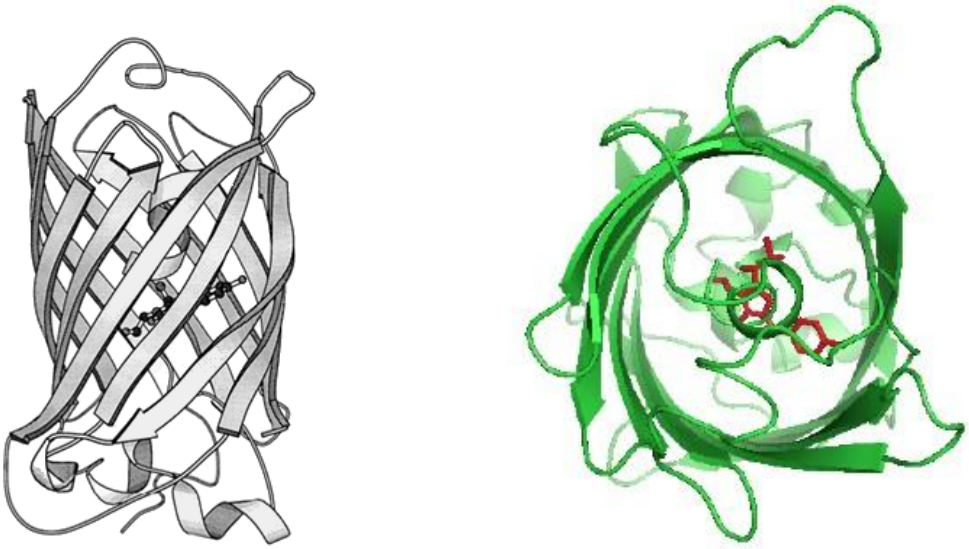
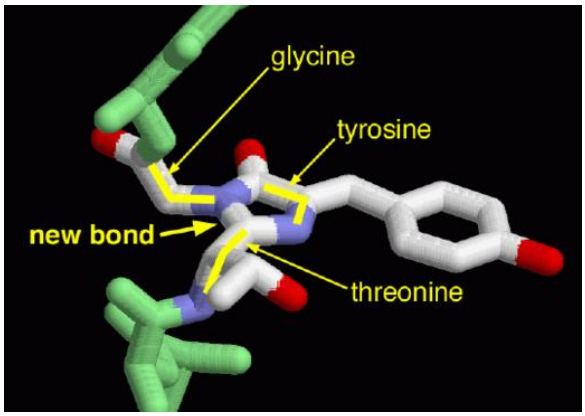


Figure 7. The tertiary structure of GFP. The central darker circles represent the chromophore, while the long flat sheets represent the barrel surrounding it.

When a protein is produced by a cell, the only part that is synthesised is a chain of amino-acids with no more structure than that. This is referred to as the "primary" structure (1°). Once completed, the amino-acid chain is folded into the right shape, forming the necessary bonds in order to hold the structure rigid. In the case of GFP, this folding brings the **amino acids necessary for the chromophore close enough together to enable it to react in a way as to produce the actual chromophore**.



Why is GFP important?

So how does all this make GFP a molecule worthy of Noble Prize winning research? Many peptide chains require enzymes to aid in the complex folding that occurs in producing the correct-shaped protein. Many proteins also use enzymes to operate, for example, the luciferin/luciferase combination mentioned earlier. **In GFP, however, the complex folding operation occurs automatically and the only thing required for the protein to fluoresce is atmospheric oxygen for the structuring of the chromophore**.

So why is this important?

- organisms, other than those in which GFP naturally occurs, can be **genetically engineered to have a gene that produces GFP and it will still work without multiple other genes being implanted**. When Chalfie first made glowing green *E. coli*, it opened wide **the possibilities for looking inside a living cell for the first time**. Figure 8 shows the Petri dish of glowing *E. coli*, under a UV light, compared to a regular sample, while figure 9 shows the first example of what makes GFP so amazing. It is a photograph of a round-worm, *C. elegans*, which has had one of its genes replaced with the genetic code for GFP. This resulted in GFP being expressed in the worm, but only in the places in which the original gene would have been expressed.
- **Neither organism suffered toxic effects** from the protein and the experiment showed that both prokaryotes (bacteria) and eukaryotes (almost everything else) can be made to express GFP. Since then it has been used in organisms as diverse as fruit flies, mice, rabbits, tobacco plants and human cells.
- As well as replacing genes, the relatively small size of GFP, for a protein, **enables it to be used as a tag, or reporter gene**, involving adding the genetic code for GFP onto the end of the gene for the protein that needs to be tagged and growing the organism. This results is the protein being produced with a small tag that doesn't affect the organism or function of the protein at all. The protein can then be seen, identifiable by the green fluorescence enabling the pin pointing of genetic expression.

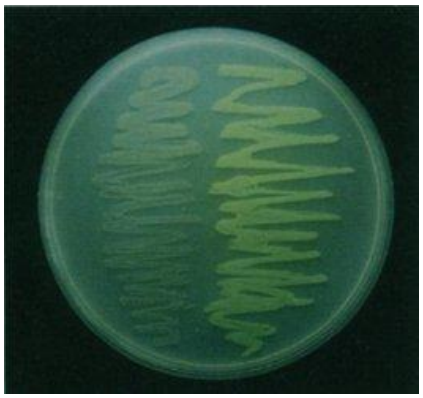


Figure 8. A photograph of the genetically altered *E. coli* (right) next to a non-altered variety.

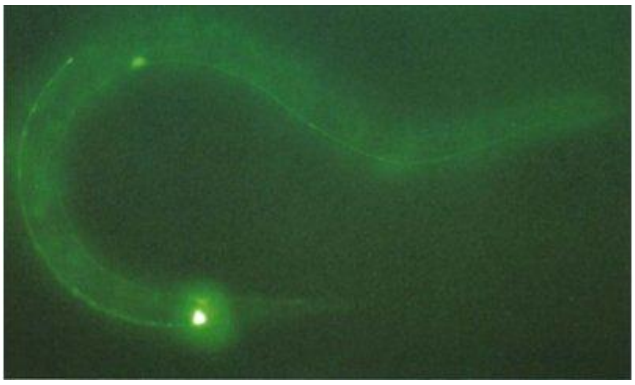


Figure 9. *C. elegans* with GFP replacing a touch receptor protein. The bright spots are where the protein would normally be most expressed.

How has it been improved?

When Roger Tsien first heard about the coding of the GFP gene, instead of wondering how he could implant it into an organism, he wondered how he could make it better - the GFP protein acquired from *A. victoria* (known as wild-type or wtGFP), sometimes being less than ideal for research. For instance, wtGFP has broad excitation peaks, that is, they absorb multiple colours of light, making it unsuitable for FRET. They are also slow in the formation of the chromophore, taking over 2 hours for the final oxidation to occur. They also have tendency to form dimers and trimers, increasing the molecule in weight massively, which can inhibit not only the function of proteins they are attached to but also the function of the GFP itself. The increased size of the dimerised and trimerised GFP molecules can inhibit the movement of tagged proteins around the cell and through membranes.

Re-engineering GFP to increase its range of color and application

Almost as soon as its sequence was elucidated, scientists began engineering new versions of GFP through mutagenesis in order to improve its physical and biochemical properties. In 1995, Roger Y. Tsien described an S65T point mutation that increased the fluorescence intensity and photostability of GFP. This also shifted its major excitation peak from 395 nm to 488 nm, effectively ameliorating the deficiencies found in the wildtype protein and facilitating its widespread use in research. Many other mutations have since been introduced to GFP and new iterations of fluorophores are constantly being engineered. **Table 1 below** lists a few common fluorescent proteins and their mutations relative to wildtype GFP. Although not listed here, many permeations within each color also exist with only slight variations separating them.

Fluorescent Protein	Mutations Relative to Wildtype GFP
EGFP	F64L; S65T
EYFP	S65G; V68L; S72A; T203Y
mYFP	S65G; V68L; Q69K; S72A; T203Y; A206K

Citrine	S65G; V68L; Q69K; S72A; T203Y
ECFP	F64L; S65T, Y66W; N146I; M153T; V163A
mCFP	F64L; S65T, Y66W; N146I; M153T; V163A; A206K
Cerulean	F64L, S65T, Y66W, S72A, Y145A, H148D, N149I, M153T, V163A
EBFP	F64L, S65T, Y66H, Y145F

Please note that many fluorescent proteins found on the red side of the spectrum are not GFP derivatives, but are instead related to the dsRed protein (called RFP) isolated from Discosoma sp. Similar work has been done to expand the red-fluorescent protein repertoire resulting in a wide spectrum of usable fluorescent proteins, shown in figure 10.

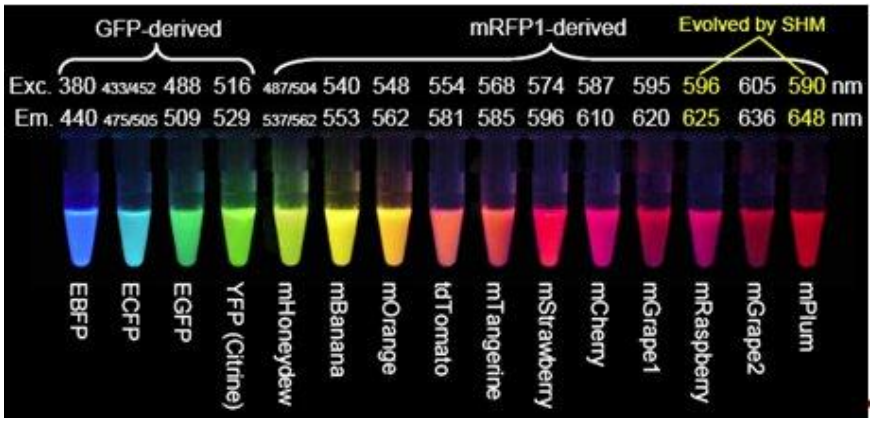


Figure 10. The wide varieties of GFP and variants that have been produced. mRFP1 is an already altered version of the *Discosoma* protein. Exc. stands for excitation and is the peak wavelength at which excitation occurs, while Em. is the peak emission wave length. E before the name of protein signifies enhanced brightness.

A multitude of applications

Due to its size and ease of use, GFP and other fluorescent proteins have become a mainstay in molecular biology. Scientists can easily utilize GFP-containing plasmids as a means to many functional ends. We've listed our favorites below, but many other uses currently exist, and new GFP technology is constantly being developed!

- **Fusion tagging:** One of the most common uses, GFP can be fused to the N- or C-terminus of a protein, which allows the scientist to visualize when and where the gene is expressed.
- **Transcription Reporter:** Placing GFP under the control of a promoter of interest can be used to effectively monitor gene expression from that promoter in a given cell type. This type of transcription reporting was among the earliest uses of GFP.
- **Förster resonance energy transfer (FRET):** This is used to study the interactions between two proteins or between two domains of a protein that undergoes conformational change.

Typcially two fluorescent proteins with overlapping excitation/emission spectra are used; one fused to each protein or domain being tested.

- **Split EGFP:** An alternative to FRET, split EGFP has also been used to study protein-protein interactions. In this case, two portions of EGFP are fused to the proteins of interest, and when they come into close proximity, the two halves of EGFP undergo folding, maturation, and fluorescence.

- **Biosensors:** A wide array of GFP-based fluorescent biosensors has been designed to detect a variety of intracellular conditions, including ion (such as Ca2+) concentrations and pH, using a range of strategies such as FRET, calmodulin, and others.

- **Optogenetics:** Scientists can use light to detect, measure, and control molecular signals, cells, and groups of cells in order to understand their activity and visualize the effects of alterations to this activity.

- **Cell marking/selection:** Expression constructs like plasmids often include GFP as a marker to help identify which cells have successfully taken up the plasmid. This can serve as an alternative to antibiotic selection. Plasmids of this type may have the GFP under the control of an additional promoter from that of the gene of interest, or expressed from the same transcript as the gene of interest, but after an internal ribosome entry site (IRES). This is oftentimes used in conjuction with FACS (see below).

- **Fluorescence-activated cell sorting (FACS):** This is a type of flow cytometry that separates mixtures of cells into distinct populations based on fluorescent signal. Thus, FACS can be used to separate cells expressing GFP from cells that are not.

- **Developmental/transgenic uses:** Because of its stability, GFP can be used in lineage tracking capacities in cell fate studies. It can also be used, when put under control of promoters of interest, to visualize the developmental stage at which these promoters are active. Further, GFP can label transgenically modified ES cells, which can then be used for implantation and generation of transgenic mice.

- **Purification:** GFP can be used as a general epitope tag for protein purification and a number of commercial antibodies to GFP are available.

- **Others:** We've really just scratched the surface of the potential applications for GFP. It has also been used to identify particular cell populations in drug screens, to visualize micrometastases in nude mice in cancer studies, act as a reporter for DNA double strand break repair, and to label pathogenic intracellular microbes to visualize host/pathogen interactions.

2.2 Luciferase.

Luciferase and **luciferin** are a non-specific enzyme and its substrate, respectively, that upon reaction in the presence of ATP, Mg^{2+}, and O_2 generate bioluminescence. Different genes or cDNAs encoding luciferases have been isolated from different organisms, such as unicellular seaweed, marine bacteria, and fish. Due to the specificity of the reaction, many luciferases have been used as reporter proteins either in prokaryotic or eukaryotic cells.
Firefly luciferase (LUC) is one of the most widely used. The oxidation reaction produces oxyluciferin, CO2, and a photon. The intensity of light emitted during the reaction is measured with a luminometer.

Luciferase-based techniques are sensitive, simple, rapid, inexpensive, safe (organic solvent or radiolabeled compounds are not required for the test), and applicable to a wide range of cell lines. One of the most recent advances constitutes the development of highly sensitive techniques that make the detection of luciferase activity in living cells and organisms feasible.

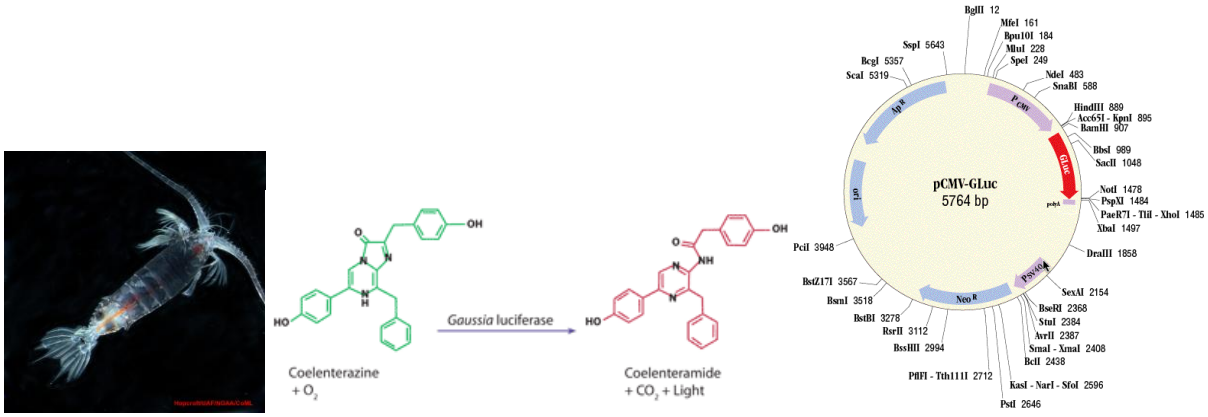
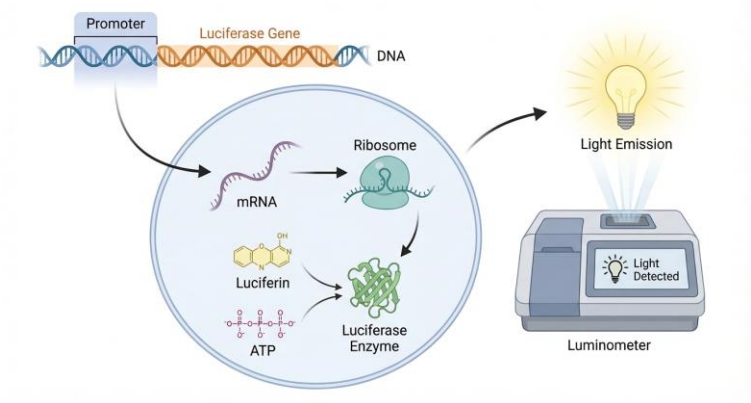


Fig 11.

The most common reporters are bioluminescent reporters whose detection is based on the enzymatic reaction with an enzyme, Luciferase, with the substrate Luciferin. The most commonly used luciferase enzymes are derived from firefly (*Photinus pyralis*) and Renilla (*Renilla reniformis*). The bioluminescent signal emitted from this reporter enzymatic reaction can be quantified easily.



These cell-based assay systems are successful in measuring the expression of a gene with high sensitivity. The signal produced can be measured with a simple luminometer consisting of a series of photomultipliers or a charge-coupled device (CCD) camera.

Luciferase Assay Types
— Overview of Main Assay Types

Assay	Primary Use	Substrate(s)	Key Features
Single Luciferase	Promoter analysis, gene expression in bacteria or eukaryotic cells	D-luciferin + ATP	7–8 log range; requires manual normalization
Dual Luciferase	Gene regulation, signaling pathways, HTS of bioactive compounds	D-luciferin + coelenterazine	Gold standard; normalized by internal RLuc control
Multicolored Luciferase	Large-scale, simultaneous multi-gene monitoring	D-luciferin only (all beetle luciferases)	One-step, one sample; different emission spectra
Real-Time Luciferase	Longitudinal in vivo imaging, circadian rhythm, tumor models	D-luciferin (stable, cell-permeable)	Quantitative + temporal resolution; in vitro & in vivo

Table 1. Comparative overview of the four main luciferase assay formats. Each assay type is optimized for specific experimental scales and biological questions.

Single Luciferase Reporter Assay:

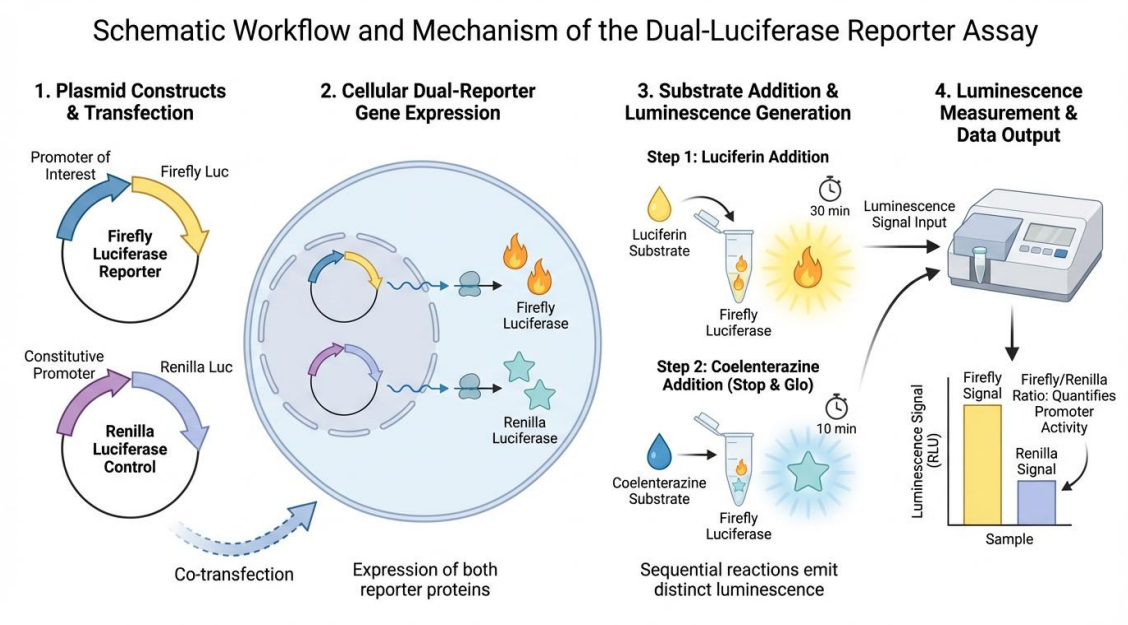
This is used to analyze gene expression and promoter analysis in bacteria or eukaryotic cells. This assay requires an excess of luciferin and ATP. It involves classical luciferase protocol in which the chimeric plasmid (luciferase gene and the target promoter) is transfected into the cells followed by lysis for some time. The expression of luciferase protein along with the targeted gene is estimated by the light intensity in in-vitro. This method is highly sensitive and has a broader linear response (in the magnitude of 7-8). It is suitable for high throughput screening because of its simplicity and usefulness in wide areas of gene expression, protein-protein interactions, and post-transcriptional. The caveat of this assay is normalization of internal enzyme activity to ensure the accuracy of the result.

Dual-Luciferase Reporter assay:

This system utilizes an internal control reporter to enhance **experimental accuracy and minimize internal experimental variability** at different parameters in terms of variability of number and viability of the transfected cells, the efficiency of transfection, lysis of the cells and harvest time. For most of the cell biology experiments, firefly luciferase is used as the experimental reporter and Renilla luciferase is used as an internal control fused with housekeeping or constitutively expressing promoters such as SV40 or CMV promoter.

From the cell sample, activities of both of the luciferases are measured sequentially depending upon their substrates. In the first reaction, D-luciferin is used to assess the activity of firefly luciferase followed by Renilla luciferin for Renilla luciferase with a parallel signal quenching of firefly luciferase signal. The final activity is measured as firefly luciferase activity normalized by internal Renilla luciferase activity.

This assay is useful for high throughput screening of bioactive compounds and gene expression but for large-scale sampling, this is not the best available method.



Dual Luciferase Assay — Step-by-Step Workflow

The Dual Luciferase Reporter (DLR) assay is **considered the gold standard for promoter analysis** and gene expression studies, as it integrates an internal control to minimize experimental variability.

Dual Luciferase Sequential Measurement Protocol

#	Step	Description
1	Transfection	Chimeric plasmid (FLuc + target promoter) + internal control plasmid (RLuc + SV40/CMV) co-transfected into cells
2	Cell Lysis	Cells lysed after incubation; lysate collected for sequential luminometry
3	FLuc Measurement	Add D-luciferin substrate → measure firefly luciferase signal (λ ~562 nm)
4	Signal Quenching + RLuc	FLuc signal quenched; add coelenterazine → measure Renilla signal (λ ~480 nm)
5	Normalization	Final result = FLuc / RLuc ratio → corrects for transfection efficiency and cell viability

Sequential workflow of the Dual Luciferase Assay. Firefly luciferase (FLuc) is measured first with D-luciferin, then quenched; Renilla luciferase (RLuc) is measured with coelenterazine. Final readout = FLuc / RLuc ratio.

Sources of variability controlled by the dual system include: transfection efficiency, number and viability of transfected cells, cell lysis efficiency, and harvest timing.

Single vs. Dual Luciferase — Comparative Analysis

Single vs. Dual Luciferase: Head-to-Head Comparison

Parameter	Single Luciferase	Dual Luciferase
Internal control	None	RLuc (housekeeping promoter)
Normalization	Manual / problematic	Automatic (FLuc / RLuc ratio)
Experimental variability	High	Reduced
Accuracy	Lower	Higher
Large-scale HTS	Suitable	Less suitable
Substrates required	D-luciferin only	D-luciferin + coelenterazine
Operational complexity	Low	Moderate

Figure 4. Direct comparison of Single and Dual Luciferase assay systems across key experimental parameters. Green cells indicate the superior option.

Key limitation of Single assay: manual normalization is required to account for endogenous enzyme activity; without it, results may be inaccurate across replicate samples.
Key limitation of Dual assay: sequential measurement adds operational steps; not ideal for very large-scale automation.

Multicolored Luciferase Reporter assay:

This assay is a one-step method for simultaneous monitoring of many extracts. It is useful for large-scale data interpretation in lesser time and amount of sample. Different beetle luciferases are utilized to emit different colors using modified luciferases and all the emissions can be detected separately. Luciferases (CBGluc, CBRluc, ELuc, SLR, SLG, and SLO) from different beetle species are used in a wide spectral range. Most of these genes are different from classical luciferase gene by point mutations. Since only one substrate (firefly D-luciferin) is required for the activation of all these genes, many targeted genes can be assessed by just one-step reaction in only one sample.

Real-Time Luciferase Reporter Assay:

Using a real-time monitoring photomultiplier system, luminescence can be measured in multi-dimensions (both quantitatively and longitudinally) with high resolution both in the in-vitro and in-vivo system. D-luciferin can penetrate well inside the cells and tissues and is very stable, therefore, used in combination with Renilla luciferin.

Luciferase-based assays are better than other reports because of the following advantages:

- Quick and real-time measurement.
- Exceptionally high sensitivity than fluorescent reporters like GFP (10- to 1,000 fold)
- Range of measurement is wide and dynamic.
- Proper controls are available to negate endogenous activity.
- Clear signals with minimal background.
- Linear response range which is better than typical enzymatic reporters such as β-galactosidase, chloramphenicol acetyltransferase, β-glucuronidase

Applications of Luciferase assays:

- non-invasive labeling of genes in live animals
- Cell viability assay
- Estimation of ATP or internal calcium in cells
- Gene expression analysis
- Promoter structure analysis
- Functional SNPs analysis for the promoter
- Studying Cell signaling pathways
- To study RNAi and non-coding RNAs