Focal Points

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The Advantages of Fluorescent Proteins over Luciferase for In Vivo Imaging

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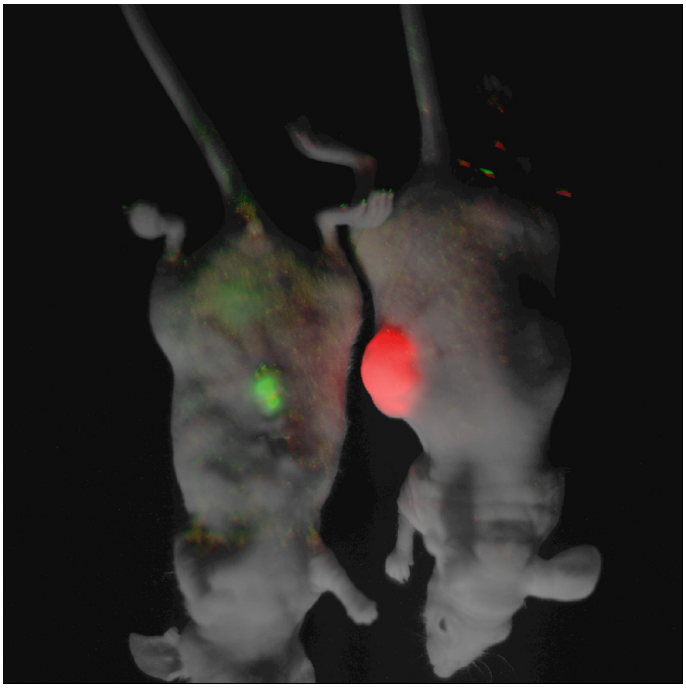

Introduction

What are Fluorescent Proteins and Luciferase?
Fluorescent proteins (FP) and luciferase are two categories of proteins that glow. Fluorescent proteins include the green fluorescent protein (GFP) series which was originally extracted from jelly fish and the cyan fluorescent protein series (CFP) which was extracted from corals. Many different versions of fluorescent proteins had been engineered by mutating the original proteins. Their glow mechanism is fluorescence which means these proteins first absorb energy of one color light (excitation), digest the energy and then emits the partially consumed energy as a different color light.

Most fluorescent proteins go by simple acronyms “xFP”, where x is one letter stating the color of the emission. Modifiers can be added to the beginning of the acronyms to emphasize the specific mutation beyond spectral variation. For example, EYFP stands for enhanced yellow fluorescent protein which has relatively enhanced brightness and photo-stability than the older version of YFP. Occasionally the creators of novel mutants prefer to give unique names to their inventions. In those cases, there are the fruit color series fluorescent proteins such as mCherry and mStrawberry and random names such as Cerulean, Emerald and DsRed.

The names and wavelength of several fluorescent proteins covering a wide spectral range are summarized in the following table[1]:

Name	Excitation	Emission
Cerulean	433	475
EGFP	488	507
Venus	515	528
tdTomato	587	610

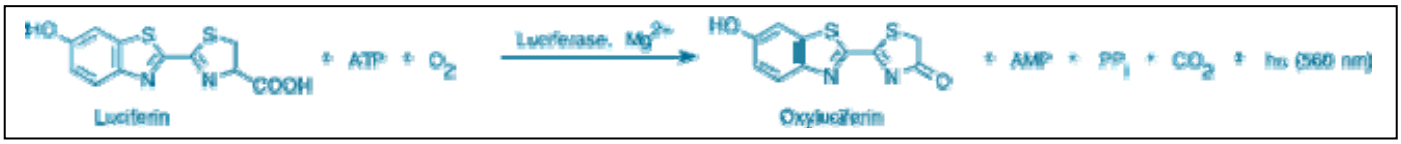

Figure 1. Reaction scheme for firefly bioluminescence generation (The Handbook, Invitrogen, Carlsbad CA)

The name Luciferase covers a type of protein which catalyzes the oxidation reaction of another molecule, called luciferin. When the reaction happens, light is produced. The light generated by this chemical reaction is called bioluminescence. Bioluminescence can be found on hundreds of life forms. The specific molecules involved, such as the structure of the luciferase and luciferin, and thus the color of light produced, can vary. Figure 1 shows one example, the firefly luciferin catalyzed by luciferase.

The commonly used luciferase are firefly luciferase (yellow color) and *Renilla* luciferase (blue, 480nm). A red luciferase developed from click beetle is also available.

How are They Introduced to Small Animal?
Fluorescent proteins and luciferase have little difference in this aspect. The common strong point of fluorescent proteins and the luciferase is that they are both light-producing proteins which can be synthesized in living subjects. When introduced to the living subject and incorporated in the cells, fluorescent proteins or luciferase are produced in the individual cells making up a tissue. Both fluorescent proteins and luciferase are referred to as “reporters” and their genes as “reporter genes” because they “report” the location and expression of the gene. When the reporter gene is next to another gene of interest-say one that is involved in cancer-the reporter gene will produce fluorescent proteins whenever the cancer gene is active. Fluorescent proteins can also mark the location and extent of a tumor.

When the probes are tagged to a protein of interest, the goal is to transform the animal with the designed DNA in a large area. Common transfection techniques were developed for in-vitro, microscopic volume sample. It can be impractically expensive to applied to small animal work as well as inefficient, because the small animal has relatively large body volume but very little surface area to be exposed to agents (chemical or viral) for transfection. A second obstacle is that the expression in this case can often be transient, due to the inability of the living tissue to regulate the excessive protein and to replicate the DNA. With the reporter gene not stably incorporated into the animal genome, the signal can have relatively very short (less than 2 days) life span.

Another way to introduce these reporter proteins in small animals is through surgical implantation of cells which had been developed to stably replicate and express the reporter genes. The probes are often not tagged to another protein and are free to diffuse within the cell membrane. The whole cells act as glowing beacons inside the animal. Many cancer cell lines, which grow uncontrollably, can be engineered to produce the probes indefinitely. The probes then provide high contrast information on the location and development of the cancerous tissue [2].

How to Image Fluorescent Proteins and Luciferase In Vivo
Fluorescence and bioluminescence originates from different mechanism and require different source of energy to generate light. To generate fluorescence, excitation light of higher energy than the fluorescent emission must travel to the location of the fluorescent probe. For an in-depth fluorescent target, the excitation light can be scattered by the tissue between the light source and the target and reduced in intensity. Shorter wavelength (bluer) excitation light tends to scatter more. The same light source used to excite fluorescent probes embedded in non-scattering medium, such as cornea, or imbedded on the surface might not be as effective when used for in-depth In Vivo application.

To generate bioluminescence from luciferase requires the presence of every reagent in the oxidation reaction. Among the reagent required for the firefly luciferase reaction as described in figure 1, ATP, oxygen and magnesium ions naturally exist in many small animals, although the concentrations can vary by location and the physiological condition of the animal. Luciferin, however, is not common found in mammals that are popular for research because their similar biology to human. In the most popular In Vivo imaging scenarios, luciferin has to be injected into the vein and delivered through the bloodstream in order to generate the bioluminescent signal. The amount of bioluminescent signal produced depends on the efficiency of delivery which is not always a dependable variable.

The UVP iBox® system was used at Anti-Cancer, San Diego, CA, to image mice with various cancerous cells expressing different fluorescent proteins. For example, the image on the cover page shows two mice, one with a RFP expressing tumor and the other with a GFP expressing tumor, in the UVP iBox system. 1mm^3 of the fluorescent, cancerous tissue was surgically implanted in the mice. The tissue can develop in size and migrate. The researchers at Anti-Cancer


iBox Imaging System

take fluorescent images of their mice in-vivo every several days for months to monitor the development of the tumor. The pictures shows that, using iBox, we can capture high quality fluorescent images, overlay them on the white light images to show the relative anatomical location and contrast different cancer tissue by their color.

Comparison of FP and Luciferase for In Vivo Imaging

The advantages of fluorescence are many. For In Vivo imaging, there are three clear, main advantages fluorescent proteins: superior brightness, innately fluorescent without injected luciferin and better controlled chemistry and biology compared to luciferase.

Brightness/Integration Time
Our actual testing results at Anti-Cancer, San Diego, CA showed that the imaging mice with GFP or RFP tumor requires much less time. The typical time scale range from 2 seconds to 50ms. The researchers were allowed sufficient time to complete thorough documentation of the animal at multiple orientation. The imaging quality was not influenced by breathing. At 50ms exposure time, we were even able to take fluorescent images without anesthesia.

In contrast, luciferases are very dim. To compensate for the low signal level, typically the image integration time is very long (10 seconds ~5 minutes) [3, 4]. For In Vivo applications, a probe requiring long integration time can be devastating. Although most In Vivo applications employed anesthesia to reduce the activity of the subject, the movement during breathing cycle cannot be suppressed and decreases the imaging quality.

Secondly, anesthesia cannot last indefinitely without causing the death of the animal. For example, the suggested duration of anesthesia by injecting Ketamine on mice is typically around 30 minutes [5]. During this time, researchers must prepare the imaging station, position the animal, take an image, adjust the exposure time until one determines an optimized imaging condition which avoids underestimating the target size(under exposure) or saturating the details. If the volume of tissue in 3D is to be measured, the orientation of the animal will be adjusted to acquire cross-section of the tissue. For each orientation the optimization steps has to be repeated. The time requires to conduct a thorough documentation of the subject labeled with luciferase easily exceeds the duration of the anesthesia.

Availability/Superior Engineering
The well understood physics of fluorescence provides a guide line for developing brighter, more stable and wider spectral variety of fluorescent proteins. Novel probes based on fluorescent proteins are constantly being released from academic sources such as Miyawaki lab in Japan, Tsien lab in San Diego, CA and Piston lab at Vanderbilt University. They commonly share their sample and detail observation on new fluorescent proteins with other academic researchers for free. There are also commercial suppliers of most fluorescent proteins DNA and lab animals. Overall, research laboratories can easily acquire and use genes for fluorescent proteins. And the better controlled chemical stability, photo-stability and molecular interaction, of fluorescent proteins assures the researchers that the signal intensity is not easily skewed by the fluctuating biochemistry of living subjects.

Although there are commercial suppliers for luciferase, the engineering of luciferase is not nearly as advanced as for fluorescent proteins [6].

Quantification
There are two aspects in quantifying the result of In Vivo imaging: the dimension and intensity. The quantitation of the dimension In Vivo is to measure the relative coordinate and the length, area, volume of the signal. These numbers can report the tissue or organ expressing the targeted gene, the development and the migration of such tissue. To accurately acquire these numbers, it is necessary to have contrast between the intensity of the signal and the background level. The researchers carefully define an intensity threshold distinguishing the signal and background based on their spectral characteristic and expected distribution. The absolute intensity number of the signal is not important for this purpose as long as there is enough contrast from the background.

The absolute intensity number can give information on the rate of gene expression and the density of the developed tissue. To quantify the intensity of In Vivo imaging is, in general, non-trivial. The proof of principle had been attempted by academic researchers and commercial instrument manufacturers in many forms and ways. Most of them are valid but remain experimental and not standard protocol. Nevertheless, the bottom line is that fluorescent signal is possible to quantify, and to quantify bioluminescent signal In Vivo involves several variables that are intrinsically impossible to control.

In principle, the intensity of fluorescent emission is linearly proportional to the intensity of excitation. Quantification of fluorescent protein signal In Vivo can involve calibrating for the effect of tissue scattering, absorption, reflection bleedthrough and auto-fluorescence. The amount of tissue scattering and absorption, which impacts both the intensity of excitation and emission, depends on the wavelength, the physical property of the tissue and the thickness of the tissue. The most straightforward method to eliminate these factors is to surgically remove the tissue covering the fluorescent target. This technique has been frequently performed by In Vivo fluorescent

microscopicist, since the microscope optics they employ often does not have enough working distance to penetrate thick tissue.

Auto-fluorescence, fluorescence emission generated by the surrounding tissue and not the fluorescent protein, is intrinsic to organic tissue and cannot be removed. Depends on the chemical composition of the tissue, auto-fluorescence can have different color. On mice, human and other warm-blooded mammals, auto-fluorescence is reddish in color. The exact spectral peak can shift due to many reasons, such as diet, subject-to-subject variations and etc.

However, the intensity of bioluminescence depends on the reaction rate which is a variable of the concentrations of reagents. They cannot be control in a live animal and can fluctuate at any moment, making quantitation extremely difficult.

Contrast
Many luciferase supporters claimed that bioluminescence imaging achieves better contrast over background [6, 7]. The reason being that bioluminescence can occur without auto-fluorescence which is a major source of background for In Vivo fluorescence imaging. On the other hand, the long exposure time required for bioluminescence imaging makes it more susceptible to detector noise.

The wide variety of fluorescent proteins also provides alternative methods which can enhance the signal-to-background ratio. In Xenogen's publication, for example, they showed that using fluorescent probes which has color different from that of the auto-fluorescence can improve the contrast.

Common Counter Points from Bioluminescence Users

People who support bioluminescence imaging for In Vivo work instead of fluorescence often believe that without using the excitation light, one can avoid having photo-bleaching, photo-toxicity and auto-fluorescence [6]. These are potential problems with fluorescence but not important for most In Vivo applications using fluorescent proteins.

- **Photo-bleaching:** Fluorescent probes can be damaged by the excitation light, due to chemical reactions which happen during excitation. The intensity drops exponentially when photo-bleaching happens at a constant rate. However, whole animal and plant In Vivo studies typically image cells and tissue which expresses fluorescent protein has very high number of fluorescent probes such that the fluorescent signal cannot be extinguished by photo-bleaching.
- **Photo-toxicity:** There is generally no concern about low amount of exposure to common excitation wavelength for GFP and RFP. For microscopic, in-vitro experiments, it had been observed that exposure to excitation light, especially UV, changes biological functions [8]. Short wavelength light sources do not penetrate most small animal tissue well and is not used for in-depth, In Vivo applications.
- **Background:** The fluorescent proteins are bright enough to be distinguished from the background for quantitative dimension measurements.

Conclusion

In short, using fluorescent proteins for In Vivo imaging, especially on small animals, have the following feature advantages:

- Short exposure time
- Wide spectral variety
- Stable chemistry
- High availability from academic researchers

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