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Fireflies, Dead Fish and a Glowing Bunny: a Primer on Bioluminescence

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Introduction and History

Luminescent organisms have been observed throughout the ages. The cause of the luminescence wasn't known until the advent of the microscope and modern science. In the oceans, sailors often remarked at the eerie glowing water generated by their vessels. Christopher Columbus, during his historic voyage across the Atlantic, noticed mysterious patches of luminescent light around the waters of his ships (Floyd, 1997). Often, the explanations of the luminescence found in the oceans were attributed to monsters of the deep or other mysteries of the unknown. During the era of great Greek philosophers, Aristotle and Pliny had noticed that the flesh of dead fish and damp wood appear to luminesce (Harvey, 1920).

The first serious scientific attempts to understand the origin of luminescence in organisms were underway by the mid 1600's. Not knowing the exact reason why, Boyle, using air pumps, determined that luminescence of dead flesh and fungi was dependent on the air (oxygen) (Kruse, 2000; Harvey, 1952). However, it wasn't until the 1830's when the German scientist, G. A. Michaelis, discovered that the luminescence from dead fish was due to something living (Harvey, 1920). The French physiologist Raphael Dubois, in 1885, was the first to isolate the light producing chemicals from clams (Alcamo, 2003; Harvey, 1920), which paved the way for the characterization of the molecular, chemical, and physiological mechanisms behind this process. Today, bioluminescence has been observed in thousands of species including bacteria, fungi, and marine animals. Only within the last 20 years has bioluminescence been harnessed as a scientific tool. Today, bioluminescence is being applied directly in experimental assays and attempts are being made to mass market bioluminescence to the general public.

What is Bioluminescence?

Bioluminescence belongs to a family of terms (including fluorescence, phosphorescence, and chemiluminescence) referring to the production of light chemicals. In fluorescence, energy, typically light of given wavelength, from an outside source interacts and excites a fluorophore – a substance that undergoes fluorescence. The energy becomes absorbed and creates an unstable "energized" fluorophore. In the attempts to regain stability, the "energized" fluorophore immediately releases the extra energy as light at a longer wavelength (Haddock et al., 2000). Once the excitation energy is removed the fluorescence is stopped. Phosphorescence is based on a similar principle to fluorescence. However, the light emitted lasts longer because of the greater stability of the energized phosphorescent substance (Haddock et al., 2000), even after the removal of the energy source. Examples of common phosphorescent products are glow-in-the-dark stickers and glow-in-the-dark facets found on watches. Chemiluminescence is based on the principle of fluorescence but the energy required to generate light comes from a chemical reaction. Unlike a burning candle, which generates light and heat, chemiluminescence, fluorescence, and phosphorescence generates little to no heat. Bioluminescence refers to light generated from within an organism. The generated light can be formed by either fluorescence or chemiluminescence and involves the use of proteins. Bioluminescence due to chemiluminescence commonly uses the enzyme "luciferase" to catalyze the oxidation of a substrate "luciferin" generating light as a by-product. Bioluminescence due to fluorescence requires an accessory protein, a fluorophore that requires the light energy generated from chemiluminescence in order to generate light.

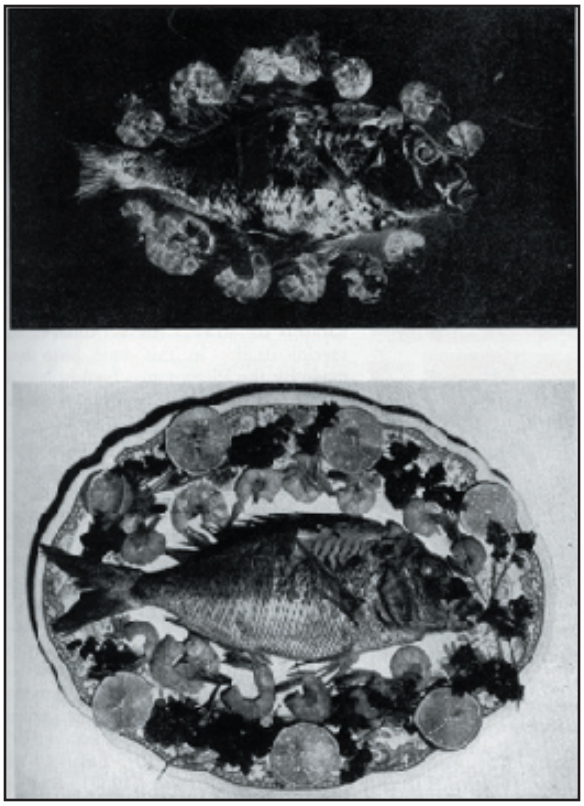

Figure 1 A dead fish and shrimp infected with luminous bacteria on a plate, photographed by their own light (above) and by daylight (below) (Harvey, 1952). The slices of lemon and parsley are not

Who Left the Lights on and Why?

The vast majority of bioluminescent organisms are found in the ocean (Meyer-Rochow, 2001). However, there are some species of organisms on land that do exhibit the phenomena including insects, bacteria, and fungi. Within the ocean, the majority of species that do bioluminesce are usually found below 800m (Meyer-Rochow, 2001). Bioluminescent marine organs include mollusks, squid, fish, plankton, and dinoflagellates. As widespread bioluminescence is, it is fairly rare within the animal kingdom. Birds, mammals, reptiles, and amphibians seemed to have been left in the dark, evolutionary wise.

Marine and terrestrial organisms generate their bioluminescence either intracellularly or with bacteria. Intracellular bioluminescence refers to the light generated from specialized cells that are assembled into light organs found in higher organisms. Examples of intracellular bioluminescence can be seen in squid, fish, and fireflies. Bacterial luminescence can be seen from two different aspects either directly, from the bacteria themselves, or indirectly, from bacteria in a symbiotic relationship with another organism. Direct bacterial bioluminescence from free-living bacteria can be seen on the flesh of dead fish (Figure 1), as Aristotle and Pliny originally observed. The function of direct bacterial luminescence isn't clear but some theories do exist. For example, it has been suggested that marine enterobacteria use bioluminescence to complete their life-cycle by attracting fish to a food source that the bacteria are growing on (Harvey, 1952). However, many of these relationships can be classified as either parasitic or symbiotic (Hastings, 1978). Indirect bacterial luminescence via a symbiotic relationship is commonly exploited by many marine organisms. For example, deep-sea angler fish have special bacterial containing light organs. Hanging in a lantern-like fashion from the angler fish, the light organ contains bacterial species related to the genus Vibro (Meyer-Rochow, 2001) that is used to attract prey.

The evolution of bioluminescence in nature has resulted in several behavioral adaptations. Although at least 20 separate functions of bioluminescence have been proposed (Harvey, 1952), the most common associated with predation, defense, and communication. Bioluminescence used in predation is observed in many species of fish. As stated earlier, the deep-sea angler fish exploits this tactic using a lure. Defensive bioluminescence can be seen in the zooplankton spp. Metridia. When swallowed by a small fish, the bright bioluminescence of Metridia can be seen through the predator's body making the smaller fish prey to bigger fish (Meyer-Rochow, 2001). Dinoflagellates, microscopic unicellular marine protists, exhibit bioluminescence at night when mechanically disturbed. The triggering of the bioluminescence in the dinoflagellate is sensitive enough to respond to minor currents in the water (Buck, 1978). If a predator of a dinoflagellate, typically small fish, attempts any movement at night, this will trigger the bioluminescence of the surrounding dinoflagellates. The bioluminescence then acts as an alarm-like beacon that can attract larger predators making the small fish vulnerable to attack. Use of bioluminescence as a tool for communication can be seen in fireflies and is possibly the earliest recognized function of bioluminescence (Buck, 1978; Bartholin, 1647). Communication between the sexes of fireflies can be used for either sex or food. The flashes of the fireflies have been duplicated experimentally and the rhythm and frequencies of the flashes are key to firefly attraction (Buck, 1978; Meyer-Rochow, 2001). However, the female of the species of some fireflies can falsely communicate flashes of attraction in order to lure and eat the unsuspecting male of other species (Lloyd, 1978).

Mechanisms of Bioluminescence

Currently, only two mechanisms of bioluminescence have been discovered. The first involves the luciferase-luciferin system and the second utilizes

the green fluorescent protein as an accessory protein. Although the principle behind each of the mechanisms of bioluminescence appear to be similar, the exact molecular basis behind each of the two separate mechanisms aren't evolutionary conserved (Hastings, 1996; Wilson and Hastings, 1998). The colours of emission of bioluminescent organisms aren't conserved and do appear to be related to the ecological location of the organism in question (Hastings, 1996). Species located in the deep ocean tend to have blue emissions. Coastal aquatic species tend to have green emissions. Terrestrial and fresh water bioluminescent species appear to have green, yellow, or orange emissions. Differences in colour emissions can also be attributed to the different molecular mechanisms used to achieve bioluminescence.

The Luciferase-Luciferin System

The luciferase-luciferin system was first characterized in 1885 by the French scientist, Raphael Dubois (Alcamo, 2003; Harvey, 1920). Using clams, Dubois was able to isolate two different substances that are required to generate bioluminescence. When Dubois ground up clams in cold water he noticed that they began to glow for several minutes. He could recharge the luminescence by adding clams that have been ground in hot water. He termed the cold water extract luciferase and the hot water extract luciferin. Luciferase and luciferin are generic terms and the organism that they are obtained from must be specified because they aren't homologous between bioluminescent species. Different species can have wildly varying sizes of luciferase ranging from 21kDa in squid to 300kDa in some worms (Wilson and Hastings, 1998). The fluorescent molecule, luciferin, can vary in size and structure depending on the species (Figure 2).

Regardless of the species of organism, the luciferase-luciferin system requires three substances to generate light. The generic form of the chemical reaction requires the luciferase to catalyze the oxidation of the luciferin with the help of the cofactor.

The result is the generation of light and a "spent" luciferin, termed oxyluciferin. The actual reaction occurs very rapidly and releases a large amount of energy. In insects, like the firefly, the luciferin (Figure 2), is synthesized from specialized cells called photocytes (Babu and Kannan, 2002) found in specialized light organs in the abdomen of the beetle. Firefly luciferase activates the luciferin using ATP and Mg2+. The luciferin then undergoes a short-lived reaction intermediary creating an excited unstable intermediate oxyluciferin (Wilson and Hastings, 1998). When the excited oxyluciferin returns to its stable form, energy is released in the form of light. In a biological setting, the release of light energy from the reaction has been calculated to be more than eight times that of the hydrolysis of ATP to ADP (Wilson and Hastings, 1998). The firefly luciferase-luciferin reaction is so efficient that 98% of the released energy is in the form of light with very little heat (Seliger and McElroy, 1960; Babu and Kannan, 2002). Incandescent light bulbs have an efficiency of about 10%, where about 90% of their energy is wasted as heat (Smith, 1995).

The Green Fluorescent Protein

Green fluorescent protein (GFP) emerged form obscurity to become one of the most popular tools in molecular biology. The study of GFP began in the mid 1950's when it was noticed that cells of the jellyfish, Aequorea victoria, fluoresced green when stimulated with ultraviolet (UV) light (Davenport and Nicol, 1955). It wasn't until the early 1960's when Osamu Shimomura began studying how and why A. victoria fluoresced. In a University of Washington lab, near Victoria, British Columbia, Shimomura attempted to extract the putative green light emitting protein (Shimomura, 1998). The extracted protein emitted blue light, contrary to the expected green. Subsequently, the blue fluorescent protein was named aequorin (Shimomura, 1998; Shimomura et al., 1962). Aequorin, a photoprotein, emits blue light when triggered by Ca2+ and doesn't fit the generic view of the luciferase-luciferin system. Aequorin contains the luciferase and the luciferin bound together covalently as a single unit (Shimomura and Johnson, 1975). In contrast, the generic luciferase and luciferin are separate entities.

By the late 1960's and early 1970's, it became apparent that the fluorescence of A. victoria was due to two separate proteins, Aequorin and a "green protein" (Shimomura, 1998). The "green protein", named green fluorescent protein (GFP) in 1971 (Morin and Hastings, 1971), absorbed the blue light, from Aequorin, and remitted green fluorescence. Nearly 20 years after Shimomura's first attempts to purify the "green protein", GFP was finally purified in 1974 (Morise et al., 1974). The mechanism of how GFP produces its green fluorescence wasn't determined until molecular biology was applied. In 1992, Douglas C. Prasher, a researcher at the Woods Hole Oceanographic Institute at the time, set out to clone the GFP gene. When Prasher completed the arduous task in 1992 (Prasher et al., 1992), a lack of funding prevented him from transforming bacteria with his GFP gene in order to confirm that he had isolated the correct cDNA (Herper, 2001a). Soon after, Martin Chalfie, a C. elegans researcher at the University of Columbia, contacted Prasher for a copy of the GFP cDNA (Herper, 2001b). Chalfie used the GFP cDNA to publish his seminal paper in 1994 (Chalfie et al., 1994). By transforming the GFP cDNA into bacteria and creating transgenic nematodes, Chal-

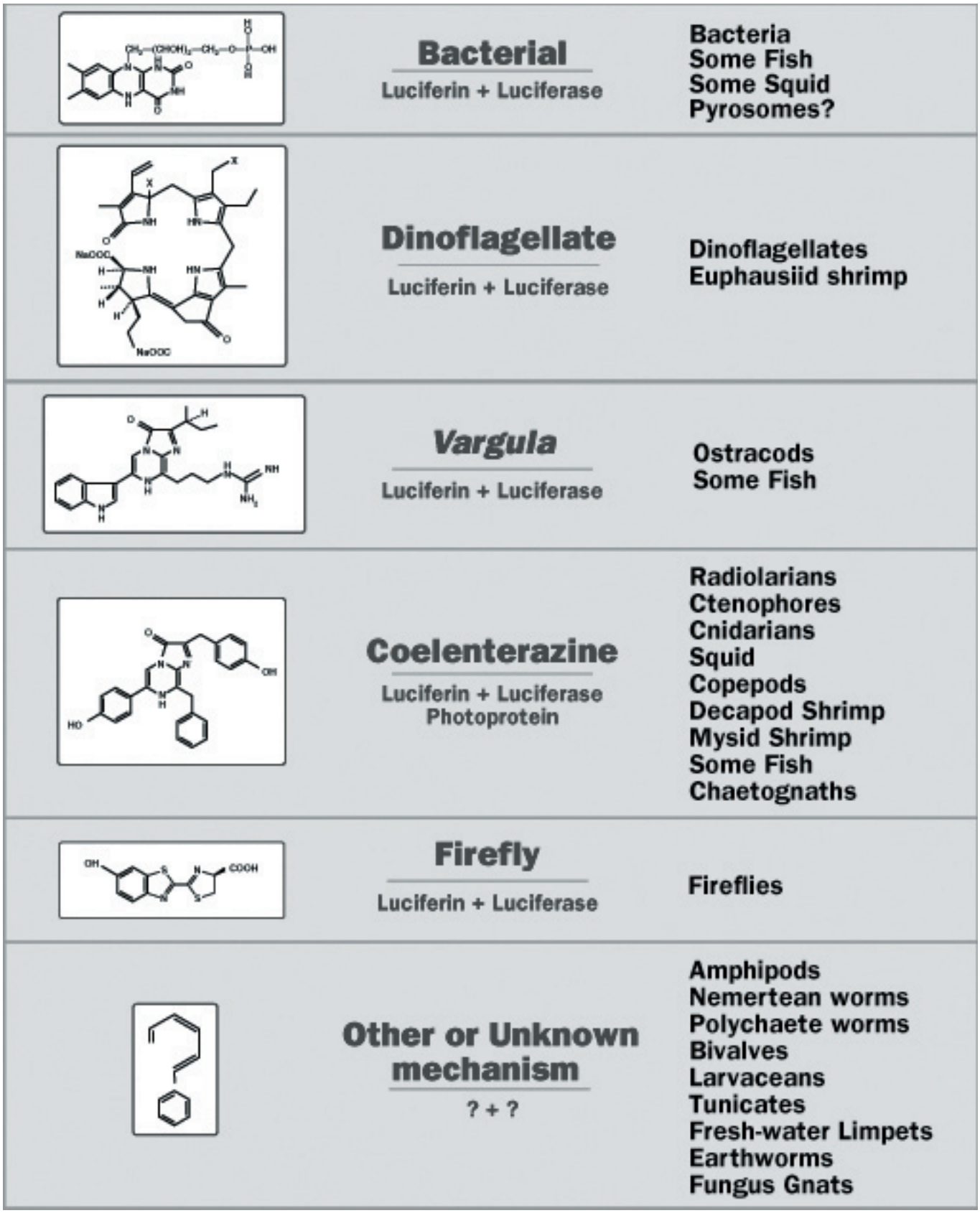

Figure 2 A survey of the different luciferins known to exist in different bioluminescent species (Haddock et al., 2000). Although the molecular architecture of the luciferins vary widely, all are comprised of complex organic compounds.

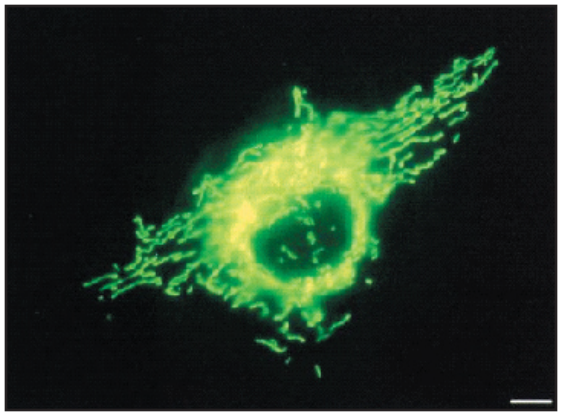

Figure 3 HeLa cells transfected with the mitochondrial signal sequence tagged GFP (Rizzuto et al., 1995). The rod-shaped fluorescent patterns were deemed to be mitochondria due to the expected shape. Scale bar = 7 μm.

fie demonstrated that GFP is a self-sufficient protein. The protein doesn't require an enzyme, substrate, or extra co-factors to generate the green fluorescence, only excitation with blue light. Furthermore, the expression and fluorescence of GFP in other species appeared to be stable and non-toxic. More importantly, Chalfie's paper was the first show that GFP can be used as a gene expression marker both in vitro and in vivo.

GFP's fluorescence is due to a chromophore created within the protein. The chromophore results from an autocatalytic event from the post-translational cyclization, dehydration, and oxidation of three residues, Ser65-Tyr66-Gly67 (Heim et al., 1994). The mechanism wasn't confirmed until the crystal structure of GFP was determined (Yang et al., 1996). The barrel shaped protein can only fluoresce once the protein has properly folded creating the chromophore. Mutational experiments have now produced different coloured variants of GFP (Heim and Tsien, 1996). Cyan, yellow and blue fluorescent proteins based on the original GFP are now commercially available. Recently, fluorescent proteins, emitting red fluorescence, that have slight homology to GFP have been cloned from ocean corals (Matz et al., 1999). The mechanism of the new red fluorescent protein (RFP) wasn't known until its crystal structure was determined (Wall et al., 2000). The structure of RFP forms a barrel shaped can, like GFP. However, RFP forms tetramer complexes in contrast to the monomer in GFP. The chromophore responsible for the red fluorescence, partially similar to GFP, is generated from the folding of key amino acids in RFP's structure. However, further chemical modification of the chromophore results in formation of special chemical bonds that contribute to the red fluorescence.

Applications of Bioluminescence

The most popular application of bioluminescence is the use of the luciferase-luciferin system and GFP as reporters of gene expression. Prior to the cloning of GFP, luciferase had been in use as a reporter gene for more than 10 years (Gould and Subramani, 1988). The bright bioluminescence generated from the luciferase assay made it ideal for sensitive non-radioactive assays. However, luciferase requires the addition of luciferin and co-factors, normally introduced after cells have been lysed, in order to measure the activity of the gene of interest. Expression of GFP in heterologous systems and demonstrated that GFP doesn't require exogenous substrates provided an excellent means of monitoring gene expression and protein localization in living cells. Shining blue or UV light onto a sample greatly simplifies the data collection process and allows GFP to be used in a variety of techniques including fluorescence-activated cell sorting (FACS) and microscopy.

The impact that GFP played in determining protein localization was demonstrated in 1995. Rizzuto et al. were one of the first groups to create a GFP protein chimera (Rizzuto et al., 1995). They successfully attached a mitochondrial signal sequence to GFP. Thus, when GFP was expressed, it localized to and extensively labeled the mitochondria (Figure 3). Three consequences resulted from their experiments. One, Blobel's signal hypothesis could finally be proved by direct visualization of tagged proteins in living cells. Two, organelle dynamics could now be studied using GFP fusion proteins. Three, protein localization and trafficking could now be extensively studied by direct visualization in vitro.

Perhaps the most spectacular use of GFP is in the generation of transgenic organisms. Stable GFP expression, as a reporter gene, has been successfully introduced in to the germ-line of mice, frogs, nematodes, flies, bacteria, and plants. Although the first demonstrated use of GFP as a transgene was by Chalfie et al. (Chalfie et al., 1994), GFP's first use in mammals was in 1995 (Ikawa et al., 1995). Ikawa et al. created transgenic GFP mice using a CMV enhancer/?-actin promoter. Traditional methods of genotyping, PCR and Southern blotting, are time consuming and require some skill. The ubiquitous expression of GFP that enabled the group too visually confirm transgenic versus non-transgenic mice. Using GFP transgenics benefits our understanding of cell lineage, gene expression, and gene function in vivo. GFP transgenics has been applied to numerous other organisms including flies, frogs, fish, and plants.

The perfect marriage between biotechnology, ethics and the application of GFP surfaced in 2001. Eduardo Kac, an assistant professor of art and technology at the School of Art Institute of Chicago, wanted

Figure 4 Alba, the GFP bunny, fluorescing GFP.

to create a GFP rabbit (Figure 4), to be used in an art exhibit, sparked a debate about the project itself, and about the practice of manipulating genes in animals for research (Kac, 2001). However, after the French group that produced an albino GFP expressing rabbit, named Alba, learned that Kac wanted to keep the animal as a pet, they refused to deliver their product. Animal rights activists complained about the needless practice of genetically manipulating animals for personal needs. Biologists acknowledged the project was frivolous and explained that the project, as far as they knew, didn't have any dangerous aspects to the rabbit. Regardless of its use, bioluminescence will continue to amaze.

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