The Use of Fluorescence in Genetically Modified Animals

The role of the enhanced Green Fluorescent Protein (eGFP) in Genetic Engineering

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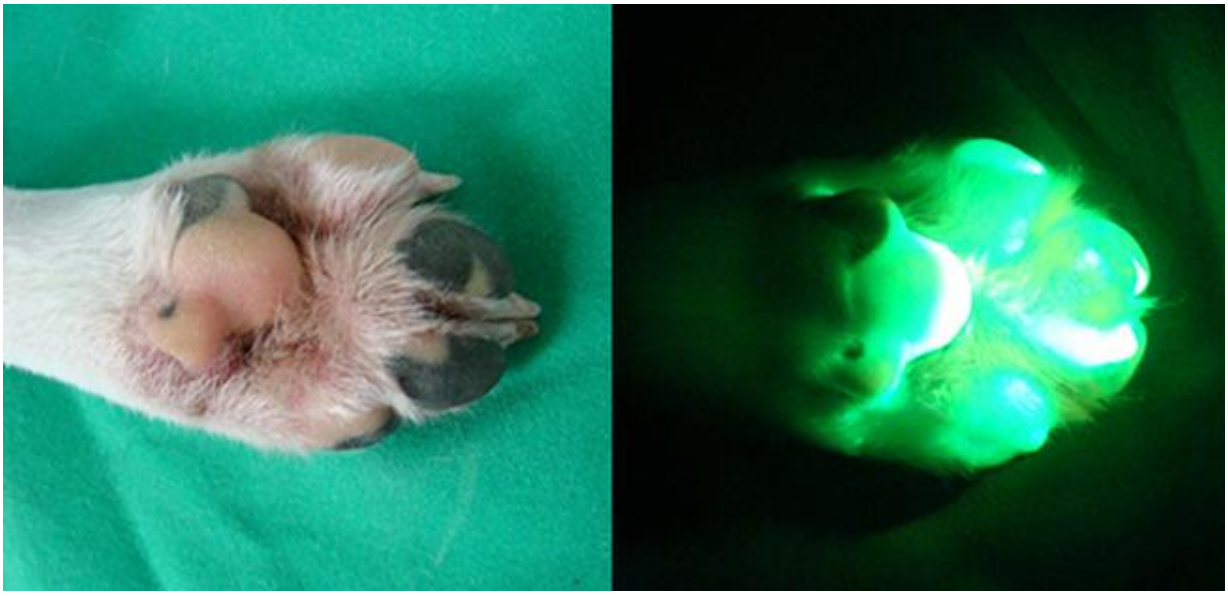

Tegon, a genetically modified dog, exhibiting eGFP

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Preface

When we were informed that we had to write a paper about Genetic Engineering, the first thought that came to mind was “Ruppy”. Ruppy was the first ever transgenic dog. She was genetically engineered so that she would glow red under UV light. We were so interested in the fact that scientists could create such animals that we began to research Ruppy and came across Tegon. Tegon was another transgenic dog created by the same laboratory. Tegon, however, expressed the enhanced Green Fluorescent Protein (eGFP). On the cover you can see a picture of Tegon’s paws when exposed to UV light. The expression of eGFP is regulated by doxycycline, so that only when it is administered, the protein is expressed and the dog can glow when exposed to UV light. The more we heard about eGFP, the more interested we became in all its possible applications in medical and biological research. eGFP (or better: the gene which encodes for it) is mainly used as a reporter gene, which means it is attached to a gene of interest and through its expression this gene is monitored. Depending on how the new genetically engineered organism is “programmed”, it can be used in multiple ways. We found it all very interesting and decided to write our whole paper on eGFP.

By writing this paper we aim to answer the following questions:

- What are some of the known uses and applications of eGFP?
- How does eGFP compare to other reporter systems, such as systems using other fluorescent proteins, β- galactosidase or the enzyme luciferase?
- How is eGFP inserted into a target organism’s DNA? What is the difference between a transgenic animal and a knock-in or knockout animal?
- How could eGFP and the use of eGFP develop in the future?
- What are some advantages and disadvantages of eGFP?

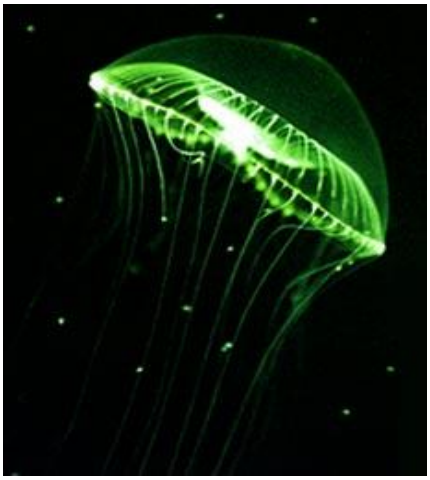

Figure 1: *Aequoera victoria*

Introduction

GFP is the abbreviation for **G**reen **F**luorescent **P**rotein. It is made up of 238 amino acids. As the name indicates, this protein gives off green light when it is exposed to blue or ultraviolet light.

Fluorescence describes the emission of light from a substance, which has just absorbed light or other types of electromagnetic radiation, of a shorter wavelength than that being emitted. Often fluorescent substances absorb ultraviolet light, or in other words light which is invisible for humans. Such substances then emit visible light, as in the case of GFP. GFP was originally found in the jellyfish *Aequoera victoria.* (**Figure 1**)

The first person who wrote about GFP was Osamu Shimomura in 1961. Then in1992 Douglas Prasher, and co-workers, isolated and cloned GFP and two years later, in

1994, they managed to use GFP as a marker for the expression of recombinant proteins. This soon became an often used method in cell and molecular biology.

In our paper we mainly discuss eGFP: “enhanced” Green Fluorescent Protein. It was first developed in two separate laboratories in 1995. There are multiple differences between GFP (also called wild type GFP or wtGFP) and eGFP. GFP is the “original” protein, while eGFP is basically the same protein, except, that it was changed so that it shines brighter and operates better at 37°C. The original wtGFP is too weak to be used in mammalian cells, and it only operates at low temperatures.
There are multiple other variations of wtGFP, which express different colors, such as cyan, blue and yellow. There are also other fluorescent proteins that have been isolated from other organisms, such as corals. To date, over one hundred fluorescent proteins in many different colors have been identified.

In 2008, Osamu Shimomura, Martin Chalfie and Roger Y. Tsien received the Nobel Prize for the discovery and the development of the GFP and it continues to be an important tool for scientific research. Only in the last month, for example, 55 articles relating to eGFP and/or using it in their research have been published[1] , ranging from the use of eGFP in cancer research to its use in protein localization. Last year, a research team for example used eGFP to track a gene, which was hoped to be useful in tackling cancer in a human carcinoma cell line.

Today eGFP can be used for many applications. It is often used as a reporter gene, to track different cells and/or proteins. This has great significance in research, because this way several processes in the body, such as cell division and specialization (differentiation) of stem cells can be tracked.
Expression profiling, the measuring of cell activity and protein synthesis, can also be done with eGFP. Protein localization, discovering where different proteins are used, can be done with eGFP too. Different promoters’ effect on protein synthesis and use can be tested with eGFP as well.
All of these applications of eGFP are helpful for drug screening, testing new medicines and for the tracking of diseases. Through the use of eGFP effective drugs can be developed.

Figure 2: GloFish

There are numerous other uses for eGFP. Pets expressing eGFP have been produced, for example: GloFish (**Figure 2**). These were actually originally developed to test how polluted the water is: they were modified, so that they would only express eGFP, if the water was polluted. eGFP was modified so that it would only glow, when other chemicals were present. In this sense eGFP was used as a biosensor.
eGFP has also been used in lineage tracing, to track which cells are formed from a stem cell. Because of this, regeneration in the intestine, tongue and brain could be traced (**Figure 3**). Scientists

[1] When accessing the link: http://www.ncbi.nlm.nih.gov/pubmed?term=(%222013%2F03%2F01%22%5BDate%20-%20Completion%5D%20%3A%20%222013%2F04%2F01%22%5BDate%20-%20Completion%5D)%20AND%20eGFP on 20.4.13 over 80 articles relating to eGFP and published over the last month could be found.

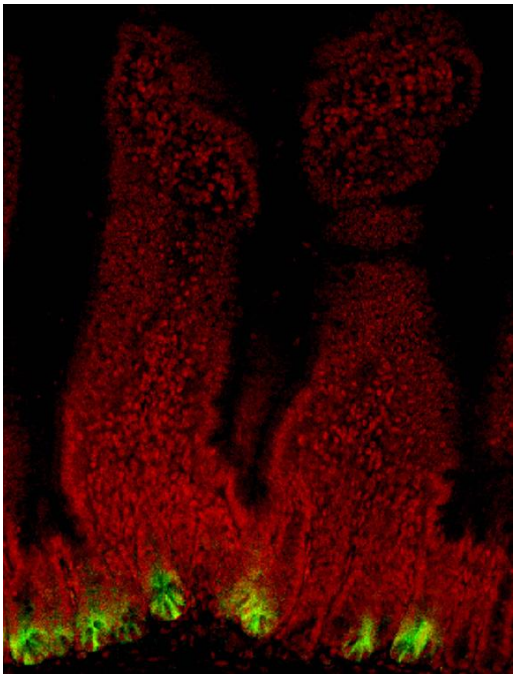

Figure 3: The expression of eGFP in stem cells of the intestine

also developed a “rainbow brain” mouse with different colored brain cells to track different cells in the brain and their development. These methods could also help with cancer research and in regenerative medicine.

Other reporter systems can also be used for these applications, such as other fluorescent proteins. These are either other modifications of eGFP or fluorescent proteins from other organisms, such as dsRed, from *Discosoma* (mushroom coral). β-galactosidase, an enzyme, encoded by the LacZ gene is another reporter system. This exhibits a blue coloring when exposed to the substrate X-gal. Yet another reporter system is luciferase, an enzyme which produces bioluminescence when it transforms luciferin. Luciferin must of course then also be injected (except for bacterial luciferase) for this reaction to occur.

Description of technique

For an organism to express eGFP, it must first be genetically modified and the gene for eGFP inserted into the organism’s DNA. This process of introducing foreign DNA into a cell is called transfection. Most of the time eGFP is used as a marker, which means it is inserted with other DNA just to show that it is there.

There are two main ways to add the gene for eGFP (and most likely other genes) into the DNA. The first: **transgenic**, is far less specific; it is unclear where the gene will end up. The second is called **knock-in** or **knockout**; this is more specific and uses homologous recombination to make sure that the new DNA sequence is inserted into the right location in the DNA (in the right locus).

For both of these main groups, however, there are multiple methods and for every method several modifications that are constantly being improved. We have just concentrated on the main methods. In **Figure 4** the main differences between transgenic and knockout and knock-in organisms is described.

A. Transgenic Animals

Transgenic animals are genetically modified and are “made” through pronuclear microinjection, or by viral transfection. This means, that nucleic acids are introduced into cells.
For pronuclear microinjection, a DNA solution is injected into the pronucleus of a newly fertilized, single-cell embryo. This is obtained from female mice by superovulation (this is when a female is brought to multiple ovulations through the administration of hormones). In the example shown (**Figure 4**) the oocyte develops into a mouse embryo which will be implanted into a pseudo-pregnant recipient mouse.

In the case of viral transfection, viruses are injected into cells or living organisms. The virus transports its genome, which has been modified, into the cell while it infects it. The genome is normally modified, so that a gene sequence, which one wants to add to the target's DNA, is added. It is also normally modified, by deleting the part of the genome, responsible for replication, to prevent the viruses from replicating and infecting more than just the part that should be modified. In this manner all of the genetic material is placed into the cells.
Tegon, for example, was created by viral transfection. In the case of Tegon, however, fibroblasts were modified and then inserted into the enucleated oocyte of another dog, and then grown in another female dog's uterus.

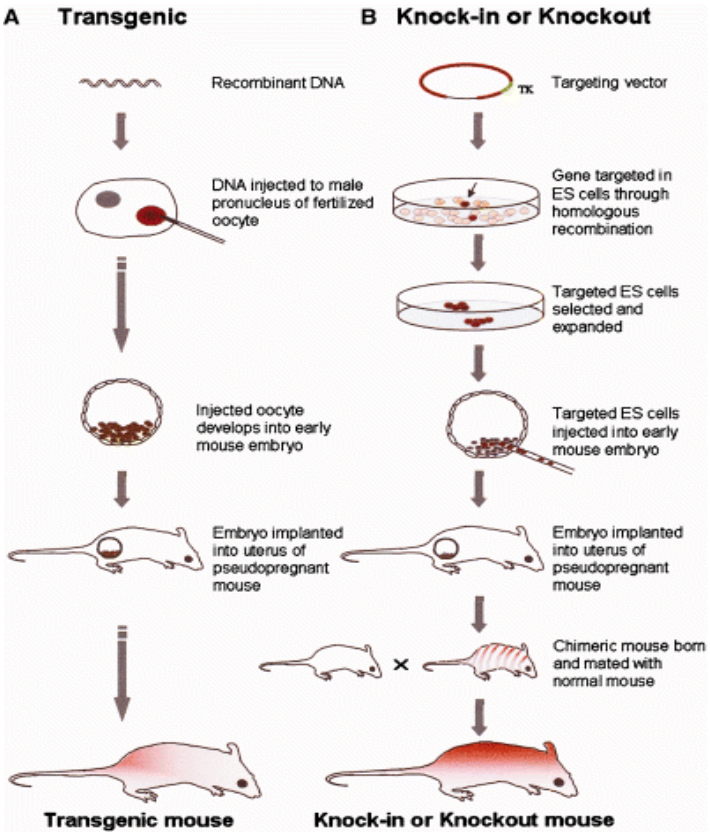

Figure 4: Main differences between Transgenic and Knock-in and Knockout mice

The disadvantage of transgenic animals is that it cannot be predetermined where the gene encoding for eGFP will end up.

B. Knock-in / Knockout Animals

The method to produce knock-in and knockout mice is practically the same. The difference is that in knock-ins the new DNA is a modification of the old gene, or a new gene and in knockouts the old gene is deleted and only biomarkers added to replace it.

To make knockout or knock-in mice, the new gene must first be designed in a targeting vector. This vector must have the new or modified gene sequence, a marker (this can be fluorescence or drug-resistance), and regions of DNA homologous to the target. Homologous regions are needed so that homologous recombination can take place between the original DNA and the targeting vector. This enables the new gene(s) to be inserted into the target DNA sequence.

Then stem cells are taken from a blastocyst of the organism (in this case a mouse), they are then grown outside of the mother for a period of time before they are transfected with the targeting vector (normally a modified plasmid). There are multiple ways to transfect a stem cell and the most common is electroporation. Electroporation or electropermeabilization is the external application of an electrical field to increase the electrical conductivity and permeability of the cell plasma

membrane. New substances including DNA can be introduced into the cell through electroporation or electropermeabilization.

After transfection, with a bit of luck, homologous recombination will occur. These stem cells will then have the new DNA sequence inserted. This insertion almost always occurs only on one of the chromosomes. The stem cells are therefore heterozygous. Using enzymes such as Cre or Flp, which are DNA-recombinases this process can be made more efficient. This is then called **RMCE (recombinase-mediated cassette exchange).** Our interview with a researcher who regularly uses this technique can be seen on page 7

Determining which stem cells have the new gene sequence inserted requires that a biomarker is present. Only the stem cells which exhibit the biomarker (i.e. for eGFP exhibit fluorescence under UV light or are resistant to the chosen drug) are reinserted into the blastocyst.

The blastocysts with modified DNA are then placed in the mother's uterus and develop further. The newborn mice are chimeras, some of their cells are derived from modified stem cells, and others are not.

These mice are then bred with other, not modified mice. If the chimera's gonads were derived from the modified stem cells, they will then possibly pass the genetic modification on to their offspring and they will be heterozygous, with all their cells having one copy of the modified gene. If these are interbred with other heterozygous mice with the new gene, mice which are homozygous for that gene may be produced.

Documentation of visited research institution:

Basel (25.03.2013)

We were able to arrange a meeting with Dr. J.[2] at a biopharmaceutical company in Basel. We were shown around the research laboratory and were able to see firsthand where the genetic modification process occurs. We were shown where and how samples are analyzed (for this a specialized microscope is needed, see **Figure 5**), where stem cells are taken from blastocysts and where the genetically modified cells are reinserted into the blastocysts (**Figure 6**).

Figure 5: Zeiss axio observer microscope, used for observing fluorescent samples.

Dr. J. also explained the process of genetic modification of the embryonic stem cells and how they use eGFP in their work. Through modification of the Rosa26 locus they can track the expression of eGFP and see what effect different promoters have on gene expression. With this method they can also track the development of different organs (lineage tracing). We could not see any transgenic animals because our presence could potentially have a detrimental effect on their health or well-being. We were also not allowed to take pictures. We have however added some photos of the machinery from the internet.

[2] Name changed.

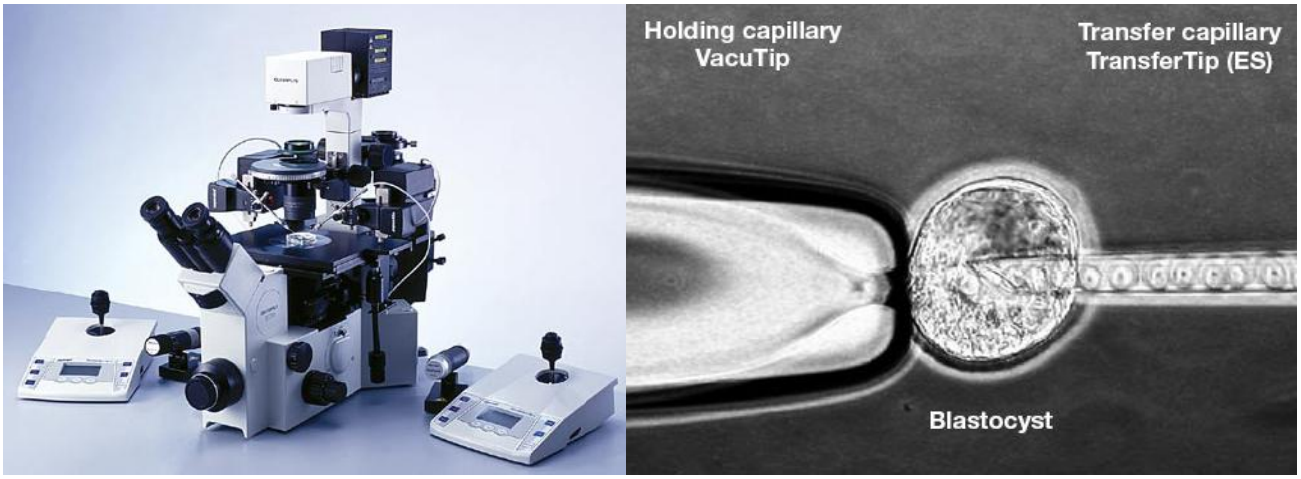

Figure 6: Equipment used in embryonic stem cell transfer into a blastocyst (because of the minute sizes, the process is highly mechanized). Shown here are: TransferMan NK2, CellTram Air, CellTram vario and an Olympus IX 70 microscope. On the right is a picture of the actual transfer.

Interview

With Dr. J. a researcher at a biopharmaceutical company in Basel (25.03.2013)

- **What, would you say are some of the most important GFP development milestones in its history as a biomarker/a reporter system?**

Well, I'd say when Roger Tsien, Osamu Shimomura and Martin Chalfie received the Nobel Prize for their work on eGFP, eGFP first gained global recognition, which was of course a big step forward. I would also say the development of ways to inhibit the expression of eGFP was the next big step, due to this we can also insert cassettes that can make sure that the cells keep on expressing the eGFP protein after it was first activated. This is important for lineage tracing, in this manner we can "mark" a cell (insert this sequence) and see what cells are formed from it.

- **What have you been able to accomplish with lineage tracing?**

With lineage tracing we have been able to see how the tongue, liver, gut and even brain are regenerated. Stem cells, located in cavities of the intestine lining for example are constantly replacing the old cells and specializing to fulfill their tasks. This can all be observed with reporter systems, such as eGFP or β- galactosidase (Dr J. showed us a short video demonstrating lineage tracing in the gut; this can be viewed via the following link: http://www.hubrecht.eu/research/clevers/research.html)

These reporter systems can also be used for drug screening (they are used as model organisms for the disease), expression profiling (the measuring of cell activity and protein synthesis) and protein localization (discovering where proteins are used).

- **Is there any limit to where eGFP can be added?**

Really any organism can be genetically modified, but of course it is best to use an organism that does not express the inserted gene naturally, because then it would be hard to distinguish between natural and modified expression.

The gene can also, in theory, be inserted anywhere. The Rosa26 locus is just such a good place to insert a gene of interest because it is an area of the chromosome, which is almost always being read and its genes are used almost in the whole organism. Deleting the original gene in Rosa26 locus also does not lead to any loss of function in the mouse. Insertion behind any promoter, would also tell where that protein is synthesized and used.

- **What are the advantages of RMCE (Recombinase-Mediated Cassette Exchange) in comparison to other methods of insertion?**

RMCE is very specific. Viral transfection is for example not easy to program, and homologous recombination takes a lot of time and relies on chance. RMCE is a method that just improves the efficiency with which the insertion occurs making it easier to create the desired recombinant organism.

- **Is the Green Fluorescent Protein the best biomarker or are other biomarkers, such as luciferase a better option?**

Well, they are all quite different and each is good for a specific purpose. Luciferase is for example, good in the respect that the animal can normally be used for longer, but it is much less specific, it just gives you a general area. eGFP, β- galactosidase and other fluorescent proteins are really the best when it comes to marking cells and using them for reporter systems and lineage tracing.

- **What are your aspirations for the future development of eGFP?**

Well I hope someday we might actually develop an even better reporter system than eGFP, maybe something using sound, which would be harmless for the organism, but still specific. Maybe it could even some day be used in human diagnostics. Maybe it could be used in cancer research and the regulation of tumor growth. For the time being, however, that's a distant hope and the best developments I see for the short term future would be the use of a combination of different markers, e.g. different modifications of wtGFP, to see exactly which cell comes from which one.

Discussion

It is amazing how the uses of GFP have developed over the years. From an unspectacular protein found in jellyfish in 1961 by Osamu Shimomura, through multiple modifications in multiple labs (the greatest of which in 1995 produced eGFP), it has become an irreplaceable research aid.

Still the advantages and disadvantages of eGFP are debatable.
In comparison to other reporter systems, eGFP has many advantages. The greatest is the fact, that it is an autofluorescent protein. There's no need for the addition of any substrates, or other cofactors, to make it visible. This is not the case for β-galactosidase or luciferase, in most cases for these reporter systems, substrates or cofactors are needed. eGFP thus allows observations of gene expression in living cells in a noninvasive manner. eGFP can also still be engineered to only express eGFP when specific inducers are administered to the organism, which allows for even more uses.
eGFP is also very stable; this facilitates the observation of cells, like embryonic stem cells over a long period of time. eGFP has a low toxicity, and so far no harmful effects over time have been identified.

With eGFP it is possible to mark and observe specific cells and even specific parts of a cell. This is not the case with luciferase, because luminescence spreads out from where the enzyme is, it is almost impossible to see exactly which cell has been marked (and locating the luciferase within cells is just impossible). Recombinant organisms expressing a luciferase reporter have other uses in biomedical research. For example because the luciferase signal can be detected in whole organisms, it can be used to track tumor growth without having to kill the host organism. Because the signal to detect eGFP expression is weaker than that of luciferase, the organism usually has to be killed and illuminated with an intense UV light source to allow the marked cells to be detected.
In comparison to other fluorescent proteins, eGFP has the advantage of extreme brightness and ability to work at higher temperatures, still its advantages over other fluorescent proteins are only slight and they are often used together. The reason why eGFP is used more than other modifications or wtGFP is, that more is known about eGFP than about the other fluorescent proteins.

eGFP is also environmentally friendly (as it is a protein that can be broken down in the environment). Genetically engineered animals, which express eGFP, are also not allowed in most countries outside of the laboratory, so their effect on the environment is minimal. GloFish, for example would not survive in nature, and are only allowed to be sold because of this (the sales are also quite restricted, e.g. not allowed in Europe and California).

Ethically, however, the genetic modification of organisms to express eGFP raises several questions. By using eGFP scientists gain an insight into the inner workings of the cell, the uses of proteins and the organism as a whole. This can have therapeutic benefits and help to produce medicines in the future. On the other hand, this information can also be used to dramatically modify an organism. How far should this be allowed to go, should people be allowed to “perfect” their genome? Also, many animals are used in these experiments and they are often killed. Is this loss justifiable by the insights gained through the experiments conducted with those animals? These are some ethical questions, which are not so easily answered.

However, for cell and molecular biology, the discovery and the many possibilities that eGFP offers is a big step forward. Scientists hope to understand the wonders of nature and of course there is also hope, that methods to visualize cancer and to understand it better could be developed with the use of eGFP. In the near future however, it is most likely that work with eGFP will be done in cell cultures and not in living animals.

Summary

In this paper the development of GFP over the years and the modification of GFP to eGFP have been discussed. Also the methods used to produce transgenic, knock-in and knockout organisms with eGFP have been explained. The multiple uses of eGFP have also been listed.
The green fluorescent protein has had an enormous impact on science. And even though it was discovered over 50 years ago, it continues to be increasingly more and more important in biomedical research.

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