How non-invasive visible light imaging can change drug discovery

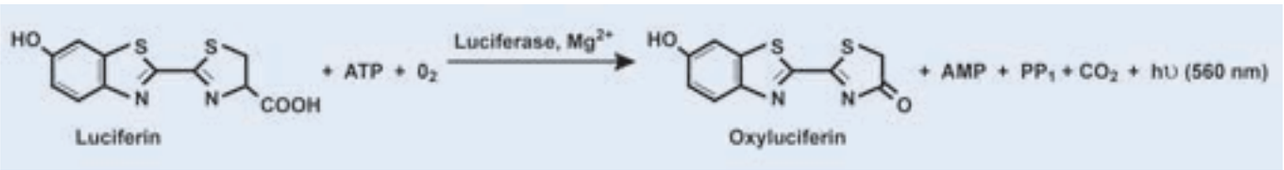
In the past decade, non-invasive visible light imaging gained acceptance not only as a valuable research tool, but also as a means of evaluating new compounds for efficacy and pharmacodynamic properties in drug discovery. It made possible routine access to animal models which were previously out of reach due to high costs, labour intensity, or animal welfare issues. Several such models have already significantly contributed to the development of approved medicines, and non-invasive visible light imaging may soon replace mammography for detection of breast cancers. In this review we will describe the already fulfilled promises, as well as future directions for this new technology.

By Dr Peter Lassota and Dr Anjani Shah

Scientists are well aware of their debt to the fruit fly for opening up the field of genetics. Now we can also give our thanks to another type of fly – the firefly. Fireflies have eased the application of non-invasive visible light imaging in biological research and drug discovery.

Non-invasive visible light imaging, also called biophotonic imaging, captures light emitted from living cells to learn about physiological processes. Most animals do not have cells which would spontaneously emit light. This allows scientists to introduce an enzyme (like firefly luciferase) which would produce visible light in the presence of its substrate, luciferin and ATP, and will allow them to follow light emitting biological processes on the background of a 'dark' animal. Depending on how the firefly luciferase is introduced to the host, the light emission can 'report' on cell locations, viability, growth and mobility, or can indicate the status of biological processes, enzyme and pathways activities, inflammatory processes, spreads of infections, injuries, etc.

What good are glowing lab mice and rats? Creating animals that emit easily-detectable light has not only enabled new kinds of assays, but has also made traditional experiments more efficient. For example, studies where animals previously had to be sacrificed to view cellular events can now be accomplished while the animal is still alive. Additionally, without the need to sacrifice the animal, more accurate data is available because cellular events can be visualised at various



time points in the same animal. This also reduces the number of animals necessary for a drug-response study, adding to cost savings. Other applications of non-invasive visible light imaging are discussed below.

Luciferase technology

Light is produced when luciferase, the enzyme discovered in fireflies, cleaves its substrate, luciferin with the help of ATP. By cloning versions of the luciferase gene into vectors such as retroviruses that can infect animal cells, cell lines and transgenic animals have been created to express the luciferase gene. When injected with the luciferin substrate, the cells expressing luciferase (the luciferase can be fused to tissue-specific promoters) in the transgenic or knocked-in animal or cell culture, will utilise the ATP to oxidise the luciferin and release light.

Image capture

Of course the light needs to be seen. Though usually not visible to the naked eye, the light is bright enough to be detected by the more sensitive charge-coupled device (CCD) cameras non-invasively, through the animal's tissue.

The *in vivo* detection system involves a black box where the animal or specimen is placed, which sometimes also serves as an anaesthesia chamber. The CCD camera sits on top so a photographic image of the subject taken first, can be superimposed upon the light emission image registered next. Newer equipment also includes the ability to emit excitation light at various wavelengths so that fluorescent probes can be used for analysis of other cellular parameters.

Technological advances

Recently the methods for introducing the luciferase gene into experimental animals have improved. Newer retroviral vectors are now available that ease the creation of transgenic and knocked-in animals so that stable expression of luciferase is possible. Transient expression of luciferase is a limitation for *in vivo* experiments because most assays are not performed until a few weeks after luciferase introduction, so its expression must be long-lived, and steady. The older, much easier methods of introducing luciferase give transient expression, what largely limits the resulting bioluminescence to short, *in vitro* assays.

The luciferase gene itself has also been reworked so that its expression levels are higher. The first round of modifications was aimed at making sure that mammalian, not firefly codons are used to produce the protein. The second round just made the enzyme making more light. Both together enabled greater light production in mammalian subjects, making the assay more sensitive. In fact, one potential limitation is that the newest constructs are able to produce so much luciferase now, that one needs to make sure the cells expressing luciferase have enough ATP for their own metabolism.

In vivo applications of bioluminescence are more versatile now also because of advances in detection equipment. The latest imager is able to perform three-dimensional reconstruction to better pin-point the source of light emissions in the animal's body. There is also a dual illumination capability (epi- and trans-illumination) which reduces background fluorescence thereby enabling more precise visualisation of the fluorescent probes. For example, both shallow and deep tumours in rodents stained with fluorescent antibodies can now be visualised in 3D.

Advanced fluorescence imaging with the spectral unmixing feature allows using multiple fluorescent reporters which can be read in addition to bioluminescence.

Non-invasive visible light imaging in development of oncology therapies

Preclinical drug development in oncology provides many illustrations of the benefit of non-invasive visible light imaging. Several anti-cancer therapies to have hit the market in the last few years, such as Sutent and Nilotinib, were developed with assays that used bioluminescent technology. A luciferase system provides the important advantage of an earlier window into the drug's efficacy.

Traditionally, a drug's effectiveness in preclinical studies is measured by its ability to shrink the size of a tumour in an animal. However this is a late

sign of the compound's effectiveness because it takes up to few weeks before cell death leads to reduced tumour size. In a rodent model of cancer with the luciferase transgene expressed in the target cells, death of the relevant cells is visible as a decrease in brightness. Such animals provide an earlier indication of the effectiveness of the anticancer agent being tested and thus speed the drug development process. From an animal welfare standpoint the technology is also helpful, particularly in models of disseminated diseases, because the rodents can be relieved earlier from suffering rather than waiting until their death, which was often an endpoint for the earlier studies of anticancer agents.

Opening new areas of study

Another way bioluminescence has helped drug development is by allowing access to animal models of diseases that previously could not be studied. An example is bone metastasis. Tumour cells in the bones were normally impossible to assay without sacrificing the animal. Thus it was hard to determine whether a potential therapy is effective or not or even to understand how an untreated cell is changing over the course of disease.

With bioluminescence technology, however, tumour cells expressing luciferase can be used in models. When animals are injected with luciferin, the tumour cells in the bones will be detectable. This enables one to follow how treatment effects the cells' survival over time.

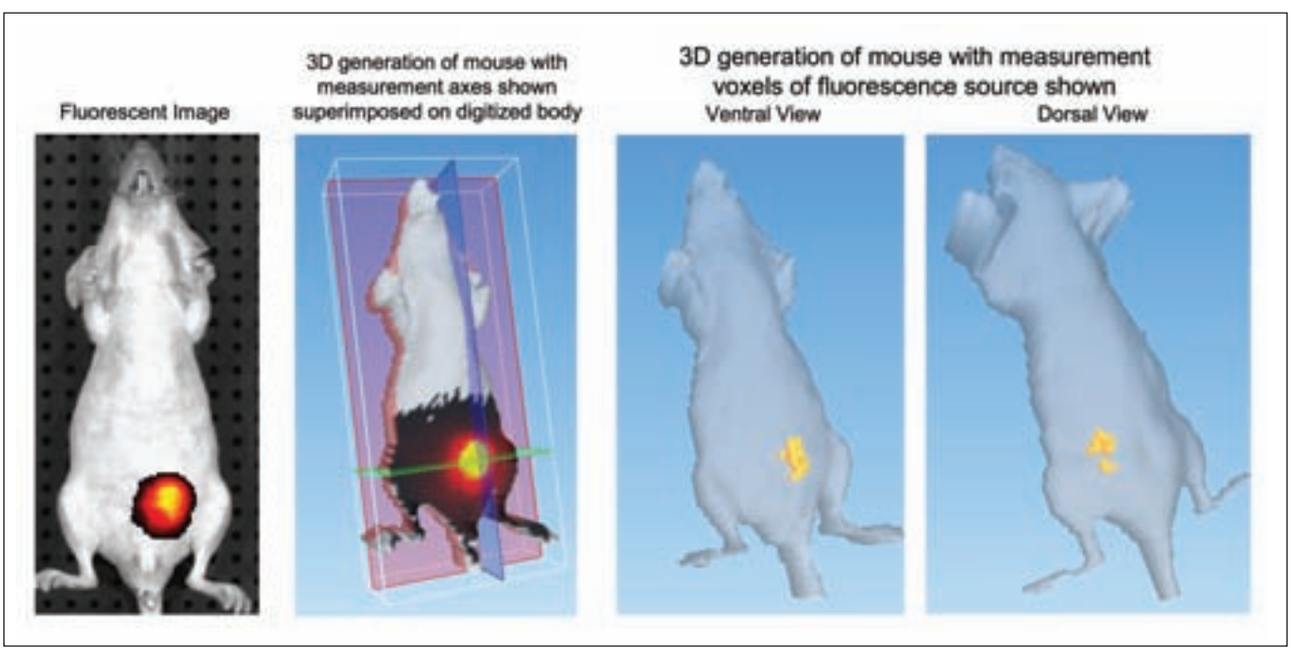
Infectious diseases

Many applications of the luciferase technology can be found in infectious disease research. Bacterial studies are especially suited for this technology because the bacteria one is studying can express not only the luciferase enzyme itself, but also its substrate luciferin, eliminating the need for delivering luciferin exogenously. By sneaking luciferase into the bacterial genome, one can monitor which host cells are infected by the bacteria over time and follow disease progression and response to treatment such as antibiotics and vaccines. One does not have to sacrifice the animal to get images of tissue for determining where the labelled pathogen went. Similar approach can be applied to most viral infections, but luciferin still has to be delivered exogenously in such systems.

Coupled with fluorescent technology

In some ways bioluminescence is similar to fluorescent technology – biological entities are labelled in order to be visualised. However, the two technologies are proving to be complementary rather than competitive and have distinct challenges and advantages.

The advantage of fluorescent visualisation is that experimental systems do not have to be modified ahead of time; no foreign gene has to be inserted. However, a probe must exist for the process one is interested in following. Another major drawback is that interpreting fluorescent signal is more



The three-dimensional reconstruction of the fluorescence image of an orthotopic prostate tumour labelled with XenoFluor-Herceptin probe

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complicated because it has to be extracted from the background, natural fluorescence of cellular components, which can be significant.

By contrast, bioluminescent technology is a bit harder on the front end (need to create the appropriate luciferase-labelled biological entities like cells, or proteins), so it is hard to imagine it will ever be used in the clinic, in humans. However, the analytical end is easier. Interpretation of the light signal is simpler because there is basically no background bioluminescence.

The latest and best imaging machines have been designed to detect fluorescent as well as bioluminescent light so that both technologies can be used in the same experiments, enabling very information-rich studies. Illuminating biological processes in a single animal with fluorescent probes as well as bioluminescent technology allows even greater real-time visual exploration and analysis of gene expression, cellular pathways, drug/target interactions and the mechanism of action of drugs.

Future applications in drug development

The main advantage of the bioluminescent imaging that has yet to be realised is the technology's continuity. The same assay can be used in a drug discovery and drug development mode. In other words, the same assay used *in vitro* to determine whether a compound is affecting a specific target, can then be applied *in vivo* to determine if the newly discovered compound affects the same target in the same way. This continuity could increase the speed of *in vivo* experiments and the quality of compounds reaching the clinic by providing a validated pharmacydynamic readout of drug activity.

The *in vitro* target approach is slightly different from the traditional *in vivo* bioluminescent experiment where one uses luciferase/light as an indicator of cell viability. Here, luciferase is fused to a specific protein whose survival (measured by light) is indicative that a drug is active. By using this same construct in an *in vivo* experiment, one can determine if the cellular phenotype one is interested in correlates with the target's response to the drug. If so, one has extended the validation of the target into the *in vivo* situation .

A more concrete scenario of using the same bioluminescence assay for an *in vitro* and *in vivo* application is illustrated by a recent study using bioluminescence to find anti-cancer compounds. Researchers at the Dana Farber Institute in Boston created an *in vitro* assay fusing luciferase to a protein, p27. This protein is normally stable in its unphosphorylated state and inhibits cell proliferation. When cells are stimulated to proliferate, a kinase, called CDK2, phosphorylates p27 and targets it for degradation by proteosome removing the brake on cell proliferation. .Using this *in vitro* set-up, the researchers screened for anti-cancer compounds that inhibit CDK2 and cause cell cycle arrest by reducing the phosphorylation of p27 and increasing its stability (assay for increase in light). By using the same p27-luciferin target in an animal experiment, researchers would then be able to determine if the activity of CDK2 as judged by change in light, correlates with reduction in tumour size in response to whatever agents made it through *in vitro* screens.

However this is hard to put into practice, mainly because it requires innovation in the way most pharmaceutical companies are structured. All the pieces are there but management needs to pay more attention to putting them together. Presently, at most companies, the teams involved in drug discovery are different and isolated from the teams involved in drug development. So there is no incentive for continuity.

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One of the newest products to market, the IVIS Spectrum, combines spectral unmixing capabilities with 3D tomography for both fluorescent and bioluminescent reporters. Available through Caliper Life Sciences. Image credit: Caliper Life Sciences

A brief scan of peer-reviewed articles published in Summer 2007 illustrates the range of applications of non-invasive *in vivo* imaging:

- An anti-cancer agent's *in vitro* activity was compared to its *in vivo* performance by fusing the agent's target, a specific gene promoter, to luciferase and creating transgenic animals. [Dhar et al, Carcinogenesis 2007 Jul 25].

- The effectiveness of an anti-cancer agent was examined in an animal model of bone cancer by determining the location of tumour cells engineered to express the luciferase construct. [Eur J Nucl Med Mol Imaging. 2007].

- The ability of an oncolytic virus to replicate *in vivo* was determined by co-infecting with a virus expressing luciferase. The *in vivo* imaging enabled the same animal to be imaged repeatedly to determine progression of the viral infection. [Guse et al, Gene Ther. 2007 14(11):902-11].

- The ability of a recently developed stem cell biomaterial scaffold to enhance *in vivo* tissue regeneration was monitored by creating mice whose stem cells expressed the luciferase gene. The presence of lighted areas in the mice indicated how well their immune systems were accepting the stem-cell scaffold. [Roman et al., Biomaterial 2007 June 28(17):2718-28].

Future applications in humans

In vivo imaging with luciferase will obviously never be an option in humans. However, advances are occurring in making sense of light propagation, dispersion and fluorescent emission by various human tissues. Progress is slow, however, because specialised knowledge and well developed databases are required to determine how the pattern of emitted light from particular areas of the body correlates with information about the cell's shape, density, or other properties. For example, reports in the literature suggest that soon knowledge of the optical properties of healthy breast tissue vs breast tumours may enable early detection of abnormalities, possibly replacing the need for mammograms. Prototypes of such clinical instruments are now functional, and the FDA approval for such approach seems to be only the matter of time. Similarly, work is in progress to use light patterns from the fluid around the eyes as an indication of the amount of glucose in circulation. This would enable easy monitoring of blood glucose levels for diabetes patients. DDW

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