An inner light

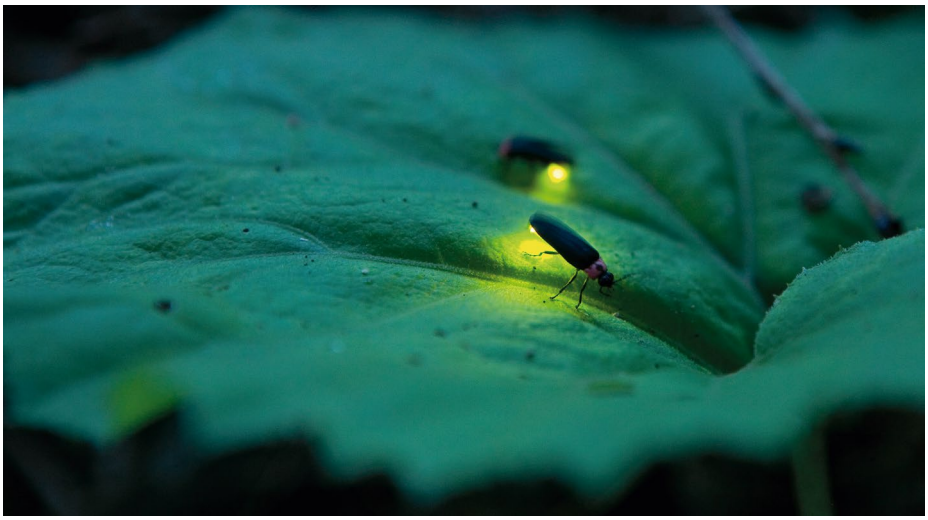
In vivo bioluminescence imaging offers a non-invasive look inside the body. Its future looks bright.

Michael Eisenstein

Shinae Kizaka-Kondoh recently had the opportunity to read a monkey's mind. Using an imaging system called 'AkaBLI', Kondoh—a researcher at the Tokyo Institute of Technology—and colleagues were able to observe neurons firing within the brain of a marmoset as it roamed about its cage. Traditional methods of looking at the cellular and molecular activity of the brain generally entail restraint or anesthesia, or require surgery or dissection. AkaBLI is a newly developed iteration of a non-invasive method called bioluminescence imaging (BLI), and it allowed the researchers to visualize brain function through the intact skull of a freely moving animal over the course of several months[1]. "It is wonderful to be able to get such information from inside the body with light," Kondoh says.

Scientists have been exploiting bioluminescence—the enzymatic reaction that produces the distinctive glow of animals like the firefly—as a visual readout of biological activities in cultured cells for decades. But more recently, researchers have come to recognize it as a simple, non-intrusive method for direct *in vivo* imaging in animal models. Newcomers are routinely taken aback by what they can observe with BLI. Gary Luker of the University of Michigan recalls showing a collaborator imaging data from mice that had been infected with bioluminescent herpesvirus back in 2002[2]. "I still remember his amazement to actually see the site of infection," says Luker. "In some immunocompromised strains, we could see that an infection that started in the footpad of the mouse may have spread to the central nervous system, which nobody really had even thought about before."

With an expanding toolbox of reagents and increasingly sophisticated detection equipment, *in vivo* bioluminescence imaging (BLI) has become a useful tool for researchers studying dynamic processes related to cancer and infectious disease. And as high-profile demonstrations like the recent work from Japan continue to emerge, many early adopters believe BLI is ready to go mainstream, offering life scientists an accessible window into the inner workings of the body.



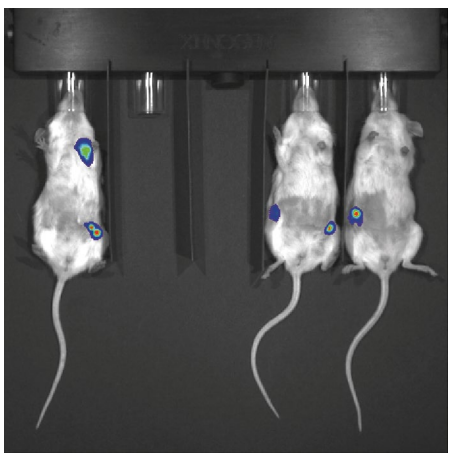
From nature to the lab: Perhaps the best known bioluminescent animal is the firefly, which has lent its glow to the lab. Other sources—natural and engineered—are improving bioluminescence imaging too. Credit: tomosang / Getty

Penetrating analyses

When it comes to bioimaging, fluorescent proteins generally hog the glory. These molecules do not shine on their own, but emit a bright signal when illuminated at a particular wavelength. Extensive efforts towards fluorescent protein discovery and engineering have produced a diverse palette of molecules that produce colors spanning the spectrum. By coupling these to different genes, one can visualize molecular-scale processes within cells, or use combinations of fluorescent proteins to track many cellular targets at once.

In contrast, bioluminescence is produced by a chemical reaction mediated by enzymes known as luciferases. In naturally bioluminescent animals, luciferase processes its substrate in a chemical reaction that results in the emission of photons, generating visible light. The firefly *Photinus pyralis* is perhaps the best-known example. Its greenish-yellow glow comes from the reaction between luciferase and a substrate molecule called D-luciferin. However, there are a host of other bioluminescent insects, including various beetle species, and numerous marine organisms ranging from plankton to fish that also use luciferase-mediated reactions to generate light. Over the past three decades, molecular biologists have identified and cloned a number of different luciferase genes.

For *in vivo* BLI, animals—in most cases, mice—are genetically modified to express one of these genes. The objective is for the



Tracking tumors: The Luker lab uses bioluminescence imaging to monitor tumor progression. Credit: G. Luker

animal to produce the luciferase enzyme in response to a particular biological event, such as the expression of a specific gene or activation of a particular cellular protein, or in a particular subset of cells. The strategy used for genetic modification is a critical component of this process, and can profoundly affect the outcome of the experiment (see Box 1). After the animal has been administered the enzyme's substrate, it will produce a measurable glow at the time and place where luciferase expression occurs—typically within a matter of seconds or minutes of the gene being switched on.

The fact that BLI does not require external illumination gives it an edge over fluorescence for some *in vivo* applications. The laser light used to excite fluorescent proteins generates considerable unwanted background glow as it illuminates and scatters off tissues, making it difficult to decipher imaging data. "It's not so much that the signal from bioluminescence is higher," explains Jennifer Prescher of the University of California at Irvine, "it's that the background noise is almost zero." If a researcher sees light with BLI, it's probably from a true biological event.

BLI can also image deeper inside the living animal, enabling a view of tissues where the excitation light required for fluorescence would be too heavily scattered to generate useful data. In practice, this means that researchers can look several centimeters beneath the skin with BLI, whereas such a feat with fluorescence would require surgery to give the microscope a clearer view. "There's a massive emphasis now in the UK on the 'three Rs' and improving animal welfare," says Simon Waddington at University College London (UCL). "This non-invasive imaging plays right into that." Animals can be routinely monitored with BLI for days, weeks or even months.

Researchers have been obtaining intriguing data from *in vivo* BLI since the first commercial imaging instruments hit the market in the early 2000s. Early studies mainly entailed tracking labeled cells, bacteria or viruses within the rodent body,

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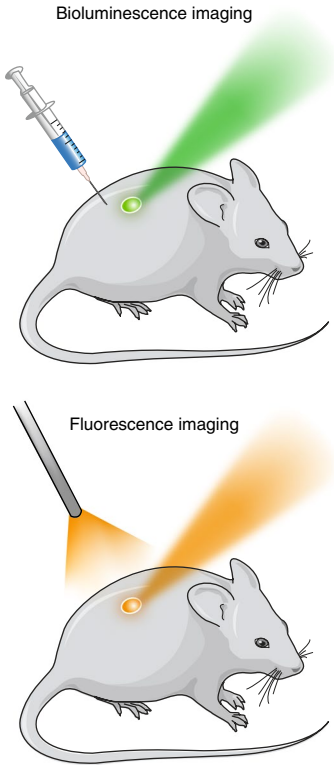


Inner vs. outer light: For *in vivo* bioluminescence imaging, animals express a luciferase enzyme. When the enzyme's substrate is injected, a chemical reaction produces visible light. The approach is less invasive than fluorescence imaging and can function deeper in tissues, but it can have a lower spatial resolution. Credit: E. Dewalt / Springer Nature

but the field has quickly evolved. "It didn't take a lot of imagination to figure out that many of the molecular biology assays that one uses bioluminescence for in cell culture could be adapted," says Luker. "Looking at what cells are doing and how they are signaling, and if drugs administered to an animal are hitting their target."

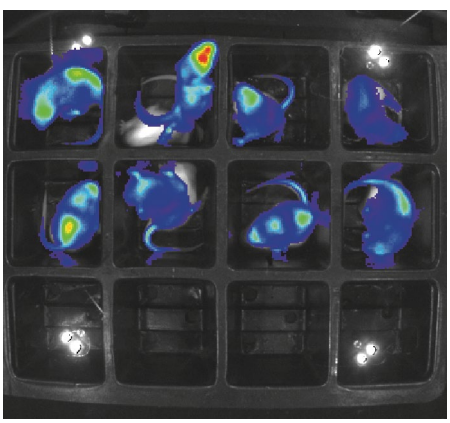
This latter aspect was a major focus of Kondoh's earliest work with BLI[3]. To investigate the function of a drug developed to target oxygen-deprived cells deep within tumors, her team generated mice that produce luciferase within cells expressing a hypoxia-responsive protein known as HIF-1. "We found that irradiation increases HIF-1 activity, which helps cancer cell survival," she says. "We also clarified that the drug removes hypoxic cells and HIF-1-expressing cells generated by irradiation."

As detectors have become increasingly sensitive, researchers can continuously collect bioluminescence data from unrestrained, unanesthetized animals—a feat that is far more technically complex with fluorescence. "We have these cameras that are very sensitive to photons, so you can get millisecond-scale acquisition of the image while the animal moves," says Laura Mezzanotte of the Erasmus Medical Center. This can be invaluable for studies of metabolic activity, which would be confounded by the physiological changes experienced by an immobilized or anesthetized rodent. Rajvinder Karda, a researcher studying gene therapy at UCL, notes that her studies of central nervous system function have benefited immensely from the elimination of anesthesia. "Isoflurane can dampen the development of the growing brain, and we are trying to monitor luciferase expression at neonatal stages of development," she says. "We're exposing them to less chemicals and it's a very quick *ex vivo* readout."

Evolving options

But BLI users must grapple with some notable limitations. Unlike the sub-cellular resolution achievable with fluorescence, bioluminescence is better suited for larger multicellular structures or entire organs. "Spatial resolution is at best a millimeter or two," says Luker, "In the context of the mouse, this is not that small." Although BLI can go deep, depth comes with compromises in terms of resolution. Photons become more scattered the farther they have to travel, such that signals emitted from deeper tissues will be greatly broadened by the time they are detectable at the surface of the skin, reducing image quality.

These constraints have sparked efforts to develop enzymes and substrates that might yield better results within the body. Fortunately, nature offers a plethora of potential options, and several groups have abandoned insect-derived luciferases, which produce luminescence through a reaction



Light moves: Using AAV to deliver luciferase, Rajvinder Karda and colleagues can image freely moving mice. Credit: R. Karda

Box 1 | Keep it specific

The success or failure of a BLI experiment ultimately hinges on whether the luciferase enzyme produces light at the right time and place in the body. Many researchers achieve this specificity by generating genetically modified mice, although this is a costly, time-consuming, and labor-intensive process. It also creates the risk of unwanted background luminescence if, for example, the luciferase-coupled gene is expressed in multiple tissues.

Viruses offer an appealing and cost-effective alternative for achieving targeted luciferase gene delivery. Lentiviruses, for example, efficiently integrate their genetic material directly into the genome of their host after a single injection. "Lentivirus expression is very site-specific, so you only get expression within the organ that we inject it into," says Karda. More recently, she and Waddington demonstrated that another viral vector, adeno-associated virus (AAV), may offer even more finely-tuned targeting of luciferase expression[5]. "There's been an awful lot of work on modifying viral capsids, so that the vector itself targets specific cell populations," says Waddington. "For example, some AAV capsids are highly specific for just neurons or astrocytes."

In many cases, a single administration is sufficient for an imaging experiment lasting the lifespan of the animal. "We can get very widespread expression over the course of the development of the mouse from just a single neonatal injection of the AAV vector carrying a luciferase transgene," says Karda. She and Waddington are now using this approach to monitor the dynamic processes that contribute to type 2 Gaucher's disease, a hereditary neurodegenerative disorder that typically proves fatal within the first two years of a patient's life. "We want to monitor different signaling pathways involved with cellular death and inflammation, and see if we can track different signaling pathways in a transgenic disease model as opposed to inducing inflammation artificially," says Karda.

Rajvinder Karda uses bioluminescence imaging in freely moving mice to help study gene therapies for treating nervous system diseases. (R. Karda, University College London)

with D-luciferin, in favor of luciferases derived from various marine invertebrates, such as the sea pansy (*Renilla reniformis*), which act upon substrates known as coelenterazines.

The two subsets of enzymes bring different assets to the table. Firefly- and beetle-derived luciferases require ATP, the fundamental energetic currency of the cell, in order to process D-luciferin. This means that they are highly effective in the ATP-rich environment of the cellular interior, but not within bodily fluids or at the outer surfaces of cells. Some researchers also worry that their ATP-burning proclivities could harm the biological processes one might hope to image. "Producing 100 photons is going to consume a lot of ATP," says Huiwang Ai of the University of Virginia, "and that could disturb cellular metabolic pathways."

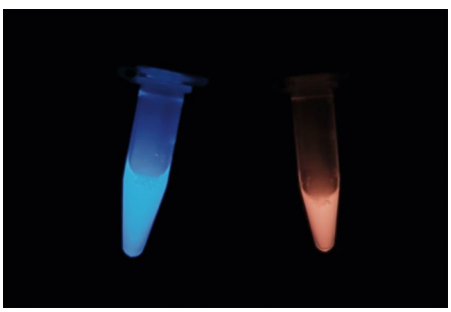
Coelenterazine-based systems require no ATP, and enzymes such as NanoLuc, a shrimp-derived protein marketed by Promega, offer an alternative to firefly luciferase. "It's much brighter," says Luker, "and we can see it readily with bioluminescence microscopy." However, coelenterazines have a tendency to spontaneously produce light at low levels even in the absence of luciferase, which can lead to higher background.

There are other important considerations as well. Every imaging session requires fresh administration of substrate, and individual substrates may behave differently in the body. For example, D-luciferin is highly water-soluble and can readily travel throughout most of the body, whereas coelenterazines tend to stay close to the injection site. Reaction rates can also differ considerably. Mezzanotte notes that reactions involving D-luciferin generally occur at a fairly stable rate, such that minor variations in the timing of animal imaging from day to day should have minimal effect on the final results. "But with coelenterazine, if you measure one day three minutes after administration and the next day after four minutes, it can change the signal by as much as 30%," she says.

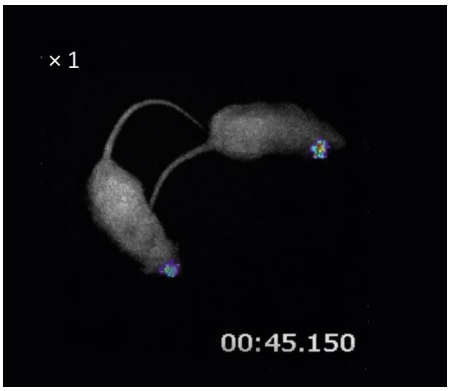
Chemists have also engineered novel luciferase substrates that outperform their natural counterparts. For example, both D-luciferin and coelenterazine have difficulty crossing the vascular tissue that insulates the brain against potentially damaging molecules or pathogens in the bloodstream, limiting the usefulness of BLI for neuroscientists. To address this, Stephen Miller's team at the University of Massachusetts Medical School team has developed substrates that can more efficiently penetrate this blood-brain barrier, including luciferin derivatives that remain inactive until they are selectively processed by an enzyme commonly found in the mammalian brain[4]. "These work extremely well in live animals," he says. "They can get into the brain and then get converted into luciferin, so we can use much lower dosesand get much brighter signals."

Modifying the luciferase enzyme in parallel with the substrate to create optimized enzyme–substrate pairs can yield further gains. One of the greatest enemies of deep-tissue imaging is the scattering of light; natural luciferase reactions typically emit short-wavelength, blue-green photons that are especially vulnerable to this problem. 'Red-shifted' reagents that produce light at the far-red or near-infrared end of the spectrum can perform much better at depth, with reduced scattering that allows emitted photons to travel farther.

It was frustration with the challenges of imaging deep within tumors using existing bioluminescence reagents that led Kondoh to team up with imaging expert Atsushi Miyawaki at Japan's RIKEN Brain Science Institute to develop AkaBLI. This heavily



Custom glow: Molecular biologists and chemists are developing enzyme-substrate combinations that glow at different wavelengths, like the blue-colored NanoLuc (left) and a red-colored derivative, Nano-lantern (right)." Credit: J. Prescher



Seeing thoughts: The engineered bioluminescent complex AkaBLI is visible through the skull of freely moving rats. Credit: RIKEN

engineered luciferase-substrate combination emits brightly in the near-infrared, and enabled Kondoh and Miyawaki to accurately image structures deep within the brains of live mice and marmosets[1]. "That's a stunning example of how sensitive these probes have become," says Prescher. "It was a real tour de force."

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A bright future

The pioneers of BLI are continuing to push the limits. For example, by combining different luciferase-substrate pairs—one that uses coelenterazine, and one based on D-luciferin—one can track multiplebiological processes in parallel. Luker's team is working with luciferase enzymes that have been broken into two fragments; these are respectively attached to a signaling molecule and its receptor, such that a luminescent readout only appears when these pieces come together. By combining these with a secondary reporter, his team can simultaneously study both the activation of a signaling pathway and its physiological consequences. "If we administer a drug that blocks that receptor, we can in real-time correlate the inhibition of the receptor with biological outcomes like metastasis," he says.

Interest in the technique is steadily growing. For example, Prescher is confident that progress in developing brain-penetrating luciferase substrates that generate far-reaching near-infrared photons will earn the technology a stronger following in the neuroscience community. "I expect them to become more and more intrigued as these probes get brighter and can be visualized through the intact skull," she says. Recent work with nonhuman primates from Kondoh and others has also demonstrated the potential of this technique in larger animal models, rather than the rodents that have largely been the mainstay of *in vivo* BLI work to date. However, these experiments will also require a costly investment in large doses of substrate, and could introduce some additional technical hurdles to clear. "The thicker the organism, the more difficult it is to get the light out," says Prescher.

Critically, the barriers to entry are low. High-resolution imaging of actively roaming animals may require state-of-the-art detectors, but more conventional bioluminescence experiments are well within reach for newcomers. "Most major universities have access to the equipment that you need for this now," says Miller. And as Luker and his collaborators learned in their early forays into viral imaging more than a decade ago, even a relatively simple imaging experiment offers remarkable opportunities for new biological insights. "That Yogi Berra cliché is true: you can observe a lot just by watching," he says. "And by just taking the time to look at what is going on with new technologies, you can discover things you wouldn't have otherwise." ❐

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