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Technoreview

Applications of bioluminescence imaging to the study of infectious diseases

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Summary

Bioluminescence imaging (BLI) has emerged as a powerful new method to analyse infectious diseases in animal models. BLI offers real-time monitoring of spatial and temporal progression of infection in the same animal, as opposed to euthanizing a cohort of animals and quantifying colony or plaque forming units at multiple time points. Pathogens or mice are engineered to express genetically encoded luciferase enzymes from bacteria, insects, or the sea pansy. The seminal study showing the feasibility of detecting microbially generated luminescence within a living mouse was published by Contag and colleagues in 1995, using *Salmonella typhimurium* transformed with the *lux* operon from *Photorhabdus luminescens*. Following this, they and others performed many studies of infection by bioluminescent Gram-negative and Gram-positive bacteria. Viruses can also be engineered to encode luciferase. Our laboratory has used bioluminescent reporter viruses to follow HSV and vaccinia pathogenesis; others have used an alphavirus or novirhabdovirus. Recently, even eukaryotic parasites *Plasmodium, Leishmania* and *Toxoplasma* have been transformed with luciferase and yielded unique insights into their *in vivo* behaviour. We expect that both the range of organisms and the molecular events able to be studied by BLI will continue to expand, yielding important insights into mechanisms of pathogenesis.

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Introduction

Conventional assays of host–pathogen interactions require that experimental animals be euthanized at multiple time points to identify sites of infection and quantify amounts of pathogen in various anatomic sites. While this approach has defined key aspects of microbial pathogenesis, there are important limitations with this conventional approach to animal models of infection. Analysing individual animals at single time points precludes real-time monitoring of spatial and temporal progression on infection in the same animal, which may reveal biologically relevant variations in host–pathogen interactions. Spread of a pathogen to an unexpected site of infection may be missed because the infected tissue is not harvested and analysed. Finally, conventional assays of pathogenesis typically require large numbers of animals to obtain statistically meaningful data at multiple time points.

Recently, *in vivo* imaging techniques have emerged as a powerful complement to conventional assays of microbial pathogenesis. In particular, there is increasing use of bioluminescence imaging (BLI) to monitor disease processes in living animals. This technology allows specific molecular and cellular events, such as cell migration, signal transduction, proliferation and apoptosis, to be investigated in the context of an intact animal. In this review, we describe the technology used for BLI, strengths and limitations of this technique, and potential future applications in small animal models of microbial pathogenesis.

Bioluminescence imaging reporter enzymes

Bioluminescence imaging detects light produced by the reaction of luciferase enzymes with a defined substrate. There are two different strategies for engineering bioluminescent reporters in pathogens or host cells. First, the *lux* operon from bacteria such as *Photorhabdus luminescens* or *Xenorhabdus luminescens* encodes genes to synthesize luciferase and the substrate luciferin. Bacteria expressing the *lux* operon constitutively produce light without the need for an exogenous substrate. The *lux* operon has been transferred stably to other species of bacteria for imaging studies. However, this operon has not

been transferred to higher species, although there are ongoing efforts to engineer the *lux* operon as a reporter system for mammalian cells.

The second strategy for bioluminescent reporters uses luciferase enzymes from organisms such as the firefly (*Photinus pyralis*) or sea pansy (*Renilla reniliformis*). Pathogens and mammalian cells can be engineered to express one or more luciferase enzymes as reporters for *in vivo* imaging. Mammalian tissues do not produce the luciferin or coelenterazine substrates for firefly or Renilla luciferases, respectively, so these molecules must be delivered systemically for small animal imaging. Luciferin rapidly distributes throughout a mouse and penetrates intact blood–tissue barriers, such as the placenta and brain, after intraperitoneal injection. Therefore, substrate availability is not a limitation for *in vivo* imaging with firefly luciferase. By comparison, bioavailability of coelenterazine, the substrate for Renilla luciferase, is limited *in vivo*, at least in part by the multidrug resistance P-glycoprotein (MDR1), which transports coelenterazine and similarly structured compounds out of or off the membranes of mammalian cells (Pichler *et al*., 2004). Thus, coelenterazine may be unavailable to the cytoplasm of some cells infected with a Renilla luciferase-expressing virus, and may also have difficulty crossing membranes.

Because firefly and Renilla luciferase use two different substrates, it is possible to use these reporters to image two different biological processes in the same mouse (Bhaumik and Gambhir, 2002). However, the kinetics of each reporter differ markedly, in part because of differences in pharmacokinetics of each substrate. After intraperitoneal injection of luciferin, bioluminescence from firefly luciferase reaches a plateau in approximately 10 min and remains relatively constant up to 30 min (Paroo *et al*., 2004). Bioluminescence from Renilla luciferase peaks rapidly at 1 min after intravenous injection of coelenterazine and then declines to near background levels by 10 min (Bhaumik and Gambhir, 2002).

It also is possible to distinguish two unique biological processes based on differences in emission spectra of various luciferases. Renilla luciferase and the bacterial *lux* operon luciferase have peak emissions at 480 and 490 nm respectively. Using appropriate wavelength filters, light from these enzymes can be distinguished from firefly luciferase, which emits at approximately 560 nm, or other red-shifted click beetle luciferases. This type of strategy is described below for imaging bacterial localization and a host response. An important caveat for multireporter studies is the greater attenuation of light from blue-green luciferases (Renilla and bacterial) relative to firefly luciferase or other enzymes that emit light greater than 600 nm. As a result, *in vivo* detection of Renilla or bacterial luciferase is much less sensitive than enzymes with red-shifted emission spectra.

Bioluminescence imaging instrumentation and imaging technique

Instruments for BLI use a very sensitive charge-coupled device (CCD) camera to detect the low levels of light emitted from luciferase reporters *in vivo*. There is minimal background from with this technique, although mouse chow containing chlorophyll or similar pigments can produce low amounts of light in the intestine. Because of the very low background signal, it is possible to detect relatively small numbers of a pathogen in mouse models. For example, we have shown that ocular infection with a bioluminescent Herpes simplex virus type 1 reporter virus (HSV-1) can be detected at an input titre as low as 1×10^2 plaque-forming units (pfu) (Luker *et al*., 2002). BLI is a rapid and relatively inexpensive imaging modality that allows large numbers of mice to be examined repetitively. In our experience, approximately 20–30 mice can be imaged in approximately 1 h. Bioluminescence can be measured by computer analysis of emitted photons, allowing relative quantification of data. Assuming that expression of the luciferase enzyme is maintained by the engineered pathogen or cell, relative numbers of viruses, bacteria, or cells correlate directly with greater bioluminescence. However, absolute values for bioluminescence produced by a defined number of pathogens will differ in various anatomic sites because of absorption and scattering of light by overlying tissues.

Although BLI is the most commonly used imaging technique for studying pathogenesis, there are important limitations to this modality. In addition to 10-fold attenuation of light by overlying tissues, bioluminescence is decreased by pigmentation of organs, such as liver and spleen, and fur. This affects studies of viral-host pathogenesis because many genetically engineered mice are developed in SV129 or C57BL/6 backgrounds. Shaving mice with electric clippers and removing fur with a depilatory agent (such as Nair) can minimize this problem. Alternatively, mice can be bred into an albino C57BL/6 background. BLI typically is a two-dimensional imaging technique with a spatial resolution of 2–3 mm, although there are three-dimensional imaging system and/or reconstruction techniques available commercially. Even with advanced instrumentation, it still may be difficult to separate photons produced by infected cells in two immediately adjacent sites, such as the eye and periocular tissues. Finally, although BLI is a powerful imaging modality in small animal models, it is unlikely to be used in humans because of the limitations in detecting light produced in deep tissues.

Validation of BLI for bacterial pathogenesis

Initial studies showing the feasibility of BLI for studying microbial pathogenesis were performed by Contag and

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colleagues (Contag *et al*., 1995). These researchers stably transformed three strains of *Salmonella typhimurium* with a plasmid bearing the *lux* operon from *P. luminescens* (*luxCDABE*). Luminescent bacteria were detected throughout C57BL/6 or BALB/c mice following infection, showing that the bioluminescent reporter signal could be detected *in vivo*. As evidence that BLI could be used for serial studies of bacterial pathogenesis, imaging was used to monitor bacterial distribution, distinguish between more and less virulent strains of bacteria, differentiate susceptibility of mouse strains to infection, and monitor antibiotic therapy. This initial study also highlighted the importance of oxygen for light produced by the *lux* operon. While there is sufficient oxygen in viable mammalian tissues to support the luciferase reaction, the ability of BLI to detect extracellular bacteria in anaerobic microenvironments, such as the lumen of the intestine, may be limited. Indeed, injection of air into the ileum and colon of *Salmonella*-infected mice caused the appearance of a luminescent signal where none previously had been detectable.

Further work validating BLI for monitoring bacterial infection was carried out by a different group a few years later (Rochetta *et al*., 2001). They set out to test, rigorously and with statistical analysis, whether BLI could be used to measure antibiotic efficacy *in vitro* and *in vivo*, using *luxCDABE*-transformed *E. coli*. They found that BLI could be used to reliably determine minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of antibiotics in broth culture. Growth curves generated by BLI correlated with curves generated by plating and counting colony-forming units (cfu). BLI measurements correlated very highly ($r = 0.98$) with colony counts for dose-dependent responses to three different antibiotics *in vitro*. This correlation extended to *in vivo* imaging in a soft tissue model of infection, where dose-dependent inhibition of bacterial growth with ceftazidime was quantified with BLI and colony counts. However, there were also differences in the magnitude of inhibition as measured by BLI or colony counts, which varied according to the time point and the drug being used. The study noted that BLI-generated curves increased or decreased more consistently than curves generated using traditional colony counts. The authors attributed this to the fact that sequential luminescence points are derived from the exact same set of animals, while cfu are derived from different groups of animals. These data demonstrate an advantage of *in vivo* imaging for quantifying responses to treatment in the same cohort of mice over time.

More recently, another study compared light from *lux CDABE*-transformed *Pseudomonas aeruginosa* with the conventional colony counts in a model biofilm (Marques *et al*., 2005). After treatment with ciprofloxacin, these authors found a discrepancy between cfu and bioluminescence. Cfu from the disrupted film decreased to a much greater degree than light intensity from the same biofilm, and the kinetics of recovery after cessation of antibiotic were different between the two methods. They offer several possible explanations for these findings, including elongation of drug-injured cells resulting in greater light output per cell and differential effects of ciprofloxacin on light-producing ability versus viability. They conclude that 'bioluminescence and viable counts measure different aspects of cell physiology and neither should be regarded as the definitive indicator of cell viability.

Bioluminescence imaging in bacterial pathogenesis

Following the initial proof-of principle study for BLI in bacterial pathogenesis, this technology has been used to study a variety of other bacterial pathogens. As evidenced by many different publications, BLI has allowed researchers to identify new features of host–pathogen interactions. Representative examples of BLI applications in bacterial infection are described below.

Wiles and colleagues transformed *Citrobacter* with a transposon plasmid to stably integrate the *luxCDABE* operon into the bacterial genome (Wiles *et al*., 2004). After confirming that the transformed bacteria were not attenuated *in vivo* or *in vitro* compared with the parent strain, they orally inoculated mice with the bioluminescent *Citrobacter* and monitored colonization and clearance of the mouse colon by BLI and colony counts. These authors made the new discoveries that *Citrobacter* colonized the caecal patch before colonizing the rest of the colon, and bacteria were cleared from the caecum before being cleared from the rest of the colon. Oxygen availability was not a confounding factor in this study because mice were dissected and their intestines moved to a Petri dish prior to imaging. In a subsequent article (Wiles *et al*., 2005), the same authors use the luminescent *Citrobacter* to show that this bacterium becomes 'hyperinfectious' after passage through a mouse, requiring a lower infectious dose to colonize naïve mice. In addition, these 'hyperinfectious' bacteria did not need to colonize the caecal patch before infecting the rest of the colon.

The *P. luminescens luxCDABE* operon is not translated in Gram-positive bacteria such as *Staphylococcus aureus* because ribosomes in Gram-positive organisms cannot bind and translate the mRNA. Early attempts at making bioluminescent *S. aureus* relied on firefly luciferase or variations of a partial *lux* operon (*luxAB*), encoding luciferase only (Corbisier *et al*., 2004) (Steidler *et al.* 1996). These approaches have the disadvantages of requiring the addition of exogenous substrate and detergent or being unstable at temperatures above 30°C (Hill *et al*., 1993). This technical challenge was overcome by

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Francis and colleagues, who modified *luxCDABE* so that it could be expressed by Gram-positive bacteria (Francis *et al*., 2000). This technical advance was accomplished by constructing a Gram-positive ribosome binding site (RBS) and inserting it upstream of each gene in the operon. The new cassette was called *luxABCDE*. *LuxABCDE* was used to transform *S. aureus*. Light emitted by the transformed *S. aureus* was bright, was optimal at 37°C, correlated with colony counts *in vitro*, and decreased upon antibiotic treatment in a mouse model of *S. aureus* thigh infection. These researchers then developed a transposon-based method for stably integrating *luxABCDE* into the genome of *Streptococcus pneumoniae* (Francis *et al*. 2001). Genomic DNA from a successfully transformed, brightly luminescent transposant was used to transform clinical isolates of *S. pneumoniae*. The transformed isolates were equal in virulence to their parent strains and allowed BLI imaging of a 7 day course of infection and response to antibiotic therapy. It was noted that luminescence dropped sharply when the bacteria reached stationary phase in culture, but this phenomenon did not affect *in vivo* results.

A recent study by Hardy *et al*. emphasizes the power of BLI to reveal unexpected new locations of microbial replication (Hardy *et al*., 2004). The authors generated bioluminescent *Listeria monocytogenes* carrying *luxABCDE* stably integrated into the chromosome. Although the transformed strain was attenuated compared with the parent strain, they used a combination of *in vivo* imaging and image-guided dissection to show that *L. monocytogenes* can live and probably replicate in the lumen of the mouse gall bladder, suggesting that it may do the same in the human gall bladder. Therefore, the gall bladder may represent an internal reservoir for *Listeria*.

Finally, a very interesting study of *S. pneumoniae* meningitis described the combined use of two different bioluminescent systems to simultaneously yield data about bacterial replication and the host response (Kadurugamuwa *et al*., 2005). The authors used a transgenic mouse that expressed firefly luciferase under control of the promoter for glial fibrillary acidic protein (GFAP). GFAP is an intermediate filament protein expressed by mature central nervous system (CNS) astrocytes, and it is an established marker for astrocyte activation. Bioluminescent *S. pneumoniae* were injected into the subarachnoid space of transgenic GFAP-firefly luciferase mice, and the resulting infection was monitored over 72 h. Bacterial luminescence could be distinguished from luminescence due to activation of the GFAP promoter because of their differing peak emission wavelengths *in vivo* (490 nm for bacterial luciferase, 610 for firefly luciferase). The reporter signals also could be distinguished because the firefly luciferase signal required the injection of its substrate, luciferin.

The authors found parallel increases in both bacterial and GFAP signals in infected areas of the brain and spinal cord. However, the onset of bioluminescence from GFAP was 3–4 h later than the onset of the bacterial signal. If infected mice were treated with the antibiotic ceftriaxone, the bacterial signal decreased to baseline. However, the effect of antibiotic treatment on the GFAP signal depended on when the antibiotic was given. If ceftriaxone was administered 11 h or less post infection, the GFAP signal never rose above baseline. At 17 h post infection, the brain GFAP signal was moderately high, and administration of ceftriaxone eliminated bacteria without affecting the GFAP reporter signal. Furthermore, if ceftriaxone was delayed until 19 h post infection, the brain GFAP signal *increased* 30- to 100-fold. Most of these mice did not survive beyond 22 h, but those that did sometimes experienced epileptic seizures. These data suggest that the host proinflammatory response is a major factor causing morbidity and mortality in this disease.

While we expect that future studies will exploit this multicolour strategy to image both a pathogen and host response, it is important to note the added complexity of this method. Shorter wavelengths of visible light are attenuated by tissues to a greater extent than longer wavelengths. Therefore, less light will be transmitted from bacterial luciferase (490 nm emission) than firefly luciferase (610 nm emission), which will affect quantification of imaging data. Because bioluminescence images are weighted towards light emitted at or near the surface of an animal, there also will be depth-dependent spectral shifts in the emitted light that can complicate analysis of data. Despite these caveats, we expect future applications of multicolour imaging to yield new insights into host immune responses to other pathogens.

Viral infections

Our laboratory has used BLI to investigate pathogenesis of HSV-1 infection in mice, using a recombinant strain KOS virus that encodes both firefly luciferase and Renilla luciferase under control of an early gene promoter (Luker *et al*., 2002). Initial research established that firefly luciferase activity from the reporter virus could be detected readily in mice after four different routes of infection. Viral titres quantified by plaque assay correlated directly with light emitted from firefly luciferase. This study also demonstrated limitations of BLI for pathogenesis studies. Because of the relatively low spatial resolution of this imaging modality, it is difficult to distinguish between viral infections in adjacent tissues. For example, bioluminescence from the site of infection in the cornea could not be separated from subsequent spread of infection to periocular tissues. In addition, light from firefly luciferase was detected readily in the infected cornea after intra-

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peritoneal administration of luciferin, but Renilla luciferase was identified only when coelenterazine was applied directly to the cornea. The latter finding emphasizes problems with bioavailability of coelenterazine due to transporters such as MDR1 P-glycoprotein.

We subsequently used this same HSV-1 reporter virus to further analyse viral-host interactions in mice with BLI. Our laboratory investigated effects of type I and type II interferon receptors on replication and spread of HSV-1 (Luker *et al.*, 2003). In wild-type mice and mice lacking receptors for type II interferons, HSV-1 infection remained localized to the site of epithelial infection and innervating sensory neurons. By comparison, we found that type I interferon signalling is essential to limit systemic dissemination of HSV-1. In mice lacking receptors for type I interferons, HSV-1 disseminated from the site of inoculation in cornea or footpads to infect multiple abdominal organs, including liver and spleen. This phenotype was exacerbated in mice lacking both type I and type I and II interferon receptors, demonstrating the critical function of innate immunity in restricting HSV-1 dissemination. The presence of abdominal activity in multiple organs also illustrated a potential limitation of BLI for anatomic localization. Because the luminescent image is two-dimensional, the precise origin of bioluminescence in the abdomen was difficult to discern from a single image. This problem was largely overcome by imaging mice at several different projections: dorsal, ventral and left lateral. Using information from these three images and knowledge of mouse anatomy, infection was localized to liver, spleen, kidneys, spine and popliteal lymph nodes. These sites were confirmed by dissection of the mice and imaging of isolated organs.

The KOS reporter virus also has been used by Burgos *et al.* to monitor intraperitoneal infection with HSV-1 (Burgos *et al.*, 2006). Based on *in vivo* and *ex vivo* imaging of abdominal organs, these authors determined that ovaries and adrenal glands were the primary sites of infection in this model. Bioluminescence correlated directly with amounts of HSV-1 DNA quantified by real-time polymerase chain reaction (PCR), further validating the reporter virus for studies of viral replication and spread *in vivo*.

As an alternative approach to image HSV-1 infection with BLI, we engineered a transgenic mouse to respond to viral infection, rather than expressing firefly luciferase from the viral genome (Luker *et al.*, 2006). The transgenic mouse expressed firefly luciferase (FL) under the control of the promoter HSV-1 thymidine kinase (tk) promoter to detect viral replication. These mice produced light at the site of HSV-1 infection, using either corneal or flank models, but corresponding sites of mock infection did not (Fig. 1). Light was produced in response to infection with three different strains of HSV-1, and increasing the viral inoculum caused a corresponding increase in luminescence intensity. Moreover, no luminescence above background was seen when the mice were infected with an unrelated tk-expressing DNA virus. This shows that the TK-FL mouse could be used to study replication and spread of HSV-1 specifically.

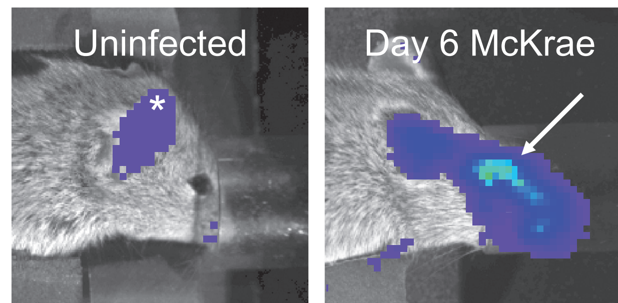

Fig. 1. Reporter mouse for HSV-1 infection. The transgenic mouse expresses firefly luciferase under the control of the promoter for HSV-1 thymidine kinase. The uninfected mouse shows background bioluminescence in the ear (asterisk), which is seen commonly in firefly luciferase transgenic mice. There is robust activation of the HSV-1 reporter transgene in the infected eye and periocular tissues (arrow) 6 days after infection with 1×10^6 pfu strain McKrae HSV-1.

The advantage of using a reporter mouse instead of a reporter virus is that multiple strains or mutants of HSV-1 can be studied without making a new recombinant virus each time. Moreover, this method avoids the possibility that the virus may be attenuated by inserting a reporter. However, there also are limitations to the reporter mouse strategy. First, the reporter mouse is approximately 10-fold less sensitive for detecting HSV-1 infection as compared with a reporter virus. This may be due to differences in number of copies, level of transcription, or level of translation of the reporter gene when it is integrated into the host genome instead of the virus genome. Second, the HSV-1 reporter mouse, similar to many other luciferase reporter animals, has background luminescence in ears, paws and tail, possibly due to the increased stability of the luciferase protein at cooler temperatures in the extremities. While this background does not preclude its use for studying HSV infections in the eye or flank, it does make it unsuitable for studying infections given in the footpad. Nevertheless, the TK-FL mouse represents a novel way to study HSV pathogenesis using BLI.

Our laboratory also has developed bioluminescent and fluorescent reporter vaccinia viruses for studying poxvirus infection *in vivo* with BLI. These reporter viruses are not attenuated relative to wild-type Western Reserve strain vaccinia virus *in vitro* or *in vivo*, allowing studies of viral pathogenesis similar to those described for HSV-1. Using BLI to monitor spatial and temporal progression of viral infection, we have shown that type I interferons signalling predominantly in parenchymal, rather than haematopoietic cells, limit systemic dissemination of vaccinia virus (Luker *et al.*, 2005) (Fig. 2). We also have used this model

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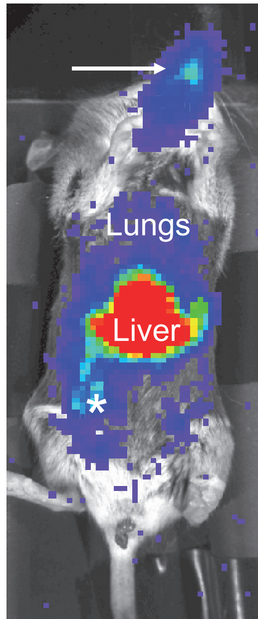

Fig. 2. Firefly luciferase vaccinia virus. A mouse lacking receptors for type I and II interferons was infected intranasally with 1×10^6 pfu of a recombinant Western Reserve vaccinia virus engineered to express firefly luciferase from a hybrid early/late promoter. Widespread systemic dissemination of virus is seen 5 days after infection. Viral bioluminescence is evident in the nasopharynx (arrow), lungs, liver, and inguinal lymph node (asterisk).

to identify a protective function for alveolar macrophages in host defence against respiratory infection with vaccinia virus (Rivera *et al*., 2007).

Both HSV-1 and vaccinia virus are DNA viruses with large genomes that can accommodate insertion of reporter genes. Insertion of luciferase reporters has been an obstacle for studies of viruses with RNA genomes, although BLI has been used to monitor a limited number of RNA viruses. For example, Cook and Griffin generated recombinant Sindbis viruses that express firefly luciferase for studies of viral entry into the CNS. BLI offers two advantages for addressing this question in the biology of alphaviruses such as Sindbis. First, Sindbis virus occurs in significant quantities free in the blood (Cook and Griffin, 2003). When an organ is excised for standard plaque assays of viral titres, virus in infected tissues cannot be distinguished from free virus in the vasculature. Because light is produced only in infected cells, confounding effects of free virus on measurements of organ infection are eliminated. However, it is important to realize that light may be produced by infected leukocytes that remain within blood vessels. Second, BLI offers real-time monitoring of viral dissemination in the same mouse, thereby allowing serial studies of viral localization. Using a foot pad model of infection, some mice developed nasal infection prior to brain infection, while other mice developed spinal cord infection. The variation between different mice may be one reason it has been difficult to determine the route of brain entry for Sindbis virus. One caveat of these studies is that the recombinant viruses were attenuated by approximately 50% in mice compared with parental virus, showing the difficulty in making reporter pathogens for some RNA viruses.

Bioluminescence imaging typically has been used to study infection in mouse models, but this imaging modality can be used effectively in other organisms. For example, Harmache *et al*. used BLI to investigate infection with infectious haematopoietic necrosis virus (IHNV), an RNA virus that is significant pathogen in young trout fish (Harmache *et al*., 2006). In particular, the study focused on the route of viral entry into fish. To enable this study, the authors constructed a recombinant IHNV that expressed a Renilla luciferase reporter. Unlike Sindbis virus, the recombinant IHNV virus was not attenuated compared with the parental strain. Juvenile trout were infected with the reporter virus and then imaged with BLI at various times post infection. Whole-body luciferase imaging of infected fish revealed the unexpected localization of virus to the fin bases. Infection of fin bases occurred prior to infection of other organs and tissues and persisted after virus had spread systemically. Imaging of a euthanized fish with the skin removed established that light from inside the body could be detected readily through the skin, suggesting the early detection of luminescence in fin bases is not simply due to the thinness of the tissue at that site. An attenuated virus lacking a non-structural protein infected the fin bases without disseminating, which further supported the conclusion that viral entry occurred through fin bases. This study established the power of BLI to study infection in non-mouse models of infection.

Parasites and fungi

Recent studies also demonstrate that BLI can be used to investigate parasitic infections. Hitziger *et al*. investigated analysed virulence of two different strains of bioluminescent *Toxoplasma gondii* (Hitziger *et al*. 2005). These authors established a high correlation between light intensity and amounts of parasite in spleen and testis, which is consistent with findings for other pathogens described above. They also concluded that deletion of various Toll-like receptors (TLR1, 2, 4, 6, or 9) did not affect susceptibility of mice to infection with this parasite. Saeij *et al*. also engineered luciferase reporter strains of *Toxoplasma* and used BLI to quantify differences in replication over time, as well as imaging reactivation of parasites in the setting of chronic infection (Saeij *et al*., 2005). It is important to note that Saeij *et al*. determined that the reporter strains were slightly attenuated compared with corresponding wild-type strains. Nevertheless, these studies

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demonstrate that luminescent *T. gondii* can be engineered and used to reveal new information about this parasite's pathology.

Bioluminescent *Leishmania amazonensis* and *Plasmodium berghei* have also been developed and used to monitor parasitic infection *in vivo*. Lang *et al*. generated firefly luciferase expressing *Leishmania* and used the reporter parasite to monitor infection and response to therapy in cell culture, excised organs from infected mice, and living mice (Lang *et al*., 2005). Franke-Fayard and colleagues (Franke-Fayard *et al*., 2005) engineered three firefly luciferase expressing *P. berghei*: one that expressed luciferase in all life stages, one that expressed luciferase under a gametocyte-specific promoter, and one that expressed luciferase under a schizont-specific promoter. Using these luminescent parasites, the authors determined that red blood cells infected with the schizont stage of the parasite are sequestered in the microvasculature of the lung and adipose tissue. Sequestration of the parasite is dependent on expression of CD36 on the capillary endothelium. These authors also demonstrated that cerebral malaria is not dependent on CD36-mediated red blood cell sequestration, which countered previous assumptions of disease pathogenesis. Importantly, the ability to monitor specific stages in the parasite life cycle is a key advance for *in vivo* BLI studies.

The development and use of bioluminescent fungi for monitoring fungal infections *in vivo* is less advanced than for other kinds of pathogens, in part due to technical challenges with engineering bioluminescent reporter organisms. One group recently transformed *Candida albicans* with firefly luciferase stably integrated into the chromosome and use the recombinant fungus as a marker of mucosal or systemic infection (Doyle *et al*., 2006a,b). Fungal bioluminescence was detected easily, and data for photon flux corresponded with cfu counts in a vaginal mucosal infection. However, fungal bioluminescence from systemic infections in intact animals was too dim to correlate well with colony counts or to give useful information about the extent of infection. It was hypothesized that this is due to a combination of attenuation of light by overlying tissues and reduced permeability of the hyphal cell wall to the substrate luciferin compared with the cell wall of the yeast form. Because yeast are slow-growing compared with bacteria, the ability to monitor mucosal infections in real time with BLI potentially can give a significant advantage in drug screens. However, the great attenuation of the light in systemic infections greatly limits the usefulness of this bioluminescent *Candida* to study *in vivo* pathogenesis.

Future directions. BLI has proven to be a powerful tool for localizing and quantifying microbial infections *in vivo*, establishing that imaging techniques can substantially advance studies of pathogenesis. In the future, we anticipate the development of imaging strategies to detect not only the pathogen, but also cellular and tissue-wide host reactions. The study by Kadurugamuwa *et al*. using multicolour bioluminescence to interrogate bacterial spread and host neuronal injury is key step towards the goal of monitoring both the host and pathogen. Because of the inherent challenges with multicolour BLI, it is likely that BLI will be combined with other structural and/or functional imaging methods, such as magnetic resonance imaging (MRI), radiotracer modalites (positron emission tomography, PET; single-photon emission computed tomography, SPECT) or computed tomography (CT). MRI, PET and SPECT each have been used to analyse trafficking of immune cells, including T lymphocytes and dendritic cells, generally in the context of cancer or autoimmunity. However, these same approaches could be applied to interrogate kinetics and localization of immune cells in response to infection.

In addition to imaging defined populations of immune cells, we envision that small animal imaging methods will be used to quantify changes in tissue architecture, permeability, or metabolism associated with infection. For example, both MRI and CT can detect sites of inflammation based on alterations in vascular permeability and/or tissue water. PET imaging with the glucose analogue ^{18}F-fluorodeoxyglucose also can be used to detect focal sites of inflammation during infection, based on increased metabolism of glucose by infiltrating immune cells. Image co-registration techniques then can be used to integrate data from multiple imaging examinations, providing an integrated data set for the pathogen and specific components of host immunity. Through these approaches, we expect imaging to become a key component of basic and translational research in infectious disease.

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