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Imaging brain structure and function, infection and gene expression in the body using light

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SUMMARY

Light can be used to probe the function and structure of human tissues. We have been exploring two distinct methods: (i) externally emitting light into tissue and measuring the transmitted light to characterize a region through which the light has passed, and (ii) internally generating light within tissue and using the radiated light as a quantitative homing beacon. The emitted-light approach falls within the domain of spectroscopy, and has allowed for imaging of intracranial haemorrhage in newborns and of brain function in adults. The generated-light approach is conceptually parallel to positron emission tomography (PET) or nuclear medicine scanning, and has allowed for real-time, non-invasive monitoring and imaging of infection and gene expression *in vivo* using low-light cameras and ordinary lenses. In this paper, we discuss recent results and speculate on the applications of such techniques.

1. DIFFUSION-BASED OPTICAL SPECTROSCOPY AND IMAGING

Medical optical imaging (MOI) and medical optical spectroscopy (MOS) use light emitted into scattering media, such as human tissue, to determine interior structure and chemical content, and have broad medical applications to the continuous, non-invasive bedside or operating room monitoring of tissue structure, oxygenation, blood flow and metabolite concentration. Also called, though not quite correctly, near-infrared imaging (NIRI) and near-infrared spectroscopy (NIRS), these techniques are based upon the fact that both visible and near-infrared light pass through the body in small amounts, and emerge bearing clues about the tissues through which they passed. Images can be reconstructed from such data. The advantages of optical measurements are that they are non-invasive, portable, non-ionizing and exhibit a contrast due to tissue composition and structure. Historically, optical concentration estimates have been rendered non-quantitative by the long, irregular photon paths caused by scattering. Similarly, *in vivo* optical images using simple transmission of light have been poor. However, if additional information about the path taken by light through the tissue is known, the concentration of physiological intermediates of deep tissues, such as the brain, can be measured, and the optical information used to map tissue structure, tissue function or both (table 1).

(*a*) *Structural imaging*

Most current medical imaging techniques focus on the mapping of tissue structure. Structural imaging aids medical diagnosis by allowing for discrimination between tissues. Even magnetic resonance imaging (MRI), which contains quantitative spectroscopic information, is normally reduced to unit-less numbers, such as T1- or T2-weighted values, developed as means to provide tissue contrast rather than tissue quantitation. It is unlikely, for example, that a clinical physician has ever requested an MRI scan in order to determine the T1-weighted value of a particular patient's liver.

Our interest in structural optical imaging is in the use of optical tomography to identify cerebral haemorrhages in neonates at increased risk for such bleeding. We previously described construction of a time-of-flight (TOF) optical imaging system (Benaron & Stevenson 1993), and tested this device in both model systems (Benaron & Stevenson 1994) and pathological brain samples (Van Houten *et al.* 1996*b*). In 1994, we converted our optical tomography device into a real-time clinical scanner, and began to compare the effectiveness of an optically based tomographic imaging system to conventional ultrasound for the detection of intraventricular haemorrhage in the premature neonate. Early optical detection of haematoma had already been shown to benefit adults after surgery for head trauma (Gopinath *et al.* 1993). For some infants, we believed that detection of cerebral haemorrhages might lead to a similar benefit, such as for infants receiving extracorporeal membrane oxygenation (ECMO) who are at high risk for bleeding due to anticoagulation protocols. Early identification of cranial haemorrhages in these infants could result in a change in the haemostasis management, whereas a missed haematoma could extend and lead to severe brain injury. We also suspected that the level of optical imaging resolution for our device was near or at a clinically useful level at the time the study was started.

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Table 1. *Differences in focus between structural and functional imaging*

	structural imaging	functional imaging
goal	tissue contrast	predictive values
resolution	maximized	coarse
imaged value	arbitrary	quantitative
use	haemorrhage, tumour, detection of physical lesions	stroke, ischaemic disease, asphyxia, quantitation of water in oedematous tissue

We studied premature infants undergoing ultrasound examination and, after informed consent, scanned these infants optically as well. For these studies, a motionless tomographic optical system was created and consisted of an imaging headband constructed from a web of fibres sewn into a soft band (Benaron *et al.* 1996*a*). This band may be wrapped around the head for extended periods without discomfort or difficulty.

Small errors in the estimated location of the fibres can lead to a substantial blurring of the image and loss of resolution. To correct for such positional blurring, the spatial locations of the fibres were recorded. In this manner, the fibre headband can be placed in the most convenient orientation, while the software automatically rotates the image into standard radiological orientation. The structural images were then processed for a variety of factors, including the differential path length factor (DPF), reduced scattering coefficient (μ_s'), absorption coefficient (μ_a), as well as for the rate of change of these variables over space ($\delta\mu_s'/\delta x$, $\delta\mu_a/\delta x$ and $\delta DPF/\delta x$). These values were estimated using a fit to a full TOF diffusion approximation. Images were also analysed for localized concentration of haemoglobin, which would be expected to be substantially lower in normal tissues than at sites of haemorrhage.

Data collection for imaging required from 1–4 h; image processing required 2 min. Two optical tomographic image haemorrhages are shown (figure 1). In the pilot study, we found that optical scanning correctly identified the site and grade of bleeding in 75% of cases (Benaron *et al.* 1996*a*, *b*; Hintz *et al.*, 1997). In infants without haemorrhage, as determined by ultrasonography, no bleeds were identified in the corresponding optical scans. Haemorrhages were seen best on images of scattering coefficient and average distance travelled by the light.

The study described above demonstrates that optical imaging can be performed in a true tomographic configuration using a portable system with clinically safe power levels. Thus, monitoring and imaging of gross brain pathology appears to be possible using time-resolved optical tomography. The optical diagnostic errors are explainable based on the different optical properties of haemorrhages of differing ages, or on a small bleed size. In the case of an ageing clot, a clot visualized in earlier optical scans of the same infant was not seen in the optical scans collected 2 weeks later, while still remaining visible in the ultrasound. Thus, similar to MRI, optical scanning may provide a method for dating bleed age via an age-dependent signal. Further, alterations in local haemoglobin concentration and oxygenation near the site of the haemorrhagic events were found, raising the possibility that such signs accompany, and perhaps precede the haemorrhagic event. Such findings may allow for a degree of haemorrhage prediction.

Time of collection remains long, though improvement is expected. Ultimately, data collection times are expected to be in the order of several minutes. Multiple groups are now working on this problem, including ours and the laboratories of Chance (University of Pennsylvania), Delpy (University College London), Gratton (University of Illinois), Sevick (Perdue) and others. We are now constructing a fast clinical system using parallel detectors, while a system constructed for maximum sensitivity is currently under construction by the London group.

(*b*) *Functional imaging*

In addition to structural imaging, there is a need for methods that allow for quantitative measurements with predictive value, such as brain oxygenation. For example, patients who are ill or undergoing surgery, particularly those who remain ill for extended periods or who must undergo procedures that affect blood supply to the brain, face a significant risk of brain injury. Such injury is currently not predictable. Two nearly identical patients may undergo a similar operative procedure, yet one patient may emerge normally, while the other emerges with major brain injuries leading to stroke, the development of retardation or cerebral palsy, or even death. Early in the course of brain injury, such as hypoxia–ischaemia or haemorrhage, clinical signs that could lead to treatment, or a change in treatment, are often absent. Thus, imaging of cerebral disease tends to occur in retrospect, long after irreversible damage has already occurred. To prevent death or minimize long-term injury, proactive early detection through use of continuous monitoring or imaging may be beneficial. Finally, imaging, as opposed to monitoring, is important as brain hypoxic pathology is often focal, rather than diffuse. Even a global asphyxia often gives way to a patchy disease. Thus, while continuous tests such as non-imaging NIRS exist, such tests yield data that are an ensemble average of brain oxygenation, devoid of localizing data, making it difficult to identify isolated regions of the brain that are critically under-oxygenated.

We measured oxygenation in models and in humans using a clinical system similar to that described in the structure studies, above. In homogenous model systems, we measured mean oxygen saturation errors of $\pm 0.4\%$ at 1 cm depth to $\pm 1.7\%$ at 6 cm depth. In humans,

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we measured oxygenation in an ischaemia model, oxygenation, in the neonatal brain, and during changes in localized brain oxygenation during motor stimulation. We found that changes in oxygenation measured optically in the ischaemia model corresponded in magnitude and direction to the expected changes, that absolute optical measures of oxygenation in neonatal brain corresponded to measured jugular or superior vena cava oxygenation as determined by standard invasive methods, and that an optical image of oxygenation in an infant with a stroke showed areas of low oxygenation that corresponded to the location of the stroke in a computerized tomography (CT) scan. In measurements during motor activity, optical monitoring of adult motor cortex during hand movement revealed focal, contralateral increases in oxygenation with motor activity, with good spatial agreement of optical imaging activation maps to images of activation as determined by fMRI (Van Houten *et al.* 1996*a*; Benaron *et al.* 1997*b*).

The functional changes involved in motor activation bear special note. The regional cerebral blood volume (rCBV), blood flow (rCBF) and haemoglobin saturation (rSO_2) of human cortex vary over time. Localized physiological changes occur over seconds to minutes during regional brain activity, with elevations in blood volume and flow occurring in excess of need over an area larger than the electrical activity, affording a regional mapping signal that colocalizes with brain activity. As such slow optical changes occur over seconds, and colocalize with fMRI vascular signals, while fast optical changes occur over milliseconds, and colocalize with EEG electrical signals, optical methods allow for a single modality with which to explore the spatiotemporal relationship between electrical and vascular responses in the brain. We are now pursuing such fMOI (or fNIRI) methods as a tool for prospectively identifying infants who subsequently go on to be diagnosed with cerebral palsy.

Finally, optical techniques for oxygen-related functional determinations rely on the colorimetric analysis of haemoglobin and other oxygen-carrying pigments. Using such signals, haemorrhages could be automatically identified and marked (figure 1). Other pigments can be monitored as well, allowing the identification of cell components (e.g. water, fat) or tissue types (e.g. nerve, artery, vein). Such tissue analysis may facilitate tissue diagnosis for what has been termed 'optical biopsy' or 'light assisted medical diagnosis', and this approach is being pursued by our group (Benaron *et al.* 1997*a*), as well as by Tromberg (University of California, Irvine), Bigio (Los Alamos National Laboratory, New Mexico), Ferrari (University of L'Aquila, Italy), Feld (Wellman Laboratory, MIT) and others.

2. LUMINESCENCE-BASED DETECTION

A limitation of the spectroscopic approaches presented in the first half of this paper is that such approaches require an inherent, identifiable optical signature. However, key molecules in many processes may not possess distinguishing features allowing them to be monitored from an external vantage point, such as the events associated with gene activation or infection. Because of this absence of characteristic signals, laboratory assays are typically limited to analysis of biopsy or postmortem tissue samples, in the absence of the relevant influences of the intact animal. This can lead to an inability to extrapolate our understanding of such processes to actions *in vivo*. For example, an antiviral agent may be active against HIV-I in the test tube, but fail to have efficacy when given to a patient. Thus, non-invasive assays for monitoring the progression of biological processes in the living, intact animal are needed.

An alternative to the use of external light sources is the use of internally generated light linked to specific biological functions, similar in concept to placing a lightbulb in the body to mark a particular place or event. This method has tremendous advantages over conventional spectroscopy methods. First, background signals are nearly non-existent. Therefore, if a photon is measured from tissue, it must have come from the luminescent probe. The detectability of such a signal is restricted only by the photon count (shot-noise) limits. This allows for an unparalleled subattomolar ($< 10^{-18}$), single-event detection *in vitro* (Hooper *et al.* 1990), and detection *in vivo* within an order of magnitude of this limit per centimetre of tissue subsurface depth. Next, this allows for linking of specific internal events, such as gene expression or viral replication, with light emission, thus allowing for localization and monitoring of functional and pathological processes not previously able to be followed. Finally, the approach can be performed using available imaging equipment.

The light beacons we use are luciferase enzymes. The firefly produces its characteristic glow by combining an energy-rich chemical with a luciferase enzyme that liberates this energy to produce light. Many eukaryotic and prokaryotic organisms use such enzymes to make light, and many different forms exist. Importantly, the chemical pathways that allow this reaction to occur exist on genes that can be moved into the cells of nearly any organism. We have moved sets of light-emitting genes from soil bacteria and fireflies into other cells to create new bacterial and animal cells that emit light. These new cells glow, much as a firefly does, and can then be detected and quantified using cooled or intensified video-type (CCD) cameras in dark rooms.

We have been investigating the use of luminescent markers placed *in vivo* to allow us to follow the course of infection and gene regulation in animal models of human diseases. These two areas are discussed below.

(*a*) *Imaging infection*

The route a pathogen takes in the infection of a living organism is influenced by characteristics of both the pathogen and the host, but methods to study infections *in vivo* are currently slow and cumbersome. To study the efficacy of a new antibiotic under development, for example, that antibiotic must be

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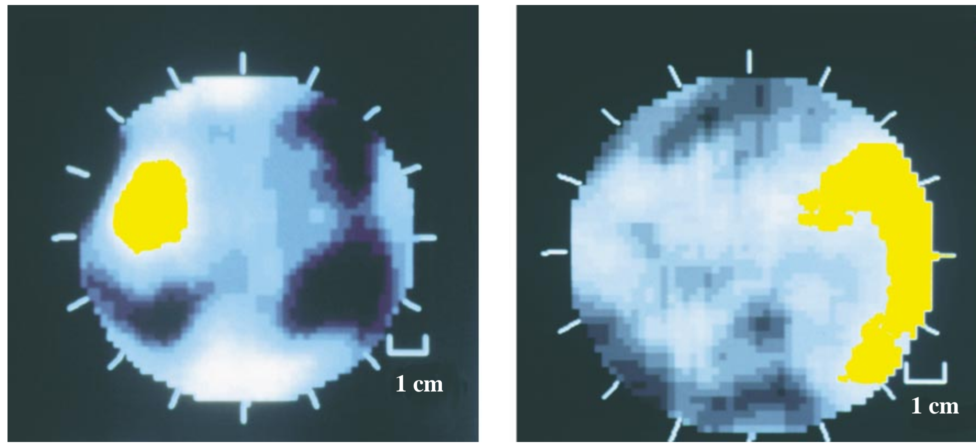

Figure 1. Optical tomographic images of intraventricular (left) and subdural (right) brain haemorrhages in infants. Using an automated diagnostic system that classifies tissue by type based upon optical properties, the haemorrhages have been highlighted in yellow.

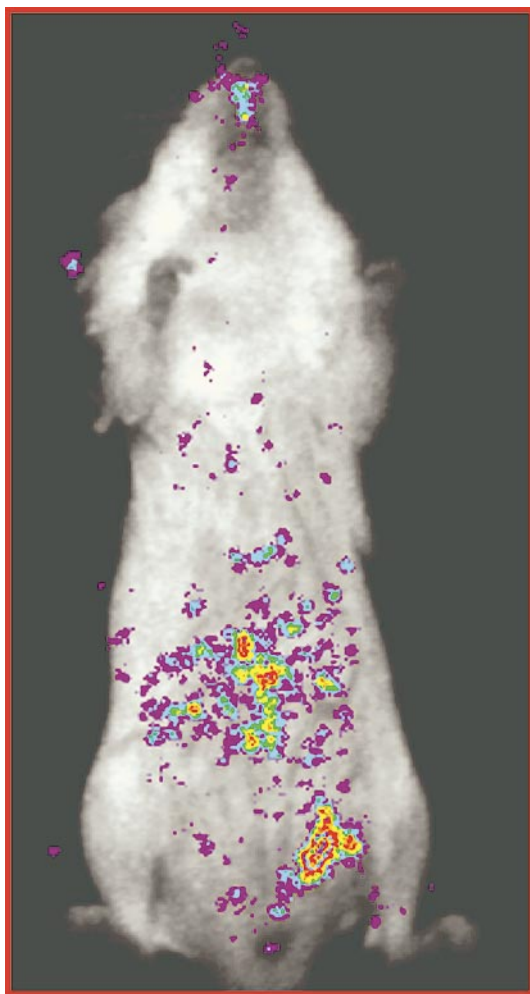

Figure 2. Image of a systemic infection with *Salmonella* (colour). The infection is widely distributed throughout the body, including the nose, after an abdominal cavity injection of bioluminescent bacteria.

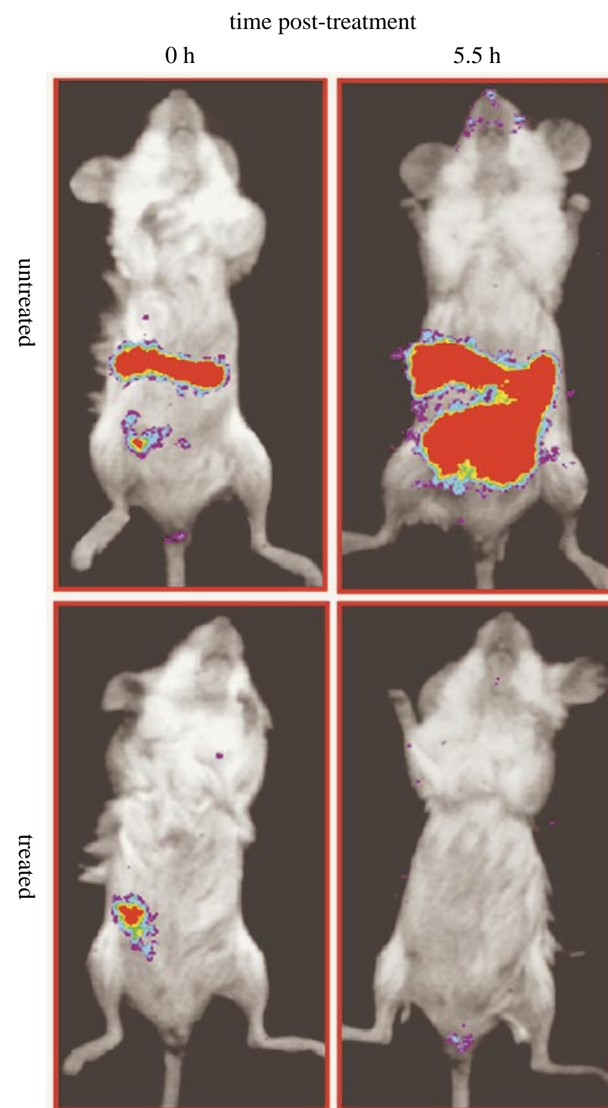

Figure 4. Images of an intestinal *Salmonella* infection (colour) with and without antibiotic treatment. The treated infection resolves and disappears, while the untreated infection spreads.

given to many animal and human subjects, and then blood and tissue samples collected and analysed in a lengthy process. Thousands of tissue samples are likely to be needed from any one series of studies, requiring weeks or months of research time. Thus, it is now difficult to rapidly screen novel drugs or test promising therapies, making the development of new treatments tedious, slow and expensive.

To non-invasively study a bacterial infection *in vivo*,

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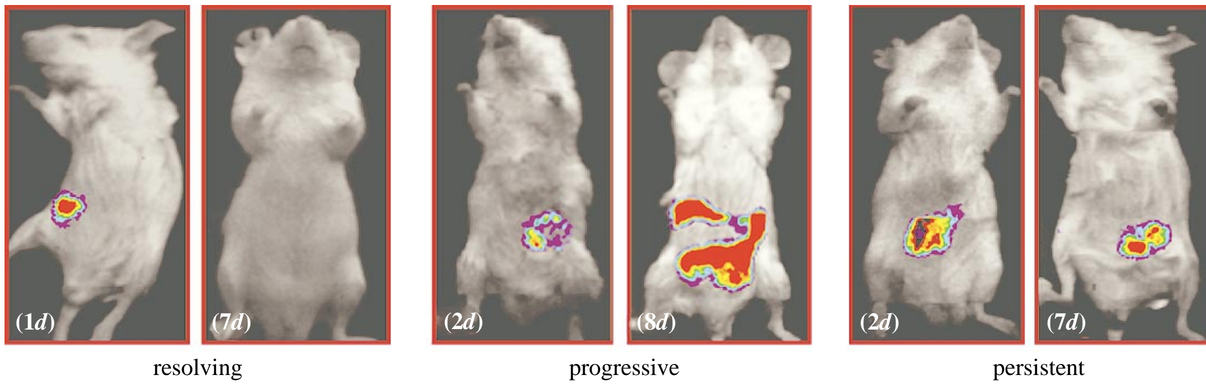

Figure 3. Images of intestinal *Salmonella* infection (colour) over time. The infection can be seen to be self-resolving, progressive or persistent after infection with different bacterial strains.

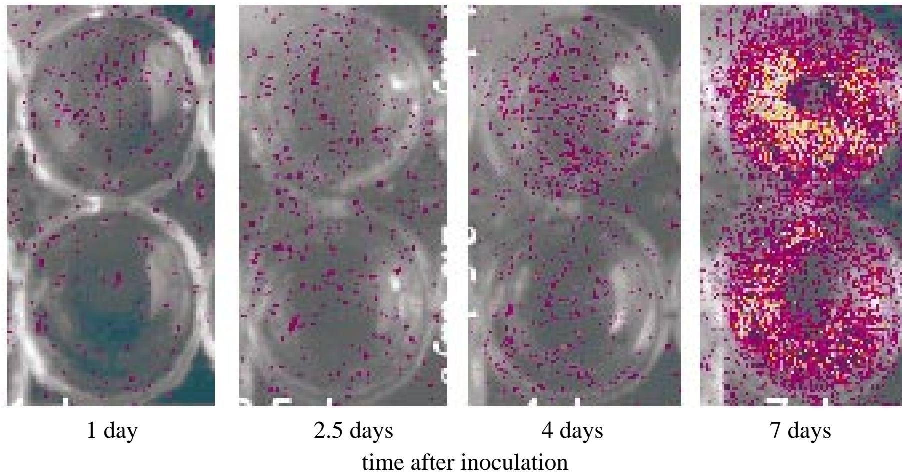

Figure 5. Images of HIV replication (colour) in a Jurkat cell culture. The replication of HIV induces bioluminescence, with signal detectable within 96 h of inoculation.

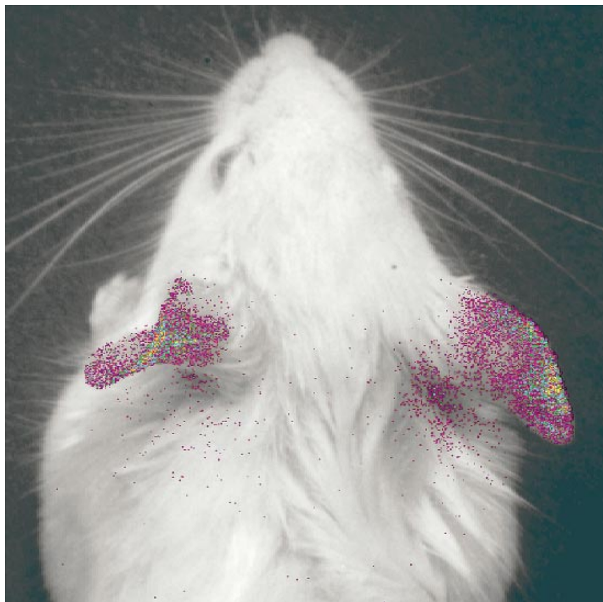

Figure 6. Bioluminescence from the ears of a transgenic bioluminescent mouse (colour) maps the sites at which a selected promoter in the skin has been locally activated using a topical chemical treatment.

we labelled bacteria with a light-emitting luciferase (Contag *et al*. 1995, 1996, 1977). This light serves as a marker of infection. Unlike standard labels, light emission is linked to bacterial metabolism, and therefore follows the infection. As the gene is incorporated into the bacterial DNA, it provides a label that persists over many bacterial generations. Infection of mice with *Salmonella typhimurium* is a well-characterized animal model of a human disease, typhoid fever, and *Salmonella* was, therefore, selected as the model with which to initially develop this bioluminescent approach. *Salmonella* were labelled through the expression of luciferase and accessory proteins obtained from the soil bacterium *Photorhabdus luminescens* (also called *Xenorhabdus luminescens*).

A few details on the particular enzymes used in this demonstration are noted here. Bacterial luciferases are heterodimeric mixed-function oxidases that emit photons during the energetic catalysis of substrate. A characterized plasmid encoding the entire *lux* operon from *P. luminescens* confers on *Escherichia coli* the ability to emit light at 486 nm in aerobic environments. Optimal bioluminescence for these transformed *E. coli* occurs at 37 °C, in contrast to the low temperature optima of luciferases from eukaryotic and other prokaryotic bioluminescent organisms. Thus, luciferase from *P. luminescens* appeared to be ideally suited for studying biological events in mammalian systems.

A variety of *Salmonella* strains differ in their ability to cause disease in mice. Susceptible (BALB/c) mice were inoculated with different bioluminescent *Salmonella* strains. These mice were evaluated using

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external monitoring of internally generated photons using an intensified CCD camera (Hamamatsu Corp., Japan) placed within a dark box (Contag *et al.* 1995).

Animals infected via direct injection into the peritoneal cavity with the virulent wild-type strain of *Salmonella* (SL1344) demonstrated widely distributed bioluminescence, suggestive of a disseminated systemic disease (figure 2). Animals infected orally with different strains showed different patterns of intestinal infection (figure 3), such that progressive courses of infection appeared distinct from those that were persistent or abortive. The distribution of bioluminescence in persistent and progressive infections suggested that the caecum may be a critical site for maintenance of *Salmonella* infection, which, under some conditions, can then lead to systemic disease. Thus, this approach may help elucidate mechanisms of disease. Last, the effect of treatment with the antibiotic Ciprofloxacin was non-invasively monitored in real time (figure 4), with a rapid reduction of signal noted within minutes of antibiotic treatment.

In summary, we put the light-emitting *Salmonella* bacteria into animals, and showed that we could follow the progress and location of infections in real time. We expect that as little as one bacteria in a sample of blood could be detectable. This method allows for the understanding of infectious processes in living animals, such as how bacteria, viruses and fungi overtake their host. Knowing the spatiotemporal distribution of pathogens and the effects of anti-infective agents on these distributions is expected to enhance our ability to intervene in disease processes.

(*b*) *Imaging gene expression*

The function and regulation of genes is an active area of research, and the use of reporter genes has contributed greatly to our understanding of how genes are expressed in cells in culture and *in vivo*. Regulation of gene transcription is mediated by complex interactions among positive and negative regulatory elements that are ultimately directed by the environment of the cell. Studies of gene regulation in mammalian cells, however, must often be performed on cells in culture, in the absence of contextual influences of the entire organism. Reporter systems currently used in animals now require *ex vivo* assays and thus are limited to biopsy or postmortem tissue samples. Further, such studies are arduous, and results may be delayed until a lengthy analysis has been completed.

We have shown that luminescence can be used to track gene activation in living tissues, without disturbing the native tissue state (Contag *et al.* 1997). In a first model system, a human T-cell line (Jurkat cells) had been earlier modified with an HIV-1 promoter gene fusion to emit light in response to intracellular replication of HIV-1 (White *et al.* 1995). We followed an HIV-1 inoculation of these Jurkat cells, and demonstrated that replication in cell culture could be detected and imaged (figure 5). In an animal model system of gene expression we used the promoter of HIV-1. This promoter was selected because of its known expression in the skin when present in cells of transgenic mice (Morrey *et al.* 1991). Earlier, this promoter had been placed in front of the firefly luciferase gene (Morrey *et al.* 1991). Fortuitously, the HIV-1 promoter also happens to be inducible using a variety of chemical and physical treatments, including the topical application of dimethylsulfoxide (DMSO) (Morrey *et al.* 1992, 1993), thus allowing gene expression to be experimentally induced in defined areas.

In this *in vivo* model, photons are produced with the activation of transcription of a specific gene, as shown in the localized induction of light in the ears of a mouse using topical DMSO (figure 6). Photon emission from internal organs was also detected in mice given luciferin systemically (not shown).

The transition from labelled bacterial cells and cells in culture, to expression of luciferase genes in mammalian cells and detection *in vivo*, required analysis of a eukaryotic luciferase *in vivo* and exogenous delivery of the substrate luciferin (the substrate for the bacterial luciferase is synthesized within the bacterial cells and, therefore, did not need to be supplied exogenously in those earlier experiments). In the model described above, we induced the expression of luciferase in the transgenic mice using topical DMSO, and delivered luciferin either systemically or topically. The two modes of luciferin delivery and the fact that photons originating from cells deep inside the animal can be detected, indicate that tissue-specific promoters of many types expressed in a variety of different tissues may be studied in small laboratory animals using this approach.

Imaging of other types of processes now appears possible with this method, such as the monitoring of tumours over time. A tumour, engineered to emit light, would allow non-invasive monitoring of tumour growth and response to antitumour agents. Metastatic cells should be detectable in very low numbers as the metastasized cells would emit light continuously and the integration periods can be very long using a non-invasive imaging system. The development of drug resistance during infection or tumours under treatment should be easily seen as a new 'bright' region in an otherwise darkening field. Many different processes may be able to be measured using this approach.

In summary, we have adapted our method of localizing infection and the expression of bioluminescent reporter genes in living mice to the study of gene regulation for real-time analyses in living animals. These studies demonstrate that real-time non-invasive analyses of pathogenic events, pharmacological monitoring and assessment of promoter activity can be performed *in vivo* using bioluminescent proteins as reporters for these processes.

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