Bioluminescence imaging of lymphocyte trafficking in vivo

Jonathan Hardy\(^a\), Matthias Edinger\(^b\), Michael H. Bachmann\(^a\), Robert S. Negrin\(^b\), C. Garrison Fathman\(^c\), and Christopher H. Contag\(^a\)

\(^a\)Departments of Pediatrics, Microbiology & Immunology, and Radiology, Stanford University School of Medicine, Stanford, Calif., USA; \(^b\)Division of Bone Marrow Transplantation, Department of Medicine, Stanford University School of Medicine, Stanford, Calif., USA; \(^c\)Division of Immunology and Rheumatology, Department of Medicine, Stanford University School of Medicine, Stanford, Calif., USA

Lymphocytes are highly mobile cells that travel throughout the body in response to a tremendous variety of stimuli. Revealing lymphocyte trafficking patterns in vivo is necessary for a complete understanding of immune function, as well as cell-cell and cell-tissue interactions in immune development and in response to insult. Although the location of cell populations in various tissues at any given point in time may be revealed by techniques such as flow cytometry and immunofluorescence, these methods are not readily amenable to the assessment of dynamic cell migration patterns in vivo. In the past 5 years, technologies for imaging molecular and cellular changes in living animals have advanced to a point where it is possible to reveal the migratory paths of these vitally important cells. Here, we review one advancement in cellular imaging, in vivo bioluminescence imaging, which addresses the problem of lymphocyte tracking. This imaging strategy has the potential to elucidate the temporal patterns of immune responses and the spatial distribution of lymphocytes within the body. © 2001 International Society for Experimental Hematology. Published by Elsevier Science Inc.

From their origin in the bone marrow to their final destination in peripheral locations, the movement of lymphocyte populations through the lymphoid organs and to their tissue targets remains elusive. Despite the advent of extremely useful reagents for the detection of lymphocyte populations, and even with the analysis of antigen-specific T cells using major histocompatibility complex (MHC) tetramers [1,2], visualization of the dynamic flow of lymphocyte trafficking has been limited. Monitoring of these cells in vivo is difficult due to the relatively small numbers within a given lymphocyte population, overlapping subsets, and the highly mobile nature of these cells.

For decades, the migration of lymphocytes has been followed using blood and tissue samples and a variety of tools. Imaginative, and sometime heroic, efforts have yielded much information, but the analyses of these processes in single animals over time has not previously been feasible. Methods that employ reagents for staining specific lymphocyte populations in tissue samples can be restricted by sampling limitations or the need to sacrifice the animals in the study. The removal of blood and tissue samples eliminates the contextual influences of living organs and tissues, with a resulting gap in temporal knowledge. The tremendous demand for information pertaining to in vivo cell migration patterns has led to such techniques as cryosectioning whole animals and imaging the distribution of labeled lymphocyte populations within the sections [3]. Valuable insights have been gleaned from such studies and they provide a solid basis for extending the static analyses to those that assess dynamic changes. Despite formidable obstacles, serial images of lymphocyte localization in response to disease states may yet be obtained, revealing the true dynamics of the immune response. Combining advances in immunology with in vivo molecular and cellular imaging techniques has already proven fruitful, and further technological advancements are certain to occur. The techniques that are being directed at these problems are either variations of those used in medical practice, or novel methods that are well suited for studying molecular and cellular processes in vivo in laboratory animals.

**PET and SPECT imaging**

The radioactive emission of radionuclides can be detected and imaged in living animals with scintillation cameras or other devices, serving to locate the radiolabeled substances in the body. Positron emission tomography (PET) and single photon emission computed tomography (SPECT) are two nuclear medicine techniques [4] with possible applica-
tion to studying immune cell populations in vivo [5,6]. Nuclides such as $^{18}$F, $^{15}$O, $^{13}$N, and $^{124}$I that emit positrons are used in PET imaging, which takes advantage of the pairs of coincident 511 keV $\gamma$ photons emitted by positron-electron collisions to decrease background and scattered signal. Radionuclides that emit $\gamma$ photons are utilized in SPECT, and their emission can be directly imaged using gamma cameras that can be rotated around a subject to detect emission from multiple angles and determine the location of the emitter in three-dimensional space. The spatial resolution of PET and SPECT are well within limits that would yield informative data, about 1 mm at optimum [7], and it is certainly possible that these modalities could be used to locate exogenously radiolabeled lymphocytes in animals and humans. The deep penetration of the high-energy emission from radionuclides and the three-dimensional images offer significant advantages for animal studies with direct application to clinical imaging. Miniaturization of clinical instrumentation for use on common animal models such as the mouse has been accomplished [8–10], and research applications for this instrumentation have been reported [11,12]. The limitations of imaging radionuclide emission of exogenously labeled cells are the relatively short half-lives of some of the isotopes used, the dissociation of detectable signal from the target cell population, some constraints in radiochemistry, and the loss of signal due to dilution during cell division. To overcome the dilution problem and limitations due to short half-lives of the isotopes, several reporter genes that concentrate radiolabeled compounds in expressing cells have been described [13–15]. This strategy holds significant promise for immune cell trafficking studies and may have clinical utility. The use of reporter genes for labeling of cells in this manner overcomes the problems of diluting the radiolabel and the short half-lives of isotopes, since the reporter replicates with cell division and the isotope can be administered repeatedly prior to image acquisition. These advancements permit the long-term analyses that are necessary for understanding lymphocyte biology.

**MRI**

Magnetic resonance imaging (MRI) has also been adapted for small-animal imaging, and is a modality where translation to clinical imaging is possible. In this technique, the subject is placed in a strong static magnetic field. Nuclei of certain elements such as spin-1/2 hydrogen have a magnetic moment that aligns preferentially along the direction of the magnetic field. Irradiation with a radio frequency magnetic field at the proper frequency (the resonance condition) causes transitions between energy states of the nuclei, resulting in net excitation of the nuclear spin system. Relaxation of the spins back to the lower energy state generates a signal (the nuclear magnetic resonance signal) that is characteristic of the nuclei and their chemical and physical environment. With suitable magnetic gradient coils and Fourier transform signal processing, these signals can be localized to produce an MR image. Many refinements of MRI have been made that permit images of unprecedented quality and resolution for the detection of tumors and a wide variety of other medical applications. Recently, MRI has been employed with the intent of localizing lymphocytes in vivo [16]. In this study, the lymphocytes were labeled with iron beads, which exhibit a MRI signal, linked to peptides derived from the HIV Tat protein to facilitate cellular uptake. These peptides efficiently labeled lymphocytes, and individual cells labeled with this strategy were detectable within bone marrow samples. Whole, living animals were not imaged in this study, but the possibility remains tantalizing.

**Optical imaging techniques**

The relative opacity of tissue permits the transmission and piping of light through the body, and thus small amounts of light emitted from weak internal biological sources escape the scattering and absorbing environment of the body and can be detected externally. As significant amounts of light (on average, 10-fold per centimeter of tissue) are lost due to absorption and scattering, this method is best suited to the study of small laboratory animals such as mice and rats [17]. Fluorescent signals can be detected in vivo [18–21] and the use of optical dyes such as fluorescein permits the reisolation of labeled cells from animals using flow cytometry. As an example of using dyes for tracking studies, exogenously labeled lymphocytes have been imaged in the rat eye in vivo using a scanning laser ophthalmoscope [22]. Unfortunately, light of the wavelengths that are needed to excite many of these fluoros, as well as the wavelength of emission, are blue and green and do not penetrate deeply into and out of mammalian tissues [23,24]. These techniques are, therefore, best applied to the study cells in culture or at superficial tissue sites in vivo. Advancements in optical detectors that employ confocal or two-photon detection modalities will greatly improve the utility of the in vivo detection of fluorescent signals as the excitation is accomplished with longer wavelengths with deeper tissue penetration.

Red light penetrates tissues more efficiently than green or blue emission; dyes that absorb and fluoresce in the red, such as indocyanin green, have been well described and used in animal models and in humans for detection of tumor nodules using light in vivo [25–27]. Some of these dyes have been coupled to proteolytic cleavage sites in juxtaposition to quenchers providing a molecular “light switch” that is activated by specific protease activity, and cleavage of such molecules in vivo has been used to assess protease activity in living animal models of neoplastic disease [21]. There are many applications in which optical imaging offers significant advantages over other modalities, especially in animal models of human biology and disease [17,28,29].

Despite the benefits of using fluorescent and other exogenous tags, cell trafficking studies employing these ap-
In vivo bioluminescence imaging

Although no single imaging modality can provide an investigator with all of the necessary information pertaining to a biological system, imaging strategies that use genetically tagged cells that express bioluminescent reporter proteins offer the advantages of fine temporal analyses, labeling versatility, accessible instrumentation, and a signal-to-noise ratio that provides high sensitivity of detection. Weak internal sources of biological light, bioluminescence, can be externally detected using sensitive charge-coupled device (CCD) cameras as low-light detection systems. This imaging modality, known as bioluminescent imaging (BLI), employs light-emitting proteins, known generally as luciferases, for real-time in vivo detection of tagged cells, thus enabling the type of temporal analysis in single animals that is necessary to image lymphocyte trafficking. Given that there are very few sources of light in mammalian cells and bioluminescent proteins do not require an excitation source, the background in BLI is nearly nonexistent. Luciferases have been studied for decades and are thus well characterized and readily applied to a variety of biological questions; this approach has been used to monitor infectious diseases, gene expression patterns, and tumor cell proliferation in vivo [17,30,31].

Luciferases are a family of proteins produced by luminous organisms such as Photinus pyralis, the common American firefly, that produce visible light from substrates known generically as luciferins. These reactions require high-energy molecules such as ATP, and thus light production is coupled to metabolic activity of the cells expressing the reporter gene. Genes coding for luciferases are useful tools for molecular and cellular biologists and, due to the penetration of bioluminescent light through mammalian tissues, have more recently been shown to be valuable for in vivo analyses [32]. The luciferase (luc) gene of Photinus pyralis or other genes encoding bioluminescent proteins, can be inserted behind the regulatory elements of various genes (promoters), thus becoming a reporter for expression of a given gene. Reporter gene constructs that employ synthetic, or natural, promoters that are constitutively expressed in a variety of cell types can be used to label immune cell populations such that the cells may remain labeled despite physiologic changes that may occur in vivo.

The luciferin that is a substrate for the Photinus pyralis luciferase is a small, water-soluble molecule that quickly penetrates the membranes of cells in tissues following intraperitoneal or intravenous injection, even crossing the blood-brain and placental barriers [33,34]. Limited penetration of luciferin into prolactin-secreting cells has been reported [35]; however, this appears not to be a general problem [30,36]. Luciferin is easily administered intraperitoneally together with anesthetic for imaging. The toxicity of this molecule, while not yet fully evaluated, appears to be low. Evidence for low toxicity has been obtained in a number of studies [17,30,33,36–39], and most notably its repeated administration to newborn mice results in no discernable effects on the health of the animals (Contag et al., unpublished results). The distribution of luciferin to tissues has been examined in a limited study [30], and more complete pharmacologic studies are underway in several laboratories. Strong correlations between in vivo signal intensity with relative luminescence of ex vivo assays, and other measures, has been obtained at a number of tissue sites, suggesting that, under the conditions reported, luciferin is present in excess in brain, fetal, and all other tissues that have been examined [33,34,40].

The instrumentation for low-light imaging is comparatively inexpensive to purchase, operate, and maintain and does not require the use of radionuclides, offering the opportunities of decentralized imaging facilities and thus enabling accelerated investigation of biological processes in vivo [41]. The sensitive CCD detectors that are required to capture transmitted photons have been widely used in biological imaging, and several camera architectures are available for imaging of extremely weak optical signals. Cooling the CCD detector to temperatures of −100 to −120°C is an effective means of reducing thermal noise and results in extremely sensitive CCD cameras that can detect light over a broad spectral range, including the wavelengths that are more readily transmitted through living tissue (650–1100 nm, [41]). At present, CCD detectors of this format offer greater sensitivity to light transmitted through living tissues (100-fold) than cameras that employ other architectures such as those that employ image intensifiers. This is, in large part, due to the broad spectral range of cooled CCDs and hence sensitivity to red light [41].

For BLI the animal is anesthetized and the substrate, luciferin, is injected, usually intraperitoneally, at the time that anesthesia is administered. After a fixed time, usually 15 minutes, the animal is placed in a light-tight chamber upon which a cooled CCD camera has been mounted [41]. Gray-scale reference images of the study subjects are obtained under weak illumination using camera settings that are optimal for this image (small lens aperture and short exposure
times of typically 1 second). A second image is then acquired, in the absence of an external light source, collecting the light that is generated internally and transmitted through the animal’s tissues. To obtain this bioluminescent image the lens aperture is maximum, and integration times as short as one second (even a fraction of a second) or as long as five minutes, depending on signal intensity, are used. In cooled CCD cameras, pixel size can be increased by “binning,” which serves to increase the number of photons represented at each point in the low-light image. Binning refers to combining a number of individual small pixels into a larger pixel, which is useful for reducing the background due to read noise and rapidly producing images with good contrast. Binning, however, results in a loss of resolution. When the signal intensity is high, binning is not necessary and the full resolution of the CCD camera can be preserved. Pixel binning of 10 × 10 is generally used to acquire the bioluminescent image; however, binning of 2 × 2 can also be used for study subjects with intense signals. The number of photons collected at each pixel is stored digitally for each exposure. These data can be presented in pseudocolor and overlaid on the grayscale reference image to localize the signals to specific tissue or organ sites. The raw data of actual photon count that have been stored digitally can be recalled for analysis and reanalysis.

The most serious limitation of BLI is the absorption and scattering of light by mammalian tissue, which reduces both the sensitivity and resolution of any optical method. The primary absorber in tissue is hemoglobin, and imaging of blood-rich organs, such as the liver and spleen, can be less efficient than more peripheral sites with less hemoglobin. As hemoglobin absorbs primarily in the blue-green region of the spectrum, much of the yellow-green emission of the firefly enzyme is absorbed by mammalian tissue [41]. The recent cloning of a red-emitting luciferase from the railroad worm [42] and the isolation of mutants of firefly luciferase that emit in the red [43] may be of help in this regard. Background signals in BLI can result from the long-lived fluorescence (phosphorescence) of contaminants on fur and plant-derived fluoros in food, but these signals are generally very low and are readily subtracted as background.

BLI has been applied to the in vivo study of neoplastic disease [31,34]. Cancer cells are relatively easy to manipulate in culture, and were among the first targets of luciferase-based in vivo imaging technology [40]. When HeLa cells were transfected with luciferase, their growth at several different anatomical sites could be easily measured [31]. As a model of minimal disease, the small numbers of cells present early in the study were visible also, indicating that BLI would be useful for studying therapies that address the minimal disease states that occur early in the disease course or after reduction of the tumor mass by other therapies [40]. The ability to detect small numbers of cancer cells in whole-body images has also been useful in localizing micrometastasis in animal models of human cancer [29]. As bioluminescence images can be rapidly obtained from multiple animals simultaneously, drug efficacy and the kinetics of tumor relapse could be readily evaluated in several study groups [40]. This provides great insight into the early events of tumor growth and response to novel therapeutic strategies such as transfer of cytotoxic lymphocyte populations [40].

**BLI in cancer immunotherapy**

The effects of an anti-cancer treatment that utilizes lymphocytes expanded ex vivo have been evaluated using BLI. Cytokine-induced killer (CIK) cells, comprised largely of CD3+CD56+ cells (NK T cells) were introduced into mice bearing a luciferase-expressing tumor. CD3+CD56+ cells display antitumor activity against a variety of tumors in culture and in vivo [44,45]. Sweeney and coworkers have demonstrated the effect of this immune cell therapy on tumor burden over time using BLI [40]. As CIK cells are CD3+, it has been possible to redirect the activity of these cells using antibody molecules with two distinct binding domains, one for CD3 and the other for a tumor-associated antigen (Sheffield, Contag, and Negrin, results under consideration for publication). These experiments were also performed in SCID mice using luciferase-expressing tumor cells, in this case a human ovarian cancer cell line that overproduces the surface protein HER2/neu (SK-OV3Luc). Bispecific antibodies directed toward CD3 and the HER2/neu oncogene increased the therapeutic efficacy of the CIK antitumor treatment, presumably due to increased adherence of CIK cells to the tumor cell targets. The quantification of emitted photons over time provides information about the kinetics of response in individual animals.

It is especially exciting that tumors below the size that can be readily identified and recovered for histological examination at autopsy can be visualized. In this manner, image data can be used to direct tissue sampling for the ex vivo assays, leading to improved analyses and more predictive animal models of human neoplastic disease. In the imaging studies used to examine the anti-tumor activity of CIK cells, the target was labeled and not the effectors; therefore the distribution of immune cells still needed to be analyzed by traditional methods, most of which involve sacrifice of the animal subjects at various time points. By labeling the effector population rather than the tumor, the dynamic migration and redistribution of these lymphocytes could be observed in vivo. Such experiments are currently underway and may reveal the properties of CIK cells that allow them to find and clear tumor cells. Labeling of immune cell populations is performed by transducing the cells in vitro [46], which is often inefficient when using peripheral blood mononuclear cells (PBMCs) as the target cells.

**Lymphocyte trafficking**

The specific genetic labeling of effector lymphocytes in vitro permits localization of these cells in animals to be imaged by BLI. When CD4+ T-cell hybridomas or primary T cells specific for known peptide antigens are transduced...
with retroviral vectors that encode multifunctional reporter genes (see below), they can be followed as they migrate to locations determined by their antigenic specificity. Employing a joint inflammation model of arthritis, T-cell hybridomas from transgenic mice that express a T-cell receptor specific for a type II collagen peptide (CII) home to the joints [47]. This localization can be observed by BLI when the specific T cells are transduced with a luciferase reporter gene and injected into mice intraperitoneally or intravenously (Fig. 1). The process of homing can be imaged in individual mice over time, revealing initial localization (within 5 minutes) in the lung, with subsequent localization (at ~24 hours) in lymph nodes, followed 48–96 hours later by signals from sites of inflammation, in this case the inflamed joints. When the T cells are derived from mice transgenic for a T-cell receptor (TCR) that recognizes myelin basic protein (MBP), signal was retained in MBP-immunized mice for at least nine days, and the labeled cells were observed to localize to the central nervous system (CNS) (Fig. 2) [37]. Interestingly, MBP-specific T cells also transiently homed to inflamed joints in the arthritis model, indicating nonspecific migration to sites of inflammation. The MBP-specific cells were only transiently retained in these sites, whereas CII-specific cells remained in inflamed joints for over seven days [47]. When the joints are preinflammatory clinically, the nonspecific localization is not observed. These data indicate that lymphocytes migrate nonspecifically to sites of inflammation but are retained in areas where they encounter the “relevant” antigen, confirming long-held models of lymphocyte migration and function.

These experiments represent the beginning of a field that is certain to expand to include the analysis of many models of autoimmunity and infection. The possibilities are manifold, including the determination of the kinetics of CTL migration and possibly even memory-cell distribution and reactivation. By understanding the trafficking patterns of lymphocytes it may be possible to deliver therapeutic or immune modulatory proteins to diseased tissues as was demonstrated in the arthritis model [47].

One of the problems that might arise in the analysis of lymphocyte migration is the background of circulating cells in the blood. Placing luciferase genes behind appropriate promoters may restrict the expression of the reporter to desired circumstances or locations. Activation markers might be a target for this procedure and could reveal areas of lymphocyte activation. Effector genes such as the granzymes also exhibit expression patterns of this type. In the study of lymphocyte function, the analysis of gene expression patterns by every means possible may be necessary. For greater versatility, multifunctional reporters that exhibit different properties can be employed. Fusions of luciferase with green fluorescent protein (GFP) [48], or related proteins, allow analyses with CCD imaging, flow cytometry, and immunohistopathology, greatly increasing the utility of the reporter gene construct. A critical advance in this regard is the development of transgenic mice that express luciferase in all their tissues, thus serving as universal donors for transplantation and trafficking studies. Stem cells from such mice may be introduced into appropriate recipients and followed throughout the life of the animal, providing unprecedented possibilities of analyses. In an initial attempt at creating such a universal labeled donor mouse, Bachmann et al. (MHB, PK, CHC, unpublished) produced transgenic mice in which a cytomegalovirus (CMV) immediate early promoter directed expression of a GFP-luciferase fusion gene (Fig. 3) [49–51]. These mice were luminescent in several tissues such as heart and pancreas, and could be used as donors of labeled tissues [38] and stem cells. However, as they do not express GFP in hematopoietic tissues (Scheffold, Edinger, Bachmann, unpublished data), cells of these lineages could not be identified by fluorescence.

Using transgenic animals that express the multifunctional reporter genes in lymphoid tissues and sorting the donor cells for such properties as antigen specificity using MHC tetramers, immune responses and memory could be examined by imaging. The migration of other populations, such as dendritic cells, might also be imaged over time using transplants. Of decided advantage would be the ability to track two or more populations, using several different luciferases. The use
Figure 2. MBP-specific transduced CD4+ T cells traffic to the CNS. Splenocytes from MBP TCR Tg mice were transduced with a GFP-luciferase retroviral vector. CD4+ cells were MACS isolated and analyzed by FACS for GFP expression, then cell suspensions with $1 \times 10^6$ GFP+ cells were transferred intravenously to PL/J recipients. $1 \times 10^6$ CD4+ cells transduced to express GFP-luciferase were transferred to naïve (A) or MBP-immunized mice prior to clinical signs of experimental allergic encephalitis (EAE) (B). Data are representative of 3 experiments with n = 2–3/group. Although the patterns were initially the same in both groups of animals, in the immunized animals the signal from the labeled lymphocytes persisted and appeared to localize to the lumbar region of the spinal cord. In the naïve animals the signal initially appeared over the lymphoid organs and was eventually lost over time. Ventral and dorsal views are shown as indicated at the 24-hour time point. This figure was adapted from Costa et al. [37].

Figure 3. Transgenic mice as a source of labeled cell populations. A GFP-luciferase fusion gene was created by amplification of GFP (S65T) from plasmid pCDM7-GFP [49], with a 19-amino acid linker [50] attached at the 3’ end. This was inserted into the plasmid pGL3 (Promega, Madison, WI, USA) upstream of the luciferase gene such that the two open reading frames were fused in frame. The fusion gene (diagrammed in the lower part of the figure) was cloned into the expression vector pJW4304 [51] downstream of a CMV immediate early promoter and intron, resulting in plasmid pJW-GyL [16]. The expression cassette was excised from the plasmid DNA and introduced into pronuclei derived from the FVB strain of mice via microinjection. Offspring were monitored for luminescence at age 4 weeks. The labeled animal (A) exhibits widespread luminescence whereas its littermate (B) is negative. Transgenic mice with ubiquitous and constitutive reporter gene expression may serve as reliable sources of labeled cell populations for immune cell trafficking studies as well as a source of labeled tissues for studies in transplantation [30].
of this procedure to track any cell of interest in real time, especially cells of the immune system, will reveal new information about the physiological behavior of cells as well as their function in different diseases. This method will provide new insight into cellular roles in infectious disease, autoimmunity, tumor immunology, and transplantation biology. The availability of a wide range of knockout and transgenic animals as lymphocyte donors will allow the study of functional roles of individual molecules in vivo, especially in regard to their influence on homing and proliferation. This method would be very useful in the analysis of anti-tumor immunology by labeling both the tumor and the effector population. To enable such studies, considerable efforts are now underway to discover and utilize novel luciferases that have different wavelengths of emission and/or use different bioavailable substrates. Should these possibilities be realized, a new dimension in optical imaging will be available.

**Imaging and hardware developments**

Not to be underestimated is the potential for further technological advancement of CCD imaging, and the refinement of imaging devices for these novel imaging strategies. The CCD cameras currently in use for the majority of optical imaging are macroscopic. There is no reason why higher-powered lenses, such as those employed in dissecting scopes or even microscopes, could not be mounted for use in imaging. The feasibility of this approach has been demonstrated in the analysis of bacterial interaction with mammalian cells in culture [52]. Such tools may lead to finer levels of analysis that can be readily coupled to the macroscopic imaging strategies. There are limitations to microscopic analysis of bioluminescent emission, in that the resolution is constrained by the scatter of light as it passes through tissue, and so the extent of these potential improvements remains to be elucidated. Currently, the images obtained by the CCD camera are two-dimensional. Modifications of the instrumentation could change this situation, yielding three-dimensional, or possibly even tomographic, images by manipulating the camera or the subject to obtain a series of images from around the animal. These images would then be combined by computer analysis into a three-dimensional representation of cell trafficking or gene expression patterns.

**Summary and future directions**

In conclusion, the field of imaging technology has advanced to the point of soon revealing secrets of lymphocyte behavior that have previously been elusive. Medical imaging techniques such as PET, SPECT, and MRI show great promise in the detection of labeled lymphocyte populations in vivo, and more improvements are certain. Optical imaging is especially promising because of the ability of luciferases to emit light without external excitation and their utility as genetic tags. Tumor models employing luciferase-based imaging already reveal the interaction of lymphocytes and their targets, the location of the tumors, and the efficacy of treatment regimens. Lymphocytes labeled in vitro have been traced to their targets in models of autoimmunity. The exploitation of these techniques in the search for the temporal movements of many lymphocyte subsets and antigen-specific clones is only a matter of time.

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