Review

# *In vivo* imaging approaches in animal models of rheumatoid arthritis

Michael L Dustin

Skirball Institute of Biomolecular Medicine and Department of Pathology, New York University School of Medicine, New York, USA

Corresponding author: Michael L Dustin (e-mail: dustin@saturn.med.nyu.edu)

Received: 14 Nov 2002 Revisions requested: 29 Nov 2002 Revisions received: 4 Apr 2003 Accepted: 10 Apr 2003 Published: 1 May 2003

*Arthritis Res Ther* 2003, **5**:165-171 (DOI 10.1186/ar768)


## Abstract

The interaction of activated leukocytes with the rheumatoid synovial environment is a key process in arthritis. Understanding this process will play an important role in designing effective treatments. *In vivo* imaging approaches combined with molecular genetics in animal models provide important tools to address these issues. The present review will focus on approaches to *in vivo* imaging, with particular attention to approaches that are proving useful for, or have promise for, research on animal models of rheumatoid arthritis. These approaches will probably shed light on the specific local mechanisms involved in chronic inflammation and provide real time monitoring approaches to follow cellular and molecular events related to disease development.

**Keywords:** arthritis, fluorescence, imaging, luminescence, microscopy

## Introduction: the *in vivo* renaissance

The early phase of exploration of the lymphoid system generated a wealth of information about anatomy and *in vivo* responses. Our ability to define molecular structures in the context of the anatomy of the *in vivo* immune response, first with antibodies and more recently with tools of molecular genetics, has increased the ability to incisively test hypotheses through *in vivo* experimentation. This is leading to a renaissance in a variety of *in vivo* studies, mostly focused around genetic manipulations. The molecular genetics tools are also complemented by new technologies to image the movements and interactions of cells *in vivo*.

The present review will focus on emerging technologies that allow *in vivo* imaging of specific cells or molecules using noninvasive methods or direct microscopic imaging of single cells in the *in vivo* environment using minimally invasive methods. Microscopic imaging has the advantage of being able to study single cells in action. Invasiveness in this case refers specifically to the need for surgical procedures to expose tissues for high-resolution imaging of cells or molecules of interest. The advantages and limitations of each approach are discussed with a specific emphasis on imaging in joints and on work directly relevant to rheumatoid arthritis. This information is summarized in Table 1.

## Whole animal imaging

Imaging of events in intact live animals is a powerful approach primarily because it allows studies over time with minimal perturbation of the experiment. These methods also couple in powerful ways with molecule genetics technologies that allow *in situ* labeling of cell populations expressing specific genes. The present review will also discuss recent studies in this area with direct relevance to animal models of rheumatoid arthritis.

### Bioluminescence imaging in intact animals [1]

The expression of luciferase has for many years been a powerful tool in gene expression studies. This is because the substrates in the luciferase reaction generate no signal (light) in the absence of luciferase. Instruments that detect luminescent reactions can be optimized for sensitivity to light without the necessity of rejecting any significant

CCD = charge-coupled device; FIAU = 2′-fluoro-2′-deoxy-1-beta-D-arabinofuranosyl-5-iodo-uracil; HSV-Tk = herpes simplex virus thymidine kinase; MHC = major histocompatibility complex; MRI = magnetic resonance imaging; PET = positron emission tomography.

**Table 1**

**Summary of *in vivo* imaging methodologies**

| Imaging mode | Invasiveness | Sensitivity, resolution, time scale | Advantages | Disadvantages |
|---|---|---|---|---|
| Bioluminescence | Anesthesia | ~100 cells, 5 mm, minutes | Noninvasive, sensitive, quantitative | Resolution, penetration |
| Micro positron emission tomography/single photon emission commuted tomography | Anesthesia | 1000 cells, 2 mm, minutes | Noninvasive, resolution | Short half-life of isotopes |
| Magnetic resonance imaging | Anesthesia | 1000 cells, 0.1 mm, minutes | Noninvasive, resolution | Sensitivity, slow |
| Intravital microscopy | Anesthesia/surgery | 1 cell, 0.2 μm, seconds | Highest resolution | Invasive, penetration limited |

background signals. Only in recent years have cameras become sensitive enough to detect the faint light emissions of the luciferase reaction from within intact animals.

The most useful detectors are back-illuminated, cooled, charge-coupled device (CCD) cameras that have very low background and very high 'quantum efficiency' (the proportion of photons hitting the detector that are converted into a usable signal). Back-illumination refers to a method of preparing the CCD sensor so that the photons directly strike the light-sensitive thinned back surface, in contrast to conventional CCDs where photons pass through nonlight-sensitive elements on the front of the CCD with a resulting loss of efficiency. These systems also have very low noise, and long exposures can therefore be used to integrate the signal over time and to obtain a usable signal.

To apply this approach, the luciferase gene can be introduced into an animal using transgenic or homologous recombination technology to place luciferase expression under the control of specific genetic elements. When transcription of luciferase is activated, the cells or tissues expressing the gene can metabolize injected substrates (luciferin in the presence of endogenous ATP), which are nontoxic. The substrate metabolism can generate a signal detected by the external camera with the only requirement that the animal is anesthetized so that it does not move during the imaging period. Breathing causes movements in the thoracic area, but these are not significant compared with the general resolution. The drawback of this method is that the light emitted from the luciferase reaction is yellow–green, and thus is highly scattered as it passes through tissues and exits the animal. The resolving power is therefore low (millimeters). However, this is certainly adequate to identify cell migration or gene expression within the joint with a detection threshold in the order of 10–100 cells.

Lymphocytes for transfer studies could be prepared from luciferase expressing transgenic mice. Luminescence imaging has been applied to studies on cell transfer in the murine autoimmune disease model experimental autoimmune encephalitis [2] and has been applied to examination of transcription factor nuclear factor-κB in inflamed mouse joints [3]. This approach has also been used to track antigen-specific T cells for gene therapy of collagen-induced arthritis in mice [4]. Application of the luminescence methodology to humans would be problematic due to the greater thickness of human skin as a barrier to photon escape and detection. Shifting the luminescent emission to the red end of the spectrum might improve these prospects [5]. Transcutaneous imaging of cells expressing green fluorescent protein and other fluorescent dyes has also been demonstrated with similar resolution to the luminescence-based imaging, but with less sensitivity owing to the greater background from autofluorescence and scattered excitation light [6].

**Radioactive tracer imaging in intact animals**

Radioactive tracer studies offer greater penetration and quantitative integrity compared with optical imaging methods because the emissions from radioisotopes have less interaction with tissues than does light. Of the available methods for radioisotope imaging, that with the best resolution for small animal imaging is positron emission tomography (PET).

PET imaging is based on isotopes such as $^{14}F$ and $^{64}Cu$, which decay by emitting positrons that, on collision with an electron, emit γ-rays at 180° to each other. Arrays of detectors surrounding an animal can simultaneously detect these γ-emissions and then determine with great precision the line along which the emission was localized. From a number of such emissions, the PET method can build an image in which the source can be localized with a resolution of ~2 mm.

The limitation of PET imaging is that the positron-emitting isotopes have short half-lives so they can only be used to follow the cell or molecule *in vivo* for a day or two at most. Within this time span, however, very important results can

be obtained. A striking recent example is a study on the interaction of antibodies to glucose-6-phosphate isomerase, a ubiquitous enzyme [7]. These antibodies transfer arthritis and are specifically produced in mice transgenic for the KRN T-cell receptor on a nonobese diabetic mouse genetic background. A mystery in this disease process is why antibodies to a generally expressed enzyme would specifically induce a joint disease. Anti-glucose-6-phosphate isomerase antibodies were labeled with $^{64}$Cu and injected into recipient mice, which were then subjected to micro-PET analysis (a PET scanner configured to produce high-resolution images of small animals). It was found that the anti-glucose-6-phosphate isomerase antibody was rapidly concentrated in distal joints (the targets of the disease), while control IgG did not show this localization [7]. Therefore, an important advance in understanding the pathological effects of autoantibodies in a rheumatoid arthritis model was made using PET imaging of molecules. PET imaging is performed with human subjects where the short-lived isotopes are considered to pose a small risk and much information is gained, particularly regarding the metabolic status of tissue [8]. *In vivo* studies on autoantibody involvement in human rheumatoid arthritis are thus possible.

An alternative mode of imaging is the use of single photon imaging of γ-emitting isotopes like $^{111}$In or $^{99}$Tc. Imaging of γ-emitting isotopes is referred to as single photon emission commuted tomography. This approach as been used to follow isotope-labeled materials in joints of arthritis patients. It has the advantage that the individual components can be radiolabeled and followed *in vivo*, but has the disadvantage that γ-emitters of sufficiently high activity also have relatively short half-lives. Cells can be labeled prior to transfer to animals or can be labeled *in situ* by injection of monoclonal antibodies labeled with appropriate isotopes [9,10]. This method has lower resolution than PET, but is simpler and utilizes isotopes such as $^{111}$In that are readily incorporated into live cells. These isotopes can also be detected with γ-cameras with similar resolution.

A drawback of both the PET and single photon emission commuted tomography methods is that the isotopes have short half-lives, making long-term tracking impractical. This problem has been partially overcome for experimental animal models through the expression of herpes simplex virus thymidine kinase (HSV-Tk) in cells of animals and then injecting the animals with 2′-fluoro-2′-deoxy-1-beta-D-arabinofuranosyl-5-iodo-uracil (FIAU), a compound that is specifically accumulated in cells expressing the HSV-Tk gene product [11]. Similar experiments have been performed with rat myocardium using other tracer compounds, but FIAU appears to be the best [12–14]. This approach allows an elegant combination of molecular genetics and noninvasive imaging: the presence of the HSV-Tk gene can mark a specific cell population in a specific state of activation based on the activity of the promoter controlling expression of the HSV-Tk gene. The animals expressing tagged cells can then be labeled with radionuclide-tagged FIAU (for either single photon emission commuted tomography or for PET imaging) on repeated occasions over a long period of time. The HSV-Tk cells can then be located as long as they are not in organs like the bladder that accumulate FIAU as part of normal metabolism and excretion of the FIAU.

#### MRI of transferred lymphocytes

A promising technology for tracking cells deep in animals is the use of paramagnetic contrast agents taken into cells using cell-penetrating peptides in conjunction with MRI [15,16]. This method uses the HIV tat peptide, a highly cationic peptide that has the ability to enter into cells through the plasma membrane in an energy-independent process and to bring along large cargo [17], linked to superparamagnetic iron [18]. *In vitro* MRI imaging of bone marrow material populated with a few cells that had taken up the paramagnetic iron shows that single cells are detected as 'signal voids'. Because this is a dark signal on a light and variable background, the actual sensitivity may not reach the single-cell level *in vivo*. However, T-cell infiltrates in nonobese diabetic mice were readily detected in the pancreas [16]. This suggests that the sensitivity is sufficient to be useful in tracking cells in inflammatory infiltrates. This contrast agent allows the detection of cells in the context of the normal high level of tissue contrast that can be attained with MRI. This method is relatively new and has not been extensively applied to autoimmune situations. One important issue will be the minimum number of cells, which can be tracked.

#### Ultrasound imaging with microbubbles

A novel type of specific tracer for noninvasive cellular imaging is the use of ultrasound to image cells specifically tagged with stable microbubbles [19–22]. These studies demonstrated that the microbubble contract agents of various surface chemistries are readily phagocytosed by leukocytes attached to inflamed blood vessels. These phagocytosed microbubbles were more stable than extracellular microbubbles and thus could be imaged with high contrast. Microbubbles could also be specifically targeted to inflamed endothelium with antibodies to P-selectin (CD62P). The tendency of microbubbles to attach to leukocytes in inflamed vessels may correlate with the utility of these contrast agents in detecting active arthritis in the knee [23]. The utility of ultrasound may be enhanced, and the mechanism of contract agent accumulation is better understood and specific targeting strategies for contrast agents developed for clinical use.

## Microscopy approaches

Microscopic approaches allow the resolution of cellular and subcellular details with high numerical aperture objec-

tives. The general drawback of these methods is that they do not allow this level of resolution transcutaneously, and therefore require surgical exposure of the organ or tissue of interest. These invasive methods must be approached with great care since the surgical procedures are well known to induce leukocyte adhesion to endothelial cells and other effects, which may render the surgical preparations different in some ways from intact tissues. Nonetheless, microscopy is essential to address questions of single cell and supramolecular dynamics *in vivo*.

**Intravital microscopy**

Fortunately for immunologists and rheumatologists interested in surgical procedures for *in vivo* imaging, there is a rich arsenal of procedures for imaging within almost all major organs of mice or rats. Almost all were developed originally for microvascular research and then adapted for inflammation research. A nonexhaustive list includes the brain, the liver, the lungs, the muscle, the spleen, the lymph nodes, the pancreas, the mesenteries and the skin [24–28]. Each of these preparations has unique strengths and caveats, and most show some effects of surgical trauma that must be considered in interpreting the results. For example, in the cremaster muscle preparation, the abundant rolling leukocytes in the venules are due to P-selectin upregulation on endothelial cells in response to surgical trauma [25].

It is important to note that there is a recently developed intravital preparation for mouse joint synovium [29]. The synovium is exposed for imaging by partial resection of the patella tendon. This preparation has been used to evaluate the effects of anti-inflammatory drugs and nitric oxide inhibition on leukocyte recruitment to rheumatoid synovium [30–32]. The important results were that inducible nitric oxide synthase was protective in acute joint inflammation but had no influence on chronic synovial inflammation. The nonconventional anti-inflammatory drug oxaceprol reduced leukocyte adherence to synovial microvessels and generally reduced the signs of inflammation. The groundwork for further studies on the dynamics of lymphocyte interactions in the synovium has thus been established.

Most of the work in intravital imaging of leukocytes has focused on the interaction of lymphocytes with endothelial cells, and has only minimally addressed the issues of what leukocytes do after they extravasate. While leukocytes in blood vessels have high contrast, the extravasated leukocytes in tissues generally lack contrast and can only be tracked by fluorescence imaging of labeled cells. Those workers studying leukocyte interactions with blood vessels have also had a very clear hypothesis in the form of the multistep paradigm, which argued for rolling, activation and arrest steps executed by selectins, chemokines and integrins [33,34]. This hypothesis created a clear framework for many studies to identify these components, or their absence, in different tissue sites for different leukocyte subsets.

A hypothetical framework for migration of leukocytes and lymphocytes in tissues is provided by the multistage guidance of leukocytes by chemokines and bacterial products [35], and by the concept that antigen receptor engagement delivers a stop signal for lymphocytes [36]. While the movement of leukocytes in blood vessels is fast and much data can be collected in a couple of minutes of recording, the migration of leukocytes in tissues is relatively slow and requires many minutes of recording to track cells. This longer imaging period requires greater stability of the preparations. A few studies have now documented that leukocyte and lymphocyte migration in the parenchyma of tissues can be followed *in vivo* by imaging in thin tissues like the mesenteries or by fluorescence intravital microscopy, but there has been very little systematic analysis of this migration at this point [37–39]. Werr and colleagues clearly established that the collagen receptor VLA-2 has an important role in the migration of leukocytes in the rat mesenteries [37]. At this point, the adhesion systems used by lymphocytes for migration in tissues are not known.

An intermediate step between *in vitro* and *in vivo* studies on tissue migration of lymphocytes is the use of organ culture systems. A very useful experimental system is based on thymic organ cultures in which positive and negative selection in thymocyte maturation can be recapitulated in long-term culture models. Imaging of fluorescently labeled thymocyte migration in thymic organ cultures demonstrates both dynamic and stable interactions that were dependent upon positive selecting MHC–peptide complexes [40]. The power of this system is that imaging results can be directly related to the functional maturation of thymocytes in the culture system.

Lymph node organ cultures are not a traditional system in immunology, yet imaging of lymph nodes from mice into which a few million fluorescently labeled T cells or B cells had been transferred was an informative experiment. The lymph nodes were excised and immediately superperfused with highly oxygenated media in an effort to maintain oxygen levels within the intact mouse lymph node, which is about 1 mm in diameter. Both T cells and B cells in the cultured lymph nodes displayed dramatic motility, which was restricted to the T-cell zones and the follicles, respectively, but was otherwise random in direction [41]. While it was not clear whether oxygen was a critical parameter for these experiments, a clearly critical parameter was temperature. The motility of T cells in the lymph node was critically dependent on the temperature being close to 37°C. The motility dropped steeply below, and also above, this level. The increased local temperature associated with inflammation in tissues may therefore play a role in optimizing leukocyte migration in the site. It is probable that this

rapid, random migration, which was not previously postulated, is a critical element in the search of lymphocytes for presenting cells with appropriate antigens. T-cell receptor engagement appeared to deliver a stop signal in both systems [41,42].

These organ culture experiments will probably serve as stepping stones to *in vivo* observations now that it is clear that there are interesting things to be learned from following migration of labeled lymphocyte populations. It was also demonstrated that a sufficiently high resolution can be obtained for imaging the distribution of molecules within individual cells, making it possible to approach analysis of formation of the immunological synapse, a specific supramolecular pattern of receptors involved in immune cell communication, *in vivo* [42,43].

**Two-photon microscopy**

One of the limitations of high-resolution optical imaging is that it is very sensitive to light scattering by biological tissues. This makes the effective imaging depth for conventional high-resolution microscopy around 0–50 μm into a tissue. Cells can still be detected for another 50 μm, but all detail on the micrometer scale is lost.

Two-photon microscopy is a powerful method for imaging deeper within tissues that takes advantage of the lower light scattering with infrared light [44,45]. This is demonstrated by the classic childhood experiment of holding a flashlight to one's hand and observing that the light that penetrates is red. Two-photon excitation is based on the excitation of fluorescence for typical visible excitation fluorophores with two photons of low-energy infrared light. The two photons have to be absorbed by the fluorophore in rapid succession such that the instantaneous intensity of light has to be millions of times brighter than that typically used for conventional fluorescence excitation. This extreme brightness is accomplished using a mode-locked titanium–sapphire laser, which emits light in fentosecond pulses. While the average power is similar to that used in conventional confocal microscopy, the peak power is $10^6$ times higher. The beam is then expanded to fill the back aperture of the objective and is focused to a diffraction-limited spot in the tissue. Only at this focal point is the density of photons sufficiently high to achieve multiphoton fluorescence excitation, resulting in a very small volume of 0.2 μm wide × 0.5 μm high. The laser beam is scanned through the specimen and all the light that is emitted is collected by a photomultiplier mounted as close to the back of the objective as possible. No pinhole is needed since the excitation volume defines the image plane. The emission can be highly scattered as it exits the tissue, but only needs to hit the detector to count toward the signal.

The practical depth of imaging achieved with multiphoton imaging depends on the objective used, on the tissue and on the exact wavelength that is used for excitation. In the brain, it is possible to image up to 300 μm with submicron resolution. Lymph nodes appear to have more background signal and scattering than the brain, but imaging over 100 μm deep is still readily achieved and cellular signals can be identified up to 200 μm [46]. Advances in technology such as gradient refractive index lenses may enable much deeper high-resolution imaging in the future.

## Future studies

A clear direction for future studies will be the direct examination of T-cell migration and cell–cell interactions in the rheumatoid synovium. This process may be studied at many levels, from cell populations by noninvasive methods to single cells by direct microscopic observation after simple surgical procedures to expose the synovium. Mice expressing fluorescent proteins in specific tissues will be valuable for these future studies.

There are a number of key questions about cell dynamics in the synovium. Do T cells form stable immunological synapses with antigen presenting cells in the synovium? Is stable synapse formation related to the assembly of ectopic secondary lymphoid tissues in the vicinity of the synovium? How do T cells interact with different types of synoviocytes – the macrophage-like type I cells and the fibroblast-like type II cells? Do T cells interact in specific ways with macrophage-like cells at sites of bone erosion? How do autoantibodies interact with tissues and immune cells, including mast cells, at the microscopic level? These and other questions can be addressed by combining molecule genetic methods with new imaging modes.

We should know in the near future the general utility of these approaches in evaluating therapeutics and disease models. It is most probable that these approaches will yield surprising results and will be highly informative in the effort to cure arthritis.

## Competing interests

None declared.

## Acknowledgements

The author thanks his laboratory group for inspiring discussions and the Irene Diamond Fund for generous support. The work is also supported by grants from the National Institutes of Health. MLD is a past recipient of an Arthritis Foundation Research Grant, which supported work on the TCR stop signal.

## References

1. Contag CH, Bachmann MH: **Advances in in vivo bioluminescence imaging of gene expression.** *Annu Rev Biomed Eng* 2002, **4**:235-260.
2. Costa GL, Sandora MR, Nakajima A, Nguyen EV, Taylor-Edwards C, Slavin AJ, Contag CH, Fathman CG, Benson JM: **Adoptive immunotherapy of experimental autoimmune encephalomyelitis via T cell delivery of the IL-12 p40 subunit.** *J Immunol* 2001, **167**:2379-2387.

3. Carlsen H, Moskaug JO, Fromm SH, Blomhoff R: **In vivo imaging of NF-kappa B activity.** *J Immunol* 2002, **168**:1441-1446.
4. Nakajima A, Seroogy CM, Sandora MR, Tarner IH, Costa GL, Taylor-Edwards C, Bachmann MH, Contag CH, Fathman CG: **Antigen-specific T cell-mediated gene therapy in collagen-induced arthritis.** *J Clin Invest* 2001, **107**:1293-1301.
5. Branchini BR, Magyar RA, Murtiashaw MH, Anderson SM, Helgerson LC, Zimmer M: **Site-directed mutagenesis of firefly luciferase active site amino acids: a proposed model for bioluminescence color.** *Biochemistry* 1999, **38**:13223-13230.
6. Mahmood U, Tung CH, Tang Y, Weissleder R: **Feasibility of in vivo multichannel optical imaging of gene expression: experimental study in mice.** *Radiology* 2002, **224**:446-451.
7. Wipke BT, Wang Z, Kim J, McCarthy TJ, Allen PM: **Dynamic visualization of a joint-specific autoimmune response through positron emission tomography.** *Nat Immunol* 2002, **3**:366-372.
8. Antonini A, Moresco RM, Gobbo C, De Notaris R, Panzacchi A, Barone P, Calzetti S, Negrotti A, Pezzoli G, Fazio F: **The status of dopamine nerve terminals in Parkinson's disease and essential tremor: a PET study with the tracer [11-C]FE-CIT.** *Neurol Sci* 2001, **22**:47-48.
9. Jorgensen C, Apparailly F, Couret I, Canovas F, Jacquet C, Sany J: **Interleukin-4 and interleukin-10 are chondroprotective and decrease mononuclear cell recruitment in human rheumatoid synovium in vivo.** *Immunology* 1998, **93**:518-523.
10. Kipper SL, Rypins EB, Evans DG, Thakur ML, Smith TD, Rhodes B: **Neutrophil-specific 99mTc-labeled anti-CD15 monoclonal antibody imaging for diagnosis of equivocal appendicitis.** *J Nucl Med* 2000, **41**:449-455.
11. Tjuvajev JG, Finn R, Watanabe K, Joshi R, Oku T, Kennedy J, Beattie B, Koutcher J, Larson S, Blasberg RG: **Noninvasive imaging of herpes virus thymidine kinase gene transfer and expression: a potential method for monitoring clinical gene therapy.** *Cancer Res* 1996, **56**:4087-4095.
12. Inubushi M, Wu JC, Gambhir SS, Sundaresan G, Satyamurthy N, Namavari M, Yee S, Barrio JR, Stout D, Chatziioannou AF, Wu L, Schelbert HR: **Positron-emission tomography reporter gene expression imaging in rat myocardium.** *Circulation* 2003, **107**: 326-332.
13. Brust P, Haubner R, Friedrich A, Scheunemann M, Anton M, Koufaki ON, Hauses M, Noll S, Noll B, Haberkorn U, Schackert G, Schackert HK, Avril N, Johannsen B: **Comparison of [18F]FHPG and [124/125I]FIAU for imaging herpes simplex virus type 1 thymidine kinase gene expression.** *Eur J Nucl Med* 2001, **28**:721-729.
14. MacLaren DC, Toyokuni T, Cherry SR, Barrio JR, Phelps ME, Herschman HR, Gambhir SS: **PET imaging of transgene expression.** *Biol Psychiatry* 2000, **48**:337-348.
15. Dodd CH, Hsu HC, Chu WJ, Yang P, Zhang HG, Mountz JD Jr, Zinn K, Forder J, Josephson L, Weissleder R, Mountz JM, Mountz JD: **Normal T-cell response and in vivo magnetic resonance imaging of T cells loaded with HIV transactivator-peptide-derived superparamagnetic nanoparticles.** *J Immunol Methods* 2001, **256**:89-105.
16. Moore A, Sun PZ, Cory D, Hogemann D, Weissleder R, Lipes MA: **MRI of insulitis in autoimmune diabetes.** *Magn Reson Med* 2002, **47**:751-758.
17. Wender PA, Mitchell DJ, Pattabiraman K, Pelkey ET, Steinman L, Rothbard JB: **The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters.** *Proc Natl Acad Sci USA* 2000, **97**: 13003-13008.
18. Lewin M, Carlesso N, Tung CH, Tang XW, Cory D, Scadden DT, Weissleder R: **Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells.** *Nat Biotechnol* 2000, **18**:410-414.
19. Lindner JR, Song J, Christiansen J, Klibanov AL, Xu F, Ley K: **Ultrasound assessment of inflammation and renal tissue injury with microbubbles targeted to P-selectin.** *Circulation* 2001, **104**:2107-2112.
20. Lindner JR, Song J, Xu F, Klibanov AL, Singbartl K, Ley K, Kaul S: **Noninvasive ultrasound imaging of inflammation using microbubbles targeted to activated leukocytes.** *Circulation* 2000, **102**:2745-2750.
21. Lindner JR, Dayton PA, Coggins MP, Ley K, Song J, Ferrara K, Kaul S: **Noninvasive imaging of inflammation by ultrasound detection of phagocytosed microbubbles.** *Circulation* 2000, **102**:531-538.
22. Lindner JR, Coggins MP, Kaul S, Klibanov AL, Brandenburger GH, Ley K: **Microbubble persistence in the microcirculation during ischemia/reperfusion and inflammation is caused by integrin- and complement-mediated adherence to activated leukocytes.** *Circulation* 2000, **101**:668-675.
23. Carotti M, Salaffi F, Manganelli P, Salera D, Simonetti B, Grassi W: **Power Doppler sonography in the assessment of synovial tissue of the knee joint in rheumatoid arthritis: a preliminary experience.** *Ann Rheum Dis* 2002, **61**:877-882.
24. Piccio L, Rossi B, Scarpini E, Laudanna C, Giagulli C, Issekutz AC, Vestweber D, Butcher EC, Constantin G: **Molecular mechanisms involved in lymphocyte recruitment in inflamed brain microvessels: critical roles for P-selectin glycoprotein ligand-1 and heterotrimeric G(i)-linked receptors.** *J Immunol* 2002, **168**:1940-1949.
25. Kunkel EJ, Jung U, Bullard DC, Norman KE, Wolitzky BA, Vestweber D, Beaudet AL, Ley K: **Absence of trauma-induced leukocyte rolling in mice deficient in both P-selectin and intercellular adhesion molecule 1.** *J Exp Med* 1996, **183**:57-65.
26. Andonegui G, Goyert SM, Kubes P: **Lipopolysaccharide-induced leukocyte–endothelial cell interactions: a role for CD14 versus toll-like receptor 4 within microvessels.** *J Immunol* 2002, **169**:2111-2119.
27. Ley K, Linnemann G, Meinen M, Stoolman LM, Gaehtgens P: **Fucoidin, but not yeast polyphosphomannan PPME, inhibits leukocyte rolling in venules of the rat mesentery.** *Blood* 1993, **81**:177-185.
28. von Andrian UH: **Intravital microscopy of the peripheral lymph node microcirculation in mice.** *Microcirculation* 1996, **3**:287-300.
29. Veihelmann A, Harris AG, Krombach F, Schutze E, Refior HJ, Messmer K: **In vivo assessment of synovial microcirculation and leukocyte–endothelial cell interaction in mouse antigen-induced arthritis.** *Microcirculation* 1999, **6**:281-290.
30. Veihelmann A, Hofbauer A, Krombach F, Dorger M, Maier M, Refior HJ, Messmer K: **Differential function of nitric oxide in murine antigen-induced arthritis.** *Rheumatology (Oxford)* 2002, **41**:509-517.
31. Veihelmann A, Landes J, Hofbauer A, Dorger M, Refior HJ, Messmer K, Krombach F: **Exacerbation of antigen-induced arthritis in inducible nitric oxide synthase-deficient mice.** *Arthritis Rheum* 2001, **44**:1420-1427.
32. Veihelmann A, Hofbauer A, Refior HJ, Messmer K: **Oxaceprol, an atypical inhibitor of inflammation, reduces leukocyte adherence in mouse antigen-induced arthritis.** *Acta Orthop Scand* 2001, **72**:293-298.
33. Lawrence MB, Springer TA: **Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins.** *Cell* 1991, **65**:859-873.
34. von Andrian UH, Chambers JD, McEvoy LM, Bargatze RF, Arfors KE, Butcher EC: **Two-step model of leukocyte–endothelial cell interaction in inflammation: distinct roles for LECAM-1 and the leukocyte beta 2 integrins in vivo.** *Proc Natl Acad Sci USA* 1991, **88**:7538-7542.
35. Foxman EF, Campbell JJ, Butcher EC: **Multistep navigation and the combinatorial control of leukocyte chemotaxis.** *J Cell Biol* 1997, **139**:1349-1360.
36. Dustin ML, Bromley SK, Kan Z, Peterson DA, Unanue ER: **Antigen receptor engagement delivers a stop signal to migrating T lymphocytes.** *Proc Natl Acad Sci USA* 1997, **94**:3909-3913.
37. Werr J, Johansson J, Eriksson EE, Hedqvist P, Ruoslahti E, Lindbom L: **Integrin alpha(2)beta(1) (VLA-2) is a principal receptor used by neutrophils for locomotion in extravascular tissue.** *Blood* 2000, **95**:1804-1809.
38. Miura S, Tsuzuki Y, Fukumura D, Serizawa H, Suematsu M, Kurose I, Imaeda H, Kimura H, Nagata H, Tsuchiya M, *et al.*: **Intravital demonstration of sequential migration process of lymphocyte subpopulations in rat Peyer's patches.** *Gastroenterology* 1995, **109**:1113-1123.
39. Grayson MH, Chaplin DD, Karl IE, Hotchkiss RS: **Confocal fluorescent intravital microscopy of the murine spleen.** *J Immunol Methods* 2001, **256**:55-63.
40. Bousso P, Bhakta NR, Lewis RS, Robey E: **Dynamics of thymocyte–stromal cell interactions visualized by two-photon microscopy.** *Science* 2002, **296**:1876-1880.

41. Miller MJ, Wei SH, Parker I, Cahalan MD: **Two-photon imaging of lymphocyte motility and antigen response in intact lymph node.** *Science* 2002, **296**:1869-1873.
42. Stoll S, Delon J, Brotz TM, Germain RN: **Dynamic imaging of T cell–dendritic cell interactions in lymph nodes.** *Science* 2002, **296**:1873-1876.
43. Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, Dustin ML: **The immunological synapse: a molecular machine controlling T cell activation.** *Science* 1999, **285**:221-227.
44. Denk W, Strickler JH, Webb WW: **Two-photon laser scanning fluorescence microscopy.** *Science* 1990, **248**:73-76.
45. Xu C, Zipfel W, Shear JB, Williams RM, Webb WW: **Multiphoton fluorescence excitation: new spectral windows for biological nonlinear microscopy.** *Proc Natl Acad Sci USA* 1996, **93**: 10763-10768.
46. Miller MJ, Wei SH, Cahalan MD, Parker I: **Autonomous T cell trafficking examined in vivo with intravital two-photon microscopy.** *Proc Natl Acad Sci USA* 2003, **100**:2604-2609.

## Correspondence

Michael L Dustin, Program in Molecular Pathogenesis, Skirball Institute of Biomolecular Medicine and Department of Pathology, New York University School of Medicine, 540 First Avenue, New York, NY 10016, USA. Tel: +1 212 263 3207; fax: +1 212 263 5711; e-mail: dustin@saturn.med.nyu.edu