Dynamic imaging of the immune system: progress, pitfalls and promise

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Abstract | Both innate and adaptive immunity are dependent on the migratory capacity of myeloid and lymphoid cells. Effector cells of the innate immune system rapidly enter infected tissues, whereas sentinel dendritic cells in these sites mobilize and transit to lymph nodes. In these and other secondary lymphoid tissues, interactions among various cell types promote adaptive humoral and cell-mediated immune responses. Recent advances in light microscopy have allowed direct visualization of these events in living animals and tissue explants, which allows a new appreciation of the dynamics of immune-cell behaviour. In this article, we review the basic techniques and the tools used for *in situ* imaging, as well as the limitations and potential artefacts of these methods.

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A fundamental characteristic of the vertebrate immune system is the motility and migratory behaviour of its haematopoietic components[1–4]. Haematopoietic cells leave the bone marrow and circulate in the blood. Some cells then enter and traffic through secondary lymphoid tissues or the thymus, re-enter the blood and either return to secondary lymphoid sites or move into tissues and organs throughout the body. The activities of the many different immune cells are often interdependent. For example, cells of the innate and adaptive immune systems communicate extensively[5–7], each guiding the differentiation of the other, to produce the proper quality and magnitude of response to a given infectious agent[8–10]. This requires that these cells colocalize in space and time, and, in most cases, engage in direct cell–cell contact.

Support for this description of the immune system comes from more than a century of analyses, almost all of which involved drawing conclusions about cell dynamics from static methods. For example, conclusions have been drawn from snapshots of *in vivo* responses taken at what were relatively long intervals of time relative to the rate at which the underlying events actually transpired or from *in vitro* cultures of dissociated cells (see REFS 4,11 for in-depth reviews, including citations of the extensive original literature). Despite advances in staining reagents[12,13], flow cytometers[14] and microscopy tools[15–18], dynamic studies of immune-cell behaviour were, until recently, limited to the following: *in vitro* imaging of cultured cells, which was used to clarify the molecular reorganization that occurs at cell–cell contacts[19–21]; and single-colour analysis of leukocyte behaviour in small vessels[22], which helped to confirm, *in situ*, the rolling, stopping and transmigration model of cell extravasation from the blood to the tissues[23].

The recent application of advanced light microscopy tools has revolutionized our ability to probe more directly how immune cells conduct their business *in vivo* (or at least something approaching their natural environment — see later). Confocal, and two-photon microscopy instruments in particular (BOX 1; TABLE 1), have been combined with new surgical methods and techniques for the fluorescent labelling of cells to allow dynamic imaging of immune cells in many tissues[24–53] (TABLE 2). Other methods such as positron emission tomography (PET), magnetic resonance imaging (MRI) and luminescence imaging also allow tracking of immune cells, but with very different spatial and time resolutions (see REFS 54,55 for a detailed comparison of these methods with microscopy approaches).

The data generated by these studies have important implications for our understanding of how, where and when cells interact during the initiation of an immune response and during the delivery of effector function at peripheral sites. A striking early observation was the unexpectedly rapid pace of lymphocyte migration in secondary lymphoid tissues[25], a finding that had important consequences for our models of how rare antigen-specific cells and antigen-presenting cells find one another to produce an effective response. *In situ* evidence has emerged showing that dynamic networks of relatively sessile dendritic cells (DCs) with highly motile processes have a central role in the ability of

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Box 1 | Technical details of two-photon imaging

The main advantage of multi-photon microscopy for immunologists is that it gives them the ability to image deep into live tissues[3,4,11,39,43,58,60–67], a capacity that depends greatly on the choice of laser system, the scanhead and the many optical components.

Lasers

- Computer-tuned Ti:Sapphire system with integrated solid-state pump laser producing output ranging from ~700–1080 nm.
- 600–800 mW of average power at 780nm; preferably 1.5 W of peak power to allow useful output at long or short wavelengths, such as those used for enhanced yellow fluorescent protein (915 nm) or indo-1 (705 nm).
- Integrated into a system that limits pulse broadening and peak power loss owing to dispersive elements such as glass lenses. 'Prechirping' the laser using adjustable diffraction gratings or prisms that retard the 'red' components of the beam relative to the 'blue' components[82,83] can offset such dispersion.

Detectors

- Usually a photomultiplier tube used without a pinhole for two-photon imaging because fluorescence radiates from a diffraction-limited focal volume and even scattered photons can contribute to a useful signal.
- Ideal detector placement is as close as possible to the back aperture of the objective; common in custom-built systems[84], but generally impractical on commercial systems that double as confocal microscopes.

Acquisition speed

- Laser scanners operating in either a raster or resonate mode[17,84,85].
- Linear raster scanning is precise but only able to generate images at a few frames per second.
- Resonant scanning is high-speed, generating 30 or more frames per second, thereby allowing real-time specimen examination and frame averaging to reduce noise.

Objectives

- Should have high infrared transmission and a large numerical aperture (NA). High NA water-dipping objectives (NA = 0.8–0.95) are preferred because they do not require the use of a cover glass, typically have long working distances and enhance fluorescence signal collection owing to their wide field of view[86].
- Need to optimize the diameter of the laser beam entering the back aperture of the lens. A good compromise for maintaining both laser power and high spatial resolution is to slightly underfill (~70%) the back aperture[87].

these cells to scan through the lymphocyte repertoire at a high rate[30,31]. The paths taken by naive lymphocytes within lymph nodes have also been analysed[25,27–30], provoking an interesting debate on the relative roles of random and guided interactions in supporting immune responses[39,56–59]. When a migrating T cell finds an antigen-laden DC, markedly different durations of cellular interactions have been reported to characterize distinct phases of the early adaptive immune responses in lymph nodes[24,26,28,29,32,34].

The results and biological implications of many of these imaging studies have been extensively reviewed[3,4,11,39,43,58,60–67] and we refer the reader to these publications for more information on these issues. In this Review, our focus is the methodology underlying this new spate of imaging studies, in particular, we discuss the following: the tools and techniques involved; the potential artefacts that occur in such experiments and how these can be recognized and avoided; the resources for analysing the data generated; and what a reader should look for when perusing papers in this area. Our intention is to make some of the 'behind the scenes' aspects of such imaging studies more accessible to those who read about them in the literature or hear talks in which such data are presented, with the goal of enhancing the critical analysis of the results. It is also hoped that this Review will provide a useful guide to the technology for those thinking about whether to incorporate such imaging experiments into their own research.

Confocal microscopy
A form of fluorescence microscopy in which out-of-focus signals are rejected by an aperture that restricts all light from reaching the detector except that originating from the focal plane of the excitation spot.

Two-photon microscopy
A fluorescence-imaging technique that takes advantage of the fact that fluorescent molecules can absorb two photons nearly simultaneously during excitation before they emit light. This technique allows all emitted photons to contribute to a useful image.

Fluorescent labelling

All the imaging methods discussed in this article rely on the use of fluorescence. This means that to visualize a cell of interest, it must be labelled using appropriate dyes or by expression of fluorescent proteins[68]. The labelling can either be done *in vitro* using isolated cells, which are then adoptively transferred into a host animal, by the introduction of targeted fluorochromes directly into the animal (for example, through the injection of labelled antibodies[69]) or by genetic means. Each method has its advantages and disadvantages and in many cases, a combination of techniques is the best solution.

Dyes. The earliest studies of *in situ* lymphocyte and DC behaviour used cells that were labelled *in vitro* with fluorescent dyes such as CFSE (5,6-carboxyfluorescein diacetate succinimidyl ester), SNARF (seminaphthorhodafluor) and CMTMR (chloromethylbenzoyl aminotetramethylrhodamine)[24–27,29,46]. This approach is technically straightforward, it allows highly purified populations of cells to be selected for labelling, and it results in dilution of the labelled cells among non-fluorescent cells so that their detection and tracking is easier than if all cells of that differentiation state were homogeneously labelled. *Ex vivo* labelling also makes it easy for the investigator to change the dyes used and to switch dyes among cell populations as a control for the affects of a particular compound on cellular function.

However, labelling with dyes also has some severe disadvantages. The most obvious is that the cells to be labelled are extracted from their normal environment, manipulated in culture and transferred into a new host. For lymphocytes, only 10–15% of the transferred cells can be recovered from lymphoid tissues and this raises questions about whether the imaged cells are representative of the starting population or are a specific subset with unique properties. Furthermore, because the dyes leak out of the cells over time and dilute with cell division[25], one cannot follow labelled lymphocytes after a few days of antigenic stimulation and it is not possible to follow cells into peripheral sites of effector function, unless already differentiated effector cells are isolated, labelled and adoptively transferred. In the case of DCs, such manipulation invariably induces some form of differentiation and/or maturation, making it nearly impossible to assess how such cells operate under true steady-state conditions. Furthermore, many of the dyes can be toxic at high concentrations and some affect membrane properties that have a role in cell mobility. These compounds are also subject to rapid bleaching and can generate toxic products on illumination.

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Positron emission tomography
An imaging method that depends on the three-dimensional detection of (positrons) radiation from a probe that is typically localized to a cell by direct *ex vivo* labelling or *in situ* metabolic conversion of a precursor compound.

Magnetic resonance imaging
A method that uses detection of changes in the alignment of protons in a strong magnetic field when they are perturbed by radio wave pulses to generate structural information about an object in that magnetic field.

Table 1 | **The advantages and limitations of single-photon and two-photon imaging**

	Advantages	Limitations
Single-photon imaging	• Shorter wavelength and higher resolution • Less expensive and easier to maintain lasers • Better performance in some tissues (such as the skin and liver)	• Limited depth of penetration in scattering tissues • Bleaching in all planes • Phototoxicity • Chromo- and fluorophore-based phototoxicity
Two-photon imaging	• Greater penetration • Longer wavelength, confined excitation and all emission detected • No bleaching of out-of-focus planes	• Longer wavelength and lower resolution • Nonlinear phototoxicity, linear heating and adsorption in dark tissues • Reflections in some tissues • Chromo- and fluorophore-based phototoxicity

Fluorescent proteins. Expression of fluorescent proteins by target cells is the other main method for making cells visible for imaging. Expression can either be obtained by DNA transfection or viral transduction of isolated cells (with the same isolation and manipulation-related problems that accompany *in vitro* labelling with dyes) with constructs encoding the fluorescent protein or, alternatively, by genetic manipulation of animals[31,49] or haematopoietic stem cells (HSCs)[24]. Using knock-in technology, mice can be generated that express a fluorescent protein in a specific cell lineage (for example, mice have been engineered to express green fluorescent protein (GFP) in place of CXC-chemokine receptor 6 (CXCR6)[38,70]). Animals with selective tissue expression of a fluorescent protein can also be made by transgenic procedures (for example, the *Cd11c* promoter has been used to drive the selective expression of a fluorescent protein in DCs[31]). However, most investigators are turning to bacterial artificial chromosome (BAC) technology for making transgenic animals because this can achieve physiological expression (in terms of protein amount, timing and tissue restriction) without the time and expense of generating knock-in animals[71]. Alternatively, high level expression of the fluorescent protein can be obtained in nearly all cells through the use of promoters such as those regulating the genes encoding β-actin and ubiquitin; in this case, the desired fluorescently labelled cell population is isolated and transferred. Last, labelled cells can be generated by retroviral or lentiviral transduction of mobilized HSCs[72], using either standard vectors for pan-expression or self-inactivating (SIN) vectors with a lineage-specific promoter, and these modified cells are then transferred into lethally irradiated recipient mice to create chimeric animals. Several weeks after transfer of the transduced HSCs, a fraction of the cells of interest in the chimeric mice will express the fluorescent protein and these can be imaged *in situ* or after adoptive transfer.

As with dye labelling, there are advantages and disadvantages to the use of fluorescent proteins. It is beneficial to have cells that do not dilute their label, because this prolongs the length of time cells can be tracked after the initiation of clonal expansion. Cells are labelled *in situ* and for certain experiments do not need to be manipulated *ex vivo* before being studied, so eliminating a major source of potential artefact. However, it is much more work to create genetically modified mice than to directly label cells *in vitro*. Even after creating a new mouse strain, it is usually necessary to conduct extensive breeding programmes to move the gene encoding the fluorescent protein onto the proper genetic background (for example, so that the mice co-express the fluorescent protein and a specific T-cell receptor (TCR)). It is also expensive and time consuming to make multiple independent lines of mice each expressing a fluorescent protein of a different colour. However, generating several lines of mice that express various fluorescent protein tags will be necessary if more than one cell or molecule is to be imaged at a time. Furthermore, the most commonly used fluorescent proteins have overlapping emission spectra[73]. One must therefore sacrifice a substantial amount of signal intensity

Table 2 | **Tissues that have been imaged**

Tissue	Conditions	Comments
Lymph nodes	Explant and intravital	• Intravital imaging of inguinal and popliteal lymph nodes • Any lymph nodes as an explant, including pancreatic • Oxygen level and perfusion important if explant is submerged
Thymus	Explant	• Intravital imaging not yet possible
Liver	Intravital one-photon (confocal) imaging	• Easily damaged by surgery • Use propidium iodide to detect dead cells
Central nervous system	Explant and intravital	• Spinal cord immobilized by stereotactic system
Bone marrow	Intravital	• Two-photon imaging through parietal bone
Skin	Explant and intravital	• One-photon imaging is better than two-photon imaging for Langerhans cells
Spleen	Explant and intravital	• Both one-photon and two-photon imaging is useful • Red pulp contains many T cells
Gut	Explant, intravital and using fibre-optic microscopy	• Low autofluorescence • Transepithelial processes of dendritic cells
Eye	One-photon imaging using fibre-optic microscopy	• Cornea functions as a transparent window so no surgery is required for imaging • Some motion artefacts
Kidney	Intravital one-photon and two-photon imaging	• Limited view of distal collecting system

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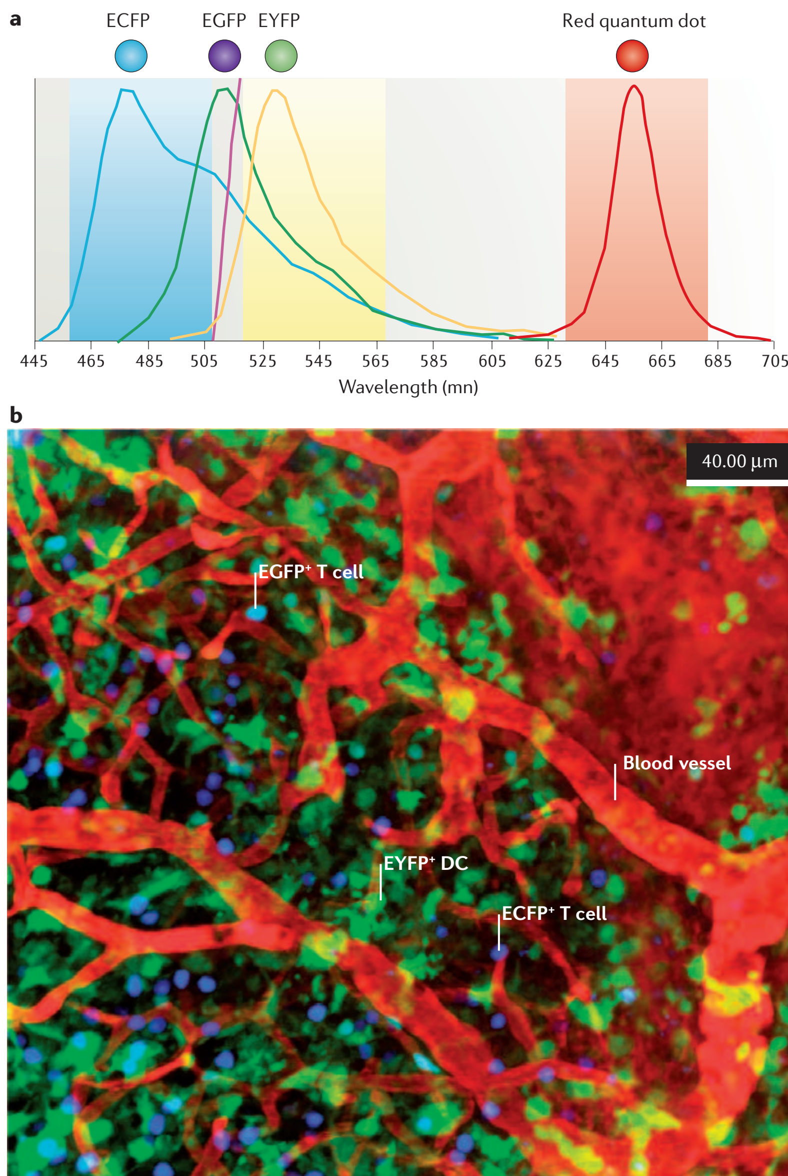

Figure 1 | **Detection of four fluorescent signals with three photomultiplier tubes.** **a** | The emission spectra of enhanced cyan fluorescent protein (ECFP), enhanced green fluorescent protein (EGFP), enhanced yellow fluorescent protein (EYFP) and red quantum dots, and the band-pass limits of the filters used for emitted light collection (grey). **b** | To generate this image, a typical CFP–YFP filter set was used together with excitation of the sample with 910 nm light from a mode locked Ti:Sapphire laser. The EGFP signal is split between the CFP and YFP channels with some loss owing to the dichroic mirror. In this image of a lymph node, ECFP (T-cell 1) is shown as cyan, EGFP (T-cell 2) is shown as blue, EYFP (dendritic cell (DC)) is shown as green and red quantum dots (injected in the blood) are shown as red.

to ensure that there is no cross-channel contamination and, because one is frequently working at the detection limit to begin with, this severely compromises the depth of imaging and the detection of subpopulations of cells that are only dimly fluorescent. Nonetheless, with high expression levels of individual fluorescent proteins in the cytoplasm it is possible to discriminate between cyan fluorescent protein (CFP), GFP and yellow fluorescent protein (YFP) using the appropriate filter sets and emission ratio analysis[34] (FIG. 1).

Even when the emission spectra are sufficiently separated[74], one is faced with the problem of very different excitation optima for commonly used fluorescent proteins. For experiments in which limited imaging depth is sufficient and confocal microscopes can be used, one can use multiple lasers to excite the different fluorescent proteins in a nearly optimal manner. However, most current two-photon instruments have only a single laser that cannot be tuned rapidly enough to conduct dynamic imaging studies at multiple wavelengths (BOXES 1,2). Under such circumstances, it is necessary to sacrifice sensitivity to obtain a balanced output from the multiple fluorescent proteins by choosing a compromise wavelength that is suboptimal for the different fluorochromes.

The limitations of using a single excitation wavelength also pose a major problem when trying to combine dye labels with fluorescent proteins in a single experiment, because most of the commonly used dyes excite optimally at a lower wavelength than fluorescent proteins. In this regard, it is better to use quantum dots in combination with fluorescent proteins, but these labels have their own limitations (discussed later). Finally, some of the brightest fluorescent proteins are oligomers that can be toxic, although newer fluorescent proteins are constantly being developed[73,74]. As more variants with distinct emission spectra become available, judicious choices should allow three, or even four, well-separated colours to be used for cell and molecular tracking experiments, with relatively little loss of signal because of filtering and suboptimal choice of wavelength.

Quantum dots. Quantum dots[75] are a recently introduced alternative to dyes and fluorescent proteins. These silicon-based materials have properties that make them potentially extremely useful for imaging using two-photon instruments. In contrast to dyes and fluorescent proteins, a quantum dot of a given diameter has a relatively narrow emission wavelength, making it easier to distinguish among different sizes (the colour detected depends on the size) of quantum dots without severely reducing overall signal strength. In addition, nearly all quantum dots can be efficiently excited at a single excitation wavelength in the range typically provided by a two-photon laser. Finally, quantum dots are extremely photostable and seem to be non-toxic.

Although these properties are all advantages, quantum dots have a severe disadvantage that limits their use in the labelling of immune cells for dynamic imaging. It remains difficult to get quantum dots into the cytoplasm of cells without also decorating the cell surface and filling the endocytic pathway. This problem allows quantum dots to be released from labelled cells, creating a background fluorescence after cell transfer that can limit data interpretation. Antibody-directed labelling with quantum dots does not solve this problem and so, although these reagents are useful for multiplex staining experiments with fixed cells, improvements in delivery are needed to make them useful for *in situ* live-cell work. At present, quantum dots are best used as

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Luminescence imaging
A technique that uses photons emitted by the process of luminescence, rather than fluorescence, to obtain an image of cells in a living animal. This method is extremely sensitive and non-invasive but generates data of much lower resolution than microscope-based fluorescent imaging.

Knock-in technology
The introduction of a transgene into a precise location in the genome, rather than a random integration site. Knocking-in uses the same technique of homologous recombination as a knockout strategy but the targeting vector is designed to allow expression of the introduced transgene under control of the regulatory elements of the targeted gene.

BAC transgenic technology
A method for creating genetically altered mice in which very large segments of mouse genomic DNA are propagated in bacteria and used to achieve physiological patterns of gene expression. This technique avoids the need to create knock-in mice by homologous recombination in embryonic stem cells.

SIN vectors
Retroviral or lentiviral vectors that contain mutations that inactivate the enhancer element in the 3′ LTR (long terminal repeat). Because the sequence of the 3′ LTR is used to reconstitute the 5′ LTR during reverse transcription, these vectors 'self-inactivate' the 5′ LTR enhancer before integration into the host-cell DNA. This allows exogenous gene regulatory sequences downstream of the 5′ LTR to control gene expression after integration.

Emission spectrum
A quantitative representation of the wavelengths (energies) of the photons emitted from a fluorescent compound after it is excited by shorter wavelength (more energetic) photons from an illumination source.

Excitation optimum
The wavelength of incident light that is best absorbed by and causes maximal emission from a fluorescent compound.

Box 2 | **Technologies for the future**

More colours

- Use multibeam devices, such as a prototype dual-beam, four-detector multi-photon microscope that rapidly alternates between two laser lines, to allow up to eight fluorescent probes to be imaged with only a small loss in temporal resolution.

Going deeper

- Cut the tissue to expose deeper regions, for example, using vibratome thick sectioning as applied to the thymus to look at Ca^{2+} signalling[67] and to lymph nodes to examine T-cell movement (L. Koo, personal communication). The main problem is tissue damage.
- Amplify laser output. Regenerative amplifiers allow excitation 1 mm into a tissue[88]. Drawbacks include a decrease in effective scan rate and increased thermal damage.
- Increase detector sensitivity. Gallium arsenide phosphide detectors promise a 2–4 fold increase in sensitivity, possibly allowing visualization of low abundance fluorescent protein chimaeras such as those used to study protein re-organization in the immunological synapse[89].
- Use needle-like gradient index lenses[90], often in combination with fibre-optic microscopy[91–94].
- Harmonic generation microscopy[95] can be used with less tissue damage, allowing more laser power to be used.

Imaging faint molecular events

- Tissue autofluorescence (such as that from flavoproteins and aromatic co-enzymes[96,97]) limits detection of faint signals by decreasing the signal–noise ratio.
- This autofluorescence problem can be overcome by exploiting the lifetime of the excited state. For example, quantum dots have a longer lifetime than organic dyes, with most emission taking place after autofluorescence emission but before phosphorescence emission[98]. A two-photon microscope able to perform fluorescence lifetime imaging over the nanosecond to microsecond timescale could distinguish these events and also perform *in situ* oxygen measurements[99].

Breaking the resolution limit

- Resolving power is based on the wavelength of the illumination beam and is ~200 nm for blue light and proportionally poorer for the infrared light used in two-photon microscopy.
- Frequency domain information from structured saturating illumination generated by intersecting laser beams can extend the limit to less than 10 nm for light microscopy[100], but the time resolution of this approach is limited and it results in greatly increased photobleaching[101]. It is also currently unable to provide three-dimensional information.
- Multi-photon Raman spectroscopy[102] might extend imaging to the molecular level.

Breaking the speed limit

- Spinning disc confocal systems offer high speed imaging at more than 100 frames per second, but they typically operate in single-photon mode with limited imaging depth.
- A reflector-based system provides multiple beamlets compatible with two-photon excitation, increasing imaging speed beyond that of resonant scanners[103]. However, this technology requires use of a camera with images that are degraded by the scattering of emitted photons, limiting the effective depth of imaging.

alternatives to fluorescent dextrans as labels for blood and lymph, situations in which their extreme brightness, narrow emission spectra and lack of bleaching are all very desirable properties.

Tissue access and physiology

Having determined how to get the cells of interest labelled, it is then necessary to choose a method for imaging the tissue sites in which these cells reside or to which they traffic. Two basic approaches have been developed, one using explants[24–26] and the other using direct intravital imaging of live animals[27,29]. Each approach has its advantages and limitations (TABLE 3).

Explants. Explants (which have been used mostly for imaging lymph nodes) are much easier to study and require much less surgical skill to get under the microscope lens. An organ or tissue sample is removed from an animal and is, typically, embedded in agarose[24] or glued to a coverslip within a heated, perfused imaging chamber[25]. The explanted tissue can be oriented in whatever manner the investigator desires. By contrast, analysis of surgically exposed lymph nodes is constrained by which portion of the organ can be made accessible to the objective without compromising blood and lymph flow. The freedom to image a lymph node from any side when using explants is especially important for visualizing regions near the hilum, which is the region of a lymph node through which blood vessels enter and through which efferent lymphatics exit[36]. The hilum is often too deep for imaging from the lymph-node surface that is accessible in a live animal. However, using delicate surgery, it is possible to perform intravital imaging of the hilum of the inguinal lymph node, but not the more commonly studied popliteal lymph node.

By contrast, it is difficult to know what changes to physiological behaviour accompany culture of an isolated lymph node, and although tracking of T-cell movement has so far not shown gross differences between well-maintained explants and intravital studies[3,11,58,62,64], this

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Table 3 | **The advantages and limitations of explant and intravital imaging**

	Advantages	Limitations
Explants	• Higher throughput • Relatively free of movement artefacts • Defined environment • Access to different surfaces of tissue • Pharmacological studies • Acute cell addition • Imaging human biopsies possible	• Vascular and lymphatics present but no flow • Lack neural innervation • Processes stop during death and are restarted by oxygenation and/or perfusion
Intravital imaging	• True *in vivo* observations • Physiological oxygen levels and metabolism • Vascular and lymphatics intact • Neural innervation	• Lower throughput • Motion artefacts from breathing and blood flow • Anesthaesia effects • Surgical trauma • Access of inflammatory cells might cause progressive damage

is only one parameter among many that reflect lymphocyte function. The lack of blood and lymph flow and the severing of nerve connections might drastically alter lymphocyte and/or DC function within the explanted organ. In addition, explants prevent one from studying the entry of lymphocytes across high endothelial venules (HEVs) and the effect of blood or lymph flow on organ function.

Intravital methods. Surgical exposure and immobilization are technically complex aspects of intravital imaging. During surgery, care must be taken not to damage blood vessels, lymphatics and nerves. Regions of the animal close to the heart and lungs are more difficult to image because of body motions that are hard to control even in anaesthetized animals. In more peripheral sites, small motions resulting from involuntary muscle contractions, as well as transmitted motions from the thoracic cavity can still degrade images. Each laboratory has worked out methods to limit this problem for the tissue they are studying, such as the use of veterinary glue, surgical pins or custom-made restraints.

The objective lenses used for these imaging studies are large relative to the mice that are being studied. This requires preparing the target tissue so that the objective, which is most often a water-dipping lens, can approach to within a millimetre of the tissue being imaged. For some tissues, such as the liver, the combination of the need for immobilization and the need for objective access are most easily met by approaching the organ from below with an inverted instrument[38,39]. In other cases, such as for lymph nodes, upright microscopes are more convenient[27,29], although inverted devices can also be used.

Other considerations. Other key aspects of specimen preparation and maintenance involve the related issues of anaesthesia, perfusion, oxygenation, pH and temperature control. Studies in sheep have indicated that various anaesthetics have adverse effects on lymphocyte migration[76] and unpublished data confirm that this as a potential problem in small animals, at least for some common injectable anaesthetics (A. Y. C. Huang, personal communication). Consistent results have nevertheless been obtained using three injectable anaesthetic mixtures: ketamine, xylazine and acepromazine[34]; ketamine and xylazine[29]; and tribromoethanol[27]. The appropriate injectable anaesthetic has to be determined for each mouse strain. For example, B10.BR mice do not tolerate the mixture of ketamine, xylazine and acepromazine but do tolerate tribromoethanol (G. Shakhar, personal communication). Although it is more expensive and difficult to implement, gas (isofluorane) anaesthesia provides another alternative. Gas anaesthesia does have the advantage of being more easily maintained over long time intervals and of limiting the need to stop data collection early because of premature emergence of an animal from its immobile state. Gas anaesthesia is typically delivered in 100% oxygen (O_2) through a nose cone, and a miniaturized device that measures pulse and blood O_2 concentration is used to help assess the animal's physiological state under anaesthesia.

Perfusion, oxygenation, pH and temperature are issues that affect both intravital and explant methods. In the case of live animals, dehydration can set in rapidly in heated imaging chambers unless the animal is given infusions of physiologically appropriate solutions. Loss of fluid volume can seriously affect blood and lymph flow. In the case of lymph-node explants, results have differed between laboratories with respect to the effects of medium exchange and gas balance on lymphocyte behaviour. In some cases, anything less than rapid, continuous perfusion of the tissue with medium equilibrated with 95% O_2 limits lymphocyte movement within the explant[3,29,48,58,61,62,65–67]. In other cases, an 8-fold lower O_2 tension (PO_2) in the culture medium is adequate but movement of the perfusate must be maintained (J. Huang, personal communication). In yet other hands, neither perfusion nor oxygenation beyond that available from room air is necessary[24,39,64], however, in this case the lymph node is maintained at an air–liquid interface, whereas in the other studies, the organ is immersed in medium. At present, the most common condition used requires a perfusate equilibrated with a high concentration of O_2 and 5% carbon dioxide to maintain the proper pH of the medium. Whether the supraphysiological concentrations of O_2 used in many explant studies disturb normal lymphocyte behaviour remains an unresolved issue in the field. The principal argument against artefacts is the comparable rate and pattern of T- and B-cell migration in explants exposed to high O_2 concentrations and in lymph nodes imaged using intravital methods.

Temperature control is another crucial parameter for both explants and intravital studies. Small (1–2°C) changes in the temperature of the tissue being imaged have marked affects on the migratory properties of lymphocytes. Combinations of heating blankets, stage and objective warmers, custom-made circulating loop heaters and thermal blocks, along with micro-temperature probes and rectal thermometers, have been used to both achieve stable temperature of the specimen and allow continuous monitoring during an experiment.

Quantum dot
A nanocrystalline semiconductor of extremely small size (10–50 nm) that results in its absorption of incident photons, followed by the emission of photons at a slightly longer wavelength. Because of a phenomenon called the quantum confinement effect, the colour (wavelength) of the emitted light is determined by the size of the nanocrystal.

Water-dipping lens
An objective lens for a microscope that is optimized for use with its front surface in contact with an aqueous solution, because there is an improved match in refractive index between the glass and buffer solution that limits spherical aberration in the image.

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Four-dimensional data collection

The underlying physics of one- versus two-photon imaging, as well as the differences in basic instrument design and emitted photon capture, have been well covered in recent reviews[61,77] and are not repeated here. Both methods involve the capture of x–y planar data from fluorescent targets in thick tissue specimens. Some information about the stromal surroundings of labelled haematopoietic cells can be obtained using a combination of fluorescent molecules, which are injected intravenously to highlight the vasculature (especially HEVs in lymph nodes), and intrinsic fluorescence, which is seen as second harmonic emission from organized tissue elements such as collagen when using two-photon illumination[4,78].

These planar data are extended to volume information by changing the focus of the microscope and sequentially capturing successive z-plane images. After collecting three-dimensional data in this manner, the imaging process is repeated to produce a four-dimensional dataset (three-dimensional volume plus time). This introduces two of the key technical issues in such work, namely the maximum depth in a tissue at which useful images can be obtained and the speed with which each set of z images (called a stack) can be collected. As discussed extensively elsewhere[4,61], one-photon methods are more limited than two-photon methods in the depth to which they can image (TABLE 1), because of scattering of the shorter wavelength laser light used in one-photon instruments. One-photon (confocal) approaches can typically obtain data to a depth of only 80 μm from the exposed tissue surface, whereas two-photon methods can extend to depths of 200–300 μm or more in lymphoid tissues.

As to the speed at which z stacks can be collected, this is a complex product of the rate of x–y scanning, the rate of z-plane movement by the microscope, the thickness of each z image and the number of images collected before returning to the original focal plane. Useful x–y scanning speed is determined by the mechanical properties of the instrument, the potential fluorescent intensity of the target and the level of laser illumination needed to produce an adequate emission output. As the rate of image collection slows, one must either sacrifice the overall depth imaged to allow each volume to be collected in a short enough time so that the cells being imaged do not move more than their diameter before the next cycle (if this condition is not met, one cannot with assurance follow the tracks of individual cells over time) or one must increase the thickness of each z image, sacrificing axial resolution.

The physical design of the microscope has a major influence on the imaging parameters just discussed. Different microscopes have widely varying maximal physical rates of laser line scanning, with instruments that use resonant scanners operating much faster in this regard than those using other types of galvanometers to control beam position (often called line or raster scan instruments). The increased speed of resonant scanners makes it possible to observe phenomena that occur on a timescale that is too short for line scanners to resolve, such as cells flowing in the circulation[27] and the rapid movement of membrane processes. For example, the dendrites of DCs can be very thin (1–2 μm in diameter) and move at more than 60 μm per minute. Using a raster scan at a rate of 2 frames per second, these dendrites would be poorly resolved and recorded as only a faint smear in the image, but these cellular features would be resolved using a resonant scan at a rate of 30 frames per second. By contrast, the short laser dwell time associated with resonant scanning usually means that several x–y frames must be averaged to provide adequate image contrast. This requirement reduces the speed gain these resonant scanning instruments have in completing an x–y collection compared with the other devices. Nevertheless, despite the requirement for averaging, resonant scanning instruments usually acquire data more rapidly than raster scan systems and have the added advantage that the raw scan data can be displayed in real time. Although the images from the real-time scan might not be of sufficient quality for data analysis, they are generally adequate for surveying the tissue for areas suitable for higher quality image collection and allow this to be done much more easily than do raster-based systems. Raster-scan systems, on the other hand, offer much more flexibility in terms of region of interest scanning and are not prone to image distortion artefacts that arise because of variability in resonant scan speed.

How deep into the tissue one can image also depends on the following: the wavelength of the laser; the number of fluorochromes per unit volume of the cells and structures to be imaged; the balance between increased laser power eliciting more emission from a given target versus causing photodamage or bleaching; the overall sample heating caused by the infrared nature of two-photon laser beams; how fast the instrument can change z focus; and the acceptable thickness of each z slice. These limitations are most pronounced in the case of fluorescent chimeric proteins that have been extremely useful for single cell *in vitro* imaging of molecular dynamics at the immunological synapse. When the chimeric protein is expressed at a similar level to that of the endogenous protein for which it is a marker, the local fluorophore density is so low that prolonged beam dwell times and/or high power are necessary to generate sufficient photon emission to get a signal above background. This results in excessive tissue heating above the plane of focus, as well as photodamage to the cell being illuminated and these problems have so far limited the use of such fluorescent chimeric proteins for *in situ* imaging studies.

Assessing sample physiology

One theme running through the preceding sections is the biological equivalent of Heisenberg's Uncertainty Principle — it is impossible to observe the immune system in detail without disturbing it[3]. Although, there are methods that are close to this goal, such as luminescence imaging[79], even in this case there is the unknown affect of expressing luciferase in cells, the issue of immunity to a foreign protein, the possible affect of the substrate on cell metabolism and the effect of anaesthesia on the animals. In addition, current whole-animal imaging techniques do not provide the spatial resolution necessary to

Second harmonic emission
The non-radiative production of frequency-doubled polarized light emission from a highly ordered (anisotropic) material on illumination by a laser beam. In practical terms, the production of polarized light emission from extracellular matrix materials such as collagen when subjected to two-photon illumination in the absence of fluorochrome labelling.

Galvanometers
A device that uses electric currents to control the mechanical displacement of an object such as a scan mirror that in turn directs a laser beam.

Heisenberg's Uncertainty Principle
The concept that measurement of the properties of an object, in particular momentum and position, cannot be accomplished with complete accuracy. Sometimes used (with some license) to encompass the 'observer effect', which indicates that the mere attempt to measure such properties changes them from their intrinsic state.

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visualize individual cells and their behaviour, much less molecular events such as protein redistribution.

With respect to one-photon and two-photon imaging, the concerns about introducing artefacts into the system are greater. Clearly physiology is disturbed using explant methods. Intravital techniques require surgery, which inevitably is accompanied by inflammation and some degree of fluid leakage. Temperature control is a potential problem, as is compression of vessels by the materials used to achieve immobilization. In all cases, the laser illumination can cause excess heating of the sample or cell damage owing to the generation of oxygen radicals and other forms of phototoxicity. Many of the deleterious effects are cumulative, limiting the time in which reliable observations can be made. In some cases, the effects are nonlinear, with a sharp threshold between physiological behaviour and cessation of the cell function of interest, for example, migration.

Various approaches have been suggested as ways of monitoring the state of a tissue sample being imaged. For intravital studies, minimal checks include blood flow, lymph flow, the mobility of DC dendrites, and the motility of naive lymphocytes in the B-cell and/or T-cell zones of the lymph node. For explants, the mobility of DC dendrites, and the motility of naive lymphocytes in the B-cell and/or T-cell zones of the lymph node are still available as controls. A further benefit of imaging blood and lymph flow, as well as stromal cells, is that it provides important anatomical context, in addition to allowing assessment of viability. In some tissues, endogenous fluorescent signals can also be used to assess physiological state. For example, in the liver there is autofluorescence that is excited by a 488 nm laser line and that seems to provide real-time feedback on tissue health in a manner directly relevant to lymphocyte motility[38].

Unfortunately, incorporating these controls into all experiments poses big problems. Even if probes of the same colour were used for lymph and blood flow, this would make one colour unavailable for the experimental part of the study. Including irrelevant lymphocytes so that their motility can be assessed would remove another colour, as would the analysis of DC migration or dendrite activity. Therefore, to use all three of these tests routinely, a minimal experiment would have four colours (three control and one experimental), which is at the current limit of multiplex capacity for these instruments. Nevertheless, inclusion of such controls is viewed as essential given the complexity of the preparations and imaging requirements.

Beyond these checks on basal physiology, there is also the issue of determining whether differences in cell behaviour noted in distinct imaging data sets arise from the planned experimental manipulation or from uncontrolled differences in the precise region of the tissue studied in each sample. The volumes imaged by these methods are small. It is therefore impossible to ensure that functionally identical regions of two lymph nodes are imaged in successive experiments. If cell behaviour varies with its location within a tissue, for which there is good evidence, then this could confound data analysis[64]. For this reason, the development of new labelling methods to highlight specific structures and microdomains within tissues is an urgent priority. Currently, one way around this limitation is to include both experimental and control cells and/or conditions in one imaging volume. This is a powerful method, as it can be striking, for example, to see that two adjacent DCs have completely different modes of interaction with the surrounding lymphocytes. The downside is that it requires the capacity to image multiple colours and to distinguish them from each other in a single field, as well as a contemporaneously collected data set. Furthermore, it increases the difficulty of incorporating the controls mentioned earlier for assessing the physiology of the organ, because one quickly runs out of available detectors.

Processing and presenting the data

The four-dimensional information from these imaging experiments is used to create movie clips that provide a time-enhanced view of the dynamics of the fluorescent entities within the imaged volume. Special software and viewing glasses are sometimes used to visualize the three-dimensionality of these data. However, the output of *in situ* imaging is most often presented as movies made by compressing the z information into a single plane, often using a method called maximum intensity projection. This process removes information about where in the z-dimension structures and cells actually lie and unfortunately often results in the impression that two cells come into contact when they actually pass over each other in space. Various methods have been devised to provide more z-dimension information to the viewer, the most useful of which is a scheme implemented after primary data collection that dynamically changes the colour-coding of a cell based on its z position within the imaging volume[27].

The rate at which the individual z stacks of a four-dimensional data set are shown on screen markedly affects the impression of mobility conveyed to the viewer. Differences in the rate of video playback can give the mistaken impression of very different cellular dynamics in two studies, when the behaviour is actually the same. The fact that it is difficult for readers or reviewers to adjust this parameter to allow side-by-side comparisons between the work of different groups can lead to the false impression that one group's data reflect 'unphysiological behaviour'. There is no 'correct' speed at which such movies should be run, but at a minimum a time stamp should be prominently displayed and the elapsed time clearly visible. As the field develops, some standardization in this aspect of data presentation will hopefully emerge.

Other software manipulations are done to prepare the data for viewing. Various filtering routines remove noise and smooth the images but can also obscure thin structures such as fine dendrites. Just as with standard static confocal images, colour intensity and hue can also be altered. This must be done carefully and using global algorithms with linear effects to avoid the generation of images that distort the underlying reality. In cases in which other manipulations are needed to allow visualization of particular image features, the alterations must be carefully detailed for the readers so that they can make a proper evaluation of the data.

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Although viewing data is important, it is equally important to obtain quantitative information[80]. Intravital data and even explant data can be subject to tracking artefacts because of either tissue movement or simple imaging noise causing small apparent movements of the cell. If data are acquired at a high rate, this jitter can register as real motion, although analysis of displacement over longer time intervals might show no migration of the cell. For this reason, it makes sense to use a displacement filter when analysing tracking data. As one example, for analysis of cells in data sets generated from intravital lymph-node imaging, the application of a displacement threshold of 2.5 μm — meaning that movement is only recorded as such when the cumulative displacements from any location along the track exceeds 2.5 μm — greatly increases the reproducibility of tracking data[34].

As four-dimensional imaging efforts become more ambitious in terms of resolution, numbers of fluorescent channels, volume and duration, the amount of data collected in a single imaging session will exceed a terabyte and the technical problems associated with cell tracking on this scale will increase substantially. Efforts are underway in several fields of investigation to develop linked experimental and analytical approaches for such large-scale data collection and processing. For example, in studies of zebrafish development, a combination of plasma membrane and nuclear labelling with fluorescent proteins was found to be the most reliable way to track cells in four-dimensions in the developing embryo. Software able to handle the difficult task of following all the cells in a developing embryo is being developed and might find application in immune-cell tracking[81].

Concluding remarks

In vivo high-resolution imaging of immune cells in complex tissues is a field that is only a few years old. In this brief period, it has advanced from single-colour data to multicolour studies that simultaneously track three, or even four, cell populations at depths of several hundred microns for prolonged time periods. Data analysis has become more sophisticated, as has the static and dynamic display of the results of these studies. Many different tissues can now be examined using these techniques, including secondary lymphoid tissues, organs such as the liver and kidney, and various epithelial surfaces. The field has allowed new insights into the rates and routes of cell migration within these tissue environments. These techniques have made it possible to appreciate the dramatic motion of lymphocytes, the contribution of DC processes to effective antigen encounter by T cells and the changing duration of antigen-dependent cell–cell contacts during an early adaptive immune response. These examples are just some of the observations that have substantially influenced thinking about how the immune system goes about its business[57].

However, to extend our understanding of the function of the immune system *in vivo*, new technologies are needed to examine cells and molecular structures that are deeper in tissues, fainter and smaller, as well as to show biochemical events that occur on a very rapid timescale. Some of the technologies that are required are already under development (BOX 2) (for example, a prototype dual-beam multi-photon microscope has been constructed at Washington University (M.J.M, unpublished observation), which theoretically can allow up to eight fluorescent probes to be imaged concomitantly with only a small loss in temporal resolution), whereas others await innovative new solutions. It is worth noting that two-photon microscopy arose from observations in physics and that it is probable that interdisciplinary efforts in physics, mathematics and computer science will be needed to overcome these imaging challenges.

Despite this bright future, no discussion of immune-system imaging would be complete without a caution. It is all too tempting to look at the pictures and movies generated by *in situ* microscopy and imagine that the coloured cells are conducting their business in an inky void, as if they were in a culture dish in which the black of the image was just watery medium. Of course, nothing could be further from the truth. A quick look at even the simplest stained tissue section from a lymph node shows that the few fluorescent cells visible in the recently generated dynamic images are operating in a densely packed environment, surrounded by other lymphocytes, DCs and stromal cells, as well as fixed tissue elements such as blood vessels and extracellular matrix components, all in the context of an unknown number and distribution of chemical mediators that might be diffusing freely or fixed to the underlying stroma. These 'dark components', which remain unseen in the data record, undoubtedly function in subtle and profound ways to influence the behaviour of the labelled cells we see. Indeed, the ability to monitor simultaneously an increasing number of cell types and tissue structures is key to developing a robust understanding of how tissue organization, extracellular factors and cell movement combine to support the development of useful immune responses.

1. Anderson, A. O., Anderson, N. D. & White, J. D. in *Animal Models of Immunological Processes* (ed. Hay, J. B.) 26–95 (Academic Press, London, 1982).
2. Butcher, E. C., Williams, M., Youngman, K., Rott, L. & Briskin, M. Lymphocyte trafficking and regional immunity. *Adv. Immunol.* **72**, 209–253 (1999).
3. Mempel, T. R., Scimone, M. L., Mora, J. R. & von Andrian, U. H. *In vivo* imaging of leukocyte trafficking in blood vessels and tissues. *Curr. Opin. Immunol.* **16**, 406–417 (2004).
4. von Andrian, U. H. & Mempel, T. R. Homing and cellular traffic in lymph nodes. *Nature Rev. Immunol.* **3**, 867–878 (2003).
5. Janeway, C. J. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb. Symp. Quant. Biol.* **1**, 1–13 (1989).
6. Banchereau, J. & Steinman, R. M. Dendritic cells and the control of immunity. *Nature* **392**, 245–252 (1998).
7. Degli-Esposti, M. A. & Smyth, M. J. Close encounters of different kinds: dendritic cells and NK cells take centre stage. *Nature Rev. Immunol.* **5**, 112–124 (2005).
8. Reis e Sousa, C. Dendritic cells as sensors of infection. *Immunity* **14**, 495–498 (2001).
9. Sher, A., Pearce, E. & Kaye, P. Shaping the immune response to parasites: role of dendritic cells. *Curr. Opin. Immunol.* **15**, 421–429 (2003).
10. Pulendran, B. Variegation of the immune response with dendritic cells and pathogen recognition receptors. *J. Immunol.* **174**, 2457–2465 (2005).
11. Halin, C., Rodrigo Mora, J., Sumen, C. & von Andrian, U. H. *In vivo* imaging of lymphocyte trafficking. *Annu. Rev. Cell Dev. Biol.* **21**, 581–603 (2005).
12. Winter, G. & Milstein, C. Man-made antibodies. *Nature* **349**, 293–299 (1991).
13. Kohler, G. Derivation and diversification of monoclonal antibodies. *Science* **233**, 1281–1286 (1986).

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14. Herzenberg, L. A. & De Rosa, S. C. Monoclonal antibodies and the FACS: complementary tools for immunobiology and medicine. *Immunol. Today* **21**, 383–390 (2000).
15. Sheppard, C. J. & Wilson, T. The theory of the direct-view confocal microscope. *J. Microsc.* **124**, 107–117 (1981).
16. Denk, W., Strickler, J. H. & Webb, W. W. Two-photon laser scanning fluorescence microscopy. *Science* **248**, 73–76 (1990).
17. White, J. G., Amos, W. B. & Fordham, M. An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy. *J. Cell Biol.* **105**, 41–48 (1987).
18. Kupfer, A. & Kupfer, H. Imaging immune cell interactions and functions: SMACs and the immunological synapse. *Semin. Immunol.* **15**, 295–300 (2003).
19. Monks, C. R., Freiberg, B. A., Kupfer, H., Sciaky, N. & Kupfer, A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* **395**, 82–86 (1998).
20. Wulfing, C., Sjaastad, M. D. & Davis, M. M. Visualizing the dynamics of T cell activation: Intracellular adhesion molecule 1 migrates rapidly to the T cell/B cell interface and acts to sustain calcium levels. *Proc. Natl Acad. Sci. USA* **95**, 6302–6307 (1998).
21. Grakoui, A., *et al.* The immunological synapse: a molecular machine controlling T cell activation. *Science* **285**, 221–227 (1999).
22. von Andrian, U. H. Intravital microscopy of the peripheral lymph node microcirculation in mice. *Microcirculation* **3**, 287–300 (1996).
23. Warnock, R. A., Askari, S., Butcher, E. C. & von Andrian, U. H. Molecular mechanisms of lymphocyte homing to peripheral lymph nodes. *J. Exp. Med.* **187**, 205–216 (1998).
24. Stoll, S., Delon, J., Brotz, T. M. & Germain, R. N. Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. *Science* **296**, 1873–1876 (2002).
One of a trio of early papers (see also refs 25, 46) reporting the dynamic imaging of immune cells in lymphoid tissues. This was the first paper to focus on the duration of antigen-induced T-cell–DC interactions, documenting a long monogamous phase of interaction followed by dissociation and rapid migration of the activated T cells.
25. Miller, M. J., Wei, S. H., Parker, I. & Cahalan, M. D. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* **296**, 1869–1873 (2002).
The first report applying two-photon technology to lymphocyte imaging in intact tissues, documenting and carefully quantitating the unexpectedly rapid movement of T and B cells in the normal lymph node.
26. Bousso, P. & Robey, E. Dynamics of CD8⁺ T cell priming by dendritic cells in intact lymph nodes. *Nature Immunol.* **4**, 579–585 (2003).
The first report extending *in situ* imaging to CD8⁺ T cells in lymph nodes, confirming the prolonged antigen-dependent association of T cells with DCs and providing the first quantitative description of the rate of lymphocyte scanning by DCs.
27. Miller, M. J., Wei, S. H., Cahalan, M. D. & Parker, I. Autonomous T cell trafficking examined *in vivo* with intravital two-photon microscopy. *Proc. Natl Acad. Sci. USA* **100**, 2604–2609 (2003).
The initial report of intravital two-photon imaging of T-cell behaviour in lymph nodes.
28. Miller, M. J., Safrina, O., Parker, I. & Cahalan, M. D. Imaging the single cell dynamics of CD4⁺ T cell activation by dendritic cells in lymph nodes. *J. Exp. Med.* **200**, 847–856 (2004).
29. Mempel, T. R., Henrickson, S. E. & Von Andrian, U. H. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature* **427**, 154–159 (2004).
This paper introduced the use of CD62L blockade as a tool for synchronizing the residence time of T cells in an imaged lymph node, and used this method in a new intravital preparation, showing a third early phase of transient T-cell–DC interaction that preceded the previously reported stable association phase.
30. Miller, M. J., Hejazi, A. S., Wei, S. H., Cahalan, M. D. & Parker, I. T cell repertoire scanning is promoted by dynamic dendritic cell behavior and random T cell motility in the lymph node. *Proc. Natl Acad. Sci. USA* **101**, 998–1003 (2004).
A careful analysis of T-cell migration, leading to the hypothesis that immune activation arises from random walk migration of T cells promoting contact with rapidly gesticulating DC processes.
31. Lindquist, R. L., *et al.* Visualizing dendritic cell networks *in vivo*. *Nature Immunol.* **5**, 1243–1250 (2004).
The first intravital imaging study of DCs using endogenous fluorescent protein labelling rather than adoptive transfer of the cells, showing an extensive, relatively sessile network of resident DCs that was joined by DCs arriving from peripheral sites.
32. Hugues, S., *et al.* Distinct T cell dynamics in lymph nodes during the induction of tolerance and immunity. *Nature Immunol.* **5**, 1235–1242 (2004).
33. Gunzer, M., *et al.* A spectrum of biophysical interaction modes between T cells and different antigen-presenting cells during priming in 3-D collagen and *in vivo*. *Blood* **104**, 2801–2809 (2004).
34. Shakhar, G., *et al.* Stable T cell-dendritic cell interactions precede the development of both tolerance and immunity *in vivo*. *Nature Immunol.* **6**, 707–714 (2005).
35. Okada, T., *et al.* Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS Biol.* **3**, e150 (2005).
The first paper using *in situ* imaging to document chemokine-directed, rather than random, migration of lymphocytes *in situ*.
36. Wei, S. H., *et al.* Sphingosine 1-phosphate type 1 receptor agonism inhibits transendothelial migration of medullary T cells to lymphatic sinuses. *Nature Immunol.* **6**, 1228–1235 (2005).
37. Tang, Q., *et al.* Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nature Immunol.* **7**, 83–92 (2006).
The first report to visualize the effect of regulatory T cells on the dynamics of effector T-cell migration and interaction with DCs in the lymph-node environment.
38. Geissmann, F., *et al.* Intravascular immune surveillance by CXCR6⁺ NKT cells patrolling liver sinusoids. *PLoS Biol.* **3**, e113 (2005).
This report extended the range of tissues imaged to the liver, documenting an unexpected behaviour of natural killer T cells in this organ.
39. Germain, R. N., *et al.* An extended vision for dynamic high-resolution intravital immune imaging. *Semin. Immunol.* **17**, 431–441 (2005).
40. Cavanagh, L. L., *et al.* Activation of bone marrow-resident memory T cells by circulating, antigen-bearing dendritic cells. *Nature Immunol.* **6**, 1029–1037 (2005).
41. Grayson, M. H., Hotchkiss, R. S., Karl, I. E., Holtzman, M. J. & Chaplin, D. D. Intravital microscopy comparing T lymphocyte trafficking to the spleen and the mesenteric lymph node. *Am. J. Physiol. Heart Circ. Physiol.* **284**, H2213–2226 (2003).
42. Grayson, M. H., Chaplin, D. D., Karl, I. E. & Hotchkiss, R. S. Confocal fluorescent intravital microscopy of the murine spleen. *J. Immunol. Methods* **256**, 55–63 (2001).
A very early report indicating that advanced microscopy tools could be applied to immune-cell imaging in complex tissues.
43. Wei, S. H., Miller, M. J., Cahalan, M. D. & Parker, I. Two-photon imaging in intact lymphoid tissue. *Adv. Exp. Med. Biol.* **512**, 203–208 (2002).
44. Zinselmeyer, B. H., *et al. In situ* characterization of CD4⁺ T cell behavior in mucosal and systemic lymphoid tissues during the induction of oral priming and tolerance. *J. Exp. Med.* **201**, 1815–1823 (2005).
45. Tutsch, E., Griesemer, D., Schwarz, A., Stallmach, A. & Hoth, M. Two-photon analysis of calcium signals in T lymphocytes of intact lamina propria from human intestine. *Eur. J. Immunol.* **34**, 3477–3484 (2004).
46. Bousso, P., Bhakta, N. R., Lewis, R. S. & Robey, E. Dynamics of thymocyte-stromal cell interactions visualized by two-photon microscopy. *Science* **296**, 1876–1880 (2002).
This study documented the different migratory behaviours of thymocytes in reconstituted thymic aggregates.
47. Bhakta, N. R., Oh, D. Y. & Lewis, R. S. Calcium oscillations regulate thymocyte motility during positive selection in the three-dimensional thymic environment. *Nature Immunol.* **6**, 143–151 (2005).
This paper introduced the use of Ca^{2+} reporter dyes for relating lymphocyte behaviour to signalling in a complex tissue environment.
48. Witt, C. M., Raychaudhuri, S., Schaefer, B., Chakraborty, A. K. & Robey, E. A. Directed migration of positively selected thymocytes visualized in real time. *PLoS Biol.* **3**, e160 (2005).
49. Kissenpfennig, A., *et al.* Dynamics and function of Langerhans cells *in vivo*: Dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. *Immunity* **22**, 643–654 (2005).
50. Rosenbaum, J. T., *et al.* Imaging ocular immune responses by intravital microscopy. *Int. Rev. Immunol.* **21**, 255–272 (2002).
51. Kawakami, N., *et al.* Live imaging of effector cell trafficking and autoantigen recognition within the unfolding autoimmune encephalomyelitis lesion. *J. Exp. Med.* **201**, 1805–1814 (2005).
This paper extended the application of two-photon imaging to peripheral sites of lymphocyte effector function.
52. Nitsch, R., *et al.* Direct impact of T cells on neurons revealed by two-photon microscopy in living brain tissue. *J. Neurosci.* **24**, 2458–2464 (2004).
53. Bajenoff, M., *et al.* Natural killer cell behavior in lymph nodes revealed by static and real-time imaging. *J. Exp. Med.* **203**, 619–631 (2006).
54. Hildebrandt, I. J. & Gambhir, S. S. Molecular imaging applications for immunology. *Clin. Immunol.* **111**, 210–224 (2004).
55. Massoud, T. F. & Gambhir, S. S. Molecular imaging in living subjects: seeing fundamental biological processes in a new light. *Genes Dev.* **17**, 545–580 (2003).
56. Wei, S. H., Parker, I., Miller, M. J. & Cahalan, M. D. A stochastic view of lymphocyte motility and trafficking within the lymph node. *Immunol. Rev.* **195**, 136–159 (2003).
57. Catron, D. M., Itano, A. A., Pape, K. A., Mueller, D. L. & Jenkins, M. K. Visualizing the first 50 hr of the primary immune response to a soluble antigen. *Immunity* **21**, 341–347 (2004).
58. Cahalan, M. D. & Parker, I. Close encounters of the first and second kind: T-DC and T-B interactions in the lymph node. *Semin. Immunol.* **17**, 442–451 (2005).
59. Castellino, F., *et al.* Chemokines enhance immunity by guiding naive CD8⁺ T cells to sites of CD4⁺ T cell-dendritic cell interaction. *Nature* **440**, 890–895 (2006).
Evidence that chemokine guidance can have a crucial role in fostering cell–cell interactions in lymph nodes.
60. Bousso, P. & Robey, E. A. Dynamic behavior of T cells and thymocytes in lymphoid organs as revealed by two-photon microscopy. *Immunity* **21**, 349–355 (2004).
61. Cahalan, M. D., Parker, I., Wei, S. H. & Miller, M. J. Two-photon tissue imaging: seeing the immune system in a fresh light. *Nature Rev. Immunol.* **2**, 872–880 (2002).
62. Cahalan, M. D., Parker, I., Wei, S. H. & Miller, M. J. Real-time imaging of lymphocytes *in vivo*. *Curr. Opin. Immunol.* **15**, 372–377 (2003).
63. Dustin, M. L. *In vivo* imaging approaches in animal models of rheumatoid arthritis. *Arthritis Res. Ther.* **5**, 165–171 (2003).
64. Huang, A. Y., Qi, H. & Germain, R. N. Illuminating the landscape of *in vivo* immunity: insights from dynamic *in situ* imaging of secondary lymphoid tissues. *Immunity* **21**, 331–339 (2004).
65. Robey, E. A. & Bousso, P. Visualizing thymocyte motility using 2-photon microscopy. *Immunol. Rev.* **195**, 51–57 (2003).
66. Witt, C. M. & Robey, E. A. Thymopoiesis in 4 dimensions. *Semin. Immunol.* **17**, 95–102 (2005).
67. Bhakta, N. R. & Lewis, R. S. Real-time measurement of signaling and motility during T cell development in the thymus. *Semin. Immunol.* **17**, 411–420 (2005).
68. Zhang, J., Campbell, R. E., Ting, A. Y. & Tsien, R. Y. Creating new fluorescent probes for cell biology. *Nature Rev. Mol. Cell Biol.* **3**, 906–918 (2002).
69. Hawiger, D., *et al.* Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions *in vivo*. *J. Exp. Med.* **194**, 769–779 (2001).
70. Unutmaz, D., *et al.* The primate lentiviral receptor BONZO/STRL33 is coordinately regulated with CCR5 and its expression pattern is conserved between human and mouse. *J. Immunol.* **165**, 3284–3292 (2000).
71. Copeland, N. G., Jenkins, N. A. & Court, D. L. Recombineering: a powerful new tool for mouse functional genomics. *Nature Rev. Genet.* **2**, 769–779 (2001).
72. Larochelle, A. & Dunbar, C. E. Genetic manipulation of hematopoietic stem cells. *Semin. Hematol.* **41**, 257–271 (2004).

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73. Miyawaki, A. Innovations in the imaging of brain functions using fluorescent proteins. *Neuron* **48**, 189–199 (2005).
74. Tsien, R. Y. Building and breeding molecules to spy on cells and tumors. *FEBS Lett.* **579**, 927–932 (2005).
75. Gao, X., *et al.* *In vivo* molecular and cellular imaging with quantum dots. *Curr. Opin. Biotechnol.* **16**, 63–72 (2005).
76. Moore, T. C. Anesthesia-associated depression in lymphocyte traffic and its modulation. *Am. J. Surg.* **147**, 807–812 (1984).
77. So, P. T., Dong, C. Y., Masters, B. R. & Berland, K. M. Two-photon excitation fluorescence microscopy. *Annu. Rev. Biomed. Eng.* **2**, 399–429 (2000).
78. Zoumi, A., Yeh, A. & Tromberg, B. J. Imaging cells and extracellular matrix *in vivo* by using second-harmonic generation and two-photon excited fluorescence. *Proc. Natl Acad. Sci. USA* **99**, 11014–11019 (2002).
79. McCaffrey, A., Kay, M. A. & Contag, C. H. Advancing molecular therapies through *in vivo* bioluminescent imaging. *Mol. Imaging* **2**, 75–86 (2003).
80. Sumen, C., Mempel, T. R., Mazo, I. B. & von Andrian, U. H. Intravital microscopy: visualizing immunity in context. *Immunity* **21**, 315–329 (2004).
81. Megason, S. G. & Fraser, S. E. Digitizing life at the level of the cell: high-performance laser-scanning microscopy and image analysis for *in toto* imaging of development. *Mech. Dev.* **120**, 1407–1420 (2003).
82. Treacy, E. B. Chirped optical pulses. *Ann. NY Acad. Sci.* **168**, 400–418 (1969).
83. Iyer, V., Losavio, B. E. & Saggau, P. Compensation of spatial and temporal dispersion for acousto-optic multiphoton laser-scanning microscopy. *J. Biomed. Opt.* **8**, 460–471 (2003).
84. Nguyen, Q. T., Callamaras, N., Hsieh, C. & Parker, I. Construction of a two-photon microscope for video-rate Ca^{2+} imaging. *Cell Calcium* **30**, 383–393 (2001).
85. Fan, G. Y., *et al.* Video-rate scanning two-photon excitation fluorescence microscopy and ratio imaging with cameleons. *Biophys J.* **76**, 2412–2420 (1999).
86. Oheim, M., Beaurepaire, E., Chaigneau, E., Mertz, J. & Charpak, S. Two-photon microscopy in brain tissue: parameters influencing the imaging depth. *J. Neurosci. Methods* **111**, 29–37 (2001).
87. Helmchen, F. & Denk, W. Deep tissue two-photon microscopy. *Nature Methods* **2**, 932–940 (2005).
88. Theer, P., Hasan, M. T. & Denk, W. Two-photon imaging to a depth of 1000 micrometers in living brains by use of a $Ti:Al_2O_3$ regenerative amplifier. *Opt. Lett.* **28**, 1022–1024 (2003).
89. Krummel, M., Wulfing, C., Sumen, C. & Davis, M. M. Thirty-six views of T-cell recognition. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* **355**, 1071–1076. (2000).
90. Levene, M. J., Dombeck, D. A., Kasischke, K. A., Molloy, R. P. & Webb, W. W. *In vivo* multiphoton microscopy of deep brain tissue. *J. Neurophysiol.* **91**, 1908–1912 (2004).
91. Bird, D. & Gu, M. Two-photon fluorescence endoscopy with a micro-optic scanning head. *Opt. Lett.* **28**, 1552–1554 (2003).
92. Gobel, W., Kerr, J. N., Nimmerjahn, A. & Helmchen, F. Miniaturized two-photon microscope based on a flexible coherent fiber bundle and a gradient-index lens objective. *Opt. Lett.* **29**, 2521–2523 (2004).
93. Jung, J. C., Mehta, A. D., Aksay, E., Stepnoski, R. & Schnitzer, M. J. *In vivo* mammalian brain imaging using one- and two-photon fluorescence microendoscopy. *J. Neurophysiol.* **92**, 3121–3133 (2004).
94. Flusberg, B. A., *et al.* Fiber-optic fluorescence imaging. *Nature Methods* **2**, 941–950 (2005).
95. Sun, C. K. Higher harmonic generation microscopy. *Adv. Biochem. Eng. Biotechnol.* **95**, 17–56 (2005).
96. Huang, S., Heikal, A. A. & Webb, W. W. Two-photon fluorescence spectroscopy and microscopy of Nad(P)H and flavoprotein. *Biophys. J.* **82**, 2811–2825 (2002).
97. Zipfel, W. R., *et al.* Live tissue intrinsic emission microscopy using multiphoton-excited native fluorescence and second harmonic generation. *Proc. Natl Acad. Sci. USA* **100**, 7075–7080 (2003).
98. Pinaud, F., *et al.* Advances in fluorescence imaging with quantum dot bio-probes. *Biomaterials* **27**, 1679–1687 (2006).
99. Torres Filho, I. P., Leunig, M., Yuan, F., Intaglietta, M. & Jain, R. K. Noninvasive measurement of microvascular and interstitial oxygen profiles in a human tumor in SCID mice. *Proc. Natl Acad. Sci. USA* **91**, 2081–2085 (1994).
100. Gustafsson, M. G. Nonlinear structured-illumination microscopy: wide-field fluorescence imaging with theoretically unlimited resolution. *Proc. Natl Acad. Sci. USA* **102**, 13081–13086 (2005).
101. Dyba, M. & Hell, S. W. Photostability of a fluorescent marker under pulsed excited-state depletion through stimulated emission. *Appl. Opt.* **42**, 5123–5129 (2003).
102. Holtom, G. R., Thrall, B. D., Chin, B. Y., Wiley, H. S. & Colson, S. D. Achieving molecular selectivity in imaging using multiphoton Raman spectroscopy techniques. *Traffic* **2**, 781–788 (2001).
103. Nielsen, T., Fricke, M., Hellweg, D. & Andresen, P. High efficiency beam splitter for multifocal multiphoton microscopy. *J. Microsc.* **201**, 368–376 (2001).

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Competing interests statement
The authors declare no competing financial interests.

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