Review
Application of *in vivo* microscopy: evaluating the immune response in living animals

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Abstract

The initiation of an immune response requires that professional antigen-presenting cells, such as dendritic cells, physically interact with antigen-specific T cells within the complex environment of the lymph node. Although the way in which antigen is presented to T cells and in particular the cellular associations involved in antigen-specific stimulation events have been extensively investigated, data on antigen presentation have come primarily from studies *in vitro* or examination of the late consequences of antigen presentation *in vivo*. However, there is increasing recognition that events defined *in vitro* might not correspond entirely to the physiological situation *in vivo*. Recent developments in imaging technology now allow real-time observation of single-cell and molecular interactions in intact lymphoid tissues and have already contributed to a more detailed picture of how cells coordinate the initiation or suppression of an immune response.

Introduction

Until recently, the only method of demonstrating antigen processing and peptide–major histocompatibility complex (pMHC) formation by antigen-presenting cells (APCs) *in vivo* was to measure antigen-specific T cell activation *in vitro* [1,2]. Although these T cell-based assay systems are very sensitive, their drawbacks are variations in the stimulatory capacity of different APC populations and the unknown activation state of the responder T cells.

Flow cytometry and tissue section imaging have been valuable methods for the investigation of antigen presentation *in vivo*. In particular, the use of pMHC-specific antibodies allows the detection of small numbers of molecules per cell, thereby permitting the analysis of antigen-specific T cell activation [3-5].

The ability of a cell to move on any substrate must represent a combination between adhesion and the ability to extend processes. However, this obviously depends strongly on the nature of the surface; results on lymphocyte motility and interactions with APCs obtained from studies *in vitro* have consequently given drastically different results depending on the experimental system used [6-8]. In contrast, studies *in vitro* have provided valuable information about the signaling cascade that leads to lymphocyte activation, thereby describing the intricate choreography of key signaling molecules that participate in the formation of the immuno-logical synapse at the T cell–APC interface [9,10]. Neverthe-less, chemokine gradients, signals from the local nervous system and circulating hormones as well as integrin inter-actions with components of the extracellular matrix are lack-ing in cell culture systems. Finally, this methodology does not allow the observation of the movement and interaction of APCs with lymphocytes within organized lymphoid tissues in real time over short intervals.

This has led several laboratories to develop imaging methods with high resolution to be able to perform spatiotemporal analysis of cell–cell interactions *in vivo* within intact lymphoid tissues.

Dynamic imaging techniques

Resolution at the cellular and subcellular levels can currently be obtained mainly by two optical techniques: confocal microscopy [11] or the more recent technique of two-photon imaging [12].

In confocal microscopy, laser light is focused in the specimen by an objective lens and is used to excite cells or structures that have been labeled with fluorescent dyes. The emitted fluorescent light is collected through the same lens and is refocused in a pinhole aperture that is designed to reject

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APC = antigen-presenting cell; CSFE = 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester; DC = dendritic cell; GFP = green fluorescent protein; HEV = high endothelial venule; IL = interleukin; MHC = major histocompatibility complex; PET = positron emission tomography; TCR = T cell receptor.

almost all light except that originating at the focal point. By raster-scanning the laser spot, a two-dimensional plane can be imaged (*x*-axis and *y*-axis), and a so-called *z*-stack consisting of several such planes can be acquired as the microscope is focused at small increments into the specimen (*z*-axis) to sample a three-dimensional volume. This process can be repeated over time to accumulate a time-lapse movie. However, confocal microscopy has two major drawbacks for live-cell imaging. First, scattering of light by most tissues limits the depth of penetration into the tissue to 80 to 100 μm on average, which for lymph nodes allows the penetration of about only one-quarter of the whole lymph node. Second, although light is imaged only from the focal spot, the laser beam excites both exogenous fluorophore molecules and endogenous chromophores in cells above and below this plane; this leads to accelerated dye bleaching and possible cell toxicity.

Two-photon microscopy provides the same optical sectioning effect as confocal microscopy, but it uses a different optical principle with the advantage of greater imaging depth and reduced photobleaching and phototoxicity. Currently it can be regarded as the method of choice. Fluorophores are excited by the near-simultaneous absorption of two infrared photons, rather than by a single photon of visible light as in confocal microscopy. Each of the two photons contributes half of the energy required to induce fluorescence. The energy of a photon decreases with increasing wavelength, so the infrared light photons together provide comparable energy to a single blue photon, and a fluorophore such as fluorescein is thus excited and subsequently emits a green photon as it would during normal fluorescence. In addition, two-photon excitation requires lasers able to emit brief (femtosecond) pulses of light with instantaneous energies high enough to achieve two-photon excitation. The advantage of two-photon excitation for microscopy is that fluorescence is excited only at the focal spot of a laser beam, whereas the density falls off rapidly above and below the focal point. Excitation is achieved with infrared light, which because of its relatively long wavelength penetrates tissues with reduced scattering, allowing imaging more deeply (on average 200 to 300 μm) into biological specimens. In addition, excitation (and hence photobleaching and photodamage) is largely confined to the focal plane, whereas regions above and below experience only the relatively innocuous infrared radiation.

Limitations of two-photon microscopy are the following: the cost of the lasers, which are far more expensive than those used for confocal microscopy; light scattering by tissues, which limits imaging depth; and the challenge of introducing informative fluorescent labels into tissues. These approaches include labeling cells with vital dyes before transferring them back into mice or explanted organs. However, currently available cell tracker dyes were developed for use with conventional one-photon microscopy and require relatively high laser powers to give sufficient fluorescence emission with two-photon excitation. However, a threshold for cell damage is abruptly reached with increasing laser intensity. Consequently there is a fine dividing line between being able to see cells and cell toxicity because of photodamage.

Biological preparations for lymphoid tissue imaging; explant versus intravital

Most of the currently used experimental set-ups rely on techniques that were established for static imaging [13]. Usually, bone marrow-derived dendritic cells (DCs), generated in the presence of granulocyte/macrophage colony stimulating factor (GM-CSF) and IL-4 [14] are used as the most potent APCs [15]. Bone marrow-derived DCs can be labeled with various intravital dyes such as dialkylcarbocyanines (DiI, DiD) or succinimidyl ester (SNRF) and are injected subcutaneously into syngeneic animals either after having been pulsed with a defined antigenic peptide or left unpulsed. Similarly, T cells from transgenic animals expressing the cognate TCR are dye-labeled with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CSFE) or 5-(and-6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine (CMTMR) and injected intravenously (for a more extensive list of dyes that can be used for intravital microscopy, see the review by Cahalan and colleagues [16]). At various time points after cell transfer, lymph nodes draining the site of DC injection are analyzed by four-dimensional microscopy (space and time).

Alternatively, methods have been developed for injecting vital dyes *in situ* [17]. In these methods, resident DCs are labeled in the skin by injecting CSFE together with antigen and adjuvant. CSFE+ DCs are then detected after migration in the draining lymph nodes and the interaction with antigen-specific adoptively transferred T cells can be analyzed. DCs are expected to carry physiological concentrations of pMHC complexes and to enter the LNs at the appropriate stage of maturation.

More recently, by using green fluorescent protein (GFP) derivatives such as retroviruses or transgenes, methods have been developed that allow the tracking of specific cell types such as endogenous DCs in the steady state [18].

The ideal goal, of course, is to image single immune cells within their undisturbed environment in an intact living animal; however, this goal is still almost impossible to achieve. Currently, two methods are mainly being used that try to mimic the situation *in vivo* as closely as possible. One 'semi-intravital' method is the preparation of explanted intact organs. The excised lymph nodes, thymus or spleen are imaged while being perfused in warm medium with or without oxygenation [19-22]. This preserves the structural integrity of the natural tissue, but normal vascular and lymphatic circulation are severed. A second approach is the intravital imaging of lymph nodes. In these experiments animals are

anesthetized and lymph nodes are surgically prepared [23-25]. Easily accessible is the inguinal lymph node of the mouse by folding back a broad flap of abdominal skin or the popliteal lymph nodes of the feet [25]. A rubber ring is glued on the inner surface of the exposed skin flap with tissue adhesive. Thereby a watertight chamber filled with phosphate-buffered saline is formed into which a water-immersion objective is lowered for imaging. Both mouse and chamber are warmed and kept at 35 to 36°C.

In principle, both methods have certain limitations. One concern with explanted tissues has been the maintenance of physiological oxygen tension. Whereas some investigators have studied explanted lymph nodes in culture medium perfused with 95% O_2 and 5% CO_2 [20], others have argued that normal oxygen tension in lymph nodes may be low [19,26,27] and culture conditions perfused with 95% O_2 might represent unphysiological conditions causing abnormal lymphocyte motility. However, recent experiments [23] have reported similar T cell mobility in lymph nodes of living animals breathing either room air or 95% O_2/5% CO_2.

In contrast, manipulations involved in the intravital approach including the trauma associated with anesthesia and surgery could also introduce considerable artefacts, and data are still too limited to estimate the impact on subtle cell–cell inter-actions. Finally, the anatomical situation of certain tissues itself may limit the amount of data one can collect because the available field of view is sometimes diminished in comparison with the explant method, in which the isolated tissue can be analyzed from multiple imaging angles.

In general, however, the results reported so far have shown a remarkable concordance for both approaches with respect to the motility rates of different cell types, the dynamics of cell movement and antigen-dependent T cell–DC contacts. These results suggest that explant and intravital imaging techniques, at least for lymph nodes, can provide conditions that are physiologically appropriate.

Anatomical considerations
Whenever one is imaging fluorescently labeled cells within the natural environment of lymph nodes or other lymphoid tissues, one has to keep in mind that such labeled cells are of course not 'swimming' freely in a dark background of empty and unobstructed space. Within the lymph node there is a great excess of 'invisible' resident, unlabeled lymphocytes and other motile cells along with fixed structures such as the complex network of stromal elements and reticular fibers, together with high endothelial venules (HEVs) or blood vessels. Some of these structures, such as collagen-rich fibers or biological membranes, can be revealed by second-harmonic imaging, which is an additional three-dimensional microscope contrast mechanism that does not require the excitation of fluorescent molecules [28]. In addition, blood flow can be verified by the intravenous injection of rhodamine

dextran; alternatively, HEVs can be directly stained *in vivo* with fluorescent-conjugated MECA-79 antibody [29]. These unseen structures and cells undoubtedly influence the observed behavior of labeled cells but their true impact will remain unknown until better detection methods have been developed.

Imaging T cells and DCs
Recirculation of naive T cells between the blood and secondary lymphoid organs is critical for the detection of foreign antigens in various tissues of the body [30-32]. Within secondary lymphoid organs, T cell motility is required for migration within the T cell zone and for interaction with APCs. After activation, motility permits T cells to leave the lymph nodes and enter peripheral tissues to exert effector cell function [33]. Until recently these dynamic events could not be studied *in vivo*, and studies *in vitro* reported striking differences in T cell–APC interaction dynamics and activation requirements depending on the culture system [6-8]. Thus, only studies *in vivo* as outlined above now permit the study and understanding of lymphocyte function as it occurs in the natural environment.

T cell–DC interaction in the absence of specific antigen
In the absence of specific antigen, T cells were found to migrate autonomously in the T cell area and B cells likewise in the follicle, apparently providing no evidence for the directional guidance of putative chemokine gradients [22,23]. T cells moved in cycles of repetitive lunges with a period of about 2 min. Peak velocities of as high as 25 µm/min have been observed with a mean velocity for naive T cells of about 10 to 12 µm/min [22,23,34]. Similar values were obtained when explanted lymph nodes were used for imaging [20]. This is in contrast with results obtained by confocal microscopy, in which T cells were nonmotile in the absence of antigen, moving only after becoming activated [19]. The overall movement of T cells has been described as not collectively but rather autonomously, with each cell taking an independent trafficking path [23]. However, the potential role of pervasive chemokine gradients within this concerted action cannot be finally answered until data from studies with T cell or DC populations selectively deprived of distinct chemokine receptors become available. Nevertheless, this question gains considerable importance as soon as antigen-specific T cells are supposed to interact with antigen-bearing DCs. Antigen recognition may rely on a solely stochastic process with chance encounters between highly motile T cells and antigen-bearing DCs or, alternatively, chemokine gradients and the expression pattern of chemokine receptors may be required to orchestrate this interaction. Another hypothesis has suggested that T cells might use extracellular matrix elements such as the fibroblastic reticular cell network [35] as a guidance system for T cell migration [36].

Various populations of resident DCs have been described in the lymph node with the help of an enhanced yellow

fluorescent protein reporter under the control of the CD11c promoter [18]: subcapsular DCs with few dendrites and multiple large ruffles; DCs in the T cell zone; DCs in the B cell follicles; and perifollicular DCs, well positioned to acquire antigen from the lymph.

DCs in the B cell zone moved the fastest (about 4 µm/min), followed by subcapsular DCs (about 2 to 3 µm/min) and perifollicular DCs (about 2 µm/min), whereas DCs in the T cell zone showed the lowest mobility (less than about 1 µm/min).

When adoptively transferred, lipopolysaccharide-stimulated mature DCs were analyzed they were found to settle at the interface between the B and T cell zones and were present throughout the T cell area at 24 hours and at later time points (48 to 72 hours). They moved faster than steady-state DCs, particularly between 24 and 72 hours after transfer [18]. These data are in line with other published reports describing a random DC 'crawling' with average speeds of 2.7 to 6.6 mm/min [23,25,37,38].

At all time points immigrant DCs joined the endogenous DC network and became sessile. The higher motility of mature DCs probably functions to distribute DCs and the antigen(s) they carry throughout the T cell zones, thereby maximizing the likelihood of antigen-specific T cell–DC interactions.

Immigrant, tissue-derived DCs were described to localize preferentially in the vicinity of HEVs, where they formed clusters with antigen-specific T cells [39]. A similar high concentration of interacting T cells and DCs was observed in the interfollicular region ('cortical ridge'). Immigrant DCs seemed to accumulate first in the subcapsular sinus, from which they penetrated into the 'cortical ridge' region [40]. This distribution of antigen-bearing DCs could most efficiently ensure their encounter with incoming T cells.

In contrast, and unlike mature DCs, steady-state (immature) DCs are not preferentially associated with HEVs [18] although a selective affinity for the 'cortical ridge' has been demonstrated as well [40].

It has been estimated that, in the absence of antigen, each DC interacts with 500 to 5,000 different T cells per hour, and antigen-unspecific T cell–DC interactions were found to be short-lived (less than 1 hour) for both bone marrow-derived DCs [23,37] and resident DCs [18].

T cell–DC interaction in the presence of specific antigen
Cognate T cell interactions with antigen-bearing DCs seem to last significantly longer: stably interacting CD4+ T cells and DCs (more than 1 hour and up to 15 hours), preceding T cell activation, were first described in the superficial area of explanted lymph nodes by Stoll and colleagues [19] using confocal microscopy. With the use of two-photon micros-copy, CD8+ T cell–DC interactions were observed in the

range of hours [25,37]. Subtle differences in the exact duration of T cell–DC interactions might be explained by differences in the experimental set-up (oxygen perfusion versus no perfusion; different time points of analysis; differences in cell tracker dyes used), the type of cells being examined (CD4+ versus CD8+ T cells; bone marrow-derived DCs versus freshly isolated splenic DCs) or the method of detection (confocal versus two-photon microscopy with different limitation in the depths that can be analyzed). This prolonged T cell–DC interaction is in line with data in vitro demonstrating that more than 10 hours of TCR signaling is required for the initiation of naive T cell proliferation [41] and argues against a serial encounter model based on a 'digital' counter mechanism inside T cells that would initiate T cell proliferation only whenever multiple short encounters of cells exceed a certain threshold [8].

Interestingly, short-lived T cell–DC interactions have also been observed at an early time point after cell transfer (less than 8 hours), and this occurred even in the presence of antigen. These encounters of rapidly migrating T cells with DCs occurred preferentially in the vicinity of HEVs [25]. The role of these early and short-lived interactions is still under discussion. However, it has been suggested that DCs might line up around HEVs in strategic positions for the interaction with incoming T cells.

In summary, these observations have led to the proposal of a multi-phasic model of T cell activation in which the T cells collect signals from multiple short contacts with antigen-bearing DCs before forming a long-lasting interaction that initiates the production of IL-2 and interferon-γ. This is followed by a third phase in which T cells resume their rapid migration and short contacts with DCs and finally start to leave the lymph nodes [19,25]. Apparently, even in the absence of specific antigen, T cells seem to follow this three-phase itinerary when they traffic through lymphoid tissues. Without antigen, however, phase two is abbreviated and T cell–DC contacts do not result in the expression of activation markers (CD25), cytokine production (IL-2) or cell division but do induce TCR signaling, which might represent TCR interaction with self-MHC ligands required for optimal foreign antigen reactivity [42].

Nevertheless, one should keep in mind that the few live tissue-imaging studies performed so far have monitored cellular interactions occurring at a particular time point because it is still not possible to follow an individual T cell from the time of its initial encounter with an APC to the time at which it begins to produce IL-2 and to proliferate. Thus, additional experiments are required to determine whether T cells that are subject to distinct patterns of encounter with DCs (short-lived versus long-lived) will end up with different functional capabilities. Finally, it will be important to analyze T cell–DC interactions in various mouse models of infectious and autoimmune diseases because different infectious or

autoimmune processes influence the phenotype, number and functional capacity of DCs and thus certainly influence the way in which they interact with T cells.

Future directions and challenges for imaging *in vivo*

Future improvements of cell tracking techniques *in vivo* requires, among others, the development of specifically designed new fluorophores with improved two-photon absorption cross-sections and the optimization of microscope objectives and detector light paths to maximize the collection of emitted fluorescence photons. Thus, the challenge of obtaining a sufficiently bright signal to allow detection deep within scattering tissues is likely to continue to pose limits on this technique. Moreover, the development of fluorescent fusion proteins and other indicators of signaling and differentiation events would allow the characterization of the functional capacity of DCs at distinct differentiation stages or signaling in T cells upon interaction with cognate antigens. The use of a CD43–GFP reporter construct to indicate T cell–DC immunological synapse formation *in vivo* has demonstrated the feasibility of this approach [19,43]. Signaling events can be studied further by following the subcellular localization of fluorescent fusion proteins over time and by using calcium indicator dyes. GFP reporter transgenes driven by promoters restricted by tissue or cell type could be used to track specific cell types within tissues and to monitor gene expression. Thereby measurement of protein–protein association below the limit of light microscopy could be performed through fluorescence resonance energy transfer involving the cyan and yellow variants of GFP [44]. T cells with fluorescence protein expression controlled by gene-regulatory regions of cytokine or chemokine receptor expression [45,46] can be used to track the development of effector activity and changes in chemokine receptor expression that control T cell homing to the lymph node or migration to peripheral sites of effector function.

Recently, a three-photon fluorescence technique has become available that uses a femtosecond laser with a wavelength of 1,200 to 1,300 nm and offers enhanced penetration capability, improved spatial resolution and a wider selection of fluorescent labels. The combination with third-harmonic generation provides a general structural imaging modality and can be used to map the cellular structure down to a few hundred nanometres [47].

Dynamic four-dimensional (space and time) imaging *in vivo*, especially when performed over extended periods, generates considerable amounts of data because hundreds of individual cells, potentially interacting with each other, are revealed at several time points. Therefore, to monitor the migratory paths of cells and cell–cell contacts over time, specialized imaging and data processing as well as software programs for statistical analysis are required. Some of these software programs have already been developed and are used for tracking the movements of single cells and for the calculation of cell speeds during migration [48]. Data from imaging programs, some of which allow semi-automated tracking of cell movement, further permit the calculation of individual cell trajectories and motility coefficients that are required for quantitative data on migration pattern or the significance of T cell–DC interactions under different immunological settings [25,49]. However, further efforts in the refinement of methods for data analysis obtained from dynamic microscopy *in vivo* are of utmost importance for the exploitation of the maximum information that can be obtained from these experiments.

However, microscopic imaging cannot be used for the quantitative tracking of T cell migration out of the lymph node and into sites of inflammation or for tracking T cells over prolonged times *in vivo*. Finally, it cannot be applied to use in humans. Other methods of imaging *in vivo* might therefore be found useful in the future. In general, most of these techniques have not been suitable for small-animal models because of resolution limitations. However, micro-positron emission tomography (PET) with a resolution of 1 microl should be available soon [50]. Together with PET reporter genes that overcome the problem of dilution of the radiolabel during cell divison and in combination with micro-computed tomography to overlay anatomic resolution, it might be used for antibody imaging and to monitor T cell trafficking and T cell activation [51]. However, its limitations are the inconvenience of expensive short-lived tracers that also require extensive coordination with respect to the scheduling of the animal model, tracer preparation and access to the scanner as well as some constraints in radiochemistry.

Another approach is bioluminescence imaging *in vivo*. This imaging strategy uses genetically tagged cells that express bioluminescent reporter proteins such as luciferase that can be detected externally with sensitive charge-coupled device cameras as low-light detection systems [52]. With the use of bioluminescence imaging, antigen-specific T cells that had been transduced with retroviral vectors encoding multi-functional reporter genes were efficiently tracked in a joint inflammation model of arthritis [53]. In addition, transgenic mice that express luciferase in all their tissues have been developed and can serve as universal donors for trans-plantation and cell trafficking [54].

Finally, methods based on magnetic resonance imaging have to be adapted for imaging analysis *in vivo*. In particular, the use of efficient intracellular cell labeling methods with HIV Tat-peptide-derivatized magnetic nanoparticles now allows the tracking of systemically injected cells with magnetic resonance imaging *in vivo* at near-single-cell resolution and in three-dimensional reconstructions [55].

Conclusion
Dynamic optical imaging studies are providing a fresh look at the behavior of lymphocytes and APCs *in vivo* and allow the

most elegant monitoring of distinct immune cell–cell interactions in lymphoid tissues. However, many of the results obtained so far have merely confirmed pre-existing views generated with traditional methods of immunological investigation and have therefore only complemented established immunological theories. Nevertheless, the combined employment of various imaging techniques together with the right kind of accompanying studies *in vitro* will certainly provide a deepening understanding of the complex cellular choreography that is required for the initiation of a coordinate and appropriate immune response.

Competing interests

The author(s) declare that they have no competing interests.

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