TWO-PHOTON IMAGING IN INTACT LYMPHOID TISSUE

Sindy H. Wei, Michael D. Cahalan, Mark J. Miller, and Ian Parker*

1. INTRODUCTION

For imaging in living tissue, the two-photon technique presents several advantages over conventional confocal fluorescence imaging, including reduced photobleaching, minimal phototoxicity, and the ability to resolve detail at greater depths. We have established a two-photon imaging method that permits five-dimensional (x, y, z, t, emission wavelength) *ex vivo* imaging in intact murine lymphoid organs of viable T lymphocytes, B lymphocytes, and antigen-presenting cells (APC) up to a depth of about 300 μm. Three-dimensional reconstruction of lymph node revealed B cells in shallow, discrete subcapsular follicles, T cells in interfollicular regions, reticular fiber networks, and migratory dendritic cells. Additionally, we have imaged lymphocyte activity within the periarteriolar lymphocyte sheath in the intact spleen. In both lymph node and spleen, distinct modes of B and T cell movement may reflect specific antigen search programs. In this paper we review the capabilities of our experimental system, and hope to demonstrate the importance of live tissue imaging studies at the cellular level.

1.1. Why Tissue Imaging is Important

Lymphocyte motility and T cell-APC interactions have been studied extensively using *in vitro* techniques, or by following histological changes over time. Although the use of extracellular substrates and matrices attempts to simulate physiological tissue environments, there still remains the question of whether the behavior of cells observed outside of the native environment is relevant to cellular functions such as trafficking, chemotaxis, antigen presentation, activation, proliferation, and effector functions in the context of the intact immune system.

* Sindy H. Wei, Mark J. Miller, Michael D. Cahalan, Department of Physiology and Biophysics, University of California, Irvine, California 92697-4561, USA. Ian Parker, Department of Neurobiology and Behavior, University of California, Irvine, California 92697-4561, USA.

Lymphocytes do not passively flow between the circulatory and lymphatic systems, but require the development of motile characteristics in their perpetual search for antigen, and afterward, to act as effectors in both lymphatic and peripheral tissue. Although extravasation and homing have been thoroughly examined by *in vitro* and *in vivo* approaches, there exists only limited information on lymphocyte passage through and antigen presentation within lymphoid organs. This can be attributed to difficulties in replicating the myriad factors that affect motility of naïve lymphocytes, including the three-dimensional extracellular matrix (ECM), cytokine and chemokine gradients, and cell-cell interactions. Live tissue imaging with two-photon laser microscopy permits the study of dynamic lymphocyte behavior in the native environment, and may help to resolve questions regarding the role of ECM and cell-cell interactions in modulating T cell motility *in vivo*.

T cells must encounter multiple positive signals in order to generate an immune response. This process can be broken down into several distinct phases: positive selection in the thymus, initial encounter with antigen in the lymph node, proliferation and expression of activation markers, homing to inflammatory sites, and induction of effector function upon restimulation with antigen. These events are influenced by factors in the native environment that are difficult, if not impossible, to reconstitute in experimental systems. The chemokines constitute a large family of proteins that mediate chemotaxis and extravasation of leukocytes, and in some cases act as a costimulatory signal. Chemokines bind to seven-transmembrane G-protein coupled receptors and can trigger a variety of intracellular signaling pathways (Zlotnik and Yoshi, 2000). Lack of diffusible or deposited chemokines may render the results of lymphocyte activation, intracellular signaling, and chemotaxis studies in an *in vitro* system difficult to interpret.

In addition, lymphocyte motility and interaction with APC differ drastically depending on the experimental system. T lymphocytes crawl dramatically slower when plated on two-dimensional substrates, compared with when they are cultured in a three-dimensional collagen gel matrix or studied *in vivo* (Negulescu et al., 1996; Gunzer et al., 2000; Miller et al., in press). Furthermore, the motility pattern of primary B lymphocytes have not been well characterized until recently, perhaps due to the complex requirement of cells for soluble or structural components of the follicular environment to develop motile behavior (Clinchy et al., 1993; Miller et al., in press). T cell commitment to activation requires interaction with antigen-specific APCs, but the nature of this interaction (stable or transient) remains controversial. At present there exist two models for primary T cell activation: The single-encounter model requires the formation of a stable, long-lived immunological synapse between a T cell and an APC bearing the proper peptide-MHC complex (Dustin et al., 2001), while the serial encounter model involves serial "scanning" of multiple APC surfaces by a T cell to acquire a sum of activation signals (Friedl and Gunzer, 2001). Both modes of interaction result in TCR engagement, downstream signaling, and proliferation despite the fact that stable interactions were observed in a 2D system without ECM, while transient encounters were observed in a 3D collagen matrix. The environment of lymphoid tissue comprises a complex 3D structure, an extensive reticular fiber (collagen) network covered by a sheath of fibroblastic reticular cells, and endogenous APCs that send out chemical signals. During the initial antigen encounter in lymphoid tissue, collagen fibers are not expected to destabilize the T cell-APC interaction since they are isolated from cellular contact (Dustin et al., 2001). However, our studies of lymphocyte response to antigen priming in intact lymph node reveal both stable and scanning clusters one day after adoptive transfer

(Miller et al., in press), implying that the lymphoid environment may be modified in a compartment- or time-dependent manner upon first encounter with APCs, and that physiological antigen response is not easy to predict or reconstitute *in vitro*. *In vivo* imaging will allow us to sort out differences in experimental observations, and to assess the physiological significance of phenomenon observed *in vitro*.

1.2. Confocal Versus Multiphoton Imaging

In recent decades, conventional video and confocal microscopy methods have allowed researchers to study physiology at the single-cell level, and have inspired the rapid development of fluorophores and specialized indicators to probe intracellular structures, localization, signaling and function. Confocal microscopy was a drastic improvement over conventional fluorescence microscopy, achieving high spatial and temporal resolution by rapidly scanning a laser across the sample and then selectively transmitting in-focus signals through a pinhole to acquire thin optical slices (Periasamy, 2001). In addition, it is able to resolve structures at depths of tens to one hundred microns deep. Serial acquisition of optical slices through a sample in the Z-direction permits subsequent three-dimensional reconstruction of the sample.

However, serious concerns of viability arise when conventional confocal technique is used to study live specimens by continuous imaging over periods of time. The small confocal aperture rejects most of the fluorescence emitted from the sample, and thus requires high laser output or long scanning times to achieve a decent signal-to-noise ratio. Unfortunately these properties result in photobleaching of fluorophores (within minutes) and increased phototoxicity from free-radical formation, compromising tissue integrity as well as cellular function.

Many problems associated with live-cell imaging can be diminished by the use of multiphoton excitation microscopy. Two-photon excitation microscopy is based on the principle that fluorophores can be excited by the near simultaneous absorption of two lower energy photons (longer wavelength), instead of using one photon of higher energy (shorter wavelength). The standard Ti:Sapphire laser emits high intensity light, but in extremely short pulses that yields an overall low mean laser power and prevents thermal damage in tissue. Two-photon excitation occurs as a highly nonlinear function of intensity and therefore results in an inherently confocal image. By limiting excitation to the focal plane, two-photon microscopy does not require pinholes to reject out-of-focus fluorescence and greatly reduces photobleaching and phototoxicity (Denk et al., 1990). Another advantage is the ability of multiphoton microscopy to image tissue sections hundreds of microns deep at greater resolution, due both to the lack of detection pinhole limitations and the use of deep-penetrating infrared or near-infrared excitation wavelengths.

2. METHODS

Figure 1 describes the general paradigm that we have adopted to image lymphocytes and APC in intact lymphoid organs. These methods are discussed in greater detail below as they relate to the study of lymphocyte motility and antigen presentation.

[insert figure 1 here in this line only]

Figure 1. Reconstitution of fluorescently-labeled lymphocytes and APC in mouse lymph node and spleen. Isolated lymphocytes or cultured APC were purified by magnetic bead depletion, labeled, and adoptively transferred into a recipient mouse. Cells were allowed to home for variable times before nodes or spleen were excised for imaging under physiological conditions.

2.1. Adoptive Transfer of Fluorescently Labeled Cells

In order to image the behavior of living cells inside intact lymphoid organs we have used adoptive transfer of isolated, purified subsets of T lymphocytes, B lymphocytes, and dendritic cells (or other APC) from BALB/c mice that are labeled *in vitro* with Cell Tracker dyes (Molecular Probes), following established methods (Ingulli et al., 1997). We isolated naive BALB/c T and B cells from lymph node and spleen, respectively. After applying magnetic bead purification to >98% purity, 10-30 million cells naïve T and B cells were labeled with fluorescent CellTracker (Molecular Probes) dyes such as CFSE (green) or CMTMR (orange-red) and adoptively transferred into a recipient BALB/c. Transferred cells were allowed 6 hours to 5 days to home to peripheral LN or spleen prior to imaging. For antigen presentation studies we isolated ovalbumin-specific T cells from transgenic DO11.10 mice (Murphy et al., 1990) on a BALB/c background for adoptive transfer into antigen-primed mice. To visualize cells interacting with macroscopic components in their environment, we counterstained lymph nodes to label structures like reticular fibers by incubating intact lymphoid organs with fluorescent dyes, including the CellTrackers (Miller et al., in press).

2.2. Antigen Presentation

Endogenous APCs can be primed (but not visualized) by s.c. injections of antigen with adjuvant and TNF-alpha into BALB/c mice, at sites that drain directly to peripheral lymph nodes. APCs presumably take up and transport the antigen to local lymph nodes. 24 hours later, antigen-specific, naive T cells were adoptively transferred into antigen-primed mice, and excised nodes were imaged 1-5 days after antigenic challenge. In the presence of antigens, T lymphocytes are observed in stable or actively swarming clusters (Miller et al., in press). Alternatively, dendritic cells may be cultured from bone marrow with appropriate cytokines (Ingulli et al., 1997), primed with antigen in the presence of their maturation factor TNF-alpha, labeled, injected into the footpad, and visualized within lymphoid tissue. Our preliminary studies of dendritic cells employ an endogenous labeling and priming method, and show large dendritic cells migrating rapidly within the lymph node, actively probing the cellular environment with long, complex dendrites (data not shown).

2.3. Imaging

Five-dimensional imaging was performed under physiological conditions using a custom-built two-photon microscopy system (Nguyen et al., 2001), and image analysis was carried out as described previously (Miller et al., in press). This system is able to acquire high-resolution optical sections through a volume of 200 x 200 x 50 μm, corresponding to 2 nanoliters, at intervals of 10 seconds/frame. Acquisitions at intervals

of greater than 20-30 seconds do not yield high enough time resolution to track individual lymphocyte paths. With this *in vivo* reconstitution system, we have observed significantly increased T and B lymphocyte velocities (up to 35 μm/min for T cells) in lymph node in contrast to observations made on 2D substrates, as well as random migration in the unprimed node. Both B and T lymphocytes exhibit a high degree of temperature dependence, developing maximal motility near physiological temperature (approximately 36°C). For motility studies it is crucial to implement steady temperature control, as slight cooling of 2-4°C can decrease velocities drastically (Miller et al., in press). Fluorescence intensity did not degrade appreciably due to photobleaching for at least one hour, while rapidly crawling lymphocytes were observed for at least 6 hours.

3. RESULTS AND DISCUSSION

3.1. Time-Lapse Imaging of T cells in Different Regions of the Lymph Node

Even though the unseen factors within lymphoid tissue plays a significant role in the homing and motility patterns of lymphocytes, there is evidence for an intrinsic B or T cell antigen search program that is independent of the local environment. T cell regions are highly fibrillar in nature, whereas B cell regions express their own distinct set of cytokines, chemokines, and ECM. As the lymph node is highly compartmentalized, T cells rarely wander into B cell regions, and vice versa. Assuming that lymphocytes employ a "random walk" mode of movement, analysis of T and B cell motility patterns within their proper location in the lymph node show the motility coefficient of T cells to be five- to six-fold greater than that of B cells (Miller et al., in press). Panels 1-6 in Figure 2 represent sequential time-lapse images of a T cell that has crossed into a B cell zone within the primary follicle. Even though the chemical, cellular, and structural environment of the follicle differs dramatically from that of the interfollicular space, the T cell intruder appears to maintain its basic motility characteristics. These include a high motility coefficient, periodic changes in the shape index (a long, stretched-out form alternating with rounded morphology), and lunging motility. The lone T cell can be contrasted and compared with B cells (triangular morphology) within the follicle, and other T cells outside of the follicle. In looking at the cells within the tissue, volume imaging is the only method to be certain that a given cell is in a particular spatial relationship with other cells. This will be important in examining regional variations of cell behavior, defining the extent of contact between T cells and APC, and tracking cell proliferation.

3.2. Conduits for *Z*-Axis Motion in the Lymph Node

We acquired stacks of images through a volume of tissue at 10 seconds/frame, and then compressed the fluorescent signals in all Z-stacks so that all lymphocytes within that imaging volume are represented. Tracking of lymphocyte motility in a video in the plane parallel to the lymph node capsule demonstrated the presence of distinct regions within the tissue structure that allow rapid movement of lymphocytes perpendicular to the capsule (Fig. 3A). There appear to be two modes of Z-movement throughout the 50μm depth: slow drift beyond, and a fast dive into or out of the planes of focus. Blue and red dots mark locations where T cells moved into or out of the imaging volume respectively,

in a direction perpendicular to the capsule. In this field, two regions of active locomotion in the *z*-axis are discernable, while all other regions appear to favor movement in the *x-y* plane parallel to the capsule. These putative conduits give us some idea of how lymphocytes may transit through this highly compartmentalized organ in search for antigen.

3.3 Imaging in Spleen

Similar to murine lymph node, the spleen is comprised of a dense collagen capsule with a reticular fiber network. Figure 3B demonstrates the use of two-photon imaging within spleen; polarized, motile lymphocytes (green) are revealed within a network of reticular fibers (red). In the spleen, lymphocytes reside within the white pulp, more specifically in the periarteriolar lymphocyte sheath (PALS), while erythrocytes constitute the major cell population within the splenic cords and sinuses of the red pulp. As in the lymph node, the lymphatic tissue of the PALS is organized into diffuse T cell and nodular B cell regions. In both spleen and lymph node there is concern that organ removal from the circulation may disrupt necessary chemical gradients that affect homing, cell motility, and behavior. To address with this issue, an intravital imaging approach should be developed.

4. CONCLUSIONS

We have shown that our system reveals living lymphocytes within lymphoid tissue and can most likely be applied to all secondary lymphoid tissues if the proper homing system is developed. We have shown that 2-photon laser microscopy volume imaging can determine location, structure, motility pattern, and the timing and dynamics of antigen presentation. It is important in *ex-vivo* imaging of intact lymphoid tissue to keep physiological conditions of oxygenation and temperature. We anticipate that these methods will enable characterization of immunological synapse formation, antigen-stimulated Ca^{2+} signaling, and gene expression *in vivo*. We hope that this technique will create demand for the development of *in vivo* or genetically-encoded indicators that will be stable over the time course of an immune response. In conjunction with existing animal models for autoimmune disease and sites of tissue injury and inflammation, we hope to extend this cell imaging technique to elucidate mechanisms of tissue damage as well as the action of candidate immunomodulatory agents.

5. REFERENCES

Clinchy, B., Elenstrom, C., and Moller, G., 1993, The effect of T cell-derived cytokines on B cell motility in vitro, *Cell. Immunol.* **146**:62.

Denk, W., Strickler, J. H., and Webb, W. W., 1990, Two-photon laser scanning fluorescence microscopy, *Science* **248**:73.

Dustin, M. L., Allen, P. M., and Shaw, A. S., 2001, Environmental control of immunological synapse formation and duration, *Trends Immunol.* **22**:192.

Friedl, P., and Gunzer, M., 2001, Interaction of T cells with APCs: the serial encounter model, *Trends Immunol.* **22**:187.

Gunzer, M., Schafer, A., Borgmann, S., Grabbe, S., Zanker, K. S., Brocker, E. B., Kampgen, E., and Friedl, P., 2000, Antigen presentation in extracellular matrix: interactions of T cells with dendritic cells are dynamic, short lived, and sequential, *Immunity* **13**:323.

Ingulli, E., Mondino, A., Khoruts, A., and Jenkins, M. K., 1997, In vivo detection of dendritic cell antigen presentation to CD4(+) T cells, *J. Exp. Med.* **185:**2133.

Miller, M. J., Wei, S. H., Parker, I., and Cahalan, M. D., Two-photon imaging of lymphocyte motility and antigen response in intact lymph node, *Science*, in press.

Murphy, K. M., Heimberger, A. B., and Loh, D. Y., 1990, Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo, *Science*, **250:**1720.

Negulescu, P. A., Krasieva, T. B., Khan, A., Kerschbaum, H. H., and Cahalan, M. D., 1996, Polarity of T cell shape, motility, and sensitivity to antigen, *Immunity* **4:**421.

Nguyen, Q. T., Callamaras, N., Hsieh, C., and Parker, I., 2001, Construction of a two-photon microscope for video-rate Ca(2+) imaging, *Cell Calcium* **30**:383.

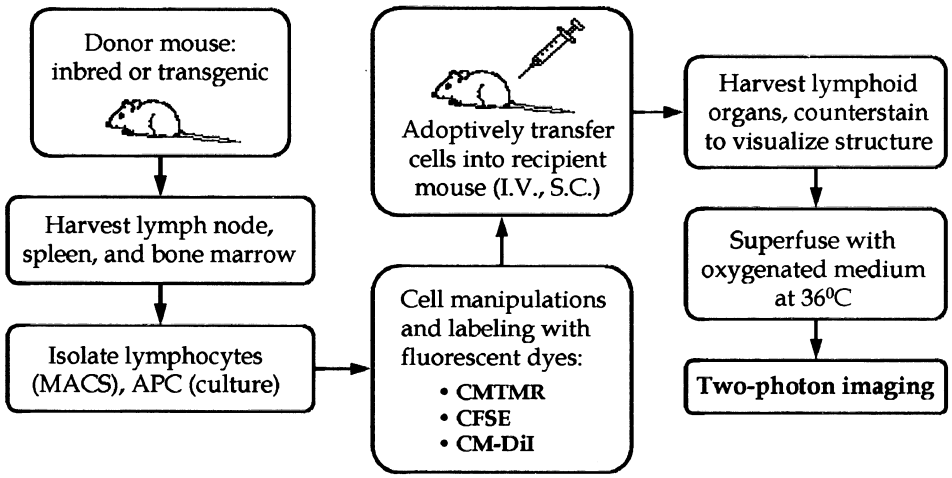
Periasamy, A., 2001, *Methods in Cellular Imaging*, Oxford University Press, Oxford, pp. 66-67.

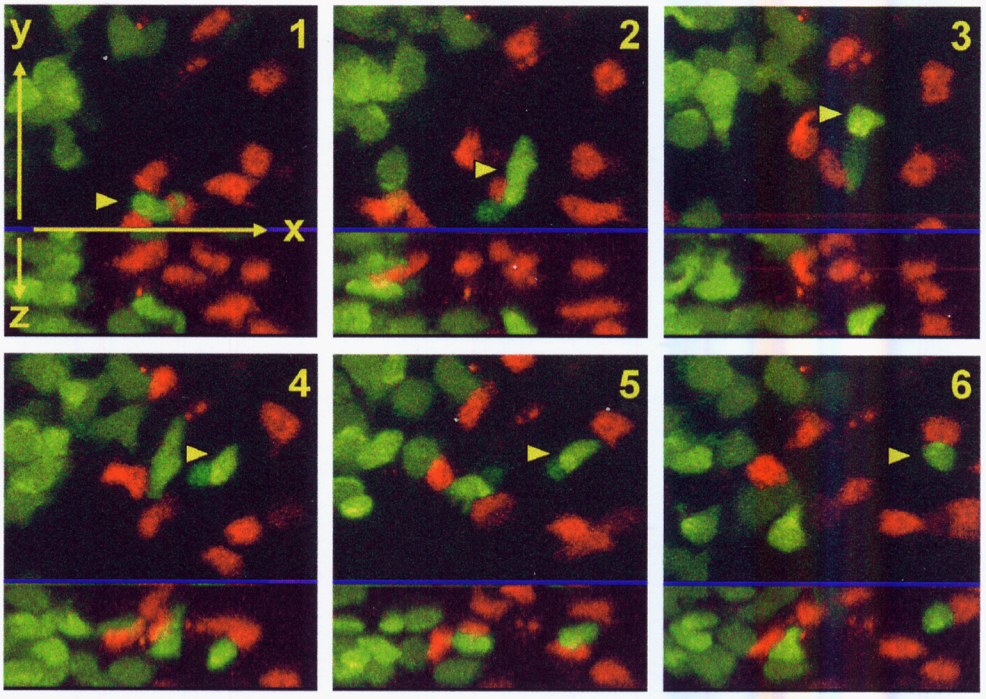
Zlotnik, A., and Yoshie, O., 2000, Chemokines: a new classification system and their role in immunity, *Immunity* **12:**121.

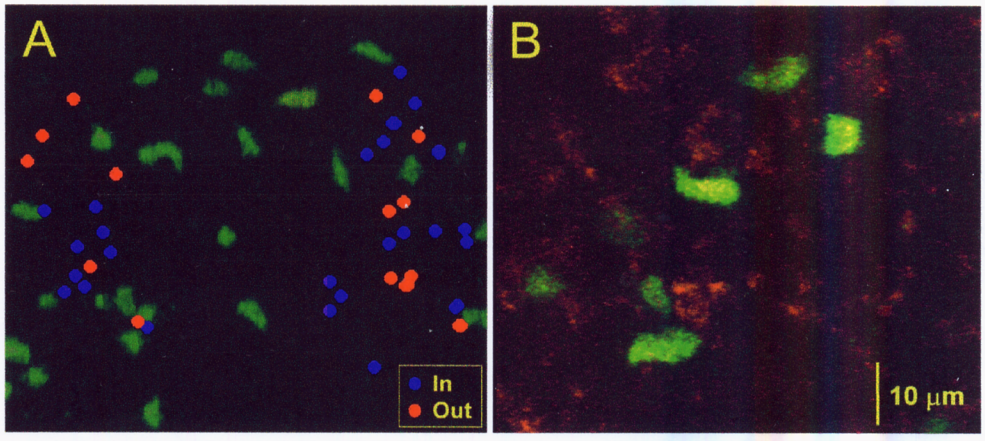
Figure 2. T cell behavior within a B cell follicle. For each of the six time points shown, Z-sections through the imaging volume were reconstructed in 3-D and compressed to create an *x-y* and *x-z* view (separated by the blue line). To ensure that the T cell (triangle) is passing through, but not above or in front of the B cell region, we must compare the location of the T cell with respect to B cells in both the *x-y* and *x-z* directions.

Figure 3. Imaging of lymphocytes within mouse lymph node and spleen. (A) A record of T cells moving through an imaging field was analyzed to determine if movement in the Z-direction occurs in specific regions. Blue and red dots represent individual events of cellular migration into and out of the imaging volume, respectively. Clusters of dots represent regions where movement perpendicular to the capsule may be favored, and may correlate to putative "conduits" of approximately 20-40μm in diameter that we have observed in separate experiments. (B) Fluorescently-labeled T cells (green) exhibit polarized, motile behavior within the spleen. Reticular fibers in the organ environment can also be counterstained with fluorescent dyes (red) to elucidate structure.

In vivo reconstitution




y
x
z
1
2
3
4
5
6


A
In
Out
B
10 μm