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Current Perspective

Recent Advances in Intravital Imaging of Dynamic Biological Systems

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Abstract. Intravital multiphoton microscopy has opened a new era in the field of biological imaging. Focal excitation of fluorophores by simultaneous attack of multiple (normally "two") photons generates images with high spatial resolution, and use of near-infrared lasers for multiphoton excitation allows penetration of thicker specimens, enabling biologists to visualize living cellular dynamics deep inside tissues and organs without thin sectioning. Moreover, the minimized photo-bleaching and toxicity associated with multiphoton techniques is beneficial for imaging of live specimens for extended observation periods. Here we focus on recent findings using intravital multiphoton imaging of dynamic biological systems such as the immune system and bone homeostasis. The immune system comprises highly dynamic networks, in which many cell types actively travel throughout the body and interact with each other in specific areas. Therefore, real-time intravital imaging represents a powerful tool for understanding the mechanisms underlying this dynamic system.

Keywords: intravital imaging, multiphoton microscopy, cellular dynamics, bone, inflammation

1. Introduction

The most unique characteristic of any biological system is its highly dynamic nature. A variety of cell types, hematopoietic as well as mesenchymal, are continuously circulating throughout the body, migrating through the peripheral tissues and interacting with each other in their respective niches. Conventional methodologies, such as histology, cell or tissue culture, biochemistry, and flow cytometry, have brought tremendous advances within this field, although the ability to investigate cellular dynamics in an entire animal remains elusive. Recent developments in optical microscopic techniques allow visualization of intact biological events that have not previously been seen. Of these, the development and improved usability of multiphoton excitation-based laser microscopy have revolutionized the biological sciences. In this brief review, we introduce the technical advantages of this novel microscopy method and provide some findings that only it could have revealed.

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2. The advantages of multiphoton microscopy

In this section, we delineate the advantages of multiphoton microscopy compared to conventional (single-photon) confocal microscopy (1 – 4). In confocal microscopy, upon excitation, a fluorophore molecule absorbs energy from a single photon and subsequently releases this energy as an emitted photon. In contrast, in multiphoton (usually two-photon) excitation, a fluorophore absorbs multiple (two) photons simultaneously. Such an event occurs rarely, and only in areas of high photon density. Based on this, multiphoton microscopy can spatially limit the excitation area to the focus point of an objective lens, concentrating excitation photons into a very narrow area. This spatiotemporally restricted excitation provides many advantages over confocal microscopy in terms of bioimaging. For one, bright and high-resolution images of regions deep inside tissues and organs can be obtained. Because the near-infrared wavelength lasers used for multiphoton excitation can pass deeper inside with less absorption or scattering than the

visible light used in confocal microscopy, depths of up to 100 – 1,000 μm can be visualized with multiphoton microscopy, whereas confocal microscopy is limited to a depth of less than 100 μm. This capacity is especially useful for imaging live tissues and organs. A broader range of tissues can be visualized using conventional microscopy if the object is fixed and thin-sectioned, but the cells in the section are already dead and not moving. In applications that involve visualization of live moving cells, the regions of interest are often present deep inside, where multiphoton excitation microscopy becomes useful. Moreover, excitation with near-infrared lasers can minimize photo-bleaching and phototoxicity, ideal for long-duration live imaging.

Nonlinear optical effects such as second-harmonic generation are another merit of multiphoton microscopy for visualization of live biological systems (4, 5). When high-intensity lasers pass through a coordinated material, a second-harmonic emission at precisely half the wavelength of the original light is generated. When near-infrared lasers (800 – 1,000 nm) are used for multiphoton excitation, the second harmonic emission is in the visible spectrum (400 – 500 nm). Because many intrinsic biological structures, including collagen fibers, muscle, brain, cornea, and bone, induce second harmonic generation, these can be visualized without exogenous fluorescent labeling.

3. Bone tissue imaging — the first "non-destructive inspection" of the bone marrow cavity

Bone is a highly mineralized hard tissue that limits the penetration of excitation lasers, and it is thought to be extremely difficult to observe intact bone tissues in living animals. Classical bone histomorphological analyses provide only static information regarding cellular and molecular distribution, not temporal changes thereof. We have established a new system for visualizing intact bone tissues and bone marrow cavities in live animals using an advanced imaging technique with intravital multiphoton microscopy (6, 7). Visualization of deep bone tissues is problematic, because not only visible but also infrared light is readily scattered by calcium phosphate crystals in the bone matrix. We selected the murine calvaria bone as the observation site, which is thin enough (approximately 80 – 120 μm) to allow passage of infrared lasers for multiphoton microscopy (Fig. 1). Using this system, we revealed the regulatory mechanism underlying the migration and localization of osteoclasts and their precursor monocytes in vivo. Furthermore, we successfully visualized the function and differentiation of osteoclasts in live bones.

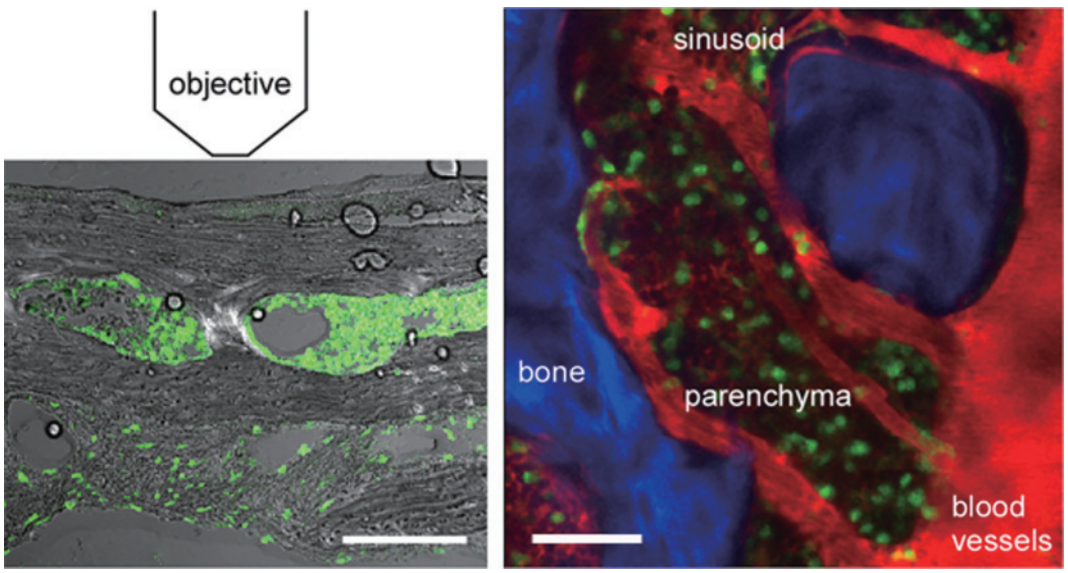

Fig. 1. Intravital multiphoton imaging of bone marrow. A vertical section of calvaria bone containing EGFP (green)-expressing bone marrow cells (left panel) and intravital imaging of bone tissues using multiphoton microscopy (right panel). Blood vessels were visualized using intravenously administered Texas Red–conjugated high molecular weight dextrans (70 kDa), and bone tissues were visualized without staining by second harmonic generation from collagen fibers (blue). CX_3CR1-EGFP-positive (green) monocytes can be seen in both bone marrow parenchyma and sinusoids. Scale bars represent 100 μm (left panel) and 50 μm (right panel), respectively.

4. Imaging of the cellular dynamics of bone-resorbing osteoclasts and their precursors in live bone

Osteoclasts are bone-resorbing giant polykaryons that differentiate from mononuclear macrophage/monocyte-lineage hematopoietic precursors (8, 9). Upon stimulation with several humoral factors, such as macrophage-colony stimulating factor (CSF-1 / M-CSF) and receptor activator of nuclear factor κB ligand (RANKL), osteoclast precursor monocytes attach to the bone surface, fuse with each other to form giant cells, and mediate bone resorption. However, how osteoclast precursor monocytes migrate to the bone surface and what controls their migratory behavior in vivo has remained elusive. Using this new intravital multiphoton imaging system, we demonstrated that sphingosine-1-phosphate (S1P), a lipid mediator enriched in blood, controls the migratory behavior of osteoclast precursors, dynamically regulating bone mineral homeostasis. Thus, we identified a critical control point in osteoclastogenesis.

S1P is a bioactive sphingolipid metabolite that regulates diverse biological functions, including cell proliferation, motility, and survival (10, 11). S1P signals via five 7-transmembrane receptors or G protein-coupled receptors (GPCRs), S1PR1 to S1PR5, previously referred to as endothelial differentiation gene (Edg) receptors (12). We found that macrophage/monocyte-lineage cells, including osteoclasts, expressed both S1PR1 and S1PR2 (6, 13), and that they were chemoattracted to S1P in vitro, a response that is blocked by pertussis toxin. To validate their in vivo significance, mobilization of osteoclast precursors by S1P stimulation in vivo was demonstrated using intravital bone imaging. For identifying osteoclast precursors in visual fields, we utilized transgenic or knock-in mice expressing EGFP under the promoter of CSF1R or CX_3CR1, both of these receptors are predominantly present in monocytoid cells including osteoclast precursors. While these cells were stationary at the steady-state in bone tissues, osteoclast precursors were stimulated and moved into vessels when a potent S1PR1-specific agonist, SEW2871, was injected intravenously, meaning that osteoclast precursors were mobilized by S1P stimulation in vivo. These imaging experiments unequivocally identified the S1P-mediated regulatory mechanism of osteoclast precursor migration in vivo.

Further analyses demonstrated the fine tuning of osteoclast precursor migration by two reciprocal S1P receptors, S1PR1 and S1PR2 (13), and the mode of osteoclastic bone resorption in vivo (under review). Bone marrow is an important tissue not only for osteoclast and bone remodeling, but also for hematopoietic differentiation and maintenance of stem cell interactions with mesenchymal/stromal cell networks. Intravital multiphoton bone imaging will be useful in a wide array of biological sciences in the near future.

5. Immune system imaging to visualize the dynamics in immune and inflammatory conditions

5.1. Second lymphoid organs (peripheral lymph nodes)

One of the earliest applications of intravital multiphoton imaging was visualization of an explanted lymph node (14). Different lymphocytes such as T and B cells can be visualized in lymph nodes when they were labeled membrane-permeable fluorescent dyes and adoptively transferred into recipient mice. Naive T cells showed higher motility than B cells in lymph nodes, which challenged the previous belief that T cells were immobile in the absence of antigen stimulation. Further analyses have proposed a model of "organized migration" of T cells, rather than "random-walking", based on the presence of unseen objects such as other cells, stroma, and the reticular network (3, 15). Live imaging has revealed that T cells change their migration behavior upon contact with antigen-presenting cells (3, 14). Once T cells encounter activated antigen-presenting dendritic cells (DCs) in a lymph node, they generate stable complexes that last for at least several hours. Thereafter, the T cells regain their motility. In contrast, T cells have a much shorter sustained contact with DCs during tolerance induction. These results clearly indicated that immune reactions such as antigen presentation between T cells and DCs are highly dynamic and their migratory behaviors and function were closely associated each other.

It is thought that B cell proliferation occurs only in the germinal center and that activated T cells, which exhibit decreased expression of C-C chemokine receptor 7 (CCR7) and increased expression of C-X-C chemokine receptor 5 (CXCR5), migrate towards B cell follicles to help promote antibody production. Interestingly, real-time imaging has revealed that B cells upregulate CCR7 expression and migrate to the follicle boundary (16). Intravital imaging has revealed spatiotemporal aspects of these dynamic cellular interactions in lymph nodes.

5.2. Thymic selection

In thymic cultures, multiphoton microscopy has demonstrated the interactions between thymocyte and stromal cells during positive and negative selection (17). $CD4^+CD8^+$ double-positive immature thymocytes localize in the outer cortex. During positive selection, they become $CD4^+CD8^-$ or $CD4^-CD8^+$ single-positive thymocytes and migrate to the central medulla. Live imaging demonstrated that thymocytes were highly motile and that their recognition of MHC molecules was associated

with both stable and dynamic contacts with stromal cells. These different interaction patterns may be associated with different signals or could correspond to different stages of selection. After positive selection, the thymocyte population displayed rapid, directed migration toward the medulla (17). Compared to those in the cortex, medullary thymocytes migrated limitlessly and more rapidly, and they made frequent and transient contacts with DCs. During negative selection, thymocytes migrated slowly and in a highly confined manner within zones of up to 30 μm in diameter (18).

5.3. Blood vessels: pathways for immune and inflammatory cells

Following activation within lymph nodes, immune cells can enter zones of inflammation through blood vessels (19). Neutrophils, normally visualized as lysozyme M – EGFP-positive cells, are the first cell types to be recruited to inflamed sites. Intravital microscopy has observed consecutive dynamic inflammatory cell events, such as tethering, rolling, crawling, and invasion. This migratory behavior is controlled by selectin adhesion molecules, such as P- and L-selectin, as well as integrins.

In addition to neutrophils, monocytes and macrophages also circulate through the vascular system, crawling over the endothelial cell surface (19). Their attachment depends on interactions between C-X_3-C-chemokine receptor 1 (CX_3CR1) and CX3C-chemokine ligand 1 (CX_3CL1), and between lymphocyte function–associated antigen1 (LFA1) and intercellular adhesion molecule 1 (ICAM1).

5.4. Autoimmune inflammatory models

Dynamic behaviors of antigen-specific pathogenic T cells have been visualized in the spinal cord of a murine encephalitis model, EIA (20). Therein, T cells are highly motile and arrest antigens upon recognition in the same manner as in lymph nodes. In a type I diabetes model using NOD mice, interactions between antigen-specific T cells and DCs were observed in a draining lymph node (21). Islet antigen-specific $CD4^+CD25^-$ T helper (Th) cells and regulatory T (Treg) cells homed to similar areas of the lymph node, and their movement patterns were indistinguishable; that is, they both swarmed and arrested in the presence of antigens. No stable interaction between Th cells and Treg cells was observed, but Treg cells interacted directly with DCs and inhibited their activation of Th cells.

6. Future perspectives in intravital multiphoton microscopy

Intravital multiphoton imaging has revealed, and continues to reveal, the dynamic features underlying various physiological and pathological conditions. Its greatest strength is its ability to generate spatiotemporal information regarding living systems, which is not possible by using conventional methods. However, current multiphoton microscopy imaging techniques have several limitations. First, we should keep in mind that not everything in visual fields is observed using multiphoton microscopy. Although fluorescence labeling and second-harmonic generation enables us to detect target cells and organs, a paucity of signal does not necessarily reflect an open field, and diverse structural constituents and cellular components may be present. To avoid misinterpretation, we must interpret our observations with caution. Second, although multiphoton microscopy is able to penetrate tissue to a greater depth than conventional confocal microscopy, the maximum is only 800 – 1,000 μm in soft tissues (e.g., brain cortex) and 200 μm in hard tissues (e.g., bone). Therefore, it may only be applied to small animals, such as mice and rats. Moreover, due to the wide scattering of light by the skin, target internal organs must be exteriorized. It is possible that the necessary operative invasion and resultant changes in oxygen concentration and humidity may influence cellular behavior. To resolve these problems, technical innovations in fluorochrome and optical systems, including improvements in light emission and amelioration of resolution problems (22), are needed.

Intravital microscopy has begun to be applied in not only observational studies, but also functional analysis and interventions. Several new fluorescence tools have recently been developed; these include cell-cycle indicators (23) and light-sensing devices such as photoactivatable fluorescent proteins (24). Although their limitations remain to be resolved, the range of in vivo imaging applications using these new probes continues to expand.

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