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Dynamic Multiplex Tissue Imaging in Inflammation Research

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Abstract

Inflammation is a highly dynamic process with immune cells that continuously interact with each other and parenchymal components as they migrate through tissue. The dynamic cellular responses and interaction patterns are a function of the complex tissue environment that cannot be fully reconstructed ex vivo, making it necessary to assess cell dynamics and changing spatial patterning in vivo. These dynamics often play out deep within tissues, requiring the optical focus to be placed far below the surface of an opaque organ. With the emergence of commercially available two-photon excitation lasers that can be combined with existing imaging systems, new avenues for imaging deep tissues over long periods of time have become available. We discuss a selected subset of studies illustrating how two-photon microscopy (2PM) has helped to relate the dynamics of immune cells to their in situ function and to understand the molecular patterns that govern their behavior in

vivo. We also review some key practical aspects of 2PM methods and point out issues that can confound the results, so that readers can better evaluate the reliability of conclusions drawn using this technology.

1. INTRODUCTION

Biologists are most interested in the experimental results obtained by two-photon microscopy (2PM) imaging, and to this end, we begin this review by providing examples of immunobiological studies in which the technical advantages of 2PM enabled conclusions that would not have been possible without this method (**Figure 1**). However, it is critical for readers to understand how the method works and its limitations, to enable informed evaluation of the existing and future literature and the conclusions drawn by investigators using this tool. For this reason, we devote the second part of the review to a description of the key optophysical principles, technical approaches, and limitations of the method.

2. TWO-PHOTON MICROSCOPY FOR THE STUDY OF PHYSIOLOGICAL AND PATHOLOGICAL ASPECTS OF IMMUNITY

The goal of dynamic imaging of peripheral tissues is to evaluate the behavior of cells and their interactions with each other, either at the level of individual cells or in the context of anatomical compartments. Despite recent advances in single-cell profiling, we still have an incomplete understanding of how these cells behave within complex tissues and how their molecular states translate into function in such environments. Intravital imaging methods allow us to assess these dynamics both in naive tissues and during the onset, propagation, and resolution of inflammatory responses.

Intravital imaging does not rely exclusively on two-photon excitation. Model organisms such as zebrafish larvae are inherently translucent and allow the use of wide-field illumination approaches on thin tissue compartments such as the dorsal fin. Confocal techniques such as spinning disk microscopy can be used to visualize dynamic processes on the surface or near the surface of visceral organs in mammals that do not have optical or physical barriers. However, for imaging opaque tissue at depths greater than 20–30 μm or under a fibrous capsule, multiphoton excitation is the method of choice. With the right model animal, experimental system, and optical equipment, we are now able to visualize the inflammatory cascade in response to various sterile or infectious challenges. The typical duration of continuous imaging found in publications is usually less than an hour but can extend to several hours (1, 2). While many pathological conditions are too complex or lengthy for complete dissection using these methods, these imaging approaches have played a crucial role in defining basic concepts of immunophysiology and the cellular events involved in acute inflammatory processes.

2.1. The Very Early Responders: Platelet Activation and Aggregation

When the integrity of our vascular system is lost in a traumatic injury, the first dynamic cellular events are the recruitment, activation, and aggregation of platelets. The controlled transition from passive flow to targeted accumulation at site of injury requires elaborate and robust mechanisms, especially in high-flow and high-pressure environments. 2PM can be used to study the dynamic orchestration and the molecular mechanisms involved in blood clotting in different models of experimental venous thrombosis (3) or of arterial damages through laser ablation (4). The key role of platelets in guiding $CD8^+$ T cells to target sites in the infected liver has been uncovered using 2PM (5).

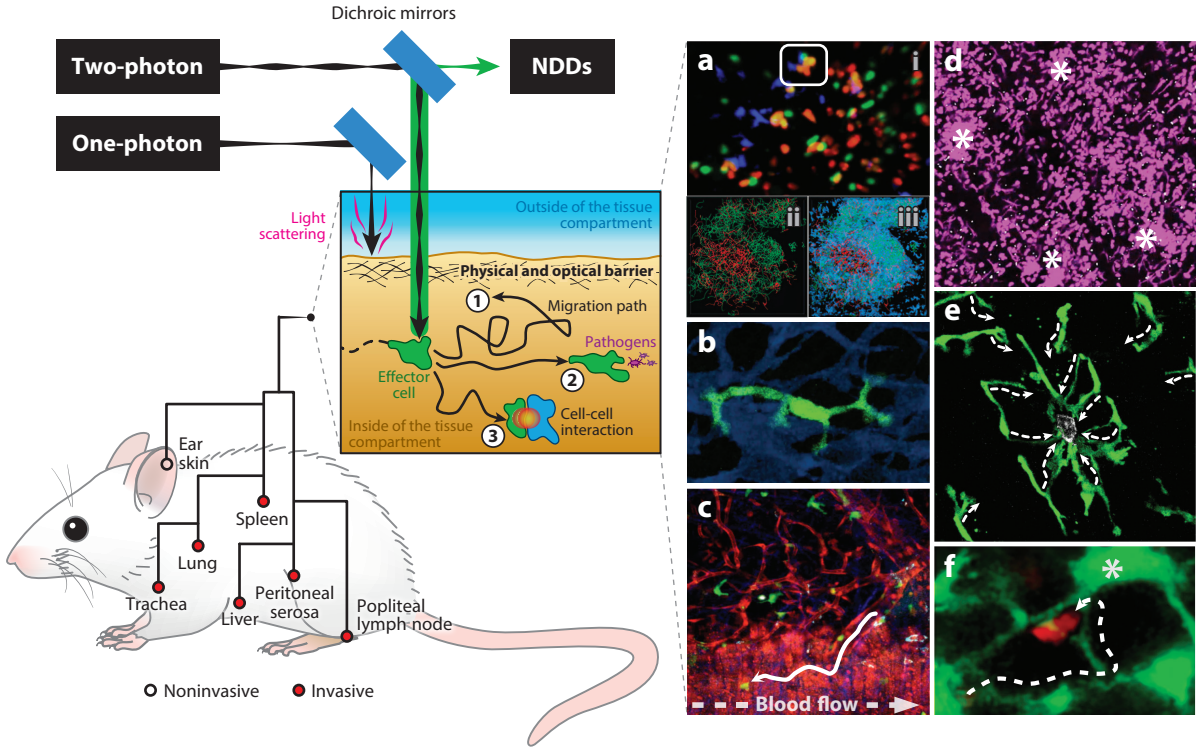

Figure 1

Schematic depiction of a selected subset of established surgically invasive or noninvasive 2PM experiments. While one-photon excitation fails to reach deep tissue compartments due to light scattering, two-photon excitation enables the excitation of fluorescent proteins expressed in immune cells, even deep in opaque tissue. Time-lapse imaging can be used to study various dynamics, including exploratory migration of sentinels such as (①) neutrophils, (②) targeting dynamics of immune cells toward sterile or infectious triggers, or (③) migration arrest and tight cell-cell interactions as in antigen presentation. In this way, 2PM enables real-time visualization of various biological aspects in vivo: (*a*, *i*) interactions between T cells (TCR-transgenic T cells in *red* and naive wild-type T cells in *green*) and cognate antigen-presenting dendritic cells (*blue*) in popliteal lymph nodes, or (*ii*, *iii*) T cell migration dynamics in germinal centers, e.g., wild-type (*red*) versus SAP-deficient (*green*) antigen-specific T cells (panels *ii* and *iii* adapted from Reference 72); (*b*) steady-state behavior of resident tissue macrophages (*green*) and stromal fibroblasts (*blue*) in the peritoneal serosa; (*c*) patrolling behavior (indicated by *white arrow*) of resident monocytes (*green*) inside the circulatory system (*red*); (*d*) swarm dynamics of extravascular neutrophils (*magenta*) in response to bacteria (*white asterisks*) (panel *d* adapted from Reference 17); (*e*) damage response (cloaking) of resident tissue macrophages (*dashed arrows* indicate directed movement of membrane protrusions toward the focus) (panel *e* adapted from Reference 17); (*f*) migratory dynamics (*white dashed arrow*) of T cells (*red*) along the fibroblastic reticular cell network (*green* and indicated by *asterisk*) inside lymph nodes (panel *f* adapted from Reference 154). Abbreviations: 2PM, two-photon microscopy; NDD, nondescanned detector.

2.2. Neutrophils: One of a Dynamic Imager's Favorite Cell Types

Neutrophils are a popular cell type for dynamic imaging due to their distinct and versatile dynamic behavior in vivo (6). However, neutrophils are particularly sensitive to disturbances in tissue integrity caused, for example, by manipulations during an experimental procedure or by light damage itself. Fortunately, with 2PM, intact tissue compartments such as the skin dermis in nonpigmented mice can be explored, while phototoxic damage is limited. Utilizing noninvasive 2PM imaging, intravascular recruitment of neutrophils (7) as well as their extravascular swarming behavior has been studied with high temporal resolution and functional granularity in vivo. The experimental versatility of model systems such as zebrafish allows the study of the molecular

mechanisms of neutrophil recruitment, activation, and clearance with high resolution (8). While the zebrafish larva's anatomy allows for the use of confocal or even wide-field imaging applications, 2PM can add additional value here, for instance, by measuring fluorescence lifetimes, which provides information about metabolic processes and the cellular redox state (9). However, for analysis of immune effector functions in the context of mammalian tissue stroma, rodent model systems are of greater relevance.

In mice, neutrophils are released en masse from the bone marrow when inflammation is triggered, a process readily visualized with 2PM in vivo (10). In peripheral tissues, neutrophils follow cytokine signals that regulate their mass movements in a highly orchestrated manner. The cremaster model has proven to be a particularly valuable system to study neutrophil recruitment and extravasation dynamics in response to specific stimuli such as tumor necrosis factor (TNF) (11). 2PM studies on footpads infected with *Escherichia coli* showed that the LTB4-BLT1 (leukotriene B4/leukotriene B4 receptor 1) axis acted in an autocrine/paracrine manner to recruit and arrest neutrophils in circulation and induce actomyosin-dependent morphological polarization of cells that eventually drove their extravasation, and that this mechanism required the release of extracellular vesicles from neutrophils (12). LTB4 is a factor of particular importance for neutrophil dynamics. 2PM studies revealed that LTB4 not only sets the pace intravascularly but also determines the behavior of extravascular neutrophils: LTB4-driven local auto-signal amplification was determined to be a key driver of neutrophil swarming in tissues (13). Short pulses of two-photon excitation were used to induce sterile tissue damage within a defined region of interest as a target for extravascular neutrophils with precise experimental control of the size, location, and timing of the damage (13–16). With this method, it was shown that the initial approach of extravascular neutrophils to a lesion follows a rather exploratory migration pattern (13). Once these "pioneer" cells come into physical contact with debris, rapid but molecularly ill-defined cell death occurs (17), leading to the formation of a signal-relay LTB4 gradient that attracts increasing numbers of activated neutrophils, manifested as swarming behavior (13, 18). Through this relay effect, activation of theoretically a single sentinel can trigger the rapid mobilization and aggregation of a multicellular infiltrate.

Ultimately, this amplification circuit must also be turned off. 2PM studies showed that negative control is mediated in part by receptor desensitization, which limits swarm spread and thus collateral tissue damage. Surprisingly, this negative regulation also is key to effective bacterial killing at the swarm site, as shown in a mouse model of *Pseudomonas aeruginosa* infection (19, 20). Recently, 2PM has been used to capture rare events of reverse transendothelial migration of neutrophils in mice as a potential mechanism to resolve inflammation, but which in turn could also "carry" inflammatory damage to distant organs (8, 21). Ultimately, neutrophils that have not died in action in vivo must be removed from the system in an orderly fashion. 2PM has revealed that migrated older neutrophils interact specifically with bone marrow resident macrophages, suggesting that they drive their own phagocytosis and thus their silent removal (22). Thus, 2PM allows us to visualize in great detail the entire life cycle of neutrophils in vivo, from their generation and release, through the fulfillment of their purpose, to their final elimination.

2.3. Resident Tissue Macrophages: Silent Safeguards of Tissue Homeostasis

2PM has documented the patrolling phenotype of nonclassical monocytes probing the luminal side of vessels to clear endothelial debris (23) as well as the recruitment dynamics of monocytes in a sterile wound and their conversion from a proinflammatory to an anti-inflammatory, reparative phenotype in situ (17, 24, 25). With appropriate labeling of the different cell types, 2PM can also be used to study the complex interactions between two or more myeloid cell types such as neutrophils and monocytes or macrophages (26, 27). 2PM allows penetration into the abdominal wall and

direct visualization of peritoneal macrophages in their free-floating state in the abdominal cavity. In this way, the cells were found to swiftly assemble at the site of peritoneal injury in a manner similar to platelets aggregating at the site of endothelial injury (28) and even to use molecular tools known for their specific function in coagulation (29).

Assessing the biology of tissue macrophages as opposed to monocytes or monocyte-derived macrophages in either healthy or pathologic environments is challenging because the surgical intervention required for optical access may interfere with their state. This may be particularly the case when using disease models or inducible challenges that exceed local regulatory circuit capacity—such situations have historically been selected for their ability to reliably trigger neutrophil-driven inflammation. Resident tissue macrophages (RTMs) that derive from embryonic seeding events (30) receive instructive and priming interactions from their immediate environment; these interactions determine their functional phenotype (31, 32). Changes in extracellular milieu can abrogate their dynamics (as discussed below) or their functionality can swiftly be eclipsed by infiltrating immune cells (33). When removed from their host tissue, they eventually lose their identity (34). Assessing the biology of RTMs in their natural environment has therefore become increasingly important.

Kupffer cells, the resident macrophages of the liver sinusoids, are also widely studied using different ex vivo or in situ approaches (35). The liver parenchyma can be visualized using one-photon excitation close to the liver surface (36, 37) or deeper in the liver parenchyma, further away from potential interfering influences using 2PM (2, 5, 38, 39). The latter approach has been used to investigate specific functions of Kupffer cells such as the intravascular clearance of opsonized particles or cells (40, 41) or to study dynamics of Kupffer cell niche turnover after their depletion (42).

Like Kupffer cells, interstitial macrophages, which are resident to virtually all mammalian tissues, are predominantly nonmigratory. Apart from smaller specialized subsets with a limited range of motion (43), most RTMs are distributed in a stationary matrix-like manner and inherently sessile as revealed by 2PM (17). Initial 2PM studies of microglia, the resident macrophages of the brain parenchyma, revealed that these stationary cells are in fact highly dynamic at their own scale; microglia continuously probe their environment by extending and retracting membrane processes at regular intervals without leaving their position (44), a behavior also observed for Langerhans cells in the mouse skin (45).This physiological dynamic comes to a sudden halt when microglia are challenged with local damage. The cells immediately stop probing and instead converge their processes around the lesion (46). Similar dynamic behavior could be found in interstitial macrophages of peripheral tissues: RTMs continuously probe their environment and take up fluids via untargeted pinocytosis (47). When faced with a sudden sterile injury they also stop sampling and converge their membrane processes around the lesion (cloaking) without leaving their position (17). Such a cloaking response protected the tissue from unwanted neutrophil-driven inflammation to microlesions.

2.4. Visualizing Adaptive Immune Responses: Translating Cell Contacts into Function

One main advantage of 2PM is the ability to investigate individual cell behavior in an anatomical context, allowing us to infer function from tissue structure and organizational aspects. 2PM has been successfully performed with TCR-transgenic T cells and cognate antigen-presenting cells (APCs) to study functional interactions within spleen and lymph nodes, revealing prototypical dynamic patterns, including increased migratory activity of T cells upon activation, their scanning of APCs, and prominent arrest after contact with their cognate antigen (48–57). Since within this process early switches are set that determine differentiation fate and the long-term outcome of the immune response, this knowledge is of utmost importance for the rational development

of vaccine strategies. Regions of antigen capture can differ from zones of antigen presentation and recognition by T cells. 2PM in vivo tracking of B cells in the spleen revealed targeted migration patterns of B cells from the marginal zone into the follicles, a mechanism by which they supplied the follicles with opsonized antigens—with direct implications for the adaptive immune response to blood-borne antigens (58). Experimental evidence now allows us to draw conclusions from migration behavior to cytokine production, linking cell dynamics to downstream effector functions, as done for T cells within the skin during delayed hypersensitivity responses or within mycobacterial granulomas (1, 38).

In both lymph nodes and spleen, different dendritic cells (DCs) present antigens to different T cell effectors in spatially separated compartments (59). Given the multiplicity of cell types and potential immune interactions, this underscores the essential importance of spatial organization and strategic positioning of all cellular players, evolved to increase the likelihood of the cell-cell contacts critical for adaptive immune responses (60–62). This positioning is tightly regulated. Direct visualization of type-2 conventional DC motility in the spleen by 2PM showed that a mechanosensing mechanism involving interactions between CD97 and CD55 on the surface of red blood cells results in the retention of APCs in lymphoid tissue that would otherwise enter the bloodstream and thus be unable to present their blood-borne antigens (63).

2PM imaging has been especially valuable in studying T cell–dependent humoral immune responses. A series of seminal studies revealed a role of subcapsular sinus macrophages in antigen capture and transfer via B cells to the follicular DC network (64–70). Using in situ photoactivation in the photoactivatable green fluorescent protein (PA-GFP) mouse model, 2PM showed that B cells that had migrated to the light zone were selected in that location by follicular T helper cells on the basis of the amount of antigen taken up, after which they returned to the dark zone for clonal expansion (71). This selection process is not random. 2PM has shown that B cells that acquire a higher affinity B cell receptor actively attract T follicular helper cells via CCL22, which in turn induces CCL22 expression in B cells in a feed-forward manner, providing them with even more "help." Without this arrangement, affinity maturation is abrogated (55). Other 2PM studies showed that a decreased duration cell-cell adhesion following loss of expression of the small adaptor SAP (signaling lymphocyte activation molecule-associated protein) in T cells prevented effective participation in the germinal center reaction but not activation by antigen-bearing DCs. These differences in lymphoid versus myeloid cell adhesion helped explain why, in addition to the humoral defect in this genetic disease, Epstein–Barr virus infections occurred in X-linked lymphoproliferative disease, presumably due to poor CD8 T cell–B cell target cell interactions (72).

Single-cell tracking in vivo is sensitive and can be used to quantitatively assess small perturbations of the system. Using 2PM, the transcription factor Bcl6 was shown to be functionally important not only for the development and survival of follicular T helper cells but also for their support of germinal center B cells (73). Inadequate help at this critical early stage may alter the overall course if the initial selection of the most potent B cell receptors does not occur. While only a few B cells are selected for survival and further maturation because of these T–B cell interactions, most cells are destined to die. Direct visualization of B cell apoptosis using a fluorescence resonance energy transfer–based apoptosis indicator dependent on caspase-3 activity has indeed shown that B cell apoptosis in the light zone is a ubiquitous feature of germinal center dynamics, evolved to select only the best effectors. Apoptosis also occurs in the dark zone, but there due to random, often deleterious mutations during the process of affinity maturation (74).

2.5. Using Infections to Understand Immunity

2PM has been used to study dynamic aspects of various infectious diseases in vivo. *Staphylococcus aureus* is a commonly used model pathogen for such imaging, and several genetic variations as

well as fluorescent reporter systems have been developed. Tracking transendothelial migration with 2PM in infected skin demonstrated that neutrophils use inflamed postcapillary venules for tissue entry in response to cytokines released by surrounding perivascular macrophages. *S. aureus* employed alpha-hemolysin to deplete macrophages and block neutrophil recruitment to promote survival (75). When local containment failed, neutrophils entered *S. aureus*–infected lymph nodes through high endothelial venules to rapidly clear the infection and prevent further spread through the lymphatic system (76).

2PM has also been used to quantify vascularization and collagen formation during the healing process (77). While the dynamics of neutrophils are key to their antipathogenic function, their motility can indeed be exploited by certain pathogens, as demonstrated for *Toxoplasma*. Following oral infection, the pathogen invaded intestinal tissue, triggered local granulocyte-rich inflammation, and selectively invaded neutrophils but was not itself eradicated. These hijacked neutrophil granulocytes remained highly migratory and were exploited to spread infection via a luminal passageway (78). Ultimately, *Toxoplasma* targets the brain parenchyma, and its passage through infected endothelial cells, which serve as replication niches prior to central nervous system lysis and infiltration, could also be visualized directly in vivo using 2PM (79). A similar process involving uptake of *Leishmania major* by neutrophils, then death of the neutrophil and reuptake of viable parasites by macrophages whose exposure to the dead neutrophils inhibited parasite killing, was also studied using 2PM (7).

Viruses have been widely used in 2PM as antigen carriers to study antigen presentation (61) and as vectors to introduce fluorogenic expression constructs into the tissue of interest (80). 2PM has also been used to study the dynamics of immune responses to viral infection itself. While recent studies have begun to address the dynamics of SARS-CoV2 (severe acute respiratory syndrome coronavirus 2) spread between cells (81), influenza viruses of different virulence have been widely used to study immune processes in vivo. Effector T cells typically need engagement by cognate antigen to mediate effector activities that contribute to viral clearance. Using 2PM, it was observed that antigen-bearing neutrophils in the acute infiltrate actively attract and are phagocytized by recruited monocytes. These monocytes in turn act as presenters of neutrophil-derived viral antigens to activate T cells, which are essential for effective viral clearance (82). 2PM imaging also showed that viral elimination is not the end of the anti-influenza immune response. Instead, sterile antigen presentation appears to continue long after virus elimination, with implications for the resolution of inflammation or sustained immunopathology (83). Humanized mouse models have also been utilized for analysis of human viral pathogens, for example, the spread of human immunodeficiency virus (HIV) (84). HIV infection of T cells resulted in strong migratory activity leading to their widespread dissemination and increased egress from lymphoid tissues, resulting in systemic viral spread. Moreover, HIV was shown to actively induce fusion between cells and creation of virologic synapses in vivo, which may facilitate cell-cell transmission. Thus, 2PM enables not only the study of physiological immune responses to pathogens but also insights into disease pathogenesis, the development of chronicity, and important switching points in the immune system. The latter may be exploited by the pathogen, but the knowledge gained can also guide therapeutic interventions.

2.6. Autoimmunity and Autoinflammation: Being Right There When Immune Regulation Goes Wrong

With the increasing prevalence of autoimmune diseases, there is growing interest in understanding the dynamics of immune cells in diseased tissues. Visualizing cellular processes in already inflamed sites is challenging: The tissue of interest is often deeply embedded in body cavities or

hidden behind a dense fibrous capsule and cannot be easily exposed experimentally without damage, dense cellular infiltrates appear chaotic and make cell tracking difficult, and compartments become difficult to delineate. In addition, many established models of autoimmune diseases based on immunizations have different penetrance depending on the mouse strain used and are difficult to synchronize, confounding facile interpretation of data. Inflammation models in which autoreactive effector cells are transferred into a susceptible host or an inflammation-inducing agent is applied systemically are advantageous here, even if this approach omits crucial aspects of the disruption of self-tolerance and only the inflammatory effector phase is assessed.

While other imaging modalities are currently more commonly used (85), 2PM has been employed to study mouse models of inflammatory arthritis that resemble human rheumatoid arthritis. Using 2PM on mouse ankles in the serum transfer model of inducible arthritis, the complement receptor C5aR was shown to be critical for neutrophil adhesion to the endothelium in inflamed joints. This adhesion was a prerequisite for subsequent LTB4-BLT1-driven extravasation (86), the universal activation cascade of neutrophil-driven inflammatory processes.

The same anatomical site was studied in an immunization-based model of collagen-induced arthritis. Although mechanistic insight was limited, it could be shown that in an inflamed mouse synovium, application of therapeutic CTLA-4 immunoglobulin directly targets macrophages and fibroblasts and that chronic inflammation causes abnormalities in synovial lymphatic drainage that may serve as a potential amplifier (87). This inflammatory process often involves the adjacent bone tissue and leads to erosive loss in both mice and humans. Simultaneous visualization of osteoblasts and osteoclasts by 2PM revealed reciprocal regulation via direct cell-cell interaction (88), and chronic arthritis disrupts such homeostatic circuits. Infiltrating RANKL-producing Th17 cells could be observed to interact directly with osteoclasts and modulate their bone-degrading function (89). In addition to dynamic assessment of cellular interactions, second-harmonic generation (SHG) in 2PM was used to evaluate extracellular matrix remodeling and collagen changes in experimental osteoarthritis of the temporomandibular joint (90). Thus, while direct, high-resolution insight into synovitis is often still limited due to anatomic obstacles, certain pathognomonic features of human disease can be recapitulated and studied in mouse models.

Another disease in which 2PM has provided great functional insights is autoimmune diabetes. The pancreas is hidden deep in the retroperitoneal compartment and is not readily mobilizable. While protocols have indeed been established for imaging the pancreas in situ (91, 92), more recently, immunological events have been studied primarily in explanted islets. Thus, it was shown that migrating DCs continuously sampled the tissue and took up islet antigens for major histocompatibility complex II–dependent presentation (93, 94). Analogous to the dynamic processes involved in the establishment of physiological adaptive immune responses in lymph nodes, autoimmune diseases such as T cell–mediated insulitis were also characterized by marked T cell arrest and direct interactions with local APCs, which differ according to the disease stage. In insulitis, local APCs limited autoreactive T cell engagement and activation via MERTK, with the absence of this molecule in turn promoting increased T cell activation and tissue destruction (95).

Some organs and tissues are especially difficult to study using 2PM, but these technical barriers have been overcome to yield important information, with the lung being a prime example. Sophisticated protocols have been established to preserve physiologic conditions and minimize organ movement during respiration (96). With such technical advances, investigators have been able to study the interplay between alveolar macrophages and DCs in capturing and processing *Bacillus anthracis* spores, as just one relevant example (97). From a technical point of view, it is noteworthy that the authors here use the SHG collagen signal for image registration and motion correction in postprocessing. In addition to infectious models, which include influenza and *S. aureus*, 2PM revealed that the lung harbors unexpected cells—megakaryocytes—in its microcirculation.

Direct visualization confirmed that these cells actively produce and release substantial numbers of platelets during their lung passage (98). The arrival and subsequent fate of circulating tumor cells could also be observed using 2PM lung imaging (99). It was possible to witness directly how they interacted with various resident and recruited myeloid cells, which together formed an early metastatic niche that can show either tumor-promoting or tumor-suppressive properties. Given the major importance of the tumor microenvironment in tumor growth and progression, understanding the dynamic processes of its cellular players is crucial for the contextual development of therapeutic strategies not only in mice but also, especially, in humans. One 2PM study showed how natural killer (NK) cells rapidly kill metastatic tumor cells in the lung microcirculation until accumulation of shed ligand for a key activating NK receptor forms a shield against further NK cell activation and tumor lysis (100). In another report, 2PM imaging of human melanoma tumor sections revealed that NK cells within the tumor microenvironment are an important source of critical survival factors (Flt3L) for antitumor DCs required for an antitumor immune response. Here, time-lapse imaging revealed frequent and long-lasting interactions between NK cells and $XCR1^+$ DCs as a dynamic expression of this cellular communication (101). Finally, several studies have used 2PM to investigate lymphocyte-myeloid cell interactions in the tumor microenvironment, revealing opposing effects of interactions with macrophages versus DCs on T cell activity (102) or perivascular niches where a conjunction of chemokines and cytokines promotes $CD8^+$ T cell effector function (103).

3. PRINCIPLES OF TWO-PHOTON MICROSCOPY

The brief overview above of the many ways 2PM imaging has provided new insights into physiological and pathological immune cell behavior in vivo makes clear the value of this technology. But at the same time, many readers of studies utilizing this method are unaware of the many potential technical pitfalls involved in conducting 2PM, as well as the details of the methods necessary to garner useful information. We thus feel it is valuable to spend the remainder of this review providing information on these topics, so that readers can make more sophisticated judgments about the likely validity of conclusions drawn from 2PM studies that have been published or presented in lectures.

The most fundamental point is that if our cells did not refract and scatter visible light, we would all be transparent. The probability that an electromagnetic wave such as light will be scattered by tissue is inversely proportional to its wavelength. The same principle applies to sound waves and is easily observed in medical ultrasound applications: Sonographic examination of deeper organs, such as the kidneys, requires longer wavelengths than, for example, examination of peripheral joints. This also applies to the light used in optical microscopy. Light with wavelengths in the visible range is scattered by a wide range of molecules and structures, starting with the first cell layer of our skin. Conventional optical systems therefore fail when it comes to making deeper structures or cells visible.

3.1. Two-Photon Absorption and Excitation

To better penetrate tissue and deliver light into deeper compartments, one can increase the wavelength of the light toward the infrared spectrum. However, most fluorescent molecules commonly used in various biomedical approaches were developed to be excited by the visible light spectrum (e.g., GFP). As we move away from the visible spectrum toward the infrared, photons become less energetic and we quickly lose the ability to excite these molecules, with the consequence that we do not see anything at all. Here, one can make use of a natural, albeit extremely rare, phenomenon called two-photon absorption (2PA). When two photons strike an excitable molecule

simultaneously or within an exceedingly short interval, both photons can be absorbed by the same molecule, adding up their respective energies. However, the time frame in which this summation can take place is in the pico- or femtosecond range. Thus, to reliably achieve 2PA, a photon density that provides a sufficiently high probability of 2PA occurring is required. Such density would allow imaging using infrared light that penetrates deeper into the tissue but is still able to excite different fluorescent molecules. Conventional lasers are unable to generate such high photon densities. 2PA requires special laser systems with pulsed outputs. Since the simultaneous absorption of the second photon must occur before the state excited by the first photon is lost, ultrashort but very intense laser pulses in the femtosecond range are necessary. This can be achieved, for example, with mode-locked titanium-sapphire lasers, which have a typical excitation range of 680 to 1,400 nm. This range allows light to penetrate deep into tissue, up to 300 or even 500 μm below the surface.

3.2. Minimizing Off-Focus Effects with Two-Photon Excitation

2PM uses point scanning for image acquisition, and 3D imaging is achieved by adjusting the focus up or down in the axial direction. In regular optical microscopy, single-photon excitation occurs not only in the focal plane but also in the tissue above and below this plane, resulting in significant out-of-focus excitation and emission that produces a blurred image in wide-field microscopy. To avoid this blurring and get closer to the actual optical section of interest, the emitted light can be refocused through confocal apertures (pinholes)—a process called descanning that physically blocks the blurred emission and by which confocal microscopes achieve sharp images. 2PA is nonlinear (i.e., the probability of its occurrence is proportional to the square of the light intensity). Consequently, 2PA takes place only in the actual focal plane of the optical system—2PM is therefore inherently confocal, as only the focused tissue area is excited, eliminating out-of-focus emission. While 2PA-emitted light can be detected with the same internal system used for confocal imaging, most two-photon applications use external detector units [nondescanned detectors (NDDs)] that are not shielded by confocal apertures but collect as much light as effectively as possible—both inside and outside the optical focus, as scattered photons can now also contribute to the generation of a useful image since they originate only from the plane of focus where the excitation laser intensity is adequate. In this way, a significant increase in performance can be achieved using NDDs, without a decrease in spatial resolution. This imaging method is similar in principle to stereotactic radiotherapy. The latter method spares healthy tissue by concentrating smaller doses of irradiation specifically within the tumor bed where they add up to a therapeutically effective radiation dose. The use of longer wavelength excitation light in 2PM likewise prevents phototoxic damage outside the focal plane, as we reach sufficient energy levels only within it. This makes 2PM an excellent optical tool for studying biological processes in living tissue without risking too many out-of-focus effects. One trade-off is that two-photon lasers create substantial heat in the imaged tissue, and this must be taken into account in experimental design to avoid tissue damage from this aspect of the setup.

3.3. One-Photon Versus Two-Photon Excitation of Fluorophores

In 2PA, the energetic difference between the excited state and the ground state of the absorbing molecule is equal to or smaller than the sum of the energy of the two absorbed photons. This difference depends on the specific absorption properties of the fluorescent molecule. If both photons are completely absorbed and their energies add up exactly, an excitation with approximately twice the one-photon excitation can be expected. However, a molecule's capacity to absorb one or two photons can differ: Two fluorescent molecules that exhibit similar characteristics in one-photon

microscopy may show very different behavior in 2PM. For example, the commonly used fluorescent cell dye CMFDA (5-chloromethyl fluorescein diacetate) and GFP are both excited with a 488-nm laser in conventional light microscopy and emit light at approximately 500–520 nm. While their fluorescence behavior is similar in single-photon excitation, the differences in their 2PA spectra produce a two-photon excitation maximum of CMFDA at approximately 800 nm, while GFP is best excited at 920 nm. While there are valuable sources detailing the two-photon properties of certain dyes and molecules, researchers should always familiarize themselves with the optical behavior of the dyes used in their own experimental system (e.g., excitation and emission range, photostability, etc.).

4. PRACTICAL CONSIDERATIONS FOR INTRAVITAL IMAGING

4.1. Surgical Access to Imaging Target

In mammalian model systems (i.e., rats or mice), tissues of interest are securely shielded by barrier tissues such as skin and the peritoneal wall. While 2PM can be used to image through skin, the depth of penetration into opaque tissue is still limited due to the scattering of even two-photon excitation light, and such barriers can significantly reduce the available focal range. In most cases, therefore, deep imaging of tissues of interest first requires physical access at their location in the organism: the popliteal lymph nodes inside the popliteal cavity, the inguinal lymph nodes in the subcutaneous fat pads of both flanks, the cremaster muscle inside the scrotum, etc. To provide surgical access to the tissue for imaging, a precise knowledge of the macro- and microanatomy is essential—one must know where to cut (e.g., within avascular structures) and where not to cut (e.g., across large vessels) to avoid major tissue damage, arterial bleeding, or disruption of lymphatic vessels. However, it will be inevitable that surgical incisions will cause some tissue damage, especially of difficult-to-visualize nerves whose importance to the behavior of the immune system is now obvious. This may result in both local and systemic reactions that must be acknowledged and controlled as much as possible (104). It is crucial that at least the tissue compartment of interest itself, for example, the lymph nodes or the peripheral joints with their respective capsules, remains intact and unaffected to maintain as physiological a state as possible. This applies also to image stabilization. While smaller and more homogeneous drifts of the imaged area in lateral directions can be corrected in postprocessing (drift correction), motion artifacts such as animal breathing or muscle twitching need to be effectively suppressed from the start, which is done with the help of tissue glue and often custom-made clamps or body shields. Care must be taken when stabilizing tissue, as there is a risk of directly damaging the tissue or interrupting the microcirculation to such an extent that not only the tissue of interest stops moving but also the cells of interest lose the capacity for migration.

4.2. Tissue-Specific Environmental Factors

Similar considerations apply when studying tissue-resident cells in their natural environment. Disrupting the extracellular milieu can substantially alter cellular behavior such as that of tissue macrophages, by diluting or depleting extracellular ions such as calcium (105) or key cytokines. Both volume-related dilution effects and the composition of the buffers used are thus important. For the study of thick brain tissue sections, special formulations of artificial cerebrospinal fluid have been developed to maintain a physiological extracellular ionic environment, which is particularly crucial when studying intercellular communication via ion fluxes. The same applies to imaging of peripheral tissues. For example, if the tissue is exposed to too much "physiological saline" without calcium and magnesium ions, resident macrophages in different tissues cease their continuous probing dynamics, fail to take up fluorescent particles by pinocytosis, and don't

execute various homeostatic functions (our own unpublished observations). Similarly, when examining cells in peripheral tissues, the actual temperature in the tissue should also be considered. While imaging chambers are usually set at 37°C, certain tissues such as the outer skin do not regularly experience temperatures typical of the body core under physiological circumstances—in fact, this could be interpreted locally as a systemic fever reaction. In this case, laser thermometers can be used to determine the actual temperature of the tissue of interest, and the incubator should be set to specifically maintain this temperature while monitoring the animal's core temperature.

4.3. Animal Anesthesia

Adequate anesthesia and analgesia are essential for intravital experiments. Our labs have long-standing experience with gas anesthetics such as isoflurane. In our hands, isoflurane provides an easily controlled level of anesthesia that can be quickly adjusted to the animal's vital signs, such as breathing rate. Prolonged imaging experiments require close monitoring of vital parameters such as body temperature, blood oxygen levels, and respiratory rate; while the latter can be observed visually, pulse oximeters for small animals provide reliable information on the health status of the anesthetized animal. When gaseous anesthetics are used, the problem of environmental exposure must be solved by safe and sealed tubing and special isoflurane traps. Injection of other anesthetic agents such as ketamine may be a valuable alternative, for example, if the experimental setup does not allow the use of an animal face mask, but undesirable off-target effects must be considered. Immune-competent cells themselves express NMDA (*N*-methyl-D-aspartate) receptors and may directly respond to ketamine (106) or may be affected by necessary analgesic or antimuscarinic medications. Further, it requires repeated injection to maintain a level of sometimes-hard-to-control anesthesia.

4.4. What We Do in the Shadows. . .

In general, researchers need to be aware that the observed cell behavior may be a consequence of their intervention and not necessarily a physiological process. Any invasive procedure may have local or even systemic effects that need to be carefully considered. Particularly noteworthy in the study of real tissue is the fact that the black areas in an image are almost never empty—we just don't make the contents visibly fluorescent. Somewhat contrary to the usual dogma that "seeing is believing," when studying the dynamic nature of individual cells, what happens in the shadows can be a crucial factor that is easily overlooked. A neutrophil that has stopped on its way to a focal point may not have become inactive but may have encountered an impenetrable wall of collagen, other matrix components, or a nerve or blood vessel (107). Reporter systems that provide information about the seemingly empty space, such as ubiquitously expressed DsRed (108) or differential expression of red or green fluorescence as a function of Cre activity, such as the mT/mG mouse model (109), are particularly useful for gaining insights into how the tissue microanatomy influences immune cell behavior.

Surgery to expose the tissue to be imaged can frequently lead to macroscopically invisible but significant tissue damage that triggers an inflammatory reaction with an influx of large numbers of neutrophils—all in the "dark" without the researcher noticing. We suggest that experimenters, especially when approaching a new in vivo model, practice the surgical interventions in animals with myeloid reporter systems (e.g., LysM-GFP mice) that would readily reveal neutrophil infiltrates as a state of unwanted inflammation associated with iatrogenic tissue damage. The use of blood tracers such as conjugated dextrans can also be used to monitor for vascular leak, again a sign of undesired inflammation. In peripheral tissues, even cells lining the surface of tissues and organs such as the liver (38), the visceral pleura of the lungs (110), or the serosa of the peritoneum (17) may

undergo substantial cell death in response to mechanical pressure, disruption of microcirculation, or the nonphysiological stiffness of the coverslip used for acute imaging or for chronic windows in long-term imaging experiments. Superfusion with propidium iodide visualizes random cell death that will otherwise soon become evident indirectly, for example, by neutrophil recruitment and activation.

4.5. Imaging Organ Explants

Extensive surgical procedures were developed to access a variety of deep organs including those in the visceral abdomen, such as the pancreas (91). However, when capturing dynamic processes that may be widely distributed in an organ and easily missed when the field of view is restricted by a surgical window (111) or when the tissue in question is not directly accessible to a large microscope objective due to its anatomical nature or location, removing the organ or tissue from the body and establishing an ex vivo culture system may be necessary. In this case, continuous supply with an oxygenated, tissue-optimized buffer is just as crucial as removal of molecular waste products to maintain an environment as physiological as possible. However, this inevitably exposes the cells to previously unknown environmental conditions: Interstitial cells may come into direct contact with serum contents that they normally sense only in vascular injury, epithelial cells of the lung normally exposed to air may now be exposed to fluids that would indicate pathologic pulmonary edema, or tissues normally exposed to shear stress, particular environmental pressures or viscosities, or even vacuum may experience nonphysiological conditions. Therefore, it is essential to compare dynamic data with metrics obtained in controlled in vivo experiments, such as the velocity of naive T cells or neutrophils. These parameters are an important indicator for the assessment of the physiological state of not only an explant but also of an in vivo approach and thus for the reliability of the protocol (112).

5. EXPERIMENTAL DESIGN IN TWO-PHOTON MICROSCOPY

5.1. Fluorescent Reporter Systems Used in Two-Photon Microscopy

The development of reliable reporter model systems, ex vivo or in vivo labeling protocols, and sophisticated tracking tools that allow different cell populations to be distinguished enabled insightful application of 2PM methods in a variety of biological fields. 2PM approaches have been used to explore dynamic interactions among multiple cell types, migration patterns, or real-time detection of specific activation states or even signaling activities such as calcium flux or transcription factor translocation (113). An essential prerequisite for all these analyses is that both the anatomical environment and the cells or molecules of interest are visible and traceable over time. To achieve this end, genetic reporter systems are employed that drive expression of fluorescent proteins [e.g., floxed CAG-driven red fluorescent tdTomato protein (114)] under the control of cell-specific promoters, either Cre-driven [e.g., LysM-Cre (115)] or in the actual gene locus [e.g., LysM-GFP (116)]. While the first approach can be used to study the long-term fate of cells that have activated a particular promoter at any time in the present or past, when short-lived reporter proteins are employed, knock-in approaches provide information on the current and often transient activity state of the promoter in question. Thus, they can be used not only to identify cells but also to assess a functional polarization state. Strains commonly used in 2PM include mouse models that can visualize DCs [*Zbtb46*-GFP (117), monocytes and macrophages (*Cx3cr1*-GFP/*Ccr2*-RFP) (118)], NK T cells [*Cxcr6*-GFP (119), IFN-γ-producing cells (*Ifng*-YFP) (120)], T helper cells [DPE-GFP (121)], IL10-producing cells [*Il10*-GFP (122)], regulatory T cells [*Foxp3*-GFP (123)], nonclassical monocytes or T cells [*Nr4a1*-GFP (124)], or various subsets of B cells [*Bcl6*-GFP (125), *Blimp1*-YFP (126)].

Significantly higher specificity can now be achieved by using newly developed binary (or split) Cre approaches, which require the simultaneous expression of not one but two different promoters to express a functional Cre recombinase (127). Such approaches often require costly and time-consuming breeding efforts, and the use of Cre-driven expression of a ubiquitously expressed floxed fluorescent protein gene is inherently limited to one specific cell type but may be combined with particular knock-in reporter models. Designing such imaging experiments is crucial, but it can take a long time before the desired model systems are in place and the experiments can be carried out. In addition, great care must be taken regarding the specificity of the chosen reporter system when interpreting the data, as the ability to phenotype cells in vivo is limited compared with ex vivo approaches. Rarely, a particular reporter system allows separation of two cell types on the basis of intensities, such as the LysM-GFP mouse model (17, 26).

5.2. Ex Vivo Labeling Approaches for In Vivo Immune System Imaging

For immunological and inflammation studies, there is a need to track multiple distinct cell types at once and, if possible, also assess the state or signaling activity of the cells at the same time. In seeking to achieve these goals and overcome limitations imposed by genetic models, a variety of strategies with particular applicability to immune cells were developed. This includes isolation of the cell populations of interest, ex vivo labeling, and subsequent transfer into a suitable host, such as a fluorescent reporter animal, allowing even transfer and direct comparison of different genotypes or pharmacological treatments within the same experiment (13, 128–130). However, this is feasible only for certain cell types, primarily migratory, nonresident cells such as neutrophils or T cells. Great care must be taken to apply only isolation protocols that do not activate cells or make them targets for antibody-dependent clearance or killing, for example, negative enrichment for naive T cells (131). When cell activation leads to upregulation of adhesion molecules that promote entrapment in small capillary beds such as the lung after intravenous injection, adoptive transfer is compromised. For ex vivo labeling, a wide range of dyes is available that are suitable for 2PM and can be combined with other dyes or fluorescent proteins. In addition, ex vivo labeling offers the possibility of combining cellular labeling with functional biosensors that allow, for example, the tracking of changes in intracellular calcium concentration (132), redox potential, oxidative stress, or even membrane potential.

5.3. Making Resident Tissue Cells Fluorescent

Ex vivo labeling and/or adaptive transfer of tissue-resident cells, such as macrophages, is more challenging and requires further discussion. Macrophages have usually differentiated to such an extent that they can no longer return to their own or other tissues via the bloodstream and are instead eliminated from circulation. As special cases, alveolar macrophages (133) can be isolated from the bronchoalveolar space or macrophages from the peritoneal cavity (134) can sometimes even be kept in culture for extended periods (133) and returned directly to their original environment by intraperitoneal or intratracheal injection, bypassing the bloodstream.

This does not apply to the bulk of interstitial cells. An alternative strategy here could be the transfer of progenitor cells, for example, bone marrow-derived monocytes, which are naturally able to cross the vascular barrier and invade various tissues to develop into macrophages. However, this process requires an open niche in the tissue network where the monocytes can settle and become macrophages (135). "Opening" tissue niches by specific depletion of niche-occupying tissue macrophages has been successfully used to generate triple chimeras of the resident macrophage population within the same tissues (17). However, such approaches must consider the potentially disruptive effects of initial depletion, as well as the critical aspects and ongoing debate about

whether or when progenitor cells can become "true" resident macrophages. Moreover, it depends on whether the tissue allows bone marrow–derived progenitor cells to become part of its macrophage pool, as tissues such as the brain maintain exclusivity of their resident cells (136).

The same considerations apply to bone marrow chimeras. After irradiation, the entire hematopoietic system (or parts thereof) can be replaced by (subset-specific) fluorescent reporter bone marrow from a donor by adoptive transfer. Again, the effects of irradiation as well as the state of chimerism need to be validated at the tissue level to draw meaningful conclusions from in vivo imaging data. In this way, even more sophisticated approaches can be applied: Retro- or lentiviral transduction of hematopoietic stem cells and their adoptive transfer after irradiation can generate bone marrow chimeras that selectively express mutants or fluorescently labeled signaling molecules in myeloid cells, for example, to study transient events such as activation of inflammatory pathways in vivo with sufficient temporal resolution (137).

5.4. In Vivo Labeling Approaches

Direct labeling of cells in vivo can be a practical alternative under certain circumstances. Depending on the tissue anatomy, the entire tissue area can be "painted" by topical application with general fluorescent dyes (e.g., DiI), functional reporter dyes (e.g., Fluo4), or various biosensors for pH or membrane potentials. Highly specific cell labeling in vivo can be achieved with fluorescent protein–coupled antibodies, as we have previously demonstrated using the peritoneal serosa (17). In addition, knowledge of the lymphatic drainage pathways makes it possible to deliver antibodies to the popliteal lymph nodes, for example, by subcutaneous injection into the sole of the foot. This is particularly useful to label cells along the lymphatic drainage route, such as subcapsular sinus macrophages, which are among the first to be exposed to injected antibodies directed at CD169 (138). However, there are two major points to bear in mind. First, commercially available antibody solutions often contain preservatives such as NaN_3. While systemic application of minute amounts may not be a major problem, local application can affect cell function or even viability. Therefore, it is important to remove cell-toxic substances by dialysis or other methods or, preferably, obtain reagents lacking such molecules completely. Secondly, loading cells with antibodies can influence cell function, which needs to be controlled separately in the context of the biological process under investigation. For example, using a neutrophil reporter system, we found that inflammatory neutrophils in the presence or absence of anti-Ly6G antibodies exhibited indistinguishable behavior in terms of migration as well as response to sterile tissue damage; this allowed us to use specific antibody labeling to reliably distinguish neutrophils from macrophages in a rather broad LysM-driven macrophage-monocyte-neutrophil reporter system (17).

As a side note, not all fluorophores are suitable for two-photon imaging. The choice of fluorescent molecule is crucial, as most of the dyes in the Alexa Fluor family, for example, bleach quickly when exposed to two-photon laser light (e.g., Alexa Fluor 647). Of the commonly used fluorophores, only Alexa Fluor 488, Alexa Fluor 594, and Alexa Fluor 680R have sufficient photostability to be used in 2PM experiments. Interestingly, given their susceptibility to bleaching in single-photon approaches, older fluorochromes such as FITC or PE are surprisingly photostable when exposed to two-photon laser light and may represent attractive and cost-effective alternatives, especially in experimental platforms where deep penetration of antibodies into tissues is not critical, for example, into the serosa of the peritoneum or the sinuses of the lymph nodes. With the right choice of fluorophore, 2PM can also provide additional value to other static multiparameter imaging approaches. With 2PM, high-spatial-resolution images can be obtained even from the depth of a fixed tissue to visualize and resolve even intracellular structures. In addition, one can take advantage of SHG, which is not affected by tissue fixation. This allows

researchers to visualize label-free, repetitive noncentrosymmetric structures such as collagen, which can help delineate compartments within the tissue, capsules, or ligament structures.

5.5. Optical Manipulation and Visual Mapping

The ability to obtain precise focal plane molecular excitation deep in tissues makes 2PM a useful tool for labeling spatially defined regions of tissues or even individual cells using convertible fluorescent reporter systems. Genetic approaches involving PA-GFP or photoconvertible Kaede can be implemented with the proteins expressed under control of ubiquitous promoters or driven by cell-specific Cre expression. While exposure to UV or violet light in the range of 350–410 nm induces the change in molecule conformation that leads to "activation" of an otherwise weakly emitting GFP [PA-GFP (71)] or conversion from green to red fluorescence [Kaede (139)], the same reaction can be induced with high spatial resolution using two-photon excitation in the 800-nm range. In vivo conversion using the two-photon lasers can then be used to label cells, and the induced labels can be utilized either for in vivo imaging or for subsequent sorting and transcriptional profiling, that is, NICHE sequencing (140). Similarly, two-photon lasers can benefit experimental systems that rely on precise spatial optical control. The coupling of light-sensitive molecules such as opsins to enzymes or ion channels makes it possible to activate or deactivate them with the aid of light and thus control ion fluxes or even biochemical processes to study neuronal activities and the connections of brain networks. These opsin-coupled molecules are introduced via viral vectors or genetically manipulated mouse models (optogenetics), and optical control is then usually performed with solid-state lasers or light-emitting diode devices (141). Here, multiphoton excitation can significantly improve spatial resolution (142) and can also be used in innovative experimental approaches that combine selective photostimulation with simultaneous recording of neuronal activity, for example, by calcium imaging (143). Neuroscience has pioneered optogenetic approaches, but the use of opsin- and nonopsin-based light-sensitive molecules to control immune responses in vivo is on the rise and will soon also become established in immunological research and immunotherapy (144).

5.6. Direct Functional Readouts

The standard 2PM laser scanning mode can be used to study signal events at a cellular level. While true quantitative biosensors are still lacking, the recording of relative temporal changes in intracellular calcium levels as an indicator of cellular signaling activity has become widely accepted. Calcium imaging is primarily used in the study of excitation conduction in neurons or the heart, but it can also be studied as a universal second messenger, and its cytoplasmic transients can be used to monitor various signaling pathways. The development of the first highly sensitive $Ca2^{+}$ probes (145) led to continuous advancement of the technology and improved probes with different binding kinetics and sensitivities for differential visualization of $Ca2^{+}$ in living cells, yielding a powerful tool for visualizing cell activation, studying communication in multicellular networks, and linking specific signaling patterns to dynamic behavior in vivo (132, 146, 147).

In addition to SHG-based visualization of collagenous structures, the autofluorescence of a tissue can also be translated into biological information without additional labeling. Autofluorescence is influenced by small molecules, such as the electron-shuttling molecule NADH, which act as endogenous fluorophores. The autofluorescence intensity of NADH depends on its oxidation state: While NADH fluoresces in its reduced form, NAD^{+} is not fluorescent. However, especially in tissues, small differences in fluorescence intensity may not be a reliable measure. By measuring the fluorescence lifetime, that is, the time required for an excited fluorophore to emit light and return to its ground state, one can distinguish between "free" and protein-bound

NADH, with lifetimes of approximately 400 ps and 1.0 to 4.0 ns, respectively. The ratio between free and bound NADH reflects the NAD^+/NADH ratio and thus the NAD(H) redox state: In general, glycolysis increases "free" NADH and decreases nonfluorescent NAD^+, which increases autofluorescence and shortens lifetimes—oxidative phosphorylation instead leads to longer lifetimes. With appropriate technical equipment that allows temporal resolution of the detected light, valuable information can be obtained without additional labeling, especially about relative changes in the metabolic state of a single cell and even whole tissues. Although it should be kept in mind that NADH is not the only molecule that dynamically contributes to cellular autofluorescence [e.g., NADPH, FAD, etc., as well as other confounding factors, also contribute (148)], two-photon fluorescence lifetime imaging microscopy represents an exciting modality whose further development could provide a label-free indicator of cellular redox state and mitochondrial function (149) even in complex tissue compartments.

5.7. Analyzing Dynamic Metrics

The questions that can be asked using 2PM in vivo are determined by the number and type of dynamic parameters that can be assessed in the context of tissue anatomy, the resolution of single-cell tracking that can be achieved, and the duration of the imaging session. In general, a significant number of dynamic parameters such as velocity, tracking fidelity, or displacement metrics are readily generated from 2PM imaging data, metrics that can be obtained with both commercial and open-source software. Recently, dimensionality reduction and unsupervised clustering strategies involving an extensive list of parameters describing various aspects of the dynamic behavior of individual neutrophils in vivo allowed the authors to define specific functional states of cells (6). Other parameters such as radial velocity with respect to a particular focus (13), cell-cell interactions, or clustering metrics require additional calculations or custom computational approaches.

Identifying and tracking individual cells is key to analyzing dynamic data. This can be particularly challenging in dense and crowded environments, such as neutrophil infiltrates: Single neutrophils in active clusters soon become indistinguishable. Therefore, choosing the right "inflammatory load" as well as the right setting for the research question is crucial for the subsequent data analysis. Both spatial resolution and acquisition speed are critical to track individual cells reliably and continuously from frame to frame and still identify them as the same cell. Until recently, segmentation of cells into individual trackable objects was mainly performed using thresholding approaches, which allow cells to be defined as spots based solely on fluorescence intensity. Such spots then represent the respective cell as individual points with x, y, and z coordinates in the Euclidean space of the imaging volume. Segmenting cells as individual objects with a 3D surface representation of their true morphology is considerably more difficult, especially over time. Region- and intensity-based segmentation algorithms such as water shedding often cannot reliably separate overlapping objects in dense tissue volumes (i.e., instance segmentation). Recent computational advances have created exciting interfaces between object tracking and machine or deep learning–based cell segmentation (150), promising more accurate and reliable generation of dynamic data with significantly less effort and manual correction.

6. PERSPECTIVE

Functional intravital imaging remains a demanding task, requiring knowledge of normal tissue anatomy and physiology, often complex animal models to achieve the necessary fluorescent labeling of cell populations, surgical skill, and some level of expertise in technical aspects of microscopy and data analysis. But with the right tools, the assessment of biological processes in a living system is a powerful tool, not only for the study of immunology, and new technological developments

are now permitting ever faster, longer, and deeper imaging. For instance, a creative redesign of the conventional 2PM light path has recently been developed to minimize phototoxicity, which is particularly important for continuous long-term imaging (151). In the future, we will be able to penetrate even deeper into tissues, as optical systems that enable three- or even four-photon excitation will become more widely available (152). Technological advances are producing increasingly sensitive detectors that can be combined with more flexible multiband spectrophotometry systems, enabling more extensive multiplexing experiments.

However, we will always be limited by the available reporter systems and fluorescent probes that allow us to visualize biological processes. While CRISPR technology has made the creation of genetic models easier than ever, subsequent crossbreeding programs and animal husbandry remain laborious. Beyond simply recording migration patterns of cell populations or dynamic changes in cell morphology, there is an urgent need for quantitative biosensors that provide a direct functional readout of various aspects of cell state that can then be linked to dynamic behavior. While this could be achieved at the genetic level, there is always the risk that the reporting system will interfere with the pathways it is supposed to record. Such biosensors could be delivered to cells in vivo in a targeted fashion (e.g., directly into the macrophage compartment via receptor-based uptake) and specifically activated chemically or via optical means.

Knowledge of the in vivo behavior of cells must be integrated with other techniques to obtain a comprehensive understanding of the relationship between dynamics and function. This remains an experimental challenge and requires the development of new methodological approaches. In addition to existing NICHE sequencing approaches, computational advances in multimodal registration of anatomical features in three dimensions (e.g., vascular structures) allow researchers to combine dynamic in vivo imaging with subsequent multiplexed volume imaging of the same fixed tissue compartment using optical clearing, thus combining dynamic data, spatial positioning, and information about the tissue microenvironment (153).

The use of artificial intelligence will have a significant impact on the future of dynamic imaging. Content-sensitive restoration of noisy images using neural networks will reduce phototoxicity by reducing laser power and increasing scanning speed without compromising image quality. Restoration methods will also make it possible to improve image stabilization as well as lateral and especially axial resolution at a predictive level. Machine learning approaches have already made inroads into the analysis of dynamic data and will continue to dominate it using deep learning–assisted segmentation and automated tracking of cells in even highly complex cellular environments such as inflammatory infiltrates. These rapidly developing advances in each of the technologies needed for conducting intravital imaging and interpreting the resulting data hold substantial promise for gaining deeper insight into immune function and immunopathologic processes in the very near future.

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