Recording the wild lives of immune cells

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Abstract

Intravital microscopic imaging can uncover fundamental aspects of immune cell behavior in real time in both healthy and pathological states. Here we discuss approaches for single cell imaging of adaptive and innate immune cells to explore how they migrate, communicate and mediate regulatory or effector functions in various tissues throughout the body. We further review how intravital single cell imaging can be used to study drug effects on immune cells.

INTRODUCTION

We are at a point in immunology where we appreciate the complexity of the relationships between immune cell populations and disease states. Unravelling this complexity requires a palette of research tools to investigate immune cell subpopulations and their functions. Selecting the proper tools for a specific application and model requires knowledge of each technology's strengths and limitations. The immunology field has historically focused on classifying immune cells by first isolating and then studying them using multiplexed cytometry. More recently, the introduction of single cell transcriptomics has given us the ability to collect unbiased information on all cell populations. In contrast to these ex vivo approaches, intravital imaging enables direct visualization of immune cells and their functions in various tissues in vivo, without the need for isolation and selection procedures that can introduce bias. Intravital imaging is particularly suited to dynamically visualize immune cells over time, often revealing previously unknown behaviors. Indeed, many immune functions are heavily dependent on cell migration and cell-cell contacts, both of which can be faithfully captured by single cell resolution intravital imaging. Spatial organization can dictate the effective functionality of immune cells, which is readily apparent from imaging studies but may not be detected using traditional cell profiling methods. Furthermore, the ability to quantitatively measure kinetics of behaviors provides key insights into immune processes and even potentiates mathematical modeling of

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emergent behaviors. Here, we focus on how immunologists can use intravital single cell imaging approaches to complement super-resolution imaging in isolated cells (1) and imaging at the whole body level (2).

THE TOOLBOX

A number of intravital imaging approaches exist and their basics and general implementations have been covered already (3–5). This section therefore focuses on specifically adapting these technologies to observe how immune cells travel, function and interact in live mice.

What are the different intravital imaging approaches available, and what are their advantages and limitations?

Today's two main approaches for single cell imaging utilize either confocal laser scanning or multiphoton microscopy. The systems are generally implemented on upright microscopes, and several configurations are now commercially available. Most intravital imaging setups for mice now have an upright configuration, although in some cases, inverted systems are also used. The latter may be beneficial for dual purpose cell/tissue imaging systems or for exteriorized organs which assume a flat configuration on the glass. Confocal microscopy set-ups are usually less expensive and represent a good all-around technique for much of the imaging done today (6). These systems use an array of solid state lasers for excitation and matched laser/filter sets can demultiplex fluorophore signals, similar to flow cytometry. The downsides of confocal imaging are higher autofluorescence and scattering, which generally limit imaging depths to <100 μm, and typically in the range of 20–50 μm. Furthermore, shorter wavelength channels have higher phototoxicity, although this is often a lesser concern for in vivo imaging than it is for in vitro imaging.

Multiphoton laser scanning microscopy (7) bypasses the limitations of confocal microscopy using more expensive and tunable Ti:sapphire lasers that operate in the near-infrared range. Localized nonlinear excitation based on two-photon absorption allows for superior tissue penetration at higher wavelengths and less out-of-focus excitation. One of the major ways in which multiphoton imaging reduces phototoxicity and improves resolution is by inherent optical sectioning due to the more restricted photon excitation volume. Typical penetration depths are in the 200–300 μm range for most organs, except in the brain and cleared tissues where deeper imaging depths can be achieved. Multiphoton microscopy can induce photobleaching but again, this is often less of a concern for in vivo imaging. One minor disadvantage of the multiphoton system is that many of the fluorophores used in flow cytometry, epifluorescence and confocal microscopy experiments have not been characterized in the multiphoton setup.

What are the key requirements for a single cell imaging experiment?

To perform state-of-the-art single cell 3D imaging one requires i) an integrated imaging system in a dark and appropriately cooled room (see supplemental information in (4)); ii) suitable fluorescent reporter mouse models with either exteriorized organs (8) or implanted window chambers (9); iii) motion suppression techniques (10); iv) physiologic support

modules and v) data processing and analytical software (Fig. 1). Physiologic support, including feedback temperature controls, are critical to maintaining homeostasis and hydration in immobilized and ventilated animals. This support is most commonly achieved by using warming plates, immobilization chambers and continuous vital sign monitoring while the animal is anesthetized. Temperature control in exteriorized organs is especially critical to preserve cell motility.

Quality control measurements include calibrating the imaging system with fluorescent bead phantoms, correcting artifacts, detecting channel bleed-through, determining the stability of reporter expression levels and properly managing data outliers. Data processing and analysis is a major part of a single cell imaging experiment. Various software packages automate the analysis of high-dimensional data, although manual curation is still often necessary. Common software packages that support image processing, segmentation, cell measurements and profiling features include CellProfiler, Fiji, Imaris and microscope vendor packages. Matlab and Python are programming languages that are also utilized to implement post-processing algorithms and quantitative analysis, whereas widely available packages such as GraphPad Prism or CellProfilerAnalyst are used for statistical analyses of extracted features across study groups, findings and datasets.

Common approaches

Prior to performing an imaging experiment, the questions to be answered should be clearly outlined and all experimental variables considered. What type of information can be retrieved, and how many parameters can be measured simultaneously? The answers to these questions will vary from organ to organ, but it is generally accepted that up to four (confocal) or six channels (multiphoton) can be acquired during a typical 3D acquisition. Most often, these channels are reserved for different immune cell populations (11), structural content (collagen fibers, vessels) or labeled drugs (12). Inhalation-anesthetized mice bearing window chambers generally tolerate repeat imaging well and can be analyzed on subsequent days unless non-survival surgery is performed on exteriorized organ preparations, in which case it is important to maintain the homeostatic environment with respect to blood flow and oxygen supply in addition to temperature. The typical imaging session varies in length but often lasts one to two hours unless slower phenomena are being studied. The most commonly imaged organ systems are tumors, lymph nodes and the skin but also include brain, bone marrow, kidney, liver, lungs, pancreas, reproductive tract, spleen and yolk sac (Fig. 2A–B).

Window chamber models (9, 13) are particularly suited for intravital imaging because they can be easily accessed and immobilized. They also offer the possibility to investigate a wide range of pathological conditions, including autoimmunity (14), infection (15) and cancer (16). The installation of these immobilizing devices requires administration of a post-operative analgesic, often buprenorphine. Antibiotics or antimycotics can also be given to mitigate the risk of infection at the surgical site. Additional considerations may be taken concerning the housing and handling of the mice to avoid damaging the chamber if the animal is to be monitored over repeated imaging sessions. Typically, imaging is initiated several days after the chamber is implanted, i.e. when surgery-induced inflammation has

resolved. However, it is always critical to asses whether the procedure used to prepare the mouse affects the readouts to be recorded during the imaging session; this should be done, at least in part, by imaging mice that underwent sham surgery. Detailed methods are available for intravital imaging of various organs such as lymph nodes (17), tumors (13, 18, 19), ear skin (20), abdominal tissues (21–23), lung (9) and heart (24).

INTRAVITAL IMAGING TO STUDY ADAPTIVE IMMUNITY

Cell migration and cell-cell interactions play pivotal roles in adaptive immunity. For instance, circulating naive T cells continuously home to secondary lymphoid organs, where they physically engage with antigen-presenting cells. Local encounters with cognate antigen can produce activated T cell progeny, including effector, central and tissue resident memory subsets, which gain distinct migratory and functional abilities. Cells that traffic to sites of inflammation can further interact with antigen-bearing and other cells, and mediate effector functions. Single cell imaging enables the direct study of all these dynamic processes in complex microenvironments and various tissues.

T cells

The least invasive methods to image T cells use genetic mouse models in which endogenous T cells selectively express a fluorescent protein reporter (Table 1). This approach can assess T cell infiltration in various tissues (11, 25) and bypasses ex vivo cell manipulation and transfer, which may affect cell behavior and fate. Indeed, endogenous T cells may behave differently from adoptively transferred ones. Ideally one would want to image endogenous T cells using mouse models that have T cell-specific reporters whereas adoptive transfer should be considered in special cases. Genetic mouse models can also be used for tracking T regulatory (T_{reg}) cells, which has been performed in lymph nodes (26), bone marrow (27) and non-lymphoid tissues (28). In practice, however, intravital T cell tracking still often relies on adoptive cell transfer approaches. Here, T cells of interest are first purified from mice ubiquitously expressing a fluorescent protein reporter and then administered into recipient mice, so transferred cells and their progeny can be visualized within a tissue of interest (Table 1). This approach is convenient because it allows one to control the quantity of visible (exogenously added) cells in a given field of view. By contrast, the density of endogenous T cells in tissues such as lymph nodes is often quite high making it difficult to distinguish individual cells.

Intravital imaging of adoptively transferred T cells has uncovered how naive (29, 30) and central memory (31, 32) T cells sense antigens and become activated in lymph nodes. For instance, imaging has revealed that naive lymph node CD8^{+} T cells are initially motile, which allows them to undergo multiple transient contacts with antigen-presenting DCs, but can then progressively decrease their motility to form stable contacts with DCs, at which time they also start producing effector cytokines. The T cells eventually resume their migration, begin to proliferate and can emigrate from the lymph node (29). The type of interaction that a CD8^{+} T cell undergoes with an antigen-presenting DC depends at least in part on the antigen dose. Whereas low antigen dose conditions can functionally prime CD8^{+} T cells, only high antigen dose may trigger stable T cell-DC contacts (33); these longer

contacts may be necessary for the generation of full-fledged immune responses. Intravital lymph node imaging has further revealed that helper $CD4^+$ and $CD8^+$ T cells are largely compartmentalized during priming reactions; however, during later phases of lymphocyte maturation, these cells are brought together by some DCs. This process enables the delivery of $CD4^+$ T cell help to $CD8^+$ T cell responses, which is important for the generation of $CD8^+$ T cell memory (30).

Intravital imaging in the context of vaccinia virus infection has further revealed the compartmentalization of naive and memory $CD8^+$ T cells in lymph nodes. Whereas naive $CD8^+$ T cells primarily locate in the cortex, memory $CD8^+$ T cells distribute along the periphery of the lymph node (34). This distinction is likely relevant because it brings memory $CD8^+$ T cells in close proximity to subcapsular sinus (SCS) macrophages, which are among the first lymph node cells to capture lymph-borne viruses. Therefore, it appears that memory $CD8^+$ T cells have preferential access to antigen upon viral reinfection (31, 34). Other imaging studies have tracked T cells in nonlymphoid tissues. For example, skin and female reproductive tract imaging have defined antigen-specific T resident memory cell (T_{RM}) activation and proliferation in response to viral infections (35, 36). T_{RM} responses in the female reproductive tract can occur in the absence of DCs (36); however, it is unknown whether T_{RM} responses in other tissues require these cells.

Additional imaging tools extend our ability to study T cell communication with neighboring cells at a deeper level, such that specific molecular outcomes can be identified (Table 1). For example, transgenic mice harboring a T cell receptor (TCR)-GFP fusion protein enable the visualization of TCR clustering and internalization following antigen encounter (37). Translocating nuclear factor of activated T cells (NFAT) from the cytoplasm to the nucleus is a sensitive readout of TCR signaling and can be assessed by intravital imaging using fluorescently labeled NFAT (38). The NFAT reporter is often used in combination with fluorescently labeled histone protein H2B, a nuclear marker. Other cell signaling mechanisms such as calcium flux can be detected using transgenic mice expressing the calcium sensor protein GCaMP6s to read out features such as in vivo T cell activation (39) or target cell killing (40).

$CD8^+$ T cells can also be "caught in the act" of effector activities. For instance, intravital imaging approaches have shown blood circulating $CD8^+$ T effector cells to adhere onto liver sinusoids and engage in immune surveillance (41) and cytotoxic T cells to kill virus-infected cells in lymph nodes (40). The latter study used color-labeled viruses to visualize virus-infected cells and identified that T cell-mediated target cell killing *in vivo* may be much slower than anticipated from prior *in vitro* studies. Interestingly, the proximity of T_{reg} can dramatically slow the kinetics of $CD8^+$ T cell killing in vivo (42). Cytokines such as interferon-gamma (IFN-γ) and interleukin-10 (IL-10), both produced by T cells, can also be detected by intravital imaging. Because IFN-γ has a rapid on/off cycling (43), a common readout typically requires restimulation of T cells in vitro with an antigenic trigger. This method is useful to define whether given cells are equipped to produce IFN-γ but it cannot assess whether these cells actually produced the cytokine in vivo. By contrast, intravital imaging of IFN-γ-internal ribosome entry site-YFP (IFN-γ-IRES-YFP) reporter mice detects YFP, which is expressed by cells that have turned on IFN-γ production (25). YFP

remains detectable even after IFN-γ production is turned off, which makes intravital imaging a particularly useful tool to detect IFN-γ pathway activation in vivo (Fig. 2C), although it cannot identify cells that are currently producing the cytokine. Imaging is also useful to detect cells producing IL-10, which was identified as a factor important for both viral containment and tissue protection following vaccinia virus infection (44). IL-7, which is required for T cell development, survival and memory development, can also be tracked in IL-7 reporter mice. Imaging of IL-7 reporter systems has provided useful information of the location and composition of IL-7-dependent immune cell niches (45).

B cells

Intravital B cell tracking, like T cell tracking, usually involves transgenic reporter mice or adoptively transferring fluorescent B cells into recipient mice (Table 1). Both approaches enable the study of B cells in different body compartments. In lymph nodes, intravital imaging has shed light into how B cells interact with various cellular and molecular components, including subcapsular sinus (SCS) macrophages (46), T follicular helper cells (T_{FH}) (47) and type I interferon (39, 48), and how these dynamic interactions regulate the initiation of humoral immune responses. For example, in the context of infection, intravital imaging studies have revealed that lymph-borne viruses preferentially infect SCS macrophages, which then present viral particles to B cells to initiate humoral immune responses (46). Intravital imaging studies further identified B cells can express intercellular adhesion molecule 1 (ICAM1) and ICAM2, which are critical for forming stable contacts between B cells and T_{FH} and subsequent development of humoral immunity (47). By contrast, imaging studies have shown that type I interferon inhibits antiviral B cell responses in lymph nodes, for example by recruiting inflammatory monocytes that produce nitric oxide (48) and by activating cytotoxic T lymphocytes that kill antiviral B cells (39). Indeed, not all T cell-B cell interactions in lymph nodes stimulate humoral immunity, as cytotoxic T lymphocytes can kill B cells under certain contexts (39, 42).

Intravital imaging has been further used to study B cells in other tissues. For example, intravital imaging revealed how splenic B cells shuttle antigens from the marginal zone and into B cell follicles (49). B cell imaging has also been extended to various bone compartments using novel imaging platforms that allow for long-term, longitudinal studies of bone marrow dynamics (50) and to non-lymphoid tissues such as the lungs and liver (51). For example, pulmonary intravital imaging has revealed that B cells can physically interact with aged neutrophils and contribute to their clearance locally (51).

By transferring B cells carrying the photoactivatable reporter PA-GFP, it is possible to tag these cells by multiphoton photoactivation within distinct micro-anatomical tissues such as the germinal center of a lymph node (52). This photoactivation system in situ was used to interrogate migratory behavior of B cells within germinal centers and identify that cell proliferation occurs in the dark zone of the germinal center after B cells are instructed by T cells in the light zone of the germinal center (52). The photoactivated cells can be monitored in vivo for up to several days or purified for additional analysis in vitro. In principle, this approach can be used to tag any adoptively transferred cell, regardless of its type, and be further extended to photoconvertible fluorescent reporters, such as Kaede and Dendra2,

which shift from green to red fluorescence upon exposure to light of appropriate wavelength (4). These types of experiments enable investigators to have finer spatial resolution of migratory phenotypes as photoconversion of cells can identify distinct migratory properties that would otherwise be difficult to study. In addition, photoactivation models can be used in combination with single cell sequencing approaches to retain spatial information in high dimensional sequencing technologies (53).

INTRAVITAL IMAGING TO STUDY INNATE IMMUNITY

An increasing number of tools are available to image a variety of innate immune cell types, including dendritic cells (DCs), monocytes, macrophages, neutrophils and innate lymphocytes. Single cell imaging is most often used to understand innate immune cells' interplay with their adaptive counterparts or to reveal their functions in development, steady state, health, and disease.

Dendritic Cells

Both genetic reporter and adoptively transferred DCs can be tracked by intravital imaging (Table 1). *CD11c*-YFP reporter mice have been used to yield insight into how endogenous DCs are spatially organized in lymph nodes, collect antigens, and promote T cell responses to these antigens (30, 54). However, other cell types including macrophages may express CD11c. Endogenous DCs can also be imaged using *Flt3*-BFP2 (11) or *XCR1*-Venus (55) reporter systems. Typically all DC subsets express Flt3, whereas macrophages and other cells usually don't or do so at much lower levels. XCR1 expression is more selective for a subtype of DCs, whereas CD11c is expressed by DCs but also some macrophages and T cells. An *XCR1$^{KiKGR/+}$* genetic mouse model further enables photoconversion of XCR1$^+$ DCs in vivo (56). These mice are convenient for tracking migratory DCs and distinguishing them from neighboring resident DCs. For instance, skin XCR1$^+$ DCs photoconverted by violet-blue illumination can be tracked upon migration to skin-draining lymph nodes and discriminated from non-photoconverted DCs (56). This approach identified that skin XCR1$^+$ DCs that migrate to draining lymph nodes gradually accumulate in deep medullary regions of the T cell zone, where they can prime naive CD8$^+$ T cells (56). Painting the skin with a fluorescent dye, such as TRITC, may also distinguish recent skin DC migrants, which carry the dye, from other lymph node DCs (57), although it is formally possible that some dyes may percolate to lymph nodes independently of DCs or be transferred from one cell type to another. In the context of HSV-1 infection, TRITC-labeled DCs accumulate in the T cell zone of draining lymph nodes, where they interact predominantly with naive CD4$^+$ but not CD8$^+$ T cells. CD8$^+$ T cell activation occurs approximately one day later and involves XCR1$^+$ (TRITC$^-$) DCs, suggesting that distinct DC subsets activate CD4$^+$ and CD8$^+$ T cells sequentially. Eventually, clusters of XCR1$^+$ DCs and activated CD8$^+$ T cells recruit the pre-activated helper CD4$^+$ T cells, which provide licensing signals to enhance CD8$^+$ T immunity (57). The formation of a "ménage-à-trois" between DCs, CD4$^+$ and CD8$^+$ T cells can also be promoted by clusters of DCs and CD4$^+$ T cells that recruit naive CD8$^+$ T cells (58).

Some DC functions can also be imaged. For example, a transgenic mouse reporting expression of the chemokines CXCL9 and CXCL10 (which bind the chemokine receptor

CXCR3, expressed by T cells) has demonstrated that DC-derived CXCL10 facilitates interactions between DCs and naive CD4$^+$ T cells in the T cell zone. Both CXCL10 and CXCL9 (produced by stromal cells) further guide activated CD4$^+$ T cells away from the T cell zone and into interfollicular and medullary zones of the lymph node. This process is important for the development of the Th1 subtype of CD4$^+$ T cells, presumably because it enables CD4$^+$ T cells to receive stimuli from cell populations located in the periphery of the lymph node, including SCS macrophages (7, 32). Cytokines such as interleukin-12 (IL-12), which can be produced by DCs to activate T cells, is also detectable by intravital imaging (Fig. 2D).

Monocytes and Macrophages

Intravital imaging has been used to examine macrophages in various tissues, including lymph nodes (59), tumors (25, 60, 61), liver (62), lung (63), pancreas (11), kidney (64) and the brain (65). Macrophages can be viewed using genetic reporters such as MerTK-GFP (11) or after in vivo labeling with fluorescent nanomaterials (66) (Table 1). Furthermore, imaging has been employed to study several macrophage immune functions, including microenvironmental surveillance (67), bacteria capture (68), adaptive immune response stimulation (59), extracellular matrix remodeling (69) and tissue repair (62), as well as perhaps less expected functions such as shielding injured cells (70), exchanging cytoplasm with neighboring cells (71) and promoting vasculature bursting (72).

Macrophages that accumulate in inflammatory sites typically originate from circulating monocytes, which can be detected using CX3CR1 reporter mice (73). The same mice can be used to track splenic reservoir monocytes and their deployment to the circulation in response to inflammatory cues (74). Additionally, CX3CR1-GFP;CCR2-RFP mice can be used to distinguish between monocyte/macrophage subsets that express these chemokine receptors at different levels (62). These mice were used to show that mature macrophages from the peritoneal cavity can migrate to the liver after acute injury (62), challenging the paradigm that macrophages are sessile immune cells. In contrast to macrophages in inflammatory sites, many macrophages found in resting adult tissue are established before birth. Imaging can also be used to track the development of the immune system through embryonic precursor populations by using CX3CR1 reporter models. These approaches have shown how yolk sac pre-macrophages migrate to the developing embryo and seed macrophage pools (75).

Neutrophils

Neutrophils have been imaged in various tissues such as liver (76), lung (77), skin (78), spleen (79), joints (80), trachea (81) and tumors (82). Methods to track neutrophils rely either on tagging them with fluorescent antibodies, such as anti-Ly-6G mAbs, or using genetically encoded reporters, such as LysM-GFP (Table 1). The antibodies used to tag neutrophils are given at lower doses than for cell depletion studies, and the neutrophils are imaged shortly (~15 min) after intravenous mAb administration. Such approaches require testing that the injected mAbs label the targeted cells without substantially altering their fate. Also, care must be taken in interpreting LysM-GFP studies because macrophages can also express LysM. Neutrophil extracellular traps (NETs), which are composed of DNA and are

released by neutrophils to eliminate extracellular pathogens, can be visualized using nucleic acid-labeling agents (e.g. SYTOX Green) or antibodies against extracellular histones or neutrophil elastase (83). In sterile liver injury models, photoactivatable cell tracking approaches have started to reconstruct the journey of neutrophils. These studies identified that neutrophils, initially produced in the bone marrow, are recruited to the injury site where they mediate key effector functions, and then reenter the circulation and relocate to the lung and eventually the bone marrow where they are eliminated (76). The implications for neutrophil egress from injury sites, and homing back to the sites where they were produced, requires further study.

Other Hematopoietic Cells

Hematopoietic stem cells (HSCs), megakaryocytes, platelets, eosinophils and innate lymphocytes can also be tracked in living animals (Table 1). For example, intravital imaging of the mouse calvarium has helped characterize the location and composition of HSC niches in the bone marrow (27, 84). HSC progeny can also be tracked in extramedullary tissues (85). Additionally, CD41-YFP and Pf4-cre:mTmG transgenic mice are useful for detecting megakaryocytes and the platelets they produce. Intravital imaging studies have revealed platelets' contributions to host defense. For example, they can promote the release of NETs to protect host cells from virus infection (86), collaborate with Kupffer cells to encase bacteria (87), help circulating T cells to probe for the presence of antigens (41), and migrate at sites of vascular injury to collect bacteria (88). Intravital imaging also identified the lung as an unexpected site of platelet production (89). Imaging has also been used to track eosinophils within mouse airways (85). Lastly, imaging studies have started to assess innate lymphocytes in various settings such as tissue repair (90), stroke (91), skin homeostasis (92), tumors (93), and patterning immune responses in lymph nodes (94); Kastenmüller et al., 2013, #93282}. Innate lymphocyte imaging largely relies on CXCR6 reporter mice; iNKT cells and ILC2 express this chemokine receptor, though other cell types may as well.

INTRAVITAL IMAGING TO STUDY DRUGS AND THEIR EFFECTS ON IMMUNE CELLS

A major development in single cell imaging has been the ability to track drugs in complex tissue microenvironments (12). When combined with immune cell reporters, single cell imaging can uncover pharmacokinetics (PK) and pharmacodynamics (PD) at the single cell level with unparalleled insight into drug resistance (25) and mechanism of action (95) (Fig. 3A). For PK studies, fluorescently labeled drugs are imaged over time to follow their distribution and cell tropism. The approach requires validation that the fluorescent drug retains biological activity, which appears to be the case for antibody drugs and can also be achieved for small molecules. For example, single cell imaging has been used to uncover that the immune checkpoint blocker anti-PD-1 mAb initially binds its intended targets (i.e. PD-1 expressed on the surface of T cells) but can then be quickly captured by neighboring macrophages, which limits therapeutic efficacy (25) (Fig. 3B). For PD studies, all reporter systems presented in Table 1 could be deployed to assess a drug's impact on immune components while additional reporters are emerging (12). For example, since IFN-γ is a key factor required for anti-tumor immunity, IFN-γ-eYFP reporter mice can be used to assess a

central readout of successful immunotherapy response (25). PD studies have also shown that radiation therapy can prime tumor-associated macrophages to induce vascular bursts, which enhance drug uptake in the tumor microenvironment (Fig. 3C), and that chemotherapeutics can induce tumor infiltration by myeloid cells, which contribute to chemoresistance (96, 97). Currently, intravital imaging of drug responses focuses mostly on cancer, though existing approaches can be adapted for studies of host defense or autoimmunity.

NEEDS AND EMERGING APPROACHES

Further improvement of intravital single cell imaging for an improved understanding of immunology will be possible with technological, biological and computational advances. Technological needs include active and passive methods of motion suppression (24) to image an increasing number of tissues; new chambers and surgical exteriorization methods (9, 19) for imaging in orthotopic organs; photoacoustic microscopy (98–100) for body-wide imaging; infrared imaging and three-photon imaging (98, 101) for deeper imaging (beyond 200 μm); and implantable microscope optics (102, 103) for imaging over days. Biological needs include new mouse models with fluorescent reporters in multiplex compatible formats for visualizing several immune cell (sub)types simultaneously, new signaling reporters—also in multiplex format—for more detailed understanding of immune cells' molecular activities, as well as labeled antibodies and nanoprobes that are validated for intravital microscopy and could also be useful for clinical translation. Computational needs include the development of more efficient analytical software, which could be achieved by adapting deep learning and artificial intelligence solutions (104) and could also be used for whole organ mapping (105, 106). The combination for single cell imaging with single cell RNA sequencing or FISH should also offer deeper integration of physiological data at the cellular and molecular levels (53). Beyond these needs, a number of emerging technologies are on the horizon, including tissue clearing for whole organ imaging, photoactivatable optogenetic reporters and photoacoustic microscopy. These methods have considerable potential and applications for immune cell imaging. Tissue clearing methods (e.g. CLARITY, CUBIC, C_e3D) reduce light scattering and thus enable deep tissue imaging (107). These methods are still limited to excised and perfused whole organs but nevertheless represent a powerful method for 3D reconstruction of immune cell populations at the whole organ level (108, 109). Finally, photoacoustic microscopy is an innovative high-resolution, high-speed imaging methodology that can provide deeper, larger field-of-view optical contrast, especially when combined with cell-specific exogenous labeling agents. While much development has happened at the meso- and macroscopic scales (110), future developments will surely advance the field of immune cell imaging.

ACKNOWLEDGEMENTS

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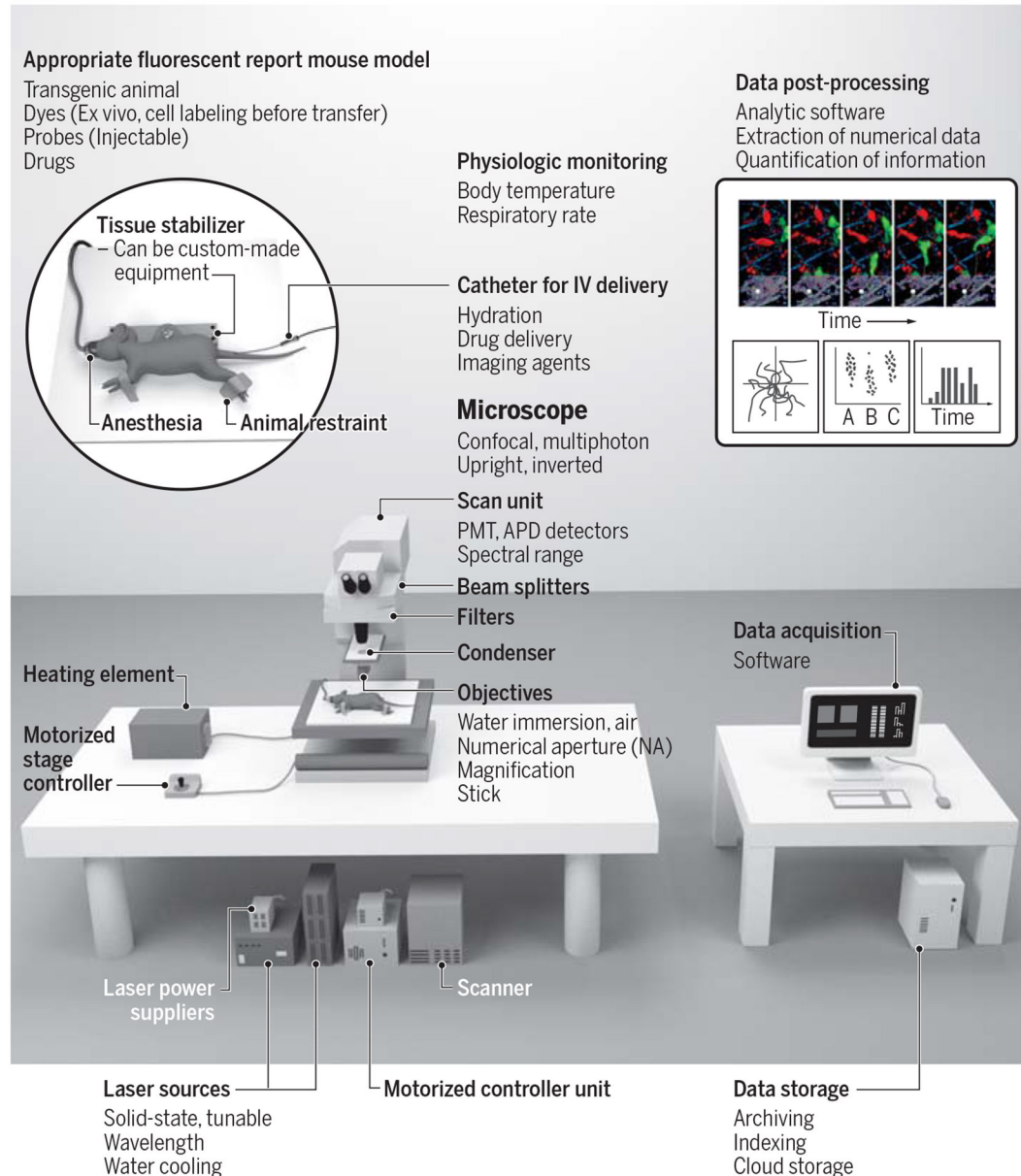
Dark, ventilated, cool room (~200–300 sq. ft.)

Appropriate fluorescent report mouse model
Transgenic animal
Dyes (Ex vivo, cell labeling before transfer)
Probes (Injectable)
Drugs

Data post-processing
Analytic software
Extraction of numerical data
Quantification of information

Tissue stabilizer
– Can be custom-made equipment
Anesthesia Animal restraint

Physiologic monitoring
Body temperature
Respiratory rate

Catheter for IV delivery
Hydration
Drug delivery
Imaging agents

Time ⟶

A B C   Time

Microscope
Confocal, multiphoton
Upright, inverted

Scan unit
PMT, APD detectors
Spectral range

Beam splitters

Filters

Condenser

Objectives
Water immersion, air
Numerical aperture (NA)
Magnification
Stick

Heating element

Motorized stage controller

Data acquisition
Software

Laser power suppliers

Scanner

Laser sources
Solid-state, tunable
Wavelength
Water cooling

Motorized controller unit

Data storage
Archiving
Indexing
Cloud storage

Fig. 1 –. Diagram of an intravital imaging setup.
Left: the equipment required for conducting an intravital imaging experiment includes an appropriate fluorescent mouse model, a microscope (shown here is an upright multiphoton microscope), laser sources, physiological monitoring, an appropriate anesthesia setup, and a tissue stabilizer to prevent motion artifacts during imaging. This image illustrates imaging in a dorsal skinfold window chamber; other tissues can be imaged (see Figure 2), each of them requiring their own methods of tissue preparation, stabilization and monitoring. Right: data acquisition, followed by data storage and data post-processing to extract quantitative information. Credit: A. Kitterman/AAAS
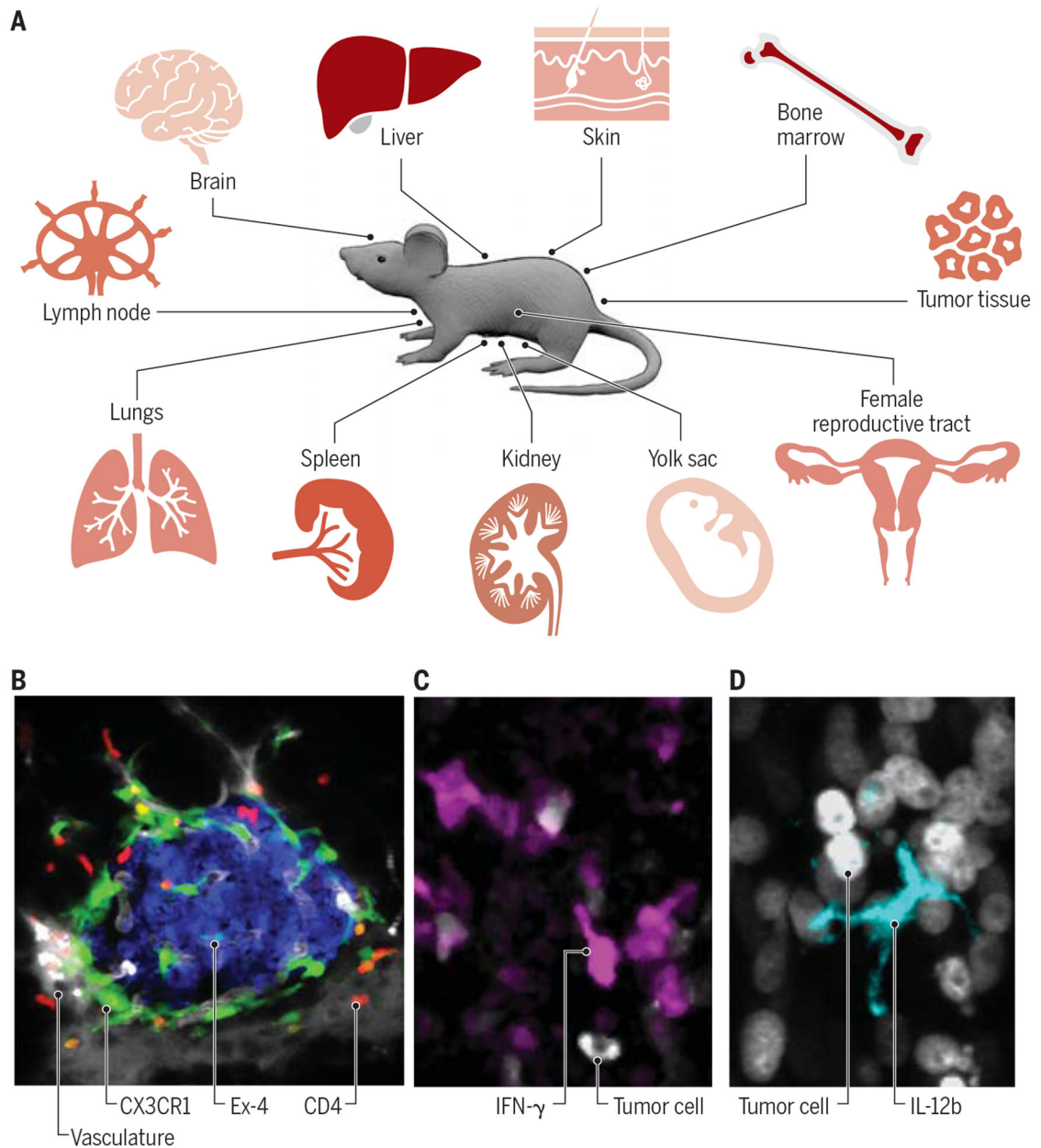
Fig. 2 –. Existing applications of immune cell imaging in various tissues.

(**A**) A wide range of mouse tissue sites is adapted for single cell imaging. (**B**) Intravital micrograph showcasing detection of two immune cell types, namely CD4^{+} T cells (red) and CX3CR1^{+} macrophages (green) in pancreatic islets. β-cells (blue) were visualized with exendin-4–like neopeptide conjugated to the fluorescent dye Se-Tau-647 and the vasculature was detected with 500 K MW dextran conjugated to a Pacific blue dye (grey). (**C-D**) Intravital micrographs showcasing detection of cytokines produced by immune cells in live mice, here in tumor tissues. Panel C shows IFN-γ-producing lymphocytes (magenta) and panel D shows IL-12b-producing myeloid cells (cyan). Tumor cells are also shown (grey). All scale bars represent 10 μm. Credit: A. Kitterman/AAAS
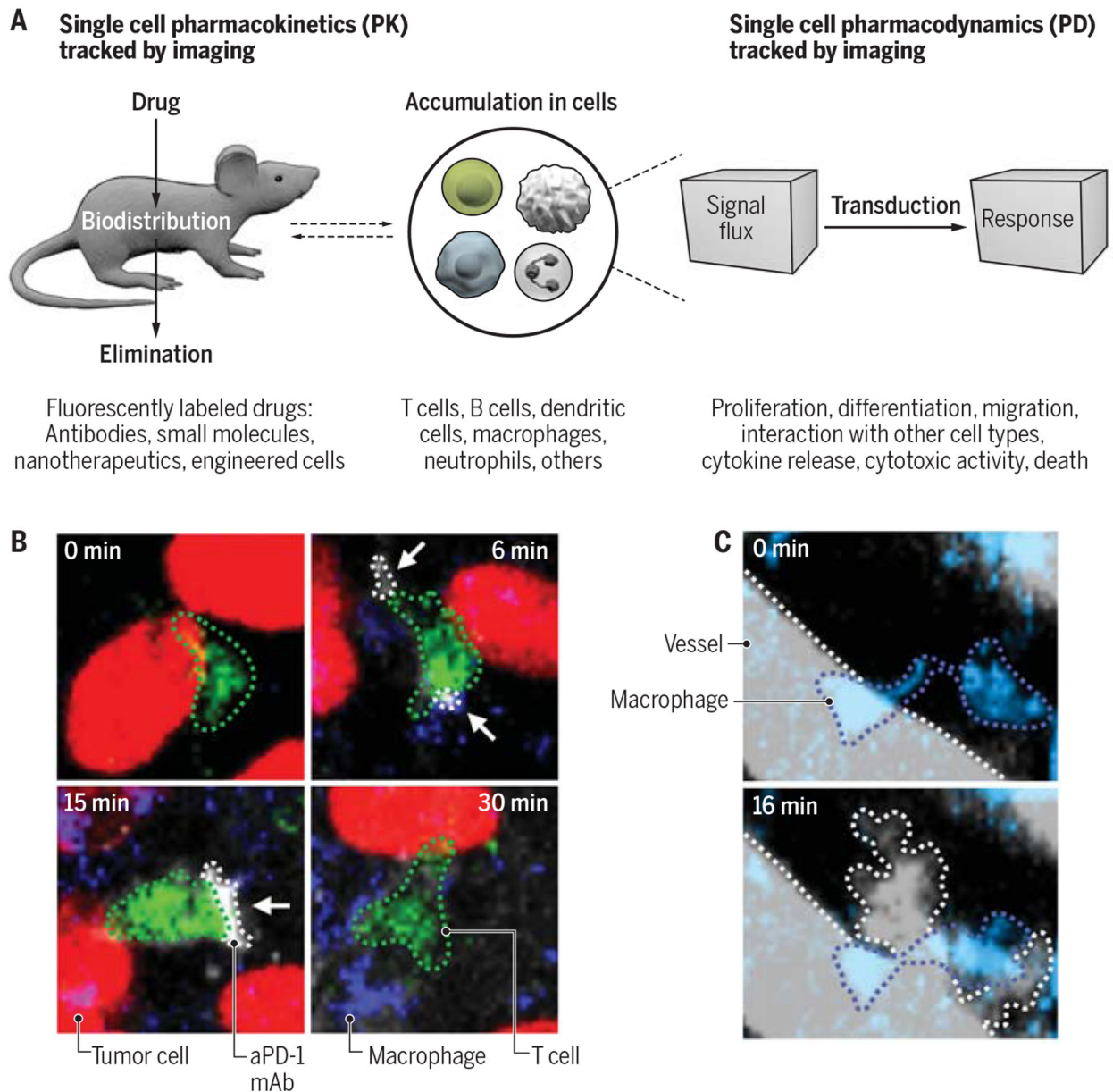
Fig. 3 –. Single cell imaging of drugs and their effects on immune cells.
(**A**) Intravital imaging can reveal drugs' PK (left) and PD (right) at single cell resolution. The examples listed below indicate which types of drugs, cells and responses can be detected and are relevant to immunology. (**B**) A PK study reveals that the immune checkpoint blocker anti-PD-1 mAb (aPD-1 mAb, gray) binds to T cells (green) only transiently in the tumor stroma (25). (**C**) A PD study reveals that radiation therapy primes tumor-associated macrophages (blue) to initiate vascular bursts (courtesy of M. Miller, Massachusetts General Hospital, Boston). Credit: A. Kitterman/AAAS

Table 1:

Reporter systems to track different immune cells and their functions by single cell imaging[*]

Cell Type/Other	Reporter	Notes	Examples
T Cells	GFP, CFP, DsRed	Requires adoptive cell transfer	(29)
	PA-GFP	Photoactivatable fluorescent reporter useful for cell tracking; requires adoptive cell transfer	(52)
	DPE-GFP	A pan T cell marker, can also label pDCs	(25)
	NFAT-GFP, NFAT-YFP;H2B-mCherry	Readout of TCR signaling	(44)
	IFN-γ-YFP	Subset of activated T cells; can also be produced by NK cells	(25)
	IL-10-GFP	Subset of T cells; can also be expressed by macrophages	(44)
	FOXP3-GFP, FOXP3-mRFP	Labels regulatory T cells; FOXP3 is on the X chromosome, be aware of X chromosome inactivation in females	(27)
	Nur77-GFP	Labels T cells and also B cells upon antigen receptor engagement	(111)
	GranzymeB-TdTomato	A a marker of granule exocytosis in cytolytic T cells and NK cells	(112)
	Tbx21-ZsGreen	For assessing Th1 responses in T cells	(113)
	CD2-eGFP	Labels peripheral T cells and some NK subsets	(114)
	IL-17F-Cre[eYFP]	Many variants exist for studying Th17 cells in vivo	(115)
	IL-4-eGFP	Used to assess Th2 immunity	(116)
	IL-21-GFP	Used to study the role of T_{FH} cells	(117)
B Cells	GFP, CFP, DsRed	Requires adoptive cell transfer	(118)
	PA-GFP	Photoactivatable fluorescent reporter useful for cell tracking; requires adoptive cell transfer	(52)
	CD19-tdRFP	Expressed by numerous B cells, adoptive transfer may be necessary if tissues are densely infiltrated by B cells	(50)
	Bcl6-YFP	A B cell marker already validated for 2-photon microscopy; also labels T_{FH} cells	(119)
	Blimp-1-YFP	Expressed by B cells and highly expressed in plasma cells	(120)
	AID-GFP	Subset of B cells	(121)
Dendritic Cells	CD11c(ITGAX)-YFP, CD11c-mCherry	Cells other than DCs can express CD11c	(122)
	XCR1-Venus/+	Homozygous mice are null for XCR1	(56)
	XCR1-KIKGR/+	Xcr1 replaced with photoconvertible fluorescent protein Kikume Green-Red (KikGR)	(56)
	CXCL9-RFP, CXCL10-BFP	These chemokines can also be expressed by other cell types, including monocytes, macrophages, granulocytes and non-immune cells	(32)
	SIGLEC-H-GFP	Expressed by pDCs	(55)
	Flt3-BFP2	This construct has also been incorporated into a genetic model used to visualize DCs, macrophages, CD4^{+} and CD8^{+} T cells simultaneously (Flt3-BFP2, Mertk-GFP-DTR, Cd4-tdTomato, Cd8a-tdTomato)	(11)
	Zbtb46-GFP	A cDC marker that may be expressed in erythroid and endothelial populations	(123)
Monocytes/ Macrophages	CX3CR1-GFP/+	This chemokine receptor can also be expressed by T cells, NK, DC, and macrophages. Homozygous mice are null for CX3CR1	(124)
	CCR2-RFP/+	Homozygous mice are null for CCR2	(62)
	CX3CR1-GFP/+;CCR2-RFP/+	Homozygous mice are null for CX3CR1 and/or CCR2	(62)
	Mertk-GFP-DTR	Homozygous mice are null for MerTK	(11)

Cell Type/Other	Reporter	Notes	Examples
	c-fms-GFP	c-fms is also known as Csf-1 receptor	(61)
	MacBlue	A gal4-responsive UAS-ECFP cassette also under the Csf-1 receptor promoter	(125)
	Dye-conjugated Ferumoxytol	Administered to mice several hours before imaging	(25)
	Dye-conjugated dextrans and dextran nanoparticles	Administered to mice several hours before imaging; can be used to label blood vessels in short-term after administration	(72)
	Dye-conjugated anti-CD169 mAb	Used to label SCS macrophages	(126)
	Dye-conjugated anti-F4/80 mAb	A pan macrophage marker in mice	(62)
Neutrophils	Dye-conjugated anti-Ly-6G mAb	Imaging is performed shortly after administration (~15 min)	(127)
	Dye-conjugated anti-Gr-1 mAb	Imaging is performed shortly after administration (~15 min)	(127)
	LysM-GFP	Can also be expressed by macrophages	(78)
	Dye-conjugated anti-neutrophil elastase mAb	Imaging is performed shortly after administration (~ 15 min)	(86)
	CXCL12-mRFP	Can also be expressed by stromal cells	(81)
	SYTOX Green	Used to visualize NETs (extracellular DNA labeling); also demarcates cell death	(86)
Innate Lymphocytes	GFP, CFP, DsRed	Requires adoptive cell transfer	(93)
	CXCR6-GFP	Can be used to track ILCs and iNKT cells but is also expressed by some T cells	(90)
Megakaryocytes and Platelets	PF4-cre tdTomato	Labels megakaryocytes as well as platelets, which are defined based on their smaller size	(89)
	Dye-conjugated anti-CD49b mAb	Labels all platelets	(86)
	CD41-YFP$^{ki/+}$	Labels a fraction of platelets	(86)
HSC-Derived Cells	GFP, CFP, DsRed	Requires adoptive cell transfer and is used to track HSCs and their progeny	(85)
Non-Immune Components	Dye-conjugated anti-Lyve-1 mAb	Labels lymphatics	(46)
	AngioSPARK	Labels blood vessels	(72)
	Second Harmonic Generation (SHG)	Applicable to multiphoton (not confocal) microscopy; enables visualization of collagen fibers	(92)
	IL-7-eCFP	Expressed by stromal cells in thymus and bone marrow	(45)
	GCaMP6s	Sensor of calcium flux; relevant in the context of e.g. virus-infected cells	(40)
	qDots	Bright, highly tunable fluorophores surface-engineered to maximize circulation	(128)
	Isolectin	Used to label endothelial wall of blood vessels	(129)
	Hoechst33342 and CellTracker dyes	Indiscriminately labels cell nuclei or cytosol; often used for adoptive cell transfer	(42)

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This list serves as a general survey of intravital imaging labeling strategies across cell types, however numerous Cre strains can additionally be incorporated into useable reporter systems.