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**Tumor immunosurveillance and immunotherapies:
a fresh look from intravital imaging**

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Understanding the complex interactions between the immune system and the tumor microenvironment is an essential step toward the rational development and optimization of immunotherapies. Several experimental approaches are available to tackle this complexity but most are not designed to address the dynamic features of immune reactions including cell migration, cellular interactions and transient signaling events. By providing a unique means to access these precious parameters, intravital imaging offers a fresh look at intratumoral immune responses at the single-cell level. Here, we discuss how *in vivo* imaging sheds light on fundamental aspects of tumor immunity and helps elucidate the mode of action of immunotherapies. We conclude by discussing future developments that may consolidate the unique contribution of intravital imaging for our understanding of tumor immunity.

Decoding mechanisms of tumor immunity

Recent years have witnessed multiple successes for tumor immunotherapies. Despite these breakthroughs, we are far from fully understanding how the immune system acts within the complexity of the **tumor microenvironment** (see **Glossary**) and how to best harness immune cells to eradicate distinct types of tumors. To date, most of the available immunotherapies are only effective in a fraction of patients and in a subset of cancers. A full mechanistic understanding is therefore essential for increasing the efficacy of these treatments, for developing new strategies or for combining them together. Multiple approaches are available to conduct these mechanistic studies. In preclinical models, manipulation of immune subsets (using knock-out models or antibody-based depletion for example) or genetic manipulation of tumor cells are routinely used. From human samples, high dimensional flow cytometry or single-cell RNA sequencing are powerful technologies to establish important correlations and to suggest putative mechanisms that could be further explored. These types of approaches have

provided important insights into the differential modes of action of blocking T cell co-receptors relevant to immunotherapies, such as *via* anti-CTLA-4 (cytotoxic T lymphocyte-associated protein 4) or anti-PD-1 (programmed cell death 1) [1]. Most of these strategies however have not directly addressed the most dynamic aspects of immune reactions such as intratumoral cell migration, establishment of cell-cell interactions and any associated signaling events. Understanding how these dynamic events are modulated by immunotherapies is likely to help unlock some of the secrets behind their modes of action *in vivo*.

Intravital imaging with single-cell resolution is a technique of choice to address many of these questions *in vivo* and, in the recent years, has helped identify a number of previously unappreciated mechanisms operating in the tumor microenvironment. Here, we provide an overview of models and tools currently available for *in vivo* imaging. We next review how *in vivo* imaging sheds light on fundamental aspects of tumor immunosurveillance and immunosuppression. We also address how dynamic imaging helps elucidate the mode of action of immunotherapies and discuss future technical developments that will reinforce the use of intravital imaging in preclinical models of cancer.

Using intravital imaging to assess tumor immunity

At the beginning of the 20th century, pioneering experiments by Elie Metchnikoff using optical microscopy demonstrated that immune reactions occurred in transparent organisms, such as *Daphnia magna* and starfish larvae [2]. As described elsewhere, progress in imaging technology and biological tools have reinvigorated the use of *in vivo* imaging in the last few decades [3,4]. To date, spinning-disk confocal microscopy, laser scanning confocal microscopy, and **two-photon microscopy** are the three main high-resolution methods for intravital imaging. Each has its own benefits and drawbacks and should be chosen depending on the biological constraints. Briefly, spinning-disk confocal imaging is preferred for its rapid

image acquisition while conventional laser scanning confocal microscopy can generate images of higher resolution. Finally, two-photon microscopy is most often used for deep tissue imaging as it relies on fluorophore excitation by low-energy infrared light.

Intravital imaging in mouse models has provided significant insights into basic immune mechanisms of lymphocyte activation or effector immune responses in a variety of contexts. Applied to preclinical models of cancer with relevant fluorescent labeling, *in vivo* imaging approaches can therefore offer access to numerous dynamic processes in the tumor microenvironment, with the potential to reveal new mechanisms of immune cell activity during tumor development, or during the course of tumor immunotherapies. For instance, by offering crucial information on cellular interactions, cell motility and cell signaling, intravital imaging has proven useful for identifying cellular partners as well as determining whether cells are engaged in antigen-specific interactions (**Figure 1, Key Figure**). The typical workflow for assessing host-tumor interactions in animal models using intravital imaging is described in **Box 1**. Of note, a number of *in vivo* imaging studies have examined the role of the tumor vasculature in influencing indirectly immune responses; we refer the reader to excellent published reviews on that topic [5,6].

Models and tools for intravital imaging of tumor immunity

A large and constantly expanding toolbox is available for intravital imaging of antitumor immune responses. Both human and mouse models have been developed to microscopically explore antitumor immunity. Tumors derived from patients can be transplanted, **orthotopically** or **ectopically**, into immunodeficient mice (such as NSG (NOD *scid* gamma) mice) directly (patient-derived **xenograft** (PDX) models) or following *in vitro* culture (cell line-derived xenograft (CDX) models). In addition, transgenic mice engineered to develop spontaneous cancers (such as models of breast cancer in **MMTV-PyMT** mice [7] and **B-cell lymphoma** in

Eμ-myc mice [8,9]) are suitable to visualize immune responses during the course of tumor development. Strategies to fluorescently label spontaneously developing tumors have been incorporated in some models to facilitate imaging approaches. Alternatively, these spontaneous tumors can be isolated, fluorescently labeled and reinjected into secondary recipients. Finally, **syngeneic** mouse tumor cell lines expressing genetically-encoded fluorescent proteins or reporters have been used extensively for imaging purposes.

In tumor-bearing mice, intravital imaging has been performed in numerous anatomical sites, including the skin, lungs, mammary fatpads, digestive tract, liver, kidney, lymph nodes, bone marrow and brain. Moreover, **window chambers** offer the opportunity to image one particular site at multiple time points, thus giving insights into the progression of any given immune reaction in the same organism [10–15]. Alternatively, techniques using human tumor slices (e.g. from lung, ovarian tumor biopsies) obtained from patients can provide valuable information regarding immune cell behavior in the complex tumor microenvironment. **Table 1** summarizes various tumor models, anatomical sites and immune cells that have been or are currently the focus of intravital imaging studies.

While a wide range of fluorescent proteins is available to label immune and tumors cells, their combination with reporters that measure functional parameters provides an additional layer of information. Examples of genetically-encoded functional reporters used to dissect host-tumor interactions are shown in **Figure 2**. For example, tumor cells can express probes to visualize apoptosis [16,17] and proliferation [18] in the tumor microenvironment. Tumor **multicolor barcoding** provides a means to study tumor clonal heterogeneity, and can give insights into tumor spatial organization [19,20]. In addition, immune cell activity can be tracked using gene reporters for signal transduction, including calcium signaling [17], and nuclear factor of activated T cells (NFAT) nuclear translocation [21], or for cytokine production, using for instance, interferon gamma (IFN- γ) or interleukin 12 (IL-12) reporters [11]. Of note, additional

fluorescent reporters, such as oxygen probes and redox ratios, have been used in mice to image cancer development or responses to chemotherapy and could be potentially applied for the study of tumor immunity [5,22]. The continuous development of new genetically-encoded fluorescent probes to track conditions such as metabolic activity, cell phenotype and functional states can help foster new concepts in tumor immunity.

Mechanisms of tumor immunosurveillance and immunosuppression

Tracking tumor infiltration.

Tumor immunosurveillance requires migration and infiltration of effector cells into the tumor, and intravital imaging studies have helped unveil mechanisms where tumor encounters are either promoted or limited. For example, cytotoxic T lymphocytes (CTL) have been visualized: migrating, stably interacting with, and directly killing antigen-expressing subcutaneous thymoma cells in mice *in vivo* [16,23,24]. These studies have highlighted the role of cognate antigen expression on tumor cells in promoting CTL migration and intratumoral infiltration. Additionally, interactions between extracellular matrix proteins and CD44 expressed on CTL have been reported to be key determinants of interstitial migration in subcutaneous tumors [25]. A patrolling T cell behavior can be key to maintain cancer-immune equilibrium as shown in a melanoma model for resident memory T cells in the epidermis [26]. Of note, distinct effector immune cells may exhibit different immunosurveillance strategies; for example, NK cells tend to form less stable conjugates with tumor cells than T cells, a feature that may favor their intratumoral dissemination [27].

However, several mechanisms have also been shown to limit intratumoral CTL infiltration. Specifically, imaging studies of human lung and ovarian cancer slices revealed how stromal collagen fibers could limit T cell access to tumor cells [28–30]. CTL retention can also be promoted by macrophages [31] and dendritic cells (DCs) [32] in the form of unproductive stable

interactions. Finally, CTL sequestration by apoptotic tumor cells may also limit intratumoral dissemination and reduce killing rate [16]. These studies highlight the need to delineate new strategies for promoting effective T cell-tumor cell encounters.

Identifying cellular partners.

Visualizing dynamic cellular interactions can reveal putative cooperations and crosstalk between distinct immune cell types during antitumor immunity. For example, melanoma-derived antigens can be captured by subcapsular macrophages in the tumor-draining lymph node and then deposited on follicular dendritic cells, thus promoting the humoral immune response [33]. At the tumor margin, in a fluorescent mouse model of spontaneous breast cancer, rare activating CD103⁺ DCs can compete for T cell interactions with tumor-associated macrophages and favor tumor elimination [34,35]. Moreover, previously unsuspected stable NK-DC conjugates in the mouse melanoma microenvironment appeared decisive for effective antitumor responses [36].

Delineating the role of immunosuppressive cells.

Intravital imaging can also shed light on immunosuppressive mechanisms operating in the tumor microenvironment. For instance, **regulatory T cells (Tregs)** have been shown to limit the killing ability of CTLs by reducing granule exocytosis rather than by altering their capacity to form conjugates with target cells [37]. As an additional suppressive mechanism, Tregs have been suggested to kill DCs in tumor-draining lymph nodes, thus limiting the onset of CTL responses [38]. At the tumor site, Tregs can establish interactions with antigen-presenting cells (APCs), decreasing the surface expression of costimulatory molecules such as CD80 and CD86 on APCs and subsequently promoting CTL dysfunction [39]. Similarly, in an orthotopic pancreatic ductal adenocarcinoma model, Treg depletion promoted the accumulation of

intratumoral DCs and their expression of costimulatory molecules, which subsequently favored prolonged contacts between CTLs and DCs [40]. Moreover, Tregs appeared to form a ring around B16 melanoma tumors that could be disrupted following cyclophosphamide administration (a chemotherapeutic agent used as a lymphodepleting, conditioning regimen for **adoptive cell transfer** protocols), thus favoring the infiltration of adoptively transferred CTLs [41]. Finally, the process of immunosuppressive cell recruitment can also be studied with *in vivo* imaging approaches. In this context, the **transmigration** of immunosuppressive Ly6C^{lo} monocytes in tumors has been reported to rely on the CX3CL1/CX3CR1 axis [14]. Altogether, these imaging studies suggest potential avenues for blocking the recruitment and activities of immunosuppressive cells in the tumor microenvironment.

Uncovering spatial heterogeneity in the tumor microenvironment.

An important outstanding question is whether immunosurveillance can occur uniformly across different tumor areas, or whether it varies due to local constraints, therefore possibly creating niches for tumor escape. Tumor-antigen specific CD8⁺ T cells have been shown to traffic differently in zones containing distinct lymphoma subclones in the bone marrow, as revealed by multicolor tumor barcoding and intravital imaging [20]. As an additional factor promoting spatial heterogeneity in the immune infiltrate, intratumoral hypoxia can be inversely correlated with T cell motility, especially within the core of solid tumor compared to the tumor margin [42]. Similarly, tumor-associated macrophages tracked with an Arginase-1 reporter can also exhibit distinct behaviors and morphology depending on whether they are located at the center or at the periphery of solid tumors [43]. These studies all highlight the importance of tumor microanatomy in the regulation of immune cell activity.

Visualizing tumor metastasis.

Intravital imaging can also provide mechanistic insight into tumor cell intravasation and metastasis. These processes have been visualized for example using the MMTV-PyMT mouse model of breast cancer, revealing a role for macrophages in inducing loss of vascular junctions [44]. Another study imaged how pioneer melanoma cells dynamically generate **tumor microparticles** in the lung vasculature when injected intravenously in mice [13]. Specifically, ingestion of these tumor microparticles by monocytes and CD103⁺ DCs recruited in the lungs elicited pro- and anti-metastatic effects, respectively. Finally, neutrophils could contribute to tumor metastasis through the generation of **neutrophil extracellular traps (NETs)** that were observed surrounding metastatic cells in the lungs [45].

As illustrated in this section, the use of intravital imaging has opened up new opportunities to reveal dynamic tumor-immune cell interactions at various stages of tumor development, to pinpoint novel cellular partnerships and to identify new putative targets that might be assessed to ideally boost immunosurveillance or circumvent immunosuppression.

Toward a single-cell understanding of the mode of action of tumor immunotherapies

With the intense efforts to develop new and efficient antitumor immunotherapies, more than ever it is essential to fully understand how these therapies precisely work *in vivo*. Thus, the use of intravital imaging can be decisive in confirming, clarifying or challenging other data, or perhaps preconceived ideas -- obtained most often with *in vitro* experiments-- about the modes of action of specific immunotherapeutic drugs.

Tumor-targeting antibodies.

Directly targeting tumors *via* **monoclonal antibodies (mAb)** can be an effective strategy to reduce tumor burden, as illustrated by the clinical use of anti-CD20, anti-EGFR (epidermal

growth factor receptor) or anti-HER2 (human epidermal growth factor receptor 2) mAb for treating lymphoma, colorectal and breast cancer respectively [46]. Multiple modes of action have been proposed for mAb, including Fc-independent effects as well as Fc-dependent mechanisms such as **complement-dependent cytotoxicity (CDC)**, **antibody-dependent cellular cytotoxicity (ADCC)** or **phagocytosis (ADP)** [47]. Yet, the respective contribution of each of these mechanisms *in vivo* is not well characterized. It is also unclear whether different mechanisms operate at distinct anatomical sites. Intravital imaging has proven to be an invaluable tool to directly address these key questions. For example, two independent studies provided evidence that ADP by **Kupffer cells (KC)** in the liver was a major mechanism for mAb-mediated tumor cell depletion [48,49]. Specifically, KC were visualized and shown to engulf circulating normal and malignant B cells within minutes following anti-CD20 administration. Similarly, KC eliminated circulating B16 melanoma tumor cells and C26 colon carcinoma cells upon mAb therapy thus preventing liver metastasis [49]. Notably, glycoengineering of mAb lowered the threshold for KC-mediated depletion during anti-CD20 therapy [50]. While these studies have focused on circulating tumors, the modes of action of tumor-targeting Ab in solid tumors or in tumors established within lymphoid tissues remain to be identified. To date, ADCC by NK cells has rarely been visualized in mouse models of tumor-targeting mAb treatments despite being largely reported in *in vitro* studies. In one study using subcutaneously injected EL4 thymoma cells, Fc-mediated recognition of tumor-bound mAb by NK cells was not sufficient to induce target killing, but was required to stabilize interactions with target cells thus facilitating cell killing through the **NKG2D** activating receptor [51].

From another angle, **bispecific antibodies** have been engineered to promote contacts between tumors and effector cells. A recent study using a bispecific Ab targeting CD3 and the carcinoembryonic (CEA) tumor antigen in a colon carcinoma xenograft, confirmed that

treatment with the bispecific Ab promoted T cell interactions with tumors, thus resulting in a greater cytotoxic activity at the tumor site [12].

Immune checkpoint blockade.

Immune checkpoint blockers (ICB), including mAb that target CTLA-4, PD-1 or PD-L1 (programmed death ligand 1), have shown great promise for the treatment of some cancers [52], yet a complete understanding of their *in vivo* modes of action is lacking.

In vivo imaging has revealed that ICB can have a strong impact on intratumoral T cell dynamics. Specifically, treatment with anti-CTLA-4 mAb appeared to increase overall melanoma-specific CD8⁺ T cell motility in the tumor microenvironment, although the underlying mechanism is unclear [53,54]. Another study reported that PD-1 blockade could restore CTL conjugation to target cells and their subsequent killing activity in lymph nodes in the context of **graft versus leukemia effects** [55]. Anti-PD-1 activity may also benefit from releasing CD8⁺ T cells engaged in unproductive interactions with macrophages as observed in a spontaneous murine model of breast cancer [31].

Imaging studies have also been key in uncovering unexpected aspects of anti-PD-1 therapy. For example, visualizing the fate of labeled anti-PD-1 mAb in mice bearing MC38 colon carcinomas revealed that after binding to T cells, these reagents are rapidly captured by neighboring macrophages in a Fc receptor-dependent manner [56]. Blocking this capture process increased anti-PD-1 activity by prolonging mAb binding time on T cells. Intratumoral imaging using reporters for IFN- γ and IL-12 cytokines contributed to identify a key cellular crosstalk mediating anti-PD-1 therapeutic effects. In this model, IFN- γ released by anti-PD-1-sensitized T cells was essential to induce IL-12-production by DCs thereby increasing antitumor activity [11]. Notably, anti-PD-1 treatment might not only act to reduce tumor burden

but might also finely tune its subclonal heterogeneity [20], a phenomenon that should be considered when designing combination therapies.

Targeting macrophages.

Targeting of tumor-associated macrophages (TAMs) may offer ways to release T cells and promote their dissemination in the tumor as mentioned previously. In addition, targeting macrophages using anti-CSF1 (colony stimulating factor 1) mAb was shown to restore tumor blood vessel patterning and function as visualized by longitudinal multiphoton imaging in a mouse glioma model [15]. Additionally, intravital confocal imaging was used to better characterize the impact of using depleting anti-CSF1R mAb on the myeloid cell compartment, revealing depletion of cells harboring markers of TAMs and DCs in a mouse model of breast cancer [57]. Finally, targeting the protease-dependent mesenchymal mode of migration of TAMs in a subcutaneous fibrosarcoma mouse model can limit their recruitment and subsequently reduce tumor growth [10].

Cell therapy.

Infusing expanded **tumor-infiltrating lymphocytes (TILs)** or genetically engineered T cells that express a tumor-specific TCR or a **chimeric antigen receptor (CAR)** can represent a very effective strategy to control tumor burden [58]. Among the important outstanding questions is how efficiently transferred effectors infiltrate the tumor and at which rate they kill tumor targets. Strategies to visualize tumor killing in real-time can provide insights into these important parameters. In a model of solid tumor using EL4 thymoma, adoptively transferred CTLs were found to kill tumor cells at a relatively low rate (1 killing event per CTL every 6hrs on average) and their accumulation at high density was essential to induce tumor regression [16]. Combining therapies might boost killing activity as illustrated by the use of an agonistic

anti-CD137 (a T cell co-receptor) mAb which promoted the cytotoxic activity of transferred CTLs [59]. Notably, CTL activity can vary extensively depending on the anatomical site. In a model of graft versus leukemia effect, CTL recognition was specifically silenced in lymph nodes due to widespread PD-L1 upregulation as visualized by two-photon imaging [55]. CTL activity could be modulated over time as illustrated by the observation that their ability to stably engage targets decreased with their residence time in the tumor microenvironment [60].

Real-time imaging of CAR T cell killing and signaling using **Förster resonance energy transfer (FRET)**-based probes for apoptosis and calcium signals in a model of B cell lymphoma revealed extensive heterogeneity in CAR T cell activity [17]. Nevertheless, a subset of CAR T cells could engage, kill and detach from tumor targets in a short period of time (25 min) and these events were sufficient to eliminate the bulk of tumor cells [17]. Finally, imaging may help validate strategies to overcome tumor escape from CAR T cell therapy such as the use of T cells expressing two distinct CARs directed against the CD19 and CD123 molecules that retain the ability to interact with CD19-negative leukemia cells [61].

Overall, intravital imaging not only improves our mechanistic understanding of these treatments but also provides clues about how to improve their activity *in vivo*.

Concluding Remarks

In sum, by providing dynamic information at the single-cell level in the tumor microenvironment, intravital imaging represents a powerful approach to identify fundamental mechanisms of tumor immunosurveillance and immunosuppression. In addition, this approach is refining, when not redefining, our understanding of how immunotherapies might actually be acting *in vivo* (**Figure 3**). This knowledge is essential to identify ways to improve or combine immunotherapies effectively. For this reason, one should consider including intravital imaging experiments as an essential step in preclinical development of new immunotherapies.

Moreover, intravital imaging can be combined with other techniques. For instance, **dynamic *in situ* cytometry (DISC)** relies on cell surface labeling to link phenotypic markers and motility parameters [62]. Photoactivation of fluorescent proteins can be used to pinpoint some of the cells imaged and subsequently analyze them by flow cytometry [63], or single-cell RNA sequencing (**Niche-Seq**) [64]. Imaging can also be coupled to techniques that mark cell-cell interactions such as the **LIPSTIC (Labeling Immune Partnerships by SorTagging Intercellular Contacts)** approach [65]. Many new questions and challenges lie ahead (**OUTSTANDING QUESTIONS**). New mouse models of cancer relevant for human diseases, emerging immunotherapies as well as novel functional reporters and optogenetic strategies can be used to manipulate immune cells during imaging (**Box 2**); these approaches can further establish intravital imaging as a unique and essential tool enabling a deeper understanding of immune cell activities in the tumor microenvironment, and informing putative immunotherapeutic approaches to treating malignancies.

Box 1. Technical workflow for intravital imaging

A typical intravital imaging experiment proceeds through the following steps:

Choosing the relevant model to image

- Select a genetically-engineered spontaneous or transplantable fluorescent mouse model of cancer
- Use fluorescent reporter recipients to visualize immune cells of interest and/or adoptively transfer fluorescently labeled immune cells

Reaching the site of imaging

- Insert window chamber for longitudinal imaging
- Surgically expose the site of interest for immediate imaging

Image acquisition and data collection

- Maintain optimal and physiological conditions for animal welfare and imaging quality: anesthesia, analgesia, temperature, oxygen...
- Choose the correct mirrors and filters to visualize fluorescent cells
- Choose the appropriate laser wavelength for the excitation of the distinct fluorescent proteins and dyes
- Determine acquisition parameters (laser power, 3D volume, scanning rate and time intervals)
- Define length of the recording

Data extraction and analysis

- Choose a relevant software for time-lapse movie analysis
- Determine the parameters to be quantified in the regions of interest (speed, confinement, shape, signal intensity, contacts...)
- Proceed to automated (eg. cell tracking) or manual parameter quantification
- Graph the extracted data
- Assemble the time-lapse movie as 2D projection of the 3D volume (legends, time display, scale bar)

Box 2. Combining intravital imaging and optogenetics to manipulate individual immune cells

The use of light sensitive-proteins has allowed the engineering of multiple genetically-encoded systems to manipulate cellular function with exquisite spatiotemporal control (referred to as **actuators**) [66,67]. The technologies, termed optogenetics have been used extensively in neuroscience but have only emerged recently as a new tool for immunologists. For example, optogenetic tools have already been used to manipulate trafficking of immune cells *in vivo*, by using a system in which CXCR4 signal is triggered by light exposure [68]. Calcium actuators that trigger aggregation of **stromal interaction molecule 1 (STIM1)** can provoke DC activation or augment CD8⁺ T cell cytotoxicity [69,70]. Chemokine secretion [71] or TCR signaling [72,73] can also be manipulated with light. To date, most optogenetic approaches with immune cells have been conducted *in vitro* or *in vivo* by bulk photoactivation of the whole tumor, but combined with intravital imaging, optogenetic actuators might also potentially allow the manipulation of single immune cells as we observe them. This may offer a unique ability to understand the functional contribution of specific cells and/or the consequence of a given cellular function in the tumor microenvironment. Among the challenges facing the use of single-cell optogenetics in tumors are the ability to design actuators with low background activity and high response rates, to introduce actuators in primary immune cells and to photoactivate cells located deeply inside tumors. Yet, controlling single immune cells in tumors and assessing the functional consequences of such manipulations may represent an important milestone in the field of in intravital imaging.

Glossary

Actuators: Genetically-encoded tools for light-activated control of proteins regulating cellular functions.

ADCC: A cytotoxic immune response often elicited by NK cells following the recognition through Fc receptors of antibody-bound tumor cells. This results in the delivery of their cytotoxic content toward the target cell and its subsequent death.

Adoptive cell transfer: A therapy based on the isolation and the infusion of immune cells into the same or another individual.

ADP: The mechanism by which macrophages can recognize, through their Fc receptors, antibody bound to tumor cells, resulting in cell engulfment and destruction.

B cell lymphoma: B cell-derived tumors developing in lymphoid organs.

Bispecific antibodies: Synthetic antibodies that can simultaneously bind two distinct antigens.

CAR T: T cells genetically engineered to express synthetic receptors composed of intracellular signaling domains fused to an antibody-derived recognition domain specific for surface target antigens.

CDC: A lysis mechanism triggered by the recruitment of molecules of the complement pathway upon antibody binding to its target cells.

DISC: A technique combining advantages of intravital imaging and flow cytometry analysis to link cell behavior and phenotype *in vivo*.

Ectopic transplantation: Transplantation in a different anatomical site than the original location in the donor.

E μ -myc mice: Genetically modified mice that express the oncogene c-myc driven by the immunoglobulin heavy chain enhancer and that develop spontaneous aggressive B-cell lymphoma.

FRET: An energy transfer between a pair of adjacent fluorophores. FRET is used to monitor proximity between protein domains, conformational changes or protein cleavage.

Graft versus leukemia effect: An immune response against host leukemic cells mediated by transplanted donor immune cells.

ICB: A strategy that aims at increasing immune responses through the blockade of immunoreceptors that inhibit the activity of immune effectors.

Kupffer cells: Resident macrophages found in the liver.

LIPSTIC: A technique that enables the identification of receptor-ligand interactions between cells by using an intercellular enzymatic labeling. When a ligand and its receptor interact, the enzyme mediates the transfer of a substrate onto the tagged receptor.

MMTV-PyMT model: A transgenic mouse model of breast cancer. The mammary tumor virus long terminal repeat (MMTV-LTR) drives the expression of mammary gland specific polyomavirus middle T antigen (PyMT), resulting in a rapid development of highly metastatic tumors.

Monoclonal antibody: A specific antibody that recognizes a unique epitope.

Multicolor barcoding: A series of approach to randomly label individual tumor cells with one of multiple fluorescent proteins to follow subclonal progenies. Barcoding can be performed *ex vivo* by transducing tumor cells prior to transfer or directly *in vivo* using Cre-based random genetic recombination in transgenic mouse models.

NETs: DNA meshes associated to cytotoxic enzymes that are released in the extracellular space by neutrophils and that can trap, neutralize and kill pathogens.

Niche-Seq: A technique that uses photoactivatable fluorescent proteins to sort and analyze gene expression of cells located in selected areas of specific tissue.

NKG2D: An activating receptor expressed by NK and T cells.

Orthotopic transplantation: Transplantation in the same anatomical site where cells or tissue were originally extracted from the donor.

STIM1: A transmembrane protein, mainly localized in the endoplasmic reticulum, involved in the control of calcium entry within the intracellular space.

Syngeneic: Sharing the same genetic background.

TILs: Lymphocytes localized in the tumor microenvironment.

Transmigration: Migration of cells across the endothelium vessel wall.

Tregs: A subset of T cells specialized in the suppression of T cell responses.

Tumor microenvironment: The environment that surrounds and feeds tumor cells. This includes blood vessels, immune cells, fibroblasts, extracellular matrix and signaling molecules.

Tumor microparticles: Microscale blebs that shed from a tumor cell.

Two-photon microscopy: A fluorescence imaging technique that uses two photons of low-energy infrared light to excite fluorophores. This greatly improves imaging depth compared to confocal microscopy and reduces phototoxic effects associated with visible light.

Window chamber: Glass-covered structure surgically implanted into the recipient to enable repeated imaging of a tissue by intravital microscopy.

Xenograft: A tissue graft originating from a different species than the recipient.

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Table 1. Tumor models, anatomical sites and immune cells used in intravital imaging studies.

Site of imaging	Tumor model	Immune cells	Window chamber	Refs.
Skin	EL4 or EG7 (OVA ⁺) murine thymoma s.c.	T		[16,23–25]
		NK and T		[27]
		NK		[51]
	B16 murine melanoma s.c or epicutaneous	T _{RM}		[26]
	B16 murine melanoma s.c.	Tregs, CTL, DC, N		[41]
		CTL		[53,59]
	B78 murine melanoma i.d.	NK, DC		[36]
	4T1 murine breast cancer s.c.	CTL		[54]
	MCA-OVA murine fibrosarcoma s.c.	CTL, DC		[32]
	LBP murine fibrosarcoma s.c.	TAM	Dorsal window	[10]
	CT26 murine colon carcinoma s.c.	CTL		[21]
		CTL, Tregs, APC		[39]
	MC38 murine colon cancer s.c.	T, TAM		[43,56]
T, DC		Dorsal skinfold	[11]	
Human colorectal cancer CEA ⁺ s.c.	T	Dorsal skinfold	[12]	
Lung	4T1 murine breast cancer orthotopically	N		[45]
	MCA-205 murine fibrosarcoma cells i.v.	T		[42]
	B16.F10 murine melanoma i.v.	N, Mo, DC	Intercostal window	[13]
Mammary fatpads	Spontaneous breast tumors in MMTV-PyMT mice	T, DC		[35]
		TAM		[44]
		TAM, DC		[57]
Pancreas	Kras ^{G12D} -PDEC orthotopically	CTL, Tregs, DC		[40]
Lymph nodes	B16.F0 murine melanoma s.c.	TAM, FDC, B		[33]
	CT44 murine colon carcinoma f.p	CTL, Tregs, B		[37]
	MCA-OVA murine fibrosarcoma s.c.	CTL, Tregs, DC		[38]
	E μ -myc murine B-cell lymphoma i.v.	CTL		[55]
Bone marrow	E μ -myc murine B-cell lymphoma i.v.	CTL		[20]
		CAR T		[17]
	Human B-cell acute leukemia xenograft i.v.	CAR T		[61]
	Spontaneous B-cell acute leukemia BCR-ABL ⁺	T		[42]
Liver	E μ -myc murine B-cell lymphoma i.v.	KC		[48,50]
Kidney	EL4 murine thymoma i.v.	CTL, DC		[32]
Cecum	SL4 or CT26 murine colorectal cancer orthotopically	Mo	Cecum window	[14]
Cranial	CT2A and GL261 murine glioma intracranially	TAM	Cranial window	[15]
Human cancer slices	Breast cancer	TAM		[10]
	Lung cancer	TAM, CTL		[29,31]
	Ovarian and lung cancer	CTL		[30]

s.c.: subcutaneous; i.d.: intradermal; i.v.: intravenous; f.p.: footpad; T: T cells; B: B cells; CTL: cytotoxic T lymphocytes; T_{RM}: tissue-resident memory T cells; Tregs: regulatory T lymphocytes; CAR T: chimeric antigen receptor T cells; NK: natural killer cell; APC: antigen-presenting cell; DC: dendritic cell; FDC: follicular dendritic cells; TAM: tumor-associated macrophages; Mo: monocytes; N: neutrophils; KC: Kupffer cells.

Figure legends

Figure 1, Key Figure. Intravital imaging provides access to dynamic parameters within the tumor microenvironment.

This figure illustrates the diversity of parameters that can be collected using two-photon microscopy. Cell migration, cellular interactions, cell division or phagocytosis are readily visualized using fluorescently-labeled cells but the introduction of functional reporters has extended the list of measurable parameters including cell signaling, cell death or gene expression. For instance, nuclear translocation of nuclear factor of activated T cells (NFAT) [21] and calcium (Ca^{2+}) probes [17,74] have been used as readouts of TCR signaling and signal transduction respectively. Cytokine gene expression can be monitored using for example interferon gamma ($\text{IFN-}\gamma$) or interleukin 12 (IL-12) reporters [11]. A fluorescent probe reporting caspase-3 activity enables the detection of apoptosis in real time and *in vivo* [16,17]. Combining several of these readouts can provide unique spatiotemporal clues on immune and tumor cell activity within the tumor microenvironment.

Figure 2. Examples of genetically-encoded fluorescent reporters for the study of host-tumor interactions.

The left column depicts examples of reporters expressed by tumor cells. Tumor apoptosis has been visualized in real-time using a Förster resonance energy transfer (FRET)-based reporter for caspase-3 activity. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) are linked by the DEVD peptide, which is cleaved upon caspase-3 activation, resulting in loss of FRET in apoptotic cells [16,17]. Based on genetic recombination [19] or on multicolor *ex vivo* labeling of tumor cells [20], multicolor barcoding strategies have been employed to investigate intratumoral spatial architecture and clonal heterogeneity. As example for genetic

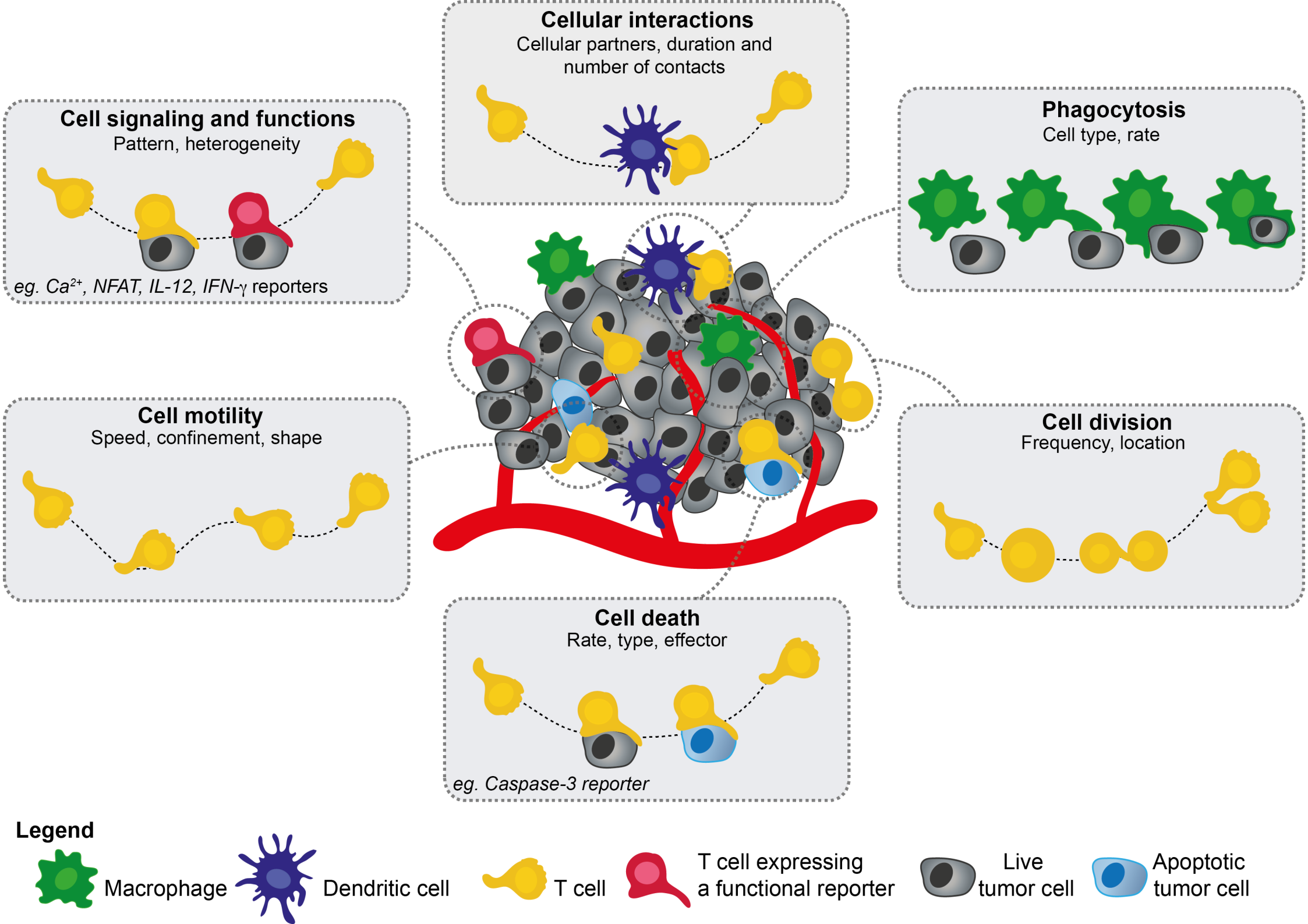
recombination, a multicolor reporter is depicted [75]. Upon Cre recombinase activation, various fluorescent proteins (nuclear green fluorescent protein (GFP), YFP, red fluorescent protein (RFP) and membrane CFP) are randomly expressed. A proliferation sensor, called fluorescence ubiquitination cell cycle indicator (FUCCI) enabled visualization of cell-cycle dynamics in real-time in individual tumor cells [18].

The right column shows some reporters expressed by immune cells. Fluorescent sensors for calcium signaling can be used to detect productive T cell contacts. An example is the Twitch-2B calcium indicator in which FRET occurs upon calcium-binding to troponin C (TnC) [17,74]. Reporter mice have been developed to monitor gene activation at the single-cell level. For example, IL-12 and IFN- γ reporters use an internal ribosome entry site (IRES) to express enhanced YFP (eYFP) following cytokine gene activation [11]. Finally, nuclear translocation of NFAT-GFP has been used as a readout of TCR signaling [21].

Figure 3. Examples of the use of intravital imaging to assess mechanisms of immunosurveillance or the effects of immunotherapies *in vivo* in mice.

This figure compiles time-lapse images from different studies illustrating mechanisms of intratumoral immune activity identified by intravital two-photon imaging. Images are representative of A) Immunosuppression by regulatory T cells (Tregs) forming transient contacts with antigen-presenting cells (APCs) as visualized by two-photon imaging using a dorsal skin-fold chamber. Scale bar, 10 μ m. Adapted from [39]. B) A crosstalk during anti-PD-1 mAb therapy involves IL-12-producing dendritic cells and IFN- γ -producing T cells as visualized by two-photon microscopy using cytokine reporter mice. Arrows highlight IL-12p40-eYFP-expressing cells. TAM, tumor-associated macrophages. Scale bar, 30 μ m. Adapted from [11]. C) *In vivo* two-photon imaging has been used to track the fate of anti-PD-1 mAb (aPD-1) revealing rapid capture by TAM. Yellow arrows indicate sites of anti-

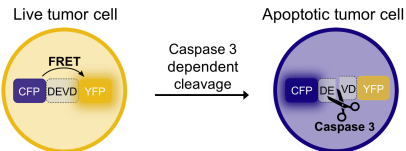
PD-1 mAb binding to CD8⁺ T cells at 15 min. At 30 min, anti-PD-1 mAb is internalized in macrophages. Scale bar, 30 μ m. Adapted from [56]. D) Multicolor tumor labeling can help visualize the extent of clonal heterogeneity in a developing lymphoma in the bone marrow. Scale bar, 50 μ m. Adapted from [20]. E) Visualizing tumor cell apoptosis can reveal rapid and direct killing activity of chimeric antigen receptor (CAR) T cells in the bone marrow. Red arrowheads show a CAR T cell coming into contact with a tumor cell (dotted circle) that subsequently undergoes apoptosis. Scale bar, 20 μ m. Adapted from [17]. F) Two-photon imaging after treatment with an anti-CD20 antibody highlights rapid and efficient B cell phagocytosis by the resident macrophages of the liver. Scale bar, 25 μ m. Adapted from [50]. G) Time-lapse images reveal tissue-resident memory T (T_{RM}) cells dynamically surveying B16 melanoma cells in the skin. Second harmonic generation (SHG) is a label-free signal that can help visualize collagen fibers. Scale bar, 25 μ m. Adapted from [26]. H) Cytotoxic CD8⁺ T lymphocytes and NK cells exhibit distinct behaviors during target cell killing in lymph nodes. Individual micrographs (effectors in green, targets in red) are shown for cells establishing contacts, thus revealing that CD8⁺ T cells maintain contacts with moving targets (superimposed tracks) whereas NK cells transiently interact with their targets (intercrossed tracks). Scale bar, 40 μ m. Adapted from [27].



Tumor cells

Cell death sensor

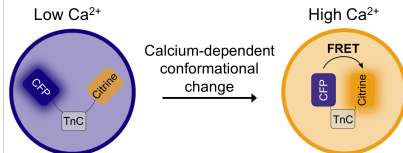
eg. reporter of caspase 3 activity



Immune cells

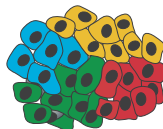
Calcium sensor

eg. Twitch



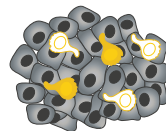
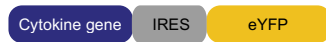
Multicolor barcoding

Genetic recombination
Multicolor ex vivo labeling



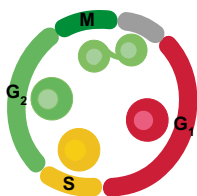
Cytokine reporter

eg. IL-12-eYFP
IFN- γ -eYFP



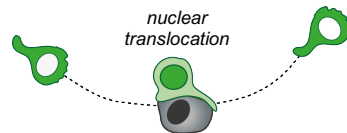
Proliferation sensor

FUCCI

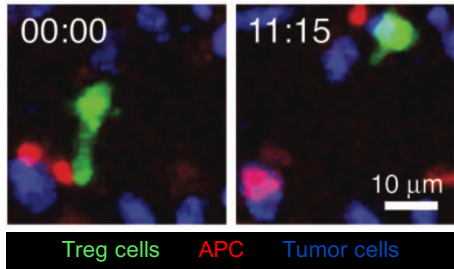


Signaling reporter

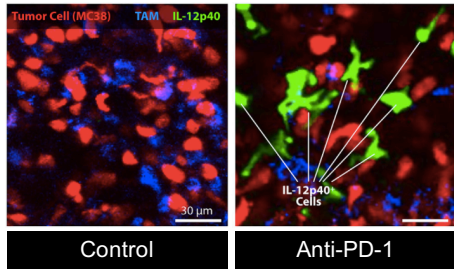
eg. NFAT-GFP nuclear translocation



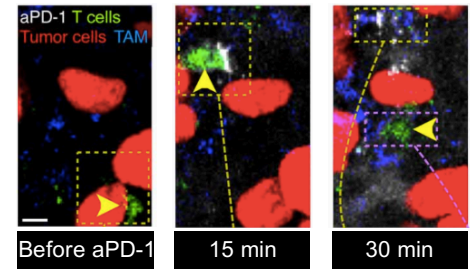
A. Defining mechanisms of immunosuppression



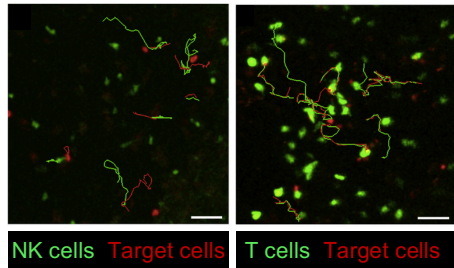
B. Uncovering cellular crosstalk



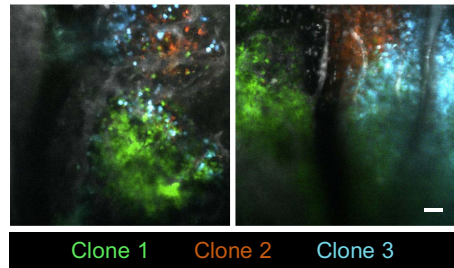
C. Tracking the fate of immunotherapeutic drug



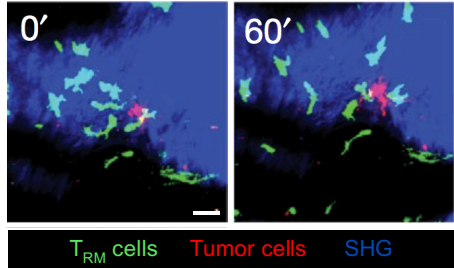
H. Unraveling specific behavior of tumor-infiltrating immune cells



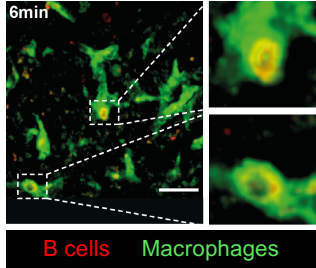
D. Revealing intratumoral clonal heterogeneity



G. Characterizing actors of immunosurveillance



F. Identifying cellular effectors during immunotherapy



E. Determining immune antitumor activity

