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Intravital imaging of stromal cell dynamics in tumors

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Summary

Tumor stroma, consisting of the extracellular matrix and multiple cell types such as immune cells, fibroblasts and vascular cells, contributes to the malignancy of solid tumors by a variety of mechanisms. Intravital imaging by different microscopy techniques, especially by confocal and multi-photon microscopy, has proven to be a powerful method for analyzing the cell-cell and cell-matrix interactions in the dynamic tumor microenvironments. Intravital imaging has fostered the acquisition of data on parameters such as motility of different cell types in distinct tumor regions or manipulated with defined challenges, kinetics of tumor cell killing by T cells or macrophage-assisted tumor cell extravasation, functionality of the vasculature, protease activity and metabolic state. Achieving the direct observation of intact tumors offered by intavital imaging provides unique insights into tumor biology that will continue to deepen our understanding of the processes leading to malignancy and of the ways they can be targeted.

Introduction

It is now widely recognized that the stromal microenvironment of mutated tumor cells is a major determinant of malignancy in epithelial cancers (reviewed in [1]). Composed of a variety of extracellular matrix (ECM) components and several different cell types, the stroma of solid tumors varies according to tumor type, location and stage of disease progression. The stromal cells include carcinoma-associated fibroblasts (CAFs) and the blood and lymphatic vascular cells and other tissue type-specific mesenchymal cells [1], as well as infiltrating immune and inflammatory cells. Infiltrates contain adaptive immune cells, but are dominated by innate immune cells such as macrophages, mast cells, granulocytes, dendritic cells and natural killer (NK) cells that can account for a significant part of the tumor volume. During cancer progression, dynamic interactions between tumor cells, stromal cells and the surrounding ECM are required for the tumor cells to exploit the functionality of stromal cells and generate a microenvironment favorable to malignancy.

In recent years, research has elucidated the distinct roles that stromal cells can play in tumors. The link between inflammation and cancer has long been recognized, and there are striking parallels between tumor stroma and wound healing tissue [2]. Infiltrates of innate immune cells such as macrophages and mast cells correlate with poor prognosis in cancer patients and, along with other myeloid cells such as neutrophils, these cells promote experimental cancers by producing growth factors, cytokines and proteases that stimulate angiogenesis and tissue remodeling, by generating free radicals that induce DNA damage and in some cases by

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suppressing antitumor immunity [3]. On the other hand, natural killer (NK) cell and lymphocyte infiltrates can correlate with favorable prognosis, and these cells have antitumor activity in experimental systems [3]. However, adaptive immune cells such as regulatory T cells, B lymphocytes and CD4+ helper T cells have also been shown to have tumor-promoting functions, illustrating the importance of the context in which these cells function in determining their properties [3,4].

CAFs also have many cancer promoting functions, through their extensive ECM synthesis and remodeling capacity, as well as through their secretion of growth factors and chemokines that can be directly mitogenic to tumor cells or stimulate angiogenesis or immune cells [5,6]. Contact with CAFs can induce otherwise nontumorigenic cells to form tumors, whereas 'normal' fibroblasts may suppress tumor formation; but the mechanisms by which CAFs regulate tumor formation and progression are still incompletely understood [5]. The concerted research effort on the mechanisms of tumor angiogenesis has led to the development of anti-angiogenic cancer therapeutics, three of which are currently approved for clinical use in a range of cancers. However, it has become evident that tumors do evade anti-angiogenic therapy [7]. The potential for targeting lymphatic vessels to stop metastasis [8], as well as the mechanisms that tumors use to evade anti-angiogenic therapy [7], are currently under intense investigation.

The dynamic nature of cell-cell and cell-matrix interactions in the tumor stroma has led to the need for direct observation of the events in tumor tissue to develop a better understanding of the processes involved and to observe the effects of experimental manipulation. With the development of intravital imaging techniques with subcellular resolution, it has become possible to analyze cell behavior in tumor tissues *in situ*. Intravital microscopy of experimental animals has been used to analyze cell movement and interactions since the transillumination brightfield observations of blood flow and leukocyte rolling in the 19th century, and techniques have evolved through epifluorescence to confocal and multi-photon microscopy (MPM) and second harmonic generation (SHG) microscopy of various *in vivo* preparations with different visualization methods (reviewed in [9–11]). Table 1 lists the major challenges that must be addressed to obtain meaningful and/or quantifiable information by live imaging of the tumor microenvironment, and summarizes some of the approaches that can be taken to meet these challenges. The references given are some examples of studies relevant to cancer biology where these techniques were used, and the list is by no means exhaustive. In this review, we highlight the recent advances in our understanding of tumor-stroma interactions that have resulted from the application of intravital microscopy to animal models of cancer.

Dynamics of inflammatory cells in tumor infiltrates

Multi-photon microscopy has been the intravital imaging method of choice for immunologists analyzing dense tissues such as lymph nodes and tumors (reviewed in [9,12,13]). MPM uses short pulses of near-infrared light for excitation, and the combined energy of two or more photons is required to excite the fluorophore (i.e., two photons at twice the normal excitation wavelength). Unlike one-photon confocal microscopy, one excitation wavelength is typically used for multiple fluorophores. Near-infrared light also has the ability to produce SHG signals from non-centrosymmetric organic structures such as fibrillar collagen. MPM has intrinsic confocality, since the probability of near-simultaneous excitation by multiple photons is extremely low anywhere other than at the focal point. This property eliminates the need for a pinhole, and minimizes out-of-focus photobleaching and phototoxicity. Near-infrared light also has superior tissue penetration and less scattering compared to shorter wavelengths. For an excellent overview of the principles of multi-photon microscopy and its application in tumor biology, with special emphasis on techniques such as fluorescence lifetime imaging microscopy (FLIM), spectral-lifetime imaging microscopy (SLIM) and Förster (or fluorescence) resonance energy transfer (FRET) that have been used to study cellular and

molecular interactions as well as the metabolic status of tumors see Provenzano et al. [14]. Table 2 presents a comparison between the different microscopy methods used for intravital imaging of tumors.

Cytotoxic T lymphocytes (CTLs) can infiltrate into tumors, recognize tumor antigens and kill tumor cells. Boissonnas and coworkers used intravital MPM of subcutaneous tumors to analyze the dynamics of adoptively transferred, T cells expressing green fluorescent protein (GFP), and found profound differences in CTL motility and infiltration depending on the expression of their cognate antigen by the tumor cells [15]. In the same experimental model, the actual tumor cell killing by T cells was observed by an elegant strategy, where tumor cells express a cyan fluorescent protein (CFP)-yellow fluorescent protein (YFP) construct with a caspase-cleavable linker, resulting in disruption of FRET between CFP and YFP in apoptotic tumor cells that can be quantified by MPM [16]. MPM has also been used to show that macrophages, visualized by transgenic expression of GFP under specific promoters or by their ability to take up fluorescent dextran leaking from blood vessels, are directly involved in the intravasation of tumor cells in mammary tumors. Moreover, this process depends on a paracrine signaling loop between macrophages and tumor cells involving the epidermal growth factor (EGF) and colony-stimulating factor (CSF)-1 [17].

Despite the advantages of MPM listed above, single photon confocal microscopy techniques are popular for intravital imaging as they are considerably less expensive, user-friendly and allow more convenient use of a wide range of different fluorophores (Table 2). Spinning (Nipkow) disk confocal microscopy is particularly useful for long-term continuous imaging of the tumor microenvironment. The advantages of this system include very rapid image acquisition, which combined with sensitive detection by an intensified charge-coupled device (ICCD) allows continuous imaging with minimal photobleaching [18,19]. The imaging can be done in a lit room, which aids in the observation of the mouse necessary for long-term anesthesia, and the rapid acquisition helps to minimize motion artifacts resulting from respiratory movement of the animal [18,19]. Taking advantage of labeling, it is possible to visualize tumor cells, macrophages, fibroblasts or regulatory T cells through transgenic fluorescent protein reporters, blood vessels and macrophages by intravenous injection of fluorescent dextrans and $Gr1^+$ myeloid cells by injecting a labeled anti-Gr1 antibody [18]. In the mouse mammary tumor virus (MMTV) promoter-polyoma middle T antigen transgenic breast carcinoma model, analysis of the dynamics of at least four different cell types in parallel over periods exceeding 24 hours has indicated distinct cellular behaviors in different areas of the tumor and tumor microenvironments [18]. Figure 1 presents a series of images of MMTV-PyMT tumor nodules with surrounding blood vessels and macrophages, obtained with a spinning disk confocal microscope in a time-lapse experiment. Irrespective of method, the data acquired by intravital imaging of immune cells in tumors have demonstrated that *in vitro* systems or analysis *ex vivo* samples cannot give us a complete picture of the mechanisms by which these cells exert their complex and sometimes paradoxical effects in the tumor stroma.

Functional probes for protease activity

ECM remodeling proteases, such as matrix metalloproteinases (MMPs), serine proteinases and cysteine cathepsins, contribute to cancer progression by multiple mechanisms, including the facilitation of angiogenesis and tumor cell invasion by ECM remodeling and regulation of the bioavailability and activity of growth factors, cytokines and chemokines [20]. Autoquenched protease substrates that fluoresce upon cleavage were first used to image tumor protease activity *in vivo* in 1999 [21], and since then various autoquenched or FRET-based probes, including a near-infrared FRET probe for MMP-7 [22], have been developed for detection of MMP activity (reviewed in [23]). The group of Roger Tsien has developed fluorescently labeled activatable cell-penetrating peptides (ACCPs) that translocate into cells upon cleavage

by MMP-2 or MMP-9, allowing accumulation in tumor cells in the vicinity of protease activity and intravital visualization [24,25]. In agreement with previous data for MMP-2/9 activity, the highest uptake of the peptide in MMTV-PyMT tumors is observed in the tumor-stroma interface. Importantly, small (100 μm) metastatic foci in the lungs are also visualized [26]. These tools can be used to localize regions of protease activity during tumor progression and to assess the efficiency of protease inhibitor treatments. They could be modified for other extracellular proteases as well. ACPPs can also be used for clinical applications, such as magnetic resonance imaging, by conjugating them to contrast agents; they could be used to carry therapeutic payloads into tumors with high protease activity [24,25]. In another recent example of the utility of protease probes for cancer research, the autoquenched near-infrared cathepsin B probe ProSense 680, used in an intravital imaging study of a mouse model of hereditary polyposis, has shown that cathepsin activity is localized to $CD11b^{+}Gr1^{+}$ myeloid cells, and is important for their tumor-promoting activity [26].

Windows to vasculature and metastasis

All of the studies introduced thus far were done in rodents where minimal surgery, namely the generation of a skin flap, is used to expose the tumor. However, following tumor development over a longer period of time with repeated imaging sessions raises additional challenges. Implanted imaging windows allow access to the tumor without the need for repeated surgery. The dorsal skin fold chamber [27] and the cranial window [28] have been used for a long time, especially for examining tumor angiogenesis, permeability and diffusion (reviewed in [11]), and imaging windows over the inguinal mammary gland have been developed more recently [29,30]. Kedrin and coworkers recently used the mammary imaging window to follow tumor cells expressing the photoswitchable protein Dendra2, which allows subpopulations of tumor cells in different microenvironments to be switched from green to red fluorescent when exposed to blue light [30]. This study showed that tumor cells, which are close to blood vessels, are more motile that those further away from vasculature, and noted that the perivascular microenvironments are rich in macrophages and ECM [30]. Migration of tumor cells along blood and lymph vessels and collagen fibers was also observed in a slightly modified dorsal skin fold chamber model [31].

Window chamber models have also been used in combination with imaging to evaluate the response of tumors to anti-vascular therapy. The results show that the therapy leads to normalization of the disorganized tumor vasculature, resulting in better penetration of large molecules such as therapeutics into the tumor [11,32]. In the cranial window model, a functional diamonofluorescein probe that reacts with nitric oxide (NO) was used to monitor NO gradients after silencing of tumor cell NO synthase [33]. Normalization of tumor vasculature was observed when the NO gradient is restricted to perivascular area [33]. Furthermore, platelet-derived growth factor (PDGF)-C was identified as a potential factor in the resistance to current anti-angiogenic therapy in a cranial window model of glioblastoma [34].

A new model of lymphatic metastasis, where a small tumor grown in the tip of the mouse ear can be directly imaged by MPM, is an example of a completely non-invasive method for intravital tumor imaging [35]. Similarly to the dorsal window, however, the tumor size is very limited and the location is non-orthotopic for most tumors.

Conclusions and Perspectives

Application of intravital microscopy is still a recent event in the effort to understand tumor biology. However, it has become clear that observation of the dynamics of tumor cells and stromal cells in context is essential for complete understanding of tumor development,

metastasis and responses to therapy. The combination of intravital imaging in small animal models with *ex vivo*, *in vitro* and clinical analyses of the players involved in cancer and their interactions will enable the development of more powerful strategies for treatment of human cancer, possibly by combinatorial approaches that target both tumor cells and the stroma.

Technological advances in instrumentation and molecular tools for intravital imaging have been rapid during the last couple of decades, and this development shows no signs of slowing down. MPM is moving from near infrared to infrared excitation, allowing the use of red fluorophores and fluorescent proteins [36]. Infrared MPM achieved by using an optical parametric oscillator to convert a shorter wavelength pump beams into two tuneable longer wavelength beams was demonstrated to cause less photobleaching and photodamage than near infrared MPM, while being capable of markedly improved tissue penetration and better SHG [36]. Additionally, a recently developed infrared fluorescent protein engineered from a bacterial phytochrome will allow better deep tissue imaging of reporters also by single photon excitation [37]. Miniaturised epifluorescence and 2-photon fiber optic microscopes that can be used to image even freely moving animals, and confocal endomicroscopy in humans are bringing the advantages of intravital microscopy to the clinic, and offer less invasive imaging solutions also for small animal models [38–41]. A second-generation optical coherence tomography technology termed optical frequency domain imaging can generate truly impressive time lapse images with measurable parameters of blood- and lymphatic vasculature, or viable versus necrotic tumor tissue, over much wider regions than feasible with confocal microscopy [42]. As technology improves, we will obtain a more and more accurate view of the actual real-time events in tumors that combine and collaborate to result in malignancy. The promise that this information can be translated to enable earlier detection, better treatment selection, and eventually better therapy for human cancer is just beginning to be realized.

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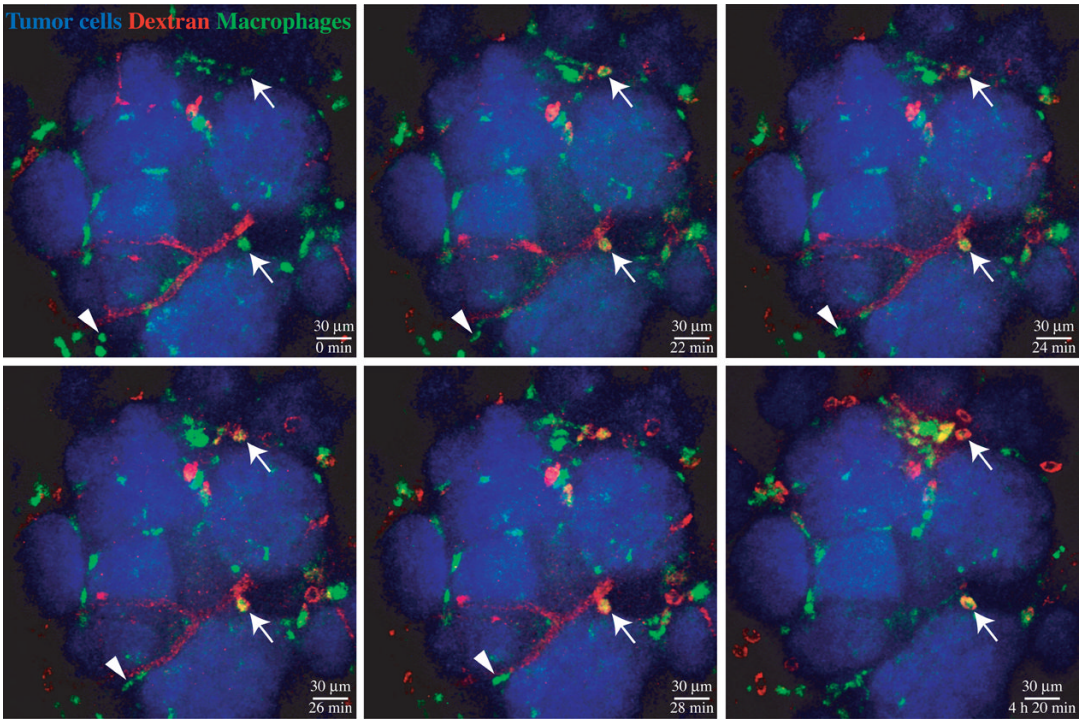

Figure 1.

A series of stills from a time lapse movie of over 4 hours of an early MMTV-PyMT tumor taken with a spinning confocal microscope. Tumor cells are visualized by the high transgenic expression of CFP under the β-actin promoter (blue). The *c-fms* promoter directs GFP expression to myeloid cell, mostly macrophages (green). Rhodamine-dextran (red) injected into the tail vein immediately before imaging accumulates first in the blood vessels, slowly leaks and is taken up by stromal macrophages, and eventually disappears from the blood vessels over time. Macrophages that have ingested dextran (arrows) remain sessile, whereas some other myeloid cells, especially on the tumor margins, are seen to migrate (arrowheads). The panels are maximum intensity projections of three z planes at 4 m intervals. The images have been modified to improve brightness and contrast.

Table 1

Challenges and solutions for intravital microscopy

Challenge	Possible solutions	Refs.
Visualization of tumor components	• Endogenous contrast • Genetic labeling with fluorescent proteins • Transplantation of tracker-labeled cells • Injection of labeled antibodies or other targeted tracers • Injection of functional probes for enzyme activity or changes in e.g. metabolic microenvironment • Injection of high molecular weight tracers to visualize the vasculature and assess permeability (e.g. dextran, nanoparticles) • Injection of tracers to visualize uptake by endocytosis/phagocytosis • Second harmonic generation to visualize ECM components • Combination of multi-color excitation and/or detection, or multi-photon fluorescence combined with second harmonic generation microscopy to visualize multiple components simultaneously	[42–44] [19,30,45,46] [47,48] [19,31,45,49] [22,25,26,33,50] [19,35,47,51] [17,19,51] [46,52,53] [19,31,36,45,46]
Minimal out-of-focus signal detection	• Confocal microscopy (spinning disk/point scanning) • Multi-photon microscopy	[19,54] [14,51]
Minimally invasive tissue preparation	• Imaging of transparent tissues (e.g. mesentery, ear) • Minimal surgery such as skin flap • Imaging windows such as dorsal skin fold chamber, cranial or mammary imaging windows (best for repeated imaging sessions) • Aseptic technique in surgery	[35,43,44] [19,45,54] [27–30]
Long-term anesthesia	• Humidified gas anesthesia for long-term imaging • Careful observation of vital signs • Heating element to keep the mouse warm • Saline injection to compensate for loss of fluid	[19]
Deep tissue imaging	• Multi-photon microscopy, near-infrared or infrared excitation • Infrared fluorescent proteins	[14,36,51] [37]
Minimal photobleaching and phototoxicity	• Rapid image acquisition, low local excitation intensity and sensitive detection • Multi-photon microscopy, near-infrared or infrared excitation • Suitable high-quality objective lenses	[19] [14,36,51]
Minimal motion artefacts	• Rapid image acquisition and sensitive detection • Immobilisation of tissue where possible	[19]

Challenge	Possible solutions	Refs.
Ability to image several regions in the same mouse	• Electronic computer-controlled piezo stage or objective collar • Rapid image acquisition and sensitive detection	[19]

Table 2

Comparison between different microscopy modalities for intravital imaging

Modality	Depth*	Advantages	Limitations
Wide-field epi- fluorescence	20 μm	• Low cost • Very user friendly	• High background • Very limited depth • Phototoxicity and bleaching
Confocal (point- scanning)	50–100 μm	• Very high resolution • Very low out-of-focus signal • Flexible set-up with a wide range of excitation and fluorophore options for multicolor detection	• Limited depth • Phototoxicity and bleaching
Spinning disk confocal	50–100 μm	• High resolution • Low out-of-focus signal • Lower local excitation intensity (lower phototoxicity and bleaching) • Rapid acquisition • Can be combined with very sensitive detection • Flexible set-up with a wide range of excitation and fluorophore options for multicolor detection	• Slightly decreased resolution in z • Fixed pinhole size is optimal for only one magnification (slightly decreased resolution for lower magnification) • High-power illumination of selected regions not possible (e.g. photobleaching, photoconversion)
Near-infrared multi-photon	400–1000 μm	• High resolution • Minimal out-of-focus signal • Minimal out-of-focus excitation (minimal out-of-focus toxicity and bleaching) • Better deep tissue penetration and resolution	• High cost • In-focus phototoxicity and bleaching can still be high
Infrared multi-photon	Increased from near infra-red	• High resolution • Minimal out-of-focus signal • Minimal out-of-focus excitation	• Very high cost • Custom-built systems only

Modality	Depth*	Advantages	Limitations
		(minimal out-of-focus phototoxicity and bleaching) • Reduced in-focus toxicity and bleaching • Improved deep tissue penetration and resolution • Increased range of available fluorophores	• Still in development
Optical frequency domain imaging	> 1 mm	• No need for exogenous contrast agents • No phototoxicity or photobleaching • Very good tissue penetration • Rapid imaging of very wide areas	• Very high cost? • Custom-built systems only • Still in development

*Maximum depth for achieving sufficient resolution depends on the tissue and fluorophores used References: [14,36,42,55,56]