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Textures of the tumor microenvironment

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Abstract

In this review, we present recent findings on the dynamic nature of the tumor microenvironment (TME) and how intravital microscopy studies have defined TME components in a spatiotemporal manner. Intravital microscopy has shed light into the nature of the tumor microenvironment, revealing structural details of both tumor cells and other TME co-habitants *in vivo*, how these cells communicate with each other, and how they are organized in three-dimensional space to orchestrate tumor growth, invasion, dissemination and metastasis. We will review different imaging tools, imaging reporters and fate-mapping strategies that have begun to uncover the complexity of the tumor microenvironment *in vivo*.

A. Introduction

The word *texture* is derived from the latin word *textura*. By definition, a texture is described as the organization of the different fibers contained in a fabric that produce a visual effect. Tumors are dynamic entities where multiple architectural components (blood and lymph vessels, extracellular matrix (ECM)) and different cell types (tumor cells, immune cells, fibroblasts, adipocytes, and endothelial cells) form the “tumor microenvironment” (TME). Immunohistochemistry and electron microscopy studies¹ first defined the basic components of the TME. Recently, the development of live-cell imaging approaches and high-resolution imaging has helped to redefine the TME as a dynamic entity. Recent studies point towards tumors as an evolving ecosystem where tumor cells interact with multiple cell types as the disease progresses². Importantly, it is now accepted that the TME is an active player and has a significant impact on all stages of tumor progression: from growth, dissemination, response to therapy and metastasis formation. We can liken the TME to a tapestry of textures, in which tumor cells, ECM, immune cells, fibroblasts, vasculature and other components are the textures that form the tapestry (Fig. 1).

Initial studies of tumors utilized microscopy and histology tools and described the features observed in tumor specimens with minuscule detail. In 1890, Ramon y Cajal pioneered the study of what we now call tumor heterogeneity and plasticity in one of his seminal works, “*Manual de Anatomia Patologica*”³. While inspecting a mammary tumor, he notes “Deben mencionarse, por frecuentes, las formas celulares en huso, pera, y particularmente en estrella.... formas en estrella que semejan especie de estroma celular” (Ramon y Cajal,

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Manual de Anatomia Patologica, p.254)³. When translated to English: *It is worth mentioning the different cellular shapes found in a tumor such as spindle, pear and star ...[and continues] cells with a star shape looks similar to the cellular stroma* (translated by authors).

As imaging technology has evolved, new techniques have expanded our view of tumors from static to changing entities; this view has been instrumental in translating cancer biology from the bench to the bedside. One imaging approach that has revolutionized our understanding of tumor cell behavior and the TME is intravital microscopy. The possibility of imaging tumors with single cell resolution in living animals has expanded our understanding of tumor phenotypes and helped to define the role of the different TME components in the process of tumor progression.

i) Intravital microscopy of tumors

Intravital microscopy or intravital imaging refers to the technique used to perform microscopy of tissues in organs of a living animal (i.e. mouse, rat, rabbit, zebrafish, chicken embryos). Early studies by Dr. Rakesh Jain showed the potential of using animal models and intravital imaging to study the transport of macromolecules in tumor microcirculation⁴ and angiogenesis⁵. He was one of the first to use imaging windows, such as the mouse dorsal window developed by Algire⁶ for longitudinal experiments in human tumor studies⁷. In these studies, the authors showed the dynamic nature of tumor vessels during tumor progression⁸. The Jain lab used multiphoton laser scanning microscopy to image tumors due to: a) improved signal-to-noise ratio; b) greater imaging depth; c) possibility of longer imaging sessions and d) reduced photobleaching and phototoxicity when compared to confocal scanning microscopy⁹. Additional contributions to the field of tumor intravital imaging were done by the Condeelis lab, who pioneered the study of tumor cell motility using intravital microscopy. They were the first to describe different motility patterns of highly metastatic tumors *in vivo*¹⁰. The authors used GFP-labeled tumor cells to track real-time cancer cell movement, opening a new avenue of tumor biology to further explore how motile behaviors are controlled *in vivo*. These studies also defined tumor cell protrusions and their role in regulating the motility of cancer cells^{11,12}. More recent intravital microscopy work by the Condeelis lab, as we will describe below, identified a unique tri-cellular structure in tumors that is required for tumor cell intravasation¹³ which has been translated to the clinic¹⁴. From the very early intravital microscopy studies to now, there has been an increasing amount of literature on the use of intravital microscopy combined with optical devices to increase temporal and spatial resolution, as well as novel fluorescence reporters in order to define the different components of the TME.

ii) Longitudinal intravital experiments

As mentioned earlier, dorsal windows were used to perform longitudinal studies in tumors⁷. Several anatomical windows have been developed afterwards that have facilitated the study not only of the TME at the primary site (i.e. breast¹⁵, ovary¹⁶) but also in metastatic organs. Examples of these devices include a recently developed permanent lung window to study extravasated cancer cells in the lung¹⁷, a lymph node window to study dissemination in the lymph node¹⁸, and an abdominal window developed by Dr. Jacco Van Rheenen and his lab¹⁹. In their studies they described the different window implantation procedures, their

advantages and disadvantages²⁰. These devices, combined with two-photon microscopy have revealed that cell types and structures in the TME can be dynamically remodeled as tumors and metastasis evolve (see Fig. 2 for examples of imaging windows).

Also, different studies used non-invasive implantation of cancer cells in the mouse ear to study tumor behavior. The thinness of the ear tissue coupled with 2-photon microscopy allowed for longitudinal intravital experiments to record cancer cell motile behaviors. For example, work by Dr. Goetz and his group used this imaging approach to visualize breast cancer cell extravasation²¹. Although ear imaging decreases the risk of tissue inflammation when compared with window implantation, it limits the relevance of the study because of the absence of a proper tumor microenvironment for the type of tumor studied (with the exception of melanoma where implantation is considered orthotopic²²). Paget's seed and soil" hypothesis²³ highlighted the importance of the microenvironment (soil) in determining dissemination patterns of cancer cells (seed) - using imaging windows opens up the possibility to study cancer cell behavior in their orthotopic microenvironments.

iii) Fluorescence reporters to track signaling pathways

Motility of tumor cells is controlled by the activation of signaling pathways that remodel the actin cytoskeleton, regulating the formation of tumor cell protrusions such as lamellipodia, invadopodia or filopodia^{24,25}. Among the signaling pathways that control tumor cell motility, RhoGTPases have been shown to be main players in the formation of tumor cell protrusions²⁶. Several imaging tools, such as the RhoGTPase FRET-based biosensors, have been developed in order to define the activation of RhoGTPases during tumor cell invasion at subcellular resolution²⁷. Examples of these reporters include single chain biosensors for RhoA²⁸, RhoC²⁹⁻³¹, Rac1³², Rac3³³ and Cdc42³⁴. These studies uncovered the complex regulation of different GTPases at subcellular invasive protrusions made by tumor cells, named invadopodia, which are required for intravasation (Fig. 3). In the studies on RhoC GTPase, authors showed how RhoC activity localized to areas outside the invadopodium core confining actin polymerization³⁰. This technology has been expanded into *in vivo* mouse models expressing reporters of several GTPases. Rac1-FRET³⁵ and RhoA FRET³⁶ mice were developed by the Welch and Timpson labs to study the activation of Rac1 and RhoA in different tumor types. In these studies, the authors combine intravital imaging, FRET-FLIM and imaging windows to study the activation of RhoA and Rac1 in tumors. They showed in pancreatic tumors that Rac1 increases as tumors progress. In mammary tumors, the authors showed that RhoA activity increases as tumors progress and in pancreatic tumors, decreases as the tumor develops.

It is also worthy to note the use of cell-fate mapping imaging tools, such as the photoconvertible Dendra2, to follow the fate of tumor cells from the primary tumor¹⁵. This approach marks tumor cells in the primary tumor by photo switching regions from green to red, tracks these cells over time in the primary site³⁷ and can be visualized in different metastatic organs *post mortem*.

The studies described have changed our understanding of how tumors regulate their intracellular signaling. In the following sections we will comment on recent studies that describe the different TME components by using intravital microscopy.

B. The textures of the tumor microenvironment at primary tumor sites

i) Tumor cell motility phenotypes

Motile vs non-motile cells: Intravital imaging of primary tumors has been used to study the motile behavior of cancer cells. It helped to identify a substantial heterogeneity in the different phenotypes that cancer cells display (Fig. 4). A basic classification of tumor cells *in vivo* comes from the first intravital imaging experiments in tumors where cells have been classified as motile and non-motile¹⁰.

Tumor cell phenotypes are diverse and recent studies have shown different types of tumor cell movement:

- i. Single cell motility: where tumor cells move as solitary units extending lamellipodium protrusions³⁸ (Fig. 4a).
- ii. Multicellular Streaming: where tumor cells and macrophages co-migrate together towards blood vessels^{38,39} (Fig. 4b)
- iii. Collective migration^{40,41}: where tumor cells forming cell-cell junctions migrate as cohesive strands.

A combination of intravital microscopy with imaging windows has been used to access different tumor sites, including the breast¹⁵, brain⁴², and pancreas⁴³ and to study how the motility of tumor cells is regulated. Time-lapse intravital microscopy and fate-mapping reporters as previously described have facilitated tracking of tumor cells over time to understand their behaviors *in vivo*. What are the functions of these different modes of migration? We can hypothesize that while all these different phenotypes may coexist in a tumor, they may also play different roles at different stages of tumor progression. For example, studies of early lesions of mammary tumors showed that tumor cells move only in a single-cell mode⁴⁴. Also, intravital imaging work has shown that multicellular streaming is required for tumor cell movement towards blood vessels to facilitate intravasation³⁹.

Recently, it has been shown that in mammary tumors, collective migration could be a source of single-cell release⁴⁵. These studies highlight that different modes of migration may play specific roles during tumor progression. From a translational point of view, intravital imaging studies in patient-derived xenografts³⁸ will shed light into how the different modes of migration are represented in patients from specific tumor types. This information could be used in the future to test drugs that may interfere with a mode of migration highly represented in a tumor type and to assess therapeutic response.

ii) The textures of the ECM

The ECM is a complex meshwork where tumor cells reside. Intravital microscopy studies have applied second harmonic generation imaging (SHG) to describe the three-dimensional architecture of the matrix (Fig. 5). Fibrillar collagen, because of its noncentrosymmetric structure, can produce SHG photons^{46,47}. The Jain lab showed the potential of using SHG to assess tumor collagen architecture. They analyzed how ECM architecture can be modified upon pharmacological intervention⁴⁸ to increase diffusion in tumors and proposed that loosening of the ECM can increase penetrability of therapeutics drugs. Seminal work by Dr.

Patricia Kelly used SHG imaging to define collagen signatures depending on the tumor stage and used these signatures to predict breast cancer patient outcome^{49,50}.

SHG signals come primarily from fibrillar collagens. There are several fibrillar collagens in tissues (types I, II, III, V, XI, XXIV and XXVII⁵¹). Recent work by the Campagnola lab has implemented SHG methods to determine the changes in Collagen I/Collagen III composition in ovarian tumors^{52,53}. While SHG imaging of tumors refers to fibrillar collagens, the ECM is composed of many other proteins. The catalog of ECM and ECM-related molecules has been organized into “the matrisome”⁵⁴, which consists of 1056 genes in the human genome (accounting for 4% of the proteome). Based on the number of genes in the “matrisome,” one can assume that tumor microenvironments are quite complex with unique 3D architectures that may affect the SHG signals generated from the collagen matrix.

iii) The immune microenvironment.

The macrophage component: Macrophages in the primary tumor play an important role in regulating the dissemination of breast cancer cells. Elegant intravital imaging studies in mammary tumors using two-color animal models have shown that there are different types of tumor-associated macrophages *in vivo* that display different behaviors⁵⁵. Streaming macrophages represent a population of macrophages that can be found co-migrating with tumor cells in “streams” and guide tumor cells towards blood vessels³⁹ (Fig. 4b). A paracrine signaling loop (EGF/CSF-1) between tumor cells and macrophages drives migration toward blood vessels⁵⁶. Perivascular macrophages contribute to tumor cell intravasation by associating with both a Mena-expressing tumor cell and endothelial cells, forming a structure called TMEM (tumor microenvironment of metastasis)⁵⁵. Physical interaction of tumor cells and macrophages induces the formation of invadopodia in tumor cells through a NOTCH1-dependent pathway³⁷. TMEM structures promote transient permeabilization of blood vessels and facilitate tumor cell intravasation¹³. Intravital imaging studies revealed the role of chemotherapy in the formation of TMEMs. In this study, the authors show that chemotherapy increases the density and activity of TMEM sites, promoting distant metastasis⁵⁷. Clinical studies have also shown that TMEM structures correlate with increased metastasis in patient samples. All of these studies combined reveal the translational aspect of basic cell biology discoveries^{14,58}.

Tumor-infiltrating lymphocytes: By using multicolor imaging and breast cancer mouse models Egeblad et al.⁵⁹ showed the behavior of T-regs, dendritic cells and myeloid cells *in vivo*. By using intravital microscopy and multicolor imaging, they found that Tregs move in the proximity of blood vessels and that migratory behavior is sensitive to oxygen levels.

Neutrophils and their NETs: Neutrophils have also gained attention as key players in the TME regulating tumor progression. Intravital imaging from the Egeblad lab showed that activation of NET (neutrophil extracellular traps) formation in neutrophils by cancer cells can stimulate tumor invasion and migration, promoting metastasis⁶⁰. Recently, her group also showed how upon inflammation, neutrophil extracellular traps can awaken dormant cells and influence metastasis outgrowth⁶¹.

iv) Adipose tissue

Adipocytes are an abundant cell type within the TME of tumors such as breast or melanoma; however, little attention has been paid to the interaction between tumor cells and adipocytes. A recent study from the White lab⁶² shows that co-culture of melanoma cells with adipocytes increases the matrix degradation capacities of tumor cells, further suggesting that adipocytes mediate the activation of an invasive program in melanoma resulting in the formation of invasive protrusions and invasion. This process is mediated by the transfer of fatty acids from the adipocytes to the tumor cells through the FATP1 receptor. By using intravital imaging and THG (third harmonic generation is a label-free scatter process that is elicited by water-lipid and water-protein interfaces, including intra- and extracellular membranes, and extracellular matrix structures⁶³), work by Peter Friedl's lab showed that tumor cells are able to move along adipocytes, extending lamellipodia-like protrusions. They showed in melanomas *in vivo*, invasion through adipose tissue by multiple leader cells followed by a multicellular tumor mass widens the space and favors collective invasion⁶⁴. While the role of adipocytes in metastasis and tumor development is gaining more attention, it will be interesting to define how and when tumor cells, such as melanoma cells, migrate through different layers of the skin and how adipocyte-contact can modulate the modes of tumor cell migration.

C. The textures of the tumor microenvironment at metastatic sites

The behavior of tumor cells in distant organs has been a great mystery and its understanding is still at the beginning. Once tumor cells disseminate, they must survive in the circulation, extravasate and seed in distant organs. The difficulty of imaging organs such as the lungs or the liver during long periods of time has made it difficult to define the stages of tumor seeding and the behavior of tumor cells at metastatic sites. However, recent work using intravital imaging and new imaging windows is starting to dissect the steps of the metastatic cascade at the distant sites.

i) Lung imaging windows and pulmonary metastasis.

Intravital microscopy of the lungs had been challenging because of the difficulty of accessing the tissue as well as the motion derived from mice breathing (requiring extensive post-acquisition processing in order to correct the data for motion artifacts). Early work in the field of pulmonary dynamics used vacuum-based windows to image the lungs and to study pulmonary microcirculation⁶⁵. In 2016, work by the Krummel lab developed an intercostal window that allow for ~12 hours of continuous imaging⁶⁶. With this imaging approach, they were able to show that within minutes, when circulating tumor cells (CTCs) enter into the lung, tumor cells shed tumor microparticles. These CTC fragments attract myeloid cells that accumulate in the lung, promoting the development of successful metastasis. Entenberg et al. showed by using a vacuum window, highly protrusive tumor cells were able to extravasate in the lungs⁶⁷. More recently, a new window for high-resolution imaging of the lung (or WHRIL) was developed by the Condeelis lab¹⁷. This permanent window allows for the visualization of tumor cell arrival and extravasation into the lungs. The advantage of this new device is that longitudinal experiments can be

performed and tumor cell growth can be followed over a period of days. Multiple efforts have been made to correct for the artifacts generated by breathing motion^{68–70}.

ii) Imaging lymph node metastasis

Work by the Skobe lab using melanoma tumor models showed that tumor entry into the lymph node cortex requires active tumor cell migration⁷¹. By using intravital imaging and tracers to label the lymphatic vessels they were able to show that tumor cells migrate through the lymphatic vessels into the lymph node. Once they reach the subcapsular sinus, tumor cells move slowly towards the cortex, independently of the lymph flow. Recent work by Olmeda et al. using intravital microscopy also showed that melanoma cells within metastatic lymph nodes display a motile phenotype⁷². In order to achieve temporal resolution and image specific areas during long periods of time, Meijer et al. developed a chronic lymph node imaging window (or CLNW) to perform intravital imaging of the inguinal lymph node in mice for up to 14 days¹⁸. Work by the Padera lab using a CLNW showed that tumor cells can move towards blood vessels as single cells and invade the vascular basement membrane. By using fate-mapping technology (i.e. the photoconvertible protein Dendra2 to identify specific tumor cell areas and follow them over time and space) and intravital imaging, they were able to show that tumor cells in the lymph node invade lymph node blood vessels, enter the blood circulation, and colonize the lung.

These imaging studies highlight the metastatic lymph node as an active site where tumor cells display unique motility behaviors and suggest that motility of tumor cells within this site may be rewired over time in order to favor cross-seeding at distant sites.

What are the modes of migration in the lymph node? Are invadopodia required for tumor cells to leave the lymph node as shown for tumor cell intravasation in the primary site^{73,74}? Do immune cells facilitate tumor cell movement? All of these are interesting questions that will need to be addressed in order to understand the motility behavior of tumor cells at lymph node sites.

iii) The bone marrow niche

In several tumor types, such as prostate and breast, tumor cells disseminate to the bone and form metastasis. Several reports have described the biology of tumor cells in the bone marrow at multiple anatomical locations. The mouse tibia was used to image myeloma cells that colonize the endosteal niche in the bone marrow. In this work, intravital imaging was used to show how osteoclasts promote proliferation of myeloma cells by remodeling the endosteal niche⁷⁵. The calvarial bone marrow (BM) had been used in order to understand the biology of disseminated breast cancer cells in the bone marrow. The calvarial BM contains two distinct anatomical sites: a) sinusoidal-enriched region concentrated around the central vein and b) a lateral or caudal region that lacks sinusoidal vasculature. Intravital imaging of the calvarial BM by Pierce et al. shows that proliferation of tumor cells occurs at the lateral regions while cells that remain dormant (non-proliferative) reside in the sinusoidal areas. By using time-lapse intravital imaging they were able to show that E-selectin regulates the homing of breast cancer cells in the bone marrow and that the blockade of the CXCR4 receptor mobilizes dormant cells from their niches⁷⁶.

There are some caveats associated with imaging the bone marrow niche and bone metastasis *in vivo*. The most important is the accessibility to the areas of interest and the need in some cases to perform invasive surgeries to access long bones (i.e. tibia⁷⁷). As the cortex of the mouse skull is relatively thin, the calvarial BM is easier to access for imaging with minimal manipulation to study BM metastasis. In the case of brain metastasis, this bone area has to be removed to implant window devices and perform intravital imaging of the brain⁴².

iv) Visceral metastasis and the abdominal window

As mentioned before, accessing the internal organs requires extensive surgery and devices that allow for access to internal tissue for imaging. To image the visceral organs (liver, pancreas, spleen), Ritsma et al. developed an abdominal window (Fig. 2). This new window allows for the visualization of cancer cells in the liver for long periods of time (14 days) and has been used to define the stages of liver colonization¹⁹. This device has also been used to image other internal organs in order to study pancreatic cancer cell behaviour⁴³ and to study colorectal cancer progression⁷⁸.

Summary and perspective

Stopping cancer from spreading is the holy grail for many metastasis researchers who focus their research on understanding how tumor cells escape from the primary tumor. The body of work presented in this review highlights multiple angles that can be exploited to mitigate tumor dissemination. For many years, the cancer field has had a primary tumor-centric view and has prioritized targeting tumor cells to prevent cancer spread; recent work suggests that other alternatives may also provide better outcomes. With this idea in mind, targeting the TME is an appealing idea already supported by multiple studies and presents potentially druggable targets that could be explored further to prevent cancer from spreading. Intravital imaging has helped to uncover potential targets for therapeutic intervention as we have discussed above. However, other aspects of tumor biology, such as therapeutic response, can benefit from intravital imaging. The study of therapeutic response to drugs using imaging is a new area of research that could potentially have implications on clinical treatments, as shown in recent reports in pancreatic cancer⁷⁹. In addition, the study of nanoparticle⁸⁰ delivery to tumors could benefit from intravital imaging studies that can monitor the uptake of nanotherapeutics in real time by immune cells or other cell types in the tumor microenvironment.

The new biology uncovered by innovative imaging techniques (i.e. intravital imaging) holds promise in finding targets that can stop cancer spread by targeting the “textures” of the TME.

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Figure 1. The tumor microenvironment.

A) Artist representation of the tumor microenvironment showing a tumor, blood vessels and invasive cancer cells. b) Intravital imaging of a breast tumor showing tumor cells in green (rat adenocarcinoma MTLn3 cells expressing GFP), SHG imaging of the ECM (blue) and blood vessels labeled with 155 kDa TRITC dextran (red).

Figure 2. Imaging windows.

A) Example of a mammary imaging window implanted in a mouse. An intravital image of a mammary tumor is shown. MTLn3 cells expressing GFP in green and blood vessel labeled with 155 kDa TRITC dextran in red. B) Example of an abdominal window implanted in a mouse. An intravital imaging of the liver vasculature is shown. Blood vessel labeled with 155 kDa TRITC dextran in red and SHG imaging of the ECM (green) is shown.

Figure 3. Invasive protrusions.

A) Artist representation of a tumor cell intravasating into the blood stream. B) Intravital imaging of a tumor showing a tumor cell extending an invasive protrusion (invadopodia) towards blood vessel (arrow). MTLn3 cells expressing GFP are shown in green and blood vessels labeled with 155 kDa TRITC dextran are shown in red. C) Example of a tumor cell intravasating into a blood vessel (red). Her2-CFP expressing tumor cells pseudocolored in green and blood vessels labeled with 155 kDa TRITC dextran in red are shown.

Figure 4. Motility modes in vivo.

A) Example of single tumor cell motility. Arrows point towards single tumor cells escaping the tumor. B) Example of multicellular streaming. White arrows point towards tumor cells. Yellow arrows point towards macrophages that can be visualized as dark shadows. Her2-CFP expressing tumor cells pseudocolored in green and blood vessels labeled with 155 kDa TRITC dextran in red are shown.

Figure 5. The extracellular matrix.

Second harmonic generation two-photon imaging (purple) of a breast tumor and different organs in a mouse.