Intravital Imaging in the Study of Tumor Microenvironment and its Role in Tumor Growth

Master Thesis
Cancer Genomics and Developmental Biology
15th November 2011

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1. Introduction

Despite much advancement in treatment of malignant diseases, there are still no curative therapies available. In case of breast cancer, about 40% women relapse despite chemotheraphy. Many cancers, such as pancreatic, liver of brain cancers, have a very low 5-year-survival percentage and these statistics have not improved much over the recent decades [1]. There is still a need for new approaches to treating cancer. Recent findings of the tumor microenvironment's molecular mechanics and its role in tumor progression and metastasis give us promise of finding those new potential therapeutic targets that could improve cancer patients' chances of survival. The purpose of this literature study is to present and discuss the most recent findings concerning the effect of tumor microenvironment on tumor progression as well as the intravital imaging technique as a state-of the art tool used to research the mechanisms involved.

Cells that are cancerous have acquired an ability to survive and proliferate endlessly, via a number of genetic mutations that have damaged their DNA. Those damages, which often include an oncogene activation and/or tumor suppressors deactivation, have an impact on many basic cellular processes, most of all the regulation of the cell cycle. In a non-malignant system, a cell which DNA has been damaged would either undergo repair, or if impossible – cell death. The cancerous cell cycle checkpoints are often ineffective and a damaged cell is able to continue proliferating. There is still a long way for such cell to become metastatic, which is a process defined by cell detachment from the primary tumor, and outgrowth at a secondary site. There are a number of steps and processes that need to take place. First, the occurrence of certain mutations in a supportive microenvironment enables the cells to become motile and invade through the stroma into the blood or lymphatic system. The intravasation is considered a rate-limiting step in tumor progression. Next, the cells need to survive in the bloodstream and seed into the target tumor site, extravasate; a process, which is often stopped via senescence or apoptosis. Once successfully seeded, the malignant cells continue dividing, which enables a growth of a clinically relevant metastasis [2, 3].

The process of cells shifting from healthy cell to cancerous and then to metastatic, as well as its ability to escape the many repair/apoptotic processes is facilitated by the permissive, pro-metastatic microenvironment. What is being referred to as the microenvironment is actually a number of stromal elements such as a range of immune cells surrounding the tumor, fibroblasts, adipocytes, epithelial cells etc., as well as the extracellular matrix and its physical properties such as fiber stiffness. By furthering the understanding of processes in which the microenvironment influences cancerous growth and metastasis, it might be possible to find new therapeutic approaches to cancer treatment.

Until recently, there have not been many tools to take a closer look at the tumor and its surrounding tissue. Now, by means of bioluminescence and fluorescent probes it is possible to visualize tumor distribution and the movement of cells within tumor tissue. Whole body fluorescence/bioluminescence methods are non-invasive techniques which enable detection of a few hundred cells expressing a fluorescent probe from an engineered tumor tissue. The more invasive intravital imaging implements confocal/epifluorescent/multiphoton microscopy, in which smaller regions of tumor tissue are imaged,

enabling visualization of subcellular structures and obtaining three-dimensional information about the area studied [4]. Importantly, such methods enable the examination of cell motility *in vivo*, providing information which could not be obtained by *in vitro* methods. By studying biological processes in living animals it is more likely that mechanistic information obtained is more clinically relevant and could be used in providing more efficient therapeutic approaches.

1. Intravital Microscopy – state of the art imaging techniques

Intravital microscopy refers to a number of imaging techniques which are implemented to study a living organism. With the progression of our scientific knowledge, the need to visualize living tissues and mechanisms regulating the cells within them in physiological setups grew stronger. Especially in cancer biology, where the tumor cells as well as the tumor supporting cells are characterized by large plasticity and motility, studying these characteristics outside of physiological context is not likely to provide reliable information. A number of approaches have been already implemented to image tumors and their surroundings *in vivo*.

Since tumor growth takes place over weeks, even months, it is important to be able to study these processes over equally long periods of time and preferably in the same animal subject. Intravital imaging so far has been mostly performed in rodents by means of minimally invasive surgeries in which tumors were exposed via generation of a tumor-exposing skin flap. Unfortunately, animals which undergo this surgery are not being used repeatedly as it renders them too weak; they are euthanized after the experiment. Therefore, a surgical preparation involving an implantation of imaging windows that provide access to the tumors without repeated surgeries was invented. The most commonly known imaging windows are dorsal skin fold chamber [5], the cranial window [6] and an inguinal mammary window [7, 8]. They were used mostly in the studies of tumor angiogenesis (e.g. tumor response to anti-vascular therapy), permeability and diffusion [9]. An interesting example of intravital imaging window used in tumor studies is by Kedrin et al. with tumors expressing Dendra2 photoswitchable protein [8]. The subpopulations of tumor cells in different microenvironments could be followed as their fluorescence could be switched from green at blue light exposure. This study revealed that tumor cells in the vicinity of blood vessels are more motile than those further away. Also, it noted the high numbers of macrophages and ECM in the perivascular microenvironments [8].

Certain technical considerations are necessary while making a successful surgical preparation. Especially in the case of skin flap approach, it is of vital importance that a clean tumor is positioned under the microscope; contact with e.g. hair or other solid contaminations may induce an immunological response, especially unwanted in the intravital studies of immune reactions in tumor stroma. Moreover, the tumor needs to be positioned in as stable manner as possible. All movement taking place under the microscope, including breathing, will be recorded by the camera and if not reduced to a necessary minimum – may render the collected data unusable [10].

Not only did the preparation of animals for *in vivo* studies make great advances in the recent years, the imaging platforms used in intravital microscopy became more reliable too. As early as in the nineteenth century, the observations of blood flow and leukocyte rolling pioneered the development of more advanced imaging platforms, such as epifluorescent microscopy. Later, confocal and multi-photon/single photon microscopy technologies [4, 11] were developed.

The use of fluorescence in tumor microscopy dates back to 1924, when autofluorescence of endogenous porphyrins was observed in tumors illuminated with ultraviolet light [12]. What followed was development of fluorescent labels and probes that could provide information on specific tissue components. The principle behind fluorescence microscopy is connected to the properties of fluorescent compounds which label tissue components to be imaged. An examined sample is illuminated with light filtered at a specific wavelength. Then, the sample emits fluorescent light which is at a different (longer) wavelength than the illumination one. This light is detected through microscope objective and an image is created. In epifluorescent microscopy, light passes first through the objective lens and only then through the specimen. The emitted fluorescent light is focused to the detector by the same objective. Since the excitation light is absorbed by the tissue, almost only the emitted light is detected, which enables lower signal to noise ratio [13]. A number of microscopic setups have utilized fluorescence; it is nowadays a most common choice in biological studies due to its availability and efficiency. In case of long term intravital imaging studies simple fluorescence microscope is, however, not a sufficient tool. Due to long and intense light exposure of examined tissue, it suffers from high phototoxicity. Phototoxicity not only can seriously damage the tissue, but also, even in small amounts, drastically reduce picture quality.

Multiphoton microscopy (MPM) is, in case of intravital imaging, a more common platform choice. In comparison with a simple fluorescence microscope, it provides higher data resolution with minimal phototoxicity. Moreover, as tumor tissues are often very dense, ability to have an increased imaging depth, as enabled by MPM, is a great advantage. MPM is in fact a subtype of laser scanning confocal microscopy (LSCM). Unlike in standard laser scanning confocal microscopes, MPM uses short-pulsed near-infrared lasers instead of continuous light. This enables low intensity illumination with the same average power from near infra-red lasers. The light is focused by the objective lens, leading to fluorophore excitation by absorbing two or more low energy infrared photons, generating fluorescence without a need for a confocal pinhole. The photons can further be efficiently gathered by highly sensitive field non-descanned detection, which records signals from even the deep tissues. Such a setup limits phototoxicity only to the focal plane without reducing detection efficiency. The infrared heating can be limited by using low average laser powers, which is crucial for long-term high quality signal detection as is necessary during time-lapse intravital imaging [4, 14-16].

Another platform frequently used in *in vivo* studies is single photon microscopy – confocal laser scanning microscopy, which has undergone a lot of development in recent years. In this method, fluorescent images are enabled by means of the excitation of fluorophores having single-photon absorption in the ultraviolet range with a stream of strongly focused subpicosecond pulses of red laser light [17]. In comparison with MPM, it enables higher image resolution and even lower phototoxicity, as the

Modality	Depth [µm]	Advantages	Disadvantages
Wide field epifluorescence	20	• Low cost • Very user friendly	• High background • Very limited depth • Phototoxicity and bleaching
Confocal (pinhole)	50-100	• Very high resolution • Very low out of focus signal • Flexible setup with a wide range of excitation and fluorophore options for multicolor detection	• Limited depth • Phototoxicity and bleaching
Confocal Spinning Disk	50-100	• 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 setup with a wide range of excitation and fluorophore options for multicolor detection	• Decreased resolution in z-plane • Fixed pinhole size is only optimal for one magnification (decreased resolution for low magnifications) • High power illumination of selected regions not possible
Near Infrared multiphoton	400-1000	• High resolution • Minimal out of focus signal • Minimal out of focus excitation (very low toxicity and bleaching) • Better deep tissue penetration and resolution • Flexible setup with a wide range of excitation and fluorophore options for multicolor detection	• High cost • In focus phototoxicity and bleaching can still be high
Infrared Multiphoton	Increased from near infrared	• High resolution • Minimal out of focus signal • Minimal out of focus excitation (very low toxicity and bleaching) • Reduced in focus toxicity and bleaching • Improved deep tissue penetration and resolution • Increased range of available fluorophores • Flexible setup with a wide range of excitation and fluorophore options for multicolor detection	• Very high cost • Still in development
Optical Frequency domain imaging	>1000	• No need for exogenous contrast agents • No phototoxicity or photobleaching • Very good tissue penetration • Rapid imaging of very wide areas	• Possibly very high cost • Custom built systems only • Still in development

Figure 1 Overview of microscope modalities (adapted and revised from review [4])

fluorescence emission requires much lower excitation intensity. Rapid resonant scanning laser scanning confocal microscopy (RRS-LSCM) and spinning disk confocal microscopy use much shorter laser beam dwells, which allow the excited triplet states to dissipate before the photochemical fluorophore is inactivated [15, 18]. Moreover, the spinning disk microscope uses a more efficient, camera-based detection which greatly enhances the picture quality. Deep tissue imaging, though still more available than in a simple confocal microscope, is not as good as in MPM platform. Summarizing, both single photon and multiphoton platforms have the subcellular spatio-temporal resolution that is necessary to study the interactions between individual cells in tumor microenvironment and thus are a popular choice for such studies.

A variety of laser scanning microscopy techniques, e.g. such as multiphoton microscopy, have enabled an advancement in tumor biology studies, elucidation of pathways involved in cancer progression and aided the establishment of novel therapeutic strategies. Nevertheless, these methods still have large limitations. They allow a rather superficial visualization of tumor and its environment's structure (300-400 µm depth) over a limited area and few positions. Moreover, the application of longitudinal studies is still limited due to an accumulation of exogenous contrast agents [19]. Therefore, a different approach to intravital imaging is still necessary to compensate for these disadvantages and provide new information. A recent development of second generation optical coherence tomography (OCT), named optical frequency domain imaging (OFDI) is one of the solutions that circumvents the MPM's technical limitations. In OFDI, the use of very long excitation wavelengths enables deep penetration into the scattering medium producing an image without the use of any exogenous agents. This technique was shown capable of performing high-resolution, wide-field and deep imaging of tumor vasculature; morphological and fractal characterization of vascular networks; contrast-free functional lymphangiography and characterization of tissue viability, which are all key to the understanding of tumor progression [19]. With such advancements becoming more available to tumor biologists it can be expected that our understanding of tumor and its environment will encompass the microscopic processes taking place in a whole body subject. This could for example provide vital information on tumor heterogeneity in the same animal subject and allow us to discern universal mechanisms from individual traits.

1.1 Specialized imaging techniques in intravital studies

Additional techniques were developed to assist specific experimental requirements within the previously described imaging platforms. Among them, the most favored include second harmonic generation microscopy (SHG), fluorescence lifetime imaging microscopy (FLIM), Förster Resonance energy transfer (FRET) and spectral-lifetime imaging microscopy (SLIM), which will be introduced further [9, 11]. The main advantage of these methods comes from their providing smart solutions for improving imaging resolution, identification of various structural components and biological processes taking place in the tissue. Also, they may enable longer imaging periods by reducing light toxicity. All these features are made possible by exploiting intrinsic properties of examined tissues and fluorophores used.

In one example, certain extracellular matrix (ECM) components, such as fibrillar collagen and elastin together with some cellular metabolites can be imaged using MPM without labeling due to specific

properties of these molecules; collagen fibers and other structures with a highly repetitive chiral structure interact with femto-second light pulses, which leads to non-linear light scattering interference. This is called second harmonic generation (SHG), in which visible light of exactly half the wavelength of the illumination is produced [20]. This method can be used both in thick tissue studies as well as during *in vivo* imaging. The second harmonic generation can be acquired in both forward and back-scattered directions, thus, producing a lot of signal which is obtained by objectives with high numerical aperture [15].

Elastin and other cellular metabolites, which include nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) can also be studied using SHG. They emit intrinsic autofluorescence, of a very specific to each of them wavelength of a fairly large spectrum, which is excitable by means of two-photon microscopy [21]. The overall ECM structure together with tumor hypoxia regions can be revealed by combining SHG with autofluorescence imaging. The latter is enabled by imaging the intracellular redox states – NAD/FAD ratios [15].

Along SHG there have been developed other new methods which can be used as an addition to traditional microscopy setups – from single, two-photon to multiphoton platform. Fluorescence lifetime imaging microscopy (FLIM) is a method which produces an image by mapping the fluorophore decay rate, i.e. the spatial distribution of excited state lifetimes from endogenous or exogenous fluorophores [10]. As the method mostly relies on fluorophore lifetime, rather than its emission intensity, it is a good method for detection of weaker signals. Multiphoton FLIM is well suited for an accurate detection of metabolic changes, e.g. using redox ratio of NAD/FAD or increased glycolysis. These can be then used for detecting tumors as well as monitoring tumor progression and metastasis[22]. The complex interactions that regulate oncogenes and cell migration are important processes in cancer biology, which also can be studied using FLIM. For example, epidermal growth factor receptors (EGFRs) which have been implicated in many human cancers have been studied using FLIM. The study elucidated spatial and temporal characteristics of EGFR activation and reported novel findings regarding lateral signal propagation at the cell membrane [23].

Förster resonance energy transfer (FRET) is a technique that enables detection of protein-protein interactions, protein-DNA interactions and protein conformational changes by measuring the distance between the molecules on a scale of a few nanometers [10]. The measured molecules are tagged with a donor fluorophore and an acceptor fluorophore respectively, and if in close proximity (1-10nm), the emission of an acceptor is being detected and a picture is obtained [10]. An example of FRET use in cancer biology is in the study of cytotoxic T cells (CTLs), implicating their important role in tumor killing; it was shown that cancer cell apoptosis occurs most likely in those cancer cells which interacted with at least one CTL, suggesting CTLs as the mediators of tumor killing mechanism [24]. A combination of FRET with FLIM is a powerful tool, as it achieves deeper tissue imaging. It allows visualization of the spatial and temporal activation states of the studied molecules *in vivo*. An example of such combined use is a study of Src oncogene and its interaction with actin cytoskeleton in human colon cancer cell line. Using FRET, the emergence of a Src-β-actin complex responsible for actin dynamics regulation near the cell periphery was demonstrated [25].

Much of the efforts in improving current imaging methods are focused on increasing the imaging depth by signal amplification or more sensitive detection. Another approach is to exploit all the properties of fluorescence, for example by combining the spectral-lifetime data [26]. In spectral-lifetime imaging microscopy (SLIM), it is possible to collect and analyze fluorescence lifetime data simultaneously within separate defined channels [13]. Images can be then generated examining FLIM from each channel. This method diminishes noise from adjacent spectra, as well as enables an easier fluorophore identification [26]. It is an important tool that facilitates fluorescence lifetime measurements which could lead to improvements in the understanding of tumor formation and progression.

Other than taking into consideration the enhancements in signal detection, proper preparations of tissues being examined by intravital imaging are crucial. Intravital imaging involves living animals, which have been tagged with specific markers enabling detection of proteins/cells/tissues of interest. Meticulous surgical preparation protocols and application of a variety of high-quality probes are an inherent part of such experiments, necessary to ensure both animal's comfort and high data quality.

Visualizing particular cell subsets of interest *in vivo* requires proper labeling. Two strategies are commonly applied for this purpose. In the first approach, purified cell populations are labeled ex vivo by fluorescent dyes or genetically-encoded fluorescent proteins, to be later transferred to synegeic mice. In the second approach, fluorescent reporter transgenic mice are used. As the chemical dyes dilute due to cell proliferation, such labeling is inefficient in the long term. This can be avoided by means of stable expression of fluorescent proteins; lineage-specific fluorescent protein reporter transgenic mice used as tumor recipients can have their endogenous host cells followed indefinitely, also allowing for a quantification of these populations [15].

Visualizing vasculature, its deregulation and leakage, is another important component of studying tumor microenvironment, requiring its own markers. An intravenous (i.v.) injection of high molecular weight dextrans or quantum dots enables such imaging. In another approach, vasculature is visualized in reporter mice expressing green fluorescent protein (GFP) under Tie-2 promoter [27]. As angiogenesis is in many tumors important for enabling tumor growth, it is often useful to visualize the growing, nascent vessels. They can be imaged using for example the nexin promoter GFP-mouse strain [28].

Using functional probes is yet another approach in tumor microenvironment studies. ECM remodeling, a process accompanying cancer progression, involves protease activity of e.g. metalloproteinases (MMPs), serine proteases and cysteine cathepsins. Autoquenched protease subsets which fluoresce upon cleavage were first used for *in vivo* studies in 1999 [29] and since then, a number of FRET-based probes (e.g. for MMP-2, MMP-7 or MMP-9) have been introduced [4]. Visualizing the protease activity provides information about the most active tumor regions. It could also be used in the clinic in assessing the efficiency of protease inhibitor treatments.

Despite the wide choice of fluorescent and functional probes available, there is still a need to find other solutions for molecular staining which would enable more accurate and more extensive studies. One of major problems in using fluorescent dyes is that their emission spectra overlap, and one marker may be

detected in multiple color channels. An interesting new strategy for studying cancer cells and their environment, named quantum dots-based quantitative multiplexed imaging, has been recently developed by Peng et al. [30]. Quantum dots (QDs) are engineered nanoparticles which have unique optical and electronic properties, such as size and composition-turnable light emission, enhanced signal brightness and resistance to photobleaching [31]. The greatest advantage of the method is that a single light source can excite different QDs colors with very low spectral overlapping, providing images of very high resolution [31]. This method was used in studying human cancer cells/microenvironment co-evolution and has provided new spatiotemporal information that enabled a distinction of specific invasion patterns [30].

As was presented, there are many different imaging platforms and techniques available for the *in vivo* study of metastasis. As every approach has its strength and weaknesses, it is important to choose techniques appropriate for the researched questions and balance the drawbacks with efficient data collection. It is wise to know beforehand, whether deep tissue imaging, or large imaging areas/multiple positions are a priority – necessary to accurately answer particular scientific questions. It may be a better solution to give up such enhancements in exchange for better resolution or reduced tissue phototoxicity. An overview of various microscopic modalities and their advantages and disadvantages is presented in **Figure 1**, as was reviewed by M. Lohela in 2010 [4].

2. Tumor microenvironment's effect on tumor growth

Stroma of a group of tissues being either a healthy organ or a tumor has the same role – to provide a supportive network, an environment in which these tissues will thrive. In healthy organs this is a desired property, especially in organ development [32]. In case of tumors, the microenvironment's supportive role is actually damaging to the entire organism. During cancer progression, tumor stroma undergoes dramatic morphological changes where a number of stromal components are being implicated. Their role and influence is often very specific to the tissue type and tumor stage and thus understanding of the processes in which they're involved is very important.

Stromal microenvironment is rich in a variety of components, which can be grossly divided into the extracellular cell matrix (ECM) and various stromal cells. The ECM is mainly formed by proteoglycans, hyaluronic acid, fibrous proteins: collagen, fibronectin and laminin. Among the stromal cells there are the cells of vascular system, those of an immune system and mesenchymal supporting cells such as fibroblasts and adipocytes. Additionally, stroma contains various growth factors, chemokines, cytokines, antibodies and metabolites [3]. There is also a distinction between tumor promoting and tumor inhibiting microenvironments – each of them having certain dominating features. Among the most common tumor promoting stromal components are, for example, carcinoma-associated fibroblasts (CAFs),type I collagen and the immune cell infiltrate [33]. Tumor growth restricting microenvironments contain tumor suppressors; molecules which maintain normal tissue architecture and keep cancer cells quiescent. Using intravital imaging to obtain detailed information about these environments such as characteristics and dynamics of cells occupying tumor microenvironment is a valuable tool for

understanding of tumor progression. **Figure 2** [3]provides an overview of tumor stroma components many of which will be discussed further.

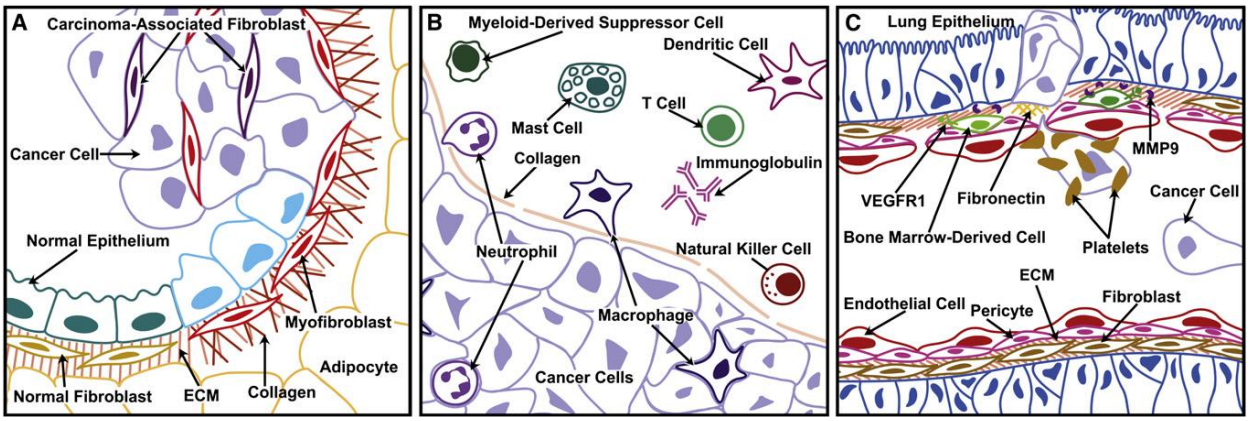

Figure 2 Overview of tumor and stromal components interactions. A. Interactions with mesenchymal cells. B. Recruitment of cells of innate and adaptive immune compartment to the carcinoma. C. Components involved in metastases formation [3].

2.1.1 Cancer-Associated Fibroblasts (CAFs)

As was mentioned, one of the mainly tumor-promoting stromal components is cancer-associated fibroblasts (CAFs). Fibroblasts are mesenchymally derived cells, which are present in the stroma of most tissues. In development, they are involved in epithelial-mesenchymal crosstalk, necessary for proper organ formation. In adults, they are activated by tissue injury and play a role in extracellular matrix (ECM) turnover. CAFs, are fibroblasts present in the tumor microenvironment; they share characteristics with embryonic fibroblasts and or mesenchymal progenitors [34, 35]. They have been reported to stimulate a number of processes necessary for tumor progression: cell growth, inflammation, angiogenesis, and invasion. In hyperplasias, they have a contrasting, tumor-inhibiting effect which indicates their ambiguous role in the tumor progression [35].

CAFs are being recruited to the cancer site during tumor growth. Regular fibroblasts do not have a tumor promoting effect to the same extent as CAFs, fibroblasts overexpressing transforming growth factor-β (TGF-β), or those that express hepatocyte growth factor (HGF) [35, 36]. One regulation method of CAF activation is via genetic alteration, as has been shown in case of tumor suppressor Pten ablated in mammary stromal fibroblasts; this modification resulted in an increase in fibrillar collagen, angiogenesis, macrophage infiltration and malignancy of epithelial mammary tumors [3, 33]. Also, the CAF activation signals may come from mediators such as cancer-cell secreted platelet-derived growth factor (PDGF), which recruit TGF-β secreting macrophages [37]. CAFs themselves secrete certain tumor promoting factors, such as growth factors and chemokines [38].

Some of the fibroblasts in tumor microenvironment may in fact be preadipocytes. Adipocytes secrete endocrine factors, adipokines (e.g. adiponectin), which are proangiogenic and, thus, may stimulate tumor growth [39]. In another tumor-supporting mechanism, they secrete ECM protein type VI collagen

that binds to NG2 collagen receptor on cancer cells, which in turn activates Akt and β-catenin and stabilizes cyclin D1 [40], disrupting normal cell cycle progression.

As mentioned, CAFs are commonly present in tumor microenvironment, and thus are often a subject of intravital imaging studies. Fibroblasts-like cells can be induced by tumor cells to express vascular endothelial growth factor (VEGF), a signaling protein associated with wound healing in normal tissues [9]. Multiphoton laser scanning microscopy was implemented in studying this process. Intact tissue imaging showed that VEGF-active fibroblasts formed an up to 50-µm thick, dense layer around tumors and up to 200-µm thick layers around angiogenic vessels [41]. Moreover, CAFs have been suggested as playing a role in relaxin-dependent collagen fiber remodeling, a process which is largely dependent on β1 integrin and matrix metalloproteinases (MMPs) [42]. These studies, although hinted at CAFs involvement, have not yet provided a clear evidence of CAFs being crucial modulators of tumor cell migration.

2.1.2 Extracellular Matrix (ECM)

Tumor associated changes of the extracellular matrix (ECM) are in large part caused by microenvironmentally activated fibroblasts [35, 37]. Among these changes there are ECM synthesis upregulation, ECM posttranslational modifications and an extensive remodeling of ECM proteins by proteinases such as matrix metalloproteinases (MMPs) [43]. Those alterations promote tumor progression by architectural and signaling pathways.

Epithelial cells attach with their basal surface to ECM and the basement membrane which is composed of laminins, type IV collagen and proteoglycans [44]. During tumor progression, especially once the tumor acquires invasive properties, this membrane is broken. An important role of laminins has been discovered in the studies of mammary basal membrane during development. During mammary gland development, myoepithelial cells together with stromal cells regulate the basement membrane synthesis, via regulating luminal epithelial cell polarity, branching and differentiation. The myoepithelium has a tumor suppressor activity, by means of regulation of protease activation [45]. This activity is lost during cell to tumor transition and is likely due to a loss of an ability to synthetise laminin-111 that takes place in this process[45].

Another ECM category is an interstitial matrix, composed of fibrillar collagens, fibronectin and proteoglycans. Additionally to providing structure and elasticity to the tissue, it binds growth factors and cytokines. The architecture of type I collagen is majorly affected in tumors, as its synthesis and remodeling increases; the collagen fibers change from being curly and oriented in parallel to cellular epithelium to being straighter and more perpendicular to the tumor border [46, 47]. This enhances integrin signaling and enables cell migration along the collagen fibers, promoting invasion, an important step in tumor progression [47, 48]. Integrins are major ECM receptors and tumor progression mediators. Blocking β1 integrins attenuates the malignant phenotype both in culture and *in vivo* [49]. On the other hand, ectopic expression of an activated β1 integrin mutants weakens multicellular tissue morphogenesis and enhances tumorigenesis [46].

A number of ECM proteins play a role both in embryonic organ development and during tumor progression. One example is Tenascin-C which is present in ECM in the proximity of angiogenic blood vessels where it enhances endothelial cell migration, proliferation, sprouting and elongation in embryonic development [50]. Its presence at the tumor invasion border is a predictor of cancer recurrence. Another example is fibronectin, which is expressed both in embryonic development and in tumors, where it facilitates angiogenesis [34].

Apart from direct ECM signaling, the matrix also has an indirect regulatory role, e.g. via proteinase activity. By cleaving ECM molecules, proteinases release growth factors and cytokines, such as FGF-2 and TGF-β [3]. MMPs cleave type I collagens and laminins, which generates fragments promoting tumor cell survival and migration [51]. Type XVIII and type IV collagens have an opposite, tumor-inhibiting effect [35].

The described changes in the architecture of ECM largely influence its physical properties. Tumors are characterized by larger stiffness than normal tissue and present as defined, hard nodules. Tissue stiffness has a pronounced effect on cellular function; which has been exemplified by various studies. In one of them, mammary epithelial cells were cultured in collagen matrices of varying stiffness. The culture started with polarized acini which were later treated with lactogenic hormones that induced differentiation. It was shown that with increasing collagen stiffness, the cells transition to proliferating colonies of compromised polarity [52]. When the stiffness reaches the level of tumor rigidity, nonmalignant epithelial cells become disorganized and invade [53].

Collagen cross-linking is an underlying mechanism that causes ECM stiffening. Once combined with oncogene activation it promotes invasive behavior. For example, in an *in vitro* study, sustained activation of ErbB2 oncogene was not sufficient to drive epithelial cell invasion in mammary tumors, unless it took place in stiff, cross-linked collagen. Reducing collagen cross-linking in an *in vivo* study resulted in a lower mammary tumor incidence in mammary mouse carcinoma model [47]. In clinical breast carcinoma studies, poor prognosis was associated with high expression levels of integrins and MMPs, and low laminins' expression levels; while overexpression of proteins inhibitors correlates with a favorable outcome [54].

As already mentioned, microscopic imaging techniques have been widely used in the studies of the ECM's role on tumor growth and have provided important information. Moreover, intravital imaging was used to study increased deposition of collagen, or desmoplasia (growth of fibrous connective tissue) in cancers at different clinical stages [55]. By means of SHG and tissue autofluorescence based multiphoton imaging; an analysis of ECM in esophageal cancer at different clinical stages was performed. It was shown that desmoplastic collagen fibers lost their typical fine structure and their area was reduced [21]. On the other hand, the amount of elastin in the stroma was increased, presenting shorter, more fragmented elastin fibers compared to healthy tissues. Such tumor associated collagen signatures were also studied in breast cancer model, with specific profiles identified for possible use as markers of tumor expansion [56]. Cancer-affected tissues changed their morphologies - their intrinsic femtosecond pulse-excited autofluorescence. This property could be identified using intrinsic contrast MPM. At least

five autofluorescence-detectable features diagnostically different from normal epithelium, were spotted in 7,12-dimethylbenz(a)anthracene-inducible hamster cheek squamous carcinomas: the nuclear density ratio, the keratin layer, epithelial thickness, and the fluorescence intensity in the keratin and epithelial layers [57].

Studying stromal cells in relation to the ECM structure involves a closer look at the metabolism which takes place in tumor microenvironment. Rapid tumor growths together with poor vascularization often lead to hypoxia in these tissues. In such case, cells switch from oxidative phosphorylation to glycolysis [58]. MPM imaging can be used in the study of altered cellular respiration as it is capable of measuring the intracellular rations of the reduced form of NAD (NADH) and the oxidized form of FAD. Moreover, intracellular redox states affect fluorescence lifetimes, measurable with pulsed excitation microscopy [59]. These methods were used in differentiating precancerous and cancerous cells from the surrounding tissues, both in primary and metastatic sites, without the use of additional fluorescent labeling [60, 61]. Because no additional labeling is required, SHG and intrinsic autofluorescence MPM provide a possibility of a noninvasive imaging method for imaging human tissue biopsies and maybe even *in vivo* subjects. Also, adding the cell tracking function to SHG enables broadening our understanding of cellular dynamics with relation to the ECM [62].

Yet another process with a large involvement of ECM has a large impact on tumor progression – tumor motility and invasion. Intravital multiphoton imaging revealed two distinct modes of cancer cell motility, which was distinguished by morphological changes during motility [63]. Mesenchymal or fibroblastic migration, characterized by spindle-like shape of migrating cells, proceeds by means of pseudopod protrusion at the leading edge, followed by focal attachment to the ECM and detachment of the trailing edge. Cancer cells are capable of creating openings between the fibers of the ECM network and squeeze through them without causing excessive distortion. These opening present as tube-like tracks lined with ECM degradation products. Activities of MMPs at the focal attachment points, as well as integrins driven adhesion regulate ECM degradation. Once MMP activity is inhibited, cancer cell motility mode transformed from mesenchymal to amoeboidal, with more spherical morphology and no ability to digest its way through the ECM [64]. This movement was associated with smaller adhesiveness, and thus, higher velocities. The process of transition into the amoeboidal motility model could be involved in increasing tumor invasiveness, and if the cells crosses into the blood circulation – even metastasis [65].

2.1.3 Vasculature and Lymphatics

Vasculature recruitment via an angiogenic switch is an essential step in both normal organ development and tumor growth [32]. This process can take place in various tumor stages and can be implemented via different mechanisms. Angiogenic sprouting with the recruitment of perivascular cells is often involved [66]. Another mechanism is more dependent on vasculogenesis – the recruitment of bone marrow derived endothelial precursor cells [67]. Proangiogenic factors can be secreted by cancer cells, as well as by tumor-infiltrating myeloid-derived cells, which are recruited by factors secreted by hypoxic cancer cells (e.g. CXCL12 or VEGF) [68]. VEGF family as well as the FGF-2 are important factors in the angiogenic response [66].

Vessel density in tumors is not a good indicator of tumor malignancy. Highly angiogenic tumors like grade I pilocytic brain tumors are slow growing and non-invasive, while pancreatic adenocarcinomas are very aggressive despite low vessel density [66, 69]. Also, some tumors, like astrocytomas prefer to use existing blood vessels and grow alongside them [66]. Tumor vasculature is characterized by irregularity, dilation and presence of dead ends in the vessel. Also, the perivascular cell attachments to the endothelium is very loose, which altogether results in leaky blood vessels and extravasation of the excess fluids and proteins that are transported back to the bloodstream by the lymphatic system. Increase in density of tumor lymphatic vessels is correlated with lymphatic metastasis in human cancers, and is largely influenced by the activity of VEGF family members [70]. In the clinical context, targeting this process is compromised by the necessity of the lymphatic system to reduce interstitial fluid pressure in the tumors, as it is necessary for efficient drug delivery.

Vasculature and its role in tumor progression have also been studied using intravital imaging. In a study by Kienast et al. [71] two critical, vasculature dependent phases of metastasis development were identified – the angiogenesis-independent growth of perivascular micrometastases and the VEGF and angiogenesis-dependent transition from micro- to macrometastases. This information was derived from an observation of the early stages of melanoma and lung carcinoma metastasis establishment under a chronic cranial window preparation. Initially in both melanoma and carcinoma case the cancer cell would be arrested at microvascular branches. Three days later, they would extravasate and stay close to the vessel wall maintaining direct contact with albuminal endothelial cells, which was shown to be essential for their survival. Then, melanoma metastases could grow up to about 350µm without triggering vascularization, while carcinoma metastases triggered vascularization after growing up to about 150µm. This difference was attributed by production of VEGF by carcinoma but not melanoma cells. Melanoma metastases instead of promoting vascularization would distort microvessels into loops increasing their metastasis to vessel contact area.

Another intravital study was also successfully used in examining the pharmacological function of antiangiogenic therapeutic factors. These medications aim at slowing down tumor growth by depriving the tumor of blood supply and thus, nutrients [72]. Sunitinib is a compound that belongs to this group; it is a tyrosine kinase inhibitor which interferes with VEGF receptor and other Stat-3 dependent growth factor receptor signaling. Its mechanism of efficacy was studied *in vivo* in a renal carcinoma model with an intravital annexin V staining to reveal apoptosis. It was found out that tumor apoptosis took place even before the anti-angiogenic effects on the vasculature took place, showing that the drug had previously unappreciated tumor killing properties [72]. With this example the use of intravital imaging has proven highly appropriate for answering clinically relevant tumor biology questions.

2.1.4 Inflammatory cells of the tumor microenvironment

Chronic inflammatory responses and infections are often associated with many cancers. Among them are liver and pancreatic cancer after chronic alcohol abuse, mesothelioma caused by asbesthos exposure, gastric cancer after *Helicobacter pylori* infection or cervical cancer after human papilloma virus (HPV) infection [73]. Chronic inflammation is more commonly a facilitator to cancer initiation rather than a direct cause. In some cases, however, reactive oxygen and nitrogen agents which are

secreted by the immune cells can also damage the DNA directly and the incidence of random mutations is higher in inflamed than in normal tissues [74].

Inflammatory cells are important microenvironment components present both in normal tissues and in tumors. While they regulate various processes involved in development, epithelial growth, branching and disposal of apoptotic cells; in tumor cells they play a role in cancer initiation and progression [75]. The composition of immune cell infiltrate changes along with the growing tumor by means of complex interactions of cancer cells with those of an adaptive and innate immune system [76]. Just as a chronic inflammatory environment may facilitate tumor progression, tumors without inflammatory cause have been reported to instigate an immune response themselves, often via genetic events, e.g. activation of common oncogenes RET, Ras and Myc, which normally are involved in tissue remodeling and regeneration [77, 78].

Both innate and adaptive immune systems play a role in malignant progression. It is mostly the innate immunity that is responsible for inflammation. Among the most important players of this system are myeloid-derived cells, such as macrophages, neutrophils and mast cells, which are both the first defense against pathogens and facilitators of tissue development and repair. In tumors, they also play a role in angiogenesis regulation and metastatic spread. Tumor-associated myeloid cells have many subpopulations which play different roles in different tumor regions, the invading edge, along blood vessels or in the hypoxic regions [79].

Macrophages are monocyte derived cells with an important role on angiogenesis regulation in wound healing and organ development, in which the latter was mostly studied in the bone and in mammary gland [75]. They are commonly divided into two activation states, an M1 and M2, which are also referred to as a classical and an alternative state [80]. M1 macrophages are monocytes which have been polarized by cytokines from T helper 1 (TH1) cells; most prominent ones include IFN-γ, TFN-α and granulocyte-monocyte-colony stimulating factor (GM-CSF). These classical macrophages are likely to have an anti-tumor activity: although they produce reactive oxygen and nitrogen intermediates, inflammatory cytokines, they also have a cytotoxic activity against cancer cells [80]. M2 macrophages emerge from monocyte exposure to T helper 2-secreted cytokines, such as IL-4 and IL-2, which are commonly present in tumors. These macrophages play a role in parasite killing and tissue remodeling and are a subtype which closely, however, not exactly resembles tumor-associated macrophages (TAMs). TAMs have a number of properties that do not fall into M1/M2 categories [75]. Accumulation of TAMs is associated with poor prognosis, as they release cytokines, growth factors, ECM-degrading enzymes, angiogenic factors, as well as suppress cytotoxic T-cell activity which promotes tumor growth, invasion, metastasis and angiogenesis [48, 75]. The major mechanism of tumor dissemination by TAMs involved epidermal growth factor (EGF) and a receptor activator of NF-κB ligand (RANKL) [48, 81]. NF-κB signaling inhibits polarization of macrophages to a M2-like phenotype which results in reduced tumor growth [82].

Another element of innate immune system playing a role in tumor progression is neutrophils. They are rather short-lived granulocytes with an antimicrobial activity. Their classification is similar as in the case of macrophages; there is an N1 inhibiting and an N2 cancer promoting phenotype. Neutrophils are

polarized to a pro-tumor phenotype by TGF-β; and its inhibition results in phenotype shift into the tumor inhibiting N1 [83]. The tumor suppressing activity of N1 neutrophils takes place via activation of cytotoxic CD8+ T cells and possible dendritic cells [83], while the tumor promoting activity of N2 phenotype is conferred by producing angiogenic factors and ECM-degrading enzymes [84]

Among other important tumor promoting myeloid cells there are myeloid derived suppressor cells (MDSCs), which accumulate with an increased tumor burden; CCR1+ myeloid cells, mostly involved in tumor invasion, VEGFR1+ cells found at metastatic sites, and Tie2 receptor expressing monocytes (TEMs) with a major role in angiogenesis [3]. Also, there are mast cells, infiltrating hyperplasias and carcinoma fronts, where they induce angiogenesis via MMP9 activation [85].

The adaptive immune system, which includes primarily B-cells and T-cells, has a primarily tumor suppressing role which is conferred via tumor immune surveillance, elimination of early tumors and regulation of the initiated cancer cells [80]. Patients with chronic compromised adaptive immune system, e.g. transplant patients or those with AIDS, have an increased risk of virus-associated cancer development, such as cervical cancer due to HPV infection [86]. It has been shown that depletion of T-cells in carcinogen treated, but tumor-free mice leads to tumor development, even when the depletion took place 200 days after carcinogen exposure [87]. Also, tumor antigen-specific cytotoxic T-cell response is important for an efficient reaction to chemo- or radiotherapy; dying cancer cells release a signal to the dendritic cells, which results in efficient cytotoxic T-cell activation [3].

On the other hand, patients with a suppressed immune system have a lower risk for non-virus associated epithelial tumors, such as breast and prostate cancer, which indicates that adaptive immune system may have a role in tumor promotion [86]. In a skin cancer mouse model, B cells initiated chronic inflammatory response via antibody deposition, which was responsible for epithelial hyperplasia [76]. Cytokines produced by TH2 cells promote tumors via polarizing M2 type TAMs that are known for increasing invasive behavior in breast cancer [88]. Certain subtypes of immunosuppressive T helper cells (e.g. CD+ CD25+ T-Regs) can accumulate in tumors and suppress antitumor activity of specific cytotoxic T cells. Depletion of these cells enhances antitumor responses and in experimental setups, leads to tumor regression [86].

Many of the questions concerning tumor immunology are being addressed using intravital imaging. For example tumor associated macrophages (TAMs) are one of the most reliably found stromal components, which are easily labeled *in vivo* by fluorescent dextrans and other cell-ingestible contrast agents. Intravital microscopy has shown that they tend to concentrate at the tumor margin, as well as associate with blood vessels inside the tumors as single cells or small clusters [79]. Time lapse recordings showed that TAMs are very motile, moving with the median speed of 2.9 μm/min, which is faster than the speed of tumor cells – 1.8 μm/min, but slower than activated tumor antigen specific CTLs: 4.2 μm/min [89]. TAMs regulate their mobility in both paractine and autocrine manner by releasing hemotactic factors EGF, which is similar to cancer cells' modulation by means of colony stimulating factor 1 (CSF1) [79].It is possible that TAMs assist tumor cells in migrating through the vascular endothelium: they engage in close interaction with tumor cells and more importantly, the perivascular TAMs coincided with

intravasating tumor cells [8]. These and other studies have reinforced the notion that TAMs are critical regulators of tumor growth and invasiveness.

Other tumor-immunological questions addressed by means of intravital imaging concern for example, the mechanism and specificity of T cell recruitment and T-regs in tumor progression. In studies by Ali et al. [90, 91] tumor mice were used in examining the relative roles of T cell receptor (TCR) specificity versus non-specific interactions by means of following fluorescently labeled adoptively transferred T cells. The relative retention of polyclonal tumor-immune cytotoxic T cells (CTL) or naïve T cells in tumor vasculature was followed in real time [90]. It was discovered that tumor specific, but not the irrelevant peptide-specific, CTLs adhered to the tumor endothelium and extravasated in much higher numbers than the non-tumor specific ones. Yet, in the following study non-tumor specific CTLs accumulated in tumors in similar numbers to the tumor specific CTLs and it was suggested that rather than TCR specificity, the state of T cell activation and adhesive interactions were critical for T-cell extravasation into the tumors [92]. Although the intravasation levels were similar for both types, it was the motility of cells that differed, with tumor specific CTLs being more motile after intravasation. This was attributed to the TCR-mediated antigen recognition.

This dynamics was furthered studied in more detail [92] in tumor mice thymoma model, in which the ovalbumin (OVA) neoantigen-expressing EG7 or the parental EL4 thymoma cells were implanted subcutaneously followed by adoptive transfer of naïve CD8+ OVA-specific OT-1 T cells. The T-cell injection eventually resulted in the rejection of the OVA-expressing tumor. The early rejection phase and also late, tumor shrinking phase were imaged. At the early phase it was observed that CTL motility decreased within antigen expressing EG7 tumors as compared with the EL4 ones. An antigen-dependent contact between the CTLs and tumor cells was indicated as the CTLs would slow down or even stop at tumor cell proximity. Interestingly, at the late phase when EG7 tumor underwent apoptosis, the CTL cells resumed their migration and behaved alike in the EL4 tumor, indicating that during the slowdown they have participated in apoptosis induction. Moreover, the CTLs relation to the blood vessel proximity was studied, with their presence in EG7 tumors more pronounced at tumor periphery at the early tumor phase and this gradient disappearing later. In EL4 tumors, CTLs accumulated only at tumor boundary. It was suggested that a likely trigger for the influx of tumor-specific CTLs into tumor center is a positive inflammatory feedback between tumor killing and an increased permeability of tumor vessels.

The mechanisms of interaction of various T cell subtypes with tumor and its microenvironment is still not fully understood. Intravital imaging has so far provided a lot of new information about immune responses in cancer and will hopefully give answers to many of the remaining questions.

3. Conclusions and Future Prospects

A remarkable progress in the field of tumor biology and in particular, the understanding of stromal cell interactions regulating tumor progression has been made. By means of imaging studies it was shown how cancer cells move, progressing from a primary mass into an invading, and then metastasizing tissue. Mechanistic insight into these processes was studied by means of pharmacological modulators affecting the tumor as well as its stromal components. Also, tumor-tissue interactions have been linked with anti-tumor immune responses in the studies of architectural ECM remodeling and vascular changes. Nevertheless, there are still many mechanisms involved in tumor growth to be uncovered and many of the ones we know need clarification.

Once the malignant tumor cells are in the bloodstream it is a matter of time for them to seed and form metastases. It is still surprising, however, that despite high numbers of these cells many of them will not seed and form growths. The reason why this happens is unclear; it is still poorly understood how and by which mechanisms the immune system is suppressed in this situation. Is there an effect on T-regs recruitment? How do the innate and adaptive immune mechanisms limit metastasis engraftment? These are only a few questions out of many awaiting answers. Providing plausible explanations by experimental studies can aid finding possible therapeutic targets to impede cancer.

The studies of tumor progression have been largely enabled by the use of intravital imaging techniques in a variety of imaging platforms. In the recent centuries microscopy has immensely progressed enabling high quality resolution and low tissue phototoxicity, as well as the possibility of imaging living animals. Each imaging platform and technique used has its limitations, which need to be taken into account while designing experimental studies. For every disadvantage there are enhancements available, as presented in a short overview in **Figure 3** (adapted from [4]). Using complementary technologies such as FLIM, SHG or FRET could also solve problems in particular experimental platforms. With an increasing popularity of intravital imaging, further improvements as well as development of new platforms is both necessary and unavoidable.

Multiphoton microscopy is one of the most common imaging technologies used in *in vivo* studies. Among other qualities, it enables deeper tissue imaging than confocal laser scanning microscopy. However, even this imaging depth does not usually allow studies of the events happening in tumor core. This issue could be possibly resolved by means of long wave infrared lasers and optical parametric oscillators, which would extend the spectral range of femtosecond lasers beyond 1,100nm [15, 93]. Unfortunately, such adjustment would also result in image degradation caused by defocusing due to tissue curvature and refractive index variations. Improvements in applications of fast adaptive optics and software adjustments would be thus necessary to correct the image [94].

The imaging resolution can be further enhanced by improving spectral excitation sensitivity, which could be obtained by incorporating more than one femtosecond laser in an interline-switched configuration. Such setup has been reported to successfully separate CFP, GFP, YFP and RFP as well as enable

sensitized, emission based, heterologous FRET [15, 95]. This method is currently being calibrated and awaits further improvements.

Another challenge for the future of intravital imaging is managing the vast amounts of 3D multiplex information obtained using high-resolution time-lapse microscopy. Currently available software, although provides excellent visualization tools, lacks sufficient performance and instruction necessary to perform high throughput quantitative analysis of multicellular interactions. Biomatemathicians' involvement is necessary, for generation of new tool for such an analysis.

Challenge	Possible Solution
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 metabolic information • Injection of high molecular weight tracers to visualize vasculature and assess its permeability • Injection of tracers to visualize uptake by endocytosis/phagocytosis • SHG to visualize ECM components • Combination of multi-color excitation and/or detection, or multiphoton fluorescence combined with SHG to visualize multiple components simultaneously
Minimal out of focus signal detection	• Confocal microscopy (spinning disk/point scanning) • Multiphoton microscopy
Deep tissue imaging	• Multi-photon microscopy, near infrared or infrared excitation • Infrared fluorescent proteins
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
Minimal motion artifacts	• Rapid image acquisition and sensitive detection • Immobilization of tissue when possible •
Ability to image several regions in the same animal	• Electronic computer-controlled piezo stage or objective collar • Rapid image acquisition and sensitive detection

Figure 3 Problems and solutions for intravital imaging – short summary (adapted and revised from review [4] and references therein).

Despite developments enabling imaging without fluorescent tissue labeling, costs of gentler multiphoton lasers (e.g. Ti-Sapphire)and microscope scanners is still very high. Most laboratories will choose less expensive methods, which will likely lead to improvements in technology of fiber-based

femtosecond lasers and simplified laser scanners. Moreover, as MPM and other high resolution *in vivo* imaging techniques require fluorescent dyes and reporters, there will likely be improvements in their quality or overall a new marker approach will be implemented. These would include solutions for small molecule chemicals, genetically encoded fluorescent proteins, biosensors or specific promoter-driven constructs that enable *in vivo* visualization of apoptosis, intracellular metabolism, signaling, cytokine production, receptor occupancy, open channel activity or electrical potential [15]. Along new cell and cell activity markers, new animal models will continue to be developed, e.g. cell lineage specific fluorescent reporter mice, which would enable a closer look at specific cellular subpopulations such as MDSC, DC subsets.

Intravital imaging is a powerful tool, which will foster the understanding of tumor biology in combination with immunological, metabolic, biophysical and other biomedical studies. Cell-cell, cell-matrix and molecular interactions can now be imaged providing valuable information on the pathogenesis, dynamics and various interactions taking place in tumor microenvironment. As intravital imaging enables studies in maximally physiological conditions, it provides data which is likely clinically relevant. By expanding our knowledge on the workings of cancerous disease there is a possibility for development of more efficient and successful therapeutic approaches.

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