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Intravital microscopy as a tool to study drug delivery in preclinical studies

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Abstract

The technical developments in the field of non-linear microscopy have made intravital microscopy one of the most successful techniques for studying physiological and pathological processes in live animals. Intravital microscopy has been utilized to address many biological questions in basic research and is now a fundamental tool for preclinical studies, with an enormous potential for clinical applications. The ability to dynamically image cellular and subcellular structures combined with the possibility to perform longitudinal studies have empowered investigators to use this discipline to study the mechanisms of action of therapeutic agents and assess the efficacy on their targets *in vivo*. The goal of this review is to provide a general overview of the recent advances in intravital microscopy and to discuss some of its applications in preclinical studies.

Keywords

Intravital microscopy; non-linear microscopy; drug delivery; cancer

1. Introduction

One of the key factors in the development and successful utilization of therapeutic and diagnostic agents is the thorough understanding of the mechanisms regulating their delivery to the targeted tissue combined with the ability to provide a clear readout of their effectiveness. To this aim, several imaging techniques have been employed in live animals such as magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), and whole-body imaging using bioluminescence [1–5]. Although some of these techniques offer a high degree of sensitivity and provide the possibility to quantitatively study drug pharmacokinetic and pharmacodynamic, they lack spatial resolution at the tissue and cellular level. Intravital microscopy (IVM), which encompasses a series of techniques based on fluorescence light microscopy, has overcome this issue by enabling the image of several biological processes in live organisms at a subcellular resolution [6–9]. Although IVM has been introduced at the beginning of the last century, its major advancements have occurred in the last twenty years with the development of non-linear microscopy that has enabled performing deep tissue imaging. IVM is now a widely utilized tool that provides information on tissues such as morphology, cellular architecture,

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metabolic status, and accessibility to exogenously administered molecules in a dynamic fashion. Even though it is primarily used in preclinical studies and basic research [9–15], IVM has recently been applied in clinical settings either alone or in combination with other imaging modalities [16–20]. In this review we will provide a general overview of IVM and we will discuss how this technique has been successfully utilized to study some aspects of drug delivery in preclinical studies.

2. Intravital microscopy

2.1 Non-linear microscopy

Fluorescence light microscopy is based on the generation of contrast by the excitation of the energy levels of a molecule, generally referred as fluorophore. The excitation is achieved by illuminating the specimen with a light source such as a mercury lamp or a laser, which provides photons that have wavelengths ranging from the ultraviolet (UV) to the infrared (IR). There are two main modalities of excitation: linear and non-linear. The former, which is utilized in conventional instruments such as epifluorescent and confocal microscopes, is based on the fact that the emission intensity is linear with respect to the intensity of the excitation light [21]. The latter is based on more complex and non-linear interactions between the incident light and the specimen, which either absorbs or scatters and recombines two or more photons [15,22–27]. The diversity and unique properties of the non-linear processes has led to the development of multiple imaging modalities that have been exploited to perform high resolution imaging in live organisms (Table 1) [27–31]. We will start by briefly introducing the general principles of some of these techniques and due to space limitations we will recommend a series of more specialized articles to the reader interested in more technical details.

Multiphoton microscopy (MPM)—Multiphoton emission is based on the fact that a fluorophore can be excited by the almost simultaneous absorption (between atto- and femto-seconds, 10^{-18} – 10^{-15} sec) of two or three photons that have a half or a third of the energy required to fill the gap between two of its energy levels [23,27]. For this reason, multiphoton excitation requires IR light instead of the UV or the visible light that is commonly used in linear single photon microscopy. The major advantage of IR light is that it can penetrate biological tissues deeper than visible light, making it the ideal choice for deep tissue imaging [32]. Indeed, MPM enables imaging of biological specimen up to a depth of several hundred microns, whereas in confocal microscopy the imaging depth is restricted to 50–60 μm (Fig.1A–C). Notably, areas of the brain were imaged up to 1–1.5 mm by using longer excitation wavelengths or regenerative amplifiers [6,33,34]. Another important characteristic of MPM is that the excitation process requires a high flux of photons that is spatially and temporally focused to the focal point. This is achieved by using high numerical aperture objectives, which restrict the excitation, and consequently the emission, to a small volume ($1 \text{ fl} \sim 1 \mu\text{m}^3$) [23,27]. Since there is no off-focus emission all the emitted photons can be utilized to generate the image and since absorption occurs only at the focal point both photo-bleaching and photo-damage are reduced, enabling to extend the duration of the experimental observations. Finally, due to their broad multiphoton absorption spectra, multiple fluorophores can be excited simultaneously using a single excitation wavelength. This avoids the use of multiple lasers, which would increase the risk of photodamage [35,36] (Fig. 1D).

Second and third harmonic generation (SHG and THG)—Second and third harmonic generation do not involve any energy absorption since the incident photons are scattered and recombined into a single photon in a process without energy loss [24,28]. Molecules that generate second harmonic signals such as, collagen, microtubules, and

muscle myosin are usually assembled in highly ordered structures with non-centrosymmetric symmetries, whereas third harmonic signals are typically generated at the interface between optically heterogeneous biological materials [28,37–39]. Recently, THG has been used to image lipid bodies in small organisms or to study early embryogenesis dynamics in zebrafish [37,40]. Due to the nature of harmonic emission, SHG and THG can be combined with MPM expanding the repertoire of information that can be acquired (Fig. 1D and 1E). By using spectral un-mixing techniques, up to six intrinsic signals coming from both multiphoton and harmonic emissions were resolved, providing very detailed information about the architecture of the skin in live nude mice [41].

Coherent anti-Stokes Raman Scattering (CARS) microscopy—CARS microscopy is one of the most promising techniques to study processes in label-free samples. It is based on the use of two laser pulses (the pump and the Stokes) that are tuned to match the energetic gap between two vibrational levels in the molecule of interest generating a strong signal, which generates the contrast to image the specimen [30]. For example, the CH₂ vibrational bands have been exploited to image molecules such as lipids, either within the membrane bilayer or in intracellular storage compartments, and myelin fibers. Furthermore, CARS has been utilized to analyze more complex biological structures such as arterial walls and atherosclerotic lesions [42–45]. Since CARS provides the means to perform imaging in label-free specimen and can be complemented with MPM and harmonic signals, it may soon become a powerful technique in live animal studies [46].

Fluorescence Lifetime Imaging Microscopy (FLIM)—FLIM is based on the measurement of the lifetime that a given molecule spends in an excited state. Since this parameter is sensitive to modifications of the environment FLIM has become a powerful tool in the quantitative analysis of parameters such as pH, oxygen levels, ion concentrations, and the metabolic state of various biomolecules [7,29]. Recently, FLIM has been used for the identification of cancer cells *in vivo*, providing a potential aid for surgical tumor resection [47]. FLIM can also be combined with multiphoton excitation and harmonic generation, thus providing valuable information on the cellular microenvironment in deep tissue [48].

Optical Frequency Domain Imaging (OFDI)—OFDI is a technique derived from Optical Coherence Tomography (OCT) [49,50]. A laser beam (wavelength in the range of 1220–1360 nm) is focused into the tissue and the light reflected across all depths is combined with a reference beam and recorded as a function of the wavelength. The analysis of the interference signal is utilized to derive the optical scattering properties enabling to determine structural information on the tissue [31]. OFDI ensures a deeper tissue penetration (1 mm), a faster acquisition time, and does not require any exogenous labeling when compared with MPM, which exhibits a higher spatial resolution. Recently, a high resolution three-dimensional analysis of the tumor microenvironment and its response to a targeted therapy has been reported in the absence of any exogenous labeling highlighting the enormous potential of this technique for diagnostic purposes [31,51].

2.2 Labeling molecules and the target tissue for intravital microscopy

In order to study drug delivery in live animals it is essential to selectively and simultaneously label the molecule(s) of interest and the target tissue(s) both at the cellular and subcellular level. The major goal is to maximize the signal to noise ratio without compromising the efficacy of the drug or perturbing the normal physiological functions in the animal. To this aim, a combination of various strategies has been used such as conjugating the molecule of interest with small fluorophores and labeling the tissue either

using fluorescently tagged proteins or exploiting the intrinsic signals that are generated by each tissue in a living organism.

Small fluorophores—In the last few decades a tremendous effort has been devoted to create probes that are exceptionally photostable, bright, suitable for animal work and readily available for conjugation with the molecule of interest. New directions are constantly explored particularly to create novel probes specifically tailored for non-linear applications such as organoboron compounds or molecules based on a fluorene ring [52–54]. So far, the most promising and versatile probes are those based on nanocrystals. The first nanocrystals utilized to image biological specimen are the quantum dots (QDs). QDs are based on a semiconductor core formed by CdSe coated with a ZnS shell. The core is enclosed in a biopolymer that serves two purposes: 1) to prevent cellular toxicity, and 2) to provide the substrate for the chemical modifications necessary for the conjugation with biomolecules. QDs have several advantages such as photostability, the emission wavelengths exquisitely depending on their size (1–50 nm), broad excitation spectra, and the brightness that has been exploited to perform single molecule analysis [55–57]. For these reasons, QDs have been used extensively in numerous studies in live animals to acquire detailed information on the vasculature or to follow the delivery of potential therapeutic agents particularly in the tumor microenvironment [58–62] (Fig. 2A). Among the novel nanoprobe recently developed, the surface enhanced Raman scattering (SERS) nanoparticles offer a series of improvements with respect to the QDs [63]. They are based on a gold core encapsulated in silica and can be produced with different spectral signatures or flavors. SERS nanoparticles are less toxic, have narrower spectral peaks and because of their unique spectral fingerprint they can be easily resolved. Furthermore, their sensitivity *in vivo* is in the picomolar range, by far higher than the QDs, whose sensitivity is in the nanomolar range [63]. Recently, SHG nanoprobe based on BaTiO₃ crystals have been developed for *in vivo* imaging [64]. These particles overcome some of the shortcomings of the QDs. First, they do not blink, second the emitted signal does not saturate by increasing the excitation power, and finally, they exhibit a narrower emission peak. Furthermore, since the excitation occurs without any absorption, the risk of photodamage or generation of toxic products is substantially reduced [64]. Finally, considerable efforts have been devoted in developing probes that can be used to perform simultaneously IVM and other imaging techniques that are more sensitive in terms of quantitative readout [65], providing with the opportunity to further expand the field of multimodal analysis.

Fluorescently-tagged proteins—The possibility of using genetically encoded fluorescent proteins has created the opportunity to study the expression of a specific protein, its subcellular localization, and to follow the dynamics of specific cell populations [66–68]. Three strategies have been utilized so far in live animals: 1) the generation of transgenic models expressing fluorescent reporters under the control of specific tissue promoters, 2) the specific delivery of genes into adult animals, and 3) the orthotopic implant or the systemic injections of cell lines engineered to express fluorescent molecules.

Transgenic mice expressing fluorescent proteins in specific tissues such as subpopulations of neurons [26,69,70], pancreatic beta cells [71] and the endothelium in various organs such as the kidney and the spleen [72–74] have been extensively utilized for intravital imaging. Immunocompromised mice ubiquitously expressing soluble GFP have been successfully used to study tumor-host interactions [1,13], whereas mice ubiquitously expressing soluble GFP, DsRed or a membrane-targeted peptide fused with the fluorescent protein Tomato have the potential to provide useful structural information on various tissues [75,76] (Fig. 2B). Finally, a very elegant combinatorial approach has been used to generate the so-called rainbow mice in which neurons were labeled with different colors enabling to analyze *in vivo* neuronal circuitry [77].

The expression of genes into adult animals via either viral or non-viral mediated gene transfer has been also utilized for IVM [9]. The main advantages of this strategy are, first, the possibility to express genes in a relatively short amount of time, thus avoiding compensatory mechanisms or effects during the development, and second, the possibility to target specific tissues. One of the first study using IVM was performed in the endothelial cells of the kidney that were transduced with either GFP-actin or GFP-cofilin by using micro-puncture techniques together with adenoviral vectors [78,79]. In the skeletal muscle in live mice various proteins were expressed and imaged such as: 1) a sensor to measure the protease activity of calpain by using fluorescence resonance energy transfer (FRET) [80], 2) the two sensors Cameleon and Epac to measure calcium and cAMP levels *in vivo* respectively [81], and 3) the glucose transporter GLUT4 to study its translocation to the sarcolemma in response to insulin [82]. Recently, plasmid DNA was successfully delivered via non-viral gene transfer to the salivary glands of live rats. The expression and the dynamics of various subcellular organelles such as the Golgi apparatus or the endosomal system were imaged in cells of the salivary gland epithelium [83] (Fig. 2C). Notably, this study has opened the door to address several cell biological questions in a physiological context, utilizing approaches that so far were restricted primarily to cell cultures.

Cells engineered to express fluorescent molecules have been utilized extensively both in tumor biology and immunology. High resolution intravital imaging of GFP-expressing cancer cells has elucidated details such as the kinetics of tumor growth, the formation of protrusions at the level of single cells, the modality of migration *in vivo*, the expression of specific tumor markers during the metastatic process, and the contribution of stromal cells to cell migration [10,84–89] (Fig. 2D). Furthermore, cells simultaneously expressing markers for multiple cellular compartments (e.g. nuclei and cytoplasm) or different cell populations each expressing a different fluorescent protein have provided novel and valuable information on the metastatic process and the interactions between cancer cells and the immune system [10,90]. The latter approach has been extensively exploited to study the dynamics of cellular immunity *in vivo* (reviewed in [91–94]). A number of immunological questions have been addressed, spanning from the analysis of B lymphocytes and T cells movements in the intact lymph nodes [95–98], T cells activation [97,99], formation of mycobacterium-induced granulomas in the liver [100], T cells infiltration and elimination of solid tumors [90,101], the extrafollicular activation of B cells [102], and migration of dendritic cells [103]. Furthermore, the same approach has been used in stem cell research to track individual hematopoietic stem cells over time in the calvarium bone marrow of living mice [104].

It is clear that the development of fluorescent proteins with novel features and the generation of novel transgenic models will greatly advance IVM. The recent development of proteins that naturally emit in the IR [105] and of novel photo-activatable and photo-switchable probes [66–68] will enable to selectively image and track subpopulations of cells in well-defined temporal windows providing a plethora of novel information.

Label-free imaging—Several molecules emit in the visible or the UV range when excited via non-linear processes [38]. This approach has provided information on the metabolic status and the architecture of tissues *in situ* without using contrasting reagents [9,38]. NAD(P)H is one of the molecules that have been extremely useful in this respect. For example, data on the tissue metabolic status under pathological conditions were derived by imaging and measuring the levels of NAD(P)H in the jejunum, liver, kidney, and in the skin [106–108]. The architecture of tissues such as the skeletal muscle was analyzed in both live animals and humans exploiting the high concentration of mitochondria along the sarcomere Z-discs [14,109,110]. In the salivary glands of both live rats and mice, the fine architecture of both the acini and the large ductal structures has been imaged at a level of resolution

comparable to that obtained by classical histology from paraffin-embedded sections (Fig. 1E and 1F) [9,83,111]. Collagen fibers, which generate a strong SHG signal, have also been widely utilized to analyze tissue architecture *in vivo* [28,38,112–116] (Fig. 1E). In the context of tumor migration and invasion, SHG imaging of collagen fibers has been instrumental to correctly locate and orient tumors that are repeatedly imaged over long periods of time [89,117–119] (Fig. 2D). Other molecules have been imaged by using different modalities, such as elastin in the skin (TPM), myosin fibers in the skeletal muscle (SHG), myelin fibers in the corpus callosum (CARS) or lipid-enriched structures (CARS and THG) [18,19,42,43,109]. Recently, a high resolution three-dimensional and temporal analysis of the tumor microenvironment (OFDI and MPM, [31]) and of the embryonic development in zebrafish and drosophila (SHG and THG,[40,120]) have been presented, highlighting the power of multimodal analysis. The use of intrinsic fluorescence is a powerful tool for diagnostic purposes, which avoids the use of contrasting agents and their potential side effects [18,20]. Hence, the characterization of the patterns of intrinsic fluorescence generated in different tissues under both physiological and pathological conditions is a crucial area that would require further exploration and development.

2.3 Gaining access to the target tissue in live animals and controlling motion artifacts

A fundamental challenge in IVM is to access continuously the target tissue in ways that do not compromise its integrity and physiology. Furthermore, for high-resolution imaging, it is crucial to devise strategies aimed at minimizing the motion artifacts due to heartbeat and respiration. To this aim, some ingenious approaches have been designed such as synchronizing the scanning system to the heartbeat [121] or developing specific tools to immobilize the exposed organ [111,122,123] (Fig. 3A). In order to combine accessibility, stability, and to enable long term studies two main strategies have been devised: 1) the implant of optical windows in various areas of the body and 2) the development of miniaturized probes.

Optical windows—The first optical window was implanted in the 1920 s in the rabbit ear to image the microvasculature *in vivo*. Since then similar approaches have been developed to address several biological questions. For example, cranial windows have been extensively utilized to image the dynamics of dendritic spines up to several months, to measure blood flow in the brain cortex and as a model for angiogenesis in bone tumors [70,124–126]. A very successful and widely utilized example of optical window is the dorsal skin chamber installed in the back of rats, normal or immunocompromised mice [10–13,127,128]. The dorsal skin chamber is formed by two frames in which a skinfold is sandwiched in between (Fig. 3B and 3C). To perform IVM, a portion of the skin is removed on one side of the frame and replaced with a coverslip. The coverslip can be removed to implant the tumor cells or to locally deliver molecules [10,129]. The early models of dorsal skin chambers were made of titanium or other alloys, whereas the introduction of lighter biocompatible materials has improved the posture and the general conditions of the animal [130]. Dorsal skin chambers have provided with the opportunity to perform chronic studies *in vivo* that have shed novel light into understanding the metastatic processes [10], the role of the lymphatic system and the vasculature during tumor progression [31,118,131,132], and the dynamics of the delivery and accumulation of molecules in the tumor microenvironment [61,62,133]. Recently, an optical window has been implanted in the mammary fat pad, a set up that is very promising to study breast cancer in the proper microenvironment [84,85]

Microlenses and microendoscopes—The other strategy utilized to gain access to live tissue in a minimally invasive fashion is based on the use of miniaturized optical devices. Multiple gradient refractive index (GRIN) microlenses have been introduced enabling the development of miniaturized endoscopes (350–1000 μm in diameter) [134,135]. These

devices have been utilized in a wide range of applications such as imaging hippocampal neurons in anesthetized mice, Purkinje cells, the surface of rat kidneys, and the liver [19,109,136–138]. Microendoscopes were also utilized to perform time-lapse imaging in the brain of freely moving mice and to image the dynamics of the sarcomere contractions in skeletal muscles in humans [109,139]. The major drawbacks of these microdevices are the smaller field of view and a lower resolution when compared with conventional optics. However, several improvements have been recently reported making the use of miniaturized probes one of the most promising developments in IVM [135,136,138].

3. Imaging the delivery and the uptake of molecules in live animals

3.1 From the vasculature to the target tissue

The efficacy of molecules that are administered systemically in a live organism depends on multiple factors such as: 1) their clearance from the circulation, 2) their ability to diffuse out from the vasculature, and 3) their ability to diffuse through the interstitial space to reach the target organ. Hence, direct imaging and measurements of blood flow and vascular permeability in the area of interest are crucial. The behavior of fluorescent probes in the vasculature has been studied by most of the investigators who utilize IVM and as a consequence a large body of information are currently available. Systemic injections of fluorescently labeled bovine serum albumin (BSA) or dextrans of different sizes have enabled studying and measuring the behavior of the vasculature bed in various organs under both physiological and pathological conditions (Fig. 3D and 3E). For example, glomerular filtration and tubular reabsorption were imaged and quantitatively measured in the kidney both in normal and ischemic conditions [140–142], blood flow patterns were determined in the islet vasculature bed in the pancreas of live mice [71], the vascular flow has been imaged and measured either in normal conditions in the olfactory bulb and the skeletal muscle, or under acute ischemic damage in the brain cortex [34, 143–148].

Major efforts have been directed towards the analysis of the properties of the vasculature in the tumor microenvironment. In this respect, the dorsal skin chamber has been instrumental in performing longitudinal studies to unravel the characteristics of the blood vessels in the tumor and the machinery regulating tumor-induced angiogenesis. It has been shown that neoangiogenesis is an early event triggered when the tumor size is roughly 100–300 cells and involves the migration of individual cells towards the existing vasculature [131]. Blood vessels in the tumors are poorly differentiated with an irregular architecture and aberrant branching, whereas the normal vasculature exhibits highly organized patterns [11,12,31,61,132,149,150]. A very detailed three-dimensional analysis on the vascular morphology has been performed in various tumors measuring parameter such as vascular tortuosity, interstitial distances, diameter and surface area of the vessels [31,132]. Furthermore, blood vessels in the tumors are leakier and exhibit a higher permeability than normal vessels generating a high interstitial pressure that represents a major obstacle for drug delivery [132,149,151,152]. By using fluorescently labeled dextrans of different sizes it has been reported that molecules with a molecular weight between 40–70 kDa (4–6 nm Stoke s radius) show maximal accumulation in the tumor due to an optimal balance among permeability, penetration and clearance from the vasculature [149,153]. Furthermore, the kinetics of extravasation and tumor penetration has been characterized for receptor-targeted drugs *in vivo* [58,153] and the machinery regulating vascular permeability has been studied in conditions mimicking the tumor microenvironment, such as upon increased levels of growth factors and chemokines [154].

The following step is to image the fate of a molecule after leaving the vasculature and reaching the target tissue. In this respect, the first studies were pioneered by the Molitoris group in the kidney where the delivery of molecules such as dextran and BSA and the

internalization of fluorescently labeled folic acid into the endosomal system were imaged dynamically [155–157]. Furthermore, the same group has investigated the delivery and trafficking of therapeutic agents such as the antibiotic gentamicin and the filtration, accumulation, and efficacy of siRNA at the subcellular level [158]. Recently, our group has imaged and studied the internalization and trafficking of various molecules through the endo-lysosomal system at a very high resolution in the salivary glands of live rats [111] (Fig. 3D). This approach has also been utilized to track the internalization of fluorescently labeled plasmid DNA in real time, opening novel avenues to unravel the machinery regulating gene transduction *in vivo* [83]. The delivery and internalization of molecules have also been studied in the context of the tumor microenvironment. For example, the distribution and uptake of liposomes were measured in murine melanoma *in vivo* showing that the TNF- α improves their uptake leading to intracellular degradation [133]. By using a dorsal skin chamber, single QDs conjugated with anti-VEGFR antibodies were tracked in real time in tumors implanted in mice [62], RGD peptides conjugated to QDs were imaged in dorsal xenografts [60] and the internalization of nanotubes conjugated with EGF and QDs was imaged in tumor cells highly expressing EGFR that were implanted in the back of immunocompromised mice [58].

3.2 Assessing the efficacy of a therapy by intravital microscopy

Although several techniques provide real time quantitative information on the efficacy of a drug, IVM can still provide unique information on various cellular and molecular aspects. A clear example comes from studies on the antiangiogenic therapy using the dorsal skin chamber. The antiangiogenic therapy affects substantially the properties of tumor vessels in terms of vessel diameter, shape irregularities, and permeability. In various tumors anti-VEGFR antibodies decrease the interstitial fluid pressure by normalizing the morphology of the blood vessels and improving drug penetration. Furthermore, they improve the oxygenation of the environment, creating a temporal window in which the tumor acquires sensitivity to radiotherapy [151,152]. In this respect, a very interesting study has addressed the hemodynamic and the metabolic status of the tumor microenvironment. These are key factors in regulating cyclic hypoxia that is one of the characteristic features of many tumors, which determines their aggressiveness and resistance to therapy [127]. Antiangiogenic molecules other than those targeting the VEGFR were also investigated leading to a series of novel findings. For example, the antiangiogenic activity of the poly-L-lysine (PLL) dendrimer molecules was shown to be associated with a significant reduction of the tumor volume and delayed tumor growth [159]. Furthermore, antagonists of low-molecular-weight heparins (LMWHs) were found to confer a survival advantage in cancer patients and, by using IVM, it was established that these molecules decreased the vascular area fraction and the microvessel density [160]. Finally, the vascular-disrupting agent, 5,6-dimethylxanthone-4-acetic acid (DMXAA) was found to significantly reduce the tumor volume in murine colon adenocarcinomas by increasing vascular permeability [161]. IVM has also been utilized to monitor the efficacy of immunotherapy as shown by an elegant study using fluorescently cytotoxic T lymphocytes, which elicited tumor clearance [90,101]. All these examples highlight the power of IVM to assess the efficacy in cancer therapy. Additionally, similar approaches have begun to be utilized to study the efficacy of drugs in other dysfunctions such as in the kidney, where decreased expression of p53 by siRNA conferred protection against injury due to ischemia-reperfusion [162] and in the brain where systemic injections with curcumin reduced the amyloid plaques in a mouse model for Alzheimer's disease [163].

4. Conclusions and perspectives

In conclusion, IVM provides a formidable tool to study physiological and pathological processes *in vivo* and to provide valuable information for the design of effective therapeutic

agents in preclinical studies. One of the main directions to pursue is the use of IVM in conjunction with other imaging techniques to fully exploit the power of multimodal approaches. To this aim much effort has to be devoted towards the generation of multimodal probes and the realization of multimodal platforms in order to overcome the shortcomings of a single approach. Furthermore, the next step of development for IVM applications should be towards its use in patients. For organs that are easily accessible some clinical applications have been already implemented, such as for the diagnosis of skin cancer or inflammatory and neoplastic lesions in the gastrointestinal tract [16,18,20]. The development of miniaturized optics will be instrumental in this direction to expand further the clinical applications.

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Figure 1. Multiphoton microscopy and second harmonic generation in live animals

A - Comparison between confocal and multiphoton microscopy. The salivary glands of a transgenic mouse expressing soluble GFP and a membrane-targeted peptide fused with the tandem-Tomato protein were imaged by using single-photon confocal microscopy (left panel, excitation wavelengths of 488 nm and 561 nm) or MPM (right panel, 930 nm excitation wavelength). Volume rendering of a z-stack of the first 60 μm from the surface of the glands (60x water immersion objective-Olympus). Bar 10 μm . **B, C** - Imaging the vasculature in live animals. 70 kDa Texas red was injected into the tail artery of anesthetized rats and then the salivary glands (**B**) or the brain cortex (**C**) were exposed and imaged by two-photon microscopy (excitation wavelength of 930 nm, 25X water immersion objective - Olympus). Volume rendering of a z-stack of the first 100 μm (**B**) and 700 μm (**C**) from the surface of the organ. Scale bars 10 μm (**B**) and 100 μm (**C**). **D**-Simultaneous excitation of multiple fluorophores by MPM. Salivary glands of anesthetized mice were exposed and Alexa 488 dextran and 594 transferrin were injected in the stroma. The glands were imaged by MPM (excitation wavelength of 740 nm, 60x water immersion objective-Olympus). Dextran (green) and transferrin (red) are accumulated in the stroma and internalized by stromal cells. In blue the endogenous fluorescence excited by MPM reveals the architecture of the acinar cells. Bar 50 μm . **E** MPM and SHG imaging of the salivary glands. The salivary glands of anesthetized mice were imaged to reveal the parenchyma of the glands

(green) or the second harmonic signal generated by the collagen fibers (red) on the surface of the gland (excitation wavelengths of 720 nm and 800 nm, 20x water immersion objective Zeiss). Maximal projections of a z-stack from the first 100 μm below the surface are shown. **F** Endogenous fluorescence in live animals. Rat salivary glands were exposed and imaged by MPM (excitation wavelength of 740 nm, 60x water immersion objective-Olympus). A series of large intercalated duct are shown. Scale bar 40 μm .

Figure 2. Strategies for labeling molecules and the target issue for IVM

A QDs internalization in stromal cells. Transferrin conjugated with QDs (625 nm emission) were applied topically on the exposed salivary glands and after 20 minutes imaged by MPM (excitation wavelength 930 nm, 60x water immersion objective-Olympus). Transferrin is in red and collagen fibers in cyan. Bar 40 μ m. **B** -Transgenic mice ubiquitously expressing soluble GFP (left), soluble Ds-Red (center) and tm-Tomato fused with a membrane targeted peptide (right) were imaged using confocal microscopy (left, 488 nm and right 561 nm excitation) or MPM (excitation wavelength of 930 nm). The left panel shows the submandibular salivary glands, the center panel tendon cells, and the right panel the liver. Bars 40 μ m. **C** Gene delivery in rat salivary glands. Plasmid DNA encoding for mCherry TGN38, a marker for the trans-Golgi network (left panel, red) or GFP-actin (right panel green) were infused into the submandibular salivary glands and imaged as described in [83]. Bars 10 μ m. **D** Cells engineered to express fluorescent proteins. Squamous cell carcinomas cells (OSCC3) were engineered to express the nuclear marker H2B fused with GFP (left panel), soluble mCherry, or the small GTPase Rab25 fused with GFP (right panel). Cells were orthotopically implanted in the tongue of immunocompromised mice and imaged by MPM after 30 days (excitation wavelength of 930 nm). Left panel shows x-z maximum intensity projection from the first 70 μ m below the surface of the tongue OSCC3 cells (green), stromal cells labeled by injection of Texas red dextran (red), and collagen fibers are shown in cyan. Right panel shows OSSC3-mCherry (red) and OSSC3-GFP-Rab25 (green) from a single plane at 100 μ m below the surface of the tongue. Bars 10 and 30 μ m.

Figure 3. Devices for facilitating intravital microscopy in live animals

A- Custom-made holders for the stabilization of the externalized organs during IVM procedures (left and center holders are designed for the salivary glands, the right for the kidney). **B** Frames for the dorsal skin chamber made of the biocompatible polyacetal resin Duracon [130]. **C**- Dorsal skin chamber implanted in the back of a nude mouse. The vasculature is clearly visible. **D** Imaging the vasculature in the salivary glands of live rats. The salivary glands of an anesthetized rat were exposed, placed in a custom-made holder, and imaged by MPM as described in [111]. First, Hoechst (blue) and 500 kDa FITC-dextran (green) were administered systemically (upper panel) followed by a second injection of 70 kDa Texas Red-dextran (red). Note that after 20 minutes from the second injection, the larger dextran is retained in the vasculature, whereas the smaller one diffuses into the stroma and is internalized by stromal cells (arrows) (excitation wavelength of 820 nm, 60x water immersion objective-Olympus). Bar 30 μ m. **E** The tumor vasculature imaged through the dorsal skin chamber. OSCC3 cells were implanted in the dorsal skin chamber mounted on the back of a nude mouse. 70 kDa Texas Red dextran was injected in the tail vein of the mouse and imaged by MPM (excitation wavelength of 930 nm, 20X water immersion objective from Olympus).

Table 1

Comparison between the imaging techniques used for intravital microscopy

Imaging Technique	Advantages	Limitations	References
Single photon microscopy	High temporal resolution High spatial resolution	Tissue penetration (50–60 μm) Phototoxicity and photobleaching Off-focus emission	[21]
Multiphoton microscopy (MPM)	Deep tissue penetration (hundreds of microns) No off-focus emission Reduced phototoxicity and photobleaching	Temporal resolution	[6,15,22–27,35,46]
Second and third harmonic generation (SHG and THG)	Imaging of endogenous molecules (collagen, myosin, lipids) No off-focus emission Reduced phototoxicity and photobleaching	Temporal resolution Tissue penetration	[24,28,37–41]
Coherent anti-Stokes Raman scattering (CARS) microscopy	Imaging of endogenous molecules (lipids, myelin) Reduced phototoxicity and photobleaching	Temporal resolution Tissue penetration	[30,42–46]
Fluorescence lifetime imaging microscopy (FLIM)	Deep tissue penetration (same as MPM) Metabolic information on tissue microenvironment	Temporal resolution	[7,29,47–48]
Optical frequency domain imaging (OFDI)	Deep tissue penetration (more than 1 mm) Fast acquisition of the data Reduced phototoxicity and photobleaching No need for exogenous labeling	Lower spatial resolution than MPM	[31,49–51]