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Intravital Microscopy Imaging Approaches for Image-Guided Drug Delivery Systems

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Abstract

Rapid technical advances in the field of non-linear microscopy have made intravital microscopy a vital pre-clinical tool for research and development of imaging-guided drug delivery systems. The ability to dynamically monitor the fate of macromolecules in live animals provides invaluable information regarding properties of drug carriers (size, charge, and surface coating), physiological, and pathological processes that exist between point-of-injection and the projected site of delivery, all of which influence delivery and effectiveness of drug delivery systems. In this Review, we highlight how integrating intravital microscopy imaging with experimental designs (*in vitro* analyses and mathematical modeling) can provide unique information critical in the design of novel disease-relevant drug delivery platforms with improved diagnostic and therapeutic indexes. The Review will provide the reader an overview of the various applications for which intravital microscopy has been used to monitor the delivery of diagnostic and therapeutic agents and discuss some of their potential clinical applications.

Keywords

Biological barriers; drug delivery; therapeutic efficacy; intravital microscopy; vascular transport

1. INTRODUCTION

Integration of biological imaging into the development therapeutic and diagnostic agents can provide invaluable readouts regarding biological barriers encountered during transit of agents from point-of-injection to the projected site of delivery and provides a means to assess concentration and effectiveness of delivered agents [2]. This information can be used in an iterative approach to develop optimized drug delivery systems (DDSs) that can ably negotiate barriers, ensuring preferential accumulation at target sites and improved therapeutic efficacy. To this end, several imaging techniques including magnetic resonance

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

imaging [3], computed tomography (CT), positron emission tomography (PET), and whole-body imaging bioluminescence have been employed to monitor the delivery and obtain clear readouts regarding accumulation at target site [4–6]. While these imaging modalities offer a high degree of sensitivity and enable quantitative measurements of drug pharmacokinetic and pharmacodynamic, they lack spatial resolution at the tissue and cellular level [7].

Fortunately, rapid advances in the field of non-linear microscopy and ultra-fast scanning mechanics have made intravital microscopy (IVM) an important tool with high spatiotemporal resolutions that enables imaging and dynamic visualization of biological processes in live animals and at a subcellular resolution [2, 8, 9]. IVM has become a widely utilized tool including live-animal imaging applications in cell biology [10], immunology [11, 12], neurobiology [13], and tumor pathophysiology [8]. While it has primarily been used in preclinical studies in basic research [8, 9, 14], it is gaining applications in clinical settings either alone or in combination with other imaging modalities [15–17]. In regards to drug delivery, IVM imaging can provide unique information regarding existing biological barriers and properties of drug carriers (size, charge, and surface coating) that can be integrated into an interactive feedback loop approach for rational design and optimization of drug delivery carriers with improved delivery and efficacy profile. In this review we will discuss how IVM imaging has been utilized to acquire quantitative information that can be integrated to inform the rational design of drug delivery systems particularly focusing on the nanomedicines. Specifically, the various applications will be placed in the context of our own research efforts which have centered around the development of multiscale silicon vectors (MSVs) for diseased-specific drug delivery platforms. Examples on the various IVM applications are provided including tabulated summary of key observations in each category (Table 1).

2. INTRAVITAL MICROSCOPY

Fluorescence light microscopy has been a fundamental tool of biological discovery for several decades where it has been used to study dynamic processes [18], study and visualize cellular interactions with particulates [19, 20], and elucidate subcellular processes *in vitro* [21]. Most of these studies have been performed using *in vitro* model systems such as cells grown on solid substrates or in 3D matrices, explanted embryos, and organ cultures [22]. Fluorescence microscopy is based on the generation of contrast by the excitation of the energy levels of fluorophore molecules. The excitation is achieved by illuminating the specimen with a mercury lamp or laser light source, which provides photons in wavelengths ranging from the ultraviolet to the infrared light (IR). Recent advances in non-linear optical microscopy and high-speed scanning mechanisms have led to enormous improvements in temporal and spatial resolution, enabling deep tissue imaging. Thus, this has led to a wider deployment of fluorescent microscopy to quantify dynamic biological processes in live animals [10].

Intravital microscopy encompasses various optical microscopy techniques used to visualize biological processes in live animals. It allows continuous and fast acquisition of events (30–400 frames per second (fps)) [14] and is well suited for monitoring biological processes that can elucidate existing biological barriers, reveal cell-particle interactions, and shed light on

cellular responses to therapy under conditions closely approximating those of physiological environments [10]. The ability to dynamically image cellular and subcellular structures combined with the possibility to perform longitudinal studies have enabled investigators to use IVM to elucidate varying biological functions that include assessment of blood flow dynamics [23], vascular morphology and permeability [8, 24], and response to therapy [25]. IVM imaging also provides structural and functional information with subcellular resolution, sufficient to identify intracellular organelles [26], identify and study trafficking of cells [27], and monitor transport of drug delivery systems of different sizes [14, 28]. The advent of multiphoton microscopy has enabled deep-tissue imaging that the generation of the second- and third-harmonic imaging used for label-free imaging.

2.1. Multiphoton Microscopy

Multiphoton microscopy (MPM) is a collective term used for a variety of imaging microscopy techniques that include nonlinear excitation of a sample by using two or more photons. The nonlinear photons excite fluorescence only within a thin focal plane which precludes unnecessary energy loss and laser-induced tissue damage. It is based on the simultaneous excitation of a fluorophore using two or three photons that are half or third of its energy level [29]. For this reason, MPM imaging requires IR light instead of UV or visible light which is commonly used in a linear single-photon microscopy. IR light can penetrate biological tissues deeper than visible light, making it suitable for deep tissue imaging [30]. Thus, MPM enables deeper tissue and multicolor imaging in a complex *in vivo* environment that includes imaging of tissue explants and in live animals. Another important characteristic of MPM is that it reduces photobleaching and photodamage since photon absorption only occurs at the focal point, facilitating extended real-time *in vivo* imaging [31]. The use of long wavelength IR excitation photons enables biological deep-tissue imaging up to 200 μm compared to an imaging depth of 50–60 μm for conventional confocal microscopy [32]. MPM is now increasingly used for biological imaging as the best noninvasive means of fluorescence microscopy when combined to appropriately chosen source of fluorescent indicators.

2.2. Second and Third Harmonic Generation

While two-photon excited fluorescence is usually the primary signal source in MPM, second and third-harmonic generation (SHG, THG) have been used for multiplexed color imaging [26]. SHG and THG do not involve any energy absorption because the incident photons are scattered and recombined into a single photon in a process that ensures no energy loss [33]. SHG has enabled label-free imaging of biological molecules including collagen [34], microtubules, and muscle myosin while THG has been used to image lipid-based molecules in small organisms or to study early dynamics of embryogenesis in zebrafish [35]. The combination of MPM with second and third generation imaging has expanded the repertoire of information that can be acquired by intravital microscopy.

3. LABELING OF DRUG MOLECULES AND TARGET TISSUE FOR IVM IMAGING

To visualize and monitor the delivery of therapeutic and diagnostic agents in live animals, it is essential to selectively label molecules of interest (i.e. endothelial walls) and the target tissue both at the cellular and subcellular level. This ensures maximum signal-to-noise ratio without compromising imaging capabilities, ability to track fate of drug carrier and its efficacy, and without perturbing the normal physiological functions in a live animal. To this end, combinations of exogenous and endogenous fluorescent labeling strategies have been used.

3.1. Small Fluorescent Dyes

In the last few decades a tremendous effort has been devoted to create probes that are exceptionally photostable, bright, and suitable for animal work and readily available for conjugation with the molecules of interest. For example, photostable injections of fluorescently labeled proteins (i.e. dextrans, albumin) and fluorescent nanoparticles such as quantum dots (QDs) have been used to delineate vascular walls [36, 37]. Our group has used both endogenous and exogenous sources of contrast agents to provide structural and functional references for studying cell-particle interactions (Fig. 1). For example, systemic injections of fluorescently labeled dextrans and bovine serum albumin (BSA) of different sizes (5kDa - 2MDa) are examples of exogenous labeling strategies we have used to label the vascular network. To label macrophage cells, fluorescent antibodies specific for a target cell population such as Kupffer cell marker (F4/80) have been used [38–40]. To label macrophage cells that reside in liver and spleen, we have used fluorescently labeled red blood cells (RBCs) that are adoptively-labeled, systemically injected, and in turn taken up by Kupffer cells [14]. Other groups have used fluorescent nanoparticles such as QDs to non-selectively label macrophage cells while other efforts have utilized targeted QDs for receptor-mediated labeling of immune cell population [41, 42].

3.2. Endogenously Expressed Fluorescent Proteins

The ability to generate genetically encoded green fluorescent proteins (GFP) in live animals has provided a facile strategy with which to provide contrast for imaging the dynamics behavior of specific cell populations and particulates [27, 43, 44]. This technique is uniquely suitable for immunological studies utilizing IVM to observe dynamic cell-cell interactions where the use of exogenous contrast agents is less amenable [45–49]. Thus far, three different strategies have been utilized including; 1) the generation of transgenic models expressing fluorescent reporters under the control of specific tissue promoters, 2) the orthotopic implantation or the systemic injections of cell lines engineered to express fluorescent molecules, and 3) targeted gene delivery in adult animals [7, 48]. In other cell-particle interaction studies, research efforts have used transgenic mouse strains that express fluorescent reporters under the control of endothelial promoters, such as Tie2-GFP mice [43] and their athymic equivalent [50] used to delineate vessel walls (Fig. 1). Transgenic mouse strains that express a variety of fluorescent promoters including fluorescent Kupffer cells, dendritic cell, and T-cell fluorescent labels are also used to visualize cell-cell interactions,

study gene expression in tumor micro-environment, and evaluate transport and effect of therapeutic agents [46, 47, 51, 52].

3.3. Label-Free Imaging

Aside from the use of contrast agents, autofluorescence generated by both cellular and extracellular structures and the use of second-harmonic (SHG) signal have proven useful for multiplexed IVM imaging. For example, organs including liver [53] and blood [54] are known to auto fluoresce and provide endogenous contrast which are particularly useful for identifying fields of view (FOVs) to be compared across healthy and diseased animal models [55]. SHG signal has been used to image collagen and myosin fibers, both of which are widely used to detect and characterize the architecture of microenvironment in diseased and well as healthy tissue. The use of SHG signal has enabled multicolored intravital imaging used to characterize tumor microenvironment under varying physiological and histopathological conditions without the need for fluorescent agents (Fig. 2) [56].

3.4. Fluorescent Labeling of Drug Delivery Systems

Selecting appropriate set of fluorescent labels is critical in ensuring that drug-loaded particulates and target tissue can be tracked by IVM imaging from point-of-injection to the projected organs. Commercially available fluorescent molecules that are reactive to varying functional groups and less prone to photobleaching have been used to label nanoparticles including silica and iron oxide nanoparticles, polymeric nanoparticles, and liposomal nanoparticles [57]. For instance, our group has employed tetramethylrhodamine-succinyl ester Alexa Fluor™ dye to fluorescently label silica nanoparticles derivatized with amine functional groups, resulting in photostable particles that allowed prolonged IVM imaging [14, 28].

The use of cells to evaluate blood flow and vascular flow dynamics in live animals has been facilitated by the advent of robust lipophilic fluorescent dyes that allow facile labeling without compromising their viability. For example, red blood cells (RBCs) extracted from mice were fluorescently tagged with DiD dye (ex/em: 575nm / 635nm) through a 30-min labeling protocol and used to indirectly label liver and splenic macrophage cells [57]. Such appropriately chosen set of fluorescent dyes allows simultaneous imaging of particles and cells in respective target tissue [14]. Elsewhere, labeled RBCs have been used to evaluate tumor blood flow dynamics and were stained using similar lipophilic dyes [58].

4. VISUALIZE AND EVALUATE VASCULAR TRANSPORT

The vascular endothelium is a major barrier that impedes the distribution of intravenously injected therapeutic agents (i.e. nanoparticles) from access to tumor cells [59]. However, this barrier and the general transit of nanoparticles from the vessel to the extravascular space remains largely misunderstood. Understanding this vascular obstacle is critical to elucidating the delivery of therapeutics to target site (i.e. cancer) [60]. The efficacy of systemically administered therapeutic agents depends on multiple factors such as: 1) their clearance rate from circulation, 2) ability to attach and extravasate through vascular barriers, and 3) ability to diffuse through the interstitial space to reach the target organ. Direct visualization of all

these processes including existing biological barriers is crucial in the development of agents that can successfully negotiate through barriers and deliver therapeutic agents to projected organs [61]. For instance, vascular transport differentials in tumor microenvironment dictates the transport and efficacy of chemotherapeutic agents and, thus, understanding and appropriately tailoring DDSs that can take advantage of these transport characteristics is key to improving patient outcome [62]. Our group has attempted to use IVM imaging to elucidate mechanism of enhanced transvascular transport and develop accompanying drug delivery vehicles that are better at shuttling therapeutics across the endothelial walls into the projected organ.

4.1. Fluorescent Proteins to Characterize Vascular Properties

As mentioned above, small fluorescently labeled proteins have been widely used to evaluate vascular dynamic behavior of blood flow and vascular morphology (geometric arrangement, diameter, length, and number of blood vessels) in normal vessels and in inflamed vasculature [63]. Understanding transport parameters present in inflamed vessels is critical in determining effectiveness of therapeutic agents [64]. We have used IVM imaging to delineate and characterize tumor vascular networks that dictate chemotherapeutic delivery into tumor microenvironment using fluorescently labeled dextrans of different sizes (Fig. 3). Upon systemic injection, fluorescent dextran molecules (70kDa) perfuse and mostly remain in the vascular space due to relatively bulky size, providing a clear delineation of vascular network from the interstitial space and other underlying tissue. The degree of tissue vascularity influence amount of therapeutics that can be transported into projected organs whereby insufficient vascularization hinders the optimal transport of cell nutrients, oxygen, and drugs to cancer cells in solid tumors and, thus, such analyses provide insightful information regarding transport network in specific organ.

Since IVM allows enhanced spatio-resolution imaging, it has been utilized to characterize and automate vascular perfusion measurements at a single vessel level using the systemic injection of fluorescent dextrans [37]. Such findings can enable the modeling of therapeutic delivery and can be integrated into design in order to optimize delivery into target site. Some of our other efforts have exploited fluorescent contrast agents and IVM imaging to measure changes in blood flow patterns in the breast tumor in live mice before and after mild hyperthermia treatment [58]. These findings have demonstrated that localized mild hyperthermia treatment transiently increases blood flow and provides an optimal “time-of-opportunity” with which to improve delivery of macromolecules, including chemotherapeutic agents.

Intravital microscopy has also been used to construct detailed three-dimensional analysis of vascular morphology describing parameters such as vascular tortuosity, interstitial distances, diameter, and surface area of vessels using fluorescent contrast agents as vessel tracers [65]. Additionally, high-speed IVM imaging have enabled quantitative assessment of localized tumor dynamics including vascular permeability and clearance rates where fluorescent of different-sized dextrans are used to model therapeutic delivery in selected physiological diseased states such as in the tumor microenvironment [36, 66, 67]. Our laboratory has used this technique to reconstruct an automated method to evaluate localized flow kinetics

whereby fluorescent tracer was used to measure shear rates, tissue permeability, and blood volume fraction on a vessel-by-vessel basis, potentially leading to insights into how local variations in perfusion can affect drug delivery and treatment response [37]. This is akin to that use of MRI to quantify extent of organ perfusion, except IVM analyses provide improved spatial resolution [68].

Likewise, we have conducted IVM studies that aim to design personalized nanotherapeutics that exploits transport differentials in cancer, termed “transport oncophysics” to improve the delivery and treatment of cancer therapeutics [61, 69, 70]. For instance, *Yokoi et al.* [62] used fluorescent dextrans to evaluate permeability of tumor vasculature and identified predictive serum biomarkers that can predict patients most likely to respond pegylated liposomal doxorubicin (PLD) based on degree of vascular permeability. The findings showed the serum biomarkers expression can be used to predicted vascular permeability whereby highly permeable tumors were more likely to retain PLD treatment based on enhanced permeation and retention effect (EPR) effect, and consequently more responsive to therapy.

Furthermore, intravital microscopy studies have proven useful in the study and characterization of tumor properties that directly affect drug delivery such as interstitial fluid pressure (IFP), a phenomena known to hinder chemotherapeutic delivery into tumor microenvironment [71, 72]. For example, *Simonsen et al.* [65] captured dynamic parameters of macromolecules in xenografted melanoma tumors and established relationships between high IFP and tumor angiogenesis. However, the study established no correlation between IFP and tumor geometry or angiogenic activity as the existence of IFP is complex and differ substantially among human tumors of the same histological type.

4.2. Nanoparticles and Vascular Interactions

IVM-based imaging techniques have been developed and used to assess and evaluate *in vivo* flow dynamics of individual particles [28, 73]. For example, we have studied dynamic flow and particle bio-distribution in a variety of organs acutely or longitudinally using surgical manipulations including the skin, liver, spleen, and solid tumors by coupling advanced image-processing algorithms to high-resolution microscopes with optical sectioning capabilities [28]. Nanoparticle accumulation in blood vessels, in circulation, and in specific organ of interest can be dynamically visualized with the appropriate selection of fluorescent tracer or the use of transgenic animal strains to delineate the vessel walls [57]. IVM imaging has been used to visualize and capture the flow velocities, flux, trajectories of particles of varying sizes and shapes (i.e. silica particles, liposomes, MSVs) using high-speed image acquisition (up to 35 fps). Due to their ability to remain in circulation for prolonged periods, fluorescently red blood cells (RBCs) have been preferred as a surrogate to evaluate blood flow dynamics of tumor microvasculature, enabling the rendering of dynamic flow behavior in the tumor microenvironment. For instance, using high-speed IVM imaging acquisition we demonstrated that localized mild hyperthermia treatment increases in-flux of MSVs particles (1000 × 400 nm) which was assessed on a vessel-by-vessel basis using fluorescently RBCs as a surrogate to evaluate blood flow dynamics (Fig. 4) [58]. The analyses showed that mild hyperthermia led to a 2-fold increase in blood flow in tumor microenvironment when

assessed 5 h after treatment which directly correlated to transient elevation in the in-flux of MSVs and thereby providing a means with which to enhance tumor localization of nonthermally sensitive particles [58]. Other efforts have used IVM imaging to determine effect of surface modification on *in vivo* circulation such as PEGylation of hydrogels which was demonstrated to neutralize surface charge on the hydrogels, resulting in prolonged circulation relative to unmodified counterparts [73].

One of the major challenges in the development of effective nanomedicine is the design of particle that can preferentially adhere firmly to vessel walls of the diseased tissue. To this end, IVM-based imaging techniques have been used to evaluate cell-particle interactions that occur along vessel walls of inflamed vasculature with potential clinical implications that span areas in cardiovascular, cancer therapy, and inflammation [74–76]. These efforts have informed the optimal design of particles by fine-tuning their physicochemical properties (i.e. size and shape) and evaluating the effectiveness of vascular targeting agents. For example, *van de Ven et al.* demonstrated through time-course IVM analyses that discoidal MSVs (1000×400 nm) preferentially adhered to the vessel walls of melanoma tumors than their smaller counterparts (600×200 nm), suggesting that taking advantage of hydrodynamic and interfacial interactions can be an effective strategy that exploits “transport oncophysics” in cancer to improve the delivery and efficacy of cancer therapeutics (Fig. 5).

The use of vascular targeting agents to improve selectivity of therapeutic agents and reduce “off-target” toxicity has become widespread [77–79]. Therefore, IVM imaging has also been used to visualize and evaluate the efficiency of targeting agents such as arginine-glycine-aspartic acid (RGD) to target $\alpha_v\beta_3$ integrins predominantly expressed in inflamed vasculature such as in tumor microenvironment [80]. Through these analyses, up to a 3-fold enhancement in accumulation of RGD-targeted discoidal MSVs (1000×400 nm) was observed in melanoma tumor-bearing mice as compared to untargeted particles (Fig. 5) [28]. Other research efforts have exploited IVM imaging to examine the effectiveness of arginine-glycine-aspartic acid (RGD) peptide conjugated to quantum dots [77], carbon single-wall nano-tubes [78], and liposomal nanoparticles [79].

Increased leukocyte-endothelial interactions are intimately related to a wide-range of inflammatory pathophysiological conditions that include cardiovascular disease, neurophysiology, and cancer [20, 52, 81, 82]. In the context of developing “smart” drug delivery systems, IVM imaging has been used to visualize and study the leukocyte–endothelial cell adhesion chemistry in inflamed tumor endothelium that can facilitate transvascular transport of drug payload [83]. Thus, recent efforts have evaluated the preferential binding of silica particles coated with leukocyte-derived membrane to selectively deliver therapeutic payload to tumor microenvironment whose vasculature is inflamed. For instance, *Parodi et al.* [83] designed that hybrid silicon particles coated with biomimetic leukocyte membrane, termed as “leukolike vectors”, that leveraged self-recognition mechanisms to bind inflamed endothelium which facilitated transport across the endothelial layer *in vivo*. Other particles that are being investigated and developed to take advantage of this leukocyte-endothelium cell mechanism include PEGylated biodegradable particles, lipid-polymer nanoparticles, and curcumin-loaded nanoparticles [82, 84].

Another application for which IVM imaging has become a vital tool is in cardiovascular research where it has been used to identify potential drug targets through assessment of critical signaling processes that occur in diseased vasculature [85, 86]. For example, *Thunemann et al.* [87] has recently described an IVM-based method used to evaluate the role and signaling processes of cyclic guanosine monophosphate (cGMP) on the vascular cells. cGMP is an important signaling molecule and drug target in the cardiovascular system. It is well known that stimulation of the vascular nitric oxide (NO)-cGMP pathway leads to vasodilation. However its spatiotemporal dynamics, concentrations within specific cardiovascular cell types in health, disease, and during pharmacotherapy are largely unknown [88].

5. VISUALIZE AND EVALUATE DRUG/PARTICLES IN RETICULOENDOTHELIAL SYSTEM

One of the biological barriers that limits effective delivery of systemically administered drugs and nanoparticles is the reticuloendothelial (RES) system which consists of monocytes and macrophages cells residing primarily in the liver and spleen [61]. Therapeutic efficacy of drug delivery systems is largely determined by the ability to delay sequestration by the RES system. For example, many different types of therapeutics accumulate at target sites at low efficiency because a larger proportion of circulating drugs are taken up by macrophages cells and specialized monocytes cells residing in the liver, spleen, and bone marrow [89]. Early efforts to prolong systemic circulation and reduce macrophage uptake involved coating the surfaces of the drug carriers with nonionic surfactants and polyethylene glycol polymers [90, 91]. These innovations improved the pharmacokinetic and pharmacodynamics of nanoparticle-based chemotherapeutics, resulting in enhanced efficacy compared to ‘naked’ drugs.

5.1. Particle Design and Avoidance of Reticuloendothelial System

The invent of IVM imaging techniques have allowed assessment and study of how nanoparticles interact with the RES system and devise novel strategies to delay capture, providing a vital tool for the development of “smart” delivery strategies, particularly for cancer and other diseases characterized by pathological disruptions of mass transport [69]. Our group has developed IVM-based techniques to study dynamic behavior of particle transport and cell particle interactions in the liver, an important repository of macrophage cells [14, 28, 83]. Resident macrophages of the liver (Kupffer cells) are the predominant cells responsible for phagocytosis of many classes of circulating nanoparticles [92]. For example, *van de Ven et al.* recently described a detailed step-by-step protocol, experimental design, and animal preparation including abdominal incision and time-lapse assessment of individually-identified and tracked particles within the liver vasculature [14]. In healthy mice, IVM studies of nanoparticle dynamics are used to determine how chemicophysical particle properties, such as particle size, surface charge, and coating affect their capture and uptake by Kupffer cells. These cells are readily visualized within the liver sinusoids through a small midline abdominal incision and following labeling of cells using indirect RBCs labeling strategy [14]. Adhesion of fluorescently labeled particles to the vasculature and subsequent internalization by Kupffer cells can be monitored in real-time using a fast

acquisition IVM imaging set-up (30 fps). Frame-by-frame analyses of IVM videos yield time-lapse quantitative information regarding the degree of particle capture by Kupffer cells, enabling the tailoring of particle properties to reduce avoid RES clearance and prolong *in vivo* circulation (Fig. 6). In subsequent efforts, we showed that coating silicon particles with biomimetic leukocyte-derived membranes transiently delayed Kupffer cell mediated capture and reduce the overall quantity of particles internalized by the liver, resulting in enhanced tumorotropic accumulation of leukolike vectors [83]. In the most recent efforts, IVM studies was used to evaluate the design of biomimetic liposome-like vesicles that can be loaded with a wide spectrum of therapeutics [93]. For instance, *Molinaro et al.* demonstrated successful assembly of a biomimetic liposomal-based drug delivery system termed as “leukosomes” prepared by manipulation of a biological proteolipid material (i.e., proteins derived from the plasma membranes of leukocytes) and using IVM studies to visualize relative accumulation in RES system as well as preferential tumorotropic accumulation [93]. In other research groups, IVM studies have been used to improve the design and efficiency of varying types of drug carriers [38, 73]. For instance, *Kim et al.* recently showed, through IVM analyses, that coating drug delivery systems with hyaluronic acid enhances liver specific targeting, and, thus provides target-specific derivative for treatment of liver disease [94]. Thus, IVM imaging has been used to glean information regarding the particle surface properties (size, shape, and surface) in order to optimize and reduce the rate of particle capture [28] as well as the quantity of particles captured by Kupffer cells [95].

5.2. Antibody and Mechanism of Action

The use of monoclonal antibodies (mAbs) as therapeutic agents has increased dramatically in the last decade and is now one of the mainstream strategies for cancer therapy [96, 97]. To this aim, liver imaging via intravital microscopy have been used to elucidate the mechanism through which monoclonal antibodies (mAbs) eliminate circulating tumor cells [92]. Through real-time imaging, tumor cells were shown to be rapidly recognized and arrested by liver macrophages (Kupffer cells), leading to the conclusion that antibody-dependent phagocytosis (ADPh) by macrophages is a prominent mechanism for removal of tumor cells from circulation in a murine tumor cell opsonization model. IVM imaging has also been utilized to elucidate mechanisms through which specific antibodies function to reduce disease burden through the detoxifying liver functions [98, 99]. A clear example involved dynamic IVM imaging of liver sinusoids to elucidate the mechanism through which anti-CD44 antibody reduced systemic inflammatory burden 4 h after LPS injection [98].

6. VISUALIZE THERAPEUTIC DELIVERY AT TUMOR MICROENVIRONMENT

Extraordinary advances in IVM imaging have enabled visualization of blood-borne therapeutic molecules, particles, and cells as they make their way through blood vessels, across the vessel wall, and into tumor interstitial space [100]. IVM imaging has been exploited to gain insights into existing biological barriers in tumor microenvironment and then integrating these findings with *in vitro* analyses [20, 101] and mathematic modeling [102, 103] to design drug carriers that preferentially accumulate at diseased tissue by taking advantage of the Enhanced Permeability and Retention (EPR) effect.

6.1. Therapeutic Delivery in Tumor Microenvironment

Major efforts have been directed towards evaluating effectiveness of therapeutic delivery in the tumor microenvironment. Our group has developed IVM-based techniques used to assess preferential accumulation of particles in live animals bearing breast, pancreatic, melanoma, and liver tumor models in efforts to evaluate the effect of particle size, shape, and surface coating on therapeutic accumulation [23, 28, 83, 104]. For instance, IVM analyses of melanoma tumor model revealed that large discoidal MSV particles ($1000 \text{ nm} \times 400 \text{ nm}$) were more likely to accumulate within tumor microvasculature than their smaller counterparts ($600 \times 200 \text{ nm}$) which extravasated out of the vascular walls through fenestrations into interstitial space [28]. Additional analyses also evaluated the influence of surface modifications on preferential particle accumulation in tumor microenvironment. The study showed that MSV particles derivatized with arginine-glycine-aspartic acid (RGD) peptide, known to specifically attach to $\alpha_v\beta_3$ integrins expressed along tumor endothelial walls [80], preferentially accumulated in the tumor microenvironment than uncoated particles. In a follow-up study, IVM imaging showed that the biomimetic leukocyte-derived membranes coating on discoidal MSV particles, termed as leukolike vectors (LLVs), enhanced tumorotropic accumulation due to ability to attach to inflamed vasculature [83]. In efforts seeking drug delivery strategies for pancreatic cancer treatment, IVM-based techniques have been used to evaluate and quantify efficiency of targeted MSV particles using orthotopic models of human pancreatic cancer. By leveraging IVM studies, *Yokoi et al.* demonstrated that receptor-mediated targeting of Ly6C-conjugated discoidal MSV particles led to a 10-fold increase tumor-specific accumulation of particles 4 h after intravenous injection [105].

IVM imaging has also been used to evaluate the dynamic changes that occur in the tumor environment upon application of external stimuli such as mild hyperthermia and radiofrequency treatment [24]. These stimuli are increasingly becoming common methods with which to improve chemotherapeutic delivery by “normalizing” tumor vascular blood flow and improving transvascular transport [106, 107]. For instance, IVM analyses revealed that mild hyperthermia treatment causes a prolonged (up to 36 h) enhancement in transvascular transport of larger dextran dye (54 nm) without causing occlusive vascular damage. This study indicated that localized mild hyperthermia treatment provides a transient window used to augment macromolecule transport with potential to improve therapeutic efficacy (Fig. 7) [24]. Other applications of IVM imaging to assess and confirm therapeutic delivery include; 1) assessment of drug-loaded liposomal carriers application of external stimuli (i.e. mild hyperthermia, photodynamic therapy), 2) visualizing influx of immune cell population (i.e. dendritic and T cells) after immunotherapy treatment, and 3) visualization of particle delivery in lung and liver metastatic tumor models [47, 108–112].

7. ASSESSING THERAPEUTIC EFFICACY OF DRUG DELIVERY SYSTEMS

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 used to assess the efficacy of antiangiogenic therapy on tumor microenvironment. Through IVM analyses, antiangiogenic therapy has been shown to

significantly affect the properties of tumor vessels in terms of vessel diameter, shape irregularities, and permeability. For instance, anti-VEGFR antibodies are shown to reduce the interstitial fluid pressure by “normalizing” the morphology of blood vessels and improving drug penetration in various types of tumor [113]. Furthermore, they improve the oxygenation of the environment, creating a temporal window in which the tumor acquires sensitivity to radiotherapy [113, 114]. The effect of other antiangiogenic molecules such as poly-L-lysine (PLL) dendrimer molecules which caused significant reduction of the tumor volume and delayed tumor growth have also been investigated [115].

Other groups, such as the Jain’s Group, have incorporated IVM imaging to characterize the effects of combinational tumor normalizing and chemotherapeutic treatment strategies [34, 116–120]. For instance, *Liu et al.* used IVM to elucidate the mechanism with which TGF- β blockade (with an inhibitor) improved breast tumor growth after treatment with doxorubicin, a convectional chemotherapeutic agent [121]. Dynamic vascular measurements demonstrated that improved tumor suppression from the combination of TGF- β inhibitor and doxorubicin was due to improved blood vessel coverage which led to enhanced chemotherapeutic delivery into the tumor microenvironment. Other therapeutic agents including the use of a vascular-disrupting agent, 5,6-dimethyl-xanthenone-4-acetic acid (DMXAA) have been studied and demonstrated to significantly reduce tumor volume in murine colon adenocarcinomas by increasing -vascular permeability [122]. 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 [123].

IVM imaging has been exploited to evaluate therapeutic efficacy in other diseased states. A clear example comes from studies that evaluated the effect of vascular-disrupting agent given to patients ailing from age-related macular disorder [112]. Localized IVM imaging revealed that the treatment prolonged vascular leakage (~2 days) and improved therapeutic delivery. IVM imaging has also found vital applications in immunotherapy. For example, dynamic imaging of tumor environment was shown by an elegant study using fluorescently cytotoxic T lymphocytes, which elicited tumor clearance [45, 124]. Similar approaches have begun to be utilized to study the efficacy of drugs in other dysfunctions in the brain, heart, and kidney. IVM imaging have shown that. For example, IVM dynamic imaging, systemic injections of curcumin reduce the size of the amyloid plaques in a Alzheimer’s disease mouse model and expression of p53 by siRNA conferred protection against injury due to ischemia–reperfusion in the kidney animal model [125]. All these examples highlight the power of IVM as is used to assess the efficacy of cancer chemotherapeutics.

CONCLUSION AND PERSPECTIVE

In conclusion, IVM is an invaluable tool that is widely deployed to study physiological and pathological processes in live animals and to monitor delivery of agents during transit from point-of-injection to the desired organ of delivery. IVM imaging has provided clear readouts regarding material properties (size, and shape) that affect preferential accumulation in the tumor microenvironment as well as the effect of vascular targeting agents on accumulating at the diseased tissue sites. One of the main directions to pursue is the full integration of IVM

imaging with experimental designs so that unique information acquired from imaging can be incorporated into the design of drug delivery systems through an iterative “feedback loop” approach. To this aim, much effort has to be devoted towards the generation of multimodal probes and labeling strategies that do not affect the properties of drug carriers in order to fully model their physiological behavior in patients. Additionally, development of miniaturized imaging optics to allow facile imaging of respiratory systems including the lung and heart of live mice would dramatically expand the reach of this technology in many different research fields (i.e. cardiovascular research). Continued miniaturization of imaging optics would also further expand its clinical applications towards uses in human subjects. For easily accessible organs, some its clinical applications are already underway such as for the diagnosis of neoplastic lesions in the gastrointestinal tract and skin cancer [16, 17]. In the near future, intravital microscopy promises to be a powerful tool that can be fully harnessed by researchers to develop “smart” drug delivery systems, and used in clinical practice to guide patients’ treatment and monitor efficacy.

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TAKE HOME MESSAGE

Intravital microscopy is an indispensably important imaging platform that should be incorporated into the design and evaluation of drug delivery systems. It provides critical information regarding nature of existing biological barriers that hinder effective therapeutic delivery and provide real-time imaging capability used to monitor therapeutic delivery from the point-of-injection to the intended organ while providing clear readouts about amount and effectiveness of therapeutic agents. Taken together, intravital imaging is becoming a vital platform that is continually integrated into design of novel drug delivery systems in preclinical studies to achieve improved therapeutic indexes.

Fig. (1). Examples of labeling strategies used to define vascular space and label other cells. Exogenous contrast agents (40kDa fluorescent dextran) is used to label liver sinusoids and endothelial GFP expressed in transgenic mice. Scale bar = 50 μ m respectively.

Fig. (2). Identification of collagen fibers by second-harmonic signal IVM imaging

A) Second-harmonic signal in melanoma tumor grown on skinfold chamber.; **B)** SHG signal with vessels labelled with i.v. injection of red dextrans and collagen in green (pseudocolor).

Reference: Brown *et al.*; Nat Med, 2003; 9: 796–800.

Fig. (3). Vascular transport properties of pancreatic tumor as characterized by IVM dynamic imaging

A) IVM imaging used to define and study vascular density upon i.v. injection of 70 kDa fluorescent dextran(green); **B)** combination of different size of fluorescent dyes used to evaluate tumor permeability whereby a 70kDa dextran (green) is used as tracer while smaller 3-kDa dextran serves as a macromolecule with which to study and model vascular permeability and diffusion across the tumor microenvironment; **C)** Quantitative analyses clearly showing difference in vascular properties across the different tumors. Scale = 503m.

Fig. (4). Illustrations on use of IVM to monitor vessel flow dynamics on a vessel-by-vessel basis after mild hyperthermia treatment;

A) Fluorescently labelled MSVs (1000 $\times$ 400 nm) monitored by IVM imaging B, C) Time-lapse quantitative analyses of fluorescently-labeled MSVs in tumor vasculature showing increased initial and total number MSVs in tumors receiving MHT treatment versus control groups. 5-fold enhancement was observed in SUM159 and 3-fold for MCF7 tumor; D) Representative time-lapse images from videos acquired over 60 min, showed decreased number of MSVs with time and adherent particles shown after 60 min. Curves represent number of particles monitored in animals (n = 6) injected with 56108 particles (1000x400 nm, 50 mL PBS-did you inject 50 ml or you wanna say “56108 particles/50ml PBS”) and monitored over 60 min. Error bars represent standard deviation from (n = 6) collected over 8 FOVs per animal. Reference: Kirui *et al.* PLoS ONE, 2013; 9(2): e86489

Fig. (5).

Example of use of IVM imaging to evaluate delivery to tumor microenvironment.

Accumulation of untargeted and RGD-targeted plateloid particles in melanoma tumors. **a**, Intravital microscopy data on the time-dependent accumulation of 600×200 nm (left), 1000×400 nm (center), and 1800×600 nm (right) plateloid particles. **b**, ICP-AES data on the cumulative particle uptake expressed as percentage of the injected dose normalized by the tumor mass. **c**, Stills extracted from intravital videos reveal that 1000×400 nm particles accumulate within the tumor vasculature (top left), whereas some 600×200 nm particles extravasate out of the vasculature (bottom left). The SEM sections clearly show the fenestrations in the tumor vasculature and their size relative to the plateloid particles (scale bar, $1.0 \mu\text{m}$). **d**, Representative histological images of the tissue surrounding the tumor vasculature. The vessel wall is poorly organized with gaps between endothelial cells (top). The tumor cells are poorly packed, resulting in gaps between cells (bottom) (Scale bar, $1.0 \mu\text{m}$). Reference: *van de Ven, J. Control. Release, 2012; 158(1):148–155*

Fig. (6). IVM to visualize and assess effect of particle coating on clearance by liver of live mice
A) The left lobe of the liver is readily exposed via a small midline incision. A coverslip may be gently placed against the tissue surface to allow high magnification and high resolution microscopy without impacting liver movement or blood flow; **B)** Fluorescent 100-nm particles (red), injected retroorbitally, can be identified and tracked within the liver microvasculature (green) using a high speed intravital microscope equipped with optical sectioning capabilities. Particle accumulation within the vasculature, as well as particle uptake by Kupffer cells (blue) are visualized from the time of injection. Scale bar, 50 μm ; **C)** Quantitative analyses derived from time-course monitoring of particle accumulation over 60-min imaging intervals (shown here as every 10 seconds).

Fig. (7). IVM imaging used to assess tumor microenvironment transport after localized treatment;

A, B) time lapse IVM images showing increased dextran accumulation in tumor interstitial space after mild hyperthermia treatment; **C)** mean average fluorescent intensities with distinct R values between the treated and untreated group; **(D)** average intensity acquired over 20 min (1 h post-heat) and fitted to a mass transport model with a higher Y_{max} and R for treated group; **(E)** comparison of treatment groups (1 and 5 h post-treatment) showed similar average fluorescent intensities and transport enhancement properties with $Y_{max} = 3816$ and 3661 fluorescent units [1] and ($R = 0.325$ and 0.458 min^{-1}), respectively; **(F)** histological analyses confirm that MHT caused little morphological alterations to tumor microenvironment while thermal ablation caused cellular damage. *Reference: Kirui, Nanomedicine (London), 2014; 10: 1487–1496.*

Table 1Examples on use of IVM imaging to monitor fate of drug delivery systems *in vivo*.

Category	Types	Key Observations	Refs.
Preparation & materials for IVM imaging	Nanoparticles, microparticles liposomes, cells	- Facile fluorescent conjugation strategies enabling particle labeling (i.e. varying sizes, geometry & surface functional groups)	[14, 28, 55]
Staining of Vasculature	Fluorescently-labeled proteins, i.e. dextrans & Quantum dots	- Delineation and definition of vascular networks in normal and diseased tissue	[36, 37]
	Endogenously-expressed fluorescent reporters engineered into transgenic mice	- Exquisite labeling of endothelial walls enabling facile visualization of particle-wall interactions	[27,41–43, 47, 45, 50]
Evaluation of Vascular Transport	Fluorescent proteins of varying sizes (dextrans, 5kDa – 2MDa)	- Characterization of vascular and transvascular transport i.e. perfusion measurements used to model delivery of therapeutics in tumor microenvironment	[37, 60, 64, 65]
	Fluorescent red blood cells (RBCs)	- Measure alterations in blood flow dynamics and establish correlations to drug transport	[55, 56]
Visualizing interactions along endothelial wall	Drug carriers such as targeted particles vs. bare i.e., MSVs, NPs	- Targeting of vascular RGD enhanced accumulation and delivery	[28, 70, 79, 104]
	Cells, biomimetic coated with leukocyte membrane systems – i.e. “leukolike”	- Mimics of leukocytes interactions along inflamed vasculature – i.e. biomimetic leukocytes coating on particles, liposomal drug carriers	[20, 50, 78, 80]
Visualizing and imaging of RES system	Nanoparticles, microparticles, RBCs, and cancer cells	- Red blood cells endogenously labeled with lipophilic cell staining i.e. DiD, DiI	[14, 55, 83, 93]
	Antibody labeling of macrophage cells	- Anti- (F4/80) antibody used to label monocytes and macrophages in liver and spleen	[38–40]
Monitoring particle delivery and clearance in the RES system	Nanoparticles, microparticles, and functionalized particles	- Evaluate effect of surface coating on ability to avoid/delay of particle sequestration by Kupffer cells	[38, 70, 83, 93, 91]
	Therapeutics i.e. antibody therapy (mAbs)	Elucidate mechanisms with which mAbs function to reduce infection and disease burden	[89, 95, 96]
Imaging of tumor microenvironment (i.e. barriers, transvascular transport & therapeutic efficacy)	Fluorescent dextrans	- Evaluate vascular transport barriers - Assess alterations in transport before and after treatment	[23, 50]
	Drug-loaded particles & chemotherapeutics	Visualize extent of delivery in tumor microenvironment Evaluate effect of therapy	[56, 71–73, 118–120]
Other imaging applications (i.e. cardiovascular & immunology)	Cardiovascular signaling molecules, cGMP	- Elucidate signaling pathways associated with cardiovascular diseases	[74–76, 81, 82, 88]
	Immune cells i.e. dendritic & T cells	- Visualize and elucidate mechanisms of actions of immunotherapy	[45, 105–109]