TOPICAL REVIEW

Probe-based intravital microscopy: filling the gap between in vivo imaging and tissue sample microscopy in basic research and clinical applications

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Abstract
Non- and minimally invasive imaging technologies have become indispensable in preclinical studies using animal models to understand biological processes and assess novel therapeutic strategies within the complex context of living organisms. Various imaging modalities can provide anatomical, functional or molecular information on the organ- or whole-body level, however, there exist a need to obtain dynamic information on the microscopic level in living animals to bridge the gap with microscopical analysis of processed tissues. To fulfill this need, intravital microscopy (IVM), which allows imaging at cellular and subcellular resolution in living animals, was developed. The emergence of different imaging modalities, advances in fluorescent labeling methods and the transition from optical windows to fiber-optical probes to obtain direct access to sites deep inside the animal, have supported its widespread use. This review provides an overview of these technological advancements and specifically of the application of fibered confocal fluorescence microscopy in preclinical research. In addition, the implementation of probe-based confocal laser scanning endomicroscopy in clinical research on gastrointestinal and lung diseases and possible applications in drug development are described. Finally, drawbacks and possible advances that can broaden the potential of this technique and what can be learnt from IVM in a context of multimodal imaging are highlighted.

1. Introduction

Animal models are indispensable in many research areas, as their use enables the study of complex biological processes and the dynamic interplay between disease development and the host immune response that cannot be reproduced in vitro. Since ex vivo analytical methods often require the animals to be sacrificed, the possibility to obtain dynamic information from individual animals is lost. For this reason, non-invasive or minimally invasive imaging techniques have become of great importance as they enable longitudinal investigation of the same animal over a relevant time scale [1]. Distinct imaging modalities that can provide valuable anatomical, functional or molecular information on the organ- or whole-body level are now widely available. Among the optical imaging modalities, bioluminescence imaging (BLI) and whole-body fluorescence imaging respectively visualize the enzymatic production of visible light or fluorescence by genetically engineered cells, that can be tracked within small laboratory animals to obtain temporal and spatial information on the whole-body level in real time [2–5]. BLI enables for example the study of infectious diseases in animal models through the localization of genetically engineered pathogens enabling
infection monitoring over the disease course and therapy efficacy [4, 6–11]. Compared to these whole-body imaging techniques, magnetic resonance imaging (MRI) and computed tomography (CT) typically provide a smaller imaging scale, zooming in on a specific part of the body or organ with a higher imaging resolution and yielding tomographic, three-dimensional data. MRI uses a strong magnetic field while CT is based on x-ray transmission to provide information on both the physiological and anatomical changes of tissues and organs. These techniques are applied both in a preclinical or clinical context for diagnosis, staging and the evaluation of treatment effects [4, 12–15]. Given the inherent imaging capabilities and properties linked to each imaging modality, multimodal imaging, i.e. the combination of different imaging techniques, has proven to be extremely valuable to obtain complementary information and overcome the limitation of the individual techniques [1].

However, a gap remains between these non-invasive in vivo imaging techniques that typically offer dynamic information at the macroscopic level and ex vivo histological microscopic examinations of small tissue samples yielding incredible detail [3, 16]. To bridge this gap, intravital microscopy (IVM) has been developed. IVM is an optical imaging technique aimed at the visualization of biological processes in living animals at cellular and sub-cellular resolution [17–19]. Different from conventional histological microscopy, IVM allows to simultaneously collect both morphological and functional information, in a time-dependent manner in the complex environment of a living animal [20–22]. In the last decade, IVM has constantly evolved in combination with the development of several light microscopy techniques, including epifluorescence microscopy, spinning disc, confocal and two-photon microscopy. Advances in surgical strategies to expose and maintain the target tissue under physiological conditions, while minimizing motion artifacts due to respiration and heartbeat and the use of microscopes equipped with high-magnification optics and higher spatial and temporal resolution, significantly improved the quality of the acquisition [23–25]. The development of probe-based imaging systems, such as fibered confocal fluorescence microscopy (FCFM), has made it possible to access deeper sites in a minimally invasive way [26]. In addition, major advances have also been made in fluorescent labeling techniques, as well as the generation of animal models genetically modified to endogenously express multiple fluorescent reporters. In preclinical research, IVM has become important to address fundamental biological questions. Tracking interactions between the host and pathogens, studying drug delivery, monitoring cancer progression or understanding vaccine responses are just a few examples of the many possibilities [27–30]. In parallel to these developments, IVM has been implemented in human clinical settings as well. The idea to use confocal laser scanning to examine tissues without the need for tissue biopsy, referred to as ‘optical biopsy’, originated at the end of the eighties of the previous century. By now, probe-based confocal laser scanning endomicroscopy (pCLE) has found applications in digestive and respiratory medicine, urology and beyond [31–33].

This review gives an overview of the technological advancements in IVM including the main imaging modalities, the transition from imaging windows to fiber-optical probes and the use of fluorescent markers in both preclinical and clinical settings. In addition, the major benefits of IVM and what it can contribute in a multimodal imaging context are discussed. Furthermore, the review focuses on the implementation of FCFM in preclinical and pCLE in clinical research on gastrointestinal and lung diseases and the application in drug development. To conclude, the unexplored possibilities and potential of these techniques in other biomedical research fields are highlighted.

2. Technological advancements in IVM

2.1. From confocal to multi-photon microscopy (MPM)

Confocal and MPM are the most widely used modalities in IVM. In laser-scanning confocal microscopy, molecules absorb a single photon in the visible range of the light spectrum. Since excitation and emission occur in relatively large volumes around the focal plane, the out-of-focus emission is eliminated by the presence of a pinhole, thus allowing a 3D optical sectioning of the tissue [34]. However, the use of high energy single-photon excitation can result in photobleaching/phototoxicity and significantly limits light penetration in tissue (up to 50–60 µm, depending on tissue density and structure) [22]. In the last decade, the development of non-linear optical microscopy techniques, including MPM, led to a strong increase of IVM applications in different fields, including immunology and cancer [19, 20, 35]. In MPM, fluorescent molecules are excited upon the simultaneous absorption of two or multiple photons: since the total energy of the two or three photons required for the molecule excitation equals the energy required in single-photon illumination, the laser wavelength used for two- or three-photon microscopy is approximately two or three times longer, respectively, than the one required for single-photon microscopy [36]. As a consequence, multi-photon excitation is provided by pulsed laser emitting light in the near-infrared or infrared spectrum: the longer wavelength and the lower energy source result in lower light scattering, less photobleaching and deeper tissue penetration (up to 300–500 µm, depending on tissue density and structure, and up to 1.5 mm
in brain) [37, 38]. In addition, since the photon density required for multi-photon excitation is obtained only in proximity of the focal point, no out-of-focus emission is detectable and the presence of a pinhole is not needed, resulting in a higher-speed tissue scanning [39]. Finally, the multi-photon absorption spectrum of several molecules is wider compared to single-photon excitation, allowing the illumination of multiple molecules with a single excitation wavelength [19]. Although multi-photon and confocal microscopy are the most widely used, other imaging modalities have been used successfully for intravital imaging. For example, innovative techniques such as a label-free multicolor optical surface tomography, fluorescence lifetime microscopy (FLIM), optical coherence tomography and widefield fluorescence or spinning disk microscopy allow a very fast image acquisition and are suitable when deep imaging is not required [24, 40, 41].

2.2. The development and improvement of imaging windows
To gain imaging access to deep tissues, imaging sites need to be surgically exposed. However, due to its invasiveness, a surgical approach is not suitable for long-term repetitive imaging, even when the animal’s vital functions are tightly measured and controlled. To prolong imaging over days or months, imaging windows have been developed to expose tissues to microscopic analysis, while at the same time covering and protecting them, allowing the animals to recover from anesthesia between different imaging sessions [23]. These imaging windows are a valuable tool to gain access to tissues of interest such as the intestines, lungs and brain. The first surgically implanted imaging windows for the digestive tract were focused on the small intestines [42], since imaging of the colon appeared to be more challenging because of the distension and sensitivity to obstruction. Recently, a successful imaging window was developed based on a three-dimensional printed titanium platform and an implanted ferromagnetic scaffold (figure 1(A)) [43]. This allowed stabilization of the colon and imaging of the same location in the organ over two weeks, which in turn allowed imaging of luminal content, immune cells, epithelial cells and bacteria. Such technological advances are extremely useful for gaining further insight into cellular interactions in diseases of the lower gastro-intestinal tract. On an important note, for all in vivo procedures and in particular for longitudinal experiments, all surgical aspects including post-operative care must be considered. In addition, the surgical procedure and the insertion of an imaging window could influence the tissue of interest.

Imaging windows were also developed to study the intact lungs [44, 46–49]. The fact that the lungs are in constant motion was a major hurdle and many methods were tested to address this challenge [50]. The most successful was the development of a vacuum stabilized imaging window [47, 48]. The first vacuum stabilized imaging windows were mostly limited up to 6 to 12 h of imaging because of the high invasiveness of these surgeries (figure 1(B)) [44]. This limitation and the accompanying artifacts such as compression of blood vessels made it impossible to image metastatic sites in lung tumor research over days or weeks [46]. Recently, a minimally invasive window to study the murine lungs was developed using a transparent ribcage, closing the thoracic cavity and protecting the lungs. This allowed the recovery of the mouse, thereby extending imaging to a few days or weeks [46]. To obtain access to the brain for imaging, three main types of optical windows were developed: a closed cranial window, a thinned skull cranial window and an open skull window [29]. The brain was imaged through the intact skull in a closed window leading to only minimal invasiveness and low complexity. However, penetration depth and resolution are limited [51, 52]. A more invasive open skull window allowed a large imaging area and higher penetration depth; however, this was accompanied by an inflammatory response which requires the need for immunosuppression [53, 54]. To overcome these limitations, the thinned skull cranial window was developed in which the skull remained intact but was thinned to improve the resolution and imaging depth [55]. This has been successfully applied to study neuronal networks, the intact cortex and synaptic connections [56–58]. In addition, interactions between the host and pathogens, such as Plasmodium species in the brain, have also been of major interest for IVM [29, 59]. The combination of reporter mice with transgenic parasites, such as Toxoplasma gondii, showed the invasion and replication of the pathogen in endothelial cells in the brain vasculature [60]. IVM of the heart in animal models is highly affected by the rapid contraction of the heart which leads to a low image resolution. Therefore, motion compensation and tissue stabilization are mostly obtained by surgical exposure of the heart. However, the nature of this surgery only allows imaging up to two hours and often induces tissue damage [61]. A new technique for motion compensation was introduced by combining mechanical stabilization and cardiorespiratory gated acquisitions [62]. In this way, the beating murine heart was imaged for up to 4 h over several days without tissue damage. The migration of exogenously administered GFP-labeled cells, isolated from the bone marrow of donor mice, could be tracked in a ischemia reperfusion injury mouse model [62].

2.3. From imaging windows to fiber-optical probes
A non- or minimally invasive alternative for imaging windows to gain access to the tissue of interest are fiber-optical probes [26]. In this tool, the microscope objective is replaced by a thin-diametered flexible
Figure 1. Examples of IVM in preclinical applications. (A) Imaging of immune cell activation using multiphoton IVM in a murine colitis model via a colonic window. Innate dendritic cells and inactivated monocytes (green) activated monocytes (blue) and the vasculature (red) were imaged in the same location one day (left panel) and three days (right panel) after treatment with dextran sodium sulfate [43]. (B) Multiphoton IVM was used to image the lungs up to 12 h via a vacuum stabilized imaging window. Mice were injected intravascularly with fluorescently labeled high molecular weight dextran (red), to label the vascular lumen of the lung [44]. (C) Probe-based confocal endomicroscopy of labeled colonic mucosal vasculature (white arrow) in mice immediately (left panel) and 10 min (right panel) after fluorescein isothiocyanate (FITC)-dextran administration [45]. (D) Bronchoscopic FCFM was used to image Aspergillus fumigatus fungal lung infection. Mice were infected with green fluorescent protein (GFP)-expressing A. fumigatus (green) and imaged by inserting the probe via the mouth and trachea into the lungs [3]. Figure 1(A) is adapted from Rakhilin and colleagues and is licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/) [43]. Figure 1(B) originates from Rodriguez-Tirado et al [44]. Figure 1(C) originates from Mielke and colleagues and is licensed under the Creative Commons CC-BY license [45]. Figure 1(D) is adapted from Vanherp and colleagues and is licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/) [3].

Fiber-optic bundle which allows direct access of sites deep inside the animal [23, 63]. These fiber-optic bundles range in diameter size from about 100 µm up to several millimeters. Different types of fiber-optical probes have been developed such as flexible endoscopes to image hollow cavities and micro-endoscopes for minimally-invasive imaging within deep tissues [64, 65]. The main areas of application are limited to the digestive and respiratory tract; however, probe-based imaging has also been applied in preclinical investigations of the bladder, pancreas, liver and the male and female genital tract [66–68]. 'pCLE techniques, referred to as FCFM in the preclinical context,' permit the study of inflammation and tumor development in individual animals over time [28, 69].

Because these fiber-optic microscopic bundles fit inside the working channel of an endoscope, pCLE was developed for clinical settings. This allows, for example, high quality imaging of the gastro-intestinal mucosa to detect cellular or vascular changes upon gastric cancer with endoscopic procedures [31]. In clinical endoscopy, devices that can be used in combination with medical endoscopes have been developed, giving rise to so-called ‘in vivo endomicroscopy’ [70]. A first modality of the technique is ‘probe-based’ using a semi-flexible probe with a cross-sectional diameter of 1.2 mm that can be introduced through the working channel of a flexible bronchoscope. The probe consists of 30 000 small optical fibers that guide the laser emission, enabling real-time video imaging (optical area 1.13 mm², frame rate 12 images per second, lateral resolution 3 µm, depth of focus of 0–50 µm). By guiding the laser beam in an alternating manner through every single optical fiber consecutively, laser scanning is performed at the tip of the probe. As a result of excitation, the generated signal is then guided back up through the same fibers and confocal microscopy is processed in an external unit, referred to as ‘proximal scanning’. In an alternative ‘endoscope-based’ method, the scanning and microscopy itself is performed in the tip of the endoscope, limiting the amount of artifact and image distortion that is caused by the guidance through the long optical fibers of the probe-based version. This method of ‘distal scanning’ has a better lateral resolution (<1 µm) and a depth of focus reaching 250 µm, enabling high-end three-dimensional imaging. A major drawback of this technique is its
relatively large size and hence restricted use to digestive endoscopy [71]. Apart from applications for imaging of the digestive tract [72], pCLE of the respiratory tract (proximal scanning) was available as of 2008 and is capable of three-dimensional imaging during flexible bronchoscopy [32]. The technique uses a thin, semi-flexible probe similar to what is used in digestive endoscopy. This probe is inserted through the working channel of a bronchoscope to be advanced within the human airways. However, the optical characteristics of respiratory mucosa differ from the digestive tract and hence the clinical implementation of pCLE in the respiratory tract is less obvious.

2.4. Improvement of the signal-to-noise ratio

In order to implement pCLE in a broad clinical context, some technical hurdles should still be overcome. During recent years, efforts have been made to optimize the technical specifications of the probe-based systems and to provide better resolution in a compact system at lower production costs. Signal-to-noise ratio plays an essential role when using pCLE in image acquisition, analysis and interpretation in clinical settings. pCLE imaging generates noise as a result of several factors. First of all, the laser light is focused at a selected depth in the tissue of interest and the fluorescent signal of the tissue is directed back towards the system where it is focused by the same lens. The tissue signal is scattered during its ascend through the optical fibers, resulting in noise but as a result of the pinhole, the scattered component of the tissue signal is excluded from detection. The pinhole is able to optimize the signal-to-noise ratio; however, this decreases the strength of the tissue signal. A second factor limiting the resolution of confocal microscopy is the relative low photon count in fluorescence microscopy leading to a lower signal-to-noise ratio. This is compensated through the use of more sensitive photodetectors or through the increase in the intensity of illumination source, potentially bleaching the specimen of interest, which is a third cause of noise especially important when comparing fluorescence intensity between different experiments [73]. A fourth factor responsible for signal noise is the fact that the pCLE probe is composed of a fiber bundle containing thousands of separate fibers. Fiber-to-fiber differences in transmission, the number of fibers and the space between adjacent fibers result in an inhomogeneous honeycomb-like fiber-pattern and determine both the field-of-view and the resolution. Finally, signal noise originates from the practical implementation of the pCLE technique. The lack of or the inconsistency in contact of the fiber with the tissue of interest blurs the image. A similar effect results from ‘biofouling’, i.e. the soiling of the probe tip with mucus, fluid or micro-organisms [73]. When the probe is moved through the airways, the spatial movement throughout a recorded sequence in relation to the temporal rate of acquisition is characterized by a significant variability, caused by respiratory movements of the patient and the variable speed at which the probe is travelling through the airways. This result in motion artifacts, e.g. deformed anatomical structures, discontinuity in image acquisition, uninformative frames. To partially amend all these factors of signal noise and to overcome problems caused by image distortion, image computing algorithms on raw pCLE data, hardware and software improvements have been extensively developed. The implementation of these algorithms should result in the automatic segregation of uninformative frames and frames with adequate structural information [73]. Kernel methods and more specifically support vector machines are used for machine learning and data mining. Such methods can be used to implement computerized pattern recognition, which has already been explored as a means to enhance the diagnostic performance of pCLE [74]. Through the use of these mathematical models, healthy from diseased individuals and smoking from non-smoking subjects could be discriminated [75].

2.5. The innovation of fluorescent markers

Fluorescence imaging in vivo requires tissues, cells or molecules of interest to be fluorescently labeled for detection. The labeling should provide a sufficient long-term and stable signal, without causing tissue toxicity. Exogenous fluorescent dyes are chemicals which interact with specific cellular components and are used for in vitro staining of cells or pathogens and subsequent injection or for in vivo staining through coupling of target moieties. There is a wide variety of colors available and they are easy to use, however, possible toxicity and dilution of these dyes upon cell division do not allow long-term in vivo imaging [76]. Examples of fluorescent dyes used for intravital imaging are carboxyfluorescin succinimidyl ester, cell tracker orange and BODIPY [77, 78]. In addition to exogenous fluorophores, several molecules, such as retinol, riboflavin, indoleamines, nicotinamide adenine dinucleotide (NADH) and flavin adenin dinucleotide (FAD), can be visualized in a label-free modality, taking advantage of their intrinsic autofluorescence. In particular, the autofluorescence generated by NADH and FAD can be imaged by two-photon microscopy and correlated with cellular metabolic activity in vivo [79]. More recently, other non-linear optical imaging techniques have been generated, including second and third harmonic generation (SHG and THG respectively). In SHG and THG, photons interact with tissue and recombine into photons...
having half/third the incident wavelength, allowing the label-free visualization of several structures, such as collagen and muscle myosin (by SHG) or myelin and lipids (by THG) [80, 81]. These label-free techniques are usually combined with the detection of exogenous fluorophores, increasing the number of molecules and structure that can be imaged by IVM [82]. In addition, FLIM has been successfully applied to IVM. FLIM measures the time that a fluorophore spends in its excited state before emitting a photon: since fluorophore lifetime is influenced by environmental parameters, including temperature, pH or the presence of a quencher, FLIM offers the possibility to provide dynamic information on a specific cellular process that cannot be elucidated by the analysis of the fluorescence intensity alone [83]. In particular, the intrinsic lifetime of NADH and FAD has been related to changes in cellular metabolism: NAD(P)H lifetime is significantly shorter in the free state of the molecule, compared to the protein-bound state. The opposite has been observed for FAD, showing longer lifetime in free state compared to protein-bound state [83–85]. More recently, measurement of intrinsic NADH lifetime has been used to visualize changes in the anaerobic glycolysis associated with regions of acidic pH and hypoxia in a model of wound healing and cancer. In addition, several exogenous FLIM-based probes, with the ability to change their lifetime based on the environmental conditions have been generated [86].

The development of genetically encoded fluorescent reporters, in which the fluorescent protein is constitutively produced by the target cells, has made a lot of progress with the advances in molecular biology [87]. Advances in genome engineering, such as viral vector transduction and clustered regularly interspaced short palindromic repeat (CRISPR) technology, significantly facilitate the genetic introduction and endogenous expression of fluorescent proteins, thereby allowing long-term imaging throughout organ development [87, 88]. Genome engineering is often used for the in vivo imaging of infectious diseases by genetically engineering pathogens such as bacteria and fungi to express a fluorescent reporter gene [89]. This allows the localization and proliferation of pathogens and the visualization of the interaction with host immune cells. For in vivo imaging of host factors, transgenic animals engineered to express genetically labeled host cells, and in particular immune cells, can be used. Rodents and zebrafish with expression of GFP in different cell lineages are now widely used for IVM experiments [76, 90]. For example, a mouse strain with GFP attached to a lysozyme which is specifically expressed in neutrophils and macrophages can be used for the visualization of these immune cells during inflammation [91]. However, the generation of transgenic animals takes time, even with the advancements in CRISPR technology, and is often associated with high costs. Therefore, a more versatile alternative is offered by activatable or smart fluorescent probes that are designed to only elicit the fluorescent signal after binding a specific target [92]. Compared to standard dyes, these smart probes have the advantage of a minimal background signal and therefore a high sensitivity [93].

The development of dyes and fluorescent proteins in both the visible and near-infrared range has made it possible to visualize multiple fluorophores with IVM techniques, opening up applications towards the simultaneous monitoring of different cell populations or processes. In an in vivo setting, the simultaneous excitation of all these fluorophores is crucial but not trivial [94]. Multicolor imaging is now available in both a multi-photon and confocal fluorescence microscopy approach [95]. Currently, up to two colors can be visualized in a FCFM set-up (figure 2), whereas more colors can be imaged with MPM. The ability to visualize multiple colors has a lot of potential in the imaging of polymicrobial infections or interactions between pathogens and cells of the host immune system [96].

2.6. From preclinical to clinical fluorescent markers

In contrast to the many possibilities of fluorescent labeling and unlimited use of transgenic cells genetically labeled with fluorescence reporter genes in preclinical settings, IVM in clinical settings is limited to the use of approved fluorescent dyes and contrast agents which can be administered for labeling specific target cells. The implementation of non-cytotoxic, fluorescent molecular markers that can be used topically or systemically and are designed to be directly detected by pCLE imaging, potentially heralds a new era in advanced imaging of the respiratory tract. The use of such fluorescent markers and other future developments could have a direct impact on the clinical diagnostic value of pCLE. Until now, no targeted probes for the in vivo use in humans have been developed, although several reports have been published on the development of fluorophore-labeled antibodies and nanoparticles in animal models [99–101]. Real-time and in vivo pCLE visualization of diseased areas during colonoscopy has been reported, using fluorescent antibodies against membrane-bound tumor necrosis factor in patients with Crohn's disease. In the most affected areas, the amount of fluorescent immune cells correlated with the probability of clinical response to adalimumab treatment [102]. Such spectacular reports suggest that a broader implementation of these fluorescent tracers in daily-life-endoscopy could only be a matter of time.

One of the most challenging technical adaptations will be the introduction of a platform to allow for in vivo multicolor imaging in humans. This technique will enable the use of multiple wavelengths for the
excitation of a single location. As a result, differential spectral analysis will provide more optical information from the different structural components of the examined tissue, eventually leading to an increase in diagnostic accuracy. Notwithstanding that, the exploitation of autofluorescence from tissues and macromolecules in combination with approved dyes for specific target labeling already opens up a plethora of relevant possibilities for IVM in the clinic.

3. Preclinical, clinical and translational applications of probe-based IVM

3.1. Applications in gastrointestinal diseases
To study diseases of the digestive tract, different genetic or chemically induced animal models were developed, for example, mouse models of inflammatory bowel disease or colorectal cancer [103]. In pre-clinical settings, pCLE techniques enable the detection of cancerous lesions or tumors without the need for a biopsy. This is performed easily via insertion of the probe into the rectum of mice or rats [45]. This technique has been successfully used to image yellow fluorescent protein-labeled epithelial cells of transgenic mice and the mucosal vasculature after administration of FITC-dextran over the course of colitis progression in mice (figure 1(C)) [45]. In addition, colitis-associated cancer was studied in mice using a confocal fiber-optic laser probe [28]. The probe was advanced through the working sheath of an endoscope and positioned on the area of interest. By obtaining microscopic images of the vasculature after injection of the fluorescence imaging agent IntegriSense it was revealed that upon inhibition of vascular endothelial growth factor (VEGF), the mucosal vasculature was more organized compared to the control groups, pointing out the importance of VEGF in tumor growth and colon cancer [28]. Furthermore, endomicroscopy has proved to be a valuable tool in the detection of physical or chemical damage to the colorectal epithelial barrier in a mouse model [104]. These injuries are dangerous for the translocation of pathogens, such as bacteria and viruses, from the lumen into the intestinal mucosa. pCLE has been optimized in pigs to evaluate the vasculature blood-flow by injection of fluorescein sodium which allows the imaging of blood vessels. With this technique, intestinal ischemia, i.e. a restriction in blood supply to intestinal tissues, could be detected [105]. The use of this technology found its way to clinical settings as well.
In clinical settings, pCLE was first introduced in gastroenterology. The use of confocal laser imaging during colonoscopy can predict the presence of dysplastic and neoplastic lesions with confident accuracy [106]. It can be used in combination with topical fluorophores like methylene-blue, acriflavine hydrochloride and cresyl violet. Alternatively, intravenous administration of fluorescein sodium and indocyanine green are used to analyze living cells in situ and in vivo. These nonspecific agents are used to induce contrast enhancement optimizing the optical aspects of the mucosal crypt structure, epithelial cells and connective tissue, thereby enabling virtual histology as a screening tool for neoplastic changes [106, 107]. As a result, the proximal scanning pCLE technique is able to diagnose dysplasia and Barrett’s esophagus, i.e. an abnormal change in the mucosal cells lining the esophagus by administration of fluorescein during esophagoscopy [108]. High-resolution white light endoscopy in combination with pCLE enhances the detection of neoplasia in patients with Barrett’s esophagus compared to stand-alone white light endoscopy [109]. A recent multicenter study compared pCLE with random tissue biopsy in Barrett’s detection [110]. Optical biopsy was able to detect more patients with intestinal metaplasia than tissue biopsy, showing a promising advance in the clinical implementation of pCLE. In 2019, criteria for the clinical grading of pCLE in Barrett’s esophagus were generated and validated showing good reproducibility and interobserver agreement [111]. pCLE has also been investigated in other gastroenterological disorders such as ulcerative colitis [112]. Changes in colonic mucosal blood flow in patients with ulcerative colitis were investigated using pCLE after administration of fluorescein sodium (figure 3(A)). Furthermore, needle-based instead of pCLE allows the probe to be advanced through a biopsy needle and has been used in order to reach anatomic locations beyond the wall of the digestive duct. Recently, this technique was used for more accurate diagnosis of pancreatic cysts and to provide an added value to ultrasound-guided fine-needle aspiration [75].
3.2. Applications in lung diseases

In the earliest preclinical investigations using probe-based imaging for the lungs, larger animals were used because the existing probes were too large in diameter for non-invasive imaging in small laboratory animals. IVM studies on acute lung injury or acute respiratory distress syndrome have been performed in porcine using the probe-based method [116]. Regional alveolar changes and the alveolar structure could be successfully imaged in this way. pCLE imaging also demonstrated that it is feasible to quantify the velocity of red blood cells within intrapulmonary capillaries after intravenous administration of FITC-dextran in pigs and assessed changes in pulmonary capillary blood flow induced by different ventilator regimens [117]. This technique helps to understand changes in pulmonary capillary perfusion in a minimally invasive approach. Later, fiber-optical probes were used in a preclinical model of acute lung injury in rats via an intratracheal catheter [99]. FCFM was used for intravital lung tumor detection in rabbits. In combination with CT-guided bronchoscopy, the probe could be directed to the site of interest [118–120]. FCFM is also used to study infectious diseases in the lungs. Fluorescent bacteria in the lungs were imaged using fibered micro-endoscopy after surgical exposure of the lungs and invasive pulmonary aspergillosis infections were studied in rats for the first time using FCFM [121]. For these studies, at first, thoracotomy was needed to surgically gain access to the lungs [101, 122]. The further miniaturization of fiber-optical probes allowed the insertion in natural openings such as the airways, to study pulmonary diseases. The next step was taken recently, when fungal lung infections were repeatedly imaged in vivo using FCFM in a non-invasive manner in mouse models of pulmonary aspergillosis and cryptococcosis (figure 1(D)) [3]. In addition, the use of a multimodal imaging approach in which FCFM was combined with MRI or CT allowed the visualization of lung lesions at both microscopic and macroscopic resolution in a mouse model of pulmonary aspergillosis [123].

In clinical settings, generation of the optical image mainly depends on the autofluorescence characteristics of elastic fibers situated beneath the basal membrane of the bronchial mucosa or at the level of the pulmonary acinus. Although variations in intensity are observed, spectral shapes were found to be similar on different bronchial sites in the same patient and even between different patients. In contrast to what is reported in the field of gastroenterology, currently CE-labeled and commercially available devices do not allow for the direct visualization of epithelial cells in the human airways without the addition of fluorophores [32]. However, differential cell count in broncho-alveolar lavage and ex vivo spectrometry showed that tobacco smoke particulates (tar) containing macropores appear highly fluorescent when excited at 488 nm during pCLE [124]. pCLE imaging with topical methylene-blue, which is absorbed by the bronchial lymphatics, and intravenous fluorescein were used to visualize bronchial epithelial cells at the level of the pulmonary acinus [125, 126]. In a pilot study concerning the use of pCLE in the detection of solitary pulmonary nodules (SPN), radial endobronchial ultrasound, virtual bronchoscopic navigation and transbronchial forceps biopsy were combined in order to correlate histopathology with pCLE imaging. This report described the feasibility and utility of pCLE in vivo micro-imaging of SPN using so called dual-band imaging, i.e. combining pCLE imaging with a laser source alternating at both 488 nm wavelength for elastin network and 660 nm wavelength for cellular imaging [127]. Furthermore, pCLE imaging was used in specific respiratory disorders. In emphysema, endomicroscopic morphology could be correlated with lung functional parameters of airway obstruction and hyperinflation [128, 129]. Salaün et al reported on the pCLE characteristics of amiodarone related lung injury and compared pCLE findings within cases of interstitial lung disease [130]. One case report describes the pCLE characteristics of invasive pulmonary aspergillosis (figure 3(B)) [113]. The pCLE images from this patient showed the destruction of the elastin network of the alveolar wall. In two cases, the correlation between histopathology and pCLE imaging of endobronchial hamartomas was described [131]. The endomicroscopic characteristics of pulmonary alveolar proteinosis were confirmed and the use of pCLE as a monitoring tool during whole-lung lavage therapy was proposed [132]. Feasibility and safety data show that the pCLE technique is perfectly safe for use through the airways when it is performed during flexible bronchoscopy in spontaneously breathing patients [133]. Inter- and intra-observer agreement was studied and seemed moderate to good for image brightness, poor for fiber thickness at the bronchiolar level and moderate for fiber thickness at the alveolar level [129, 134].

3.3. Applications in other diseases

For probe-based imaging of the brain in preclinical studies, the probe can be inserted stereotactically to image deep brain structures or can be introduced through the nasal cavity to study olfactory receptor neurons [135, 136]. In this way, neural degeneration and regeneration can be imaged in real time in vivo. Due to their flexibility and small diameter, the probes can be positioned precisely which is a major advantage to study neural structures and function in a living animal. pCLE is also able to visualize the striatum and neural
structures in humans and has been described to accommodate brain tumor surgery (figure 3(C)) [114, 135]. Microscopic imaging of the heart using FCFM in animal models provides additional detailed information on cardiac microstructural arrangements with high resolution [137, 138]. In this regard, heart surgery associated injuries of the nodal tissues were successfully imaged by FCFM [139]. These are interesting applications toward open heart surgery, however, most of these preclinical studies still rely on the excision of the heart.

In urology, the diagnostic accuracy of pCLE for the diagnosis of bladder cancer and interobserver agreement were found to be moderate [33]. In addition, the technique was already successfully used to guide transurethral surgery with providing in real-time bladder tumor histology (figure 3(D)) [115]. In ophthalmology, clinical applications of external confocal laser microscopy have been used for more than 20 years. They allow for the diagnosis of eye infections such as Acanthamoeba and deep fungal keratitis and they are used to measure the residual corneal bed thickness after laser keratolysis [140]. Scanning laser ophthalmoscopy is now broadly used to examine retinal abnormalities and allows for longitudinal follow-up [141]. In dermatology, confocal laser scanning microscopy is able to examine the dermal layers resulting in optical biopsy of some skin tumors like malignant melanoma with excellent correlation to histopathology [142, 143].

3.4. Applications in immunology and drug development

The rational design of vaccines or therapy with respectively optimal immunogenic or therapeutic potential requires profound understanding of immune activation by the pathogen or disease. To this end, IVM has been increasingly used to visualize dynamic interactions between host and pathogen or between immune cells in their natural environment [144]. For example, studies using IVM in bone marrow, lung, spleen, lymph nodes and skin have significantly contributed to our knowledge about neutrophil functioning, migration and interactions, and have disproved previous dogmas about neutrophils [145]. IVM is thus able to reveal unknown complexity levels of immune responses, enabling the development of more targeted therapies [145]. This was illustrated by performing intravital confocal microscopy on an exposed lung lobe in mice to identify an adhesion receptor for neutrophil recruitment in pulmonary capillaries after lipopolysaccharide challenge, uncovering a potential therapeutic target in neutrophil-driven lung inflammation such as in acute lung injury or acute respiratory distress syndrome [145, 146]. Spinning-disk IVM was also used in mice on the exposed liver to identify an intracellular niche of Methicillin-resistant *Staphylococcus aureus* (MRSA) in Kupffer cells which was not reached by conventionally administered vancomycin, allowing them to rationally design a liposomal formulation of vancomycin efficiently targeting intracellular MRSA [147, 148]. However, commonly used IVM techniques in mice such as multi-photon and confocal microscopy are invasive and not compatible with larger non-transgenic animals such as nonhuman primates which might be more relevant models towards vaccine development. Therefore, IVM of immune populations in nonhuman primates has been performed using FCFM [30]. FCFM was performed on the skin surface of nonhuman primates to non-invasively and longitudinally study the density of epidermal Langerhans cells after intradermal injection of fluorescently labeled antibodies (figure 4(A)). This method allowed to validate electroporation as an effective vaccine delivery system and adjuvant for intradermal DNA vaccines in macaques [30, 149]. In internal organs, such as the airways of baboons, bronchoscopy-coupled two-channel pCLE was used to visualize the interaction between GFP-producing *Bordetella pertussis* and fluorescently labeled antigen-presenting cells in the trachea (figures 4(B) and (C)), providing new insights in *B. pertussis* pathogenesis and enabling detailed testing of candidate vaccines [150]. FCFM and its endoscopic applications such as pCLE are thus also of interest in translational research on larger animals to test vaccines, therapy or therapeutic delivery systems.

Towards infectious diseases, when used in a multimodal imaging setting, FCFM can provide valuable and complementary readouts in addition to *in vivo* three-dimensional structural and functional information obtained from CT, MRI, positron emission tomography, single photon emission computed tomography or BLI, further expanding this multifaceted toolbox to study pathogenesis and treatment effect. The benefits of such a multimodal imaging approach have been illustrated in a mouse model of cryptococcosis. BLI could follow the spatial and temporal distribution of viable cryptococci throughout the body, allowing to quantitatively and qualitatively observe infection dynamics and response to treatment. MRI and CT were able to analyze tissue damage induced by the fungal infection, such as edema and inflammation, allowing to quantitatively monitor disease progression in the lung and brain [7, 8]. These anatomical techniques may
therefore be useful in showing disease prevention, for example after vaccine administration. In cryptococcomas, magnetic resonance spectroscopy was used to study fungal cell metabolism, leading to the identification of a quantitative biomarker for diagnosis and treatment follow-up, and FCFM visualized fungal density [136]. Moreover, organ- and whole-body imaging techniques could be helpful in identifying interesting sites at interesting time points for potential cellular and molecular events regarding pathogenesis or treatment effect, which can then be visualized using FCFM, thus closing the gap between longitudinal in vivo macroscopic and microscopic techniques. The implementation of IVM and especially FCFM in a multimodal imaging setting could be an added value in the rational design of vaccines and therapy with increased insight in their mechanism of action, possibly leading to earlier differentiation between drugs with or without clinical potential, increasing the success rates of phase I clinical trials [151–154].

4. Conclusion

All individual imaging modalities have their own advantages and drawbacks (table 1). Whole-body imaging techniques provide a large field of view but miss the cellular details. On the other hand, IVM can provide this cellular information with high resolution but in a very limited field of view. Therefore, multimodal imaging will be important to overcome the limitations of the individual modalities.

The development of MPM allowed for higher tissue penetration, the visualization of multiple colors, the detection of SHG and THG and at the same time less toxicity and photobleaching. However, it mostly requires invasive surgery and is associated with a lower resolution compared to confocal microscopy. Therefore, the implementation of fibered-optical probe-based approaches is a valuable non-invasive alternative to optical windows to interrogate deeper sites inside the body with cellular resolution, thereby closing the gap in the imaging toolbox for a plethora of basic biomedical research applications. Future translational research should provide guidance on how to develop imaging agents and optical probes that have multiple characteristics. They should be able to meet a specific clinical need within an existing diagnostic approach, should be easy to synthesize on a larger scale and meet high standards of safety and quality control. Moreover, their diagnostic accuracy should be validated in a large clinical cohort and in the end should facilitate or even enhance the current clinical diagnostic algorithms [155].

Figure 4. Examples of two-channel IVM in nonhuman primates. (A) In vivo FCFM of the skin in macaques, 2 h after intradermal injection of fluorescent monoclonal antibodies, visualizing anti-CD1a-AF488 labeled Langerhans cells at 488 nm (green) and anti-HLA-DR-F647 labeled Langerhans cells at 660 nm (red) [30]. (B) In vivo bronchoscopic pCLE image of the trachea in baboons infected with B. pertussis B1917-GFP, 2 h after topical application of anti-HLA-DR AF647 antibodies in the trachea, showing B1917-GFP bacteria at 488 nm (green) and anti-HLA-DR AF647 labeled antigen-presenting cells (red). (C) The pCLE probe (arrow) in the trachea as seen by the bronchoscope camera [150]. Figure 4(A) is adapted from Todorova and colleagues and is licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/) [30]. Figures 4(B) and (C) are adapted from Naninck and colleagues and are licensed under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/) [150].
| Table 1. Overview of in vivo imaging modalities. |
|-----------------|-----------------|-----------------|-----------------|
| **Modality** | **Principle** | **Strengths** | **Limitations** |
| Whole-body imaging | BLI | Enzymatic production of visible light | No need for external illumination source | Transgenic modification |
| | | | No background signal | Low resolution |
| | | | High specificity | Limited penetration |
| | | | | Substrate required |
| | Fluorescence imaging | Fluorescence | Multiple colors | Transgenic modification/fluorescent contrast agent |
| | | | | Autofluorescence |
| | | | | Phototoxicity |
| | | | | Limited penetration |
| Whole-body and organ imaging | MRI | Strong magnetic field | High spatial resolution | Slow acquisition |
| | | | High penetration depth | High cost |
| | | | Clinical applications | Contrast agent may be required |
| | CT | X-ray transmission | High spatial resolution | Low-dose radiation exposure |
| | | | Fast acquisition | Contrast agent may be required |
| | | | High penetration depth | |
| | IVM | Confocal | Fluorescence | Clinical applications |
| | | | High spatial resolution | Limited tissue penetration |
| | | | Multiple colors | Photobleaching |
| | | | FCFM: imaging deeper tissues | Phototoxicity |
| | | | pCLE: clinical applications | Out-of-focus background light |
| | MPM | Fluorescence | Better penetration | Lower resolution |
| | | | Less photobleaching | High cost |
| | | | Less phototoxicity | |
| | | | No out-of-focus emission | |
| | | | Multiple colors | |

BLI, bioluminescence imaging; MRI, magnetic resonance imaging; CT, computed tomography; FCFM, fibered confocal fluorescence microscopy; pCLE, probe-based confocal laser scanning endomicroscopy; MPM, multi-photon microscopy.

Data availability statement

No new data were created or analyzed in this study.

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