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Review Article: Modern Trends in Imaging II

Point-of-care pathology with miniature microscopes

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Abstract. Advances in optical designs are enabling the development of miniature microscopes that can examine tissue *in situ* for early anatomic and molecular indicators of disease, in real time, and at cellular resolution. These new devices will lead to major changes in how diseases are detected and managed, driving a shift from today's diagnostic paradigm of biopsy followed by histopathology and recommended therapy, to non-invasive point-of-care diagnosis with possible same-session definitive treatment. This shift may have major implications for the training requirements of future physicians to enable them to interpret real-time *in vivo* microscopic data, and will also shape the emerging fields of telepathology and telemedicine. Implementation of new technologies into clinical practice is a complex process that requires bridging gaps between clinicians, engineers and scientists. This article provides a forward-looking discussion of these issues, with a focus on malignant and pre-malignant lesions, by first highlighting some of the clinical areas where point-of-care *in vivo* microscopy could address unmet needs, and then by reviewing the technological challenges that are being addressed, or need to be addressed, for *in vivo* microscopy to become a standard clinical tool.

Keywords: *In vivo* microscopy, molecular imaging, biomedical optics, microendoscopy, endomicroscopy, optical sectioning, optical biopsy, biomarker, cancer, dysplasia, image-guided therapy, telepathology

1. Introduction

1.1. Rationale for in vivo microscopy

Microscopic observation of tissue specimens—prepared according to established protocols in histology for fixation, embedding, sectioning and staining—is regarded by the medical community as a highly reliable method for the diagnosis of disease and is the current standard of care. The science and art of histopathology is based upon the observation of deviations from normal cellular and nuclear morphology, cellular orientation (polarity) and density, as well as an assessment of overall tissue architecture and cellular composition. Immunohistochemistry and in situ hybridization enable high-resolution localization of protein or nucleic acid markers of disease, respectively, with reasonable specificity and sensitivity. While molecular testing in the absence of morphology, via PCR or flow cytometry, for example, also has value in the detection, diagnosis, and monitoring of human malignancy, morphologic visualization of

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human tissue will continue as the clinical standard for diagnosis of cancer and pre-cancerous conditions for the foreseeable future.

Despite its acceptance as the standard-of-care, there are limitations to conventional histopathologic diagnosis. First is the need to perform an invasive biopsy or resection procedure. Second is its dependence on the ability of the human observer (typically, the pathologist) to make reliable judgments, both as a single observer over time ("intra-observer variation") and between different observers ("inter-observer variation"). A third issue is the sampling of tissue itself, which depends on removing the appropriate tissue from the patient and then selecting and microscopically reviewing the most relevant portions of the biopsied material. A fourth concern relates to possible artifacts introduced during processing that can impair morphological and/or molecular interpretation. These artifacts include sample desiccation, shrinkage caused by fixation and embedding in non-aqueous materials, and differential loss or alteration of molecular constituents. In short, devitalized tissue sections mounted on glass slides provide pathologists with a limited two-dimensional cross-section of what was once living three-dimensional tissue.

In contrast, we will describe the potential advantages, challenges, and implications of what can be called "point-of-care pathology (POCP)," which consists of real-time morphologic examination, at the cellular level, of living tissues in their native context. Due to space constraints, we will not discuss non-image-based techniques, such as varieties of light-scattering characterization, or reflectance and fluorescence spectroscopy.

In situ examination circumvents the need for *ex vivo* tissue processing, a key source of variability in the era of molecular diagnostics. While *in situ* sampling error remains a potential problem, and *in vivo* molecular imaging has its own set of challenges, POCP has the potential to provide immediate assessment of disease status, improve diagnostic accuracy, guide tissue biopsies, and accelerate diagnostic and therapeutic processes. This development may even permit identification of new molecular and functional signatures of cancer. For example, real-time microscopic visualization of blood flow or the transport of contrast agents could provide early physiological evidence (functional biomarkers) of malignant transformation prior to the onset of altered morphology [1]. In addition, techniques that require no exogenous molecular probes—relying only on the intrinsic optical properties of tissue such as autofluorescence, light-scattering, or fluorescence lifetime—are highly sensitive to environmental properties such as hydration, pH, oxygenation, and electrolyte concentration, and are ideally performed *in situ* [2–6].

If exogenous agents are to be used for molecularly specific diagnosis, staging, and typing of diseases, they can be chosen from a large number of probes under development [7–9]. Advanced, activatable probes have the desirable property of only switching "on" (i.e., generating a signal) when they have reached their target, thereby improving signal-to-background ratios [7, 10–14]. In addition, optical imaging is ideally suited for the simultaneous detection of multiple probes, each emitting and/or absorbing light at a different wavelength [15]. These advanced agents can provide optical contrast and report on both the molecular content and environment of living tissue, and thus have the unique potential to advance the field of point-of-care pathology. However, given the enormous scope of this specific topic, including the challenges posed by obtaining regulatory approval, and the fact that such reagents have recently been reviewed elsewhere [7–13], we will not discuss them further.

1.2. Clinical paradigms

The simplest view of *in vivo* pathology is that the proceduralist (endoscopist, surgeon, or other) obtains diagnostic morphologic information in real time and relies only on the *in vivo* morphologic findings to make on-the-spot decisions about patient management and intervention. *In vivo* microscopy would thus move morphologic assessment from post-procedure to intra-procedure, fundamentally changing the interactions of the healthcare team with the patient. In the case of endoscopy, either the endoscopist (e.g., gastroenterologist or surgeon) and the pathologist would have to interact during the procedure in ways that do not impose delays, or the endoscopist would have to make her/his own assessments:

1) In the first instance, the endoscopist and the pathologist are independent practitioners. Short of having the pathologist in the procedure room, as has been practiced for rapid diagnosis of needle aspirations, this model demands a telepathology approach, whereby digital image data are transmitted to a remote location for processing and interpretation (Fig. 1) [16, 17]. In

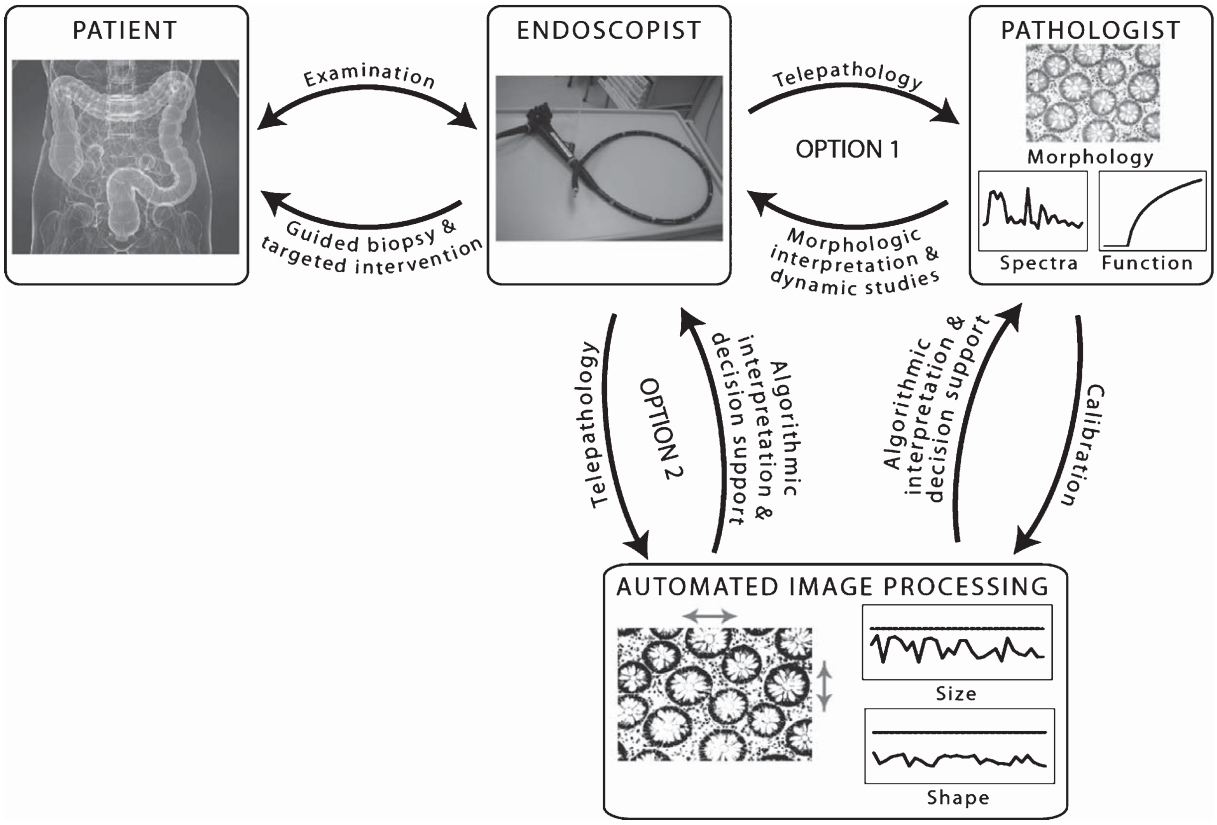

Fig. 1. An example of how point-of-care pathology, via endoscopic microscopy, may be incorporated into the clinical workflow for the detection of gastrointestinal diseases. Option 1 depicts the proceduralist and pathologist working as a team. Option 2 depicts the proceduralist working by her/himself, possibly with the aid of automated image processing tools. See text for details.

this scenario, the endoscopist must manage the patient during the procedure, utilize the *in vivo* microscopy instrumentation, and respond to interpretations from an off-site colleague. This first model provides for a dedicated and specialized pathologist reviewing the stream of morphologic data arising during the course of a procedure. This first model also has the advantage of a team of physicians – either a proceduralist and pathologist, or a team of off-site pathologists – conferring on a diagnosis.

2) In the second scenario, the endoscopist also serves as the on-site morphologist. This model assumes that there are sufficient advantages in having a single individual filling both roles. Three fundamental concerns arise: would the single individual have sufficient morphologic expertise; would that individual be able to apply that expertise competently in the midst of managing the patient during the procedure; and what quality-control review processes would be in place? Regardless of the model, medical management could be essentially instantaneous, including the decision to obtain a confirmatory tissue biopsy and/or to institute intra-procedural treatment. Under either scenario, a key disadvantage is that point-of-care pathology, and possibly intervention, would extend the duration of the procedure, exposing the patient to prolonged physiologic stress and extended anesthesia time, and reducing the productivity of the proceduralist and pathologist alike. This disadvantage would have to be outweighed by the value of real-time diagnosis and intervention, so that the patient would obtain more timely care and might avoid additional invasive procedures.

Major efforts are underway to develop automated image-processing algorithms for the analysis of digital pathology images, including those obtained on fresh tissues with miniature microendoscopes [18–20]. Such approaches have the benefit of being able to integrate multiple parameters, either contained within a given

image, or accessed across multiple imaging modalities (Fig. 1). Adding to the potential value of an automated evaluation, clinical and biochemical data can also be folded into decision-support algorithms [21]. Part of the motivation for these efforts is the concept that *in vivo* pathology may contain morphologic and functional information beyond what one individual trained in traditional morphologic methods can interpret. In addition, integrating multiple information sources quickly enough to guide intra-procedural decision making could be taxing even for two collaborating physicians (proceduralist and pathologist) on a case. With that in mind, it is worth noting that relevant real-time optical information obtained during the procedure should be captured for possible post-review, either as "snap-shots" and/or as a continuous digital record.

Regardless of clinical practice model, applications in which *in vivo* pathology could add value may be broadly categorized as either image-guided biopsy or image-guided therapy:

1) Image-guided biopsy is an obvious step towards transitioning from traditional histopathology to *in vivo* pathology, as it uses the latter to enhance the former rather than to replace it. An example application is screening patients with Barrett's esophagus, in which a geographic survey involving potentially dozens of random biopsies is currently used as a means to screen for the dysplastic transformations that precede esophageal cancer [22]. Beyond the fact that these "saturation" biopsies create a whole set of iatrogenic esophageal ulcers, cost concerns related to the reimbursement of multiple histopathologic tissue samples have also led to a pernicious issue: pooling of multiple tissue biopsies into sample jars. Such pooling reduces the opportunity to track the location of each biopsy. Identification through *in vivo* microscopy of tissue sites of greatest concern would lessen the need for a high number of physical biopsies and reduce the need for pooling of biopsy samples. *In vivo* microscopy would allow more sites to be noninvasively sampled in real time, thus reducing sampling errors. Lastly, image-guided biopsy would potentially draw advantage from both the real-time assessment of *in vivo* microscopy, and the reliability of follow-up histopathology.

2) Image-guided therapy (i.e., definitive treatment) currently relies on correlating pre-procedure MRI and CT images with findings inside the patient at the time of procedure. While the optimal spatial resolution of MRI and CT is on the millimeter scale, disease-to-normal contrast is only modest. Furthermore, significant spatial deformations often occur between the time of MRI and CT imaging and a procedure. Hence, the ability of such imaging to guide a therapeutic intervention is limited [21]. In contrast, *in vivo* microscopy has the potential to improve therapeutic/surgical accuracy in a direct fashion, through the real-time observation of cellular and subcellular morphology, function, and molecular expression, and the ability to more precisely obtain biopsy specimens if they are required.

However, it is important to consider the potential limitations of *in-vivo* microscopy when medical decisions are made in the absence of confirmatory histologic evaluation of tissue. For example, if the intra-procedural decision is not biopsy but immediate treatment (e.g., through localized freezing or heating), the imaged tissue may be obliterated. Intra-procedural diagnostic error may thus remain undetected, leading either to inappropriate or unnecessary therapeutic intervention, and/or the failure to follow a different management path of greater benefit to the patient. Even with the development of highly reliable and validated technologies for *in vivo* microscopy, rigorous quality management must be instituted, to ensure both appropriate use of the technology, and the competence of the healthcare practitioners (proceduralist and pathologist alike) in an ongoing fashion. Second, the inherent artifacts of histopathology, such as tissue shrinkage, are an almost unconscious part of the knowledge foundation for pathologists. Morphologic assessment of living tissues will require establishment of a new interpretive skill set, particularly since the artifacts introduced by devitalization and fixation will be absent. Until new image-to-disease correlations are established, there is potentially enhanced risk of interpretive error of *in vivo* microscopic images.

1.3. Bridging the gap between technology and practice

There is often a disconnect between the engineers and scientists who develop new medical technologies – and who are often largely technically driven – and clinical end-users, whose potential adoption of new methods will be guided by very different incentives. Therefore, for the remainder of this article, we seek to do two things. First, we attempt to survey and project forward, based on organ sites, particular applications that could benefit from point-of-care pathology. Second, we discuss the technical advances

in optical hardware that are needed to translate *in vivo* pathology into the clinic. Many of these advances leverage the application of lasers to various forms of imaging (and simultaneous treatment), and thus exploit novel or unfamiliar optical contrast mechanisms. Possibly the furthest along into the clinic is optical coherence tomography (OCT) and its relative, optical coherence microscopy [23, 24]. These reflectance microscopy techniques have overcome the engineer-clinician chasm, achieving enormous penetration in select areas. As they have been thoroughly reviewed in the literature, we will not discuss them in depth in this review. Photo-acoustic methods for deep-tissue imaging, which rely upon the detection of acoustic signals generated by optical absorption of brief laser pulses [25, 26], is another extremely promising technology being translated into clinical use, but will for the same reason not be covered here.

Additional novel microscope technologies being applied clinically, but not for *in vivo* use, are also worthy of mention. First, there are efforts to develop *ex vivo* three-dimensional pathology devices for rapid bedside microscopic imaging of fresh biopsy samples. Such devices could significantly improve clinical care, for example, by expediting the performance of Mohs micrographic resection of skin tumors [27, 28] or the detection of residual disease at the surgical margins of breast cancer patients [29]. Efforts are also ongoing to develop technologies for off-site pathology diagnostics, with the potential to influence global health challenges in resource-limited regions. Examples include the utilization of cell-phone cameras and mobile networks for diagnoses of diseases [30] such as malaria [31–33] and cervical cancer [34].

2. Specific clinical applications

Numerous academic and corporate research groups are advancing the field of *in vivo* microscopy to address point-of-care clinical needs, each with unique challenges in terms of imaging hardware, contrast agents, regulatory approval, integration of these technologies into the clinical and reimbursement workflow, and patient acceptance. Such potential applications can be roughly grouped into three categories: imaging of accessible surfaces, endoscopic imaging of hollow organs, and imaging during surgery or through incisions. Table 1 provides examples of each, along with a list of technical requirements that are relevant for each application. Note that ophthalmic applications have deliberately been left off of this list, as techniques such as scanning laser ophthalmoscopy (SLO) have enjoyed great success in the clinic and are not considered here as an "unmet need" but rather as a model for translating technologies to the clinic.

Common themes appear in Table 1 in terms of both clinical and technical needs. As summarized in the introduction, the predominant clinical paradigms include the detection of diseases, image-guided biopsy, and image-guided surgery or therapy. An additional aim is to monitor responses to therapy, and to track the recurrence of disease, through morphological and/or molecular imaging of targeted contrast agents.

A major technical challenge is the miniaturization of the devices to fit within small hollow organs, within endoscope instrument channels, trocars (for laparoscopy or core-needle biopsy), and within surgical cavities. As far as detection is concerned, large fields of view are always desired, either in individual image frames, or through the stitching together (mosaicing) of overlapping image frames. In certain cases, deep imaging depths may be desired – for example, to monitor the physical penetration of a topically applied chemical therapeutic, or to visualize the entire mucosal (epithelial) layer of a hollow organ. The technical challenges listed in Table 1 are being addressed in various ways, as described in the following section. Figures 2 through 4 show examples of images obtained in clinical and preclinical settings, from accessible organs (Fig. 2), hollow organs (Fig. 3), and during surgical procedures (Fig. 4).

3. Technical advances

Technical improvements specifically in microscopy-based approaches will be required to advance *in vivo* pathology, with requirements dictated by the geometric constraints imposed by the organ or tissue site, as well as the distinguishing characteristics of the disease targets. Important parameters include the dimensions of the optical head, the flexibility of the tether, the tissue imaging depth, the frame rate, spatial resolution, field of view, detection modality, and the capability to handle individual or multiple wavelengths. The following section outlines various design considerations along with some current and future approaches towards addressing unmet clinical needs. This section assumes a basic understanding of confocal and multiphoton

Table 1
Unmet clinical needs

Organ systems	Clinical applications	Technical requirements
Accessible organs e.g. skin, oropharynx	• Tumor margin detection (e.g. Mohs surgery) • Monitoring response to topical therapies (e.g. siRNA delivery) • Detection and staging of lesions	• Large fields of view • Multi-color imaging of contrast agents targeted to various disease biomarkers • Improved label-free methods for obtaining image contrast (e.g. reflectance and fluorescence spectroscopy, fluorescence lifetime imaging) • Large penetration depth for monitoring delivery of therapeutics • Ergonomic and flexible designs for ease of use
Hollow organs via endoscopy e.g. esophagus, stomach, rectum, intestine, ampulla of vater, uterine cervix, bladder, lungs	• Detection of dysplasia (pre-cancer) • Staging of lesions • Monitoring response to therapy (e.g. RF ablation, photodynamic therapy) • Image-guide biopsy and/or resection	• Endoscope-compatible design (miniaturization) • Large fields of view • Multi-color imaging of contrast agents targeted to various disease biomarkers • Improved label-free methods for obtaining image contrast • Large penetration depth for interrogating full-thickness mucosa (∼0.5 mm) • High frame rates for monitoring physiological processes (e.g. blood flow or transport of contrast agents)
Surgically-accessed organs e.g. prostate, ovaries, muscle, bone, breast, brain	• Detection and staging of disease • Image-guided biopsy • Image-guided resection at the margins	• Laparoscope-compatible design • Large fields of view • Multi-color imaging of contrast agents targeted to various disease biomarkers • Methods for minimally invasive insertion • Improved label-free methods for obtaining image contrast (e.g. autofluorescence, second-harmonic generation) • Incorporation with surgical-positioning robotics • Integration with therapy tools (e.g. suction catheters, photodynamic therapy)

microscope technologies, as described in numerous introductory and review articles [35–41]. While multiphoton approaches have been popular for pre-clinical investigations, confocal approaches have been pursued more aggressively for clinical applications. This is in part due to the fact that confocal designs tend to be simpler, utilizing inexpensive low-power lasers as opposed to the high-peak-power pulsed lasers and dispersion-compensating optics and fibers required for multiphoton-based systems.

3.1. Resolution, contrast, and imaging depth

In terms of *in vivo* pathology, the specific application will dictate the requirement for spatial resolution. For example, if detecting nuclear atypia is required, then achieving sub-cellular resolution will be important. Alternatively, if molecular imaging of cell-surface proteins with targeted reagents is the goal, then cellular-level resolution may be sufficient. As with resolution, image contrast is a critical variable to consider.

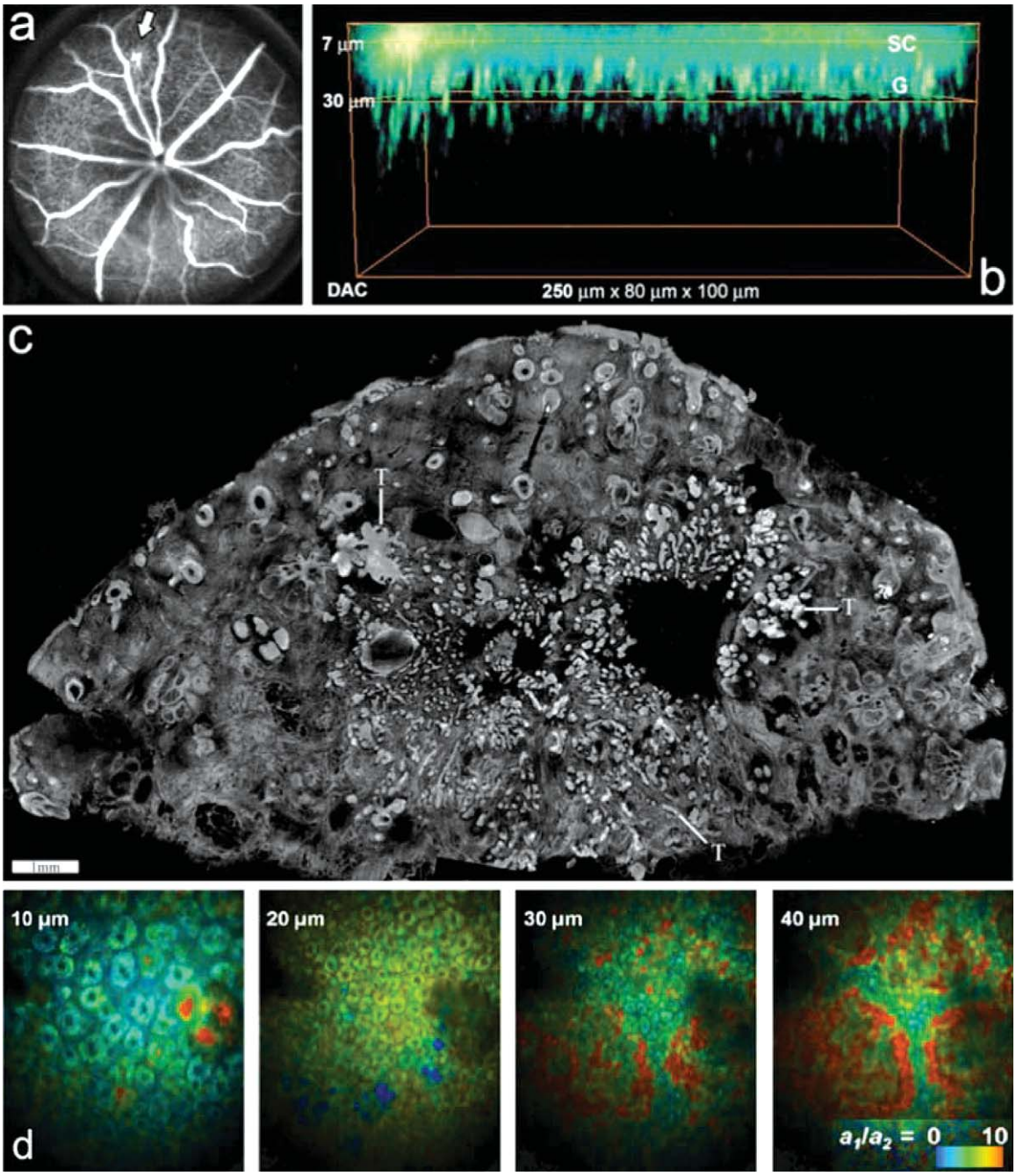

Fig. 2. a: Neovascularization in a mouse model (with fluorescein angiography). Image taken *in vivo* with a Heidelberg Engineering HRA I scanning laser ophthalmoscope. Arrow points to a well-defined lesion [110]. b: Microscopic imaging of the skin of a transgenic mouse model with GFP expression in the footpad. *In vivo* image taken with 10-mm diameter, dual-axis confocal (DAC) microscope [111]. c: Confocal mosaic of a micronodular basal cell carcinoma (BCC) obtained *ex vivo*. Individual images (430 by 430-µm field of view) were combined to create a large image of excised tissue with a 10- to 20-mm field of view. Small tumor nests (T) are shown as bright spots. Fluorescence emission detected from acridine orange-stained nuclei using a VivaScope 2000 by Lucid, Inc. Scale bar indicates 1 mm [112]. d: Multiphoton microcopy of intrinsic fluorescence contrast from healthy human skin at various depths. The false coloring shows the ratios of the amplitudes (a_1/a_2) of the double-exponential fluorescence lifetime decay, a presumed indicator of metabolism [6].

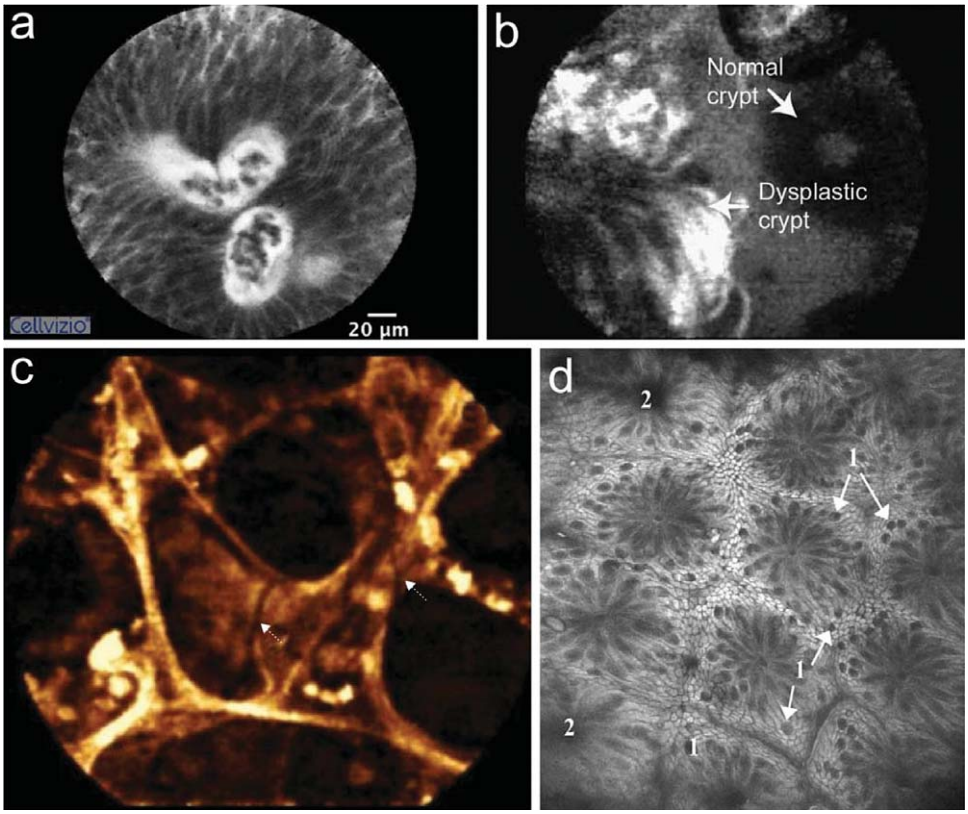

Fig. 3. a: Endomicroscopic image of a low-grade papillary tumor in a human bladder taken with a fibered confocal microscope (Mauna Kea Technologies) consisting of a 2.6 mm diameter flexible probe. Laser illumination was transmitted through an imaging bundle of more than 30,000 optical fibers. The image was taken at a depth of 60 μm with a 240-μm diameter FOV. Scale bar indicates 20 μm [113]. b: *In vivo* confocal fluorescence image of the border between colonic adenoma and normal mucosa, showing peptide binding to dysplastic colonocytes. Image taken in a human patient with the Cellvizio-GI imaging system by Mauna Kea Technologies, based on a fiber-bundle design [114]. c: Fibered confocal fluorescence microscopy imaging of the alveoli in the lungs of a smoker during bronchoscopy. A 1.4-mm diameter Cellvizio-Lung prototype was used (Mauna Kea Technologies), based on a fiber-bundle design. The field of view is 600 μm in diameter. Scale bar represents 50 μm [115]. d: Confocal image of the subsurface microvasculature of normal, descending human colon, collected *in vivo* following intravenous injection of sodium fluorescein. The confocal microscope, developed jointly by Pentax and Optiscan, is permanently integrated into an endoscope and utilizes a single fiber that is mechanically scanned with piezoelectrics. FOV = 500 × 500 microns [116].

Reflectance imaging often provides limited contrast, since only structures with large differences in refractive index compared to the surrounding medium, such as cell membranes and nuclei, can be easily visualized. Fluorescence imaging of exogenously applied fluorophores can generate enhanced contrast of selected targets, provided that they bind specifically to those targets and with high affinity, and that unbound probes in the background are effectively cleared, or compensated for [42]. As mentioned previously, activatable probes that emit optical signals only when they bind to or find themselves in the target region have great potential for increasing achievable signal-to-background [7, 10–14]. However, in general, enthusiasm for exogenous labeling approaches have to be tempered by considerations of cost and time required to achieve FDA approval and the difficulties of obtaining adequate return on investment for their developers.

In certain clinical applications, imaging below the surface may be necessary. For example, in tumor resection, molecular reagents may label subsurface cells more accurately than surface cells that have been surgically perturbed. Another example would be to visualize dysplastic changes throughout the epithelial layer of organs such as the colon, where the mucosa is 500 μm thick. Multiphoton microscopy, in particular,

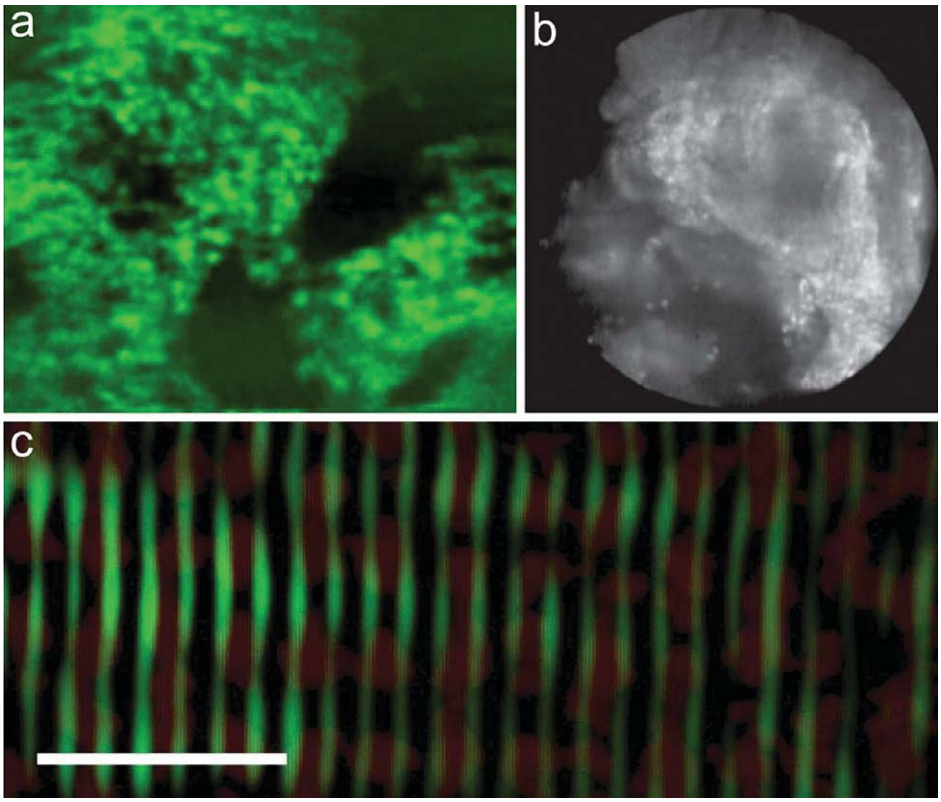

Fig. 4. a: *In vivo* image of GFP-expressing medulloblastoma in a mouse taken by a miniature dual-axis confocal microscope designed to guide brain tumor resection (image depth is 30 μm). The design features a biaxial scanning MEMS mirror and a 2-mm diameter GRIN relay lens [46]. b: *In vivo* images depicting epithelial cells that have an abnormal appearance, diagnosed as serous cystadenoma. FOV is 450 microns. This image was taken with a confocal microscope designed into a laparoscopic device [117]. c: Image of a cultured mouse muscle fiber with two-photon autofluorescence (red) and second-harmonic generation (green) from sarcomeres. A GRIN relay lens was used to access subsurface muscle. Scale bar indicates 10 μm [61].

has demonstrated an ability to image relatively deeply in a variety of tissues types. Where deep optical sectioning is not required, however, conventional confocal microscopy has been utilized to acquire high-contrast microscopic images near the tissue surface (<100 μm deep). A variant of conventional confocal microscopy that utilizes low-NA lenses to image in both reflectance and fluorescence modes is termed dual-axis confocal (DAC) microscopy. This technique has demonstrated improved optical sectioning with penetration depths approaching that of multiphoton microscopy [43–45]. The DAC architecture can enable laser-scanned imaging over relatively large fields of view with the use of biaxial-scanning MEMS mirrors [46–48]. An alternative approach for imaging at shallow depths is to use structured illumination to distinguish between signal generated at the focal plane vs. signal generated elsewhere [49–53]. This is a full-field imaging approach that does not require beam scanning and is thus able to achieve high frame rates, albeit at limited imaging depths in tissues due to issues related to dynamic range and shot noise.

Table 2 and Fig. 5 summarize some of the major approaches for achieving optical sectioning in tissues.

3.2. Approaches towards miniaturization

The greatest constraint in the design of *in vivo* microscopes is size. Optical-sectioning microscopes, which are generally necessary for high-contrast high-resolution cellular imaging in thick tissues, are mature technologies as embodied in large bench-top laboratory instruments. However, adaptation of

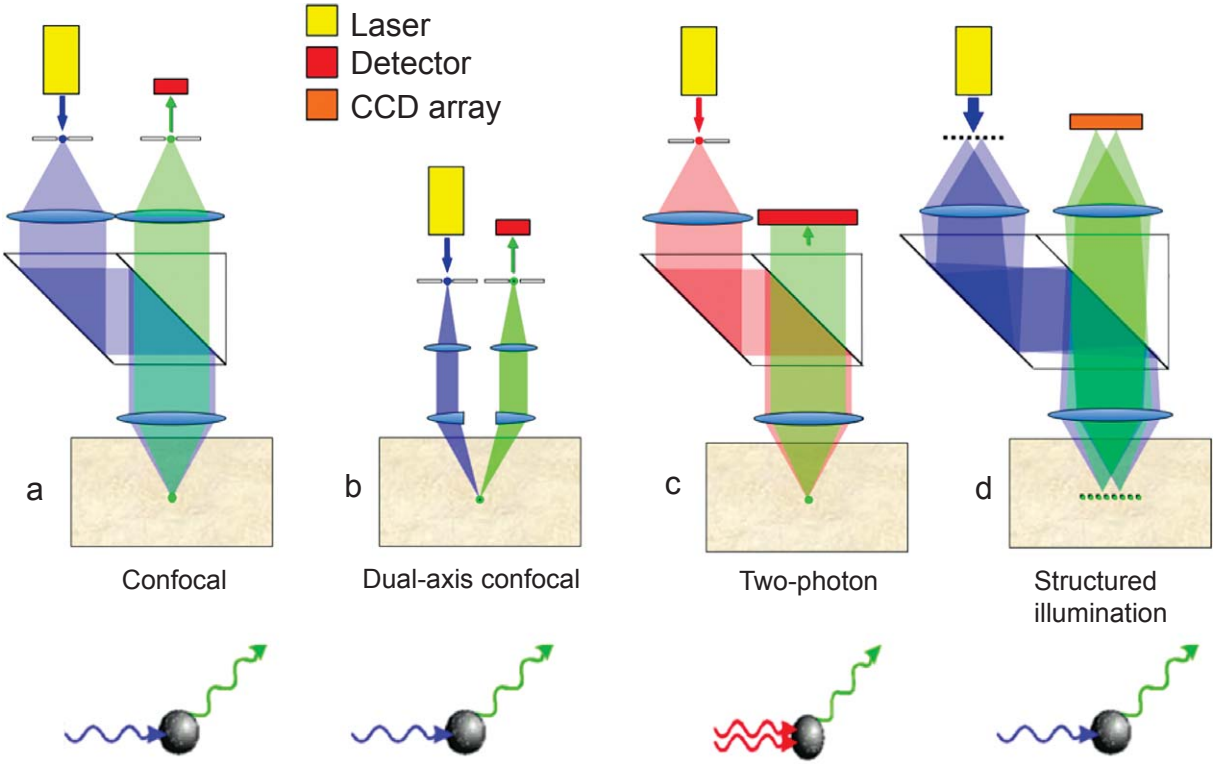

Fig. 5. a: The conventional confocal microscope, using a high-NA (0.8–1.4) objective and two pinholes to provide a point source and point detector. Images are collected by scanning the focal spot within the specimen in either reflectance or fluorescence (shown) modes. Excitation light (blue) is absorbed by the fluorescent molecules in the specimen, and fluorescence emission (green) is collected by the point detector. b: The dual-axis confocal microscope, using two low-NA (0.1–0.2) objective lenses and two pinholes to provide a point source and point detector. The excitation (blue) and emission (green) beams are spatially separated within the specimen, except where they overlap at the focus. c: The multiphoton microscope using a high-NA (0.8–1.4) objective and a single pinhole to provide a point source. The red beam represents the long-wavelength excitation light (high-peak-power pulsed laser), which is non-linearly absorbed by the fluorescent molecules in the specimen. The efficiency of two-photon absorption, for example, scales as the square of the intensity of the light, and is therefore confined to just the focus of the excitation beam. The green beam represents the shorter-wavelength fluorescence emission light collected by a large area detector. d: The structured illumination microscope can be used to gather full-field (single-shot) 2D images by a photodetector array (CCD) in either reflectance or fluorescence (shown) modes. The images are quasi-optically-sectioned due to the fact that the structured pattern is only imaged with high contrast at the focal plane within the specimen. By spatially modulating the illumination pattern, out-of-focus (low-contrast) light may be distinguished from, and digitally filtered away from the in-focus light that is modulated with high contrast. The multiple blue beams represent a pattern of excitation light beams emanating from a structured light source, which is absorbed by the fluorescent molecules in the specimen. The respective green beams represent the fluorescence emission light collected by the CCD.

optical-sectioning microscopes for use in clinical settings has necessitated creative approaches towards miniaturization. In general, to achieve high spatial resolution in microscopy, light must be focused sharply into specimens using high-numerical-aperture (NA) objectives, which are engineered to enable aberration-free imaging over a field of view (FOV). Multi-element compound miniature optics have been developed, using conventional glass lenses as well as injection-molded aspheric lenses, to enable uniform telecentric imaging [54–56]. Gradient-index (GRIN) optics, which are cylindrical components with small diameters on the order of 1 mm, have also been utilized for confocal endomicroscopy and multiphoton imaging but are currently limited in NA to about 0.6 [46, 57–62]. Hybrid designs utilizing conventional optics and GRIN lenses have been developed with larger NAs of up to 0.8 [63], enabling both greater light collection and higher spatial resolution, while still enabling a small overall probe size.

Table 2
Major microscope modalities

Approach	Pros	Cons
Multiphoton	High resolution and contrast; deep tissue penetration	Expensive laser source; slow point scanning; need for dispersion compensation
Structured illumination	Fast full-field imaging with a camera; inexpensive light source; no moving parts	Limited contrast and imaging depth in tissue due to high shot-noise and limited dynamic range of detector
Conventional confocal	Impressive miniaturization is possible through proximal scanning into fiber bundles; inexpensive light source; commercial devices exist	Limited contrast and imaging depth in tissue; high-NA optics lead to short working distances and increased potential for aberrations
Dual-axis confocal	Efficient rejection of out-of-focus scattered light for high contrast and deep tissue imaging; long working distance; relatively isotropic resolution	Low-NA optics limit sensitivity; alignment of dual beams may be challenging
Coherence gating	Efficient rejection of out-of-focus scattered light for high contrast and deep tissue reflectance imaging with a long working distance	Limited to detection of coherence reflected light; low-NA focusing limits lateral resolution
Non-sectioned	Epi-fluorescence imaging provides fast full-field imaging with a camera; inexpensive light source; no moving parts	Poor contrast and imaging depth in tissue (no optical sectioning)

In terms of packaging, a number of design approaches have been explored to bring miniature microscopes into the clinic, some of which rely on optical fiber components.

3.2.1. *Non-fibered approach*

One approach towards *in vivo* microscopy has been to retrofit large, non-fibered systems with long-working-distance objectives or miniature optics, such as GRIN lenses, for imaging in animals and humans. This approach, commonly termed intravital microscopy, has been instrumental in enabling a number of landmark studies in immunology, stem-cell biology, cancer, and other diseases [61, 64–68]. Surgically implanted window models have been developed in animals to provide long-term optical access to tissues; alternatively, miniature GRIN-lens microendoscopes have been inserted into deep tissues for long-term imaging studies. One recent application of intravital microscopy has been to perform line scans across individual vessels, or groups of blood vessels, to monitor the passage of fluorescently labeled cells over time [65, 69–71]. This "*in vivo* flow cytometry" technique has been used for multicolor investigations of various cell types, including circulating tumor cells, immune cells, and hematopoietic stem cells. The large size and reduced flexibility of these non-fiber-based approaches has made the clinical translation of such devices challenging, except for certain applications in which optical access is unrestricted, such as in the skin and eyes.

3.2.2. *Fiber-bundle vs. single-fiber approaches*

Two major forms of miniature flexible microscope designs have emerged: those that utilize an imaging fiber-bundle approach and those that utilize a single optical fiber for delivering laser illumination and for signal collection. Fiber bundles are useful in that the optical scanning mechanism can be located at the proximal end of the system and thus outside of the patient or animal subject. Each individual fiber in the bundle is imaged to a unique point within the sample. By illuminating and collecting light from one fiber at a time, a 2D image may be reconstructed. The fiber-bundle approach has the advantage of requiring few or no moving parts within the distal optical head, which allows for impressive miniaturization of the endomicroscope to sub-millimeter dimensions in some cases [1, 5, 54, 55, 72–77]. However, such devices generally image at a single depth, since axial scanning would require the addition of a linear actuator within the distal scan head to adjust the position of the focusing optics, thus limiting the extent of miniaturization [56, 78]. Several non-confocal versions of this technology have been developed for rapid full-field (non-scanned) camera-based imaging of tissue surfaces [5, 19, 79, 80]. This approach is a favorable choice if high-speed, compact, and inexpensive imaging of a tissue surface is preferred rather than high-contrast subsurface imaging.

In contrast, single-fiber approaches rely upon micro-optics and scanning mechanisms integrated into the distal optical head of the endomicroscope to enable 2D

Table 3
Approaches to miniaturization

Approach	Applications	Pros	Cons
Non-fibered	– Chronic intravital microscopy with window preparations – GRIN lenses inserted in tissue – *In vivo* flow cytometry	– Diffraction-limited and achromatic optics – Limited requirements for miniaturization	– Accessibility into the body is limited – Poor clinical portability
Fiber bundle	– Proximally scanned confocal microscopy – Nonconfocal, full-field microscopy of tissue surfaces with fast cameras	– Proximal scanning – High degree of miniaturization – Rapid full-field imaging is possible	– Fixed imaging depth at the surface or at one working distance – Fiber spacing may limit lateral resolution
Single fibers	– Distal scanning with MEMS mirrors and resonant mechanical scanners – Spectrally encoded scanning	– Diffraction-limited distal scanning; axial scanning may be possible	– Distal scanning limits the extent of miniaturization

or even 3D imaging. Two major strategies have been used to accomplish this: micro-electro-mechanical systems (MEMS) scanning mirrors [46, 48, 81–87], and mechanical fiber/optical component deflectors [88–92]. An alternative method for achieving distal scanning has been achieved in a miniature microscope through the use of spectral encoding, in which the wavelength components of a broadband light source are mapped along one dimension or two dimensions with a diffractive optical element such as a prism or grating [93–96]. This eliminates the need for scanning along those dimensions. However, it has been largely limited to reflectance imaging since fluorescence emission is broadband, where spatial positions cannot efficiently be encoded by wavelength components [79, 97].

Table 3 and Fig. 6 summarize some of the major approaches towards the miniaturization and scanning of *in vivo* microscopes.

3.3. Achromatic designs

The ability to simultaneously image at multiple wavelengths is extremely important in many applications. Multi-color fluorescence microscopy allows for the observation of interactions and correlations between labeled cells and tissue components [64–68, 98–101] as well as for the ratiometric quantification of image intensities [42, 102–104]. Color-corrected micro-objectives have been developed for *in vivo* devices [54–56]. Diffractive optical elements may also be used to compensate for the effects of chromatic dispersion and are being explored for the development of achromatic GRIN lenses [B. Messerschmidt, GRINTECH GmbH, personal communication] and microscope systems. Often, these approaches may require a sacrifice in diffraction-limited performance and resolution in order to achieve effective color correction.

3.4. Clinical deployment

Clinical considerations such as ease of use and ergonomics cannot be overlooked in the design of instruments for *in vivo* pathological assessment. As these technologies continue to mature, various refinements will be necessary to encourage clinical adoption, such as the integration of POC microscopes into existing devices. For example, in image-guided surgery applications, miniature microscopes could be integrated with therapeutic tools such as tumor suction catheters for co-localized image detection and treatment. In other cases, POC microscopes could be designed to be deployed through existing platforms, such as the instrument channels in conventional endoscopes and laparoscopes. Additional clinical considerations include the ease of sterilization, the lifetime of the device, and the availability of disposable components. Robotic-assisted surgery, although not yet shown to improve outcome, is maturing and is predicted to increase in clinical use. Integration of *in vivo* microscopes with surgical robots would be

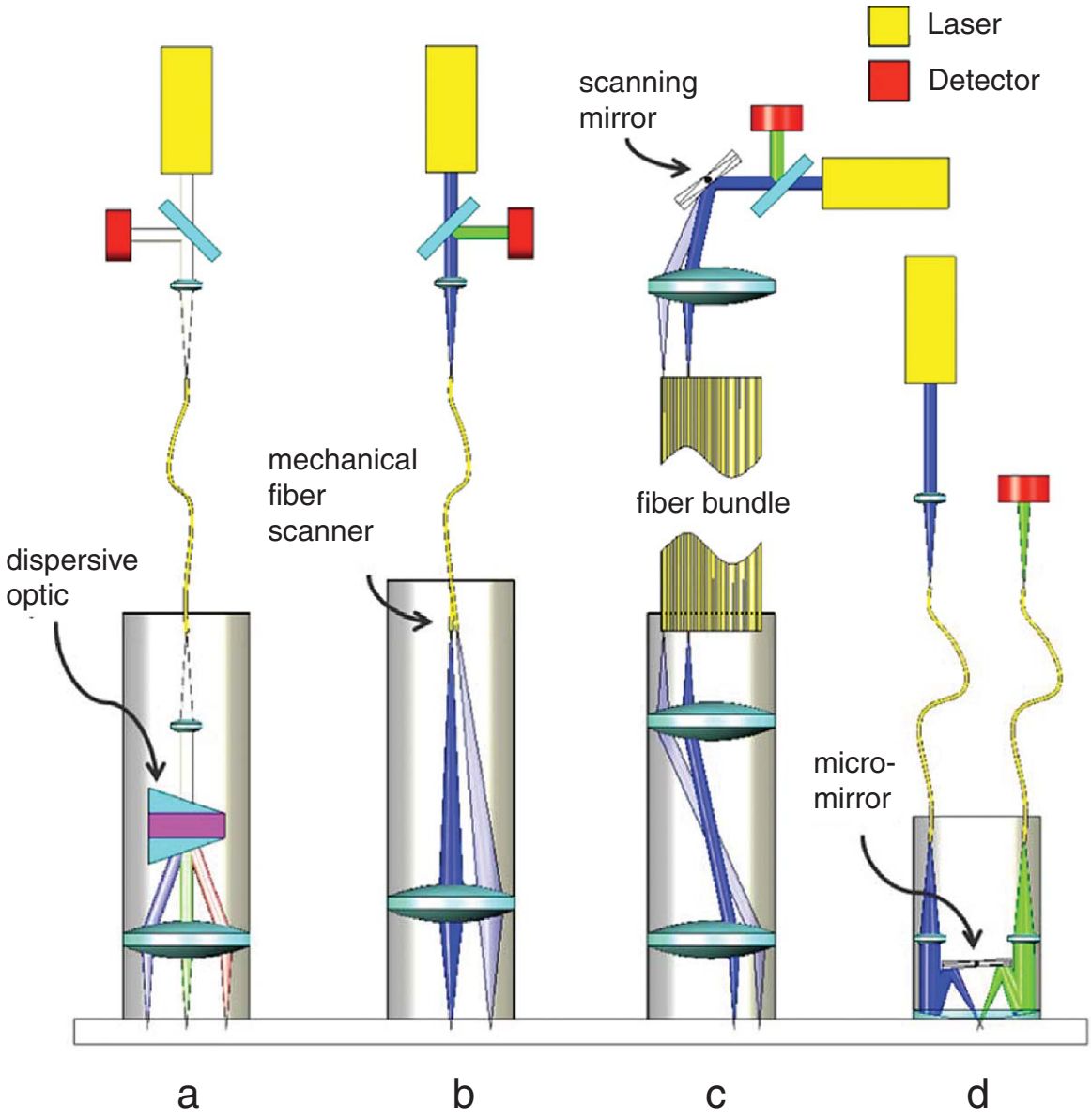

Fig. 6. a: The spectrally-encoded scanning mechanism disperses a broadband beam into multiple beams, based on wavelength, which are projected at different angles towards the objective lens, which in-turn produces a sequence of focused points along a linear path within the specimen. A line scan is achieved rapidly in one direction by either scanning the wavelength of the laser source, or by using a broadband light source. In the latter case, the reflected light that is collected must be analyzed with a spectrograph in order to decode spatial location from the wavelength components of the signal. b: The resonant fiber scanning mechanism uses the end of a single-mode fiber to serve as both the point source and point detector of a confocal microscope. This system can be used to gather an image by providing a mechanism for vibrating (deflecting) the end of the fiber in a resonant sinusoidal pattern to scan the focal spot within the specimen. The blue beam represents the excitation light, which is absorbed by the fluorescent molecules in the specimen. The green beam represents the fluorescence emission light collected by the end of the fiber and then directed to a detector. c: The fiber-bundle scanning mechanism uses the ends of multiple fibers to provide multiple point sources and point detectors for a large number of parallel confocal microscopes, each imaging a single point (pixel) within a 2D image. Beam scanning is performed on the proximal end of the fiber bundle (away from the patient) where each fiber is illuminated sequentially in time. d: A miniature dual-axis confocal microscope is depicted, where a fast electrostatically driven MEMS scanning mirror steers the excitation and emission beams along two orthogonal directions for 2D imaging. Two separate single-mode fibers act as a point source and point detector, respectively.

especially beneficial since motion-induced imaging artifacts could be greatly attenuated.

While the subject of software development is beyond the scope of this review, key components in the clinical translation of any *in vivo* imaging device will include optimizing what is presented to the proceduralist and pathologist (if involved), and the development of image-processing algorithms to assist in interpretation. Among the greatest challenges that will confront imaging scientists, particularly in biomedicine and regardless of modality or dimensional scale, will be to understand how best to combine the virtues of automated analysis with the power of a trained human visual observer. In a field as variable and complex as pathology, for instance, our goal, in the short- to medium-term, should not be to entirely replace human image interpretation, but rather to find ways to optimize the presentation of image data to reduce observer bias and to increase the accuracy and efficiency of analysis.

3.5. Translational considerations

Optical coherence tomography (OCT) is an example of the successful translation of a non-invasive optical technology to broad application in the medical practice market [105, 106]. This market extends beyond the field of ophthalmology into gastroenterology, dermatology, urology, otolaryngology, and others. Lessons learned from its successful implementation include: (a) approval by the Food and Drug Administration (FDA) for clinical utilization; (b) inclusion of the FDA-approved technology in Medicare and Medicaid reimbursement schedules; (c) ease of use and incorporation into the clinical workflow; (d) development of professional and technical expertise amongst healthcare providers; (e) development of a strong evidence base for successful clinical management; and (f) robust interest from the commercial sector in continued investment in this technology.

Any novel technique of *in situ* microscopy will encounter four major challenges [107]:

1) Analytical utility: does the technology measure what it claims to measure?
2) Clinical relevance: does the technology answer the clinical question being asked?
3) Clinical effectiveness: does use of the technology lead to better patient outcomes [108]?
4) Social value: does the technology meet ethical, economic, and legal standards?

Extensive effort is currently underway to answer the first three questions of the "validation" issue. The overall translational challenges are discussed by Wells et al. [109], who noted that, until primary evidence from the field can be developed, optical technologies must depend on histopathologic evaluation as a "gold standard" for the validation of analytical and clinical utility. Hence, the potentially flawed technology of histopathology remains as a validation portal through which optical technologies, including point-of-care pathology, must pass.

4. Summary

The field of *in vivo* pathology, enabled by advances in optics, sensors and electronics, is rapidly evolving and being translated into various clinic arenas. Since there is often a disconnect between the clinical end users and the engineers and scientists developing the core technologies, we have attempted to do two things: to outline some of the major clinical needs and scenarios for which *in vivo* pathology can be effective, as well as to summarize the major technical approaches and challenges in meeting those needs. Each specific clinical application will necessitate its own set of design choices that will require extensive communication between physicians and those developing the technologies. Since complex issues are not easily summarized, this article discusses a limited set of challenges, including the integration of these techniques into the clinical workflow, the use of contrast agents, regulatory approval, reimbursement, robotic aids, and an infrastructure for telepathology. Nonetheless, we hope that discussions such as this can provide a useful framework for progress along this exciting new frontier.

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