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ABSTRACT

Many bioimaging studies, including those in engineered tissue constructs, intravital microscopy in animal models, and medical imaging in humans, require cellular-resolution imaging of structures deep within a sample. Yet, many of the current approaches are limited in terms of resolution, but also in invasiveness, repeatable imaging of the same location, and accessible imaging depth. We coin the term micro-endomicroscope to describe the emerging class of small, cellular-resolution endoscopic imaging systems designed to image cells in situ while minimizing perturbation of the sample. In this Perspective, we motivate the need for further development of micro-endomicroscopes, highlighting applications that would greatly benefit, reviewing progress, and considering how photonics might contribute. We identify areas ripe for technological development, such as micro-scanners and small lens systems, that would advance micro-endomicroscope performance. With the right developments in photonics, many possibilities exist for new minimally invasive translatable imaging tools across the scientific, pre-clinical, and clinical spectrum: from longitudinal studies of engineered tissue constructs, to tracking disease progression in animal models, to expanding the ability to diagnose and develop treatments for diseases without the need for invasive medical procedures.

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I. INTRODUCTION

Laboratory studies are an important tool to understand the underlying mechanisms associated with biological processes, both homeostatic (i.e., normal) and pathological. Yet, many challenges exist in translating results from the laboratory to meaningful health outcomes. Laboratory studies are often highly artificial—isolating individual cell types or separating cells from their environment. While studying cells on their own has been proven highly informative, it is generally accepted that in these artificial environments, cells behave differently than they do in their native environment.1 The ability to address many biological questions, and applications that derive from answering them, will rely on the ability to observe cells in their unperturbed native environment. Understanding interactions between cells and their environment is a key aspect to understanding how changes at the cellular level influence changes we see at the larger length scales of tissues, organs, and whole organisms. In order to study this, we need cellular-resolution imaging tools that can be adapted to multiple scenarios along the translational imaging pipeline, from laboratory studies to human medical imaging, so
that we can correlate what scientists see in the laboratory with what doctors see in their patients.

Two important developments in this translational progression are the ability to create highly complex, three-dimensional (3D), tissue-mimicking environments and engineered tissues for in vitro cell studies and intravital microscopy, which refers to imaging of cells in situ in a living animal model (often longitudinally, i.e., at the same location for multiple time points). Both of these developments have opened up new possibilities of studying cells in their environment.\(^2\)–\(^7\) Growing cells in 3D scaffolds has reduced the gap between two-dimensional plate-based cell culture and tissue-based studies, enabling a better understanding of how cell–cell and cell–matrix interactions influence cell behavior and proliferation.\(^8\) Imaging in fresh excised tissues, which can provide highly representative information of in vivo biology, can also be supplemented using organotypic explant cultures, which extend the life of excised tissues for longer-term studies. Spheroid- and organoid-based systems (see the caption of Fig. 1 for definitions), in particular, have shown much potential because they can contain the same variety of cell types as, and display similar functionality to, a whole organ.\(^9\),\(^10\) Engineered tissues can build upon organoid research by incorporating further realistic features, such as vascularization and immune cells, enhancing their suitability for identifying cellular and molecular contributors to disease and providing a platform for studying treatments in human tissues without relying on excised samples.\(^11\)

While 3D model systems exhibit the geometry of live organisms, some components of the extracellular matrix and many signaling molecules are often missing. Thus, intravital microscopy remains an invaluable tool for studying biology and has enabled many advances including the following: observation of intercellular dynamics; functional imaging of immune response; and longitudinal studies of disease progression and drug efficacy.\(^2\),\(^12\)–\(^14\)

Many different imaging techniques have been used to access cellular-level information in 3D cultures and in vivo.\(^15\)–\(^17\) A major limitation of most is the limited penetration depth of image

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**FIG. 1.** A schematic overview of 3D cell culture types benefitting from micro-endomicroscopy. Each type of culture can be imaged by inserting a micro-endomicroscope into the sample. While fresh excised tissue is not typically considered a type of tissue culture, we include it here as a commonly used alternative approach that can benefit from probe-based imaging in the same manner as cultured tissue. Left: the primary types of 3D cell cultures. Multilayer cultures can be formed by plating multiple cell types in a 3D scaffold environment and can be useful models for epithelial tissues, in particular, and other layered structures. Engineered tissue constructs are complex organizations of cells arranged in a scaffold to mimic a biological tissue. Spheroids and organoids are both clumps of one or more cell types formed in a cell-culture platform under non-adherent conditions (i.e., cells will adhere only to other cells and not to the culturing environment). Spheroids comprise mainly tumor cells, whereas organoids are made from stem cells or organ progenitor cells that self-organize and function similarly to the original organ in a tissue culture environment. Organotypic explants are excised portions of an organ or tissue kept alive and intact in a cell culture environment. Compared with fresh excised tissue, explant cultures can be imaged over longer time periods. Observation of 3D tissue constructs throughout their growth represents a novel opportunity for the application of micro-probes. Structures can be formed and maintained either in a liquid culture or in a scaffold. Many scaffolds are available with different optical, mechanical, and functional properties to simulate different biological environments (right). Understanding the optical properties of scaffold materials is essential when designing imaging experiments; image formation is more challenging in fibrous and porous environments. The size of these structures can vary over several orders of magnitude based on the cell types used and the size and complexity of the overall structures. Spheroids and organoids typically range in diameter from \(\sim 100 \, \mu m\) to several millimeters. Engineered tissue constructs and explant cultures can be even larger depending on the model tissue. An example of an explant culture is a segment of human artery that could be centimeters long with walls several millimeters thick.\(^1\),\(^2\),\(^3\),\(^6\),\(^7\)
formation in tissues. Tissue is highly scattering, which makes it difficult to image more than a few hundred micrometers below any tissue surface. In many intravital microscopy-based studies, the animal subjects undergo surgery to implant a glass window or otherwise expose the relevant organ to avoid the challenge of imaging through the highly scattering skin.\textsuperscript{14} While highly informative, such studies can be limited in terms of invasiveness (windows are often more than 10 mm in diameter), their ability to image the same location over long periods, and the large number of animals required. Small endoscopes or fiber-optic probes can be used as an alternative, complementary approach. Such probes can and should be designed and optimized for specific applications in terms of size, resolution, imaging technique, and desired contrast. They can have small diameter (much smaller than most windows), user-defined length, and high mechanical flexibility, which facilitate the development of implantable and wearable probes to enable minimally invasive longitudinal imaging.\textsuperscript{8–21} Most importantly, such probes can be placed many millimeters to centimeters deep in tissues, thereby overcoming—in a practical way—the limitations of light scattering on high-resolution microscopy.

In most cases, such imaging probes have been classified as "endomicroscopes," probes, which have microscopic resolution but are not necessarily small, or "microendoscopes," which have a diameter of typically less than 1 mm but do not necessarily have microscopic resolution.\textsuperscript{22} If the aim is to build adaptable tools for longitudinal studies in cell and tissue cultures, animal models, and humans, a micro-endomicroscope—an imaging probe that is both small (which we define as having a diameter of 0.5 mm or less—as we justify in Sec. II B) and images with cellular resolution—is required. For convenience here, we define cellular resolution as 2 μm or better in at least two of the three spatial dimensions. While many imaging probes developed to date satisfy one of these criteria, as we catalog in Sec. III D, only a small number of imaging probes simultaneously satisfy both these criteria, and their adoption has thus far been limited due, mainly, to technical challenges impeding their development. In this Perspective, building on recent reviews of endoscope technologies,\textsuperscript{22–26} we will discuss progress and future directions in the development of micro-endomicroscopes. With further development, micro-endomicroscopes promise the following: imaging deep inside 3D constructs and tissue samples to observe cells in their native environment without confounding boundary effects; a new suite of experiments including much less invasive imaging in animal models and humans; and opportunities for longitudinal imaging in animal models below the surface of organs, including as long-term wearable imaging probes.

The achievement of that further development, in maintaining a small probe size without sacrificing imaging resolution or contrast, presents a variety of interesting technical challenges. Traditional lenses are limited in terms of the link between light collection and lens diameter. Advances in optical design, including the use of metamaterials, novel fiber designs, and 3D-printed or freeform optics, will be required to push the boundaries of what is achievable. Furthermore, the current size of micro-scanners is limiting miniaturization, particularly for probes designed for volumetric imaging. New advances in nano-3D-printed scanner devices would enable more compact and versatile micro-endomicroscope designs.

This article is organized in two main sections. In Sec. II, we will motivate the need for minimally invasive, cellular-resolution longitudinal imaging deep in tissues—for which we have coined the term micro-endomicroscopy. The emphasis will be on implantable/in situ imaging probes; we will allude to "from the surface" approaches for comparison but will not focus on them. In Sec. III, we will discuss the challenges and trade-offs in the design of small, cellular-resolution probes. We will briefly highlight existing probe designs and their performance based on confocal fluorescence and nonlinear imaging, optical coherence tomography (OCT), and Raman spectroscopic imaging. Each of these imaging techniques has unique design requirements and considerations for minimizing the probe diameter. Finally, we will highlight several opportunities for further research to advance the design and implementation of micro-endomicroscopes throughout the translational imaging pipeline. Through this article, we hope to promote new applications of photonic materials and device technologies in biomedical research, as well as identify opportunities for technological advancement that will bring us closer to versatile, translational micro-endomicroscopy.

II. THE NEED FOR MICRO-ENDOMICROSCOPES

We first consider the wide range of opportunities for micro-endomicroscopes spread across three stages of the translational imaging pipeline, from minimally invasive laboratory studies in cellular and tissue constructs, to preclinical imaging in animal models, and finally to clinical imaging in humans. We then examine in more detail the justification for less than 0.5-mm diameter probe size in minimizing invasion, the resolution required (sub-2 μm), and how contrast affects this.

A. Micro-endomicroscopy in engineered tissues, in animals, and in humans

The idea of “optical biopsy,” i.e., the ability to diagnose diseases entirely using optical imaging and without the need to excise any tissue, has been a goal of the photonics community for more than 20 years\textsuperscript{27,28}—enabling cellular-resolution in vivo optical imaging is a crucial part of this goal. Compared with medical imaging techniques, such as magnetic resonance imaging and ultrasonography, one of the main limitations of optical imaging is the shallow penetration of image formation in tissues. Depending on the tissue type, light propagating more than a few hundred micrometers into scattering tissue has undergone severe wavefront aberration and multiple scattering events that make image formation problematic.\textsuperscript{29} Such challenges are being tackled by a large community of imaging scientists, but the basic problem has yet to be overcome.\textsuperscript{30–32} Meanwhile, imaging micro-probes, frequently based on fiber optics, allow us to examine deeper structures than would be accessible from the tissue surface while minimizing aberrations, illumination power, and perturbation of the sample.\textsuperscript{22,24} The effectiveness of imaging micro-probes has been demonstrated somewhat, particularly for intravital imaging in the mouse brain, in some explant tissue cultures and, more recently, for clinical imaging in humans, which we describe below. Many of these probes use gradient-index (GRIN) rod lenses that exceed 0.5 mm in diameter. Very few imaging probes have been developed, which satisfy our criteria to be classified as micro-endomicroscopes (maximum outer diameter of 0.5 mm and resolution of 2 μm or
better). Although delivering the micro-endomicroscope to the site of imaging may be invasive, it is less invasive than the alternatives: implantation of windows, which may or may not be feasible depending on the location and depth; surgical exposure; or excision. Micro-endomicroscopes will take us a step closer to the goal of imaging cells in their unperturbed, native environment, and utilizing such tools in multiple imaging applications—from engineered tissues, to intravital microscopy, and to imaging in humans—will better connect investigations at these different stages. Below, we briefly consider the opportunities presented by each stage, in turn, noting the state of the art in microscopy, the limited extent to which micro-endomicroscopy has been conducted to date, and the opportunities that micro-endomicroscopy might offer in the future.

1. **3D tissue constructs, engineered tissues, and fresh excised tissues**

First, we consider the opportunities for imaging with micro-endomicroscopes in 3D tissue constructs, engineered tissues, and fresh excised tissues. Biological microscopy, practiced with sophisticated laboratory benchtop microscopes on thin layers of cells or tissue on glass slides, has provided us with exquisite resolution and functionality, down to the molecular level. However, cells exist in native 3D environments, and there is now a good deal of evidence showing that cells behave differently in 2D and 3D environments. Thus, research is transitioning from 2D imaging on slides to 3D spheroids, organoids, and engineered tissues. An overview of each of these techniques is presented in Fig. 1. With the move toward 3D tissue constructs and larger excised tissue samples comes the need to image within such samples. One motivation (relevant to fresh, cultured, or engineered tissues) is to observe cells sufficiently far away from artificial surfaces and boundaries to avoid artifacts. This requires the use of larger tissue constructs (such as spheroids and organoids, which can be larger than 1 mm in diameter) and is, thus, very likely to extend to the regime where cellular-resolution imaging cannot be achieved throughout the entire sample; therefore, imaging would be facilitated by the use of probes. Scaffolds for engineered tissues can be even larger—on the order of several millimeters—with specialized internal regions designed to support the growth of particular cell types. Imaging tissue constructs throughout their growth also represents a novel opportunity for imaging with micro-probes. Currently, time-lapse studies of large, engineered tissue constructs require freezing and sectioning at several time points. Probes can be implanted within the growth medium to facilitate repeated imaging of the same location over multiple imaging sessions within the same sample. To our knowledge, this type of study has not yet been demonstrated. Particularly for engineered tissue constructs that model disease progression, micro-probes represent a novel approach for longitudinal monitoring.

Increasingly, 3D tissue constructs of the types shown in Fig. 1 are being used to improve early validation of drug efficacy. Drug discovery is a lengthy and costly process that requires many stages of experimentation, validation, and clinical trials before a drug can be approved for use. The earliest stages of research include high-throughput screening to identify candidate drugs. Traditionally, these studies are performed in 2D cell cultures that do not accurately represent the complex microenvironment encountered in a patient; this has been found to be a significant contributor to the low percentage of clinically trialed drugs that actually make it to approval. Using micro-endomicroscopes could facilitate testing in engineered tissue constructs that more closely mimic the patient microenvironment and enable longitudinal monitoring of drug activity over time. If primary cells from a patient are used to engineer tissues and organoids, an added benefit is the ability to design and study personalized therapies based on the clinical phenotype of a particular patient. Monitoring drug efficacy in such cultures could enable doctors to identify the best treatment without subjecting patients to unnecessary risk.

2. **Intravital microscopy and animal models**

Next, we will discuss the progress and opportunities for utilizing micro-endomicroscopes in intravital microscopy. Many preclinical studies of cells using animal models rely on ex vivo imaging of excised tissue samples from animals sacrificed at each stage of the study. The introduction of several techniques enabling longitudinal imaging of the same animal (including implantable imaging windows and imaging probes) has removed many disadvantages of studies involving multiple animals. Utilization of imaging windows has enabled cellular-resolution imaging in vivo in animal models and has had a huge impact on our understanding of tissue-level dynamics and disease progression, as indicated in the range of representative images in Fig. 2. Yet, if imaging below the surface of organs is desirable, window-based studies alone are insufficient; micro-probes can provide a complementary approach to enable less invasive imaging below the organ surface. Imaging micro-probes are already a valuable part of the toolbox for intravital microscopy, particularly for imaging the brain. Yet many opportunities exist to improve their performance, expand their use to new imaging targets, and enable access to hard-to-reach areas, such as inside blood vessels, alveoli in the lungs, and crypts in the gastrointestinal (GI) tract. Many of these targets cannot be imaged with an implantable window, so an imaging probe is the only way to access them in a living animal. Figure 2 provides organ dimensions to indicate the depth required to access some of these targets using intravital microscopy. To date, most intravital microscopy studies using endomicroscopes have relied on large diameter (greater than 1 mm) devices (with the exception of studies in the brain) or do not achieve image quality on par with window-based measurements. Many further opportunities exist for exploring less-invasive deep-tissue imaging using micro-endomicroscopes—in particular, with the aim of achieving image quality and resolution comparable with those achieved using implantable windows while minimizing perturbation of the imaging target.

3. **Clinical imaging in humans**

Opportunities for micro-endomicroscopy in human medical imaging with cellular resolution are centered on diagnosis, guidance of surgical procedures, and connection to preclinical studies. Although probe-based imaging may or may not be less invasive than surgery, it potentially performs diagnosis in situ, informs or guides biopsies to improve targeting (diagnostic yield), or aids in surgical guidance. The ability to visualize cells has been shown to provide a versatile capability, albeit with the level of invasion limited by the large size of endoscopes, rarely as small as 1 mm in diameter. The smallest commercially available endoscope for imaging in...
humans is currently the Cellvizio AQ-Flex 19 (Mauna Kea Technologies, Paris, France), a 0.85-mm fiber bundle mounted inside a 19-gauge needle with a total outer diameter of 1.067 mm.\textsuperscript{56–58} Imaging probes designed for use in humans can be classified into two groups based upon their delivery method: flexible endoscopes for imaging in hollow organs and rigid needle probes for imaging in solid tissue. While some flexible endoscopes are in common clinical use, they are typically several millimeters in diameter and, thus, are reserved for larger hollow organs, such as the colon and esophagus. Many new opportunities exist for the development of smaller flexible probes targeting smaller hollow organs, such as ducts, alveoli, arterioles, and lymphatic vessels. For example, the GI tract is already a primary target for imaging probes, but current approaches could be supplemented with higher resolution imaging options. In particular, clinicians could benefit from the ability to monitor epithelial neoplasia \textit{in vivo}. Even within hollow organs, some areas can require specialized micro-probes to access sub-structures, such as intestinal crypts.\textsuperscript{29} The base of the crypts contains immune cells and stem cells; imaging within the crypts has implications for better understanding of GI-related autoimmune disorders, such as Crohn’s disease and colitis. Imaging the stem cells within the crypts, the “stem cell niche,” could help build a better understanding of how adult stem cells differentiate and has implications in the development of organoid cultures.\textsuperscript{60} Other applications, including imaging muscles and imaging in the brain, require the ability to deliver light several millimeters into solid tissue in order to avoid or guide invasive biopsies. Some of these novel targets and the imaging depth required to reach them are highlighted in Fig. 3.\textsuperscript{60,61} One example is tumor grading; observation of whether epithelial cell growth has penetrated the basement membrane is a criterion for determining malignancy and treatment strategy.\textsuperscript{62} The cellular architecture at the basement membrane cannot be easily visualized with existing tools, particularly in the many hard-to-reach areas, such as the oral cavity and bile ducts in the pancreas. Many other diseases can be tracked by observing variations in the thickness of tissue layers and other morphological changes: early atherosclerosis diagnosis can be aided by monitoring of the tunica intima thickness in arteries;\textsuperscript{63} morphological changes in muscle can aid diagnosis of neuromuscular disorders;\textsuperscript{64} variations in the thickness of neocortical layers can have implications for the development of Alzheimer’s disease.\textsuperscript{65} Nuclei in the brain—dense clusters of neurons in the deep, non-cortical regions of the cerebral hemispheres—also represent a novel imaging target, only accessible by probes, which can aid in the study of how the neural structure affects behavior.
FIG. 3. Schematic diagrams of some targets of interest for micro-endomicroscopy in humans, indicating structures of primary interest and imaging depth required.\(^6\) The diagrams are not to the same scale, but sizes are indicated, where available. (a) The artery wall comprises three distinct layers. The tunica intima, the innermost layer, is composed of a single layer of endothelial cells on top of connective tissue and an internal elastic lamina. The tunica media is composed of smooth muscle cells, elastic tissue, and collagen arranged in periodic lamellae and makes up the majority of the vessel wall.\(^6\) (b) The neocortex in the brain is composed of periodic neocortical columns. These columns can be divided into six layers based on the cell types and neuronal connections present. The proportional thicknesses of the layers and the overall thickness of the neocortex vary between different brain regions.\(^6\) (c) The skin is composed of three layers. The outermost layer, the stratum corneum, is composed mainly of corneocytes—a type of non-living cell. It varies greatly in thickness across different parts of the body and is thickest on the palms of the hands and soles of the feet. The epidermis is composed of stratified epithelial cells (keratinocytes) and is sometimes further divided into three sublayers based upon the differentiation of keratinocytes: the granular layer, the spinous layer, and the basal layer. The basal layer is a single layer of cells directly attached to the basement membrane and also contains melanocytes (depicted in brown). The dermis is composed of two layers, the papillary dermis and the reticular dermis. Both layers are composed mainly of connective tissue, but the reticular dermis is denser and contains more elastic and reticular fibers. Hair follicles, sweat glands, nerves, blood vessels, and lymphatic vessels are all contained within the dermis. Between the skin and muscle is a subcutaneous layer of connective tissue composed of the hypodermis (also known as the superficial fascia) and the deep fascia. The hypodermis is a layer of loose connective tissue composed mostly of adipocytes. The deep fascia is a highly fibrous layer of dense connective tissue that surrounds all muscles and organs. Imaging through the skin will be required for many wearable probes as well as imaging of capillary networks and muscle. (d) The wall of the gastrointestinal tract can be divided into six layers. The innermost layer, the epithelium, is mainly cellular. In most regions of the gastrointestinal tract (excluding the esophagus, pharynx, and anus), the epithelium consists of a single cell layer. The innermost layer, the epithelium, is mainly cellular. In most regions of the gastrointestinal tract (excluding the esophagus, pharynx, and anus), the epithelium consists of a single cell layer. The lamina propria is a layer of loose connective tissue containing fibroblasts (green) and several types of cells related to the immune system (blue). The muscularis mucosae is a thin layer of smooth muscle. These three layers are sometimes collectively referred to as the mucosa. The submucosa is a layer of dense irregular connective tissue containing nerves, blood vessels, and lymphatic vessels. The muscularis externa is composed of two layers of smooth muscle. The inner layer is oriented with the muscle fibers around the circumference of the gastrointestinal tract, and the outer layer is oriented longitudinally. The serosa is a layer of connective tissue and adipose that surrounds the organs. The basement membrane [green arrow in (c) and (d)] is an important imaging target since epithelial invasion of the basement membrane is a marker of tumor malignancy.\(^6\)

B. Invasiveness: Reducing probe diameter reduces perturbation of the sample

In Sec. II A, we made the case that the use of imaging micro-probes for less invasive imaging deep in biological samples could have widespread application. However, the probe diameter and means of access (needle, instrument channel, hollow organ, etc.) must be considered when evaluating invasiveness. From a basic science standpoint, minimizing perturbation of the sample is crucial for probing real life without distorting the result through the process of measurement. For intravital microscopy and imaging in humans with needle-based imaging micro-probes, smaller probes minimize excessive tissue trauma. Especially for short-term studies, one issue is damage caused by insertion. The tip geometry and insertion rate of the needle have an impact on the direction and magnitude of tissue displacement and potential damage.\(^6\) A sharp-tipped needle has been shown to require 2–3 times less pressure than a blunt-tipped needle to penetrate a tissue of the same thickness, will create a smaller crack for the needle to pass through, and will deform a smaller area of tissue. Faster insertion rates are associated with less direct puncture damage but are more susceptible to friction; thus, the optimal insertion rate must be determined based on the sample geometry and mechanical properties.\(^6\) For permanent or
semi-permanent implantation of imaging probes, any probe must be small and light. For probes designed for use in hollow organs, the probe must be sufficiently small and flexible to fit within the lumen of the target organ without distorting the tissue structure.

When determining the appropriate size of an imaging probe, several factors must be considered. First, for a needle-based probe, how does the size of the probe compare with the size of the structures being imaged? The probe tip and surface area must be small compared with the imaging target to minimize perturbation of the sample. For a probe designed for imaging in a hollow organ, how does the probe diameter compare with the lumen diameter? A second factor is the required imaging resolution: since numerical aperture (NA) scales with lens diameter, reducing the overall probe diameter limits the maximum resolution achievable when using refractive lenses. Thus, for conventional linear optics, the approximate resolution requirement $\frac{\lambda}{NA}$ determines the minimum probe diameter. This will be discussed further in Sec. III B. Finally, the probe durability and mechanical strength (which scales with the area) must be balanced against invasiveness and weight.

Several studies using implanted GRIN rod lenses have demonstrated long-term intravital microscopy, particularly in the mouse brain. While these GRIN lenses are fairly small (around 1 mm in diameter), this is still large compared with the size of the mouse brain. Insertion of such probes can cause significant damage and tissue displacement. Proponents of this technique argue that this is acceptable as long as the insertion of the rod does not impact the behavior they are trying to study, and sufficient time has passed after implantation for the associated inflammation to diminish. It is clear, however, that minimizing the probe diameter is optimal in terms of minimizing discomfort of and behavioral changes to the animal models being studied. Ohayon et al. found that a probe diameter of 0.1 mm leads to negligible tissue displacement, whereas probe diameters larger than 0.5 mm cause significant distortion to tissue. This is crucial to consider for needle-based probes, which assess the area immediately adjacent to the probe. Thus, we have chosen an outer diameter of 0.5 mm as a reasonable upper bound for micro-endoscopy probes designed for imaging in solid tissue.

C. Cellular resolution: Visualizing cells is crucial

In Secs. II A and II B, we have mainly motivated the need for micro-endoscopes, emphasizing the importance of small size in accessing deep sites with low invasion, with the ability to visualize cells implicit. However, early detection of disease depends on the ability to see cells. In this section, we explicitly consider the importance of acquiring cellular-resolution images comparable to histological microscopy images as a crucial aspect of minimally invasive optical biopsy.

The ability to observe biology on different length scales conveyed by imaging has highlighted the benefits of observing macroscopic diseases on the cellular and subcellular level. The prospect of performing endomicroscopy in situ in animal models and in living humans with comparable resolution to in vitro techniques is slowly coming into view but remains challenging. An important question is what resolution is needed for a particular study, which depends on the size scale of the relevant information. This is especially important for micro-endomicroscopes due to limitations on resolution imparted by the physical probe size. A companion question also relates to contrast in an image as visualization of features depends on both. Thus, in this section, we consider both examples of cellular-resolution imaging without the use of probes and consider sources of contrast and the interplay between contrast and resolution as issues that will arise in developing future micro-endomicroscopes.

Figure 4 indicates the resolution required for imaging representative structures of interest. In general, resolution better than 10 μm is required for imaging cells depending on the cell type. However, as can be seen by comparing the images in Fig. 4, a resolution of 1–2 μm is required to identify individual cells and observe how they interact with each other and their environment. Sub-micrometer resolution is required to resolve extracellular structures (such as collagen fiber bundles) and to observe subcellular processes and organelles. Confocal microscopes can easily achieve (lateral) resolution better than 2 μm and have become a mainstay of modern experimental biology, mostly using fluorescence imaging but also Raman spectroscopic imaging. Confocal fluorescence imaging and multiphoton microscopy with 1–2-μm resolution have enabled tracking of individual cells in vivo, whereas Raman imaging in this range can be used to identify cell types using biomarkers such as the rate of glucose uptake. Recent biomedical spectroscopy studies have noted a marked improvement in specificity by improving imaging resolution. Super-resolution microscopy (SRM) techniques are ever more commonly being used to reach the sub-few 100 nm resolution range; in vivo SRM studies have demonstrated an approach for studying intracellular dynamics in native conditions.

OCT imaging does not readily achieve isotropic cellular resolution, although some examples have demonstrated cellular resolution in at least one spatial dimension. Axial resolution in OCT is determined by the bandwidth of light used for imaging; axial cellular resolution (2 μm or better) requires a bandwidth of at least 140 nm at a central wavelength of 800 nm and at least 370 nm at a central wavelength of 1300 nm. Visible light OCT, with a shorter central wavelength, can achieve better axial resolution but with limited penetration depth into the tissue compared with longer wavelengths due to much stronger scattering. At the same time, achieving high lateral resolution over an extended axial range is incompatible with how OCT functions in capturing a full axial scan (to be discussed further in Sec. III B). Technological advantages can be leveraged to optimize OCT resolution including supercontinuum sources with notably large bandwidths, shaped beams, or coherent confocal geometries. Some of these approaches can be adapted for improving the resolution of micro-endomicroscopes for OCT.

The resolution and sensitivity of the optical system and the intrinsic contrast obtainable from a sample combine to determine the size of structures resolvable in an image. The intrinsic contrast derives from the particular cross section of substructures in the imaging volume interacting with the illumination light to generate a signal. The response of the imaging system is described by the optical transfer function (OTF). Beyond a spatial frequency threshold where the normalized value of the OTF is less than one, small features will be rendered in the image with lower contrast than large features of the same inherent sample contrast, as highlighted in Fig. 5. A consequence is that low-contrast features within the resolution range of the instrument may not be visible. Likewise, features with very high contrast may be detectable in an image
despite their sub-resolution size—this principle is why fluorophores with high absorption cross sections are required for super-resolution microscopy. In biological samples, the quality of an image with low contrast can be improved either by imaging with better resolution or by enhancing sample contrast; without perturbing the sample, improving resolution is generally more achievable—although it is more challenging in micro-endomicroscopes than in benchtop imaging systems.

One approach to mitigate the limited scattering-based contrast in biological tissues without introducing external contrast agents is to leverage other interactions between light and the sample to generate different forms of contrast, which we briefly now consider. In many cases, these interactions can provide information about structures that are not directly resolvable by the imaging system, for example, when using polarization-sensitive OCT (PS-OCT), second-harmonic generation (SHG) microscopy, or Fourier holography based on the directionality of scattering [Figs. 5(h) and 5(i)]. In the cornea, for example, the stromal lamellae may be as small as a few micrometers in thickness with relatively low contrast and are typically not resolvable by even high-resolution OCT systems. However, the fibrillary structure means that the layers will modulate the polarization of light; thus, adding polarization sensitivity allows them to be resolved, even if the contrast and resolution are both insufficient in the scattering-based image. Similarly, phase-sensitive OCT can enable the detection of the lamellae through variation in mechanical properties even if they were unresolvable in the structural image. Another approach using phase-sensitive OCT to detect modulation of the phase of light from interaction with the tissue has been used to differentiate between cone types in the retina, a challenging task using reflected intensity alone.
spectroscopy can be used to image sub-cellular and sub-resolution structures using an approach similar to super-resolution microscopy techniques, leveraging statistical methods to localize the position of chemical compounds within the cell. For nonlinear microscopy, structures within the sample can have a further impact on the contrast, for example, SHG microscopy can provide contrast based on crystal orientation and molecular organization based on structures that are smaller than the resolution limit. Each of these techniques has enabled some recent studies of fibrillar organization in tissues without direct resolution of the fibers themselves. Leveraging these additional mechanisms of contrast can help mitigate the need for better resolution and may be particularly useful when limitations on size for micro-endomicroscopes limit the ability to improve the imaging resolution.

III. PHOTONICS FOR FUTURE MICRO-ENDOMICROSCOPY

In Sec. II, we have made a strong case for micro-endomicroscopy—in terms of prospective applications and in terms of the critical need for such cellular-resolution, minimally invasive approaches to deep access in samples. In this section, we will discuss the challenges that inhibit the design and performance of micro-endomicroscopes for particular applications. We will discuss trade-offs in design parameters that must be considered when developing new probes. Throughout, we will draw from the existing state of the art and summarize imaging micro-probe technology today—mainly in Sec. III D—clearly indicating the gap that must be bridged to routinely perform micro-endomicroscopy. In so doing, we will highlight the specific needs for several different imaging techniques and propose some photonics solutions that would advance the performance of these probes in the future. In first presenting the design trade-offs before the existing state of the art, we hope to put what has been achieved in context, emphasize how the available technology has limited what has been achieved thus far, and will continue to guide micro-endomicroscope development in the future.

A. Volumetric imaging in micro-endomicroscopy

1. Point scanning

Volumetric imaging with a probe supporting a point-by-point imaging method requires a suitable scanning mechanism, which can be proximal or distal, as indicated in Fig. 6. If distal, the scanning
FIG. 6. Schematic of an archetypical micro-endomicroscope comprising three main components: a relay system (gold box), beam focusing system (green box), and a scanning system (navy boxes). The relay system is typically composed of optical fibers but could also be composed of sufficiently small bulk lenses and exists to transport light within the probe. Focusing elements could include one or more of the following: GRIN lenses or GRIN fibers, ball lenses, metalenses, and freeform/3D printed optical elements. Ball lenses, metalenses, and 3D printed optics can be manufactured independently or on the end of an optical fiber. The micro-endomicroscope includes either a proximal scanner or distal scanner. Proximal scanning systems are external to the probe and typically comprise a motor, which rotates the probe, and a fiber-optic rotary joint, which forms the optical connection between the probe and the stationary imaging system. Another realization of a proximal scanner involves using an external mirror system to scan the beam across the back aperture of the probe. In the case of scanning-free multimode fiber probes, a spatial light modulator is used to modulate the point-spread function in place of a mechanical scanning system. Distal scanning systems are included within the probe itself and include either MEMS or piezoelectric scanners implanted in the probe tip or a nano-scanner printed on the tip of an optical fiber. The probe is typically connected to the imaging system using optical fibers (black). Probes can be either front-viewing or side-viewing, with an additional element required to achieve the latter. The spot size (s) determines the imaging (lateral) resolution; the confocal parameter or depth of field (DOF) determines the imaging range. The working distance (WD) is defined as the distance from the probe to the focal spot.

Mechanism (usually microelectromechanical systems (MEMS) or piezoelectric based) is contained within the probe itself. While these point beam-scanning approaches have several advantages, including the potential for high scan speed, low inertia, and high precision, they are, thus far, inadequate for the development of microprobes (less than 0.5 mm in diameter). MEMS scanners typically limit the probe diameter to several millimeters, often developed with a target diameter of less than 5.5 mm to ensure compatibility with instrument channels in commercial endoscopes. High-resolution 3D printing has emerged as a promising approach for minimizing the size of MEMS devices; while several micro-optical components have been printed, including silicon photonic devices, lenses, and resonators, 3D printing has yet to be applied to the development of micro-optical scanners. Recent advances in micromachining and lithography have enabled the preliminary development of nano-mirror systems; however, further development is required before these devices can be used for bioimaging applications due to challenges involving torque and stability. Few, if any, functional and reliable scanner options exist for diameters smaller than 500 μm. It seems that one remaining challenge is bridging the gap between the ultra-small components created by nanoscale 3D printers, which require nanometer-scale manufacturing precision, and the hundreds-of-micrometers size of the final devices. However, the prospect of printing a scanning system on the tip of an optical fiber is certainly within reach, and its achievement would represent a promising step forward for distal volumetric scanning in micro-probes. For ultra-high-speed imaging applications, the repeatability of scanning patterns must also be considered. Fast scanning (faster than ~10 fps) with MEMS or piezoelectric scanners typically relies on resonant scanners, but increased imaging speed comes at a cost—resonant scanners generate non-uniform or non-repeatable spatial sampling that impacts the overall image resolution and signal-to-noise ratio (SNR). This issue must be taken into account if these scanners are to be widely adopted for use in fast imaging. In a promising development, intravascular imaging using a distal micromotor-based scanning system has been achieved with a 4000 kHz radial frame rate. This micromotor is 2 mm long and 1 mm in diameter and rotates a microprism in the distal tip of the probe. A separate proximal pullback motor system was used to generate volumetric scans. Currently, proximal scanning probes tend to be smaller than distal scanning probes because the scanning systems are external.
and, therefore, do not limit the probe size. Proximal scanning techniques include translating the beam across the back aperture of the imaging probe, a common approach for forward-viewing probes, or incorporation of an external rotation/pullback system that moves the whole probe.\textsuperscript{12} The rotation/pullback system is driven by car-
diac and intravascular applications of optical coherence tomography and was pioneered for intravascular ultrasound imaging.\textsuperscript{12} It requires the use of a fiber-optic rotary joint (FORJ) to maintain connections between rotating and stationary segments of optical fiber. However, the FORJ can introduce intensity fluctuations due to imperfect coupling between fibers during rotation. There is also the issue of non-uniform rotation distortion (NURD), especially when using long fibers. NURD can be somewhat mitigated by minimizing the distance between the motor and the end of the probe and/or by compensation in post-processing.\textsuperscript{128}

2. Focus scanning

In order to acquire volumetric images with 2D \textit{(en face)} imaging techniques, such as confocal and nonlinear microscopy, the ability to adjust the depth of the focus position within the sample must be incorporated. While this can be accomplished using distal MEMS scanners, this approach is thus far limited in terms of maintaining a small probe size (as discussed above). Other approaches include using acousto-optic or liquid crystal tunable lenses\textsuperscript{129–131} or incorporating tunable structures, such as shape memory alloys and piezoelectric benders, into the probe body to modulate the separation between lenses.\textsuperscript{132–134} Recent advances in these technologies are enabling faster volumetric scanning and miniaturization,\textsuperscript{135} although imaging micro-probes based on these technologies have yet to be developed. While the lenses themselves may be sufficiently small to be included within micro-endomicroscopes, pistons, piezoelectric devices, or electronic components are typically required to modulate the lenses, and inclusion of these components in the distal end of the probe would require an increase in diameter. Additionally, the need for multi-element lenslet arrays limits the overall lens size; individual lens components might be 50–100 μm in diameter, resulting in an overall lens array size larger than 1 mm.\textsuperscript{136,137} Micropatterning techniques discussed in Sec. III C combined with liquid crystal lenses open up the possibility for adjustable-focus Fresnel lenses, metalenses, and other adjustable complex lens geometries as well as further miniaturization.\textsuperscript{138}

3. Scanning-free volumetric imaging with multimode fibers

One promising recent approach to volumetric imaging with imaging micro-probes is single-fiber lens-free imaging with multimode fibers (MMFs) or multi-core fibers (MCFs).\textsuperscript{139–142} A similar technique has been used with fiber bundles, but using MMFs is most promising in terms of maintaining small probe diameters. MMFs typically have an outer diameter of 125 μm; MCFs are typically 250 μm or larger in diameter, although smaller MCFs are currently being developed.\textsuperscript{142} The approach is based on measuring the transmission matrix of the fiber and using this information in conjunction with a spatial light modulator (SLM) to modulate the output point-spread function from the fiber. The achievable resolution is limited by the NA of the fiber and the density of modes; more excited modes enable higher resolution (similarly, increasing the number of fibers in a fiber bundle enables higher resolution since adding fibers constitutes adding additional modes).\textsuperscript{143} The number of modes determines the number of resolvable features within the core area and approaches the diffraction limit as the number of modes is increased.\textsuperscript{139} For MMFs, depending on the wavelength and core diameter, diffraction-limited performance can be readily achieved if all available modes are used. Using this approach, beam focusing and volumetric scanning are achieved, without using any lenses, by illuminating the back of the fiber with the correct pattern of light. Micro-endomicroscopic imaging \textit{in vivo} in an animal model has been demonstrated using this technique. Volumetric fluorescence imaging of a mouse brain with 1.35-μm lateral resolution was achieved using lens-free imaging through a multimode fiber with an outer diameter of 125 μm.\textsuperscript{144} The researchers were able to resolve dendritic spines and axonal boutons, although the field of view (FOV) was limited to the diameter of the fiber core, 50 μm in this case. FOV improvements were demonstrated using a fiber with a larger core at the expense of a larger outer diameter. The results from this study compared with a tabletop confocal microscope [Fig. 9(d)] demonstrate impressive imaging performance. Another study was able to achieve 1-μm lateral resolution in the mouse brain.\textsuperscript{145} Raman spectroscopic imaging with diffraction-limited res-
olution has also been achieved using this technique, although it has yet to be applied to complex samples.\textsuperscript{146} In contrast, this tech-
nique is not readily compatible with high-resolution OCT imaging, as each illuminated mode within the MMF corresponds to a different optical path length; this, without compensation, results in the broadening of the axial point-spread function (PSF) and a reduction in sensitivity.

Compared with other approaches, lensless MMF probes show a lot of promise in terms of being able to achieve cellular resolution without increasing the probe diameter. Volumetric scanning and depth sectioning can be achieved without the need for micro-optical scanning systems, although depth sectioning in cur-
tent approaches is limited by the fiber core diameter. In comparison to distal scanning probe designs (in which the scanning systems are the limiting factor in terms of the probe diameter), MMF probes are limited only by the diameter of the optical fiber itself. Compared with pullback-based techniques, using an SLM and computation for focusing enables axial scanning of the focus without perturbation of the sample. Additionally, by using such a means of scanning, designs are not restricted by the availability of small focusing optics, thereby offering more versatility in terms of PSF control compared to GRIN lenses or micro-ball lenses.

However, several problems remain before these probes can be widely adopted. First, the lateral FOV is limited by the core diam-
eter of the fiber; a maximum FOV of a few hundred microme-
ters has been achieved thus far. While this is comparable to some other techniques, the axial FOV is limited compared with pullback-based techniques resulting in a relatively small imaging volume overall if the axial position of the probe is not physically scanned (which may introduce problems regarding changes to the transmis-
sion matrix). The maximum and minimum distance of the focus from the end of the fiber and the collection efficiency are also limited by the diameter and NA of the fiber. These trade-offs limit the applications to imaging over small volumes. Second, the trans-
mission matrix is variable and depends on many factors including the conformation of the fiber. This presents a challenge for lengthy and flexible probes, in particular, or imaging in moving subjects.
Either changes in the transmission matrix must be avoided or the ability to actively measure the transmission matrix for each measurement is required. Most approaches for measuring the transmission matrix require access to the distal end of the fiber, making real-time measurement impossible. One approach involves using a reflective meta-surface to actively measure the transmission matrix during measurements. Determining the transmission matrix is computationally intensive, so the ability to perform the computation sufficiently rapidly might be an impediment to real-time imaging. Some evidence suggests that it may be possible to predict changes in the transmission matrix for given perturbations of the fiber, and these predictions could be used to reduce the computation time. Alternatively, novel engineering solutions could be developed for moving the probe with sufficient accuracy so as not to change the transmission matrix. These issues will need to be addressed before MMF probes can be applied to imaging of awake animals, imaging studies with probes implanted in a living animal, such as those tracking disease progression, or studies with longer, flexible probes.

B. Probe design trade-offs for micro-endomicroscopy

A main consideration when designing a probe with cellular-resolution is the link between the design parameters of probe size, resolution, SNR, working distance, FOV, and depth of field/optical sectioning (DOF). In most instances, optimizing the design for one of these parameters sets limits on the range of the other parameters. We briefly consider these trade-offs.

The NA describes the link between the diameter of an objective lens and the achievable resolution: \( NA = \frac{n \sin \theta}{\lambda} \), where \( n \) is the index of refraction of the medium in which the light is being focused, \( D \) is the diameter of the lens aperture, and \( f \) is the focal length. The minimum size of objects that can be resolved, according to the conventional diffraction limit, is \( s = \frac{0.611}{NA} = \frac{1.22\lambda}{nf} \). In order to achieve cellular resolution (2 \( \mu \text{m} \) or better), \( NA > \sim 0.15–0.45 \) is required depending on wavelength. This relationship is key to consider when designing an endoscope as it sets the achievable limits using conventional Gaussian-beam optics, as presented in Fig. 7(b).141,149–168 as the endoscope diameter decreases, it becomes more challenging to achieve high NA and high resolving power. This challenge is exacerbated in endoscopes based on GRIN fiber elements because the beam diameter is limited to the fiber core diameter, but the excess diameter of the cladding can be appreciated.169 One approach for overcoming this limitation is the use of materials for focusing elements with a higher index of refraction than silica, such as sapphire (\( n = 1.763 \)) or barium titanate (\( n = 2.369 \)), thereby improving the achievable NA for a given probe diameter.160,161 Sapphire optics are further advantageous due to their large transmission bandwidth and low Raman signal.141,162

Another trade-off is the link between the resolution and working distance; cellular-resolution probes tend to have a shorter working distance, as can be derived from the formula for NA mentioned above. This trade-off can present another design challenge, particularly for volumetric imaging with small probes. In side-viewing probe designs, a minimum working distance is required in order for the focal spot to be outside of the imaging probe. This problem is exacerbated if the imaging probe is encased within a needle or catheter, thereby extending the minimum working distance required. For some applications, the working distance has become a limiting factor in terms of the minimum achievable resolution with a particular probe design. Figure 7(b) indicates the possible parameter combinations for imaging at a wavelength of 800 nm with a Gaussian beam in trading off working distance, resolution, and probe diameter. One approach for extending the working distance could be incorporating multiple focusing elements similar to approaches used in the design of long-working-distance objective lenses.165

The signal collection efficiency must also be considered as the probe diameter is decreased. Collection efficiency is especially important for Raman spectroscopic imaging due to the low signal intensity and can also be important in fluorescence imaging. In both cases, the excitation beam is focused to a tight spot, determining resolution, but the emitted light is largely isotropic, and so the collection numerical aperture is key in optimizing the overall signal strength. For Raman spectroscopy, most probe designs rely on multiple fibers to optimize signal collection.141 In theory and if focusing elements are used, single-probe designs have better collection efficiency than designs with separate illumination and collection paths. However, both signal collection and noise collection are enhanced—so improved collection efficiency alone does not necessarily translate to improved SNR.165 One of the benefits of separate illumination and collection paths is the ability to reject background scattered light from within the fiber itself. New advances in double-clad fibers (DCF) could provide an alternative to the N-around-1 designs, which can maintain a small probe diameter but still separate the illumination and collection path.164 For fluorescence microscopy, the use of a DCF has demonstrated the dual benefits of the smaller spot size achievable from a single-mode fiber by using the core for illumination, while leveraging the multimode inner cladding to improve collection efficiency,163 at the expense of confocal axial sectioning, which is then foregone. In addition to optimizing collection efficiency and separating illumination and collection paths, DCFs are also useful for developing dual-modality imaging probes.166–168 This is especially true when combining OCT with fluorescence- or Raman-based imaging techniques since the single-mode core is used to collect the OCT signal, while the inner cladding is used to collect the fluorescence signal without the need for additional filtering components.

Another important factor to consider is the depth of field (DOF), which defines the depth range over which an image is considered to be in focus. In most microscopy techniques, DOF determines the optical sectioning or axial resolution of the image. When imaging with a Gaussian beam, the DOF is fixed for a given spot size and varies with it. The standard definition of the DOF (when imaging with a Gaussian beam) is twice the Rayleigh range, \( z_B \), where the Rayleigh range is the distance from the beam waist to the point where the cross section has doubled: \( DOF = 2z_B = \frac{2\omega_0^2}{\lambda} \). Practically, this means that the DOF decreases for smaller spot sizes; in other words, better transverse resolution corresponds to better axial resolution for most microscopy techniques. However, for OCT imaging, the axial resolution is not determined by the DOF; the DOF instead determines the depth of the imaging range. As the resolution of the OCT imaging system is improved, the corresponding decrease in DOF limits the volumetric imaging range. This poses a particular problem for OCT systems with lateral resolution better
than 5 μm. This is true in general, not just for imaging with endoscopes; however, this trade-off can still have an impact on aspects of the design. This limitation is apparent in Fig. 7(a); for this reason, probes designed for OCT do not typically aim for diffraction-limited performance.

One approach for overcoming this limitation is the use of alternative beam shapes, particularly Bessel and related extended beams. Bessel-like beams maintain their diameter over a larger axial distance compared to a Gaussian beam with the same spot size, enabling the relationship between DOF and resolution to be modified based on the beam’s Fresnel number [Fig. 7(c)]. Consequently, the SNR is reduced in the focal plane but tends to be improved toward the limits of the DOF as compared with Gaussian beams. Extended DOF imaging has been achieved using fiber probes using a few different methods and will be discussed in more detail in Sec. III D. Airy beams have been proposed as an alternative approach for extending the DOF for OCT imaging and light-sheet microscopy. The main benefit of Airy beams is the improved SNR for the same DOF extension compared to a Bessel-like beam. Achieving OCT imaging with an Airy beam is nontrivial due to the asymmetric beam shape (deconvolution is required to extract the sample structure), so the potential is unclear. However, for light-sheet microscopy, the use of an Airy beam has demonstrated subcellular resolution over a tenfold larger FOV compared with a Gaussian beam.

C. Small, high NA lenses in micro-endomicroscopy

Recent advances in lens design and engineering are creating opportunities for the development of small, cellular-resolution optics for integration into miniaturized optical devices, including endoscopic probes. Flat diffractive optics and metalenses (described below) allow for miniaturization while maintaining high resolution; two-photon polymerization-based 3D printing and advances in photolithography enable the development of complex microlens structures that provide enhanced PSF control without the increased lens size. In addition to many opportunities for miniaturization of complex optical systems, these techniques share the potential for mass production, which could facilitate the low-cost fabrication of imaging probes. Examples of several approaches are shown in Fig. 8.

Fresnel lenses and other diffractive optics provide an interesting opportunity for miniaturization because they inherently require less material to develop. While traditional Fresnel lenses have suffered from aberrations due to limitations in fabrication techniques, recent advances in focused ion-beam milling and photolithography have enabled the fabrication of Fresnel lenses directly on the end of optical fibers achieving sub-micrometer resolution and near-diffraction-limited performance. Diffusive optics can be designed to form single-focus, multi-foci, or extended-DOF PSFs (for example, using a diffractive axicon). Using photopolymerization or 3D printing, additional materials can be deposited on the end of the fiber to create Fresnel lenses with different refractive indices for immersion applications, such as in vivo imaging. Fresnel lenses are typically very sensitive to chromatic aberration, but the performance over wide optical bandwidths, if required, can be improved using higher-order diffractive lenses. Fresnel lens fiber probes have been used for optical trapping and manipulation but have yet to be developed for imaging applications.

Metalenses are a special type of flat diffractive lens with subwavelength features that can produce a desired equivalent bulk-optical effect. Their design is based on the Huygens principle: a grid of nanoantennas built on a substrate modulates the phase of light such that the superposition results in the desired PSF. In addition to modulating the phase of light, the polarization can also be controlled. Thermo-optic tunability of the focal length has been demonstrated, which could facilitate volumetric scanning. A metalens has recently been developed with achromatic performance over a 600-nm bandwidth in the visible and near-infrared range, opening up application to imaging techniques requiring broadband bandwidths. Recent work has explored the possibility of developing...
metalenses on the end of optical fibers, although this work is typically limited to short working distances. The versatility of metalenses positions them to lead a revolution in the miniaturization of imaging devices.

Fabrication by 3D printing provides the opportunity to design and realize precisely shaped microlenses for specific applications. Highly complex configurations, including multi-element objectives and lens arrays, are achievable using multiphoton polymerization-based printing systems. 3D-printing techniques can also be used to fabricate diffractive lenses and metalenses. Tadayon et al. have developed a new approach for fabricating cascaded multi-lens endoscopes using 3D printing and photolithography; they have demonstrated a probe size of 100 μm with a 1-μm resolution. Another recent study has investigated building a photonic chip on the tip of an optical fiber. 3D printing has also enabled the fabrication of freeform optics directly on the tip of optical fibers, enabling the creation of complex beam shapes. One recent study achieved ex vivo OCT imaging in a human coronary artery and a mouse aorta using an imaging micro-probe with a 3D-printed lens and lateral resolution of 12.4 μm. To our knowledge, none of the cellular-resolution (2-μm or better) 3D-printed lens designs have yet been applied to biological imaging either in vivo or ex vivo. This wide array of new options for miniaturized high-performance focusing elements presents an excellent platform from which to develop future sub-systems and systems for micro-endomicroscopy.

D. Toward micro-endomicroscopes—progress to date

While many probes have been developed for various imaging techniques, most are either endomicroscopes or microendoscopes; very few imaging micro-probes with cellular imaging resolution (and none with isotropic cellular resolution) have been definitively demonstrated, as also reviewed elsewhere. Most approaches use either GRIN rods, which typically range from 350 μm–1 mm in diameter, or fiber-based approaches, which are typically 80–250 μm in diameter. In this section, we aim to highlight endoscope designs for a variety of imaging techniques, which, at least in part, meet the specifications of having an outer diameter less than 0.5 mm and a resolution better than 2 μm in at least two spatial dimensions, as introduced in Secs. II B and II C. A summary of probes that satisfy these criteria is presented in Table 1. Some probes that do not meet the criteria, but with noteworthy characteristics, have also been included as benchmarks for what has been achieved. Figure 7(a) shows several notable examples and their imaging performance. Note that no published probe meets the demanding criteria of sub-2-μm resolution in each dimension for volumetric imaging in a probe of less than 500-μm diameter.

1. Confocal fluorescence, MMF fluorescence, and multiphoton imaging

Confocal fluorescence and multiphoton imaging represent the imaging techniques with the most developed micro-endomicroscope research, mainly driven by intravitral neuroscience applications. The aim in such applications is to achieve resolution comparable to imaging with a benchtop microscope and approaching the diffraction limit (1 μm or better) while exceeding imaging depths accessible using multiphoton imaging from the surface (deeper than 1.3 mm). Several designs achieving this goal for confocal fluorescence imaging have been developed using GRIN rods, particularly for neural imaging in mice. One example using
| Microscopy modality | Probe diameter (μm) | Axial resolution (μm) | Lateral resolution (μm) | Image depth (μm) | FOV (μm) | Distinguishing features/ Applications | References |
|---------------------|---------------------|-----------------------|------------------------|------------------|----------|--------------------------------------|------------|
| Wide field          | 100                 | 2                     | 5 (max)                | 65               |          | Fabricated using photolithography; *in vivo* in mouse brain | Tadayon et al.\textsuperscript{191} |
|                     | 100                 | 1                     | 3 (max)                | 60               |          | Fabricated using photolithography; *ex vivo* in mouse brain slices | Tadayon et al.\textsuperscript{187} |
|                     | 125                 | 12.7                  | 1.5                    | 8.8              | 24 (axial) | GRIN fiber; *ex vivo* in chicken tendon | Sato et al.\textsuperscript{156} |
| Confocal fluorescence| 350                 | 11.5                  | 0.7                    | 15               | 70       | GRIN rod lens; *ex vivo* in bovine muscle tissue | Pillai et al.\textsuperscript{157} |
| MMF fluorescence     | 125                 | 20.2                  | 1.35                   | 1.8              | 50       | MMF; *in vivo* in mouse brain | Vasquez-Lopez et al.\textsuperscript{144} |
|                     | 125                 | 1.18                  | 2                      | 50               |          | MMF; *in vivo* in mouse brain | Turatev et al.\textsuperscript{145} |
| SHG, auto-fluorescence | 350              | < 1.5                 |                        |                  |          | GRIN rod lens; *in vivo* in human and mouse muscle tissue | Llewellyn et al.\textsuperscript{192} |
| 2-photon excited fluorescence | 500 | 8.6 | 0.85 | 5 | 200 | GRIN rod lens; *in vivo* in mouse brain | Bocarsly et al.\textsuperscript{158} |
|                     | 500                 | 53–68                 | 1.25                   | 1.55             | 220      | GRIN rod lens; Bessel focus scanning; *in vivo* in mouse brain | Meng et al.\textsuperscript{150} |
| Spontaneous Raman spectroscopy | 80 | 10 | 10 |  |  | Ball-lens microendoscope with a notably small diameter; imaging not yet demonstrated | Yamanaka et al.\textsuperscript{193} |
| Coherent anti-Stokes Raman spectroscopy | 250 | 5 | 5 |  |  | Dual GRIN fibers; highest resolution CARS microendoscope; *ex vivo* in primate brain | DePaoli et al.\textsuperscript{194} |
| Brillouin spectroscopy | 1800 | 5 | 1 |  |  | Single and dual GRIN fibers; only published Brillouin probe; imaging not yet demonstrated | Kabakova et al.\textsuperscript{195} |
| OCT                 | 410                 | 2                     | 5                      |                  |          | Needle probe; *ex vivo* in porcine pancreas | Joo et al.\textsuperscript{151} |
|                     | 500                 | 2                     | 3                      | 1000 (axial)     |          | GRIN rod lens and MMF; biological imaging not yet demonstrated | Yin et al.\textsuperscript{152} |
|                     | 500                 | 1                     | 3                      | 400 (axial)      |          | GRIN rod lens and MMF; *ex vivo* porcine esophagus | Yin et al.\textsuperscript{153} |
|                     | 500                 | 1.5                   | 3                      | 1000 (axial)     |          | GRIN rod lens and MMF; *ex vivo* in human coronary artery | Yin et al.\textsuperscript{196} |
|                     | 70                  |                       |                        |                  |          | BaTiO\textsubscript{3} ball lens; notably small probe diameter; *ex vivo* mouse brain | Marrese et al.\textsuperscript{160} |
|                     | 520                 | 1.7                   | 5.7                    | 1.23             |          | Diffractive lens; *in vivo* in mouse brain | Yuan et al.\textsuperscript{156} |

\*Lateral FOV except where indicated as axial.
a GRIN rod with a diameter of 350 μm and 488-nm excitation for confocal fluorescence imaging achieved a lateral resolution of 700 nm.\textsuperscript{197} A similar GRIN micro-endomicroscope was used to image sarcomere contractile dynamics in mice and in humans.\textsuperscript{192} This same probe was used for SHG microscopy and imaging of NADH autofluorescence (NADH is a molecule involved in metabolism). Designs based on lensless imaging using multimode fibers for fluorescence imaging have also achieved impressive resolution with even smaller diameter probes; with a 125-μm fiber, imaging deep in a mouse brain was achieved with a lateral resolution of 1.35 μm and axial resolution of 20.2 μm.\textsuperscript{144} Volumetric scanning was achieved over a distance of 100 μm from the fiber tip using digital refocusing. As mentioned in Sec. II, confocal fiber bundles developed by Mauna Kea Technologies (Paris, France) have enabled cellular-resolution confocal fluorescence imaging in humans and are commercially available.\textsuperscript{158} These probes are particularly versatile since they are long and flexible without impacting imaging performance. Developed probes have achieved 1-μm resolution; however, these probes all have a diameter greater than 2 mm. Alternatively, their smaller 1-mm diameter probes are limited to 3.5-μm resolution. It is challenging to reduce the probe diameter without reducing the number of fibers in the bundle, which then negatively impacts the resolution. The growing number of FDA-approved fluorescent compounds for use in humans is improving the prospects of wide adoption of in vivo confocal fluorescence imaging as a diagnostic (and interventional) tool, although there are still challenges in optimizing their use.\textsuperscript{197, 199} Fluorescent dyes can be delivered as a topical spray, such as acriflavine, or systemically, such as fluorescein.

Multiphoton and other nonlinear microscopies can often achieve a similar resolution to confocal fluorescence microscopy meanwhile utilizing longer wavelengths to image deeper. This is because the (usually quadratic or cubic) intensity dependence of the nonlinear process results in 3D spatial confinement of the focal volume. While confocal microscopes rarely achieve their theoretical resolution limit, multiphoton microscopes do, thus minimizing the penalty of operating at a longer wavelength.\textsuperscript{200} GRIN lens- and multimode fiber-based designs similar to those used for confocal fluorescence imaging have been developed for multiphoton imaging (Fig. 9). Subcellular multiphoton imaging with 850-nm lateral resolution was achieved in a mouse brain using a 500-μm diameter GRIN lens.\textsuperscript{144} The lens was inserted into a 600-μm diameter guiding cannula to enable minimally invasive repeatable imaging of the same location. Multiphoton imaging of a mouse brain and fluorescently labeled microcapillaries was achieved with a 600-μm diameter GRIN rod, demonstrating a lateral resolution of 825 nm using an excitation wavelength of 830 nm.\textsuperscript{17} However, the working distance was less than 50 μm and the FOV was a 58-μm diameter circle, which limited the capability of this design for volumetric imaging. To overcome this limitation, Meng et al. proposed using a Bessel beam for microendoscope-based two-photon microscopy.\textsuperscript{150} By scanning the Bessel beam focus in two dimensions, volumetric imaging over a FOV of 370 μm × 370 μm × 52 μm was achieved. The ability to resolve axonal boutons with 1.25-μm resolution was demonstrated with a higher volumetric throughput than could be achieved with conventional two-photon microscopy at the expense of axial resolution. The loss of optical sectioning was determined to be unimportant, particularly in sparse samples, although

FIG. 9. Some examples of micro-endomicroscopes for fluorescence and two-photon microscopy. (a) Photographs of some singlet and doublet GRIN microendoscopes.\textsuperscript{20} (b) Two-photon microscopy images of neurons acquired in vivo in anesthetized mice with a 500-μm diameter GRIN endoscope.\textsuperscript{20, 158} (c) Images of sarcomere contractile dynamics in vivo in a human using SHG microscopy through a wearable probe based on a 500-μm GRIN microendoscope.\textsuperscript{21} (d) Fluorescence images of dendritic spines acquired using a lensless multimode fiber endoscope.\textsuperscript{144} Reproduced with permission from Barretto et al., Nat. Med. 17, 223 (2011). Copyright 2011 Springer Nature; Bocarsly et al., Biomed. Opt. Exp. 6, 4546 (2015). Copyright 2015 The Optical Society; Sanchez et al., Neuron 88, 1109 (2015). Copyright 2015 Elsevier; and Vasquez-Lopez et al., Light Sci. Appl. 7, 110 (2018). Copyright 2018 Springer Nature.
deconvolution could be applied to this technique in more densely labeled samples.

2. Linear and non-linear biomedical spectroscopic microscopy

Beyond the design constraints discussed for confocal fluorescence and multiphoton microscopy, additional design challenges must be considered for Raman and Brillouin spectroscopic imaging through fiber-based micro-probes. While Raman spectroscopy provides access to the chemical fingerprint of a sample and Brillouin spectroscopy provides information on its viscoelastic properties, the engineering considerations for designing microendoscopes for these two techniques are similar: in both cases, the challenge stems from isolating the desired signal from the much stronger undesired background. For Raman spectroscopic imaging, the desired Raman signal must first be separated from the background signal composed of Rayleigh scattered light, luminescence (phosphorescence and/or fluorescence), and Raman signal from the fiber itself (silica generates a strong Raman intensity signal several orders of magnitude larger than that from the sample). Additionally, the signal collection must be optimized for collection of the weak Raman signal, especially from a small interaction volume within the sample when highly focused excitation is used. The typical approach to isolating the Raman signal involves the use of multiple, separate multimode collection fibers at the cost of miniaturization. Raman spectroscopic imaging with benchtop microscopes has demonstrated the improved specificity to be gained by imaging with better resolution, yet, to our knowledge, no probes have been developed for Raman spectroscopic imaging, which satisfy the criteria for both diameter and resolution laid out at the start of this section—all designs achieving resolution below 2 μm exceed 1 mm outer diameter and most developed probes have millimeter-scale resolution. An imaging micro-probe, with 80-μm diameter and with separate illumination and collection paths, was realized using two 30-μm diameter fibers; however, no focusing elements were used resulting in a resolution of 23 μm. Micro-endomicroscopes for Raman imaging remain an open area for research.

Stimulated (nonlinear) Raman imaging, particularly coherent anti-stokes Raman scattering (CARS) and stimulated Raman scattering (SRS), may be more important for biological imaging than spontaneous Raman imaging. Stimulated Raman scattering events occur with orders of magnitude higher probability than spontaneous ones, so images can be acquired at higher rates without sacrificing SNR; as such, non-linear Raman techniques have a greater potential for rapid volumetric imaging applications. Moreover, with the stronger signal, the collection efficiency of the non-linear Raman probe is slightly less important, which relaxes the requirements in terms of probe design.

Initial investigations into novel fiber types report promise for the development of Raman imaging probes. Hollow-core photonic crystal fibers generate significantly less Raman background signal than do silica fibers. Although the utility of these fiber types for Raman imaging has been demonstrated, they have yet to be used to create a cellular-resolution imaging probe. In particular, the double-clad Kagome fiber presented in Ref. shows great potential because it would allow for the separation of illumination and collection paths to minimize the collected Rayleigh scattered light in addition to air guidance to minimize the Raman background from the fiber. This is an area for potential future development.

Benchtop Brillouin microscopy has also demonstrated a notable improvement in specificity by improving imaging resolution. Similar to Raman spectroscopic imaging, the performance of Brillouin microscopy hinges on the ability to isolate the desired Brillouin signal from the background signal dominated by Rayleigh scattering. Brillouin imaging through fiber faces even greater challenges than Raman imaging. The small few-gigahertz magnitude of the Brillouin shift from the excitation light makes it even more difficult to separate from the Rayleigh scattered light, although this can be achieved by spectrometers with sufficient resolution, such as tandem Fabry–Pérot devices and virtually imaged phased arrays. Additionally, the Brillouin signal from the fiber is a significant source of background noise; while the frequency shift of the Brillouin signal in glass is sufficiently distinct from the shift in tissue, the long interaction length in an optical fiber generates a signal orders of magnitude larger and negatively impacts the dynamic range of the measurement. Even so, the prospect of achieving Brillouin imaging through a fiber probe is intriguing and an area that merits further study. To our knowledge, the two probes presented in Ref. represent the only realized fiber probes for Brillouin microscopy; thus, we include them here although they do not satisfy the criteria laid out at the start of this section; both the designs utilize a 1.8-mm diameter GRIN lens. Additionally, only single-point measurements have been demonstrated. The first steps to achieving fiber-based Brillouin imaging have been taken, but much more work in this area is required.

3. Optical coherence tomography

Achieving OCT imaging with a lateral resolution on the scale of 1–2 μm or better is challenging both in benchtop systems and in fiber probe designs, mainly due to the link between DOF and axial imaging range that is specific to OCT. While some probes with a diameter less than 500 μm and axial resolution better than 2 μm have been developed for OCT imaging, no probes that fully satisfy the criteria for micro-endomicroscopes (resolution of 2 μm or better in at least two dimensions) have been developed for OCT (Fig. 10). We focus here on notable, realized probe designs approaching this criterion. Since the axial and lateral resolution are decoupled for OCT, it is possible to have an imaging system with micrometer-scale resolution in the axial dimension but worse resolution in the lateral dimension (or vice versa, although less commonly). For example, a probe developed for needle-based imaging of neoplasia in the pancreas with an outer diameter of 410 μm demonstrated an axial resolution of 2 μm but a lateral resolution of only 5 μm. The imaging application determines whether isotropic cellular-resolution is required or whether high axial resolution alone is sufficient, although visualizing cells typically requires sufficient resolution in at least two dimensions. Interestingly, this is the converse for confocal fluorescence imaging, which typically has better lateral resolution than axial resolution. Other small diameter probes developed for cellular-resolution OCT include one developed for intravascular imaging with an outer diameter of 0.5 mm and a 3-μm lateral and 1.5-μm axial resolution. A 70-μm diameter probe was developed incorporating a barium titanate ball lens glued to a tapered fiber tip, although the reported sensitivity of 83 dB is insufficient for high-quality imaging in biological samples.
Several approaches have been proposed to overcome the limited DOF in high-resolution OCT fiber probes including phase masks and fiber axicons created through polishing or chemical etching. None of the approaches have yet achieved a probe with better than 2 μm resolution and sufficient DOF, although a probe with a resolution of 2.5 μm and a DOF twice as long as a Gaussian probe with the same spot size has been created using phase masks. OCT imaging through an axicon-based fiber probe has proven challenging; the light passes through the axicon in both the illumination and collection path, resulting in a doubling of the one-way SNR penalty and a lateral PSF with strong side lobes, which significantly impedes the ability to image biological samples. This can potentially be overcome by using modified axicon-like beam shapes, for example, an axicon with a flat-polished tip. Alternatively, decoupling the illumination and collection paths eliminates the need for the collected light to pass through the beam-shaping components. This has been accomplished in an endoscope using a GRIN rod as a relay lens to image the Bessel beam onto the sample. The challenge of achieving sufficiently extended DOF to match the volumetric collection of OCT with high resolution, especially with a small probe, has yet to be achieved. Extending the DOF without sacrificing SNR is key to achieving cellular-resolution probe-based imaging with OCT.

IV. SUMMARY

We have highlighted the promise of micro-endomicroscopes in applications spanning basic discovery science to clinical translation. We have summarized the state of photonics devices and subsystem technologies today and identified challenges to be addressed and opportunities to attain the identified necessary performance. In this section, we draw these perspectives together and reiterate areas that are most ripe for advancement. The development of micro-endomicroscopes is very much in its infancy; while some micro-endomicroscopes have been developed for fluorescence and multiphoton imaging, none have yet been demonstrated for Raman spectroscopic imaging or OCT. Imaging micro-probes with a diameter less than 0.5 mm and with isotropic resolution better than 2 μm have yet to be demonstrated, despite the progress outlined in this Perspective.

Promising application areas for cellular-resolution imaging with micro-endomicroscopes, at whatever stage of the translational imaging pipeline, require imaging in situ or at the site of the disease in order to record what is happening in a real, living sample. Longitudinal (time-lapse) imaging using probes implanted in 3D tissue constructs will advance the study of disease progression and the development of personalized treatments. The use of micro-endomicroscopes in intravital microscopy will enable new access to hard-to-reach areas, including inside of small blood vessels, ducts, and alveoli, with minimal damage. In humans, minimally invasive diagnosis and monitoring, especially of cancer in hard-to-reach areas not accessible using currently available imaging tools, such as the oral cavity and bile duct in the pancreas, will be hugely advanced through the availability of micro-endomicroscopes.

Key to advancing micro-endomicroscopy is addressing the design trade-offs we have highlighted, beginning with the primary
limiting factor of realizing high-NA optics with small diameters. As we have seen, resolution better than 2 μm has been achieved either in the lateral or axial dimension, but not isotropically, which is the most versatile scenario. Beam-focusing elements that are more sophisticated than simple refractive lenses will feature prominently in the next phase of micro-endomicroscope development. Metalens systems have already begun to enable complex beam shaping in small probes and show great promise. 3D printing directly on the tip of optical fibers is starting to enable the design of more complex lens geometries and compound lenses for application to micro-endomicroscopes.

As isotropic cellular resolution becomes achievable, attention to developing the microscanner technology will be necessary to enable volumetric scanning. Progress in the development of 3D-printed nano-scanners has demonstrated our ability to design sufficiently small scanning devices, although limitations to how much torque they can apply present an obstacle to incorporating these devices directly into micro-endomicroscopes. Advances in 3D-printing technology to bridge the gap between standard MEMS devices and nano-printed components (likely, the printing of micro-scale devices with nanoprinting techniques) would impact on reducing probe diameters. Similarly, lensless scanning approaches using an SLM and MMFs have shown a lot of promise for combined beam shaping and volumetric scanning, but further investigation is required to improve the robustness of these methods. Preliminary studies using liquid crystal and electro-optic tunable lenses have indicated that these devices, too, could make possible practical focus-scanning-based micro-endomicroscopes in the future.

Reducing background signals to improve image SNR will advance the field of micro-endomicroscopes based on confocal fluorescence and spectroscopic imaging. Progress has been made in this direction by designing novel types of optical fibers, such as photonic crystal fibers, which have been specifically optimized for these applications. Further advances in this area would help accelerate the development of the single-fiber probe designs required for micro-endomicroscopes.

Advances in photonics will drive advances in micro-endomicroscopy, enhancing our capability to monitor cellular and sub-cellular processes in vivo, in real-time, and in realistic settings. This capability will advance our understanding of how cells impact tissue-scale processes, contribute to human clinical applications, and finally bring minimally invasive all-optical biopsy within our reach.

**DATA AVAILABILITY**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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