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Optical-Based Analysis of Soft Tissue Structures

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Abstract

Fibrous structures are an integral and dynamic feature of soft biological tissues that are directly related to the tissues' condition and function. A greater understanding of mechanical tissue behavior can be gained through quantitative analyses of structure alone, as well as its integration into computational models of soft tissue function. Histology and other nonoptical techniques have traditionally dominated the field of tissue imaging, but they are limited by their invasiveness, inability to provide resolution on the micrometer scale, and dynamic information. Recent advances in optical modalities can provide higher resolution, less invasive imaging capabilities, and more quantitative measurements. Here we describe contemporary optical imaging techniques with respect to their suitability in the imaging of tissue structure, with a focus on characterization and implementation into subsequent modeling efforts. We outline the applications and limitations of each modality and discuss the overall shortcomings and future directions for optical imaging of soft tissue structure.

Keywords

tissue optics; optical imaging; optical histology; tissue structure

1. INTRODUCTION: OPTICAL-BASED ANALYSIS OF SOFT TISSUE STRUCTURE

1.1. Background

Fibrous structures play an important role in the function and behavior of biological tissues, both healthy and diseased. For example, structural changes in collagen and elastin fibers are particularly important in biomechanical remodeling of many dense connective tissues. In wound healing and scar tissue generation, collagen fibers are deposited regularly across wound boundaries to support antagonistic biomechanical forces and ensure closure of the wound. Similarly, throughout the body, these fibers provide adaptive structural support in response to changes in mechanical stresses, such as those that arise during growth or injury. A wide array of procedures use this phenomenon to artificially induce remodeling of the geometry and stiffness of native tissues in order to improve their performance. However, these techniques currently use entirely mechanistic approaches, as quantitative tissue structure data generally require excision of tissue, thereby skewing the measurements.

Although biological tissues are often modeled as homogeneous, single-phase materials, difficulties in structural quantification persist due to problems in tissue accessibility and density, as well as the presence of layers and macroscopic structures. Additionally, being subjected to constantly fluctuating physical, chemical, and biological stresses causes a tissue's structure to be highly dynamic. Analyses of tissue structure using traditional techniques are often limited because measurements and sample preparation necessarily alter the tissue's natural configuration (1). Better techniques for analyzing tissue structure in vivo and in real time can enable improved observation and quantification of the normative physiological state and functional behavior of tissue, which can ultimately lead to the development of more complete computational models and more accurate evaluation and diagnostic capabilities.

Optical techniques for analyzing tissue structure have several important benefits (2). Light within the visible and IR spectrum is nonionizing and can generally be employed noninvasively, allowing tissue to be analyzed in its functional setting. Hydration, loading, and temperature are only a few of the dynamic changes that can affect the analysis of excised tissues, resulting in misleading and incorrect conclusions. Optical sectioning techniques allow the sample to remain in situ, rather than necessitating physical sectioning. When used in clinical applications such as screening, evaluation, and diagnostics, optical techniques can be performed in vivo with little to no harm or discomfort to the patient while offering the advantage of rapid and high-throughput use. Additionally, optical techniques provide a wide range of scales, resolutions, and sources of contrast, making them highly versatile for an array of applications. Importantly, optical techniques offer degrees of freedom beyond spatial variation of intensity with which to make quantitative measurements. Polarization-, wavelength-, and time-dependent measurements can allow quantification of a greater variety of metrics, including molecular composition, fiber alignment, and mechanical properties. These factors make optical techniques ideal for analyzing the structure of tissue.

In this review, we provide a brief overview of traditional techniques used for analyzing tissue structure and composition, then discuss the drawbacks of their use and necessary improvements. Most of this article explores several contemporary optical techniques that improve the imaging quality and content of traditional histological and nonoptical techniques; we emphasize optical imaging modalities that are well suited to analyses of tissue structure and function. Rather than explore the theory and instrumentation of these various techniques, we focus on their capabilities and appropriate areas for their application. Finally, we discuss the fundamental limitations of current optical systems and areas of focus for future technologies.

1.2. Key Soft Tissue Structures

The term soft tissue broadly encompasses a wide variety of distinct tissue types, ranging from tendon to muscle to brain matter. These soft tissues vary in their constitutive structures and composition, yielding vast differences in characteristic form and function between types. However, in soft tissues there are surprisingly few of the base structural components that significantly affect gross biological or mechanical function. Constituents can typically be

classified as either extracellular or cellular. For most connective tissues with load-bearing function, the proteins collagen and elastin are paramount.

Type I collagen is the most abundant protein in the body and is the major determinant of dense connective tissue mechanical behavior (3, 4). At the molecular level, tropocollagen molecules are composed of a triple helix of three α chains that arrange themselves into a quarter-staggered array to form collagen fibrils (3, 4). Collagen fibrils form the functional subunits of the collagen fibers, as described by the Hodge–Petruska model (3, 5). Like the fibrils from which their functional properties are derived, collagen fibers exhibit high tensile but low flexural stiffness (6). Thus, the fiber longitudinal axial direction is the primary determinant of the stiffness of the tissue composite. Interestingly, collagen fibers typically have a stiffness of 1 GPa (7–10) and extend by no more than 4–5%. To increase tissue-level compliance, collagen fibers at the macroscopic scale are sinusoidally crimped (3). Mechanically, tissue-level stress does not occur in the fiber–fibril complex until the fiber-level crimp has been straightened. Moreover, the distribution of fiber-straightening strains is the mechanism underlying tissue nonlinearity at large strains (11, 12).

In contrast to collagenous structures, elastin forms complex cross-linked fibrous networks of comparatively low modulus that are thought to assist in tissue recoil (13, 14) and in the maintenance of collagen fiber crimp (15, 16). Finally, glycosaminoglycans (GAGs) are known to affect water retention in soft tissues (17); the loss of GAGs reduces thickness and diminishes rehydration capacity. However, GAGs are not a primary load-bearing component. Instead, they appear to function to maintain tissue volume and as a dampening agent to smooth rapid motions (18). In muscular tissues, the muscle cells have a direct role in function; myofibers (multinucleated, actin-and myosin-dense cells) determine active and passive tissue mechanical behaviors. Much research has been devoted to this topic, especially for the heart (19).

1.3. Why Structure Is Important

As in any system, determining how the constituents make up that system and their arrangement provides great insight into organ-level function. Structure determines function; by gaining an understanding of the structure of soft tissues, we can better understand tissue function (20). However, the soft tissue microenvironment is one of constant activity—cell growth, cell death, differentiation, propagation, protein production, signaling, and mechanical stimulation. The health and behavior of the cell populations in soft tissue are directly linked to properties of the extracellular matrix (ECM), and vice versa. In addition, the study of soft tissue structure is a study of scale. Both ECM and cell behavior factor into organ-level function; conversely, organ-level factors, such as injury, affect structure. Ultimately, the fundamental relationship between tissue function and performance is the underlying structure, which we can attribute primarily to structural proteins.

1.4. Qualitative Technique Assessment

From the above considerations, the goal of any soft tissue imaging technology is to enable accurate, nondestructive, and quantitative analysis of tissue structure in vivo across a wide range of scales. This goal requires a somewhat qualitative and relative assessment of the

different techniques in comparison to histology and nonoptical techniques, which is represented by the pentagonal radar chart in the table accompanying each modality reviewed. This section outlines our evaluations of each of the five major metrics.

1.4.1. Resolution—One of the most important requirements of a tissue imaging system is that it must be able to resolve the smallest tissue features, including cells, structural proteins, and membrane components. For our purposes, optimal spatial resolution is satisfied at 10 μm or better, although higher resolutions are required to image some structural proteins on the order of single micrometers. The resolution is considered for all dimensions; therefore, we analyze techniques on both their lateral (image plane) and axial (depth) resolutions. Techniques that integrate signal spatially over any dimension are considered to have maximum dimensional resolution equal to the imaging depth. We also consider contrast resolution (the ability to detect image signal from noise/background).

1.4.2. Scale—Depending on the specific tissue or feature being investigated, the absolute sizes of structures are often quite variable. Ideally, an imaging system should be capable of imaging tissues throughout an entire organ without loss of resolution. Scanning hardware is generally capable of observing large lateral and axial fields of view, but depth attenuation in most optical systems severely limits the axial field of view (21). Therefore, we emphasize the capability of imaging deep within tissue ($>1\text{ mm}$) without the need for physical sectioning of the sample.

1.4.3. Quantitative measurement—Most existing analyses of tissue structure are based largely on qualitative morphological techniques. More quantitative measurements of tissue, especially mechanical tests, are limited to characterizing bulk tissue, not fine tissue elements or structures. In this review, we consider quantitative measurement to be the ability to assign a characteristic metric to a tissue feature relevant to structure, such as fiber direction or composition. Labeling is a minimum requirement, and correlation to physical characteristics of the tissue constituents is desirable. Measurements that are empirical or relative are advantageous, but ultimately those with direct analytical relationships to physical properties are desired.

1.4.4. Tissue preparation and destructiveness—As discussed above, many imaging techniques require physical sectioning of the tissue, which is undesirable. However, we treat physical sectioning as an issue in scaling capability, rather than a destructiveness issue. Techniques that physically, chemically, or otherwise damage tissue during preparation or measurement (other than physical sectioning) are considered destructive. We emphasize techniques that allow samples to be left in their native environment during imaging and do not adversely affect the tissue or its structure as a result of measurement. Even if the measurement technique itself is nondestructive, any preparation—including chemical fixing, optical clearing, and use of extrinsic contrast agents—is considered undesirable.

1.4.5. Functional imaging—Biological tissues are dynamic in nature. They can exhibit very different behavior over a range of temporal scales, and functional imaging that can record temporal changes in tissue structure, composition, and function and relate these changes to tissue physiology is particularly relevant to modern diagnostic and research tools

(22, 23). Although functional imaging is often associated with monitoring brain activity in response to a stimulus, it also has significant application in linking a tissue's macroscopic function to its microstructure. There is a large body of research investigating dynamic fiber reorientation due to loading, growth, or remodeling in cardiac tissue, with applications in producing better bioprosthetic materials (24, 25). Fast acquisition times of full data sets (three spatial dimensions plus one time dimension) are the ultimate goal of tissue imaging systems. Although we discuss two-dimensional (2D) functional imaging, three-dimensional (3D) functional imaging capability is considered the ultimate goal.

1.5. Traditional Standards

Many different optical and nonoptical techniques exist for studying tissue structure. This section addresses their current usage and inherent drawbacks.

1.5.1. Histology—Light-based microscopy has historically been one of the most powerful tools for understanding biological organisms (26, 27). The greatest advantage of light microscopy is that the images it produces are intuitively interpreted with minimal training. Also, its versatility in application is nearly unmatched—it can function as either a simple low-level magnifier or a probe of cellular scales.

Bright-field microscopy is the simplest and most familiar form of light microscopy (28). A thin sample is placed on a slide between an illumination source and an objective lens, which relays the transmitted image to the viewing ports. Contrast is provided through the attenuation of light as it passes through the sample, and a “shadow” of the image is created. A serious limitation of this technique is that it relies almost entirely on intrinsic absorption, which often results in low contrast in prepared thin samples. Histological stains—dyes that bind to specific tissue or cellular components—are often necessary to achieve suitable contrast in bright-field microscopy. This technique requires pathological conditions for viewing excised tissues, and is still a mostly qualitative assessment.

Different methods of contrast enhancement have been investigated in order to use wide-field light microscopy more effectively. Phase contrast, differential interference contrast (DIC), and dark-field microscopy all use different sources of contrast in order to show tissue structure in greater detail (29). Each technique is particularly effective when integrated into a single microscope stage so that the sample can be imaged using different modalities. The main limitations of bright-field microscopy—namely resolution and sectioning capability in thick samples—also apply to these optical methods. The main advantage of these techniques is their ability to use a variety of contrast mechanisms to identify different structures and chemical signals within tissues. However, these mechanisms are viable only when samples can be fixed and thinly sectioned, and the results are not always representative of functional, in vivo tissue.

1.5.2. Nonoptical methods—Nonoptical techniques, such as magnetic resonance imaging (MRI), computed tomography (CT), and ultrasound, have been widely adopted as modern imaging standards in medicine, particularly in the areas of large-scale morphology, tissue differentiation, and biomechanics. These methods enjoy success mainly because of their relatively large fields of view, intrinsic contrast mechanisms, and broad in vivo utility.

However, they also suffer from fundamental limitations in the local, micrometer-level structural information they provide. Ultrasound, which detects differences in tissue densities, is a form of localized tomography that is nondestructive and easily administered to a wide variety of tissues (30, 31). Although high-resolution ultrasound is available, it is largely unachievable in highly heterogeneous tissues because the acoustic signal suffers from shadowing artifacts and depth attenuation, which limit sectioning detail beyond modest depths (32, 33). Additionally, density differences at the microstructural level of tissue are often too insignificant to yield meaningful contrast. MRI and other nuclear imaging techniques that use electromagnetic fields of individual atoms as a source of contrast have inherently broad fields of view (34). Although MRI is especially well suited to hemodynamic functional imaging, it requires extensive acquisition times for smaller-scale, static structural data, and the spatial resolution limits are around 0.5–1 mm for conventional systems due to poor contrast resolution (35, 36). Higher overall resolution can be achieved by enhancing the signal-to-noise ratio considerably, but doing so requires instrumentation that is either prohibitively costly or ill suited to the imaging of biological tissue (37). For example, a high-magnetic field MRI, currently under development, that is designed to attain 0.1-mm resolution in a conventional MRI format will cost more than US\$250 million to produce (38).

Conventional X-ray CT is the technique that is best able to obtain micrometer-level resolution while maintaining large (<40-cm) imaging depths and fields of view (39, 40). Additionally, as a technique using electromagnetic radiation, CT is capable of quantitative spectroscopy. However, the high-energy ionizing radiation associated with X-rays can severely damage biological tissues, limiting CT as a method for extended, in vivo use (41, 42).

1.5.3. Drawbacks—Clearly, traditional optical histology techniques are limited in their use. Specifically, the need for histology techniques to physically section tissue leads to extremely low scales for use, destruction of the native tissue, and an inability to perform functional imaging (Figure 1). Nonoptical techniques represent a significant advance in large-scale, nondestructive functional imaging of entire tissues, but they suffer from much poorer resolution and low levels of intrinsic contrast and capability for tissue differentiation at the meso- to microscale. Advanced optical techniques that improve upon the relatively low resolution of both of these traditional imaging modalities will be required for more accurate, quantitative analyses of tissue structures.

2. CONTEMPORARY OPTICAL TECHNIQUES

This section highlights several different modalities currently used for imaging tissue structure. Because a comprehensive description of optical imaging modalities would warrant an entire volume of its own, we have chosen a small subset of techniques on the basis of their relevance for investigations of tissue structure, as outlined in the preceding section. These modalities represent contemporary alternatives to histology that are more suitable to studying tissue structure in a less invasive manner, providing greater detail through enhanced contrast/resolution and more quantitative metrics. We have sorted these techniques into groups according to the method used to achieve imaging contrast.

Presented with each modality is a table unique to that particular modality, representing a summary of its imaging capabilities. Note that although the numbers listed describe expected performance metrics for contemporary systems, they should not be taken as universal. Instead, they represent the general limitations of the imaging technique that the user can expect in practice. However, many of the imaging techniques can and should be adapted for specific applications, which can result in better (or worse) performance metrics. The goal of each section is to provide the reader with an idea of what applications each modality is best suited for. More information regarding theoretical background and instrumentation for each of these imaging systems can be found in the cited papers.

2.1. Bulk Constituent Structure: Signal Amplitude Differentiates Between Tissues and Tissue Interfaces

This subsection covers the modalities that most closely mimic traditional optical histology techniques but have better resolution and sectioning capabilities. The main sources of contrast for these techniques are tissue interfaces in which increased scattering occurs because of a mismatch in the index of refraction, or absorption attenuates the incident light. These are some of the most mature contemporary techniques and have already found widespread academic use and some level of commercial success, making them suitable for many general tissue imaging applications requiring high resolution, variable contrast, and precise sectioning capability. We discuss confocal reflectance/fluorescence microscopy, nonlinear techniques, and optical coherence tomography (OCT) with respect to their tissue imaging capabilities. Superresolution techniques that surpass traditional diffraction limited microscopy are omitted because their resolution is orders of magnitude below that relevant to tissue structure and they are generally used for single-molecule localization.

2.1.1. Confocal reflectance/fluorescence laser scanning microscopy—Confocal microscopy is one of the most adaptable imaging techniques (Table 1). The contrast mechanism is widely variable for different applications, and the fundamental method enables high resolution and optical sectioning (43, 44). The confocal principle itself is a method of filtering out any light except that reflected from the focal plane—a major problem in traditional light microscopy when investigating thick ($>10\text{-}\mu\text{m}$) samples with low endogenous contrast (45, 46). Consequently, the axial and lateral resolutions of the system become entirely dependent on its optical components, primarily the numerical aperture and confocal pinhole size. Therefore, confocal microscopy has become particularly relevant with the advent of lasers, which can be focused much more precisely than incoherent light sources. Practical limits have increasingly approached the theoretical limits of submicrometer resolution in both the axial and lateral dimensions. In practice, commercially available objectives with a numerical aperture of at least 0.9 can be expected to achieve axial and lateral resolution on the order of $0.5\text{--}5\text{ }\mu\text{m}$ (44).

Confocal microscopy can use intrinsic contrast from changes in refractive index or absorption; in fact, certain biological molecules, such as melanin, provide particularly good contrast (47, 48). However, this technique has been particularly effective when paired with fluorescent-mode imaging (49). Fluorophores can be used as markers to highlight specific areas of tissues and cells, and multiple fluorescent channels can be imaged simultaneously

while maintaining exact registration (45). This powerful technique is responsible for some of the most striking images of tissue and cell structure.

A drawback of the confocal method is related to spatial filtering. Spatial filtering requires point scanning, resulting in much longer acquisition times. Line scanning and raster scanning techniques can yield much faster imaging speeds, but usually at some cost to the resolution and imaging field (50, 51). Additionally, spatial filtering, which eliminates out-of-focus light, also rejects light that has been multiply scattered en route to and from the sample. Therefore, the depth penetration of confocal reflectance microscopy operating near ideal confocal performance is limited to approximately three to four optical depths in order to obtain an adequate signal-to-noise ratio (52). The optical depth depends on many factors, including tissue type and laser wavelength; for reference, confocal microscopy is generally limited to under 200–300 μm in human skin tissue (53). Note that fluorophores emitting light in the visible spectrum further limit the penetration depth for confocal imaging (44).

Over the past few decades, confocal microscopy has become a universal standard for imaging tissue structures, exemplified by its inclusion as a standard modality on many commercial biological microscopes (54–56). Its simultaneous, multichannel acquisition of fluorescent markers is very advantageous in component-specific labeling, contrast, and registration. Despite the limited penetration depth, confocal microscopy should be considered one of the most versatile and accessible techniques for imaging tissue structure, specifically as a high-resolution histology technique with precise sectioning and labeling capabilities.

2.1.2. Nonlinear techniques (two-photon microscopy/second harmonic generation)—Nonlinear microscopy techniques, namely two-photon microscopy and second harmonic generation (SHG), have several advantages over linear reflectance- and fluorescence-based techniques (Table 2). In traditional fluorescence, a single incident photon results in emission of a similar-energy photon in fluorophores. In multiphoton fluorescence, two (or more) photons incident on a single molecule result in the emission of a photon with higher energy, leading to a higher frequency and a lower wavelength (57, 58). Because this phenomenon occurs only when the photons arrive nearly simultaneously at the molecule, it confines the effect to the focal volume of the system, resulting in submicrometer imaging resolution (59). SHG achieves a similar effect, although through a different physical mechanism (60).

Nonlinear microscopy techniques have several advantages over fluorescence microscopy (61, 62). Longer excitation wavelengths in the red and near-IR regions allow much deeper penetration (up to 1 mm) than most other imaging techniques while maintaining submicrometer resolution (63, 64). Additionally, multiphoton systems require much less illumination power, as no pinhole is necessary for optical sectioning, resulting in less light wasted through filtering as well as less photobleaching to the sample (65). Emission wavelengths can be easily distinguished from the excitation wavelength, yielding a huge improvement of contrast when used as an imaging method (66).

Most importantly, two-photon microscopy and SHG can take advantage of intrinsic contrast mechanisms from native tissue structures. Many structurally relevant molecules, including collagen and elastin, can be specifically identified using two-photon excitation or SHG (67). Contrast enhancement and specific labeling of other tissue regions and constituents can also be achieved through the use of fluorophores similar to those employed in confocal fluorescence imaging (68, 69).

Multiphoton microscopy has some notable drawbacks. Excitation wavelengths in the near-IR region have inherently larger diffraction limited resolutions; however, this loss in resolution is mostly an issue in cell imaging, and the technique remains capable of resolving the fine detail of tissue structure. Whereas a depth of up to 1 mm is considered thick in optical imaging, the imaging depth is inherently limited for larger fields of view without physical sectioning of samples or confinement to superficial layers (70). Finally, high-speed scanning can result in video-rate (30-Hz) acquisition for single, 2D planes, but for larger, high-resolution, volumetric data collection, longer acquisition times are necessary (71).

Multiphoton microscopy is most relevant when high resolution and high contrast are necessary in optically turbid tissue up to 1 mm thick. It is capable of visualizing the bulk microstructure and even the cellular structure of tissue, as shown in Figure 2, which depicts images of neurons extending nearly 1 mm from the surface of the brain. It can also specifically label important structural and functional tissue constituents, and is suitable for in vivo imaging of cells (72). Two-photon microscopy can offer significant improvements over confocal microscopy when photobleaching, depth attenuation, and intrinsic contrast are immediate concerns.

2.1.3. Optical coherence tomography—OCT originated in the early 1990s and has rapidly been adapted for clinical use and commercial systems (Table 3) (73). OCT is analogous to ultrasound, although it uses light, rather than sound, as its probing mechanism. OCT uses low-coherence interferometry to identify regions of high backscattering, which indicates interfaces within the sample. One axial scan results in a one-dimensional (1D) data set in which changes in intensity indicate changes in tissue composition. Additional lateral scans allow 2D and 3D morphology to be rendered at high resolution.

Axial resolution of OCT relies mostly on the bandwidth of the laser and is normally on the order of 10–15 μm (74). There is a trade-off between lateral resolution and axial field of view over which OCT can resolve an image—higher lateral resolution limits the depth of focus, whereas higher depths of focus can be achieved only by compromising lateral resolution (75). Imaging depth is also limited by light attenuation, although most near-IR wavelengths used are capable of low attenuation. OCT can achieve a 6-mm axial field of view while maintaining 10- μm lateral resolution (76). Lateral field of view, in any case, is limited only by the scanning instrumentation used.

Newer implementations of OCT, namely swept-source and Fourier domain OCT, have enabled much higher axial scanning speeds, and video-rate 3D scans with relatively large fields of view have been accomplished (77). In addition, newer lasers have given rise to micro-OCT (μOCT), a technique that can improve axial resolution to $<1 \mu\text{m}$ and lateral

resolution to $<2\ \mu\text{m}$ (78, 79). OCT can also be employed using fiber optics, enabling imaging probes with diameters of $\sim 1\ \text{mm}$ (80). The greatest demand for OCT is in the field of ophthalmology, as it allows retinal morphology to be easily accessed and evaluated for certain diseases. OCT is also a convenient endoscopic tool, especially in intraluminal imaging. μOCT methods are capable of cellular and subcellular imaging.

Overall, OCT is a robust, highly adaptable method for imaging tissue morphology. Its main limitation is the shadowing effect, which can obscure deeper tissue regions and decrease contrast; however, it is still suitable for many imaging tasks in which features near the surface of the tissue are important. It is useful in turbid, highly scattering tissues, as it is capable of much higher penetration than confocal or nonlinear techniques. OCT benefits from good resolution in both the axial and lateral directions, offers high-speed acquisition capability for functional imaging, and can image much more deeply into turbid media while maintaining fine resolution in comparison to most other optical imaging techniques.

2.2. Substructure Orientation: Signal Amplitude Is a Function of Underlying Structural Orientation

The orientation of tissue constituents, especially within orthotropic tissues with regular fiber arrangements, plays an important role in the growth, development, and remodeling of tissues. Imaging techniques that can provide preferred and induced fiber orientations, especially after the application of controlled mechanical stresses and strains, can yield important information about the biomechanical function of the tissue. This section outlines two techniques, polarization imaging (PI) and small-angle light scattering (SALS), which provide high-contrast information about the localized arrangement of tissue microfibers on a macroscopic scale.

2.2.1. Polarization imaging—PI is less remarkable as an independent imaging technique than it is as a method of enhancing existing imaging techniques (Table 4) (81). PI exploits the orientation of the electromagnetic field of light to allow filtering and gating of light. In PI, light from an illumination source is filtered so that its electromagnetic field has a well-defined orientation as it propagates to the sample. Certain tissue structures (especially cylindrical structures) are birefringent, causing anisotropic scattering that depends on the polarization of the incident light and therefore influences the amount of light that maintains its polarization as it propagates through the tissue (82). Comparing the initial light polarization with the orientation of the reflected or transmitted light allows the underlying optical and structural anisotropy of the material to be quantified.

The power of PI is that it can be adapted to nearly any scattering-based imaging technique and can provide additional information about the tissue structure (83). Notably, PI has been successfully paired with OCT, reflectance spectroscopy, and spatial frequency domain imaging (SFDI) for application to biological tissues (84–86). Although it can be used as a stand-alone imaging method with standard imaging and microscopy equipment to either eliminate or isolate specular reflection from the superficial layers of a sample, PI shows particular promise when incorporated with different imaging techniques (87–90).

2.2.2. Small-angle light scattering—SALS was originally used to study and characterize polymer chains suspended in solution by analyzing the behavior of light scattered from the medium (Table 5) (91). More recently, the technique was adopted as a means of quickly acquiring sample-wide soft tissue microstructure information (92). SALS techniques for collecting structural properties work by exposing samples to a nonpolarized beam of low-power laser light. Light passing through the tissue is either transmitted or scattered, such that the spatial distribution of scattered light (the SALS pattern) represents the sum of all structural information within the light beam envelope (92). Photons scattered by tissue microstructural elements are characterized by two values: the scattering angle and the azimuthal angle. The scattering angle is measured from the direction of the incident beam and is determined by the physical characteristics of the scattering structure, whereas the azimuthal angle is perpendicular to the orientation of the scattering structure in the plane of the tissue sample, as dictated by cylindrical scattering theory. The SALS pattern is the collection of all scattered photons by a photosensor or projection screen; highly aligned structures produce high-eccentricity elliptical patterns and randomly distributed fibers yielding circular patterns. From these patterns, the angular distribution of microstructure fibers can be directly obtained. A full-field fiber orientation map can then be constructed for entire tissues (Figure 3).

By raster scanning the sample through the path of the laser beam, one can create large field-of-view microstructure maps that denote fiber properties, including mean fiber direction, index of fiber alignment, and mean fiber diameter. The lateral spatial resolution of these maps is determined only by the raster step size and the diameter of the laser beam. SALS can resolve properties from collections of fibers with diameters on the order of the illumination wavelength ($\sim 0.5\text{--}20\ \mu\text{m}$).

Note that, unlike some imaging modalities, SALS cannot resolve structures axially through the sample. SALS point scans yield structural information that is inherently an average of individual fiber properties across the area of the incident beam and through the depth of the sample. Also, because of the transmission-based nature of SALS, the technique requires translucent samples: If the sample is not adequately translucent, histologic sectioning or chemical clearing will be required.

SALS imaging is best used in cases in which tissue-wide microstructure is required and damage to the sample is a nonissue. SALS can be used on soft tissues following mechanical testing or fixation under load to study microstructure-derived mechanical properties, or in the characterization of healthy and diseased tissue. In constitutive model development, SALS is a powerful experimental complement to mechanical testing of soft tissues with the direct inclusion of these microstructure maps (93).

2.3. Composition/Spectroscopy: Signal Amplitude Is Related to the Optical Properties of Tissue Constituents

Spectroscopy techniques have emerged as powerful tools for molecular identification. Spectroscopy utilizes the large amounts of information encoded in both the amplitude and frequency of light after interaction with matter.

2.3.1. Diffuse reflectance/fluorescence/Raman spectroscopy—In its most general form, spectroscopy is a method of observing interactions between light and matter—specifically, how light is absorbed and scattered by different materials (Table 6). The absorption and scattering characteristics depend strongly on the properties of both the material and the incident light (94). Therefore, prior knowledge about the incident light allows both empirical and analytical differentiation of tissue composition.

Diffuse reflectance spectroscopy (DRS) uses inverse models of light diffusion in heterogeneous tissue to calculate the bulk scattering and absorption of different regions. The signal has a distinct signature of diffuse reflectance from the tissue across a broad wavelength spectrum, which allows one to identify the specific scattering and absorption properties of the sample and attribute them to different tissue constituents (95, 96). Similarly, laser-induced fluorescence spectroscopy (LIFS) uses a fluorescence spectrum to differentiate tissue regions, similar to confocal fluorescence but across a larger, more highly resolved spectrum (97, 98).

Raman scattering spectroscopy investigates nonelastic scattering in tissue. Nonelastic scattering occurs when scattered light interacts with high-frequency vibrations within the individual molecules, resulting in an increased or decreased wavelength (99). The signal is several orders of magnitude weaker than in DRS or LIFS, but individual molecules elicit very specific Raman scattering spectra, which can enable more reliable identification of tissue constituents. Additionally, the signal can be greatly enhanced through the use of surface-enhancement techniques applied to specific nanoparticles (100, 101).

Each of these modalities can be adapted as point-scanning techniques; however, the information thereby obtained pertains to bulk local tissue characteristics, rather than being confined to a focal volume. The result is generally higher a lateral resolution, typically defined by the source–detector separation in the probes (on the order of 100 μm). Additionally, the signal is integrated along the entire axial dimension sampled, meaning that the axial resolution is an average measurement of reflectance or fluorescence across this sampling depth. Probe design can influence the depths at which the tissue is sampled but cannot result in true optical sectioning, only averaged depth sectioning.

These spectroscopy techniques are important because of their abilities to differentiate and empirically determine bulk tissues. Despite the capability of point-scanning paradigms, this added information comes at the cost of resolution and volumetric confinement of the signal. Its best use is for investigations of larger tissue structures or broader spectroscopic analyses of bulk tissue regions to understand the underlying constituents of the tissue.

2.3.2. Spatial frequency domain imaging—SFDI is a wide-field spectroscopy technique that can be used to obtain scattering and absorption values of material across wide fields of view (Table 7). Whereas most other spectroscopy techniques measure diffuse scattering of narrow illumination beams (essentially a point source), SFDI uses structured illumination of an entire planar field (102, 103). The spatial pattern is typically a sinusoid, the frequency of which controls the integration depth of the image. Demodulation

techniques enable the attenuated illumination patterns to reveal quantitative optical scattering and absorption properties of the material (104, 105).

SFDI is particularly well suited to tissue differentiation. However, it has two major limitations. First, the wide-field technique requires three different spatial patterns to be projected and imaged. The sample is assumed to remain constant between each of the three exposures, which can lead to errors in observing rapid tissue dynamics. Second, SFDI does not automatically determine the scattering and absorption values as a function of wavelength. If single-wavelength illumination is used, then the values are specific to that wavelength; if a broadband source is used, then the values are a composition of the scattering and absorption values of the entire illumination spectrum. This means that there is less information to empirically identify tissue constituents.

SFDI is a less specific but more robust tool for empirical tissue differentiation. Although it requires multiple exposures per image, it eliminates 2D scanning and its depth sampling is more easily controlled, making it better suited to both higher resolution and more rapid, functional imaging in comparison to other spectroscopy techniques. Finally, it can be easily combined with other light-gating techniques; for example, a polarized SFDI system exploits the integration depth control of SFDI and the directional information of PI to observe tissue fiber orientation as a function of depth (Figure 3) (86). Although relatively young, SFDI shows promise as a spectral imaging technique to quantitatively differentiate regions of heterogeneous tissue.

2.4. Coupled Mechanical Measurements: Signal Is Sensitive to the Mechanical Characteristics of Different Materials

A relatively young field of imaging uses the mechanical properties of tissue to act as both a source of structural contrast and a quantitative contrast metric. These imaging methods generally achieve contrast through photoacoustic interactions in which light propagation is influenced by the mechanical properties of the constituent materials. These interactions often occur through photon–phonon interaction, in which an incident photon's path or energy is influenced by an acoustic event. Whereas some of these methods can rely on endogenous contrast, such as thermoelastic fluctuations, most benefit by using artificially induced acoustomechanical waves to attain an adequately noise-free signal. This is usually achieved through ultrasound transduction or photoacoustic stimulation of shock waves. The resulting data are usually related to the speed at which acoustic waves can travel through the material, which indicates that material's stiffness. This information can be processed to provide maps of relative stiffness or specific values of one (or more) of the materials' elastic moduli.

The main benefit of optical determination of mechanical properties is that it allows localized measurements on a much finer scale compared with traditional mechanical testing, which can provide only bulk mechanical characterization. Additionally, this technique does not require one to excise a sample entirely from its functional environment, and it leaves the tissue intact and relatively undisturbed. The optical techniques described in the following subsections enable high-resolution, localized, functional measurements of tissue mechanical properties. This information can be used to pair tissue structure with mechanical behavior

and can be employed to model biomechanical structures more accurately, develop more robust engineered tissues, and differentiate between tissues in a quantitative rather than qualitative way.

2.4.1. Photoacoustic tomography/ultrasound-modulated optical tomography—

Photoacoustic imaging is a combined imaging method that uses energy from light to generate acoustic waves and subsequently detect the acoustic wave propagation (Table 8). In photoacoustic tomography (PAT) the acoustic wave is detected by an array of ultrasound transducers to reconstruct the volumetric data set, whereas in photoacoustic microscopy (PAM) the excitation beam itself is scanned and the acoustic wave is detected by a single transducer attached to the optical probe (23, 106). In each case, the acoustic signal depends both on absorption of the excitation light by the material and on the mechanical properties of acoustic wave propagation within the material.

Other photoacoustic techniques use alternative imaging methods (including OCT and laser speckle) to observe the acoustic wave propagation and relate the speed directly to the elastic properties of the material (107, 108). The additional mechanical data that these methods glean are generally a bulk or averaged measurement of tissue properties across the focal volume and are not on the scale of the actual imaging resolution of those modalities; however, an added quantitative metric can still be useful in some applications.

Photoacoustic methods are limited by both photonic and ultrasonic attenuation. When IR excitation is used, ultrasonic attenuation is usually a limiting factor to a depth of ~3 mm for high-frequency PAM, while resolution in the tens of micrometers is maintained (109). Although much higher depths can be obtained for PAT at lower frequencies, resolution degrades rapidly to the order of hundreds of micrometers to millimeters.

The addition of both optical excitation and acoustic signal transduction can yield much higher depth penetrations. Image information is decoupled from the optical signal that would originally be recaptured by scattered light. Instead, photoacoustic events occur only at areas where the focal point of the excitation system and that of highly absorptive molecules (such as blood or melanin) overlap. Although initiating these photoacoustic often requires extrinsic contrast agents, it is useful for imaging deep-lying tissue structures. Finally, the high temporal resolution of this technique allows tremendous functional imaging capabilities across many scales, particularly when detecting hemodynamic response (23, 106, 110). The additional mechanical information that can be obtained from photoacoustic techniques is desirable both for research purposes and for clinical applications in quantitative differentiation of tissue constituents.

2.4.2. Brillouin microscopy—Brillouin microscopy is a method to indirectly measure the mechanical properties of materials without the need for mechanical excitation (Table 9). Brillouin scattering is a type of inelastic scattering, similar to Raman scattering, that occurs due to density fluctuations of molecules within a structure (111, 112). The frequency of these acoustic fluctuations (phonons) relates to the elastic characteristics of the material. The shift in light frequency due to interaction with phonons, known as a Brillouin shift, can be

recorded at many points to provide a volumetric map of the tissue mechanical properties (113), as shown in the elastic strength map of a bovine cornea in Figure 4.

This technique has significant advantages over existing mechanical testing methods. First, most traditional techniques in biomechanics involve measurement of bulk structures excised from tissue samples. Brillouin microscopy enables in vivo measurement without excision of samples, providing more accurate measurements of physiological tissue properties. Additionally, although stimulated Brillouin microscopy can also increase signal strength, no extrinsic mechanical stimulation is necessary to achieve imaging contrast (114). Brillouin microscopy also uses confocal imaging instrumentation, yielding a resolution similar to that of confocal microscopy and enabling measurement of mechanical property distributions within single cells (115).

A significant drawback of Brillouin microscopy is that signal attenuation is very high in all but the most translucent samples. Although imaging depth in transparent ocular components can provide full depth characterization, imaging of opaque and turbid tissues is constrained to a depth of well under 100 μm . Therefore, the use of Brillouin microscopy is limited to natively transparent or optically cleared tissues. However, as this technique develops further and optical clearing techniques increase in reliability, it will become a powerful tool for quantitative imaging of tissue structure (116).

3. DISCUSSION

3.1. Comparison of Techniques

In this section, we compare the abilities of the optical modalities presented above.

3.1.1. Analysis—This subsection presents a qualitative analysis of the above-described modalities on the basis of the assessment criteria established in Section 1.4.

3.1.1.1. Resolution: Existing techniques used to study tissue structure in vivo are often limited in their ability to resolve features relevant to tissue structure. MRI, CT, and ultrasound cannot resolve heterogeneity at the microstructure level, an area sorely lacking in the study of tissue structure. Bright-field and other transmissive light microscopy techniques have better lateral resolution, but physical sectioning limits their axial resolution. More advanced, specialized optical techniques can achieve resolution on the order of micrometers, well above the resolution needed to image tissue structure. Two-photon and confocal microscopy have the highest resolutions out of all the modalities discussed here, although OCT, SALS, and Brillouin microscopy are all capable of resolving fine tissue structure.

3.1.1.2. Scale: Traditional histology techniques are heavily limited by light scattering, which restricts imaging of all but thinly sectioned samples (on the order of 5–10 μm thick) (28). More advanced optical techniques are capable of some level of optical sectioning, but only to certain depths before both scattering and absorption obscure the image. In these systems, longer illumination wavelengths that are less affected by scattering and absorption in tissue can attain deeper penetration, but image contrast and resolution are often correspondingly lower. Longer exposure times can sometimes help in these situations, but both spatial and

contrast resolution suffer as deeper sections of tissue are probed. New techniques will need to detect subtle interactions of light and tissue, which occur only at confined focal volumes, and the encoded information must remain unaltered throughout the imaging depth. OCT is currently the best option for deep imaging greater than 1 mm in turbid tissue with adequate resolution. Wide-field techniques, such as SFDI, can provide much greater transverse fields of view in comparison to point-scanning techniques.

3.1.1.3. Nondestructiveness: Nondestructive imaging methods can be interpreted in two ways. The first regards a nondestructive measurement as one in which the act of physical measurement does not affect the sample in any way. Optical techniques are extremely well suited to this purpose, as their level of interaction with tissues is relatively low energy and usually does not cause any major damage when used properly. The second, more rigorous interpretation requires that all preparation involved be equally nondestructive. Many of the preparation methods for optical imaging—including physical sectioning, optical clearing, fixing, and use of contrast agents—can limit in vivo application due to their harmful nature. This is especially true with respect to physical sectioning required for larger fields of view in nearly all optical systems. Systems that enable one to resolve images at greater depths by mitigating signal attenuation, or by relying on nonindigenous contrast agents, can eliminate the necessity of physical sectioning. Most of the optical techniques discussed here are relatively nondestructive during measurement, but none has the scale of nonoptical techniques (MRI and CT) that do not require physical sectioning of the material.

3.1.1.4. Quantitative measurement: Most histological and nonoptical imaging techniques tend to rely entirely on qualitative analyses of sample morphology, usually performed by a trained physician or researcher. This leaves room for error on the basis of both variances in the observer's background/experience and general tissue variances between individual patients, tissue types, and especially tissue regions. Although there have been many attempts to analyze tissue using more quantitative morphometry techniques, these methods still rely on image processing techniques that are less robust and cannot be universally applied to large, varying databases of sample images (118). This is especially true when the image data do not correspond to contrast that is directly related to small-scale differences in tissue composition. More quantitative optical metrics of tissue structure can allow more objective thresholding of boundaries and features during digital processing of tissue data, which will lead to more impartial diagnostic and screening methods for physicians and more accurate inputs to computational models for researchers. Quantitative image information can ultimately give rise to more standardized and robust analyses of tissue structure. In advanced optical techniques, information about tissue can be encoded within light to ascertain more quantitative metrics, including optical, chemical, and mechanical properties that can ultimately lead to better differentiation and characterization of tissue and tissue structure (2). The spectroscopy techniques presented here, along with Brillouin microscopy, provide an incredible amount of additional quantitative information that can be used for both research and clinical purposes.

3.1.1.5. Functional imaging: As mentioned above, functional imaging is key to gaining an understanding of tissue beyond homeostatic or in vitro conditions. Traditional wide-field

optical techniques are generally not limited by image acquisition speeds, but newer techniques that utilize point scanning require rapid scanning mechanisms (21). The scale of temporal change in tissues should be gauged in order to determine how rapidly systems need to acquire image data. For observations of tissue structure during stress relaxation, image acquisition speed must vary throughout the experiment—initial relaxation is rapid (requiring more than 100-Hz acquisition), then rapidly slows down, requiring an image frame only every 10 s or more (119). There will always be trade-offs among field of view, resolution, and scanning speed, and functional imaging speed is often the least important consideration. Therefore, although imaging at video rate or faster is desirable, it is often infeasible for many circumstances. Of the techniques discussed here, OCT and SFDI have the most capable functional imaging speeds.

3.1.2. Trade-offs—Here we discuss the inherent trade-offs among the assessment criteria.

3.1.2.1. Resolution/field of view/imaging speed: In any imaging system, resolution and field of view are inherently coupled with image acquisition time (119–121). Because most high-resolution imaging systems scan a single point at a time, large fields of view necessarily have longer acquisition times. This is a huge consideration for functional imaging—if high temporal resolution is paramount, then spatial resolution or field of view must be limited to compensate. Currently, imaging techniques are limited by their ability to physically scan throughout the field of view. Future systems aiming to achieve functional imaging while maintaining large fields of view and high spatial resolution must increase overall signal strength to reduce exposure times and enable higher rates of scanning, or they must adopt wide-field paradigms to reduce the number of dimensional scans needed.

3.1.2.2. Field of view/destructive sectioning: Many of the techniques discussed above have limited optical sampling depths, and image integrity is reliable only for shallow depths due to the attenuation of light by either scattering or absorption. For larger depths to be probed, the sample must be sectioned to thicknesses equal to the imaging depth of the system. This process is obviously destructive to the tissue and requires that it be removed from its native environment, restricting accurate functional imaging. Future optical techniques will benefit by confining their measurements to depth-independent focal volumes.

3.2. Applications

In this section, we describe applications of optical imaging of tissue structure.

3.2.1. Bioengineering—Below we present applications in the engineering and development of biomimetic biomaterials.

3.2.1.1. Computational modeling: Computational models of tissues and living tissue systems have given rise to a greater understanding of mechanistic behavior and response to disease and disrepair. However, the inputs regarding structure, composition, and material properties must be very precise for these models to accurately simulate real tissues (122). Some of the most successful models used to describe tissue growth and remodeling require additional inputs about individual fiber families and their orientation, necessitating good

knowledge of microscale tissue structure (93, 123). One of the most common types of morphological data for biomechanical modeling is the fiber orientation distribution function, which can be expressed as either a 2D or 3D function. There are several standard ways to determine various quantities (e.g., preferred directions, degree of orientation, skew, kurtosis) (134–137). In order to represent the fiber distribution at each point in the tissue, transformation of the distribution into a fourth-rank structure tensor is desirable so as to minimize requirements for data storage and computational intensity in large data sets. These structure tensors act as concise mathematical descriptors of the fiber orientation properties and enable close-fit reconstruction of the original orientation distribution function (ODF) by use of well-defined operations. From the ODF one can compute all fiber orientation–related parameters, such as preferred direction and orientation index, a measure of in-plane fiber splay.

Computational modeling can also be applied to spatially accurate, 3D nodal data sets, from which one can obtain valuable sample-specific information about spatial variation in microstructural properties via gradients of scalar, vector, and tensor values throughout the transmural thickness of the tissue. Additionally, 3D reconstructed data can then be visualized using isosurfaces, vector fields, and tensor glyphs in the ParaView application (created by Kitware, Clifton Park, New York). All of these analysis methods have been validated using 3D data sets in which the ODF varies locally by spatial position. This information is combined with mechanical property databases to model and predict the structure–function behavior of various tissues. Actual databases of tissue structure information, such as the one presented in Figure 5, are now available for modeling and simulation studies. To date, this method has been used to characterize the soft tissue microstructure of bovine tendon, porcine aortic valve leaflets, infarcted porcine myocardium, and porcine sclera.

3.2.1.2. Tissue engineering: Information about native tissue structure and material properties is necessary to achieve truly biomimetic synthetic tissues that can sustain functionality in a highly dynamic biological environment (2, 124). More quantitative, high-resolution measurement techniques are necessary to differentiate heterogeneous tissue constituents at microstructural levels. Additionally, these techniques enable one to validate the structure and composition of synthetic tissues and to more easily compare them with the native tissues that they aim to replace.

3.2.1.3. Growth, development, and remodeling: Knowledge about tissue structure and composition is vital to understanding the processes underlying tissue growth and remodeling (125). Growth and remodeling are driven by a combination of factors, including cyclic loading, initial states of structure and composition, external forces, and intrinsic biochemical factors that regulate growth (126). Improved measurements of tissue structure, composition, and morphology are necessary to better understand how tissue develops during growth and reforms after injury.

3.2.2. Diagnostics—In this section, we discuss applications of optical imaging in clinical settings.

3.2.2.1. Screening: There are major, obvious benefits of so-called virtual biopsies, which would allow physicians to discern malignant tumors without needing to perform invasive biopsies and subsequent histology. Tissue structure and morphology have long been used to identify and differentiate between malignant tumors, benign tumors, and healthy tissue (127, 128). Screening and diagnostic tools that are nondestructive to the tissue are vital both for increasing patient comfort and for decreasing residual care costs. Additionally, virtual biopsy can allow more comprehensive screening coverage, resulting in fewer false negatives, which often occur with random biopsies (129, 130).

3.2.2.2. Image-guided therapy: Real-time tissue imaging can allow surgeries and procedures to be treated as dynamic exercises, rather than static protocols, in which the surgeon can assess progress more reliably throughout the process (131–133). Modalities that can differentiate tumors with high precision can more explicitly identify disease boundaries, which can often be achieved through structure-sensitive contrast.

3.3. Future Directions—Below we discuss anticipated trends in the development of optical imaging tools to assess tissue structure.

3.3.1. New (multimodal) technologies—Future imaging techniques must meet two major, nontrivial goals: to eliminate signal attenuation as a function of depth and to increase image acquisition speeds for appreciable fields of view. The main problem with both tasks is that the same tissue features that contribute to signal contrast from scattering and absorption also ultimately contribute to attenuation. Diffusely/multiply scattered light generally causes information loss related to specific structures, instead providing only a signal integrated along the entire scattering depth. Most advanced optical modalities are capable of high resolution, nondestructiveness, and highly quantitative measurements. Their trade-offs and limitations involve mainly the inability of optical systems to probe much beyond a few millimeters in turbid media, limiting their capability beyond superficial tissue regions. New technologies must confine measurements to finite volumes (one envisions something similar to two-photon absorption) and avoid signal attenuation both to and from the measurement area. Moreover, more rapid imaging methods are necessary both for functional imaging and for high-resolution/large field-of-view imaging of whole tissues. These efforts for improved optical imaging technology start with improved image contrast (to decrease exposure times), better scanning instrumentation, and wide-field modalities to decrease scanning times and increase field of view.

3.3.2. Desired functionalities—In research related to the study of soft tissue, including tissue engineering and biomechanics, characterization of tissue composition and quantification of structural properties are very important. As research problems become more specific and advanced, researchers will increasingly need ways to understand and describe the roles of structural elements in the multiscale behavior of soft tissues. Researchers primarily need imaging techniques that maintain the resolution of current systems while providing additional quantitative characterization, such as tissue composition or fiber structure, across larger scales. Of secondary importance are additional functional

imaging techniques that achieve the rapid image acquisition rate required to observe dynamic events related to tissue structure, such as fiber realignment and stress relaxation.

In the clinical environment, the role of imaging modalities is that of a diagnostic tool. Invaluable clinical imaging systems will incorporate the ability of optical techniques to noninvasively probe soft tissue structure and the ability to quickly and correctly make diagnoses or surgical decisions. Medical applications of optical techniques include rapid screening, patient-specific therapies, and real-time image-guided surgery. Future clinical imaging technologies need to characterize in situ tissue states at various scales and depths without compromising nondestructiveness. The high-resolution features of some modern optical techniques may become desirable in certain fields of medicine, but typically the resolution of microstructure elements is not crucial in a clinical setting. Most critically, optical techniques for soft tissue structural analysis should be “smart” imaging tools that can diagnose disease through identification of specific structural indicators.

Finally, new imaging technologies should be integrated into areas of existing practice. New techniques, especially those replacing older histology methods, must to some extent mimic the older processes. Both physicians and researchers are much more comfortable when new technologies emulate the usage of older methods because that allows relative comparison between new techniques and existing standards. Therefore, new imaging modalities will be most successfully integrated into current practice when their image data are represented in ways that are familiar to the field of traditional histology.

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Figure 1.
Comparison among standard imaging modalities in terms of observing biological tissue.

Figure 2.

In vivo two-photon imaging in an intact neocortex. The brain surface (*left*) has been imaged to a depth of 800 μm (63).

Figure 3.

(a) Grayscale image, (b) SALS image, and (c) polarized SFDI image of a pulmonary heart valve leaflet showing directional fiber orientations. Note that the polarized SFDI has much higher resolution than the SALS image (86). Abbreviations: SALS, small-angle light scattering; SFDI, spatial frequency domain imaging.

Figure 4.

Brillouin imaging of the cornea. (a) A cross-sectional Brillouin image of a bovine cornea, revealing the decreasing modulus with depth. The horizontal and vertical spans are 5 mm×0.5 mm. (b) En face Brillouin image of the cornea optically sectioned at a shallow depth. (c) A Brillouin image of a deeper section. The frequency shift is directly correlated to the stiffness of the material, and the longitudinal bulk modulus can be derived from the measurement (117).

Figure 5.

(a) Apex view of the endocardial surface of intact infarcted ovine myocardium. (b) Transmural cross section of tissue with endocardium at the top. (c) Three-dimensional visualization of transmural microstructure map, showing the preferred fiber direction.

Table 1

Summary of confocal reflectance/fluorescence laser scanning microscopy

Source of contrast	Interfaces of tissue components (scattering due to refractive index change), absorption
Imaging technique	Point or line scanning, reflective
Resolution	Axial and lateral, <1 μm (diffraction limited)
Field of view	Axial, <0.3 mm; lateral, >1 mm
Functional imaging speed	Less than video rate (30 Hz) for meaningful, high-resolution applications
Major application areas	Histology and diagnostic pathology, cellular imaging, biochemical and molecular labeling
Radar chart

Table 2

Summary of two-photon microscopy and second harmonic generation

Source of contrast	Nonlinear photon absorption/emission by fluorophores
Imaging technique	Point scanning
Resolution	Axial and lateral, <1 μm (diffraction-limited focal volume)
Field of view	Axial, <1 mm; lateral, >1 mm (hardware limited)
Functional imaging speed	Less than video rate (30 Hz) for small fields of view
Major application areas	Intrinsic contrast imaging of cells and tissues; structural and functional molecule labeling
Radar chart

Table 3

Summary of optical coherence tomography

Source of contrast	Interfaces of tissue components
Imaging technique	Point scanning
Resolution	Axial, 1–10 μm (coherence limited); lateral, 10 μm (diffraction limited)
Field of view	Axial, <5 mm; lateral, >1 mm (hardware limited)
Functional imaging speed	Less than video rate (30 Hz)
Major application areas	Ophthalmology, optical biopsy, intra-arterial imaging
Radar chart

Table 4

Summary of polarization imaging

Source of contrast	Directional scattering induced by structural/fiber orientation
Imaging technique	Can be adapted to most imaging techniques
Resolution	Depends on paired imaging technique
Field of view	Depends on paired imaging technique
Functional imaging speed	Requires acquisitions for each polarization angle
Major application areas	Identification of fiber orientation, birefringent structures; specular reflection isolation

Table 5

Summary of small-angle light scattering

Source of contrast	Directional scattering of laser light due to microstructural/fiber orientation
Imaging technique	Point or raster scanning
Resolution	Axial, depth averaged; lateral, <0.5 mm (limited by beam diameter and raster step size)
Field of view	Lateral, >1 cm
Functional imaging speed	Requires raster scanning for large field of view, >20 min
Major application areas	Soft tissue microstructural properties, quantification of fiber orientation, index of fiber alignment
Radar chart

Table 6

Summary of spectroscopic techniques (Raman, diffuse reflectance, laser-induced fluorescence)

Source of contrast	Absorption, scattering, and fluorescence by molecules
Imaging technique	Probe, point scanning
Resolution	Axial, integrated along depth; lateral, >100 μm
Field of view	Axial, <1 mm (limited by depth penetration)
Functional imaging speed	Long exposure times for Raman
Radar chart

Table 7

Summary of spatial frequency domain imaging

Source of contrast	Absorption and scattering of light
Imaging technique	Wide-field structured illumination
Resolution	Axial, integrated along depth; lateral, >100 μm (optics dependent)
Field of view	Axial, <1 mm (limited by depth penetration)
Functional imaging speed	Less than video rate (30 Hz)
Radar chart

Table 8

Summary of photoacoustic techniques

Source of contrast	Optical absorption
Imaging technique	Point scanning
Resolution	Axial and lateral, $>20\ \mu\text{m}$
Field of view	Axial, $>3\ \text{mm}$
Functional imaging speed	Less than video rate (30 Hz) for high-speed, functional imaging
Major application areas	Imaging of tissue structures with high optical absorption (blood vessels, melanin), hemodynamics, bulk mechanical characterization
Radar chart	<p>The radar chart displays the performance of photoacoustic techniques across five metrics: Nondestructiveness, Resolution, Scale, Quantitative, and Functional imaging. The chart features a red pentagon representing the technique's performance, which is relatively balanced across all metrics, with a slight emphasis on Nondestructiveness and Functional imaging.</p>

Table 9

Summary of Brillouin microscopy

Source of contrast	Inelastic scattering of light due to acoustic phonons
Imaging technique	Point scanning, reflective/transmissive
Resolution	Axial and lateral, <10 μm (diffusion limited)
Field of view	Axial, <5 mm; lateral, >1 mm (scanning hardware limited)
Functional imaging speed	<1 Hz for two-dimensional images
Major application areas	Imaging of optical structures (cornea, lens); optically clear tissue imaging and elastography
Radar chart