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Journal

American Journal Of Pathology, 192(2)

ISSN

0002-9440

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Publication Date

2022-02-01

DOI

10.1016/j.ajpath.2021.10.010

Peer reviewed

The American Journal of Pathology, Vol. 192, No. 2, February 2022

The American Journal of
PATHOLOGY
ajp.amjpathol.org

REVIEW

Slide Over

Advances in Slide-Free Optical Microscopy as Drivers of Diagnostic Pathology

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Accepted for publication
October 18, 2021.

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Conventional analysis using clinical histopathology is based on bright-field microscopy of thinly sliced tissue specimens. Although bright-field microscopy is a simple and robust method of examining microscope slides, the preparation of the slides needed is a lengthy and labor-intensive process. Slide-free histopathology, however, uses direct imaging of intact, minimally processed tissue samples using advanced optical-imaging systems, bypassing the extended workflow now required for the preparation of tissue sections. This article explains the technical basis of slide-free microscopy, reviews common slide-free optical microscopy techniques, and discusses the opportunities and challenges involved in clinical implementation. *(Am J Pathol 2022, 192: 180—194; https://doi.org/10.1016/j.ajpath.2021.10.010)*

Histopathology is a crucial technique used to evaluate disease state at the tissue level.[1] In a typical clinical pathology assessment, a clinician collects a biopsy sample or surgical resection from a patient. The sample is sectioned and placed on microscope slides in a histology laboratory and then evaluated by a pathologist using a microscope. Tissue processing and staining for histopathology generate optical contrast that can be useful in distinguishing cell nuclei, cytoplasmic components, extracellular matrix structures, and other microscopic features. These microscopic morphologic structures provide useful information about the pathologic condition of the tissue, which a trained pathologist can interpret to diagnose a disease, understand the severity of a condition, or assess the status of a surgical margin.[1]

Sample preparation is the crucial first step of histopathology examinations. In the most commonly used approach to histopathology, formalin-fixed, paraffin-embedded (FFPE) permanent slides are generated.[2] In this almost universally used procedure, formalin-fixed tissue is first dehydrated with a serial solvent exchange, and then is embedded in paraffin, which has mechanical properties that support thin sectioning. The processed sample is then manually embedded in additional paraffin to make a solid block and cut into thin slices with a microtome. The resulting sections are mounted on glass slides, rehydrated, and stained with dyes to generate color contrast visible under a bright-field microscope.[3] In most major hospitals, associated histology laboratories are responsible for this routine process.

Assessment of histology slides by a pathologist can be time-sensitive in certain situations, such as when a surgeon wants to determine the presence of cancer foci at the margins of tumor resection during a procedure. Intraoperative consultation (IOC) is conducted by an on-site surgical pathologist, who examines tumor resections rapidly

Supported by NIH grant R01EB028635 (R.M.L.).
Disclosures: Y.L. and M.W.J. are cofounders of OpsiClear LLC; R.M.L. is a cofounder of HistoliX Inc. and inventor of microscopy with UV surface excitation and fluorescence imitating bright-field imaging in patents.

https://doi.org/10.1016/j.ajpath.2021.10.010

prepared via cryosectioning (also called frozen sectioning) as a quick alternative to FFPE-based microtome sectioning.[1,4] Freezing tissue is much faster than is FFPE, and a typical IOC can take between 20 and 60 minutes, depending on complexity and the number of samples examined.[5] However, the results of IOC are often less reliable than are permanent slides due to the presence of artefacts associated with cryosectioning.[6] Usually, one or more FFPE permanent slides are consulted in succeeding days to re-examine the margin status.

The primary imaging modality for both FFPE and cryosections is bright-field microscopy, designed to visualize thin specimens mounted on glass slides.[7] Bright-field microscopes depend on white light transmitted through specimens only a few microns in thickness to generate contrast based on the binding patterns and absorbance properties of chemical stains that highlight different tissue and cellular components. For over a century, bright-field microscopy has remained the most affordable, convenient, and reliable strategy for histology visualization.[8]

More recently, the development of digital pathology has further improved the utility of anatomic pathology.[9] Automated slide scanners equipped with digital cameras and robotic slide handling can be used to rapidly capture entire microscope slides as high-resolution digital images.[10] The data are uploaded to cloud-based health information−management systems, and pathologists can review the images anywhere and anytime.[11,12] Empowered by advanced computational methods, digital pathology also enabled the development of various automated data-analysis tools that promise to assist pathologists in interpreting the resulting images more efficiently and accurately.[13]

However, there are some intrinsic limitations to conventional histopathology techniques. For the FFPE permanent slides, the sample-preparation procedure is laborious and time-consuming. Although laboratory automation has simplified some tissue-processing steps (eg, solvent exchange, staining) in large clinical laboratories, a significant portion of the process (eg, embedding and sectioning) is still performed by skilled personnel, essentially limiting speed and throughput.[14] Even with the more rapid cryosectioning procedure, surgeons still need to pause the operations before the IOC results become available. The wait can lengthen the procedure time for the patient and contribute to a significant portion of the operating-room charges.[15] Especially for IOC, there is a need for faster and more effective pathology analysis.

A straightforward way to improve the efficiency of clinical pathology is to reengineer histopathology workflows (Figure 1) to bypass the tedious sectioning process. In this article, slide-free histopathology is defined as any imaging method that is not dependent on slide-based microscopy.[16] In the past, it was difficult to visualize thick tissue samples under a microscope due to a lack of good optical-section capability to generate in-focus images from optical slices representing just a few microns in thickness.[17] It was

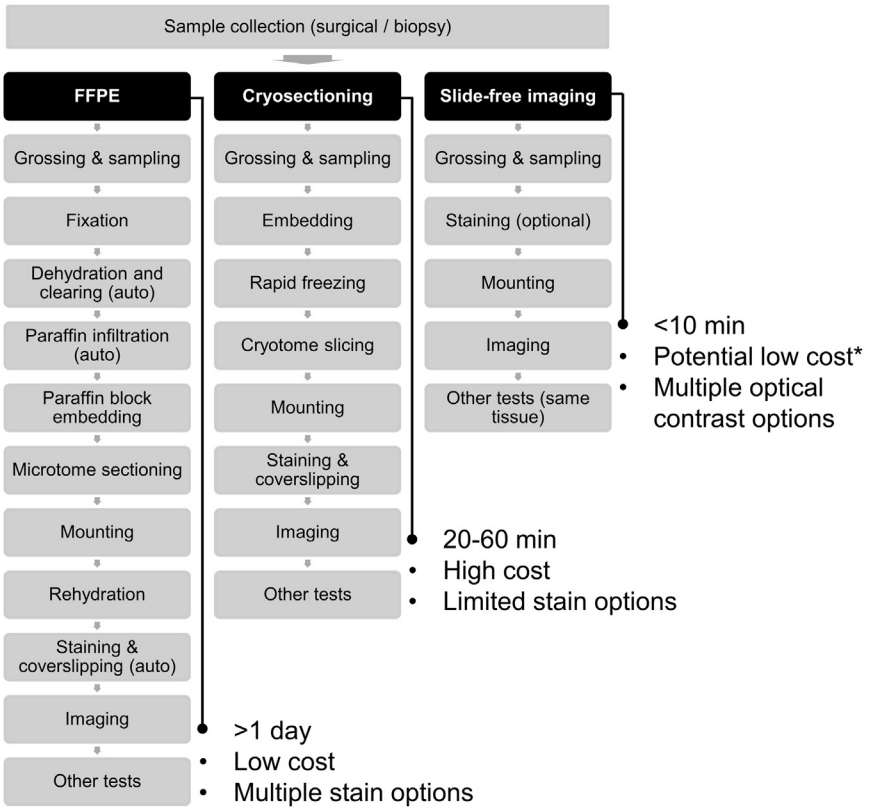

Figure 1 Comparison of histopathology workflows. *The low-cost aspect applies to certain techniques (eg, microscopy with UV surface excitation) that use inexpensive setup. FFPE, formalin-fixed, paraffin-embedded.

also difficult to record and process the data before digital imaging became widely available.[9] This situation has largely changed over the past few decades. Advancements in sensors, light sources, optics, computing power, and algorithmic methodologies have drastically broadened the strategies available for optical microscopy, enabling multiple effective technical paths to meet the emerging need for slide-free histopathology. Especially in recent years, these new technologies have become increasingly competitive in cost, speed, availability, functionality, and performance. They also demonstrate great potential to disrupt the century-old histopathology workflow. This review introduces common design considerations of microscopy-based slide-free histopathology technologies, describes emerging slide-free histopathology imaging modalities, and discusses the future clinical implementation of these technologies. (Note: Discussion of other, nonoptical methods such as X-ray-based slide-free histopathology is not included.)

Design Considerations

Developing slide-free histopathology is a complicated task involving sample preparation, optical imaging, data handling, and clinical workflow integration. Although slide-free histopathology can be accomplished with various optical-imaging techniques, they share many common design considerations. This section highlights some universal technical challenges and aspects relevant to all slide-free histopathology methods.

Optical Contrast

Optical contrast is the difference in signal intensity that makes microstructures distinguishable in a microscopy image.[18] It can be generated by the intrinsic optical properties of the tissue or induced by extrinsic chemical treatment (eg, stains). In optical microscopy, image contrast can be categorized by either the nature of the optical signal (eg, fluorescence) or the type of emphasized histology structures (eg, cell nuclei). In principle, any optical contrast between tissue microstructures can be used to interpret and understand the microanatomy of the tissue. Some types of optical contrast, such as nuclear contrast, are particularly salient for disease detection and classification.[19] Historically, optical contrast in microscopy originated from chemical stains and is visible to the naked eye. Today, optical imaging no longer depends on visibly appreciable properties, but can rely on fluorescence,[20] absorption,[21] scattering,[22] nonlinear optical properties,[23] photoacoustic effects,[24] and combinations of multiple optical contrasts.

Sample Preparation

A simple sample-preparation procedure is the key advantage of many slide-free histopathology techniques. Most such techniques require one or two quick processing steps to prepare whole-mount tissue samples. The first step, tissue resampling, involves removing unwanted tissue, cutting the tissue sample into appropriate dimensions, and exposing the region of interest. These procedures are often performed by a pathologists' assistant.[25] For extrinsic optical contrast, the second step is to label the sample surface with dyes. This process is usually performed by rinsing the sample with a dye solution, followed by a few washes. Overall, this process is considerably simpler and faster than FFPE or cryosectioning. In addition to imaging the surface of the tissue, some slide-free histopathology methods (eg, light-sheet microscopy) generate three-dimensional (3D) histology data in a tomographic format.[26,27] A special sample-preparation technique, optical clearing,[28] increases the transparency of the samples and enables optical imaging deep into the tissue.

Sample Containment

Mounting tissue specimens in a slide-free imaging system is not trivial. Unlike conventional slide-based microscopy, in which thin-sectioned samples are flattened between a slide and coverslip, slide-free imaging often involves thicker, irregularly shaped tissue pieces that require specialized sample holders to stabilize the sample, flatten the sample surface, and align it to the focal plane of the imaging system (eg, US patent 9797816B2).[29] For pathology applications, it is also important to keep the sample in the desired orientation and to prevent unwanted motion during imaging. At the microscopic scale, the surface of the tissue is never perfectly flat. A well-designed sample holder can apply compression across the tissue specimen to eliminate large surface fluctuations and prevent associated image artefacts.[30] A sample holder also serves as a reliable fluid reservoir in certain slide-free imaging systems in which the sample is immersed in a fluid (eg, US patent publication US20210124162A1).[31] Special mechanical design measures need to be implemented to enable hazard-free fluid handling.

Resolution and Field of View

Different pathologic applications pose different imaging requirements. Some applications require a large field of view (FOV) to cover the entire sample, while others need high resolution to uncover detailed subcellular morphology.[32] In the design of a slide-free imaging system, the intended use affects such design inputs. As with conventional slide-based microscopy systems, the optical resolution and FOV of slide-free imaging systems are also primarily determined by the properties of the microscope objectives used. There is always a trade-off between resolution, FOV, and imaging speed.[33] Unlike conventional slide-based microscopy systems, in which it is easy to switch between several microscope objectives, some more complex imaging systems have design constraints (eg, the

need for precise positioning of the back-focal plane of the objective) that limit lens interchangeability.

Optical Sectioning

Optical sectioning describes various optical methods that eliminate out-of-focus signals, improving image contrast and signal-to-noise ratio.[17] Without the help of physical sectioning, optical sectioning is a fundamental technical feature of a vast majority of slide-free imaging techniques. Until recently, the most commonly used optical-sectioning strategy was point-scanning–based imaging such as confocal microscopy. Optical signals are collected from single focal spots, and the out-of-focus signals can be easily removed by spatial filtering or other optical methods. More recently, advanced optical techniques such as interferometry (eg, full-field optical coherence tomography),[34] specialized illumination (eg, UV fluorescence[35] and light sheet[36]), and computational methods (eg, deconvolution[37]) are implemented in slide-free histopathology to provide optical-sectioning capability with simple, cost-effective camera sensors. These optical-sectioning techniques will be discussed in *Imaging Modality*.

Depth of Field

Depth of field (DOF) is defined as the distance between the deepest and the shallowest objects that can be sharply imaged under a microscope.[38,39] For high-resolution imaging systems that use high–numerical aperture Gaussian optics, the DOF is often thinner than the topologic excursions of the whole-mount sample surface (10-μm DOF versus 100-μm excursions). Moreover, many slide-free histopathology imaging systems are designed to image a large FOV by tiling many images together. Different imaging locations often have a slightly different focal level due to small tilts and curvatures of the sample surface. It can take extra effort and time to keep the entire scan area in focus. The easiest way to improve the DOF is to use lower numerical aperture-detection optics, but this can impair lateral resolution. The most straightforward extended DOF method involves collecting multiple Z-stack images, but this comes with a significant speed penalty.[39] To overcome the limitation, advanced optical techniques such as dynamic focusing,[40] wavefront coding,[41] and computational deconvolution[42] can also improve the DOF without a major impact on imaging speed. An imaging system with good DOF performance will be more accommodating with respect to sample-flattening issues, potentially simplifying the whole-mount sample-mounting process.

Imaging Speed and Throughput

Superior imaging speed is crucial for most slide-free histopathology techniques, as most of them are developed to compete with conventional slide-based histopathology imaging. Speed can be affected by multiple factors, but typically one issue dominates in each system configuration.[43] Identifying and addressing the key speed-limiting factor can lead to improved performance.

It is worth highlighting a few commonly encountered issues in most slide-free histopathology imaging systems. First, exposure or pixel dwell time is the major speed-related limitation of systems with a limited photon budget. Second, for point-scanning–based systems, achievable speed of the scanning device (eg, galvo mirror) limits the frame rate. Third, occasionally the repetition rate of a pulsed light source or a detector sampling rate can be limiting. Fourth, in camera-based systems, speed is frequently affected by achievable data-transfer rates (eg, 600 MB/second for USB 3.0). Fifth, with high–frame rate imaging systems, mechanical movement (eg, focusing, stitching, and filter switching) is another common speed-limiting factor. Sixth, in some techniques, multiple images need to be acquired and processed in real time. Computationally intensive calculations could also limit the speed of such systems. With simple, fast sample preparation, the throughput of a slide-free histopathology imaging system is directly limited by the imaging speed and the number of devices implemented.

Image Processing and Rendering

There are several common image processing tasks in slide-free histopathology. Most slide-free histopathology techniques have the capacity to combine multiple FOVs into a single large image. This process often requires automated image-stitching algorithms that correct field distortion, normalize intensity, align images, and merge them into a single matrix.[44,45] Each imaging-system design may require a specialized stitching algorithm that is adjusted for that specific optical design. As with conventional whole-slide digital pathology, the stitched slide-free histopathology images are usually very large and thus challenging to display with minimal lag on a computer screen. Implementing a pyramid/multiscale large data format file system is necessary to manage and display such stitched images. Image contrast can be distinct in different dynamic ranges, and may be better visualized after high–dynamic-range correction. Unfamiliar optical contrast generated by some imaging systems may be difficult for untrained pathologists to interpret. Color-remapping algorithms can be implemented to combine and translate novel contrasts into pathologist-familiar color schemes such as hematoxylin and eosin (H&E).[46]

Imaging Modalities

Imaging modality refers to a type of imaging technology based on an underlying physical principle that produces images. Imaging modalities can be classified in various

Table 1 Major Slide-Free Histopathology Imaging Modalities and Key Technical Aspects

Imaging modality	Optical contrast mechanism	Light source	Detector	Sample format*	Staining method	Optical-sectioning mechanism	Extended depth-of-field mechanism	Primary speed-limiting factor	Selected references
Bright-field microscopy/ whole-slide scanners	Absorption	Incoherent white light	Camera	Microscope slide	Color dyes	N/A	N/A	Sample stage movement	9,10
Confocal microscopy	Elastic scattering, fluorescence	CW laser	Point detectors (PD, PMT, APD)	Whole-mount surface, limited volume	Intrinsic light scattering, fluorescent dyes optional	Spatial filtering	Stacking	Scanner speed	47–51
Nonlinear microscopy	2P, 3P, SHG, THG, SRS, CARS	Ultrafast laser	Point detectors [PD, PMT, APD, lock-in amplifier (SRS)]	Whole-mount surface, limited volume	Intrinsic tissue property, dyes optional	Energy density threshold	Stacking	Scanner speed, low SNR	23,52–56
Structured illumination microscopy phase mask detection	Fluorescence	Incoherent visible light	Camera	Whole-mount surface	Fluorescent dyes	Structured illumination, computation	Stacking, computational methods	Sample stage movement	57–59
MUSE	Fluorescence	UVC LED	Camera	Whole-mount surface	Fluorescent dyes	Shallow UV penetration	Stacking, computational method	Sample stage movement	35,60,61
FIBI	Fluorescence absorption hybrid	405 nm LED	Camera	Whole-mount surface	Color and fluorescent dyes	Surface staining	Stacking	Sample stage movement	F. Fereidouni, R.M. Levenson, unpublished data
FFOCT	Elastic scattering	Low-coherent white light	Camera	Whole-mount volume	Intrinsic light scattering in tissue	Interferometry	Stacking	Sample stage movement	62–64
Light-sheet microscopy	Fluorescence	CW laser	Camera	Whole-mount volume	Fluorescent dyes	Light-sheet illumination	Stacking	Camera speed, stage movement	26,36,65
Photoacoustic microscopy	Photo-induced sound wave	Nanosecond laser	Ultrasound transducer	Whole-mount surface, limited volume	Intrinsic light absorption in tissue	Energy density threshold	Stacking	Laser repetition rate	66,67

*Indicates what has been demonstrated in slide-free histopathology and may not represent a fundamental limitation (eg, limited volume versus volume).

2P, two-photon; 3P, three-photon; APD, avalanche photodiodes; CARS, coherent anti-Stokes Raman scattering; FFOCT, full-field optical coherence tomography; FIBI, fluorescence imitating bright-field imaging; MUSE, microscopy with UV surface excitation; N/A, not applicable; PD, photodiodes; PMT, photomultiplier tubes; SHG, second harmonic generation; SNR, signal to noise ratio.

ways, such as optical-contrast mechanisms and specific hardware configurations. Table 1[47–67] provides a list of common imaging modalities for slide-free histopathology and summarizes their technical signatures. In the following section, common imaging modalities are grouped into two categories: camera-based microscopy and scanning-based microscopy. Under each category, some popular techniques are highlighted.

Camera-Based Modalities

Before the invention of cameras, conventional microscopes were designed to relay visible light from the focal plane of the microscope to the observer's retina. Most of these microscopes were wide-field microscopes, which illuminate and image the entire FOV from either above or below the sample. The bright-field microscope used in clinical

pathology, for example, is a simple form of a wide-field microscope that use white transillumination. The wide-field microscope family also includes microscopes that uses more advanced contrast techniques such as fluorescence, dark-field, phase, and differential interference. At subcellular resolution, most wide-field microscopy techniques are optimized for thin samples.

Today, digital-camera sensors have become highly available and affordable. Digital cameras are faster and more sensitive than are human eyes. They have broader spectral coverage from UV to near-infrared and require simpler optical elements (eg, no eyepieces) to acquire images from wide-field microscopes. Image acquisition can be precisely timed with electronic control signals. Acquired images can be easily processed with a computer and projected to the screen in desired formats. By coordinating imaging acquisition with specialized illumination and hardware control, digital imaging has further enabled workflows and functionalities previously impossible with nondigital microscopes, including the acquisition of meaningful pathology images from nonsliced tissue samples.[68]

Exciting Tricks—Microscopy with UV Surface Excitation and Fluorescence Imitating Bright-Field Imaging

Fluorescence microscopy is an imaging technique widely used in biomedical research. It is also used in clinical pathology for advanced tasks such as multiplexed immunohistochemistry (IHC) imaging.[20] As with bright-field microscopy, wide-field fluorescence microscopy has limited performance in imaging whole-mount tissue specimens. The illumination excites fluorophores from both the surface and the interior of a specimen, resulting in a strong subsurface signal that blurs and degrades the image.[17] A standard fluorescence microscope requires thin samples to achieve high resolution due to limited optical sectioning. Hence, it is not ideal for slide-free histopathology.

A simple and effective epifluorescence illumination strategy to address this problem is the use of an excitation wavelength with limited tissue-penetration depth.[60] Most clinically relevant tissue specimens are semitranslucent in the UV−visible−near-infrared spectrum, which is widely used in conventional fluorescence microscopy. However, the attenuation coefficient of the tissue drastically increases below approximately 300 nm as a result of strong UV absorption by organic compounds. Therefore, optical sectioning at the tissue surface can be easily achieved using UVC illumination because most UVC light can effectively penetrate only a few micrometers below the tissue surface.

Exploiting this useful tissue-optical property, microscopy with UV surface excitation enabled slide-free histopathology through an extremely simple optical configuration: a whole-mount sample compressed against a UV transparent optical window, a simple wide-field microscope focusing on the flattened sample surface, and an accessible UVC LED placed between the sample and the microscope objective.[35,60] This versatile design can be implemented in microscope systems in various scales, from benchtop systems with automatic focusing and stitching functionalities[60,69] to an ultra-compact smartphone-based portable microscope.[61]

Microscopy with UV surface excitation has several additional advantages in slide-free histopathology. Notably, while 280- to 300-nm UV illumination can excite many common fluorescent dyes (eg, Hoechst, rhodamine, eosin), many common optical materials (eg, borosilicate glass) are not transparent below 300 nm.[35] Therefore, light emitted at different visible wavelength ranges can be detected and unmixed with a simple RGB camera, and the UV excitation light is excluded without the use of specific emission filters. Images acquired by microscopy with UV surface excitation are directly useful for understanding the microanatomy of the tissue. They can also be color-remapped into virtual-H&E images to help pathologists understand the contrast.[70]

Recently, another novel epifluorescence-based design also demonstrated promising imaging performance for slide-free histopathology using a minimally modified conventional fluorescence microscope, a more common excitation wavelength, and pathology-relevant tissue stains. Fluorescence imitating bright-field imaging employs an epifluorescence microscope with 405-nm excitation and detects the emitted light using an RGB camera (F. Fereidouni, R.M. Levenson, manuscript in preparation). The sample is prepared by staining the surface with an H&E solution—the most common stains used in conventional clinical pathology. After a few quick washes, the tissue is gently compressed against a conventional coverslip (glass) window and imaged directly using the microscope.

The superficial H&E staining plays a crucial role in facilitating the fluorescence imitating bright-field imaging contrast. Eosin strongly fluoresces under 405-nm excitation, and it, along with tissue autofluorescence, combines to generate a uniform, bright background light inside the thick specimen that is remitted to the collection lens. Cell nuclei stained by hematoxylin at the tissue surface absorb some of this remitted light to generate histologic contrast similar to that of a conventional H&E slide. By optimizing the concentration of the dyes, staining/washing time, and acquisition parameters, the strategy can generate histologic contrast useful for pathology analysis. The standard clinical pathology workflow is minimally affected given that H&E staining is also used for standard histology.

Hacking the Microscope—Structured Light and Wavefront Coding

With computational image reconstruction, slide-free histopathology can also be accomplished using specialized illumination and detection configurations. When combined with hardware synchronization and image processing, structured illumination can be useful in removing unwanted background and refining the signal from a desired focal plane, improving the resolution and optical sectioning of the system. By manipulating the wavefront using special optical

elements (eg, phase masks), spatial features at different imaging depths can be modulated with wavefront coding and decoded with post-processing. These strategies have provided multiple technical paths to performing slide-free histopathology imaging based on conventional bright-field microscopes with minor modifications.

Structured illumination refers to the projection of light using user-defined patterns during image acquisition, allowing users to extract additional information from the images with post-processing.[71] This method has many in research-, industry-, and consumer-related applications. In biomedical optical imaging, structured illumination microscopy is well-known for super-resolution microscopy.[72] In slide-free histopathology, the more salient feature of structured-illumination microscopy is its superior optical-sectioning capability.[57] In an optical-sectioning, structured-illumination microscopy system, the structured-illumination pattern is generated by a movable illumination mask or a programmable spatial light modulator at the conjugate focal plane of the microscope, projecting defined strip patterns.[73] To produce an optically sectioned image, the system takes a few images with varying illumination patterns and reconstructs them into a single image.[57] The image-reconstruction algorithm uses simple math and can be streamlined to support a high frame rate. For instance, recent studies have demonstrated that such structured illumination microscopy–based, slide-free histopathology imaging systems could be used for imaging a multicentimeter, large FOV with micron-level resolution within a minute.[58,59]

Another technique that can augment imaging performance of camera-based microscopy systems is modulation of the detected light wavefront, often referred to as wavefront coding.[74] By introducing specially designed optical phase-masks at the pupil plane or the conjugate pupil plane, the system can encode spatial information of the detected signals in the image plane.[41] The encoded information can be later decoded with algorithms to generate an image with improved resolution and DOF. This technique was recently implemented in a slide-free histopathology technique, DeepDOF, to facilitate single-shot, extended DOF imaging of uneven whole-mount tissue surfaces.[37] With the help of deep learning, DeepDOF simultaneously optimizes the optical phase-mask design and the decoding convolutional neural network algorithm in the design stage. This design improves the DOF fivefold (from 40 to 200 μm) while preserving the near-diffraction–limited resolution required for visualizing subcellular features such as cell nuclei. The extra DOF eliminates the need for taking multiple images and significantly improves the speed of the system.

Seeing the Invisible—Light-Sheet Fluorescence Microscopy

Nondestructive 3D histopathology is a special category of slide-free histopathology with enormous clinical potential.[27] In conventional histopathology, 2D imaging provides only a single cross-section within a bulk specimen. Most of the sample volume is disregarded in this process. Although this 2D information is adequate for primary clinical pathology analyses, the ignored 3D spatial information is an under-explored gold mine of histopathology data. Understanding the 3D organization of the tissue at the microscopic level can potentially bring novel insights about disease states, prognostic outcomes, and potential treatments.

With conventional thin-slice–based microscopy techniques, generating a microscopic 3D reconstruction of a pathology specimen is complicated. Researchers have to prepare microscope slides from every single layer of the tissue, image the slides one by one, and reconstruct the series of 2D images into a 3D volume.[75] Although this process can be largely automated by serial sectioning block-face imaging, it is still unsuitable for routine clinical uses. There is a need for more robust methods to image whole-mount histopathology samples in 3D.

Recent advances in optical clearing provide an alternative technical path for 3D histopathology imaging. Optical clearing refers to sample-preparation methods that make tissue samples optically transparent by eliminating light-scattering microstructures in the tissue and homogenizing the refractive index of the tissue.[76] The optically cleared tissue samples can be nondestructively imaged in 3D using various fluorescence imaging techniques. Although optical clearing was originally developed for research, some methods have recently been developed specifically for applications in 3D histopathology.[27,28,36] (It is worth noting that optical clearing is different from the clearing step in the conventional pathology workflow.)

Light-sheet fluorescence microscopy (LSFM) is an effective technique for imaging optically cleared tissue samples and uses a fluorescence microscope with a special illumination setup.[77] The excitation light illuminates a thin sheet coming from the side of the cleared specimen, aligned to the focal plane of the detection optics. Given that the sample volume outside the focal plane is not exposed to the excitation light, the sample is effectively optically sectioned via the light sheet. By mechanically translating the sample in small steps, an LSFM system can acquire a 3D image of the entire whole-mount sample.

LSFM has a broad design-parameter space. Applications of LSFM range from subcellular to whole-organ imaging.[78] In virtual 3D histopathology, the desired specification is a medium to large FOV (>0.5 mm) with a capacity to resolve individual cell nuclei (eg, <1 μm lateral resolution) and at least two fluorescence channels to capture both nuclear and cytoplasmic contrasts.[26] From a sample-preparation standpoint, the specimen is often whole-mount stained with at least a nuclear dye (eg, DRAQ5) and optically cleared.[36] The cytoplasmic contrast can be generated with either a structural stain (eg, eosin) or intrinsic autofluorescence. Smaller samples (eg, <1 mm thickness) are better suited for achieving more manageable processing times and quality control in a clinical setup.

Mounting cleared tissue samples in a conventional LSFM can be a challenge. In most conventional LSFM systems, a

cleared sample is mounted on a mechanical holder with super glue or the sample is embedded in delicate containers. The mounted sample needs to be carefully positioned in a fluid chamber. This process can significantly complicate the workflow of a routine clinical procedure. The open-top light-sheet microscope design addresses this problem using a convenient inverted-microscope geometry, allowing cleared tissue samples to be dropped into a fluid reservoir with a large, accessible opening at the top of the system.[36] The light-sheet and the detection optics are oriented at 45 degrees about the horizon below the sample. With two-axis lateral scanning, a 3D volume of the sample can be easily reconstructed. With additional multimagnification, multi-immersion, and rapid optical-clearing features, this optical design has demonstrated extraordinary performance and usability in 3D histopathology samples with a wide range of geometries.[65,79]

Deep, Vibrant Cells—Full-Field Optical Coherence Tomography

Fluorescence microscopy often depends on extrinsic staining for the generation of optical contrast. Although the staining procedures of most slide-free histopathology techniques are simple, fast, and minimally affect the quality of the sample, any extra manual steps present potential burdens in a clinical process. Therefore, several slide-free histopathology techniques have been developed that rely on intrinsic tissue optical properties for generating contrast. Full-field optical coherence tomography (FFOCT) is one of the most effective methods of camera-based, label-free, slide-free histopathology.[80]

The optical design of a conventional FFOCT system is based on a Linnik interference microscope and low-coherence interferometry.[80] The FFOCT system is basically a Michelson interferometer with one mirror replaced by the sample and the other (reference) mirror mounted on a piezo stage. The optical path length from the light source to the sample focal plane is equal to the optical path length from the light source to the reference mirror. The reflected light from the sample and the reference mirror are combined at the camera, producing a 2D interferogram. This configuration provides excellent optical-sectioning capability determined by the coherence length of the light source. In the case of a tungsten lamp, it is often less than a few micrometers.

The interferogram can be processed in several ways to generate different types of image contrast. In one method, the reference mirror is axially translated by the piezo stage to produce interferograms from slightly different depths. A few consecutive interferograms are computationally reconstructed into one image. The optical contrast represents the intrinsic reflectance of the different tissue microstructures, suitable for mesoscale histologic assessment over a large FOV.[62] However, it provides few feature details about the morphology and organization of individual cells. A second method uses the same hardware configuration but a different image-acquisition procedure for probing the subcellular activities of individual cells. This contrast mechanism is often referred to as *dynamic cell imaging* or *dynamic FFOCT*.[63] Dynamic FFOCT measures the temporal response of the fringe fluctuations in the 2D interferograms, corresponding to small movements within the sample. Multiple raw images are acquired at a fixed reference-mirror location at a high frame rate. Temporal properties of each pixel or small area can be extracted to generate various dynamic contrasts.

In fresh tissue samples such as surgical resections, most cells are still alive even after the tissue is removed from the body. The subcellular activities of different cells tend to generate distinct movement patterns and generate different temporal responses.[64] Time-domain signatures such as mean power spectrum density, mean frequency, and temporal SD can potentially be used for classifying cell types.[63] They can also be used to identify cell boundaries when combined with the scattering-based FFOCT morphologic contrast. These functional improvements significantly enhance the usefulness of FFOCT for identifying cancer cells in fresh surgical resections.

Scanning-Based Modalities

Scanning-based imaging modalities are also popular in slide-free histopathology. Unlike a wide-field microscope that images an entire field of view simultaneously, a laser scanning microscope uses a focused laser beam to detect a single focal point at a time. The location of the focal spot can be rapidly translated across the sample by opto-mechanical devices such as galvanometer scanners and linear actuators. The resulting signals from the points are detected with a point detector (eg, photodiodes, photomultiplier tubes, avalanche photodiodes), and mapped to corresponding scan locations to form an image.

Old but Gold—Confocal Microscopy

Confocal microscopy was the first imaging modality implemented in slide-free histopathology[49,81] and demonstrates excellent spatial resolution and optical sectioning. The lateral resolution is primarily determined by the point-spread function of the laser focal spot, which often approaches the diffraction limit of the optical system. A pinhole before the detector rejects out-of-focus light and provides robust optical sectioning, allowing important histology features to be imaged at the plane of focus while rejecting unwanted signals from the rest of the tissue.

Reflectance and fluorescence are two commonly used modes of contrast in confocal microscopy. A reflectance confocal microscope directly detects reflected light from the sample. Because reflectance is typically an intrinsic tissue optical property, reflectance confocals can be used with unprocessed fresh-tissue samples. Reflectance contrast varies with the illumination wavelength. A confocal

fluorescence microscope uses an incident beam to induce fluorescence emission. Before detection, an optical filter removes reflected excitation light, passing only the resulting fluorescence signal.

Most clinically relevant tissue specimens have limited natural (auto-) fluorescence. Therefore, the surface of the tissue is often stained with fluorescent dyes such as acridine orange and eosin before confocal imaging. Today, most conventional confocal microscopes can be used for detecting multiple fluorescent and reflection wavelength ranges by a change of the illumination and filter settings. A few optical contrasts with different implications in pathology can be combined in a single image to improve histologic visualization. The contrast can be further enhanced by advanced image-processing strategies, for example, color remapping to mimic the appearance of an H&E-stained slide.[46]

Confocal microscopy is disadvantageous compared to camera-based microscopy techniques in several ways, including slower imaging speed, a requirement of more maintenance, and higher instrumentation costs.[82] Nonetheless, confocal microscopy has the longest history in slide-free histopathology imaging. It has been thoroughly tested and supported by many clinical studies, especially for use in dermatology procedures,[49,83,84] and is already widely available among major hospitals. Without complicated processing, data generated by confocal microscopy is directly useful for pathology evaluations. These advantages make confocal microscopy a prominent candidate for a broad range of slide-free histopathology applications.

Colorful, Label-Free—Nonlinear Microscopy

Nonlinear microscopy refers to advanced laser-scanning microscopy based on various nonlinear optical effects. Four common types of nonlinear optical contrast in slide-free histopathology include multiphoton excitation, second (or third) harmonic generation (SHG), stimulated Raman scattering (SRS), and coherent anti-Stokes Raman scattering (CARS). Nonlinear microscopy enabled several new types of optical contrast for slide-free histopathology, including label-free contrasts that are useful for identifying specific tissue microstructures of great importance in pathology.

Two-photon (2P) fluorescence excitation and SHG are the two most commonly used nonlinear microscopy methods implemented in the same optical configuration.[23] In 2P fluorescence excitation, targeted fluorophores simultaneously absorb two photons, enter into an excited state, and then emit fluorescence at a wavelength shorter than that of the excitation beam.[85] In SHG, two photons of the same energy level are combined into a single photon with doubled frequency, usually in a nonlinear optical material. A commonly used light source is a Ti-sapphire laser emitting within the wavelength range of 750 to 800 nm—suitable for relatively deep tissue penetration. As the nonlinear effects occur only at the focal point of the light, optical sectioning can be achieved without spatial filtering. The emission is spectrally filtered to separate the 2P and SHG signals. As with 2P and SHG, three-photon and third harmonic generation signals can similarly be utilized.[86]

The 2P−SHG combination is good for use in slide-free histopathology.[52,53] The improved imaging depth and optical-sectioning capability make imaging of irregular tissue surfaces easy. In addition, they generate unique optical contrasts for differentiating various histologic structures. 2P absorption is a robust way of exciting extrinsic labels such as cell nuclei stains. It can also be used for detecting intrinsic fluorochromes, such as nicotinamide adenine dinucleotide and flavin adenine dinucleotide—metabolic signatures useful for characterizing certain types of cancers.[87] SHG provides a valuable contrast for (some) collagen fibers, an important molecular constituent relevant in characterizing many pathologic conditions.

Coherent Raman contrast, including SRS and CARS,[88,89] is also deployed for use in slide-free histopathology. *SRS* and *CARS* probe vibrational states of molecules.[90,91] As abundant biomolecules, such as proteins and lipids, have distinct chemical compositions, their intrinsic chemical signatures can be differentiated using SRS and CARS, without the need for extrinsic chemical stains.[89] SRS and CARS use an illumination design more complex than those of 2P and SHG, as two synchronized ultrafast laser sources (pump and stroke) need to be used. However, it is straightforward to incorporate combined 2P and SHG in these coherent Raman imaging systems. The multiplexed contrast contains an abundance of histologic information that is not available from conventional histopathology. A notable application of SRS in slide-free histopathology is the imaging of fresh tissues in brain cancer resections.[56] As brain tissue has a high abundance in lipid and protein, the SRS contrast can be used for effectively differentiating neural fibers and lipid-containing structures. In clinical studies, SRS was used for accurately determining malignancy status and for differentiating the grade of brain tumors.[92]

A limitation of nonlinear microscopy is instrument complexity and its attendant cost. Nonlinear microscopy often uses a beam scan scheme similar to that of confocal microscopy, but the illumination setups are often more involved. Most nonlinear optical effects happen at a high-power density and thus rely on high−peak-power ultrafast lasers to generate special optical contrast. Fortunately, recent advancements in ultrafast laser designs have reduced the challenge of high design complexity by the implementation of more compact fiber-based femtosecond laser systems.[56]

Listen to the Light—Photoacoustic Microscopy

Photoacoustic microscopy (PAM) is an emerging hybrid imaging technology that combines optical illumination with acoustic detection.[93] Photoacoustic describes a physical phenomenon that occurs when a material absorbs light: The

absorbed energy is converted into heat and causes a local thermal expansion that generates a wide-band acoustic wave. PAM typically exploits this effect using a scanning-microscopy configuration using nanosecond lasers as the light source and an ultrasonic transducer as the detector.

PAM is more advantageous than other point scanning–based imaging systems in several ways.[24,93] Although the most well-known strength of PAM is its outstanding imaging depth, its most prominent advantage in slide-free pathology is its capacity to characterize light absorption. In a reflectance confocal microscope, the back-scattered signal is a mixture of reflectance and absorption, with little specificity of the structure type and only a weak dependence on the wavelength. In contrast, the PAM contrast is mostly dependent on the absorption of the light, strongly associated with the spectral absorptivity of the tissue. Therefore, PAM contrast can be more biologically meaningful at specific wavelengths.[94] For example, nucleic acids absorb UVC light more strongly than do proteins and lipids.[66] Using a 266-nm laser, PAM can be used to generate nuclei contrast in fresh, unstained tissue, making it an ideal option for visualization in histopathology.[67]

Alone, PAM generates only one type of optical contrast. A multimodal configuration of PAM and other optical-imaging modalities can be used for generating a fuller picture of tissue micromorphology.[95] Because a scanning-based imaging setup is used in PAM, the functionality of confocal fluorescence microscopy can be easily integrated into a PAM system.[96] The integration of PAM with other scanning-based imaging modalities such as Fourier-domain optical coherence tomography has also been reported.[95]

Clinical Implementation

Slide-free histopathology is an emerging technical concept with great potential to revolutionize clinical pathology and improve the overall efficiency of health care. The growth of these novel imaging technologies comes with both enormous opportunities and critical challenges. The clinical implementation and adaptation of slide-free histopathology require close collaboration between scientists, engineers, clinical professionals, and regulatory experts. There is a need for workable business (reimbursement) models as well.

Clinical Workflow Integration

Although slide-free and conventional histopathology procedures have many of the same intended uses, the slide-free workflow is drastically different from the conventional methods. The implementation of these disruptive technologies in practice may be met with significant resistance from the well-established clinical pathology infrastructure, which is almost completely based on slide-based microscopy. Most histology laboratories are designated to process FFPE or cryosectioning slides, and pathologists are trained to read these standard products. Implementing slide-free histopathology in a standard clinical setup will require additional personnel, space, training, and knowledge. To ensure a smooth transition from the slide-based process to a slide-free process, innovators in this space should consider clinical-integration strategies ahead of time.

After preliminary laboratory validation of the technology, researchers often conduct clinical pilot studies by collaborating with medical professionals in research hospitals. The same sample is then processed with standard histopathology, and the results are compared. Such side-by-side comparisons serve multiple important functions for new slide-free histopathology methods, including: i) validating the new design by verifying the usefulness and benefits of the new information; ii) linking the new information and image contrast to existing knowledge on pathology; iii) preparing data for the future regulatory approval; and iv) generating insight into human factors, user interface, and user experience considerations.

In the future, as the technologies are thoroughly validated, demonstrate effectiveness, and achieve regulatory approval, they may be used for primary pathology assessments in certain applications. At first, clinician end-users may still expect a standard FFPE-based confirmation to validate results. Therefore, when developing technologies for slide-free histopathology, most developers ensure that the new sample-preparation procedures do not interfere with the standard clinical histopathology workflow. Most slide-free histopathology techniques work with fresh and minimally processed tissue samples, which minimally affect downstream FFPE and cryosectioning procedures. Some slide-free imaging techniques do involve tissue-processing steps (eg, staining) that could potentially affect the outcome of the standard procedures (eg, PCR analysis). It is necessary to verify the quality of the tissue after these chemical processes so that the same samples can be reused in other downstream clinical procedures for primary diagnosis or confirmation. Currently, many clinical tests (eg, IHC, PCR, and other molecular bioassays) are performed on FFPE slides. To completely replace conventional methods such as FFPE, it is also necessary to develop new workflows for these additional tests.

Finally, it is important to understand the target end-users and envision the potential clinical workflow of slide-free histopathology. For instance, IOC is a common target application of many slide-free histopathology techniques. In a conventional workflow, surgeons request intraoperative histology studies, technicians prepare the sample, and pathologists analyze the images. This process involves sample transfer between the operating rooms and surgical pathology laboratories, requiring communication between all parties to ensure proper sampling and processing of the specimen. Slide-free histopathology technologies will face a situation similar to that of cryosectioning-based IOC. Therefore, in addition to advancing the imaging technologies themselves, there are essential needs for developing new training

materials, user interfaces, and user experience to educate, connect, and coordinate all user groups.

Integration of Other Technologies

Slide-free histopathology can be integrated with many existing technologies in digital pathology, notably telemedicine[12] and automated pathologic analysis supported by artificial intelligence.[13] Conventional surgical pathology consultations, especially IOC, are conducted in hospital settings. A pathologist has to be physically present in a clinical setup to read slides under the microscope. By digitally scanning the data and uploading it to health information systems on the cloud, pathologists can now conveniently review the data remotely on computers or mobile devices. This approach drastically improves turnaround time and productivity in clinical pathology evaluation. The well-established digital pathology infrastructure can be especially beneficial for the clinical implementation of slide-free histopathology technology. Data from slide-free histopathology are naturally digital images. Therefore, the existing remote digital pathology infrastructures can be readily repurposed for most slide-free histopathology techniques, presenting the data in pathologist-familiar formats and telepathology workflow. As pathologists become increasingly familiar with digital interactions with data and consultations, it also reduces the effort for training the users. Artificial intelligence–empowered digital-pathology workflow is also quickly becoming a reality. These advanced computational methods can be useful in automating histopathology-based diagnosis, segmentation, prediction, and scoring in large pathology data sets, supporting decision-making and report generation for pathologists.[97] Although most of today's artificial-intelligence algorithms are developed for use with standard pathology data such as H&E images, the principles of these algorithms can easily be applied to slide-free histopathology data through new network training or transfer learning.[98] Artificial intelligence could further be useful in improving the visualization of histopathology data by reducing image artefact, enhancing image definition, and remapping the image to more pathology-familiar color contrast. Still, it is worth noting that artificial intelligence–based image processing, especially that involving generative networks (eg, cycleGAN), can potentially introduce nonreal information. It is necessary to thoroughly validate these methods before implementing them in clinical applications.

In today's clinical setup, IHC, in-situ hybridization, and flow cytometry (liquid pathology) have already become commonly used diagnostic techniques. However, these techniques often require tissue destruction, generating lower-dimensional data, and leaving valuable 3D information behind. By introducing advanced 3D slide-free histopathology (eg, light-sheet microscopy), molecular pathologists can reveal the spatial distribution of pathology-relevant molecules by imaging the intact whole-mount labeled tissue sample and generating information not available from conventional 1D/2D data.[27] This information may provide additional knowledge about the pathology of the disease. Some slide-free histopathology techniques may also work with other emerging biomedical technologies, such as advanced molecular biomarkers (eg, aptamer, small peptides) and personalized tissue cultures. These new technologies often involve significant histopathology imaging. Compatible slide-free imaging techniques can serve as fast and efficient imaging platforms for these technologies, helping clinicians better understand the disease state of the tissue, predict the potential risks, and determine the prognostic outcomes.

Regulatory Concerns

Slide-free histopathology imaging systems are novel medical devices, often requiring extensive regulatory reviews by local authorities before broad clinical implementation. In the United States, the Food and Drug Administration (FDA) regulates the sale of medical devices. In most cases, FDA clearance must be obtained before imaging systems are entered into the market for clinical use and diagnosis. There are three potential pathways for a slide-free histopathology imaging device under the current FDA regulatory framework: Class I (low-risk devices, general control); Class II (intermediate-risk devices, general controls, and special controls); and laboratory-developed tests (tests developed by clinical laboratories that are not considered as medical devices). Class I classification is the simplest regulatory pathway. Most imaging systems can be classified as Class I medical devices under the category of Microscope and Histology Supplies, exempted from FDA Premarket Notification 510(k). One example is the stimulated Raman histopathology system (Invenio Imaging, Santa Clara, CA). The imaging system received a 510(k) exemption in 2017 under the code IBM (Microscopes and Accessories), and the sample holder received a 510(k) exemption in 2019 under the code KES (Tissue Processing Equipment). A similar example is the Vivascope 2500 confocal microscope (CaliberID, Andover, MA), which was classified as Class I in 2018. However, these approvals do not cover any intended use of systems for primary diagnosis. A slide-free imaging system may also be approved for a specific intended use, potentially as a Class II device. In this case, a novel slide-free imaging system must be reviewed under the *de novo* pathway if there is not a substantially similar product on the market. Substantial laboratory testing and clinical trials are often required for this regulatory pathway. Currently, there is no slide-free histopathology system approved as a Class II device. Alternatively, some slide-free histopathology applications can avoid the medical-device regulatory process by marketing the technologies as laboratory-developed tests. Currently, the regulatory burden of laboratory-developed tests is much lower than that of medical devices.

However, they can be used only in Clinical Laboratory Improvement Amendments–certified laboratories, limiting the penetration of these technologies in clinical procedures.

The ultimate ambition regarding slide-free histopathology is the replacement of conventional slide-based pathology. However, the regulatory pathway is much more challenging and unpredictable. As there are many similarities between whole-slide imaging (WSI) and slide-free histopathology technologies in terms of the indications of use and potential health risks, the regulatory history of WSI systems provides some insight into this uncertainty.[99] In 2017, the FDA approved the first WSI system, IntelliSite Pathology Solution (Philips Healthcare, Andover, MA), as a Class II medical device for use in reviewing surgical pathology slides prepared from biopsied tissue. This process took more than a decade. A clinical study in around 2000 surgical pathology cases from various anatomic sites was useful in establishing FDA approval. During the process, regulators once considered classifying WSI as Class III in 2009. As suggested by the *de novo* review (DEN160056; *https://www.accessdata.fda.gov/cdrh_docs/reviews/DEN160056.pdf*, last accessed December 16, 2021), the major potential risks of WSI include delayed results and misdiagnosis due to inaccurate or missing results. According to Code 21 CFR864.3700 (*https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?fr=864.3700*, last accessed December 16, 2021), premarket notifications of WSI systems need to include results for component-level bench testing, system-level performance testing, and product-level evaluation (Table. 2).

Table 2 Detailed Regulatory Requirement for Whole-Slide Imaging as Described in Code 21 CFR864.3700

Premarket notification items	Detailed requirements
Indications for use	Specify the tissue specimen
Component-level testing	Slide feeder, light source, imaging optics, mechanical scanner movement, digital imaging sensor, image processing software, image composition techniques, image file formats, image review manipulation software, computer environment, and display system
System-level testing	Color reproducibility, spatial resolution, focusing, whole-slide tissue coverage, stitching error, and turnaround time
Product-level performance demonstration	Precision variability, data reproducibility, clinical studies, and human factor engineering

Economic Concerns

With fewer tissue-processing steps, lower labor costs, and faster turnaround times, slide-free histopathology may prove to be more economical than standard histopathology even if the imaging instrumentation is expensive. However, although some slide-free histopathology techniques demonstrated the potential to replace slide-based histology for specific histopathology applications, conventional slide-based histopathology will remain the gold standard for primary clinical decisions for years to come. Implementing slide-free histopathology technologies to supplement the existing pathology infrastructure will thus require additional investment in capital equipment, facilities, and personnel. Multiple devices may be required for redundancy and process parallelization, further increasing the cost. In some countries, regulations require long-term storage of patient data. It can also take extra resources to store and manage the extra slide-free histopathology data, especially if microscope glass slides are completely abandoned. The cost and data maintenance are open questions to be addressed in the future.

Special clinical motivations for a slide-free histopathology technology, such as faster IOC for certain surgeries, may be required to facilitate early market penetration. It will be important to demonstrate quantifiable reduction in cost of care and improvement in procedures by introducing a new product into clinical workflow. For instance, health care cost reduction may result from decreased usage of other hospital resources (eg, operating room time, space, other pathology equipment) or improved health care quality (eg, diagnostic accuracy and outcomes). Reducing the potential occupational hazards (eg, exposure to formaldehyde) and waste (eg, disposable materials) of the present methods are also potential benefits. Overall, it will be crucial to ensure that the added benefits outweigh the added costs.

Summary

Slide-free histopathology is an emerging area. Faster turnaround time, simpler operating procedures, and opportunities to generate novel clinical insights will be useful in determining its potential impact. Combining slide-free histopathology with advanced data-processing methods can make clinically relevant pathology workflow more efficient and affordable, improve quality, decrease cost, and potentially revolutionize anatomic pathology even in the near future. Currently, most slide-free histopathology technologies are in the early stages of development. Before broad clinical adaptation can occur, these technologies must be thoroughly validated to ensure their safety and efficacy, a task requiring close collaboration of all stakeholders in this field.

Author Contributions

Y.L. wrote the manuscript. M.W.J. and R.M.L. provided significant guidance and edited the manuscript.

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