

A new wide field-of-view confocal imaging system and its applications in drug discovery and pathology

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ABSTRACT

Conventional widefield light microscopy and confocal scanning microscopy have been indispensable for pathology and drug discovery research. Clinical specimens from diseased tissues are examined, new drug candidates are tested on drug targets, and the morphological and molecular biological changes of cells and tissues are observed. High throughput screening of drug candidates requires highly efficient screening instruments. A standard biomedical slide is 1 by 3 inches (25.4 by 76.2 mm) in size. A typical tissue specimen is 10 mm in diameter. To form a high resolution image of the entire specimen, a conventional widefield light microscope must acquire a large number of small images of the specimen, and then tile them together, which is tedious, inefficient and error-prone. A patented new *wide field-of-view* confocal scanning laser imaging system has been developed for tissue imaging, which is capable of imaging an entire microscope slide without tiling. It is capable of operating in brightfield, reflection and epi-fluorescence imaging modes. Three (red, green and blue (RGB)) lasers are used to produce brightfield and reflection images, and to excite various fluorophores. This new confocal system makes examination of large biomedical specimens more efficient, and makes fluorescence examination of large specimens possible for the first time without tiling. Description of the new confocal technology and applications of the imaging system in pathology and drug discovery research, for example, imaging large tissue specimens, tissue microarrays, and zebrafish sections, are reported in this paper.

Keywords: Confocal, drug discovery, fluorescence, imaging, microscope, microscopy, pathology, tissue, wide field-of-view, microarray

1. INTRODUCTION

1.1. Optical Microscopy

An optical microscope is an instrument designed to help observe fine details of the specimen. It must have three features, *i.e.*, high magnification – producing a magnified image of the specimen; good resolution – distinguishing the neighboring fine details; and high contrast – making the details visible to the human eye or imaging device. The usefulness of any microscope depends not only on its ability to magnify, but more importantly, upon its ability to resolve details.

Optical microscopy refers to the techniques and applications of light microscopes, and can be classified as lens-based and lensless microscopy.¹ The ultimate imaging performance of lens-based microscopy is limited physically by optical aberrations, wavelength and diffraction of light, and out-of-focus light in the image. Aberration corrections can be made to the microscope optics, more importantly the objectives; light diffraction sets the optical resolution limit; and out-of-focus light in the image degrades both the resolution and contrast. Two optical techniques are available to reduce the out-of-focus influence in the image: confocal microscopy²⁻⁸ which employs a point-like illumination and a detection pinhole aperture, and nonlinear microscopy including two photon and multiphoton fluorescence microscopy,⁹⁻¹⁵ and second and third harmonic generation.¹ Near-field microscopy falls into the lensless category.¹ Computer image processing techniques can also be used to enhance the image quality, for example, deconvolution analysis of conventional widefield images to remove out-of-focus blur.

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Two types of optical microscopes are in common use today, *i.e.*, conventional widefield microscopes¹⁶⁻¹⁸ and the more recent confocal microscopes.²⁻⁸ Both of these have relative different advantages and disadvantages.^{6, 19, 20}

1.1.1. Conventional widefield light microscopy

The name of widefield microscopy comes from the fact that the entire field of view is uniformly illuminated and observed. In both brightfield (transmission) or epi-fluorescence imaging, the specimen needs to be sufficiently thin (*e.g.*, 2 - 5 μm), so that structures at different sections can be clearly observed to obtain maximum resolution. Absorption difference by the structures as light passes through the specimen and fluorescence emission difference from the structures when excited produce contrasts that reveals the details.

In widefield light microscopy, specimen staining is a routine process. Most living biological specimens are virtually transparent, and often remain so after fixation, especially after being sliced into thin sections.²¹ Imaging contrast can be realized by treating them with natural or synthetic dyes, a process called staining.^{21, 22} The commonly used hematoxylin and eosin staining colors the nuclei of cells blue-purple and other tissue components pink.^{23, 24} Generally, dyes are stains used in brightfield microscopy, and fluorophores in fluorescence microscopy. The term “stain” refers to both dyes and fluorophores.²³ A dye is an organic aromatic molecule containing the requisite groups that provide visible color and permit molecular binding to a material. A fluorophore (fluorochrome) is a fluorescence agent capable of fluorescing under illumination. Some fluorescence agents absorb only in the ultraviolet and therefore are colorless when viewed in visible light.

1.1.2. Confocal laser scanning microscopy

There are two technical aspects in confocal laser scanning microscopy (CLSM),²⁻⁶ *i.e.*, confocality and scanning. In an ideal confocal imaging system, both the illumination and the detection are confined to a diffraction-limited spot. The image is recorded by precisely controlled raster-scanning of the focused laser spot across the specimen plane or *vice versa*. The scanning can be realized in various ways, such as x-y stage-stage scanning, x-y beam-beam scanning, x-y beam-stage scanning, or objective lens scanning.²⁵

In a basic confocal imaging configuration, the point illumination source, the specimen, and the detector pinhole are located at conjugate focal planes, hence the name “confocal”. Although the practical confocal imaging systems today are somewhat different from the basic form, the operating principle is still the same, *i.e.*, both the detection pinhole and objective lens take part in the image formation. The confocal effect comes from the confocal pinhole aperture, which prevents out-of-focus light from being detected. In reflectance and fluorescence imaging, the rejection of such optical “noise” is realized in two ways. The point illumination at the specimen reduces and the confocal pinhole at the detector further rejects the “noise” from the areas adjacent to the detection volume being imaged and the sections away from the focal plane. Therefore, confocal microscopy offers greater spatial resolution (a theoretical improvement of a factor of 1.4) than conventional widefield microscopes. With confocal microscopy, it is possible to look deep into cells and tissues without being affected by the “noise” of out-of-focus light. Therefore, it enables 3D imaging through the stacking of digitally captured images of optical sections, and allows the visualization of a thick specimen without the need for physical sectioning. This is the optical sectioning or depth discrimination capacity of confocal microscopes.

For fluorescence imaging, confocal microscopy has a number of advantages compared to conventional epi-fluorescence microscopy. The two most important ones are the ability to eliminate out-of-focus noise (better resolution and contrast) and increased detection sensitivity.²⁰ In addition to light detectors of high sensitivity, the confocal system has the advantage of being able to accumulate (or average) images over time. Also, because of the controllable laser source, there is a reduced risk of specimen bleaching.²⁰

1.1.3. Fluorescence microscopy

By using appropriate fluorochromes, it is possible to identify cells and sub-cellular components with a high degree of specificity amidst non-fluorescing entities or those with different fluorescence properties; and the availability of hundreds of fluorescent labels with known excitation and emission curves and well-understood biological structure targets has accelerated the application of fluorescence microscopy in both clinical laboratories and research.

The basic task of a fluorescence microscope is to provide excitation light to irradiate the specimen, and then separate the much weaker ($\sim 10^{-3}X$) fluorescent light from the excitation light, so that only the emission is observed or detected. Conventional widefield microscopy and confocal microscopy are also the two key techniques for fluorescence imaging.^{26,27} Fluorescence signal emanating from above and below the focal plane creates an out-of-focus haze in the image, which can be removed by using confocal microscopy or through widefield deconvolution microscopy.²⁸ The choice of CLSM and widefield microscopes largely depends on the type of sample, with CLSMs performing better on thick samples and widefield microscopes on thin ones if no deconvolution is applied.

1.1.4. Comparison of widefield and confocal microscopy

Confocal and widefield microscopy differ in light source, illumination mode, sensitivity, resolution, specimen thickness, light exposure, 2D/3D images, data acquisition time, and computing required.¹⁹ A detailed comparison of widefield and confocal techniques can be found in the references.^{6,19} For optical sectioning of thick tissue specimens, although conventional widefield microscopy with image deconvolution analysis²⁹ can also provide good results, CLSM is still the key technique commonly used. However, both the conventional widefield and confocal microscopes in use today are based on standard microscopy objectives. Due to the limited field-of-view of the standard objective lenses, it is necessary to take small images and use tiling techniques for large specimen imaging.

1.2. Pathology, Drug Discovery and Microarray Technologies

Light microscopy has always been a fundamental research tool in pathology. It is the means for observing both morphologic changes and molecular biological changes in cell. Especially, fluorescence confocal microscopy allows both high-resolution analysis and observation of spatially extended areas.

The drug discovery process seeks to develop a biological or chemical entity that, when administered to a patient, will improve disease symptomatology or actually treat the underlying pathophysiological basis of a particular disease state.³⁰ A drug target is a biological entity, usually a gene, mRNA or protein molecule, with which a pharmaceutical interacts. Advances in drug delivery require an understanding of drug design, drug stability and metabolism together with the complexities imposed by the biological system such as cell/tissue penetration, drug-target interaction, and the pharmacodynamic consequences. Fluorescence microscopy provides a comprehensive tool for investigating many of these aspects of drug delivery in single cells and whole tissue.³¹

Microarray techniques, especially DNA microarray and tissue microarray technologies developed recently have made a great contribution to drug discovery and pathology. Fluorescence microscopy is the key technical tool in such applications. A high throughput optical microscopy system that can perform fast, large field-of-view, and high resolution fluorescence and brightfield imaging will be valuable for both pathology and drug discovery, to match the high throughput of the microarray technologies.

1.2.1. DNA microarray

Microarray analysis is based on standard molecular biology, with a principal advantage being higher throughput and greater precision than traditional filter and blotting techniques.³² DNA microarray technology provides a global analysis of gene expression at the level of transcription. It combines standard molecular techniques with high throughput screening. Through the use of microarrays, a single experiment can assay gene expression (transcriptional profiles) across thousands of genes or even the entire genome under experimental or clinical conditions.^{33,34} DNA microarrays are comprised of a library of genes immobilized in a grid on a glass microscope slide. The relative intensities of the two colored signals on individual spots are proportional to the amount of specific mRNA transcripts in each sample, enabling an estimate of the relative expression levels of the genes in samples and control populations.³⁵

1.2.2. Tissue and tissue microarray

Tissue specimens are usually prepared on a standard microscope slide after fixing, embedding, and staining, for the study of anatomical or molecular biology changes in diseased tissue.³⁶ The tissue microarray (TMA) technique enables researchers to extract small cylinders of tissue from histological blocks and arrange them in a matrix configuration in a recipient paraffin block, such that hundreds or even thousands can be analyzed simultaneously

for morphological or molecular changes.^{37,38} The advantages of TMA technology are that it allows simultaneous analysis and high-throughput data acquisition, and amplification of limited tissue resources by providing the means for producing large numbers of small core biopsies, and each specimen can be treated in an identical manner.^{34,39}

Most of the TMA technology comes from the field of cancer research.³⁷ TMAs can be used for clinical investigation of candidate genes and their proteins identified by molecular biology techniques, such as DNA microarrays.³⁸ By revealing the cellular localization, prevalence and clinical significance of candidate genes, TMAs are ideally suitable for genomics-based diagnostics and drug target discovery.

1.2.3. Pathology, drug discovery, and light microscopy

Light microscopy is routinely used in the examination of diseased tissues, cells and fluids on glass slides, for assessing the pathologic changes, and much of the pathology involves the study of cells.³⁶ Since the invention of compound microscopes in the 18th century, and the development of microtomes and the techniques of fixation, embedding and staining in late 19th century,⁴⁰ with continued technical development including lenses of higher numerical aperture, magnification and contrast methods, light microscopes have been important tools for the biological studies of cells and tissues, and identifying micro-organisms causing diseases.¹⁶⁻¹⁸ In fact, the cell theory was started based on microscopic observations. Application of CLSM started to increase rapidly in the 1990s, due to the increased availability of confocal systems thanks to advances in optics, electronics and computer technology, and newly developed fluorochromes with better matching laser lines and brighter fluorescence.⁴¹ Applications of CLSM include fluorescence detection of gene expression profiling using cDNA microarrays,⁴² fluorescence volume investigation and reconstruction of tissue samples,⁴³ identification of diagnostic markers,⁴⁴ and so on.

In molecular drug discovery, DNA and tissue microarrays are playing more and more important roles. The availability of multi-tumor analysis helps overcome the issues of time consumption, intensive labor, and cost in validation of multiple pathogenic markers on multiple cancer tissue specimens.³⁸ Although the technique of TMAs provides the potential of high-throughput data acquisition, only through highly efficient imaging systems with microscopic resolution can the potential be realized.

1.3. Wide Field-of-View Confocal Microscopy

The *wide field-of-view* confocal imaging system presented in this paper* is capable of confocal imaging of large area specimen in a single scan, *e.g.*, imaging an entire standard microscope slide (1 by 3 inches in size) without tiling, while a conventional widefield light microscope or confocal microscope using microscope objectives must acquire a large number of small images of the specimen, and then tile them together, which is tedious, inefficient and error-prone.

Microscopic imaging of large specimens is of considerable interest to life science, partly because of the high throughput efficiency it offers. However, there are only a few academic investigations of wide field-of-view microscopes reported elsewhere, *e.g.*, microphotography with a thin white light slice,⁴⁵ using single spatial frequency grid pattern,⁴⁶ and objective lens array based large field-of-view imaging.^{47,48} The sizes of specimen that can be imaged with conventional widefield microscopes are limited by the field-of-view of the microscope objectives. With the increase of power (magnification) and resolution (numerical aperture), the field-of-view becomes smaller and smaller. When using standard microscope objectives for scanning laser imaging, except for stage-stage scanning, in both the beam-beam and beam-stage scan methods, the scan size is limited by the field-of-view of the objective lenses. In many clinical studies, it is desirable to acquire images of whole diseased tissue sections, such as those of tumors, while retaining microscopic resolution.⁴⁹

The *wide field-of-view* confocal microscopy imaging system reported here can be used for the examination of morphology and molecular biology images of clinical tissue specimens, and high throughput screening in drug discovery through efficient imaging of microarray slides. It is capable of operating in brightfield, reflection and epifluorescence imaging modes. A red, green and blue laser is used to produce fluorescence, brightfield and reflection

*This confocal instrument has been commercialized by Biomedical Photometrics Inc., under the trade name of "TISSUEScopeTM". Website: www.confocal.com.

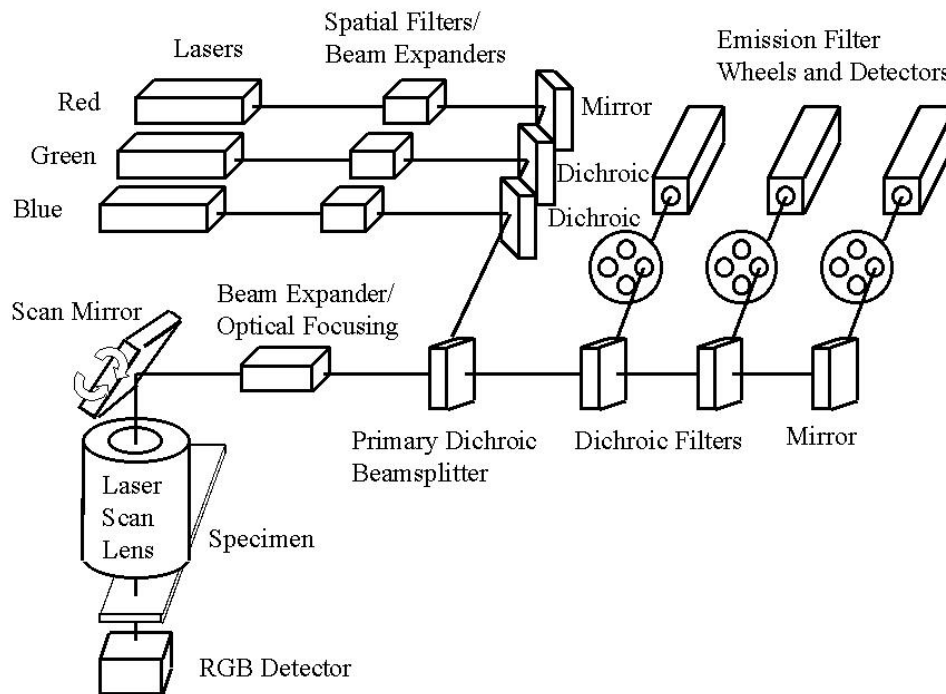


Figure 1. Optical Schematic of Wide Field-of-View Confocal Imaging System

images. This new confocal system makes fluorescence examination of large specimens with microscopic resolution possible for the first time without tiling. The *wide field-of-view* will help fully realize the high throughput potential provided by TMA technology in drug discovery research by offering the capability of imaging the whole slide efficiently with a single scan and using the same data acquisition condition, so that TMA users will not have to look at the array spots one by one using other imaging techniques.^{37,38}

This new confocal microscope technology enabling confocal fluorescence and brightfield imaging of large biomedical specimens is described in the next section. Applications of the *wide field-of-view* confocal imaging system in pathology and drug discovery research, for example, imaging large tissue specimens, tissue microarrays, and zebrafish sections, are also reported in this paper.

2. WIDE FIELD-OF-VIEW CONFOCAL LASER SCANNING SYSTEM

The wide field-of-view confocal imaging system consists of an optics module, an electronics module and a computer and software. The optics module is the main part of the system, through which the unique wide field-of-view imaging function of the system can be realized. The electronics module provides system control, signal processing and data acquisition. The computer and software coordinate the imaging system through the electronics, providing such functions as user interface, and image display and data archiving.

The optical schematic of the system is shown in Fig. 1. The raster scan of the focused laser spot across the specimen is realized by beam scanning in the X (lateral) direction and stage scanning in the Y (longitudinal) direction of the specimen, to produce images in the standard tagged image file format (TIFF). The main components of the optical module are the laser scan lens, excitation/illumination optics, and fluorescence and RGB (red, green and blue) detection optics. The system is capable of confocal imaging in reflection and fluorescence modes. In addition, an RGB brightfield (non-confocal) imaging mode is also available.

2.1. Laser Scan Lens

One of the most important and expensive components of the *wide field-of-view* imaging system is the custom-designed laser scan lens. Currently, two proprietary scan lenses have been designed and built, with specimen magnification, excitation NA and fluorescence collection NA of 10X/0.20/0.35 and 40X/0.50/0.50, respectively. They have a working distance, lateral and axial resolution of 6mm/2 μ m/25 μ m and 3mm/0.5 μ m/4 μ m, respectively.

The laser scan lenses are color-corrected, infinity-corrected, telecentric and F^*Theta (or $F * \Theta$) lenses with the entrance pupil outside the lens system. A infinity-corrected lens allows greater flexibility in the imaging system design. Various dichroic beamsplitters and other accessories can be readily inserted into the collimated beam. With the entrance pupil located outside the scan lens, it is possible to use a scanning mirror of the smallest size to fill the entrance pupil with the excitation beam. On the contrary, the entrance pupil of a standard microscope objective is located inside the lens system. If used for scanning imaging, it is necessary to use relay optics to place the pivot point of the scanning beam at the entrance pupil position.⁵⁰

The laser scan lens has *telecentric* and F^*Theta properties, which are essential for optimal imaging flat specimens and performing linear scan. In a telecentric scan lens, the chief ray always emerges parallel to the optical axis during the scan, *i.e.*, the chief ray of the converging cone of light always is perpendicular to the specimen plane/focal plane. Telecentricity guarantees that the optical measurement is independent of the imaging position across of the field-of-view.^{51, 52}

In an F^*Theta lens, the image height is $H = F * \Theta$, where F is its focal length, and Θ is the scan angle, *i.e.*, the angle formed by the incoming beam with the Z-axis of the scan lens. An F^*Theta lens focuses the collimated beam entering with an angle Θ with respect to the optical axis at a distance $F * \Theta$ from the optical axis. The scanner moves at a uniform angular velocity. For the pixel positions to be uniformly spaced across a flat slide, it is necessary that the image height be proportional to the scan angle.^{51, 53} The $F * \Theta$ property makes it possible to scan the focused spot with constant linear velocity; and a constant data acquisition rate results in equally spaced measurements on the specimen. An F^*Theta lens makes the image data acquisition process easier, because the pixel rate can be synchronized with the angular velocity of the scanning mirror. In an ordinary microscope objective, the pixel position is $F * \tan\Theta$. If the scan angle is small enough, as it usually is in a confocal microscope with a small field-of-view, then $F * \tan\Theta \approx F * \Theta$, so it approximates an F^*Theta lens.

2.2. Excitation/Illumination Optics

The optical schematic of the *wide field-of-view* confocal instrument is shown in Fig. 1. Three solid state lasers, a red laser with a wavelength of 635 nm, a green laser of 535 nm, and a blue laser of 488 nm with an output power of approximately 25 mW each are used as the light sources. The lasers are used as excitation sources for fluorescence imaging, and illumination sources in reflectance and brightfield imaging. Each of the laser beams passes through a spatial filter composed of two positive lenses and a pinhole to filter out high spatial frequency components from the laser source. The spatial filters also operate as beam expanders and collimators. The output from the solid state red laser is not a single line. Therefore, it is necessary to use an excitation filter to clean its output. A circular aperture is placed at the output of each Spatial Filter/Beam Expander, to select only the central portion of the laser intensity of a normally Gaussian intensity profile, so that the entrance pupil of the scan lens is filled with relatively uniform illumination and the maximum numerical aperture (resolution) of the lens is realized.

The three collimated lasers are made colinear with a beam combiner composed of a regular mirror and two different long pass dichroic mirrors. The regular mirror is used for deflecting the red laser to the combiner. One dichroic mirror reflects the shorter wavelength green laser and passes the red laser. Similarly, the other dichroic mirror reflects the blue and passes both the green and red lasers. The colinear laser beams are then reflected by the Primary Dichroic Beamsplitter and fed through the Beam Expander/Optical Focusing Mechanism, which is composed of two apochromatic lenses.

The Beam Expander/Optical Focusing Mechanism further enlarges the beam diameter so that it fills the entrance pupil of the scan lens, to maximize its numerical aperture, and hence the resolution limit. By controlled

adjustment of the axial distance between the two beam expander lenses, the collimated laser beam can be tuned to be slightly diverging or converging, which helps to move the focal plane up and down along the optical axis of the scan lens, to bring thin specimens with different mounting media (e.g., coverslip) in focus. Optical sectioning can be realized by collecting stacks of images at different focal plane positions in thick specimens, which can be used for 3D reconstruction of structures.

2.3. Fluorescence Detection Optics

Fluorescence microscopy provides many opportunities to understand biological structures and processes, such as the possibility of selective excitation in a mixture of different molecular species, the high sensitivity which allows a relatively small number of fluorescence molecules to be detected, with the availability of a variety of fluorescence indicators, and the ability of imaging fluorescence reporter proteins.⁵⁴ In addition, the advantages of higher sensitivity, optical sectioning, and 3D analysis of images available due to confocal microscopy provide more information about biological objects.⁵⁴

For fluorescence imaging, the fluorescence emission from the specimen is collected by the laser scan lens, while the raster scanning (beam scanning and stage scanning) is taking place, and the detected fluorescence signal is de-scanned simultaneously by the scanning mirror. With its diameter reduced by the backward beam expander, the fluorescence light passes the Primary Dichroic Beamsplitter, gets reflected by one of the detection dichroic filters or the detection mirror depending on the wavelengths of the fluorescence band, and passes through the respective emission filter on the filter wheel, before it is focused with the detection lens (achromatic focusing lens, not shown) and arrives at the detection pinhole (not shown) in front of the detector, which helps to reject out-of-focus light so that the confocal effect is realized. Due to the confocal effect, only the light signal from the detection volume located in the focal plane of the specimen can reach the photon detector. The dichroic filters in the detection path are long pass filters, which reflect light of shorter wavelengths and pass longer wavelengths.

The respective emission filters (barrier filters) on the filter wheel stop excitation light from entering the detectors, so that only the fluorescence signals are detected. The emission filters on the motorized filter wheels can be chosen through software. It is important to have efficient optical filters to block the excitation light from entering the detectors, because the fluorescence light is about 1,000 times weaker than the excitation. The three detection channels can be used in simultaneous or sequential acquisition modes. For example, simultaneous fluorescence detection can be used for colocalization analysis; sequential detection can be used to decouple fluorescence spectra when multiple fluorophores are present. Although more time consuming, sequential detection has the advantage of reducing bleed-through of the emission spectrum of one fluorochrome into that of another (cross talk), which results in false positive imaging.

In fluorescence microscopy of thin specimens, although there is little interference from neighboring focal planes within the specimen, a confocal system does provide higher sensitivity and offers sharp images because of its ability to reject glare from the slide substrate and stray light from other sources.

2.4. Reflection Imaging

Confocal microscopy is mostly used for fluorescence imaging in biomedical research, in consideration of its optical sectioning power combined with the specificity of multiple fluorescence labeling to produce improved images of cells and tissues. However, the confocal imaging system can also be used in reflectance microscopy to gather additional information from a specimen. Confocal reflectance microscopy provides information on unstained tissues and those labeled with probes that reflect light. For example, unlabeled cells can be detected in a population of fluorescently labeled cells, or the interactions between fluorescently labeled cells growing on an opaque and patterned substrate can be imaged.⁵⁵

In the *wide field-of-view* confocal system presented in here, the reflectance imaging mode shares the same illumination and detection optics with the fluorescence mode. Neutral density filter attenuation of laser power at the detection path is used for reflectance imaging, in place of the fluorescence emission filters. In reflectance imaging, the laser light reflected/scattered from the detection volume of the specimen is collected by the laser scan lens, descanned by the scanning mirror, and detected by the same confocal detector used in fluorescence imaging (with appropriate filter changes).

2.5. Transmission Imaging

Transmission (brightfield) microscopy is the most commonly used method in the examination of histological samples, based on the absorption of the illumination light by structures in the specimen. In a conventional widefield microscope, the commonly used light sources are tungsten filament lamps with a continuum of blackbody radiation, or xenon arc lamps with a continuum between 300 and 800 nm.²¹ However, in the brightfield detection mode of the *wide field-of-view* imaging system presented in this paper, the red, green and blue (RGB) lasers are used as the illumination source to focus simultaneously through the specimen. RGB imaging is based on the color perception theory. In general, a mixture of relative amounts of red, green and blue light can present most colors to the human eye. For example, the perception of yellow (*e.g.*, 560 nm) can be evoked by either the presence of that wavelength or the presence of comparable intensities of red (600 nm) and green (530 nm). Broadly speaking, when transilluminated as in brightfield microscopy, if a sample appears green, the red and blue lights are absorbed.

As discussed in Sect. 1.1.1, in brightfield microscopy, the tissue sections are usually stained with one or more chromophores to add contrast to such sections. The end result is to produce an amplitude object from a transparent phase object, so that the illumination light is absorbed differently across the specimen. The three lasers have wavelength lines of 633 nm (red), 535 nm (green) and 488 nm (blue). The common dyes used for tissue staining are mostly broadband dyes. Thus their absorption spectra will overlap with one or more laser lines, which makes RGB imaging using the three laser sources possible.

In confocal reflectance and epi-fluorescence imaging, the signal light is descanned by the scanning mirror so that the detection aperture tracks the focused laser spot at the specimen, and the full advantage of a confocal system, *i.e.*, the two way rejection of out-of-focus light, is realized. In a confocal transmission configuration, however, the descanning is more technically challenging. The published solutions include the use of a second synchronized scanning system,^{2,3} a complicated beam path to redirect the transmitted light back to the scanner,⁵⁶ and the use of a nonlinear crystal as confocal pinhole for the detection of transmitted signals through second-harmonic generation without descanning.^{57,58}

The transmission imaging mode in the *wide field-of-view* system described in this paper does not implement a confocal pinhole, the transmitted light is simply detected using an RGB detector. However, the point illumination of the specimen in the scanning system offers theoretically better signal-to-noise ratio than a conventional widefield microscope. The point-illumination scanning scheme does reduce the lateral background, and reduces photobleaching in the specimen. As the brightfield mode is not implemented in a confocal configuration, it is only suitable for applications in thin and stained tissue imaging.

3. APPLICATIONS IN DRUG DISCOVERY AND PATHOLOGY

The imaging applications of the new *wide field-of-view* confocal imaging system in drug discovery and pathology are described in this section. Selected imaging specimens include a large tissue section, a tissue microarray, a slide of zebrafish larva sections, a whole mouse embryo slide, a DNA microarray slide. Most of the images are presented in three formats, an overview of the whole slide or a major part of a whole slide, a magnified portion of the specimen, and a portion of the actual pixel (original size) image of the specimen. The overview images show the large field of view of the *wide field-of-view* imaging system; the zoomed in images display some interesting structures in the specimens; and the actual pixel images indicate the resolution limit of the microscopy imaging system. The images shown in this section are all computer screen captures of 1024 by 768 resolution. The pixel resolution is twice that of the optical resolution based on the Nyquist Theorem. For example, a pixel resolution of 1 μm measured at the specimen is required to correctly capture and display an image from a microscope whose optical resolution is 2 μm . Most of the images presented in this Section are taken at 2.0/1.0 μm optical/pixel resolution, except for the images shown in Figure 11 to 13, which are taken at 0.5/0.25 μm optical/pixel resolution.

3.1. Tissue, Tissue Microarray and DNA Microarray Imaging

In pathology and drug discovery, confocal microscopy and conventional widefield light microscopy will continue to play an important role, for both morphological investigations and molecular biological diagnostics. Morphological

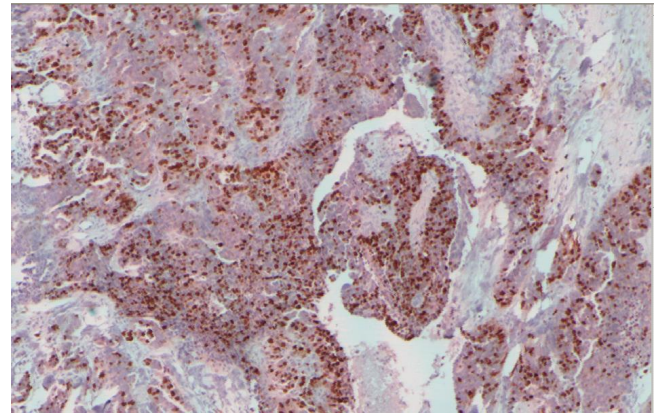
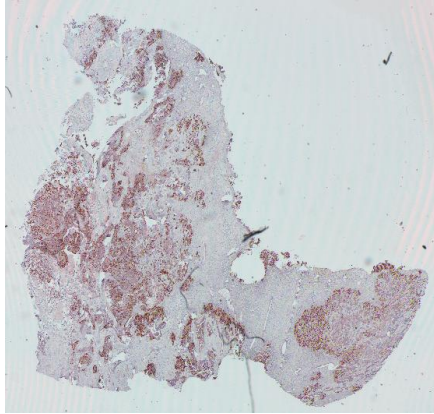


Figure 2. Screen captured image of a tumor tissue section (10.5 by 10 mm) immunostained for the proliferation marker MIB-1 (Ki67). RGB brightfield image. Section Overview. **Figure 3.** Magnified view showing the proliferating cells of the previous tumor section image. Dark round spots indicate nuclei of cells positive for the proliferation marker MIB-1 (Ki67). RGB brightfield image.

diagnosis, as the gold standard in soft tissue diagnostic pathology, is facing a challenge from molecular diagnostics which is providing the basis for reclassification of some well-entrenched morphological entities,⁵⁹ and fluorescence microscopy is crucial to molecular biological investigations.

Figures 2 - 4 show RGB brightfield images of proliferating cells in a tumor tissue section 10.5 by 10 mm in size, immunostained for the proliferation marker MIB-1 (Ki67). These images were taken at a pixel resolution of 1 μm , using the laser scan lens with an optical resolution of 2 μm . Fig. 3 is a magnified view of part of Fig. 2, in which the round dark spots are nuclei of cancerous cells immunostained with the MIB-1 marker. Figure 4 is a screen capture of part of the image at the actual pixel size with 1 μm pixel resolution. Cancerous cells immunostained with the proliferation marker MIB-1 (Ki67) are clearly shown.

Figures 5 - 7 display fluorescence images of a tissue microarray, recorded with a blue (488 nm) laser excitation and a 570/50 nm emission filter at 2 μm optical (1 μm pixel) resolution. The fluorescence images are intentionally inverted in greyscale, which makes bright fluorescence object appear dark, for ease of printing in black and white. The slide label part of the image is not shown. Figure 6 is a magnified image of a part of Fig. 5, with nine tissue core sections in it. Figure 7 is the actual pixel image of one of the tissue core sections in Fig. 5 and Fig. 6.

Figure 8 shows the fluorescence image of a DNA microarray slide, with the excitation lasers being green (535 nm) for Cy3 fluorophore and red (633 nm) for Cy5. The spots are 150 μm in diameter. Figure 9 is a fluorescence image of a Cy3 DNA calibration biochip with features of 15 μm squares, detected with green (532 nm) laser excitation and a 570/50 nm emission filter.

3.2. Imaging Zebrafish Slide

Zebrafish, as a vertebrate model organism for studying gene function and drug effects in humans, is increasingly being used for testing in drug discovery. Zebrafish have all of the equivalent organs found in mammals, and most human genes have homologues in zebrafish.⁶⁰⁻⁶²

Figures 10 - 13 are inverted fluorescence images of coronal sections of zebrafish larvae stained with H & E, plastic sectioned at 1 μm thickness. Figure 10 is an overview image of the slide taken using a green (535 nm) laser and a 700/75 nm bandpass emission filter. There are six larva sections in it. Figure 11 is an overview of the inverted fluorescence image of one of the zebrafish larva sections in Fig. 10, depicting entire organism in a coronal section. It is recorded with green laser (532 nm) excitation and a 570/50 nm bandpass emission filter, at 0.5/0.25 μm optical/pixel resolution. Figure 12 is a transposed and magnified part of the fluorescence image in Fig. 11. This section view depicts lower coronal section of the transition region from pharyngeal portion to abdomen of the zebrafish larva. Figure 13 is the actual pixel image of an eye of the zebrafish larva shown in Fig. 11. The overlaying cornea, round lens and retinal layers are observed clearly.

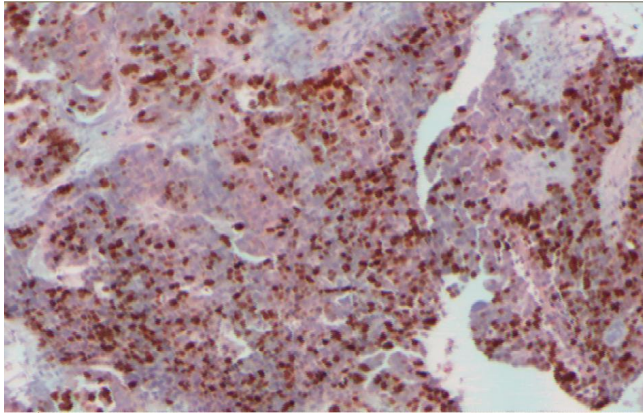


Figure 4. Details of the previous slide with cancerous cells clearly marked with the proliferation marker MIB-1(Ki67). Actual pixel image at $2.0/1.0 \mu\text{m}$ optical/pixel resolution.

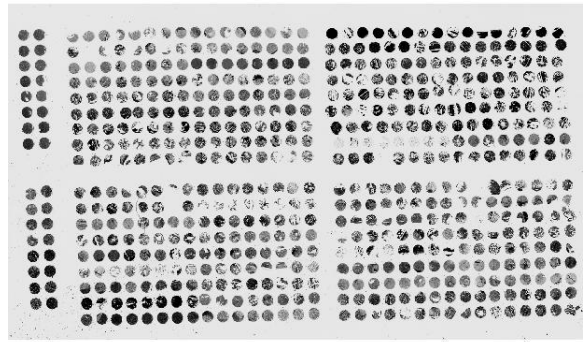


Figure 5. Overview of a tissue microarray slide. Fluorescence image with 488 nm laser excitation and 570/50 nm emission filter. Inverted greyscale. Slide label not shown.

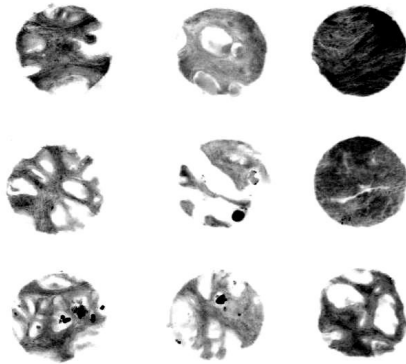


Figure 6. A magnified part of the fluorescence image shown in Fig. 5. Blue (488 nm) laser excitation and 570/50 nm emission filter. Inverted greyscale. image at $2.0/1.0 \mu\text{m}$ optical/pixel resolution.

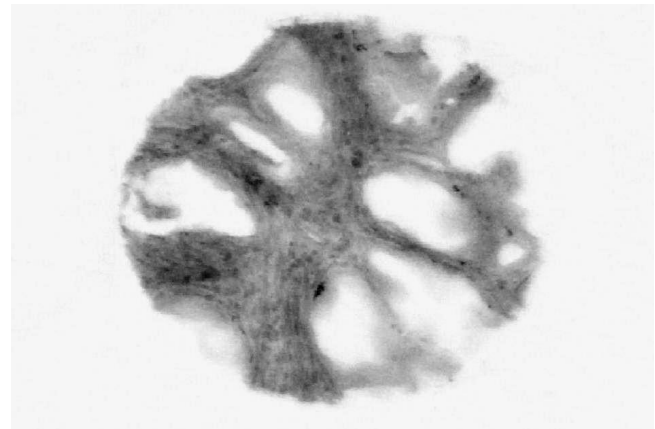


Figure 7. One tissue core section in Fig. 6. Actual pixel image at $2.0/1.0 \mu\text{m}$ optical/pixel resolution. Inverted greyscale.

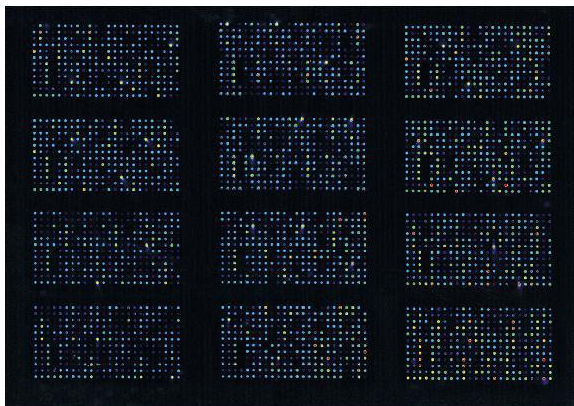


Figure 8. A $2 \mu\text{m}$ pixel resolution fluorescence image of a DNA microarray slide, with green excitation laser (535 nm) for Cy3 fluorophore and red (633 nm) for Cy5.

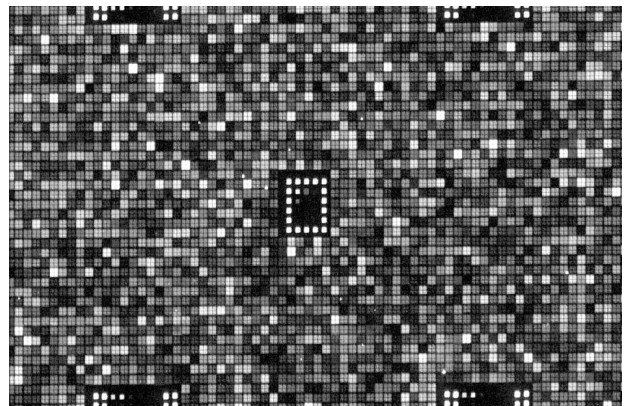


Figure 9. Fluorescence image of a Cy3 DNA calibration biochip with features of $15 \mu\text{m}$ squares. Green laser 532 nm excitation and 570/50 nm emission filter.

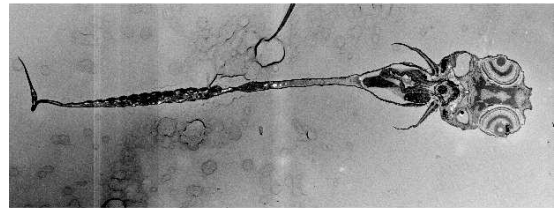
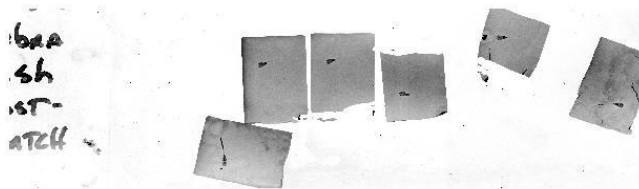


Figure 10. A fluorescence image of a slide of plastic sections of zebrafish larvae. Green (535 nm) laser excitation and (532 nm excitation and 570/50 nm emission filter). Slide Overview.

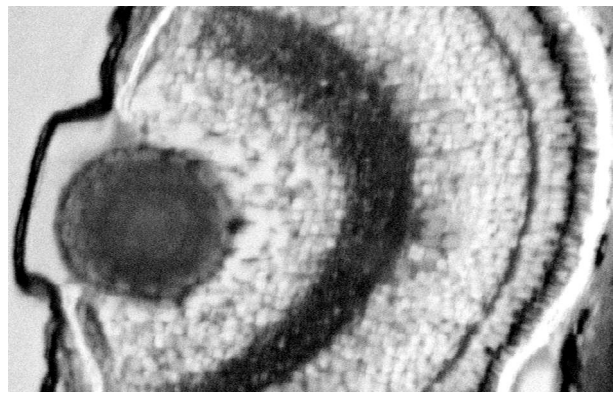
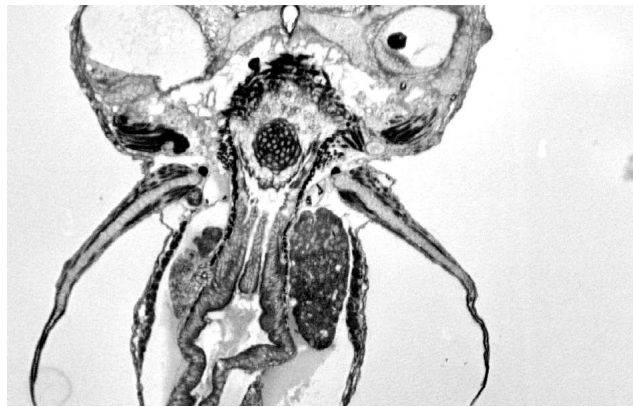


Figure 12. A transposed and magnified fluorescence image of a part of Fig. 11. Taken at 0.5/0.25 μm optical/pixel resolution.

Figure 13. An actual pixel fluorescence image of an eye of the zebrafish larva shown in Fig.11. Taken at 0.5/0.25 optical/pixel resolution. Transposed laterally.

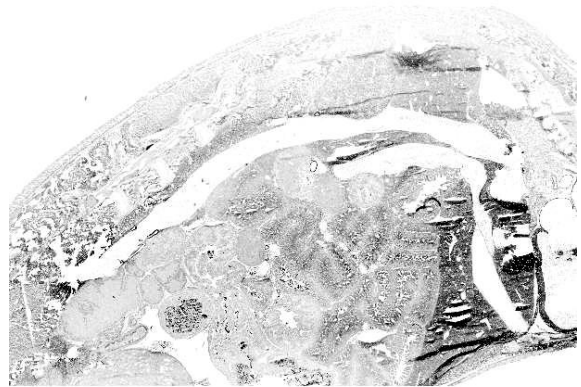
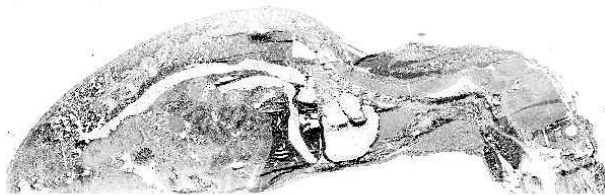


Figure 14. Overview of a whole mouse embryo section **Figure 15.** An inverted fluorescence image of 20 by 30 mm (20 by 65 mm). Inverted fluorescence image. Thioflavin-S in size, sagittal view zoomed on thoraco-abdominal region of stained. Green laser (535 nm) excitation, 700/75 nm emission filter.

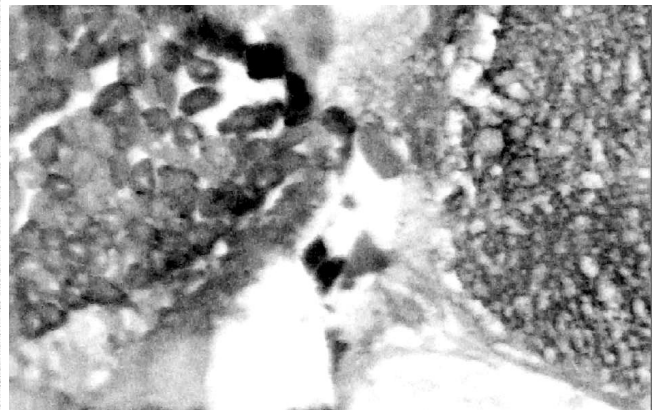
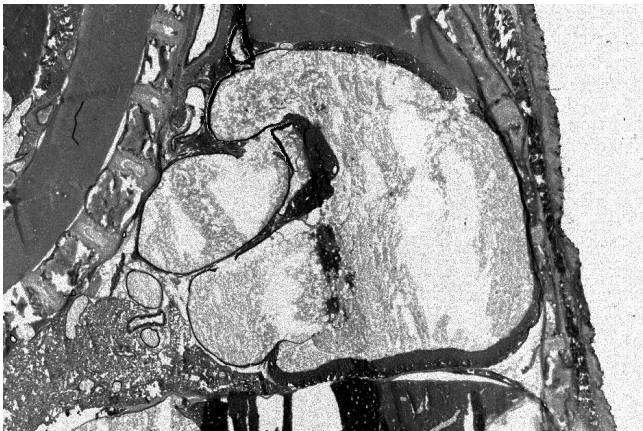


Figure 16. An inverted fluorescence image showing the heart of the whole mouse section. **Figure 17.** Actual pixel image at 2.0/1.0 μm optical/pixel resolution. A tiny part of Fig. 14.

3.3. Whole Mouse Section Imaging

Figures 14 - 17 are inverted fluorescence images of or a part of a sagittal whole mouse section stained with Thioflavin-S. The images were recorded using green (535 nm) laser excitation and 700/75 nm bandpass emission filter. Figure 14 is an overview of a 20 by 65 mm whole mouse section on a standard microscope slide (25.4 by 76.2 mm) at 2.0/1.0 μm optical/pixel resolution, showing the entire sagittal section with the anterior (head) end on the right and the (posterior) tail end on the left side. Figure 15 is a fluorescence image of a 20 by 30 mm area of the whole mouse section, displaying the thoraco-abdominal region of the mouse embryo from Fig. 14. In Fig. 16, details of cardiac region are observed. The image has been rotated to the left with respect to Fig. 14 and Fig. 15. Heart valves can be seen in the middle of the section, which shows mainly the atrial region of the heart. A chain of thoracic vertebra sections can be seen on the left. Figure 17 is an actual pixel image of a tiny part of Fig. 14 at 2.0/1.0 μm optical/pixel resolution.

4. CONCLUSIONS AND FUTURE DIRECTIONS

The new *wide field-of-view* confocal microscopy imaging system will have wide application in pathology and drug discovery, because of its capability of imaging large tissue samples and entire slides of tissue and DNA microarrays with microscopic resolution in a single scan, in both confocal fluorescence and reflectance imaging,

and in RGB brightfield. The proprietary laser scan lenses, auxiliary optics, electronics, and computer and software for the confocal imaging system make the image acquisition more efficient than other methods. By using the new microscopy system, it is possible to perform high throughput screening of drug effects, *e.g.*, using slides of whole zebrafish embryos. Many researchers have started to use zebrafish embryo models for large scale whole organism screening.⁶³ By using this new confocal imaging system, for example, zebrafish embryos held in microwell plates that have been subjected to various treatments across the wells could be mounted on a single microscope slide and evaluated together in a single scan, to pinpoint differences in gene expressions through fluorescence in situ hybridization, or morphology through routine H & E staining. With a little modification, the *wide field-of-view* confocal microscopy imaging system can be turned into a high resolution wellplate reader. In combination with the capability of imaging large area specimens, automated image acquisition and analysis, the *wide field-of-view* system can be equipped with multiple slide holders to realize a higher degree of imaging automation. In addition, more efficient methods of image archiving and large image handling and browsing will be developed.

The *wide field-of-view* confocal imaging system presented in this paper has three excitation lasers and six detection channels, three for fluorescence and reflected-light imaging, and three for bright field. In applications where multiple fluorescence probes and a wider variety of dyes are used, separation of the spectra on the basis of emission wavelengths may not be specific enough, and excitation wavelength must also be selected. Therefore, methods such as the use of an acousto-optic tunable filter (AOTF) for the selection of laser lines may need to be adopted.⁶⁴ AOTF simultaneously selects the wavelength and controls the degree of attenuation applied.⁶⁴ When using an AOTF as a tunable dichroic beamsplitter, multiple specific wavelengths can be deflected towards the objectives simultaneously or in sequence. A polarizing beamsplitter can also be used in place of the primary dichroic beamsplitter, which preferentially reflects polarized light (the laser light), while transmitting the nonpolarized fluorescence light to the detection optics.⁶⁴ When using dyes with overlapping spectra, selection of excitation wavelengths rather than emission wavelengths must also be made. In most cases this can be adjusted sufficiently rapidly to allow “line by line” sequential scanning, where each line is scanned twice - once using one excitation wavelength and one detector, the next using another excitation wavelength and detector.⁶⁴

Fluorescence detection with dichroic mirrors and barrier filters can be highly specific, but lacking in versatility. Therefore, spectrometric detection strategies have recently come into vogue.^{64,65} Spectral imaging and multispectral imaging are necessary for the detection of wideband fluorescence emissions of specimens stained with multiple fluorophores. Spectral imaging is the capture, processing, display and interpretation of images with a high number of spectral channels. Multispectral imaging is the simultaneous or serial acquisition of imaging data from two or more discrete spectral bands. The whole spectrum of a fluorescence emission can be determined by using spectral imaging. The overall shape of a spectrum can provide more information than just a few wavelengths. In principle, spectral imaging is like having a large number of spectrophotometers working in parallel. Preliminary results from a spectrally-resolved version of this instrument have been reported by Constantinou *et al.*⁶⁵

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