

# Histology and Slide Scanning for Light Microscopy

Application Compendium



# Table of Contents

<b>Introduction</b>	<b>3</b>
<b>Histology Best Practices</b>	<b>4</b>
Sample preparation	4
Detection	6
<b>Instrumentation</b>	<b>8</b>
<b>Color Brightfield and Fluorescence Imaging Applications for Histology, Tissue Microarrays, and Slide Scanning</b>	<b>9</b>
Hematoxylin and eosin-stained tissue	9
Automated color imaging of tissues	15
Applied robotics forenhanced throughput options in microscopy as demonstrated by automated tissue microarray (TMA) imaging	22
<b>References</b>	<b>30</b>

# Introduction

Today, histology and the study of microanatomy continue to advance not only in the targeted design of pathogenic models, the sectioning capabilities and staining selectivity, but also in the automation and computational methods for sample acquisition and analysis. The variety and flexibility of methods for tissue preparation and staining allows researchers to optimize their protocols and adapt them for available instrumentation and imaging resources. These staining techniques utilizing colorimetric, chromogenic, or fluorescent labeling of fixed samples can each provide unique insights into tissue components and biochemical processes.

Researchers commonly follow general practices for histology, but many modify techniques and protocols to best suit their tissue of interest. Consistent steps must be taken to minimize variability among samples throughout the protocol to ensure reproducibility. If there are observed differences between stained samples, they are most likely due to irregular processing throughout the histology workflow. In addition, manual assessment of staining differences between samples can often be tedious, inaccurate, or unintentionally biased. Instead, automated imaging and defined parameters can provide an accurate comparison for consistent results and analysis.

Methods from fixation to staining histological specimens for light microscopy can present a vast number of options. However, the most successful researchers learn which methods provide consistent results allowing for repeatable immunohistochemical analyses. In addition, the techniques for image acquisition and quantification can be optimized for high-content microscopy. The Agilent BioTek imaging systems, including the Agilent Biotek Lionheart automated microscope and Agilent Biotek Cytation cell imaging multimode reader lines, provide the ability to easily automate the acquisition of slides and create saved protocols with reproducible analytical procedures.

The goal of this application compendium is to introduce various imaging assays utilizing Agilent BioTek's high-content imaging instruments and image analysis software to characterize staining expression on histological sections and samples.

This compendium also provides insight into an optimized histological workflow to reduce assay variability among samples by recommending best practices from sectioning, to staining, to imaging and analysis. Large tissue imaging is facilitated by using a montage collection of tiles at a designated magnification that creates a single composite image, enabling efficient comparison of different tissue samples. Automated image analysis is implemented in the Agilent BioTek Gen5 microplate reader and imager software to provide a range of data parameters. Within Gen5, a number of readouts can be reported (such as size, area, and shape) and measurements of signal (pixel intensity) within areas of tissue sections to measure expression among samples.

# Histology Best Practices

## Sample preparation

Processing a histological sample begins with the collection of a fresh sample either from a donor or a research animal. To preserve the sample for study, specimens are typically treated with chemical fixatives to preserve the cellular structures by irreversibly cross-linking proteins, hardening the tissues. Fixatives consist of various formulations of aldehydes, such as the commonly used formalin or formaldehyde solutions. Common methods of fixation of lab animals include either perfusion of fixative after exsanguination and saline, allowing full organs to rapidly be fixed, or post-dissection where they can be immersed in solution for diffusion of fixative.<sup>1</sup> Depending on the thickness of the tissue, it may require a longer duration in fixative to penetrate fully. It is critical to allow both a sufficient time in fixative as well as the prevention of over-fixation of samples allowing for viable downstream tissue processing in the histology workflow. Once samples have completed this process, tissues can be segmented into smaller sections, typically to fit the boundaries of small embedding cassettes of a few centimeters, to expose regions of interest within an organ in anticipation of sectioning efficiency.

The fate of tissue processing for histology can proceed in one of two ways, depending upon the sample and the goals of the immunostaining application. For tissues that can be processed slowly and at higher temperatures, paraffin embedding is an optimal choice and is noted as formalin-fixed paraffin embedded samples, or FFPE.<sup>2</sup> If the tissue sample is time-sensitive, and the aim is to examine non-proteinaceous components, or enzymatic detection is present, it can be rapidly frozen instead of embedding at warmer temperatures.

FFPE specimens must undergo a dehydration process to allow the paraffin to infiltrate the tissue, replacing water for sectioning. Typically, a series of graded ethanol concentrations, increasing from 70% to 100% EtOH, are used in the dehydration procedure followed by several rounds of a solvent such as xylene to enable the embedding media to penetrate the tissue sample. Immunohistochemistry (IHC)-grade wax is the most commonly used paraffin for the embedding process, although there are several alternatives if the samples are fragile or do not have the appropriate stiffness. In addition, resins could be beneficial if antibody labeling is required. Tissue samples are best oriented into a favorable position for minimal adjustments of the block on a microtome for sectioning.

Frozen samples are common among human samples once extracted, and is typically the case with tissue from a subject destined for histopathology. Tissues and organs of research animals may also be prepared for frozen embedding. Although tissues destined for frozen sectioning do not undergo a dehydration process, they are subjected to a series of sucrose/glycerol concentrations before the embedding. During this cryogenic procedure, the fixed tissue is often trimmed to depths of several millimeters and embedded into a gel-like substance within a plastic mold. Optimal cutting temperature compound (OCT), is a convenient medium composed of polyethylene glycol and polyvinyl alcohol and is typically used for embedding frozen samples. At room temperature it exists as a gel, facilitating the removal of bubbles within the media in OCT-samples by vacuum to minimize tears and poor sectioning downstream. Once the OCT compound is exposed to temperatures below -20 °C, the media will solidify, encasing the embedded tissue within.

Preparation of FFPE or frozen specimens for sectioning requires some adjustments both before and after the block is secured in the microtome or cryostat. For frozen samples, the microtome is built inside of the cryostat, and maintains a temperature-controlled environment for cryosectioning. Careful trimming of the embedded block, at 90 degree angles and creating slopes from the block surface to the cassette holder- similar to that of a pyramid, allows the edge of the block to lay parallel with the length of the microblade. Small adjustments to the sample holder of the microtome or cryostat also assures the face of the block, with the surface of the tissue to be sectioned, is in the correct orientation where the blade will generate consecutive serial sections in the form of a ribbon, in the case of paraffin sections. Tissue sections are then cut to the preferred micron thickness, a common 3-8  $\mu\text{m}$ , and mounted onto slides. Removal of the embedding medium from the tissue section is typically required before staining and detection of samples.

## Detection

Once samples have been sectioned and mounted onto slides, staining for a biological component or process is carried out. There are numerous detection methods that will depend on the aims of the study, tissue, and available reagents.

Colorimetric staining is one of the oldest scientific methods and remains a commonly used histological assay. Varying stains and dyes with different tissue and cellular specificities are used to increase the contrast of features and cells of interest. Hematoxylin and eosin (H&E) staining is one of the most commonly used labels for histology as nuclei are stained blue with hematoxylin giving cell locations, and other cellular and tissue structures are labeled pink from the eosin (Figure 1A).<sup>3</sup> A detailed explanation of H&E staining is described in the application note “Hematoxylin and eosin-stained tissue” later in this compendium.

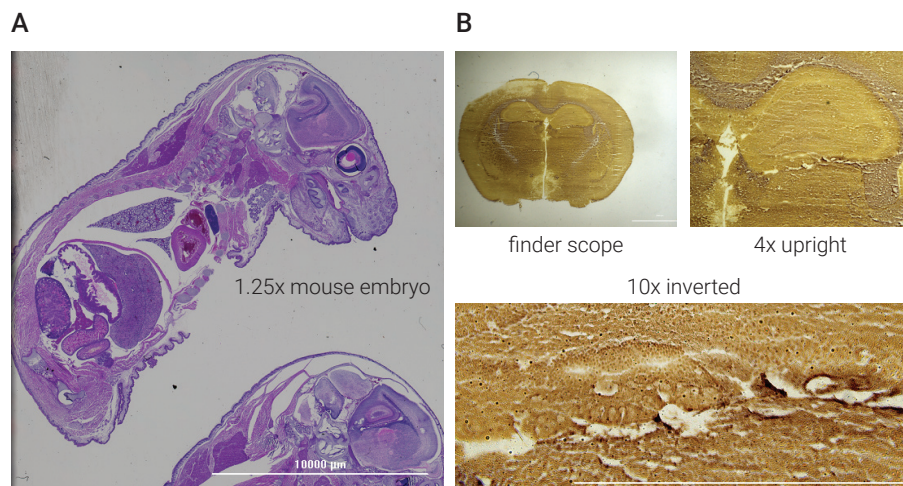
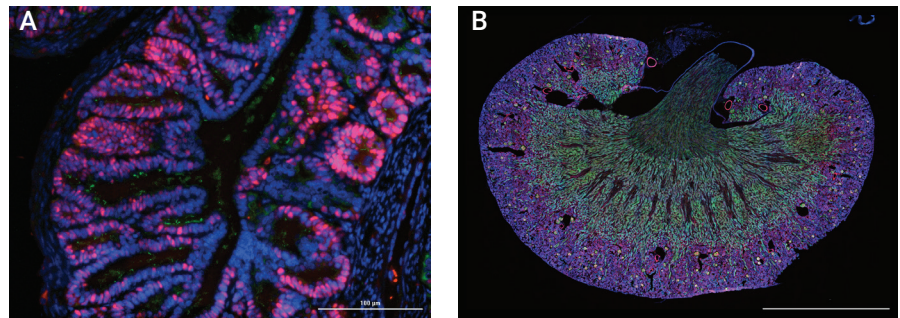


Figure 1. Colorimetric stained tissues. (A) H&E staining of mouse embryo at 1.25x color brightfield (NIH Bethesda, MD). (B) DAB staining of mouse brain section using the upright finder scope, 4x, and at 10x with the inverted microscope (BioTek – a part of Agilent Technologies, Inc.).

Several fluorescent dyes have the selective capability of binding to tissue and cellular locations and provide a clear quantifiable signal. The most common fluorescent nuclear stain for fixed tissues is DAPI, while Hoechst stain is common for live cells. These stains are not only used to identify cell count but also to quantify their DNA content and thus cell cycle stage. Nuclear stains can also be utilized to address cell viability. Propidium iodide (PI) cannot cross the cell membrane of living cells, but when viability is lost and cell membranes become permeable, nucleic acids become labeled and fluoresce. Ethidium bromide (EB) is another stain that only labels the nucleus when cell membranes become permeable during cell death. Acridine orange (AO) can stain the nucleic acids of viable cells and is used in tandem with EB to assess the percentage of viability. Fluorescent dyes such as fluorescein, rhodamine, and others can be used to differentially label tissue structure.



**Figure 2.** Fluorescently stained tissues. (A) Fluorescent staining of mouse reproductive oviducts (fallopian tube equivalent) of an ovarian cancer model with T-Antigen expression (red) in epithelial cells, ciliated epithelial cells labeled with GFP (green), and nuclei stained with DAPI (blue); imaged on a Agilent BioTek Lionheart FX automated microscope. (Fox Chase Cancer Center Philadelphia, PA). (B) Mouse kidney- FluoCells prepared slide #3 (mouse kidney section with Alexa Fluor 488 WGA, Alexa Fluor 568 Phalloidin, and DAPI, Thermo Fisher Scientific) imaged at 4x with montage capture on a Agilent BioTek Cytation 5 cell imaging multimode reader.

For greater precision in the detection of tissue, cellular structure, and biochemical processes, specific antibodies are used for immunohistochemistry and immunofluorescence. The tracking labels can be attached to primary antibodies or secondary antibodies to increase the signal.

Immunohistochemistry employs antibodies conjugated to enzymes such as horseradish peroxidase (HRP), alkaline phosphatase (AP), or beta-galactosidase (BGAL) to localize the activity of chromogenic reporters to your target of interest. The use of a conjugated antibody like HRP reacts with an appropriate substrate system, such as DAB, in the presence of hydrogen peroxide, DAB will oxidize and generate a brown precipitate identifying the target (Figure 1B). Protein signal can be amplified when using a biotinylated secondary antibody, where the biotin binds up to four avidin or streptavidin - proteins that are conjugated with HRP. Traditionally, hematoxylin is regularly used as a counterstain for nuclear detection alongside DAB staining. Fixed and processed IHC slides are stable and will keep their color stain indefinitely. Further details are provided in the application note "Automated color imaging of tissues" below.

Immunofluorescence utilizes secondary antibodies conjugated to with fluorophores such as FITC or the Alexa Fluors to create a fluorescent signal localized to your target protein (Figure 2B). With the flexibility and range of fluorescently labeled secondary antibodies, paired with the optimal imaging filter cube, fluorescent signals can provide a greater quantifiable dynamic range and identification of protein expression; however, they tend to be less stable long-term. An example of automated fluorescence slide scanning is found in the application note "Applied robotics for enhanced throughput options in microscopy" as demonstrated by automated tissue microarray (TMA) imaging as a TMA prototype application.

In this application compendium, several examples of fluorescent and color brightfield histology sections are presented using Agilent BioTek Cytation and Lionheart imaging systems. Precise descriptions of specific assay methods, images, and quantification are described in the application notes below.

# Instrumentation

Once the samples have been prepared and mounted on slides, light microscopy is utilized to examine the results. As histology slides are typically fixed under a coverslip, both upright (Cytation 7) and inverted (Cytation 5, 7, or Lionheart) microscopy are applicable. However, the shorter working distances of inverted microscopes will typically yield a better resolution for higher magnification objectives. The Agilent BioTek microscopes have several imaging modalities to assure that samples can be imaged with the appropriate tools.

## Agilent BioTek Cytation cell imaging multimode readers



The Agilent BioTek Cytation 5 and Agilent BioTek Cytation 7 combine automated digital microscopy and conventional microplate detection in a modular, upgradable platform that is automation friendly. The inverted microscopy module offers up to 60x magnification in fluorescence, brightfield, high contrast brightfield, color brightfield. The Cytation 5 has the additional option of phase contrast imaging, while the Cytation 7 upright camera provides both reflected and transmitted light for color imaging at lower magnifications and can be optimized for slide scanning. Moreover, the Cytation 7 upright imaging color camera allows for region of interest selection and acquisition speed, where one can examine each individual RGB channel from the resulting composite.



## Agilent BioTek Lionheart FX automated microscope

The Agilent BioTek Lionheart FX automated microscope is a compact, inclusive microscopy system with an open stage for fluorescence, brightfield, color brightfield, and phase contrast channels for maximum application reach. A broad variety of imaging workflows can be imaged with objectives ranging from 1.25x to 60x air, and the additional 60x and 100x oil immersion.



## Agilent BioTek BioStack microplate stacker

The Agilent BioTek BioStack microplate stacker is a compact and versatile microplate stacker compatible with Agilent BioTek washers, dispensers, readers and imaging systems. Once an automated imaging and acquisition protocol has been established in Agilent BioTek Gen5 software, it is very simple to step up to a high throughput setting. BioTek is well known for its robust and easy-to-use robotics that can feed both microplates and adapters to the Agilent BioTek Cytation line of microscopes. The Agilent BioStack rapid plate and adapter exchange speeds increase throughput and enhance productivity, accommodating multiple slides in adapters. This enables many slides to be scanned with image acquisition for automated analysis within the Gen5 software.



# Color Brightfield and Fluorescence Imaging Applications for Histology, Tissue Microarrays, and Slide Scanning

## Hematoxylin and eosin-stained tissue

### Assay introduction

Chromogenically stained tissues have been the hallmark of pathology and cancer research for nearly a century. Fixed tissue samples are routinely embedded in paraffin and used as a method of long-term data storage. Thin slices of tissue are cut from the paraffin block, immobilized on slides, and subsequently stained, commonly with hematoxylin and eosin, and sealed with a cover slip. Examination of the histology slide requires the use of a multi-objective microscope to make observations. After examination, the slide along with the paraffin embedded tissue are routinely archived. If reexamination is required, the slide can be retrieved or the paraffin tissue block is reprocessed to generate a new slide for observation (Figure 3). With advancements in digital imaging and data storage colored imagery is becoming more desirable.

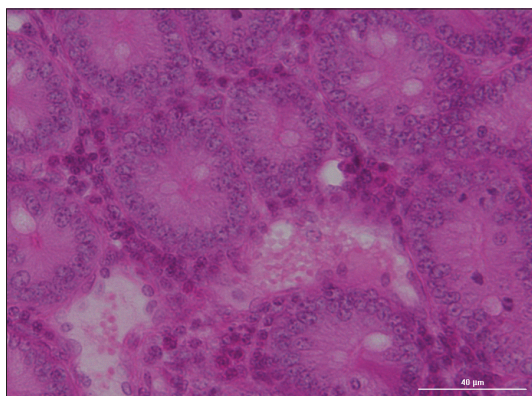


Figure 3. Hematoxylin and eosin-stained normal human small intestine tissue.

Hematoxylin or, more correctly, its oxidized form hematin binds with a mordant (typically  $Al^{3+}$ ) to stain DNA in cellular matter. It is thought to bind with the negatively charged phosphate groups that comprise the DNA backbone and then undergo complex coordination or conjugation to become a permanent stain of the nucleus. Together with its  $Al^{3+}$  mordant, the dye produces a blue color in neutral to basic conditions (Figure 4).

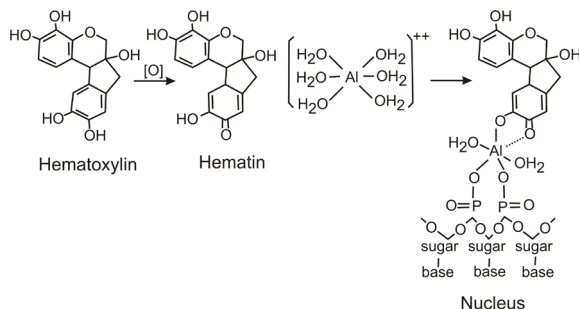
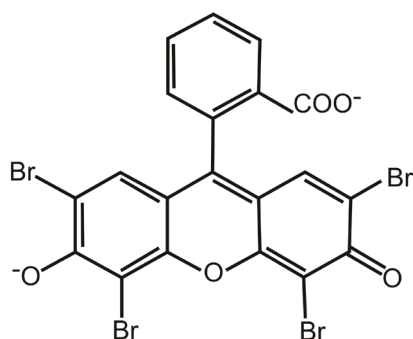


Figure 4. Hematoxylin (hematin) binding to DNA in the cell nucleus.



**Figure 5.** Structure of the dianion, eosin Y used in H&E staining.

Conversely, the anionic eosin Y will bind to positively charged groups on proteins, such as amino groups. Lysine residues, for example, have an  $\epsilon$ -amino group with pKa's in the range of 10, such that they will remain as positive ions throughout the staining process (Figure 5).

While other stains and protocols for staining cells and tissue sections have been developed, the original method developed almost 140 years ago remains relatively unchanged. Hematoxylin is not synthetically produced, but is instead extracted from the logwood tree. Even so, H&E staining remains the most common staining protocol for applications in histology.

This application note demonstrates the utility of a new automated digital microscope for the imaging of H&E stained tissue sections. Low to high magnification of sections is possible by the software controlled selection of microscope objectives ranging from 2.5x to 60x. Focusing and exposure settings are all automated resulting in simple operation. Real time images are viewed on a computer monitor and desired images can be saved and downloaded as a number of different data files, including TIF and PNG simplifying data sharing.

## Materials and methods

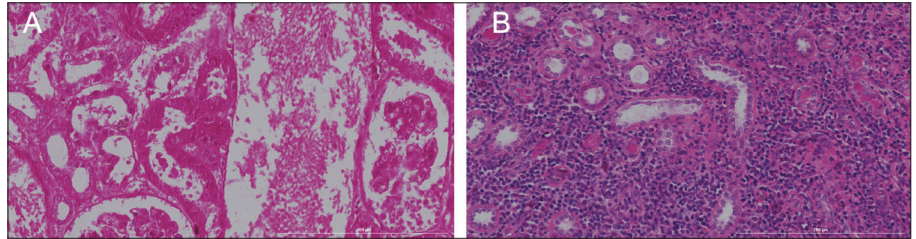
Commercially available fixed and stained tissue slices of normal human intestine and kidney, as well as human kidney with chronic nephritis were purchased from Konus. Slides were imaged using a Cytation 5 cell imaging multimode reader.

Montage arrays is a feature in Gen5 allowing multiple fields of view (FOV) or a larger area than a single image frame FOV to be captured. When separating the images from one another, arrays can be used to obtain individual tiles from different regions of the slide, and each would be examined and analyzed separately. Alternatively with slight overlap, the discrete images can be combined to create a much larger contiguous composite. With increasing magnification, more information is obtained, albeit on a much smaller region. The amount of slide area examined is dependent on the magnification used. Montage spacing overlap was set to the default for stitching in Gen5 software. The individual image tiles captured for a color brightfield montage (12 x 8) were stitched together using a linear blend along overlapping portions of each tile. The registration channel selected for stitching was applied on the red channel, and the resultant file was reduced to 25% of maximum size.

## Results and discussion

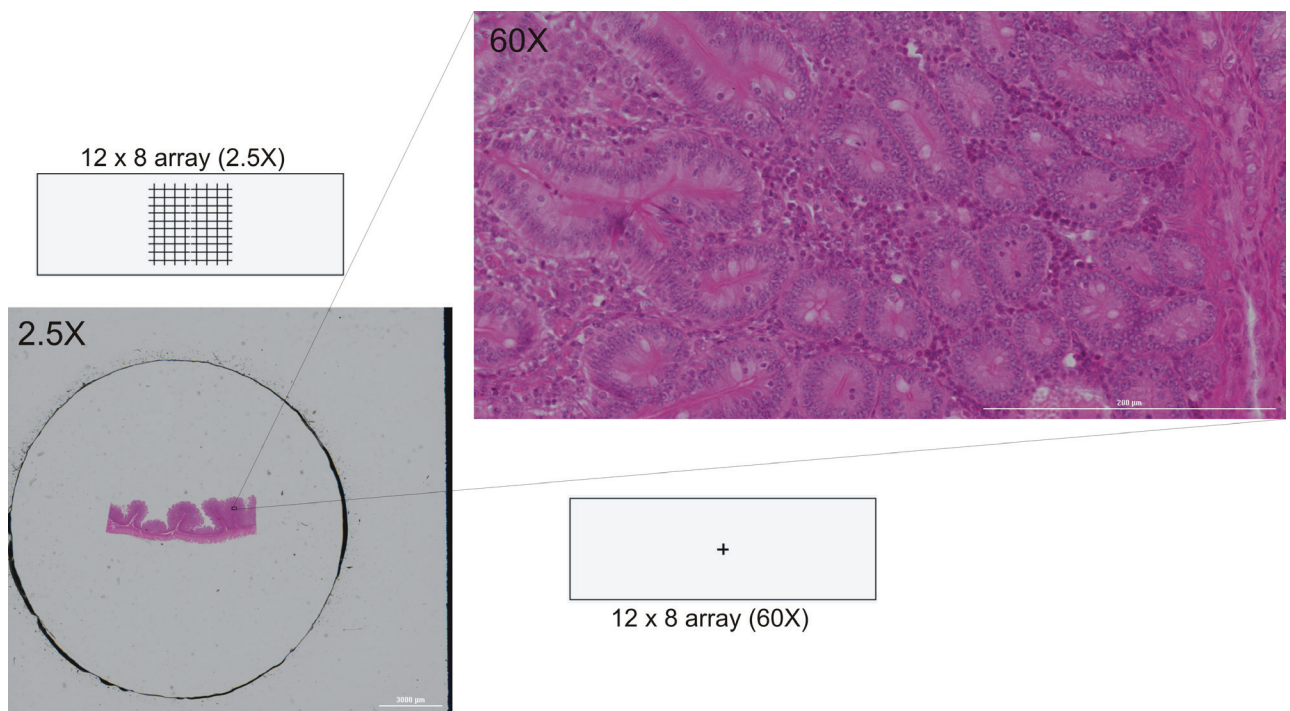
The capability of the Cytation 5 to color brightfield image fixed and H&E stained tissue slices is demonstrated in Figure 6, where normal and diseased kidneys are compared. Using a 60x objective montage, images of slides were captured, recorded and the individual image tiles stitched to form single contiguous image files for each slide. Marked infiltration of lymphocytes in the interstitial spaces is

identified with significant hematoxylin blue staining. Hyalinization of the glomeruli is indicated by amorphous pink eosin staining in the Bowman's capsules. Normal kidney tissue glomeruli show open Bowmen's spaces and no inflammatory cells.



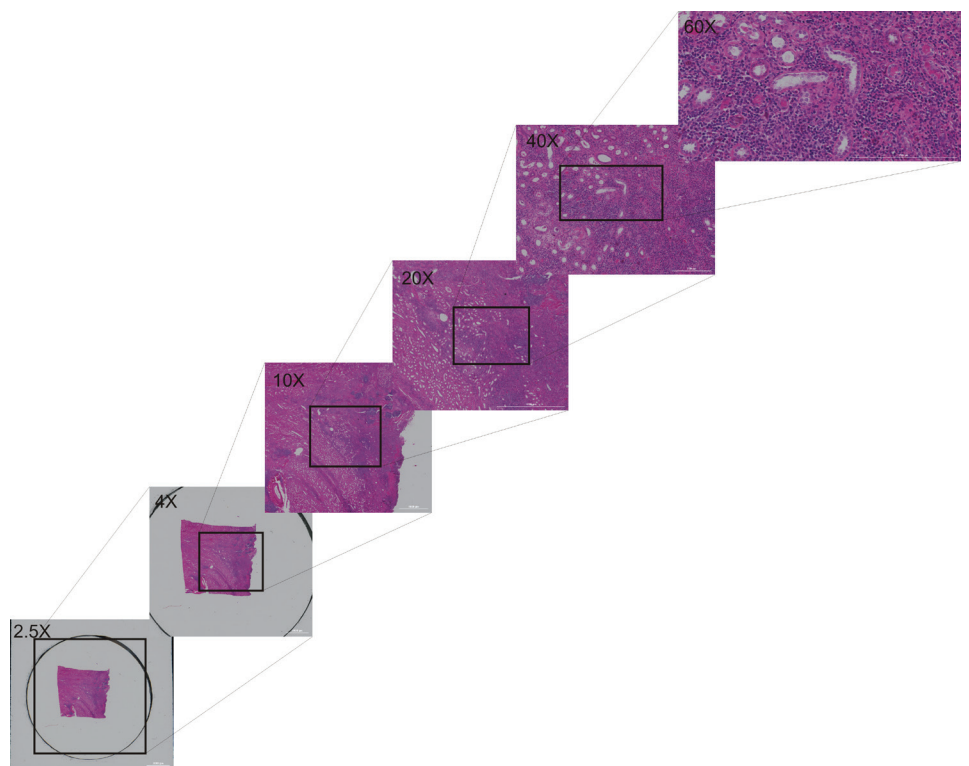
**Figure 6.** Comparison of normal and diseased human kidney. Hematoxylin and eosin-stained tissue from (A) normal kidney and (B) chronic nephritis kidney. Images represent a stitched 12 x 8 montage made using a 60x objective. Scale bar represents 200  $\mu\text{m}$ .

The degree to which the Cytation 5 can magnify a region of a slide is demonstrated in Figure 7, where a montage of a human intestinal wall tissue slice was imaged using the 2.5x and 60x objectives. The 2.5x objective montage depicts the entire tissue slice as a small portion of the total image. The edge of the round cover slip is also discerned. The imaging FOV on the slide is depicted as crosshairs within a schematic representation by the Gen5 software. The 60x objective montage is a very small subsection of the 2.5x image. The location of this image in context with the entire tissue slice is indicated, along with the much smaller region of the slide.



**Figure 7.** Maximum magnification using the Agilent BioTek Cytation 5 cell imaging multimode reader. Fixed and stained tissue slice of human intestinal wall immobilized on a 1" x 3" slide was imaged with 2.5x and 60x objectives to generate a 12 x 8 montage. The area of tissue from 2.5x image magnified with 60x objective is indicated, as well as the area of the slide imaged with each montage with slide schematic.

The convenience of the multi-objective turret system of the Cytation 5 is demonstrated in Figure 8. The same tissue slide of diseased human kidney tissue was imaged using a color brightfield montage (12 x 8) with six different magnifications (2.5x to 60x) without intervention. The area imaged by the increasing magnification is identified for each image. Because the Cytation 5 uses a six position turret system, different magnification steps can be programmed for slide regions for automatic positioning, focus and imaging without any user intervention. As with Figure 7, the entire tissue slice can be captured with lower magnification. With increasing magnification, greater resolution of microscopic details emerge. The best magnification for subsequent analyses can then be selected after viewing stitched images.



**Figure 8.** Macroscopic and microscopic structure of human chronic nephritic kidney tissue. A series of montage (12 x 8) images at increasing magnification were recorded and stitched from the same tissue slice. Boxed area represents the area imaged in the next higher magnification image. Objective magnification and scale bar are present for each montage.

## Results and discussion

The Cytation cell imaging multimode reader, in combination with Gen5 software, has several features that assist in both manual and automated imaging of stained tissue slides. Manual imaging allows the investigator to have complete control over the carrier x,y position while it is inside the reader using either the Gen5 software controls or an external joystick. Once the imaging region has been identified, single or montage color brightfield images can then be captured, stitched, and analyzed within the Gen5 manual mode session. Alternatively, slides can be imaged using an automated routine. Slide location, magnification objective, and montage array (up to a 15 x 15 matrix) can all be programmed prior to imaging. In addition, multiple imaging steps can be implemented to utilize different objectives, and/or specific regions of the slide to be imaged within the same experiment by setting up Beacons or custom ROI contours with Gen5 3.08 and higher.

An important attribute of color brightfield is white balancing. White balancing is the process of removing unwanted color casts such that objects that are white are rendered white in the image. Correct balancing needs to account for the color "temperature" or relative warmth or coolness of the light source. The human eye is much more adept than a digital sensor at judging white under a variety of light conditions so it is important that it be automatically balanced in order to achieve correct color rendition during color brightfield imaging. Color balance is achieved by measuring and adjusting the output of the three color brightfield channels independent of the actual sample to insure that they are equivalent. This process is done automatically by Gen5 for every set of three color images taken. Once white balancing has been performed, illumination levels are fixed and unchanged during the read step.

Low magnification objectives such as the 2.5x and 4x have long depths of field. When these objectives are employed for color brightfield imaging montages, the Cytation 5 will use autofocus prior to the first image only, saving considerable amounts of time with large montages. Higher magnification objectives, having much shorter depths of field, require autofocus with each image, as small deviations of the slide flatness or specimen thickness can result in out-of-focus images. When manually imaging the specimen, the user will naturally adjust the focus with each move of the imager. Conversely, with unattended operation the instrument needs to be able to adjust the focus without intervention in order to achieve good images necessary for further analysis. The autofocus process is different between fluorescence and color brightfield microscopy.

Fluorescence-based autofocusing uses an image-based statistical algorithm that assesses contrast by measuring the standard deviation and/or correlation between adjacent pixels to determine optimal focus.<sup>4</sup> As the instrument approaches the focus point, the difference in signal between the true image and background pixels increases dramatically, thus the standard deviation of all the pixels increases. A plot of pixel standard deviation vs. z-axis height results in a sharp peak at the point of focus.<sup>5</sup> The advantage of using a statistical algorithm, such as standard deviation, is the width of the feature. The contrast of the image begins to increase far from focus, yielding a wider range within which searching results are successful in finding optimal peak contrast.<sup>5,6</sup>

While fluorescence imaging has very sharp differences between background and image, color brightfield autofocus presents unique challenges with autofocusing. Brightfield imaging has considerably less overall contrast, as well as less well defined target edges and multiple focal planes from reflections, meniscus and thick samples. With color brightfield imaging, a scan of standard deviation vs. z-axis objective height reveals a double peak when the sample thickness exceeds the depth of field, which is common for objectives 10x and greater. To ensure uniform images, the same peak needs to be selected. The Cytation 5 uses a “dual peak” algorithm to select the higher focal plane of the right hand higher z-axis peak. Briefly, a scan is performed using either contrast or edge detection. The derivative of the scan is calculated, and the zero crossing corresponding to the trough between the two peaks is selected. The algorithm now looks for the peak “edge detection” value to the right of the zero crossing. The image is then taken at the higher z-axis maximum of the edge detection calculation. This procedure ensures that the same focus point is always used for subsequent images, making the focus sharp and repeatable.

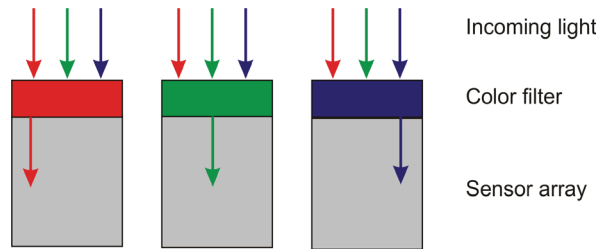
## Automated color imaging of tissues

### Assay principle

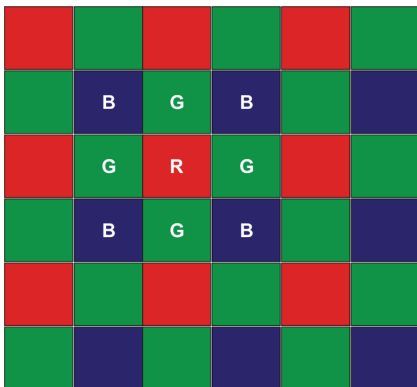
Chromogenically-stained samples, usually tissue, have been the mainstays for research microscopy almost from the moment light microscopes were invented. In order to improve contrast, differentiating stains were developed to treat the samples prior to examination. Over time, the recording of images obtained from brightfield microscopy has progressed from hand drawings to photographic material and, more recently, to digital imagery. In all these technologies the recordings were first black and white (i.e. grayscale) and then, as technology advanced, color. Just as black ink drawings were replaced by multicolored ink, silver reduction-based black and white film was replaced by multilayered color processing providing a true representation of the subject. The same is true in digital microscopy, where digital grayscale brightfield imagery has been supplanted by color brightfield for the examination and data recording of color microscope slides.

The brightfield microscope uses white light absorption by the specimen as its principle mechanism for contrast formation. While some samples contain significant amounts of chromogen (i.e. plants), animal cells are for the most part transparent and cannot provide sufficient contrast for the microscope-assisted human eye to observe fine cellular structure. This led to the development of stains used for the visualization of cellular matter. One of the most commonly used stains is hematoxylin and eosin (H&E stain). Its primary use is in recognizing tissue morphology.<sup>3</sup> Hematoxylin requires oxidation and complexation with a mordant to be effective as a cellular stain. Once properly treated, the dye stains nucleic acids with a deep blue-purple color, while eosin is pink and stains proteins nonspecifically. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining. Specific proteins can also be identified within tissues through the use of immunohistochemistry, where antibodies specific towards antigens, primarily protein epitopes, are employed. Visualizing an antibody-antigen interaction can be accomplished in a number of ways, the most common being the use of an antibody-enzyme conjugate. The enzyme, usually horseradish peroxidase (HRP) or alkaline phosphatase, can catalyze color-producing reactions when incubated with an appropriate chemical substrate. The epitope of interest is either directly labeled with a conjugated primary monoclonal antibody or more commonly labeled with a secondary antibody conjugate that is directed against the primary antibody.

In order to image and digitally record brightfield imagery, essentially two different types of digital cameras have been developed; color and grayscale. In a digital grayscale camera, each pixel on the sensor captures information regarding the intensity of light striking it, regardless of wavelength. Each pixel records a numerical value from 0 (i.e. black) to its maximum value (i.e. white), with the intermediate numbers representing the range or scale of gray between the extremes. A color digital camera uses the same set of pixels, except that each pixel has a red, green, or blue color filter in front such that the intensity measurement for only the intensity of specific wavelengths are recorded (Figure 9). The filters, (red, green, and blue) only allow specific wavelengths of light to pass through to the sensor array pixel with the result in measuring the intensities of the primary colors on separate pixels.



**Figure 9.** Schematic cross section of individual pixels of a digital color sensor. A specific color filter allows only the intensity of a specific wavelength to pass through to the sensor array.

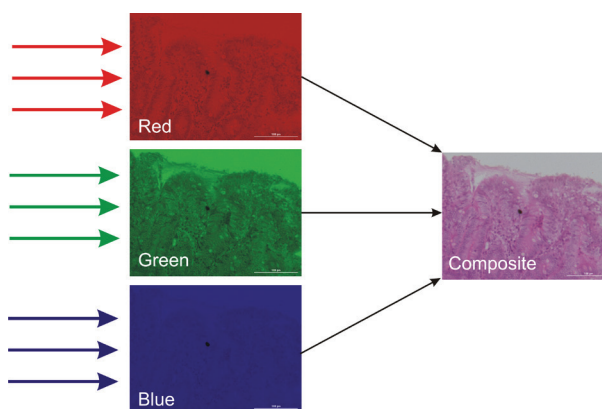


**Figure 10.** Bayer color filter array of pixel arrangement on a grayscale sensor. Each pixel is a single primary color with the other two colors being calculated as the average of the four adjacent pixels. In this example, the green value for the red pixel (R) would be the average of the 4 green pixels (G), while the blue value for the same red pixel would be the average of the adjacent blue pixels (B).

The most common array arrangement is the Bayer pattern (Figure 10), such that there are twice as many green pixels as either blue or red, as a means to compensate for the eye's increased sensitivity towards the color green. With this arrangement, the numbers of pixels remains the same as the grayscale camera, but each color is divided up from the total. In order to reconstruct an output image of equal resolution to the original camera resolution, a process called demosaicing is used. Essentially, this process interpolates the two color values for each pixel that are missing by looking at neighboring pixel values for that color. While there are many different interpolation algorithms, one of the simplest is bilinear interpolation. In this method, neighboring pixels are used to interpolate light intensities filtered out by the CFA. As an example, the red pixel labeled R in Figure 10 can be considered. The CFA allows only red wavelengths of light to be transmitted to the grayscale camera at that pixel. Blue intensity for that R pixel position is calculated from the mean intensity of 4 neighboring B pixels at the corners of the R pixel. Similarly green intensity at the R pixel is calculated from the mean intensity of the 4 neighboring G pixels at the sides of the R pixel.

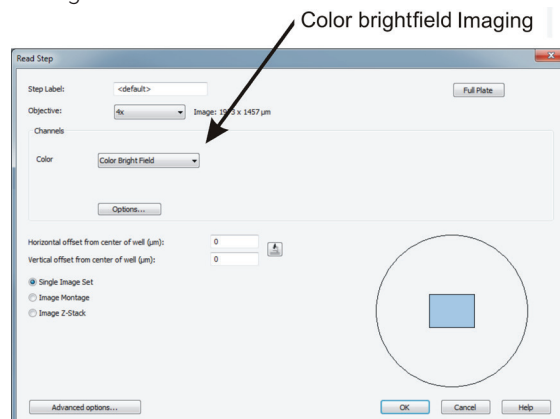


The primary tradeoff between these two technologies is one of spatial vs. color resolution, with grayscale being superior in providing spatial resolution and a color digital camera providing color. The Cytation 5 uses a mixture of these two technologies to provide color brightfield imaging with full spatial resolution without the need for demosaicing processing. Rather than limiting colored light exposure to each pixel of the digital camera using a CFA, the Cytation 5 illuminates the sample and the camera sensor with three different light sources made up of the primary colors (red, green, and blue) in quick succession, producing full resolution images with each color separately. The three images are then overlaid with each other, resulting in a composite color brightfield image (Figure 11).



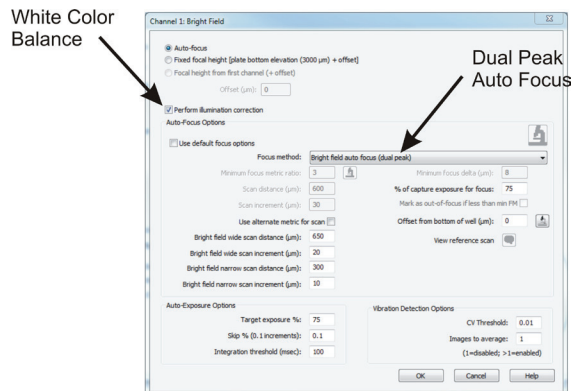
**Figure 11.** Composite overlay image from three separate RGB filtered brightfield images. LED light sources with different color profiles (red, green, and blue) are used to illuminate the entire digital sensor array in quick succession. The three images are then combined to generate a composite color brightfield image.

Selection of color brightfield reading is made from the Imaging Read step screen using the pull down menu for color (Figure 12). Selection of Color Brightfield activates the red, green and blue emitting LEDs in the Cytation 5 for light illumination during the read.



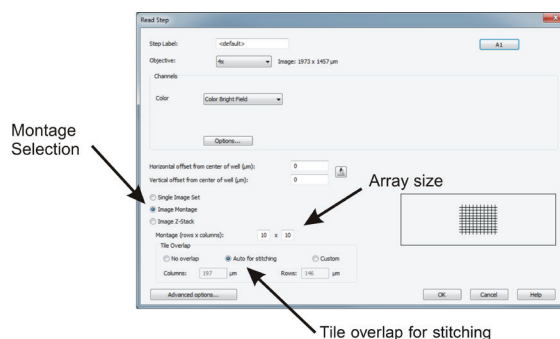
**Figure 12.** Gen5 Read Step identifying color brightfield imaging. The images are collected using a 4x objective with color brightfield selected as the imaging color.

Selecting Color Brightfield as the imaging color enables a number of default features viewable under the “options” button. White color balance is automatically enabled with color brightfield imaging as part of the illumination correction and cannot be deselected in the options screen (Figure 13). In addition, the dual peak method for autofocus is automatically chosen as the default (Figure 13).



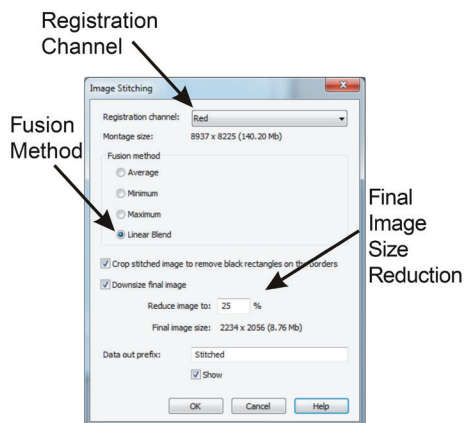
**Figure 13.** Gen5 imaging options field with Color Brightfield. Identification of white color balance and dual peak autofocus defaults.

Samples being examined by color brightfield microscopy can often exceed the field of view of the objective, particularly at higher magnification. In order to image the complete area, a series of images or montage are taken that encompasses the entire sample. For example, the area of a typical fixed and stained tissue section is significantly larger than a single image from the 4x objective. However, a montage of several overlapping images will encompass the entire section. Moreover, tissue sections have gross and microscopic structures that are best assessed using a montage series at different magnifications to capture tissue and cellular structures. The montage is generated with a series of images or tiles in a serpentine fashion to accommodate the desired number of rows and columns necessary to create the full picture, which is then stitched together using Gen5 software.



**Figure 14.** Gen5 Read Step identifying montage imaging. The color brightfield images are collected using a 4x objective with Image Montage selected and a 10 x 10 array with tile overlapping set to auto for stitching.

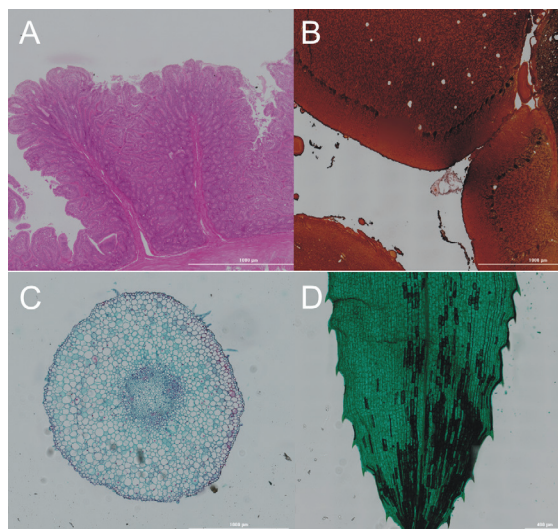
Montage imaging is selected from the Read Step screen in Gen 5. After the color and slide position have been chosen, the “Image Montage” radial button is selected. This activates the montage parameters regarding the number of rows and columns of the image array and tile overlap (Figure 14). If there is no interest in stitching the montage together, selecting “No overlap” will result in a series of images encompassing the maximal amount of image area, with image tiles



**Figure 15.** Gen5 Image Stitching data reduction step. The registration channel (red, green, or blue) is selected along with the fusion method to be employed. The final image size can be reduced in size saving disc storage space.

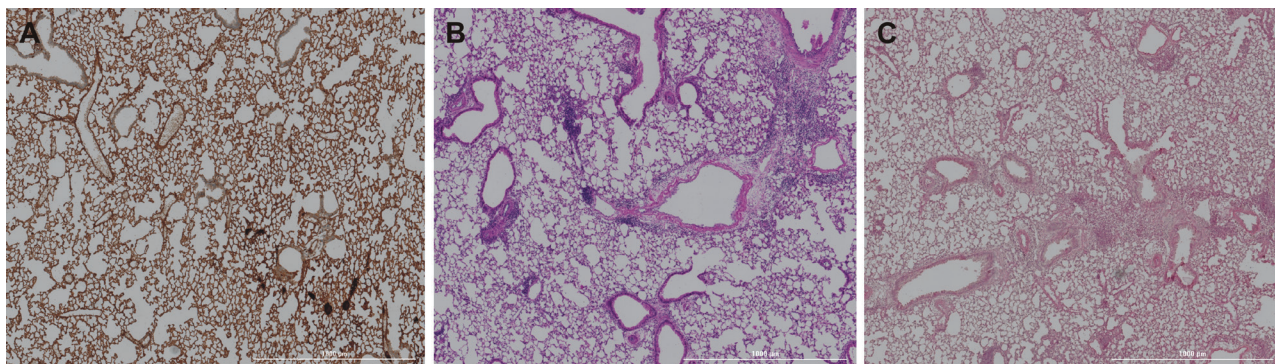
immediately next to one another, and all image tiles will be analyzed separately. If however, the desire is to stitch the images into a single montage file that can be analyzed or saved as a single image file, there needs to be some overlap with each digital image in order to properly register and fuse them together (Figure 14). Unless there are unusual features to the image, the default overlap is suggested. A custom overlap can be selected that allows the user to increase or decrease the amount of image tile overlap. The stitching fusion method needs to be selected, with the linear blend normally being the best option (Figure 15). It is best to select the channel with the most complexity to use a registration channel for optimal image fusion. The increased granularity resulting from image complexity results in easier alignment of the image tiles during the stitching process. Due to the potential for enormous file size, Gen5 software allows the user to compress the final file to more manageable size levels. Those interested in maximum detail for analysis can maintain full image size and resolution, while those interested in a smaller composite image can elect to substantially reduce image resolution. The default name of “stitched” can be used if only one file is being produced, but alternative names with more precise significance can also be entered (Figure 15).

Faithful color rendition is a key element in color brightfield imaging. Currently, no official color standard exists that allows one to “calibrate” the color output of the digital camera. Therefore, the ability to render multiple colors faithfully is critical. The Cytation 5 uses a white color balance procedure as part of the illumination correction that is automatically enabled. While many tissue slices are stained with hematoxylin and eosin (H&E stain) for its ability to identify nuclei and proteins, other stains or the natural color of samples are also visualized microscopically. As demonstrated in Figure 16, the vivid green from chlorophyll present in plant leaves or the vasculature of growing plant roots cross sections can also be imaged.

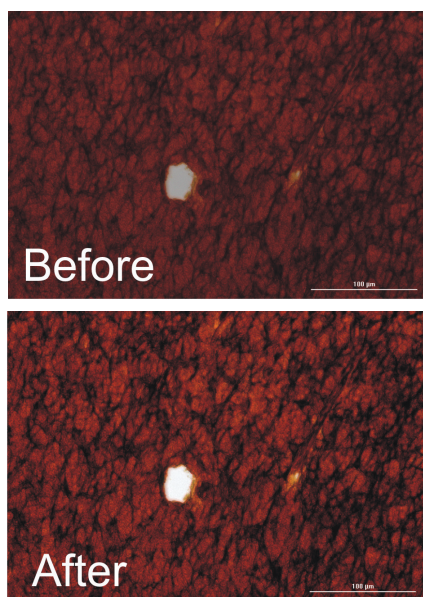


**Figure 16.** Series of color brightfield images from different tissues. (A) H&E stained human intestinal wall imaged with a 20x microscope objective; (B) Human cerebellum tissue imaged with a 20x microscope objective; (C) Young bean root cross section imaged with a 20x microscope objective; (D) Hydrilla verticillata water plant leaf imaged with a 20x microscope objective. All images are stitched montage composites.

Tissue slices can be stained for increased contrast or to identify specific cellular proteins using a number of different methods. Antibody-based methods typically employ a specific antibody conjugated to a reactive enzyme such as horseradish peroxidase or alkaline phosphatase. These enzymes react with provided substrates to form chromogenic compounds that stain the tissue where the antibody is bound, yielding spatial and expression level information of antigen. Horseradish peroxidase conjugates convert the substrate 3, 3' diaminobenzine (DAB) to form a black to brown product, while alkaline phosphatase converts 3-amino-9-ethylcarbazole (AEC) forming pink shaded products. Figure 17 illustrates mouse lung tissue stained using an HRP conjugated antibody and reacted with DAB substrate, resulting in brown stained lung parenchyma. Likewise, alkaline phosphatase conjugated antibodies result in pink staining. A comparison to the familiar H&E stain is also shown.



**Figure 17.** Mouse lung tissue stained using various methods. Lung tissue was stained using (A) HRP antibody conjugate reacted with DAB to form a brown reporter color; (B) H&E dyes; and (C) Alkaline phosphatase conjugate reacted with AEC to form pink products.



**Figure 18.** Use of color contrast and brightness adjustments to improve image quality. Color brightfield image (20x) of human brain tissue before and after contrast and brightness adjustment. Images represent red, green, and blue color images overlaid.

Individual images can often be improved by altering brightness and contrast of each color separate from one another. Due to differences in perception of the human eye compared to the digital camera sensor, particularly with green coloration, slight adjustments to the brightness and or contrast of the image can result in a sharper looking image. This is particularly true with higher magnification. As demonstrated in Figure 18, an increase in the green contrast results in a sharper image.

## Results and discussion

The Cytation 5 is a cell imaging multimode reader which is equipped to perform optical microscopy and PMT-based fluorescence detection. The microscopy components are digitally-based consisting of LEDs for illumination, filter cubes, microscope objectives (1.25x to 60x) and a grayscale CCD camera. Most microscopy components are located in the lower half of the Cytation 5, except for phase contrast illumination, which resides in the same upper space as the

PMT-based optics of the conventional microplate reader. The design is also modular allowing selection of microscopy and/or microplate reader optics as desired. Various sample vessels can be used with the microscopy modules including microscope slides, a broad range in microplate densities (6 to 384-well), petri dishes (35, 60, and 100 mm) and tissue culture flasks (T-25 cm<sup>2</sup>).

The Cytation 5 cell imaging reader interacts with Gen5 software to provide optimal results depending on the read modality chosen. For color brightfield imaging, multicolored LED illumination within the Cytation 5 provides for the resolution of a high performance grayscale digital camera sensor with the color capability normally seen with color digital cameras. Correct color adjustment, critical for correct color rendition, is automatically enabled in the software when color brightfield is selected with no user intervention required. Montage color brightfield imaging utilizes a sophisticated autofocus software algorithm to optimize image focus. The autofocus routine is automatically performed at the initiation of a montage for all objectives and repeated with each image of higher magnification objectives (10x to 60x). Lower magnification objectives (2.5x and 4x), having a larger depth of field, do not require repeated refocusing during montage reads and autofocus is only performed at the initiation of the image step, saving time during montage imaging.

Image acquisition, processing, and analysis are performed by Gen5 software available with the Cytation 5. The software is a critical component in conjunction with the Cytation 5 hardware in rendering crisp and useful images. Most commonly, regions of the slide that are imaged are larger than a single capture. In these instances, multiple images are taken in a montage that encompasses the region to be examined. In order to align or analyze the series of images faithfully, they need to be combined into a single contiguous image by a process referred to as stitching. Because digital images are at their most basic levels, an array of numerical values, mathematical algorithms can be utilized to combine separate files. The separate tiles or images are fused at the area of overlap using a number of different user selectable methods: linear; average; minimum; and maximum. The linear blend method typically provides the best result. It is a calculated average, with each point weighted by its distance to the closest border. This method often produces the smoothest transition from one image tile to another. The other methods, as their name implies, use the average, minimum, or maximum intensity values of the overlapping regions for the output.

## **Acknowledgments**

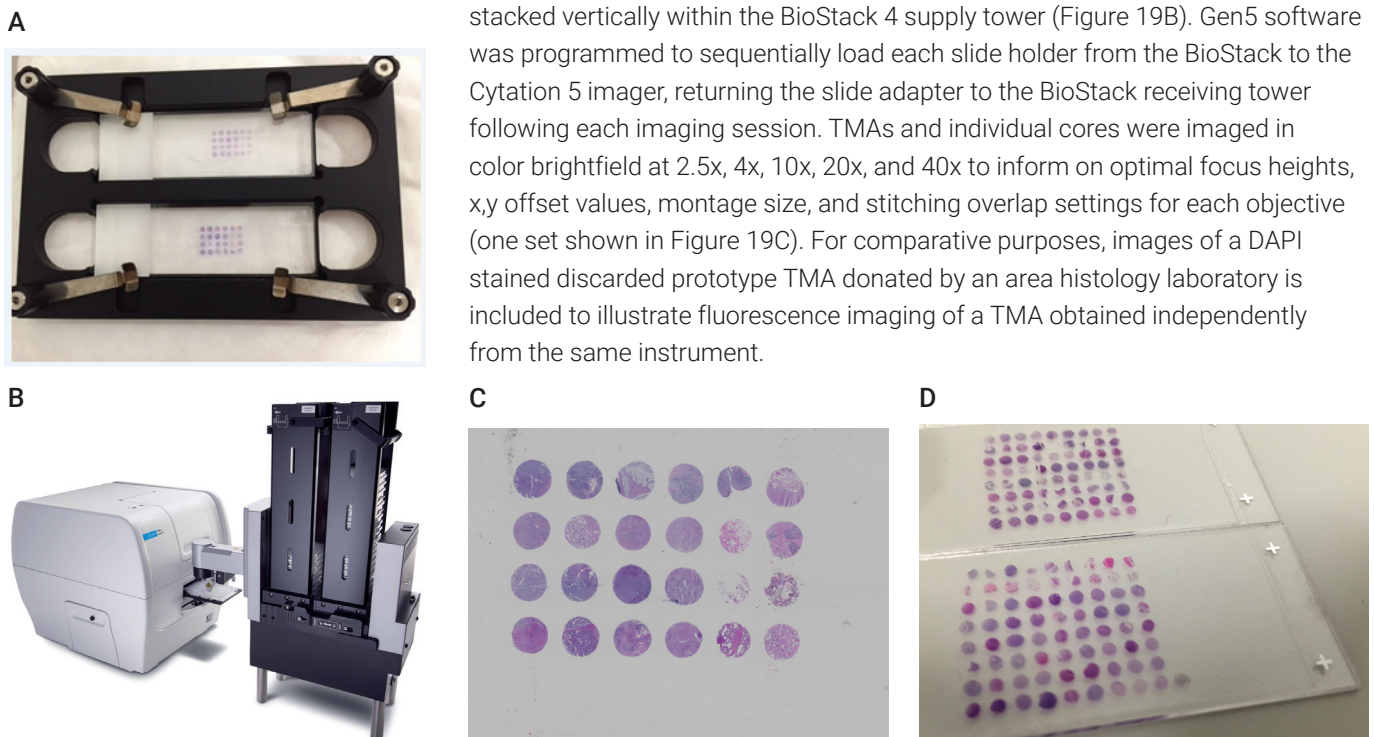
We would like to thank Dr. Yvonne Janssen-Heninger and Dr. Doug Taatjes from the Department of Pathology at the University of Vermont, School of Medicine for their kind donation of stained tissue slides.

## Applied robotics for enhanced throughput options in microscopy as demonstrated by automated tissue microarray (TMA) imaging

### Assay introduction

Historically, microscopy has been a hands-on method that can limit higher throughput options in sample processing. More recently, options for sample processing in microscopy have expanded from both the introduction of automated digital microscopes, and the adaptation of microscopy slide techniques to imaging in microplates or other high-throughput formats. An example of one of these adaptations is the tissue microarray (TMA), a technique where hundreds of individual tissue cores as small as 0.6 mm in diameter and 2 to 5  $\mu\text{m}$  thick can be arrayed on a single microscopy slide, allowing increased throughput in a number of common histology procedures. TMAs (Figure 19) have proven useful in many applications such as bio banking and archiving of biopsy and other tissue samples; diseased state examination, classification and grading; quality control; antibody and staining optimization during assay development.<sup>7</sup>

To demonstrate optimal image acquisition settings designed to overcome the challenges of automating TMAs at high throughput, a set of two H&E stained 1.5 mm 72-core TMA slides and six H&E stained 1.5 mm 24-core TMA slides were loaded into 4 Agilent BioTek microscopy slide holders (Figure 19A), and stacked vertically within the BioStack 4 supply tower (Figure 19B). Gen5 software was programmed to sequentially load each slide holder from the BioStack to the Cytation 5 imager, returning the slide adapter to the BioStack receiving tower following each imaging session. TMAs and individual cores were imaged in color brightfield at 2.5x, 4x, 10x, 20x, and 40x to inform on optimal focus heights, x,y offset values, montage size, and stitching overlap settings for each objective (one set shown in Figure 19C). For comparative purposes, images of a DAPI stained discarded prototype TMA donated by an area histology laboratory is included to illustrate fluorescence imaging of a TMA obtained independently from the same instrument.



**Figure 19.** Microarray slides. (A) Two TMA slides are positioned in a slide holder compatible with robotic interface to a multimode imaging reader (B). The software was preprogrammed to sequentially load each slide holder from the robot to the imager that then captured 15 x 15 images of each slide using automatic montage and stitching features to illustrate the final array (C), shown on one of the TMAs at 4x. (D) Two 9 x 8 tissue microarrays (TMAs), shown with different offset positions on the microscopy slide. The slide on the bottom includes a marker core, often embedded into arrays as a control and to assist with identifying panel locations, but also adding to asymmetric positioning between arrays. Customized x,y offsets and montage and stitching options available in Agilent BioTek's Gen5 software can be used to compensate for these geometric anomalies during imaging on an Agilent BioTek Cytation 5, allowing mixed size TMAs to be run together in higher throughput via the Agilent BioTek BioStack 4 microplate stacker.

TMA were chosen as a model for demonstrating montage and stitching image optimization at high throughput due to their unique geometry and mounting technique. Following microtome sectioning, paraffinized TMA slices are placed in water and the microscope slide is dipped under the sheet and lifted up out of the water bath to capture the array. Although there is a window of time when the TMA can be repositioned on the slide, the result of this technique is that even panels of the same number and size of cores may not be mounted with the same center offset on a microscope slide (Figure 19D). Additionally, some TMAs may or may not have marker cores that can be offset from the remaining cores. Further, although the Agilent BioTek microscopy slide adapters have been designed for standard 25 x 75 x 1 mm microscopy slides, some tolerance should be included when calibrating x,y offsets for imaging to compensate for possible slide movement during robotic transfers and/or image carrier positioning.

## **Materials and methods**

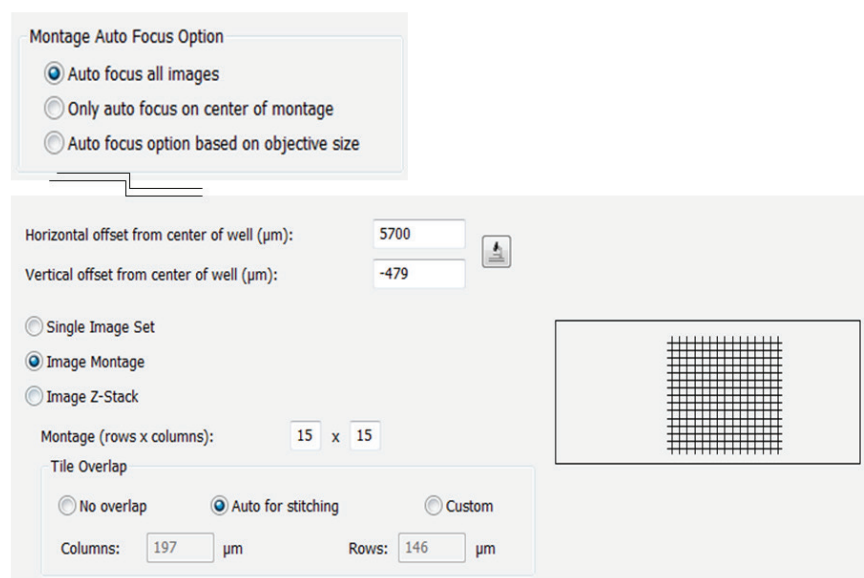
### *Materials*

- 1 set FDA Human Normal Organ Tissue, US Biomax, Inc. (p/n DA802 H&E stained, Microarray Panels) and Core Specification Sheets: [www.biomax.us/tissue-arrays/Multiple\\_Organ/FDA802](http://www.biomax.us/tissue-arrays/Multiple_Organ/FDA802)
- Qty 6 Top 4 Types of Cancer Test Tissue, US Biomax, Inc. (p/n TP242 H&E stained, Microarray Panel) shown by Figure 25, Core Specification Sheet: [www.biomax.us/tissue-arrays/Multiple\\_Organ/TP242](http://www.biomax.us/tissue-arrays/Multiple_Organ/TP242)
- Discarded prototype DAPI stained TMA slide donated by area histology laboratory (Thyroid cancer and adenoma tissue array, US Biomax, Inc. p/n TH641 unstained)
- Agilent BioTek slide adapters (p/n 1220548)
- Agilent BioTek integration kit for Cytation 5 – BioStack 4 interface (p/n 7310053)

### *Method*

Optimal image acquisition settings for automating the imaging of TMAs were determined using an H&E stained 72-core TMA. The TMA was placed into a slide adapter, then x,y offsets and montage parameters were established using manual mode within the protocol definition interface of Gen5 software at 4x magnification in color brightfield. A second 72-core TMA was placed on the holder, and both slides were imaged with the parameters defined. Following the imaging session, one of the TMAs was slightly offset and the other was centered. The x,y offset optimization was performed on the second slide (Figure 20). Two read steps were then programmed into the protocol so that all TMAs would be two different geometric configurations. Six H&E stained 1.5 mm 24-core TMA slides were loaded into three additional Agilent BioTek microscope slide holders, and all four holders were stacked vertically within the BioStack 4 supply tower. Gen5 software was

programmed to sequentially load each slide holder from the robot to the Cytation 5 imager, returning the slide adapter to the stacker receiving tower following each imaging session. TMAs and individual cores were imaged using the defined protocol. Following the run, images were processed using a linear blend montage method on the red channel and a downsized image size of 7.35%. The same process was used to image select cores on three TMAs at 20x and 40x. For comparative purposes, a DAPI stained discarded prototype TMA donated by an area histology laboratory was also optimized using the same procedure at 4x in the fluorescence imaging channel, and is included to illustrate fluorescence imaging of a TMA. In that case, the BioStack 4 was still used, but for a single slide transfer to and from the same Cytation 5 imager allowing walk-away imaging.

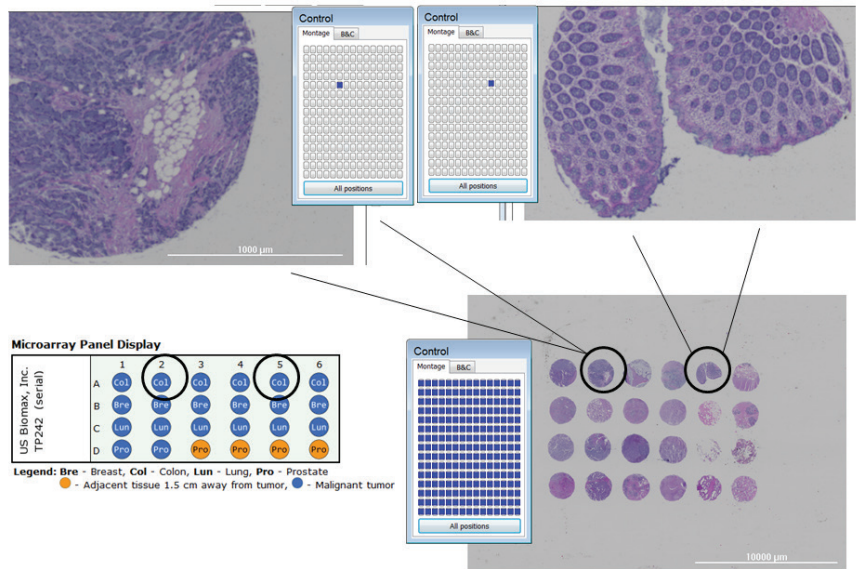


**Figure 20.** Agilent BioTek Gen5 capture procedure parameters. An example of the x,y offsets, focus setting, and montage and stitching options that were used to sequentially image a batch of eight TMAs of two different panel sizes at 4x in color brightfield on an Agilent BioTek Cytation 5. Slides were transferred back and forth to the imager using the Agilent BioStack 4.

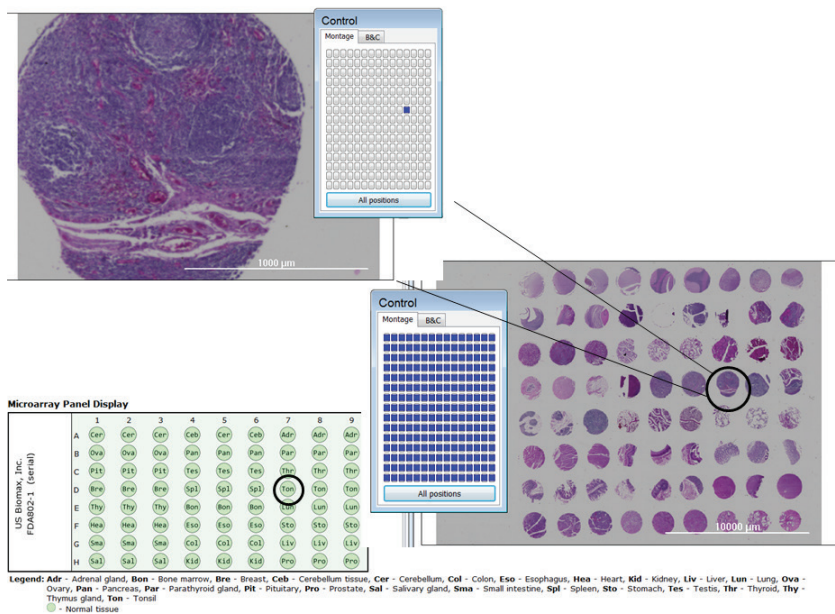
## Results and discussion

The ability of Cytation 5 to image H&E stained TMAs is demonstrated in the following range of figures. The Cytation 5 microscopy module uses an inverted microscope orientation, thus TMAs must be imaged through the bottom of the TMA to ensure correct core identification in the TMA panel. Thus, slides were oriented on the holder with the label up. Figure 21 illustrates a 24-core sample TMA, while Figure 22 shows the ability to image a 72-core sample TMA using lower resolution microscopy with a 4x objective.



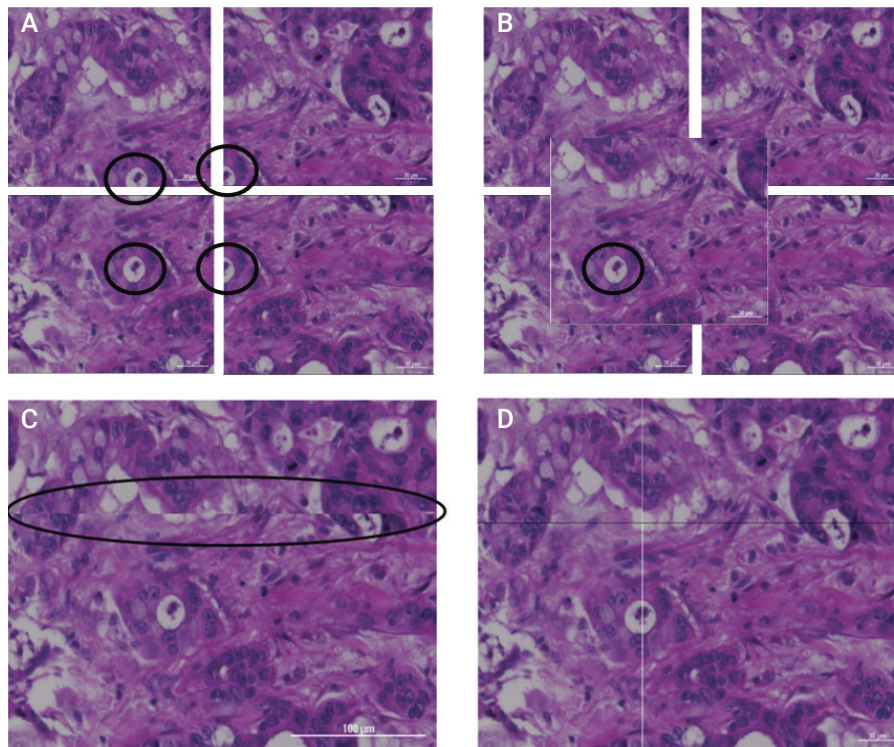


**Figure 21.** A 24-core tissue microarray. Imaged on an Agilent BioTek Cytation 5 at 4x, using montage and stitching options available in Agilent BioTek Gen5 software. This slide was run in a batch of eight mixed sized TMAs using Agilent BioTek BioStack 4. The bottom right image shows the entire array displayed using the montage interface option to view all tiles (All Positions mode). At the top are two different cores from the array shown in single tile view. The case on the left is diagnosed as colon mucoid adenocarcinoma grade III malignant from an 81 year old female, and on the right a case of cancer adjacent colon tissue type malignant from a 35 year old male. Because Cytation 5 images from the bottom, positioning the slides on the slide holder in the correct orientation facilitates tissue identification so the final image matches the TMA panel (lower left). In this case, slides were oriented on the holder with the label up.



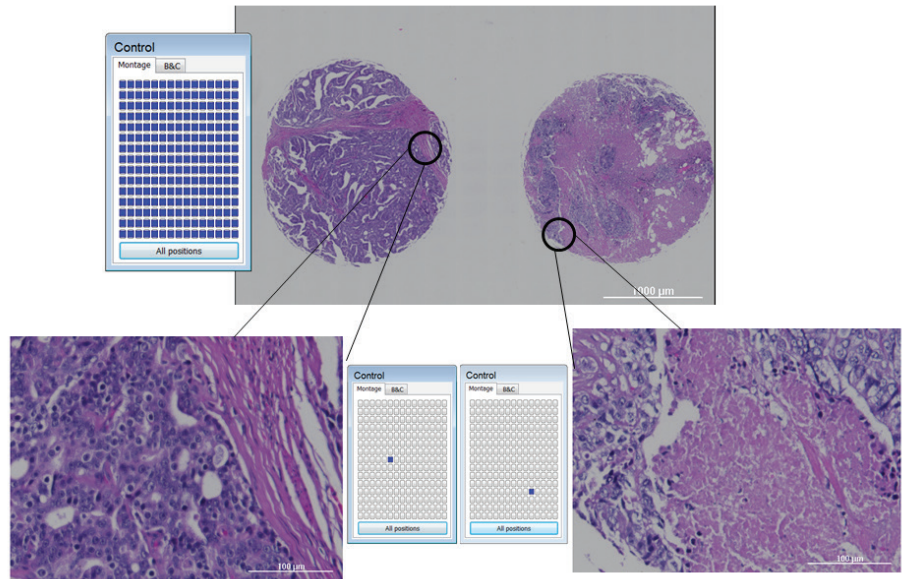
**Figure 22.** A 72-core tissue microarray of human normal organ tissue. Imaged on an Agilent BioTek Cytation 5 at 4x using montage and stitching options available in Agilent BioTek Gen5 software. This slide was run in the same batch of eight mixed-sized TMAs as the array in Figure 21 using the same settings. At the top, the entire array is displayed using the montage All Positions view. A core of normal tonsil tissue is shown in single tile view, selected by clicking on an individual tile in the montage control panel, or doubleclicking directly on the montage image.

Several TMA examples have been imaged using the Cytation 5 imager, demonstrating the ability to perform higher resolution microscopy to enable tissue examination. With default or custom overlap settings, selected before image acquisition, optimized stitching parameters can be modified to provide the final image for analysis (Figure 23).

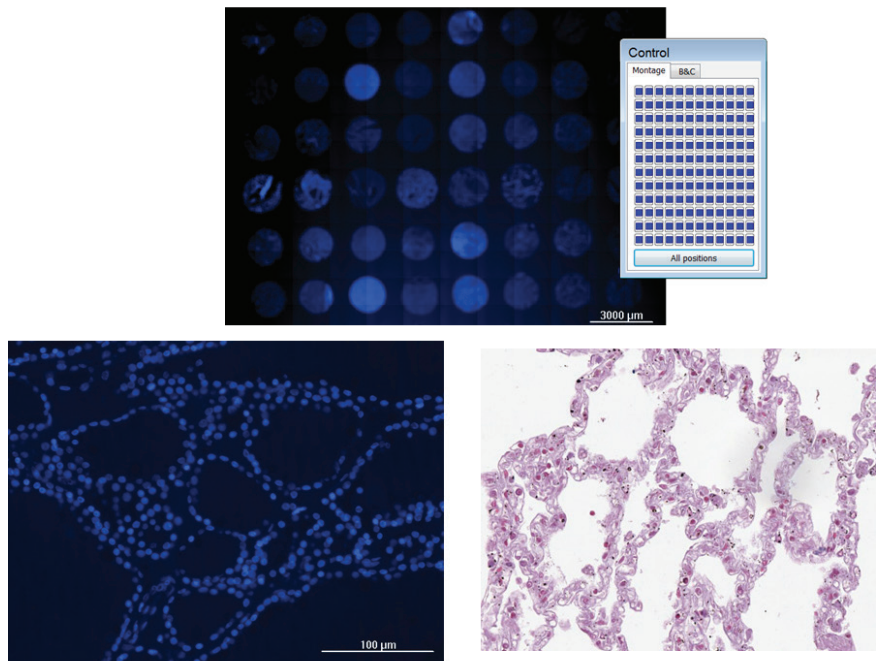


**Figure 23.** (A) Images of individual tiles of a 2 x 2 montage at 40x taken from a TMA core. The circles show where each tile is imaged in relationship to the others using a distinguishable marker on the tissue. (B) A single image using the same center offset as the montage at 40x overlaid on the four tiles shown on the left. The image was centered over the marker in the lower left tile (circled) to illustrate that increased total surface area at the same magnification can be obtained from 4 individual tile images using montaging. (C) Default stitching overlap settings are asymmetric along the horizontal row (circled). Tile overlap values can be customized to help correct this. Vertical (column) offsets are well aligned. (D) Illustration of optimal 2 x 2 montage tile overlap for stitching at 40x.

Finally, Figure 24 represents an example of TMA when comparing normal versus diseased tissue for researcher interpretation, while Figure 25 displays the ability to also perform fluorescence microscopy on TMAs using the Cytation 5.



**Figure 24.** Prostate tissue. 20x montage (top center) with area of detail for individual tiles shown. The bottom left tissue is malignant prostate tumor; the bottom right is prostate adjacent tissue 1.5 cm away from tumor. These cores are from two different cases.



**Figure 25.** A discarded prototype TMA. Donated by an area histology laboratory stained with DAPI and imaged at 4x on an Agilent BioTek Cytation 5 using montage and stitching in Agilent BioTek Gen5 software (top). Background haze is likely attributed to incomplete deparaffinization. The bottom left is a single image taken at 20x from the same array, while the bottom right is an H&E stained normal lung tissue at 20x from another slice of the same array imaged by an independent source on another microscope for comparison (US Biomax p/n TH641).

## Conclusion

The ability of the Agilent BioTek Cytation 5 cell imaging multimode reader, and the Agilent BioTek BioStack 4 microplate stacker for the automation of workflows for imaging H&E stained TMAs for high-throughput histology applications is demonstrated in this application note. There are several key parameters that can be optimized for the ideal image for analysis and interpretation. For the TMA assay, it's critical to remember that the Cytation 5 images from under the slide, therefore to facilitate tissue ID, slides should be oriented label side up in the slide adapter – the upwards facing side of the slide when placed in the slide holder should match the geometric IDs of the microarray panel.

The built-in stitching and montage algorithms available in the Agilent BioTek Gen5 microplate reader and imager software are ideally suited for imaging tissue microarrays in color brightfield and fluorescence modes at any throughput. Optimizing the montage size, tile overlap dimensions, x,y offsets and focus options enhances customized image acquisition useful for arrays of the same or different sizes to be run in a single batch. The montage control panel allows a view of either the entire stitched image or a close-up of each individual tile of the montage, allowing more detailed examination of stained tissue cores. Individual tiles can also be displayed by double-clicking directly on the area of interest in montage 'All positions' view.

When needed, the ability to include multiple read steps within a single protocol allows "parallel montaging" at multiple magnifications or geometric configurations, overcoming the challenge of imaging arrays of different sizes or with different x,y offsets at higher throughput, particularly when images of larger surface areas are desired at higher magnifications. Imaging at 4x using a 15 x 15 montage grid with autofocus on each image and custom x,y offsets configured to the largest array was found most favorable for capturing all cores of 72 and 24 1.5 mm TMAs. Higher density arrays may require 2.5x magnification to capture all cores in a single stitched montage image. At 20x, a 15 x 15 montage was found optimal for imaging pairs of 1.5 mm cores, offering enhanced detail via a larger field of view useful, for example, to image a malignant tissue core and a core with adjacent tissue 1.5 cm away from tumor side-by-side. With the higher magnification at 40x, a 2 x 2 montage provided four tiles that taken together covered a larger field of view than a single image at the same magnification on a portion of one 1.5 mm core sample. Custom tile offsets may be required to optimize tile overlap alignment if a final stitched image is desired from a 40x montage.

By means of any of the multimode imaging options available on the Cytation 5, including brightfield, color brightfield, phase contrast, digital phase contrast, and fluorescence, the techniques shown here can be performed with many microscopy slide applications using fixed human or animal cell lines, single tissue slices, partial or whole specimen mounts, or bacteria and yeast smears for example.

Histological sections are commonly stained with H&E for structural identification. Recent publications using BioTek microscopes for color brightfield imaging for histology and tissue section applications include:

1. Becker, W., Nagarkatti, M., and Nagarkatti P.S., miR-466a Targeting of TGF- $\beta$ 2 Contributes to FoxP3+ Regulatory T Cell Differentiation in a Murine Model of Allogeneic Transplantation. *Front. Immunol.* 9, 688, **2018**.  
<https://doi.org/10.3389/fimmu.2018.00688>
2. Randolph,G.J., Bala, S., Rahier, J-F, Johnson, M.W., Wang,P.L., Nalbantoglu,I, Dubuquoy,L, Chau, A., Pariente, B., Kartheuser, A., Zinselmeyer,B.H., and Colombel, J-F, Lymphoid Aggregates Remodel Lymphatic Collecting Vessels that Serve Mesenteric Lymph Nodes in Crohn Disease. *American J Path.* 186, 12; 3066-3073, **2016**. <https://doi.org/10.1016/j.ajpath.2016.07.026>

All of Agilent BioTek imagers possess fluorescence microscopy capabilities that can acquire and quantify fluorescently stained and immunofluorescent prepared slides. In addition, the selection of over 20 different preset filter cubes and optimized LED coupling, along with the ability to custom make filter sets, allows for a variety of fluorophores to be selected for imaging needs. Four-channel images can be acquired in each read step for multiplexing with various fluorophores and immunofluorescent labeling. Publications using the Agilent BioTek inverted microscopy for fluorescent detection include:

1. Antes, T.J., Middleton, R.C., Luther, K.M., Ijichi, T., Peck, K.A., Liu, W.J., Valle, J., Echavez, A.K., and Marbán, E., Targeting extracellular vesicles to injured tissue using membrane cloaking and surface display. *J. Nanobiotechnol.* 16:61, **2018**.  
<https://doi.org/10.1186/s12951-018-0388-4>
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6. Yeo, T., Jayasooriah, R., Autofocusing for Tissue Microscopy. *Image Vis. Comput.* 11, 629-639, **1993**
7. [www.biomax.us](http://www.biomax.us)



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