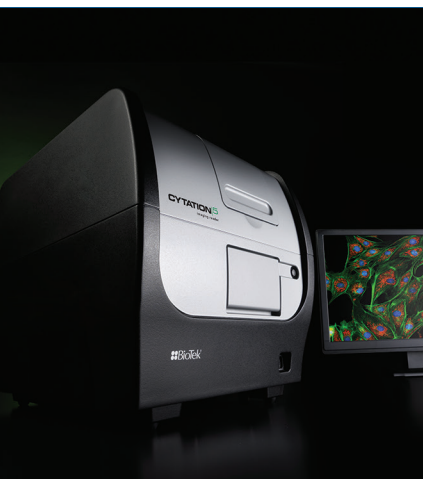


# Automated Color Imaging of Tissues

## Using Color Brightfield Microscopy and Image Stitching to Examine Fixed and Stained Tissues on Microscope Slides

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The microscopic examination of samples and tissues is one of the most commonly used tools to investigate cellular structure of plant and animal tissues and is the hallmark of clinical diagnostic pathology. The tissues are typically stained with compounds to improve contrast resulting in colored specimens that can be optically examined. Here we describe the use of the Cytation™ 5 Cell Imaging Reader to perform color brightfield imaging on fixed and stained tissues on microscope slides.

### Introduction

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 multi-layered 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, (e.g. 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 [1]. 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 horse radish 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 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 max 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 1). 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.

### Key Words:

Hematoxylin

Eosin

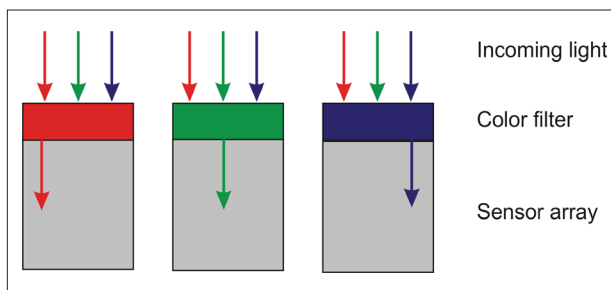
Stained Tissue

Color Brightfield

Montage

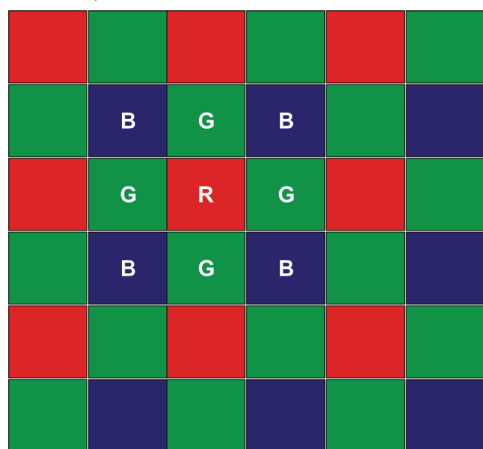
Microscope Slides

H&E



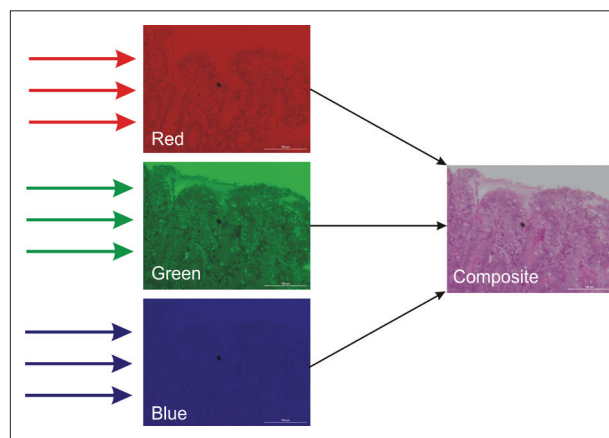
**Figure 1.** Schematic cross section of individual pixels of a digital color sensor.

Typically the color filters are laid out in a checkerboard-like array called a color filter array (CFA). The most common array arrangement is the Bayer pattern 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, considers the red pixel labeled R in Figure 2. 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.



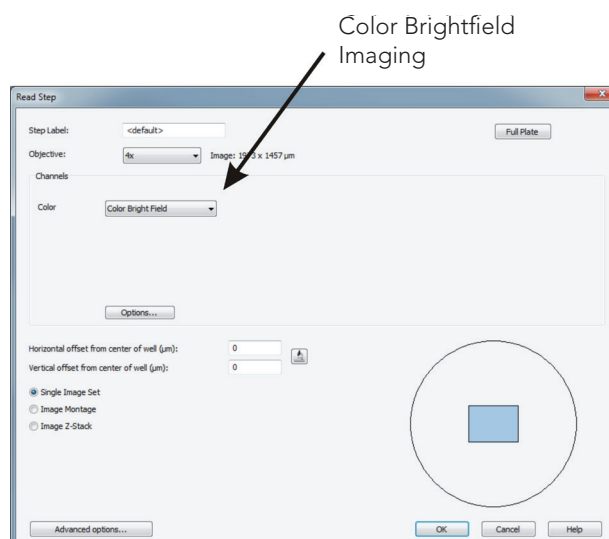
**Figure 2.** Bayer color filter array of pixel arrangement on a grayscale sensor. Each pixel is a single primary color with the other two colors being calculated as the average of the four adjacent pixels. In this example the green value for the red pixel (R) would be the average of the 4 green pixels (G), while the blue value for the same red pixel would be the average of the adjacent blue pixels (B).

The 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 3).



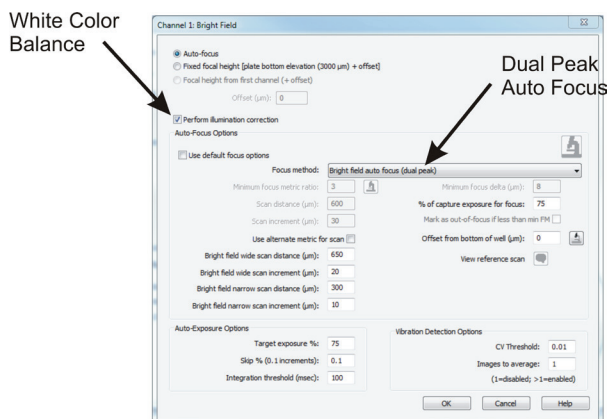
**Figure 3.** Composite overlay image from three separate RGB filtered brightfield images. LED light sources with different color profiles (red, green, and blue) are used to illuminate the entire digital sensor array in quick succession. The three images are then combined to generate a composite color brightfield image.

Selection of color brightfield reading is made from the Imaging Read step screen using the pull down menu for color (Figure 4). Selection of Color Brightfield activates the red, green and blue emitting LEDs in the Cytation 5 for light illumination during the read.



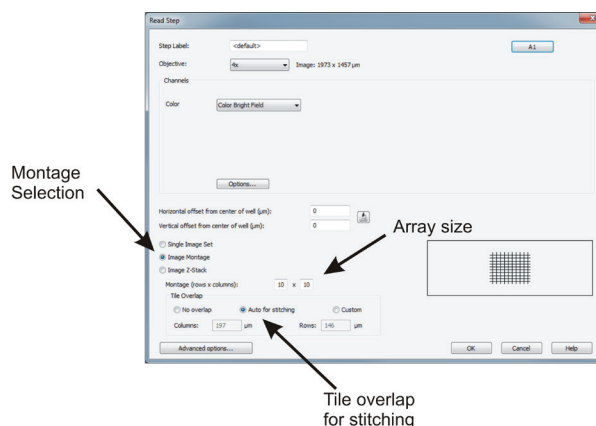
**Figure 4.** Gen5 Read Step identifying color brightfield imaging. The images are collected using a 4x objective with color brightfield selected as the imaging color.

Selecting Color Brightfield as the imaging color enables a number of default features viewable under “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 5). In addition the dual peak method for autofocus is automatically chosen as the default (Figure 5).



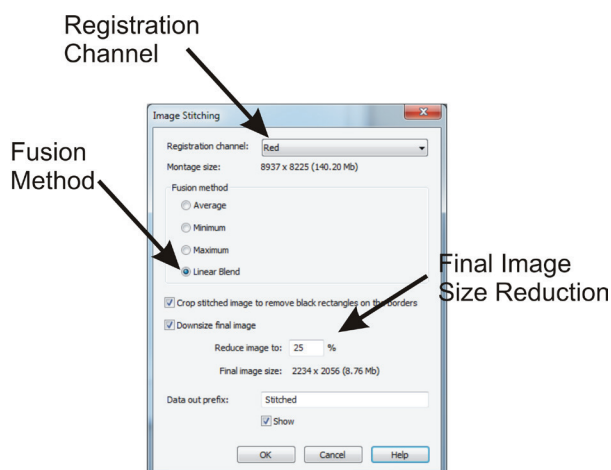
**Figure 5.** Gen5 Imaging options field with Color Brightfield. Identification of white color balance and dual peak autofocus defaults.

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™.



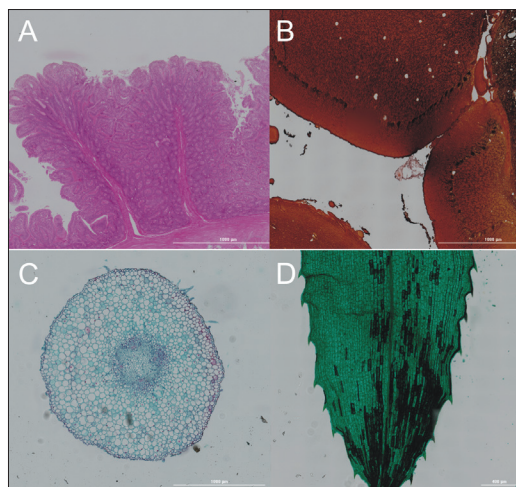
**Figure 6.** Gen5 Read Step identifying montage imaging. The color brightfield images are collected using a 4x objective with Image montage selected and a 10 x 10 array with tile overlapping set to auto for stitching.

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 6). 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 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 6). 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 7). 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 7).



**Figure 7. 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.

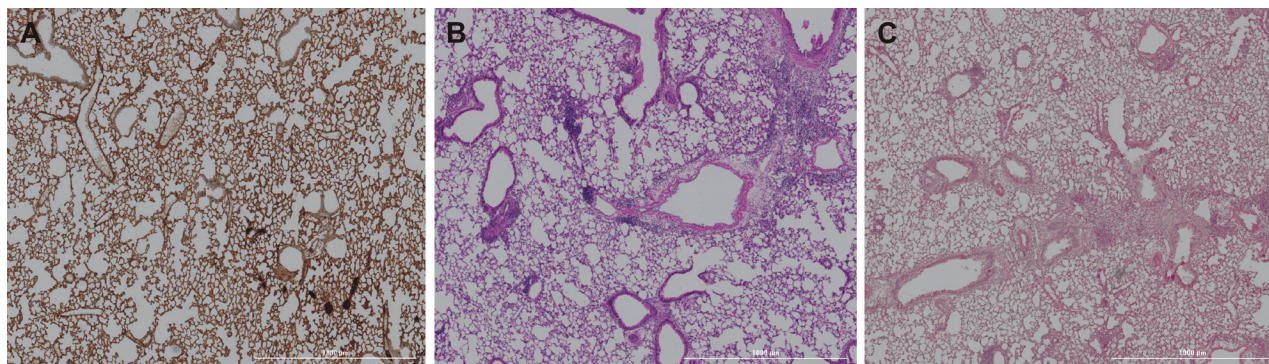
Faithful color rendition is a key element in color brightfield imaging. Currently no official color standard exists that allows one to “calibrate” the color output of the digital camera. Therefore the ability to render multiple colors faithfully is critical. The Cytation™ 5 uses a white color balance 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 8, the vivid green from chlorophyll present in plant leaves or the vasculature of growing plant roots cross sections can also be imaged.



**Figure 8.** 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.

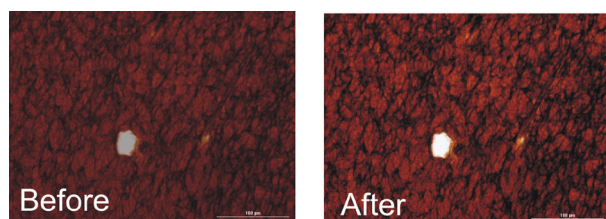
Tissue slices can be stained for increased contrast or to identify specific cellular proteins using a number of different methods. Antibody based methods typically employ a specific antibody conjugated to a reactive enzyme such as horse radish peroxidase or alkaline phosphatase. These enzymes react with provided substrates to form chromogenic-compounds that stain the tissue where the antibody is bound yielding spatial and expression level information of antigen. Horse radish 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 9 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 9. 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.

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 10, an increase in the green contrast results in a sharper image.



**Figure 10. 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.

## Discussion

The Cytation™ 5 is a new cell imaging multi-mode 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 (2.5x – 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>).

Cytation 5 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 auto focus routine is automatically performed at the initiation of a montage for all objectives and repeated with each image of higher magnification objectives (10x-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. Usually 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.

## References

1. Wissowzky A. (1876) Ueber das Eosin als reagenz auf Hämoglobin und die Bildung von Blutgefäßen und Blutkörperchen bei Säugetier und Hühnerembryonen. Archiv für mikroskopische Anatomie; **13**:479-496.

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