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Hematoxylin and Eosin Stained Tissue

Using color brightfield imaging with the
Agilent BioTek Cytation 5 to image fixed and
stained tissue

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Abstract

The imaging and analysis of labeled fixed and chromogenically stained cells has traditionally been accomplished using manual examination of microscopic slides with a multi-objective staged microscope. With the advent of digital color brightfield imagery, samples can be automatically imaged and the data stored for examination independent of the microscope. This application note describes the use of the Agilent BioTek Cytation 5, a low-cost high-value combination cell imager and microplate reader, to rapidly image hematoxylin and eosin-stained tissue slides.

Introduction

Examination of chromogenically stained tissues has been the hallmark of clinical diagnostic pathology and cancer research for nearly a century. Fixed and stained tissue on microscope slides has been the routinely used method of data storage. Pathological samples to be viewed were routinely fixed and embedded in paraffin for long term storage. Thin slices were then made from the paraffin samples, immobilized on slides, dewaxed, and subsequently stained, usually with hematoxylin and eosin, and sealed with a cover slip. Examination of the pathological slide required that the examiner use a staged multi-objective microscope to make their observations. After examination, the slide along with the paraffin embedded tissue was routinely archived for later reexamination. If reexamination was required, the slide had to be retrieved or the paraffin fixed tissue had to be reprocessed and a new slide made prior to reexamination. With advancements in digital imaging and data storage, colored imagery is becoming more desirable.
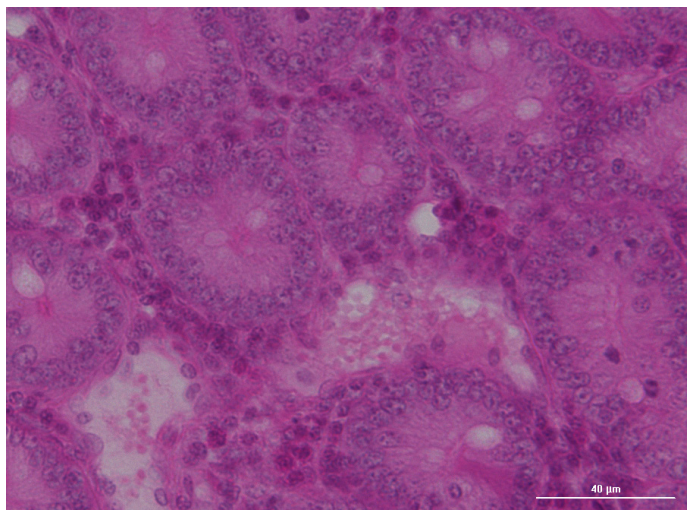

Figure 1. Hematoxylin and eosin-stained normal human small intestine tissue.

The combination of the hematoxylin and eosin (H&E) dyes was first used to stain tissues in 1876 by the chemist Wissowzky.[1] 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, 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 2).
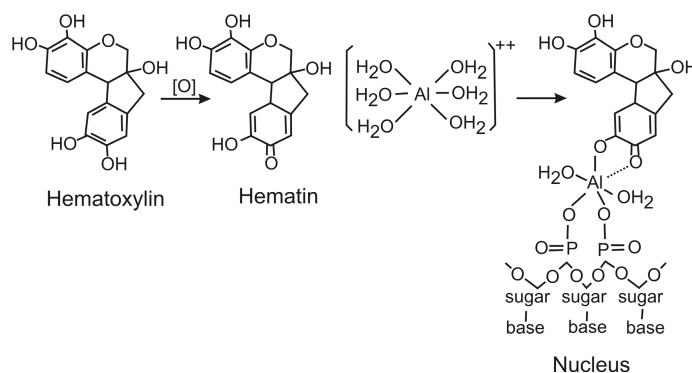

Figure 2. Hematoxylin (hematin) binding to DNA in the cell nucleus.

Conversely, the anionic eosin Y will bind to positively charged groups on proteins, such as amino groups. Lysine residues, for example, have and ε-amino group with pKa's in the range of 10, such that they will remain as positive ions throughout the staining process (Figure 3).
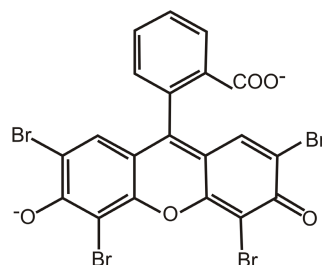

Figure 3. Structure of the dianion, eosin Y, used in H&E staining.

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 even 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 an 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 2.5 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 an Agilent BioTek Cytation 5 cell imaging reader. Montage spacing overlap was set to the default for stitching in Agilent BioTek Gen5+ software. A series of color brightfield image montages (12 × 8) and the individual image tiles were stitched using linear blending along overlapping portions of each image tile. Registration was made using 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 4, where normal and diseased kidneys are compared. Using a 60x objective, montage images of slides were captured and 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.
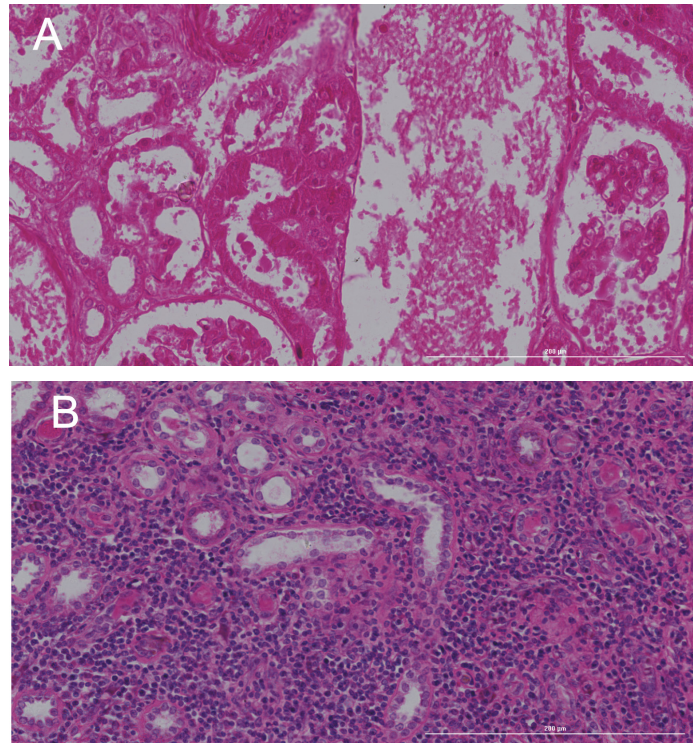

Figure 4. Comparison of normal and diseased human kidney. Hematoxylin and eosin stained tissue from (A) normal kidney and (B) chronic nephritis kidney. Images represent a stitched 12 × 8 montage made using a 60x objective. Scale bar represents 200 µm.

Montage arrays allow a larger area than a single image frame to be captured. By separating the images from one another, arrays can be used to obtain individual tiles from different regions of the slide. 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. The degree to which the Cytation 5 can magnify a region of a slide is demonstrated in Figure 5, where a tissue slice of human intestinal wall was montage 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 can also be discerned. The region of the slide imaged is depicted on the slide graphic presented 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.
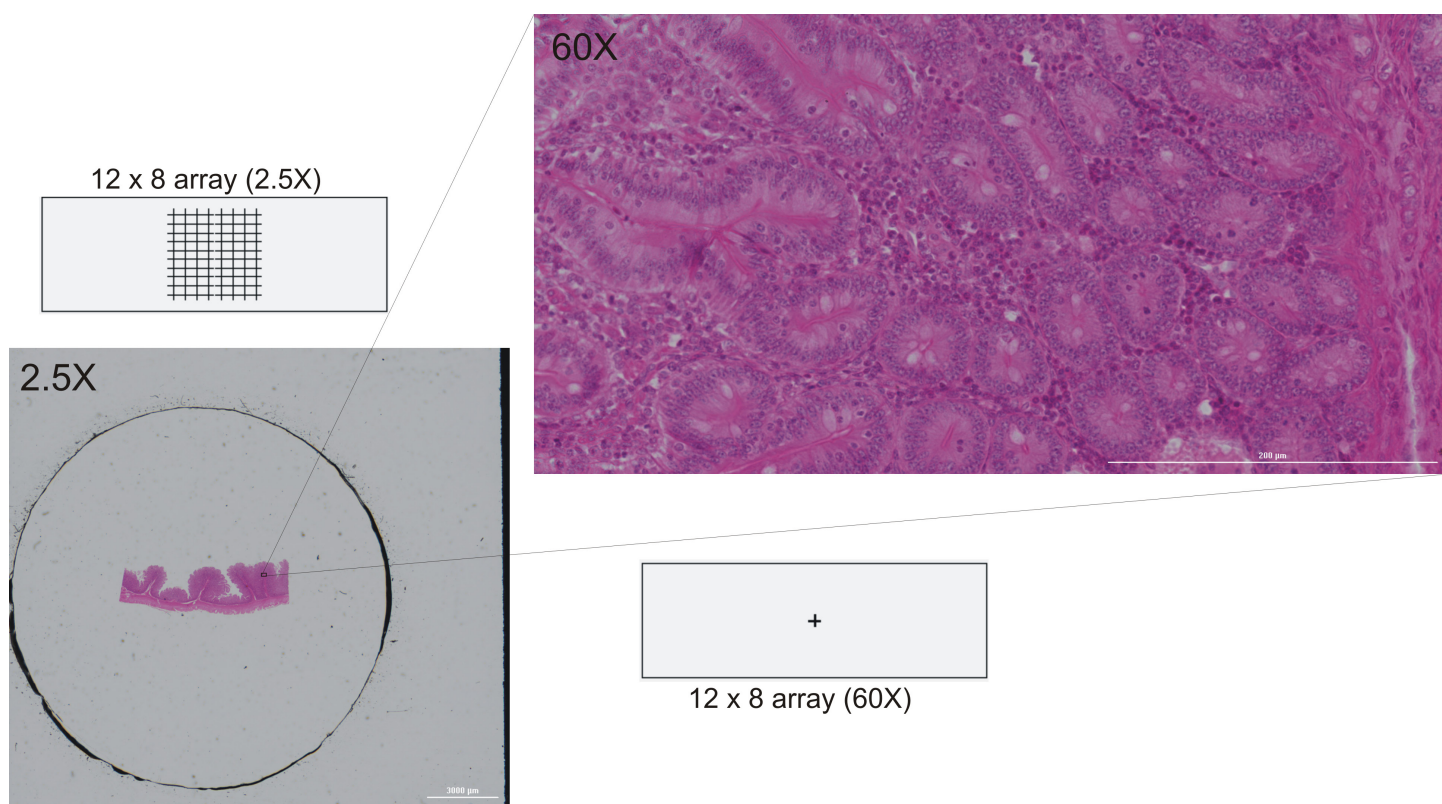

Figure 5. Maximum magnification using an Agilent BioTek Cytation 5 cell imaging multimode reader. Fixed and stained tissue slice of human intestinal wall immobilized on a 1" × 3" slide was imaged with the 2.5x and the 60x objectives to generate a 12 × 8 montage. Montage tiles were then stitched using linear blend method with registration of the red channel. Subsequent image file was reduced to 25% of maximum. 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 schematic of slide.

Figure 6 demonstrates the convenience of the multi-objective turret system of the Cytation 5. The same fixed and stained tissue slide of diseased human kidney tissue was imaged using a color brightfield montage (12 × 8) at six different magnifications (2.5x to 60x) without intervention. The area imaged by the next higher magnification is identified for each image. Because the Cytation 5 uses a 6-position turret system, different magnification steps can be programmed for slide regions for automatic positioning, focus and imaging without any user intervention. As shown in Figure 5, the entire tissue slice can be observed in low resolution with the lower magnification images. With increasing magnification, greater resolution of microscopic details emerge. The best magnification for demonstration purposes can then be selected after viewing the results.
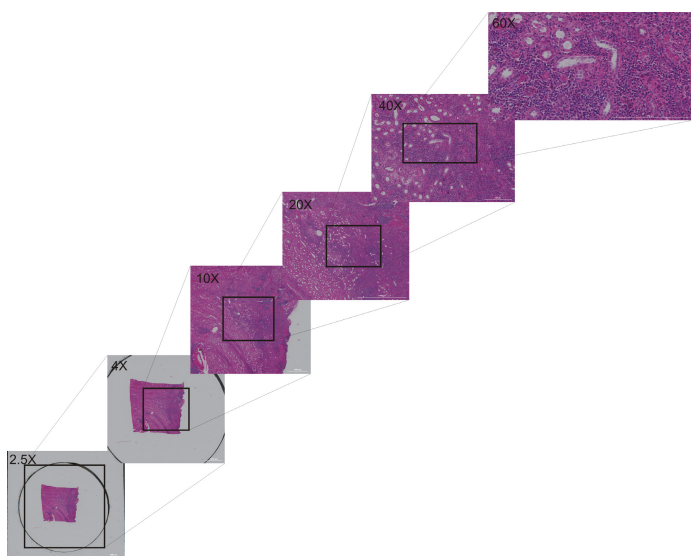

Figure 6. Macroscopic and microscopic structure of human chronic nephritic kidney tissue. A series of montage (12 x 8) images at increasing magnification were recorded and stitched from the same tissue slice. Boxed area represents the area imaged in the next higher magnification image. Objective magnification and scale bar are present for each montage.

The Cytation 5 cell imaging multimode reader, in combination with Gen5 software, has several features that assist in the automated imaging of stained tissue slides. Slides can be imaged both manually and using an automated routine. Manual imaging allows the investigator to manually manipulate the carrier while it is inside the reader using either the software controls of Gen5 or an external joystick. Once the region of interest has been identified, single or montage color brightfield images can then be captured and saved and images stitched and analyzed within the Gen5 manual-mode software fields. Alternatively, slides can be read using an automated routine. Slide location, magnification

objective, and montage array (up to a 15 × 15 matrix) can be programmed prior to imaging. Multiple read steps can be used to define different objectives and/or different regions of the slide to be imaged within the same experiment.

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 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 used 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 to achieve good images necessary for further analysis. The autofocusing 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.[2] 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 versus Z-axis height results in a sharp peak at the point of focus.[3] 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.[3,4]

Conclusion

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 versus 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 Agilent BioTek Cytation 5 cell imaging multimode reader 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.

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