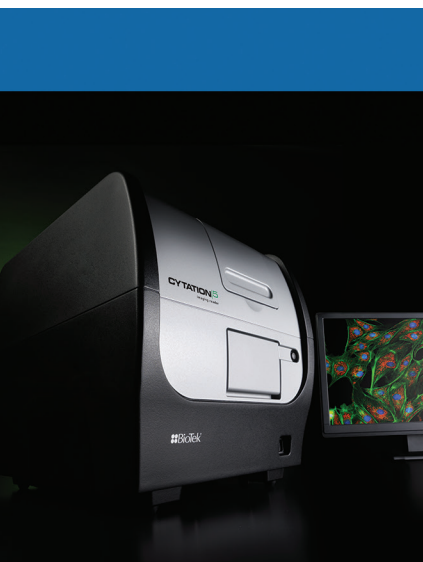


Applied Robotics for Enhanced Throughput Options in Microscopy

As Demonstrated by Automated Tissue Microarray (TMA) Imaging

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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 0.6mm in diameter and 2-5µm thick can be arrayed on a single microscopy slide allowing increased throughput in a number of common histology procedures. TMAs (Figure 1,2) have proven useful in many applications such as bio banking and archiving of biopsy and other tissue samples; disease diagnosis, classification and grading; quality control; antibody and staining optimization during assay development; and for meeting criteria such as the 'Validation and Verification of Immune Reactivity of All Classes of IHC's with a Panel of Normal Tissues or Cells' required by the U.S. FDA regulatory document *Guidance for Submission of Immunohistochemistry Applications to the FDA; Final Guidance for Industry*^[1,2].

TMAs 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 2). Additionally, some TMAs may or may not have marker cores that can be offset from the remaining cores (Figure 2). Further, although the BioTek microscopy slide adapters have been designed for standard 25 x 75 x 1mm 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.

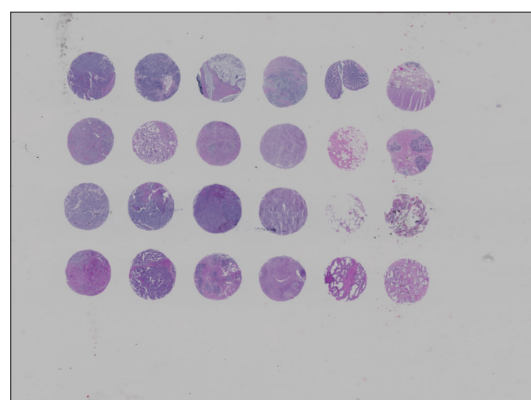


Figure 1. (Top) Two TMA slides are positioned in a slide holder compatible with robotic interface to a multi-mode imaging reader (center). Software was preprogrammed to sequentially load each slide holder from the robot to the imager that then captured 15 x 15 images of each slide using automatic montage and stitching features to illustrate the final array, shown here on one of the TMAs at 4x (bottom).

Key Words:

Tissue Microarray

Histology Imaging

Robotics

Slide Imaging

H&E

Montage

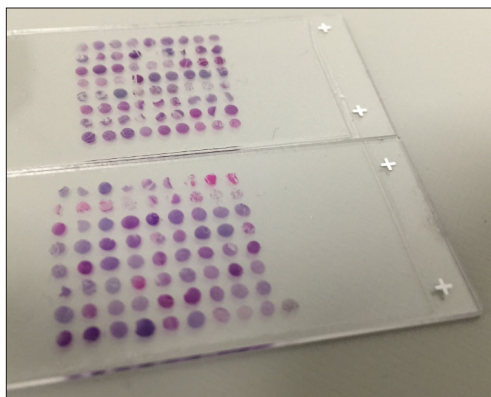


Figure 2. Two 9 x 8 tissue microarrays (TMAs) shown with different offset positions on the microscopy slide. The slide on the bottom includes a marker core, often embedded into arrays as a control and to assist with identifying panel locations, but also adding to asymmetric positioning between arrays. Customized x-y offsets and montage and stitching options available in BioTek's Gen5™ software can be used to compensate for these geometric anomalies during imaging on a Cytation™ 5, allowing mixed size TMAs to be run together in higher throughput via the BioStack™ 4 robot.

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 BioTek microscopy slide holders (Figure 1), and stacked vertically within the BioStack 4 supply tower (Figure 1). 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 by Figure 4). For comparative purposes, images of a DAPI stained discarded prototype TMA donated by an area histology laboratory is included to illustrate fluorescent imaging of a TMA obtained independently from the same instrument.

Materials and Methods

Materials

- 1 set FDA Human Normal Organ Tissue, US Biomax, Inc. p/n DA802 H&E stained, Microarray Panels (one shown by Figure 7) and Core Specification Sheets: 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 6, Core Specification Sheet: 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, including TNM and clinical stage, US Biomax, Inc. p/n TH641 unstained)
- BioTek Slide Adapters p/n 1220548
- BioTek Integration Kit for Cytation 5 – BioStack 4 Interface p/n 7310053

Equipment

Cytation™ 5 Cell Imaging Multi-Mode Reader

Cytation™ 5 is a uniquely integrated, configurable system that combines automated digital widefield microscopy with conventional multi-mode microplate detection. This instrument replaces multiple modules and software interfaces, yet is simple to setup and operate. The microscopy module provides high-quality cellular and sub-cellular imaging in fluorescence, brightfield, color brightfield, and phase contrast channels with up to 60x magnification, and a somewhat controlled turret that can house 6 onboard objectives. The multi-mode detection module features BioTek's patented Hybrid Technology™, which incorporates variable bandwidth monochromator optics and high sensitivity filter-based detection optics. Shaking, temperature control to 65 °C, plus available CO₂/O₂ control and dual reagent injectors optimize conditions for live cell kinetic imaging and detection. After activating the BioStack 4 in the Gen5 software, it can be enabled or disabled for use with Cytation 5 via a checkbox in the Gen5 v2.07 Instrument Configuration interface. A menu will appear at runtime allowing either manual or robotic mode for slide holder transfers. Other options include electing to process all plates from the supply stack automatically, or only processing a user-specified number of plates. The slide adapters can either be restacked into the supply stack after processing, or left in the output stack as defined by the user at runtime. These options are shown in Figure 4.

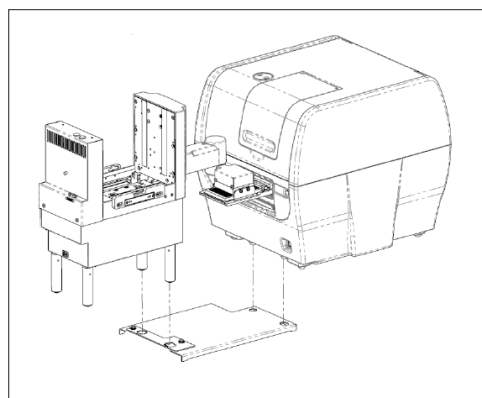


Figure 3. The Cytation 5 imager and BioStack 4 are interfaced using an Integration Kit (BioTek p/n 7310053) as shown.

BioStack™ 4 Microplate Stacker

BioStack™ 4 is a compact and versatile microplate stacker compatible with BioTek's washers, dispensers, detectors and imaging systems. The unique carrier design of BioStack provides rapid transfer speeds to increase throughput and enhance productivity. BioStack 4 has a rotational gripper for ergonomically friendly instrument panel access. The patent pending BioStack 4 offers plate de-lidding and re-lidding capability, ideal for sensitive cell-based assays. The BioStack 4 is compatible with a microscope slide adapter (BioTek p/n 1220548), shown in Figure 1. Walk-away batch microscopy slide imaging is easily accomplished with Cytation™ 5. BioStack models are available with 10, 30, or 50 carrier storage stacks, all removable and interchangeable to accommodate individual throughput needs. The 30 carrier stack was used for the applications presented here. Instructions for calibrating the unit with a Cytation 5 are found in the Operator's Manual (BioTek p/n 7311000) and associated Install-Operate BioStack with Cytation chapter. Of note, when integrating the Cytation 5 imager and BioStack 4 both units should be on equal, level footing. In place of the Cytation 5 isolation table, a BioStack integration kit should be used, making physical calibration seamless between the two units (Figure 3). During z-height calibration of the robotic arm to the Cytation 5 carrier, the grippers should be calibrated 100-400 steps beneath the bottom of the Cytation 5 carrier to snugly snap the slide adapter into place during transfers. When loading microscopy slide adapters to the robotic supply tower, the slide holder should be loaded so the A1 marker is at the back left corner of the supply tower when loaded and locked into place on the BioStack 4.

Gen5™ v2.07 Software

Robotics control, image capture, data collection, and image and data analysis are driven by Gen5™ software. For the applications described here color bright field, fluorescence, and digital phase contrast were used at 2.5x, 4x, 10x, 20x, and 40x magnifications either individually or in parallel. Image montage and stitching and single imaging features available in Gen5 were utilized as described in the method section. Figure 4 shows the user interface panels for adding a BioStack 4 to Gen5, enabling imager interface for use with robotics, and runtime options available in Gen5 when BioStack is enabled.

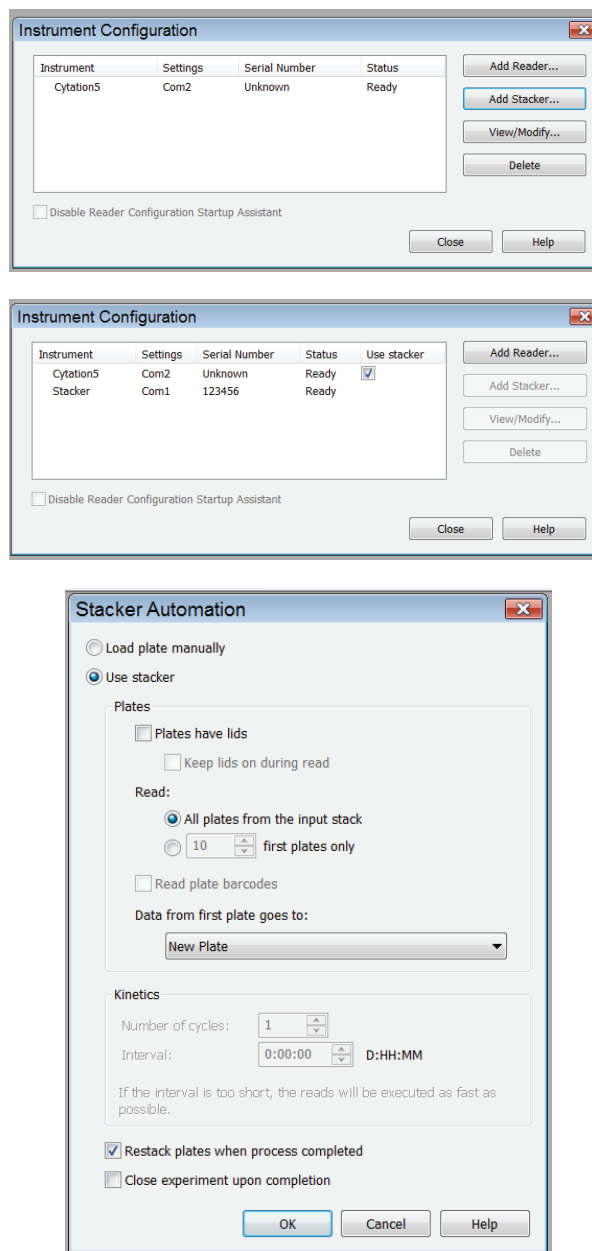


Figure 4. Gen5 user interface for integrating the BioStack 4 and Cytation 5 imager (top and center), and options available at runtime when the BioStack 4 is enabled (bottom).

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 (Figure 5). 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. X/y offset optimization was performed on the second slide. Two read steps were then programmed into the protocol so that all TMAs would be imaged at two different geometric configurations.

Six H&E stained 1.5 mm 24 core TMA slides were loaded into 3 additional BioTek microscope slide holders, and all 4 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 3 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 fluorescent imaging channel, and is included to illustrate fluorescent 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 walkaway imaging.

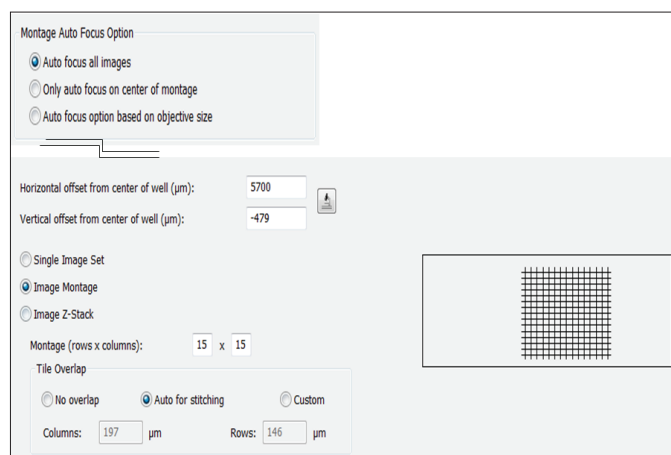


Figure 5. An example of the x-y offsets, focus setting, and montage and stitching options that were used to sequentially image a batch of 8 TMAs of 2 different panel sizes at 4x in color brightfield on a Cytation 5. Slides were transferred back and forth to the imager using the BioStack 4.

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 6 illustrates a 24 core sample TMA, while Figure 7 shows the ability to image a 72 core sample TMA, each using lower resolution microscopy with a 4x objective.

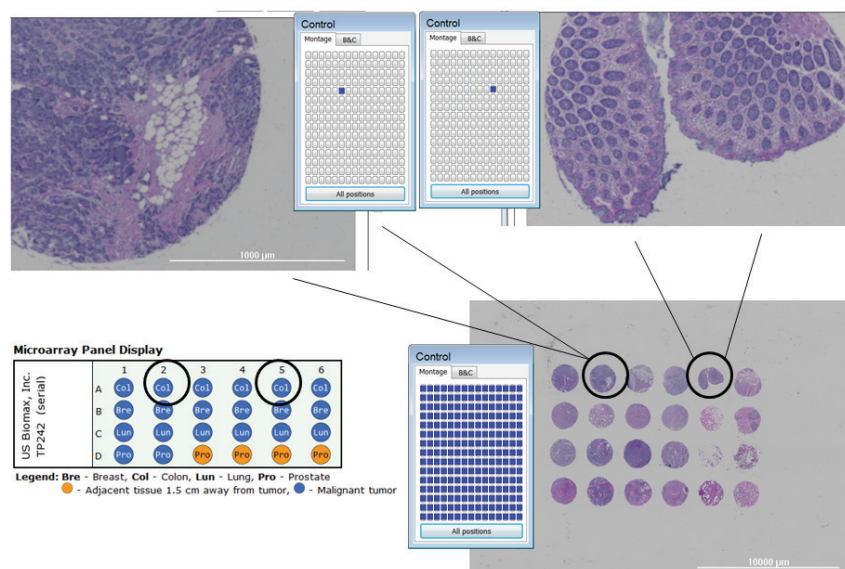


Figure 6. A 24 core tissue microarray imaged on Cytation 5 at 4x using montage and stitching options available in Gen5 software. This slide was run in a batch of 8 mixed sized TMAs using BioStack 4. The bottom right image shows the entire array displayed using the montage interface option to view all 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 yr old female, and on the right a case of cancer adjacent colon tissue type malignant from a 35 yr 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.

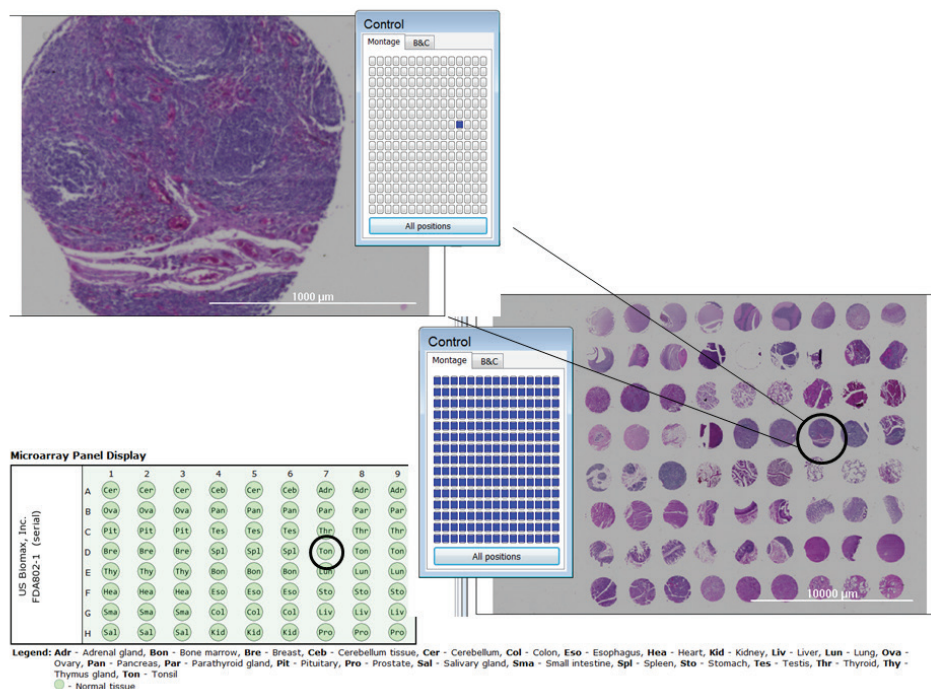


Figure 7. A 72 core tissue microarray of human normal organ tissue imaged on Cytation™ 5 at 4x using montage and stitching options available in Gen5™ software. This slide was run in the same batch of 8 mixed sized TMAs as the array in Figure 6 using the same settings. The entire array is displayed using the montage All Positions view at bottom right. A core of normal tonsil tissue is shown in single tile view at top left, selected by clicking on an individual tile in the Montage control panel, or double clicking directly on the montage image.

Figures 8 – 10 demonstrate the ability to perform higher resolution microscopy with Cytation 5 which enables disease diagnosis. Finally, Figure 11 displays the ability to also perform fluorescence microscopy on TMAs using the Cytation 5.

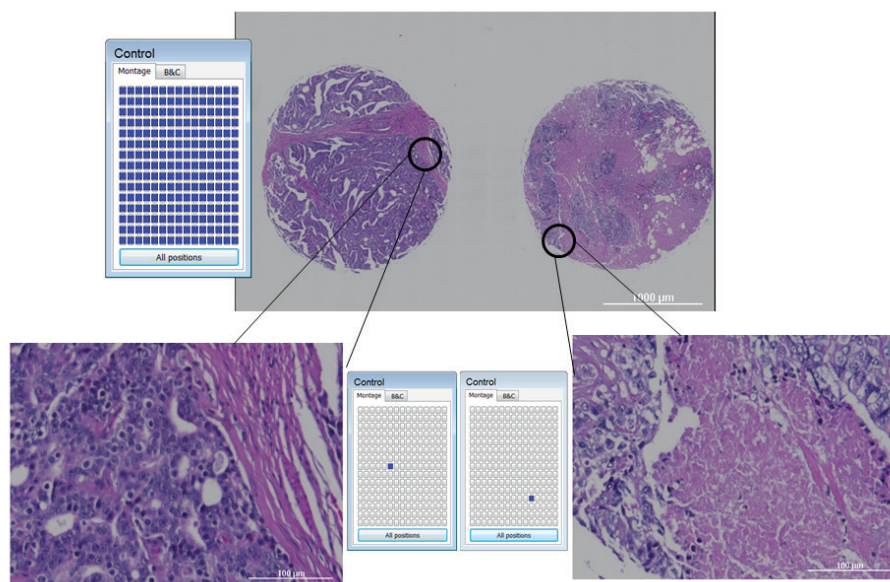


Figure 8. 20x montage (top) with area of detail for individual tiles shown. The left tissue is malignant prostate tumor, on the right is prostate adjacent tissue 1.5 cm away from tumor. These cores are from two different cases.

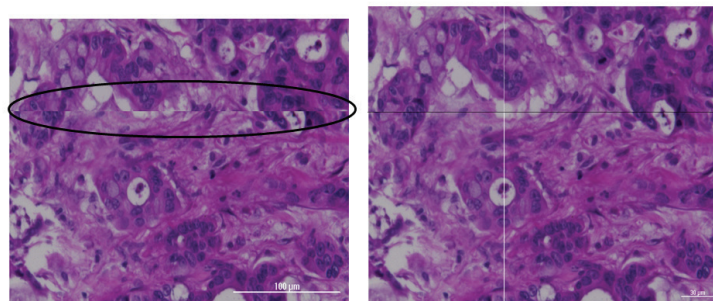


Figure 9. Default stitching overlap settings are asymmetric along the horizontal row (circled). Tile overlap value can be customized to help correct this. Vertical offsets (column) are well aligned. Illustration of optimal 2 x 2 montage tile overlap for stitching at 40x, shown right.

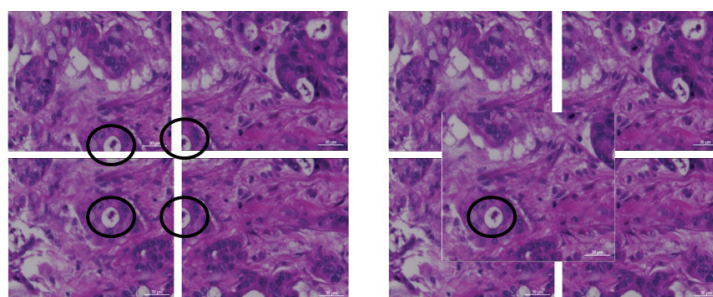


Figure 10. (Left) Images of individual tiles of a 2 x 2 montage at 40x taken from a TMA core. The circles show where each tile is imaged in relationship to the others using a distinguishable marker on the tissue. These are images of the individual 4 tiles of the 2 x 2 montage shown by Figure 8. (Right) A single image using the same center offset as the montage at 40x overlaid on the four tiles shown 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 montage.

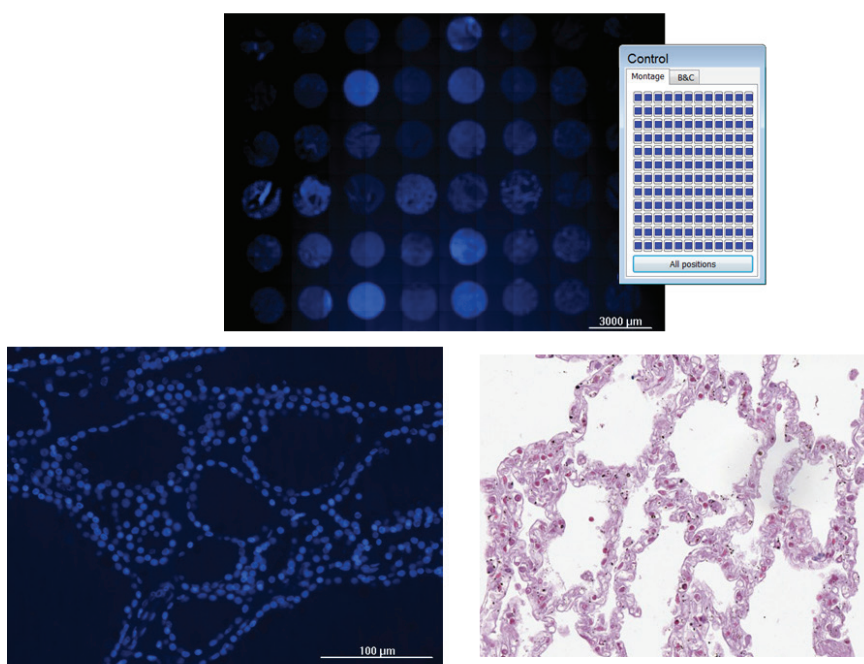


Figure 11. A discarded prototype TMA donated by an area histology laboratory stained with DAPI and imaged at 4x on Cytation™ 5 using montage and stitching in Gen5™ software (top). Background haze is likely attributed to incomplete deparaffinization. Bottom left is a single image taken at 20x from the same array, and bottom right is 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).

Conclusions

Below are the pertinent conclusions from this work which demonstrate the ability of Cytation™ 5 and BioStack™ 4 for the automation of work flows for imaging H&E stained TMAs for high throughput histology applications.

- The built-in stitching and montage algorithms available in Gen5™ v2.07 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.
- 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.
- 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.
- 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.
- At 40x a 2 x 2 montage provided 4 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.
- Imaging at 4x using a 15 x 15 montage grid with auto focus 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.
- By means of any of the multi-mode imaging options available on the 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.

References

1. www.biomax.us
2. <http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm094002.htm>