Application Note

Histology and
Slide Scanning

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Applied Robotics for Enhanced Throughput Options in Microscopy

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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 0.6 mm in diameter and 2 to 5 µM thick can be arrayed on a single microscopy slide allowing increased throughput in a number of common histology procedures. TMAs (Figures 1 and 2) have proven useful in many applications such as biobanking and archiving of biopsy and other tissue samples, clinical disease diagnosis, classification and grading, quality control, antibody and staining optimization during assay development. TMAs are also used to help meet 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]

Figure 1. (A) Two TMA slides are positioned in a slide holder compatible with robotic interface to an Agilent BioTek multimode imaging reader (B). Software was preprogrammed to sequentially load each slide holder from the robot to the imager that then captured 15 × 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 (C).

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 Agilent BioTek microscopy slide adapters have been designed for standard 25 × 75 × 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.

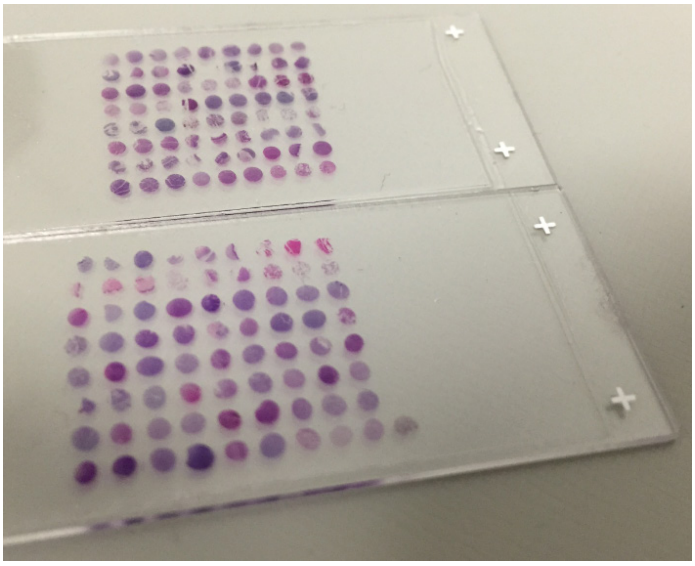


Figure 2. Two 9 × 8 tissue microarrays (TMAs) shown with different offset positions on the microscopy slide. The slide on the bottom includes a marker core, often embedded into arrays as a control and to assist with identifying panel locations, but also adding to asymmetric positioning between arrays. Customized x,y-offsets and montage and stitching options available in Agilent BioTek 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 robot.

To demonstrate optimal image acquisition settings designed to overcome the challenges of automating TMAs at high throughput, a set of two hematoxylin and eosin (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 microscopy slide holders (Figure 1A), and stacked vertically within the Agilent BioTek BioStack 4 supply tower (Figure 1B). Agilent BioTek Gen5 software was programmed to sequentially load each slide holder from the Agilent BioTek BioStack microplate stacker to the Agilent BioTek 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 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

- One set FDA Human Normal Organ Tissue, US Biomax, Inc. (part number FDA802) H&E-stained, Microarray Panels (shown in Figure 7) and Core Specification Sheets: https://www.biomax.us/FDA802-1
- Six Top 4 Types of Cancer Test Tissue, US Biomax, Inc. (part number TP242) H&E-stained, microarray panel shown in 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. (part number TH641 unstained)
- Agilent BioTek slide adapters (part number 1220548)
- Agilent BioTek integration kit for Cytation 5 – Agilent BioTek BioStack 4 interface (part number 7310053)

Equipment

Agilent BioTek Cytation 5 imaging multimode reader

Cytation 5 is a uniquely integrated, configurable system that combines automated digital wide field microscopy with conventional multimode 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 s/w controlled turret that can house 6 onboard objectives. The multimode detection module features Agilent BioTek Hybrid Technology, which incorporates variable bandwidth monochromator optics and high sensitivity filter-based detection optics. Shaking, temperature control to 65 °C, plus available CO_2/O_2 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.

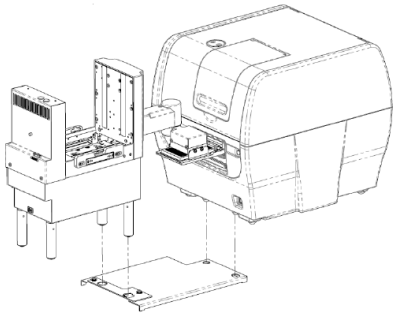
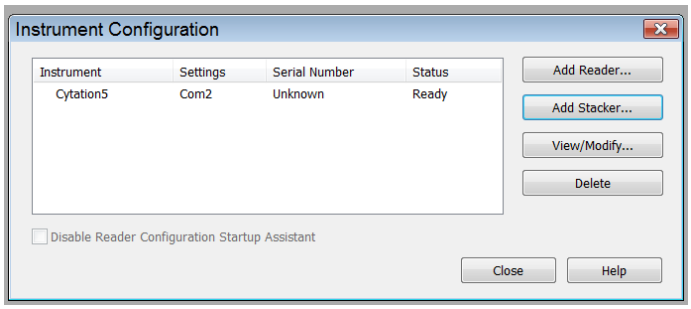


Figure 3. The Agilent BioTek Cytation 5 imager and the Agilent BioTek BioStack 4 are interfaced using an Agilent BioTek integration kit (part number 7310053), as shown.

Agilent BioTek BioStack 4 microplate stacker

Agilent BioTek BioStack 4 is a compact and versatile microplate stacker compatible with Agilent BioTek washers, dispensers, detectors, and imaging systems. The unique carrier design of BioStack 4 provides rapid transfer speeds to increase throughput and enhance productivity. BioStack 4 has a rotational gripper for ergonomically friendly instrument panel access. The patented BioStack 4 offers plate delidding and relidding capability, ideal for sensitive cell-based assays. The BioStack 4 is compatible with a microscope slide adapter (part number 1220548), shown in Figure 1B. 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 (part number 7311000) and associated *Install-Operate BioStack with Cytation* chapter. 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. During z-height calibration of the robotic arm to

A



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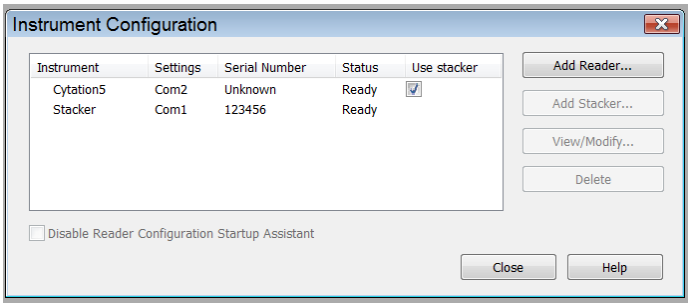

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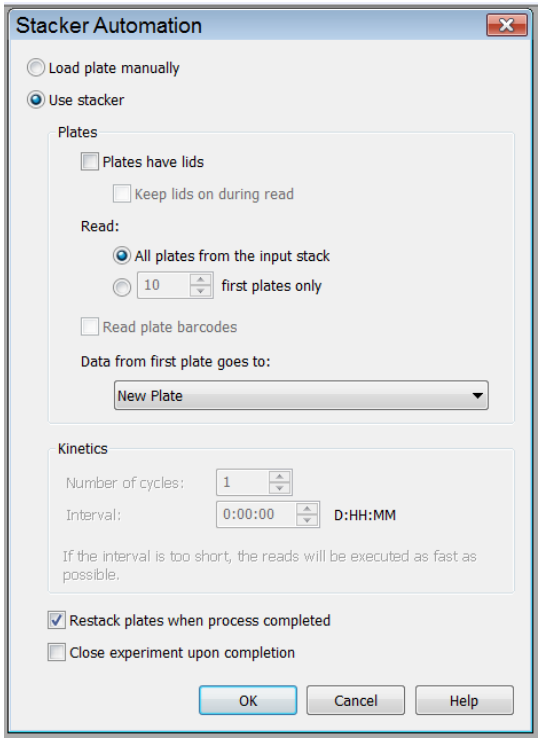

Figure 4. Agilent BioTek Gen5 user interface for integrating the Agilent BioTek BioStack 4 and Agilent BioTek Cytation 5 imager (A and B), and options available at runtime when the Agilent BioTek BioStack 4 is enabled (C).

the Cytation 5 carrier, the grippers should be calibrated 100 to 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.

Agilent BioTek 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 brightfield, 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 used 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.

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.

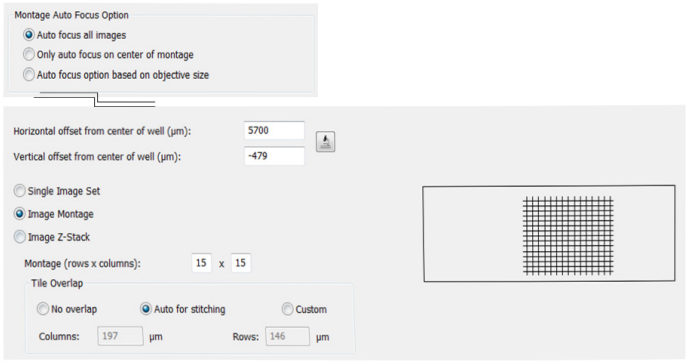

Figure 5. An example of the x,y-offsets, focus setting, and montage and stitching options that were used to sequentially image a batch of 8 TMAs of 2 different panel sizes at 4x in color brightfield on an Agilent BioTek Cytation 5. Slides were transferred back and forth to the imager using the Agilent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

Six H&E-stained 1.5 mm 24 core TMA slides were loaded into 3 additional Agilent 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.

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 using lower resolution microscopy with a 4x objective.

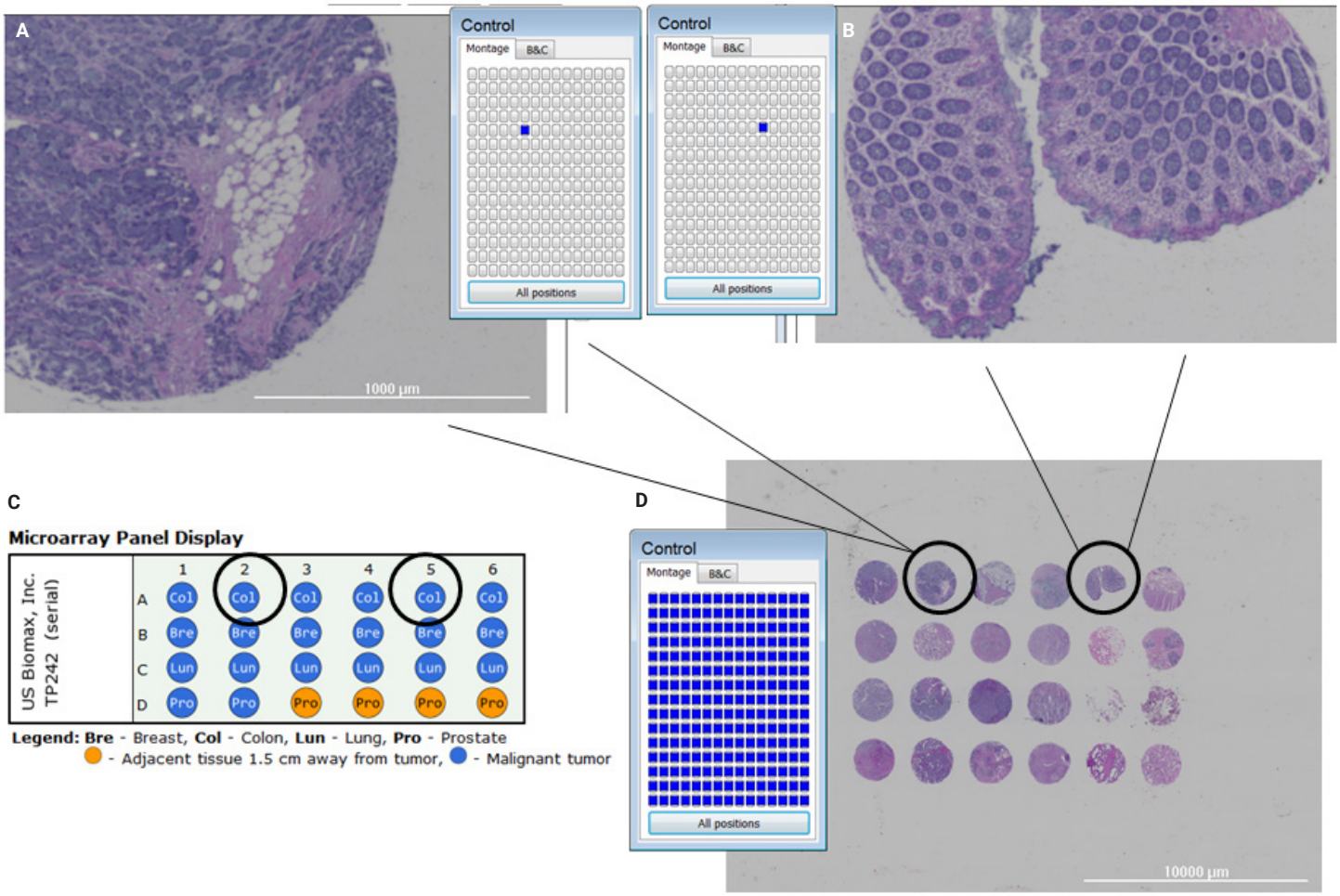

Figure 6. A 24-core tissue microarray imaged on an Agilent BioTek Cytation 5 at 4x using montage and stitching options available in Agilent BioTek Gen5 software. This slide was run in a batch of 8 mixed sized TMAs using Agilent BioTek BioStack 4. (D) The entire array displayed using the montage interface option to view all tiles (All Positions mode). (A,B) Two different cores from the array shown in single tile view. (A) is diagnosed as colon mucoid adenocarcinoma grade III malignant from an 81-year-old female, and (B) is a case of cancer adjacent colon tissue type malignant from a 35-year-old male. Because Agilent BioTek 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 (C). In this case, slides were oriented on the holder with the label up.

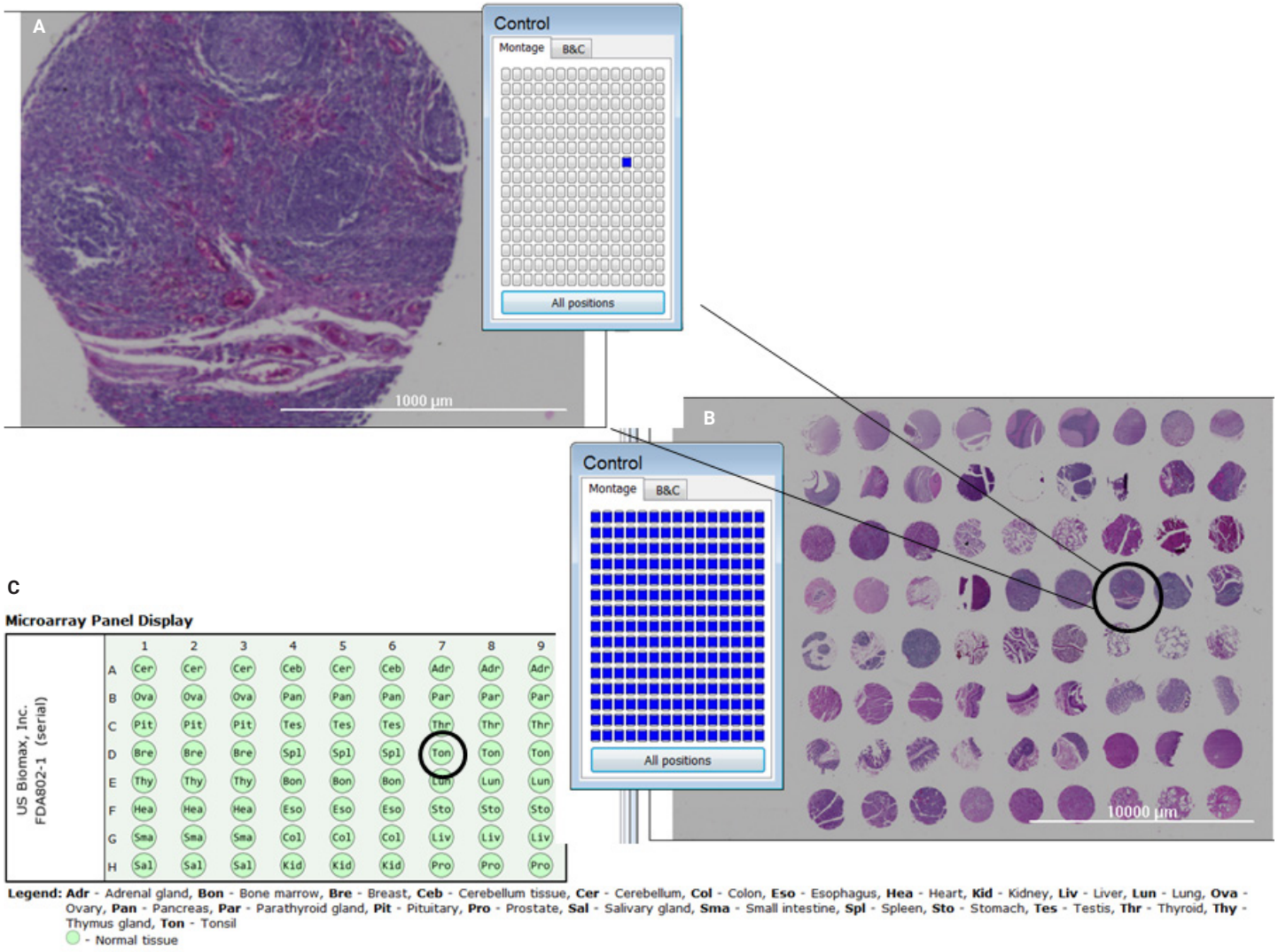

Figure 7. A 72-core tissue microarray of human normal organ tissue imaged on an Agilent BioTek Cytation 5 at 4x using montage and stitching options available in Agilent BioTek Gen5 software. This slide was run in the same batch of 8 mixed sized TMAs as the array in Figure 6 using the same settings. (B) The entire array is displayed using the montage All Positions view. (A) 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 double clicking directly on the montage image.

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

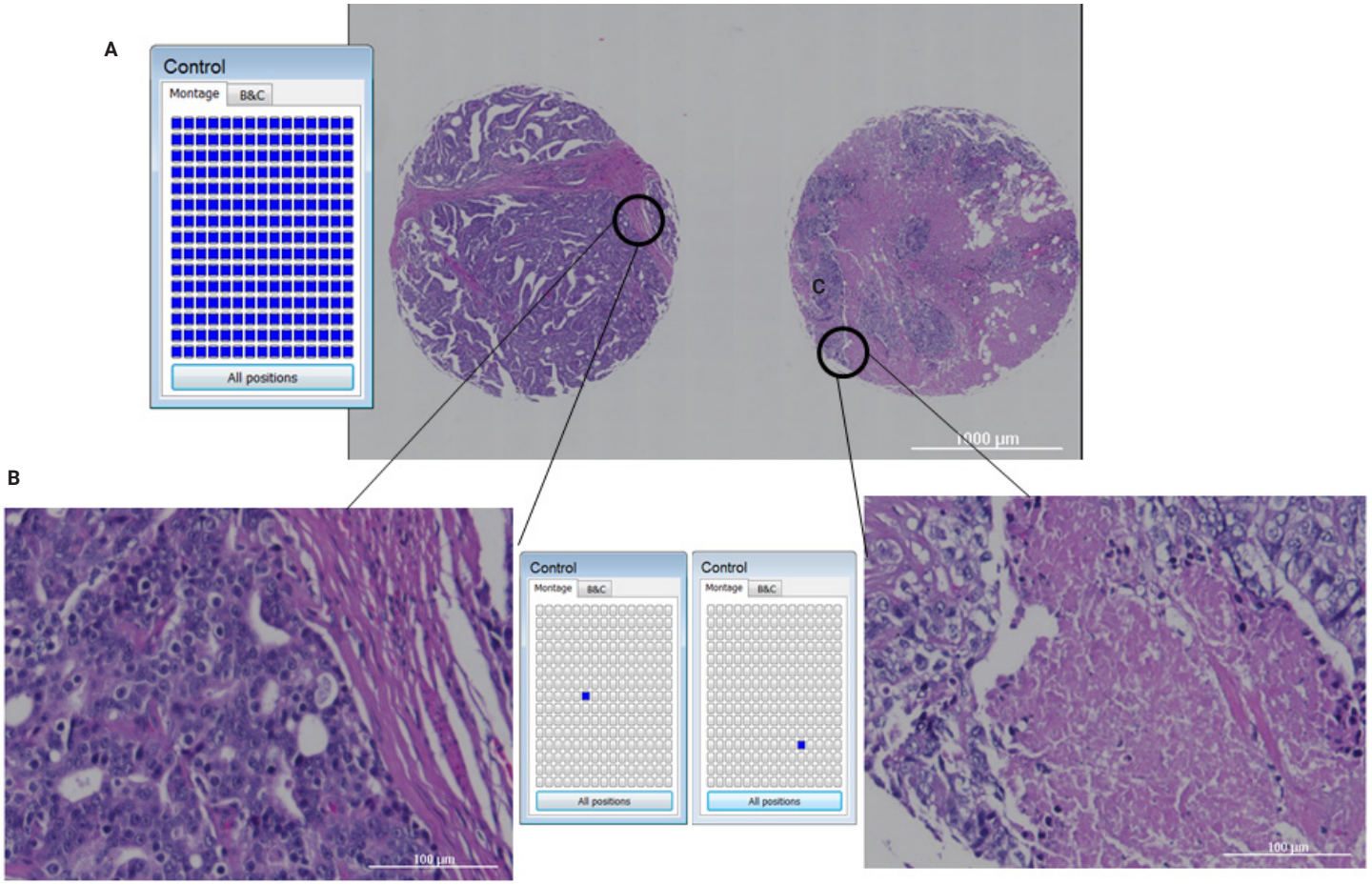

Figure 8. 20x montage (A) with area of detail for individual tiles shown. The left tissue (B) is malignant prostate tumor. Panel (C) is prostate tissue 1.5 cm away from the tumor. These cores are from two different cases.

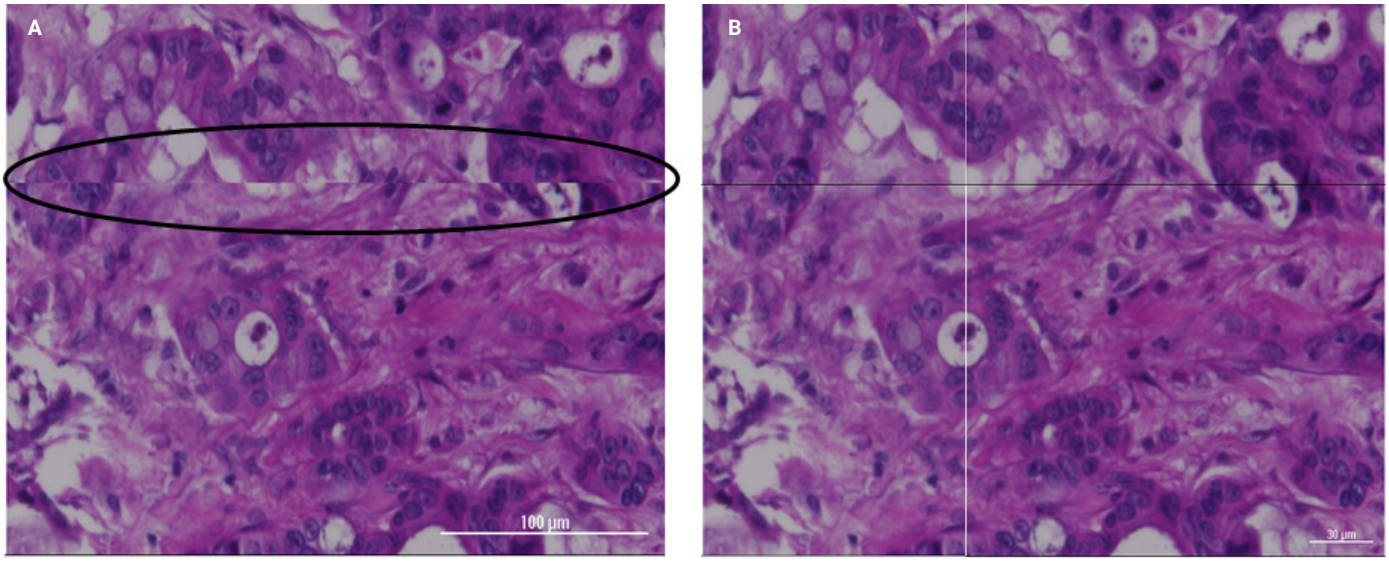

Figure 9. (A) Default stitching overlap settings are asymmetric along the horizontal row (circled). Tile overlap values can be customized to help correct this. Vertical offsets (column) are well aligned. (B) Illustration of optimal 2 × 2 montage tile overlap for stitching at 40x.

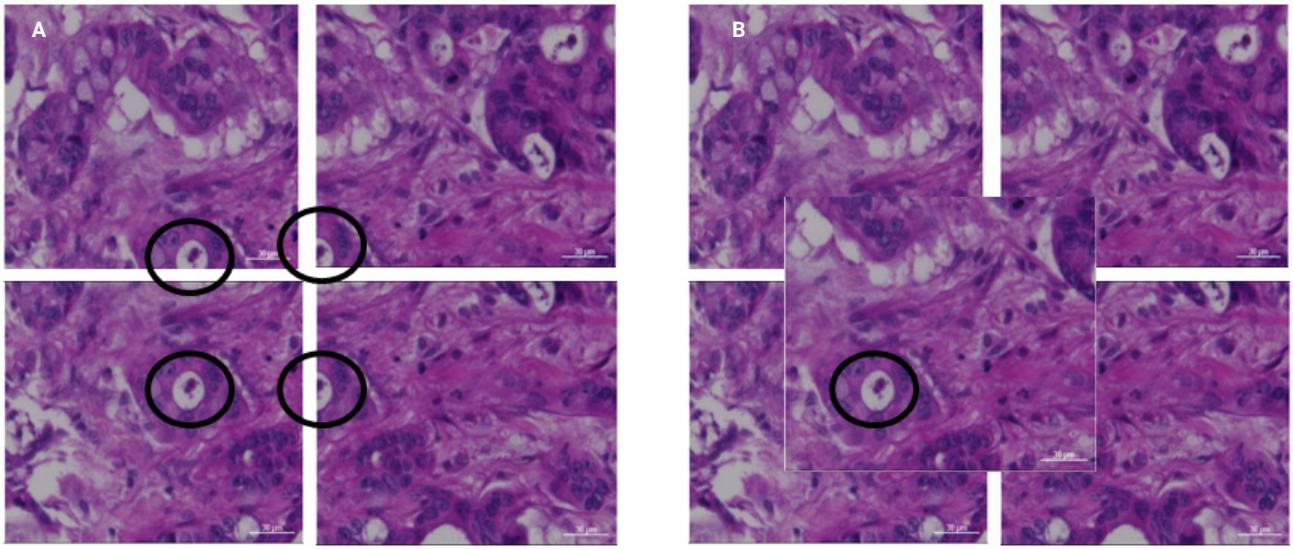

Figure 10. (A) Images of individual tiles of a 2 × 2 montage at 40x taken from a TMA core. The circles show where each tile is imaged in relationship to the others using a distinguishable marker on the tissue. These are images of the individual 4 tiles of the 2 × 2 montage shown in Figure 8. (B) A single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

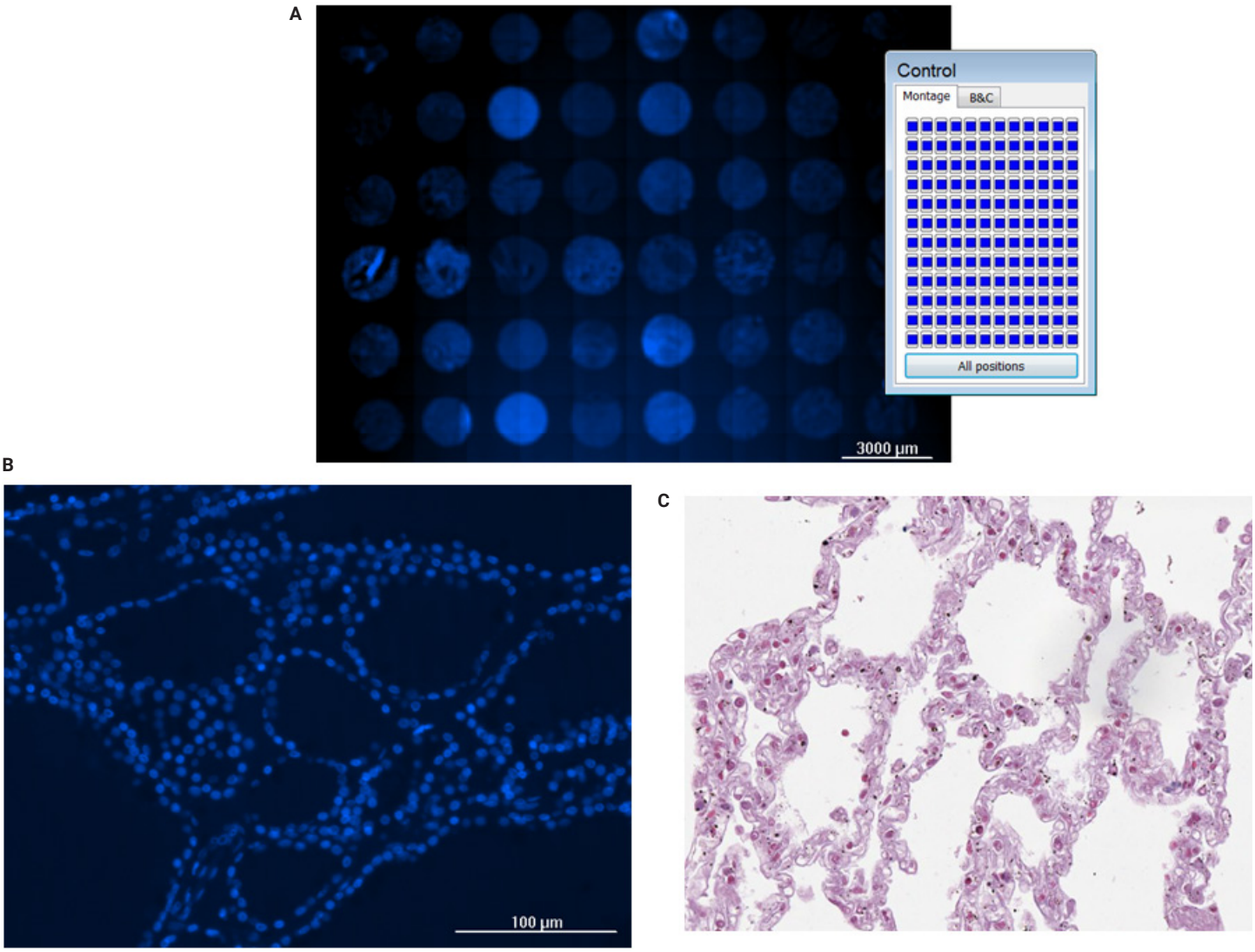

Figure 11. A discarded prototype TMA donated by an area histology laboratory stained with DAPI and imaged at 4x on an Agilent BioTek Cytation 5 using montage and stitching in Agilent BioTek Gen5 software (A). Background haze is likely attributed to incomplete deparaffinization. (B) A single image taken at 20x from the same array. (C) 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 part number TH641).

Conclusion

Below are the pertinent conclusions from this work that demonstrate the ability of Agilent BioTek Cytation 5 and Agilent BioTek BioStack 4 for the automation of workflows for imaging H&E-stained TMAs for high-throughput histology applications.

- The built-in stitching and montage algorithms available in Agilent BioTek Gen5 v2.07 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.
- 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 × 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 × 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 × 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 multimode 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

www.agilent.com/lifesciences/biotek

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