NIH NATIONAL CANCER INSTITUTE

LHTP003.07.25: 20X Whole Slide Image Capture of IFA Tumor Biopsy Slides using a ZEISS Axioscan 7 Microscope Slide Scanner

Effective Date: 03/11/2025

Please check for revision status of the SOP at
http://dctd.cancer.gov/drug-discovery-development/assays/validated-biomarker-assays

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VERSION INFORMATION

1. Approvals

Technical Reviewer: Angie Dull Date: ANGIE B. DULL -S (Affiliate) Digitally signed by ANGIE B. DULL -S (Affiliate) Date: 2025.05.13 08:51:20 -04'00'

PADIS Approval: Deborah Wilsker Date: DEBORAH F. WILSKER -S (Affiliate) Digitally signed by DEBORAH F. WILSKER -S (Affiliate) Date: 2025.05.13 09:50:12 -04'00'

NCLN Approval: Li Li Date: LI LI -S (Affiliate) Digitally signed by LI LI -S (Affiliate) Date: 2025.05.13 10:32:46 -04'00'

LHTP Approval: Katherine V. Ferry-Galow Date: KATHERINE V. FERRY-GALOW -S (Affiliate) Digitally signed by KATHERINE V. FERRY-GALOW -S (Affiliate) Date: 2025.05.13 12:23:02 -04'00'

DCTD OD Approval: Toby Hecht Date: Toby T. Hecht -S Digitally signed by Toby T. Hecht -S Date: 2025.05.13 12:53:13 -04'00'

2. Change History

Revision	Approval Date	Description	Originator	Approval
--	3/11/2025	New Document	AD/LL	TH

OVERVIEW OF IMMUNOFLUORESCENCE ASSAY FOR BIOPSIES

SOP340507:

Tumor Frozen Needle Biopsy Specimen Collection, Handling and Shipping for PADIS, Frederick National Laboratory for Cancer Research (FNLCR)

OR

SOP340567:

Tumor Frozen Needle Biopsy Specimen Collection, Handling and Shipment to EET Biobank

- Collect tumor needle biopsies in 1.5 mL tubes
- Immediately place in liquid nitrogen or on dry ice/ethanol to flash freeze within 2 min of collection
- Ship to biopsy processing laboratory or biorepository

↓

SOP340550:

Tumor Frozen Needle Biopsy Preparation for Pharmacodynamic Immunofluorescence Assays Utilizing Murine Testis and/or Jejunum Control Tissues

- NBF fix and paraffin embed tumor needle biopsies and control tissues
- Section biopsies for use in IFA
- Stain slides by H&E for standard histology analysis

↓

Assay Specific Staining SOP

e.g., LHTP003.07.23: γH2AX, pNBS1, pKAP1 IFA Staining with β-Catenin Segmentation for Tumor Biopsy Slides

- Load biopsy and control slides into Bond-RX Processing Module
- Multiplex Bond-RX automated staining of slides with assay specific critical reagents
- Stain slides with DAPI and mount cover slips

↓ **Image within 72 h**

LHTP003.07.25:

20X Whole Slide Image Capture of IFA Tumor Biopsy Slides using a ZEISS Axioscan 7 Microscope Slide Scanner

AND Assay specific addendum

e.g., LHTP003.07.25-ADM-01 Addendum to LHTP003.07.25 for Use With γH2AX, pNBS1, pKAP1 IFA with β-Catenin Segmentation

- Capture images of stained biopsy and control slides using a Zeiss Axioscan whole slide image scanner

↓

Assay Specific Image Analysis SOPs:

e.g., LHTP003.07.26: Image Analysis of Tumor Biopsy Slides from γH2AX, pNBS1, pKAP1 IFA with β-Catenin segmentation

- Quantitate markers from captured images of stained biopsy and control slides using Indica Labs HALO and HALO AI software

1.0 PURPOSE

To standardize the whole slide image acquisition of formalin-fixed paraffin-embedded (FFPE) tissue biopsy sections multiplex stained to detect and quantify biomarkers for pharmacodynamic (PD) evaluations of drug response using a Zeiss Axioscan 7 scanner. The goal of this SOP and associated training is to ensure consistency of biomarker measurements between operators and clinical sites.

2.0 SCOPE

This procedure applies to all personnel involved in the whole slide image acquisition of multiplex-stained tumor biopsy slides from patients participating in clinical trials. This SOP outlines the recommended procedure for whole slide image capture of stained, paraffin-embedded tumor biopsy sections using a Zeiss Axioscan 7 scanner. Assay specific addendum procedures are supplemental to this document and provide information detailing scan profile settings, marker specific exposure time ranges, target background intensities and LED lamp intensities for each associated multiplex assay.

3.0 ABBREVIATIONS

Cy5	=	Cyanine 5, a far-red fluorescent dye
Cy7	=	Cyanine 7, a near-infrared fluorescent dye
DAPI	=	4',6-Diamidino-2-Phenylindole
DDR9	=	γH2AX, pNBS1, pKAP1 IFA Staining with β-Catenin Segmentation
DCTD	=	Division of Cancer Treatment and Diagnosis
FFPE	=	Formalin-fixed paraffin-embedded tissue
FITC	=	Fluorescein Isothiocyanate, a green fluorescent dye
ID	=	Identification/Identifier
IFA	=	Immunofluorescence Assay
LED	=	Light Emitting Diode
LHTP	=	Laboratory of Human Toxicology & Pharmacology
NCLN	=	National Clinical Laboratory Network
PADIS	=	Pharmacodynamic Assay Development and Implementation Section
ROI	=	Region of Interest
S/N	=	Serial Number
SOP	=	Standard Operating Procedure

4.0 INTRODUCTION

PADIS develops and implements multiplexed immunofluorescence assays to quantify pharmacodynamic biomarkers in clinical trial biopsy sections. These multiplex assays also include a tissue segmentation marker, most often β-Catenin. The tissue segmentation marker, together with pathologist annotation, serve to define the areas in which biomarkers are quantitated. Additionally, DAPI stain is used to identify and segment cellular nuclei.

5.0 ROLES AND RESPONSIBILITIES

Laboratory Director/Supervisor	The Laboratory Director/Supervisor directs laboratory operations, supervises technical personnel and reporting of findings, and is responsible for the proper performance of all laboratory procedures. The Laboratory Director/Supervisor oversees the personnel who follow the SOPs within the laboratory and is responsible for ensuring the personnel are certified and have sufficient experience to handle clinical samples.
Certified Assay Operator	A Certified Assay Operator may be a Laboratory Technician/ Technologist, Research Associate, or Laboratory Scientist who has been certified through DCTD training on this SOP. The Certified Assay Operator works under the guidance of the Laboratory Director/Supervisor. This person performs laboratory procedures and examinations in accordance with the current SOP(s), as well as any other procedures conducted by a laboratory, including maintaining equipment and records and performing quality assurance activities related to performance.

5.1 It is the responsibility of the Laboratory Director/Supervisor to ensure that all personnel have documented training and qualification on this SOP prior to the actual analysis of samples from clinical trial patients. The Laboratory Director/Supervisor is responsible for ensuring the Certified Assay Operator running the SOP has sufficient experience to perform whole slide image capture of stained clinical trial biopsy slides.

5.2 The Certified Assay Operator for this SOP should be well versed and comfortable with the operation of the ZEISS Axioscan 7 Microscope Slide Scanner.

5.3 The Certified Assay Operator responsible for conducting the assay is to follow this SOP with associated addendum and complete the required tasks and associated documentation. The Batch Record (Appendix 1) must be completed in ***real-time*** for each experimental run, with each page ***dated and initialed.***

5.4 All responsible personnel are to check the DCTD Biomarkers website (http://dctd.cancer.gov/drug-discovery-development/assays/validated-biomarker-assays.htm) to verify that the most recent version of this SOP is being used.

6.0 MATERIALS AND EQUIPMENT REQUIRED

6.1 ZEISS Axioscan 7 Microscope Slide Scanner (Carl Zeiss Microscopy)

6.2 ZEN 3.7 blue edition (Carl Zeiss Microscopy)

6.3 Kimwipes (e.g., Fisher Scientific, Cat#: 06-666A)

6.4 ZEISS Lens Cleaning Spray (e.g., Amazon, Item model number#:740.000.00146)

NOTE: Carl Zeiss Microscopy no longer offers this item through its catalog and recommends purchase from Amazon using the specific item model number listed above.

6.5 Bond-RX stained clinical biopsy and control slides (such as those processed according to SOP LHTP003.07.23)

7.0 OPERATING PROCEDURES

7.1 The Batch Record (Appendix 1) for image capture should be completed for a single Bond-RX staining run (up to a total of 30 slides).

7.1.1 Record the clinical protocol numbers, Specimen IDs, Assay Names and Slide #s in the Batch Record (Appendix 1).

7.2 On the Batch Record (Appendix 1, Section 1) record the name of the Laboratory, Assay Operator, the date the slides were stained, the date the images were captured, the S/N or ID of the Axioscan being used for this run, and the name of the server where the images will be saved.

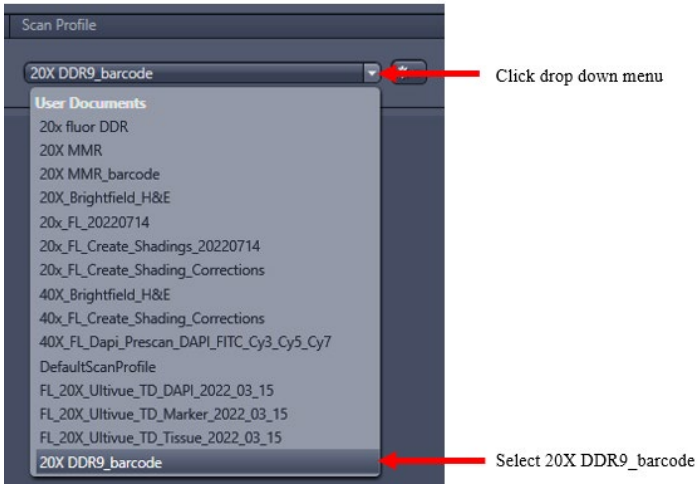
7.3 Turn on the Axioscan 7 Microscope Slide Scanner. Detailed procedures for the Axioscan 7 scanner startup process are provided in Appendix 2.

7.4 Load stained slides onto the Axioscan 7 Microscope Slide Scanner. Detailed procedures for the slide loading process are provided in Appendix 3.

7.5 From the ZEN software, under the "**Scan**" tab, go to the "**Magazine**" page as shown in (A) below. Select the appropriate assay specific scan profile from the dropdown menu as shown in (B) below. Use the "**Mark All"** button as shown in (C) below to acquire all slides using the selected assay specific scan profile as shown in (D) below.

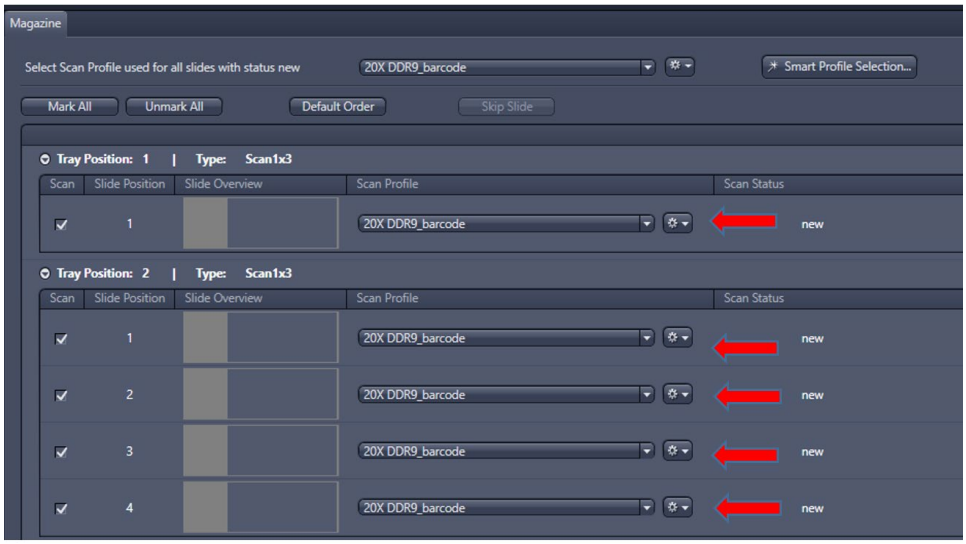
A.

B.



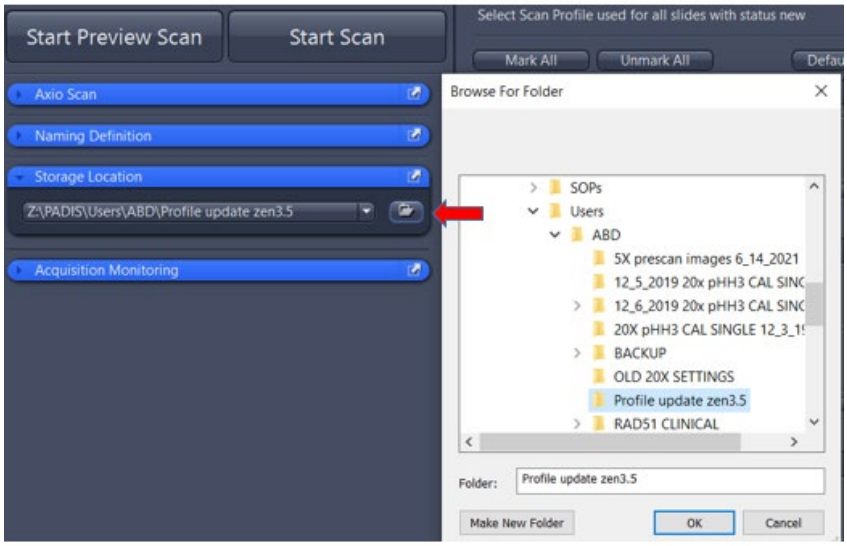
C.

D.



NOTE: The "**Unmark All"** button shown in (C) above can be used to unselect all slides then select specific slides for scanning.

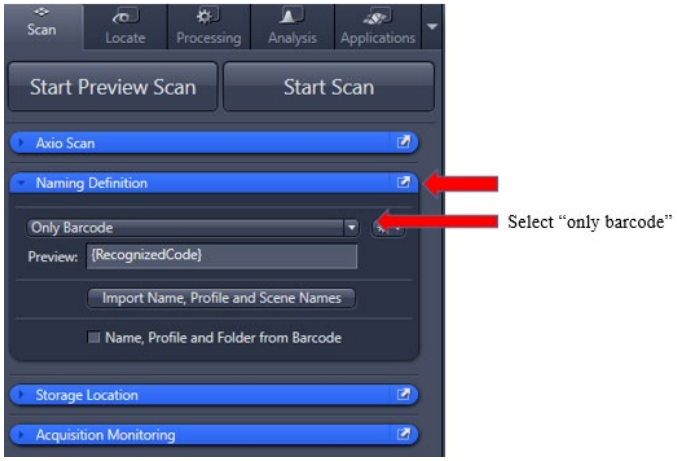
7.6 Under "**Storage Location**", click the folder icon to select the appropriate location mapped to the Axioscan 7 to store the scanned images as shown below.



NOTE:

- Do not save images to the local computer.
- Clinical specimen images should be saved to an appropriate clinical folder.
- Preclinical specimen images should be saved to an appropriate folder designated for preclinical images.

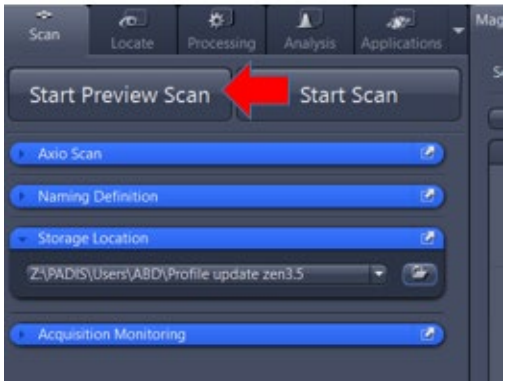
7.7 Under "**Naming Definition"**, select "**Only Barcode**" as shown below. Using this option, the 2D barcode on the slide label will be used as the slide name. If there is no 2D barcode on the label, the user can manually change the slide name in the "**Preview**" field.



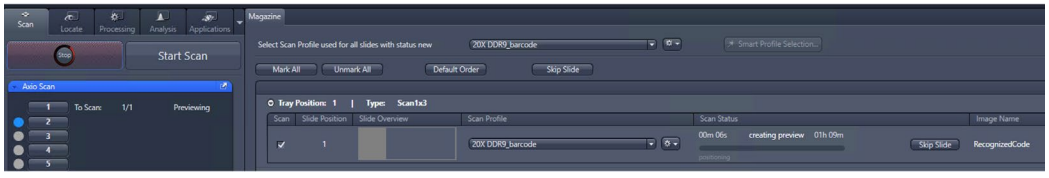
NOTE: It is important for slides to be named before the **Preview Scan**. For slides without barcodes, select "**Manually**" under **"Naming Definition"** and enter the slide names.

7.8 Click the "**Start Preview Scan"** button as shown in (A) below to start the 5X pre-scan. The progress for the preview scan will show up in the "**Magazine**" page for each slide as shown in (B) below.

A



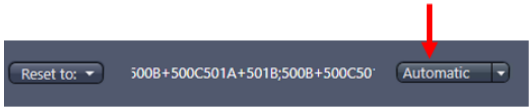
B



NOTE: Once the "**Preview Scan**" is started, the remainder of the set up can be performed by remoting into the system using the **Remote Desktop Connection** app. In the login screen as shown below, use the password "zeiss" to login.

7.8.1 If the slide label has a barcode, the slide name will be automatically generated according to the barcode. To modify the slide name, change the slide name setting from "Automatic" as shown in (A) below to "Manually" as shown in (B) below and enter the slide name in the field provided as shown in (B) below.

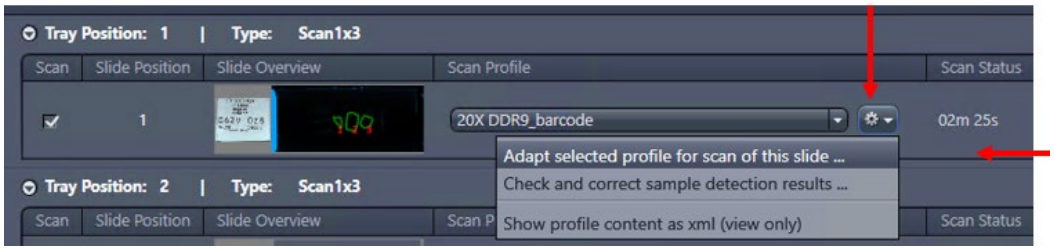
A



B

NOTE: Slides should be named before the "**Preview Scan**", however the slide names can be modified after the "**Preview Scan**" if necessary.

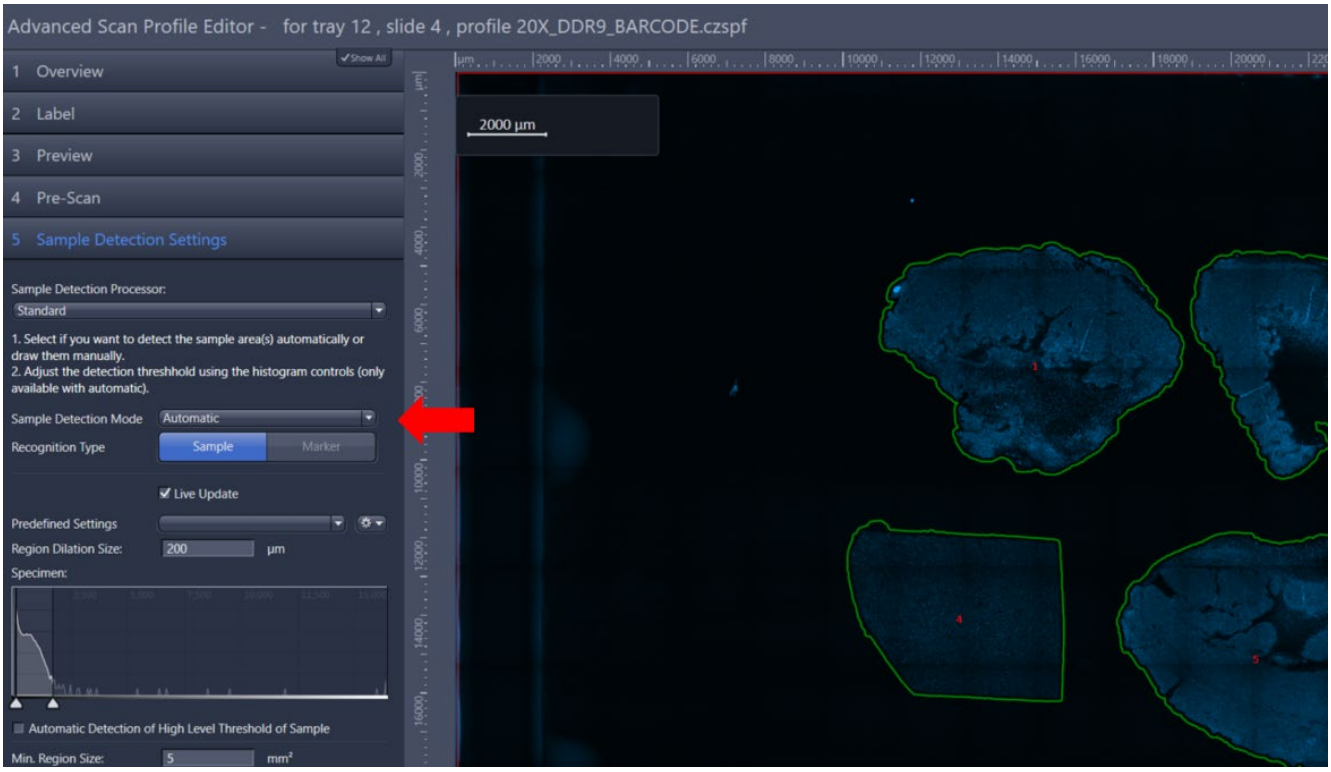
7.9 After the "**Preview Scan**" is complete for all slides, click on the gear icon next to the selected scan profile name and select "**Adapt selected profile for scan of this slide**" as shown below. Initiate a "**20X Scan**" for each slide in the "**Advanced Scan Profile Editor"** window following Section 7.9.1 to Section 7.9.5.



7.9.1 Set up "**Sample Detection Settings"** following instructions below.

7.9.1.1 For xenograft tissues, select "Automatic" as the "**Sample Detection Mode**" as show in (A) below and adjust the histogram to visualize DAPI if needed as shown in (B) below. Delete ROIs not needed using the delete key on the keyboard.

A

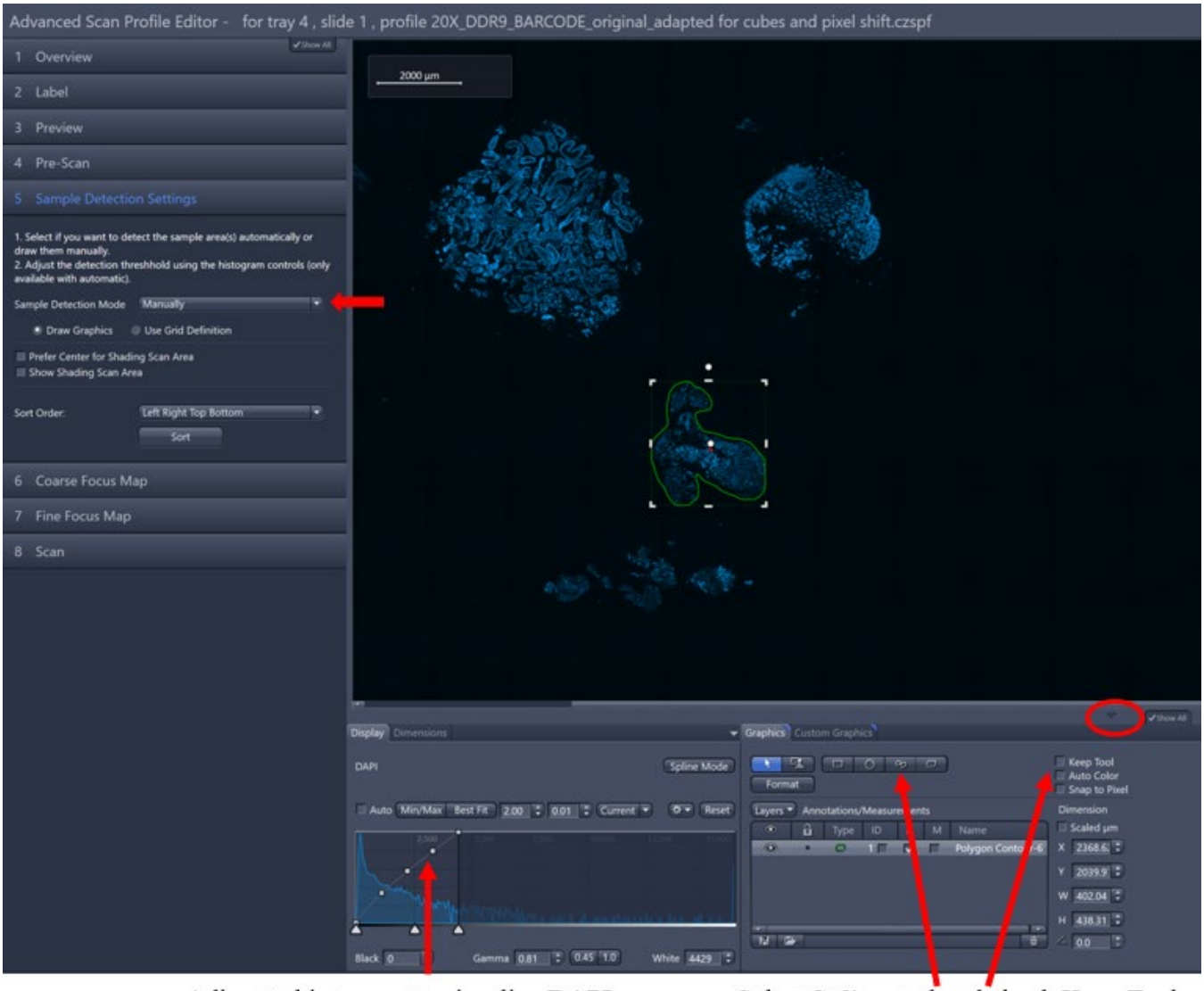


B

NOTE: The automatic tissue detection usually works well for xenograft tissues and more regularly shaped tissue pieces, but not for tumor biopsies.

7.9.1.2 For biopsy tissues, select "Manually" as the "**Sample Detection Mode**" and manually draw ROIs on the images following the steps shown below: A) Adjust histogram to visualize DAPI signal; B) Select "**Spline**" tool and check "**Keep Tool**" box; C) Click mouse and hold down to draw ROIs around the tissues of interest, then right click to end drawing. ROIs can be deleted by "**backspace**" on the keyboard or by selecting the trash icon.

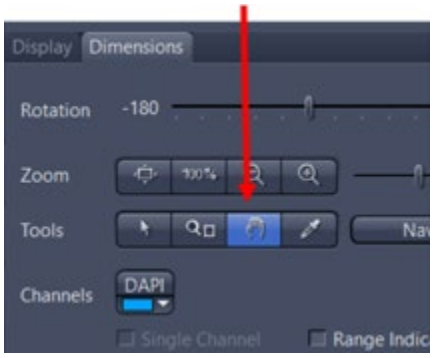
7.9.1.2.1 For fragmented biopsy specimens, draw one ROI for each biopsy fragment with tumor according to the nearest H&E image. This will facilitate the optimization of ROI number and fine focus points as detailed in Section 7.9.4 .



NOTE:

- Use mouse wheel to zoom in/out of the image.

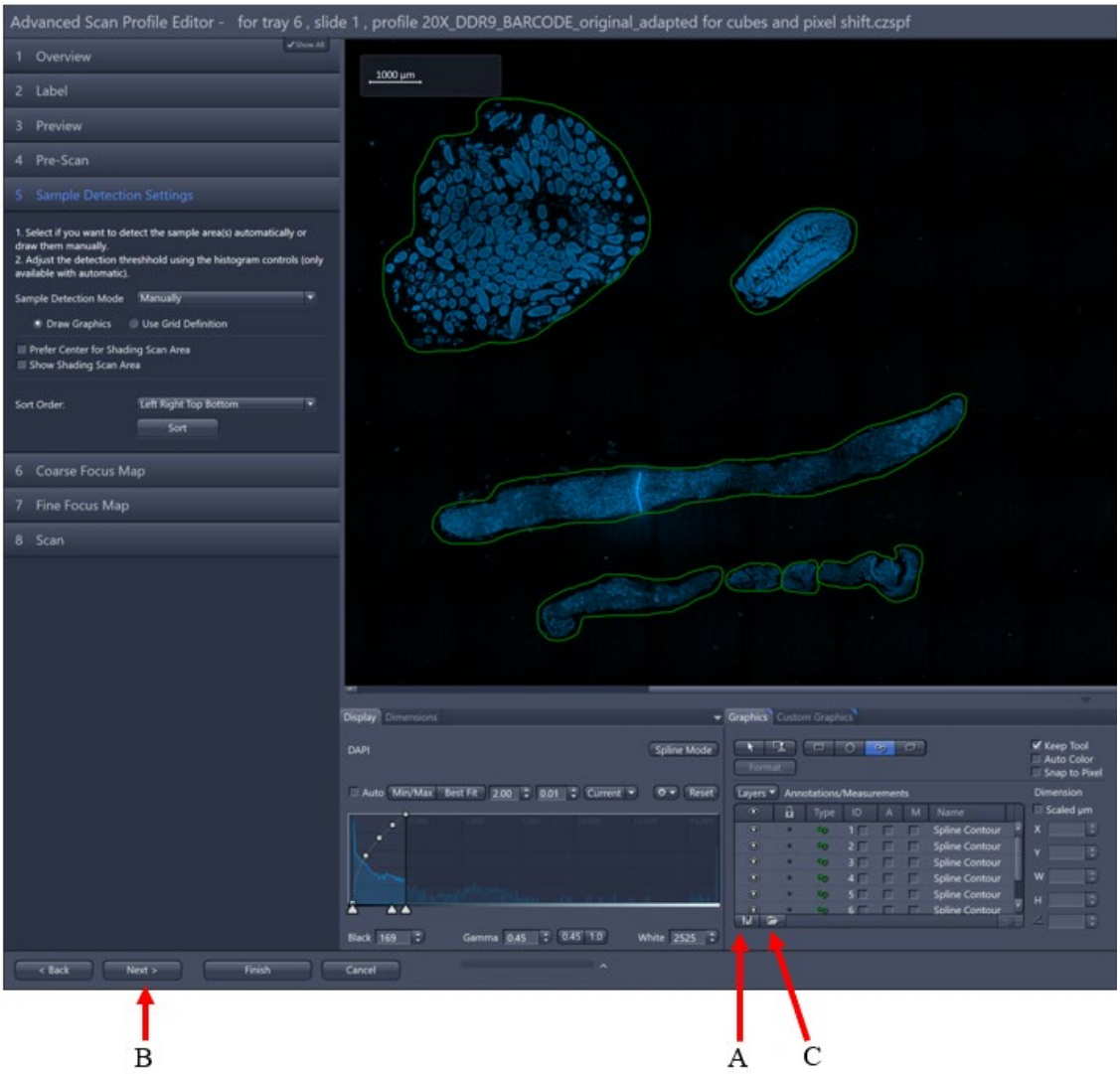
- Use the arrow circled in the image above to hide the Graph Tools to view a larger image if needed. However, in general it is recommended to check the "**Keep Tool**" box, so that multiple ROIs can be drawn on an image.
- The "**Panning**" tool (hand) shown below under the "**Dimensions**" tab can be used to navigate around the slide.



7.9.1.3 Once all the ROIs have been drawn, save the ROIs by clicking the "**Save**" icon as shown by arrow (A) below then "**Next**" as shown by arrow (B) below. The ROI files should be saved in the designated folder for the image project in the appropriate image folder location.

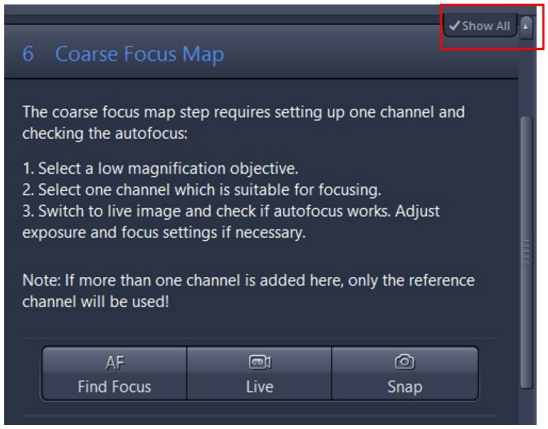
7.9.1.4 ROIs can also be imported using the **Open Folder** icon as shown by arrow (C) below.

NOTE: The ROIs can be imported only if they were already drawn and saved on another slide or if the software crashes. The saved ROIs can be imported upon restart of the software.



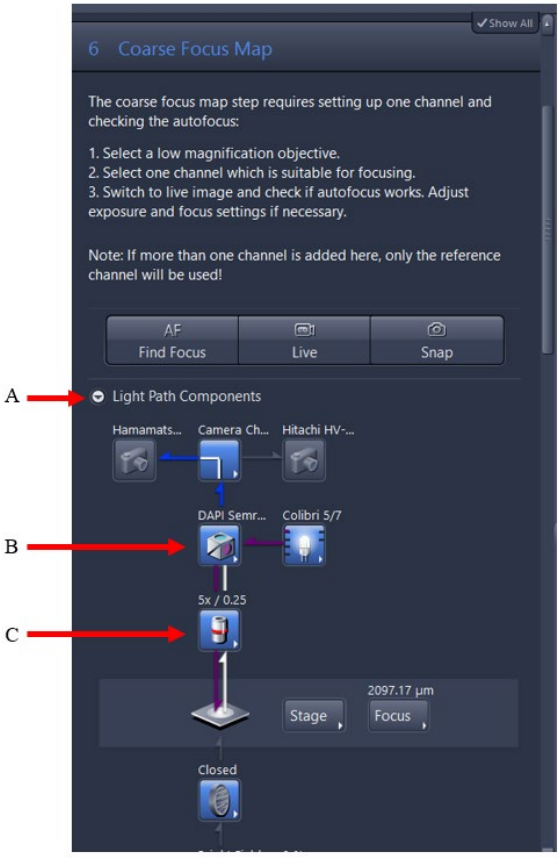
7.9.2 Set up the "**Coarse Focus Map**" settings following instructions below.

NOTE: Make sure the "Show All" box is checked before proceeding through steps in this section as shown below.

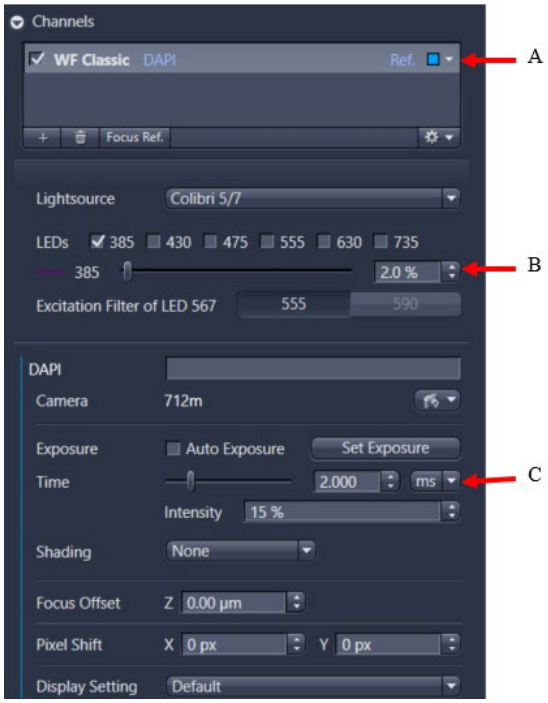


7.9.2.1 Under the "**Light Path Components**" section, click the small triangle next to "Light Path Components" to open this section as shown in (A) below. Select "DAPI" channel and "5x/0.25" objective as shown by arrows (B) and (C) below. Once the components are confirmed, close the "**Light Path Components"** settings by clicking on the small triangle shown by arrow (A) below.

NOTE: Depending on the reflector used on a specific Zeiss Axioscan, the DAPI channel may be named as "DAPI Shemrock" or "SR Blue".



7.9.2.2 Click the small triangle next to "**Channels**" to open options for "**Channels**" settings. The "**Channels"** settings should be set to "WF Classic DAPI" as shown by arrow (A) below, with 2% LED lamp intensity as shown by arrow (B) below and 1-2 ms exposure time as shown by arrow (C) below.

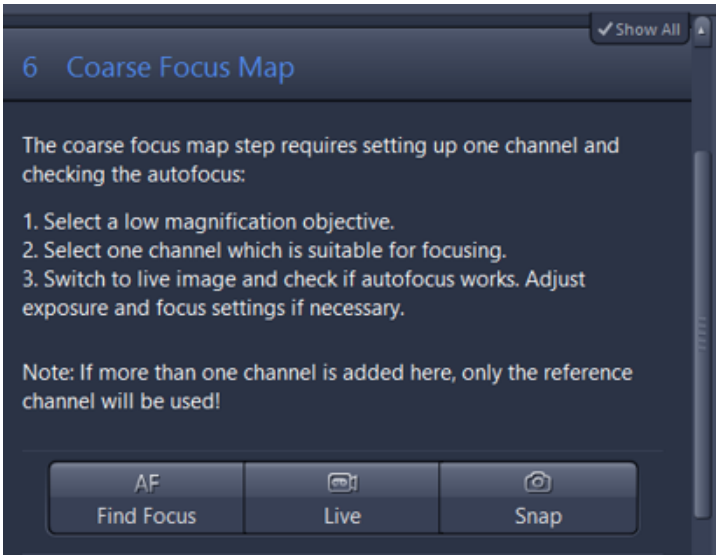


NOTE:

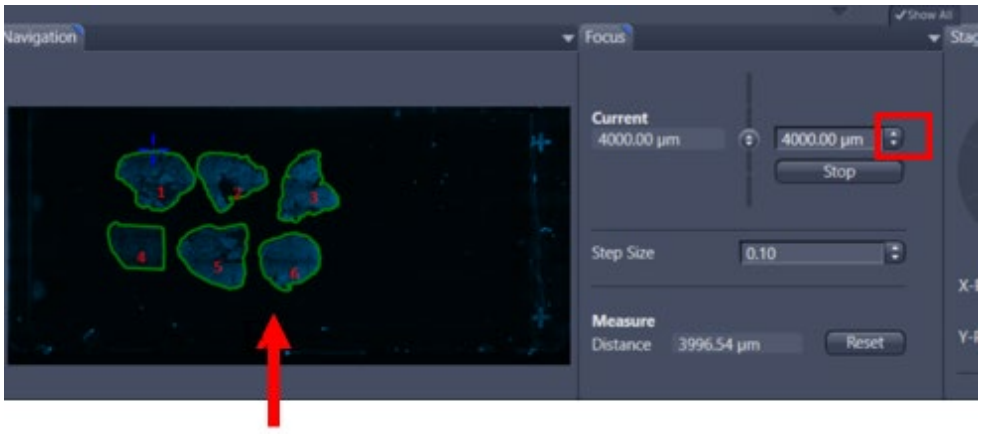
- The exposure time can be adjusted. The typical exposure time for this coarse focus setting is 1-3 ms.
- Do not check the "**Auto Exposure**" box or click on "**Set Exposure**".

7.9.2.3 To set up "**Coarse Focus Map**", click "**Find Focus**" as shown in (A) below and wait until the focus is complete. Alternatively, click on "**Live**" as shown in (A) below and focus manually using the button circled in (B) below. After the focus is complete, click "**Snap**" as shown in (A) below. Click on each tissue on the slide as shown by arrow in (B) below to test the exposure settings. Follow Section 7.9.1.1 to adjust the DAPI intensity to visualize the tissues if needed.

A



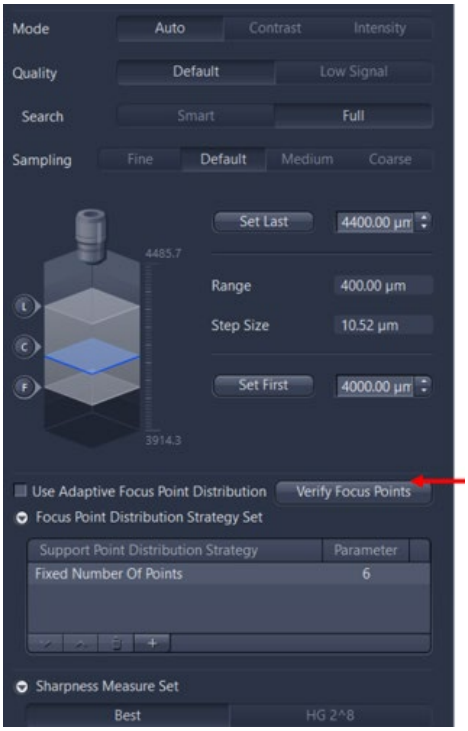
B



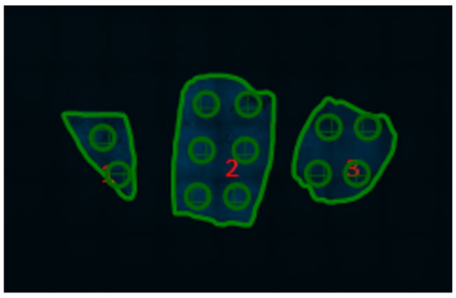
NOTE: If the exposure time needs to be adjusted, change the exposure value, and click "**Snap**". Exposures for DAPI should be adjusted to minimize the underexposed nuclei while preventing appearance of overexposed nuclei to give an accurate representation of the sizes of the nuclei. Overexposure of the signal will have a "swelling" effect on the area, giving a larger "false-positive" area. If the position on the tissue has been changed, select "**AF Find Focus**", and click "**Snap**".

7.9.2.4 Keep the assay specific settings in the scanning profile selected in Step 7.5. Refer to the assay specific addendum to make sure settings in the profile are consistent with assay specific settings. Click on "**Verify Focus Points**" as shown in (A) below. Ensure focus points are placed on tissue in the next window as shown in (B) below. Use the arrow tool as show in (C) below to click on the focus points for moving. Click "**Next**" at the bottom of the window to proceed to the next step after all settings have been confirmed.

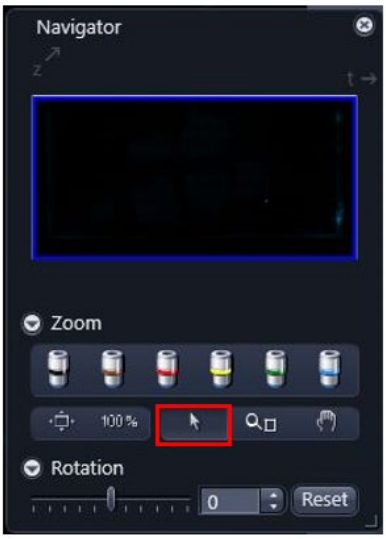
A


Mode
Auto
Contrast
Intensity
Quality
Default
Low Signal
Search
Smart
Full
Sampling
Fine
Default
Medium
Coarse
Set Last
4400.00 µm
4485.7
Range
400.00 µm
Step Size
10.52 µm
Set First
4000.00 µm
3914.3
Use Adaptive Focus Point Distribution
Verify Focus Points
Focus Point Distribution Strategy Set
Support Point Distribution Strategy
Parameter
Fixed Number Of Points
6
Sharpness Measure Set
Best
HG 2^8

B


1
2
3

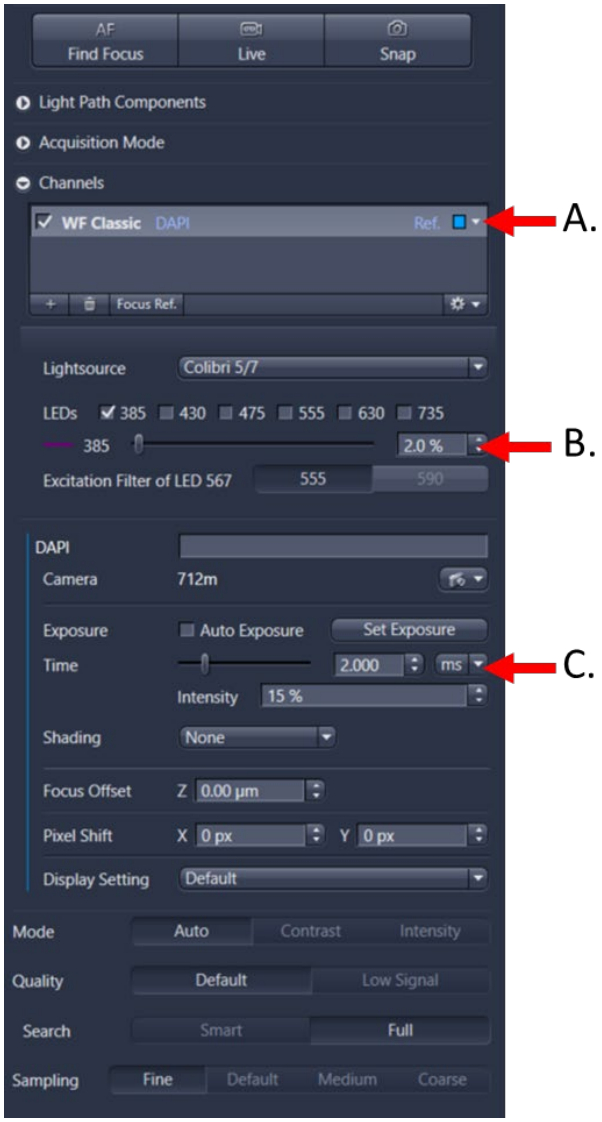
C


Navigator
Zoom
100 %
Rotation
0
Reset

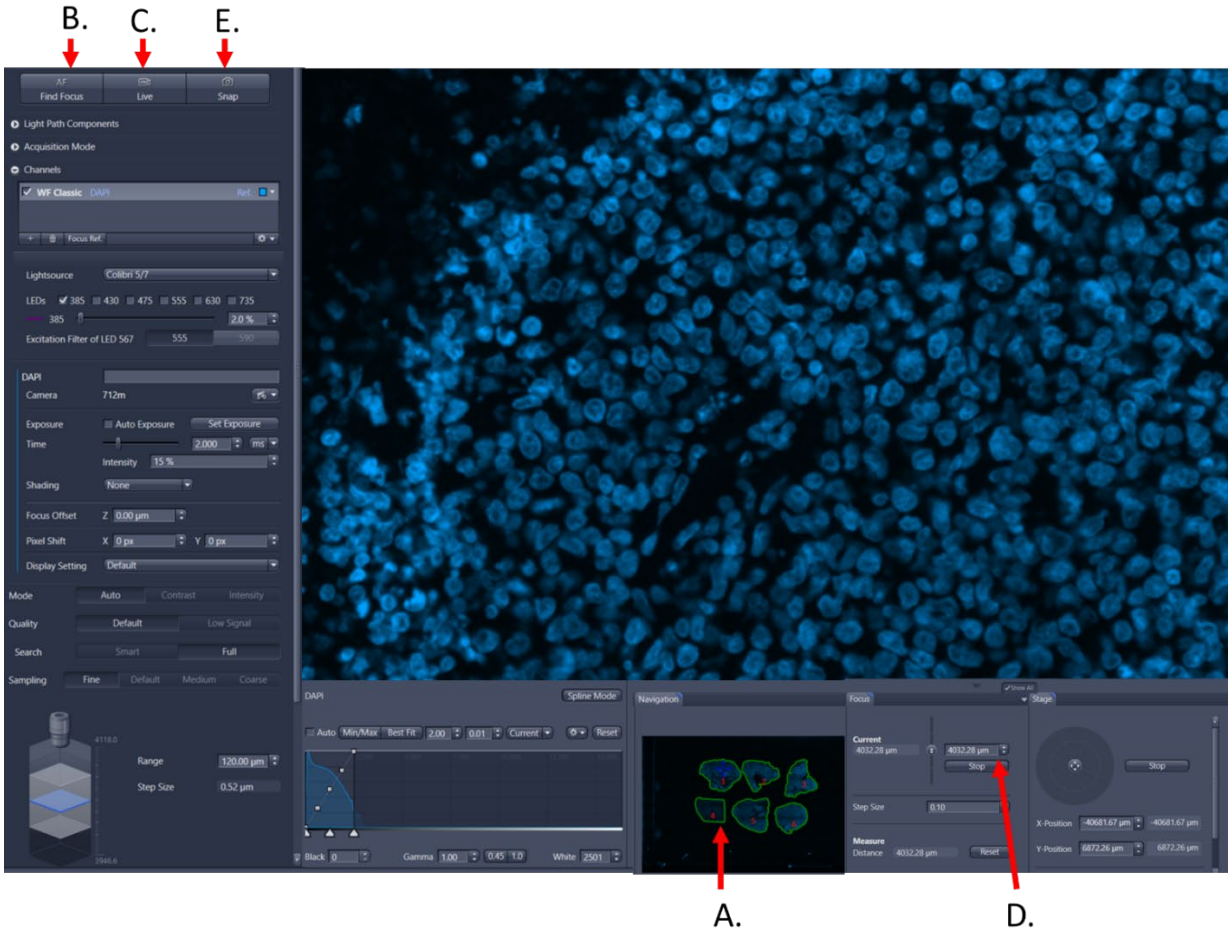
7.9.3 Set up "**Fine Focus Map"** settings following instructions below.

7.9.3.1 Under "**Light Path Components**" section, select "DAPI" channel and "20x/0.8" objective as shown by arrows (A) and (B) below. Once the components are confirmed, close the "**Light Path Components**" settings by clicking on the small triangle as shown by arrow (C).

7.9.3.2 "**Channel**" settings should be set to "WF Classic DAPI" with 2% lamp intensity and exposure should be 1-3 ms as shown by arrows (A), (B) and (C) below, respectively.

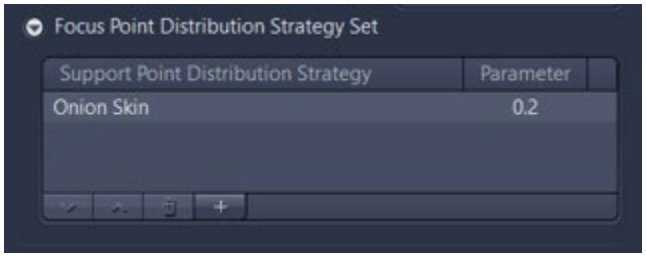


7.9.3.3 To set up "**Fine Focus Map**", test exposure by clicking on the tissue as shown by arrow (A) below. Then either click on "**AF Find Focus**" as shown by arrow (B) below or click on "**Live**" as shown by arrow (C) below and manually focus using the button shown by arrow (D) below. Click on "**Snap**" to view image as shown by arrow (E) below and ensure the image is in focus.

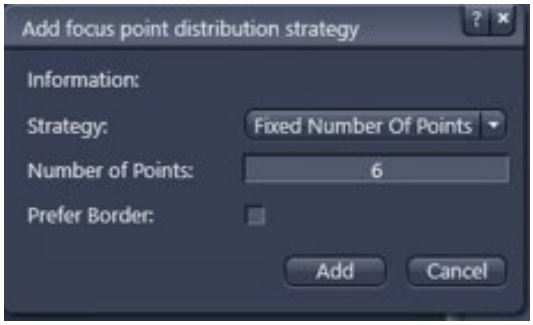


7.9.3.4 Scroll down to autofocus strategies, keep the settings in the assay specific scanning profile selected in Step 7.5. Refer to assay specific addendum to ensure settings in the profile are consistent with assay specific settings. Change "**Support Point Distribution Strategy**" by double clicking on "Onion Skin" as shown below.

7.9.3.5 For more regularly shaped tissues such as xenograft and control tissues, use "Onion Skin" and "Density 0.2" as shown below.

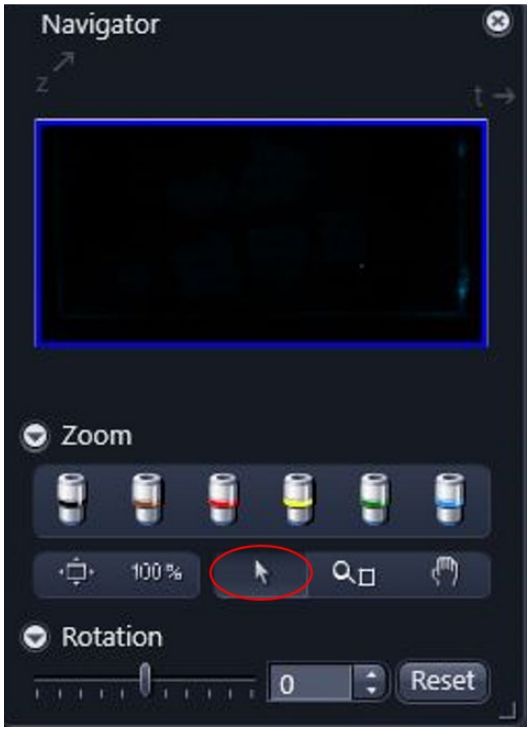


7.9.3.6 For biopsy tissues, switch the strategy to “Fixed Number of Points” from the drop-down menu as shown below. Number of points will vary depending on the size and tumor content of biopsy specimens. Refer to Section 7.9.4 for details on Focus Point and ROI Optimization.

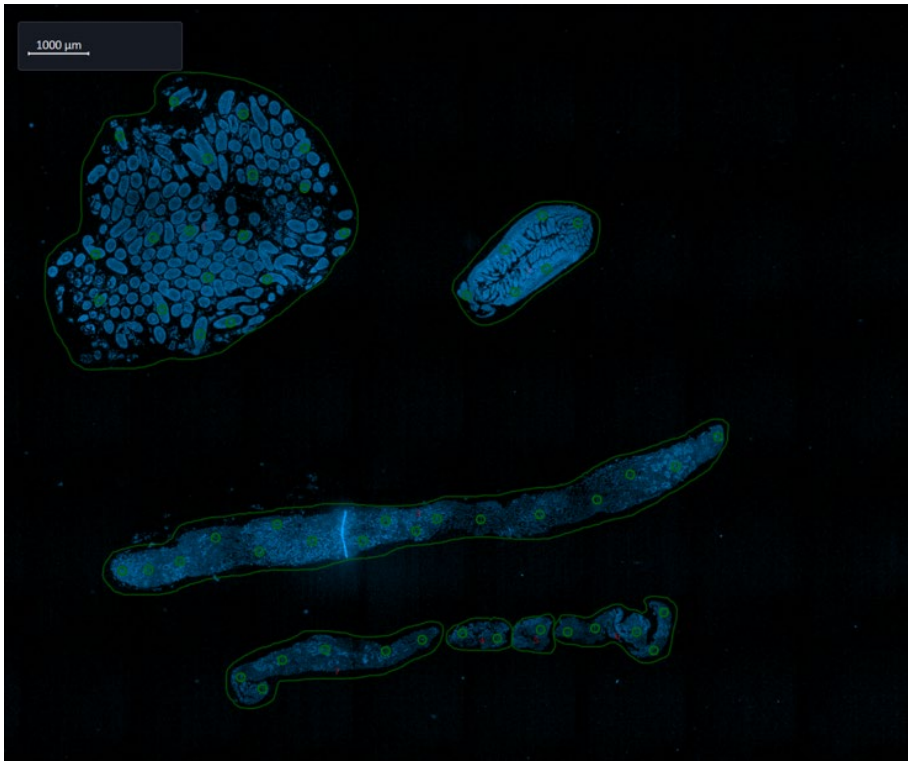


7.9.3.7 Click on “**Verify Focus Points**” as shown below.

7.9.3.8 In the Navigator window, click the arrow circled below.



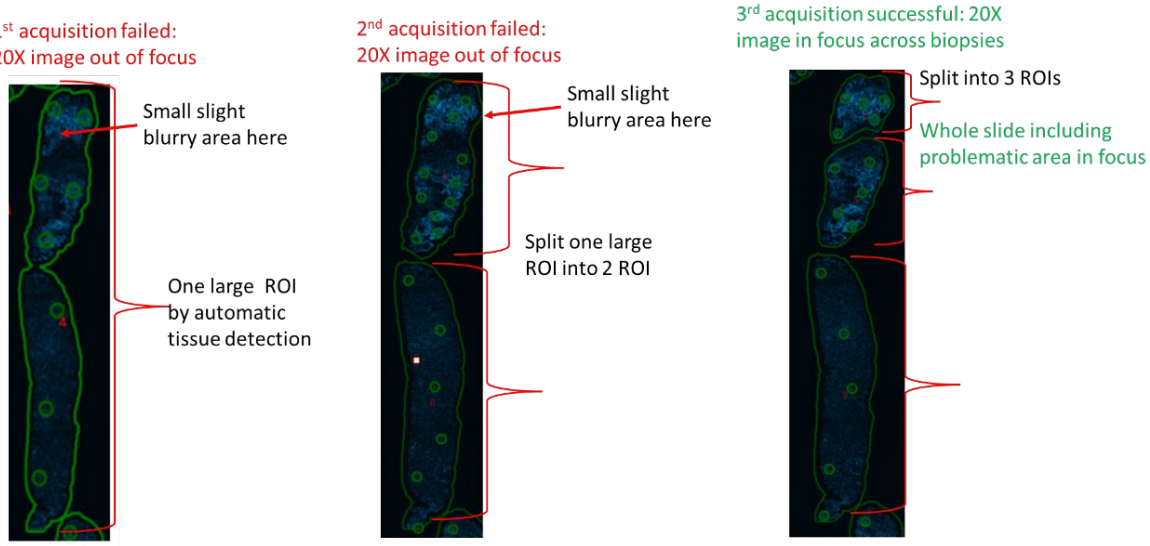
7.9.3.9 In the window shown below, add or move focus points or create smaller tissue ROIs as needed.



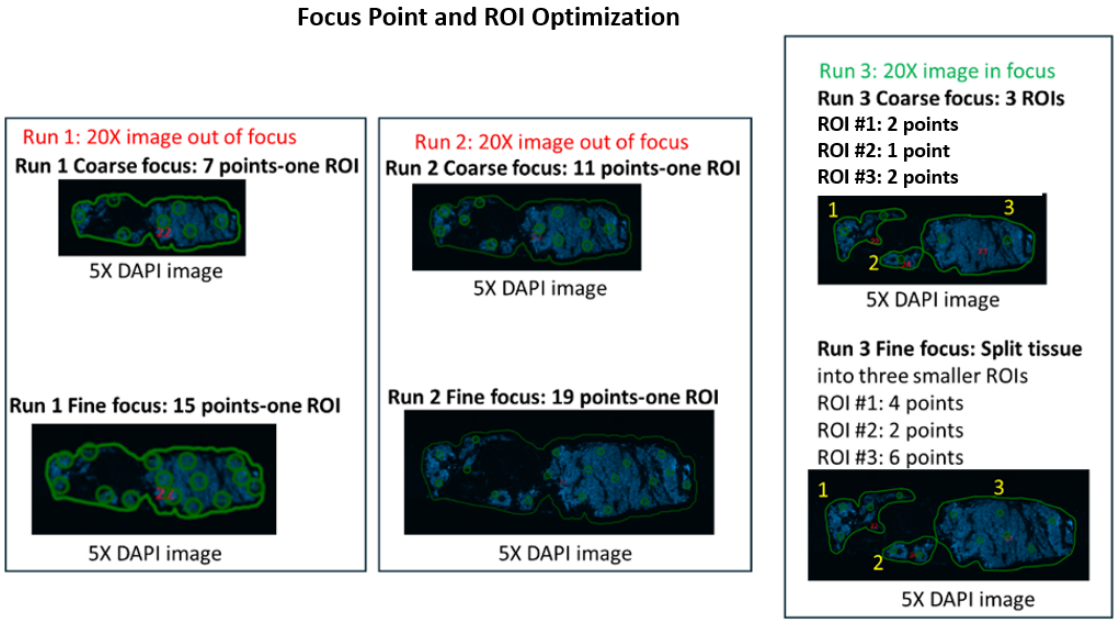
7.9.3.10 Click on "**Next**" at the bottom of the window when finished.

7.9.4 Number of fine focus points, point position and number of ROIs for a single biopsy are important parameters for specimens to be in focus post-20X acquisition and should be adjusted for each biopsy specimen. For the example shown in (A) below, breaking a single biopsy pass into multiple ROIs and positioning of more fine focus points are both important to maintain focus in the 20X acquisition. For the example shown in (B) below, a combination of using additional ROIs and adjusting focus point position with less points results in a 20X image that is in focus.

A



B



7.9.5 To set up the "**Scan**" setting, click "**AF Find Focus**" if you have moved within the tissues since the last step.

7.9.5.1 Under "**Channels**" settings, ensure all channels are selected according to the assay addendum. An example of assay channel selection for the DDR9 assay (γH2AX, pNBS1 Immunofluorescence Assay with β-Catenin Segmentation) is shown by arrow (A) below.

NOTE:

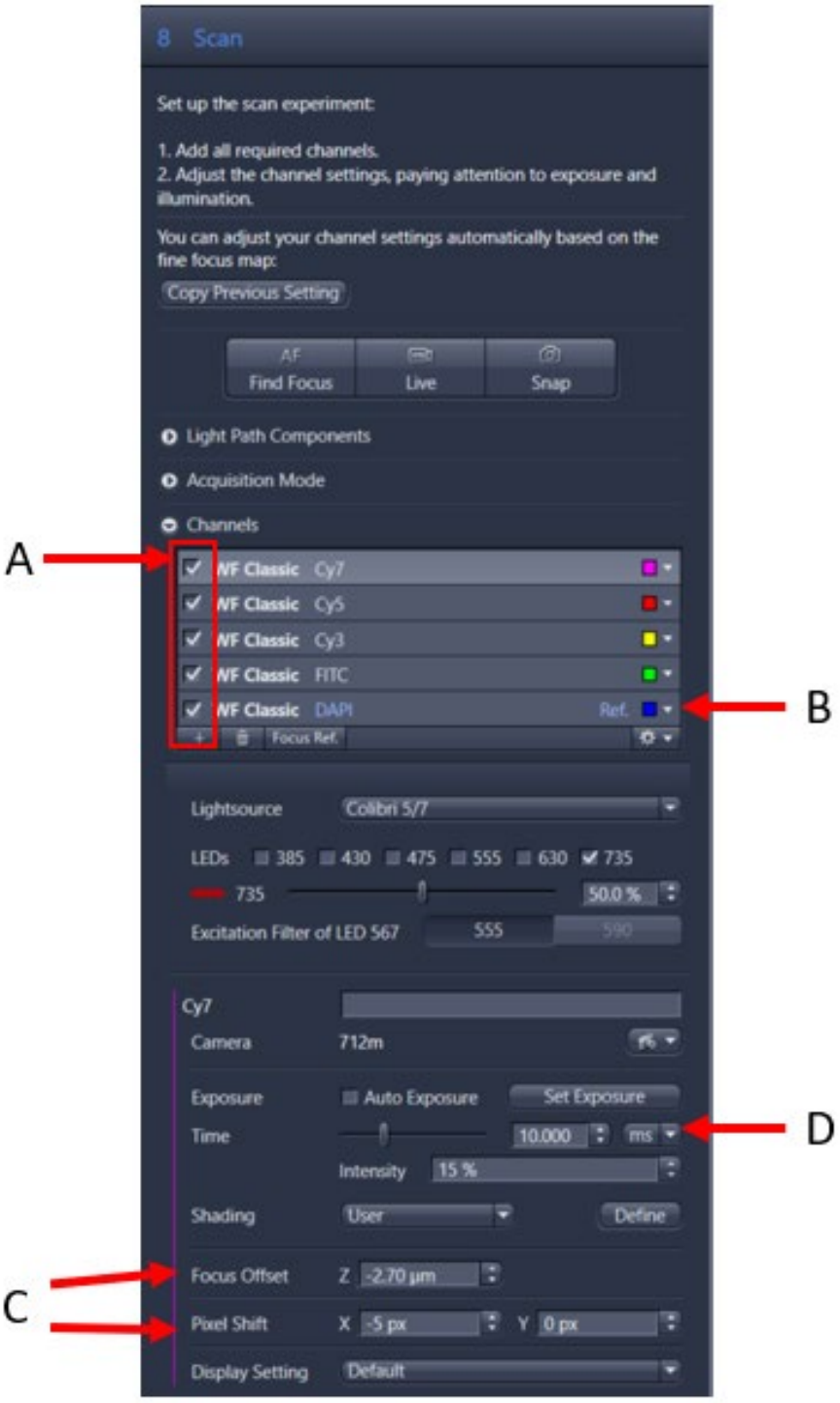
- DAPI should be the reference as shown by arrow (B) below.
- Confirm that shading is set to "user" for each channel.

7.9.5.1.1 Click on each channel to display the channel setting as shown below.

7.9.5.1.2 Check "**Focus Offset**" and "**Pixel Shift**" for each channel and confirm setting match the calibration performed by ZEISS as shown in by arrow (C) below.

NOTE: Each Zeiss Axioscan 7 has specific "**Focus Offset**" and "**Pixel Shift**" set by ZEISS. Please refer to instrument specific information.

7.9.5.1.3 Adjust exposure time (ms) as needed as shown by arrow (D) below.

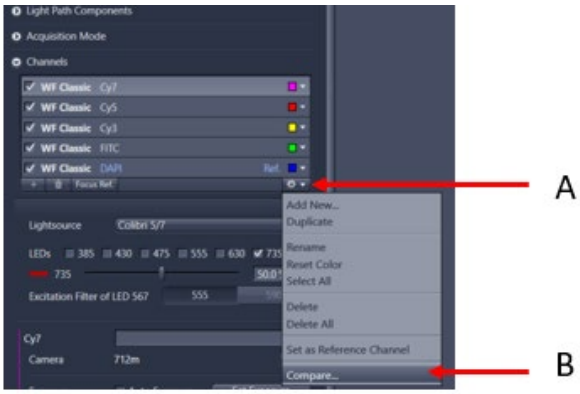


NOTE:

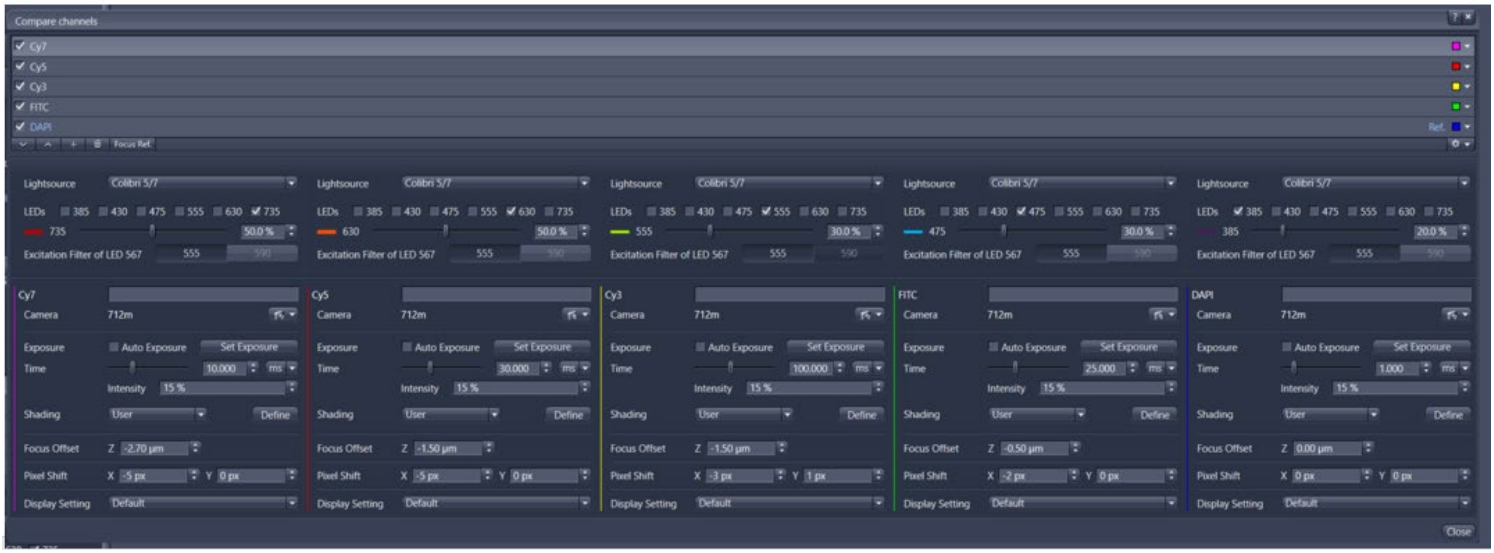
- It is important to check multiple areas of a tissue and multiple slides to identify proper exposure times before starting the 20X scan following instructions in Section 7.9.6.

- Slides from the same patient should be scanned using the same exposure settings, evaluate all slides from the same patients or the same preclinical studies to confirm the settings are adequate for each slide. Adjust the exposure settings for all slides as needed following methods in Section 7.9.6.

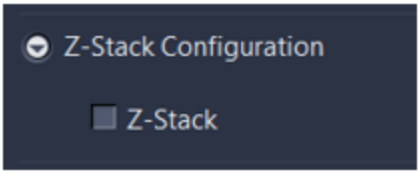
7.9.5.1.4 Click on gear icon and select "**Compare**" as shown by arrow (A) below to check all channel settings including LED power, exposure, focus offset (z) and pixel shift (x, y) as shown by arrow (B) below.



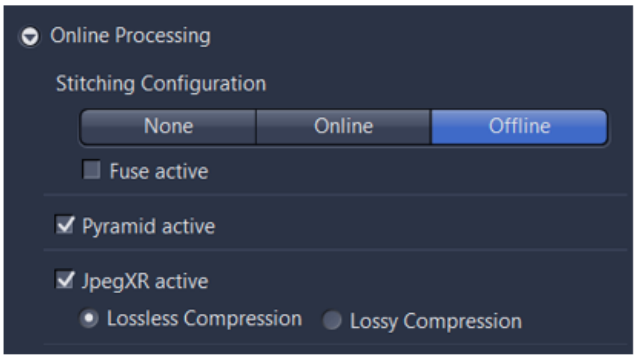
7.9.5.1.5 The detailed acquisition settings for all channels will be displayed. An example of DDR9 assay (γH2AX, pNBS1 Immunofluorescence Assay with β-Catenin Segmentation) acquisition setting for all channels is shown below.



7.9.5.2 Under "**Z-Stack Configuration**", make sure the box is unchecked as shown below.

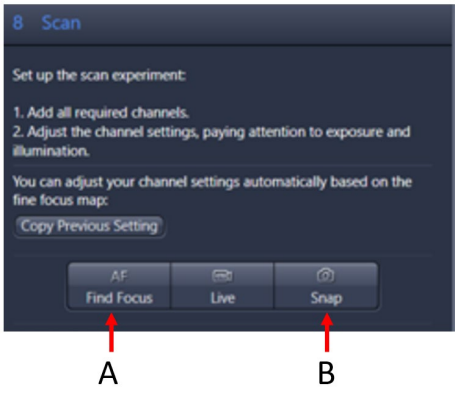


7.9.5.3 Under "**Online Processing**", make sure that "Offline" is selected as shown below. This means that the image will automatically be stitched in the background after the image finishes acquiring with no input from the user. Additionally, make sure that the boxes for "Pyramid active" and "JpegXR active" are checked and that "Lossless Compression" is selected as shown below.



7.9.6 Refer to assay addendum for the recommended exposure time ranges, target background intensities and LED lamp intensity settings for each channel for each assay. Additionally, the signal to background ratio should be > 2. Check the biomarker staining on all slides for appropriate signal and to ensure that the staining is not over- or under-exposed. Control slides and clinical patient specimen slides may have different exposures, but all control slides from the same staining run must have the same exposures and all individual slides from the same patient must have the same exposures. Slides from different patients may have different exposures.

7.9.6.1 For each tissue to be scanned on the slides, click "**Find Focus**" to focus across the tissue as shown by arrow (A) below. Then, click "**Snap**" to snap images across the tissue as shown by arrow (B) below.



7.9.6.2 Start the evaluation of exposure time using a qualitative method for each snapped image.

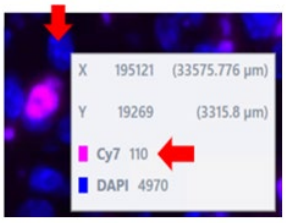
7.9.6.2.1 Go to "**Dimensions**" tab, enable "**Show All**" as shown by arrow (A) below, then select "**Show Values**" tool as shown by arrow (B) below to view channel intensity values.

7.9.6.2.2 Hover the mouse over positive and negative nuclei to view channel intensity values. Examples of channel intensity values for positive and negative nuclei in γH2AX Con High (A) and Con Low (B) tissues are shown below.

NOTE: Select negative nuclei near nuclei with positive signal.

A

Negative Nuclei in CON High



Positive Nuclei in CON High

B

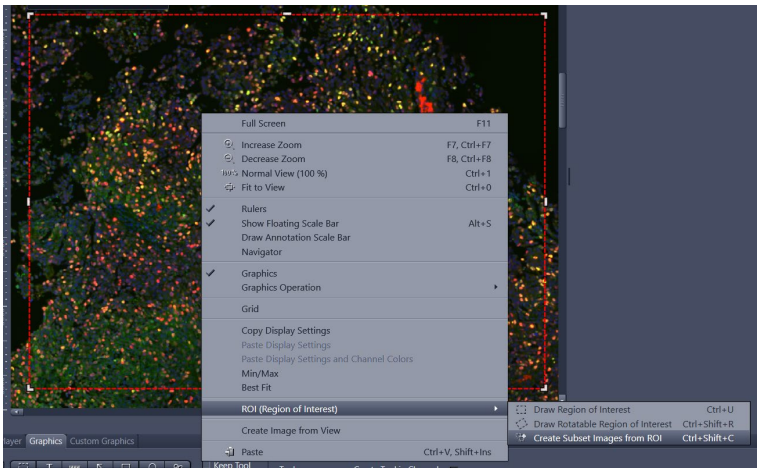
Negative Nuclei in CON Low

Positive Nuclei in CON Low

7.9.6.2.3 Snap preview images across the entire slide on all biopsies and across slides and qualitatively evaluate the background signal intensity and signal-to-background ratio with the "**Show Values**" tool. Adjust the exposure time to ensure that the average intensity for negative nuclei and signal to background ratio meet the requirements defined in the assay specific addendum for each channel.

7.9.6.3 After all slides have been evaluated using the "**Show Values**" tool, snap one image per tissue on both pre- and post-dose biopsy passes on one slide to create image ROIs for quantitative assessment following the steps below.

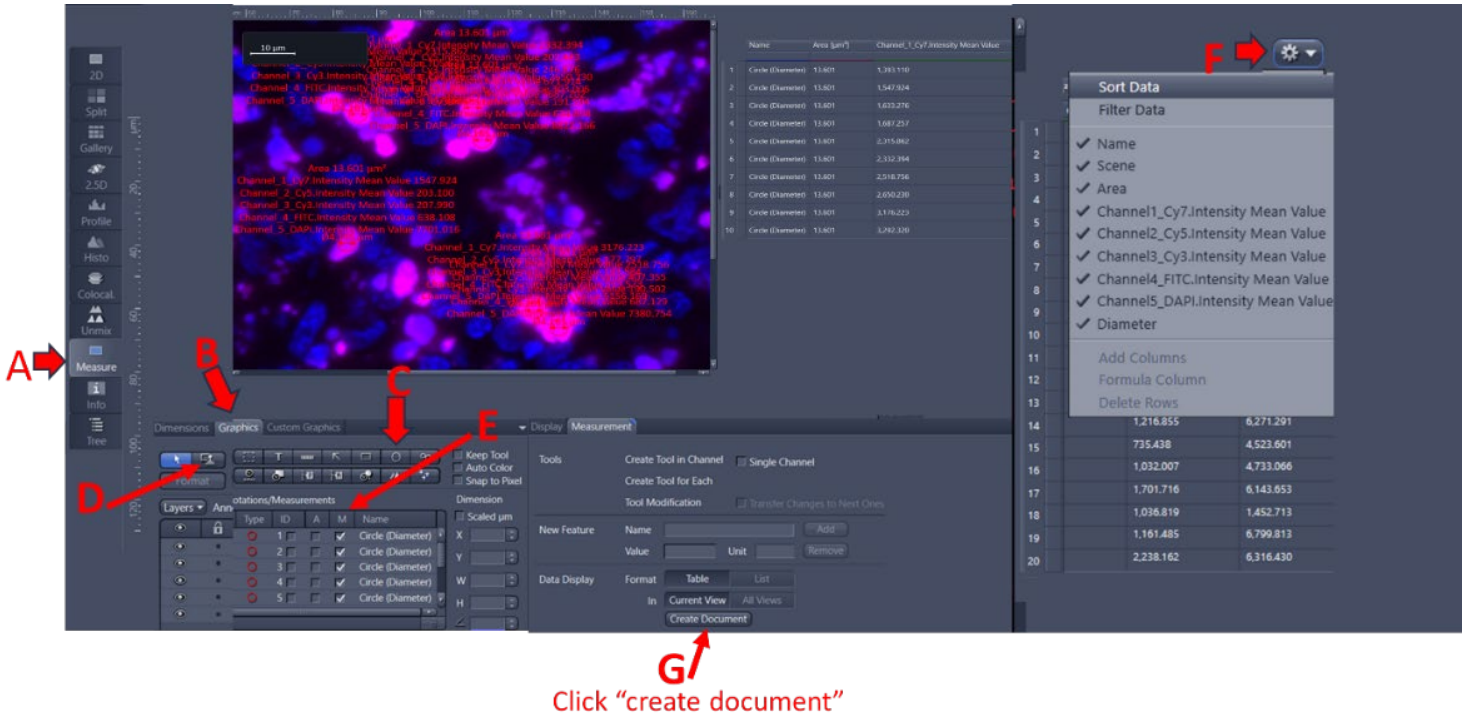
7.9.6.3.1 Draw a red ROI box in the snapped image, then right click within the ROI and select "Create subset images from ROI" from the dropdown menu as shown below. Repeat the process and draw ROIs following the guidelines below.



a. For calibrator images, draw ROIs on both Cal High and Cal Low tissues.

b. For clinical images, draw ROIs on both the pre and post-dose tissues.

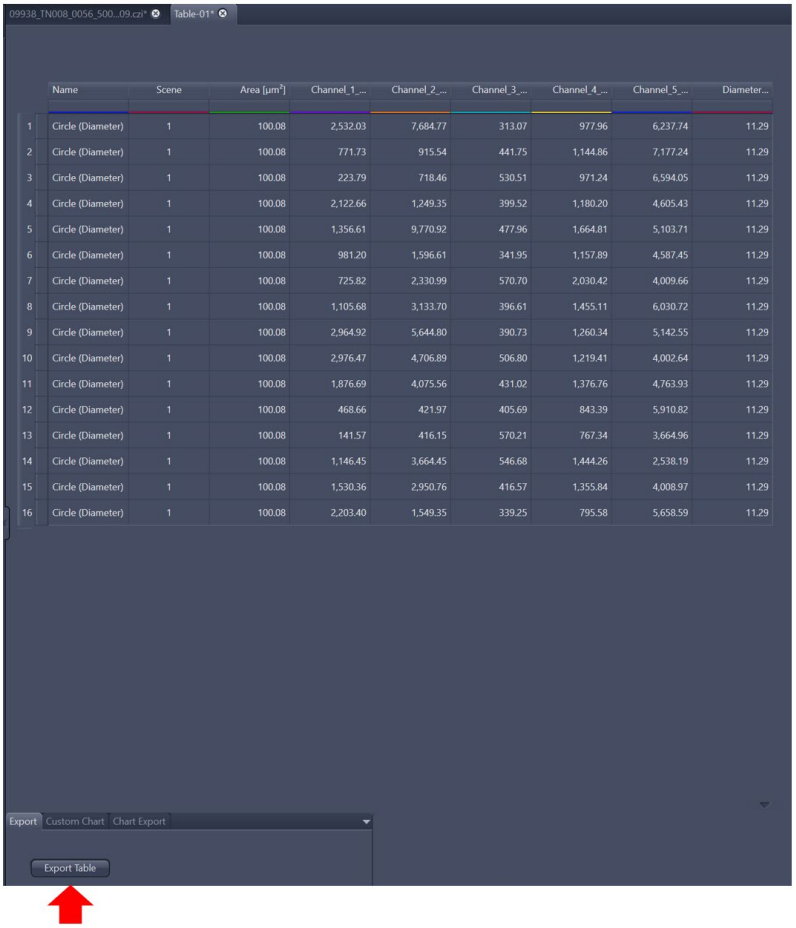
7.9.6.3.2 In the image created for each ROI, use the annotation tools to measure biomarker signal intensity values in biomarker-negative and biomarker-positive tumor nuclei. Draw small circular ROIs on the image for signal intensity value evaluation following the steps below: (A) go to "Measure" section of the image created from the ROI; (B) go to the "Graphics" tab of the control panel; (C) draw a circular ROI around one nuclei using the "Circle" tool; (D) use the "Clone" tool to create more ROIs of the same size around more nuclei (A total of 10-20 ROIs total for both positive and negative nuclei should be created for each tissue per slide.); (E) click on the "M" column to check all the ROIs; (F) click on the gear icon to check/uncheck the data to be included in the table; (G) select "Create Document" to see measurement table.



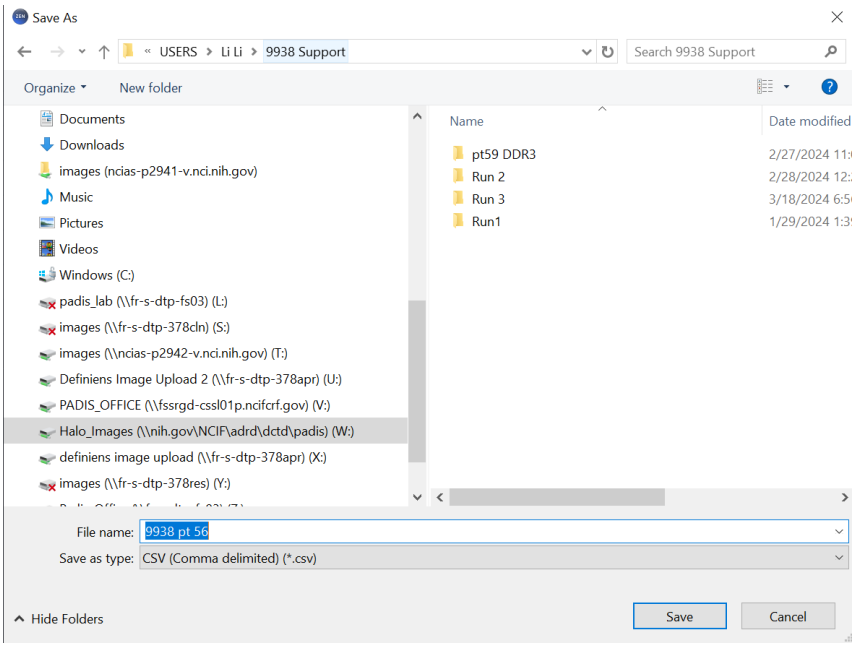
NOTE: The text boxes next to the ROIs can be hidden by unchecking the “M” column under the annotation table as shown by arrow (E) above, but the table will disappear when the “M” column is unchecked.

7.9.6.3.3 Click on “Export Table” in the window that appears after “Create Document” is selected as shown in (A) below. Save the exported table as a csv file as shown in (B) below.

A


09938_TN008_0056_500...09.czi*
Table-01*
Name
Scene
Area [µm²]
Channel_1_...
Channel_2_...
Channel_3_...
Channel_4_...
Channel_5_...
Diameter...
1 Circle (Diameter) 1 100.08 2,532.03 7,684.77 313.07 977.96 6,237.74 11.29
2 Circle (Diameter) 1 100.08 771.73 915.54 441.75 1,144.86 7,177.24 11.29
3 Circle (Diameter) 1 100.08 223.79 718.46 530.51 971.24 6,594.05 11.29
4 Circle (Diameter) 1 100.08 2,122.66 1,249.35 399.52 1,180.20 4,605.43 11.29
5 Circle (Diameter) 1 100.08 1,356.61 9,770.92 477.96 1,664.81 5,103.71 11.29
6 Circle (Diameter) 1 100.08 981.20 1,596.61 341.95 1,157.89 4,587.45 11.29
7 Circle (Diameter) 1 100.08 725.82 2,330.99 570.70 2,030.42 4,009.66 11.29
8 Circle (Diameter) 1 100.08 1,105.68 3,133.70 396.61 1,455.11 6,030.72 11.29
9 Circle (Diameter) 1 100.08 2,964.92 5,644.80 390.73 1,260.34 5,142.55 11.29
10 Circle (Diameter) 1 100.08 2,976.47 4,706.89 506.80 1,219.41 4,002.64 11.29
11 Circle (Diameter) 1 100.08 1,876.69 4,075.56 431.02 1,376.76 4,763.93 11.29
12 Circle (Diameter) 1 100.08 468.66 421.97 405.69 843.39 5,910.82 11.29
13 Circle (Diameter) 1 100.08 141.57 416.15 570.21 767.34 3,664.96 11.29
14 Circle (Diameter) 1 100.08 1,146.45 3,664.45 546.68 1,444.26 2,538.19 11.29
15 Circle (Diameter) 1 100.08 1,530.36 2,950.76 416.57 1,355.84 4,008.97 11.29
16 Circle (Diameter) 1 100.08 2,203.40 1,549.35 339.25 795.58 5,658.59 11.29
Export
Custom Chart
Chart Export
Export Table

B


Save As
USERS › Li Li › 9938 Support
Search 9938 Support
Organize
New folder
Documents
Downloads
images (ncias-p2941-v.nci.nih.gov)
Music
Pictures
Videos
Windows (C:)
padis_lab (\\fr-s-dtp-fs03) (L:)
images (\\fr-s-dtp-378cln) (S:)
images (\\ncias-p2942-v.nci.nih.gov) (T:)
Definiens Image Upload 2 (\\fr-s-dtp-378apr) (U:)
PADIS_OFFICE (\\fssrgd-cssl01p.ncifcrf.gov) (V:)
Halo_Images (\\nih.gov\NCIF\adrd\dctd\padis) (W:)
definiens image upload (\\fr-s-dtp-378apr) (X:)
images (\\fr-s-dtp-378res) (Y:)
Name
Date modified
pt59 DDR3 2/27/2024 11:
Run 2 2/28/2024 12:
Run 3 3/18/2024 6:5
Run1 1/29/2024 1:3
File name: 9938 pt 56
Save as type: CSV (Comma delimited) (*.csv)
Hide Folders
Save
Cancel

7.9.6.4 Follow steps below to assess the biomarker exposure quantitatively.

7.9.6.4.1 Determine intranuclear biomarker intensities by measuring positive signal intensities in biomarker-positive tumor nuclei following instructions in Section 7.9.6.3.

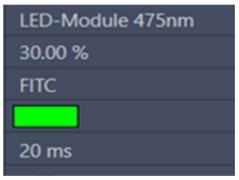
7.9.6.4.2 Determine background intensity by measuring marker signal intensities in biomarker-negative tumor nuclei, not intensity of glass following in Section 7.9.6.3.

7.9.6.4.3 Calculate the average marker background intensities in the negative nuclei and calculate the signal to background ratio. Use signal to background ratio to evaluate exposures per biomarker. A minimum signal to noise ratio of > 2 is desired.

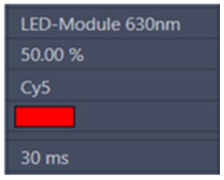
7.9.6.4.4 When making adjustments, increase exposure time first, if required, and only increase LED intensity if the exposure time is already > 200 ms.

NOTE: If LED intensity adjustment is necessary, make small incremental increases as needed (5% step).

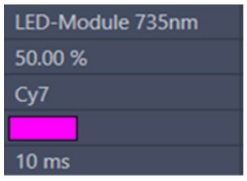
7.9.6.5 An example of successfully acquired DDR9 image settings and calculations are shown below for reference. For this example, the exposure settings meet the established specifications for LED lamp intensity, exposure range, target background intensity and S/B as outlined in the assay specific addendum. Specifically, pKAP1 (FITC) signal to background ratio is 6 and the mean target background intensity of the negative nuclei is 489.8, so this exposure meets the criteria for minimum S/B and target background intensity for FITC according to the assay addendum. The pNBS1 (Cy5) signal to background ratio is 13.45 and the mean target background intensity of the negative nuclei is 270, so this exposure meets the criteria for the minimum S/B and target background intensity for the Cy5 according to the assay addendum. The γH2AX (Cy7) signal to background ratio is 17.4 and the mean target background intensity of the negative nuclei is 130, so this exposure meets the criteria for minimum S/B and target background intensity for the Cy7 according to the assay addendum.



pKap1 pos nuc	pKap1 neg nuc
3655.024229	555.1219512
3527.121739	517.5991379
2424.846983	477.5886214
2704.485839	519.9517544
2700.274336	421.1846154
3751.844789	536.9422222
2937.403084	484.4678492
2484.151982	472.6884532
2800.78022	490.3237885
2505.766885	421.9780702
mean	mean
2949.170009	489.7846464
s/b	
6.021360674	



pNbs1 pos nuc	pNbs1 neg nuc
2828.239382	274.6507177
2679.432225	284.1666667
3893.692503	319.0445247
3380.145729	306.2845138
5447.612167	291.2375152
5925.166667	257.2493976
3455.243622	234.9384615
2763.183569	302.4677033
2888.860104	222.9126794
3053.673831	206.6686747
mean	mean
3631.52498	269.9620855
s/b	
13.4519815	



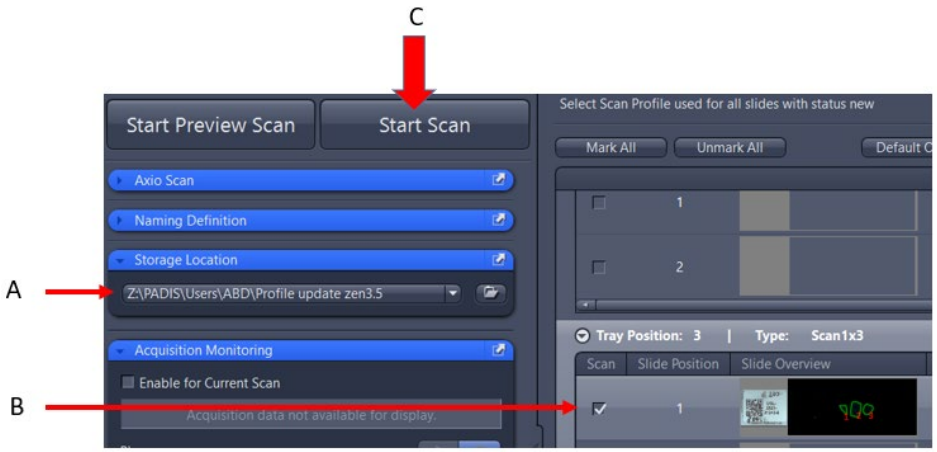
gH2AX + Nuc	gH2AX - Nuc
3292.319527	137.8514056
3176.22334	185.5571429
2573.045082	115.6369168
2650.229508	139.8531746
2332.393878	101.7263581
2315.862069	116.6166329
1457.094378	130.199211
1687.257028	127.0165289
1633.27621	108.884058
1547.924335	138.988
mean	mean
2266.562535	130.2329429
S/B	
17.40391091	

7.9.6.6 After this evaluation, complete the Specimen ID, Channel Names, Biomarker Names, Average Positive Nuclei Intensity, Background Intensity and Signal to Background Ratio in the Batch Record for each tissue (Appendix 1 Section 4). The measured values should meet the assay specific requirements specified in the assay addendum.

7.9.6.7 A successfully captured image should meet all the requirements specified in the assay addendum. For example, a successfully captured image for pNBS1 biomarker from the DDR9 assay must meet the following requirements specified in the assay addendum: 1) pNBS1 (Cy5) channel exposure time must be between 25-50 milliseconds (ms); 2) LED lamp intensity must be 50%; 3) the mean intensity for pNBS1 (Cy5) channel across 10-20 negative nuclei per tissue per slide (40-80 nuclei for a patient specimen with 4 tissues on one slide) should be below 300 intensity units (IU or pixels). Additional details for evaluating marker exposure time within the exposure range, minimum signal to background ratio and previously established target background intensity values (biomarker negative tumor nuclei) can be found in the assay addendum.

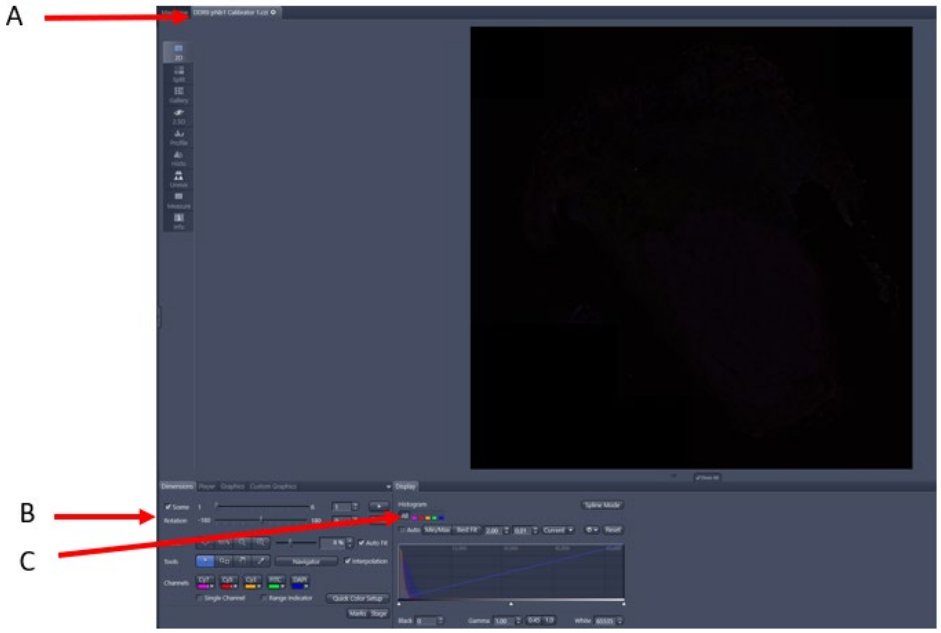
7.10 Double check that the correct storage location has been selected as shown by arrow (A) below and that the correct slides are checked as shown by arrow (B) below. Then click "**Start Scan**" as shown by arrow (C) below.

NOTE: Do not save the script or overwrite the default assay specific scan profile.



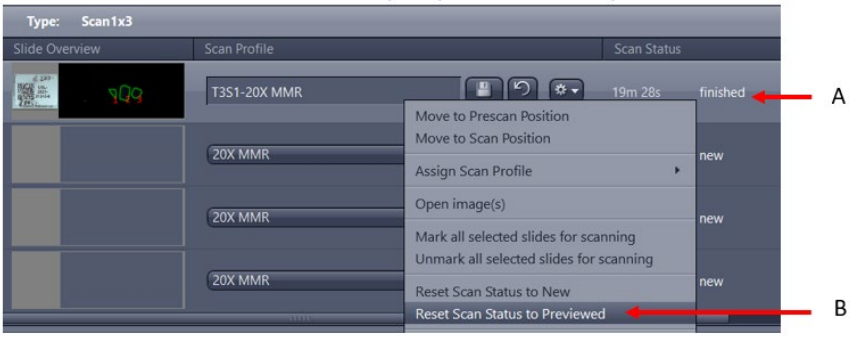
7.10.1 When the scan status shows the scan is complete as shown by arrow (A) below and no other slides are being actively acquired, double click on the slide image to open it.

7.10.2 In the new window that opens as shown by arrow (A) below, uncheck the "**Scene**" box as shown by arrow (B) below to view the entire slide. Click on each channel as shown by arrow (C) below to independently adjust the histogram for each channel.

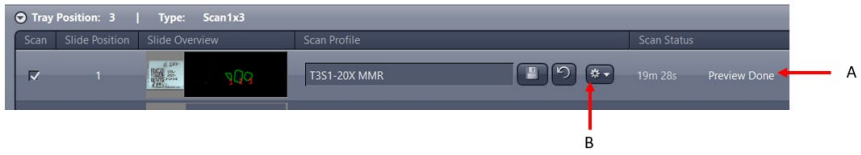


7.10.3 If there is a focus or exposure problem, reset the acquisition, adjust the settings (by moving focus points and/or altering ROIs), and reacquire the image.

7.10.3.1 To reset the acquisition, right click near "finished" scan status as shown by arrow (A) below, then select "Reset Scan Status to Previewed" as shown by arrow (B) below.

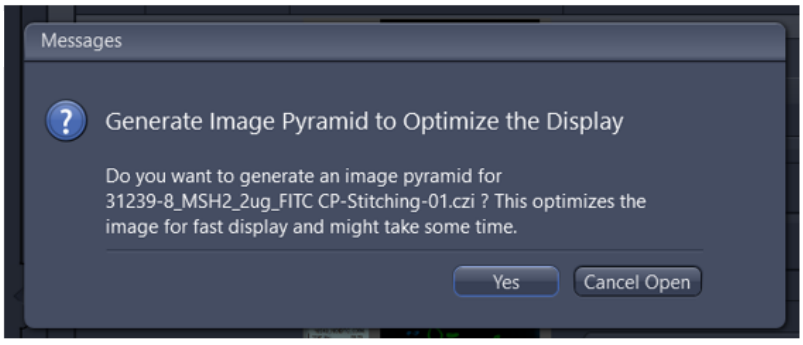


7.10.3.2 When the scan status is set to "Preview Done" as shown by arrow (A) below, click the gear icon as shown by arrow (B) below to go to the "Adapt Selected Profile" to adjust the setting for focus, exposure, then repeat the scan.

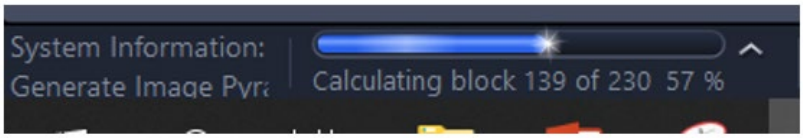


7.10.4 When opening the saved image from its storage location, a prompt to generate an image pyramid will show up. Click "**Yes**" in the popup window as shown in (A) below and save the image after the image pyramid is created as shown in (B) below.

A



B



7.11 Review and finalize the Batch Record and document **ANY** and **ALL** deviations from this SOP in the Batch Record (Appendix1 Section 2).

7.12 The Laboratory Director/Supervisor should review and sign/date the Batch Record affirming the data contained within the reports are correct (Appendix1 Section 3).

APPENDIX 1: BATCH RECORD

Protocol #	Specimen ID	Assay Name	Slide #

1. Image Capture Record

Facility/Laboratory Name: _____

Assay Operator: _____

Date of Slide Staining: _____

Date of Image Capture: _____

S/N or ID for Axioscan: _____

Name of Image Server: _____

2. Notes, including any deviations from the SOP:

3. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (PRINT)

_____ (SIGN)

Date: _____

4. Exposure Time Evaluation Record

Specimen ID	Channel Name	Biomarker Name	LED Intensity	Exposure Time	Average Positive Nuclei Intensity	Background Intensity	Signal to Background Ratio

Specimen ID	Channel Name	Biomarker Name	LED Intensity	Exposure Time	Average Positive Nuclei Intensity	Background Intensity	Signal to Background Ratio

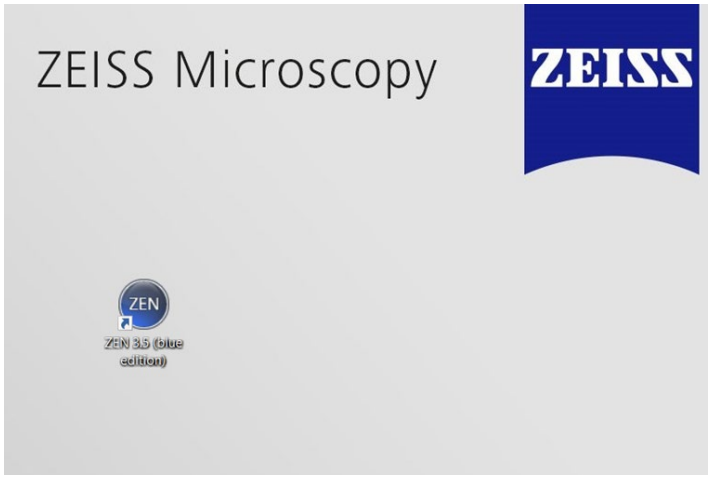
APPENDIX 2: AXIOSCAN START UP PROCEDURE

1. Turn on Axioscan using the on/off switch shown below.

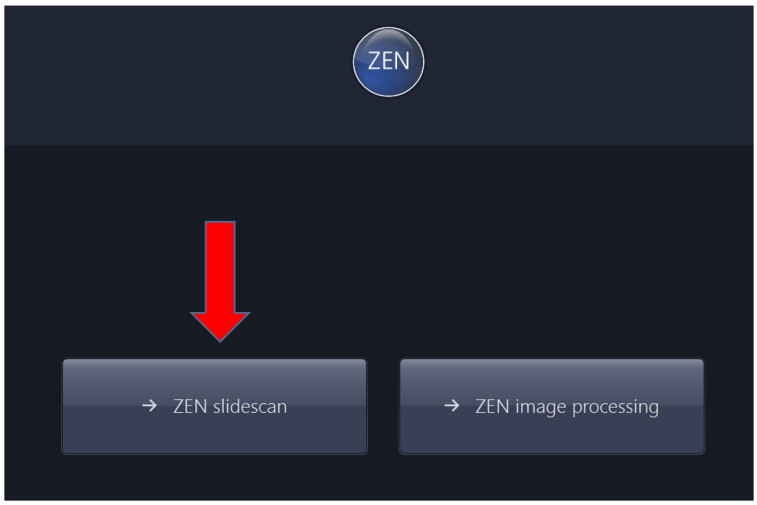
2. Turn on the control computer for the Axioscan.
3. Wait until the light on the Axioscan turns green as shown below before launching the software.

4. Log into the ZEISS control computer (log on screen is shown below).

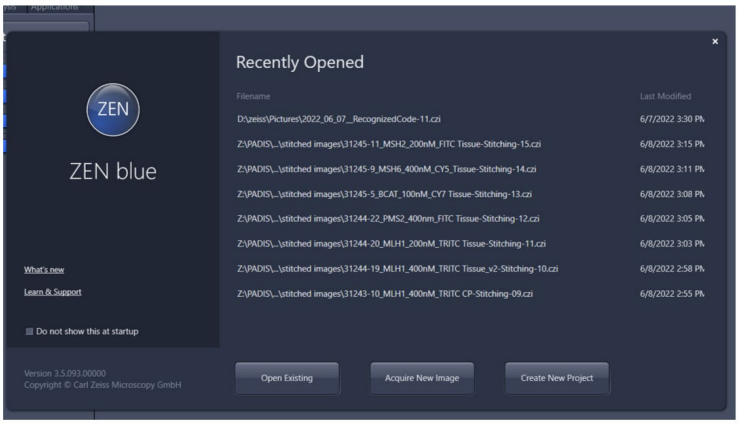
5. Open the ZEN software (software icon is shown below).



6. Select "**ZEN slidescan**" as shown below.

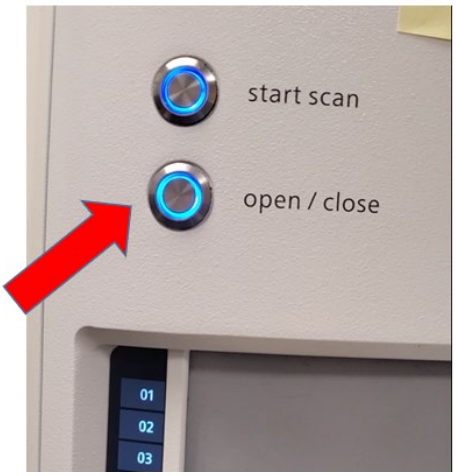


7. Close the "**Recently Opened**" window shown below.



APPENDIX 3: INSTRUCTIONS FOR ADDING SLIDE TO THE AXIOSCAN

1. Open the tray hotel using the "**open/close**" button as shown below.



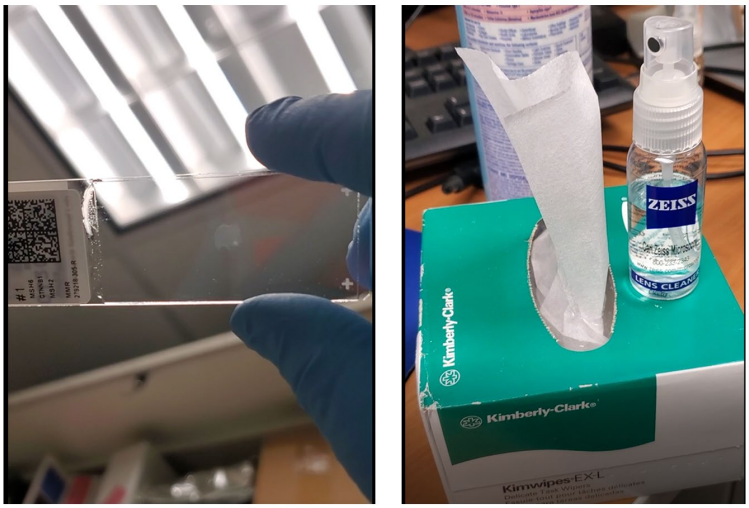
2. There are 25 trays in the tray hotel. Each tray holds 4 slides.
3. Add slide holder into slide holder tool and make sure the spins on tool fit into slots on the slide holder as shown by the red arrow below.

4. Push forward handle on the slide of the tool as shown in (A) below. This will open the individual slide locations as shown in (B) below

A B

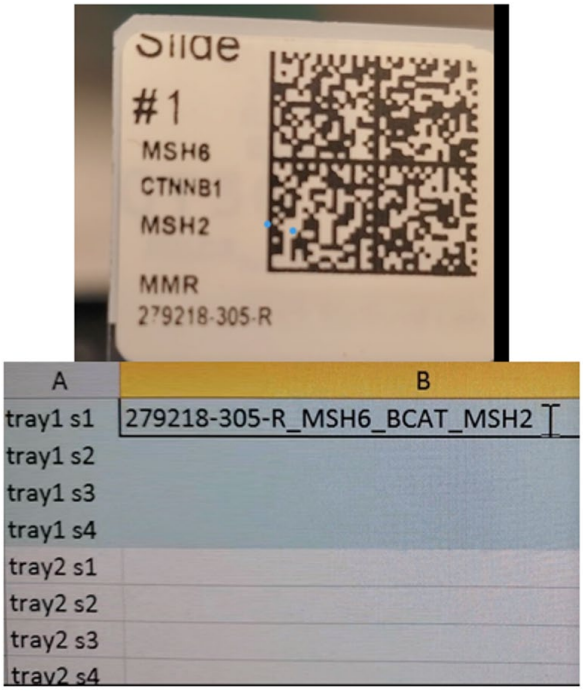
5. Hold slides up to light and make sure they are free of dust, debris and mounting media as shown in (A) below. If needed, the slides can be cleaned using Kimwipes and ZEISS cleaner as shown in (B) below.

A B



6. Add slides to the slide holder. Make sure the slide is inserted label slide facing up and seated flatly into the slot and is pushed to the back as shown below.

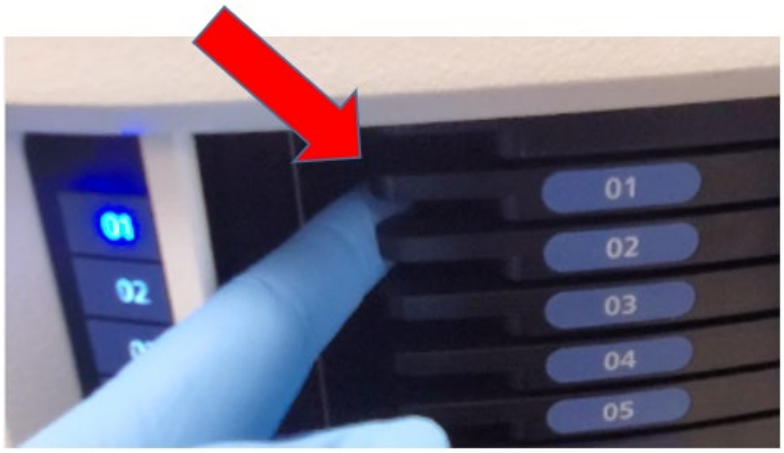
7. Record slides and corresponding locations in the Excel Axioscan Slide Tray Template as slides are being added to the slide holders. An example is shown below.



8. Pull the level back on the slide holder tool to release slides holder as shown below.

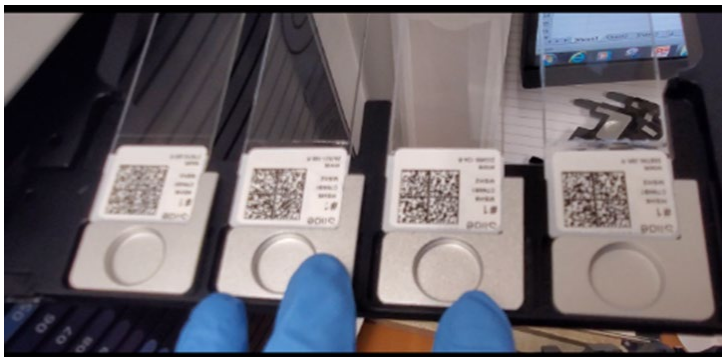
9. Pull the left corner of the tray out to open a slide tray as shown in (A) below. Image of an open tray is shown in (B) below.

A



B

10. Add slides to the tray holder and make sure they are flat and pushed back as shown below.



11. Push the corner of the tray holder to close tray as shown below.

12. Close the Axioscan door by pressing the "**open/close**" button as shown below.