NIH NATIONAL CANCER INSTITUTE

LHTP003.07.26: Whole Tissue Analysis of Tumor Biopsy Slides from γH2AX, pNBS1, pKAP1 IFA with β-Catenin Segmentation using HALO® Image Analysis Software

Effective Date: 03/18/2025

Please check for revision status of the SOP at

http://dctd.cancer.gov/drug-discovery-development/assays/validated-biomarker-assays.htm

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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 08:41:48 -04'00'

PADIS Approval: Deborah Wilsker Date: DEBORAH F. WILSKER -S (Affiliate) Digitally signed by DEBORAH F. WILSKER -S (Affiliate) Date: 2025.05.13 10:00:25 -04'00'

NCLN Approval: Li Li Date: LI LI -S (Affiliate) Digitally signed by LI LI -S (Affiliate) Date: 2025.05.13 10:36:21 -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 12:23:57 -04'00'

DCTD OD Approval: Toby Hecht Date: Toby T. Hecht -S Digitally signed by Toby T. Hecht -S Date: 2025.05.13 12:51:16 -04'00'

2. Change History

Revision	Approval Date	Description	Originator	Approval
--	03/18/2025	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

↓

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:

Whole Slide Image Capture of IFA Tumor Biopsy Slides using ZEISS AxioScan 7 Microscope Slide Scanner

AND

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

↓

LHTP003.07.26: Whole Tissue Analysis of Tumor Biopsy Slides from γH2AX, pNBS1 IFA with β-Catenin Segmentation using HALO® Image Analysis Software

- 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 tissue analysis of multiplex immunofluorescence Zeiss Axioscan-acquired whole slide images (WSI) of formalin-fixed paraffin-embedded (FFPE) tissue biopsy sections stained to detect and quantify histone H2AX phosphorylated at serine 139 (ɣH2AX), NBS1 phosphorylated at serine 343 (pNBS1) and KAP1 phosphorylated at serine 824 (pKAP1) using β-Catenin tumor segmentation for pharmacodynamic (PD) evaluations of DNA damage repair status. The goal of the SOP and associated training is to ensure consistency of biomarker measurements between operators and clinical sites.

This document details the procedure for image analysis using HALO® software including Artificial Intelligence (AI) Classifier use for detection of tumor areas, AI nuclear detection and segmentation of DAPI stained nuclei, and biomarker analysis to accurately detect and quantitate pKAP1, pNBS1 and ɣH2AX.

2.0 SCOPE

This procedure applies to all personnel involved in the use of the ɣH2AX, pNBS1, pKAP1 IFA with β-Catenin Segmentation for Tumor Biopsy Slides from patients participating in clinical trials. This SOP outlines the recommended procedure for image analysis from whole slide images of stained, paraffin-embedded tumor biopsy sections. This SOP outlines the recommended procedure for developing and executing all aspects of image analysis and data reporting.

3.0 ABBREVIATIONS

Ab	=	Antibody
AI	=	Artificial Intelligence
DAPI	=	4',6-Diamidino-2-Phenylindole
DCTD	=	Division of Cancer Treatment and Diagnosis
DDR9	=	ɣH2AX, pNBS1, pKAP1 IFA with β-Catenin Segmentation
FFPE	=	Formalin-fixed paraffin-embedded tissue
γH2AX	=	Histone H2AX Phosphorylated at Serine 139
H&E	=	Hematoxylin and Eosin
IFA	=	Immunofluorescence Assay
LHTP	=	Laboratory of Human Toxicology & Pharmacology
NCLN	=	National Clinical Laboratory Network
NucSeg	=	Nuclear Segmentation
PADIS	=	Pharmacodynamic Assay Development and Implementation Section
PD	=	Pharmacodynamics
pNBS1	=	NBS1 phosphorylated at serine 343
pKAP1	=	KAP1 phosphorylated at serine 824
QC	=	Quality Control
ROI	=	Region of Interest
SOP	=	Standard Operating Procedure
VM	=	Virtual Machine
WSI	=	Whole Slide Image
OARS	=	Oncology Automated Reporting System

4.0 INTRODUCTION

The DDR9 (γH2AX, pNBS1, pKAP1) IFA with β-Catenin segmentation is a multiplexed immunofluorescence assay developed to quantify these biomarkers within tumor tissue in support of pharmacodynamic studies. β-Catenin staining is analyzed with an AI classifier to provide tumor area masking which, together with pathologist annotation, defines the tumor areas within which the biomarkers are quantitated. Additionally, DAPI staining is used to identify and segment tumor 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 analyze clinical samples.

5.2 The Assay Operator responsible for conducting the assay is to follow this SOP and complete the required tasks and associated documentation. The Batch Record (Appendix 1) must be completed in real-time for each experimental run.

5.3 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 HALO® Image Analysis Software v3.4 or higher, Indica Labs

6.2 Microsoft Excel software

6.3 Zeiss Axioscan whole slide images of DDR9 stained clinical and control slides imaged according to SOP LHTP003.07.25 and LHTP003.07.25-ADM-01

6.4 Microsoft Excel Master Templates for DDR9 Clinical Data Report

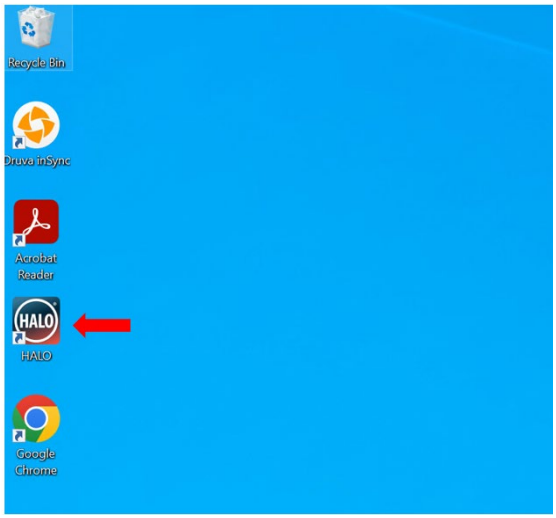
7.0 OPERATING PROCEDURES

NOTE: HALO User Guides can be accessed following instructions in Appendix 3.

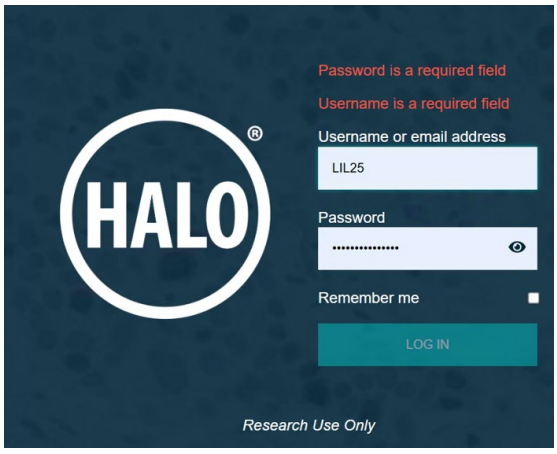
7.1 Preparing for Image Analysis

7.1.1 Log into a virtual machine (VM) following instructions in Appendix 2 or a HALO workstation. From the HALO VM or workstation desktop, open the software as shown in (A) below. Upon opening, enter your institutional credentials (username not email address) in the pop-up HALO-link login window to log into HALO as shown in (B) below.

A



B



7.1.2 Study Folder Creation in HALO_Image share

7.1.2.1 If this is the first time using the HALO software, map the Halo_Images share to the HALO VM or workstation.

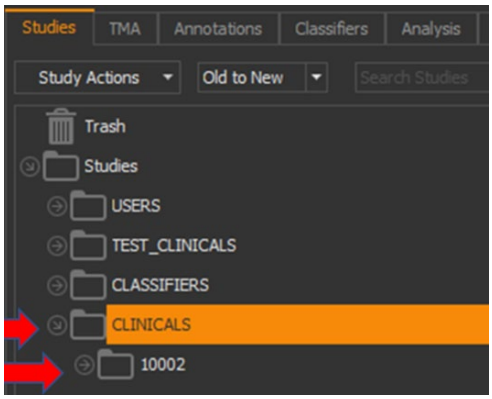
- HALO Share for PADIS: \\nih.gov\NCIF\adrd\dctd\padis\Halo_Images
- HALO Share for NCLN PD IFA laboratory: \\mdanderson.edu\mdadata\DPLM

7.1.2.2 All images to be analyzed should be stored in these Halo_Images share or comparable location within your institution.

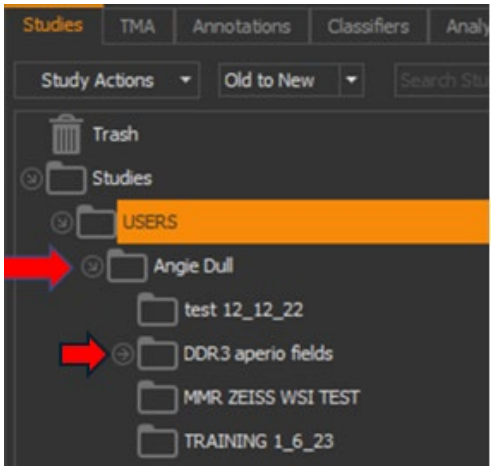
7.1.2.3 Clinical images should be stored in a designated patient study folder within the "CLINICALS" folder in the Halo_Images share, e.g., Halo_Images > CLINICALS > specific trial folder > DDR9. The patient study folder should include the Patient ID (e.g., Halo_Images\CLINICALS\10002\DDR9\10002_NCIDTC_0029).

7.1.2.4 HALO stores all associated images, files and settings in a project's study folder. If this is the first time specimens from a clinical or preclinical study will be analyzed, a new study folder should be created to organize that study's files. In PADIS, clinical specimens should be organized by trial folders under the "CLINICALS" folder as shown in (A) below. Preclinical analysis should be organized by study under individual user folders as shown in (B) below. View subfolders by clicking on arrow next to the study folder as shown in (A) and (B) below.

A

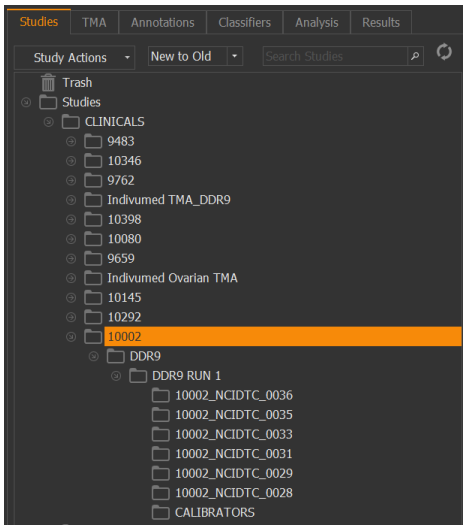


B



7.1.2.5 To create a new study folder for a patient's specimens, go to "**Studies**" tab and open the "CLINICALS" folder > corresponding clinical protocol folder > corresponding assay folder as shown below. Under the assay sub-folder, create a new folder for each patient's specimens, by selecting " **Study Actions**" > " **New Study**" or right clicking on the assay folder and selecting "**New…**" from the dropdown list. In the

pop-up window, type in the patient ID and click "**OK**". The final suggested clinical study structure should be organized in HALO as shown below.



7.1.2.6 To modify a study's properties, from the "**Studies**" pane, select the study to modify. To rename a study, select "**Study Actions**" > "**Rename…**" or right click the study > "**Rename…**". In the pop-up window, type a new study name. Click "**OK**".

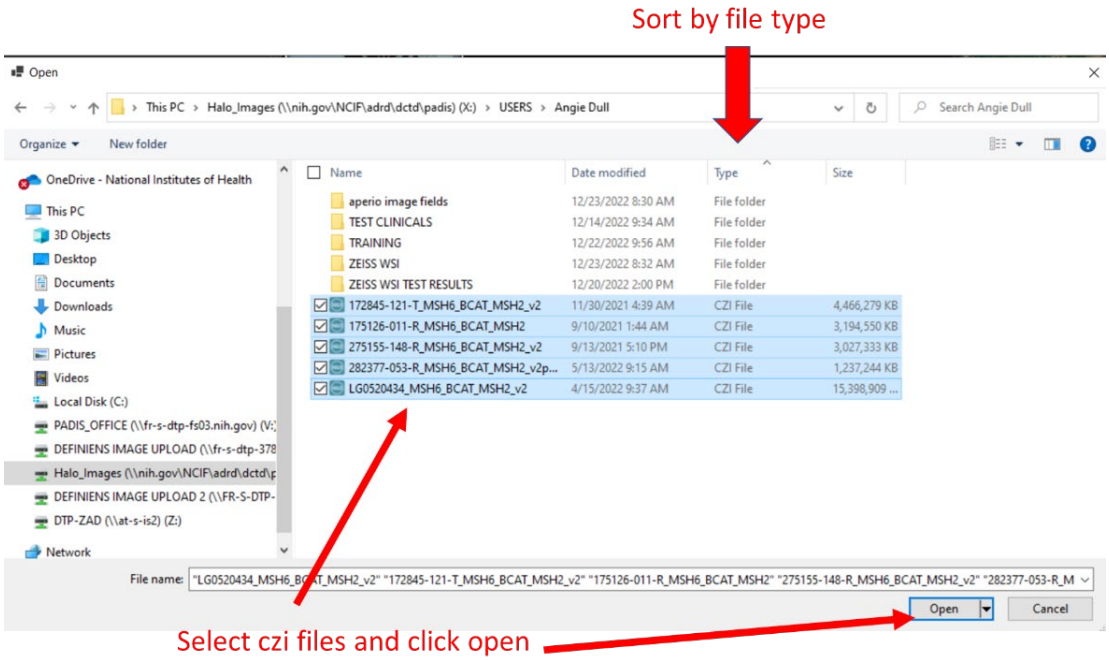
7.1.2.7 To move a study under a parent folder, select "**Study Actions**"> "**Move…**" or right click the study > "**Move…**". In the pop-up window, type a new folder name. Click "**OK**".

7.1.2.8 To delete a study under a parent folder, select "**Study Actions**" > "**Delete…**" or right click the study > "**Delete …**". Confirm by clicking "**OK**".

7.1.3 Importing Images Into HALO

7.1.3.1 All images to be analyzed should be stored in the HALO_Image share according to Step 7.1.2. HALO utilizes this established file path during the image import process for image analysis data, so in the HALO_Image share be sure not to change the name or location of images that have been imported into HALO.

7.1.3.2 To add images to the study folder in HALO, select "**File**" > "**Open Images**". In the pop-up window, sort the files for ".czi" images and select the czi images to open as shown below. Alternatively, highlight the images in File Explorer and drag them to the study folder. In the pop-up window, select the study folder to assign the image files to and click "**OK**".



7.1.3.3 To copy images into another folder in HALO, copies of the images should be made and renamed in the new folder of the Halo_Images share.

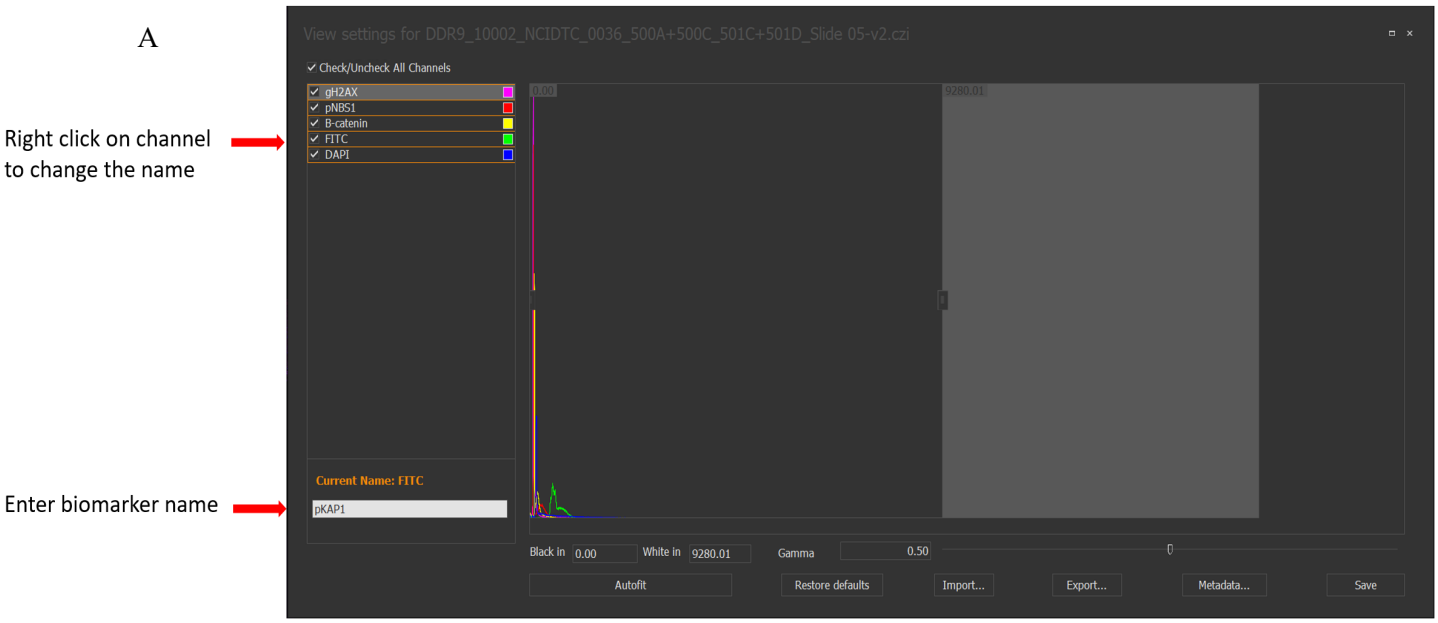
7.1.3.4 To remove images from a study in HALO, right-click on the image or select "**Move to Trash…**". This will not delete the image from the Halo_Images share but will only remove it from HALO study structure.

7.2 Image View Settings

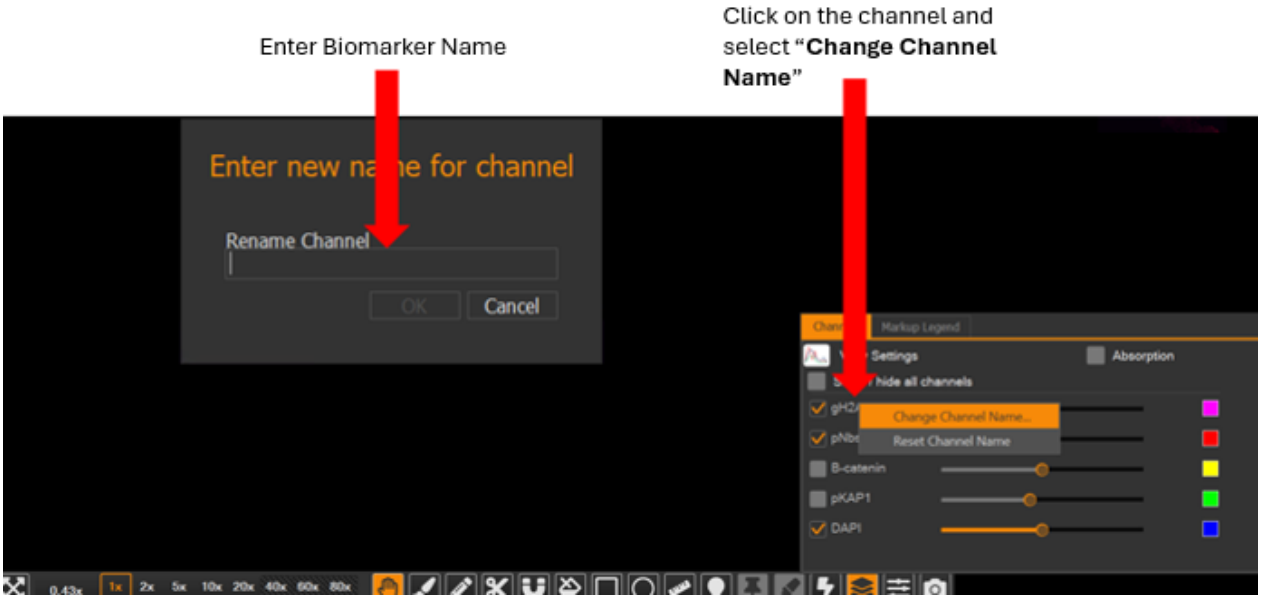
7.2.1 Open the image of interest by double clicking the image icon in the study folder in HALO. To open multiple images, hold down the control key to select the images and right click > select "**Open**". From the navigation menu**,** select **"View" > "View Settings…".**

7.2.2 Channel names should be edited to the DDR9 marker names in the view setting by right clicking on the channel names and entering DDR9 marker names in the window that appears at the bottom left of the screen and press "**Enter**" as shown in (A) below. Specifically, "FITC" should be edited to "pKAP1", "Cy5" to "pNBS1", "Cy7" to "gH2AX", and "Cy3" channel to "B-catenin". Alternatively, image channel names can be changed by right clicking the channel names in the "**Channels**" tab at the bottom right of the screen as shown in (B) below and select "**Change Channel Name…**" from the dropdown list and enter the channel name in the "**Enter new name for channel**" window.

A



B

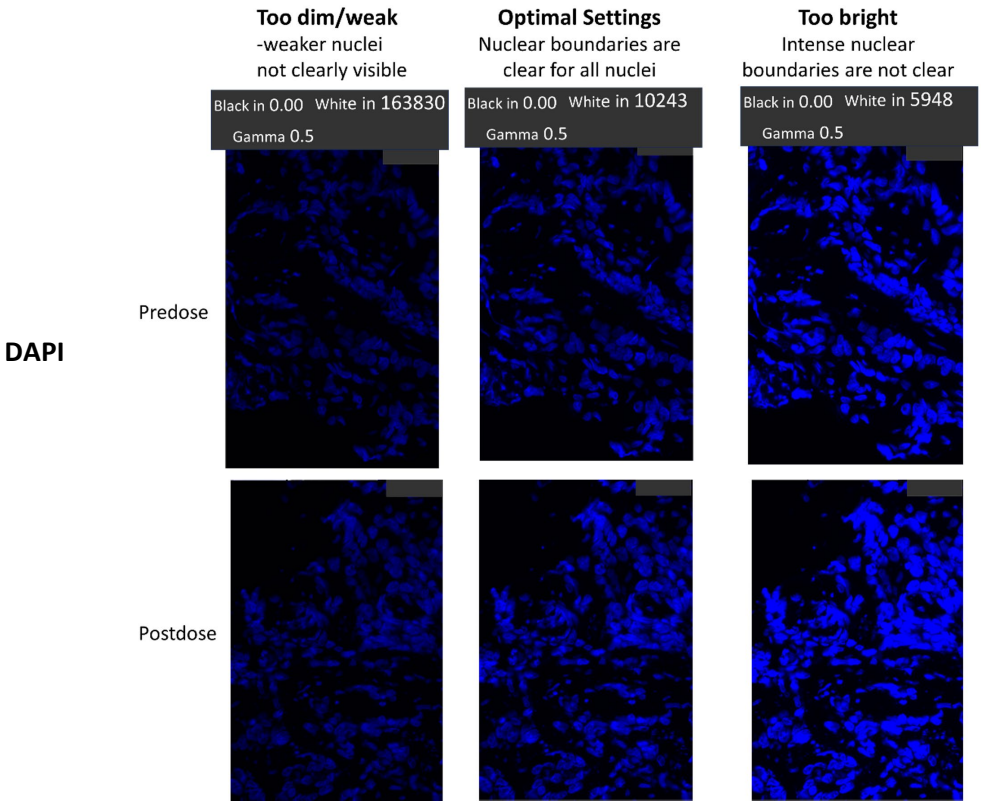


7.2.3 The color-coded histogram graph in the "View Settings" window allows for changing "**Black In**", "**White In**" and "**Gamma**" as shown in (A) to (C) below. "**Black in**" and "**White in**" adjusts the intensity range (view only). "**Black in**" adjusts the low end of the intensity range, and the "**White in**" adjusts the high end of the intensity range. Adjusting the "**White in**" allows the image to appear brighter, while adjusting the "Black in" will remove some signal on the low end where there may be background signal. The "**Restore Default**" button will revert to default image settings. View the

histogram for each marker independently to optimize view settings for each channel by only checking the box next to only one channel at a time as shown below. Evaluate pre-dose and post-dose specimens on each slide and across a patient's slide set before finalizing a view setting. Ensure that tumor-positive areas are being viewed while optimizing the view settings.

7.2.4 For the DAPI channel adjustment, adjust "**Black in**" and "**White in**" until nuclei are clearly visible with clear nuclear boundaries as shown in the "Optimal Settings" examples below.

7.2.4.1 "**Gamma**" should not be adjusted for the DAPI channel.

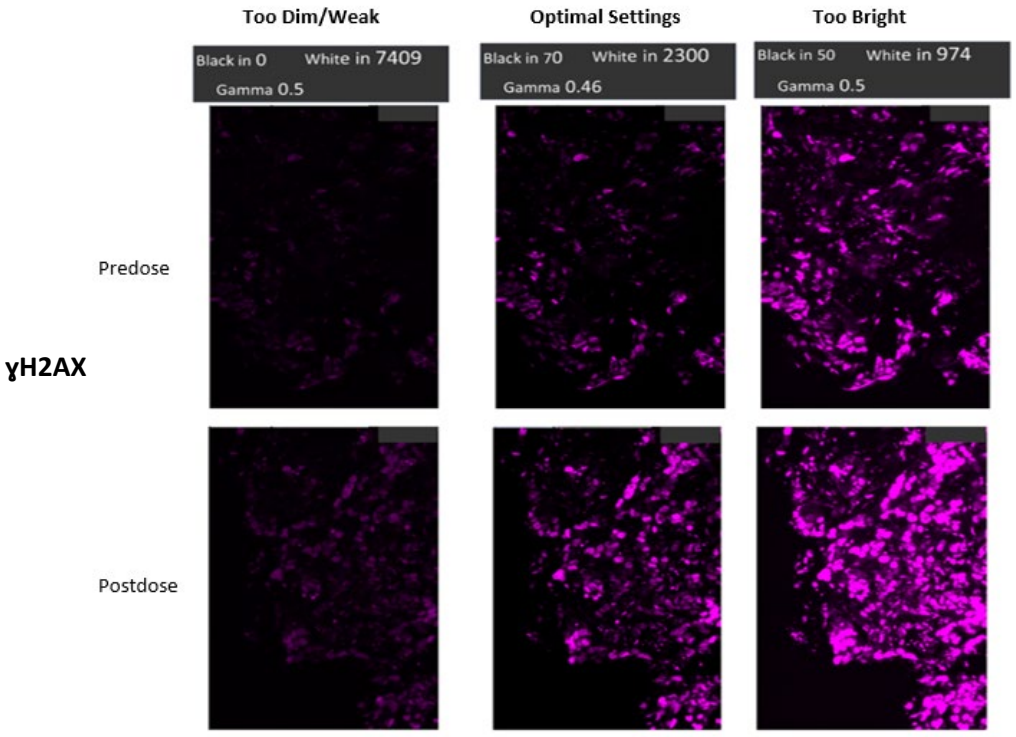


7.2.5 For each biomarker channel adjustment, adjust "**Black in**", "**White in**" and "**Gamma**" until both the weak and bright signals are visible and distinguishable from the background as shown in the "Optimal Settings" examples for ɣH2AX (A), pNBS1(B) and pKAP1 (C) shown below. If the signals are too dim/weak as shown below in the "Too Dim/Weak" examples for ɣH2AX (A), pNBS1(B) and pKAP1 (C), these signals will be too weak to be accurately distinguished from the background. If the signals appear too bright, the positive areas will either have a swollen appearance and expand outside of the nuclear boundaries as shown in the "Too Bright" examples for ɣH2AX (A) and pNBS1(B) below, or the background will be too high to distinguish the weak signal from the background as shown in the "Too Bright" examples for pKAP1 (C) below.

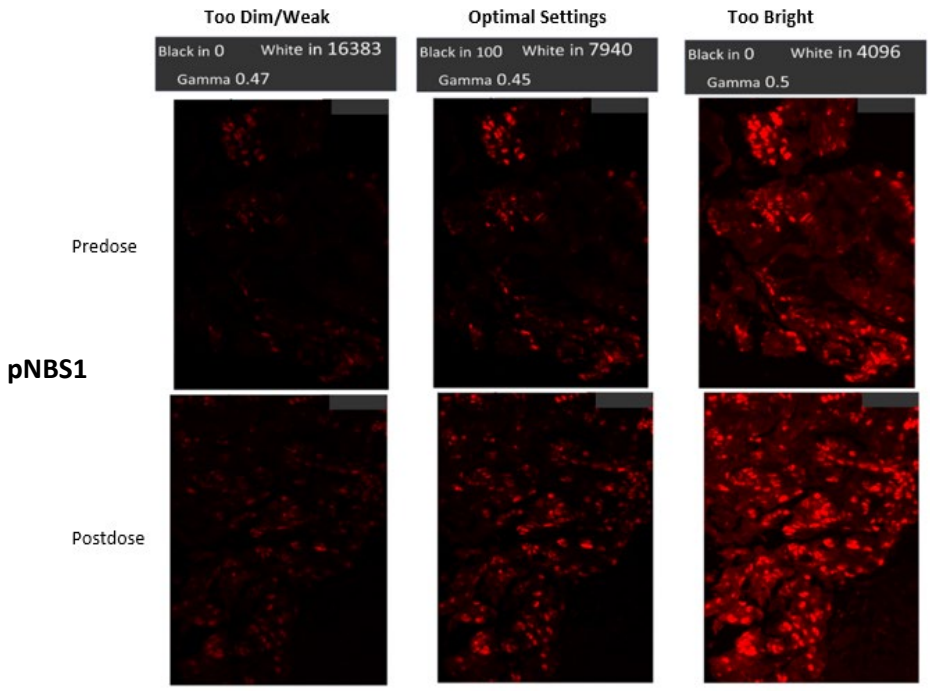
7.2.5.1 Use caution when adjusting the "**Black in**" and "**Gamma**" parameters to minimize background but not lose visible weak signal.

7.2.5.2 Some visible background is acceptable.

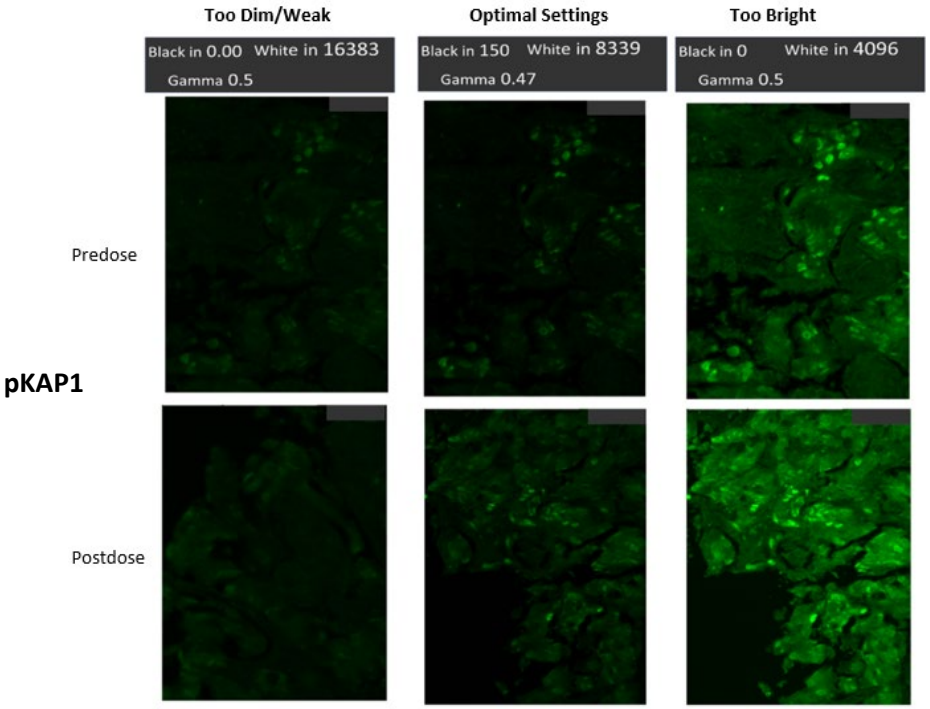
A


Too Dim/Weak
Optimal Settings
Too Bright
Black in 0 White in 7409
Gamma 0.5
Black in 70 White in 2300
Gamma 0.46
Black in 50 White in 974
Gamma 0.5
Predose
γH2AX
Postdose

B


Too Dim/Weak
Black in 0 White in 16383
Gamma 0.47
Optimal Settings
Black in 100 White in 7940
Gamma 0.45
Too Bright
Black in 0 White in 4096
Gamma 0.5
Predose
pNBS1
Postdose

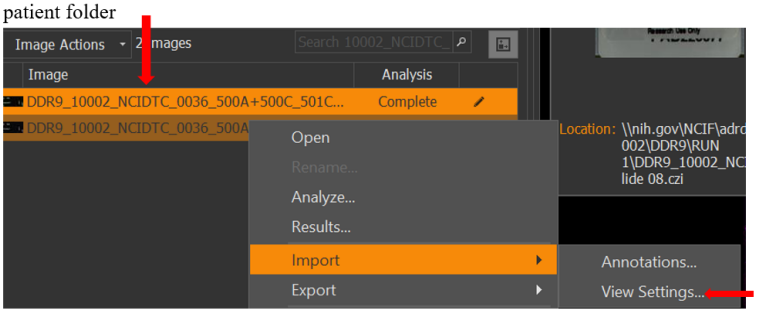
C


Too Dim/Weak
Black in 0.00 White in 16383
Gamma 0.5
Optimal Settings
Black in 150 White in 8339
Gamma 0.47
Too Bright
Black in 0 White in 4096
Gamma 0.5
Predose
pKAP1
Postdose

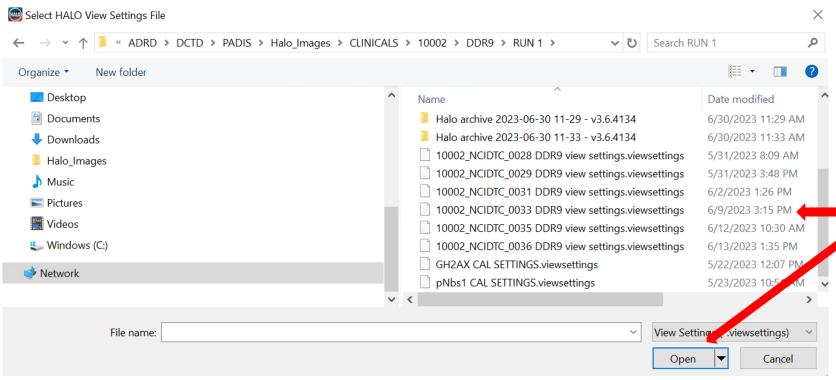
7.2.6 After the channel name changes and histogram adjustments, click "**Save**" before closing the "**View Settings**" window as shown below. Export the view settings by clicking the "**Export…**" button. From the pop-up window, name the view settings file as "Patient ID_DDR9 view settings" and save the view settings file in a patient's image folder in HALO_Image share drive.

7.2.7 The same view settings should be applied to all images of a patient's specimen set. Follow these steps to import saved view settings to multiple slides of the same patient's specimens or preclinical specimens from the same study as shown below, (A) select the images of interest in the images pane in the "**Studies**" tab of HALO, (B) right-click on the images, select "**Import**" > "**View Settings…**" from the dropdown list, (C) from the pop-up window, select the desired view settings file and click "**Open**". The selected view settings will be applied on all selected images.

A. Select all images within a clinical patient folder



B. Right click on selected images and select "**Import**"> "**View Settings…**".



7.3 Image Annotations

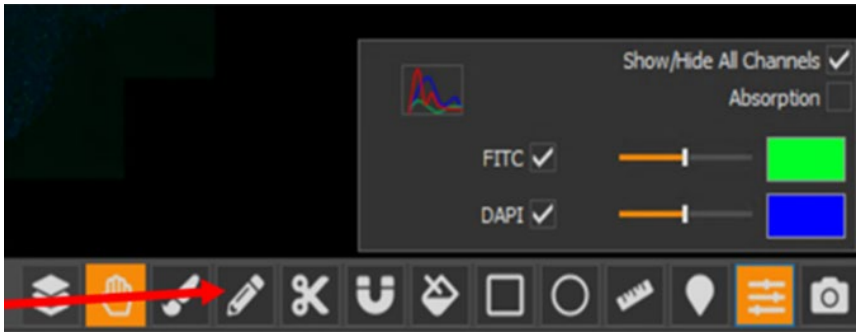
7.3.1 Annotations are used to define the region of interest (ROI) by including the area for analysis and excluding artifacts or invalid tissue regions in the analysis. Limiting the area of analysis reduces the analysis time required and improves accuracy of the biomarker results.

7.3.2 Creating Image Annotations in HALO

7.3.2.1 Create a new annotation layer for each specimen to be annotated and name the layer. To do this, open the image to be annotated. Click on the "**Annotations**" tab, create an annotation layer by selecting "**Layer Actions**" > "**New Layer**". Select "**Layer Actions**" > " **Rename Layer**". In the "**Rename Annotation Layer**" pop-up window, name the layers as specified for the DDR9 control and clinical slides in Section 7.3.3.

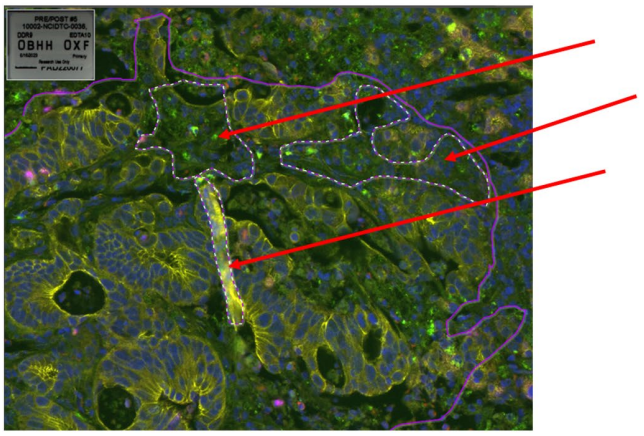
- **To modify annotation layer properties**, select the layer of interest from the drop-down menu next to the "**Layer Actions**" button.
- **To toggle layer visibility**, select "**Layer Actions**" > "**Visible**". A check mark next to the option will update to reflect the layer's visibility status.
- **To rename a layer**, select "**Layer Actions**" > "**Rename…**". Enter a layer name in the pop-up window. Click "**OK**".
- **To change a layer's color**, select "**Layer Actions**" > "**Color…**". Select the desired color or click on "**More Colors…**" to define your own color.
- **To delete a layer**, select "**Layer Actions**" > "**Delete Layer**". Confirm by clicking '**OK**' in the pop-up window.
- **To edit layer name**, click on "Layer Actions" > "Rename…"

7.3.2.2 To draw new annotations, select the layer of interest from the drop-down menu next to the "**Layer Actions**" button. To draw a new annotation, select one of the annotation tools at the bottom right of the screen as shown below.



NOTE:

- For "**Pen**" tool, click and hold the left mouse button to draw the annotation and stop holding down the mouse button when finish.
- For "**Flood Fill**" tool, click and hold the left mouse button to draw the annotation and stop holding down the mouse button when finish.
- For "**Magnetic Pen**" tool, click and hold the left mouse button to draw the annotation and click the space bar to stop drawing.
- To exclude areas from analysis, hold the "**Ctrl**" key while drawing. This will create a dotted line expressing exclusion/negative annotations as shown below.



Exclusion annotations are dotted lines.

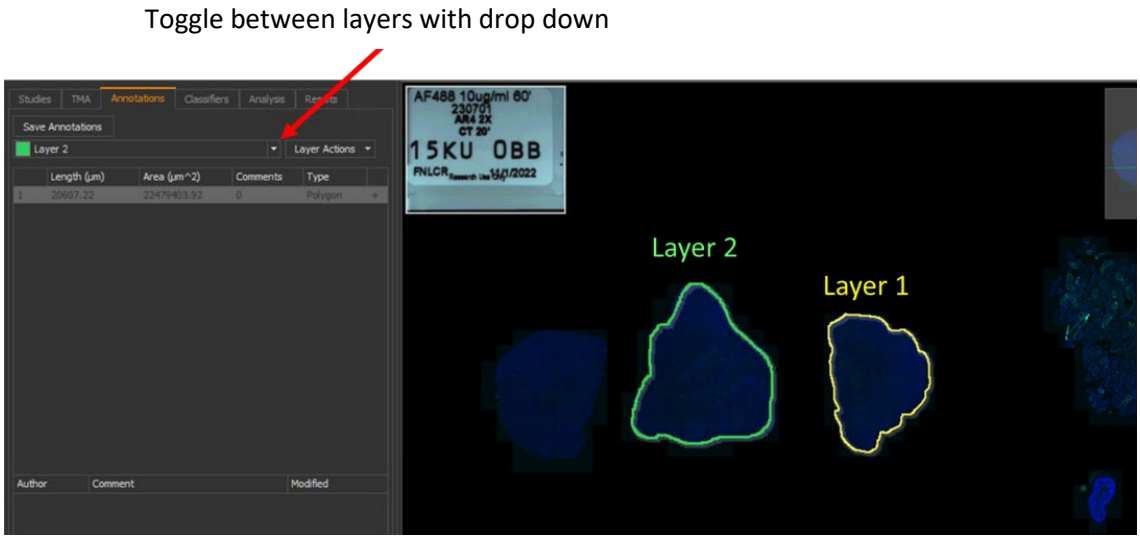
7.3.2.3 To modify annotations**,** select the "**Pan**" tool from the annotations toolbar. Right-click on an annotation on the image to access the annotations dropdown menu and select the desired operation. Most annotation tools (pen, scissor, and brush tools) will let you adjust them by clicking and drawing on the annotation layer with the tool. Annotation layers can also be copied and pasted within the same or different image.

7.3.2.4 **Exporting annotations** allows you to export and then import the same set of annotations into another image and allows you to share the annotation files on a shared drive. Select "**Layer Actions**" > "**Export**", this will export every annotation layer for this image to the annotation file. From the pop-up window, name the annotation file as the image name and save file in the according study folder in HALO image share.

7.3.2.5 **Importing annotations**, select "**Layer Actions**" > "**Import**", or right-click on the desired image in the study field and select "**Import**" > "**Annotations…**". From the pop-up window, select the desired annotation file. Click "**Open**".

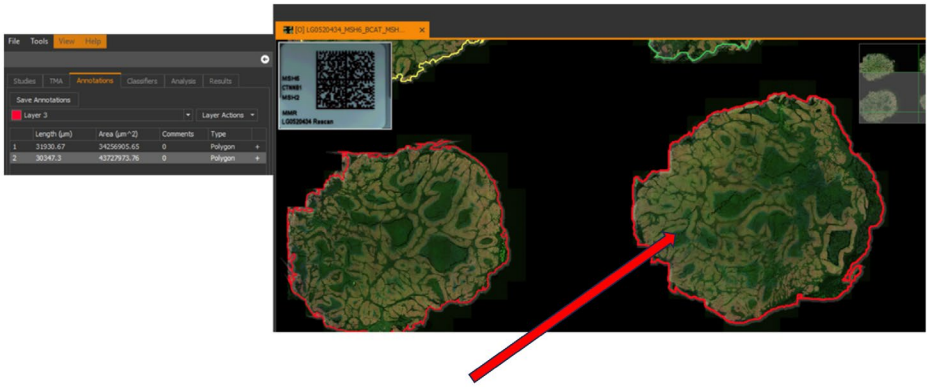
7.3.2.6 When moving to the next annotation, instead of selecting the "**pan**" tool, the user can keep with the annotation tool and right click to navigate to the next tissue on the slide.

7.3.2.7 Use the button next to layer name as shown below to toggle between annotation layers.



7.3.2.8 If the user accidentally draws an annotation on another tissue without creating a new corresponding layer, the new annotation can be moved to a new layer instead of deleting the current layer following the steps: (1) Create a new layer following instructions in Section 7.3.2.1., (2) Navigate back to the layer with tissue as shown by the arrow in (A) below, (3) select the "**Pan**" tools, right click on the new annotation and select "**Send to Layer**" as shown in (B) below, (4) select the layer where the annotation needs to be moved to in the pop-up window and click "**Confirm**", click "**Yes**" in the next pop-up window to confirm moving of the annotation to a new layer as shown in (C) below. The annotation will show up in a new color as shown in (D) below.

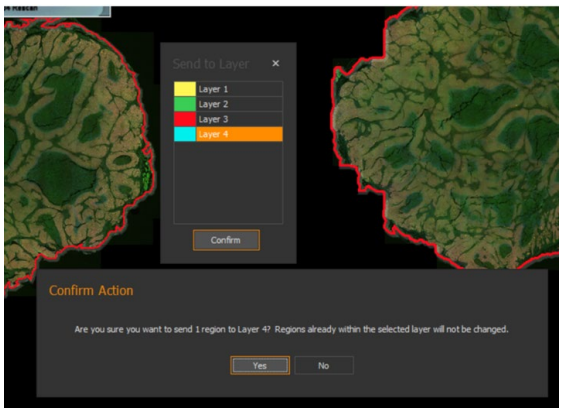
A



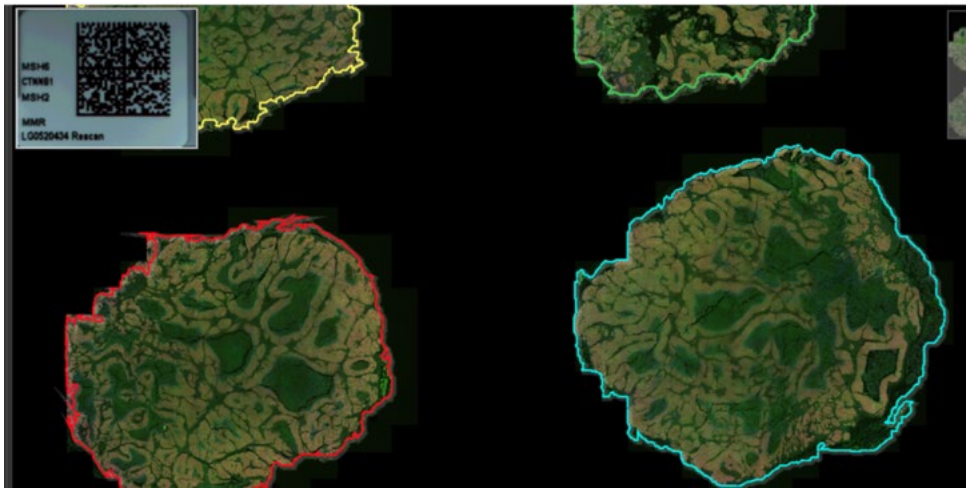
B


Copy Ctrl+C
Paste Ctrl+V
Paste XY Ctrl+D
Move Ctrl+M
Rotate
Grow Ctrl++
Shrink Ctrl+-
Invert Ctrl+I
Send to Layer
Crop
Advanced
Delete Del
Send to Layer
Send selected regions to a different layer

C


Send to Layer
Layer 1
Layer 2
Layer 3
Layer 4
Confirm
Confirm Action
Are you sure you want to send 1 region to Layer 4? Regions already within the selected layer will not be changed.
Yes
No

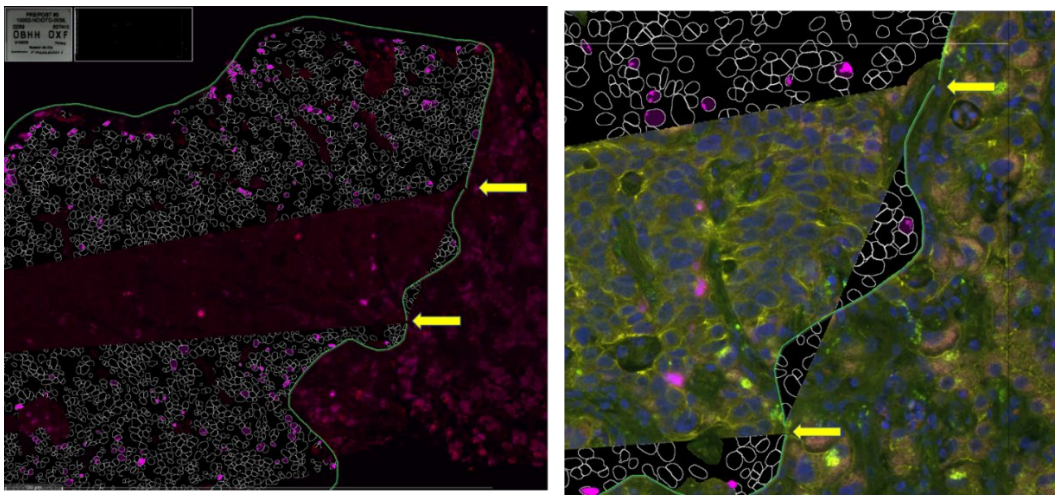
D



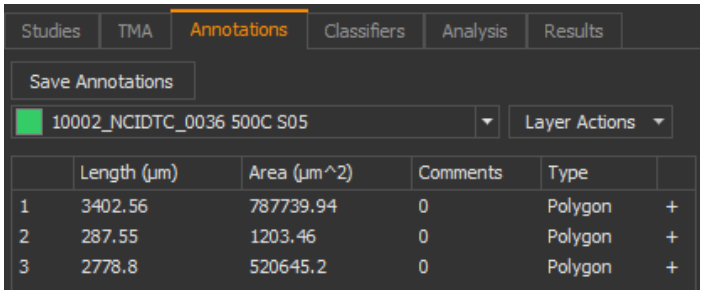
7.3.2.9 When HALO is slow, there might be breaks in the annotations, which will affect the analysis. When this happens, breaks in the annotations can be visualized upon zooming in as shown in (A) below and multiple annotations are visible instead of one annotation under the "**Annotations**" tab as shown in (B) below. The annotation can be edited by deleting the smaller annotations in the "**Annotations**" tab as shown in (C) below and redrawing one annotation. To redraw the annotation, select the annotation and the "**pen**" tool and continue drawing the annotations until only one ROI is visible when finished as shown in (D) below.

- If HALO runs slowly when using a VM, try restarting the VM, or wait for 30+ minutes and try again later.

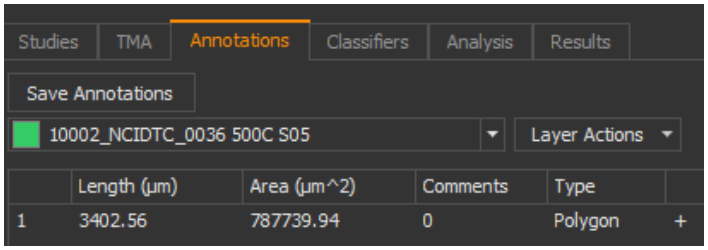
A



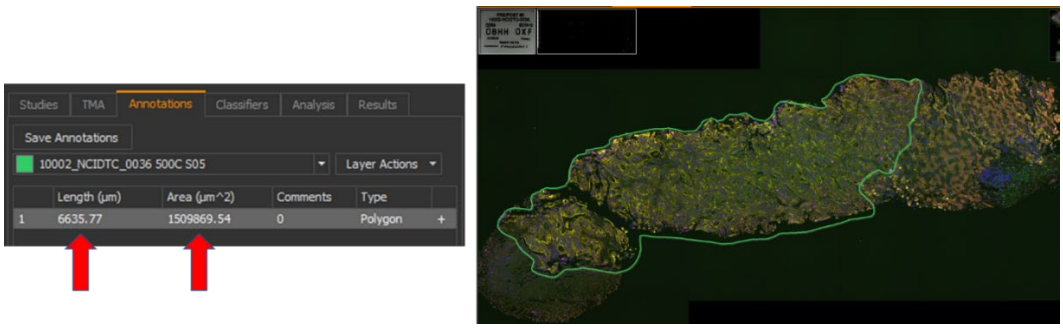
B



C



D



7.3.3 Creating Image Annotations for the DDR9 Assay

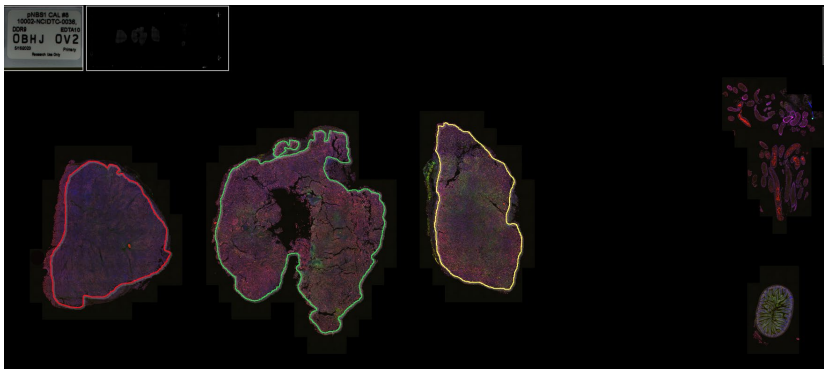
7.3.3.1 For the control slides, create one annotation layer for each control tissue and name each tissue as "Marker *space* Con#" (e.g., GH2AX Con2). For the ɣH2AX control slides, the 3 levels of controls will be named as Con1 (for Control Low), Con2 (for Control Mid) and Con3 (for Control High). For pNBS1/pKAP1 control slides, the 2 levels of controls will be named as Con1 (for Control Low) and Con3 (for Control High).

7.3.3.2 For clinical specimens, create one annotation layer for each biopsy core and name each biopsy core as "Protocol#(5 digits)_Site#(6 digits)_Patient#(4 digits)_Tissue ID(4 digits) space Time Point(5 digits) space Slide#(2 digits) (e.g., 10002_NCIDTC_0036_500C C1D01 05). There may be multiple annotations for one specimen. If the annotation needs to be adjusted for a specific marker to exclude nonspecific signal or tissue edges, the biomarker name should be added as a suffix to the annotation (e.g., "10002_NCIDTC_0036_500C C1D01 05_pNBS1").

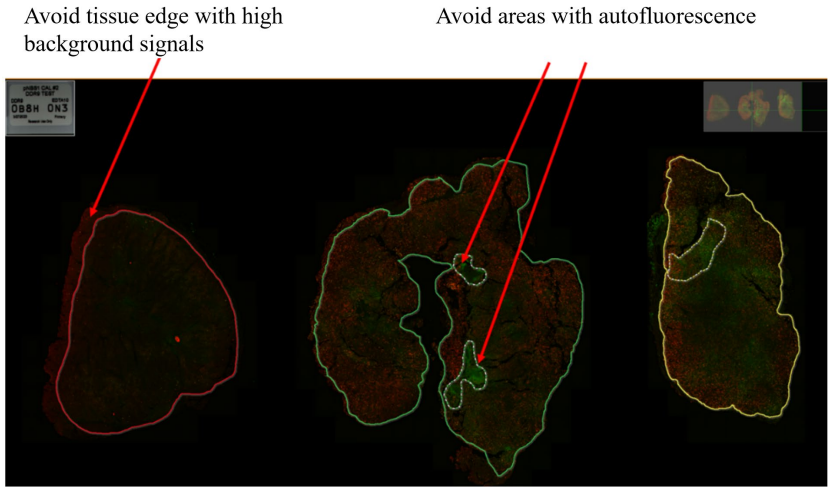
7.3.3.3 For each tissue on the control slides draw an annotation around the tissue in the corresponding annotation layer as shown in the example of an annotated pNBS1 control slide image in (A) below.

7.3.3.4 pNBS1 and pKAP1 control analyses both utilize the pNBS1/pKAP1 control slides (PADIS/IQC Part# 60006). Annotations can either be drawn to accommodate both markers or for pNBS1 and pKAP1 separately, if required. If separate annotations are needed for pKAP1 and pNBS1, add "pKAP1" or "pNBS1" as a suffix to the annotation names for differentiate during analysis and post-analysis result QC. Use exclusion annotations to avoid/exclude areas where there are edge effects, tissue folds, or tissue autofluorescence as shown in (B) below. This is especially important for pKAP1 in the FITC channel and pNBS1 in the Cy5 channel where there may be areas of autofluorescence or high background to be excluded from analysis.

A



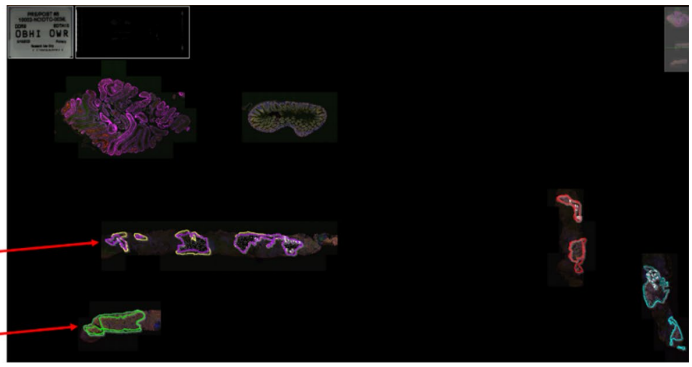
B



7.3.3.5 For clinical specimens, draw the annotation covering the entire analyzable tumor area using the nearest adjacent pathologist-annotated H&E image as a guide. Any unanalyzable areas including areas of abnormal staining, high background, necrosis, or tissue folds should be excluded from the analysis using an exclusion annotation.

7.3.3.6 The users may have multiple annotations for the clinical specimens: one annotation for ɣH2AX and annotations for pNBS1/pKAP1 to avoid areas with high background or autofluorescence. An example of an annotated clinical specimen image is shown below.

Multiple annotations might be needed to exclude areas for pNBS1/pKAP1.



7.3.3.7 Remember to create a new layer before drawing annotation on another tissue following instructions in Section 7.3.2.1.

7.4 General DDR9 Image Analysis Information

7.4.1 The following image analysis procedure details the process to identify the tumor nuclear areas positive for a particular biomarker of interest. This process involves optimizing relevant detection thresholds to accurately assess the true positive nuclear areas.

7.4.2 The analysis algorithm used for the DDR9 IFA in HALO is FISH (Fluorescent In Situ Hybridization). This algorithm was chosen during algorithm development as the nature of the morphology and biomarker staining pattern for the DDR9 nuclear markers were optimally evaluated using this analysis approach. This module incorporates DAPI as a nuclear dye for nuclear detection, and specifies ɣH2AX, pNBS1 or pKAP1 biomarkers as FISH probes. Any current FISH analysis module version can be used for the analysis.

7.4.3 For the purposes of mask visualization during analysis QC, a SINGLE algorithm setting should be used for each marker (ɣH2AX, pNBS1 or pKAP1) following instructions in Section 7.5 below.

7.5 Analyzing the biomarkers

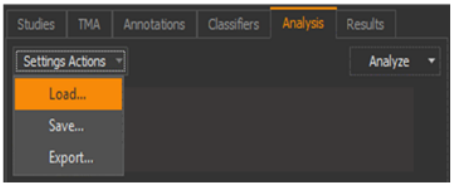
7.5.1 Set up analysis algorithm for each biomarker (ɣH2AX, pNBS1 or pKAP1) separately following Section 7.5.2 to Section 7.5.14.

7.5.2 Open an image, ensure that the biomarkers are named properly in the view settings according to Section 7.2.2 prior to opening a saved Biomarker (ɣH2AX, pNBS1 or pKAP1) Algorithm or the default FISH algorithm. Go to the "**Analysis**" tab, select "**Settings Actions**" and click on "**Load…**" as shown in (A) below. Select the saved algorithm for a biomarker in the "**Saved Settings**" tab of the "**Select Algorithm**

Setting" pop-up window as shown in (B) below or load a new FISH algorithm if creating a new analysis from the "**Default Settings**" tab as show in (C) below. An example of loaded ɣH2AX FISH Algorithm is shown in (D) below.

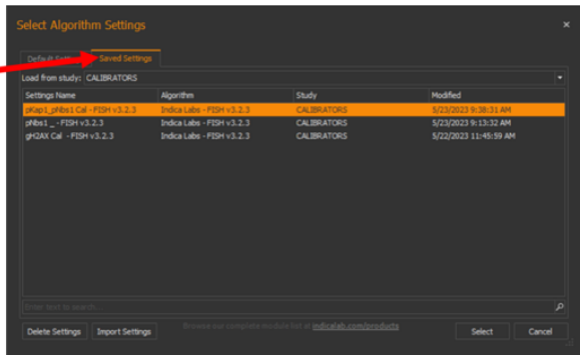
A

Click "**Settings Actions**"> "**Load..**"



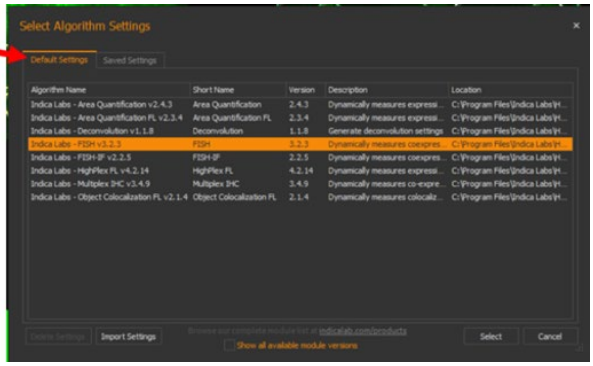
B

Open saved DDR9 algorithm from "**Saved Settings**" tab.



C

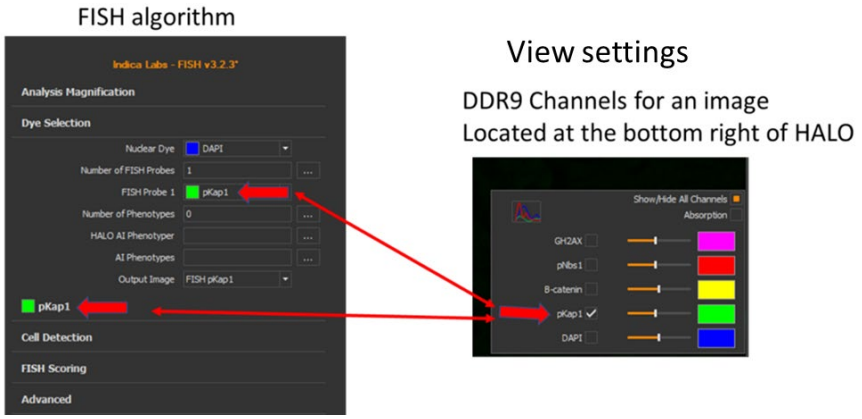
Open default FISH algorithm from "**Default Settings**" tab.



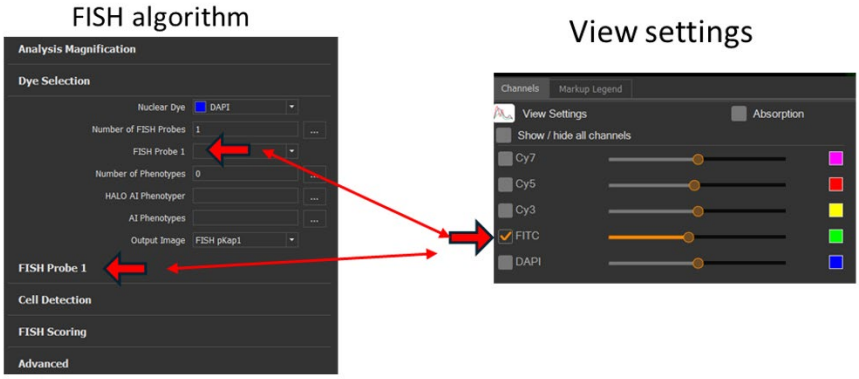
D

7.5.2.1 It's important to make sure an image is open with the correct view settings channel/biomarker names adjusted according to Section 7.2.2 prior to opening a saved script. If the channels have been named correctly according to Section 7.2.2, the **FISH Probe 1** field will be assigned the biomarker name being analyzed as shown in (A) below. If the channels have not been named according to Section 7.2.2 from default, the **FISH Probe 1** field will be "unassigned" as shown in (B) below.

A



B



7.5.3 **"Real-time Tuning"** is used to view live changes to the algorithm settings during optimization of thresholds. In the "**Analysis**" Tab, click the "**Analyze**" dropdown menu and select "**Real-time Tuning**" as shown in (A) below. Alternatively, analyze by "**Field of View**" as shown in (B) below to see larger areas analyzed to facilitate setting marker thresholds. "**Real-time tuning**" is a good tool to get rough draft parameter settings, but there can be differences in the results between "**Real-time Tuning**" and the final analysis, so it is important to run "**Field of View**" prior to finalizing the script parameters.

7.5.3.1 Tumor segmentation will only be visible using "**Field of View**" analysis but not visible with "**Real-timing Tuning**".

NOTE: Cy3 channel name must be changed to "B-catenin" in the view settings for the tumor classifier to function correctly.

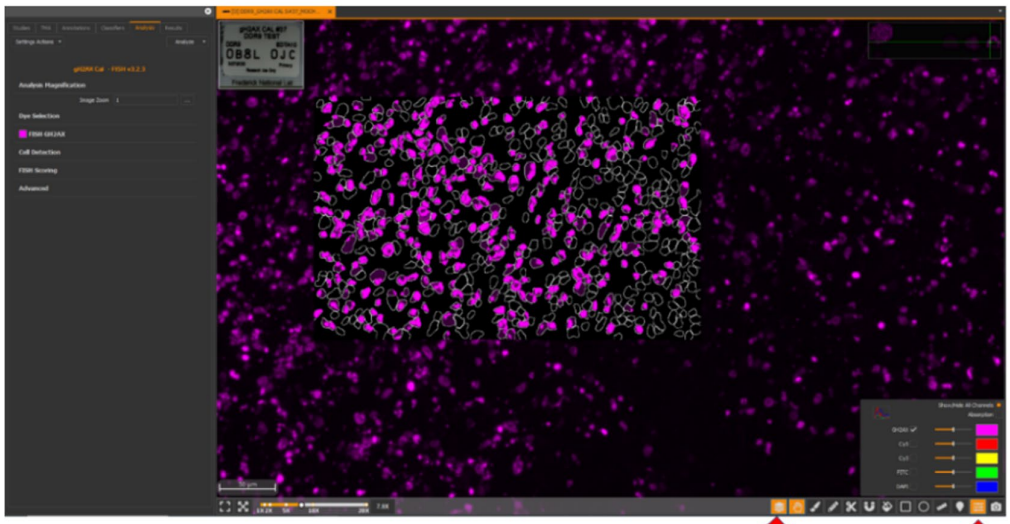
A

Select "**Real-time Tuning**"

B

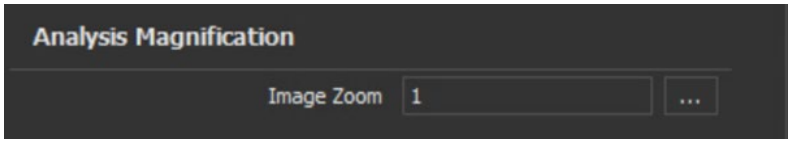
Select "**Field of View**"

7.5.4 Once "**Real-time Tuning**" is selected, a new window will appear displaying the mask for the current settings in the selected area. The mask in this window can be toggled on and off using the layer tool as shown below. The channels can be toggled on and off using the channel tool as shown below. For QC purposes, turn off all channels except for the biomarker to be analyzed so that the threshold values can be optimized for the specific biomarker mask.



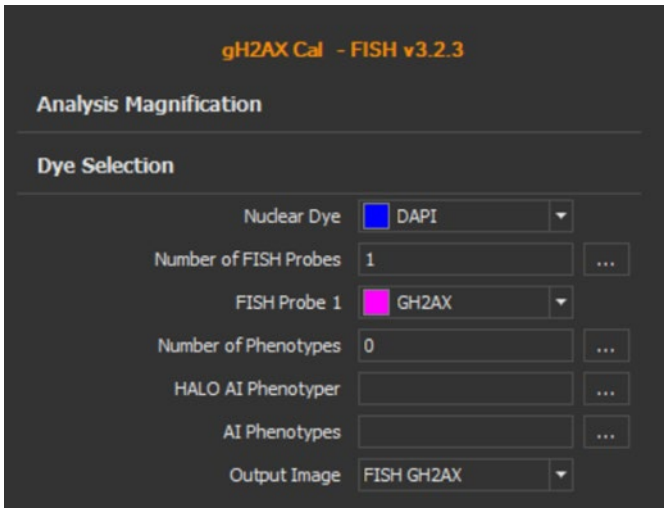
Toggle marker mask Toggle channel selection

7.5.5 **Analysis Magnification** is kept at default, Image Zoom = 1 during this process.



7.5.6 Dye Selection

7.5.6.1 An image from the study needs to be open for this section to pre-populate. Enter the following information within this section: (1) select "DAPI" from the drop-down for "**Nuclear Dye**", (2) enter "1" for "**Number of FISH Probes**" which will create dropdowns for selecting a channel in the image, (3) select the biomarker to be analyzed for "**FISH Probe 1**"(GH2AX, pNBS1 or pKAP1), (4) enter "0" for "**Number of Phenotypes**", as no phenotype will be used in this study, and (5) Set "**Output Image**" to "FISH Biomarker" (e.g., FISH GH2AX as shown below).



7.5.7 FISH Biomarker (e.g., FISH GH2AX) is used to adjust the thresholds to identify positive staining for a biomarker. The numbers shown below should be used only as a guide and the "**Real-Time Tuning**" and "**Field of View**" analysis features should be used to assess and appropriately adjust these parameters. Editable parameters are "**Biomarker Contrast Threshold**" (e.g., **GH2AX Contrast Threshold**), "**Biomarker Signal Minimum Intensity**" (e.g., **GH2AX Biomarker Signal Minimum Intensity**) and "**Biomarker Spot Size**" (e.g., **GH2AX Spot Size**), occasionally the "**Biomarker Spot Segmentation Aggressiveness**" (e.g., **GH2AX Spot Segmentation Aggressiveness**) may need to be adjusted. This assay measures positive nuclear areas for biomarkers, so it's very important to adjust these parameters so that the biomarker masks match the positive biomarker signal areas as closely as possible. Refer to Section 7.5.8 to Section 7.5.10 for examples of under-thresholded, over-thresholded and correctly-thresholded image masks for each biomarker.

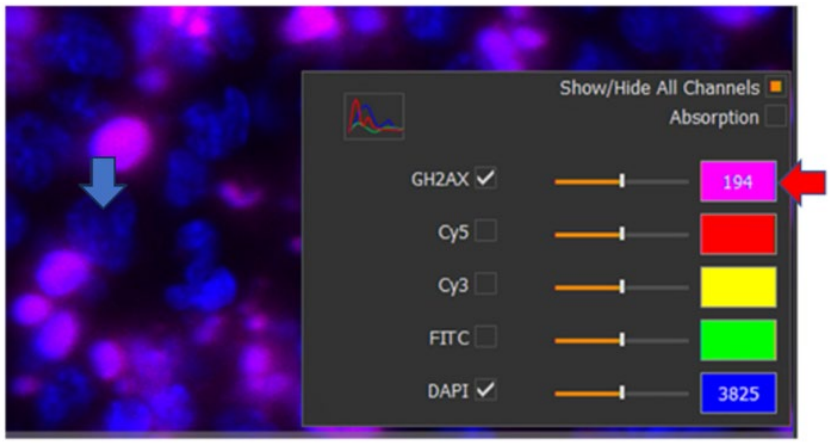
7.5.7.1 The "**Biomarker Contrast Threshold**" and "**Biomarker Signal Minimum Intensity**" are fractions, to translate into gray values, multiply the fraction by 16,384, which is the range for the Axioscan 7 images.

7.5.7.2 "**Biomarker Contrast Threshold**" is a parameter that evaluates pixels compared to the average intensity of neighboring pixels. Raising this number will reduce the number of pixels counted; while reducing it will raise the number of pixels counted. The default for this parameter is 0.5. This parameter should be adjusted as needed for individual patient slide sets.

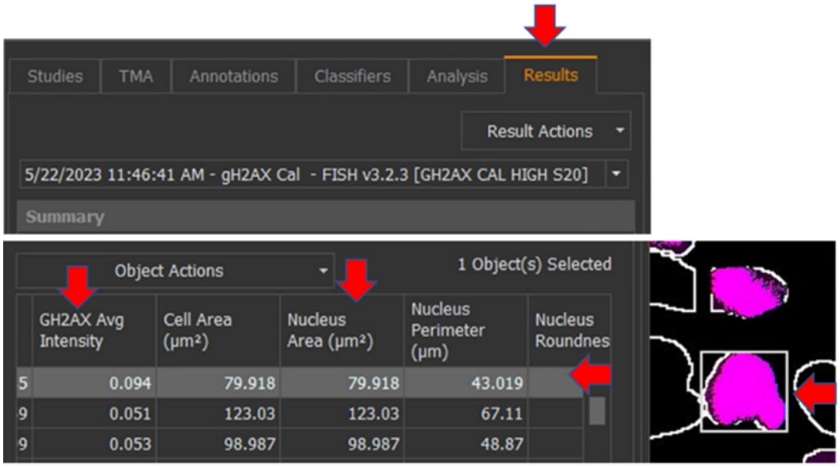
7.5.7.3 "**Biomarker Signal Minimum Intensity**" is a parameter that determines the minimum intensity that pixels need to be counted as positive. It is on a relative scale from 0-1, lowering it will include more pixels as positive. The default is 0.25. This parameter should be adjusted as needed for individual patient slide sets.

7.5.7.3.1 Adjusting the "Biomarker Signal Minimum Intensity" setting may help to eliminate background signal. In the screenshot of Section 7.5.7., the "GH2AX Signal Minimum Intensity" is 0.018. As 0.018 x 16,384 = 294.9, with this setting, intensity values above 294.9 gray values will be measured as positive signal, whereas signal intensity below this value will be considered background.

7.5.7.3.2 To determine the appropriate background signal intensity, go to "**View**" > "**Display Pixel**" > "**FL Channel Intensities**". In the example for GH2AX biomarker shown below, place the mouse over biomarker negative nuclei as pointed to by the blue arrow below, and the biomarker intensity associated with the selected negative nuclei will display in the bottom right of the screen next to the GH2AX biomarker name as pointed to by the red arrow below. Convert the value to a fraction to determine the appropriate "**GH2AX Signal Minimum Intensity**" value, which should be 0.012 (194/16384 = 0.012) in this case. Use careful assessment when setting the GH2AX minimum signal value in order to prevent elimination of true signal.



7.5.7.3.3 After running the "**Field of View**" analysis, measured values for individual nuclei can be found in the "**Object Data**" table of the "**Results**" tab as shown below. These values can also be used to determine "**Nuclear Size**" ranges and "**Minimum Nuclear Intensity**" values in Section 7.5.12.



7.5.7.4 "**Biomarker Spot Size**" is a parameter that sets a size threshold for the minimum and maximum size range in μm^2. The default is "0.5, 20". This parameter should be adjusted as needed for individual patient slide sets.

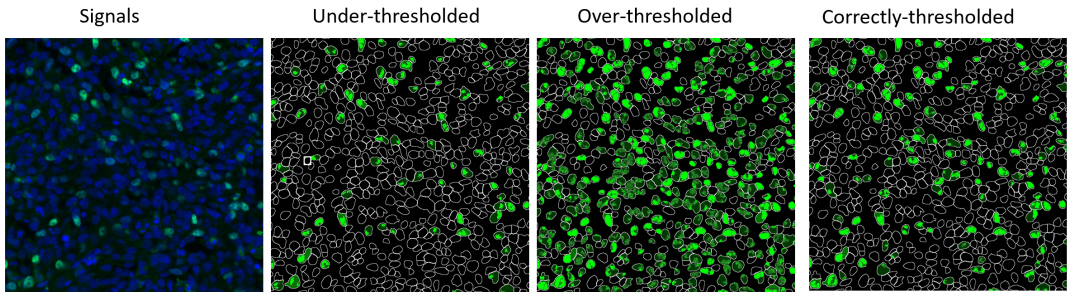
7.5.7.5 "**Biomarker Copy Intensity**" is a parameter that defines how to break up clusters of signals into counts and relies on relative intensity of spots. It is on a relative scale from 0-1. The default is 0.15 and should be changed to **1** for DDR9 assay.

7.5.7.6 "**Spot Segmentation Aggressiveness**" is a parameter that adjusts the aggressiveness of the segmentation of signal clusters. It is on a relative scale from 0-1 and raising it

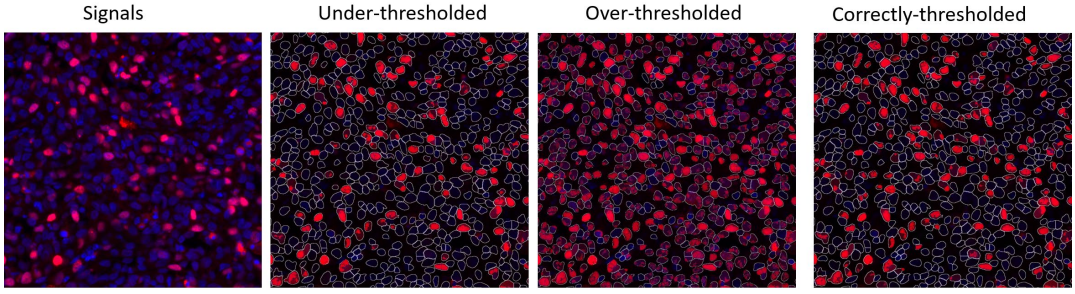
will increase how aggressive the spot segmentation is. The default is 0.95. This parameter should be adjusted as needed for individual patient slide sets.

7.5.7.7 The "**Output Image**" dropdown should be on the "FISH Biomarker" (e.g., FISH GH2AX) option.

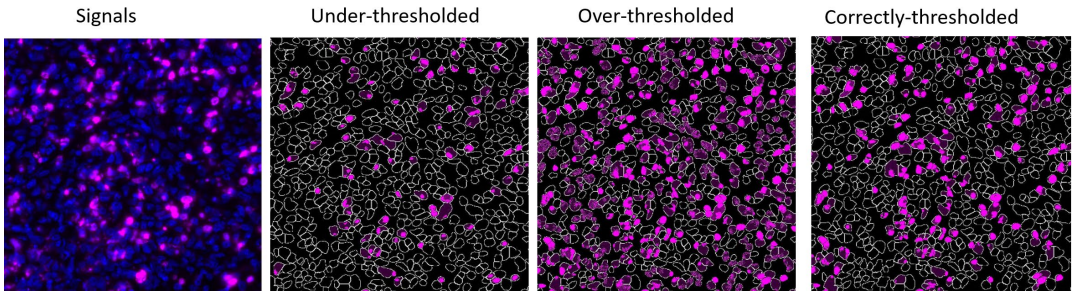
7.5.8 Examples of under-thresholded, over-thresholded and correctly-thresholded image masks for pKAP1 are shown below.



7.5.9 Examples of under-thresholded, over-thresholded and correctly-thresholded image masks for pNBS1 are shown below.



7.5.10 Examples of under-thresholded, over-thresholded and correctly-thresholded image masks for ɣH2AX are shown below.



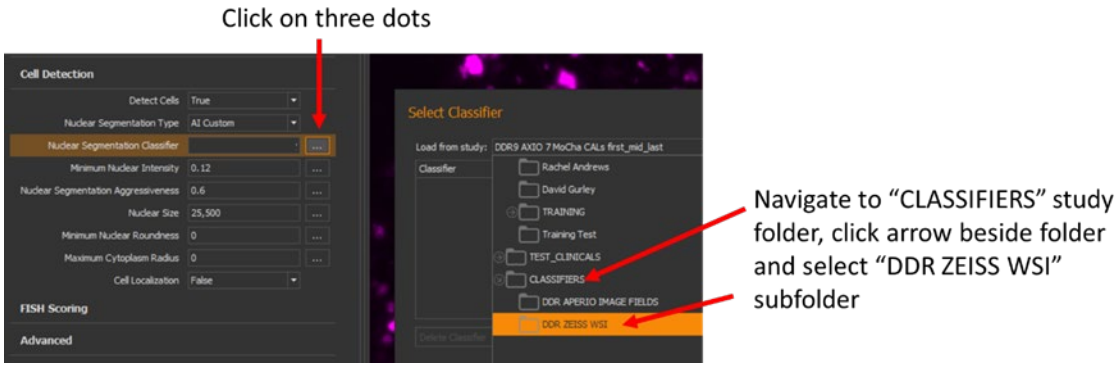
7.5.11 **FISH Scoring** is a parameter that adjusts how many copies of a FISH signal are necessary in a cell for it to be classified as 1+, 2+, 3+ or 4+. **This was not necessary for DDR9 assay so all values should be left at default and not adjusted.**

7.5.12 The **Cell Detection** window is used to select the settings for nuclei detection. Select "True" for "**Detect Cells**". Under "**Nuclear Segmentation Type**", select "AI Custom"

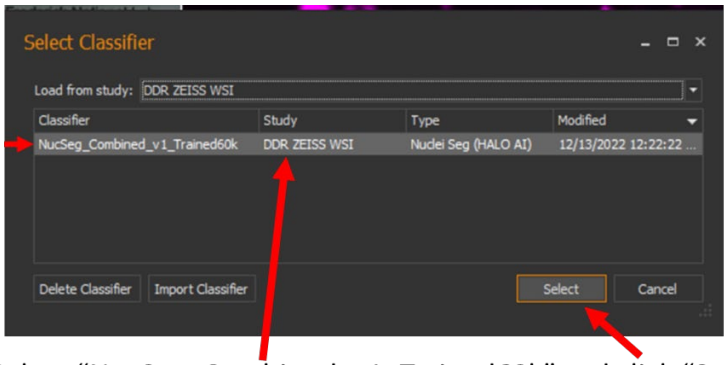
as shown in (A) below. Once selected, under "**Nuclear Segmentation Classifier**", press the "**…**" as shown in (B) below, which will prompt you to select your AI nuclear segmentation algorithm. Select the final Algorithm "**NucSeg_Combined_v1_Trained60K**" in the "**Select Classifier**" pop-up window as shown in (C) below. An example of final "**Cell Detection**" settings is shown in (D) below.

A

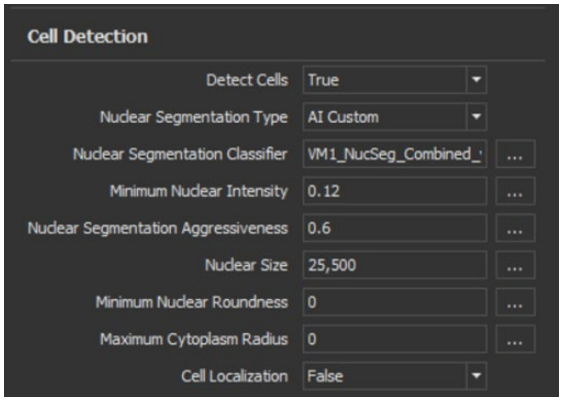
B



C



D



7.5.12.1 There are options of "Traditional", "AI Default", and "AI Custom" under "Nuclear Segmentation Type". "Traditional" will use the DAPI channel and non-AI nuclear segmentation. "AI Default" will use a pre-trained AI nuclear segmentation algorithm based on the DAPI channel. "AI Custom" allows to plug in a custom trained AI nuclear segmentation algorithm. This should be selected for DDR9 assay, as a custom trained AI nuclear segmentation algorithm has been developed for the assay.

7.5.12.2 "**Minimum Nuclear Intensity**" is a parameter to change the minimum intensity of DAPI necessary to be called a true nucleus. The default value is **0.095**, which should be adjusted as needed to detect weakly stained nuclei.

7.5.12.3 "**Nuclear Segmentation Aggressiveness**" is a parameter to change how aggressive the algorithm will segment the nuclei. Increasing this value will allow for more aggressive cell segmentation, which is helpful in nuclei dense areas. The default is **0.65**, which was decreased to **0.6** in the DDR9 algorithm since the AI network segments very well. This can be adjusted further as needed to properly segment nuclei.

7.5.12.4 "**Nuclear Size**" is a parameter that represents the minimum and maximum nuclear area in microns squared that will be called as nuclei. Objects smaller or larger than these two numbers will be ignored. The default is "11.3, 571.7". **It is recommended to change these values to "25, 500" for the control slide, but they should be adjusted as needed for patient slide sets**.

7.5.12.5 "**Minimum Nuclear Roundness**" is a parameter used to exclude elongated and irregularly shaped nuclei. The default is **0**, which should not be changed for the DDR9 assay.

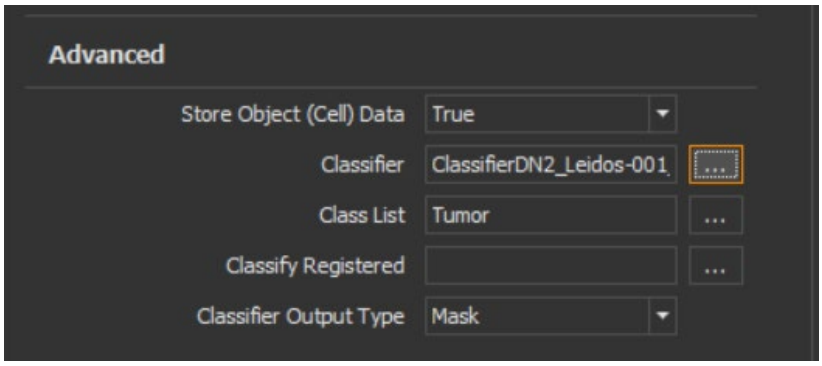
7.5.12.6 "**Maximum Cytoplasm Radius**" is where to adjust the cytoplasmic radius of the cells to be detected, and to adjust the aggressiveness of membrane segmentation. For DDR9 analysis, no cytoplasmic radius is used since only nuclear markers are involved and cytoplasmic and membranous staining are not important to analysis.

7.5.12.7 **Important:** Apply the same **Cell Detection** settings to each marker analysis within a single patient image set.

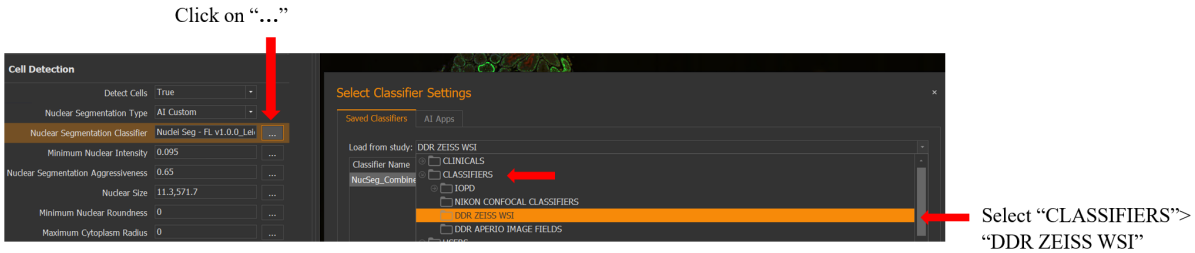
7.5.13 The "**Advanced**" window is used to set up tumor segmentation. Select "True" under "**Store Object (Cell) Data**" as shown in (A) below. Load the tumor segmentation

classifier by clicking on the "**…**" next to "**Classifier**" field as shown in (A) below and selecting "ClassifierDN2_Leidos-001_Zeiss WSI_ v8_.8_34K" for specimens with appropriate β-Catenin signal from the "CLASSIFIERS" study folder in the "**Select Classifier**" pop-up window as shown in (B) and (C) below. Select "Tumor" under "**Class List**" as shown in (A) below and select "Mask" from the dropdown list for "**Classifier Output Type**" as shown in (A) below. For control slides or specimens lacking appropriate β-Catenin signal, the "**Classifier**", "**Class List**" and "**Classifier Output Type**" fields should be left empty.

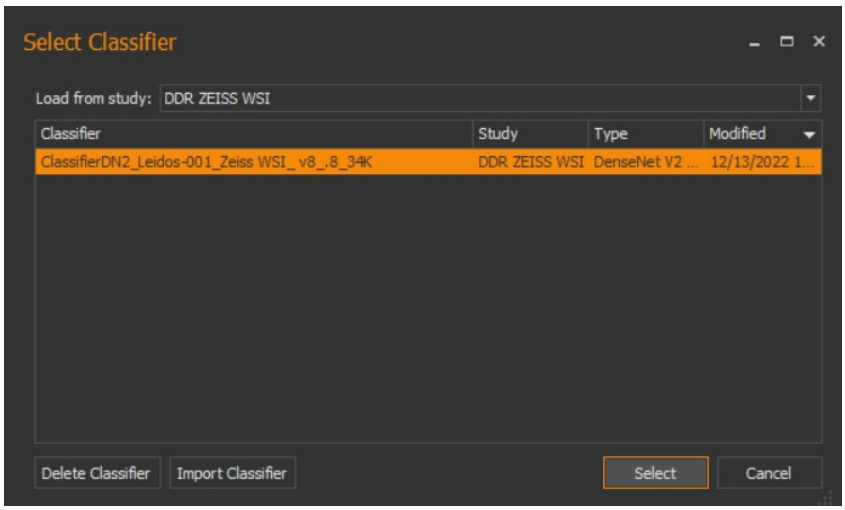
A



B



C

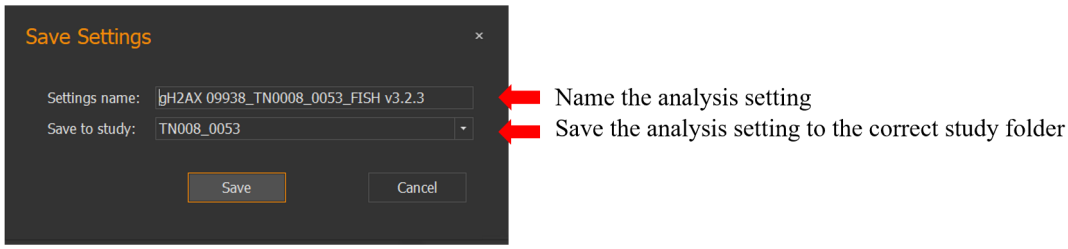


7.5.14 Save the analysis setting within the study folder. Click "**Settings Actions**" then select "**Save…**". In the pop-up window ensure the correct "**Settings name**" and the correct "**Save to study**" folder is populated as shown below.

7.5.14.1 For control slides stained with each patient slide set, name the "**Settings name**" as "Marker name *space* con *space* Protocol #(5 digits)_Site#(6 digits)_Patient#(4 digits)" (e.g., GH2AX con 10346_ NCIDTC_0005), then save it to the control slide study folder for the corresponding patient.

7.5.14.2 For patient specimen slides, name the "Settings name" as "Marker name *space* Protocol #(5 digits)_Site#(6 digits)_Patient#(4 digits)" (e.g., GH2AX 10346_NCIDTC_0005) and save it to the appropriate patient study folder.

7.5.14.3 For clinical images to be reported using the "Master Template DDR9 Clinical Data Report" for data reporting, it's very important to save "Settings name" following this rule.



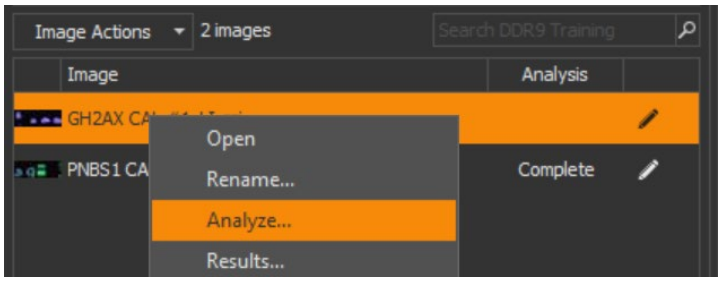
7.5.15 Repeat the procedure from Section 7.5.2 to Section 7.5.14 for each biomarker (γH2AX, pNBS1 or pKAP1) separately until all three biomarkers have been analyzed.

7.6 Batch Image Analysis of the DDR9 Biomarkers

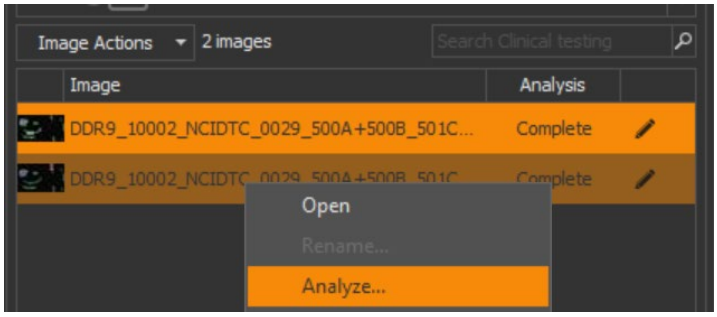
7.6.1 The batch image analysis should be performed using each biomarker's algorithm following Section 7.6.2 to Section 7.6.6.

7.6.2 Navigate to the "**Studies**" tab and select the images for analysis.

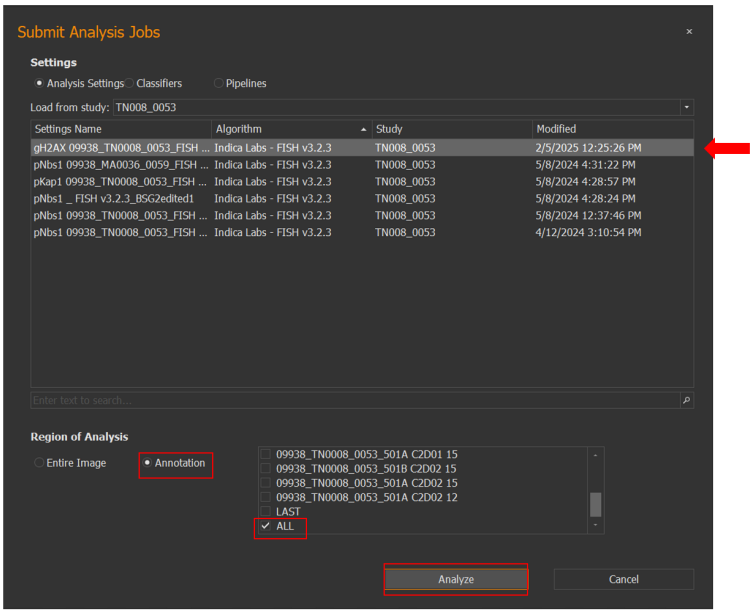
7.6.3 To analyze control slides, select an individual control slide image, right click and select "Analyze…" as shown below:



7.6.4 To analyze clinical specimens, select all slide images from the same patient, right click and select "**Analyze…**" as shown below:

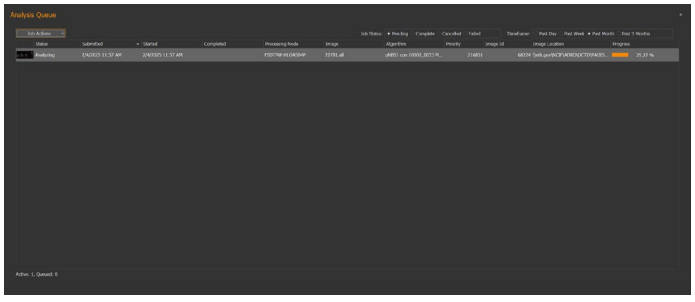


7.6.5 Select the saved analysis settings in the correct study. Ensure that the correct study is selected from the "**Load from study**" drop-down menu as shown below. Under "**Region of Analysis**", select "**Annotation**" and scroll down to select "**ALL**" the layers. Then click "**Analyze**" as shown below.



7.6.6 The analysis queue window will appear as shown below and can be closed.

IMPORTANT: DO NOT close the HALO software.



7.7 **To export the Image Analysis Settings**, make sure the analysis setting of interest is open and click on the "**Analysis**" tab, under the "**Settings Actions**" drop down, select "**Export…**". In the

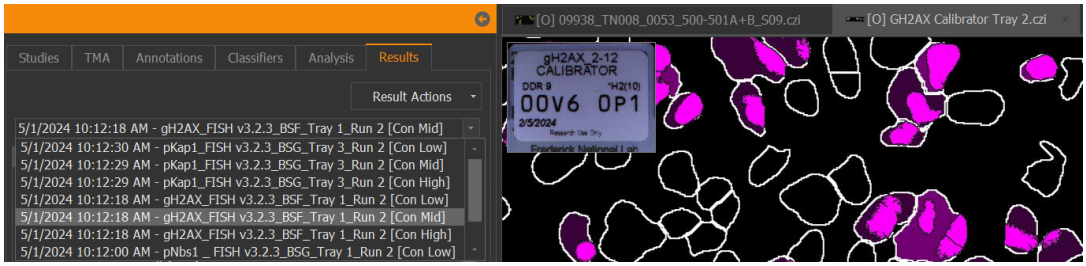
pop-up window, navigate to the study folder in the HALO_Image share and save the analysis setting as "Marker name space Protocol #(5 digits)_Site#(6 digits)_Patient#(4 digits)" (e.g., GH2AX 10346_NCIDTC_0005).

8.0 QUALITY CONTROL, DATA ANALYSIS AND REPORTING

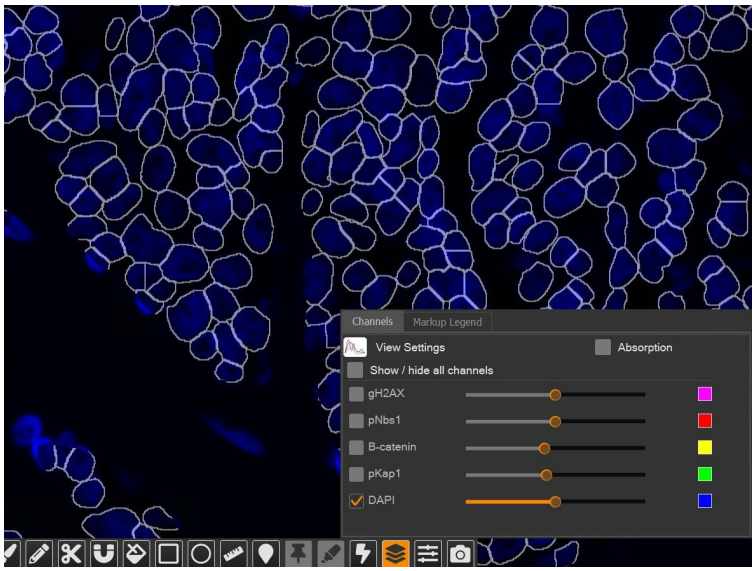
8.1 Images can be evaluated for appropriate detection parameters via a QC process defined below either in HALO-link or HALO. Performing these steps in the HALO software will allow for an easier process to adjust threshold settings.

8.2 DDR9 QC Process in HALO

8.2.1 Select an image that needs review of analysis thresholds and go to the "**Results**" tab. Click on the "**Result Actions**" dropdown menu to select analysis for the marker of interest and the annotation ROI as shown below.



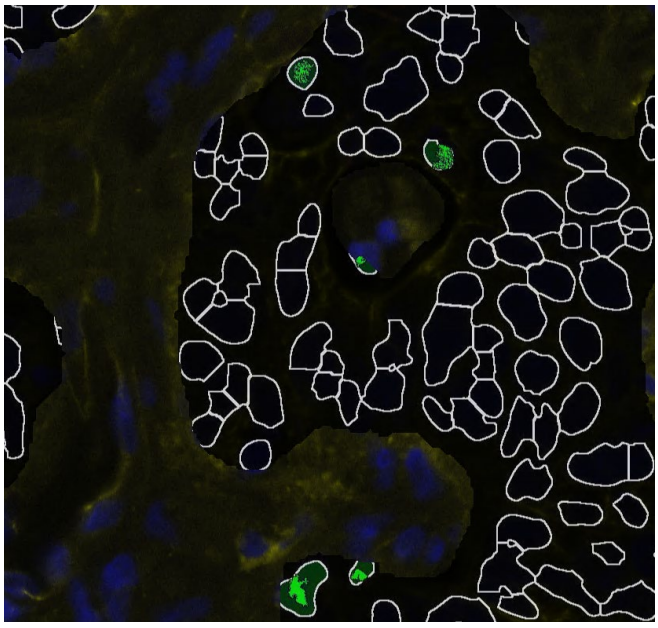
8.2.2 **Review nuclear segmentation** by turning off all channels except for the DAPI channel as shown below. Make sure all nuclei have been segmented properly or adjust as needed.



8.2.3 **Review Tumor Classifier Mask** with "AlgorithmMarkup".

8.2.3.1 **Reminder:** Tumor segmentation is not required for the DDR9 control slides. Tumor segmentation using β-Catenin is used for appropriate clinical specimens.

8.2.3.2 Under the "AlgorithmMarkup", tumor area mask is shaded in black and tumor cells are outlined in white.

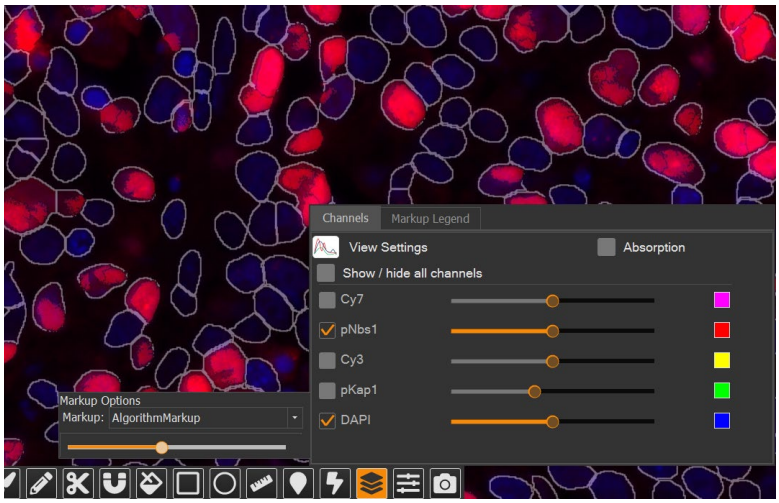


8.2.3.3 If there is any issue with tumor segmentation by the default DDR9 tumor classifier "ClassifierDN2_Leidos-001_Zeiss WSI_ v8_.8_34K", try additional tested DDR9 classifiers and record the classifier used in the Comments" section of the Batch Record (Appendix 1). If all the DDR9 classifiers fail, manually adjust the annotation according to the steps below and record this deviation in the "Comments" section of the Batch Record (Appendix 1).

- If a β-Catenin negative area is misclassified as tumor, use exclusion annotation to exclude the misclassified area and re-run the analysis.
- If a β-Catenin positive area is not classified as tumor appropriately, manually annotate the β-Catenin positive areas, using the nearest pathologist annotated H&E slide as a reference, and re-run the analysis without a tumor classifier.
- When using manual annotation and analyzing without any classifier, it's crucial to only include tumor area in the annotation.

8.2.4 **View Marker Analysis** by clicking on the layer tool and selecting "AlgorithmMarkup" in the drop-down list. To adjust the overlay, utilize the "**Transparency**" slider bar found

in the layer tool as shown below. Turn off all channels except for the marker channel that requires image QC. Toggle marker masks on/off to assess marker thresholds. Repeat the process to complete QC for all the DDR9 biomarkers.

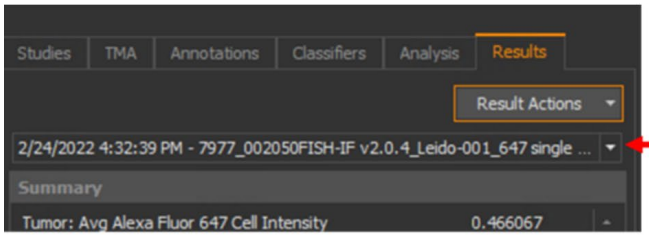


8.2.5 To adjust the marker threshold, go to the "**Analysis**" tab, load the analysis setting, and edit the parameters. Re-save the analysis setting, re-run the analysis and repeat the image QC process. The user may also need to modify the annotation to avoid auto fluorescent regions, areas with high background or other image artifacts. Remember to save the annotations after modification prior to repeating analysis.

8.2.6 Once the final thresholding is established, the previous analysis results should be removed using one of the two methods below.

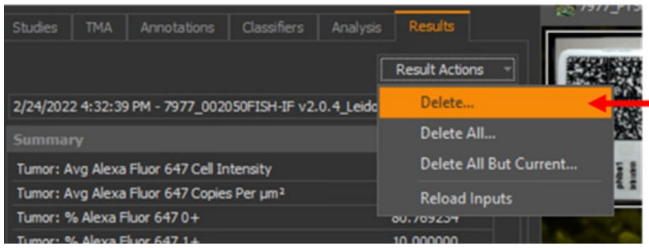
8.2.6.1 Navigate to the "**Results**" tab and select the analysis that needs to be removed as shown in (A) below. Click "**Results Actions**" and select "**Delete…**" as shown in (B) below. This will result in the removal of the currently displayed results.

A



Select analysis that needs to be reset/removed

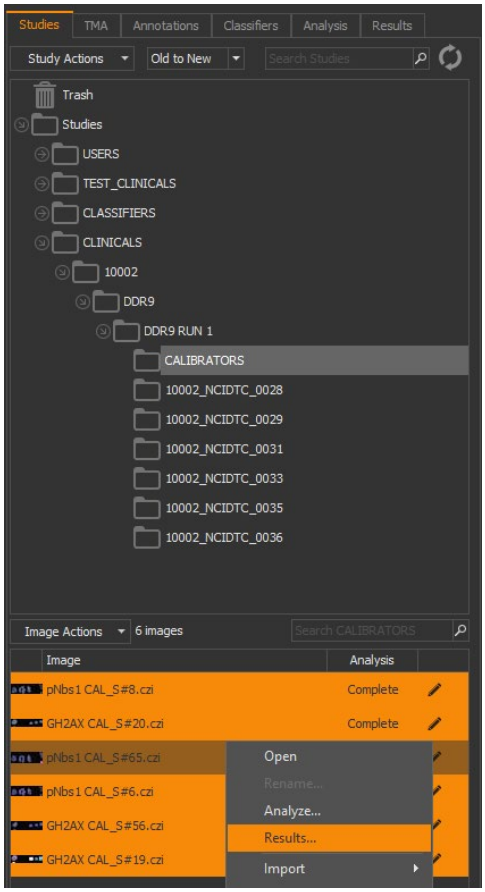
B



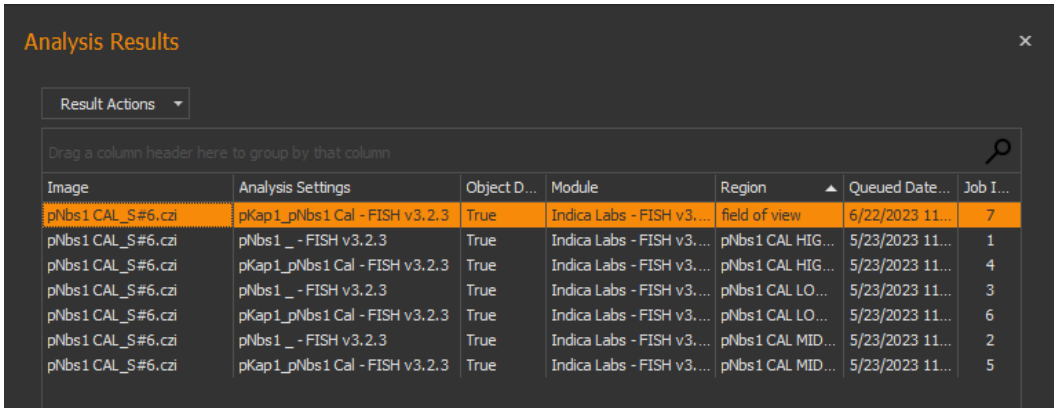
Select "**Delete…**" from dropdown menu

8.2.6.2 If there are multiple results to be removed, go to the "**Studies**" tab, and select images where there are results that need to be removed and right click and select "**Results…**" as shown in (A) below. In the pop-up window, select the analysis that needs to be deleted as shown in (B) below. Select "**Delete**" from the dropdown list of "**Result Actions**" as shown in (C) below.

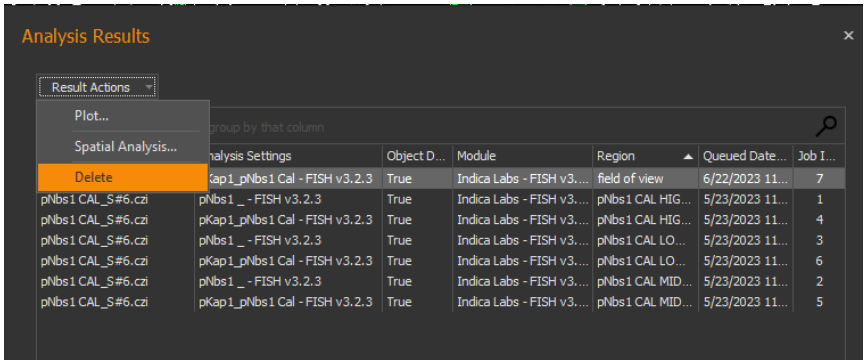
A


Studies
TMA
Annotations
Classifiers
Analysis
Results
Study Actions
Old to New
Search Studies
Trash
Studies
USERS
TEST_CLINICALS
CLASSIFIERS
CLINICALS
10002
DDR9
DDR9 RUN 1
CALIBRATORS
10002_NCIDTC_0028
10002_NCIDTC_0029
10002_NCIDTC_0031
10002_NCIDTC_0033
10002_NCIDTC_0035
10002_NCIDTC_0036
Image Actions
6 images
Search CALIBRATORS
Image
Analysis
pNbs1 CAL_S#8.czi
Complete
GH2AX CAL_S#20.czi
Complete
pNbs1 CAL_S#65.czi
pNbs1 CAL_S#6.czi
GH2AX CAL_S#56.czi
GH2AX CAL_S#19.czi
Open
Rename...
Analyze...
Results...
Import

B


Analysis Results
Result Actions
Drag a column header here to group by that column
Image
Analysis Settings
Object D...
Module
Region
Queued Date...
Job I...
pNbs1 CAL_S#6.czi pKap1_pNbs1 Cal - FISH v3.2.3 True Indica Labs - FISH v3.... field of view 6/22/2023 11... 7
pNbs1 CAL_S#6.czi pNbs1 _ - FISH v3.2.3 True Indica Labs - FISH v3.... pNbs1 CAL HIG... 5/23/2023 11... 1
pNbs1 CAL_S#6.czi pKap1_pNbs1 Cal - FISH v3.2.3 True Indica Labs - FISH v3.... pNbs1 CAL HIG... 5/23/2023 11... 4
pNbs1 CAL_S#6.czi pNbs1 _ - FISH v3.2.3 True Indica Labs - FISH v3.... pNbs1 CAL LO... 5/23/2023 11... 3
pNbs1 CAL_S#6.czi pKap1_pNbs1 Cal - FISH v3.2.3 True Indica Labs - FISH v3.... pNbs1 CAL LO... 5/23/2023 11... 6
pNbs1 CAL_S#6.czi pNbs1 _ - FISH v3.2.3 True Indica Labs - FISH v3.... pNbs1 CAL MID... 5/23/2023 11... 2
pNbs1 CAL_S#6.czi pKap1_pNbs1 Cal - FISH v3.2.3 True Indica Labs - FISH v3.... pNbs1 CAL MID... 5/23/2023 11... 5

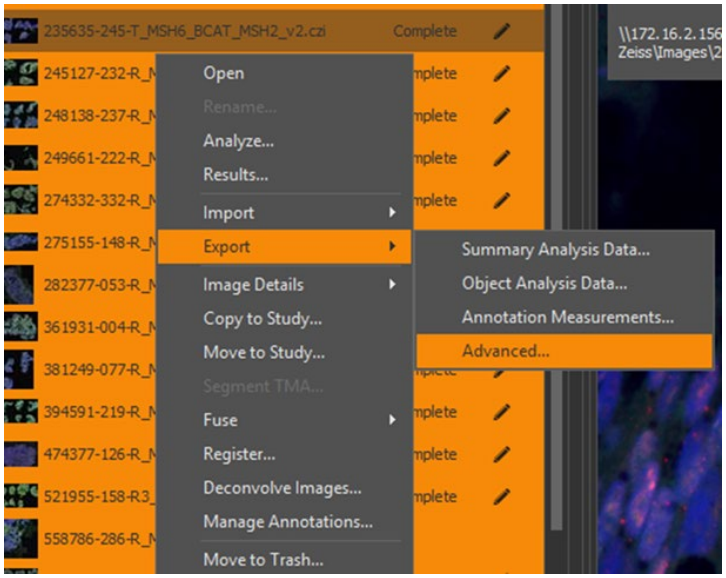
C



8.2.6.3 **NOTE:** It is very important to remove the data that is not final so as to maintain an accurate audit trail for the final biomarker data reported.

8.3 DDR9 Data Export in HALO

8.3.1 After QC is complete, the analysis results can be exported by selecting the desired images in the "Studies" tab and right clicking to select "**Export**" > "**Advanced…**" as shown below.



8.3.1.1 For control slides, the results from each control slide should be exported separately.

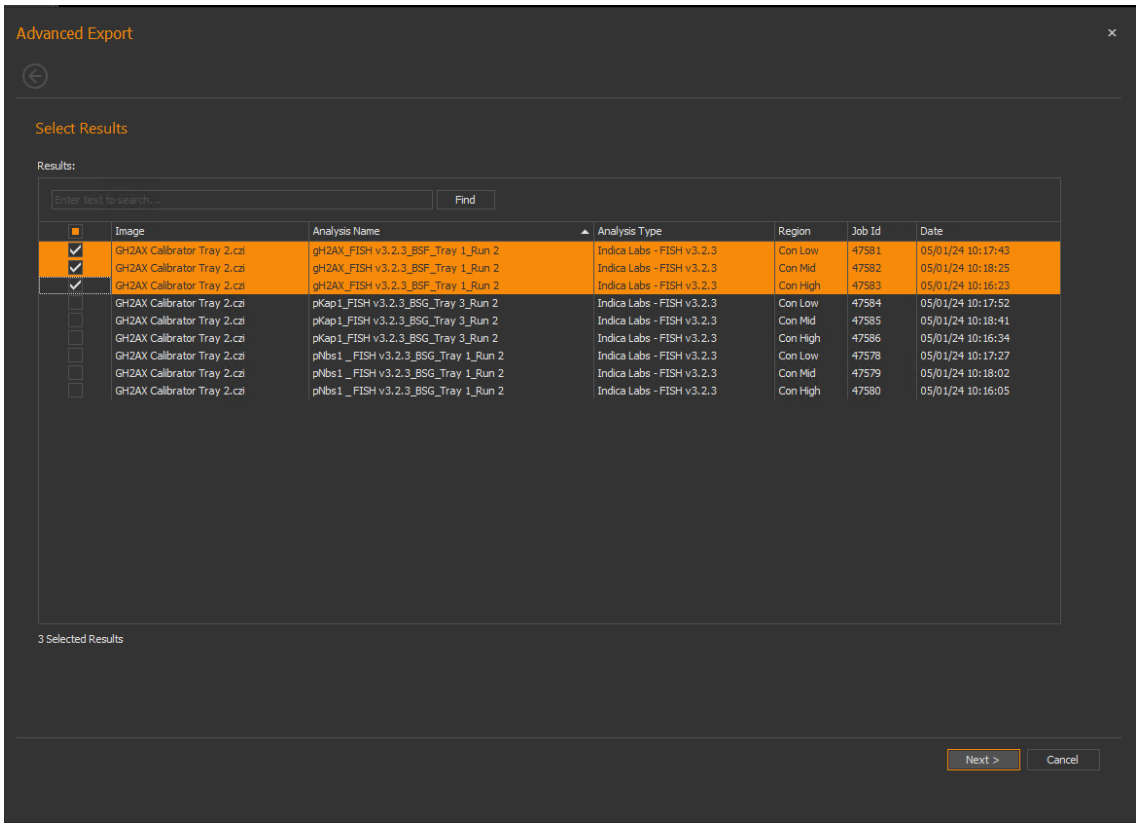
8.3.1.2 For preclinical slides, the results from different slide images from the same study should be selected for export together.

8.3.1.3 For clinical slides, the results from different slide images from the same patient specimens should be selected for export together.

8.3.2 In the pop-up "**Advanced Export**" window as shown below, sort the results based on the analysis name to group the analysis by ɣH2AX, pNBS1 or pKAP1.

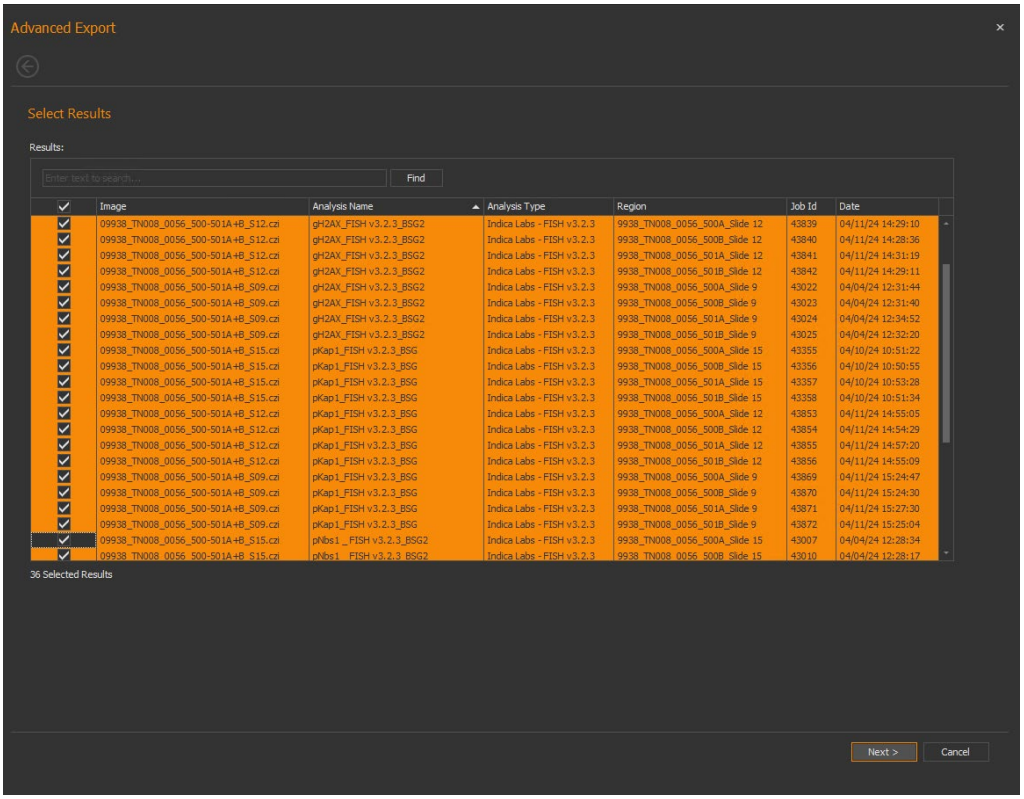
8.3.2.1 For control or preclinical specimen slides or any data reporting without the Master Template for DDR9 Clinical Data Report, export the analysis results for each marker

independently. In the example of ɣH2AX data export as shown below, sort results by "**Analysis Name**" , select the results for all ɣH2AX control tissues on the ɣH2AX control slide and click "**Next**" as shown below.

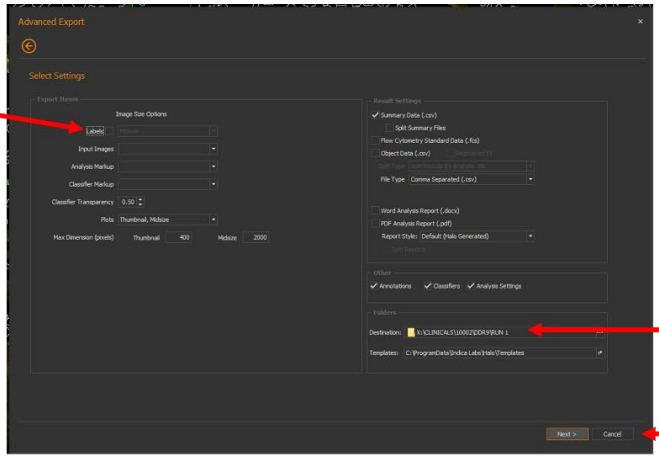


8.3.2.2 For clinical specimen slides to be reported using the Master Templates for DDR9 Clinical Data Report, export the all specimens analysis results from one patient for all biomarkers across all slides together. It is very important to sort results by "Analysis Name" so that the results are organized in the order of gH2AX > pKAP1 > pNBS1 as shown below for the Master Template for DDR9 Clinical Data Report to work. Select all the final results and click "Next" as shown below.

- **Important:** Ensure all non-final results have been removed according to Section 8.2.6 before the export.



8.3.3 In the "**Export Manager**" window as shown below, several parameters should be adjusted as shown below.



8.3.4 The "**Export Items**" section is for exporting recreated images from the study including "**Input Images**", "**Analysis Markup**", "**Classifier Markup**", and "**Plots**". Uncheck the box next to "**Labels**" in this section.

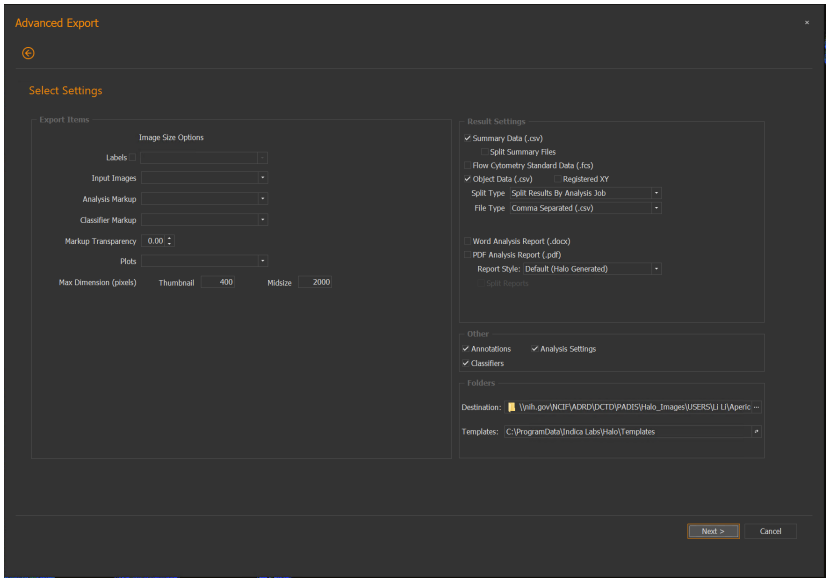
8.3.5 The "**Result Settings**" section is for selecting the type of data to be exported, select "**Summary Data**" for DDR9 data export in this section.

8.3.6 The "**Other**" section is for selecting the archive files to be saved. Check the boxes for "**Annotations**", "**Classifiers**" and "**Analysis Settings**" in this section.

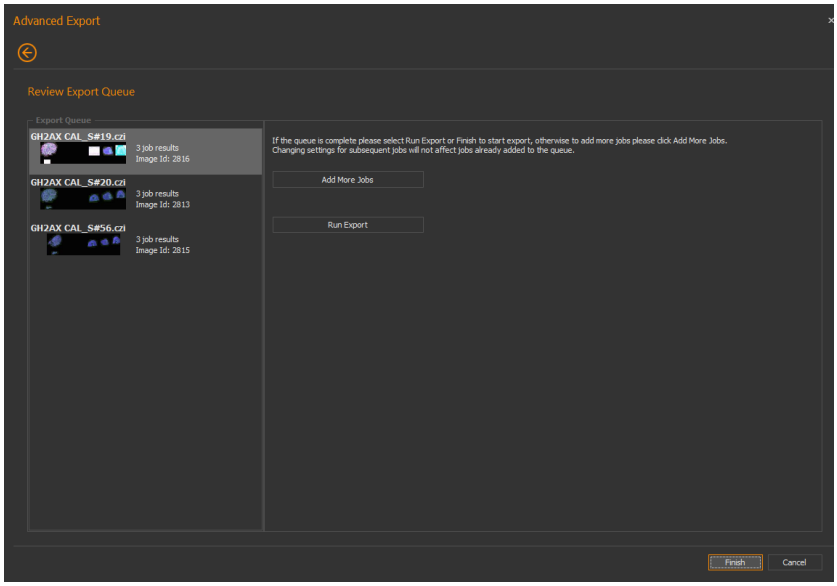
8.3.7 The "**Folders**" section is for selecting the location to save Archive files. Under "**Destination**", select "**…**" and navigate to the corresponding study folder where the results should be saved.

8.3.8 Click "Next" after finishing the setup for the export.

8.3.8.1 An example of a completed export using Advanced Export settings is shown below.

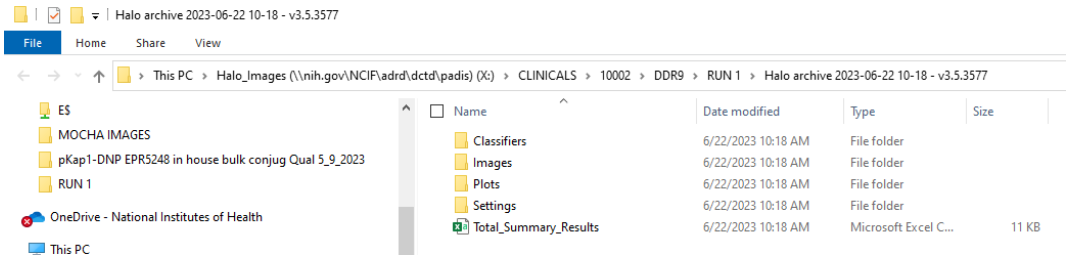


8.3.9 Click "**Finish**" in the "**Review Export Queue**" window as shown below.



8.4 DDR9 Data Report Process

8.4.1 After the data export is completed, the location of the exported file will open as shown below. Open the "Total_Summary_Results" csv file in the folder.



8.4.2 Follow Section 8.4.2.1 to Section 8.4.2.4 below to calculate biomarker %NAP values for DDR9 data reporting without the Master Templates for the DDR9 Clinical Data Report.

8.4.2.1 Columns needed for the control slide image analysis summary are as follows: "**Image Tag**", "**Algorithm Name**", "**Analysis Region**", "**Total Cells**", "**Total GH2AX** (or **pKAP1** or **pNBS1**) **Area (µm²)**" and "**Total Cell Area (µm²)**". All other columns can be hidden. An example of control slide data is shown below.

C	D	F	M	O	AA
Image Tag	Algorithm Name	Analysis Region	Total Cells	Total GH2AX Area (µm²)	Total Cell Area (µm²)
GH2AX CAL_S#20.czi	gH2AX Cal - FISH v3.2.3	GH2AX CAL HIGH S20	55756	436596.4063	2735245.5
GH2AX CAL_S#20.czi	gH2AX Cal - FISH v3.2.3	GH2AX CAL MID S20	78961	247104.5469	4089331.5
GH2AX CAL_S#20.czi	gH2AX Cal - FISH v3.2.3	GH2AX CAL LOW S20	61773	9670.635742	3141231.75

8.4.2.2 Create a new column to calculate the %NAP for the control slides using the following equation:

- ɣH2AX (or pKAP1 or pNBS1) %NAP = Total γH2AX Area (or pKAP1 or pNBS1) (µm²)/ Total Cell Area (µm²) * 100

8.4.2.3 Columns needed for clinical slide image analysis summary are as follows: "**Image Tag**", "**Algorithm Name**", "**Analysis Region**", "**Tumor: Total Cells**", "**Tumor: Total gH2AX** (or **pKAP1** or **pNBS1**) **Area (µm²)**" and "**Tumor: Total Cell Area (µm²)**". All other columns can be hidden. An example of clinical slide image data is shown below.

C	D	F	AL	AN	AZ
Image Tag	Algorithm Name	Analysis Region	Tumor: Total Cells	Tumor: Total gH2AX Area (µm²)	Tumor: Total Cell Area (µm²)
09938_PA015_0068_500-	gH2AX 09938_PA0015_0068_	09938_PA0015_0068_500A C1D01 12	2083	88.609215	119670.1953
09938_PA015_0068_500-	gH2AX 09938_PA0015_0068_	09938_PA0015_0068_500B C1D02 12	3356	491.068634	180563.4219
09938_PA015_0068_500-	gH2AX 09938_PA0015_0068_	09938_PA0015_0068_501A C2D01 12	10129	917.050537	592232.875
09938_PA015_0068_500-	gH2AX 09938_PA0015_0068_	09938_PA0015_0068_501B C2D02 12	2153	162.702042	121282.4922
09938_PA015_0068_500-	pKap1 09938_PA0015_0068_	09938_PA0015_0068_500A C1D01 12-pk	2088		119809.6406
09938_PA015_0068_500-	pKap1 09938_PA0015_0068_	09938_PA0015_0068_500B C1D02 12_p	3268		175823.2188

8.4.2.4 Create a new column to calculate the %NAP for the clinical slides using the equation provided in Section 8.4.2.2.

8.4.3 Follow Section 8.4.3.1 to Section 8.4.3.10 below for data reporting using the Master Templates for DDR9 Clinical Data Report.

8.4.3.1 The NCLN PD lab should use Master Templates DDR9 Clinical Data Report to report DDR9 data for clinical specimens.

8.4.3.2 There are two Master Templates DDR9 Clinical Data Report.

8.4.3.2.1 For clinical specimens analyzed using tumor segmentation, open "Master Template DDR9 Clinical Data Report_TumorSeg" and save it as Master Template DDR9 Clinical Data Report_TumorSeg_Patient ID (e.g, "Master Template DDR9 Clinical Data Report_TumorSeg_ 09938_MA0036_0064") in the patient study folder in the Halo_Image share.

8.4.3.2.2 For clinical specimens analyzed without tumor segmentation, open "Master Template DDR9 Clinical Data Report_NoTumorSeg" and save it as Master Template DDR9 Clinical Data Report_NoTumorSeg_Patient ID (e.g., "Master Template DDR9 Clinical Data Report_NoTumorSeg_09938_PA0015_0055") in the patient study folder in the Halo_Image share.

8.4.3.3 In the "Specimen Info" tab, enter the Specimen ID, Time Point, RAVE Specimen ID, RAVE Universal Patient ID and biopsy Pass ID as shown in the example below.

Specimen Information	PADIS Specimen ID	Time Point	RAVE Specimen ID	RAVE Universal Patient ID	Pass ID
1st time point specimens	09938_MA0036_0064_500A	C1D00	09938-1874GJ41-1	1874GJ41	A
	09938_MA0036_0064_500B	C1D00	09938-1874GJ41-1	1874GJ41	B
2nd time point specimens	09938_MA0036_0064_501A	C1D02	09938-1874GJ41-5	1874GJ41	A
	09938_MA0036_0064_501B	C1D02	09938-1874GJ41-5	1874GJ41	B
3rd time point specimens					

8.4.3.4 Open the "Total_Summary_Results" csv file exported according to Section 8.3.2.2.

8.4.3.5 For specimens analyzed using tumor segmentation, copy and paste all data into the "Total_Summary_Results" tab of the Master Template DDR9 Clinical Data Report_TumorSeg.

8.4.3.6 For specimens analyzed without tumor segmentation, copy and paste all data into the "Total_Summary_Results" tab of the Master Template DDR9 Clinical Data Report_NoTumorSeg.

8.4.3.7 Copy the exported control slide data from "Total_Summary_Results" csv file exported according to Step 8.3.2.1 for each biomarker and paste the appropriate data into the "gH2AX CAL", "pNBS1 CAL" or "pKAP1 CAL" tab of the Master Templates DDR9 Clinical Data Report.

8.4.3.8 Go to the "Cal Summary" tab and enter the control slide lot numbers and corresponding Low and High limits in the fields highlighted by the red arrows below. The associated limits for the control slides are established by lot and may be found on the manifest that is provided with the reagents.

Control Summary

gH2AX Control	NAP%	Total #Nuclei	Pass/Fail
gH2AX Low	0.47	58351	Pass
gH2AX Mid	4.47	61237	Pass
gH2AX High	17.31	37244	Pass

gH2AX Lot#	23C000683
Limits	
Low	2
Mid	N/A
High	14

pNBS1 Control	NAP%	Total #Nuclei	Pass/Fail
pNBS1 Low	1.50	95361	Pass
pNBS1 High	17.40	80287	Pass

pNBS1 Lot#	21J000413
Limits	
Low	3
Mid	N/A
High	7

pKAP1 Con	NAP%	Total #Nuclei	Pass/Fail
pKAP1 Low	3.001646071	110902	Pass
pKAP1 High	13.14860983	98899	Pass

pNBS1 Lot#	21J000413
Limits	
Low	4
Mid	N/A
High	9

8.4.3.9 Go to the "DDR9 Summary" tab and enter the assay operator, analysis date and report folder name as shown in (A) below. Record any deviation in the "Additional Information" section as shown in (B) below.

A

gH2AX, pNBS1 and pKAP1 PD Analysis (DDR3)

Assay Operator:	Bella	Date:	1/1/2024
Patient ID:	09938_TN0008_0056	Protocol#:	09938
Report folder Name:	Halo archive 2024-05-08 16-49 - v3.6.4134		

B

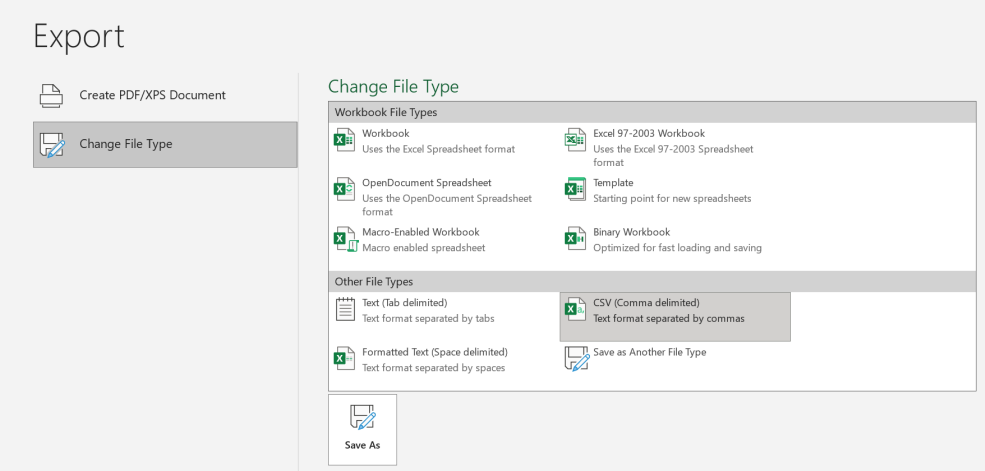
Additional Information

8.4.3.10 The DDR9 Clinical Data Report should be completed by the operator including entering the operator's name, date of the report and report folder name. The report should be reviewed by the operator and also the laboratory manager for accuracy and completeness. An example of completed DDR9 Clinical Data report can be found in Appendix 5.

8.4.4 DDR9 data reporting through Oncology Automated Reporting System (OARS)for NCLN PD studies.

8.4.4.1 For NCLN PD studies, DDR9 data should be reported through the Oncology Automated Reporting System (OARS).

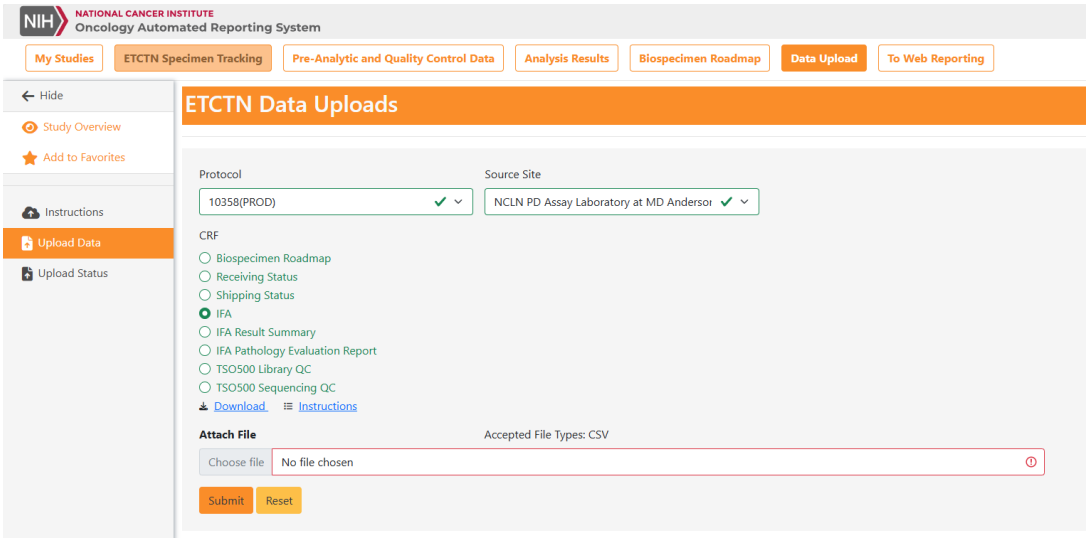
8.4.4.2 To prepare csv files for the upload, go to the "IFA_Data_Upload" tab of Master Templates DDR9 Clinical Data Report and export the tab as a csv file as shown below. Name the csv file as " Patient ID_DDR9_IFA_Data_Upload" and save it to the patient's study folder in the Halo_Image share for data upload into OARS.



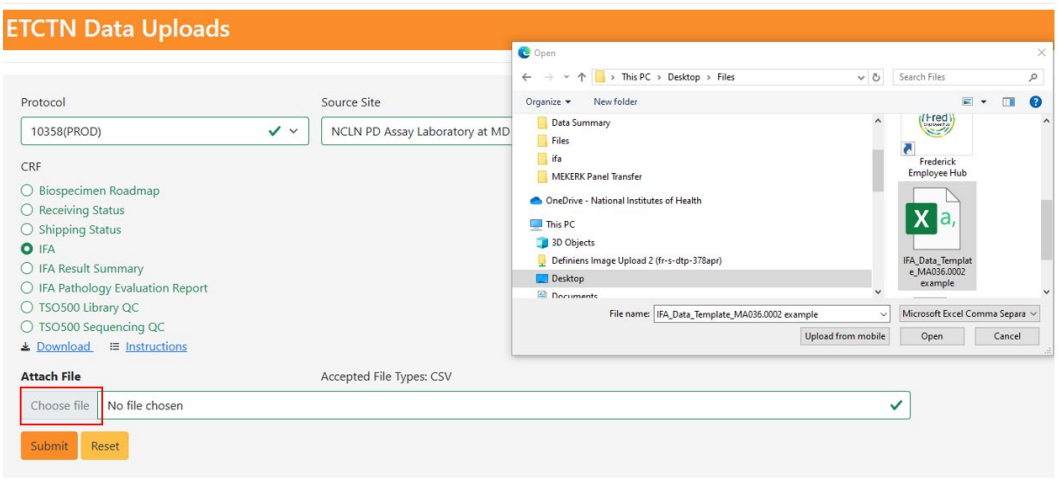
8.4.4.3 Go to the "IFA_Result_Summary_Upload" tab and export the tab as a csv file as shown following instructions in Section 8.4.4.2. Name the csv file as " Patient ID_DDR9_IFA_Results Summary_Upload" and save it to the patient's study folder in the Halo_Image share for data upload into OARS.

8.4.4.4 Log into OARS through nci-oars.com

8.4.4.5 Select "Data Upload" > "Upload Data". On the "Upload Data" page, select either "IFA" or "IFA Result Summary" for data upload, then select corresponding Protocol and Source Site "NCLN PD Assay Laboratory at MD Anderson" from the drop-down list as shown below.



8.4.4.6 Click "**Choose file**" button as circled below and select the csv file to be uploaded from the pop-up window as shown below. Click "**Submit**" once the file has been uploaded successfully.



8.4.4.7 Check the "Upload Status" tab to make sure the status of upload is "Success" to make sure the upload is successful.

8.5 DDR9 Data analysis QC and reporting can also be performed in HALO Link when HALO Client is not working. Refer to Appendix 6 for details about the process.

APPENDIX 1: BATCH RECORD

1. Image Extraction and Analysis

Facility/Lab Name:		Date:	
Operator Name:			
CTEP #:		Patient ID:	
Slide #'s:		Calibrator Lot #s:	
Name of Study Folder:			
Name and Location of Result Folder			

2. Comments:

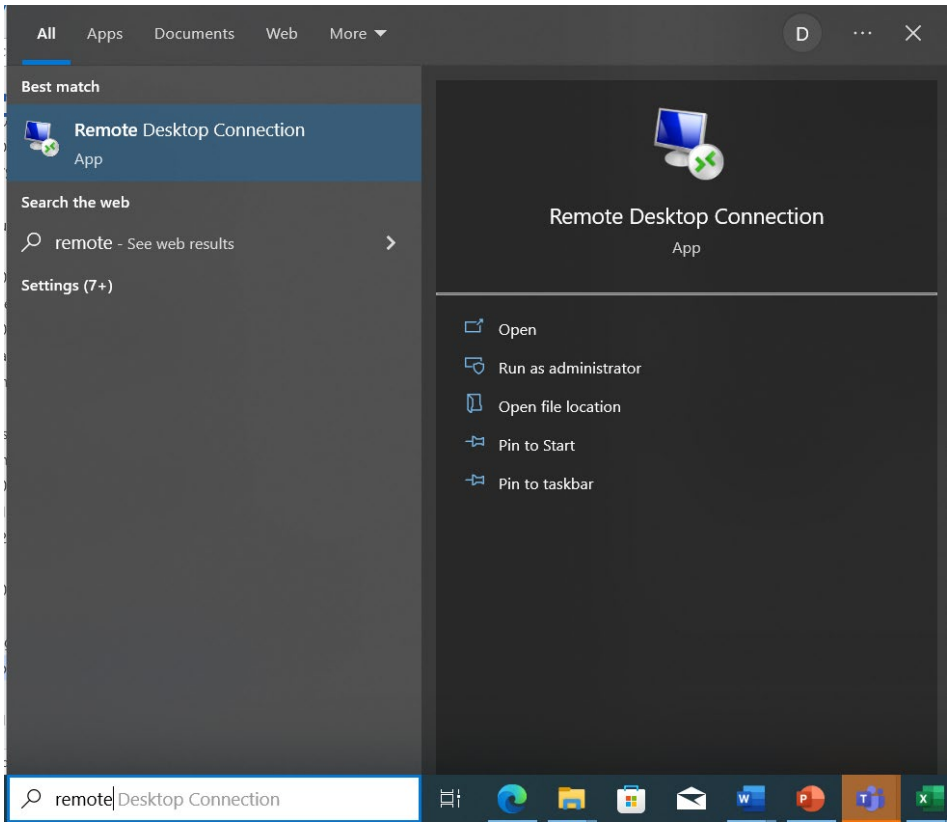
3. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (PRINT)

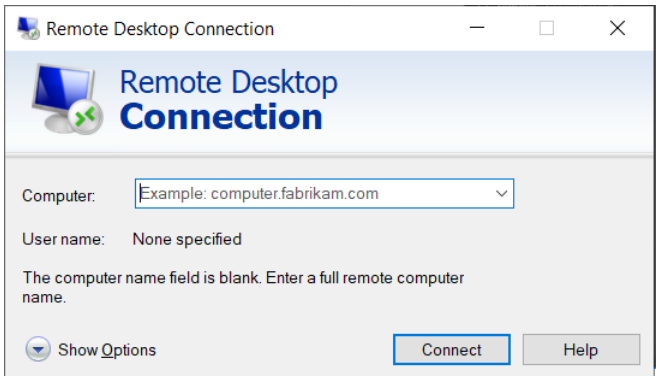
_____ (SIGN)

APPENDIX 2: INSTRUCTIONS FOR LOGGING INTO USER'S HALO VIRTUAL MACHINE

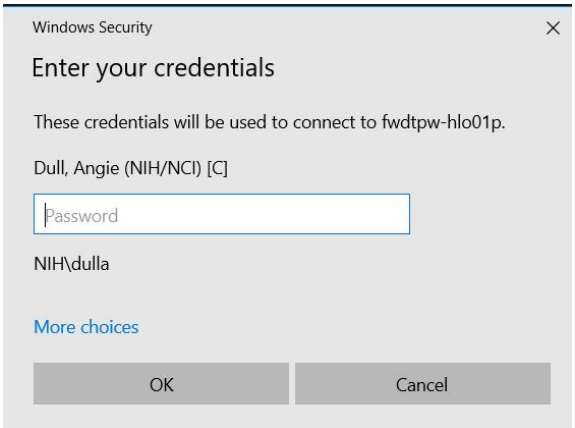
1. Go to remote desktop by typing "remote" into search bar at bottom left of your screen and select "**Remote Desktop Connection**" as shown below.



2. Type in your assigned VM name at the computer prompt and select "Connect" as shown below.

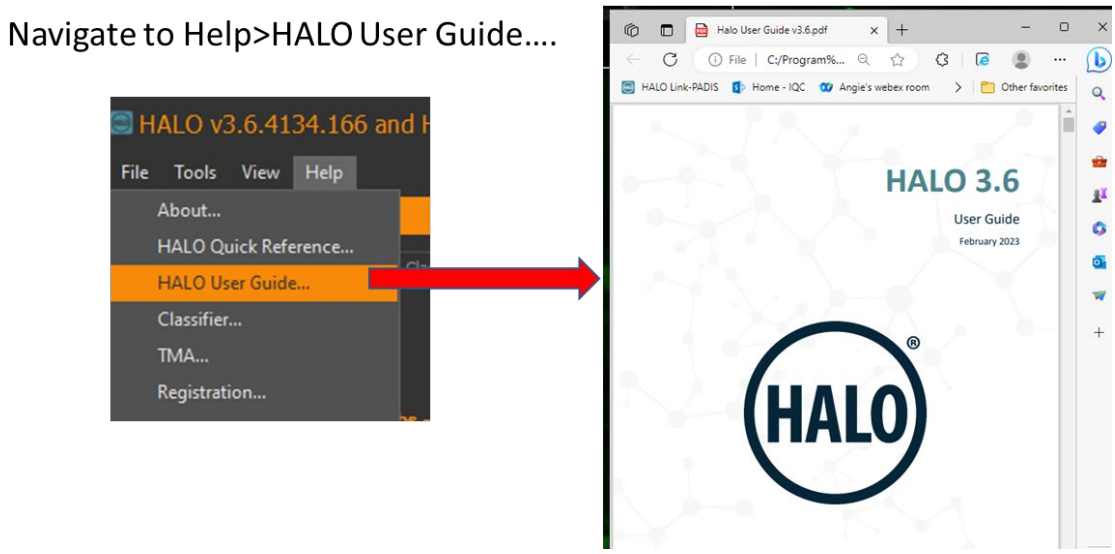


3. For PADIS user, sign in using your NIH credentials in the pop-up window as shown in below to log in.

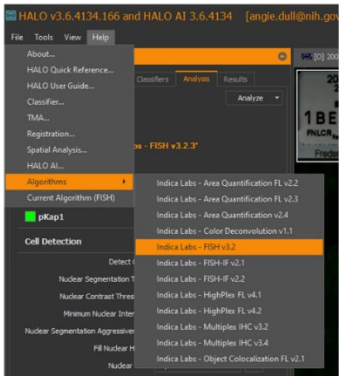


APPENDIX 3: INSTRUCTIONS ABOUT HALO USER GUIDE

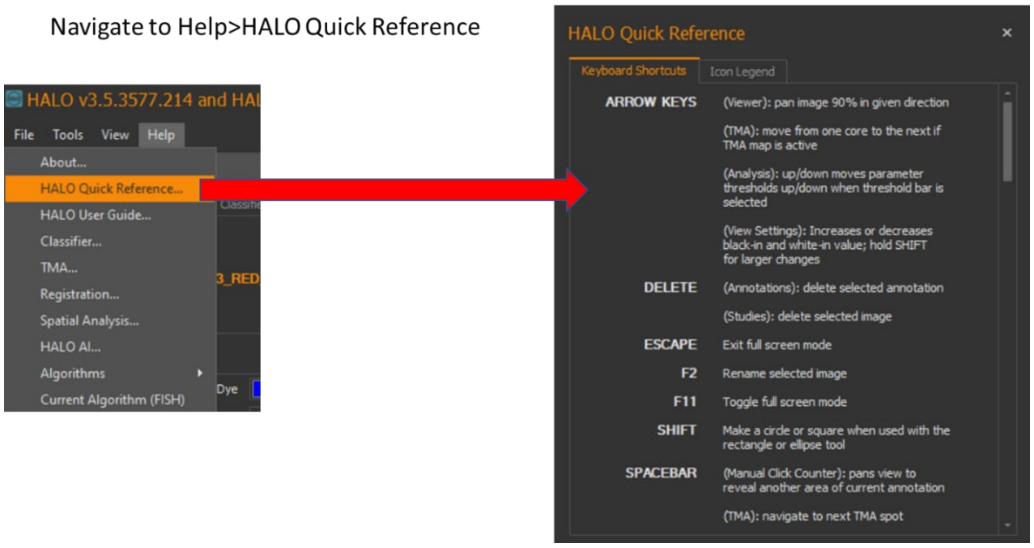
1. To access HALO User Guide, navigate to "Help" > "HALO User Guide" as shown below.



2. To access HALO algorithms, navigate to "Help" > "Algorithm", then select algorithm from the dropdown list as shown below.



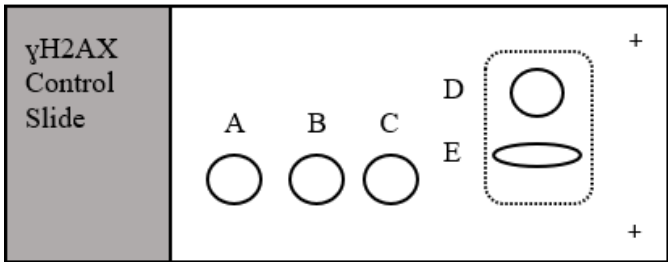
3. To access quick reference, navigate to "**Help**" > "**HALO Quick Reference**" as shown below.



APPENDIX 4: CONTROL SLIDES AND CLINICAL SLIDES

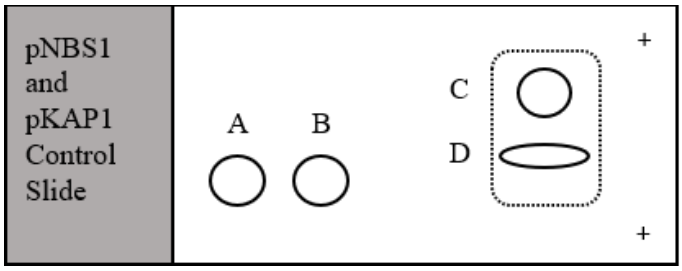
1. Control Slides

A. One γH2AX control slide is required for each clinical Bond-RX run. The layout of the γH2AX control slides used for this SOP are shown below.



Sections & Tissue – γH2AX Control Slides	
Cal-Low (A)	Xenograft (Vehicle-treated)
Cal-Mid (B)	Xenograft (Treatment A)
Cal-High (C)	Xenograft (Treatment B)
Tissue Control (D)	Mouse testes
Tissue Control (E)	Mouse jejunum

B. pNBS1 and pKAP1 biomarker control slide is required for each clinical Bond-RX run. The layout of the pNBS1 and pKAP1 control slides used for this SOP are shown below. pNBS1 and pKAP1 control slides are used for both pNBS1 and pKAP1 biomarker evaluation.

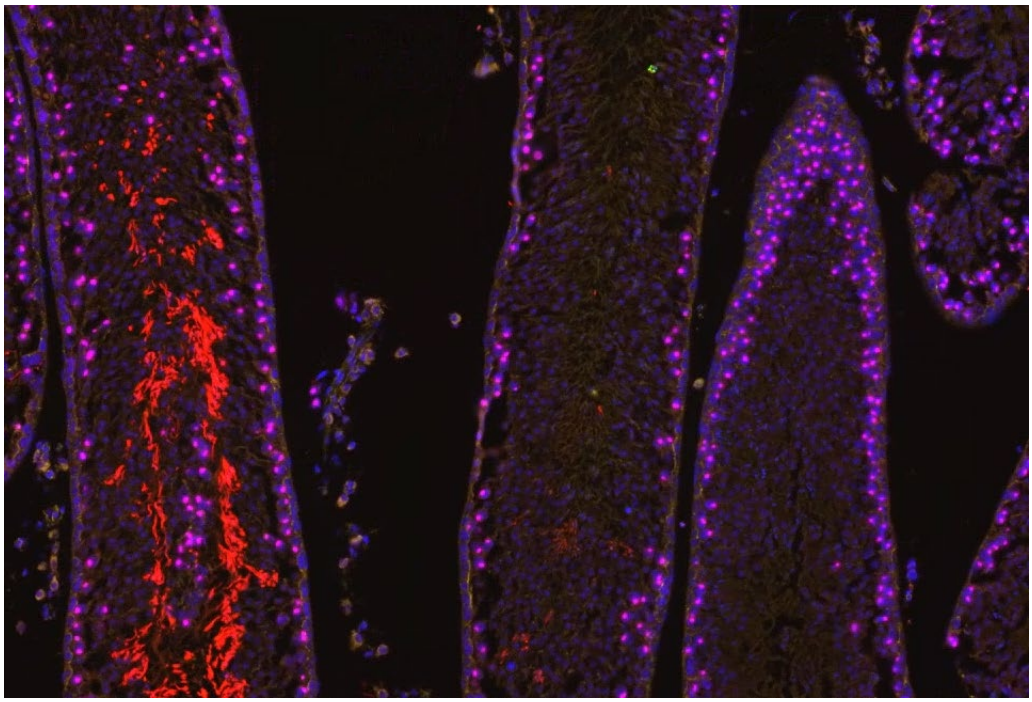


Sections & Tissue – pNBS1 and pKAP1 Control Slides	
Cal-Low (A)	Xenograft (Vehicle-treated)
Cal-High (B)	Xenograft (Treatment A)
Tissue Control (C)	Mouse testes
Tissue Control (D)	Mouse jejunum

C. Controls included on the control slides are evaluated qualitatively.

a) The jejunum should be very low or negative for γH2AX, pNBS1 and pKAP1.

b) The mouse testis should be negative for pNBS1 and pKAP1 in the nuclei throughout. Some non-nuclear staining may be observed in the center of a small subset of tubules. The spermatogonia, spermatocytes, and spermatids should be intensely stained for γH2AX. An image of the testis control showing intense staining of γH2AX is shown below.



D. γH2AX and pNBS1 control slide criteria

a) γH2AX control sections are generated from NBF-fixed, paraffin-embedded mouse xenograft tumor quadrants derived from vehicle and drug treated animal representing three different levels of biomarker expression (Controls [Con]: (1) Low, (2) Mid, and (3) High). The controls serve as a visual reference standard for drug effect on target.

b) pNBS1 control sections are generated from NBF-fixed, paraffin-embedded mouse xenograft tumor quadrants derived from vehicle and drug treated animal representing two different levels of biomarker expression (Controls [Con]: (1) Low and (2) High). The controls serve as a visual reference standard for drug effect on target.

c) The specifications for acceptable biomarker levels in the controls are determined by the control slide lot.

d) An example of control slide data is shown below.

Control Summary

gH2AX Control	NAP%	Total #Nuclei	Pass/Fail
gH2AX Low	0.47	58351	Pass
gH2AX Mid	4.47	61237	Pass
gH2AX High	17.31	37244	Pass

gH2AX Lot#	23C000683
Limits	
Low	2
Mid	N/A
High	14

pNBS1 Control	NAP%	Total #Nuclei	Pass/Fail
pNBS1 Low	1.50	95361	Pass
pNBS1 High	17.40	80287	Pass

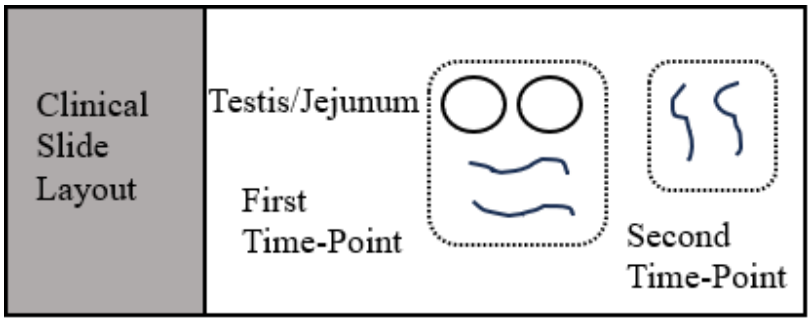
pNBS1 Lot#	21J000413
Limits	
Low	3
Mid	N/A
High	7

pKAP1 Con	NAP%	Total #Nuclei	Pass/Fail
pKAP1 Low	3.001646071	110902	Pass
pKAP1 High	13.14860983	98899	Pass

pNBS1 Lot#	21J000413
Limits	
Low	4
Mid	N/A
High	9

2. Clinical Slides

A. Clinical samples for this assay will be frozen needle biopsies collected according to SOP340507 or SOP340567. A representative clinical slide layout is shown below. In some cases, there may be only one pass embedded for each time point.



B. Two or three slides are pre-stained with H&E and are used to evaluate the tissue quality prior to ɣH2AX, pNBS1, pKAP1 and β-Catenin staining.

C. Typically, slides submitted for IFA staining represent 2-3 nonconsecutive sections from the range of slides determined to be optimal based on visual inspection and H&E evaluation.

D. Backup slides are available for use if the first slide set does not meet QC criteria for biomarker staining and analysis.

APPENDIX 5: EXAMPLE OF A COMPLETED DDR9 CLINICAL DATA REPORT

gH2AX, pNBS1 and pKAP1 PD Analysis (DDR3)

Assay Operator:	Bella	Date:	1/1/2024
Patient ID:	09938_MA0036_0054	Protocol#:	09938
Report folder Name:	Halo archive 2024-05-07 10-39 - v3.6.413		

Time Point	C1D02					
Biomarker	gH2AX		pKap1		pNbs1	
PADIS Specimen ID	09938_MA0036_0054_501A	09938_MA0036_0054	09938_MA0036_0054	09938_MA0036_0054	09938_MA0036_0054	09938_MA0036_0054_5
RAVE Specimen ID	09938-1874GJ41-1	09938-1874GJ41-1	09938-1874GJ41-1	09938-1874GJ41-1	09938-1874GJ41-1	09938-1874GJ41-1
25	0.30	0.09	0.03	0.03	0.07	0.12
36	0.42	0.10	0.13	0.02	0.17	0.15
23	0.28	0.09	0.06	0.05	0.19	0.21
Specimen Average	0.33	0.09	0.08	0.04	0.14	0.16
Total Specimen Nuc	9779	46995	9735	46834	9698	46132
Time Point Average	0.21		0.06		0.15	
Time Point Std	0.14		0.04		0.05	
Total Time Point Nu	56774		56569		55830	

Time Point						
Biomarker	gH2AX		pKap1		pNbs1	
PADIS Specimen ID						
RAVE Specimen ID						
Specimen Average						
Total Specimen Nuc						
Time Point Average						
Time Point Std						
Total Time Point Nu						

Time Point						
Biomarker	gH2AX		pKap1		pNbs1	
PADIS Specimen ID						
RAVE Specimen ID						
Specimen Average						
Total Specimen Nuc						
Time Point Average						
Time Point Std						
Total Time Point Nu						

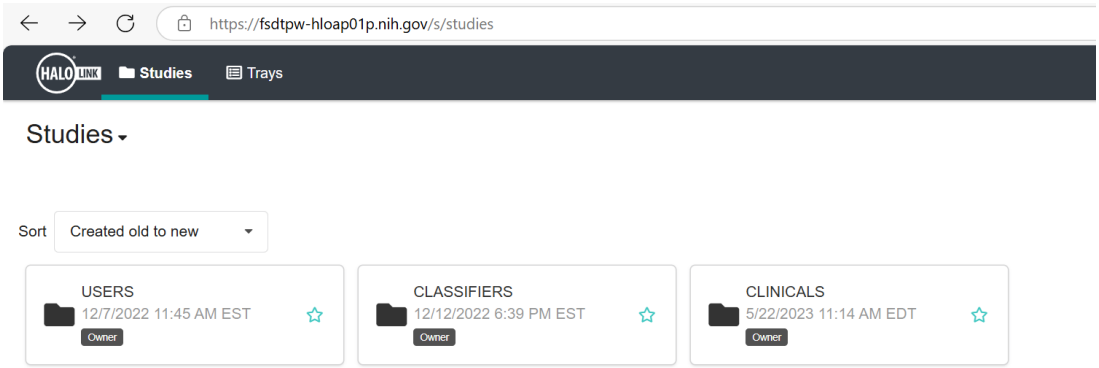
Control Slide Data Summary		
gH2AX control		
	%NAP	Pass/Fail
Low	0.47	Pass
Mid	4.47	Pass
High	17.31	Pass
pNBS1 control		
	%NAP	Pass/Fail
Low	1.50	Pass
High	17.40	Pass
gKAP1 control		
	%NAP	Pass/Fail
Low	3.001646071	Pass
High	13.14860983	Pass

Additional Information

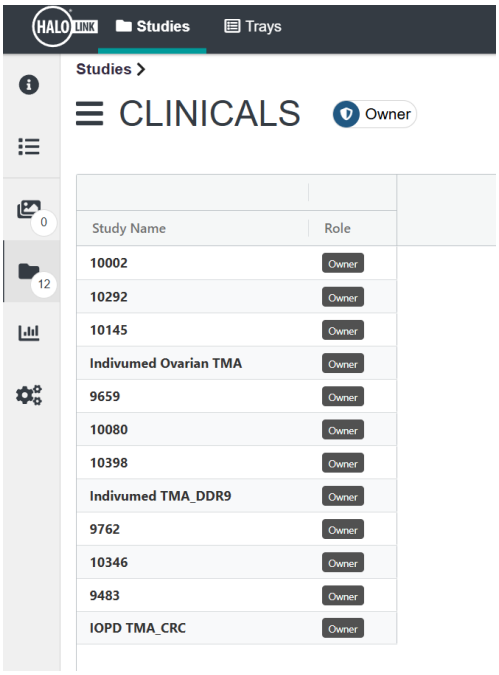
APPENDIX 6: HALO LINK DATA ANALYSIS QC PROCESS

1. Log into HALO Link in Chrome or Microsoft Edge (https://fsdtpw-hloap01p.nih.gov/s/studies), open the appropriate "Group Folder" and navigate to the appropriate "Studies" folder to locate a specific analysis. Click on "Study Folder" as shown in (A) below to enter study and click on the folder ion on the left to view the study subfolders as shown in (B) below. Click the image icon on the left to view the list of images in the subfolder as shown in (C) below. Double click on an image thumbnail to open the image and review results.

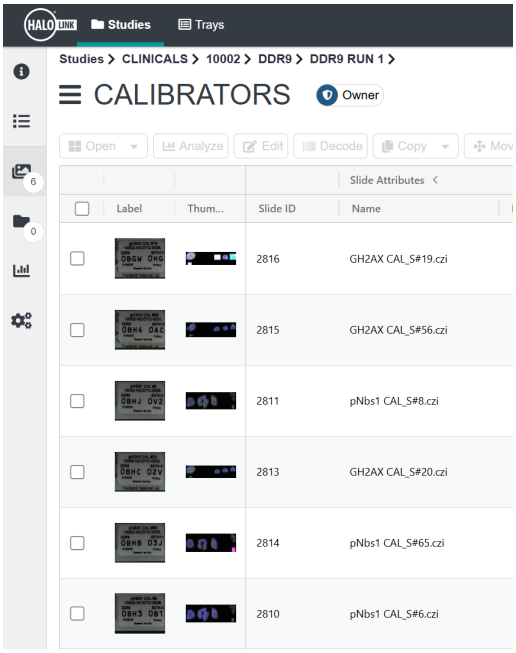
A



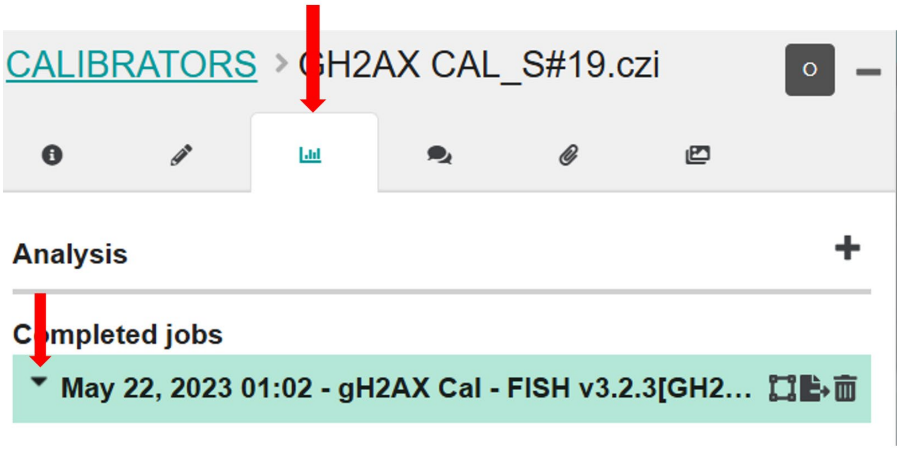
B



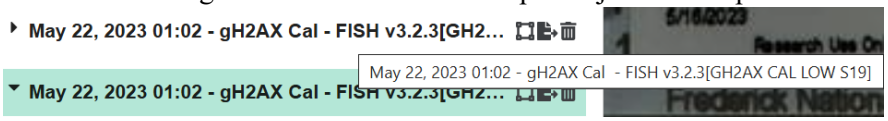
C



2. "Analysis" tab can be used to view the analysis results per image. Click on the "Analysis" tab icon to view completed jobs and click the triangle before the complete analysis name to select the appropriate analysis for QC as shown in below.

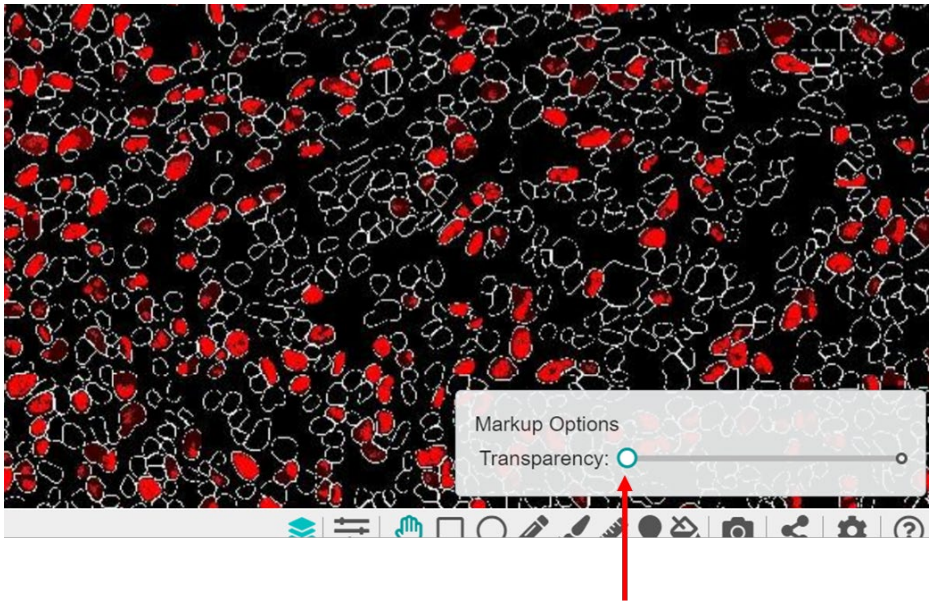


NOTE: Hovering the mouse over each completed jobs will expand the full name of the analysis.



A. View **Tumor Classifier Mask** by clicking on the layer tool and selecting " **Analysis Mark-up** " and follow guideline in Section 8.2.3 to review tumor segmentation and make adjustments in HALO when any segmentation issues have been identified.

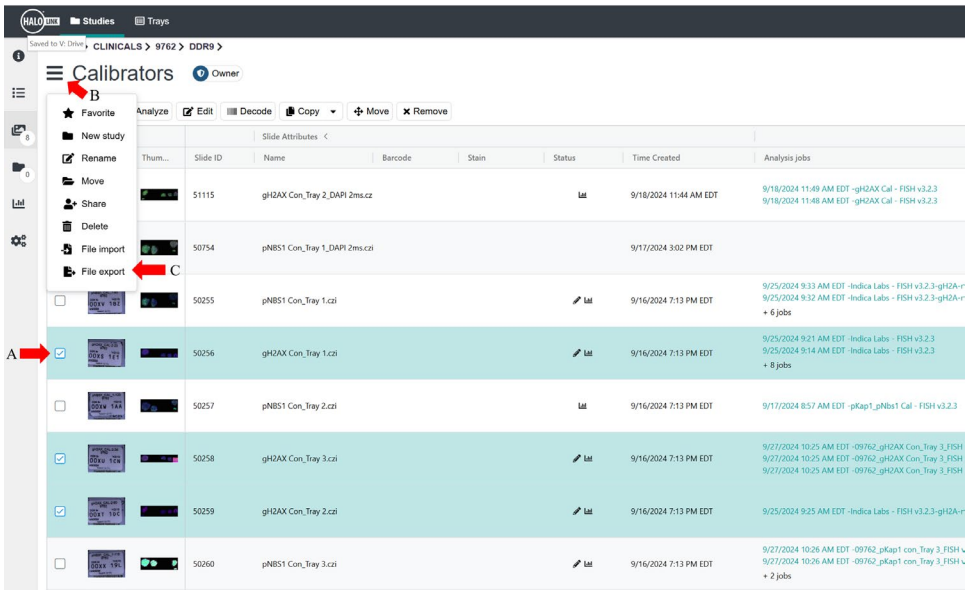
B. **View Marker Analysis** by clicking on the layer tool and selecting "**Analysis Mark-up**" in the drop-down list as shown below. To adjust the overlay, utilize the "**Transparency**" slider bar found in the layer tool as shown below. Turn off all channels except for the marker channel that requires image QC. Toggle marker masks on/off to assess marker thresholds. Follow guideline in Section 8.2.4 to review marker mask and make adjustments in HALO® when issues with marker masks have been identified.



C. Repeat process to complete QC pKap1, pNbs1, and γH2AX.
D. If the image analysis mark-up is over-thresholding or under-thresholding for the marker, the user can re-analyze the data following Section 8.2.5, and then remove the unwanted analysis in HALO following Section 8.2.6.

3. Summary Data Export from HALO Link

HALO Link Export is typically done to include manifest data corresponding to selected images in the study. Select an image to export data by checking the box in front of image as shown by arrow (A) below. Click the button in front of the study name as shown by arrow (B) below and select "File Export" from the dropdown list as shown by arrow (C) below. This will prompt a dialogue box listing what types of data will be exported, select the "**Export Study**" button and a .csv file will be downloaded.



4. Data Report from HALO Link

Open the downloaded csv file and analyze the data following instructions in Section 8.4.2.
NOTE: Data exported from HALO Link will not be compatible with Master Templates for DDR9 Clinical Data Report.