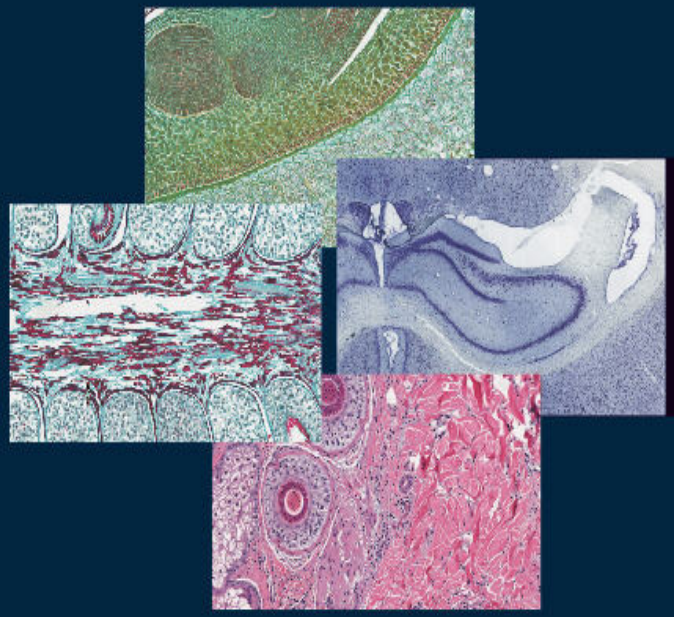


APERIO UNIVERSITY

Image Analysis 101

Course Objectives

Upon conclusion of this training session, learners will be able to complete the following tasks related to their job:

- Describe how Aperio's Image Analysis tools work
- Describe Aperio's Image Analysis tools for Area, Cell, and Event Quantification
- Understand the input parameters for each Image Analysis tool
- Understand the output parameters for each Image Analysis tool
- Complete the steps for running and Aperio Image Analysis tools
- Calibrate stain color using Color Deconvolution
- Create a macro and register it with Spectrum
- Run a macro using Spectrum Plus batch analysis option
- Export Image Analysis results using Spectrum's Export Data tool
- Describe Aperio's Scalable Architecture

Several Aperio algorithms have been cleared by the FDA for clinical use when used on ScanScope models that are labeled as approved medical devices, and are intended for research use for other applications.

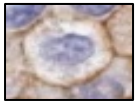
Terminology Table

Image Analysis	The extraction of meaningful information from digital images by means of digital image processing techniques.
Deconvolution	Image processing technique that removes the effects of convolution from measured data.
Colocalization	Analysis technique that determines the contribution of each stain at every pixel location.
Intensity	The measure of brightness of the pixel. Intensity ranges from 0 = black to 255 = bright white.
Color Vector	The normalized optical density for the Red (R), Green (G), Blue (B) components that represent a specific color (stain).
Immunohistochemistry (IHC)	A method for staining cells; antibodies to specific proteins are used to analyze specimens and identify specific types of cells, especially for certain types of cancer.
Angiogenesis	The formation of new blood vessels.

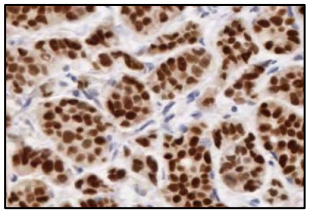
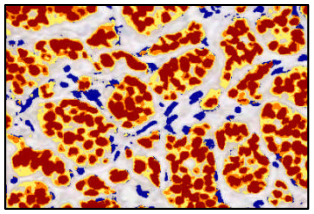
Image Analysis Illustrated

Image Analysis extracts meaningful data:

- **Stains are used to highlight features.**
  - Tissue and cell components are revealed.
    - Nuclei, membranes and cytoplasm.

  - Specific protein expressions such as, ImmunoHistoChemistry panels.
    - HER2, ER and PR.
- **Image analysis can help answer questions.**
  - Where and how much staining is there?
  - Where and how many objects are there?
    - Tumor cells.
  - How much staining is there on different objects?
    - Cell nuclei.
    - Cell membranes.

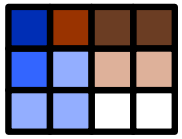
Original ***Markup***

Image Analysis tools can present a markup image to highlight analysis results.

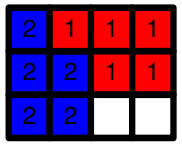
Image Analysis Illustrated

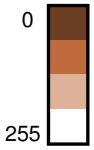
How Image Analysis works:

- **Analyzing a digital slide by applying an algorithm directly to the digital slide or selected region of the digital slide.**
  - Stained slides result in a multicolored image. Image analysis acquires the RGB color of the pixels.

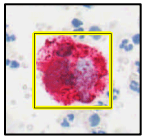


- **Image Analysis tools organize the color (RGB pixels) of stains.**
  - Classifies pixels by color (stain).
  - Measures pixel (stain) intensities.
  - Indentifies objects by color, shape or size.
  - Measures object (stain) intensities.





Intensity: 0 = black & 255 = white



Rare Event

Nucleus

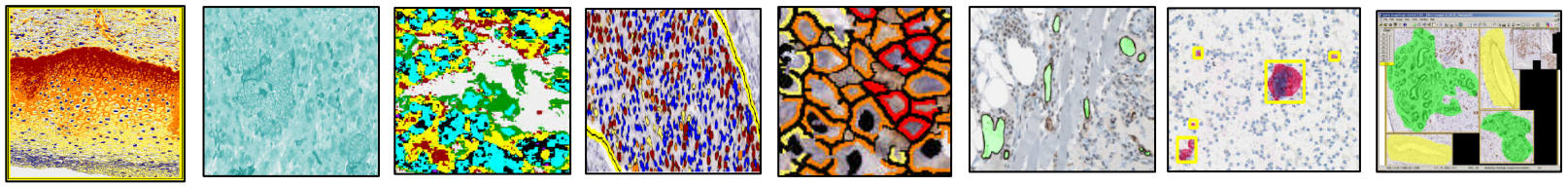
Membrane

Image Analysis Tools

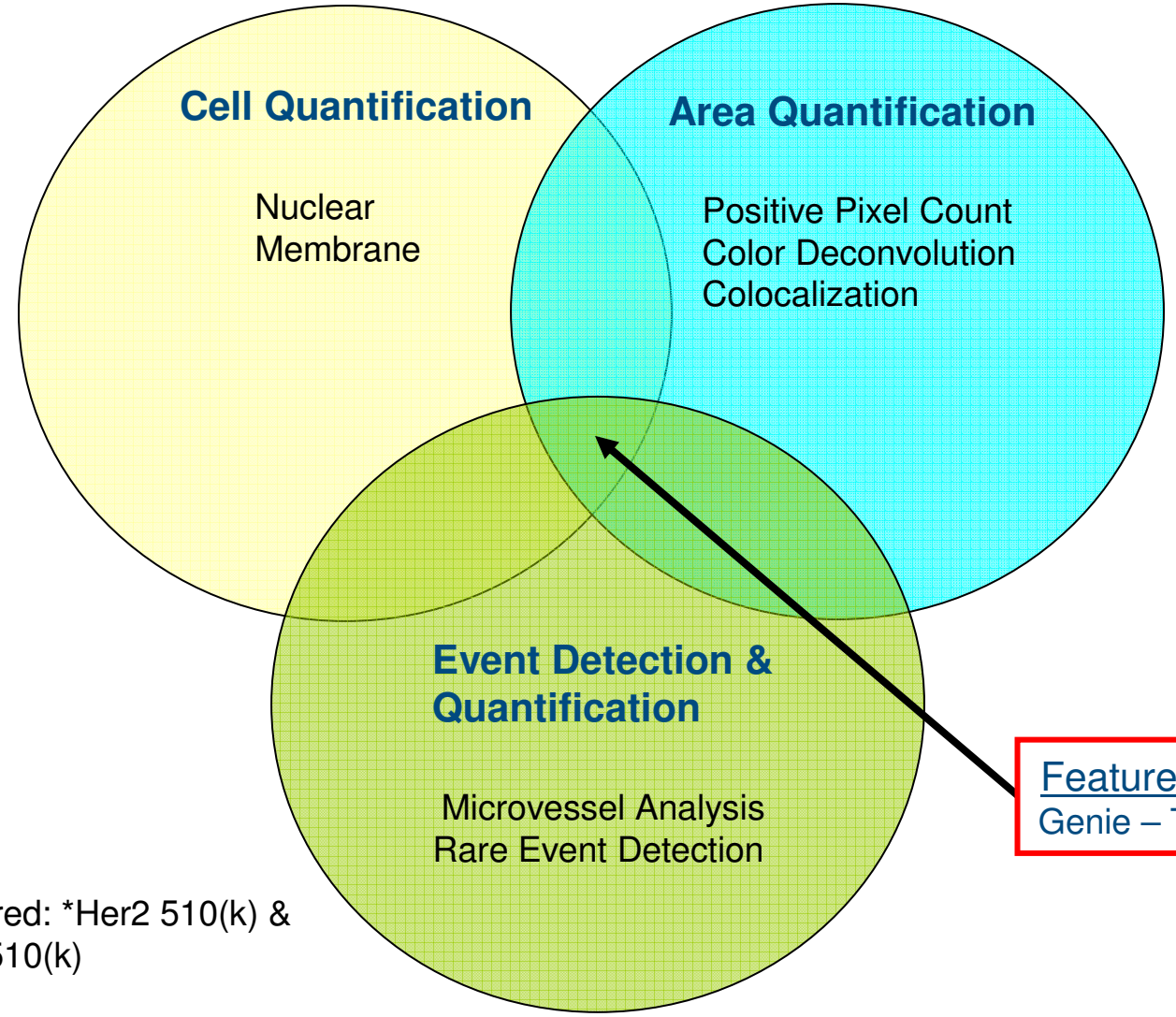
Image Analysis tools and their specific applications:

- **Positive Pixel Count* –** quantifies the area and intensities of positive and negative staining.
- **Color Deconvolution** –** accurately separates up to 3 stains and measures area and intensity separately, resolving multi-stain colocalization problems.
- **Colocalization –** quantifies the area and intensity of “colocalized” markers.
- **Nuclear –** quantifies nuclear staining and intensity.
- **Membrane –** quantifies membrane staining and intensity.
- **Microvessel Analysis –** detects and quantifies microvessels.
- **Rare Event Detection –** detects micrometastasis of tumor cells.
- **Digital IHC Analysis** – streamlined workflow for IHC analysis.
- **Genie** – smart tissue classifier.

*Positive Pixel Count is licensed without fee with other Aperio Software.
**Color Deconvolution is our professional version of our Positive Pixel Count algorithm.



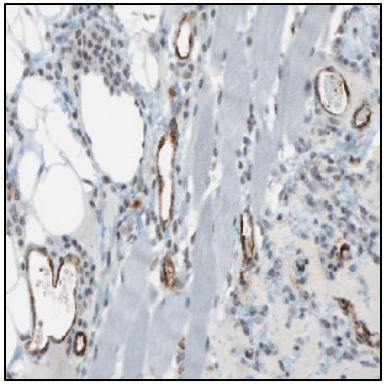
Aperio Image Analysis Capabilities



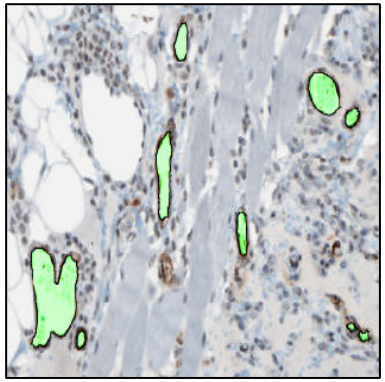
FDA Cleared: *Her2 510(k) &
**ER/PR 510(k)

Image Analysis Applications

Blood Vessels

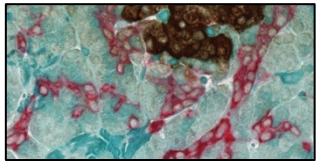


Original

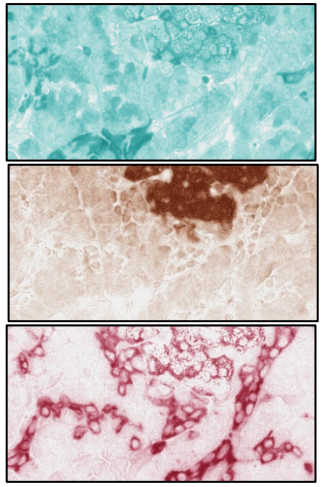


Markup

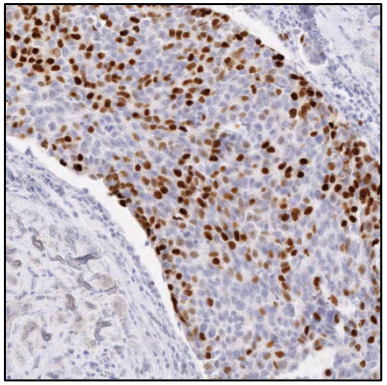
Stain Separation



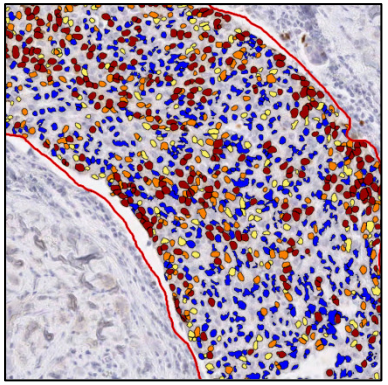
3 colors



Nuclear Staining Quantification

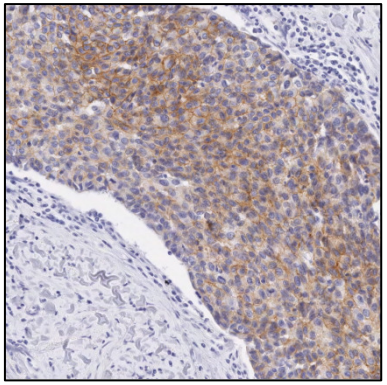


Original

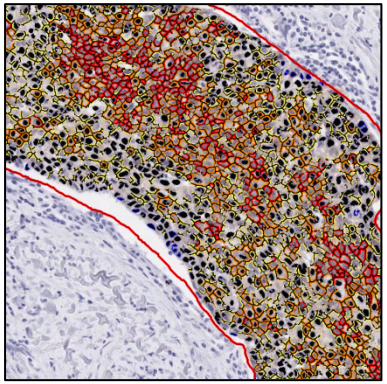


Markup

Membrane Staining Quantification



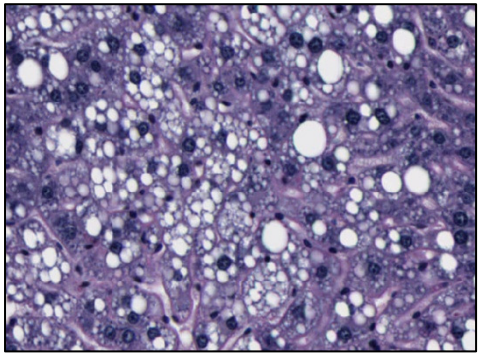
Original



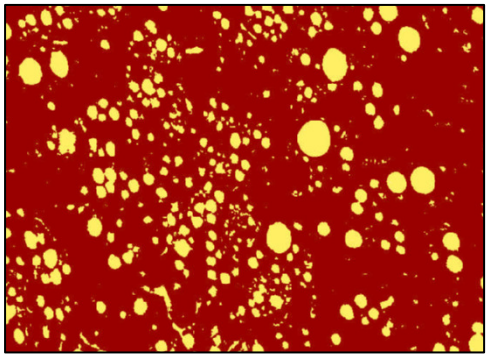
Markup

Image Analysis Applications

Fatty Vacuoles

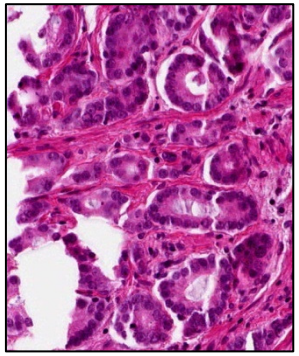


Original

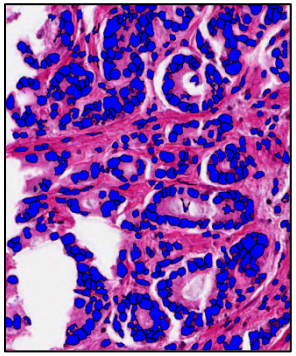


Markup

H&E Nuclear Counting

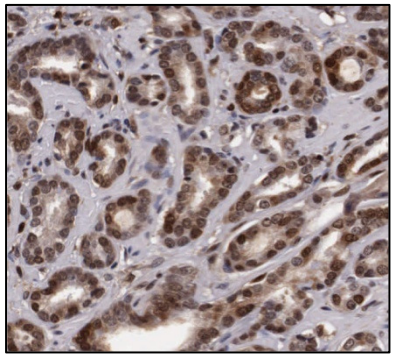


Original

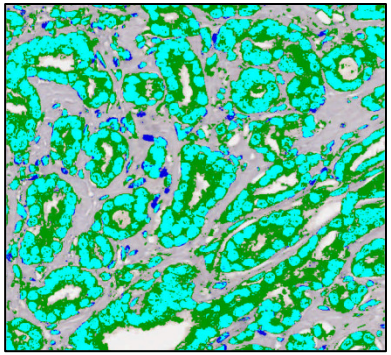


Markup

Cytoplasmic Quantification

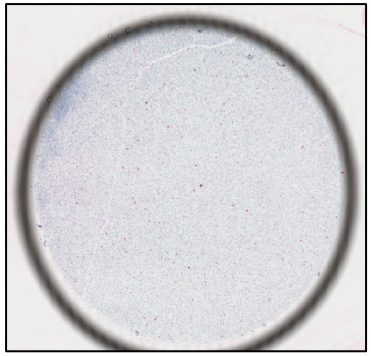


Original

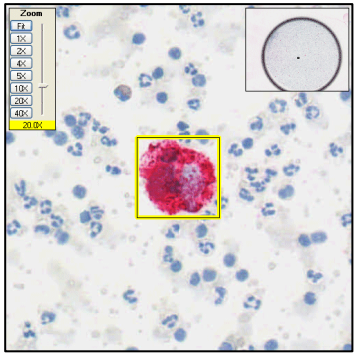


Markup

Rare Event Detection



Original



Markup

Performing Analysis

Run Analysis several ways.

- **ImageScope Analysis**
  - Use the Analysis command of ImageScope to analyze either a local digital slide image (that is, an image on your workstation or on the network where your workstation can see it via Microsoft file sharing).
  - Use ImageScope to connect an image on an Aperio ImageServer.
- **Spectrum Plus Analysis**
  - Analyze a single digital slide image that resides on the ImageServer by using Spectrum's **Analyze** command.
- **Batch Analysis**
  - Analyze a batch of digital slide images that reside on the ImageServer by selecting multiple images and using Spectrum's **Analyze** command.

In most cases, it is expected that a digital slide will be opened in Spectrum. For the purpose of training all images will be opened in Spectrum.

Performing Analysis

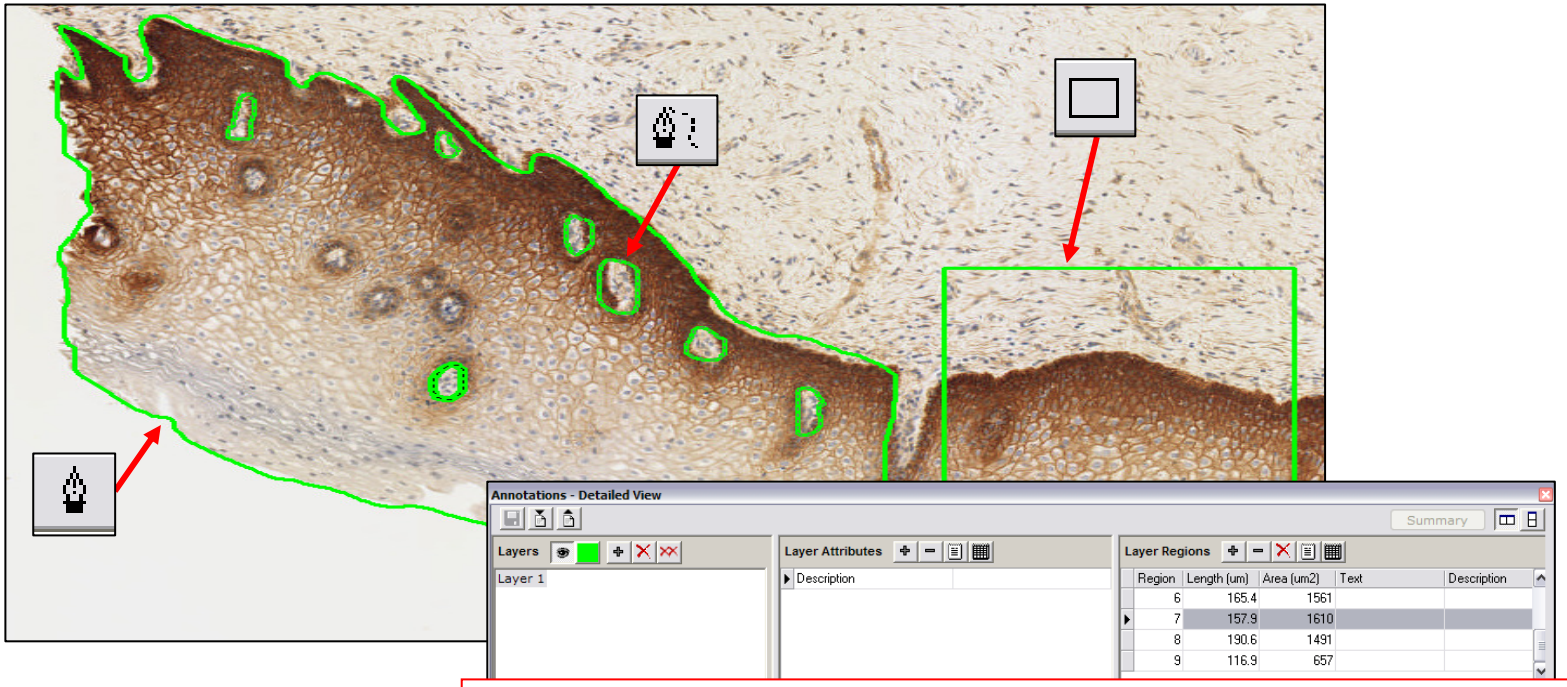
Selecting Areas to Analyze

- Analyze entire digital slide or selected areas.
- Use ImageScope drawing tools to select or exclude areas to analyze.


Pen - draw free-form area of interest.


Negative Pen – draw free-form area to *exclude* from analysis.


Rectangle – draw a rectangular area of interest.

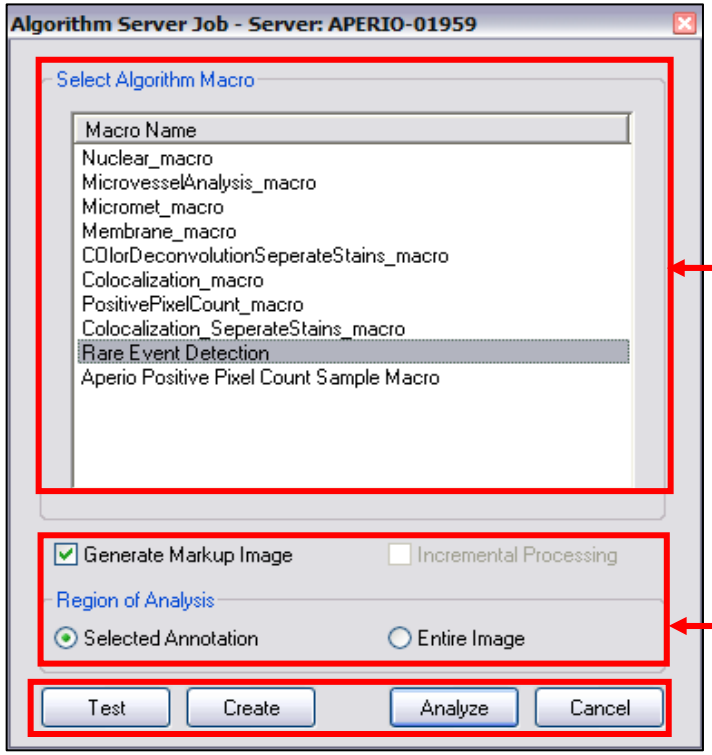


View annotation information in ImageScope's Annotations window

Performing Analysis

Analysis Window

- When performing analysis via Spectrum the Algorithm Server Job displays registered macros.



List of macros

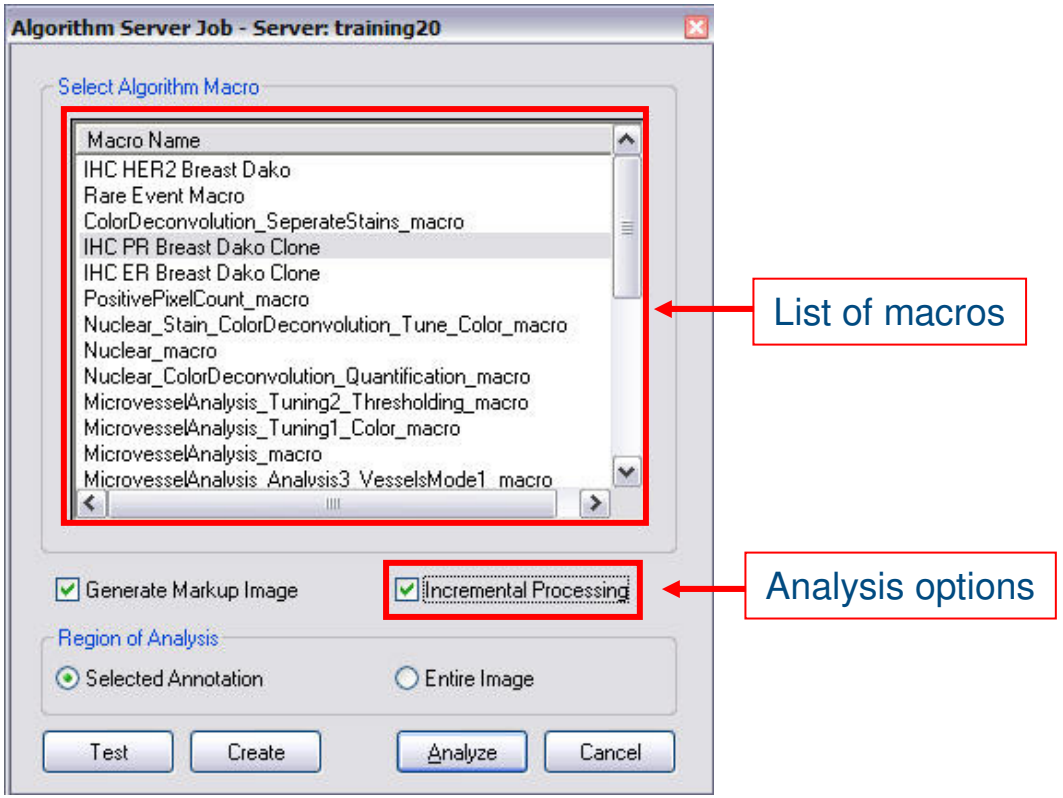
Analysis options

Analysis Buttons
Test – modify existing macro for selected algorithm and test before saving.
Create – creates new macro based on the original algorithm.
Analyze – run analysis.
Cancel – cancel current analysis job.

Performing Analysis

Incremental Processing

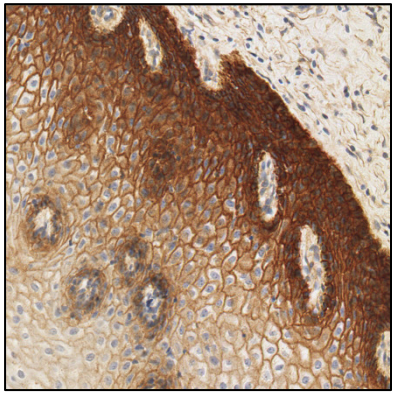
- Some Aperio algorithms support incremental processing.
- Incremental processing allows the algorithm to analyze only regions added after the initial analysis without re-analyzing the previously analyzed regions.



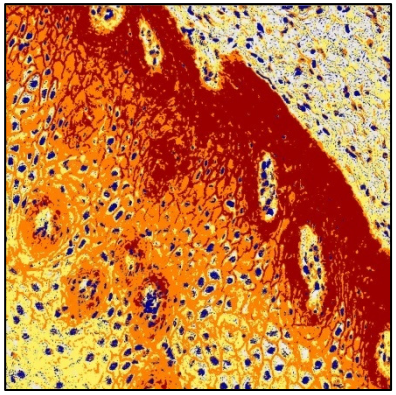
Positive Pixel Count

PPC quantifies the area & intensity of two stains.

- Positive Pixel Count (PPC) looks for positive, negative and neutral areas of staining by analyzing the slide staining against user defined values.



Original



Markup

The markup image color-codes the analyzed pixels in the following format: **Negative, Weak, Medium, Strong Positive**

- **Outputs:**
  - Negative, Weak, Medium, Strong Positive Staining
    - Number of pixels
  - Intensity sum
  - Average intensity of weak positive pixels
  - Ratio of strong/total number

Positive Pixel Count

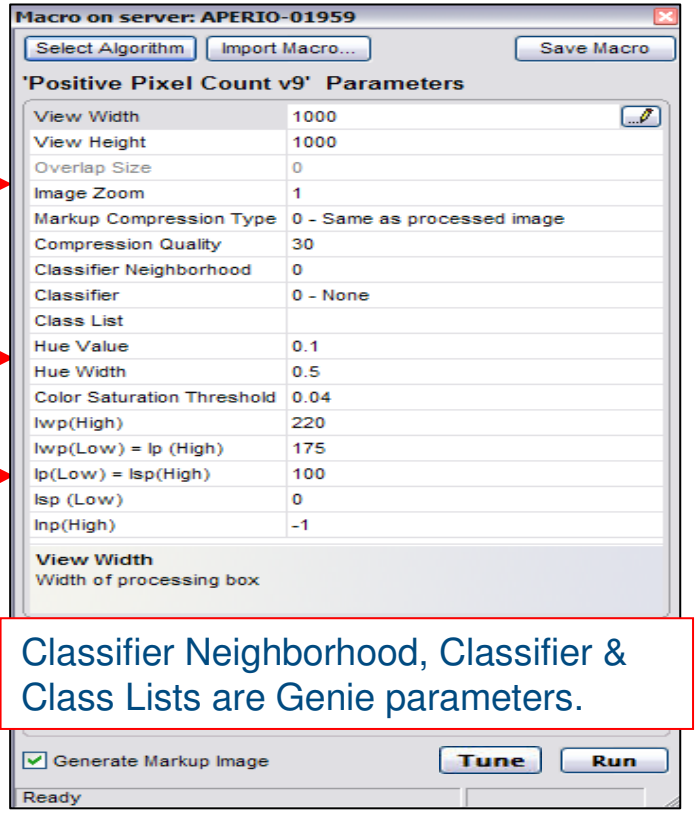
PPC has a set of input parameters.

- Input parameters allow the user to define the positive stain color and intensity thresholds for the positive stain using **HSI (Hue, Saturation, Intensity) color model**.
  - The user will specify a color (range of hues and saturation) and three intensity ranges (weak, positive, and strong).
  - PPC input parameters are defaulted for brown color quantification.

Default parameters for ScanScope images

Color defining parameters

Intensity ranges parameters



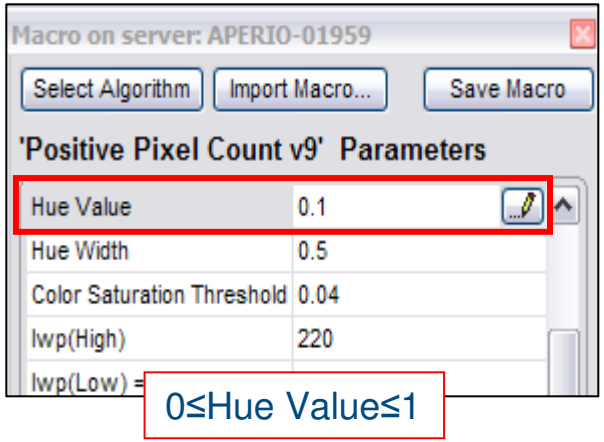
Classifier Neighborhood, Classifier & Class Lists are Genie parameters.

Positive Pixel Count

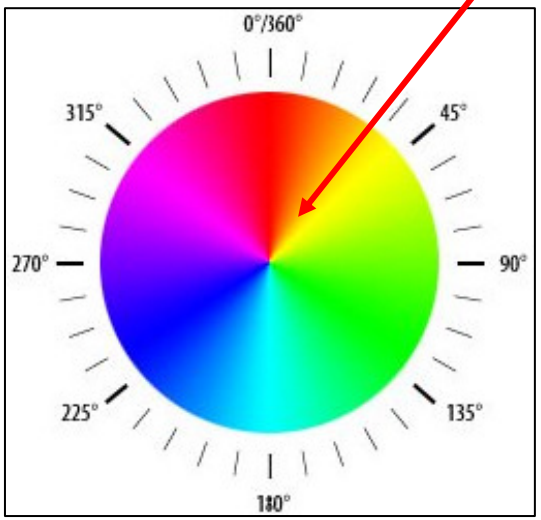
PPC input parameters:

- The **Hue Value**, and **Hue Width** can be adjusted to define the color to match the specific stain being used.
  - Hue Value – position on the color wheel for positive color.
  - This parameter defines the color of the positive stain.

Color: position on color wheel/360 = Hue Value



Brown: 36/360 = 0.1

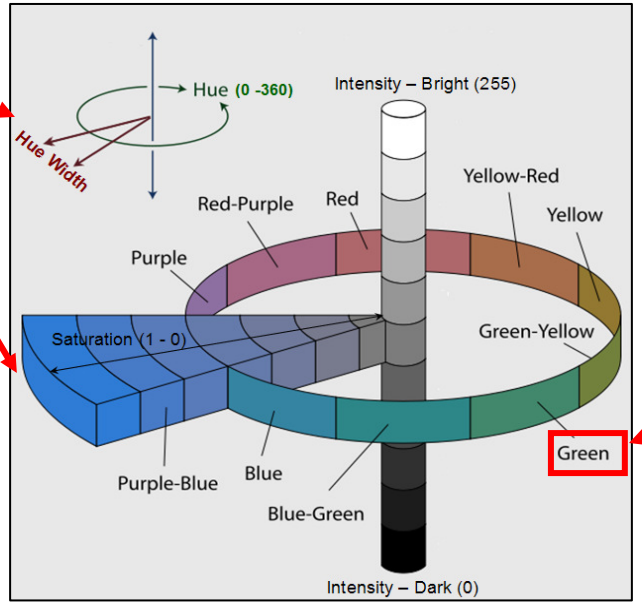


Positive Pixel Count

PPC input parameters:

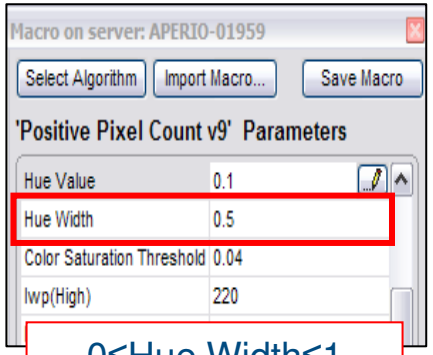
- Adjust the **Hue Width** to define the ranges of hues for analysis.
  - Hue Width is the range of hues, centered on the Hue Value that will be analyzed.
  - The larger the Hue Width, a larger range of Hues will be included for positive color.
  - The lower the Hue Width, a smaller range of Hues will be acceptable.

Hue Width



A Hue Width between 0.33 and 0.5 is usually reasonable.

Hue Value

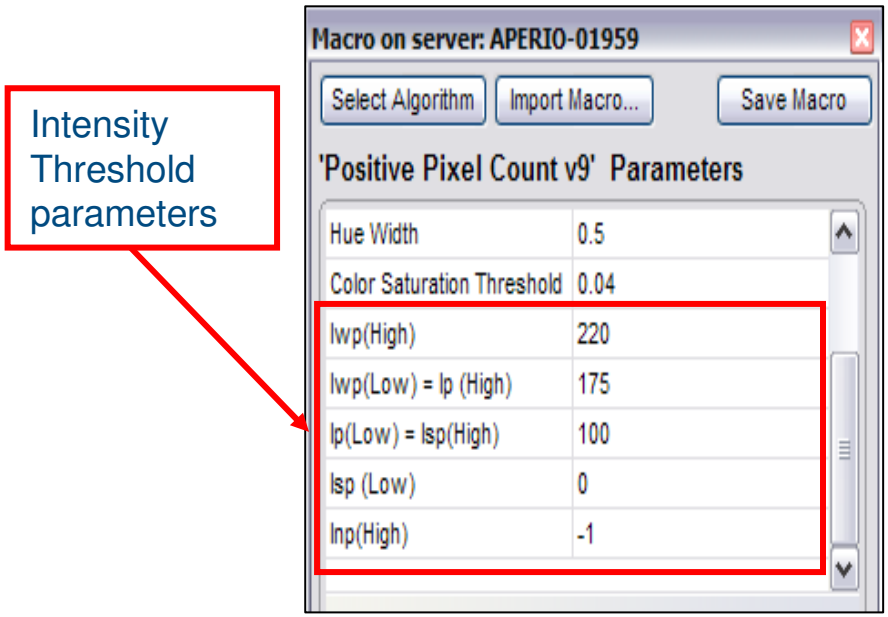


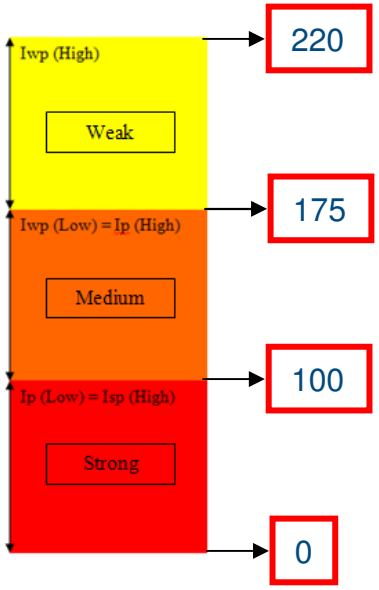
0≤Hue Width≤1
0=Narrow & 1=Wide

Positive Pixel Count

PPC input parameters:

- PPC classifies each pixel as either negative, weak positive, positive, strong positive or non-tissue (clear glass) based on its color and intensity.
- Positive stain color intensity results are separated into three color coded ranges.
  - Intensity weak positive (Iwp) = yellow
  - Intensity positive (Ip) = orange
  - Intensity strong positive (Isp) = red

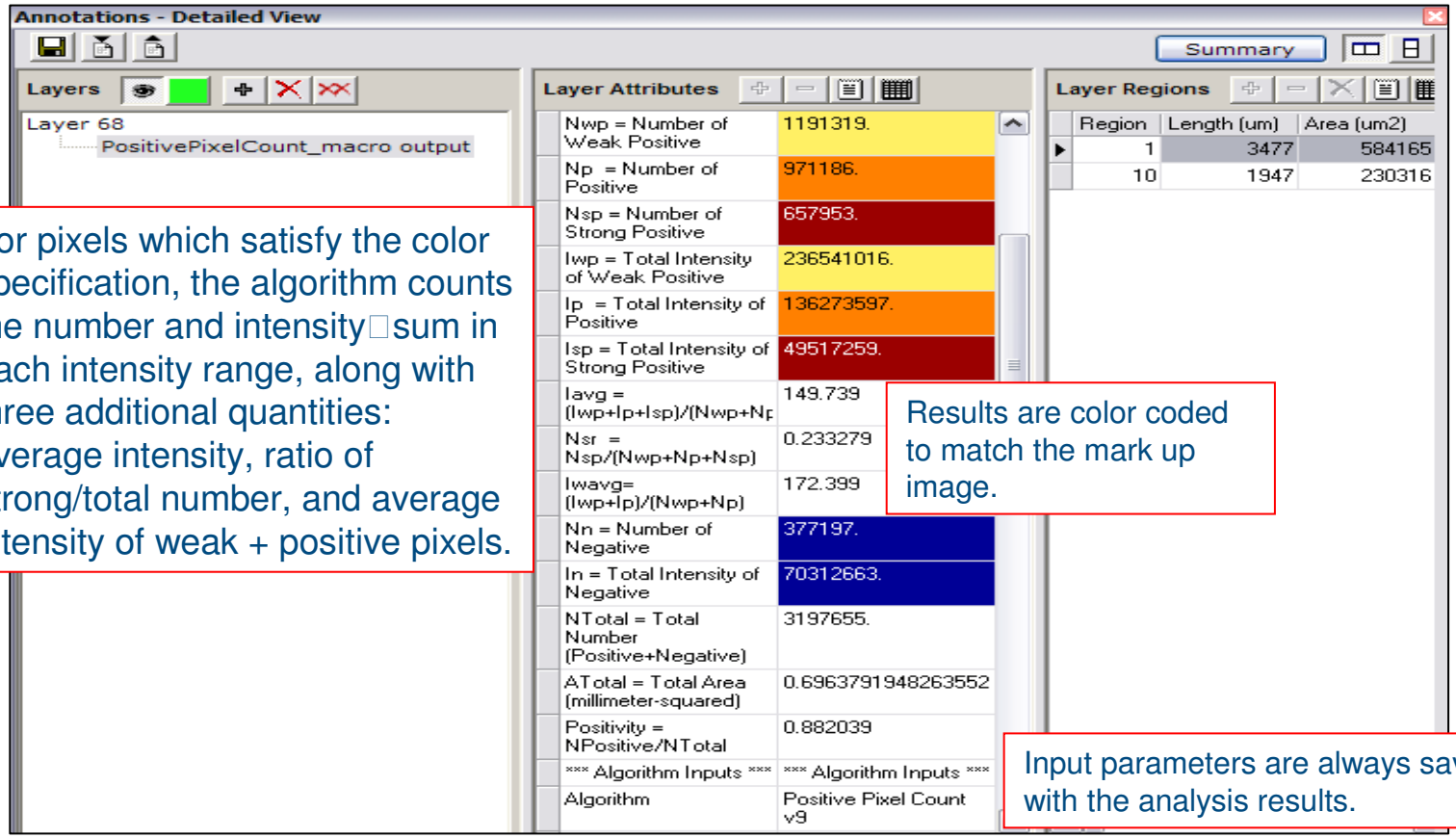




Positive Pixel Count

PPC analysis results:

- Analysis results are viewed from the **Annotations Window** in ImageScope.
- PPC analysis results are area based quantification and intensity values.
  - If running analysis in ImageScope, a false color Markup image can be generated.



For pixels which satisfy the color specification, the algorithm counts the number and intensity sum in each intensity range, along with three additional quantities: average intensity, ratio of strong/total number, and average intensity of weak + positive pixels.

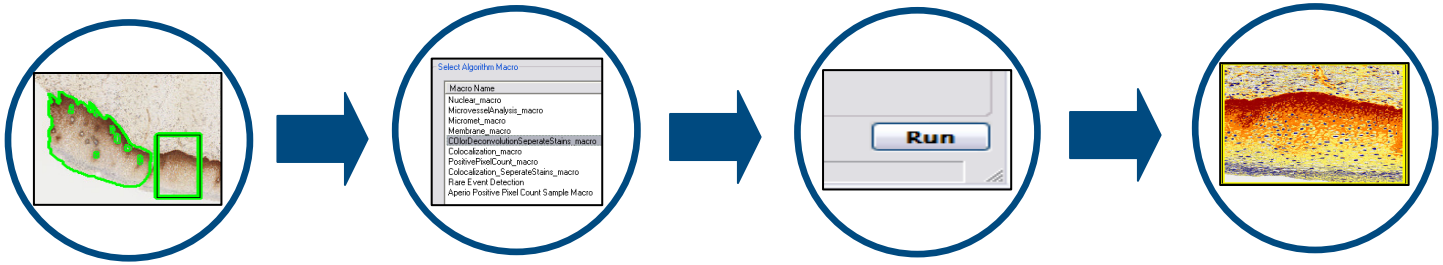
Results are color coded to match the mark up image.

Input parameters are always saved with the analysis results.

PPC Workshop

Watch Instructor demo the tasks below and perform them using the steps beginning on the next page:

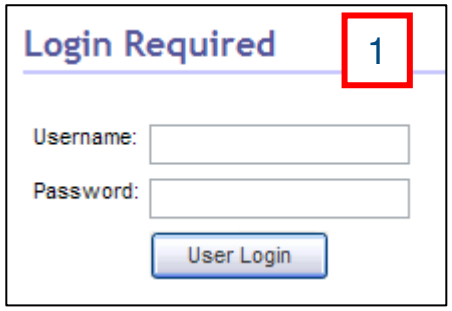
- Open PPC demo file
- Select PPC macro
- Run analysis
- View Results



Open Image Select Macro Run Analysis View Results

Positive Pixel Count Workshop

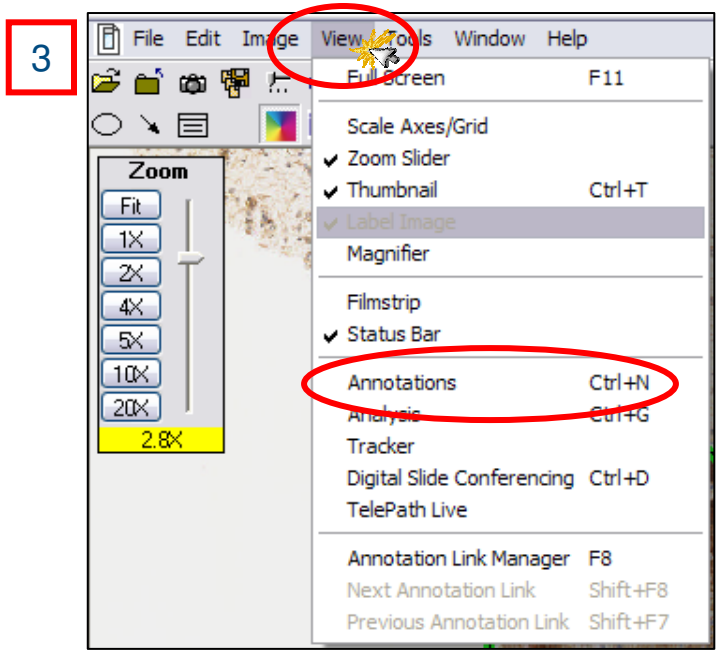
1. Log into Spectrum with a username and password provided by instructor & select the specified **Research Role**.
2. Navigate to the digital slide list, open the **Positive Pixel Count Demo** slide as shown below.



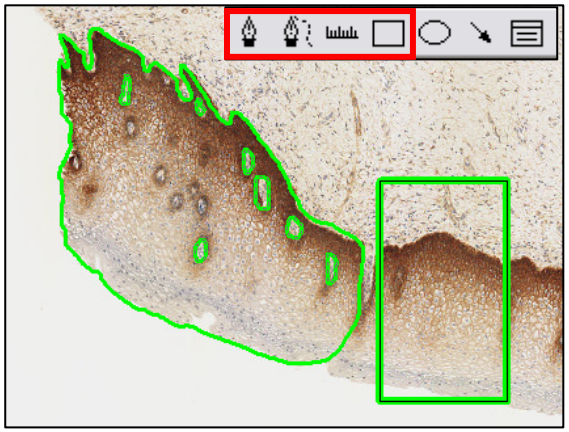
Click thumbnail image to open ImageScope

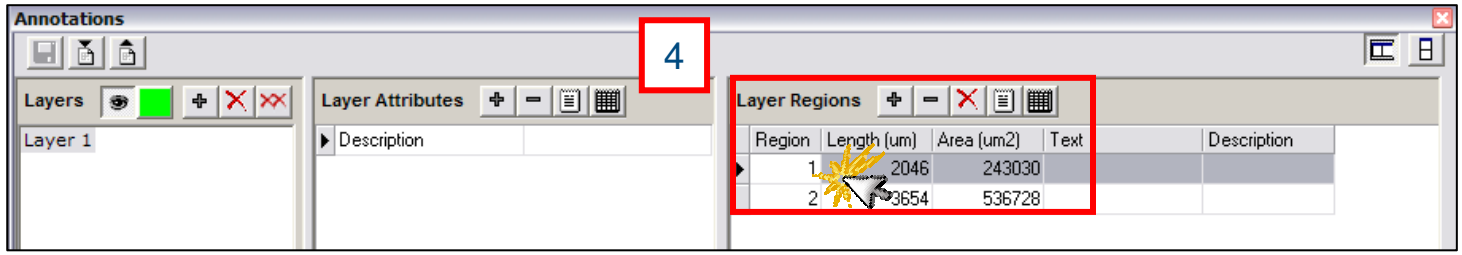
Positive Pixel Count Workshop

3. Click **View,** select **Annotations Window (Ctrl N)**.
4. Click **Region 1** in the Annotations Window under the Layer Regions section to select the area for analysis. (For this exercise please use region 1).



Note: You can create your own region of analysis by using any of these annotation tools in ImageScope. The negative pen tool will exclude regions for analysis.

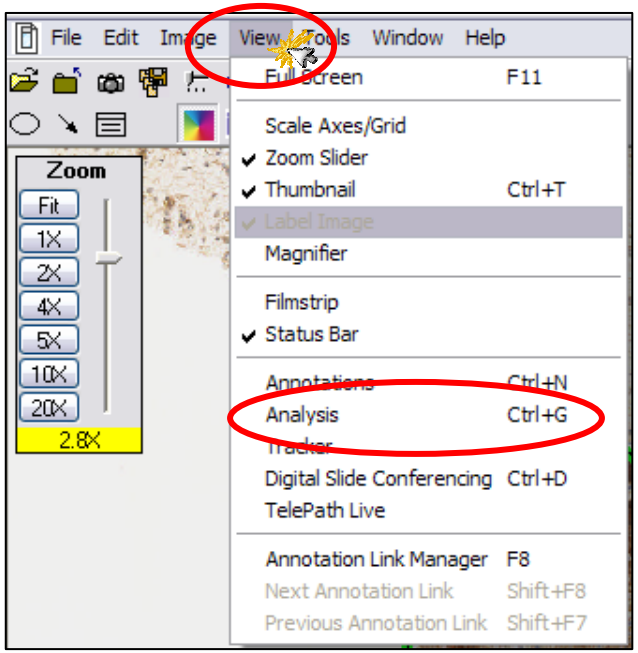




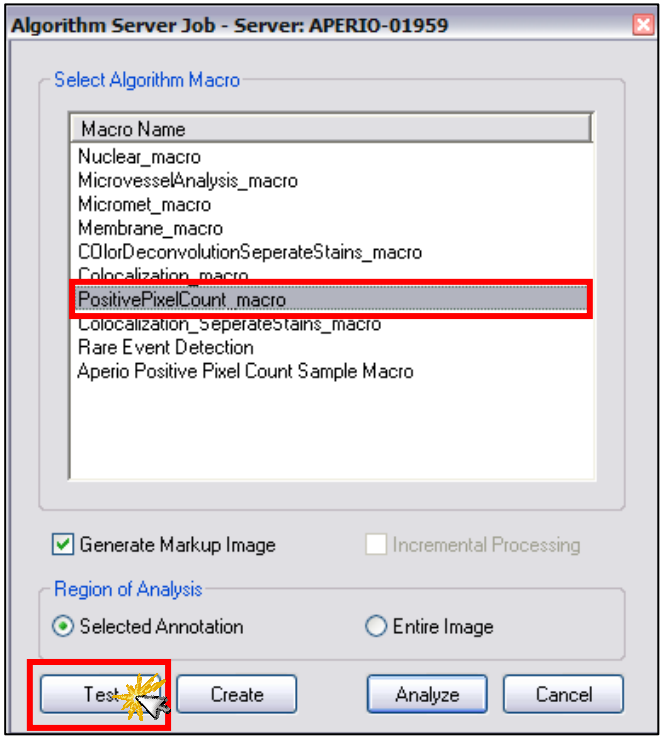
Positive Pixel Count Workshop

5. Click **View**, Select **Analysis (Ctrl G)**.
   The Algorithms Window is now displayed.
6. Select **PositivePixelCount_macro** & click **Test.**
   The saved input parameters are now displayed.

5



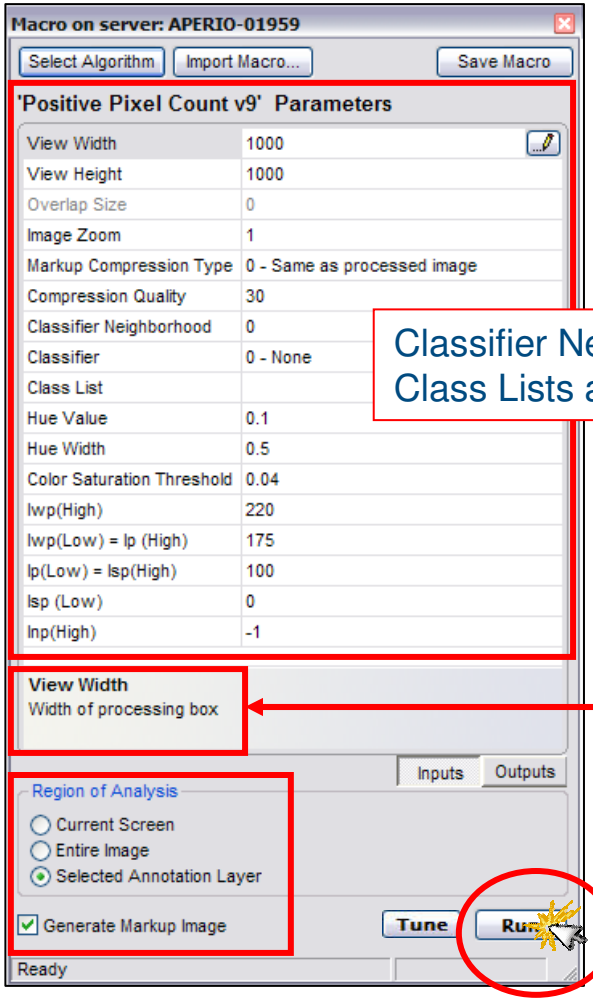
6



Positive Pixel Count Workshop

7. Review the parameters, set Region of Analysis to **Selected Annotation Layers**, & **Generate Markup Image**.
8. Click **Run**.

The default color for Positive Pixel Count is brown.



7

Classifier Neighborhood, Classifier & Class Lists are Genie parameters.

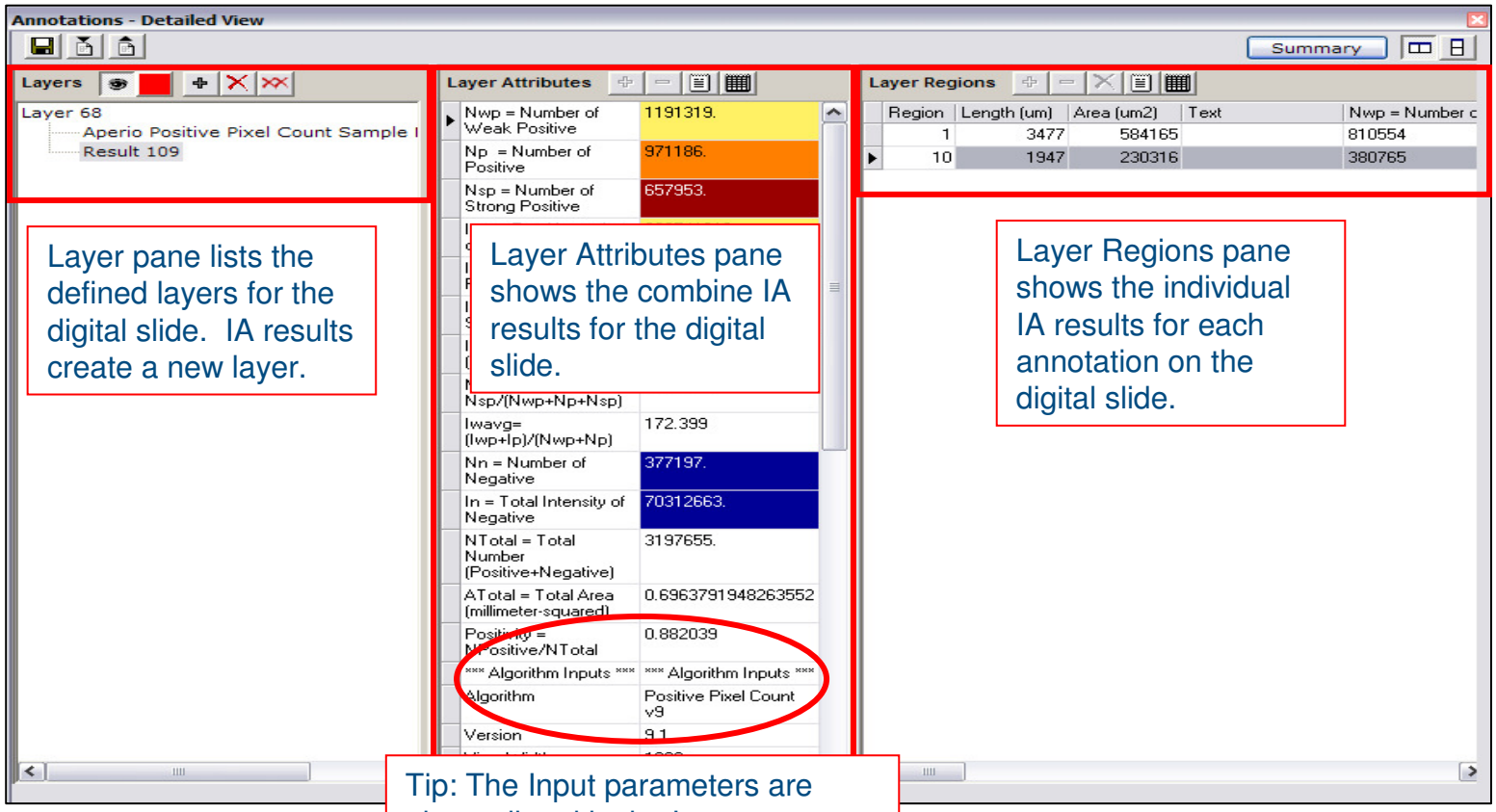
Tip: Click on a single parameter value and see the definition below.

8

Positive Pixel Count Workshop

9. View analysis results in the **Annotations window** under **Layer Attributes** section.

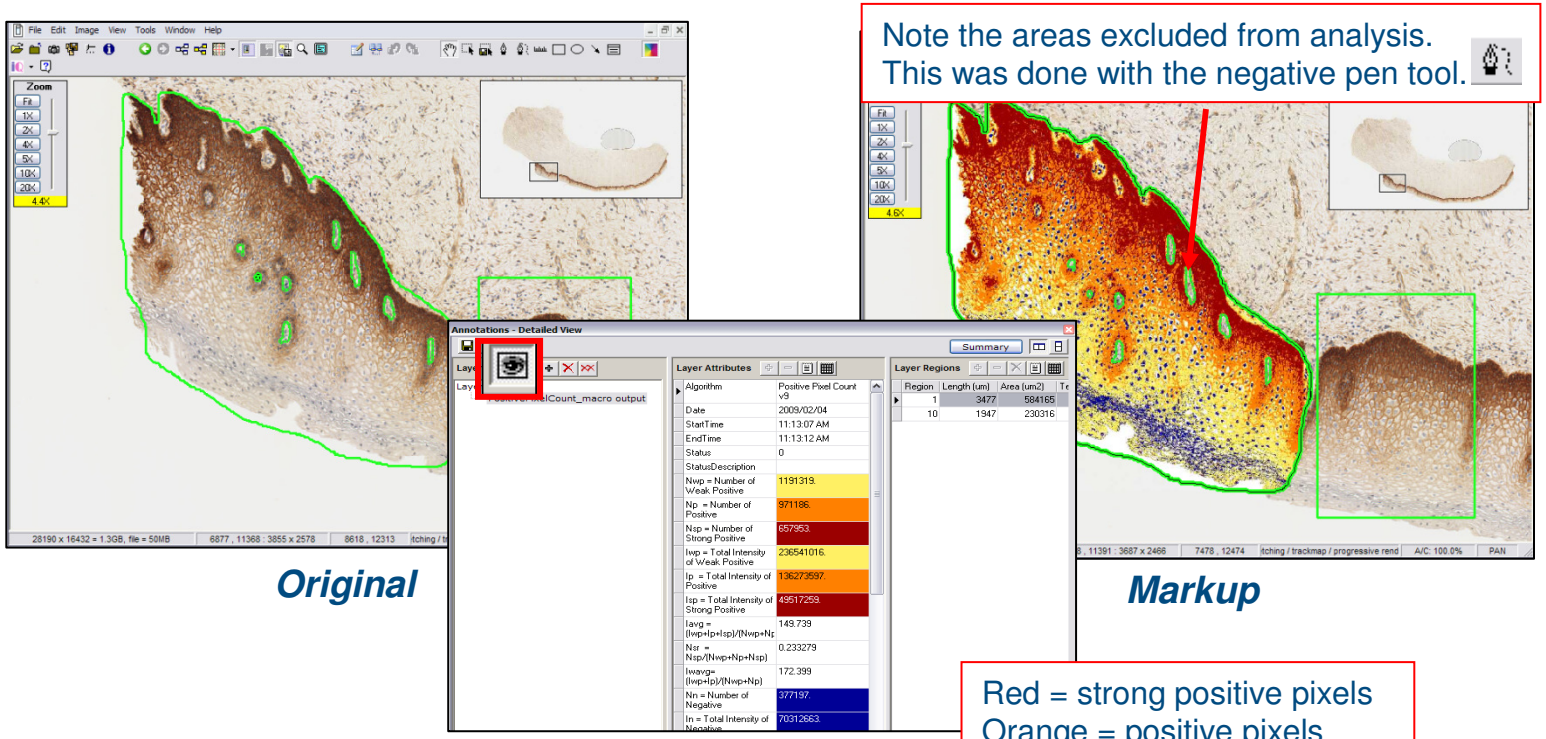
9



Positive Pixel Count Workshop

10. View **Markup Image**. Click **Show/Hide Layer Icon**  in the Annotations window to compare analysis results to the original digital slide image.

10



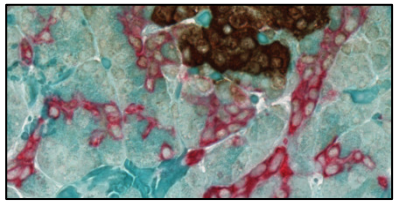
Knowledge Check

1. Positive Pixel Count provides outputs for area quantification?
   a) True
   b) False
2. The user must adjust the color hue of the stain if using anything other than a brown stain?
   a) True
   b) False
3. What is the default color quantification for Positive Pixel Count?
   a) Blue
   b) Red
   c) Green
   d) Brown
4. What parameter value is defaulted for ScanScope images?
   a) Hue Value
   b) Iwp
   c) View Width
   d) Hue Width

Color Deconvolution

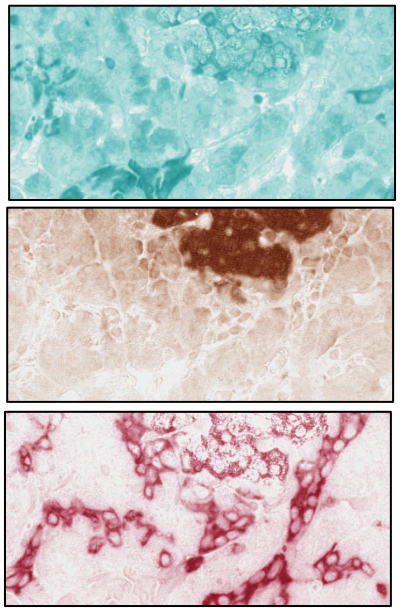
Color Deconvolution accurately separates stains, resolving multi-stain colocalization problems.

- **Accurate Stain Separation**
  - Separates the image into 3 channels corresponding to the actual colors used.
  - Allows accurate measurement of the area for each stain individually, even when the stains are superimposed at the same location.
- **Automatic and precise training of stain colors.**
  - Eliminates guesswork and trial and error.



Original

This example is the result of running analysis three times once for each color channel.

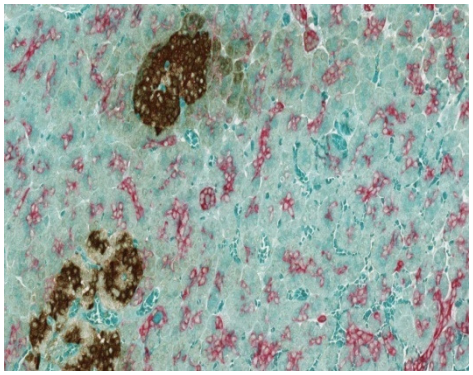


Separated Stain Markup Images

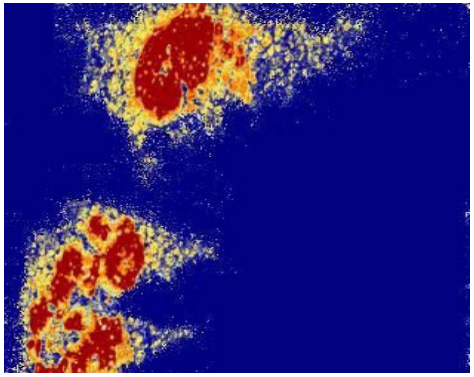
Color Deconvolution

Also quantifies the area & intensity of separated stains.

- Color Deconvolution does more than just present a visual separation of stains, it also accurately calculates the area and intensity for each individual stain.



Original



Intensity Ranges Markup

The markup image color codes analyzed pixels in the following format:
Negative, Weak, Medium, Strong Positive

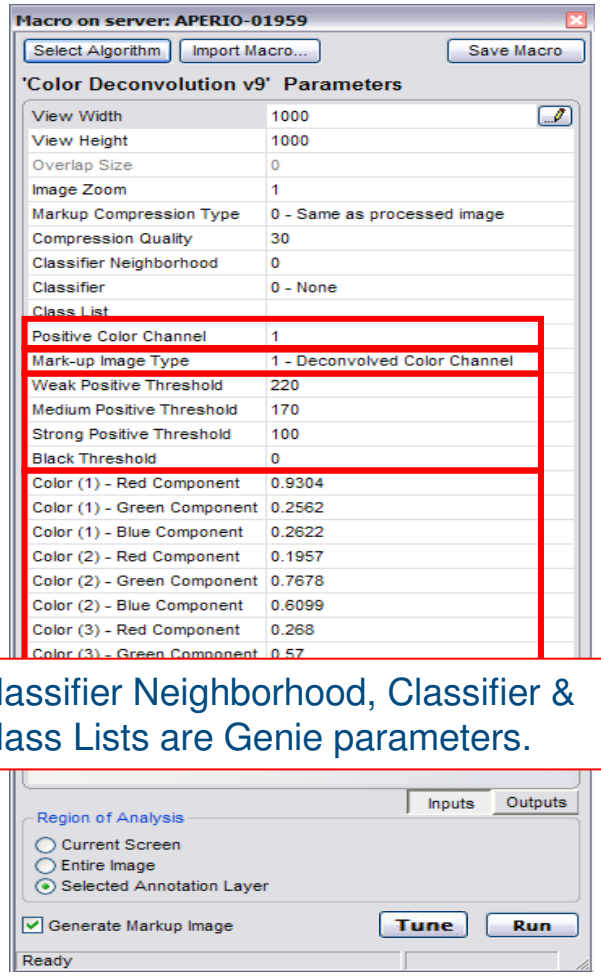
Outputs:

- Negative, Weak, Medium, Strong Positive Staining
  - Percentages
  - Average intensity
- Area
- Other statistical measurements

Color Deconvolution

Color Deconvolution input parameters:

- Color Deconvolution analysis performance is controlled by a set of input user define input parameters
- Default color channels are
  - Color (1) = Hematoxylin
  - Color (2) = Eosin
  - Color (3) = DAB
- **Positive Color Channel**-Particular stain to be analyzed (1, 2, or 3).
- **Markup Image Type**-
  - Deconvolved Color Channel
  - Intensity Ranges
- **Intensity Thresholds**-
  - Weak Positive
  - Medium Positive
  - Strong Positive
  - Black Threshold
- **Color (1, 2 or 3)**-Normalized Optical Density values for the Red, Green, Blue components for the corresponding color channel. If only 2 stains are present set color 3 values to 0.
- **Clear Area Intensity**-Default value for white balance.

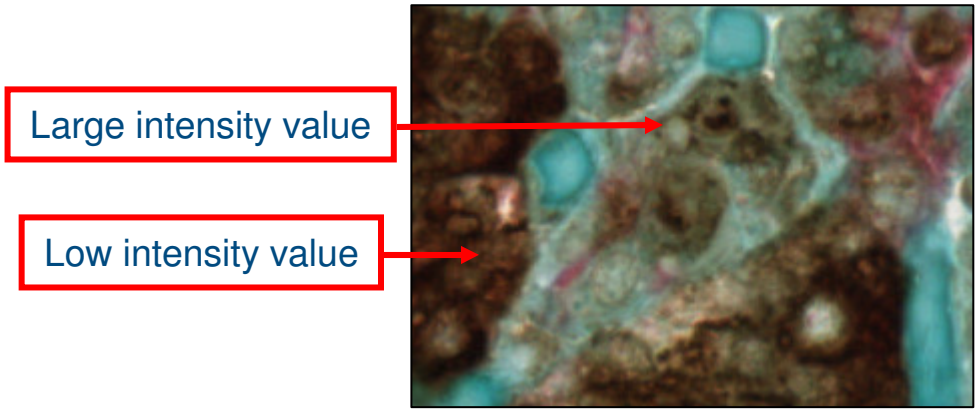


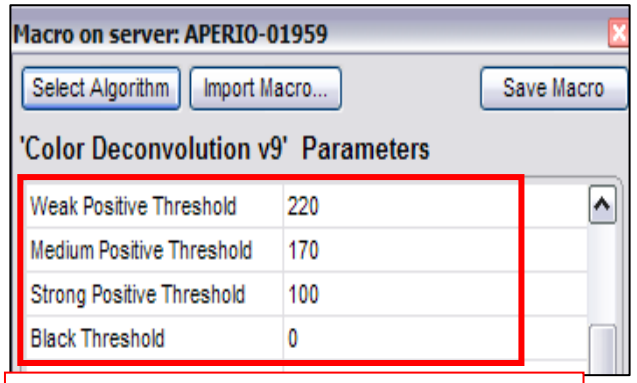
Classifier Neighborhood, Classifier & Class Lists are Genie parameters.

Color Deconvolution

More on intensity:

- **Intensity** – Raw image data are in RGB format. Intensity is the average of RGB channels in the raw data: (R+G+B)/3.
  - RGB value of (255,255,255) corresponds to bright white.
  - RGB value of (0,0,0) corresponds to black.
  - Large intensity is bright and corresponds to very light staining.
  - Low intensity is very dark and corresponds to dark staining.



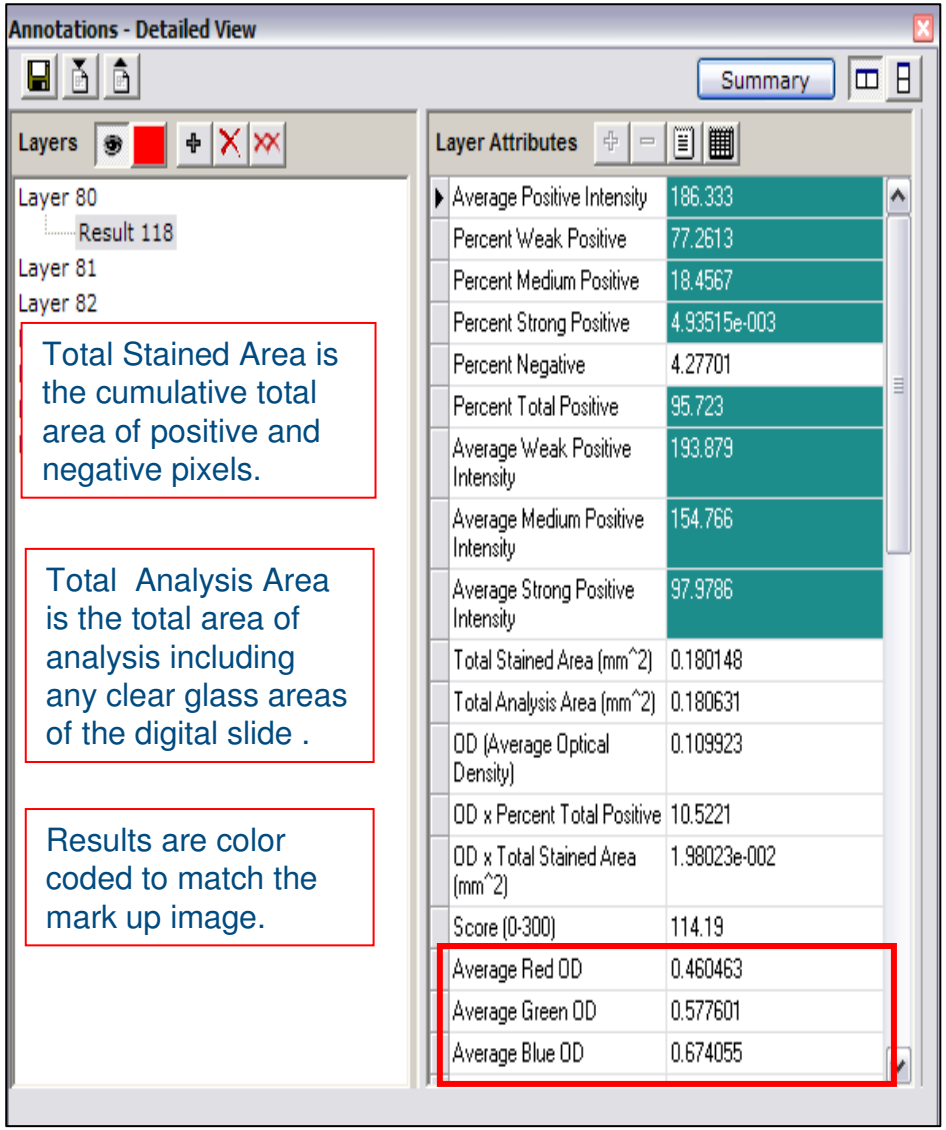


Note: Change the intensity input values to determine what intensity will be enumerated.

Color Deconvolution

Color Deconvolution Results:

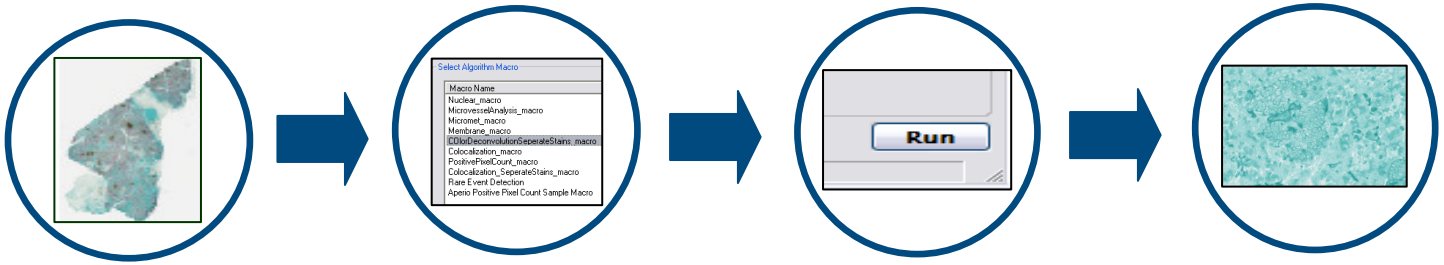
- Color Deconvolution results are area based quantification and intensity values.
  - A **positive** result applies to pixels that **are stained** in the Positive Color Channel specified in the analysis inputs.
  - A **negative** result applies to pixels that **are not stained** positive.
  - In addition, the Average Red, Green and Blue OD values are given.
    - These results can be used for color (stain) calibration.



Color Deconvolution Workshop

Watch Instructor demo the tasks below and perform them using the steps beginning on the next page.

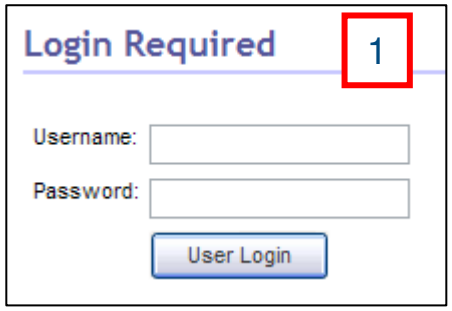
- Open Color Deconvolution Demo File
- Select Color Deconvolution Macro
- Run Analysis
- View Results



Open Image | Select Macro | Run Analysis | View Results

Color Deconvolution Workshop

1. Log into Spectrum with a username and password provided by instructor & select the specified **Research Role**.
2. Navigate to the digital slide list, open the **Color Deconvolution Demo** slide as shown below.



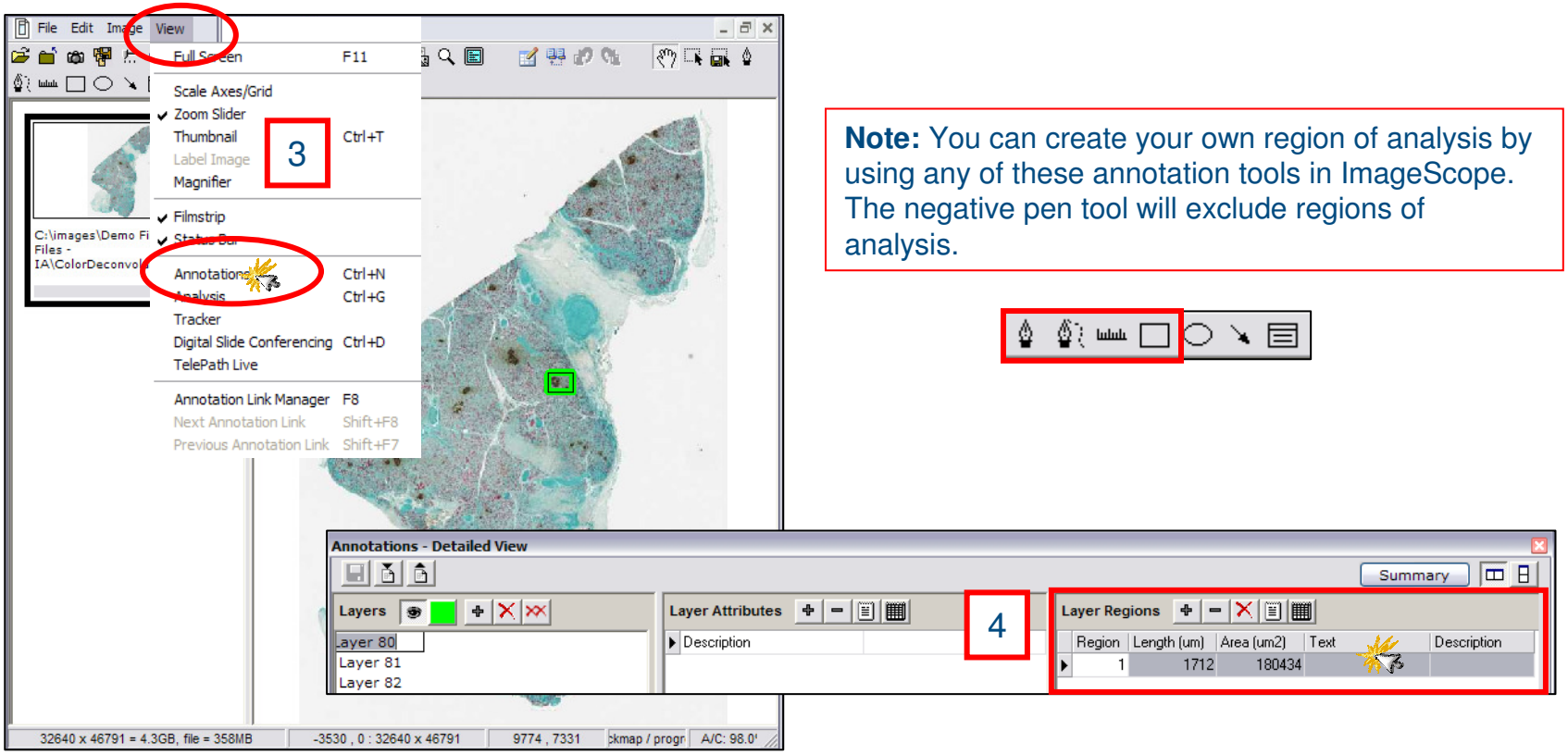
Click thumbnail image to open ImageScope

Color Deconvolution Workshop

3. Click **View**, select **Annotations Window (Ctrl N)**.

   The annotations window is now displayed.

4. Click **Region 1** in Annotations Window under the Layer Regions section to select a region for analysis. (For this exercise use Region 1).



Note: You can create your own region of analysis by using any of these annotation tools in ImageScope. The negative pen tool will exclude regions of analysis.

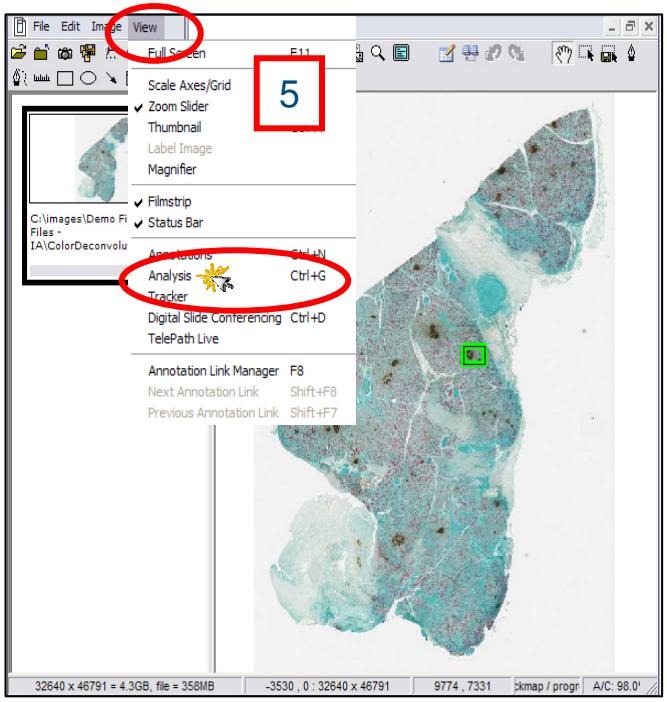
Color Deconvolution Workshop

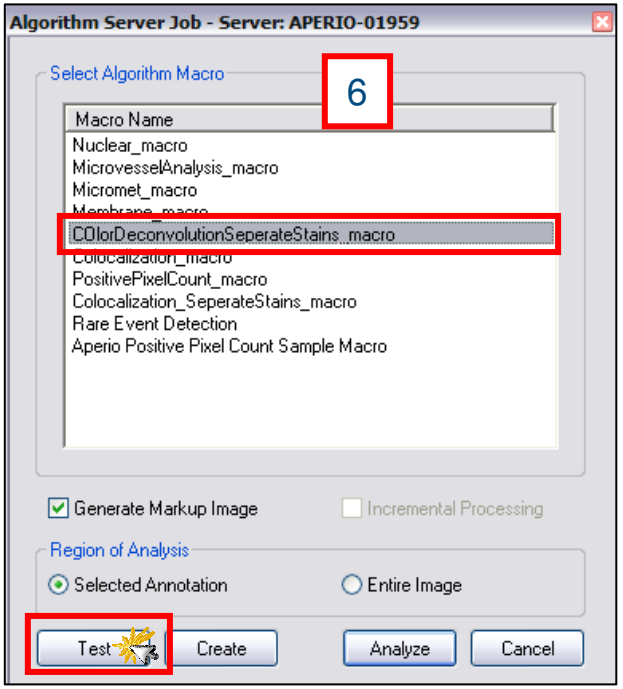
5. Click **View**, select **Analysis (Ctrl G)**.

   The Algorithms Server window is now displayed.

6. Select **ColorDeconvolutionSeperateStains_macro** & click **Test.**

   The saved input parameters are now displayed.

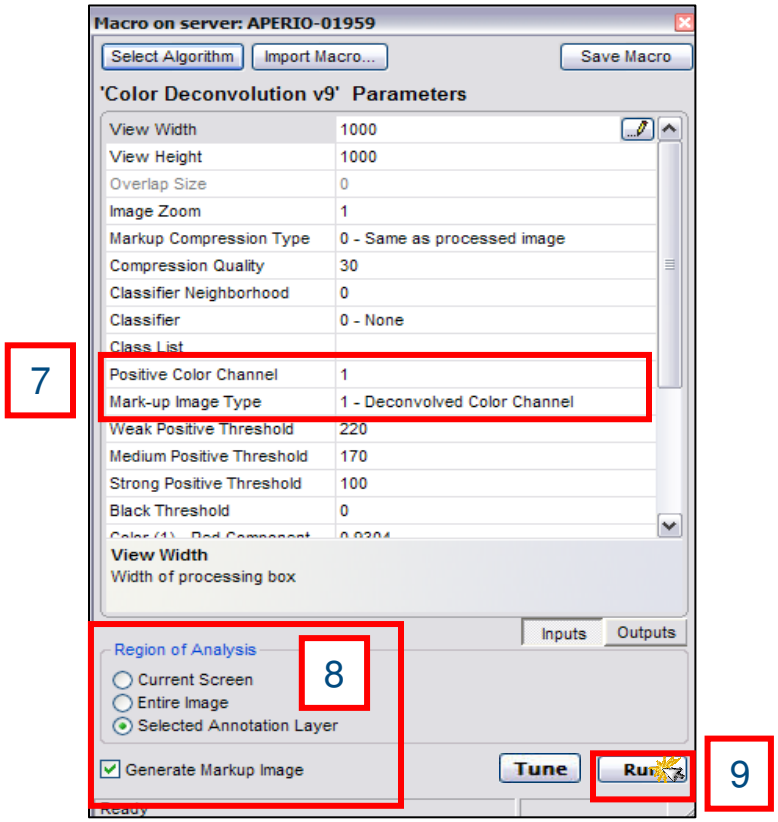




Color Deconvolution Workshop

This macro has been saved with the parameter settings for the specific stains in our sample image.

7. Verify that **Positive Color Channel** is set to **1**, the **Markup Image Type** is set to **Deconvolved Color Channel**.
8. Select **Selected Annotation Layer** & **Generate Markup Image**.
9. Click **Run**.



Color Deconvolution Workshop

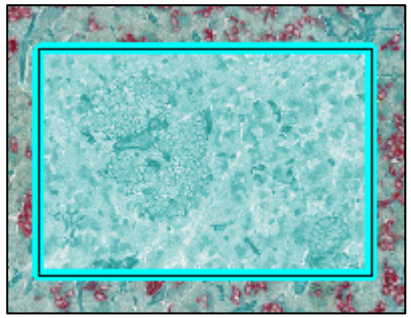
10. View the **Color Channel 1** markup image.

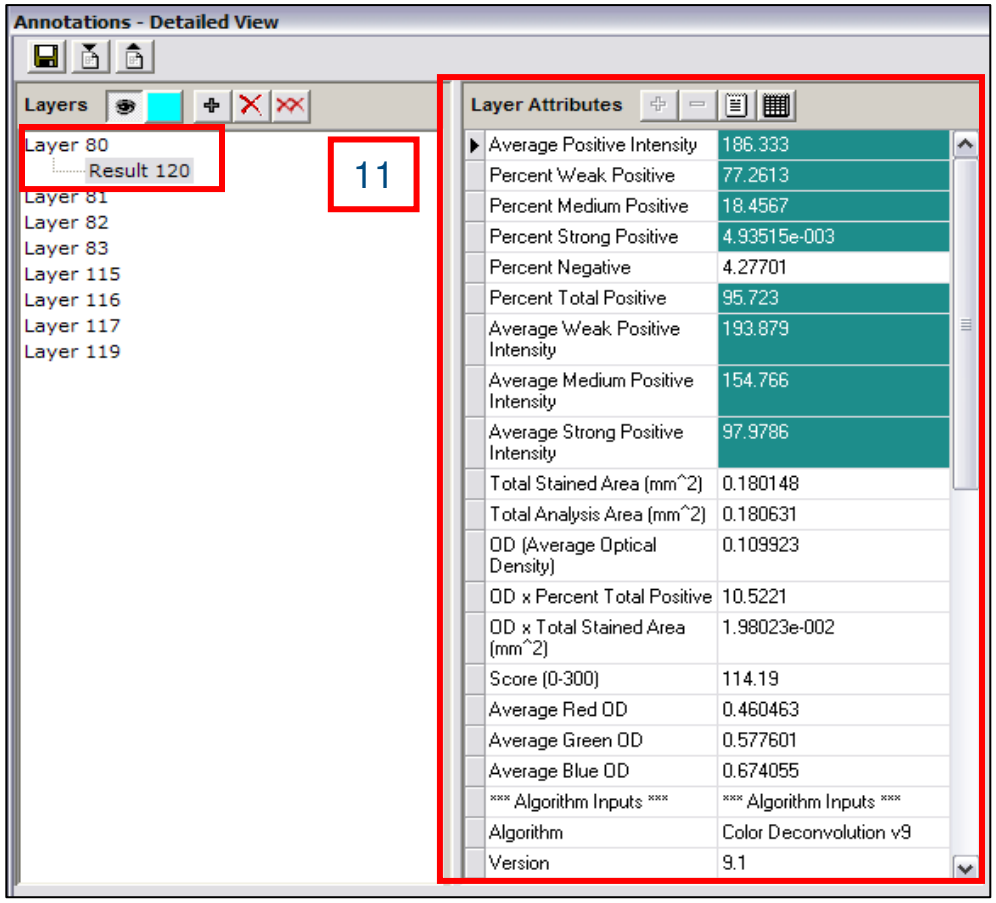
    *The markup image should display automatically if **Generate Markup Image** was selected.*

11. To review numerical results click **View** & select **Annotations**.

    Positive results are color coded.

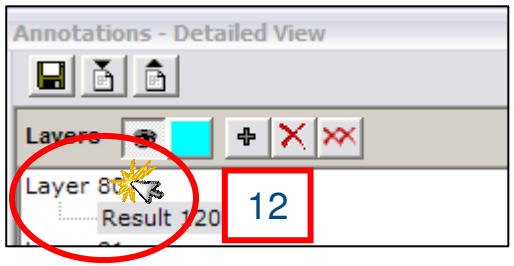
10

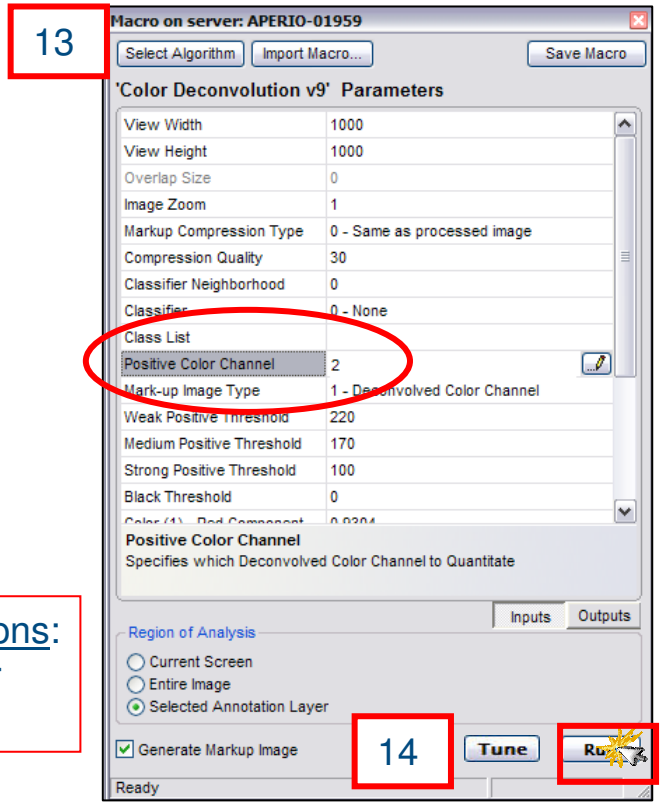




Color Deconvolution Workshop

12. Select the original **Annotation Layer** in the Annotations Window.
13. Change **Positive Color Channel parameter to 2** in the Analysis Window.
14. Click **Run**.



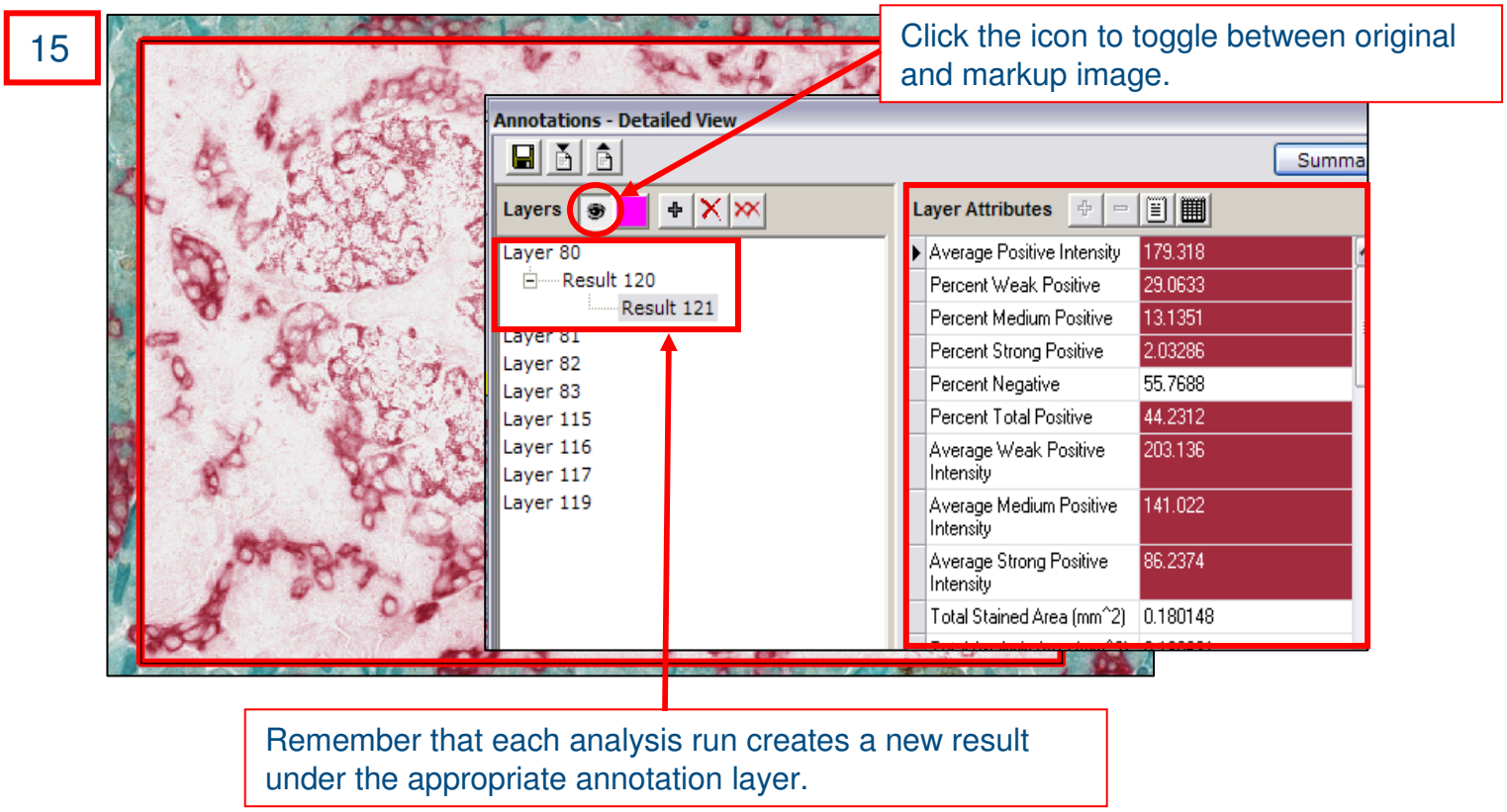


Region of Analysis selections:
Selected Annotation Layer
Generate Markup Image

Color Deconvolution Workshop

15. View **markup image & results**.

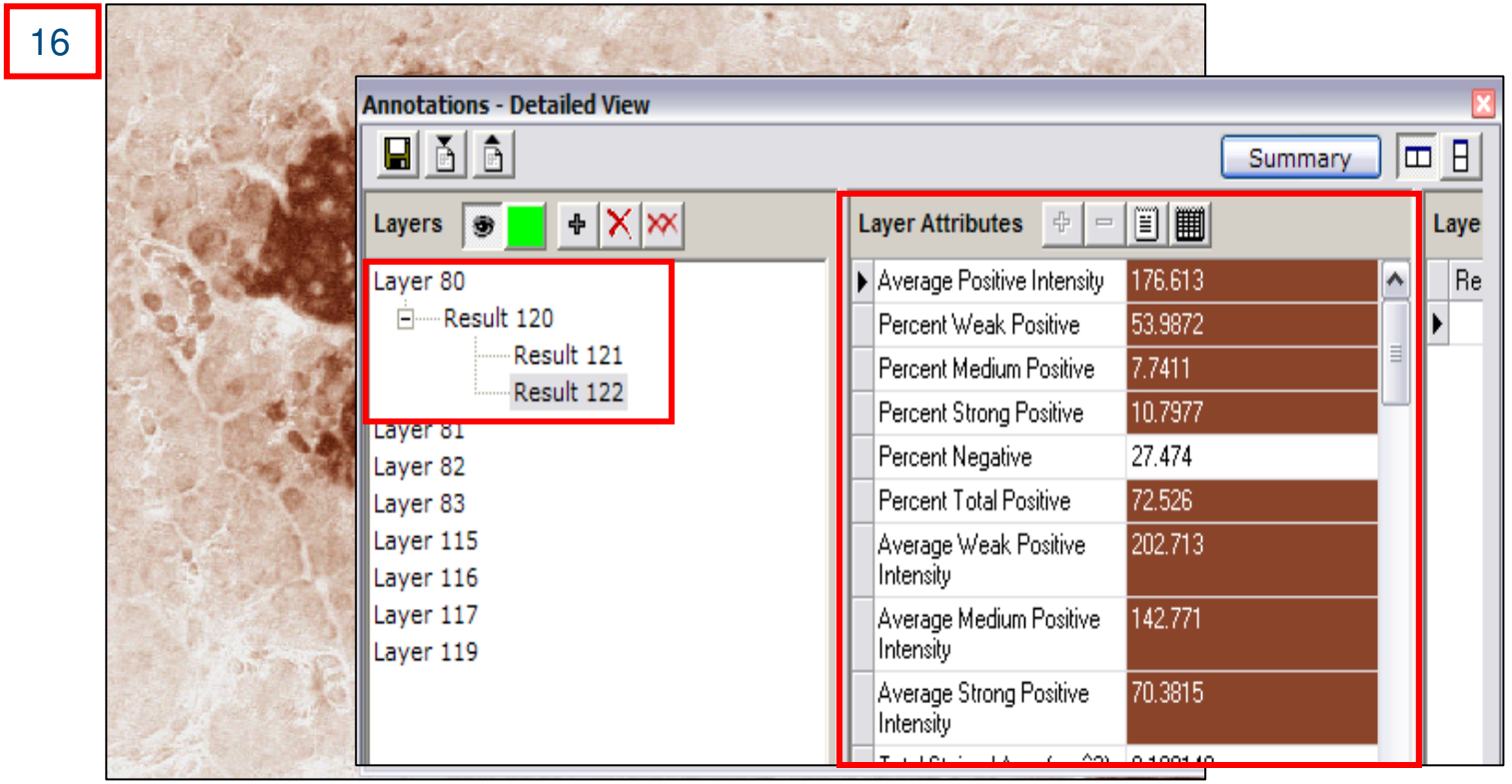
Now the red color markup image of stain #2 is displayed.



Color Deconvolution Workshop

16. Repeat **steps 12 – 15**; changing the **Positive Color Channel parameter to 3**.

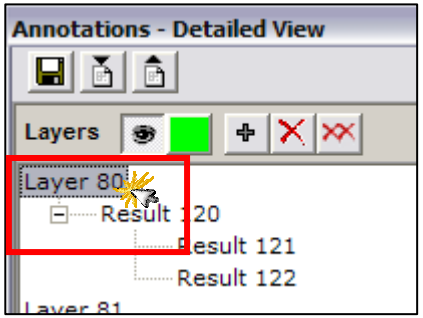
Now the brown color markup image of stain #3 is displayed.



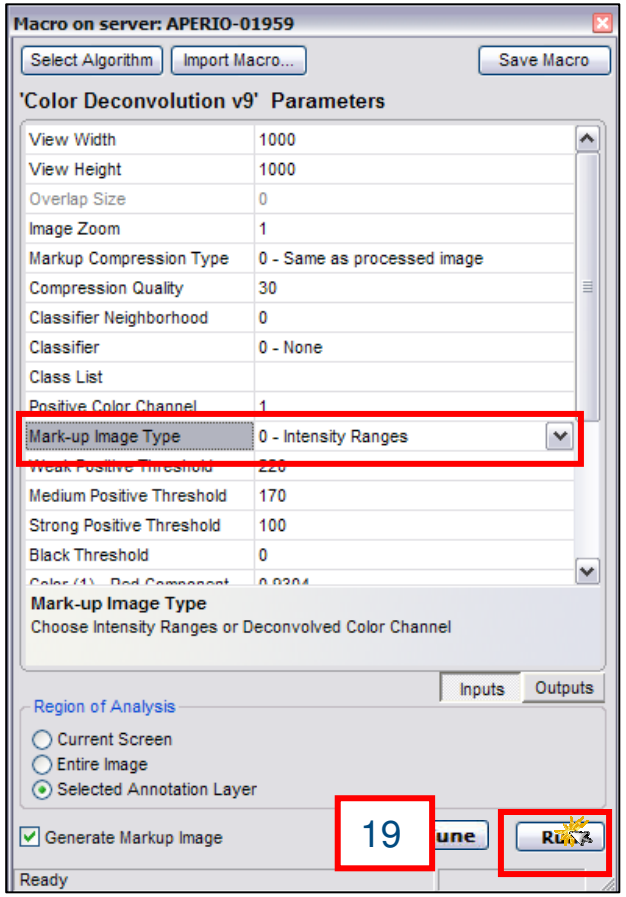
Color Deconvolution Workshop

17. Click **Original Layer** in the **Annotations Window**.
18. Change the **Markup Image Type to Intensity Ranges** in the Analysis Window, leaving everything else the same.
19. Click **Run**.

17



18

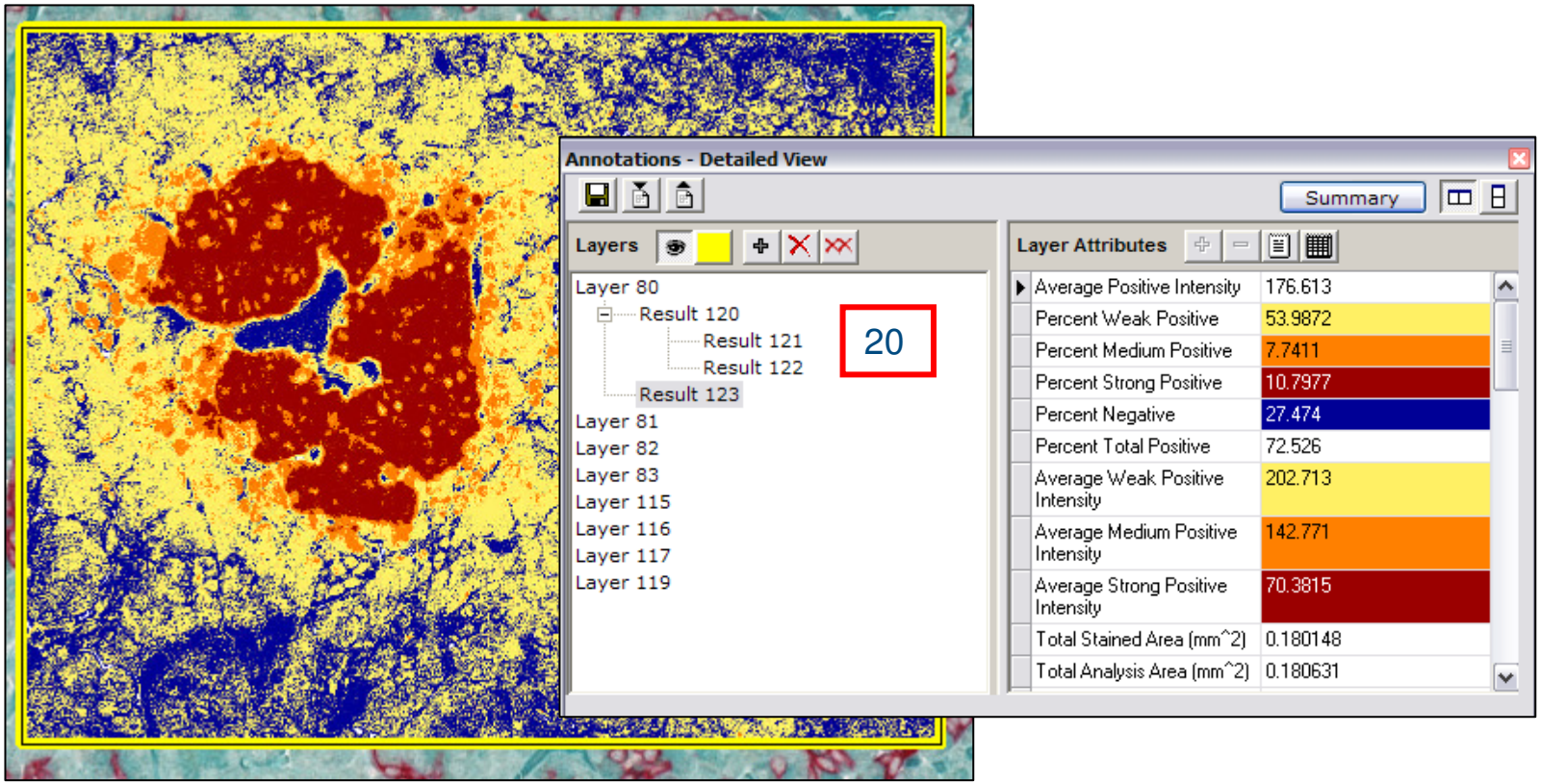


Region of Analysis selections:
Selected Annotation Layer
Generate Markup Image

Color Deconvolution Workshop

20. View **markup image & results**.

Notice that the markup image for intensity for Color Channel 3 (DAB) is displayed.



Note: The choice of markup image type does not change the numerical results. Verify this by toggling between the Channel 3 deconvolved results and the intensity ranges (in this example results 4 & 5).

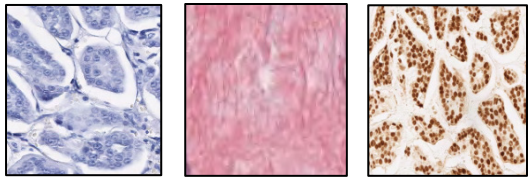
Knowledge Check

1. What does Color Deconvolution quantify?
   a) Cell quantification
   b) Area and intensity of separated stains
   c) Area and intensity of colocalized markers
2. Color Deconvolution can only separate up to 2 stains.?
   a) True
   b) False
3. Color Deconvolution uses color channels to determine stain analysis?
   a) True
   b) False

Color Deconvolution – Color Calibration

Color Deconvolution can be used for stain color calibration.

- Color Calibration defines the stain color vector (red, green and blue components) so stained cells will be correctly identified by the analysis tool.
  - Default colors vectors are:
    - Color 1 – Hematoxylin
    - Color 2 – Eosin
    - Color 3 – DAB

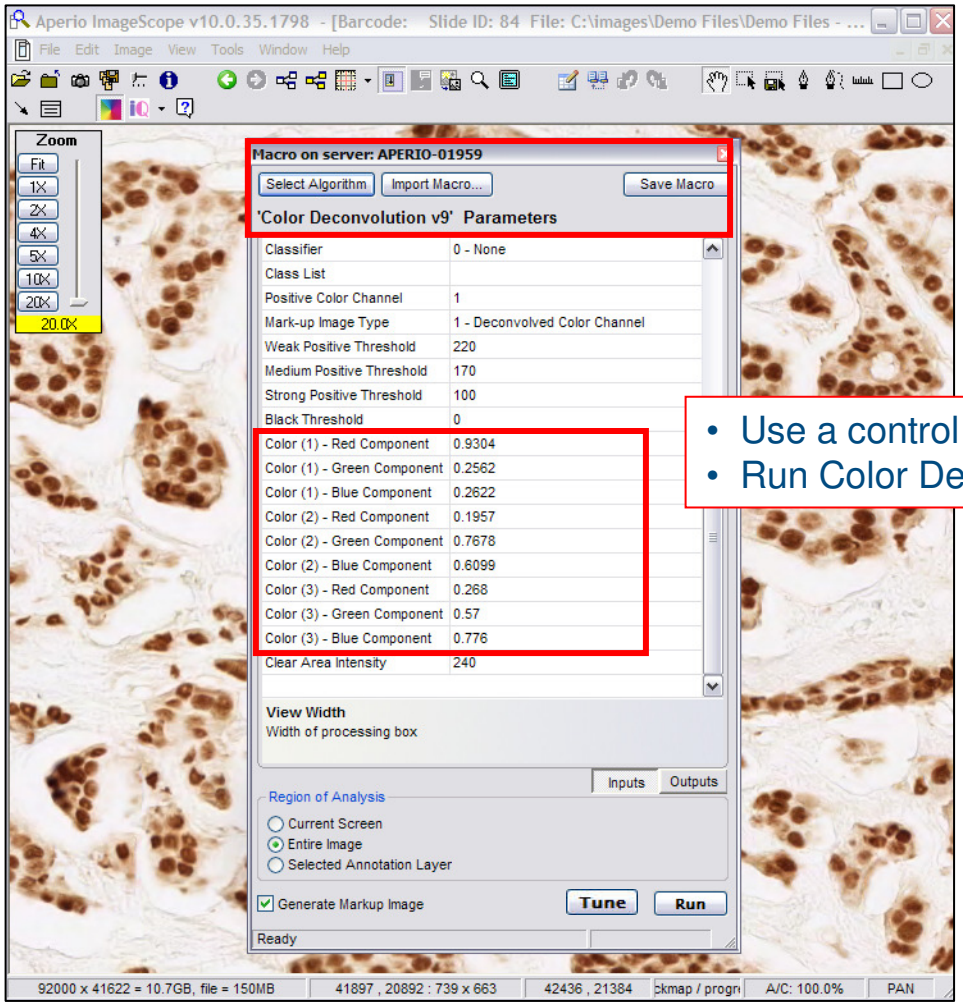


  - Color vector numbers must be changed if different stains are used.
    - The color for each stain is calibrated separately for each stain that differs from the defaults.
    - Separate control slides for each stain should be used.
    - After calibration is complete, the modified parameter settings can be saved as a macro.

Color (1) - Red Component	0.65
Color (1) - Green Component	0.704
Color (1) - Blue Component	0.286
Color (2) - Red Component	0.072
Color (2) - Green Component	0.99
Color (2) - Blue Component	0.105
Color (3) - Red Component	0.268
Color (3) - Green Component	0.57
Color (3) - Blue Component	0.776

Color Calibration

Calibration Outputs become input parameters.

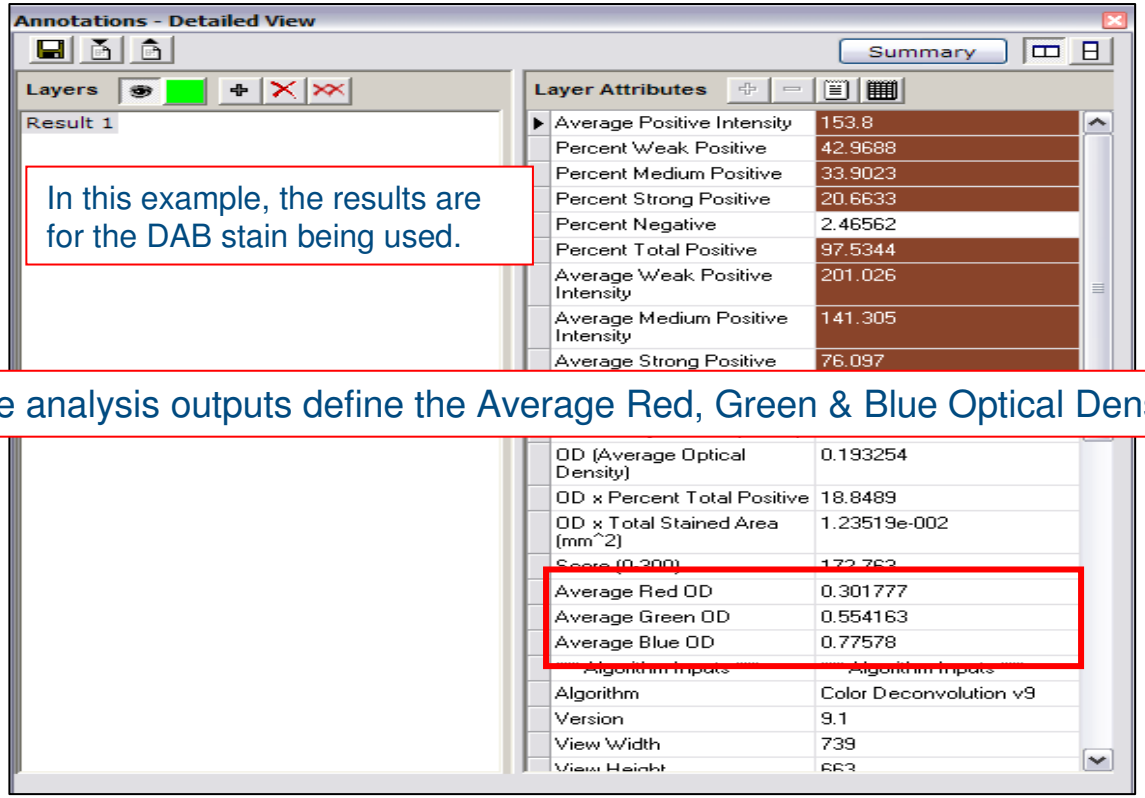


- Use a control slide with a single stain.
- Run Color Deconvolution Analysis

Color Calibration

Calibration Outputs:

- By using a control slide with one color, you can guarantee the Average Optical Density for the stain's RGB color components will be accurate.
- These results will become the Color Channel inputs (red, blue & green components) for that stain when running Color Deconvolution or Colocalization.

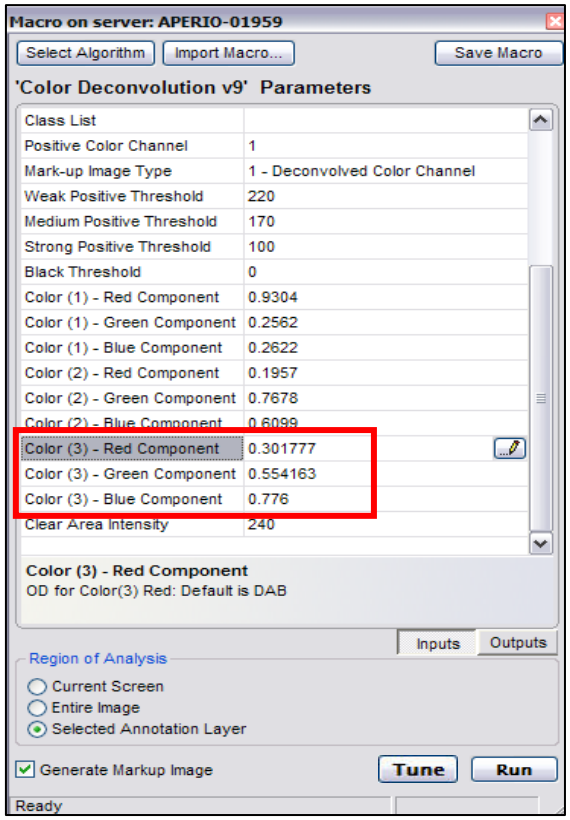


The analysis outputs define the Average Red, Green & Blue Optical Densities for control stain.

Color Calibration

Calibration Inputs:

- Performing color calibration on a control slide, results in the Average Red, Green & Blue Optical Density for that stain.
  - The output optical densities are the color vectors for the specific stain being used.
  - These become the Red, Green and Blue Components for the Color Channel inputs for the stain.

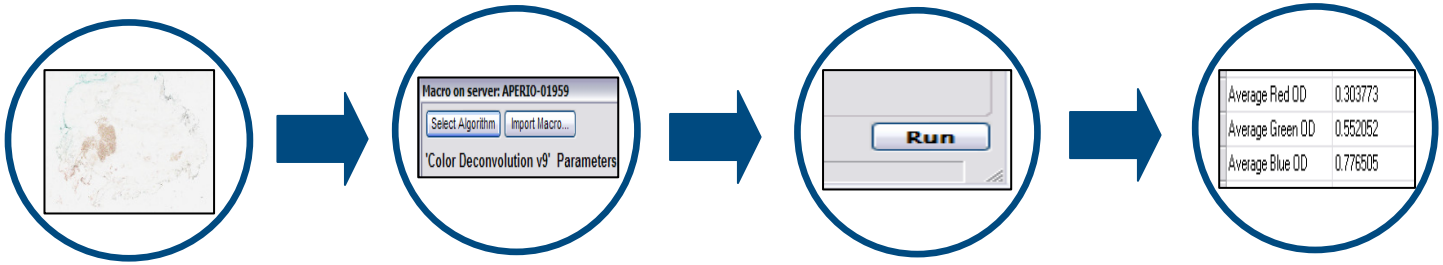


Color Calibration takes the guesswork out of the trial and error method of running Color Deconvolution and Colocalization.

Automatic Stain Calibration

Watch Instructor demo the tasks below and perform them using the steps beginning on the next page.

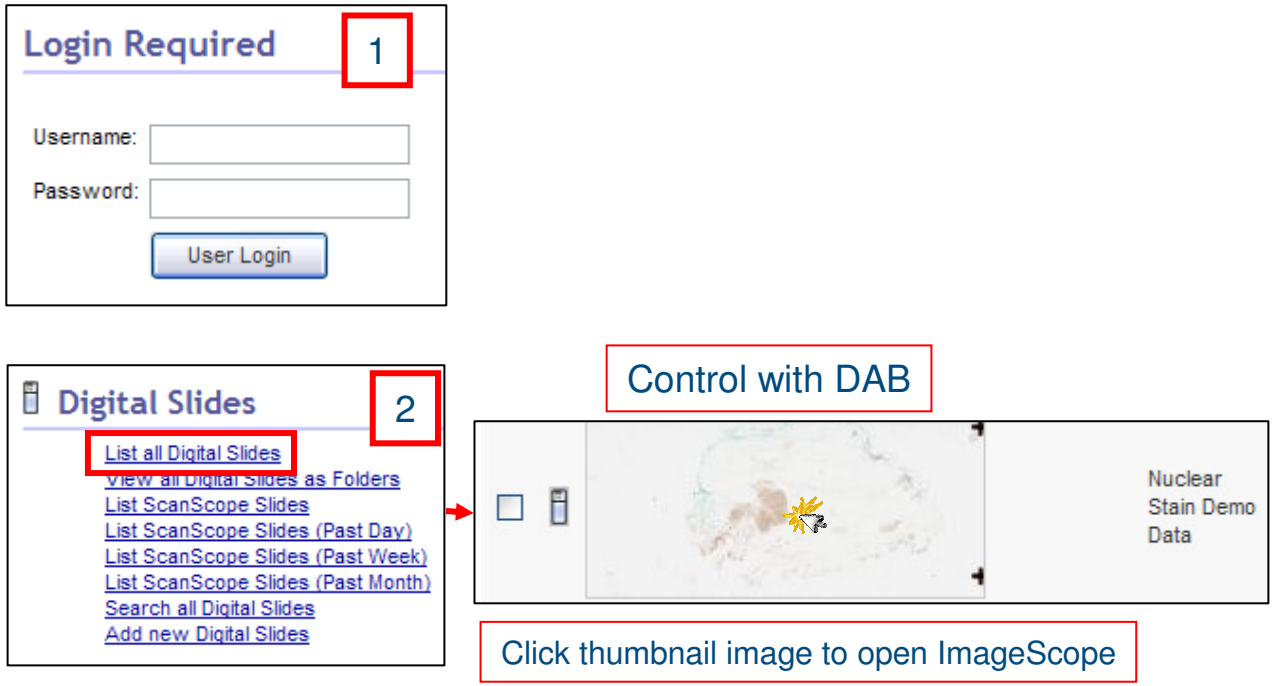
- Open Nuclear Stain demo file
- Select Nuclear Stain Tuning macro
- Run analysis
- View Results



Open Image | Select Macro | Run Analysis | View Results

Stain Calibration Workshop

1. Log into Spectrum with a username and password provided by instructor & select the specified **Research Role**.
2. Navigate to the digital slide list, open the **Nuclear Stain Demo** slide as shown below.



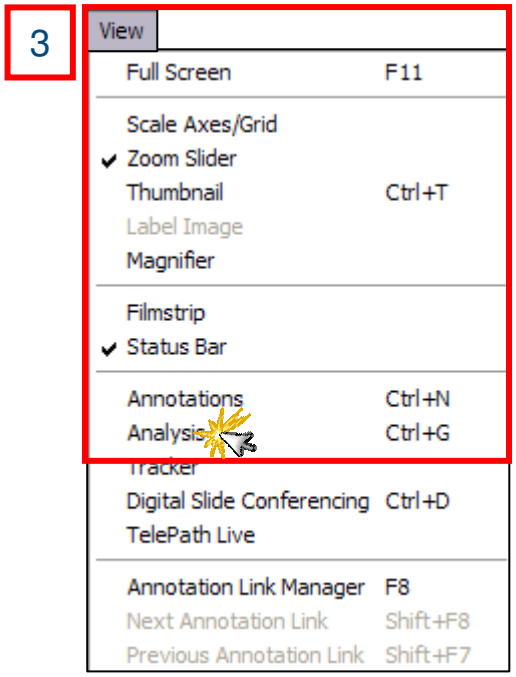
Stain Calibration Workshop

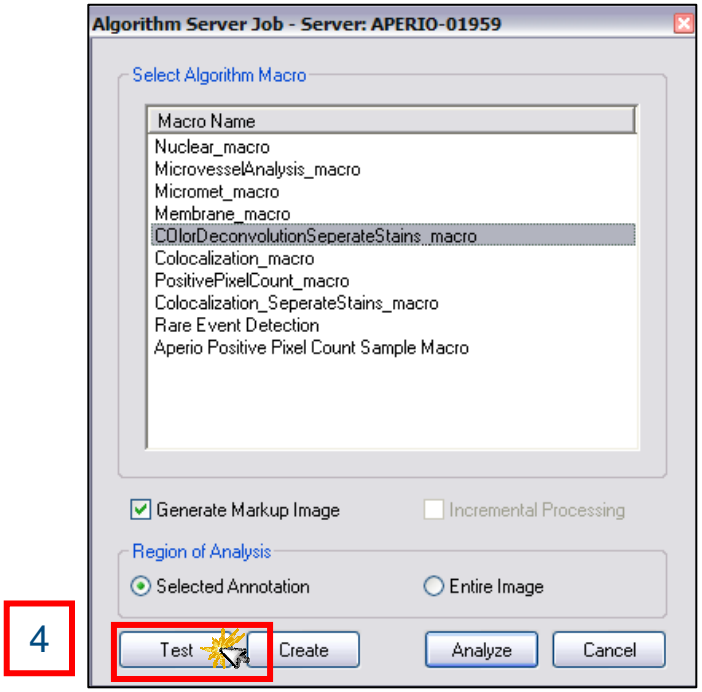
3. Click **View** and Select **Analysis (Ctrl G)**.

   The Algorithms Server window is now displayed.

4. Click **Color Deconvolution macro** in the Algorithms Server window & click **Test**.

   The algorithm parameters are now displayed.

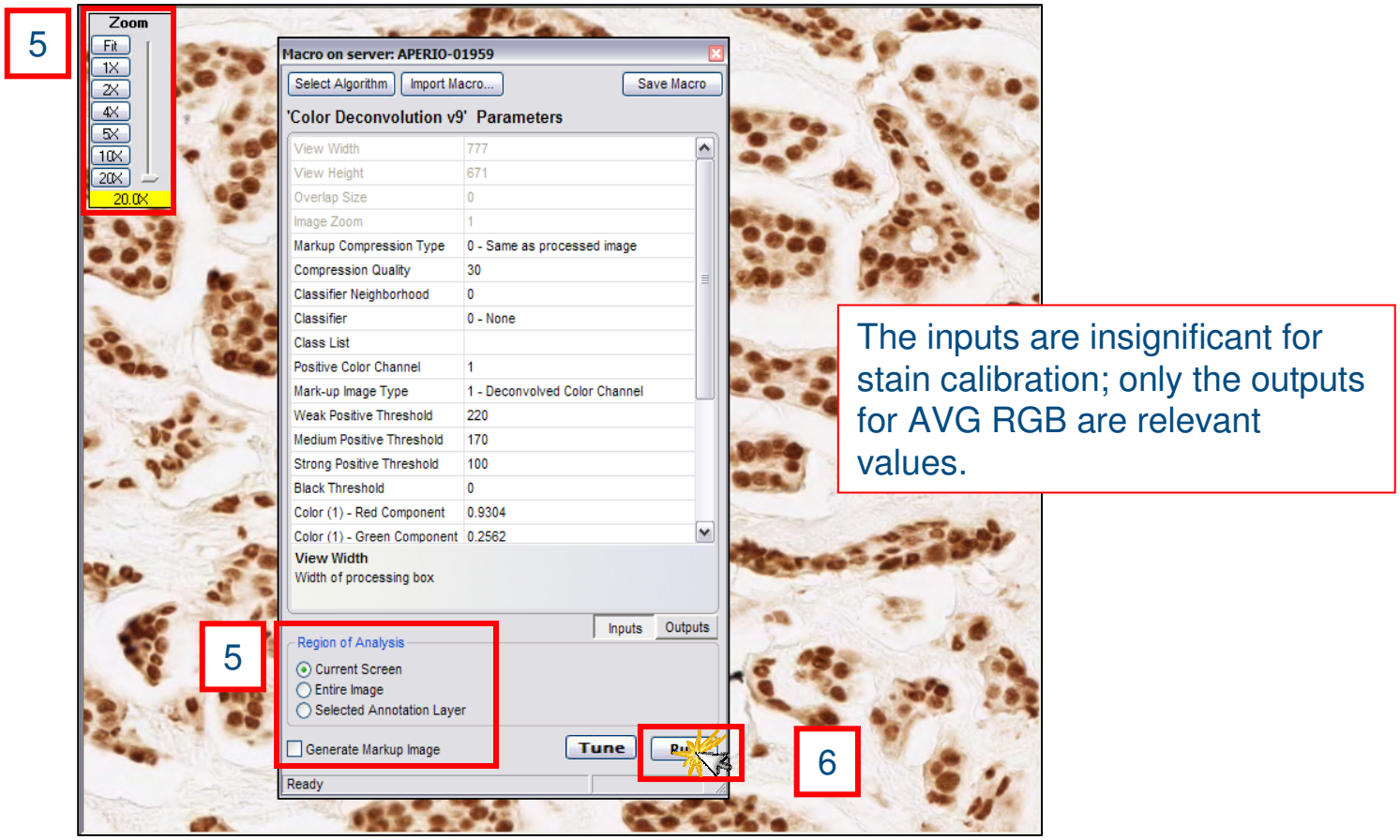




Stain Calibration Workshop

5. Zoom to an area that represents the stain being calibrated. Set the Region of Analysis to **Current Screen** & uncheck the **Generate Markup Image** box.
6. Click **Run**.

Color Deconvolution is run with its erroneous Color settings and the output for the control slide is obtained.

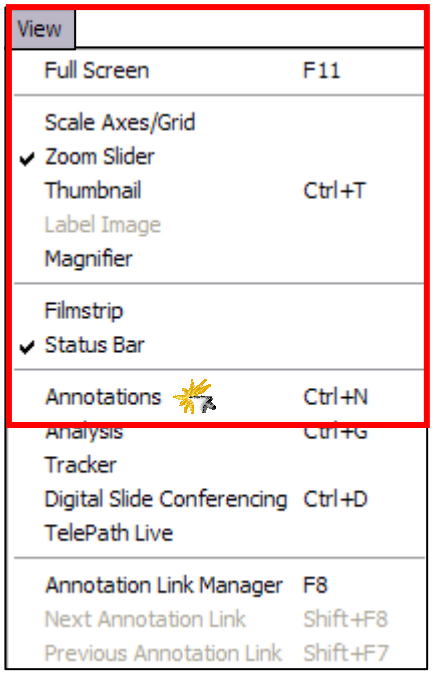


Stain Calibration Workshop

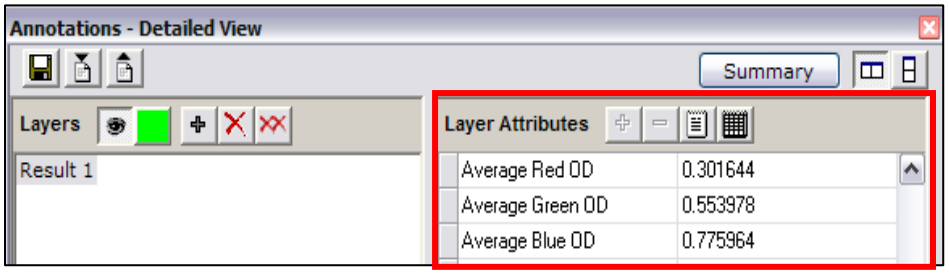
8. Click **View**, select **Annotations (Ctrl N)**.
9. View **Layer Attributes** in **Annotations Window**.

Color Deconvolution provides the Average, Red, Green and Blue Optical Density (OD) values of the image it analyzes.

8



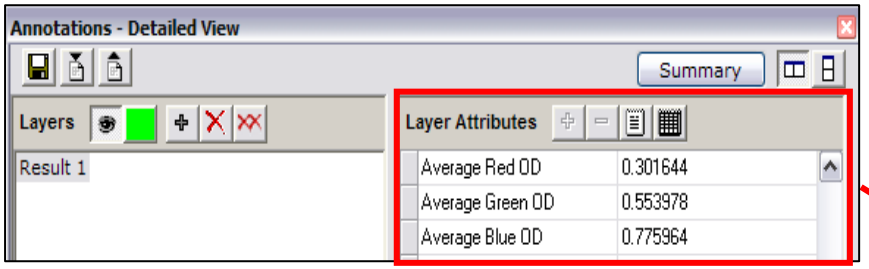
9



The Average Red, Green & Blue OD is the RGB color component for this stain. Use these values as inputs when saving macros for this particular stain.

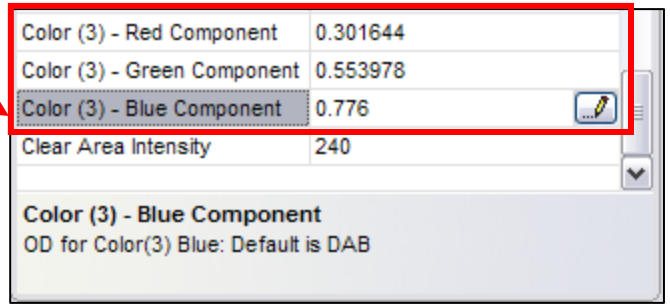
Stain Calibration Workshop

10. Now that you have the Red, Green & Blue OD values you can use these outputs as parameter inputs every time this stain is used. As an example:



These outputs become the Red, Green & Blue Component inputs for the appropriate color channel every time this stain is used. This is done by manually adjusting the Red, Green & Blue Components in the Algorithms Window.

10



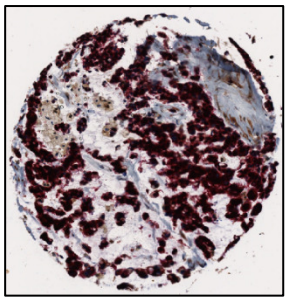
Knowledge Check

1. Stain calibration can be completed using Color Deconvolution.
   a) True
   b) False
2. Calibration outputs become analysis input parameters.
   a) True
   b) False
3. What are the important output results for stain calibration?
   a) Percent Weak Positive
   b) Percent total Positive
   c) Average Red, Green and Blue OD

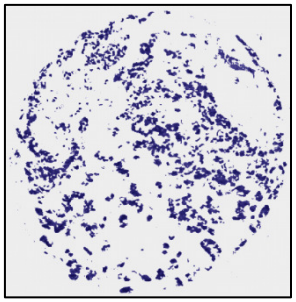
Colocalization

Quantifies the area & intensity of "colocalized" markers.

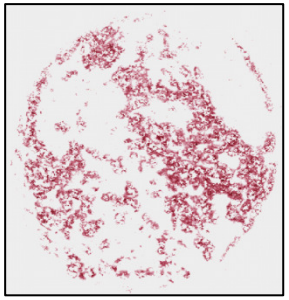
- Colocalization Analysis calculates the contribution of each stain at every pixel location in the image as either part of a single stain or representing a combination of staining.
  - Analysis results are based on the separated stain's intensities.
  - For IHC, it determines where specific proteins are present and to that extent the proteins are colocalized.



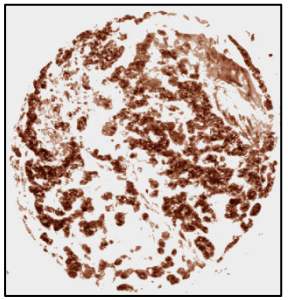
Original



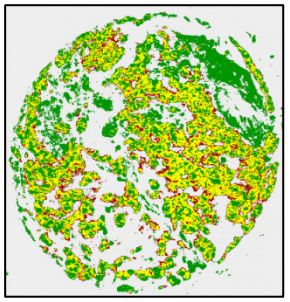
Hematoxylin



Marker A (Fast Red)



Marker B (DAB)



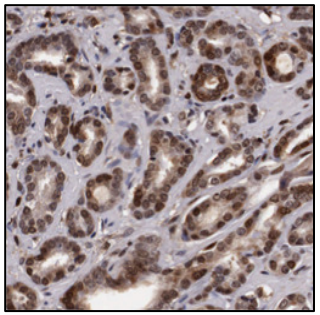
Marker A, Marker A+B, Marker B

- **Outputs:**
  - A, B, A+B: 3 possible stain combinations for two stains.
  - A, B, C, A+B, B+C, A+C, A+B+C: 7 possible combinations for three stains.
    - Percentages
    - Average Intensities
  - Area

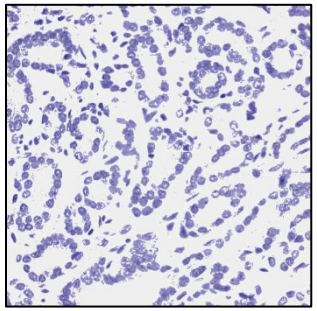
Colocalization

Cytoplasmic Quantification by area.

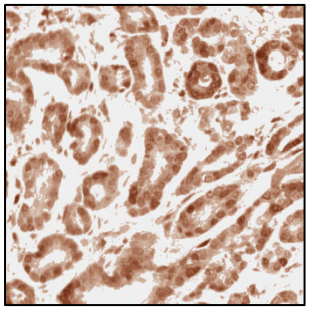
- Colocalization can be used for Cytoplasmic analysis by measuring only the cytoplasmic component of staining.



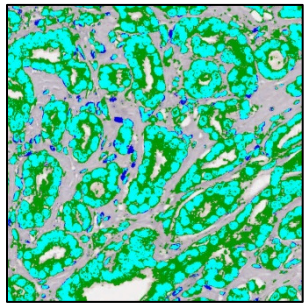
Original



Hematoxylin (Nuclear) Markup



DAB (Nuclear + Cytoplasm) Markup



DAB Cytoplasmic Staining (Green) Markup

- **Outputs:**
  - A, B, A+B: 3 possible stain combinations for two stains.
  - A, B, C, A+B, B+C, A+C, A+B+C: 7 possible combinations for three stains.
    - Percentages
    - Average Intensities
  - Area

Colocalization

Colocalization input parameters.

- Colocalization analysis performance is controlled by a set of input parameters allowing users to define:

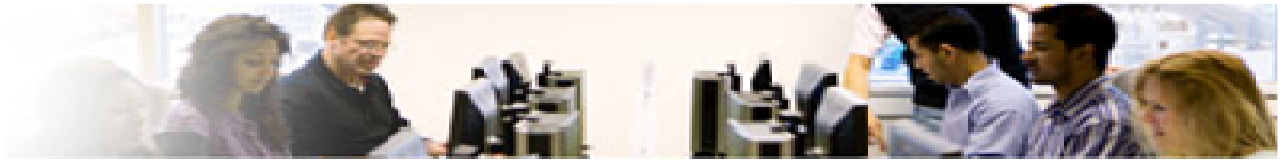
- **Markup Image Type**-
  - Colocalization
  - Deconvolved Color Channel 1, 2 or 3
- **Mode**-
  - Colocalization
  - Counter-stain, Double Label
- **Threshold for intensity ranges**-
  - Upper and Lower threshold for colors 1, 2 & 3
- **Color (1, 2 or 3)**-Normalized Optical Density values for the Red, Green, Blue components for the corresponding color channel. If only 2 stains are present set color 3 values to 0
- **Clear Area Intensity**-Default value for white balance

'Colocalization v9' Parameters

View Width	1000
View Height	1000
Overlap Size	0
Image Zoom	1
Markup Compression Type	0 - Same as processed image
Compression Quality	30
Classifier Neighborhood	0
Classifier	0 - None
Class List	
Mark-up Image Type	0 - Co-Localization
Mode	0 - Co-Localization mode
Color (1) Threshold	200
Color (1) Lower Threshold	0
Color (2) Threshold	200
Color (2) Lower Threshold	0
Color (3) Threshold	200
Color (3) Lower Threshold	0
Color (1) - Red Component	0.65
Color (1) - Green Component	0.704
Color (1) - Blue Component	0.286
Color (2) - Red Component	0.072
Color (2) - Green Component	0.99
Color (2) - Blue Component	0.105
Color (3) - Red Component	0.268
Color (3) - Green Component	0.57
Color (3) - Blue Component	0.776
Clear Area Intensity	240

Classifier Neighborhood, Classifier & Class Lists are Genie parameters.

Colocalization Workshop

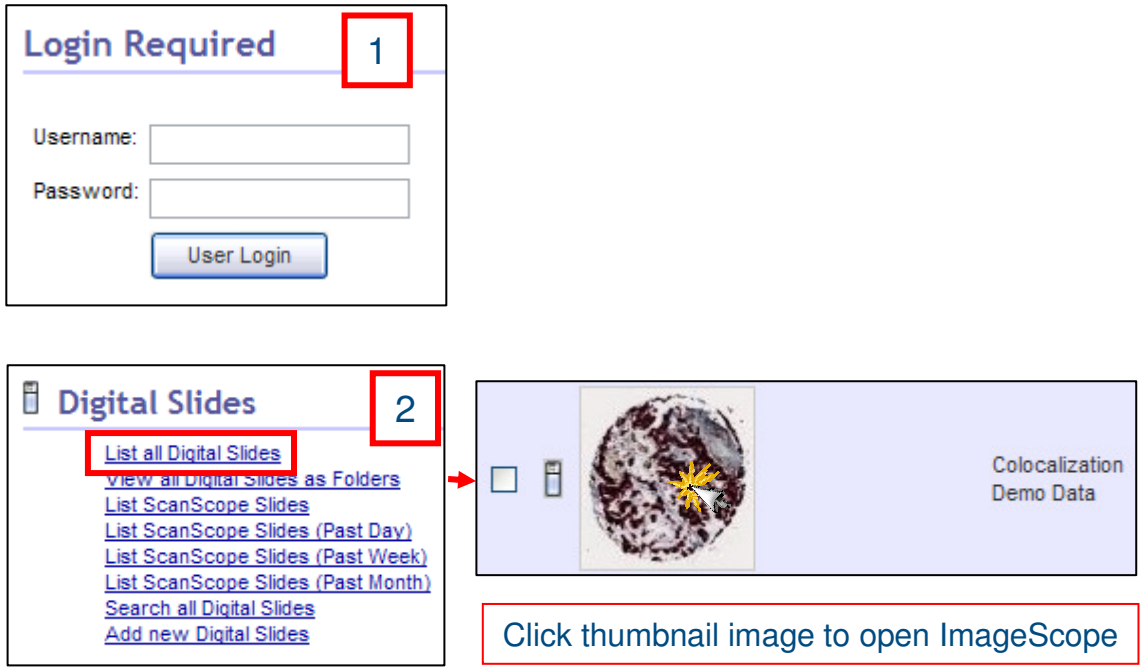


Watch Instructor demo the tasks below and perform them using the steps beginning on the next page.

- Open Colocalization demo file
- Select Colocalization macro
- Run analysis
- View Results

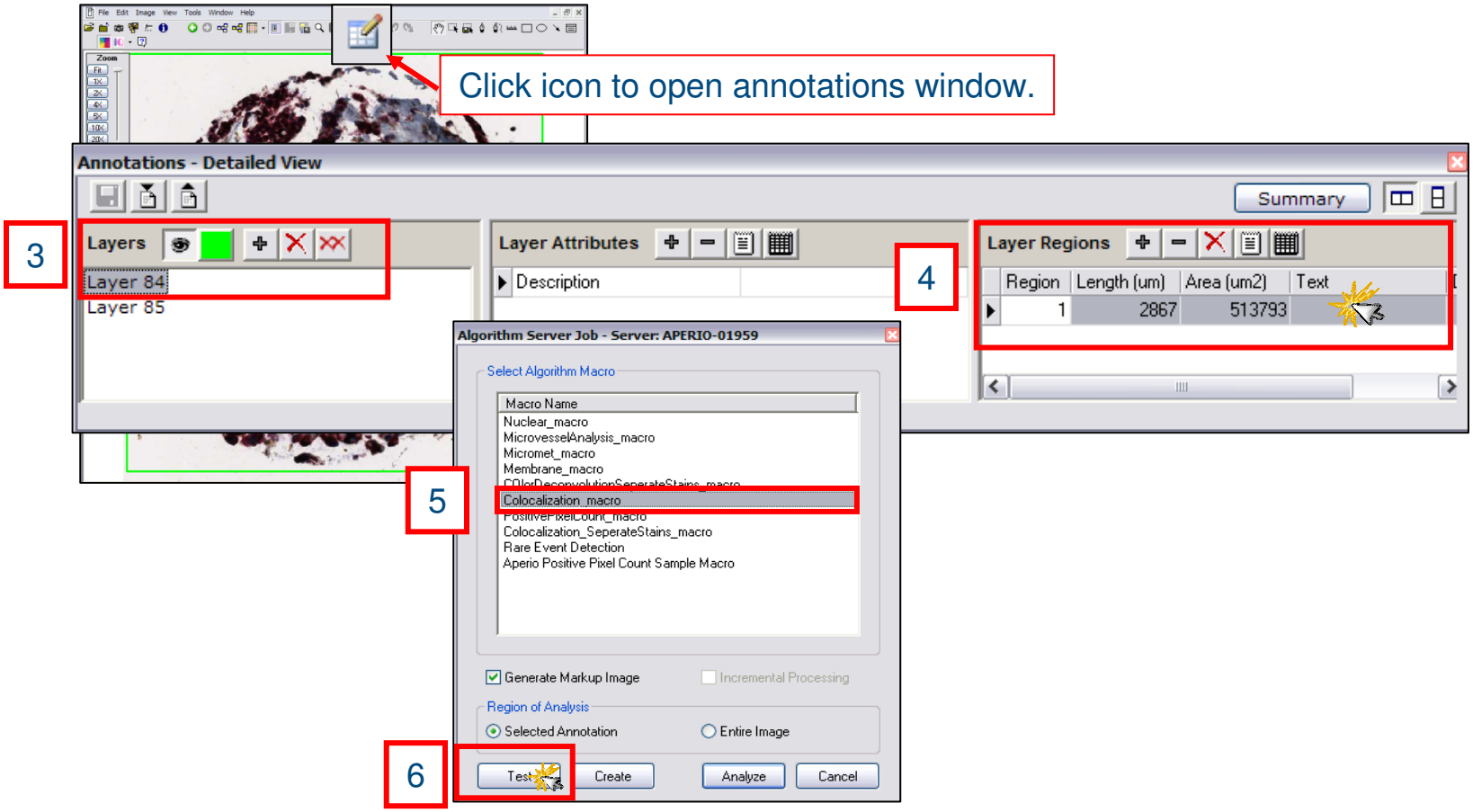
Colocalization Workshop

1. Log into Spectrum with a username and password provided by instructor & select the specified **Research Role**.
2. Navigate to the digital slide list, open the **Colocalization Demo Slide** as shown below.



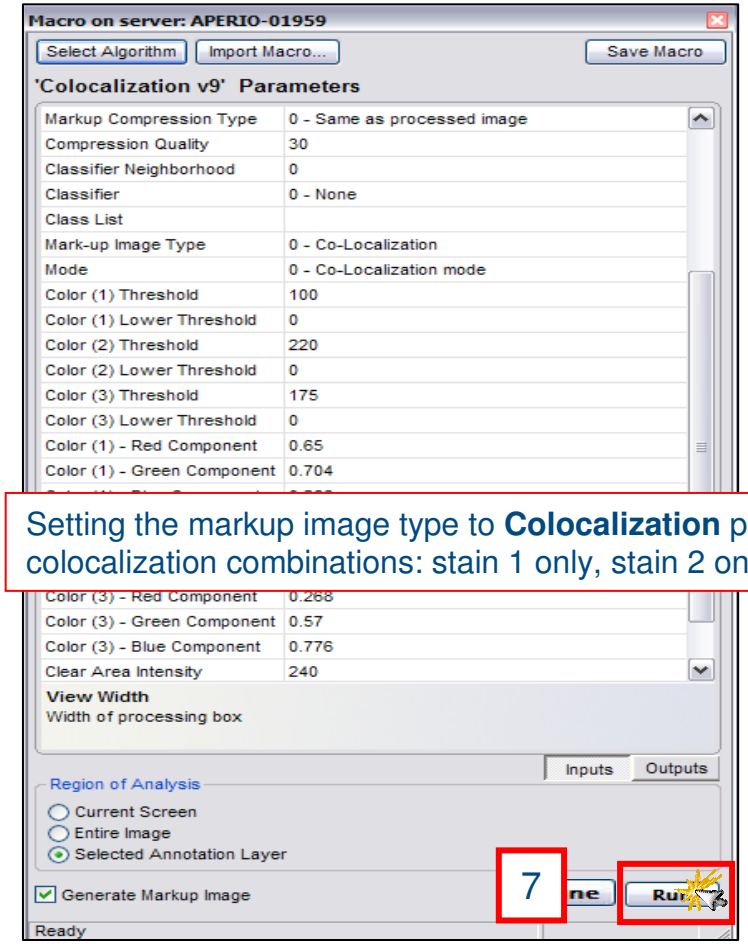
Colocalization Workshop

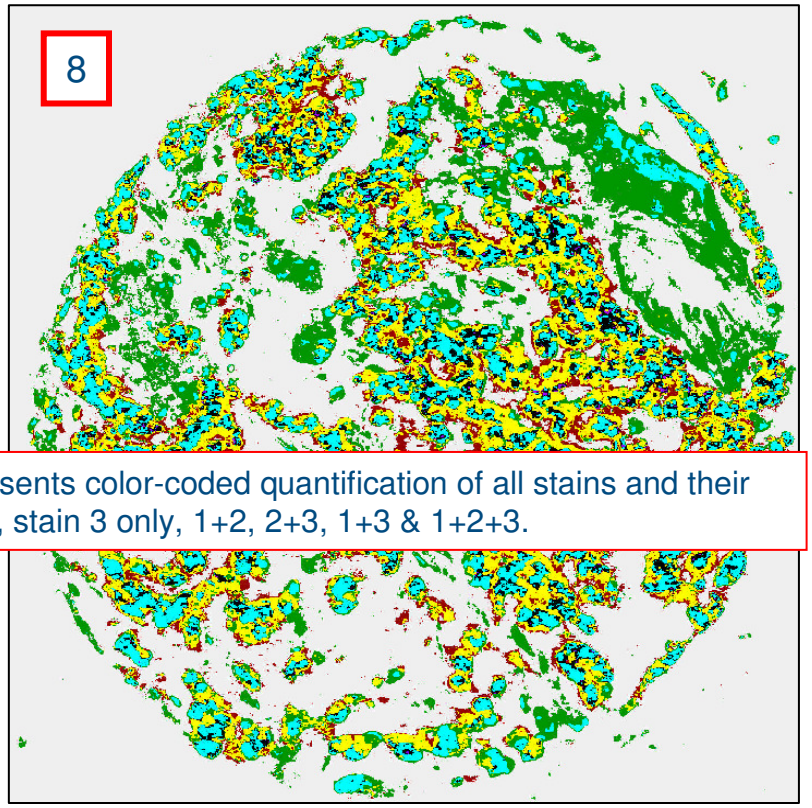
3. Open the Annotations winnow (Ctrl+N) & select the 1st **Layer.**
4. Click **Region 1** in the **Annotations** window under the **Layer Regions** section.
5. Open the Analysis window (Ctrl+G) & select the **Colocalization macro**.
6. Click **Test** to display the algorithm parameters.



Colocalization Workshop

7. Click **Run** in the **Algorithms** window.
8. View **markup image**.



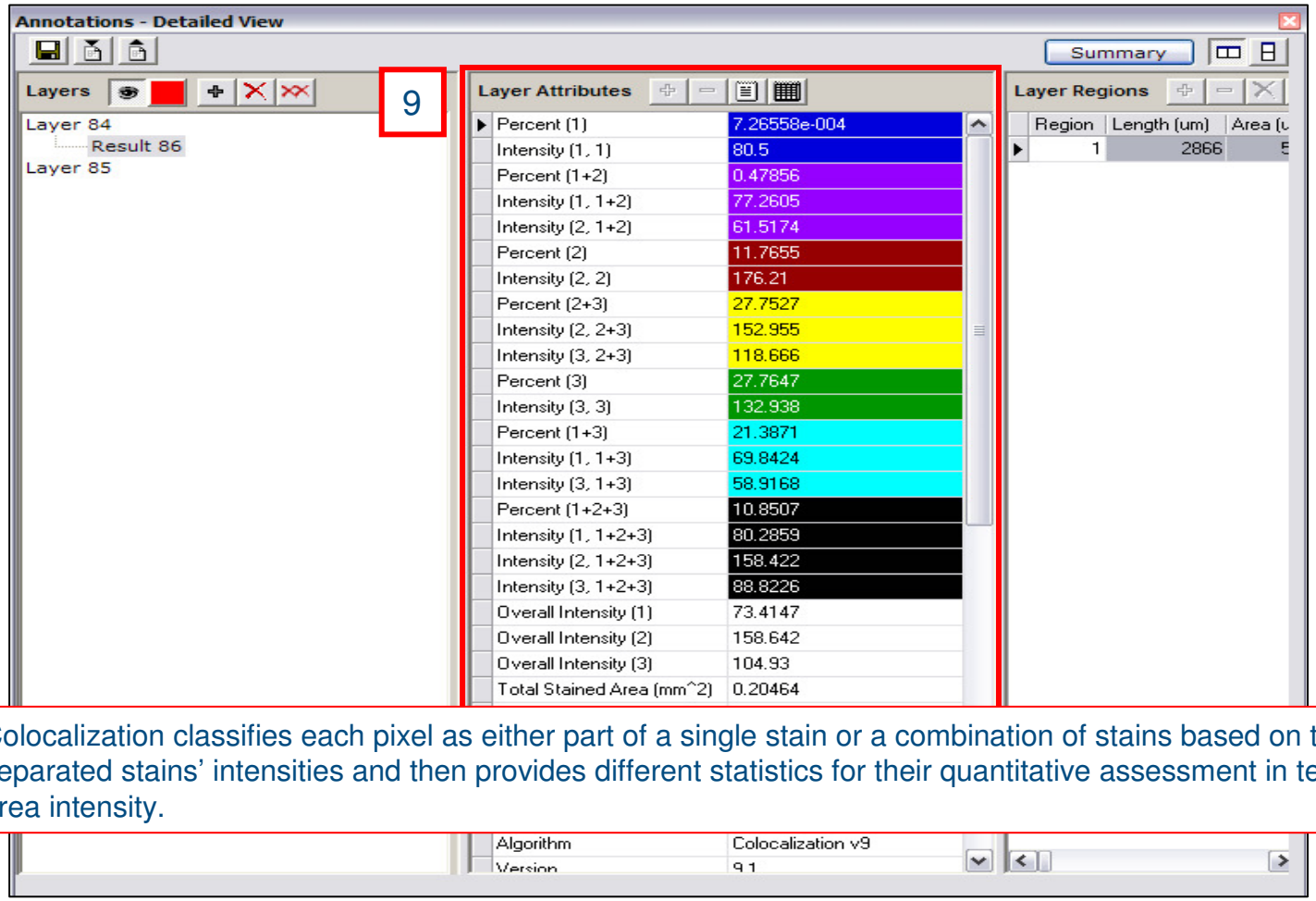


Setting the markup image type to **Colocalization** presents color-coded quantification of all stains and their colocalization combinations: stain 1 only, stain 2 only, stain 3 only, 1+2, 2+3, 1+3 & 1+2+3.

Region of Analysis selections:
Selected Annotation Layer
Generate Markup Image

Colocalization Workshop

9. View **results** (Ctrl N will display Annotations window) under **Layer Attributes** in the **Annotations** window.



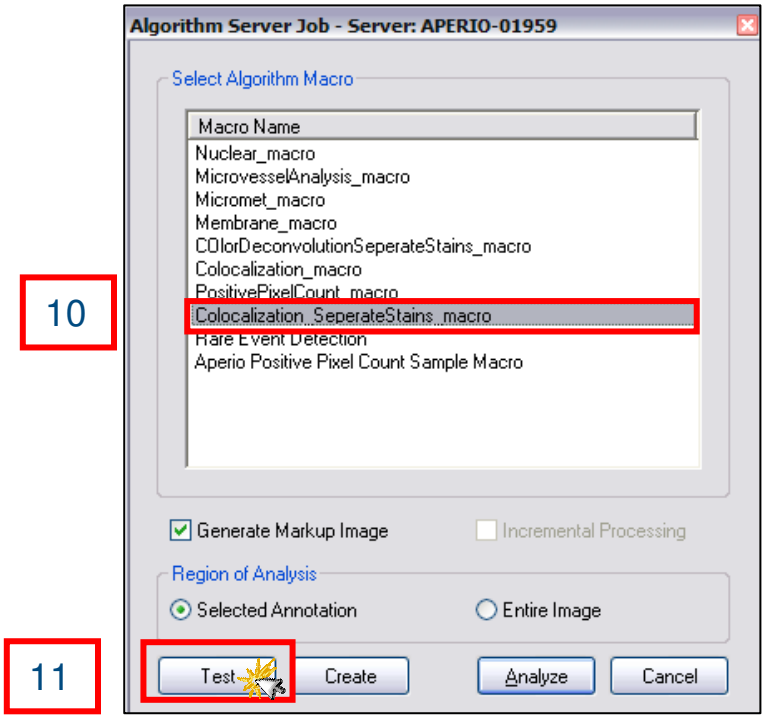
Colocalization classifies each pixel as either part of a single stain or a combination of stains based on the separated stains' intensities and then provides different statistics for their quantitative assessment in terms of area intensity.

Colocalization Workshop

Demonstrate that the different stains have been separated properly.

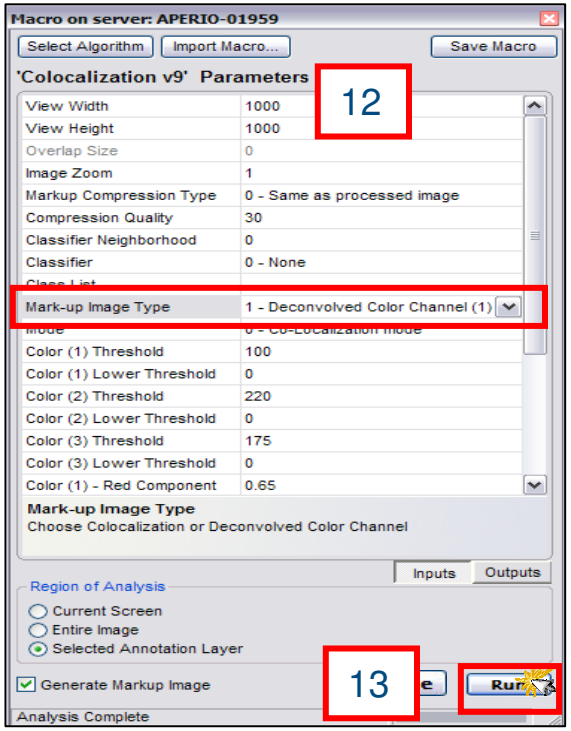
- *Hematoxylin*
- *Marker A (Fast Red)*
- *Marker B (DAB)*

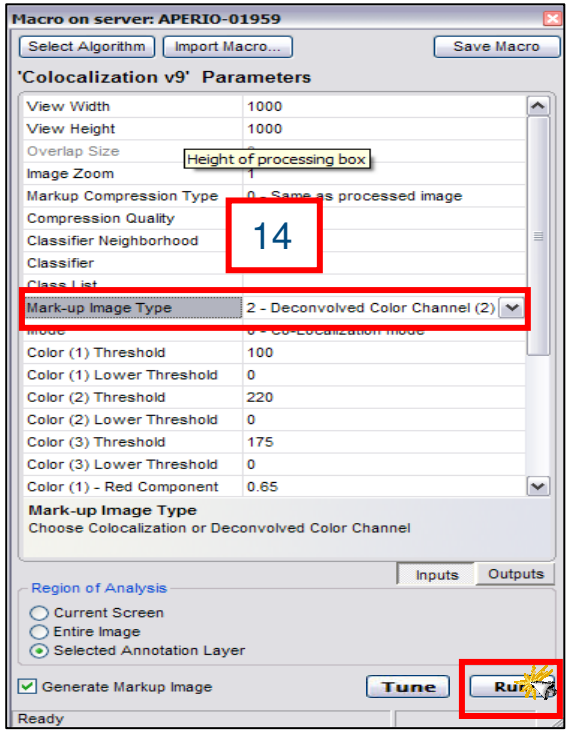
10. Reopen the **Analysis** window (Ctrl G) & select the **Colocalization_SeperateStains_macro**
11. Click **Test** to load parameter values.



Colocalization Workshop

12. Change **Markup Image Type to 1 – Deconvolved Color Channel (1)**.
13. Click **Run** in the **Algorithms window**.
14. Repeat steps 12-13 for the **Deconvolved Color Channels 2 & 3**.

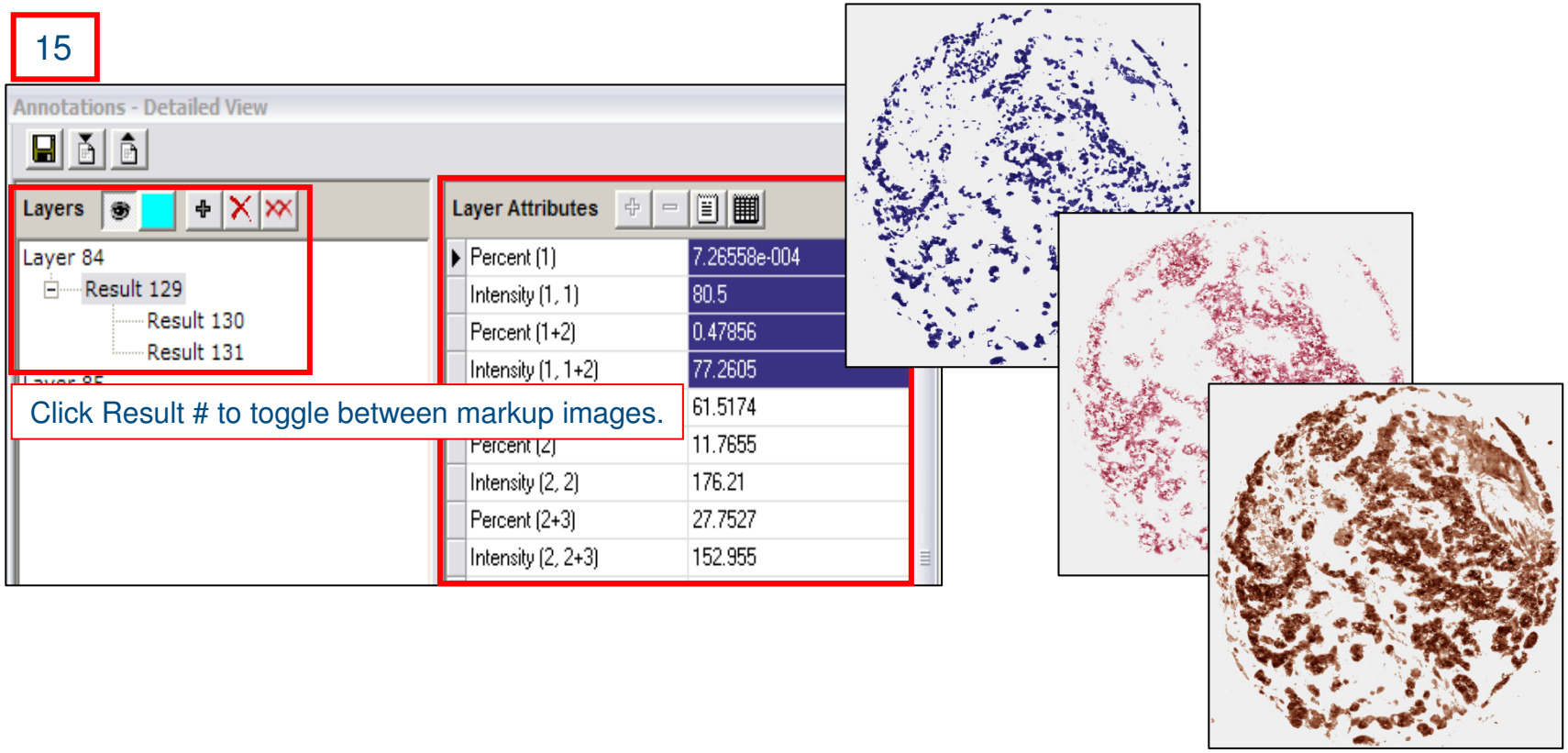




Region of Analysis selections:
Selected Annotation Layer
Generate Markup Image

Colocalization Workshop

15. Show **Markup Images** & view **results** in the **Annotations window**.



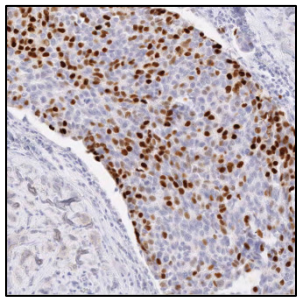
Knowledge Check

1. Colocalization Analysis determines to what extent proteins occur separately or in combination with each other.
   a) True
   b) False
2. What does Colocalization Analysis quantify?
   a) Cell quantification
   b) Membrane quantification
   c) Area and intensity
   d) Nuclear quantification
3. When using 3 color channels there are 7 possible output combinations?
   a) True
   b) False

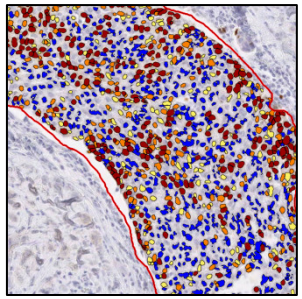
Nuclear

Nuclear analysis quantifies nuclear staining and intensity.

- Markup image highlights the detected nuclei which are color-coded according to their classification.
- Nuclear staining is classified as follows and is based on nuclear staining intensity: **Blue = 0, Yellow = 1+, Orange = 2+, Red = 3+**



Original



Markup
0, 1+, 2+, 3+ Nuclei

Nucleus = 0 when it has no staining.
Nucleus = 1+ when it has weak staining.
Nucleus = 2+ when it has moderate staining.
Nucleus = 3+ when it has intense staining.

- **Outputs:**
  - Percentage Positive Nuclei (0.0 – 100.0)
  - Average Intensities (0, 1+, 2+, 3+)
  - Percentages of 0, 1+, 2+, 3+
  - Other statistical measurements

Nuclear

Can be tuned for IHC ER and PR stained breast tissue.

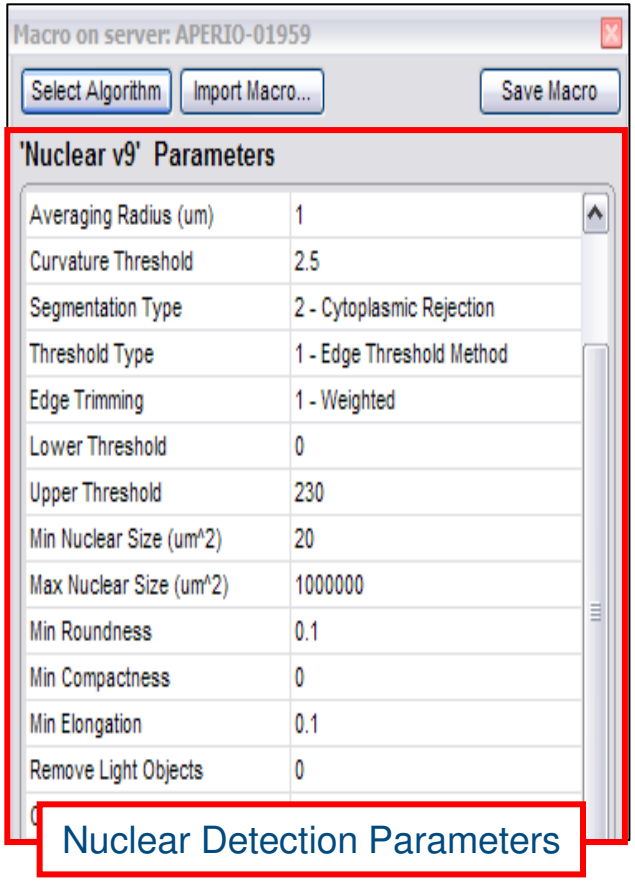
- Detects the nuclear staining for target chromogen for individual cells and quantifies intensity.
- Performs same complex analysis as pathologists.
- Detects positive staining for individual nuclei.
- Quantifies nuclei by staining intensity.
- Classifies nuclei as 0, 1+, 2+ and 3+.
- Automatic cytoplasmic stain removal.
- Automatic lab/reagent stain tuning.
- Supports different scoring schemes: Allred, HScore.
- Can be used for other tissue type by changing input parameters.

Nuclear

Nuclear Analysis input parameters.

- Nuclear Analysis is controlled by a set of input parameters.
- Cell feature detection and scoring scheme parameters are handled separately.

- **Avg. Radius**-Radius for noise reduction.
- **Curvature Threshold**-determines the level of de-clustering for touching nuclei.
- **Seg Type**- method of nuclear segmentation
  - Intensity, Cytoplasmic Reduction.
- **Threshold Type**-Determines method of edge separation (where one cell ends and another begins).
- **Min/Max Nuclear Size**-Nuclei outside of range will not be counted
- **Min Roundness**-Nuclei with roundness < value will not be reported.
- **Min Compactness**-Nuclei with compactness < value will not be reported.
- **Min Elongation**-Nuclei with elongation < value will not be reported.
- **Remove Light Objects**-Allows the removal of light objects.



Nuclear Detection Parameters

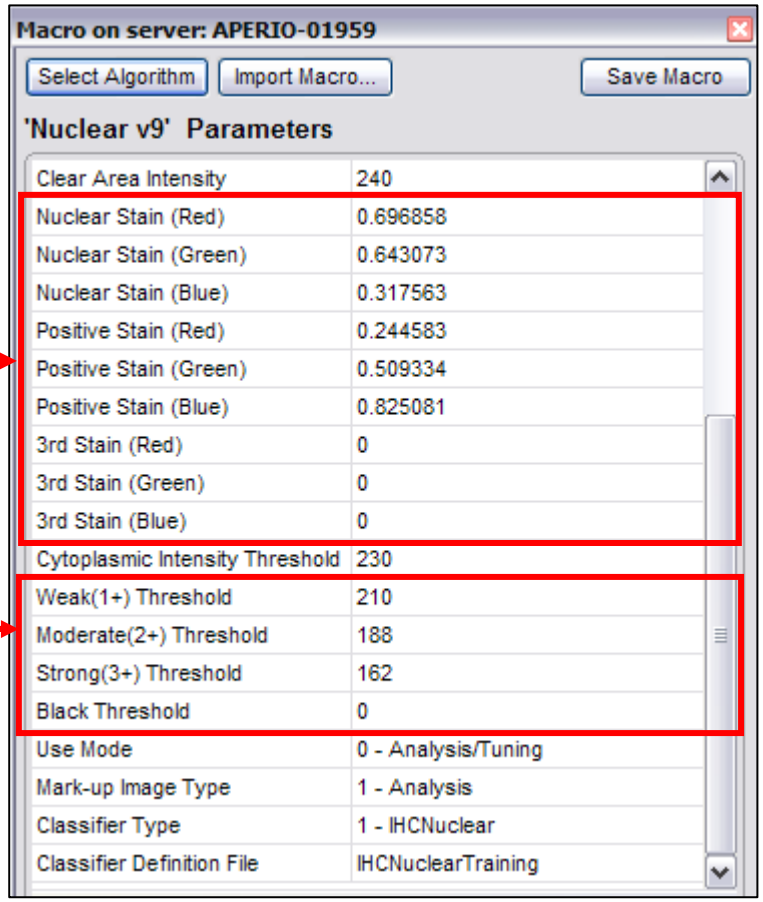
Nuclear

Nuclear Analysis input parameters.

- Stain color parameters can be calculated using Color Deconvolution's stain calibration process.

Stain Color Parameters

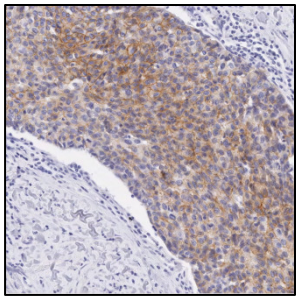
Scoring Scheme Parameters



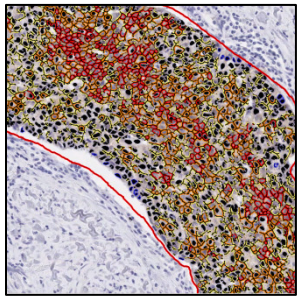
Membrane

Membrane Analysis quantifies membrane staining and intensity.

- Markup image highlights the detected cell features and the membrane staining which is color-coded according to cell classification.
- Membrane staining is classified as follows and is based on membrane staining, intensity and completeness: **Blue = 0, Yellow = 1+, Orange = 2+, Red = 3+**

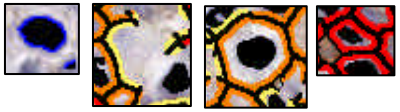


Original



Markup

0, 1+, 2+, 3+ Cells



Negative = 0 when it has no membrane staining.
Tumor Cell = 1+ when it has partial or weak staining.
Tumor Cell = 2+ when it has moderate & complete staining.
Tumor Cell = 3+ when it has intense & complete staining.

- **Outputs:**
  - HER2 Score (0, 1+, 2+, 3+)
  - Percentages of 0, 1+, 2+, 3+ cells
  - Other statistical measurements

Membrane

Can be tuned for IHC HER2 stained breast tissue.

- Detects membrane staining for individual tumor cells and quantifies the intensity and completeness of the membrane staining.
- Performs same complex analysis as pathologists.
- Detects positive membrane staining for individual cells.
- Quantifies cells by membrane staining, intensity and completeness.
- Classifies cells as 0, 1+, 2+, 3+.
- Applies HER2 scoring scheme.
- Automatic lab/reagent staining tuning.
- Supports different scoring schemes: HScore.
- Can be used for other tissue types and membrane stains by changing input parameters.

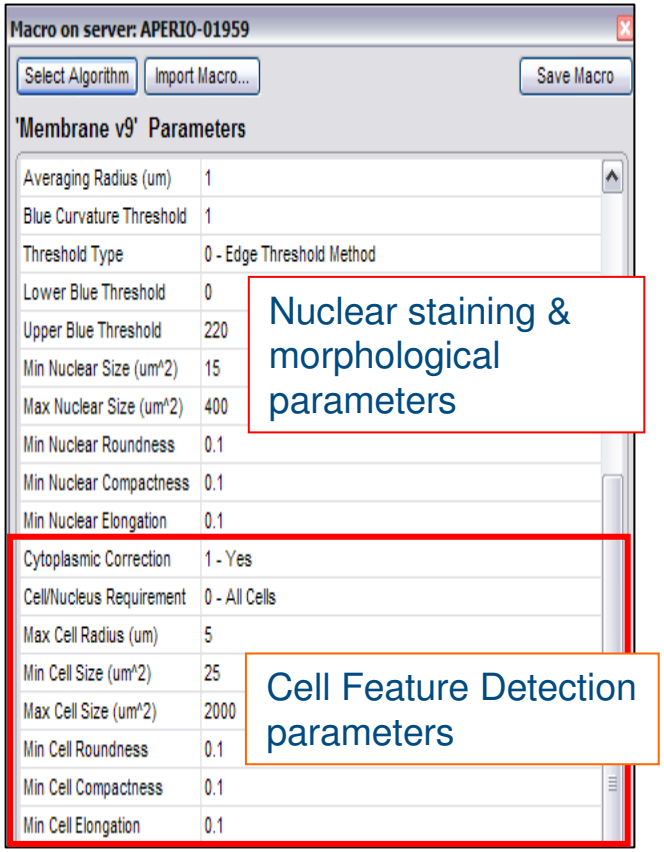
Membrane

Membrane Analysis input parameters.

- Membrane Analysis is controlled by a set of input parameters.
- Cell feature detection and scoring scheme parameters are handled separately.
  - Cell feature parameters specify cell feature detection thresholds and methods as well as size and shape and constraints of nuclei to distinguish tumor cells from normal, lymphocyte and stroma cells.

Cell Feature Parameters

- **Cytoplasmic Correction**-If set to yes, background staining is weeded out.
- **Cell/Nucleus Req.**-Include/exclude cells without nucleus.
- **Max Cell radius**-Determines cell size for cells that have a nucleus but no membrane staining.
- **Min/Max Cell Size**-Cells outside this range will not be counted.
- **Min Roundness**-Cells with roundness less than this value will not be reported.
- **Min Compactness**-Cells with compactness less than this value will not be reported.
- **Min Elongation**-Cells with elongation less than this value will not be reported.

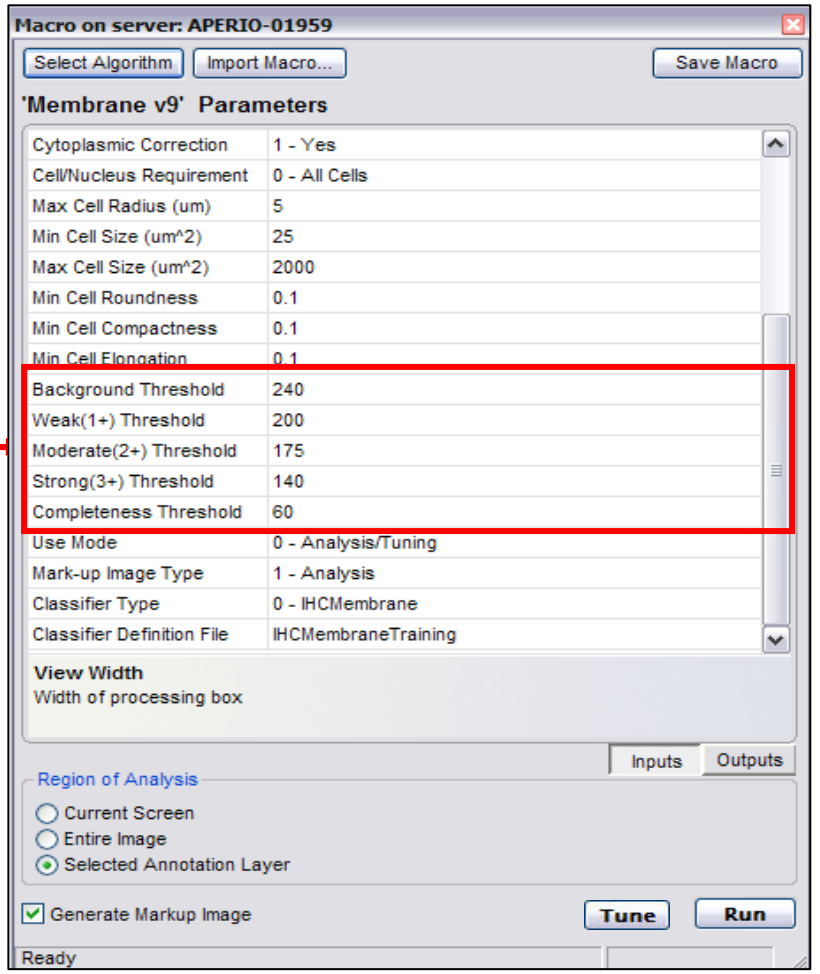


Membrane

Membrane Analysis input parameters.

- The scoring scheme parameters specify the staining intensity and membrane completeness thresholds that determine the individual tumor cell classification.

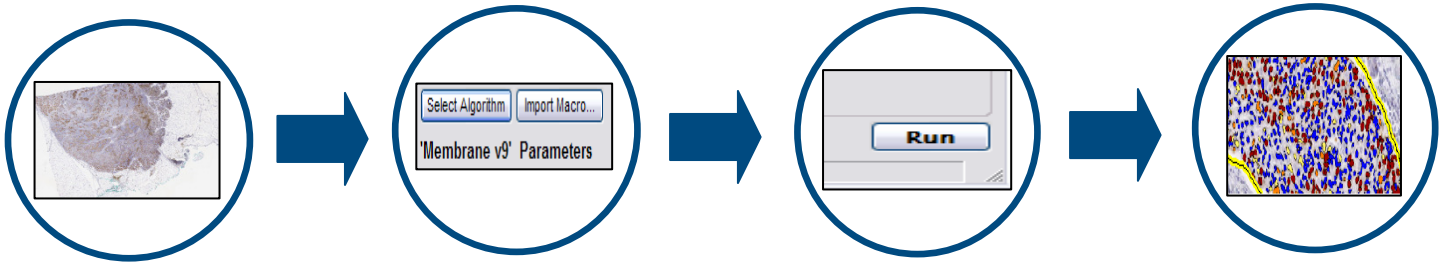
Scoring scheme classification thresholds



Nuclear & Membrane Workshop

Watch Instructor demo the tasks below and perform them using the steps beginning on the next page.

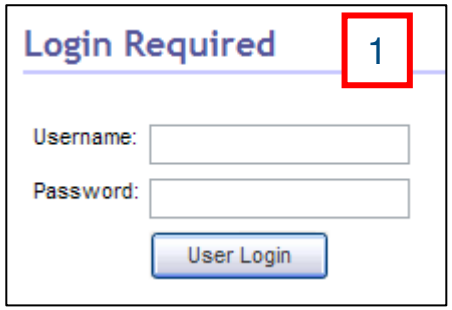
- Open Nuclear & Membrane demo files
- Select Nuclear & Membrane macros
- Run analysis
- View Results



Open Image | Select Macro | Run Analysis | View Results

Nuclear Workshop

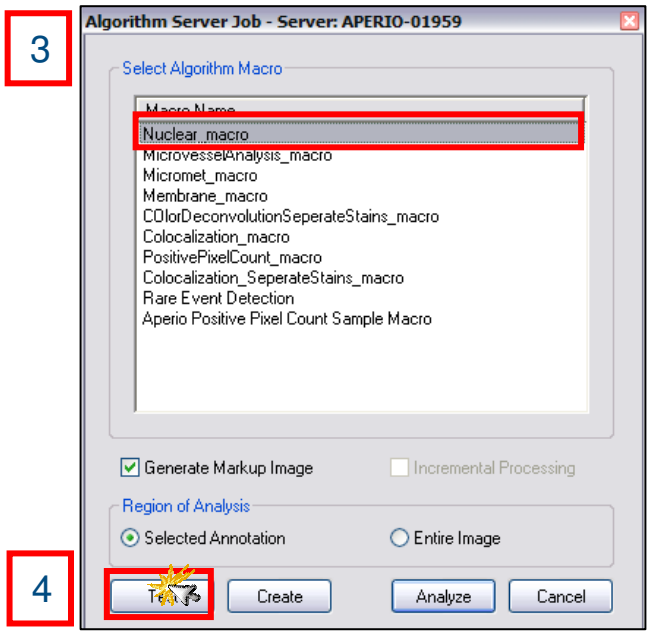
1. Log into Spectrum with a username and password provided by instructor & select the specified Research Role.
2. Navigate to the digital slide list, open the **Nuclear Demo** slide as shown below.

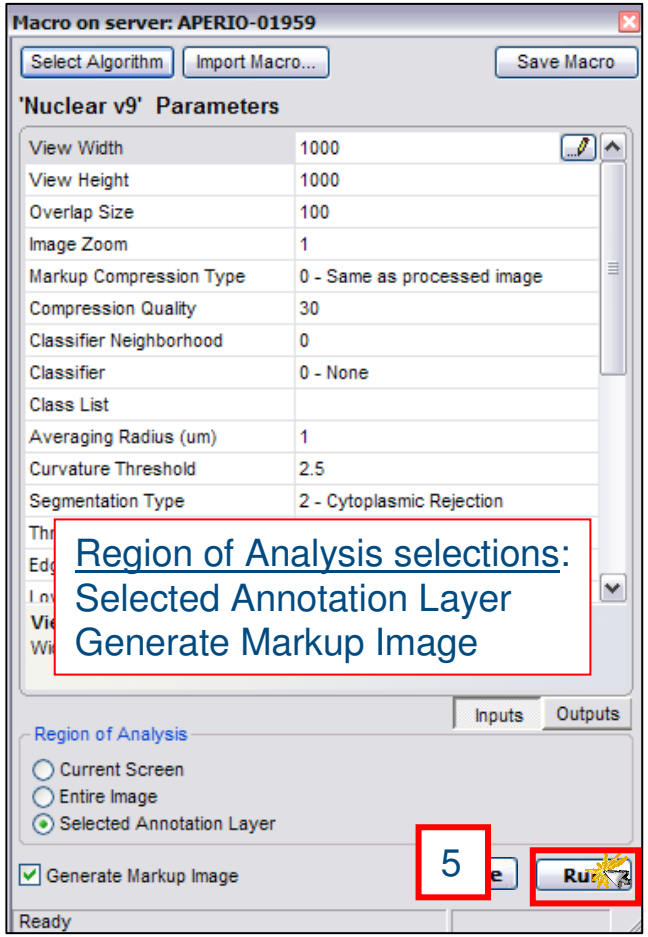


Click thumbnail image to open ImageScope

Nuclear Workshop

3. Open the **Analysis window (Ctrl G)** & select the **Nuclear macro**.
4. Click **Test** to display the parameters.
5. Review the parameters & click **Run** in the **Algorithms window**.

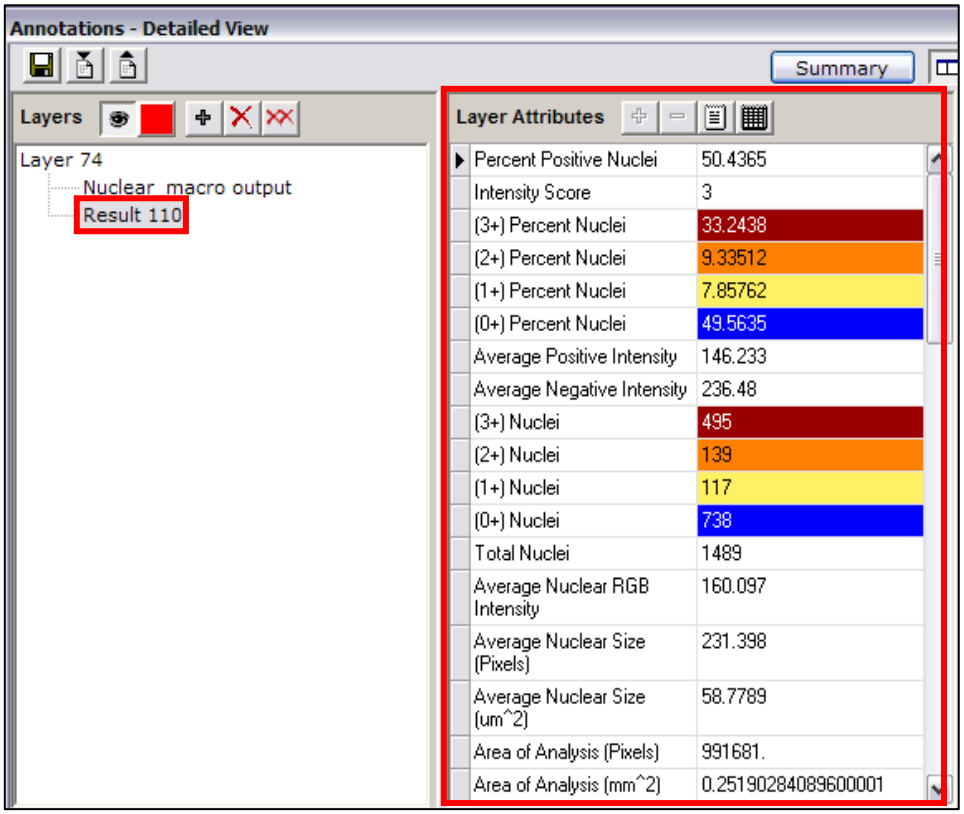


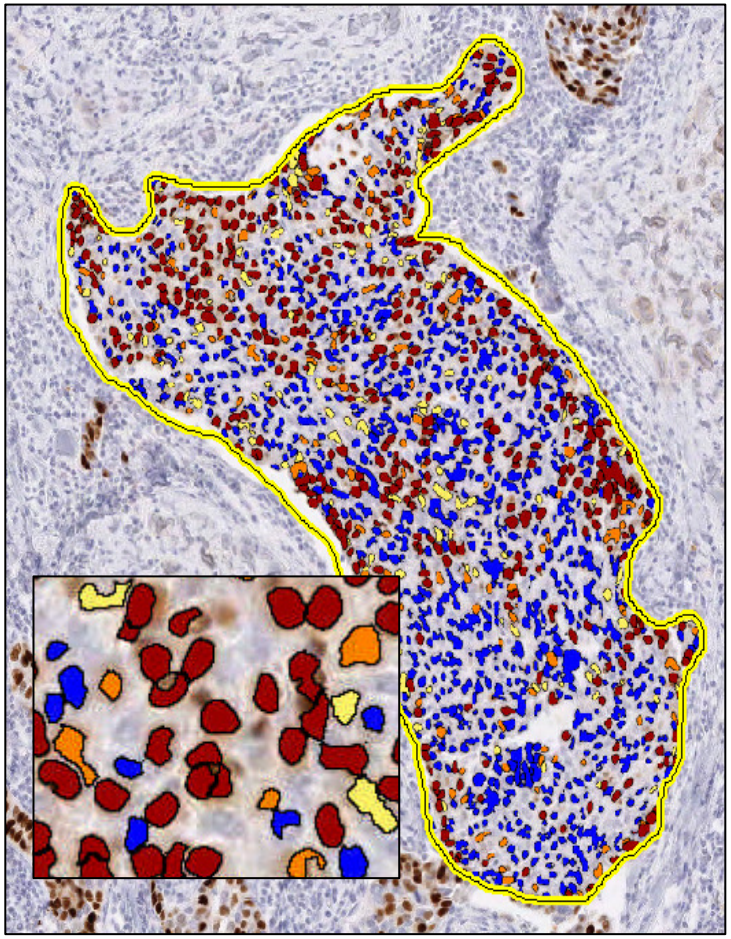


Nuclear Workshop

6. View **Markup Image** & **results** in the **Annotations window**.

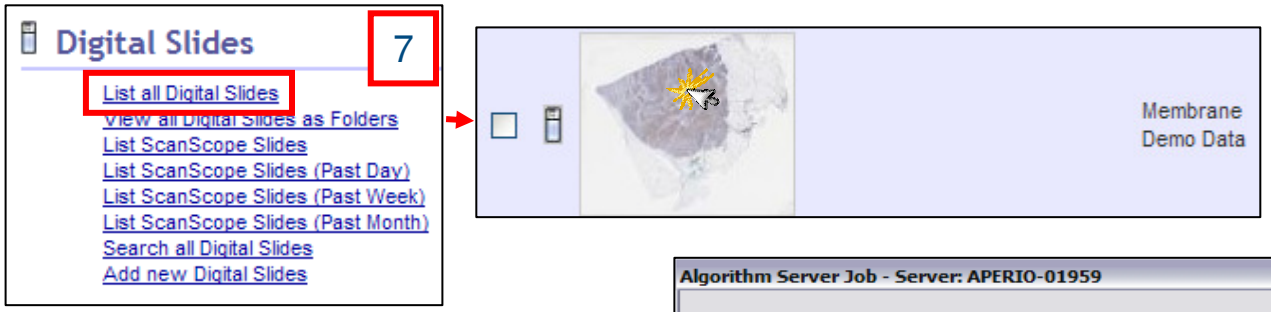
6

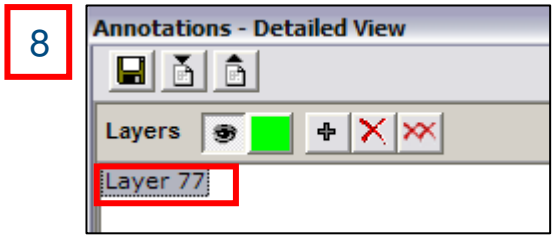


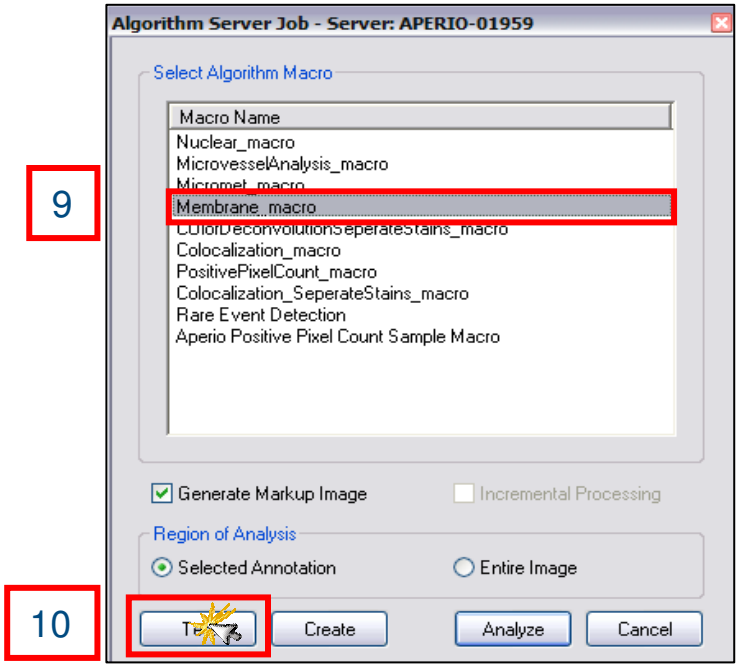


Membrane Workshop

7. Return to Spectrum and open the **Membrane Demo** slide as shown.
8. Open the **Annotations window** (Ctrl N), and select the first **Layer**.
9. Open the **Analysis window (Ctrl G)** & select the **Membrane macro**.
10. Click **Test** to display the input parameters.

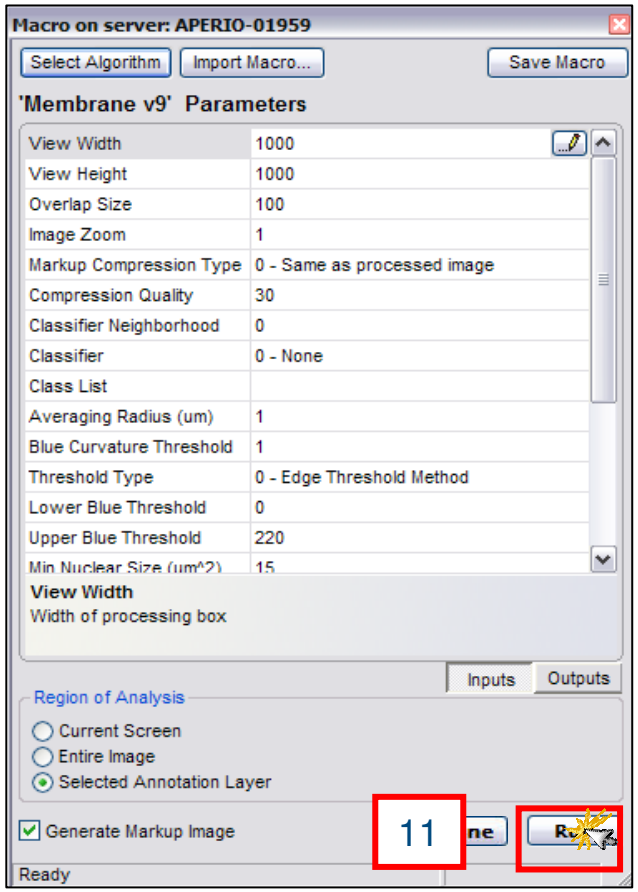






Membrane Workshop

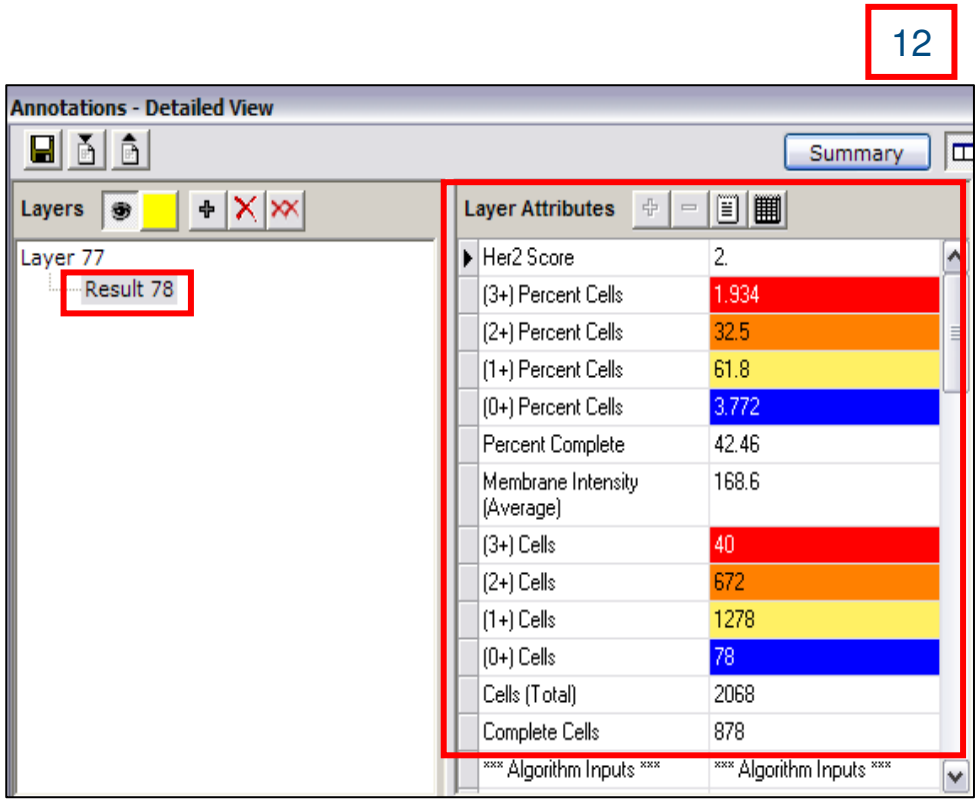
11. Review the parameters and click **Run**.

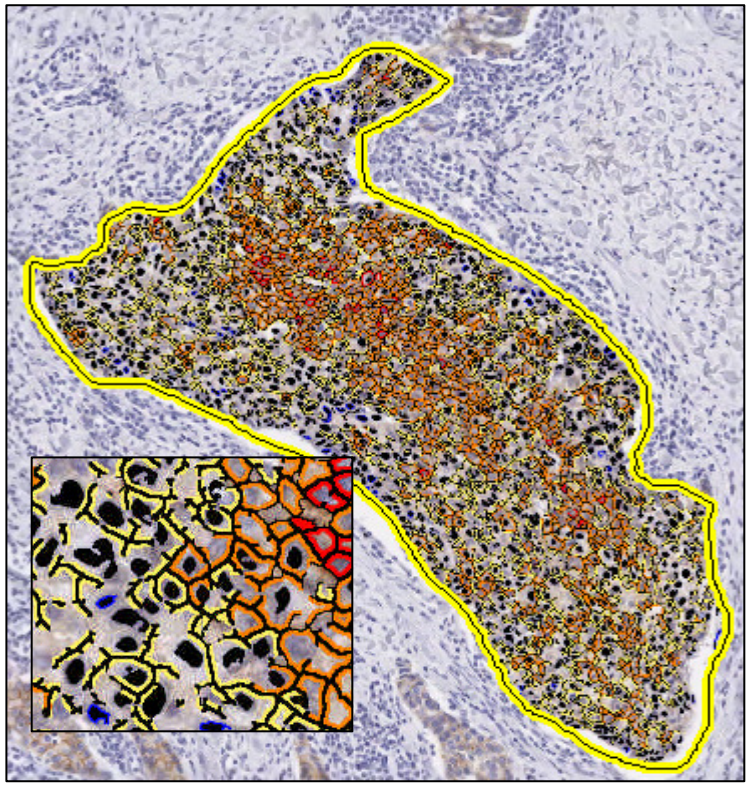


Region of Analysis selections:
Selected Annotation Layer
Generate Markup Image

Membrane Workshop

12. View **Markup Image** & **results** in the **Annotations window** (Ctrl+N).





Based on the percentage of 0, 1+, 2+ & 3+ cells, a score of 0, 1+, 2+, or 3+ is determined according to the HER2 scoring scheme.

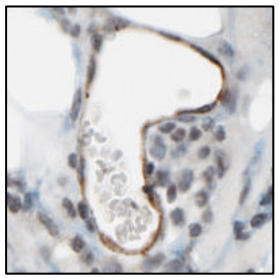
Knowledge Check

1. Nuclear and Membrane Analysis can be tuned for applications other than breast tissue.
   a) True
   b) False
2. Nuclear and Membrane markup images use what colors to code analysis results?
   a) Cyan, Magenta, Yellow
   b) Red, Green, Blue
   c) Blue, Red
   d) Blue, Yellow, Orange, Red
3. Curvature threshold parameter is an example of a clustering parameter?
   a) True
   b) False
4. What is the purpose of the Averaging Radius parameter?
   a) Establish nuclear or membrane morphology
   b) Area quantification
   c) Averages the color intensity across nuclei
   d) Decluster nuclei

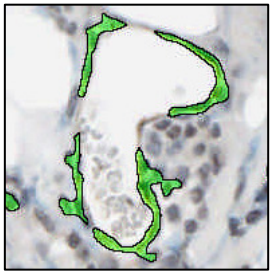
Microvessel Analysis

Detects and quantifies microvessels on slides stained with endothelial markers.

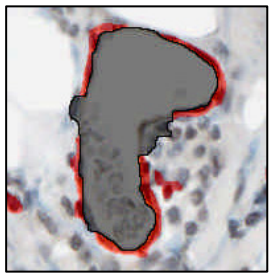
- **Applications include:**
  - In vivo angiogenesis assays in cardiovascular research.
  - Vascular morphology changes in oncology xenografts.
  - Microcapillary measurements in rodent models in ophthalmology.
- **Analysis results:**
  - Number of vessels.
  - Vessel Area.
  - Vessels Density.
  - Stain area and intensity.



Endothelial cells



Grouped to make regions



Vessel perimeter drawn in middle of vessel walls

Vessel Area

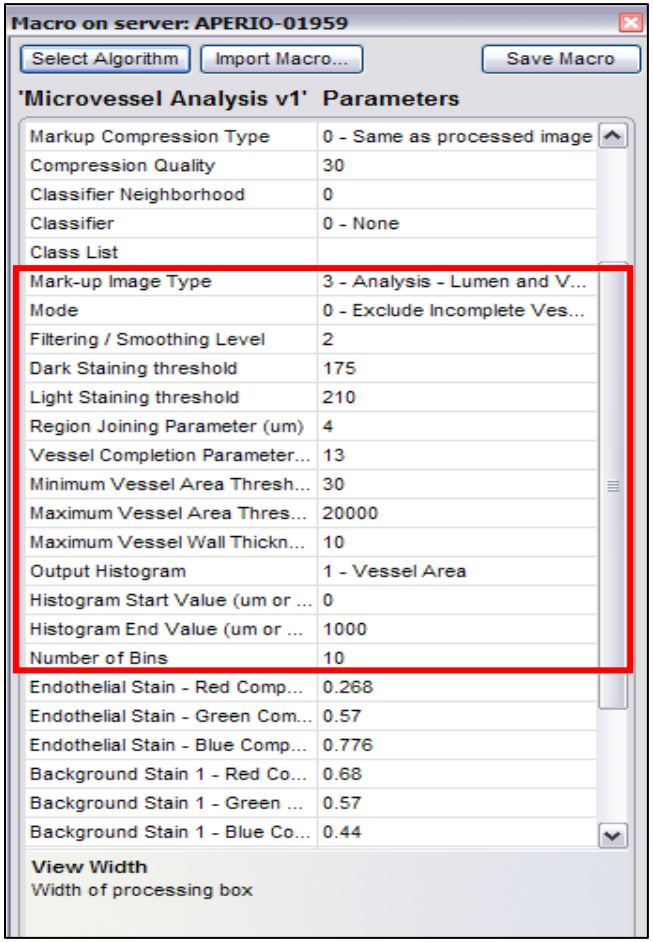
Red = vessel wall, vascular area
Green = vessel area
Black = vessel perimeter
Gray = lumen

Microvessel Analysis

Microvessel input parameters.

- Microvessel has a set of input parameters, which determines the analysis to be performed.

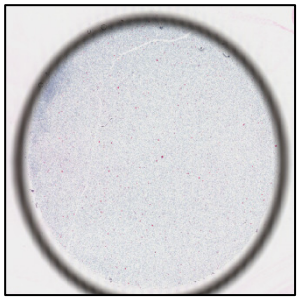
- **Mode**-Choose to include or exclude incomplete vessels.
- **Filter/Smoothing Level**-Specify the amount of filtering on the deconvolved vessel channel.
- **Intensity Thresholds**-
    - •Dark Staining
    - •Light Staining
- **Vessel Completion Thresholds**-Specify the distance in microns between pieces of vessel walls and the ends of the vessels walls that are joined to complete the vessel.
- **Min & Max Vessel Area Thresholds**-Specify internal area size to eliminate vessel from numeric results.
- **Histograms**-Specify histogram details.



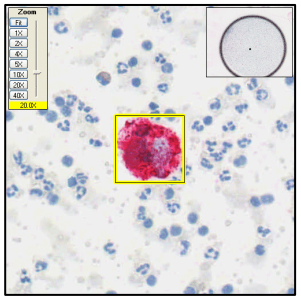
Rare Event Detection

Rare Event Detection finds specific events on a digital slide.

- Define the objects you are looking for by color (defined in the Hue Saturation Intensity color space) and size.
- An example:
  - Detect micrometastasis of tumor cells found in circulating blood.
  - Uses color segmentation and morphological image processing methods to detect and count objects of interest.



Original



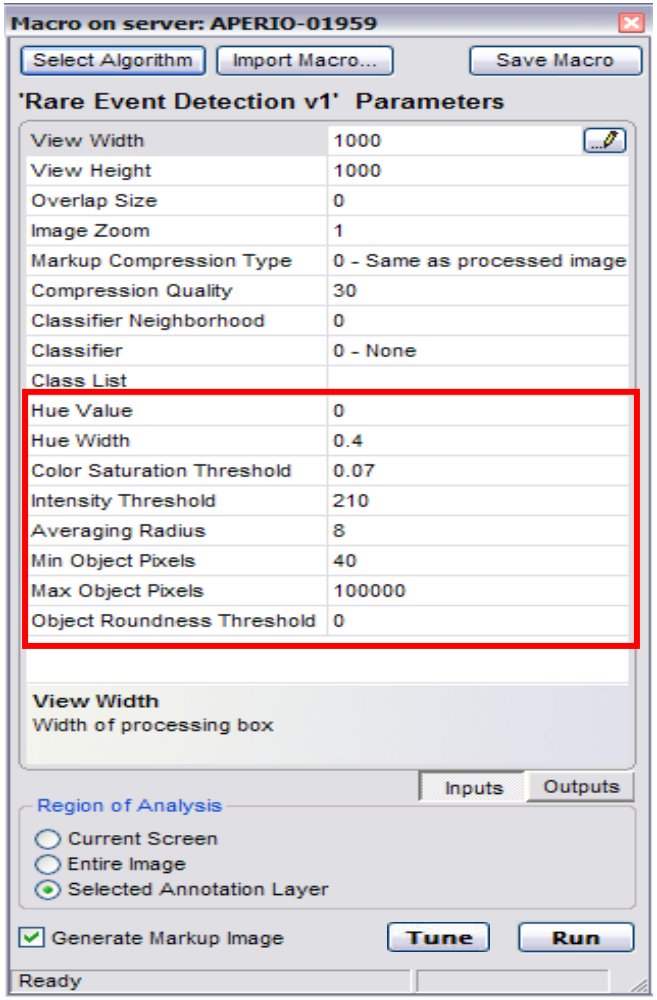
Markup

Rare Event Detection

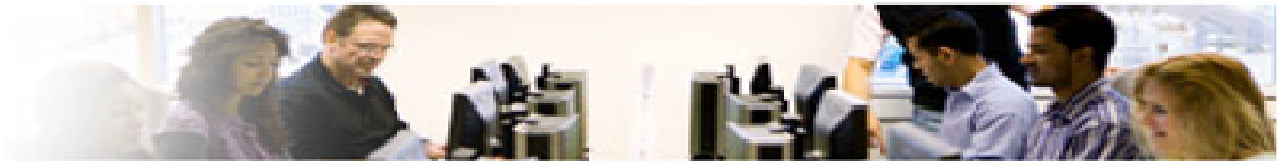
Rare Event input parameters.

- Default parameter set is used to detect micrometastasis of tumor cells in circulating blood.

- **Hue Value** – defines the color of the objects of interest.
- **Hue Width** - selects the range of hues, centered on the Hue Value, that will satisfy the hue detection process.
- **Color Saturation Threshold** – the required saturation of the detected object.
- **Intensity Threshold** - the intensity threshold of positive pixels.
- **Averaging Radius** - A smoothing parameter used by the morphological process to remove small□scale noise structure (value > 0).
- **Min Object Pixels** – min pixel size of object.
- **Max Object Pixels** – max pixel size of object.
- **Object Roundness Threshold** - Objects with roundness less than this value are not reported (0.0 < Value < 1.0). Circular objects will have a roundness=1, while elongated objects will have a smaller value (a line has roundness=0).

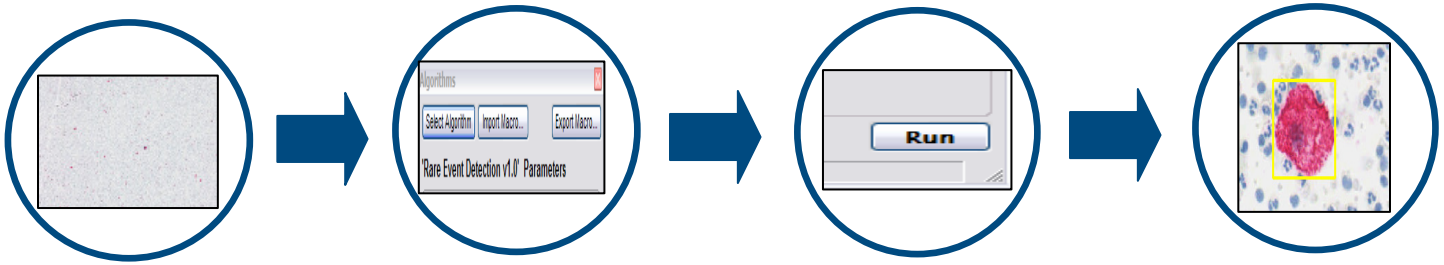


Microvessel & Rare Event Workshop



Watch Instructor demo the tasks below and perform them using the steps beginning on the next page.

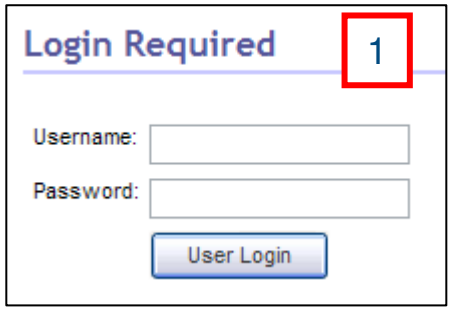
- Open Micro Vessel & Rare Event demo files
- Select Micro Vessel & Rare Event macros
- Run analysis
- View Results



Open Image | Select Macro | Run Analysis | View Results

Microvessel Workshop

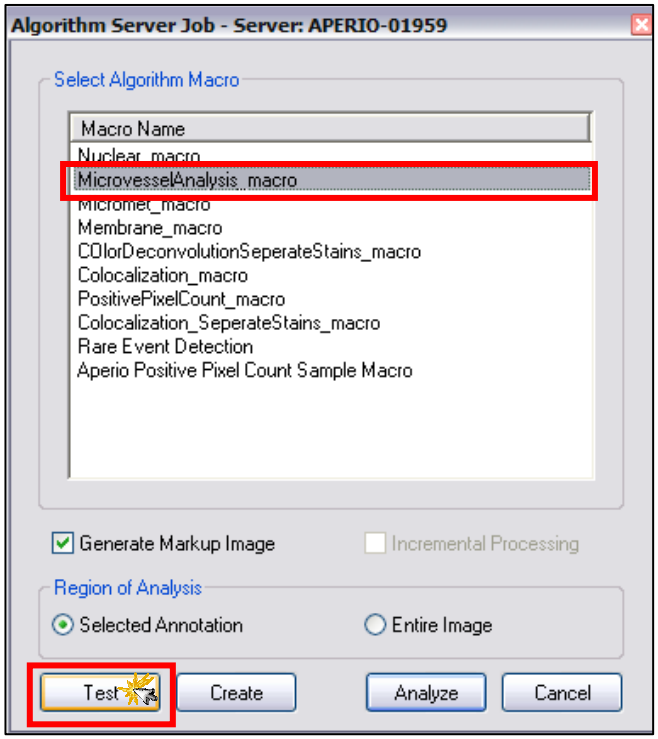
1. Log into Spectrum with a username and password provided by instructor & select the specified Research Role.
2. Navigate to the digital slide list, open the **Microvessel Demo** slide as shown below.



Microvessel Workshop

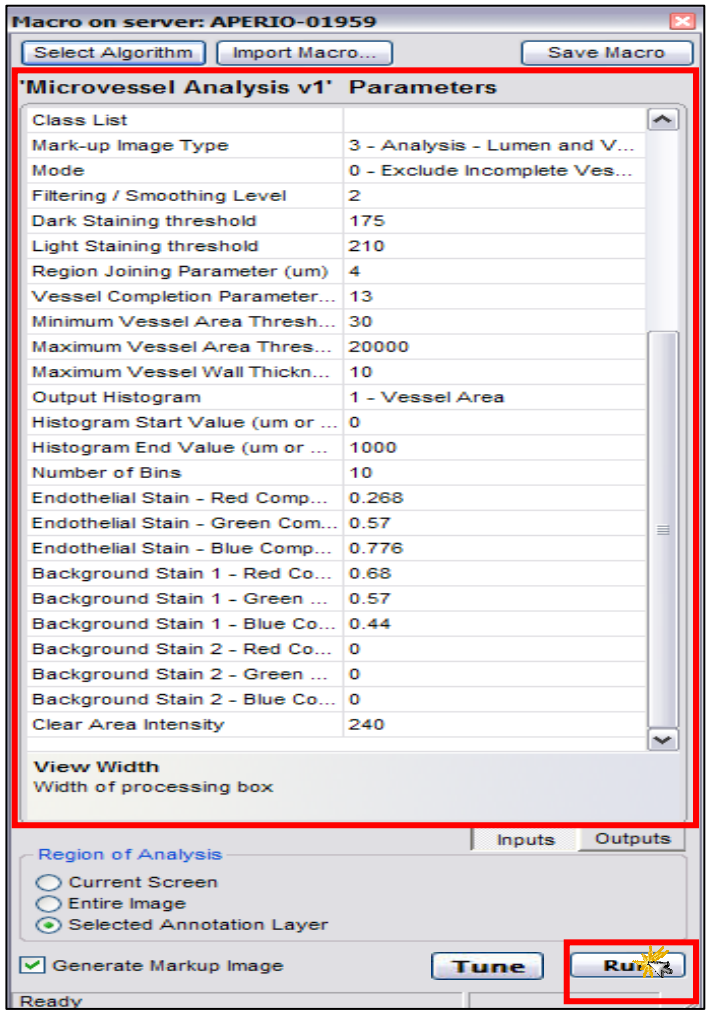
3. Open the **Analysis window (Ctrl G)** & select the **MicrovesselAnalysis_macro**.
4. Click **Test** to display the parameters.
5. Review the parameters and click **Run**.

3



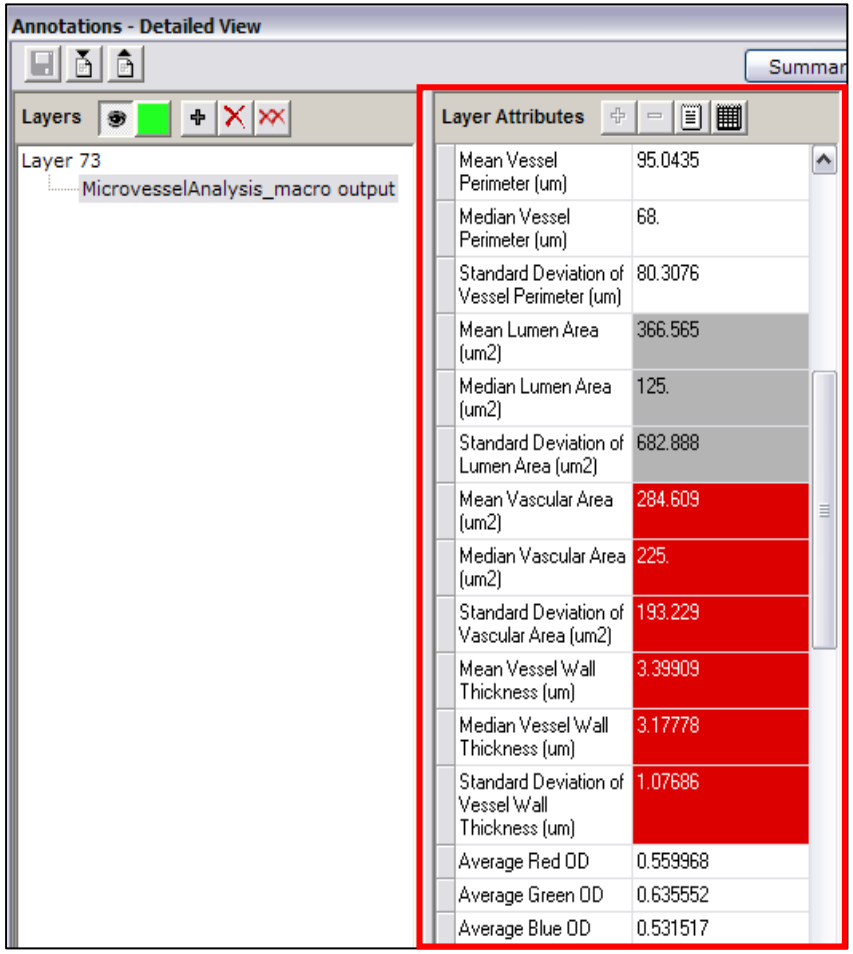
4

5

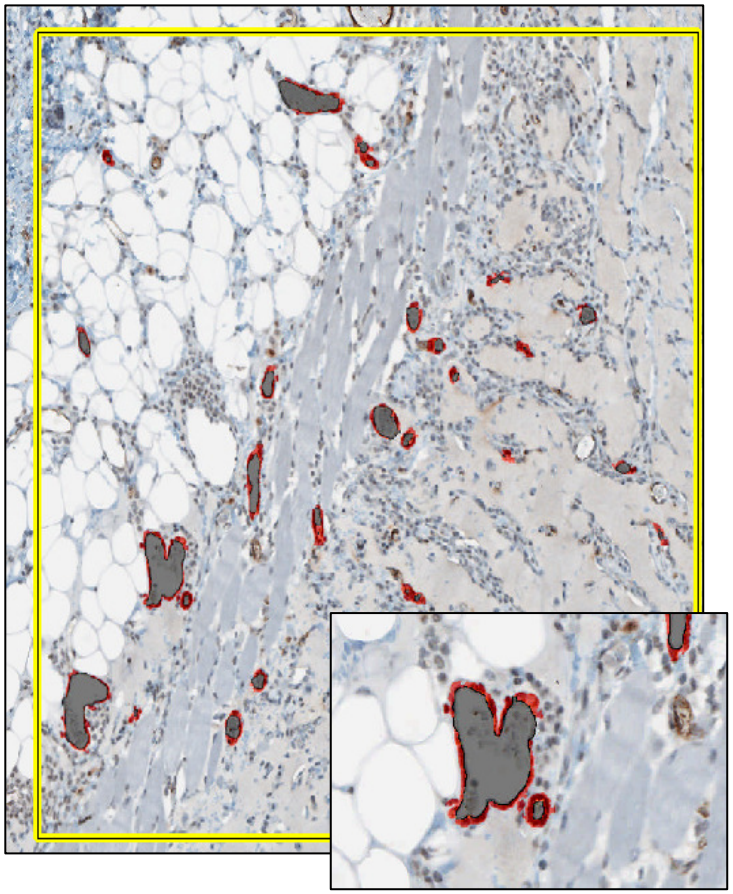


Microvessel Workshop

6. View **Markup Image** & **results** in the **Annotations window** (Ctrl+N).



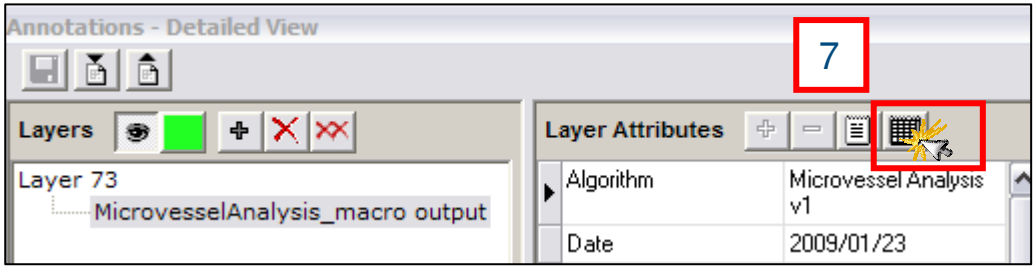
6

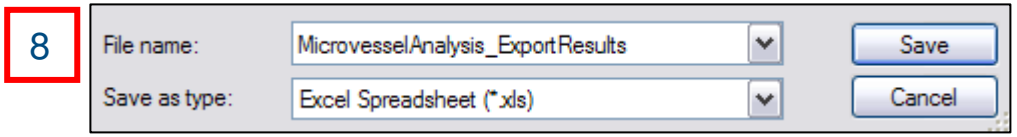


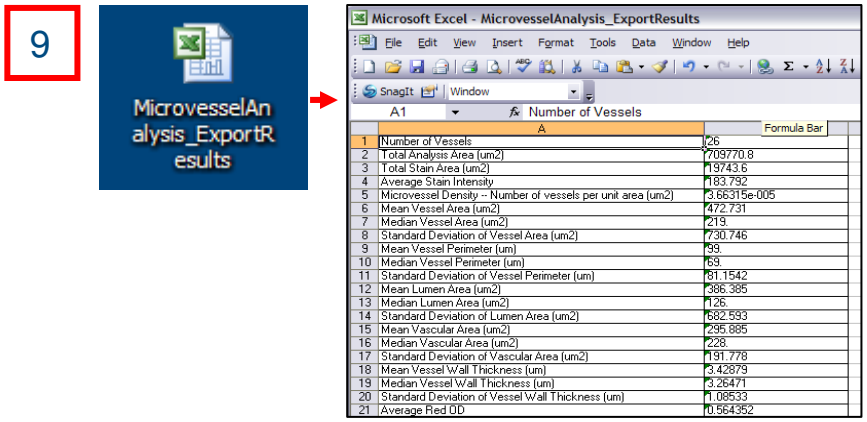
Microvessel Workshop

Export results and histogram values into an Excel Spreadsheet.

7. Click **Export Grid to Excel Spreadsheet button** in the **Layer Attributes section**.
8. Save the values, **File name: MicrovesselAnalysis_ExportResults** to the desktop.
9. Open the file and view the Report.

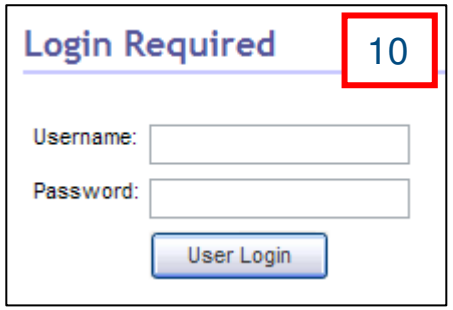






Rare Event Workshop

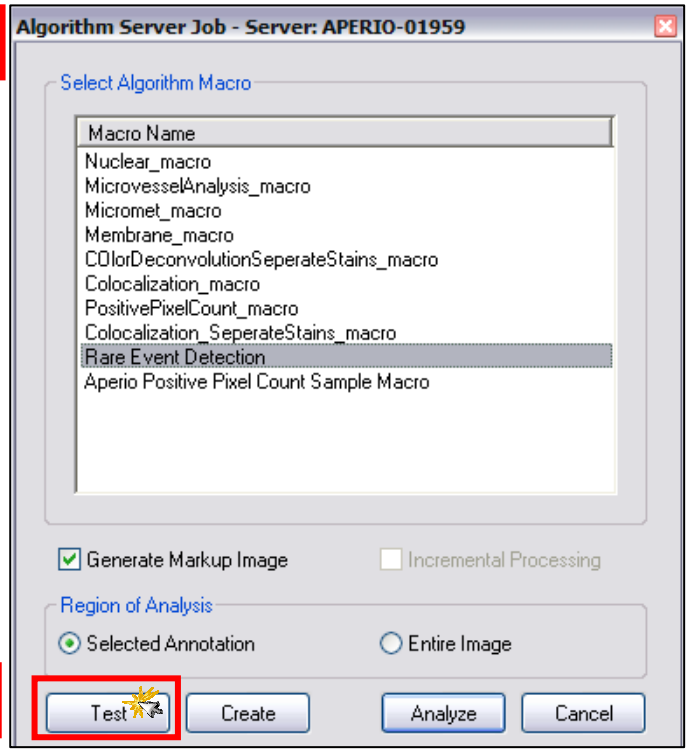
10. Log into Spectrum with a username and password provided by instructor & select the specified **Research Role**.
11. Navigate to the digital slide list, open the **Rare Event Demo** slide as shown below.



Rare Event Workshop

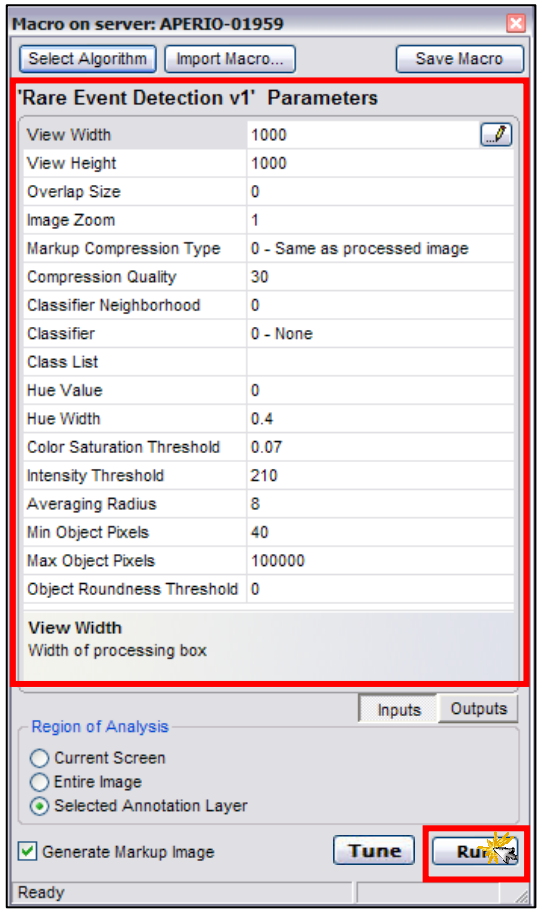
12. Open the **Analysis window (Ctrl G)** & select the **MicrovesselAnalysis_macro**.
13. Click **Test** to display the parameters.
14. Review the parameters and click **Run**.

12



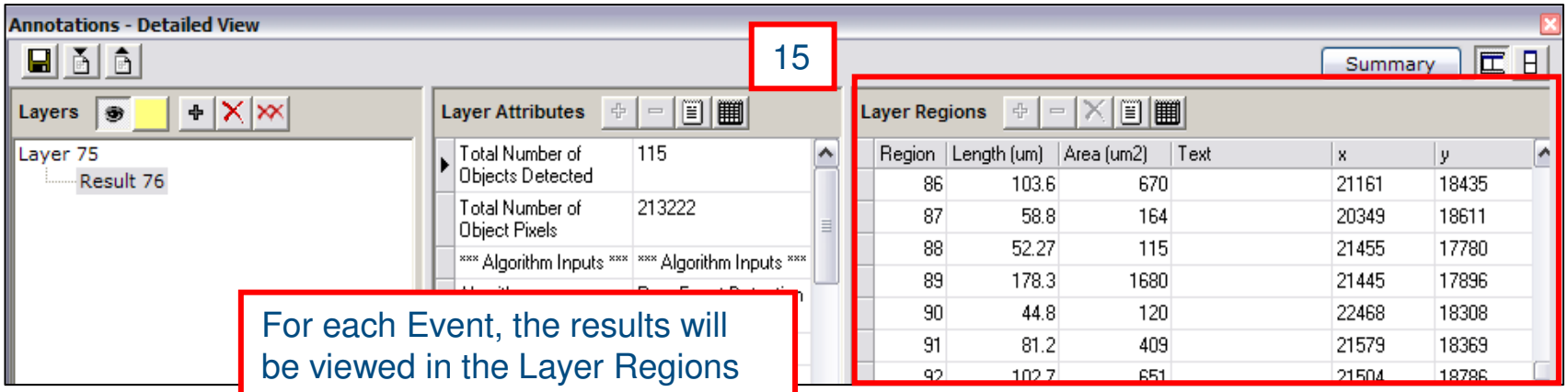
13

14

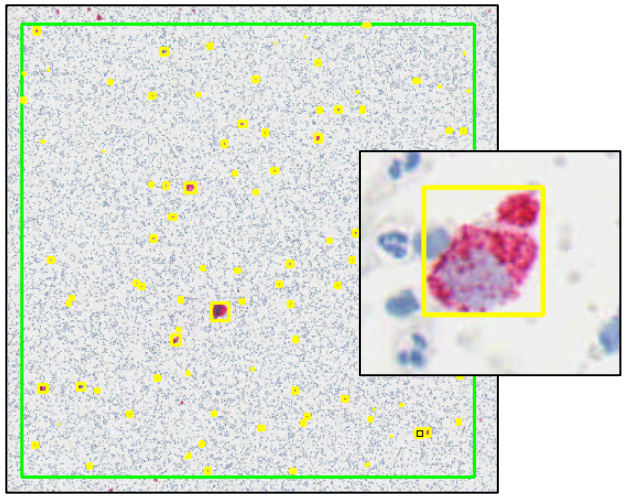


Rare Event Workshop

15. View **Markup Image** & **results** in the **Annotations window**.



For each Event, the results will be viewed in the Layer Regions pane of the Annotations window.

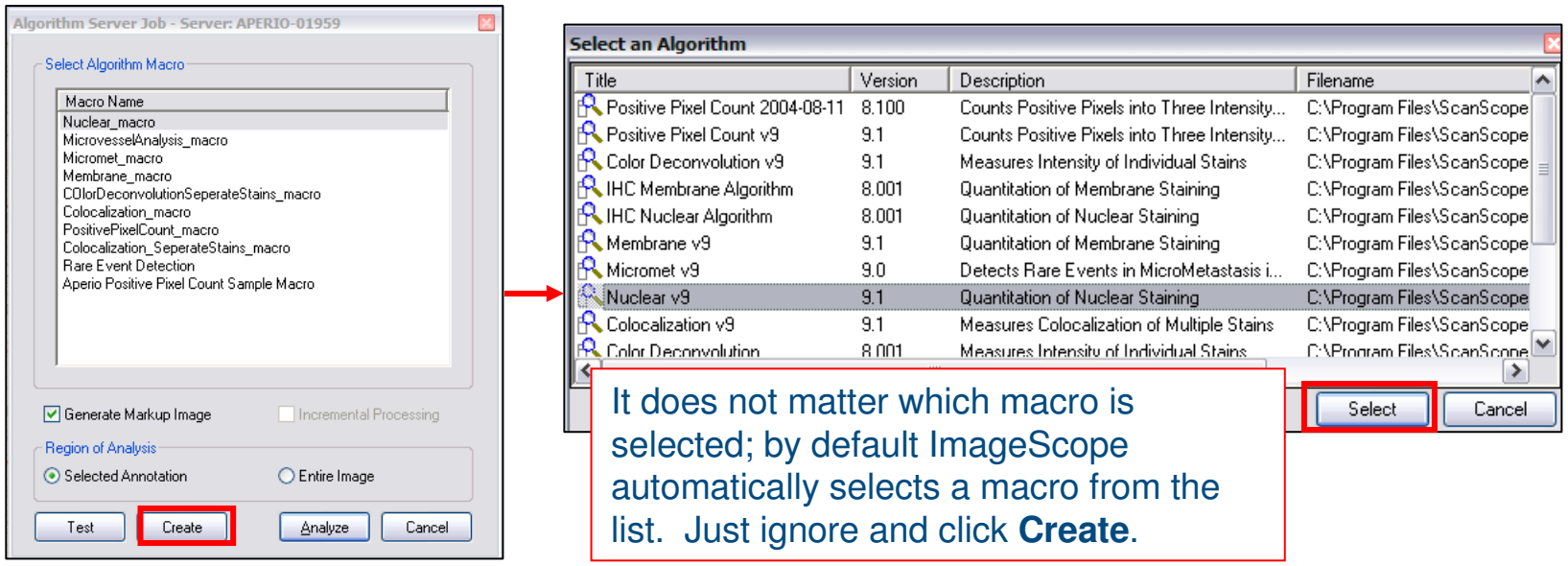


Knowledge Check

1. Rare Event Detection is used for cell quantification.
   a) True
   b) False
2. Rare Event Detection uses the HIS (Hue, Saturation & Intensity) color model?
   a) True
   b) False
3. Microvessel has a set of parameters that join incomplete vessels into complete vessels. What are these parameters?
   a) Filtering/Smoothing
   b) Minimum Vessel Area Threshold
   c) Region Joining
   d) Vessels Completion
   e) c & d

Creating New Macros

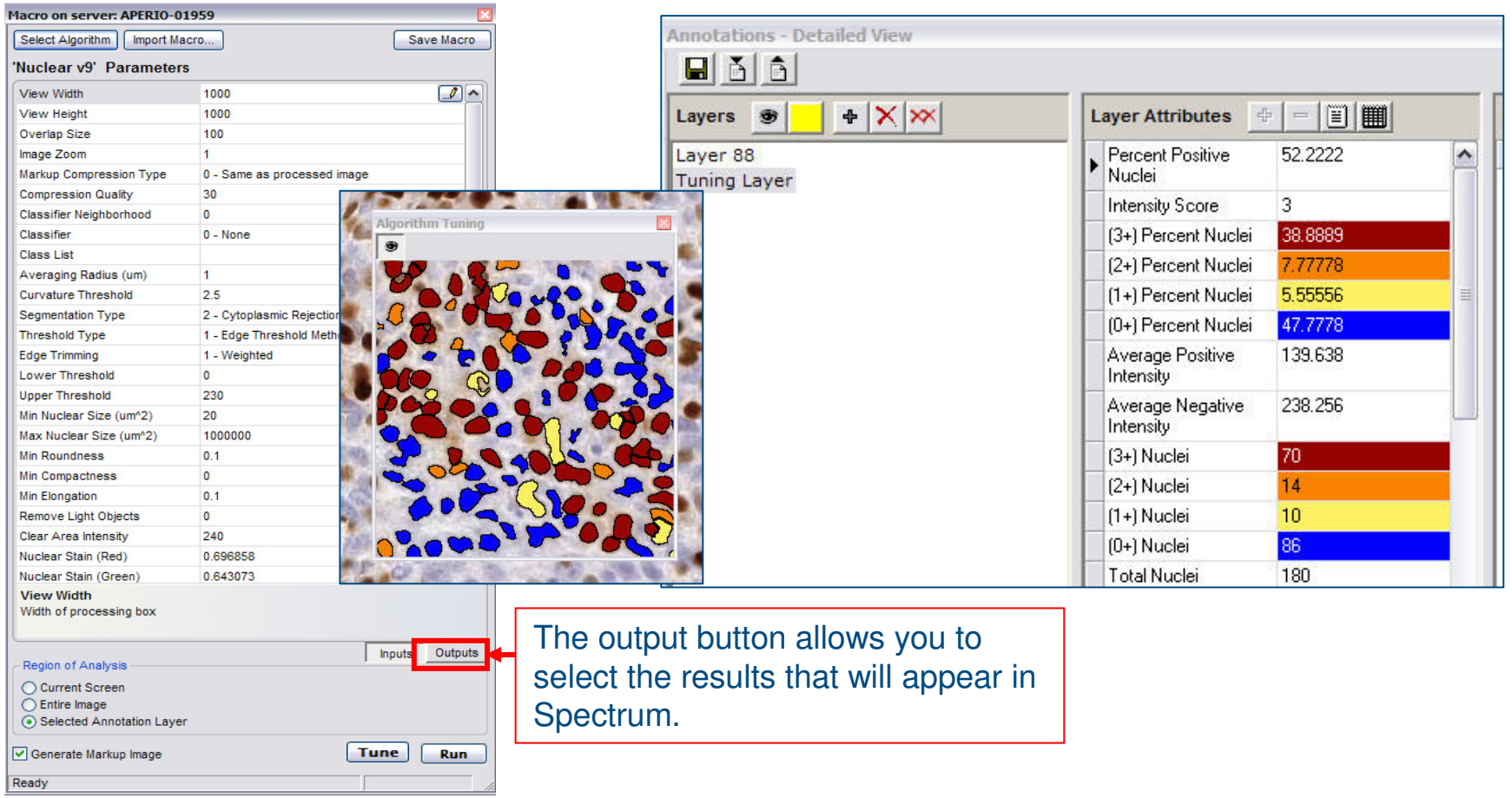
- Only users with the appropriate Spectrum administrator permissions can create new macros and register them in Spectrum.
  - After opening a digital slide in ImageScope, open the **View** menu and select **Analysis**.
  - Click **Create**, the Select an Algorithm window appears.
  - Select the Algorithm you want to create a macro for, the Analysis window appears.



Additional Topics

Creating New Macros

- Modify the parameters.
- Use the Tuning window to see the effect of the parameter changes made.
- Continue adjusting the inputs until desired results are achieved.

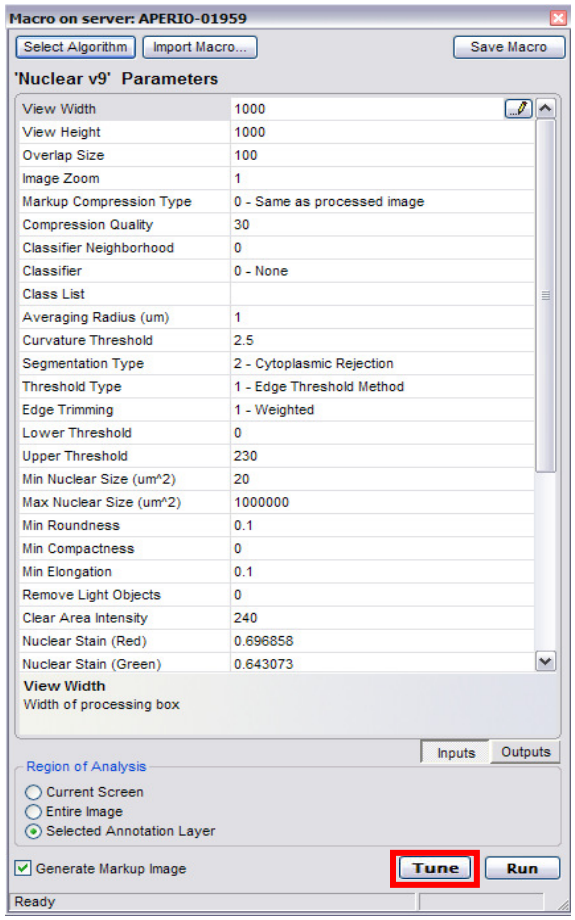


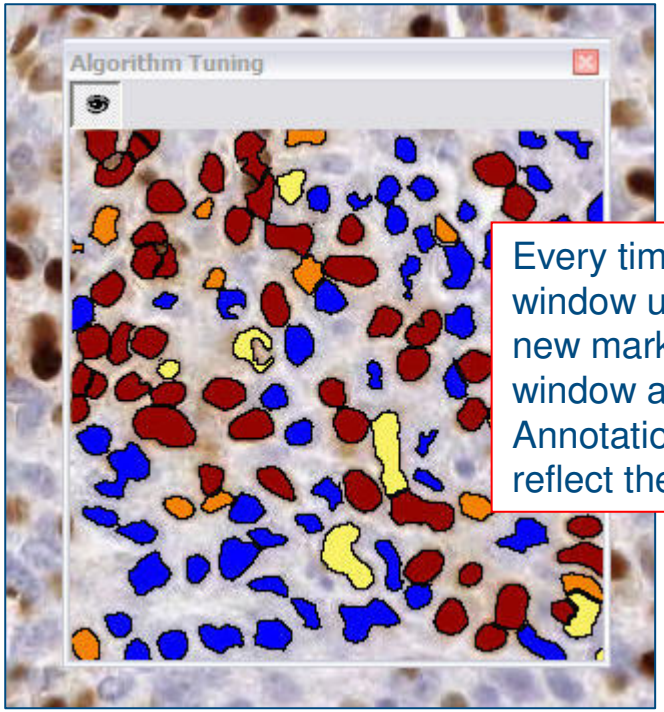
The output button allows you to select the results that will appear in Spectrum.

Additional Topics

Algorithm Tuning Window

- Quickly see the results of analyzing an area of an image or test changes made to the algorithm parameters.

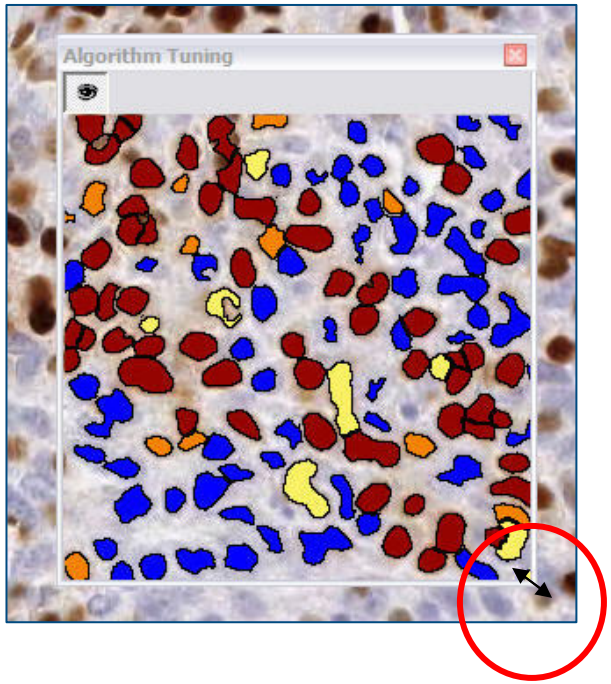


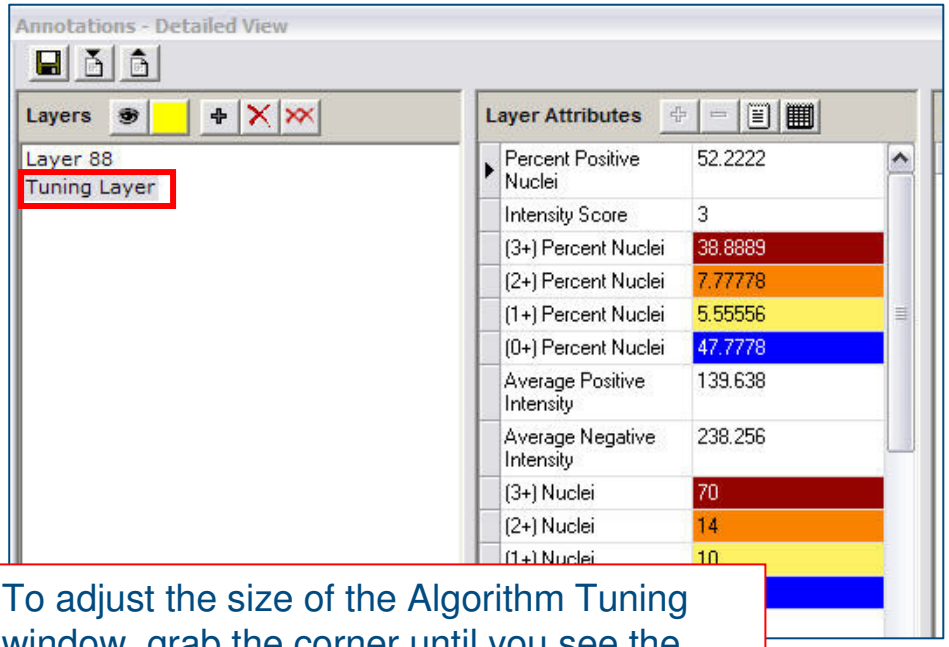


Every time the Algorithm Tuning window updates the analysis, a new markup image appears in the window and the numeric data in the Annotations window changes to reflect the new analysis.

Algorithm Tuning Window

- As the parameters are changed, the Tuning window will display the markup image for current parameters.
- The Annotations window will display the numeric results of the analysis in a new **Tuning Layer**.

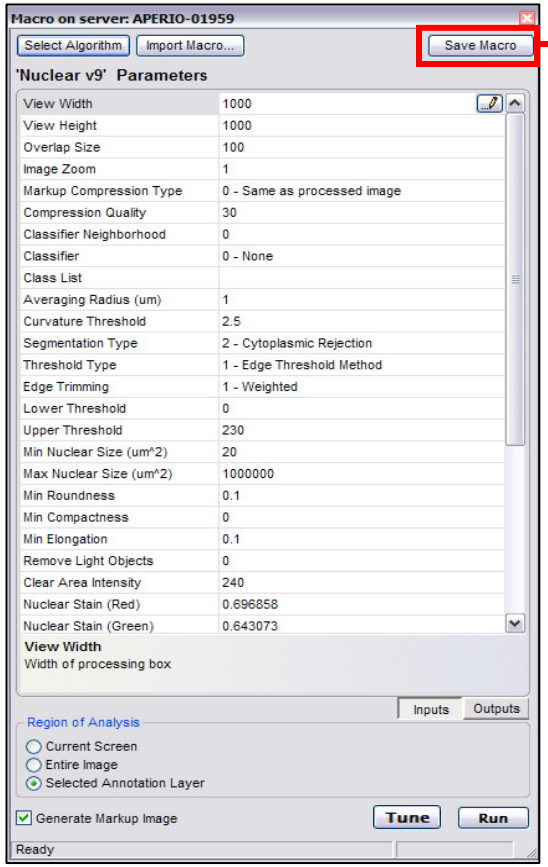


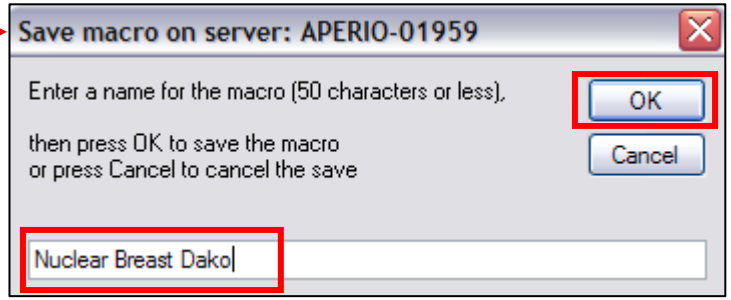


To adjust the size of the Algorithm Tuning window, grab the corner until you see the double headed arrow and pull the corner to the desired size.

Saving and Registering Macros with Spectrum

- After the macro has been created, save it to register it to Spectrum.
  - From the Analysis window click **Save** to register it to Spectrum.
  - Name the macro with a naming convention that helps identify it.

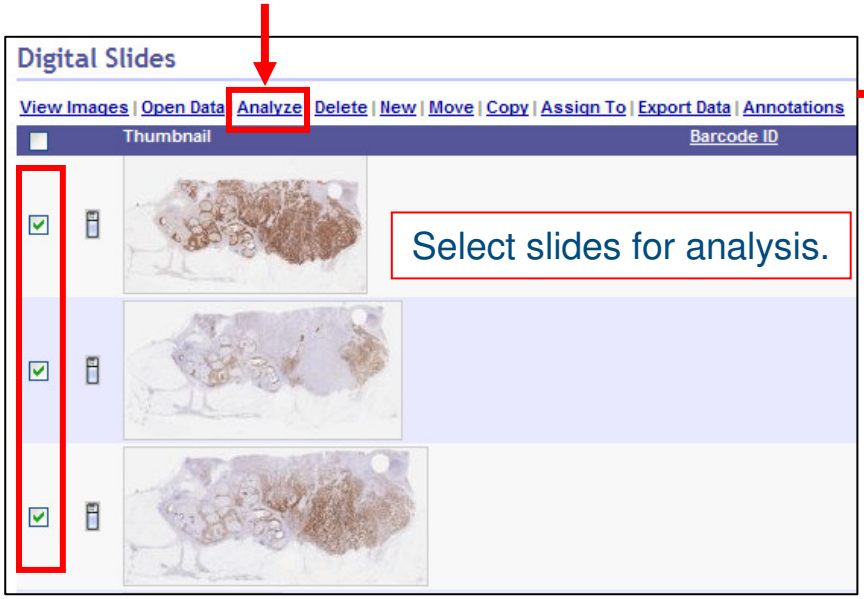


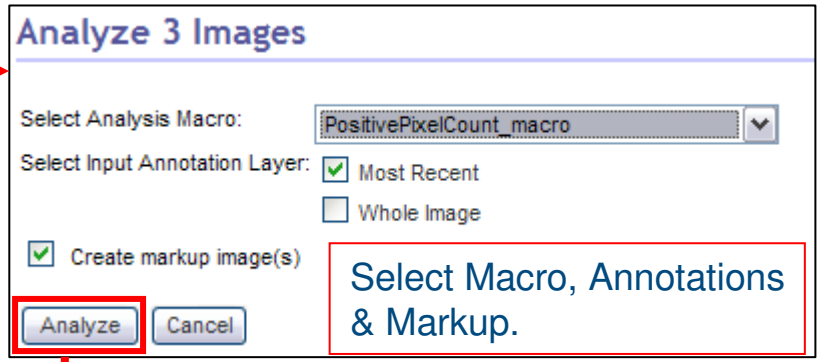


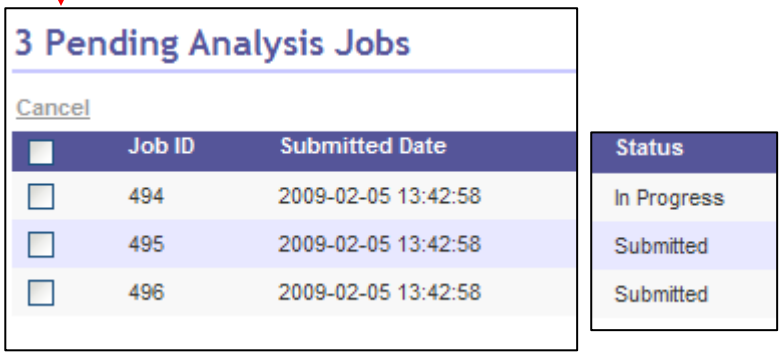
Now, when you bring up the Analysis window, you will be able to select your macro by clicking Select Algorithm because your macro will appear in the list.

Batch Analysis in Spectrum

- Spectrum provides a convenient tool for batch analysis of slides.
- The Spectrum administrator must first create a macro for each image analysis algorithm used for analysis.
- Select digital slides to be analyzed from Spectrum, click **Analyze**.
- From the Analysis page, determine the analysis details and click **Analyze**.
- Slides chosen will be added to the Job Queue.







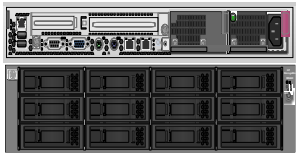
From the job queue, click **F5** to check job status or select cancel jobs.

Scalable Processing Architecture

Analysis Accelerator

- Provides the ability to run Whole Slide Analysis at an accelerated speed.
- Provides a multi-user system with a single-user response time.
- Capable of processing up to 7 Image Analysis jobs in parallel.
  - Scalable for 7, 14, 21…job as needed.

Image Server



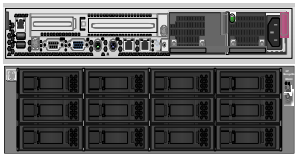
Analysis Accelerators

Up to 7 Analysis Jobs in parallel

Scalable Processing Architecture

A scanned Slide every 2 min.

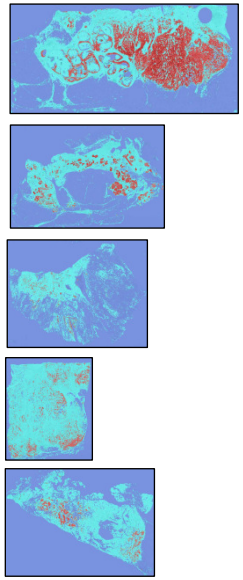
Image Server



Analysis Accelerators

Example: 4x AA
Up to 28 jobs in parallel
- with a slide every 2 min.
WSA can take up to 1 hour

A Whole Slide Analysis every 2 min.



2 min.
2 min.
2 min.
2 min.
2 min.
2 min.
2 min.
2 min.

Course Summary

Upon conclusion of this training session, learners are able to complete the following tasks related to their job:

- Describe how Aperio's Image Analysis tools work
- Describe Aperio's Image Analysis tools for Area, Cell, and Event Quantification
- Understand the input parameters for each Image Analysis tool
- Understand the output parameters for each Image Analysis tool
- Complete the steps for running and Aperio Image Analysis tools
- Calibrate stain color using Color Deconvolution
- Create a macro and register it with Spectrum
- Run a macro using Spectrum Plus batch analysis option
- Export Image Analysis results using Spectrum's Export Data tool
- Describe Aperio's Scalable Architecture

Several Aperio algorithms have been cleared by the FDA for clinical use when used on ScanScope models that are labeled as approved medical devices, and are intended for research use for other applications.