Algorithm User Guide:  
Nuclear Quantification

Use the Aperio algorithms to adjust (tune) the parameters until the quantitative results are sufficiently accurate for the purpose for which you intend to use the algorithm. You will want to test the algorithm on a variety of images so its performance can be evaluated across the full spectrum of expected imaging conditions. To be successful, it is usually necessary to limit the field of application to a particular type of tissue and a specific histological preparation. A more narrowly defined application and consistency in slide preparation generally equates to a higher probability of success in obtaining satisfactory algorithms results.

Aperio algorithms provided by Human Tissue Resource Center:
Positive Pixel Count  
Colocalization  
Color Deconvolution  
**Nuclear Quantification**  
Membrane Quantification  
Microvessel Analysis  
Rare Event Detection

The Nuclear algorithm detects the nuclear staining for target chromogen for individual cells and quantifies the intensity. It performs the same complex analysis as pathologists and is able to detect positive staining for individual nuclei. This algorithm quantifies nuclei by staining, intensity and provides automatic cytoplasmic stain removal. Each nucleus is classified as 0, 1+, 2+, or 3+.

The first 6 input parameters on every macro should NOT be changed. The next 3 parameters (Classifier Neighborhood, Classifier, and Class Lists) are Genie parameters and should be changed.
1 – Algorithm Input Parameters

A. Input Parameters for Nuclear Algorithm

- **Averaging Radius** – Radius (microns) for Noise Reduction \([0 < \text{Value} < 100]\). The radius of a smoothing filter, which reduces noise resulting in smoother object edges.

- **Curvature Threshold** – Curvature threshold for nuclear de-clustering \([0 < \text{Value} \leq 25]\).

- **Segmentation Type** – Use nuclear stain, intensity, or cytoplasmic rejection to segment nuclei \([0 – \text{Nuclear Stain Only}, \ 1 – \text{Intensity}, \ 2 – \text{Cytoplasmic Rejection}]\). Nuclear objects are identified using intensity. The staining components are combined into a single intensity image in one of three ways:
  - Nuclear stain uses the nuclear counter-stain only, after separating and removing other stain components. This works well if counter-staining is dark.
  - Intensity uses the total intensity, the sum of all staining components. This works best when the counter-staining is very light and cytoplasmic staining is also small.
  - Cytoplasmic rejection should be used when cytoplasmic staining is present. Analysis is performed on the statistics of the positive staining channel for a bimodal distribution and the lighter mode is removed.

- **Threshold Type** – Threshold method \([0 – \text{Amplitude Threshold Method}, \ 1 – \text{Edge Threshold Method}, \ 2 – \text{Manual Threshold}]\). A threshold must be applied to the intensity image in order to find the edges of the nuclei. Threshold type specifies the method to be used to determine the intensity thresholds:
  - The amplitude threshold method automatically adjusts the threshold according to the mean intensity of all pixels. The threshold is set to one sigma above the mean, which is an approximation to reject background staining.
  - The edge threshold method automatically adjusts the threshold according to the mean of edge pixels. The algorithm uses an edge finding method to identify edge pixels and uses the average of these pixel values to determine the threshold.
  - The manual threshold method is the simplest method, which uses the prescribed intensity thresholds (lower and upper below) to eliminate unwanted background. This method will not automatically adjust to compensate for lighter or darker staining between slides.

*Note that an intensity value of 0 corresponds to black and a value of 255 to white (as bright as possible).*

- **Edge Trimming** – Trim light edges of objects \([0 – \text{None}, \ 1 – \text{Weighted}, \ 2 – \text{Unweighted}]\). This parameter allows the light edges of nuclei with dark centers to be removed. Histogram analysis is done and a threshold is automatically set using bimodal analysis:
  - None disables the method and no edge trimming will be done.
  - Weighted weights the threshold by a quality factor which judges the quality of the analysis. Histograms which are not bimodal will then not do very much edge trimming.
  - Unweighted gives the largest effect and uses the full threshold estimate.

- **Lower Threshold** – Lower limit for intensity thresholding \([0 < \text{Value} \leq 255]\). This value can be changed when using the manual threshold type. Since very dark nuclei are possible, a value of 0 is usually used. Increasing this value will ignore very dark pixels.

- **Upper Threshold** – Upper limit for intensity thresholding \([0 < \text{Value} \leq 255]\). This value can be changed when using the manual threshold type. A large value (e.g., 240) represents very faintly stained pixels. Lowering this value to 200 will ignore faintly stained pixels and require nuclear pixels to have lower intensities (darker).

- **Min Nuclear Size** – Minimum area for detectable nuclei (micron-squared) \([\text{Value} > 0]\). Nuclei smaller than this area limit will not be counted.

- **Max Nuclear Size** – Maximum area for detectable nuclei (micron-squared) \([\text{Value} > 0]\). Nuclei larger than this area limit will not be counted.

- **Min Roundness** – Nuclei with roundness less than this are not reported \([0 \leq \text{Value} \leq 1]\). Roundness is the ratio of the object area to the area of a circle that fully encloses that object. Circular objects will have a value of 1. Small values indicate non-circular objects.
• **Min Compactness** – Nuclei with compactness less than this are not reported \([0 \leq \text{Value} \leq 1]\). Compactness is the ratio of area of the object to the area of a circle that has a circumference equal to the perimeter of the object. Circular objects will have a value of 1. Small values indicate noncircular objects.

• **Min Elongation** – Nuclei with aspect ratios less than this are not reported \([0 \leq \text{Value} \leq 1]\).

• **Remove Light Objects** – Value = 0 removes no nuclei, Value = 1 removes all nuclei \([0.0 < \text{Value} < 1.0]\). This parameter allows removal of light objects. A value of 0.0 causes no objects to be removed. A value of 1.0 will remove all objects. Values between these two limits define a dividing line between the lightest and darkest object found and removes objects with average intensity above this line (lighter objects). For example, value = 0.5 defines the dividing line at the midpoint between lightest and darkest object found.

• **Clear Area Intensity** – Intensity of clear area \([0 < \text{Value} \leq 255]\).

• **Nuclear Stain (Red)** – OD for nuclear red \([0.0 \leq \text{Value} \leq 1.0]\).

• **Nuclear Stain (Green)** – OD for nuclear green \([0.0 \leq \text{Value} \leq 1.0]\).

• **Nuclear Stain (Blue)** – OD for nuclear blue \([0.0 \leq \text{Value} \leq 1.0]\).

• **Positive Stain (Red)** – OD for positive red \([0.0 \leq \text{Value} \leq 1.0]\).

• **Positive Stain (Green)** – OD for positive green \([0.0 \leq \text{Value} \leq 1.0]\).

• **Positive Stain (Blue)** – OD for positive blue \([0.0 \leq \text{Value} \leq 1.0]\).

• **3rd Stain (Red)** – OD for color (3) red \([0.0 \leq \text{Value} \leq 1.0]\).

• **3rd Stain (Green)** – OD for color (3) green \([0.0 \leq \text{Value} \leq 1.0]\).

• **3rd Stain (Blue)** – OD for color (3) blue \([0.0 \leq \text{Value} \leq 1.0]\).

• **Cytoplasmic Intensity Threshold** – Adjust the nuclear Intensity of Cytoplasm < Threshold. \([0 \text{ Disables}]\).

• **Weak(1+) Threshold** – Nuclear intensity threshold for weak (1+) \([0 < \text{Value} \leq 255]\).

• **Moderate(2+) Threshold** – Nuclear intensity threshold for moderate (2+) \([0 < \text{Value} \leq 255]\).

• **Strong(3+) Threshold** – Nuclear intensity threshold for strong (3+) \([0 < \text{Value} \leq 255]\).

• **Black Threshold** – Intensity threshold (lower limit) of strong positive nuclei \([0 < \text{Value} \leq 255]\). Nuclei with positive intensity less than the black threshold are not counted. Usually this value is set to 0.0 and defines absolute black. Positive intensity is calculated as \((R+G+B)/3\) of the deconvolved positive channel.

• **Use Mode** – Select Training or Analysis/Tuning Mode \([0 – \text{Analysis/Tuning}, 1- \text{Training}]\).

• **Mark-up Image Type** – Choose type of markup image \([0 – \text{Tuning}, 1 – \text{Analysis}]\).

### 2 – Algorithm Results

#### A. Understanding the Results

• Nuclear staining classified as 0, 1+, 2+ and 3+ is based on nuclear staining intensity.
  - A nucleus is classified 0 when it has no nuclear staining.
  - A nucleus is classified 1+ when it has weak nuclear staining.
  - A nucleus is classified 2+ when it has moderate nuclear staining.
  - A nucleus is classified 3+ when it has intense nuclear staining.

• Based on the percentages of 0, 1+, 2+ and 3+ nuclei, the percentage of positive stained nuclei as a percentage of 0 to 100% and the average staining intensity of the positive nuclei as a score of 0, 1+, 2+ or 3+ is determined.

For questions please contact: Lei-Ann Arceneaux, larceneaux@bsd.uchicago.edu
http://pathcorebsd.uchicago.edu
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Note: The first section of the Layer Attributes pane displays the algorithm results; the second portion (labeled “Algorithm Inputs”) repeats the input parameters you specified.

B. Algorithm Output Parameters
- Percent Positive Nuclei
- Intensity Score – The H Score: The score is obtained by the formula: \((3 \times \text{percentage of strongly staining nuclei}) + (2 \times \text{percentage of moderately staining nuclei}) + \text{(percentage of weakly staining nuclei)}\), giving a range of 0 to 300.
  - (3+) Percent Nuclei
  - (2+) Percent Nuclei
  - (1+) Percent Nuclei
  - (0+) Percent Nuclei
  - Average Positive Intensity
  - Average Negative Intensity
  - (3+) Nuclei
  - (2+) Nuclei
  - (1+) Nuclei
  - (0+) Nuclei
  - Total Nuclei
  - Average Nuclear RGB Intensity
  - Average Nuclear Size (Pixels)
  - Average Nuclear Size (um²)
  - Area of Analysis (Pixels)
  - Area of Analysis (mm²)

3 – Correcting Background Staining

A. Background Staining
- Cytoplasmic or background staining can create problems for the correct quantification of the staining because it increases the overall staining intensity of the slide. Cytoplasmic or background staining can also create problems for image analysis algorithms because segmentation of the nuclei becomes more difficult.
- Positive nuclei have a dual stain and counter-stain co-localization problem which makes it difficult to truly determine the amount of positive stain on the nuclei, especially for faint positive staining.
- Color Deconvolution is used to calibrate the stain colors for more accurate quantitative data.
B. Stain Color Parameters
- Stain color parameters can be calculated using Color Deconvolution’s stain calibration process.

C. What Is Color Calibration?
- By defining the stain color vectors, you are identifying to the Color Deconvolution algorithm which color identifies which stain.
- The default color vector values are as follows:
  - Color 1 – Hematoxylin
  - Color 2 – Eosin
  - Color 3 – DAB
- The color vector numbers must be changed if different stains are used. The color for each stain is calibrated separately.
  - If possible, use a separate control slide for each stain you want to analyze.
  - If this is not possible, look for several areas of the digital slide that are mostly stained with stain of interest and select them by using the ImageScope drawing tools. Pick an area of light staining of only this color. Avoid selecting darker, overstained areas.
- If you are using only two stains, not three, set the color vector values for the third color to zero.

D. Performing Color Calibration
- Annotate the digital slide. Be sure to annotate an area on the slide stained ONLY by that particular color. Open the Analysis window and click Create.
- Select Color Deconvolution v9.
- Choose the appropriate layer and select Selected Annotation Layers.
- Click Run.
- Open the Annotations window.
- Search the results and look for the Average OD (optical density) values for Red, Green, Blue.

<table>
<thead>
<tr>
<th></th>
<th>Average OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Red OD</td>
<td>0.540967</td>
</tr>
<tr>
<td>Average Green OD</td>
<td>0.630966</td>
</tr>
<tr>
<td>Average Blue OD</td>
<td>0.55609</td>
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</tbody>
</table>

- Type these values into Input parameters corresponding color component for Color 1, 2, or 3.

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<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Color (1) - Red Component</td>
<td>0.65</td>
</tr>
<tr>
<td>Color (1) - Green Component</td>
<td>0.704</td>
</tr>
<tr>
<td>Color (1) - Blue Component</td>
<td>0.286</td>
</tr>
</tbody>
</table>

- Repeat these steps for the other stains if necessary.
4 – Nuclear Analysis

A. Running Analysis
- Annotate the slide. Open the Analysis window and click Create.
- Select Nuclear v9.
- Adjust the input parameters.
- Select Selected Annotation Layer to analyze only selected areas of the image. Check the Generate Markup Image box.

- Click Run.
- Open the Annotations window to view results.