The Pathology Company

Leica BIOSYSTEMS

Nuclear Algorithm

User's Guide

Research Use Only

MAN-0338, Revision A | 5 August 2015

Nuclear Algorithm User's Guide (RUO)

This document applies to eSlide Manager Release 12.3 and later.

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Customer Resources

- For the latest information on Leica Biosystems Aperio ePathology products and services, please visit www.LeicaBiosystems.com/ePathology.

Disclaimers

- Use normal care in maintaining and using Aperio ePathology servers. Interrupting network connections or turning off the servers while they are processing data (such as when they are analyzing eSlides or generating an audit report) can result in data loss.
- This manual is not a substitute for the detailed operator training provided by Leica Biosystems Imaging or for other advanced instruction. Leica Biosystems Imaging Field Representatives should be contacted immediately for assistance in the event of any instrument malfunction. Installation of hardware should only be performed by a certified Leica Biosystems Imaging Service Engineer.
- ImageServer is intended for use with eSlides created by scanning glass slides with the scanner. Educators will use Aperio ePathology software to view and modify eSlides in Composite WebSlide (CWS) format.

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Contact Information – Leica Biosystems Imaging, Inc.

Headquarters	Customer Support	General Information
Leica Biosystems Imaging, Inc. 1360 Park Center Drive Vista, CA 92081 USA Tel: +1 (866) 478-4111 (toll free) Direct International Tel: +1 (760) 539-1100	US/Canada Tel: +1 (866) 478-3999 (toll free) Direct International Tel: +1 (760) 539-1150 US/Canada/Worldwide Email: TechServices@LeicaBiosystems.com	US/Canada Tel: +1 (866) 478-4111 (toll free) Direct International Tel: +1 (760) 539-1100 Email: ePathology@LeicaBiosystems.com

REF 23S9152WS, 23N-IA, 23IHCT, 23IAW, 23AW-N-IA

Customer Service Contacts

Please contact the office for your country for technical assistance.

Australia:
96 Ricketts Road
Mount Waverly, VIC 3149
AUSTRALIA
Tel: 1800 625 286 (toll free)
Between 8:30 AM-5 PM, Monday-Friday, AEST
Email: lbs-anz-service@leicabiosystems.com

Austria:
Leica Biosystems Nussloch GmbH
Technical Assistance Center
Heidelberger Strasse 17
Nussloch 69226
GERMANY
Tel: 0080052700527 (toll free)
In-country Tel: +43 1 486 80 50 50
Email: support.at@leicabiosystems.com

België/Belgique:
Tel: 0080052700527 (toll free)
In-country Tel: +32 2 790 98 50
Email: support.be@leicabiosystems.com

Canada:
Tel: +1 866 478- 999 (toll free)
Direct International Tel: +1 760 539 1150
Email: TechServices@leicabiosystems.com

China:
17F, SML Center No. 610 Xu Jia Hui Road, Huangpu District
Shanghai, PRC PC:200025
CHINA
Tel: +86 4008208932
Fax: +86 21 6384 1389
Email: service.cn@leica-microsystems.com
Remote Care email: tac.cn@leica-microsystems.com

Danmark:
Tel: 0080052700527 (toll free)
In-country Tel: +45 44 54 01 01
Email: support.dk@leicabiosystems.com

Deutschland:
Leica Biosystems Nussloch GmbH
Technical Assistance Center
Heidelberger Strasse 17
Nussloch 69226
GERMANY
Tel: 0080052700527 (toll free)
In-country Tel: +49 6441 29 4555
Email: support.de@leicabiosystems.com

Eire:
Tel: 0080052700527 (toll free)
In-country Tel: +44 1908 577 650
Email: support.ie@leicabiosystems.com

España:
Tel: 0080052700527 (toll free)
In-country Tel: +34 902 119 094
Email: support.spain@leicabiosystems.com

France:
Tel: 0080052700527 (toll free)
In-country Tel: +33 811 000 664
Email: support.fr@leicabiosystems.com

Italia:
Tel: 0080052700527 (toll free)
In-country Tel: +39 0257 486 509
Email: support.italy@leicabiosystems.com

Japan:
1-29-9 Takadannobaba, Sinjuku-ku
Tokyo 169-0075
JAPAN

Nederland:
Tel: 0080052700527 (toll free)
In-country Tel: +31 70 413 21 00
Email: support.nl@leicabiosystems.com

New Zealand:
96 Ricketts Road
Mount Waverly, VIC 3149
AUSTRALIA
Tel: 0800 400 589 (toll free)
Between 8:30 AM-5 PM, Monday-Friday, AEST
Email: lbs-anz-service@leicabiosystems.com

Portugal:
Tel: 0080052700527 (toll free)
In-country Tel: +35 1 21 388 9112
Email: support.pt@leicabiosystems.com

Sweden:
Tel: 0080052700527 (toll free)
In-country Tel: +46 8 625 45 45
Email: support.se@leicabiosystems.com

Switzerland:
Tel: 0080052700527 (toll free)
In-country Tel: +41 71 726 3434
Email: support.ch@leicabiosystems.com

United Kingdom:
Tel: 0080052700527 (toll free)
In-country Tel: +44 1908 577 650
Email: support.uk@leicabiosystems.com

USA:
Tel: +1 866 478 3999 (toll free)
Direct International Tel: +1 760 539 1150
Email: TechServices@leicabiosystems.com

Contents

1 Introduction

This chapter introduces the Nuclear algorithm.

*The primary source for information on creating, testing, and saving image analysis macros is the **Aperio Image Analysis User's Guide**. That guide also contains details on running image analyses from within eSlide viewers and batch analyses from within eSlide Manager, and viewing and exporting analysis results.*

*The Aperio Image Analysis Workstation provides a streamlined image analysis workflow on your local workstation; if you are using that product, please refer to the **Aperio Image Analysis Workstation User's Guide** for instructions on creating, testing, and saving image analysis macros That guide also contains details on running image analyses from within ImageScope, running batch analyses, and viewing and exporting analysis results.*

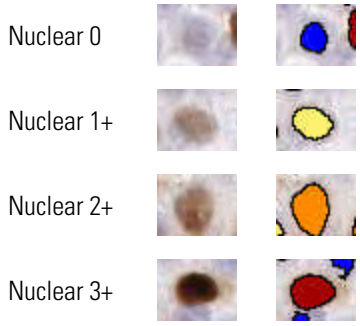
About This Guide

This guide for this image analysis algorithm discusses how to set the algorithm parameters to suit your image analysis needs. After tuning the parameters, you will save the settings as an algorithm macro. The macro can then be used by you and other users to analyze specific eSlides (digital slide images).

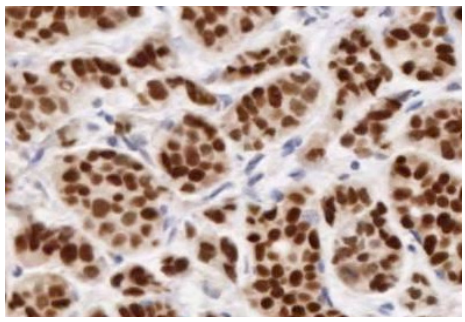
The guide works in concert with the *Aperio Image Analysis User's Guide* or the *Aperio Image Analysis Workstation User's Guide* to present the complete picture of Aperio image analysis.

The Nuclear Algorithm

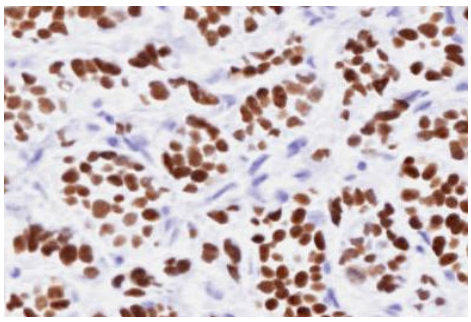
The Nuclear image analysis algorithm detects the nuclear staining for a target chromogen for the individual cells in those regions and quantifies their intensity. Nuclear staining classified as 0, 1+, 2+ and 3+ is based on positive (Biomarker 1) staining intensity. A nucleus is classified 0 when it has no positive (Biomarker 1) staining. A nucleus is classified 1+ when it has weak positive staining. A nucleus is classified 2+ when it has moderate positive staining. A nucleus is classified 3+ when it has intense positive 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.



Cytoplasmic or background staining can create problems for the correct quantization 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. Slides that exhibit high cytoplasmic or background staining due to the staining process (rather than due to a biological cause) should be caught by the laboratory's quality control process. In any case, image analysis algorithms should be able to deal with a certain degree of cytoplasmic or background staining. The Nuclear image analysis algorithm detects cytoplasmic staining and corrects for it in the staining intensities and in the segmentation of the nuclei.



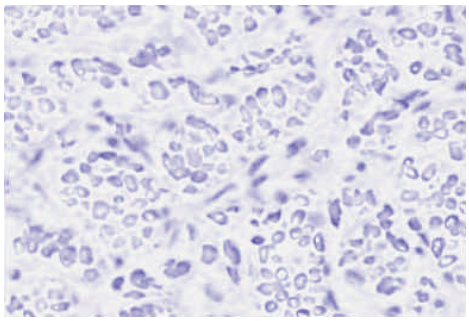
Cytoplasmic Staining



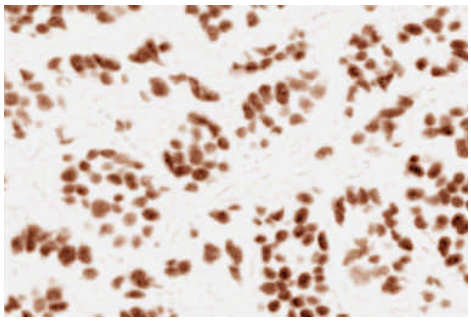
Cytoplasmic Staining Removed by Algorithm

Positive nuclei have a dual stain and colocalization problem which makes it difficult for the researchers or any image analysis system that is based on a color space classification system to truly determine the amount of positive stain on the nuclei, especially for faint positive staining. The Nuclear image analysis algorithm uses a technique called color deconvolution that separates up to three different color stains, thereby providing a true stain separation otherwise only achievable with multi-spectral imaging systems.

Different labs may use different colors for the staining. Using the color deconvolution tuning step, the colors of the stains can automatically be calculated and used to calibrate the stain colors in the Nuclear image analysis algorithm.



Counterstain



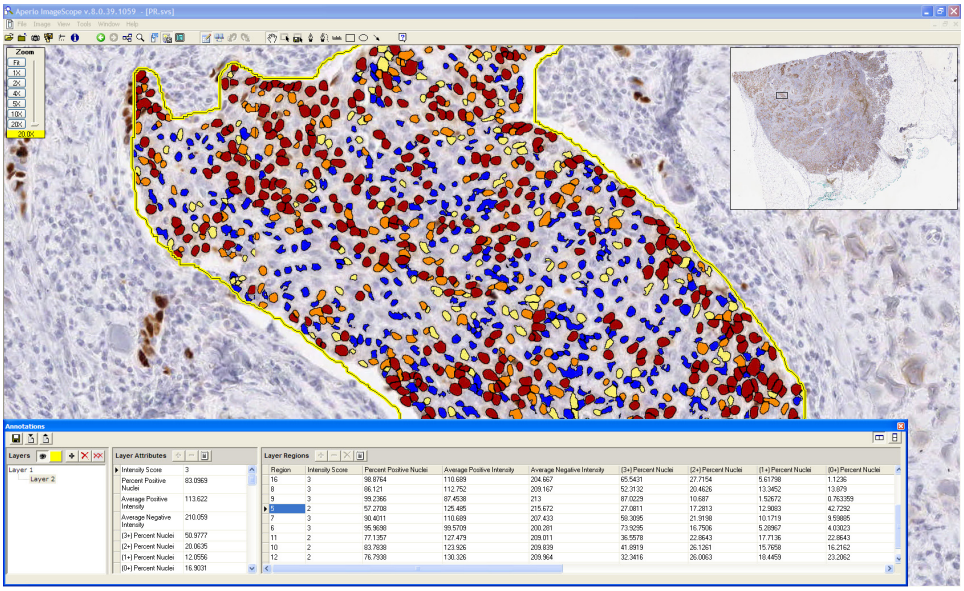
Positive Nuclear Stain

Researchers perform a complex analysis of stained cell features when scoring slides. The Aperio image analysis algorithms are designed to process the same cell features (nuclei and membrane) and follow the same scoring schemes as the researchers when assessing a slide under a microscope. Being able to do this requires high quality eSlides taken at least with a 20x objective.

As with any image analysis algorithm, the Nuclear image analysis algorithm must be set up for its specific application by tuning its input parameters. Specific applications may vary in the tissue type, staining process and/or scoring standard. Cell feature detection parameters and scoring scheme parameters are handled separately. The cell feature detection parameters specify cell feature detection thresholds and methods as well as size and shape constraints of nuclei to distinguish tumor nuclei from normal, lymphocyte and stroma nuclei. The scoring scheme parameters specify the staining intensity thresholds that determine the individual cell nuclear classification.

The Nuclear image analysis algorithm is typically used from within ImageScope, but can also be used from eSlide Manager or TMALab (Tissue Micro Array). Once a researcher has outlined the regions of interest and runs the algorithm, the algorithm provides a mark-up image and an annotation window as its outputs.

The mark-up image highlights the detected nuclei which are color-coded according to their classification (blue = 0, yellow = 1+, orange = 2+, red = 3+). The annotation window provides the percentage of positive stained nuclei, the average staining intensity of the positive nuclei, the percentages of 0, 1+, 2+ and 3+ nuclei and other image analysis statistics. The other image analysis statistics include: average positive and negative staining intensity as intensity value 0 to 255, number of 3+, 2+, 1+ and 0 nuclei and total number of nuclei, average nuclear RGB intensity, average nuclear size in pixels and μm^2 and area of analysis in pixels and mm^2 Providing not only the percentage of positive nuclei and the intensity score, but also the percentages of 3+, 2+, and 1+ nuclei gives the researcher a very detailed assessment of the slide and makes it easier for him/her to identify and assess border line cases.



The performance of the Nuclear image analysis algorithm should be validated following standard laboratory practices for the specific applications before being used for analysis.

The researcher using the Nuclear image analysis algorithm should verify its proper operation on each slide analyzed.

Prerequisites

The Nuclear algorithm requires that you use eSlide Manager Release 12.3 or later.

Because Aperio eSlides are by design high resolution and information rich, for best results you should use a high quality monitor to view them. Make sure the monitor is at the proper viewing height and in a room with appropriate lighting. We recommend any high quality LCD monitor meeting the requirements recommended in the *Aperio ePathology System Requirements*.

Intended Use

For research use only. Not for use in diagnostic procedures.

Algorithms are intended to be used by trained researchers who have an understanding of the biological characteristics or significance of biomarkers.

Each algorithm has input parameters that must be adjusted by an expert user who understands the goal of running the analysis and can evaluate the algorithm performance in meeting that goal.

You will adjust (tune) the parameters until the algorithm results are sufficiently accurate for the purpose for which you intend to use the algorithm. You should 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 tissue type 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 algorithm results.

If you get algorithm analysis results that are not what you expected, please see the "Troubleshooting" information in the *Aperio Image Analysis User's Guide* for assistance.

Installing the Algorithm

In most cases you install the algorithm on the eSlide Manager server only. (In fact, Technical Services may install the algorithm for you on your server.) This is because you typically fine-tune and save the algorithm parameters on the eSlide Manager server.

For the case that you need to fine-tune the algorithm parameters on your local workstation by using a local image, refer to the *Aperio Image Analysis User's Guide* for installation and use instructions.

For More Information

For details on tuning the Nuclear algorithm to create an algorithm macro, go to *"Chapter 2: Tuning Algorithm Parameters" on page 11.*

See the *Aperio Image Analysis User's Guide* for information on:

- Installing an algorithm
- Creating a new algorithm macro or modifying an existing one
- Saving or exporting a macro
- Selecting the areas of an eSlide to analyze

- Running an analysis on a single eSlide through the eSlide viewer or running an analysis on one or more eSlides using eSlide Manager batch analysis
- Viewing analysis results quantitatively and visually
- Exporting analysis results

For details on using ImageScope to view eSlides, see the *ImageScope User's Guide*.

2 Tuning Algorithm Parameters

The Nuclear image analysis algorithm needs to be set up for its specific application by tuning its input parameters. Specific applications may have different tissue types, staining processes and/or scoring standards. In the setup, we distinguish between cell feature detection parameters and scoring scheme parameters.

Parameter tuning must be done by a skilled user knowledgeable in image analysis and the biochemistry of the application. If you want help with the setup of the algorithm, please contact Leica Biosystems Imaging for image analysis services.

Labs use different IHC reagents and kits (for example: Dako and Ventana) with different staining characteristics. Different labs also have slightly different staining processes with different staining characteristics. Keep in mind that to assure the accuracy of the image analysis algorithms, the lab needs to follow the quality control instructions recommended in the manufacturer's labeling of the reagent or kit.

If changes are made in the staining procedure, repeat tuning and validation of the algorithm may be necessary.

Tuning Parameters

To create a macro for the algorithm or to modify an existing macro:

1. In ImageScope, open an eSlide you want to use to tune the algorithm parameters. (Refer to the *ImageScope User's Guide* for instructions.)
2. In ImageScope, open the algorithm and choose to create a new macro or open an existing macro to modify it. (Refer to the *Aperio Image Analysis User's Guide* for instructions.)

   You now see the parameters for the algorithm (these are listed later in this chapter).
3. Now adjust the parameters in the Analysis window as discussed below and move the Algorithm Tuning window on the image to see a mark-up image of the results.

After adjusting the input parameters and selecting the output parameters as discussed in the following sections, move the Algorithm Tuning window to various areas on the eSlide to see an approximation of the analysis results. The results appear in the Annotations window and as a mark-up image in the ImageScope main window.

You can open the ImageScope Annotations window to see the algorithm tuning results displayed numerically.

When saving the algorithm macro by clicking  , you can choose whether to save the macro locally on your workstation or, if connected to eSlide Manager, to save the macro remotely on eSlide Manager. You are asked to supply a name for the macro that will help you identify it in the future. If saving the macro to eSlide Manager, you are also asked to specify which data group the macro will be associated with; only eSlide Manager users who have permission to use that data group will be able to choose that macro for analysis.

Algorithm Parameters

Nuclear analysis algorithm performance is controlled by a set of parameters which determine many different types of analyses. Note that the parameters are grouped so that related parameters can be adjusted together. In general, you will want to proceed through the grouped parameters in sequence; for example, adjust the Stain parameters before adjusting the Nuclei Identification parameters.

Stains

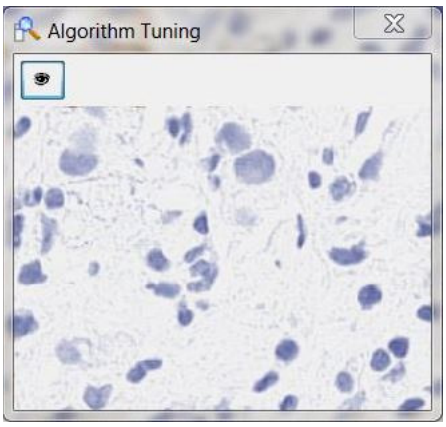
Up to three visible stains on the slide can be identified using the parameters listed below. Each stain has a different section in the algorithm parameters. Note that the mark-up image in the Algorithm Tuning window is influenced by the changes in the color vector for other stains, and therefore may not show the optimal image until you have adjusted all of the stains.

A separate section is included in the parameters for each stain and the parameter names have prefixes corresponding to the stain (S1 for Stain 1, for example).

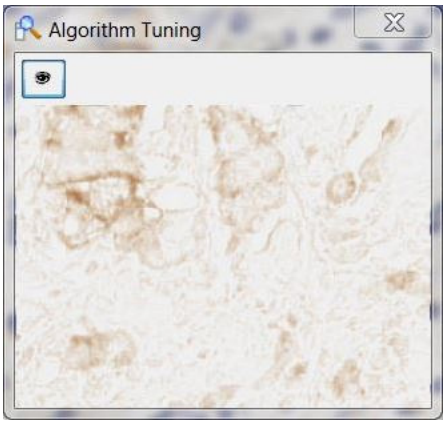
Parameter	Default	Description
Number of Visible Stains	2	0 to 3. This value can be changed in any of the Stains sections.
Target	Counterstain	Counterstain, Biomarker 1, Biomarker 2. Labels the currently selected stain. Each channel needs to have the correct label for the algorithm to work properly. The current stain is displayed in the Algorithm Tuning window.
Color	Lock	Lock or Train. The Lock setting uses the previously computed or default values for the stain. To compute new values, select **Train**. See *"Determining Stain Color" on page 13* for information on adjusting color values.
Values	Hide	Hide or Show. The Show setting displays the numeric values of the stain color vectors. You may manually adjust these values. See *"Determining Stain Color" on page 13* for information on adjusting color values.

These are examples of the mark-up image in the Algorithm Tuning window when adjusting stain parameters.

This mark-up image shows Stain 1 with the target set to Counterstain using the default color vector for the nuclear stain.



This mark-up image shows Stain 2 with the target set to Biomarker 1 and uses the default color vector values.



The Nuclear algorithm contains an automatic stain color finder that can be used to calculate the stains on the slide. See the next section for details. Note that the colors displayed are inter-dependent. If one of the stain values is incorrect, it will add or remove energy from the other stains, resulting in incorrect deconvolution for all stains and incorrect colors being displayed in the Algorithm Tuning window.

Determining Stain Color

The stain color finder feature of this algorithm locates the most unmixed pixels from each stain in the Algorithm Tuning window, providing automatic color deconvolution. By default, this feature is turned off so that the algorithm uses the same default color values as previous versions of this algorithm.

To use this feature for each stain:

1. Move the Algorithm Tuning window to an area where the stains are well separated.
2. Set the Number of Visible Stains for the number of stains visible in the area of the slide shown in the Algorithm Tuning window. (This may be fewer than the total number of stains shown on the entire slide.)
3. Set the Color parameter to **Train**. The algorithm computes the color values and the corresponding stain mark-up image is shown in the Algorithm Tuning window. These values are computed then saved as the default.
4. Set the Values parameter to **Show**. You see the numeric values for the stain colors.
5. Set the Target to the appropriate value to adjust the stain color values for that stain.

Repeat these steps for the other stains shown in the Algorithm Tuning window.

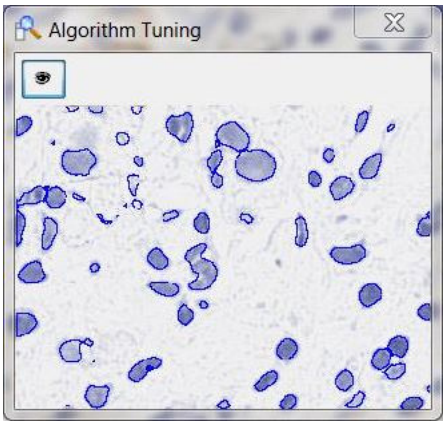
If you know the exact color vector numbers (from previous experience with the stain, for example), you can type the numbers directly into the Values parameter rather than having the algorithm calculate them.

Nuclei Identification

This stage segments the nuclei from the eSlide.

Parameter	Default	Description
Type	Default	Selects the stain for detecting nuclei. (Values are Counterstain, All Stains, Default) Nuclear objects are identified using intensity. The staining components are combined into a single intensity image in one of three ways: **Counterstain** uses the nuclear counterstain only, after separating and removing other stain components. This works well if counterstaining is dark. **All Stains** uses the total intensity, the sum of all staining components. This works best when the counterstaining is very light and cytoplasmic staining is also small. **Default** includes Counterstain combined with Biomarker 1.
Method	Automatic	(Values are Average, Automatic, Manual) A threshold must be applied to the intensity image in order to find the edges of the nuclei. **Automatic** automatically adjusts the threshold according the mean intensity of all pixels. The **Manual** 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.
- Threshold Lower Limit	0	Lower limit for nuclear auto thresholding (0 < Value ≤ 255). This value can be changed when using the Manual Method. Since very dark nuclei are possible, a value of 0 is usually used. Increasing this value will ignore very dark pixels.
- Threshold Upper Limit	230	Upper limit for nuclear auto thresholding (0 < Value ≤ 255). This value can be changed when using the Manual Method. 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 (being darker).
Smoothing (um)	1	A pre-processing parameter, this sets the radius of a smoothing filter, which reduces noise to result in smoother object edges.
Merging	2.5	Defines nuclear declustering (0 < Value ≤ 25). Merging determines the level of declustering used to separate touching nuclei. Increasing the value reduces the effect of the declustering logic – this may be necessary if single nuclei are being split and counted more than once. Decrease this value if more declustering is needed to separate nuclei.
Trimming	Low	Sets amount of cell boundary reduction by trimming light edges of objects (Values are Low, Medium, High). This parameter allows the light edges of nuclei with dark centers to be removed.

This is an example of the mark-up image in the Algorithm Tuning window when adjusting these parameters. This mark-up image shows the segmented nuclei outlined in blue.

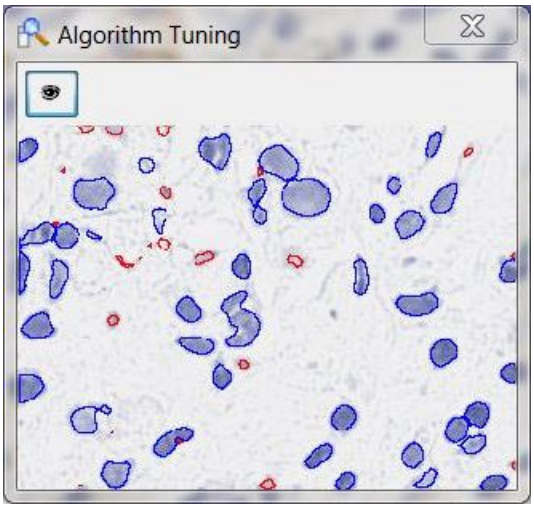


Nuclei Exclusion

This stage filters the nuclei identified during the previous stage based on the measure of nuclear size and shape.

Parameter	Default	Description
Min Size (um^2)	20	Minimum area for detectable nuclei (micron squared). Value > 0.
Max Size (um^2)	1000000	Maximum area for detectable nuclei (micron squared). Value > 0.
Roundness	0.1	Nuclear objects with roundness less than this value are excluded. (0 < Value < 1). Roundness is the ratio of the object area to the area of a circle that fully encloses that object. Move the value towards 1 to exclude non-circular objects. Circular objects will have a value of 1.
Compactness	0	Nuclear objects with compactness less than this value are excluded. (0 < Value < 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. Move the value towards 1 to exclude non-compact objects such as objects with ragged edges.
Elongation	0.1	Nuclear objects with aspect ratios less than this value are excluded. (0 < Value < 1). Elongation is the ratio of the length and width of the object. Circular objects will have a value of 1. Small values indicate objects which are long and thin.
Remove Light Objects	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. 0 removes no nuclei, 1 removes all nuclei.

This is an example of the mark-up image in the Algorithm Tuning window when adjusting these parameters. This mark-up image shows the excluded nuclei outlined in red along with the segmented nuclei from the previous stage.

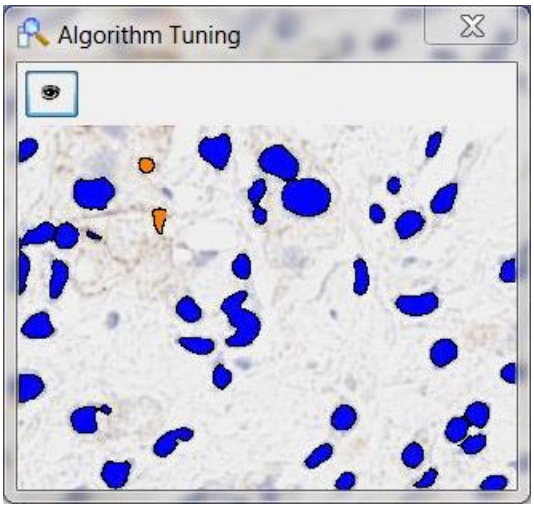


Scoring Criteria

This is the final stage of the tuning workflow, and it ranks the nuclei segmented from the previous stage into Weak, Moderate, and Strong categories.

Parameter	Default	Description
Cytoplasmic Correction	230	Reduces nuclear staining by the cytoplasmic amount.
Weak (1+) Threshold	210	Nuclei intensity threshold for weak (1+) (0 < Value ≤ 255). Nuclei with an intensity value above the threshold but below the Cytoplasmic Correction threshold will be ranked as 1+.
Moderate (2+) Threshold	188	Nuclei intensity threshold for moderate (2+) (0 < Value ≤ 255). Nuclei with an intensity value above the threshold but below the Cytoplasmic Correction threshold will be ranked as 2+.
Strong (3+) Threshold	162	Nuclei intensity threshold for strong (3+) (0 < Value ≤ 255). Nuclei with an intensity value above the threshold but below the Cytoplasmic Correction threshold will be ranked as 3+.
Dark Nuclei Removal	0	Nuclei darker than this value will be removed. Intensity threshold (lower limit) of strong positive nuclei (0 < Value ≤ 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.

This is an example of the mark-up image in the Algorithm Tuning window when adjusting these parameters. This mark-up image shows the amount of Biomarker 1 within nuclei in orange (corresponding to the Moderate category). The nuclei highlighted in blue represent the absence of Biomarker 1 within the nuclei.



Plots

This parameter selects whether analysis results are available as data plots.

Parameter	Default	Description
Display Plots	No	Select whether or not to display plots of the algorithm result data (Yes or No).

For details on displaying and interpreting plots, see the *Aperio Image Analysis User's Guide*.

Advanced

These parameters control how the algorithm is processed.

Parameter	Default	Description
Image Zoom	1	Zoom level to be used; a higher zoom results in faster algorithm run but less accurate results.
Mark-up Compression Type	Same as processed image	You may select "Same as processed image," JPEG, or JPEG2000.
Compression Quality	30	Higher quality takes longer to process and yields larger image files. Does not apply to all compression types.
Classifier Neighborhood	0	(Used if you are using Genie classifiers to preprocess the image.) Size (in microns) of the neighborhood to pad the boundary of each view, as required by the classifier.
Classifier	None	(Used if you are using Genie classifiers to preprocess the image.) Choose from the list of classifiers, if available.

Parameter	Default	Description
Class List		(Used if you are using Genie classifiers to preprocess the image.) Pick the classes to retain for further processing (if available).
Clear Area Intensity	240	Intensity threshold for the glass slide. For images scanned from the Aperio ScanScope scanner, this value is 240.

Outputs

The Output parameters section shows all of the outputs that can be shown in your analysis results in eSlide Manager. To remove an output from the analysis display in eSlide Manager, clear the check box next to it.

The basic goal of this algorithm is to detect and classify nuclei according to the relative positive staining present.

Parameter	Description
Intensity Score	The input thresholds are applied to the average intensity (positive staining only) of the positive nuclei.
Percent Positive Nuclei	Percentage of cells having staining in the nuclei. 100 * [(1+) + (2+) + (3+) Nuclei/Total.
Average Positive Intensity	Average intensity of the deconvolved positive channel for the (1+), (2+), and (3+) nuclei. This is an average over number of objects, not an area average over pixels. This is the intensity that is used by the Intensity Score above.
Average Negative Intensity	Average intensity of the deconvolved positive channel for the (0+) nuclei. Note that this number should always be higher (lighter) than the Weak (1+) threshold, since this is the threshold used to divide the (0+) from the (1+) nuclei. This is an average over number of objects, not an area average over pixels.
(N+) Percent Nuclei	100 * (N+) Nuclei/Total
(N+) Nuclei	The number of strong-stained (3+) nuclei, moderately-stained (2+) nuclei, weak-stained (1+) nuclei, or negative nuclei as defined by the input thresholds.
Total Nuclei	The sum of (3+), (2+), (1+), and (0+) nuclei.
Average Nuclear RGB Intensity	Average intensity of input image pixels for all nuclei.
Average Nuclear Size (um^2)	Average size in pixels of nuclei detected.
Area of Analysis (Pixels)	Total area analyzed, including any white areas enclosed by the regions drawn in number of pixels.
Area of Analysis (mm^2)	Total area analyzed, including any white areas enclosed by the regions drawn in millimeters squared.

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Symbols

- The following symbols may appear on your product label or in this user's guide:

	Manufacturer
	Date of manufacture (year - month - day)
	European Union Authorized Representative
	In vitro diagnostic device
	Serial number
	Catalog number
	Relative humidity range
	Biological risks
	Storage temperature range
	Electronic and electrical equipment waste disposal
	The exclamation point within an equilateral triangle is intended to alert you to the presence of important operating and maintenance (servicing) instructions. *Le point d'exclamation dans un triangle équilatéral vise à avertir l'utilisateur qu'il s'agit d'instructions d'utilisation et d'entretien importantes.*
	The lightning flash with arrowhead symbol within an equilateral triangle is intended to alert you to the presence of uninsulated "dangerous voltage" within the product's enclosure that may be of sufficient magnitude to constitute a risk of electric shock to persons. *Le symbole de l'éclair avec la pointe de flèche dans un triangle équilatéral vise à avertir l'utilisateur que le boîtier du produit présente une « tension dangereuse » non isolée d'une amplitude suffisante pour constituer un risque d'électrocution.*
	The flat surface with waves symbol within an equilateral triangle is intended to alert you to the presence of hot surfaces which could cause burn damage. *Le symbole d'une surface plane et de vagues dans un triangle équilatéral vise à avertir l'utilisateur de la présence de surfaces chaudes qui peuvent causer des brûlures.*
	The UV lamp within an equilateral triangle is intended to alert you to the presence of UV light within the product's enclosure that may be of sufficient magnitude to constitute a risk to the operator. *La lampe UV dans un triangle équilatéral vise à avertir l'utilisateur de la présence de rayonnement UV dans le boîtier du produit qui peut être d'une amplitude suffisante pour constituer un risque pour l'utilisateur.*

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