The Pathology Company

Leica BIOSYSTEMS

Cytoplasmic Algorithm

User's Guide

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Cytoplasmic Algorithm User's Guide

This document applies to eSlide Manager Release 12.3 and later.

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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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Contents

1 Introduction

This chapter introduces the Cytoplasmic algorithm. For general information on using an algorithm, please see the *Aperio Image Analysis User's Guide*.

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.

About the Cytoplasmic Algorithm

When a pathologist or researcher needs to measure protein expression within immunohistochemisty (IHC) stained tissue using brightfield microscopy, the tissue is illuminated with visible white light and protein expression is typically observed with diaminobenzidine (DAB), a brown stain. In most cases, the darker the stain the greater the protein expression. Counterstains such as Hematoxylin, a blue stain, are applied for morphologic detail of the surrounding tissue to help study the tissue and draw conclusions.

The Cytoplasmic algorithm completes the Aperio cell-based analysis module for analyzing IHC-stained tissues. Together with the membrane and nuclear algorithms, all major sub-cellular compartments can now be analyzed on the Aperio platform. The Cytoplasmic algorithm by default is set to analyze DAB staining intensity and provide the percentage of cells containing stain within in the nucleus and the cytoplasm. The intensity for the positive stain is divided into four score classes with the

ability to add additional classes (up to 10 total). In addition, the algorithm provides an H-Score, a co-localized result and a histogram for the IHC staining of the cytoplasm and nuclei.

The ability to differentiate between staining in the two sub-cellular compartments is an important feature of the Cytoplasmic algorithm. Translocation between the cytoplasm and the nucleus is a common method to regulate the activity of proteins in the cell, especially transcription factors and cell cycle proteins. Under circumstances where certain cellular pathways are disrupted, such as cancer, sub-cellular localization of these proteins may be altered. Therefore, pathologists or researchers often evaluate the respective localization of these proteins in the nucleus or the cytoplasm in order to gain insight into the status of cells and tissues. The Cytoplasmic algorithm can facilitate this analysis.

Prerequisites

The Cytoplasmic algorithm requires that you be using Aperio 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 pathologists who have an understanding of the conditions they are testing for in running the algorithm analysis.

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

For More Information

For a discussion of the Cytoplasmic algorithm parameters, refer *"Chapter 2: Tuning Parameters" on page 9*.

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*.

If the analysis results are not what you expect, see the *Aperio Image Analysis User's Guide* section on troubleshooting for assistance.

2 Tuning Parameters

The Cytoplasmic 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 IHC reagent or kit.

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

Tuning Algorithm 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

Cytoplasmic algorithm performance is controlled by a set of input parameters. 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.

You will want to fine-tune the parameters to suit your analysis needs and the characteristics of your slides, and then save the algorithm macro with the adjusted 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).

If you are not using a third stain, make sure the values in the Stain 3 section are set to zero.

The Cytoplasmic algorithm contains an automatic stain color finder that can be used to calculate the stains on the slide. See the next section for details.

Parameter	Default	Description
Number of Visible Stains	2	0 to 3. This value can be changed in any of the Stains sections.
Target	Counterstain (for Stain 1)	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"* below 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"* below for information on adjusting color values.

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 under 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.

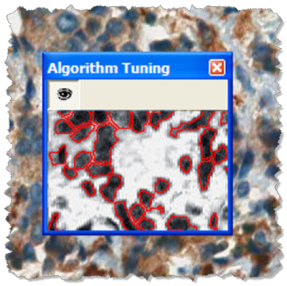
Repeat these steps for the other stains shown in the Algorithm Tuning window.

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 Identifications

The Nuclei Identification parameters filter and remove unwanted signals by smoothing (Smoothing), removing background (Type), and removing small nuclei (Min Size).

When adjusting these parameters, the Algorithm Tuning window shows the nuclei circled with cytoplasm stain removed. This gives a visual check that the algorithm is successfully finding nuclei.



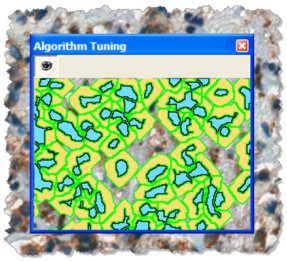
Parameter	Default	Description
Type	0	Select stain for detecting nuclei. 0 is Counterstain only; move value toward 1 for Counterstain plus Biomarker 1. Nuclei can stain imperfectly, picking up some brown stain. This parameter specifies how much brown stain to include in nuclei identification. A value of 0 gives the strictest definition of what will be considered nuclei by only considering a stain vector color of blue; a value of 1 allows both blue and brown stains to be considered. A value of .2 is a good compromise, allowing a small amount of brown stain to be included in the definition.
Method	Automatic	(Automatic or Manual). Automatic adapts to variations in nuclear staining within the threshold limit. Manual uses the defined threshold (below).
-- Threshold Upper Limit	210	Upper limit for nuclear thresholding (0<Value<255).
Min Size (um^2)	20	Minimum area for detectable nuclei (micro squared). This is used to exclude small nuclei and for declustering neighboring nuclei. When nuclei are clustered together, decreasing this value allows the algorithm to separate them.

Parameter	Default	Description
Smoothing (um)	0.5	Amount of smoothing performed (um). The larger the value, the more smoothing of the object is done. This parameter smooths out variations in nuclei size. It corrects improper segmenting of nuclei caused by spotty staining by smoothing pixels and improves nuclear counting accuracy.

Cytoplasm Segmentation

Parameter	Default	Description
Cytoplasmic Distance (um)	6	Defines the maximum allowable distance for cytoplasm surrounding nuclei.

This parameter in microns defines how far from the nucleus the cytoplasm can be yet still be reported as cytoplasm that surrounds the nucleus. When adjusting these parameters, the Algorithm Tuning window gives you a visual check of this value. In the example below, the blue areas are nuclei and the yellow areas are the cytoplasm that surround the nuclei.



Adjust the Cytoplasmic Distance parameter until the yellow regions surrounding the nuclei are the desired size.

Positivity Thresholds

Positivity thresholds define thresholds for the positive stain.

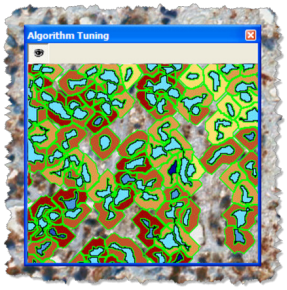
The scores for average cytoplasm intensity for the selected region are calculated based on these thresholds.

If you wish greater granularity in your results, you can define additional positive thresholds.

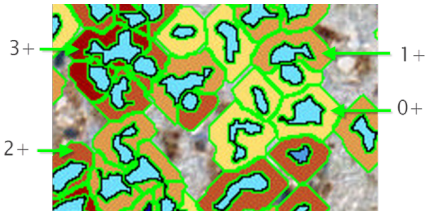
Parameter	Default	Description
Number of Positive Thresholds	3	Select the number of positive thresholds you want to use.
(1+) Threshold	210	Weak positive
(2+) Threshold	180	Moderate positive
(3+) Threshold	150	Strong positive

The Number of Positive Thresholds defines how many cytoplasm scores you want to be reported. The default thresholds are 1 (weak positive), 2 (moderate positive), and 3 (strong positive).

The individual threshold parameters define how much cytoplasm stain has to be present to be reported as +1, +2, and so on. When adjusting these parameters, the Algorithm Tuning window gives you a visual check of the different thresholds. For example:



In the Algorithm Tuning window, the following cytoplasm score colors are displayed:



The definitions of these color codes are presented in the final analysis results displayed in the Annotations window.

Advanced Parameters

The Advanced 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	JPEG	You may select "Same as processed image," JPEG, or JPEG2000. This sets the compression type for the algorithm mark-up image. Choose better compression if you need the image for a special purpose.
Compression Quality	70	Higher quality takes longer to process and yields larger image files. Does not apply to all compression types.
Classifier Neighborhood	25	(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.
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, defining white balance for the image. For images created by the Aperio ScanScope scanner, this value is 240.

Algorithm Outputs

The Outputs parameter 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 checkbox next to it.

The Annotations window shows quantitative results of the analysis, while the slide image shows a mark-up image that gives a visual representation of those results. Most of the results show threshold scores, defining the intensity with which the nuclei and cytoplasm were stained.

Parameter	Description
Cytoplasm H-Score	A cytoplasmic intensity score derived from the average intensity of the staining of the cytoplasm (cellular average) according to the threshold intervals set in the algorithm macro. For example, if there are three thresholds defined, this score equals = (%1+) + 2*(%2+) + 3*(%3+) + ... For the usual case where there are 3 thresholds, the score is between 0 and 300, where 300 represents 100% of cells being 3+.
Cytoplasm Average Positive Intensity	Average intensity of staining in cytoplasm (cellular average). This is the average of positive cells only—0+ cells are not counted in the average.
Cytoplasm: Percent Positive Cells	Percentage of cells having staining in the cytoplasm.
Nucleus: H-Score	A nuclear intensity score derived from the average intensity of the staining of the nuclei (cellular average) according to the threshold intervals set in the algorithm macro. For example, if there are three thresholds defined, this score equals = (%1+) + 2*(%2+) + 3*(%3+) + ... For the usual case where there are 3 thresholds, the score is between 0 and 300, where 300 represents 100% of cells being 3+.
Nucleus: Average Positive Intensity	Average intensity of staining in nuclei (cellular average). This is the average of positive cells only—0+ cells are not counted in the average.
Nucleus: Percent Positive Cells	Percentage of cells having staining in the nuclei.
Cytoplasm: Percent (N+)	For each cytoplasm threshold defined in the algorithm, the percentage of cells having staining in the cytoplasm.
Nucleus: Percent (N+)	For each nucleus threshold defined in the algorithm, the percentage of cells having staining in the nuclei.
Number of Cells	Total number of cells analyzed.
Percent Colocalized	Percentage of cells having staining in both the cytoplasm and nucleus.
Area of Analysis (mm2)	Area of the slide that was analyzed.
Cytoplasm Area (mm2)	Total area identified as cytoplasm
Nuclear Area (mm2)	Total area identified as nuclei.

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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
	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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