

Color Deconvolution Algorithm

User's Guide



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User Resources

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ImageServer is intended for use with the svx file format (the native format for digital slides created by scanning glass slides with the ScanScope scanner). Educators will use Aperio software to view and modify digital slides in Composite WebSlide (CWS) format.

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Introduction

This chapter introduces Aperio algorithms in general and the Aperio Color Deconvolution Algorithm in particular.

Aperio Algorithms

Analyzing digital slides helps you to examine slide staining to find patterns that will tell you more about the slide. Using an algorithm to look for these patterns provides precise, quantitative data that is accurate and repeatable. The process of analyzing a digital slide is done by applying algorithms directly to the digital slide or selected regions of the digital slide.

Several algorithms developed for specific applications are available, including:

- **Positive Pixel Count** – This algorithm looks for:
 - Positive areas and shades them orange
 - Negative areas and shades them blue
 - Neutral areas—areas that are neither positive nor negative—and shades them white.

The intensity of colors reflects the intensity of the staining.

- **Nuclear** – Used, for example, for ER, PR slides
- **Membrane** – Used, for example, for HER2 slides
- **Micrometastasis** – The micrometastasis algorithm is a rare event detection algorithm, specifically developed to detect micrometastasis of tumor cells found in circulating blood.
- **SnapShot Generator** – This algorithm creates a series of snapshot images that can be analyzed by a separate application.
- **Color Deconvolution** – This algorithm separates the image into three channels, corresponding to the actual colors of the stains used. This allows the pathologist to accurately measure the area for each stain separately, even when the stains are superimposed at the same location.

The Positive Pixel Count algorithm is licensed without charge with other Aperio software. Other algorithms are available from Aperio for a license fee. Algorithms have also been developed by third parties and tools are available from Aperio for creating your own algorithms—contact Aperio for details.

These algorithms all have control parameters—for example, intensity and hue settings—that allow the algorithm to be tailored to your specific needs.

Performing Analyses

You can run an analysis using an algorithm in several ways:

1. Use the **Analysis** command of the ImageScope viewer 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) or a remote image on an Aperio ImageServer¹.
2. Analyze a single digital slide image that resides on the ImageServer by using the Spectrum Information Manager **Analyze** command.
3. Analyze a batch of digital slide images that reside on the ImageServer by selecting multiple images and using the Spectrum Information Manager **Analyze** command.

For information on using algorithms to perform analysis, refer to the *ImageScope User's Guide* and the *Spectrum Information Manager Operator's Guide*.

Intended Use

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.

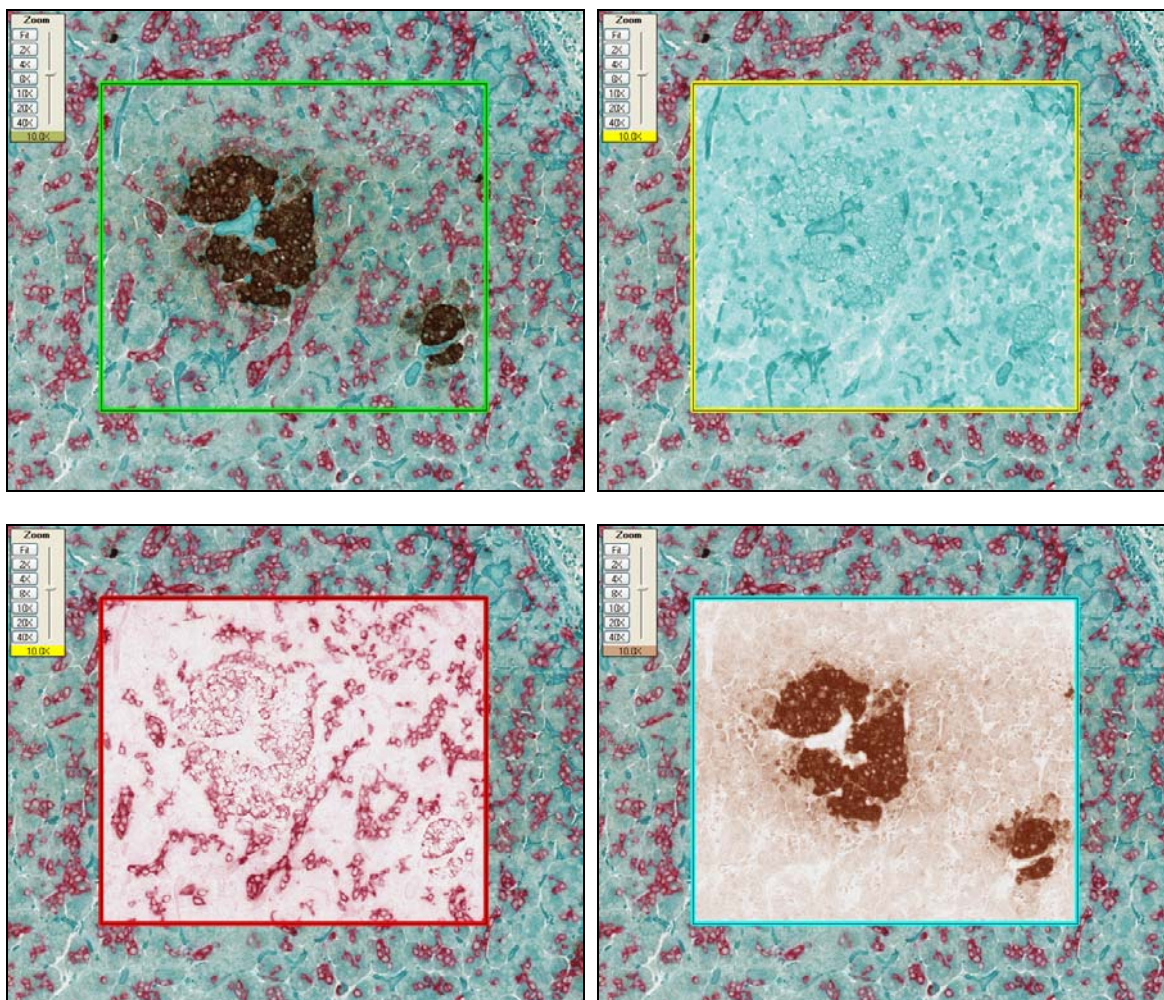
You will adjust (tune) the parameters until the algorithm results are sufficiently accurate for the purpose for which you intend to use the algorithm. You will want to test the algorithm on a variety of images so its performance can be evaluated across the full spectrum of expected imaging conditions. 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 Appendix A, "Troubleshooting" on page 21 for assistance.

¹ Educational customers can analyze digital slide images locally on their own workstations, but cannot analyze remote images via the ImageServer.

The Color Deconvolution Algorithm

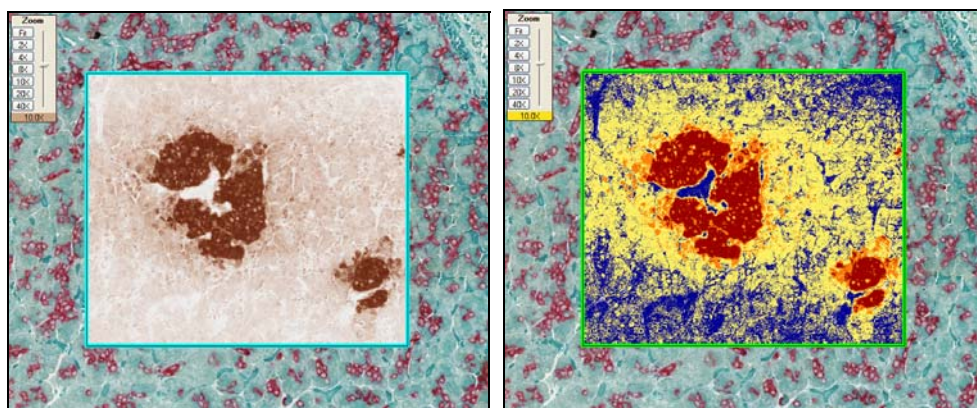
Most image processing algorithms for pathology use color to segment different types of tissue and cellular structures and for detection of specific proteins. The goal of *deconvolution* is to separate the image into three channels, corresponding to the actual colors of the stains used. This allows the pathologist to accurately measure the area for each stain separately, even when the stains are superimposed at the same location. In the image below, three stains can be seen: Crystal Light Green, Fast Red, and DAB. The scanned image, along with the three deconvolved color channels, are shown.



Deconvolution example. Scanned image with rectangular ROA (upper left); Channel 1 , Crystal Light Green (upper right); Channel 2, Fast Red (lower left); Channel 3, DAB (lower right).

It is clear in the illustration above that Crystal Light Green is present nearly everywhere, while the other two stains are more specific to certain areas. In addition, the Fast Red and DAB have some areas in common. The deconvolution algorithm does more than just present this separation visually, it also accurately calculates the areas for each individual stain as will be shown below.

The example shown in the illustration above is actually a result of running the algorithm three times, once for each color channel and shows the stain for each channel as it would be seen if the other two stains were removed. The image generated in this way is referred to as a *markup image*. There is a second type of markup image that is more useful when measuring areas of different staining density. This image is referred to as the *Intensity Ranges markup image* and is shown below along with the *Deconvolved Color Channel markup image* for DAB.



Deconvolved Color Channel (left) and Intensity Ranges (right) for Channel 3, DAB.

The Intensity Ranges (right) show four colors: Red indicates strong staining, Orange indicates moderate staining, Yellow indicates weak (background) staining in the DAB channel, and Blue indicates staining in one or both of the other two channels. The thresholds that determine the boundaries between weak, medium, and strong staining are set by the pathologist as input parameters to the algorithm.

The area for each of these four staining categories is also given as numerical output shown in the illustration on the right. The weak positive (Yellow) comprises 53.4% of the total stained area. The medium (Orange) and strong positive (Red) comprise 7.2% and 10.0% respectively. The negative staining (Blue) comprises 29.2% of the total area and corresponds to those pixels that did not receive any positive stain (DAB in this case). The total stained area is 0.19 mm², from which the actual area for any of the other categories can be easily calculated. Similar results are obtained for the other two channels.

Algorithm	Color Deconvolution
Version	8.001
Average Positive Intensity	177.988
Percent Weak Positive	53.4537
Percent Medium Positive	7.26969
Percent Strong Positive	10.0327
Percent Negative	29.2439
Percent Total Positive	70.7561
Average Weak Positive Intensity	203.063
Average Medium Positive Intensity	142.122
Average Strong Positive Intensity	70.3819
Total Stained Area (mm ²)	0.193882

Numeric Results for Channel 3, DAB.

Algorithm Installation

The instructions in this section apply to all Aperio algorithms.

Installing the Algorithm

Installing an algorithm is a simple matter of using Windows Explorer to navigate to the location of the algorithm installer file and double-clicking the file name. This file may be given to you on a CD or your network administrator may have placed it on your network.

Follow the instructions of the algorithm installer, clicking **Next** when instructed to do so.

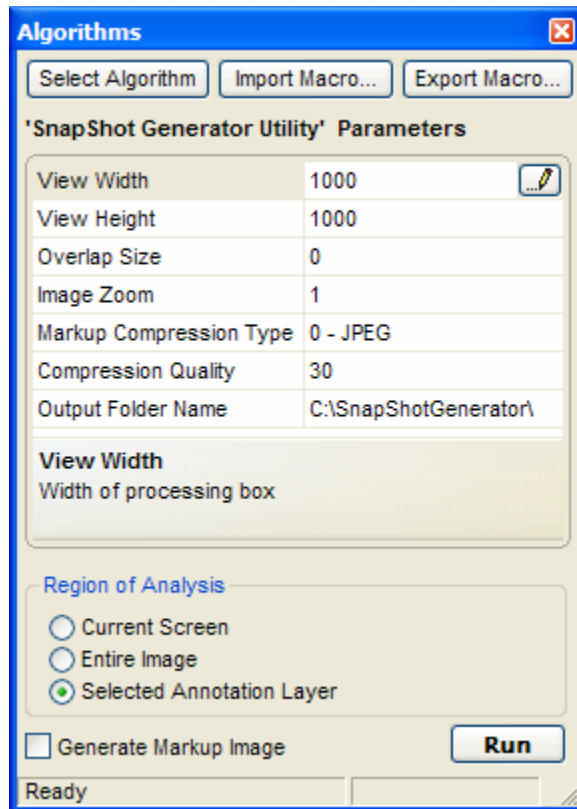
Where to Install the Algorithm

Where you install the algorithm depends on how you are going to be analyzing digital slides:

- **Analyzing local digital slides** – If you will be using the algorithm to analyze local digital slides (that is, digital slides that are located on your workstation or on your network where they can be accessed by Microsoft file sharing), install the algorithm on your workstation.
- **Analyzing remote digital slides** – If you will be using the algorithm to analyze remote digital slides (that is, digital slides that are located on your Aperio ImageServer), install the algorithm on the workstation on which the ImageServer is located.
- **Analyzing digital slides through Spectrum Information Manager** – If you are going to be using the Spectrum Information Manager's analysis features to analyze a single digital slide or a batch of digital slides, you will need to install the algorithm on both your local workstation and on the workstation that contains your ImageServer. See "Registering the Algorithm with the Spectrum Information Manager" below for details.

Registering the Algorithm with the Spectrum Information Manager

1. Follow the instructions above to install the algorithm on your local workstation *and* on the workstation that contains your ImageServer.
2. Open a local digital slide on your workstation in ImageScope.
3. Go to the **View** menu and select **Analysis** to open the Algorithms window.





4. Click **Select Algorithm** on the Algorithms window and choose the algorithm you want to register from the list of installed algorithms if it is not shown at the top of the Algorithm window. (The sample above shows that the SnapShot Generator is the currently selected algorithm.)
5. Adjust the algorithm parameters as desired (see later in this document), and click **Export Macro** to save the algorithm + settings to the \Macro folder on the workstation on which the Spectrum Information Manager is installed. (Save the image analysis algorithm with the name that will be used for uploading the macro in the Spectrum Information Manager.)
6. Log onto the Spectrum Information Manager as an administrator or ask your Spectrum administrator to log on and follow the instructions in the *Spectrum Information Manager Administrator's Guide* to install the algorithm macro in the Spectrum Information Manager.

Accessing a Digital Slide in Spectrum Information Manager

Cases, specimens and digital slides are managed using Aperio's Spectrum Information Manager. A pathologist who wants to access a digital slide first needs to log into Spectrum Information Manager and navigate to the case and the specimen that shows the list of its associated digital slides.

Please ask your Spectrum Information Manager administrator for the Spectrum Information Manager web-address, your logon name, and a password.

1. Open Microsoft Internet Explorer (or another web viewer like Firefox) and enter the Spectrum Information Manager web-address. You will see a logon page.
2. Log onto Spectrum Information Manager by entering your user name and password.
3. Navigate to the list of cases by clicking **List all Cases**.
4. Open the case you want to review by clicking on the open data icon  for the case.
5. Open the specimen digital slides list by clicking on the open data icon  for the case specimen.
6. Open the digital slide in ImageScope by clicking on its thumbnail in the specific digital slide list or select the slide and use the Spectrum Information Manager **Analyze** command to analyze the digital slide in a background job.

Patient Data Confidentiality

Spectrum Information Manager provides a full security system that ensures that users can only access data they are authorized to see, thus protecting patient data from unauthorized access.

Digital slides and related meta-data reside in the Spectrum Information Manager database. Spectrum Information Manager uses an administrator/user structure that restricts security-sensitive actions to an authorized administrator. The administrator can:

- Set up users with passwords. Users must log into Spectrum Information Manager with their user name and password and be authenticated before they can access data.
- Set up data groups that restrict access to specific users.
- Set users' access permissions to grant them full access, read-only access or no access to the defined data groups.

For More Information

For details on using Aperio algorithms to analyze digital slides, see:

- *Spectrum Information Manager Operator's Guide* for information on using algorithms to analyze individual digital slides or batches of digital slides.
- *Spectrum Information Manager Administrator's Guide* for information on installing algorithm macros so analysis can be done by Spectrum Information Manager users.
- *ImageScope User's Guide* for information on using algorithms to analyze local digital slides on your workstation or local network, or remote digital slides on an ImageServer.
- For an example of performing an analysis using ImageScope on a local digital slide, see "Sample Analysis" on page 15.

Algorithm Inputs

The Color Deconvolution algorithm performance is controlled by a set of input parameters, which determine the thresholds for the intensity ranges, the channel to be analyzed, the type of markup image to be presented, and calibration data that defines the exact colors for the three stains. The default colors are Hematoxylin, Eosin, and DAB. The colors used in the example above were obtained using a calibration procedure described later in this document.

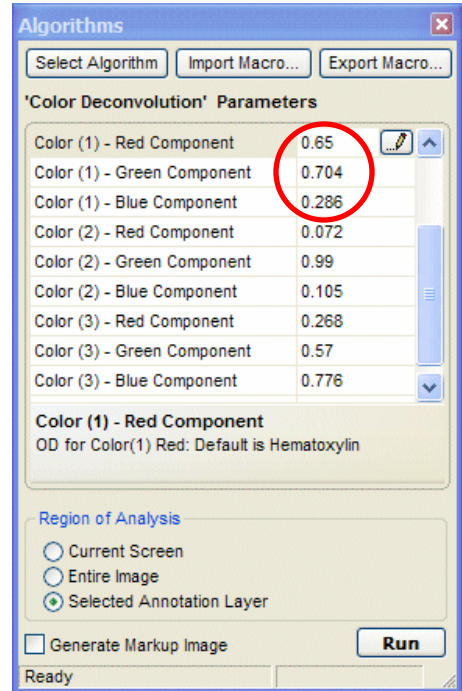
- **Intensity** – The raw image data are in RGB format. An RGB value of (255,255,255) corresponds to bright White, while an RGB value of (0,0,0) corresponds to Black. Intensity is the average of the RGB channels in the raw data: $(R+G+B)/3$. A large intensity value is very bright and corresponds to very little staining in the histological sample. A low intensity value corresponds to dark staining. Intensity is the opposite of density, in which larger values are darker.
- **Thresholds** – The input thresholds are usually in descending order, since weak intensity is greater than medium intensity which is greater than strong intensity.
- **Positive Color Channel** – Color channel to be analyzed: 1, 2, or 3. The color specification for each channel is given below. Each color channel corresponds to one of up to three possible stains.
- **Markup Image Type** – May be either “Intensity Ranges” or “Deconvolved Color Channel.” The choice of markup image does not change the numerical results in any way.
- **Weak Positive Threshold** – Upper intensity limit for the weak positive pixels and may be in the range (0 – 255).
- **Medium Positive Threshold** – Upper intensity limit for medium positive pixels and may be in the range (0 -255).
- **Strong Positive Threshold** – Upper intensity limit for strong positive pixels and may be in the range (0-255).
- **Black Threshold** – Intensity value for Black and is usually set to zero.
- **Color (1) Red, Green, Blue Components** – Normalized optical density values for Channel (1). Default for Channel 1 is Hematoxylin. These values are calculated by a calibration procedure described below.
- **Color (2) Red, Green, Blue Components** – Normalized optical density values for Channel (2). Default for Channel 2 is Eosin. These values are calculated by a calibration procedure described below.

- **Color (3) Red, Green, Blue Components** – Normalized optical density values for Channel (3). Default for Channel 3 is DAB. These values are calculated by a calibration procedure described below.
- **Clear Area Intensity** – This is the intensity for a clear area on the slide. This value is always 240 for ScanScope generated images.

Color Calibration

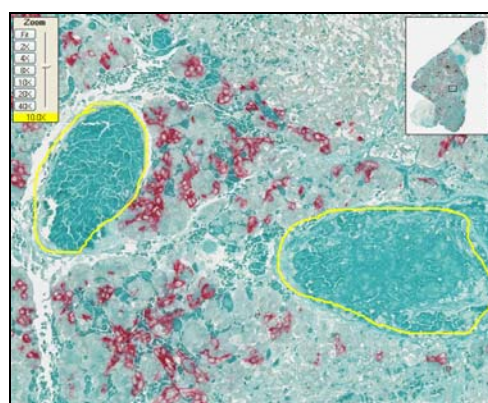
Three color components for each of the three channels must be specified in the input parameters to the algorithm—nine numbers total, as shown in the illustration on the right. If only two stains are present, the three numbers for one of the colors can be set to zero. Each channel also has a default color. The default color for channel 1 is Hematoxylin, shown enclosed by the red circle. The default colors for channels 2 and 3 are Eosin and DAB.

These numbers must be changed if different stains are used. The color for each stain is calibrated separately, using a separate image for each stain having only that color present. If three areas within a single image can be identified that are each dominated by a single stain, then these areas may also be used.



Algorithm input window showing the nine color values.

To calibrate Color 1 for Crystal Light Green, an area of the image was found (illustration below, left) that has two regions which are stained in only that color (enclosed by yellow lines). The Color Deconvolution algorithm is run with its erroneous Color 1 settings and its output for those regions, shown at the right, is obtained. To adjust the input parameters to detect Crystal Light Green on the Color 1 channel, copy the resulting average OD values back to the Color 1 parameters in the algorithm settings. This procedure must be repeated for each color that differs from the default settings. After calibration is completed, the modified parameter settings may be saved by clicking on the **Export Macro** button in the Algorithms window and specifying a macro name that is easily associated with this staining combination.



Algorithm	Color Deconvolution
Version	8.001
Average Positive Intensity	152.492
Percent Weak Positive	25.0493
Percent Medium Positive	74.7004
Percent Strong Positive	0.110772
Percent Negative	0.139525
Percent Total Positive	99.8605
Average Weak Positive Intensity	191.002
Average Medium Positive Intensity	139.66
Average Strong Positive Intensity	97.3206
Total Stained Area (mm ²)	0.0733236
Score (0-300)	174.762
Average Red OD	0.925687
Average Green OD	0.266027
Average Blue OD	0.26895

(Left) Image area containing two regions (yellow lines) with primarily Crystal Light Green stain. (Right) Analysis output with color calibration values (red circle).

Algorithm Results


Many of the algorithm results have been discussed above. The term *positive* in the result name indicates that the result applies to pixels that are stained in the Positive Color Channel specified in the algorithm input. The term *negative* indicates that the result applies to pixels that are not stained positive. The total stained area is then the combined area spanned by the positive and negative pixels.

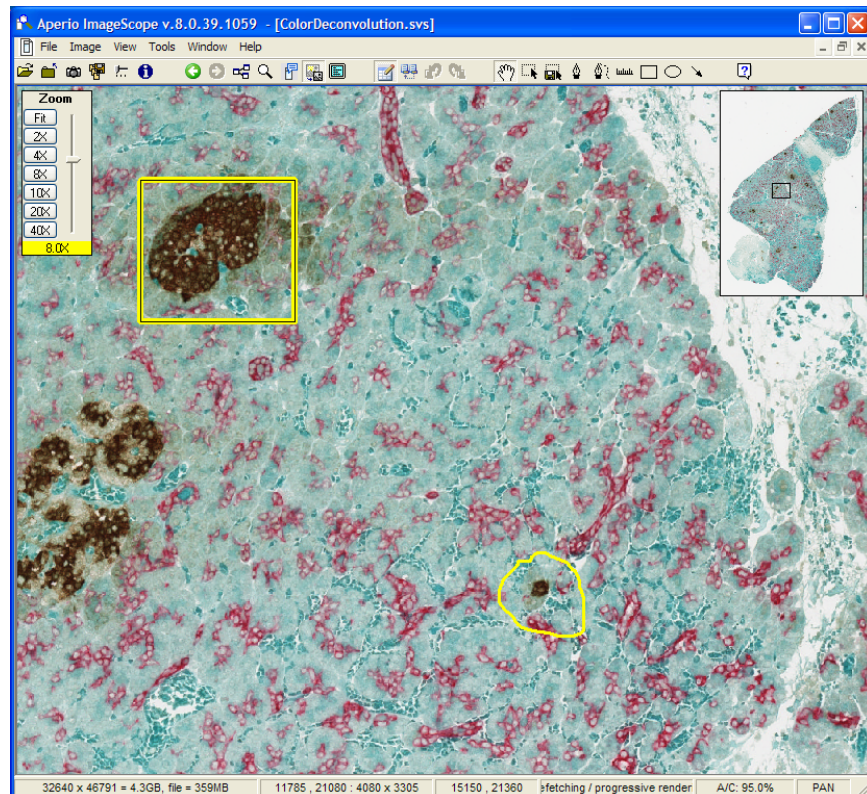
- **Average Positive Intensity** – Average intensity $(R+G+B)/3$ for all positive pixels.
- **Percent Weak Positive** – Percent of positive pixels that are weakly stained. Weak is defined by: $(\text{Weak Threshold}) > \text{Intensity} > (\text{Medium Threshold})$.
- **Percent Medium Positive** – Percent of positive pixels that are moderately stained. Medium is defined by: $(\text{Medium Threshold}) > \text{Intensity} > (\text{Strong Threshold})$.
- **Percent Strong Positive** – Percent of positive pixels that are strongly stained. Strong is defined by: $(\text{Strong Threshold}) > \text{Intensity} > (\text{Black Threshold})$.
- **Percent Negative** – Percent of pixels that are not positive, but have intensity defined by: $(\text{Weak Threshold}) > \text{Intensity} > (\text{Black Threshold})$.
- **Percent Total Positive** – Weak + Medium + Strong positive percentages.
- **Average Weak Positive Intensity** – Average intensity $(R+G+B)/3$ for all weak positive pixels.
- **Average Medium Positive Intensity** – Average intensity $(R+G+B)/3$ for all medium positive pixels.
- **Average Strong Positive Intensity** – Average intensity $(R+G+B)/3$ for all strong positive pixels.
- **Total Stained Area (mm²)** – Cumulative total area of combined positive and negative pixels in square-millimeters. The area for any other staining category may be obtained by multiplying the relevant percentage times the total area.
- **Score** – The score is calculated by a simple formula involving the positive percentages. $\text{Score} = 1.0 * (\% \text{Weak}) + 2.0 * (\% \text{Medium}) + 3.0 * (\% \text{Strong})$.
- **Average Red OD** – Average OD (optical density) of the Red component for all pixels analyzed. This value is used for calibration of input color specifications.
- **Average Green OD** – Average OD (optical density) of the Green component for all pixels analyzed. This value is used for calibration of input color specifications.

- **Average Blue OD** – Average OD (optical density) of the Blue component for all pixels analyzed. This value is used for calibration of input color specifications.

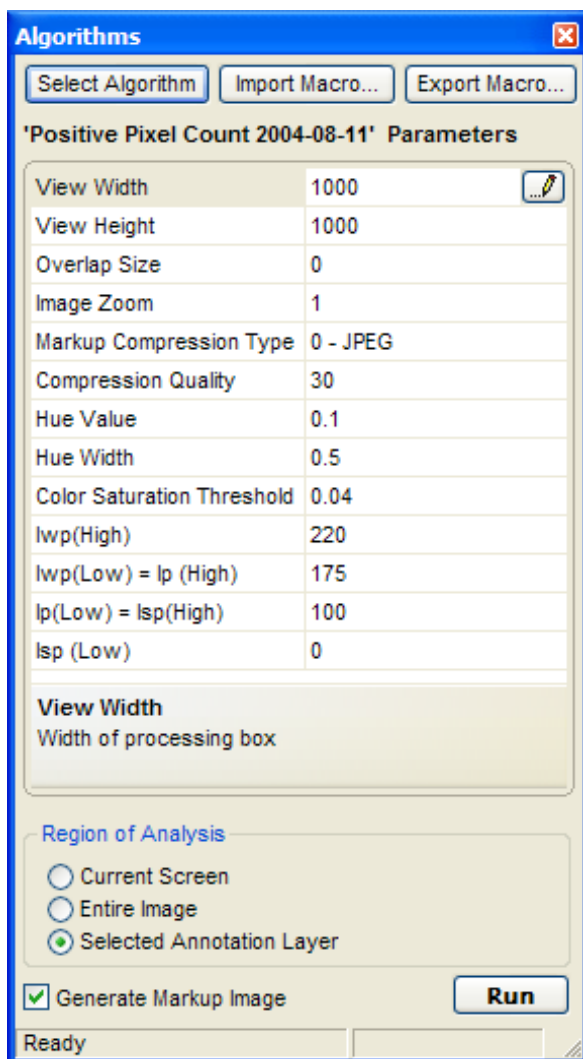
Sample Analysis

Although you can use several different tools to perform an algorithm analysis, this section shows a simple analysis using ImageScope to analyze a local digital slide that resides on your workstation or local network.

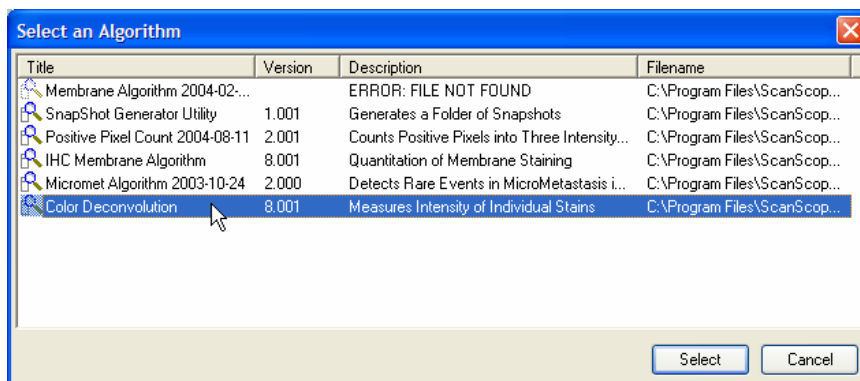
1. Open a digital slide in ImageScope.
2. If you want to limit the analysis to specific areas on the digital slide, use the ImageScope free-hand pen  or rectangle tool on the ImageScope toolbar to draw boundaries around those areas:



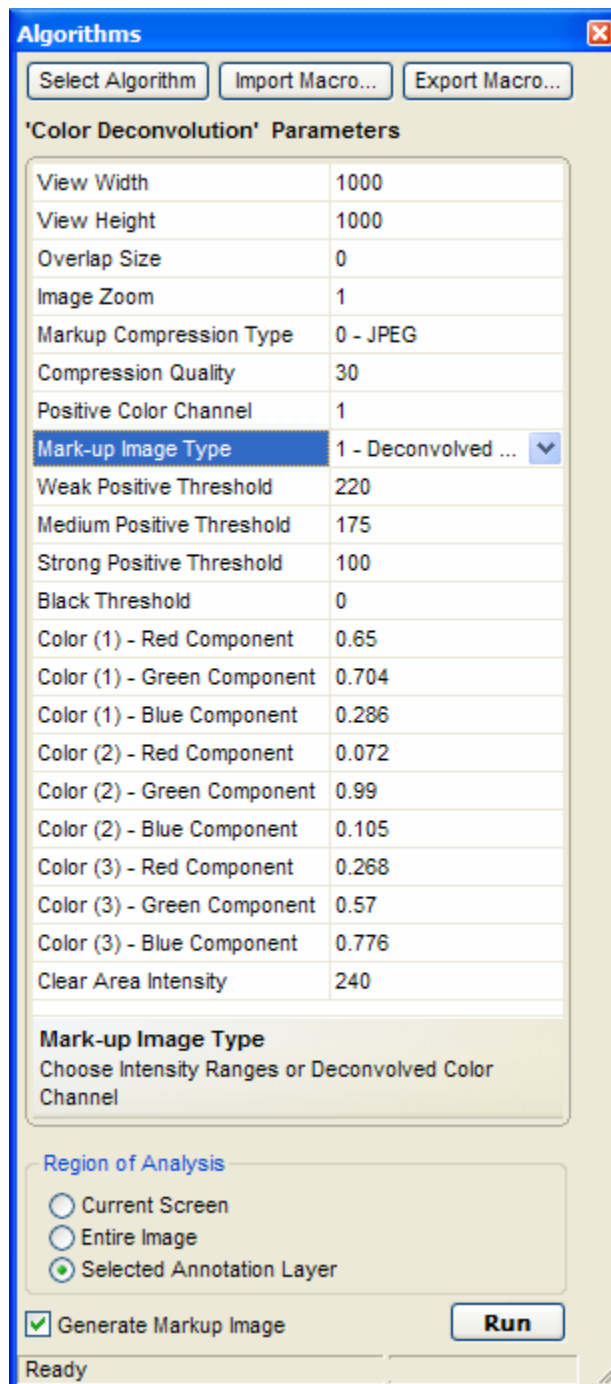
3. Go to the ImageScope **View** menu and select **Analysis**. The Algorithms window appears.



- a) If **Color Deconvolution** is not displayed at the top of the Algorithms window, click **Select Algorithm** to see the list of algorithms that are installed on your workstation and select **Color Deconvolution**.



The Algorithms window now contains the parameters for the Color Deconvolution algorithm:



The Algorithms window displays the parameters for the Color Deconvolution algorithm. The window has a title bar with a close button. Below the title bar are three buttons: 'Select Algorithm', 'Import Macro...', and 'Export Macro...'. The main section is titled 'Color Deconvolution' Parameters. It contains a table of parameters with their values. The 'Mark-up Image Type' parameter is highlighted with a blue background and a dropdown arrow. Below the table is a section titled 'Mark-up Image Type' with the instruction 'Choose Intensity Ranges or Deconvolved Color Channel'. Below this is a section titled 'Region of Analysis' with three radio buttons: 'Current Screen', 'Entire Image', and 'Selected Annotation Layer'. The 'Selected Annotation Layer' radio button is selected. At the bottom left is a checkbox labeled 'Generate Markup Image' which is checked. At the bottom right is a 'Run' button. The status bar at the very bottom says 'Ready'.

'Color Deconvolution' Parameters	
View Width	1000
View Height	1000
Overlap Size	0
Image Zoom	1
Markup Compression Type	0 - JPEG
Compression Quality	30
Positive Color Channel	1
Mark-up Image Type	1 - Deconvolved ...
Weak Positive Threshold	220
Medium Positive Threshold	175
Strong Positive Threshold	100
Black 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

Mark-up Image Type
Choose Intensity Ranges or Deconvolved Color Channel

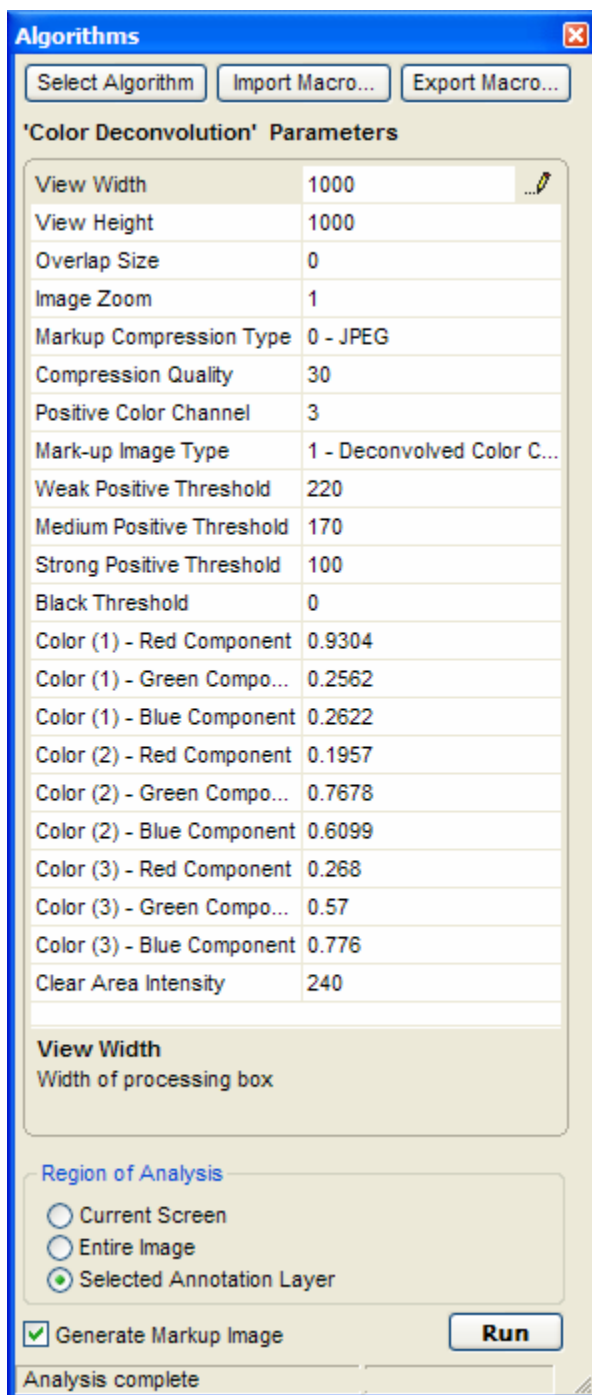
Region of Analysis

☐ Current Screen
☐ Entire Image
☒ Selected Annotation Layer

☒ Generate Markup Image **Run**

Ready

4. Adjust the algorithm parameters in the Algorithms window as discussed earlier in this document by clicking on a number and typing a new value or using the slider if one is provided for that parameter. In this case, we have changed the values so that the analysis will show the presence of DAB.



Algorithms

Select Algorithm Import Macro... Export Macro...

'Color Deconvolution' Parameters

View Width	1000
View Height	1000
Overlap Size	0
Image Zoom	1
Markup Compression Type	0 - JPEG
Compression Quality	30
Positive Color Channel	3
Mark-up Image Type	1 - Deconvolved Color C...
Weak Positive Threshold	220
Medium Positive Threshold	170
Strong Positive Threshold	100
Black Threshold	0
Color (1) - Red Component	0.9304
Color (1) - Green Component	0.2562
Color (1) - Blue Component	0.2622
Color (2) - Red Component	0.1957
Color (2) - Green Component	0.7678
Color (2) - Blue Component	0.6099
Color (3) - Red Component	0.268
Color (3) - Green Component	0.57
Color (3) - Blue Component	0.776
Clear Area Intensity	240

View Width
Width of processing box

Region of Analysis

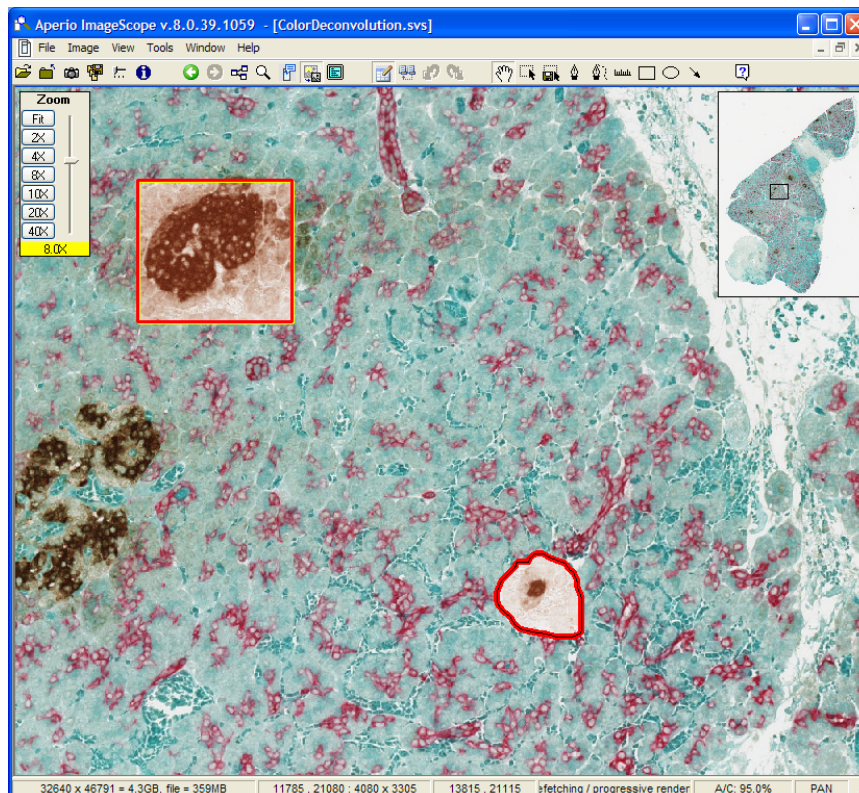
☐ Current Screen
☐ Entire Image
☒ Selected Annotation Layer

☒ Generate Markup Image **Run**

Analysis complete

5. If you want to see a visual representation of the analysis as well as a quantitative one, select **Generate Markup Image** on the Algorithms window.

6. To analyze just the annotated areas, select **Selected Annotation Layer** under the Region of Analysis section of the Algorithms window.
7. Click **Run**. Because we requested a markup image, the ImageScope main window shows the results of the analysis in the annotated areas:



8. To see the quantitative results, go to the ImageScope **View** menu and select **View Annotations**. Click on a Region number to see that annotation region centered on the ImageScope window:

The screenshot shows the 'Annotations' window in ImageScope. It is divided into three main panels: 'Layers', 'Layer Attributes', and 'Layer Regions'.

Layers Panel: Shows a tree view with 'Layer 1' expanded, containing 'Layer 2' and 'Layer 3'.

Layer Attributes Panel: Displays a list of quantitative metrics for the selected layer. The metrics are as follows:

Attribute	Value
Algorithm	Color Deconvolution
Version	8.001
Average Positive Intensity	142.847
Percent Weak Positive	38.8878
Percent Medium Positive	20.1357
Percent Strong Positive	27.8645
Percent Negative	13.112
Percent Total Positive	86.888
Average Weak Positive Intensity	196.481
Average Medium Positive Intensity	140.601
Average Strong Positive Intensity	69.6177
Total Stained Area (mm ²)	0.0395225
Score (0-300)	162.753
Average Red OD	0.418965
Average Green OD	0.555495
Average Blue OD	0.718257
*** Algorithm Inputs ***	
View Width	1000
View Height	1000
Overlap Size	0
Image Zoom	1
Pixel Area (millimeter-squared)	0.000000063504
Color Channel to Quantitate	3
Markup Image Type	1
Weak Positive Threshold	220
Medium Positive Threshold	170
Strong Positive Threshold	100
Black Threshold	0
Color(1) Red OD	0.9304
Color(1) Green OD	0.2562
Color(1) Blue OD	0.2622
Color(2) Red OD	0.1957
Color(2) Green OD	0.7678
Color(2) Blue OD	0.6099
Color(3) Red OD	0.268
Color(3) Green OD	0.57
Color(3) Blue OD	0.776

Layer Regions Panel: Shows a table of regions with their average positive intensity values.

Region	Average Positive Intensity
2	136.653
1	192.63

A

Troubleshooting

This appendix provides tips on identifying problems with glass slides that may contribute to unsatisfactory analysis results.

In “Intended Use” on page 2, we discussed the importance of understanding algorithm parameters so that you can fine-tune them so the analysis provides the results you expect and want. Even after adjusting algorithm parameters, a number of factors can arise that may cause the algorithm to yield unsatisfactory results for your application. Detection of these conditions must be done by a trained expert user who can identify these situations and take the appropriate corrective action. In some cases, the appropriate action may be to mark the glass slide as defective. In other cases, it may be that new tuning of the algorithm input parameters is required or the staining/preparation process must be repeated.

This appendix contains a partial list of conditions that may result in unsatisfactory analysis results.

Problem	Cause	Effect	Corrective Action
Defective slide	Broken slide	Blurry image and/or artifacts at crack.	You should reject the slide.
	Air pockets under coverslip.	Image very blurry, objects too light and not easily identifiable. The algorithm will likely miss information in this area.	You can exclude these areas from analysis by using the ImageScope negative-pen annotation tools
	Pen marks	Areas will be dark and will obscure underlying information.	You can exclude these areas from analysis by using the ImageScope negative-pen annotation tool.
Wrong tissue type	Algorithm tuned for one type of tissue (for example, breast cancer) and operator runs it on another type of tissue (for example, liver).	The algorithm analysis will still give results, but the results will likely be meaningless.	Choose a different algorithm or input parameter set.

Problem	Cause	Effect	Corrective Action
Wrong regions designated for analysis	For nuclear analysis, stromal and lymphocytes were included in the designated regions.	Nuclear counts will be too large.	Operator must recognize these areas and annotate them to exclude them from analysis.
Bad histology preparation	Tissue sections too thick.	Image will be blurry all over.	Prepare new slide.
	Folds in tissue.	Blurry areas and cells that are superimposed on each other.	Prepare new slide or annotate these areas to exclude them from analysis.
Variations in staining process	Staining is too light.	Cells are not solidly stained and the algorithm may over count by subdividing single cells into multiple cells.	Adjust input parameters if possible or prepare a new slide.
	Staining is too dark.	Cells which are close together become merged and counted as a single cell.	Adjust input parameters if possible or prepare a new slide.
	Staining is not specific to the features being analyzed.	For nuclear analysis, significant cytoplasmic staining may cause cells to be misidentified.	Prepare new slide.

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