Colocalization Algorithm

User's Guide

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This document applies to software versions Release 9.0 and later.

User Resources

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ImageServer is intended for use with the SVS 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.

Aperio products are FDA cleared for specific clinical applications, and are intended for research use for other applications.

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1 Introduction

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

The process of analyzing digital images begins with the ScanScope, which creates digital slides by scanning glass slides. Using Aperio image analysis algorithms to analyze digital slides provides several benefits:

- **Increases productivity –** Image analysis automates repetitive tasks.
- **Improves healthcare –** 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.
- **Development of new computer-based methods –** Image analysis helps you answer questions that are beyond the capabilities of manual microscopy, such as "What is the significance of multiple stains at the cell level and colocalization of stains?"
- **Workflow integration –** The Spectrum digital pathology information management software suite integrates image analysis seamlessly into your digital pathology workflow, requiring no additional work by the lab or pathologist. With the click of a button, the algorithm is executed while you review the digital slide.

The Colocalization Algorithm

In histology and cytology, a variety of staining methods are used to target different types of tissues and cellular structures and for detection of specific proteins. In an H&E stain, for example, Hematoxylin preferentially stains the nucleus, while Eosin stains both nucleus and cytoplasm. In IHC analyses, different stains mark the presence of one or more proteins within the cell.

The colocalization algorithm calculates the contribution of each stain at every pixel location in the image. For IHC, it determines where specific proteins are present and to what extend the proteins are "colocalized"—that is, whether they occur separately or in combination with each other.

Detecting and measuring the colocalization of multiple proteins is an important part of larger scientific studies, which seek to determine a correlation between the occurrence of these proteins and the outcome of a specific disease treatment.

The colocalization algorithm classifies each pixel as either part of a single stain or representing a combination of stains based on the separated stains' intensities.

Analysis Steps

1. The first step of colocalization analysis is to calibrate the stain color vectors so that the algorithm can accurately detect the stains. This step is covered in Chapter 3, "Color Calibration" on page 9.
2. The next step is to set stain thresholds. This is also discussed in Chapter 3.
3. The final step is to analyze the digital slide to obtain colocalization data. This is discussed in Chapter 4, "Colocalization Analysis" on page 13.

Prerequisites

The Colocalization algorithm requires that you be using Aperio Release 9 or later.

Because Aperio digital slides 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 following requirements:

Display Type:	CRT minimum, LCD (flat panel) recommended
Screen Resolution:	1024(h) x 768(v) pixels minimum, 1920 x 1050 or larger recommended.
Screen Size:	15" minimum, 19" or larger recommended
Color Depth:	24 bit
Brightness:	300 cd/m^2 minimum, 500 or higher recommended
Contrast Ratio:	500:1 minimum, 1000:1 or higher recommended

For More Information

For a quick reference to the colocalization algorithm input parameters and results, see Chapter 2, "Quick Reference" on page 5.

For examples and details on using the algorithm, begin with Chapter 3, "Color Calibration" on page 9.

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

- Installing an algorithm
- Opening a digital slide to analyze
- Selecting areas of a digital slide to analyze

- Running the analysis
- Exporting analysis results

For details on using the Spectrum digital slide information management system (for example, for information on running batch analyses), see the *Spectrum/Spectrum Plus Operator's Guide*.

For details on using ImageScope to view and analyze digital slides and using annotation tools to select areas of the digital slide to analyze, see the *ImageScope User's Guide*.

FDA Cleared Image Analysis Algorithms

Several Aperio algorithms have been cleared by the FDA for clinical use when used on ScanScope models that are labeled as approved medical devices, and are intended for research use for other applications. These algorithms have their own user guides. Please see the Intended Use section of the user guides for the specific cleared applications you wish to use for details on in vitro diagnostic use.

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 the appendix "Troubleshooting" in the *Aperio Image Analysis User's Guide* for assistance.

2 Quick Reference

This chapter contains a quick reference to all colocalization algorithm inputs and outputs. See the following chapters for details on using the algorithm.

If you are already familiar with using the colocalization algorithm, and need just a reminder of the different algorithm input and output parameters, please refer to the sections below. For more detailed information on using the algorithm, see the following chapters.

Algorithm Input Parameters

Colocalization algorithm performance is controlled by a set of input parameters, which determine many different types of analysis.

- **View Width –** Width of processing box.
- **View Height –** Height of processing box.
- **Overlap Size –** Size of the overlap region for each view. This should be at least as big as the average object size.
- **Image Zoom –** Zoom level to be used; a higher zoom results in faster algorithm run but less accurate results.
- **Markup Compression Type –** 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 –** A higher quality takes longer and yields larger files. This selection does not apply to all compression types.
- **Mark-up Image Type –** There are two types of mark-up images – Co-Localization (used for colocalization analysis) and Deconvolved (used to calibrate stain color vectors).
- **Mode** – Choose **Colocalization mode** or **Counter-stain, Double Label mode**.

  Double-label immunohistochemistry analysis is frequently used to identify cellular and subcellular colocalization of independent antigens, and is a special case of the more general colocalization analysis. In the case of double-label analysis, Color 1 represents the counterstain, for which you want information only for where Color 1 occurs by itself, not where it occurs in combination with Color 2 and Color 3. Colors 2 and 3

are used to identify specific protein markers. See Chapter 4, "Colocalization Analysis" on page 13 for examples of both types of analysis.

- **Color (1) Threshold –** Intensity threshold (upper limit) for color channel 1.
- **Color (1) Lower Threshold –** Intensity threshold (lower limit) for color channel 1.
- **Color (2) Threshold –** Intensity threshold (upper limit) for color channel 2.
- **Color (2) Lower Threshold –** Intensity threshold (lower limit) for color channel 2.
- **Color (3) Threshold –** Intensity threshold (upper limit) for color channel 3.
- **Color (3) Lower Threshold –** Intensity threshold (lower limit) for color channel 3.
- **Color (1) Red Component –** OD (optical density) for color 1 Red (default is Hematoxylin stain).
- **Color (1) Green Component –** OD (optical density) for color 1 Green (default is Hematoxylin stain).
- **Color (1) Blue Component –** OD (optical density) for color 1 Blue (default is Hematoxylin stain).
- **Color (2) Red Component –** OD (optical density) for color 2 Red (default is Eosin stain).
- **Color (2) Green Component –** OD (optical density) for color 2 Green (default is Eosin stain).
- **Color (2) Blue Component –** OD (optical density) for color 2 Blue (default is Eosin stain).
- **Color (3) Red Component –** OD (optical density) for color 3 Red (default is DAB stain).
- **Color (3) Green Component –** OD (optical density) for color 3 Green (default is DAB stain).
- **Color (3) Blue Component –** OD (optical density) for color 3 Blue (default is DAB stain).
- **Clear Area Intensity –** This is the intensity for a clear area on the slide. This value is always 240 for ScanScope generated images.

Algorithm Results

The algorithm results appear in the ImageScope Annotations window (go to the ImageScope View menu and select **Annotations**).

The first section of the annotations window displays the algorithm results; the second portion (labeled "Algorithm Inputs") repeat the algorithm input parameters you specified when you ran the algorithm.

The results give information on all the permutations of the colors detected. And the different colors in the mark-up image reflect those data.

Understanding the Results

As an example of interpreting the results listed below, the color magenta in the mark-up image shows all pixels that contain both Color 1 *and* Color 2. The intensities listed under "Percent (1+2) MAGENTA" in the results give the intensity of Color 1 in all areas that contain *both* Color 1 and Color 2 and the intensity of Color 2 in all areas that contain both Color 1 and Color 2.

- **Percent (1) BLUE –** Percent of the analyzed area that contains Color 1. Shown in blue in the mark-up image.
  - **Intensity (1,1) –** Intensity of Color 1 in areas consisting of only Color 1.
- **Percent (1+2) MAGENTA –** Percent of the analyzed area that contains Color 1 and Color 2. Shown in magenta in the mark-up image.
  - **Intensity (1, 1+2) –** Intensity of Color 1 in areas consisting of Color 1 and Color 2.
  - **Intensity (2, 1+2) –** Intensity of Color 2 in areas consisting of Color 1 and Color 2.
- **Percent (2) RED –** Percent of the analyzed area that contains Color 2. Shown in red in the mark-up image.
  - **Intensity (2,2)** – Intensity of Color 2 in areas consisting of only Color 2.
- **Percent (2+3) YELLOW –** Percent of the analyzed area that contains Color 2 and Color 3. Shown in yellow in the mark-up image.
  - **Intensity (2, 2+3) –** Intensity of Color 2 in areas consisting of Color 2 and Color 3.
  - **Intensity (3, 2+3) –** Intensity of Color 3 in areas consisting of Color 2 and Color 3.
- **Percent (3) GREEN –** Percent of the analyzed area that contains Color 3. Shown in green in the mark-up image.
  - **Intensity (3, 3)** – Intensity of Color 3 in areas consisting of only Color 3.

- **Percent (1+3) CYAN –** Percent of the analyzed area that contains Color 1 and Color 3. Shown in cyan in the mark-up image.
  - **Intensity (1, 1+3) –** Intensity of Color 1 in areas consisting of Color 1 and Color 3.
  - **Intensity (3, 1+3) –** Intensity of Color 3 in areas consisting of Color 1 and Color 3.
- **Percent (1+2+3) BLACK –** Percent of the analyzed area that contains Color 1, Color 2, and Color 3. Shown in black in the mark-up image.
  - **Intensity (1, 1+2+3) –** Intensity of Color 1 in areas containing Color 1, Color 2, and Color 3.
  - **Intensity (2, 1+2+3) –** Intensity of Color 2 in areas containing Color 1, Color 2, and Color 3.
  - **Intensity (3, 1+2+3) –** Intensity of Color 3 in areas containing Color 1, Color 2, and Color 3.
- **Overall Intensity (1) –** Overall intensity of Color 1 in the analyzed area.
- **Overall Intensity (2) –** Overall intensity of Color 2 in the analyzed area.
- **Overall Intensity (3) –** Overall intensity of Color 3 in the analyzed area.
- **Total Stained Area (mm^2)** – Total area (in mm^2) that is stained.
- **Total Analysis Area (mm^2)** – Total area (in mm^2) that was analyzed.
- **Average Red OD** – Average optical density of Red.
- **Average Green OD** – Average optical density of Green.
- **Average Blue OD** – Average optical density of Blue.

3
Color Calibration

Calibration defines the stain color vectors so that stained cells will be correctly identified by the algorithm.

By defining the stain color vectors, you are identifying to the colocalization 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, using a separate image for each stain in which only that color is present.

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.

For instructions on installing the algorithm, opening digital slides, selecting the algorithm, registering the algorithm on Spectrum, selecting areas of the image to analyze, running the algorithm, saving algorithm parameters, and saving and exporting algorithm results, see the *Aperio Image Analysis User's Guide.*

1. Open the digital slide in ImageScope, go to the View menu and select **Analysis**.
2. Click **Select Algorithm** to select the colocalization algorithm or to create the Colocalization algorithm macro if it is not listed. (See the *Aperio Image Analysis User's Guide* for details on creating an algorithm macro.)
3. Use the ImageScope drawing tools to select the areas you want to run the algorithm on (start with representative areas that show Color 1).

	Freehand pen – Use to draw a free-form area of interest.
	Negative freehand pen – Use to draw an area to *exclude* from the analysis. Note that you can use this in combination with the other drawing tools to first select an area of interest and then exclude areas within the selected area that you do not want to analyze.
	Rectangle tool – Draws a rectangular area. If you want to select a square, hold down the Shift key while drawing.

4. On the Algorithms window, select **1 – Deconvolved Color Channel (1)** from the **Mark-up Image Type** drop-down list.
5. On the Algorithms window, select **Selected Annotation Layer** so that only the selected areas will be analyzed.
6. Click **Run**.
7. Go to the View menu and select **Annotations**.
8. On the Annotations window, go to the area of the results that shows the OD (optical density) values for Red, Green, and Blue.

Average Red OD	0.540967
Average Green OD	0.630966
Average Blue OD	0.55609

9. Type those values into the Algorithms window corresponding color component lines for Color 1.

Color (1) - Red Component	0.65
Color (1) - Green Component	0.704
Color (1) - Blue Component	0.286

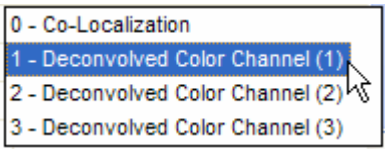
10. Now repeat these steps for the other colors used, selecting the correct deconvolution choice from the **Mark-up Image Type** drop-down list.

Thresholding

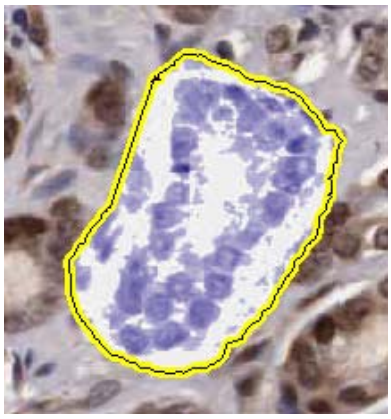
By setting upper and lower stain thresholds, you are selecting feature detection thresholds. For example, if you increase the background lower threshold, you will exclude very dark areas. Change the thresholds to pick up just the range of color you need.

For each color being used:

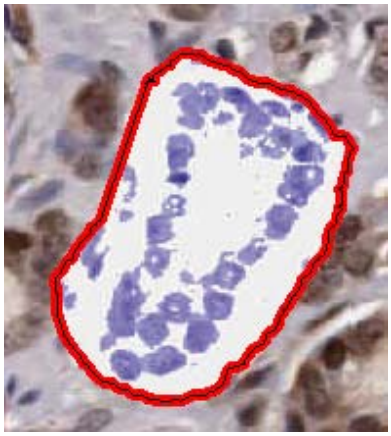
1. Select **Deconvolved Color Channel** for the color you are working with from the **Mark-up Image Type** drop-down list. In this case we select **1 – Deconvolved Color Channel (1)** because we are going to set the thresholds for Color 1:



2. Now set the thresholds for that color to maximize feature detection. For example, in the sample below we want to quantify cytoplasm cells separately from nuclei. Nuclei are stained with Hematoxylin, while nuclei *and* cytoplasm are stained with DAB. Running with **1 – Deconvolved Color Channel (1)** selected and the default, calibrated color vectors results in a mark-up image like this:



3. To maximize detection of nuclei, change the upper threshold for Color 1 to a lower number (0 = darkest; 255 = lightest), say from 200 to 180. Now more nuclei and less cytoplasm are detected:



Now to minimize picking up background, set the Mark-up Image Type to **3 – Deconvolved Color Channel (3)**. (Remember, we aren't using Color 2) and try reducing the upper threshold to limit the amount of background detected.

If you are not using a color (for example, the slide has been stained only with two stains), then set the unused color thresholds to zero.

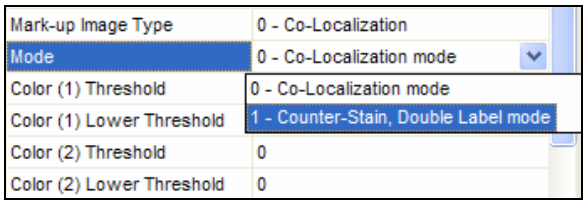
4
Colocalization Analysis

This chapter contains information on using the Colocalization algorithm, and gives some examples of its use.

After stain color vectors have been calibrated and staining thresholds set (see the previous chapter), you can run the algorithm in analysis mode to determine the percentages and intensities of stains that occur alone in the image and in combination with other stains.

To analyze colocalization of stains:

1. Use the ImageScope drawing tools to select the areas of the digital slide you want to analyze.
2. Go to the ImageScope View menu and select **Analysis** to open the Algorithm window.
3. If the Colocalization algorithm does not appear in the Algorithms window, click **Select Algorithm** and select it. If the algorithm does not appear on the Select Algorithm window, you will need to create an algorithm macro. (See the *Aperio Image Analysis User's Guide* for details on creating an algorithm macro.)
4. Once the Colocalization algorithm appears in the algorithms window, from the Colocalization parameters select **0 – Co-Localization** in the **Mark-up Image Type** drop-down list and select the analysis mode you want to use from the **Mode** drop-down list.



5. Select **Selected Annotation Layer** to analyze only the selected areas of the image.
6. Select the **Generate Markup Image** check box.
7. Click **Run**.

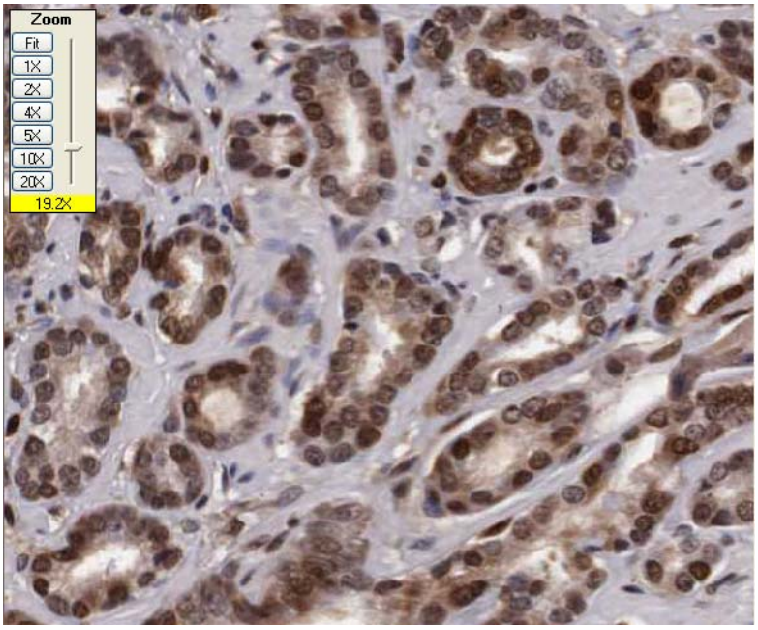
To view the numerical results of the analysis, go to the View menu and select **Annotations**. See Chapter 2, "Quick Reference" on page 5 for information on algorithm inputs and results.

Cytoplasmic Analysis Example

In the following cytoplasmic example, Hematoxylin was used as the counter-stain with DAB as the cytoplasmic stain.

The objective was to measure only the cytoplasmic component of the DAB staining. Since the DAB stains both cytoplasm and nuclei, this is a difficult task for most algorithms.

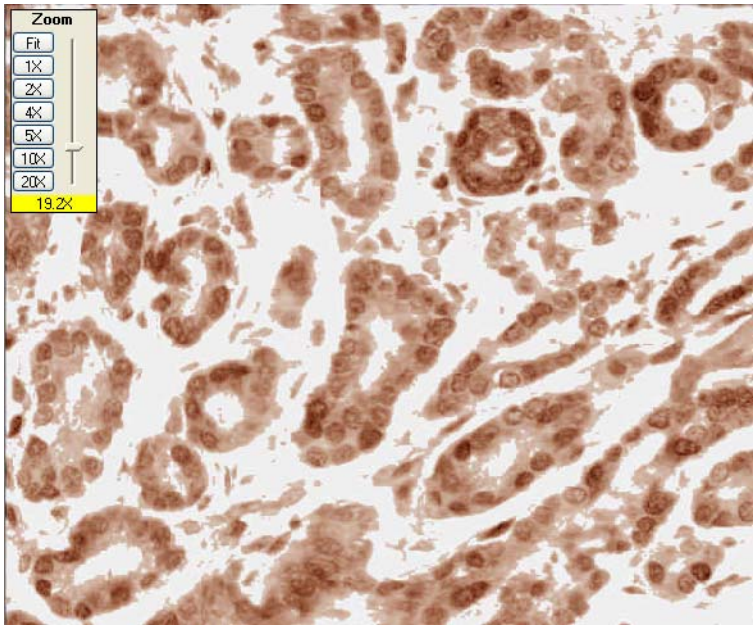
The original image of the digital slide looks like this in the ImageScope main window:



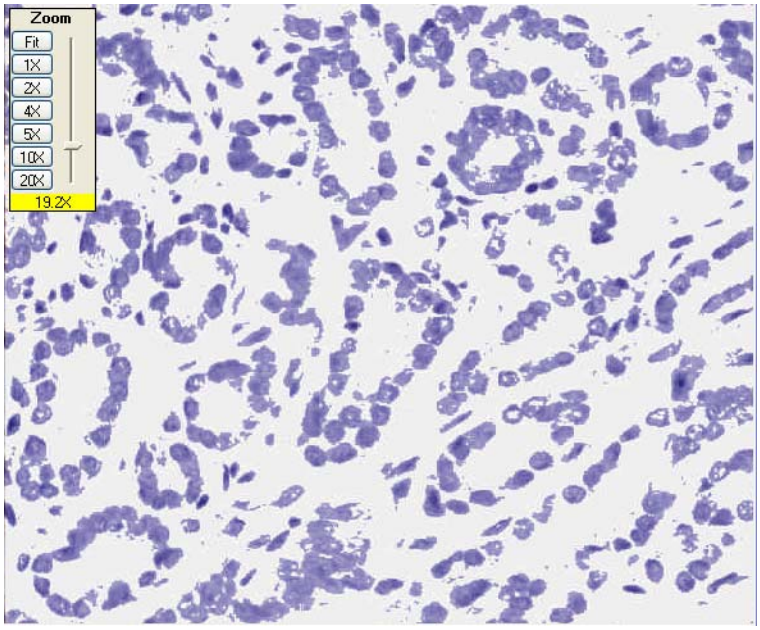
Colocalization separates the two stains and the cytoplasmic component is identified as the area where DAB only is present without Hematoxylin staining—this is the green component in the mark-up image.

The algorithm reports the percentage of the area that is comprised of cytoplasm along with the intensity of the cytoplasm staining.

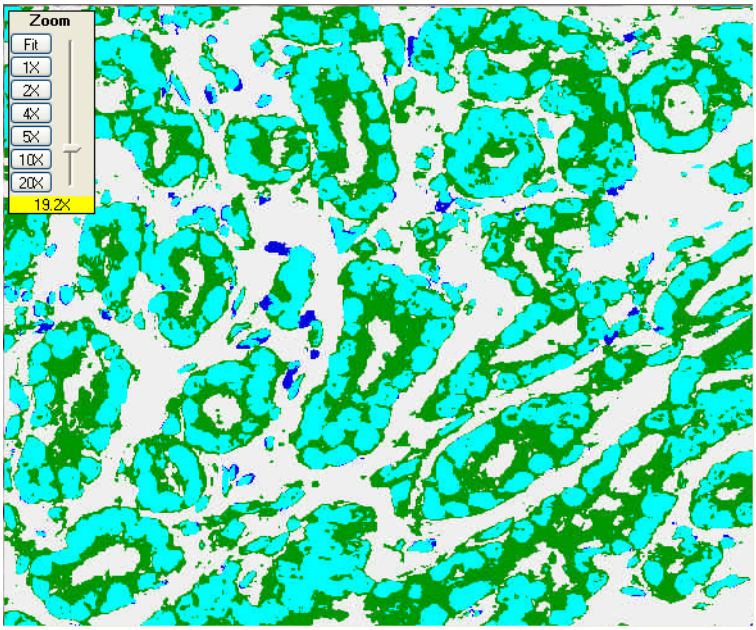
Using the Color 3 deconvolution selection in the **Mark-up Image Type** drop-down list results in a mark-up image of just the DAB stain (nuclei and cytoplasm):



Using the Color 1 deconvolution selection results in a mark-up image of just the Hematoxylin stain (nuclei):



And running the algorithm in **0 – Colocalization** analysis mode results in a mark-up image that shows all the combinations of stains present:



Nuclei – shown in cyan; Cytoplasm–shown in green.

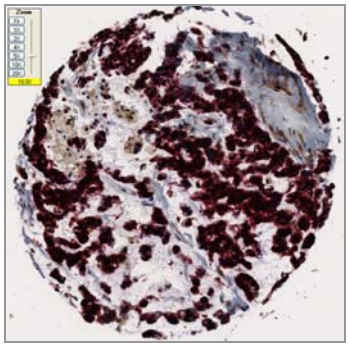
Double Labeling Example

Double-label immunohistochemistry analysis is a special case of the more general colocalization analysis. In the case of double-label analysis, Color 1 represents the counterstain, for which you want information only for where Color 1 occurs by itself, not where it occurs in combination with Color 2 and Color 3. Colors 2 and 3 are used to identify specific protein markers.

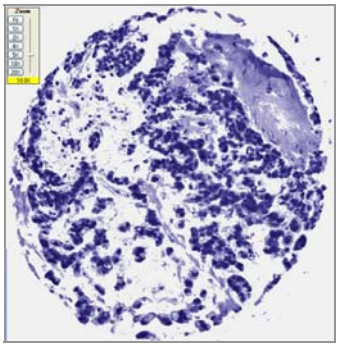
In the following double-labeling example, Fast Red and DAB were used as marker stains with Hematoxylin as the counter-stain.

The Colocalization algorithm separated the stains and reported the percentage of the stained area in which the stains occur separately (Color 2), (Color 3), and together (Colors 2+3). These three states are shown as different colors in the mark-up image.

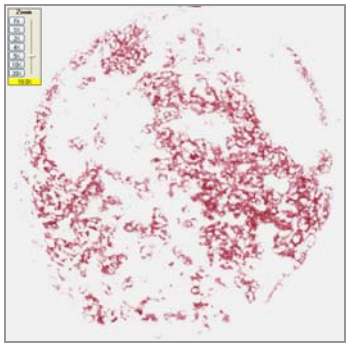
The intensity of each stain was also reported for each of the three states. The intensity information provides a measure of the protein concentration, with darker intensity corresponding to more protein.



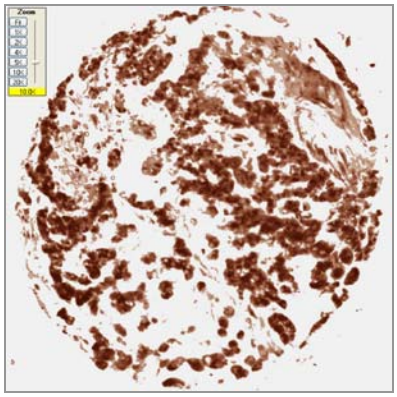
Double-labeled TMA



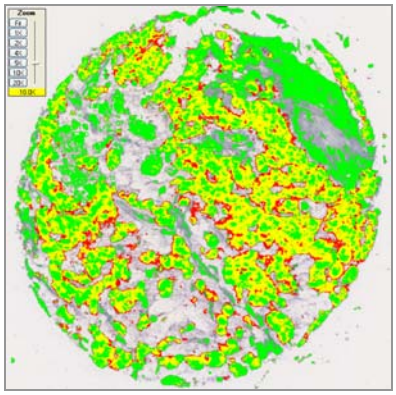
Color 1 – Hematoxylin



Color 2 – Fast Red



Color 3 – DAB



Mark-up Image

Marker	Color	Percent Area
Color 2	Red	10.1
Color 3	Green	50.8
Colors 2+3	Yellow	39.1

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