Positive Pixel Count Algorithm

User's Guide

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User Resources

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Disclaimers

Use normal care in maintaining and using the Spectrum servers. Interrupting network connections or turning off the Spectrum and DSR servers while they are processing data (such as when they are analyzing digital slides or generating an audit report) can result in data loss.

This manual is not a substitute for the detailed operator training provided by Aperio Technologies, Inc., or for other advanced instruction. Aperio Technologies 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 Aperio Technologies Service Engineer.

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. For clearance updates, visit www.aperio.com

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Contact Information

Headquarters	Europe Office	Asia Office
Aperio Technologies, Inc.	Aperio UK Ltd	Aperio Technologies, KK
1360 Park Center Drive	The Vineyard	UZ Building 5F
Vista, CA 92081	Axbridge	3-3-17, Surugadai
United States	Somerset BS26 2AN UK	Kanda, Chiyoda-ku
		Tokyo, Japan 101-0062

United States of America	
	Tel: 866-478-4111 (toll free)
	Fax: 760-539-1116
Customer Service	Tel: 866-478-4111 (toll free)
Technical Support	Tel: 866-478-3999 (toll free)
Email	Email: support@aperio.com
Europe	
	Tel: +44 (0) 1934 733679
	Fax: +44(0) 1934 733660
Customer Service & Technical Support	Tel: +44 (0) 1934 733679
Email	Email: europesupport@aperio.com
Asia	
	Tel: +81-3-3259-5255
	Fax: +81-3-3259-5256
Customer Service	Email: asiainfo@aperio.com
Technical Support	Email: asiasupport@aperio.com

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Introduction

This chapter introduces you to the Aperio Positive Pixel Count Algorithm. For general information on using any 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 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.

The Positive Pixel Count Algorithm

The Positive Pixel Count algorithm can be used to quantify the amount of a specific stain present in a scanned slide image. You will specify a color (range of hues and saturation) and three intensity ranges (weak, positive, and strong). For pixels which satisfy the color specification, the algorithm counts the number and intensity-sum in each intensity range, along with three additional quantities:

average intensity, ratio of strong/total number, and average intensity of weak+positive pixels.

The algorithm has a set of default input parameters when first selected—these inputs have been pre-configured for Brown color quantification in the three intensity ranges (220-175, 175-100, and 100-0). Pixels which are stained, but do not fall into the positive-color specification, are considered negative stained pixels—these pixels are counted as well, so that the fraction of positive to total stained pixels is determined.

The algorithm is applied to an image by using ImageScope, Spectrum or TMALab. These programs allow you to select an image Region of Analysis (set of spots in TMALab), specify the input parameters, run the algorithm, and view/save the algorithm results. When using the ImageScope program, a pseudo-color markup image is also shown as an algorithm result. The markup image allows the user to confirm that specified inputs are measuring the desired color and intensity ranges. Once a set of algorithm inputs has been confirmed, the settings can be saved in a macro file for subsequent repeated use.

To use the Positive Pixel Count algorithm, you need to install ImageScope (free download)—the Positive Pixel Count algorithm will be installed as part of the ImageScope installation. Running the algorithm in TMALab is not covered in this document.

Prerequisites

The Positive Pixel Count algorithm requires that you be using Aperio Release 10 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 positive pixel count 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, "Positive Pixel Count Analysis" 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 positive pixel count algorithm inputs and outputs. See the following chapter for details on using the algorithm.

Algorithm Input Parameters

The following inputs are accepted by the Positive Pixel Count algorithm:

- **View Width –** Width of processing box.

- **View Height –** Height of processing box.

- **Overlap Size –** Size of the overlap region for each view. The image is processed in blocks (views) and overlap is provided to ensure that regions are processed only once

- **Image Zoom –** 1.0 (recommended) for processing of all pixels. Can be reduced to 0.5 for faster processing; however, the results may not be as accurate.

- **Markup Compression Type –** You can select among "Same as processed image," JPEG, or JPEG2000 for the markup image.

- **Compression Quality –** For the compressed markup image, you can select a compression quality of 0 to 95. Higher quality takes longer and yields larger files.

- **Classifier Definition List –** If you are using this algorithm in conjunction with the Genie image analysis program, you can select Genie classifier definition lists here. See the Genie user guide for details.

- **Class List –** If you are using this algorithm in conjunction with the Genie image analysis program, you can select Genie class lists here. See the Genie user guide for details.

- **Hue Value –** This is hue position on the color circle for Positive color, ranging from 0 to 1. It can take on values between 0.0 and 1.0. Red = 0.0, Green = 0.33, Blue = 0.66, Brown = 0.1. For more information on determining this value based on the color you want to detect, see "Color Concepts" on page 9.

- **Hue Width –** This value selects the range of hues, centered on the Hue Value, that will satisfy the hue detection process. By increasing this number, you specify that a larger range of hues will be accepted for

specifying the Positive color band. By decreasing this number, you "tighten" the range of hues that will be acceptable. The number can range between zero and 1, where zero is a narrow hue width and 1 selects the entire range of hues. A value between 0.33 and 0.5 is usually reasonable.

■ **Color Saturation Threshold –** This is the required saturation of the Positive color. RGB values are represented as gray + color. The value can be between 0.0 and 1.0, with 1.0 corresponding to no gray component (fully saturated). Pixels with saturation less than this value are not reported.

■ **Iwp (High) –** Upper limit of intensity for weak-positive pixels. Iwp is also used as an intensity threshold for negative stained pixels–pixels which do not meet the hue/saturation limits, but have an intensity less than Iwp, are counted as negative pixels.

■ **Iwp (Low) = Ip (High) –** Lower limit of intensity for weak-positive pixels, upper limit of intensity for positive pixels.

■ **Ip (Low) = Isp (High) –** Lower limit of intensity for positive pixels, upper limit of intensity for strong-positive pixels.

■ **Isp (Low) –** Lower limit of intensity for strong-positive pixels.

■ **Intensity = (R+G+B)/3**

The intensity limits establish three intensity ranges for classifying and summing pixel values. The greater the intensity value, the brighter the pixel. For more information on intensity ranges, see "Intensity" on page 10.

Weak-Positive Intensity:	Iwp (High) <= Intensity < Iwp (Low)
Positive Intensity:	Ip (High) <= Intensity < Ip (Low)
Strong-Positive Intensity:	Isp (High) <= Intensity < Isp (Low)

Algorithm Results

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

The algorithm calculates the following quantities for each region of analysis, as well as the sum of all regions, for each layer that is analyzed. Results are stored in an annotation layer attached to the image and can be viewed in ImageScope.

■ **Nwp –** Number of Weak-Positive pixels (Yellow in mark-up image)

■ **Np –** Number of Positive pixels (Orange in mark-up image)

■ **Nsp –** Number of Strong-Positive pixels (Red in mark-up image)

■ **Iwp –** Sum of Intensity values for all Weak-Positive pixels

■ **Ip –** Sum of Intensity values for all Positive pixels

- **Isp** – Sum of Intensity values for all Strong-Positive pixels

- **Iavg** – Average Intensity of all pixels: Iavg = (Iwp+Ip+Isp)/(Nwp+Np+Nsp)

- **Nsr** – Ratio of Strong-Positive pixels to total pixels: Nsr = Nsp/(Nwp+Np+Nsp)

- **Iwavg** – Average Intensity excluding Strong-Positive pixels: (Iwp+Ip)/(Nwp+Np)

- **Nn** – Number of Negative pixels (Blue in mark-up image)

- **In** – Sum of Intensity values for all Negative pixels

- **NTotal** – Number of Total pixels, Positive+Negative (Nwp+Np+Nsp+Nn)

- **Positivity** – Total number of positive pixels divided by total number of pixels: (NTotal – Nn)/(NTotal)

- **ATotal** – Total area in square millimeters of all pixels counted in the NTotal result.

If the results of the analysis are not what you expect or are otherwise unsatisfactory, see the appendix "Troubleshooting" in the *Aperio Image Analysis User's Guide* for some tips on identifying problems.

3
Positive Pixel Count Analysis

Although you can use several different tools to perform an algorithm analysis, this section shows a simple analysis using ImageScope to analyze digital slide that resides in the Spectrum digital slide information system.
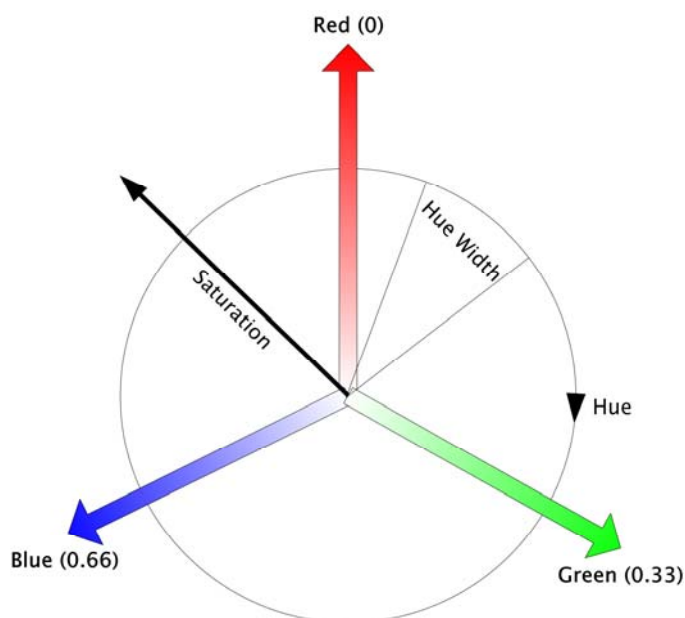
Note that the Color Deconvolution algorithm is our professional version of the Positive Pixel Count that allows automatic and precise training of stain colors, eliminating trial and error, and accurate stain separation, resolving the multi-stain colocalization problem.

Color Concepts

The Positive Pixel Count algorithm detects pixels that match the input parameters set for the algorithm. An important group of these parameters relate to color.

This section discusses some of the concepts behind these parameters.

You are probably familiar with the common artist color wheel. The Rare Event Detection algorithm uses a form of it called the HSI (Hue, Saturation, Intensity) wheel that quantifies the RGB (red, blue, green) color space:

In the example above, imagine every color residing on this wheel, with the color red being assigned the value zero. The actual color is called the *hue*. As you move around the rim of the circle, you move from one hue to another. Each hue has a numeric representation on this wheel. Green is 0.33 (as it is a third of the way around the circle from red, which is 0.00), and Blue is 0.66 (two thirds of the way around the circle). Each hue on the circle has a number assigned to it. Brown, which is almost halfway between Red and Green, has a value of 0.1.

The *Hue Value* parameter used by this algorithm is the number associated with the hue you want to use based on its position on the wheel.

Saturation represents the "purity" of the color, with the rim of the wheel representing complete saturation. For example, fully saturated Red is the color on the rim of the wheel, a less saturated Red (for example, Pink) resides on the red vector, but closer to the center of the wheel.

Hue Width is the wedge on the wheel that represents all hues that will satisfy pixel detection based on the Hue Value. The smaller the Hue Width, the more restrictive is the definition of the hues that are acceptable. For example, if you want pixels to be detected **only** if they are precisely Brown, then you might specify a Hue Width of zero. If slightly reddish brown to slightly greenish brown are all acceptable to identify a pixel, then you might specify a Hue Width of .5. You might think of this as a hue threshold value.

Intensity

Another value that can be used to detect a pixel is the *Intensity*. Not represented on the wheel above, intensity is the measure of brightness of the pixel and is the average of R+G+B values of the pixel.

Intensity ranges from zero (black) to 255 (bright white), so that a large intensity value means that the pixel is brighter. Intensity is the opposite of density. Intensity is proportional to the amount of light **transmitted** through the slide, while density is proportional to the amount o f light that is **blocked** by the stained tissue.

The input parameters allow defining the positive stain color and the weak-positive, positive and strong-positive intensity thresholds for the positive stain using the HSI color model.

The Positive Pixel Count algorithm allows you to specify three ranges of intensity:

■ **Iwp** – Weak-positive intensity.

A high Iwp value is the upper limit of intensity for weak-positive pixels. Pixels which do not meet the hue/saturation limits, but have an intensity less than Iwp, are counted as negative pixels.

A low Iwp value is the lower limit of intensity for weak-positive pixels and the upper limit of intensity for positive pixels—Iwp(Low) = Ip(High).

■ **Ip** – Positive intensity.

Ip is the lower limit of intensity for positive pixels. It is the upper limit of intensity for strong-positive pixels—Ip(Low) = Isp(High).

■ **Isp** – Strong-positive intensity. This is the lower limit of intensity for strong-positive pixels.
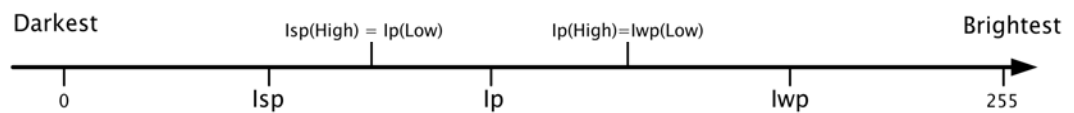
Running an Analysis

1. Open a digital slide in ImageScope. If you are using the Spectrum digital slide information system, one way to do this is to go to the main Spectrum page and select **List All Digital Slides** to see the list of digital slides, and then click on a digital slide thumbnail to open it in ImageScope.

(For information opening and analyzing local digital slides that reside on your workstation or local area network, see the *ImageScope User's Guide*. For information on accessing digital slides in other ways in Spectrum, see the *Spectrum/Spectrum Plus Operator's Guide*.)

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:
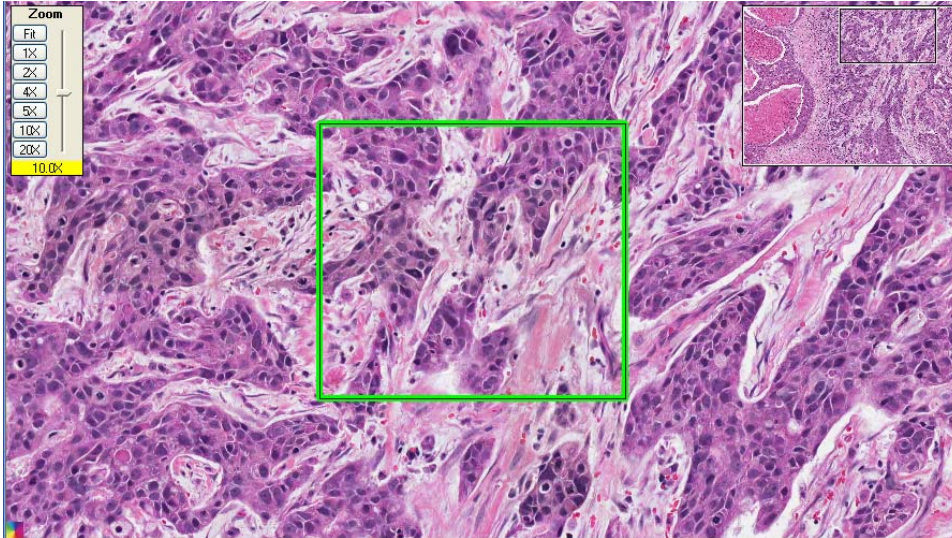
Note that you can also use the ♟ negative free hand pen to *exclude* areas from analysis. In the example below, the dotted line shows the area drawn by the negative pen:
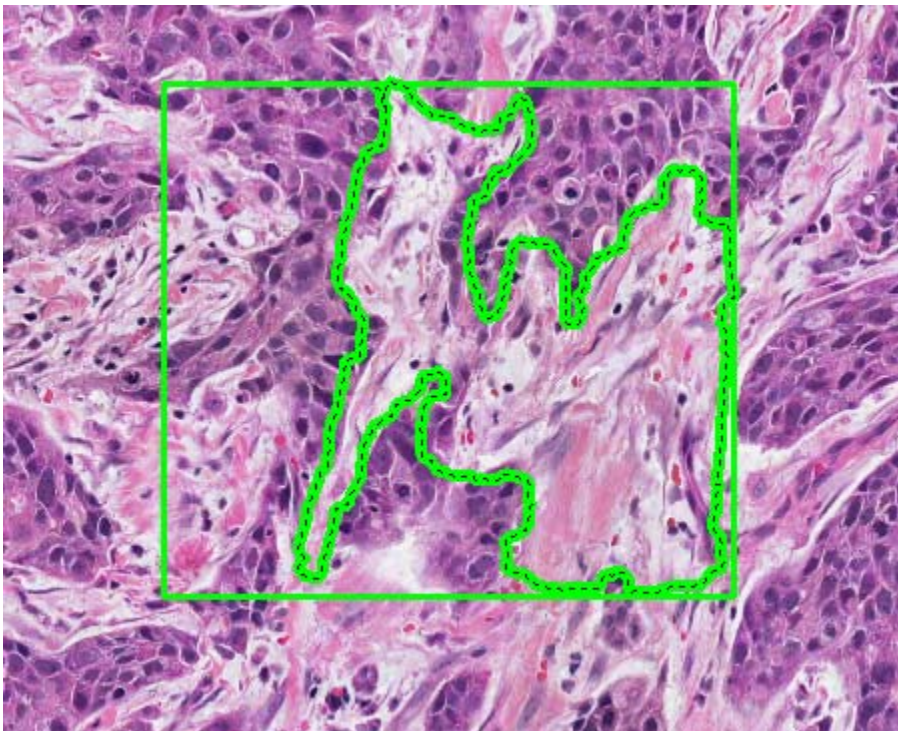
3. Go to the ImageScope **View** menu and select **Analysis**. The Server Job window appears:
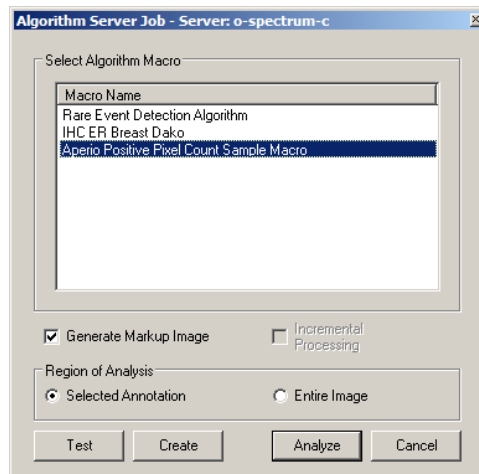
If an algorithm macro does not already exist for the Positive Pixel Count algorithm, you or the Spectrum administrator will need to create one—see the *Aperio Image Analysis User's Guide* for details on doing so.

4. Click **Test** to fine-tune the algorithm input parameters (if you know they are already correct for your application, you can just click **Analyze** to run the algorithm).

5. After clicking **Test**, the Algorithm window appears:
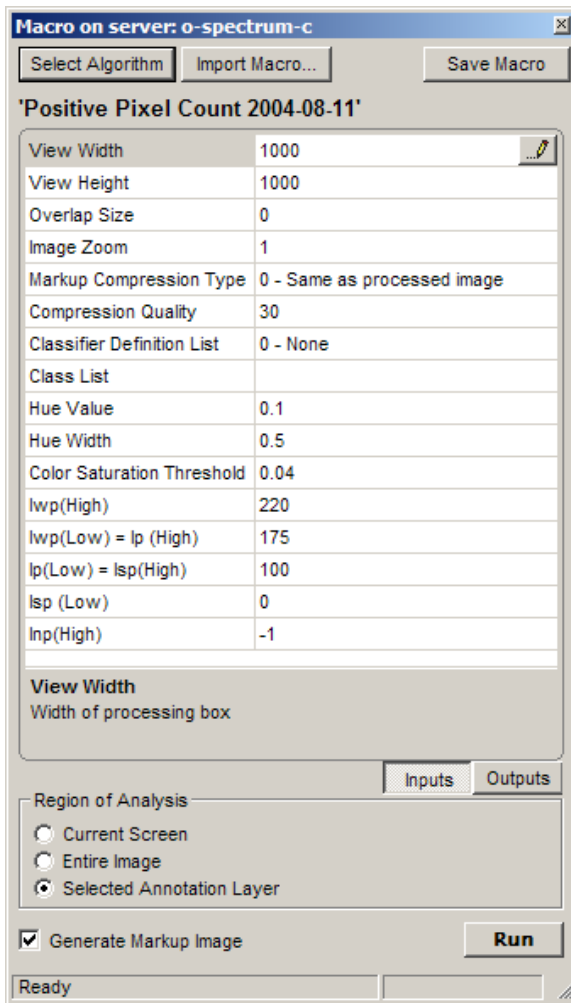
6. Click **Inputs** and adjust the algorithm parameters in the Algorithms window as discussed earlier in this document by clicking on a number and typing a new value or using the slider if one is provided for that parameter.

7. Click **Outputs** and select which results will display in Spectrum by clearing the check boxes next to the results you don't want to display.
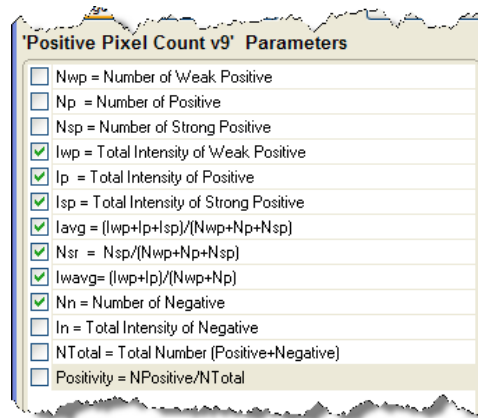
8. If you want to see a visual representation of the analysis as well as a quantitative one, select **Generate Markup Image** on the Algorithms window.

9. To analyze just the annotated areas, select **Selected Annotation Layer** under the Region of Analysis section of the Algorithms window.

10. Click **Run**. Because we requested a mark-up image, the ImageScope main window shows the results of the analysis:
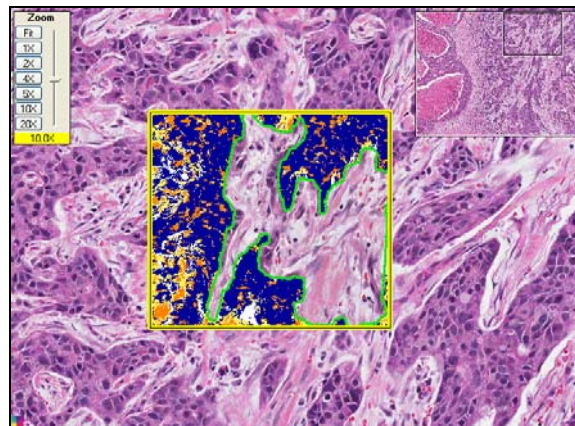
11. To see the quantitative results, go to the ImageScope View menu and select **Annotations**. The summary results are shown on the left and measurements for individual regions are shown on the right:

The colors shown next to the results correspond to the colors in the mark-up image (see "Algorithm Results" on page 6).

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