An Instructional Guide for

Computer-based Leaf Color Analysis

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Abstract

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Changes in foliar color have long been used as a valuable indicator of plant nutrition and health. Leaf color is commonly measured with visual scales and inexpensive plant color guides that are easy to use, but not quantitatively rigorous, or by employing sophisticated instrumentation including chlorophyll meters, reflectometers and spectrophotometers that are costly and may require special training. In contrast to these traditional methods, digital color analysis has become an increasingly popular and cost-effective method utilized by resource managers and scientists for evaluating foliar nutrition and health in response to environmental stresses. Working with colorful autumn samples of sugar maple (*Acer saccharum* Marsh.) leaves, we developed and tested a new method of digital image analysis that uses Scion Image or NIH image public domain software to quantify leaf color. This publication provides step-by-step instructions for using this software to measure the percent green and red in leaves, colors of particular importance for the assessment of plant health. Comparisons of results from digital analyses of 326 scanned images of leaves and concurrent spectrophotometric measures of chlorophyll *a*, chlorophyll *b* and anthocyanins verify that image analysis provides a reliable quantitative measure of leaf color and the relative concentrations of underlying plant pigments.

Keywords: chlorophyll, anthocyanin, leaf color, digital image, image analysis, Scion Image, NIH Image

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Comparison of Percent Color versus Pigment Concentration

Introduction

Foliar color has always been of great interest and value to resource managers and scientists as a visual indicator of plant health. Prior to the use of digital cameras, health assessments have often relied upon simple, visual scales to determine foliar color (Townsend and McIntosh 1993, Strimbeck 1997). Because this approach is somewhat subjective, charts depicting gradients of leaf color, including the Munsell Plant Tissue Color Chart (Sibley et al. 1995, Innes et al. 1996), the Globe Plant Color Guide and the Leaf Color Chart have been developed to help standardize color. These affordable guides are readily available for purchase and are commonly used to estimate foliar nitrogen status and evaluate seasonal changes in leaf color. Other more precise methods of foliar color analysis include those that measure chlorophyll content and spectral properties of leaves (i.e. absorbance- and reflectance-based measurements). Chlorophyll meters, spectrophotometers and reflectometers are commonly used throughout the scientific community to evaluate plant physiological processes such as leaf development (Woodall et al. 1998), leaf senescence (Lee et al. 2003, Feild et al. 2001, Merzlyak and Gitelson 1995, Boyer et al. 1988, Ishikura 1973), foliar nutrient status (Buscaglia and Varco 2002, Lopez-Cantarero et al. 1994, Lawanson et al. 1972) and fruit maturation (Reay et al. 1998). Pigment change due to environmental stresses such as high light (Merzlyak and Chivkunova 2000), ultraviolet exposure (Dixon et al. 2001, Klaper et al. 1996, Burger and Edwards 1996), water stress (Ommen et al. 1999) and low temperature (Pietrini et al. 2002, Krol et al. 1995) can be measured using this sophisticated instrumentation. Advantages of this technology include greater accuracy of measurements and, in some cases, portability of equipment for field applications. However, this analysis can also be expensive and may require access to and training with specialized equipment, potentially hazardous chemicals, and other laboratory supplies.

Recently, utilization of digital imagery has become a new trend in plant color analysis. Digital cameras or scanners in combination with computers and appropriate software can be used to photograph, scan and evaluate leaves for color with relative ease and at an affordable cost. In agriculture, digital technology has been used to characterize color in apples (Schrevens and

Raeymaeckers 1992), distinguish weeds from crops (Perez et al. 2000, Woebbecke et al. 1995, Zangh and Chaisattapagon 1995), identify storage-associated color change in chickory (Zhang et al. 2003) and apple (Vervaeke et al. 1993) and to evaluate senescence rates in spring wheat (Adamsen et al. 1999). An important application of digital image and color analysis has been its use in evaluating the role of environmental stress on foliar health. Examples include, water and nitrogen stress (Ahmed and Reid 1996), low temperature (Bacci et al. 1998) and disease such as coffee leaf rust (Price et al. 1993), maize streak virus (Martin and Rybicki 1998) and powdery mildew infection of sweet cherry (Olmstead et al. 2001) and cucumber (Kampmann and Hansen 1994).

Research conducted by U.S.D.A. Forest Service and University of Vermont scientists has expedited the use of digital image analysis to evaluate seasonal color change in sugar maple (*Acer saccharum* Marsh.) leaves. One study in particular found that sugar maple leaves with low nitrogen turned red earlier and more completely than those with high nitrogen (Schaberg et al. 2003). This group has devised an inexpensive method using a computer, scanner and public domain software called Scion Image (Scion Corporation, www.scioncorp.com) or NIH Image (developed at the U.S. National Institute or Health and available at http://rsb.info.nih.gov/nih-image) to quantify green and red coloration in leaves. This digital image processing software is free and can be easily downloaded from the internet. Development of Scion Image for PC users followed that of NIH image for Macintosh users. Both are widely used throughout the medical, scientific and educational community.

This publication provides step-by-step instructions for the use of Scion Image software to measure the percent of green and red in leaves as an indicator of plant nutrition and health. These instructions will guide the reader through the entire process of computer-based leaf color analysis, including scanning of leaves and the acquisition and use of image analysis software. Although the illustrations presented depict specific steps using Scion Image, these same instructions are applicable to analysis via NIH image. With a little practice users will become

proficient in a method for quantifying leaf color that requires no specialized equipment or chemicals.

Acquiring Scion Image

Scion Image can be downloaded free of charge from the internet at www.scioncorp.com. Scion Image for Windows is available for Windows 95/98/ME/NT/2000/XP. A Macintosh version of Scion Image exists for MacOS 7.5 to 9.x. Macintosh users can also use a free version of NIH image which can be found at http://rsb.info.nih.gov/nih-image.

Scanning foliage

In the following example, leaves were scanned using an Epson Perfection 1200U flatbed scanner and Adobe Photoshop 5.0. A Power Macintosh G3 with OS 8.6 and a 17" Apple ColorSync Display were used to acquire, save and analyze leaf images for percent color. Other combinations of computers and scanners can also be used. Petioles were removed and leaves were placed facedown on the scanner bed. After the lid was closed, leaves were scanned as a Color Photo at a resolution of 240 dpi and a scale of 100%. Images were saved in Tag Image File Format (TIFF).

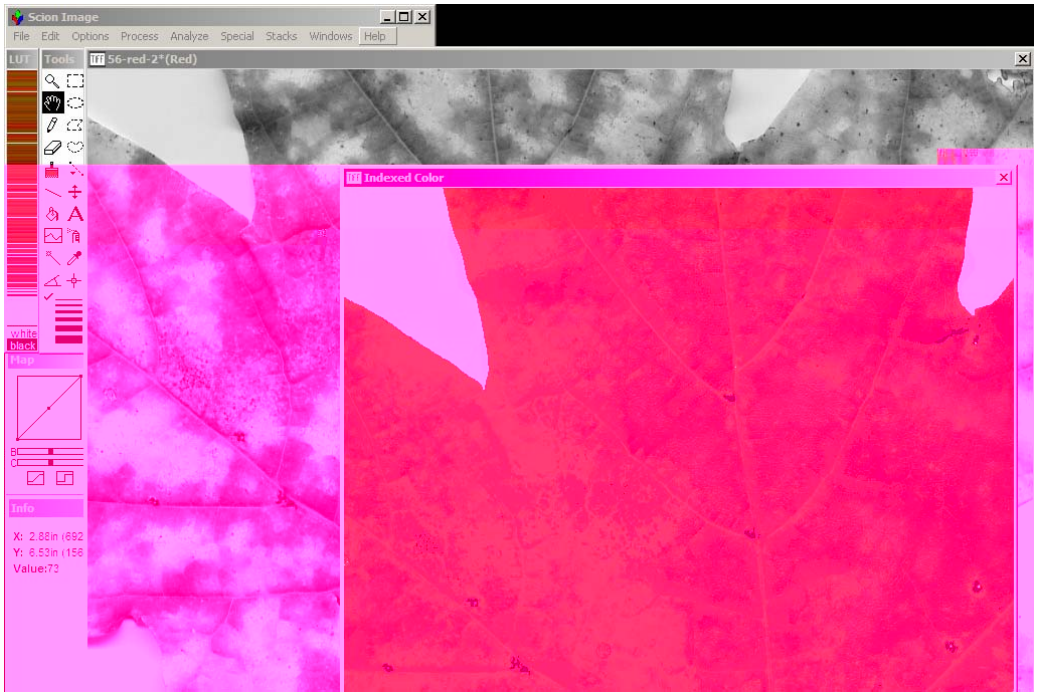
Analysis and quantification of leaf color

Getting started

1. Launch Scion Image. To open the first leaf image, choose File, then Open.

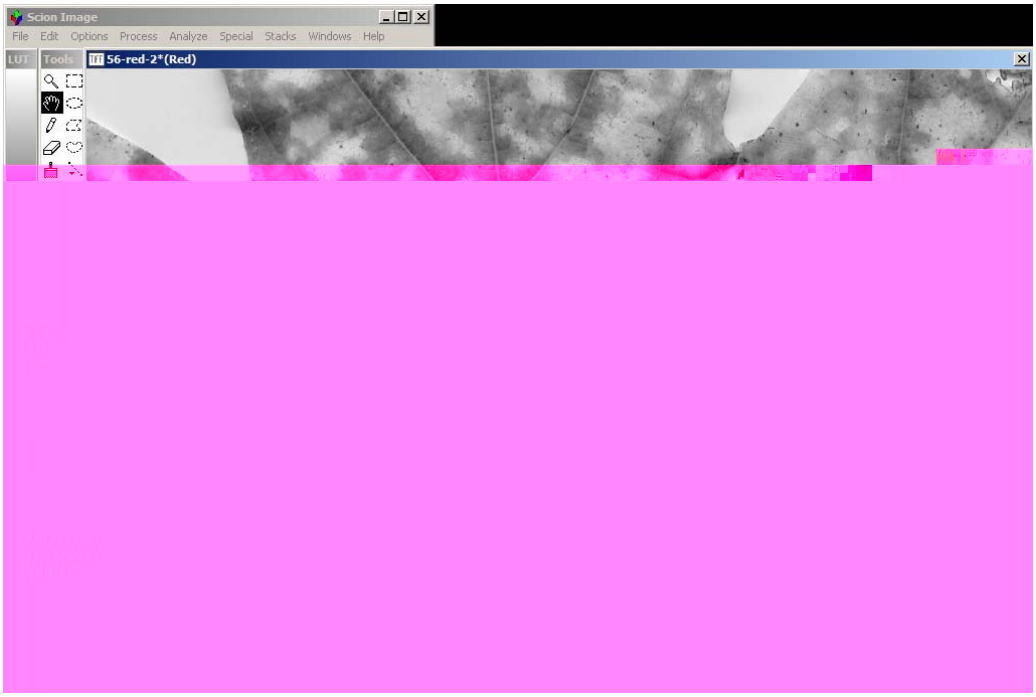
2. Select the file name for a leaf image and choose Open.

3. For each image, two windows will appear – a black and white window and an Indexed Color window. These two windows will be partially superimposed on each other.

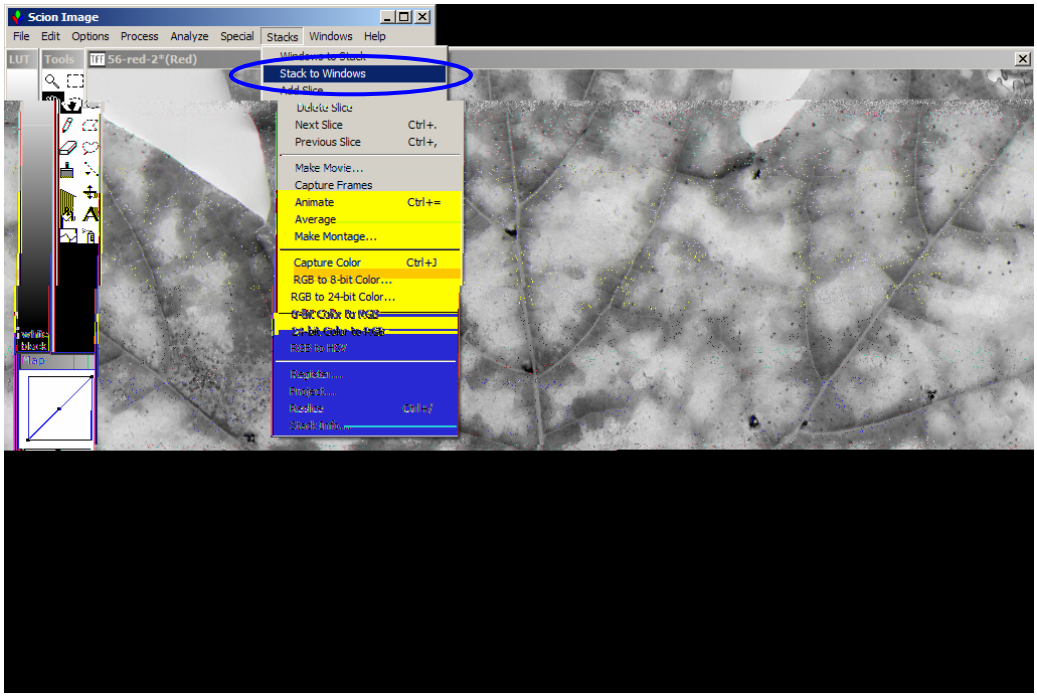


Preparing the black and white image

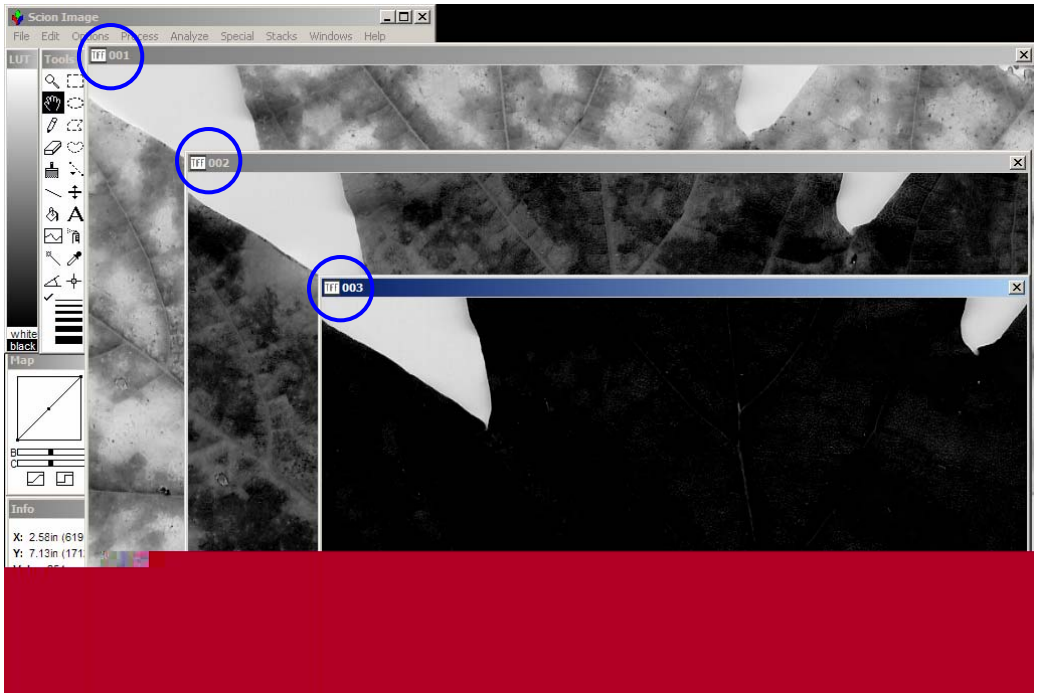
4. Selecting the black and white window.



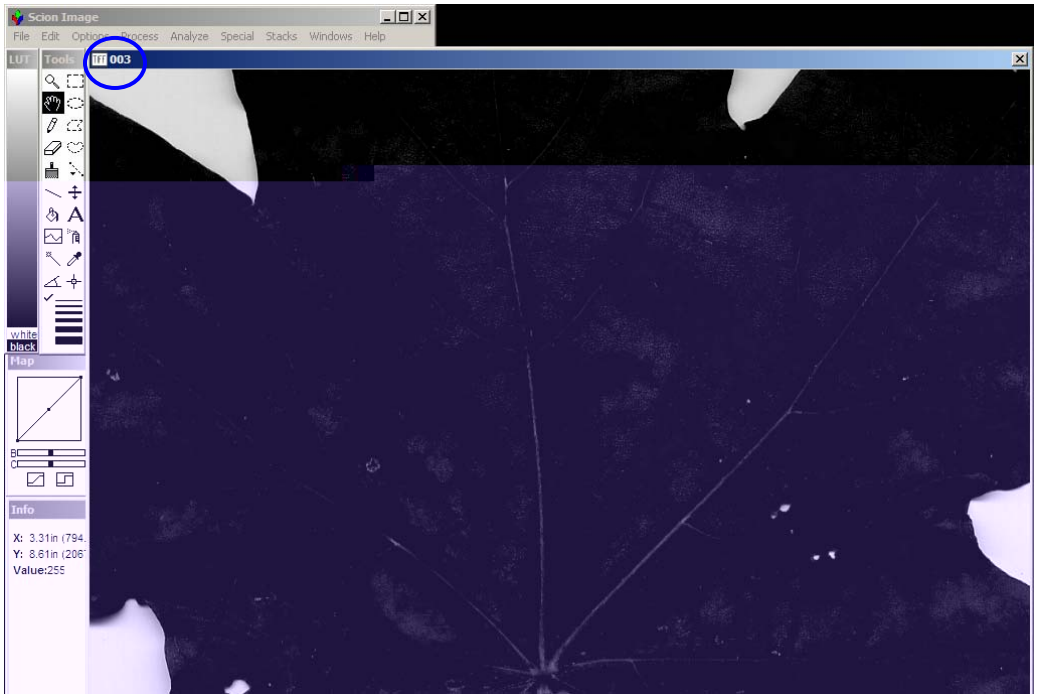
5. Go to Stacks and select Stack to Windows. Three windows will pop up displaying the leaf image in shades of black and white.



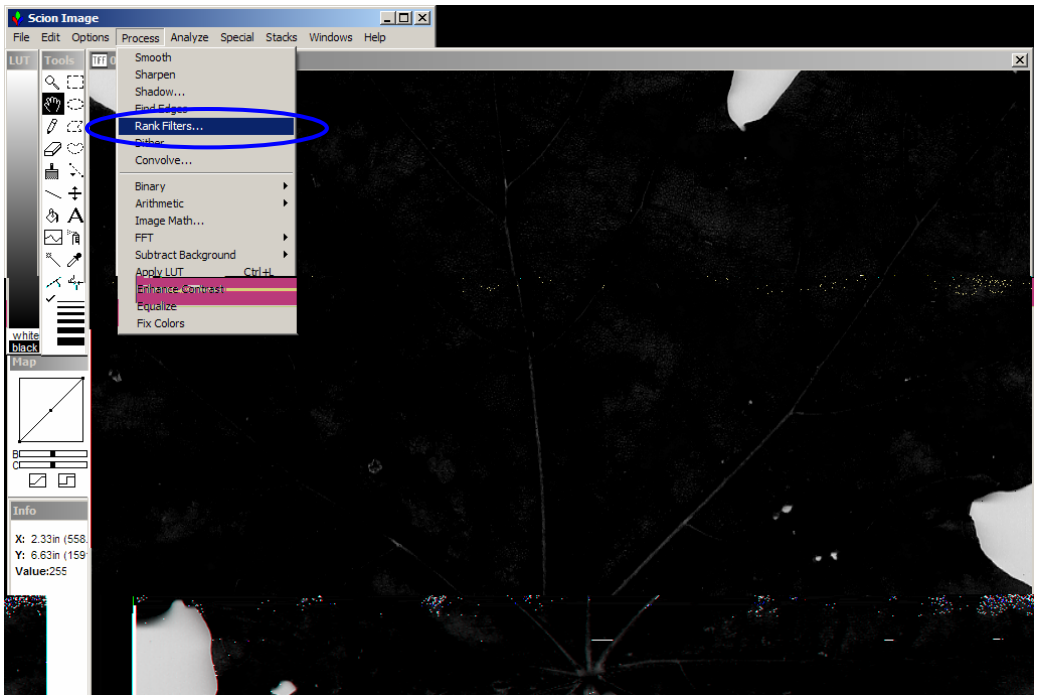
6. Three windows will appear labeled 001, 002 and 003. Window 003 will be superimposed on window 002. And window 002 will be superimposed on window 001. It is not necessary to see all three windows at the same time.



7. Go to Windows and choose 001. This will bring window 001 to the forefront. Close this window. Go to Windows again and choose 002. This will bring window 002 to the forefront. Close this window. Only window 003 will be visible now. **DO NOT** close this window.

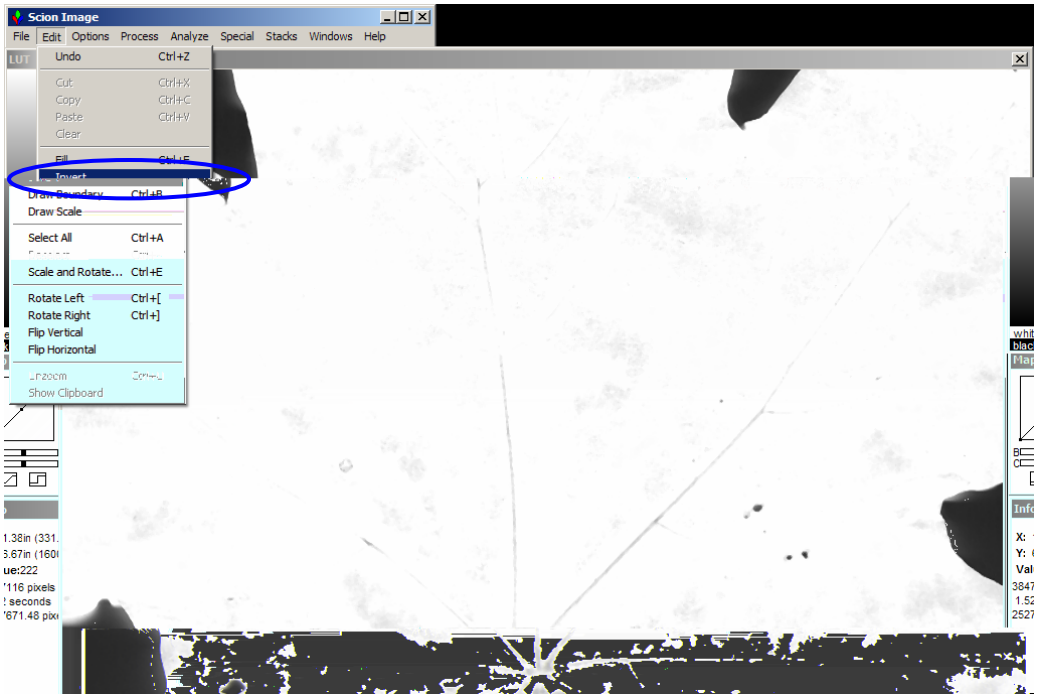


8. With window 003 selected, go to Process. Choose Rank Filters.

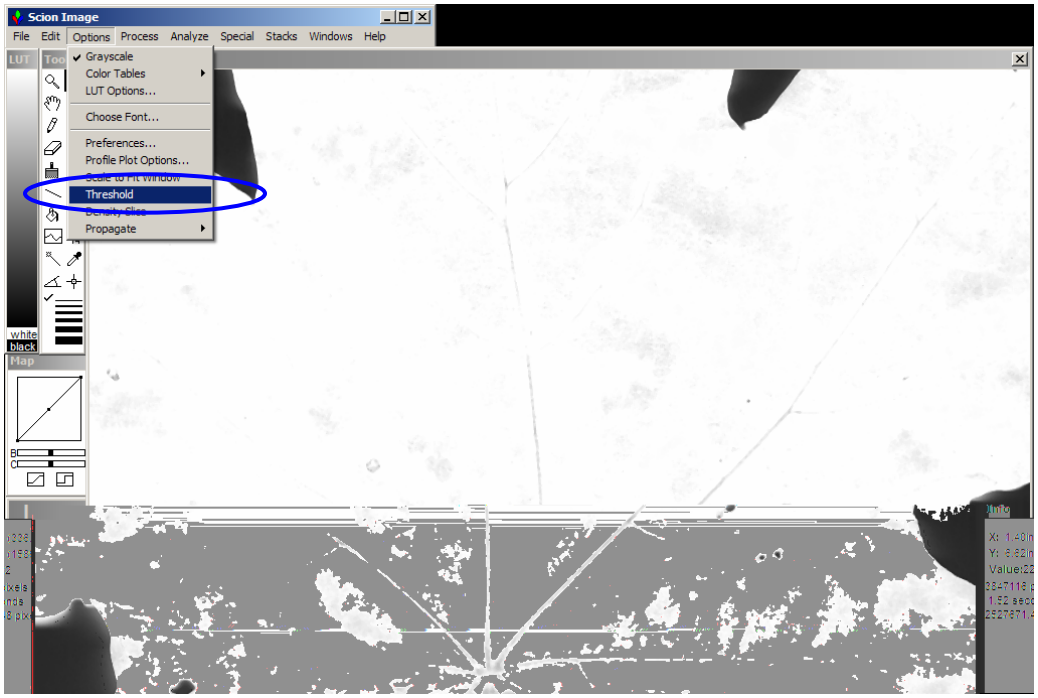


9. A box will pop up with a list of options. Median (Reduce Noise) should already be selected along with Iteration of 1. Select OK.

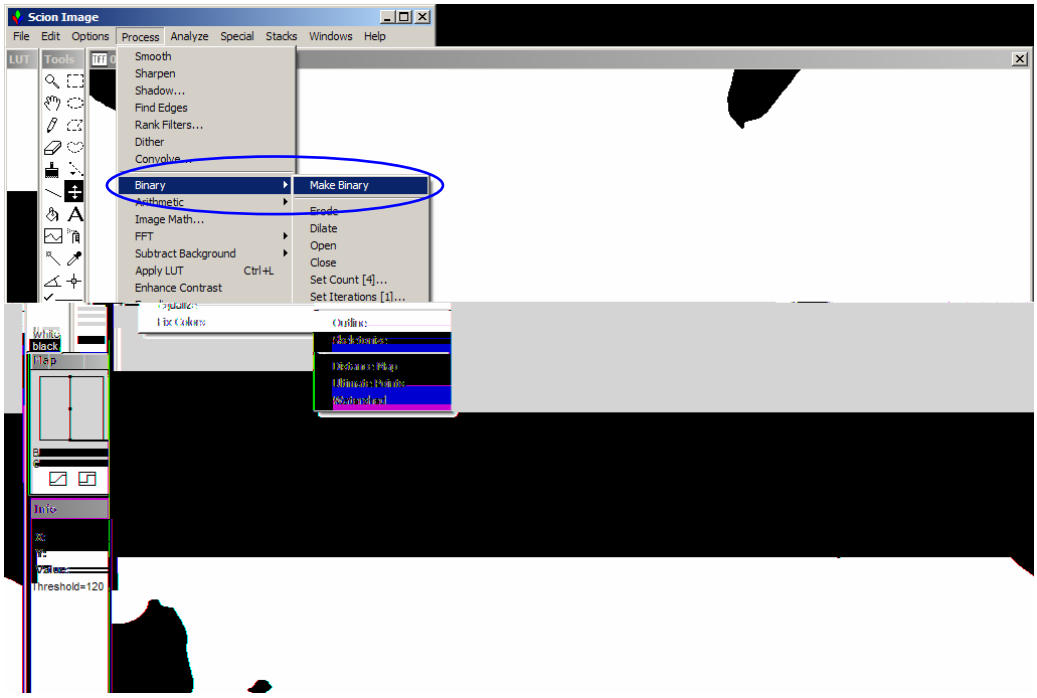
10. Select Edit and then Invert.



11. Go to Options and then Threshold.

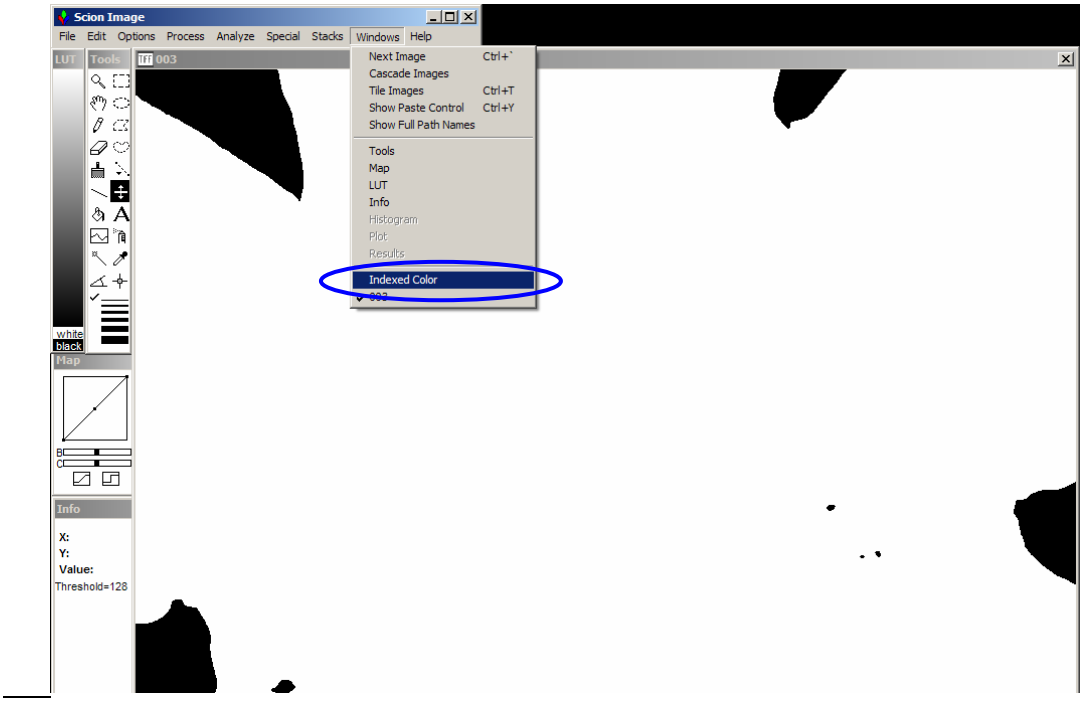


12. Go to Process and then Binary and then Make Binary. At this point, processing of the black and white image is complete. Do not close the window because it will be needed later.



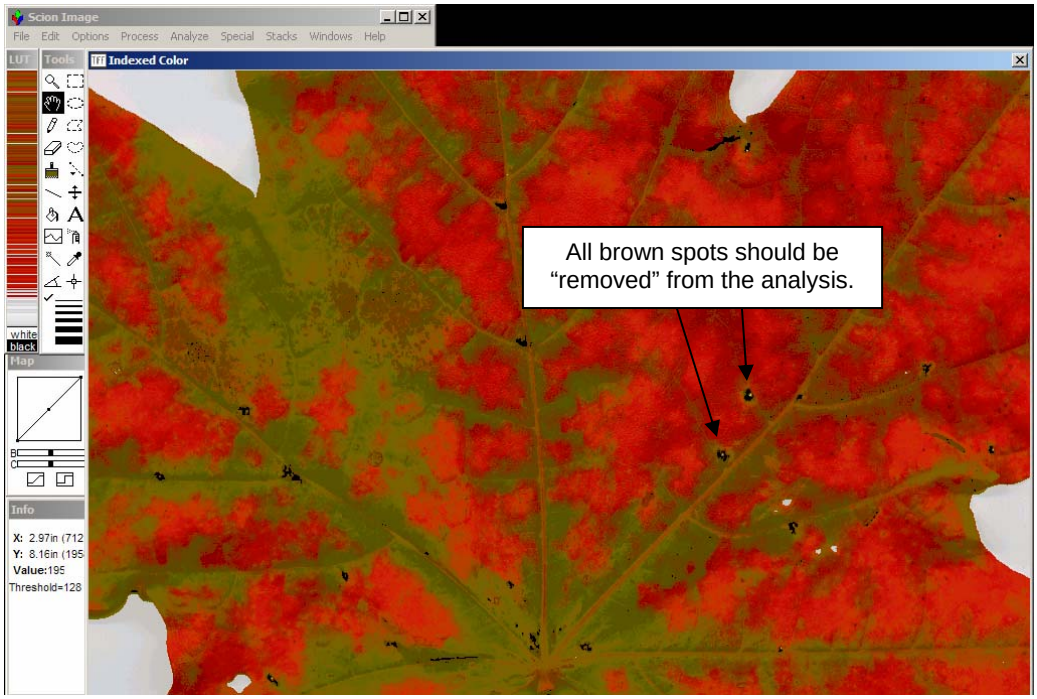
Preparing the color image

13. Select the Indexed Color window. Go to Windows and choose Indexed Color.



Removing brown areas using the paintbrush tool

14. If there are areas of brown in the leaf, they should be removed so they are not measured as red.



15. To remove brown, proceed as follows:

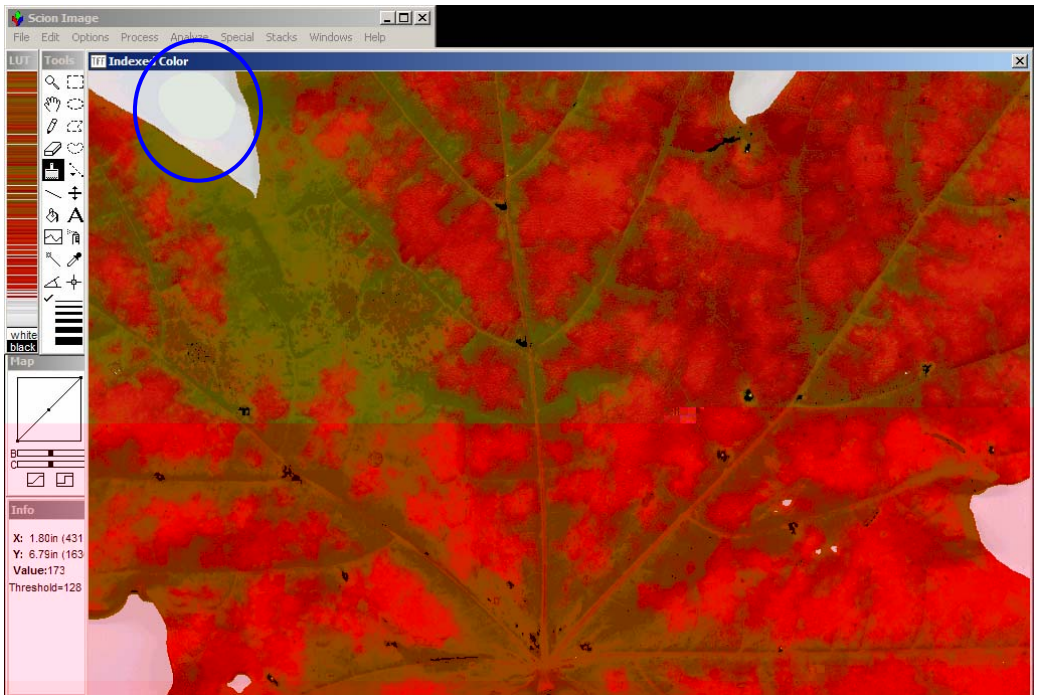
Using the paintbrush tool

a. Click on the paintbrush tool () from the Tools window found at the upper left of the screen. Move it into the LUT window which can be found to the left of the Tools column. It will turn into the eyedropper tool (). From the mosaic of colors shown, find a light grey or light blue and click on the color once.

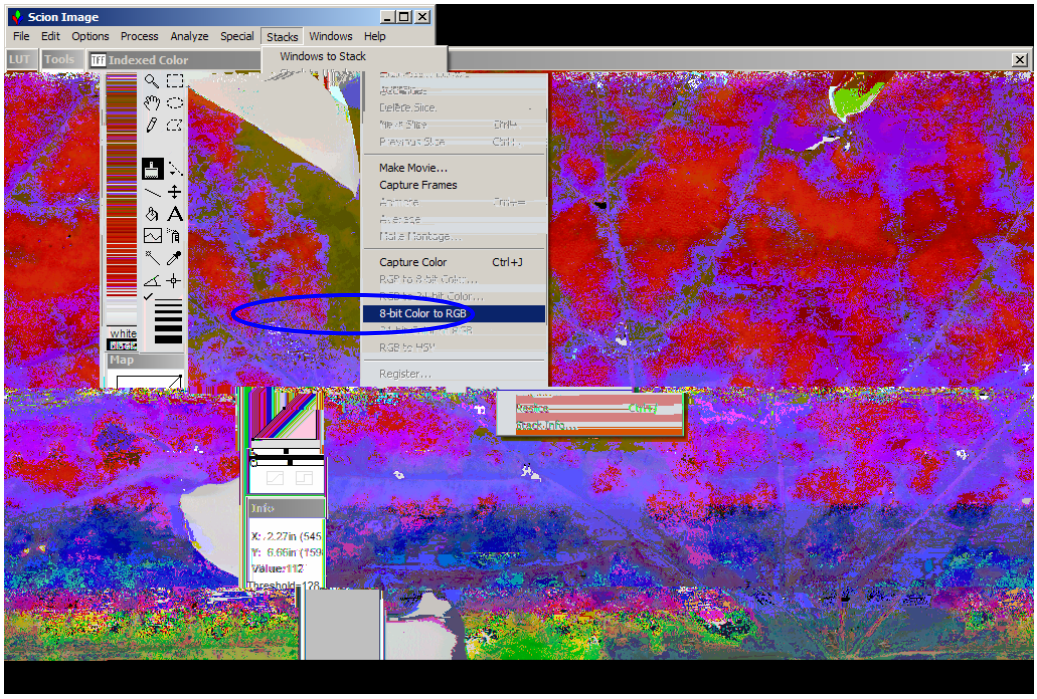
b. Notice that the paintbrush tool ( ) has changed to the color chosen from the LUT window. Note: the paintbrush can be resized to cover smaller or larger areas by double clicking on the tool and changing the pixel size.

Practicing with the paintbrush tool

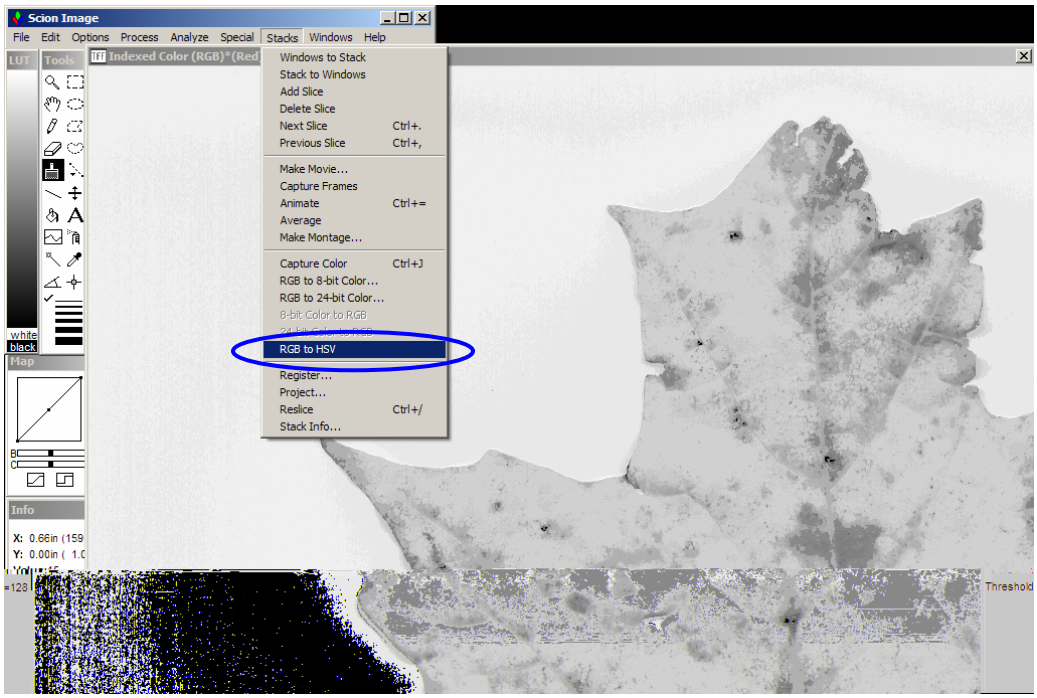
c. Anywhere in the background, or “non-leaf” area, of the indexed color window, click and hold while drawing a small test circle. Notice that the curser is now a circle when used within the Indexed Color window.



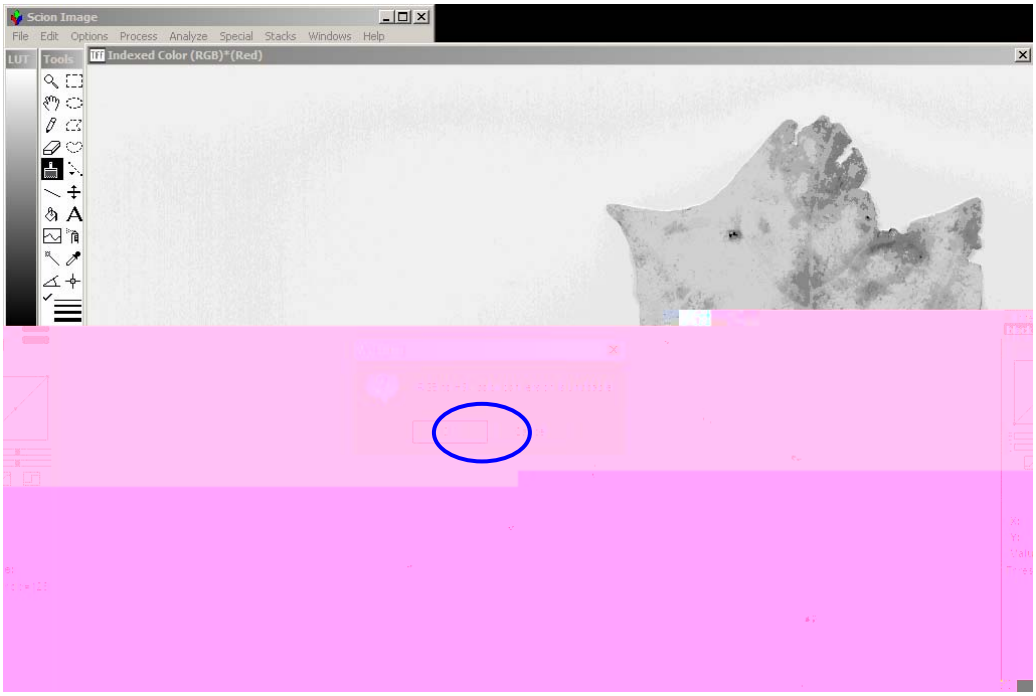
d. Go to Stacks then 8-bit Color to RGB.



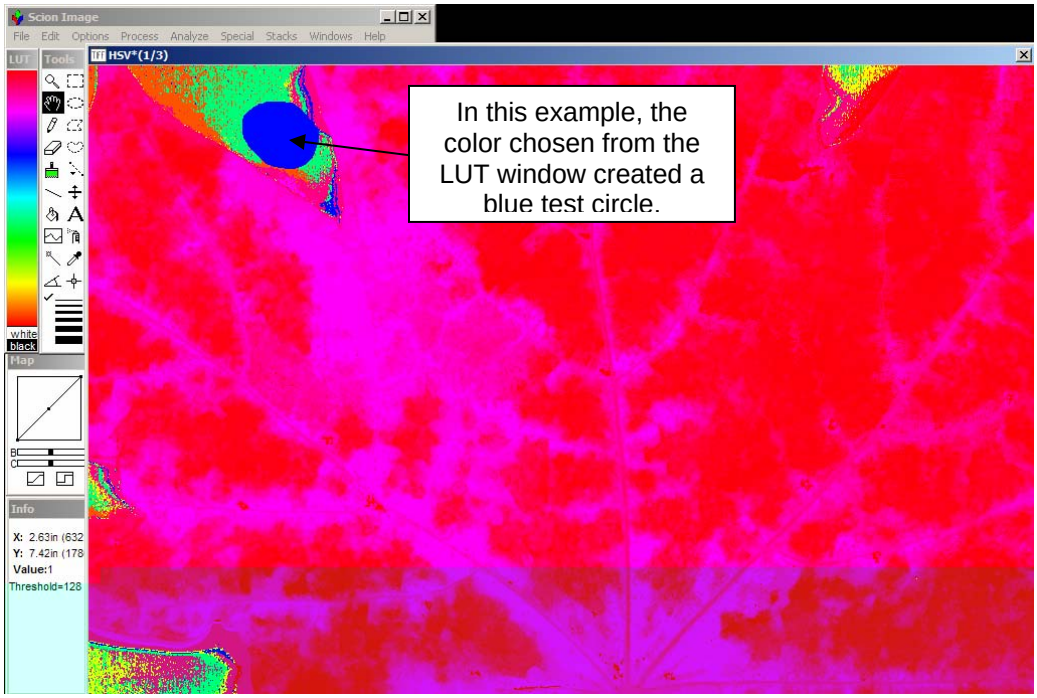
e. Go to Stacks again then RGB to HSV.



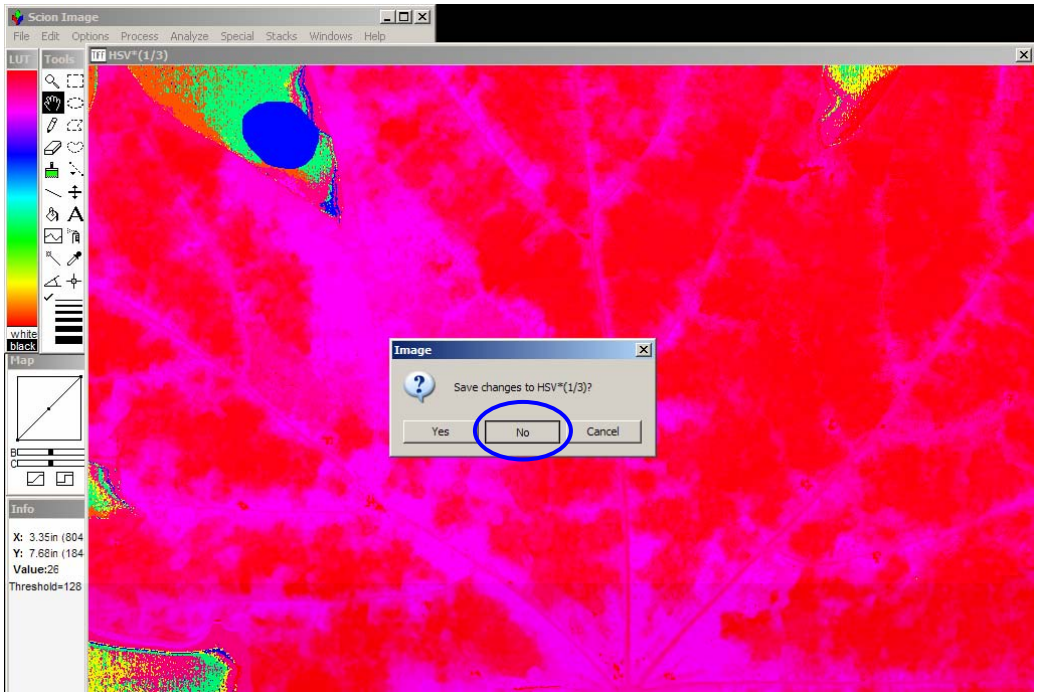
f. Select OK to the warning message.



g. Then look for the test circle and notice the color change. It should now be a shade of blue or yellow. If it is not blue or yellow, close the window and choose another color from the LUT window in step 15a and repeat steps 15b-15f until the test circle is blue or yellow.

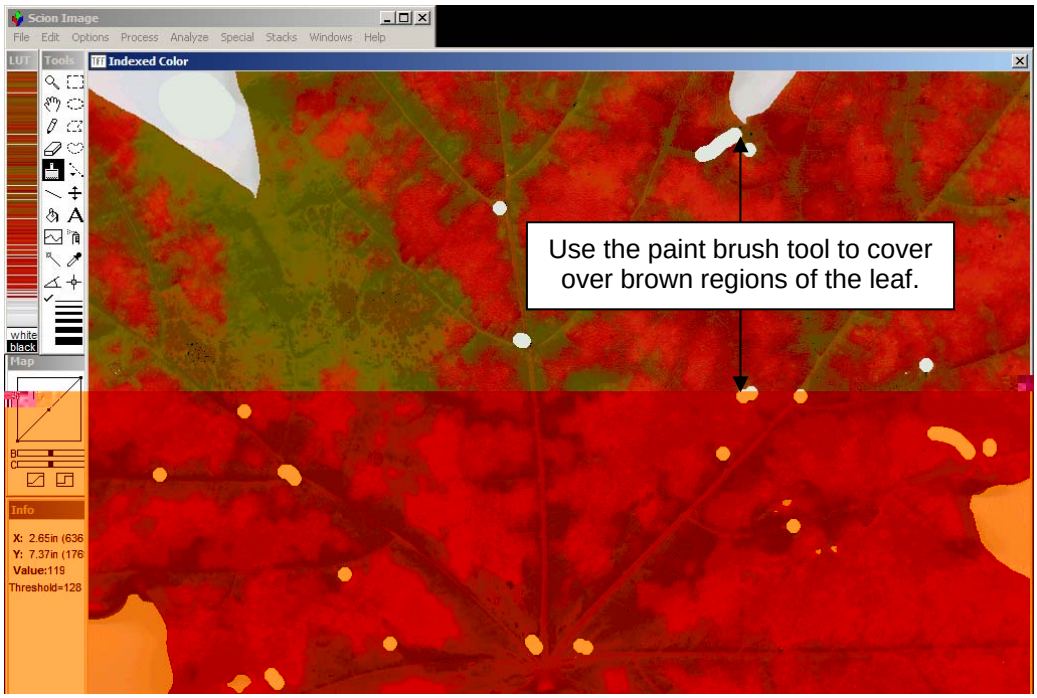


h. Close the HSV window. Do not save.

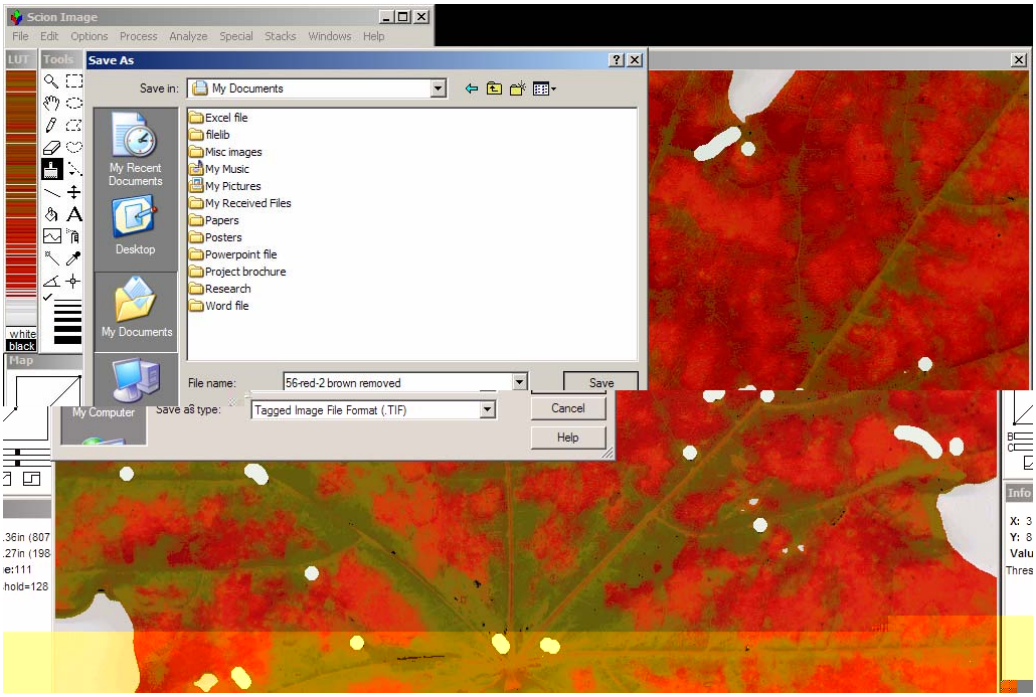


Masking brown areas

i. The paintbrush tool should still be selected. Click and hold to color over most brown spots or other areas that should be excluded from the color analysis. It may not be necessary to exclude small areas of brown because their inclusion minimally alters the results of the color analysis. Use the hand ( ) tool to move within the window. Choose the hand tool, click in the window, hold and move. Click on the paintbrush tool again to resume coloring over brown regions.

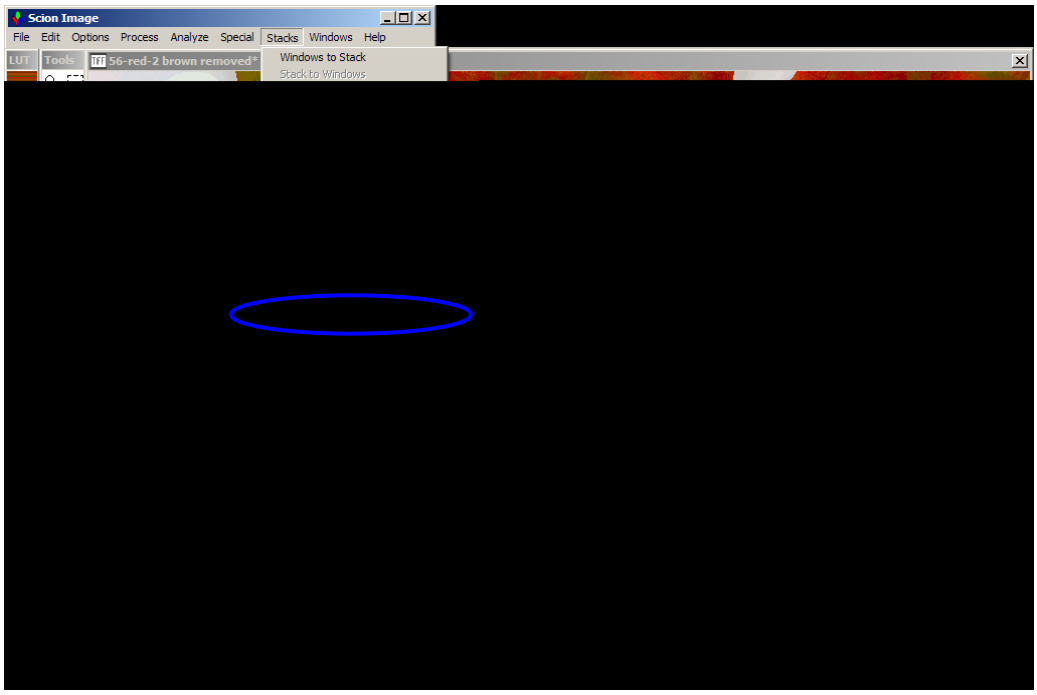


j. Some users prefer to save this image because coloring over brown areas can be a very time-consuming step. By saving the image, the hassle of repeating preparatory steps can be eliminated should leaf color analysis on the same image be needed again. To save, go to File and then Save As. Assign the image a descriptive new file name such as: the old sample name with “brown removed” added, e.g., “56-red-2 brown removed”.

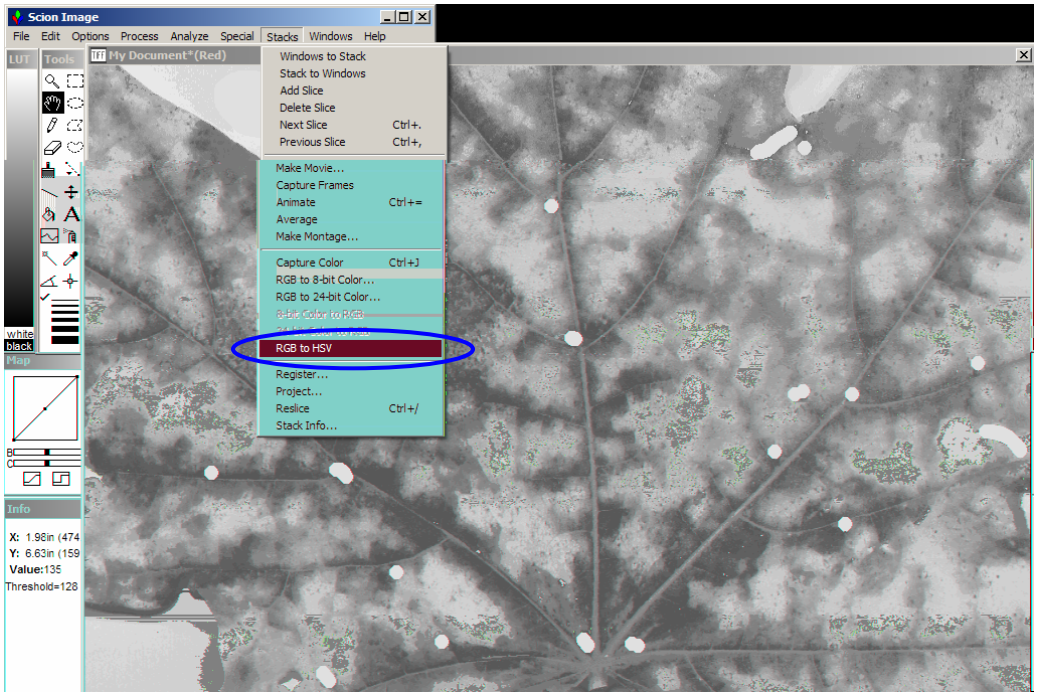


Converting color image to hue, saturation and value slices

16. With the new “brown removed”/indexed color window selected, go to Stacks and then 8-bit Color to RGB.

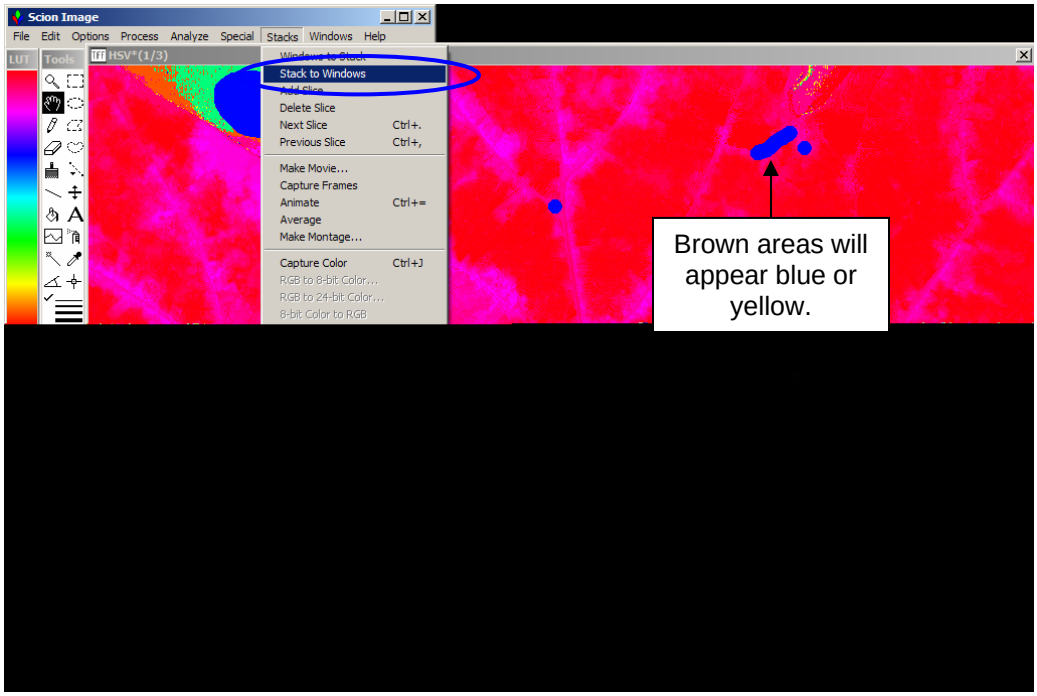


17. Go to Stacks and then RGB to HSV.

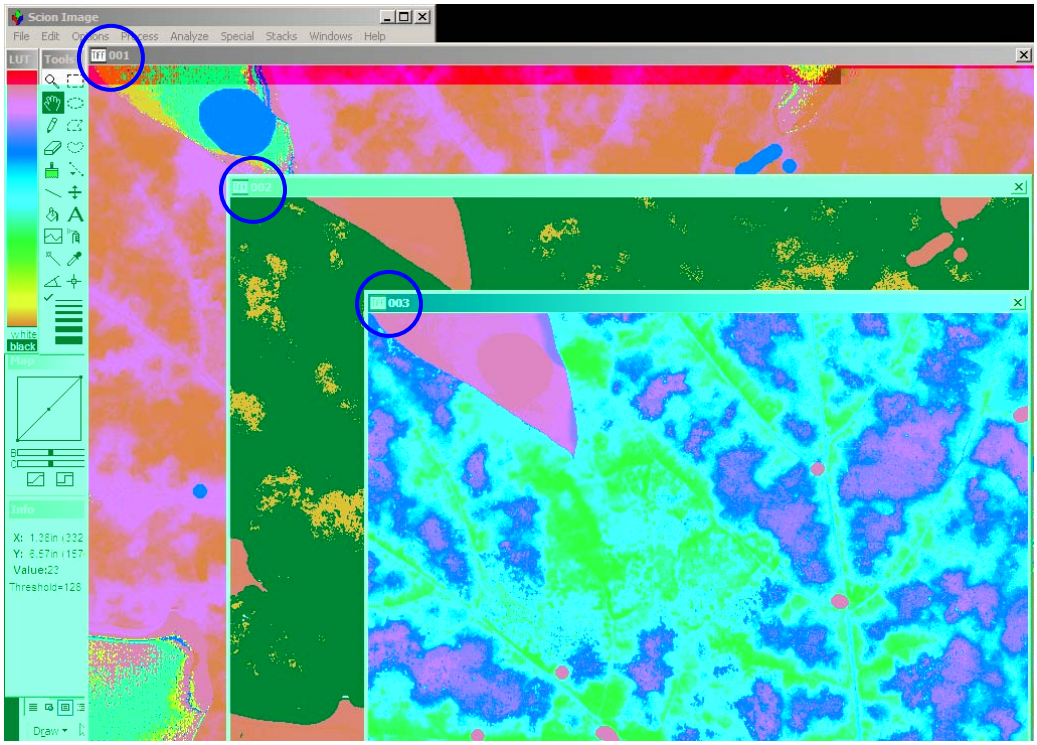


18. Select OK to the message that pops up.

19. Go to Stacks again then Stack to Windows.

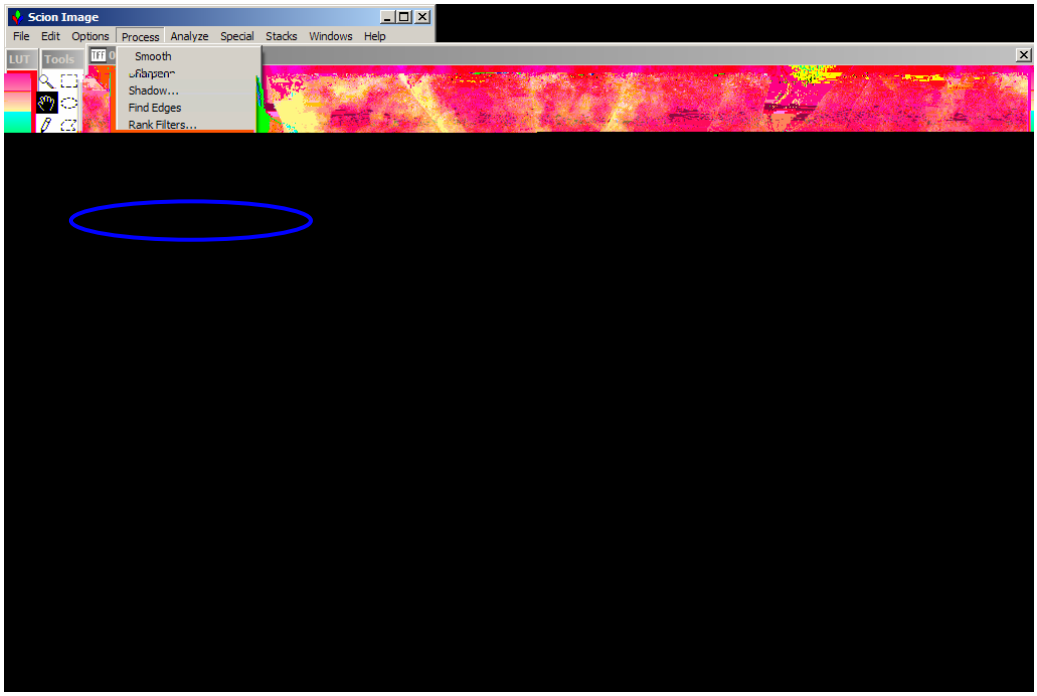


20. Three superimposed windows will come up (labeled 001, 002 and 003). Close windows 002 and 003. Only window 001 should be visible now.



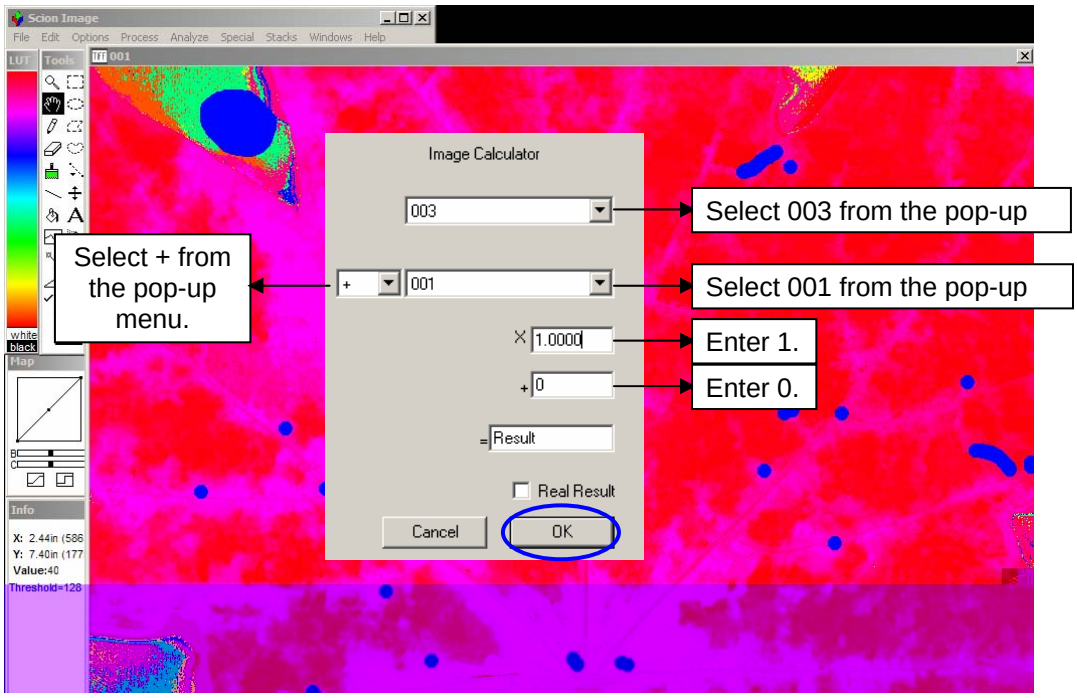
Combining images using the image calculator

21. Go to Process then Image Math.



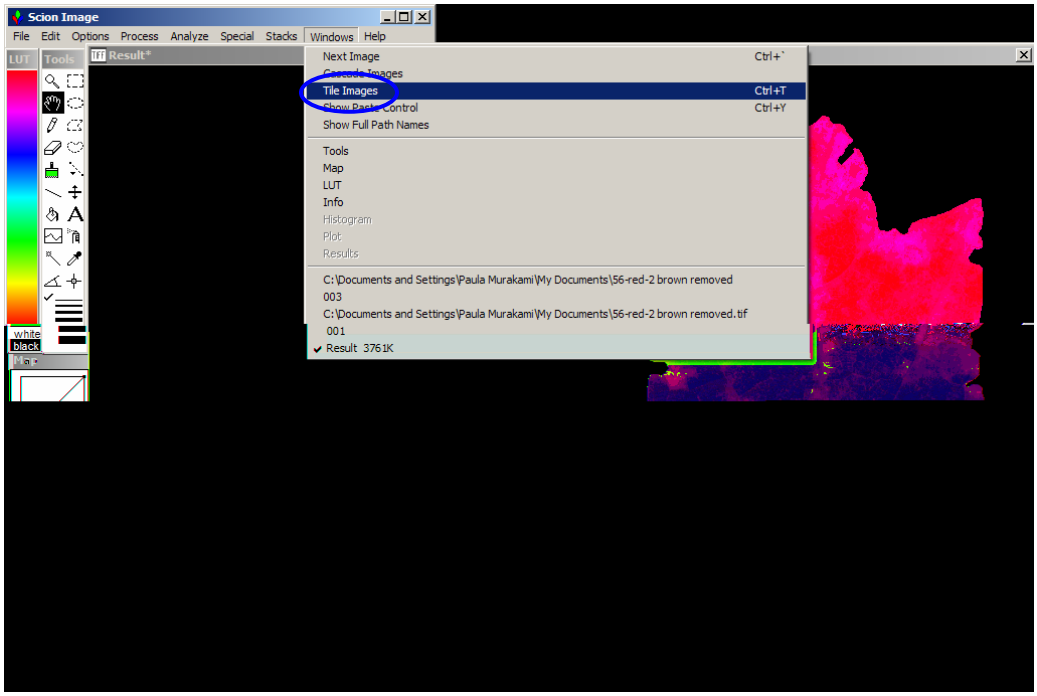
22. Using the Image Calculator that appears, set up the equation ***exactly*** as shown below.

    Real Result should not be selected. Choose OK.

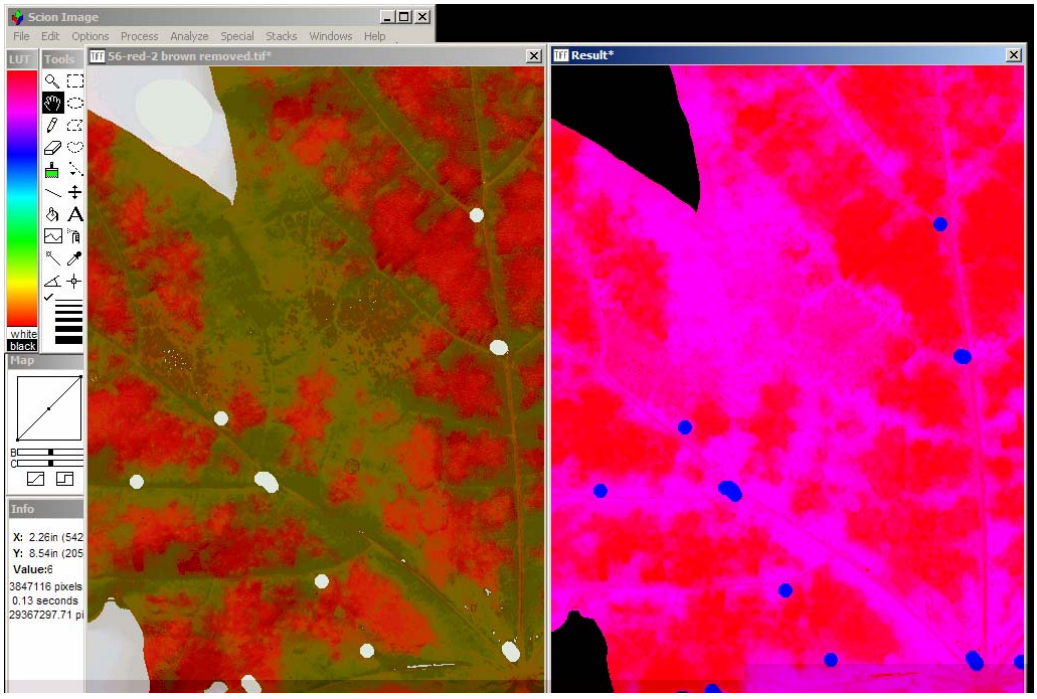


Viewing indexed color and results windows simultaneously

23. Go to Windows and then Tile Images.

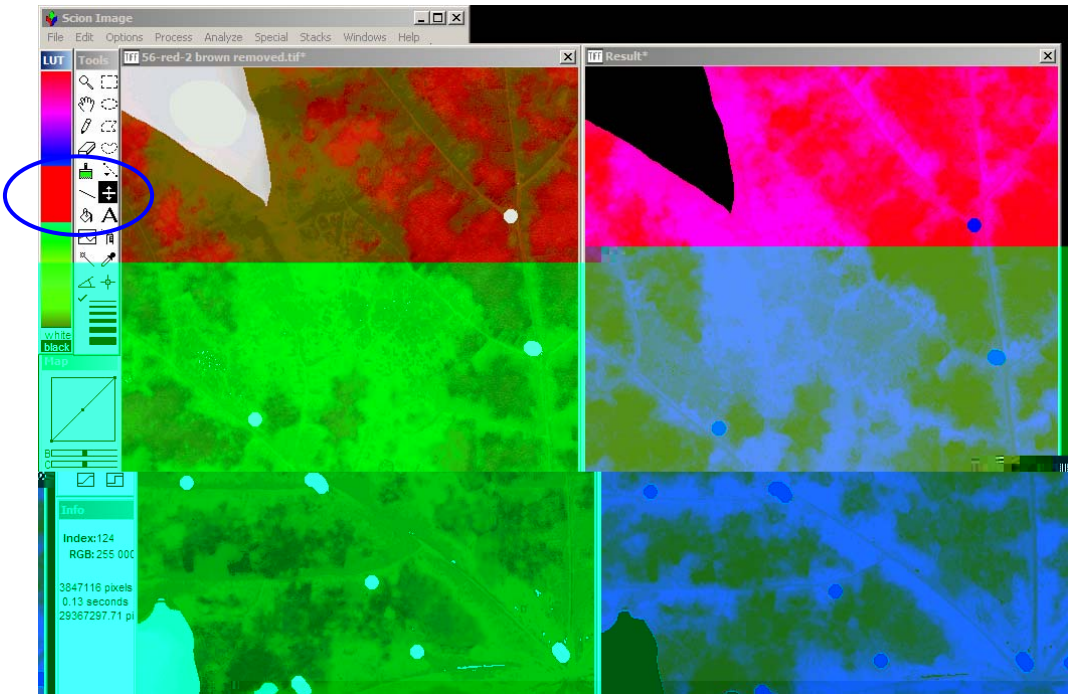


24. Extend the “brown removed” and result windows by dragging the top or bottom of each window until they are side by side.

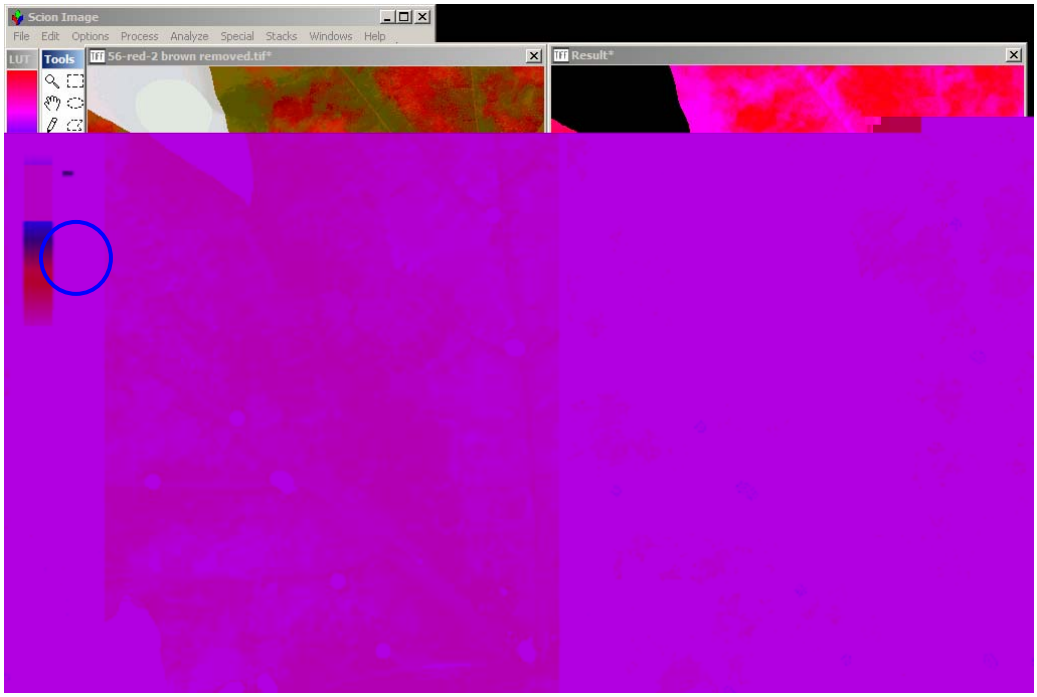


Changing the color of the LUT bar

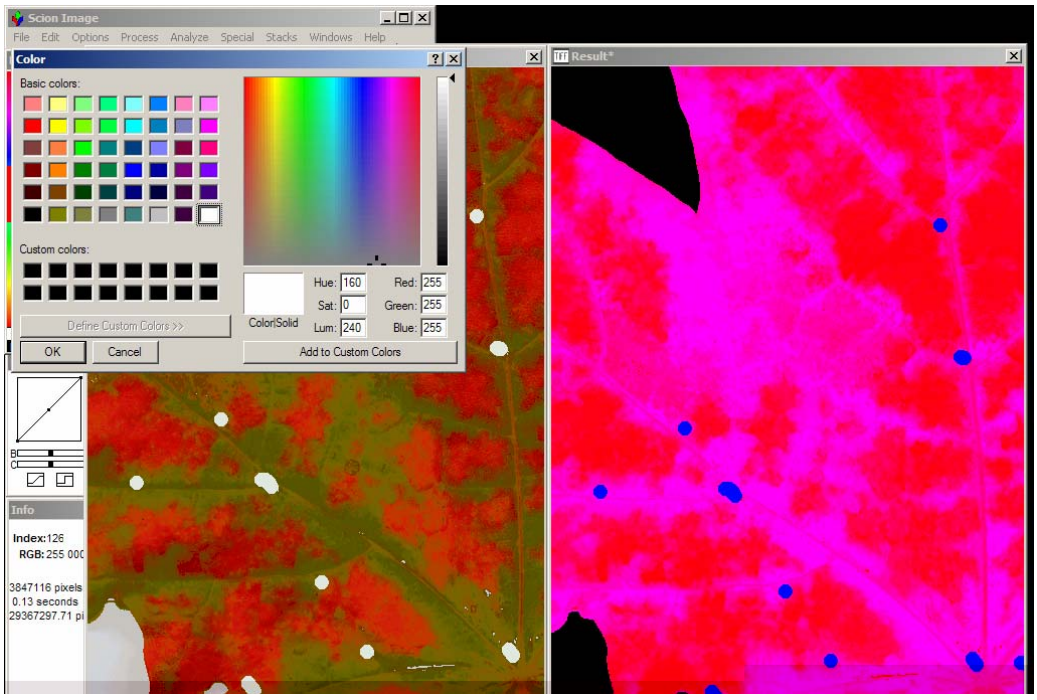
25. With the results window selected, double click on ✢ in the tools window. A solid red bar of color will show up in the LUT window. This will need to be changed to a different color. Anytime Scion Image is initially opened, this bar will be red.



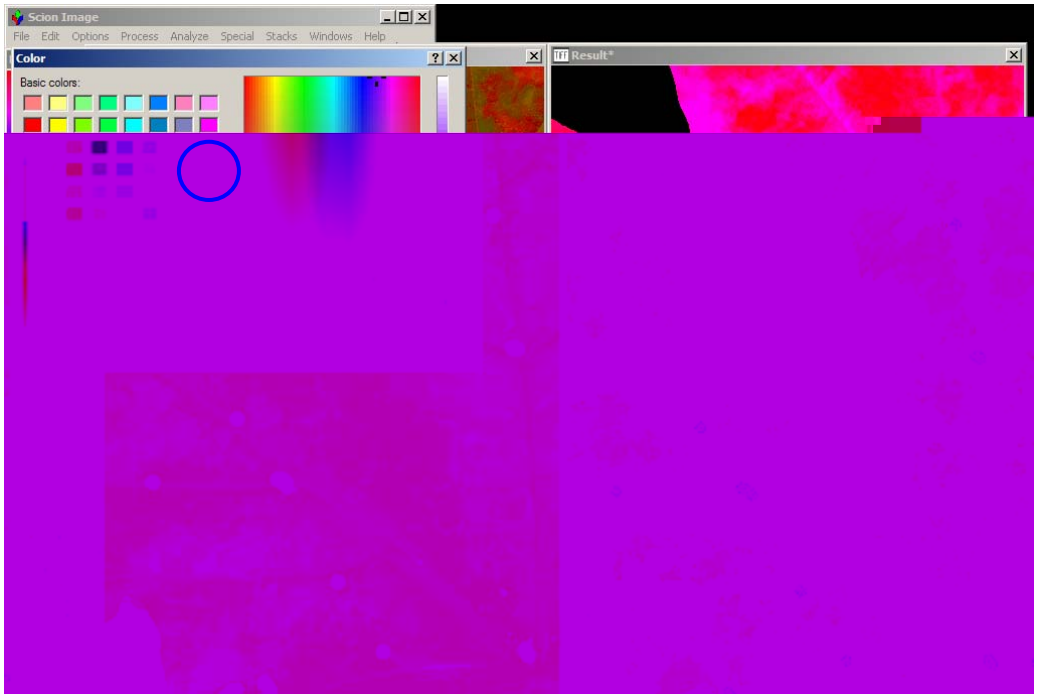
26. Click once on the eyedropper tool ( ).



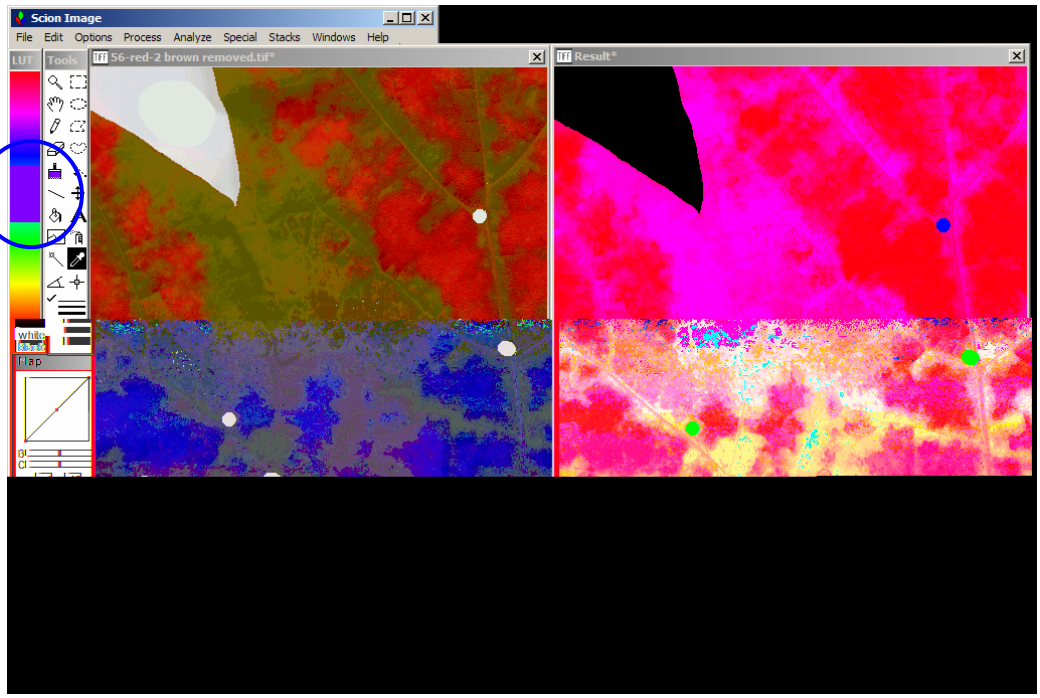
27. Double click in the red bar and a color window will pop up.



28. Select a new color to replace the red bar (purple works well). Choose OK.



29. Notice that the paintbrush and red bar are now purple.



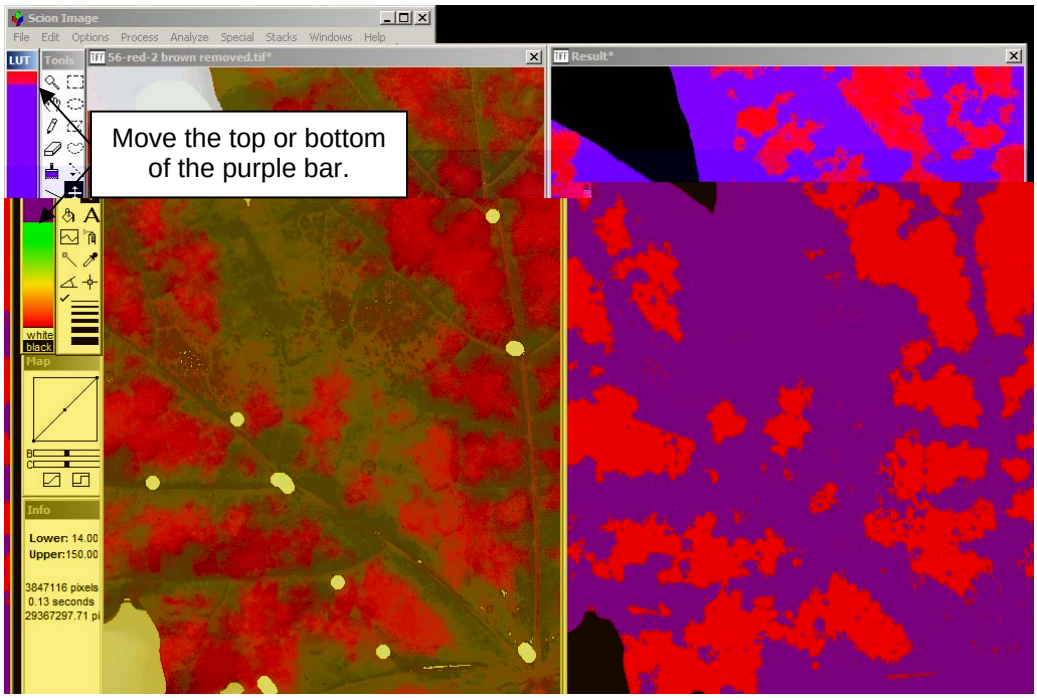
Aligning the images

30. Be sure that the same areas of the leaf are visible in both the indexed color window and the results window. If they are not similar, move the two images using the hand () tool. Choose the hand tool, click in the window, hold and move. You should see the leaf image move within the window. Align the leaves within both windows. It is important to see the same areas of the leaf in order to successfully identify areas of color in each image. It is also helpful to see some blue or yellow regions in the result window which cover brown areas in order to make sure they are not included in the analysis.

Measuring green

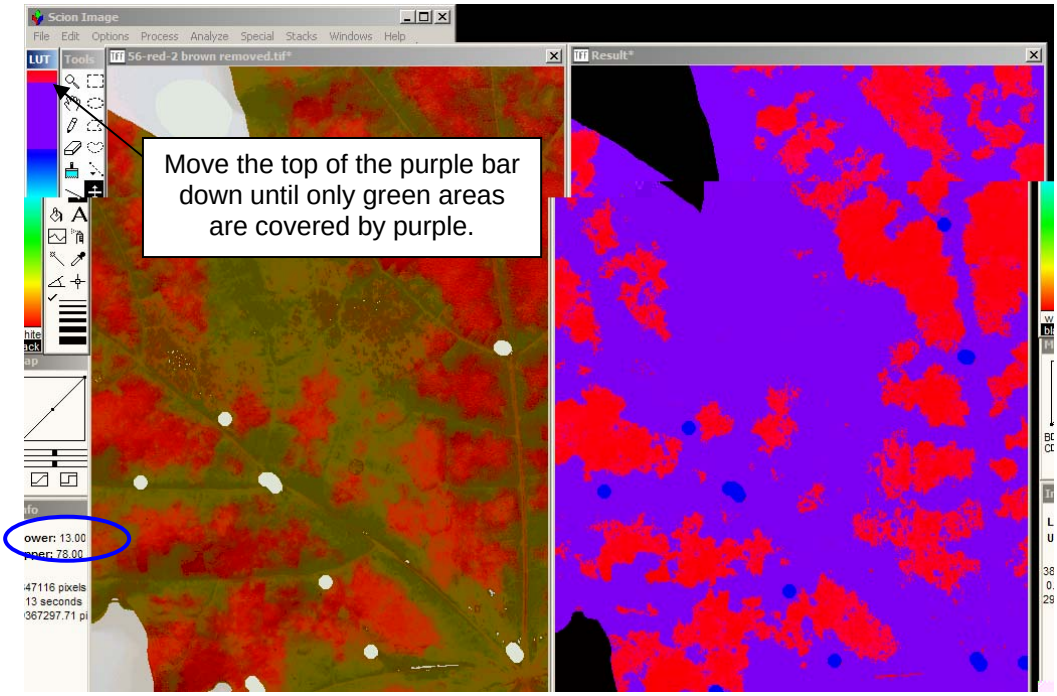
31. Select the results window.

32. Click on ✢. It may be necessary to double click on this tool in order to see the purple bar. Experiment with moving the top and bottom limits of the purple bar. Click on the top or bottom of the bar, hold and move up or down. Notice how it covers areas of the leaf in the results window with purple. Note: when the limits of the purple bar are dragged ***all the way*** to the top or bottom of the LUT window, click the top or bottom of the bar to "let go" of it. Otherwise the bar will continue to move up or down as the cursor is moved.

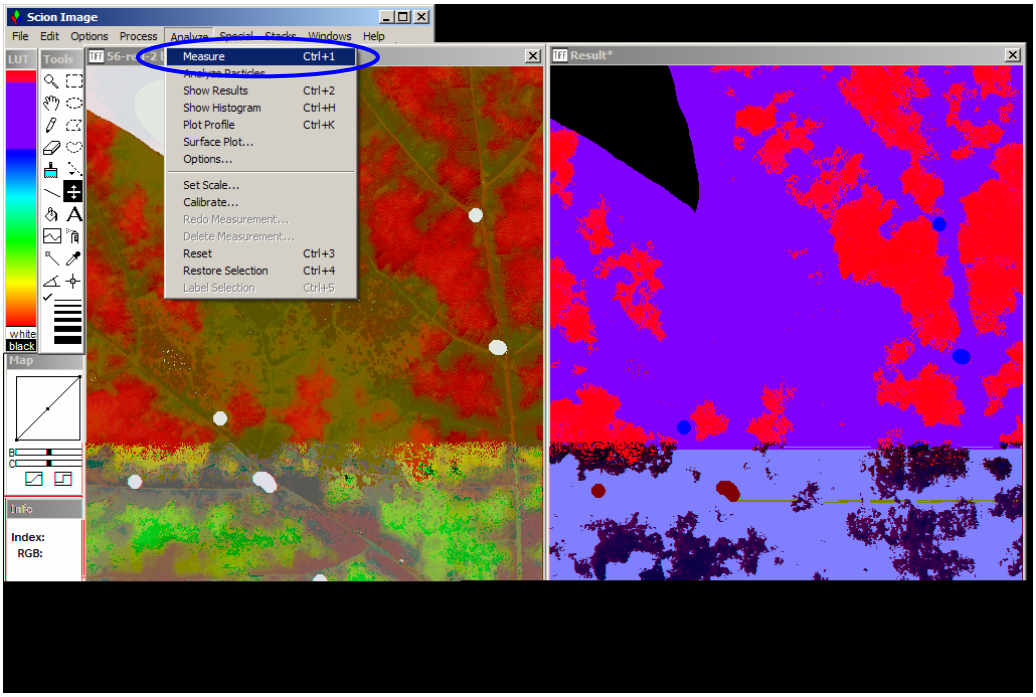


33. To measure green, click in the middle of the purple bar and move the whole bar to the top of the LUT window. This will cover most of the leaf in the results window with purple. Now move the bottom of the bar up or down until the whole leaf is purple, ***except for the brown areas which are blue or yellow***.

34. Now go to the top of the purple bar and move it down until you have covered only the green areas of the leaf. Compare with the green areas of the leaf in the “brown removed” window. Also, record the Lower number in the Info box in the bottom left-hand corner of the screen. In this example it is 13. This number will be referred to again when measuring red.

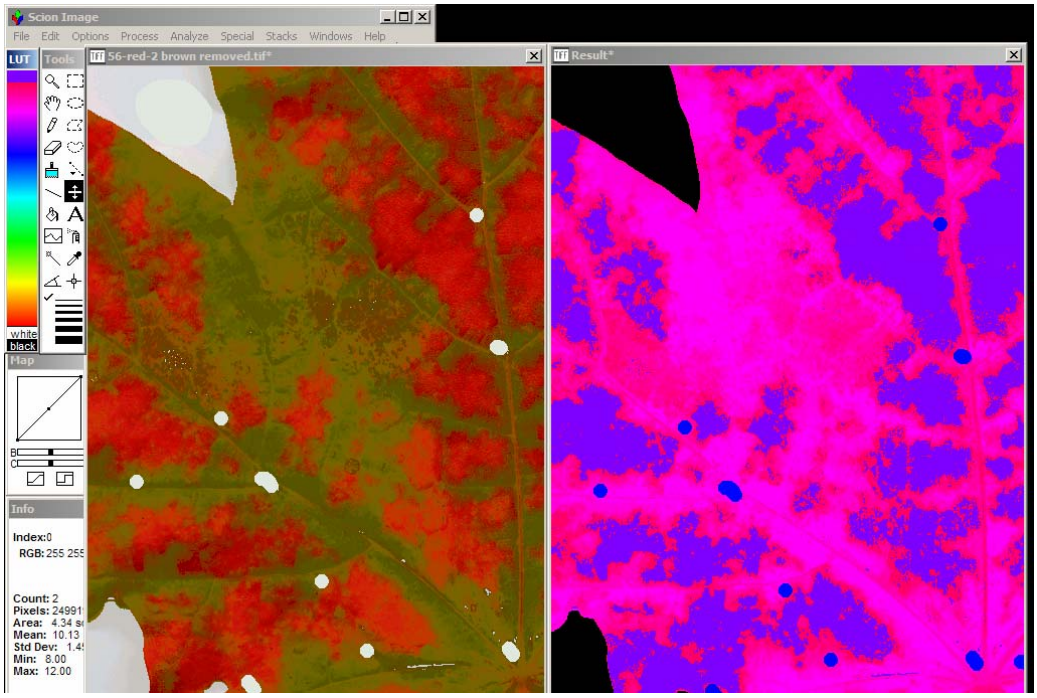


35. When all green areas of the leaf are highlighted by purple, it is time to measure. To do this, click Analyze and then Measure.

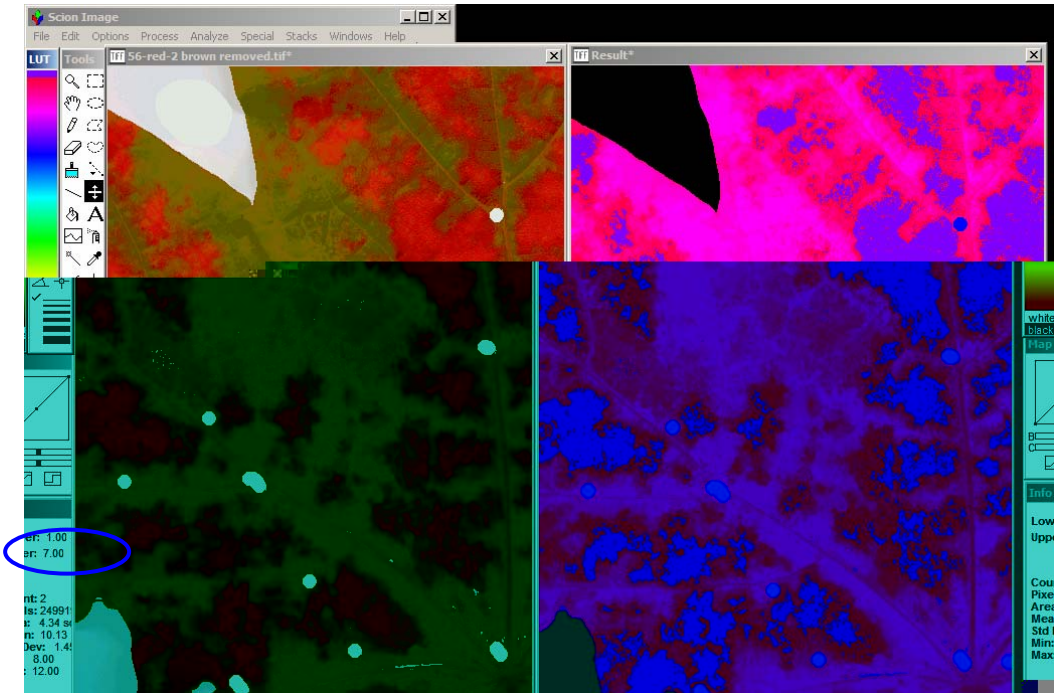


Measuring red

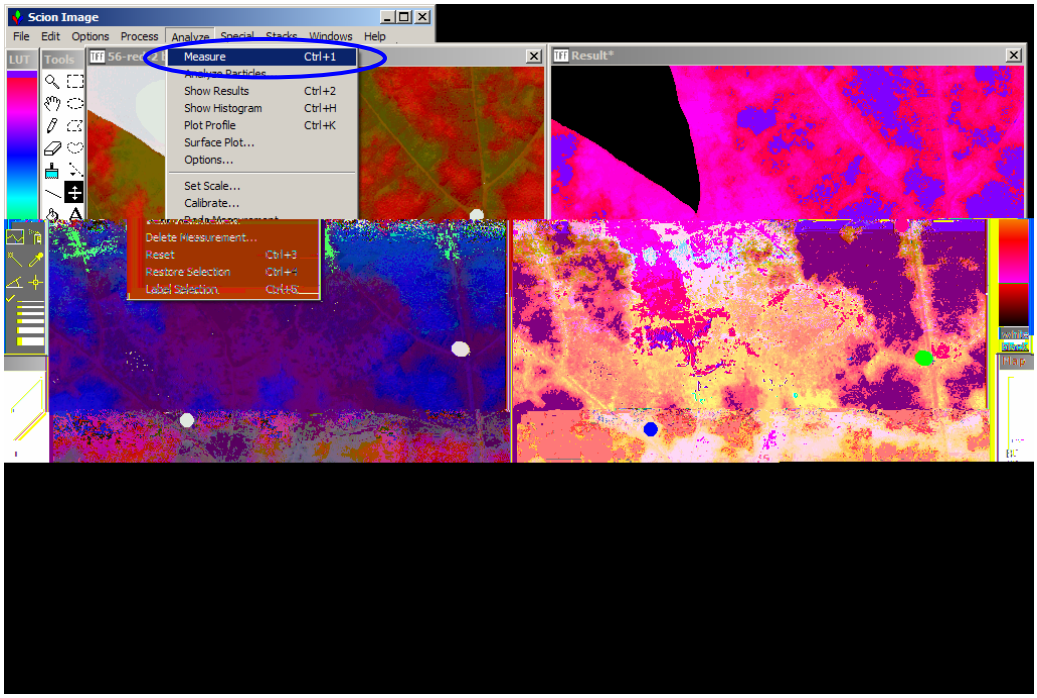
36. To measure red color, once again move the top part of the purple bar to the top of the LUT window.



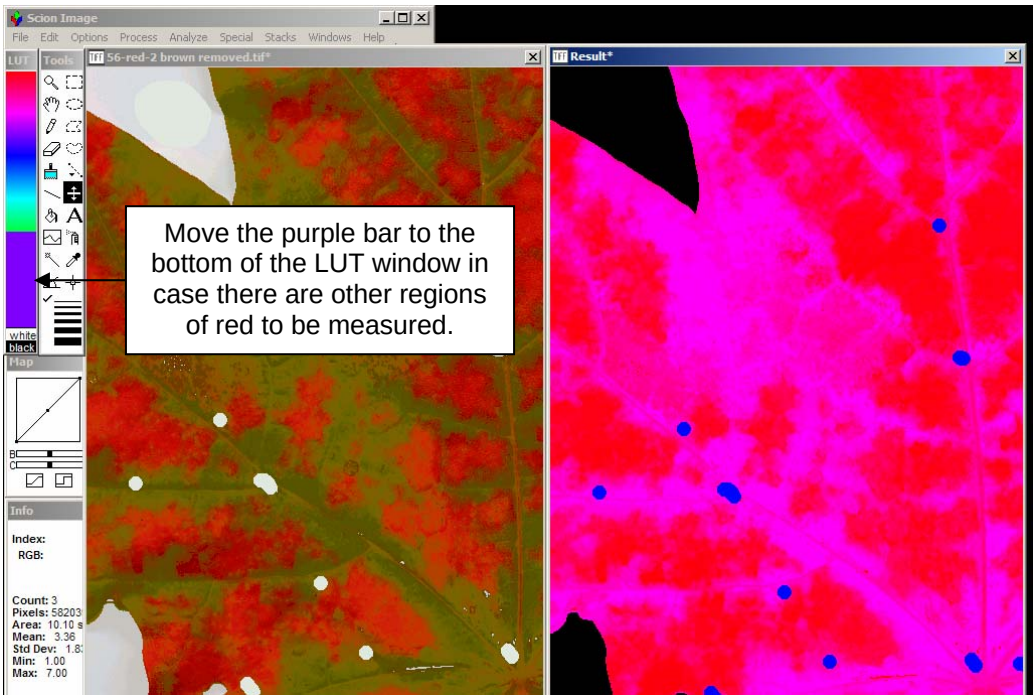
37. Now move the bottom of the purple bar up until the red areas of the leaf are highlighted by purple. Note the Upper number in the Info box. In this example it is 7. This number must be ***less*** than the Lower number recorded earlier in step 34.



38. Once again Analyze, then Measure.

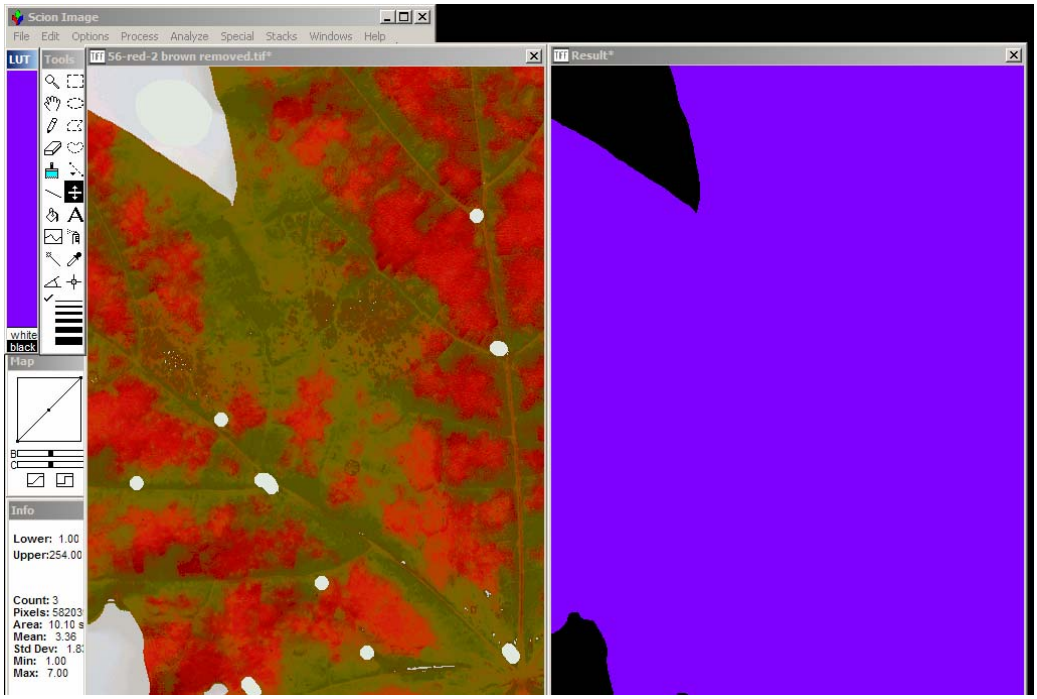


39. Sometimes red can also be measured in the lower region of the LUT bar, so it is good practice to move the purple bar all the way to the bottom of the LUT window. In doing so, look for additional red areas of the leaf to be covered by purple in the results window. If red areas are highlighted by purple, then Analyze and Measure. ***Important:*** if measuring red in this region, avoid inadvertently measuring the brown areas. Before analyzing and measuring, move the top of the purple bar down until brown areas are blue or yellow, not purple. In this example there are no other red areas to be measured.

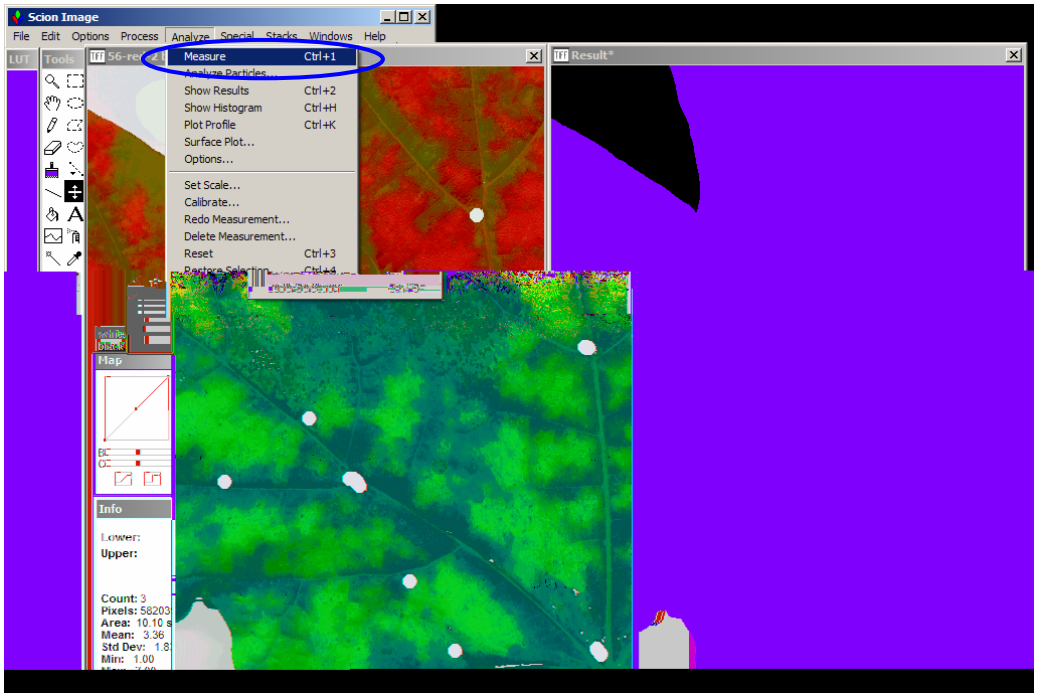


Measuring total leaf area

40. When green and red have been measured, move the top and bottom of the purple bar so that the entire LUT window is purple.

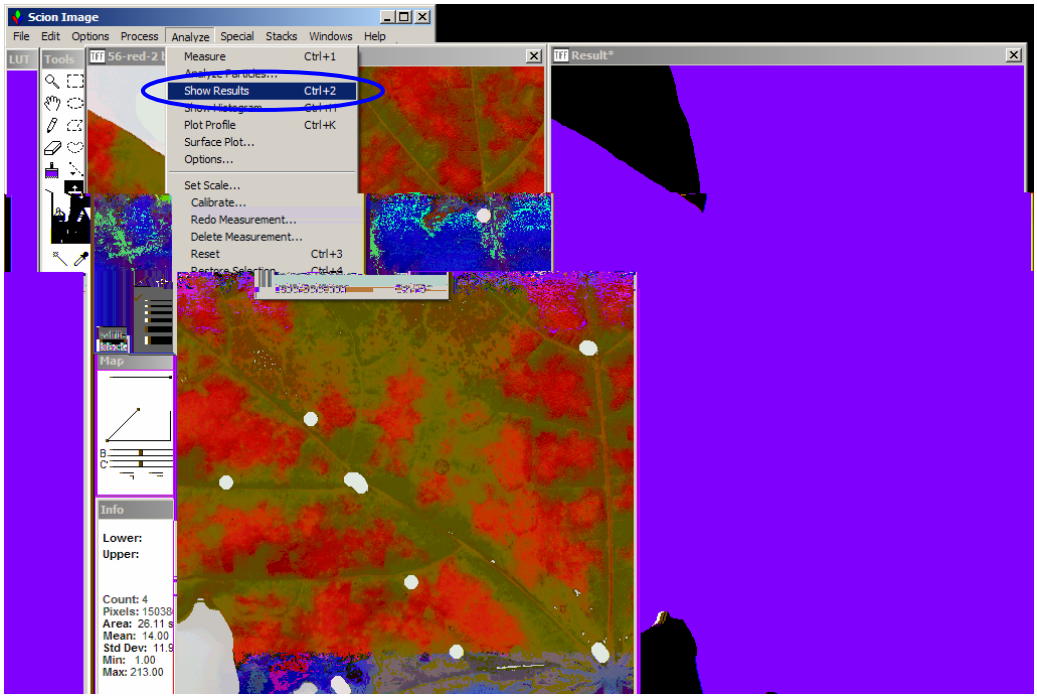


41. Click on Analyze and then Measure. By doing this you will be measuring the total leaf area (including brown areas).

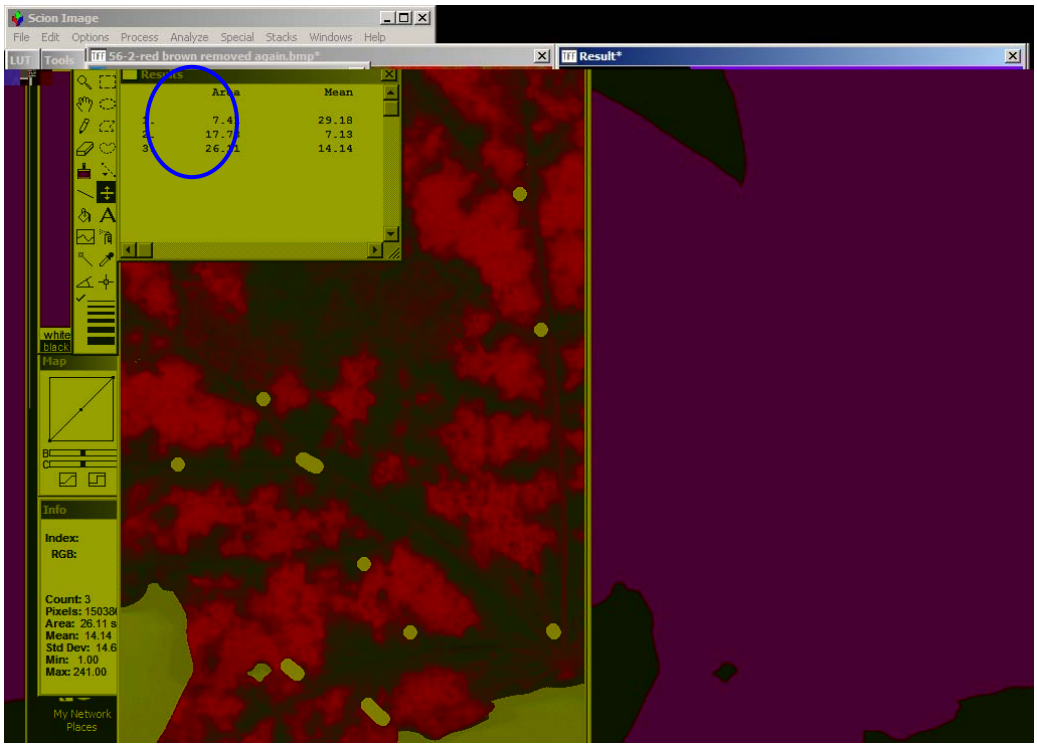


Viewing results

42. To view actual values of measured leaf areas, choose Analyze and then Show Results.



43. Record the values in the “Area” column for each color measured and the total leaf area. 1. is the green area, 2. is the red area and 3. is the total area. Make sure that the combined areas of green and red do not exceed the total area. If they do, then it is likely that the measured areas of green and red overlapped and will need to be re-measured.



44. Using these numbers, calculate the percent green and red area for each scanned leaf image.

Comparison of percent color versus pigment concentration

In order to assess the accuracy of using digital image color analysis as an indicator of foliar pigment concentrations, percent leaf color and pigment content were determined for 326 sugar maple leaf images. Fresh leaves were scanned and leaf disks were carefully removed. Punches were shredded using a razor blade and placed in either acetone/H_2O for extraction of chlorophylls or HCl/H_2O/MeOH for extraction of anthocyanin according to the methods of Gould et al. (2000). Pigment content was quantified using a Spectronic Genesys 8 spectrophotometer (Cheshire, England). Chlorophyll concentrations were calculated using the equations of Lichtenthaler and Wellburn (1983). Absorbance of anthocyanin was measured at 530 nm and the overlap of chlorophylls (0.24 x A_{653}) was subtracted (Murray and Hackett 1991). Correlation analyses were conducted to determine the relationship between percent leaf color and pigment concentration (Table 1). Linear relationships were uniformly significant. However, because entirely green or red leaves could only be measured as 100% green or red regardless of how much chlorophyll or anthocyanin accumulated, a saturation in percent color was sometimes observed. Due to this saturation effect, associations between percent color and pigment concentration were better accounted for by polynomial fits (cubic) relationships (table 1).

Analyses with sugar maple leaves indicate that digital image analysis can provide an accurate means of quantifying foliar color and estimating pigment concentration at the leaf level. Although this method has value, it is not intended to replace other more precise means of measuring color. However, it does provide resource managers and scientists with an alternative means of quantifying leaf color and aspects of plant health that do not require specialized equipment or potentially hazardous chemicals.

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Table 1--Linear and cubic relationships between percent color and pigment (chlorophyll and anthocyanin) concentrations for sugar maples leaves

	Percent green			
	Linear relationship		Cubic relationship	
Pigment	r^2	*P*	r^2	*P*
Chlorophyll *a*	0.766	<0.0001	0.918	<0.0001
Chlorophyll *b*	.580	<0.0001	.893	<0.0001
Total chlorophyll	.719	<0.0001	.919	<0.0001

	Percent red			
	Linear relationship		Cubic relationship	
Pigment	r^2	*P*	r^2	*P*
Anthocyanin	0.724	<0.0001	0.828	<0.0001