



United States
Department of
Agriculture

Forest Service

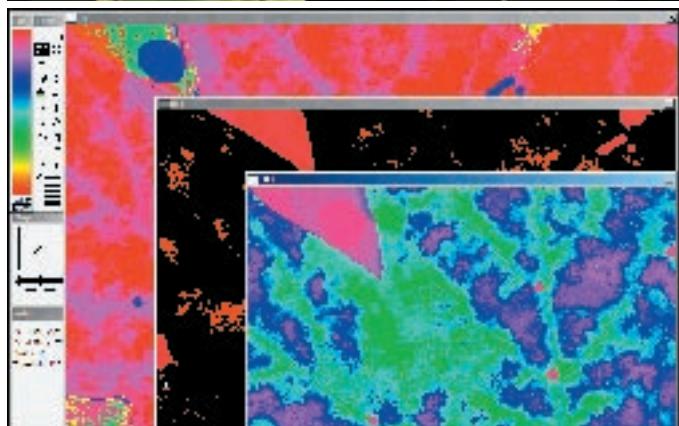
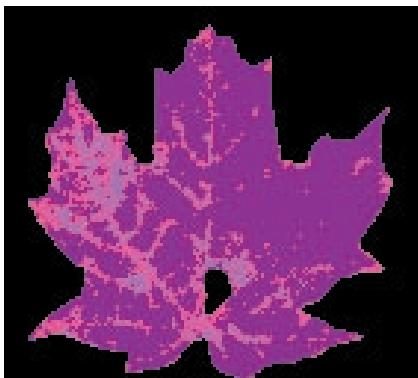
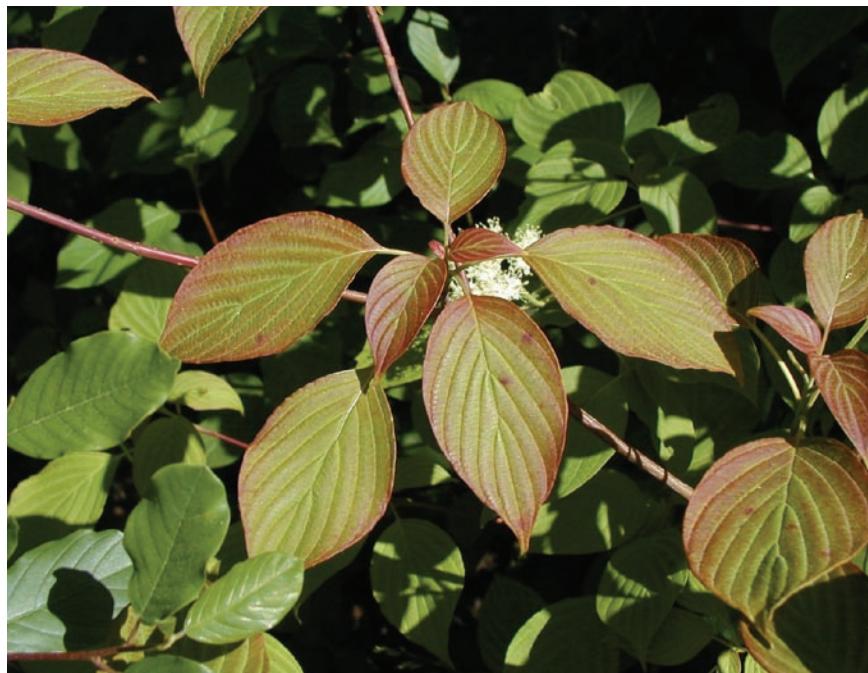
Northeastern
Research Station

General Technical
Report NE-327



An Instructional Guide for Leaf Color Analysis using Digital Imaging Software

Paula F. Murakami
Michelle R. Turner
Abby K. van den Berg
Paul G. Schaberg



Absbract

Changes in foliar color are a valuable indicator of plant nutrition and health. Leaf color is 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. 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 percentage 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.

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Introduction

Foliar color has 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 often relied on 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 were developed to help standardize color/assessments. These affordable guides are available for purchase and commonly are used to estimate foliar nitrogen status and evaluate seasonal changes in leaf color. More precise methods of foliar color analysis include those that measure chlorophyll content and spectral properties of leaves (i.e. reflectance- and transmittance- based measurements). Chlorophyll meters, spectrophotometers, reflectometers, and spectroradiometers are commonly used in 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), light absorption (Neill and Gould 1999), 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 also can be expensive and may require access to and training with specialized equipment, potentially hazardous chemicals, and other laboratory supplies.

Recently, 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. 1994), and evaluate senescence rates in spring wheat (Adamsen et al. 1999). An important application of digital image and color analysis has been in evaluating the influence 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. Department of Agriculture 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 found that sugar maple leaves with low nitrogen concentrations turned red earlier and more completely than those with high nitrogen concentrations (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 of Health and available at <http://rsb.info.nih.gov/nih-image>) to quantify green and red coloration in leaves.

These software packages are free and can be easily downloaded from the internet.¹ 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 to use Scion Image software to measure the percentage of green and red in leaves as an indicator of plant nutrition and health. These instructions guide the reader through the process of computer-based leaf color analysis, including the 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 practice, users will become proficient in a method for quantifying leaf color that requires no specialized equipment or chemicals.

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-inch Apple ColorSync Display were used to acquire, save, and analyze leaf images for percentage color. Other combinations of computers and scanners also can be used. Petioles were removed and leaves were placed adaxial (upper) side down 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 percent. Images were saved as a Tagged Image File Format (TIFF).

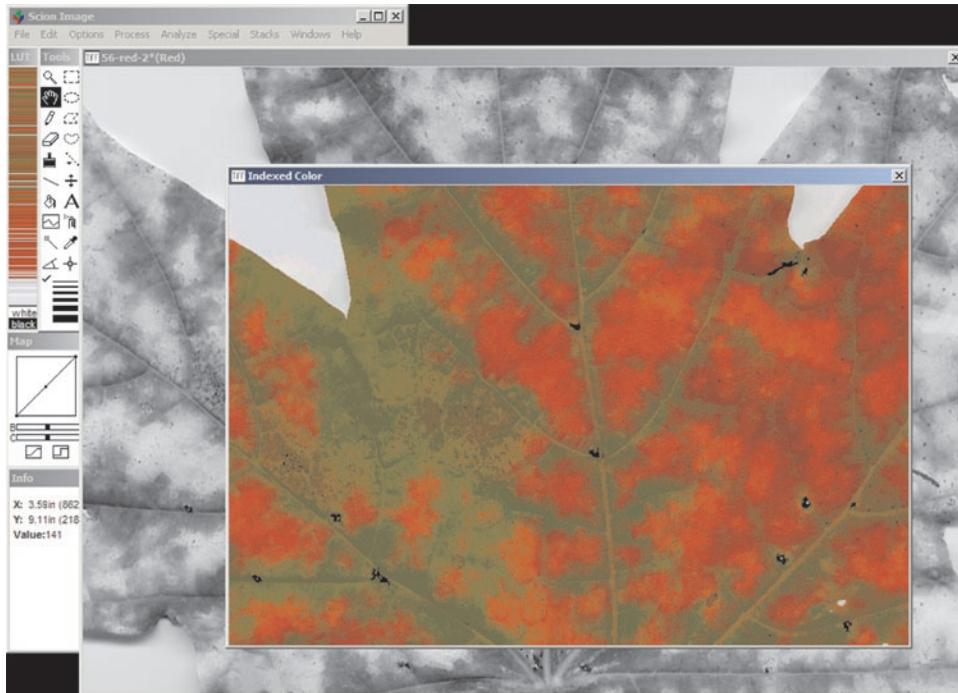
Analysis and Quantification of Leaf Color

The following instructions guide the user through a detailed series of steps from launching Scion Image through calculating the percentage of green and red leaf areas for scanned images. In the example provided, a colorful sugar maple leaf is used for demonstration purposes. When the digital image of this leaf is first opened, two windows appear. One window shows a black and white version of the leaf. The other shows the original color version of the same leaf. These images are modified then combined for final color analysis. A glossary has been provided (page 29) to define terms specific to Scion Image software. More information also can be obtained from the Scion Image website, www.scioncorp.com.

¹Scion Image for Windows is available for Windows 95/98/ME/NT/2000/XP. NIH image for Macintosh is available for system 7.0 or later. The use of trade or firm names in this publication is for the reader information and does not imply endorsement by the U.S. Department of Agriculture of any product or service.

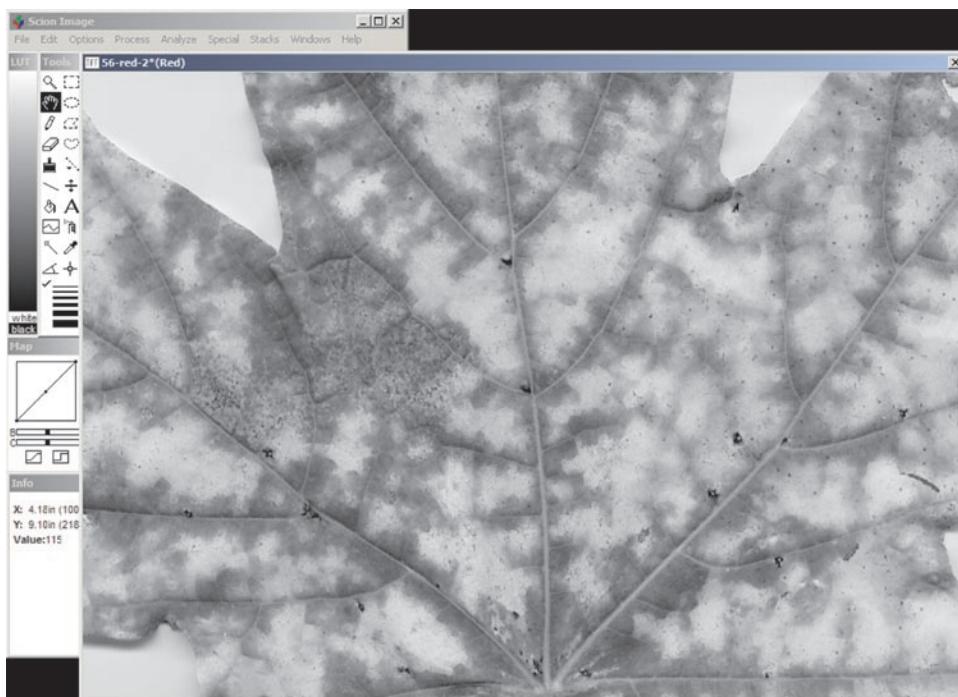
Getting Started

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 appear—a black and white window and an Indexed Color window. These two windows are partially superimposed on each other.

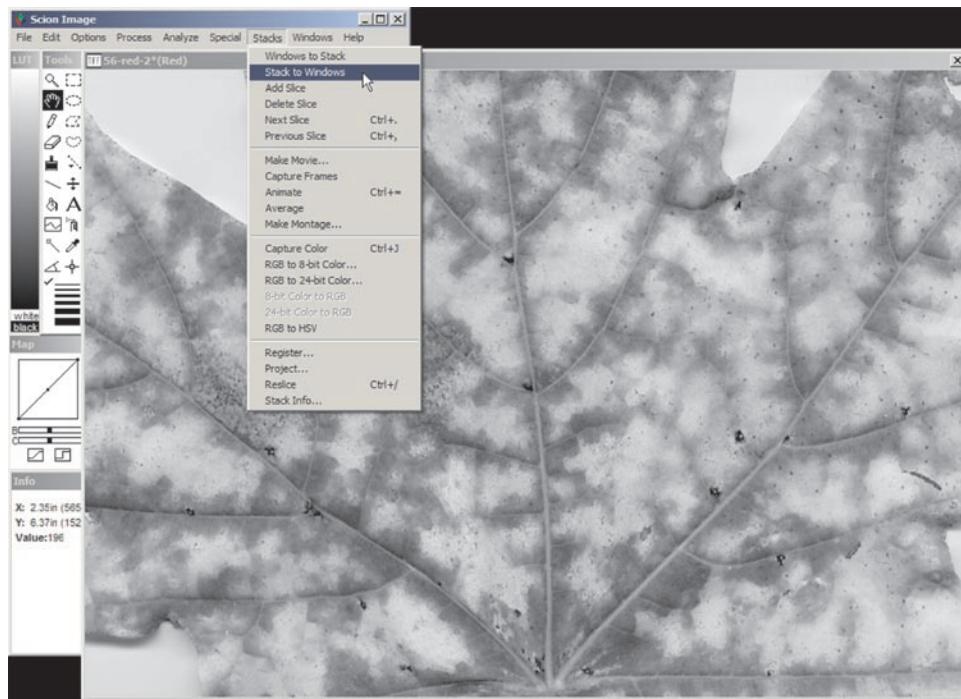


Preparing the Black and White Image

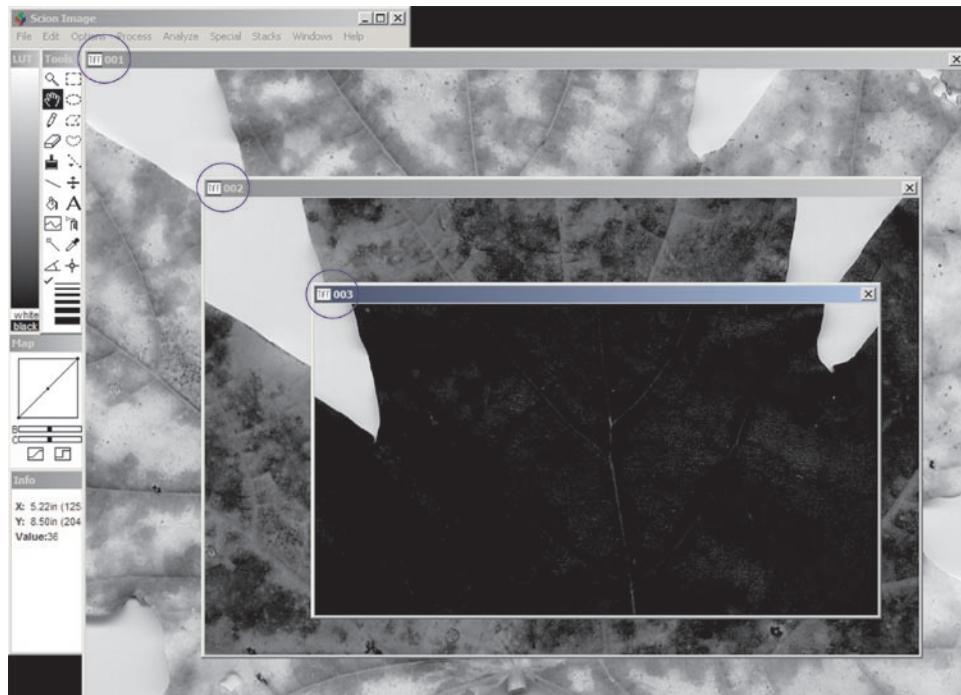
4. Select the black and white window bringing it to the foreground.



5. Go to *Stacks*² and select *Stack to Windows*. Three windows are displayed showing the leaf image in shades of grey.

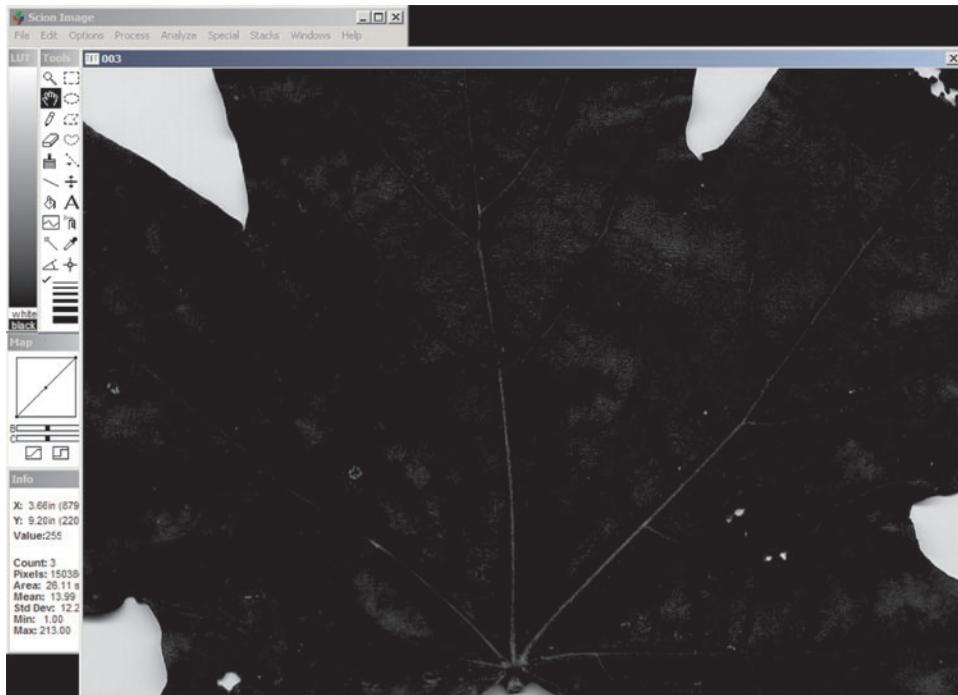


6. Three windows appear labeled 001, 002, and 003, respectively. Window 003 is superimposed on window 002 and window 002 is superimposed on window 001. It is not necessary to see all three windows at the same time.

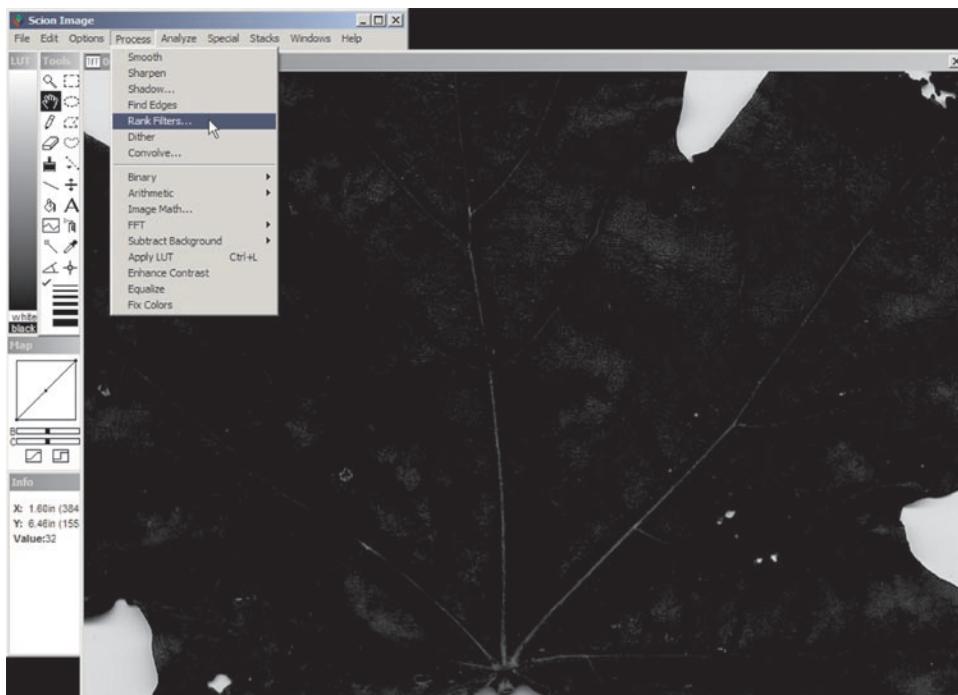


²Words in italics are defined in glossary on page 29.

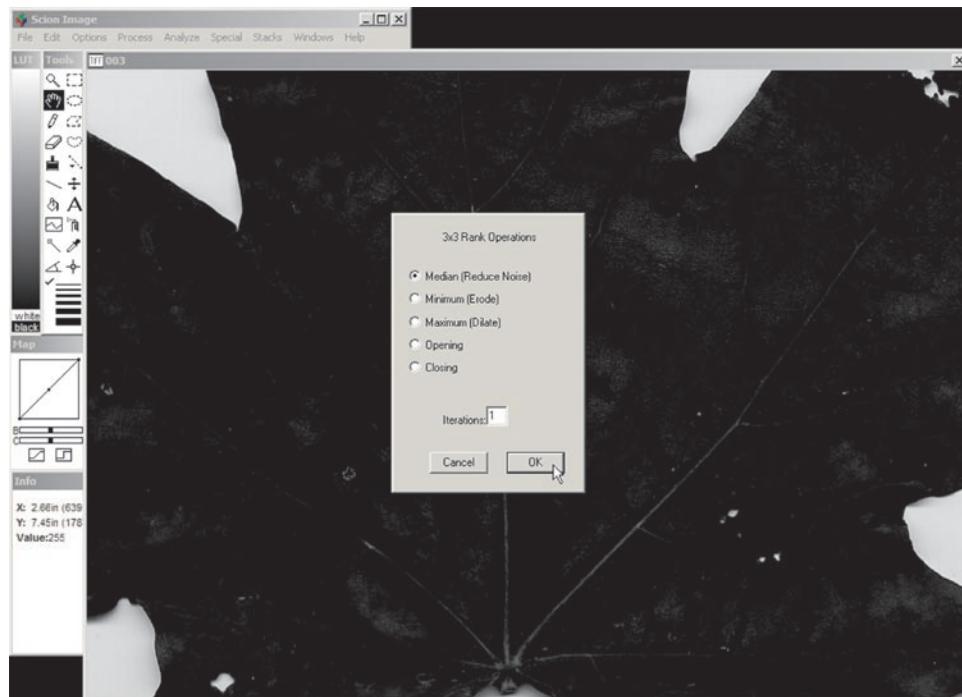
7. Go to Windows and choose 001 bringing window 001 to the forefront. Close this window. Go to Windows again and choose 002 and bring window 002 to the forefront. Close this window. Only window 003 is visible now. DO NOT close this window.



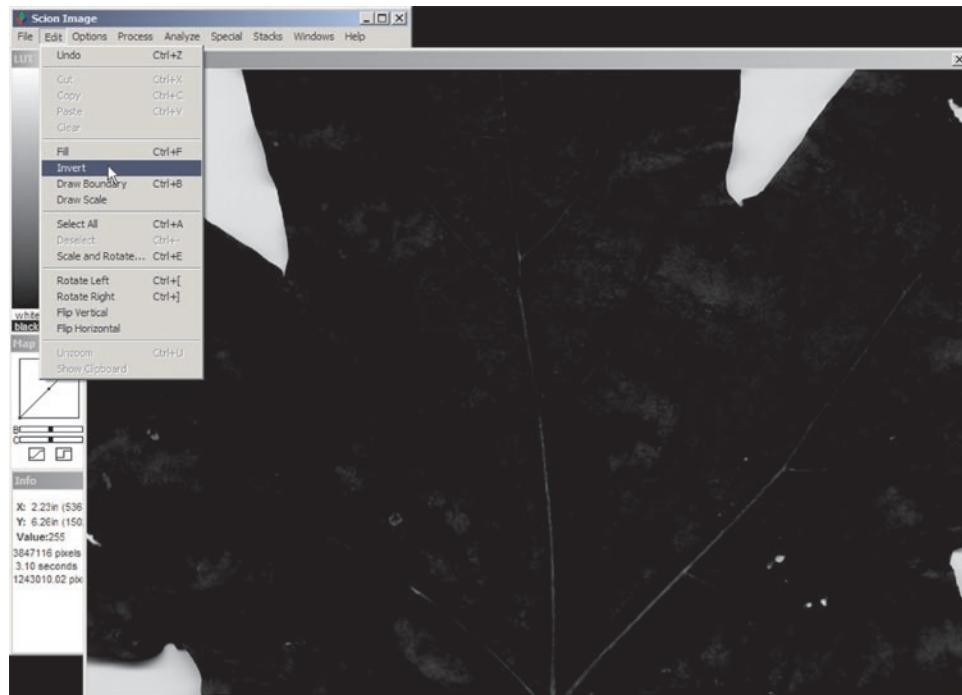
8. With window 003 selected, go to Process. Choose Rank Filters.



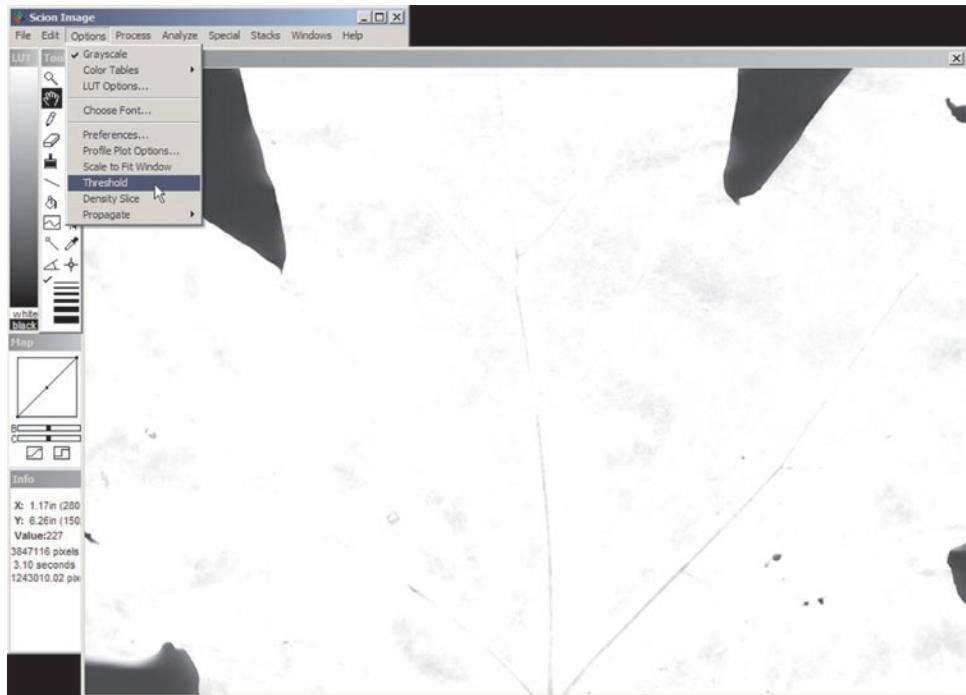
9. A dialog box is displayed containing a list of options. Median (Reduce Noise) should already be selected along with Iteration of 1. Select OK.



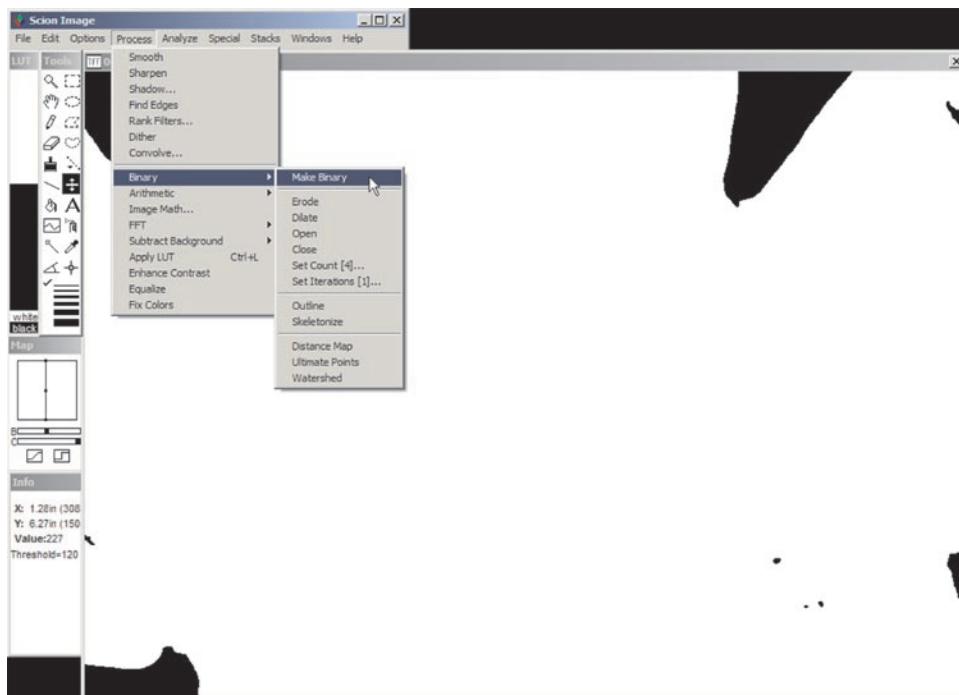
10. Select Edit and then Invert. This reverses the white and black sections of the leaves on the screen.



11. Go to Options and then Threshold. This separates objects of interest from background based on grayscale.

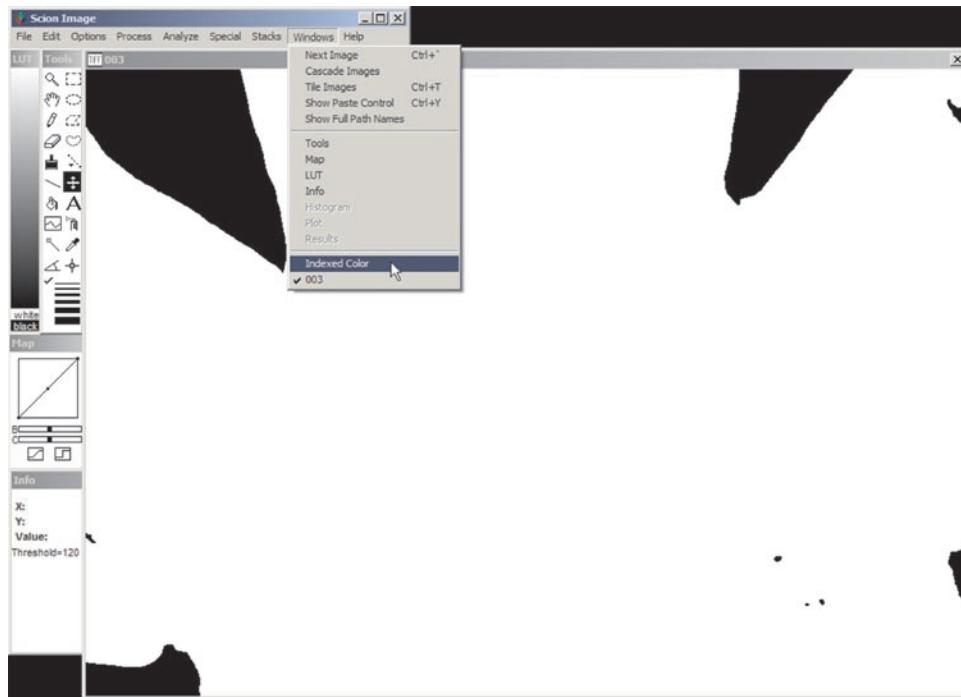


12. Go to Process and then Binary and then Make Binary. At this point, processing of the black and white image is complete. Do not close the window because it is needed later.



Preparing the Color Image

- To select the Indexed Color window, go to Windows and choose Indexed Color.



Removing Brown Areas Using the Paintbrush Tool

Areas of brown on the leaf caused by insect damage or necrosis should be removed so they are not measured as red. To remove the brown, proceed as follows:

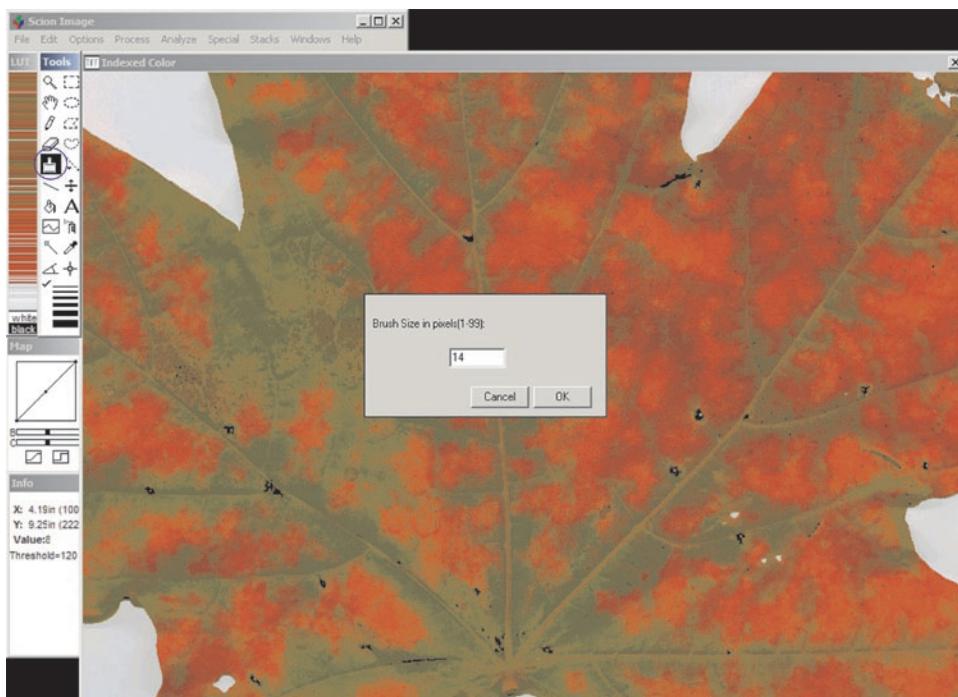


Using the Paintbrush Tool

14. a. Click on the paintbrush tool (█) from the Tools window found at the upper left of the screen. Move it into the *Look-Up Table* (LUT) window which can be found to the left of the Tools column. It turns into the eyedropper tool (☞). From the mosaic of colors shown in the LUT window, find a light gray or light blue and click on the color once.

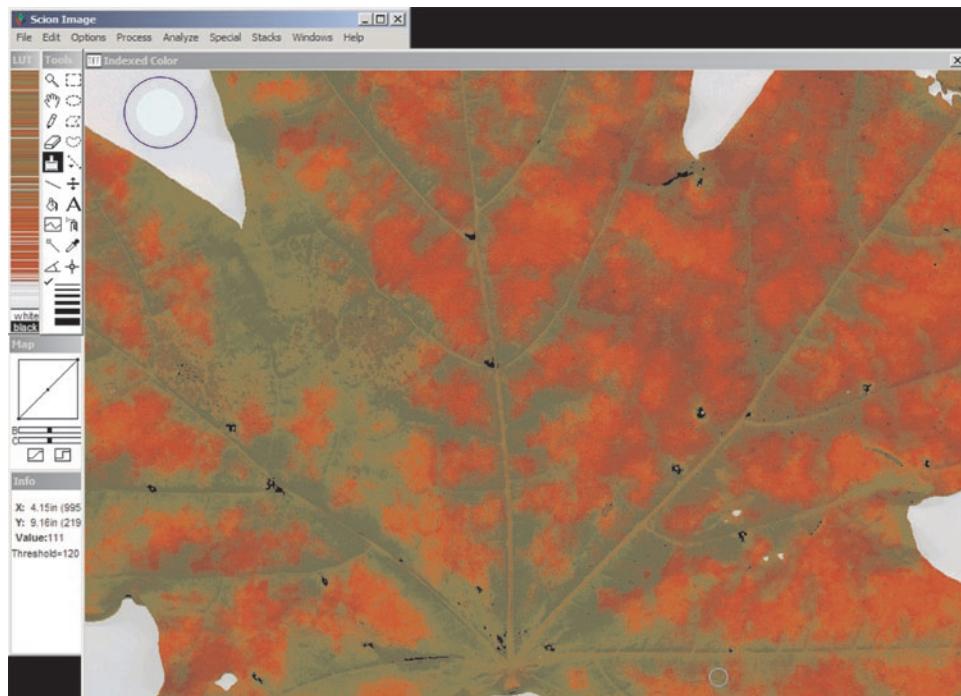


- b. The paintbrush tool (█) changes to the color chosen from the LUT window. The paintbrush can be resized to cover smaller or larger areas by double clicking on the tool and changing the pixel size.

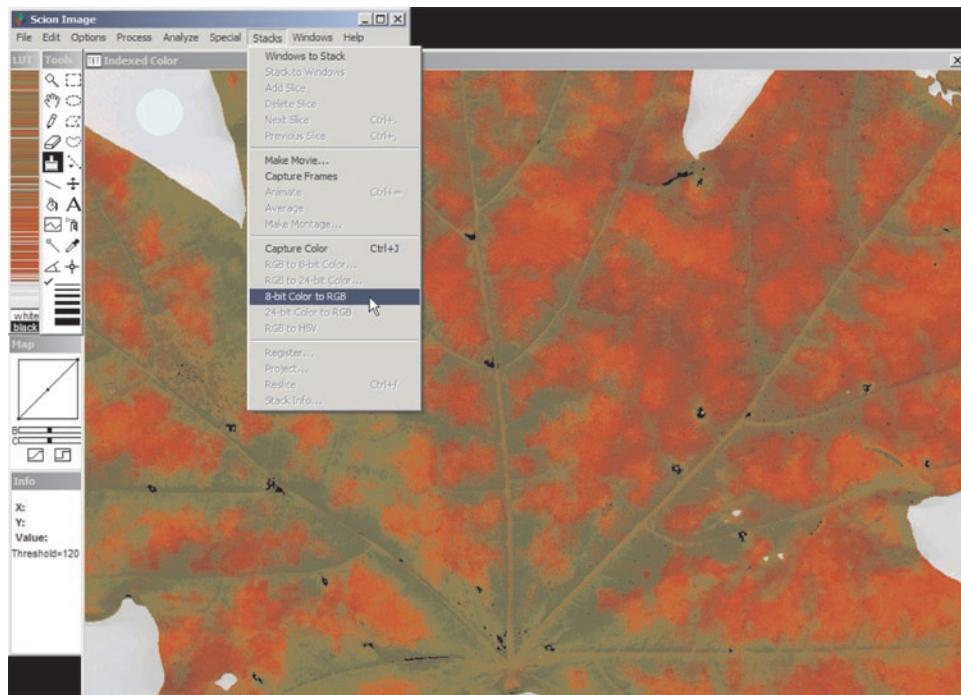


Practicing with the Paintbrush Tool

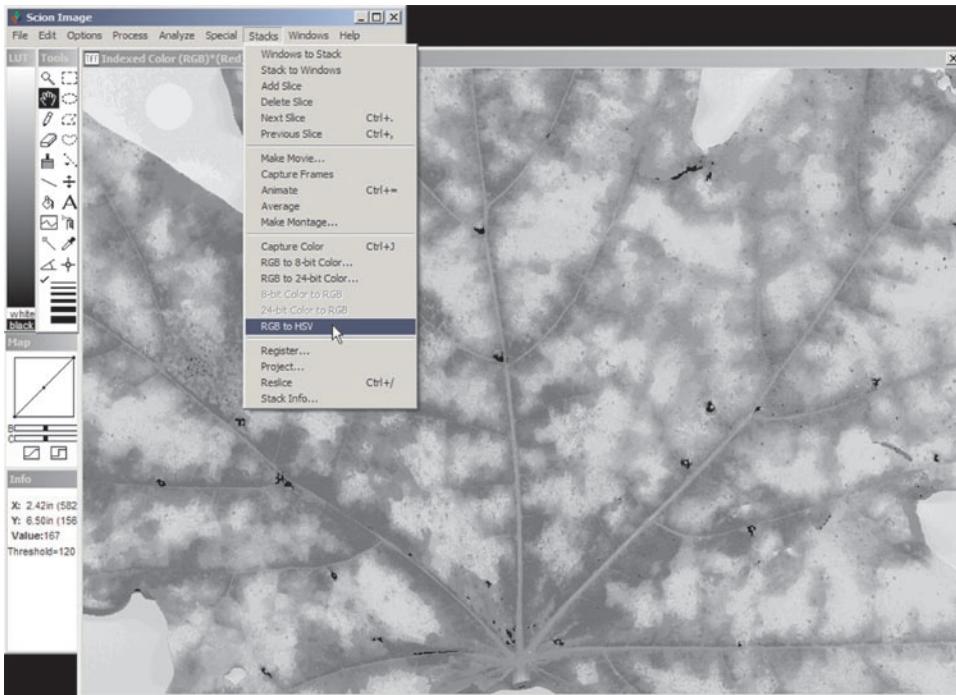
- c. Anywhere in the background, or “non-leaf” area of the Indexed Color window, click and hold while drawing a small test circle. Notice that the cursor is now a circle when used within the Indexed Color window.



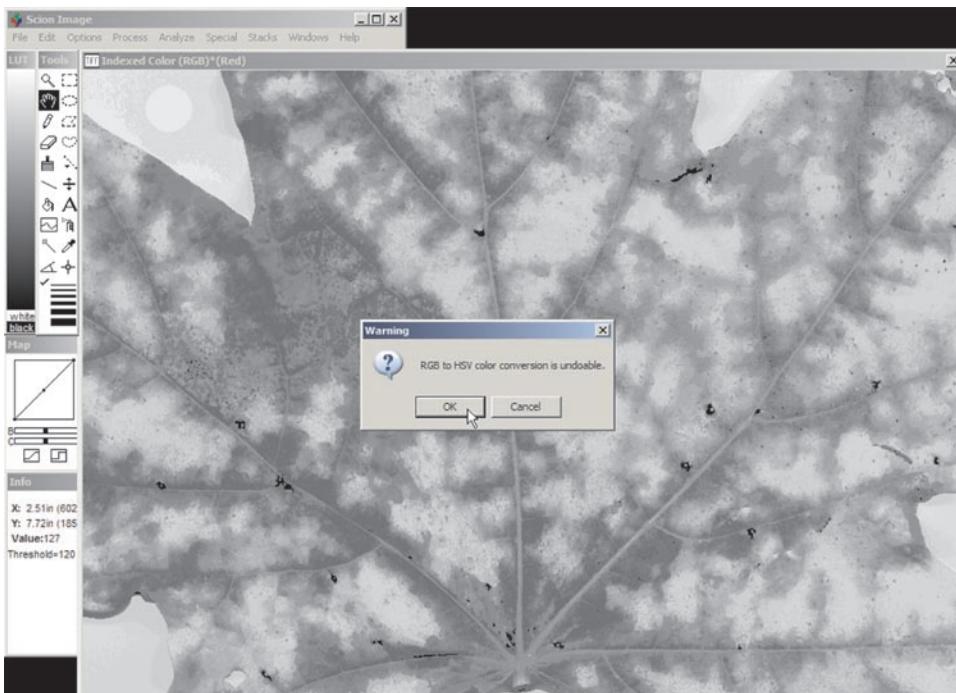
- d. Go to Stacks then 8-bit Color to RGB.



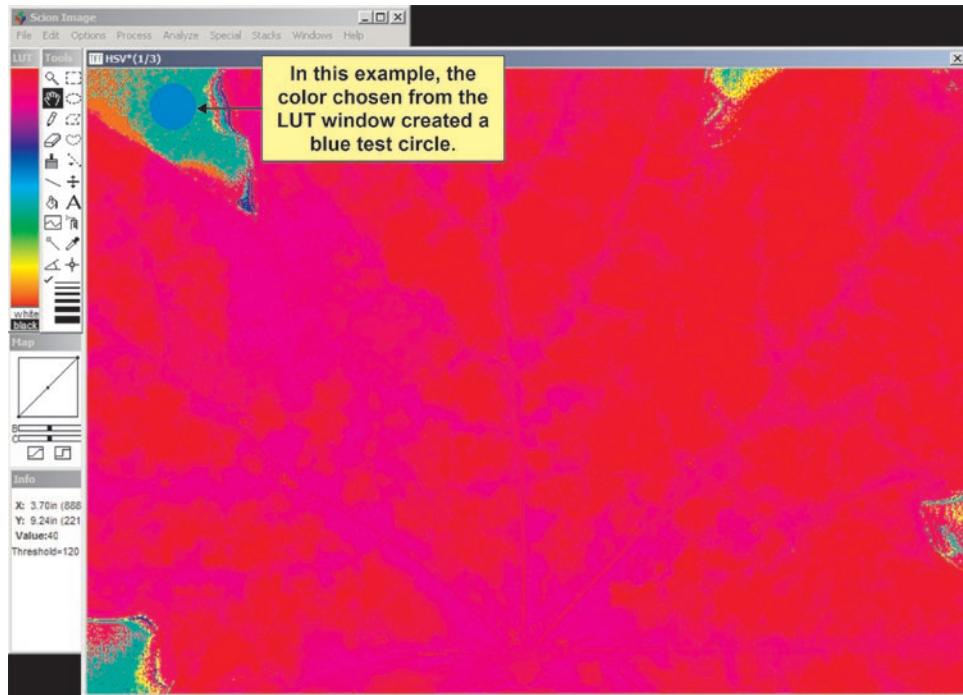
e. Go to Stacks again then RGB to HSV.



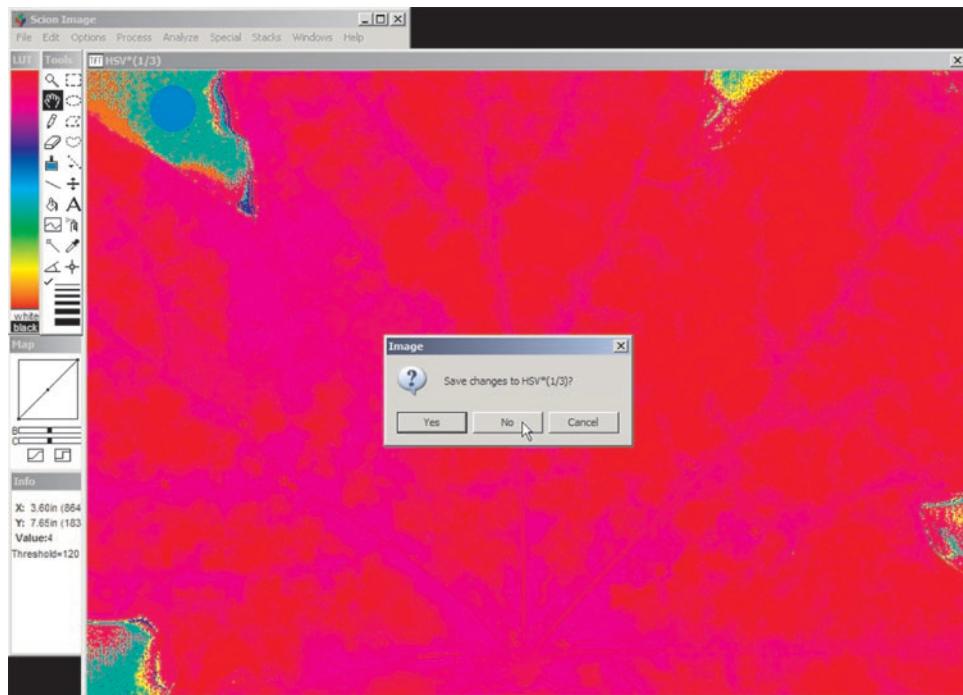
f. Select OK to the warning message.



g. 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 14a and repeat steps 14b-14f until the test circle is blue or yellow.

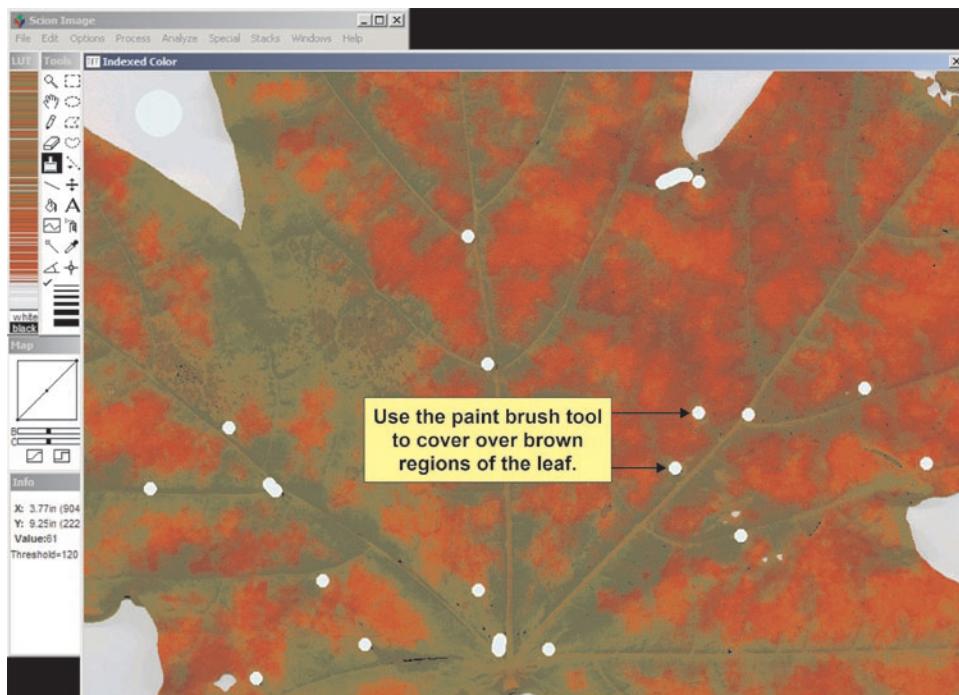


h. Close the HSV window. Do not save.

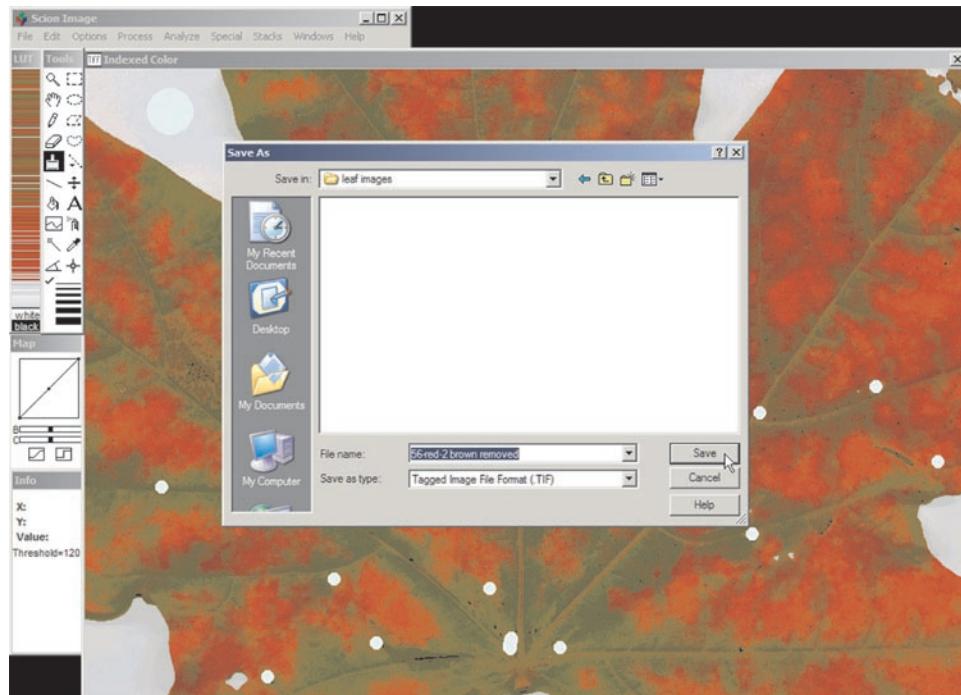


Masking Brown Areas

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 very 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.

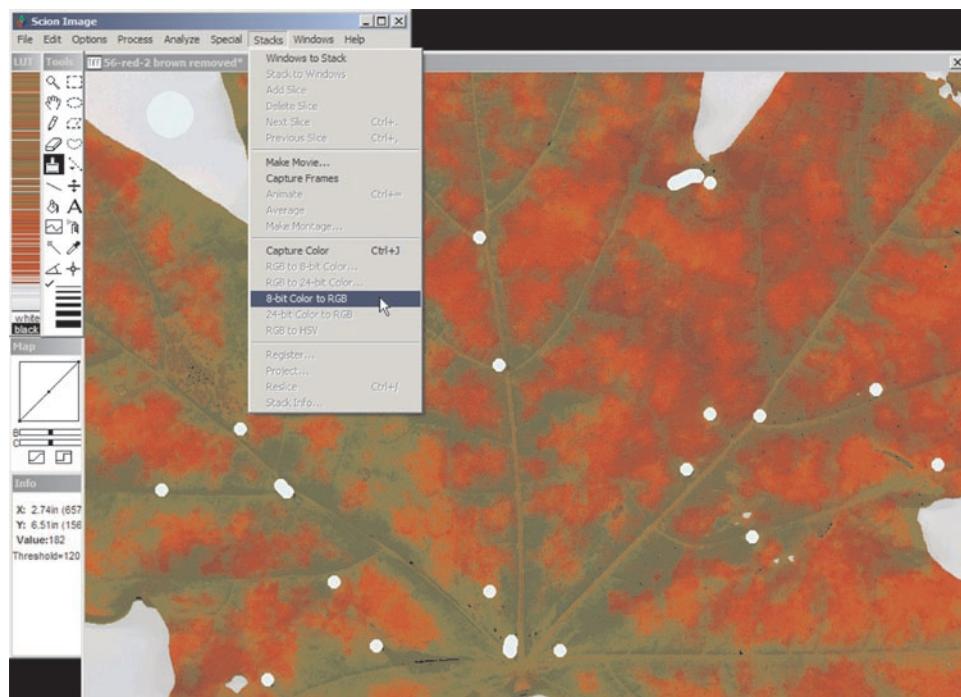


j. Some users prefer to save this image because coloring over brown areas can be very time consuming. By saving the image, the hassle of repeating preparatory steps can be eliminated should leaf color analysis on the same image be needed again. To save, go to File and then Save As. Assign the image a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

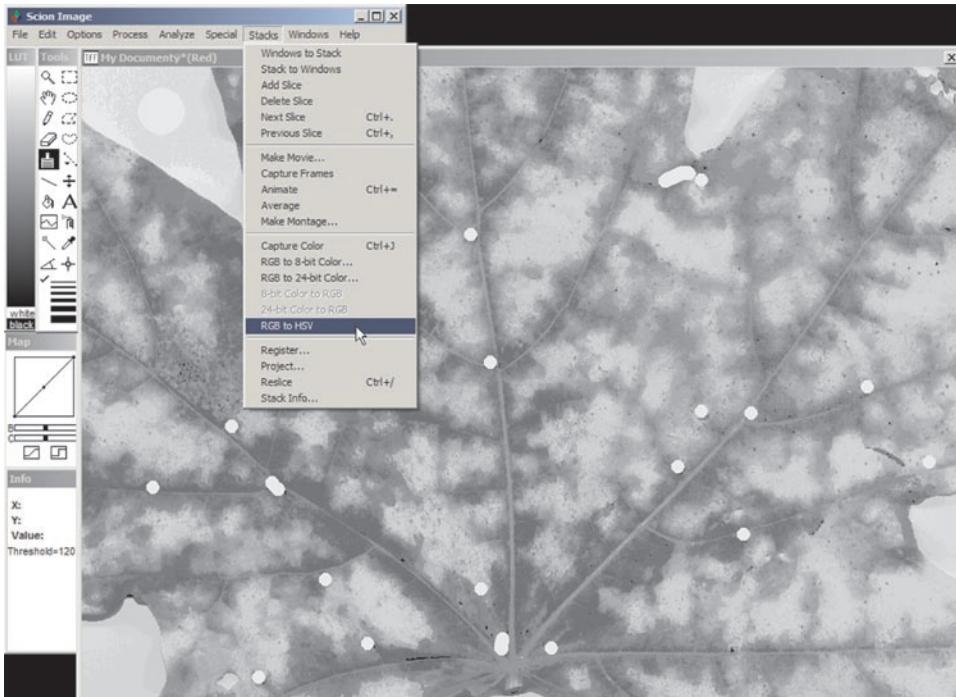


Converting Color Image to Hue, Saturation, and Value Slices

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



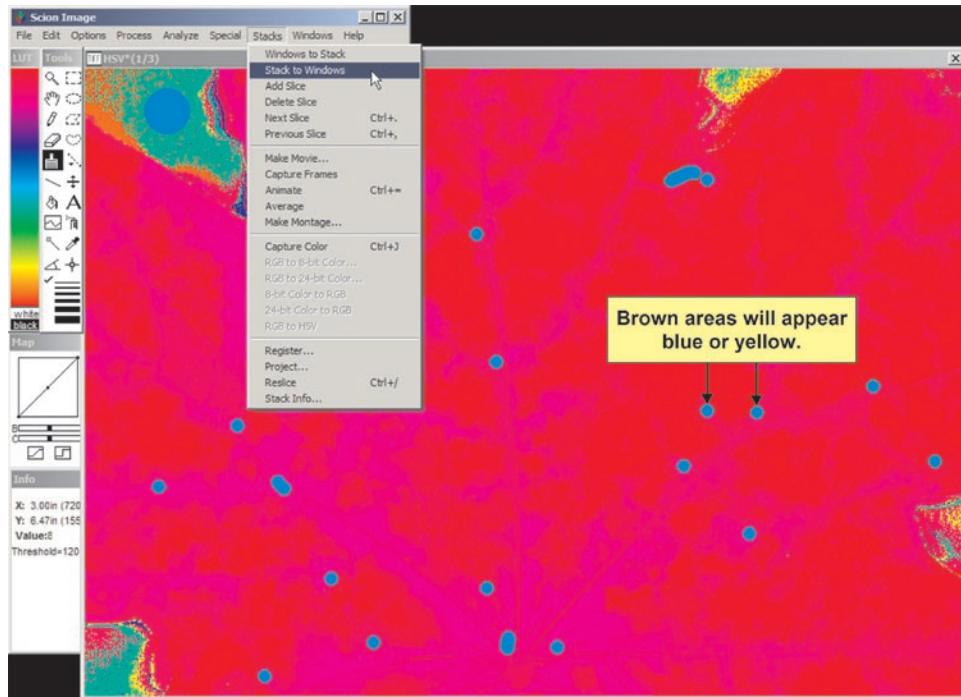
16. Go to Stacks and then RGB to HSV.



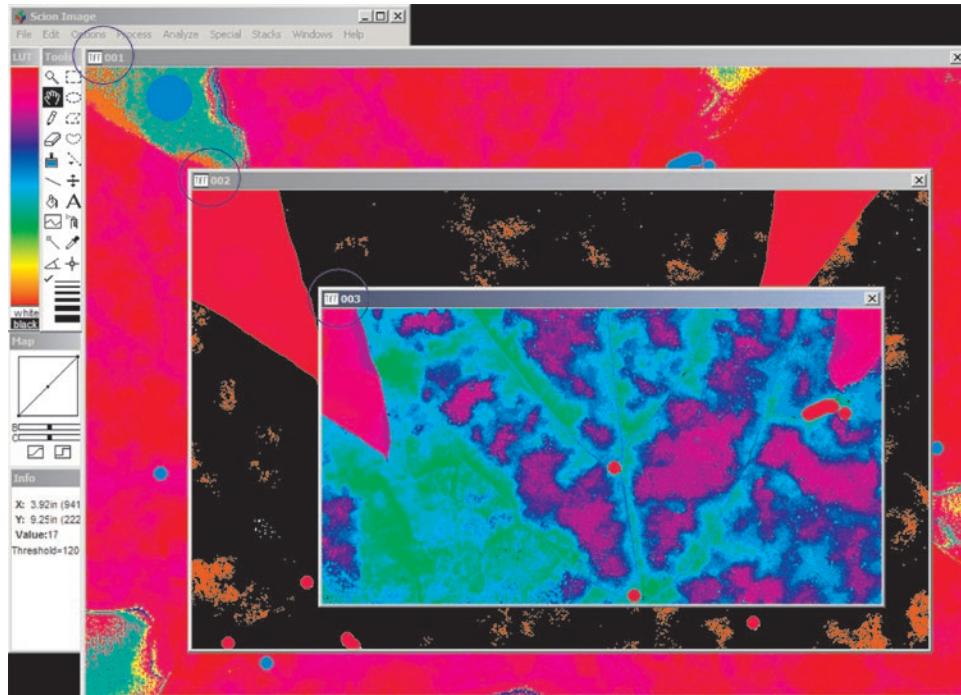
17. Select OK to the warning that is displayed.



18. Go to Stacks again then Stack to Windows.

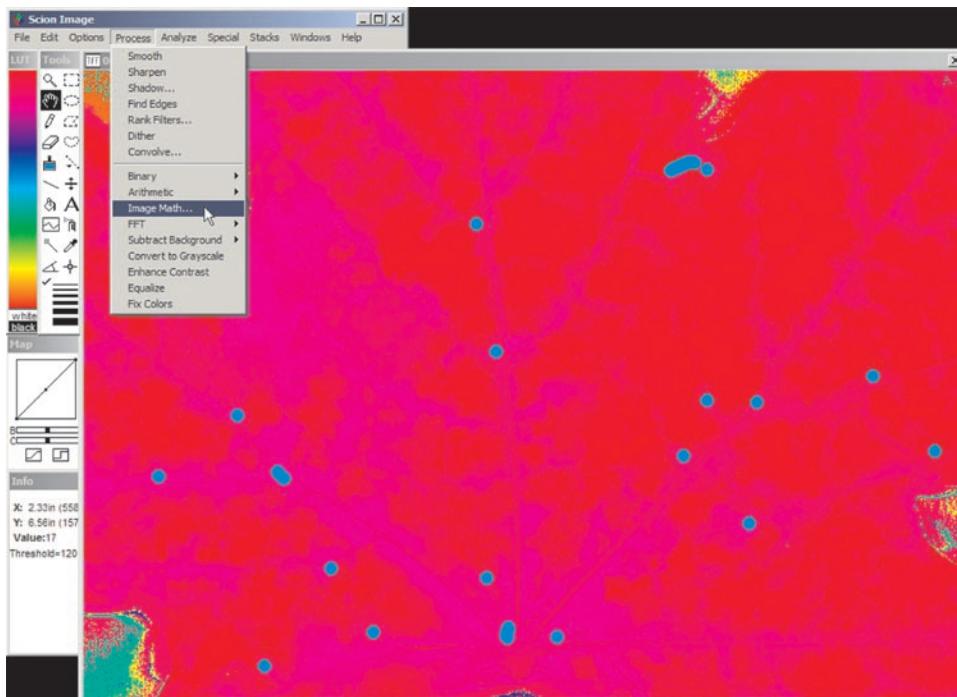


19. Three superimposed windows appear (labeled 001, 002, and 003). Close windows 002 and 003. Only window 001 should be visible now.

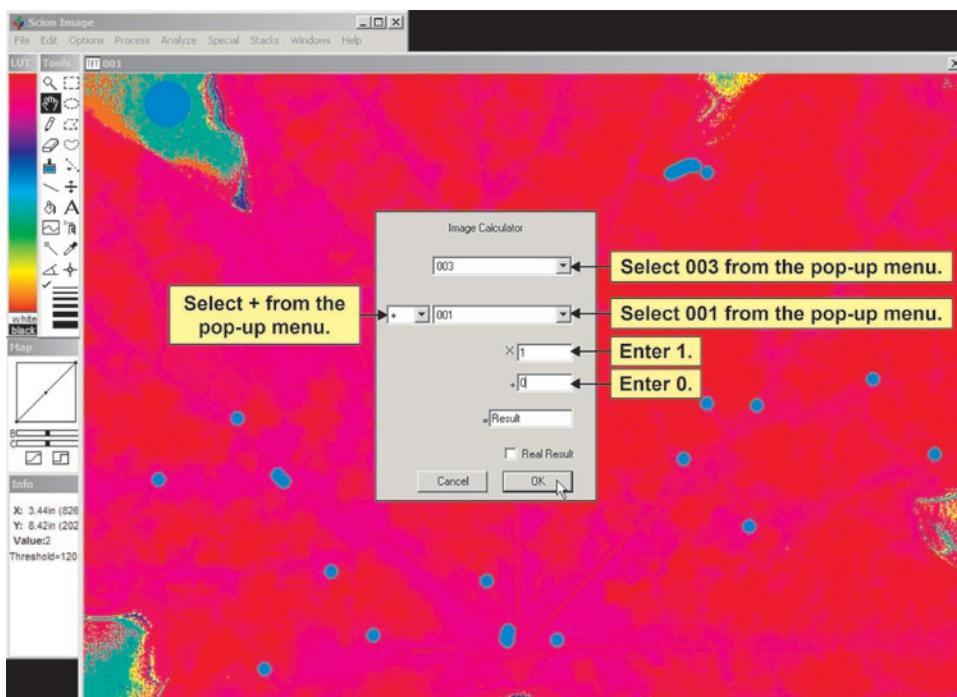


Combining Images Using the Image Calculator

20. Go to Process then Image Math.

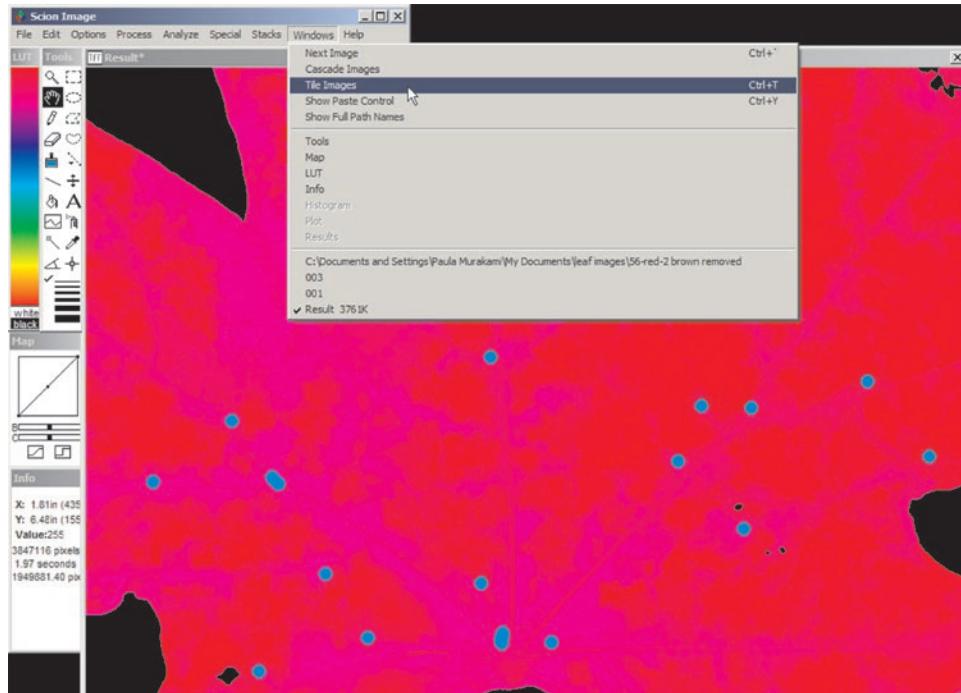


21. Using the Image Calculator that appears, set up the equation **exactly** as shown below. Real Result should not be selected. Choose OK.

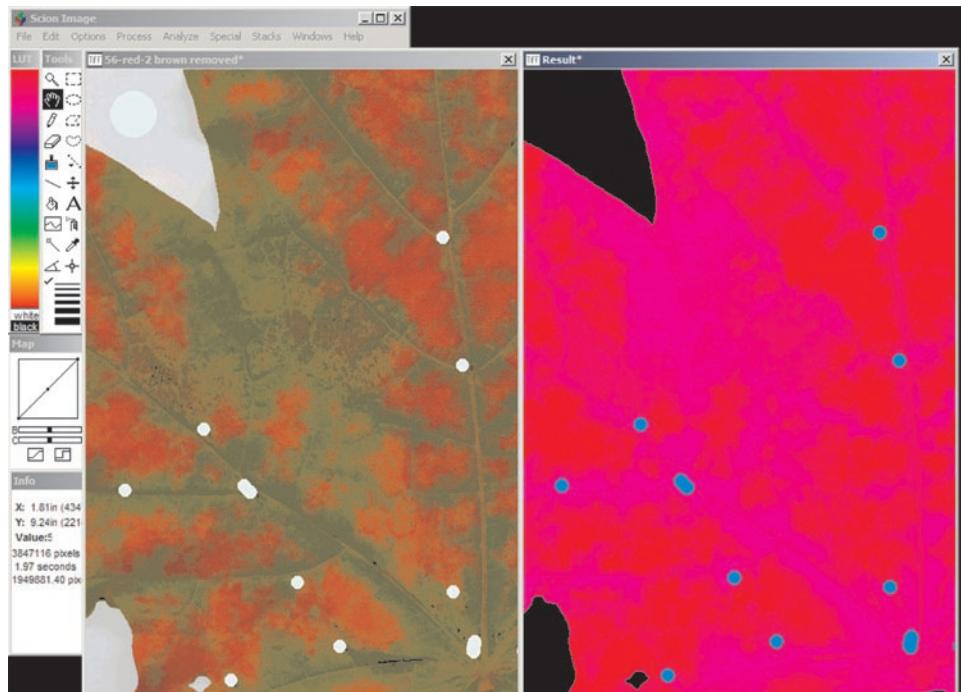


Viewing Indexed Color and Result Windows Simultaneously

22. Go to Windows and then Tile Images. This will reduce all open windows and display them simultaneously.

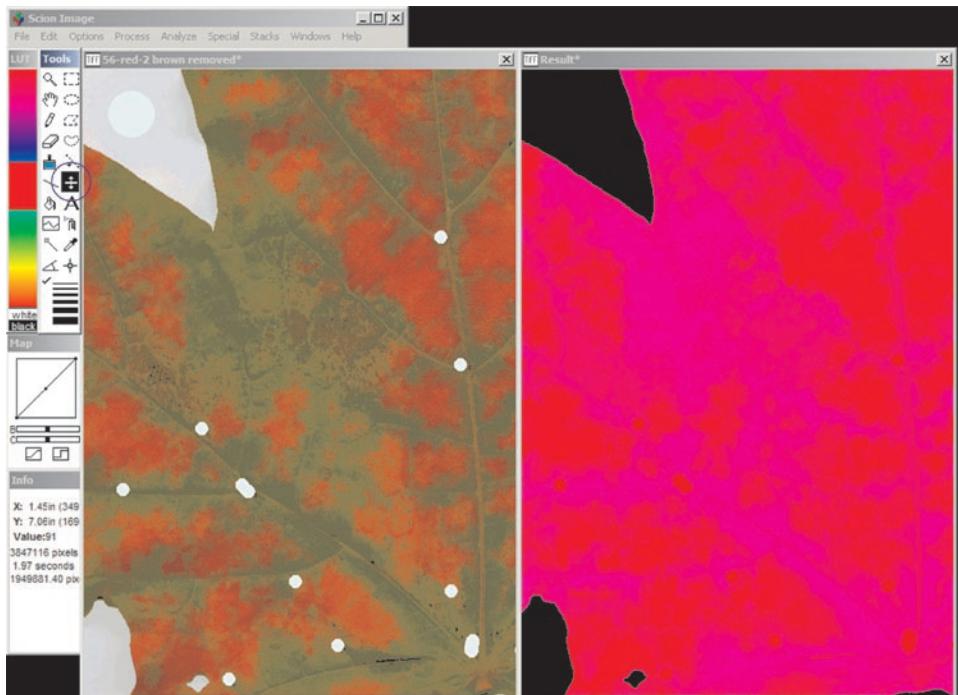


23. Extend the “brown removed” and Result windows by dragging the top or bottom of each window until they are adjacent.

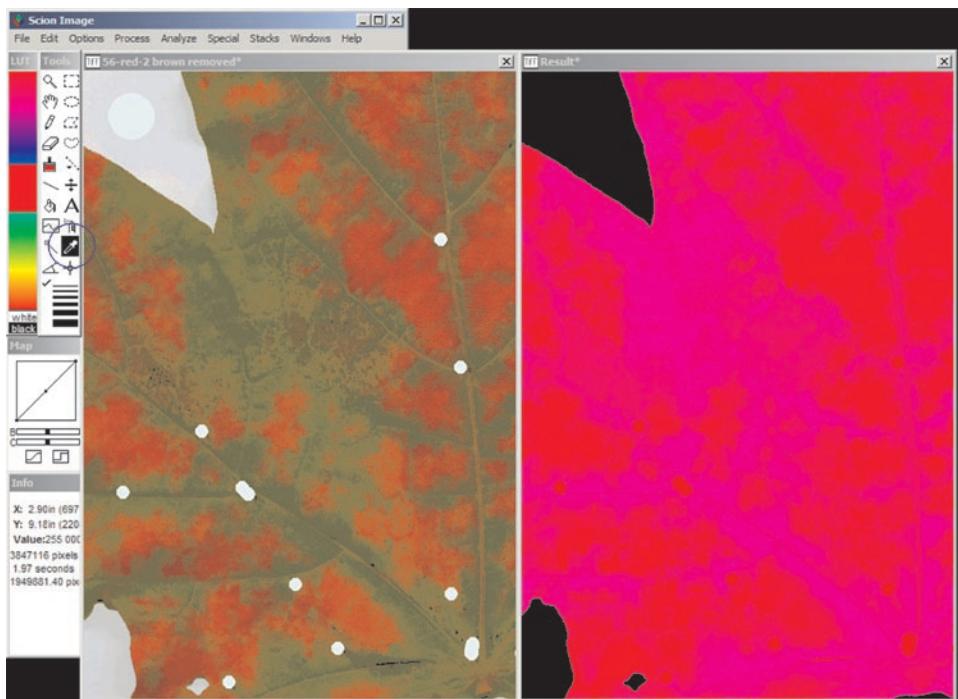


Changing the Color of the LUT Bar

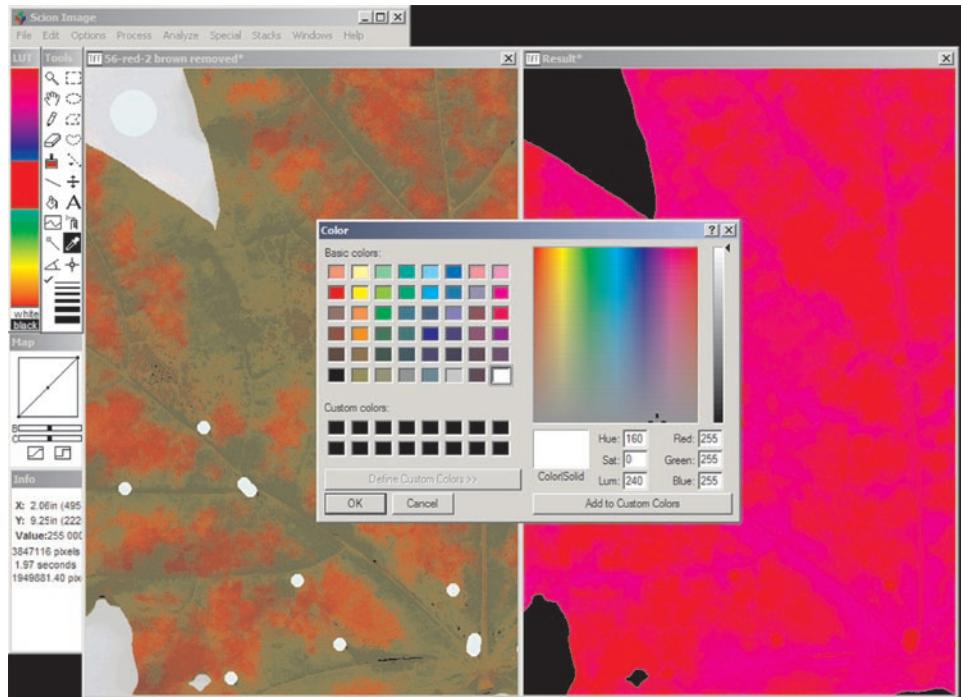
24. With the Result window selected, double click on the LUT tool () in the Tools window. A solid red bar of color is displayed in the LUT window. This needs to be changed to a different color. Each time Scion Image is initially opened, this bar is red.



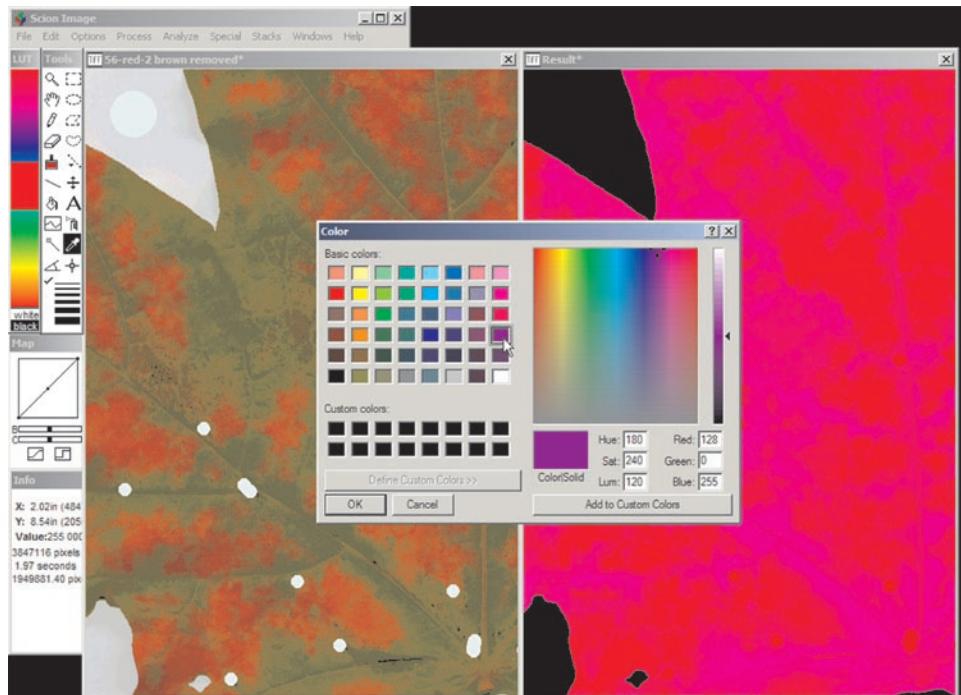
25. To change the color of the solid bar, click once on the eyedropper tool ().



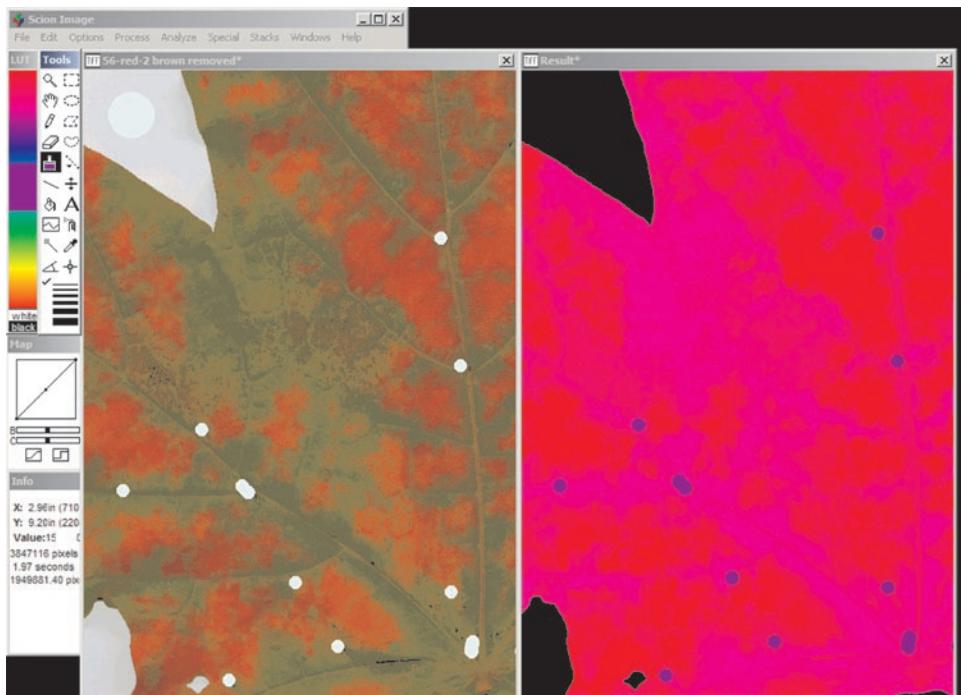
26. Double click in the red bar and a Color window will be displayed.



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



28. Notice that the red bar is now purple. Click on the paintbrush tool and it, too, turns purple.

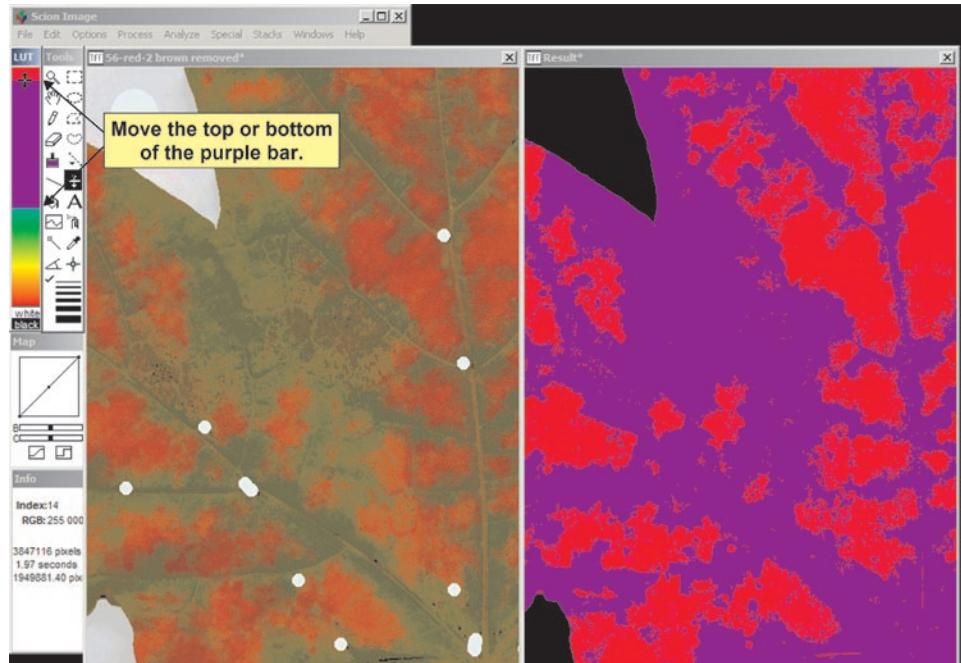


Aligning the Images

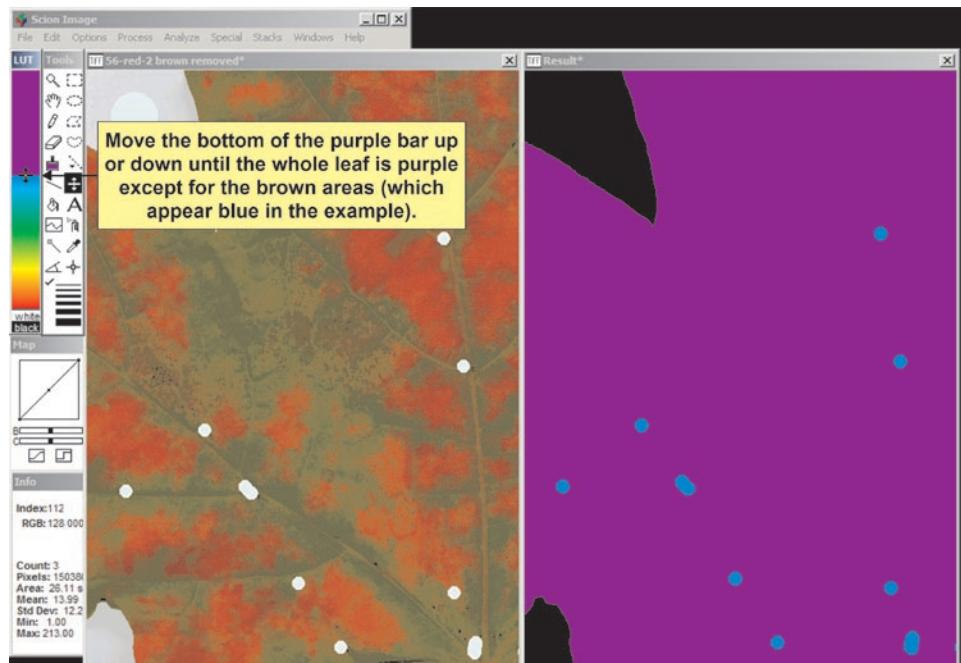
29. Be sure that the same areas of the leaf are visible in both the indexed color window and the Result window. If they are not similar, move the two images using the hand tool (). Choose the hand tool, click in the window, hold and move. You should see the leaf image move within the window. Align the 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 which cover brown areas in the Result window to make sure they are not included in the analysis.

Measuring Green

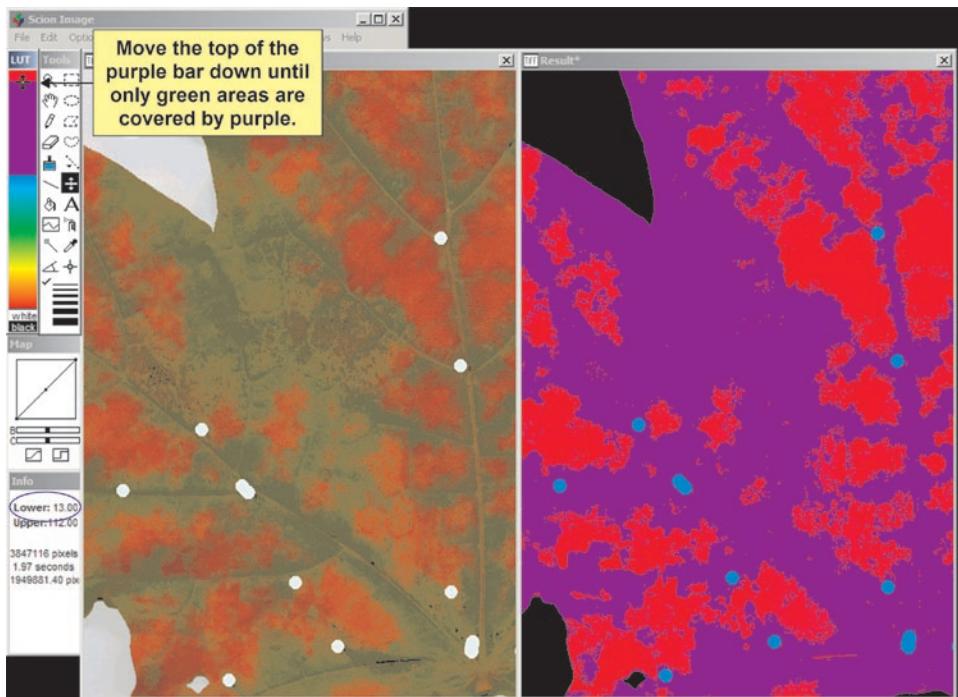
30. Select the Result window.
31. Click on the LUT tool (). It may be necessary to double click on this tool to see the purple bar. Experiment with moving the top and bottom limits of the purple bar. Click on the top or bottom of the bar, hold and move up or down. Notice how it covers areas of the leaf in the Result window with purple. 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.



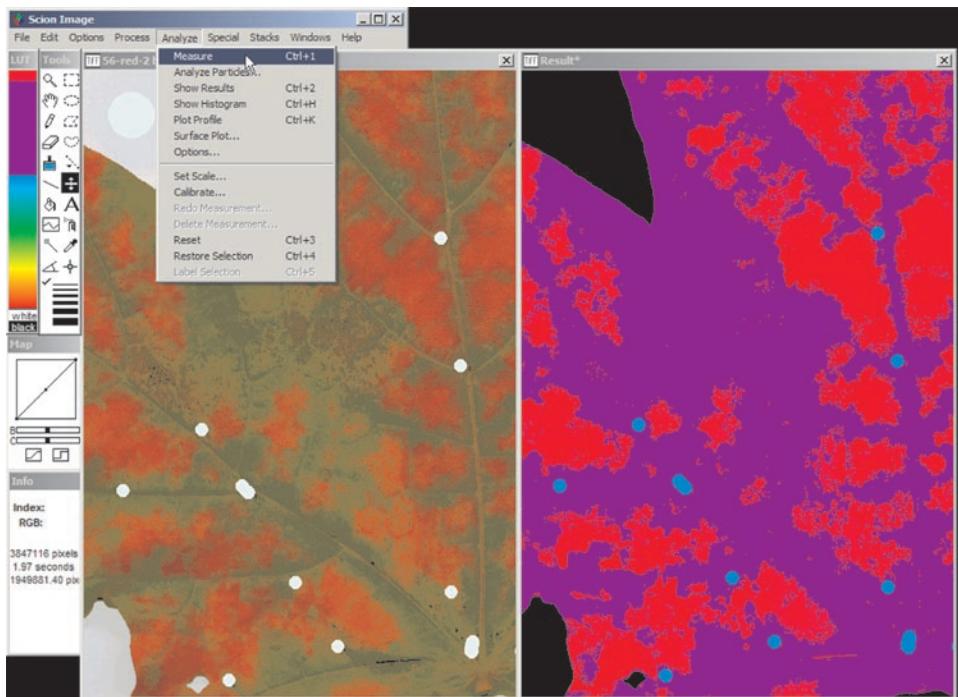
32. To measure green, click in the middle of the purple bar and move the whole bar to the top of the LUT window. This will cover most of the leaf in the Result window with purple. Now move the bottom of the bar up or down until the whole leaf is purple, *except for the brown areas which are blue or yellow.*



33. 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 is referred to again when measuring red.

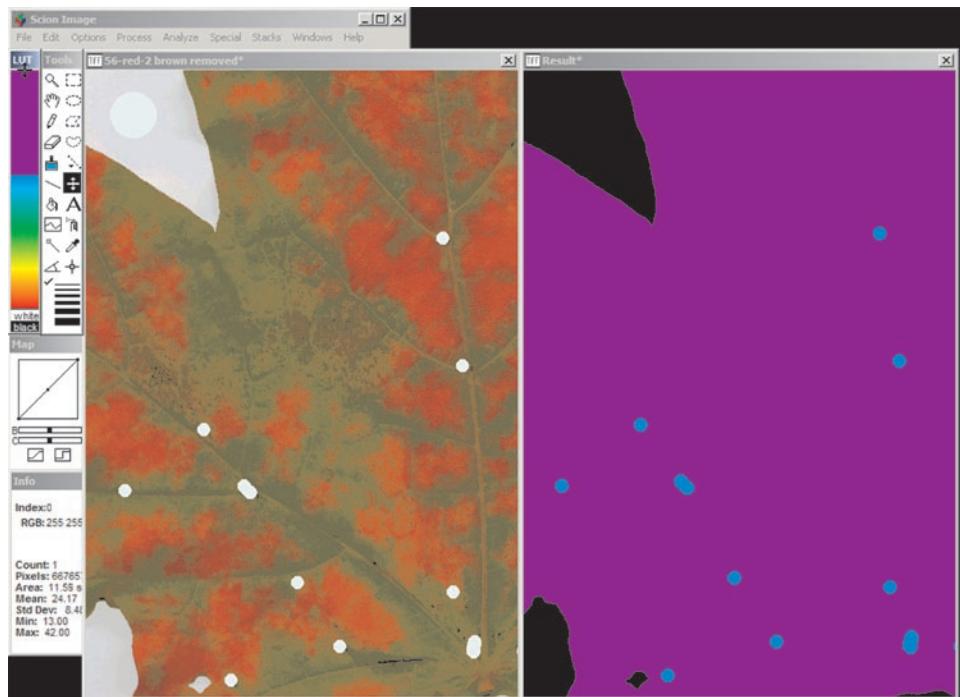


34. When all green areas of the leaf are highlighted by purple, it is time to measure. To do this, click Analyze and then Measure.

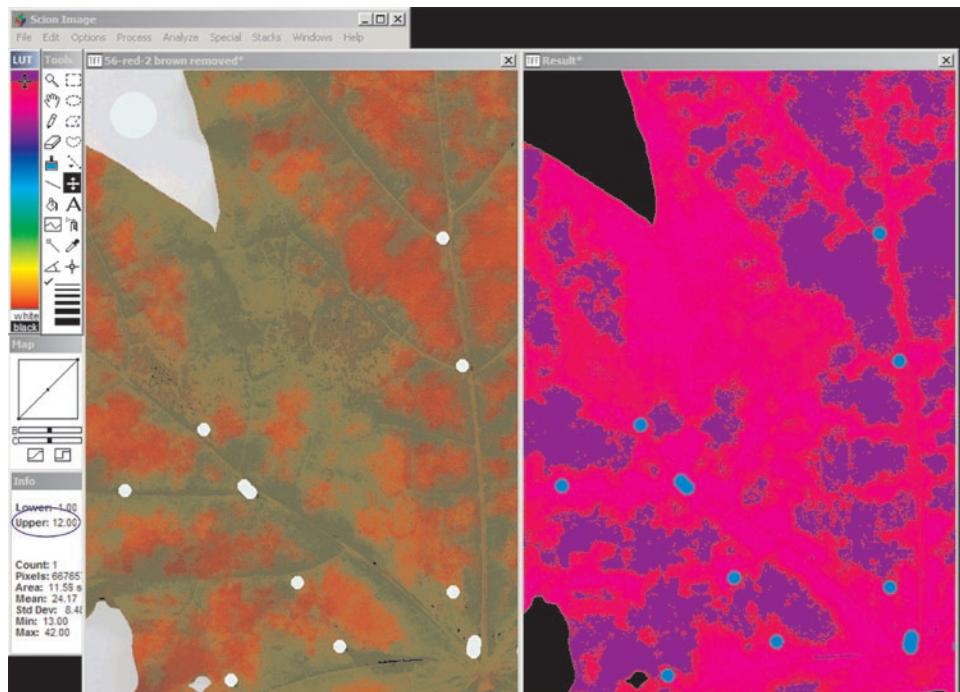


Measuring Red

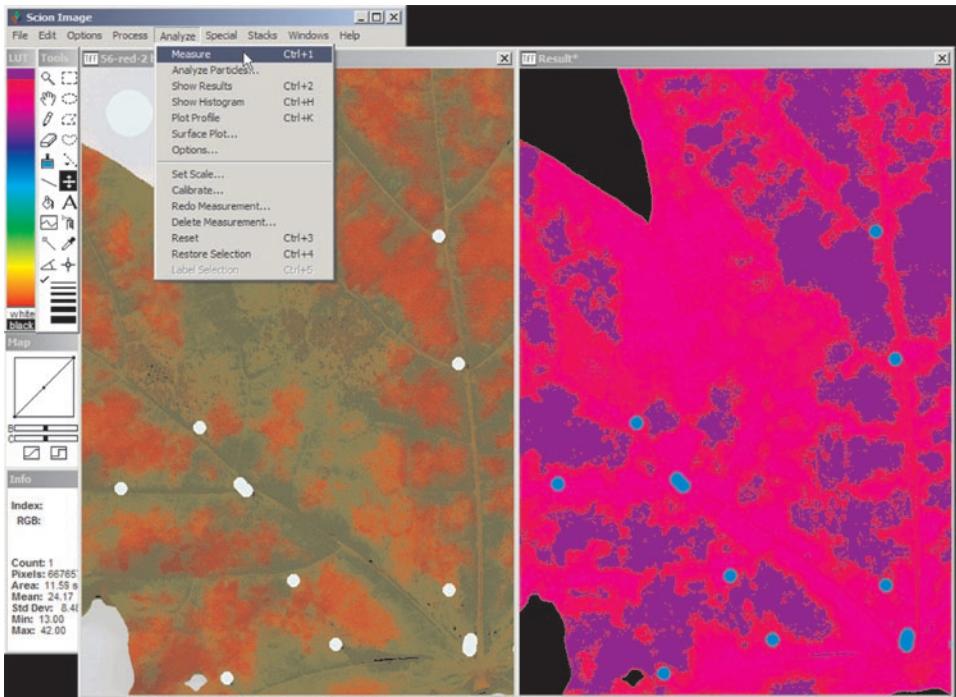
35. To measure red color, once again move the top part of the purple bar to the top of the LUT window.



36. Move the bottom of the purple bar up until the red areas of the leaf are highlighted by purple. Observe the Upper number in the Info box; in this example it is 12. This number must be **less** than the Lower number recorded in step 33 to avoid measuring areas that have already been analyzed.



37. Once again Analyze, then Measure.

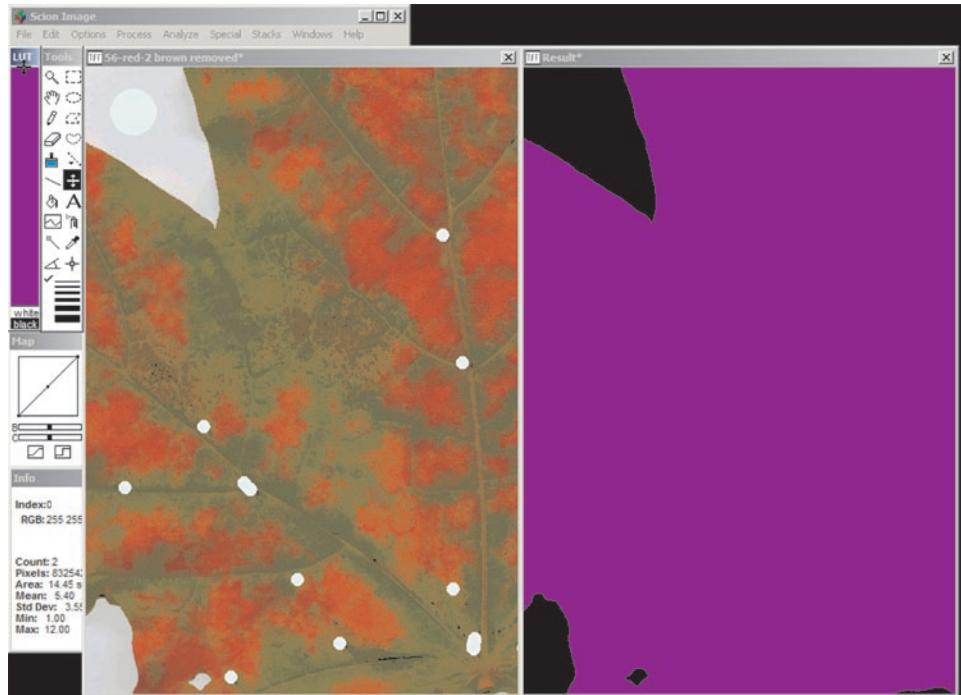


38. Sometimes red can also be measured in the lower region of the LUT bar, so it is good practice to move the purple bar all the way to the bottom of the LUT window. In doing so, look for additional red areas of the leaf to be covered by purple in the Result window. If red areas are highlighted by purple, 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.

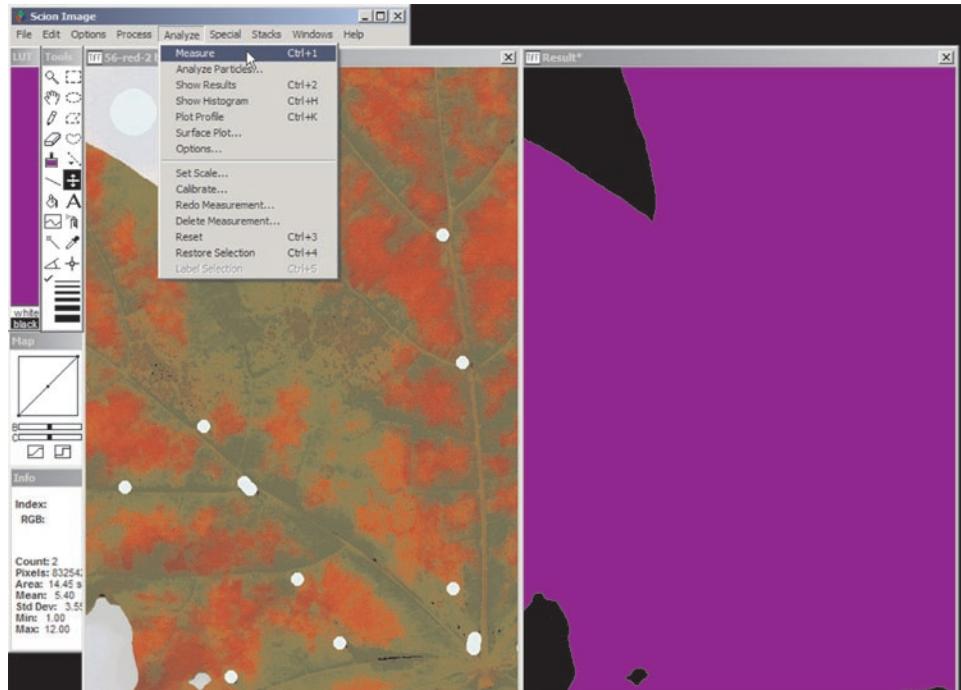


Measuring Total Leaf Area

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

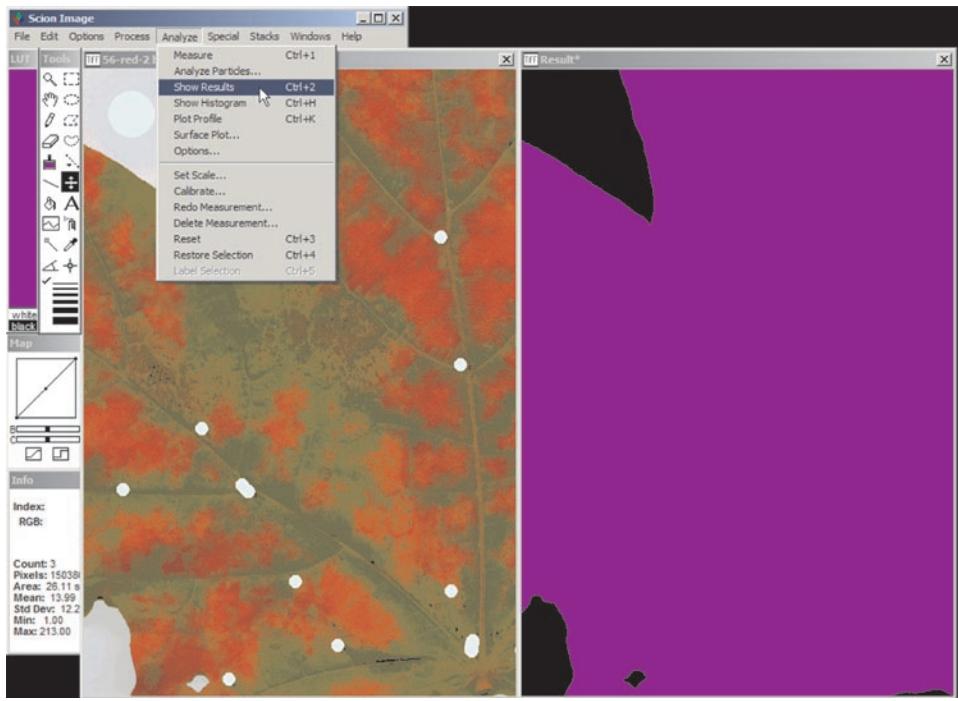


40. Click on Analyze and then Measure. This measures the total leaf area (including brown areas).

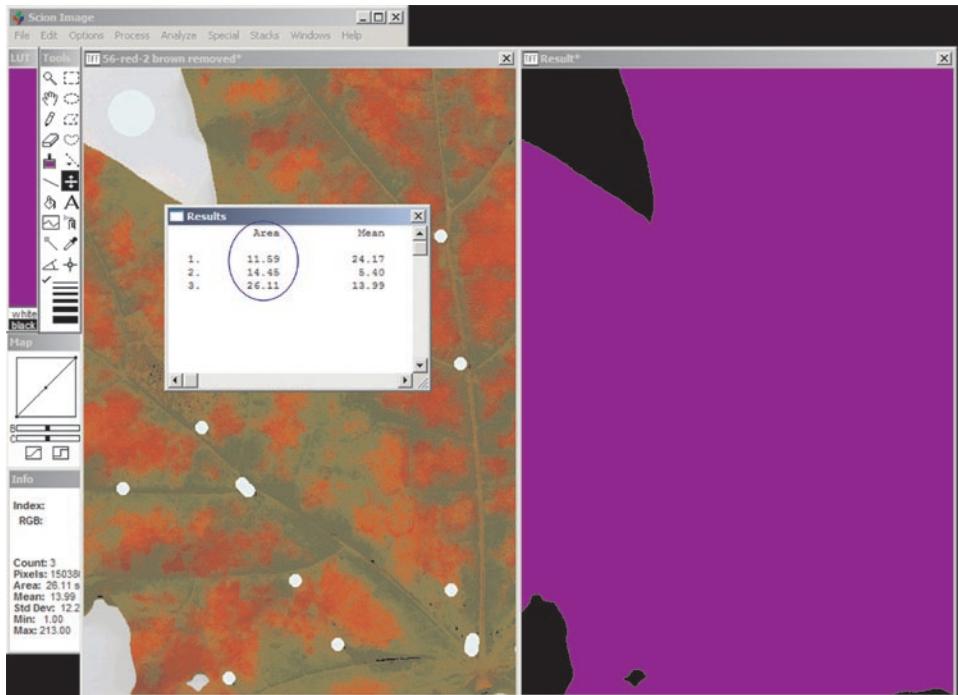


Viewing Results

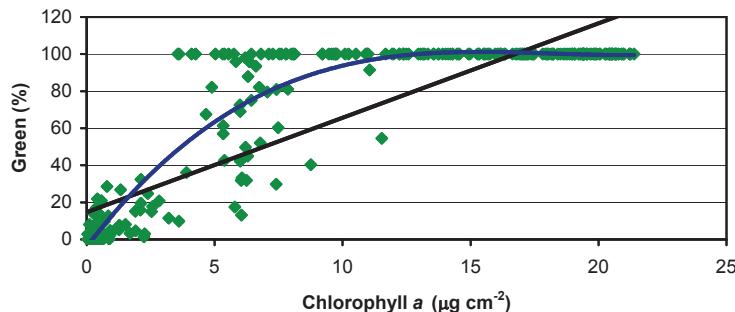
- To view actual values of measured leaf areas, choose Analyze and then Show Results.



- 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 the combined green and red areas 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 remeasured.

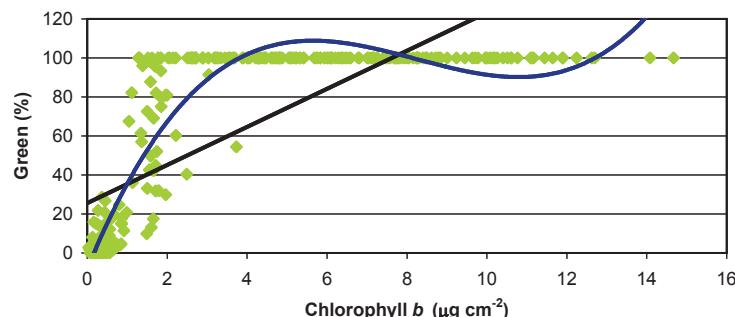


- Using these numbers, calculate the percentage green and red area for each scanned leaf image.



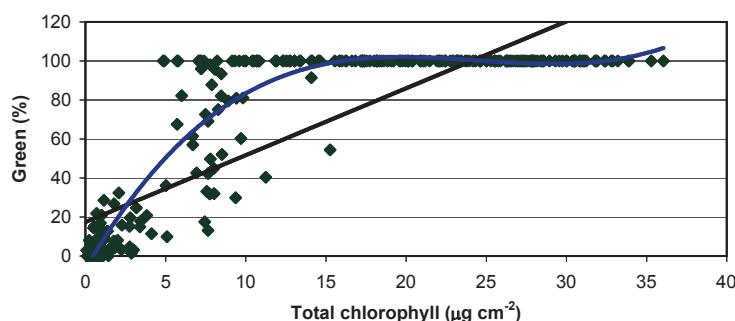
$$R^2 = 0.77; \text{Green} (\%) = 14.69 + 5.09(\text{chlorophyll } a)$$

$$R^2 = 0.92; \text{Green} (\%) = 52.44 + 4.17(\text{chlorophyll } a) - 0.51(\text{chlorophyll } a - 9.11)^2 + 0.02(\text{chlorophyll } a - 9.11)^3$$



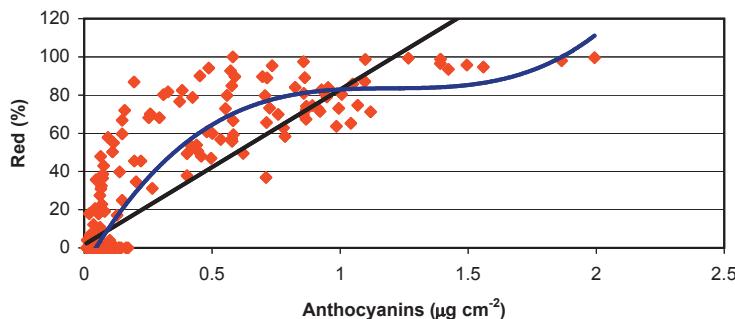
$$R^2 = 0.58; \text{Green} (\%) = 25.53 + 9.77(\text{chlorophyll } b)$$

$$R^2 = 0.89; \text{Green} (\%) = 54.96 + 11.85(\text{chlorophyll } b) - 3.78(\text{chlorophyll } b - 3.64)^2 + 0.28(\text{chlorophyll } b - 3.64)^3$$



$$R^2 = 0.72; \text{Green} (\%) = 17.39 + 3.43(\text{total chlorophyll})$$

$$R^2 = 0.92; \text{Green} (\%) = 57.36 + 2.84(\text{total chlorophyll}) - 0.29(\text{total chlorophyll} - 12.75)^2 + 0.01(\text{total chlorophyll} - 12.75)^3$$



$$R^2 = 0.72; \text{Red} (\%) = 1.62 + 81.28(\text{anthocyanin})$$

$$R^2 = 0.83; \text{Red} (\%) = -1.64 + 153.65(\text{anthocyanin}) - 156.78(\text{anthocyanin} - 0.23)^2 + 53.66(\text{anthocyanin} - 0.23)^3$$

Figure 1.—Linear and cubic relationships between percentage color and pigment (chlorophyll and anthocyanin) concentrations for 326 scanned images of sugar maples leaves.

Comparison of Percentage Color Versus Pigment Concentration

In order to assess the accuracy of using digital image color analysis as an indicator of foliar pigment concentrations, percentage 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/H₂O for extraction of chlorophylls or HCl/H₂O/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, UK). 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 ($0.24 \times A_{653}$) was subtracted (Murray and Hackett 1991). Regression analyses were conducted to determine the relationship between percentage leaf color and pigment concentration using the least squares method (SAS 2002) (Fig. 1). Linear regressions for each color were significant at $P<0.05$. However, because entirely green or red leaves could only be measured as 100 percent green or red regardless of how much chlorophyll or anthocyanin accumulated, a saturation in percentage color was sometimes observed. Due to this saturation effect, associations between percentage color and pigment concentration were better accounted for by polynomial regressions (Fig. 1).

Conclusion

Analyses with sugar maple leaves indicate that digital image analysis provides an accurate means of quantifying foliar color and estimating pigment concentration in multicolored leaves. 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.

Glossary

Note: a more detailed description of terms and specific functions can be found in the User Manuals on the Scion Corporation website: www.scioncorp.com.

8-bit Color to RGB: creates a stack with three slices (red, green, blue) from an 8-bit indexed color image.

Binary, Make Binary: creates a binary (black and white) image from a grayscale image.

Image Math: executes an arithmetic operation between selected images.

Invert: inverts pixel values.

Look-up Table (LUT): all images have a concurrent look-up table which displays pixel values (0-255) as colors.

Rank Filter (Median): reduces noise by replacing all 9 pixels in a “3 x 3 neighborhood” with the median value of that neighborhood.

RGB to HSV: creates a HSV (hue, saturation, value) image from a RGB image.

Stack: a three-dimensional image comprised of two or more slices.

Stack to Windows: converts a stack into windows which correspond to the individual slices of that stack.

Threshold: displays objects of interest and background based on gray level. Background pixels are ignored when measuring area.

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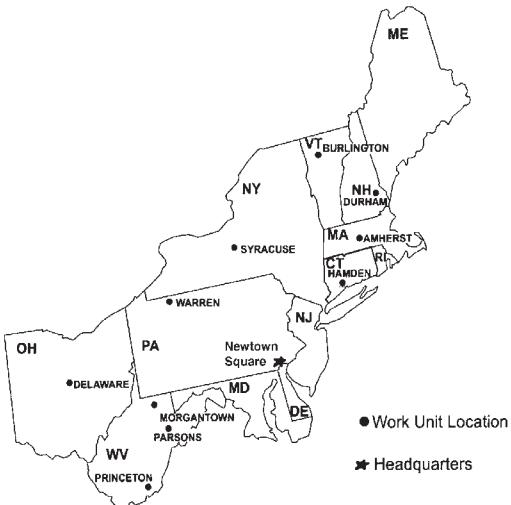
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Digital color analysis has become an increasingly popular and cost-effective method utilized by resource managers and scientists for evaluating foliar nutrition and health in response to environmental stresses. We developed and tested a new method of digital image analysis that uses Scion Image or NIH image public domain software to quantify leaf color. This publication provides instructions for using this software to measure the percentage 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 software, NIH Image software



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