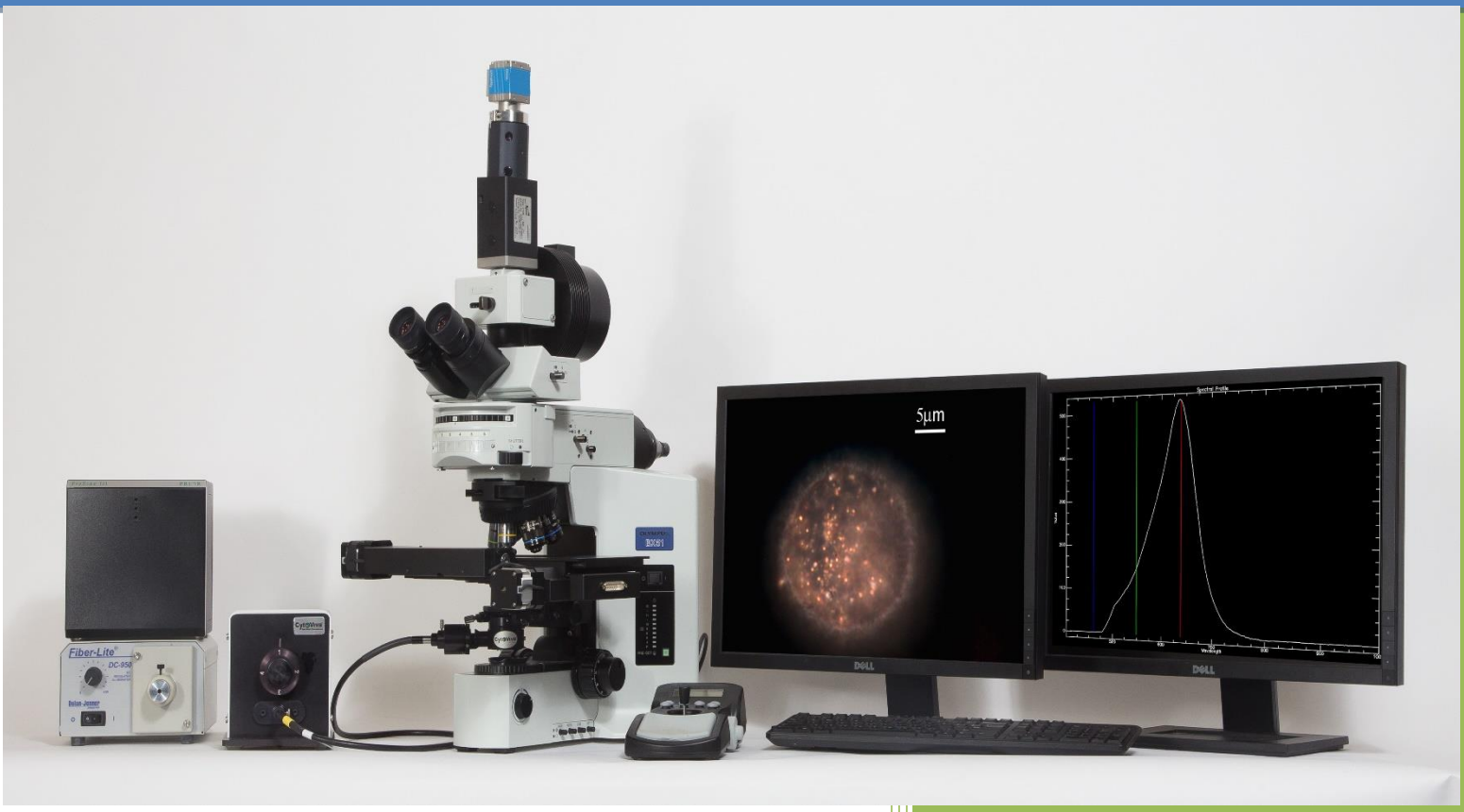
CytoViva®

Hyperspectral Microscope User Manual



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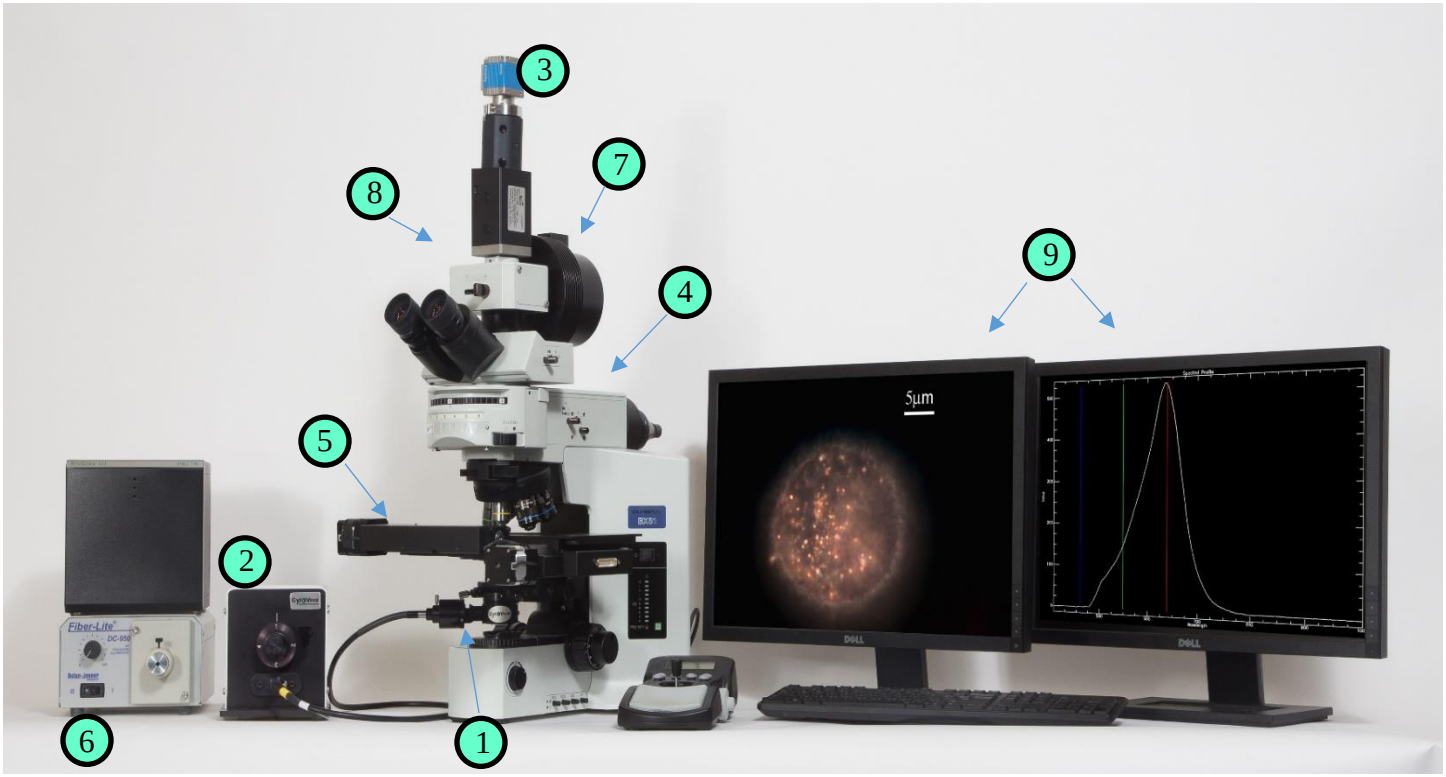
Section 1 CytoViva Hyperspectral Imaging System Installation

1.1 Parts of the Hyperspectral Imaging System



1. CytoViva Enhanced Darkfield Illuminator
2. CytoViva Dual Mode Fluorescence (DMF) Module
3. VNIR Spectrophotometer with Integrated Camera
4. Optical Microscope
5. Motorized Stage
6. 150W Halogen Light Source
7. Optical Camera
8. Dual Port
9. Computer

Section 2 Acquiring an Image & Basic ENVI Features

2.1 Starting CytoViva ENVI Software

- Open ENVI 4.8.
  - Select the CytoViva Hyperspectral Microscope tab.
  - Select CytoViva HSI Microscope Controls (Figure 2.1a).
  - The CytoViva HSI Microscope Controls GUI will open (Figure 2.1b).

Figure 2.1a

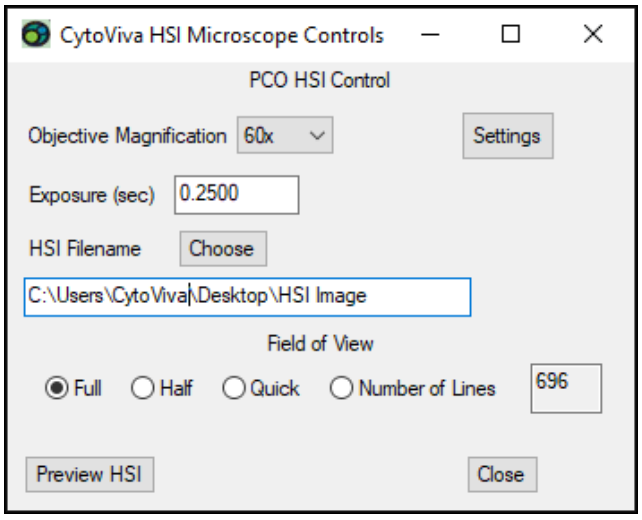


Figure 2.1b

- Select the Objective Magnification that you are going to scan the sample with. The default list contains 10X, 20X, 40X, 50X, 60X, 100X.

- Skip to the HSI Filename and select Choose.

- Once the file window opens, browse to the location where the data will be saved.
  - Enter the file name.
  - Click Open.
    - If the file name already exists, the user will be asked if they want to overwrite the file.

- Select the appropriate field of view (default is Full).
  - Full = the scan will produce an image with equal height and width.
  - Half = the scan will produce ½ of the full length image.
  - Quick = the scan is 21 lines ← this is useful to obtain a quick check of the recording settings.
  - Number of Lines = prompts the user to select the number of lines in the scan. Regardless of how many lines are scanned, the image will be created from the center outwards.

- Preview HSI: CytoViva HSI Microscopy Preview Window - Figure 2.1c.

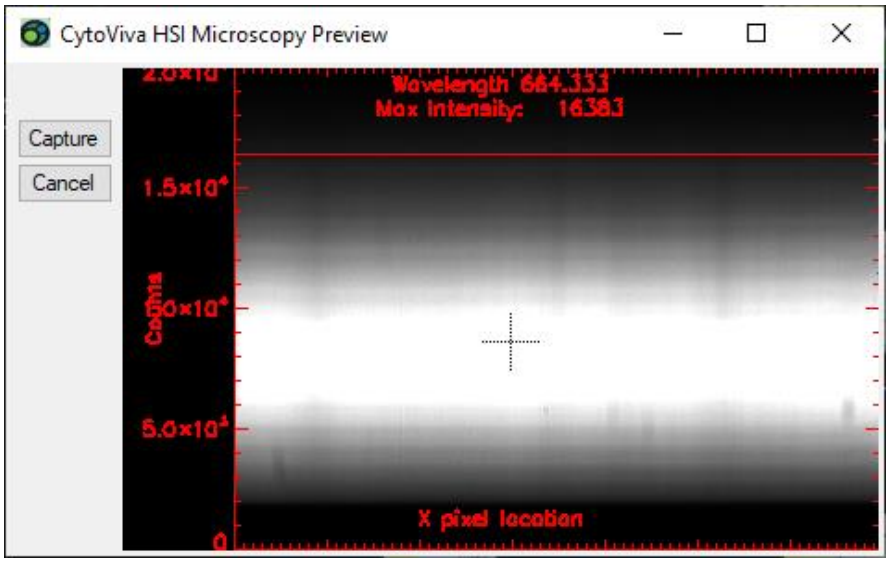


Figure 2.1c

2.2 Starting the Scan

- After entering the camera settings, the HSI scan is started by clicking Preview HSI at the lower left.

- The CytoViva HSI Microscopy Preview window will open (Figure 2.1c). This information is used to:
  - Confirm the placement of objects in the field of the scan.
  - Aid in adjusting focus.
  - Confirm that camera exposure is appropriate.

- To use the CytoViva HSI Microscopy Preview window, place the cursor inside its borders. If the cursor is outside the window, some information will not be displayed. The upper panel of the Preview shows a histogram containing the number of image pixels that have values between zero and the maximum value (16383). Under the histogram, the panel displays a live spectral image that is being captured by the camera. In this image, the horizontal axis represents the X axis of the HSI image that will be recorded. The vertical axis represents the wavelengths of the spectra captured in the HSI image. In the Figures 2 and 3, the vertical streaks are spectra from objects that are in the view of the HSI camera. This image shows that several bright objects are in the view. Overlaid on the spectral image is an intensity plot. Placing the cursor at any point on the image corresponding to a spectral wavelength causes the intensities of all the points across the image, at that wavelength, to be plotted.

- Focus: Typically if the objects are in focus in the eyepiece of the microscope then they are in focus to the HSI camera. Often the thickness of the specimen makes it difficult to properly adjust for best focus. This can be done more easily with the Preview image. When focused correctly, the spectra become sharp (Figure 2.2a). You can adjust this focus by using the fine focus knob to obtain the minimum width of the spectra. If the focus is poor, the width of the spectra lines increases (Figure 2.2b). Focusing with the Preview window insures that the HSI scanned image will be sharp.

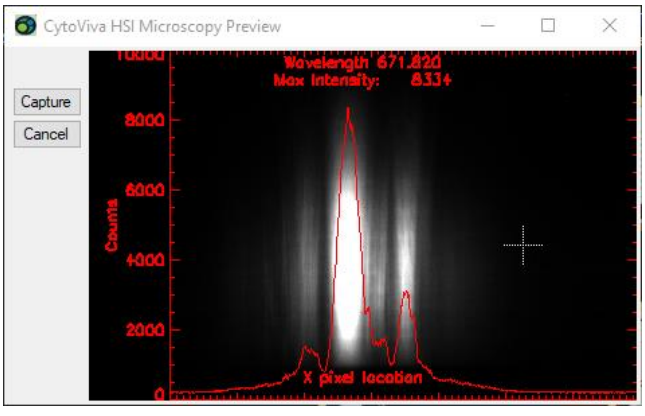


Figure 2.2a. Frame out of focus

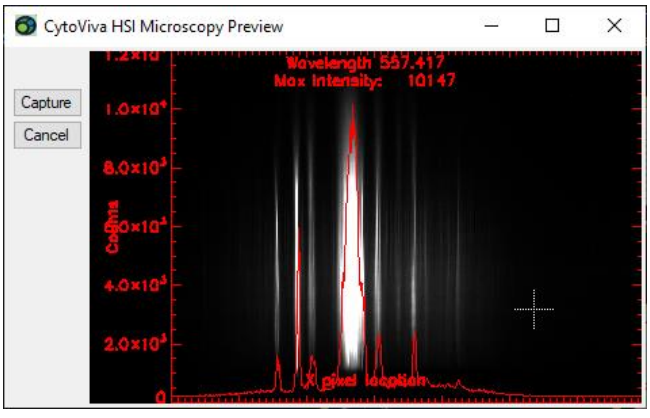


Figure 2.2b. Frame in focus

- Camera exposure: To ensure you have chosen the optimal exposure time, scroll over the brightest area of the spectral lines and check the Max Intensity value at the top of the histogram. Max Intensity values over ~16383 exceed the capabilities of the camera and will result in clipping, where the peak of the spectra is not recorded. Since the duration of the scan will lengthen with exposure time, it is necessary for the sample to be stable for several minutes and for external lights to be off when the exposure is over a couple of seconds. Please note that fluorescent lights can be picked up in the recording, causing the appearance of sharp spectral lines. If these are seen, turn off the lights.

- After you have viewed the CytoViva HSI Microscopy Preview window and have determined any adjustments that need to be made, click the Cancel button. The CytoViva HSI Microscopy Preview Window has to be closed for any changes in the Control window to reset.

- To make changes to exposure time, return to the CytoViva HSI Microscope Control window and type in the number needed. Click Preview HSI to see the changes in the CytoViva HSI Microscopy Preview window. The best exposure will be obtained when the intensity of bright features is <= 3000. Intensities of a count under 200 don't yield sufficient spectra data. Intensities will clip around 16383. The intensity values appear on the plot coordinate. The plot scale will adjust to more and less intense parts of the spectral image as you move the cursor. The most intense readings will occur at the spectral peaks, and thus the cursor should be placed at the brightest portion of the spectra to insure that the HSI image will not flat top any of the recorded spectra. Usually the spectral peak is near the center wavelength, so the cursor would be placed halfway between the lower and upper ends of the spectrum.

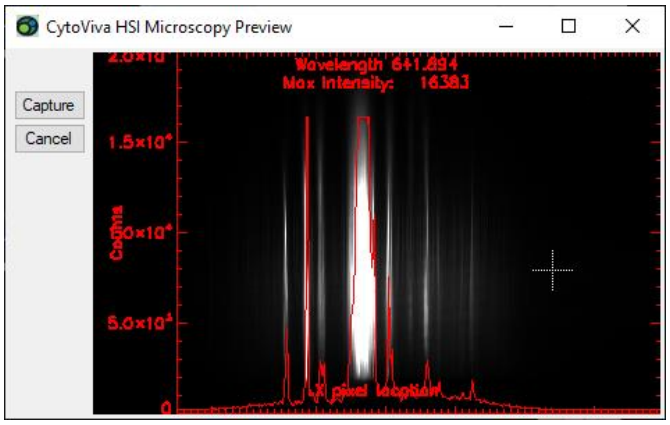


Figure 2.2c: Intensity plot showing a flat top or clipping

- Make changes to the camera exposure by canceling the Preview window and returning to the Camera Settings dialog.

- After making a change, go back to the Live Preview by pressing Preview HSI and review the new intensities.

- When you are satisfied with the Preview, press Capture to start the scan. The following message will appear (Figure 2.2d). Do not press OK yet.

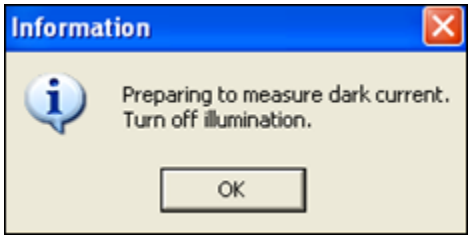


Figure 2.2d

- At this point, divert the light path from the HSI camera to the microscope eyepiece by pushing the slide bar in all the way on the trinocular head (you should hear/feel two clicks when pushing the slide bar all the way in) (Figure 2.2e).

Figure 2.2e

- Then press OK. Do not turn off or change the brightness of the illuminator. The dark image that will be used is the average of the recorded dark frames. The following dialog appears (Figure 2.2f). At this point divert the light path from the eye pieces to the HSI camera by pulling the slide bar all the way out on the trinocular head (Figure 2.2g).

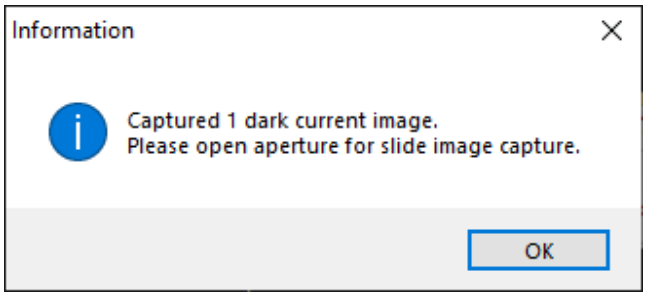


Figure 2.2f

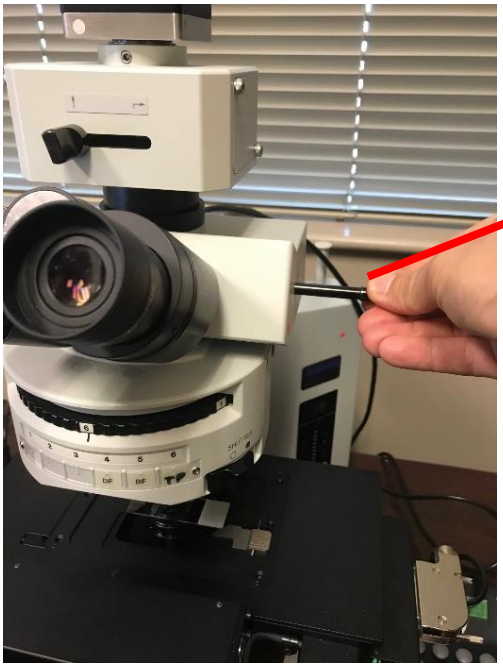


Figure 2.2g

- After completing the action in Figure 2.2g, click OK to start the scan.
- A window will appear which shows the data cube being built in real-time as well as an associated spectral intensity graph (Figure 2.2h). The stage moves to the beginning of the scanning area and then the individual lines of the HSI image are acquired, until the stage has moved to the end of the scan area.
- After the scan finishes, the HSI image automatically opens in ENVI.

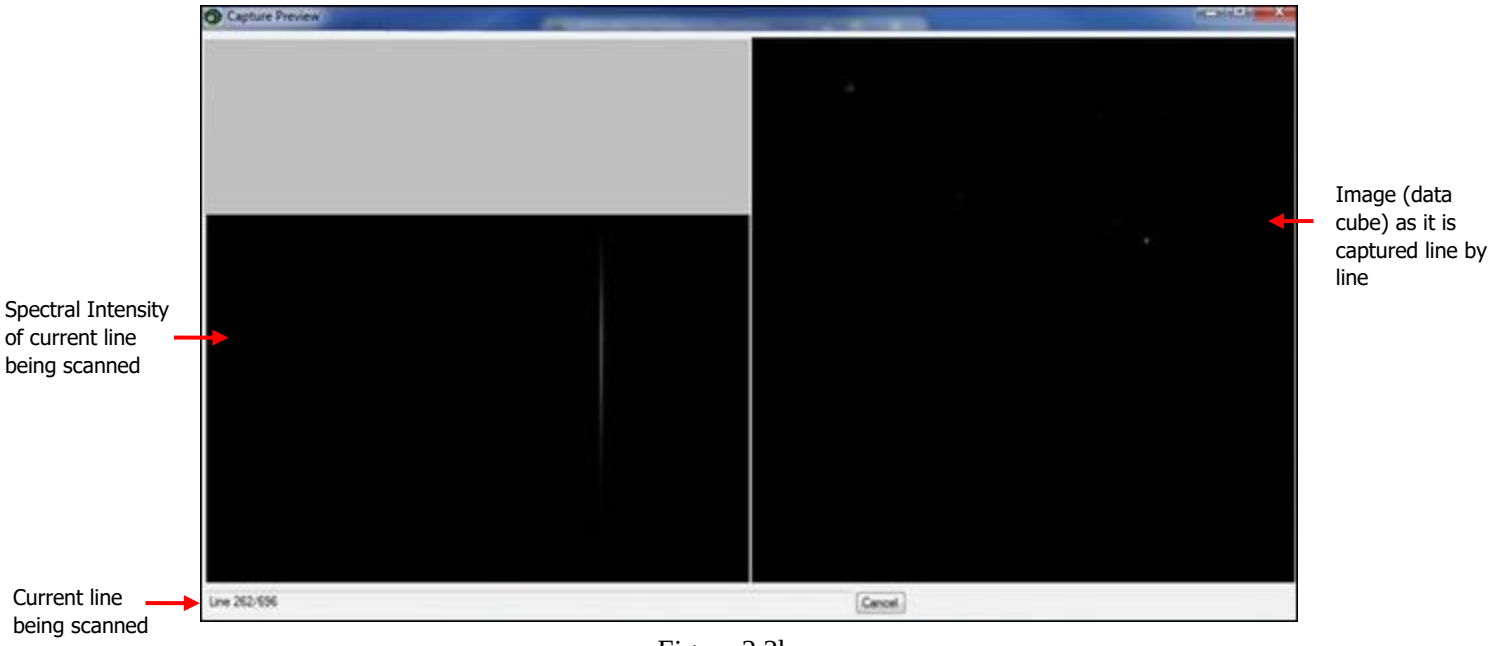


Figure 2.2h

- If an image was rescanned using the same file name as a previous scan, the old data is lost. However, the display still contains the old images. If this happens, remove the old data from the Available Bands List by selecting Close All Files and open newly scanned image into the list.

- To remove the old data, highlight the old data in the List. Press File in the main image and click on Close Selected File. Click through the display warning and the old data will be removed

- Open the newly scanned File to place it in the List and obtain the new image. See Open an Existing File below for more details.

2.3 Displaying HSI Images

- The HSI image display includes three windows:
  - **Image**: This window is sized to include a user selected area of the full HSI image. The default is a square window of 400 x 400 pixels. The image can be resized by pulling at the lower right corner with the cursor.

  - **Scroll**: This window contains the full HSI image. The size and position of the Image window is marked with a red square. The red square can be moved to enter different parts of the full HSI image into the Image window.

  - **Zoom**: A sub region of the Image window appears in the Zoom window. The red square marks the shape and the position of the zoom area in the Image window. This window can also be resized and moved within the Image window. The zoom scale can be increased (+) or decreased (-) at the lower left corner of the window.

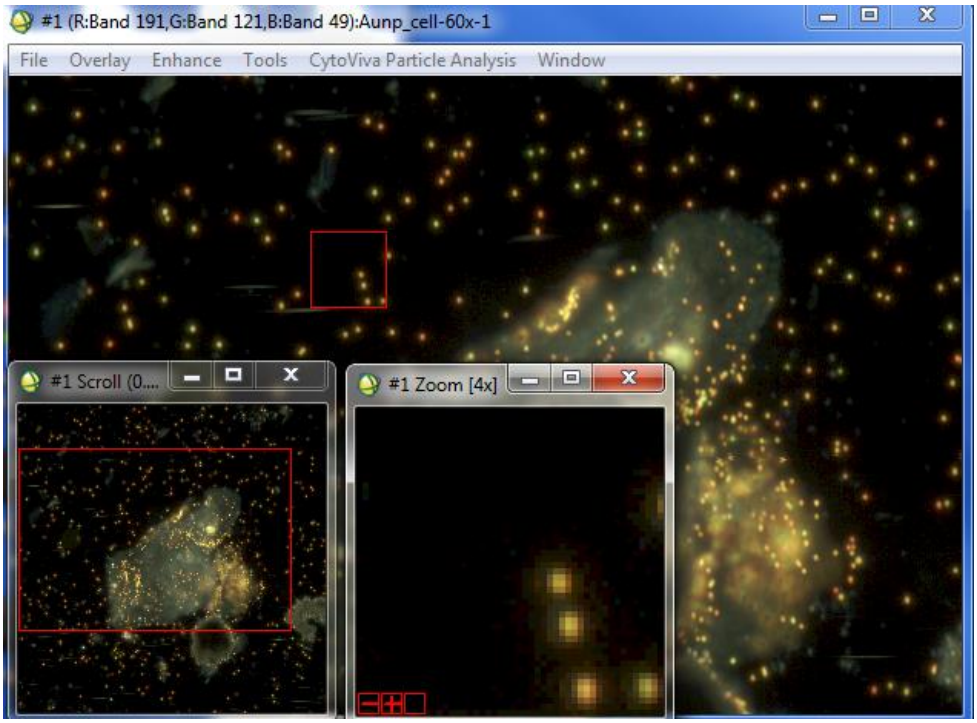


Figure 2.3a. ENVI Display of a HSI Scan of Au Nanoparticles and Cells that Includes: Image, Scroll, and Zoom displays

2.4 Opening an Existing File

- Using the Main Menu bar, previously recorded HSI images can be opened from the File/Open dialog. The HSI image is automatically loaded into three displays: Image, Scroll and Zoom windows.

- Upon opening an image file, the file name appears in the Available Bands List (Figure 2.4a).

- Under the name, the individual HSI image bands are listed, showing the associated wavelengths. Individual bands can be selected and displayed.

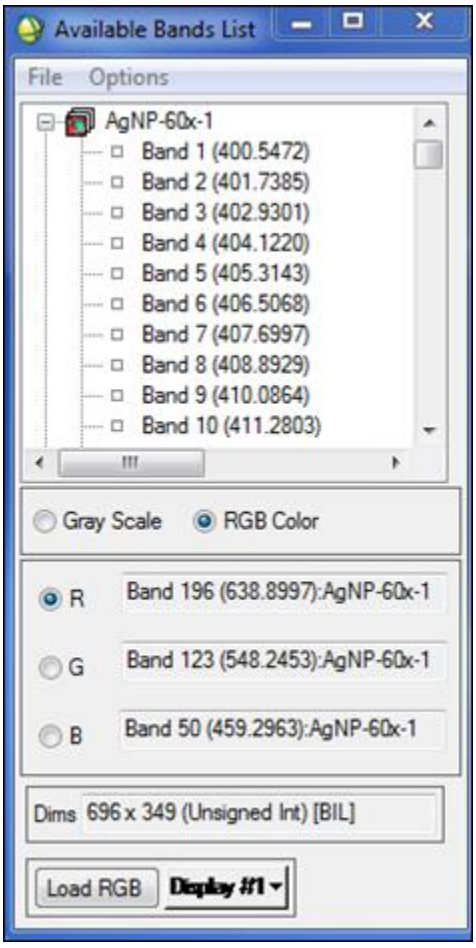


Figure 2.4a. Available Bands List

2.5 Selecting Display Bands

Images opened in ENVI can have different display properties. The images are displayed in Color format by default, but can also be changed to Gray Scale.

- When the image is first loaded, ENVI automatically picks which image bands to use for red, green and blue. The default bands give a true color rendering of the image.
    - To confirm that true color is used:
        - Right click next to the file name at the top of the available bands list.
        - Select Load True Color to Current.
        - The newly displayed image will revert to the default bands if they are not already being used (Figure 2.5a).

- Other bands can be selected for red, green and blue. One reason for selecting new bands is if the image is known to contain no intensity at one or more of the default bands. Changing to a different combination of bands will result in false color rendering. However, it may be desirable to do so in order to create image contrast based on the features of the recorded spectrum.
    - Choose new bands by clicking to the left of red, green or blue.
    - Scroll to the desired band in the window above and click on it. After changing the image band associated with one of the colors, ENVI automatically moves to the next color.
    - When you are done selecting the bands, press the Load RGB button (Figure 2.5b).

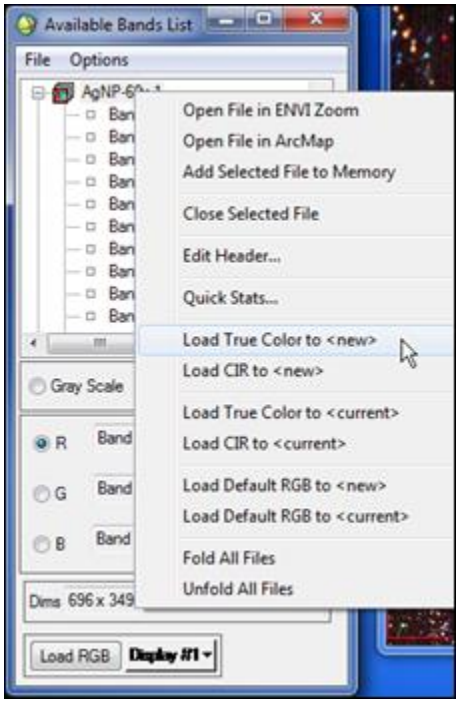


Figure 2.5a. Load True Color menu bar

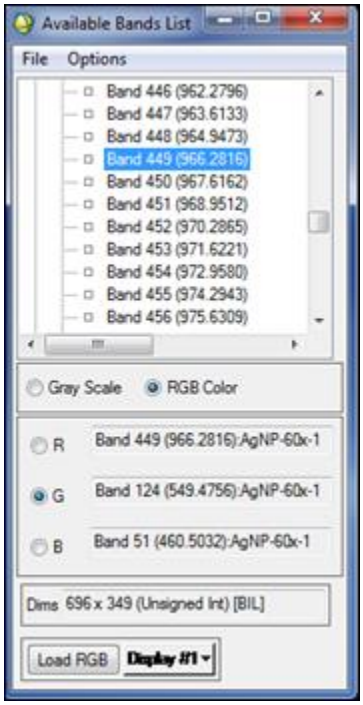


Figure 2.5b. Automatic RGB bands

2.6 Opening New Displays

- Multiple HSI images that have already been opened, and appear in the Available Bands List, can be displayed in separate windows.
    - To open multiple images from the Available Bands List (Figure 2.6a).
        - Right click on the desired image.
        - Load True Color to <new>, will load the image into a new window.
        - Load True Color to <current>, will load the image over the current image.
        - Load Default RGB to <new>, will reset RGB values and open a new image window.

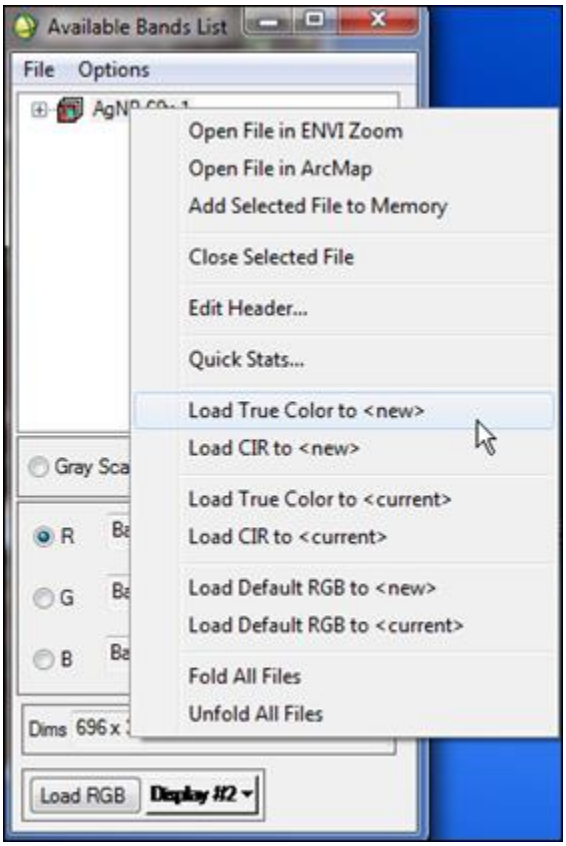


Figure 2.6a. Imaging open options

2.7 Image Enhancements

The intensity scale of the image can be stretched or compressed by using several built-in options in the Enhance drop down menu on the Image window.

- Stretch Option:
  - First, select the window containing the HSI image. Select the Enhance menu. A drop down list shows the built in options (Figure 2.7a).
  - Stretch options can be selected for any display windows: Image, Zoom, or Scroll.
  - The stretch option will be applied to the selected window, and also to the other windows. However, only the selected window will fully adhere to the rules for the stretch option.
  - Linear: Causes the full range of the display to span between the lowest and highest intensity values of the image. This type of image shows the full intensity range without clipping.
  - Linear 0-255: Only displays values between 0 and 255. This display is used more for 8 bit images, but it can also be used for 16 bit images to see fainter objects.
  - Linear 2% (default): Displays between 0 and the 2% point on the input histogram. This display emphasizes very faint objects.
  - Gaussian, Equalization and Square Root: The stretch options Gaussian, Equalization, and Square Root perform a nonlinear transformation of the input histogram and the output histogram. Square Root is most useful for visualizing both faint and bright objects together, and is recommended for displaying cell and tissue samples.

- Use the stretch options to obtain the best contrast of objects against the image background. Stretching can also reduce the appearance of vertical stripes that are caused by noise in the HSI images. Going clockwise (Figure 2.7b), these four stretches show the gold nanoparticles at increasing contrast against background. The strength of the stripes also increases. The Square Root stretching makes both faint and bright objects more visible. Stretching the display does not affect the image data. Linear 2% and Equalization stretching show the faintest objects.

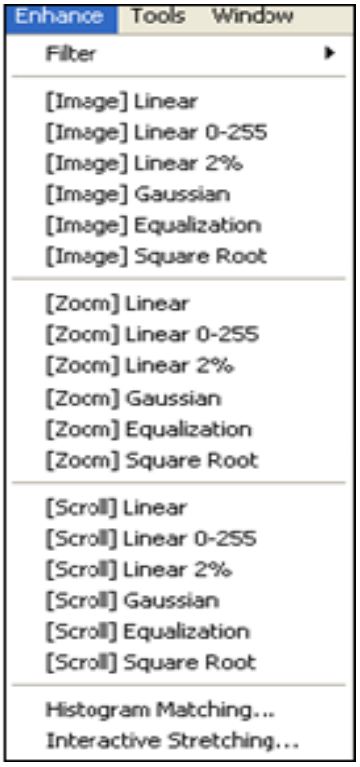


Figure 2.7a. Enhance drop down menu

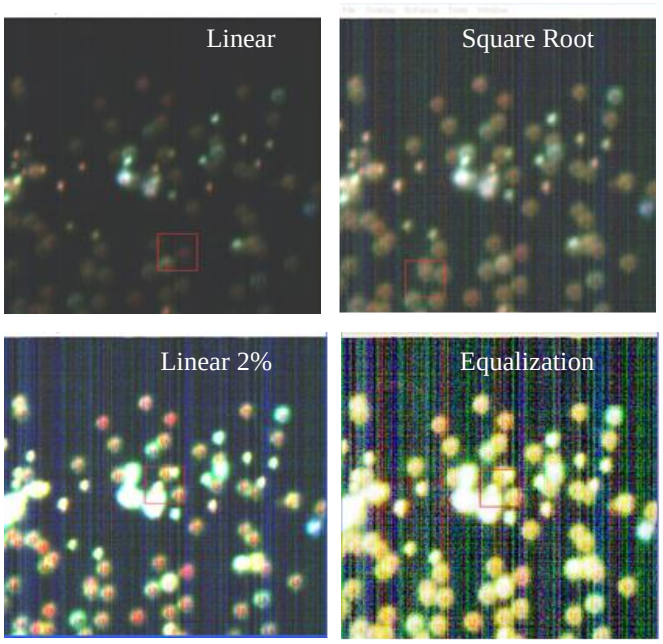
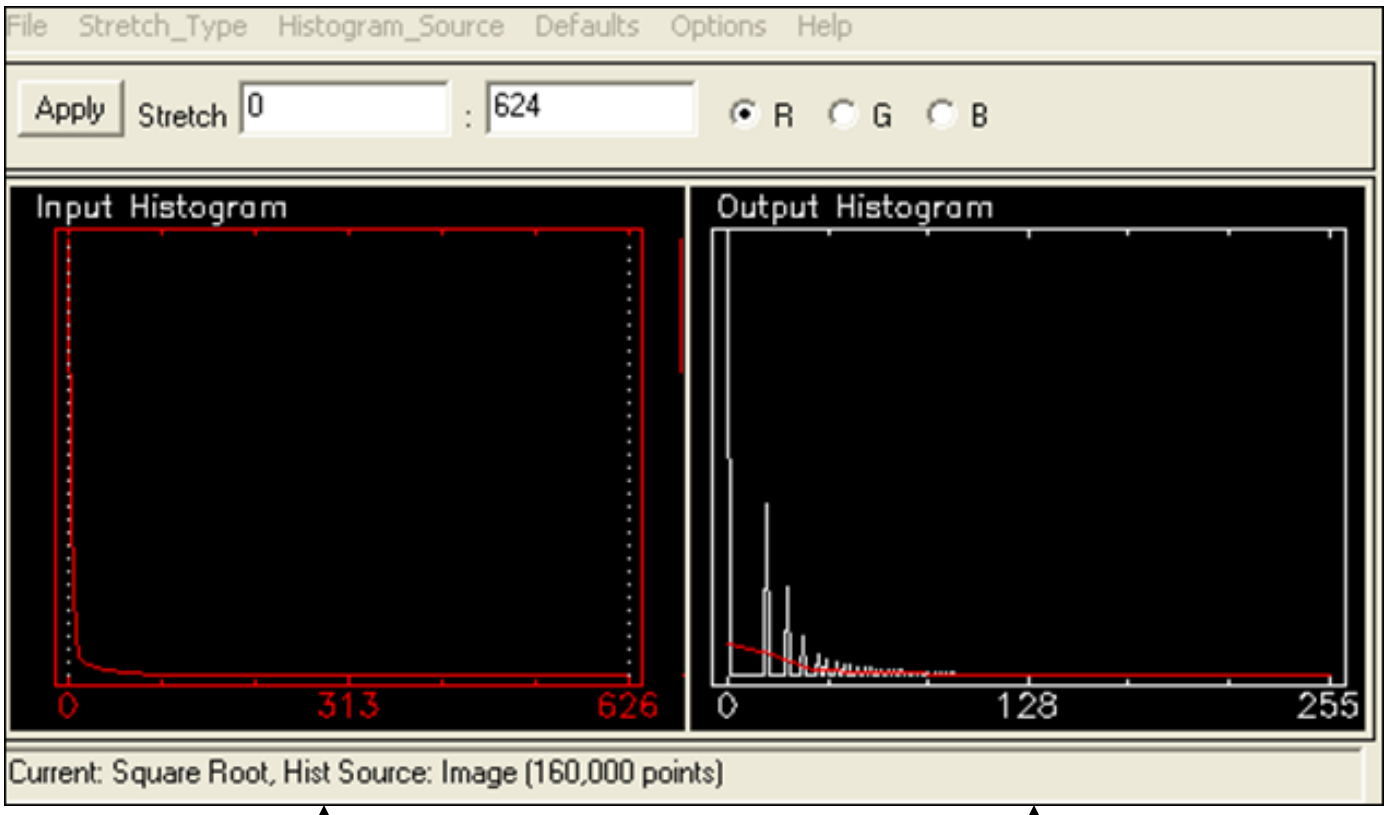


Figure 2.7b. Examples of image with faint and bright features using different stretch options

- Interactive Stretching: ENVI also allows the image contrast to be set interactively. This mode is selected at the bottom of the Enhance drop down menu: Interactive stretching.
    - Interactive Stretching with Input & Output Histograms:
        - The display can be adjusted by changing the relationship between the input and output histograms (Figure 2.7c). This option provides greater flexibility to adjust the image display.
        - The vertical dashed lines at the left and right edges of the input histogram can be moved to reset the bottom and top of the display range.
        - Alternatively, these boundaries can be set by typing the new values into the stretch boxes
        - The new stretch is made effective by pressing Apply.
        - Image noise is effectively removed from the display by setting the lower boundary value between 5 and 20.
        - For RGB images, the process is repeated for each color by clicking the R, G, or B.

Figure 2.7c. Input and Output Histogram



Input histogram of the red image band for the red channel

Output display for the red channel

2.8 Plotting

Intensity profiles along the X, Y and Z (spectral) axis can be plotted using the profile tool at the top of the main image window.

- Click on Tools, then highlight Profiles (Figure 2.8a)

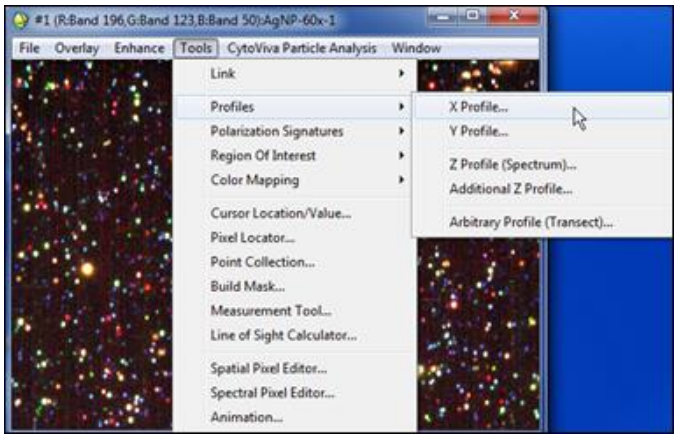


Figure 2.8a. The profile list is under tools in the image tool bar

- Select a profile (X, Y or Z). In this example of silver nanoparticles, the X profile was selected and then the Z profile was selected following the same procedure.
    - When a Profile is selected, a cross hair appears on the Image and the intensity plot is displayed
    - The plot will contain the image intensities along the horizontal crosshair (X) or the vertical crosshair (Y), or the Spectral Profile that is contained in the HSI image pixels at the crosshair intersection (Figure 2.8b).

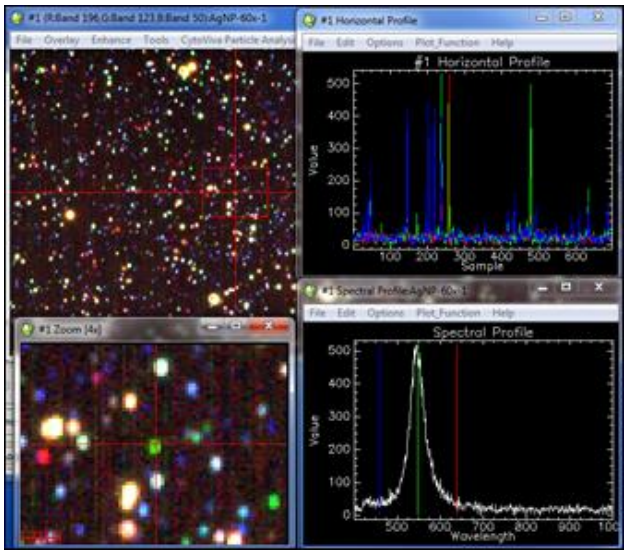


Figure 2.8b. Plot profiles

- To view the Plot Key, right click in the Spectral Profile Window.
  - Then click on Plot Key.

- To display spectra from multiple regions of the image, right click in the Spectral Profile window
  - Select Collect Spectra. A new curve is added to the plot each time a new area of the image is clicked (Figure 2.8c).
  - You can go back to the Zoom Window and select pixels to collect spectra.
  - In the Zoom window, clicking (+) increases the zoom while (-) decreases it. It is easier to center the cross hair on image features using a higher zoom setting.

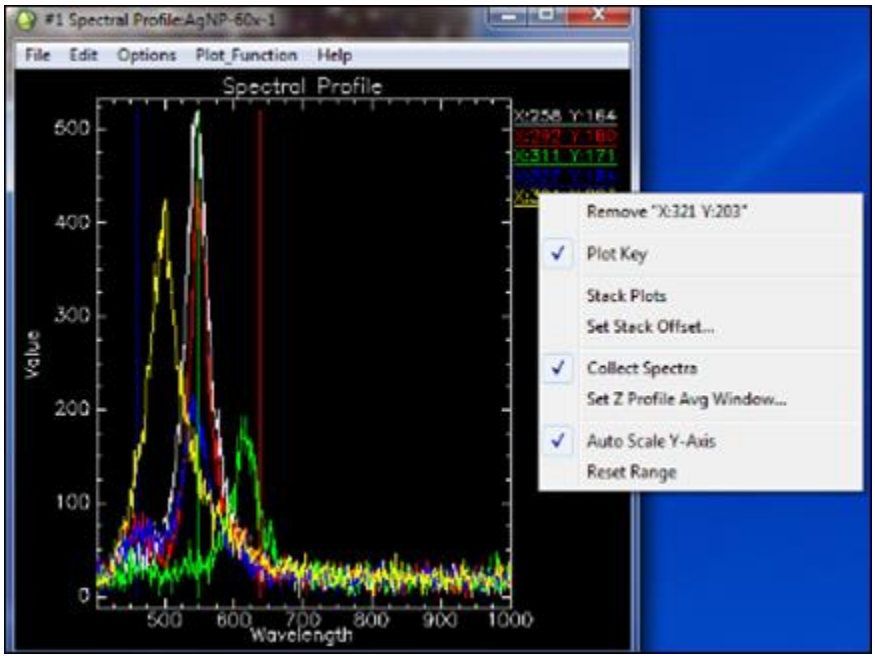


Figure 2.8c. Window to collect spectra from multiple regions

- Noise Reduction: Noise Reduction can be applied to the Spectral Plot by averaging pixels.

- Averaging Pixels:
  - To reduce noise in the spectrum, right click in the Spectral profile
  - Select Z profile average window (Figure 2.8d). When you change the Z Profile Average larger than 1 x 1 the pixel in the zoom window is the bottom right pixel of the area (Figure 2.8e).

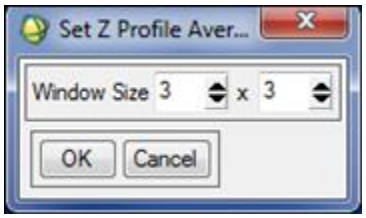


Figure 2.8d. Z profile average

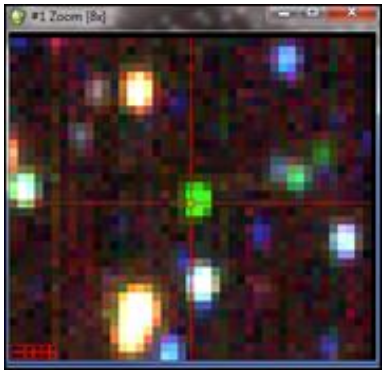


Figure 2.8e. Zoom window with 3x3 Z profile average

- By increasing the average window size to 3x3 this will significantly reduce the noise on the spectrum. Larger window sizes can be used to further reduce noise if the spectrum of interest comes from a feature that is at least this size. Excessively large window sizes will begin to include spectra from areas unrelated to the feature, changing the spectrum. Use smaller window sizes for smaller materials of interest.

- The Z Profile is useful for quickly sampling the spectrum from any part of the image.

- The spectral profile (Figure 2.8f) is marked with red, green and blue lines that show the wavelengths of the RGB display bands. The HSI band and wavelength are shown below the plot when the user clicks on the line. The default bands are true color wavelengths: red = 640nm, green = 550 nm and blue = 460 nm. Images will appear with normal colors using the default setting.

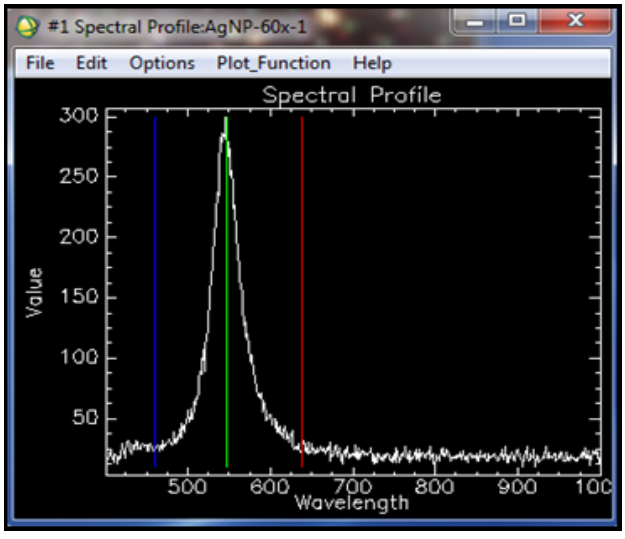


Figure 2.7f. Spectral profile, wavelength display

2.9 Saving Images

The hyperspectral image data is saved when the image is scanned, before an image is displayed. This is why the file name for the HSI file must be given before scanning. There is no need to save this data again. ENVI can open images from files and display them, or bring selected HSI bands into a gray scale or RGB image display. These display images can be saved in various formats. It is possible to save the main image and the zoom image.

- In the main ENVI bar:
  - Click on File.
  - Then select Save Image As.
  - Then select Image File.
  - The Output Display to Image File dialog opens (Figure 2.9a).

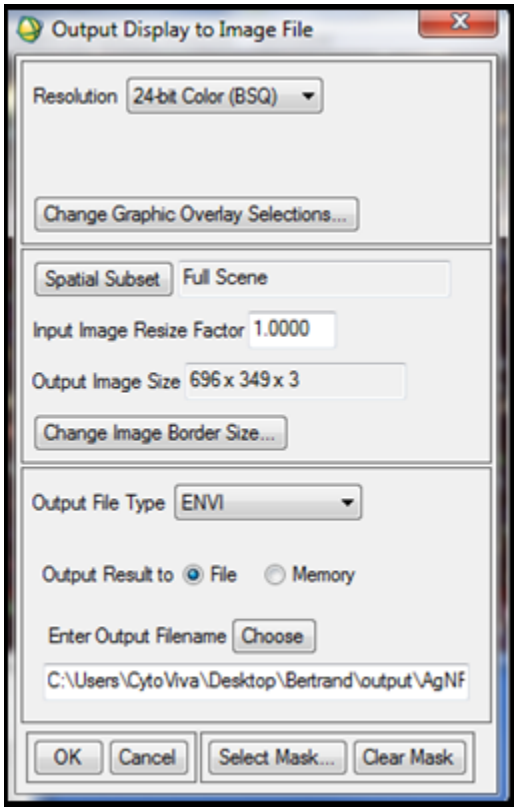


Figure 2.9a. Output Display to Image File

- If the image is color, use 24 bit BSQ resolution. For gray scale images use 8 bit.
- A portion of the image can be saved using Spatial Subset. The number of samples (NS) is equal to the number of image columns. The number of lines (NL) is equal to the number of lines scanned, and to the number of image rows.
- Click the Image button to create the portion desired. You will see a red border around the image. Grab the corner and resize the border to the desired image size, then click OK.
- Click OK in the Select Spatial Subset Window.
- The Output File Type gives a list of image types supported by ENVI. Select one of your choosing (ex. JPG, TIF, etc.).

- To enter the file name and folder.
  - Select Choose.
  - Then enter the File Name.
  - Click Open.
  - Then click OK in the Output Display to Image File.

- The Zoom window can be saved in exactly the same manner.
  - Choosing Save Zoom Image.
  - Selecting Image File.

Section 3 Extraction of Spectral Profiles

3.1 Opening Test Image

- In this example, we illustrate how you can extract spectra from silver nanoparticles that appear in different colors in the RGB display.
    - Open the ENVI Software by double clicking on the ENVI icon on the desktop.
        - Click on File.
        - Select open image file.
        - Select the folder HSI Example Files located on the desktop (All example files are provided in this folder).
        - Select **Aunp_cell-60x-1.**
    - Load the image into a new color display (Figure 3.1a).
    - Set the display enhancement.
        - Using the Image Menu click on the Enhance drop down list.
        - Click on Image.

- Observe that there are small particles spread over the image and cells. These particles are mostly yellow/orange. The colors viewed in the display are determined by which bands are chosen to represent red, green, and blue in the available bands list. Although the particles appear to have different color properties, we need to see if the spectra of the different colored particles are similar or very different.

- In the figure below, the Zoom window area is marked by a red square.

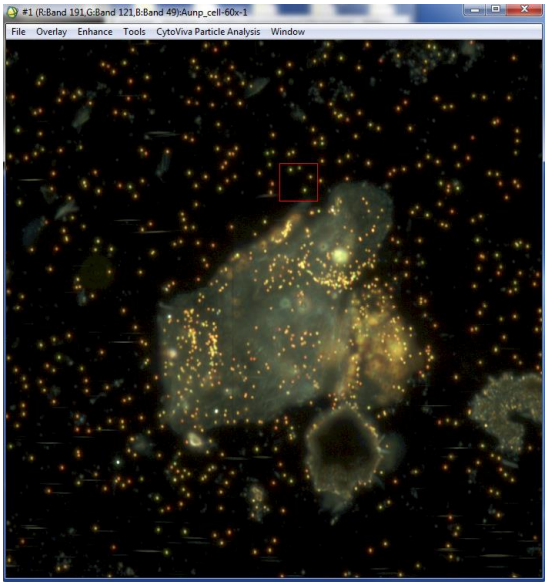


Figure 3.1a. Au nanoparticles and cells

3.2 Acquiring and Labeling a Spectral Profile

Spectra can be plotted from image features that are selected by the cursor. This action uses the Z Profile feature.

- Enlarge the Zoom window so that it contains some particles (see Figure 3.2a).
- Increase the zoom factor so that individual pixels are observed by using the (-) and (+) tabs in the zoom window.
- To turn on and off the crosshairs use the button to the right of the (+) in the bottom left corner of the zoom window. The crosshair cursor appears below in the Zoom window (Figure 3.2a).

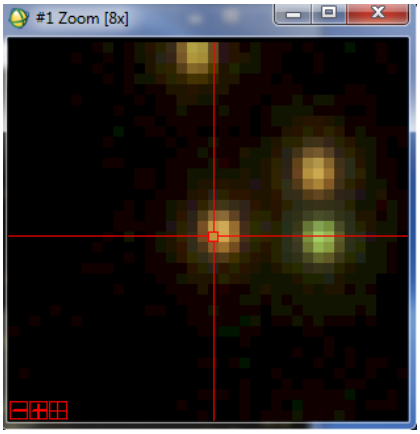


Figure 3.2a. Zoom window with crosshair enabled and centered on a particle

- Pulling up the plot window:
  - Using the Image Menu Bar.
    - Select Tools.
    - Select Profiles.
    - Then select Z Profile (Spectrum).
  - Or right click in the Zoom window and select Z Profile (Spectrum).
- In the Zoom window, move the cursor, now in a crosshair format, over a red object.

- Left click in the zoom window to obtain the spectra of the pixel under the cursor in the spectral profile window (Figure 3.2b).
    - The arrow keys on the keyboard will move the crosshair to the desired position.

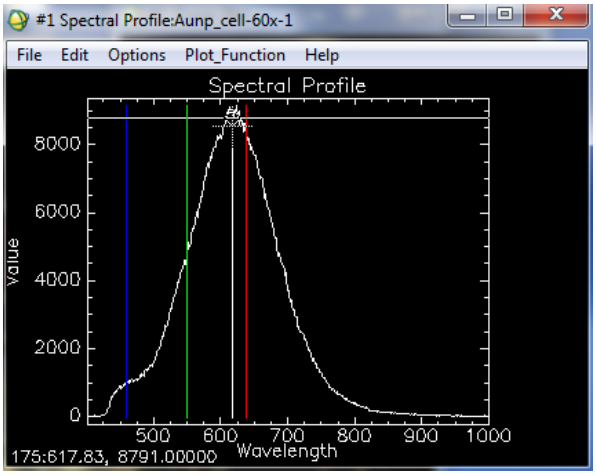


Figure 3.2b. Plots using the spectral profile tool

- Then right click in the plot window and select Set Z Profile Average Window.
- Adjust the two window size boxes to 3.
- Click OK. You will see that there is a decrease in the noise of the plotted spectrum as the spectrum is now averaged over 9 pixels centered on the crosshair.

3.3 Reading the Data

- The spectrum contains data from all the image bands at a specific set of pixels chosen in an ROI or a spectral profile. The band number, wavelength, and spectral intensity can be displayed at the bottom left corner of the plot (Figure 3.3a).

- Read the information from specific locations on the spectrum by left clicking and holding the button down as you move in the plot window. A line cursor follows your movement, and the band, wavelength and intensity are updated in the lower left.

- In this example (Figure 3.3a), the data show that the cursor is at band 175, the wavelength is 617.83nm and the intensity of the spectrum is 8791.00 units.

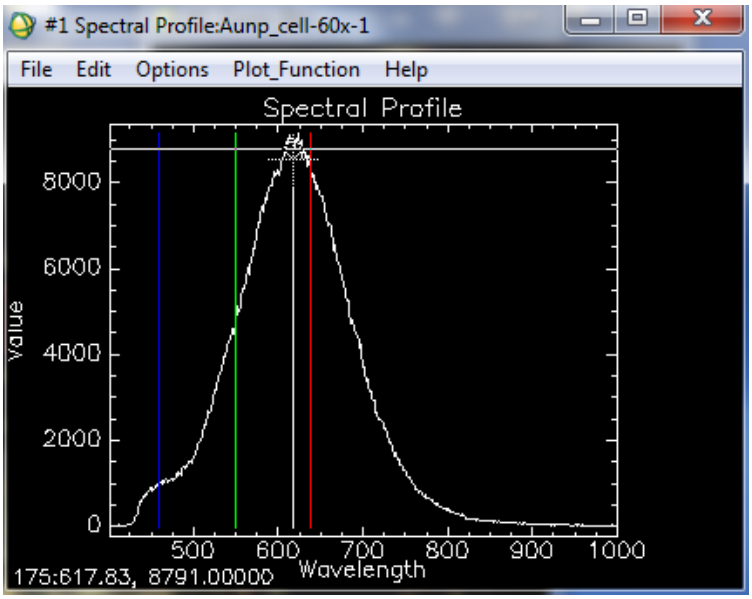


Figure 3.3a. The spectral profile with vertical cursor and data readout

3.4 Changing the Plot Key Parameters

- In the plot window right click.
- Select Plot Key – you will see the spectral curve label name in the right margin.
- To change the Plot Key names, line color and style (so that can be identified later) go to the plot window and on the image menu bar:
  - Select Edit.
  - Then select Data Parameters.
  - In the Edit window, select the label and below enter the new name in the box below.
  - To change the plot color and line style right click on the box and select the desired line color. For this example we named the particle spectrum red.
  - Click Apply and then close the edit window (Figure 3.4a).
- You can also select Plot Parameters from the Image Bar Menu to change the X & Y Axis, Title of the Plot, and Background and Foreground color. If you don't click apply the new spectral curve label will not be changed.

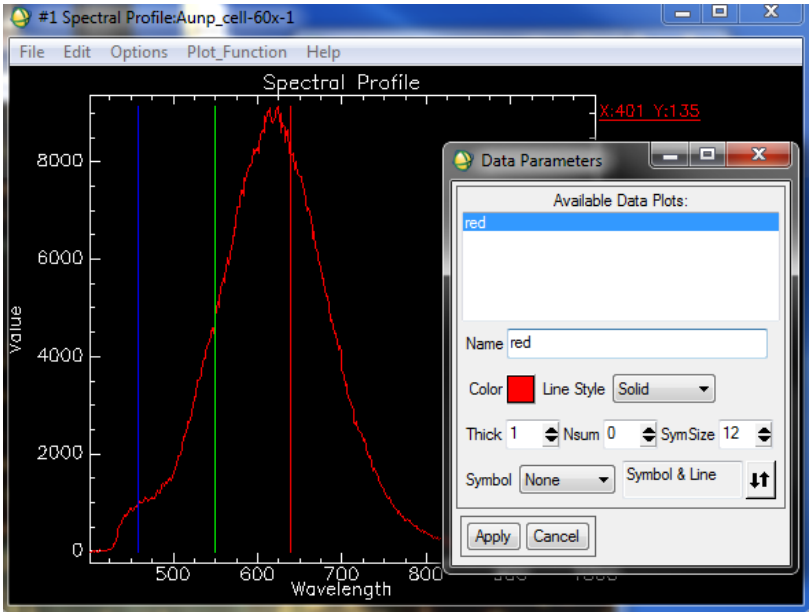


Figure 3.4a. Labeled Plot

3.5 Collecting Spectra in the Same Field of View

- In the Plot Window right click and select Collect Spectra. This has just collected the spectra of your red particle in the sample. When the Collect Spectra is selected, ENVI will collect spectra from any point clicked in all windows (Image, Scroll or Zoom Window).

- Now to collect spectra of another particle.
    - In this case we will collect the spectrum of the particle that appears yellow. Put the crosshair cursor on the desired particle by left clicking in the zoom window and moving to desired location
    - The new plot and its label appear together with the red spectrum and label.

- Repeat again for another particle.

- You may end up with three very distinct curves (Figure 3.5a).

- Finally, change the names and colors of these new spectra so they can be recognized later (Refer to Part 4: Changing the Spectral Curve Label name).

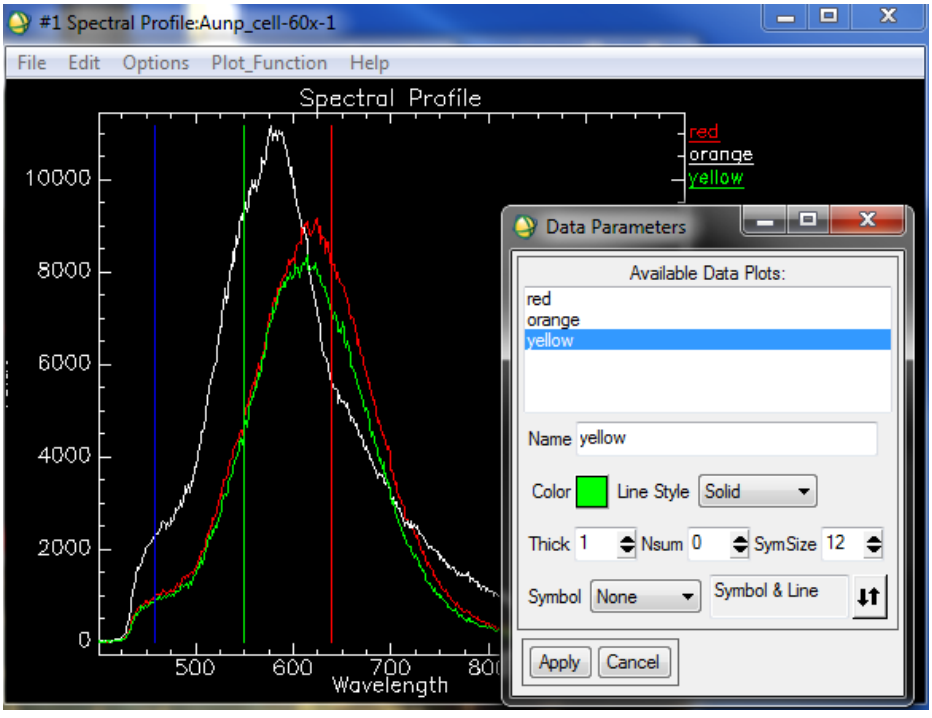


Figure 3.5a. Spectral curves from three nanoparticles

3.6 Collecting Spectra from Different locations in the Image

Multiple spectra can be plotted in the same window. This provides an easy way to visually compare the spectral curves of different features. Although points for spectra can be chosen from any of the display window, it is easiest to use the Zoom window, where individual pixels can be identified.

- In the Plot Window right click and select Collect Spectra. We have already collected the spectrum of the red particle. When Collect Spectra is selected, ENVI will collect spectra from any point clicked in all windows (Image, Scroll or Zoom Window).

- Now collect spectra of other particles.
  - We will collect the spectrum of the particle that appears yellow.
  - Put the crosshair cursor on the desired particle by left clicking in the zoom window. The image moves to place the desired feature at the center of the crosshair.
  - The new spectrum from this location, and its label, appear together with the red spectrum in the plot.

- Repeat again for a green particle.

- You may end up with three very distinct curves (Figure 3.6a). Note that the color of the spectral curve is assigned by ENVI and does not reflect the color of the feature in the display.

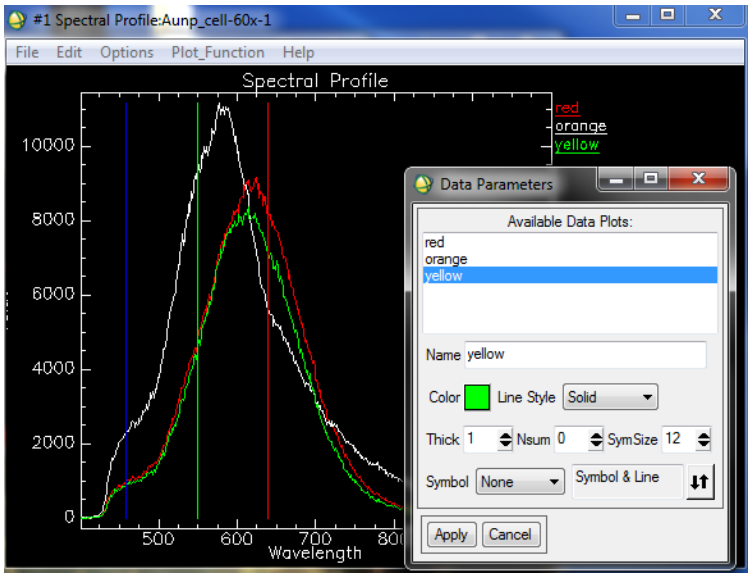


Figure 3.6a. Spectral profiles from three image features plotted together

- After collecting spectra:
  - Right click in the plot window.
  - Click plot key. This will display the names of each spectra collected. The default names will be the X and Y locations of the pixel selected for the spectra collected.
  - Then go to edit plot parameters. This will allow you to change the title of the plot, X and Y axis title.
  - By selecting Data parameters you can change the line color, line style and the plot key names.
  - By selecting editing plot parameters you can assign the spectra to different colors.
  - Finally, change the names and colors of these new spectra so they can be recognized later.

3.7 Deleting Unwanted Spectra from the Plot

If you decide to remove a spectral curve, this can be done without restarting the plot.

- In the plot window, right click on the label of the unwanted spectra collected. The first spectrum, at the top of the list, cannot be removed.
- Then click on Remove label (Figure 3.7a).
- The selected curve will be removed.

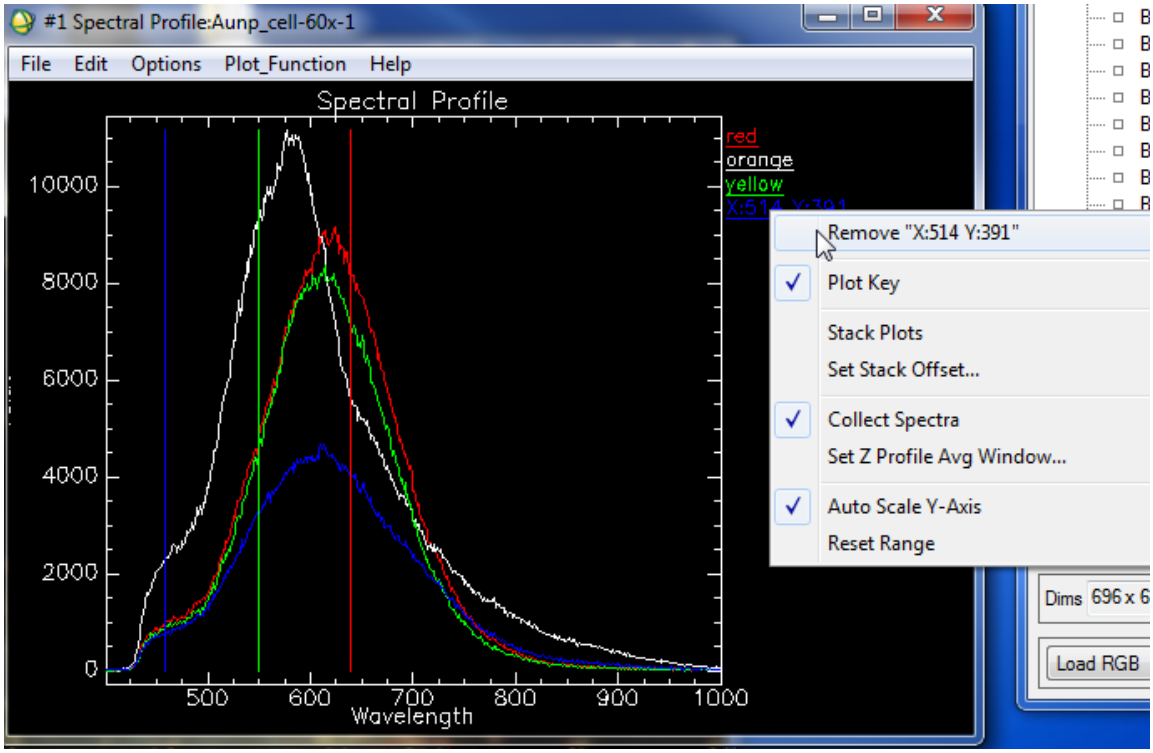


Figure 3.7a. Option list obtained by right clicking on the plot label

Section 4 Saving and Viewing Profiles in the Spectral Library

4.1 Creating an Output Folder

When doing spectral analysis on scans you will need to create several different types of output files which could include, but are not limited to:

- Spectral Library Files.
- Header Files.
- Spectral Angle Mapper (SAM) Files.

These are files of your spectral data that you will want to access later to compare against new samples. The Output folder will contain files from scanned images.

- To create your output folder, choose a location within your Images Folder.
    - Click on Create New Folder.
    - Label the folder Output (Figure 4.1a).

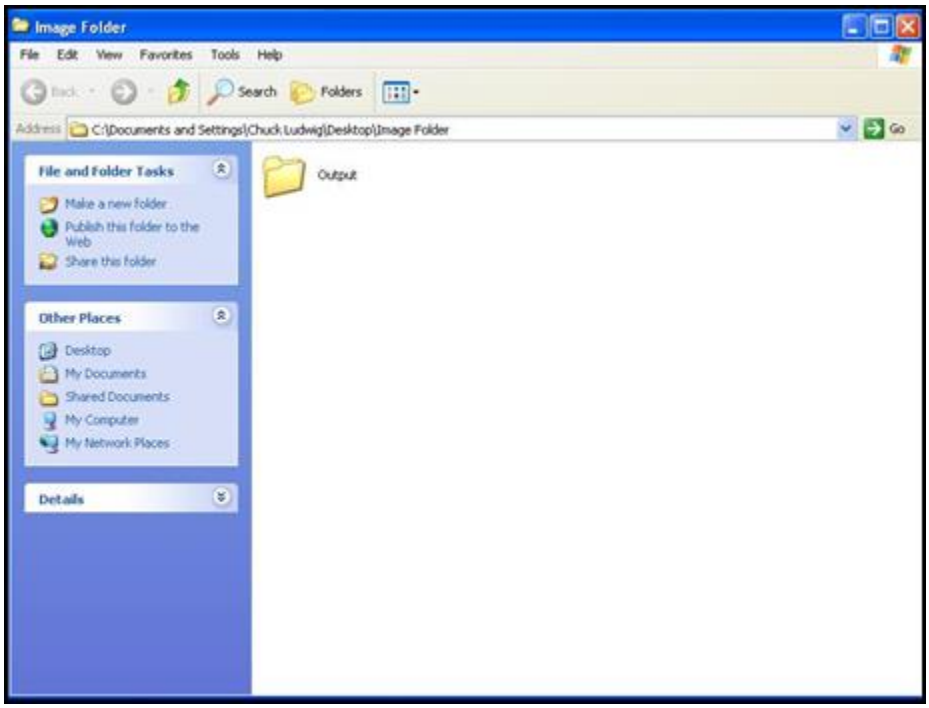


Figure 4.1a. Output folder for saving image work in the images folder

4.2 Saving and Viewing Profiles in the Spectral Library

- After you have created your output folder (refer to Part 1: Creating an Output Folder), the red, yellow, green and blue particle spectra (collected earlier) can be saved to a spectral library.

- From the spectral profile window, select File.
  - Then select, Save Plot As.
  - Then select Spectral Library.

- You will see the Output Plots to Spectral Library dialog box open.
  - Select all of the plots listed in the window by clicking the Select All Items button.
  - Then click OK.

- You will see the Output Spectral Library dialog box open.
  - Click the Select All Items button to put all spectra into the spectral library. Also individual spectra can be collected and saved into the spectral library as well.
  - Then click OK, the Output Spectral Library window will open.

- In the Output Spectral Library window, enter the baseline intensity and the highest y axis value of the three spectral curves in the Z Plot Range boxes.
  - If curves came from imagery where the dark background had automatically been subtracted, enter -20. This value will allow the variation around zero to be included.
  - Do NOT click OK yet. This information is needed for scaling spectral library curves to the size of other curves. For now, the other boxes should not be changed.

- In the Output Spectral Library box, click the Choose button (located beside the enter output file name) to select where the data will be saved.
  - Go to your Output Folder and type in the chosen file name.
  - You do not need to add an extension to the file name.
  - Click Open.
    - The library file and a header file are automatically saved in the Output Folder.
    - The library is also added to the top of the available bands list.
  - Then click OK.

4.3 Viewing the Spectral Library File

The Spectral Library Viewer allows you to view the collected spectra individually or together.

- In the Available Bands List Window right click on the file name of the desired spectral library file.
  - Choose Spectral Library Viewer.
  - In the Spectral Library Viewer select the red spectrum.
  - A plot window opens with the spectral curve.
  - Select the other spectra in the Viewer - they are added to the plot. Note that curves saved in the spectral library are also referred to as endmembers in automated spectral classifications. The use of endmembers will be discussed next.

Section 5 Spectral Angle Mapper Classifications (SAM)

5.1 Automated Comparison of Unknown Spectra with Spectral Libraries

The Spectral Angle Mapper Classification (SAM) an automated procedure for determining if a known material is present in the input image, and locating which pixels contain the material. SAM accomplishes these tasks by comparing unknown spectra in hyperspectral imagery with known spectra for the material in question. The degree of match between the unknown and known spectra from each image pixel is displayed in a grayscale image known as the Rule image. The Rule image shows, on a scale of light to dark, the relative degree to which unknown spectra in each image pixel match the known spectrum. The scale is reversed from convention, so that best matches produce the darkest pixels. Since by convention, high correlations are scaled toward the bright end of the scale in image processing, the grayscale of the Rule image is often reversed to create this effect. Additionally, a criterion for the match between known and unknown spectra can be given. When this is requested, SAM produces a binary classification image showing locations of pixels with spectra that are within the criterion.

The SAM method works by comparing unknown spectra from each image pixel with the known spectrum (for example, in a spectral library) at each of the N wavelength bands recorded in the image. SAM first determines a vector in N dimensions that represents the distance from the origin (dark) to the light intensity recorded in each band of the unknown spectrum. The direction of this vector in N-dimensional space is used to define a unit vector representing the unknown spectrum. The same procedure is performed for the known spectrum. SAM then determines the angle between the two unit vectors. The best spectral match occurs when the angle between these vectors is the smallest.

The selection criterion that was used to make the classification image is actually a threshold for the spectral angle. Image pixels with spectral angles that are smaller than the threshold are classified as containing material belonging to the known spectrum. In plot shown below (Figure 5.1a), the unknown spectrum was not classified as belonging to the known spectrum since the angle turned out to be larger than the criterion allowed. The angle of each pixel is output to the Rule image. Because the method is based only on the direction of these vectors, and not on recorded light intensity, the SAM classification is insensitive to the illumination of the sample in the recording. Therefore, it is important that dark values are removed from the data before using SAM. Since sample illumination varies over the field of recording and between images, the SAM tool is a highly useful classification method.

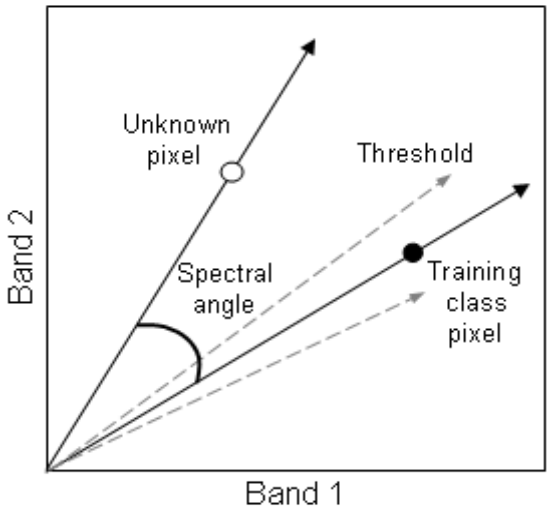


Figure 5.1a. Threshold for classification of pixels based on spectral angle

5.2 Using the Spectral Angle Mapper (SAM)

This example examines an image of gold nanoparticles in a cell. The object of this exercise is to automatically locate the AuNP objects within the image. First, the known spectrum for AuNP is acquired and saved in the spectral library. Spectra that are used as a signature for identifying a material are called endmember spectra. The SAM classification feature then is used to find the locations of objects matching the spectrum of AuNP in the image.

- Acquiring the endmember spectrum.
  - In the Main Menu Bar, use the Open Image File to load the input image.
  - Click on the file: **Aunp_cell-60x-1** (preloaded in the "CytoViva Example Files" folder on your desktop).
    - To optimize the display contrast, click on Enhance in the Image Window.
    - Then click Linear 0-255 – this will enhance the display contrast and brightness.
  - Move the Zoom area over the cluster of particles near the left of the tissue section (Figure 5.2a).
  - Use the box in the Scroll Window to locate the area of the cluster of particles.
  - Then use the zoom box in the Image window to better locate/focus the cluster of particles so that they appear in the zoom window.

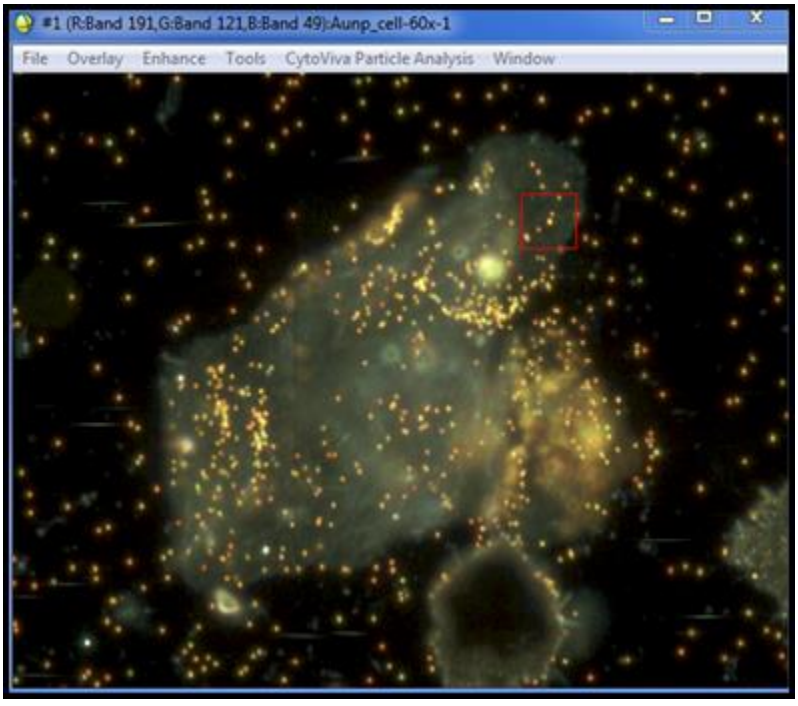


Figure 5.2a. AuNPs (inside square) in/on a cell cluster

- Using the Zoom Window, select a clear, distinct particle using the crosshair cursor (Figure 5.2b). We will use this particle to represent the AuNPs that are distributed throughout this image.

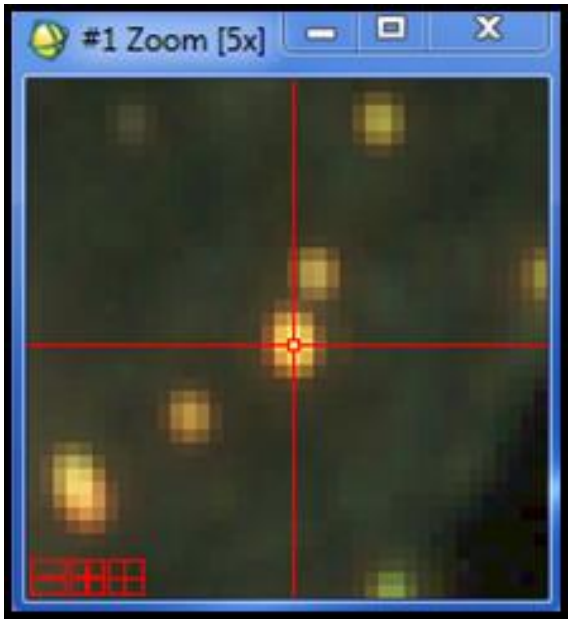


Figure 5.2b. Crosshair centered over particle in zoom window

- Now to plot the spectrum of the AuNP.
  - In the Zoom Window, right click and select Z Profile (Spectrum).
  - Before acquiring the profile, right click in the plot window and set the Z Profile Average Window to a size that fits within the borders of the particle. Here a 3x3 rectangular pixel average is selected.

- Move the crosshair cursor over the center of the particle (Figure 5.2c) and left click to obtain the endmember spectrum that will be used to classify the image.

- Now give the plot key label a new name (Refer to Section 4, Part 4 – Changing the Plot Key Parameters) that allows you to find it in the future. Here we chose AuNP_in cell (Figure 5.2c).

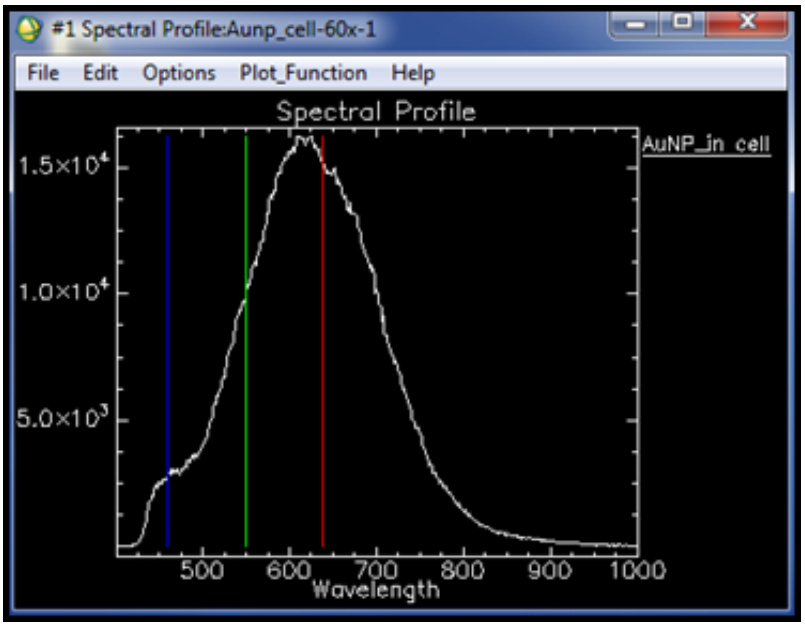


Figure 5.2c. Spectral profile of particles with label in plot key

- Saving the endmember to the spe7ctral library – 2 methods.
  - Method 1: Using the Main Menu.
    - From the Main Menu bar in the Image Window, select Spectral.
      - Then select Spectral Libraries.
      - Then select Spectral Library Builder.
      - You will see the Spectral Library Builder dialog box open.
      - Then select First Input Spectrum and click OK.
      - In the plot window click and drag the label (AuNP_in cell) to the spectrum list in the Spectral Library Builder dialog (Figure 5.2d).

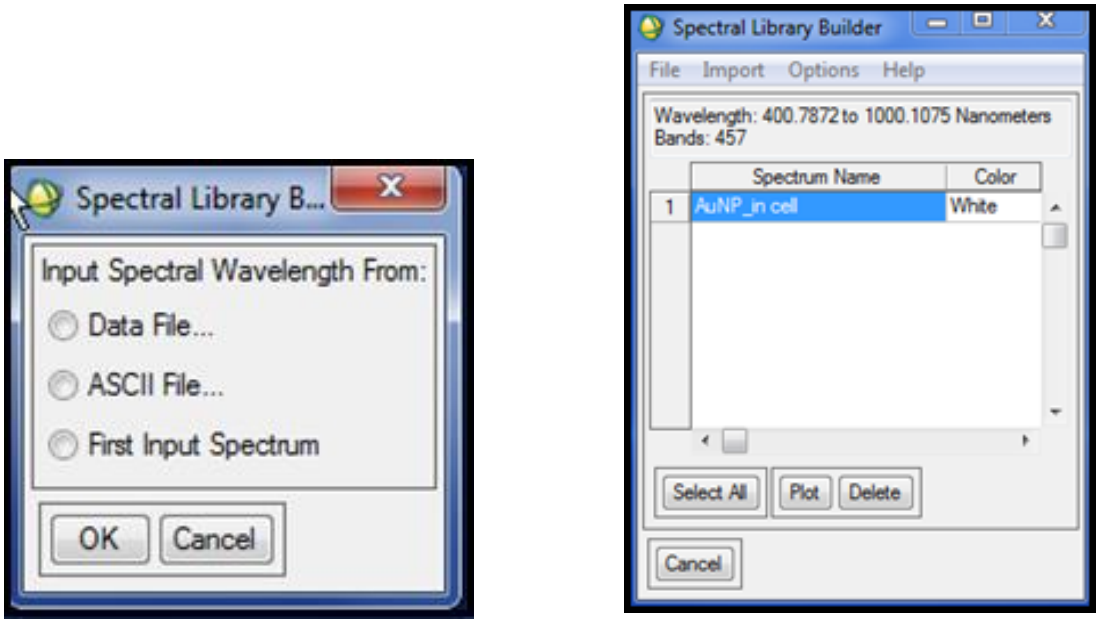


Figure 5.2d. Spectral library builder dialog windows

- Click Plot to create a plot window that is used to accumulate the Endmember Collection Spectra (Figure 5.2e). In this case we have a single Endmember.

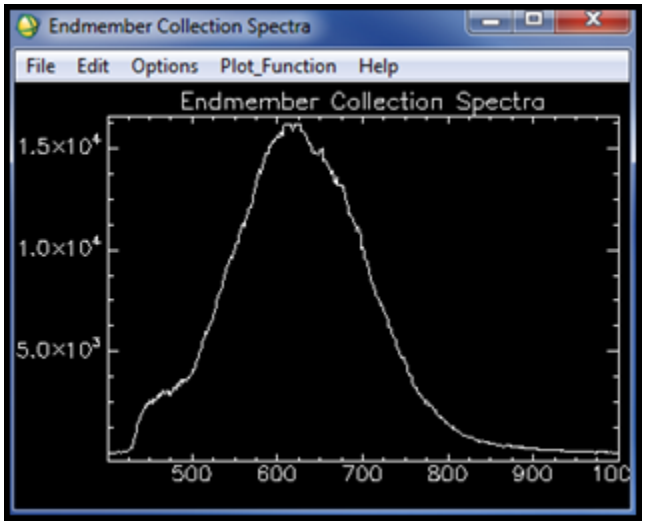


Figure 5.2e. Plot containing the spectrum of the endmember

- Method 2: Using the Endmember Collection Spectra Window.
  - In the Endmember Collection Spectra window click on File.
    - Then select Save Plot As.
    - Then select the Spectral Library (Figure 5.2f).

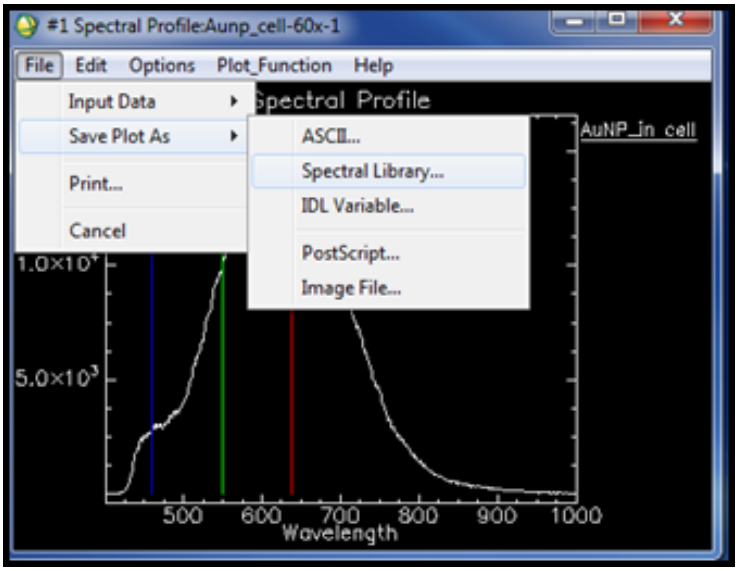


Figure 5.2f. Saving endmember to spectral library file

- The Output Plots to Spectral Library dialogue box opens.
- Click Select All Items.
- Then click OK.
- The Output Spectral Library dialogue box opens.
  - In this example, for Output Result, select Memory.
  - Your spectral library is now present in the Available Bands List as data in memory.
  - To view the Endmember plot.
  - Right click on Memory in the Available Bands list (Figure 5.2g).

  Then select Spectral Library Viewer. This now shows the plot for the Endmember(s), in this case we only have one (Figure 5.2h).

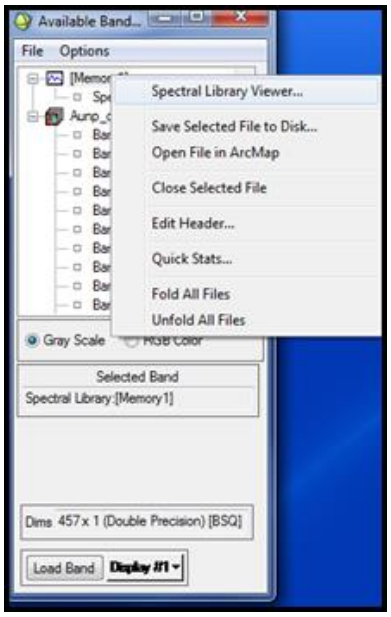


Figure 5.2g

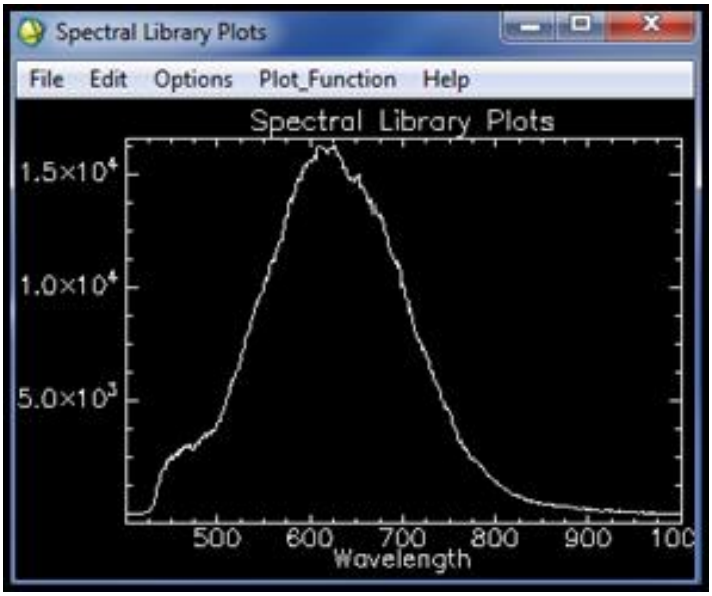


Figure 5.2h. Plot containing the spectrum of the endmember

- Step 3: Performing the SAM Classification.
  - We will use SAM to create a classification image that shows how well each pixel of the input image matches the endmember spectrum. Then, we will create binary images showing which pixels exceeded the threshold for matching the endmember.
  - In the main menu bar, select Spectral.
    - Then select Mapping Methods.
    - Then select Spectral Angle Mapper.
    - Then select the file in the list to use as the input image. For this exercise select **Aunp_cell-60x-1** (Figure 5.2i).

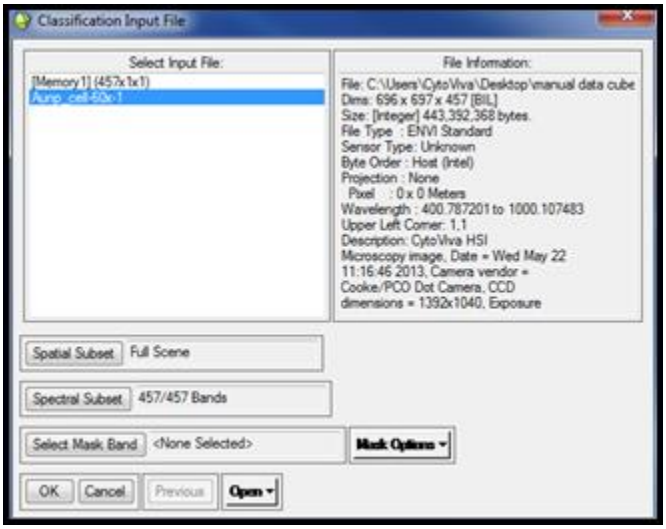


Figure 5.2i. Selecting the input file for classification

- For this specific example wavelengths above 800 nm provide no information for this sample image. So to exclude the bands covering wavelengths above 800 nm, use the Spectral Subset feature. SAM will create a 400-dimensional vector from 400 nm to 800 nm.

- Click on the Spectral Subset button.

- All bands are highlighted by default.

- First, remove the selection from all bands by clicking Clear.

- Then in the boxes to the left of Add Range enter 1 in the far left box and 312 in the box just to the right (this band corresponds to 800 nm) (Figure 5.2j).

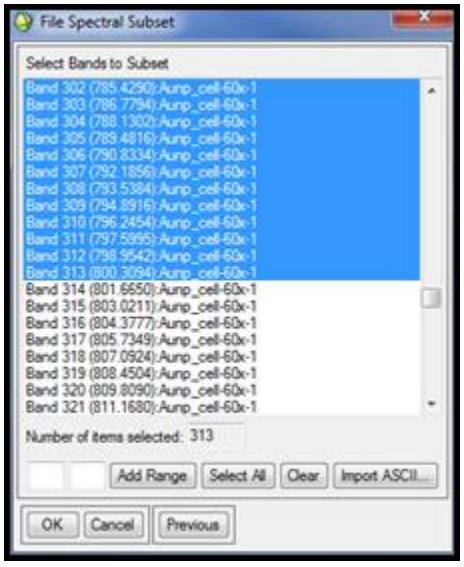


Figure 5.2j. Select the bands by using the Add Range boxes

- Then click Add Range.
- Then click OK.
- The Spectral Subset now shows 312 out of 468 bands are selected (Figure 5.2k).

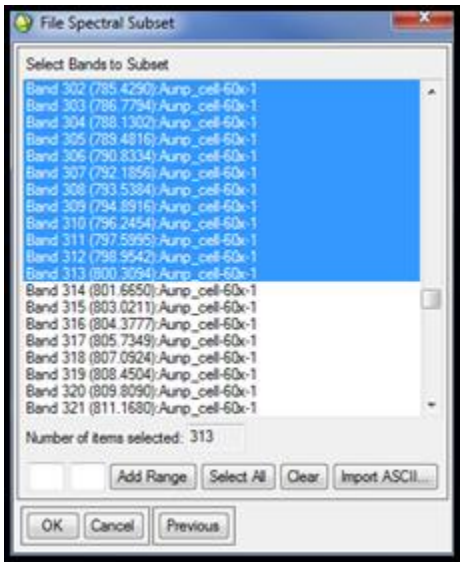


Figure 5.2k. Selection of a subset of the spectral bands

- Click OK again.
- You will see the Endmember Collection: SAM dialog box open.
- From the Plot Window, drag the label AuNP_in cell to the Endmember Collection: SAM box. It is highlighted automatically in the list (Figure 5.2l).

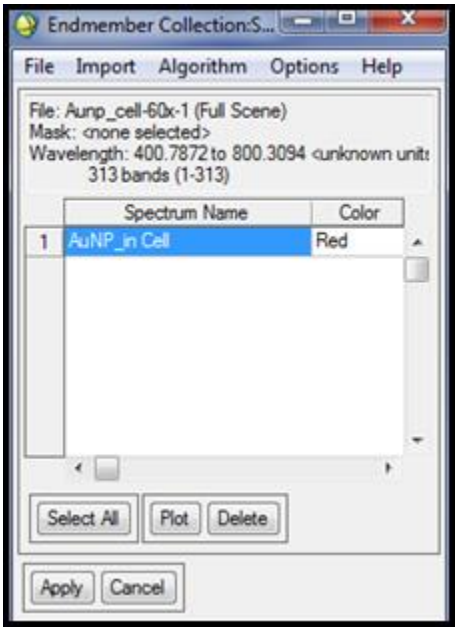


Figure 5.2l. Endmember Collection dialog with entry from label dragged from plot

- Click on Apply.
- You will see the Spectral Angle Mapper Parameters dialog box open.
- For Set Maximum Angle (radians), click on single value.
- Keep the Maximum Angle (radians) at 0.1. We will use this threshold first for the spectral angle.
- For Output Result, select Memory. This will send the classification image to the available bands list. The Output Result image can also be saved as a file. The file name for this image should be chosen as: filename_SAM.dat.
- For Output Rule Image, select No. Since the Rule image is not necessary to process the classification. The Rule image can help you understand how well the classifier is working – see ENVI help.

- If you want to Preview the Classified Mapped Image, click Preview (Figure 5.2m) from the Spectral Angle Mapper Parameter window. This will show you what pixels are going to be classified before saving.

Figure 5.2m. Window to preview

- After you click Preview, the Preview window is added to the SAM Parameter window (Figure 5.2n). The overview window ONLY shows the pixels of a section of the entire image that are classified by the SAM. Unclassified portions of the sample will not be shown.

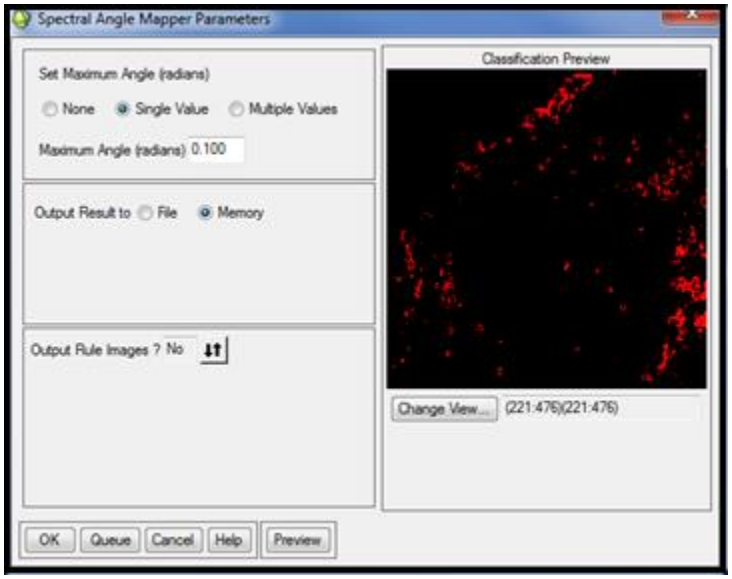


Figure 5.2n. Spectral Angle Mapper Parameters window with the preview window

- To change the preview area, click the Change View button (located under the Classification Preview window). The Select Spatial Subset dialogue box opens.
- Click the Image Button, the Subset by Image box opens.
- Move the red square to the desired area (Figure 5.2o).

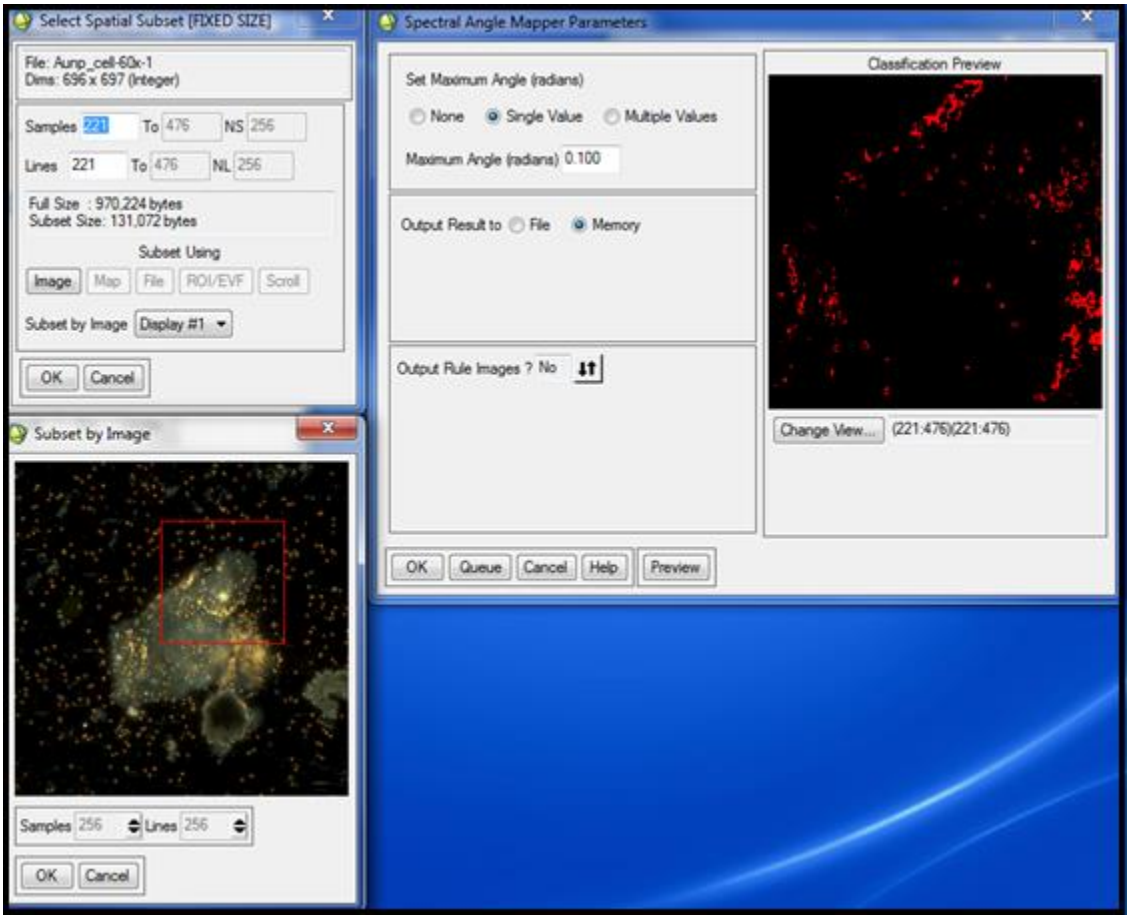


Figure 5.2o. Spectral Angle Mapper Parameters box, Select Spatial Subset box, Subset by Image

- Then Click OK in the Subset by Image Box and the Select Spatial Subset box.
- Once you have found the desired classification, click OK.
- The SAM files are now added to the Available bands list. Now there are two images added to the top of the available bands list.

- To load each image in a new display, right click on the Rule Image. Then select Load Band to New Display.
    - An image will open.
    - Then right click on the SAM image.
    - Then select Load Band to New Display.
    - Another image will open.
    - You should now have the original image, the original image and the SAM classification image shown on your screen. For future SAM classifications, you can save Output Results and Endmember Spectra by choosing the File option rather than the Memory. If you close out the ENVI software, this file will be lost since it was saved to memory.

5.3 Re-Loading Images from Available Band Lists Window

In some cases you may load one image over another by accident. This usually happens by clicking Load Band to Current Display rather than Load Band to New Display. If this happens it will load the new image over the original image. You will need to reload the old image to a new display.

- To reload the original image (Figure 5.3a), scroll through the Available Bands List to find the original image. In this exercise the image name is **Aunp_cell-60x-1.**
- Click on the file name to highlight it.
- Right click and select the option Load True to <new>.

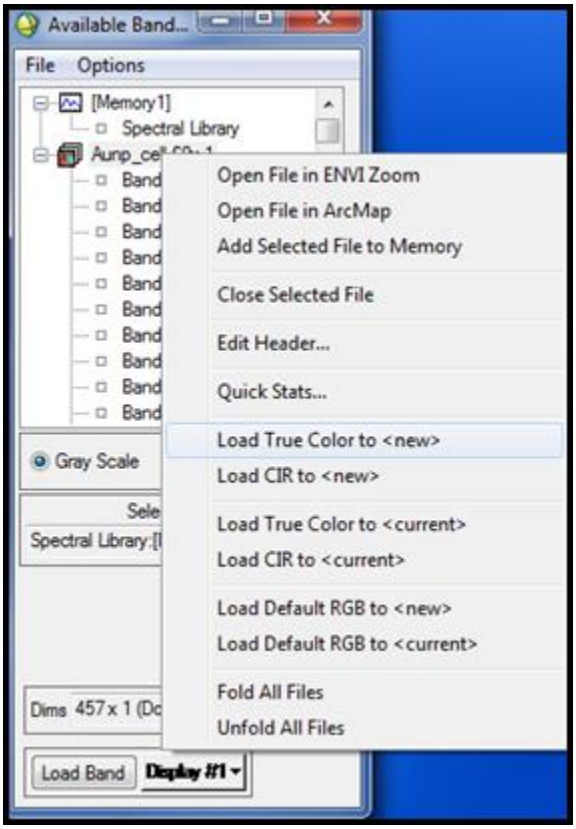


Figure 5.3a. Available Bands List

5.4 The Classification (SAM) Image:

The classification image shows the location of pixels whose spectra match that of the endmember. You will see that using a value of 0.1 for the maximum angle causes only pixels in the selected particle to be classified as a match for the endmember spectrum. The zoom window rectangle surrounds this particle, as shown in Figure 5.4a. In the zoom window, directly below the main image you can see that three pixels were classified. The rest of the pixels within the particle contained sufficiently different spectra, as did those of every other particle, and they were rejected as matches for the endmember. This outcome suggests that the spectral angle chosen for the classifier is too small.

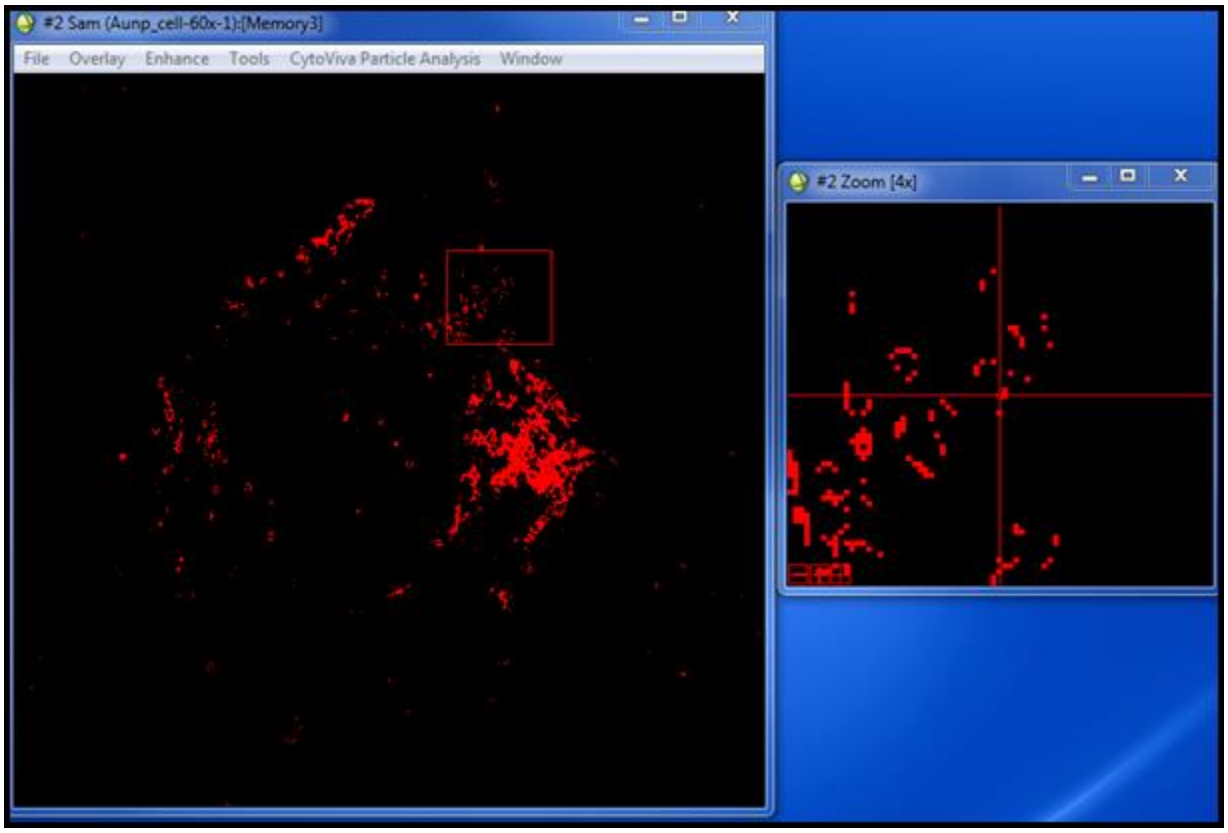


Figure 5.4a. Classification image using a low maximum angle in SAM

5.5 Re-Classifying the SAM

For this example we believe other AuNP particles contained in the image have spectra that are similar to the spectra collected for the first particle. Thus, we will try to add more of the particles into the classification.

- Go back to the Spectral Angle Mapper Parameters window.
- To return to the Spectral Angle Mapper Parameters window click apply in the Endmembers Collection: SAM window.
    - The Spectral Angle Mapper Parameters dialogue box opens.
    - Change the Maximum Angle from 0.1 to 0.12 (We recommend a default angle of 0.1).
    - Now click Preview. The new classification result is shown to the right of the dialog. You can see that more particles are classified this time.
    - For Output Result, select Memory.
    - For Output Rule Image, select No.
    - For Output Result, select Memory.
    - Then click OK.
    - From the top of the available bands list, right click on the new image.
    - Then click on Load Band to New Display. The new classification (#2 SAM) images (Figure 5.5c) with the original image (Figure 5.5b) and the original classification (#1 SAM) Image at the side (Figure 5.5a). When you compare the first and second classification images, you will see that additional particles scattered about the cell are classified as AuNPs when the angle was set to a greater value. The correct value for the angle must be found by experimenting with different values.

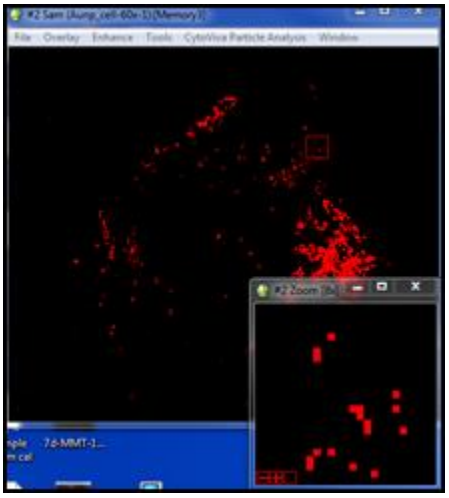


Figure 5.5a. Original Classification (#1 SAM) Image Obtained Using 0.1 Maximum Angle

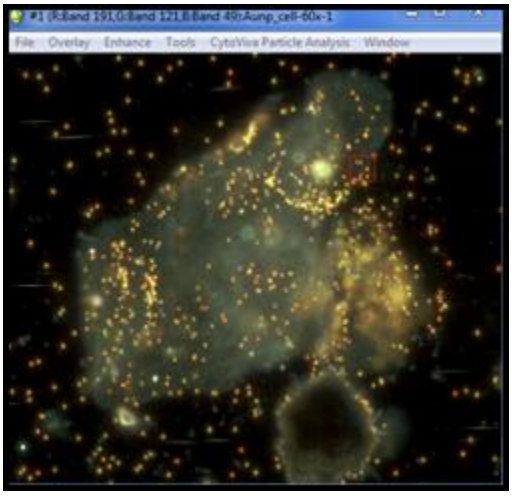


Figure 5.5b. Original Image of the AuNPs in Cells

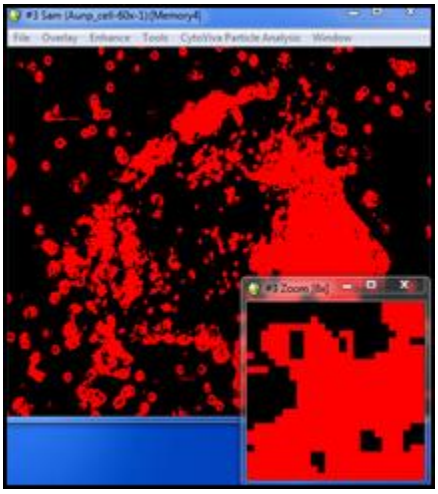


Figure 5.5c. New Classification (#2 SAM) Image Obtained Using Maximum Angle 0.3

5.6 Linking the Displays

This feature allows you to link both the original image and the classified image to view the same areas.

- Right click in the Original Image Window.
  - Select Link Displays.
  - Link Display dialogue box opens.
  - No changes are needed so click OK.
  - Now when you move the Zoom box in any of the three images of the original or classified image they move in sync.
  - If you left click and hold in any window it will display what is in the opposite window (Figure 5.6a).

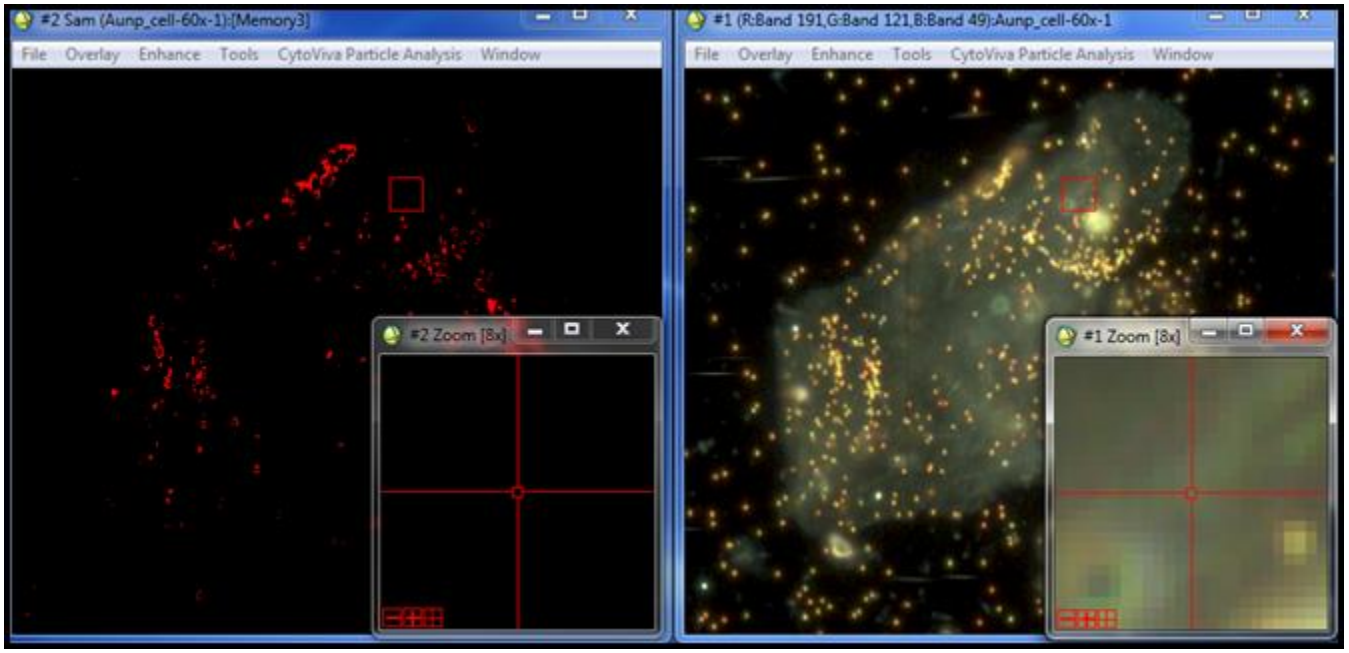


Figure 5.6a. Linking the two displays

  - To unlink the original image and classification image right click on the original image. Then select Unlink Displays.

5.7 Overlaying the Classification Image

This feature provides you with the capability to overlay the classification image onto the original image. It will enable you to illustrate where the classification pixels are in the context of the original image.

- In the original image window select Overlay from the menu bar.
  - Then select Classification.
  - The Interactive Class Tool Input File dialogue box opens (Figure 5.7a).

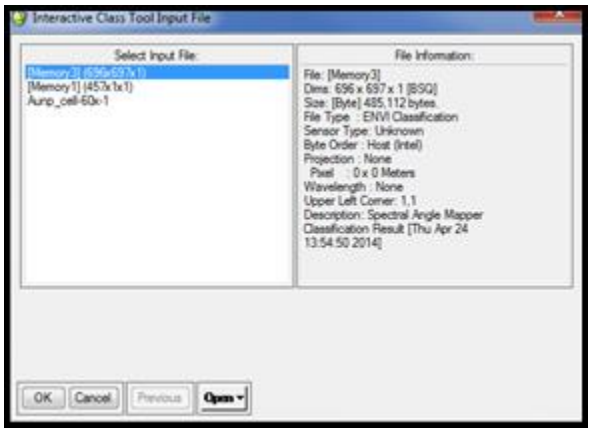


Figure 5.7a. Selecting image to overlay

  - Select the classification file created from the SAM classifier.
  - For this example select Memory 3 (or samples will have a file name).
  - You can look at the Available Bands List to help determine which one is the SAM image.
  - Click OK.
  - The #1 Interactive Class Tool dialogue box opens.
  - Click on the tab beside the red box marked AuNP in cell (Figure 5.7b).

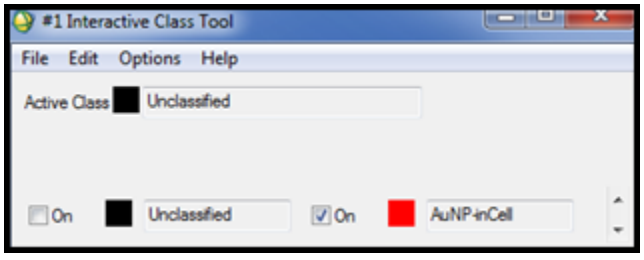


Figure 5.7b. Activating the overlay feature

- Now you will see an overlay image of your classified picture and the original image (Figure 5.7c).

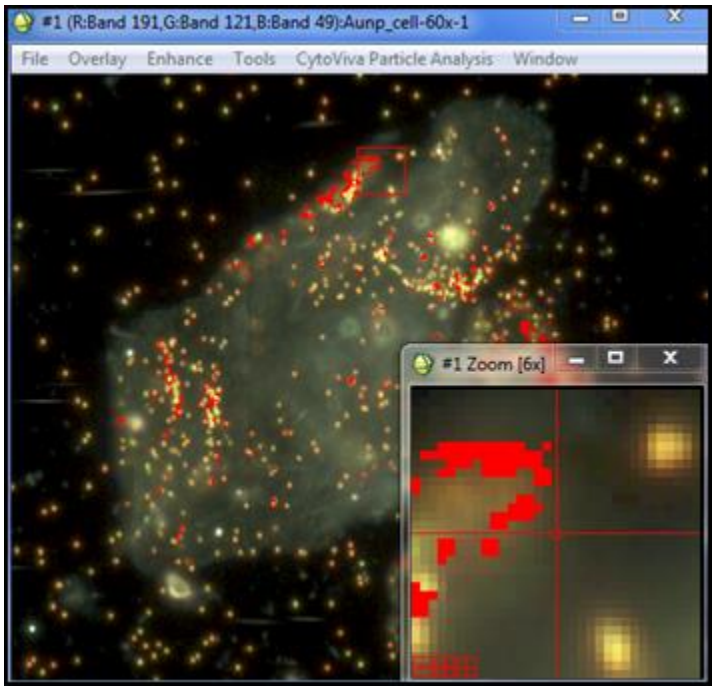


Figure 5.7c. View of the classification overlay

5.8 Merging Colors in a Classification Image

Sometimes multiple spectra are used to classify particles in a sample. This will give an overlay that has multiple colors. Merging will allow you to combine the multiple colors into a single color.

- Open the image to be classified (Figure 5.8a), scroll through the Available Bands List to find the original image.

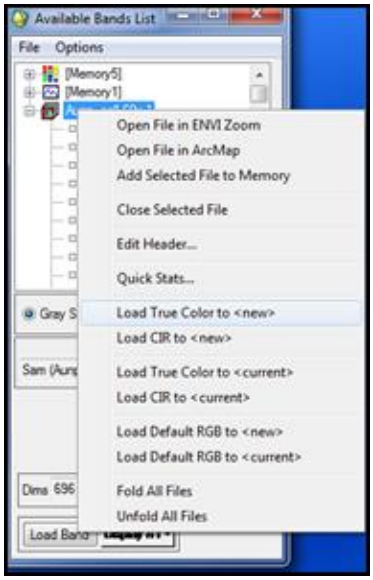


Figure 5.8a. Available Bands List

  - Click on the file name to highlight it.
  - Click Display # and select New Display.
  - Click Load Band to display the image.
  - In the picture window (Figure 5.8b).
    - Choose Overlay.
    - Click Classification.

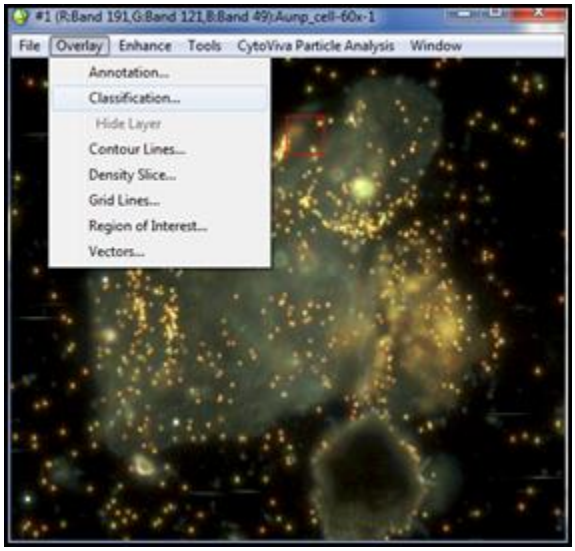


Image 5.8b. Image to be given an overlay

- The Interactive Class Tool Input File window appears (Figure 5.8c).

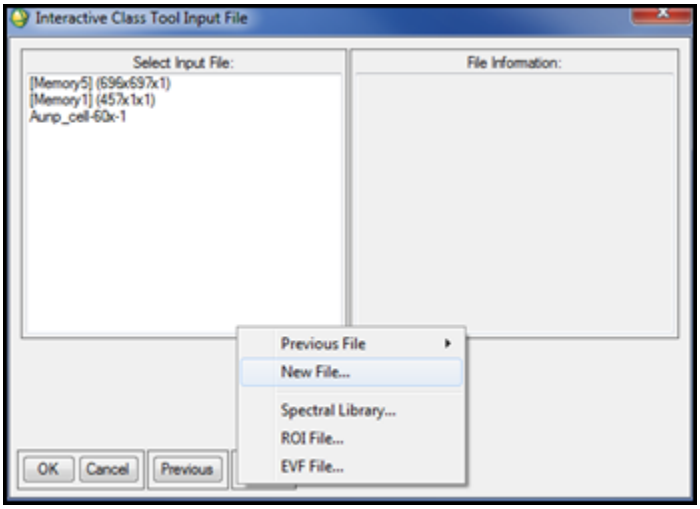


Figure 5.8c. Interactive Class Tool Input File window

- Select file, then click Okay. If the classification file is not in the Select Input File list, then click Open and select New File.
- An Interactive Class Tool window will appear.
- To show the overlay.
  - Right click on any of the colored squares to activate and overlay all classes (Figure 5.8d).
  - In the Interactive Class Tool window, click Options.
  - Select Merge classes (Figure 5.8e).

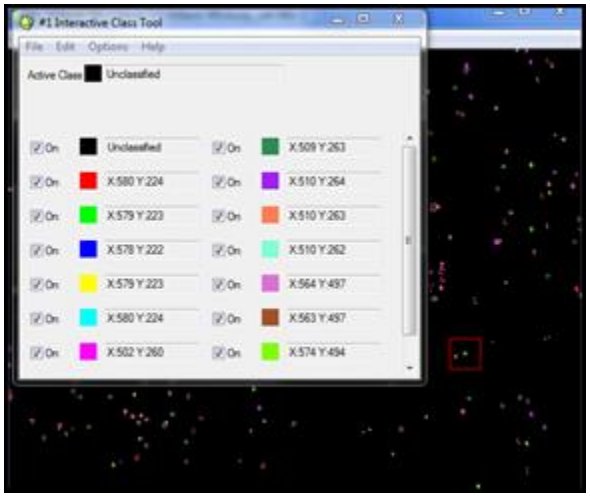


Figure 5.8d. Overlay Image with Interactive Class Tool Window

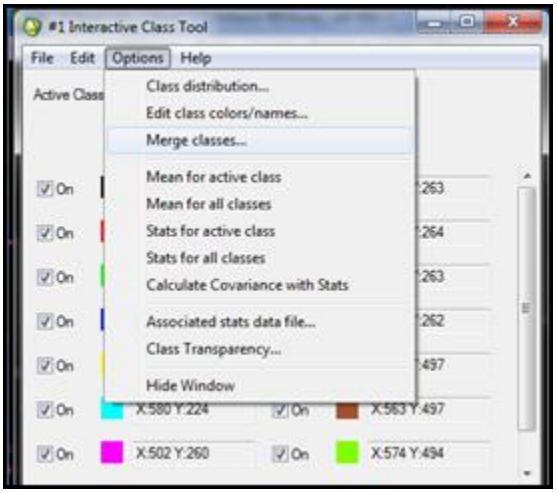


Figure 5.8e. Options Drop-Down Menu

- In the Interactive Merge Classes window (Figure 5.8f).

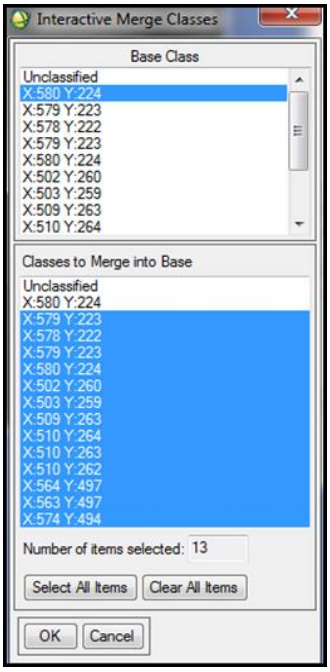


Figure 5.8f

- Select one coordinate (X###:Y###) in the Base Class list (The first is automatically defaulted to red).
- In the Classes to Merge into Base list.
- Select all other coordinates (Do not select the coordinate chosen in the Base Class list).
- Click Okay.
- The classification file will automatically update to a single color (Figure 5.8g).

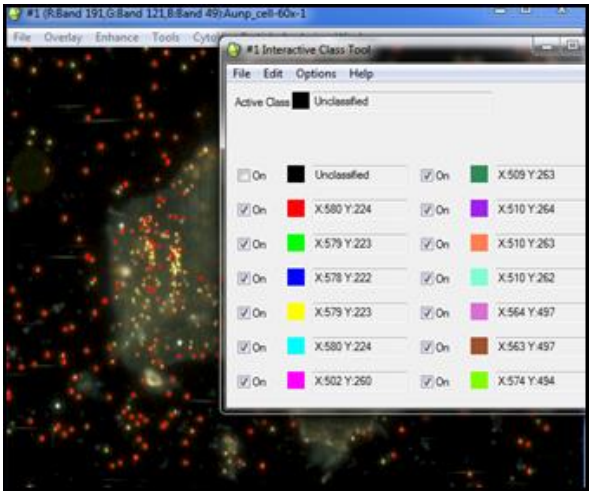
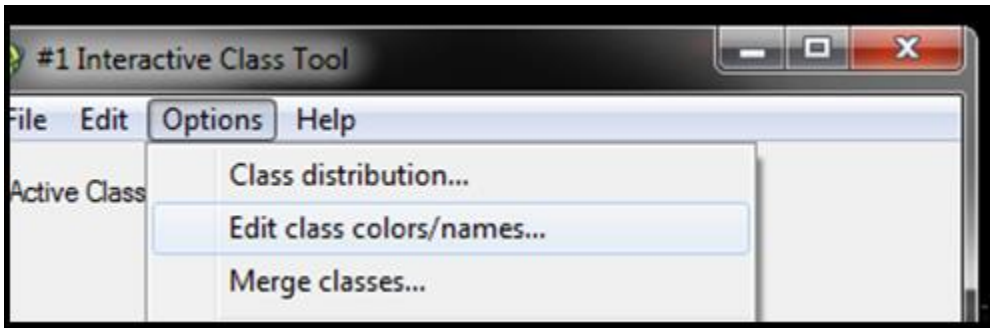


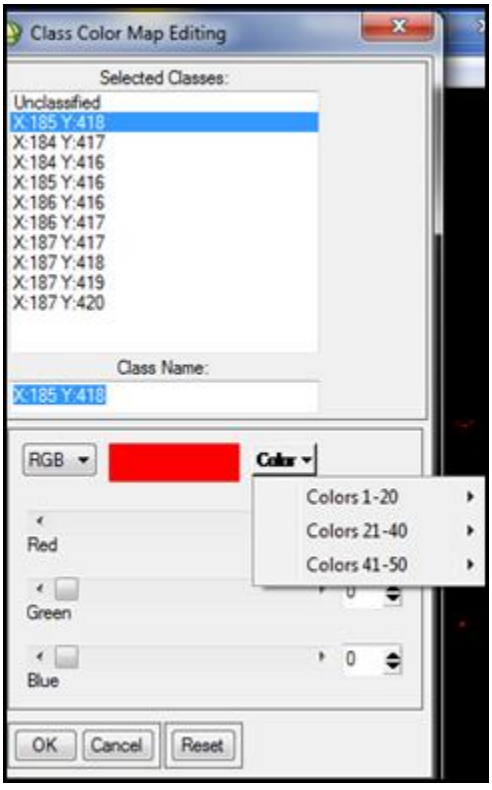
Figure 5.8g. Classification spectra unified to a single color

5.9 Editing Colors in Classification Image

- In the Interactive Class Tool window, select Options, click Edit class colors/names…



- In the Selected Classes box, choose the coordinate to be changed. You can rename this coordinate in the Class Name box. Click on Color, select desired color. Click OK.



5.10 Removing Unwanted Spectra from Data Analysis

This feature provides you with the capability to remove spectra once data analysis is complete and a classification file is created.

- In the original image window select Overlay from the menu bar.
  - Then select Classification.
  - Select the appropriate file.
  - The Interactive Class Tool window appears (Figure 5.10a).

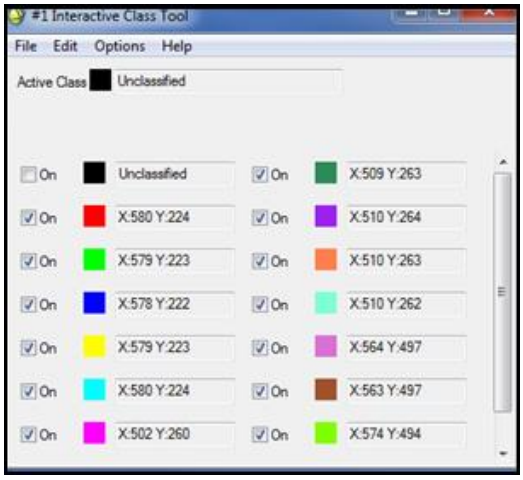


Figure 5.10a. View of the overlay image

  - Select Options.
  - Click on Class Distribution (Figure 5.10b). The Classification Distribution window shows the percent reactivity of each individual spectra. You must print this window or manually record the data to be removed, as this window will minimize when you navigate away.

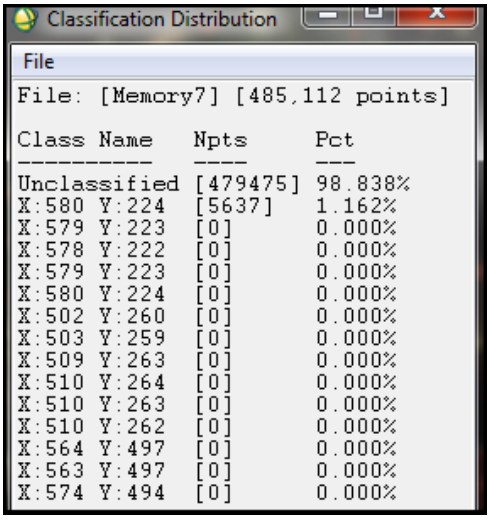


Figure 5.10b

- Open the spectral library (Figure 5.10c).

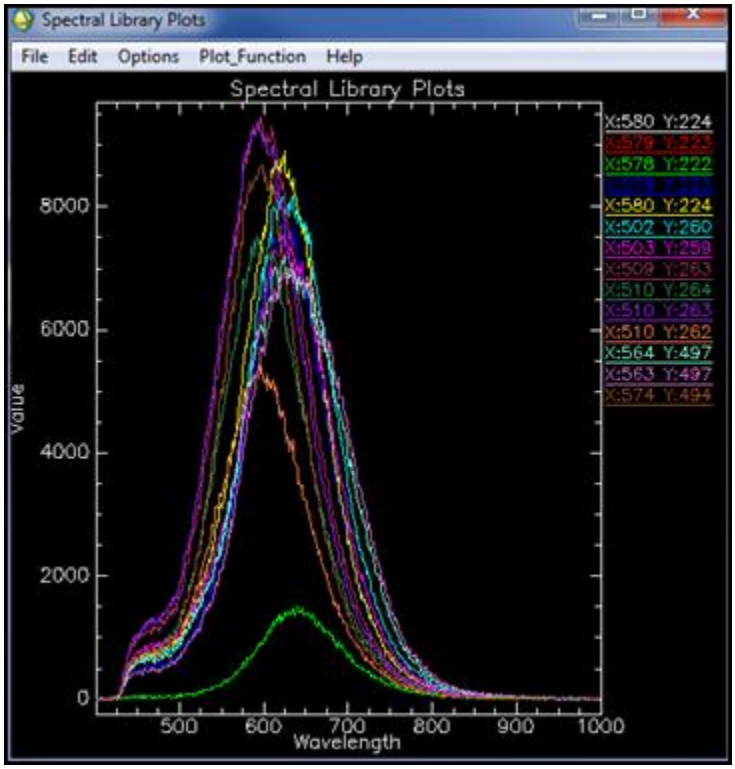


Figure 5.10c. Spectral library viewer window used to create the spectral library plot

- In the ENVI task bar, select File, click Open Image File.
- Select spectral library file to be edited. A Spectral Library Viewer window will open.
- Click on each spectra to create a spectral library plot.
- In the Spectral Library Plots window, select Options.
- Click on show plot key and all spectra will be listed to the right of the spectral plot.
- Right click on the spectra to be removed.

- Then select Remove X: ### Y:###, for all spectra chosen (Figure 5.10d).

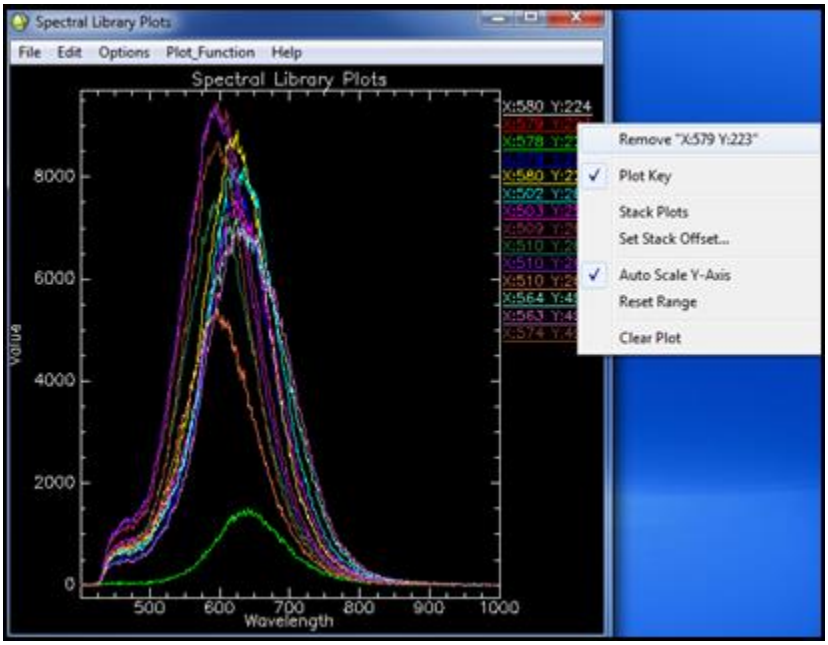


Figure 5.10d. Selection of spectra from plot key to be removed

- Save the file as a Spectral Library (Figure 5.10e).

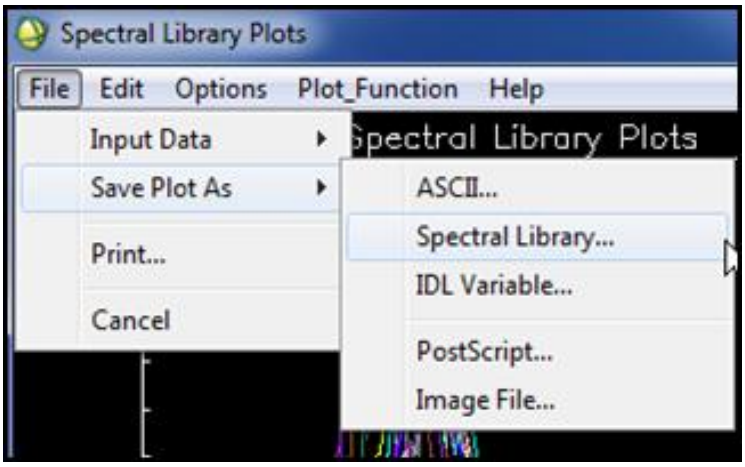


Figure 5.10e. Saving Spectral Library

- Click File.
- Select Save Plot As.
- Click Spectral Library.
- Click Select all Items in Output Plots window (Figure 5.10f).

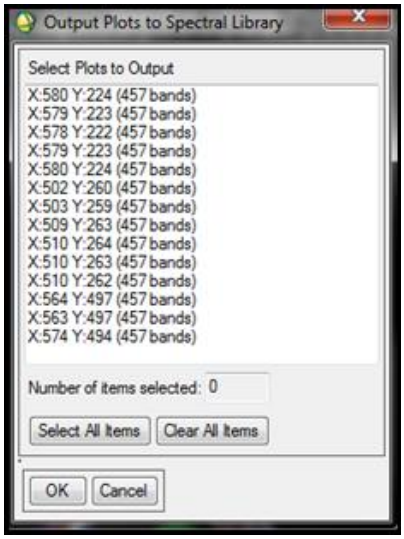


Figure 5.10f

- Then click OK.
- Click Choose in the Output Spectral Library window (Figure 5.10g).

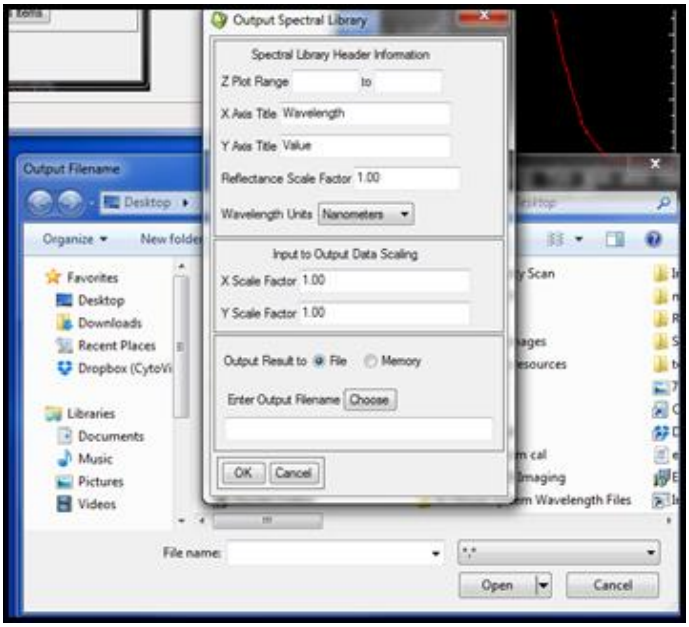


Figure 5.10g. Saving Output Spectral Library

- Enter file name in Output Filename window, click Open.
- Click okay in Output Spectral Library window.

5.11 Mean Spectra Analysis and Normalization

Mean Spectra Analysis and Normalization allows the user to compare spectra from separate samples. This is done by making all the intensities equal to 1.

- Mean Spectra Analysis:
  - In the Image Window menu bar select Tools (Figure 5.11a).

Figure 5.11a. Opening the ROI Tool

  - Select Region of Interest.
  - Click ROI tool.
  - Highlight the Region of Interest needed (refer to Section 2, Part 10: Working with Regions of Interest ROIs).
  - Then Click the Stats radio button (Figure 5.11b).

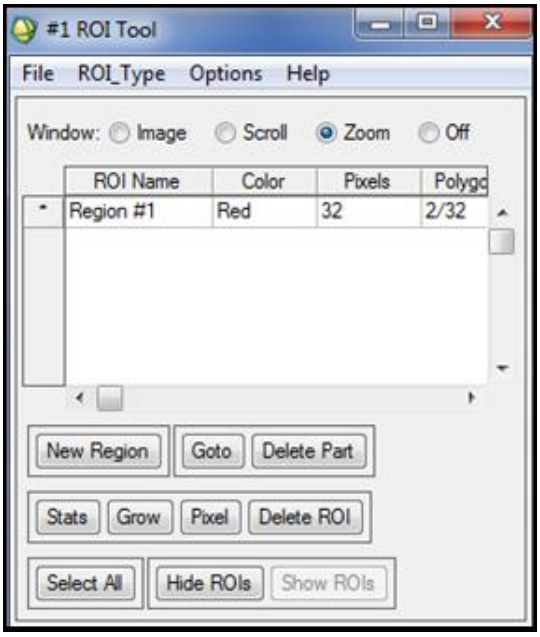


Figure 5.11b. ROI Tool Window

- The ROI Statistics Results window appears.
- Right click on the graph.
- Click Plot Key.
- Right click on the graph again.
- Select Options.
- Click New Window: Blank (Figure 5.11c).

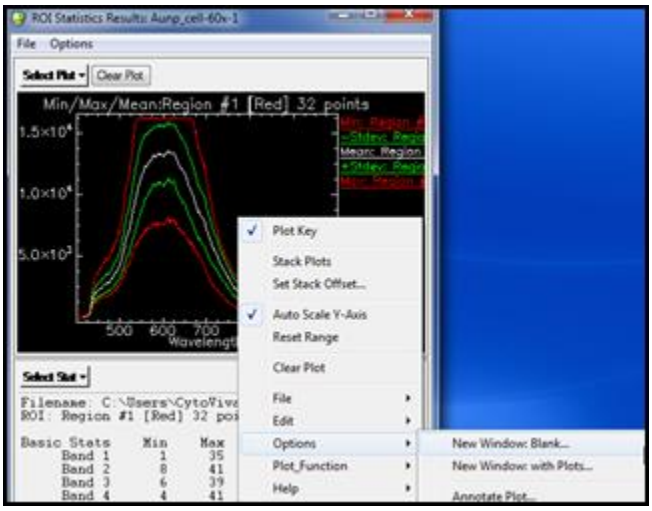


Figure 5.11c. ROI Statistics Results

- A new ENVI Plot Window opens (Figure 5.11d).

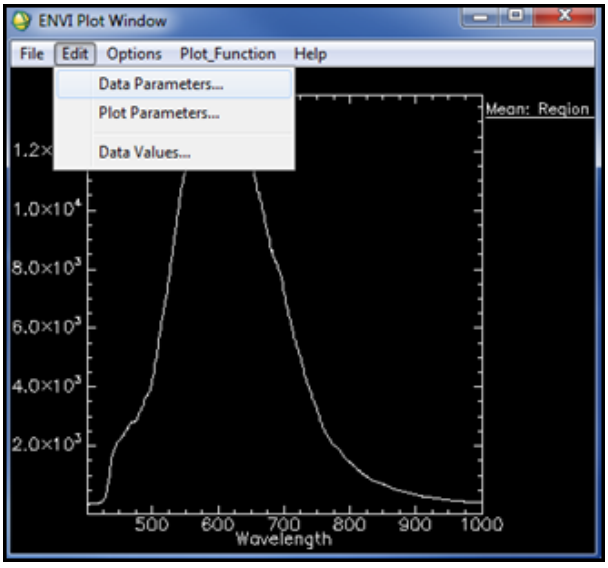


Figure 5.11d. ENVI Plot Window

- To load the mean spectra into the New ENVI Plot Window.
- Click and drag the Mean: Region of Interest from the ROI statistics window into the new ENVI Plot Window. Multiple graphs can be loaded into this window for comparison by dragging and dropping the required data.
- Click Edit from the menu bar and select Data Parameters.
- The Data Parameters Window opens. From here you can (Figure 5.11e).

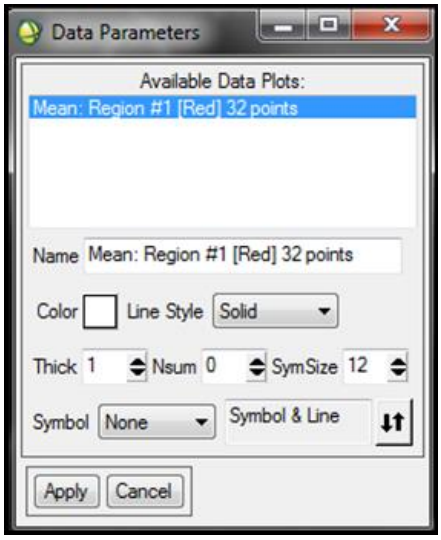


Figure 5.11e. Data Parameters Window

- Label the graph.
- Rename the selected region.
- Change the color of the plot: (Figure 5.11f).

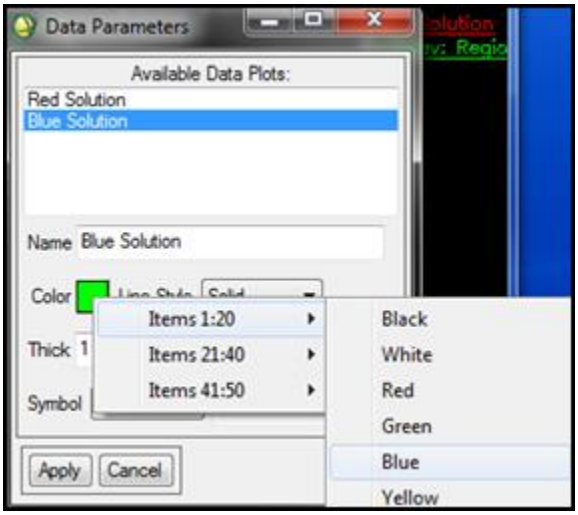


Figure 5.11f. Changing Graph Color

- To change the color, right click on the color square.
- Move mouse over Items: ##:##.
- Select a color.
- Repeat these steps to for other ROIs that you want to normalize for analysis.
- Normalizing: Using the ENVI Plot Window that has the spectra to be normalized open (Figure 5.11g)

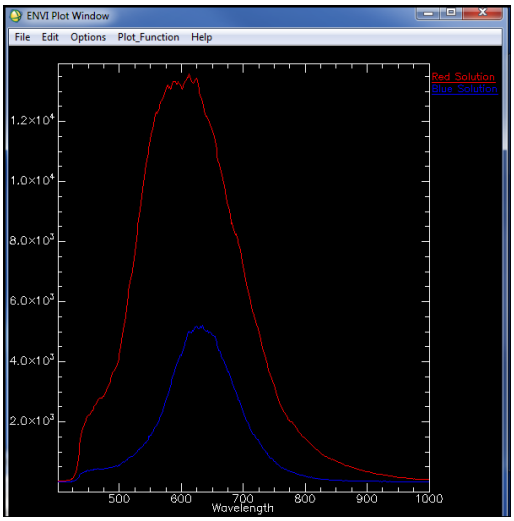


Figure 5.11g. Spectra to be normalized

- In the main ENVI toolbar.
- Select Spectral.
- Then select Spectral Math.
- The spectral math window will appear (Figure 5.11h).

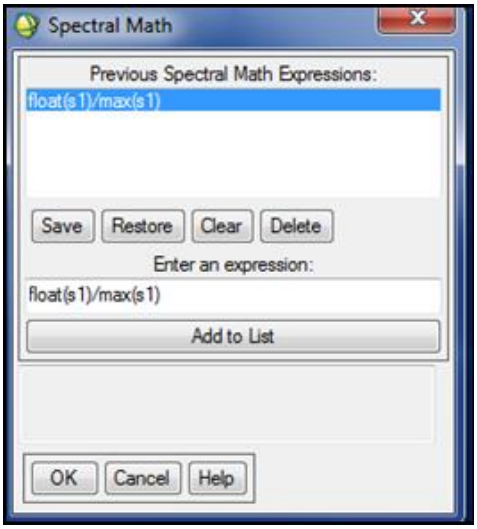


Figure 5.11h. Normalizing expression in Spectral Math window

- Enter the normalizing expression: float(s1)/max(s1).
- Click OK.
- A Variables to Spectra Pairings window will appear.
- In the Available Spectra list, click the curve to be normalized.
- Click the double arrows to change Output Result to New Window.
- Click OK (Figure 5.11i).

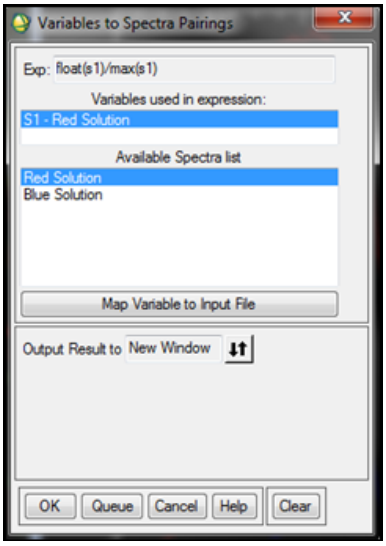


Figure 5.11i. Variables to Spectra Window

- Repeat for all desired spectra.
- A new graph will appear with a vertical axis set to 1.
- Use Edit menu to change colors and labels (Figure 5.11j).

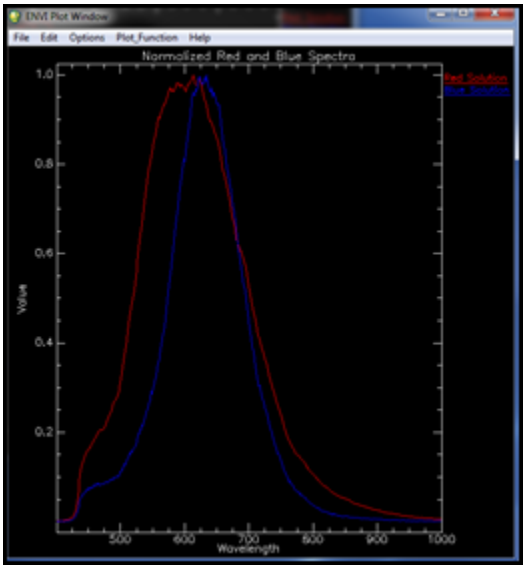


Figure 5.11j. Normalized Spectra

5.12 Resizing an Image

Resizing an image allows the user to focus on an area of interest and cut extraneous parts of the image.

- In the ENVI toolbar.
- Select Basic Tools.
- Click Resize Data (Spatial/Spectral) (Figure 5.12a).

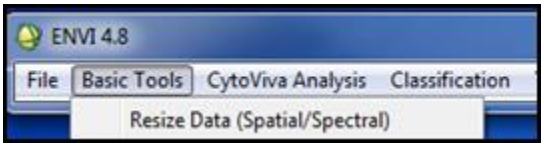


Figure 5.12a. Opening the Resize Window

- The Resize Data Input File box Opens (Figure 5.12b).

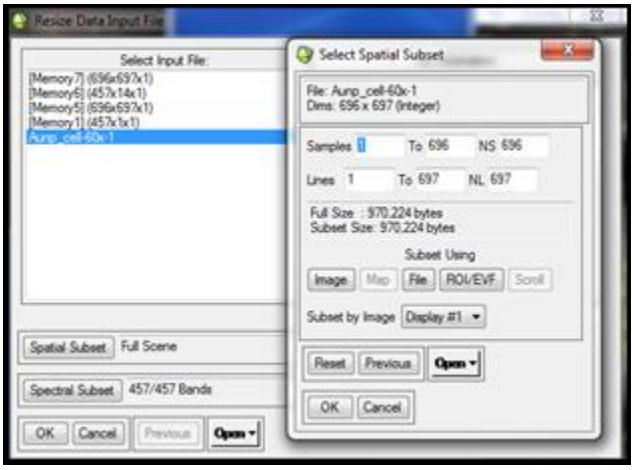


Figure 5.12b. Select Spatial Subset window

- Select file to resize.
- Click Spatial Subset radio button.
- The Select Spatial Subset window appears.
- Click the Image radio button.

- A Subset by Image window appears. The whole scan is shown in this window. There is also a red box that appears (Figure 5.12c).

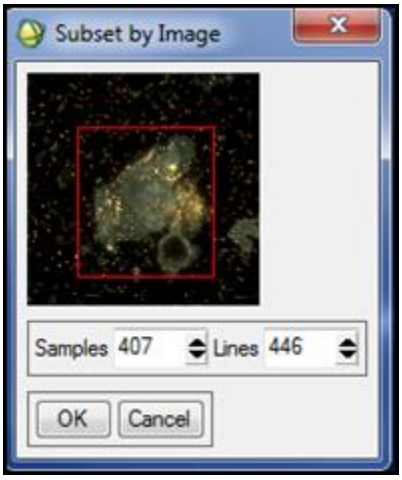


Figure 5.12c. Subset by Image Window

- Click and drag the corners of the red box to the preferred size.
- Click OK to exit Subset window.
- Click OK to exit Spatial Subset window.
- Click OK to exit Resize Data window.
- Save file accordingly and the newly resized image will appear.

5.13 Spectral Sub-setting an Image

Spectrally sub-setting an image allows the user to focus on specific wavelengths of light. This is useful in removing second order harmonics and other known reflective contaminants.

- In the main ENVI toolbar.
- Select Basic Tools.
- Click Resize Data (Spatial/Spectral) (Figure 5.13a).

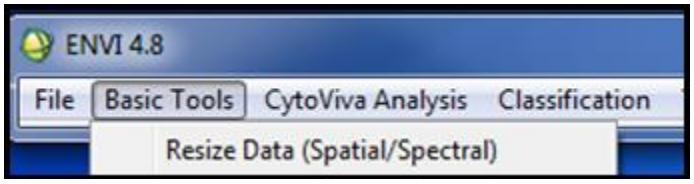


Figure 5.13a. Opening the Resize Window

- Choose image to resize in the Select Input File list.
- Click the Spectral Subset radio button (Figure 5.13b).

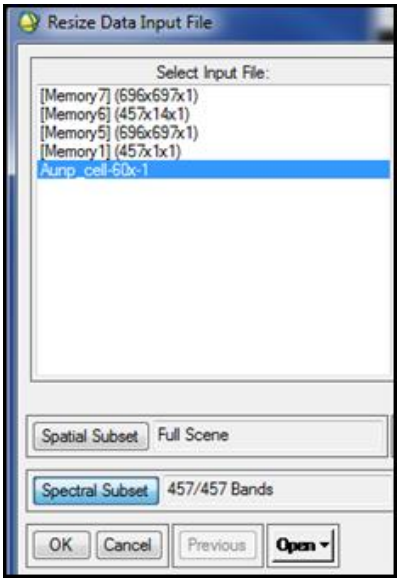


Figure 5.13b. Resize Data Input file window

- The File Spectral Subset window appears.
- Select the range of spectra to become the subset by clicking and scrolling (Figure 5.13c). To select non-contiguous values hit the Ctrl key.

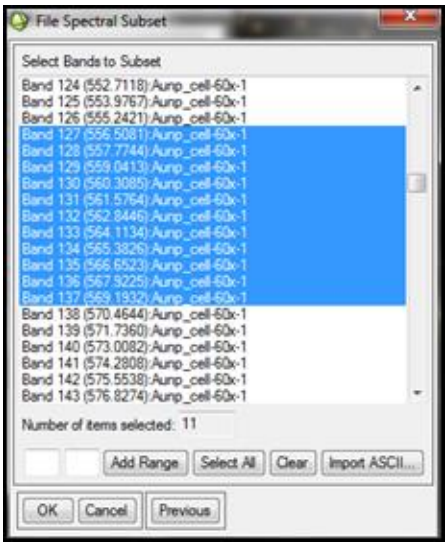


Figure 5.13c. File Spectral Subset Window

- Click OK to exit Spectral Subset window.
- Click OK to exit Resize Data window.
- Save file accordingly and the newly sub-setted image will appear.

Section 6 Quantitative Spectral Analysis

In a hyperspectral scan, the spectral features are produced by specific light scattering and absorption properties of the specimen materials that are recorded at each image pixel. Light scattering is strongly influenced by surface chemistry, whereas the light absorption will depend on internal as well as surface properties of the specimen. The recorded spectrum also contains features that are properties of the light source that are used to illuminate the specimen, and also of optical filters that alter the light reaching the specimen. The recorded data actually shows the spectrum of the light from the source, going to the sample where it is modified by specific spectral features from the specimen. For example, light absorption by a specimen over certain wavelengths may reduce the recorded light intensity at those wavelengths, but leave what is essentially a spectrum of the light source at the other wavelengths. This occurs when the sample contains light absorbers such as dyes and pigments, like melanin, that are intrinsic to the sample, or if there are dyes that are added to create contrast from sample structures. For example, the hematoxylin and eosin stains will modify the spectrum differently by virtue of their distinct light absorption properties. This situation is common for all of the microscope methods that directly sample the illumination, such as bright field, reflected bright field and dark field modes.

The situation is different for spectra of nanoparticles made from noble metals. For these, recorded spectra show light scattered within narrow peaks that are caused by plasmon resonances, where nanoparticle electrons resonate at frequencies that are influenced by the dielectric materials properties surrounding the particle. When recorded with the dark field method, the recordings are exclusively from the scattered light and do not contain any of the light absorption features. So, instead of recording the light source spectrum after it has being modified by features of the specimen, the recording contains discrete resonance peaks whose strengths are set by intensity of the light source at the resonance wavelengths. Of course if the specimen contains dyes, pigments and nanoparticles, both kinds of effects can occur together in the recorded spectrum.

For all types of HSI spectra, simple procedures can be used to improve accuracy and repeatability so that results can more easily be compared between recordings. One of the simplest methods is spectral normalization. This procedure is able to counter the effects of the camera and spectrograph on the spectral recording. Both of these components act to reduce the strength of the recorded spectrum at longer wavelengths. The idea of spectral normalization is to compensate for these effects so that spectral characteristics of the sample are clearly seen in the data. To perform this, it is first necessary to have the light source spectrum that was obtained with your HSI system.

6.1 Recording of the Light Source

- This step is described using the CytoViva dark-field condenser.
- Prepare a calibration slide with two samples under different cover slips. Put the samples close to each other. Use clean glass slides.
- The first sample (#1) should be blank, with just media (such as water or microscope oil) and coverslip on the glass slide.
- The second sample (#2) should include particles under the cover slip that are stable and that you normally have available.
- Add a small amount of immersion oil to the cover slip above each sample.
- Begin by aligning the CytoViva condenser and 10X oil objective using the particle sample (sample #2). Make the adjustments just as you would for normal viewing of the sample.
- Switch to the 100X oil objective. Adjust for best focus. Then open the objective iris fully. You will see a bright field of illumination. This happens because the numerical aperture (NA) of the objective exceeds the NA of the darkfield condenser.
- Move the stage to sample #1 (blank sample). You will again see the bright illumination, this time with no contribution from particles. The steps that you have taken ensure that the glass and oil are present just as they would be for actual recordings. Also make sure the Dolan Jenner light source is at 100% power.
- Record 50 lines from the blank sample using the normal HSI settings. Make sure the camera exposure is short enough that the plotted line is not clipped in the preview mode. Normally 0.005 seconds is a good camera exposure time for recording the lamp but you may need to decrease or increase the exposure so that the maximum intensity value on the Y axis is between 1000 and 2000 (Pixelfly PCI camera) or between 4000 and 8000 (Pixelfly USB camera and Andor cameras).
- Name the file lamp and append today's date to the file. This file is an HSI data cube containing a pure recording of the lamp (Save the calibration slide for later use).
- If the halogen light source is used every day, it is best to record the lamp once per month since aging of the lamp can affect the spectrum of the output. With light to moderate usage, a new lamp recording should be made every three months. If you halogen light source has an aluminum reflector lamp installed, the output dial should be set to full output.

- After the HSI files opens in ENVI you will see an image of a bright field of pixels. Use the ROI tool to draw a region of interest in the center of the image (Figure 6.1a):

Figure 6.1a. Image from the lamp recording

- A new region appears in the ROI list. Click on Stats.
- A plot of the lamp spectrum appears in the ROI Statistical Results window (Figure 6.1b). Right click and select Plot Key. The white curve labeled Mean: Region is the spectrum of the lamp that you will use:

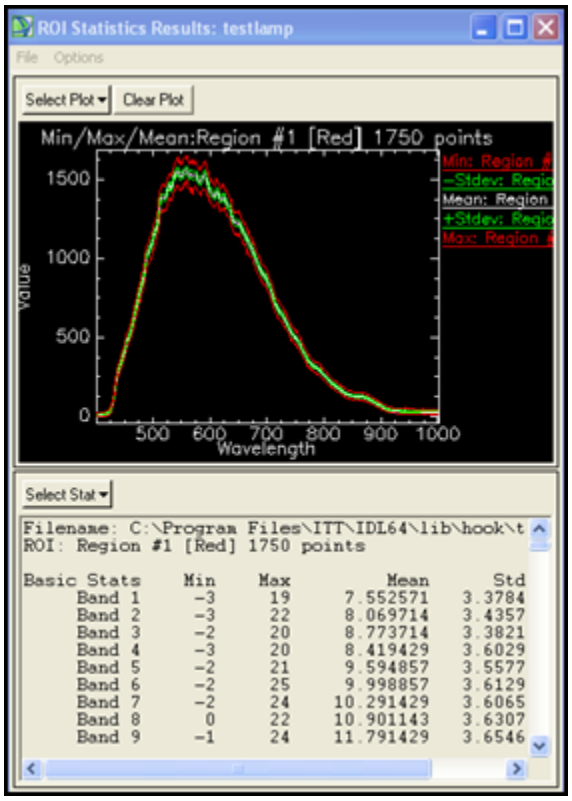


Figure 6.1b. Spectrum from lamp recording in ROI Statistics Results

- Now we create a Correction Spectrum from the spectrum of the lamp. In the main ENVI menu select Basic Tools > Spectral Math. Click the Restore button and then click on the file correction.exp. This file has been installed in the folder named hook, which is the first folder opened by the Restore button. The expression float(s1)/max(s1) opens in the Expressions list of the Spectral Math window (Figure 6.1c). Click on the expression and click OK.

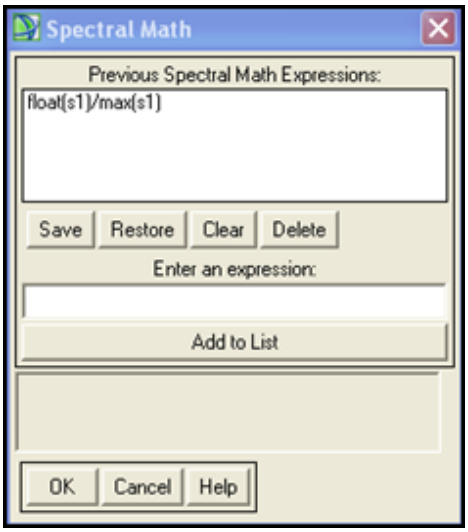


Figure 6.1c. Spectral Math input window

- Assign the spectral curve labeled Mean: Region #1 to the expression by clicking on it in the Variables Used in Expression list (Figure 6.1d). Change the Output Result to New Window and click OK. The Correction Spectrum opens in the Spectral Math Result Window.

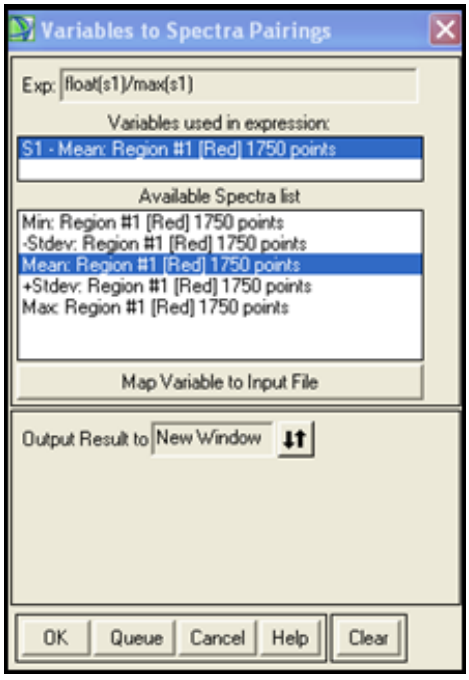


Figure 6.1d. Variable Assignment Window

- Save the Correction spectrum as a Spectral Library using the File > Save Plot As > Spectral Library tool. Use the file name Correction Spectrum - SL, which identifies it as the current correction spectral library. Save it in your imaging folder where it can be accessed easily with the Normalized for Lamp Spectrum feature. Note the maximum value of the curve is 1.0 (Figure 6.1e).

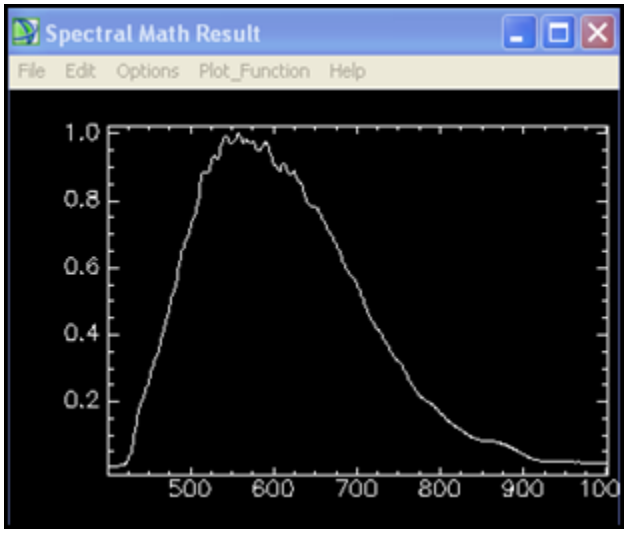


Figure 6.1e. Correction Spectrum

- The Correction Spectrum will be used to correct data cubes so that the unique spectral properties of the lamp, camera response, and spectrograph do not influence the spectrum of the sample material. Before correction, the relatively low strength of the illumination and low response of the camera at both ends of the wavelength range produce an uneven response in the sample spectrum. This uneven response is removed from the sample spectrum after correction.

6.2 Spectral Normalization of the Lamp

This procedure removes the effect of the lamp spectrum so that sample features are seen more clearly.

- First set the value of the highest point on the lamp spectrum to unity. If you recorded a new lamp spectrum, go to Basic Tools located on the main ENVI menu, select Spectral Math.
- Type in the expression: float(S1)/max(S1) and add it to the list of expressions. Select the expression and hit OK.
- Use the Save button to save this expression to a special folder where you will keep expressions. You can use Restore to bring the expression back for another session.
- Assign the lamp spectrum to the variable S1. If the lamp spectrum is already open in a plot window or saved as a spectral library, you will see the spectrum in the list. Click on the name to select it.
- Choose to save the results in a new plot. Click OK.
- The normalized lamp spectrum is plotted (Figure 6.2a).

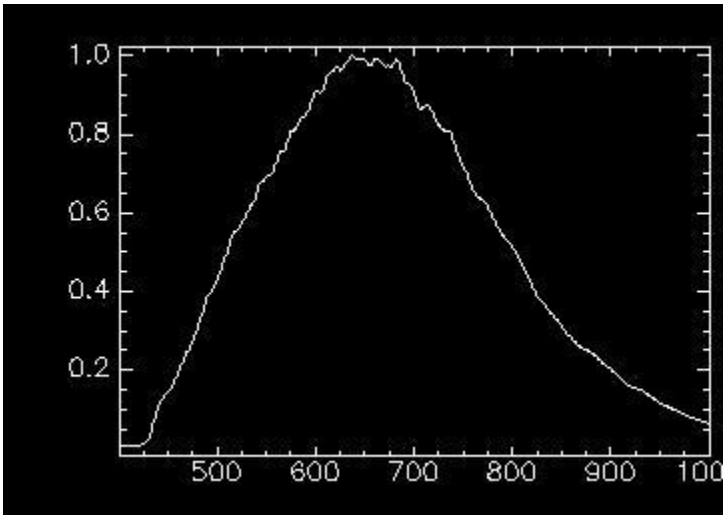


Figure 6.2a. Normalized lamp spectrum

6.3 Spectral Normalization of the Specimen

Now you have a lamp spectrum for your HSI system. Next, we will use it to correct your specimen spectra for instrumental effects. The correction is to divide the specimen spectrum by the normalized lamp spectrum. There are different ways to do this. There is a routine in the CytoViva Analysis feature called Normalize for Lamp Spectrum that is covered later. The following way is a manual way to do this to correct a single spectrum or a whole date cube from a Z profile, or from an ROI.

- Go to the Main Menu bar (Figure 6.3a).

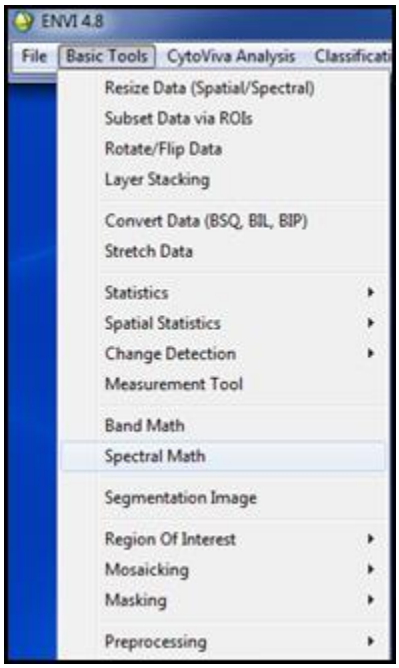


Figure 6.3a. Main menu bar

- Click Basic Tools.
- Click Spectral Math.
- Enter or Restore the correction expression: float(S1)/float(S2). Add it to the list of expressions and save it to use later. Select the expression and click OK.
- Select S1 and make one of the following assignments.
- To correct a single specimen spectrum, select the curve you want from the list. It will appear there if it is also in a plot window.
- To correct a data cube, use the file assignment option to browse for the specimen's HSI data file. If you do this, the entire image will be corrected.
- Choose a new window to plot the corrected spectrum. A new file name for the corrected data cube can be chosen.

- In the assignment window, select the normalized lamp spectrum for S2 (Figure 6.3b/c).

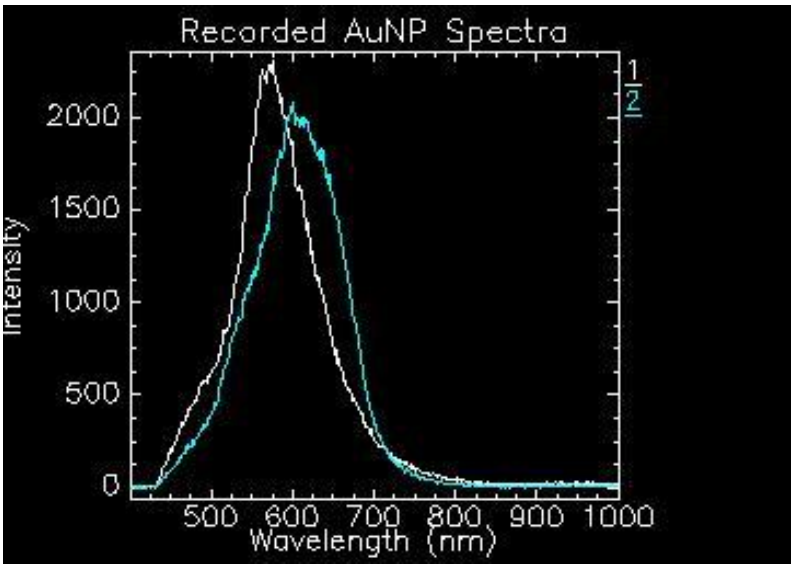


Figure 6.3b. The uncorrected spectra

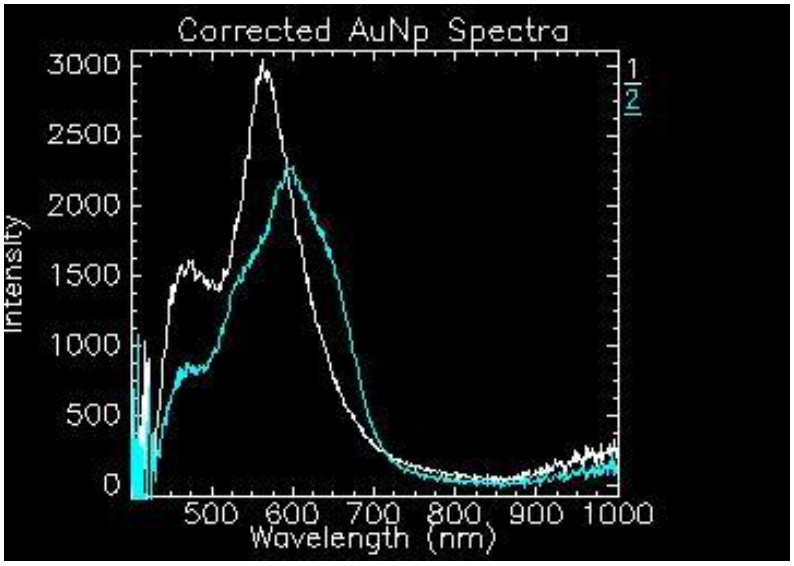


Figure 6.3c. After correction for instrumental two nanoparticles effects

- Note that the correction results in noise where the uncorrected spectrum had no intensity. This noise is not related to features of the particles.

6.4 Correction for Second Order Diffraction

The range of the spectrograph used with CytoViva is from 400 nm to 1000 nm. Since the spectrograph is a grating type, the spectrum starting at 400 nm also starts again at 800 nm because of the second order diffraction of the grating. This means that there are spectral values between 800 nm and 1000 nm that mirrors the values from 400 nm to 500 nm. Values between 400 nm and 800 nm are always obtained from the fundamental wavelengths, i.e. the first order diffraction pattern, and are never contaminated by the second order.

- To obtain true values out to 1000 nm, we recommend using a 500 nm long pass filter to block the light between 400 nm and 500 nm which will produce interference past 800 nm. This gives two free spectral ranges:
  - 400 nm - 800 nm without the filter.
  - 500 nm to 1000 nm with the filter.

- Since the long pass filter does not transmit all of the light past 500 nm, it is necessary to also record the light source spectrum when using the filter to do spectral normalization.

6.5 Conversions to Reflectance and Absorbance Manually

The light absorbance properties of materials can be determined from the absorption spectrum that is recorded as light passes through the material. Using HSI, the absorption of materials on glass slides can be found and classification of the material based on the absorption property can be carried out.

- Transmitted Light: To obtain the absorption spectrum of a specimen on transparent surface, use a bright field illumination which sends light directly through the glass slide into the microscope objective.
    - Locate the area of interest on the specimen and center it in the scan area.
    - Draw an ROI around the region.
    - Save the mean spectrum in a plot.
    - After recording the specimen, replace it with a blank slide, or a region of the same slide that does not contain the specimen.
    - Open the ROI tool and use the same region to obtain the mean spectrum of the region.
    - Save this to a plot.
    - Calculate the absorption spectrum using the spectral math expression:
    - alog(float(S1)/S2). Assign S1 to the spectrum of the blank and S2 to the spectrum of the specimen. Choose a new window to create the absorption spectrum. The light absorption is maximum at the peak of this spectrum, where also the light reflectance is minimum.

- Reflected Light: To obtain absorption spectrum of an opaque sample/material, use a reflected light illumination which sends light that bounces off the sample back into the microscope objective.
    - Locate the area of interest on the specimen and center it in the scan area.
    - Draw an ROI around the region.
    - Save the mean spectrum in a plot.
    - After recording the specimen, replace it with a reflectance target. A suitable target is made by LabSphere (model SDM-100-DU or SDM-050-DU).
    - Open the ROI tool and use the same region to obtain the mean spectrum of the region.
    - Save this to a plot.
    - Calculate the percent reflectance spectrum using the spectral math expression:
    - 100*float(S1)/S2. Assign S1 to the spectrum of the specimen and S2 to the target spectrum. Choose a new window to create the absorption spectrum. The light reflectance is maximum at the peak of this spectrum, where also the light absorption is minimum.

6.6 Conversions to Reflectance and Absorbance using the CytoViva Analysis Tool

- This feature converts a data cube into absorbance or reflectance units using the CytoViva Analysis Tool Convert to Absorbance or Reflectance.
    - Example 1: Stained Tissue with CNTs.
    - In the tool bar, click the CytoViva Analysis tab.
    - Then click the Calibration and Correction tab.
    - Then click the Convert to Absorbance or Reflectance (Figure 7.3a) and the Convert to Absorbance or Reflectance box will open (Figure 7.3b).

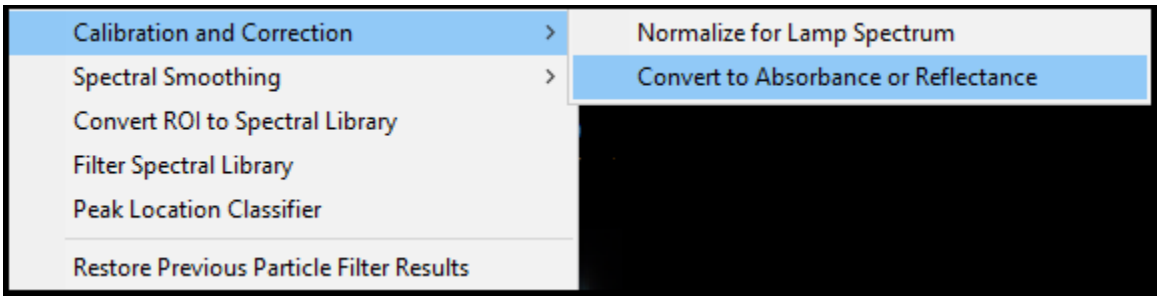


Figure 7.3a. CytoViva Analysis Tab

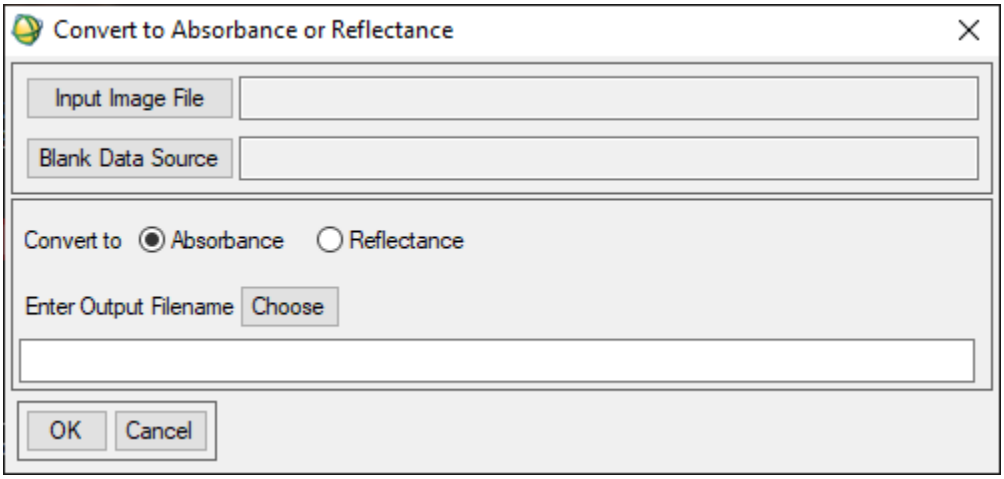


Figure 7.3b: Convert to Absorbance or Reflectance Dialog

- Select the image that you want to convert using the Image Input File button from the Convert to Absorbance or Reflectance box. For this example, use the file, Sample-100X located in the Absorbance and Reflectance Folder. Click Open, click OK. We chose a stained sample which will show how the dye absorption spectrum can be obtained with this feature.

- For the Blank Data Source, use file name Blank-slf located in the Absorbance and Reflectance Folder. Click Open, select New file, select File, click Open, click OK.
- Check Absorbance box to select the type of conversion.
- If the absorbance is chosen the blank data source file must be a preloaded lamp spectra, if reflectance is chose, the blank data source must be from a perfect reflector.
- Click Choose to enter an output file name. By convention, use the Input file name with ABS appended to the name. These appendages remind you that the data has been converted to absorbance or reflectance units.
- Click Open. Click OK.
- The Input file will be converted and the new image will automatically open in the Available Bands List and in a display. Below are the Zoom image from the original scan and the converted absorbance scan (Figure 7.3c).

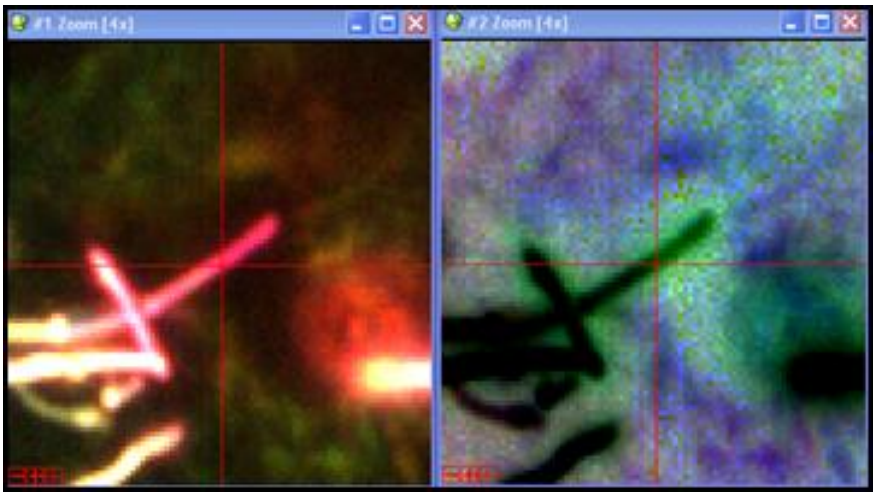


Figure 7.3c: Original image (left) and converted absorption image (right)

- The spectrum of the original sample, at the cross-hair and the absorption spectrum, at the cross-hair of the converted image (Figure 7.3d).

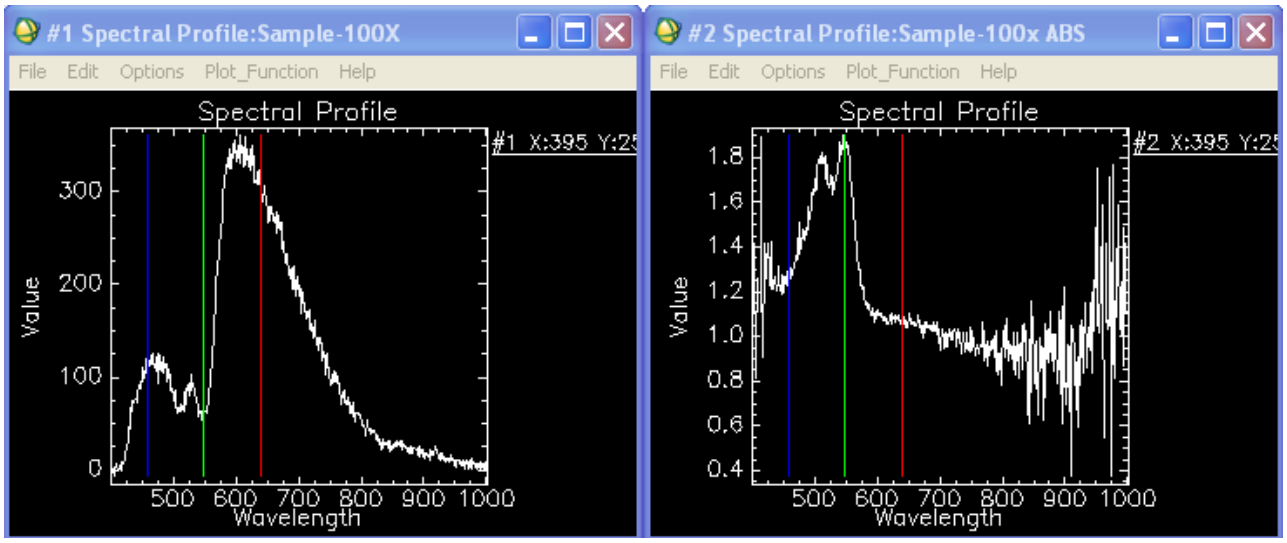


Figure 7.3d: Original image spectra (left) and converted absorption image spectra (right)

- If you try to open the original datacube after running this feature, you will need to manually assign it to a new display. You will see that the converted image opens in the new display. You can get the original image back by right clicking on the file name in the available bands list and selecting Load Default RGB to Current. This is a known bug which will be fixed in the future.

Section 7 Calibration and Correction

7.1 Correction for the Instrument Spectral Response

- The CytoViva Analysis feature Normalize for Lamp Spectrum is used to correct data cubes for the uneven spectral response of the microscope optics. This process uses a recording of light from the lamp, and thus the feature is termed Normalize for Lamp Spectrum in ENVI.
  - In the tool bar, click the CytoViva Analysis tab.
  - Then click the Calibration and Correction tab.
  - Then click the Normalize for Lamp Spectrum (Figure 7.1a) and the Normalize for Lamp Spectrum box will open (Figure 7.1b).

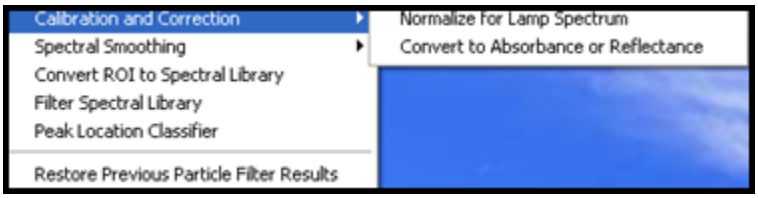


Figure 7.1a. CytoViva Analysis Tab

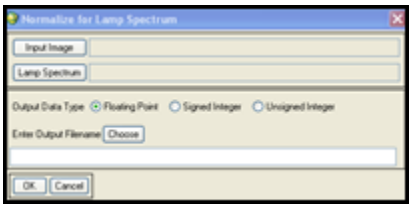


Figure 7.1b. Lamp Spectrum Button

  - Select the image that you want to correct using the Input Image button in the Normalize for Lamp Spectrum box. Click Open, then click new file, for this example, select the Ag-100X file. Click OK. If the file is already in the Input list, select the file, click OK.
  - Next click the Lamp Spectrum button to enter the Correction Spectrum. Use NORM-lamp-SL for the example, click Open, and then click OK.
  - Keep the default Data Output Type. Click Choose.
  - Give the name of the output file using the input file name with COR appended to it. COR reminds you that the file is a corrected version of the data. Click open. Click OK.
  - A corrected data cube file is saved and is opened in the available bands list and displayed. Now open the original input image in the main menu bar, click File, Open Image File, and then select the file (in this example Ag-100X). It will be displayed in the Available Bands List, select the file, right click and select Load True Color <to new>.
  - The corrected image will look virtually identical with the input image. Use the Square Root Enhancement for the best image.

- In the menu bar of the input image, select Tools, then link, then Link Displays. The Link Displays dialogue box opens (Figure 7.1c).

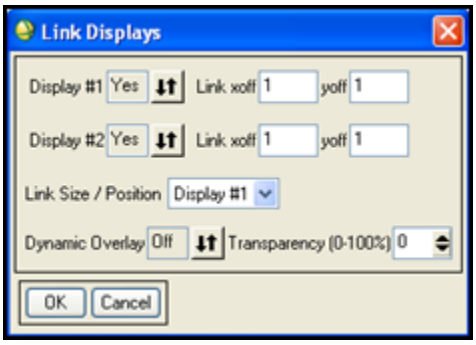


Figure 7.1c. Link Displays dialogue box

- Select Yes for both displays and Off for Dynamic Overlay. Click OK.

- Open a Z-axis profile for both images by right clicking on the image and selecting Z-Profile. As you click on different particles in the input image, the cursor moves to the same location in the corrected image since these images are linked. The curve in the plot windows correspond to the same pixel in both images. You will see how Lamp Normalization changes the input spectra. Turn on the Collect Spectra option to show multiple curves on the same plot.

- When the input image is normalized by the lamp spectrum, spectra in the new image are corrected for the uneven spectral response of the instrument. This compensates for the relatively weak illumination and camera response at each end of the instrument's spectral range. The input and the corrected spectra are most alike where the lamp spectrum is near its peak, which is between 500 and 600 nm.

- For the example with silver nanoparticles (Figure 7.1d), the input image is shown (arrows point at nanoparticles with spectra of interest). Figure 7.1e shows the uncorrected spectra from the nanoparticles on the left, and corrected spectra of the same particles on the right.

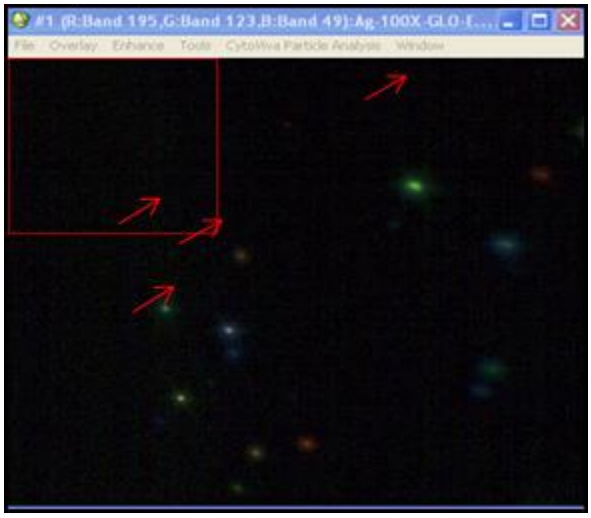


Figure 7.1d. Main image window with four nanoparticles of interest

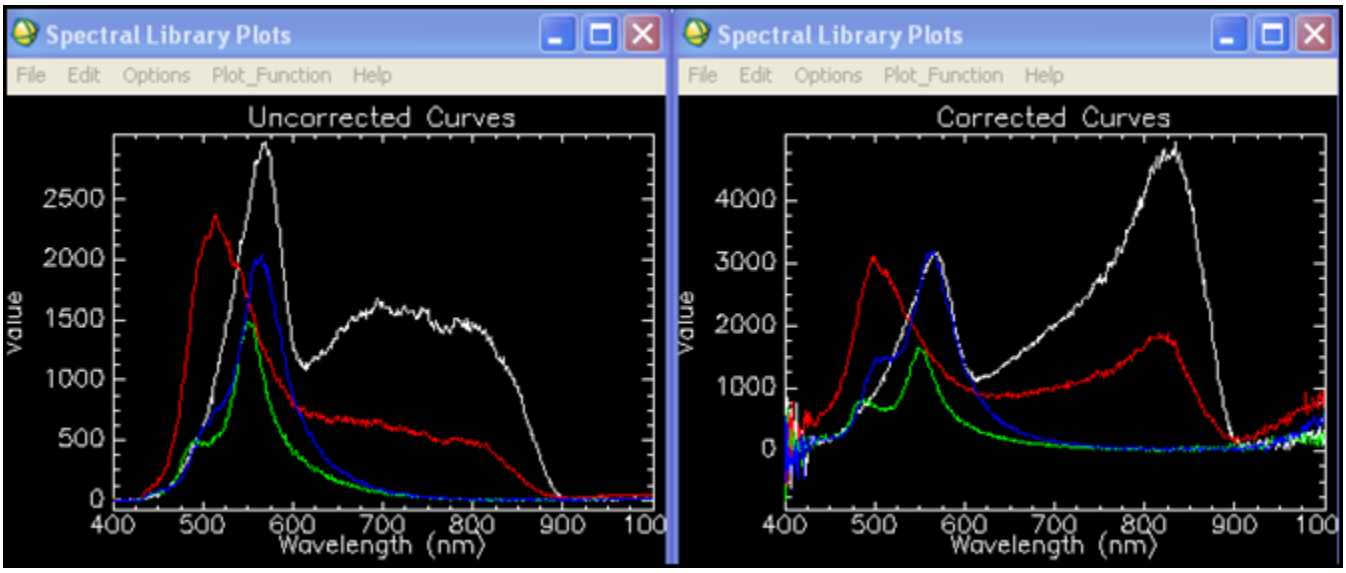


Figure 7.1e. Left: Uncorrected Spectra of Particles, Right: Corrected Spectra Using Lamp Normalizing Feature

- Correction can bring out the presence of new spectral features that were masked in the uncorrected data, and change the relative amplitudes of the features.

Section 8 Particle Filter

This new feature is used to automate the selection of particles in an image. The Particle Filter determines where there are particles or circularly-shaped objects that match criteria given by the user. The results are presented in a Review dialog box that allows for refining the particle selection criteria. It is one of the most useful new features of CytoViva ENVI 4.8.

- In the main menu toolbar, select File, click Open Image File, in the ENVI 4.8 folder in the particle filter file, select AgNPs-PF-100x. Click OK. Image opens in a new display.
    - In the image menu bar, select the Particle Filter Analysis (Figure 8.1a). Click Particle Filter.
    - The Particle Filter Params dialog box will open (Figure 8.1b).

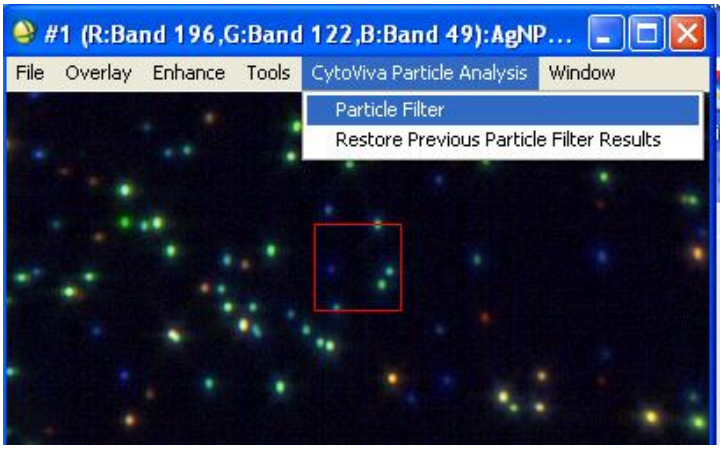


Figure 8.1a. Particle filter drop down menu

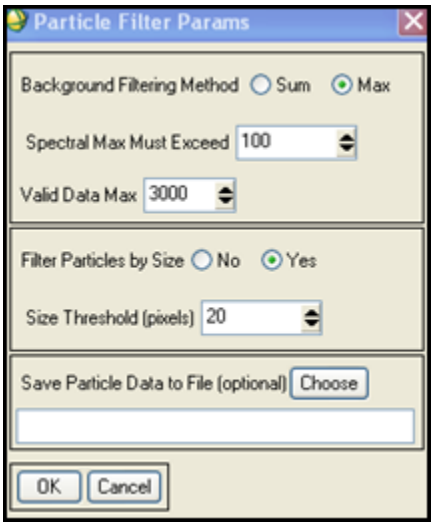


Figure 8.1b. Particle Filter Params box

- Select the Background Filtering Method. For this example, select Max.
- The default method (Max) is best to use if the particles have simple peaks in the spectrum. This choice tells the Background Filter to use the maximum value from the spectrum at each pixel of the data cube to detect particles. If you select Sum, it uses the sum of all spectral bands to detect particles. You will notice that the default value of the threshold parameter Spectral Max Must Exceed becomes 10000 when Sum is selected. If the particles have complex spectral shapes, Sum may be a better choice.
- Enter the threshold value for Spectral Max Must Exceed (Intensity counts). This is the minimum value that the filter must come up with using either the Max or the Sum Background Filtering method in order for a pixel to be found in a particle. In this example the value was set above the baseline values of spectra at 100.

- Enter the value for Valid Data Max. This is the highest value allowed for the maximum spectral value of a pixel in order for that pixel to be assigned to a particle. This value stays the same for both Background Filter Methods. The recommended setting is a value higher than the peak value of particles you want to find. Those particles having higher peak values in the data cube will be excluded. For this example we used 3000.

- Choose whether to also use the particle size as a filter parameter. The default setting uses this option. Small values cause the filter to exclude pixels around the edges of particle. In this example, the size has been set very small to 20.

- Once particles have been found using the filter, the spectra of edges of those particles can be easily found.

- (You can) enter a save file for the results if you desire. Click OK to run the Particle Filter.

- The Particle Filter Review Tool box opens (Figure 8.1c) and the detected particles are circled in the main image window (Figure 8.1d). With this example using the settings given above, the Particle Filter automatically found and circled several smaller, less bright particles.

- The option to Sort the list of particles by ascending wavelength was chosen in the Review Tool window by clicking on MaxWL and then choosing Ascending from the Sort button. The mean and maximum spectrum from the pixels forming the first listed particle are shown in the plot. Sort options make it simple to select particles according to size, wavelength or brightness. Try using the different Sort options.

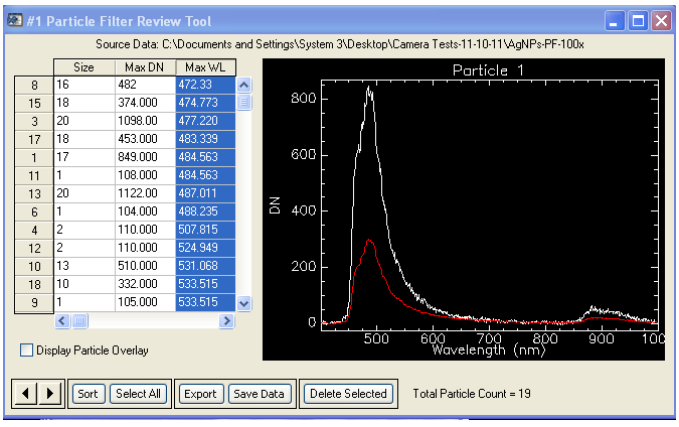


Figure 8.1c. Particle Filter Review Tool Box

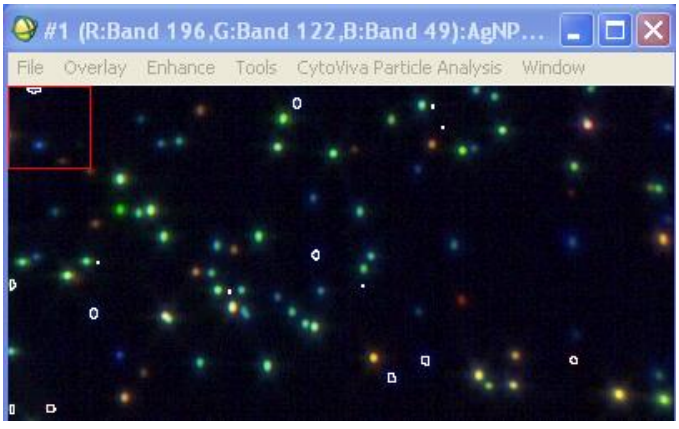


Figure 8.1d. Main Image Window for Particle Filter

- You can export particles that you select to either a Region of Interest or a Spectral Library (explained below).

- To save the data from the Review Tool Box, click Save Data.

- Saving to Particle Data creates a file that contains the last state of the Review Tool. This file can be restored by choosing Restore Previous Particle Filter Results in the drop down menu under CytoViva Particle Analysis in the main image window.

- Saving to Table Data to ASCII causes the particle filter data to be written to a text file (Figure 8.1e). Make sure to add a .txt extension to the file so it will open correctly in Notepad or Word. In both cases all of the particle data from the session is saved, not just the selected particles.

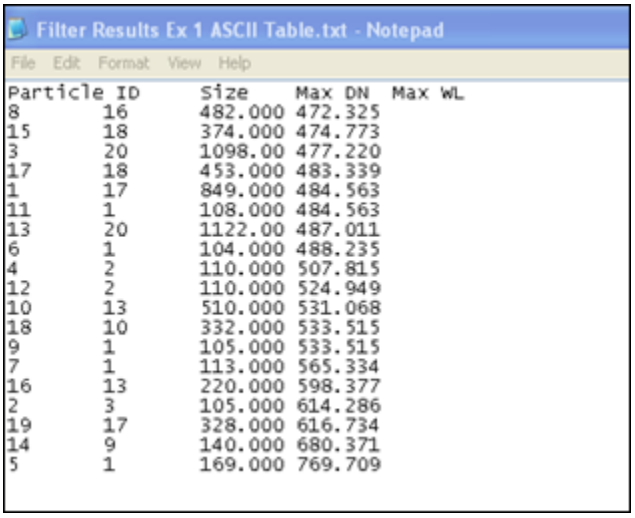


Figure 8.1e. Filter Results to ASCII file

- How Selection Criteria Are Used By Particle Filter.

- Example 1: Spectral Library Export.

- In the Particle Filter Review Tool you could make a Spectral Library for each particle that was found over a range of wavelengths between 475 nm and 525 nm by selecting the appropriate rows in the list. Then choose Export, then To Spectral Library.

- The Export particles to Spectral Library dialog appears (Figure 8.1f).

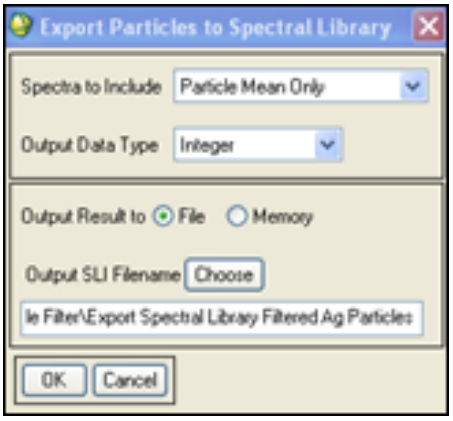


Figure 8.1f. Export Particles to Spectral Library

In this example only the mean spectrum from each particle is going to be saved to a Spectral Library. Click Choose for an output SLI file name. Use the defaults for other settings. Click OK. The file is saved and added to the top of the Available Bands List.

From the Available Bands List, right click the file and select the Spectral Library Viewer and the Spectral Library Viewer dialogue box opens (Figure 8.1g). The spectral curves of the particles that have wavelengths between 475 nm and 525 nm are listed. Click on the Means of each particle and a spectral library plot of these is shown (Figure 8.1h). Right click on the plot and select plot key (if needed).

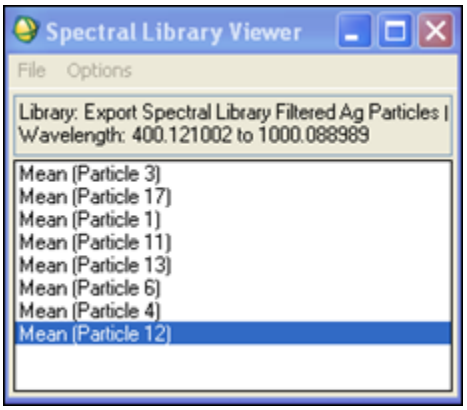


Figure 8.1g: Library Viewer

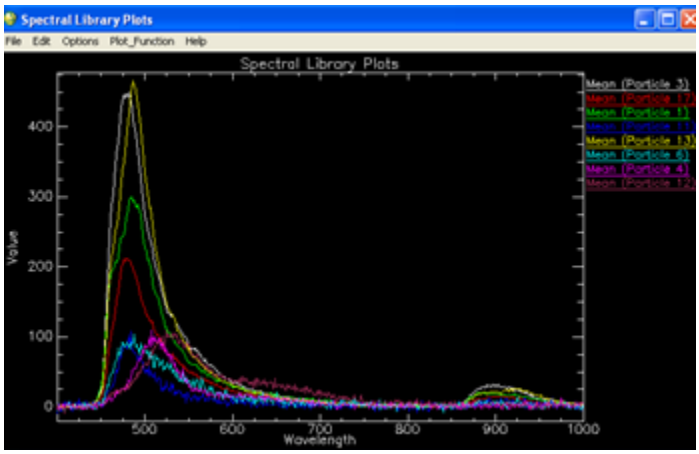


Figure 8.1h: Spectral Library Plots

- Example 2: Region of Interest (ROI) Export:
  - In the Particle Filter Review Tool you could make a Spectral Library for each particle that was found over a range of wavelengths between 475 nm and 525 nm by selecting the appropriate rows in the list. Then choose Export, then To Region of Interest and the ROI Tools Dialogue Box opens (Figure 8.1i).
  - The ROI Tool opens with the particles listed as individual ROIs which you can apply to the image. You can create different colors for the ROIs by right-clicking Color and choosing Assign default colors (Figure 8.1i).

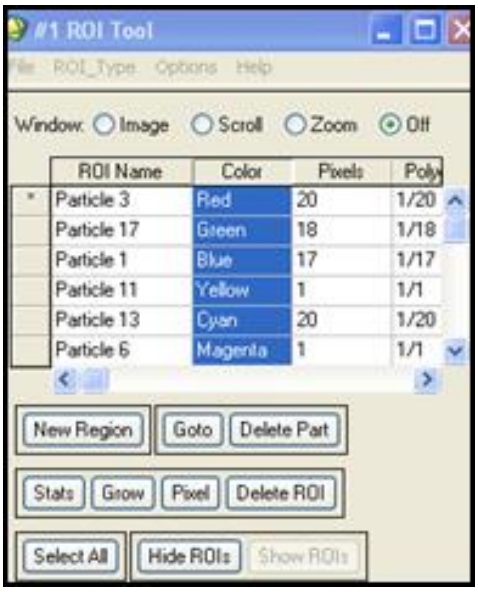


Figure 8.1i. ROI Tool box

Section 9 Filter Spectral Library

The Filter Spectral Library provides an automated process for removing spectra from a library that are different from the spectra that you want to match to an input file. A library of spectra that have similar, but not necessarily the same, properties as the undesired spectra is needed. The process removes the undesired spectra from the input library by performing a statistical comparison of spectral properties using the Spectral Angle Mapper (SAM). This is a versatile filter. An example of use of the Spectral Library Filter would be to reduce a spectral library that was created from objects that have either one or both of two different attributes, such as a range of particle size or peak wavelength, to a library that contains objects with only one of the attributes, or contains only the particles that have both attributes.

- Selecting a Spectral Library to Filter.
  - Starting from the main menu tool bar, click the CytoViva Analysis tab.
  - Then click the Filter Spectral Library (Figure 9.1a).

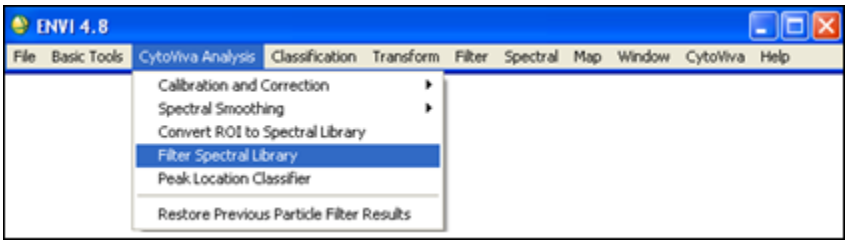


Figure 9.1a: CytoViva Analysis Tab

  - Select the Spectral Library to Filter dialogue box opens. The user selects a spectral library file from the input list or opens a new one from this window using the Open tab (Figure 9.1b). This file should contain all of the spectra from the analysis, including both the desired and undesired spectral curves.

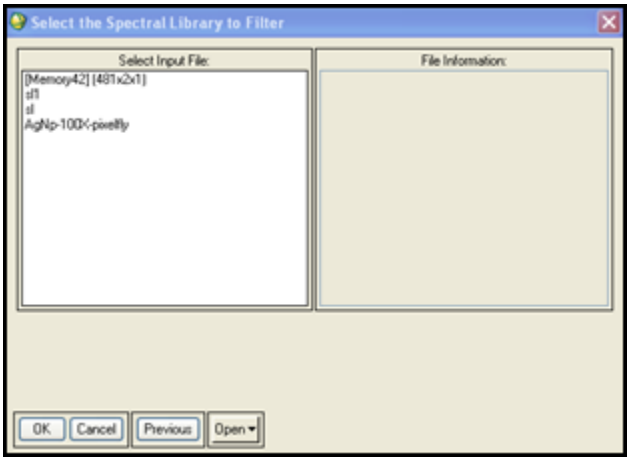


Figure 9.1b: Spectral Library to Filter Box

- Example 1: Simple Separation of Spectral Curves.
  - This example will remove two spectra, deemed undesirable from a spectral library. Once the Select the Spectral Library to Filter dialogue box is open, select the file sl. If the input file isn't in the list click open and then select the file. The input file sl contains four spectra (Figure 9.1b), located in the ENVI 4.8 Examples folder. Click OK.

  - The Filter CytoViva Spectral Library box appears (Figure 9.1c).

  - In the Filter CytoViva Spectral Library box (Figure 9.1c), starting at the top the input file name is shown in the Base Library box.

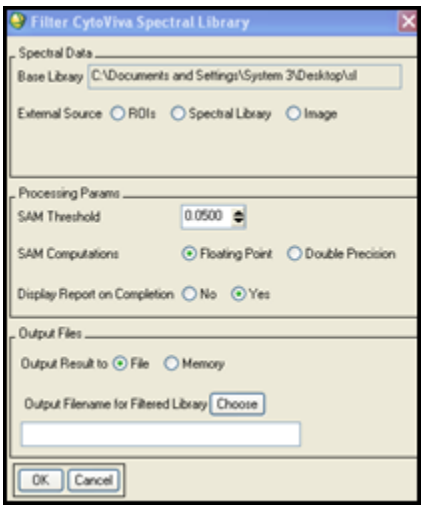


Figure 9.1c: Filter CytoViva Spectral Library Box

  - Next, choose the external source (source of undesired curves). You can choose from an ROI, Spectral library or an Image. The source for this example is Spectral Library.

  - Next is the Processing Params. The SAM threshold can be decreased if matches with the external source need to be more precise.

  - Default settings should be used for the rest of the input.

  - The Display Report on Completion will produce a window showing how many spectra were removed from the input library and how many remain.

  - Save the new library to memory for review until the desired outcome is achieved, then save it to a file. Click Choose, create a file name for filtered library. Click Open, then click OK.

- The Select external source Spectral Library box opens. Select which input file to use for the external source (Figure 9.1d). Click OK.

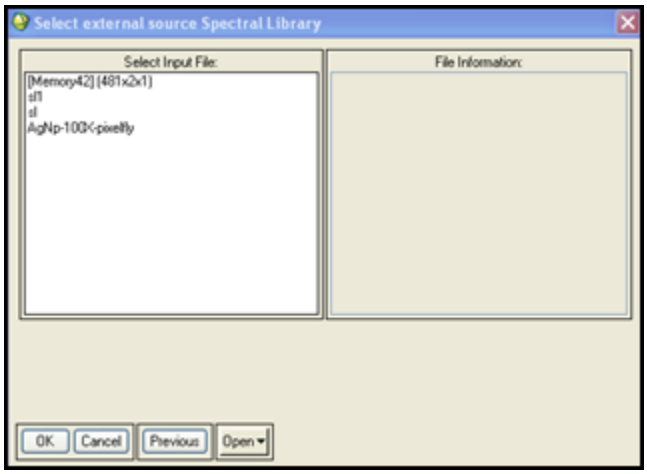


Figure 9.1d: Selecting the External Source Input File

- In this example choose: sl1.

- It contains two curves that are already in the input library, but have been deemed undesirable, and are to be removed. Since they match curves in the input library, SAM will not fail to find them. In most cases where you will use the Filter Spectral Library feature, the External Source library of undesired spectra are similar but not identical to the spectra you want to remove. If SAM is able to match any spectra in the External Source with spectra in the Base Library, the matching spectra will be removed from the Base Library. After running the filter, the results are shown (Figure 9.1e).

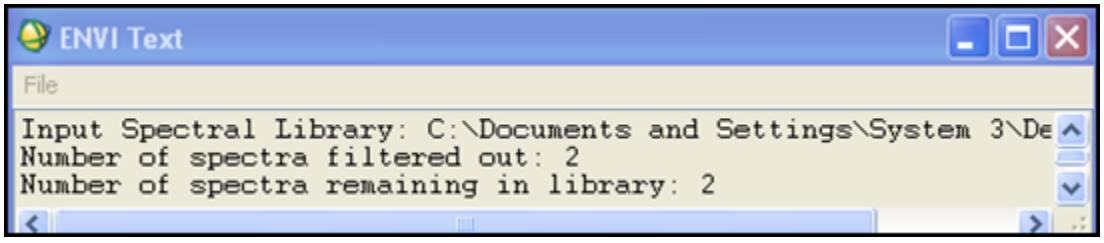


Figure 9.1e. Results of the filtered spectra

- To view the resulting spectral library, in the Available Bands List, right click on file name, the click Spectral Library viewer. The Spectral Library Viewer box opens. Click on each curve to load the spectra into a new ENVI Plot Window.

- Below are the spectral curves from the input Base Library, External Source Library and the Filtered Library (Figure 9.1f). The Input Base Library (left) containing four curves, two which are undesired, the External Library (middle) containing only the undesired curves, and the filtered Library (right) containing only the desired curves.

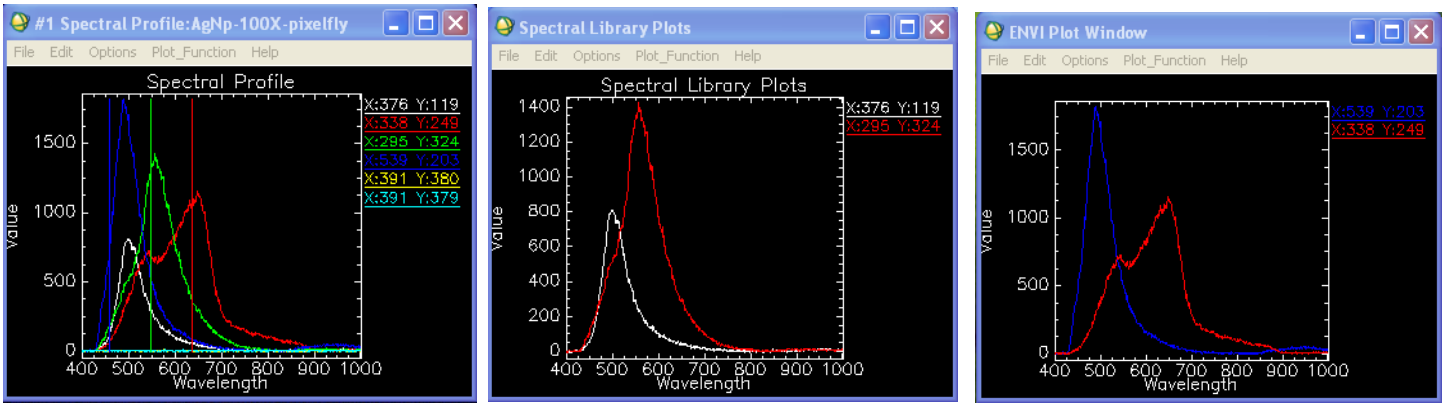


Figure 9.1f. Input Base Library, External Source Library and Filtered library

Section 10 Convert ROI to Spectral Library

This feature expands the abilities in ENVI to create spectral libraries from regions of interest (ROI) associated with open images. In this example we will be using the AgNP-100X-Pixelfly data cube.

10.1 Working with Regions of Interest (ROI)

- In the main menu bar, click File, Open Image File, then select: AgNP-100X-pixelfly.
- Expand the Zoom window and move it around the four NPs at lower right area in the main image.
- Right click on the zoom window, click ROI Tool. Add circular ROIs over the three largest NPs as shown (Figure 10a). The ROI tool box opens. In the zoom image, draw a circle around the lower left particle, then double right click to complete circle and a red overlay appears. In ROI tool box click New Region. Draw a circle around the middle particle, then double right click to complete circle and a green overlay will appear. Repeat as necessary.

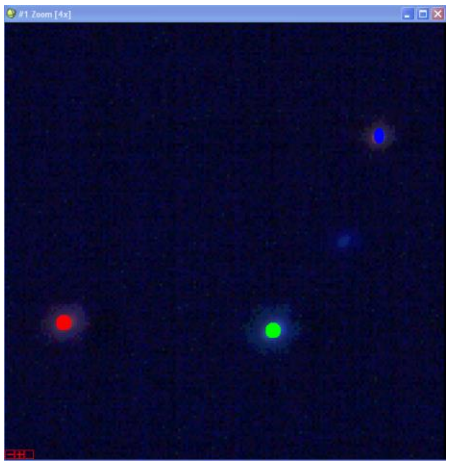


Figure 10a. Zoom Window with the ROIs (red, green, and blue)

- In the main tool bar, click the CytoViva Analysis tab, click on the Convert ROIs to Spectral Library. A standard input file dialog appears.
- Select the image file, for this example choose: AgNP-100X-pixelfly and click OK.

- The Convert ROIs to Spectral Library box opens and shows the ROIs that were created for this image. Check all boxes (Figure 10b).

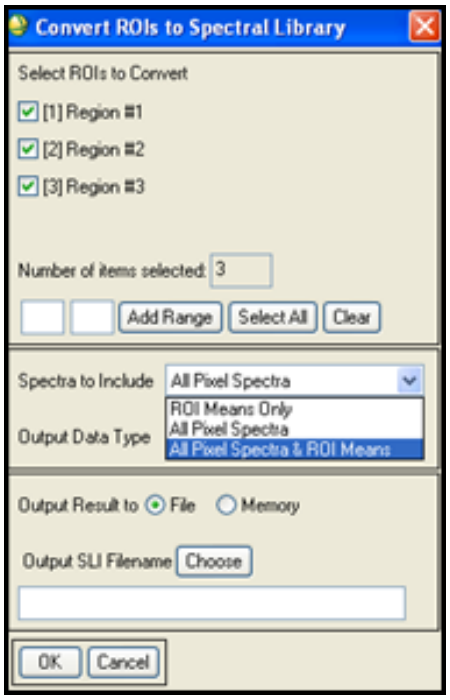


Figure 10b. Convert ROIs to Spectral Library

- For Spectra to Include, select All Pixel Spectra & ROI means. This will save spectra from every pixel in each ROI along with the mean spectrum of each ROI.
- Accept the other default settings.
- Save the output to a spectral library file. Click choose, enter filename, click open, Click OK.

- The file will show up in the available bands list. Right click on the file and select the Spectral Library Viewer to open the spectra into the list (Figure 10c).

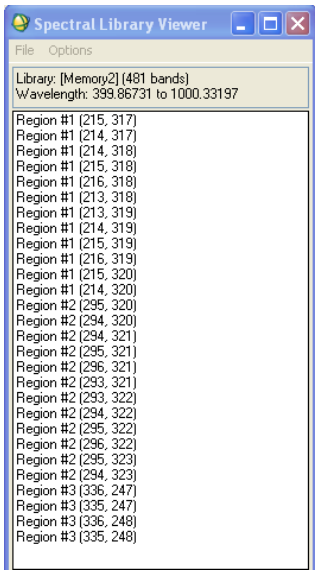


Figure 10c. Spectral Library Viewer window

- View the spectra by clicking the first entry, which opens the first spectrum into a plot window.

- The rest of the entries can be plotted by either hitting the keyboard's down arrow, or in the menu bar on the spectral plot by going to menu bar in the Spectral Library Plot box, selecting Input Data and selecting Spectral Library (Figure 10d). The Mean is the average of each ROI and the regions listed below are what make up the Mean.

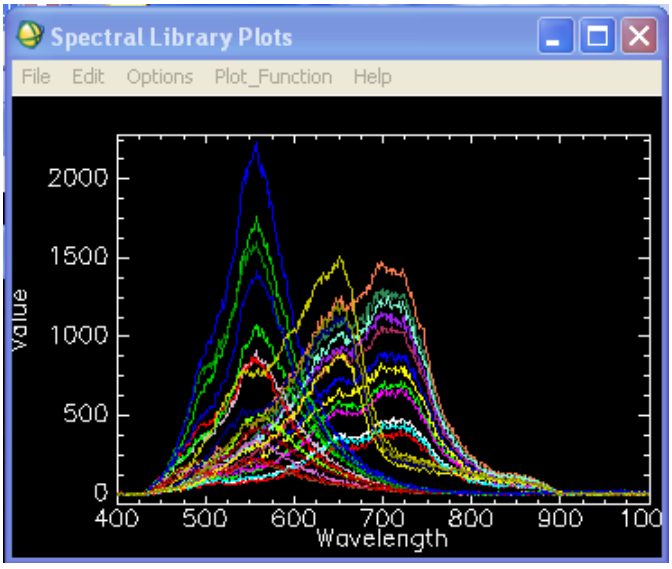


Figure 10d. Spectral Library Plots of the individual pixel spectra from the ROIs

Section 11 Spectral Smoothing

This feature is used to reduce the noise in spectra from single pixels or regions of interest. Noise reduction should be used to enhance the shape of spectral curves, such that small features of the curves are more apparent. It can also be used before spectral mapping in order to improve results. There are three smoothing filters.

- Boxcar Filter: This filter averages the spectrum across a number of bands, putting the average of each segment of the original spectrum into a new band. The number of bands in the new data is reduced by this process. The boxcar filter will significantly reduce the size of the filtered data cube.

- Adjacent Band Averaging: The spectrum is averaged across a number of bands and written to a new band in the output data. The filter moves over by one band and repeats to create a new average which is put into a second band in the output image. The number of bands in the output and input images are equal and sizes of the data cubes are the same.

- Savitsky-Golay Curve Fit Smoothing: Noise is removed from the spectrum by fitting smooth curves over segments of the spectrum. This method removes random noise to the greatest degree. It is possible for smoothed curves to have a reduced amplitude compared to the original curve amplitude. Default settings for this feature should be used to best retain the original amplitude.

- Smoothing can only be done on an image. Not on a single spectra.

- Part 1: Boxcar Filter Smoothing
  - In the main menu bar, click File, Open Image File, then select: AgNP-100X-pixelfly.
  - In the main tool bar go to CytoViva analysis tab, select Spectral Smoothing, click Boxcar Filter.
  - Select Input File box opens. Select from list or choose Open, New. Click OK (Figure 11a).

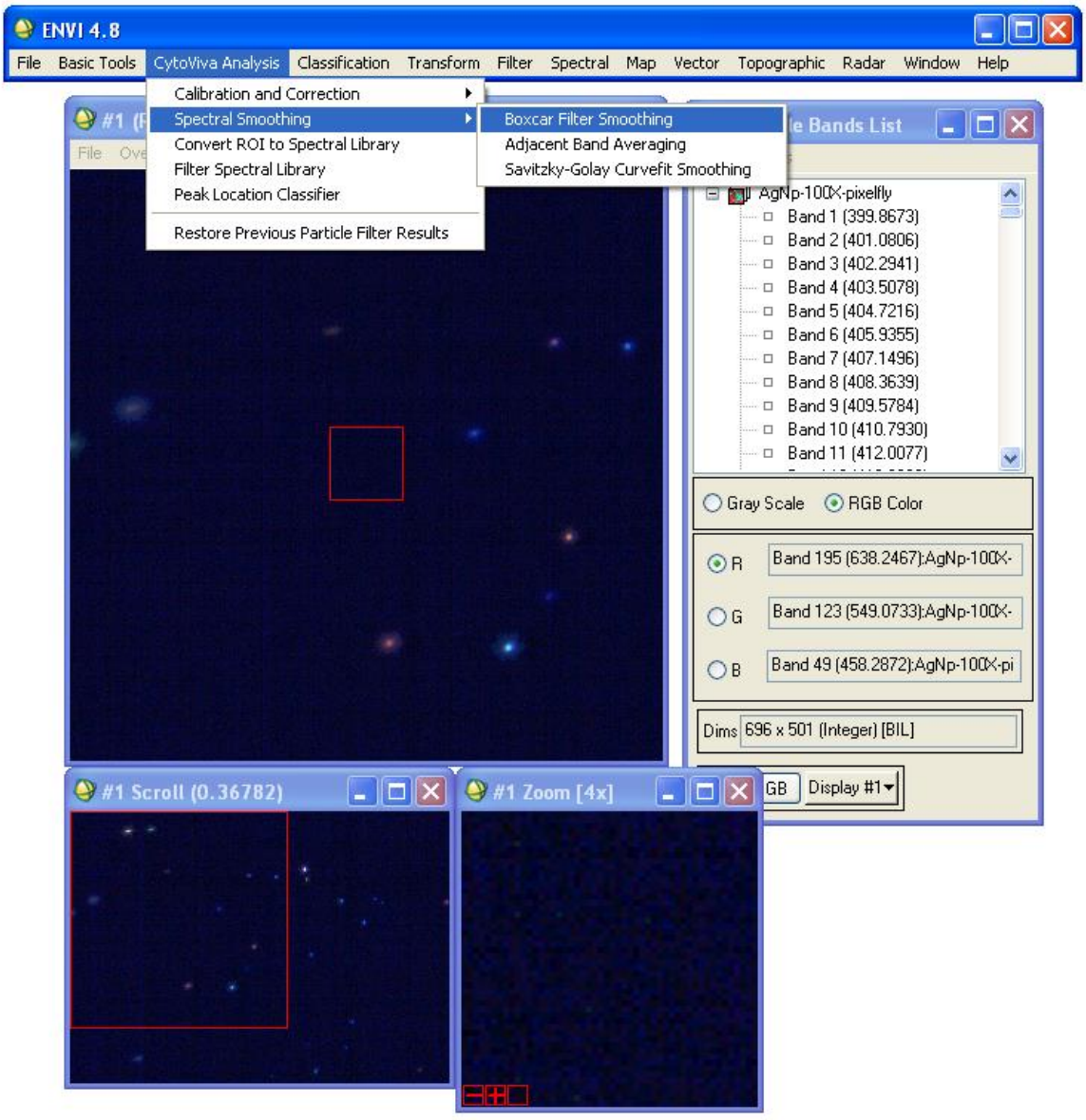


Figure 11a. CytoViva Analysis Tab

- The Boxcar Filtering Params box opens (Figure 11b). The number you will enter determines how many bands the new data will have. A larger number will cause a smaller degree of noise filtering. Select number of output bands or use the default of 50, and then click Choose.

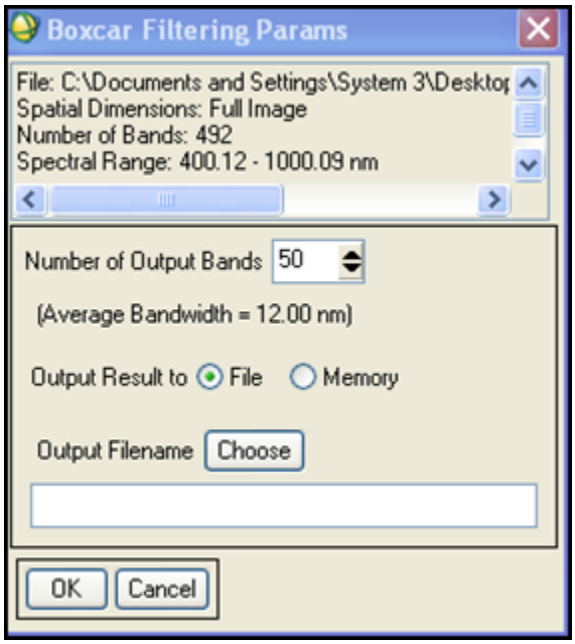


Figure 11b. Boxcar Filtering Params Box

- Output Filename window opens. Enter file name, click Open, and then click OK.

- A new image opens. On the left is the original image and on the right is the smoothed image, see (Figure 11c). In the original image, right click, click Z-profile. Spectral profile will open, select pixel of interest. Repeat for new image.

Figure 11c: Original and Filtered Image

- The spectral curves of a single pixel (Z-axis profile) are shown for the original (Figure 11d) and filterer data (Figure 11e).

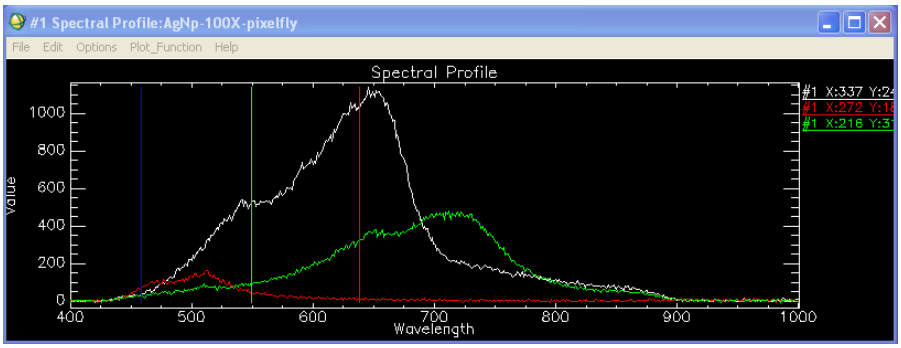


Figure 11d: Spectral Profile for Unfiltered Image

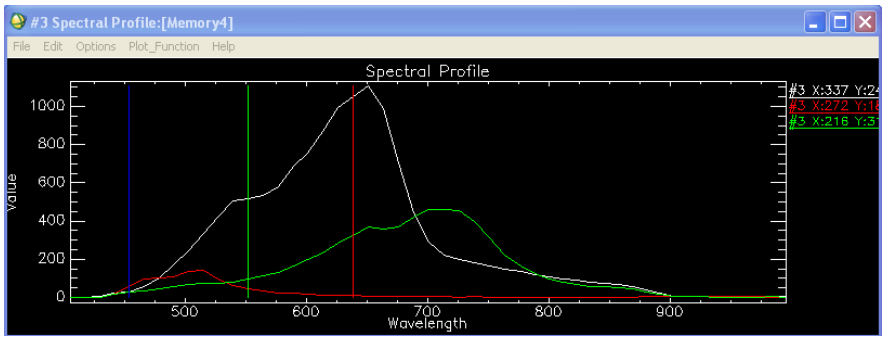


Figure 11e. Spectral Profile for Boxcar Filtered Image

- Part 2: Adjacent Band Averaging
  - In the main menu bar, click File, Open Image File, then select: AgNP-100X-pixelfly.

  - In the tool bar, click the CytoViva Analysis tab, click Spectral Smoothing, and then select the Adjacent Bands Averaging (Figure 11f).

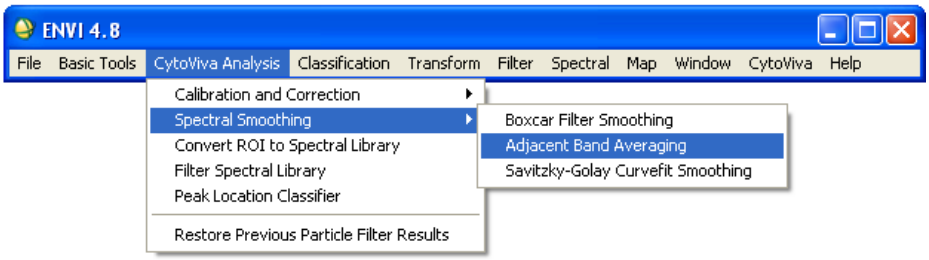


Figure 11f: CytoViva Analysis Tab

  - Select the image in the Select input file dialog, or click Open, New. Click OK. The Moving Average Filter Params box will open (Figure 11g). Keep all image bands, to avoid the mistake of creating files of differing numbers of bands.

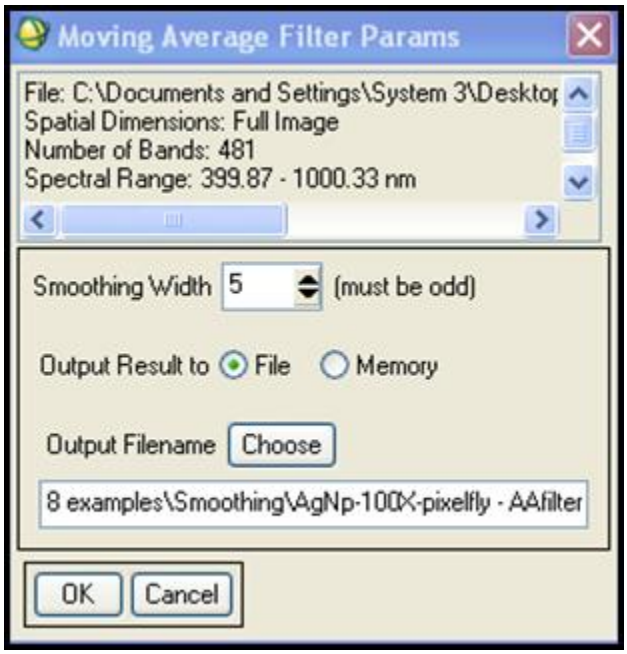


Figure 11g. Moving Average Filter Params Box

- Enter a value for the Smoothing Width. A larger value averages the curve over a longer portion.
- Save your new data to either a file. Click Choose. Output Filename window opens. Enter file name, click Open, and then click OK.
- The filtered data cube will open (Figure 11h).

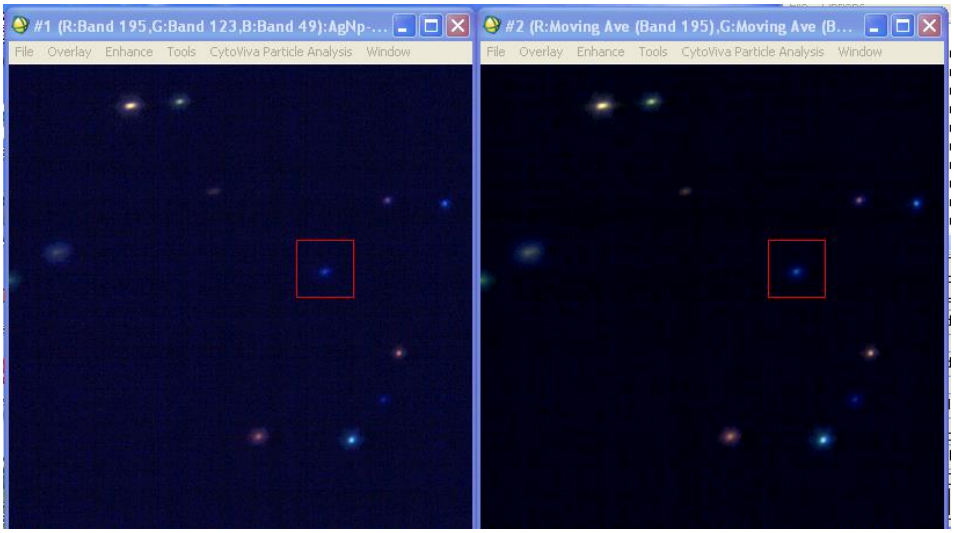


Figure 11h. Original Data Cube (left), Smoothed Data Cube (right)

- On the left is the original image and on the right is the smoothed image (To differentiate the images, in the title of the smoothed image the heading Moving Ave has been added).
- The spectral curves of a single pixel (Z-axis profile) are shown for the original and filtered data (Figure 11i).

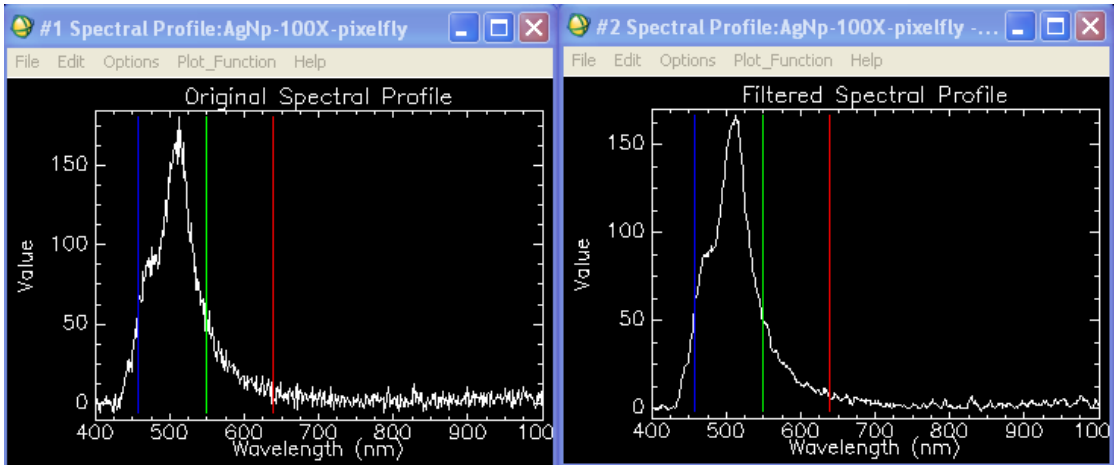


Figure 11i. Original Spectral Curve (left), Smoothed Spectral Curve (right)

- Part 3: Savitsky-Golay Filtering
  - In the main menu bar, click File, Open Image File, then select: AgNP-100X-pixelfly. In the tool bar, click the CytoViva Analysis tab, click Spectral Smoothing, and then select the Savitsky-Golay Curvefit Smoothing (Figure 11j).

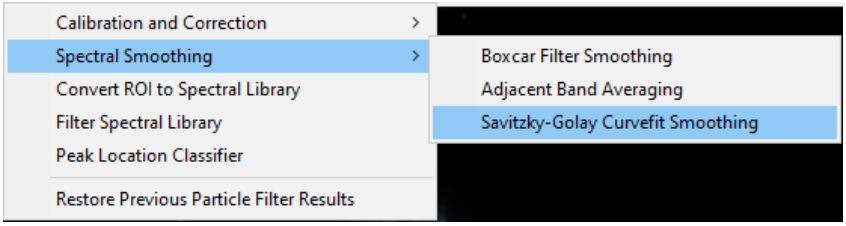


Figure 11j. CytoViva Analysis Tab

  - Select the image in the Select input file dialog, or click Open, New. Then click OK. The Sav-Gol Curvefit Smoothing Params box will open (Figure 11k).

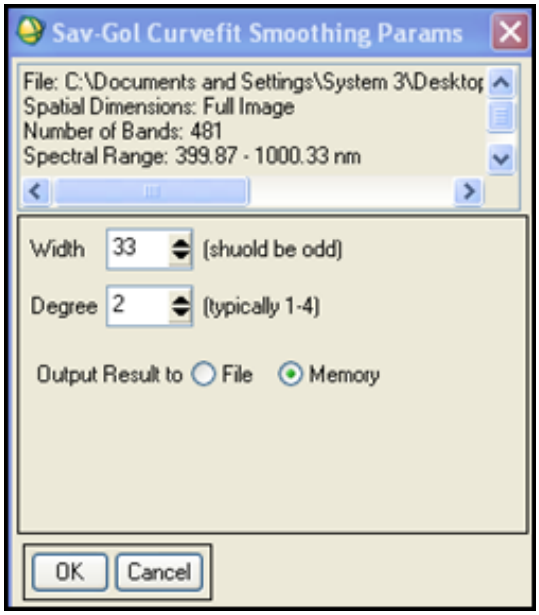


Figure 11k. Sal-Gov CurveFit Params Box

  - The Width sets how many bands are included in each fitting operation. A large width tends to keep the amplitude of the filtered data the same as that of the original data. The Degree setting determines how well the smoothed data can fit curves with narrow peaks or rapidly changing signal. A small value can manage the fast changes where a large value gives better overall noise reduction. It is best to keep the default for Width and experiment with the Degree.

- Save your new data to a file. Click Choose. Output Filename window opens. Enter file name, click Open, and then click OK.

- The filtered data cube will open. On the left is the original image and on the right is the smoothed image (see Figure 11L).

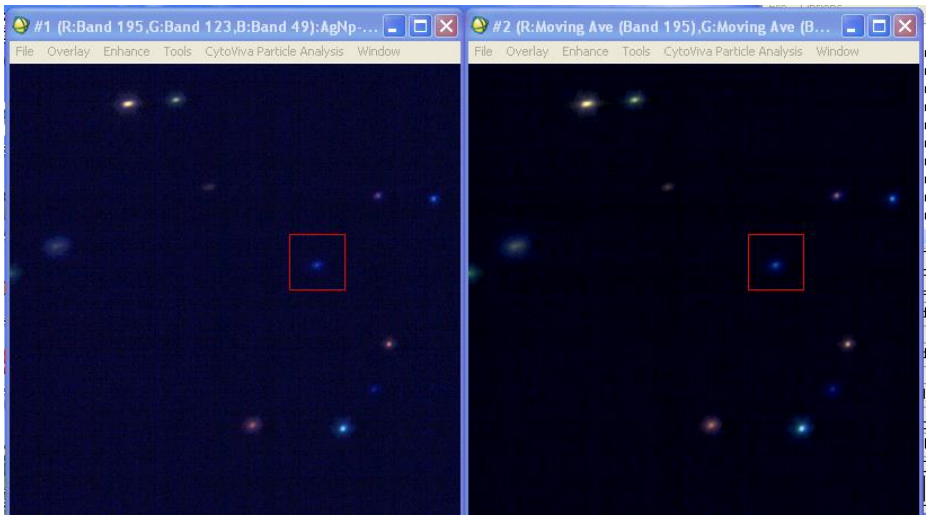


Figure 11L. Original and Filtered Image

- The spectral curves of a single pixel (Z-axis profile) are shown for the original (Figure 11m) and smoothed (Figure 11n).

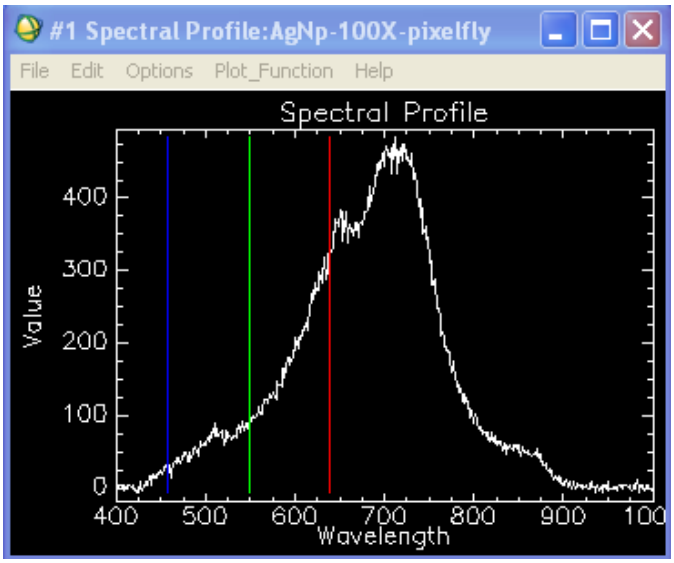


Figure 11m. Original Spectral Profile

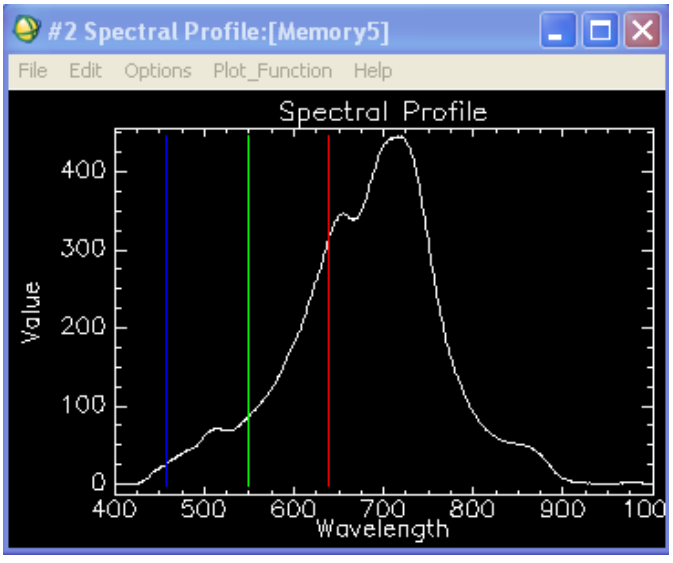


Figure 11n. Smoothed Spectral Profile

Section 12 Peak Location Classifier

This features finds all pixels in an image that have a specified peak wavelength. It operates like other classifier tools such as SAM. The Peak Location Classifier can be used to search an image for objects or regions that have a peak in the spectrum that matches a known spectral characteristic.

- Go to the CytoViva Analysis tab, Select peak Location Classifier from the drop down menu (Figure 12a).

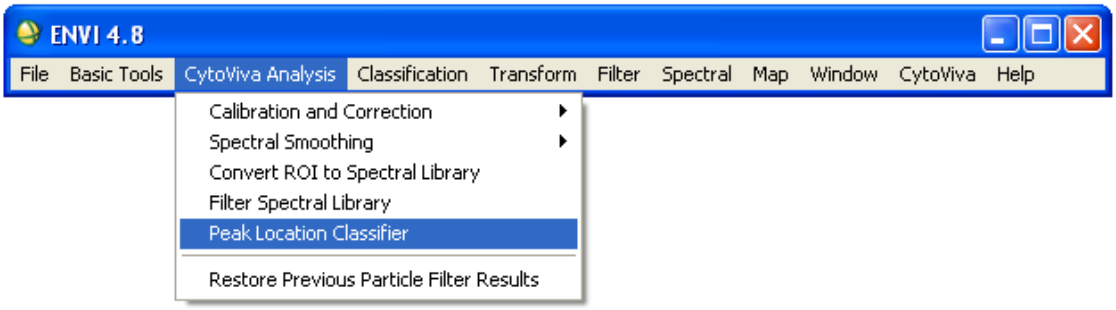


Figure 12a. CytoViva Analysis tab

- In the Select input image dialog, use Open to browse for the HSI data cube that you wish to classify and enter it. If the image was already opened it will appear in the Select Input File list. For this example we will use AgNPs-PF-100x (Figure 12b).

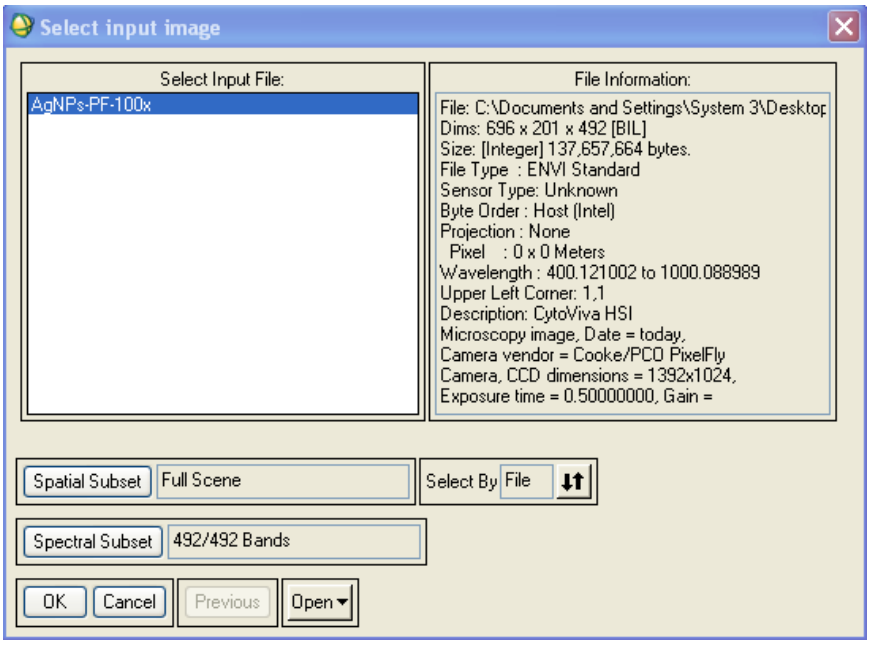


Figure 12b. Select Input Image box

- Select the data cube. Generally you will keep the default values for Spatial and Spectra Subset. You may subset the spatial size of the datacube. If Spectral bands are selected, be sure that they include the wavelengths of the peak that you are searching for and then click OK.

- The Peak Location Classifier Params dialog opens (Figure 12c). Here you will enter several parameter values that are used to decide how to process the image.

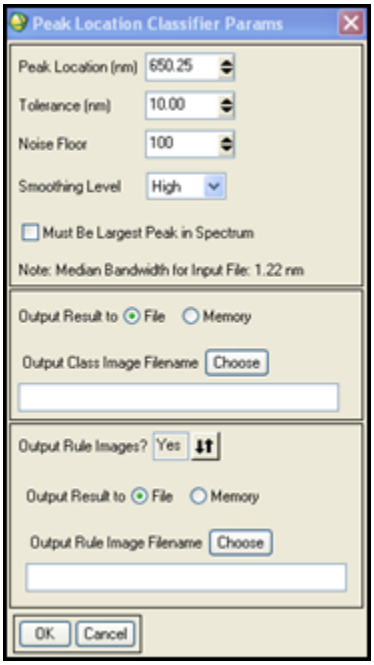


Figure 12c. Peak Location Classifier Params dialog

- In the Peak Location box, enter the wavelength of the spectral peak of interest.

- Next, enter the Tolerance, this is the range around the wavelength that you will accept for deciding to include the pixel in the classification.

- Then enter the noise floor which should be set to a value that is below the amplitude of expected spectral peaks.

- Next, choose a Smoothing Level. Choose a high level of smoothing to obtain best results (default).

- Lastly, You can check the box if you only want the largest peak in the spectrum to be used, or leave it unchecked to accept the pixel even if there is a larger peak at a different wavelength.

- The Median Bandwidth shown in the NOTE is the spectral resolution of the input file. In the example, we will accept the default values.

- Check whether you wish to save the results in memory or to a file.

- Generally select No for Output Rule Image. However, if you want to see how close each pixel of the image comes to having the desired peak, you can select Yes. If this is done, two grayscale images are written to the available bands list that you can display.

- Select OK and the results of the Peak Classifier open (Figure 12d).

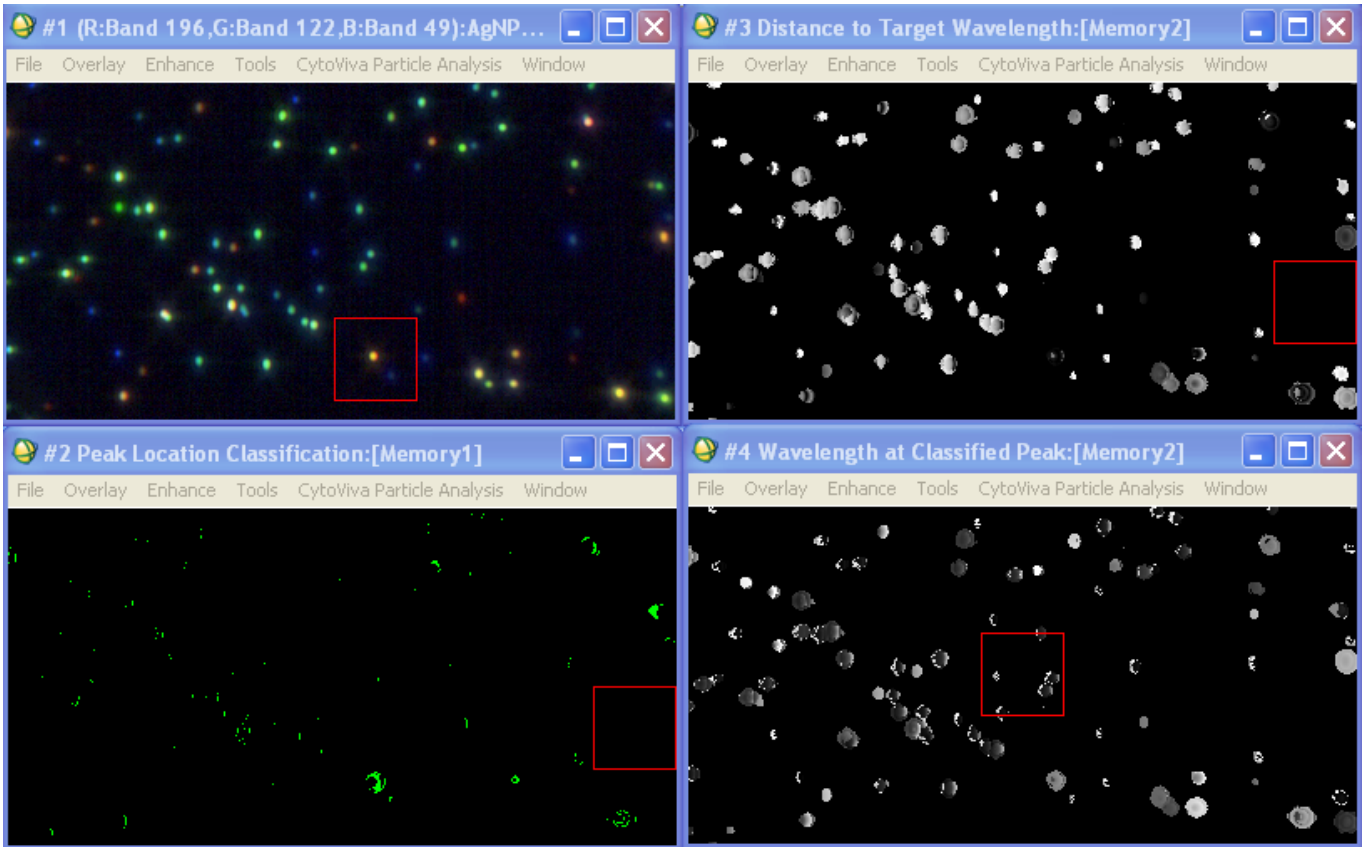


Figure 12d. (Clockwise from upper left) AgNP datacube, Distance to Target Wavelength, Wavelength at Classified Peak, Classification Image

- AgNP Image: the original data cube.

- Classification Image: Shows the pixels that are within +/- 10nm of the desired peak wavelength of 650.25nm.

- Distance to Target Wavelength Image: shows bright regions if the pixel has a peak that rises above the noise floor. The brightness of the pixel will increase as the distance of the peak from the desired value increases. The value of these pixels can be read using the Cursor/Location Value tool. The value is the separation of the peak from the desired wavelength in nanometers.

- Wavelength at Classified Peak Image: shows an image where every pixel containing a peak above the noise floor is displayed on a gray scale. The brightness of the pixel increases as the wavelength increases. This Rule Image is useful for finding other peak wavelengths and how much variability there is in the spectrum of all objects.

Section 13 Clean-up and Supplies

To properly take care of the CytoViva hyperspectral system there are a few steps that are necessary when you are done using the system to keep the system performing at its optimum level.

13.1 Daily Clean-Up of the System

- Use a Kimwipe or lens paper to wipe off any excess oil from the CytoViva condenser and the objectives. You can leave a clean Kimwipe wadded up on the condenser to protect it when not in use and this will also wick up any excess oil missed from cleanup.
- Use lens paper to wipe off any oil on the oil immersion objectives.
- Power off the light source.
- Turn the optical camera off by using the switch or unplugging the power cord connected directly to the top of the camera.
- Optional – Turn the stage controller unit off using the switch on the back of the controller box.
- Cover the microscope with the CytoViva microscope cover.
- Use cleaning solution about every 2-3 months to clean the CytoViva condenser and the objectives.
- Letting the immersion oil sit and dry on the condenser and the objective lenses can cause a gum-like film which can cause damage to the lenses and internal mirror of the illuminator.

13.2 CytoViva Supplies

Below are supplies that are recommended when using the CytoViva system:

- Fisher Scientific Items:
- Pre-cleaned slides: 1 x 3mm plain glass slide, Part #: 12-544-1
- Pre-cleaned cover slips: .17mm thickness, No. 1 slides (two sizes below)
    - 18mm x 18mm: 12-542A
    - 55mm x 24mm: 12-544-18

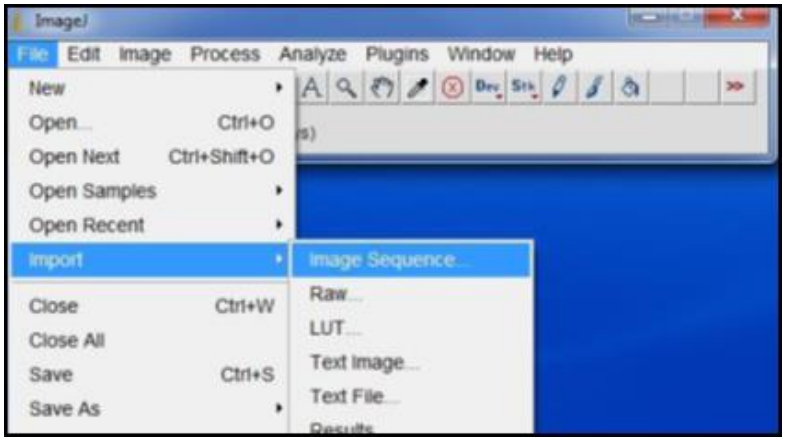
- Cargille Immersion Oil: Type A (two sizes below)
- 4 fl. oz.: 12-370A
- 16 fl. oz.: 12-368B
- TexWipes: Part # 18-315A
- Fisher Brand Optical Lens Cleaner: used for slides and cover slips, part #: 22-143-974
- Fisherbrand™ Easy Reader™ Plastic Centrifuge Tubes: part #: 05-539-5
- Fisherbrand™ Standard Disposable Transfer Pipettes: part #: 13-711-7, quantity of 500.
- Fisherbrand™ SureFit™ Ergo-F Pipet Tips: part #: 02-681-469, quantity of 1,000 per bag.
- Gilson PIPETMAN Classic: part #: F123600G, Adjustable-volume; Gilson PIPETMAN Classic; Model P20; Direct volume reading in decimal digits; Stainless-steel and PVDF construction; Vol.: 2 to 20uL; Compatible w/Gilson tips D200, DF30ST

- Cleanroom cleaned slides and coverslips:
    - Applied Microarrays, (Dist) LLC.
      Global Product Manager
      nexterion@appliedmicroarrays.com
      (480) 244-9395
    - Item# 1098576 - NEXTERION® Glass D CUSTOM COVERSLIPS, Cleanroom Cleaned
      Nexterion® Glass D type#1 D263
      Borosilicate Glass for Microarrays
      Size 22.0 mm x 22.0 mm +/- 0.015 mm
      Thickness .0145 mm +/-0.1 mm
      Edges: cut
      Surface: as down draw
      Cleanroom cleaned
      25 pcs per pack
      Cleaned glass pieces are packed in a box with spacers between each piece
      Item# 1025087 - NEXTERION® Glass B cleanroom cleaned Slides
      50 pieces per pack

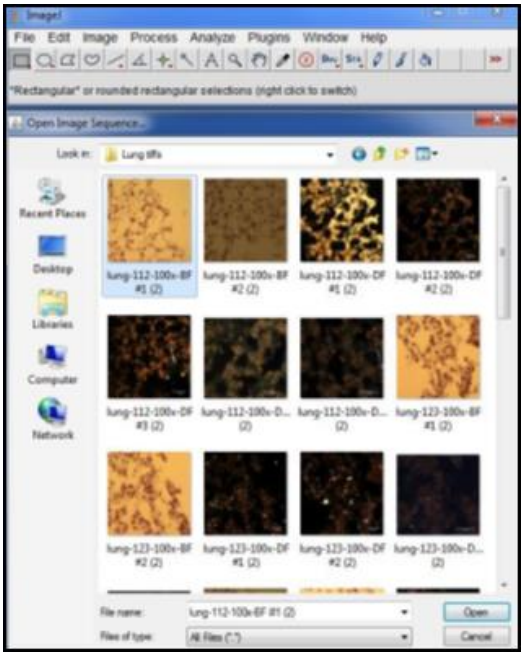
- Cleaned glass pieces are packed in a box with spacers between each piece
- Steps for adding scale bars to images using Image J:
  - Open Image J
  - Click File, Open, Select the image you want to work with.
  - Click Analyze, choose Set Scale.
  - Distance in Pixels is dependent on the magnification and type of image; optical or spectral. Use the table below to select the appropriate number.
  - Set the Known Distance and Pixel Aspect Ratio to 1.
  - Set Unit of Length to microns (um)
  - Check box next to Global. This will save your settings for subsequent images. Click OK.
  - Click Analyze, select Tools, and click Scale Bar.
  - Scale bar will appear in lower right corner. Select scale bar length.
  - Click File, Save As. Save image.

Magnification	Optical (R6)	Spectral (Pixelfly)	Spectral (Andor)	3D or Optical (R1)
10x	2.2	0.79	0.625	6.45
20x	4.40	1.58	1.25	12.9
40x	8.81	3.17	2.50	25.8
50x	11.01	3.96	3.13	32.25
60x	13.21	4.76	3.75	38.7
100x	22.02	7.93	6.25	64.5

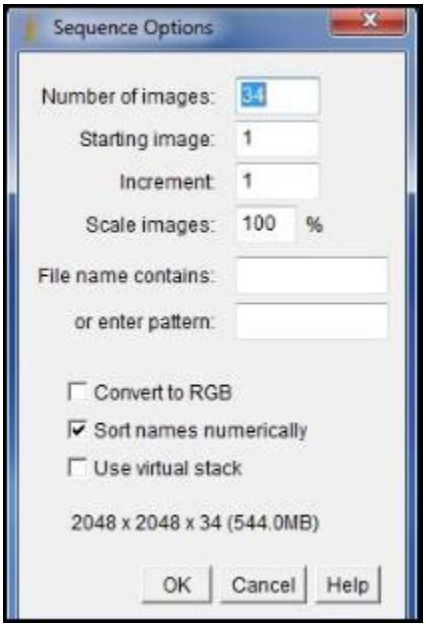
- Making an AVI from images using Image J:
- Open Image J. Go to File, Import, Image Sequence



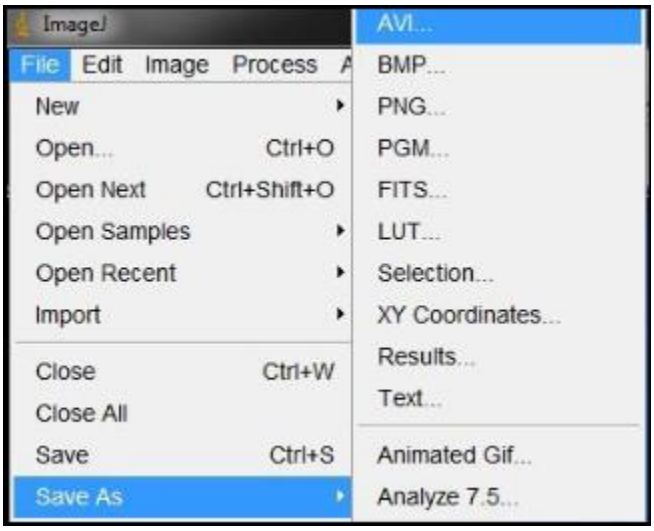
- Select the first image of the time capture results.



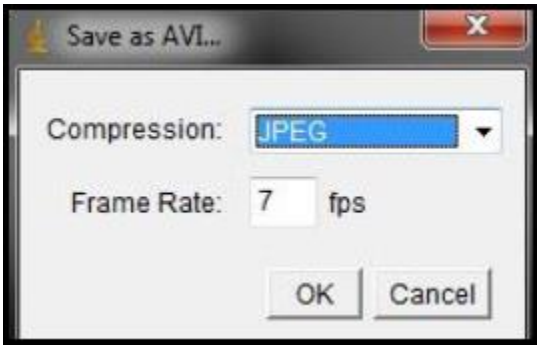
- Click Open. A Sequence Options window will open. The number of images automatically imports. Choose desired settings. Click OK.



- The AVI is then compiled in Image J. In the main Image J toolbar, Click File, Select Save As, Click AVI.



- Choose file type and compression options and frame rate. Click OK.



- File is saved in the folder with the chosen images.

Section 14 Glossary

- Image File:  This is the file of the original image scanned with the spectrophotometer.
- Spectral Library File:  This file contains the different spectra that the user will collect from the scanned image.
- Rule Image File:  This file contains the Rule image of the sample.
- Classification (SAM) File:  This contains the classification (SAM) image of the spectra that matched the library. It may also indicate a Peak Location Classifier (PLC) image.
- Rule Image: The rule image is the displayed grayscale image showing the degree of matching between the known and unknown spectra from each image pixel. The Rule image is shown on a scale of light to dark the relative degree to which unknown spectra in each image pixel match the known spectra.
- Spectral Angle Mapper Classification (SAM): an automated procedure for determining if a known material is present in the input image, and the pixels containing the material. SAM accomplishes these tasks by comparing unknown spectra in Hyperspectral imagery with known laboratory spectra for the material in question.
- Classification (SAM) Image: this image shows the location of pixels whose spectra match that of the endmember.
- Header File: A separate file created with the Hyperspectral image file, with the same name. This file must be copied with the image file when data is transferred. The header file includes information for CytoViva-ENVI to allow the image program to be opened and used.
- Endmember: This is any spectra that have been chosen as important for identification purposes in a sample.
- Output File: This is a file that you create to include several different output files such as: Spectral Library Files, Header Files, Spectral Angle Mapper Files, etc. This file will allow you to easily access this information when needed.
- Z Profile Average Window: Setting the size windows will either decrease or increase the noise in the plotted spectrum.
- Plot Key: This is the key that contains the labels of your spectral curves chosen.

- X Axis (Default): The X-Axis default is the Wavelength. By moving the cursor over the point of the peak you can see the wavelength of the spectra in the selected location.

- Y Axis (Default): The Y-Axis is the Value. This provides the intensity levels of the spectra.

- Data Parameters: This can be found in the plot key by click on the image menu bar and selecting edit, then select Data Parameters. The Data Parameters will allow you to change the Key Plot names, line style (dotted, thickness) and color.

- Plot Parameters: The Plot Parameters will allow you to change the title, X & Y axis labels and scaling, background and foreground colors, and the font. This can be found in the spectral plot by clicking Edit then Plot Parameters.

- Spatial Resolution: The minimum spatial separation that can be detected in the image. The control of spatial and spectral resolutions work the same way.

- Spectral Resolution: The minimum separation in wavelengths that can be detected. The Low spectral resolution setting is equal to half that of the Hi setting. Therefore if the Hi resolution were 1.5 nm, the Low resolution would be 3 nm.

Section 15 Troubleshooting

- What is the number for Technical Support?
  - 1-888-737-3130

- After the scan has finished the Image on the screen is Black.
  - One of the slide bars wasn't opened or closed at the proper time. Refer to the Manual, Section 2: Quick Start Guide, Part 3: Starting the Scan, numbers 6 & 7.

- The scanned image is out of focus.
  - Before you scan your sample be sure to double check that the sample is in focus. Sometimes the slightest movement can make the sample move.

- When I rescan an image the original image is still showing.
  - Go to the Available Bands list and choose close all files and then in the Available Bands list and open the new image you just scanned. You can refer to the Manual in Section 2: Quick Start Guide, Step 3: Starting the Scan, and number 8 which provides more detail.

- There is a black area at the right or left end of my scanned image.
  - This is due to the slit not being pushed in all the way on the spectrophotometer. Call Technical Support.

- There is no spectral signal past 750 nm.
  - CytoViva offers a different bulb for the Dolan Jenner to be used with hyperspectral imaging. The bulb is an aluminum reflector bulb that will provide light output to 2000 nm. This bulb is preinstalled at the CytoViva office, however the original dichroic bulb is still included as a back-up. If your aluminum bulb has blown, please call CytoViva at 888-737-3130 to order a new one.
  - There are also two different light guides that may come with the system depending on the configuration. One is all black and is used for fluorescence imaging out to about 750 nm. The other has a yellow tag and is used for hyperspectral imaging from 420 – 1,700 nm (VNIR and SWIR). The yellow tag light guide has a UV filter to protect the user.
  - If the improper bulb and/or light guide is being used, spectral data may not be transmitted at all wavelengths.

- My image quality is not as good as it used to be.
  - The system may be in need of maintenance. Light guides extinguish over time and should not be used for over 3 years. If oil has been allowed to build up on the condenser, image quality may be compromised.

- Call Technical Support for assistance. CytoViva offers service/maintenance visits and will also service certain parts in-house.

IMPORTANT: PLEASE READ CAREFULLY

CytoViva®

Limited Warranty

CytoViva warrants for a period of one (1) year from the date of purchase from CytoViva, Inc. or an authorized agent of CytoViva, Inc. (the "Warranty Period"), that the unmodified CytoViva® Enhanced Darkfield Illuminator and/or Dual Mode Fluorescence Module (the "Equipment") when new, and subject to normal use and service, shall be free of defects in materials and workmanship and shall perform in accordance with the manufacturer's specifications.

If any component of the Equipment does not function properly during the Warranty Period due to defects in material or workmanship, CytoViva will, at its option, either repair or replace the component without charge, subject to the conditions and limitations stated herein. Such repair service will include all labor as well as any necessary adjustments and/or replacement parts. If replacement components are used in making repairs, these components may be remanufactured, or may contain remanufactured materials. Repair or replacement without charge is CytoViva's only obligation under this warranty. This warranty is NOT transferable from the original purchaser of the Equipment.

Limitations

Other components of the product package, specifically including, but are not limited to, the light source(s), light source power transformer(s) and cord(s), liquid light guide(s), optical filters, spectrophotometer, camera(s), software, microscope part(s), and motorized stage are warranted based on the individual original manufacturer's warranties and policies. There is no warranty whatsoever on the contrast filters, bulbs or the coil (on the motorized stage) purchased as part of the Equipment.

This warranty does not cover circumstances beyond CytoViva's control, breakage, or a malfunction that has resulted from improper or unreasonable use or maintenance, accident, tampering, misuse, neglect, improper installation, modification, improper maintenance or service, cleaning procedures, shipping or repacking of Equipment, or service or parts to correct problems where such service or parts are performed or provided by anyone other than CytoViva or an authorized agent of CytoViva; service required as the result of unauthorized modifications or service misuse or abuse; failure to follow CytoViva's operating, maintenance or failure to use items supplied by CytoViva. This warranty is also void if the light source, light source power transformer and cord, or liquid light guide is not used in accordance with the original manufacturer's instructions, recommendations, or documentation.

Warranty service will not be provided without a dated proof of purchase. [Please return the Warranty Registration Card together with a copy of the original receipt, within thirty (30) days of purchase.] It is the purchaser's responsibility to return the Equipment to the authorized agent from whom it was purchased. If the Equipment was purchased directly from CytoViva, it should be returned, postage paid, along with the original dated receipt to CytoViva, Inc., 300 North Dean Road, Suite 5 PMB-157, Auburn, AL 36830. Your repaired item or replacement product will be returned to you postage paid. In the event the purchaser returns Equipment to CytoViva and it is determined by CytoViva that the Equipment has been returned without cause, the purchaser will be notified and the Equipment returned at the purchaser's expense.

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