

The Paper Project Guide to
**Confocal Imaging at
Home & in the Classroom**

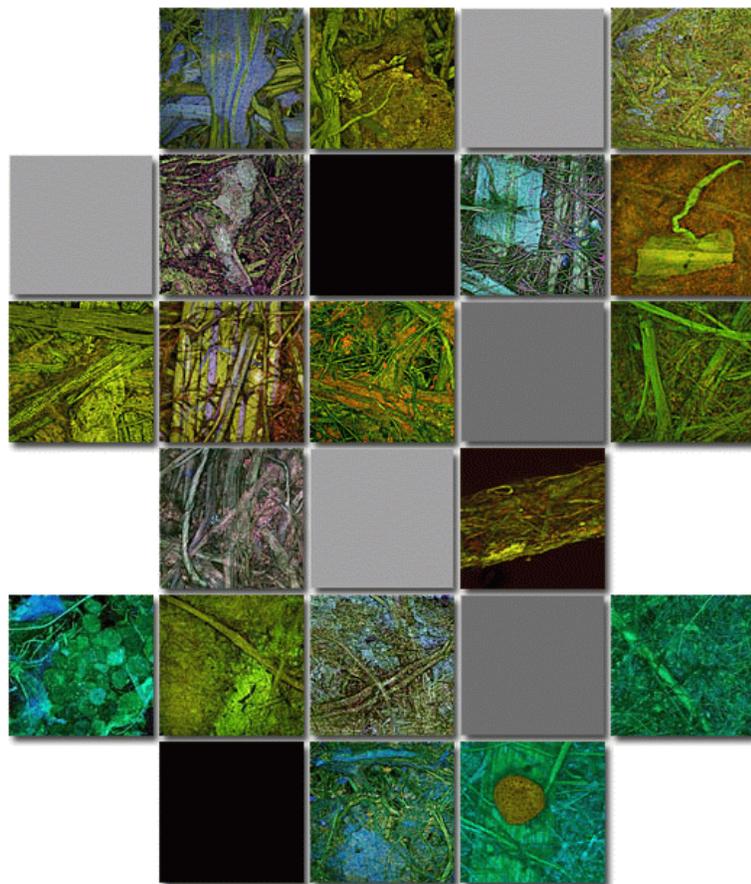


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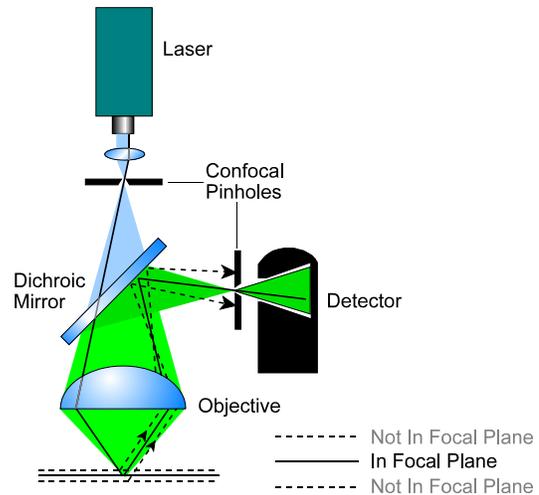
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Introduction

Okay, we are not going to be able to put a confocal microscope in your home or classroom, but you will be able to use some of the data sets from the Paper Project to build images and movies in two and three-dimensions. That's in itself worth downloading the small program called *Confocal Assistant* along with some of our own *Paper Project* data sets. Once you have experimented with the different ways you can view images from a confocal microscope you will have better idea of how powerful a tool it is in biological imaging.

A brief background about confocal microscopes

Many people will remember their experience with microscopes. Even though the object was magnified, certain details always appeared to be fuzzy. At work is a basic principle of optics that prevents both magnification and the ability to view all the details of a thick specimen. The term used to describe the thickness of the sample that can be viewed in sharp detail is called depth-of-field. Up to the mid 1980's depth-of-field limited the ability to see objects in detail. This is especially true at high magnifications. Early in the 1980's inexpensive lasers, high speed computers, and a very ingenious microscope design were combined to create the first commercial scanning-laser confocal microscope.



Confocal imaging - what is it?

In the confocal microscope all areas that are out of focus are eliminated at image formation. This is possible because the object is not being illuminated and imaged entirely at the same time, but rather one single point after the other. As shown in the diagram, this is obtained by the arrangement of diaphragms, which act as a point source and as a point detector respectively (solid line). Rays from out-of-focus areas are prevented from being imaged by the final confocal pinhole opening (dashed lines).

Using a confocal microscope eliminates out of focus points while retaining the sharp sample areas. Part of the problem for microscopes has been solved. Depth-of-field has been circumvented and sharp images can be viewed, but only for very thin slices.

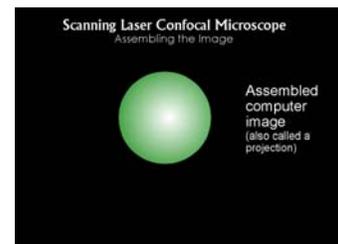
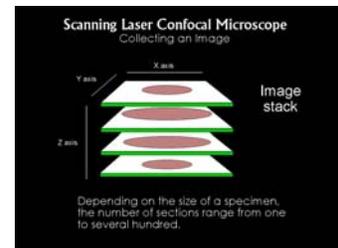
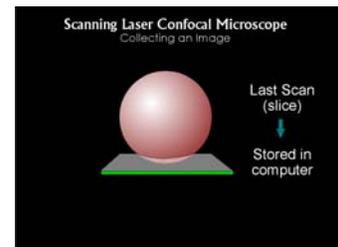
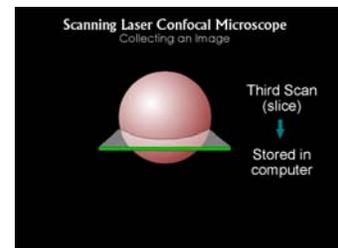
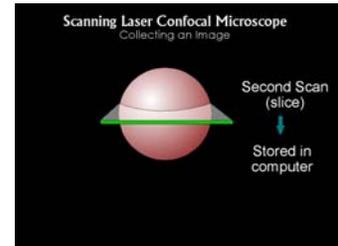
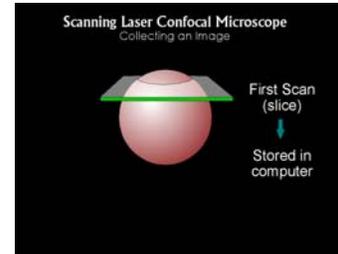
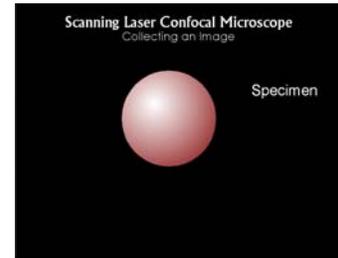
What about thick materials? The next important development was high speed inexpensive graphic computers and 3-D software.

Using a computer, multiple slices of in-focus images are collected and saved. These slices as a group are called a stack. You can think of a stack as a deck of cards. Each image is a card and the entire deck is the stack. Each card has very little depth, while the whole deck has a lot.

Using 3-D software, the stack is reassembled into one sharp image (also called a projection). Take a look and see how this works.

Building a Projection

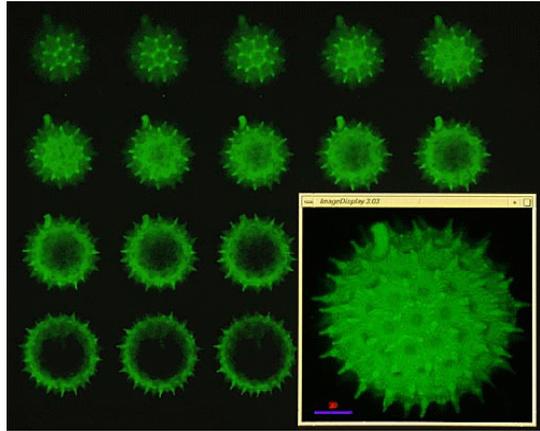
- Step one** Hypothetical specimen under a confocal microscope.
- Step two** The initial scan collects an optical slice of the image and then stores that image in the computer.
- Step three** The stage is moved in the Z direction and another optical section is taken. The digital image is again stored in the computer.
- Step four** Each optical slice taken is within the acceptable range of depth-of-focus. This means each slice appears sharp to the eye.
- Step five** The final optical slice is scanned and stored in the computer.
- Step six** Once all the slices are stored in the computer an image stack can be created. The number of slices in a stack can be from 1 to 200.
- Step seven** From the image stack an image is produced called a projection. For thicker specimens, more sections provide greater Z-resolution. This image is a projected from the top and is called an XY projection.



Notice in our hypothetical scan that the projection color is not the same as the image color. This is due to two factors.

1. The excitation wavelength cannot be the same as the emission wavelength
2. The actual colors of an image are assigned in the computer and can be set to any color. We call this pseudo (false) color. In general, images are set to resemble the actual color that would be result from a typical excitation/emission image.

To the right is a collection of individual slices called a mosaic. The series of slices were taken of a pollen grain. Each slice shows only a portion of the entire sample. The lower right image is a projection image made from the series.



What does a confocal microscope look like?

The microscope used for many of the Paper Project's images is the Leica TCS NT. A universal Laser Scanning Confocal Microscope System for the bio-medical and materials research environment. The system includes four lasers that can be used simultaneously. The lasers and laser lines include, an Argon (488nm), Krypton (568), RHeNe (633), and an Argon UV (363).



There are two separate microscopes (upright and inverted) that can accommodate the scanning head, which makes the system flexible for various research needs.

Downloading and using the data sets and software

You can download image data sets from the Paper Project. Using the Confocal Assistant software along with the data sets will give you chance to experience what it is like to use such a microscope. Details about downloading are at each web address.

Data Set and Image Software address: ??

- Confocal Assistant Software address (PC only)
- Alternate Image software addresses
- NIH image (Mac only)
- Scion image (PC only)

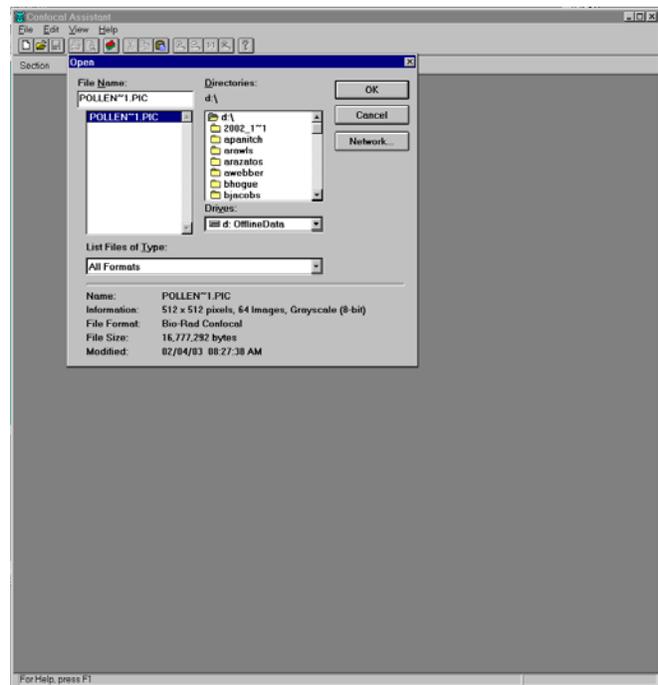
Using Confocal Assistant

We recommend Confocal Assistant for PC users since it is very small and focuses on mainly processing confocal image data sets. It does not have a manual. So we have developed a basic guide to get you up and running. Even though you do not have a confocal microscope of your own, this is really where the fun begins for those of us that do have one.

To open an image stack

Either go to the "File" drop-down menu and select "Open" or press the "Open" button.

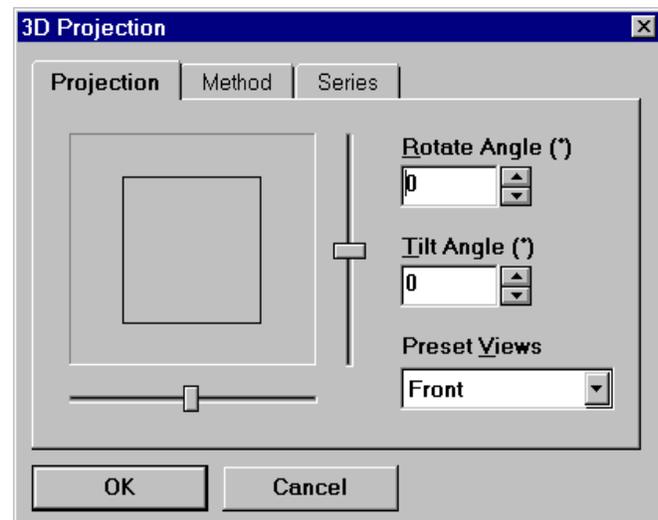
In the Open File dialog window, select the correct drive letter, find the image stack (.PIC file) you wish to open, and press "OK". Confocal Assistant only reads PIC file formats. The data sets we include have been saved in PIC format because Confocal Assistant only reads PIC files.



To make a projection

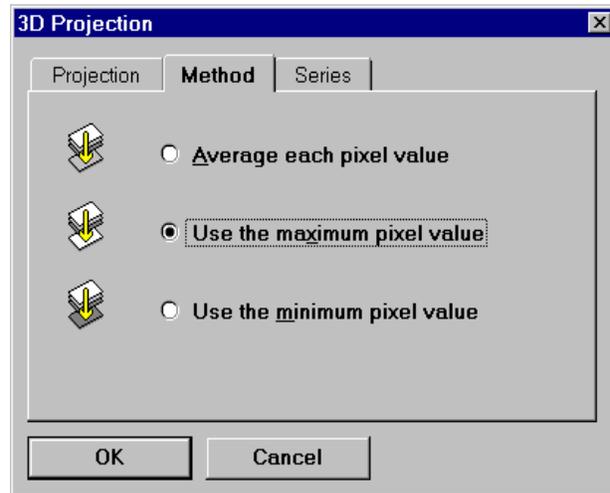
A projection is a way of displaying an image stack in so that all layers are visible at once. To make a projection, select "Projection" from the "Series" drop-down menu. The "3D projection" window will then open. This window has three tabs: projection, method, and series.

The projection tab allows you to choose a rotation angle and a tilt angle for your projection. If you leave these angles at their default value of zero, the projection will be created with a face-on view (the same angle at which the images were collected). If you wish to change these values, you can either enter the angle numerically or use the sliders or arrow buttons. If you enter a numerical angle, press enter to register the changes or the program may either ignore your values or crash.



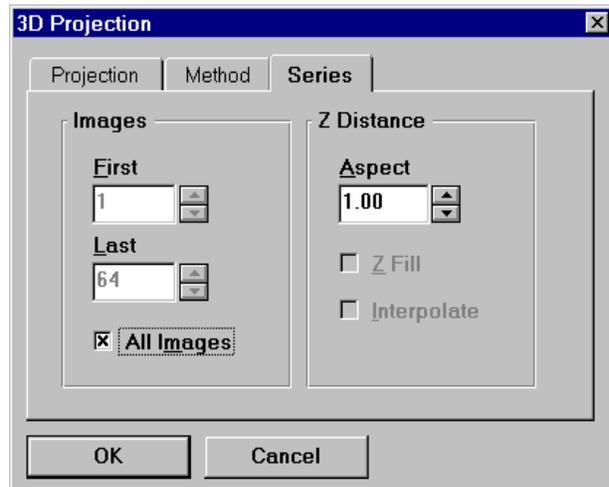
Method tab

The method tab allows you to choose what type of projection you wish to create. Using the average method, the value of each pixel in the final projection is an average of all of the pixel values at that position in the stack. Using the maximum method, the value of each pixel in the final projection is equal to the brightest pixel present at that position in the image stack. Using the minimum method, the value of each pixel in the final projection is equal to the darkest pixel present at that position in the image stack. The maximum method is usually the most useful.



Series tab

The series tab allows you to choose what images from the stack are included in the projection. The default is to use all of the images in the stack for the projection. If you wish to use only a subset of the stack, de-select the "All Images" box and then change the values in the "First" and "Last" windows. The right side of this window allows you to set the aspect of the stack. The default value is 1.00, which means that the software assumes that the distance between layers in the image stack is equal to the distance between pixels in any single image of the stack. Raising the aspect to a value greater than 1.00 has the effect of stretching the image along the z-axis (perpendicular to the screen). If you have increased the aspect to a value greater than 1.00, you may then select "Z fill" or "Interpolate". "Z fill" has the effect of filling in the spaces between layers in the projection (note: this will be visible only if the rotation or tilt angle has been set to a value other than zero, and is most visible at angles close to $\pm 90^\circ$). "Interpolate" does the same thing as "Z fill", but generally looks better (and requires more processing time).



To create a rotation movie

A movie which shows the image stack rotating is a good way to display the three-dimensional structure of the sample. To make a rotation, select "Movie" from the "Series" drop-down menu. The "3D movie" window will then open. This window has three tabs: movie, method, and series.

The movie tab allows you to see what the structure looks like in three dimensions without needing to use 3-D anaglyph glasses. The rocking motion provides the need queues for the brain to interpret the structure of an object. Try it out.

Lookup tables

By default the images in Confocal Assistant are in grey scale (black and white). To change the color of your projection or movie you need use the lookup tables we have supplied with the software download.

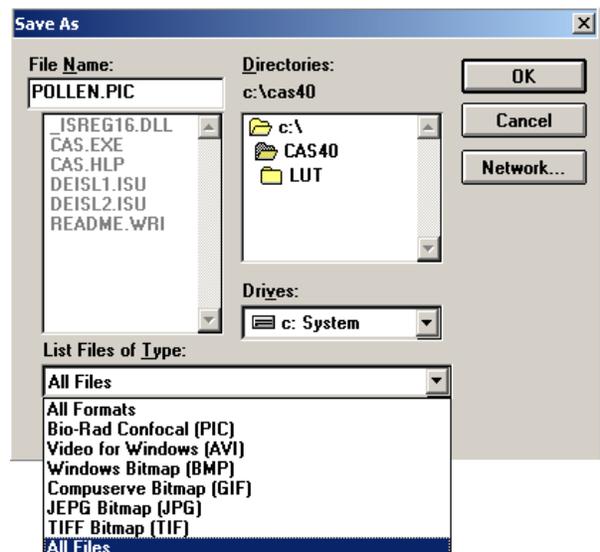
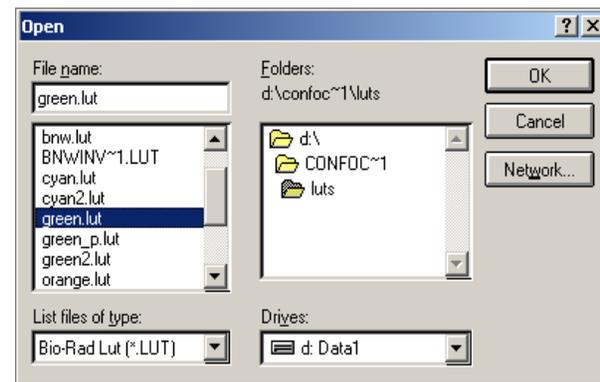
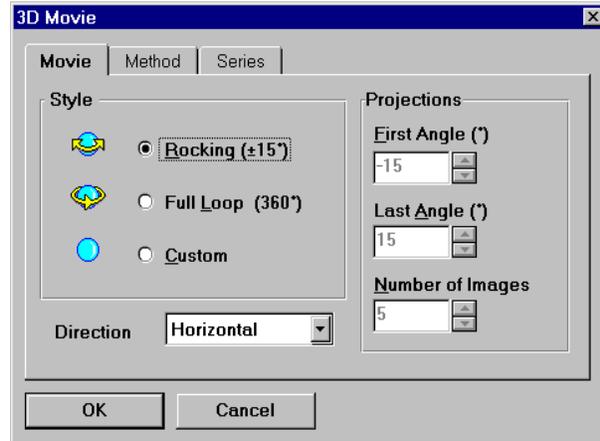
To open the Lookup Table window, select the Image menu option and click on Lookup Table. This will open the menu box where you can select the lookup colors.

You will need to move to where you stored luts folder contained in the program download file. We keep the luts folder in the Confocal Assistant directory for easy access.

It is possible to create colors that display on a computer monitor, but will not print correctly. The files labeled with an underscore p (_p) extension are those that produce printable colors.

Saving Files

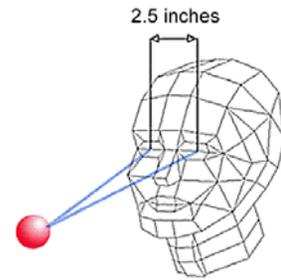
There are many file formats that you can save your files. The only short come of Confocal Assistant is it only handles files names no larger than eight plus 3 characters(8.3). Keep this in mind when naming your final images.



3-D Glasses

How do 3-D anaglyph glasses work?

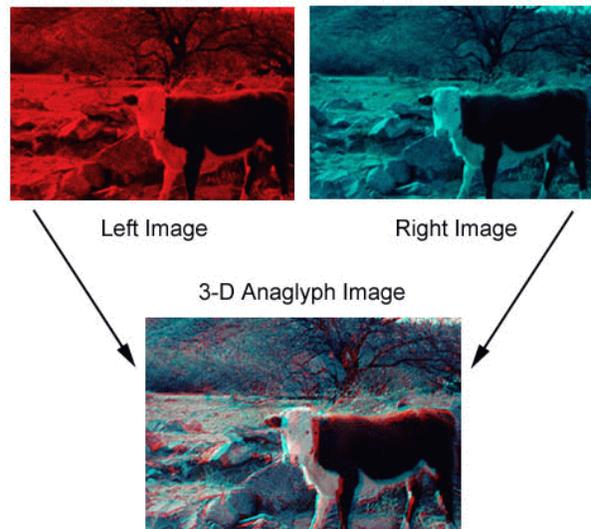
Using Confocal Assistant and other imaging software images can be made that can be viewed in three dimensions. This requires a pair of red-blue 3-D glasses also called anaglyph glasses. Just how this works and how we see three dimensions is the topic of this page.



With two eyes we have the ability to see depth, or as it is sometimes called 3-D. Depth occurs when images from two different angles are interpreted by our brain.

Our eyes are separated by a distance of about 2.5 inches, and because they are separated, we see objects from two different angles. To experience this, try this test.

3-D images mimic what our eyes do naturally. An anaglyphic 3-D picture is made from two different images. In most cases, two pictures are taken with a camera. Each image is taken at a slightly different angle similar to the angle each of our eyes normally sends to the brain. The left image is colored red and the right image is colored blue. The images are combined into one picture for 3-D viewing. At this point the 3-D picture looks blurred to the naked eye.



When you put on 3-D glasses with red and blue filters, your left eye sees only the red image and your right eye sees only the blue image. Your brain combines the images from each eye to "see" depth or 3-D.

What does the word 'anaglyph' mean?

an-a-glyph (an'-a-glif). - an image made up of two slightly different views (angles), in contrasting colors, of the same subject; when viewed using a pair of corresponding filters, the picture appears three-dimensional.