21

Presentation of Confocal Images

Georg Halder and Stephen W. Paddock

1. Introduction

Confocal microscopy is routinely used to produce high-resolution images of single, double-, and triple labeled fluorescent samples. The images are collected as single optical sections (2D imaging), as Z-series (3D imaging), as time-lapse series (2D over time), or as Z-series over time (3D over time or 4D imaging). Because the images are in a digital format, they can be further manipulated using a range of software.

This chapter covers methods of presenting confocal images for publication purposes, and is focused mainly on manipulating single optical sections or Z-series projections using relatively simple and commonly available software such as PhotoShop™ (Adobe Systems, Mountain View, CA). Many programs are available for constructing a 3D representation of a Z-series of confocal images either using proprietary software that is supplied with the confocal microscope or secondary software on a separate computer. 3D and 4D methods have been reviewed elsewhere ***(1–3),*** and *see* Chapter 18.

Most of the information contained in a confocal image of a biological specimen is related to the spatial distribution of various macromolecules. Images of different macromolecules are collected at different wavelengths. At the present time images collected at three or four different excitations are relatively routine using the laser scanning confocal microscope (LSCM) but more images at different wavelengths are theoretically possible given enough lasers and filter combinations. However, many such multiparameter images rapidly become complex and difficult to interpret when more than three of them are colorized and merged unless the images contain many regions of nonoverlapping structures, e.g., chromosomes painted with fluorescently labeled DNA probes ***(4)***. A convenient way of displaying two or three images is to use the red, green,

From: Methods in Molecular Biology, vol. 122: Confocal Microscopy Methods and Protocols
Edited by: S. Paddock Humana Press Inc., Totowa, NJ

and blue channels of an RGB color image where any overlap (colocalization of fluorescent probes) is viewed as a different additive color when the images are colorized and merged into a single three-color image ***(5,6)***.

A relatively simple method for displaying three color confocal images using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA) and a Macintosh computer is described. Photoshop is available not only for Macintosh but also for PC and UNIX machines. It is now in its fifth version, and it has evolved over the years into an extremely powerful yet affordable image manipulation program, and is used extensively in the graphic arts and publishing industries.

Several applications of the three-color merging protocol in addition to displaying confocal images are outlined below including mapping color to depth in Z-series, mapping color to time in a time-lapse series, the production of red/ green or red/blue stereo anaglyphs from Z-series, and merging confocal and transmitted light images.

These digital methods are not confined to images produced using the confocal microscope, and can be applied to any digital images imported into Photoshop collected with many different kinds of imaging devices including both light and electron microscopes, and images that have been scanned from other sources. Photoshop is currently the program of choice for compiling these images from different sources, including the confocal microscope into figures for publication. It is used by most of the journals, and therefore it is relatively easy to transfer images directly to them with a reduced risk of errors at the final publication stage, and at a resolution that is preserved from the microscope itself.

Color hard copies of the images can be produced directly from Photoshop using a 35-mm slide maker (e.g., Lasergraphics), dye sublimation printer (e.g., Tektronix Phaser IISDX), or color laser printer (Tektronix Phaser 350 or 560). These devices are controlled directly from Photoshop as Plug-ins, which avoids transferring the images to yet other programs ***(7)***.

Images of fluorescently labeled specimens were collected using a Bio-Rad MRC600 LSCM or more recently an MRC1024 LSCM (Bio-Rad Laboratories, Life Sciences Division, Hercules, CA) using previously described methods although all of these methods are compatible with digital images collected with other confocal systems. Selected images were transferred from the host IBM microcomputer of the confocal microscope to a Power Macintosh 8500/150 (with 80 MB of RAM) microcomputer via Ethernet using Fetch 3.0.1. (usually as binary files).

Images were imported directly into Adobe Photoshop (version 3.0.5, and more recently version 4.0.1), either as RAW or as TIFF files. In the case of RAW files, the actual dimensions of the Bio-Rad confocal image must be entered, e.g., a typical image size is 768×512 pixels with a 76-byte header.

The header is specific to the Bio-Rad proprietary "pic" file format, and may vary with imaging systems from different companies. This information on the image files should be freely available from each confocal company. TIFF files are opened directly into Photoshop. For example, Bio-Rad collect their images in their own *.PIC format, which can be converted into TIFF or PICT files relatively easily. The *.PIC is occasionally confused with the Macintosh PICT files by some software.

If necessary, confocal image files may be converted to TIFF files using any one of a variety of programs such as Confocal Assistant (written by Todd Brelje, Dept. of Cell Biology and Neuroanatomy, University of Minnesota Medical School and available from the BioRad web page; www.microscopy.bio-rad.com), NIH Image 1.57 (Wayne Rashband at the NIH and available at no cost from http://rsb.info.nih.gov/nih-image/), or Graphic Converter available from LemkeSoft on the web at www.lemkesoft.de. Harvey Karten has published a most valuable web page on manipulating confocal images using NIH Image: http://rsb.info.nih.gov/nih-image/more-docs/confocals.html. This page also contains many useful details of the image formats from many different confocal imaging systems.

2. Materials

2.1. Computer Hardware

1. Powermac or PC with at least 32 MB RAM (the more RAM the better)
2. Backup devices: Optical drive, Zip drive, CD writer
3. Hard copy devices: Printers, Slide Writers

2.2. Computer Software

1. Adobe Photoshop (www.adobe.com)
2. Fetch (copyright Trustees of Dartmouth College)
3. NIH Image
4. Graphic Converter
5. Confocal Assistant
6. Database (*see* Chapter 23)

3. Methods

3.1. Importing Images Using Fetch

There are several options for transferring files from the confocal computer into PhotoShop. If the files are directly compatible with PhotoShop then it is a relatively easy process of transferring the files directly to the second computer usually via Ethernet or using a floppy, optical, or CD.

1. Turn on the confocal workstation and the Macintosh computer.
2. Open Fetch 3.0.1

Usually it is best to save a shortcut to the confocal computer in Fetch, which enables rapid entry directly into the confocal images directory so that long computer addresses and passwords are not typed in each time. For example the shortcut to our MRC1024 microscope looks something like this:

Host:Internet address of the computer
User ID:Computer internet name
Password:*******
Directory:c:\ls_user\paddock\images

3. Images are best transferred as binary files directly into a folder on the Mac hard drive. Check "binary" in the Fetch menu before highlighting the files to be transferred and then check "Get File," and place the files into a specific confocal images directory on the Macintosh computer (*see* **Note 1**).

3.2. Opening Confocal Files in Photoshop

Many commonly used file formats, e.g., TIFF files, are opened directly into Photoshop. We usually transfer the *.pic files from the BioRad confocal microscope as RAW files and convert them to TIFF files using the batch conversion feature of Graphic Converter. Alternatively *.pic files can be opened as RAW files in Photoshop. Often the proprietary software that comes with the microscope has a facility for converting files or a second program can be used. We are currently using Graphic Converter because it converts files from the confocal microscope in a batch mode using the Macintosh computer. To open RAW files in *.pic format:

1. Open the file in Photoshop by opening Photoshop and selecting File and Open. Select the confocal images folder. Note that the RAW image files may not be shown if "Show All Files" is not checked.
2. Select an image to open and fill out the image dimensions, e.g.:

Width	768 pixels
Height	512 pixels
Channel	1 for a single grayscale image
	3 for a three color image made up of three grayscale images.
Check	"Not-interleaved" for both single and triple images
Depth	8 bit
Header	76 bytes for BioRad pic file

3. Select OK and a message will say "specified image is smaller than file: open anyway?" This is good and ignore it — it pertains to the header.

3.3. Merging Grayscale Images

Images of multi labeled samples collected at different wavelengths can be merged by copying and pasting between different channels within Photoshop

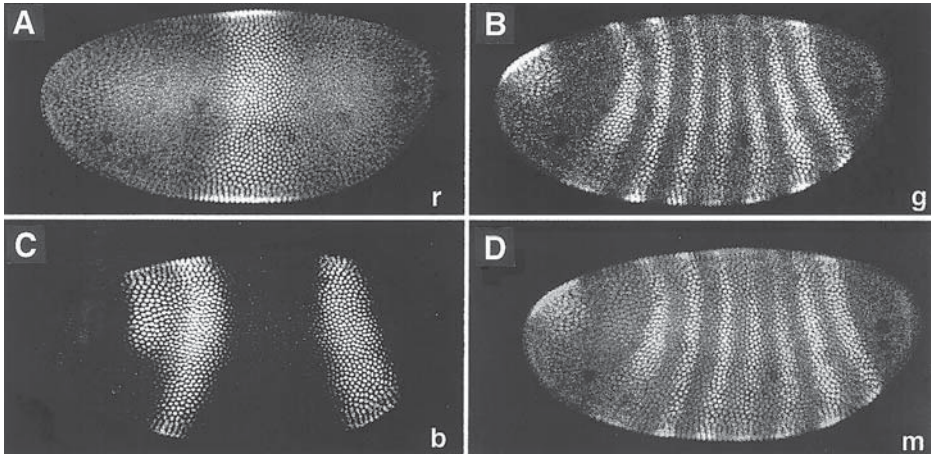

Fig. 1. A three-color image is made up of different images in the **(A)** red (r), the **(B)** green (g), and the **(C)** blue (b) channels of an RGB image. Different color combinations can be made simply by rearranging and copying the images to different channels. The merged image **(D)** is shown here in black and white and also in Color Plate I.

(**Fig. 1**). This method can also be used to rearrange the colors in a two or three -color image (*see* **Subheading 3.3.1.**).

Three-color images are produced in Photoshop by pasting each of the grayscale images from the confocal microscope into the red, the green, and the blue channels of an RGB Color image (*see* **Notes 2–4**). In the following description of the process, the location of various commands within the Photoshop program is included in (*italics*) after each operation.

1. Open three grayscale images in Photoshop.
2. Construct a blank RGB image (*File, New*) or copy from one of the grayscale images (*Select All, Copy, New, Paste*). The image must be the same size and pixel resolution as the three original grayscale images from the confocal microscope — in our case 768 × 512 pixels, and 72 pixels/inch. The size and resolution of an image can be determined and adjusted (*Image, Image Size*). This new RGB image should be black because the background in fluorescence images is usually very close to, or at black (black = 0, white = 255). Another color can be chosen for the background for cosmetic purposes, but a black mask for the actual region delineated by the image should be constructed, so that the new background color does not interfere with the information in the actual image.
3. Open the Channels palette (*Window, Palettes, Show Channels*).
4. The three grayscale images are now ready to be pasted into the newly-created RGB image. Select the first of the grayscale images (*Select, All*), and copy it into the required red, blue or green channel of the new RGB image (*Edit, Copy*), by clicking on the newly created RGB image with the mouse, and then on the required

channel in the Channels palette column. For example, for the red channel click on the red window in the column. Finally the grayscale image is pasted into the RGB image (*Edit, Paste*). The image will now appear in the Channels palette column in both the red and the RGB channel.

5. Select the second and third grayscale images, and copy and paste them into the green and blue channels of the RGB image using the same routine. The result of these manipulations is a single three color merged image, which is displayed by clicking on the RGB image line in the Channels palette column. There is no loss of bit depth information from the three original source images because three 8-bit images are merged into a single 24-bit image.

3.3.1. Rearranging Colors in an Image

1. Open the double- or triple-label image.
2. Select Window and click on Show Channels. Click on the arrow in top right of the Channels box. Select Split Channels which will split the image into three separate grayscale images comprising the red, green, and blue channels.
3. Click on the arrow in the top right of the Channels box. Select Merge Channels; select RGB Color and the Specify Channels, and then rearrange the images within the menu.
4. An alternative method is is to copy and paste images between the channels. This is especially easy for two-color images.

3.3.2. Double-Label Images

1. A double label image is simply an RGB image that is composed of grayscale image in each of the red and the green channels with a blank black image in the blue channel (**Fig. 2**). This method can be used to construct red green or red blue stereo pairs (*see* **Note 5**).

Extra colors can be included in double-label images by placing two versions of the same image into two of the three channels, with the second image of the merge in the third channel. For example, a purple and green image is produced by pasting the same image into the red and the blue channels to give purple, and the second image is placed into the green channel (**Fig. 2**). Additional color combinations are red and light blue where light blue comprises the blue and green channels or blue and yellow where yellow comprises the red and the green channels. Here overlaps of expression invariably appear white in the image because all three channels now contribute to the overlapping signals. These additional color combinations can be useful when making a multipanel figure of several double-label images where the expression patterns of more than three proteins are displayed in separate panels so that different colors are assigned to individual proteins.

3.3.3. Adding Color to a Single Grayscale Image

A grayscale image can be colorized by copying it into one of the three color channels of an RGB image and adjusting the colors using Levels. However, the

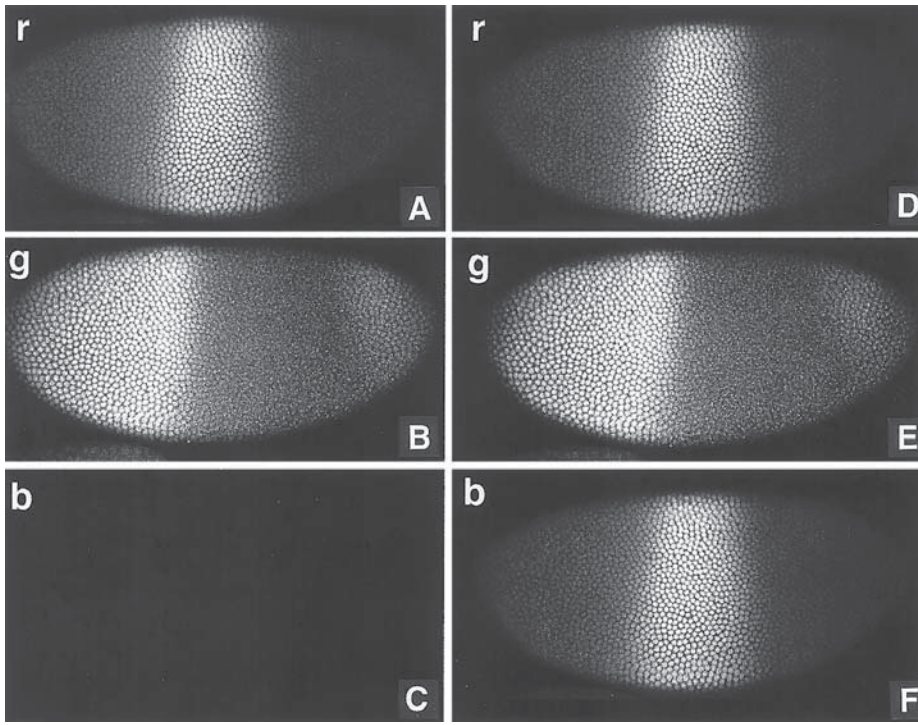

Fig. 2. Structure of double-label images. A red green image **(A–C)** is made up of two different grayscale confocal images in the red (A) and the green (B) channels with a blank black image in the blue (C) channel. A green and purple image is made by copying the same image into the red and the blue channels **(D**, **F)** with a different image in the green channel **(E)**. (*See* Color Plate I, following p. 372.)

image then appears rather flat (*see* **Note 6**). If only one color is to be used then the appearance of the image can be much improved by displaying it using a dynamic color table (or look up table, LUT). This method works well for images with many different gray values. To produce a color table ranging from black over green and yellow to white:

1. Open the grayscale image.
2. Select Indexed Color (*Image, Mode, Indexed Color*).
3. Select Color Table (*Image, Mode, Color Table*).
4. Highlight a portion of the table by dragging from the top left square inwards.
5. Slide to color and select first color (usually black) — click OK.
6. Select second color, e.g., dark green — click OK.
7. Select a second portion of the color table by dragging from the last square of the former selection.

8. The first color selected is identical to the color in the first selected square. Click OK if you want produce a continuous color table.
9. Select the second color, e.g., yellow — click OK.
10. Repeat **steps 7–9** by highlighting the last portion of the color table and assigning color values from yellow to white.
11. The color table will be displayed — click OK and view its effect on your image.
12. Specific color tables can be saved for later.

3.3.4. Transmitted Light / Confocal Image Merging

Many confocal imaging systems have the facility to collect a nonconfocal transmitted light image, which usually comes into the imaging system on one of the confocal channels as a grayscale image. For example, in the Bio-Rad system the blue channel is used. An improved merged image of transmitted light and confocal images can be produced using the following method (Harvey Karten, *personal communication*).

The following description assumes that the transmitted light image (Nomarski, phase contrast, dark field etc.) is in the blue channel with the confocal images in the red and green channels or a blank image in the red or green channels for single confocal images merged with transmitted light images.

1. Make sure that the Mode = RGB.
2. Go to the blue plane (containing the transmitted light image). Copy the blue plane image into the Copy Buffer.
3. Add an additional layer. Call it "Transmitted." Set the opacity to 40%.
4. Paste the blue plane image into the new layer, and to make it gray, paste it into separate R, G, and B channels.
5. Return to the original image and erase the blue layer. Make sure that the blue plane is replaced with a black background.
6. Play with the opacity function of the Nomarski layer to vary the ratio of the confocal image and of the background layer.

3.4. Processing Images in Photoshop

The amount of processing required to "clean up" an image is highly subjective, and is best described as an art. This phase of the process is largely up to the individual investigator and also to the information and noise in the image. Of course an ideal image will not require any processing in Photoshop. It should be remembered that most of the actions performed in Photoshop can be done in a color darkroom except much more quickly and easily. It is often tempting to over process the images, and so it is advisable to always save a basic image, and then save subsequent files as versions of the basic image. A useful feature of Photoshop 5 is the ability to undoe more than one operation.

It is always faster and more reliable to work directly from images copied to the hard disk rather than working directly from a storage device or server, provided

that the hard disk is not close to being full. It is also essential to keep track of the available RAM, and not to have too many programs open at the same time as running Photoshop. In general one should multiply the image size by at least three to estimate the amount of RAM needed to process images with Photoshop — this is because the image itself takes up memory and then Photoshop makes copies of the image each time it performs any operation on it. In addition it is a good idea to occasionaly restart the computer during a long session in order to defragment the memory.

Here we describe some of the basic operations that we routinely use on confocal images. We advise you to practice and to play with the program, and make a few test prints before deciding on any one method of manipulating the images.

1. Change the color levels in the resulting merged image using Levels (*Image, Adjust, Levels*) to adjust the red, green and the blue values in different channels of the image. The "Levels" option is extremely useful for adjusting the black and white points of the image. This is the first operation that is usually applied to an image. Here the channels are selected independently and the effects of changing levels can be viewed directly on the screen. Fine tune the brightness and contrast (*Image, Adjust, Brightness/Contrast*) of the image for presentation purposes.

Variations (*Image, Adjust, Variations*) is another useful feature in order to adjust brightness and contrast of an image. This feature displays several different settings together on the screen which enables the user to easily compare between the different effects, and to more easily make a choice.

2. Confocal images often benefit from a sharpening routine. In general "Unsharp Mask" (*Filter, Sharpen, Unsharp Mask*) works better than sharpening of the images (*Filter, Sharpen*), and is usually one of the last operations to be performed on an image (*see* **Note 10**). There are three variables, Amount, Radius and Threshold, to adjust when using the Unsharp Mask filter, and these can be adjusted interactively. Users should experiment with these on their own images. A good place to start with confocal images is a radius value of 2 and a threshold value of 1.
3. Especially useful features allow addition of graphics and compilation of the images into multipanel figures (*see* **Note 11**). Graphics are subsequently more easily edited if they are pasted into a separate layer in the image rather than permanently replacing pixels in the actual image itself. Graphics often appear much sharper when added on a different layer using a second program such as Adobe Illustrator. It is advisable to add the graphics within PhotoShop on a different layer so that changes, reformats for different journals or deletions can be made. In earlier versions of Photoshop this feature was not available so that text was added by actually replacing the pixels in the image. This meant that an unlabelled image had to be stored in addition to each of the labeled ones (*see* **Notes 12** and **13**).
4. Save the images to the hard disc of the computer in Macintosh format by selecting Save As (*File, Save As*). Images are usually saved in the TIFF format,

and LZW-compressed, which uses a lossless compression scheme. We have found that other compression methods such as JPEG compression can introduce artifacts which may be compounded each time a file is resaved. Images are eventually archived to optical disk or CD-ROM (*see* **Note 14**).

4. Notes

1. Files will not transfer if the Fetch preferences are not set correctly. The main symptom is that the file names are replaced by numbers, and the files cannot be opened. Always check the files after transfer before deleting them from the confocal computer or at least before making some form of backup!
2. Using PhotoShop it is a relatively simple task to experiment with various color combinations by rearranging the images into different channels. For example, in the triple-labeled *Drosophila* embryo, the green *hairy* stripes appear light blue in the blue *Kruppel* domain, and in a different color combination using the same images, the red *hairy* stripes appear yellow in the green *Kruppel* domain. This is achieved by rearranging the component grayscale images of the three color RGB image into different channels using Split Channels (*Window, Palettes, Show Channels, Split Channels*) and recombining the images using Merge Channels (*Mode, RGB Color*) or by copying and pasting the images between channels. Using the Specify Channels option the component grayscale images are assigned to different channels of the RGB image. The colors in the final image do not therefore always correspond to the actual colors of the specimen.

The combination of colors within a three-color merged image is important for clearly conveying the biological information collected by the microscope. The true emission colors of two of the most commonly used fluorophores, rhodamine and fluorescein are, conveniently, red and green respectively, and overlapping domains of expression are yellow. Also some of the commonly used nuclear dyes that are excited in the near ultraviolet (UV), such as Hoechst 33342, emit in the blue. These are the colors observed by eye in a conventional epifluorescence microscope equipped with the appropriate filter sets for simultaneous double-label imaging — now available from most of the microscope manufacturers. However, the third channel in a triple-label sample prepared for confocal analysis usually emits in the far red, e.g., Cyanine 5, which is conveniently shown as blue in digital images whereas the real Cyanine 5 emission is often extremely difficult to visualize by eye and not so easily depicted in a digital image. By rearranging the grayscale images, the best combination of colors that conveys the maximum amount of information, and best color balance can be achieved.

3. In addition to displaying the relative distribution of up to three different macromolecules within cells, this method of combining the three images can be used as an alternative to 3D reconstruction for displaying depth information within a specimen. Using the LSCM, a series of images from different focal planes within the specimen is collected into a single file or Z-series. These images

maintain the *X, Y,* and *Z* registration from the specimen, and are the same size and pixel resolution. The simplest method is to extract the three images from such a Z-series, export them as single image files into Photoshop, and then merge them as before, so that the colors red, green, and blue are assigned to structures at different depths within the specimen. Alternatively, to gain a more accurate representation of the Z-series it is advisable to split the images into three groups and merge each group using the confocal software or NIH image, and subsequently combine them into a three color image in Photoshop.

4. In a similar way, single images of three different time points can be extracted from a time-lapse movie sequence file collected using the confocal microscope and three color merged. These files are identical in format to Z-series files except that time has now replaced the *Z*-dimension. Here, color differences are used to summarize changes in the positions of structures over time in a single image.
5. Two-color stereo anaglyphs can be constructed in Photoshop by placing one Z-series projection in the red channel and the second offset image in the green channel for a red/green stereo pair or in the blue channel for a red/blue stereo pair.
6. Single-color images often lack contrast when printed on a dye sublimation printer. The problem can be overcome, somewhat, by producing a single RGB image, as before, and adjusting the relative levels of the three channels so that there is information in all channels for printing (*Image, Adjust, Levels*) or using a color table applied to the grayscale images.
7. Photoshop can also provide a bridge to further manipulating images because the files are compatible with many other programs. For example sequences of confocal images of development have been manipulated using Photoshop, and subsequently transferred to a commercially available morphing program such as Elastic Reality (Avid Technology, Tewksbury, MA), and processed into short animated sequences of development ***(8)***. These sequences can be further edited and compiled using Adobe Premiere or Adobe After Effects (Adobe Systems, Mountain View, CA), and viewed as a digital movie using QuickTime software directly on the computer or exported to VHS video tape for presentation purposes. For more detailed information on the production of digital movies the reader is referred to Chapter 24.
8. The presentation of time-lapse series for publication presents special problems of representing 3D and 4D data on a journal page, although with the popularity of digital publishing and the World Wide Web such video sequences can be accessed more easily.
9. In Photoshop 4 and now 5 the "Actions Palette" can be used to perform the same manipulations on a series of images.
10. Some confocal images may be contaminated with parallel lines from vibrations or a weak laser line in laser scanning systems or from a wobbling disk in the disk scanning systems. These images can be improved by using the "Blur" filter followed by Unsharp Mask.
11. In some instances a Z-series profile or *X–Z* section may appear flattened because the incorrect *Z*-step was selected. A relatively easy way of stretching such an image to the correct proportions can be performed in Photoshop using the image size feature (Image, Image Size). Here the image can be resized and stretched in

the Z direction by clicking on the "do not constrain proportions" box and entering the correct *Z* value while leaving the width the same.

12. The reorganization of panels in a composite image requires the selection of individual panels. An easy way to achieve this is by activating a channel with no image/colour information and to use the magic wand tool with a tolerance of zero. Alternatively, select the white spaces between the panels with the magic wand tool (*tolerance 0*), select Inverse (*Select, Inverse*) and then deselect unwanted panels by holding down the option key while selecting unwanted panels.
13. To align text labeling of panels in a composite image the text is first placed in the top left hand corner of each panel; it is then selected and the text is moved to the appropriate positions simultaneously.
14. It is not usually advisable to store image files on the computer hard disk or on a server because space can be limited on a multiuser confocal instrument and also hard disks are notorious for unpredicted crashes. It is therefore prudent to archive image files as quickly as possible after acquiring them. There are several options for archiving image files, including zip drives, optical drives and CD writers. Ideally copies of the most valued files should be stored in at least two different locations, and preferably with at least one copy in a fireproof safe.

Acknowledgments

The work of G. H. was supported by an EMBO fellowship. We thank Harvey Karten for advice on merging transmitted light with confocal images.

References

1. White N. S. (1995) Visualization systems for multidimensional CLSM images, in *Handbook of Biological Confocal Microscopy*, Plenum Press, New York.
2. Thomas, C., DeVries, P., Hardin, J., and White, J. (1996) Four-dimensional imaging: computer visualization of 3D movements in living specimens. *Science* **273,** 603–607.
3. Mohler, W. A., and White, J. G. (1998) Stereo-4-D reconstruction and animation from living fluorescent specimens. *BioTechniques* **24,** 1006–1012.
4. Schrock, E., du Manoir, S., Veldman, T., Schoell, B., Wienberg, J., Ferguson-Smith, M. A., Ning, Y., Ledbetter, D. H., Bar-Am, I., Soenksen, D., Garini, Y., Ried, T. (1996) Multicolor spectral karyotyping of human chromosomes. *Science* **273,** 494–497.
5. Waggoner, A. S., DeBiasio, R., Conrad, P., Bright, G. R., Ernst, L. A., Ryan, K., Nederlof, M., Taylor, D. L. (1989) Multiple spectral parameter imaging. *Methods in Cell Biol.* **30,** 449–478.
6. Paddock, S. W., Langeland, J. A., DeVries, P. J., and Carroll, S. B. (1993) Three-color immunofluorescence imaging of *Drosophila* embryos by laser scanning confocal microscopy. *BioTechniques* **14,** 42–48.
7. Kiehart, D. P., Montague, R. A., Rickoll, W. L., Thomas, G. H., and Foard, D. (1994) High-resolution microscopic methods for the analysis of cellular movements in *Drosophila* embryos. *Methods Cell Biol.* **44,** 507–532.
8. Paddock, S. W., DeVries, P. J., Buth, E., and Carroll, S. B. (1994) Morphing: a new graphics tool for animating confocal images. *BioTechniques* **16,** 448–452.

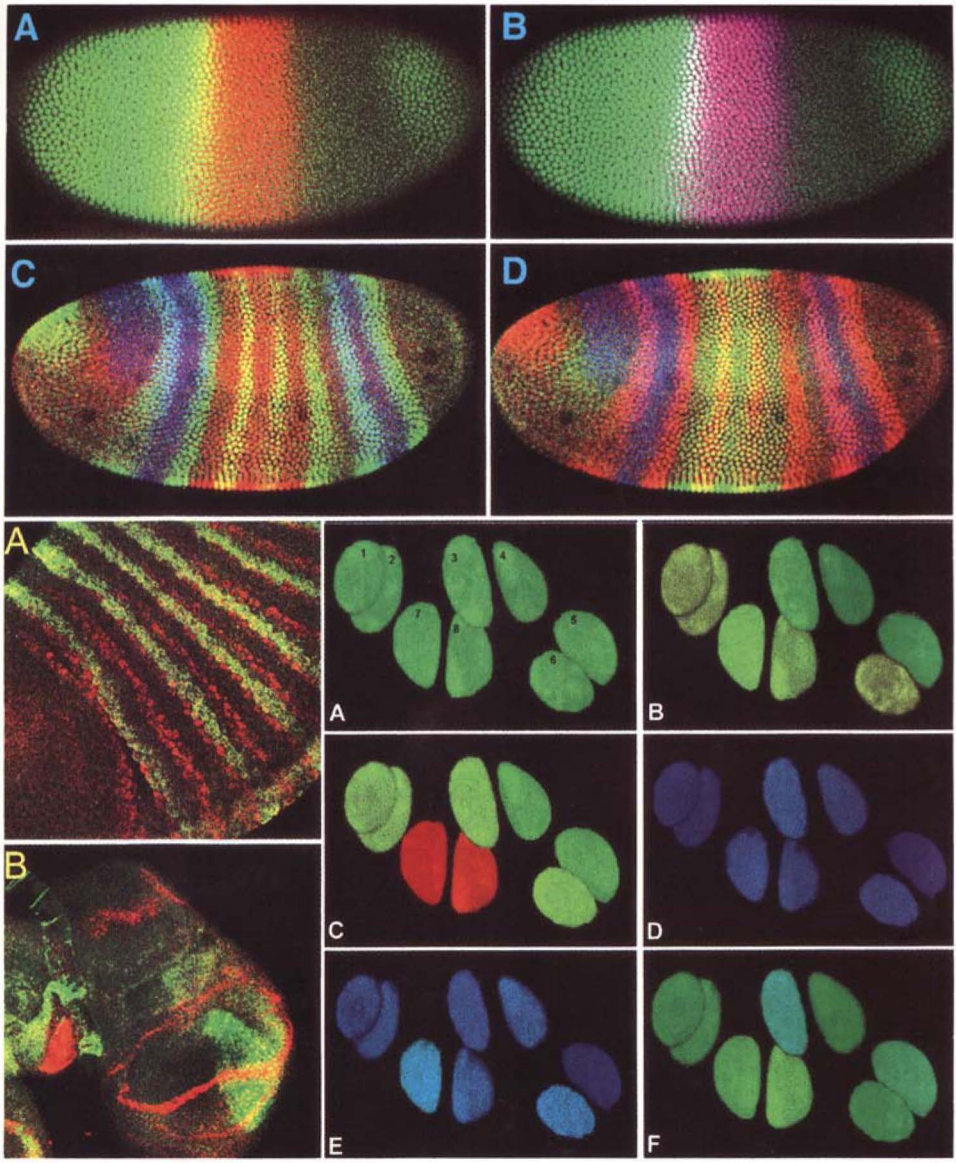

Plate I (*Top Sequence:* **[A-B]** Fig. 21-2 A-B, *see* full caption on p. 379; **[C-D]** Fig. 21-1 B, D, *see* full caption on p. 377 and discussion in Chapter 21. Lower Left Sequence: **[A-B]** Fig. 5-1 A-B, *see* full caption on p. 99 and discussion in Chapter 5. Lower Right Sequence: **[A-F]** Fig. 18-13 A-F, *see* full caption on p. 336 and discussion in Chapter 18).