Adobe Photoshop® 6.0 Distilled: Preparation of Micrographs for Publication

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A picture is said to be worth a thousand words; in the different fields of microscopy pictures are often our data as well. We interpret, analyze, and share images with our colleagues, and so image preparation, enhancement and presentation are crucial for this process. As image acquisition in microscopy moves away from capture on photographic film in favor of digital imaging methods, digital manipulation of visual data for presentation requires learning to use computer software tools. Adobe Photoshop, currently the standard in the community of both artists and scientists, is a powerful program, but it is NOT specifically designed for scientific data, and so contains dozens of features and tools that may and/or must be avoided in the preparation of micrographs for scientific publication. The learning curve for this type of software can be steep, but the steps outlined below may help to get you started.

Photoshop can be used to quickly adjust and prepare images for figures for publication and presentation. Black and white images (e.g., electron micrographs) can be prepared in a relatively straightforward manner with Photoshop. Color images (e.g., fluorescence, confocal) require an understanding of color management, not presented here.

Photoshop 6.0 has new features making labeling and layout easier to do all within the one program:

- Improved layer management
- New adjustment layers
- New vector-based type and shapes
- New Options Bar containing some palettes
- The Crop Tool is now its own tool in the Toolbox
- Better color management
- New color settings – recommend changing the default to Adobe RGB (1998)
- New batch functions for making contact sheets and web galleries
- Bundled with Image Ready, Adobe's web production tool

Set some time aside for "playing" with Photoshop 6; become familiar with the Toolbox, discover that each tool has an Options Bar at the top of the window, learn how to select colors with the squares in the bottom third of the Toolbox, how to revert to the default pure black and white, and how to switch between foreground and background colors. Learn some shortcut key strokes, find how to make palettes visible in the Window ®. Show menu, scroll through the Preferences to see what you can do, and find the improved Edit® Undo, Step Backward and History Palette to cancel all those steps made by your cat walking on the keyboard. To escape from a persistent Crop or Text tool you don't want, click on the big X on the top left of the window, or hit Escape. Most other tools and effects use Undo and/or Control Z.

Remember to always save your original images in .tiff or . tif **format and archive them**. Never work on your original files; only work on copies. Work in the native Photoshop format, .PSD for faithfulness and flexibility, then save a copy in TIFF, JPEG or

GIF formats for other applications.

Following are instructions for performing some of the more useful Photoshop actions. It is best to read these sections with Photoshop open on your computer, and to use them on an image to see their results.

Part 1: Image Adjustment

1. Start up Adobe Photoshop 6.0

2. Select **File ® Acquire** to obtain an image with a scanner or other plug-in, *or*

3. Select **File ® Open**, then navigate to and select the filename for the image you want.

4. **Rotate** the image if you wish by selecting **Image ® Rotate Canvas** and then choosing one of the options.

5. **Crop** the image if necessary
♦ Select the **Crop Tool**. While holding down the button, drag the mouse to outline the part of the image you want to keep. If you can't control the size or shape of the selection, escape the Crop tool and check that there are no entries in the Width, Height or Resolution boxes on the options bar. You may resize the crop outline (defined by the "marching ants") by dragging any of the handles (small squares) with the mouse, and you can rotate the crop rectangle by placing the mouse outside the marching ants until the cursor becomes a curved arrow, then dragging the mouse to rotate the selection.

To crop the image, you may hit **Enter**, double-click inside the crop outline, click on the **Check mark** on the top left of the Photoshop window, or go to **Image ® Crop.**

6. Adjust Image size and/or resolution
• Select **Image ® Image Size...** to see the dimensions and resolution of the image.
Pixel Dimensions, Width and **Height** of the image are given in the upper half of the dialog box.
• **Document Size** reflects the current **Width, Height and Resolution** of your image, and these are what you may change. Images obtained by various digital cameras may show a Resolution of about 72 dpi, since Photoshop opens some unknowns at that resolution. The Width and Height may be correspondingly large, but the total image size (**Pixel Dimensions**) will reflect the true image pixel dimensions.
• To see what this translates to in any given resolution, make sure **Constrain Proportions** is checked but **Resample Image** is **not** checked, then enter the desired resolution. The Width and Height will change so that the total Pixel Dimensions remains the same (desirable). You may check the **Resample Image** box to change the dimensions and resolution, but remember that resampling upwards to make a bigger image involves inventing pixels in your image and does not reflect true image data, which is undesirable! Resampling downward is not quite as critical, but involves throwing away data, also undesirable.
• Click OK when finished.

7. Adjust image tones with Levels or Curves
• With the new Adjustment Layers you can make any adjustment in its own layer for non-permanent adjustments. Generally, **Levels** is easier to use than **Curves.**
• Go to **Layer® New Adjustment Layer® Levels** and give this layer a name that helps to remind you what it is (or use the default). The other default options should be fine for now. Click on OK.
• You will see a new layer with two icons on the **Layers P alette** and also a window with the histogram of the pixel values of the image. Under the histogram itself, the left and right arrows can be moved to map your darkest and lightest pixels to black and white, and the middle arrow can be moved to change the gamma (midtones) value. A good way to begin to adjust levels is to drag the **left arrow** to the right until it is under the leftmost pixel mapped on the histogram, then slide the **right arrow** to the left until it is under the rightmost pixel in the histogram. Now slide the **middle arrow** until it is under the main peak of the histogram, typically to the left. Now you can move these arrows to give the most pleasing image. Notice the changes in values displayed in the **Input Levels** boxes above the histogram. For most printing methods an image that is a little light and flat on the monitor will print better, but this is not always the case. You may have to try a number of times to get the correct values for your output device. Click OK to continue.
• An alternative to Levels is **Curves.** Select **Layer® New Adjustment Layer® Curves** to get the New Layer dialog box. Name the layer and click OK. This is a brightness graph where you can map the brightness values in the original image to new brightness values. The horizontal axis of the graphs represents input levels. The vertical axis represents the output levels. The grayscale bar on the bottom by default shows the values ranging from white on the bottom and left to black on the top and right. To change this to the same direction as the Levels dialog box, click on the small double arrows in this bar, and the values will now reverse. With the mouse you may drag any part of the brightness curve around. When you release the mouse, a point will stay on the curve to anchor it. Then you may select other points on the curve and move them as well. The eyedropper tool can be used on the image to select specific tonal values on the curve, and these values can be manipulated. With experience, this can be a valuable tool. However, it may also lead to some very strange effects!
• You may use **Layer® New Adjustment Layer** to make additional adjustment layers, then you can compare them and keep only the layer(s) you wish and throw the others away. These **Adjustment Layers** are not permanent until you flatten the image.

8. **Sharpen** the image, if necessary
• There is currently some debate about whether using **any** Photoshop **Filter**, including those for sharpening, is ethical in presenting scientific data. Please consult your boss/editor/own ethical barometer before performing this manipulation!
• View the image in about the size it will be printed with **View® Print Size** (this may not be exact, depending on your monitor settings) or a bit more. The best sharpening is accomplished with the **Unsharp Mask** filter, named after a traditional film technique that highlights the edges in a picture.
• Make sure you have selected the **Background** image in Layers Palette, and not one of the adjustment layers. Select **Filter® Sharpen® Unsharp Mask.** You will get a dialog box which shows a small portion of your image plus some sliders. You may select a specific part of your image for the view box by clicking on that area with the mouse. Check the **Preview** box to see changes on your entire image while you are working.
• The **Amount** slider specifies the amount of contrast Photoshop will add at the edges ("edges" are defined by contrast) in the image. Values of up to 50% will produce subtle changes. Values of more than 100% (depending on the size of the image) will generally change too many pixels in your data and will begin to show odd effects.
• The **Radius** slider sets the thickness of the edge. Different output methods will require different values.

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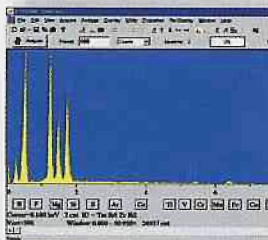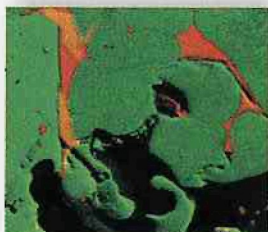*Continued from preceding page*

- For low resolution images as for the Web, or with most images saved with JPEG compression, try 0.5 pixels.
- For inkjet printing, do the best you can onscreen for glossy, but a bit less sharpening for matte paper.
- For many kinds of press, try 1.0 pixels.
- For high resolution output (*e.g.*, slides), try up to 2.0 pixels.
- A simple formula is to consider a 0.1 Radius for every 15 ppi of output resolution. To calculate, divide the intended resolution by 150 to get the Radius value.
- Generally, anything over about 2.5 pixels is considered bad taste!
- The **Threshold** slider is used to assign the amount of contrast there must be between two pixels in the image to be defined as an edge. Try values of 1 to 10 levels.
- When satisfied, or after trying several Adjustment Layers, select **File**® **Save As...** and save the adjusted image with a **new** name.

Part 2: Page Layout, Labeling, and Magnification Bars

Preparation of figure plates for publication is best done with software designed for that purpose, such as QuarkExpress, Adobe Pagemaker, or perhaps Microsoft PowerPoint. However, if you have only Photoshop in your repertoire, Photoshop 6.0 has some new changes to make these tasks easier. There are several ways to tackle the job, but this is what I use:

1. Select **File**® **New** and fill in the dialog box:
- **Name** the new plate.
- Enter the desired final page **Width** and **Height** in appropriate units (pixels, cm or inches). Follow the guides in the Instructions for Authors for the journal to which you are submitting the figures.
- Enter the desired final **Resolution**. Enter **Mode – Grayscale** for black and white, or **RGB** for color.
- Select **White** under **Contents** to simulate a white sheet of paper, then click OK.
- Go to **Edit**® **Preferences**® **Units & Rulers** to select the default units you want for the rulers around the image. **Rulers** can be turned on and off from the **View** menu or with **Ctrl+R** (PC) or **Alt+R** (Mac).

2. **File**® **Open** the images you wish to combine for the figure plate. Check **Image**® **Image Size...** for each image, make sure all your images will be of the same resolution and appropriate sizes. Be aware that images of differing resolutions will go into the new plate as different sizes unless their sizes and resolutions are adjusted.

3. Set up **Grids** and **Guides**
- You may work with **Grids** or **Guides** or both. A **Grid** is fixed; you determine the placement of **Guides**.
- Go to **View**® **Show**® **Grid** to overlay a **Grid** onto your plate. The colors and grid dimensions can be changed in **Edit**® **Preferences**® **Guides & Grid**.
- To use **Guides**, make sure the **Rulers** are on, then depress the mouse button in the ruler area (vertical or horizontal, depending on where you want your guide) and drag from the ruler line to the position you wish on the grid (if used) or on the plate. When you release the mouse, the guide will stay there. You may reposition a guide by dragging it with the mouse. Make a left vertical guide and a top horizontal guide to define the corner where you want your first image.
- Click on the image you want to place on the top left of the plate. Select the **Move Tool** from the Toolbox, then drag the image to the plate and let go of the mouse. The image should have dropped onto the plate and a new Layer should have appeared in the **Layers Palette**.
- With the **Move Tool** you may drag the image into position. Go to **View**® **Snap**. If Snap is **on**, the image will "snap" to the guideline when it gets close enough. If it is **off** (or on), you can reposition the image in pixel increments with the **arrow buttons** on your keyboard. You may need to zoom in to a higher magnifi-

cation (magnifying glass **Zoom Tool** in the Toolbox or **View**® **Zoom In**) to see how well your images line up.

• Now drag Guides from the Rulers to the other two edges of the first image. Drag another set of guides to the desired distance from the guidelines on the edges of the image, *e.g.*, 2 mm. These are the guidelines for your next image to the right and for the one below.

• Continue adding and lining up images, then dragging and positioning additional Guides to define your figure plate. You may temporarily turn off the Grid and/or Guides as you go to see how the plate looks by clicking (unchecking) in **View**® **Show**® **Grid** or **Guides**; click again to turn back on.

• To reposition any of the images, remember to use the **Layers Palette** to see which Layer you are manipulating. Select the **Layer** you want to work on by clicking on that Layer in the **Layers Palette**.

• Zoom in several steps to check your alignment.

• How to **cheat** the gap if your images are not cropped exactly right: Line up your images nicely, closer together than you really want. Make a new Layer by clicking on the bent piece of paper on the bottom of the Layers palette or by **Layer**® **New**® **Layer…** . Make sure it is at the top of the Layers palette; if not, drag it there. Choose the rectangular marquee selection tool on the top left of the Toolbox. On the options bar set Feather at 0, Style at Fixed Size, and the Width and Height of the white space you want to create. Now click on your plate to get the marching ants rectangular bar that you have defined, and drag it to the position you wish between images. You may use the keyboard arrow keys to make small adjustments. Now go to **Edit**® **Fill…** . For Contents use White, for Blending Mode select Normal, and make Opacity 100%. Click OK. This should make you a perfect white band. Use **Select**® **Deselect** to turn off the marching ants to see how it looks. You can do this for vertical and horizontal white bars. If you make each new bar as a new layer, you have complete control over their placements.

• Be sure to **Save** this plate before you continue.

4.   **Labeling with the Type Tool**

• To apply text to an image, click on the **Type Tool (T)** in the Toolbox. Your cursor will become a vertical I-beam. Make sure you are on or above the layer in the Layers Palette containing the image you want to label, then click on the image. When you do so, the cursor becomes a blinking vertical line which will be the height of the selected font, and a new layer containing the type will appear in the Layers Palette. The font, style, size, color and other features may be selected on the options bar at the top of the Photoshop window. Type the label. As long as this step is still active (I-beam or Move Tool icon present), you may edit the type by selecting it with the I beam and you may reposition the type with the move tool icon. To get the type to "stick", click on the check mark on the upper right of the Photoshop window. The color of the text, lines and arrows will be initially determined by the colors in the squares near the bottom of the Toolbox, but they can be changed.

• This text is completely editable, and sizes, fonts, colors and other attributes can be changed even within a word. To edit, select the text layer or text you want to change with the mouse, and make the changes. For fine movements, use the arrow keys on the keyboard; for larger movements, use the Move Tool.

• Start each label as a new layer on or above the desired layer as you work. If you have several labels in one layer and you try to move one, they will all move together. It is best to have each label in its own layer.

• As always, save in the **.PSD** format as you work so that you

can edit the text later.

• **Arrows** can be made with the **Line Tool.** This tool is bundled with other Shapes tools in the Toolbox with the button below the Type Tool. When you click on the Shapes Tool the bundled tools appear in the Options Bar. Select the Line Tool then click on the down arrow (next to the starburst-looking icon) to get the Arrowheads options. The options are Start, End, Width, Length and Concavity. Then select a pixel Weight on the options bar. Experiment with these to get different types of arrows. Make a New Layer for each arrow or shape to be able to manipulate then individually.

• Using the new **Drop Shadow** tool in Photoshop is an easy way to make labels that will show up well on micrographs. Select the Text layer or Shapes layer you want, then select **Layer**® **Layer Style**® **Drop Shadow…** . This will open a dialog box containing a variety of choices. You may experiment to find the best choices for your shadows. I generally begin with a **Blend Mode** of Normal, a contrasting color (generally black or white), and an **Opacity** of 100%. The **Angle** wheel can be dragged around with the mouse, or an angle can by typed into the box. You may check Use Global Light if you want *all* drop shadows on your entire image to be at the same angle, although this sometimes makes arrows look odd, so you may deselect Global for just the arrows. The slider for **Distance** determines how far away from the text or arrow the shadow falls, the slider for **Spread** dilates the boundaries of the shadow shape before blurring, and the slider for **Size** specifies the amount of blur coupled with the size of the shadow. The shadow will be crisper if the Spread and Size are 0, but try experimenting with a Size of one or two pixels and an increasing Spread. Try Contour as a straight line, Noise at 0 and check Anti-aliased. You may save these settings for future use by clicking on the **New Style** button in the options box, giving it a name and checking both the boxes. An icon will be created in the **Styles** menu, which can be accessed by clicking on Styles in the upper right portion of the Layer Style dialog box.

• **Save As…** a .PSD file with a different name, just to be safe.

5.   **Magnification Bars**
       Scale bars can be calculated and created a number of different ways, and there have been several methods discussed on the Microscopy Listserver over the past few years. Check http://www.msa.microscopy.com/MicroscopyListserver/MLInstructions.html if you wish to subscribe, and to see how to search the archives. Recently, John Russ has posted several freeware Photoshop plugins, including one for making scale bars. Remember to maintain a way to determine the magnification of each image throughout the manipulations!

• One way to produce a scale bar is to again use the **Line Tool**. Remember to turn off the Arrowheads. Create a new layer for each bar for the most flexibility. Select an appropriate Weight on the options bar. Use **Window** ® **Show Info** and hold down the mouse to choose the length in pixels (D) and angle (A) 0.00_ or 90.00_ for horizontal or vertical lines, respectively. Use the Move tool to position the bar exactly.

• If you need the same size scale bar for more images, you may make a copy of this layer for each one by dragging the selected layer in the Layer Palette to the symbol for a New Layer at the bottom of the palette, then dragging the duplicated scale bar to another image.

• Use the Text tool to label the scale bar. You may find the symbol for micrometer in the Symbols font menu.

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Using Fluorochromes To Label An Oil-In-Water Emulsion

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A question was asked on the microscopy listserver about fluorescent probes for studying oil-in-water emulsions trapped in a cellulose membrane. I don't have experience with that exact problem (I have studied chloroform/water emulsions rather than emulsions of triglycerides or alkanes), but I can make some general suggestions. The best type of hydrophobic probe to use depends on whether the probe is to be added to the hydrophobic phase before emulsification or after. If it is to be added to the preexisting emulsion as a dimethylsulfoxide stock solution, a probe with some aqueous solubility is required. Nile red and most small hydrophobic dyes have enough aqueous solubility to distribute through the water phase to the oil droplets. If this is to be done with a sample which contains a nitrocellulose filter membrane however, I expect there may be a problem with the probe transferring to the membrane and binding to hydrophobic sites there as well, adding to the background signal on the filter. Nile red has an advantage in that molecules in a polar environment do not fluoresce; the disadvantage is that it photobleaches rapidly. The hydrophobic pyrromethene dyes (such as BODIPY® 493/503 a.k.a. pyrromethene 546, BODIPY 505/515, and BODIPY 665/676) are much brighter and much more photostable, and are worth the expense in my experience. The pyrromethene dyes are said to be more specific than Nile Red, I presume because they are more hydrophobic. I haven't tried octadecylrhodamine B, DiI, etc.

If background on the membrane continues to be a problem, I would suggest a very hydrophobic probe such as a pyrromethene-labeled cholesterol ester or some other long alkylated or acylated nonpolar fluorophore. A Bodipy-labeled fatty acid, being polar, would not be the best choice. This would have to be added to the oil before emulsification. As the Molecular Probes catalog points out for labeled cholesterol esters, such probes are basically nontransferable through water, and so shouldn't bind to the membrane unless the labeled emulsion wets it. But for the same reason, they will not label oil droplets uniformly if they are added as a dimethylsulfoxide stock solution to a preexisting emulsion.

If one wants to get fancy, one can ry using a rhodamine- or pyrromethene-labeled phospholipid to label the aqueous/organic interface (again, the probe should be added before emulsification in order to get uniform labeling). This makes beautiful pictures, especially with an oil phase counterstain of a contrasting color like the far-red pyrromethene Bodipy 665/676. Phosphatidylcholine or ethanolamine derivatives usually aren't soluble in the oil phase; I haven't tried DiI etc., and don't know whether the latter would also label the oil phase. Also, for striking contrast between the phases, the aqueous phase can be specifically labeled with hydrophilic probes such as dextran conjugates or (much better, in my experience) pyrromethene 556 (BODIPY 492/515 disulfonate).

One concern, which may be minor for many users, is that the pyrromethene dyes themselves label the oil-water interface somewhat more brightly than the bulk oil phase. Apparently, the more nonpolar the oil phase (e.g., hexane), the more this effect is seen. To avoid the problem in such cases, the polarity of the probe should match that of the hydrophobic phase. Diphenylhexatriene is one possibility, although care must be taken to minimize photobleaching. Walter Niles has also suggested benzene (requiring 200 nm excitation) and decenene. Still, in many cases the pyrromethene probes will be quite suitable as a visual marker for the hydrophobic phase. ∎

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• **Save** in the .PSD format to be able to edit or remove your scale bar, arrows, or text later.

6. Finish up

You should now have a beautiful figure plate! Make sure it is saved in the .PSD format, then you may save it as well in a TIFF format to send to the publisher. Photoshop 6.0 will automatically rasterize the text and shapes when selecting the TIFF format, but will maintain the layers. It will also automatically flatten the image (compressing all your layers into the background) for formats other than .PSD or TIFF, so those layers may not be independently edited again. If you need to make any changes to any of the individual images, shapes or text you will be able to open the .PSD version again to easily edit.

These steps in Photoshop are generally considered to be acceptable for the adjustment of micrographs for scientific presentation. Additional manipulations, such as Dodge and Burn may perhaps be used with restraint, but the use of Filters, the Cloning Tool and many other options may range from inappropriate to downright fraudulent. Remember, each of the pixels in an image contributes to your data! ∎