

Laboratory for Advanced Electron and Light Optical Methods

College of Veterinary Medicine
1060 William Moore Drive
North Carolina State University
Raleigh, NC 27607
Tel: 919-513-6202
Fax: 919-513-6464
Email: Michael_Dykstra@ncsu.edu
www.cvm.ncsu.edu.research/laelom

Digital Darkroom Procedures Using Photoshop CS **(modified and expanded from R.M. Dillaman and D.M. Gay. 2004.** **Electron Microscopy Lab Manual. UNC-Wilmington)** **M.J. Dykstra 9/30/08**

Photoshop® is widely used to work with photographic images. In the popular market, images are enhanced, doctored, and changed drastically for dramatic effect. Since so many people are conversant with using the techniques available in Photoshop with their personal or artistic digital imaging efforts, they sometimes forget that the arena of scientific imaging has different rules.

It is generally accepted that the only modifications allowed for scientific images are cropping (clipping off unwanted areas of the images), adjustments of brightness and contrast, and the adjustment of gamma (the breadth of gray scales exhibited in an image). Any of these alterations of the original image should be applied **globally** to images. What is **not** allowed are **focal or localized** changes, such as removal of “dirt” in an image, insertion of pixels from one area of an image into another area of an image, and the use of filters to sharpen or otherwise change pixels in an image.

In addition, when producing images for publication, it is wise to check with the journal for their requirements first. In general, journals prefer images at 300 dpi when printed and often prefer to have image annotations (scale bars, figure numbers, letters identifying structures within the images) left as separate layers associated with the images, rather than flattened, which makes the annotations impossible to remove from the image.

On the following two pages are contemporary examples of faults in illustrations from two different journals. The first illustration is from the international journal, *Medical Mycology*. Examination of the scanning electron micrographs at the top of the page show them to be highly pixellated. Since the original images produced by modern SEMs are digital images to begin with, it is unusual to see such a pixellated image. The presumption is that they were taken into Photoshop and somehow made into the substandard pixellated images you see. In addition, the TEM images at the bottom of the page show pixellation in the image, including the numbers on the figures. Again, this tends to implicate improper use of Photoshop, resulting in degraded images.

The second set of images comes from a 2008 issue of the journal *Toxicologic Pathology*. You will note that Figure 11 has a very low-contrast, washed-out appearance compared to Figure 12. This has resulted either from improperly printed negatives, producing low-contrast images in Figure 11, or a mistake when using Photoshop. As you will see in the Photoshop instructions below, adjustments in contrast, brightness, and/or gamma would probably have allowed the authors to make Figure 11 have the same contrast and brightness as seen in Figure 12. This would have produced better images, and an over-all suggestion of professionalism.

Remember, when people are looking at the images you publish, they will not be likely to “forgive” their poor quality. No matter what quality of scientific information underlies the illustrations you use, people are going to judge the quality of your work on the images provided, at least to some extent. With luck and good execution, the Photoshop instructions below will allow you to produce great images for presentation or publication that will sway your audiences to feel that you are doing the best work out there. If they pay attention because of your excellent illustrations, you have a much better chance of selling them on your scientific ideas.

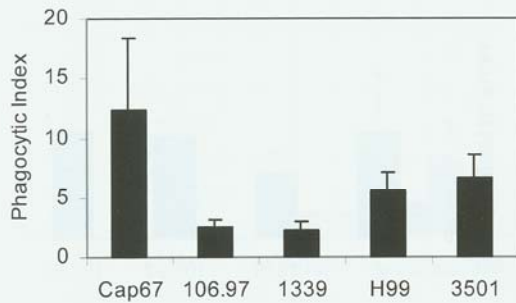


Fig. 6 Phagocytosis of cap67 cells incubated with supernatants from various strains of *Cryptococcus neoformans* by *Acanthamoeba castellanii*. For each experimental condition the number of repetitions was five. Error bars represent 1 standard deviation.

cytic effect does not appear to involve a difference in capsule growth after co-incubation with amoeba since strains from each variety were shown to manifest an increase in capsule size after exposure to amoebae. Although the mechanism for this effect is uncertain, the experiment with cap67 cells suggests that it is related to structural differences in the capsular polysaccharide of variety *gattii*. In this regard, significantly fewer phagocytic events were observed with cap67 coated with polysaccharide of variety *gattii* as compared to cap67 coated with polysaccharide from varieties *neoformans* and *grubii*. The capsular glucuronoxylomannan of *C. neoformans* var. *gattii* is more highly substituted than those of varieties *grubii* or *neoformans*, a structural difference that may translate into reduced affinity for amoeba phagocytic receptors [33].

The lower phagocytic index for *C. neoformans* var. *gattii* suggests that phagocytosis of variety *gattii* by *A. castellanii* is not essential for fungal growth or death of the amoeba, since no significant difference was seen in the percentage of dead amoebae after incubation with any of the three varieties of *C. neoformans*. Transmis-

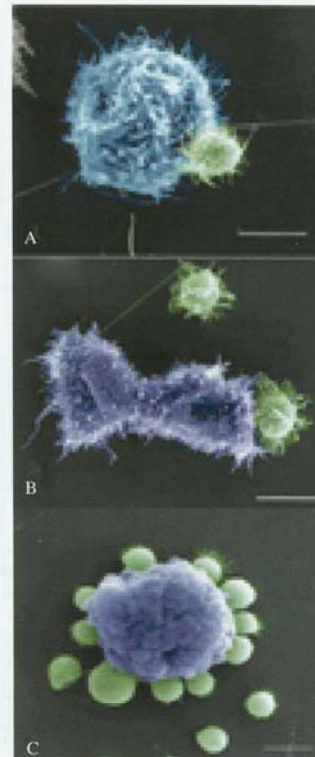


Fig. 8 Scanning electron micrographs of *Cryptococcus neoformans* var. *gattii* interacting with *Acanthamoeba castellanii* after 24 h incubation. (A) Strain I6: *A. castellanii* beginning to envelope *C. neoformans* var. *gattii*. (B) Strain 107.97: pseudopods of an amoeba beginning to encircle *C. neoformans* var. *gattii*. (C) Strain 107.97: multiple *C. neoformans* var. *gattii* adhering to the amoeba cell wall. Scale bars represent 10 μ m.

sion and scanning electron micrographs showed *C. neoformans* var. *gattii* adhering to amoeba cells, suggesting that attachment of fungal cells to the

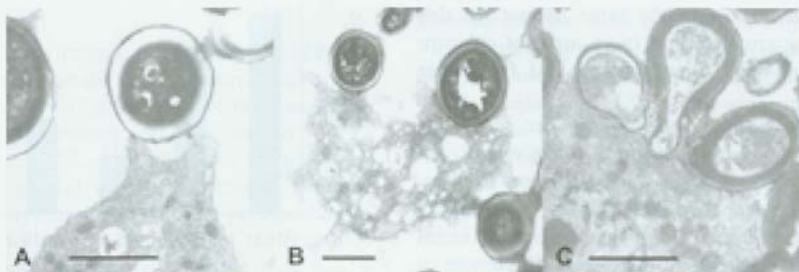


Fig. 7 Transmission electron micrographs of *Cryptococcus neoformans* var. *gattii* interacting with *Acanthamoeba castellanii*. (A) Strain 107.97: pseudopods beginning to engulf fungal cell. (B) Strain 107.97: three fungal cells adhering to one amoeba. (C) Strain I6: five fungal cells adhering to one amoeba. Scale bars represent 5 μ m

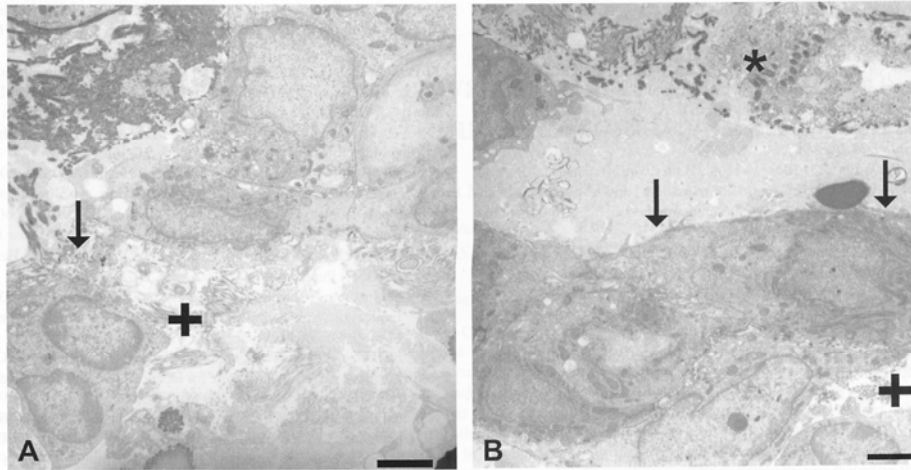


FIGURE 11.—Changes beneath the epithelium of the trachea in the exposure pattern experiment (Experiment 2): ultrastructural evidence of damage extending beneath the respiratory epithelium of the trachea in rats inhaling a TWA of 365 ppm diacetyl administered in four pulse exposures over six hours. (A) Denuding of basement membrane (arrow) and edema of the lamina propria (+). Bar = 2 μ m. (B) The normal respiratory epithelium of the trachea has been replaced by an attenuated, simple epithelium (arrows), suggesting migration and spreading of epithelial cells to cover epithelial defects. The subjacent lamina propria is edematous (+). A fibrinonecrotic membrane (*) is above the attenuated epithelium. Bar = 2 μ m.

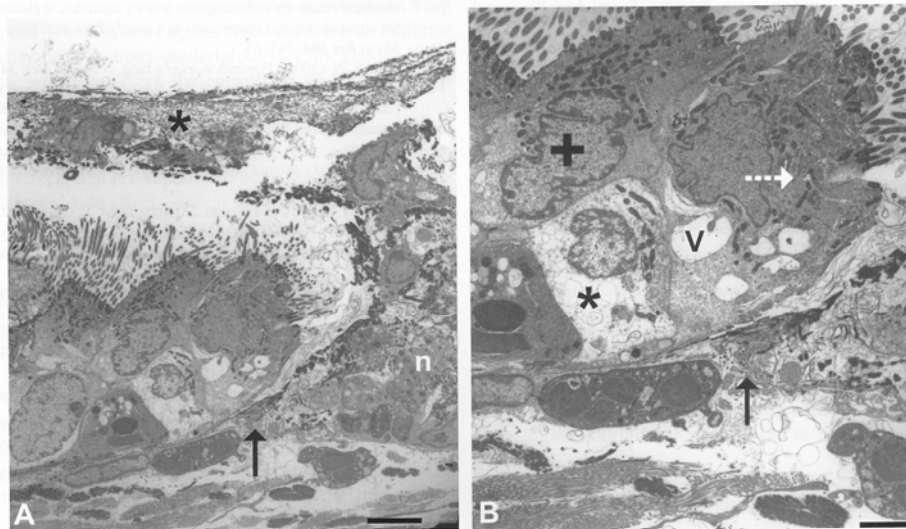
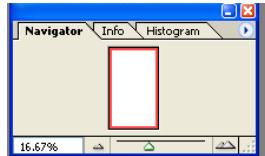


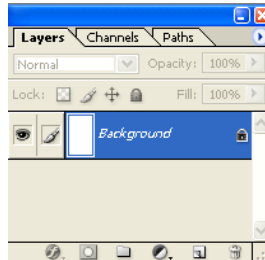
FIGURE 12.—Changes in a bronchus in the exposure pattern experiment (Experiment 2): ultrastructural changes in the right main-stem bronchus of a rat after inhaling 356 ppm diacetyl as a continuous exposure for six hours. (A) Ultrastructural changes in the bronchus include a fibrinonecrotic membrane (*), epithelial necrosis (n), and rupture of basement membrane (arrow) with edema and inflammation of the subjacent lamina propria (bar = 5 μ m). (B) A higher magnification of the ruptured basement membrane (black arrow) and degenerative changes in epithelial cells, including vacuolation (v), internalization of cilia (dashed white arrow), cytoplasmic rarefaction (*), and condensation of chromatin beneath the nuclear membrane (+). Bar = 2 μ m.

Using Photoshop to Process Images

1. Open Photoshop
2. Make sure the following pallets are on the work space:
 - a. Navigator (WINDOW>NAVIGATOR)



- b. Layers (WINDOW>LAYERS)



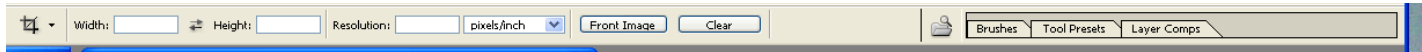
- c. Characters (WINDOW>CHARACTER)




- d. Tools (WINDOW>TOOLS)








e. Options (WINDOW>OPTIONS)



3. Check system defaults and preferences: Go to [EDIT>PREFERENCES>UNITS & RULERS] and set units to inches
4. Load an image into Photoshop: Select [FILE>OPEN] and browse for file
5. To invert image from a negative to a positive image: Select [IMAGE>ADJUSTMENT>INVERT]
6. Crop (delete) unwanted region from the image:
 - a. Select the crop tool  from the tool menu
 - b. Size the crop tool to include the region of interest
 - c. Select [IMAGE>CROP] or double click on image
7. Adjusting gray levels (gamma):
 - a. Select [IMAGE>ADJUSTMENT>LEVELS] or “ctrl + L”
 - b. Click “Preview” box ON.
 - c. Do a histogram stretch by sliding the black triangle to the right until it just meets with the first pixels of the histogram. Then slide the white triangle to the left until it just meets with the first pixels of the histogram. Click “OK”
 - d. Return to [IMAGE>ADJUSTMENT>LEVELS], click on center triangle and drag to the left to reach a gamma value of about 1.20. Click “OK”
 - e. Repeat gamma adjustment if necessary
8. Adjusting levels (brightness/contrast): **Use with caution**
 - a. Select [IMAGE>ADJUSTMENT>BRIGHTNESS/CONTRAST]
 - b. Click “Preview” box ON.
 - c. Slide the triangle below each line to the left (-) or right (+) to make brightness and contrast adjustments. Click “OK”
9. Save the image:
 - a. Select [FILE>SAVE AS]
 - b. In the “Save As” window, select folder to which you wish to save image
 - c. Rename file (negative number and initials)
 - d. Select TIFF format and save
 - e. Choose TIFF option, with no compression applied

Using Photoshop to Build Photographic Plates

1. Start Photoshop and open images to be used in plate
2. Make a copy of one of your images by right clicking on the top blue bar of the image and choosing “duplicate”

3. Adjust levels if needed (gamma, brightness/contrast)
4. Convert image to 8bits/channel: Select [IMAGE>MODE>8BITS/CHANNEL]
5. Make labels for your images:
 - a. Open the layer pallet: Select [WINDOW>LAYERS] if not open. This shows which layer is active by highlighting it with the color blue
 - b. Select label color in the color square  of the tool box. Click the curved arrow to switch from black to white or white to black.
 - c. Click on type tool  and click cursor where you want the type to appear in the image.
 - d. Photoshop automatically creates a layer for text when the window appears
 1. Select font
 2. Select size (always use “points”)
 3. Set anti aliasing  to “sharp”
 4. Type your label in the text box
 5. Click “OK”
 6. To move the location of the text:
 1. Click on the move tool 
 2. Click anywhere on the text and drag to a new location (the text layer has to be highlighted to move)
6. Making arrows and scale bars for your micrographs:
 - a. Select the line tool  in the toolbar
 - b. Go to tool presets and click on Black Arrow
 - c. Use pull down menu to turn on/off arrowhead option
 - d. Click on move tool to move the image in the direction you want it to appear. It can be moved the same as a text layer with the move tool
7. After labeling and scaling your image, save the image as a psd file. This is a photoshop document file that preserves the layer information of the image
8. Adjusting image size and resolution:
 - a. Open a psd image file and flatten the image by selecting [LAYER>FLATTEN IMAGE]. This combines all of the layers into one, decreasing file space
 - b. Decide the image size you need to each image to be included in the plate

- c. Select the crop tool from the toolbar
 - d. In the Crop Option palette, type the determined width and height and set the resolution of 300 pixels
 - e. Size the crop tool to include the region of interest
 - f. Select [IMAG>CROP]
 - g. Do this for each image to be used in plate
10. Create a new canvas for the plate:
- a. Select [FILE>NEW]
 - b. Set width to 8.5" and height to 11"
 - c. Set "Resolution" to 300 pixels and Mode to grayscale
 - d. Type name for the plate
 - e. In contents, select white (for background) and click "OK"
11. To move your image, select the move tool, click on the image, and drag it to the new canvas
- a. To make positioning easier, you can:
 - 1. Select [VIEW>RULERS] and [VIEW>SNAP TO>GUIDES]
 - 2. To create guides, click anywhere on the ruler and drag into the image area
 - 3. Position image using the move tool
12. To create a plate, insert your images into the new canvas (courtesy of Meghan Samberg):
- a. Bring up a new canvas
 - b. Add a layer for the image [LAYER>NEW]
 - c. Click "OK"
 - d. Select first image [SELECT>ALL] (Ctrl+A)
 - e. Copy image [EDIT>COPY] (Ctrl+C)
 - f. Bring up canvas and select Layer 1
 - g. Paste image [EDIT>PASTE] (Ctrl+V)
 - h. Repeat process for each additional image. Make sure to place each image in a new layer
13. When images are assembled and labeled, you can add figure legends below the figures. After you have completed them, flatten the layers and save the file

To Print, Using the Epson Stylus Photo 960 Printer:

- 1. Put image on LAELOM server:
 - a. From computer you are using, make sure you are logged onto the Novell server
 - b. Click on "My Computer"
 - c. Click on "Data on 'cvm 03fs' (G:)"
 - d. Click on "Shared" Folder
 - e. Click on "PHP" Folder
 - f. Click on "laelom" Folder

- g. Click on “EMCLASS2008” Folder
 - h. Make a new folder with your name on it
 - i. Save your image to your folder
2. Turn on Epson Stylus Photo 960 Printer
 3. Open image file from your folder in Photoshop
 4. Select [FILE>PRINT]
 5. In PRINT menu, select “Properties” to designate printer parameters
 6. In PROPERTIES menu:
 - a. Select “Media Type” (Glossy Photo paper)
 - b. Select “Black” ink
 - c. Select “Custom” box
 - d. Select “Advanced”
 - e. Set “Print Quality” to 720 dpi
 - f. Check “MicroWeave”, “High Speed”, and “Edge Smoothing”
 - g. Select “OK”

Instructions for Capturing Images From Microtek ScanMaker 8700

1. Turn on the computer and the ScanMaker 8700.
2. Open PhotoShop CS3 program.
3. Go to **File, Import, Microtek ScanWizard 5**
4. Place negatives shiny side up (emulsion side down) on the glass tray and gently slide the tray into the scanner. This will scan the negative in reversed from the normal reading side, but should reduce the probability of producing Moiré patterns from the smooth plastic side of the film interacting with the smooth glass carrier.
5. Click on **Preview**.
6. Click on **Original** and then **Film** and **Negative**.
7. Click on **Scan Type** and then **Gray**.
8. Click on **Purpose** and then **Custom** and **300 DPI**.
9. Click on the gray background of the Microtek screen.
10. Use the mouse to bring the dashed border of the **Preview Image Frame** to surround a single negative.
11. Click on **Scan** button. Make sure the bottom of the screen shows 300 DPI.
12. Move the **Frame** to the next negative and scan it, etc. until all of the negatives have been scanned.

13. Follow **PhotoShop** instructions provided to adjust levels of the images, to crop them, and to assemble them into plates of figures.

To print pictures, go to **My Computer** and select **Drive G**. Next, open **Shared** folder, then **PHP** folder, then **laelom** folder. Save your images to the class folder (**EMCLASS2008**). This is the folder where all your images should be saved. I also recommend that you save them to a personal thumb drive (stick) to make sure that they do not get lost.

Next, turn on the SEM computer and the Epson printer sitting on the console. You can then open your files on the G drive and print them. When you set up the printer, select **Black** (do not print in color unless they are light microscope images). **Print one image on regular copy paper first to make sure the jets are clean.** If the jets are clean, print the rest of your images on the photo paper on the shelf behind the SEM. Load it shiny side up in the printer and go to Properties and select **Photo Paper** and make sure you have selected **Black**.

Adobe® Photoshop® with Adobe ImageReady®

Version 7 for Windows®
QUICK REFERENCE CARD

(M) Rectangular Marquee
(L) Lasso
(C) Crop†
(J) Healing brush*
(S) Clone stamp
(E) Eraser
(R) Blur†
(A) Path selection*
(P) Pen*
(N) Notes*
(H) Hand
Foreground color
(D) Default colors
(Q) Standard mode*
(F) Standard screen mode
(F) Full screen mode with menu bar

Additional ImageReady Toolbox items
(A) Toggle image maps visibility
(Y) Preview Document

Adobe Online
Move (V)
Magic wand (W)
Slice (K)
Brush (B)
History brush (Y)*
Gradient (G)*
Dodge (O)†
Horizontal Type (T)
Rectangle (U)
Eyedropper (I)
Zoom (Z)
Switch colors (X)
Background color
Quick mask mode (Q)*
Full screen mode (F)
Jump to ImageReady (Ctrl + ⇧ + M)

Toggle slices visibility (Q)
Preview in default browser (Ctrl + Alt + P)
Jump to Photoshop (Ctrl + ⇧ + M)

Rounded rectangular marquee§
Elliptical marquee
Single row marquee
Single column marquee
Polygonal lasso
Magnetic lasso*
Patch*
Pattern stamp*
Background eraser*
Magic eraser
Sharpen†
Smudge†
Direct selection*
Freeform pen*
Add anchor point*
Delete anchor point*
Convert point*
Audio annotation*
Rectangle image map§
Circle image map§
Polygon image map§
Image map select§

Slice select
Airbrush†
Pencil (B) (Photoshop) (N) (ImageReady)
Art history*
Radial gradient†
Angle gradient†
Reflected gradient†
Diamond gradient†
Paint bucket†
Burn†
Sponge†
Vertical type*
Horizontal type mask*
Vertical type mask*
Rounded rectangle
Ellipse
Polygon*
Line
Custom shape*
Color sampler*
Measure*

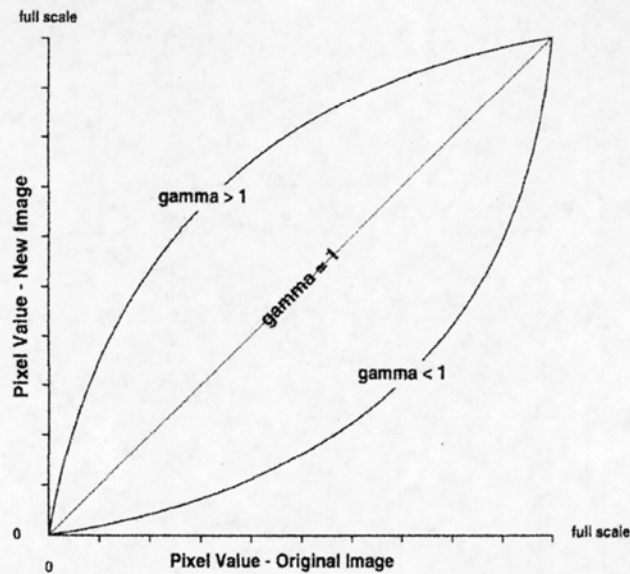
* Not in ImageReady
† In a different location in the ImageReady toolbox
‡ Tools appear in Options bar only
§ ImageReady only

Toolbar shortcuts

- Press shortcut key (in parentheses) to select tool
- Ctrl + Tab cycles through open documents
- Alt + click + tool or ⇧ + shortcut key cycles through hidden tools (except ⇧, ⇧, ⇧, ⇧)
- Select tool and press Enter to highlight options bar
- Right + click displays context-sensitive menu
- ⇧ constrains dragging or drawing to straight line or multiples of 45°
- Caps Lock displays precise cross hair for brushes
- Enter applies an operation of the magnetic lasso*; Esc cancels the operation
- / toggles shield off and on (crop tool only)

Figure 22. Adobe Photoshop 7.0 toolbar reference table.

Gamma adjustment corrects an image by creating a new version of the original. To create the new image, the Gamma Adjust function reassigns the RGB values of each pixel in the image according to the curve in the following graph:



The above graph demonstrates the basic principles of gamma adjustment:

- Black (pixel value = 0) remains black at all gamma values.
- White (pixel value = full scale) remains white at all gamma values.
- Gamma values greater than one lift the darker areas of the original image into the brighter areas of the new image.
- A gamma curve is smooth: there are no unexpected jumps or cutoffs. This means that when viewing a gamma adjusted image, you will be able to see the details (intensity differences) in both the black and white areas of the image.

Figure 23. Explanation of the gamma adjustment.