

Quick Reference Sheet

(details begin on next page)

Start-up

A. Turn on the equipment.

1. When using fluorescence, turn on the mercury lamp before anything else;
2. Turn on the Spot camera;
3. Turn on the computer;
4. Start the Spot application.

B. Set up the microscope.

1. Adjust illumination levels and filters;
2. Position and frame your sample;
3. Adjust Kohler illumination for brightfield microscopy;
4. Pull out the beamsplitter to send the image toward the camera;
5. Position the camera filter to the B/W or Color position.

C. Collect images.

1. Open Spot Basic and go through Setup;
2. Choose Manual or Automatic exposure in Spot Basic;
3. Check your focus and exposure time with the Spot tool bar;
4. Collect images;
5. Save your images as “TIFF uncompressed”.

Shut Down

A. Turn off the microscope.

1. Turn off the Spot camera;
2. Turn off the fluorescent lamp;
 - a. the mercury lamp must be on of at least 30 min. before shutting down
3. Turn off the brightfield illuminator on the microscope;

B. Finish with images.

1. Complete image processing in Spot, Photoshop or ImageJ;
2. Transfer images to back-up medium (DVD, portable drive, server, etc);
3. If not processing these images on this workstation, delete them from the hard drive;
4. Quit the Spot application;
5. Turn off computer - Select “Shutdown” from the Special Menu, or press the power button;
6. Clean up any immersion oil, pencils, notebooks, etc. and cover the microscope.

GETTING STARTED WITH THE MICROSCOPE

See the **Nikon Optiphot Guide** for additional information about the microscope.

Detailed Instructions

TURN ON THE EQUIPMENT

1. **When using fluorescence, turn on the mercury lamp before anything else.**
2. Turn on the microscope lamp.
3. turn on the Spot power supply, on the shelf directly above the microscope;
4. Turn on the computer.
 - a. press the round button on the front of the computer, next to the inventory tag.
5. Turn on the Spot camera
 - a. Press the black rocker switch at the upper right front corner of the unit.

Note: The monitor is controlled by the computer. You need not press the power button on the monitor if the computer has been shut down correctly by the last user.

B. Description of the Spot camera

This camera utilizes a type of CCD detector known as a “frame transfer” chip. The image region records an image when a mechanical shutter opens. When the shutter closes, the image is transferred to the read portion of the chip and relayed to the computer. This process is slow, about 4 frames per second. This camera is cooled to reduce noise during long exposures.

C. Color versus monochrome

Digital cameras are inherently monochrome in that they only detect photons. The concept of color in a digital camera only occurs by recording images from light that has been separated into colors. The Spot camera uses a tunable crystal to filter the microscope’s image into 3 bands of wavelengths corresponding to the human perception of “red”, “green” and “blue”. These 3 bands of light are recorded into separate images which are merged to create a color image.

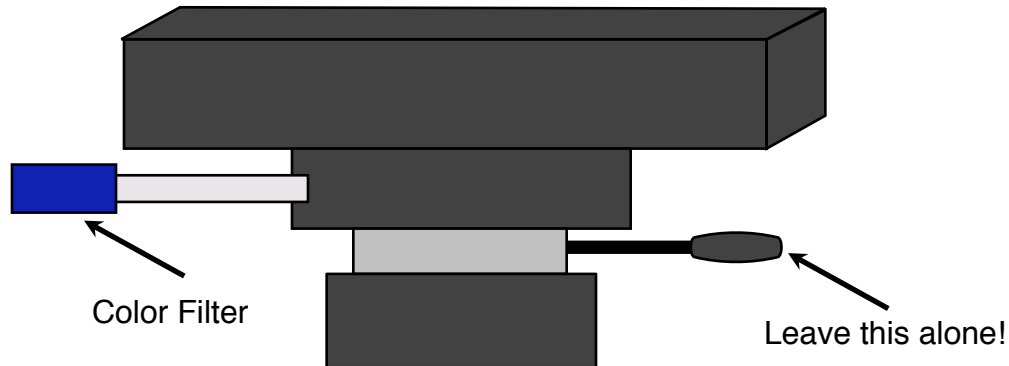
This color filter is not used to capture fluorescent images for 3 reasons. First, the fluorescence filters already separate each label. Second, the tunable filter is polarized, which means that only 50% to 60% of the randomly polarized fluorescent signal is able to pass and reach the camera. Third, the color filter slider also contains a separate filter that blocks infra-red wavelengths starting at 650 nm, which blocks the emission from far red fluorophores such as Cy5, Mitotracker Deep Red, Draq5, etc.

The RGB filter in the Spot can be inserted or removed depending upon whether you need the filter. Images from transmitted light microscopy can be acquired as monochrome (gray scale) by removing the Spot’s tunable filter.

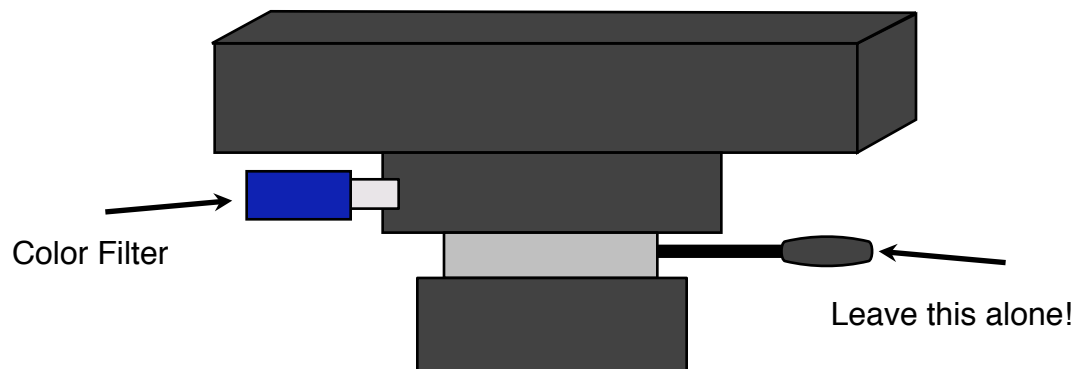
I) **Getting ready for image capture**

A. **Determine whether you would like monochrome images or color images**

1. Color Images for Brightfield - The black knob on the left side of the camera is be pulled out.



The color filter is pulled out for color images.



The color filter is pushed in for monochrome images.

2. Monochrome Images for Fluorescence or brightfield– Push the Filter Selection Rod in to remove the color filter and the infrared filter from the optical path.

The small level on the right side of the camera locks the camera to the microscope. Don't touch it!

ACQUIRING IMAGES WITH THE SPOT CAMERA

A. Open the Software

1. Open the “Spot Basic” application from the dock by a single click on the icon;
2. If the Spot application is started before the camera is turned on, an error message similar to the following will appear:

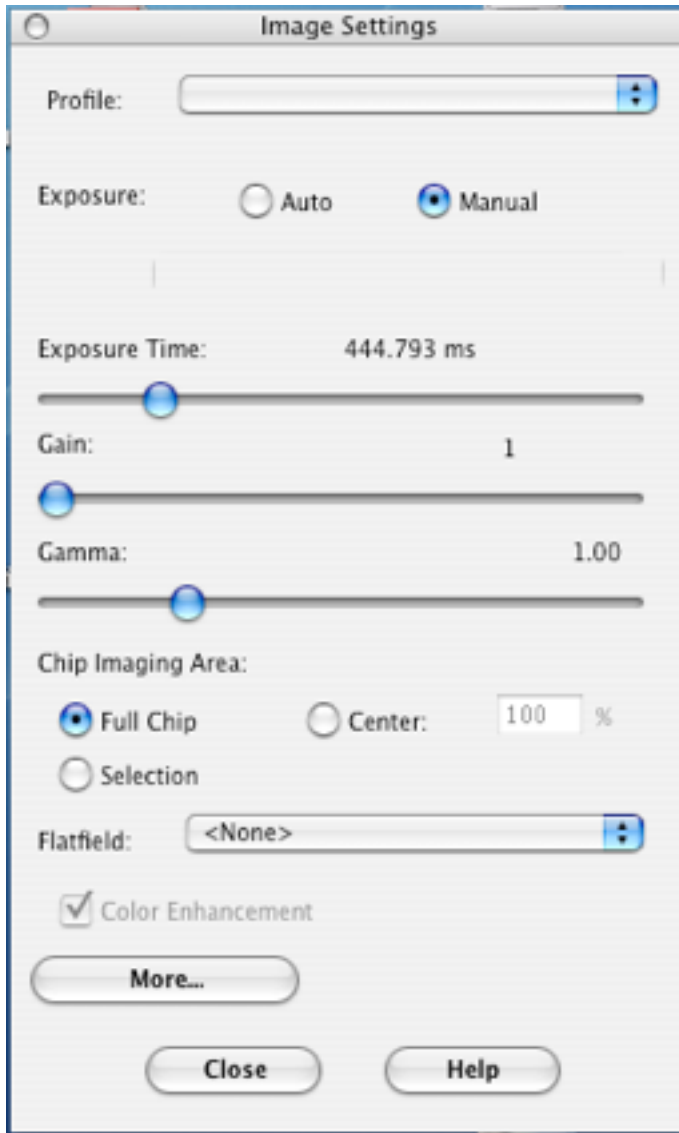


Figure 1. Error message if camera is not turned on before Spot software is started.

- (a) Dismiss the alert by clicking on “OK.”
 - (b) Turn on the power to the camera.
 - (c) Click on the “Camera” menu and release the mouse on “Initialize.”
3. Decide if you are collecting monochrome images or color images, fluorescence or transmitted light.

Image Type	Spot Filter	Application
Brightfield	Color	Color image capture Segmentation of stains by color
Brightfield	Monochrome	Densitometry Monochrome images, usually single stain, e.g. thionin, DAB
Fluorescence	Monochrome	Remove filter for brighter images Fluorescent images are inherently 1 “color” Allows capture of far red fluorophores >650 nm
Darkfield	Either	Monochrome is faster Color may be useful

Table 1. When to use tunable color filter



4. Open the 'Image Settings' window, select it from the Setup menu;

5. Ignore the Profile menu.

6. Choose 'Auto' or 'Manual' exposures.

7. "Auto" uses software to set the exposure times, but you can adjust the exposure to longer or shorter with the "Brightness" adjustment.

8. "Manual" allows you to directly set the exposure time, and is faster since test exposures aren't needed at each image capture.

9. Leave 'Gain' set to 1 for most imaging, this is only used for weak fluorescence.

10. Leave 'Gamma' set to 1, always.

11. Leave set to 'Full Chip.'

12. 'Flatfield' correction will be discussed below.

Figure 2. The Image Settings window

13. De-select 'Color Enhancement.'

14. Click on 'More...' in the lower left corner.

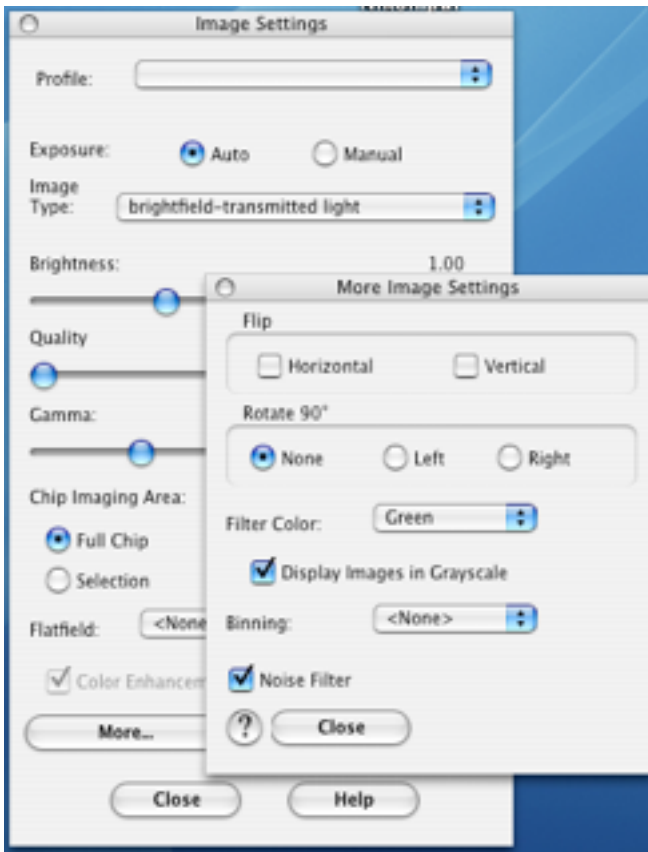


Figure 3. ‘More Image Settings’ window
Notice that in this example, the Exposure is now set to “Auto”, and the Image Type menu and Brightness control are visible.

More Image Settings

1. The ‘Flip’ options are left alone.
2. The ‘Rotate 90°’ is left alone.
3. Set ‘Filter Color’ based on table below.
4. ‘Binning’ is left set to “<None>.”
5. ‘Noise Filter’ is optional
 - a. leave off for quantiation
6. Close the More window.

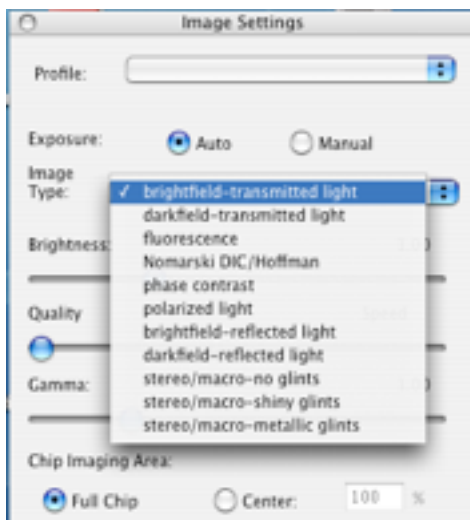
Filter Color	Application	Lever Position
<none>	Monochrome or fluorescence	In
RGB	Red, Green, Blue	Out
Red	Red wavelengths only	Out
Green	Green wavelengths only	Out
Blue	Blue wavelengths only	Out

Table 2. Choose the appropriate filter setting for the Spot filter

B. Detailed information on settings

1. Exposure: Brightness - this control only appears for auto-exposure. It sets a multiplier to adjust the auto-exposure. For example, set this to .80 and the automatically determined exposure time is reduced by 20%, if set to 1.25, then the exposure time is increased by 25%.

1. Manual exposure - you control the exposure time by sliding the button.



2. Note that the minimum possible exposure time is 80 ms.

3. Auto-exposure - The Spot software determines the exposure time by taking a series of exposures until a criterion is met.

4. The type of image must be selected for correct exposure setting. The brightest pixels are usually most important in a fluorescent image, whereas the darkest pixels are more important in a brightfield-transmitted light image. Thus, the “correct” exposure is computed differently.

Figure 4. Selecting the Auto-exposure type

5. Image Quality: this is really a setting for binning and is only used for weak fluorescence signals. The camera chip will be divided into “neighborhoods” of $n \times n$ pixels, their intensities summed and an image generated that will be n -times brighter, but with spatial resolution reduced by a factor of n .

6. View: If the RGB filter is in, the focus image will be collected with the selected color range. Green is the default, since the human eye is most sensitive to this color. If the specimen has no green color then some other color should be selected.

C. Capturing an Image

1. Push the beam splitter to the middle position or fully in to direct the image to eyepieces.
2. Frame your sample in the eyepieces and focus on the sample.
3. Set beam splitter to the middle or the fully out position to direct the image to camera.

You will frame, focus and collect images with 3 icons on the top menu bar of the Spot software (figure on next page).

4. Frame the field by clicking on the “Frame Image” icon.
 - a. Click on the “Begin” button.
 - b. Move the stage until the sample is framed in the window.
 - c. Rotate the stage, if desired. The knurled silver knob at the front of the stage locks stage rotation. **DO NOT ROTATE THE CAMERA!!!**
 - d. Stop framing by clicking on the “Close” button.
5. Focus the camera by clicking on the Focus icon.
 - a. Start focus by clicking on the “Begin” button.
 - b. Stop focus by clicking on the “Close” button.
 - c. If the image has already been captured, focus may be checked at a user-defined location.

6. Capture an image with the Image Capture icon.
7. Save as uncompressed TIFF with commands in the File Menu.

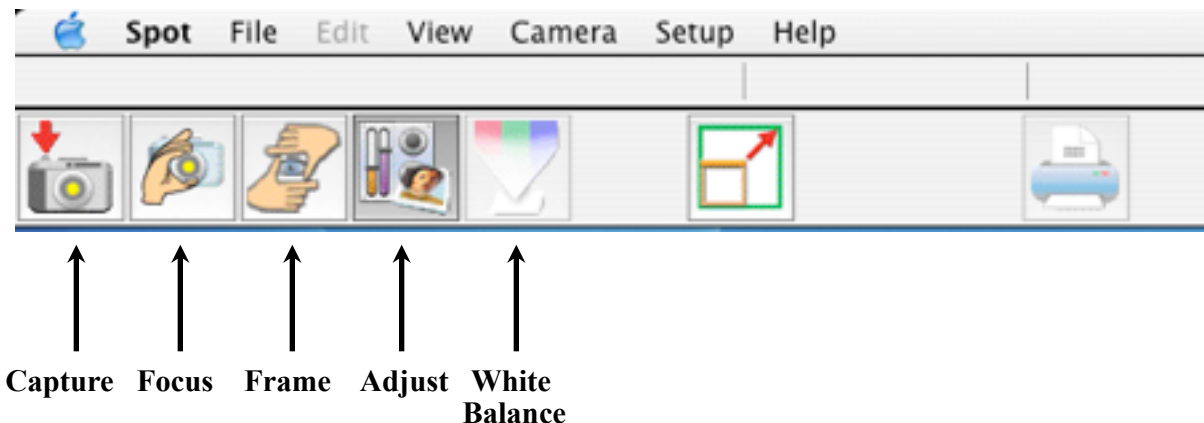


Figure 5. Spot menu icons.

A. Explanation of icons

1. **Frame** - Presents the entire camera field at 25% resolution (only 1 in 4 pixels are read out) to quickly position the field of view and for coarse focus. Click on 'Begin' to observe the camera field and click on 'Close' to dismiss the frame window.
 - a. 'Filter Color:' use only with the Spot color filter to view the field in 1 color channel,
 - b. The 'Display in Grayscale' box presents the image as monochrome regardless of Filter Color setting.
 - c. 'Speed:' controls the shutter rate during framing, with slower speeds improve image quality.
2. **Focus** - A small field from the chip is presented at full resolution and rapidly updates as you focus. Click on 'Begin' to start. By default, the focus field is placed in the center of the camera field. If an image has been captured you may drag the focus window to any point of interest.
 - a. 'Filter Color:' use only with the Spot color filter to view the field in 1 color channel,
 - b. The 'Display in Grayscale' box presents the image as monochrome regardless of Filter Color setting.
 - c. 'Binning:' works are described above to make a fluorescent image brighter.
 - d. 'Speed:' controls shutter rate during framing, with slower speeds improve image quality.
 - e. The 'Brightness' control adjusts the brightness of only this window.
3. **Capture** - Acquire the image.

E. Adjusting Settings for Focus

1. If an image has been captured, the Focus icon will present an outline on the image.
 - a. Drag the outline to encompass any structure on which to focus.
 - b. Double-click on this box to get the live focus dialog.
 - c. Click "Begin" to begin live focus and "Close" to stop and dismiss the window.
2. Setting the camera parfocal to the eyepieces
 - a. Set the microscope to the 40X air lens.
 - b. Find an object in the field of view that has high contrast or sharp boundaries.

- c. Bring this object into focus with the Focus window.
- d. Find this same object in the eyepieces, but DO NOT change the microscope focus.
- e. Adjust both eyepieces to focus on this object.
- f. Now, both camera and eyepieces should be in focus.
- g. Performing this with the 40X air lens will usually be sufficient for all lenses, but it may need to be repeated for oil immersion objectives.

F. Flatfield Correction

The flatfield correction is crucial for optimal quality in brightfield images. Illumination is often uneven across the field of view, usually with higher intensities in the center of the field that diminish toward the periphery. The problem is greatest with objectives less than 10X and with lenses that are not marked as “Plan”.

The flatfield correction uses a blank field (no specimen, no slide) to correct the illumination intensity across the field. You may create a new one, as described below, or select a flatfield image previously created.

1. Focus on your sample and set Kohler illumination (See the manual [Image Analysis with the Nikon Optiphot...](#) for additional information);
2. Select “Get Flatfield Image...” from the Camera Menu;
3. Averaging 3 frames will improve quality of the correction;
4. Move the slide from the field with the stage controls;
5. Press “Begin”;
6. Give the flatfield image a name indicating lens, RGB vs monochrome, etc.;
7. The new flatfield image will be automatically selected and applied to all subsequent captures.

Notes:

- a) The image intensity and filter selection for a flatfield correction image should resemble the conditions used for the image to which it will be applied.
- b) A separate flatfield must be collected for each objective lens.

E. White Balance – Brightfield Color Images Only

The light illuminating the specimen will change color as the lamp voltage is adjusted. A higher voltage increases intensity by raising the lamp temperature to emit more photons. A hotter lamp emits more photons with blue wavelengths and a cooler lamp emits more photons with red wavelengths. Since changes in the lamp voltage alter the color of the illumination, the color of the brightfield image will also change.

This may be corrected by using the Spot’s built-in white balance correction. Avoid constantly resetting the white balance by finding the highest voltage needed for your imaging, then using the ND filters to reduce the illumination intensity, as needed for different lenses or specimens. However, it can easily be reset when you need to change lamp voltage while collecting images.

Once the White Balance has been set, adjust illumination intensity only by sliding the ND filters into and out of the light path. The ND filters are color neutral and will not affect the color temperature of

the light passing through them. Using ND filters and the exposure time setting avoids having to repeat the color balance,

1. Click on the White Balance icon;
2. Move the slide to place a clean, clear area in the field of view;
3. Press the white balance button;
4. Make sure you have a blank region under the lens and click on “OK”.

You may want to use a blank area under the cover slip if your brightfield staining has residual background coloration on the slide itself. Such background may affect the color balance.

F. Background Correction - Fluorescence Images Only

Capturing a background correction allows subtracting the background glow sometimes found in fluorescent samples. This most commonly comes from 'leakage' of excitation light through the barrier filter or by autofluorescence in the tissue, mounting medium, or immersion medium.

1. Bring the sample into focus under the desired objective.
2. Move the slide so that only the background portion of the specimen is in view.
3. Select "Background" from the Camera Menu.
4. Click on “Begin.”
5. Provide a file name, when asked to name it.
6. Since each background image is specific for an objective or sample type, it should be named accordingly.
7. Specify the image setup that will be associated with this background image when the Background Subtraction option is selected, the current Set-Up is selected by default.

G. Collecting multiple channel fluorescent images

1. Each fluorescent label in a specimen can be captured individually and then assembled into an RGB image using ImageJ or Photoshop. This is a very simple process.
2. Collect the image for each channel and save with a filename identifying the channel, such as “488” for Alexa488 or “Cy5” for Cy5.
3. Open the channel images for a given field of view in ImageJ, such as ‘84219adapi’, ‘84219a488’ and ‘84219amito.’
4. Under the ImageJ Image menu, select ‘Color/Merge...’ and assign an image to each channel.
5. The result will be a color RGB image.
6. See the articles on the Core C website for “Making Figures in Photoshop” and “ImageJ User Guide” for additional information.

H. Quality setting for weak fluorescence

The Quality control has 5 possible settings as you move the slider, from “Quality” to “Speed”. Weakly fluorescent images may require excessively long exposure times, which, in turn, may lead to severe photobleaching. This adjusts the camera to treat reduce the “full well” capacity of the detectors and multiply the resulting signal in a corresponding manner. For example, if 60,000 photons fill a pixel to its maximum signal (full well), setting Quality = 2 will reduce this to 30,000 photons and then multiply the signal from each pixel by 2.

Setting	Gain	Fraction of Full Well	Fraction of Exposure Time	Image Noise
Quality	1	1	1	Lowest
	2	1/2	1/2	
	4	1/4	1/4	
	8	1/8	1/8	
Speed	16	1/16	1/16	Highest

Table 3. Quality-Speed settings.

Troubleshooting

1. Error message appears “Exposure time too short for camera.”
 - a) This appears with auto exposure. It means there is too much light going to the camera.
 - b) Reduce the light to the camera by reducing lamp voltage or adding a neutral density filter.
 - c) If the beam splitter is set to ‘100% to Camera’, move it to the middle position.

2. The image is snow white.
 - a) You are using manual exposure and are collecting so much light that all pixels are saturating.
 - b) It could be a sign that you are using automatic exposure and selected the wrong image type for computing the exposure.
 - c) The ‘Brightness’ setting is turned up too high for automatic exposure.

3. The image is black.
 - a) If using manual exposure there may be too little light going to the camera.
 - b) There may be too much light going to the camera it is protecting itself by blanking the chip.
 - c) First, check the beam splitter is in a position to direct light to the camera.
 - d) Check the exposure time, it may need to be longer, or shorter, go to step f).
 - e) For brightfield, make certain that the condenser is properly focused (see the Nikon guide for Koehler illumination).
 - f) Vary the lamp voltage or number of ND filters in place.

- g) Is the field stop diaphragm open?
 - h) For fluorescence, check for ND filters in the lamp path.
 - i) Is the correct filter cube in place and is the specimen in focus?
 - j) Is there excitation light striking the specimen?
 - k) Is there label in the specimen?
4. The image is snowy gray.
- a) Check that the beam splitter is delivering light to the camera.
 - b) Manual exposure - the exposure time is too short or the lamp is too low.
5. It takes too long to collect an image.
- a. There is very little signal to the camera.
 - b. Check the amount of light to the specimen.
 - c. Is the beam splitter sending 100% of the image to the camera?
 - d. Is the color filter in the light path (rod in the 'Color' position)?
 - e. Do you need the color filter?
 - f. For fluorescence, try using a lower Quality setting or binning.
 - g. Is the lens dirty?
2. The image is almost white, but there is a little color in it.
- a. Did you choose 'RGB' for Filter Color in the Setup and forget to pull the color filter into position at the camera?
2. I removed the filter slider to the "B/W" position but my images are red, green or blue.
- a. Did you set the 'Filter Color' to "<none>"?
The software thinks it should collect a color image and is applying a color lookup table.
2. I wanted RGB color but all I get is red, green or blue.
- a. Was "RGB" selected for Filter Color in the Setup?
2. How can I process my images to make them pretty?
- a. Please read the pdf file entitled "Making Figures in Photoshop" on our website. You may also wish to purchase the book Photoshop for Scientists, by Jerry Sedgewick, Kluwer Press.