Shading Correction and Background Subtraction

Abstract
With all the options available in today's image processing systems, there's a great deal of confusion regarding the use of ratiometric shading correction and background subtraction. Universal Imaging is frequently asked questions such as "do I need to correct for shading when doing ratio imaging", "what is the proper background for a fluorescence image?" and "why should I subtract the background if my image looks fine the way it is"? Fortunately, the rules are fairly easy to obey if you understand the reasons for those rules. These rules apply both to quantitative densitometry and quantitative fluorescence microscopy. Slightly different rules apply (to the same equation) for "cleaning up" DIC, phase contrast, and brightfield images. Those non-quantitative applications will be described at the end of the article. The first, and perhaps most important decision you must make is "are you going to quantify intensities in the images or are you only going to be measuring distances or generating pictures for display"? Only if you plan to quantify intensities are shading correction and background subtraction necessary, and even then, they may not always be required. So the question remains, why does one need to correct the image at all?

Instructions
The Image
To understand why you need to correct the images, you have to examine what the image on the monitor truly is. Stop and think about it for a moment. How would you fully characterize the image that you see on the monitor? Start at the light source and follow a beam of light through the entire system until it displays on the video monitor.

What you see on the monitor is the result of the combination of many factors! First, the brightness of the light source determines the amount of light that passes through the condenser and hence what passes into the sample. In virtually every illuminator, if you quantified the amount of light striking the sample, you would find that the precise amount varies for every point in the sample! Typically this will be a fairly smooth gradation from the center to the edge of the field, though dirt on the condenser as well as the specific characteristics of the filament or arc in the light source can result in a very non-uniform illumination pattern.

Stray Light
Next, note that invariably some stray light will enter the system, further altering the amount of light that
strikes the sample and which is ultimately captured by the detector, typically a video camera. So even if it were possible to measure the exact number of photons coming out of the sample, one would have to correct for the stray light and non-uniformity in the illumination system (referring to the light source, it's collector lens which isn't shown, the condenser lens and any filters that may be in the light path).

If you continue to follow the light, now emanating from the sample, you will find that it goes through more optical elements including the objective and a television projection lens (not shown) before being picked up by the detector. Each of these components in turn change the light pattern that you will measure. Dirt in the optical path will absorb light, stray light will add to the light. Moreover, the detector will have a somewhat different sensitivity at every point in the image!

**Electronics**

Now, once the light is detected, it is converted to a varying voltage signal, where the brightness striking the detector is somehow converted to a specific voltage. Note that this itself is arbitrary. Typically, the camera will output more voltage to represent a higher light level, but even this isn't guaranteed. Some cameras have contrast inverter circuitry or convert brightness to voltage in a non-linear fashion (i.e., twice as much light isn't converted to twice as much voltage). This signal then enters the image processor, and gets converted from a series of voltages into a numerical representation of these voltages through the use of a device called an analog to digital converter (ADC). Most ADC's are linear for their entire useful range within a fraction of a percent. However, there is still no guarantee that a given voltage will be converted to a given number. After some processing in the computer, the image is converted back into voltages via a digital to analog converter (DAC), and finally these voltages drive circuitry in the monitor which sets the power of an electron beam based on the voltage.

**Compensation**

Now that you can see how futile it can be to simply try to compare the brightness at different points across the image or from image to image, we will take a step back to see how you can improve your odds by compensating for many of these basic problems. Let's back up for a moment and imagine that we have a perfect detector which you can place right at the surface of the specimen to determine the brightness of the light at any given point. Recall that the illumination system is non-uniform, how can one quantify this non-uniformity? What is the brightness of a point affected by? The illuminator, the stray light, the optics, and the sample. We want some measure of the brightness or light transmission characteristics of the sample and hence want all other factors to become constants that won't affect our measurements. Given this information, we know that the amount of light coming out of any point in the sample is directly related to the illumination, i, the stray light, s, the transmittance of the lens, t, and the transmittance of the sample, a. Transmittance is a fraction from 0 to 1, where 1 represents a completely transparent object and 0 represents a completely opaque object. Illumination and stray light are arbitrary values, let's say they represent a number of photons. The resultant light coming out of the specimen, r, is then given by:

\[ r = (i + s) \times t \times a \]

The stray light can actually come in anywhere in the system and therefore could be adding in after the lens. Fortunately, the contribution from s at before the lens is usually very small compared to the actual light used for illumination, and can be considered to be 0 for now.

**Shading Correction Equations**

For each pixel, the correction equation is:
Transmittance = \frac{\text{Specimen Gray Value} - \text{Background Gray Value}}{\text{White Reference Gray Value} - \text{Background Gray Value}}

MetaMorph does arithmetic on an image by image basis, and makes a result image (rather than a fractional transmittance value), so the equation is:

\text{Corrected Image} = \text{Scaling Factor} \times \frac{\text{Specimen Image} - \text{Background Reference Image} + \text{Offset}}{\text{White Reference Image} - \text{Background Reference Image}}

The Scaling Factor is either maximum possible gray value of the image (when Acquire Image is used with an 8-bit camera, the value is 255), or the maximum gray value of the denominator. The Offset value in the numerator is typically zero. It is in the equation for those situations where you may have a few pixels in the Specimen Image that are of lower gray value than the Background Reference Image. The Offset is then used to make the numerator positive. For example without offset, 10 - 15 = -5, with an offset of 10, 10 - 15 + 10 = 5.

We can save processing time by eliminating a redundant calculation each time the above equation would be called, by pre-calculating the denominator:

\text{Shading Reference Image} = \text{White Reference Image} - \text{Background Reference Image}

The equation now reads:

\text{Corrected Image} = \text{Scaling Factor} \times \frac{\text{Specimen Image} - \text{Background Reference Image} + \text{Offset}}{\text{Shading Reference Image}}

This is the equation used in MetaMorph's Correct Shading dialog (Process Menu). The Scaling Factor is implicit in the dialog, as of version 2.0B19.

The dialog has 4 image selectors that allow you to choose the image used for each image type. The Foreground Image can be an image, the current plane of a stack, or all planes of a stack. Typically the Background Image and Shading Image are single images. We recommend using a New image for the Destination Image until you are comfortable with the Correct Shading operations. We also suggest that you get in the habit of saving the "raw" (acquired) images, and both the Background and Shading images to disk, for each experiment.

**Proof of Concept**

The simplest way to prove that the Correct Shading equation (and dialog) works is to acquire your reference images, then place a 50% neutral density filter in the light path and acquire an image of the
same field you used for the shading image. Compare the results of the image without and with shading correction. The uncorrected image is typically bright in the center and darker towards the edges; the corrected image should be uniform in brightness in all parts of the image (subject of course to some random pixel noise). Any neutral density filter will work, a 50% filter produces an image that is easy to interpret. For fluorescence images the filter should be placed in the excitation light path; for densitometry the filter can go either between the lamp and the sample, or between the sample and the camera (the former makes more sense in minimizing exposure of the sample).

The Acquisition Rules for Quantitative Fluorescence

- Acquire Background Image for White Reference: Uniformly fluorescent slide in place, epi-illumination light path blocked. Examples of such a reference include a uranium glass slide or a slide containing a uniform solution of fluorochrome. A less ideal alternative background image is to simply block all light to the camera, resulting in what we refer to as the "Electronic Bias" image. The "Background" image is preferred because it takes into account room lights and infrared light (heat) from the microscope.
- Acquire White Reference Image: Uniformly fluorescent slide, illuminated. Neutral density filters or exposure time (of cooled or integrating CCD) are adjusted so that no pixels in the camera clip (gray level 255 for a video camera) or saturate (for a cooled CCD).
- Create the Shading Reference Image: Use the Arithmetic dialog (subtraction mode) to do Shading = White Reference - Background + 0.
- Acquire Background Image for the Specimen Image: With your sample in place, epi-illumination light path blocked. See step 1 for an alternative.
- Acquire Specimen image. Be sure the camera does not clip or saturate. If the camera does, add neutral density filters or decrease exposure time. If you are acquiring multiple images you may want to acquire them to a single stack, rather than separate image windows. Save the image(s) to disk before experimenting with shading correction for the first time.
- Use the Correct Shading dialog (Process menu) where the Foreground image selector is your specimen image or stack. We recommend starting out by making a new "Corrected" image (the title is selected with the Destination image selector), rather than overwriting the specimen image(s). This way you can do side by side comparisons between the specimen and corrected images.
- Once you become very comfortable doing shading correction, you may want to do the correction at acquisition. To do this in Acquire Image, turn on the Subtract Background checkbox and select the Shading Reference image in the Shading Image: selector. For Acquire from Digital Camera [4], you need to use both the image selectors (in the More>> panel of the dialog) and turn on the checkbox(es) in the Define.
- Acquisition Settings dialog.

The Acquisition Rules for Quantitative Densitometry

For the purpose of discussion this section assumes you are acquiring images from a light box,

- Acquire Background Reference Image: Put a piece of black material (uniformly, completely exposed film or black cardboard) on the light box. Alternative is to put the lens cap on the camera, so no light enters.
- Acquire White Reference Image: Use a sheet of un-exposed developed film. Use the camera lens f-stop, neutral density filters or exposure time (of cooled or integrating CCD) so that no pixels in the camera clip (gray level 255 for a video camera) or saturate (for a cooled CCD).
- Create the Shading Reference Image: Use the Arithmetic dialog (subtraction mode) to do Shading = White Reference - Background + 0.
Acquire Specimen image. Be sure the camera does not clip or saturate. If the camera does, add neutral density filters or decrease exposure time, and re-acquire the white reference image. If you are acquiring multiple images you may want to acquire them to a single stack, rather than separate image windows. Save the image(s) to disk before experimenting with shading correction for the first time.

**Practical Tips**

- Set up your microscope for Koehler illumination. This applies to both transmitted and epi-illumination. See your microscope manual or ask for our Koehler Illumination article for information on setting up Koehler illumination.
- Maximize the dynamic range of your camera by optimizing the analog contrast settings of your camera and MetaMorph. Users of digital cameras will similarly want to optimize the exposure time for their shading reference image so that it does not quite saturate any pixel.
- Acquire appropriate background and shading reference images at the start of each imaging session for the objective (or macro lens) you are going to use. Each objective or macro lens zoom needs its own reference images. If you change analog settings you will need to acquire new reference images.
- You may need different shading images for each wavelength (or color) if you are acquiring images of multiple fluorochromes (or from an RGB camera). This is particularly true if you are not using a Plan Apochromat or Plan Fluorite objective. For multiple fluorochromes we recommend evaluating images of Molecular Probes Inc's MultiSpeckTM beads at each wavelength.
- If possible, acquire the background image first! Then acquire the 'white' reference image and immediately do a subtract from it the background image, to obtain the 'shading' reference image.

**DIC, Phase Contrast and Brightfield Images**

In order to acquire and process images as fast as possible, it is best if you can use background subtraction during acquisition to correct for non-uniformities in the image. Taking a ratio will be substantially slower (how much slower depends on the frame grabber board and/or PC).

**DIC (Differential Interference Contrast)**

DIC is an imaging method for obtaining high contrast, high lateral spatial resolution, and superb vertical spatial resolution (also known as optical sectioning). The most popular optical configuration was invented by Nomarski. Nomarski DIC uses two polarizing filters (the one between the microscope and detector is called the analyzer), a compensator and two Wollaston prisms, the first of which shears the polarized light axes apart and the second recombines them. The sheared light beams interact with the specimen producing the characteristic white and black doublets. The form of the Wollaston prisms causes a small (or large) gray level gradient across the field of view. The goal of background subtraction is to both subtract out the gradient and add a constant offset back to the image to maintain the original brightness. The subtraction will also remove any "mottle" from the image (caused by dust and dirt on the microscope optics and camera faceplate). Note that DIC is a contrast enhancing and optical sectioning method - the resolution of thin specimens in brightfield is identical to (or maybe better than!) DIC.

- Optimally adjust the specimen and optics for maximum contrast DIC by eye.
- Change the microscope port so all the light travels to the camera.
- Adjust the camera settings for maximum contrast using a high contrast feature in your specimen. Note the focus position - you will want to return to this same Z-value (i.e. use the tick marks on the focus knob or change Z using a MetaMorph focus motor).
- Go out of focus to a Z-plane with no object present (but the condenser still in Koehler illumination).
DIC with Cooled CCD:

- The typical 12-bit (4096) gray level cooled CCD’s that MetaMorph supports can be used to acquire spectacular DIC images (MetaMorph also supports up to 16-bit cooled CCD cameras that are typically used for highly quantitative fluorescence microscopy and bioluminescence imaging). We prefer to adjust the exposure time of the camera so that the average background (i.e. DIC field of view gradient) gray level is in the range of 2000 to 2800. This leaves plenty of dynamic range for white and black edges of high contrast features.
- Light level: Be sure to run the lamp at a voltage in the "Photo" range, where the lamp provides white light. The reason this is critical is that running the lamp at very low voltage causes it to flicker.
- Use a colored filter to provide monochromatic illumination to the sample. Historically green filters have been used since all objective lenses are optimally corrected for 550 nm light. The plan-apochromatic objectives are corrected for blue, green and red light and so can be used with any of these wavelengths. Blue light leads to improved resolution since it is a shorter wavelength (however, CCD's are somewhat less sensitive to blue light). Red (and infra-red) light is useful for DIC on thick specimens since longer wavelengths scatter less and therefore penetrate deeper into the sample (and DIC provides plenty of contrast). In practice, optimal alignment of the lenses and proper numerical aperture setting(s) and cleaning of the microscope and camera settings have a larger impact on image quality (i.e. resolution) than wavelength.
- Use Neutral Density filters to decrease the light level to a satisfactory exposure time.
- Optimize the exposure time for your system. The tradeoffs include:
  1. Cameras with shutters should be used with minimum cycle times of >25 ms, or the shutter will eventually overheat.
  2. Vibration can be a problem, especially with cameras far from the microscope center of gravity: longer exposure time minimizes the effects of vibrations.
  3. Keep the exposure time short enough that your living objects do not move!

DIC is a polarized light microscopy technique. If you use DIC on a regular basis it helps to understand polarized light! See Video Microscopy for details. The Royal Microscopical Society has short book on polarized light in their microscopy handbook series.

Brightfield (BRF)

This mode of transmitted light microscopy is not often used on transparent specimens because of their low contrast and the lack of optical sectioning available with DIC. However, VEC-BRF is a very handy imaging mode because of the ease with which the edges of cells can be contrast enhanced without the problems of phase halos and DIC white/black double edges. Cooled CCD's have sufficient dynamic range
and image quality that their background subtracted result images can be used after trivial "Scale 16-Bit Image" adjustments.

- Use monochromatic light (usually green light). Make sure you have a heat filter between the lamp and the color filter. CCD's are in particular sensitive to infra-red heat.
- Carefully optimize Koehler illumination, camera settings. Pay especial attention to the condenser settings: it needs to be adjusted perfectly for Koehler illumination and the Numerical Aperture diaphragm needs to be set just right. If the condenser NA is too small you generate contrast by sacrificing resolution; if too large, you lose contrast because of glare.
- Move the stage to a blank field (no objects present) and defocus slightly to avoid imaging small debris on the coverslip.
- Acquire the background image with 256 frame average (or identical exposure time to the sample, if using a cooled CCD camera). Determine the average gray level of the image (assuming it is more or less uniform brightness - if not, recheck Koehler illumination).
- Move the stage back to the specimen. Turn on background subtraction. Set the Background offset to be equal to the average gray level of the background. This way if you toggle subtraction on and off you will only see mottle disappear, but the intensity of the live image will not change.

Phase Contrast Microscopy

The first and only Nobel prize for far field light microscopy was awarded to Zernike for the discovery of phase contrast imaging methods. Phase contrast has gotten a bad reputation for poor spatial resolution because most microscopists used it for looking at live cells at low magnification through tissue culture flasks. When set up correctly on a modern research microscope with high quality (high numerical aperture) objectives, phase can give approximately as good a resolution as DIC for thin specimens. Phase contrast does not give optical sectioning and so is not very useful on thick specimens. Nikon is the only microscope company we know of that has manufactured non-DL phase objectives (the "DM" and "BM" series) that do not result in bright phase rings around the specimen. With DL objectives the rings can be eliminated by increasing the refractive index of the mounting medium (i.e. by adding BSA to your tissue culture medium). Tips are as for brightfield, with the additional requirements of:

1. Open the field diagram far enough for light to enter the phase rings.
2. Make sure the phase rings are centered, using a centering tool for the rings in the condenser turret (these are different from the knobs that center the condenser aperture!).

Conclusion

Shading correction is a powerful way to process image for quantitative intensity measurements. The correction also makes the images look nicer by making the illumination more uniform. Understanding when to do background subtraction (practically all of the time!) and when to do background subtraction and shading correction (when a ratiometric operation improves image quality) is a matter of both processing speed and result.