

## Information on Shading Correction and Background Subtraction

### Overview

This document describes what shading and background are for different types of imaging. Likewise it describes how to apply shading correction and background subtraction to images.

### Abstract

There's a great deal of confusion regarding the use of ratiometric shading correction and background subtraction on images for quantitative densitometry and quantitative fluorescence microscopy. Slightly different rules apply (to the same equation) for "cleaning up" DIC, phase contrast, and brightfield images. Shading correction and background subtraction allow you to more accurately quantify intensities and improve image quality for image display, they may not be necessary for measuring distances or counting objects.

ARTICLE #  
T20108

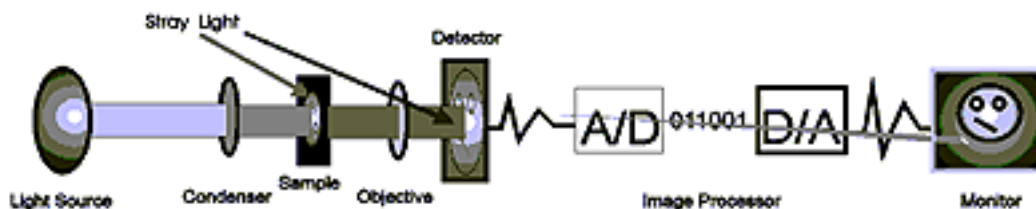
PRODUCTS  
MetaMorph®  
MetaFluor®  
MetaVue™

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### Sources of shading and background in an image

To understand shading and background you have to examine the source of the image. Start at the light source and follow a beam of light through the entire system until it is collected into an image.



The final image 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

Invariably some stray light will enter the system altering the amount of light that either strikes the sample or enters the light path after the sample but is captured by the detector. 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, its collector lens which isn't shown, the condenser lens and any filters that may be in the light path).

Following the light, emanating from the sample it goes through more optical elements including the objective and other optics before being picked up by the detector. Each of these components in turn changes 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 may have a somewhat different sensitivity at every point in the image.

## Electronics

Now, once the light is detected, it is converted to a signal, where the brightness striking the detector is somehow converted to a specific set of values (the pixel grey values). Note that this itself is arbitrary. Typically, the camera converts from a series of voltages (electrons excited by collection of photons) 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. There will be some noise introduced by the procedure and even when no light is absorbed the ADC will produce a digital signal  $> 0$ .

## Compensation

To correct / compensate for these sources of error you need to understand the source of the gray values reported at each point.

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 that can be represented by a number of photons. The resultant light coming out of the specimen,  $r$ , is given by:

$$r = (i + s) * t * a$$

The stray light can come in anywhere in the system and could be adding in after the specimen. Fortunately, the contribution from  $s$  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:

$$\text{Transmittance} = \frac{\text{Specimen Gray Value} - \text{Background Gray Value}}{\text{White Reference Gray Value} - \text{Background Gray Value}}$$

MetaMorph<sup>®</sup> software calculates on an image by image basis, and makes a result image (rather than a fractional transmittance value), so the equation is:

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

The Scaling Factor is either maximum possible gray value of the image (when acquiring with a 12 bit camera the value is 4095), 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.

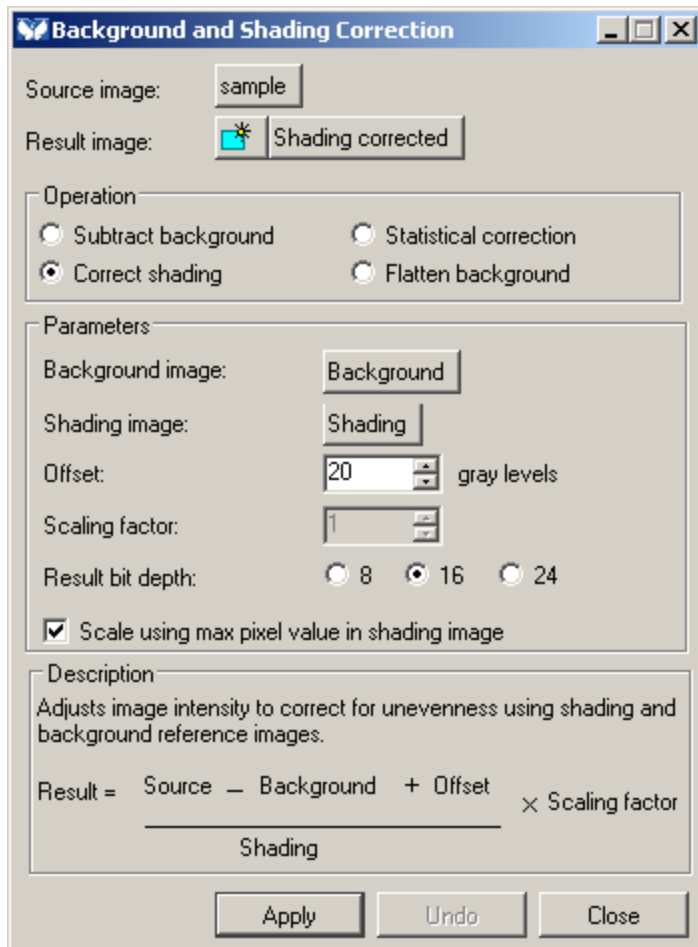
Processing is often reduced by pre-calculating the denominator:

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

The equation now reads:

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

This is the equation used in the Correct shading option of the Background and Shading Correction dialog (Process menu).



The dialog has 4 image selectors that allow you to choose the image used for each image type. The Source 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 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.

### Acquisition rules for Quantitative Fluorescence

1. Acquire the White Reference Image from a uniformly fluorescent slide. Adjust the exposure so that no pixels in the image saturate. The grey value for saturation depends upon the camera type. For video cameras the maximum grey value is 255. For 10 bit, 12 bit, 14 bit and 16 bit digital cameras the maximum grey values are 1023, 4095, 16384 and 65535.
2. Acquire a background image for the White Reference image by taking an image with the epillumination illumination turned off. 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 infra-red light (heat) from the microscope.

3. Create the Shading Image: Use the Arithmetic dialog so that the Shading image is the White Reference Image (step 1) –the White Reference Image Background (step 2).
4. Acquire a background image for the source. With your sample in place, epi-illumination light path blocked using the same exposure and other parameters that you will use with your specimen.
5. Acquire the source image of your specimen. Be sure the camera does not saturate. If the camera saturates decrease exposure time. Make sure that you retake the source background image (step 4) with the new exposure time.
6. Use the Correct shading option of the Background and Shading Correction dialog (Process menu) where source image is your specimen image or stack.
7. Once you become very comfortable doing shading correction, you may want to do the correction at acquisition. See technical note T20108

### Acquisition rules for Quantitative Densitometry

In Quantitative Densitometry the background is light and the sample is dark

1. Acquire Background Reference Image by blocking light to the camera.
2. Acquire White Reference Image: Use a slide that does not have sample or dirt on it. Adjust the exposure so that no pixels saturate.
3. Create the Shading Reference Image: Use the Arithmetic dialog (subtraction mode) to do  $\text{Shading} = \text{White Reference} - \text{Background Reference}$ .
4. 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 for information on setting up Koehler illumination.
- Maximize the dynamic range of your camera by optimizing the exposure time for their shading reference image so that it does not quite saturate any pixel.
- Acquire background and shading images using the same magnification and binning as the sample images.
- 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 any 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.
- 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

For further assistance please contact Meta Imaging Series<sup>®</sup> technical support at 800-635-5577 option 3-2-2 or email [support.dtn@moldev.com](mailto:support.dtn@moldev.com).