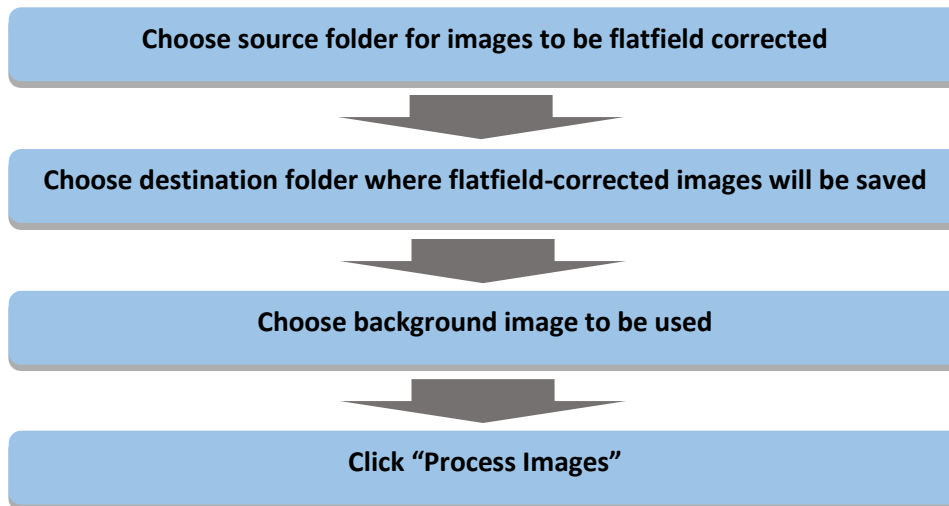


Quick Guide for FAST Flatfield Correction with CHROMACAL EZ Image Prep™



Below is important information about flatfield correction and how to generate the best images possible.

About Flatfield Correction

Digital imaging in microscopy captures not just an image or representation of the specimen, but also any shading or other anomalies in image evenness imparted by the microscope and illumination source. Flatfield correction (a.k.a. flatfielding, flat-field correction, shading correction, etc.) seeks to normalize the specimen image for these color and illumination anomalies. Flatfield correction does not typically involve white balancing, nor does it correct for inaccurate color. Figures 1(a) and 1(c) illustrate common examples of illumination and condenser alignment issues for which flatfield correction can resolve. Figure 1(b) illustrates good flatness of the field of view.

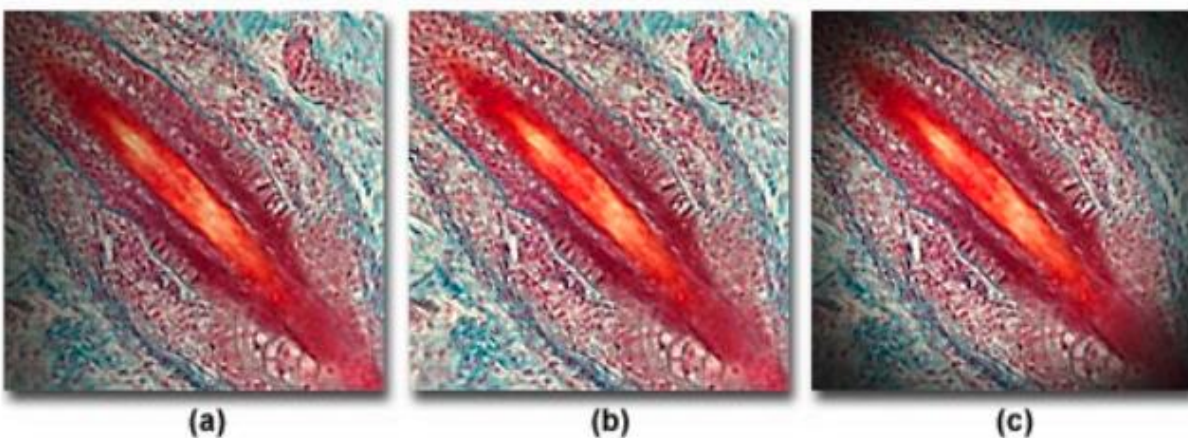


Figure 1. Examples of illumination errors in digital imaging. (a) poor alignment of the illumination. (b) good illumination alignment and evenness across the field. (c) example of vignetting. (source: <http://www.olympusmicro.com/primer/photomicrography/errors.html>)

EZ Image Prep™

Common imaging problems, such as uneven illumination or vignetting, can often be resolved with proper microscopy techniques at the start of your imaging session (see “**Ideally, Correct the Sources of Anomalies First**” in the White Paper section below). The process of flatfield correction using camera or 3rd party software is another option, but often is more challenging and cumbersome than beneficial.

For the above reasons, EZ Image Prep was developed and is the preferred choice. This CHROMACAL software feature includes a simple process to flatfield correct (if desired) and, at the same time, automatically white balance and adjust brightness to a folder of images. EZ Image Prep delivers this unique combination of adjustments using a standardized approach for consistency and repeatability, with the added benefit of batch processing. By comparison, image editing software like Adobe® Photoshop® require subjective adjustments on an image-by-image basis for each of the three components handled automatically by EZ Image Prep.

The workflow efficiency, image consistency and quality improvements with EZ Image Prep are unrivaled in the market. Here is the simple EZ Image Prep flatfield correction process:

Flatfield Correction with EZ Image Prep

1. Choose the source folder that contains your specimen images for flatfield correction.

NOTE: EZ Image Prep also automatically white balances and matches brightness for each flatfield-corrected image. These additional adjustments can also be performed without flatfield correction.

2. Choose the destination for the flatfield-corrected images.

3. Choose the background image you captured and want to be used for flatfield correction.

NOTE: See guidance provided below in the **White Paper** portion of this document,

4. Deselect the specimen images that you don't want to flatfield-correct.

5. Click Process Images.

NOTE: Images for flatfield correction must match the microscope objective magnification used with the selected background image (i.e. use a background image captured with the same objective as the specimen images).

In addition to the flatfield correction performed within EZ Image Prep, each corrected image will also be automatically white balanced and brightness matched.

The result....the best image quality possible (unless you also choose to color-calibrate with CHROMACAL!).

White Paper: About Flatfield Correction

What is Flatfield Correction?

Flatfield correction is a mathematical function applied to a specimen image, typically requiring an image of the background (sometimes referred to a flatfield image) and some compensation for brightness. Although “blind” flatfield algorithms exist (they use approximation methods in the absence of a background image), this paper discusses the process of flatfield correction using a background image.

Capturing a Background Image

1. Choose the microscope objective and area for capture. It is important to find an area on the slide, or remove the slide from the stage, so that the background image is completely devoid of any dust, debris or artifact. See “Cleaning a Microscope” below for more information.
2. White balance and adjust brightness to approximately 60% of maximum intensity.
3. Acquire and save the background image. Be sure to label the image file with the objective magnification.
4. Repeat steps 1-4 for each objective used during the imaging session.

Flatfield algorithms generally divide the specimen image by the background image on a pixel-by-pixel basis. Since the same background image can be used with a batch of images, flatfielding corrects for predictable shading across the captured image. And since the shading pattern will vary by microscope objective used, a background image must be acquired for each objective used during that imaging session. The resulting flatfield-corrected image is often darker than expected, and a brightness adjustment may be required.

Flatfielding will also correct for minor artifacts in images such as dust that appear in the same location in every image. The location of such anomalies may shift by objective, hence the importance of capturing background images with each objective.

A flatfield correction function is typically included in acquisition software, and this approach can resolve the issue during image acquisition. Other 3rd party software and applications (e.g. Adobe® Photoshop®, ImageJ/Fiji, MetaMorph, ImagePro, etc.) may provide flatfield correction during or after acquisition.

Ideally, Correct the Sources of Anomalies First

A number of factors contribute to anomalies in image evenness, some of which are never apparent to our eyes when we look at the specimen through the eyepieces. Take care to first manage the sources of anomalies, and flatfield correction will be much easier to perform.

It’s important to note that the process of Köhler Illumination and keen observation during microscope use can provide clues as to the location or source of an anomaly (e.g. a speck of dust on the camera sensor will not change position or size in the image when you rotate the camera head or change objectives; an artifact that moves out of the way when you change objectives is probably dust on the

front lens element of the objective; if after performing Köhler there is shading in the image but not when you observe the specimen through the eyepieces, the cause is likely the camera adapter (either being the wrong magnification or not optically centered with the microscope optical path and the camera sensor).

Clean the Microscope

It may seem obvious, but dust, lint, smudges and smears (collectively referred to as “schmutz”) in the microscope can lead to poor images. Clean your microscope periodically to remove grease, oils, and dust to prevent them from finding their way into your images.

Cleaning a Microscope

1. Use compressed air or a microscope “puffer” to blow dust off the stage, condenser lens(es) and field lens (sits in the microscope base, below the condenser).
2. Use lens tissue (Kimwipes are not recommended) and a warm breath to clean exposed lenses (those readily accessible and open to the air). GENTLY wipe in a circle from the inside moving outward. It is often helpful to wrap the lens tissue around the end of a cotton swab. DO NOT clean the rear objective lens element.
3. If smears, etc. persist on lenses after step 2, apply a little isopropanol or ethanol to the lens tissue before cleaning the lens – you may also use lens cleaning solution provided by the microscope manufacturer. This step is important if you are using oil immersion objectives. Repeat step 2 and evaluate if another cycle of step 3 is needed.
4. Wipe the surface of the stage with some isopropanol or ethanol and a Kimwipe to remove oils from contaminating your slides.



Align the Illumination: Köhler Illumination

Be sure to check the alignment of your microscope prior to any imaging. Köhler illumination or alignment (“Köhler”) ensures that the illumination light is evenly distributed across the entire field of view of the specimen. A microscope that is out of alignment may have unevenness in the form of shading (see Figure 1a). Once practiced, Köhler takes less than a minute to perform when you first sit down at the microscope, and only a few seconds to adjust each time you change objectives. Below is a brief summary of the process. Be sure to perform Köhler while looking through the eyepieces.

For more information on how to perform Köhler illumination, consult your microscope user manual, your microscope sales representative or various web resources (e.g. http://www.imagingandanalysis.com/koehler_illumination.html). A brief tutorial video is available at <http://scientific.datacolor.com/resources/video-library/good-microscopy-practices-video/>.

Steps for Köhler Illumination

1. Focus on a specimen.
2. Close the field diaphragm (a.k.a. field aperture or field stop) until it is visible in the field of view.
3. Focus the field diaphragm so that the edges are sharp. Use the condenser-focusing knob located behind the condenser on the condenser mount.
4. Center the field diaphragm in the field of view using the two screws towards the front of the condenser.
5. Open the field diaphragm completely.
6. Remove an eyepiece from an eye tube and look down the eye tube at the image of the condenser diaphragm (a.k.a. condenser aperture).
7. Using the adjustment knob or wheel near the front of the condenser, adjust the size of the diaphragm to approximately 85%-90% open.
8. Replace the eyepiece and proceed with observation.
9. Repeat steps 6-8 each time the objective is changed (the condenser diaphragm will have to be adjusted for each magnification), regardless of whether you are observing through the eyepieces or capturing images with a camera.

Vignetting

Vignetting is a darkening of an image towards the perimeter (see Figure 1c). It often results from improper selection of the coupler connecting the camera to the microscope, and is often more prevalent at lower objective magnifications. The best solution is to match the coupler magnification with the sensor size of the camera. As a general rule, a ½-inch sensor camera would use a 0.5X coupler, a 2/3-inch sensor camera would use a 0.65X coupler, and so on (sensor diagonal size \approx coupler magnification). This is just a guide – consult with your microscope representative for the best option.

White Balance

After ensuring that your microscope has been prepared according to the guidelines above, it is best to white balance the camera prior to specimen image acquisition. Most scientific cameras come with acquisition software, and somewhere within the software is an option for white balancing. Here are the basic steps:

1. Move to or select an area on which you want to set the white balance.
2. Click the white balance button.

Pretty simple, right? Unfortunately not. Some acquisition software allow you to choose the area or even a pixel to use to white balance, and others will perform the function automatically according to an algorithm. Regardless, you must decide whether you want to account for the slide, coverglass and

mounting medium in the white balance adjustment, or if you prefer to only account for the microscope and light source by removing the slide from the stage altogether. Either way, be sure to be consistent with your approach.

One more point – automatic white balance throughout your imaging session is not a recommended practice. Automatic white balance during acquisition may appear very convenient, and even save time on the front end, but the white balance and color of your images will vary from image to image. In the end, you'll have to spend time to subjectively edit your images using Adobe® Photoshop® or similar programs.

Of course, the **best practice** for achieving high-quality images is to use **CHROMACAL** in conjunction with your imaging system. **CHROMACAL's EZ Image Prep** feature includes a simple process to flatfield correct (if desired) and, at the same time, automatically white balance and adjust brightness to an entire folder of images. EZ Image Prep delivers this unique combination of adjustments using a standardized approach for consistency and repeatability, with the added benefit of batch processing. Further, if you desire the optimal imaging results, use EZ Image Prep in conjunction with **CHROMACAL's** color calibration feature. The overall result will be color accuracy, color consistency, as well as objectively standardize the quality of your images while avoiding subjective image-to-image adjustments.