

Dark Frame and Flat Field Corrections in Slidebook

Image Corrections.

Images collected from a microscope always contain artifactual intensity patterns due to the illumination sources, optics, camera and associated electronics. “Dark frame subtraction” and “flat field correction” are essential for quantitative analysis and very helpful with images intended for presentation. Section 9.5 in the Slidebook 5.0 Users Manual has more information along with some errors corrected in this document, as does the help section in Slidebook. A full explanation of dark frame and flat field corrections may be found on the DMC website.

Remember, these artifacts arise from the microscope and detectors, not from the specimen. Correcting them is not altering your data. However, leaving them uncorrected *is* altering your data!

Terminology:

1. Signal - intensities arising from any source, such as labeling, autofluorescence, bleedthrough, reflections or electronics.
2. Noise - random variations in a signal. These can also arise from several sources.
3. Bias current - a fixed signal level in the output from all cameras that adds to the specimen's signal. It raises every intensity by 100 to 400 arbitrary intensity units (ADU), depending on the particular camera. This signal can interfere with thresholding and introduce dramatic errors in measuring low intensity values.
4. Dark Current - from random electron motion in the camera chip, proportional to exposure time.
5. Readout Noise - created by the act of measuring the number of photons collected by the detector.
6. Shot Noise - random variation in the number of photons collected by the camera. It is proportional to the square root of the number of photons generating the signal. It is more significant for low signals than for high signals since signal from 10,000 photons has a shot noise of 100, or +/-1% variation, whereas a signal derived from 100 photons has a shot noise of 10, which is a +/-10% variation.
7. Dark Frame Image - An image collected without signal from the specimen, only intensities from current and noise generated by the camera and related electronics. Dark current is also corrected when the dark frame is collected with the same exposure time as the research image.
8. Flat Field Image - A bright, featureless image used to normalize the optical defects in specimen images. It must be collected under the same optical conditions as the research image and subjected to dark frame subtraction. A separate flat field is required for every combination of lens, camera, optical path and filter. They should be updated whenever the imaging system is modified. Flat fields for fluorescence are collected from uniformly featureless fluorescent fields.

Performing dark frame subtraction.

Dark frame correction simply subtracts a dark frame image from the data image to correct the primary components of detector artifacts: bias current, dark current and readout noise. Alternatively, the mean intensity of several dark frames can be subtracted from the data image for post-acquisition correction.

Dark frame subtraction is NOT background subtraction, which is performed after darkfield and flatfield corrections have been performed. Background subtraction is discussed in Section 9.7 of the Slidebook 5.0 Users Manual.

Creating a dark frame image in Slidebook.

When the Dark Frame option is selected, Slidebook automatically captures an image without illumination, and for the same length of exposure time as for the data image. In timelapse or z-series imaging, an initial dark frame is collected and subtracted from subsequent images.

Flat field Images.

Although the flat field correction can compensate for many optical problems, it is not a substitute for maintaining the microscope optics in optimal working order, such as ensuring objective lenses are clean and the specimen is properly prepared. Brightfield images require that Koehler illumination is correctly adjusted. Bear in mind that most images contain multiple patterns of optical and camera field aberrations. Each combination of objective lens, filter and camera will have a unique flatfield aberration.

Flatfield images on the Marianas have been collected by DMC staff and stored in Slidebook. If you have questions regarding flat field corrections, contact DMC staff.

Performing flat field correction.

Flat field correction begins with dark frame subtraction on both the research image and the flat field image. Flat field correction renormalizes intensities across an image to correct for intensity patterns that result from the optical components. The most commonly corrected problem is an illumination hotspot, where intensities are higher at the center of the image and lower at the edges and corners.

A. Setting Slidebook to correct images.

1. Select the 'Dark field' check box in the Capture Window;
 - a. dark frames are subtracted automatically as each raw image is captured
2. Select the 'Flat field' check box in the Capture Window;
 - a. flat field corrections are automatically applied to the dark corrected images
 - b. this box cannot be selected if no flat field exists for the current optical conditions

B. Flat field specimens for brightfield.

1. Focus on an appropriate specimen;
2. Focus the condenser to achieve Koehler illumination and set the field stop diaphragm;

3. Remove the specimen;
4. Set Slidebook to perform Dark Field correction only, Section A;
5. Proceed to Section D.

C. Flat field specimens for fluorescence.

1. Focus below the surface of a uniformly fluorescent specimen*;
2. Set Slidebook to perform Dark Field correction only, Section A;
3. Proceed to Section D.

*A fluorescent plastic slide or a layer of fluorescent dye (e.g. 0.01% FITC) in a coverslip well. Focus at the surface of the fluorescence then shift focus more deeply to obtain a homogeneous field. Most fluorescent plastic slides are too bright in their intended channel, it's usually best to use a slide for the 'wrong' channel to avoid ND filters or extremely short exposure times.

D. Collecting flat fields in Slidebook.

1. Ensure that the optics are clean and appropriate for the specimen;
2. Set the capture for dark field correction only, Section A;
3. Set up for fluorescent or brightfield flat field, as in Section B or Section C;
4. Open the flat field guide: Edit>Setup guides>Flat Field;
5. Set the exposure time such that the brightest pixel in the flat field is not over 85% of the dynamic range, e.g. ~3500 for 12-bits, ~13,926 for 14-bits, ~55,705 for 16-bits
6. Select the number of fields to be averaged together, usually 3 fields;
7. Select the correct filter configuration at the lower right area of the window;
8. Collect the fields to averaged;
9. Flat fields are catalogued in Slidebook by objective lens, mag. changer, date and filter.

Performing dark frame subtraction after acquisition.

Images collected without dark frame subtraction are easily corrected in Slidebook, FIJI, etc. Go to the imaging system that was used to collect the images and turn it on for at least 1 hour to bring all components to working temperature. Collect a series of 10 dark frame images at the same exposure time as the image to be corrected. These may be created by leaving the fluorescent shutter closed, redirecting the beamsplitter to direct the specimen signal away from the camera, or leaving the halogen lamp turned off. Measure the mean intensity of these images. Subtract the average mean intensity from each image to be corrected. Pay attention to imaging conditions such as room lights.

Flat field corrections may also be performed on saved images, instructions are on the DMC website.

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