

# USING THE SLIDE SCANNING WORKFLOW



In this guide, you will learn about how to use the steps in the workflow, and how to troubleshoot for the brightfield and the fluorescence modalities.

## OVERVIEW

The workflow is available from the *Acquire* ribbon bar.

Use this workflow to scan at any magnification and create high resolution 2D images or 3D stacks. Here is how it works: First, a series of individual image tiles are scanned using a motorized stage. The tiles are then merged to form a single image montage called a virtual slide.

Before you start, make sure that you have a properly calibrated system to obtain high-quality images, then follow the workflow's steps.

The workflow will vary depending on the options you select in step 1. You will be directed to the steps that are relevant to your selections.

## STEP 1: SET UP

### *Data file location*

Select a location to save the data associated with the scan.

### *Acquire type*

Select the desired scan type:

- *2D SLIDE SCAN*: Appropriate when only one image plane is desired.
- *3D SLIDE SCAN*: Appropriate when more than one image plane is needed to create an image stack. This is generally suitable for higher magnifications, but can be used at lower magnification as well (3D is only available if you purchased the 3D slide scanning module.)

### *Slide type*

Make sure you select the correct type (brightfield or fluorescence). This will affect the options available for background correction in **step 6**.

### *Scan type*

Select one of these three scanning methods:

- *Scan Inside Contour Only*: Use only closed contours for this method. Processes tiles included in the contour that was drawn. The image may have rough, jagged borders but it will be quicker and the file size will be smaller.
- *Scan A Rectangle Around The Contour*: A rectangle is generated that contains the entirety of the contour. All fields within this rectangle are scanned and included in the final output image. The final image doesn't have rough edges but acquisition time and file size are increased.
- *Scan In Grid*: Use to define the area to be scanned as a set number of fields of view in a rectangular grid. This option requires that you be positioned at the top left corner of the region to be scanned; the current field of view is the first one scanned.

Test the settings over a 3x3 grid before performing the full acquisition to ensure that the background image is satisfactory, that the trim and blend are appropriate, and to determine whether a stage delay is needed. This will save you considerable time!

## STEP 2: SET TRACING MAGNIFICATION

### *Adjust Microscope to Trace Region(s)*

- Select a lens from the *Select Low Mag Lens* drop-down menu.
- Ensure that the matching objective is selected on your hardware.

We recommend an objective with which you can view a large portion of your ROI.

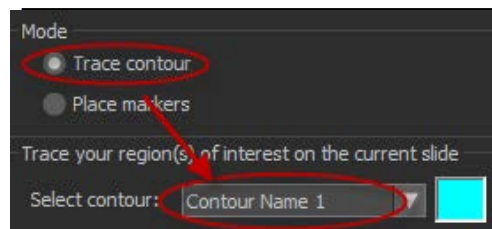
If it is difficult to distinguish the boundary of your ROI, you may need a higher magnification.

## STEP 3: TRACE

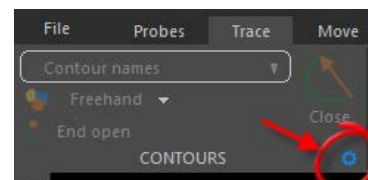
### *Trace contour*

Use this option if you selected one these scanning methods: *Scan Inside Contour Only* or *Scan A Rectangle Around The Contour* .

- Select a contour type from the *Select Contour* drop-down menu then draw the contour.



To change contour names, from the TRACE ribbon bar, click the cog icon to open CONTOUR preferences.

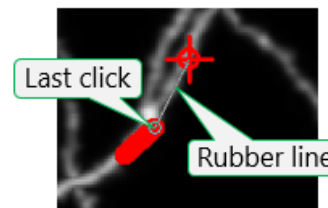


- Trace the contour around your ROI.

Optional: Check *Automatically Move The Specimen When Tracing* if your contour extends beyond the current field of view for a continuous, aligned tracing outside the displayed frame.

About the tracing methods: To use a different tracing method, right-click in the *Tracing* window and select the desired method.

- Simple click tracing:** This is the default method. Click to place a point along an object, move the cursor to a new position then click again to draw a line segment between the two points. Continue to place points until the entire object has been traced.
- Rubber line tracing:** This method is similar to simple click tracing. As you move the cursor to a new position, a "rubber line" is drawn from the last point you placed to your current cursor position.



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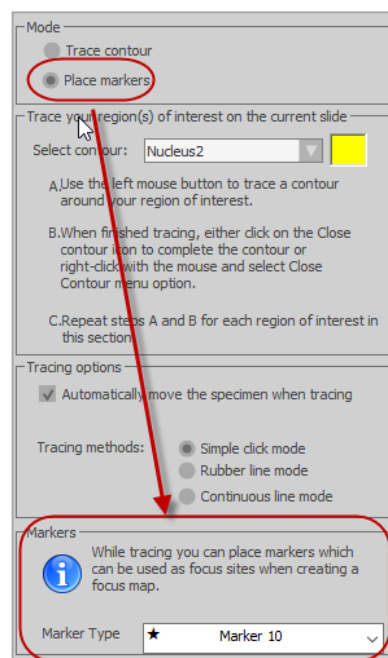


- *Continuous tracing*: Trace by dragging the mouse.
- c. When finished, right-click and select *Close Contour*.

## Place markers

You can place markers that will be used as focus sites in **step 9**.

- Select markers from the *Marker Type drop-down list* at the bottom of the panel.
- Click in the image to place markers that will be used as focus sites.



## STEP 4: SET ACQUIRE MAGNIFICATION

### ADJUST MICROSCOPE FOR ACQUIRING

- Choose the most suitable objective to see the cells of interest.
- Select the corresponding lens from the drop-down menu *Select Acquire Lens*.

#### About Joy Track

Use *Joy Track* to maintain the alignment between tracing and traced tissue when you move the stage with the joystick. The program tracks stage movement in (X, Y, Z) to maintain alignment.

## STEP 5: REGION SELECTION

Click inside a contour to select it for scanning.

If you only have one contour, it is automatically selected.

## STEP 6: BACKGROUND CORRECTION

Depending on the modality you selected in **step 1** (i.e., brightfield or fluorescent), you'll see different *Setup* options in the *Background Correction* panel.

### Background correction for brightfield

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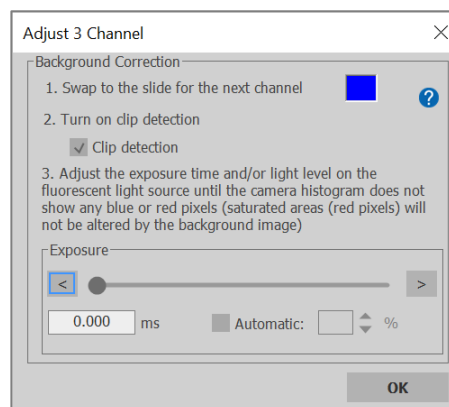
- Check *Joy Track* and navigate to an area on the tissue where some white area is visible.
- Check *Clip Detection*. This will fill any white areas that are overexposed in the field of view with dark red pixels.
- Adjust the exposure to allow for only a few scattered red pixels in the white areas (the image should be very bright but not saturated). Keep the exposure between 10 and 100 ms.
- Click the *White Balance* button to obtain automatic white balance. You also have the option to perform a manual white balance using *Camera Settings*.
- Click the *Acquire Background Correction Image* button.

Do not adjust the exposure time or light level once you have acquired your background image.

## Background correction for fluorescence

This procedure requires Chroma background correction slides. There are Chroma blank slides for each channel; these slides provide a uniform illumination and their intensity is even throughout the field of view. You can purchase those slides through Chroma or MBF Bioscience.

- Focus on your tissue so that you can get close enough to the actual focal plane you'll be using.
- Check *Clip Detection*.
- Click the *Acquire Background Correction Image* button.
- Remove the tissue slide from the stage and place the Chroma slide for the first channel on the stage.
- Because the live image is probably very bright or red (because saturated/completely white pixels are shown in red when clip detection is turned on), decrease the exposure time with the slider and/or use the remote control to turn down your fluorescent light source's intensity until only a few scattered red pixels remain visible.
- Click OK to acquire a background correction image for this channel (cf. screenshot *Adjust 3 Channel*).
- Remove the Chroma slide for the first channel, and place the Chroma slide for the second channel on the stage, then repeat steps e-g for each subsequent channel.
- Place your tissue slide on the stage again. It should still be in focus as a result of step a; if not, focus again.



After you have acquired your background correction image, adjust the exposure time and histogram settings for each channel.

- Proceed to the next step in the workflow.

When using background correction, we recommend that you acquire a sample image at **step 8** of the workflow.

## STEP 7: SCAN SETTINGS

Select the settings that will be used to scan your slide.

### *Stage Settle Delay*

If you are acquiring an image with brightfield illumination, the time delay is used to allow time for the stage in XY to stop moving and any vibration to die down.

For your test montage:

- Set this value to "zero" first
- Increase the value only if you see signs that the stage is still moving when the image is captured (e.g., blurred image).
  - If there are artifacts with a zero time delay, 100 ms is usually a sufficient delay.
  - With some of the slower stages (especially Prior stages), delays of up to 1000 ms may be needed.
  - If you are acquiring fluorescent or other low light images that require integration by the frame grabber, enter a value larger than the time of the integration (as much as two times the integration time, but start with a number only slightly larger, and increase if you see text in some individual tiles). *The software does not automatically know how long the acquisition is, so be sure to allow time for the frame grabber integration.*

This option has no effect on 3D scans.

### *Pixel trim images*

This option removes rows and columns of pixels from the edges of each field of view (image tile).

It allows you to correct for spherical aberration or a video card that acquires with a black or white strip on any of the image edges.

Try a test acquisition with all of these values set to zero, and increase them if there is a problem in the final image.

- a. Acquire a single image
- b. Zoom in on the top left and bottom right corners. Any rows or columns of bad pixels are immediately evident.
- c. Set the trim values to remove the unwanted pixels.
  - For low magnifications, 0-10 pixels is a suitable range.
  - For higher magnifications, larger trims (25-50 pixels) may be appropriate.
  - If lighting is very uneven across the field of view, large numbers of pixels may need to be removed to account for unevenly lit portions of the image

Try to correct lighting before resorting to trimming.

- When many pixels are trimmed, the fields of view in the resulting virtual slide are made smaller, and smaller stage movements are made accordingly.
- High values for trimming will slightly increase your acquisition time, since more acquisition sites are required.

## Seam blending

This option blends the edges from each field of view smoothly.

Select the number of pixels to blend at each seam. Generally, 5-10 pixels is sufficient.

## Background color

Use if the image scan type selected in **step 1** is *Scan Inside Contour Only*.

- The color (white or black) is added the edges of the image.
- Generally, white is used for brightfield imaging while black is used for fluorescent imaging.

## STEP 8: SAMPLE ACQUIRE

Use this step to test your settings.

## STEP 9: START SCANNING

Set the options as desired then click *Start Scanning At Current Z*. Details about each option are provided below:

### File location

Select a location for your scanned images.

### Keep image open

Displays the image in the *Image Organizer* when the scan is complete for immediate inspection.

### Compress tile files

Reduces the size of the image files. Use the *Compression Ratio* slider to adjust the compression. Generally a compression of 10:1 or 20:1 is adequate (meaning a compression of 20:1 will take a 100 MB image file and compress it to around 5 MB).

### Remove temporary files

Individual image files are saved in a folder until they are compiled into the final image montage at the end of the acquisition.

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Check *Remove Temporary Files* to delete the individual files once the image has been compiled (this will free up disk space).

## Postpone compilation

Individual image files are acquired and saved in a designated folder, but there is no compilation performed.

Uncheck *Keep Image Open* and *Remove Temp Files* to enable the *Postpone Compilation* option. Then use the Compiler (**Image>Compilers>Slide scanning**) later when you are ready to create the montage.

## Capture tissue

Enter the distance in microns if the image needs to be acquired below the focus map.

Useful if you want to create the focus map at the top of the tissue, but capture the image a set distance into the section thickness.

- When you designate a distance to acquire below the focus map, the first plane of the stack is acquired starting at that specified distance.
- If you enter “0”, acquisition will occur at the designated focus map locations, or at the specified top of stack.

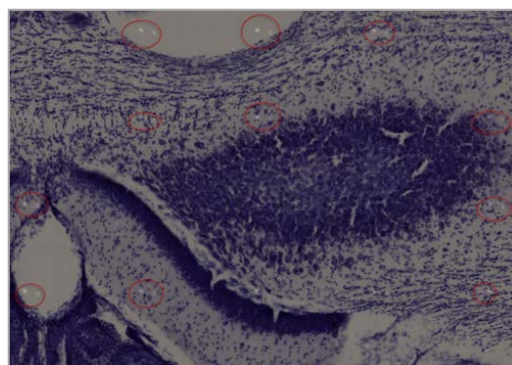
## TROUBLESHOOTING FOR BRIGHTFIELD

Several common problems and their solutions are outlined below:

**PROBLEM 1:** The image is dark which is probably due to poor lighting.

### SOLUTION

- a. Adjust light level and/or exposure level.
- b. Acquire a new background image (see **Step 6**). Ensure that live image and background image are acquired at the same light level.

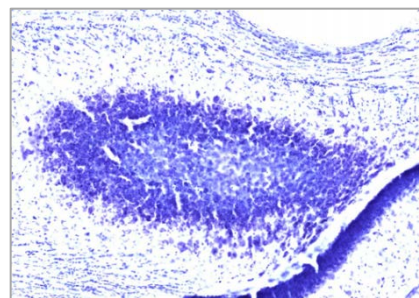


**PROBLEM 2:** There are bright specks consistently spaced throughout the image which suggests the presence of debris.

**SOLUTION:** Acquire another background image free of debris (see **Step 6**) OR acquire a quick test image in the current field of view to ensure that the background image is suitable.

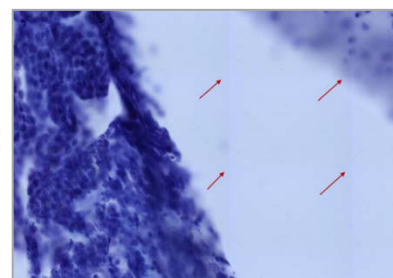
**PROBLEM:** The image appears washed out; the over-saturation is probably caused by an excessive light level.

**SOLUTION:** Turn down the light level and/or exposure and use *Clip Detection* again (see **Step 6**) and ensure that the background image and live image are acquired at the same light level.



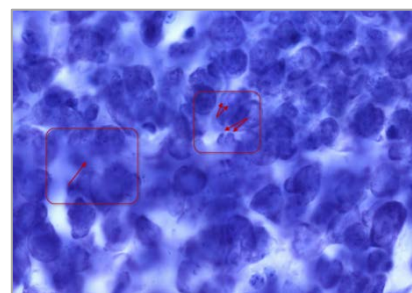
**PROBLEM:** There are distinct horizontal and vertical seam lines on every image tile because either no background image was used, or the background image has uneven lighting.

**SOLUTION:** Capture another background image (see **Step 6**) and verify that lighting is uniform, especially around the edges of the image.



**PROBLEM:** There is "doubling" or blurring along horizontal and vertical seam lines, caused by either an incorrect microscope calibration or an insufficient trim.

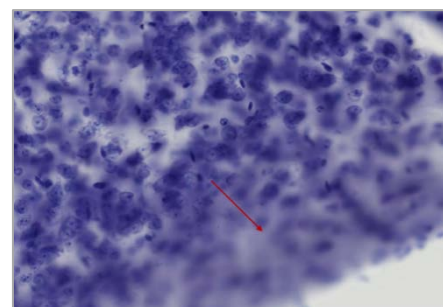
**SOLUTION:** Verify that the microscope is properly calibrated (i.e., the camera is aligned, lenses are calibrated, stage movements are functioning properly); turn up the trim on the appropriate edges using the Slide scanning settings in **step 7: Pixel Trim Images** and *Seam Blending* options; If trimming does not work, a fine-tune manual calibration may be needed.



**PROBLEM:** There are large out-of-focus areas in the image, most likely because there were not enough focus sites.

**SOLUTION:**

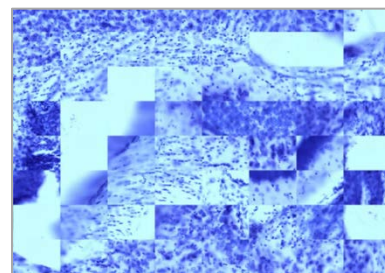
- a. Try adding more focus sites to the focus map (see **Step 9: Define focus map**).
- b. Verify that the focus sites are consistently dispersed throughout the contour or grid.
- c. If stage drift is the issue (and you allowed your microscope to warm up prior to acquisition), modify the focus adjustments settings by adding a manual focus interval. A manual focus interval enables you to adjust the focus during the imaging process, and to periodically update the focus map to compensate for drift.
  - First, try adding a manual focus 3-4 times throughout the image and see if it helps (e.g., if the image has 2000 tiles, add a manual focus every 500 sites).
  - Adjust as needed.





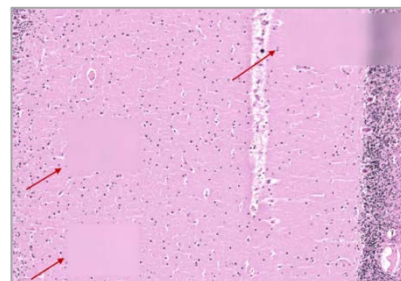
**PROBLEM:** There are very distinct tile lines and individual tiles are not lined up. This is likely caused by using mismatched objective and software lens.

**SOLUTION:** Verify that the lens selected in the software matches the objective selected on the microscope.



**PROBLEM:** There are out-of-focus tiles dispersed throughout an otherwise in-focus image. In our experience, this occurs with extremely “wavy” tissue with drastic differences in z-depth between focus sites. The focus map interpolation cannot account for these large differences resulting in out-of-focus tiles.

**SOLUTION:** Try increasing the number of focus sites in the focus map to create a tighter triangulation grid (see **Step 9: Define focus map**).



## TROUBLESHOOTING FOR FLUORESCENCE

Most common: [Uneven illumination / vignetting / tiling / lines](#)

There are multiple possible causes. We recommend that you start by acquiring a 3x3 test image with all your channels, keeping the exposure times low (less than 200 ms to limit bleaching) and examine the results.

Next, we'll address three specific aspects:

- A. Uneven illumination is visible in all channels in the test image
- B. Uneven illumination is present in some (but not all) channels in the test image
- C. The problem is with one channel only

Let's start!

- A. UNEVEN ILLUMINATION IS VISIBLE IN ALL CHANNELS IN THE TEST IMAGE

The issue is likely related to the light source. Here are some options to resolve this issue:

- Make sure that your light source is centered and aligned. Because the procedure for this varies from light source to light source, we recommend that you contact your vendor, local microscope representative, or someone from the MBF Bioscience Tech Services team.

Many direct-coupled light sources (incl. Zeiss Colibri, X-Cite 120LED, X-Cite 120 Boost) do not have centering options.

Liquid light guide light sources (e.g., Lumencor SOLA) can potentially have problems with the liquid light guide cable. Most liquid light guide cable manufacturers

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recommend replacement every two years, regardless of usage. Replacements can be purchased through the manufacturer or MBF Bioscience.

OR

- Increase the trim/blend settings in **Step 7**. Note that this may hide rather than fix the problem, will increase acquisition time, and may worsen bleaching of adjacent sites (see below). Typically a trim of 20 - 100 pixels is recommended.

OR

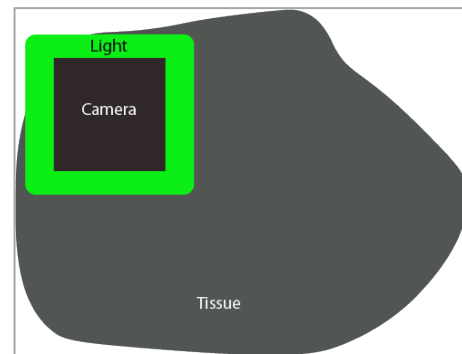
- Use background correction (**Step 6**). This may alter the signal-to-noise ratio in your image.

B. UNEVEN ILLUMINATION IS PRESENT IN SOME (BUT NOT ALL) CHANNELS IN THE TEST IMAGE

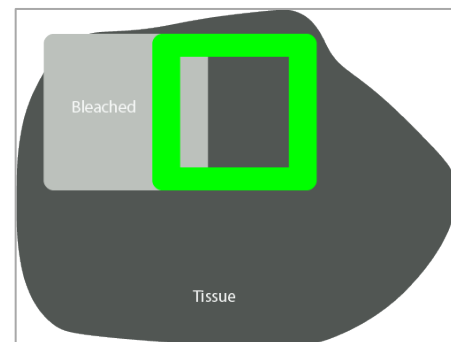
One cause is the possible bleaching of adjacent sites.

a. *Why bleaching occurs*

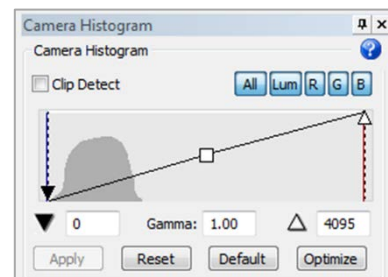
When scanning fluorescent tissue, the light source tends to cover a larger area of tissue than the camera's field of view.



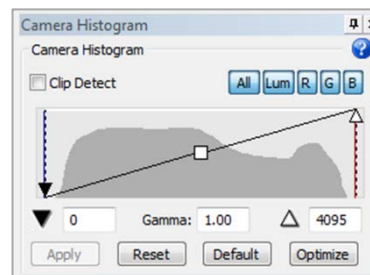
This is problematic when scanning the next sites because the area that was hit by light previously gets bleached, and it results in "vignetting" or "lines" in the scan.



In addition, the camera histogram is typically optimized to obtain a wide dynamic range. That is, if your histogram looks like the one on the right...



... you would typically increase the exposure time so that the histogram looks more like this one on the right. The problem is that increasing the exposure time with tissue that is prone to bleaching exacerbates the “adjacent tile” bleaching issue (often substantially).



## b. What you can do

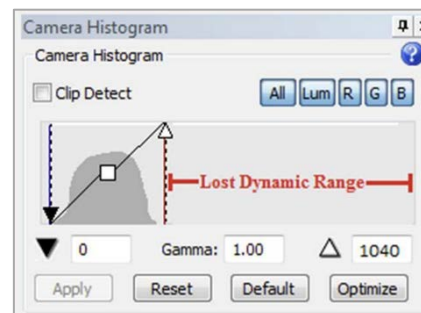
### Improve tissue preparation

This type of problem is less common with tissue that’s been properly stained and mounted so it may be worth investigating your tissue preparation protocol for any opportunity to improve the stability of your fluorophores.

If this issue occurs in the DAPI channel (usually the most robust channel), that clearly points towards a problem during tissue preparation. It is less clear for other channels; there are certainly some fluorophores that are much more prone to bleaching than others.

### Adjust the dynamic range

With cameras that have a dynamic range greater than 8-bit (e.g., Hamamatsu cameras), you can lower the dynamic range by moving the white point slider, like this on the right. This approach will allow you to use a lower exposure time, and the decreased dwell time on each site will prevent a lot of the bleaching.



This is not recommended with an 8-bit camera as it will result in a grainy image.

### Image Illumination Correction

You can use our Illumination correction tool (in the *Image* ribbon bar) to correct this issue. It is likely to work well but it greatly increases the time required for that acquisition (more than double) when applied during the acquisition, and it can’t be applied to images opened in virtual image mode (that is, to slide scans) after acquisition.

### Aperture insertion into the light path (Zeiss)

Zeiss makes two apertures: a square one (the XY) for the F slot, and a circular one for the A slot.

- If the F slot is not in use, insert the XY aperture in the F slot.
- If you have an ApoTome in the F slot, do not remove it; use the A slot instead to insert the circular A aperture.



- Only use the A slot for the aperture if you are equipped with an ApoTome.
- The A aperture may partially help with this issue, but it will not be as effective as the XY aperture.
- Closing down either of these apertures too much will exacerbate the uneven illumination.

Both apertures are shown in the photo for illustration only; they should NEVER be used together!

## Decreasing the light source intensity

It may help although LEDs typically run at 100% intensity to keep exposure times as low as possible.

Decreasing the intensity will probably require a higher exposure time, which may result in a similar amount of bleaching, and will cause slower scans – meaning that there's basically no advantage to lower light intensity in most cases.

## Background correction

Background correction will not help with this, since it is caused by bleaching of adjacent sites, and not a truly uneven illumination.

## How can you rule out bleaching?

To rule out bleaching as the cause for uneven illumination, we recommend acquiring an image with a Chroma background correction slide.

There are Chroma blank slides for each channel; these slides provide a uniform illumination and their intensity is even throughout the field of view.

You can purchase those slides through Chroma or MBF Bioscience.

## The problem is with ONE channel ONLY

This may be the result of a filter cube issue.

To resolve this, move the filter cube to a different position, make sure it's properly seated and secure, and then replace the cube.

Note that:

- With high exposure times (above 300-500 ms), a variety of artifacts can appear, especially random red/green/blue pixels on color cameras.
- Older cubes do burn out, but we generally only recommend replacing a cube if, for example, there is no trace of uneven illumination for red and green but blue shows uneven illumination, even with an appropriate exposure time and a known appropriate light source.
- Some light sources (such as the X-Cite 120 LED) use different bulbs for UV (DAPI) and other channels, so it's possible (albeit rare) to have a light source issue that occurs only in one channel.

C. THERE IS NO ILLUMINATION PROBLEM WITH THE TEST IMAGE, BUT THE FINAL ACQUISITION HAS AREAS THAT SEEM DARKER

Try using the focus map's heat map (**Step 9**) to look for large changes in Z before the acquire starts. Large Z differences between sites can cause out of focus sites that look like uneven illumination.

It is common to have some areas of the tissue that are brighter or darker than others. Make sure that you optimize your camera histogram for the brightest area you are interested in, and adjust it post-acquisition using the *Image Adjustment* panel to improve dimmer areas' appearance (gamma adjustment is typically a good option).

Increase the number of focus sites **ONLY** if certain areas are darker because they are out of focus, not simply because they're dark.

## Other issues

- A. "DOUBLING" OR POOR XY ALIGNMENT BETWEEN SITES  
To solve this issue, review your calibration.
  
- B. THE IMAGE IS TOO DARK, TOO BRIGHT, OR GRAINY  
To solve this issue, adjust the histogram before the acquisition using the *camera histogram* or after the acquisition using the *Image Adjustment* panel.
  
- C. OUT OF FOCUS AREAS  
To solve this issue, make sure that your focus map has enough sites in **step 9**.