CONTENT

Before you start, make sure that you have a properly calibrated system to obtain high-quality images.

STEP 1: OPTIMIZING YOUR WORKSPACE FOR ACQUISITION

Set up your workspace to have the tools you need ready for use. We recommend the following:

- **AutoMove**: Select **Move**>**AutoMove**.

- **Macro View**: Click the icon .

- **Image Organizer**: Select **Image**>**Image Organizer**.

- **Z-meter**: Click the icon .

- **Image Acquisition**: Select **Acquisition**>**Acquire Image Stack** to open the *Image Acquisition* window.

- **Camera Settings/Camera Histogram/Multichannel Control**: Select **Acquisition**>**Video Tool Panels**>**Show all video tools**.

> **To optimize space...**
>
> Dock the windows on the side of the screen OR, if a second monitor is available, move the Macro View and Video Tool Panels onto the second monitor to maximize the field of view on the primary monitor.

STEP 2: TRACING THE REGION OF INTEREST

Now that your workspace is ready, trace the regions that you wish to acquire with *Virtual Tissue*.

> **Are you using fluorescence?**
>
> Set up the **Device Command Sequences** before you start tracing.

1. Select the objective that will be used for the acquisition and verify that the matching lens is selected in the software.
2. Adjust the light on the microscope so that it is comfortable to look at the slide through the oculars.
3. **Live Image** mode is on (**Acquisition**>**Live Image** is checked).
4. Load the slide onto the microscope and bring the slide into focus at a low magnification (2x or 5x).
5. Using the joystick, bring the desired location into view.
6. Use **Joy Track** to maintain registration between stage movements and your contours.
7. To begin tracing, turn off the **Joy Track** mode:
8. Right-click and select **End Joystick Mode**.
9. Begin tracing a contour around the first region of interest on the slide, staying close to the tissue.
10. Continue tracing around all the sections to be acquired.

    - Trace around the entire brain section (or even multiple brain sections) or around a single structure in the brain.

    > **Virtual Tissue** creates an image file for each region delineated by a contour. To obtain an individual image for each section, trace a contour around each section.

    - Keep the contour close to the tissue to reduce acquisition time and make it easier to place focus points:
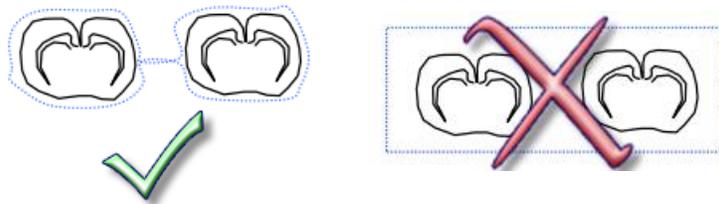
9. When finished, right-click and select **Close Contour**.

> You can't acquire regions of interest defined as **Open Contours**.

STEP 3: CAMERA (& MULTICHANNEL) SETTINGS

Before starting the acquisition process, adjust the camera settings (ALSO adjust the multichannel settings for fluorescence).

Settings for Brightfield

1. Perform a white balance to correct for any coloring imbalances.

    -   Find an area on the tissue or slide that should be white (e.g., slide background or small hole in the tissue).

    -   In the *Camera Settings* window, click the **White Balance** icon ▣ then click in an area on the live image that doesn't contain any tissue. This should result in a more appropriately colored image.

2. Adjust the camera settings: For best results, set the **Exposure** between *10 and 100 milliseconds*.

    -   If the image is too dim, adjust the light level on the microscope until it looks acceptable.

3. Verify that the camera settings are appropriate with the **Camera Histogram**:

    The histogram displays a graphical representation of the brightness and contrast of the current live image.

    -   Adjust the camera settings until a very broad histogram is achieved.

    -   The gray area should reach broadly to the edges of the graph, but should not be cut off.

    -   Use the **R, G,** and **B** buttons to view individual colors within the histogram.
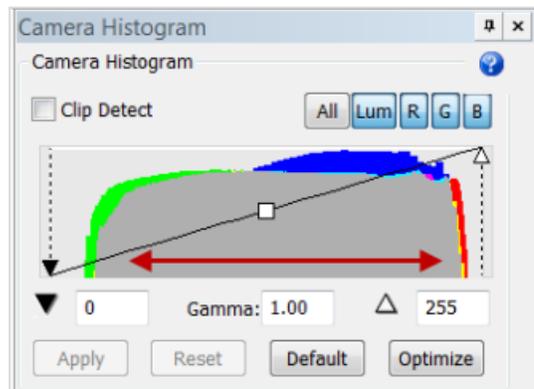
Settings for Fluorescence

1. The **Multichannel Control** panel displays the channels configured earlier.

   Optional : Click the **Setup** button to review the channel's settings.

   a. Check the **Remember Video Settings** box

   b. Click the **Device Command Sequence** button for the first channel; adjust the exposure in the camera settings until a full balanced histogram is achieved.
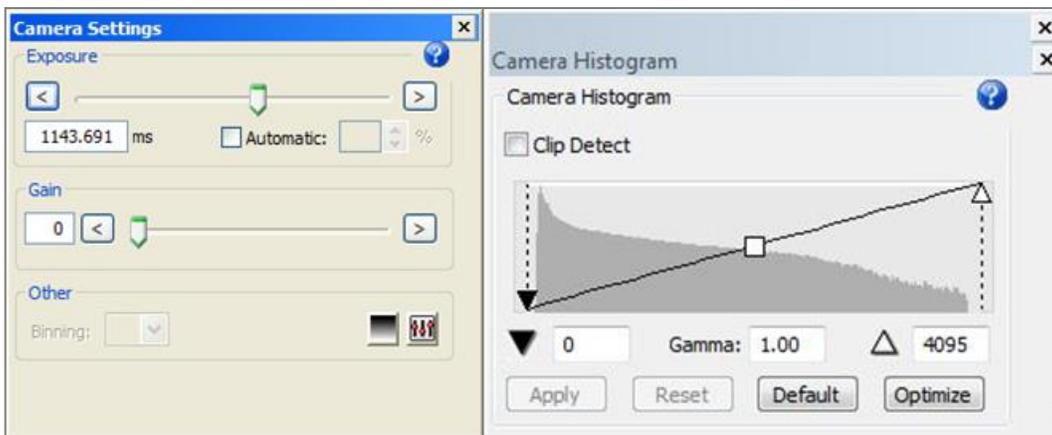
   c. Repeat **step b** for the other channels.

   d. The software has "memorized" the camera settings for each channel and is ready to apply them for acquisition.

2. Select **Acquisition**>**Acquire Multichannel Image**.

   a. The software performs the acquisition tasks defined for the first channel.

   b. The software prompts you to adjust the camera settings. Click **OK**.

   c. The software performs the acquisition tasks for the other channels defined.

3. Close the shutter to prevent the tissue from bleaching while setting up the acquisition.

4. Verify that the appropriate Device Command Sequence *Presets* have been set up.

   a. In the **Multichannel Control** panel, click **Setup**.

   b. In the **Acquire Setup** window, click the **Presets** icon 🔳 in the bottom left corner . You should see the name of your automated set of tasks.
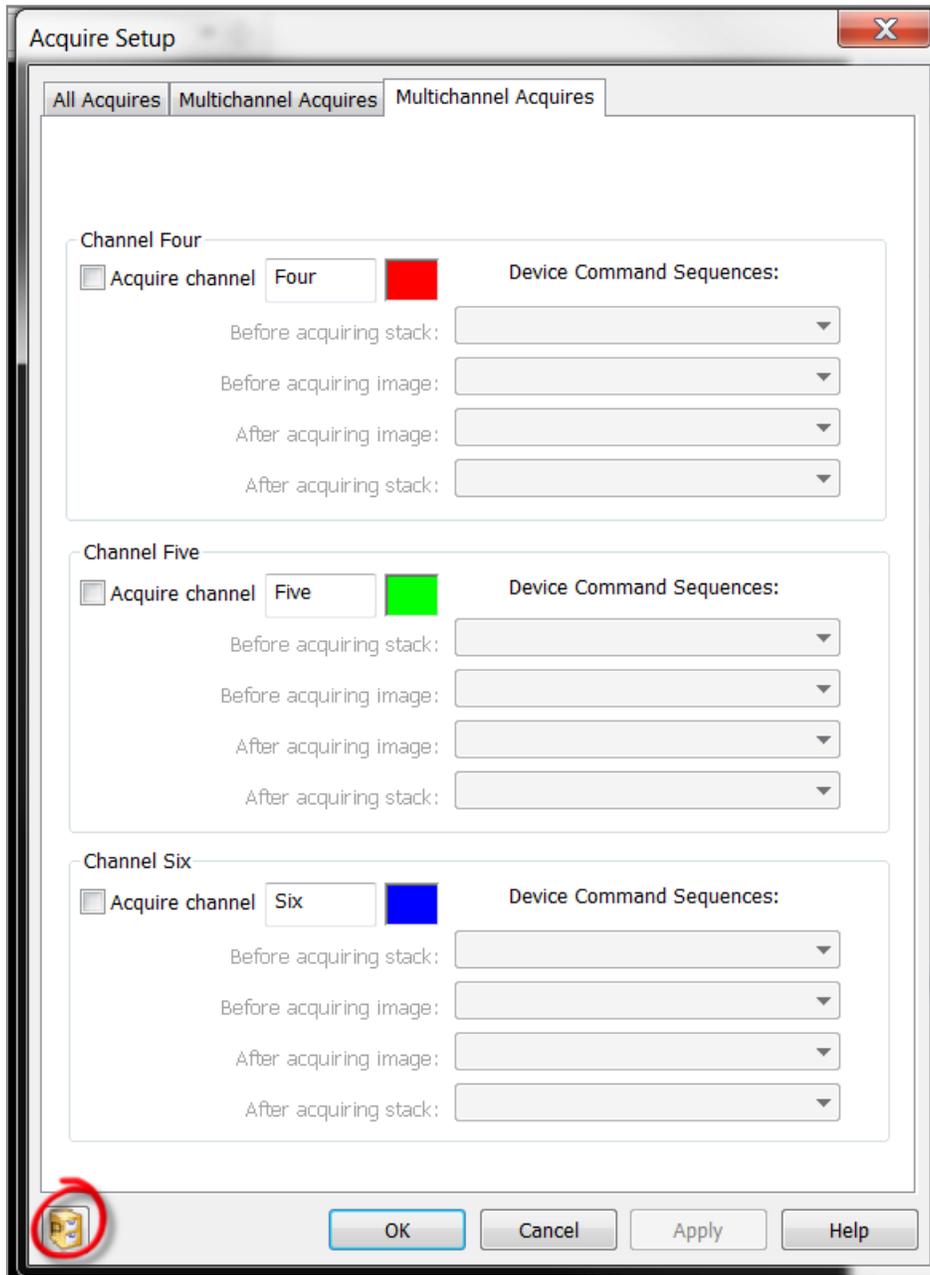
## STEP 4: ACQUIRING A BACKGROUND IMAGE (BRIGHTFIELD)

Acquiring a background correction image over an empty region of the slide allows the software to correct for uneven illumination and to reduce tiling. It ultimately yields a much smoother *Virtual Tissue* image.

1. Navigate to an area on the tissue where some white area is visible. In the **Camera Histogram** window, check **Clip Detect**.

    - **Clip Detect** fills white areas that are overexposed in the field of view with dark red pixels.
    - If nothing appears when it is enabled, raise the exposure or light level until the red pixels appear; keep the exposure between 10 and 100 ms.
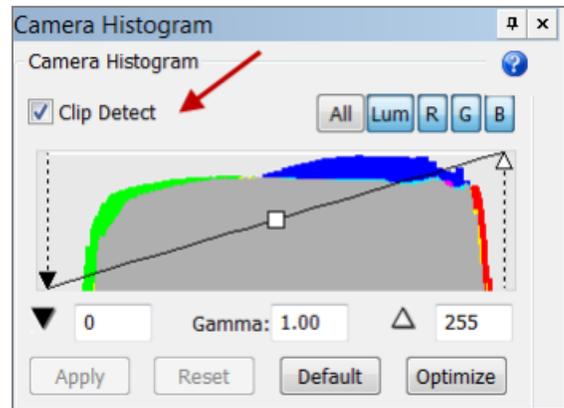
2. Adjust the **Exposure** to allow for only a "light dusting" of red pixels in the white areas.
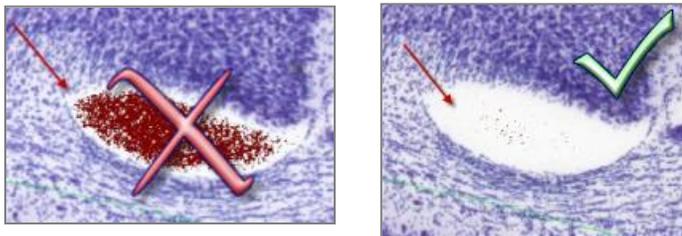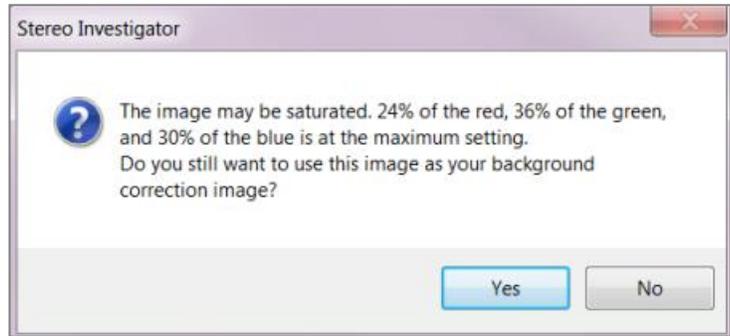

3. Once a light dusting of red pixels has been achieved, perform another white balance.

4. When the *white balance* is finished, you should still see a light dusting of red pixels in the white area.

    - If more or less pixels appear after the *white balance* is complete, adjust the *exposure* and try again until the amount of pixels is roughly the same as before the white balance.

5. When finished, uncheck **Clip Detect** to turn it off.

6. Using **Move>Joy Track** and the joystick, navigate completely off the tissue to a blank space on the slide.

    > If there is tissue or debris in the field of view, you can't obtain a background image that is completely white.

7. Click **Acquisition**>**Acquire Background Image** to acquire the background image.

8. Select the **Type of background correction**, check **Enable background correction**, and click **OK**.

- If a message appears indicating that the image is saturated, lower the light level or exposure. Make some slight adjustments and try again.

- The most recent background image taken is the one used for the acquisition, even if multiple background images appear in the **Image Organizer**.

> **Stereo Investigator**
>
> The image may be saturated. 24% of the red, 36% of the green, and 30% of the blue is at the maximum setting.
> Do you still want to use this image as your background correction image?
>
> Yes   No

9. Once the background image has been acquired, it is displayed in the **Image Organizer**.

- Ensure that it appears completely white and free of debris (sometimes debris that were not visible in the field of view can appear in the image).

- If there is debris, slightly adjust the location of the background image away from the debris then acquire the image again.

10. When the background image is satisfactory, return to the live image by clicking **Acquisition>Live Image**.

> If all the acquisitions are performed at the same magnification and with the same tissue, one background image can often be used for all images.
>
> Keep the background image "open" for each new image.
>
> A new background image needs to be acquired when there are changes to the tissue, slide, magnification, or light level.

STEP 5: ACQUISITION SETTINGS

Adjust the settings for the acquisition of the image tiles that will constitute the virtual slide.

Prerequisites

- *Live image* mode is on.
- Reference point is placed.

Process

1. Click **Acquisition**>**Acquire Virtual Tissue**.

2. The *Virtual Tissue Acquire* dialog box appears ; adjust the settings as needed.
   - When you are satisfied with the settings for acquisition, click **OK**.

3. Save the image in the desired location as prompted; name the image and select the file type from the drop-down menu:
   - 2D: .jp2 (JPEG2000 or MBF JPEG2000) or .tif (TIFF)
   - 3D: .jpx (JPEG2000 or MBF JPEG2000)
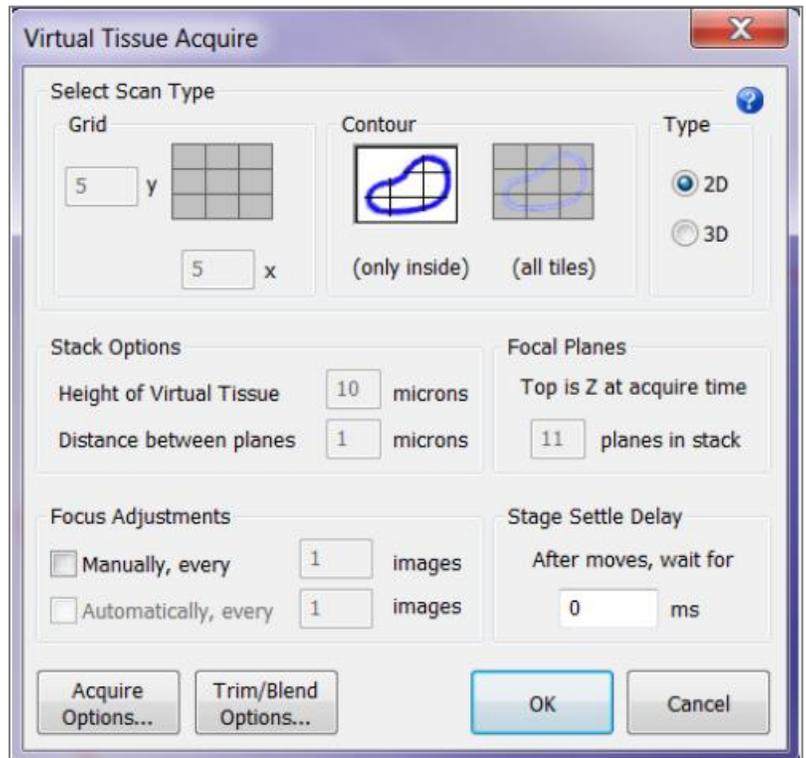
About MBF JPEG2000

- **Benefit**: Retains additional information about the image unique to the system setup (pixel scaling, raw image data, application version, etc.). Useful for troubleshooting.

- **Potential drawback**: May not open in some third-party applications.

Settings

Select Scan Type

Grid: Use to define the area to be acquired 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!

Contour: Use a closed contour.

- **(only inside):** Processes tiles included in the contour that was drawn.

  The image may have rough, jagged borders but you will save on acquisition time and file size.
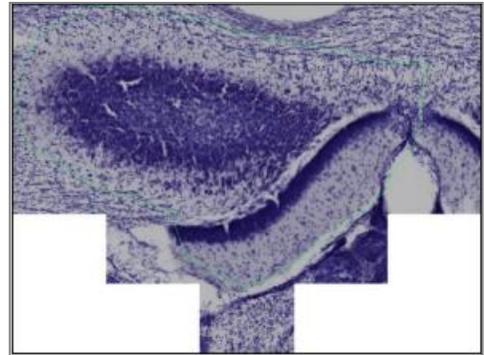
- **(all tiles)**: A rectangle containing the entire contour is generated. All fields within this rectangle are scanned and included in the final *Virtual Tissue* output.

  The final image doesn't have rough edges but acquisition time and file size are increased.
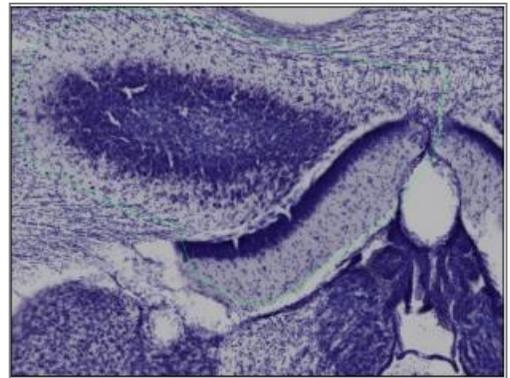
Type

2D: Appropriate for a single image plane.

3D: Appropriate when more than one image plane is needed to create an image stack.

Generally appropriate at higher magnifications, but can be used at lower magnification as well (3D is only available if you purchased the *Virtual Tissue 3D* module.)

Stack Options (3D only)

Height of Virtual Tissue: Refers to the desired stack height. The values you choose will affect the processing time.

Focal Planes (3D only)

The number of planes in the stack is determined by the **Stack Options**.

- To modify the number of planes, modify the **Stack Options**' values.

> The software automatically adds the top plane to the number of focal planes.

The total number of images to be acquired is dynamically displayed. This allows you to balance image data acquired with the time and disk space necessary to collect an image volume.

Focus Adjustments

- Uncheck all boxes to rely solely on the focus map (to be set up in the next step).

Manually, every: Check if it is necessary to manually focus the tissue periodically throughout the scan.

- Enter the number of images between each manual focus site.
- The acquisition stops at the specified increments and the software asks you to bring the tissue into focus before continuing.
- If this is being used simultaneously with a focus map, the remaining tiles on the triangulated map are recalculated after each manual focus.
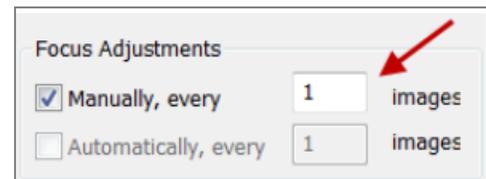
Automatically, every: This option requires special hardware. If this option is grayed out, the hardware is not installed or connected.

> **Useful for stages that drift**.
> Use manual focusing only if you need to repeat a completed scan that shows a significant loss of focus toward the bottom of the image to correct for drift.

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 stop.

For your test montage:

- Set this value to "zero" first.

- Increase the value <u>only</u> if you see signs that the stage is still moving when the image is captured (e.g., blurred image).

  - o If there are artifacts with a zero time delay, 100 ms is usually a sufficient delay.
  - o With some of the slower stages (especially Prior stages), delays of up to 1000 ms may be needed.
  - o 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 **Virtual Tissue 3D** acquisitions.

Acquire Options button

Options available for 2D and 3D

Keep Image Open: Displays the image in the **Image Organizer** when the acquisition is complete for immediate inspection.
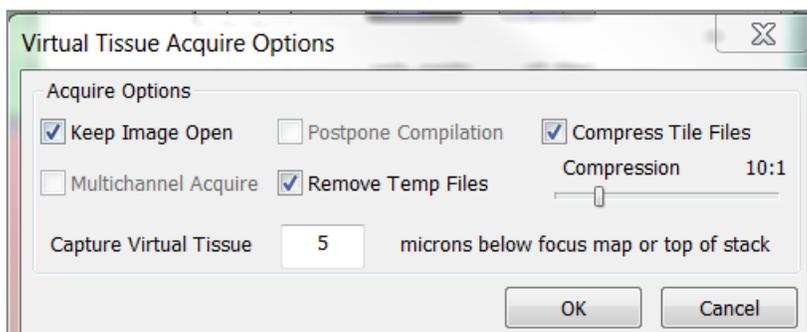
Multichannel Acquire: Uses the existing **Multichannel acquire** setup; adjust the **Multichannel Acquire** settings prior to performing a *Virtual Tissue* acquisition.

Compress Tile Files: Reduces the size of the image files.
- Use the scroll bar 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).

Options available for 3D only

Remove Temp Files: Individual image files are saved in a folder until they are compiled into the final image montage at the end of the acquisition.
- Check **Remove Temp 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.

- Use the **Virtual Tissue Compiler** when you are ready to create the montage.

Capture Virtual 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.

Trim / Blend Options

Pixel Trim Images: Removes rows and columns of pixels from the edges of each field of view (image tile).

This option 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.
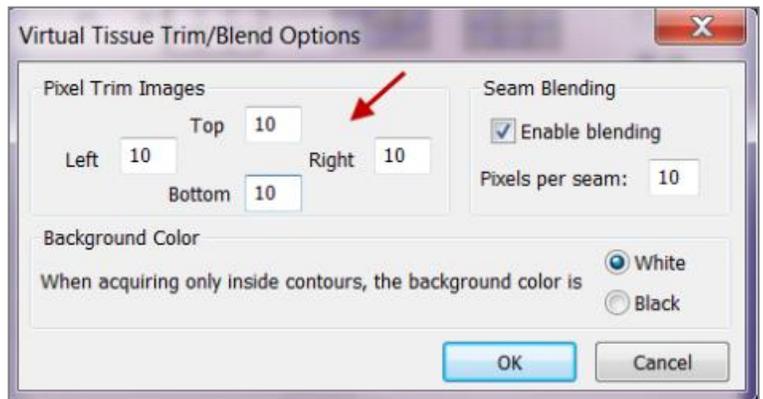
1. Acquire a single image

2. Zoom in on the top left and bottom right corners. Any rows or columns of bad pixels are immediately evident.

3. 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 smaller, and smaller stage movements are made accordingly.

High values for trimming slightly increase your acquisition time, since more acquisition sites are required.

Seam Blending: Blends the edges from each field of view smoothly.

- Use the 3x3 test first to see if your image looks acceptable without blending (try to avoid blending as it involves a slight loss of image detail).
- Select the number of pixels to blend at each seam. Generally, **5-10 pixels** is sufficient.

Background Color: Use if the image scan selected is **Contour (only inside)**.

- The color (White or Black) appears around the edges of the image (white in this example).

- Generally, white is used for brightfield imaging while black is used for fluorescent imaging.
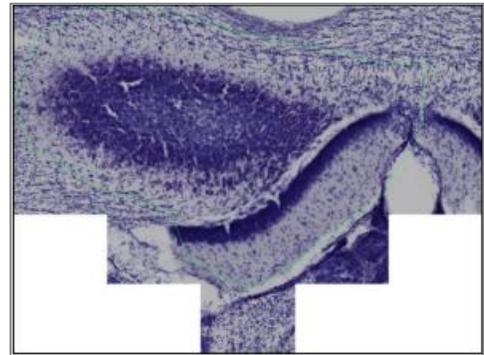
STEP 6: SETTING UP A FOCUS MAP

The acquisition settings are defined; you are now ready to create a focus map.

> If no focus map is required, and the entire scan can be acquired at the current Z -depth, right-click and select **Start Virtual Tissue Scan**.
>
> To terminate the focus map setup and return to the tracing window, right-click and select **Exit without Scanning**.

1. Once the file name and location have been specified, the contour appears inside a grid.
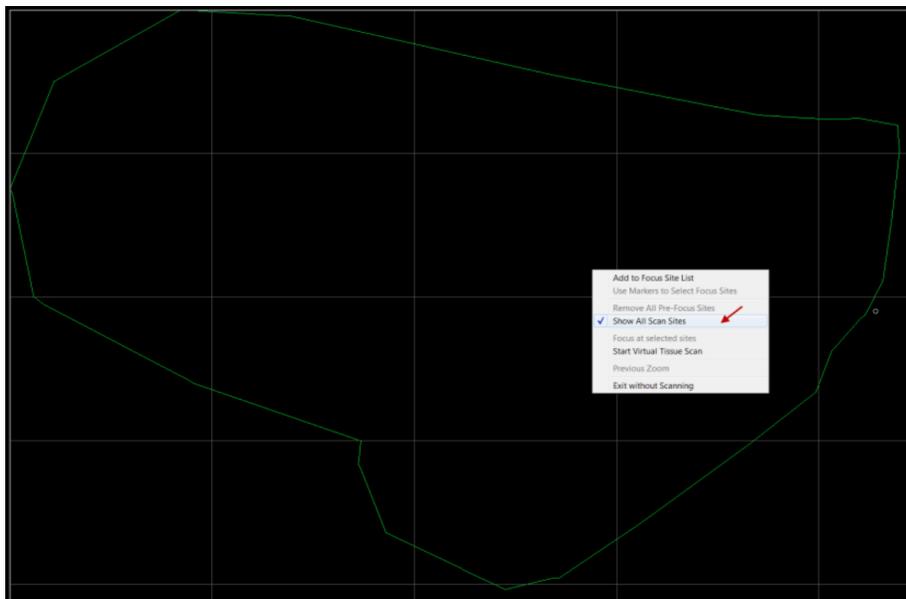

- If no grid is visible, click in the contour to display the grid.

- If clicking in the contour does not work, right-click within the tracing window and verify that **Show All Scan Sites** is checked.

Multiple contours

- For multiple contours, click the contour of interest to create a focus map for that contour.
- To select multiple contours, click each of them.
- The selection order determines order and naming for acquisition. The contour you clicked first is acquired first and its file name is *base name-1*, the contour you clicked second is then acquired and its file name is *base name-2*, and so on.
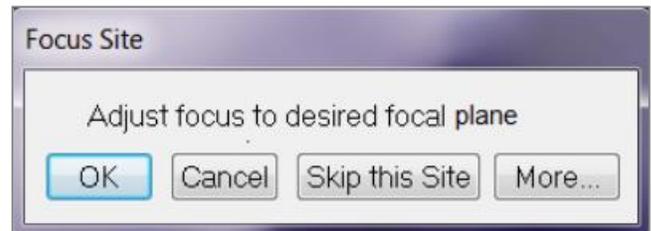
2. Create a focus map for each region of interest to be acquired.

- Hold down the **Shift** key and click several grid cells to create a triangulation pattern between the selected cells.
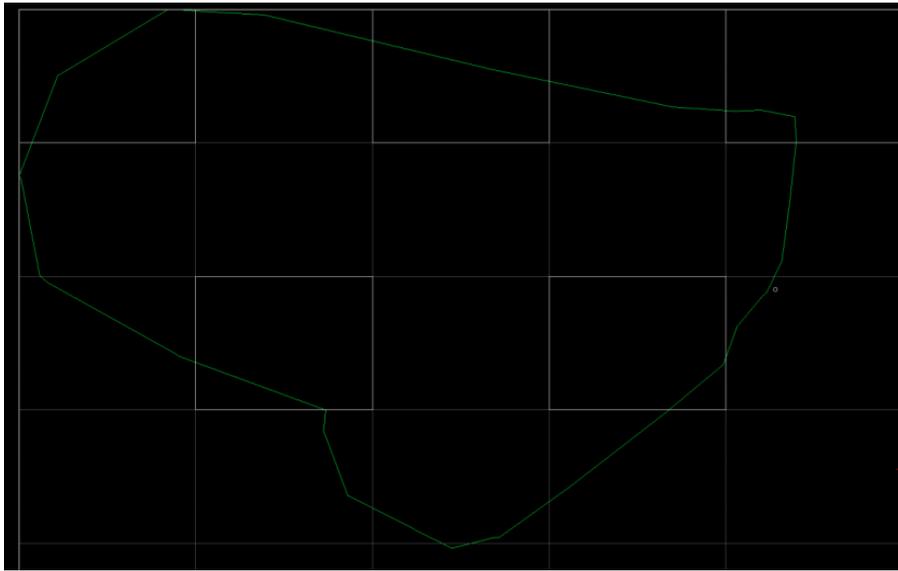
- o To remove a selected cell from the focus map, click it.
- o If the tissue is particularly wavy or uneven, use more cells for a better quality image.

3. Once all the focus sites (i.e., grid cells) have been selected, right-click and select **Focus at selected sites**.

4. The stage navigates to each selected site and the *Focus Site* dialog box opens. Bring the site into focus and click **OK** .

- To skip the current site, click **Skip this Site**; that focus site will be removed from the focus map.

- To exit the focus map, click **Cancel**.

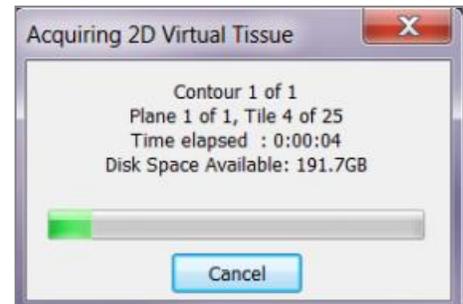- For **Device Control** options, click **More**.

5. When all the focus sites have been set, the focus grid is displayed with an interpolated focus map.

6. To add focus sites, hover the mouse over the desired grid cell, right-click, and select **Add to Focus Site List**.

   • Bring the tissue into focus and click **OK**.

7. Once the focus map is satisfactory, right-click and select **Start Virtual Tissue Scan with Pre-Focus**. The acquisition process begins.

STEP 7: ACQUIRING & COMPILING THE IMAGE

For best results, follow the following guidelines during acquisition:

- For very large/long acquisitions, allow the microscope to warm up for a few hours prior to the process; this helps mitigate stage drift during acquisition.

- Try keeping the lights in the room at a consistent level.

- Avoid touching the table supporting the microscope.

- Avoid walking near the microscope.

- Inform your colleagues that the microscope is in use to avoid disturbing the acquisition process.

1. As the acquisition begins, the software displays the progress status.

   - Click **Cancel** in the progress box at any time to cancel the acquisition.
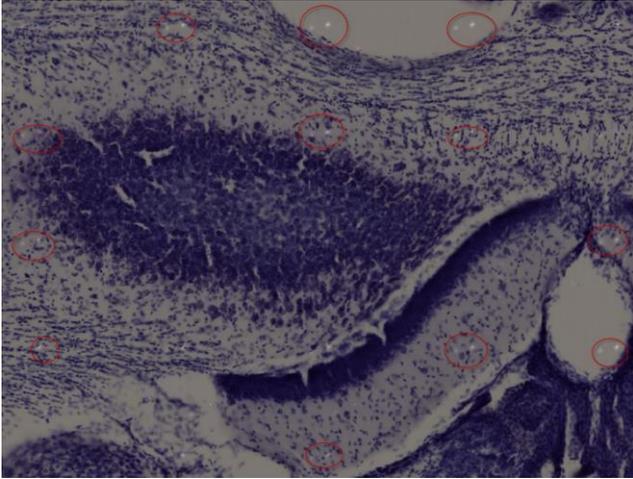
   Acquiring 2D Virtual Tissue

   Contour 1 of 1
   Plane 1 of 1, Tile 4 of 25
   Time elapsed : 0:00:04
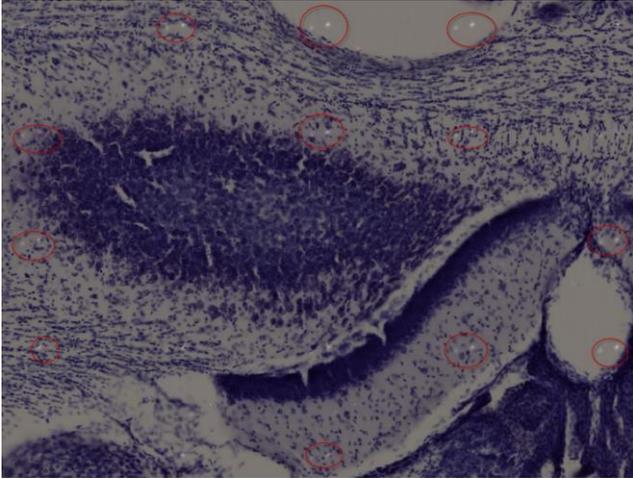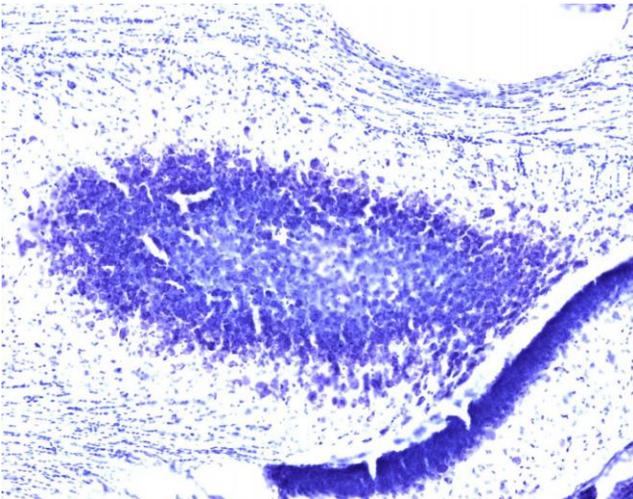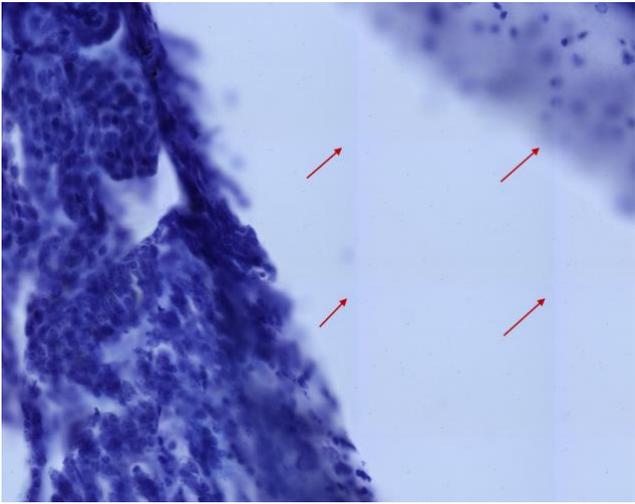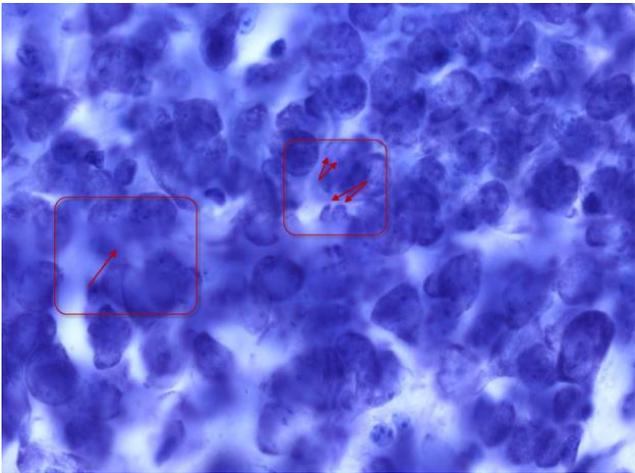   Disk Space Available: 191.7GB

   Cancel

2. The image is automatically compiled after the acquisition has been completed (unless compilation was postponed; see **Step 5: Acquisition Settings**/**Acquire Options button**/**Postpone Compilation** p.13).
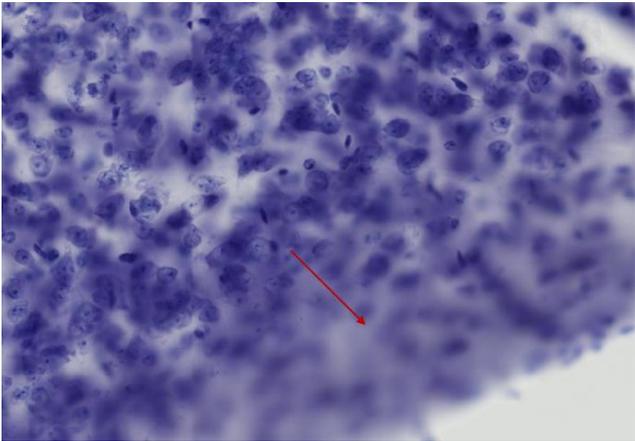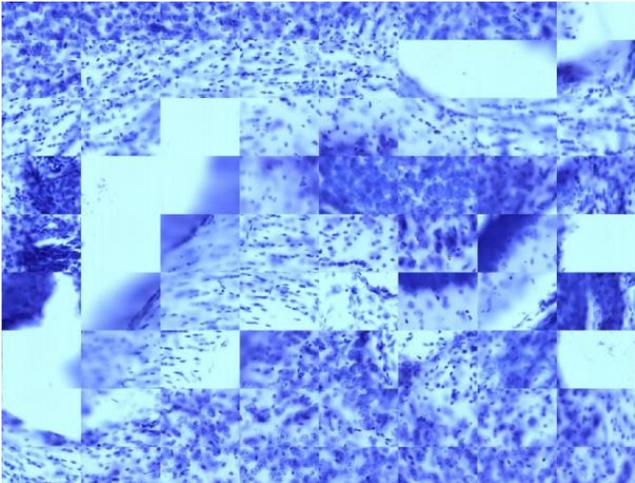
   - If the compilation was postponed (3D only), compile the image later with the **Virtual Tissue Compiler** (select **Acquisition**>**Virtual Tissue Compiler**).
   - If **Keep Image Open** was selected in the **Acquisition Settings**, the image montage is displayed in the **Image Organizer**.
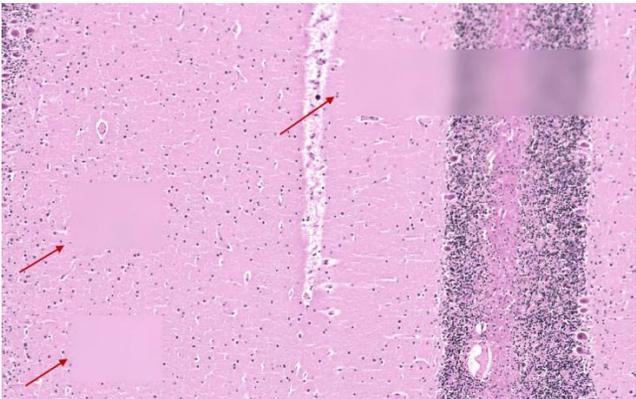   o If **Keep Image Open** was not selected, open the image manually.

   Examine the virtual tissue image upon completion to make sure that the quality is acceptable. For adjustments, return to the acquisition settings and try the acquisition again.

TROUBLESHOOTING IMAGING ISSUES (BRIGHTFIELD)

EXAMPLE	PROBLEM	SOLUTION
	Dark image, most likely due to poor lighting.	Adjust light level and/or exposure level. OR Acquire a new background image (acquire live image and background image at the same light level).
	Bright specks consistently spaced throughout the image, due to debris.	Acquire another background image free of debris. OR Acquire a quick test image in the current field of view to ensure that the background image is suitable.
	Over-saturated or "washed out" image, most likely due to excessive light level.	Turn down the light level and/or exposure and perform the **Clip Detect process** again. Acquire the background image and the live image at the same light level.

EXAMPLE	PROBLEM	SOLUTION
	Distinct horizontal and vertical seam lines at every image tile, due to absence of background image or background image with uneven lighting.	Try enabling background correction (**Acquisition>Enable Background Correction**) or acquire another background image. Verify that lighting is uniform, especially around the edges of the image.
	"Doubling" or blurring along horizontal or vertical seam lines due to insufficient microscope calibration/lack of sufficient trim.	Verify that the microscope is properly calibrated (i.e., camera is aligned, lenses are grid-tuned, stage movements are functioning properly). Turn up the trim on the appropriate edges using the *Virtual Tissue Trim/Blend Options* dialog box (see p. 13). If trimming does not work, a fine-tune manual calibration may be needed.

EXAMPLE	PROBLEM	SOLUTION
	Large out of focus areas in the image due to an insufficient number of focus sites.	Try adding more focus sites to the focus map (see **Step 6** p.15). Verify that the focus sites are consistently distributed throughout the contour or grid. 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 (see **Step 5: Settings/Focus Adjustments** p.11). With a manual focus interval, you can adjust the focus during the imaging process, and 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.
	Very distinct tile lines / Individual tiles are not lined up, due to mismatched objective and software lens.	Verify that the lens selected in the software's drop-down menu matches the objective selected on the microscope.

EXAMPLE	PROBLEM	SOLUTION
	Greatly out-of-focus tiles or strips dispersed throughout an otherwise in-focus image. In MBF's experience, this occurs with extremely "wavy" tissue with drastic differences in z-depth between focus sites. The focus map interpolation can't account for these large differences, resulting in out-of-focus tiles.	Try increasing the number of focus sites in the focus map to create a tighter triangulation grid (see **Step 6** p.15).

## TROUBLESHOOTING IMAGING ISSUES (FLUORESCENCE)

> Acquiring a fluorescent background subtraction image is problematic and should only be done as a last resort to troubleshoot tiling.
>
> Do not use this method if you intend on using the virtual tissue for pixel counting and thresholding.

Since there is no area on the slide with a uniform spread of fluorophore to reveal the variations in the fluorescent light path, one option is to obtain a slide with a uniform fluorophore coating. But it is still difficult to determine how bright the background-subtraction image should be.

Here are some tips that may help you to achieve proper illumination of your field with fluorescent imaging without performing a background subtraction image.

1. Verify that your fluorescent light source is centered and provides uniform illumination.

2. If the problem persists, try pixel trimming (see **Step 5: Acquisition Settings/Settings/Trim & Blend options** p. 13) to prevent your virtual tissue montages from looking like mosaics.

3. If the problem persists, try to darken the background using the **Camera Histogram**

    - To adjust the dark point, drag the black triangle towards the graph data but not too far from this (LEFT) to this (RIGHT).
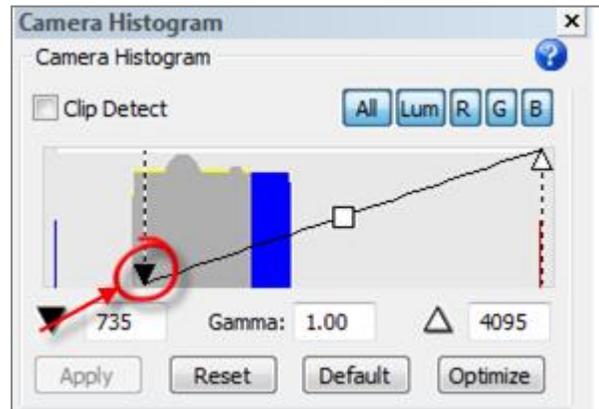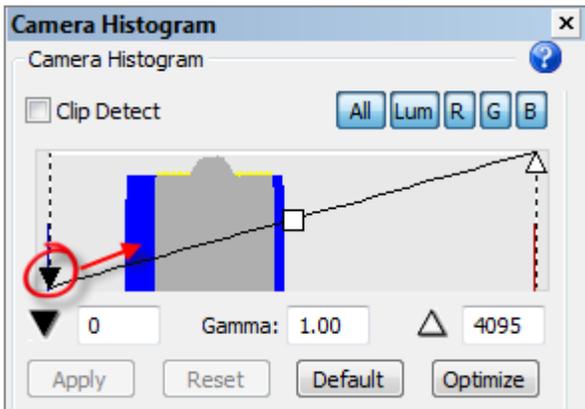
    - As a result, your background will be darker and lines in your virtual tissue will be less likely.

> Adjusting the dark point reduces the dynamic range. It may lead to data loss if you move the dark point so far right into the histogram that you start to remove signal.
>
> Pre-acquisition adjustments lead to less flexibility in performing image adjustments after acquisition (due to a reduced dynamic range).
>
> Keep in mind that images can be adjusted after acquisition to preserve the full dynamic range.