June 8, 2012

Protocol for Microscope Calibration

A properly calibrated system is essential for successful and efficient software use. The following are step by step instructions on how to calibrate the hardware using the tools provided by MBF software. Accurate execution of these guidelines is critical for proper system function.

Equipment: A microscope system (camera, stage, objectives, computer etc.), calibration grid slide, and the latest version of MBF software. Allen wrenches may be necessary for Camera to Stage Alignment.

Images for this protocol were taken using Stereo Investigator version 10.42.2.

Table of Contents:

Turn on the microscope system and the computer. Open the MBF software. Load the calibration grid slide onto the microscope.

Tighten the Objectives

1. Tighten all the objectives by hand to make sure they have not come loose. Add any new objectives to the microscope BEFORE calibrating the system.

Adding a New Objective and Lens

1. If adding new objectives, or existing lenses need to be renamed or redefined, do so at this point. Install objective, and rotate nosepiece so the objective is in the light path.

2. Go to **Tools>Define New Lens.** A box will appear to name the lens (generally by the name of the objective), record any relevant comments, select the units of measurement (micrometers is generally appropriate), select the lens type, and set the correction factor. The last step is to set the **Calibration Box** size. Click **OK** when done.

   - 250 μm (the larger grid) is appropriate for low power (2x and 5x) objectives.
   - 25 μm (the smaller grid) is appropriate for higher power (10x and above) objectives.
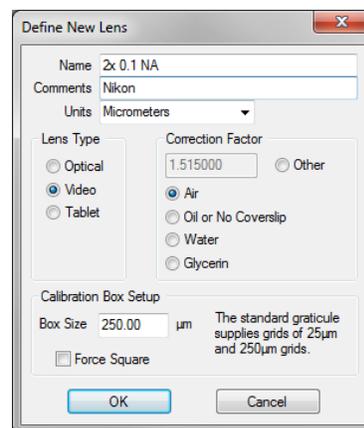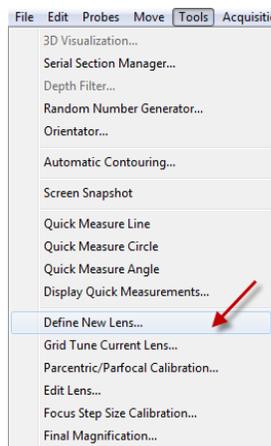
3. Click on the corner of one of the grid squares. Drag the box to the size of one grid square. Click once to set the box.
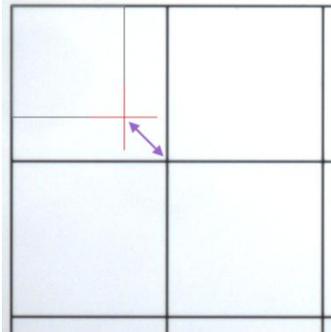
4. A perforated white grid appears with an anchor at one intersection. Drag the anchor to the top left corner of one grid square. Hover the mouse over the horizontal and vertical lines until the movement arrows appear and drag them until they outline each grid square (for a more detailed description of this technique see **Calibrating an Existing Lens** in the next section). When finished, right click and select **Finish Calibrating Current Lens.**
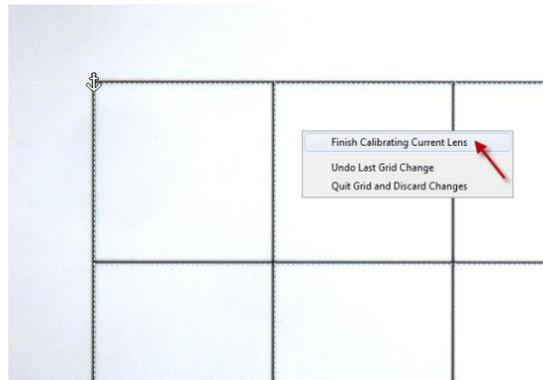

5. Continue this process until all the lenses have been properly defined.

Calibrating an Existing Lens

NOTE: For a complete visual and audio tutorial of calibrating a current lens, please visit our website at: http://www.mbfbioscience.com/tutorials.

1. Once all new and existing lenses have been properly defined, it is time to calibrate (grid tune) them. Start with the lowest power objective and move the stage (if necessary) so that the appropriate grid is visible (e.g. the 2x objective on the 250 μm grid). Move the grid to the center of the screen.

2. Click **Tools**>**Grid Tune Current Lens.** Make sure the **Calibration Box Setup** is set to the appropriate grid size.
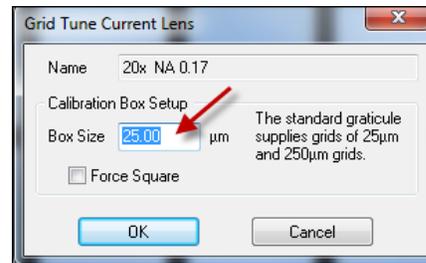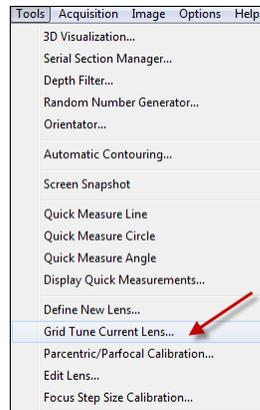
3. A perforated white grid with an anchor will appear on the screen. When working with 5x objective or lower drag the anchor to an intersection at the center of the grid. When working with 10x or higher power objective drag the anchor to have the white dotted lines rest on the side of the black grid lines (shown in figure below).

4. Hover the mouse over the grid lines for until the cursor changes to a movement arrow. Use this movement arrow to overlay the white dotted lines with the black grid lines on the calibration grid slide. Align the vertical lines and then the horizontal lines to obtain the following (note the consistency of vertical and horizontal line placement across the grid):
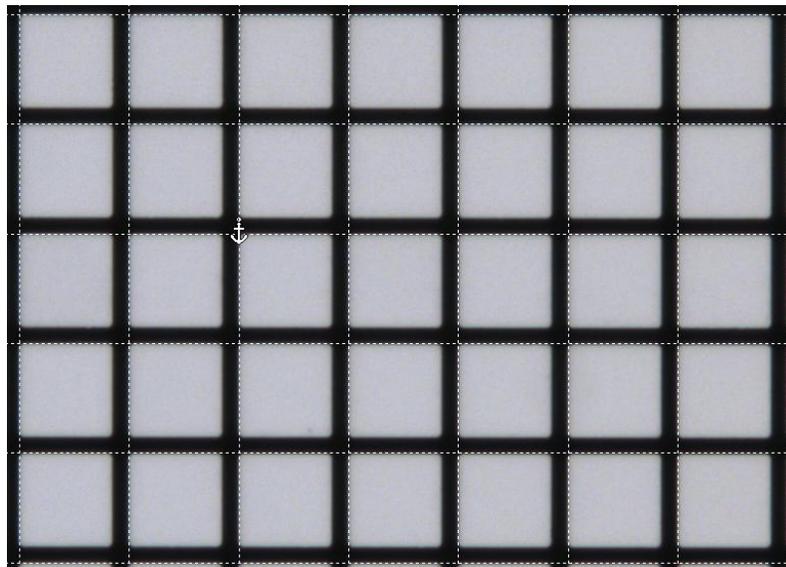
NOTE: If the black grid on the slide appears to be crooked compared to the white calibration grid on the screen please see Troubleshooting section below to check Camera to Stage Alignment.

5. When the grid is lined up as accurately as possible, right click and select **Finish Calibrating Current Lens.**
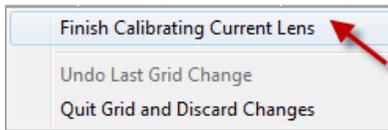
6. Continue this process for each objective in ascending order. Make sure the correct grid on the calibration slide is in the field of view and the correct grid size is specified during the grid tune. Remember:

   - 250 μm (the larger grid) is appropriate for low power (2x and 5x) objectives.
   - 25 μm (the smaller grid) is appropriate for higher power (10x and above) objectives.
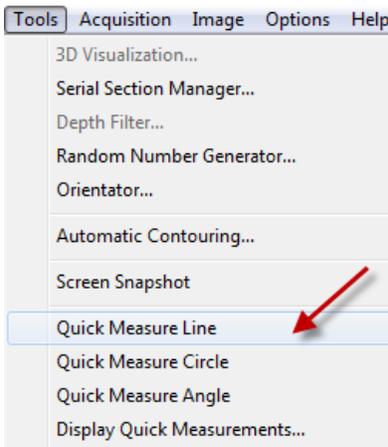
Checking the Accuracy of the Lens Calibration

To check the accuracy of the lens calibration, use a quick measure line. Check either the 250 μm and 25 μm grid (i.e. at a low power and a high power).

1. Click on the **Grid** icon to bring up the grid again.


2. Click **Tools>Quick Measure Line**

3. Draw a horizontal and a vertical line in one of the grid squares. A measurement will appear and it should read very close to 250 μm, or 25 μm, depending on what size grid is being used. If this number is significantly off, try redo Grid Tune calibration of this lenses and test again (parcentric calibration will also need to be done again if the grid tuning is changed).
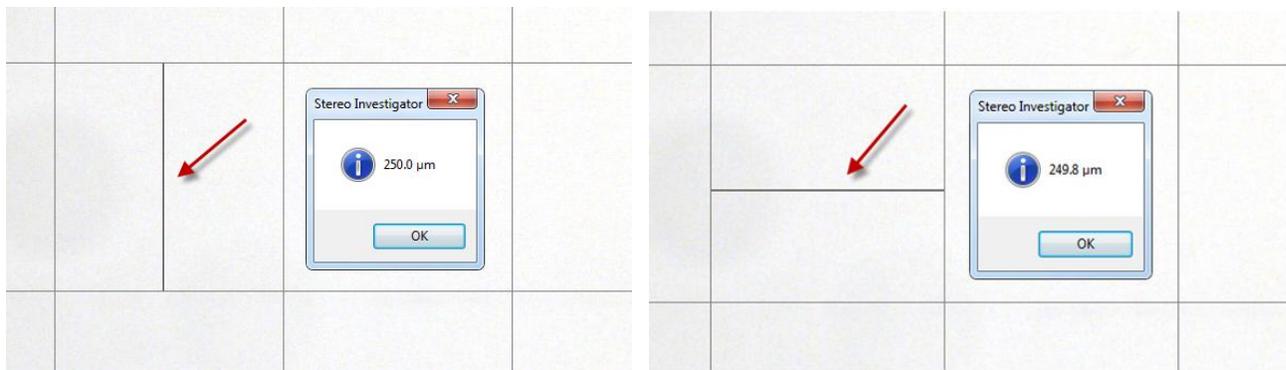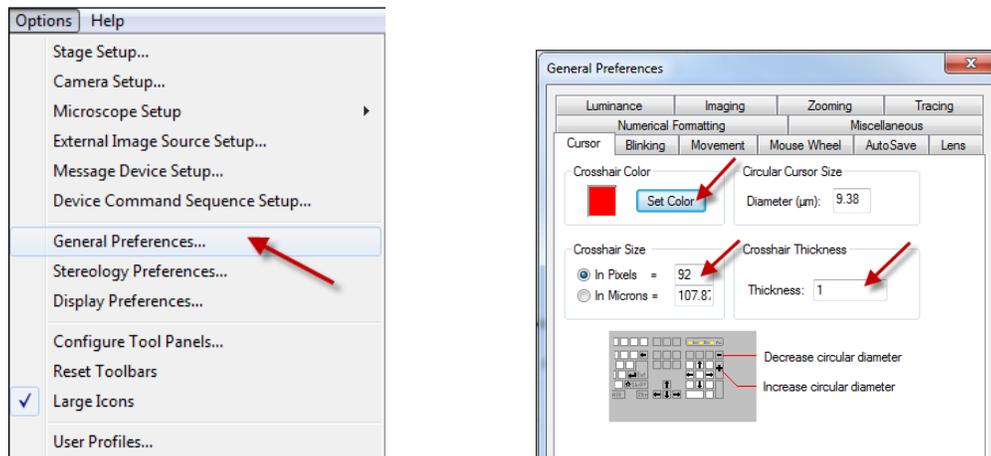
Parcentric/Parfocal

NOTE: For a complete visual and audio tutorial of Parcentric/Parfocal calibration, please visit our website at: http://www.mbfbioscience.com/tutorials.

Parcentric and parfocal calibration are both critically for maintaining proper position when changing magnification. When switching between objectives, parcentricity ensures the same XY position is found, while parfocality ensures the same spot is brought to the correct Z depth (i.e. in focus). Parcentric and parfocal calibration can be done simultaneously within the software.

NOTE: It is not necessary to use oil for oil objectives during lens calibration or parcentric/parfocal calibration as long as the grid lines on the screen can be clearly seen. The main issue with using oil is that slides tend to move when the oil is wiped off of them when switching between objectives.

1. ***Optional:*** A helpful adjustment to make for parcentric/parfocal calibration is to enlarge the size of the cursor so it is easier to see. This will help with achieving a more accurate alignment for the parcentric calibration. To do this click **Options>General Preferences**. Under the **Cursor** tab, set the color of the cursor to make it easier to see, change the crosshair size in pixels (75 or higher is a recommended length for parcentric-parfocal calibration), and change the crosshair thickness if needed (0-2 is generally an appropriate setting). Crosshair size can also be changed dynamically while using the program by pressing the CTRL key along with the up or down arrow keys. These changes simply make it easier to use the cursor for the process.
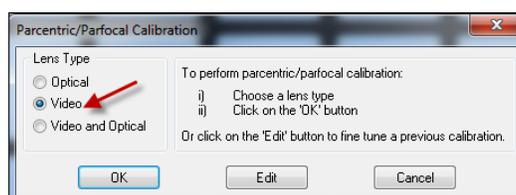
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2. Parcentric/parfocal calibration goes from the highest power objective to the lowest power in descending order. Using the highest power objective, bring a corner of the small (25 μm) grid into the center of the screen. Make sure the matching lens is selected in the lens drop down menu.

3. Click **Tools>Parcentric/Parfocal Calibration**. A succession of dialog boxes will appear instructing how to proceed:
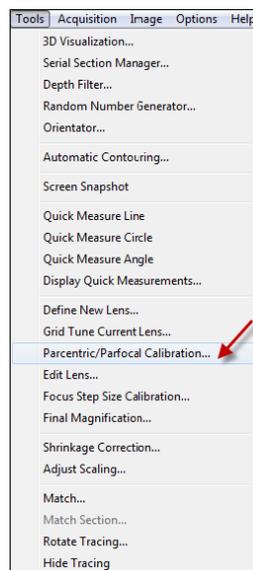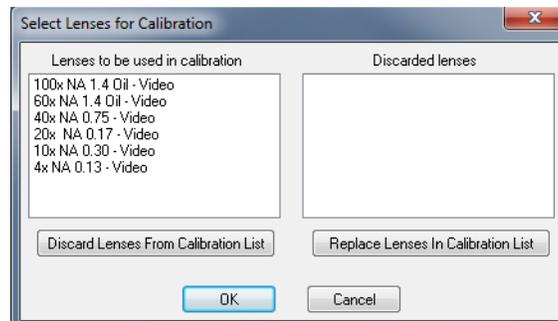
a. The first box will ask to specify the **Lens Type**. Enter **Video** and click **OK.**

b. Next, make sure the lenses are in descending order from highest to lowest. Make any adjustments by discarding improperly ordered lenses from the list and then re-inserting them in the correct order. When finished click **OK**.

c. An instruction box will appear specifying to bring the grid into sharp focus for each objective and then to click on the object. This means that for each objective, click on the **EXACT SAME SPOT** of the grid each time, regardless of where it appears on the screen. Click **OK** to proceed.
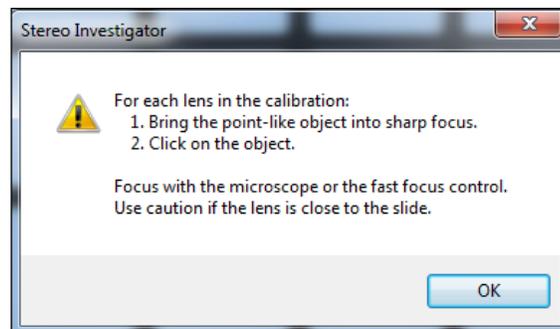

d. The next box will specify which objective to put into place. For this process, it is only necessary to switch objectives on the microscope. The lens selection in the software will automatically update at each step and does not need to be done manually by the user.
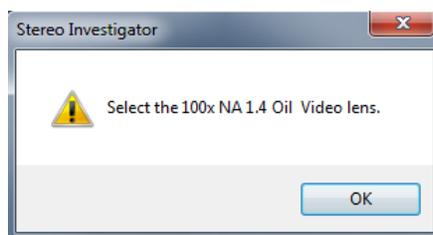
4. Between switching objectives, adjust the light level, if needed, on the microscope to accurately see the grid. If the parfocal calibration was done correctly, the tissue should be automatically brought into focus when switching between objectives. It is recommended that the user click on the upper left hand corner point of the grid to ensure the same point is being selected at each magnification. Line the crosshairs of

the cursor up to the edges of the grid as precisely as possible to be consistent between all objectives. This will ensure the best possible calibration.
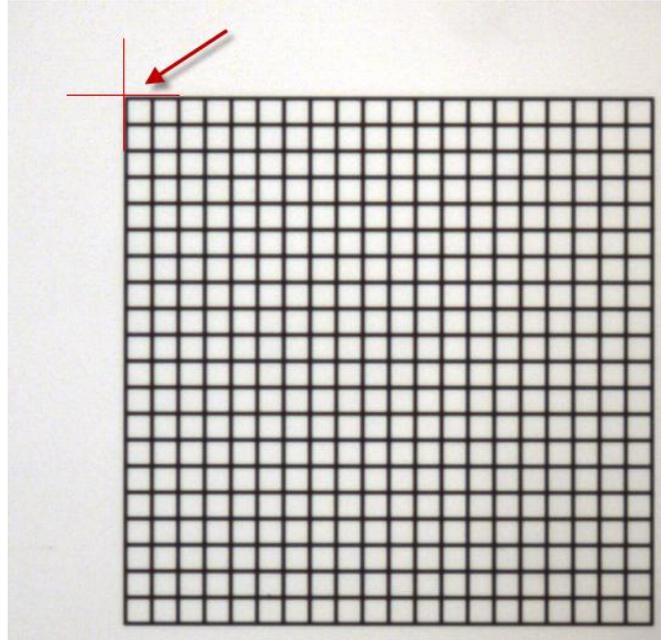
5. When the lowest power objective is finished, a final screen will appear. Check **Enable Parcentric** AND **Enable Parfocal**. Click **OK** when finished.
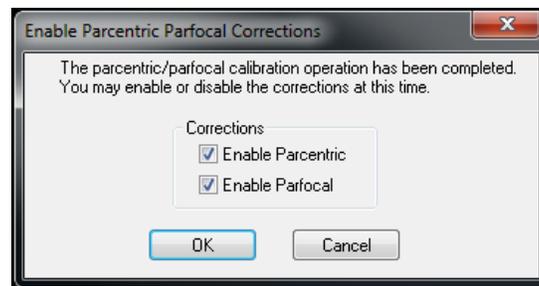
6. It is important to check the accuracy of the calibration to ensure it does not need to be repeated. Go back to the highest power objective and draw a contour in the upper left hand corner of the grid. Move down through the objectives in descending order, always making sure to match the appropriate lens in the drop down menu. When moving through the objectives, the contour drawn should remain in the same position in the corner grid square, and the grid should be automatically brought into crisp focus. If this is not the case, try this calibration again. Be sure to adjust the focus for each objective and be as accurate as possible when clicking on the same point on the grid. If, after repeated tries, you are not able to obtain parcentric or parfocal calibration please review the troubleshooting section below.

Troubleshooting

If lens calibration and parcentric/parfocal calibration failed (i.e. when the lens calibration was checked the quick line measurement was significantly off, or when parcentric/parfocal was tested the tracing did not remain in the same position between objectives), troubleshoot through the following calibrations to see if the problem can be resolved:

1. Check the camera alignment.
2. Check the stage movements.
3. Check the Z calibration.

Once those have all been examined, return to try grid tuning and parcentric/parfocal calibration again to see if any issues were resolved:

1. Re-calibrate (grid tune) the lenses and test the calibration with the quick measure line.
2. Re-calibrate parcentric and parfocal and test the calibration by drawing a contour and switching through the objectives to make sure it stays aligned.

If the camera, stage, and Z encoder have all been troubleshot and the calibration still produces insufficient results, please contact MBF Bioscience Technical Support for additional assistance. Email support@mbfbioscience.com or call 1-802-288-9290.


Camera to Stage Alignment

1. Check to make sure the camera is oriented in the proper direction. To do this, make sure what appears through the eyepieces is identical in orientation to what appears on the screen. For example (using the calibration grid slide), if the small grid is to the left of the large grid when looking through the eyepieces, then it should appear the same way on the computer screen. If it appears backwards then the camera needs to be rotated 180°. Loosen the set screw and rotate the camera. Tighten the set screw when finished.

2. Next, place a reference point on the screen (anywhere is fine). Click on the **Display Grid** icon in the **Grid Toolbar** so that a white grid appears on the screen offering horizontal and vertical reference lines. For this step THIS virtual white grid on the computer screen is what should be referred to, NOT the black grid on the grid slide (the grid size can be adjusted under **Options>Display Preference>Grid**. Adjust the X and Y Grid Spacing measurements as needed).
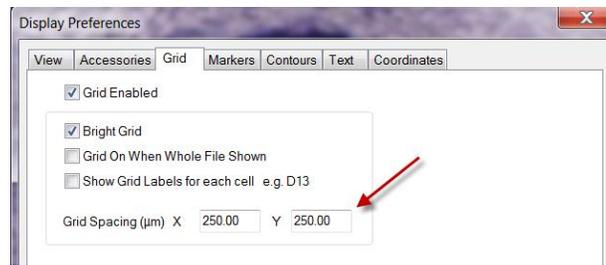
3. For a rough alignment, at a low objective (2x or 5x), find the large grid on the slide (250 μm) and line it up closely with the grid on the screen (right click and choose **Joy Track Mode** to control the slide movements with the joystick). If the two grids do not line up, the camera needs to be adjusted. To realign the camera, loosen the set screw (sometimes located at the bottom of the C-mount). Slowly, and only slightly, turn the camera in either direction until the two grids line up on the screen (make sure not to make large rotations with the camera or it may become oriented backwards - see step 1). Tighten the set screw to set it into place. ***This provides a rough starting point for camera alignment. Lining up the grids is NOT sufficient for calibration.***
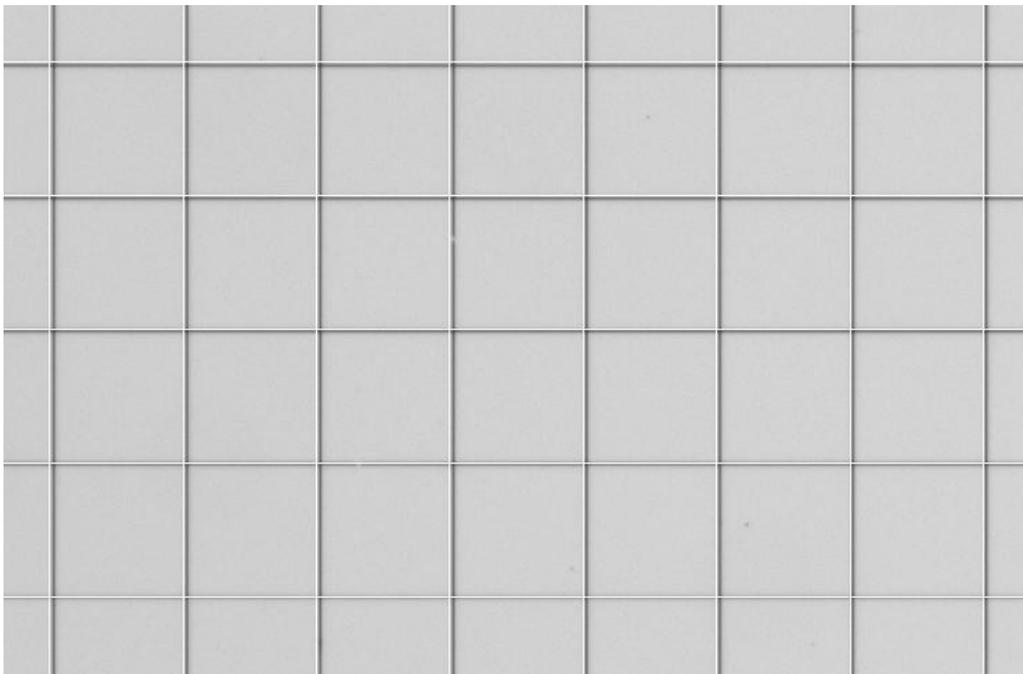

4. Now at a higher objective (10x or 20x) continue in **Joy Track** mode. Navigate around the grid slide to find a piece of dust that is of significant size (it will help if the speck has a defining mark or protrusion).
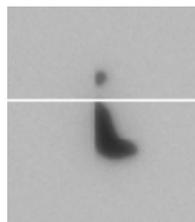
5. Move the piece of dust to the top of a white grid line on one side of the screen. Using the joystick, move it across the screen to the other side on the same grid line. With a perfectly aligned camera, the piece of dust will remain lined up with the grid line. If it has moved above or below the line, then the camera needs to be adjusted to correct for the movement. Again, loosen the set screw and adjust the camera before tightening the screw again.

6. Continue moving the piece of dust across the screen and adjusting for correction if needed. Once the dust remains constant across the screen, the camera is aligned. Move to a higher objective (i.e. 40x) and repeat this process for a more fine-tuned alignment.
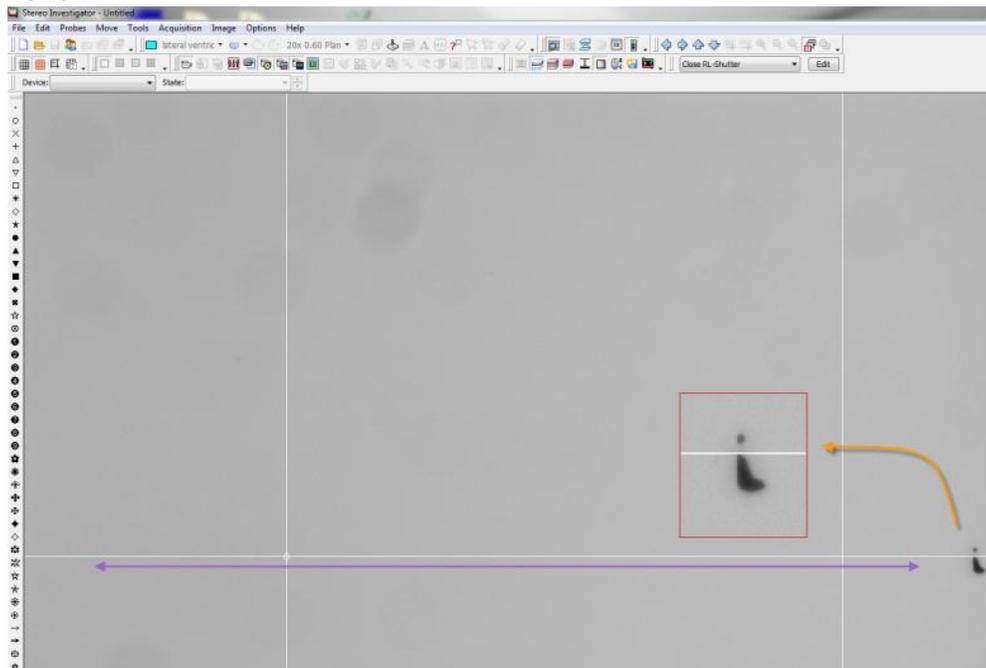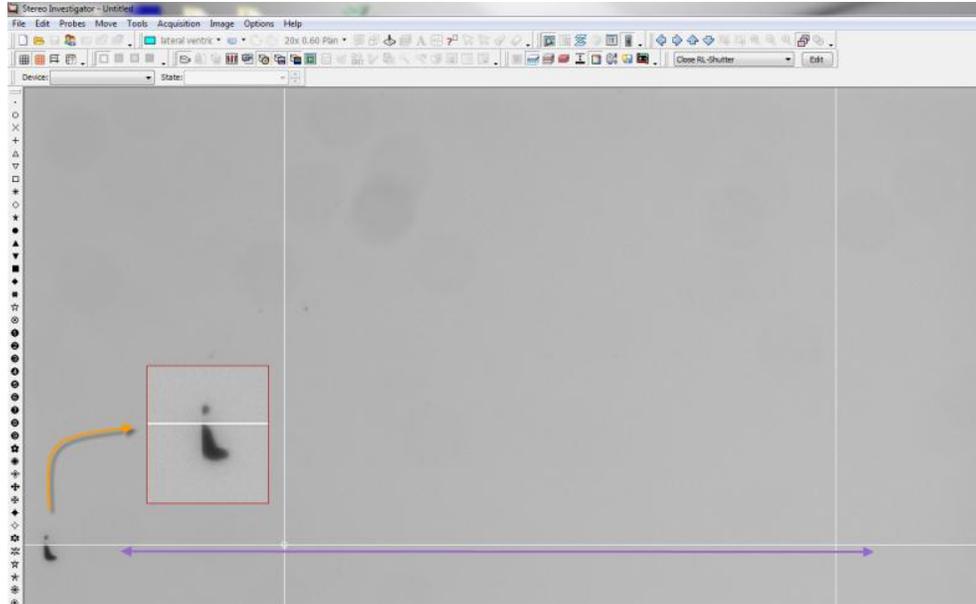
7. Click on the **Grid** icon again to turn it off.

> **NOTE:** Once the alignment is done C-mount should be tightened securely in place. As you tighten the screws C-mount may move a little bit. Please anticipate that movement as you align the camera.

Stage Movement

It is important to make sure the stage is moving the correct distance and in the correct direction. **Testing the stage movement only needs to be done at one magnification.**

1. ***Optional:*** Before checking the stage movement, it is helpful to ensure all movements will remain in the field of view on the screen. This makes calibration more convenient. To do this, click **Options>General Preferences**. Under the **Movement** tab make sure the **% of Screen Size** under **Field Movement Size** is set to 25%. Click **OK** when finished.
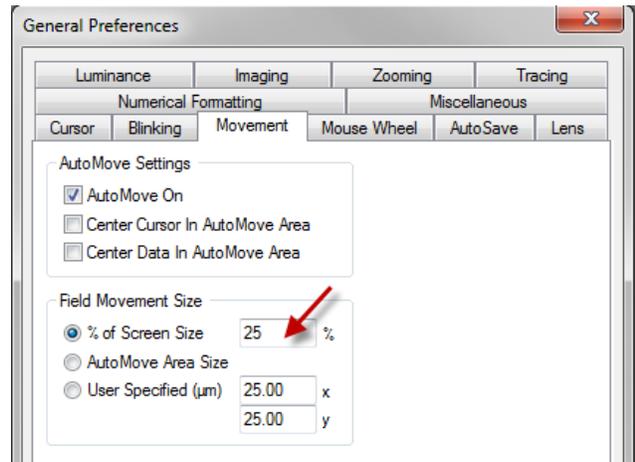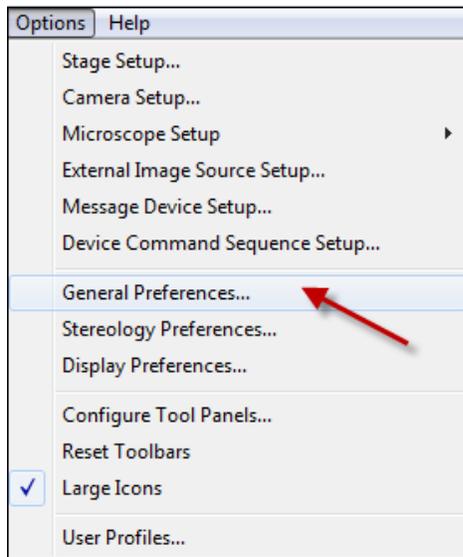
2. Bring a corner of the black grid into the center of the screen. Draw a closed contour in the corner grid square as a reference tracing. Proper stage movement will ensure that as the grid moves in any direction, the contour will move in the same direction, staying aligned with the original square. To test this, use the **Directional Arrows** in the **Movement Toolbar**.


3. Test each direction to ensure the tracing and the grid move in the same direction. If they do, the stage movements are calibrated correctly. If they **do not** move correctly (i.e. the grid moves in one direction and the tracing moves in the opposite direction, as shown below) then additional adjustments must be made.
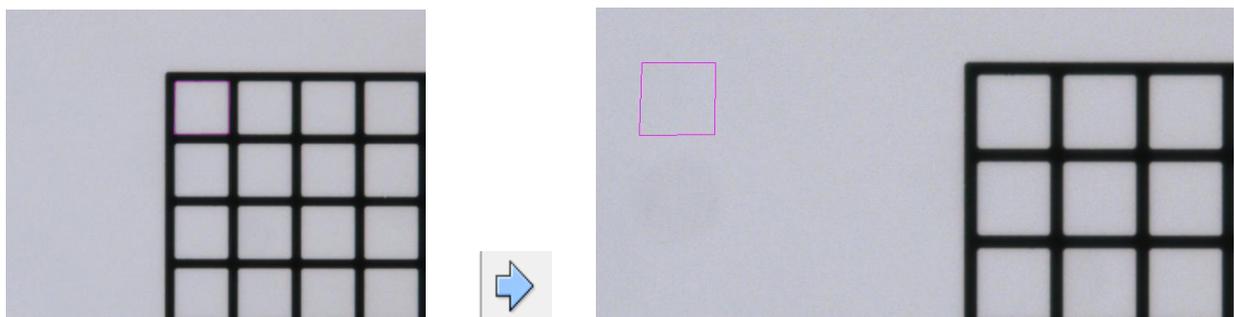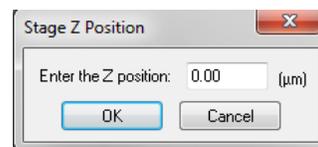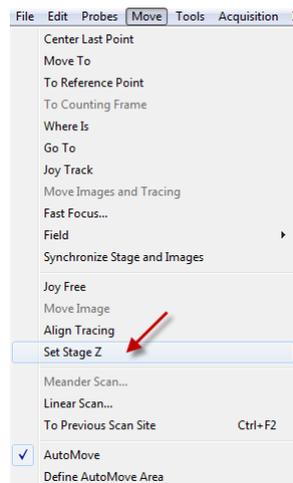

NOTE: If the movements are off or additional help is needed for any part of the set up procedure, please contact MBF Bioscience Technical Support for assistance. Email support@mbfbioscience.com or call 1-802-288-9290.

Checking the Z Calibration

1. The calibration slide is about 1.5 mm thick. If there is not already a cross drawn on the slide, one will need to be drawn. On the top of the slide, in permanent marker, draw a vertical line in an empty space. Turn the slide over and draw a horizontal line on the bottom surface to form a cross.
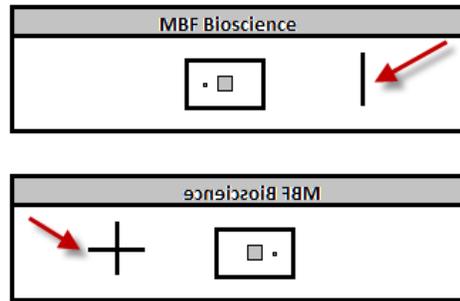
2. Re-load the slide and find where the two lines of the cross intersect.
3. Bring the top line into focus (the line that was drawn on the top of the slide).
4. Using 10x objective bring the top line in focus, set the Z meter to 0. Go to **Move>Set Stage Z.** Enter the Z position to be 0.000 μm and click **OK** (alternately, use the shortcut keys **Alt+S**. This automatically sets the current focal plane to 0.000 μm).

5. Now bring the line drawn on the bottom of the slide into focus. Once in focus, the Z meter should read approximately 1500μm.

NOTE: If the measurement is significantly off or additional help is needed for any part of the set up procedure, please contact MBF Bioscience Technical Support for assistance. Email support@mbfbioscience.com or call 1-802-288-9290.