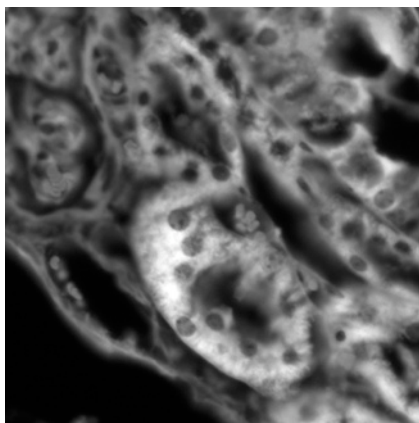


ApoTome: Users Guide

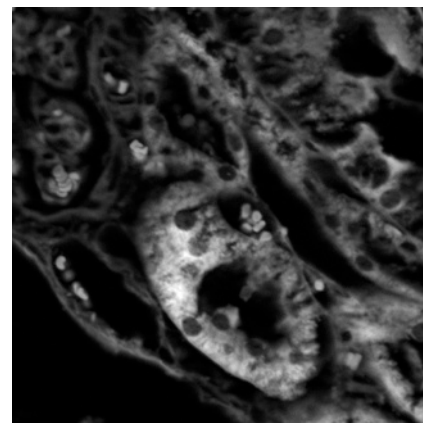
Optical Sectioning with ApoTome

ApoTome uses a moving grid to change how the slide is illuminated. By taking several images, with the grid in different positions, the ApoTome software is able to generate a single image which excludes a large amount of the out-of-focus light typically present in fluorescent images.

The resulting images look crisper than the smoother standard fluorescent image, which allows finer structures and details to be seen clearly but they are also dimmer (as there is less light reaching the camera).



GFP



GFP with ApoTome

Calibrating ApoTome

Calibration must be carried separately for each objective and filter set.

1. Insert the correct grid into the ApoTome slider

- Axio Imager:
 - 5x, 10x, and 20x objective = **L-grid**
 - 40x, 63x and 100x = **H-grid**
- Axio Observer:
 - 5x and 10x = **L-grid**
 - 40x = **D-grid**
 - 20x and 63x = **H-grid**

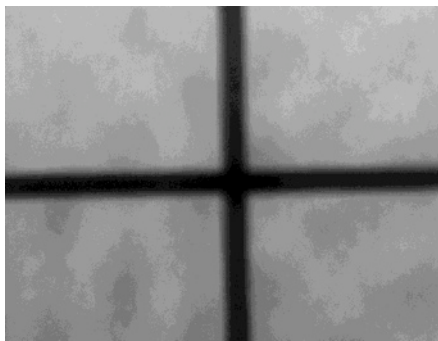
1. Unlock and remove the ApoTome slider from the side of the microscope.
2. Use the tweezers to gently remove and insert the appropriate grid.
 - When inserting, match up the white dot on the grid with the one on the slider.
 - The grid is held in magnetically so if its not sitting level move it around slightly with the tweezers until it clicks into place.
 - Once in place check it is secure by lifting the slider and *gently tapping* it over the palm of your hand.
3. Return the ApoTome slider back into the microscope
 - There are two stop positions . All the way in / "1st click" (for using ApoTome/grid) or the click before "2nd click" (for normal imaging/iris).
 - **When you finish using ApoTome remember to pull the slider back out to the "2nd click" / iris position.**

Calibrating ApoTome

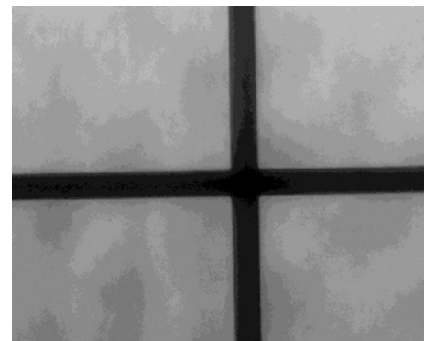
2. Phase Calibration

This calibration requires the **mirrored calibration slide**.

1. Focus on the cross-hair at the centre of the silver square on mirrored calibration slide.
 - Ensure you are using the **BF RL (brightfield reflected light) filter set** and the **HXP lamp**.
 - The light shining on the slide should appear yellow-green and down the eyepieces the cross-hair should appear black.
2. Once you have found the cross-hair switch to camera mode and ensure the cross-hair is focused as best you can.
 - If the cross-hair is out of focus you will not calibrate ApoTome correctly.

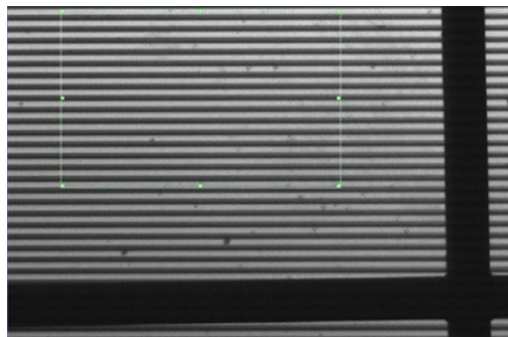


Out of Focus



Correctly Focused

3. In the top menu go to **Acquisition > ApoTome > Phase Calibration**
4. In the window that appears make sure you have the right objective selected, the correct grid size selected and that you are using the **BF RL** filter.
5. Click **Next**.
6. Now adjust the exposure of the camera for imaging the cross-hair. It should adjust automatically by clicking measure. If not, bring the exposure of the camera to a point where there is maximum brightness but no overexposure.
7. Click **Next**.
8. Push the ApoTome slider all the way into the microscope. You should be able to see grid lines across the live image.
9. Leave the exposure as it is and position the green rectangle over an area containing roughly 10 grid lines. This is the area axiovision will look at to automatically focus on the grid lines.
 - Make sure the rectangle is **not over the cross-hair**.
10. Click **Full Scan**.

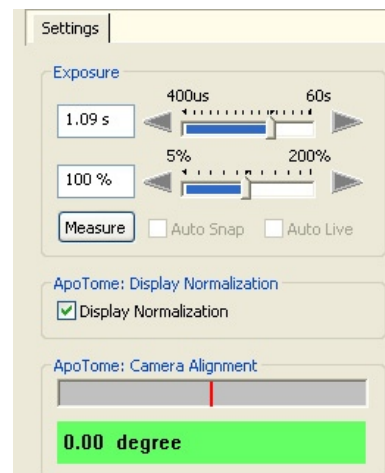
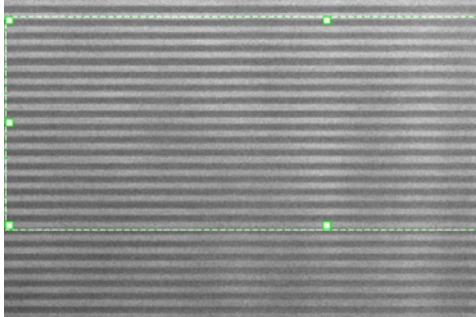


11. The Grid will move through a series of positions, until it finds a position where the grid lines are in focus. The graph showing the positions should have a defined peak at the best location.
 - **If there is no defined peak ApoTome is not calibrating correctly.**

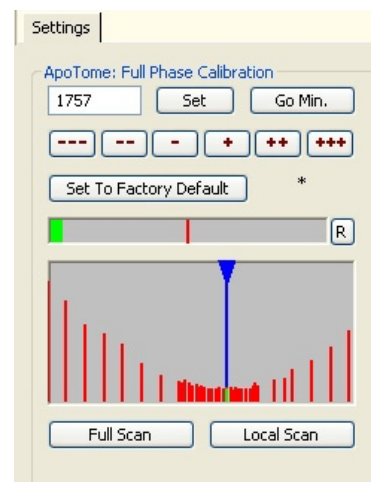
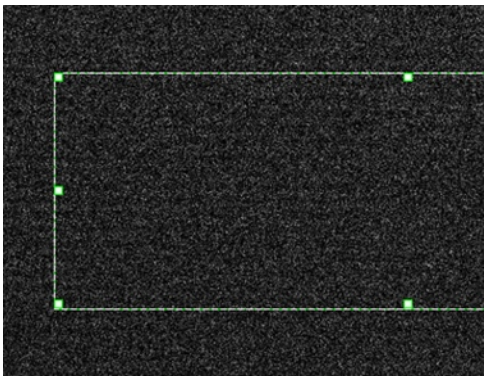
The screenshot shows the 'Settings' window for 'ApoTome: Grid Focus Calibration'. At the top, there is a '127' value with 'Set' and 'Go Max.' buttons. Below are navigation arrows and a green selection bar with a 'R' button. A histogram shows a red bar graph with a blue peak. 'Full Scan' and 'Local Scan' buttons are at the bottom of the histogram area. The 'Exposure' section has a slider from 400us to 60s, currently set at 708.6 ms, and another slider from 5% to 200%, currently set at 100%. There are 'Measure', 'Auto Snap', and 'Auto Live' checkboxes. At the bottom, 'ApoTome: Display Normalization' is checked.

Calibrating ApoTome

- Click “Local Scan” to ensure axiovision has found the best grid position. Once this is complete click **Next**.
- This step requires you to straighten the camera. Move the stage slightly so that the crosshair cannot be seen then gently twist the camera until you the angle reads 0.0 (or as close as possible).



- Click **Next**.
- You will now need to do another Full Scan. If ApoTome is working correctly the graph will look similar to below. Once complete click **Local Scan**, then click **Next**.



- The phase calibration is now finished. If you change objectives you will need to calibrate for the new objective.

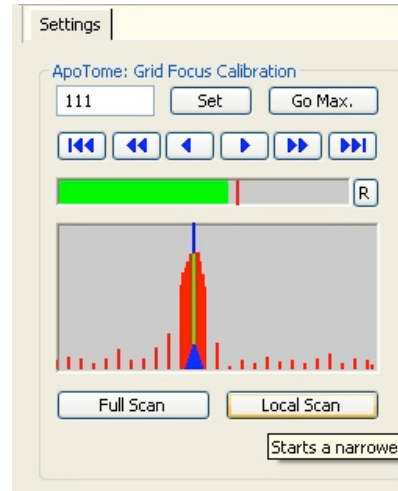
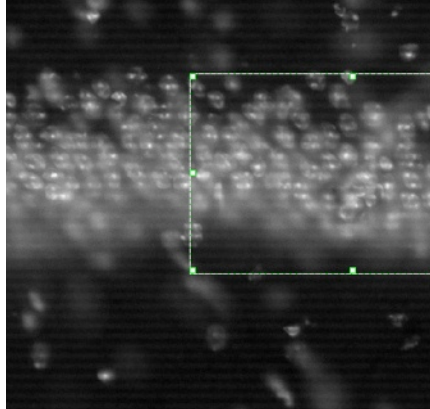
3. Grid Focus Calibration

This calibration step is to configure ApoTome for each fluorescent marker you are using.

- Remove the **mirrored calibration slide** and focus on your sample slide.
- Under the drop down menu go to **Acquisition>ApoTome>Grid Focus Calibration**.
- The first window that appears allows you to choose which filter set you wish to calibrate for (e.g. FITC or DsRed). You do not need to change any of the other settings here. Select the appropriate filter and click **Next**.
- Adjust the exposure by clicking **Measure**, then click **Next**
- Push the ApoTome slider into place – the grid should be faintly visible over the fluorescence of your sample.

Calibrating ApoTome

6. Draw the green selection rectangle over a bright region of the image (this will help axiovision focus the grid). Once you have selected a bright area click **Full Scan**.
 - **If you can't see the grid lines during the full scan, either the sample is too dim for ApoTome, or ApoTome is not calibrating correctly.**
 - If it has calibrated you will see a defined peak and the grid lines will be visible on the image.



7. Once you have completed the Local Scan click **Next**.
8. ApoTome is now calibrated for this marker. You will need to run this calibration again *for each fluorescent marker you are using*.
 - It is necessary to create a new calibration for each marker but also for each light source (i.e. separate calibrations will be required to excite GFP with the colibri LEDs and the external HXP lamp).

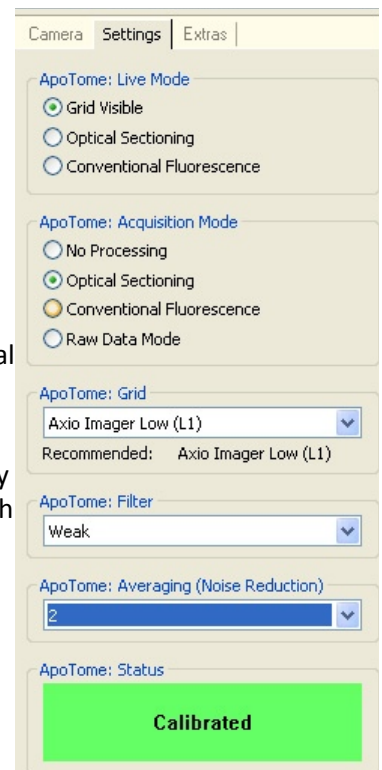
Important Settings for ApoTome

Click on **ApoTome** in the workarea to the left. Under the **settings tab** select:

1. The correct grid for the objective being used.
2. The **“Grid Visible”** option under Live Mode – this will ensure the fastest refresh rate for live mode
3. The **“Optical Sectioning”** option under Acquisition Mode – if not checked optical sectioning will not occur
4. A **weak** ApoTome filter is recommended if gridlines are still visible in the final imaged. This filter is used to remove any bands left by the grids but a good calibration should avoid this.
5. **2x** Averaging (noise reduction) is recommended as an initial setting. You may not need this if your sample is bright, likewise may need to increase this with particularly dim samples.

Under the **extras tab** ensure the **display normalization** box is ticked.

- ApoTome images are very dim as most of the light is being removed - Display Normalisation rescales how the images are displayed so you can see them properly.
- If you are using ApoTome but your images appear completely black this option is most likely disabled.



Once ApoTome has been calibrated you can image as normal (e.g. using Live/Snap or Multidimensional Acquisition experiments). ApoTome optical sectioning will occur whenever the ApoTome slider is pushed all the way into the microscope.