MetaXpress® Software Guide

Configuring Transwell plates for image acquisition

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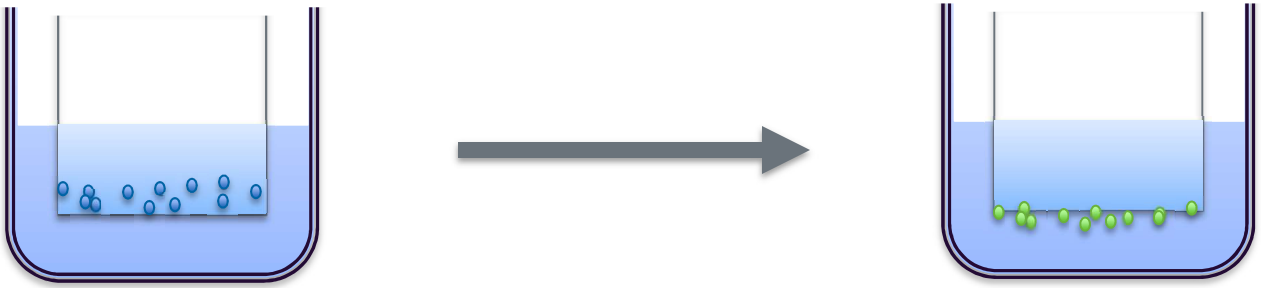
Chapter Purpose

The purpose of this chapter is to explain the process of configuring and optimizing a Transwell plate for high-content imaging with the ImageXpress system and MetaXpress software.

Transwell assay

Transwell plates have inserts with a membrane at the bottom added to each well. Also known as Boyden chambers, these are used for measuring chemotaxis or cell migration.

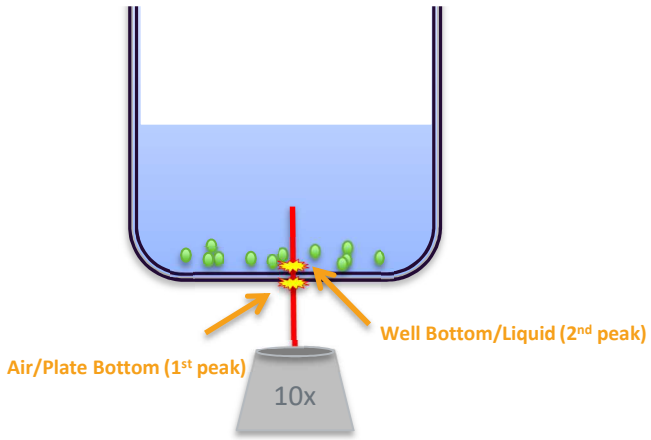
Transwell schematic

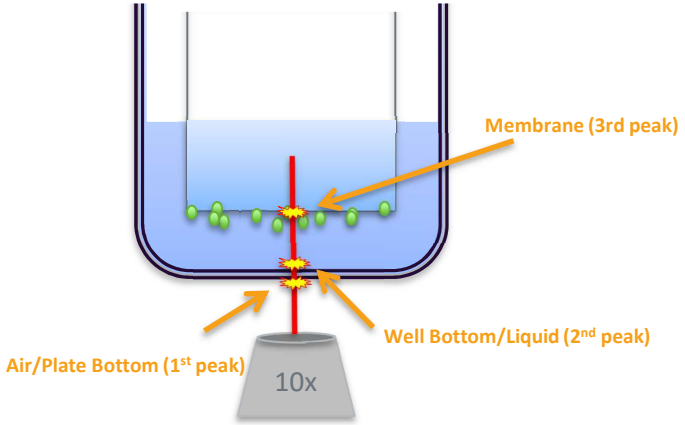


1. Insert added to well with or without chemoattractant
2. Cells added to insert
3. If chemoattractant present, cells migrate through porous membrane
4. Read fluorescent signal from below

Tip: Phenol Red in the media increases background fluorescence in the FITC channel. Use Phenol Red-free media if this interferes with measurements

Transwell plates and Laser Autofocus





Normal plates do not have an insert.

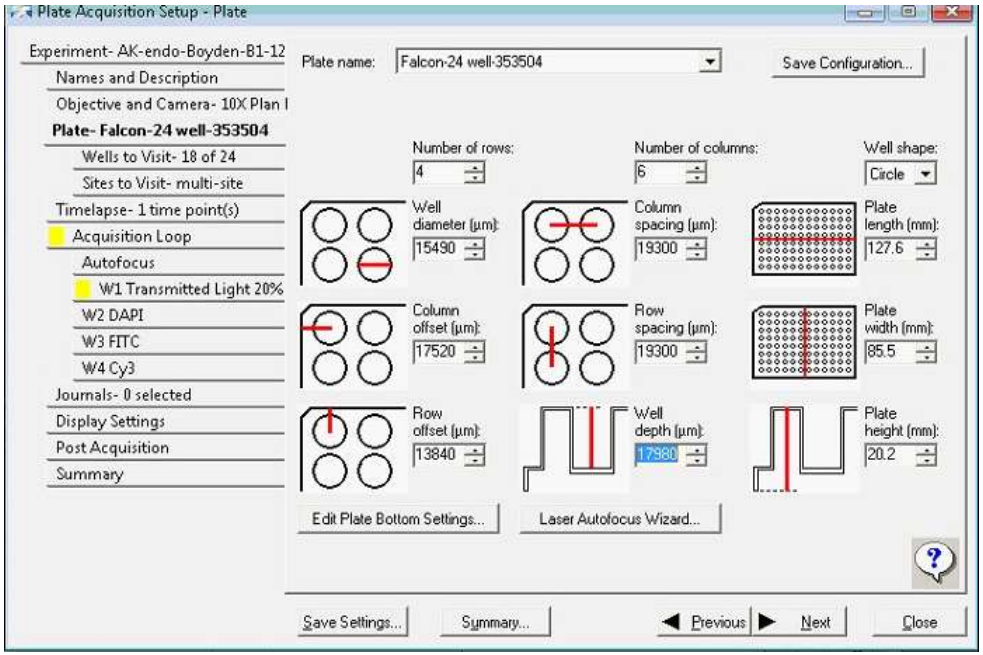
Laser Autofocus measures the plate bottom and the well bottom. A reflection ( ) is detected at each change of surface.

In a Transwell plate, the distance between the plate/well bottom and the insert may be inconsistent, causing focus issues.

Also, it can be difficult to adjust a very large Z offset from the normal plate/well bottom.

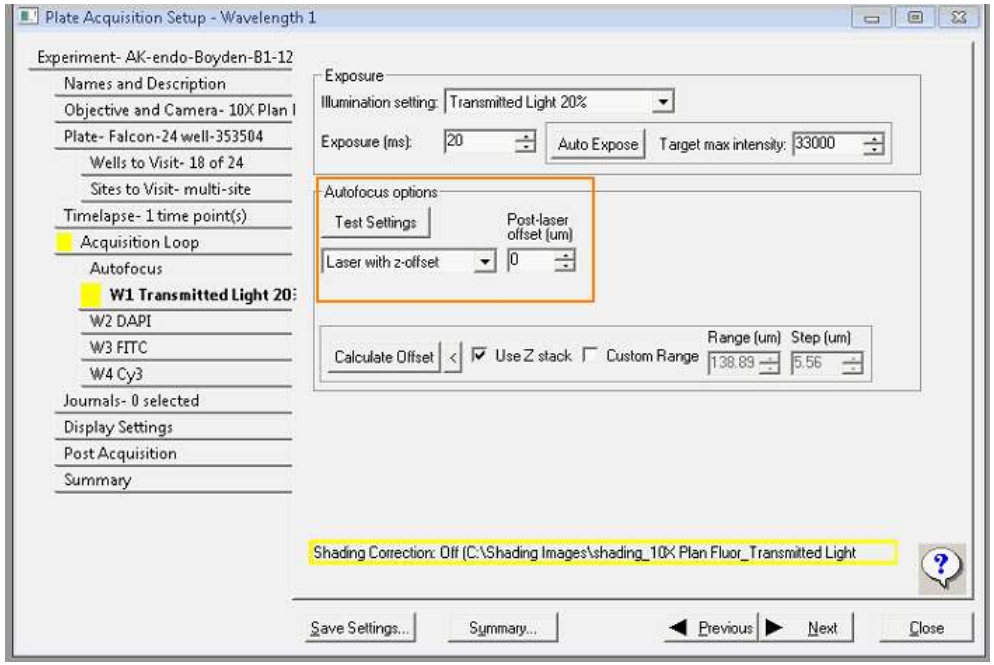
The insert introduces another reflective surface which can be read by the Laser Autofocus. The following procedure explains how to configure this.

Setting up a Transwell plate file



1. Go to **Screening > Plate Acquisition Setup**.
2. Select a long working distance objective, ideally the 10x Plan Fluor 0.3 NA. This will be used to measure the plate/insert dimensions.
3. Select or create a suitable plate file. Enter the plate dimensions as accurately as possible.
4. The Laser Autofocus Wizard will probably give incorrect results for a Transwell plate, and it is recommended to manually measure the plate bottom using this procedure, either after or instead of using the Wizard.

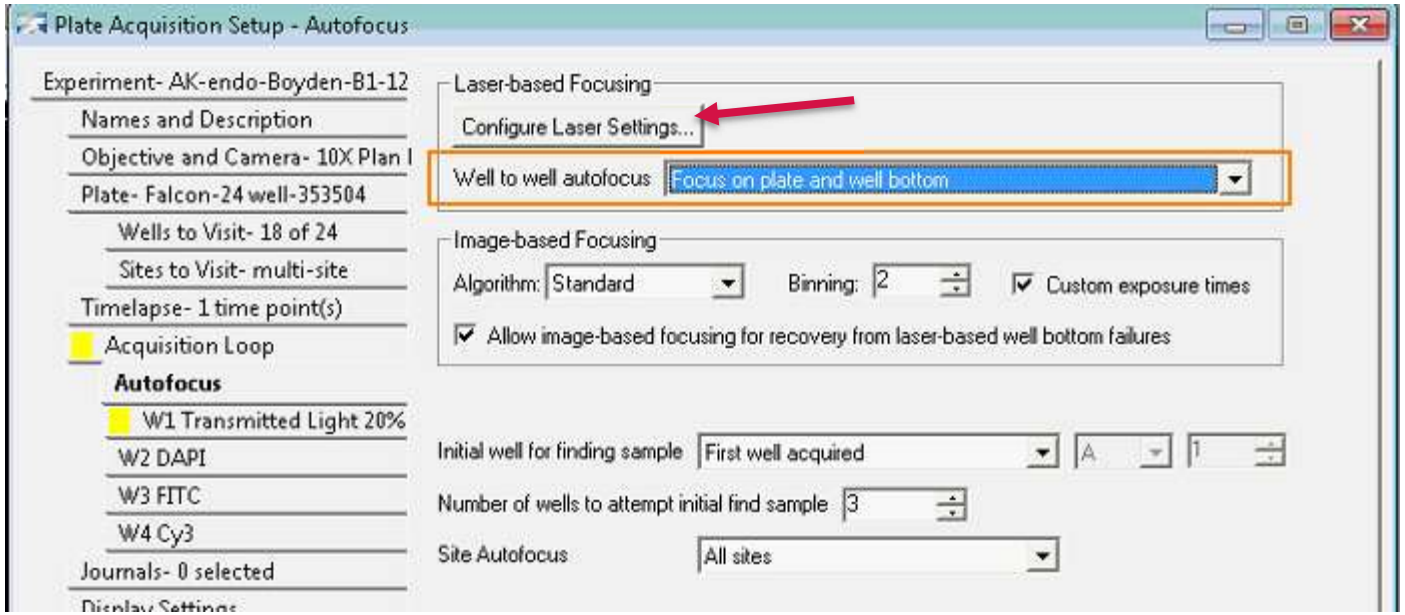
Setting up a Transwell plate file



5. Move to a well with an insert and make sure the site is towards the center, not towards the edge.
6. On the **W1** tab, select an appropriate illumination setting and wavelength. Set the Autofocus option to **Laser with z-offset** using a 0 offset.

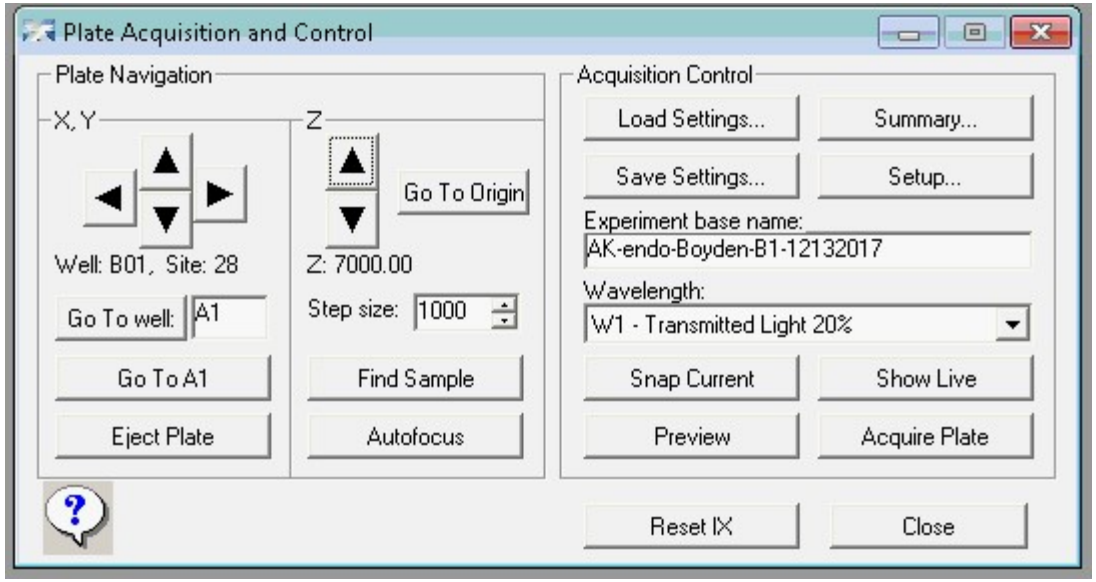
Tip: Taping the plate lid onto the plate may reduce movement of the inserts within the wells.

Setting up a Transwell plate file



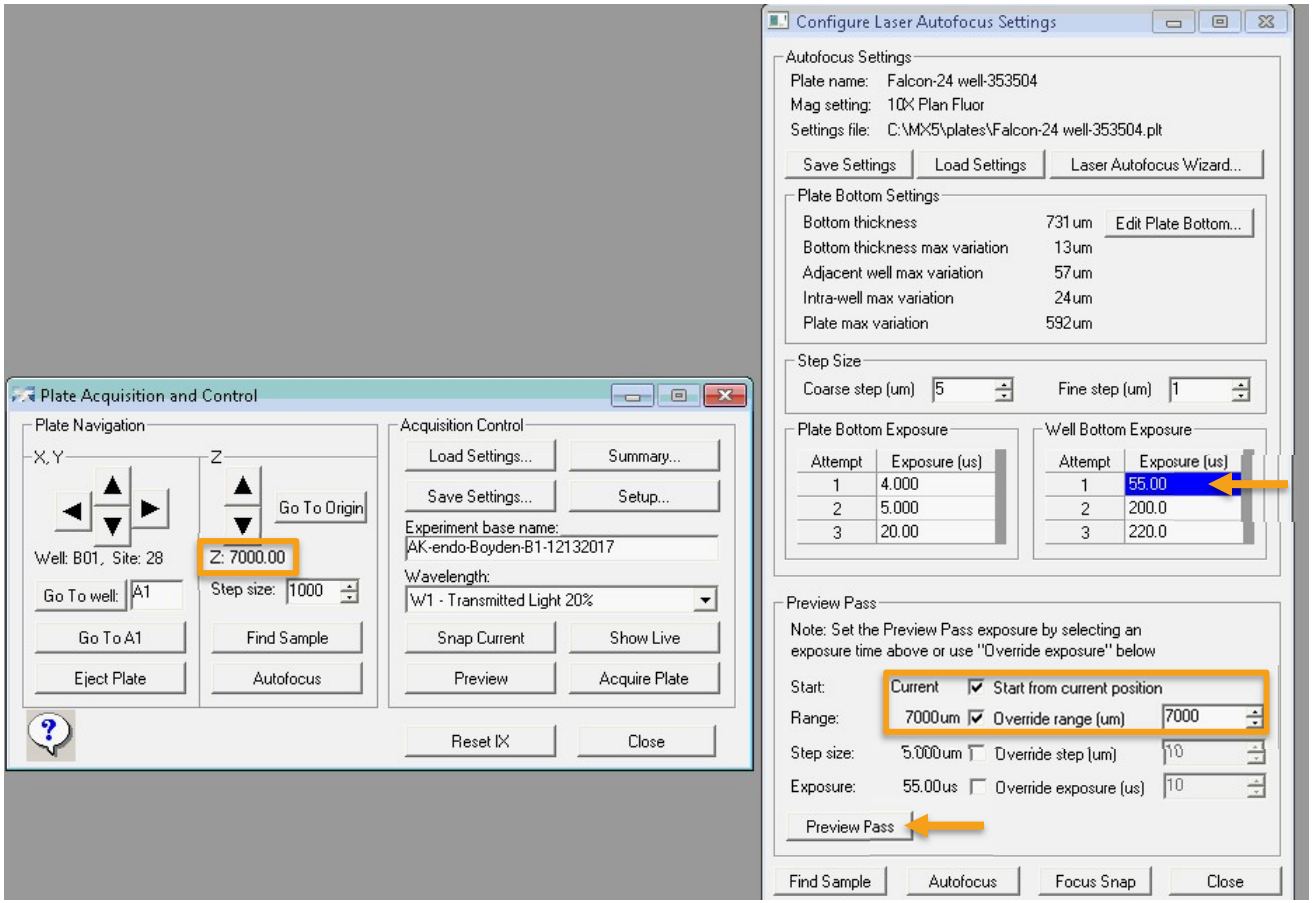
7. Go to the **Autofocus** tab and set well-to-well autofocus to “Focus on plate and well bottom”.
8. Click **Configure Laser Settings**. This opens the Configure Laser Autofocus Settings dialog.

Setting up a Transwell plate file



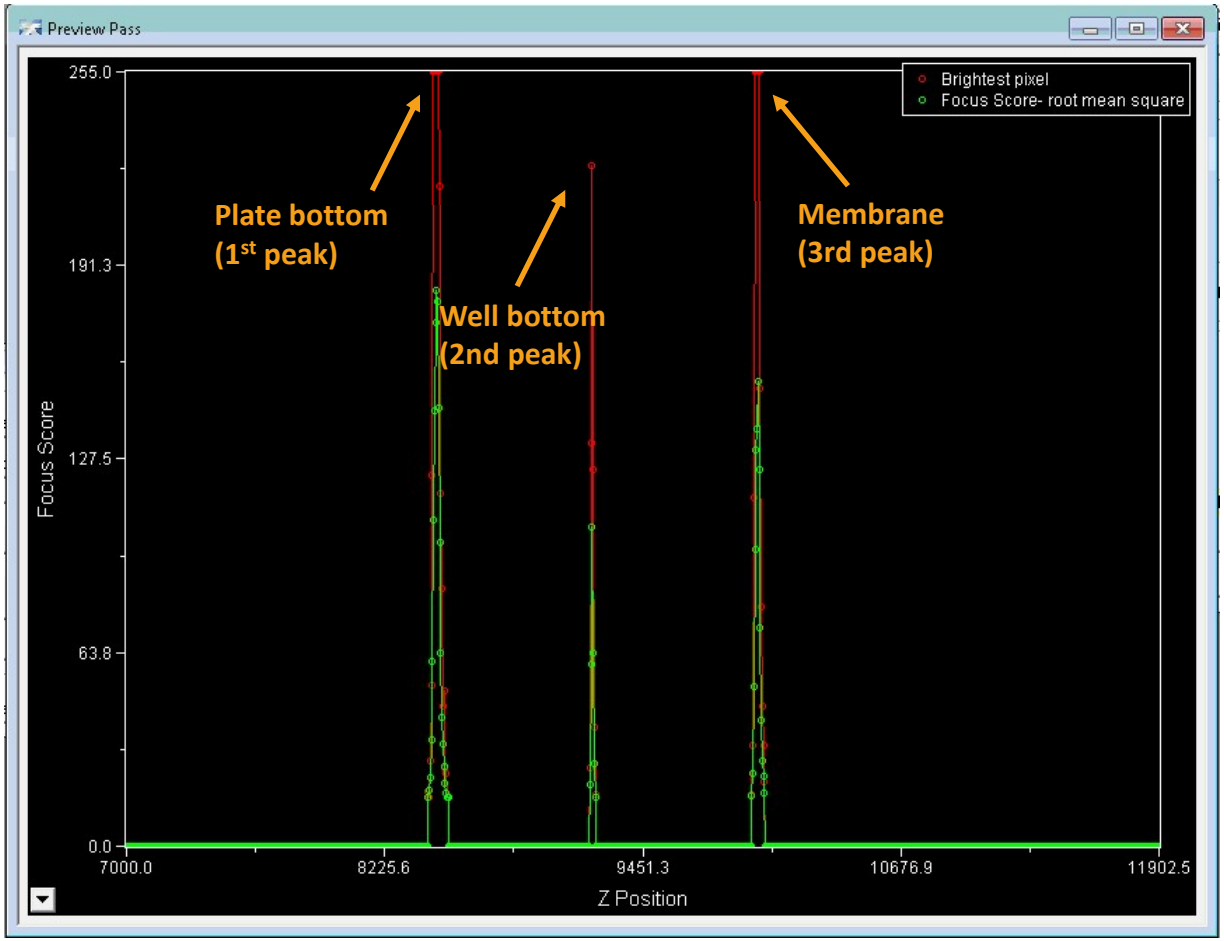
9. In addition to configuring the laser settings, you also will need to manually adjust the Z position. Go to **Screening > Plate Acquisition and Control**. This dialog will let you easily control the Z position.
10. Alternatively, you can use the **Focus** dialog from the **Devices** menu (**Control > Devices** in the simplified menu).

Setting up a Transwell plate file



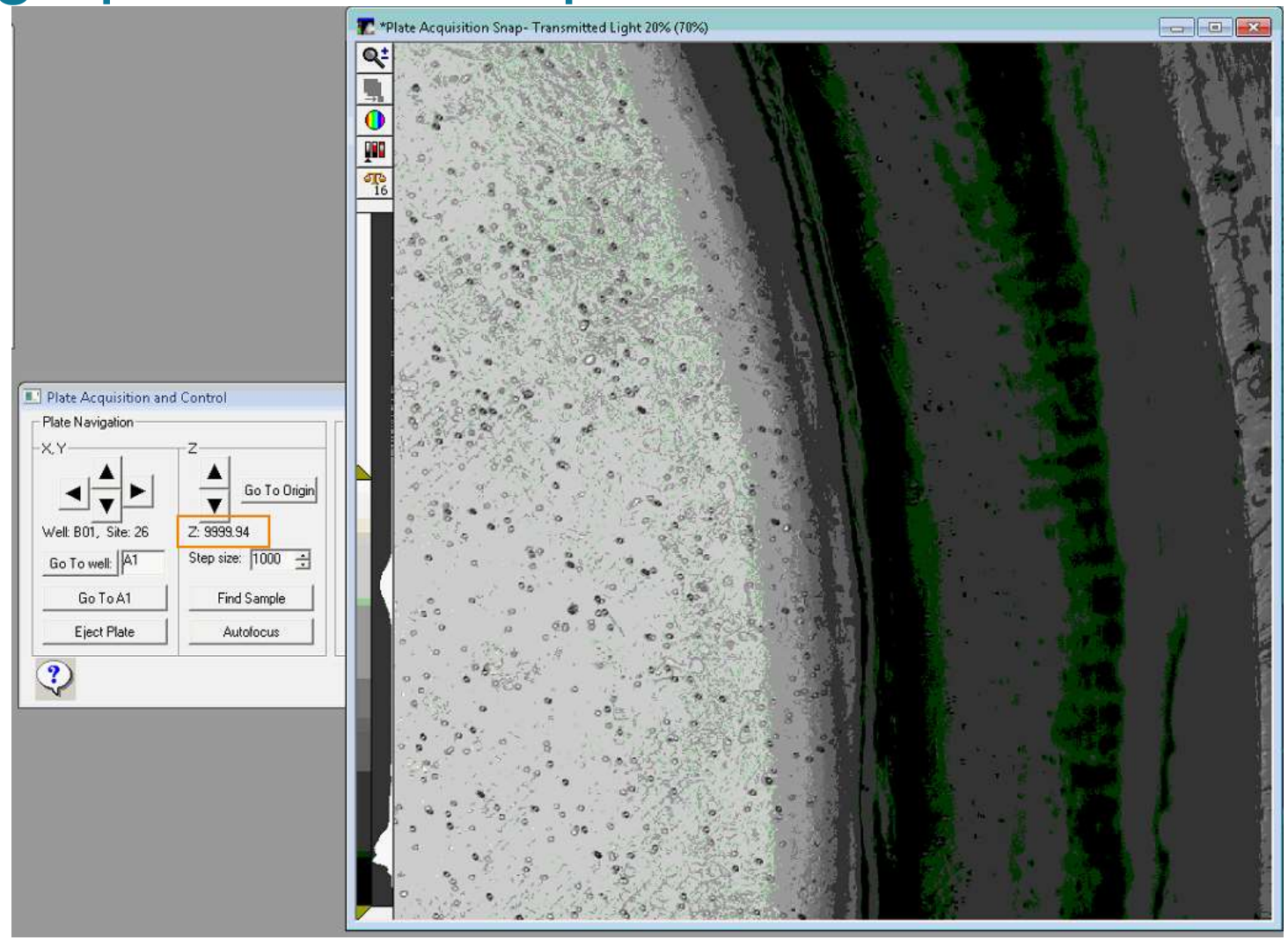
11. Adjust the Z to a setting below the plate bottom (7000 typically works).
12. In the Configure Laser Autofocus Settings dialog, override the **Preview Pass Start** to “Start from current position” and override the **Preview Pass Range** to several thousand μm.
13. Select a high exposure time (usually 50-100 μs will suffice).
14. Click **Preview Pass**.

Setting up a Transwell plate file



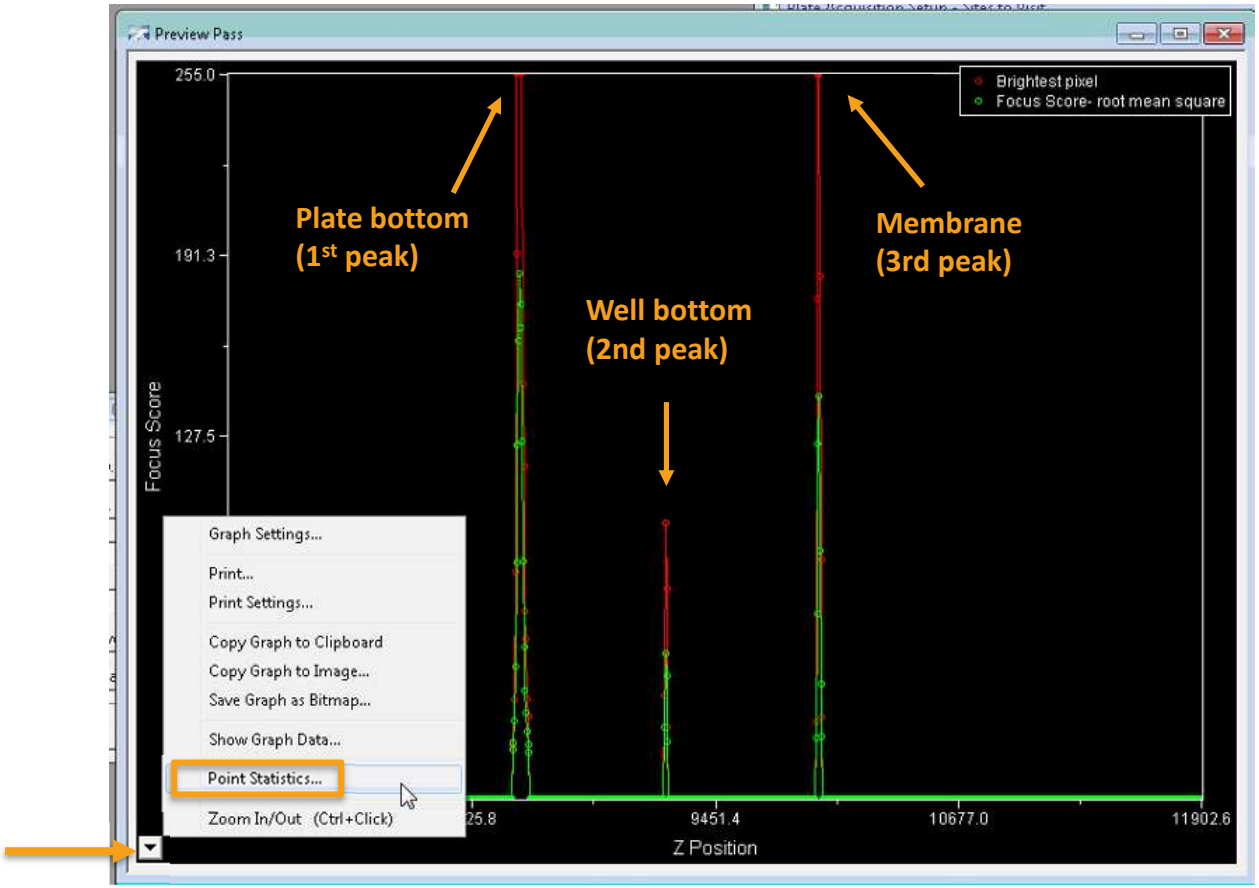
15. You should see a graph window similar to the one above, with 3-4 distinct peaks.
16. If you do not see any peaks or you only see 1-2 peaks, try increasing the exposure time and/or modifying the start Z and range. Also, try moving to other wells/sites.

Setting up a Transwell plate file



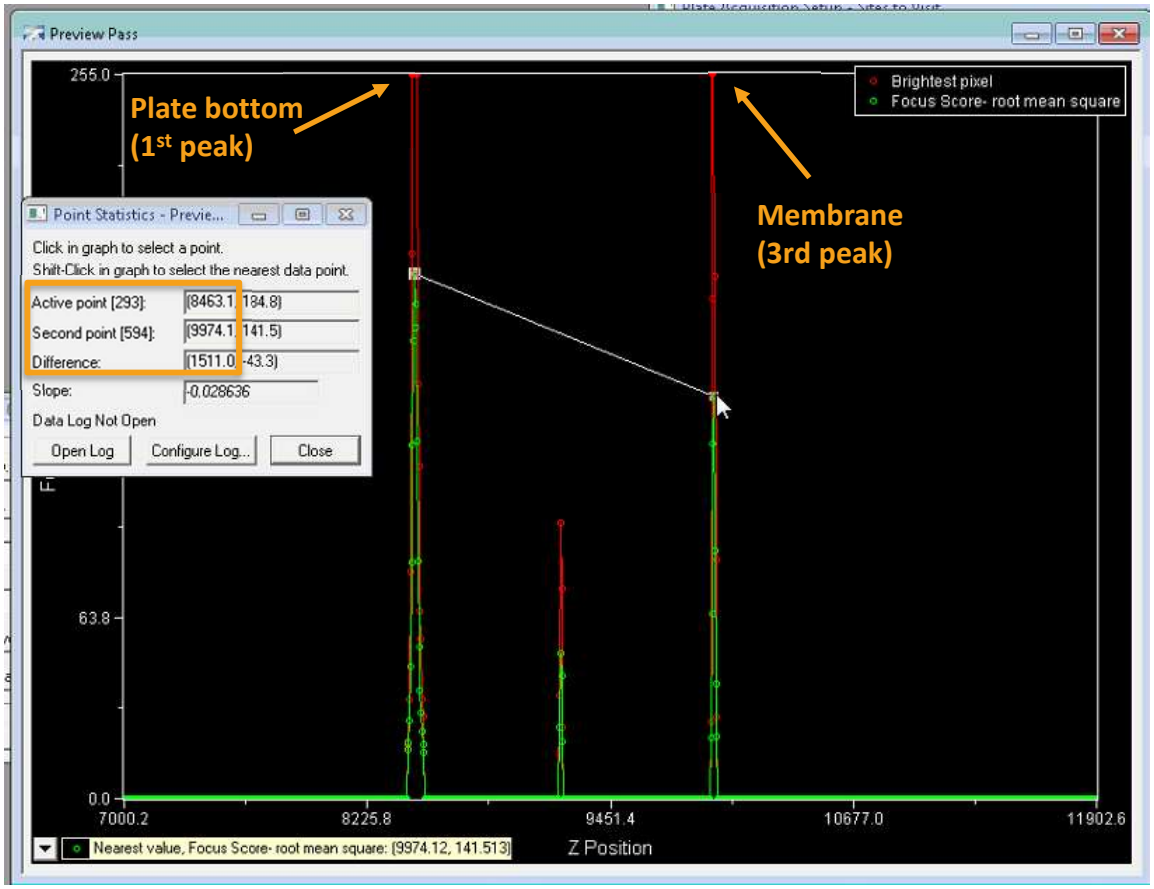
Tip: To confirm that the 3rd peak is the membrane, adjust the Z to that position and acquire an image. When you are done, return the Z to the previous setting.

Setting up a Transwell plate file



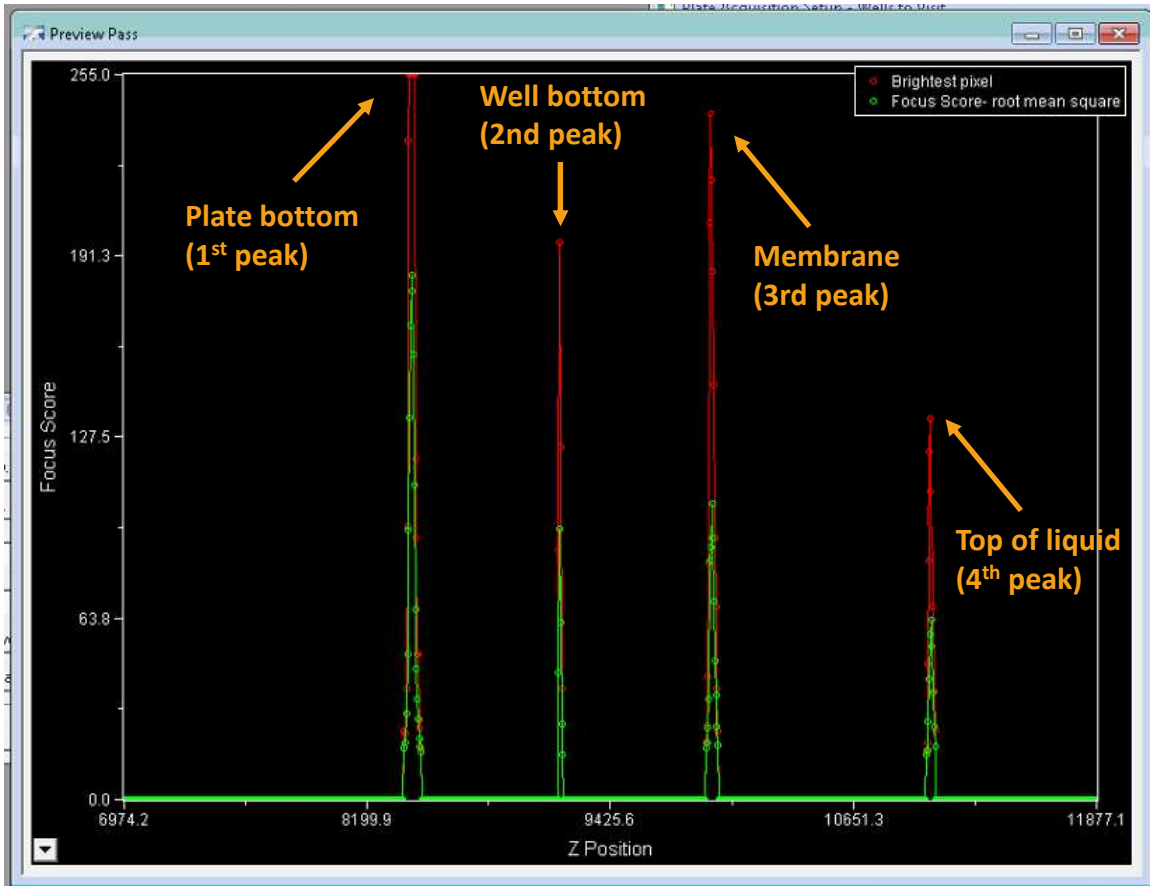
17. To measure the peaks and the difference between them, click on the icon in the lower left corner of the graph and select **Point Statistics**.

Setting up a Transwell plate file



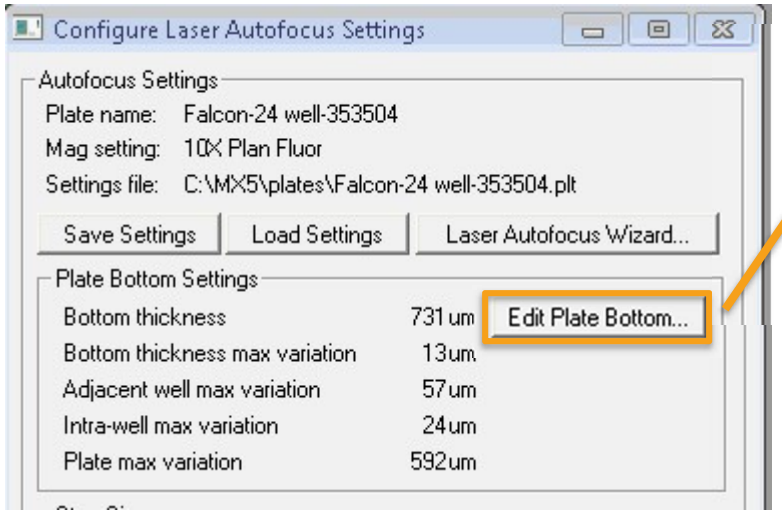
18. Click on the first and the third peaks to measure their positions and the difference between them. Usually the green peaks (focus score) are easier to measure than the red peaks (brightest pixel).
19. Note the plate bottom Z position (8463 in the example above), the membrane position (9974), and the difference (1511 μm).

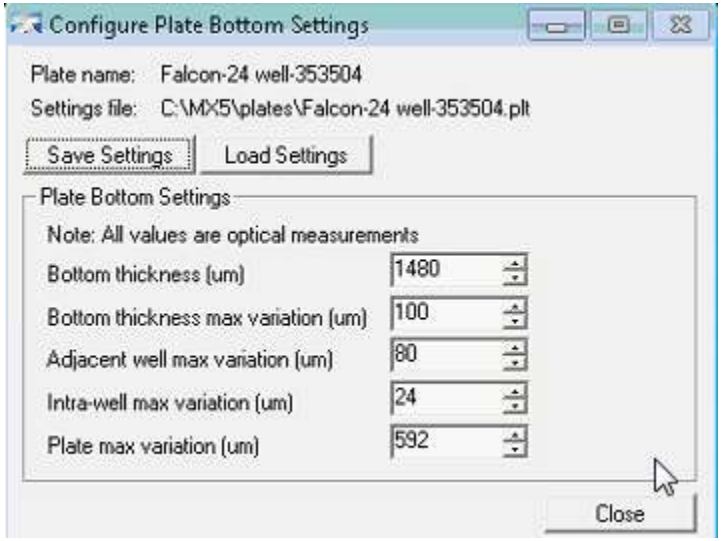
Setting up a Transwell plate file



20. Repeat for several wells so that you can determine the variability of the Z and the thickness (difference).
21. You may sometimes see a 4th peak. This is the top of the liquid and generally can be ignored. If it is too close to the 3rd peak, add more liquid to the well.

Setting up a Transwell plate file





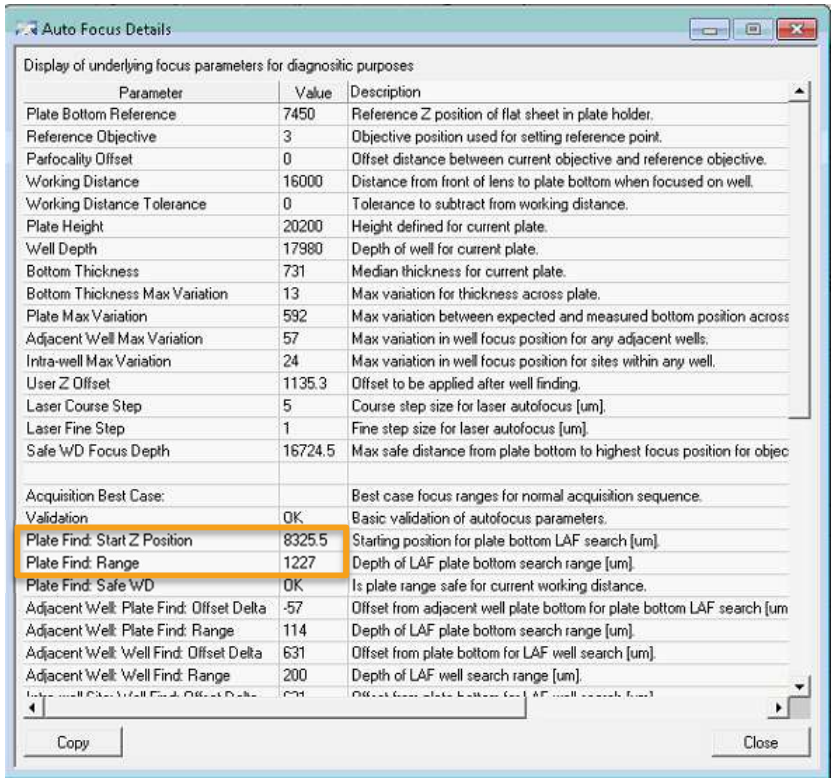
22. Once you have measured sufficient wells, click **Edit Plate Bottom**.
23. Enter the “Bottom thickness” (difference between plate bottom and membrane Z positions) and variation parameters that you estimated from your measurements.
24. Click **Save Settings**.

Tip: If the measured bottom thickness varies significantly, use a number towards the lower end and make sure the Bottom thickness max variation setting is sufficient to cover the thickness range that you measured.

Setting up a Transwell plate file

25. Changing the bottom thickness and/or the plate max variation will affect the start Z position.
26. Go to **Autofocus** tab > **View Focusing Details** to view the **Plate Find Start Z Position** and **Plate Find Range**.

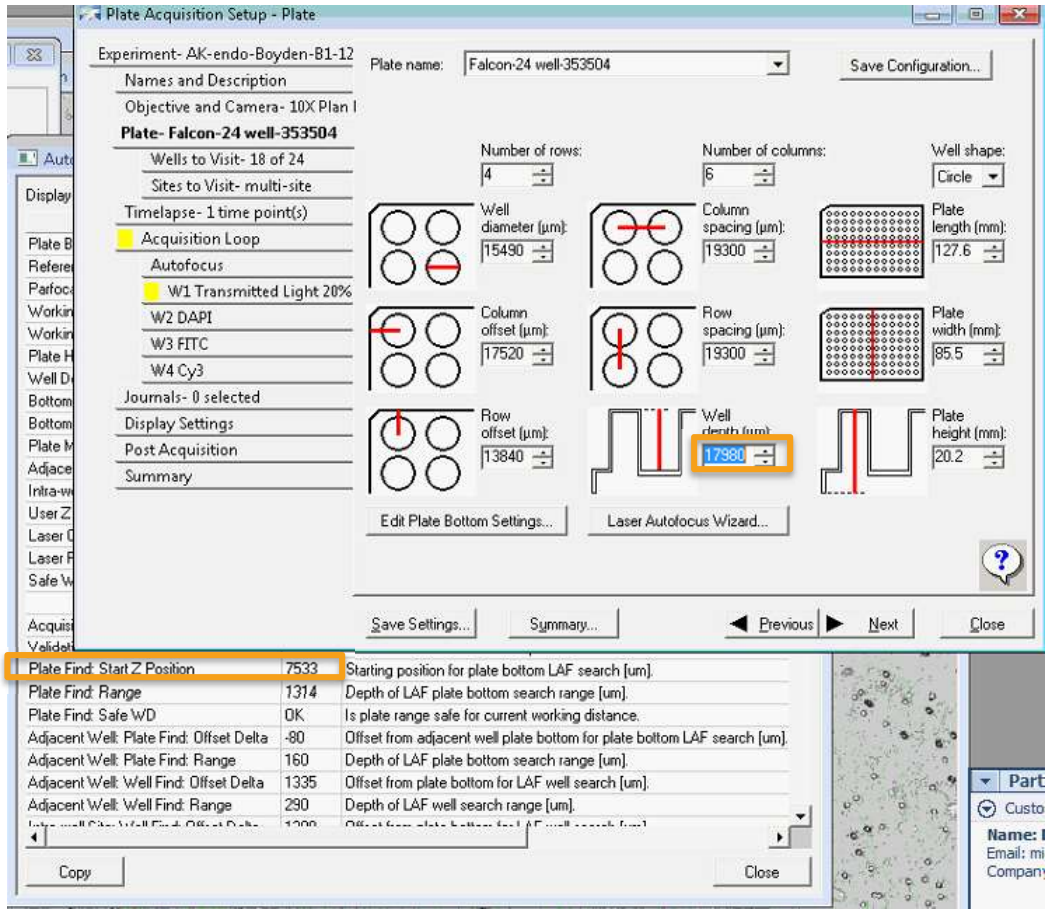
Setting up a Transwell plate file



Parameter	Value	Description
Plate Bottom Reference	7450	Reference Z position of flat sheet in plate holder.
Reference Objective	3	Objective position used for setting reference point.
Parfocality Offset	0	Offset distance between current objective and reference objective.
Working Distance	16000	Distance from front of lens to plate bottom when focused on well.
Working Distance Tolerance	0	Tolerance to subtract from working distance.
Plate Height	20200	Height defined for current plate.
Well Depth	17980	Depth of well for current plate.
Bottom Thickness	731	Median thickness for current plate.
Bottom Thickness Max Variation	13	Max variation for thickness across plate.
Plate Max Variation	592	Max variation between expected and measured bottom position across
Adjacent Well Max Variation	57	Max variation in well focus position for any adjacent wells.
Intra-well Max Variation	24	Max variation in well focus position for sites within any well.
User Z Offset	1135.3	Offset to be applied after well finding.
Laser Course Step	5	Course step size for laser autofocus [um].
Laser Fine Step	1	Fine step size for laser autofocus [um].
Safe WD Focus Depth	16724.5	Max safe distance from plate bottom to highest focus position for objec
Acquisition Best Case:		Best case focus ranges for normal acquisition sequence.
Validation	OK	Basic validation of autofocus parameters.
Plate Find: Start Z Position	8325.5	Starting position for plate bottom LAF search [um].
Plate Find: Range	1227	Depth of LAF plate bottom search range [um].
Plate Find: Safe WD	OK	Is plate range safe for current working distance.
Adjacent Well: Plate Find: Offset Delta	-57	Offset from adjacent well plate bottom for plate bottom LAF search [um
Adjacent Well: Plate Find: Range	114	Depth of LAF plate bottom search range [um].
Adjacent Well: Well Find: Offset Delta	631	Offset from plate bottom for LAF well search [um].
Adjacent Well: Well Find: Range	200	Depth of LAF well search range [um].

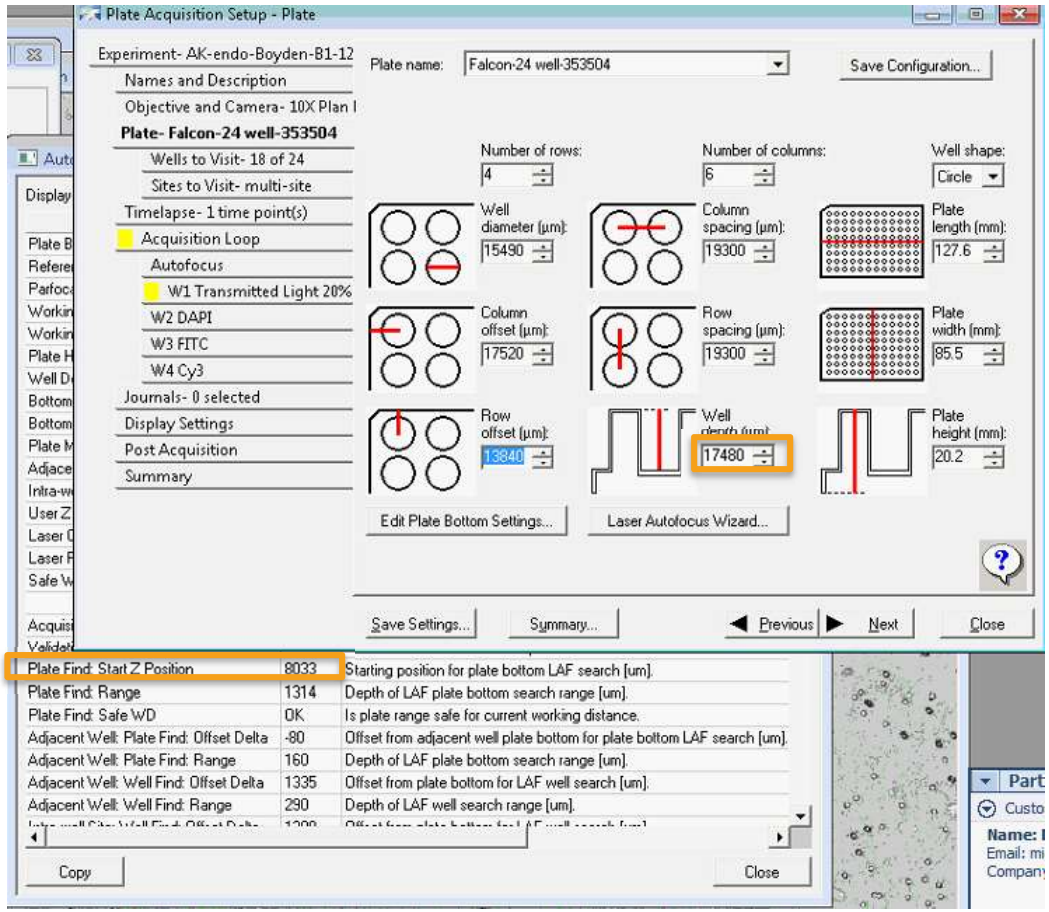
27. Compare the **Plate Find Start Z Position** and **Plate Find Range** to your measurements of the plate bottom.
28. The range must cover all of the possible plate bottom Z positions, but does not necessarily need to reach the membrane Z position.

Setting up a Transwell plate file



29. If the start Z position is inappropriate, adjust the **Well Depth**.
    a) Increase the well depth for a lower start Z position.
    b) Decrease the well depth for a higher start Z position.
30. If the range is inappropriate, adjust the **Plate Max Variation**.
31. Click **Save Configuration** after modifying plate dimensions.

Setting up a Transwell plate file



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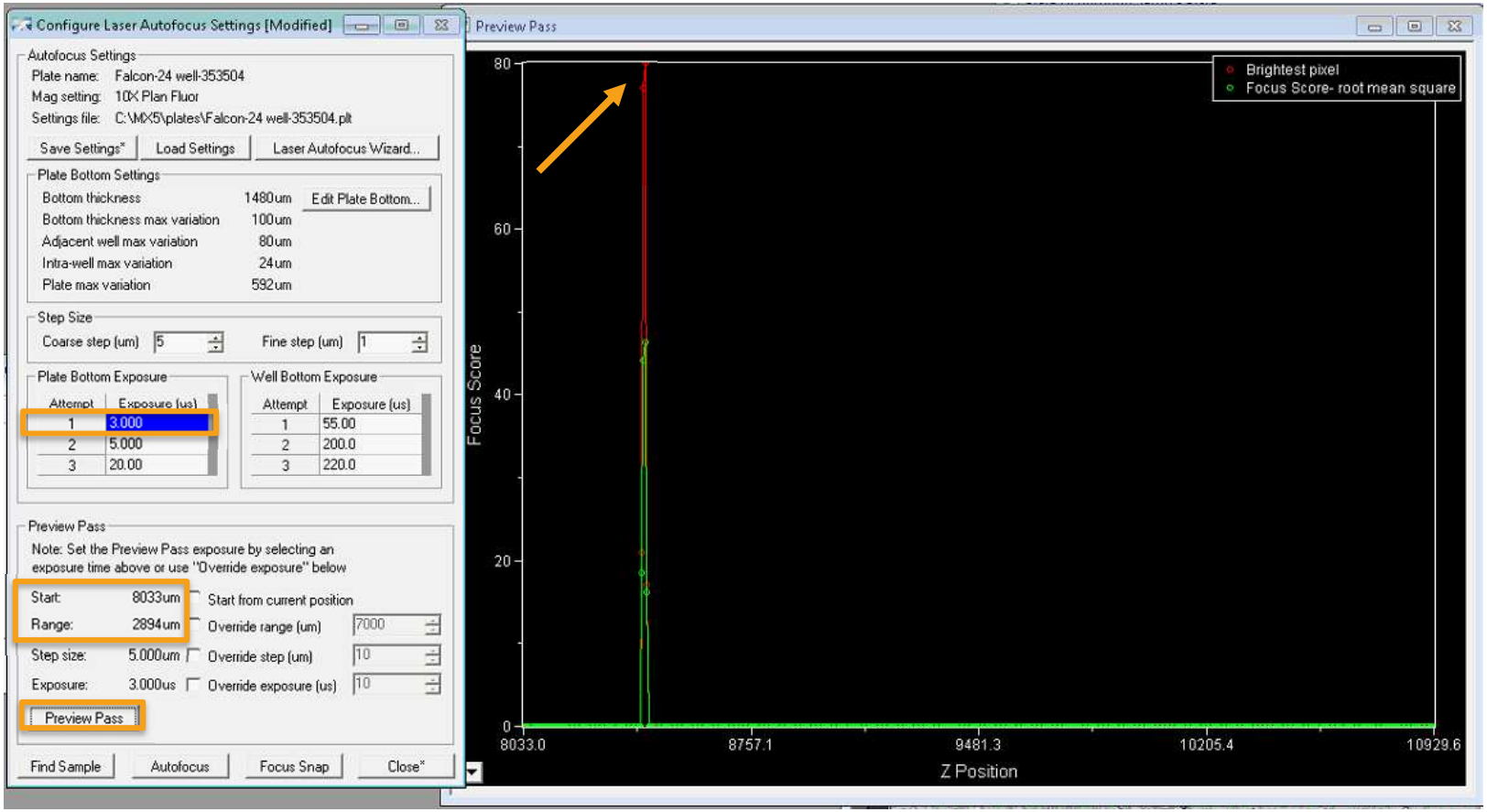
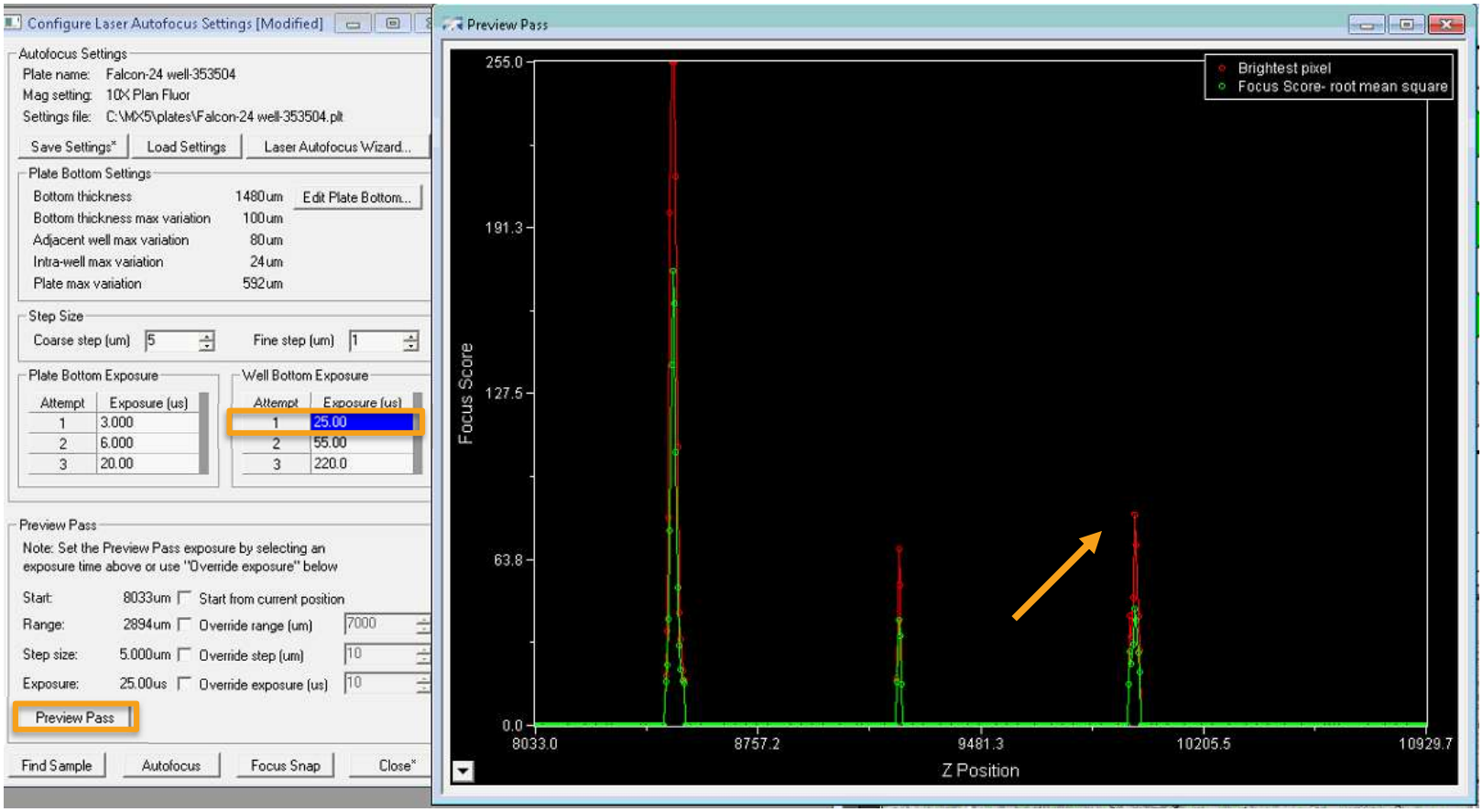


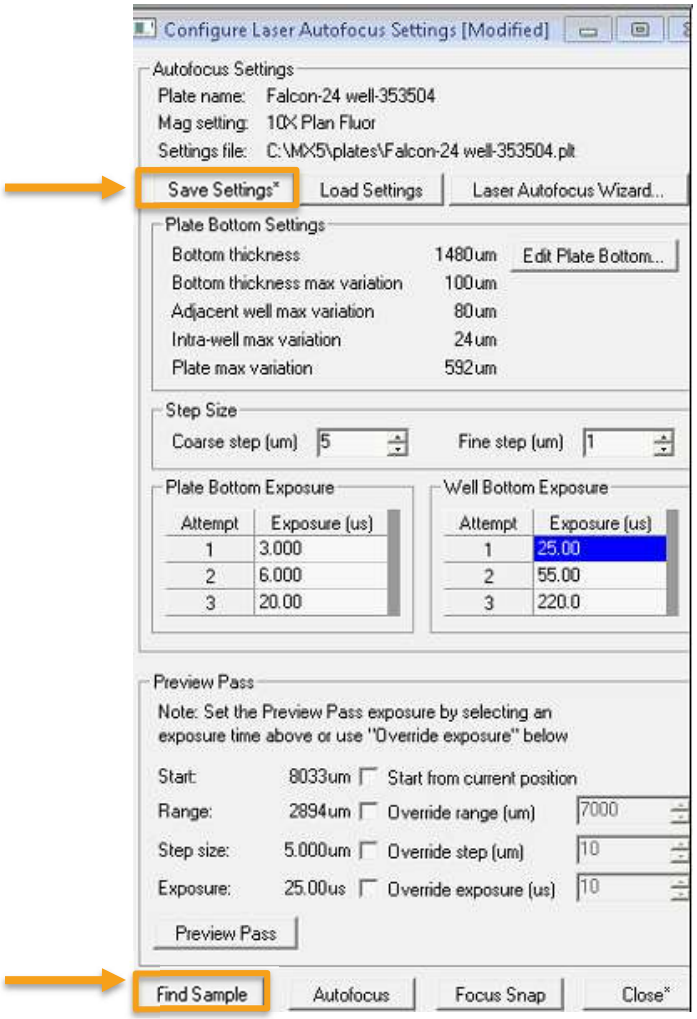
32. You can now disable the overrides in **Configure Laser Autofocus Settings** for the **Preview Pass Start** and **Range**.
33. Test and adjust the exposure times for the plate bottom. Exposure 1 should give a red peak between 50-150 for most wells. Exposures 2 and 3 should be higher as they will be used for wells with dim reflections. It is fine to only see one peak during this test.

Setting up a Transwell plate file



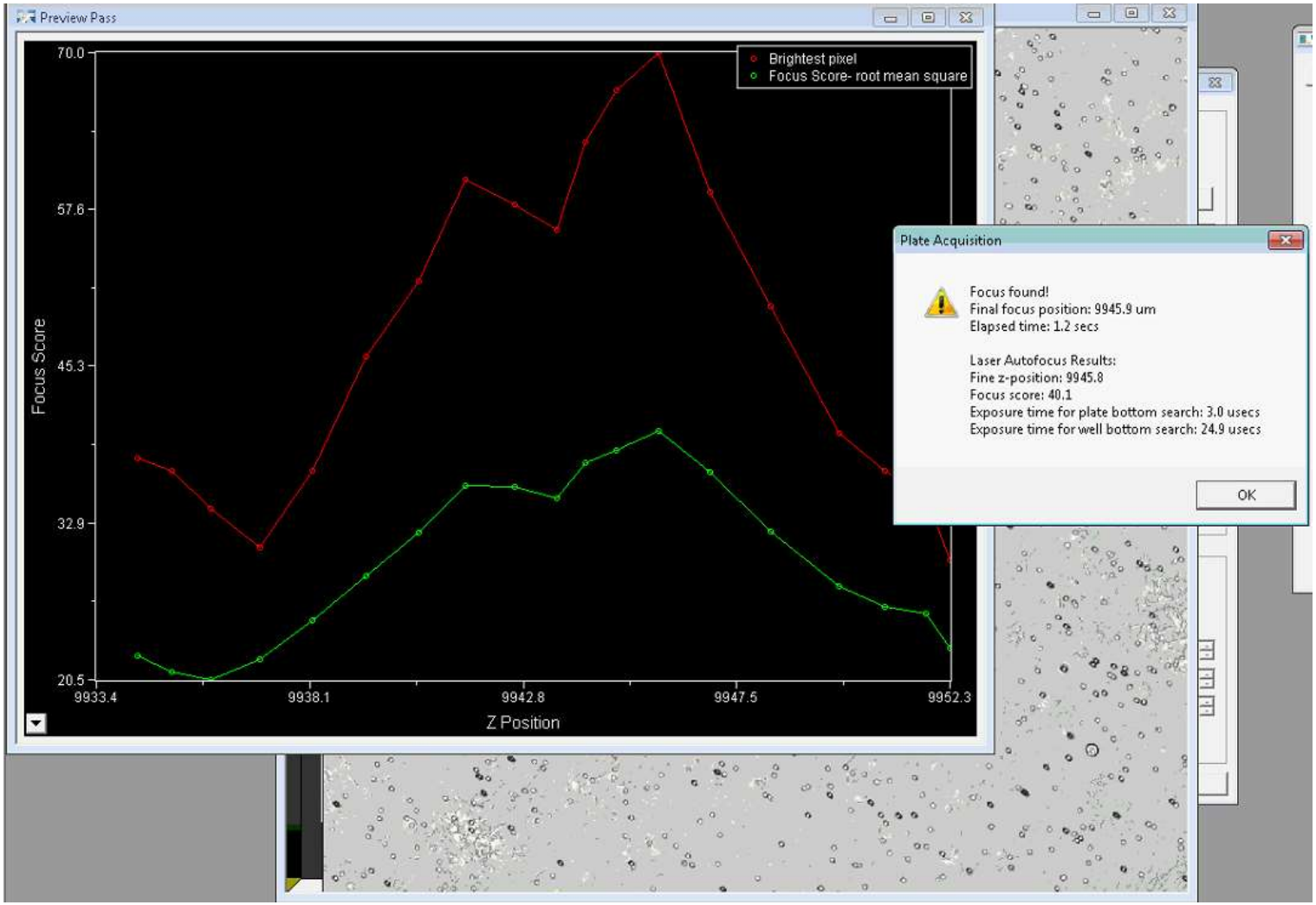
34. Test and adjust the exposure times for the “well bottom” (actually the membrane surface). Exposure 1 should give a red peak between 50-150 for most wells. Exposures 2 and 3 should be higher as they will be used for wells with dim reflections. It is fine for the other peaks to be saturating during this test.

Setting up a Transwell plate file



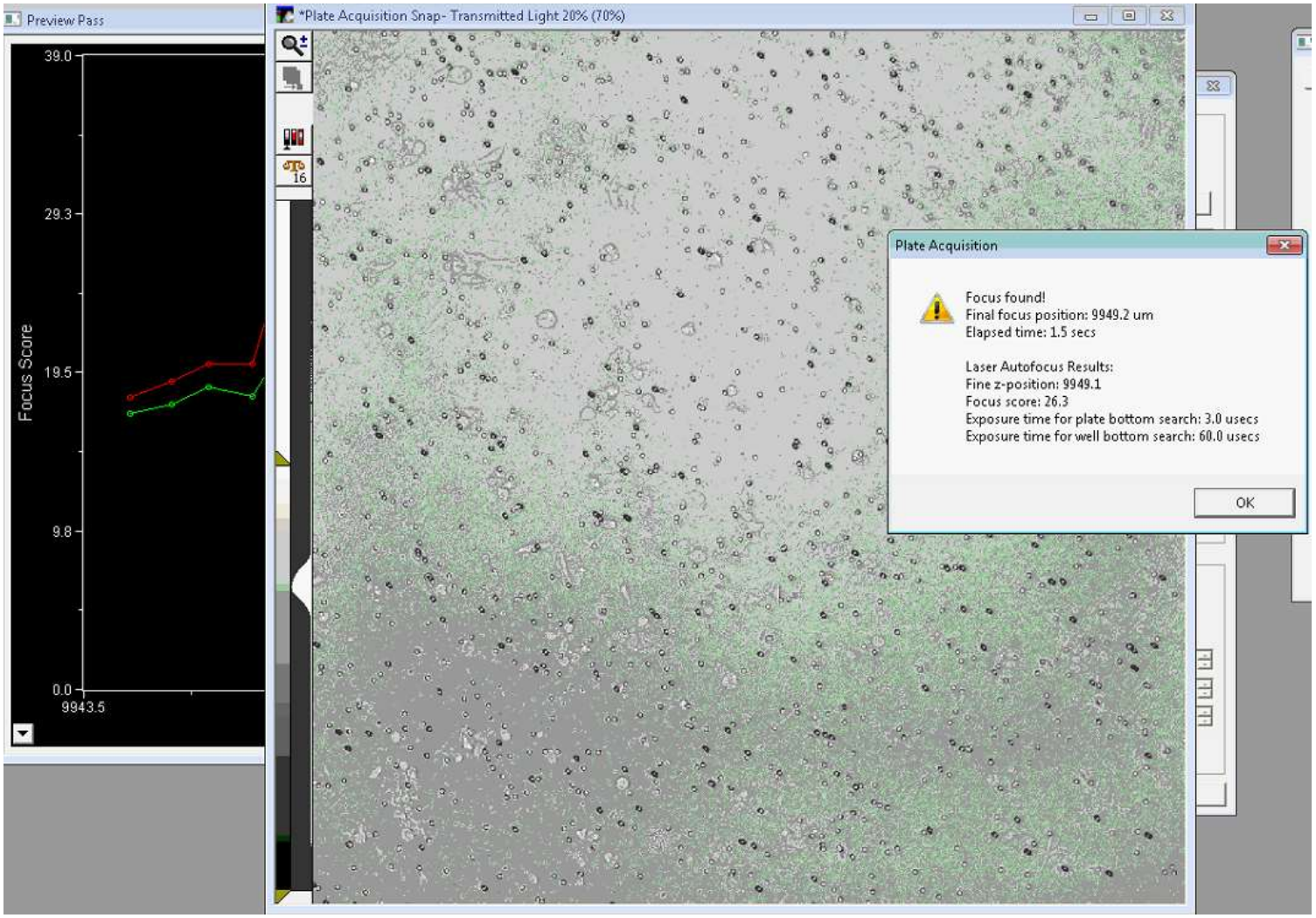
35. After adjusting settings, click **Save Settings** to update your plate file.
36. Click **Find Sample** to test the autofocus settings on the current well and site.

Setting up a Transwell plate file



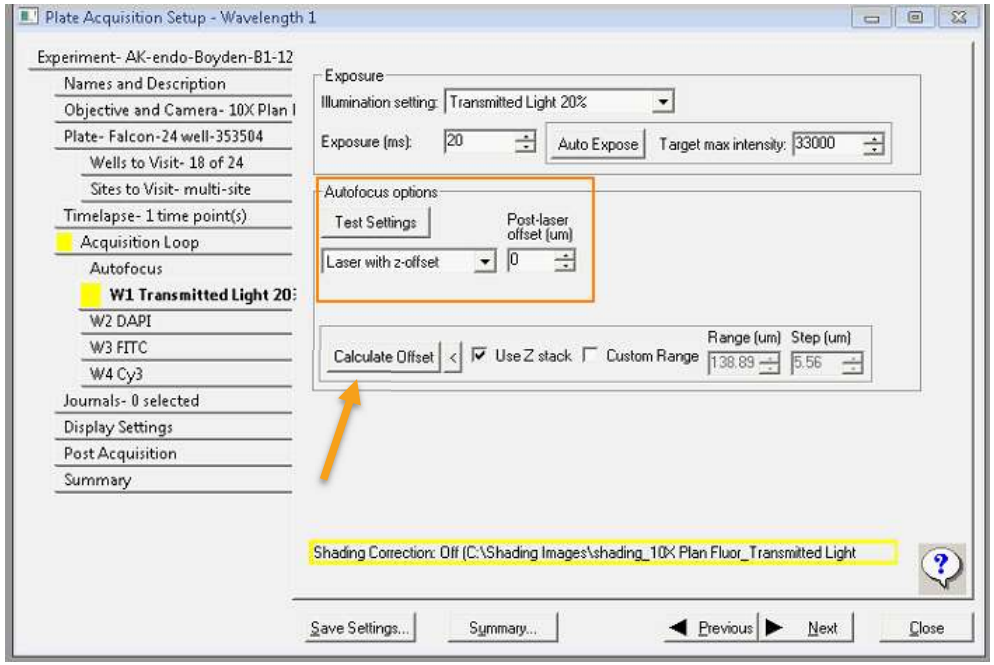
37. If the Find Sample focus succeeded, you will see a “Focus found!” message, along with a zoomed Preview Pass graph and a focused image
38. If the focus failed, adjust autofocus settings and retry. Also, try other wells.

Setting up a Transwell plate file



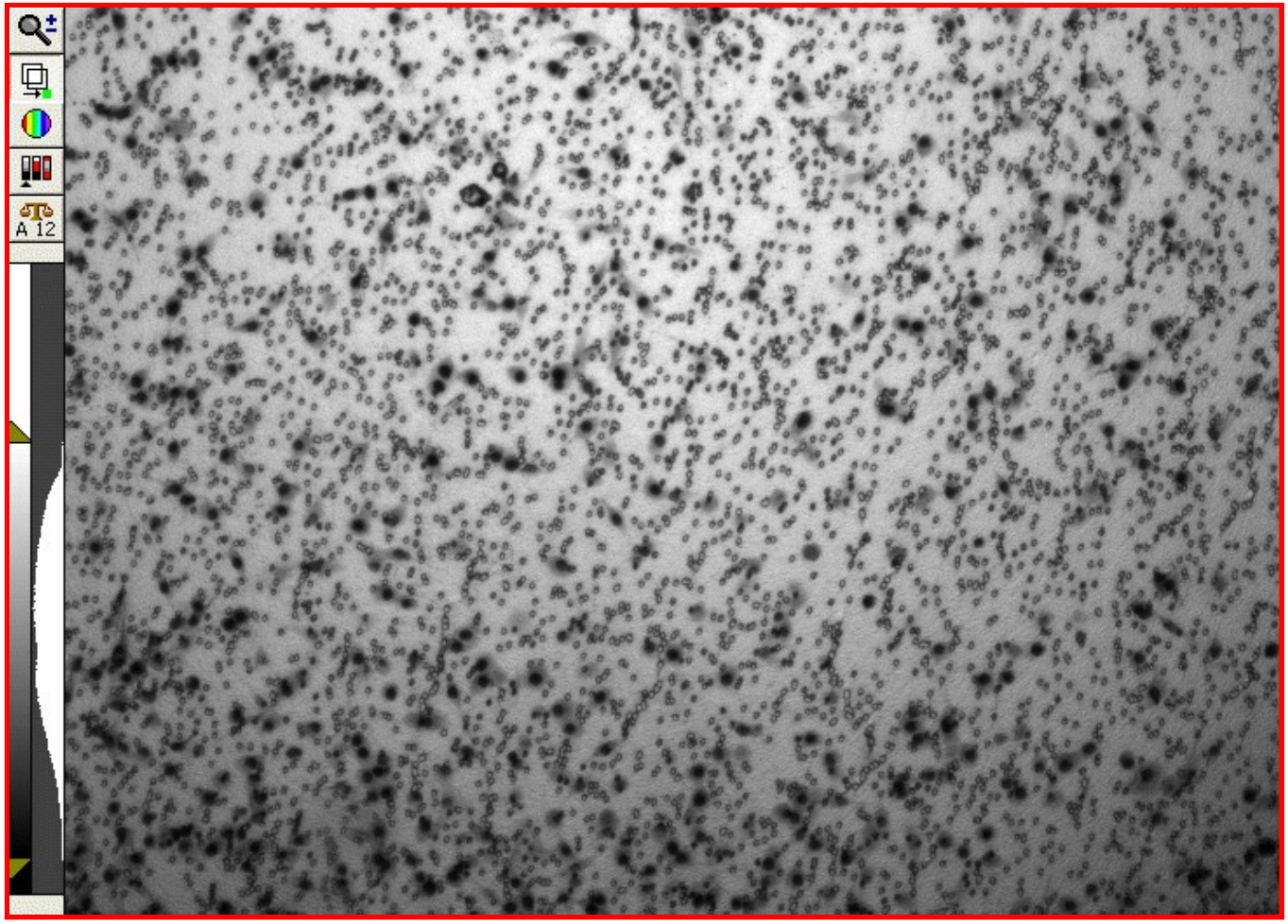
39. Repeat the exposure time optimization and Find Sample test for other long working distance objectives that you want to image with. This type of plate is incompatible with short working distance objectives.

Setting up a Transwell plate file



40. After you have verified that the autofocus is working for your objective, go to the W1 tab and Calculate Offset.
41. Verify that the exposure time is appropriate.
42. Repeat for the other wavelengths.
43. Once the settings are optimized, run a test acquisition.

Transwell assay imaging



Tip: If Transmitted Light is used, expect to see both the membrane pores and cells. A custom module or custom journal analysis is required to distinguish these. Alternatively, use Fluorescence for more accurate cell counts.

Support Resources

- F1 / HELP within MetaXpress® Software
- Support and Knowledge Base: http://mdc.custhelp.com/
- User Forum: http://metamorph.moleculardevices.com/forum/
- Email support@moldev.com
- Technical Support can also be reached by telephone:
  - 1 (800) 635-5577
  - Select options for Tech Support → Cellular Imaging Products → ImageXpress Instruments

MOLECULAR DEVICES

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