

Multi-Position Time Series with Definite Focus on the LSM780

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12/24/15

Definite focus requires the sample to be in media of a lower refractive index than glass. This is because the definite focus reflects infrared light off the interface between the cover slip and the media. Because of Snell's law, the reflection only occurs when light is going from high refractive index to low refractive index. This is the same principle as TIRF [Total Internal Reflection Fluorescence].

This is for live cell use. Definite focus will not work with mounting media.

Definite Focus works with the following objectives:

10x Dry
20x Dry
40x Water ***
40x Oil
63x Oil

***** Note: While the 40x Water objective can do definite focus the special “oil” is too viscous for multi-position experiments so should not be used.**

IMPORTANT INFORMATION TO AVOID FOCUS DRIFT

In order to stabilize the focus one must:

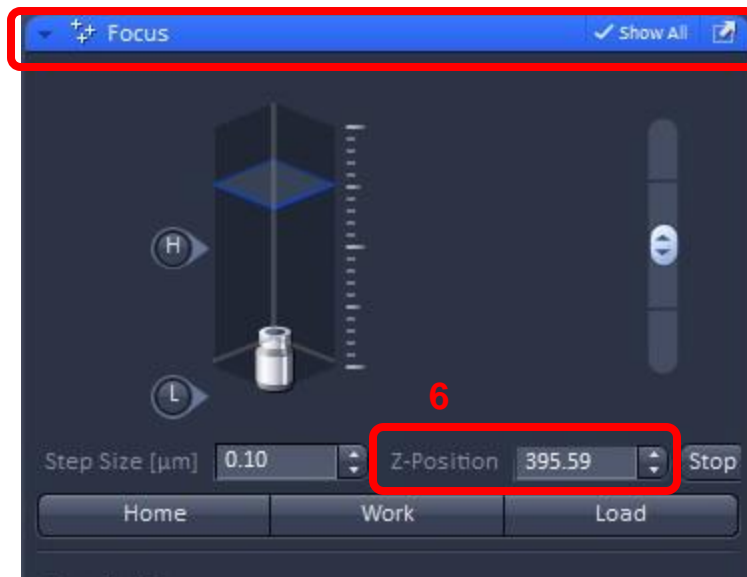
- 1. Have whole microscope on for 2 hours before starting any experiment.**
- 2. The sample should sit on the microscope for 15 minutes before you start imaging.**

If you are going to do a live cell experiment in the morning then it would be best to keep the microscope on from the day before.

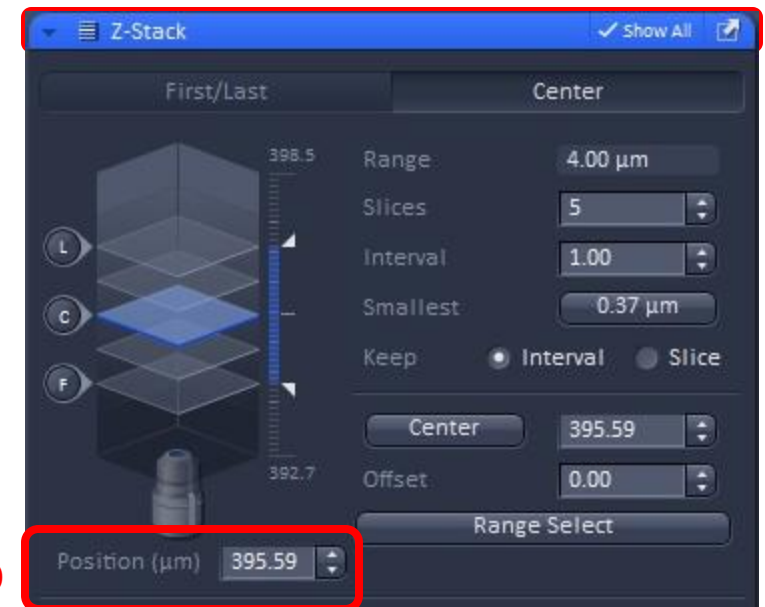
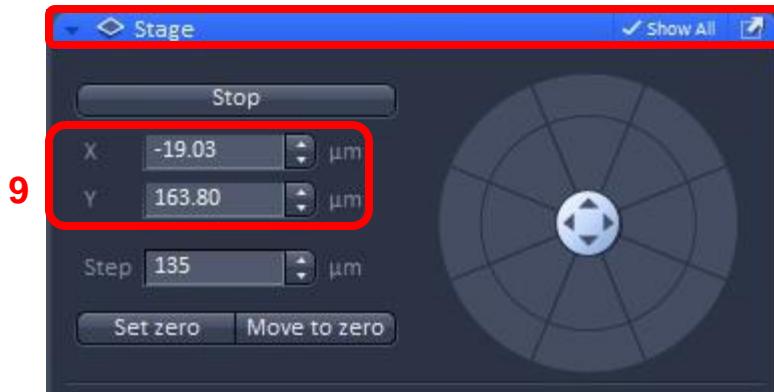
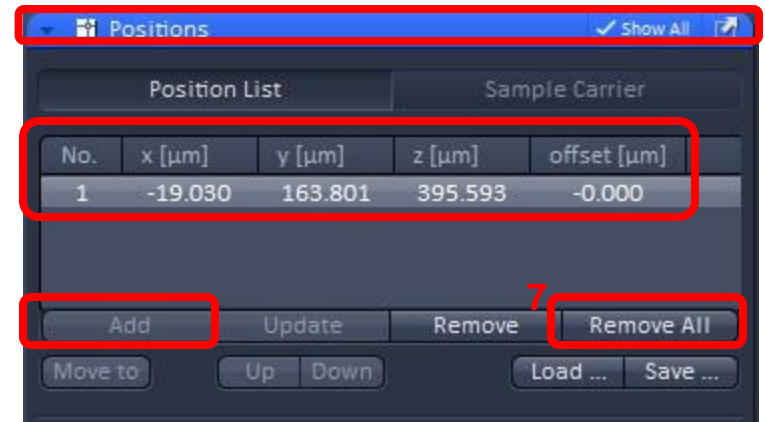
The following items that are critical to turn off in this scenario:

- 1. X-Cite Lamp power switch.**
- 2. Lasers turned off via software.**
- 3. Argon Laser toggled and key turned off.**
- 4. Computer turned off.**

1. Set up your sample as you normally would for imaging.
2. Check Z Stack, Time Series and Positions Boxes.
3. In the Z-Stack box set the Z stack by selecting Center.
4. Click the Center button.
5. Select the number of slices you want. It must be an odd number.
6. Double Check the Z position in the Z-Stack and Focus Box to make sure they match.



7. In the Positions window if there are any positions listed click Remove All.
8. Click Add to add your current position.
9. Double check x, y and z numbers in Positions window with x, y and z numbers in the Stage [x and y] and Z-Stack [z] windows. They should match.

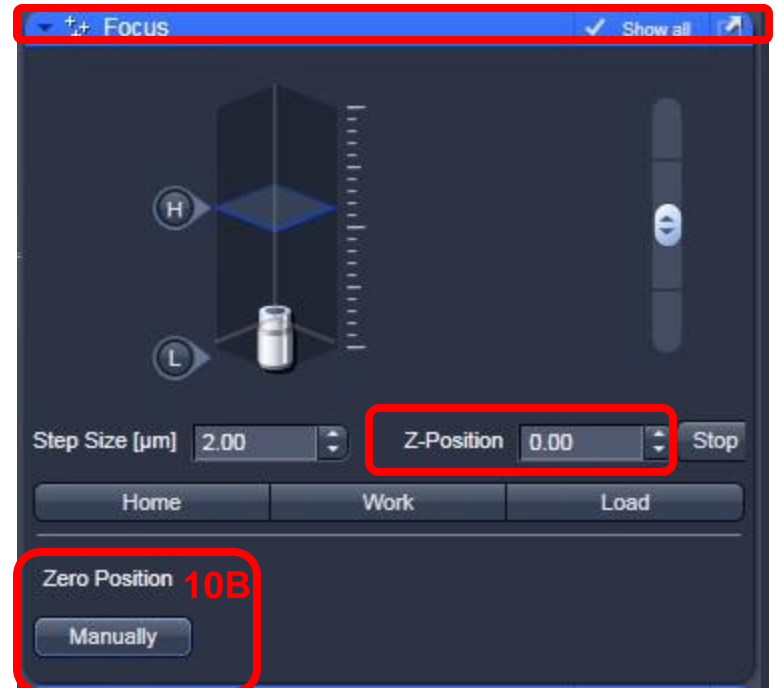
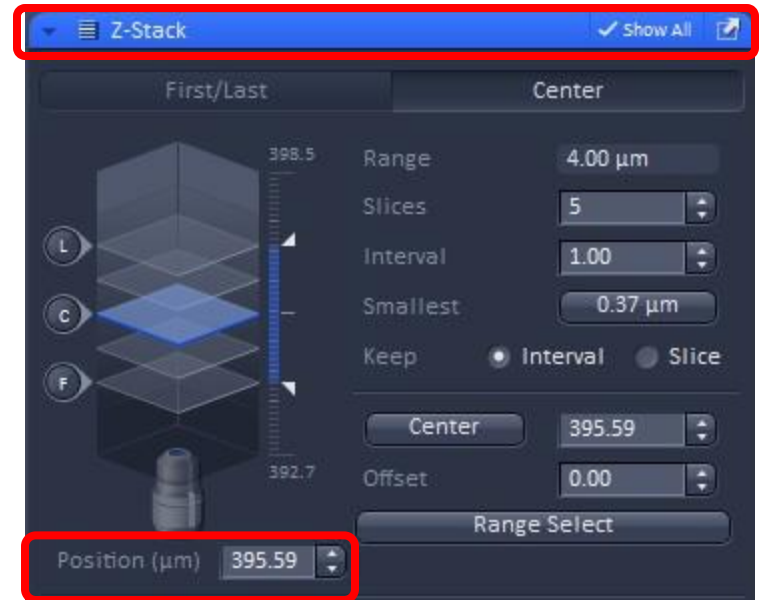


10. Before adding other positions:
- A. In the Z-Stack window check the number in the Position box.
 - B. If it is a large number, 5000 for example, then in the Focus window under Zero Position click the Manually button. This sets the Z-Position to Zero.

This enables the Z offset to function properly when you add positions.

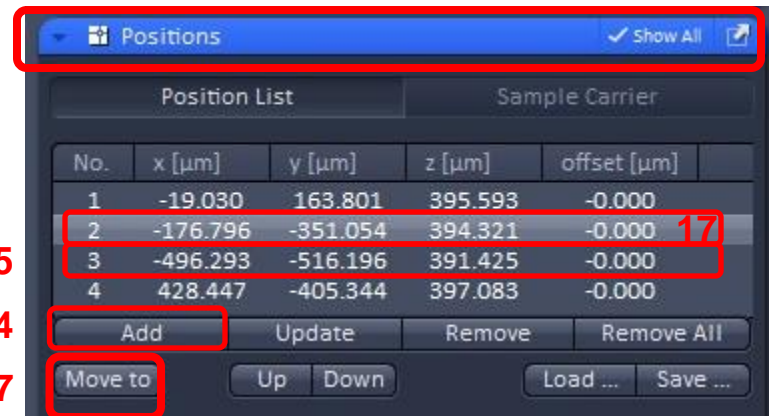
Z-Stack is ONLY set at Position 1.

10A

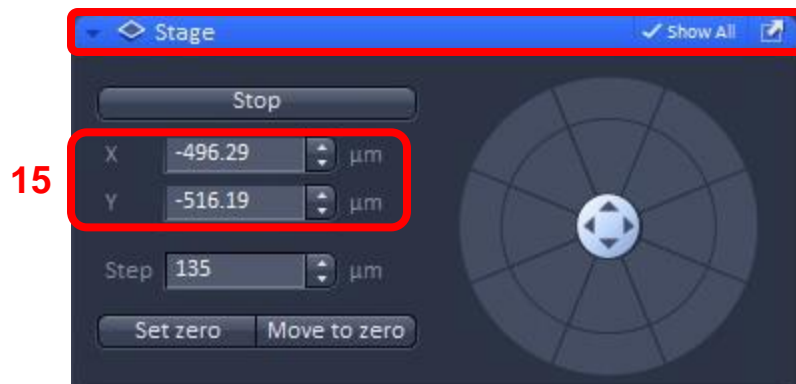


Zero Position 10B

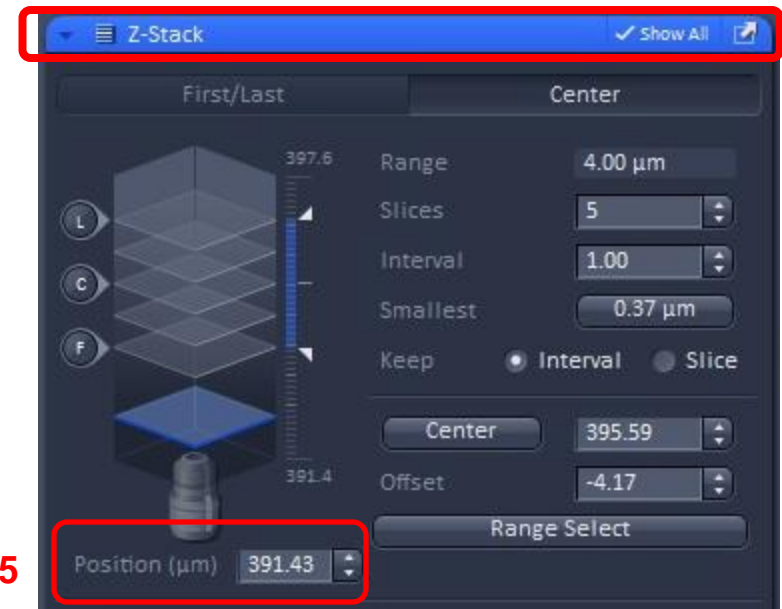
11. Move to next position.
12. Focus the sample and get your settings.
13. **DO NOT do set the Z stack.**
14. In positions box click Add.
15. Double check the X, Y and Z positions in the positions window matches the X,Y position in the Stage window and Z position in the Z-Stack window.
16. Repeat Steps 11-15 for all other positions.
17. When finished with all positions move through each one again to check that positions are correct. Highlight the position you wish to move to then click Move To in order to move to that position.



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17



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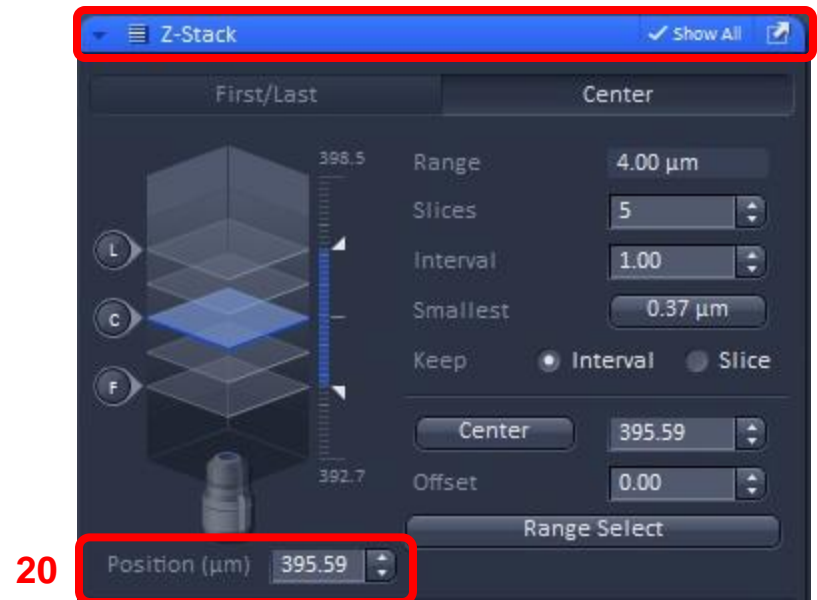
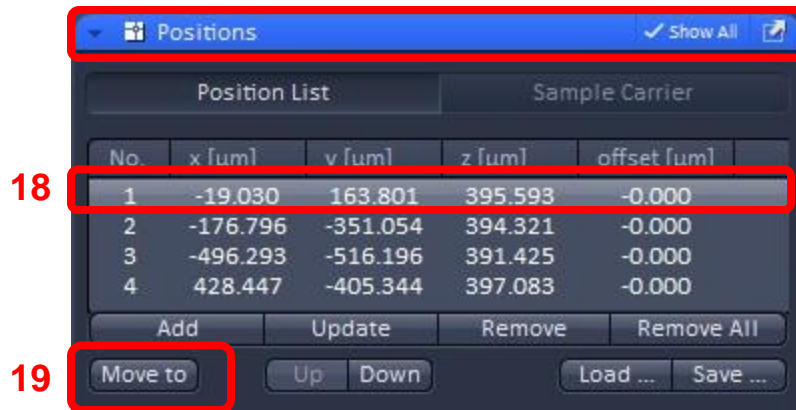
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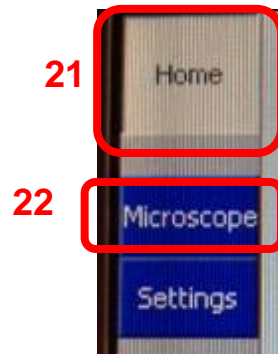
When you have set and double checked all of your positions:

18. Move back to Position 1 by highlighting the position.
19. Click Move To.
20. Check Stage and Z-Stack windows to ensure you are on Position 1.

Important Reminders Before You Continue:

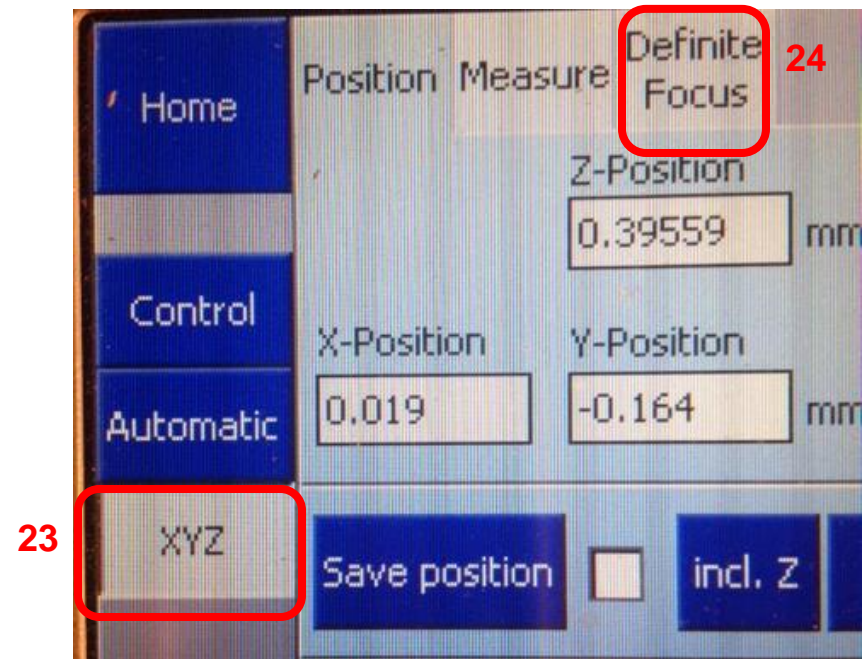
1. Z Stack is ONLY set for Position 1. Do not click anything in the Z-Stack window [Steps 3-5] for Position 2 and onward.
2. You MUST be in Position 1 when you perform ALL the steps that follow.



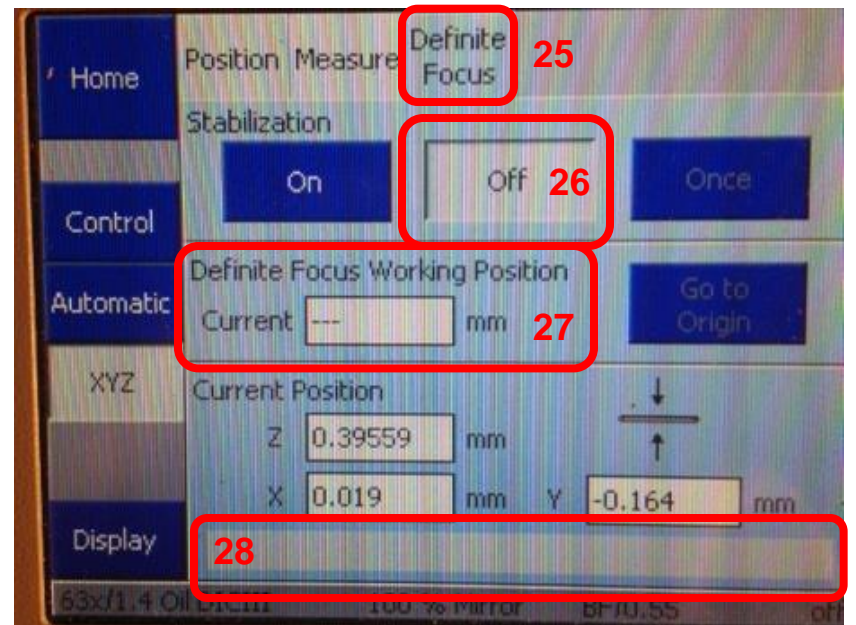


On the touch pad:

21. Select the Home screen.
22. Select Microscope.
23. Select XYZ.
24. Select Definite Focus.
25. This is what the Definite Focus Screen looks like.
26. The current position is off. [Lighter Color].
27. You will also note that under Definite Focus Working Position in the box next to Current you see no numbers. Just -----.
28. You will also see this box is blank.



23



NOTE: If you do not see the Definite Focus tab that indicates the Definite Focus Power Supply is turned off. Please see the appendix for instructions on how to troubleshoot this.

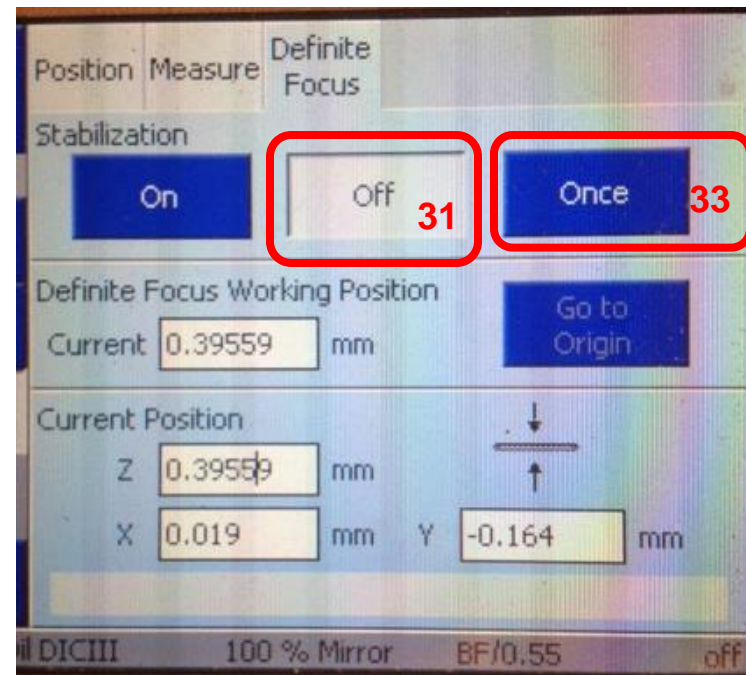
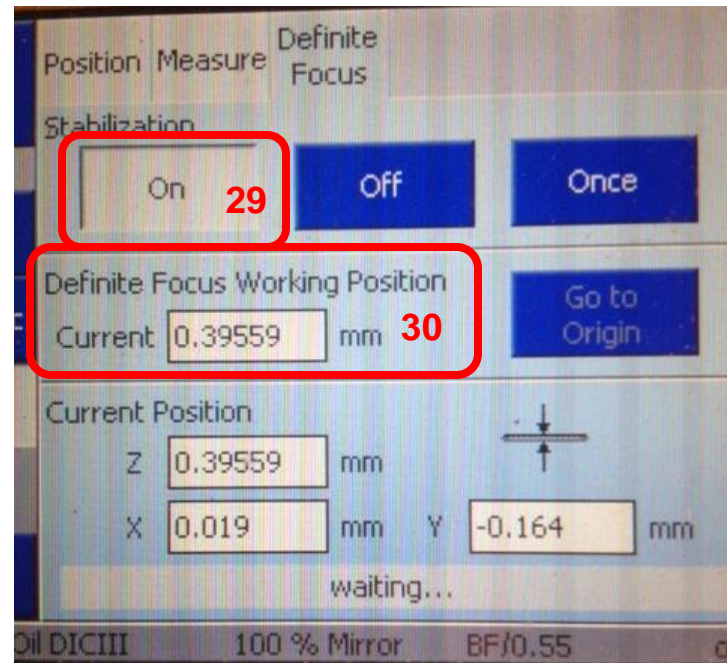
You will now need to set or “train” the autofocus:

29. Click On.
30. Wait until you see numbers populate the Current Box under Definite Focus Working position.
31. Click Off.

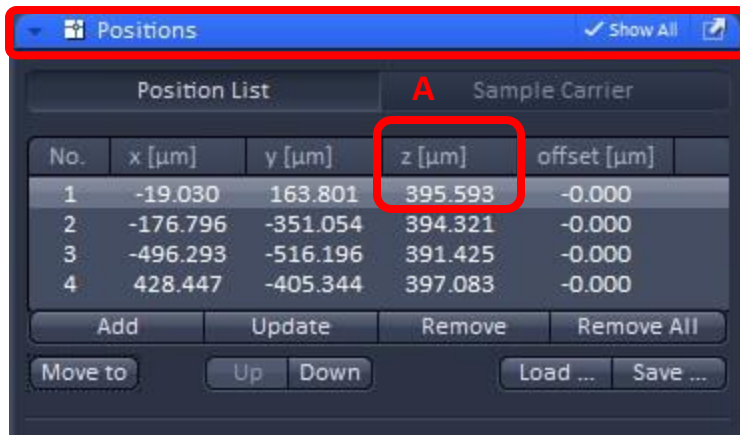
The autofocus is now trained.

If you want to test the Definite Focus:

32. Move slightly out of focus.
33. Click Once.
34. See that focus returns to proper position. If not repeat training of definite focus. Proper position is that the number in this position matches the Z position of Position 1. The next slide will illustrate this.



34. Make sure you are still on Position 1.
Confirm that the Z value in Positions [A] matches the Definite Focus Working Position Current value [B]. The Positions value is in micrometers while the Definite Focus Working Position Current value is in mm so numbers will not directly match in terms of increments.



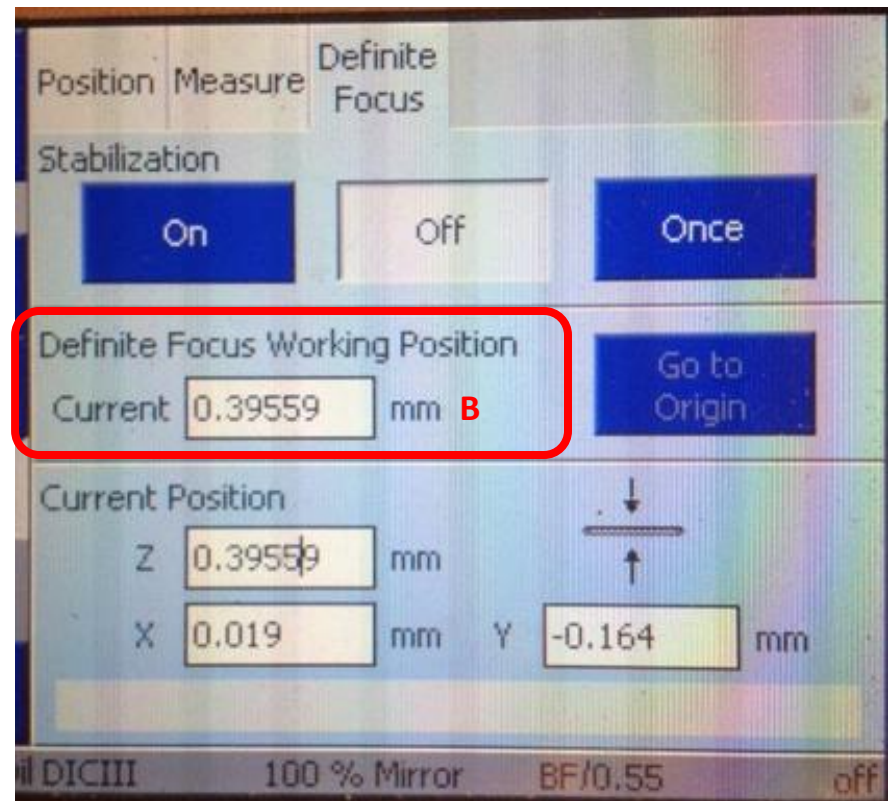
Positions ✓ Show All

Position List **A** Sample Carrier

No.	x [μm]	y [μm]	z [μm]	offset [μm]
1	-19.030	163.801	395.593	-0.000
2	-176.796	-351.054	394.321	-0.000
3	-496.293	-516.196	391.425	-0.000
4	428.447	-405.344	397.083	-0.000

Add Update Remove Remove All

Move to Up Down Load ... Save ...



Position Measure Definite Focus

Stabilization

On Off Once

Definite Focus Working Position

Current 0.39559 mm **B** Go to Origin

Current Position

Z 0.39559 mm

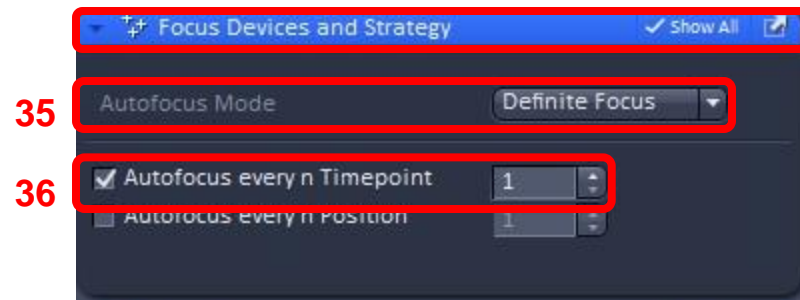
X 0.019 mm Y -0.164 mm

DICIII 100 % Mirror BF/0.55 off

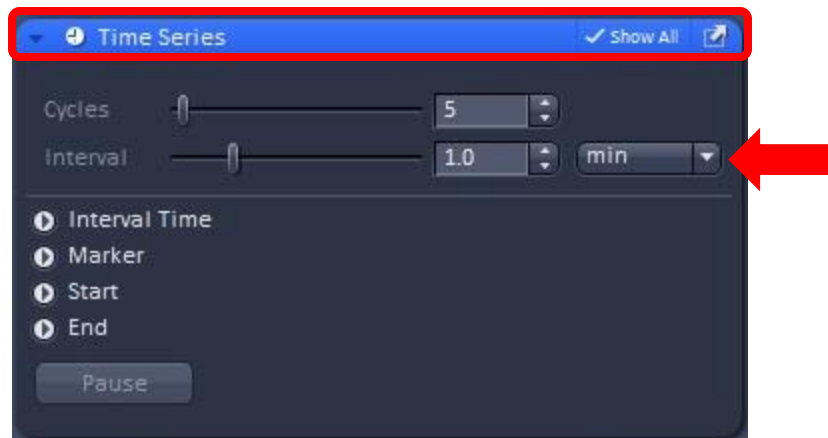
In the Focus Device and Strategy window:

35. For Autofocus Mode use the drop down menu to select Definite Focus.

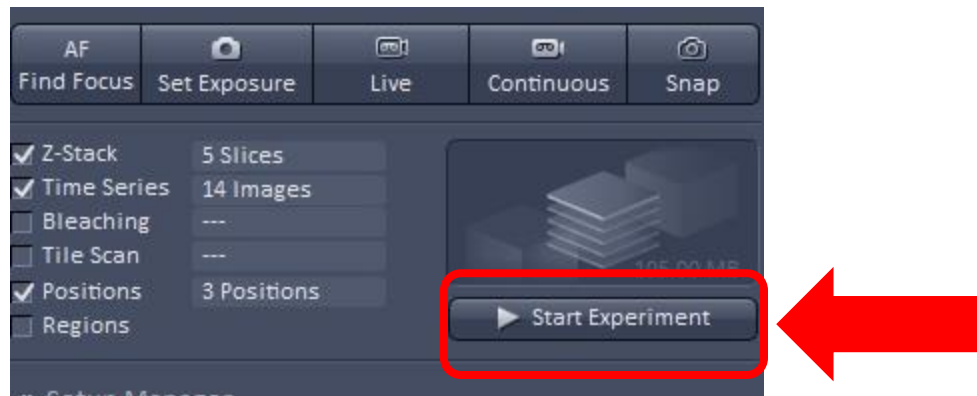
36. Check box for Autofocus every n Timepoint and enter the number 1 in the box.



37. In the “Time Series” box: Select the number of images you wish to take in “Cycles” and the time between each cycle in “Interval.” You can change the increment of interval in the pull down menu [red arrow]. Choices are msec, sec and min.

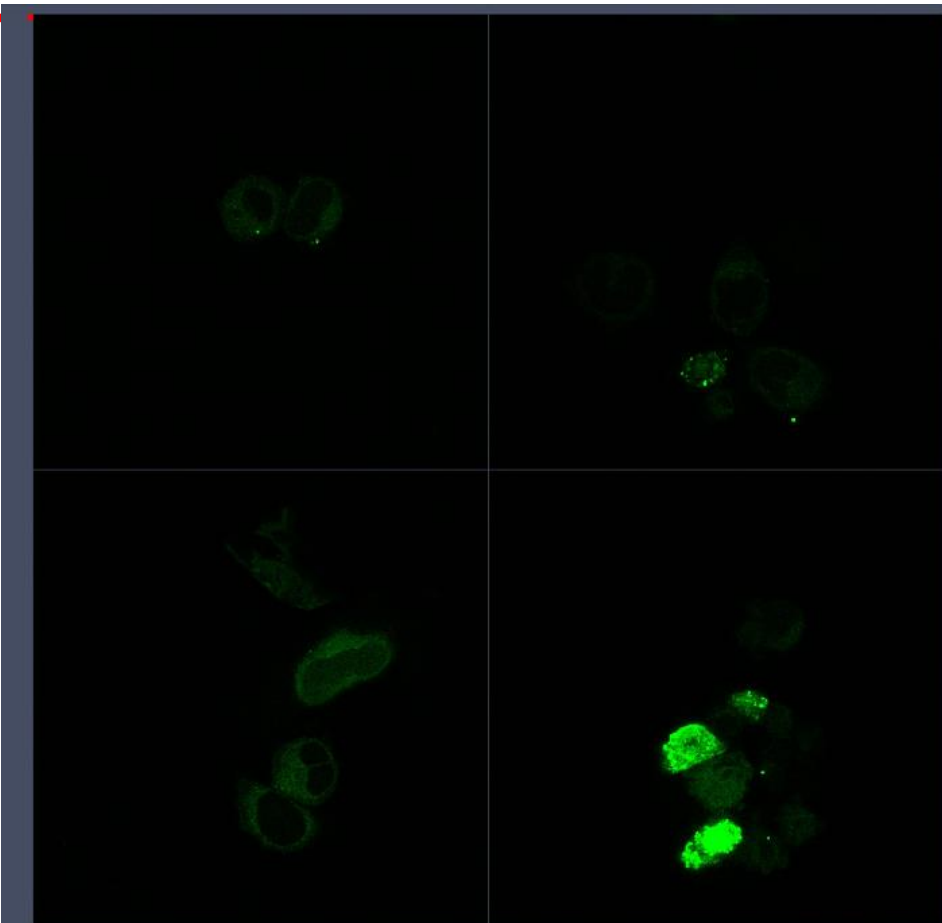


38. Click “Start Experiment” to begin imaging.





42



Looking at the results:

39. Click Dimensions Tab.

40. In Gallery Tab Click Position.

41. Back in Dimensions tab you can use the slider to move through

A. Z Position.

B. Time.

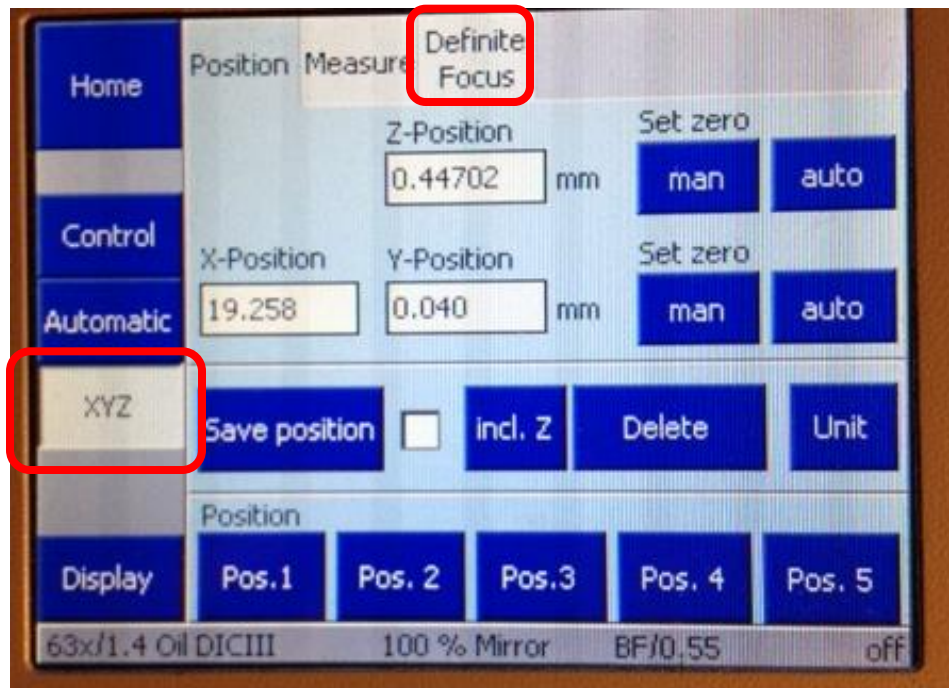
42. This small red box indicates when autofocus was performed which was before the first position of each cycle.



Appendix:

Troubleshooting Definite Focus:

If you go to the XYZ tab and do not see the Definite Focus tab then the Definite Focus Power Supply is turned off.



The power supply for the definite focus is located under the microscope table. From the front of the microscope table you can only see the side of the power supply but it is labeled with blue tape. The power switch is around the right side of the table and is seen in the right hand photo.

1. Turn on definite focus power supply.
2. Exit software.
3. Turn off computer.
4. Turn off microscope.
5. Restart microscope and computer.

