LSM780 MultiTime with Definite Focus

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12/22/15

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:

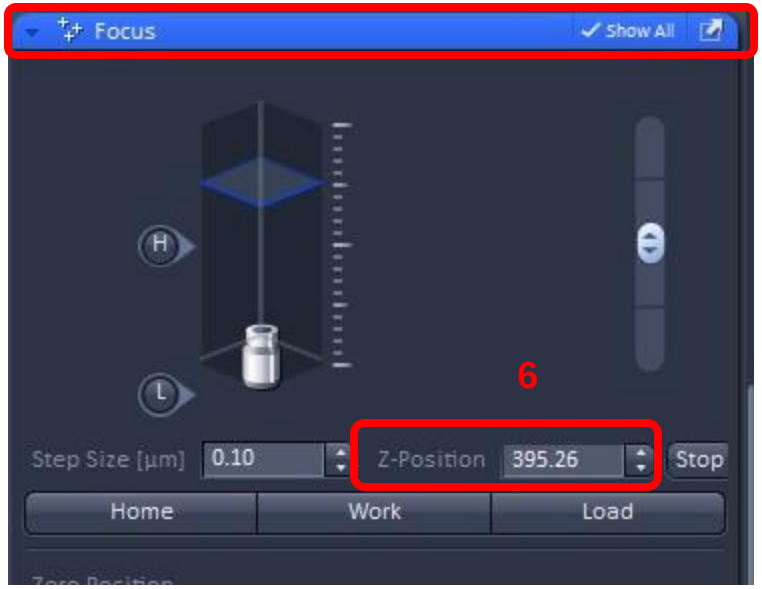
1. **Have whole microscope on for 2 hours before starting any experiment.**
2. **The sample should sit on the microscope for 15 minutes before you start imaging.**

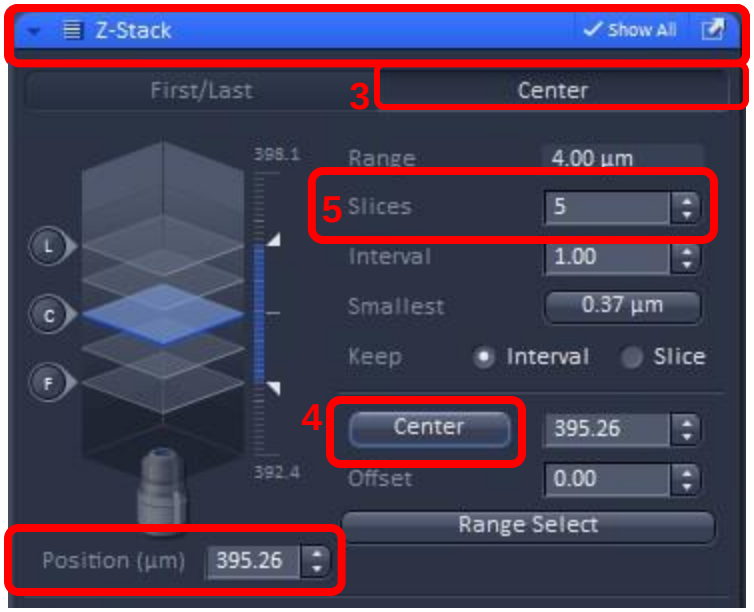
If you are going to do a live cell experiment in the morning then it would be best to keep the microscope on from the day before.

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.**

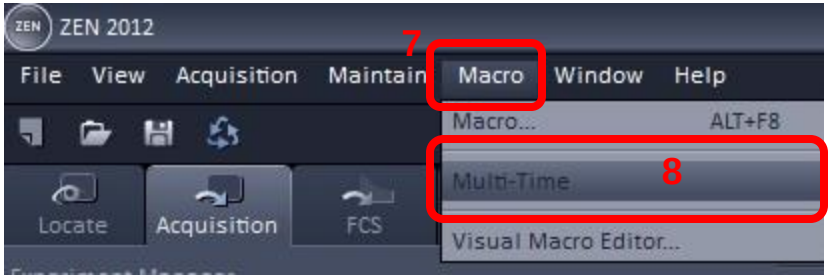
1. **Set up your sample like you normally would for imaging.**
2. **Check Z Stack.**
3. **Set the Z Stack clicking Center.**
4. **Then Click Center button.**
5. **Set number of slices. It must be an odd number.**
6. **Double check your Z position. The Z positions in the Z-Stack and Focus boxes should match.**





Open MultiTime Macro by:

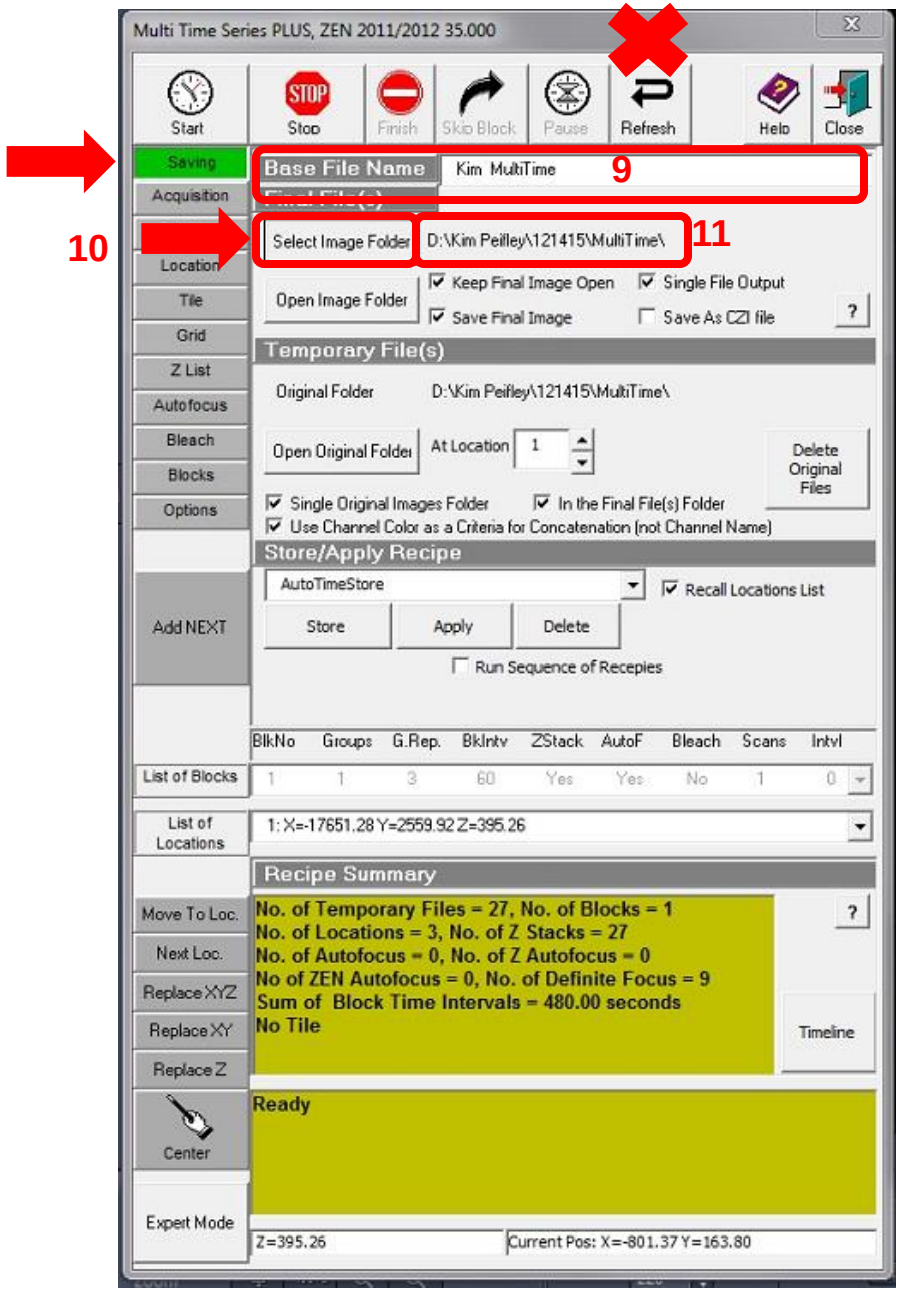
7. **Click Macro.**
8. **Click Multi-Time.**



In the Saving Tab:

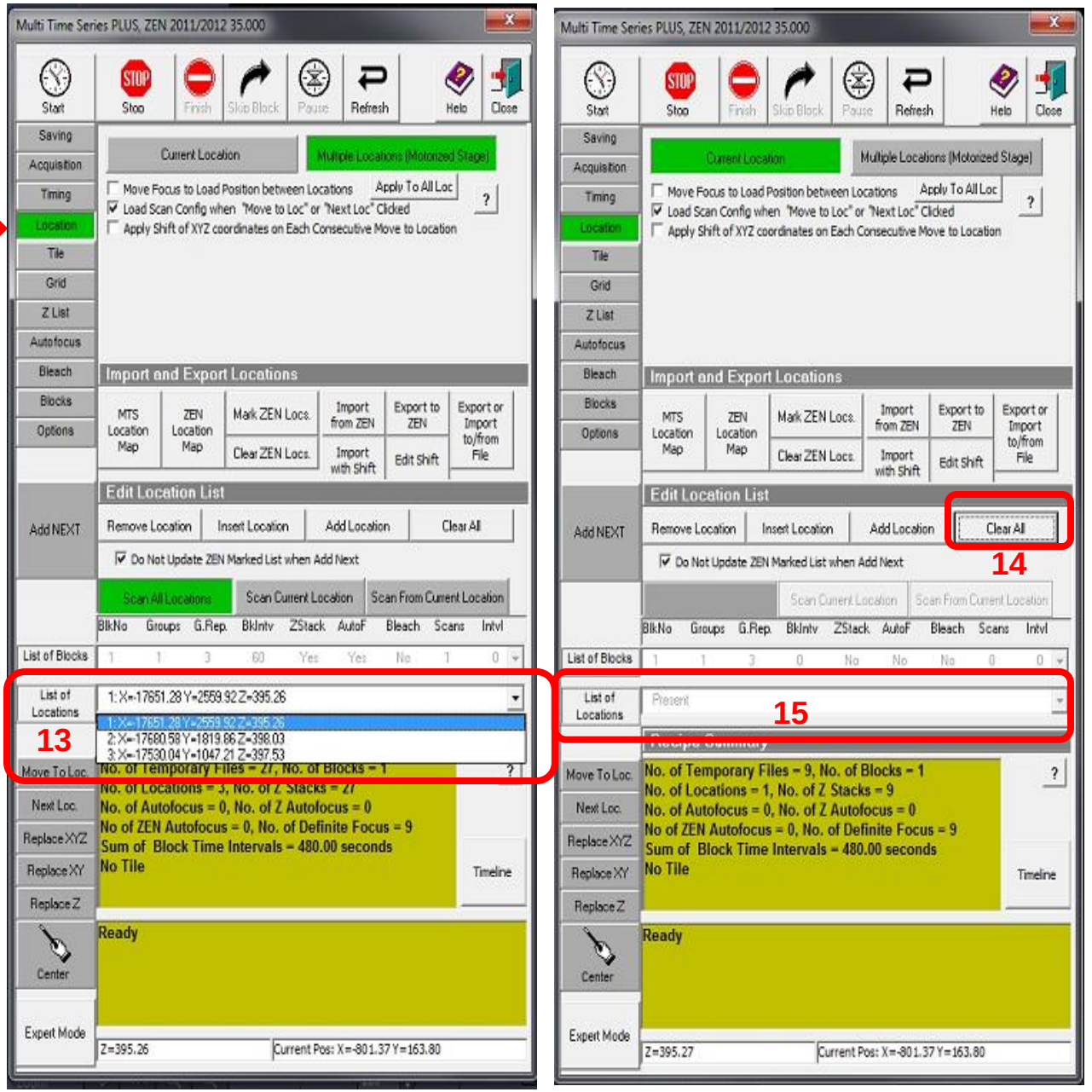
9. **Enter Base File Name. This is the name all your files will be designated.**
10. **Click Select Image Folder.**
11. **Create and Select where you wish your images to be stored. Note: Always save locally. If you save directly to the server you risk having the program freeze up.**
12. **Double check that the Base File Name window and the Select Image folder are what you selected.**

Important: DO NOT CLICK THE REFRESH BUTTON. [✖].The Refresh button is not the same as the Update button in previous versions of Multi-Time. In this version if you click Refresh it will return everything to the settings that were there when you first opened the Macro.



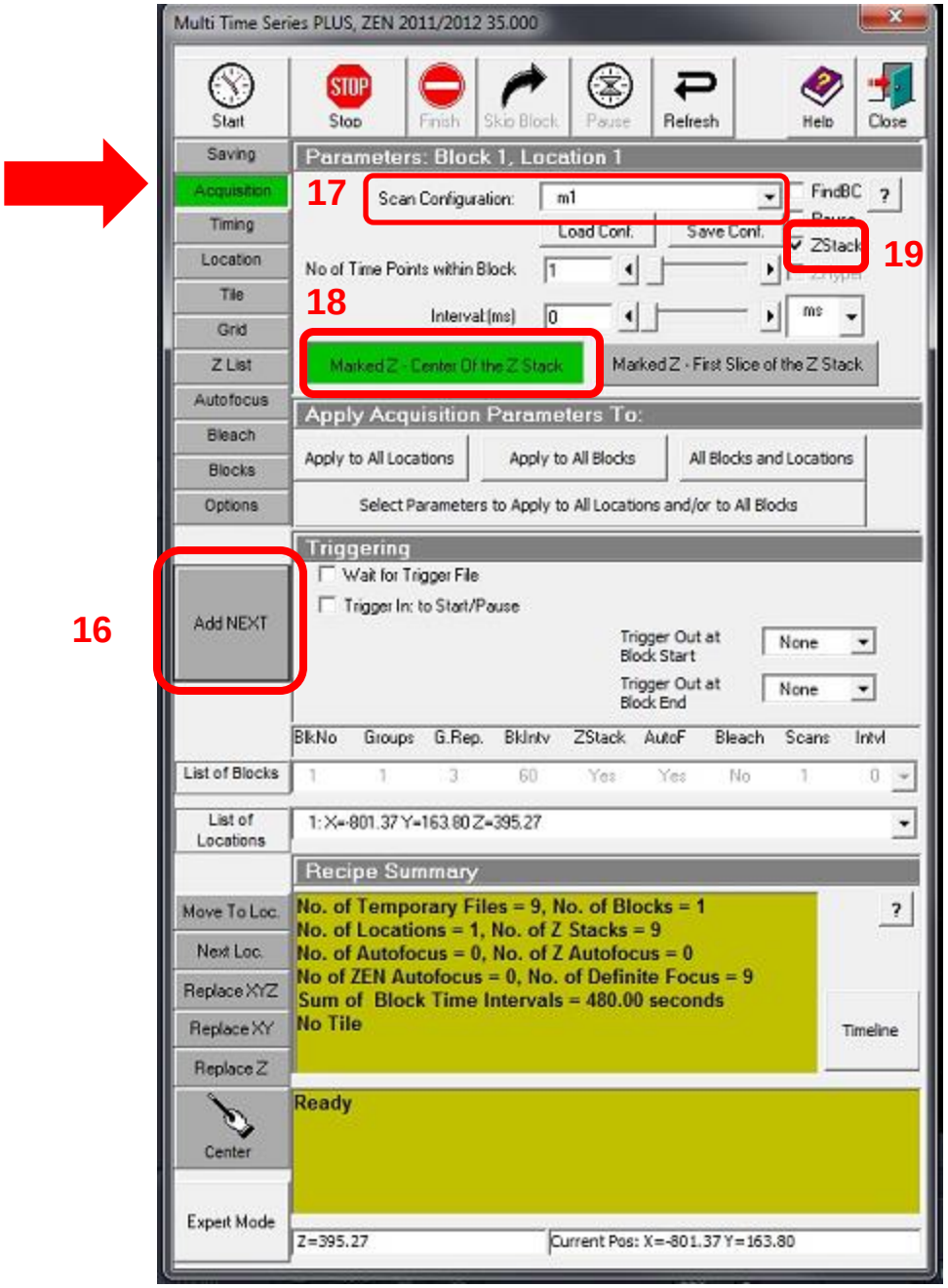
In Location Tab:

13. **Check to see if there are any locations in List of Locations.**
14. **If there are locations click Clear All.**
15. **Make sure List of Locations is now blank.**



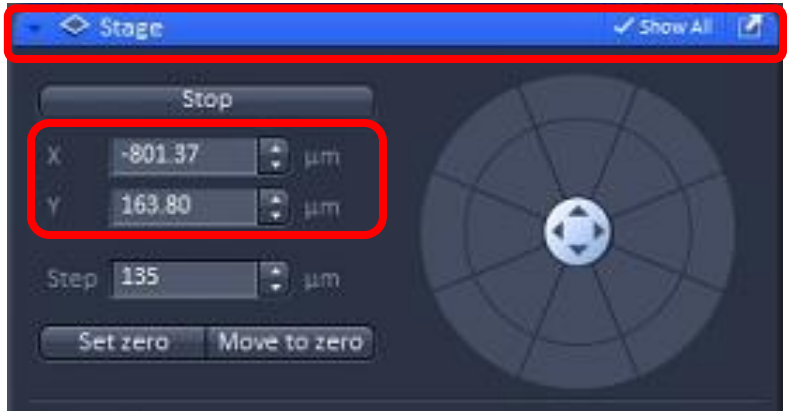
In the Acquisition Tab:

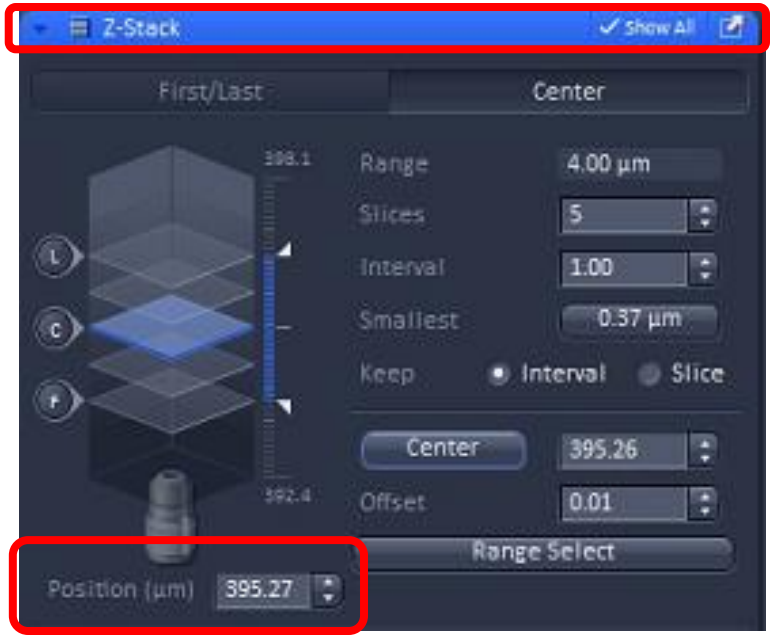
16. **To add your current position click Add Next.**
17. **Scan Configuration automatically gives it the name m1. No need to do anything more here [see below].**
18. **Make sure MarkZ-Center of the Z Stack is highlighted in Green. This means it is selected.**
19. **Make sure ZStack box is checked.**



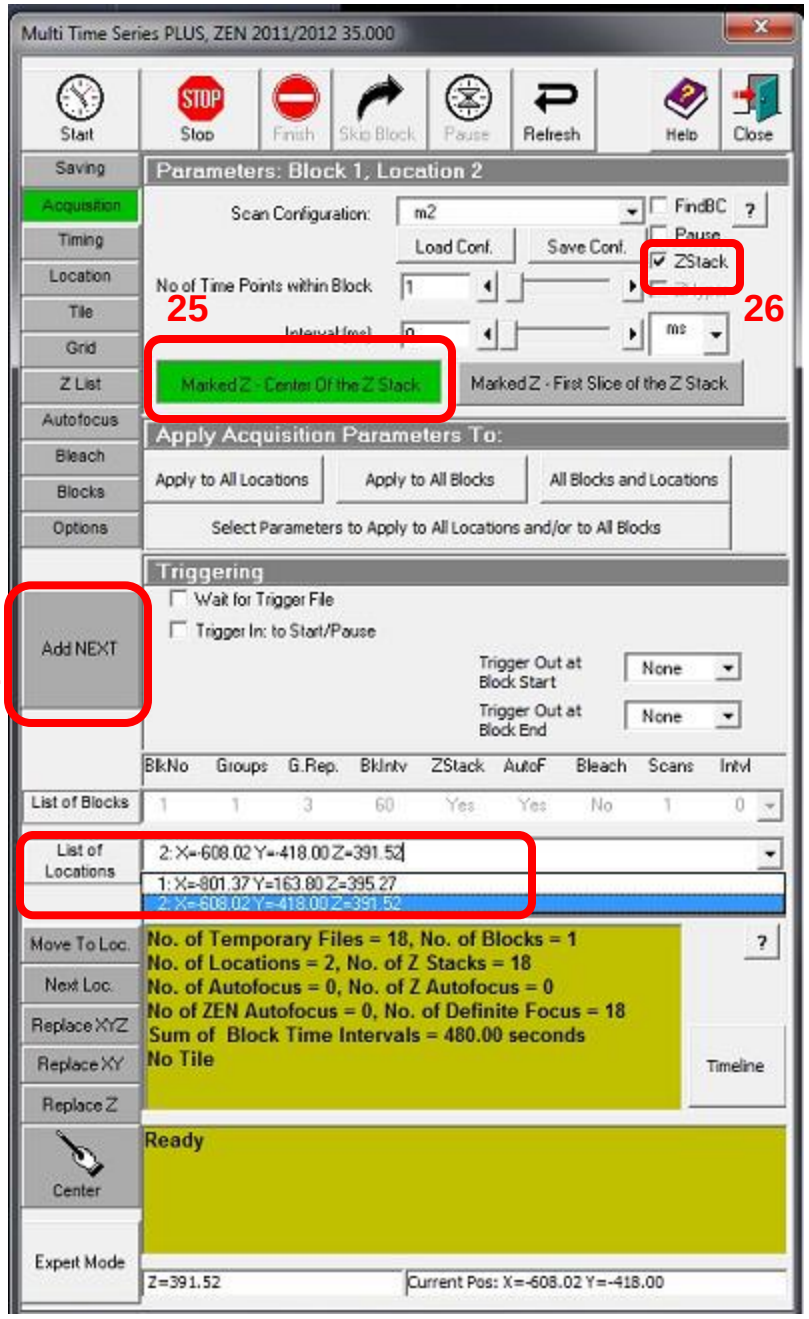
Note: In older versions of MultiTime you got your settings for a location and saved it as a configuration file. Then in Scan Configuration you would find the configuration for that location select it and click Load Configuration. There is no need to do this anymore.

20. **Check the X,Y and Z location in MultiTime with the x,y locations in the Stage window and z location in the Z-Stack window. They should match.**

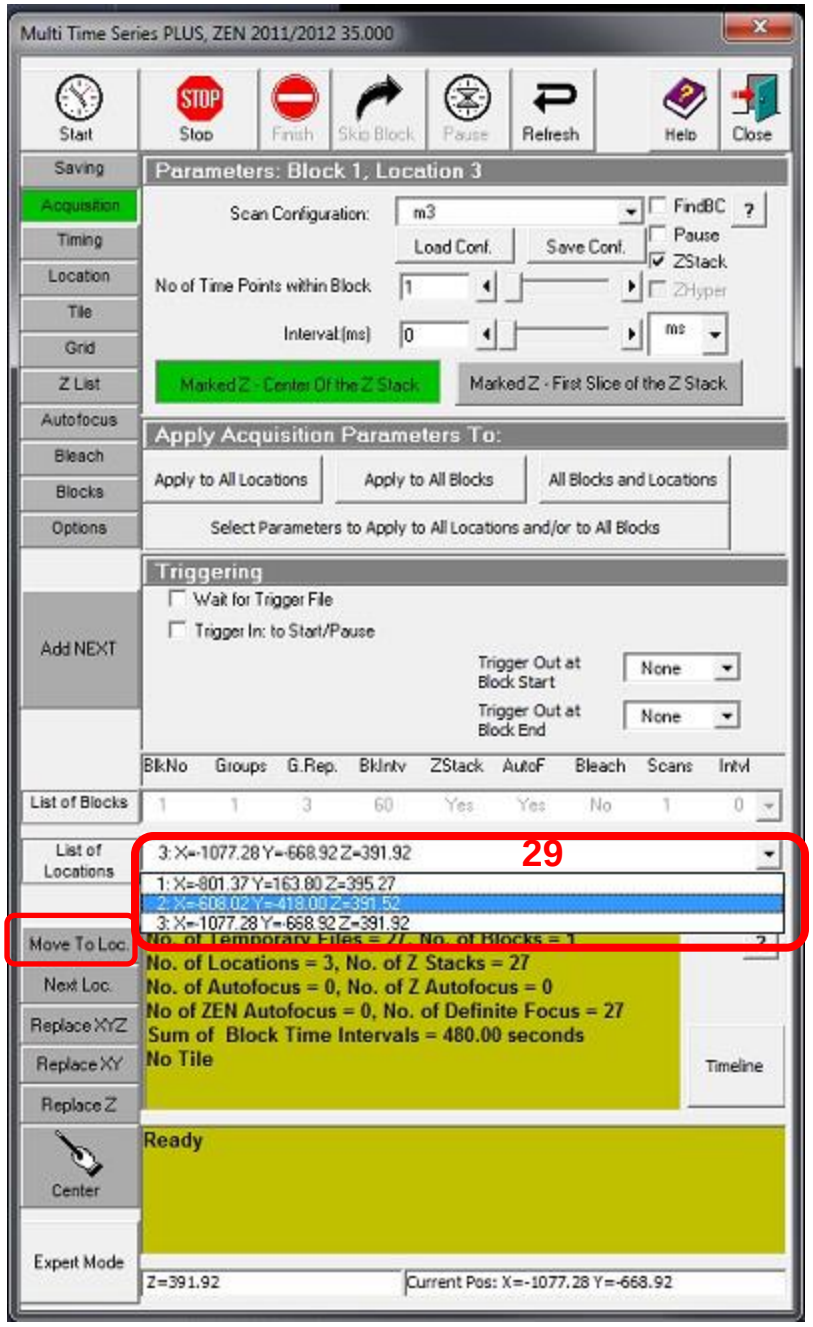




21. **Move to next location.**
22. **Get all settings.**
23. **DO NOT SET Z STACK.**
24. **Click Add Next.**
25. **Make sure MarkedZ – Center of the Z Stack is highlighted in Green.**
26. **Make sure Z Stack box is checked.**
27. **Double check locations to make sure they match.**
28. **Repeat Steps 21-27 for all locations.**

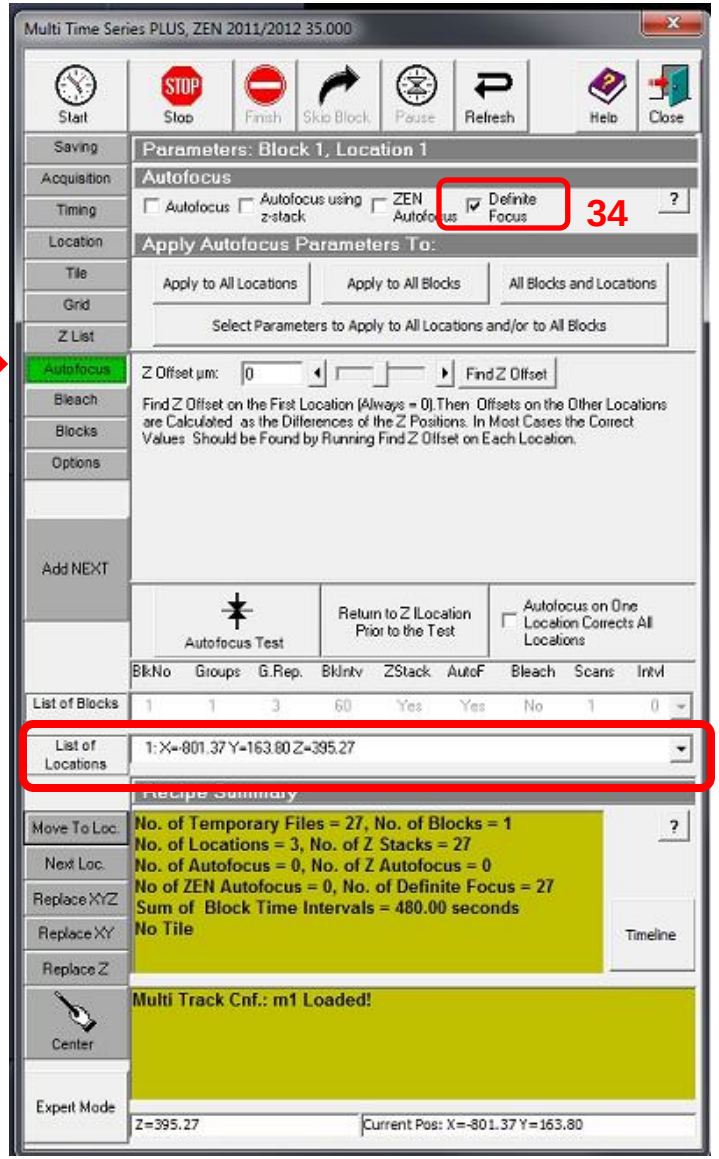


29. **Work your way back to Location 1 by highlighting location.**
30. **Click Move To Loc. to move to that location.**
31. **Double check all your settings as well as locations to make sure this is correct.**



You Must Be In Location 1 to set up the rest of the experiment.

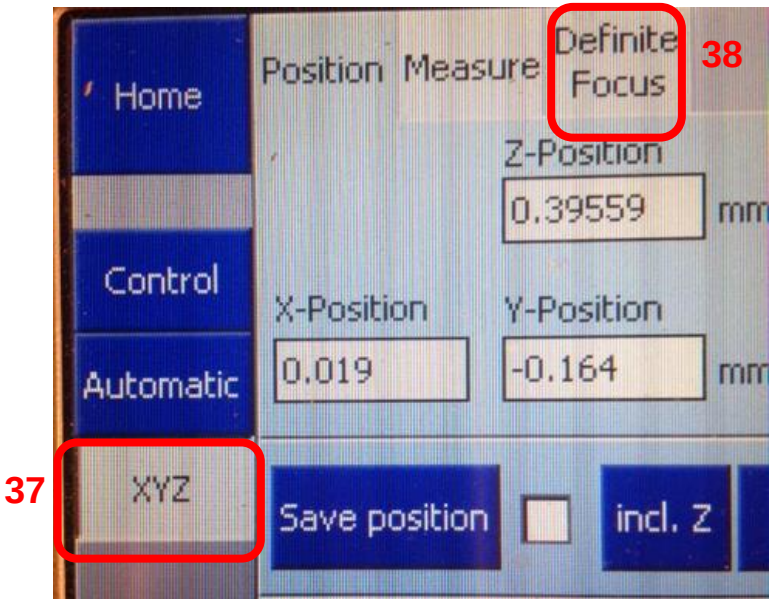
32. **Make sure you are in Location 1.**
33. **Click the Autofocus Tab.**
34. **Check Definite Focus**

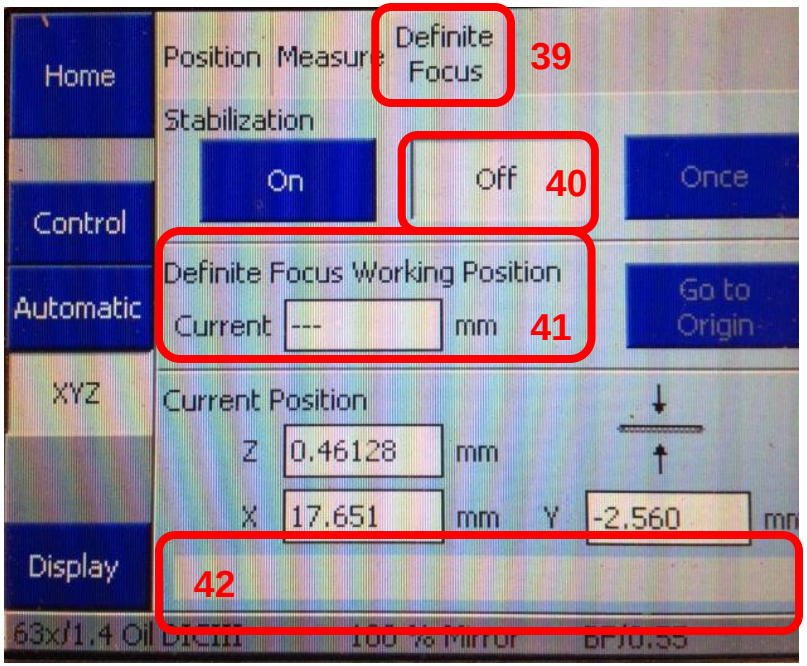


On the touch pad:

35. Select the Home screen.
36. Select Microscope.
37. Select XYZ.
38. Select Definite Focus.
39. This is what the Definite Focus Screen looks like.
40. The current position is off. [Lighter Color].
41. You will also note that under Definite Focus Working Position in the box next to Current you see no numbers. Just -----.
42. You will also see this box is blank.

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.





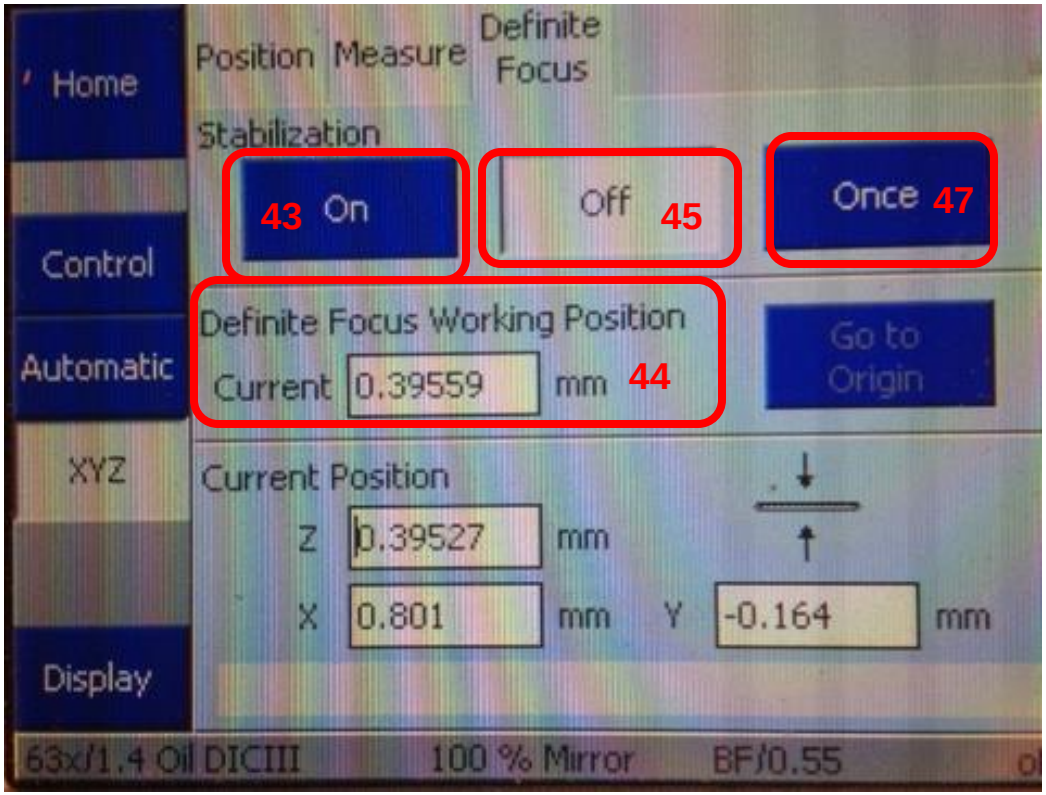
You will now need to set or "train" the autofocus:

43. **Click On.**
44. **Wait until you see numbers populate the Current Box under Definite Focus Working position.**
45. **Click Off.**

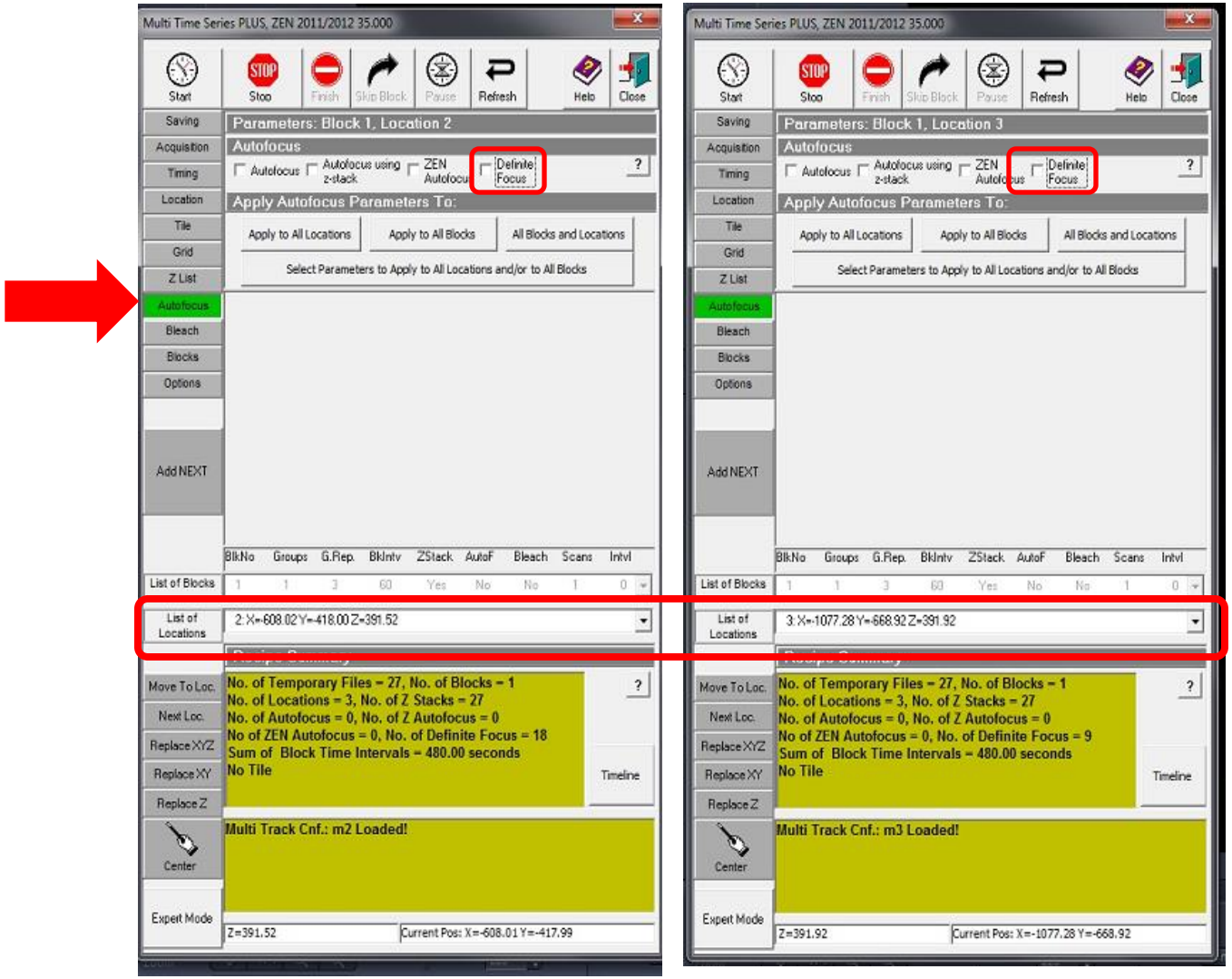
The autofocus is now trained.

If you want to test the Definite Focus:

46. **Move slightly out of focus.**
47. **Click Once.**
48. **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.**

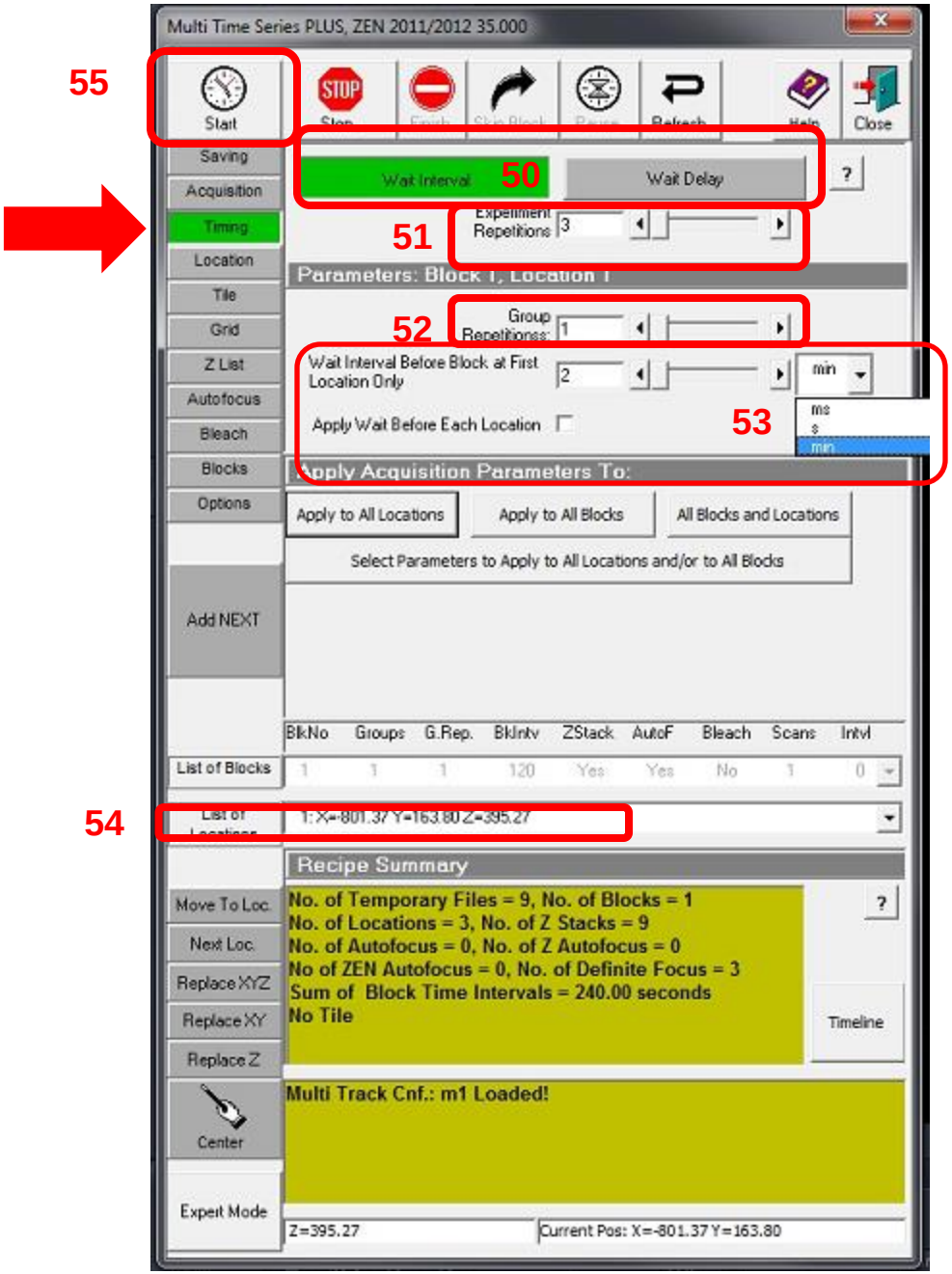


49. **Back in MultiTime in Autofocus box go through each position and make sure Definite Focus is ONLY checked for the first position.**



In the Timing Tab:

50. **Click on Wait Interval or Wait Delay. Green highlight indicates it is selected. See next slide for difference between Wait Interval and Wait Delay.**
51. **Set the number of Experiment Repetitions. This is how many times you wish to repeat the experiment.**
52. **Group Repetitions is how many times you wish to repeat the locations you set up with in the experiment.**
53. **Wait Interval Before Block at First Location only is how long you wish to wait before you repeat your Block. You can select ms, s or m.**
54. **Check to ensure you are on Location 1.**
55. **Click Start.**



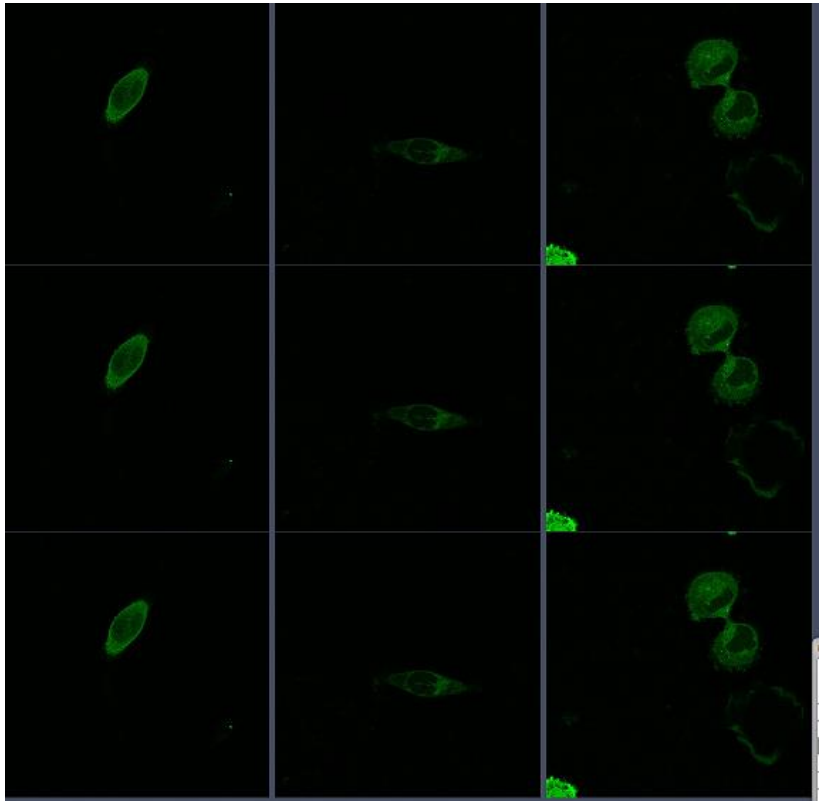
<u>Wait Interval vs Wait Delay:</u>

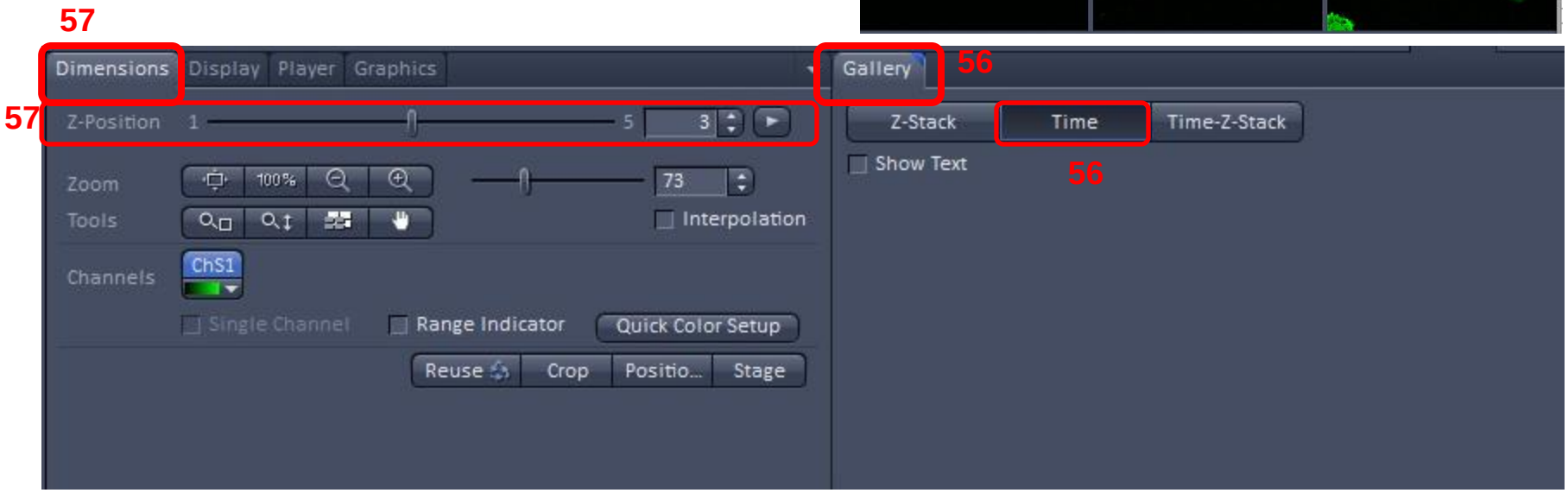
<u>Wait Interval:</u> The time frame the imaging will occur. For example 1 hour. If the experiment starts at 12pm then the locations will be imaged at 12pm, 1pm, 2pm and so on.

<u>Wait Delay:</u> The time it will wait AFTER imaging all locations before it will image again. If we use the same example from above of 1 hour starting at 12pm and the locations take 5 minutes to acquire the locations this time will be imaged at 12pm, 1:05pm, 2:10pm and so on.

If you wish to image “every hour” then Interval is your choice.

56. **In the Gallery Tab click Time.**
57. **In the Dimensions Tab you see the Z position. In this case it is set at slice 3.**
58. **In the image to the right you are seeing all 3 positions at each of the 3 time points.**

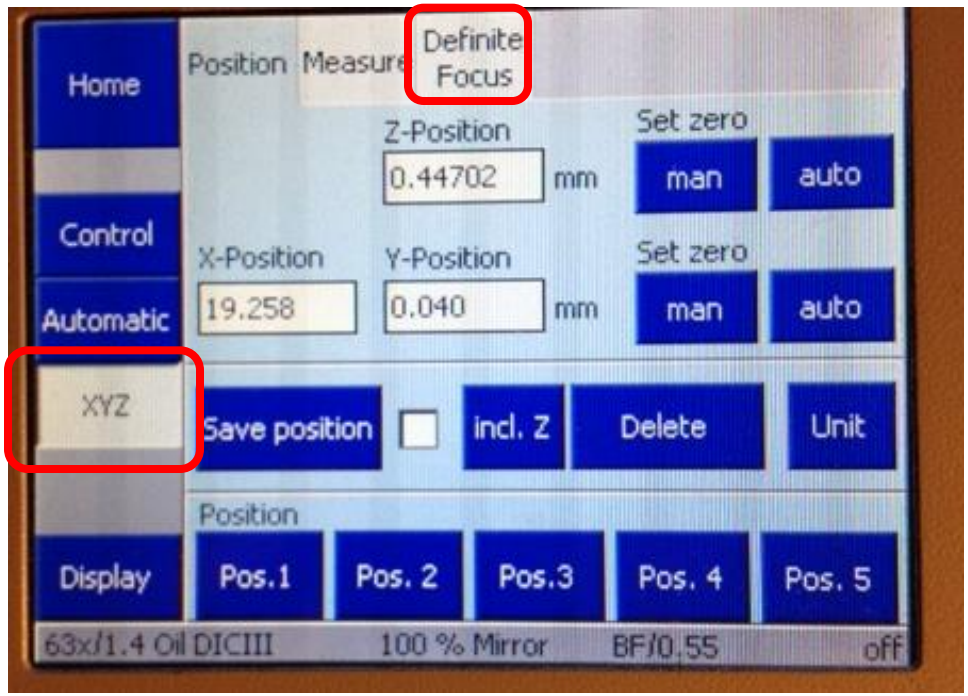


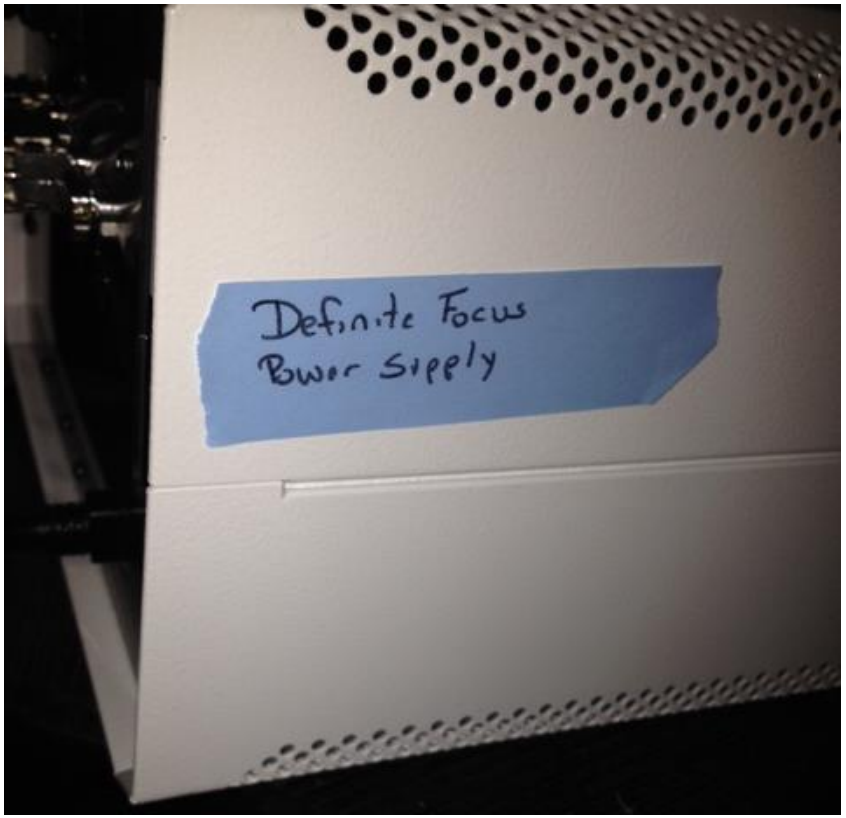


Appendix:

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.**