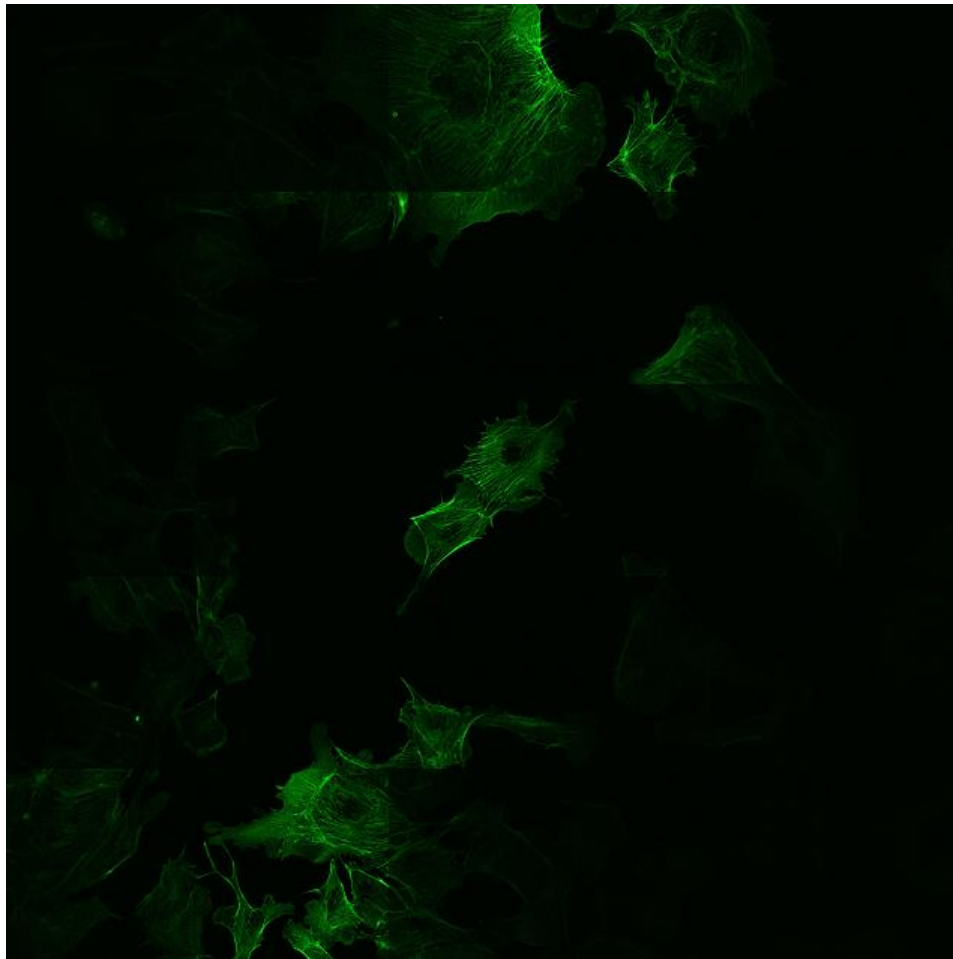


Autofocus Tiling on LSM710

Kim Peifley

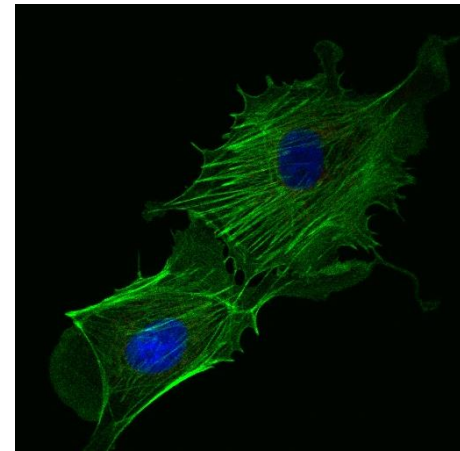
07/15/15

When tiling on the 710 you will notice that the focus is uneven throughout the tile. In order to prevent this you will have to autofocus each tile.



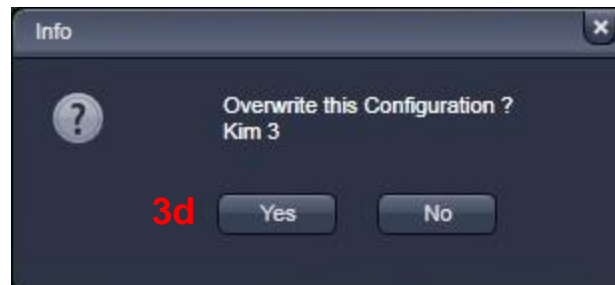
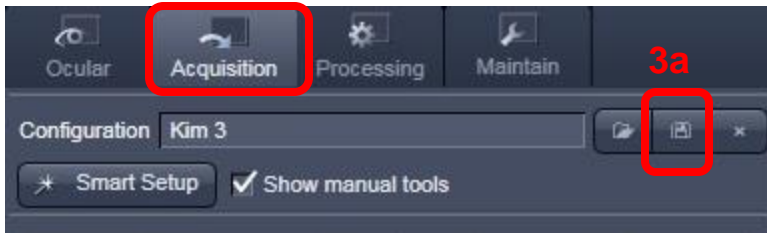
5x5 Tile with uneven focus

1



In Acquisition Tab:

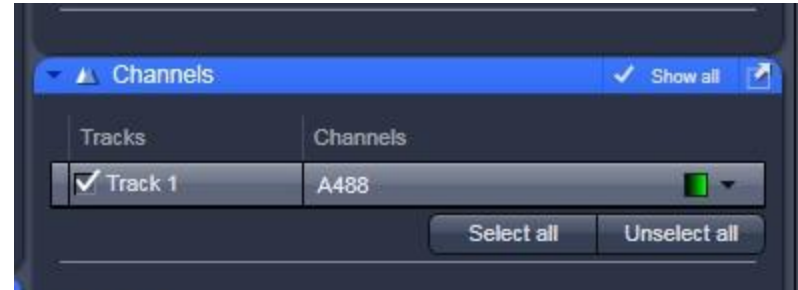
1. Get focus and settings like usual.
2. Make sure all fluorophores you wish to image are selected.
3. Save Configuration
 - a. Click the disc icon next to the Configuration box below the Acquisition tab.
 - b. Type in the name of the Configuration. Example: Kim 3.
 - c. Click OK.
 - d. If you are reusing the configuration name it will ask if you wish to overwrite. Click Yes.



Repeat this process to create the autofocus configuration.

Note:

You must have a single track, single color configuration for autofocus. If you have multiple tracks they will auto focus even if unchecked.



When naming the autofocus configuration it might help to have the word autofocus or AF in the configuration name. This might help keep things straight when you are loading configurations.



Open Multi-Time:

- Click on Macro.
- Click on Multi-Time.



In Multi Time Series PLUS:

- Click on Acquisition.
- In Scan Configuration select the configuration you created. Example: Kim 3.
- Click Load Configuration.
- Make sure Zstack box is unchecked.
- Click Update.



Multi Time Series PLUS, ZEN 2009-2010-25

Start Stop Finish End Block Pause Update 10 Help Close

Parameters: Block 1 Location 1

7 Scan Configuration: Kim 3 FindBC ?
8 Load Conf. Save Conf. ZStack ZHyper

No of Time Points within Block 1
Interval(ms) 0 ms

Marked Z - Middle Of the Z Stack Marked Z - Top of the Z Stack

Apply Acquisition Parameters To:
Apply to All Locations Apply to All Blocks All Blocks and Locations
Select Parameters to Apply to All Locations and/or to All Blocks

Triggering
 Wait for Trigger File
 Trigger In: to Start/Pause
Trigger Out at Block Start None
Trigger Out at Block End None

BlkNo	Groups	G.Rep.	BkIntv	ZStack	AutoF	Bleach	Scans	Intvl
1	1	1	0	No	Yes	No	1	0

List of Locations 1: X=-269.40 Y=-268.65 Z=-1.52 T1(5X5):1,1

Recipe Summary
No. of Temporary Files = 25, No. of Blocks = 1
No. of Locations = 25, No. of Z Stacks = 0
No. of Autofocus = 25, No. of Z Autofocus = 0
No of ZEN Autofocus = 0, No. of Definite Focus = 0
Sum of Block Time Intervals = 0.00 seconds
T1 5X5

Ready

11. Click Tile.
12. Select Tile Numbers. Example: 5x5.
13. Under Scan Configuration Select Configuration. Example: Kim 3.
14. Click Load Conf.
15. Click Create Tile Locations.
16. Click Update.



Step 15 will have to be repeated for each location in the tile. Locations can be changed under List of Locations [indicated by the star].

Note: If you are doing a large tile start off with a small test tile and load the scan configuration to each location of the test tile. When you expand to the larger tile for your experiment you will not have to load scan configuration to each new location. It would not hurt to check a random location or two to confirm this.

The screenshot shows the 'Multi Time Series PLUS, ZEN 2009-2010-25' software window. The interface includes a top toolbar with icons for Start, Stop, Finish, End Block, Pause, Update, Help, and Close. The 'Update' icon is circled in red and labeled '16'. Below the toolbar, the 'Tile' button in the left sidebar is highlighted in green and labeled '11'. The 'Tile Numbers' field is set to '5 X 5 Y' and is circled in red, labeled '12'. The 'Scan Configuration' dropdown is set to 'Kim 3' and is circled in red, labeled '13'. The 'Load Conf.' button is circled in red and labeled '14'. The 'Create Tile Locations' button is circled in red and labeled '15'. The 'List of Locations' section shows a single location: '1: X=-269.40 Y=-268.65 Z=-1.52 T1(5X5):1,1', with a red star next to it. The 'Recipe Summary' section is highlighted in yellow and shows details for 25 temporary files and 25 locations. The status bar at the bottom indicates 'Ready'.

17. Click the Autofocus tab.
18. Under Autofocus - Check the Autofocus box.
19. Click on Apply to all Locations.
20. Select configuration for Autofocus. Example Kim AF.
21. Click Load Configuration.
22. Select Z Range. Example 10.
23. Select Autofocus Z Step Size. Example 0.5.
24. Click Update.



Steps 20 and 21 will have to be repeated for each location in the tile. Locations can be changed under List of Locations [indicated by the star].

Note: If you are doing a large tile start off with off with a small test tile and load the autofocus configuration to each location of the test tile. When you expand to the larger tile for your experiment you will not have to load autofocus to each new location. It would not hurt to check a random location or two to confirm this.

Multi Time Series PLUS, ZEN 2009-2010-25

Start Stop Finish End Block Pause Update 24 Help Close

Saving Parameters: Block 1, Location 4

Acquisition **Autofocus** 18

Timing Autofocus Autofocus using z-stack ZEN Autofocus ?

Location Apply Autofocus Parameters To:

Tile 19

Grid Select Parameters to Apply to All Locations and/or to All Blocks

Z List

Autofocus 20

Autofocus Configuration Kim AF

21 Load Conf. Save Conf.

Step Z Offset μm : 0 Find Z Offset

9 Z Range μm : 10 22

Scan Speed Use Threshold 0

ZHyper

Autofocus Z Step 0.5 23

Autofocus Test Return to Z Location Prior to the Test Autofocus on One Location Corrects All Locations

BlkNo	Groups	G.Rep.	BkIntv	ZStack	AutoF	Bleach	Scans	Intvl
1	1	1	0	No	Yes	No	1	0

List of Locations

4: X=35.73 Y=187.23 Z=45.01 T1(2X2):2.2	★
1: X=-99.23 Y=52.27 Z=45.01 T1(2X2):1.1	
2: X=35.73 Y=52.27 Z=45.01 T1(2X2):1.2	
3: X=-99.23 Y=187.23 Z=45.01 T1(2X2):2.1	

Move To Loc. 4: X=35.73 Y=187.23 Z=45.01 T1(2X2):2.2

Next Loc. No. of Locations = 4, No. of Z Stacks = 0

Replace XYZ No. of Autofocus = 4, No. of Z Autofocus = 0

Replace XY No. of ZEN Autofocus = 0, No. of Definite Focus = 0

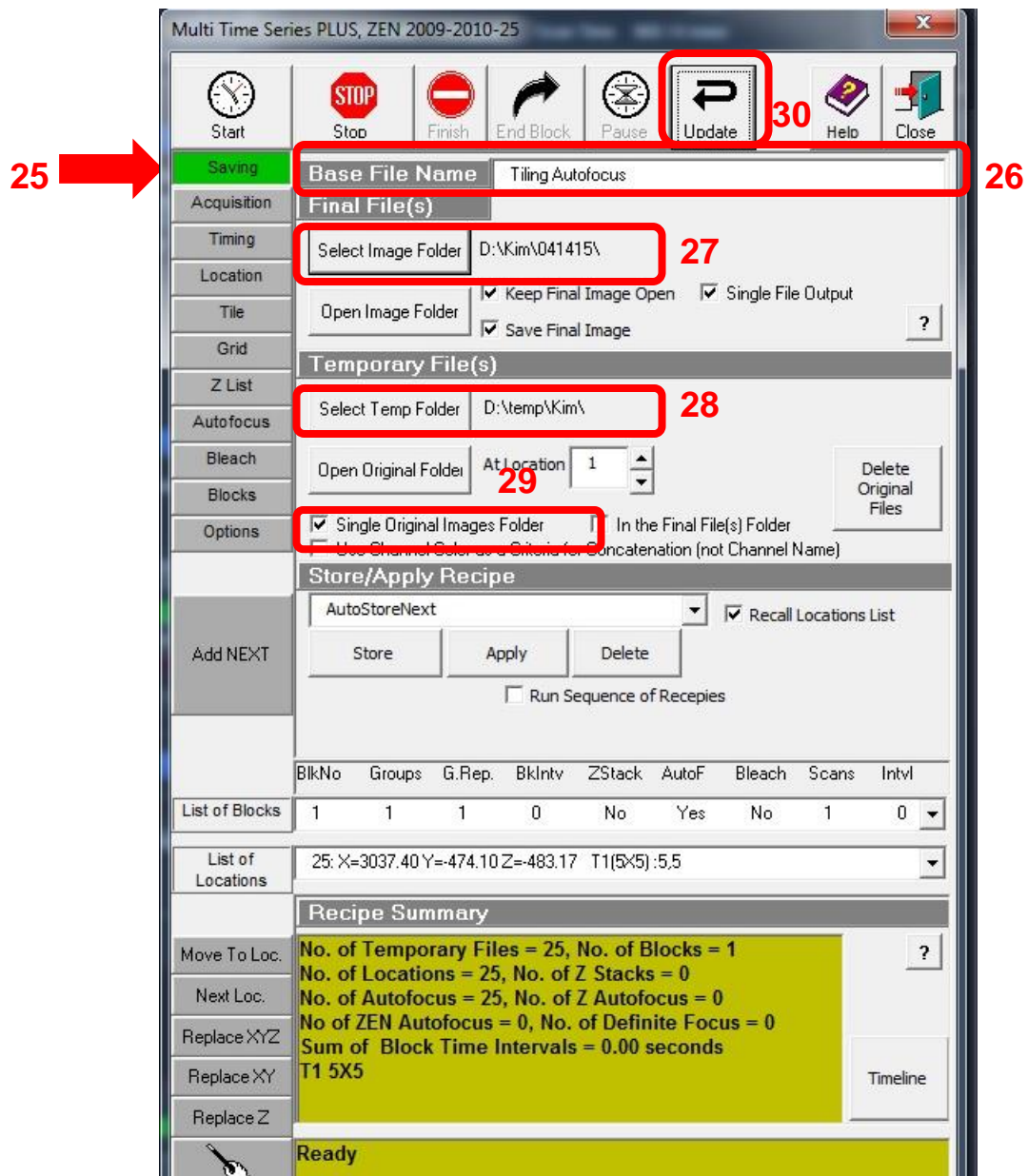
Replace Z Sum of Block Time Intervals = 0.00 seconds

T1 2X2 Timeline

Ready

Center

25. Click the Saving Tab.
26. Click on Base File Name then type in the name for the base files for this experiment. Example Tiling Autofocus.
27. Click on Select Image Folder and select where you want images to save and create a folder for the images.
28. Click on Select Temp Folder and select where you want the images to be saved
29. Check box for Single Original Image Folder
30. Click Update



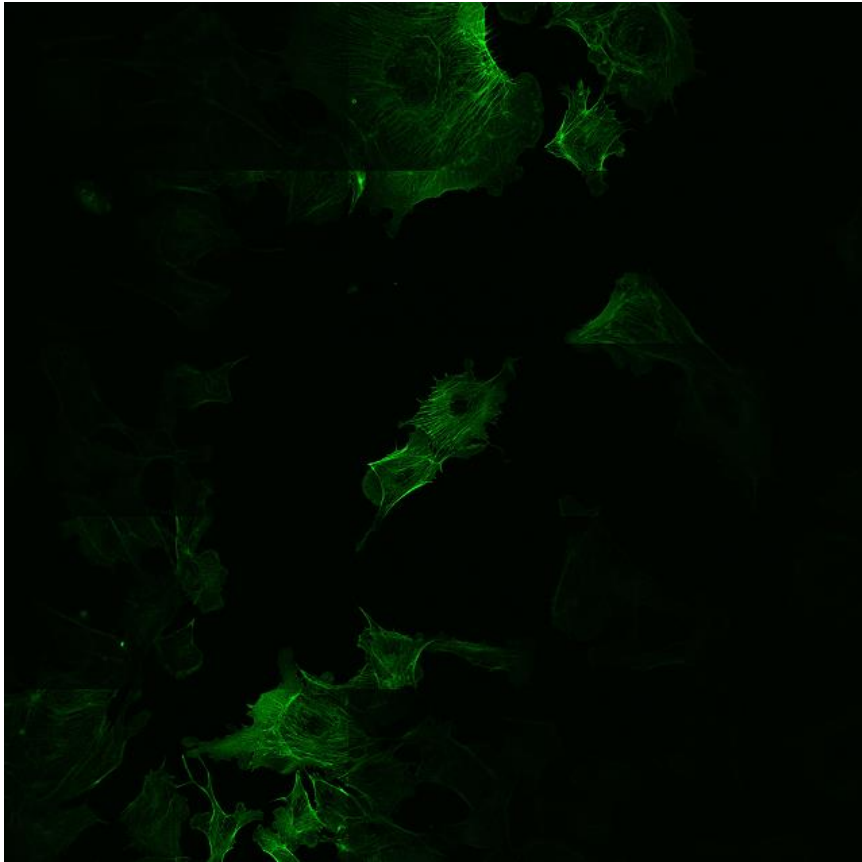
Review:

- 31. Recipe Summary: look through to see that things will be as you want them. Example No of locations is 25 which equals a 5x5 tile which is also indicated by T1 5x5.
- 32. Check that it says Yes under AutoF.
- 33. Double check that the image files will be saved where you wish them to be saved. See red stars.
- 34. Go through all the tabs to make sure the configuration you want is showing. Example Kim 3. See red arrows.
- 35. Click Update.
- 36. Click Start to start experiment.

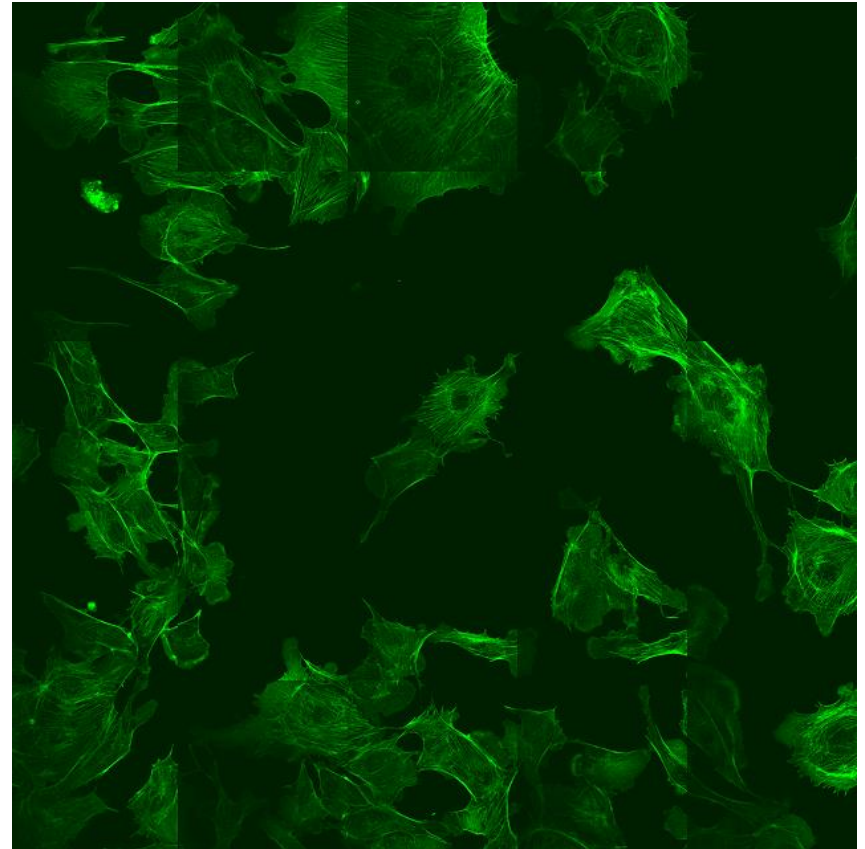
The screenshot shows the 'Multi Time Series PLUS, ZEN 2009-2010-25' software interface. The top toolbar contains icons for Start, Stop, Finish, End Block, Pause, Update, Help, and Close. The 'Update' icon is circled in red. The left sidebar has tabs for Saving, Acquisition, Timing, Location, Tile, Grid, Z List, Autofocus, Bleach, Blocks, and Options. The 'Saving' tab is selected. The main panel shows configuration fields for Base File Name (Tiling Autofocus), Final File(s), Select Image Folder (D:\Kim\041415\), Open Image Folder, Temporary File(s), Select Temp Folder (D:\temp\Kim\), and Open Original Folder (At Location 1). The 'Store/Apply Recipe' section includes AutoStoreNext, Store, Apply, Delete buttons, and a 'Recall Locations List' checkbox. A table below shows 'List of Blocks' with columns: BlkNo, Groups, G.Rep, BkIntv, ZStack, AutoF, Bleach, Scans, Intvl. The first row has values: 1, 1, 1, 0, No, Yes, No, 1, 0. The 'AutoF' cell in this row is circled in red. Below the table is a 'Recipe Summary' box with a green background, containing text: 'No. of Temporary Files = 25, No. of Blocks = 1', 'No. of Locations = 25, No. of Z Stacks = 0', 'No. of Autofocus = 25, No. of Z Autofocus = 0', 'No of ZEN Autofocus = 0, No. of Definite Focus = 0', 'Sum of Block Time Intervals = 0.00 seconds', and 'T1 5X5'. The 'Recipe Summary' box is circled in red. The status bar at the bottom shows 'Ready'. Red arrows point to the 'Saving' tab, 'Timing', 'Location', and 'Tile' tabs. Red stars mark the 'Update' icon, 'Tiling Autofocus', 'D:\Kim\041415\'', 'D:\temp\Kim\'', and the 'AutoF' cell in the table.

Final Result

Save your result just to be sure you have a saved copy.



Before

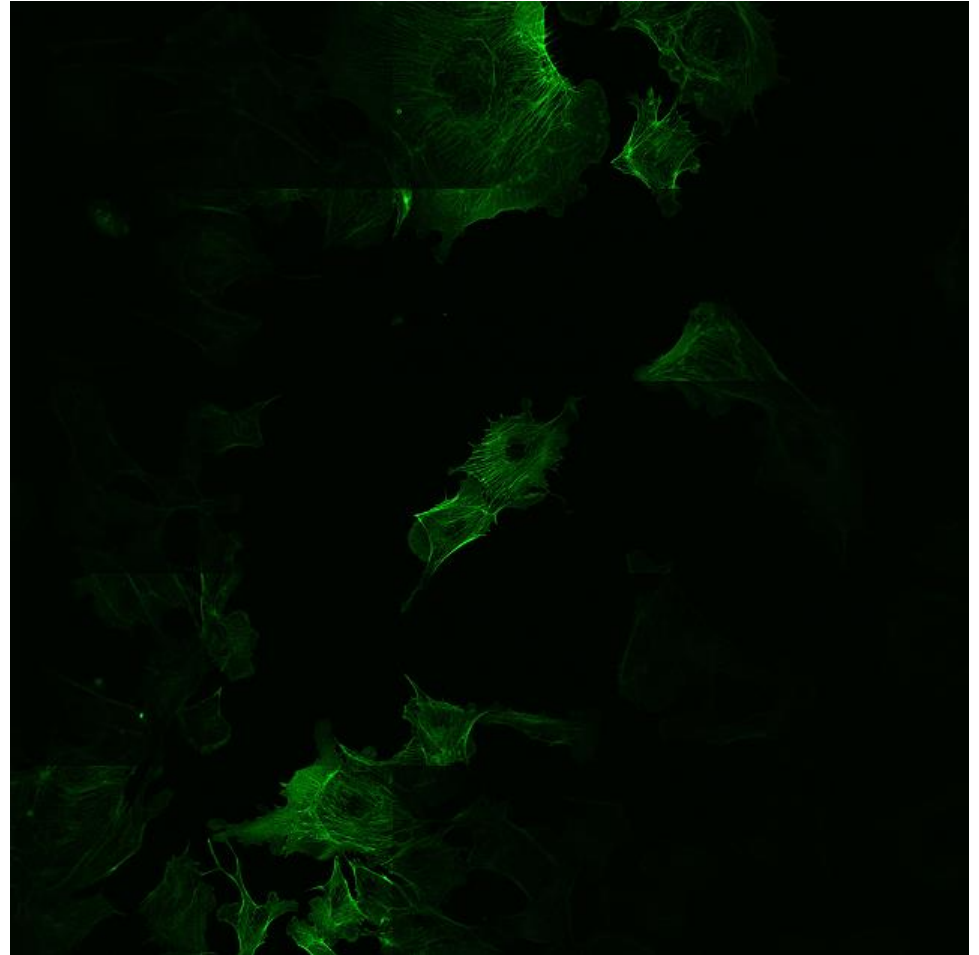


After

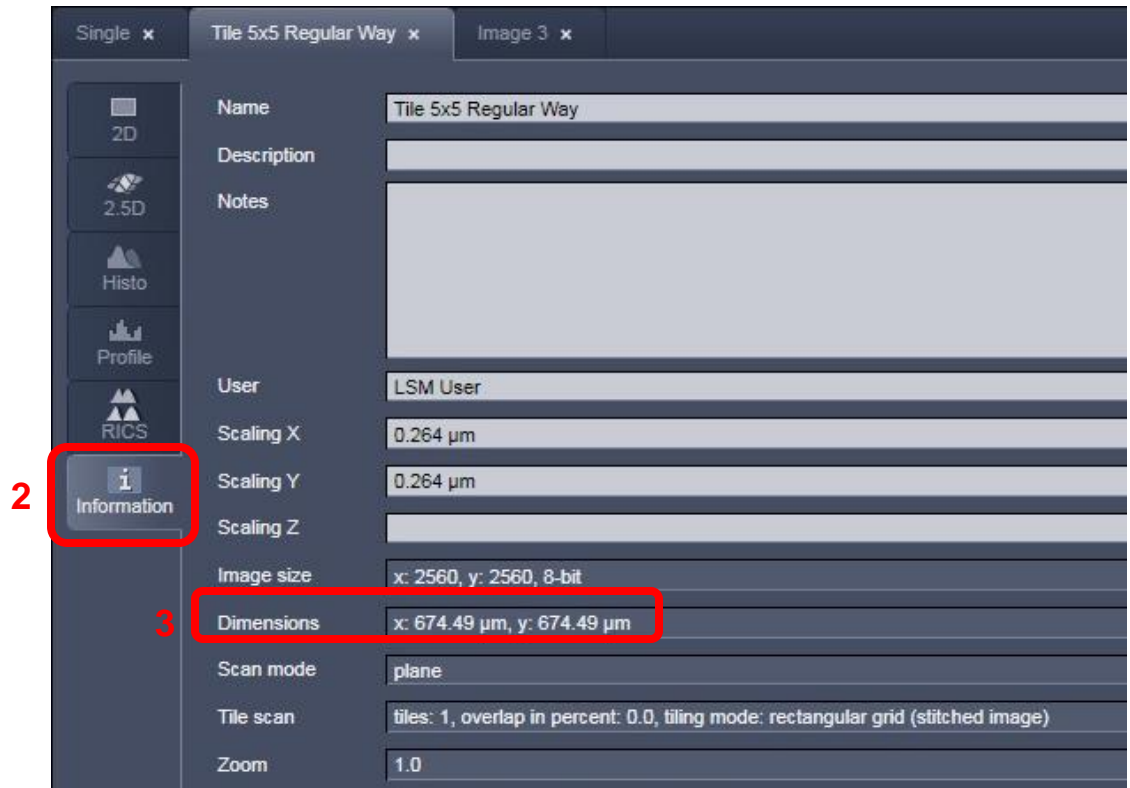
Appendix

How we went about determining the Z stack interval

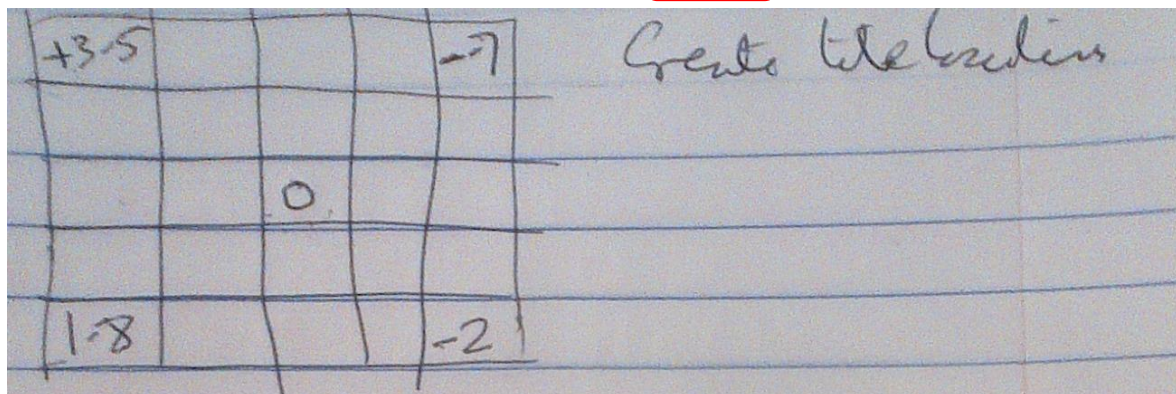
1. First we took a tiled image. Here is the 5x5 image.



- Using the Information Button we looked at the Dimensions of the tiled image. x: 674.49 micrometers, y: 674.49 micrometers
- Since the tile is 5 tiles across we divided 674.49 by 5 to get 135. This is roughly the size of each tile.

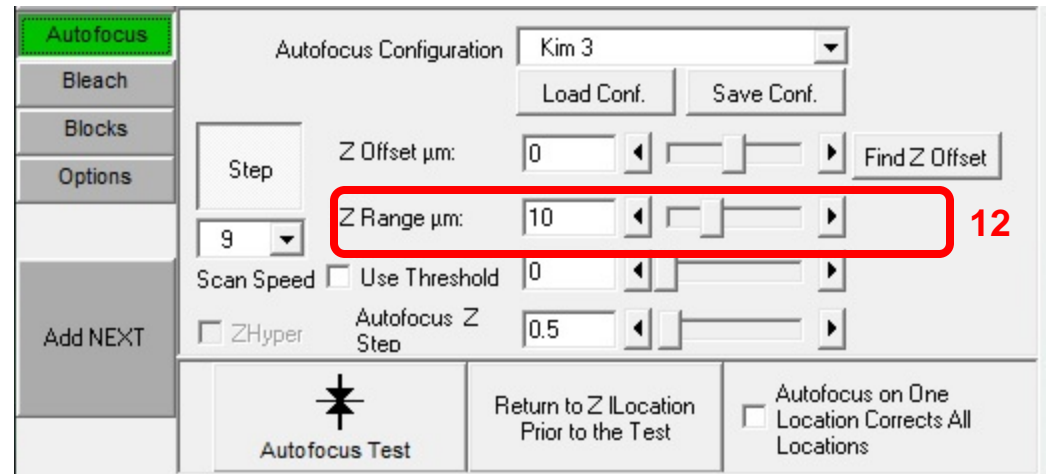
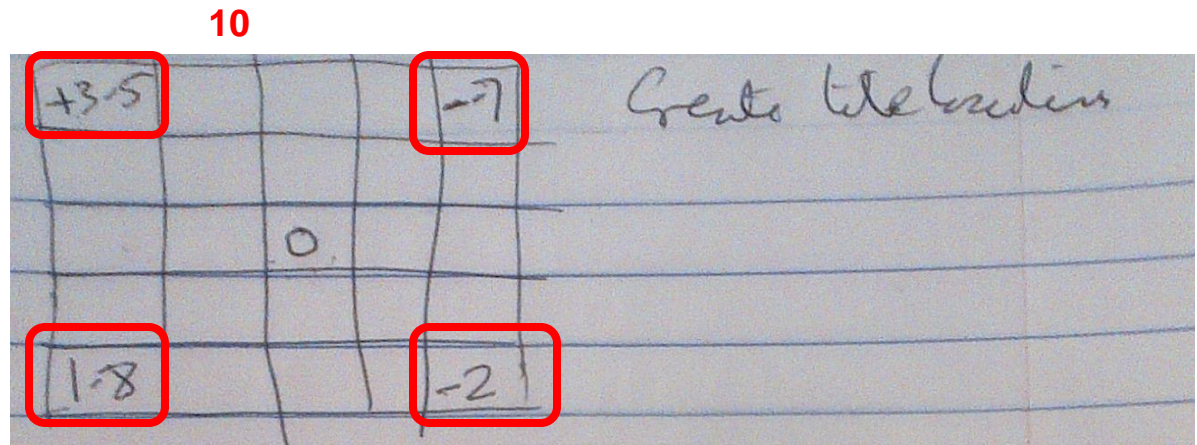


4. We made sure the sample was in focus .Thus the center of the tile was focused.
5. In the Focus window click the “Manually” button to Zero the focus position.
6. In the Stage window click Set to Zero so that the current position [center of the tile] is at zero in both x and y.
7. In Step enter 135.
8. By using the x or y up and down arrows we moved the stage until we reached a corner of the tile.
9. Sample was focused and Z position in Focus window was noted.



9

10. We had focus numbers of +3.5, +1.8, -0.7 and -2 microns.
11. To be safe we rounded this number up to 5.
12. When setting the Z Range for Autofocus the number is 10 to include 5 microns above and below focus.



13. Once you have this figured out you go through the Multitime process outlined and test it out. It's suggested that you start out with small, single color, no averaging tiles to make sure it is working before working your way up to the tile you wish. Suggest starting with 2x1 tile then 3x3 tile and so forth.