## **Protocol for Tiling Fluorescence Image**

- 1. Focus your sample and locate the field of interest.
- 2. Set the gain and exposure time for each channel under the Imaging Modes window.
- 3. Open the stage navigation window.
  - a. Select "autoscan: ox plowing" in the top-left corner of the window.

Stage Navigation				
Autoscan: 0x P 1. Log Scan Area Edges X: 65192	Log *	Slide	Mouse Down Function: Inactive For Safety 💌 🔲 Beep Near Edge	bysticks Stage
Y: 49376 Z: 0 Index: -				
2. Choose Area Shape: 3. Computed Scan Fields:	<ul> <li>Circle</li> <li>Rectangle</li> <li>Free Form</li> </ul>			
#Fields: ? Scan Time: ?	Scatter			
4. Use Scan Fields First Field Next Field + Star	rt Multi Acquire	Home Stage Calibrate Field	I Sizes	Load Save
Acquisition	Settings	Auto Focus Every: 4th Field	2012/00/2012	Field: 100 Auto
🗆 🥪 3D 🗆 🙆 Time Lapse	Save Between Fields	Ask Userto Focus	# Fields: 0	First Prev Next Last Stop

- b. The area shape is a rectangle.
- c. Move the stage to view the top-left corner of the sample. Make sure that part of the sample is visible within the field. Refocus your sample. Log this point.
- d. Move the stage to view the bottom-right corner of the sample. Make sure that part of the sample is visible within the field. Refocus your sample. Log this point.

Stage Navigation		
Autoscan: 0x Plowing		own Function: Focus Joysticks Stage
1. Log Scan Area Edges Log X: 57026 Relog 7	☐ Beep 1	Near Edge
Y: 52776 Erase Z: 0 Previous - Index: 2 of 2 Next +		
2. Choose Area Shape: Circle Circle Rectangle Free Form	Ŷ	_
3. Computed Scan Fields: # Fields: 45 Scatter Scan Time: 22.5 sec		
4. Use Scan Fields First Field Next Field + Start Multi Acquire		
	Home Stage Calibrate Field Sizes	Load Save
Acquisition Settings		eview File: Field: 100 Auto Play
□ 😂 3D 🗹 Save Between Fields		
🗌 🙆 Time Lapse 🗌 One Burst Only	Use 3 Point Pre-Focus Estimate	. Analyze First Prev Next Last Stop Start

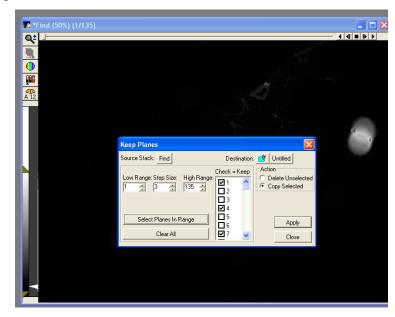
e. Check "Multi Color" in the bottom left corner.

- f. Click on "Start Multi Acquire". Specify which folder to save in, and enter the filename.
- 4. Retrieve files through the network on the Image Analysis 2 workstation.
  - a. Double-click on the "Microsrf" icon on the desktop.
  - b. Double-click on the "DM6000" icon.
  - c. Copy your files from Leica DM6000 onto your folder in the E: drive.
- 5. Create the montage using MetaMorph.
  - a. Open the entire sequence of images:
    - i. File -> Find images...
    - ii. Select the data folder through the Browse button
    - iii. Image type is "Tagged Image File"
    - iv. Click on "Find Now"
    - v. Right-click on one of the files from the list and choose "Select all files"
    - vi. Right-click on one of the files and choose "Open as stack"

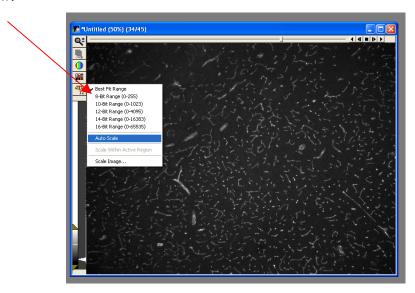
Name & Location Date Modified	Image   Wavelength   Stage	X Stage Y S	itage Label   Z	Digital Properties	Options		Find Now	
Name: ×.*						•	Stop	_
Look in: E:\Analysis Archive\	++++++(01) (+++ 2(HC))				-	Browse	New Search	
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Search Criteria: (File pattern in *.*) Af	ND (Image Type is 'Tagged Ima	ge File')						
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<_F0001_FITC.tif	E:\Analysis	2,829KB	Tagged Image File	10/31/07 3:24 PM	1	1392	1040	
×_F0001_Rhod.tif	E:\Analysis	2,829KB	Tagged Image File	10/31/07 3:24 PM	1	1392	1040	
< F0002 DAPI.tif	E:\Analysis	2,829KB	Tagged Image File	10/31/07 3:24 PM	1	1392	1040	
F0002_FITC.tif	E:\Analysis	2,829KB	Tagged Image File	10/31/07 3:24 PM	1	1392	1040	
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	E:\Analysis E:\Analysis F:\Analysis	2,829KB 2,829KB	Tagged Image File	10/31/07 3:24 PM 10/31/07 3:24 PM	1	1392 1392	1040 1040	

- b. Separate the individual channels from the image sequence:
  - i. Stack -> Keep Planes
  - ii. Ensure that the Source Stack is "Find"

- iii. For the first channel, enter "1" for Low Range. Enter the total number of images for High Range. Enter the number of channels for Step Size.
- iv. Click "Clear All"
- v. Click "Select Planes in Range"
- vi. Click "Apply"
- vii. Repeat steps 7bi-7bvii for all channels. The Low Range value will change depending on the channel.



c. Remove the Auto Scale option for each channel using the icon indicated in the image below.



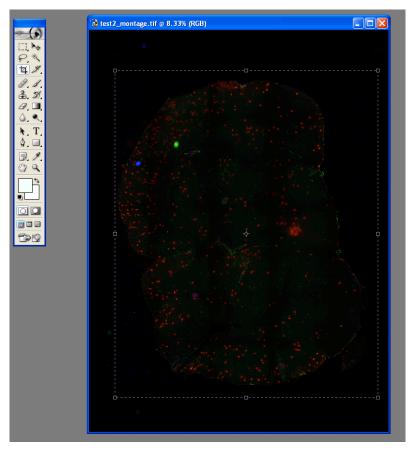
d. Click on "Color Combine" in the montage taskbar. Select "Components" under Source Type; select "24-bit" under Result Depth; choose the appropriate image stack for the corresponding color component; click on the stack icon next to each color component and select "All Planes." Finally, click "Color Combine" to merge the colors.

🗖 Color Combine 📃 🗖 🔀			
Source Type: © Components © Stack © Single Image			
Result Depth ◯ 8-bit			
RED component: Red 💋			
GREEN component: Green 💋			
BLUE component: Blue 🧧			
Destination: 📑 Color Combine			
Hue rendering:			
Enable hue rendering Coefficients			
Color Combine Cancel			

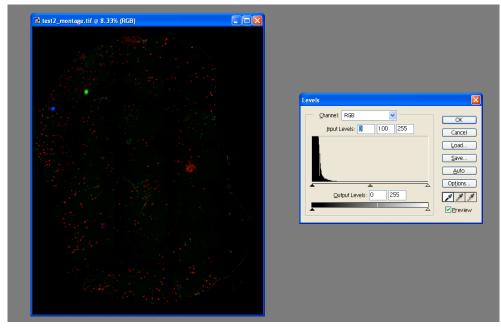
 e. Click on "Montage" in the montage taskbar. Choose the Color Combine image sequence as the source stack. Select "ZigZag – Horizontal" under Fill Order and enter the appropriate number of columns and rows. Uncheck "Stitch images". Leave every other option at the default setting. Click OK.

Montage	🛛				
Stack: Color Combine	ОК				
Dest: 🌁 Untitled	Cancel				
Planes: 45 Width: 1392 MontageWi Height: 1040 MontageHe					
Fill Order:	Columns: 🏮 📑				
C Vertical	Rows: 9 🐳				
<ul> <li>ZigZag - Horizontal</li> <li>ZigZag - Vertical</li> </ul>	Zoom %: 100 📫				
🔲 Stitch images 🛛 Ima	age Overlap %: 1 👘				
Width of separator line: 3	Color:				
Color for blank frames in the r	nontage: 0 🛨				
Draw separator line between frames					
Draw separator line around image					
🔲 Draw sequence number	255 🚊				

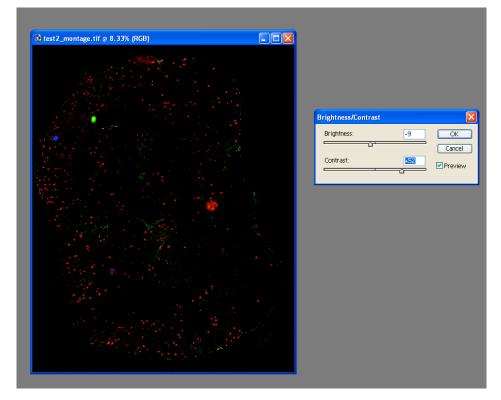
- f. Save the montage as an rgb-tiff.
- 6. Edit the image using Photoshop.
  - a. Open the image.
  - b. Crop out the field of interest: Select the crop function under the "Tools" toolbar or simply press "c". Highlight the field of interest and then double-click on the image to crop.



c. Correct the background: Image > Adjustments > Levels
 Click on the black point dropper icon on the bottom-right corner of the window.
 Click on a part of the image that is supposed to be black.



Adjust the brightness and contrast: Image > Adjustments > Brightness/Contrast
 Modify the brightness and contrast appropriately for the best results.



e. Save your changes.