Tiling on the IX81 for brightfield samples

Revised 30 July 2020

Acquisition

Initial set up

1. Turn on microscope for brightfield, open Volocity, create a new library.
2. VERY IMPORTANT:
    a. Do NOT put on your sample.
    b. Lower the objective fully (use the down arrow next to FOCUS, on the front of the microscope; you will hear a beep when the objective reaches the bottom).
3. In Video Preview mode, go to the *Stage* menu, click on *Calibrate stage…*
4. Check the spatial calibration of the objective you will use
5. Put on your sample, adjust channels as needed. Avoid saturation with and without slide. Save the settings.
6. To correct for uneven illumination you will need to take a blank image. There are two options for doing this:
    a. If your slide is transparent and your sample does not block a lot of light (you can tell if you remove it and see that the color of the image on the screen does not change, and the pixel values are not saturated): Take off the slide, take an image and name it blank. Export just the blank image, making sure to click on options to verify 'Convert to RGB' is clicked.
    b. If your slide has a slight tint to it (you can tell if you remove it and see that the color of the image on the screen changes): Go to an empty region of the slide, with as few imperfections as possible. Take an image and name it "blank". Export just the blank image, making sure to click on options to verify 'Convert to RGB' is clicked.
7. Check the straightness of the stage
8. In the *Video* menu, switch to *XY stage* view
9. In the *Stage* menu, click *Clear all points*
10. Go to the edges of the region of interest and mark positions, to generate an outline of the target area

Running a small test

11. Draw a small region somewhere in the sample that includes 2x2 squares. To do this, draw a region slightly smaller than 4 squares.
12. Go to *Acquisition setup* in the *Video* menu:
    a. Restore the MSL_tiling_BF setting
    b. Click on the *Stitch* button verify that the following settings are correct:
        i. Change XY using: Ludl XY stage
        ii. *From XY stage ROIs* should be ON
        iii. Use 10-20% overlap
        iv. *Create stitched composite image* should be OFF
        v. *Save raw files* should be ON
        vi. Select *No focusing*
13. Run the acquisition (press the record button).
14. Check to ensure there is the expected overlap between adjacent images. If not, contact Pablo

Running the full acquisition

15. Draw a region that includes the entire region of interest
16. IMPORTANT: Check the focus before starting the acquisition. To do this, focus in the middle of the region you will image, such that the image on the computer monitor is sharp. Then, move to the extreme left and right edges, and check that the focus remains good. Repeat for the top and bottom edges. If the focus is not even throughout the entire sample, consult with Pablo
17. Look at the grid and count the approximate number of squares in the X and Y dimension.
18. Run the acquisition (press the record button).
19. WAIT until Volocity finishes writing to the hard drive.

Exporting the data

20. Export images as TIFFs, using the following settings
    a. In Options:
        i. Click 'Convert to RGB'
        ii. Do NOT add 'Scale', 'Color Reference', 'Tile'

        IMPORTANT: You must go to Options every time you export. If not, there is a bug in Volocity that will format the images incorrectly.

    b. In Naming Options:
        iii. Only click on 'Append a numerical subscript'; remove all other options
            1. Count from 1 and increment by 1
            2. Pad with leading zeros to 3 digits
21. Do not put these images in the same folder as the blank file
22. Your images should be around 9MB in size. If they aren't, redo the exporting making sure to check the Options are correct.

Notes on acquisition:

- Generate a new library for each data set
- If you do not change your imaging conditions (illumination, exposure, objective), you can reuse the same blank image on a given day
- When you export your final images they should have names that are numbered: 001, 002, etc.

Stitching acquired images

1. Open FIJI
2. Use the MSL flatfielding plugin to correct for illumination.
3. Open *Plugins/Stitching/Grid/Collection Stitching*
4. Use the following settings:
    a. Grid: snake by rows
    b. Right and down
    c. Input x and y dimensions of grid (if you multiply x by y, you should get the total number of images)
    d. Input overlap you used for acquisition
    e. First file index: 1
    f. Find the directory with your flatfielded files
    g. Input the file name for your files. For example, for 001, 002, etc, input: {iii}.tif
    h. Fusion method: Min. Intensity
    i. Ensure Compute Overlap is clicked
    j. Merge method should be MIN intensity
    k. Default for other settings should work
5. Crop the part of the image of interest
6. Image Type RGB Color
7. Add white or a background color to fill in non-stitched parts, if needed. You can use the dropper and paint can tools to do this
8. Recommended: set scale and add scale bar
9. Save