Installation and Use Instructions for FocusWholeFile and FuseImages Macros

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INSTALLATION

1. Download the 6 files- FocusBlockMath4.py, ChooseFilesToFlatten.py, TileFocusingSeq.czimp, TileFocusing.czmsf, FocusWholeFile3.czmac, FuseImages.czmac
    a. You can put the 1st 4 files anywhere on your computer, but make sure you know where
    b. FocusWholeFile3.czmac and FuseImages.czmac must be placed in your Documents folder under: Documents\Carl Zeiss\ZEN\Documents\Macros\
    c. If that's the right spot, in Zen Blue (any version except Lite), you should be able to go to Tools> Customize Application > Macros >User Macros> and see the file.

2. Install Python 3 (any version) with common packages.
    a. I recommend going through Anaconda (https://www.anaconda.com/distribution/). This will have all of the packages you need.
    b. If you want to just use the basic version of python3 (https://www.python.org/downloads/release/python-380/), please also install Numpy, SciPy, Matplotlib, argparse, and pickle
    c. Find the path to your Python executable. It will look something like this: D:\Users\zeiss\Anaconda3\python.exe

3. Edit the code to include the correct paths to the files
    a. If you do not have the Zen Macro Editor:
        i. Open FocusWholeFile3.czmac in a text editor (I recommend Notepad++)
            a) Replace the paths to the 4 files, the python executable, and a temporary data file with the correct paths for your computer. The lines you need to change are listed below. The line numbers should be accurate if you use Notepad++, but it's possible a different text editor will space out the text differently. The full line of code is shown to help you find it, but you only need to change the text highlighted in red.
                - 36: Zen.Measurement.FeatureSets.ActiveFeatureSet.Load ("D:\\Users\\zeiss\\Documents\\FlatteningMacro\\TileFocusing.czmsf")
                - 37: Zen.Measurement.MeasurementSequenceSettings.ActiveMeasurementSequenceSetting.Load ("D:\\Users\\zeiss\\Documents\\FlatteningMacro\\TileFocusingSeq.czimp")
                - 108: picklefile=open(r'D:\Users\zeiss\Documents\FlatteningMacro\focusvars2.pkl','wb')
                    i. NOTE: This file will be created during the run. Change the folder path r'D:\Users\zeiss\Documents\FlatteningMacro\ to wherever you want it to be.
                - 114: pythonexe=r'D:\Users\zeiss\Anaconda3\python.exe'
                - 115: script=r'D:\Users\zeiss\Documents\FlatteningMacro\FocusBlockMath4.py'
                - 294: pythonexe=r'D:\Users\zeiss\Anaconda3\python.exe'
                    i. Should match the text in 114
                - 295: script=r'D:\Users\zeiss\Documents\FlatteningMacro\ChooseFilesToFlatten.py'
            b) Leave all odd characters (like CR LF) and all punctuation exactly as is. It is important to have the double backslash (\\) and double apostrophe (“ ”) in the first two paths and the r in front of the rest of the paths with single backslashes (\) and single quotes (‘ ’).
            c) Save the edited file as FocusWholeFile3.czmac. You can change the name, but you must give it the .czmac extension (not .txt).
        ii. Open FuseImages.czmac in the text editor

a) Replace the paths to the 4 files, the python executable, and a temporary data file with the correct paths for your computer. The lines you need to change are listed below. The line numbers should be accurate if you use Notepad++, but it's possible a different text editor will space out the text differently. The full line of code is shown to help you find it, but you only need to change the text highlighted in red.
   - 15: pythonexe=r'D:\Users\zeiss\Anaconda3\python.exe'
   - 16: script=r'D:\Users\zeiss\Documents\FlatteningMacro\ChooseFilesToFlatten.py'

b) Leave all odd characters (like CR LF) and all punctuation exactly as is. It is important to have the r in front of the paths with single backslashes (\) and single quotes (' ').

c) Save the edited file as FuseImages.czmac. You can change the name, but you must give it the .czmac extension (not .txt).

b. If you have the Zen Macro Editor:
   i. Follow all of the above instructions, but the line numbers will be different. For FocusWholeFile3.czmac, the lines will be: 33, 34, 105, 111, 112, 291, and 292. The paths in 33 and 34 will be shown in red, the rest will be in pink. For FuseImages.czmac, the line numbers will be 12 and 13, and they should be in pink.
   ii. Save the edited macros

4. The macros should be fully installed and ready to go! Within Zen Blue, you can go to Tools> Customize Application > Macros >User Macros> and double click on the edited versions to make icons appear at the top of your screen.

USING THE MACROS

1. Imaging Conditions:
   a. When imaging your slides, acquire Z stacks. The range and step size needs to be small enough that it always acquires the in-focus plane of every region of the slide. Ideally you want there to be very smooth transitions between two adjacent Z steps. I recommend 0.5 or 0.75 um steps for the 20x objective on the Axioscan.
   b. The files can have any number of channels or scenes. They must be fluorescent, though if you need this to work for brightfield images I can modify it.

2. Put all of the files that you want to process in one folder. For each batch, all files must have the same channels.
   a. For a first test of the installation, I recommend cropping a small region out of 1 or 2 files so that you can quickly test the file path edits and see what the processing looks like.
3. Run the FocusWholeFile3 macro by clicking on the icon you made in step 4 above. A window will pop up asking you to choose all of the files you want to process (you have the option to only process some of the files in a folder).
4. A second window will pop up asking which channel to use as a stitching reference. Choose whichever is the most ubiquitous (probably Hoescht). It will use this choice for all images.
5. The processing will begin, and images and tables will flash on the screen. Leave Zen alone while it thinks. When it's complete, there will be a new file in the folder called "[Name]_Flattened.czi". If there were multiple scenes in the original data file, each scene will be processed and saved separately.
   a. A full pancreas with 4 channels and 7 z steps can take 2~4 hours.
   b. In my experience, Zen gets slower with each processed file. If you process a few files overnight, I recommend restarting the computer in the morning.
6. At this point, the images will load correctly in Zen and will have a single, focused z-plane. But, they are not in the correct format to be read by BioFormats and other open-source software. The FuseImages macro converts the images into the correct format.

7. Once all of the images in the folder are flattened, run the FuseImages macro. Select just the “flattened” files. This will create new files with “_ReFused” appended to the name. This image can be opened in Fiji, QuPath, etc. NOTE: most of the metadata will be copied, but it WILL NOT have the pixel size. This must be set in your analysis software.

How the Macro works

First, it divides each field-of-view into 16 bins (4x4 in a square). For each bin, it finds the most in-focus plane by following the math of the Find_Focus ImageJ plug-in. Briefly, it applies an edge filter, then finds the Z step that has the highest variance. It stores the temporary “best” Z position for each bin in each FOV. After it has performed this calculation on all of the FOVs, it averages the results for each bin. This will give a general map of the tilt of the slide (for instance, if the top right corner of every FOV is almost always focused at a higher location than the bottom left, you know the slide is tilted along that axis). It then “subtracts out” the known offset of each bin based on this tilt from the stored results. This results in a map of which locations are best focused (based on its calculations of variation) in an unusual place compared to the slide tilt. Sometimes these differences are showing true local changes in the tissue height, but sometimes they are just noise/inaccuracies/places where there is insufficient data. It uses a smoothing filter on this map to remove noise and then puts back in the expected offset of each bin. This corrected map is used to rebuild a single plane. It goes one-by-one through each bin, calls the correct Z step, and writes those intensities into a single plane in a new image.

This whole process is repeated for each channel independently. When it has flattened all the channels, it merges them and then performs stitching based on the reference channel you selected. Afterwards, FuseImages rebuilds the pyramid.