

# Multifocal Image Analysis – User Guide

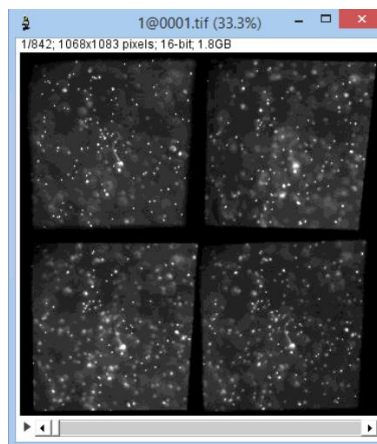
## Introduction

This user guide describes how to use and apply software published along the manuscript **Multifocal imaging for precise, label-free tracking of fast biological processes in 3D** (Jan N. Hansen, An Gong, Dagmar Wachten, René Pascal, Alex Turpin, Jan F. Jikeli, U. Benjamin Kaupp, Luis Alvarez. bioRxiv 2020.05.16.099390; doi: <https://doi.org/10.1101/2020.05.16.099390>). For more information, detailed license notes, an installation guide, etc. visit <https://github.com/hansenjn/MultifocalImaging-AnalysisToolbox>.

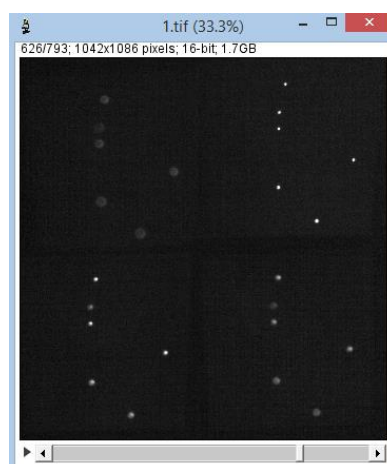
## 3D Bead tracking

### Requirements

1. Multifocal imaging recording of beads



2. A multifocal imaging piezo-generated z stack through exemplary beads to obtain a calibration between bead width and z position



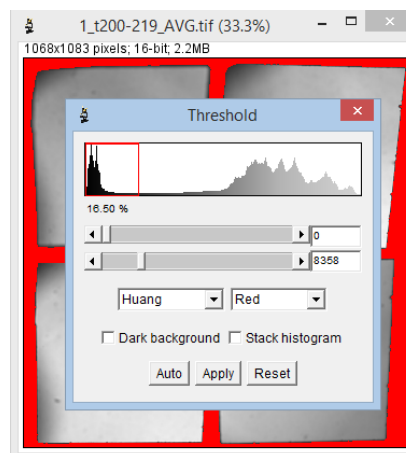
3. A reference file for intensity corrections (obtained by imaging without a specimen)



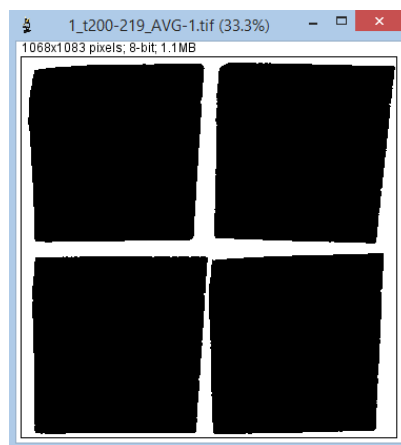
## Workflow

### Creating an intensity heat-map

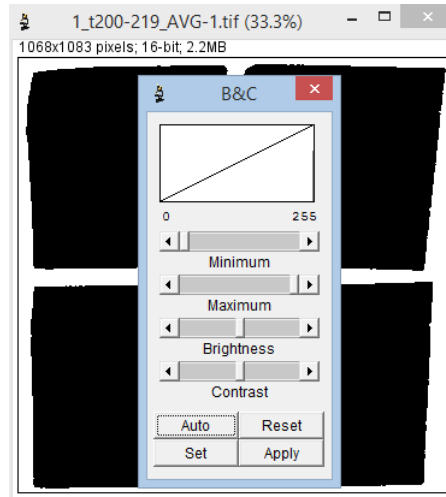
- Open the reference file for intensity corrections in ImageJ
- Duplicate the image: Image > Duplicate
- Binarize the duplicate: Image > Adjust > Threshold, select Huang, press Apply



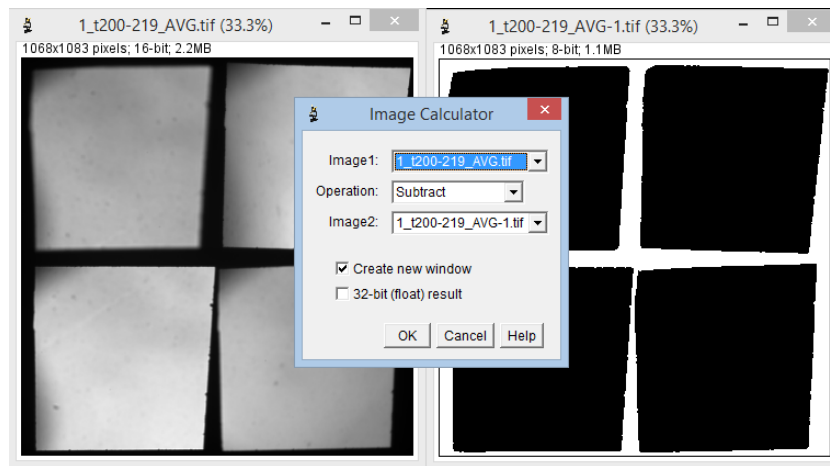
- The duplicate image will look like this:



- Convert the image to 16-bit: Image > Type > 16-bit
- Reset the intensity values: Image > Adjust > Brightness & Contrast, Press Apply



- Remove the non-plane areas from the map: Process > Image Calculator, Select the operation “Subtract”, the raw image as Image1 and the binarized duplicate image as Image2, Press OK

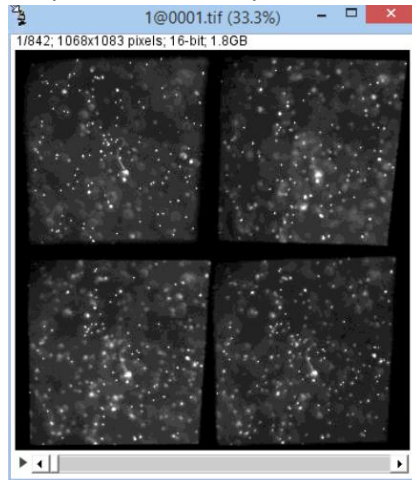


- Save the generated image as “Bead Intensity Map.tif”, it will serve as a map for intensity corrections in the plugin “Multifocal Preparation”.

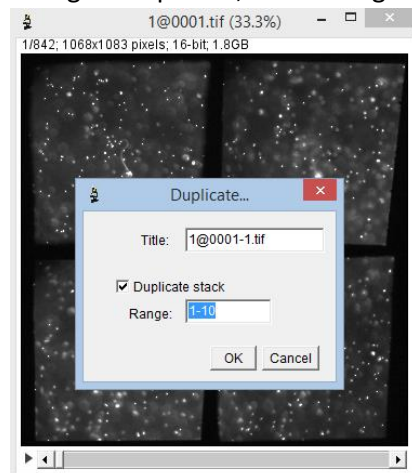


### Creating a registration file

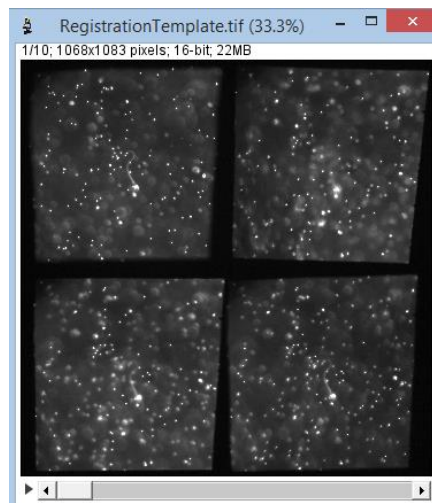
- Install the plugin MultiStackReg from Brad Busse: <http://bradbusse.net/downloads.html>
- Install the latest release from the plugin Multifocal\_Preparation: [https://github.com/hansenjn/MultiFocal\\_Preparation/releases](https://github.com/hansenjn/MultiFocal_Preparation/releases)
- Restart ImageJ
- Open the multifocal time series you want to analyze



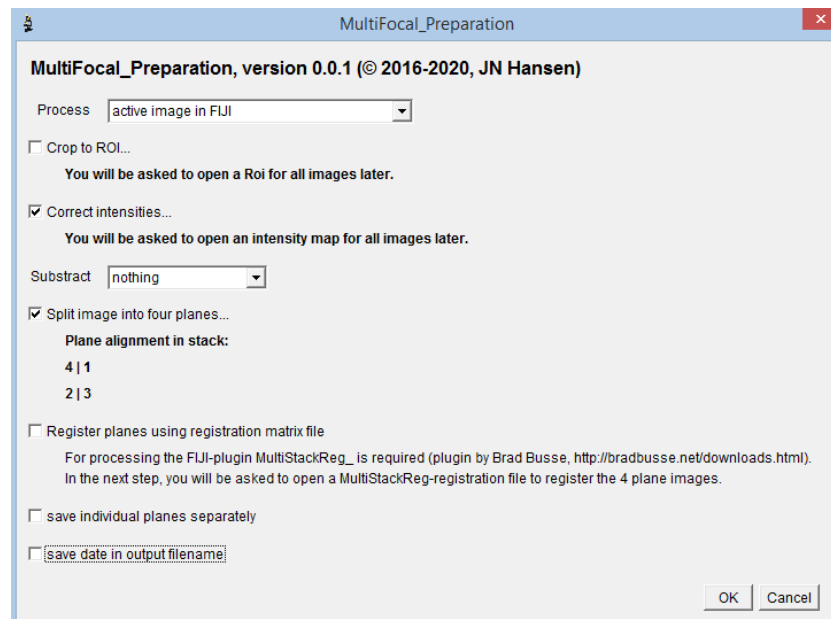
- Duplicate the first 10 frames: Image > Duplicate, select Range 1-10, press OK



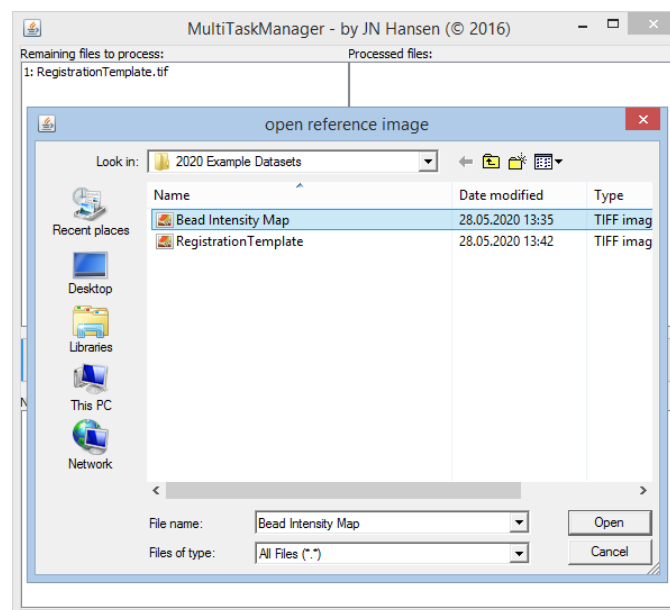
- Save the image as "RegistrationTemplate.tif"



- Process the image with MultiFocal\_Preparation: Plugins > JNH > Multi Focal > Prepare raw data for analysis, select the following options and press OK.



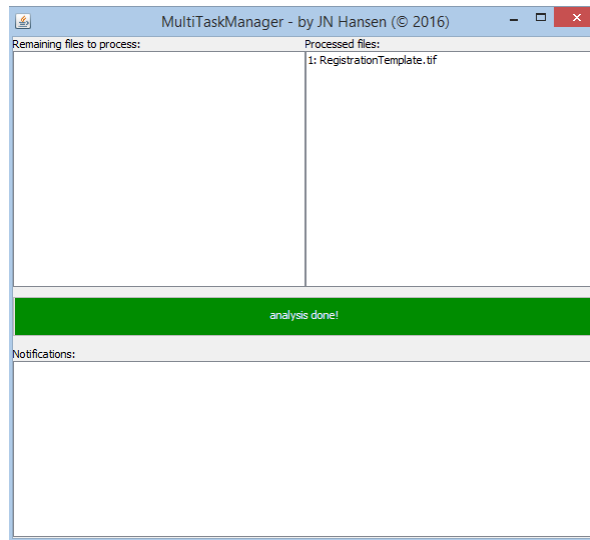
- In the upcoming dialog, select the “Bead Intensity Map.tif” produced before as a reference image:



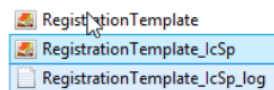
- Wait until the Processing is Done
  - In brief, the plugin will correct the intensities according to the reference image and then split the image into 4 equal quarters
  - Each quarter becomes a separate plane in the output Hyperstack
  - The conversion is as follows: the upper right quarter becomes plane 1, the lower left quarter plane 2, etc. as indicated in the settings dialog:

**Plane alignment in stack:**  
4 | 1  
2 | 3

- When the Processing is Done, the MultiTaskManager dialog looks like this:



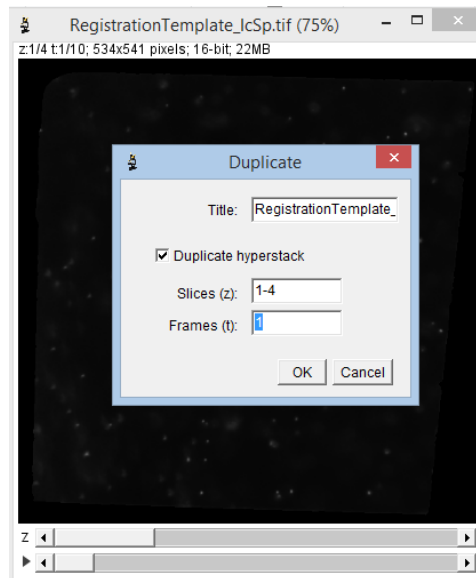
- Then, new files have been saved to the repository where the RegistrationTemplate.tif file was saved.



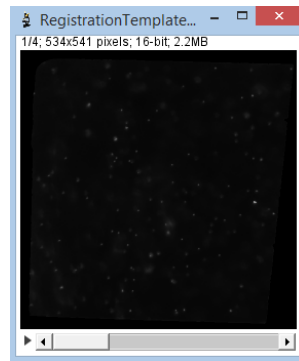
- The .tif-file with ending IcSp contains the output Hyperstack, the text file documents the processing settings of "MultiFocal\_Preparation".
- Open the RegistrationTemplate\_IcSp.tif file in ImageJ



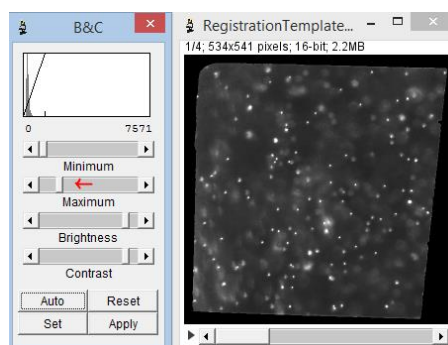
- You can see that each focal plane has become a different Z slice.
- Extract a single timepoint: Image > Duplicate, select the following settings, press OK



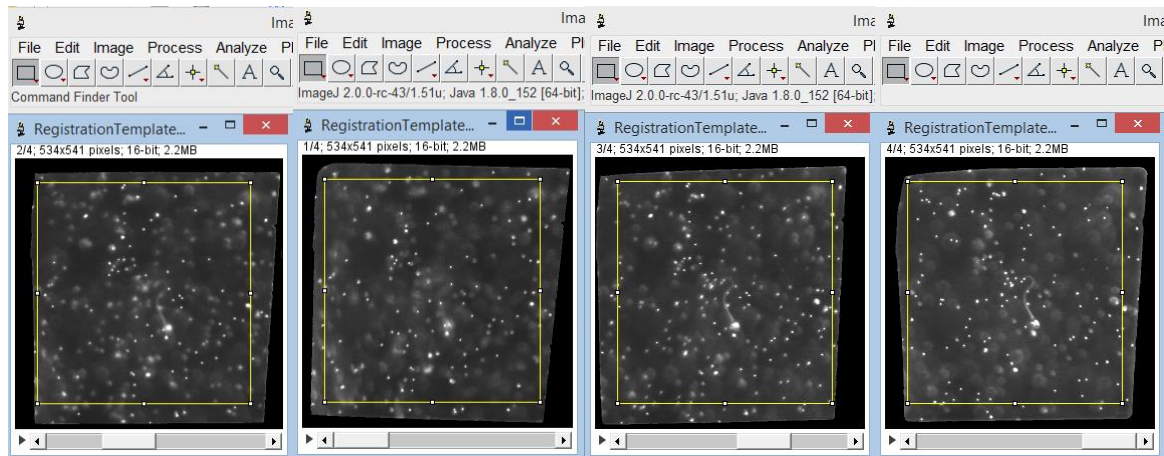
- A new image pops up containing only one time point and the four different planes



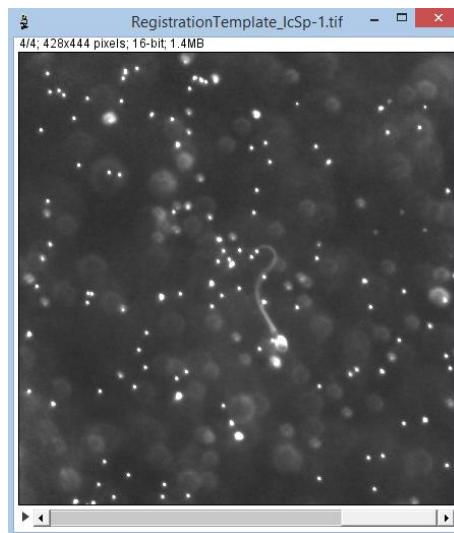
- Adapt the display range so that you can see the borders of the planes: Image > Adjust > Brightness/Contrast, drag down the maximum so that edges become apparent in the image



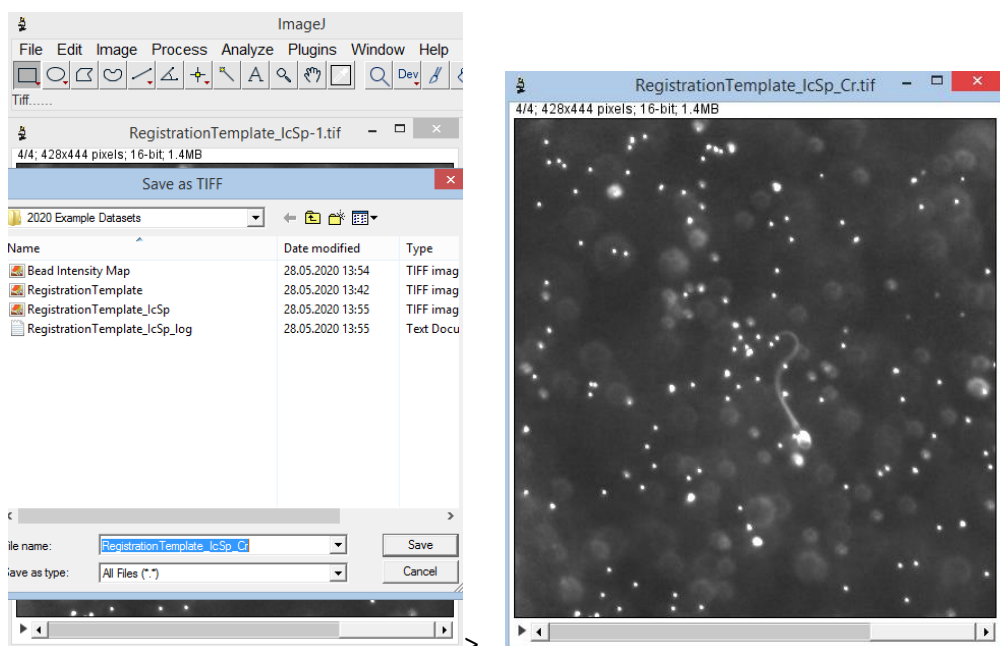
- Select the Rectangle tool in the ImageJ bar and draw a selection that is inside all planes and does not contain any black areas. Check the different planes by scrolling with the mouse wheel and adapting the selection.



- Crop the stack to the selection: Image > Crop

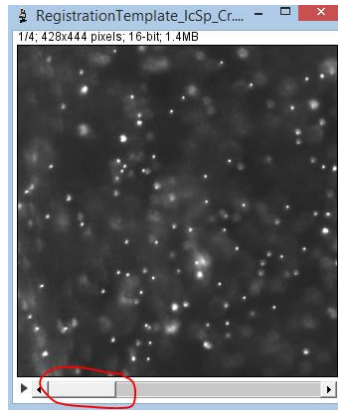


- Save the stack as RegistrationTemplate\_IcSp\_Cr: File > Save As ... > Tiff

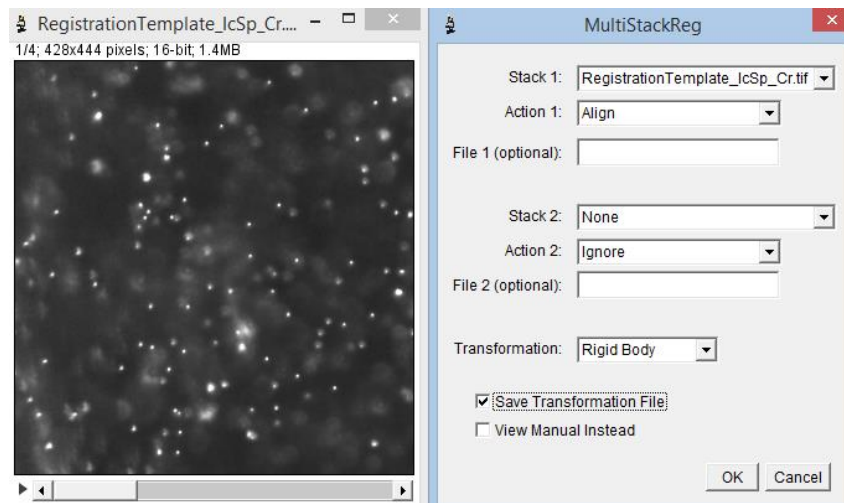




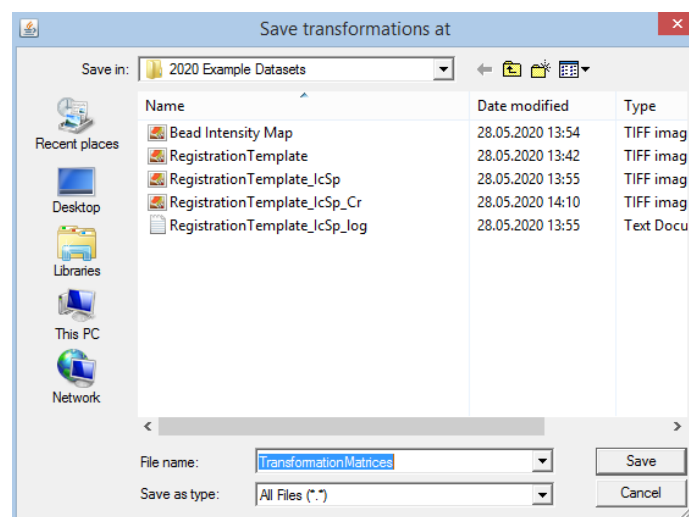
- Make sure that the first plane is selected



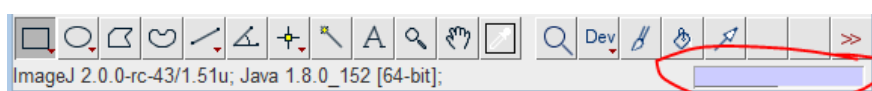
- Register the file: Plugins > Registration > MultiStackReg, select the following settings, press OK



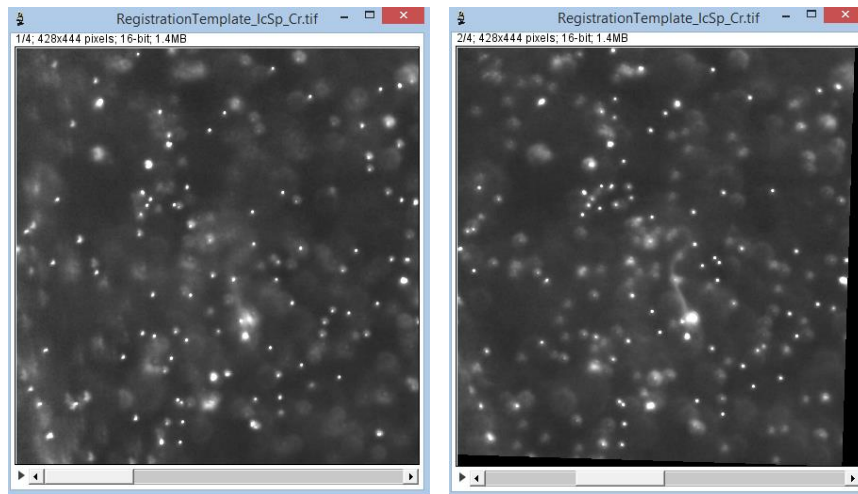
- A dialog pops up, save the transformation matrices to the folder where the image was located



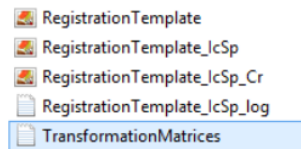
- Wait until the plugin is done and the status bar looks “resting”/normal again



- The planes in the image now have been aligned and the transformation matrix file has been saved
  - Scroll through the image to check whether the alignment is good
  - Compare for example plane 1 and plane 2:

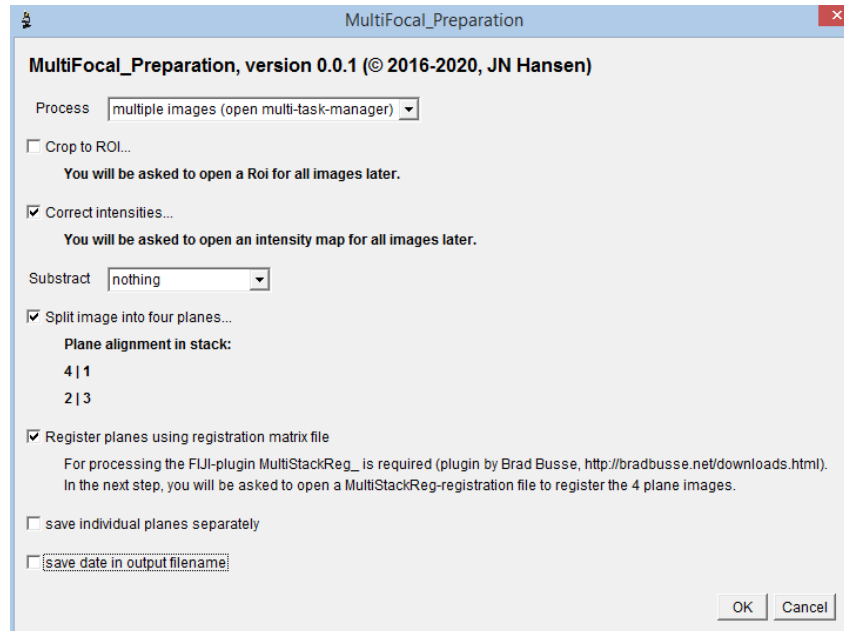


- The matrix file will be needed as a reference for registering the planes using the MultiFocal\_Preparation plugin.

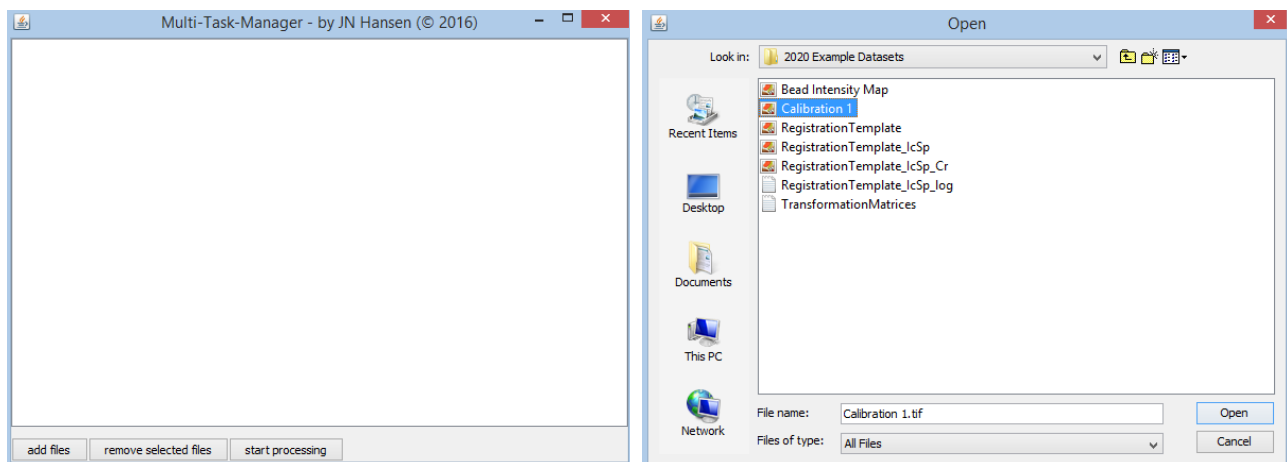


## Preparing the data for calibration and analysis (intensity correction, splitting, registering)

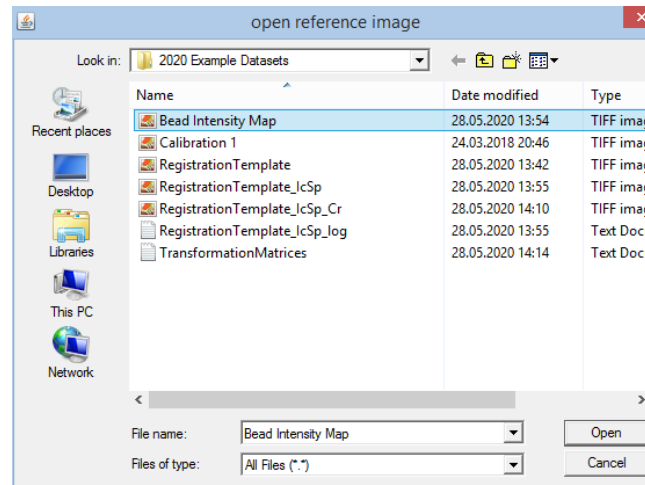
- Process all data that you want to use for calibration or subject to analysis with Multifocal\_Preparation: Plugins > JNH > Multi Focal > Prepare raw data for analysis; select the following options and press OK.



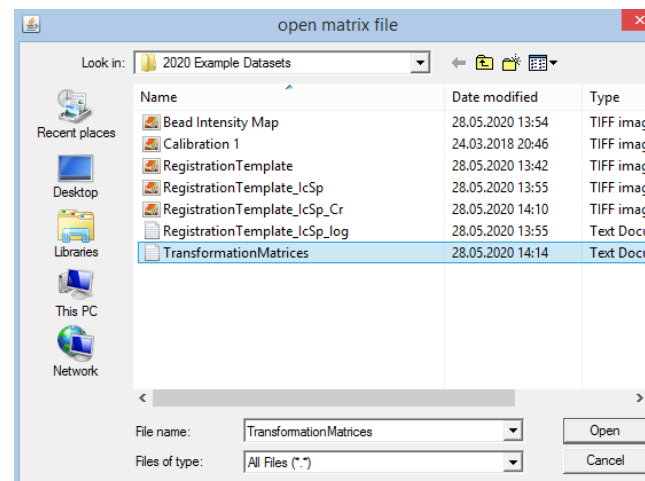
- A dialog pops up, press add files, select the files you want to analyze in your file system, press open (eventually repeat to add more files from different repositories), press start processing to let MultiFocal\_Preparation prepare the data.



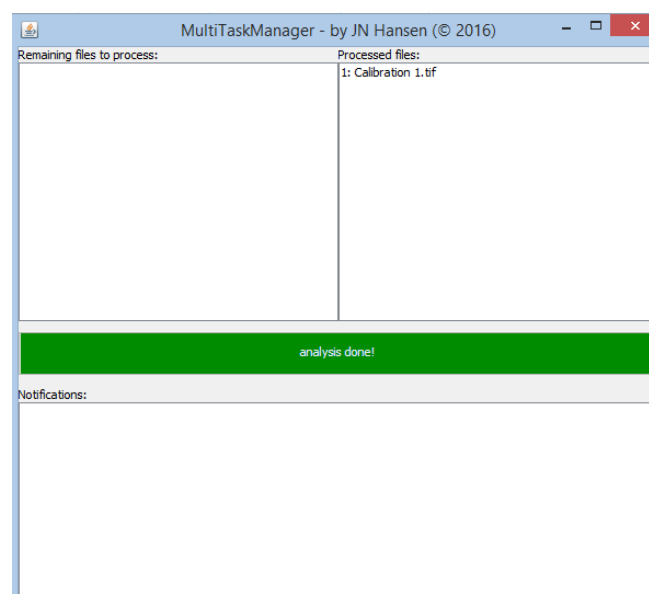
- A dialog pops up, select the Bead Intensity Map as a reference image and press open:



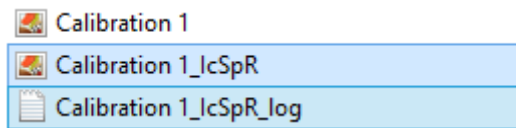
- Another dialog pops up: Select the TransformationMatrices file and press Open.



- To avoid any processing errors, don't touch the computer while processing. Some images might pop up and be hidden again during registration. Wait until processing is done.



- At the location where the template files were saved, the output files will be saved. They receive an additional ending (\_IcSpR).



- The .tif-file with ending IcSpR contains the output Hyperstack and serves as a template for data analysis, the text file documents the processing settings of “MultiFocal\_Preparation”.



Determine a calibration Look-Up-Table (LUT)

TO BE CONTINUED