Fiji/Macro Tutorial

1. System requirements
Fiji is supported on the following systems:

Windows XP, Vista, 7, 8 and 10
Mac OS X 10.8 "Mountain Lion" or later
Linux on amd64 and x86 architectures

The latest version number (used in this tutorial) is: 2.0.0-rc-69/1.52n

Installation and demo are tested on Mac OS (Mojave Version 10.14.5, 64-bit) and Windows PC (Windows 7, 64-bit), the described steps are identical for both operating systems, unless stated otherwise.

2. Installation guide

A. Download Fiji for your operating system at https://imagej.net/Fiji/Downloads

For Mac: Move the downloaded file to a directory that is easy to access. Typical install time on a "normal" desktop computer - 1 minute
For PC: Extract the zip file to a directory that is easy to access. Typical install time on a "normal" desktop computer - 5 minutes

3. Demo

The zip file contains a demo dataset "2.
Demo_DataSet_FcR1a_KO_Stimulated.lsm" which is an image taken 30 min after stimulation with anti-IgE in a heterogeneous -CRISPR-Cas9 mediated FceRI-alpha chain knockout- mast cell population.

The demo should make you familiar with Fiji, the provided macros and initial data visualisation.

Instructions to run on data:

A. Double click on Fiji (ImageJ-win64.exe on windows) to launch the program.
B. Drag the provided demo dataset "2.
Demo_DataSet_FcR1a_KO_Stimulated.lsm" into the work-bar of Fiji.
C. On the bottom-left side of the newly opened window you will find a "C" (Channel). Move the bar next to it to the right to inspect channel 2 and 3. At this point it is normal that you will see a faint signal in channel 2.
D. We will use macros to (i) enhance contrast to visually inspect the images, (ii) export images, and (iii) calculate and export MFI per cell, and correlate GFP vs. Av.SRho on this demo dataset.

The macros for time-saving semi-automated image analysis in Fiji are provide below.
To create new macro, go to Plugins, New, Macro.

i. To enhance contrast and visually inspect the images, copy the following macro into the newly opened window:

```java
Stack.setChannel(2);
run("Green");
run("Enhance Contrast", "saturated=0.35");
Stack.setChannel(1);
run("Grays");
Stack.setChannel(3);
run("Red");
run("Enhance Contrast", "saturated=0.35");
```

ii. To enhance contrast and export images, copy (note to edit the path-directory (5x) were the image has to be saved "/Users/YourDirectory/Grey-”) the following macro into the newly opened window:

```java
name=getTitle();
Stack.setChannel(2);
run("Green");
run("Enhance Contrast", "saturated=0.35");
saveAs("Jpeg", "/Users/Location/Desktop/GFP-"+name);
Stack.setChannel(1);
run("Grays");
saveAs("Jpeg", "/Users/Location/Desktop/Grey-"+name);
Stack.setChannel(3);
run("Red");
run("Enhance Contrast", "saturated=0.35");
saveAs("Jpeg", "/Users/Location/Desktop/Avidin-"+name);
Stack.setDisplayMode("composite");
roiManager("Show All");
saveAs("Jpeg", "/Users/Location/Desktop/Combined-"+name);
saveAs("Tiff", "/Users/Location/Desktop/Combined"+name);

NOTE: For Windows users, directory path should be indicated as:
```java
saveAs("Jpeg", "\GFP-"+name);
```
Change accordingly throughout the Macro (5x) (feel free to specify a specific directory on your PC).

iii. To enhance contrast, calculate and export MFI values, copy (note to edit the path-directory (1x) were the image has to be saved "/Users/YourDirectory/Desktop/"+name); the following macro into the newly opened window:

```java
name=getTitle();
run("Duplicate...", "title=Mask duplicate channels=2");
selectWindow("Mask");
run("Find Edges");
run("Threshold...");
setAutoThreshold("Default dark");
waitForUser("set treshold manually");
selectWindow("Mask");
```
run("Analyze Particles...", "size=300-Infinity pixel show=Masks 
exclude include");
run("Grays");
run("Watershed");
roiManager("reset");
run("Analyze Particles...", "size=300-Infinity pixel show=Nothing 
display exclude clear include add");
selectWindow(name);
Stack.setChannel(2);
run("Clear Results");
roiManager("Measure");
String.copyResults();
waitForUser("Export Data Chanel 2");
Stack.setChannel(3);
run("Clear Results");
roiManager("Measure");
String.copyResults();
waitForUser("Export Data Chanel 3");
//run("Channels Tool...");
Stack.setDisplayMode("composite");
Stack.setChannel(3);
run("Red");
run("Enhance Contrast", "saturated=0.35");
Stack.setChannel(2);
run("Green");
run("Enhance Contrast", "saturated=0.35");
Stack.setChannel(1);
run("Grays");
roiManager("Show All");
saveAs("Tiff", "/Users/Location/Desktop/+name");
selectWindow("Mask of Mask");
close();
selectWindow("Mask");
close();

NOTE: For Windows users, directory path should be indicated as:
saveAs("Tiff", "\C\Combined+name");
(feel free to specify a specific directory on your PC).

E. Save the 3 (edited) macros individually by going to File, Save As. Give the file an appropriate name e.g., "1.Enhance_Images.ijm"
and click save. The newly created .ijm files can be dragged directly
into the work-bar of Fiji, which opens the macro.
F. With the demo dataset still opened, load macro i into Fiji and 
click Run.
G. Switch between the channels of the demo dataset to visually 
inspect the newly enhanced images. Channel 2 should now show GFP 
positive cells (two clearly positive, other mast cells with some 
background/autofluorescence signal.
H. Next, load macro ii into Fiji and click run. In addition to 
enhancing contrast, this macro will export individual, as well as 
combined, images to the specified directory.

NOTE: If you get an error "Output has not been set", the directory
pathway is not correct and has to be adjusted.

NOTE: The macros can be executed in subsequent order (1 --> 2 --> 3) of each other, but not from from macro 3 --> 2 (due to the composite that is created in macro iii). In that case it is best to close the .tiff file and reopen the .lsm demo dataset.

I. The most important macro used in the protocol is macro iii, which enhances contrast, calculates and exports MFI values of individual mast cells. With the demo dataset opened, load macro iii into Fiji and click Run.

J. You will see different “masks” being applied to the image, followed by two pop-ups “Action Required – set threshold manually” and “Threshold”. Reduce the threshold with the upper bar until small red dots appear in the image (these will not be included in the final data analysis, due to the minimal particle size settings). For this demo dataset we recommend to set the upper bar to 7, press enter, and leaving the lower bar at the max (255). This should correlate with a threshold of about 12.02%. Click “OK”.

K. The next action required is “Export Data Chanel 2” – which contain the mean GFP MFI values per cell. Open Excel and paste the data from the clipboard to the spreadsheet, and click “OK”.

L. A new action required states “Export Data Chanel 3” – which contain the mean Av.SRho MFI values per cell. In Excel, paste the data from the clipboard to the spreadsheet, directly next to the GFP data and click “OK”.

M. Finally, a new .tif image is created (and saved to the specified directory) which visually correlates the cell numbers in the spreadsheet to cells in the image.

N. If everything went right, the visual high GFP signal in cell 2 and 7 correlate with high GFP MFI values in these cells in the Excel file. Furthermore, the visual degranulation in cell 4, 5 and 10 should correlate with high Av.SRho MFI values in these cells in the Excel file.

Expected output:

The expected output after running the 3 macros on the demo dataset are JPEG images of the individual (as well as combined) channels, an Excel file containing GFP and Av.SRho MFI values for each individual cell, and finally, a .tif file is created (and saved to the specified directory) which visually correlates the cell numbers in the spreadsheet to cells in the image.

NOTE: As with every mast cell activation assay, you can expect that not every mast cell is activated by the stimuli of choice. In addition, some dead cells may be present in the images (cell 8 may be dead, or out of focus, in the demo dataset).

Expected run time for demo on a "normal" desktop computer – 30 minutes.

4. Instructions for use
How to run the software on your data

Although our provided macros make it easy to set correct colours, enhance contrast, measure fluorescence intensity, and export ready-to-use figures and data-sets, some fine-tuning of the settings may be required depending on your individual equipment setup and configuration.

Instead of loading our provided .lsm demo dataset file, load your own image(s) -.tif, .lsm, .lif files- into the work-bar of Fiji and run a desired macro on them, similar to the demo.

We recommend to first try provided macros on your data, which should work in most cases, until a problem presents itself. A common issue is that Fiji and the supplied macros do not call individual mast cells correctly. To address this, we recommend to try these two approaches:

A. Reduce/increase the threshold with every image until you see small dots appearing around the mast cells (these will not be analysed due to the particle size settings).
B. Depending on the magnification of the image, adjust particle size parameters. We recommend a particle size of 300 for images shot at 20x, and a particle size of 100 for images shot at 10x.

In addition, when using your own data, make sure that the colours set per channel in the macro correlate with the color of your images (grays for background, green for GFP and red for Av.SRho). This is easily done by changing the "Stack.setChannel(X); per colour."