Practical work 5: Image processing with ImageJ

1. Purpose of work

The aim of this work is to learn how to use the basic image viewing and processing tools of ImageJ. Pay attention to the bolded questions, you should be prepared to answer them in the practical work summary session.

2. Background

In scientific work, image processing is not the same as making a visually pleasing picture. In addition to producing images for viewing in presentations and publications, image processing is often required for preparing images for analysis. In both cases it is critical that no information is lost or distorted because of image processing.

ImageJ is a free public domain Java program for image processing and analysis. It can be freely downloaded from the internet (http://rsb.info.nih.gov/ij/). Its functionality is largely based on plugins, either self-made, or available from various sources. In this session, we will concentrate on basic image processing in ImageJ without the use of separate plugins. If you want to download ImageJ to your own computer note that you may have to download some plugins as well.

We will view and process a few images with some of the basic tools in the "Image" and "Process" menus in ImageJ. For further help on the commands, see the ImageJ "Help" menu, or the menu printouts beside the computer.

3. Samples and software used

Instrument: computer in MIU office, room B501a
Software: ImageJ
Samples: The images are in the Images folder on your desktop

4. Tasks

1. Image Type. Open "Image_1.tif". File – Open… This is a 24-bit RGB image of cell nuclei stained with DAPI. Open a copy of the image with the duplicate command:
Image – Duplicate…
Convert one image to 8-bit greyscale by using the Type command in the Image Menu
Image – Type - 8-bit

Convert the other to three different greyscale images by splitting the RGB channels to red, green, and blue:
Image – Color – RGB Split
Save the blue channel.

Apply the blue LUT (lookup table) to both previous images:
Image – Lookup tables - Blue
Compare to the original image. **What are the differences?**

2. Background. There is often an uneven background in the image. You do not want this to affect the analysis. Therefore, background can (and should), be subtracted from the image. Pixels whose intensity values are similar to the background will be replaced with the mean background intensity value.

Open the previously saved blue channel image. As you can see, the background is uneven. Background can be subtracted using the "Subtract Background" tool:
Process – Subtract background…
The "Rolling Ball Radius" should be larger than a typical object in the image. Test using the "preview" option, start with 100 pixels. Save the background-subtracted image.

For transmitted light images, it is usually better to use a Flatfield correction, which subtracts an empty field (field without the sample itself) with the same imaging settings. If such an empty field is not available, the "Pseudo Flatfield" filter can be used.

Open Image_2.tif. This is a reflection image of cells growing on a surface. The image was taken with a confocal microscope. When this imaging technique is used, the background is often uneven. Select:
Process – Filters – Pseudo Flatfield – Kernel…
Try using different values for the "Filter kernel size" and select "Keep the flatfield". Compare the flatfield to the original image. **As the image is an 8-bit image, what limitations does such a process mean? Do these operations change the signal intensity?**

3. Threshold. Often the first step in image analysis is to separate the image into two areas: "object" and "not object". Object referes to the part(s) of the image you are interested in (e.g. cells or nuclei).

Select the blue channel of image 1 that you saved in task 1, and use the thresholding adjustment:
Image – Adjust – Threshold
First use the "Auto" button, and then manually adjust the slider to see the changes. Click "Apply" when you are happy with the image.

Now redo the thresholding using the background-subtracted image from step 2. **Are there any differences?**

4. Stacks. Open Image_3.tif. This is an image stack that consists of 44 images. The first image is out-of-focus but you can go through the stack by using the slider at the lower edge of the image. **Is there any single image in which all the strands are in focus?** Often stacks are represented as maximum projections to a single image. In the maximum projection image, the highest intensity value of the pixels with the same xy-coordinates through the stack is displayed. Select:
Image – Stacks – Z-Project… (select Max Intensity)
Does the image seem to be in focus? Why? Should you use maximum projection images for colocalization studies?

5. Filters. If thresholding is difficult you can try to filter the image data so that it is easier to threshold. There are several filters that use different algorithms.

Open Image_4 and make a duplicate as in task 1. This is a confocal image with a lot of noise. Use the median filter to reduce some noise:
Process - Filters – Median…
Test different values for the radius.

Now process the duplicated image with the mean filter:
Process – Filters – Mean…
Can you see any differences in the median- and mean-filtered images?

Sometimes unevenness in staining can make thresholding difficult as there are many peak intensities within one object. Use the mean filter to smooth the background-subtracted version of Image_1. **How does this make thresholding easier? Can it also cause negative effects? How do the "Fill holes" and "Watershed" functions affect the thresholding?**

Extra task. If you have time, you can try different image processing filters for images 1 and 4:
Process – Filters…

5. ImageJ menu commands

The ImageJ menu commands are documented on the ImageJ website (http://rsb.info.nih.gov/ij/docs/index.html). The relevant parts for this work will also be available by the workstation as a printout.

Please note that on the aforementioned webpage, there is also a link to the "ImageJ for Microscopy" manual available via the McMaster Biophotonics Facility. This is a good reference for future, collecting many of the more advanced ImageJ tools and plugins into one package.