Practical work 9. Image analysis with ImageJ

1 Purpose of the practical

In this practical you should learn the basic analysis tools of ImageJ and some theory behind them. You will also learn about automation of the analysis. **Pay attention to the bolded questions, you should be prepared to answer them in the practical work summary session.**

The practical is divided in to smaller separate sections. The topics are:

- Calculating cells using the particle analyzer
- Multi channel analysis
- Color thresholding
- Automation with macros

After each part you can close all the images and windows, they are not needed in the following parts.

2 Software and instruments

The software used in this practical is the free image analysis/processing suite ImageJ. The computers to be used are Imaging Workstations 1 and 3, found in the lobby in front of the MIU office and in room B501a (MIU office), respectively. You can find the image files required for the analysis in the folder E:\images_for_practical. Start ImageJ by double clicking the ImageJ icon on desktop.

3 Particle analyzer

3.1 Your task

The first task is to find cell nuclei in one image and do measurements on them.

3.2 Detailed information

The particle analyzer can only understand 1 channel (grayscale) images. If you load up color images, you can split the color channels using Image – Color – RGB Split and then do the analysis on the chosen channel.

Thresholding is done by choosing a pixel intensity value above which the pixel will be rated as belonging to a particle, below this it will be set as background. In this work, the threshold value is determined visually.

Several object parameters can be calculated, the most common ones being the area and mean intensity. Morphological information – meaning circularity, perimeter etc. is not that commonly analyzed even though it would often be useful. Other, sometimes valuable, information is the spatial coordinates of the objects (x and y of center point – used e.g. clustering or tracking of nuclei), and different statistical parameters calculated from the pixel intensity distributions, such as standard deviation, skewness, kurtosis etc. Knowing statistics may come in handy especially when dealing with larger amounts of data.

For setting the area restricions (default is from zero to infinite), you should estimate the area of a typical object. You may do that by drawing a circle (that has about the same area as a single object) in the image and read its width (equals diameter). Then you can calculate its area with the equation A= pi * r^2.

3.3 Instructions in short

- Load image cells1.tif by dragging the image icon onto the ImageJ bar (or use "File – Open")
- Set threshold via Image - Adjust – Threshold
    - Adjust the threshold bars so that the objects are red
    - Click set and then ok
    - **If the thresholding value is difficult to set, what options do you have? Which image processing tools?**
- Set measurements from Analyze – set measurements
    - Check: Area, Circularity, Mean gray value
- Open particle analyzer: Analyze – analyze particles
    - Set area restrictions: 0-infinite (or if there is small garbage in the mask, you can increase to e.g. 100-infinite)
    - Show: outlines
    - Check: Display results, Exclude on edges, Include holes
- Save results in E-disc as cells1_manual.txt
- **How are the Area and Mean value calculated?**
- **Do you see the difference in each object's circularity?**

4 Multi channel analysis

4.1 Your task

The basic multi channel analysis is about finding the objects in one channel, and measuring something else in the other channel. In this task, you will enlarge the objects found in channel 2, and measure the areas in channel 1.

4.2 Detailed information

In ImageJ, the structure element used for binary operators (dilation, erosion) is of fixed size – a 3x3 pixel square. The size of dilation etc. is controlled by iterations.

4.3 Instructions in short

- Load images multi_c1.tif and multi_c2.tif
- Find cells in channel 2:
    - Find threshold, set (around 28 is okay)
    - Set measurements for Area and Mean Gray Values

    - Do the particle analysis as in 3.2
- Convert the thresholded channel 2 to mask: Process – Binary – Convert to mask
- Duplicate the mask for some experimentation. (Image – Duplicate …)
- Dilate the mask:
    - Duplicate the image for comparison (Image – Duplicate…)
    - Process – Binary – Options: set iterations to 5
    - Process – Binary – Dilate
- **What was done here? What happens if you increase the number of iterations (use the duplicate)? What sort of information may be lost with more iterations?**
- Start Process – image calculator
    - Use AND command for the channel 1 image and the dilated mask created earlier
    - Move your mouse around the image and follow the number "value=nn" in the ImageJ window
    - **What do the intensity values tell you (Value=nn)?**
    - **What did AND operation do when in the other image, pixel intensity values are either 0 or 255 (binary), and in the other image, pixel intensity values can vary from 0 to 255 (8-bit image)?**
- Set threshold via Image - Adjust – Threshold. Select a range of 1-255 for the resulting image
- Do particle analysis as in 3.2
- Open the two measurement result files
- **How did the areas change?**
- **If you have time after the last task (the macro part), you can return here and try to analyze the ring around the nucleus (like at the lecture)**

4.4 Additional information

There are many other ways of transfering object information from one image into another image. One way is the ROImanager[1]. If you add particle analyzer objects into ROImanager, you can use the same ROIs in any other image. Simply activate an image and click measure in the ROImanager (Analyze – Tools – ROImanager). The manager will automatically open if you have chosen "Add to manager" in particle analyzer.

[1] ROI = Region of Interest. A sub-area of the image containing the area you are interested in. Here it is the boundaries of the particle.

5 Color thresholding

5.1 Your task

Thresholding based on something else than raw intensity data can be useful. In this part, the task is to segment the blue objects in the image. To find the best way of segmentation, you should play around with images, trying different methods and settings.

5.2 Detailed information

In principle, thresholding any information is intensity thresholding. The original data just have to be transferred into a suitable intensity range. The RGB-HSB transformation is one way of achieving this. You can try converting the image into an HSB-stack in Image – Type – HSB Stack. Then examine the image in three different layers – hue, saturation, and brightness.

5.3 Instructions in short:

- Load hist.tif
- Make a duplicate of the image so you can compare (as in 4.2)
- Start: Plugins – Segmentation – Colour based thresholding
    - Draw a square on one of the blue areas in the image
    - Click sample
    - **Explain what happened?**
    - The thresholding result should have small holes, and the background can have small garbage – they will be dealt with later on. Adjust the selection bars manually if sampling did not give optimal results (most likely it will not).
    - Mark thresholding on
- Duplicate the thresholded image and convert it to a mask (process – binary - convert to mask). This will convert the image to black and white based on the current threshold settings.
- Check that Process – Binary – Options is set to 1 iteration and 1 count
- Use open (or rather erode?) and dilate operations to enchance the objects. It depends on how your thresholded the image but probably first an open command, and then dilate once or twice will do it. Finally use the Fill Holes operation (in Process - Binary)
- **Explain what happened to the mask**
- Do particle analysis
    - Discard objects smaller than 100
    - Show: outlines
- Save the measurements as “hist.txt”

- To make comparison easier, change the “Drawing of hist” image into an RGB colour image (Image – Type – RGB). Then do an AND operation for the original image (hist.tif) and the Drawing (in Process – Image calculator)
- Compare the original image and outlines. Did you succeed in the segmentation?

6 Batch processing

6.1 Your task

Batch processing can speed up your analysis by automating parts of the process. In this part, you will create a macro to count the cells and calculate areas, circularities, mean intensities, as well as centroids in multiple images. ImageJ macros are not an easy option to start with but, once you get the basic principles, the rest is easier.

6.2 Detailed information

Making macros in ImageJ is like creating small computer programs. You can set variables, read them from functions, and do for example mathematic calculations with the parameters. All the built-in macro functions are listed at: http://rsb.info.nih.gov/ij/developer/macro/functions.html

The multithresholder uses automatic algorithms for calculating the thresholding level of image data. For this particular image, the Maximum Entropy seems to work best. Even though the algorithms are automatic, you still need to confirm visually which algorithm will give the best result. ‘Automatic analysis’ is not currently fully automatic – there is still a need for a human sensor.

6.3 Instructions in short

- Load image cell1.tif
- Start recording a macro: Plugins – Macros – Record macro
- Enter a name for the macro
- Start the: Plugins – Filters – MultiThresholder
    - Select Maximum Entropy and click set
- Go to: Analyze – Set measurements:
    - Measure: area, circularity, centroid, mean gray value
- **Can you guess why the background is segmented as objects, and the cells as background? Does it matter for your analysis?**
- Then go to analyze particles
    - Select Show: Nothing
    - Check Exclude at edges, display results, clear results, and include holes
    - Click ok
- Save your measurements in “E:\test.txt”
- Click create in macro recorder. You now have the macro in a text editor

- You have to edit the macro by hand:

Since the macro recorder only applies to the current image, you have to change the macro so that it is applicable in general case. Running the multithresholder will result in extra rows, and also the thresholding value is set to segment the background instead of objects. Here is a working example of how your macro should look like:

```
run("MultiThresholder", "Maximum Entropy");
getThreshold(lower, upper);
setThreshold(upper, 255);
run("Set Measurements...", "area centroid circularity mean redirect=None decimal=3");
run("Analyze Particles...", "size=100-Infinity circularity=0.00-1.00 show=Nothing display exclude clear include");
saveAs("Measurements", "E:\\" + File.name + ".txt");
```

The thresholding level is corrected by reading the lower and upper limits to variables called “lower” and “upper”. The original lower level is automatically set to 0. What you need to do is to segment the image from upper to 255 (setThreshold –command). Another addition is the measurement saving part. saveAs –command saves the numbers to a file denoted by a variable called “File.name”. “File.name” is the filename that was last opened in ImageJ, which means you have to do the analysis one image at the time to preserve the correct file name in the measurements file.

- Save your macro
- Close the recorder, all the images, and plugin windows
- Then try the macro:
  - Plugins – Macro – Install macro: select the macro file you just saved[2]
  - Open image cells1.tif
  - Plugins – Macro – [the name you gave for your macro]
  - Close the image
  - Check the ouput text (in e:\cell1.tif.txt)
- Repeat the macro command for cells2 and cells3
- **How accurate was the automatic thresholding level, compared to the visually set? After making the macro, you can also try the multithresholder on multi_ch2.tif – does it work with the same algorithm?**
- **Are the automatic analysis results and your manual analysis results different? (should be cell1_manual.txt and cell1.tif.txt)**
- **If you have time, you can return to 4.3 and try to analyze the ring around the nucleus (like at the lecture)**

[2] Note: If you change the macro contents, you have to install it again. Also, if you shut down ImageJ and start it up again, you have to reinstall the macro. Macros can be autoinstalled by using the “Startup Macros” menu.