**Practical work no. 11: Image analysis with ImageJ**

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**1. Purpose of the work**

Learn how stained objects such as cells are automatically counted in images.

**2. Background**

In image analysis, numerical data is extracted from an image. Numbers (and intensities) of objects of interest, for example, is a common area of analysis. Image processing is modification of an image to produce a visually enhanced final image. Image processing such as filtering is often used before image analysis to help segmentation. Before image analysis you will need to acquire an image. It is vital that you plan you image analysis before you take the image. Common problems concern background (there should be no difference in background intensities in different parts of the image ie. illumination correction should be made) and saturation (your objects of interest should have intensities within the measurable range ie. exposure time should be correct).

**3. Instrument and software used**

ImageJ is a free public domain Java program for image processing and analysis. It can be freely downloaded from Internet. Its functionality is largely based on plugins, either self-made, or available from various sources. In this session we will concentrate on basic image analysis in ImageJ without the use of separate plugins. ImageJ has an online help feature. It can be accessed from the ImageJ menu Help -> Online Docs... However, you will need an active web connection to access the documents.

**4. Sample**

In this session the sample is an image captured in the session number 2: "Fluorescence microscopy and image acquisition". U20S osteosarcoma cells were stained with Hoechst 33258 nuclear stain, which has very similar spectral properties to DAPI nuclear stain. We will count the cell nuclei.

**5. Task**

The task is to count the number of cells in the sample.

**6. Work instructions**

6.1.  ImageJ software is open. Connect your USB stick into the computer or connect to the server where your images were saved during the course. Open your DAPI (8-bit fluorescence RGB tiff) image that was captured during the work session 2. File -> Open ->


6.2.  Thresholding/Segmentation

Convert the RGB image into a gray-scale image: Image -> Type -> 8-bit

If you plan to count any particles you need to divide your image into two parts: 1) pixels that belong to objects and 2) pixels that do not belong to objects (= background). This is done by "thresholding" the image by setting all pixels above a certain intensity value (objects) to black and leaving everything else (background) white. This is called image segmentation.

Automatically: Process -> Binary -> Threshold (Fig. 1)
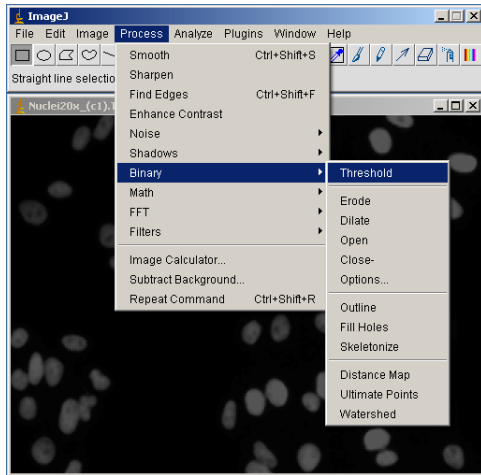

Figure 1. Thresholding automatically.
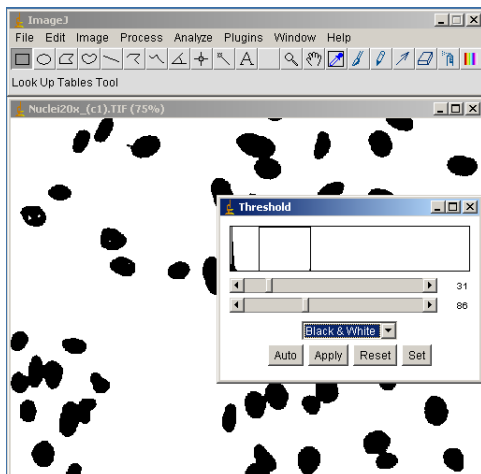
Manual: Image -> Adjust -> Threshold (Fig. 2)


Figure 2. Manual thresholding.

Test both methods (you will need to open the image again, do not save in between). Do you get same results? Continue with the automatically thresholded image.

6.3.  Watershedding

Some of your particles of interest are adjacent i.e. touching each other, and any automatic method of thresholding will recognize them as one object. They must now be separated using e.g. "watershedding". Watershed segmentation is a way of automatically separating or cutting apart particles that touch. It requires a binary

image containing black particles on a white background.  It finds pixels of maximum intensities in all objects and separates the objects at the minimum intensity between those points. Watershed segmentation works best for smooth convex objects that do not overlap too much. There are also other algorithms that can be used for separating objects.

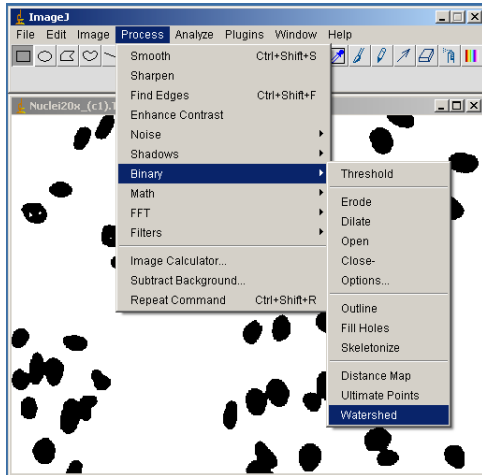Automatic: Process -> Binary -> Watershed (Fig. 3)



Figure 3. Watershedding.

6.4.  Counting objects

Automatic counting is based on different parameters (Fig. 4)

Open: Analyze -> Analyze particles
Size: What sized particles are counted? Leave "0-infinty" for now
Show: Outlines. This will create a separate image showing the outlines of the particles counted.
Exclude on edges.
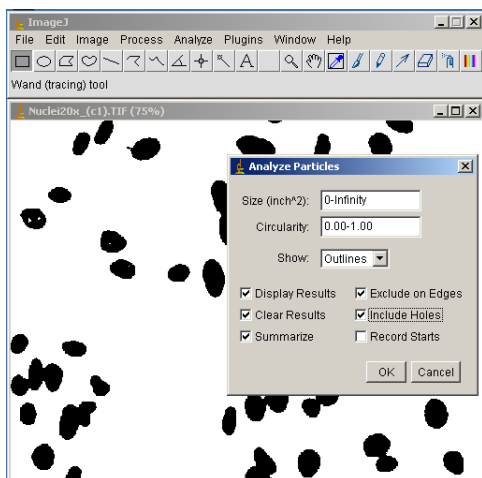Check all boxes except "Record Starts".
Click OK.



Figure 4. Counting settings.

Now you have the number of nuclei in your image, their outlines displayed in a separate window and numerical data displayed in two additional windows (Fig. 5). The number of objects is in the "Summary of ..." window on the line "Count: xx "

Experiment with different values for the size range. What are optimal values?
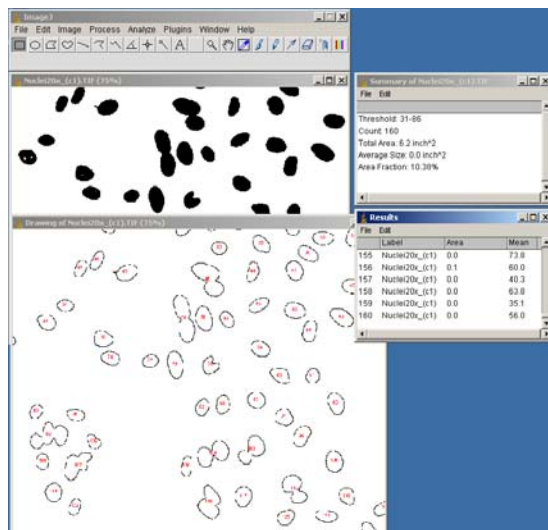


Figure 5. Summary of the cell counting values.

**Additional task**

Please write down your answers to the following steps as you will present them in the "summary" session.

1. Count the number of cells in the image above using the same methods as in the example. When you open the image you may realize that the background is uneven. You can remove this unevenness with the command:

Process -> Subtract Background...

This removes smooth, continuous variation in the background of images by measuring the intensities of background pixels just outside objects and subtracting this value from the objects' intensities and surrounding pixels. The "Rolling Ball Radius" defines the radius of the area this subtraction is performed on. This should be set just larger than the largest object of interest in the image. Use a Rolling Ball Radius of 50 and uncheck the "White Background" box.

2. As a more complicated task, you will count the number of specifically stained cells versus the total number of cells. For this, you need two images: one for total cell counting (DAPI), and one for counting positive cells (here GFP-labelled cells). You will need to do background subtraction as before on both images.

a. Count the total number of cells in the DAPI image

b. Count the number of cells in the GFP image. This is a "real-life" image, so you will need to use judgement as how to set the thresholding etc. In addition, some cells contain bright spots which make thresholding difficult. These kinds of problems can be addressed by filtering. For this exercise use simple blurring of the images. This blurs the active image or selection. This filter replaces each pixel with the average of its 3x3 neighbourhood.

Process -> Smooth

Note: you can use the Smooth function repeatedly for an increasingly blurred image.

c. Calculate the ratio of specifically stained cells / total number of cells.

Links:
Molecular Imaging Unit: http://research.med.helsinki.fi/corefacilities/Imaging/
ImageJ: http://rsb.info.nih.gov/ij/
WCIF ImageJ bundle: http://www.uhnres.utoronto.ca/facilities/wcif/download.php