**ImageJ/Fiji workshop**

1. **Loading images**

*To start with, you are going to load an example image stack into Fiji. This example data has 2 channels, which show nuclei (channel1) and cells (channel 2) across multiple focal planes.*

1. Open “ExampleData.lif” using the BioFormats reader
2. Use the sliders at the bottom of the stack window to change the focal plane and channel
3. **Adjusting brightness and contrast**

*Typically, the first thing to do when you’ve loaded an image is to change the brightness and contrast, so you can examine the detail. Contrast can be improved further by switching to a different lookup table (LUT).*

1. Select the focal plane with good focus on the nuclei (channel 1)
2. Use “Brightness/Contrast” to improve visibility of the nuclei. Try using the “auto” button within this control
3. Try applying different lookup tables (LUTs) to see how they affect the visibility of structures within the image
4. Use Zoom and Pan tools to examine image at higher zoom
5. **Selecting best images of the sample**

*Now you are going to use a couple of different approaches to extract the best images of the sample (in this case, nuclei). The first uses intensity projections (Z projection) and the second is to extract a single slice at the optimal focal plane. You’ll then choose which you think looks best – “best” in this case being bright nuclei with well-defined edges.*

1. Separate the image stack into two channels with the “Split Channels” tool
2. You don’t need the stack of cell images (channel 2), so it can be closed
3. Create a maximum intensity projection of the stack using the “Z Project” tool and rename it “MaxIP”
4. Extract the plane with best focus from the stack using “Make Substack” tool and rename it “Substack”
5. Choose which of the MaxIP and Substack images looks best (bright nuclei with sharp edges) and close the other image.
6. Save a copy of the “best” image (this is a backup for later)
7. **Subtracting background**

*You are going to compare removing background from your nuclei image using two methods: subtracting a rolling-ball background and subtracting the MinIP image.*

1. Create a minimum intensity projection of the stack and rename it “MinIP”
2. Duplicate the nuclei image from the previous exercise. You’ll want one for the rolling ball subtraction and one for minimum intensity projection subtraction
3. Select one of the duplicated images and run the “Background Subtraction” tool. Before clicking “OK”, select “Preview” and see what effect the different options have (in-particular the rolling ball radius). When you’re happy with the result click “OK”
4. Rename this image “Rolling ball”
5. Now, we’ll subtract the minimum intensity projection, so run the “Image Calculator” tool to subtract MinIP from MaxIP. Depending on the settings it will either write over the input image or create a new window entirely. Either way, rename the subtracted image “MinIP subtraction”.
6. You should now have 2 new images. Compare the results (you may need to adjust the brightness and contrast) and decide which shows the nuclei best. Save this image to your computer (with a new filename) and close the one you don’t want.
7. **Making basic measurements**

*Later-on it is necessary to know approximate nuclei sizes (areas). To do this, individual nuclei can be manually selected and measured.*

1. Open the “Set Measurements” options and make sure “Area” is ticked
2. Use an appropriate region selection tool (rectangle, oval, freehand, etc.) to highlight a single nucleus
3. Add this region to the ROI manager, then repeat for another 4 nuclei.
4. Use the ROI manager’s “Measure” function to measure all 5 nuclei ROIs simultaneously.
5. Use the Results Table “Summarize” function to calculate the mean nucleus area, make a note of this value and close the ROI manager and results window.

*The area will be specified in microns, because this is the distance-to-pixel conversion specified by the image metadata. The current distance conversion can be viewed, edited or removed at “Analyze > Set Scale…”*

1. **Image filtering**

*Thresholding and segmentation work best when noise is minimised. Next, you’re going to apply various filters to the image to investigate their effect. Some (e.g. median filter) preserve sharp edges in the image, while others are good for removing camera-induced noise (e.g. Gaussian filter).*

1. For each filter you apply, duplicate the original image and name it appropriately
2. Try applying different filters and varying the radius over which they act. Typically, filter option windows have a “preview” box, which updates the target image as you change parameters.
3. Decide which image has done the best job of supressing noise whilst maintaining sharp edges around the nuclei. Save this image to your computer (with a new filename) and close all the other images.
4. **Applying intensity thresholds for segmentation of nuclei**

*The next task is to threshold the image, so the nuclei are identified against the background. Thresholds can be set manually or automatically using a variety of algorithms; the effect of these can be evaluated using the “Try all” feature of the “Auto Threshold” tool.*

1. First, duplicate the image. This will be used later for intensity measurements of nuclei.
2. To get an idea how the threshold behaves, run the “Manual threshold” tool and adjust the slider. The threshold will be displayed in real-time on the image. Don’t apply the threshold, just close the threshold window.
3. Now, open the “Auto Threshold” tool and run using “Try all”. This will open a montage displaying examples of thresholding using 16 different algorithms. The names of each algorithm are displayed below each example (you may need to zoom in to see them).
4. Make a note of the algorithm which appears to have worked best.
5. Close the montage window and reopen the “Auto Threshold” tool. Select the best algorithm from the dropdown menu and click apply.
6. Going forward you’ll need an image with black nuclei on a white background. If necessary, images can be inverted using “Edit > Invert”.
7. Save the thresholded (“binarised”) image to your computer with a new filename
8. **Cleaning binarised images**

*Despite filtering, there will be places where there are either dark spots within a segmented nuclei or bright spots on the background. These can be corrected using the “Binary” tools. There will also be instances where separate nuclei have merged to a single object. These can be separated using the “Watershed” tool.*

1. Before running any binary tools, duplicate the image and name appropriately
2. Try applying various binary processes, such as “dilate”, “erode”, “open”, “close” and “fill holes”. It may be necessary to apply multiple processes to fix different features.
3. Once the binarised image has been cleaned, apply the “Watershed” tool to draw a line between separate nuclei that are in contact (as with all automated processes, the results may not be perfect).
4. **Analysing particles**

*Individual objects (nuclei) can be measured using the “Analyse particles” tool. Measurements can also be directed to another image; this is especially useful when measuring pixel intensities.*

1. Set the measurements to be collected (feel free to add as many as you like). “Redirect to:” should be set to the title of the non-binarised image; this is where the intensity values will be taken from.
2. Run the “Analyze Particles” tool. Set an appropriate size range based on the measurement you made earlier. Ensure “Display results” is ticked, then click “OK”.
3. The results will be displayed in a separate window. These can be copied and pasted into another program, such as Excel, for further analysis.
4. **Plotting and fitting data**

*We can now plot the data output by Analyze Particles and fit a curve to this. Here we will fit a Gaussian distribution to the nuclear areas. This requires creating the histogram with the Distribution function of results table, then plotting these points and fitting the curve.*

1. From the results table, use the “Distribution…” function to create a histogram of nuclei areas with 5 bins in the range 100-200 µm2.
2. The distribution plot doesn’t have a curve fitting option, so it’s necessary to get the histogram values and plot these as an XY plot. List the values from the generated histogram to get the bin start positions and the number of counts per bin.
3. To fit the histogram points we will have to set the new results table name to “Results”. Do this from File > Rename.
4. Plot the 5 histogram points as disconnected blue circles.
5. Fit a Gaussian curve to the 5 points. The fitting parameters should be shown in the Log window.
6. **(Optional) Recording macros**

*The macro recorder creates a record of the internal commands used to run each operation that have been implemented. This list of commands can then be re-run on different images. Making macros can be tricky, so the need to make alterations to the commands to correct errors or change image names are common.*

1. Close all open windows and images
2. Open the original image stack again (Section 1.)
3. Start the macro recorder
4. Repeat the steps for image processing and segmentation that gave the best result (i.e. don’t need to do both rolling ball and MinIP subtraction). The commands will appear in the recorder as processes are run.
5. Once complete, click “Create” to make the macro. This should open in a new window.
6. To test the macro, close any open images and reopen the original image. Run the macro using the “Run” button. This should (hopefully!) repeat the process.