

IMAGE HANDLING USING IMAGEJ / FIJI

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WHAT IS FIJI AND HOW TO GET IT?

Fiji is an open-source image processing and analysis software, it can be downloaded from here: <https://imagej.net/software/fiji/downloads>. More detailed information about the usage of Fiji and tutorials, please check the Fiji Wiki pages: <https://imagej.net/>.

Download Fiji and extract it according to the instructions on the <https://imagej.net/software/fiji/downloads> site. If working on PC do not store Fiji in programme files. Start Fiji from the Fiji/ImageJ icon. The software will ask if you want to update the software – select *Yes, please*. Restart Fiji.

HOW TO OPEN IMAGES?

Open the software and click *File -> Open*. Choose the file from your computer and click *ok*. Images can also be dragged and dropped from folder directly to Fiji.

HOW TO DUPLICATE IMAGES?

It is good practice to always start with duplication of your original image and work with the duplicate. That way you don't accidentally override the original image.

1. Open sample image "20x nucleus_high contrast" from the course package
2. Select image and click *Shift + d*

HOW TO EDIT THE BRIGHTNESS AND CONTRAST?

1. Select the image you just duplicated and choose: *Image -> Adjust -> Brightness/Contrast*. You will get a pop-up window with possibility to set the minimum and maximum gray values, the brightness, and the contrast. Auto will give you settings that the software thinks are the best; it applies an intelligent contrast.
2. If the Auto button does not produce a desirable result, use the region-of-interest (ROI) tool to select part of the cell and some background and click Auto. The stretch will then be based on the intensities of the ROI.
3. You can further modify the values by moving the bars. This can be done each channel separately.
4. The *Reset* button sets the values that were in the image when opened.
5. The true values from 0-255 (8-bit images) or 4095 (12-bit images) can be set manually from *Set* button.
6. Remember to consider if you want to click *Apply*. Pressing the *Apply* button permanently changes the actual grey values of the image. If you are analyzing image intensity **do not press this button**.

GAMMA

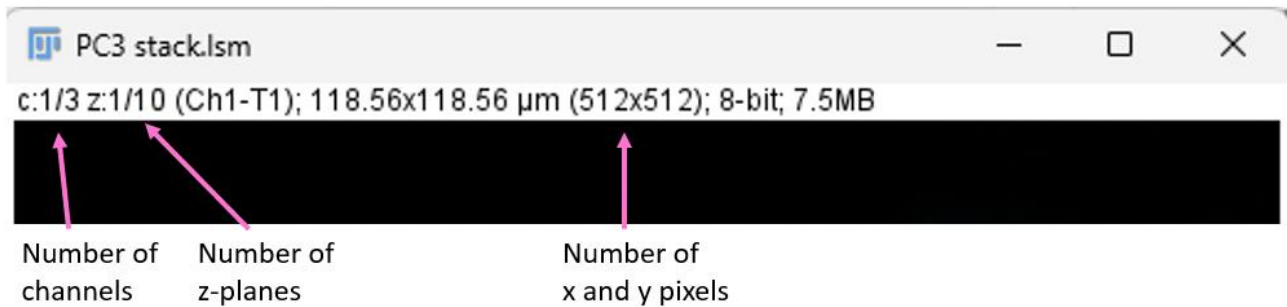
Gamma performs a non-linear histogram adjustment. If you have very faint images, you can enhance the lowest intensities with using gamma correction. Editing of gamma is considered image manipulation and is not generally accepted. If you must use gamma correction, you must mention it in the materials and methods.

Process -> Math -> Gamma...

IMAGE DIMENSIONS

An image can consist of pixel elements = pixels. Typically, they form a two-dimensional matrix. Images can also have additional dimensions, typically in microscope images there is z-dimension, time, and channels.

1. Open sample image "PC3 stack" from the course package.
2. View the dimensions at the top of the image.



3. View different dimensions by using the scrolling bars below the image.
4. Channels can be controlled through the Channels tool: *Image* › *Color* › *Channels Tool...*
5. If you want to change a channel color, change the Look up Table (LUT): *Image* -> *Lookup Tables*

HOW TO VIEW CHANNELS?

You can easily toggle channels by using the the channels slider below the image. You can also turn channels on or off using the channels tool *Image* › *Color* › *Channels Tool...* . Composite means that channels are overlayed.

HOW TO SPLIT AND MERGE CHANNELS?

Splitting of channels is needed for example when you want to delete a certain channel. Merging might be necessary when you want to prepare the image for publication or presentation.

1. Open sample image "PC3 stack" from the course package
2. Split the channels: *Image* -> *Color* -> *Split channels*
3. Merge channels: *Image* -> *Color* -> *Merge channels*. You can choose the color for each channel. Remember to choose Ignore source LUT if you want to change the colors. You can also keep the source images (recommended), so that you can then redo the merging in case something goes wrong.
4. You create a regular or a composite merge image. The regular will merge the three channels and create an RGB-image. The composite image will keep all three channels separate, and it can be again split: *Image* -> *Color* -> *Split channels*. RGB images can be split to three channels (with reservations!): *Image* -> *Type* -> *RGB Stack*

INTENSITY PROJECTIONS

Intensity projection are a way to visualize 3D stacks in one plane. For example maximum intensity projection creates an image in which pixels contain the maximum intensity value of all images in the stack at the pixel location. Only in special cases maximum intensity projections are recommended for analysis.

1. Continue with one channel from "PC3 stack" from the course package
2. *Image -> Stacks -> Z-project...*
3. You can choose from which slide to start and finish. This way unwanted frames can be removed.
4. What other projection types can you find?

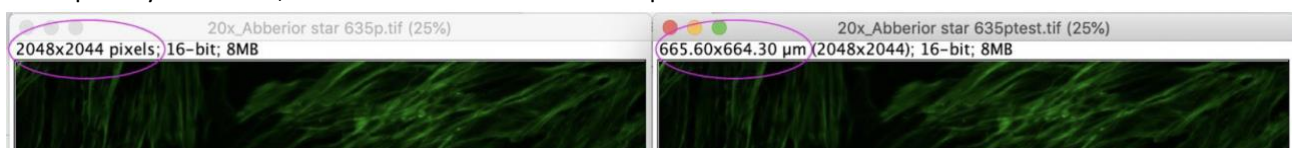
IMAGE SUB-SETTING

Sometimes an image needs to be rescaled.

1. Open sample image "20x nucleus_high contrast" from the course package
2. *Image -> Scale...*
3. Choose the scale in x-, y- or z-dimensions, 1 means the original, 0.5 is half of width in pixels (x and y) or number of slices (in z). Alternatively, you can also decide the exact pixel amount in x and y and the number of layers in z.
4. Try which interpolation methods looks best with your image.
5. You can use averaging when downsizing if needed.
6. It is recommended to create a new window (*Shift + d*). This way the original will also stay open and if you are not happy with the result, the settings can be redefined from the original image.
7. Last, choose a name for your file and click *Ok*.
8. Examine how this affected the image size and dimensions.

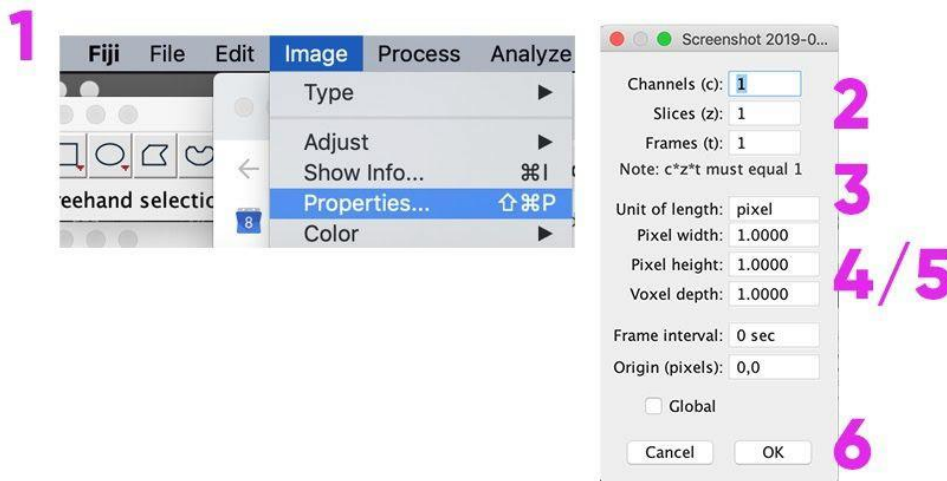
IMAGE CALIBRATION

It is important that the image file is handled properly by the and the size of the image calibrated, ImageJ doesn't always read the files properly. You can see if your image is calibrated from the from the info bar at the top of the image. If the image is calibrated, there will be μm or some other spatial unit next to the numbers. If the image is NOT spatially calibrated, the dimension will be labelled "pixels".



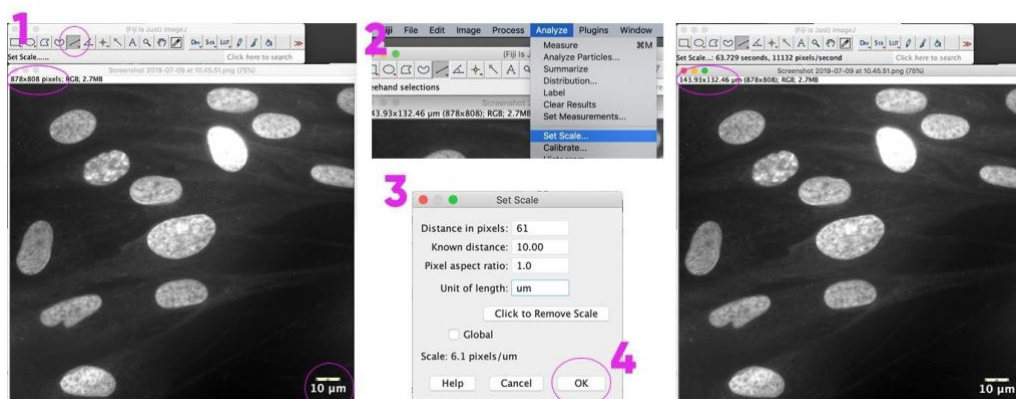
1. Open sample image "20x nucleus_high contrast" from the course package
2. Go to *Image -> Properties* to see the image settings
3. Check that the number of channels, slices, and frames match.
4. Check your unit of length and set it to μm (μm) or nm

5. Check your pixel width and height (can be usually retrieved from the metadata *Image -> Show info...* Look for PhysicalSizeX. The units are in μm .)
6. Go to *Image -> Properties* and change the pixel size
7. In 2D images the voxel depth can be ignored
8. Click *Ok*.



If you know the size of a feature (for example, a previously applied scale bar) you can use that to apply a calibration. In most image analysis software, it is possible to draw a line along the length of the known feature or scale bar and define the length and unit of the line and then apply that to the whole image. In ImageJ:

1. Using the line selection tool, draw a line along the length of the feature or scale bar.
2. Run the menu command *Analyze -> Set scale...*
3. Enter the dimensions of the object/scale bar in the "known distance" box and set the units in the "Unit of length" box.
4. Do not check Global unless you want all your images to have this calibration. Click OK.



HOW TO ADD A SCALE BAR

1. Continue with image "20x nucleus_high contrast" from the course package
2. Go to *Analyze -> Set Scale* and make sure that your image is calibrated and that the unit of length is set to μm . Click *Ok*.
3. 2. Go to *Analyze -> Tools -> Scale bar*. Set the length of the scale bar (in μm) and other settings if needed. If you intend to save the image in tiff format, remember to untick the overlay box. It will create another image layer. If saving jpeg, you can leave the box ticked, the layer will be merged with the image when exporting. Click *Ok*.

Note! Remember to do all the other modifications (e.g., merging/adjusting brightness and contrast/maximum projection) first and add the scale bar to the final image before exporting.

HOW DRAW ON THE IMAGE

To draw a line or a box on the image use the line or rectangle ROI tool from the toolbar. To stamp the line or box click *Edit -> Draw* (shortcut: d). If you're drawing the ROI on a stack, you'll be asked if you want to process all of the slices. Choosing 'yes' will draw the ROI on every slice while choosing 'no' will result in the ROI only being added to the current frame. If you use 8-bit etc images the line color will be defined by your LUT max value (in grayscale color will be white). If you want a colorful line or a box, the image must be first transformed into RGB image *Image -> Type -> RGB Color*, the color is determined by the color picker toolbar button. By double clicking the color picker button you will get a color palette. The width of the line in pixels is defined in *Edit -> Options -> Line width*. The line width must be determined before it is drawn on the image.

Adding text is done with the toolbar's text button. Double click on the button to set the font, font style, justification, font color, background color, font size and angle that the writing will be. Antialiasing can also be turned on/off here. Click the toolbar, click the image, and write the desired text. To stamp the text to the image click *Edit -> Draw* (shortcut: d).

Adding an arrow can be done by first adding the arrow drawing tool to ImageJ tool bar: *Image -> Annotate -> Arrow*. Select the arrow tool and start drawing it in an image. By double clicking the arrow button it is possible to edit the arrow. The color can be edited *Edit -> Options -> Colors -> Foreground color* and line width can be edited *Edit -> Options -> Line width*. The Foreground option in the latter will change the color of the arrow. To stamp the arrow to the image click *Edit -> Draw* (shortcut: d).

IMAGE TYPE CONVERSION

1. Converting to 8-bit from 16-bit: *Image -> Type -> 8-bit*
2. Converting to RGB: *Image -> Type -> RGB Color*

Note! Pay attention to the image bit depth. Regular image viewer programs (power point, paint, preview) are not able to read images with large bit depths. If the size of the image is 16bit, it must be converted to an 8-bit (*Image -> Type -> 8-bit*) or RGB (*Image -> Type -> RGB Color*) image before saving.

IMAGE SAVING AND EXPORTING

1. Saving images: *File -> Save as -> tiff/jpeg....*

Saving images for storage: always keep the original image data and if possible, the whole image processing and analysis workflow (macro etc). It is generally recommended to work on a duplicate of your original data to avoid any possible loss of data.

Saving images for publication: each journal has or at least should have their own image guidelines, that should be strictly followed. It is recommended to develop a workflow that is flexible and can handle a variety of formatting rules, such as 300dpi or 600dpi, raster or vector, margins, or no margins. Generally, decisions affecting the final figure should be set back in the workflow as far as possible so that starting the image editing from scratch is not necessary. The final figure files that are provided to the journal editor will be further processed regarding of where the image will be published (online/print). It might not be possible to control the figure quality loss at the journal, but it is possible to make sure that the files given to them are as high-quality as possible.

Exporting images for screens: When exporting for screens the actual screen pixels should be considered instead of dpi. Almost every monitor in the world consists of at least 1024 x 768 pixels – some monitors some are bigger, and can show you more. Most monitors are set at 72 dpi. That means if you took out a ruler and held it up to your monitor, each inch would have 72 little dots of light. Regardless of how many inches and how much resolution your image has, most of the time images that appear on web pages are going to expand or shrink to be 72dpi. If you were to view a 600 dpi image on the screen, your monitor will basically decide which 72 dots in each inch would best represent the 600 that are really there. Therefore, having higher resolution for PowerPoint or web graphics will do you no good – it will just make your slides or pages larger and they take longer to load.

High quality images are great for PowerPoint presentations, but they make file sizes huge. For most screens, a normal sized image (10 cm x 15 cm) at 150 dpi should be sufficient.

HOW TO USE THE 3D-VIEWER

2. Open sample image " PC3 stack" from the course package
3. Do any adjustments and merging needed first (split, merge, adjust brightness...)
4. When your image is looking good, go to 3d-viewer: *Plugins -> 3D viewer*
5. Choose suitable settings for your purpose and click Ok.
6. Show coordinates or surrounding box: *Edit -> Show...* Make sure that Show contents is ticked. This shows your image.
7. Practice a little how the viewer responses to mouse movements.
8. You can take a snapshot of the view: *View -> Take snapshot*
9. When you are ready to do recording go to *View -> Start Freehand Recording*
10. Move the image to your preference.
11. When ready go to *View -> Stop Freehand Recording*
12. You can now play the movie in a new window (first rendering is slow).

13. Exporting: *File -> Save as -> AVI...*

14. Choose compression (PGN is generally good for online publishing) and frame speed (24 fps the universally accepted film frame rate).

15. Choose saving location and you're done!

SEGMENTATION AND PARTICLE ANALYSIS

Image **segmentation** is the classification of pixels in an image as “pixels of interest” vs “background”. Separating a digital image into multiple segments. Assigning a label to every pixel in an image so that pixels with the same label share certain visual characteristics. Thresholding is the most common method to segment. -> results a binarized image

Global thresholding works by choosing a general cut-off value. Works well if all portions of the image have same quality and signal to noise/background, and all particles/objects have similar signal levels.

Local thresholding is computed for each pixel according to the image characteristics within a window of radius r around it

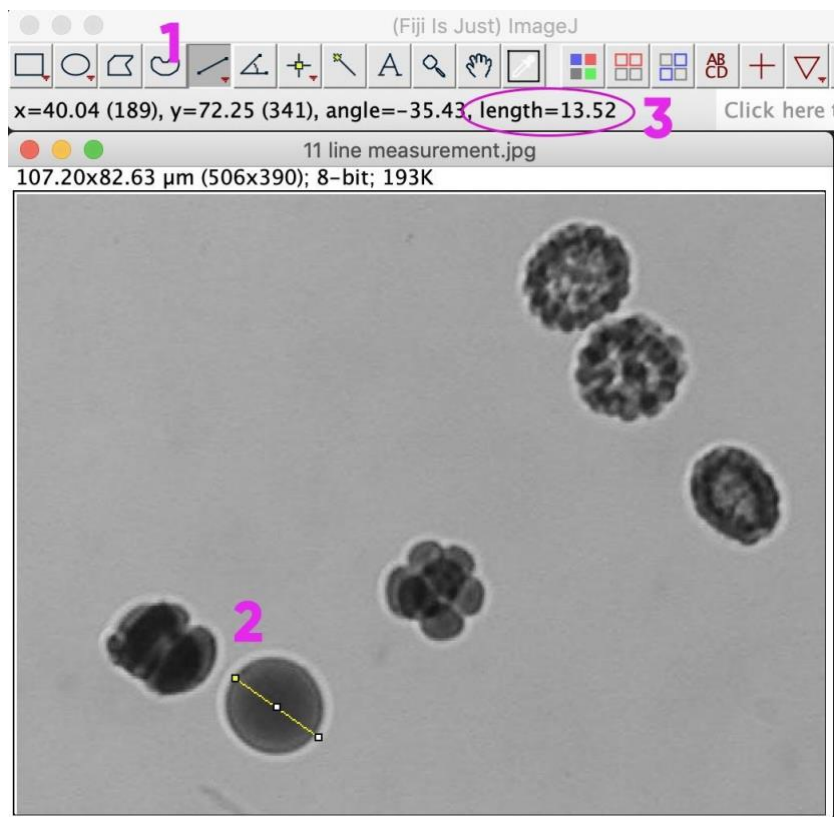
To try thresholding, try the auto-threshold plugin. It gives an overview of different thresholding methods. (This plugin binarizes 8 and 16-bit images using various global thresholding methods.)

NOTE: For historical reasons, ImageJ was setup to analyze dark particles on white background. Most analysis these days is of fluorescent particles/organelles on black background! This (still!) creates some problems during workflows, as IJ needs to know whether to aggregate bright or dark pixels for particle detection. -Re-set by *Process -> binary -> options-> “black backg”*. Always check image LUT during workflow! - some commands invert LUTs for display purposes. In the next exercise, LUT is inverted...check! -to “re-invert” LUT: *image -> Look Up Tables -> invert LUT*. (* inverting LUT is NOT the same as inverting image - it doesn't affect gray levels!)

EXAMPLE 1: SIMPLE LINE MEASUREMENT

To measure a distance in ImageJ, you can use the straight line tool. Make sure that your image is correctly calibrated first.

1. Select the straight line tool
2. Draw a line across the desired distance
3. Read the length of the line below to ImageJ toolbar or click m from your keyboard to print the result. You can also read the last coordinate of the line as well as the angle from the same place.



EXAMPLE 2: MEASURE THE SIZE DISTRIBUTION OF NUCLEI

1. Open sample image "20x nucleus_high contrast" from the course package
2. Open image & check properties (LUT state: *Process -> binary -> options...*)
3. Check if image has been calibrated *Image -> Properties*. If not, calibrate image (see Image calibration).
4. Segment *Image -> Adjust -> Autothreshold...*
5. Choose best thresholding algorithm *Image -> Adjust -> Threshold*. If the selection is wrong, tick *Dark background*. Don't click "apply" - it will change image into binary!
6. Setting the measurement parameters before measuring *Analyze -> Set Measurements*
 - Size - area, centroid, perimeter, Feret & "shape descriptors"...
 - Content - (gray values): mean, stdDev, minMax, mode...
 - Others...
 - Shape Descriptors:
 - Circ.(circularity): $(4\pi \cdot \text{area} / \text{perimeter}^2)$ 1.0 = perfect circle. Values may be unusable for small particles (few pixels)!
 - AR(aspect ratio, Fit Ellipse): the ratio of the width to the height of an ROI
 - Feret'sDiameter- Longest distance inside ROI/particle (aka "maximum caliper"). Includes also Angle (0-180 degrees) to horizontal. MinFeret= diameter perpendicular to mid Feret
 - Integrated Density- The sum of the values of the pixels in the image or selection.
 - Read more: <https://imagej.nih.gov/ij/docs/guide/146-30.html>
7. Analyze & add to ROI manager *Analyze -> Analyze Particles...* ("Filter size": 20-Infinity & Circularity: 0.5-1.0 - Display...Clear...add To...Exclude...Include... Why use these options? See here: <https://imagej.nih.gov/ij/docs/guide/146-30.html>)
8. Check distribution of measured parameters: from results window: *Results -> Distribution...*
9. *Save results* from results window: *File -> Save As...*
10. You can do simple plotting using the Results table: *Results -> Plot...* How does the area of nucleus affect its roundness? You can fit the data by using *Data >>* button from the *Plot of results* -window.

EXAMPLE 3: MEASURE THE SIZE DISTRIBUTION OF NUCLEI USING STARDIST

If you have an image with low contrast, it is not possible to segment it easily using the above-mentioned workflow. StarDist is an ImageJ/Fiji plugin for a cell/nuclei detection for microscopy images. The plugin can be used to apply already trained models to new images. Currently, the plugin only supports 2D image data.

Documentation: <https://imagej.net/StarDist> and <https://github.com/mpicbg-csbd/stardist>

StarDist needs to be installed on your Fiji. Help -> Update... . an update window will open. Click *Manage update sites* -button and select *StarDist* and *CSBDeep* from the list. Click *Close* and then *Apply changes*. Downloading starts. When done, restart Fiji.

11. Open sample image "20x nucleus_low contrast" from the course package
12. Adjust brightness and contrast, split channels and calibrate image if needed.
13. Try segmentation using the tools from Example 1 – what do you observe?
14. Start the plugin from *Plugins -> StarDist -> StarDist 2D*, dialog window opens.
15. Select a neural network model from the dropdown list, *Versatile (fluorescent nuclei)* works the best in this case
16. Adjust the postprocessing parameters
 - *Probability/Score Threshold* - higher values lead to fewer segmented objects, but will likely avoid false positives. Default setting 0.50 works well, you can try others.
 - *Overlap Threshold* - higher values allow segmented objects to overlap substantially. Default setting 0.40 works well, you can try others.
 - The segmented objects can be returned as a *Label Image* or in the *ROI Manager* (or both).
17. Click *Ok*.
18. *Analyze -> Analyze Particles...* you can use the same parameters as in the Example 1 ("Filter size": 20-Infinity & Circularity: 0.5-1.0 -Display...Clear...add To...Exclude...Include...)
19. Check distribution of measured parameters: from results window *Results -> Distribution...*
20. Try different plotting options> from results window *Results -> Plot...*

EXAMPLE 4: MEASURE SIGNAL INTENSITY IN NUCLEI

Continue from part 7. from the Example 1:

21. You have now all the objects in the ROI manager. Click *Measure* on the left – you will get a table of results.
22. Save results table *File -> Save As...*
23. To view the ROIs on the original image, open your thresholding options again *Image -> Adjust -> Threshold* and hit Reset.
24. To save generate an image with the ROIs and original image on the background click *Flatten* on the ROI manager. If you are unhappy with the ROIs, you can change them in *Properties...* in the ROI manager.

EXAMPLE 5: MEASURE SIGNAL INTENSITY OTHER CHANNEL

Continue form part 18 in example 2:

25. Once you have your ROIs in the manager activate the image with nuclei. Click *Measure* on the ROI manager. You get a result table with the values selected.
26. To get the values from the other window, select it. Untick and tick again the box *Show all* in the ROI manager. Click *Measure*.