FIJI (ImageJ)

5/11/20

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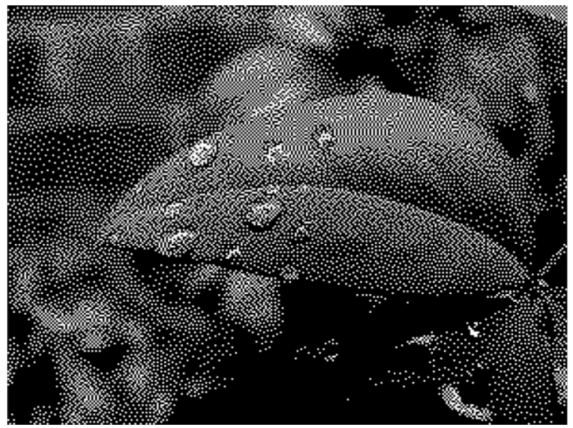
Adapted from QFM 2019 slides

Bit Size



1 bit (2 shades)

Bit size = Shades of gray a picture can display

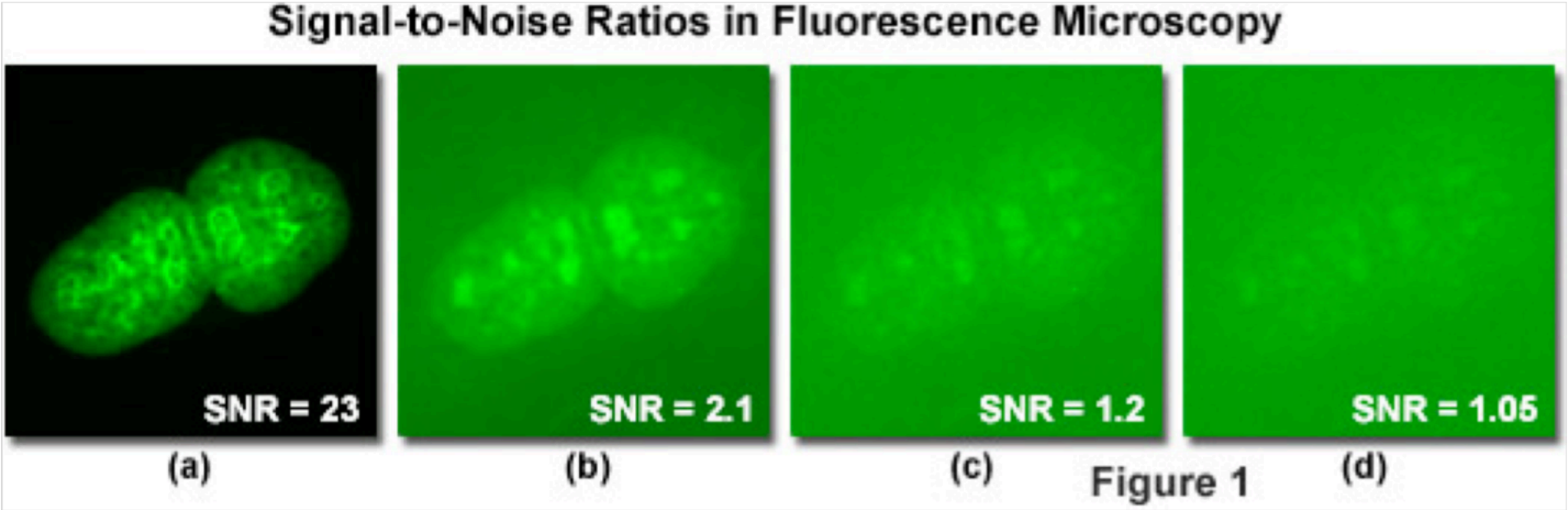
Bit size is expressed as 2^n

2 bit image = 2^2 = 4 shades
4 bit image = 2^4 = 16 shades
8 bit image = 2^8 = 256 shades

2 bit (4 shades)

8 bit (256 shades)

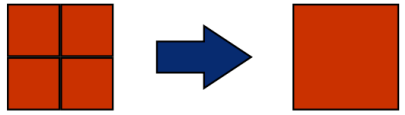
Signal to Noise Ratio



Low Signal to Noise ratio can often be a more severe limit on resolution than diffraction

Increasing SNR by Binning

This should only be done if you want to increase if you're looking at qualitative data or tracking. If you have massively oversamples, often you can bin your data and reduce your file size



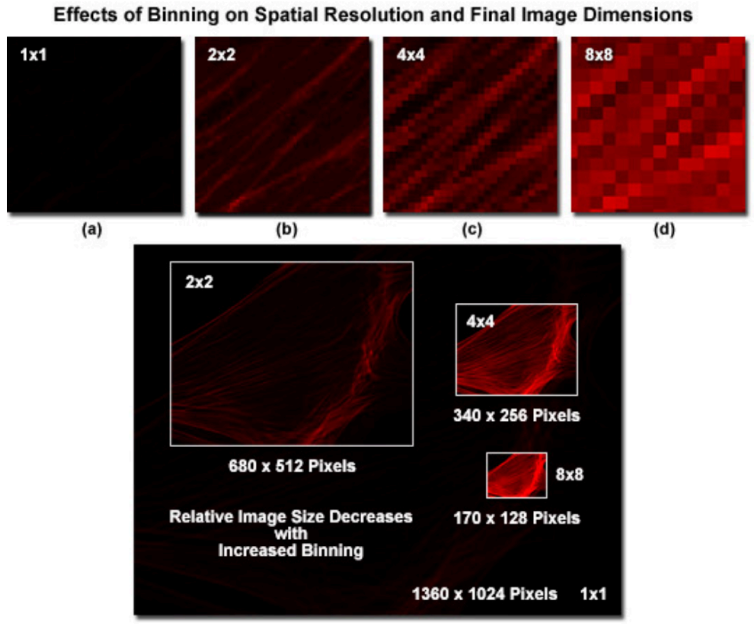


Image > Type > 32 bit
Image > Transform > Bin

2X2 binning will result in: four-fold improvement in signal
two-fold loss in resolution
two-fold increase in signal-to-noise ratio

Always Save Files as TIFF or PNG

TIFF

- Supports grayscale, indexed and true color images
- Can contain a number of images with different properties
- It is possible to store a number of variations of an image in different sizes and representations in a single TIFF file.

PNG

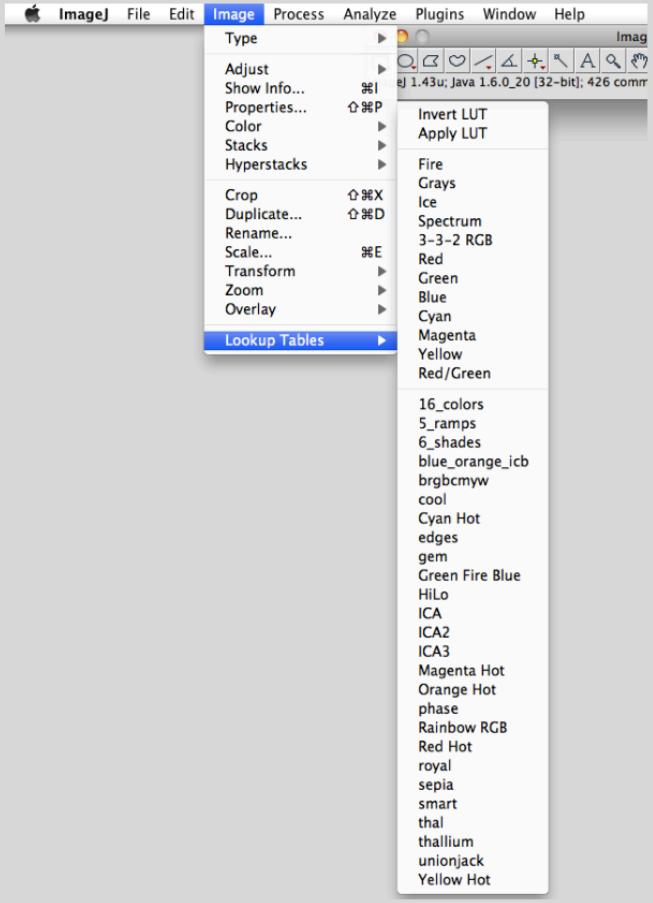
- Designed mainly for use on the **internet**
- Supports three different types of images:
  - True color (up to 3 X 16 bits/pixel)
  - Grayscale (up to 16 bits/pixel)
  - Indexed (up to 256 colors)
- Provides a PKZIP compression, no lossy compression is available

Add Color to Images

Color images are generated from the array of pixel values based on Look Up Table (LUT)

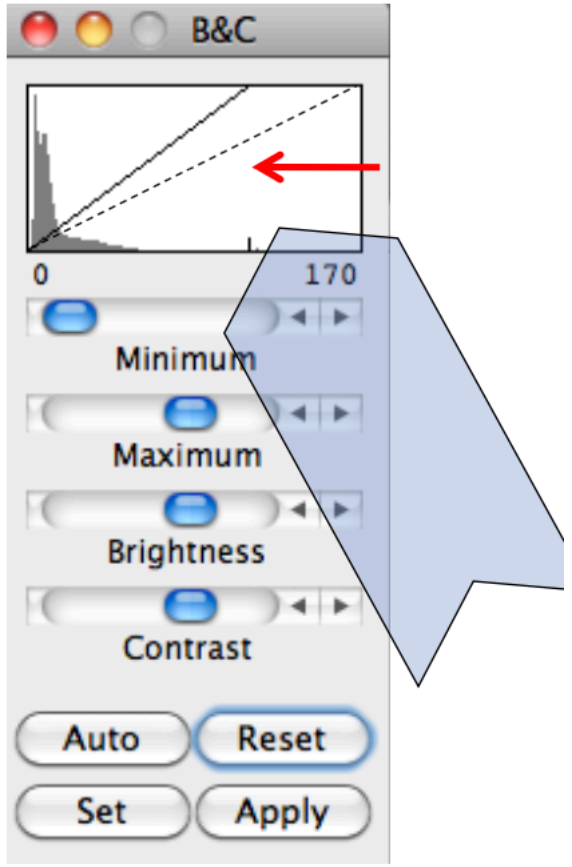
Try changing the color of your three “hela” monochromatic images.

Image > Type > 32 bit
Image > Lookup Tables > Color of choice



Know What You're Changing

- Adjusting display min/max = change individual pixel value without changing ratio
- Gamma = non-linear adjustment, it will preferentially affect the pixel values
- Image filtering is performed on **pixels** (smallest unit of digital image data) and *it will alter the original data embedded in the image*.

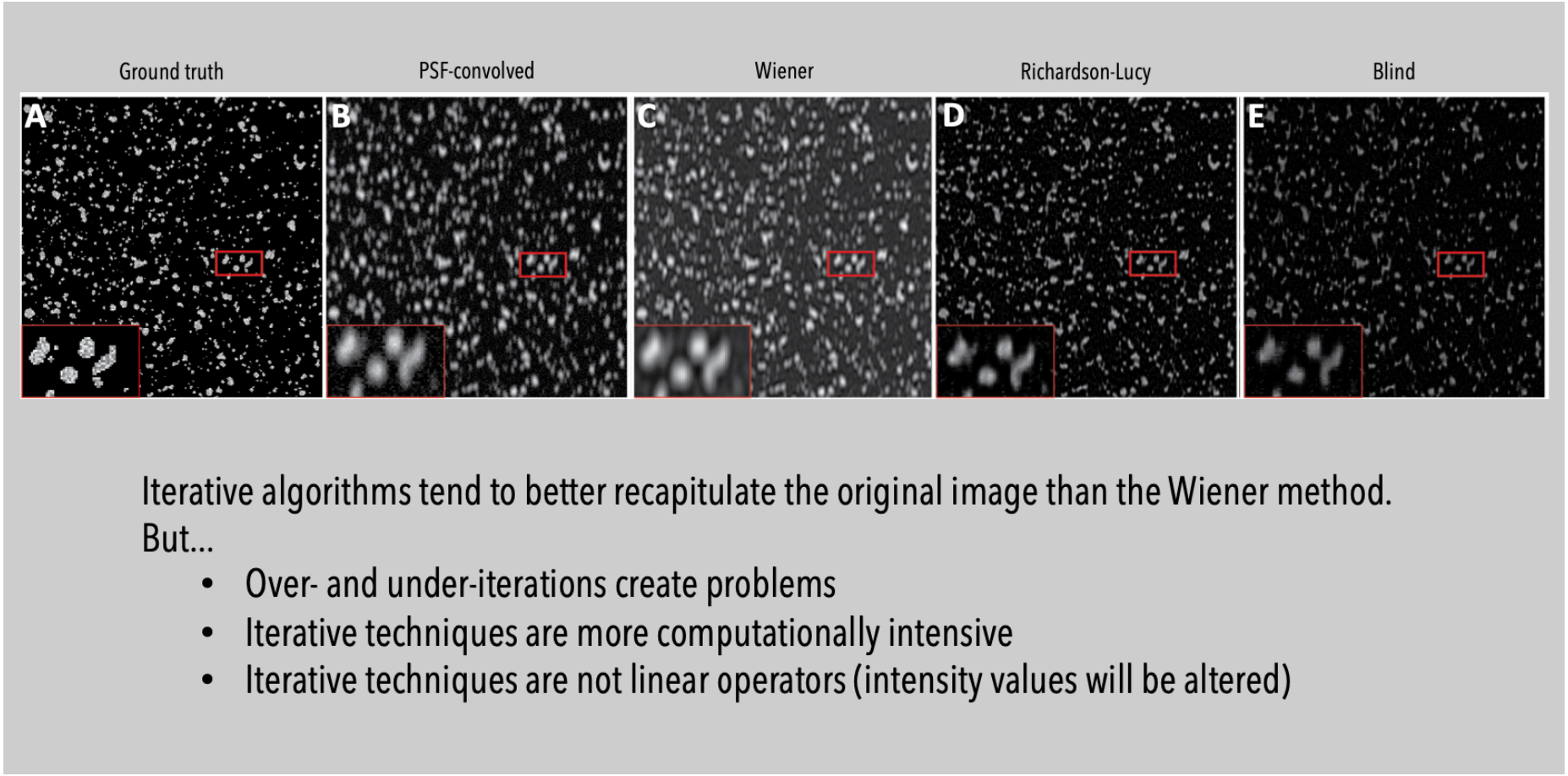


Why Use the Blur Filter?

Original Image

Process > Filter > Gaussian Blur
(sigma = 12 pixels)

Deconvolution



Iterative algorithms tend to better recapitulate the original image than the Wiener method.
But...

- Over- and under-iterations create problems
- Iterative techniques are more computationally intensive
- Iterative techniques are not linear operators (intensity values will be altered)

Wiener Filter: Divide Fourier transformed PSF with the Fourier transformed of the acquired image

Advantages:

- Relatively quick compared to other Fourier-based methods
- Linear process (good for data processing and quantification)

Disadvantages:

- Amplifies noise
- Breaks down with inaccurate PSF

Richardson-Lucy Filter: Estimates the deconvolved object (with certain constraints), blurs the estimate with PSF, and compares to raw data. Applied multiple times to arrive at final deconvolved image.

Advantages:

- Generate sharper images with less noise amplification

Disadvantages:

- Slower
- Non-linear process (relative pixel values are not maintained!)

Nearest Neighbor Deblurring: Blur the planes above and below current Z plane, subtract blurred planes from Z.

Advantages:

•Computationally simpler

•Disadvantages:

•Less accurate

•Noise from neighboring planes is added into the current Z plane

•Subtraction reduces overall signal

Local Intensities Require Finding Edges

- Successful background removal (denoising, deconvolution) brings out the desired fluorescent signals, but this is not enough.
- The intensities of biological targets are usually not homogeneous, and the boundary of biological targets may be difficult to define. So relying on absolute intensities is not sufficient for target identification.
- One important attribute of the image that could help in target recognition is **local intensity differences**
- Target recognition is usually performed by detecting the **discontinuities in intensities**

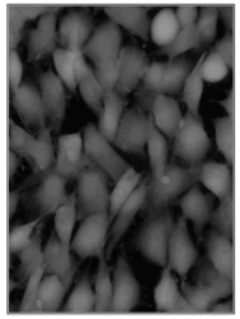
Process > Find Edges

Measure Fluorescent Intensity to Show Protein Expression Levels

Before you start – ask yourself these questions:

- Do you need to normalize fluorescent intensity to an area of interest? e.g. More cells expressing the protein at the same level vs. same number of cells expressing a higher level of protein.
- Do you need to differentiate whether the fluorescent intensity increase is due to changes in tissue biology (e.g. hypertrophy) and not necessarily protein expression level?
- If you only care about total amount of the protein of interest within the tissue – do you need imaging?

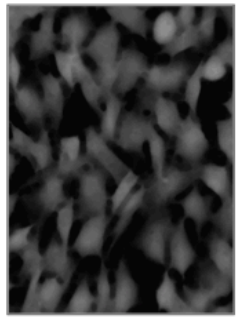
Steps for Fluorescent Intensity Measurements



Original image

Tight spaces – burring will not help

Need to find a way to highlight the thin, dark borders right away

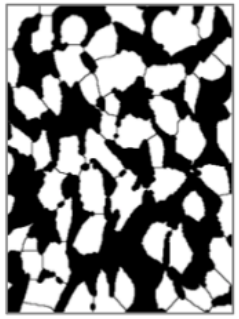


Filtering

The “minimum” filter widens the cell-cell gap and lower the intensity values

Applying Threshold

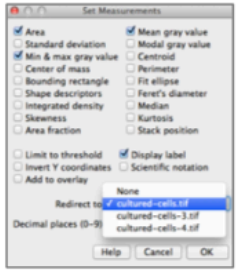
Level to be determined empirically. Strike the balance between better segmentation and better cell area coverage



Segmentation

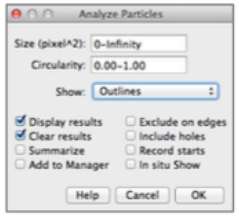
Watershed

At this point, you have segmented your objects, but you need to find a way to bring these digitized masks back to the original microscopy image.



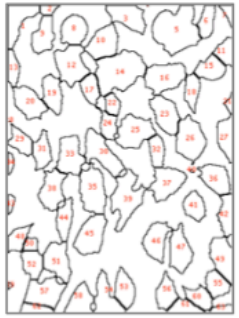
Segmentation

The key is to redirect the measurement to the image you want to measure, under “Set Measurements”



Analyze

You can further gate the size and shape factor for what you want to measure at this point.



Voilà!

Co-Localization

Step 1: We need to clean up uneven illumination in the background.

Click on C1.

[Process -> Subtract Background]

Set rolling ball radius to 50. Hit OK.

Repeat on second image.

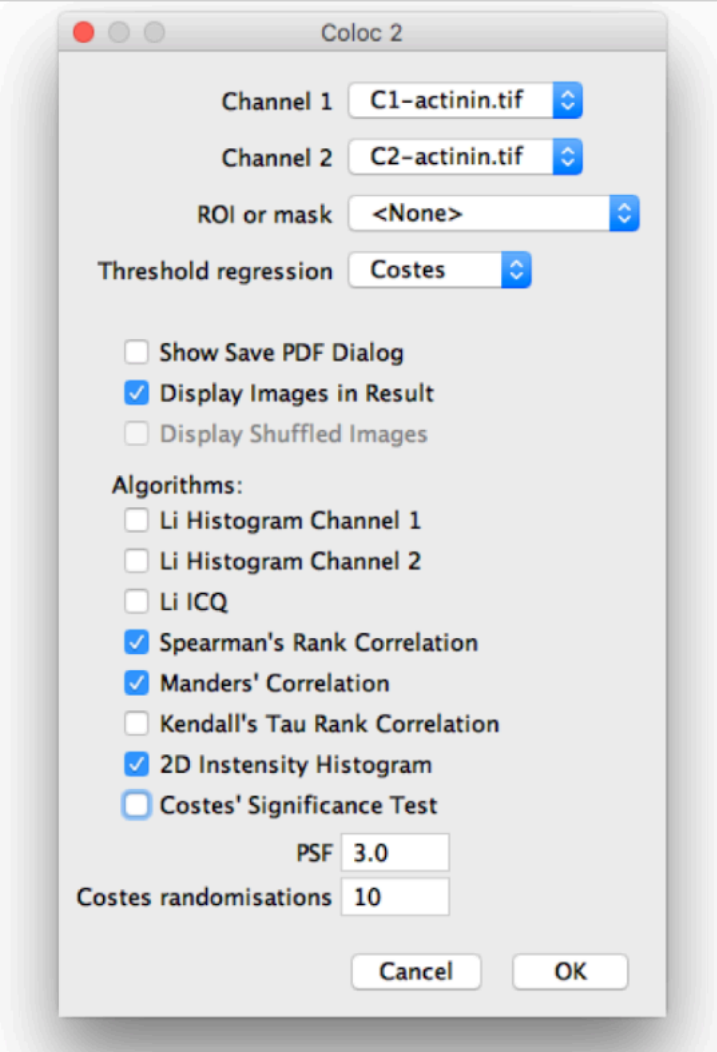
Step 2: Run colocalization.

[Analyze -> Colocalization -> Coloc 2]

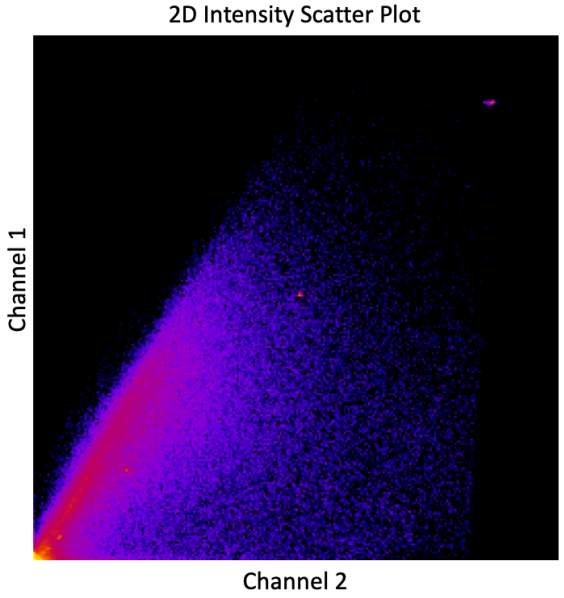
Set Channel 1 to C1, Channel 2 to C2.

Make sure these boxes are checked, as sh

Hit OK.



Name	Result
% zero-zero pixels	10.75
% saturated ch1 pixels	0.00
% saturated ch2 pixels	0.00
m (slope)	0.82
b (y-intercept)	2.00
b to y-max ratio	0.01
Ch1 Max Threshold	1.00
Ch2 Max Threshold	3.00
Pearson's R value (no threshold)	0.70
Pearson's R value (below threshold)	0.02
Pearson's R value (above threshold)	0.61
Manders M1 (no threshold)	0.988
Manders M2 (no threshold)	0.996
Manders M1 (threshold)	0.988
Manders M2 (threshold)	0.994
Costes P-value	1.00
Costes Shuffled Mean	-0.00
Costes Shuffled Std.D.	0.02
Ratio of rand. Pearsons >= actual Pearsons value	0.00



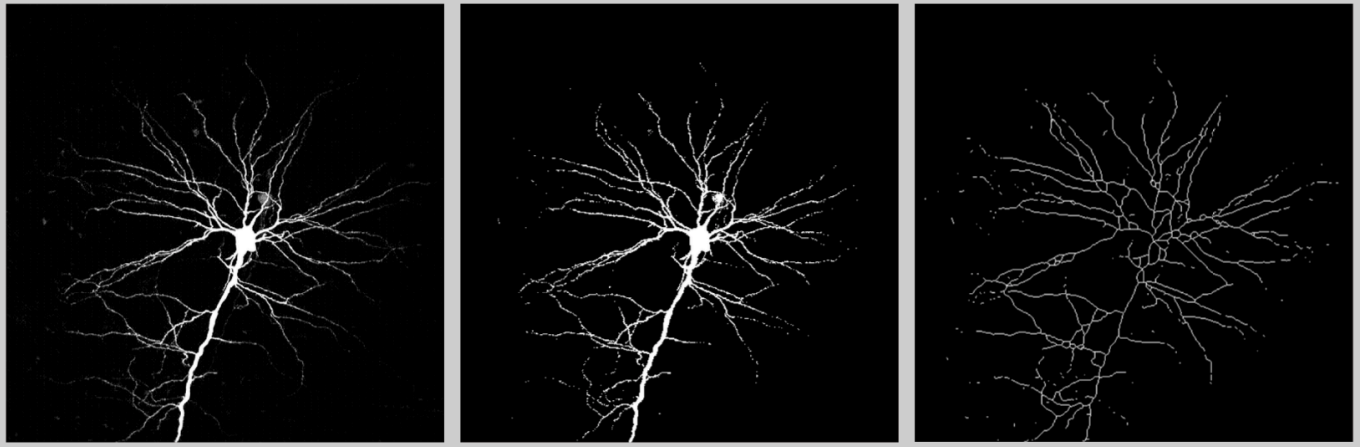
Processes Analysis

In addition to thresholding, FIJI provides you with several binary operations that are especially suitable for certain types of biological structures.

`[Process -> Binary -> Skeletonize]` creates skeletal outline of filamentous structures.

It ignores thickness of your biological samples, and draw only a pixel-thin line along thresholded areas, and is particularly powerful for analyzing branching structures such as neurons and the vasculature.

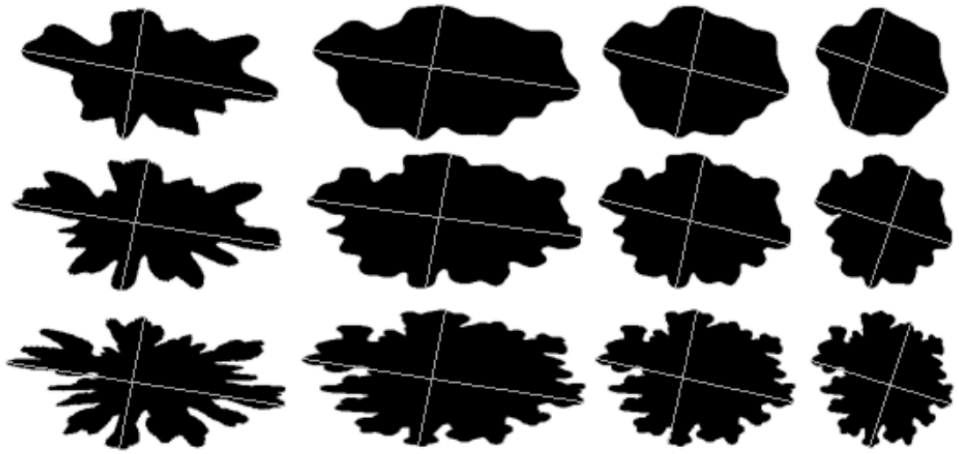
Original tif | Binary | Skeletonize



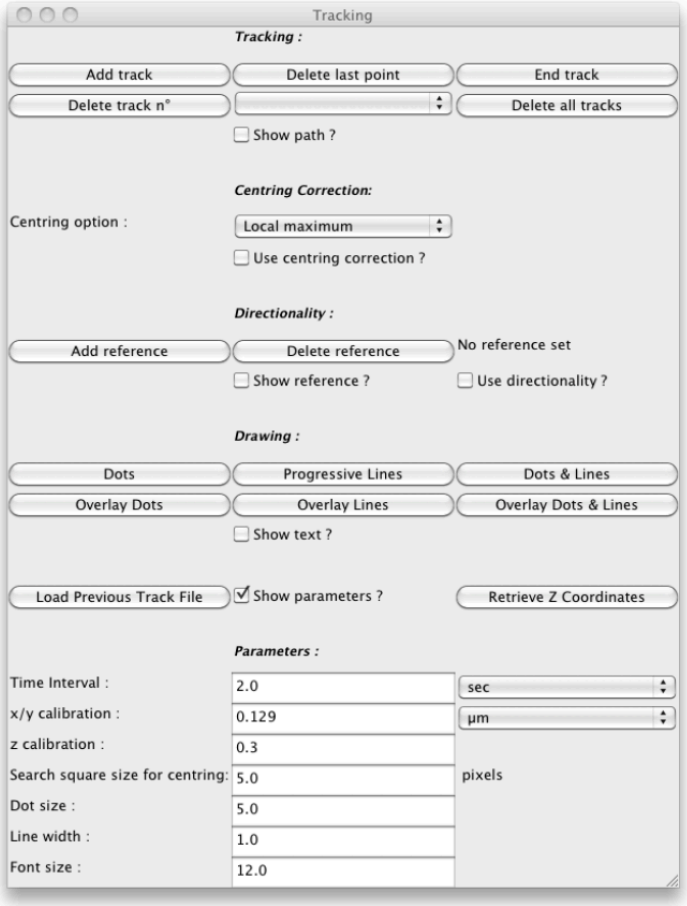
Shape Analysis

- Shape measurements are physical dimensional measures that characterize the appearance of an object.
- The goal is to use the fewest necessary measures to characterize an object adequately so that it may be unambiguously classified.
- The shape may not be entirely reconstructable from the descriptors, but the descriptors for different shapes should be different enough that the shapes can be discriminated.

Area
Perimeter
Major/minor axes
Aspect ratio
Compactness
Circularity/roundness
Convexity
Solidity



Manual Tracking



Do [plugins -> Tracking -> Manual Tracking]

A dialog box like this pops up

Check **Use centering correction**
Use **Local maximum**

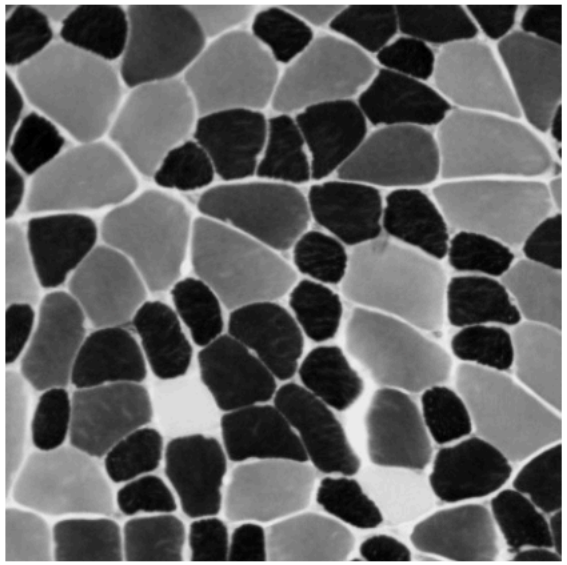
Click **Add track**
Start manual tracking
Every time you click the particle, Fiji advances to the next slice on the stack

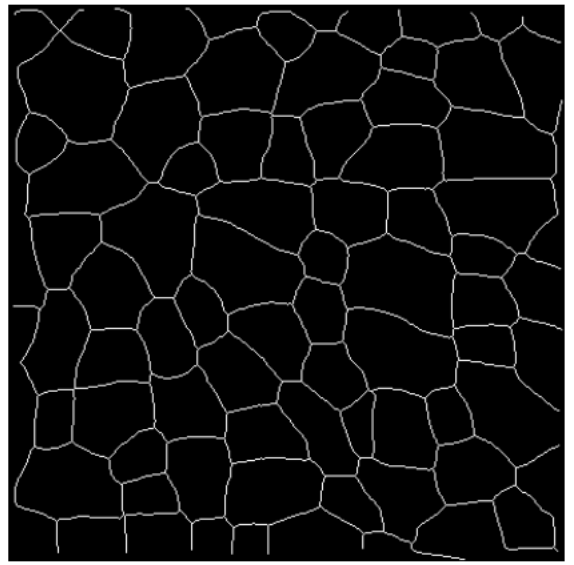
When done, click **End track**
Select the type of **Drawing** you like.

You can track different particles by repeating the steps.

Results window will list measured positions for particles etc.

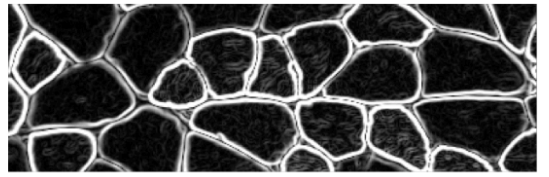
Segment Cells





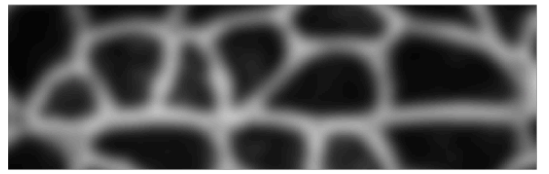
- [Process -> Find Edges]

Nice, but the edges now are comprised of two lines.
Need to make the double-layered lines into one line.



- [Process -> Filters -> Gaussian Blur]

Now, we have to make the software see the line.



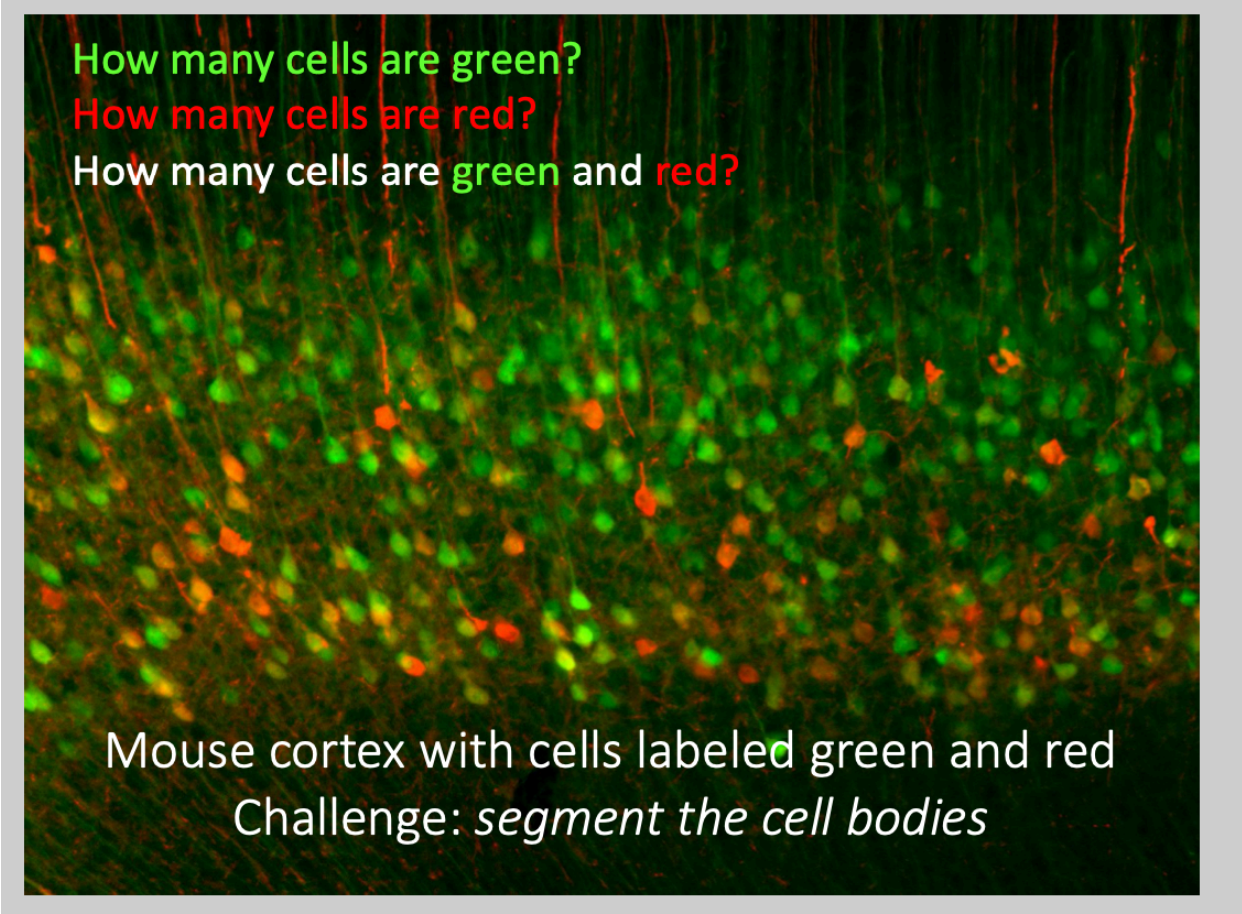
- [Image -> Adjust -> Threshold]

Nice, but the lines are really thick.

- [Process-> Binary-> Skeletonize]

Done

Measure Overlap



Split channels
Subtract background
Segment cells

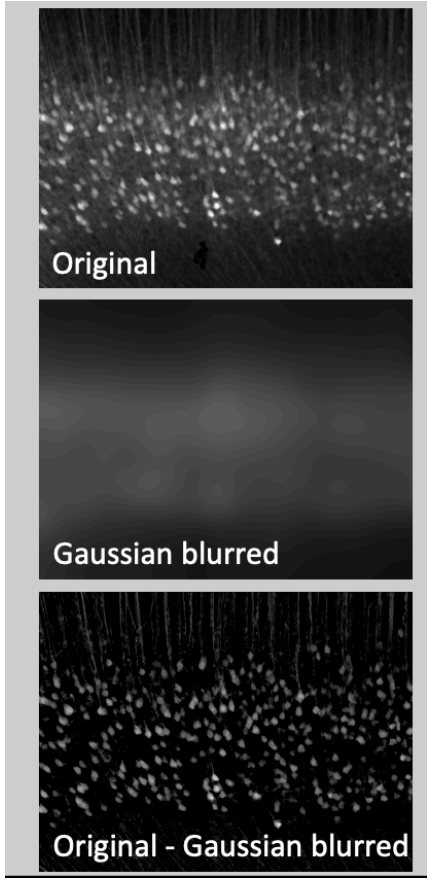


Image arithmetic on **grayscale or binary** images
• Image Boolean arithmetic (**AND**, OR, X-OR, NOT)
Process -> Image Calculator