Light Microscopy Core Facility
DUKE UNIVERSITY AND DUKE UNIVERSITY MEDICAL CENTER

CMB 551 Module 1A

Image processing and quantitative image analysis

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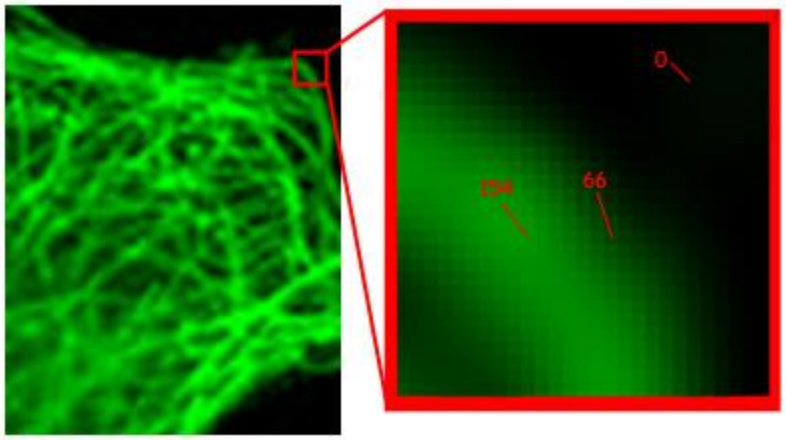
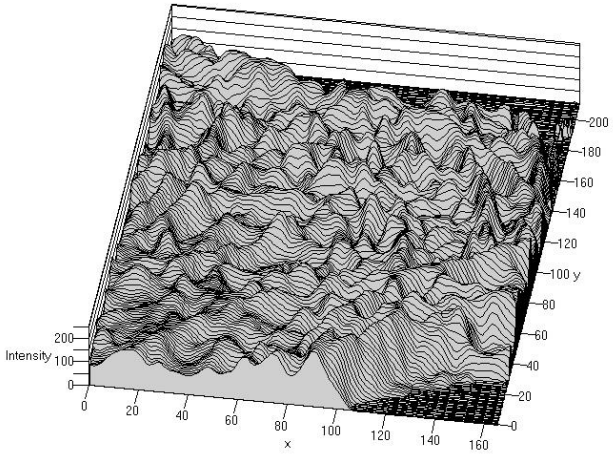
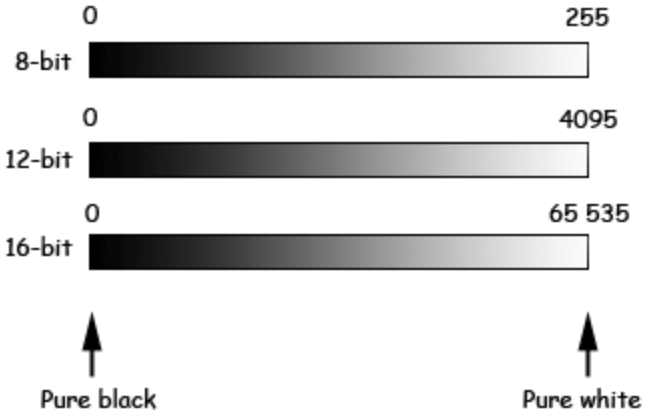
Quantification principles

Images are collections of numbers







7×8

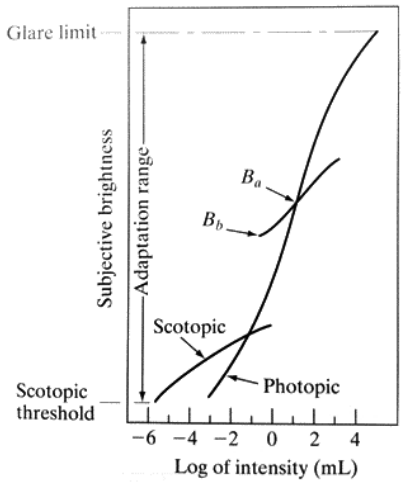
$\sqrt[3]{2350}$

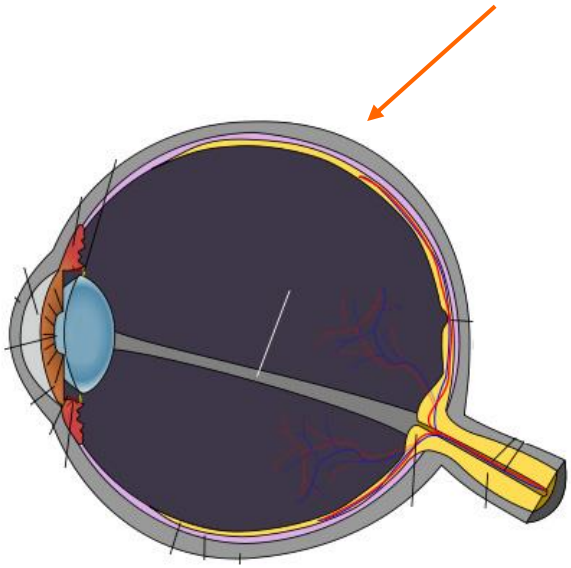
Why quantify or analyze images?

Why can't we just take images of A and B and show those?

Sometimes that is ok but . . .

- Some things are just hard to see or display well
- Quantification allows greater n numbers, unbiased, statistics vs "Representative cell shown"
- Major problems with these two things





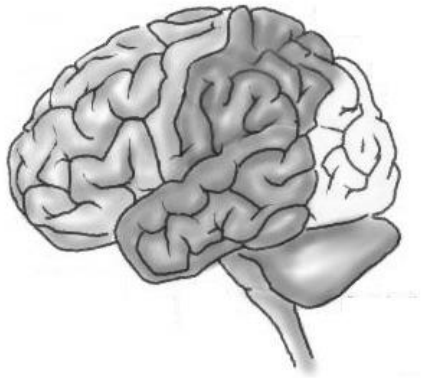
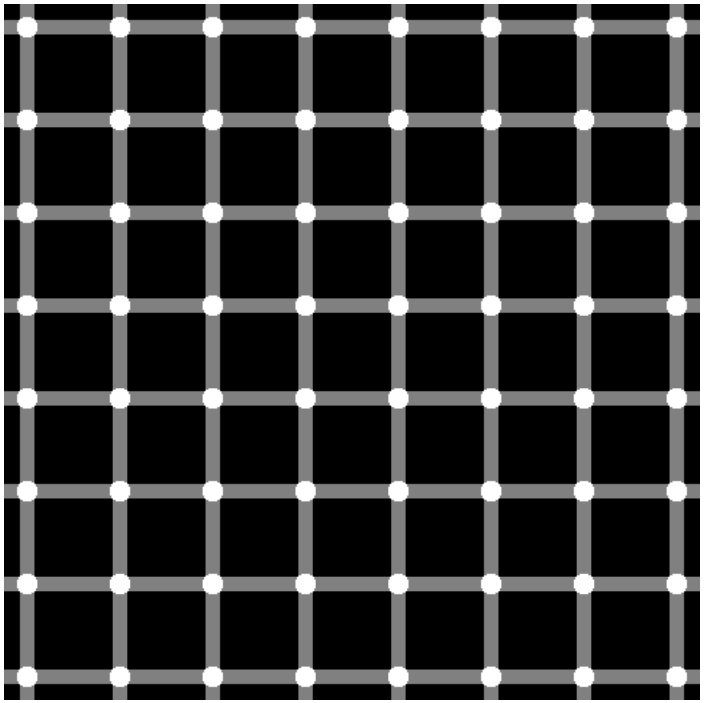
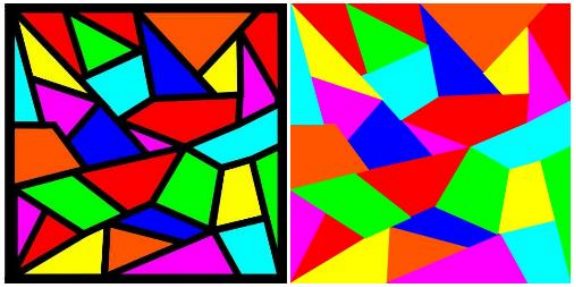
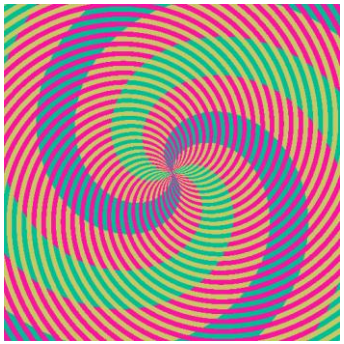
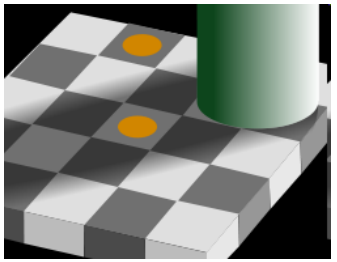


Image analysis is your friend









Seeing isn't always believing

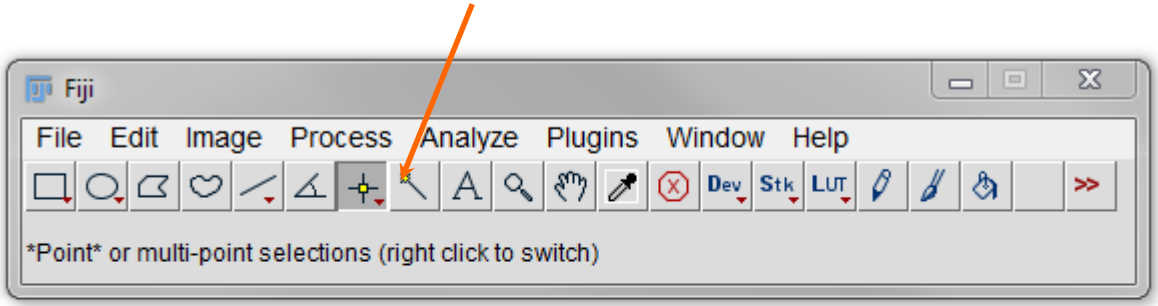
Common image quantifications

- What is the **mean intensity** of the green channel in wild-type and knockout?
- What is the **length or area** of a structure in each cell?
- How does the intensity of the GFP **change** over the time-course?
- How is the protein **distributed**, perhaps relative to a particular structure?
- How **many** cells, spots . . . are there in the image?
- How **fast and far** does an object move?
- How much does the green **colocalize** with the red?

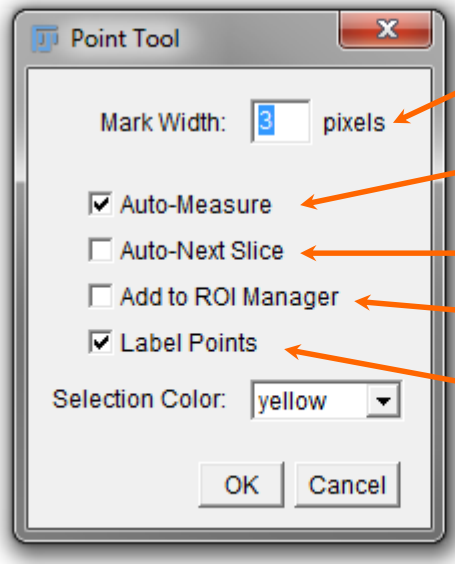
Counting . . .

. . . The old fashioned way

File: Region and count



Double-click on the point tool icon to get options . . .



Marks foreground color on image – don't save after!

Records intensity and location, and counts

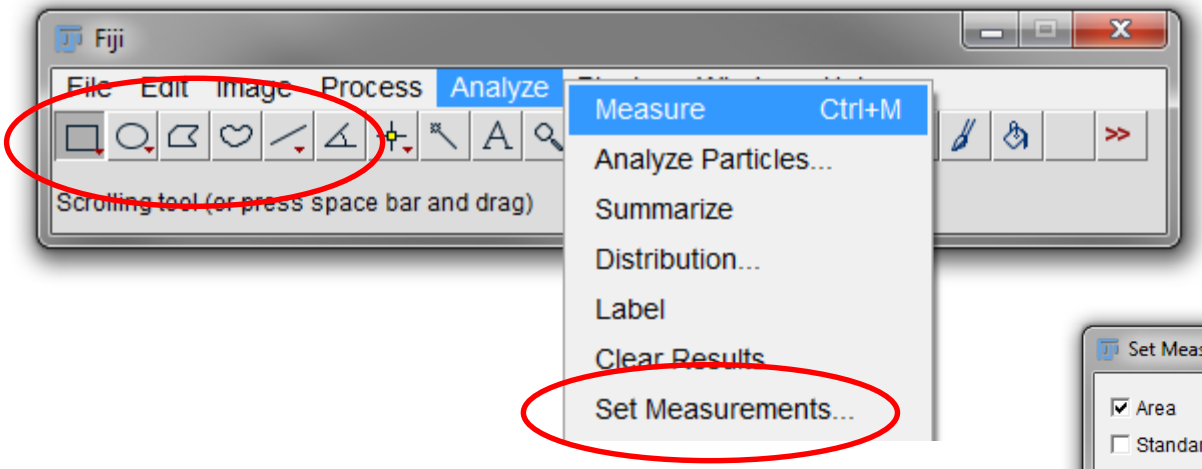
Good if you want to mark one point per slice

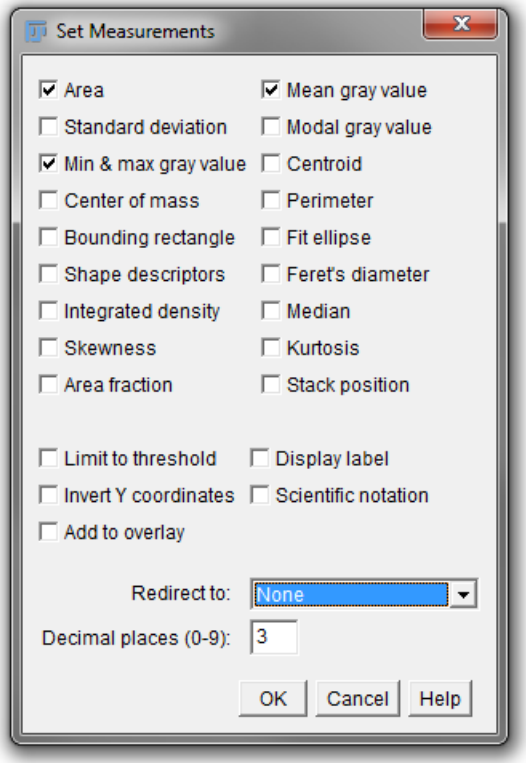
We'll come back to this

In multi-point tool (right-click on the icon) numbers are shown next to points

Region based measurements

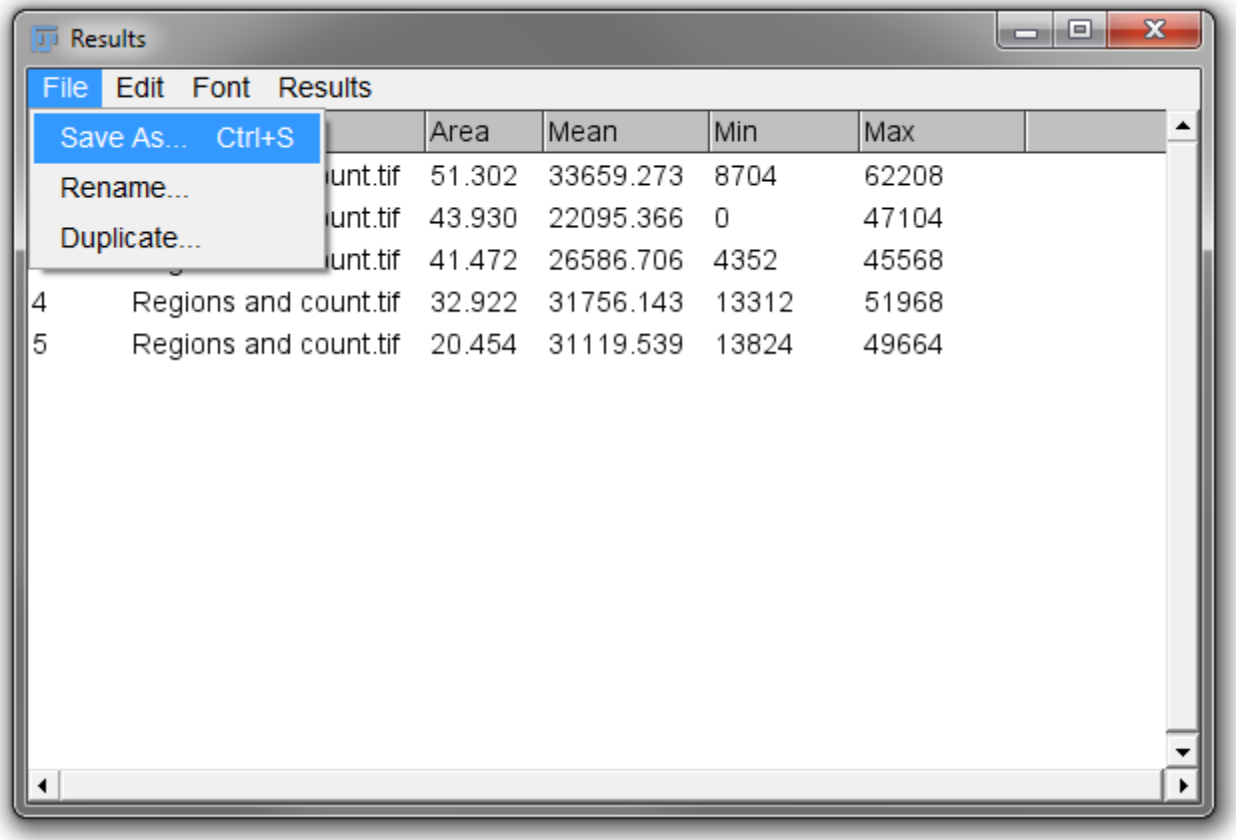
File: Regions and count





- ✓ Measure the diameter of some nuclei
- ✓ Line profiles – *Analyze/Plot Profile* or ctrl-K
- ✓ Draw Regions Of Interest around nuclei (right click on the icons with red triangles)

Output



Select and right-click on numbers to delete any mistakes/duplicates

Results/Summarize gives averages/SD/min/max for each column

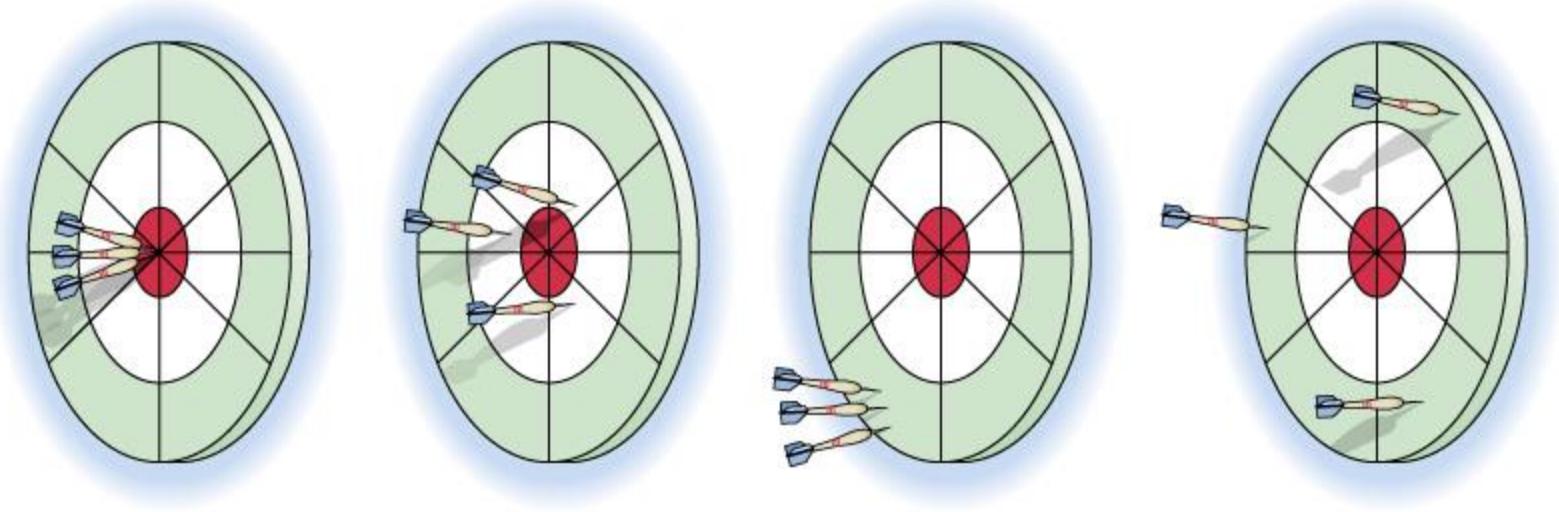
Save your measurements as .xls or .csv

What do the numbers mean?

Relative intensity, grey value = nearly arbitrary unit

- Turning grey values into photon number is possible
- Getting absolute numbers of fluorophores is much harder
  - FCS/RICS – offer ability to measure abs concentration
  - Some structures (eg viruses or centrosomes) can be present a defined average number of GFP-fusions, image and use as a calibration

Accuracy and precision



Accurate
Precise

Accurate
Imprecise

Inaccurate
Precise

Inaccurate
Imprecise

The images need to be taken correctly

- Same settings for anything settable
- Within the dynamic range (no saturation/clipping)

Which modality?
What ever works best for your sample

Limitations of the correctly taken images

- Illumination artifacts – is the field perfectly even?
- Day-to-day variation – laser power (helps to warm them up), bulb age, alignment of the optics (especially laser coupling), dirt
- Bleaching over t and z
- Antibodies – incomplete permeabilization, non-linearity. FPs don’t have these problems.

Limitations of the correctly taken images

- Fluorophore saturation (confocal)
- Bleedthrough, other fluorophore interactions (eg quenching)
- Imaging efficiency over z
- Image aberrations – eg chromatic: colocalization accuracy, spherical: intensity over z (do all your samples have the same aberrations?), intensity and resolution loss
- Reagent instability – batch variation between antibodies, mount, coverslips

Fluorescent protein oddities

Photopathologies – all might not be as simple as we might like to think – photoconversion, dark-states, odd bleaching

Valentin, G., et al., **Photoconversion of YFP into a CFP-like species during acceptor photobleaching FRET experiments**. Nature Methods, **2005**. 2(11): p. 801-801.

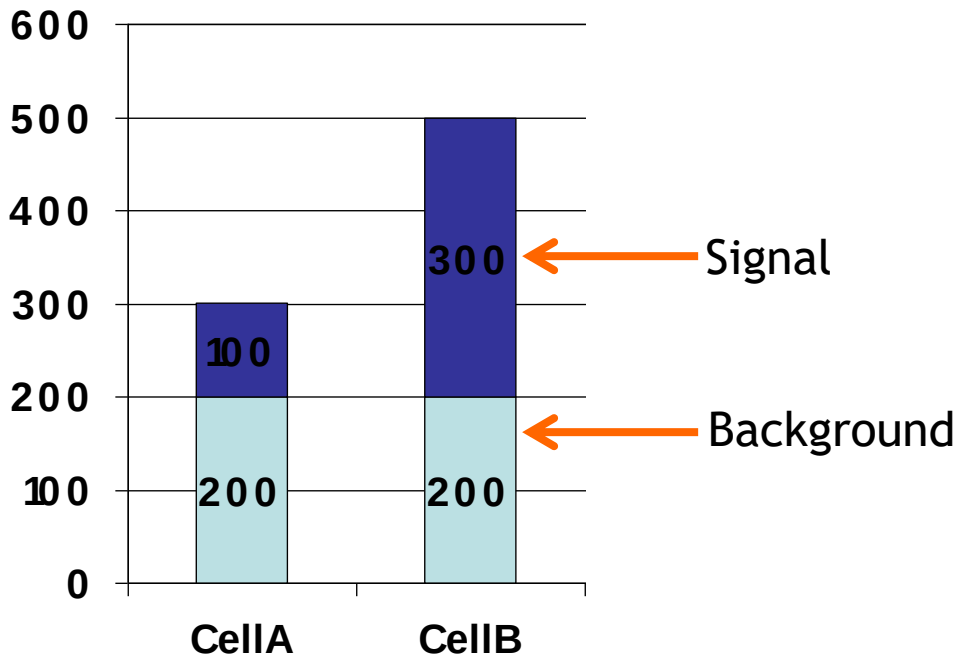
Thaler, C., et al., **Photobleaching of YFP does not produce a CFP-like species that affects FRET measurements**. Nature Methods, **2006**. 3(7): p. 491-491.

Valentin, G., et al., **Photobleaching of YFP does not produce a CFP-like species that affects FRET measurements - response**. Nature Methods, **2006**. 3(7): p. 492-493.

Verrier, S.E. and H.D. Soling, **Photobleaching of YFP does not produce a CFP-like species that affects FRET measurements**. Nature Methods, **2006**. 3(7): p. 491-492.

DAPI can PC to green

Background correction



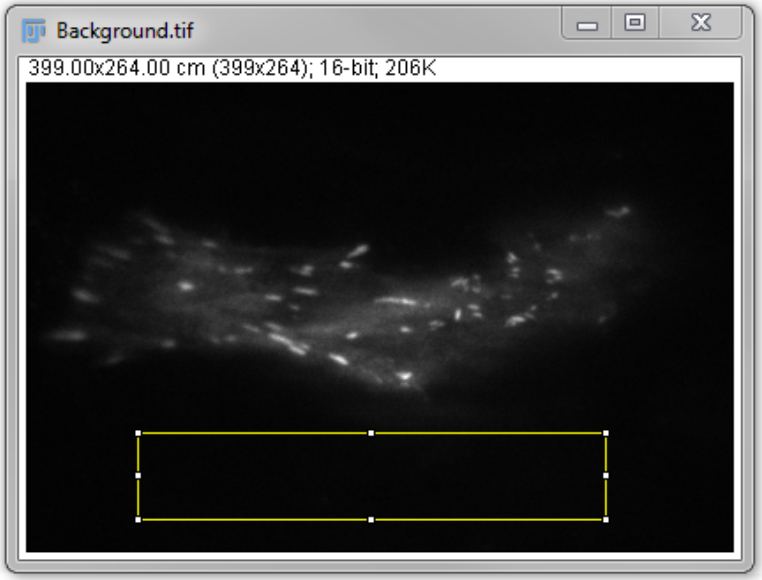
CellB is 300% of CellA, not 166%

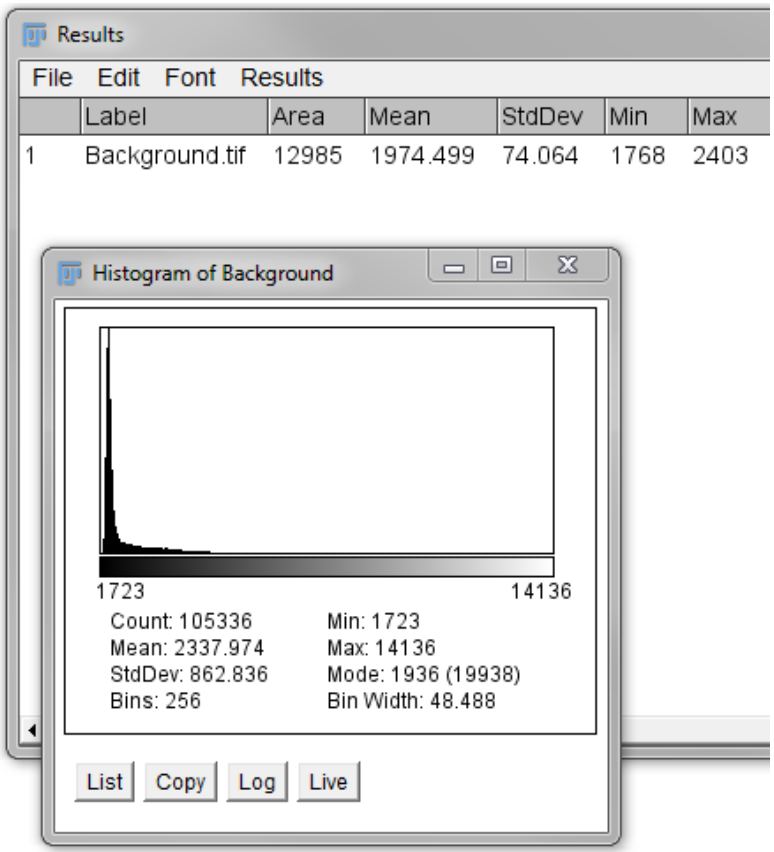
For intensity measurements (especially from CCD images) you need to take background into account, either

- Measure the mean intensity of the background and subtract that from objects
- Remove it from your images before quantification . . .

Background subtraction

File: Background



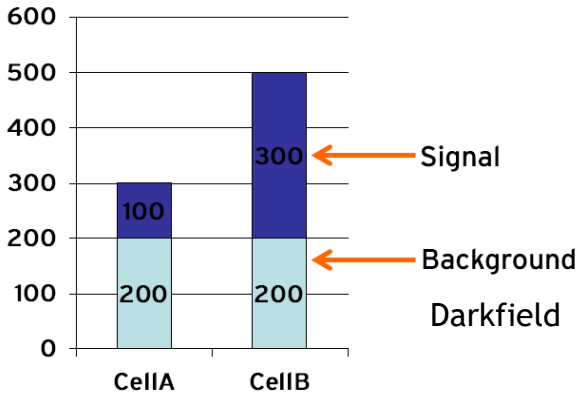


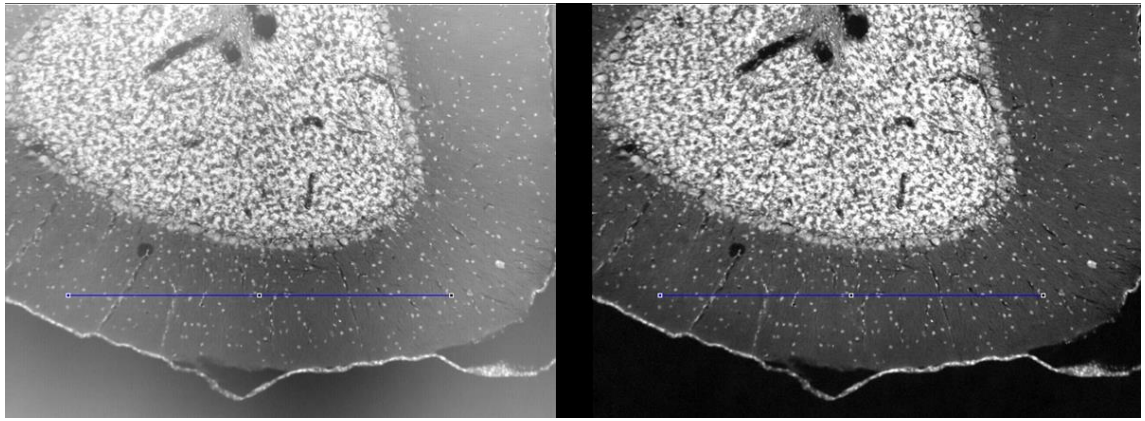
Process/Math/Subtract

Which number should you use?

You could also take an image of nothing (darkfield) and subtract that

Non-uniform background subtraction

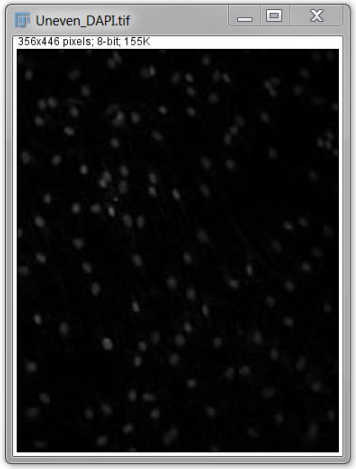


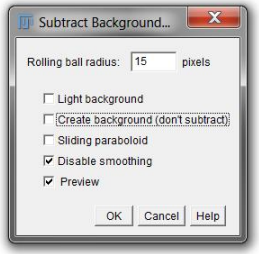


Flatfield

Process/Subtract Background . .

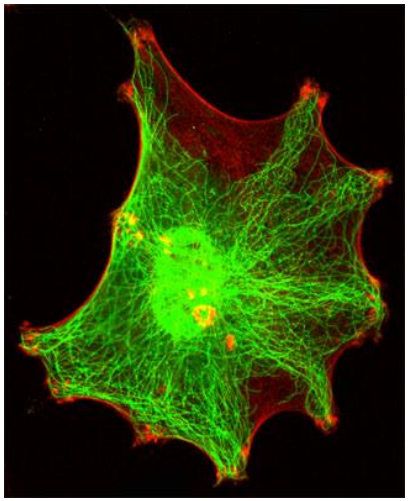
File: Uneven_DAPI

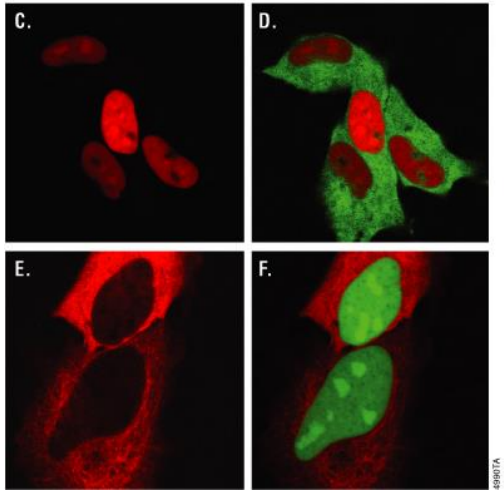


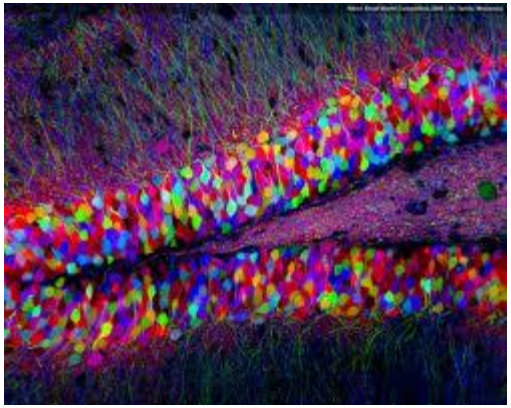


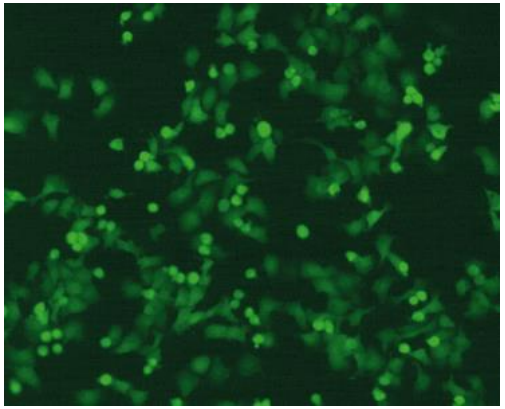
Compare by mouse-over and histogram

Choosing the subset of the image to measure

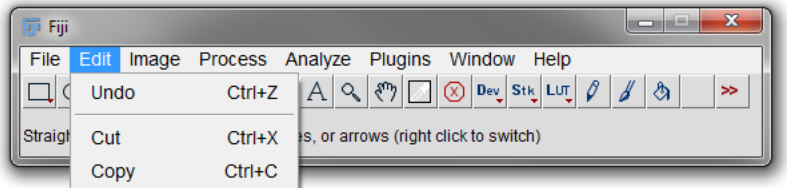


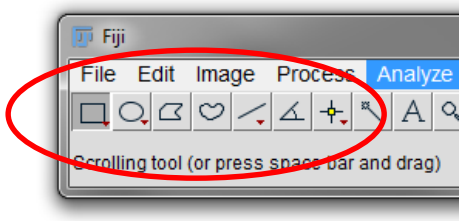


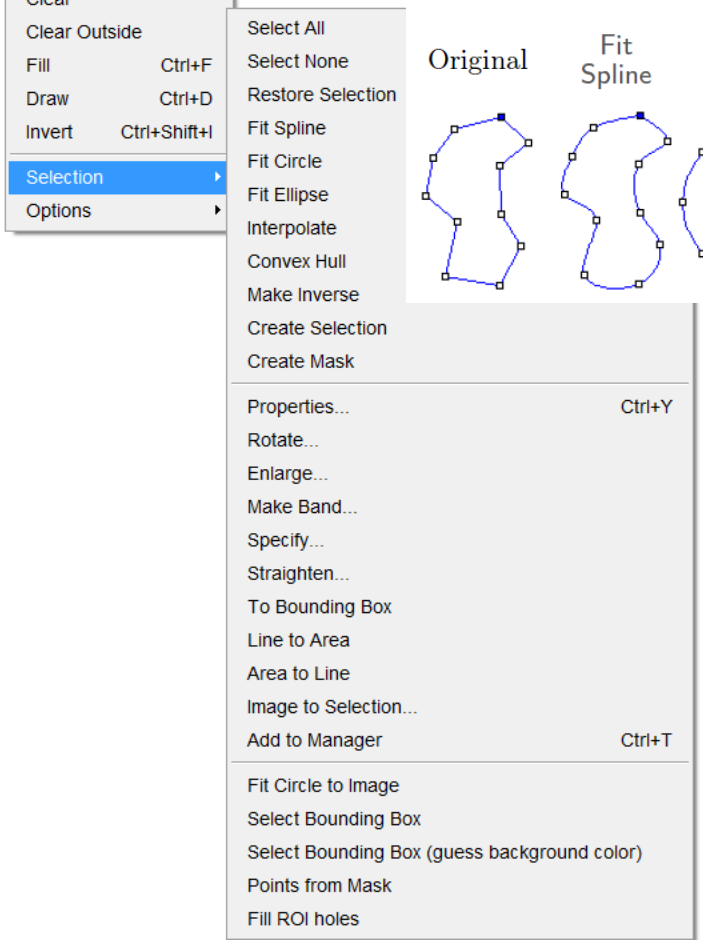


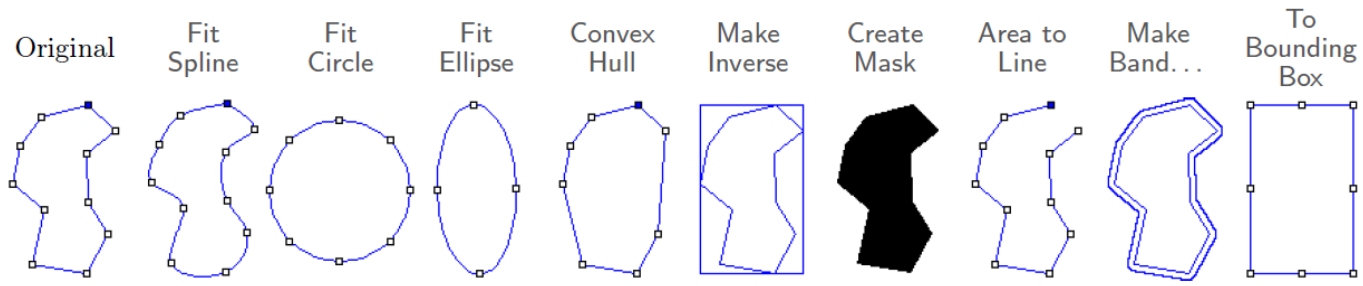


Selection tools





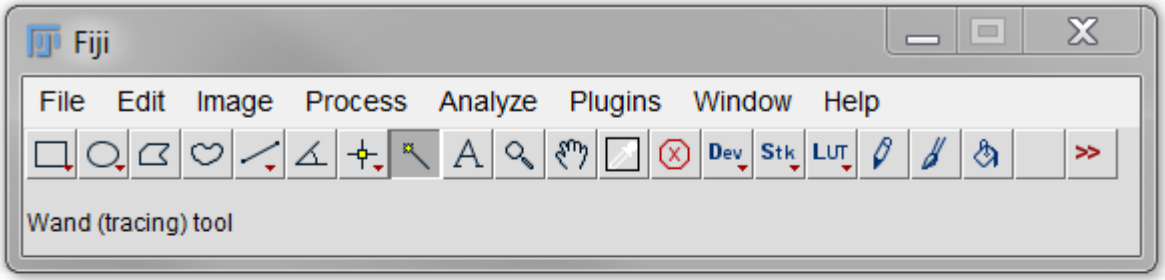


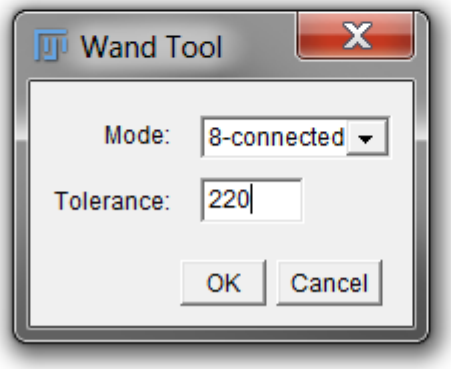


Many ways to modify a region selection

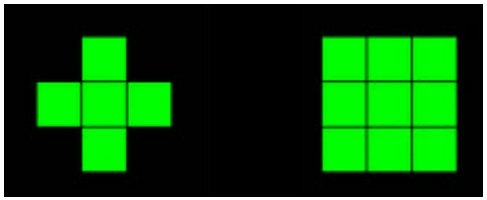
Wand

File: Regions and count





4-connected 8-connected

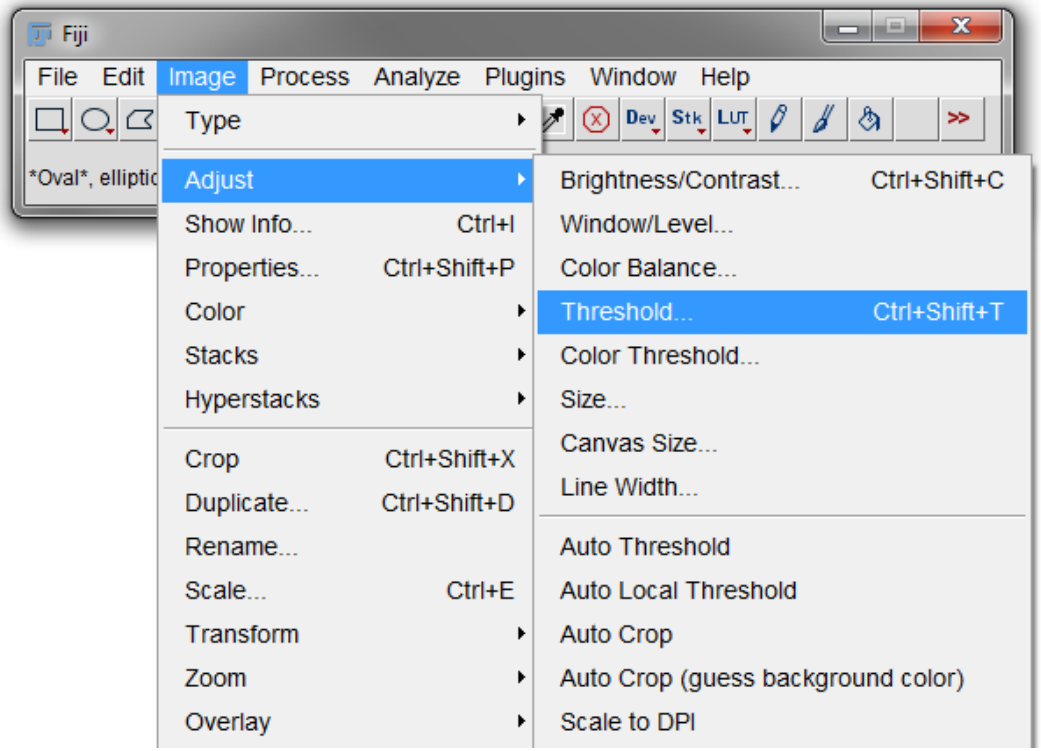


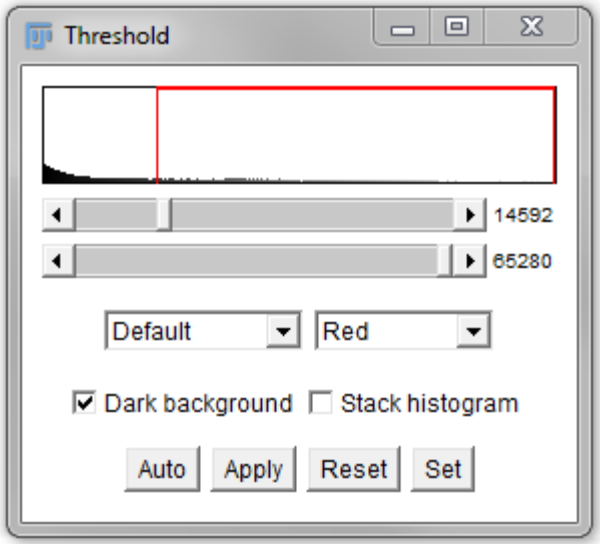
What tolerance works well?
(hint 16 bit image)

Shift-click to count several (same to add manual ROIs)

Threshold

An intensity value above which is an object, below which is background





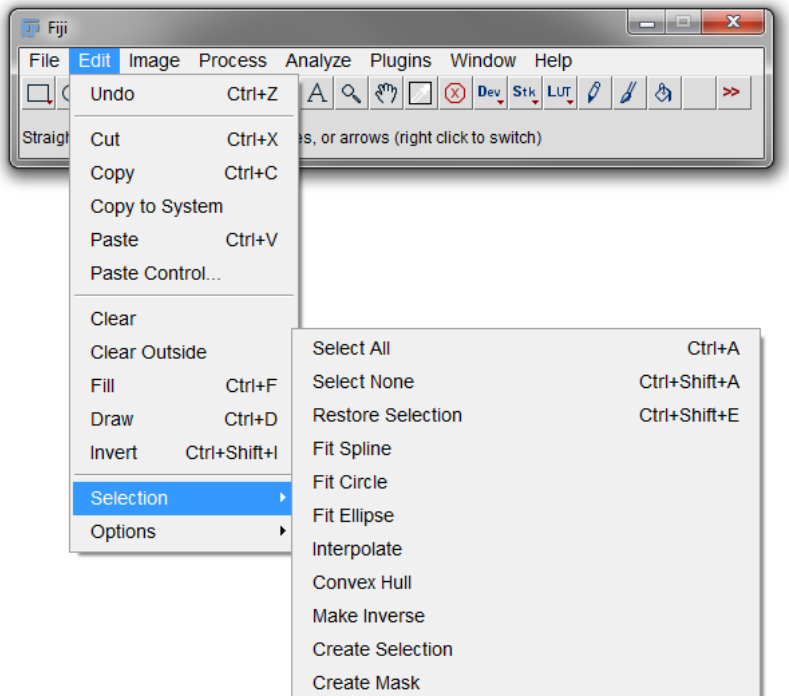
How should I set the threshold?

`File:  Regions and count`

- It’s really important
- Should I use the same value for all my images?
- Lots of different algorithms for auto-threshold - http://fiji.sc/wiki/index.php/Auto_Threshold

Selection based on the above threshold region

`File:  Regions and count`



1. Set your threshold
2. Create selection
3. Measure

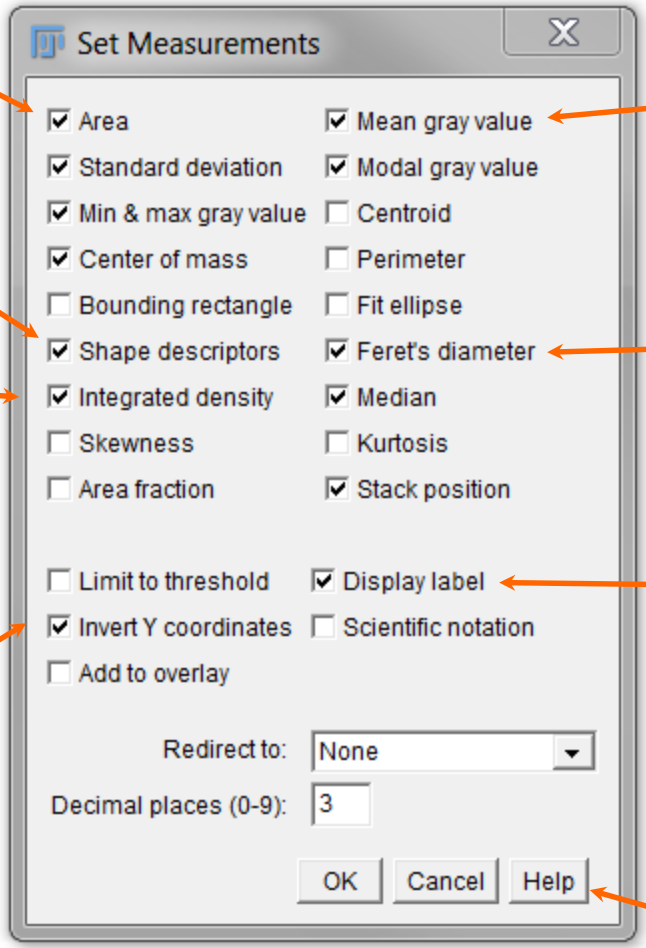
What to measure?

Pixels or microns if known

Circularity, aspect ratio, solidity

Sum of all pixel intensities in selection
RawIntDen $= \sum pixels$
IntDen = $mean \times area$

Makes 0,0 be at bottom left



Mean intensity of selection (mode and median below)

Longest straight line in selection

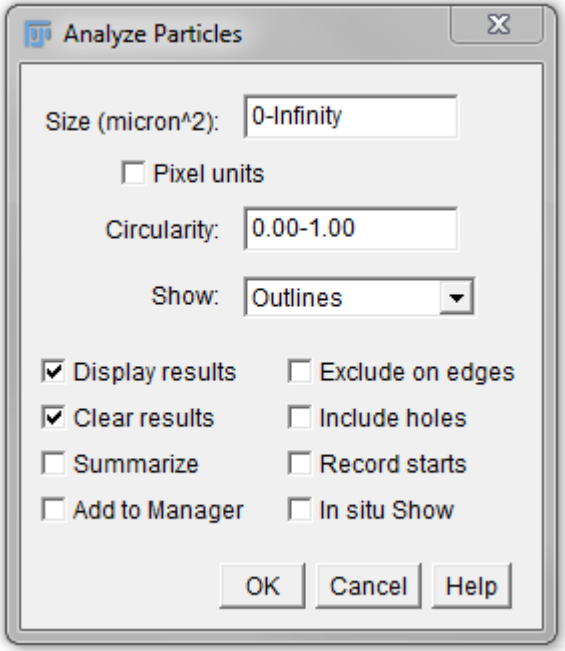
Puts image name in the results

It actually does

Analyze/Analyze particles

When you have your threshold optimally set . . .

Analyze/Analyze Particles . . . .



How many do you get?

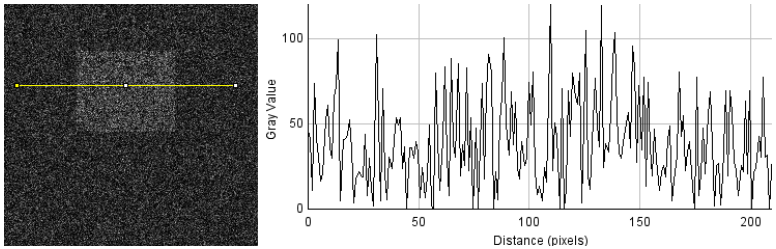
Count and measure nuclei

Optimize settings for greater accuracy

File: Regions and count

- ✓ Open image
- ✓ Threshold – repeat with a few values
- ✓ Analyze particles – try using the size range and other options
- ✓ What are the different output options – mask, overlay, outlines . . .
- ✓ How many nuclei? What is the average area and intensity?

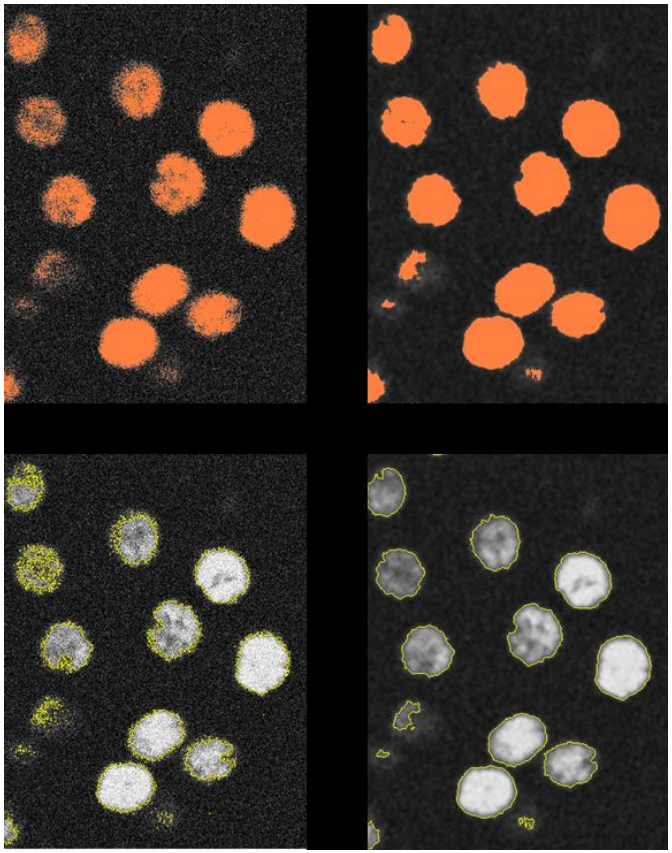
Using filters to help produce an accurate mask



Raw Smoothed

Threshold:

Outline:



Which is better?
For perimeter?
Intensity?

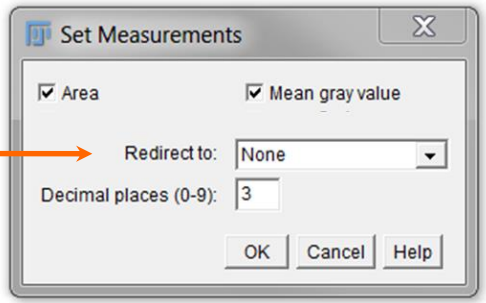
Moving selections/regions between images

Processing and filters can help, but your data is changed

Solution: Move the selections to a raw or accurately processed image

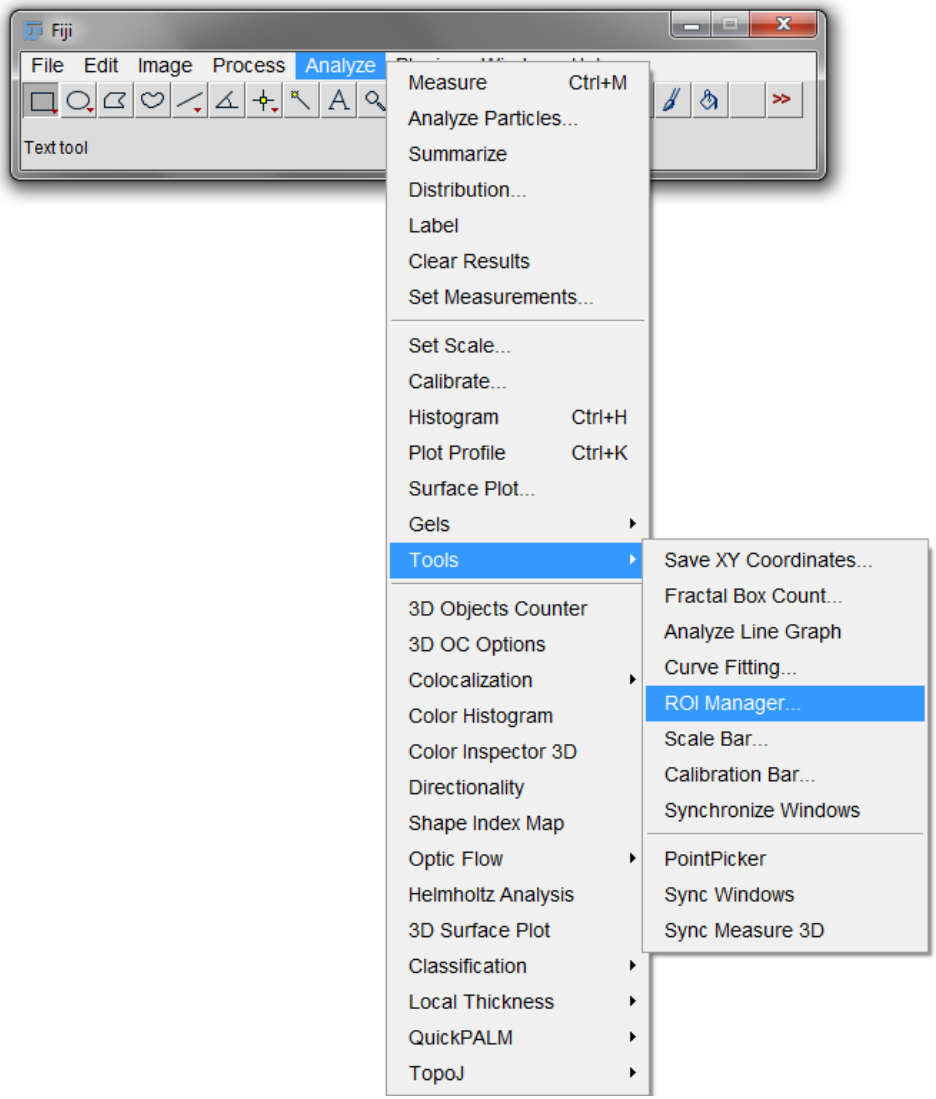
Open two copies of **`File:  Regions and count`**
Process 1, threshold, Edit/Selection/Create Selection
Select the other raw image, Edit/Selection/Restore Selection
Measure and inspect

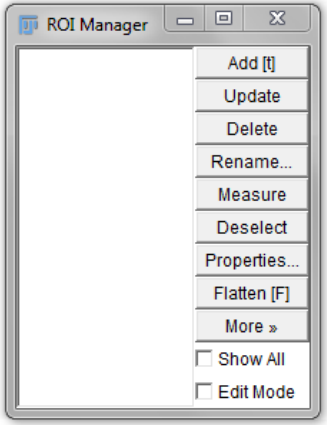
Or



FIJI region manager

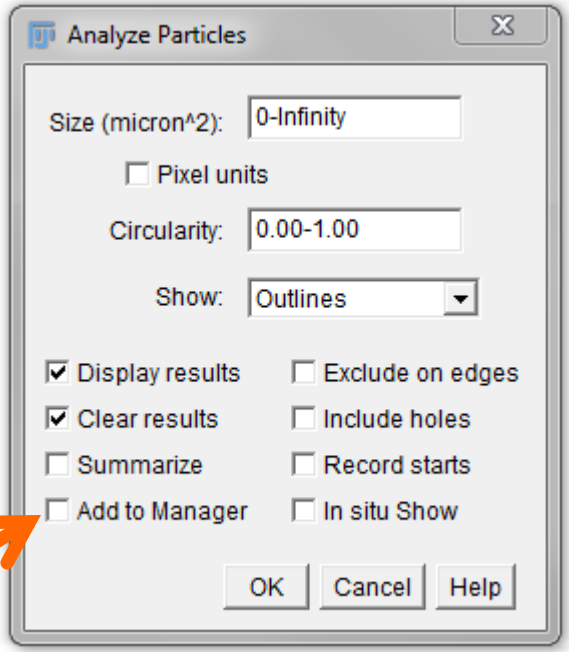
File: Regions and count





- ✓ Multiple ROIs
- ✓ Transfer
- ✓ Much more

Analyze Particles to ROI manager



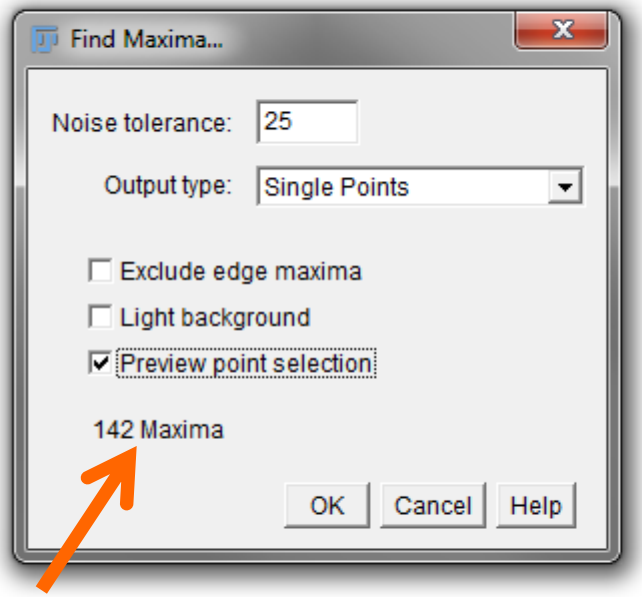
Adds generated outlines to ROI manager allowing transfer to another image

Let’s try using this, and in a 3D example

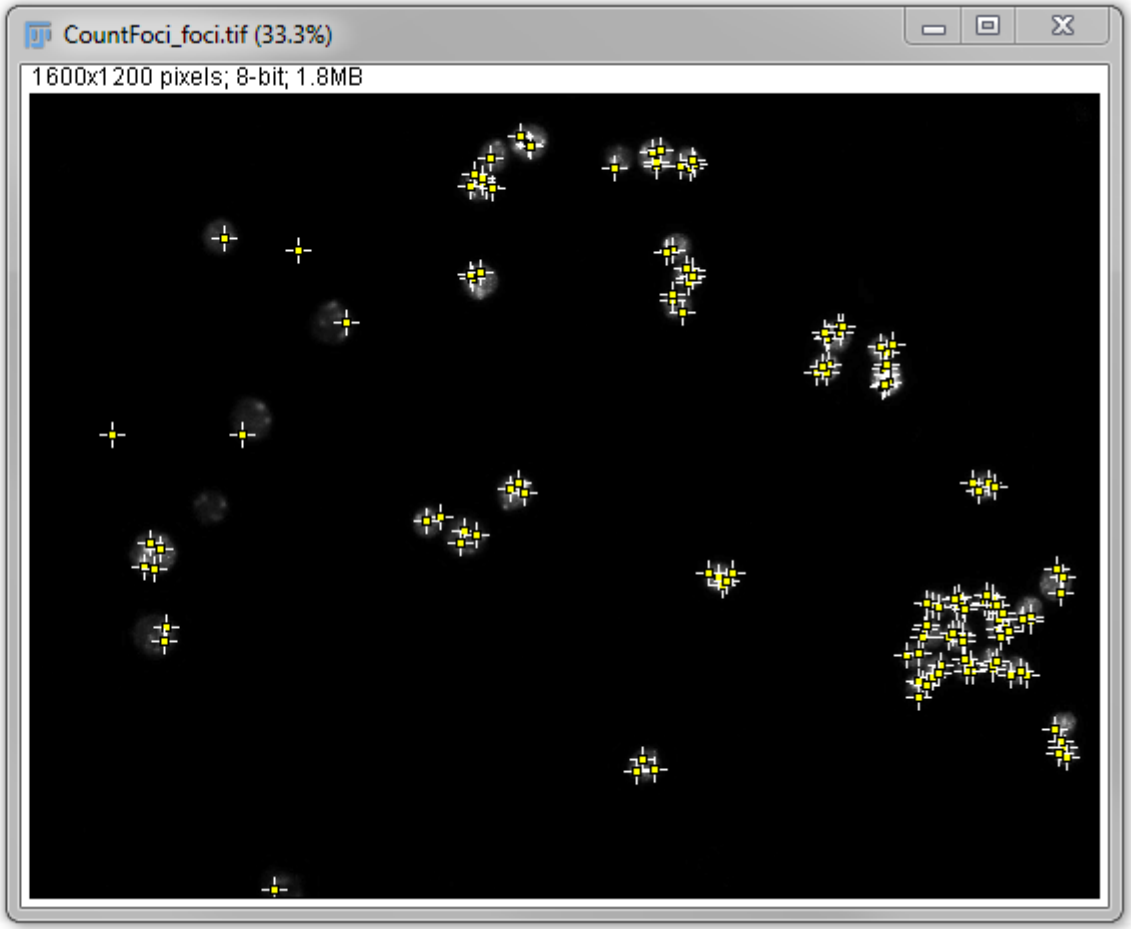
Count foci

Files:CountFoci_DAPI
CountFoci_foci

Process/Find Maxima

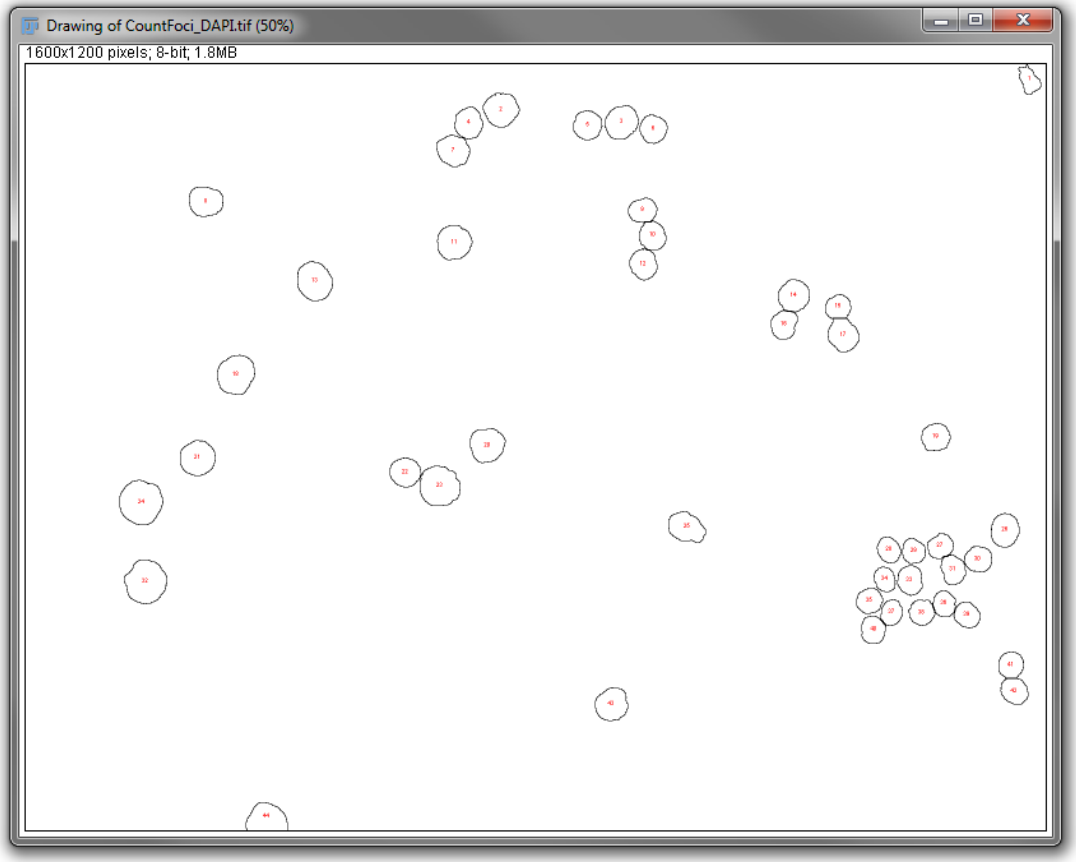


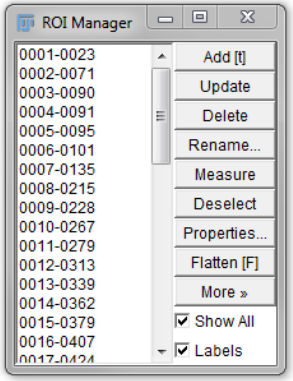
But how many per cell?



- ✓ Threshold CountFoci_DAPI (but don't press "apply")
- ✓ Process/Binary/Make Binary
- ✓ Process/Binary/Watershed
- ✓ Analyze particles and add to manager

(make sure the objects are black on a white background)

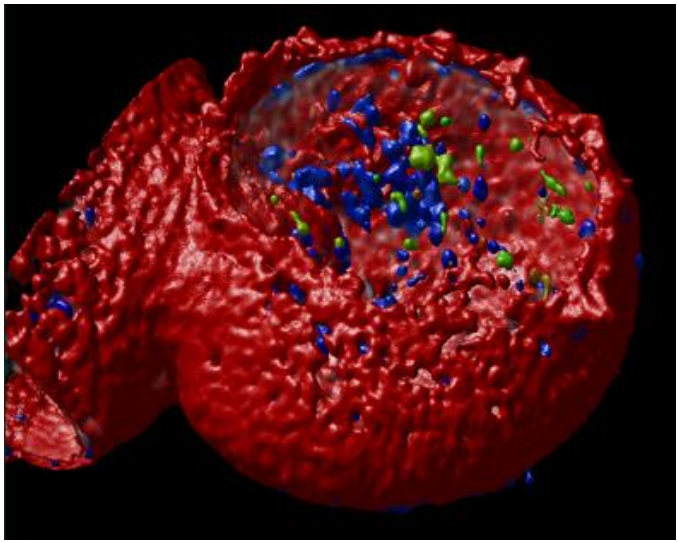


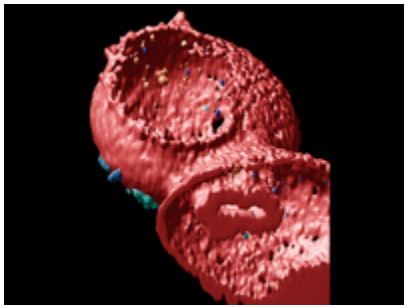


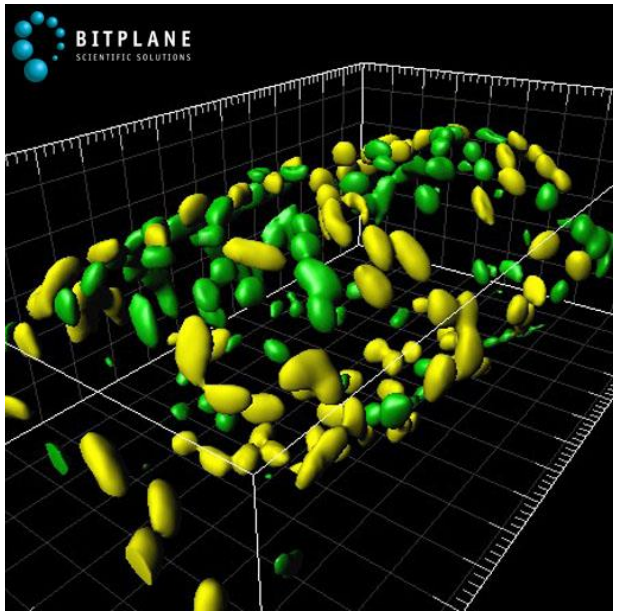
- ✓ Apply these ROIs to the find maxima output
- ✓ Measure and look at RawIntDen

Surface render for 3D measurements

Volume visualization by generating an isosurface, a 3D threshold. All points on the surface have the same intensity



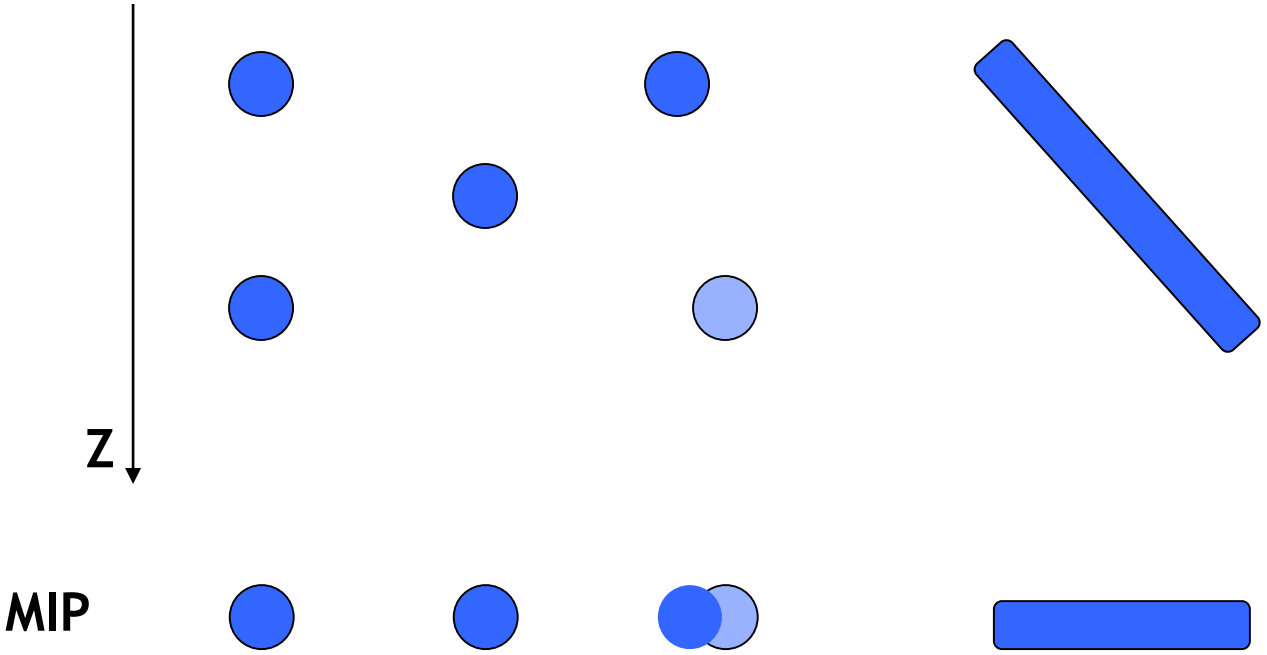




Measuring in 3D

Shall we just make a MIP and count, measure and make 2D intensity measurements with that?

No we shall not



(Sum or average projections may be ok for some things)

Measure the intensity of some yeast

```
Files: Yeast_stack_DIC
       yeast_stack_GFP
```

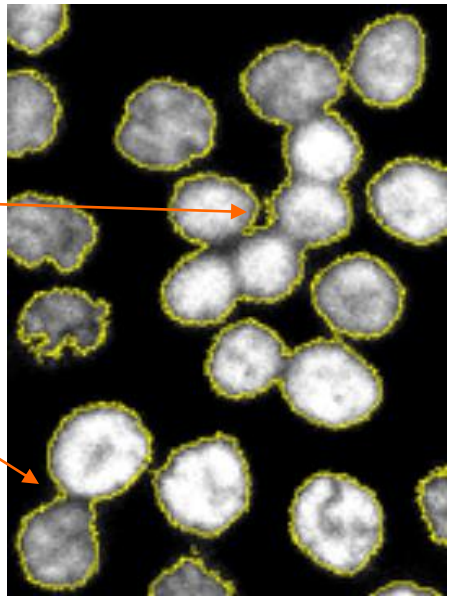
Use the DIC as a mask to measure the total GFP (why?)

Some challenges:

- DIC is hard to threshold
- Some of the yeast are touching
- The GFP images are noisy
- The GFP have a high-background
- The GFP images are 3D

What we are very good at doing

Can anybody not count how many nuclei are here?



This bit tends to be frustrating as we can see how the program could do a better job then just using a threshold

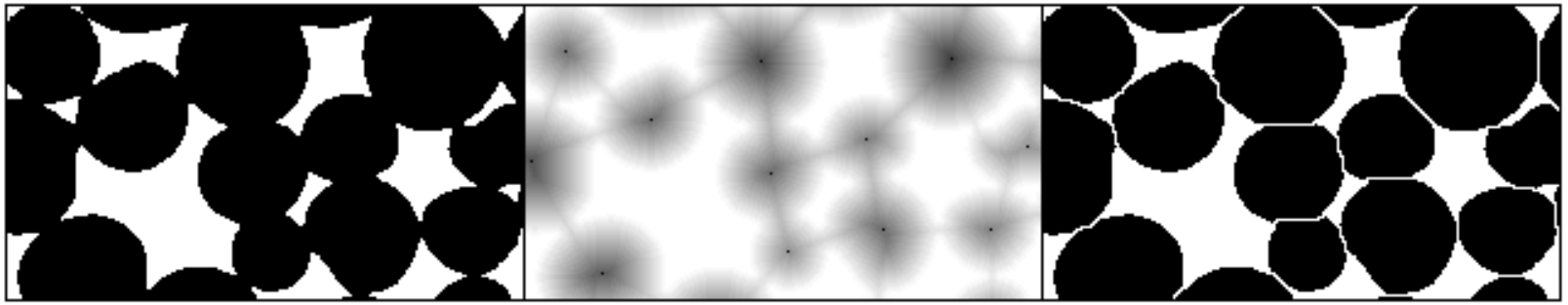
Segmentation algorithms

Many different methods to using a bit more than the threshold to choose and separate objects

http://en.wikipedia.org/wiki/Segmentation_(image_processing)

http://fiji.sc/Category:Segmentation

A really useful segmentation algorithm



Process/Binary
(when thresholded)

Or “Apply” in the
threshold box

Process/Binary/Distance Map

Process/Binary/Ultimate Points

Process/Binary/Watershed

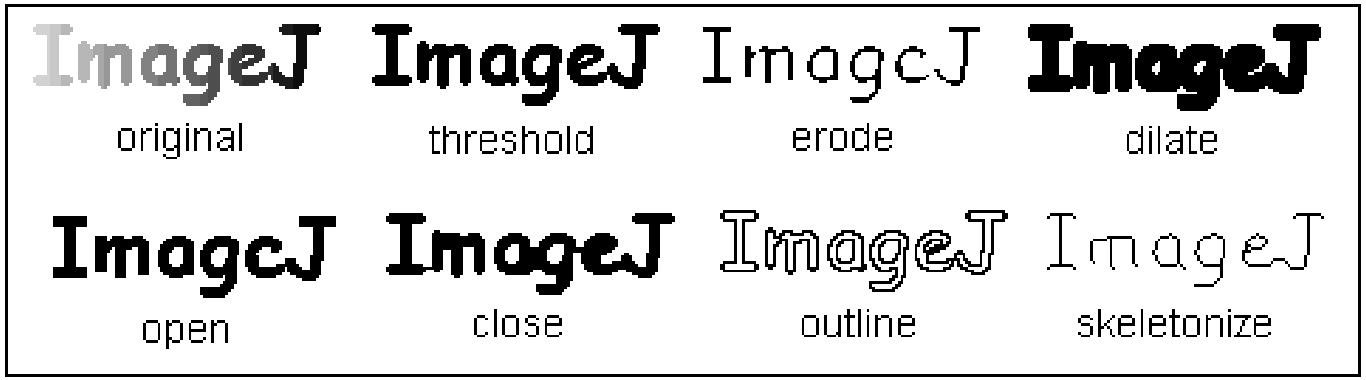
Mask modification exercise

Files: CountFoci_DAPI
CountFoci_foci

Use the DAPI image and all your skills to make a perfect mask

Process/Binary/ . . .

Fill holes



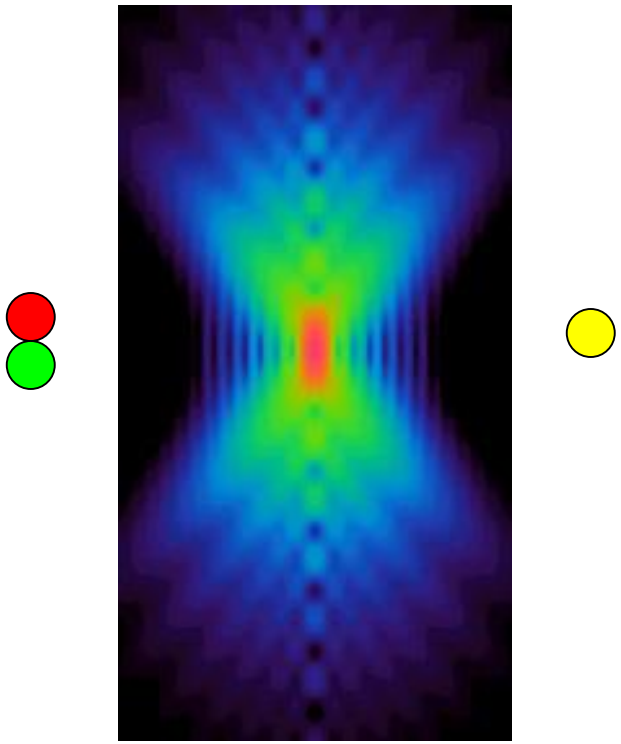
Manual changes with paint brush

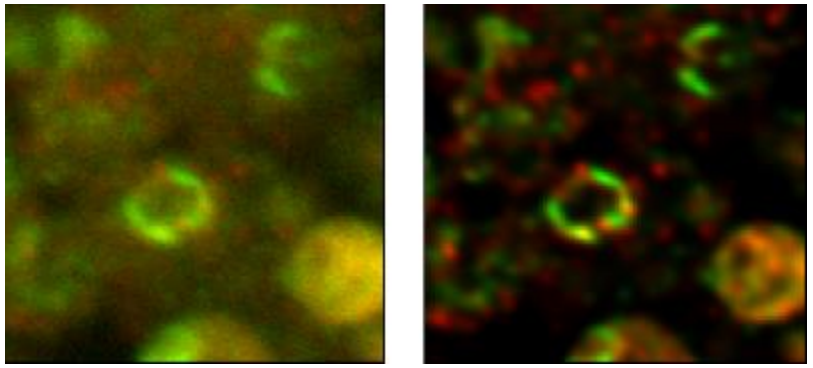
Measure the per cell intensity of the foci channel

Colocalization

Are two proteins in the same place?

All we can say is that they are in the same resolution constrained volume (and even if both are within 200 nm it doesn't mean interact or function together)





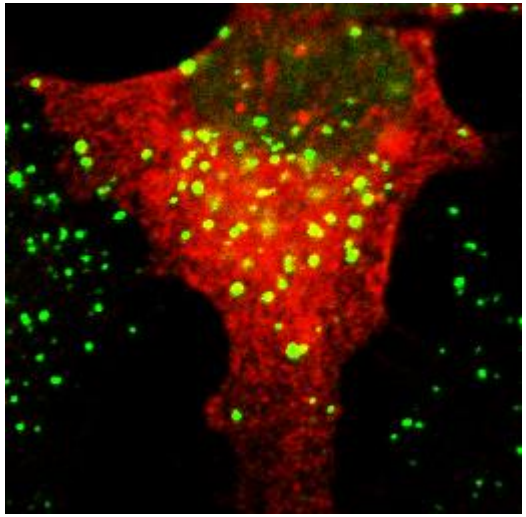
Bleedthrough = False positive (use sequential acquisition)

Chromatic aberration = False negative

The peril of yellow=colocalized

Yellow results from a similar intensity of green and red

If green and red happen to be different . . .



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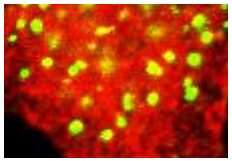
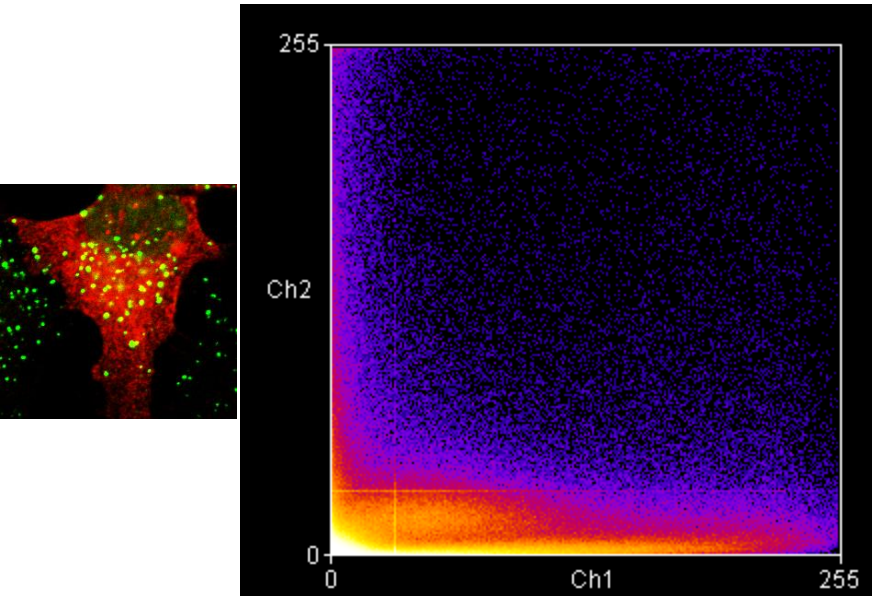
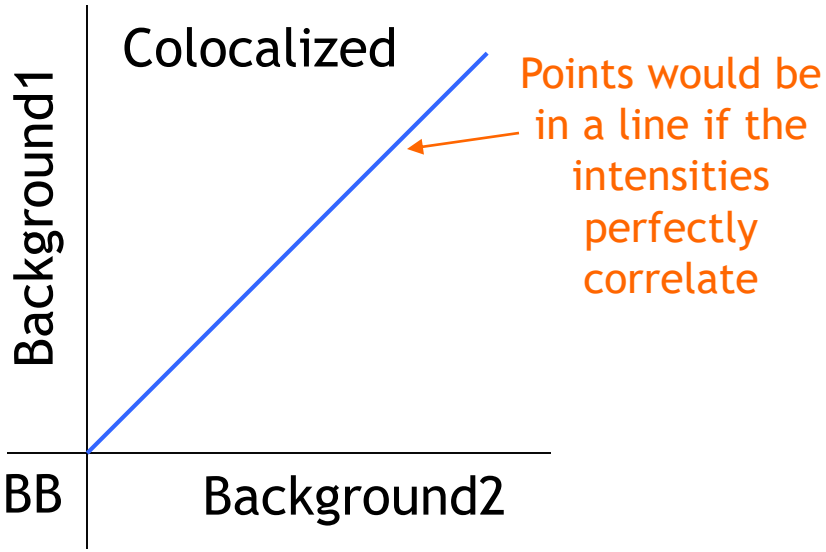


And we aren't great at judging absolute color

Statistical measures of colocalization



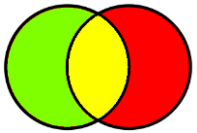


There are many statistical models of representing the extent of colocalization

http://support.svi.nl/wiki/ColocalizationTheory

Visualizing regions with both markers

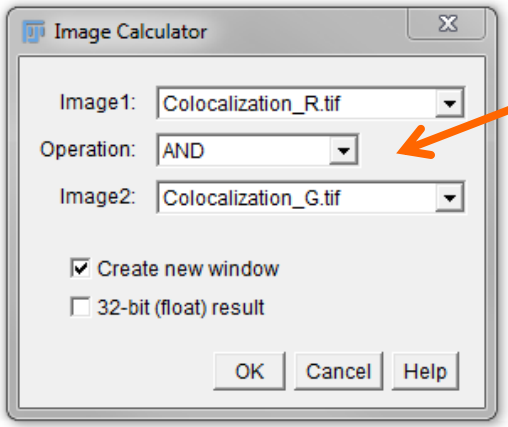
```
Files:Colocalization_G
      Colocalization_R
```



Colocalized = $G \cap R$

$I_G > T_G \cap I_R > T_R$

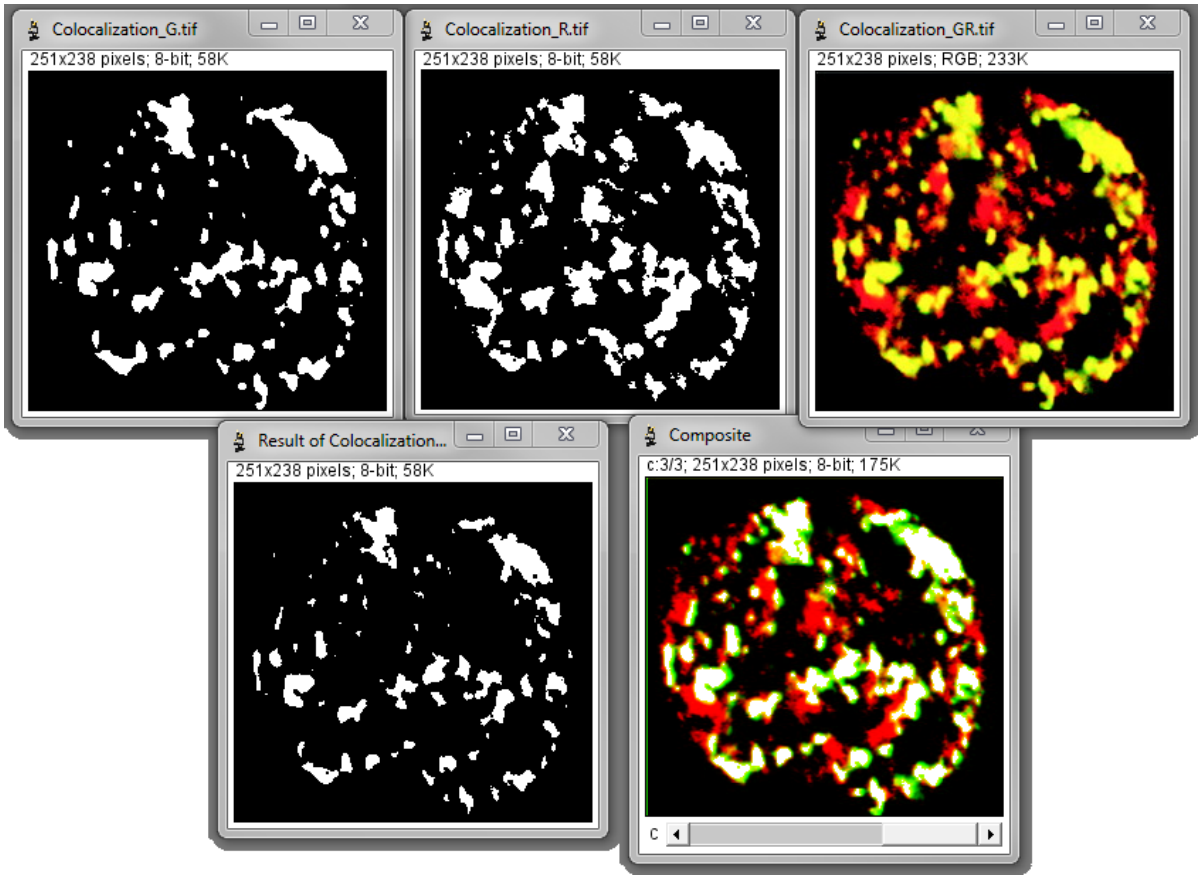
- ✓ Open the images
- ✓ Make an overlay – Green and Red=yellow? – to see what we are dealing with
- ✓ Threshold as you think best for each channel
- ✓ Process/Binary/Make Binary (mouse over black&white images)
- ✓ Process/Image Calculator



Makes a new binary image showing only regions with both G and R above threshold

(invert image or LUT if any image is negative)

- ✓ Put the AND image into the color overlay as white
- ✓ Turn on and off using the composite tools
- ✓ What do you think?

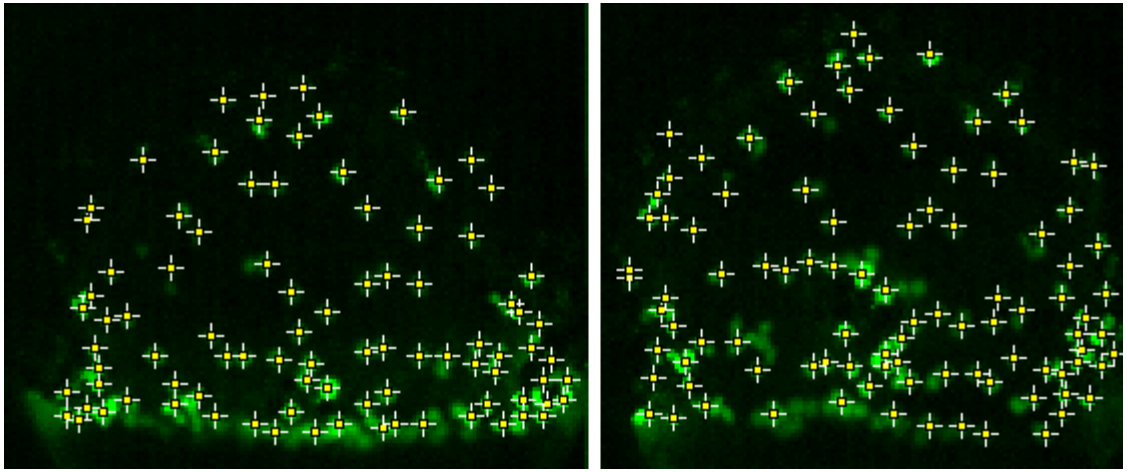


You could do trivially easy measurements for each of the above to measure the % area

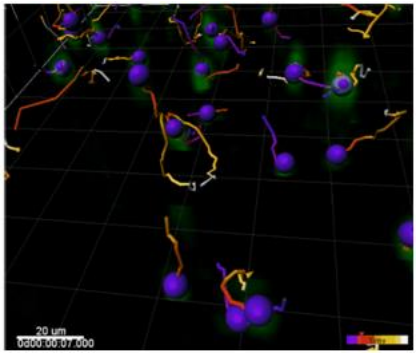
Tracking – following an object over time

1. Determine the position of the objects - similar to what we have done
2. Do that for all time points
3. Determine which of the positions over time are the same object

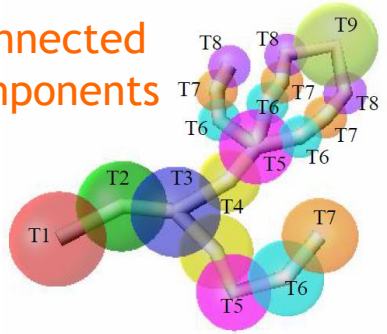
Time point 1

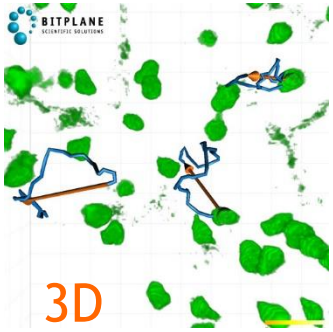


Time point 2



Connected components

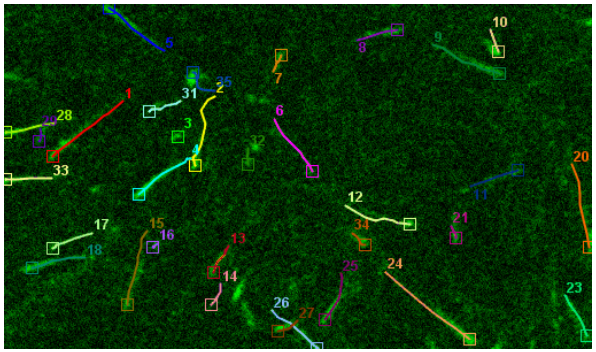




Tracking (the simple way)

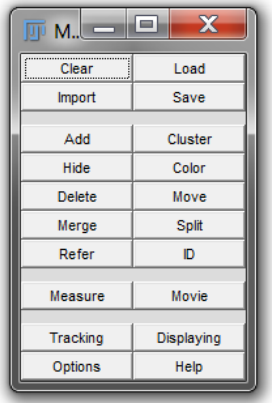
If you only have a few or they are very difficult to pick . . .

Manually click on each object over time

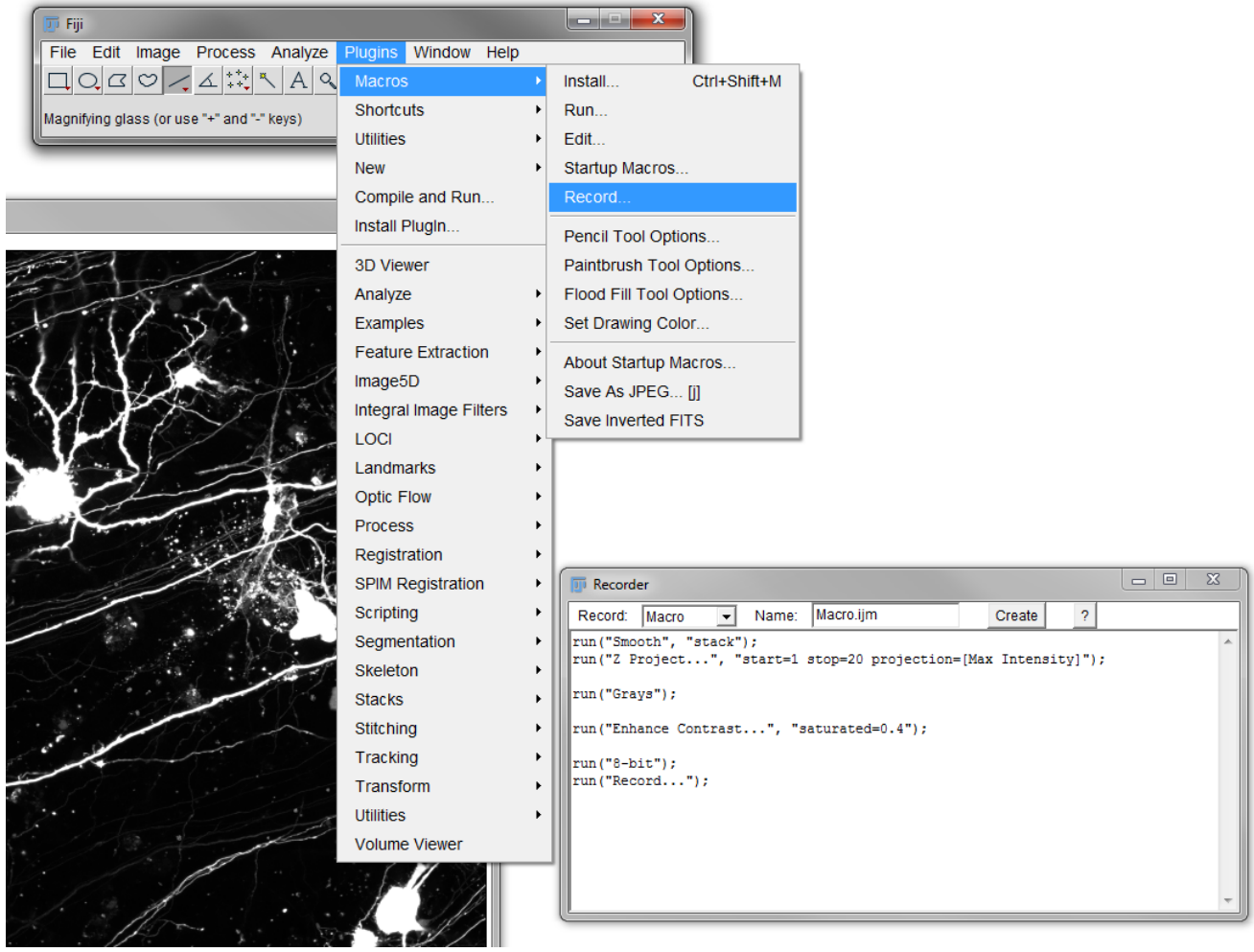


Plugins/Tracking/mTrackJ

- Add several color tracks
- Works in 4D
- Lots of measurements – eg positions, distances, velocity, angles. . .



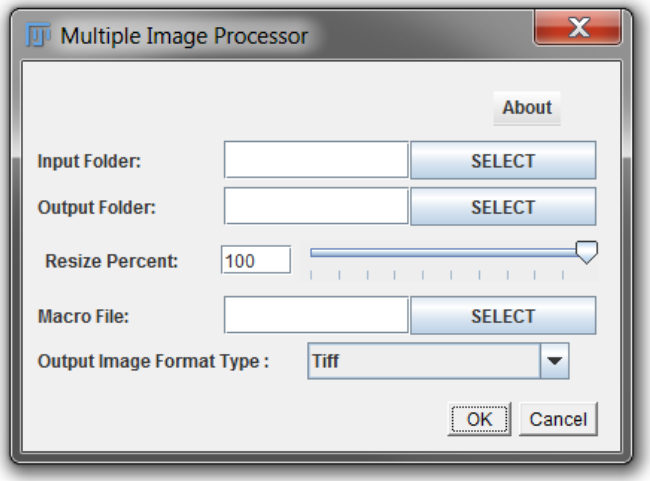
Automation with Macros



Record a sequence of operations and save mouse clicks

Macro

Process/Multiple Image Processor

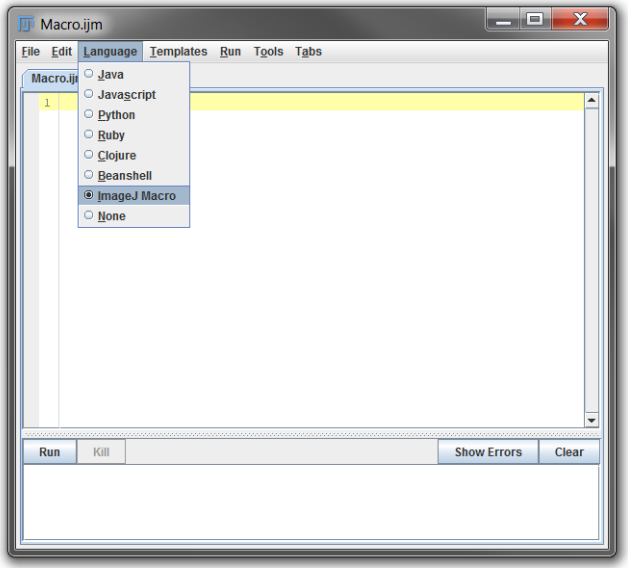


Images/Files_for_macro

Images/temp

crop_rainbow_RGB

Lots of extensibility . . .
If, else, for, while . . .



http://fiji.sc/Introduction_into_Macro_Programming

Further information

IJuser-guide – pdf covering the basics
http://imagej.nih.gov/ij/docs/user-guide.pdf

Press help and the webpage is quite descriptive on the function you are looking at, general site is here
http://imagej.nih.gov/ij/

Growing amount on the FIJI website
http://fiji.sc/wiki/index.php/Fiji