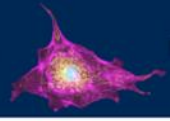
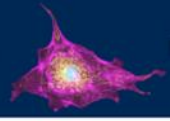


Introduction to image processing and analysis with ImageJ / Fiji. Part 4

Automating Image Analysis

Course by Dale Moulding



Session 4

1 hour 15 minutes
40 minute lecture
35 minutes exercises

Learning objectives:

- **Correct background for better analysis**
- **Develop multiple strategies for image analysis**
- **Cell counting. Manually** and automatic
- Use filters to pre-process images
- Thresholding to generate binary images and masks
- Identifying double / triple stained cells
- Explain the difference between colocalization and co-expression
- Track moving objects

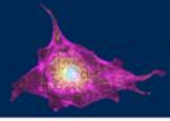


Image Processing & Analysis: Dos and Don'ts

- Manipulation should be done on a copy not the original data
- Simple adjustments to the entire image are usually acceptable
- Check journal's guidances
- Ethical guidelines: http://www.ncbi.nlm.nih.gov/pubmed/20567932
- Be honest, always describe the adjustments made
- Do not hide information (brightness, contrast, gamma)
- Do not create/destroy information (interpolation, bit-depth conversion)

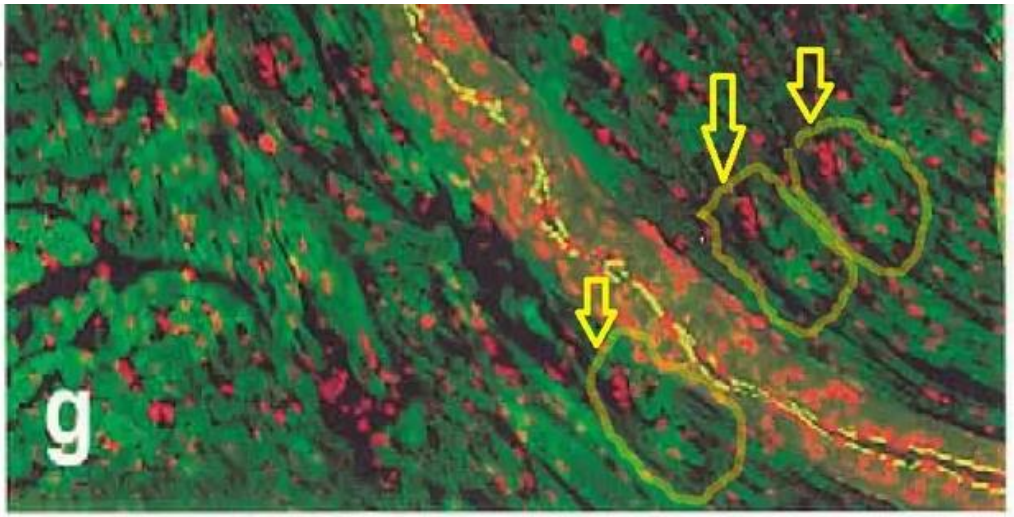
http://jcb.rupress.org/cgi/content/full/166/1/11 JCB article about image manipulation.

http://microscopy.arizona.edu/learn/digital-image-ethics
Nice article, picking the best image is not the same as picking the image that fits your hypothesis!

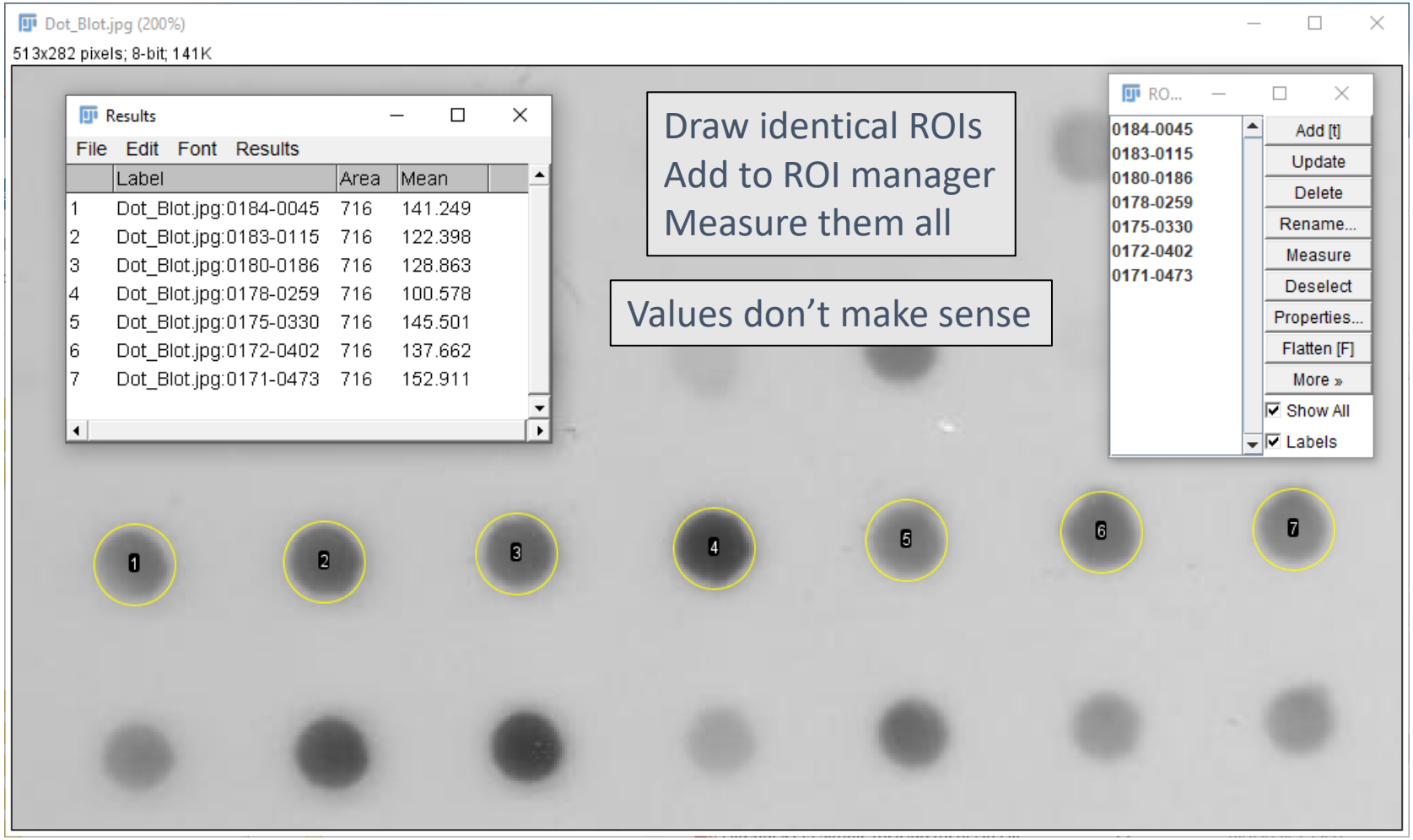
Image Processing & Analysis: Dos and Don'ts

Journals actively check submitted images for tell tale signs of manipulation.
Duplications, deletions, smudge tool, image noise, background.

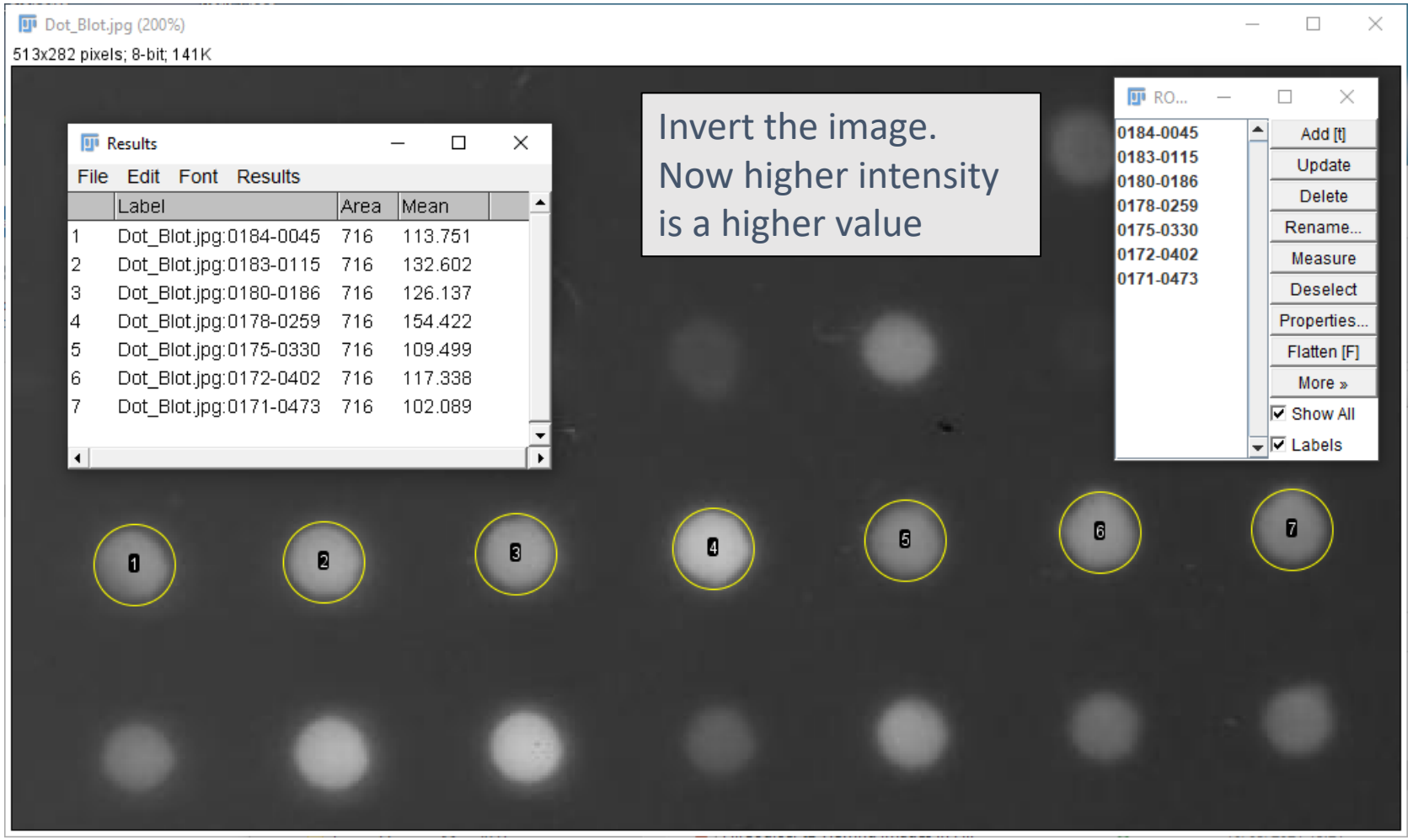
https://pubpeer.com/



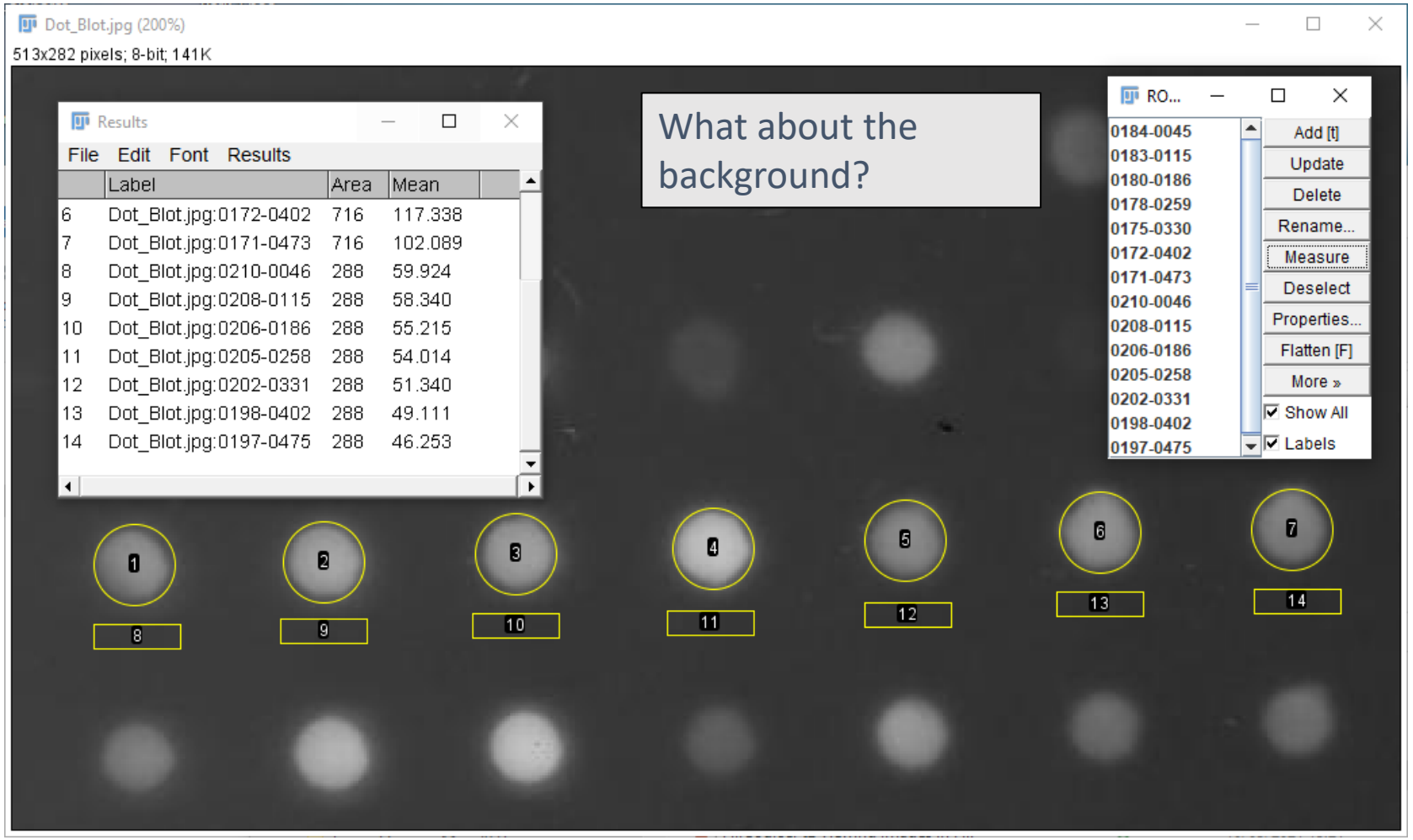
Multiple ways to make the same measurements



Multiple ways to make the same measurements

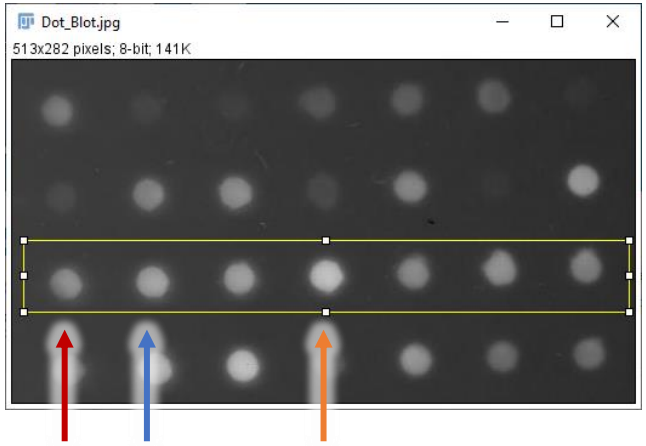


Multiple ways to make the same measurements

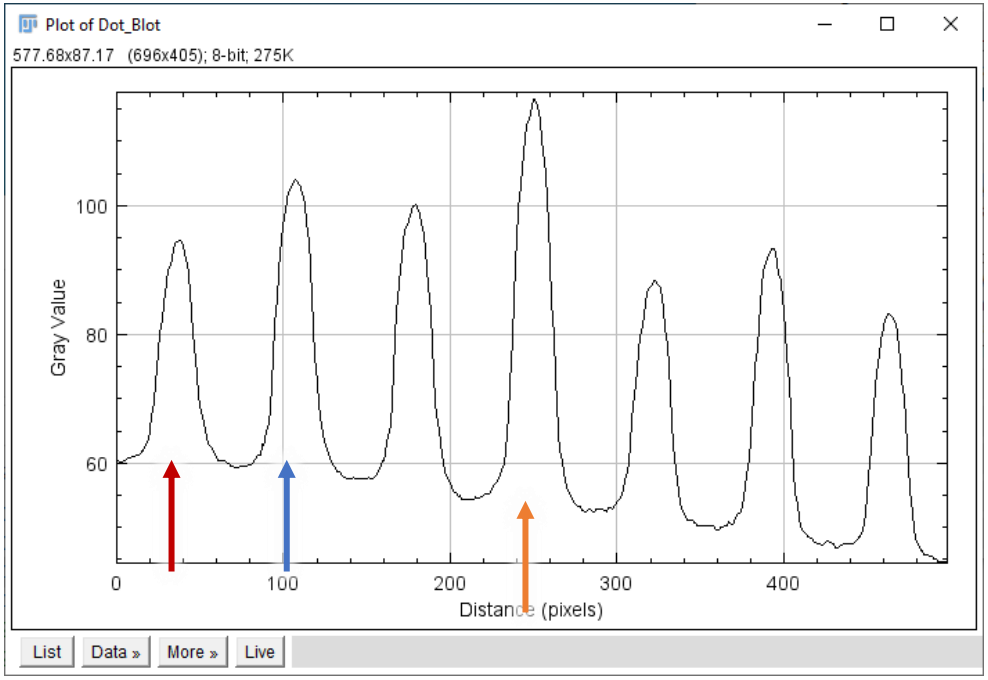


Multiple ways to make the same measurements

Analyze / Plot Profile

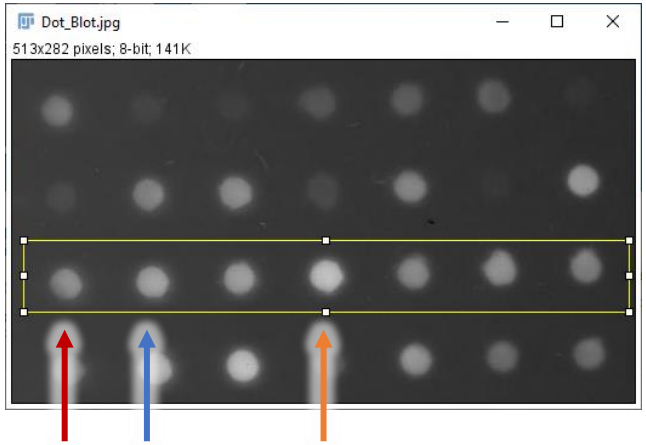


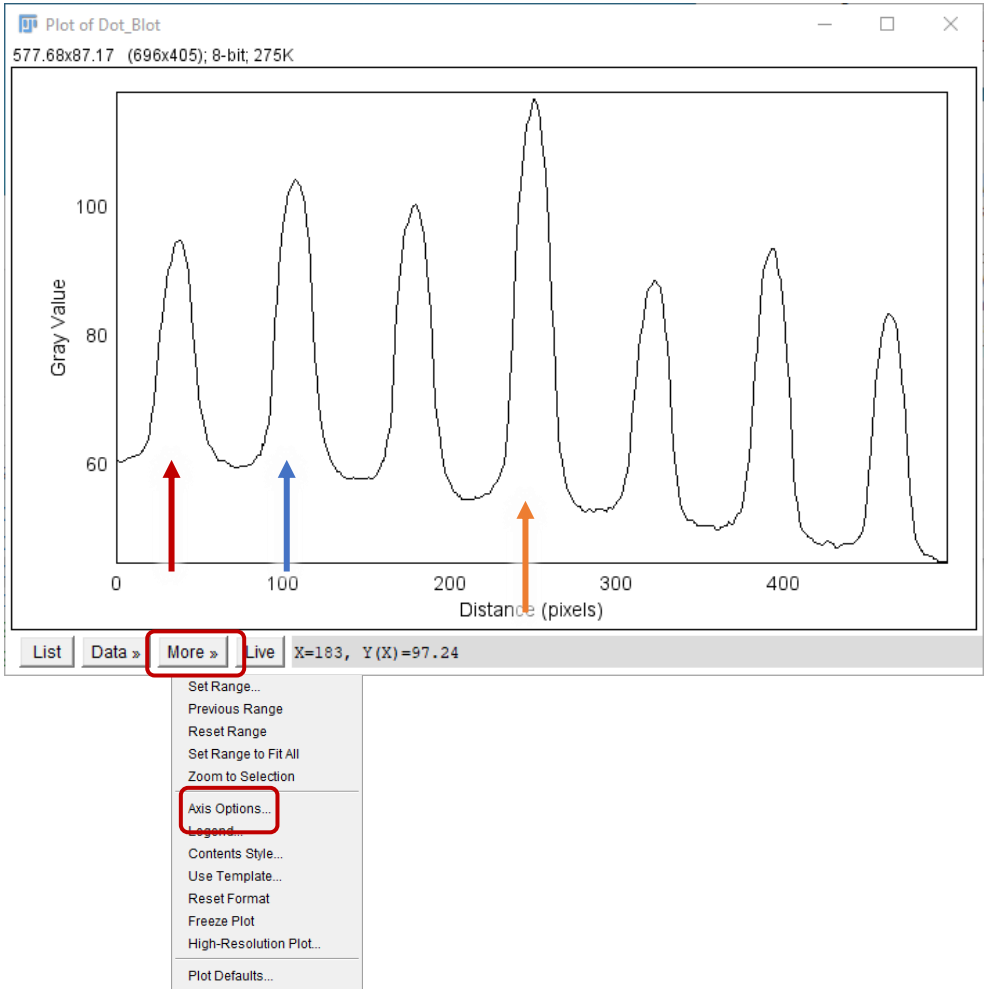
Area under each peak is proportional to the intensity of each spot



Multiple ways to make the same measurements

Analyze / Plot Profile

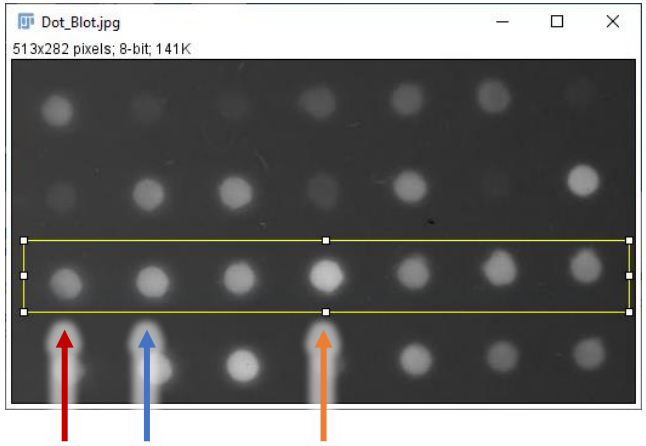




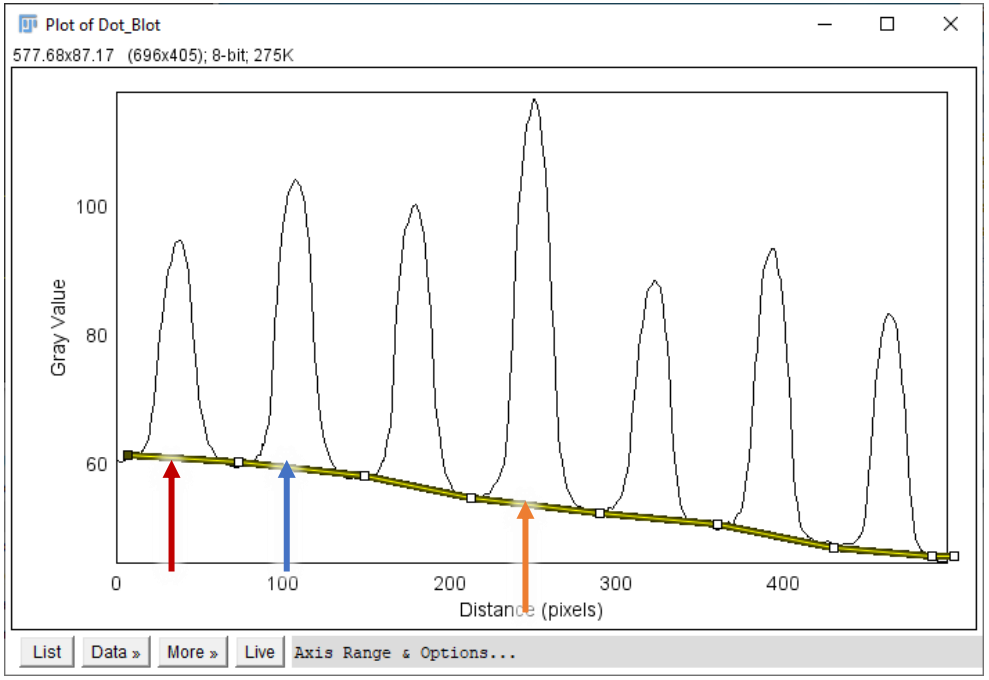
More>> Axis options.
Remove gridlines.

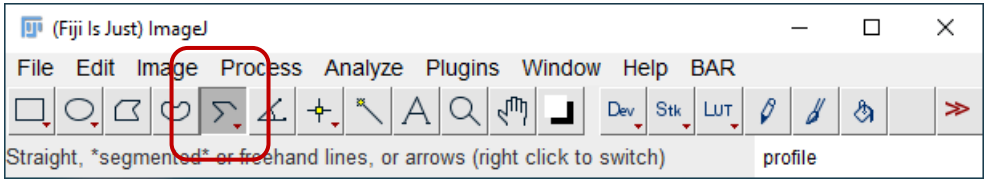
Multiple ways to make the same measurements

Analyze / Plot Profile



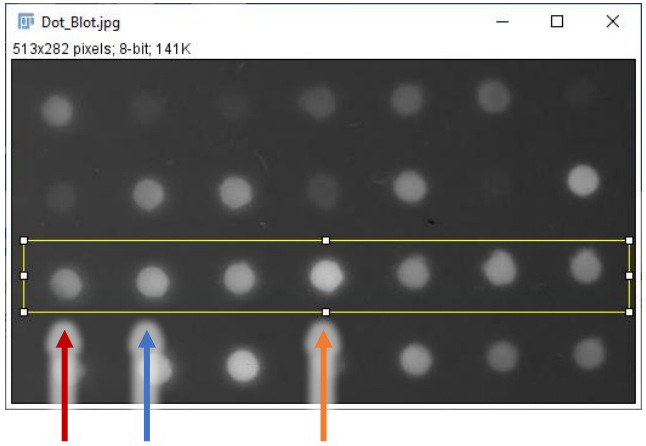
Use the line tool (switch to segmented line). Draw along the base of each peak. Press ‘Delete’ to make a black line.



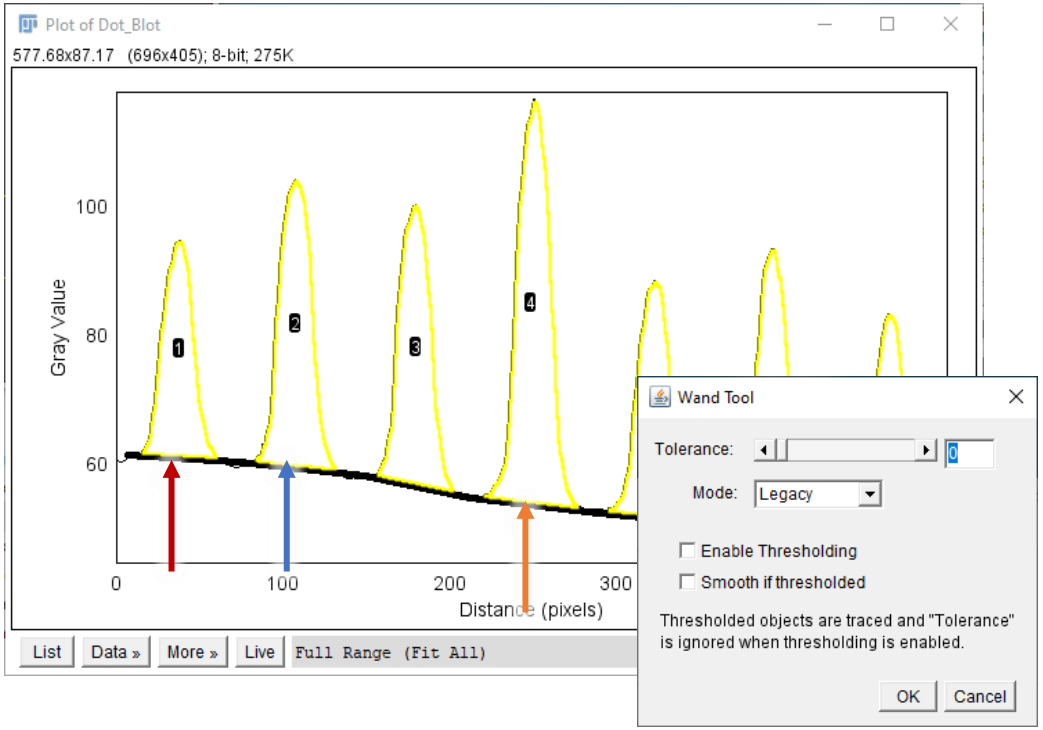


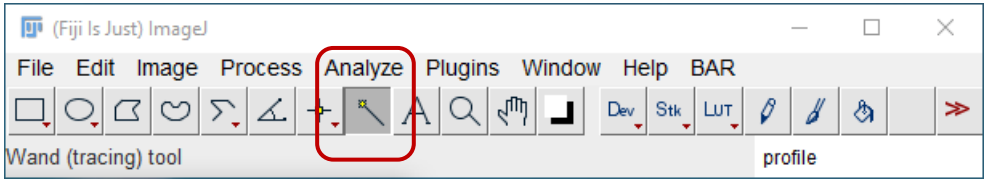
Multiple ways to make the same measurements

Analyze / Plot Profile



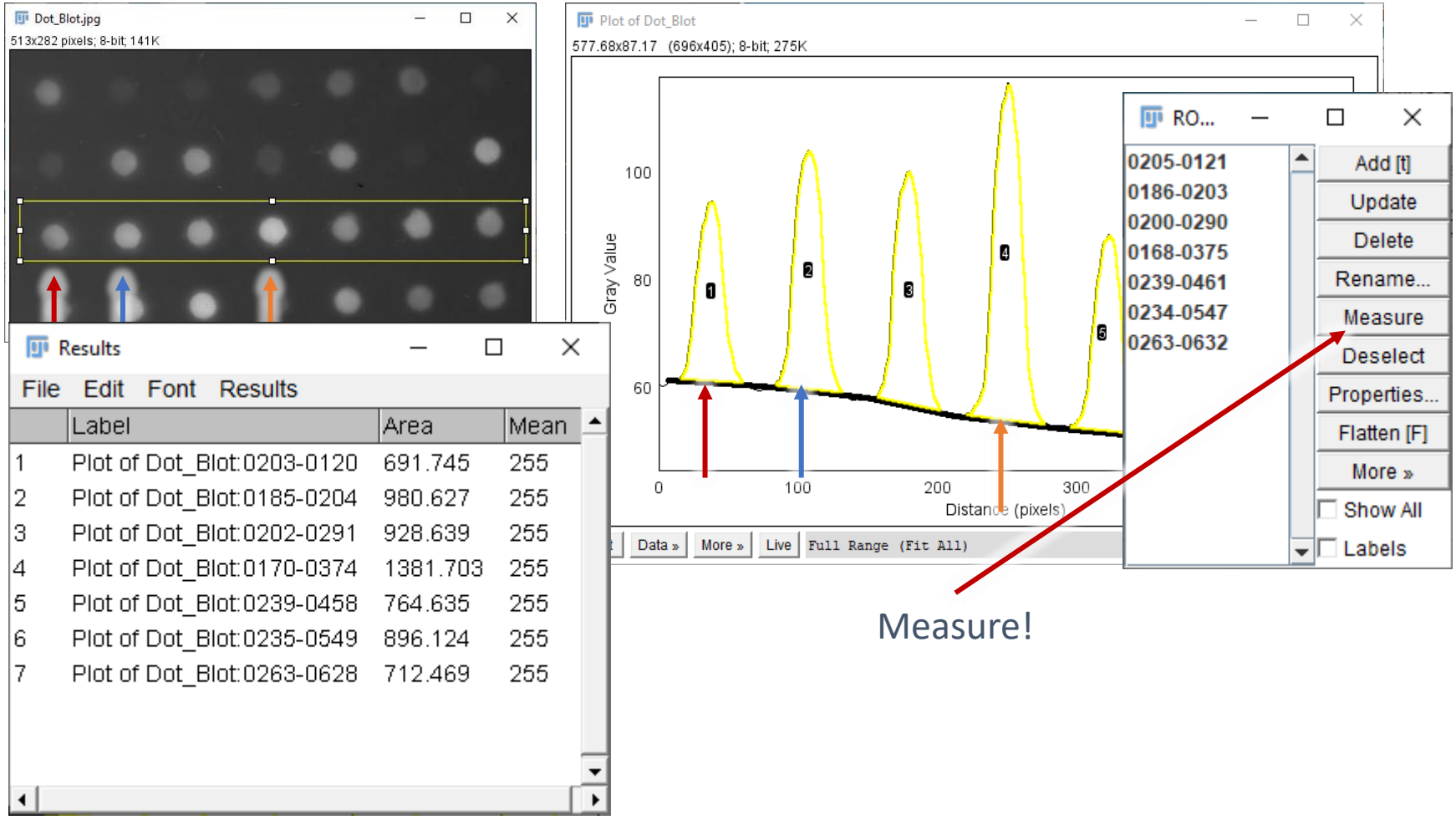
Use Wand tool (tolerance = 0).
Click in each peak.
Press ‘T’ to add to ROI manager.





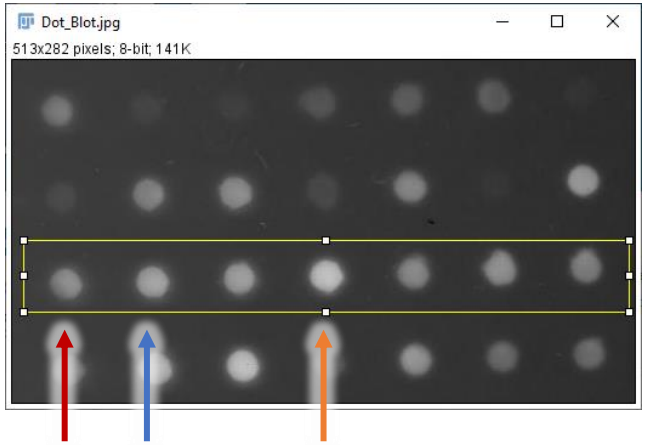
Multiple ways to make the same measurements

Analyze / Plot Profile

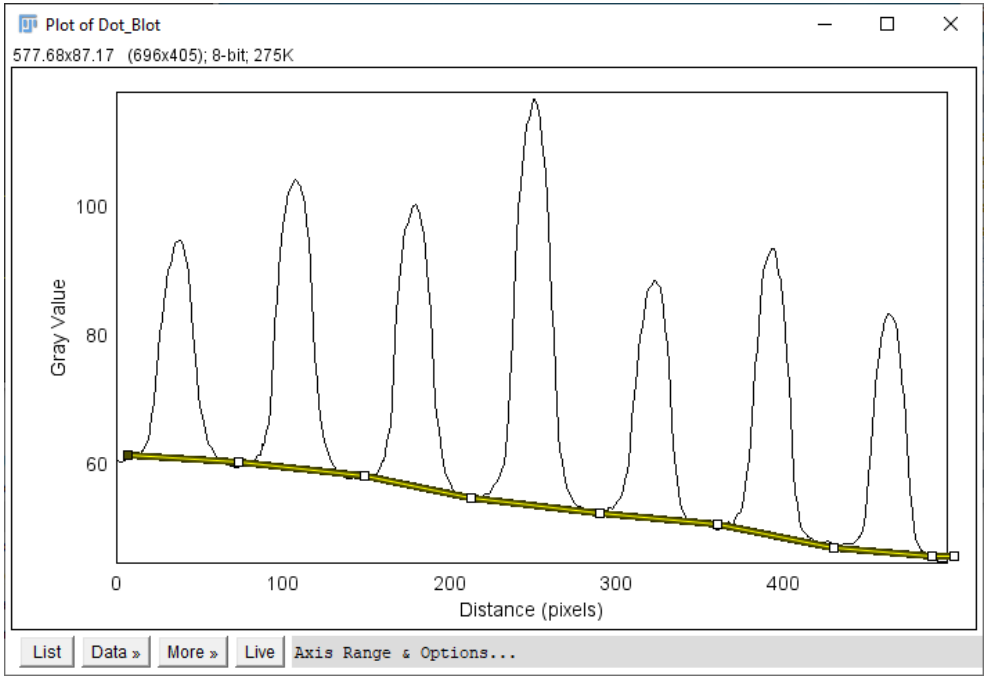


Multiple ways to make the same measurements

Analyze / Plot Profile

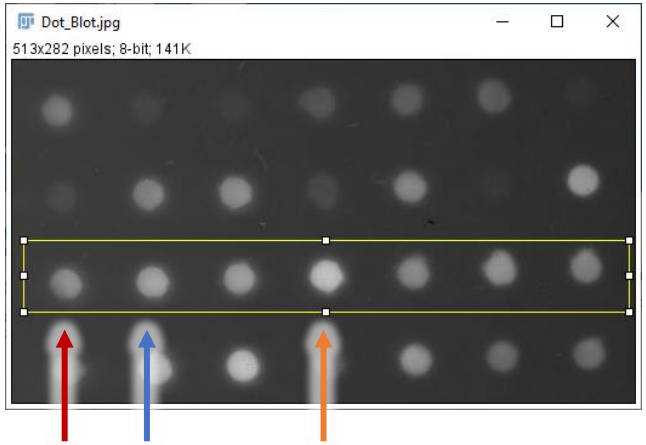


Did we just guess the background?

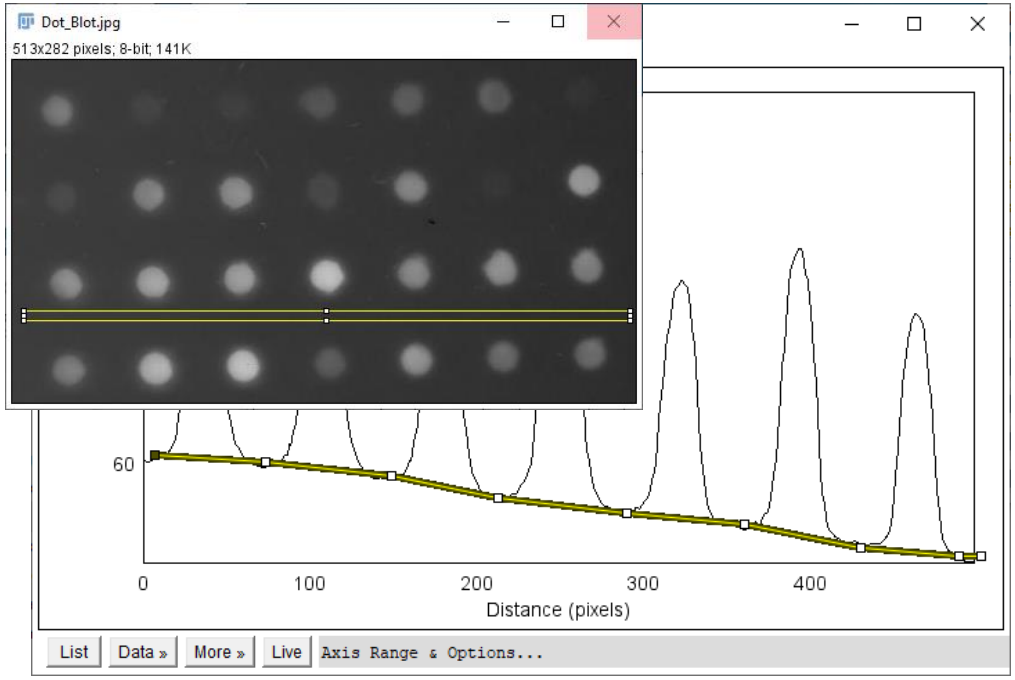


Multiple ways to make the same measurements

Analyze / Plot Profile

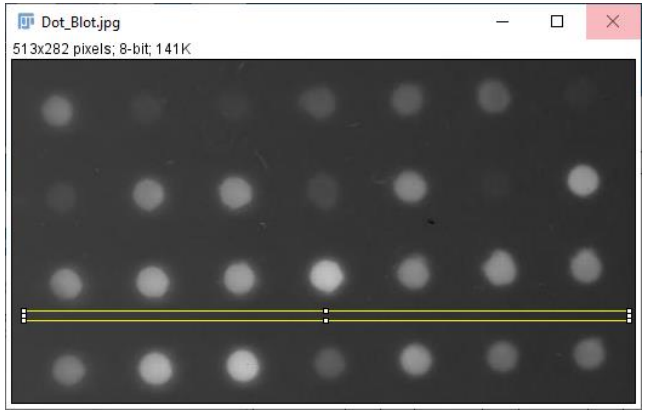


Lets measure that too…

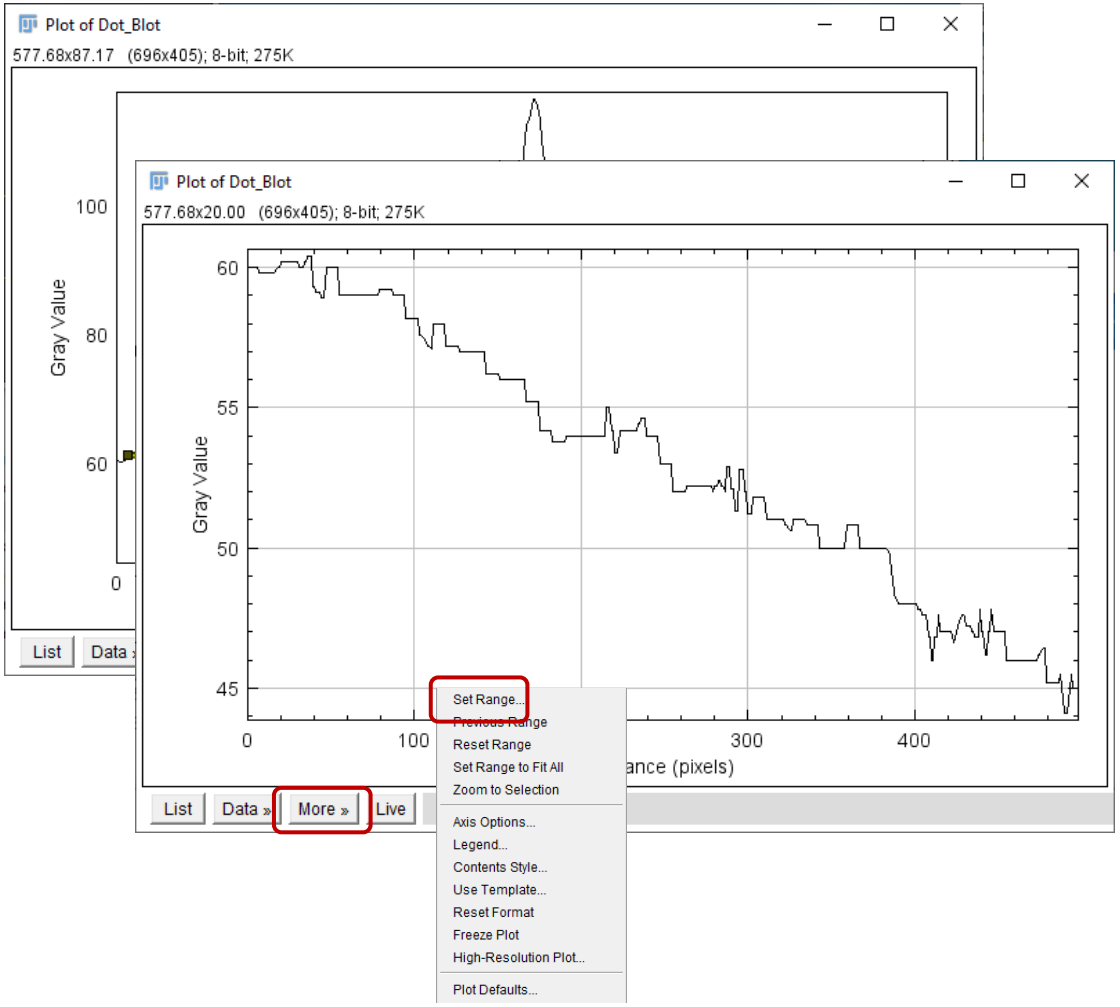


Multiple ways to make the same measurements

Analyze / Plot Profile

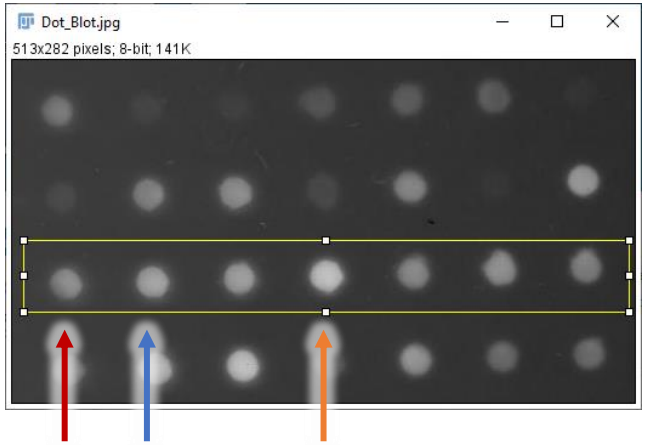


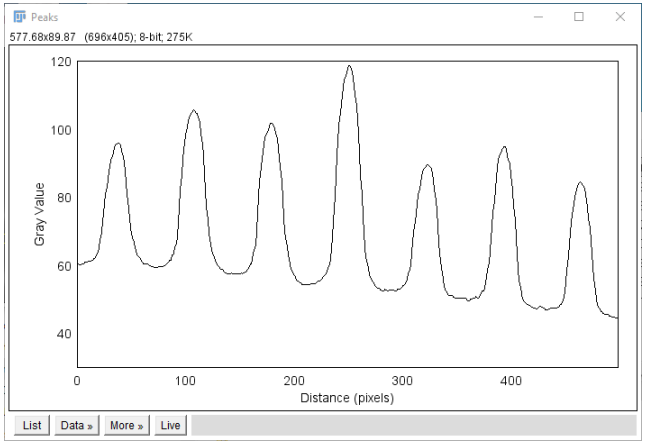
Axis has different scales.
More>> set range…

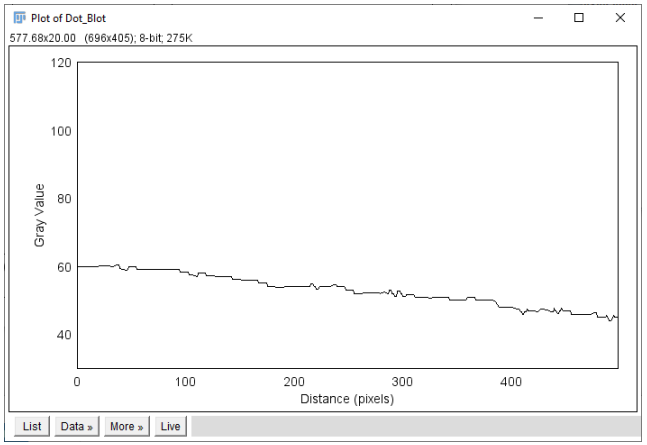


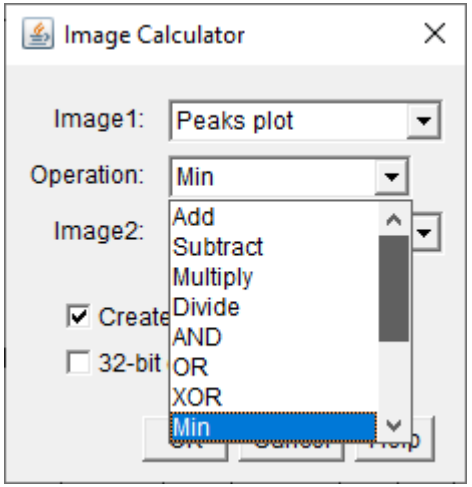
Multiple ways to make the same measurements

Analyze / Plot Profile









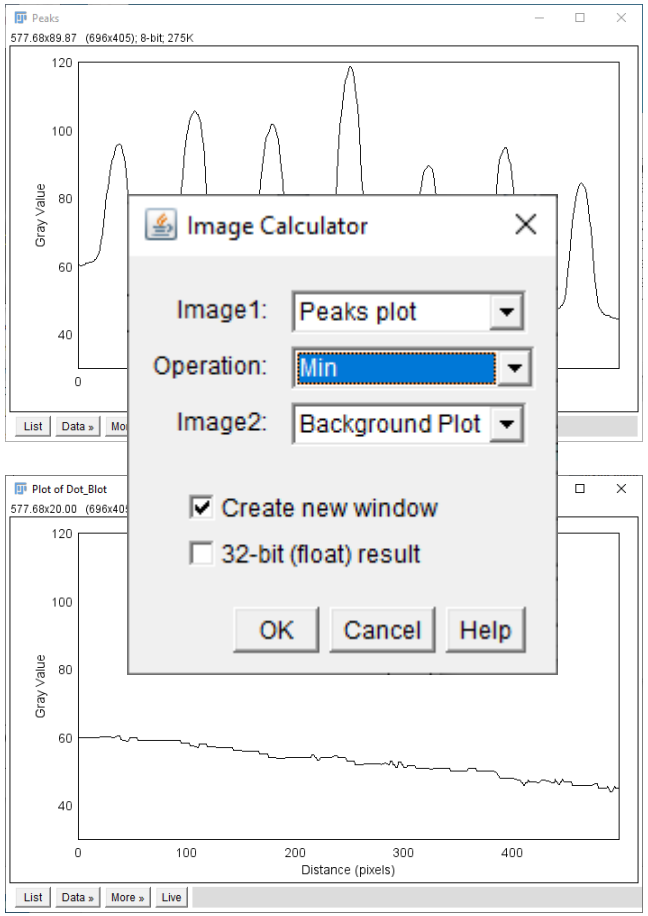
Combine the two images to have the background line below the peaks…

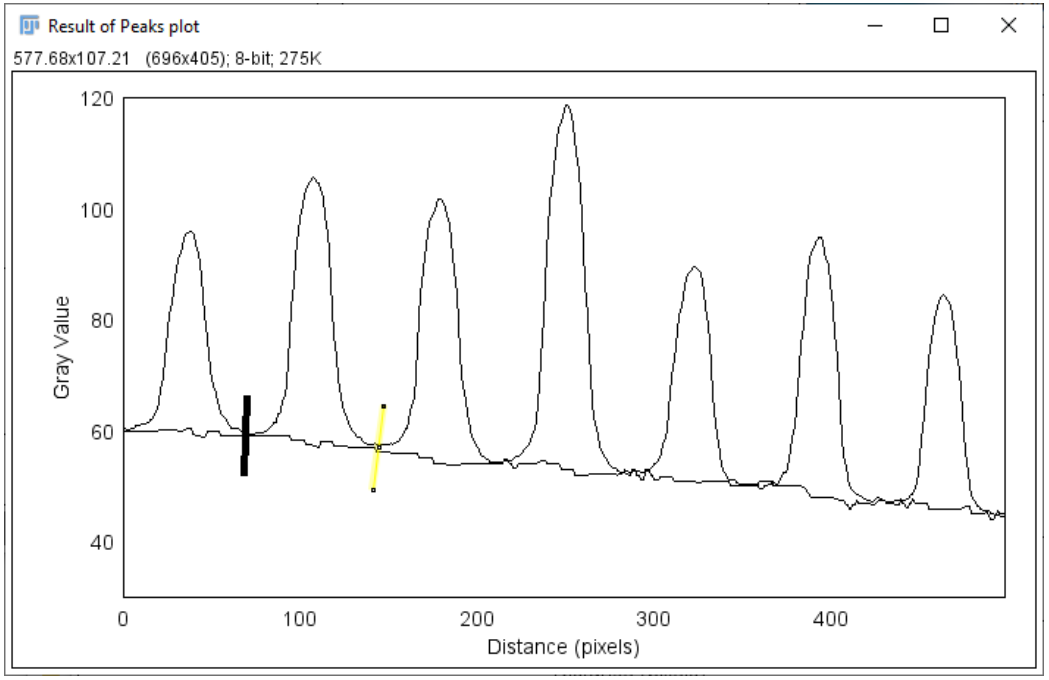
Process / Image calculator…

Combine the two images with the Operator ‘Min’. The result will have all the low value (minimum / black) pixels!

Multiple ways to make the same measurements

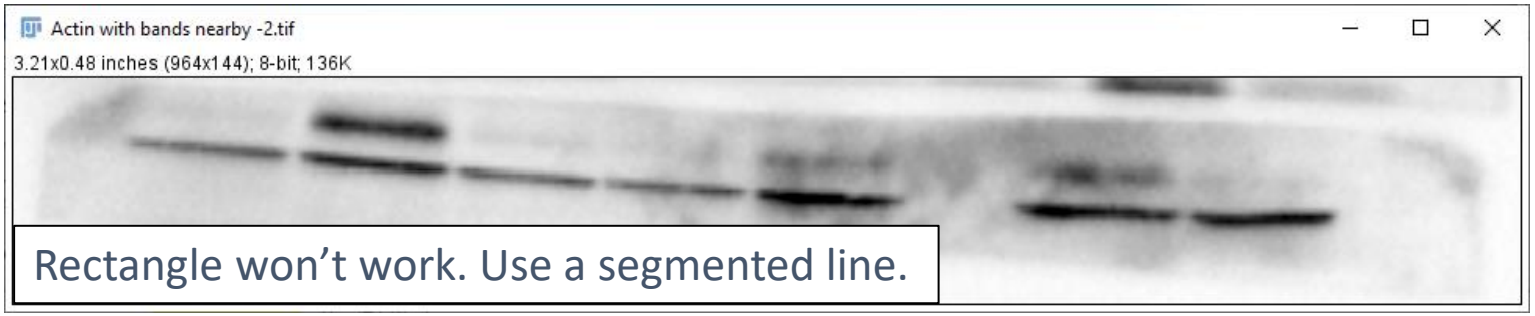
Analyze / Plot Profile

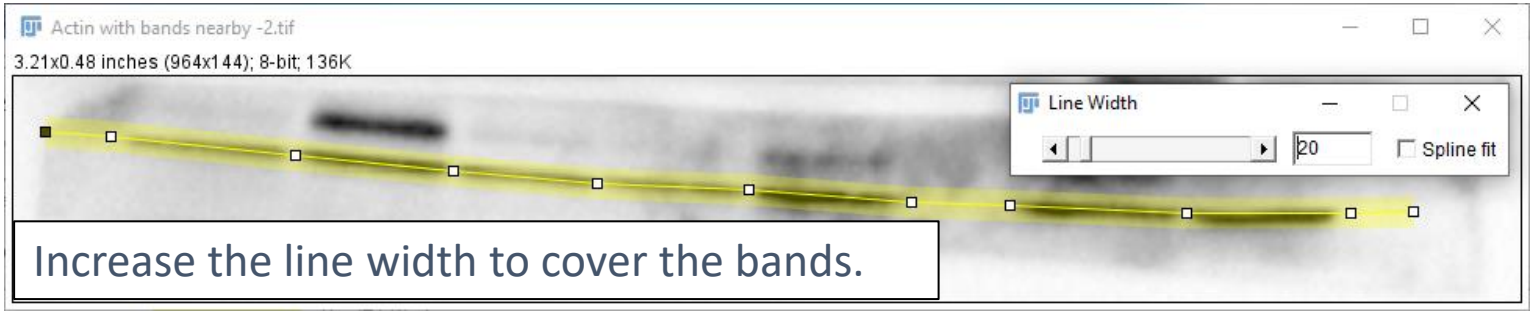


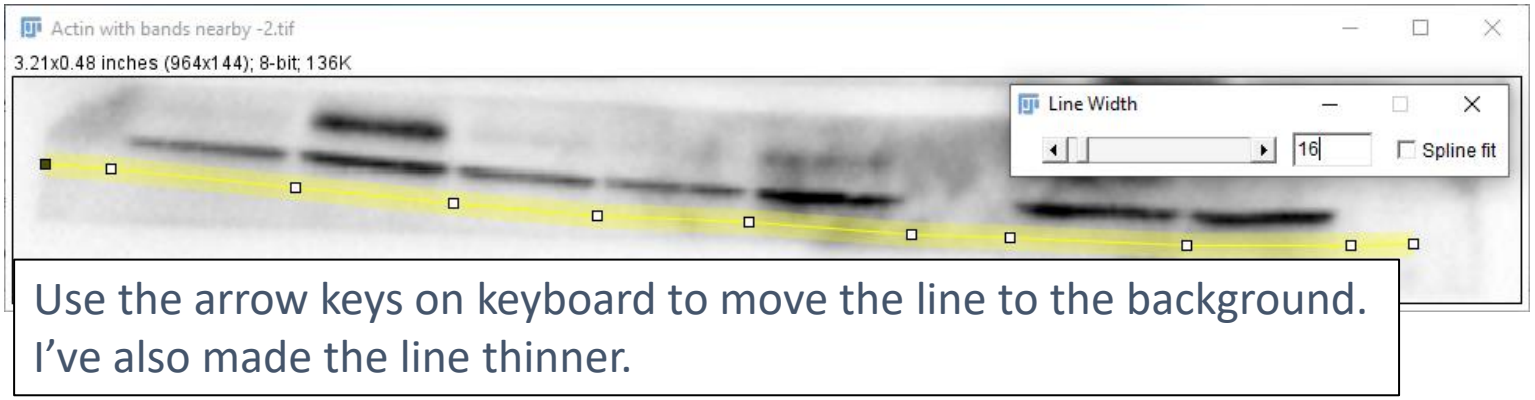


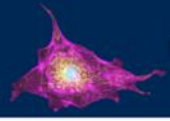
If the background line is too low, draw some lines to make each peak a single region.
Measure as before: Wand & ROI manager.

Exercise to measure western blots (15 minutes)









Background corrections

Even background across the image

Image /adjust > Brightness-Contrast

Uneven background

Process / Substract Background (rolling ball algorithm)

Flat field correction (brightfield)

Process / Calculator Plus (requires an image of just background)

Many other ways …

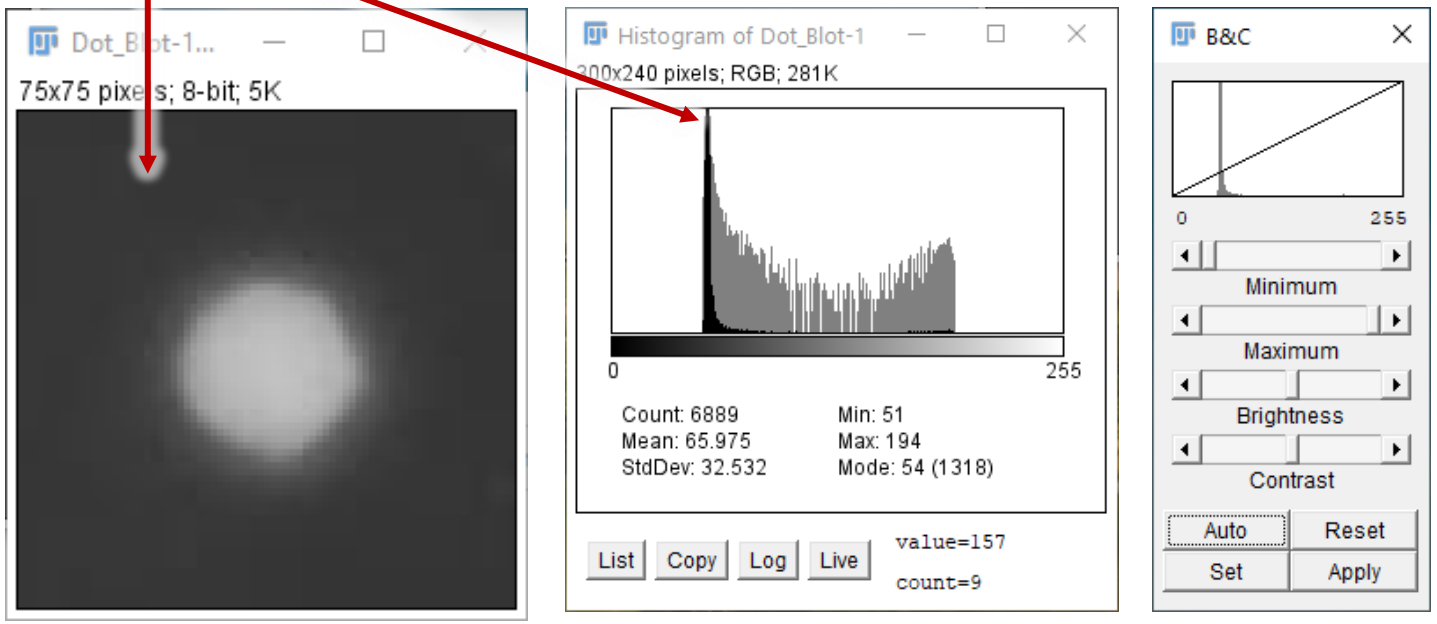
http://imagejdocu.tudor.lu/doku.php?id=howto:working:how_to_correct_background_illumination_in_brightfield_microscopy

Background corrections

Even background across the image

Image /adjust > Brightness-Contrast

Background ~ 54

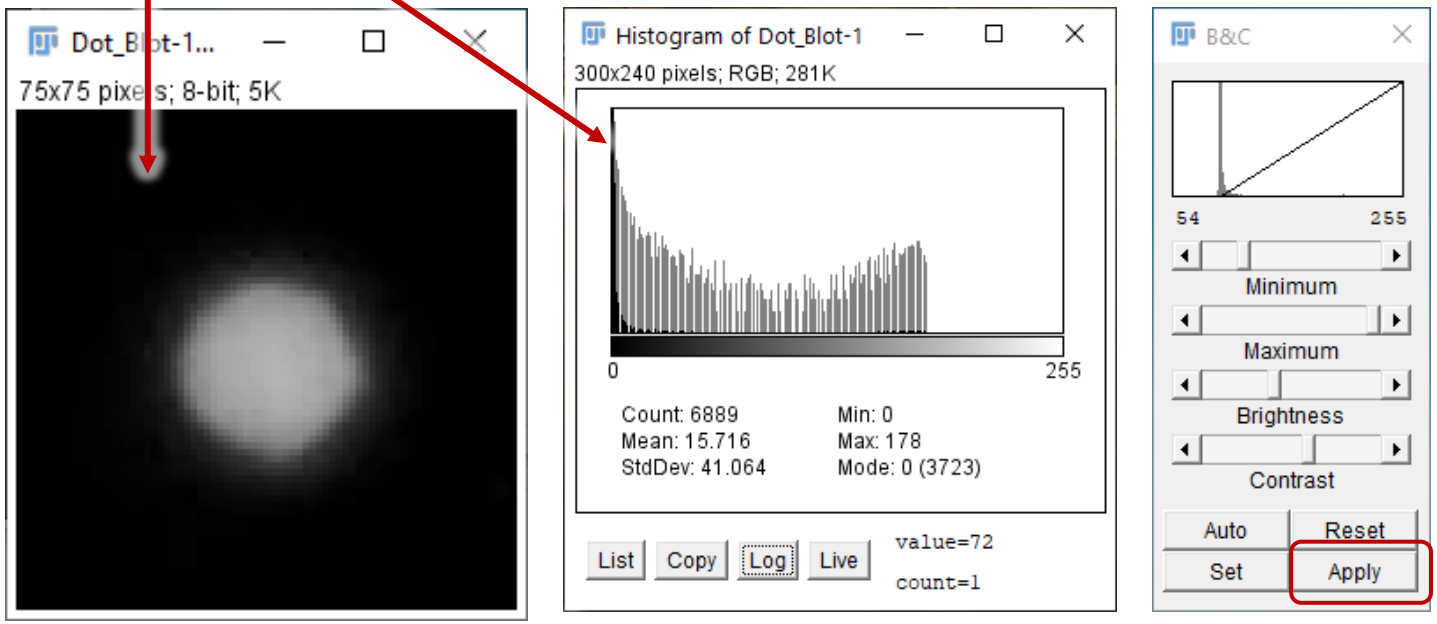


Background corrections

Even background across the image

Image /adjust > Brightness-Contrast

Background was 54, now 0.



You have changed the data, work on a duplicate.

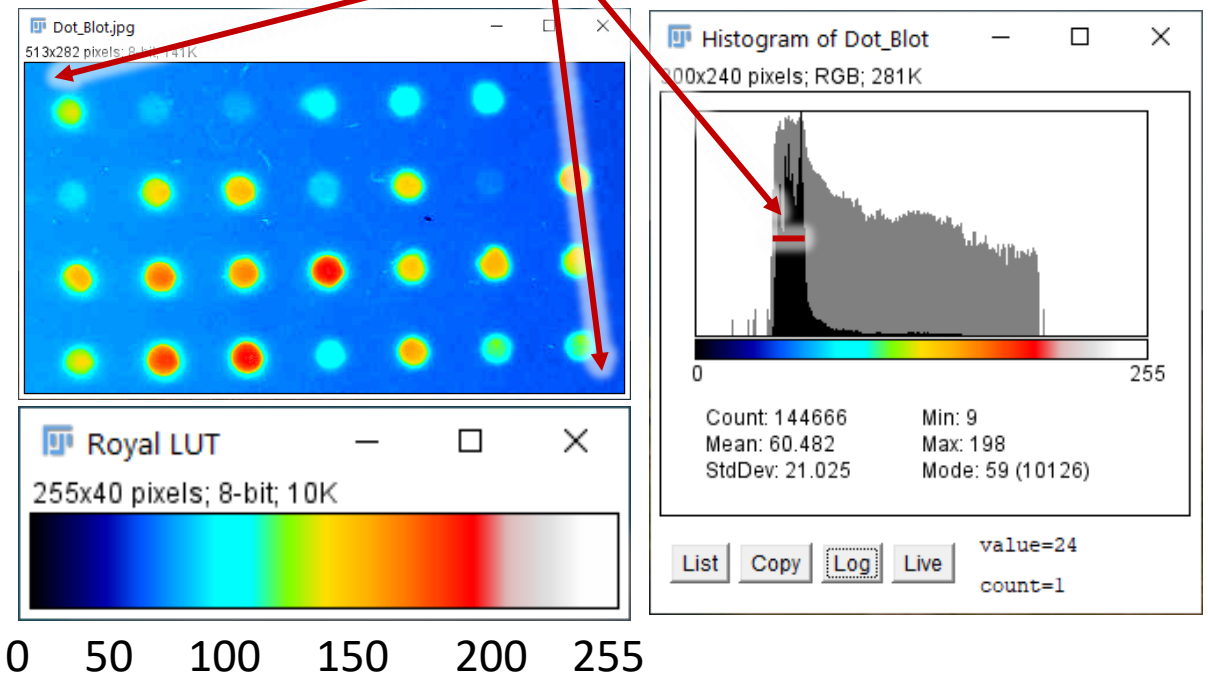
Do the exact same procedure to all images in an analysis set.

Background corrections

Uneven background

Process / Substract Background (rolling ball algorithm)

Background range ~ 60 - 46.

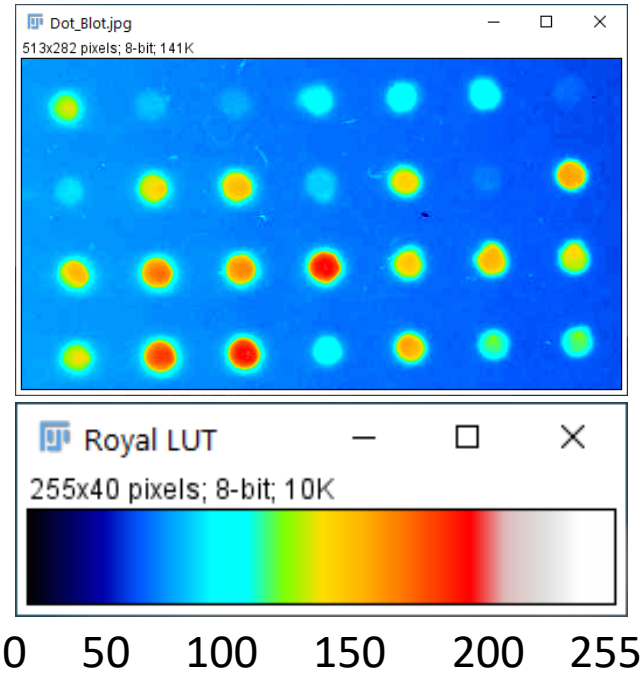


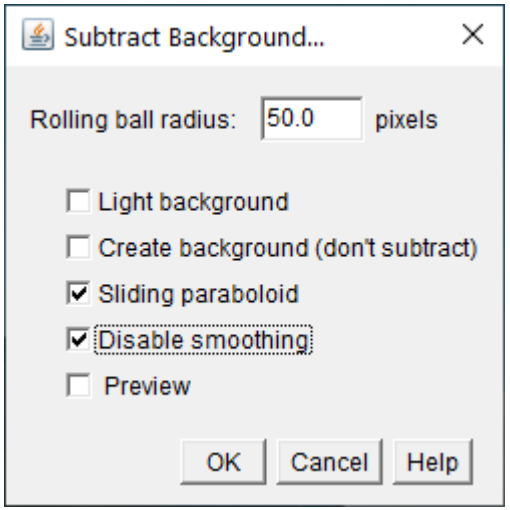
Background corrections

Uneven background

Process / Substract Background (rolling ball algorithm)

Background range ~ 60 - 46.





- Radius of ball
(bigger than image features)

- Light / dark background?

- Sliding paraboloid
Recommended, more accurate, avoids edge artefacts

- Smoothing (3x3 average)
Best disabled

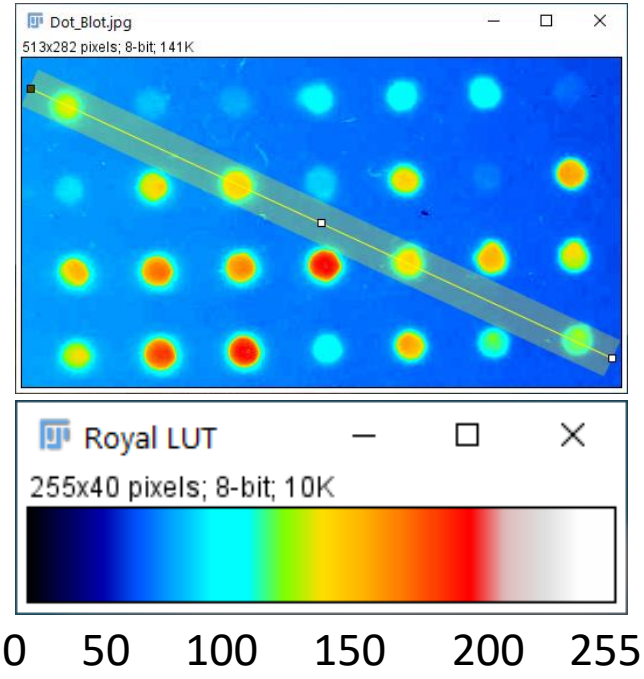
- Preview

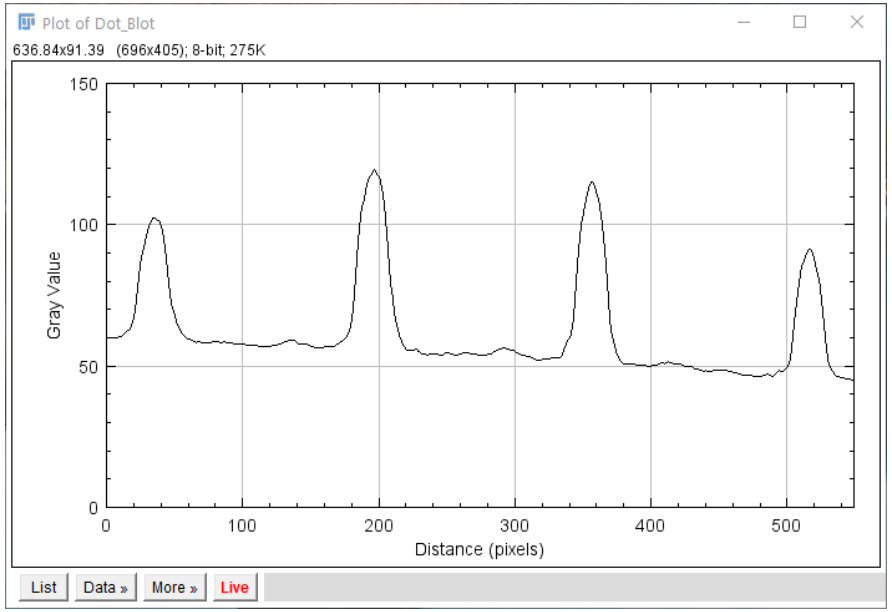
Background corrections

Uneven background

Process / Substract Background (rolling ball algorithm)

Background range ~ 60 - 46.



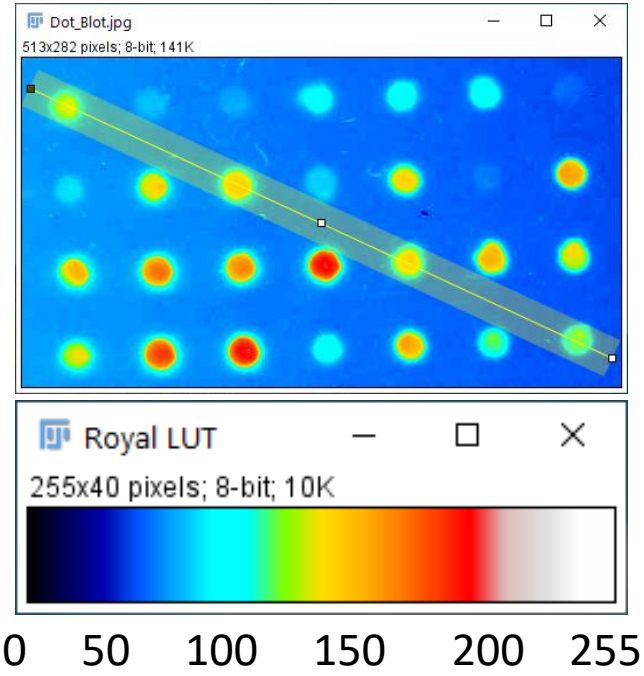


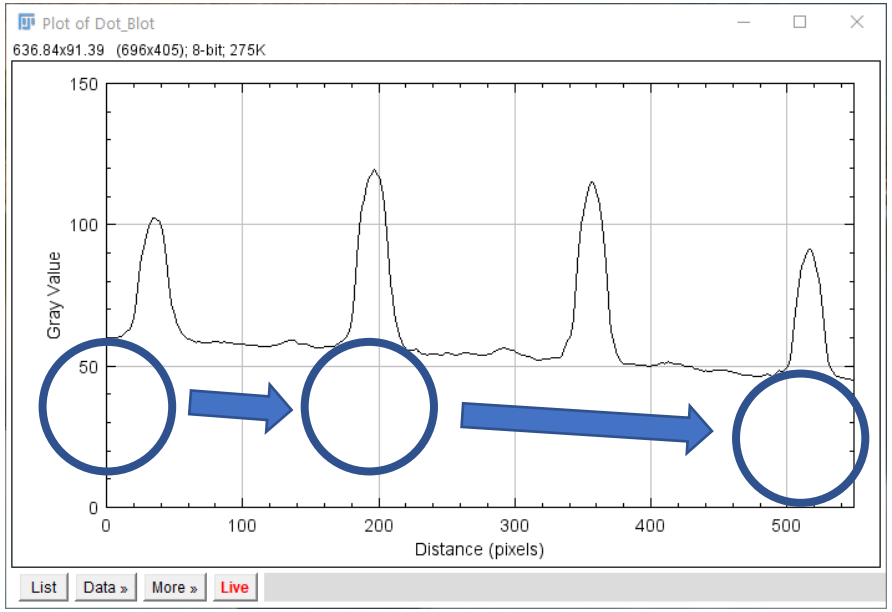
Background corrections

Uneven background

Process / Substract Background (rolling ball algorithm)

Background range ~ 60 - 46.





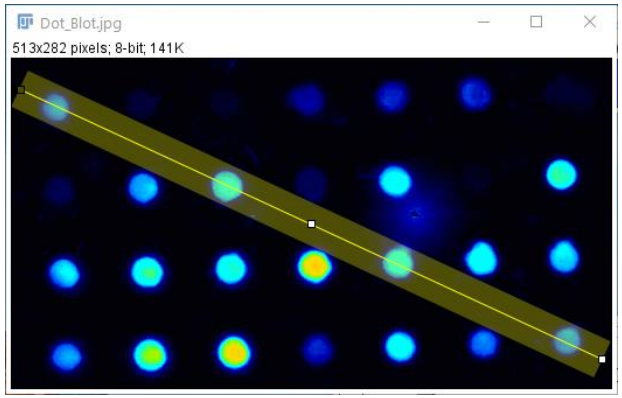
Rolling ball 

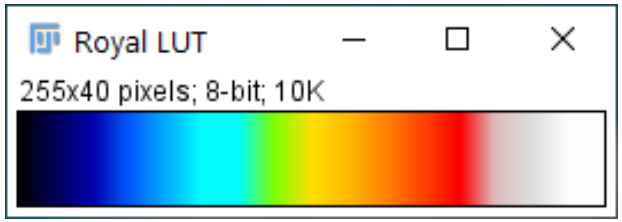
Background corrections

Uneven background

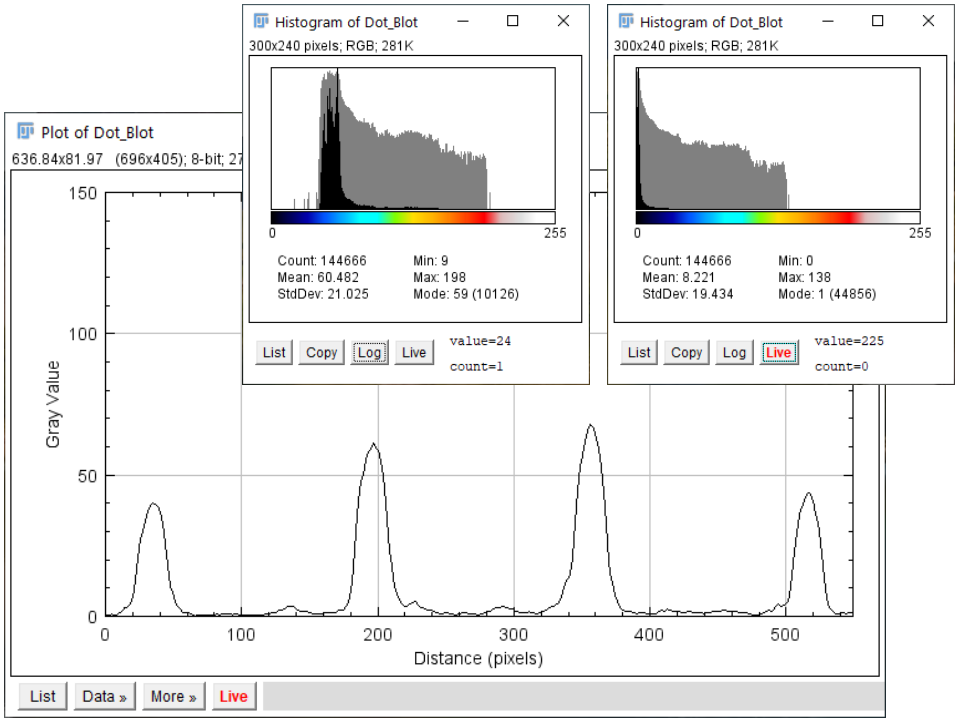
Process / Substract Background (rolling ball algorithm)

Background range ~ 1-3.





0 50 100 150 200 255



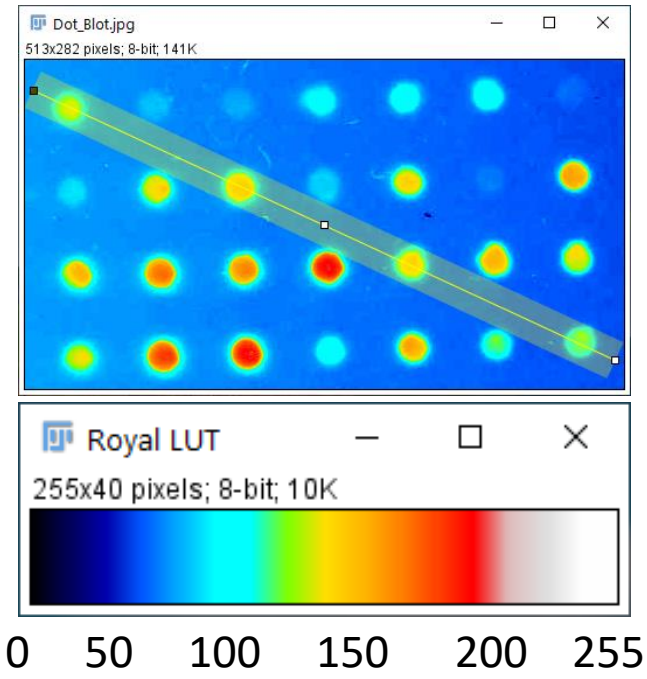
Background corrections

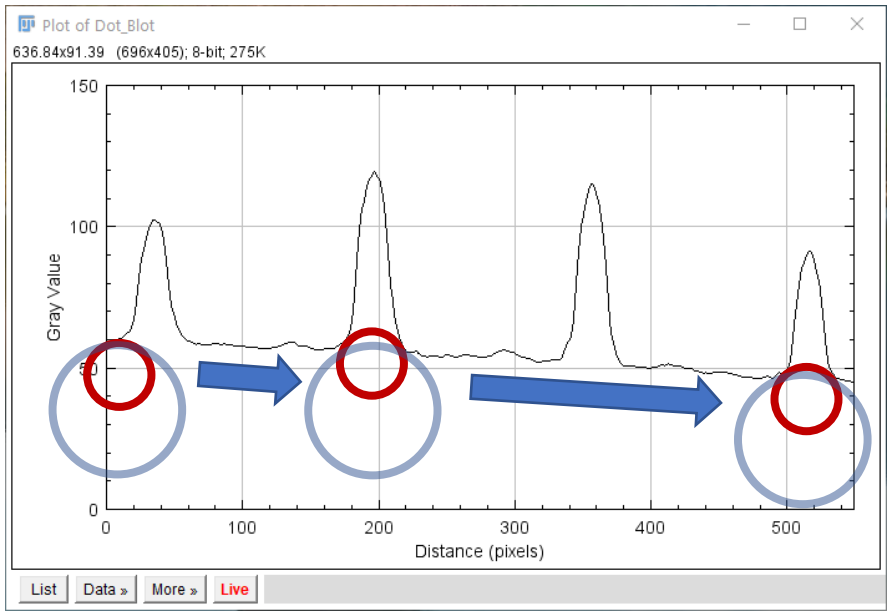
Uneven background

Process / Substract Background (rolling ball algorithm)

Background range ~ 60 - 46.

Don't make the radius too small





Rolling ball

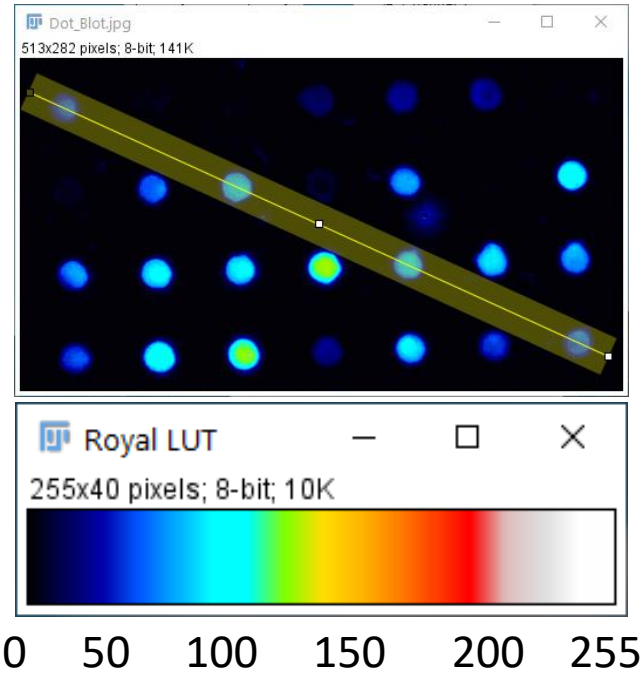
Background corrections

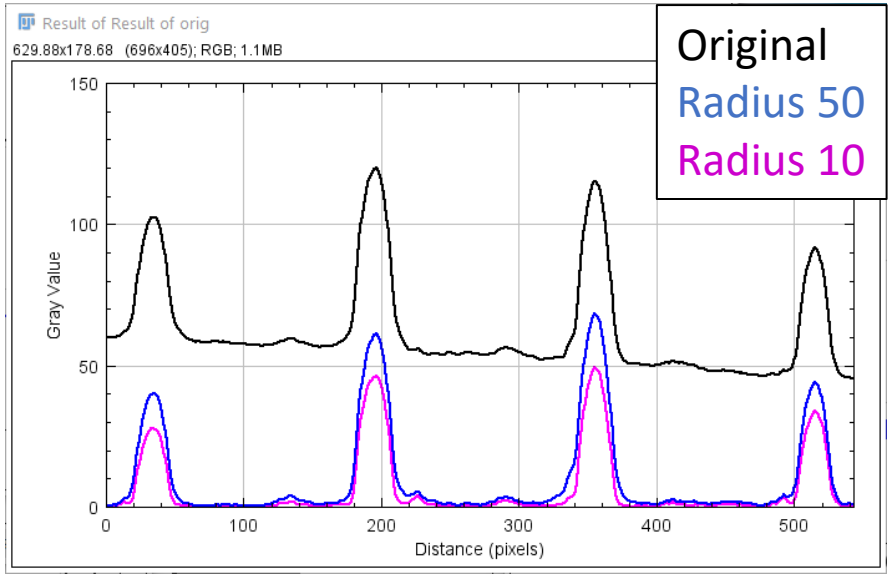
Uneven background

Process / Substract Background (rolling ball algorithm)

Background range ~ 60 - 46.

Don't make the radius too small

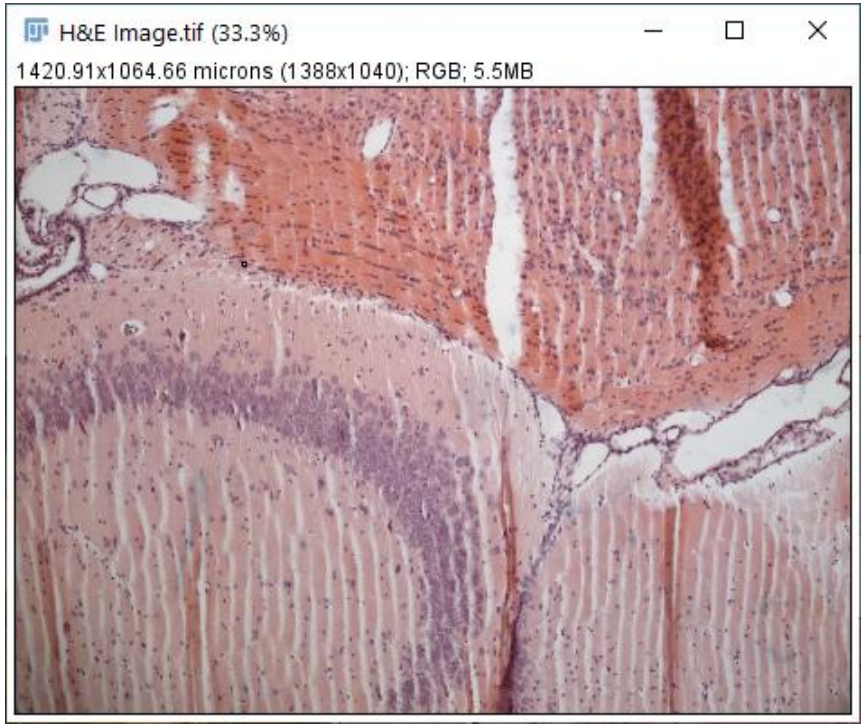




Background corrections

Flat field correction (brightfield)

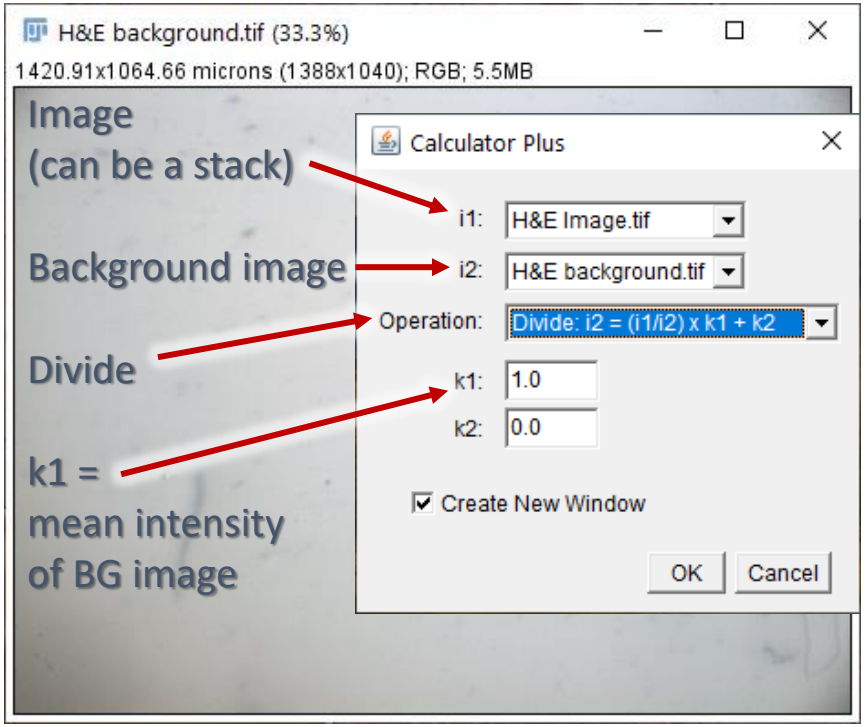
Process / Calculator Plus (requires an image of just background)

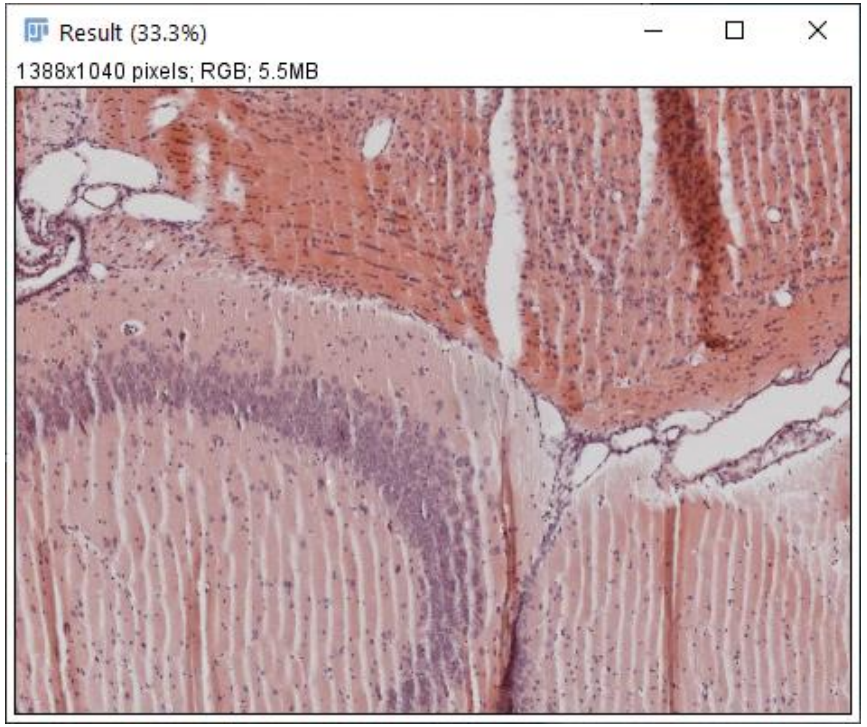


Background corrections

Flat field correction (brightfield)

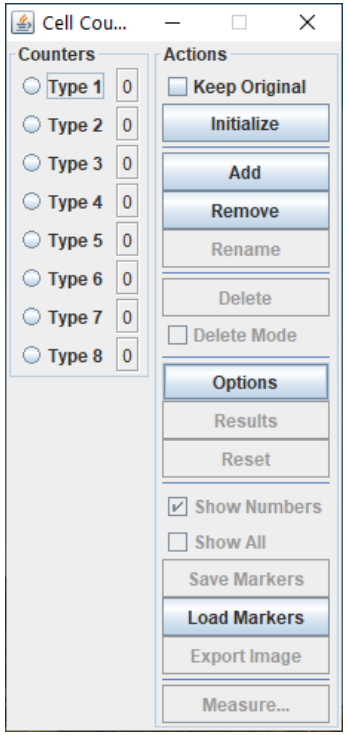
Process / Calculator Plus (requires an image of just background)

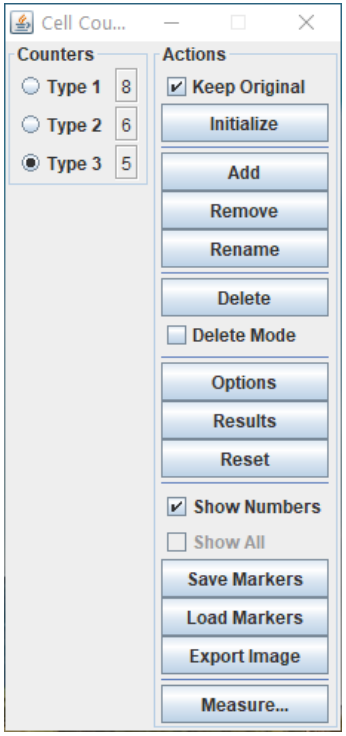




Manual Counting

Plugins / Analyze / Cell Counter





Add / remove Types

Rename types

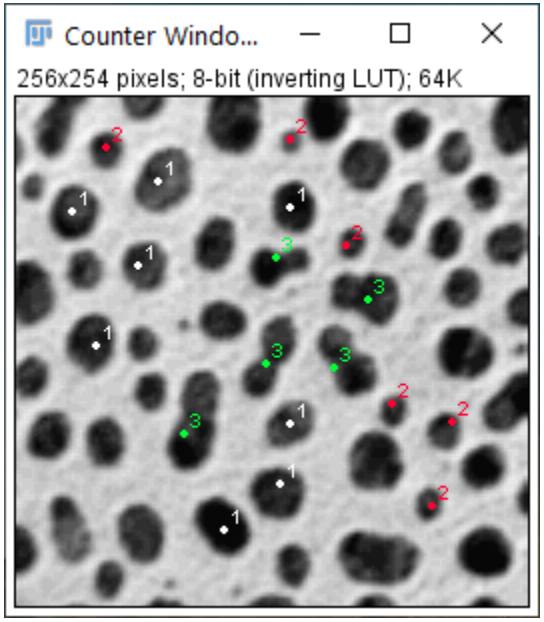
Delate last count /
Delete mode (click to delete)

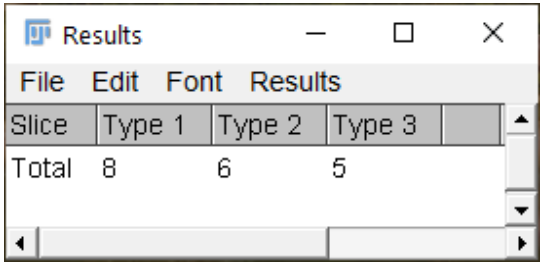
Change counter colours

Results

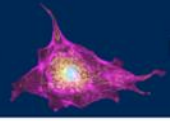
Save / Load

Measure… more results
(location & intensity)





Slow. Accurate? No double counting. Bias?



Exercises Session 4 – Automating measurements in Fiji

Making measurements and adjusting for image background…

9) Measure a dot or Western blot
   - Dot blot is easier as a first go

10) Rolling ball background correction
   - You can use the dot blot or Cell colony image
   - Histograms and Profile plots (in live mode)

11) Background corrections with a known background
   - Use the ***Process / Calculator Plus*** plugin

12) Manual cell counting