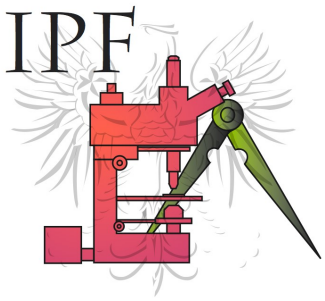
Quantitative Imaging for Colocalization Analysis

Spectroscopy,

not Photography

CBG
Max Planck Institute
of Molecular Cell Biology
and Genetics



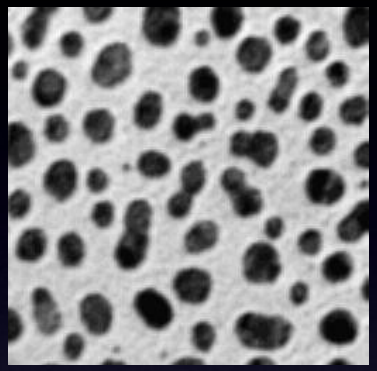
What is an Image anyway..?

- 1st: Go to Basics course slides

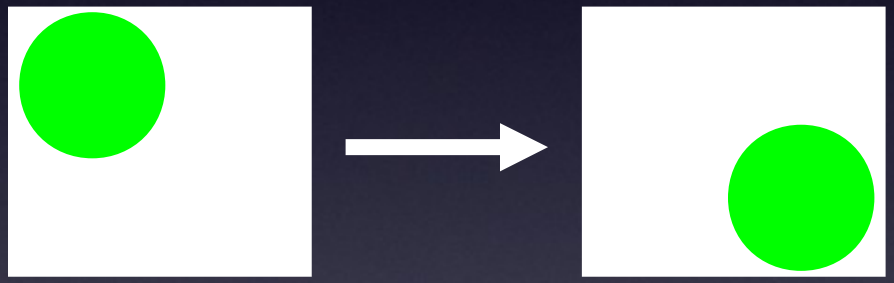
  Spatial Digitisation

Experimental Design - First Think...

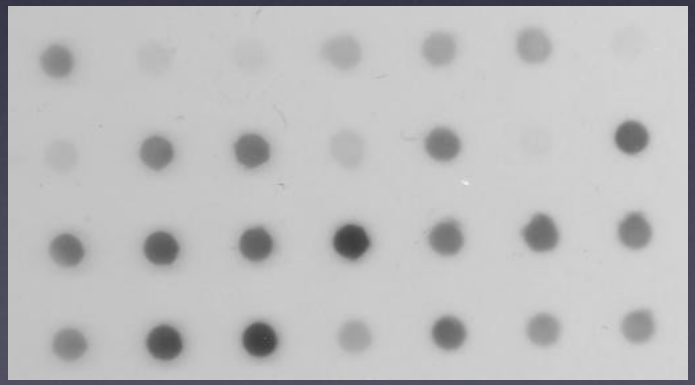
- Quantitative Experiments?
  - Am I trying to measure the size/shape of some type of object(s)



  - Am I trying to see movement over time?

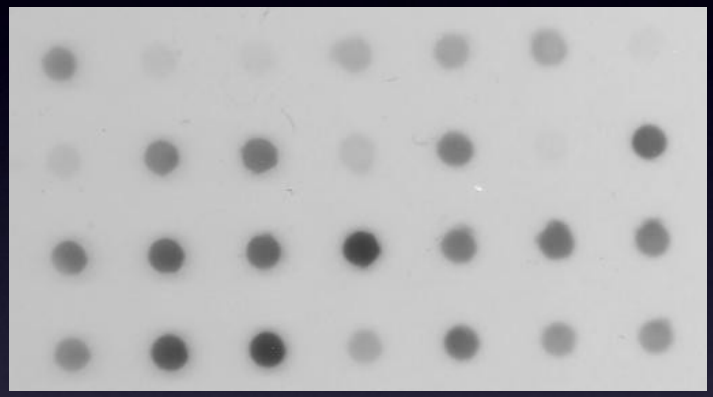


  - Am I trying to measure a number, amount or concentration?

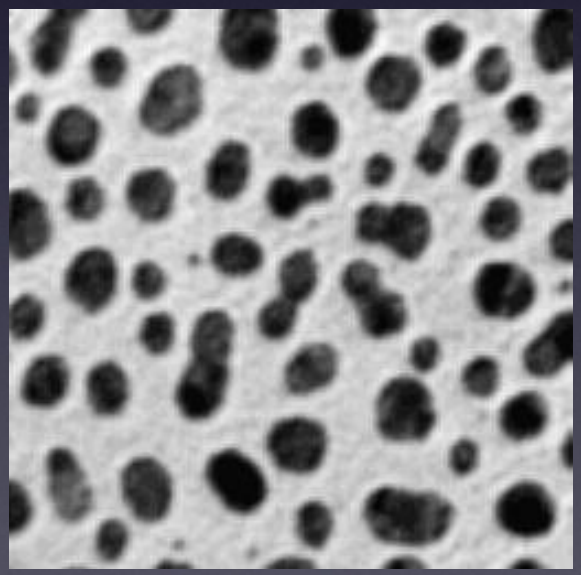


Am I trying to measure the number of some type of object?

- Can I define how my objects appear in images?
- Segmentation

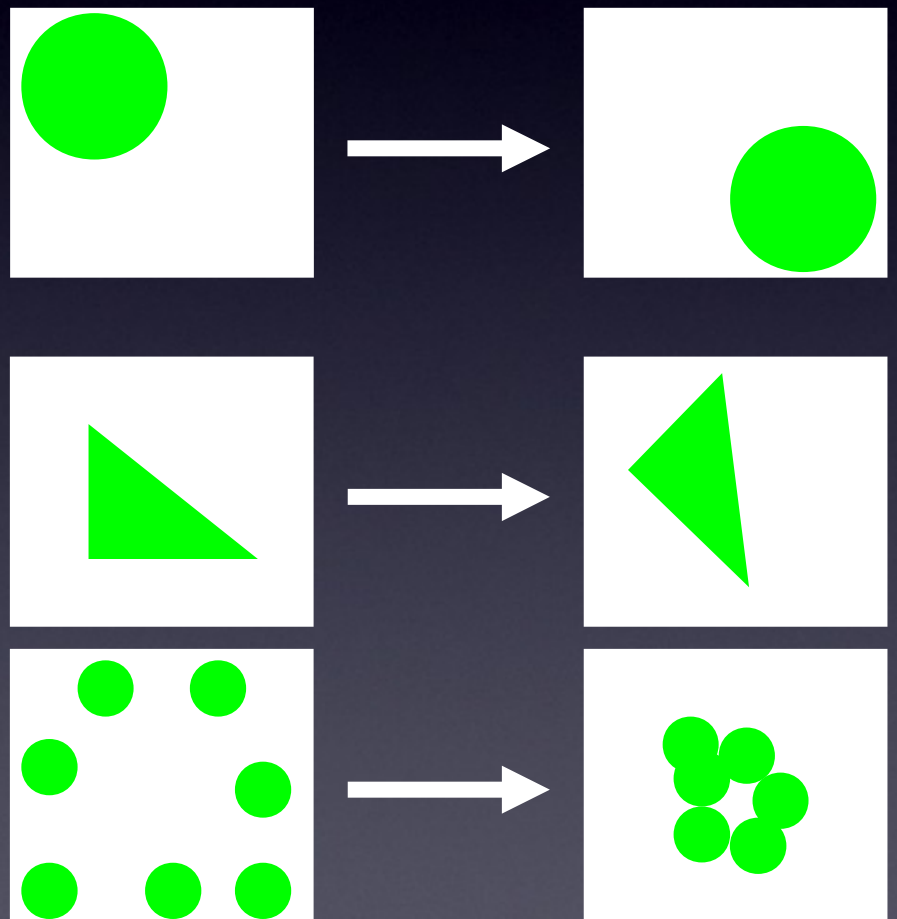


- Image intensity - threshold
- Size - threshold
- Shape - circularity etc.



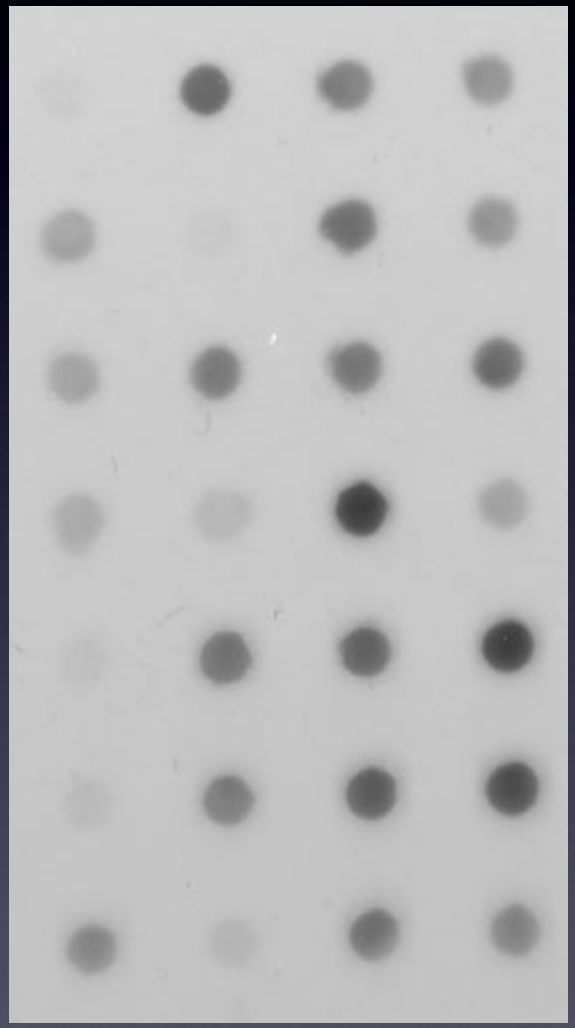
Am I trying to see something move over time?

- Can I define what movement is?
  - Linear - A to B?
    - Direction
    - Speed
    - Velocity
  - Rotation
  - Clustering



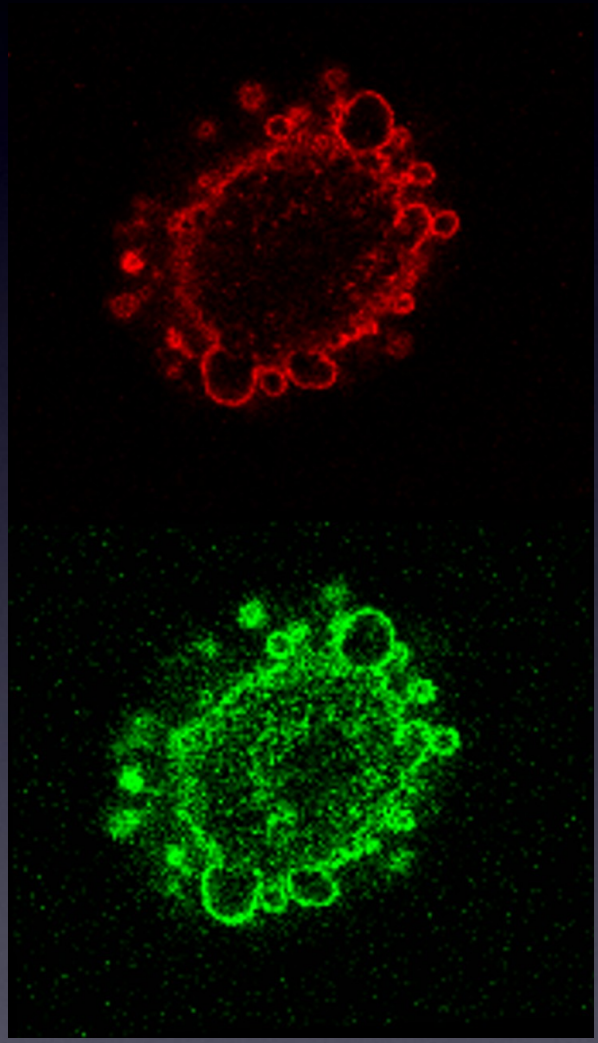
Am I trying to measure an amount or concentration?

- Does that have a Biological meaning?
- Absolute or Relative?
- Can I calibrate my image intensity vs. something else / itself?
  - eg. Fluorescence signal vs. Quantitative Assay or Baseline, Control
  - Fluorescence response might not be linear!



Am I trying to measure an "image parameter"?

- Does that have a Biological meaning?
- Absolute or Relative?
  - Total / Mean / SD of signal
  - Background
  - Signal : Noise
  - Texture (smooth/spotty)
  - **"Colocalisation"** between "colours" / channels"

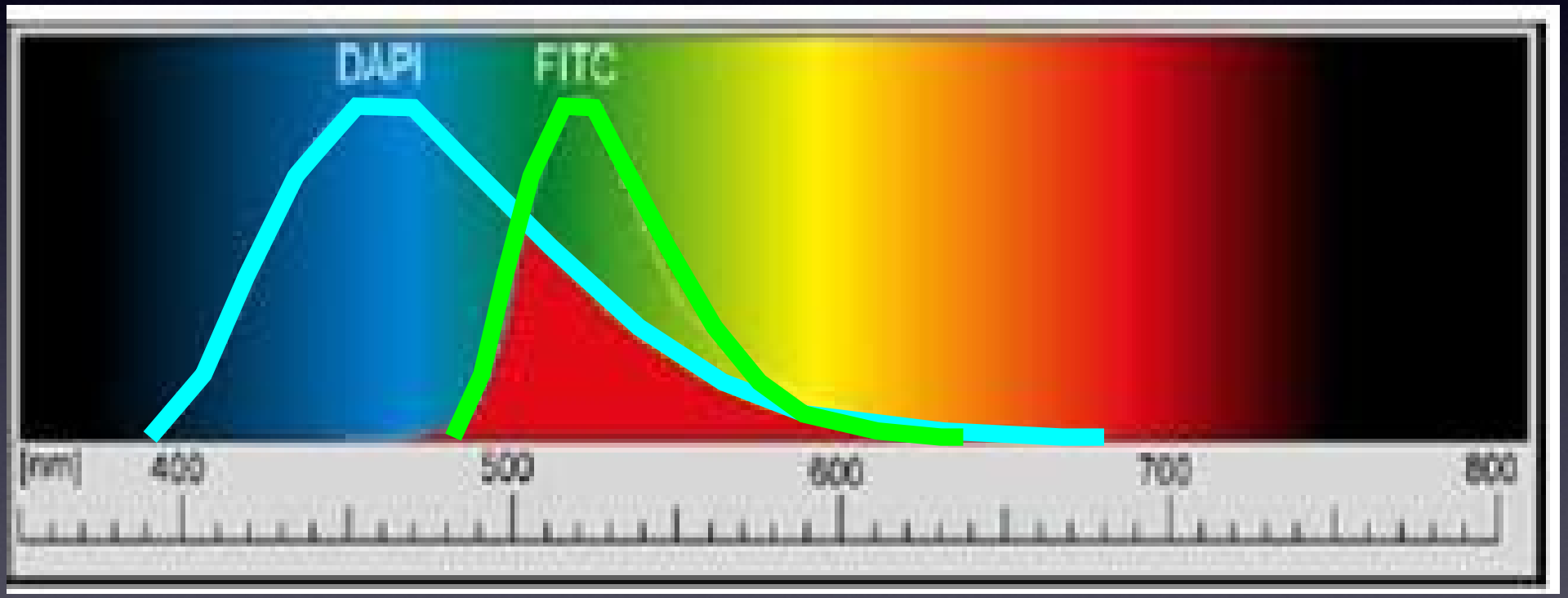


Go to Basic Course Slides

- Intensity Digitisation
- Colour Channels

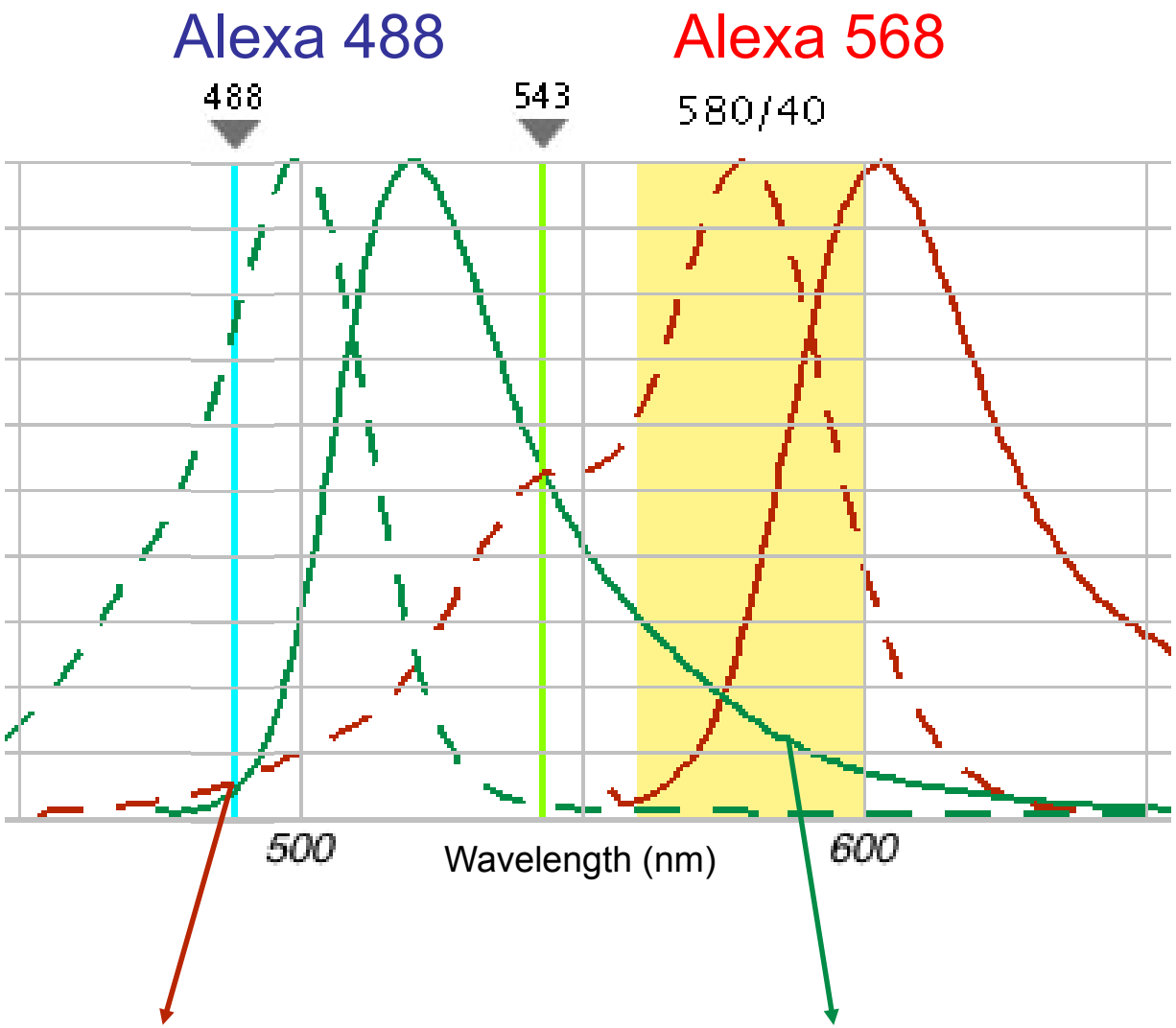
Avoid Emission Bleed Through and Crosstalk/Cross-excitation

- Dye selection / Filter selection
  - Emission bleed through and/or excitation crosstalk...
  - Means you get: Overlapping emission - Quantitative? No!



Use multi tracking (Zeiss) / sequential (Olympus)

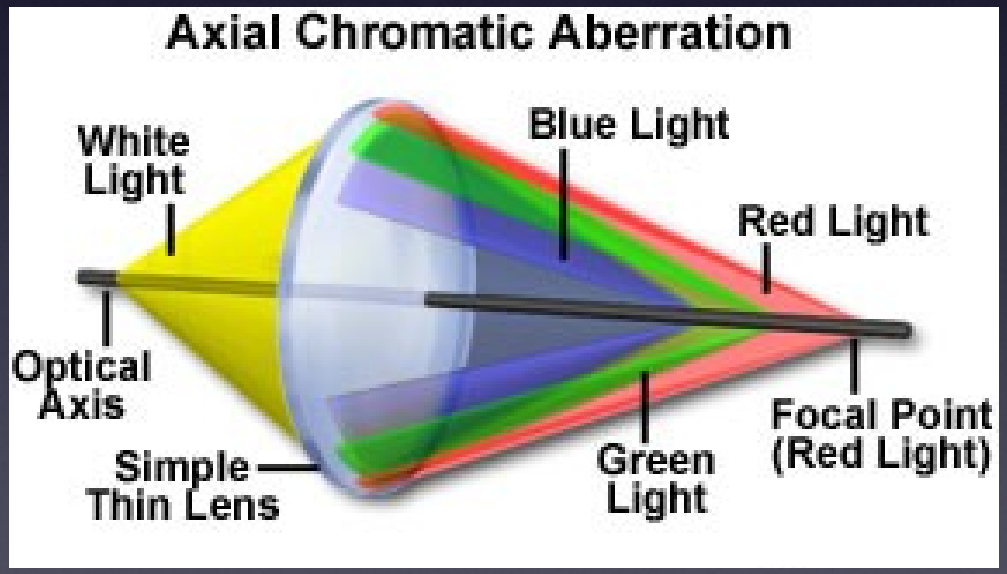
Beware ! Crosstalk and Bleed Through

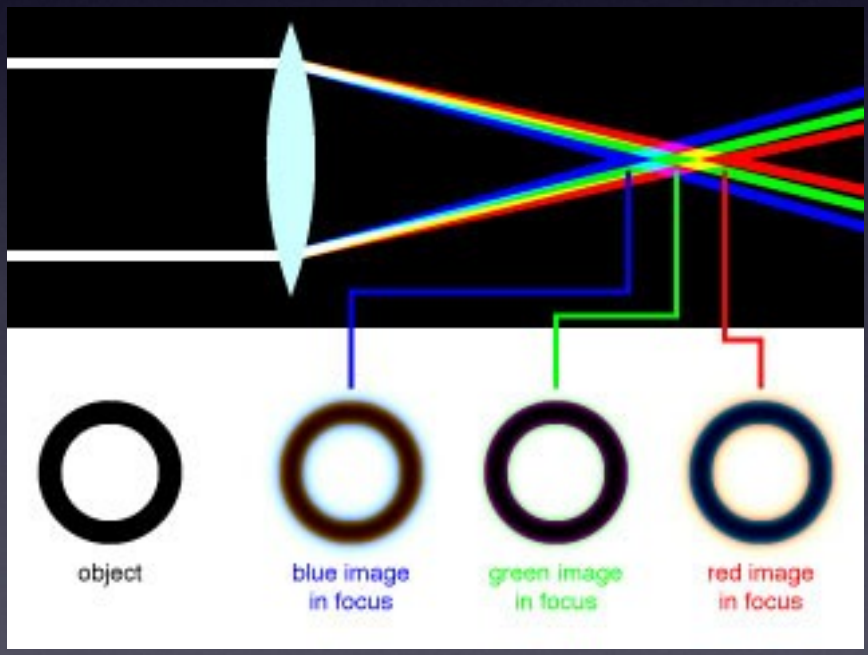


Cross talk (wrong excitation) **Bleed through (wrong emission)**

Watch Out - More Holes To Fall Into:

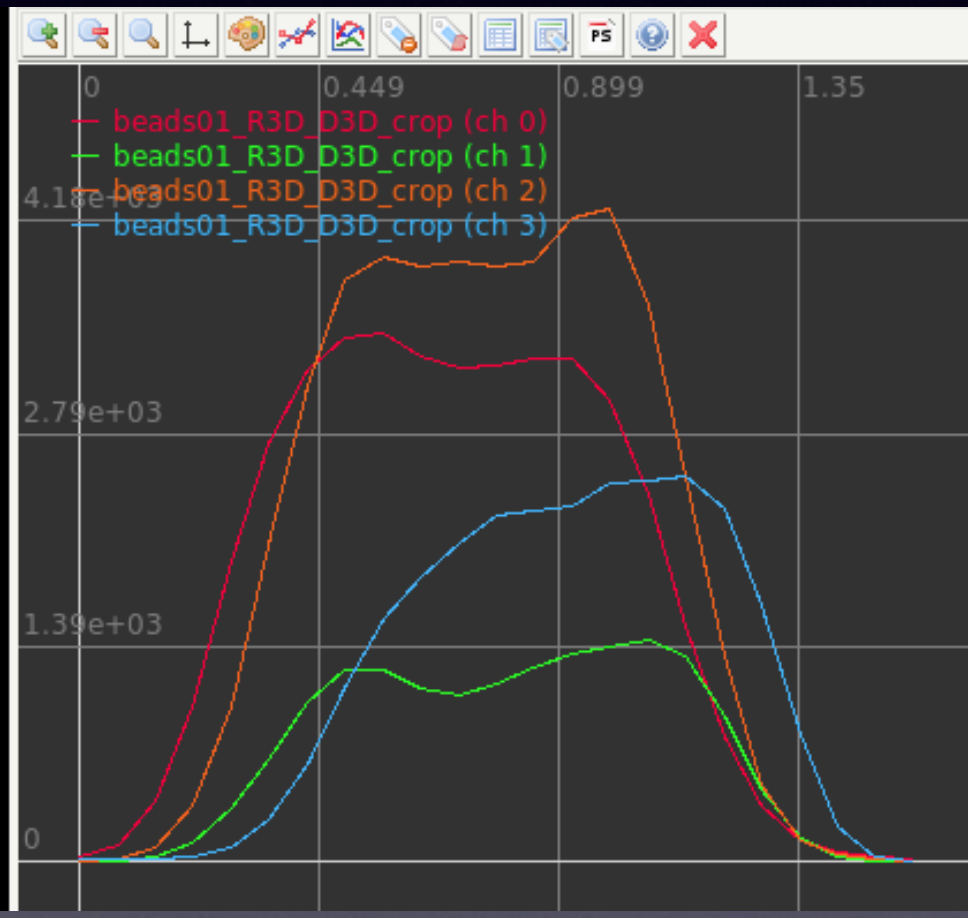
- Correct objective lens / microscope setup for task
  - N.A / Resolution.
  - Apochromat for different colours (UV)
  - Calibrate Scanner / Check with multi-colour beads





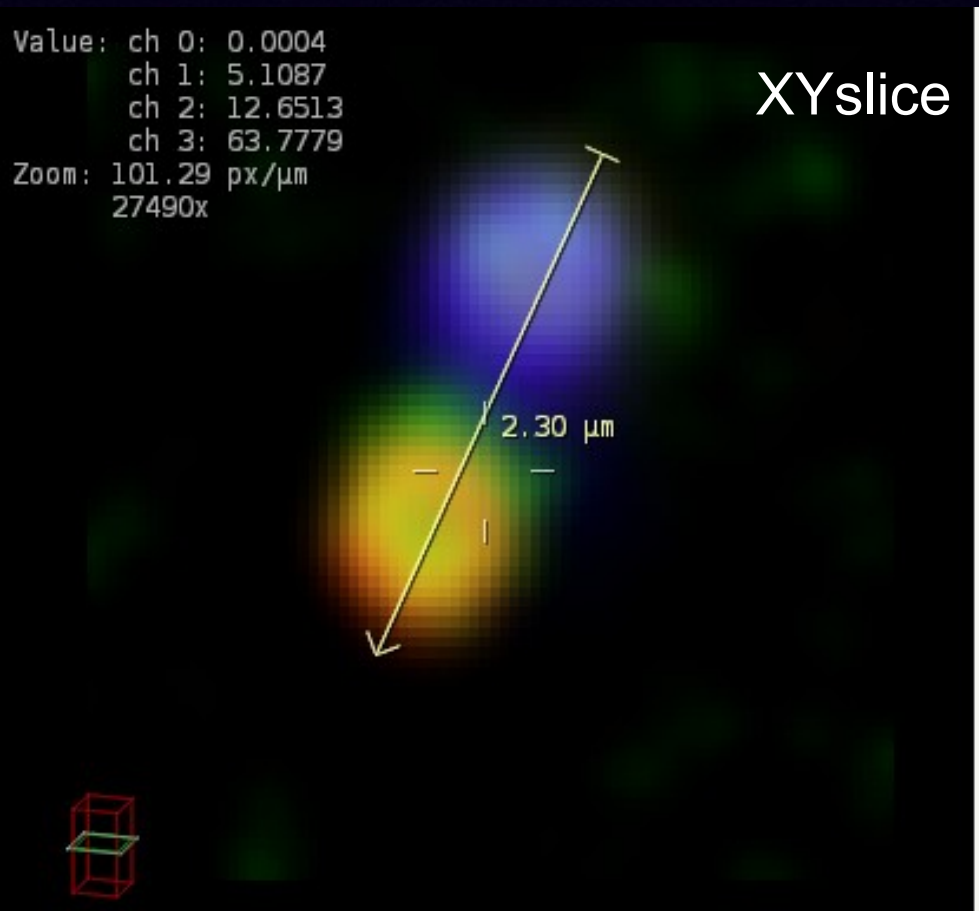
Check with multi-colour beads

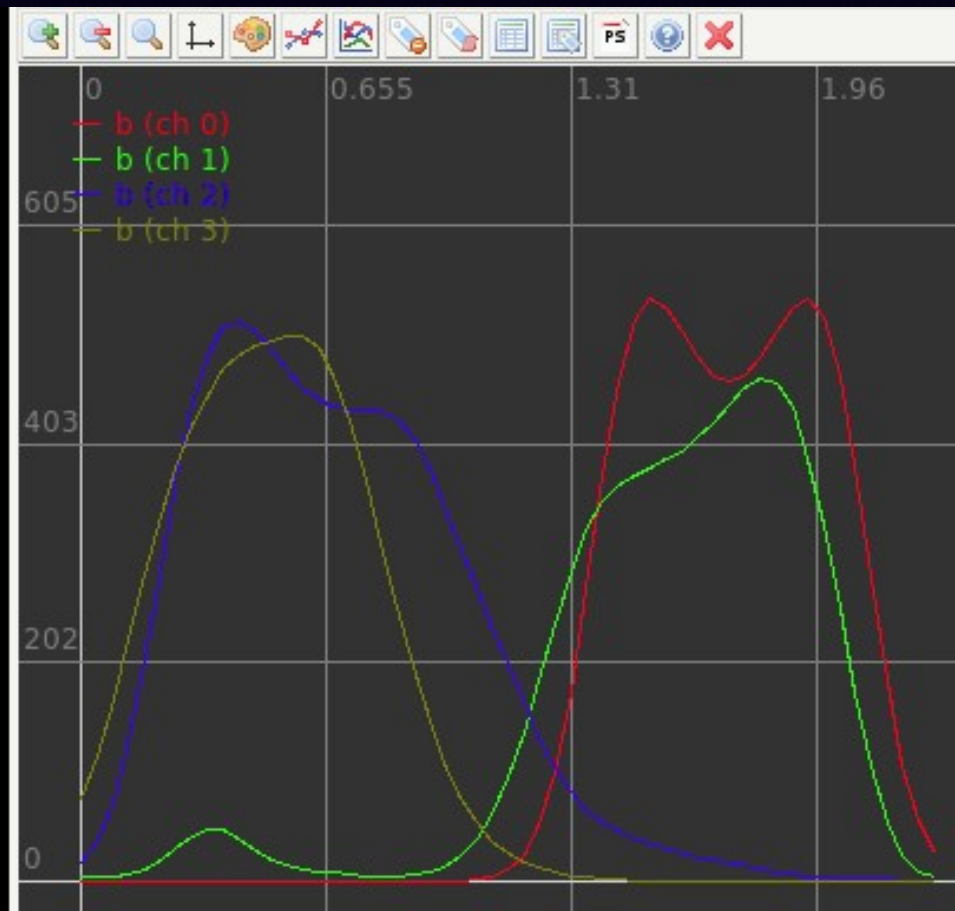
- Widefield: (Dvcore 1 micron tetraspek beads):
  - Optimise Filter alignment / angle
  - Lenses have residual aberrations, even expensive ones.



Check with multi-colour beads

- Confocal (Zeiss 510):
  - Calibrate Scanner + Align pinholes (and collimator)
- Measure error – then, correct for it!



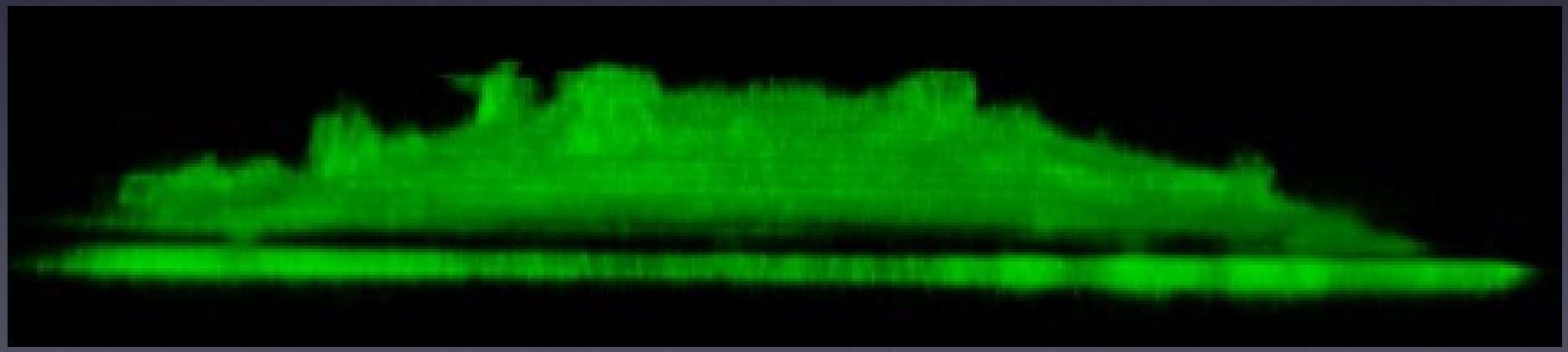


Watch Out - More Holes To Fall Into:

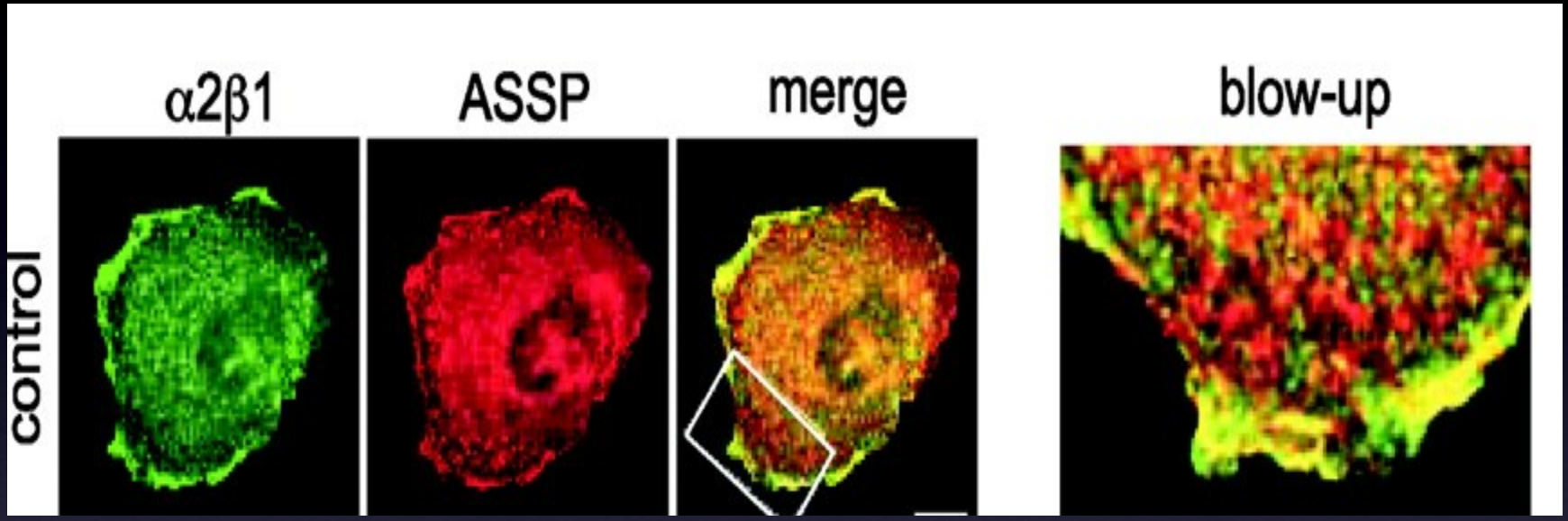
- Required bit depth - 8 bit often enough for LSCM imaging… and colocalization analysis.
  - More bits only for quantitative experiments where small intensity differences are measured.
  - 12 bit - bigger files than 8 bit.
    (Olympus... 12 bit only. Zeiss 8,12. Leica 8,12,16.)
  - 16 bit file is 2x bigger in RAM / on disk, than 8 bit !
  - CCD - many cases 12 bit might give better coloc info.

Watch Out - More Holes To Fall Into:

- Laser power - don’t bleach area before imaging it.
  - Bleached sample
    - Lower signal : noise
    - Lost information
  - Set the HV and Offset quickly (Auto HV)
  - Live imaging, bleaching - big problem
    Use low laser power (but more noise)



Colocalization / Correlation

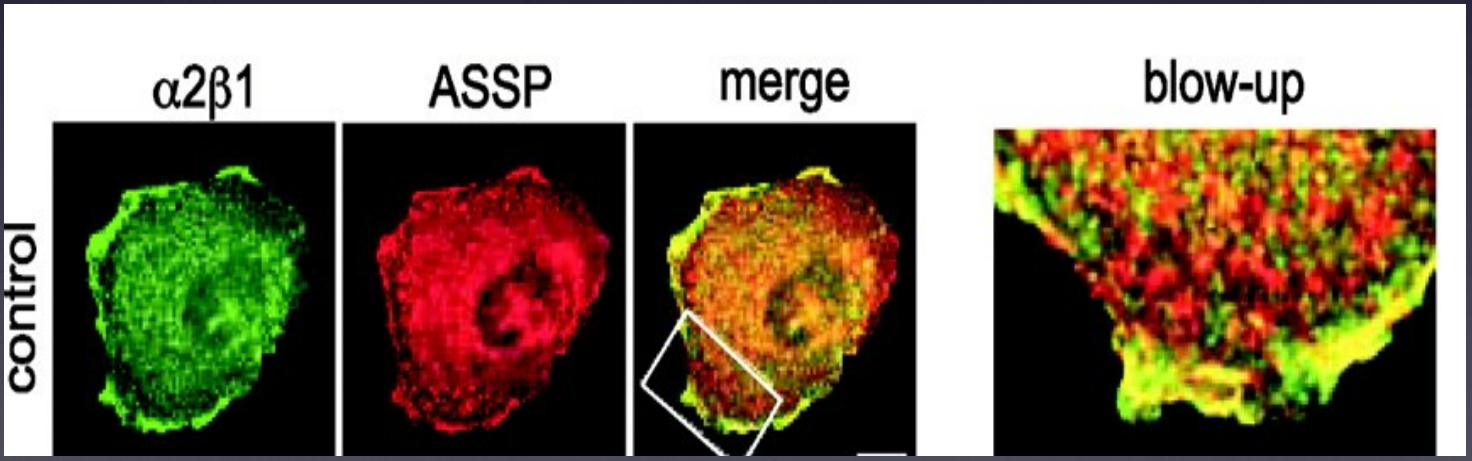


The past:
"I see yellow - therefore there is colocalization"
but published images "look" over exposed.
No colocalization definition + No stats = No Science.

From Now On: 3D. Quantification. Correlation. Statistics.
Complementary methods: BioChemical, Optical (FRET, FLIM)

Colour Merge Images? Only for Art!

- Channel Merge Images? What are they good for?
  - Apart from looking pretty... not much.
  - Scientific conclusions from the image below?
  - Colour blind people - see green and red the same!
    - **<u>Use Magenta / Green or Yellow / Blue</u>**

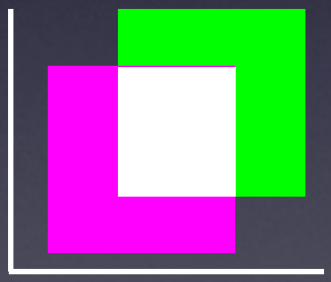


Colour Merge + Projection = Danger!

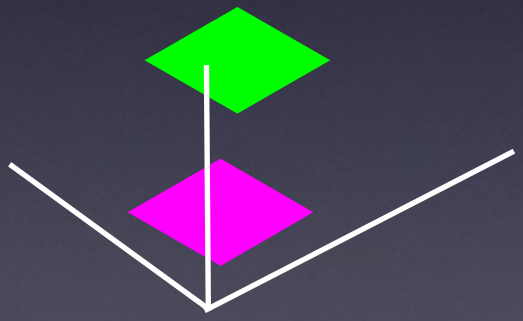
Never make colour merge / overlay images from projections of 3D / z stacks... why not?

Lose 3D info - are the objects overlapping in 3D, or is one in front of the other one, in the z-stack.

False overlaps!!! Easy to make false interpretation



colour merged projection



3 D

What does “Colocalisation” mean anyway…?

- That depends who you ask…
- … and what **BIOLOGY** you are thinking about

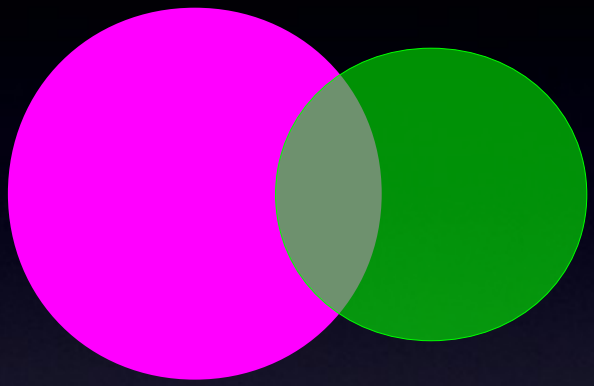
Colocalisation/Correlation? Think about the biology!

- What is the biological/biochemical question?
  - Are you looking for Co-Compartmentalisation?
  - Are you looking for exclusion / anti correlation?
  - Are you looking for interacting molecules?
    - Then you must also do biochemsitry (Immuno Co-precip, Fluo Correlation Spectroscopy)
    - FRET / FLIM might be very informative

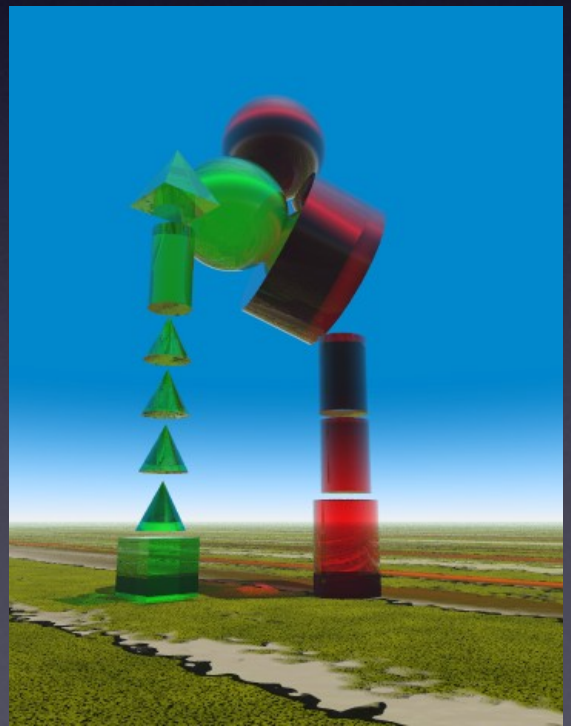
Colocalisation / Correlation / Concurrence?

"Colocalisation" covers two qualitatively different conditions:

1) that objects have both fluorophores present
(Object Based Coloc)
Segmentation needed.
Biology?



2) there is some relationship between the **intensities** of the fluorophores in a pixel.
(Pixel Intensity Based Coloc)
Interaction - BioChemistry?



Colocalisation / Correlation / Concurrence?

2 fluorophores are there in a pixel

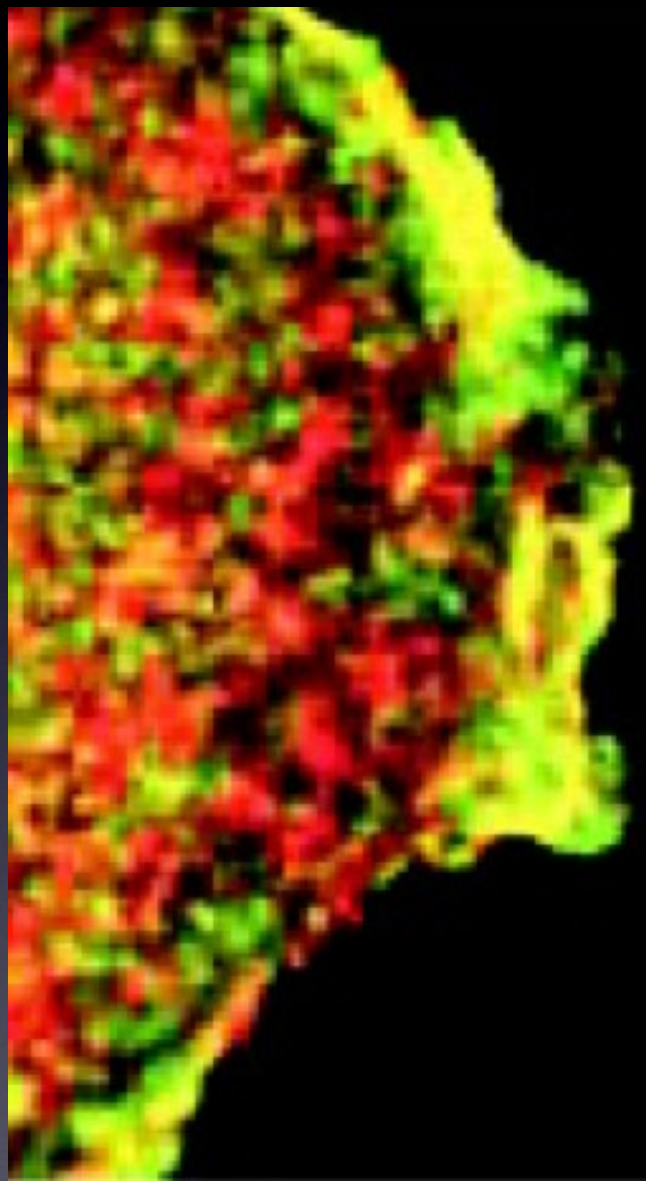
Binary information

Is it Random?

Is it Real?

Little or no biological meaning?

…unless you are confident about how to segment objects out from the background.

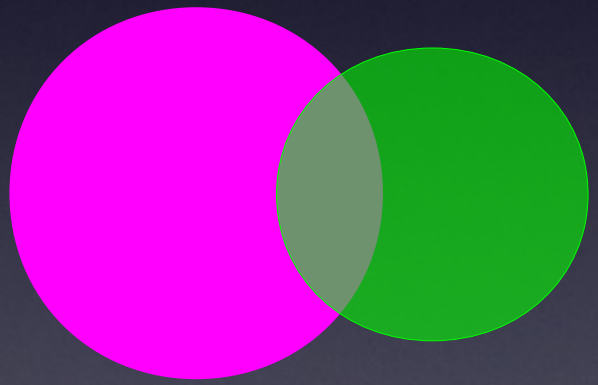


Definition of Terms

- “Concurrence” = “co-presence” “there is red and green”
- “Colocalisation” = Relationship between channel intensities
  - Eg. “Red is only found with Green”
  - Special case - “Correlation”
    - **Intensity** Correlation over Space

Define what is Colocalisation/Correlation?

Colocalisation is #1

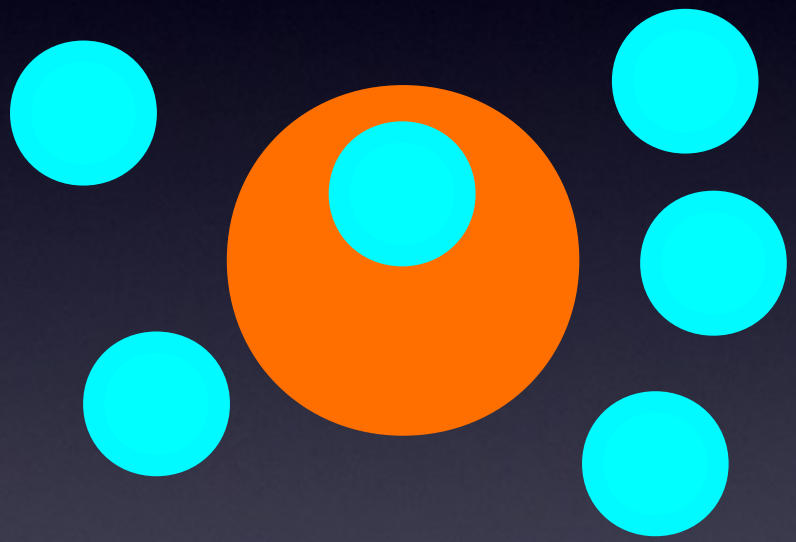


2 objects overlap
Binary information
No intensity information

Concurrence?
Image Segmentation!

Biological Meaning?

Colocalisation is #2

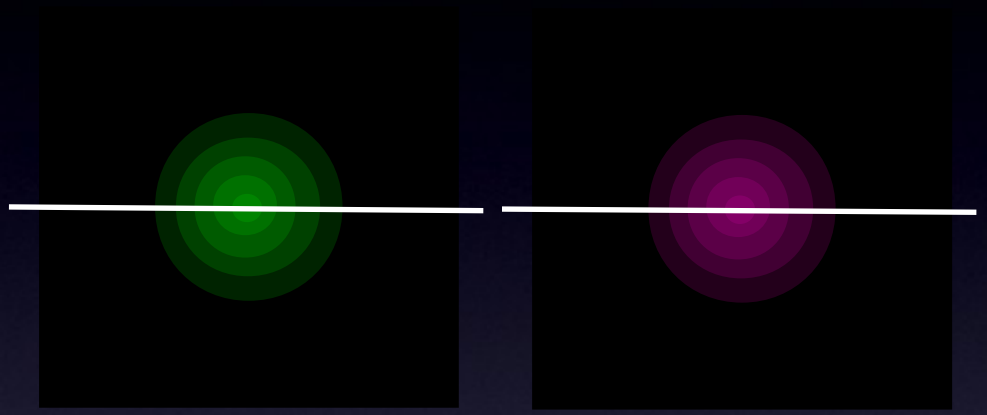


Some objects appear to overlap
with another object
Binary information
No intensity information

Colocalisation?

Biological Meaning?

Colocalisation is: #3



Intensity profiles overlap

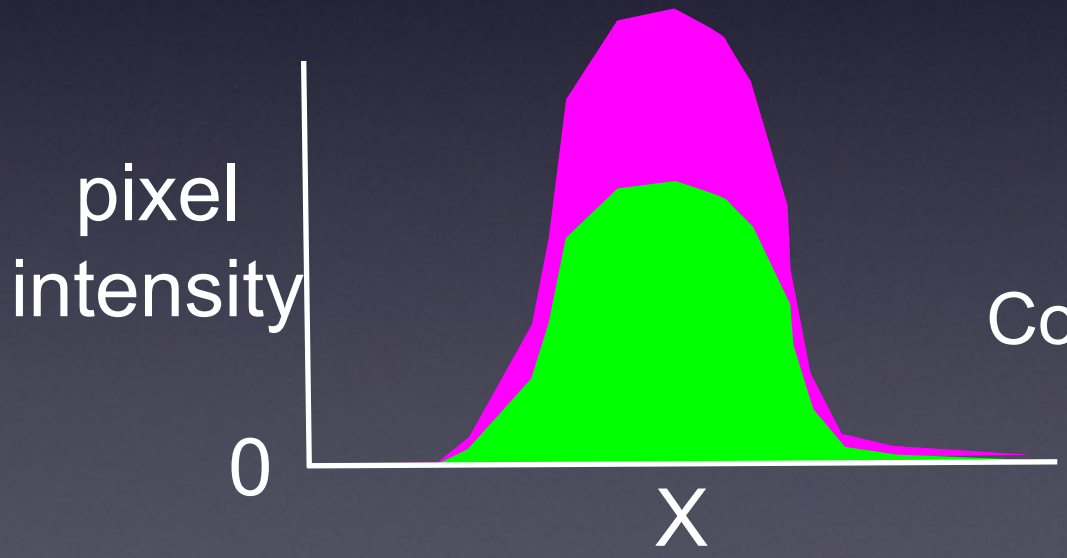


Image “Correlation”

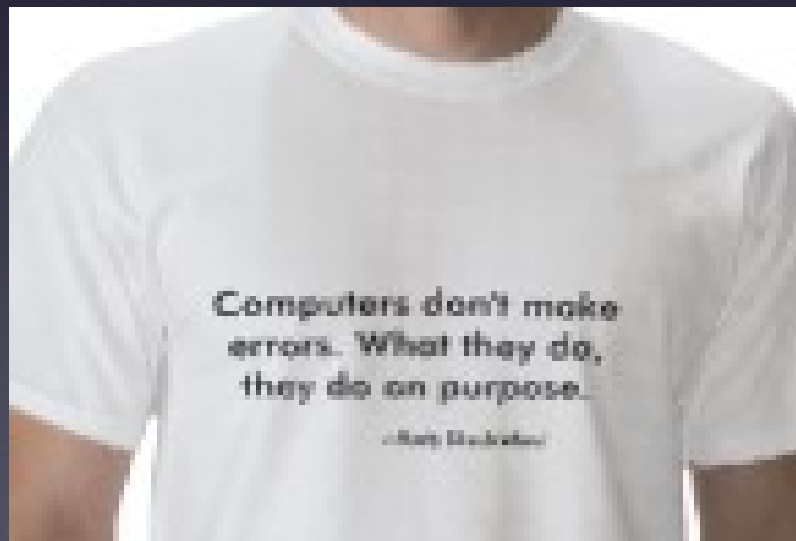
Biological Meaning?
Co-compartmentalisation?
Physical interaction?

Colocalisation/Correlation -Think about:

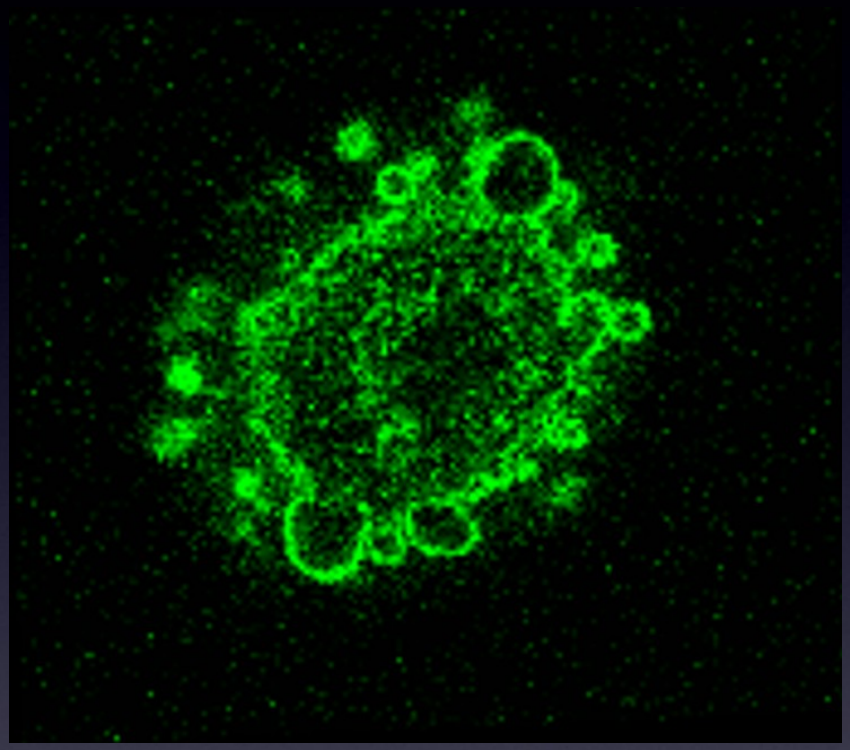
- Are your “objects” smaller than optical resolution?
  - Vesicles? Small Organelles?
    - Check channel overlap with sub resolution beads!
- Are your objects large?
  - Large single homogenous blobs?
  - Large reticular networks / membranes
  - Resolution required?
- Complementary “correlation” methods
  - Fluorescence correlation spectroscopy (FCS in live cells)
  - Flow Cytometry? Multiple markers in a cell. Good stats.

Colour Merge Images = Bad
… so what should I do instead?

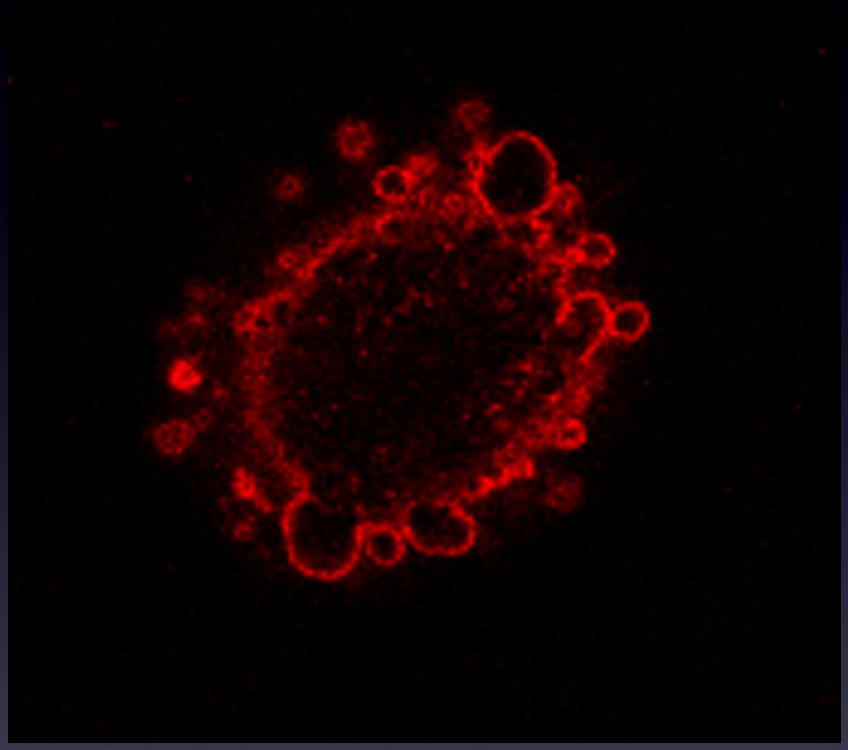
- “Colocalisation Analysis”
- Statistical Significance of Colocalisation
  - Single image - random / insignificant.
  - Statistical P value (significance), Manders coefficients, and Scatter Plot. (ImageJ, BioImageXD, Imaris and others)
- But remember…
  - Don’t merge projections of stacks (you lose 3D info, false coloc)
  - Don’t believe your eyes, they lie. Machines don’t make mistakes…



Colocalisation Analysis



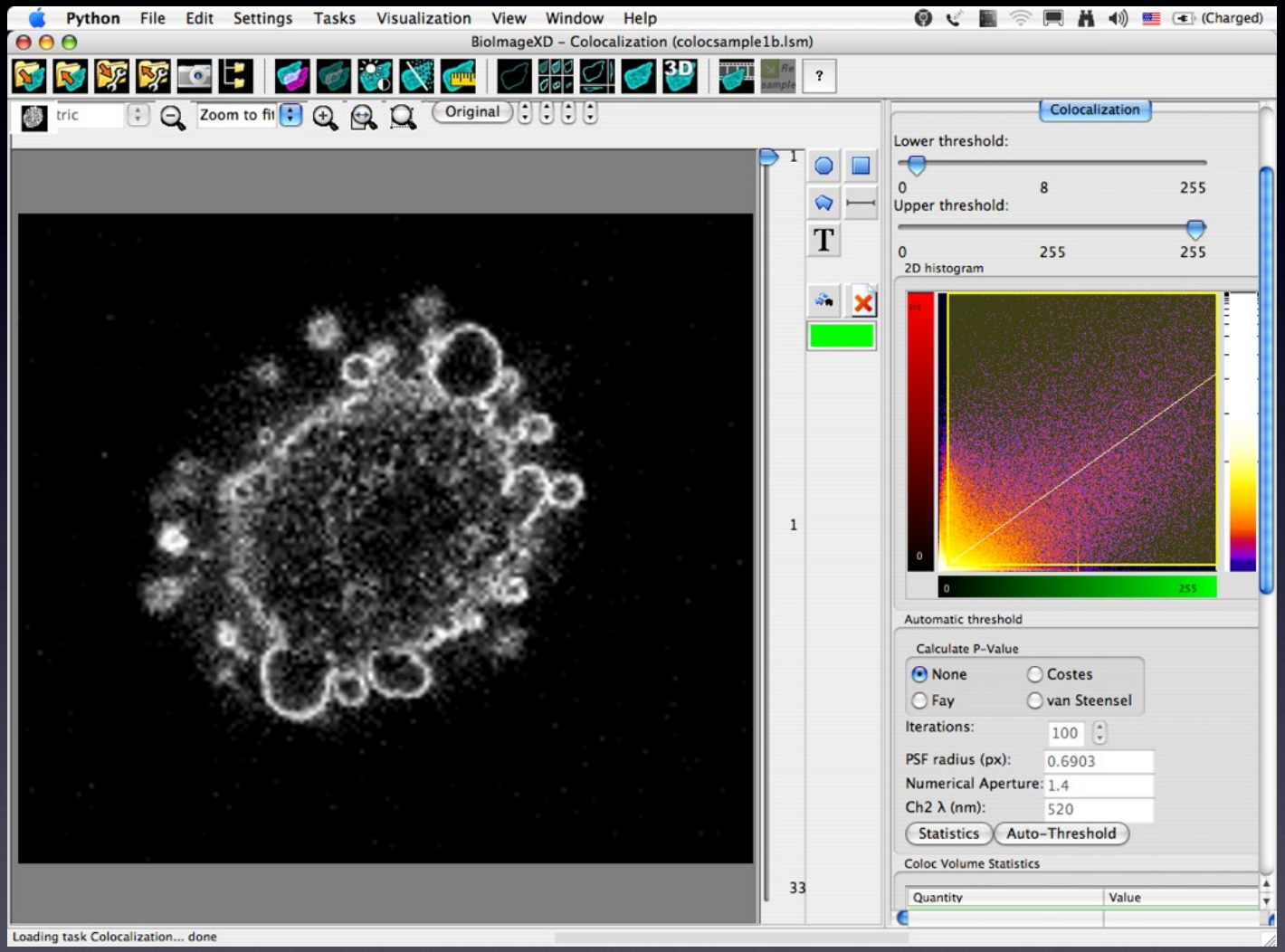
vs.



How can I measure the amount of colocalisation or rather “correlation” between these two images?

BioImageXD, ImageJ and others have methods to do that!

Colocalisation Analysis



Scatter plot
2D histogram
Publish it?

Coloc stats:
Pearsons *r*
M_1, M_2,
Costes P-val,

Automatic thresholding

Coloc Stats - Costes et al. 2004 Biophysical J. vol 86 p3993

Pearson's Image Correlation Coefficient (Manders et al., 1993)

$$r = \frac{\sum_i (R_i - R_{av}) \cdot (G_i - G_{av})}{\sqrt{\sum_i (R_i - R_{av})^2 \cdot (G_i - G_{av})^2}}$$

Don't panic - it's not that complicated!

Correlation between images, *r* ranges from -1 to +1

+1 means full correlation (images are the same)

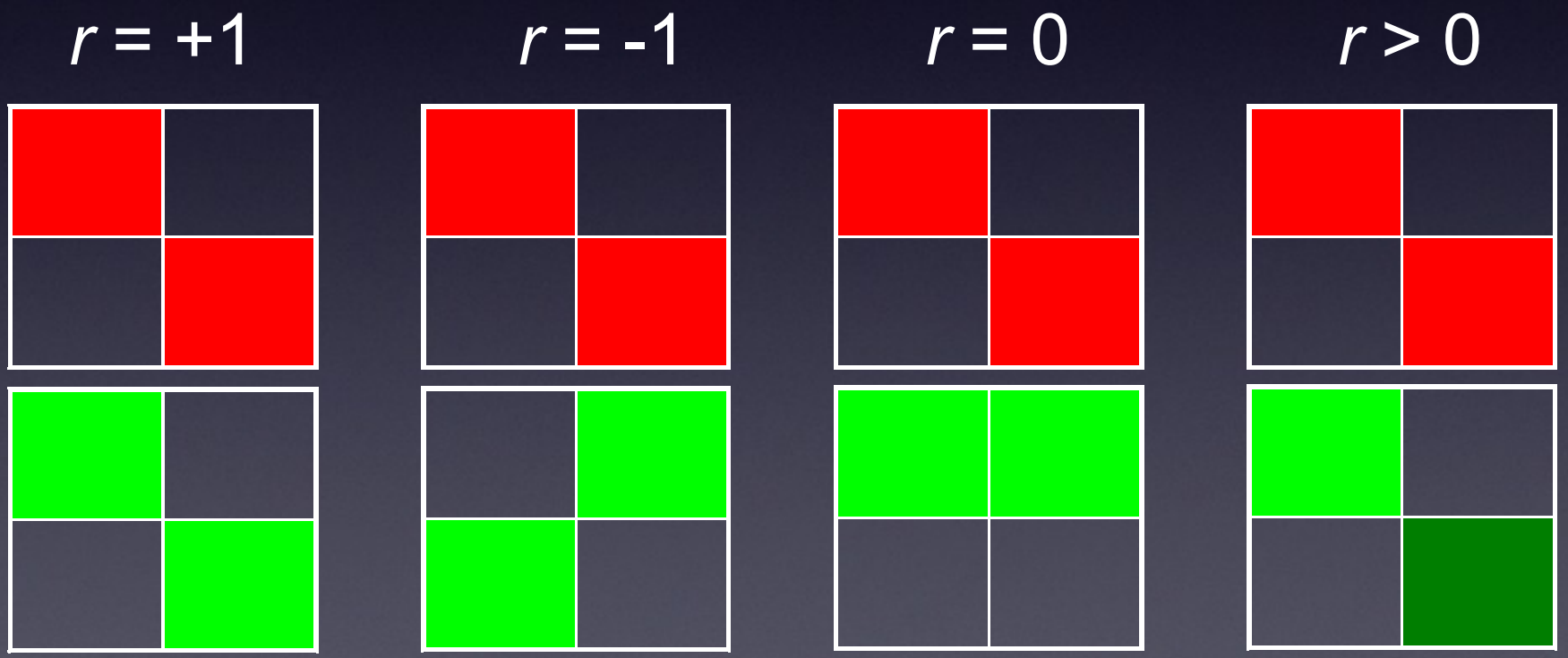
0 means no correlation (random)

-1 means full anti correlation (no red where there is green)

Pearson's Image Correlation Coefficient

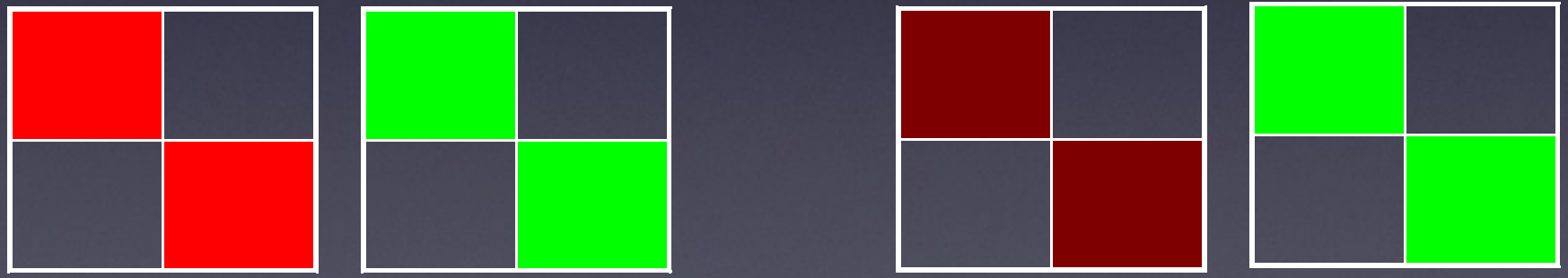
In English…per pixel and summed for the whole image:

$$r = \frac{\text{sum of (red intensity - average red) x (green intensity - average green)}}{\text{sqrt of squares of above}}$$



Pearson's Image Correlation Coefficient is…

- Insensitive to diff. intensity of the 2 images. Why?
- If red is 1/2 as bright as green…
  - Still can get $r = 1$
  - … so Pearsons r is is robust for biological imaging…



Manders Coefficients

$$M_R = \frac{\sum_i R_{i,coloc}}{\sum_i R_{i,total}}$$

$$M_G = \frac{\sum_i G_{i,coloc}}{\sum_i G_{i,total}}$$

Biologically meaningful coloc coefficients:

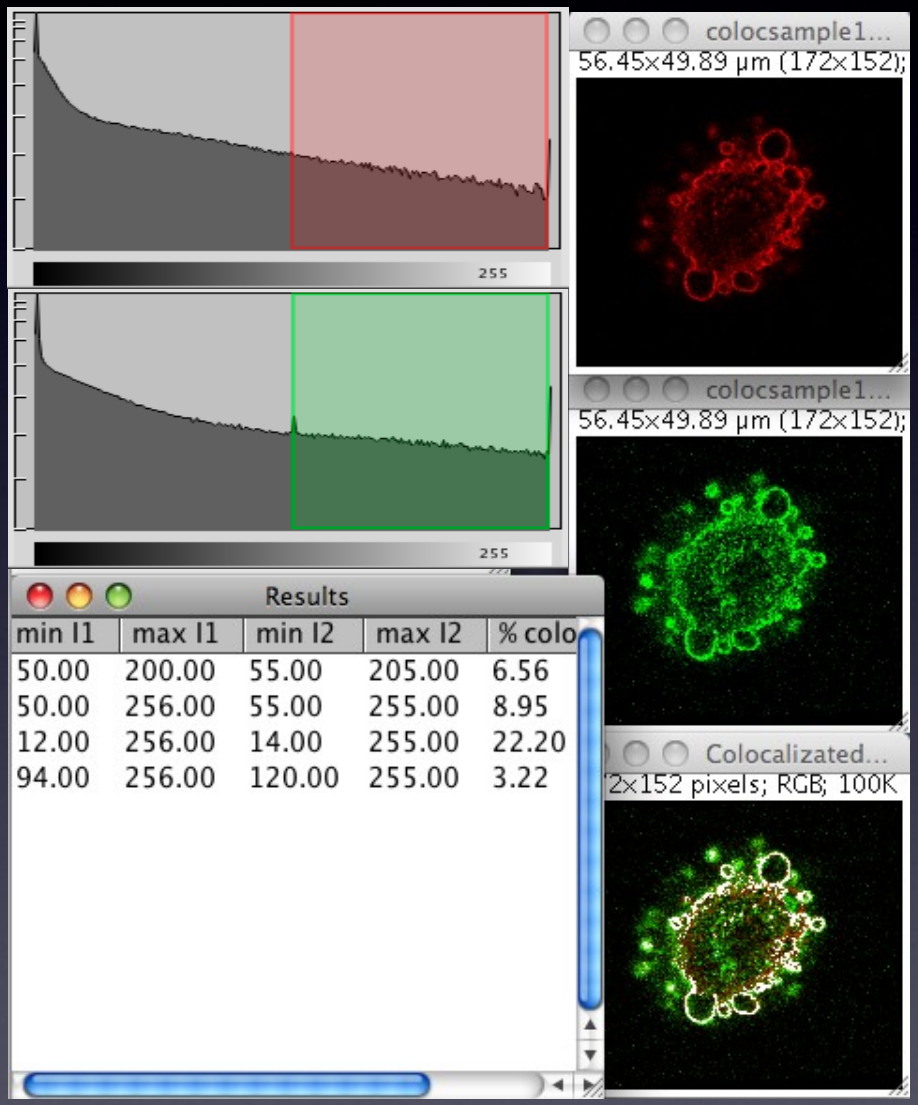
Proportion of each dye colocalised with the other (Manders et al., 1993)

$R_{i,coloc}$ = colocalised red signal

$R_{i,total}$ = total red signal

Great! … but how do I know which pixels are colocalised and which are not…?

“Thresholding” and “% colocalisation”



The calculated “% colocalisation” depends on what thresholds you set.

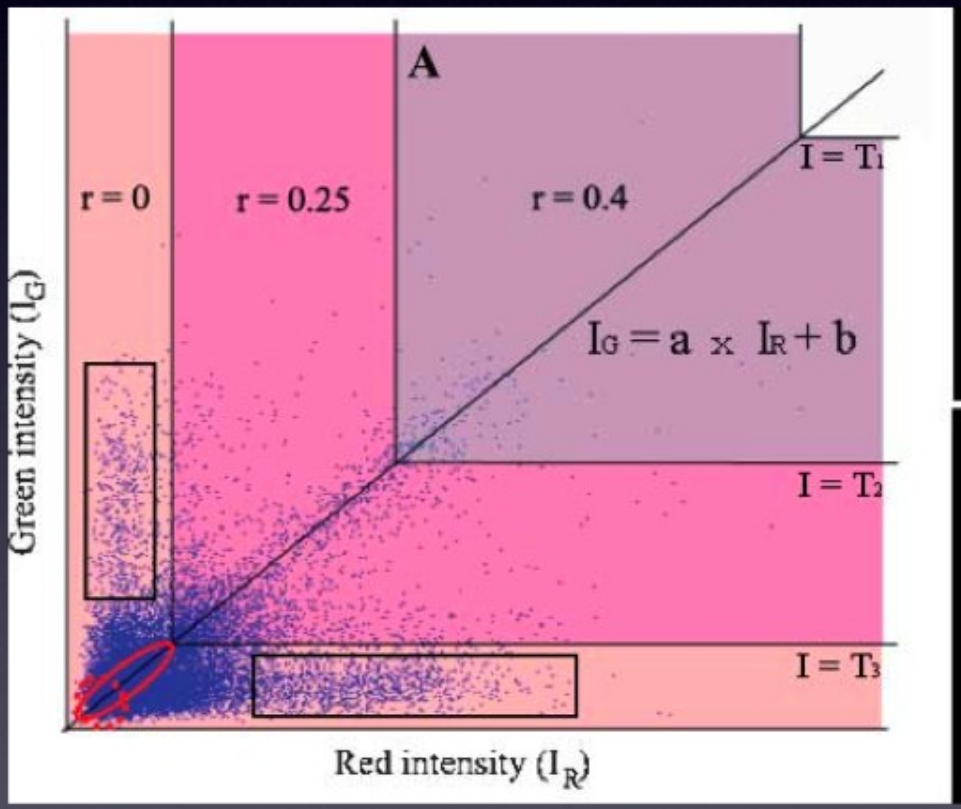
… so how should one set them?

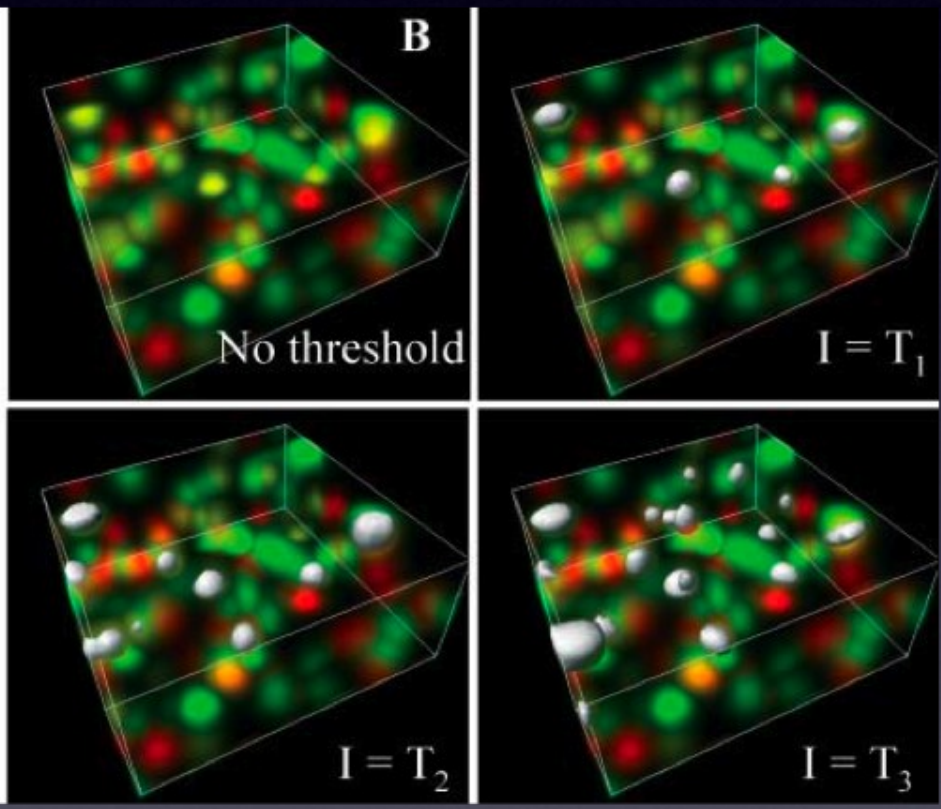
..until you get the result you want?

No science here!

Automatic Thresholding?

- How should I set the thresholds of the 2 channels?
  - Manually? No! Subjective user bias, not reproducible...
  - Need a robust reproducible method!
  - Find thresholds where Pearson correlation below thresholds <= 0

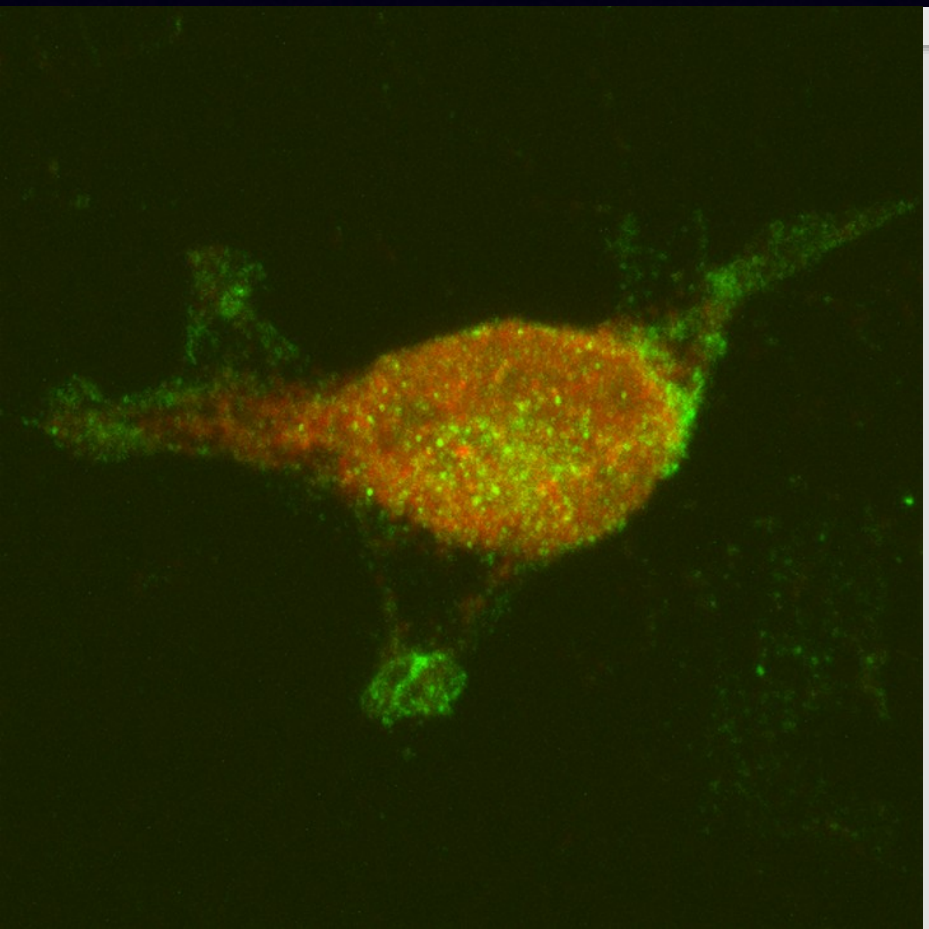


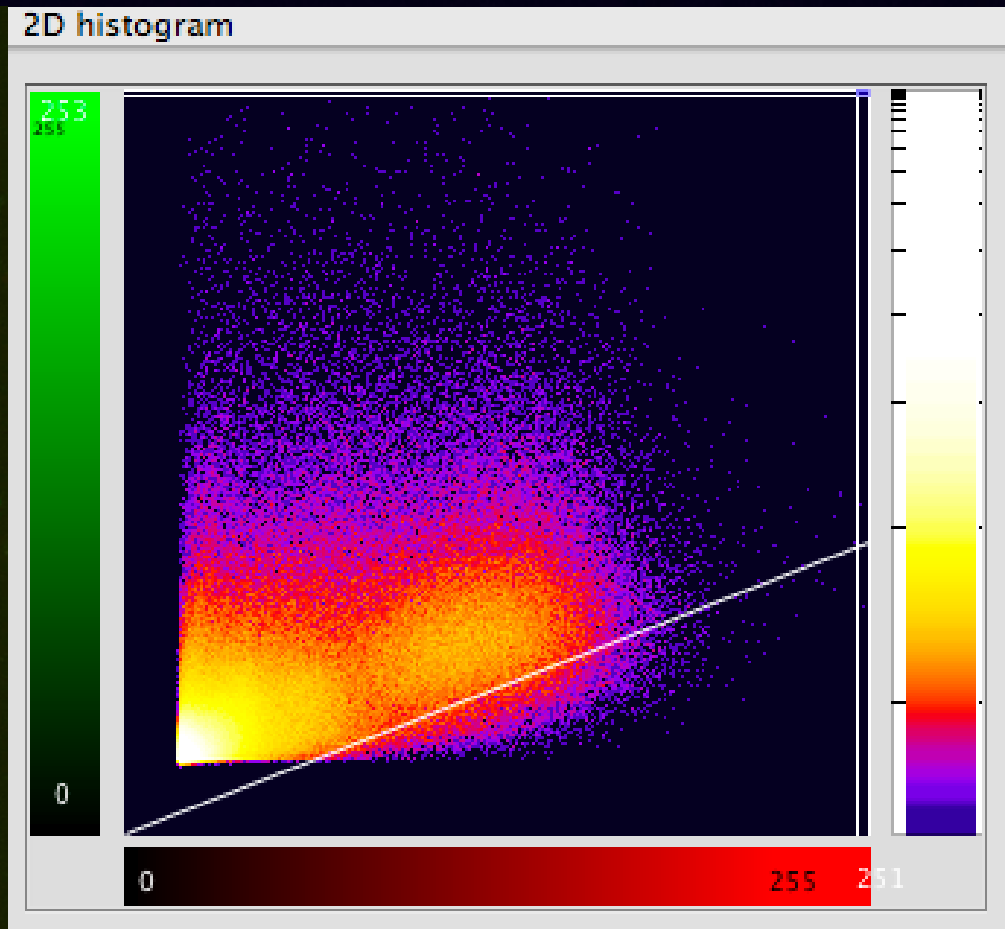


Auto Threshold - Costes et al. 2004 Biophysical J. vol 86 p3993

2D Histograms / Scatterplots

- Display 2 colour channel image data in 2D:
  - colour merge / overlay or 2D histogram?
    - 2D histogram: Ch1 - y axis (left), Ch2 - x axis (bottom)
    - Colour mapped to number of pixels with that R and G value (right)





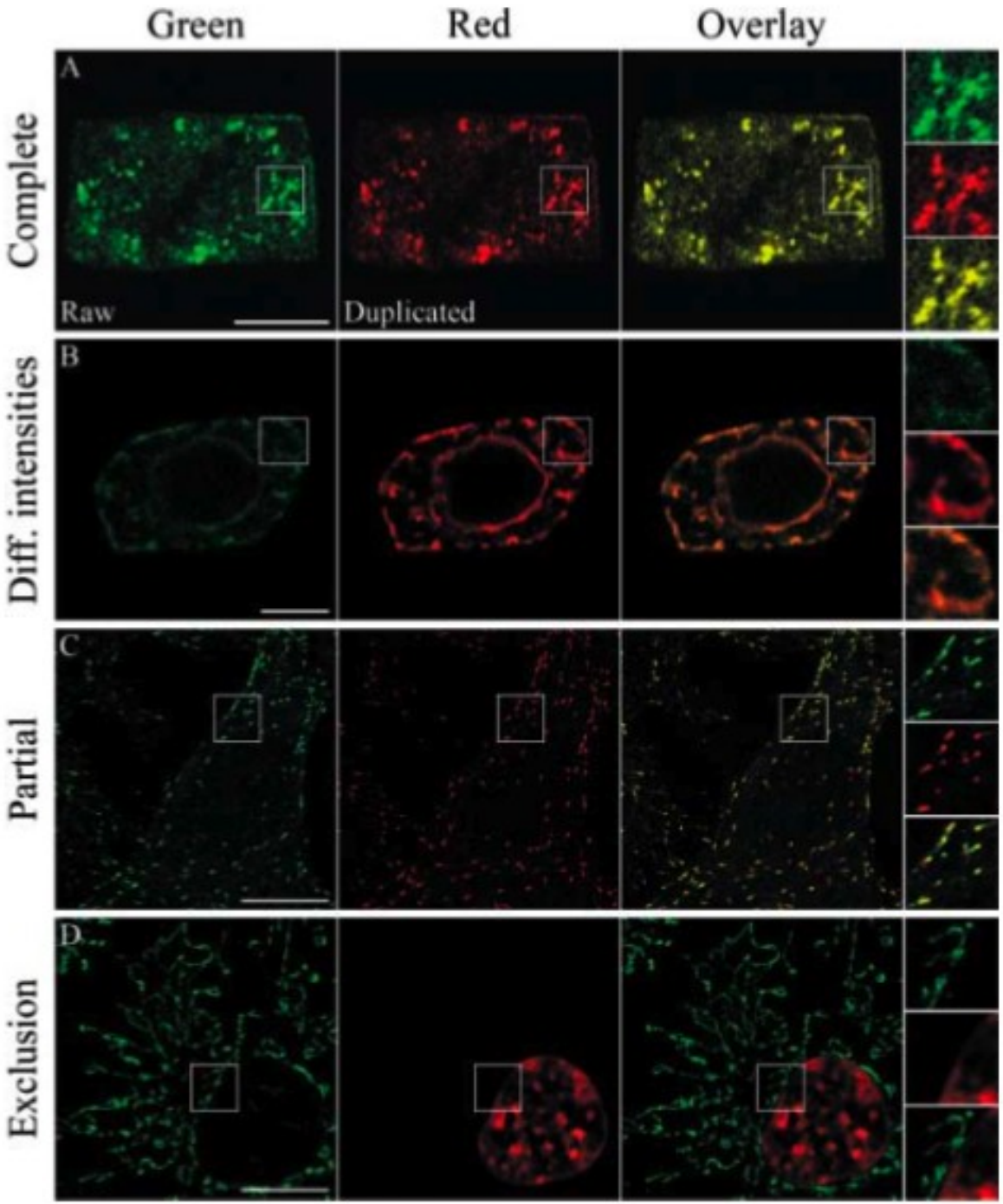


Fig. 4. Reference images for colocalization analysis. Images for colocalization analysis were acquired from fixed maize root cells with Golgi staining (A) (Boutté *et al.*, 2006) or endoplasmic reticulum staining (B) (Kluge *et al.*, 2004) and on fixed mammalian HeLa cells with microtubule plus-end tracking proteins EB1 and CLIP-170 staining (C) (Cordelières, 2003), and nuclear and mitochondrial staining (D). Scale bars, 10 μm. These images illustrate the four commonly encountered situations in colocalization analysis. (A) Complete colocalization. (B) Complete colocalization with different intensities. (C) Partial colocalization. (D) Exclusion. Grey level images of the green and red image pairs (A–D) were used for subsequent treatments with ImageJ. A zoomed view of the insets is shown on each side of the colour panels.

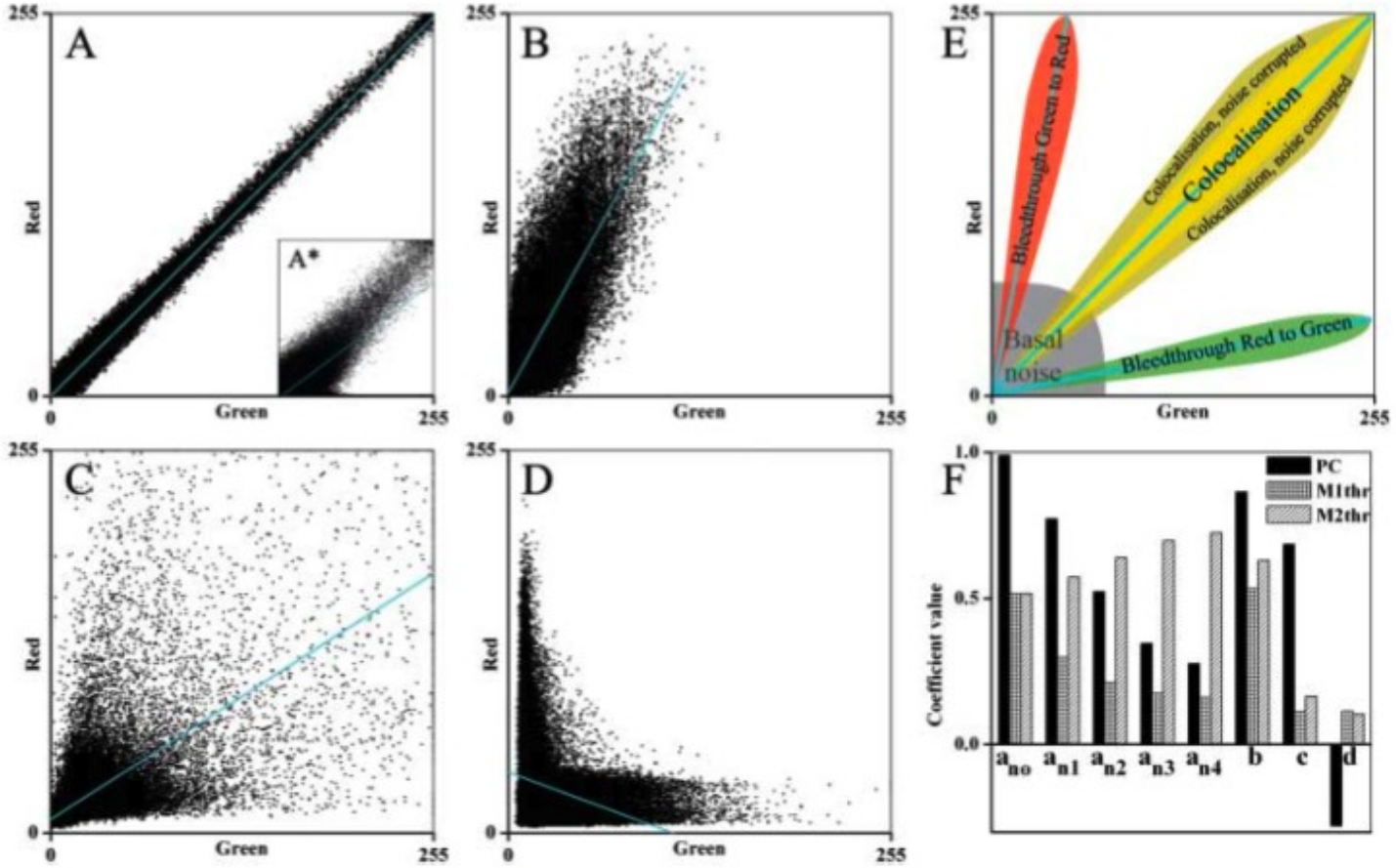
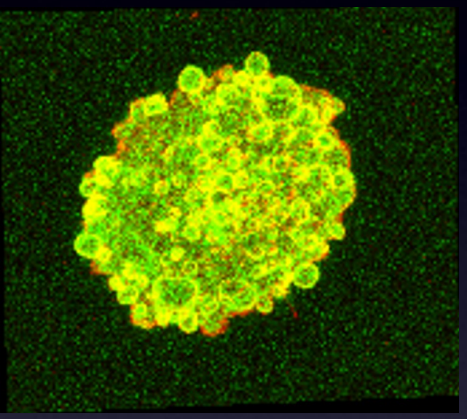


Fig. 5. Colocalization analysis with JACoP; Pearson and Manders, scatter plots and correlation coefficients. Scatter plots (A–D) correspond to the colocalization events as shown in Fig. 4. (E) Model scatter plot explaining the effects of noise and bleed-through. (F) Pearson's and Manders' coefficients in the different colocalization situations. A complete colocalization results in a pixel distribution along a straight line whose slope will depend on the fluorescence ratio between the two channels and whose spread is quantified by the Pearson's coefficient (PC), which is close to 1 as red and green channel intensity distributions are linked (F, a_{n0}, black bar). (B) A difference in fluorescence intensities leads to the deflection of the pixel distribution towards the red axis. Note that the PC diminishes even if complete colocalization of subcellular structures is still given (F, b, black bar). (C) In a partial colocalization event the pixel distribution is off the axes and the PC is less than 1 (F, c, black bar). (D) In exclusive staining, the pixel intensities are distributed along the axes of the scatter plot and the PC becomes negative (F, d, black bar). This is a good indicator for a real exclusion of the signals. (E) The effect of noise and bleed-through on the scatter plot is shown in the general scheme. (F) The influence of noise on the PC was studied by adding different levels of random noise (n1–n4)* to the complete colocalization event (A = n0, no noise). (F) Note that the PC (black bar) tends to 0 when random noise is added to complete colocalizing structures. The inset (A*) in (A) shows the scatter plot for the n2 noise level. Note that all of the mentioned colocalization events (A–D) may only be detected faithfully once images are devoid of noise. (F) Manders' coefficients were calculated for (A–D). The thresholded Mander's tM_1 (cross-hatched bars) and tM_2 (diagonal hatched bars) are shown. Compare complete colocalization (a_{n0}), complete colocalization with random noise added (a_{n1}–a_{n4}), and complete colocalization with different intensities (b), partial colocalization (c) and exclusion (d). Note that the original Manders' coefficients are not adapted to distinguish between these events, as they stay close to 1 for all situations (not shown). *Signal-to-noise ratios are: n1 = 12.03 dB, n2 = 6.26 dB, n3 = 4.15 dB and n4 = 3.52 dB.

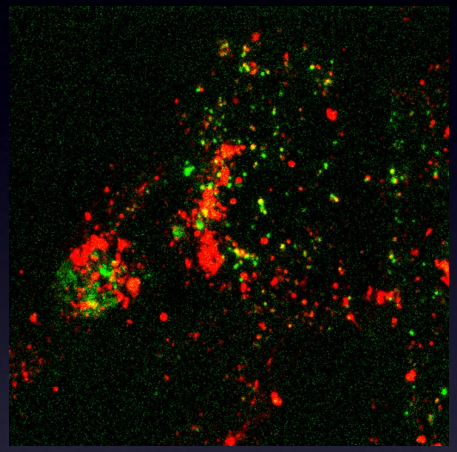
Bleed through wrong way around

2D Histograms / Scatterplots

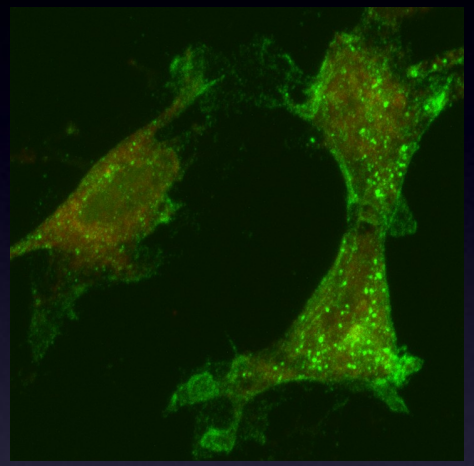
- See correlation qualitatively - better than colour merge
- See problems from imaging:



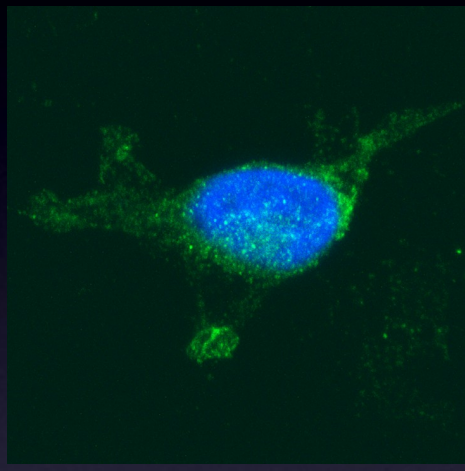
Saturated
Noisy



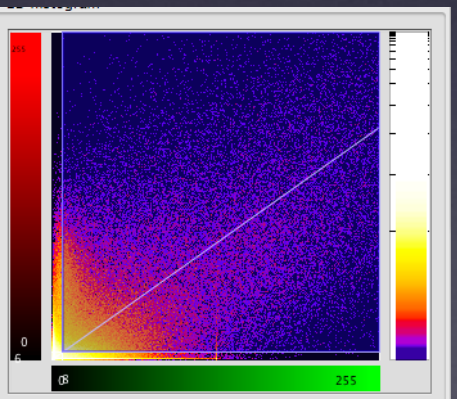
Saturated
No correlation?

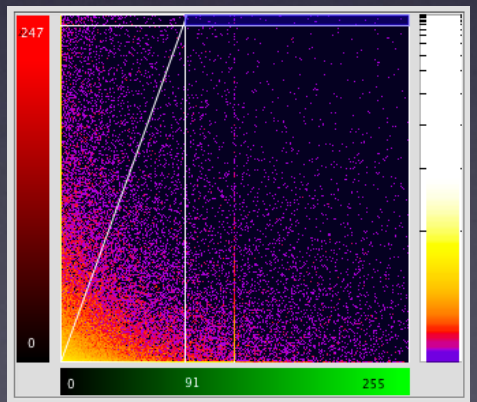


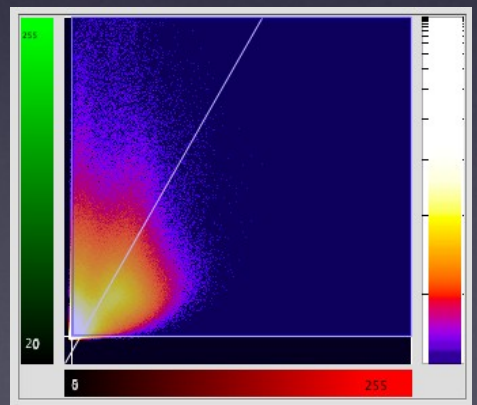
Wrong offset

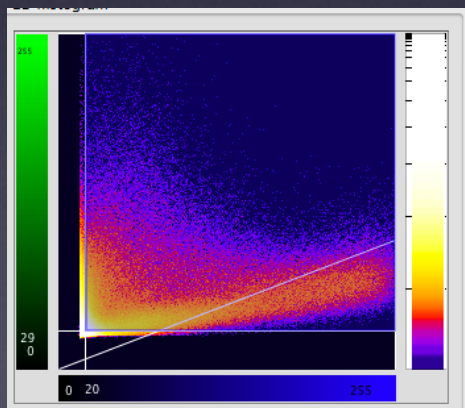


Wrong offset
Bleed through



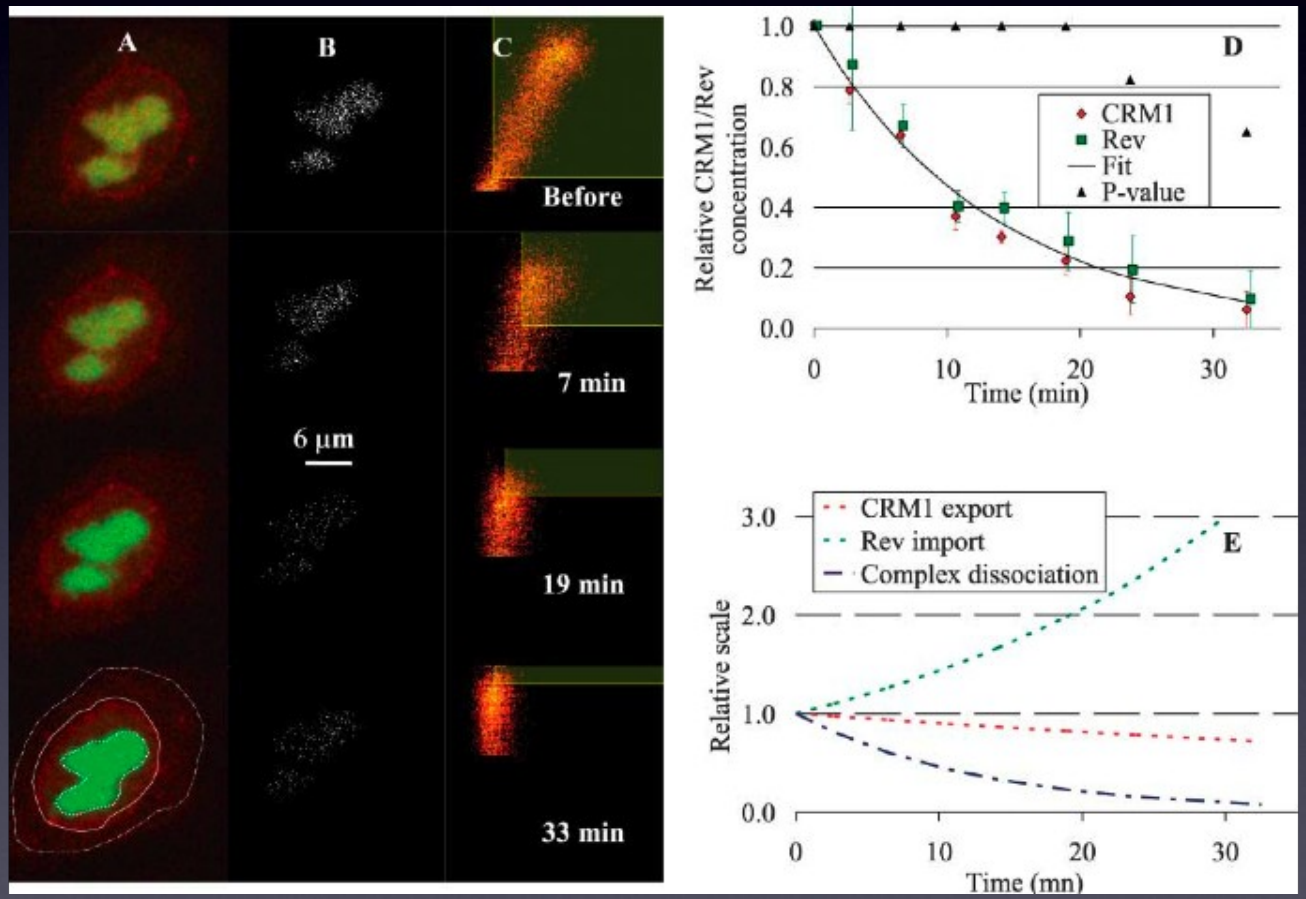






Automatic Thresholding?

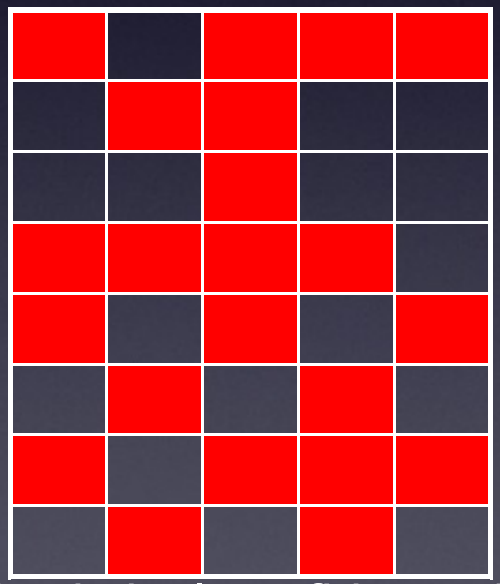
- Does it work in a biological experiment? Yes!
  - Time course of Rev-CRM1 dissociation, nucleolus to nucleus
  - The dissociation rate constant kd =1.25 ± 0.31 x 10^{-3} s^{-1}



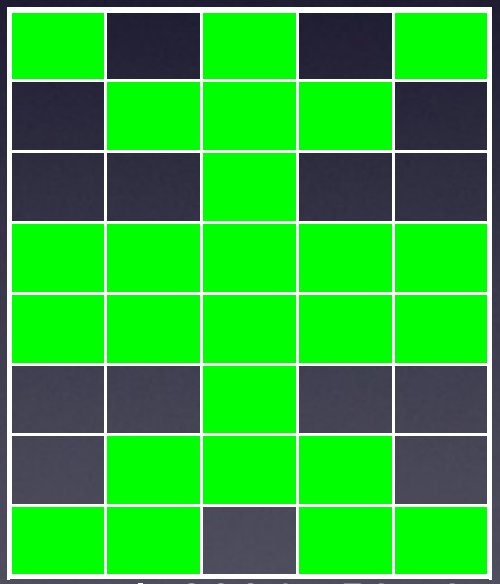
auto threshold - Costes et al. 2004 Biophysical J. vol 86 p3993

One more thing…

- Statistical significance!
  - Are coloc results better than random chance?
  - A busy image might give high correlation and Manders
    - Lots of signal = larger chance of random signal overlap.



vs.



17 / 40 pixels overlap !!!

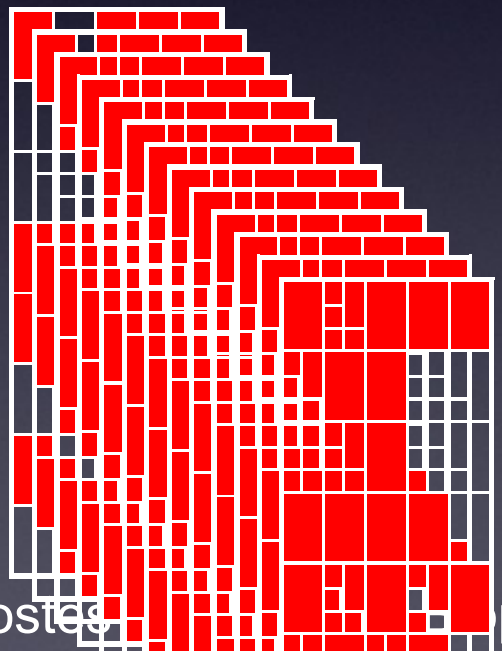
Is that significant or just random?

Statistical confidence P - Costes et al. 2004 Biophysical J. vol 86 p3993

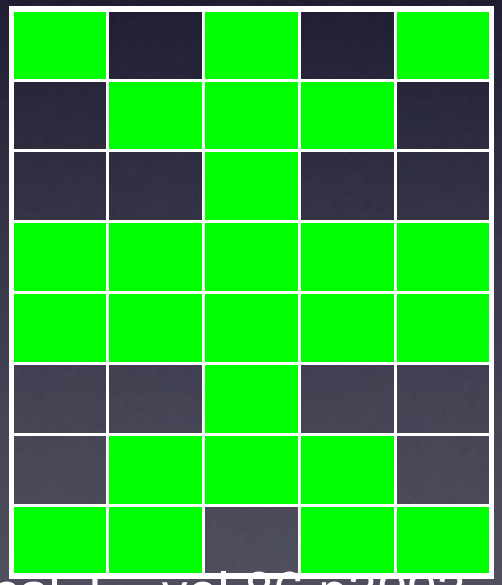
Costes Method - Randomisation…

- Measure Pearson's correlation for:
  - Randomised 1st channel image data (PSF sized chunks)
  - Repeat 100 times
  - How many randomised have <= correlation than real image.
    - If > 95% of randomised are worse, then we believe Manders.

P = 0.5 = 50% (no)
P = 0.95 = 95% (yes)
P = 1 = 100% (YES!)
confidence



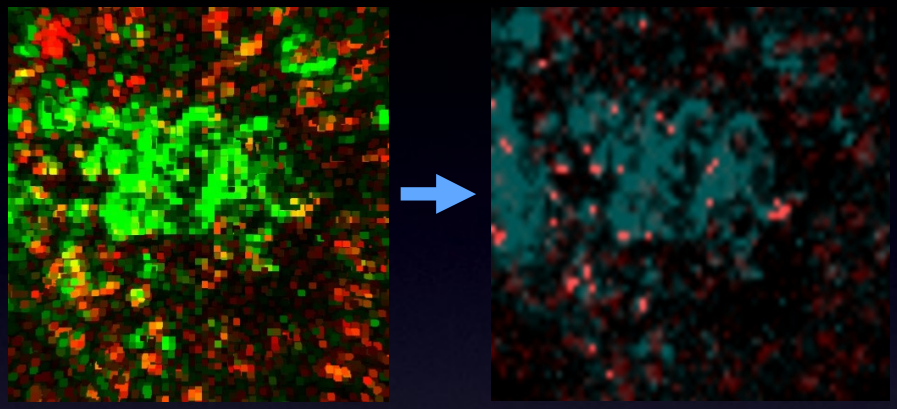
vs.



Statistical confidence P - Costes [illegible] physical J. vol 86 p3993

Colocalisation example: virus entry to caveolae

10 min P.I.

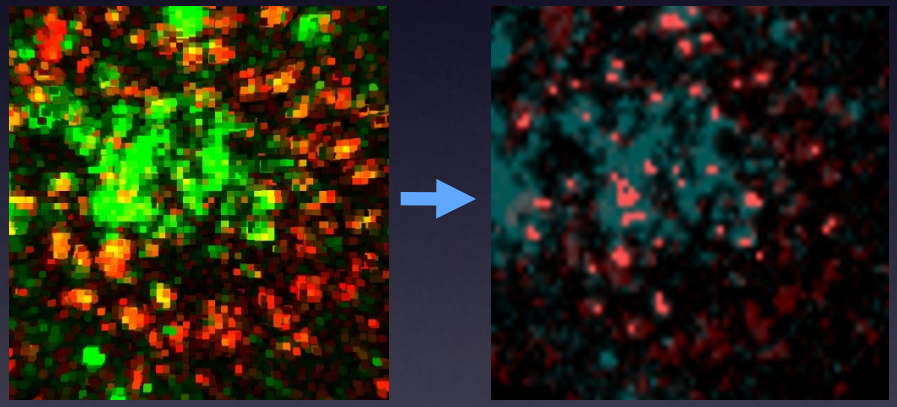


32% of virus colocalized

Costes P-value 0.00

0% chance it's real

20 min P.I.



39% of virus colocalized

Costes P-value 1.00

100% chance it's real

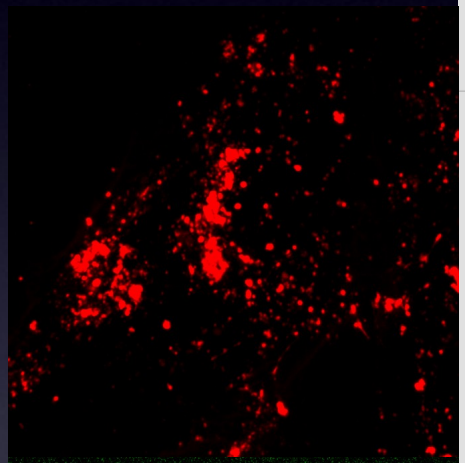
Without significance test, we wrongly assume virus is colocalised with caveolae at 10 min P.I.

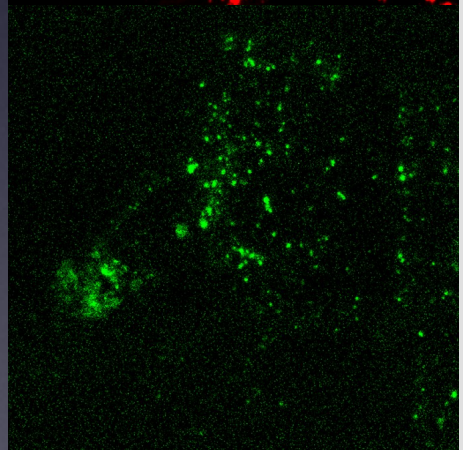
It is not! Only at 20 min is there signficant correlation.

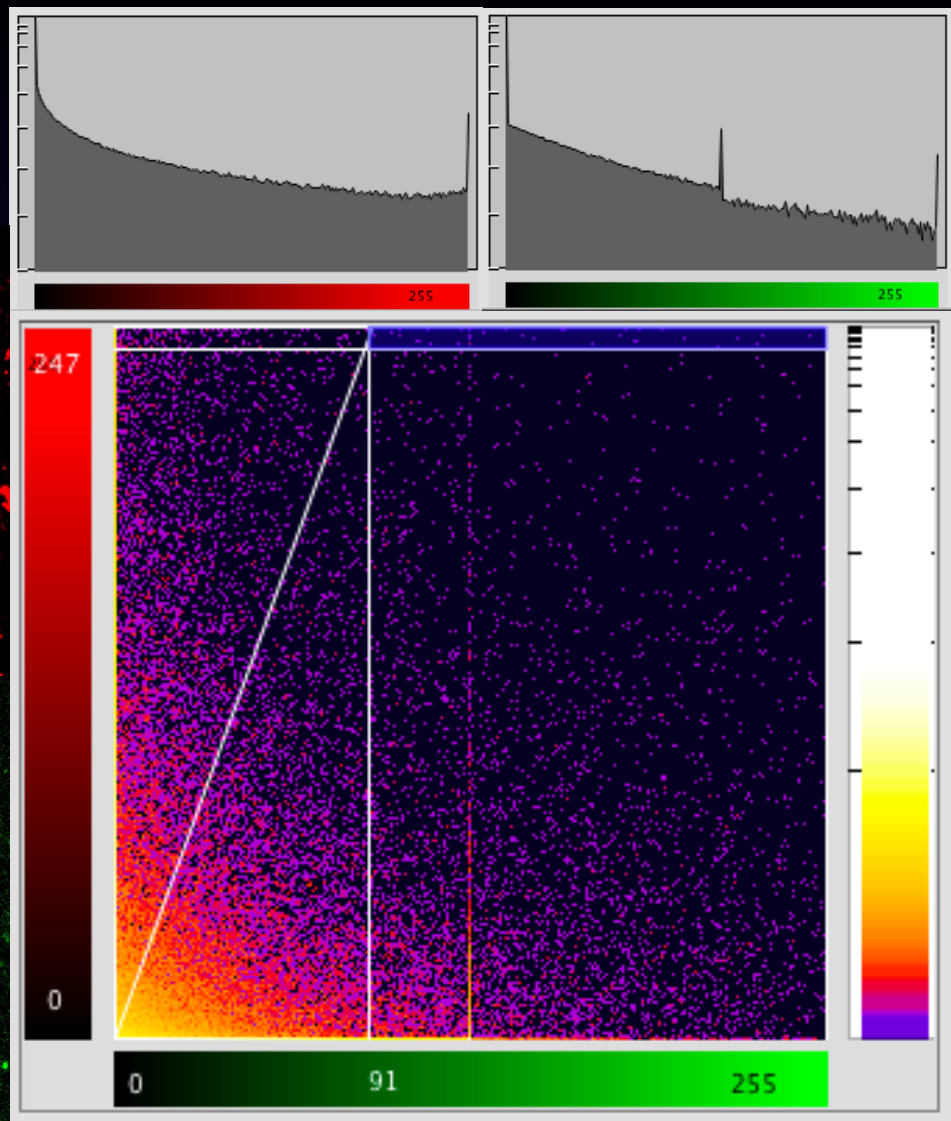
Examples:
No Correlation?

Pearson r 0.024
M1 0.0354
M2 0.0471

Why high Thresholds?





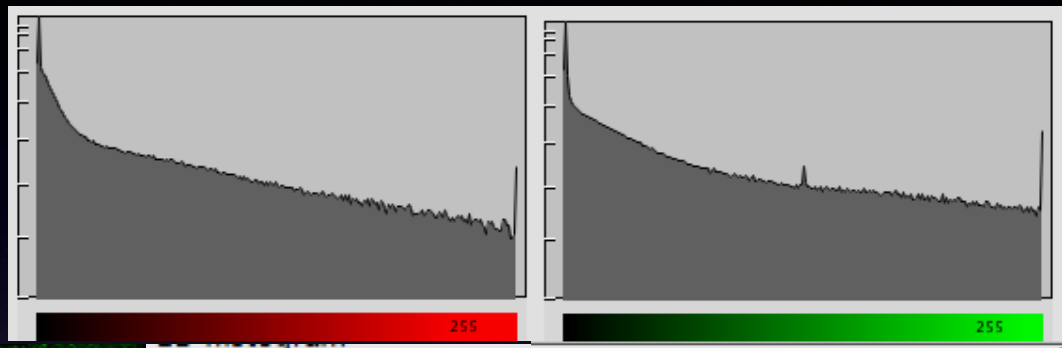


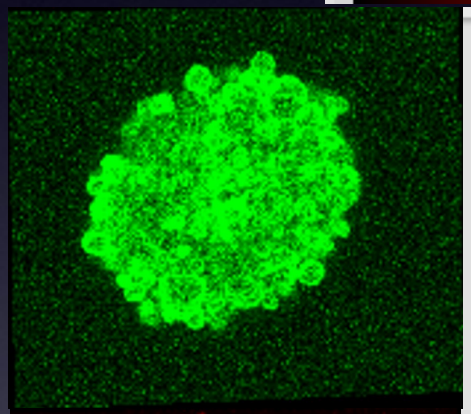
Noisy Saturated Images
Good Correlation?

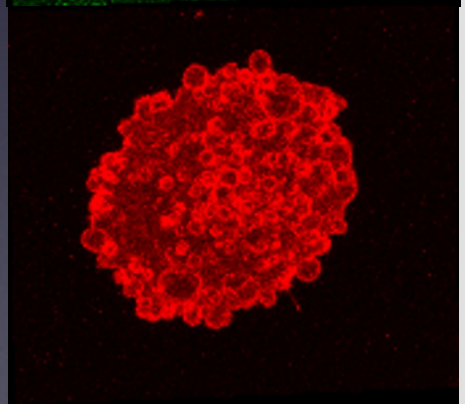
Pearson r 0.747
M1 0.7291
M2 0.7420

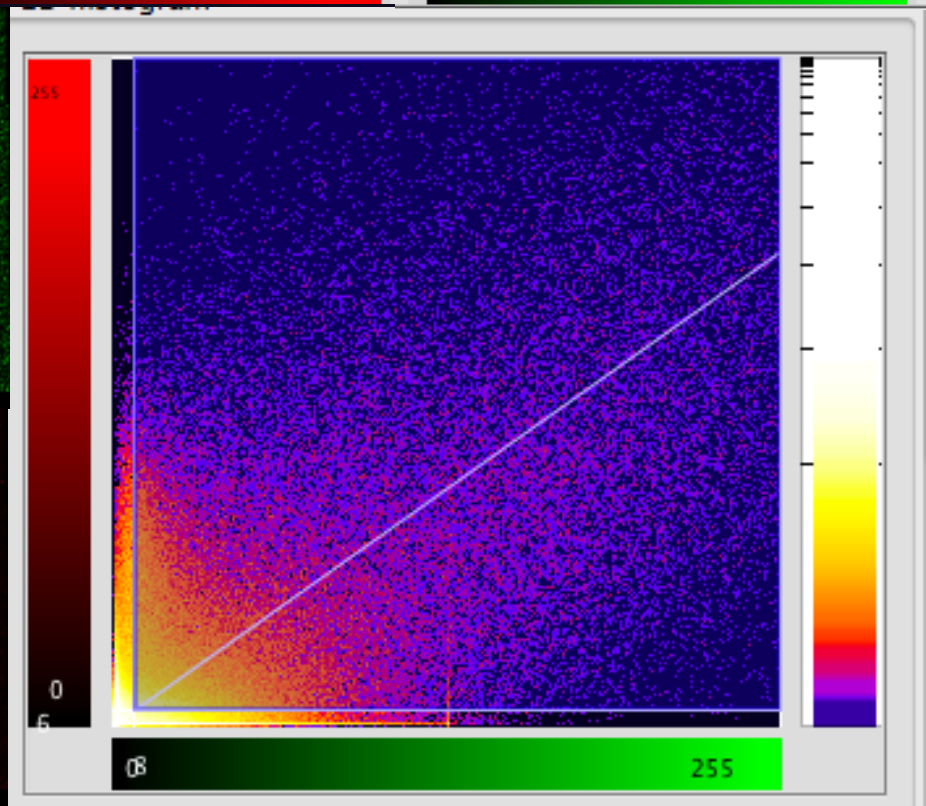
Thresholds Include noise?

Badly Saturated!







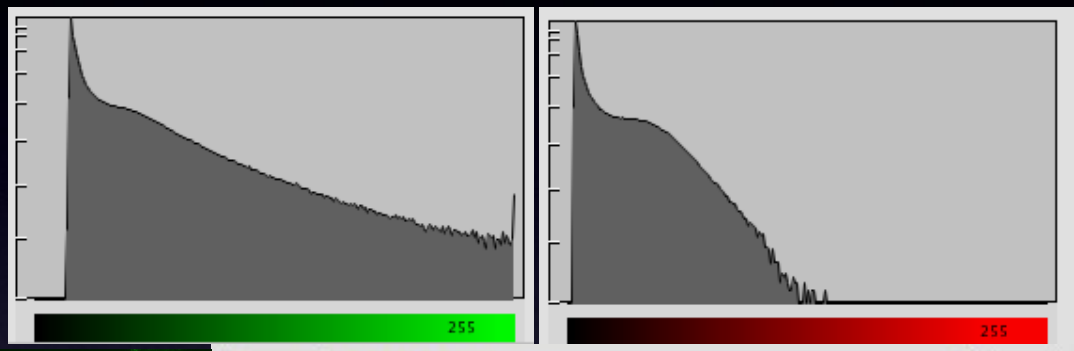


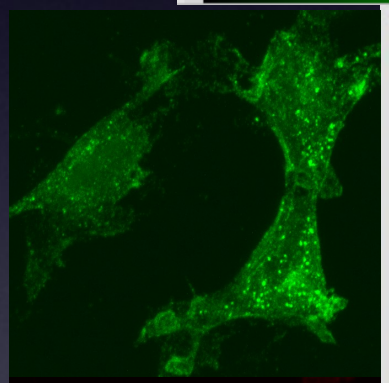
Bad detector settings
Good Correlation?

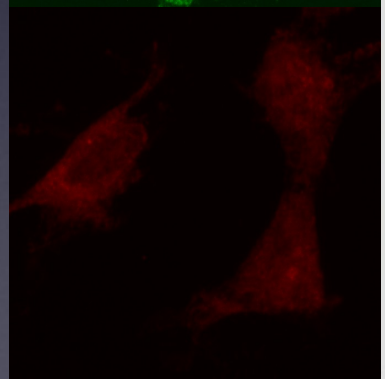
Pearson r 0.68
M1 0.77
M2 0.63

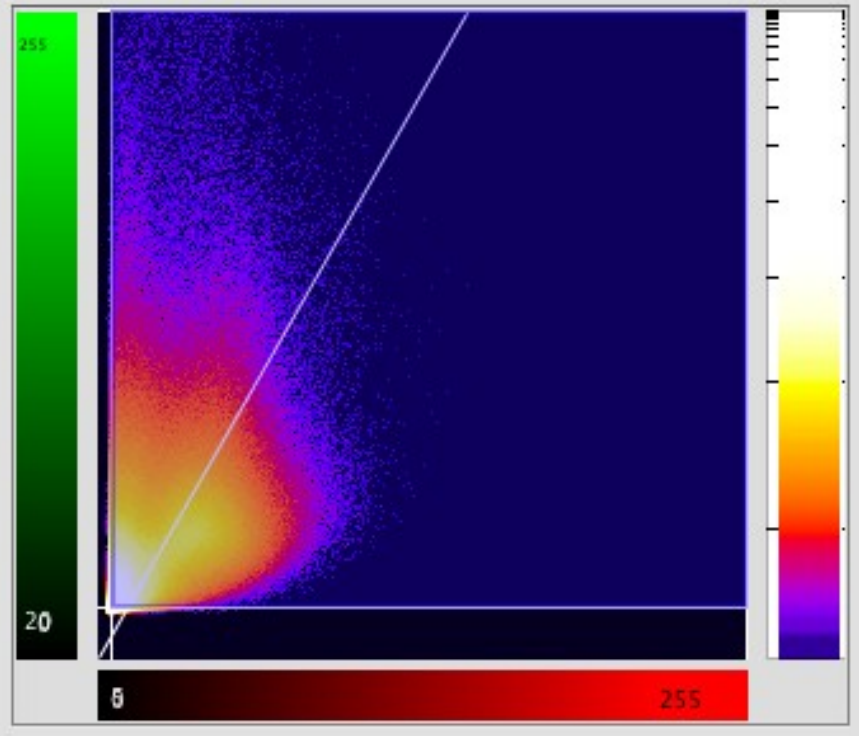
Offset wrong
+ Saturated

Thresholds
Handle it?
No?

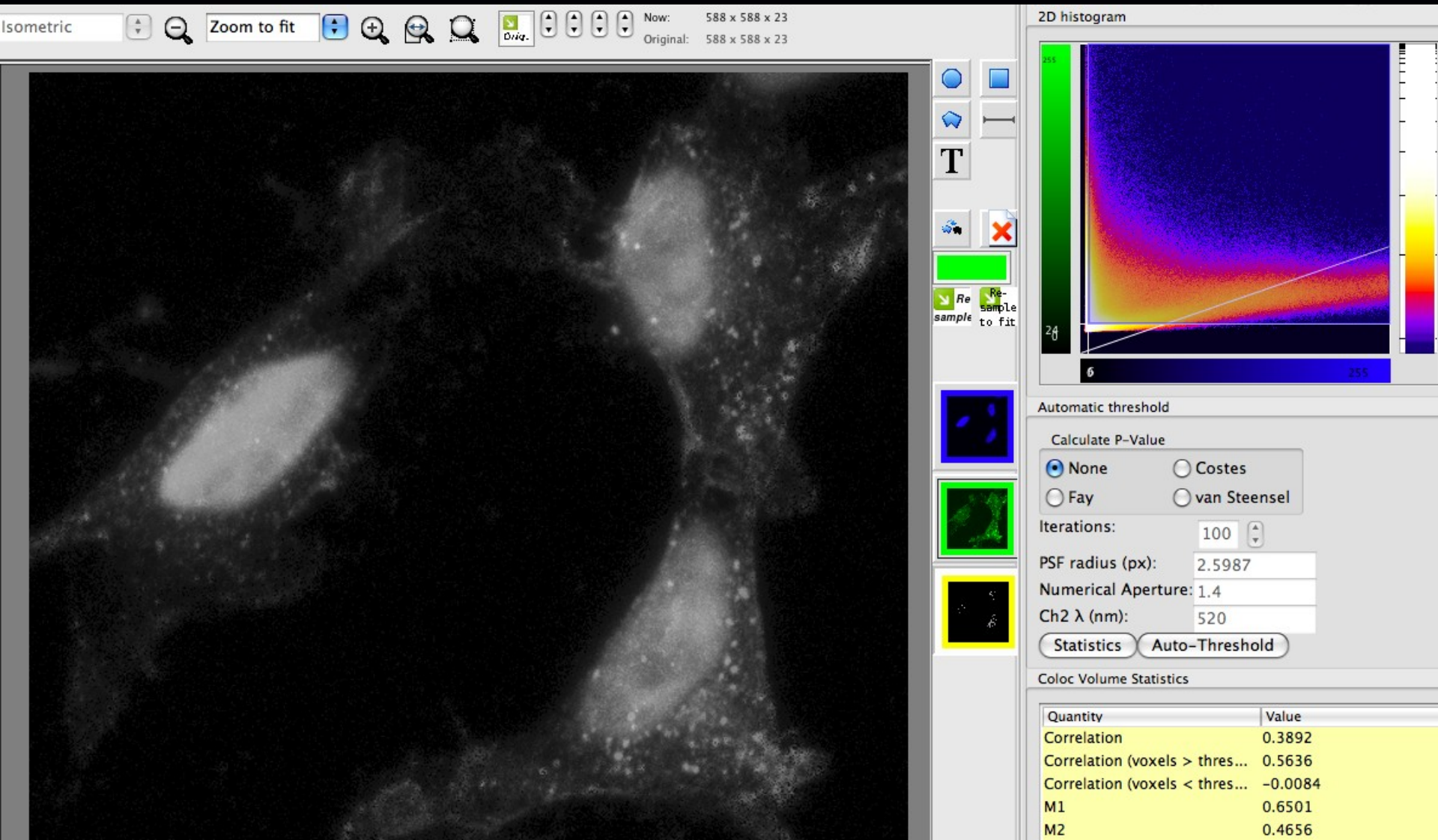




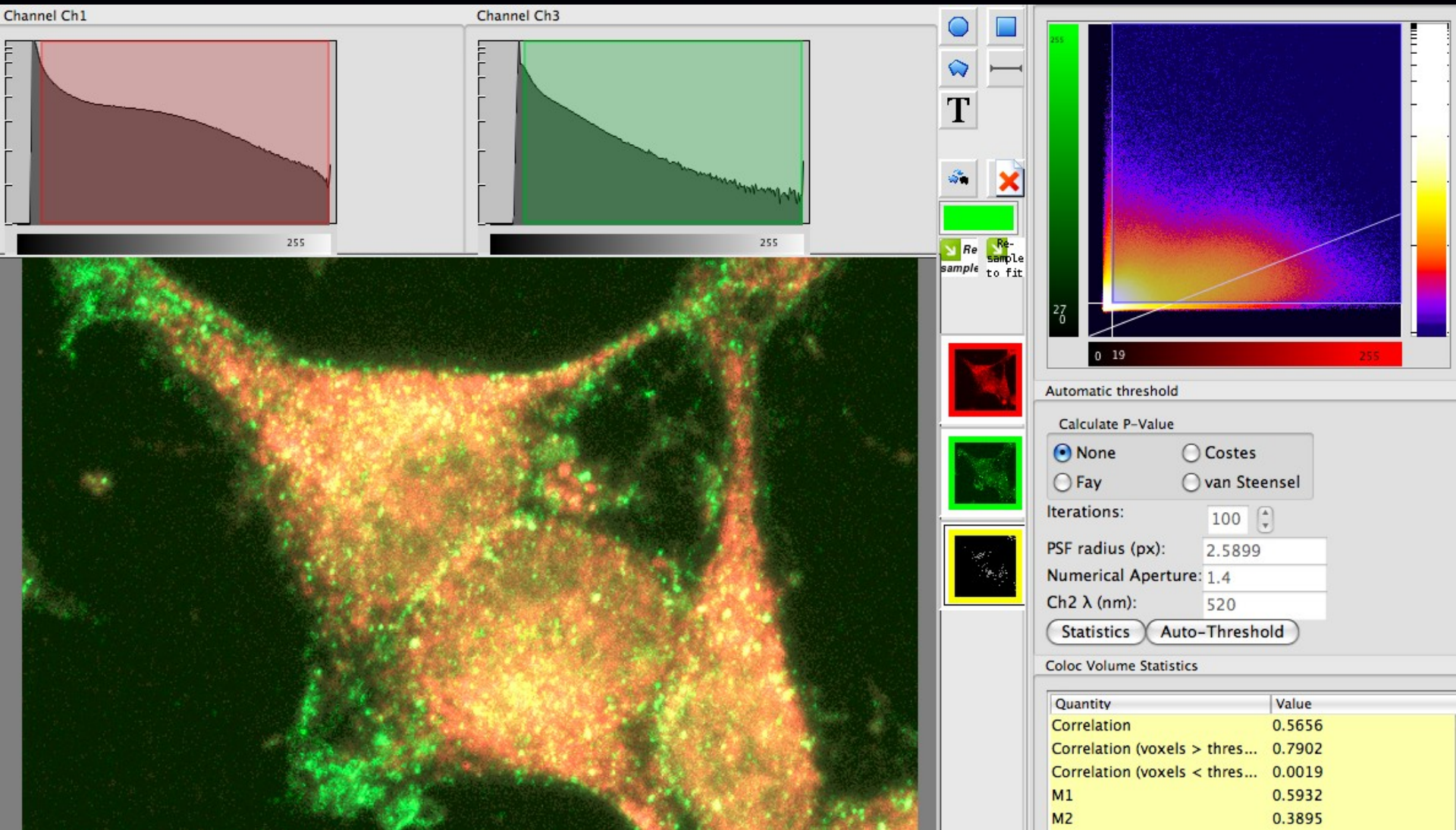




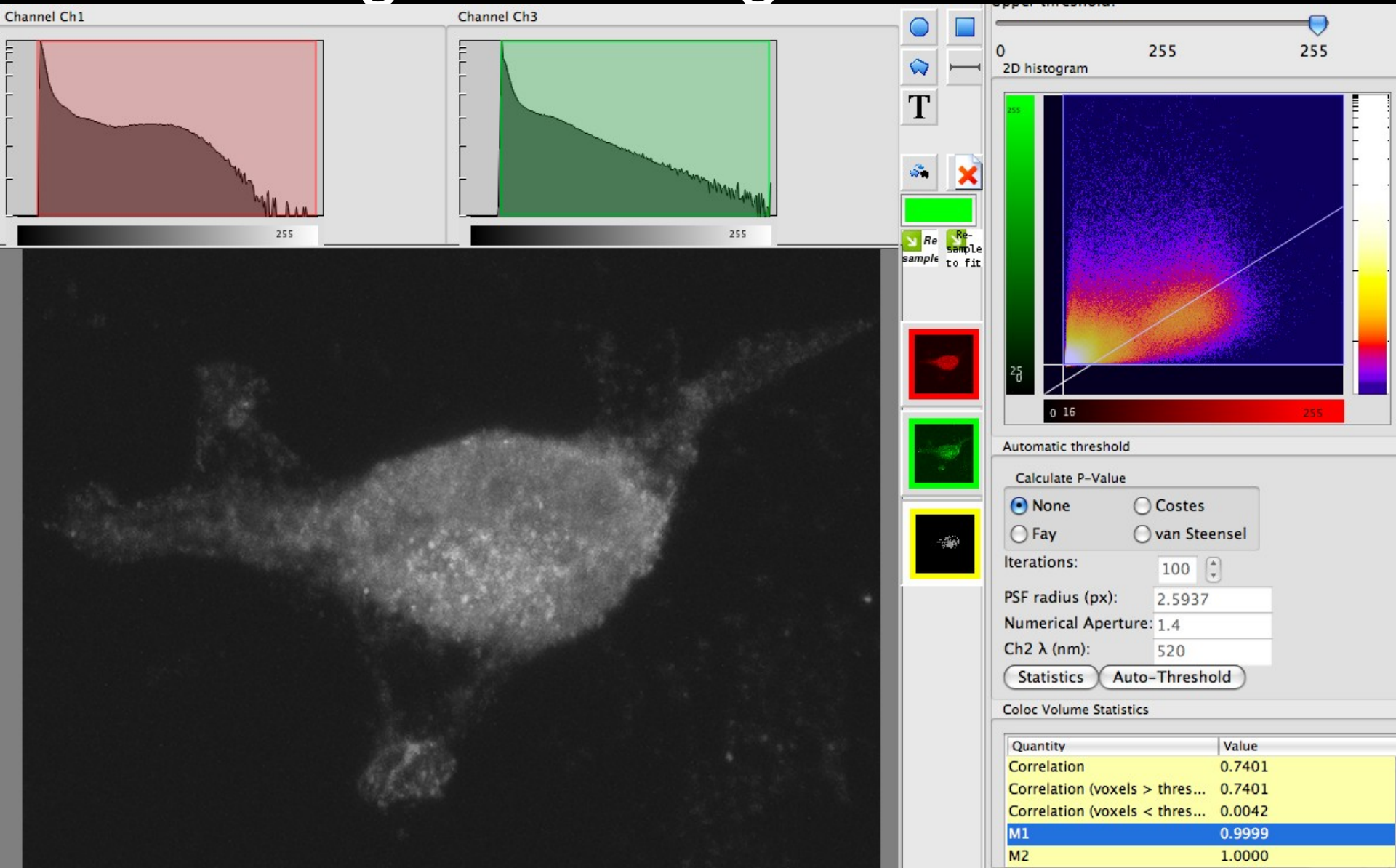
Bleed Through!
DAPI into GFP



Bad detector settings
Good Correlation? Bleed through?



Bad detector settings…
…gives wrong results!!!



Software for Colocalisation

ImageJ - Colocalisation plugins

BioImageXD (Coloc Task - Pixel Intensity and Object based methods)

Imaris (Coloc module)

Matlab (J-Y. Tinevez MPI-CBG)

Thanks to: MPI-CBG LMF and IPF
Heino, Pahajoki,
Kankaanpää, Marjomäki
Uuksalainen, Paavolainen,
TEKES,

Thanks for listening