DIGS-BB Dresden International Graduate School for Biomedicine and Bioengineering
"From Nanobiotechnology to Regenerative Medicine"

(Quantitative Imaging for) Colocalisation Analysis

… or

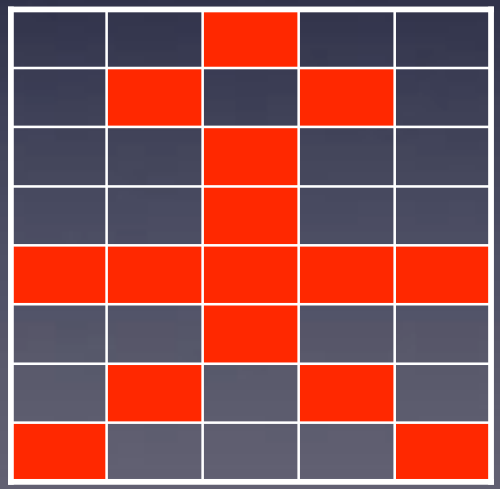
Why Colour Merge / Overlay Images are EVIL!

Special course for
DIGS-BB PhD program

CBG
Max Planck Institute
of Molecular Cell Biology
and Genetics

What is an Image anyway..?

- An image is a representation of reality (not real)
  - Image of a point is not a point (Point Spread Function)
  - Pixelated by detector (CCD or point scanner)



A digital image of ???

Image Analysis
(Brain or Computer)

A stick man?
How do I know?
How can computer know - algorithm?

What is an Image anyway..?

- Images contain information (not just pretty pictures)
  - Manipulate Image = Changed Info
    (Brightness / Contrast - Extreme Caution!!!)
  - Image data can be quantified / measured / analysed
  - You cant add lost info back.
  - Meta data (What, Where, When, How)

A digital image
How many objects?
How “bright” is it?
How big is it?
What is it?
etc.

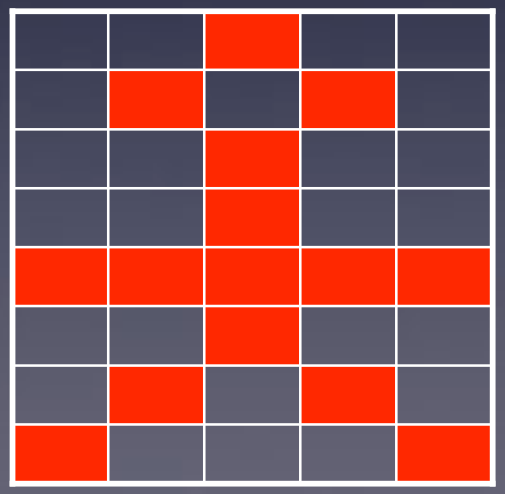
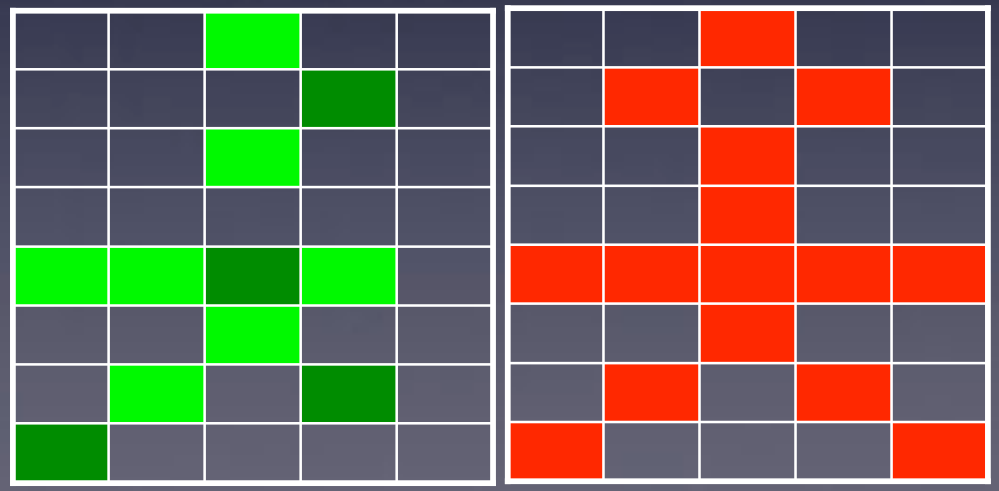


Image Data? What is it?

- Intensity is related to what? Something physical?
  - Dye concentration Or is it? Why not?
  - Noisy Images? Averaging? Pixel Time?
- Comparison of 2 colours/dyes -
  Biology / BioChemistry / Interaction ?
- Shapes, Movement, Structure?

A digital image
With 2 channels / colours

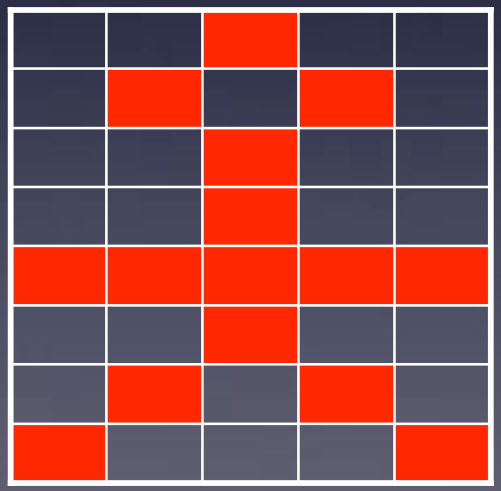
What can you say here?



Photographer or Spectroscopist?

- We can show you how to take pretty pictures (Art)
- We can teach you to get useful information (Science)

- You have to choose which you want to be!



This

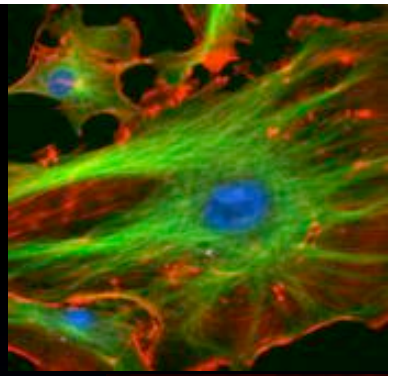
Is simply a way to "Visualise"

This

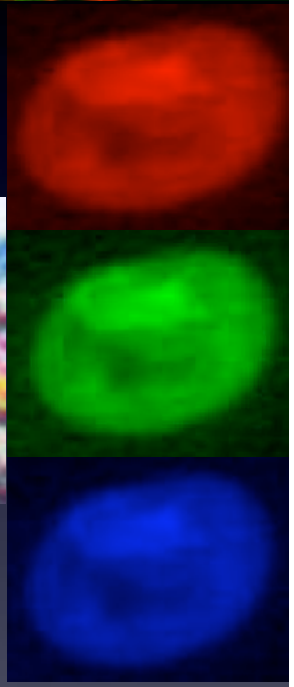
0	0	1	0	0
0	1	0	1	0
0	0	1	0	0
0	0	1	0	0
1	1	1	1	1
0	0	1	0	0
0	1	0	1	0
1	0	0	0	1

Publishing Images

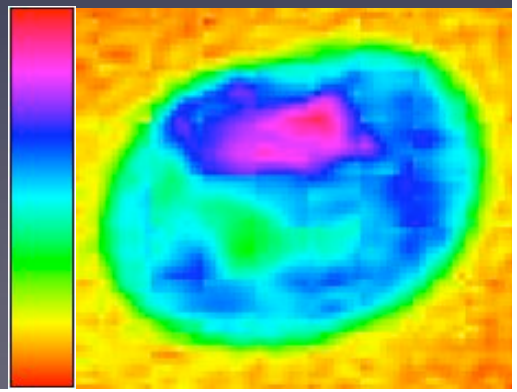
or “how Photoshop can ruin your career”



- Which image? Prettiest? Representative?
- CCD/PMT sees intensities differently than your eye/brain
  - LUT? Gamma correction? Calibrate Monitor - we have the tools!
- RBG colour space is not what we print!
  - RGB - Visualise (LCD, CRT)
  - CYMK - Print
  - Journal Image ≠ Screen Image

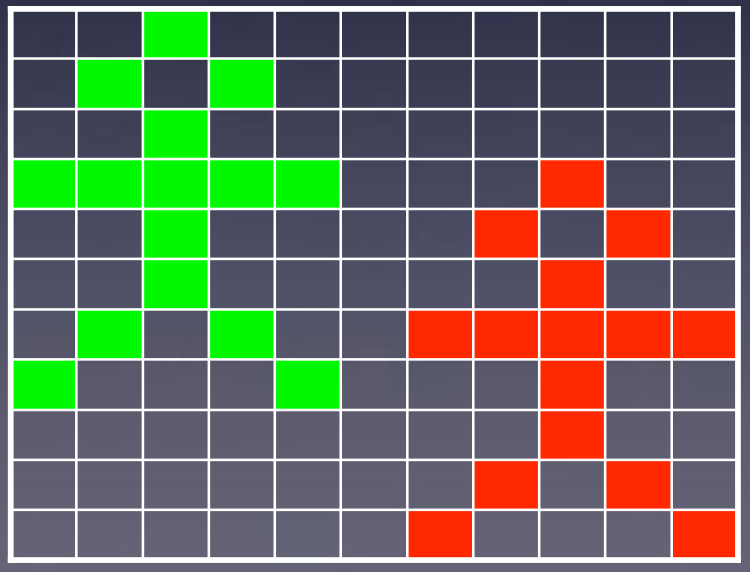


- Author instructions - image format? TIFF CYMK
- Materials and Methods - exact image processing done
- Image = data Don’t corrupt information!
  - PDF - reviewer can check image processing results!
  - Compression - Lossless ok - Lossy (JPEG) very bad
  - You wouldn’t do it to any other kind of data



Quantitative Image Analysis?
…what does that mean?

- Pretty pictures are great for journal covers...
- Movies are great for visual presentation of images...
- Interactive 3D visualisation, data exploration...
- But for meaningful biological conclusions...
  - Scientists need numerical results from image data
  - Need to measure many objects
  - Need statistics from many images
  - Computers become useful!

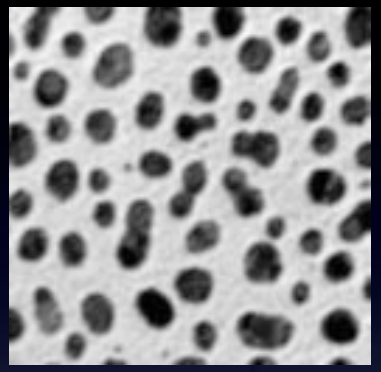


Quantitative Microscopy - First Think...

- Choosing experimental and image processing methods:
  - What BIOLOGY am I trying to see or measure?
  - Do I need 3D information? Resolution? Object size?
  - Choose / Optimise microscope system to use!
  - Statistics? How many images / data points / experiments?
  - **Controls!!!**

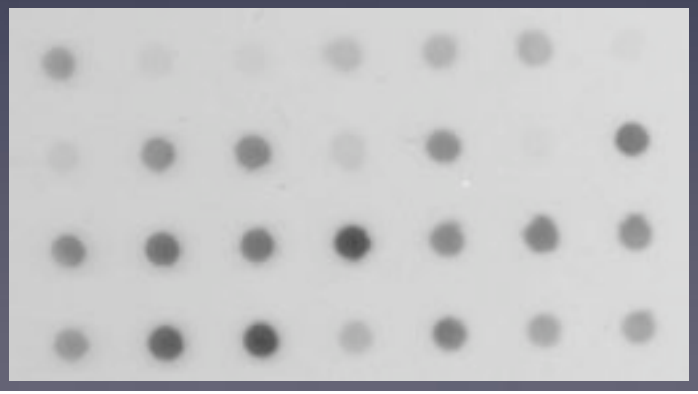
Experimental Design - First Think...

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



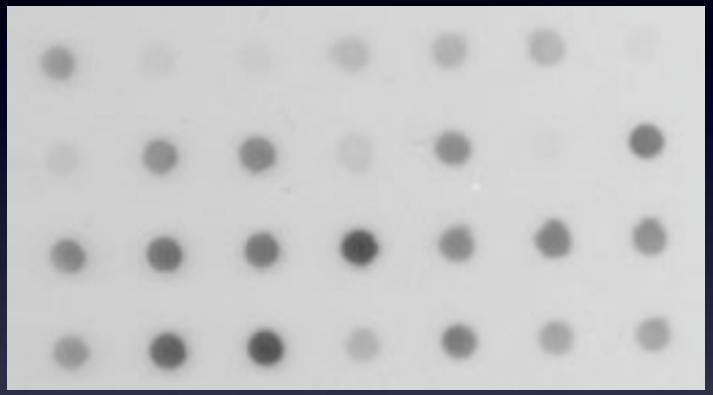
  - Am I trying to see move over time?

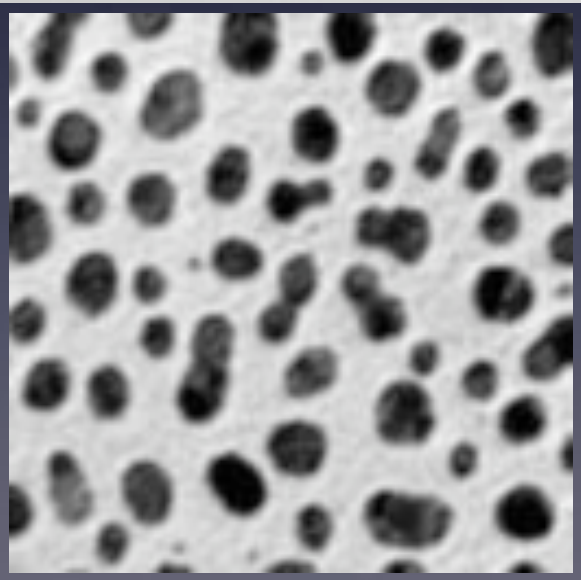
  - Am I trying to measure a number, amount or concentration?



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

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

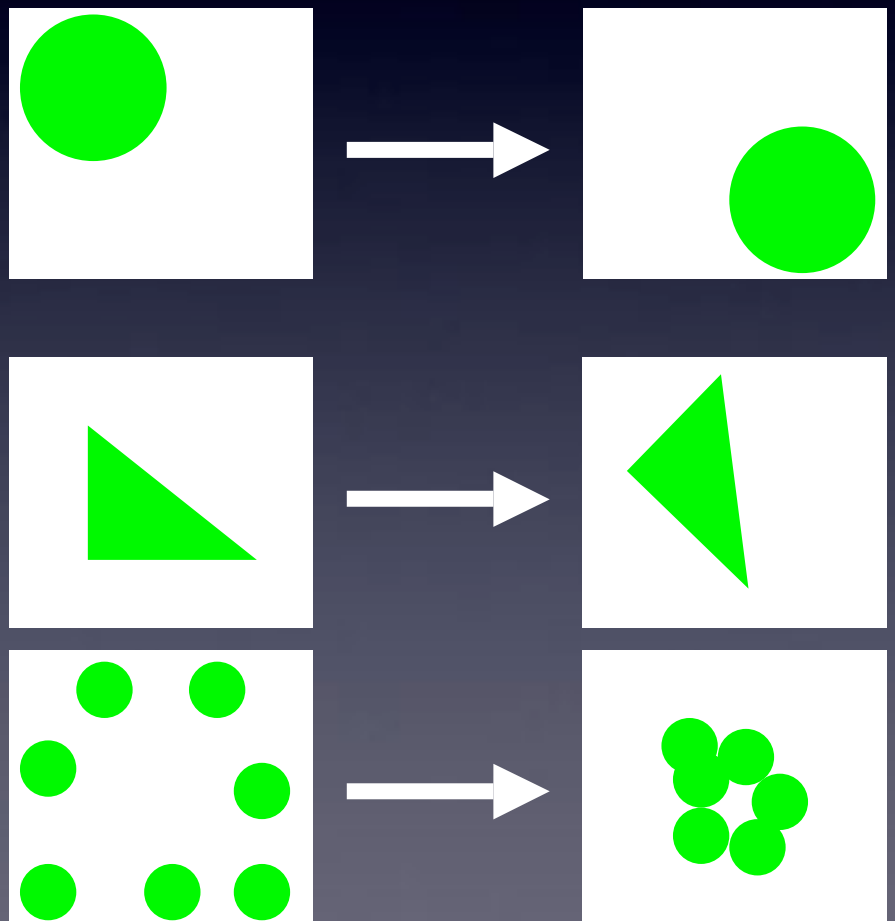




- Image intensity - threshold
- Size - threshold
- Shape - circularity etc.

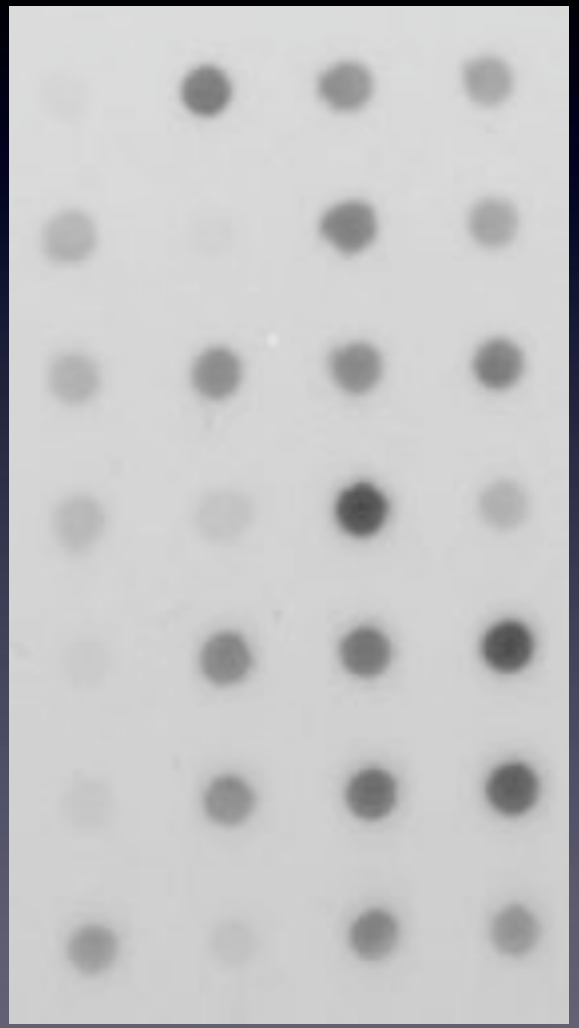
Am I trying to see something move over time?

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



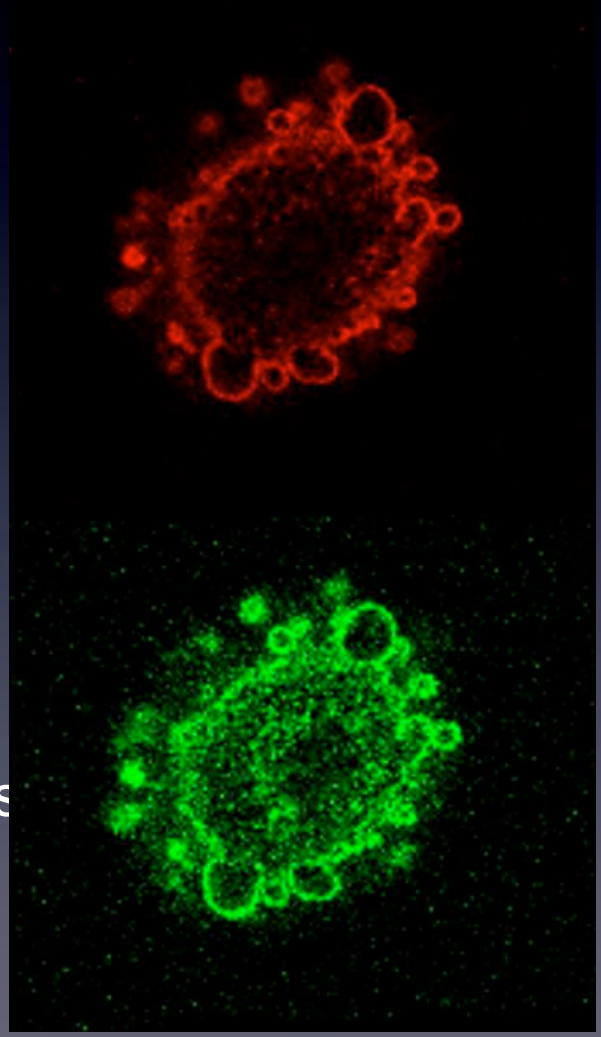
Am I trying to measure an amount or concentration?

- 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



- Signals within the range of the detector?
  - Your eyes lie! You can't see low intensities close to black! Use Range Indicator / HiLo / OU and spectrum CLUTs
  - Adjust so brightest part is within detector range.
  - Remember to check z dir. also.
  - Don't over expose the image! Why not? Lost Info!

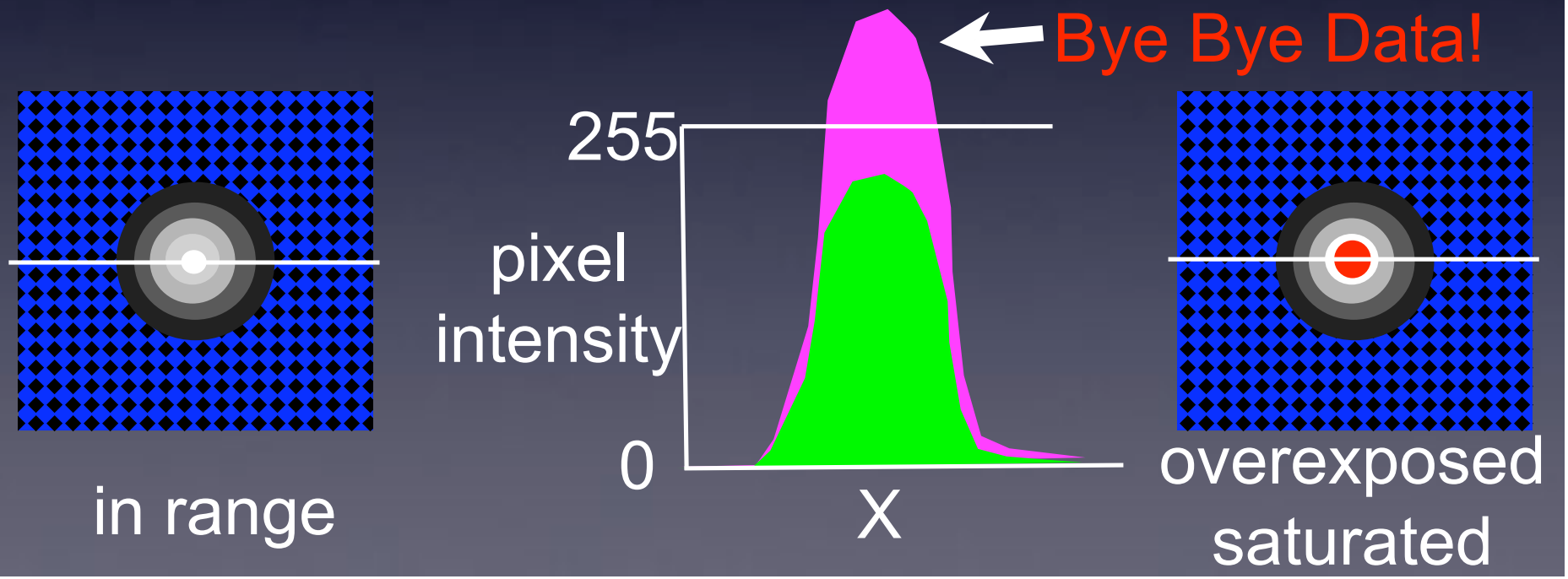
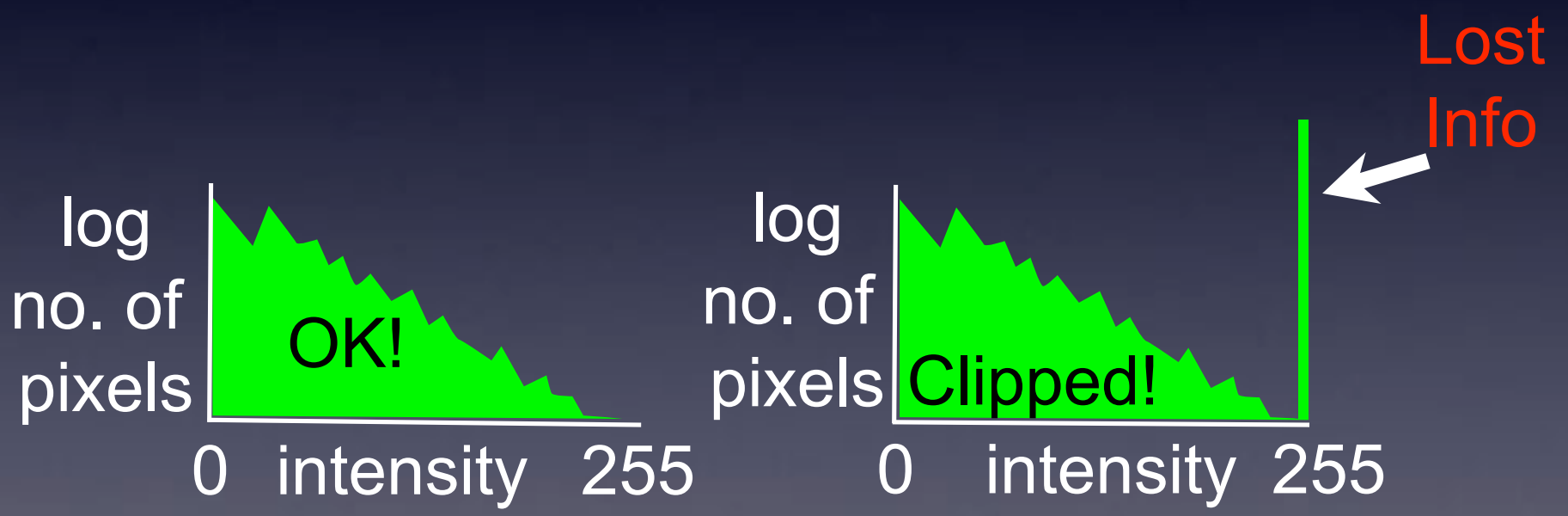
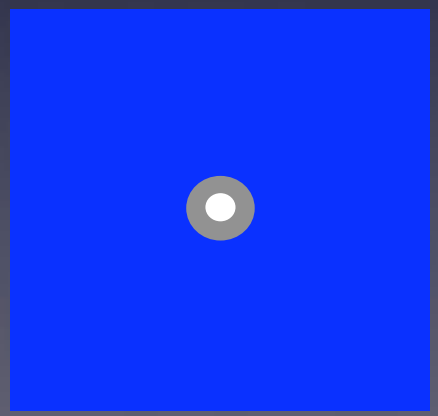


Image Histograms are your friends!

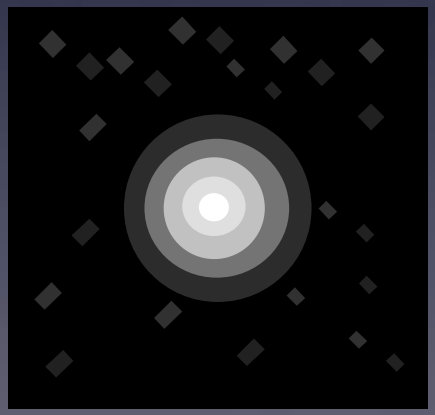
Use them!



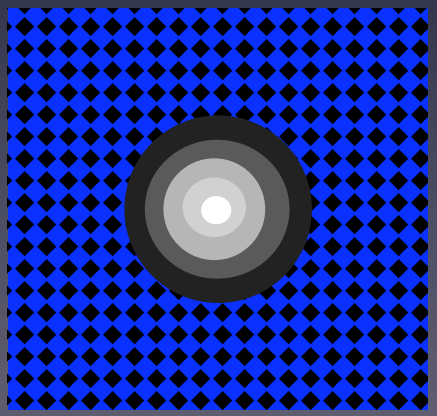
- Signal within the range of detector?
  - Offset / Zero Background - Set properly.
  - Why? “background” ≈ zero, but keep low intensity info
    - What is “Background”? You decide!
  - Range indicator / HiLo CLUT - background black and blue ~50:50
  - (0 = Blue, 1 = Black, 254 = White, 255 = Red)



too high



too low

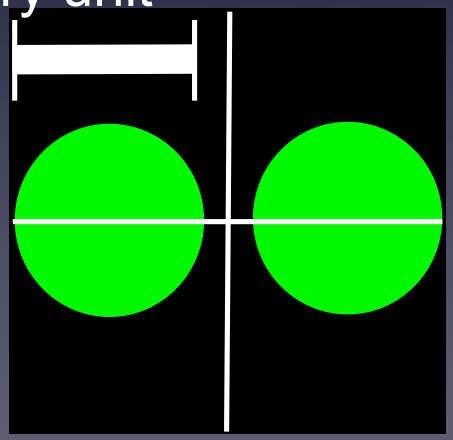


correct

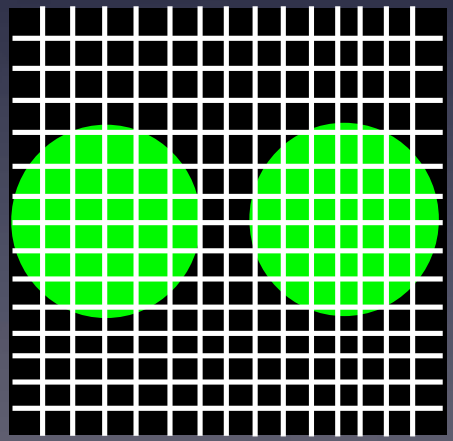
Pixel Size / Resolution

- "Correct" image size (64x64, 512x512, 2048x2048)?
  - Get all information microscope can resolve, but files not too big
  - Proper spatial sampling (Nyquist sampling theory)
  - 2.3-3 pixels over optical resolution distance. (x, y and z)
  - Adjust zoom and image size.
  - Auto Pinhole or 1 Airy unit

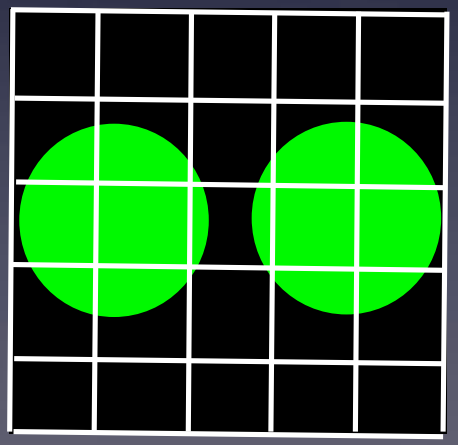
1 Airy unit



under sampled



over sampled

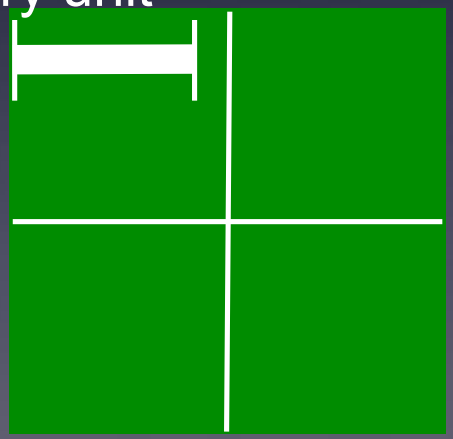


correct sampling

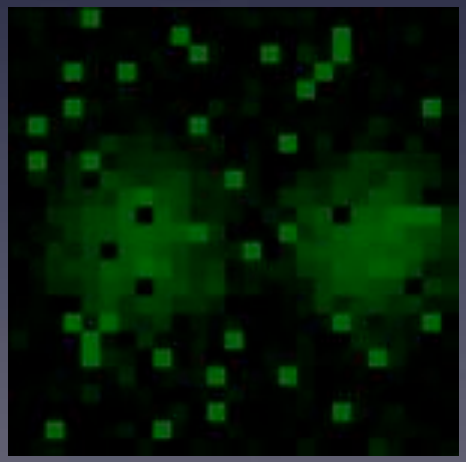
Pixel Size / Resolution

- “Correct” image size (64x64 or 2048x2048 - or something else)?
  - Get all information microscope can resolve, but files not too big
  - Proper spatial sampling (Nyquist sampling theory)
  - 2.3-3 pixels over optical resolution distance. (x, y and z)
  - Adjust zoom and image size.
  - Auto Pinhole or 1 Airy unit

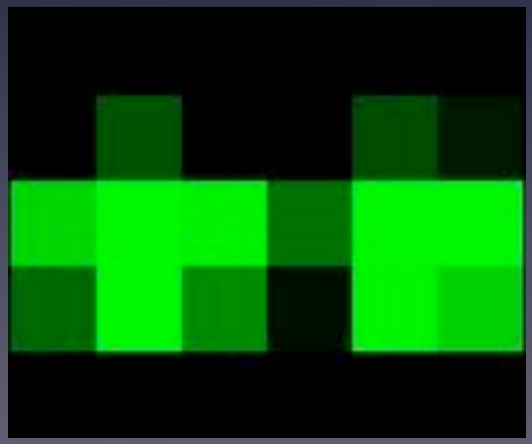
1 Airy unit



under sampled



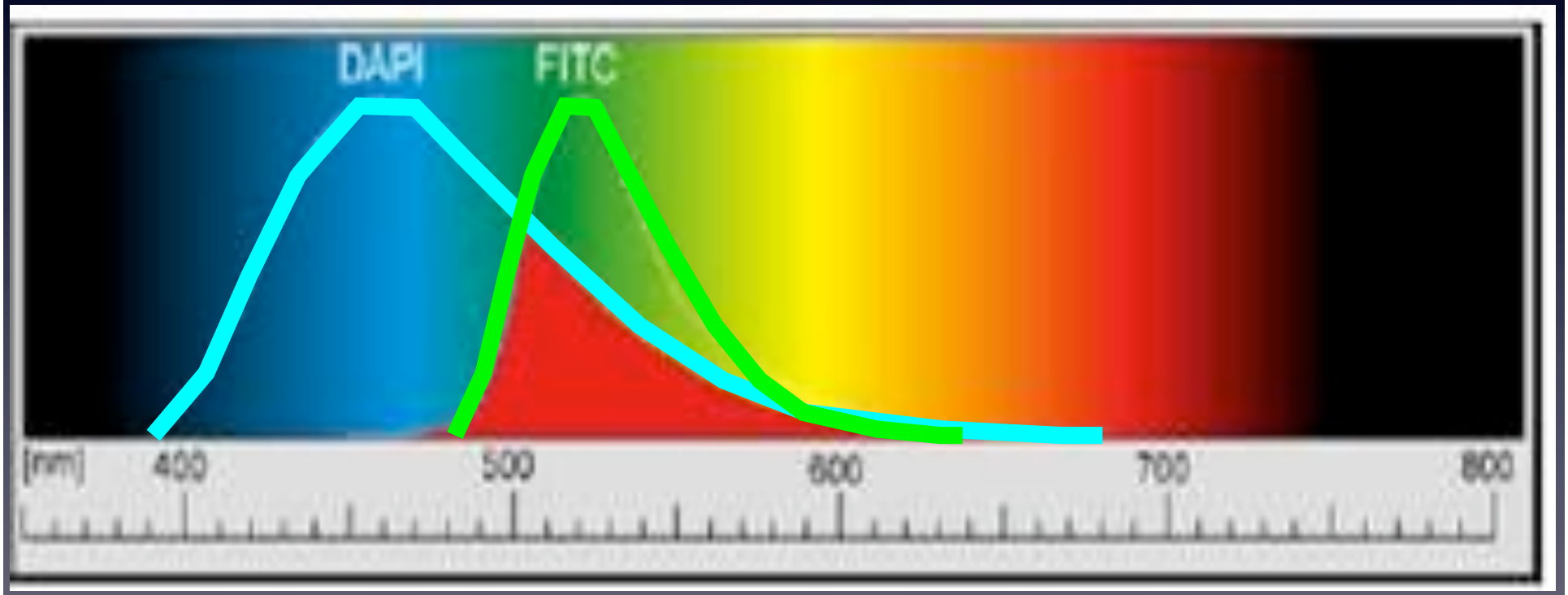
over sampled



correct sampling

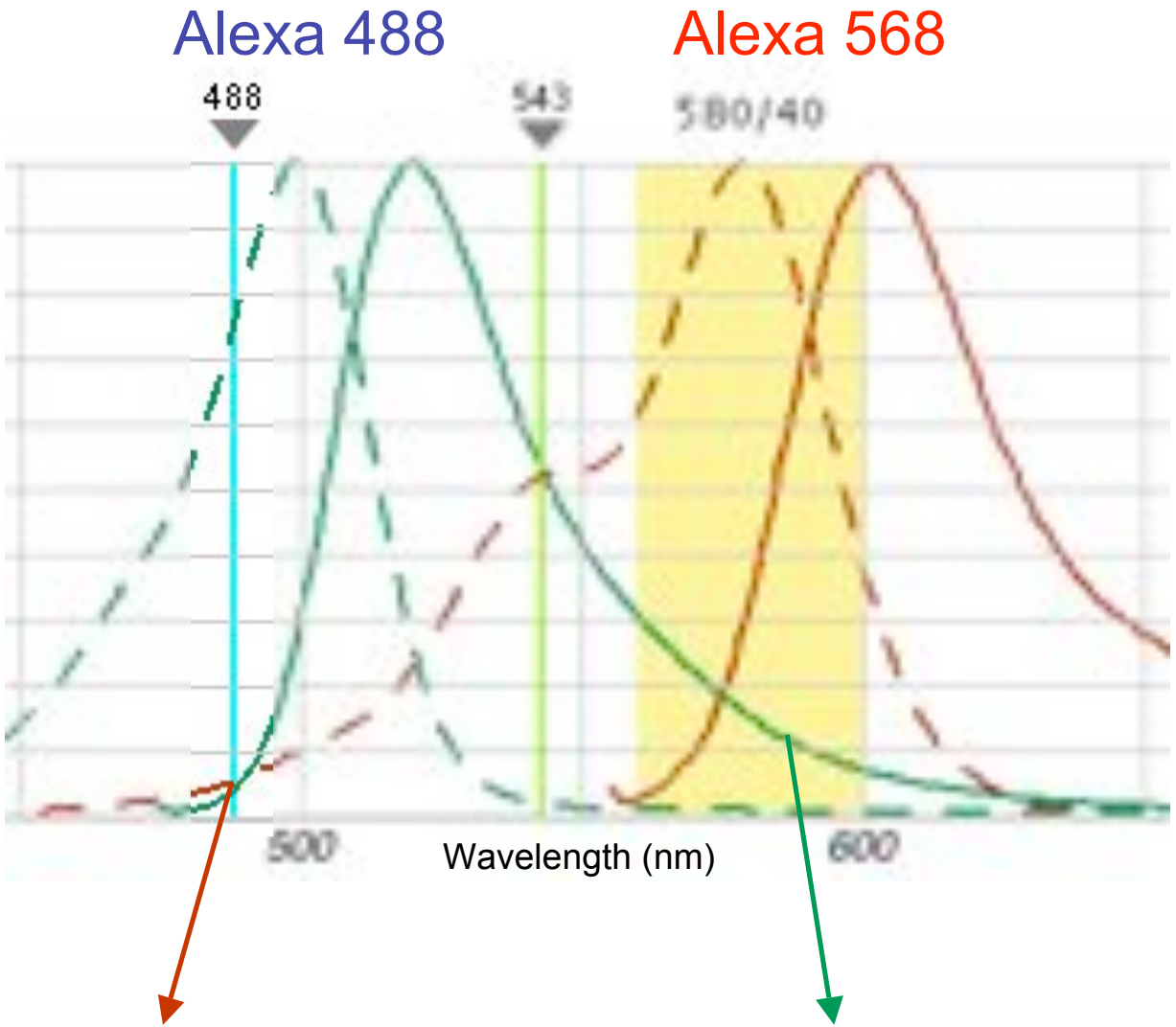
Avoid Emission Bleed Through and Crosstalk/Cross-excitation

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



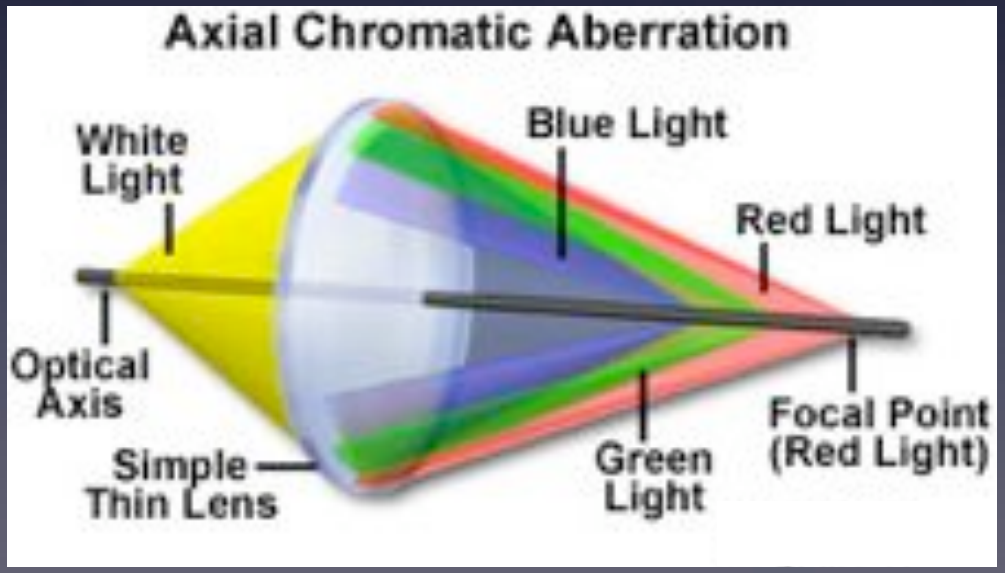
- Use multi tracking (Zeiss) / sequential (Olympus)

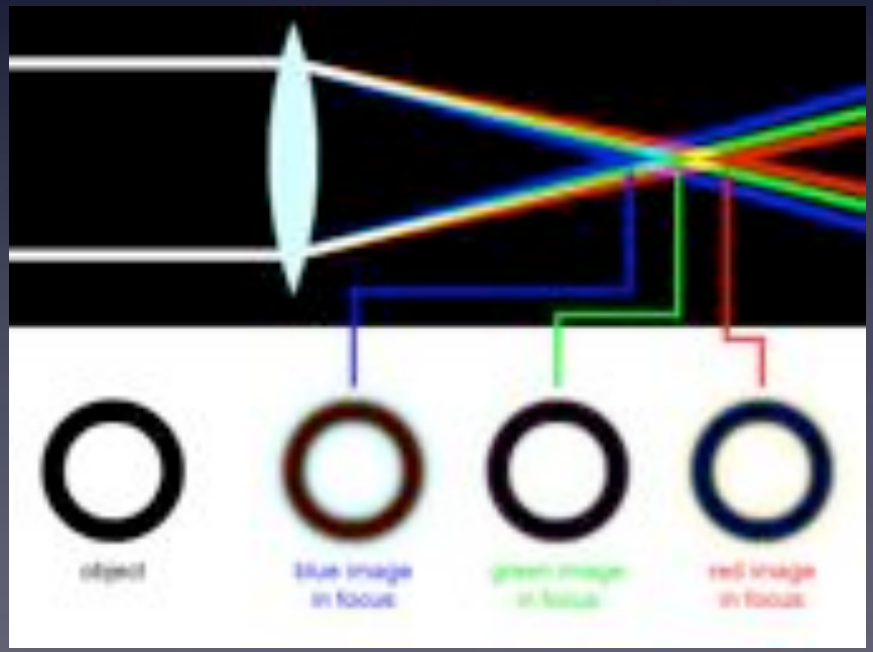
Beware ! Crosstalk and Bleed Through



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





Watch Out - More Holes To Fall Into:

- Required bit depth - 8 bit often enough for LSCM imaging… and colocalisation 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 - some 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)