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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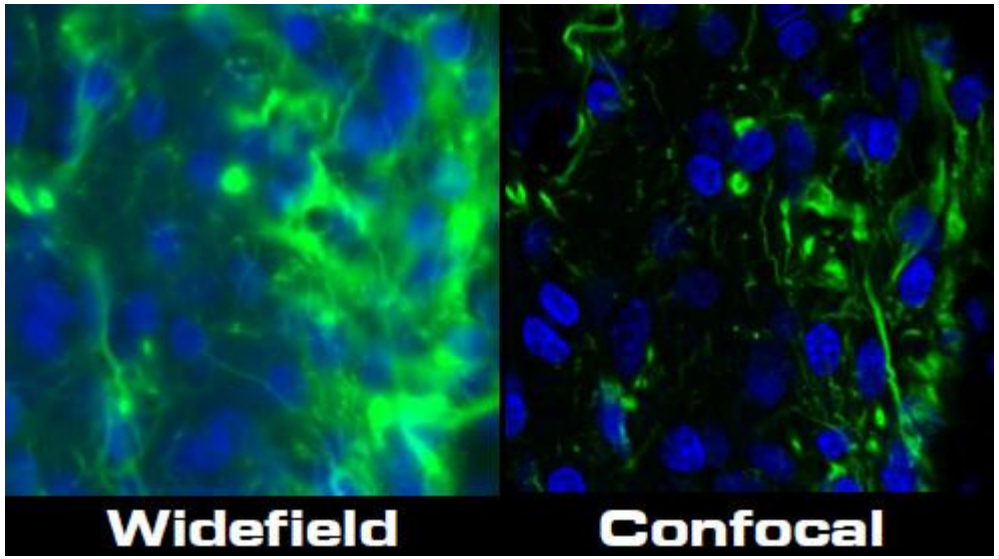
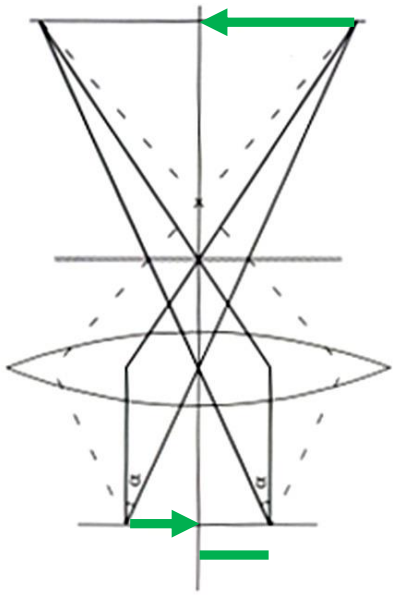
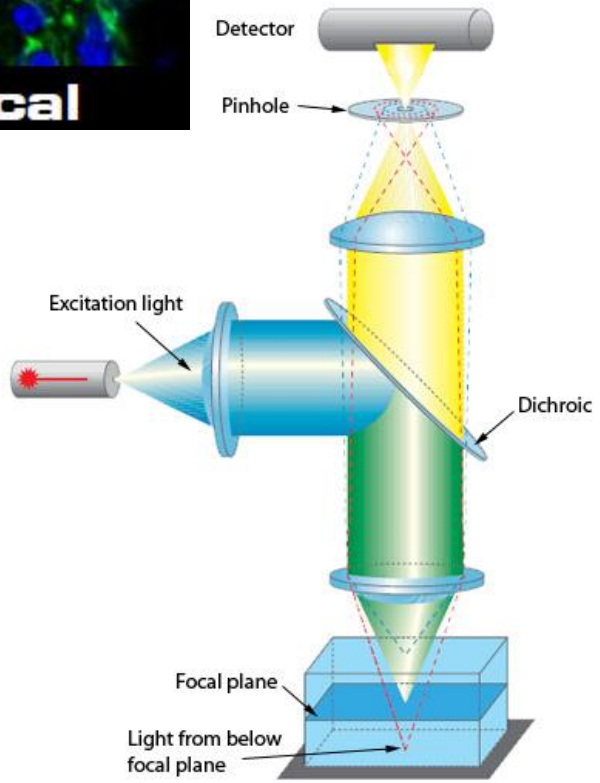
Image data of more than 2D- z, t . . .

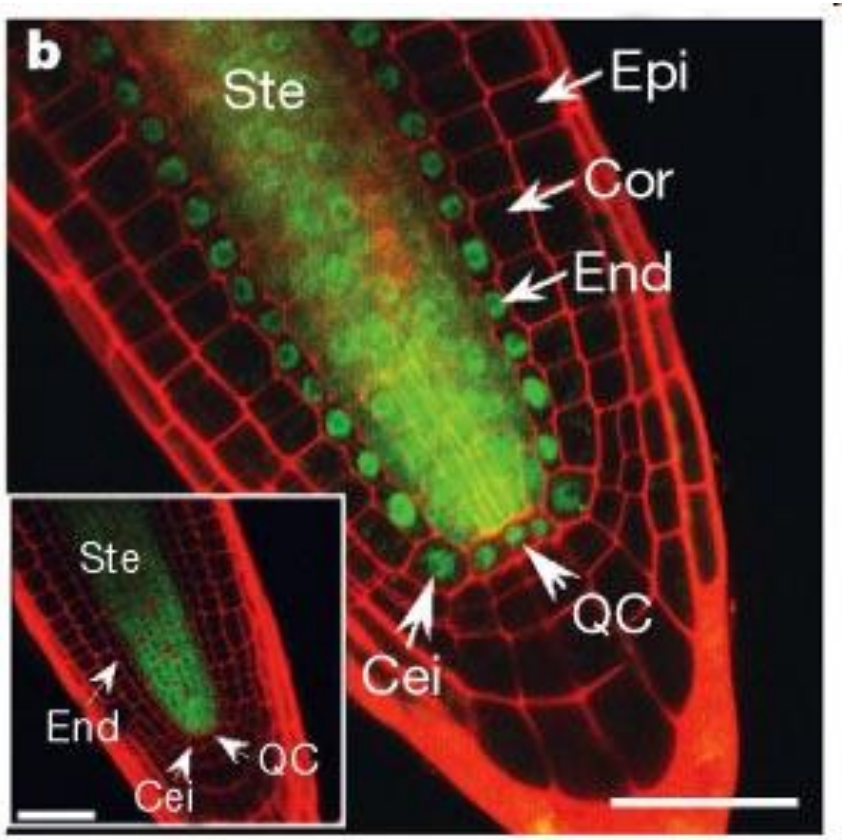
Optical sectioning

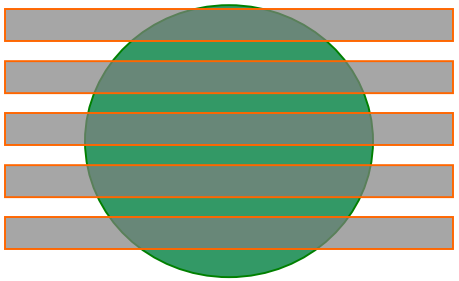




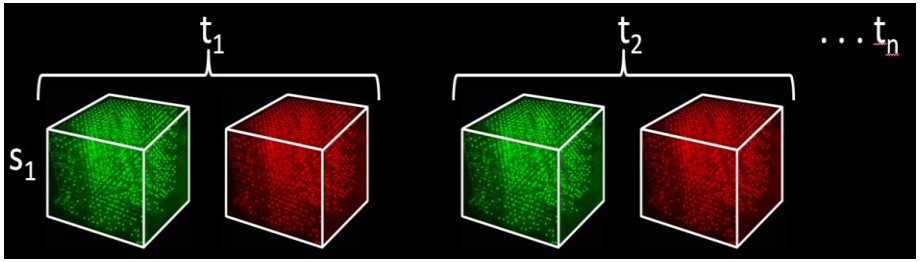


Optical sectioning and 3D imaging





When more than 2 dimensions are involved



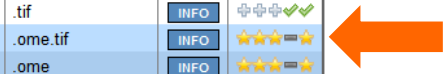
- ZTC, XYCZT, CTZ . . . order
- It’s generally all there, just need to understand it
- Bit more of a head-ache than you might think
- Watch out for mistakes
- Easy to swap (normally)

Plugins/LOCI/Bio-Formats Importer

#	Export	Format	Extensions	Details
1.	✗	Adobe Photoshop PSD	.psd	INFO
2.	✗	Alicona 3D	.al3d	INFO
3.	✗	Amersham Biosciences GEL	.gel	INFO
4.	✗	Analyze 7.5	.img, .hdr	INFO
5.	✗	Andor Bio-Imaging Division (ABD) TIFF	.tif	INFO
6.	✓	Animated PNG	.png	INFO
7.	✗	Aperio SVS TIFF	.svs	INFO
8.	✓	AVI (Audio Video Interleave)	.avi	INFO
9.	✗	Axon Raw Format (ARF)	.arf	INFO
10.	✗	Becker & Hickl SPCImage	.sdt	INFO
11.	✗	Bio-Rad PIC	.pic	INFO
12.	✗	Bitplane Imaris	.ims	INFO
13.	✗	BMP (Windows Bitmap)	.bmp	INFO
14.	✗	Cellomics	.c01	INFO
15.	✗	DeltaVision	.dv, .r3d	INFO
16.	✗	DICOM	.dcm, .dicom	INFO
17.	✓	EPS (Encapsulated PostScript)	.eps	INFO
18.	✗	Evotec/PerkinElmer Opera Flex	.flex	INFO
19.	✗	FEI	.img	INFO
20.	✗	FITS (Flexible Image Transport System)	.fits	INFO
21.	✗	Gatan Digital Micrograph	.dm3	INFO
22.	✗	GIF (Graphics Interchange Format)	.gif	INFO
23.	✗	Hamamatsu Aquacosmos NAF	.naf	INFO
24.	✓	ICS (Image Cytometry Standard)	.ics	INFO
25.	✗	Image-Pro Sequence	.seq	INFO
26.	✗	Image-Pro Workspace	.ipw	INFO
27.	✗	Improvision Openlab LIFF	.liff	INFO
28.	✗	Improvision Openlab Raw	.raw	INFO
29.	✗	Improvision TIFF	.tif	INFO
30.	✗	InCell 1000	.xdce, .tif	INFO
31.	✗	IPLab	.ipl	INFO
32.	✗	IPLab-Mac	.ipm	INFO
33.	✓	JPEG	.jpg	INFO
34.	✓	JPEG 2000	.jp2	INFO
35.	✗	Khoros VIFF (Visualization Image File Format) Bitmap	.xv	INFO

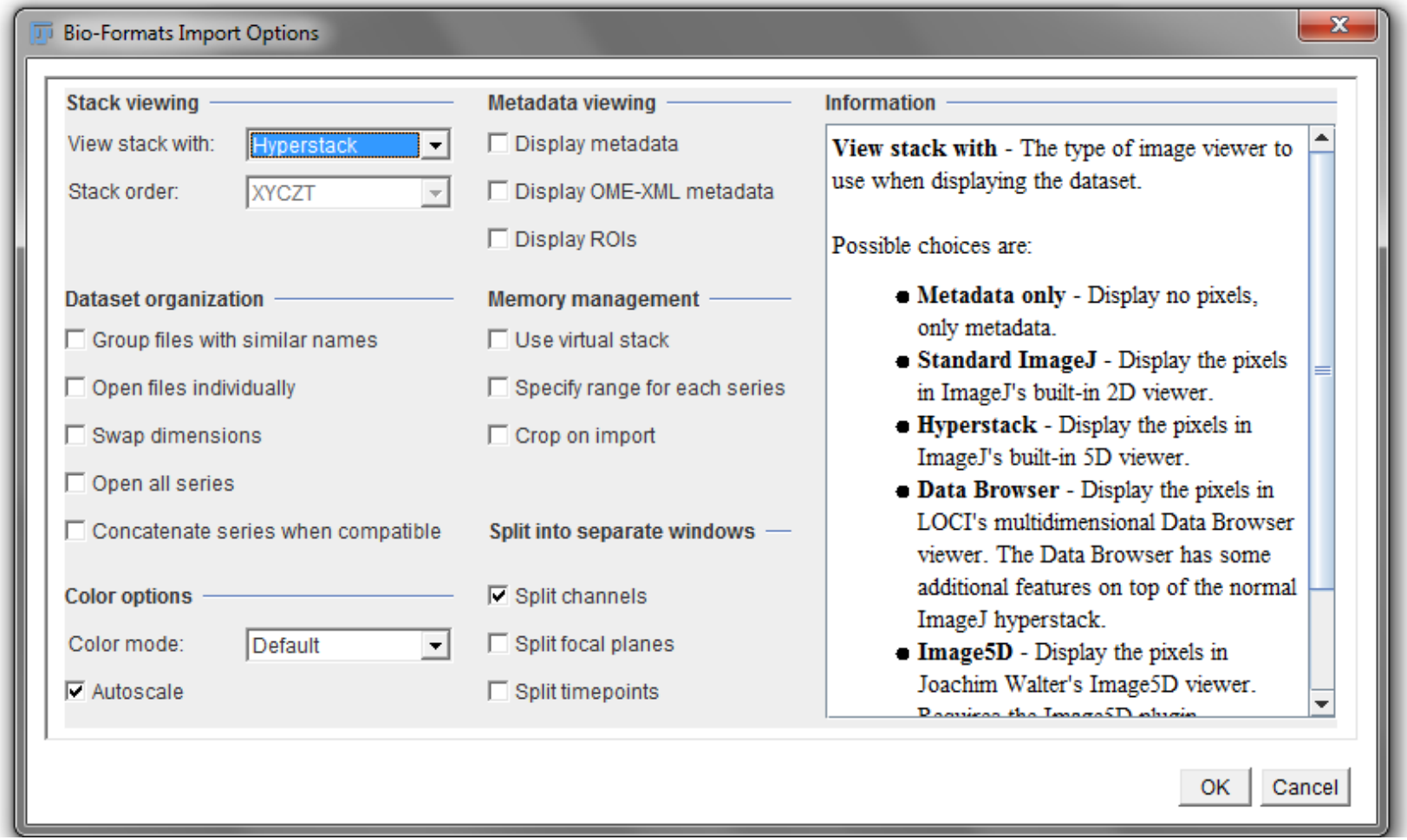
LEGEND: Pixels, Metadata, Openness, Presence, Utility

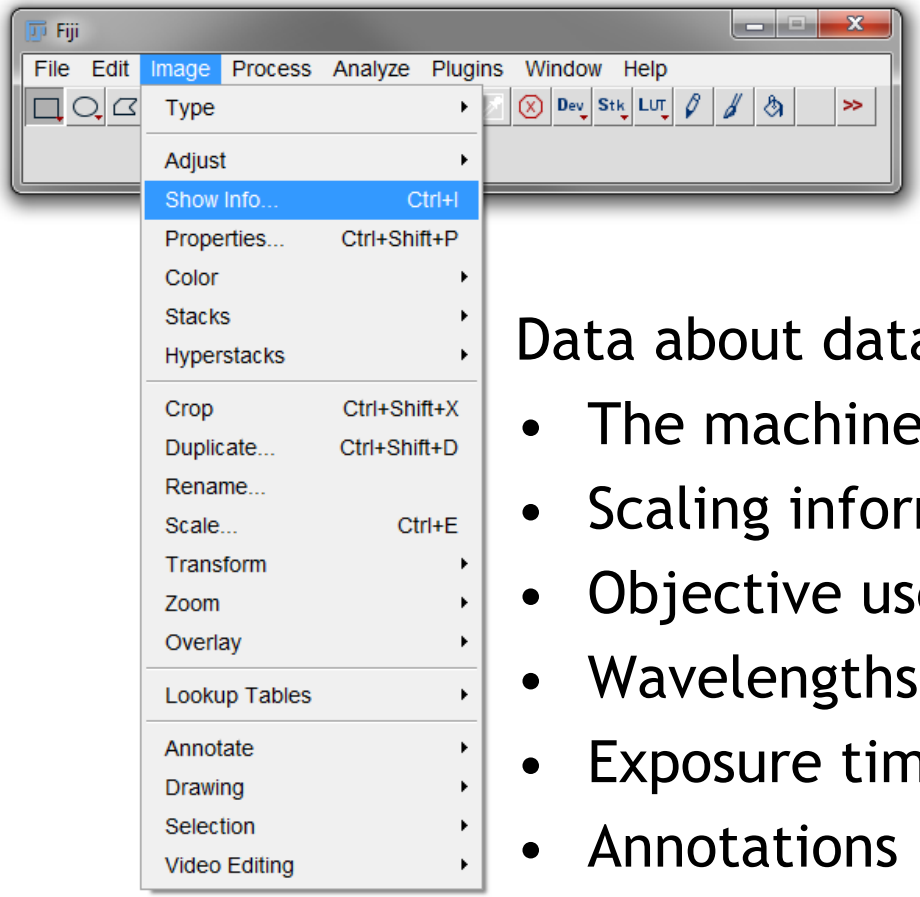
#	Export	Format	Extensions	Details
36.	✗	Leica LAS AF LIF (Leica Image File Format)	.lif	INFO
37.	✗	Leica LCS LEI	.lei, .tif	INFO
38.	✗	Li-Cor L2D	.l2d, .tif, .scn	INFO
39.	✗	LIM (Laboratory Imaging/Nikon)	.lim	INFO
40.	✗	MetaMorph 7.5 TIFF	.tiff	INFO
41.	✗	MetaMorph Stack (STK)	.stk	INFO
42.	✗	µManager	.tif, .txt	INFO
43.	✗	MINC MRI	.mnc	INFO
44.	✗	Minolta MRW	.mrw	INFO
45.	✗	MNG (Multiple-image Network Graphics)	.mng	INFO
46.	✗	MRC (Medical Research Council)	.mrc	INFO
47.	✗	NEF (Nikon Electronic Format)	.nef, .tif	INFO
48.	✗	Nifti	.img, .hdr	INFO
49.	✗	Nikon NIS-Elements ND2	.nd2	INFO
50.	✗	nrrd (Nearly Raw Raster Data)	.nrrd	INFO
51.	✗	Olympus 3i SlideBook	.sld	INFO
52.	✗	Olympus CellR/APL	.apl, .mtb, .tnb, .tif	INFO
53.	✗	Olympus FluoView FV1000	.oib, .oif	INFO
54.	✗	Olympus FluoView TIFF	.tif	INFO
55.	✓	OME-TIFF	.ome.tif	INFO
56.	✓	OME-XML	.ome	INFO
57.	✗	PCX (PC Paintbrush)	.pcx	INFO
58.	✗	PerkinElmer UltraView	.tif, .2, .3, .4, ...	INFO
59.	✗	PICT (Macintosh Picture)	.pict	INFO
60.	✗	PGM (Portable Gray Map)	.pgm	INFO
61.	✓	PNG (Portable Network Graphics)	.png	INFO
62.	✗	Prairie Technologies TIFF	.tif, .xml	INFO
63.	✓	QuickTime Movie	.mov	INFO
64.	✗	SimplePCI	.cxd	INFO
65.	✓	TIFF (Tagged Image File Format)	.tif	INFO
66.	✗	TillPhotonics TillVision	.vws	INFO
67.	✗	VisiTech XYS	.xys, .html	INFO
68.	✗	Zeiss AxioVision ZVI (Zeiss Vision Image)	.zvi	INFO
69.	✗	Zeiss LSM (Laser Scanning Microscope) 510	.lsm	INFO



http://www.loci.wisc.edu/ome/formats.html#formats

File:
Neuron_3D_time.lsm





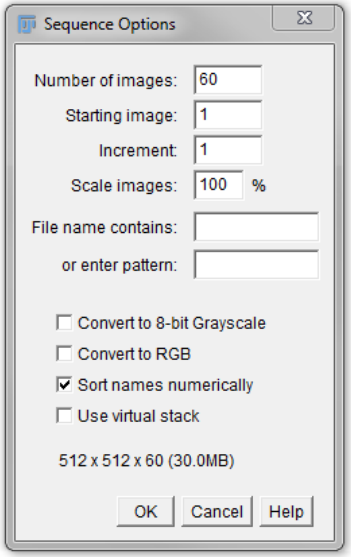
Meta-Data

Data about data-

- The machine used for acquisition
- Scaling information (size of pixels – scale bar)
- Objective used
- Wavelengths of excitation & emission
- Exposure time, Gain/offset, binning
- Annotations (sample type and preparation)
- …
- It can be wrong
- Not necessarily exported to other formats
  (good reason to keep data in native format)

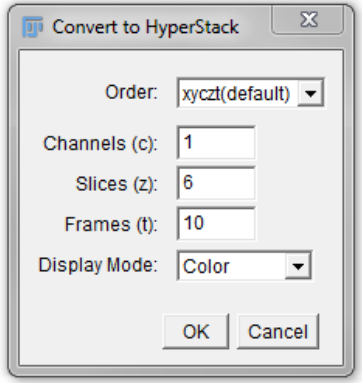
Dealing with lots of slices and dimensions: Assembling

Easy to open data in native microscopy formats, what about a tiff series like “zt_series”?



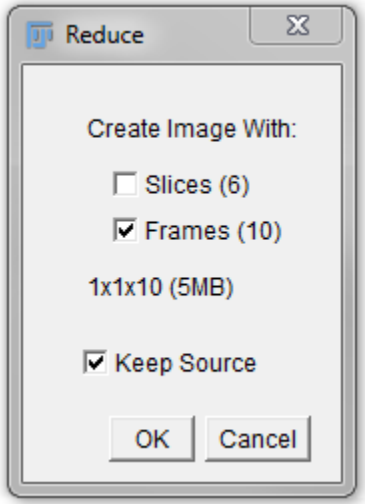
- ✓ *File/open* (or drag and drop) – one file
- ✓ *File/open* (or drag and drop) – first 6 files
- ✓ Image/Stacks/Images to Stack
- ✓ File/Import/Image sequence . . .

- ✓ Import all 60 and
- ✓ Image/Hyperstack/Stack to Hyperstack



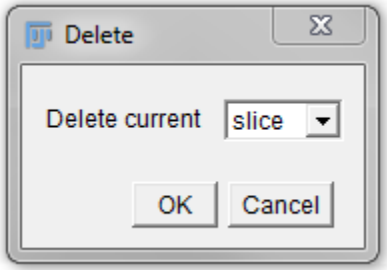
Dealing with lots of slices and dimensions: Trimming

Starting with our Hyperstack of Z=6, t=10 . . .

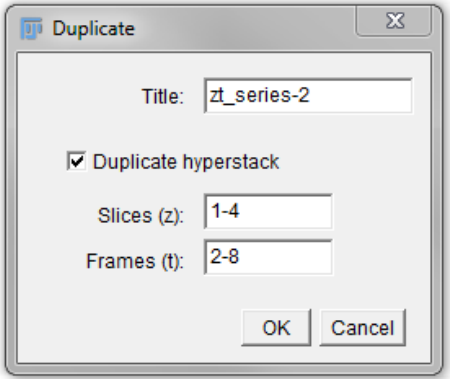


✓ Image/Hyperstacks/Reduce Dimensionality
(unchecking slices keeps current slice for all t)

✓ Image/Stacks/Delete Slice
(deletes current slice or frame)

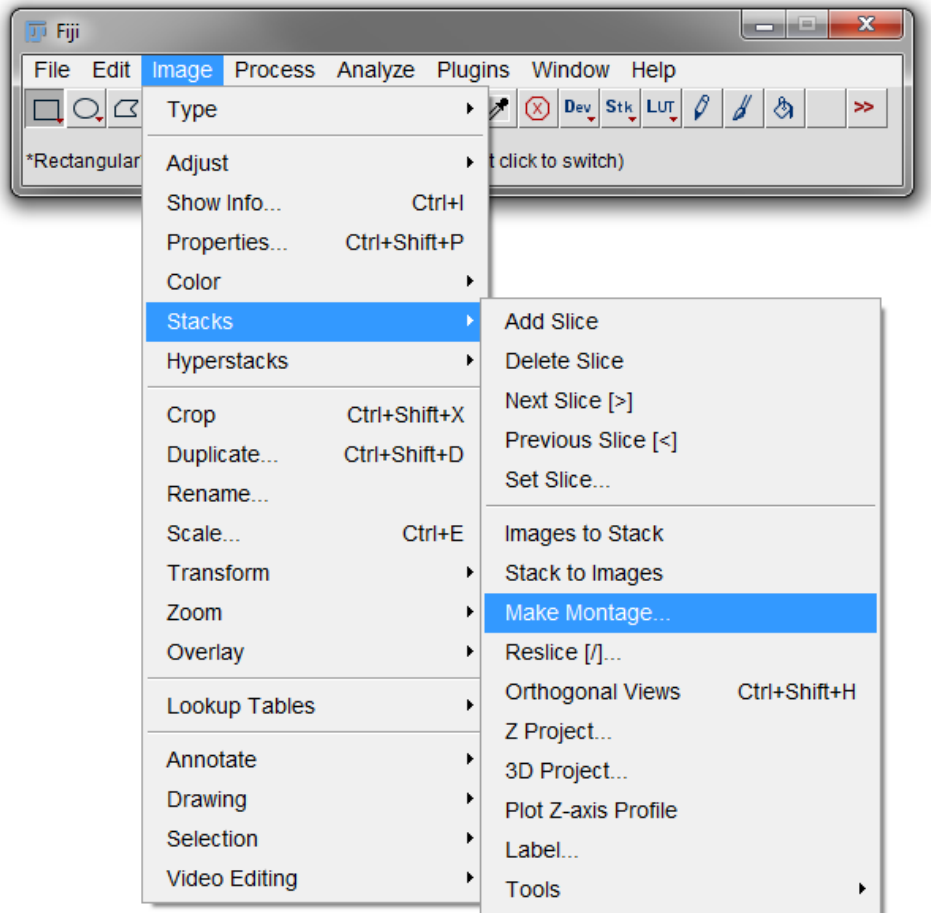


✓ Image/Duplicate
(Keep the bits you want)

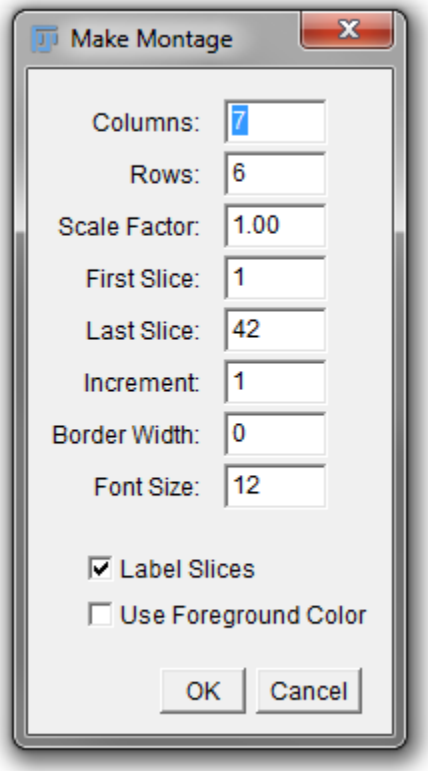


Montage

Easily make a panel of figures from a stack - Z or t



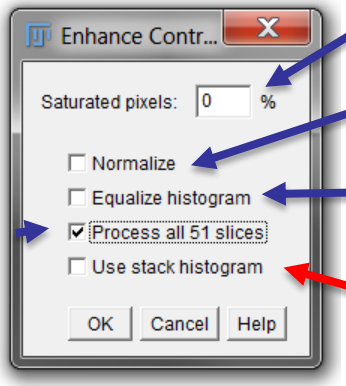
File:
Urchin_stackRGB



Digital contrast for stacks

Process/Enhance Contrast

Yes, do for all slices



Min/max set so no clipping or saturation

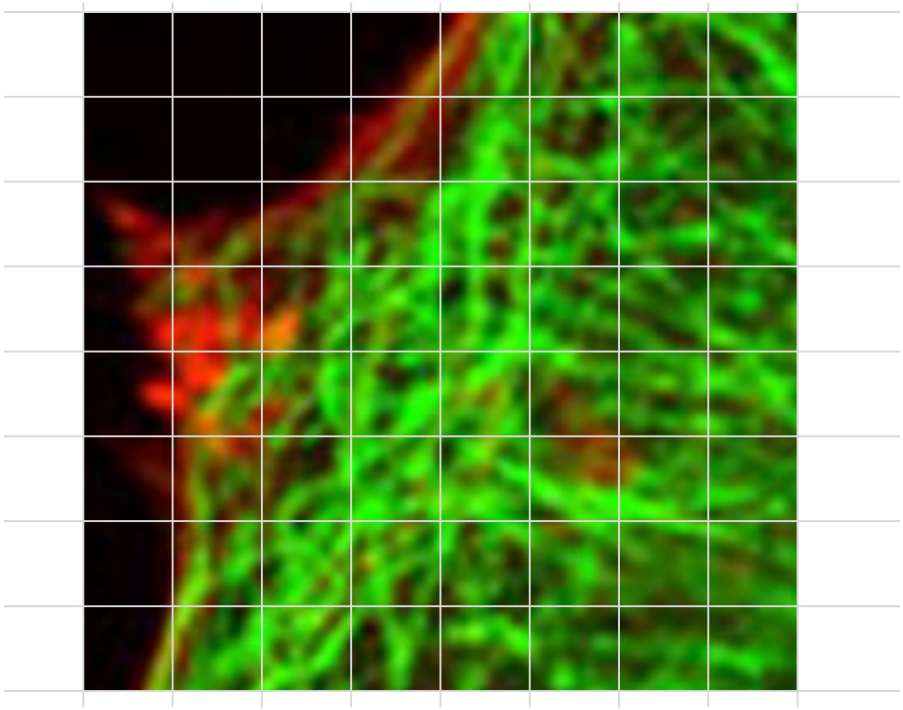
This stretches to values to full 8 bit/16bit

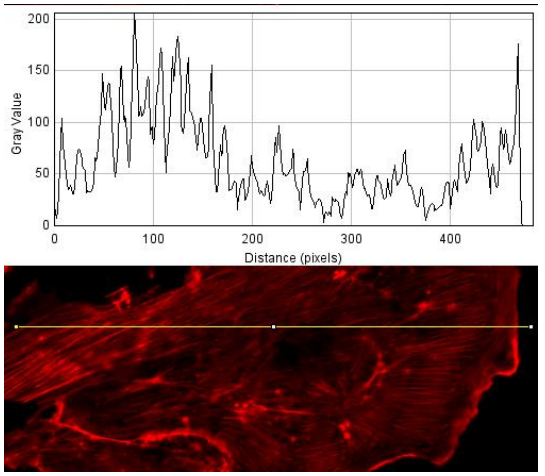
Don't do this

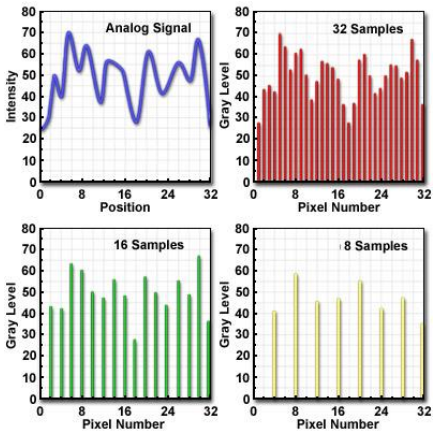
If checked, it uses the min max value of the stack for each image
If unchecked, it uses the min/max value of the individual image plane

What is a pixel?

Digitization Discretization Quantization

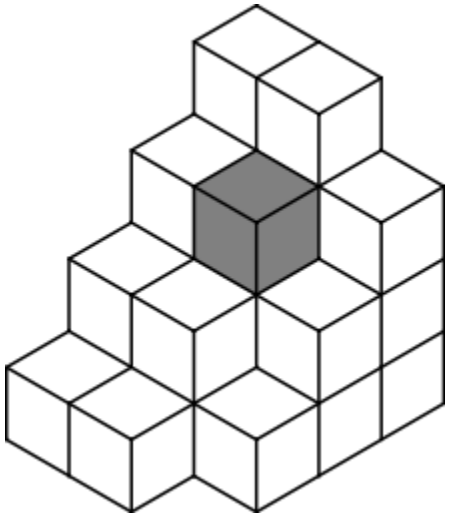






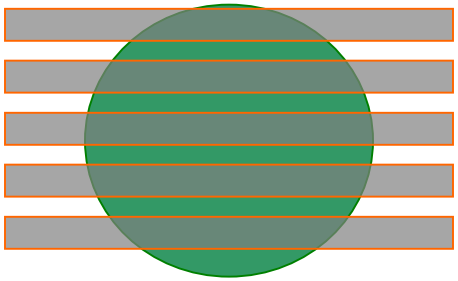
What is a pixel?

A voxel

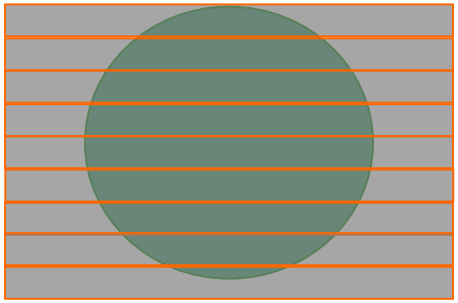


How big in z is a voxel?

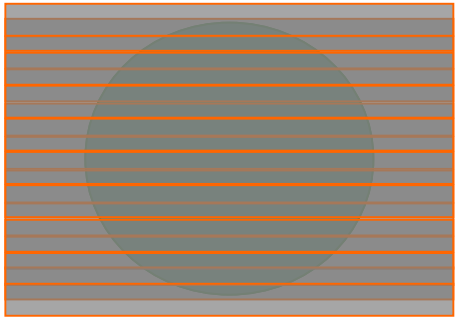
- Optical section thickness?
- Sampling?
- PSF size?



Some regions not imaged



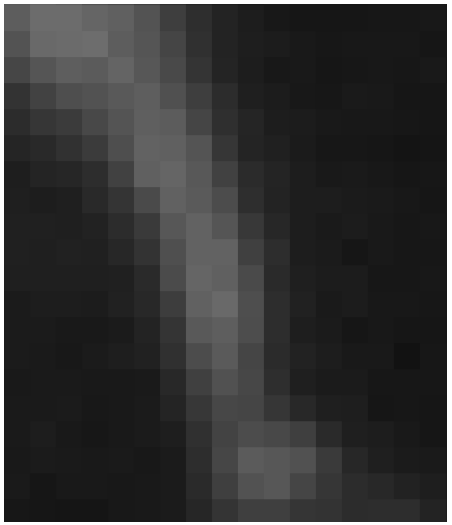
Covered



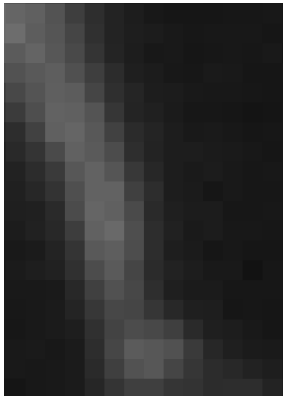
Covered and well sampled

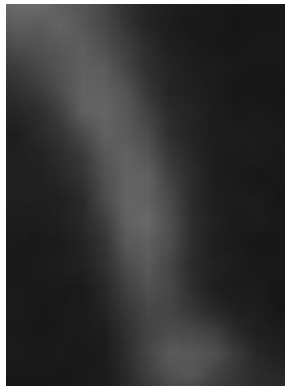
What is a pixel?

Two alternative view of pixels/voxels



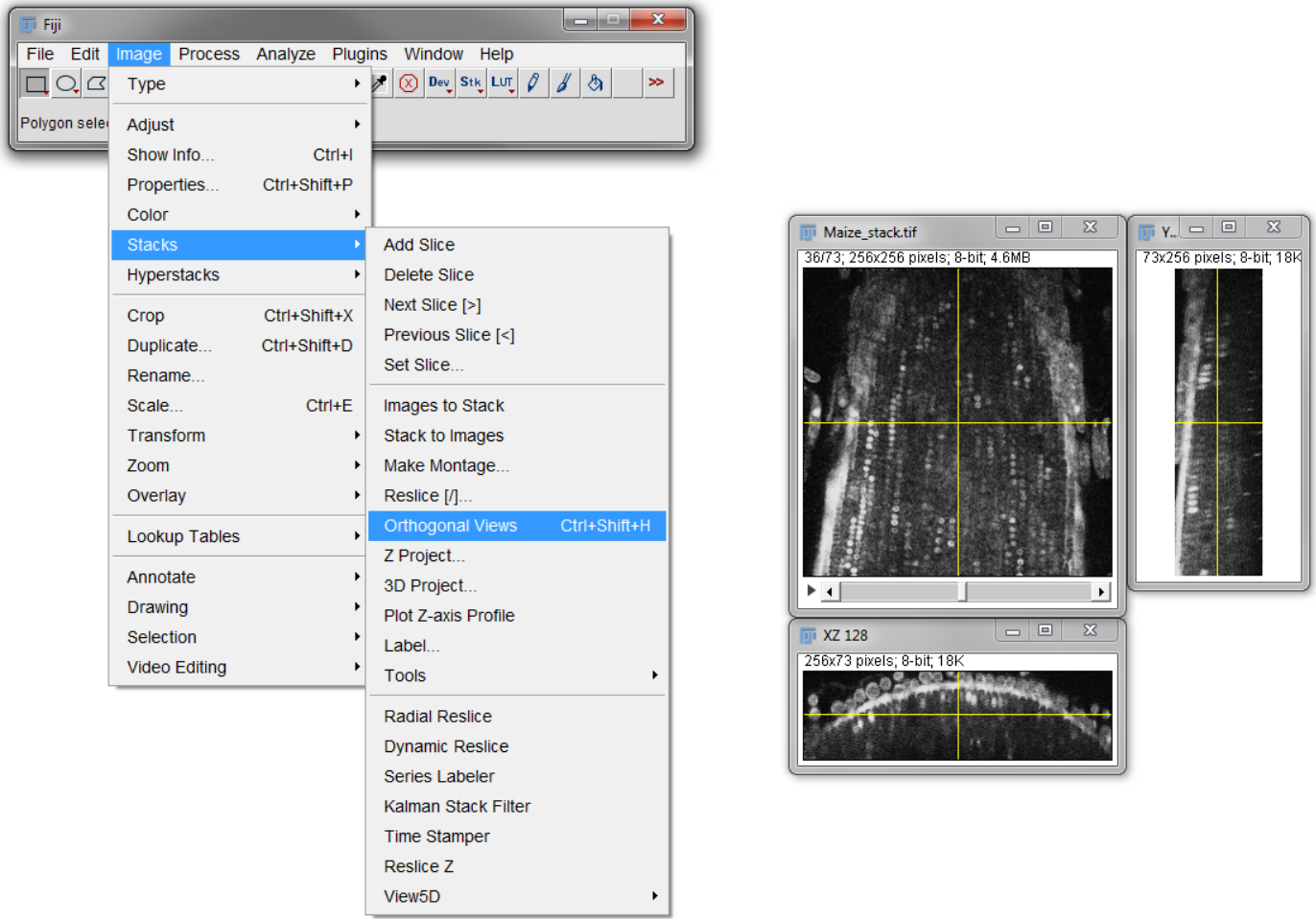
A Pixel Is Not A Little Square, A Pixel Is Not A Little Square, A Pixel Is Not A Little Square! (And a Voxel is Not a Little Cube) (Smith, AR 1995)





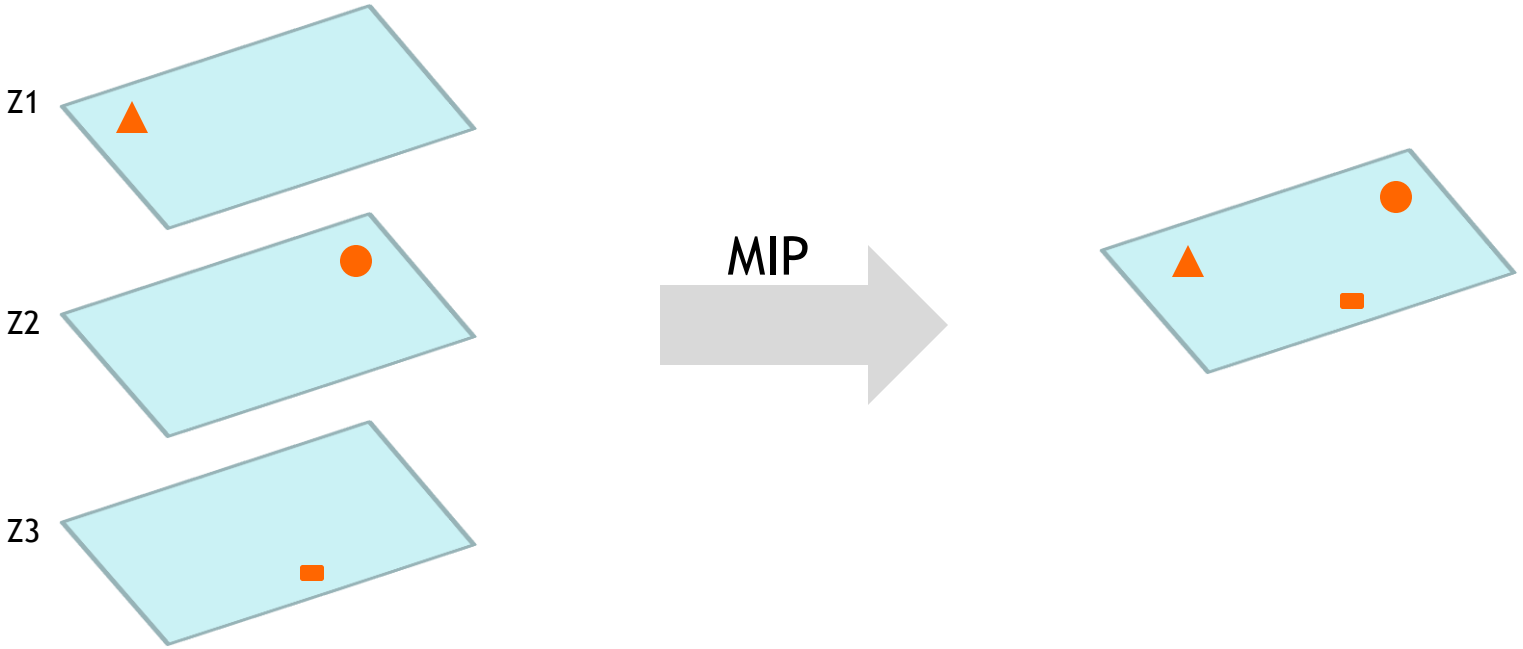
Orthogonal slices

File:
Maize_stack.tif

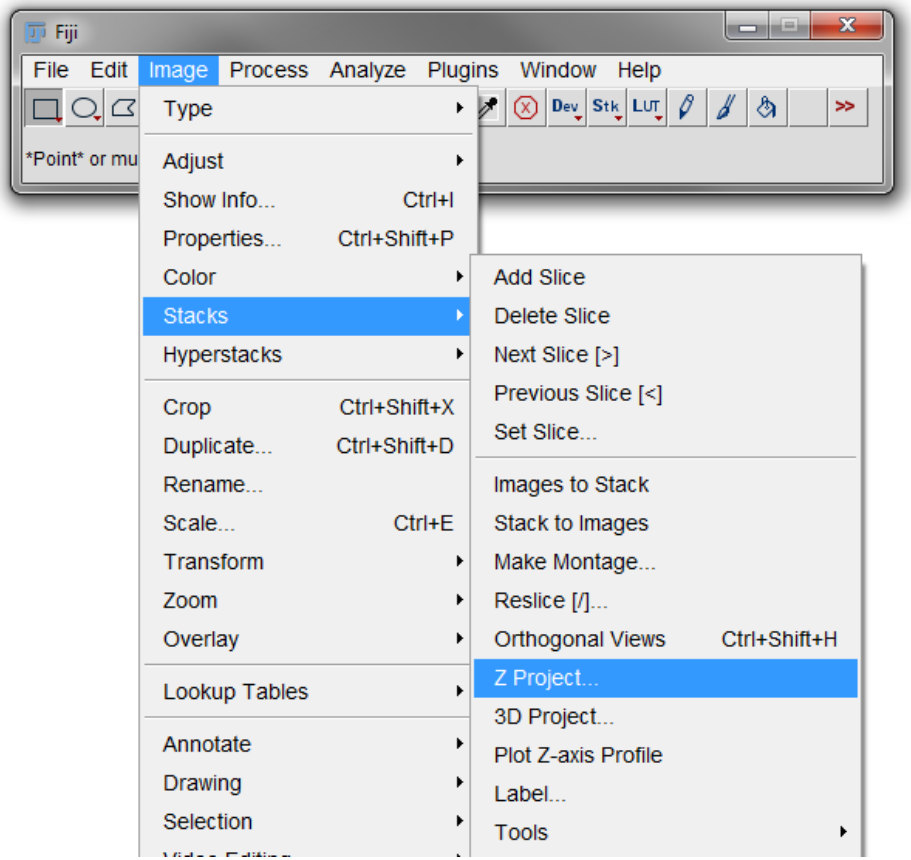


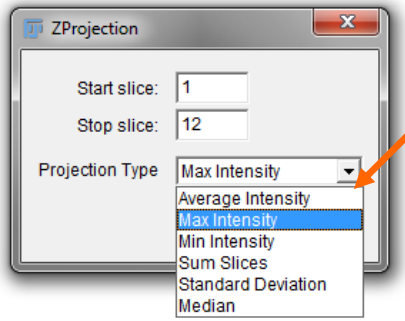
Basis of projection

Maximum Intensity Projection: The brightest pixel value of all the z-planes for each XY pixel is selected and a single plane image produced



Projections in FIJI

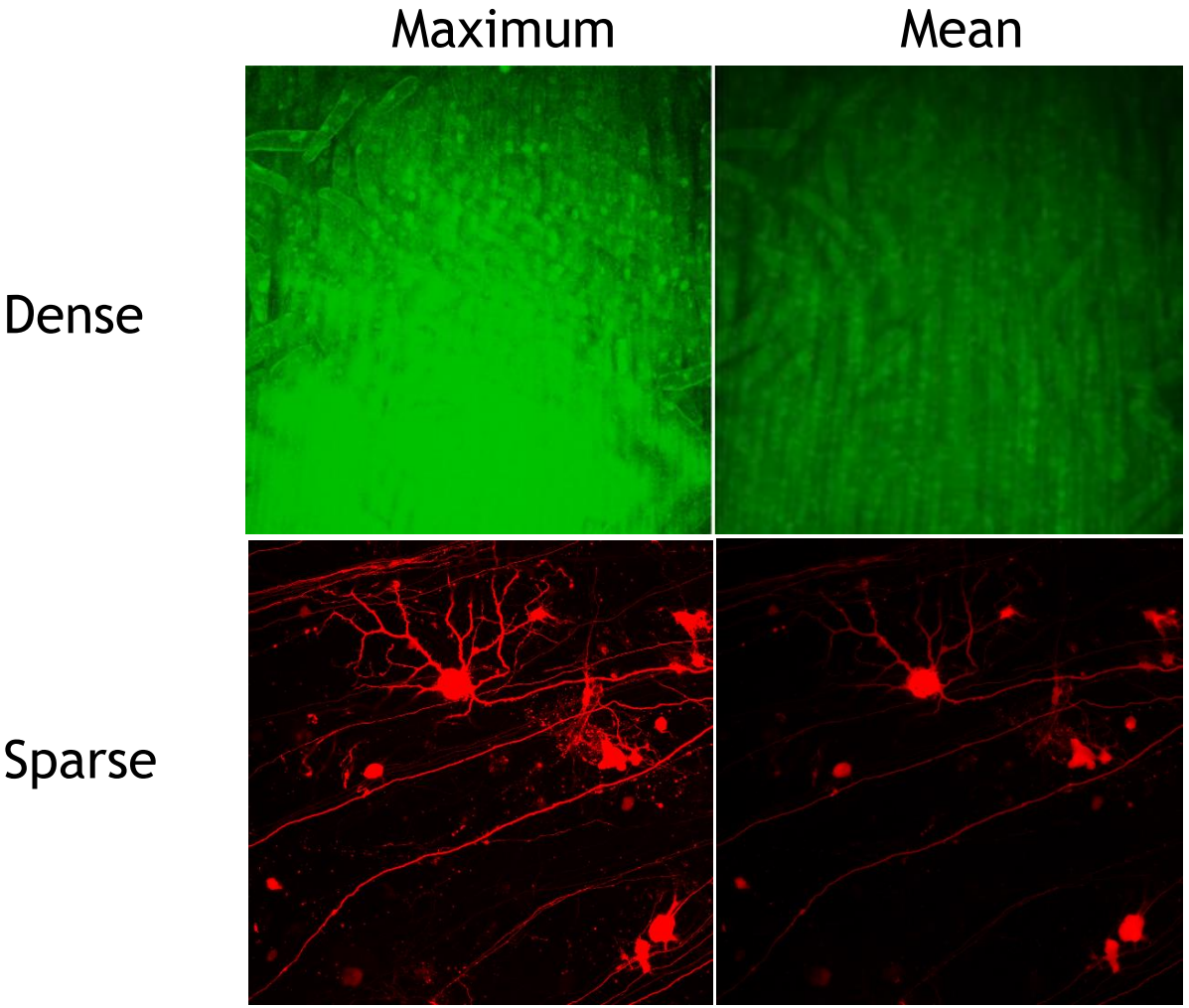




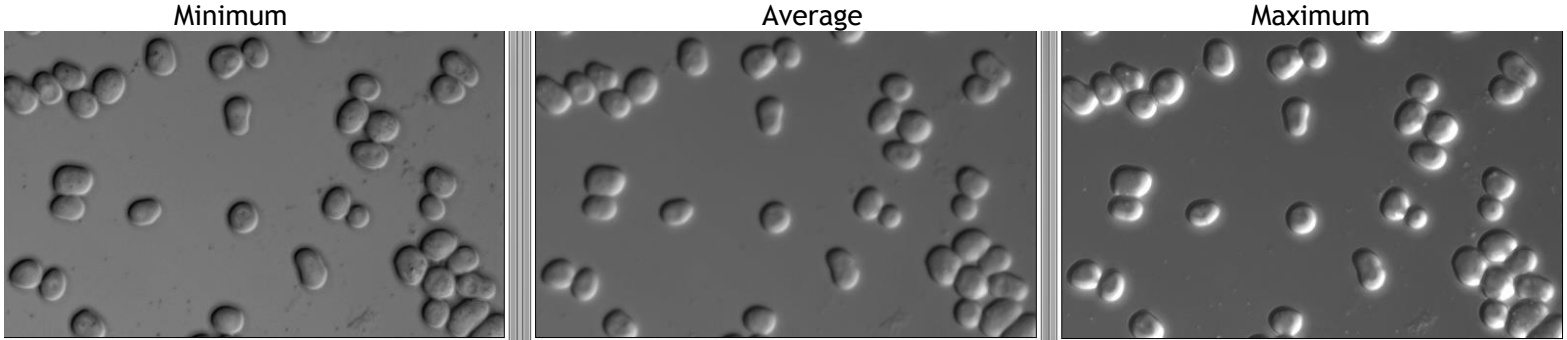
Other options for factors to project over the z range for each pixel

Files:
Urchin_stackRGB
Maize_stack
NeuronStackSparse
Yeast_DIC_stack.tif

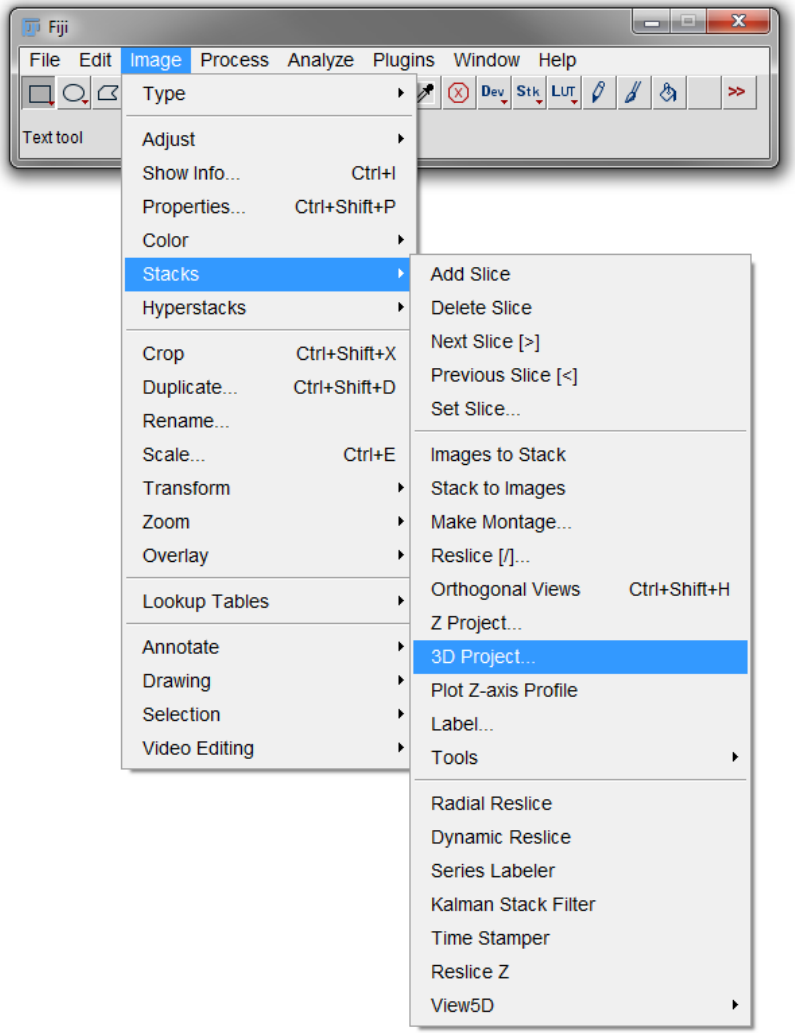
Try the different types of projections, see what works well for each dataset
Look at the histogram of the projections



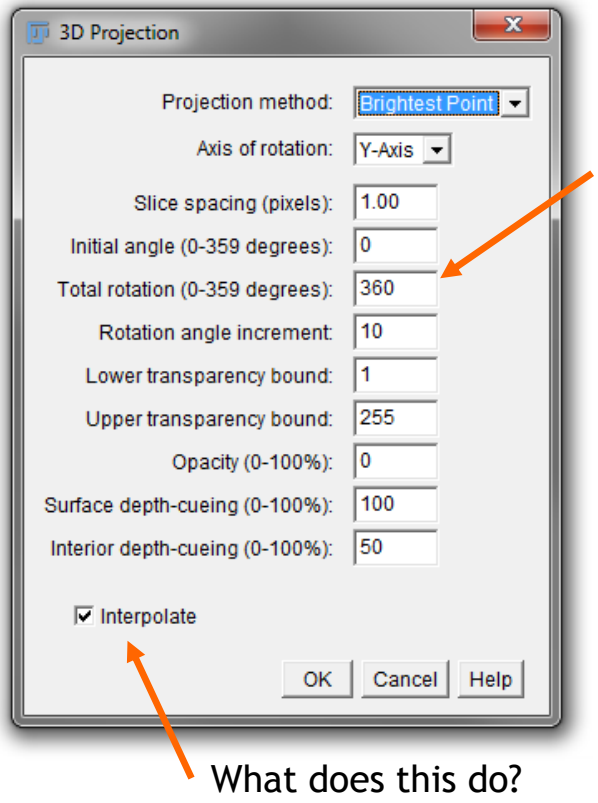
Brightfield etc



Using a projection series to appreciate the 3Dness



File:
Urchin_stackRGB

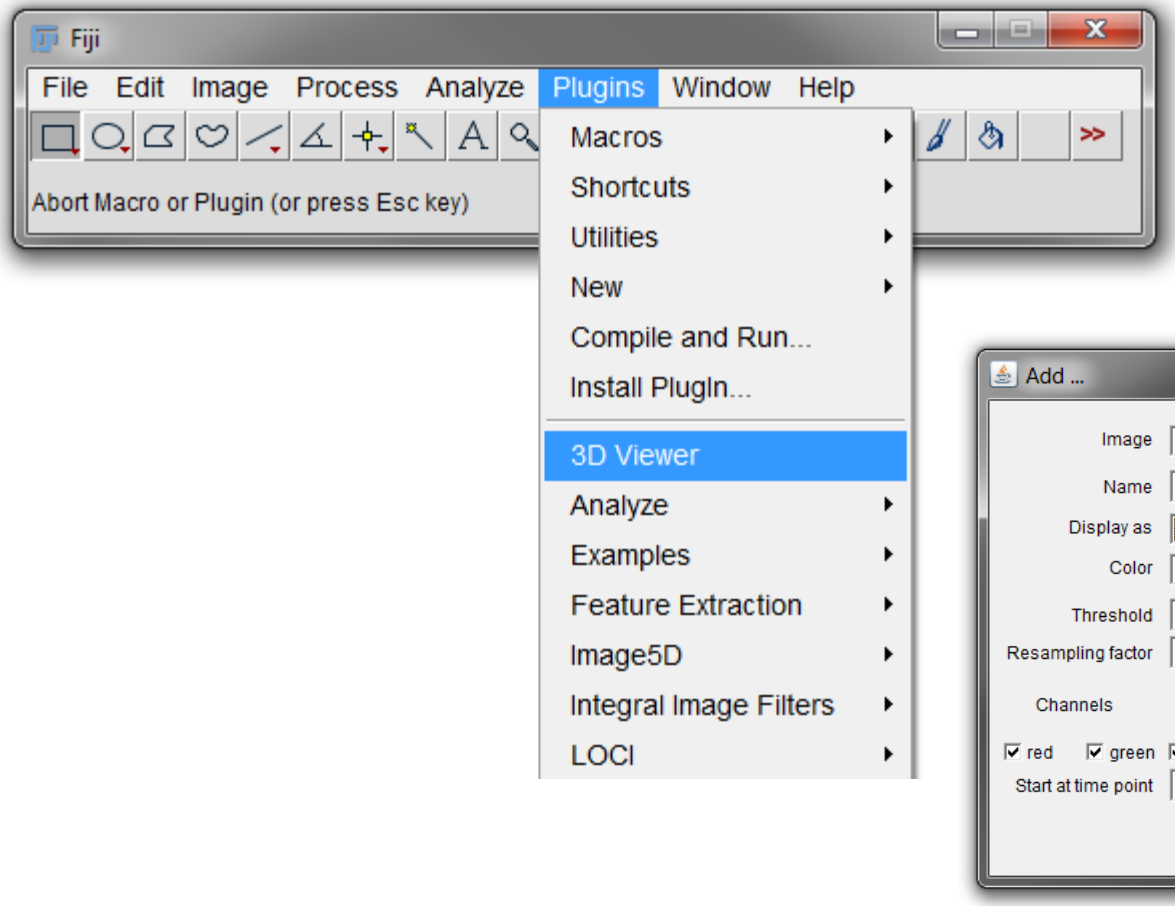


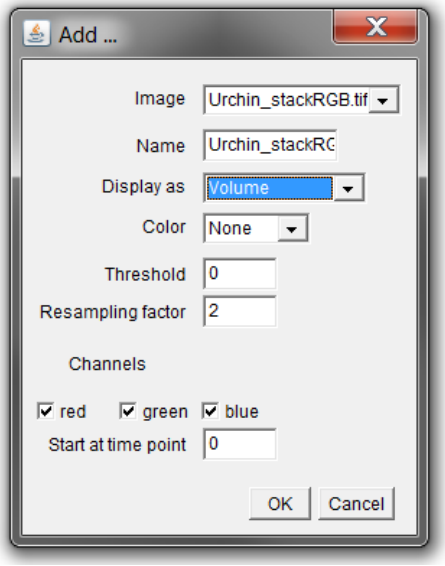
Less than 360˚ might be enough

What does this do?

A more interactive 3D viewer

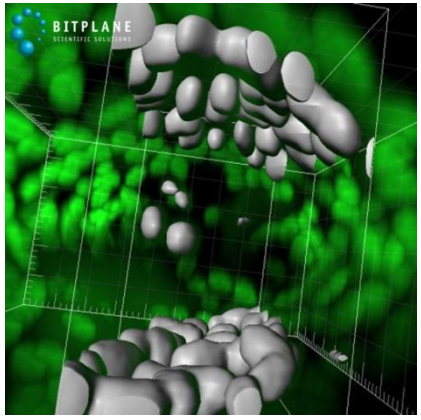
File:
Urchin_stackRGB

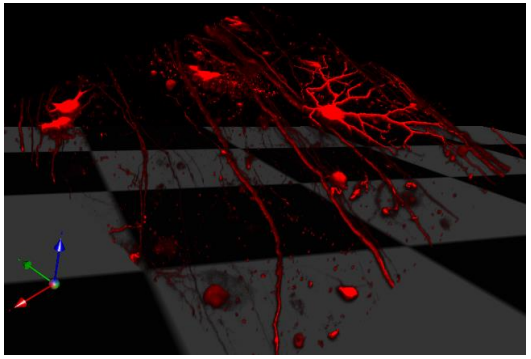


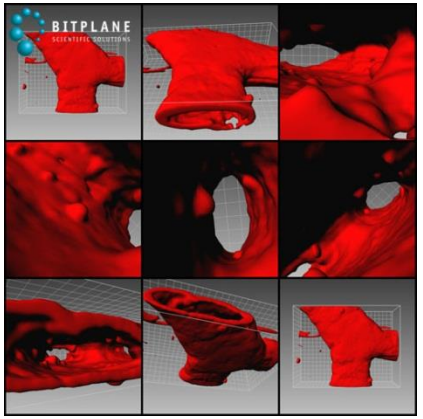


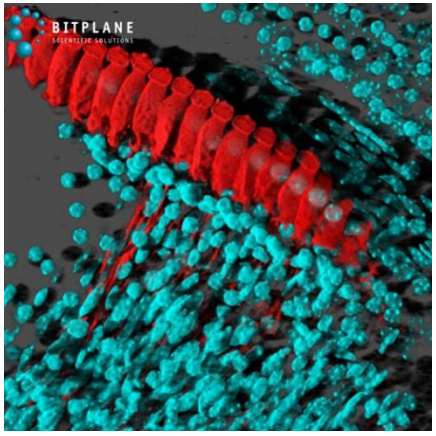
Other software for 3D

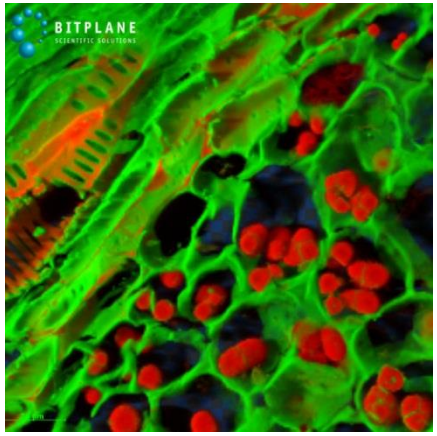
Imaris offers more powerful features of visualization and analysis in 3D
Efficient GPU rendering
Interactive
$$ Not free $$ (but no charge to use)
We have two workstations in LMCF with Imaris

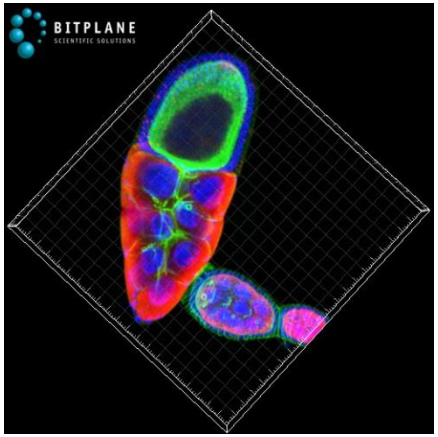


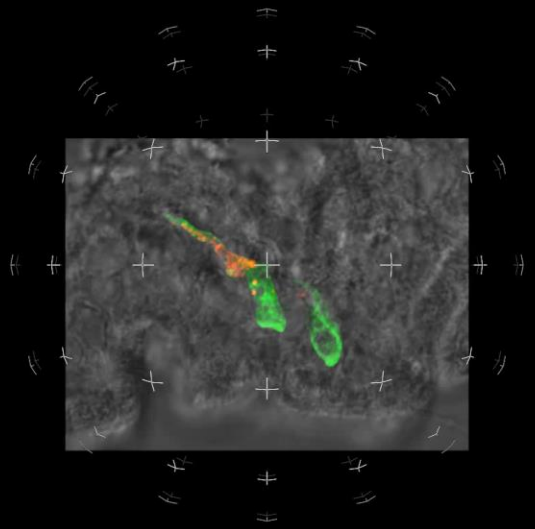


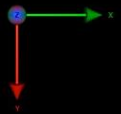






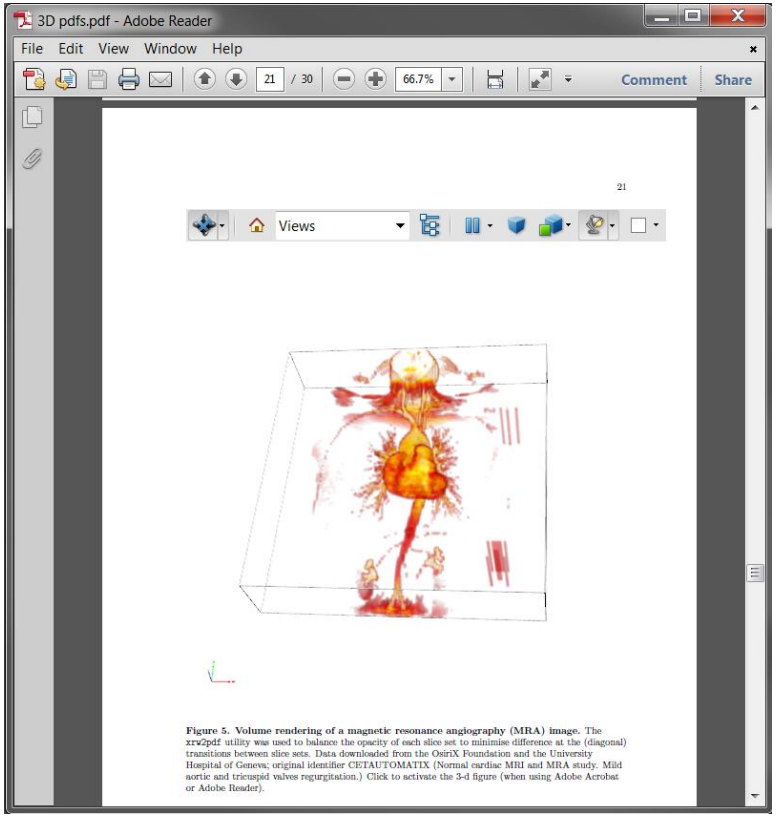





z
x
y

3D PDFs

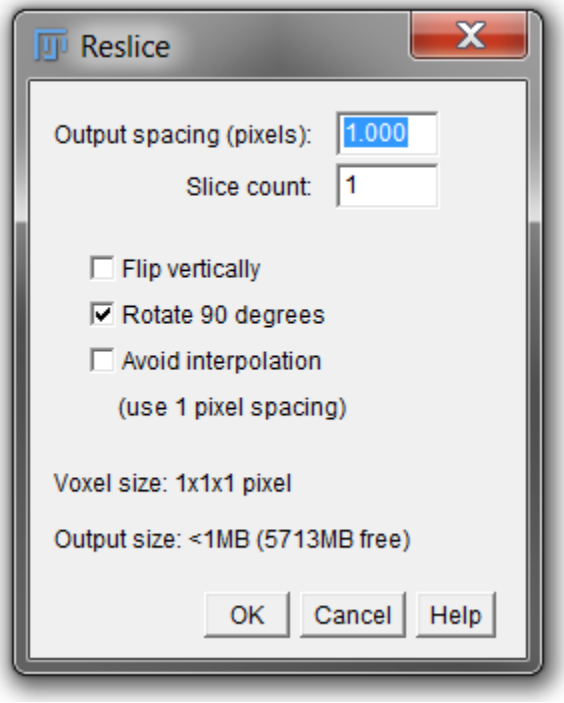
Embedding and Publishing Interactive, 3-Dimensional, Scientific Figures in Portable Document Format (PDF) Files



Kymograph

Line profiles over time

File:
Kymographstack.tif

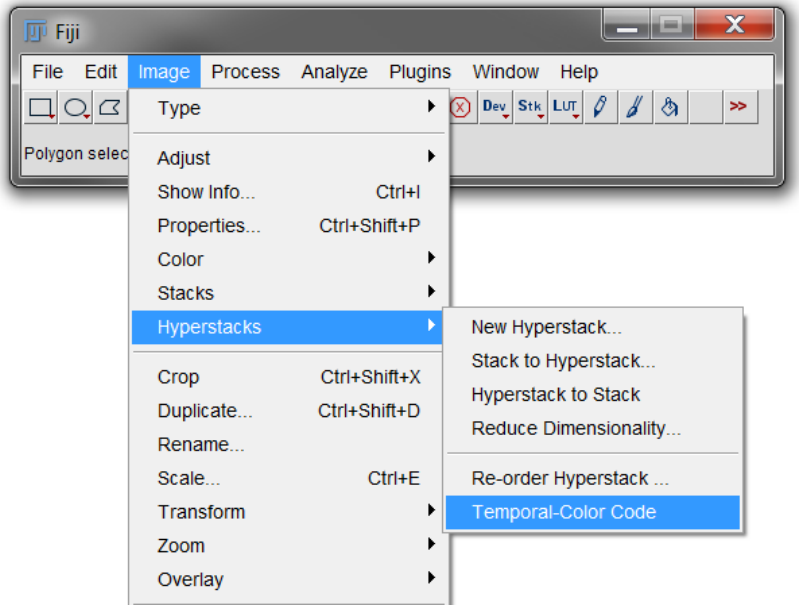


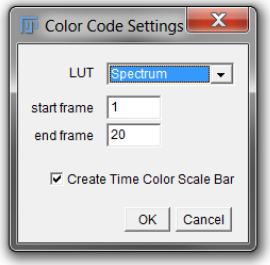
Image/Stack/Reslice

PC keyboard shortcut: /

Stack color-coding (z or t)

Files:
NeuronStackSparse
Kymographstack.tif





What colour are things that don't vary or move?

Movies: XYT

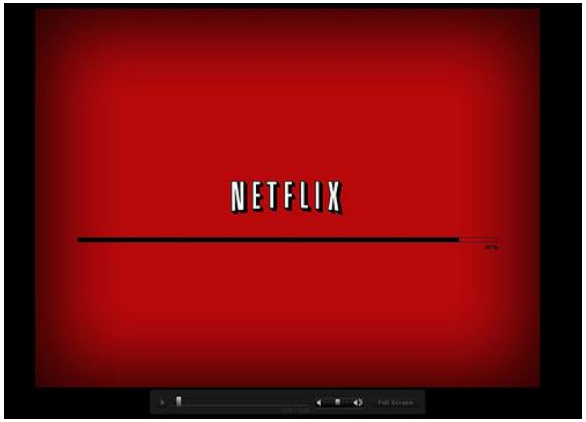
Formats such as quicktime (.mov) or avi are generally compressed and for display only

5GB powerpoint files aren't too useful – as well as compression you might need to crop, resize or lose frames as well as compress

It is useful to think in terms of frames and frame-rates . . . .

- 6000 frames = 200 seconds @30 fps
- 600 frames = 20 seconds @30 fps
- 6 frames = 200 mseconds @30 fps

Compression



Video compression

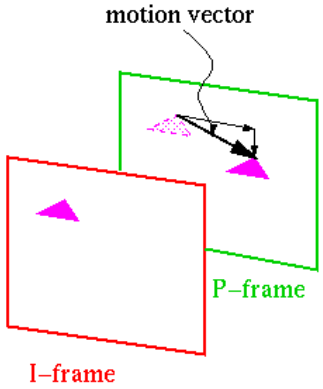
Codecs – lots available, nearly always lossy

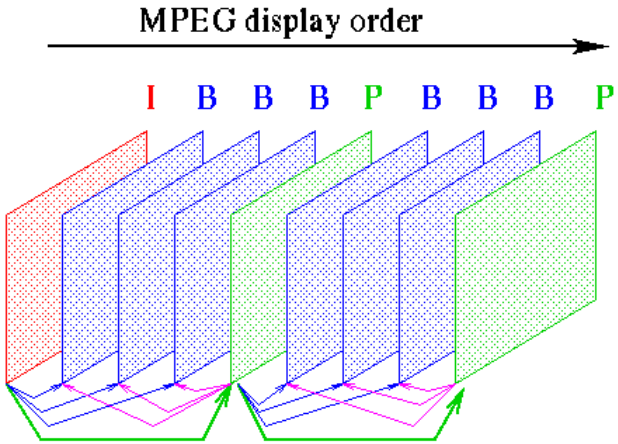
Encoder is algorithmic
i.e it does different things
according to nature of input

Decoder is determenistic,
i.e it always does what the
bitstream tells it to do

Complex to Make

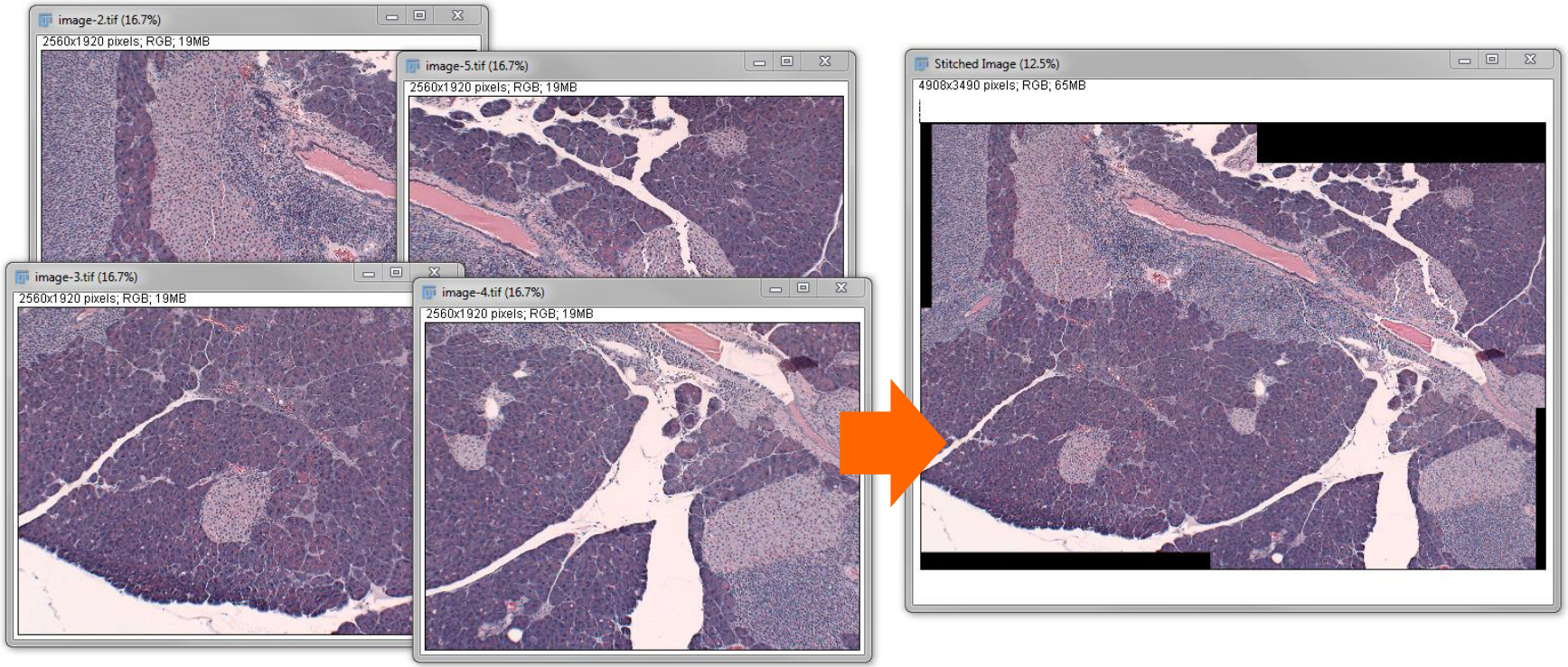
Simple to Make





Advanced assembly

Stitching - joining images together



Various methods –

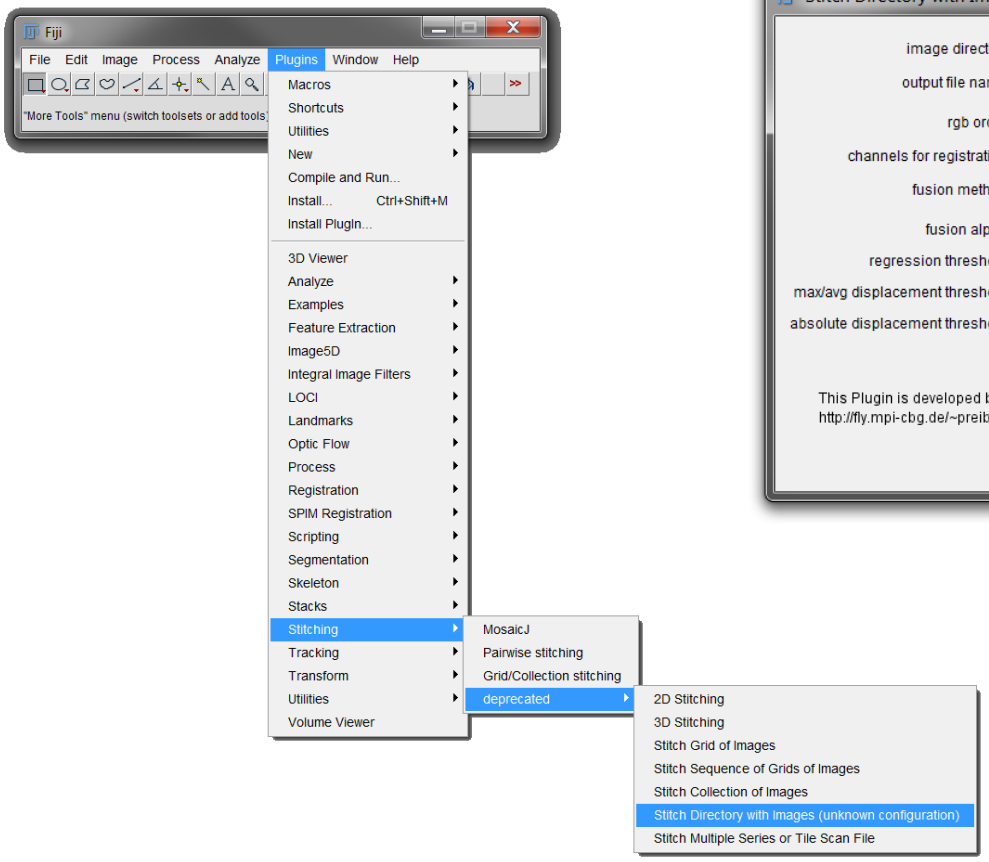
Place images adjacently in a known order

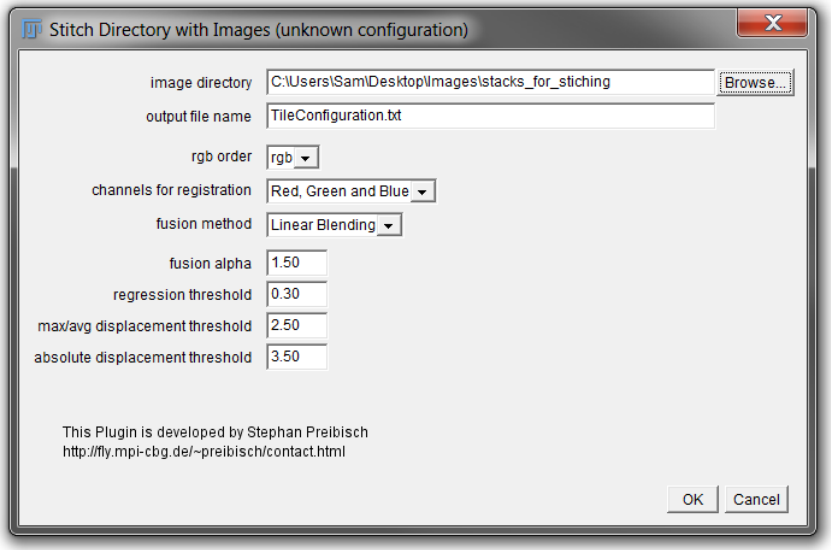
Overlap and blend - coordinates or from image appearance (or a bit of both)

2D or 3D (ie align stacks of images)

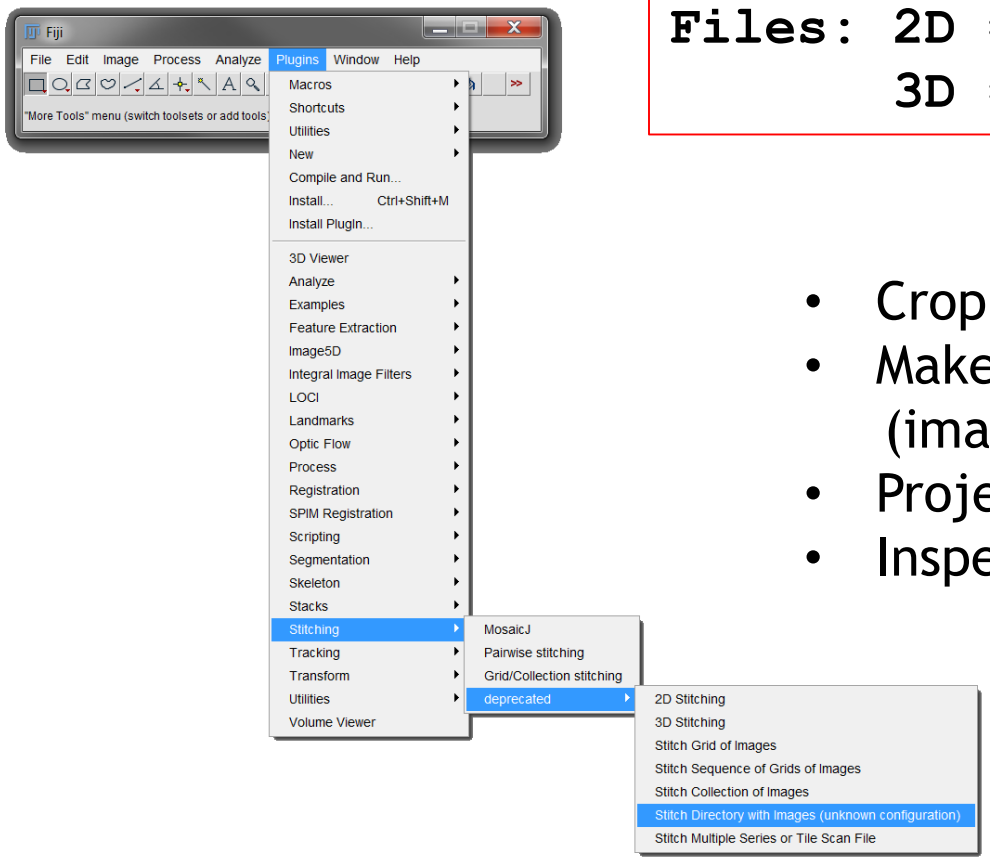
http://fiji.sc/wiki/index.php/Image_Stitching

One of the stitching methods





Stitch these

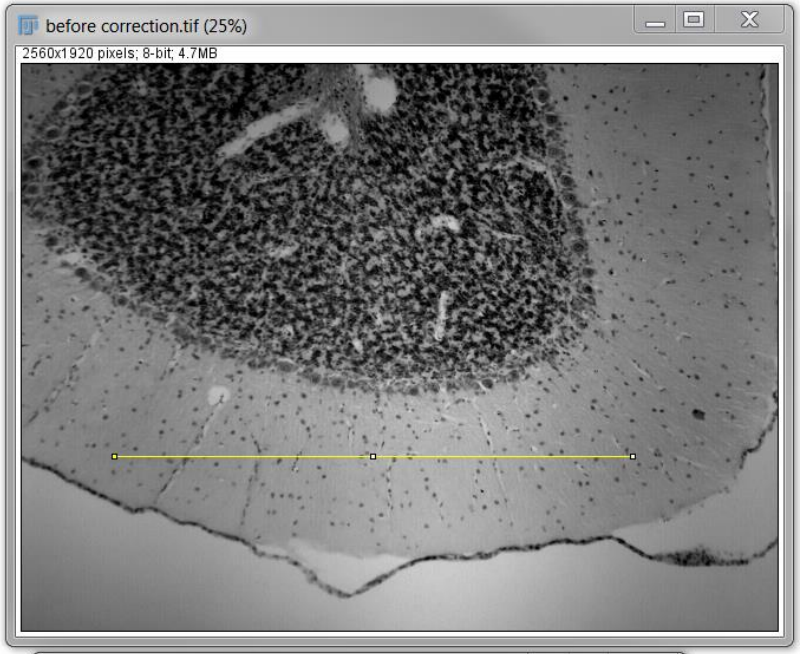


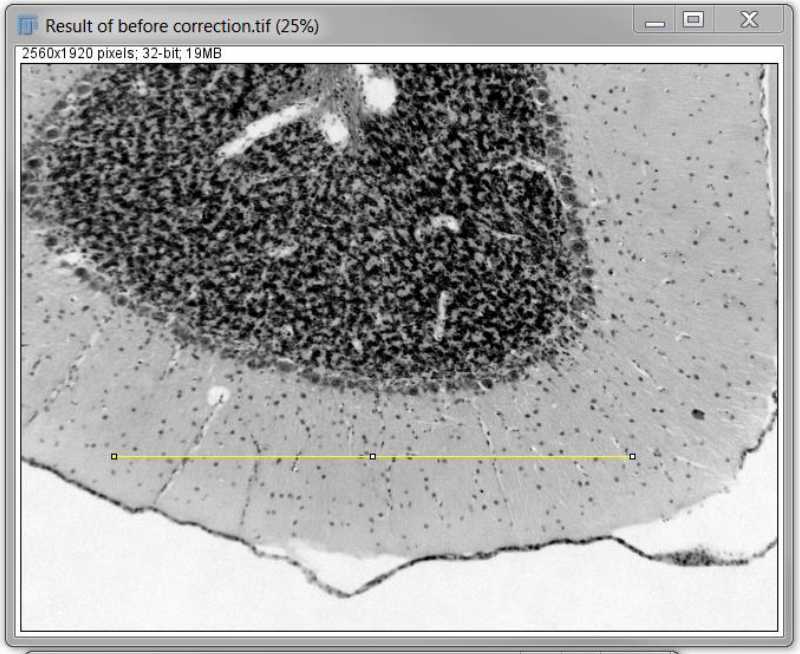

```
Files:  2D = /for_stitching
        3D = /stacks_for_stitching
```

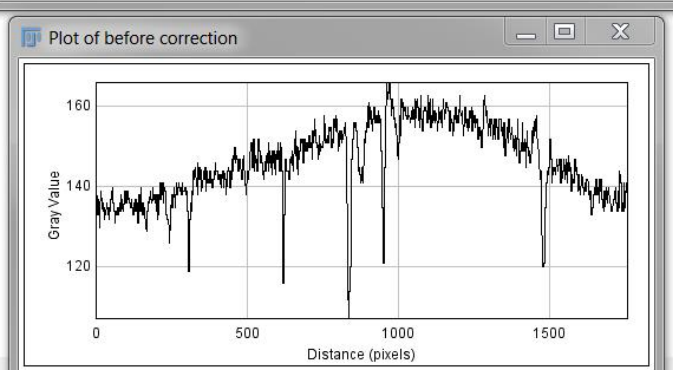
- Crop the final image to a rectangle
- Make an overlay of the 3D (image/stacks/tools/deinterleave)
- Project them?
- Inspect – can you see the joins?

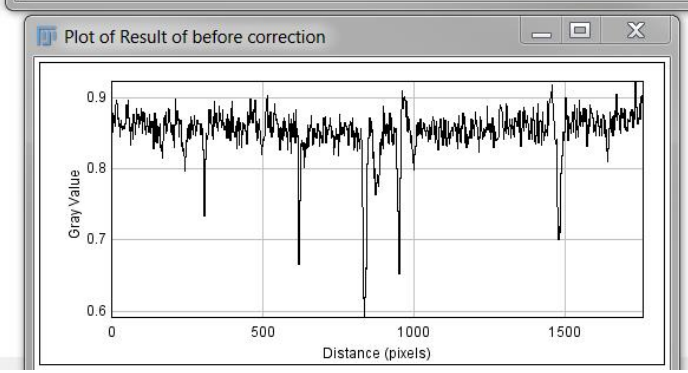
Flat-field or shading correction

Corrects for non-uniform illumination or field-curvature







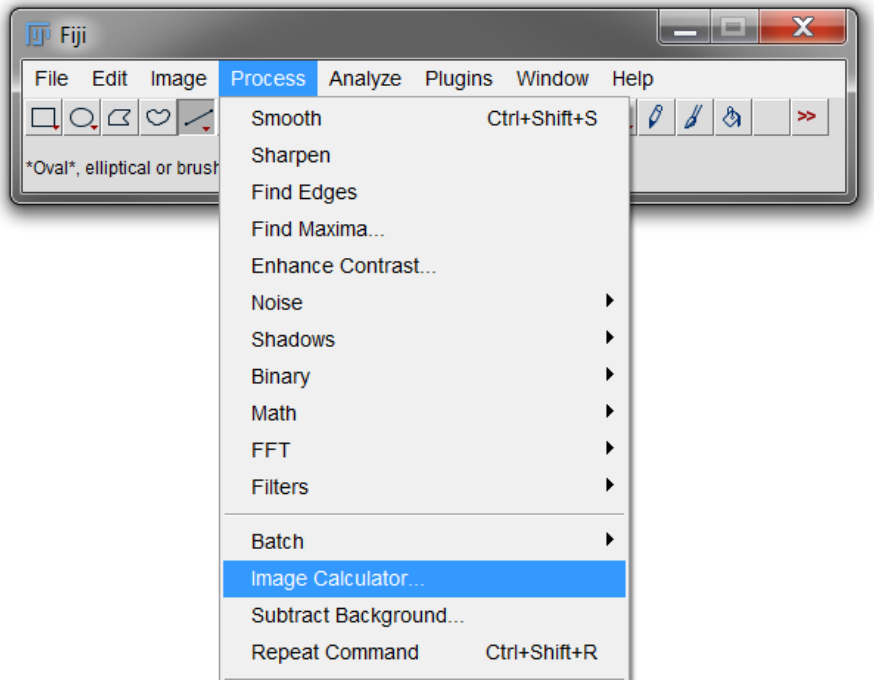


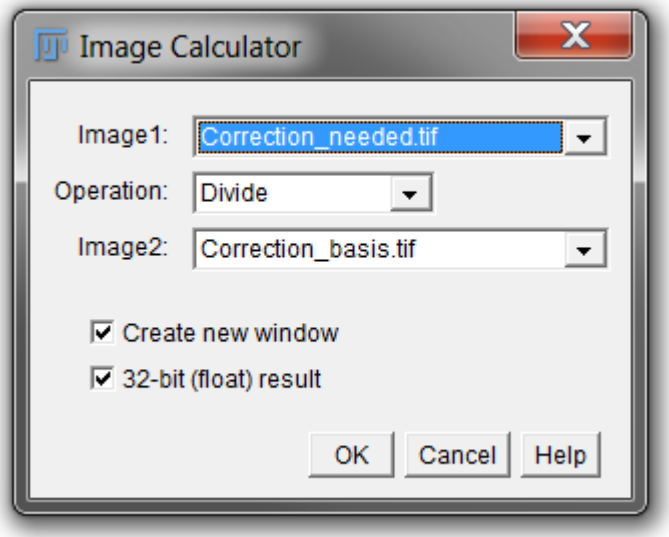
The correction calculation

File: Correction_needed.tif
Correction_basis.tif

A monochrome transmitted image suffering from non-uniformity

An averaged, sample-less image by which it can be divided

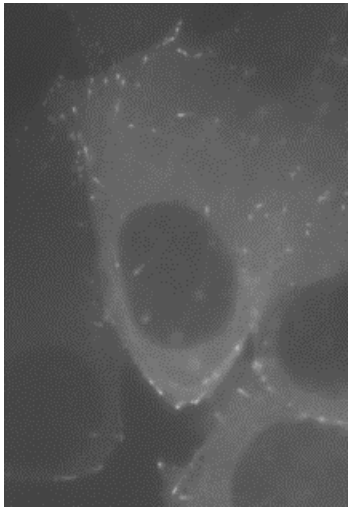




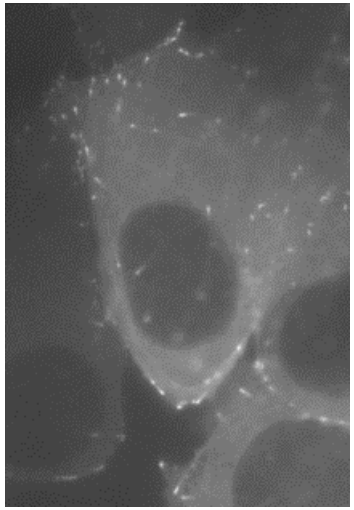
Alignment plugin

File: Shifting_timelapse.tif

Plugins/Registration/StackReg



Original

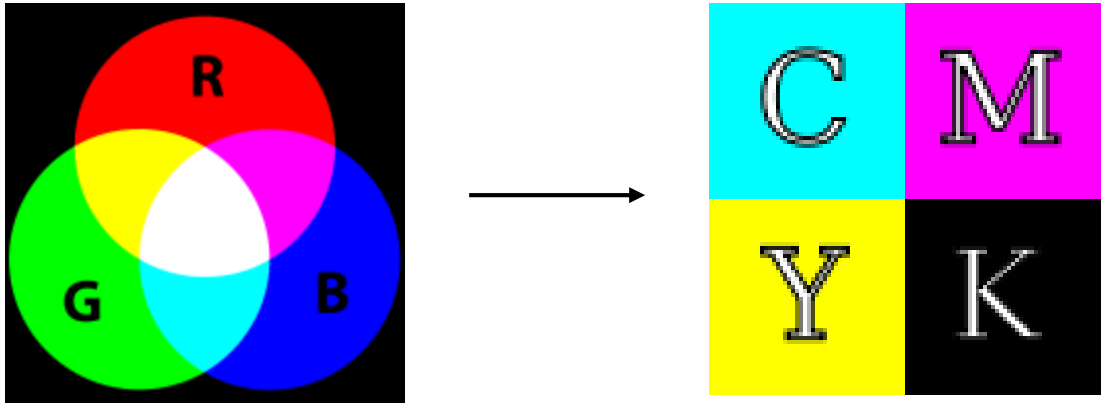


Aligned

Image processing & Making figures

Printing

Need more pixels per area (dpi) for a nice printout than a nice image on a computer screen



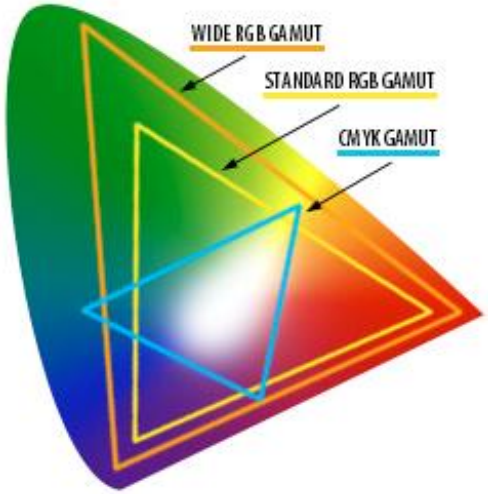
Pixels

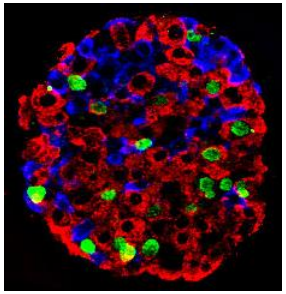
Inks

Accurate conversion to print format

RGB is the origin, the print out should look like the screen image (not that they are any immutable constant)

CMYK color gamut is smaller than RGB's





- Paper dependent
- Printer dependent (and we don't have one like the printing press)
- Trial and error

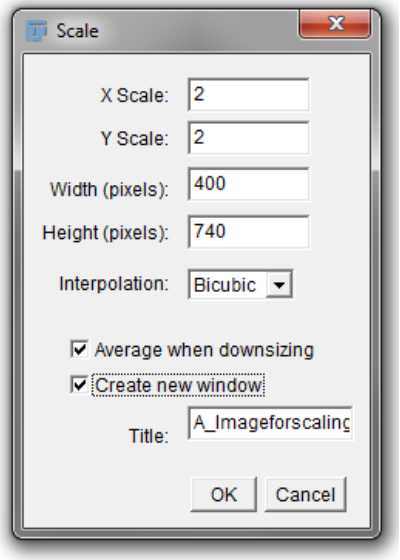
- Photoshop has a CMYK mode to mimic a CMYK version to help with adjustments - eg it warns when out of gamut

Journal formats

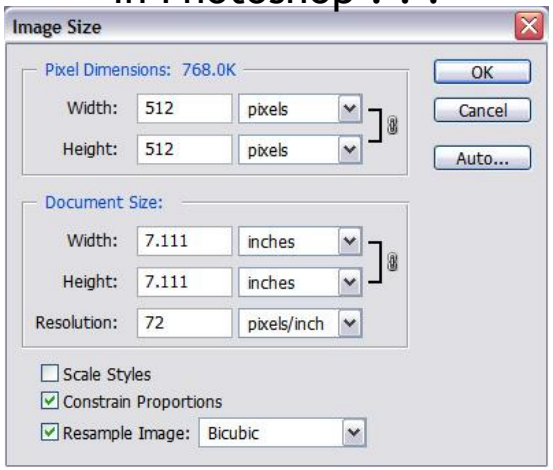
(They may ask for a hardcopy to give them something to check their CMYK conversion against, but RGB is becoming the default)

Journals may actually require you to do something you shouldn't really be doing! (eg ask for a page-sized image @600 dpi).

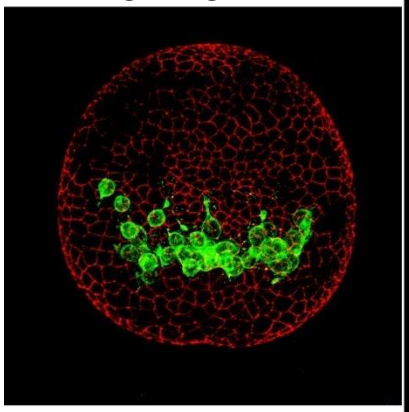
Beware of interpolation = adding pixels



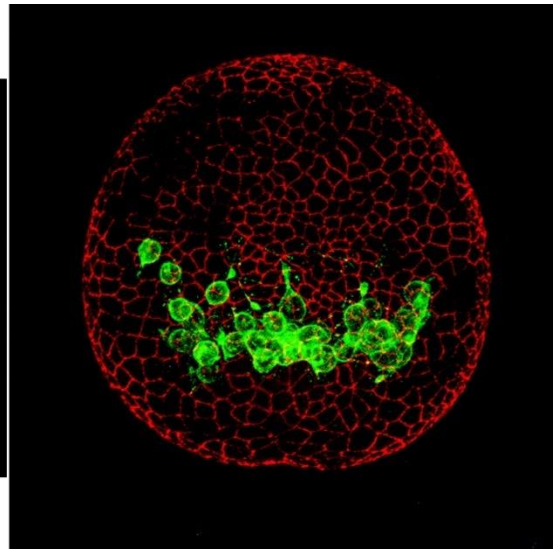
In Photoshop . . .



512*512

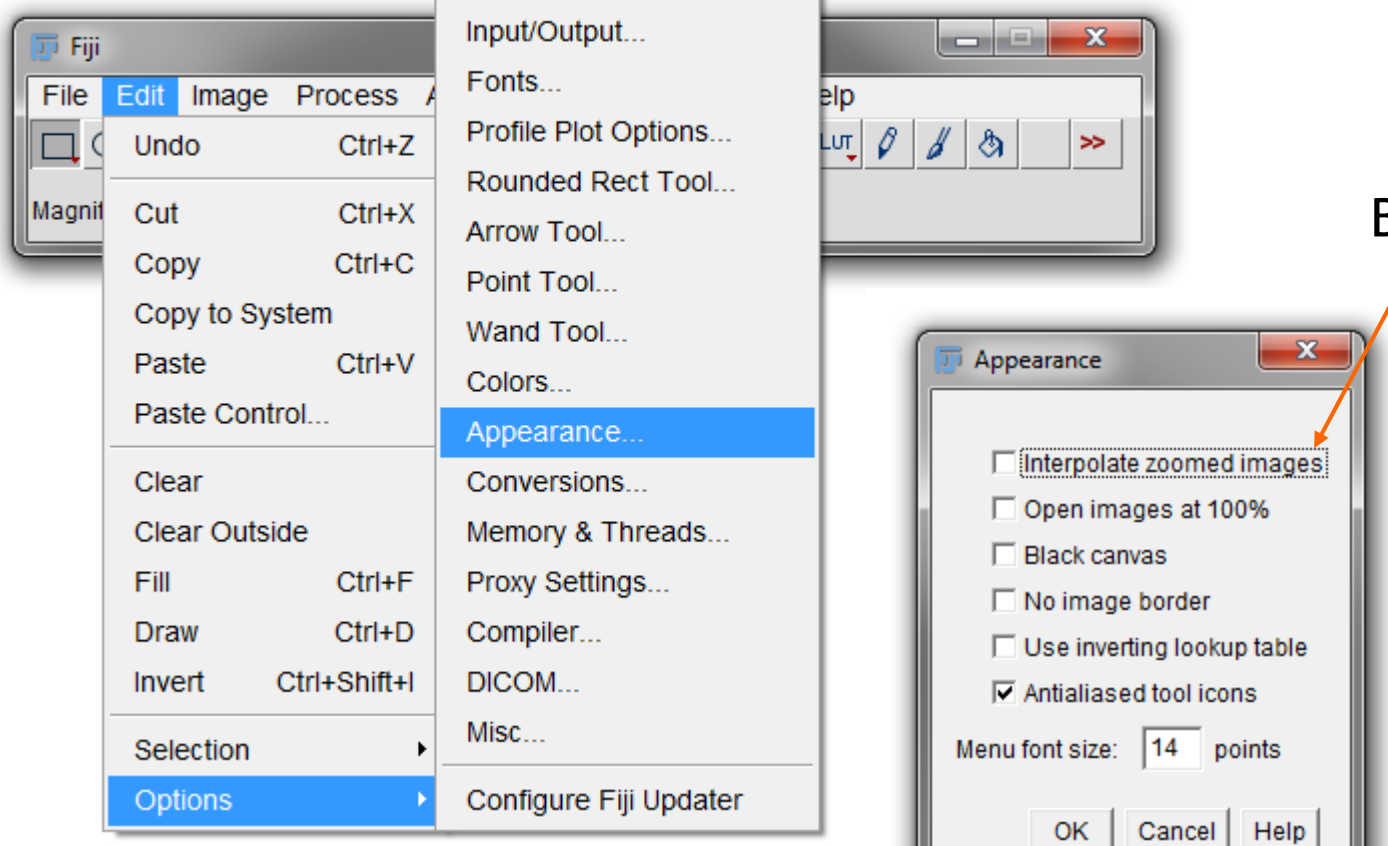


700*700



Reducing the number of pixels is ok, but rarely useful

Interpolated view in FIJI

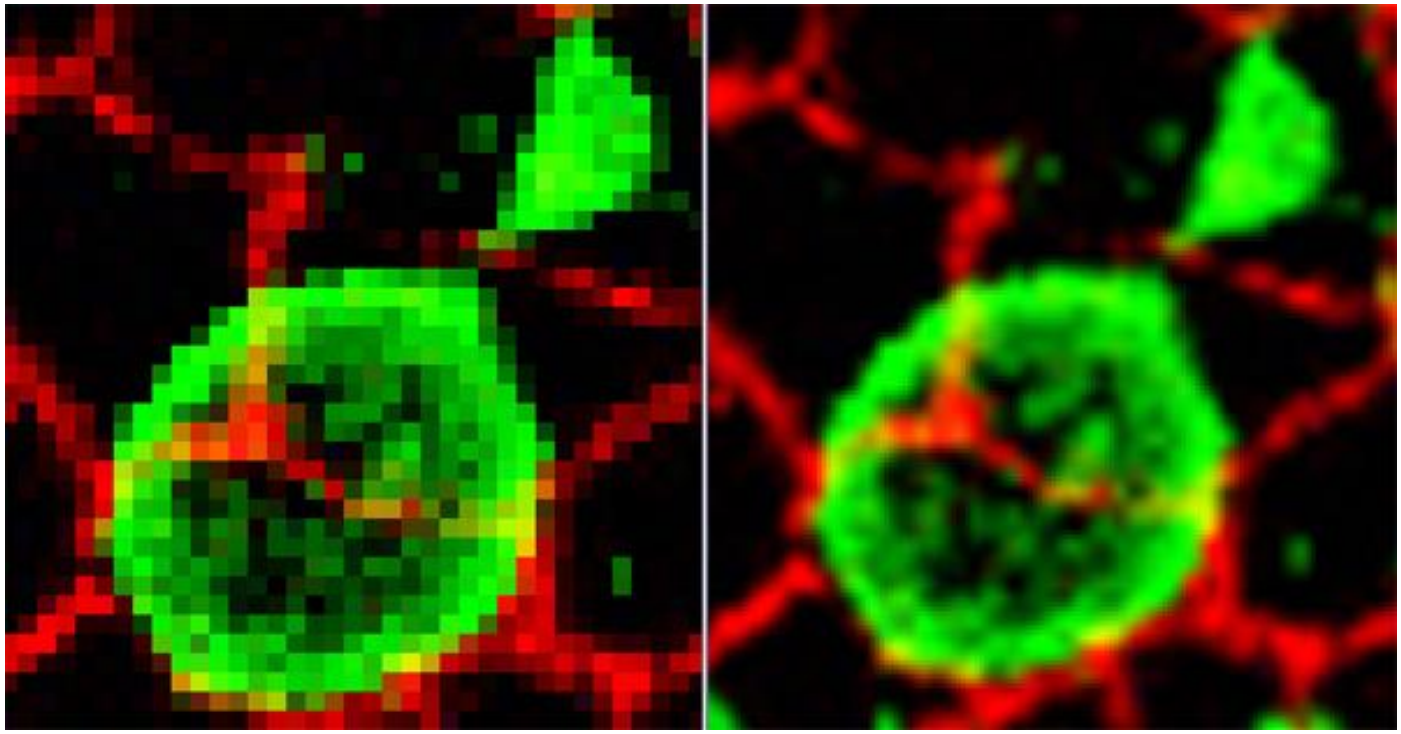


Beware a false certainty

Does the object really look like little squares?

Beware of interpolation = adding pixels

Close-up: Original Pixels added



Where did this extra information come from? Is it valid?

Ok to crop and change canvas size, both will change pixel dimensions but not the raw data

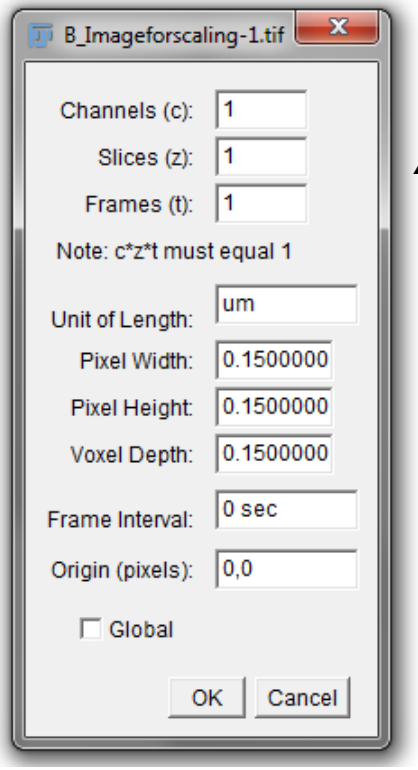
What to say about your images

- **Type of system/microscope** (eg Zeiss 510 confocal mounted on an Axio Observer microscope stand)
- **Objective** you used (eg 63x 1.4 NA oil Plan-Apochromat)
- **Wavelengths** of excitation and emission (eg 488 nm line of Argon laser with a longpass 500nm filter)
- **Camera** (eg Coolsnap ES2 from Photometrics)
- **Software** for acquisition (eg MetaMorph 7.5) and general typical settings (eg ND, exposure time, binning, interval in t and z)
- Conditions – (eg temp, CO2 buffering)
- Details of any image processing or analysis procedures – raw images may be required also.

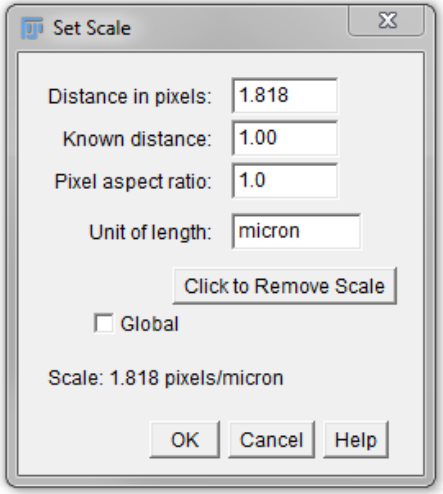
Scale bar

Analyze/Tools/Scalebar

Image/Properties . . .



Analyze/Set Scale . . .



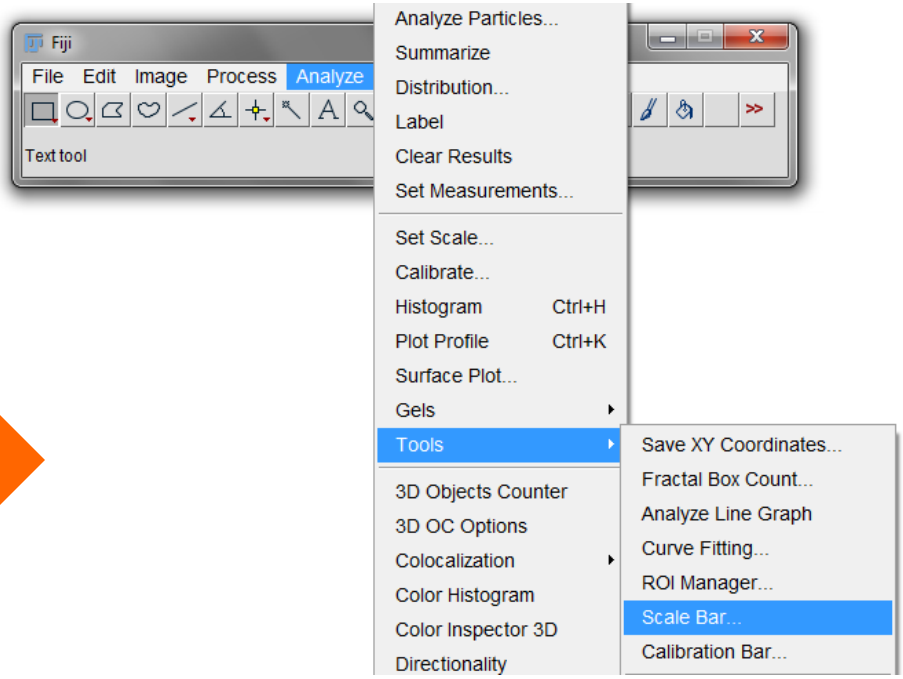
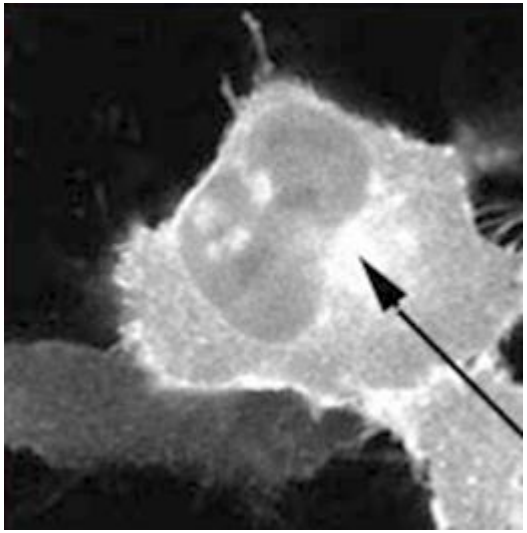
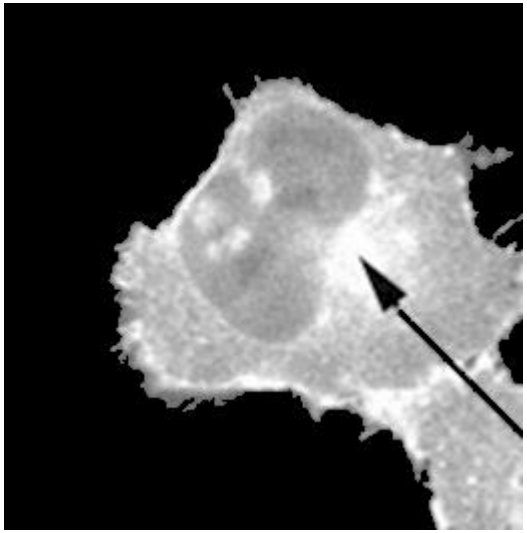


Image processing ok/not-ok's





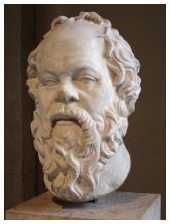
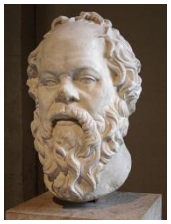


Image processing ethics



Not too much should need doing or be done

Digital contrast to best convey reality

What is done, should be done to the whole image

Understand what you're doing

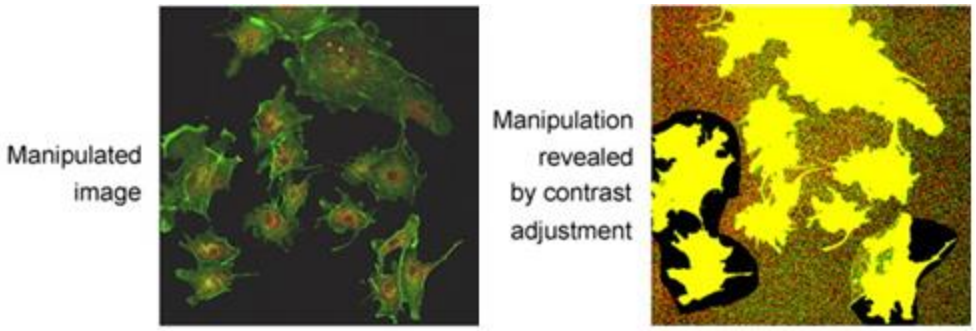
http://www.nature.com/authors/policies/image.html

JCB: What's in a picture? The temptation of image manipulation

Image processing ok/not-ok's

Scaling comparability between dataset

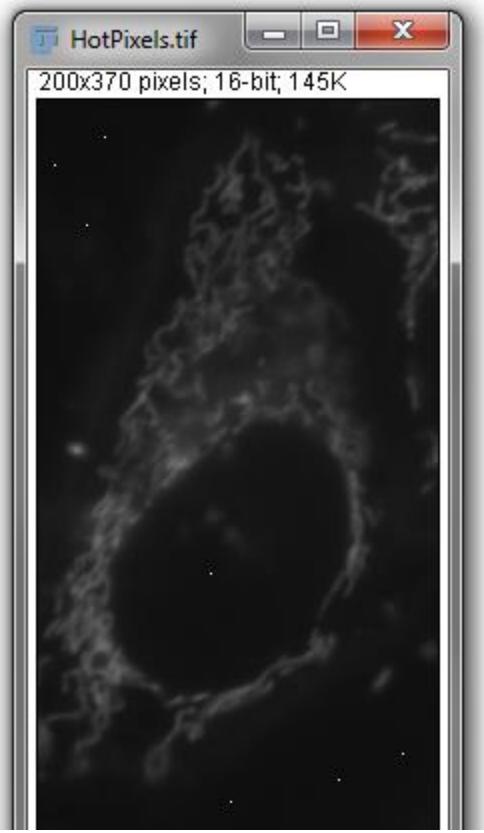
(ie if you are showing a similarity or difference in intensity, same acquisition, scaling etc. Not for unrelated subsets)

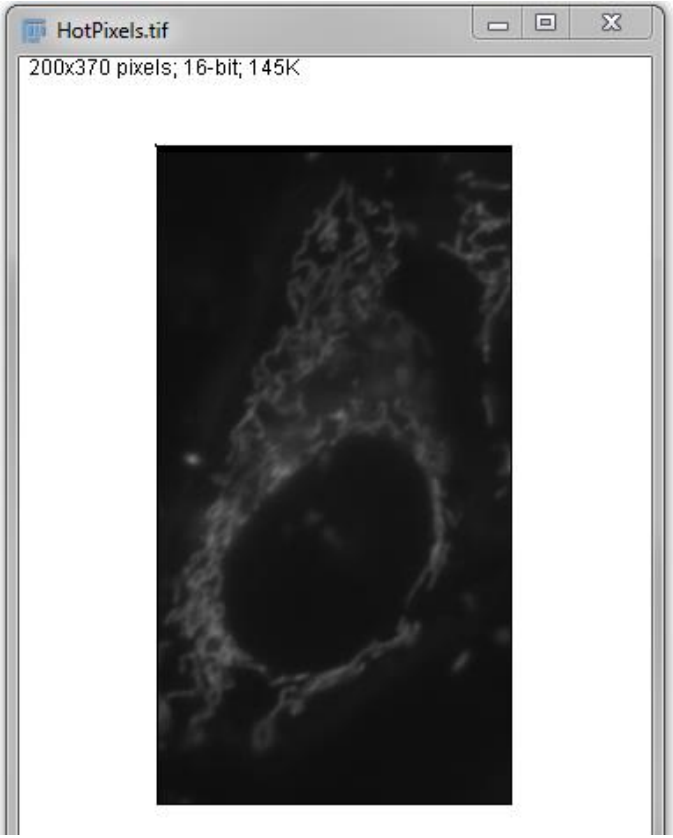


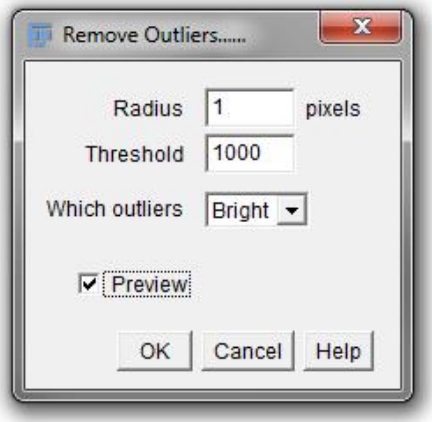
Hot pixel correction

- Few pixels of spurious intensity
- *Process/Noise/Remove outliers*

File: HotPixels

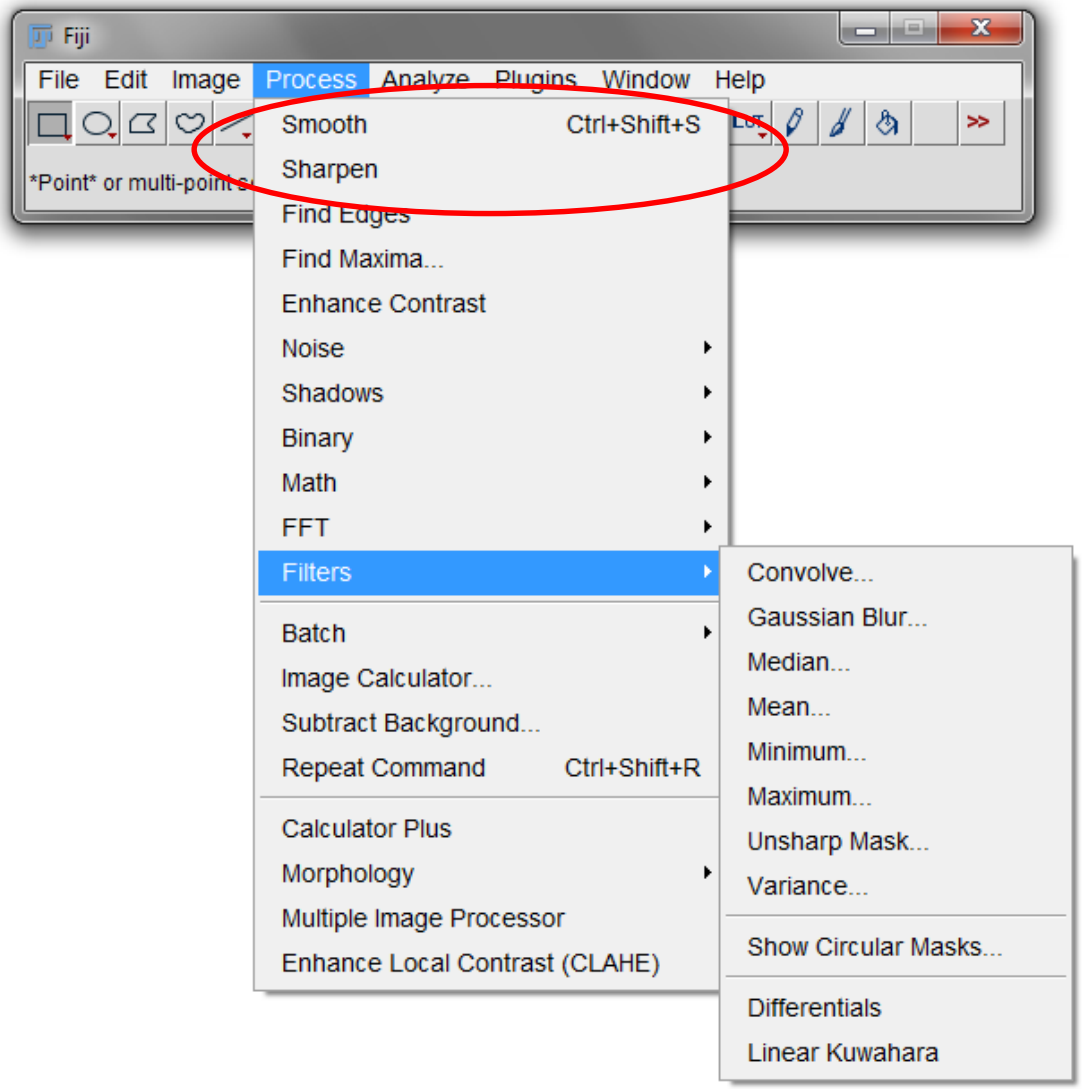






Best done with pixels smaller than the PSF

Filters

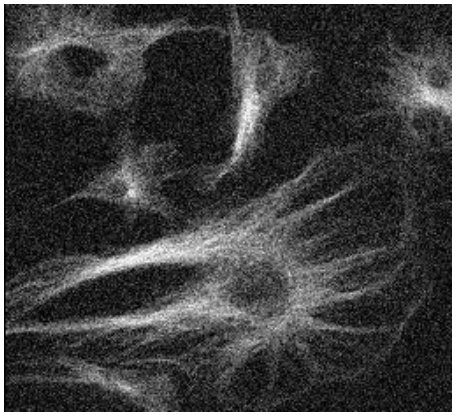


Files: Sharpen noise

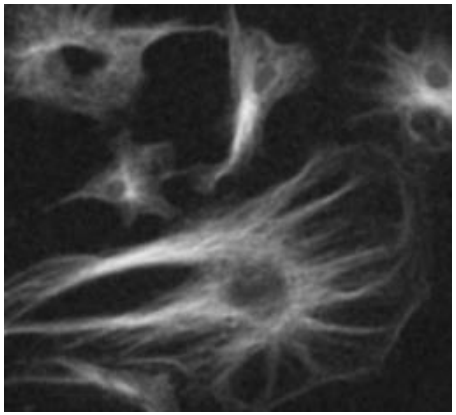
- ✓ Blur
- ✓ Median
- ✓ Sharpen

Their effect on noise and structure

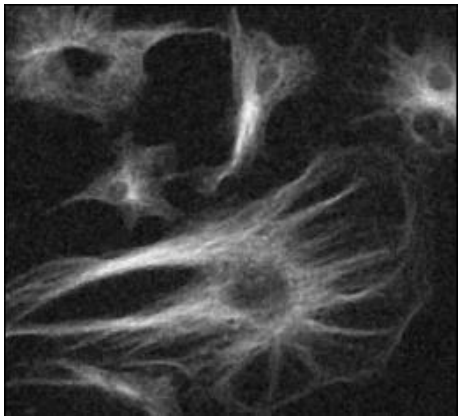
Original



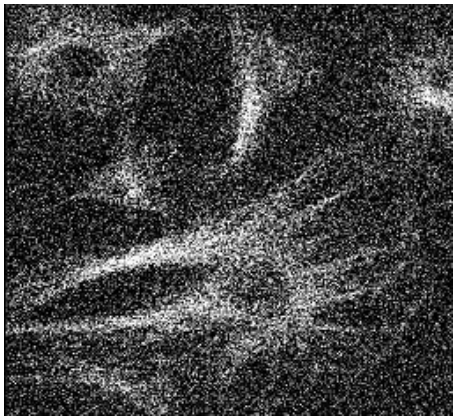
Blur filter

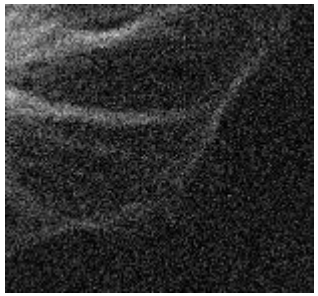


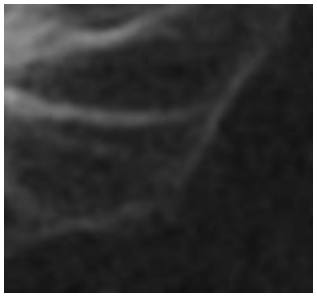
Median filter

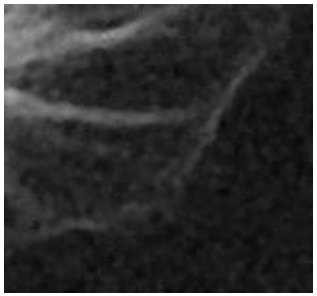


Sharpen filter



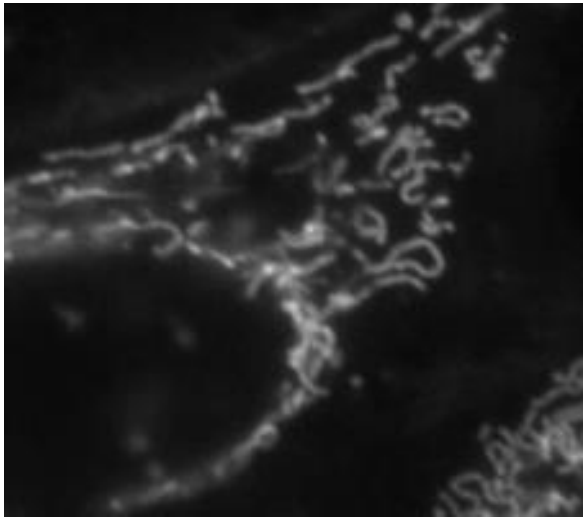


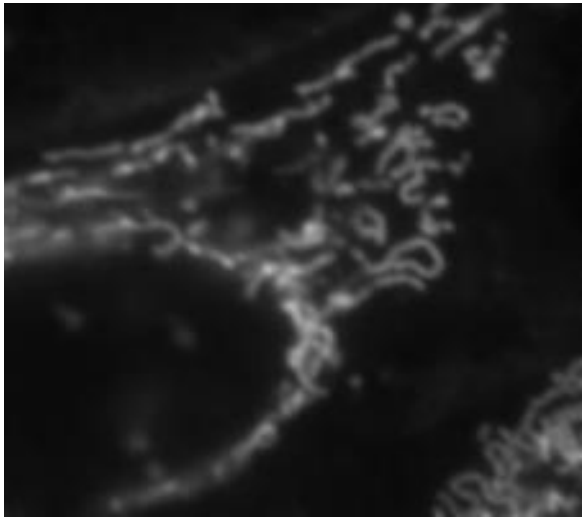




Their effect on noise and structure

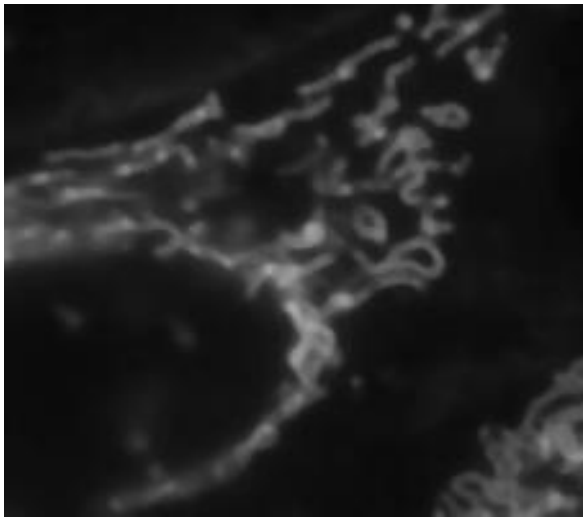
Original

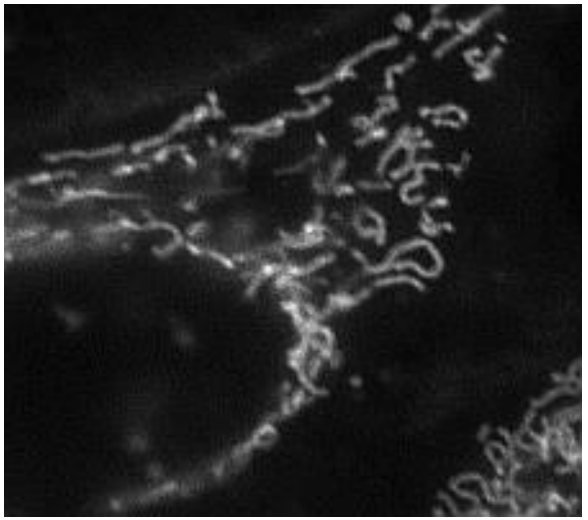




Blur

Median



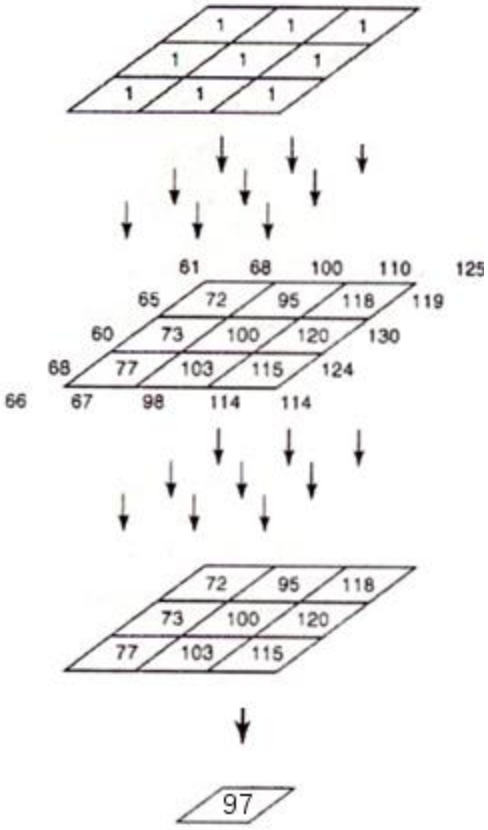


Sharpen

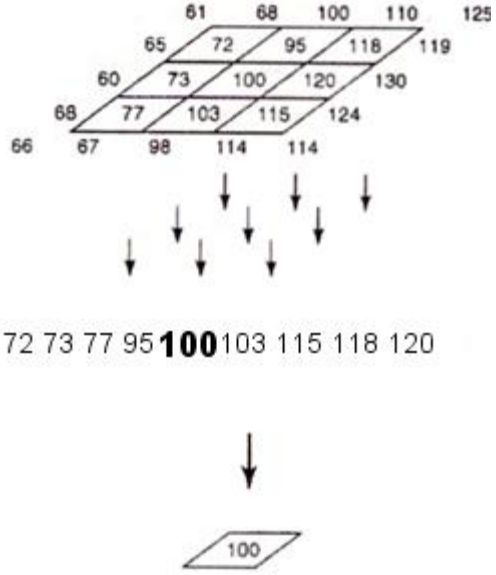
- Blur and median help noise but degrade structures
  - Sharpen helps structures, makes noise worse

What is a filter and how do they work?

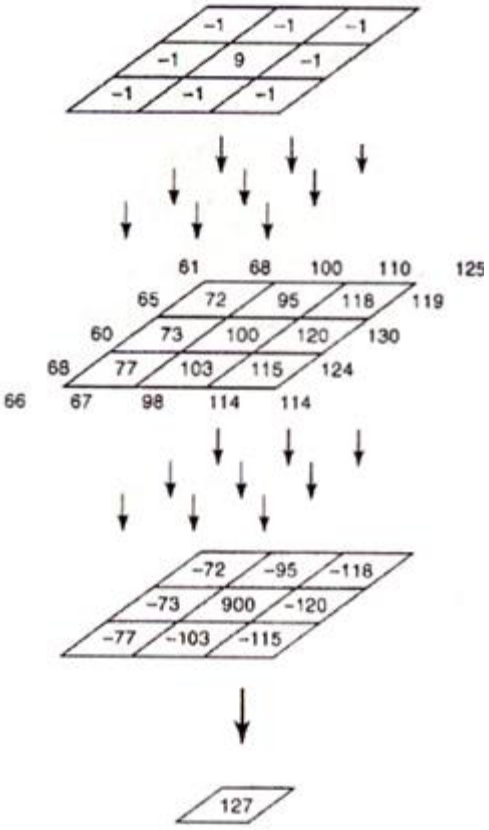
Blur filter



Median filter

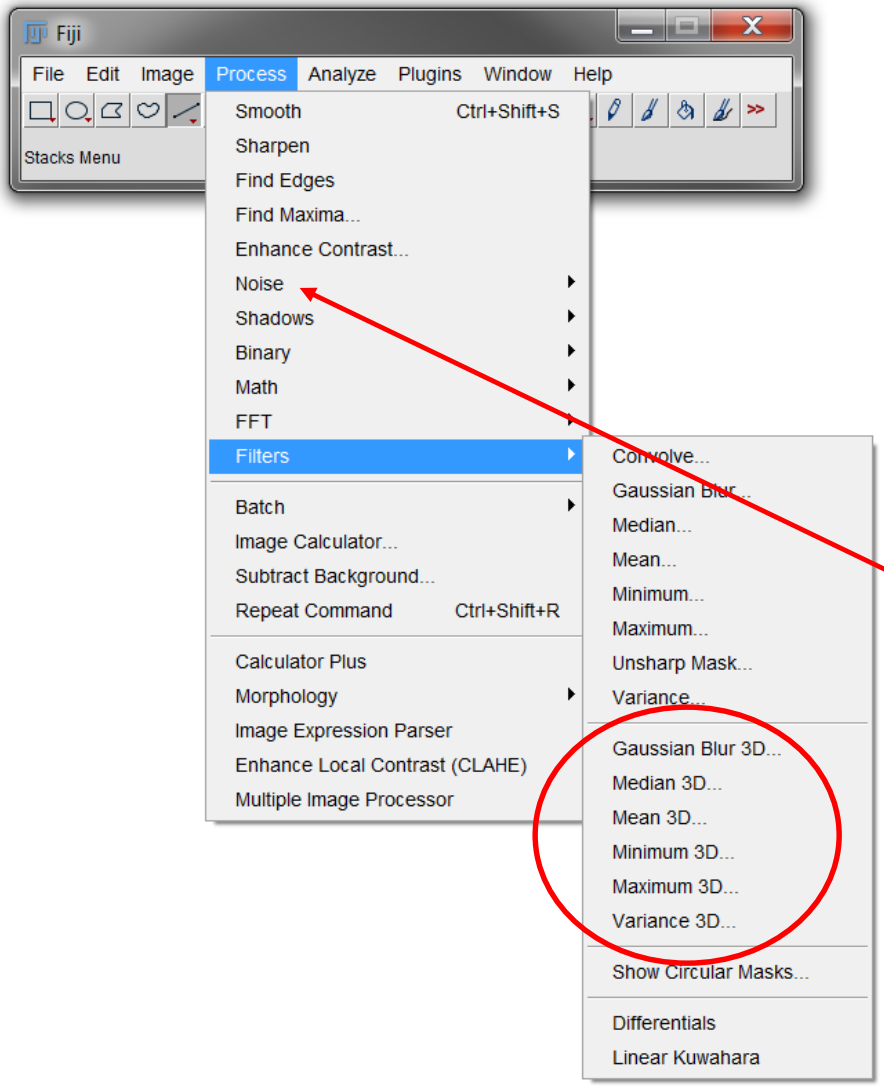


Sharpen filter



The kernel is used to compute a new value for every pixel in the original image based on the value of its neighbours

Filtering beyond one image: z and t



Kalman filters = over time

Image/Stacks/Kalman Stack filter

Try some of these, open say Maize_stack.tif, duplicate, add some noise, fix and compare Gaussian | Salt&Pepper

Analyze/tools/Synchronize windows
To compare before and after stacks

Shadow

$$\begin{matrix} -1 & 0 & 1 \\ -2 & 1 & 2 \\ -1 & 0 & 1 \end{matrix}$$

File: Sharpen

Process/Shadows/East

or define your own at
Process/Filters/Convolve . . .

Look at the histograms of before and after

1D example

$$\begin{matrix} -1 & 0 & 1 \\ -2 & 1 & 2 \\ -1 & 0 & 1 \end{matrix} \qquad \begin{matrix} -1 & 0 & 1 \\ -2 & 0 & 2 \\ -1 & 0 & 1 \end{matrix}$$

2 2 2 2 4 6 8 10 10 10 8 6 4 2 2 2 2

Edge enhancing filters

1	2	1
0	0	0
-1	-2	-1

1	0	-1
2	0	-2
1	0	-1

File:
Yeast_DIC_stack
(Maybe just the mid plane)

Vertical derivative

Horizontal derivative

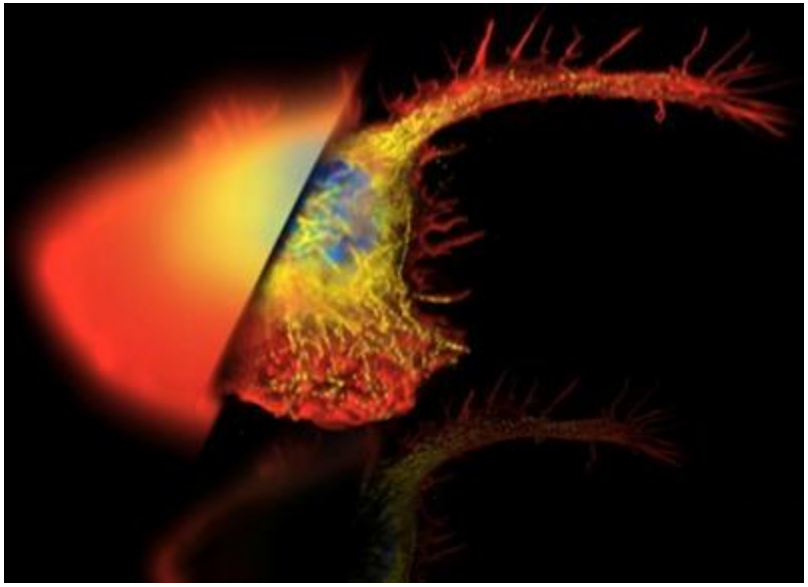
$$Sobel = \sqrt{H^2 + V^2}$$

Process/Find Edges

How does it cope with the bright and dark edges?

Deconvolution

A mathematical post-acquisition processing of images to reduce the blur from out of focus light. This can increase the signal to noise ratio and resolution of the image.



Convolution

Object
3 small, perfectly
spherical green things

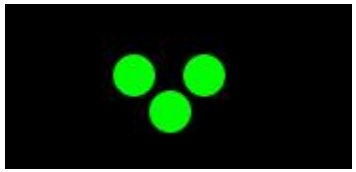
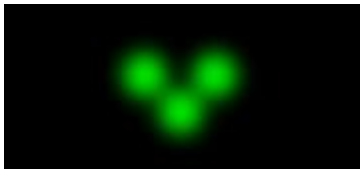
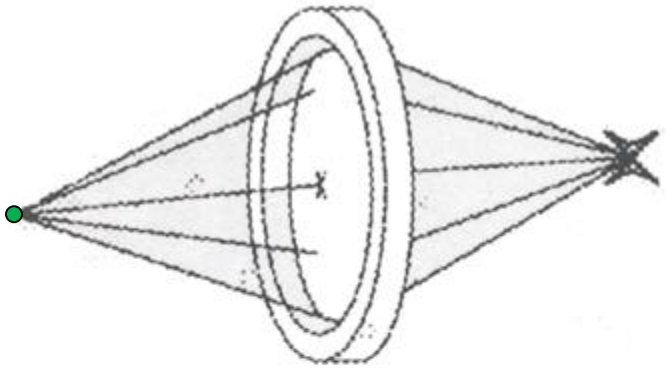


Image
of 3 small, perfectly
spherical green things

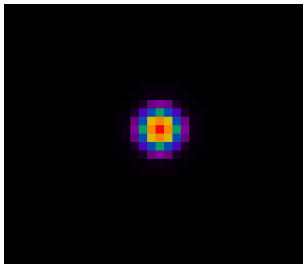


The Point-Spread Function and image formation

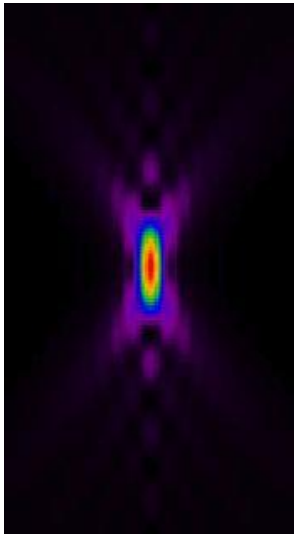


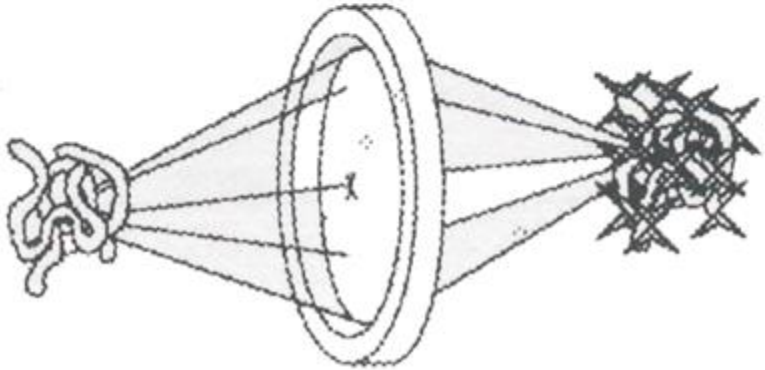
PSF

XY



XZ





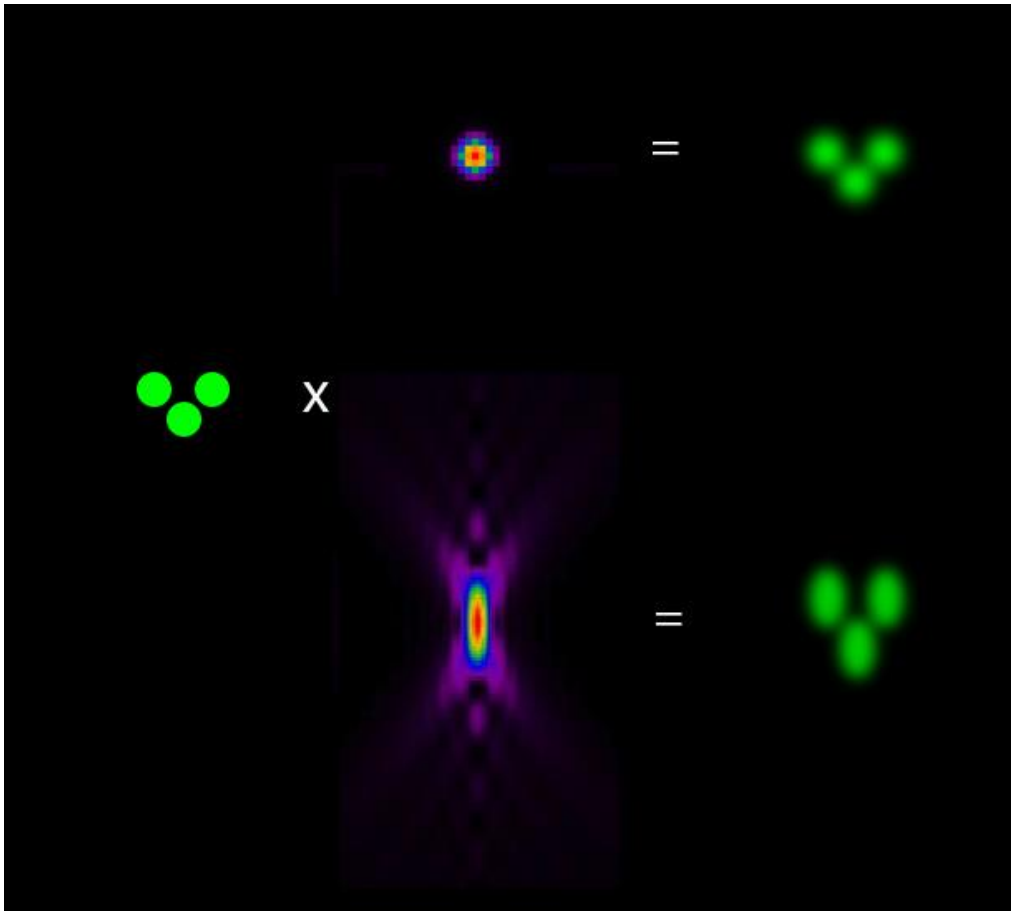
Image

The image is the sum of all blurred point images

Convolution

An image is a convolution of the object:

Object ⊗ PSF = Image

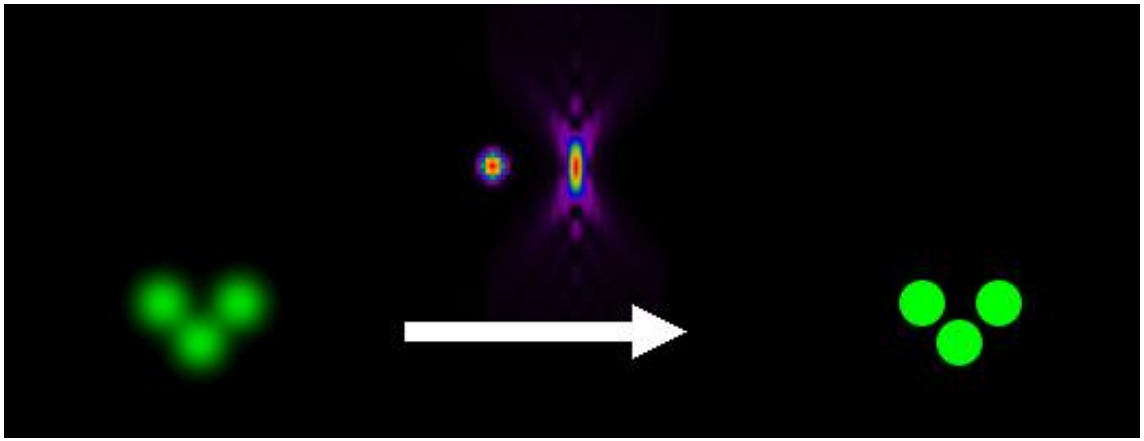


The aim of De-Convolution

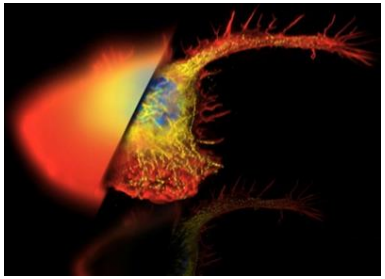
Image(s)

Knowledge of imaging psf

Underlying object



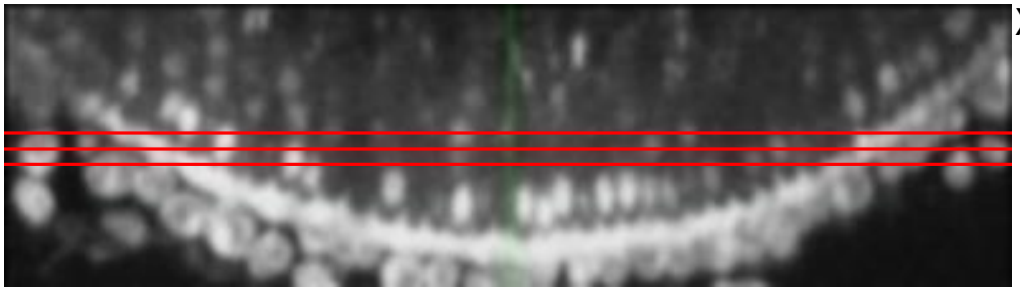
The PSF can be measured or predicted



Two types of deconvolution

1. Deblurring/nearest neighbor/2D deconvolution

- Estimates the blur from other focal planes and removes it
- Sharpens the image but is non-quantitative
- Very fast, real time

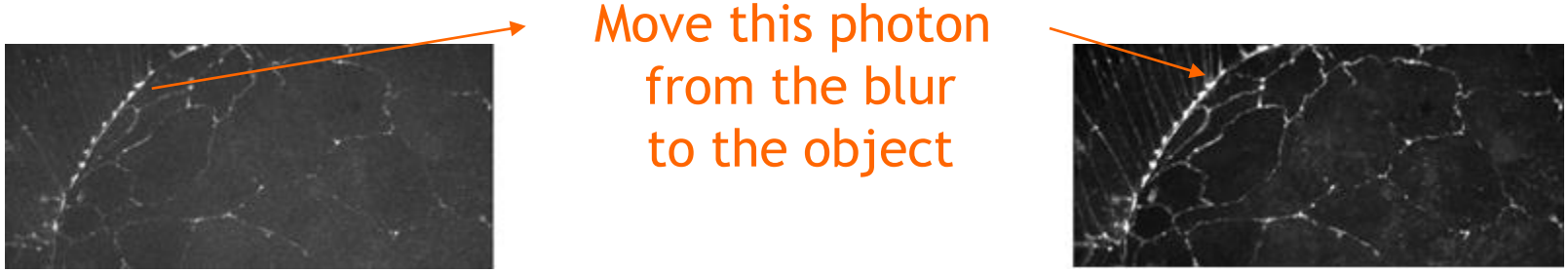


Blur the image from here and remove it from the images above and below

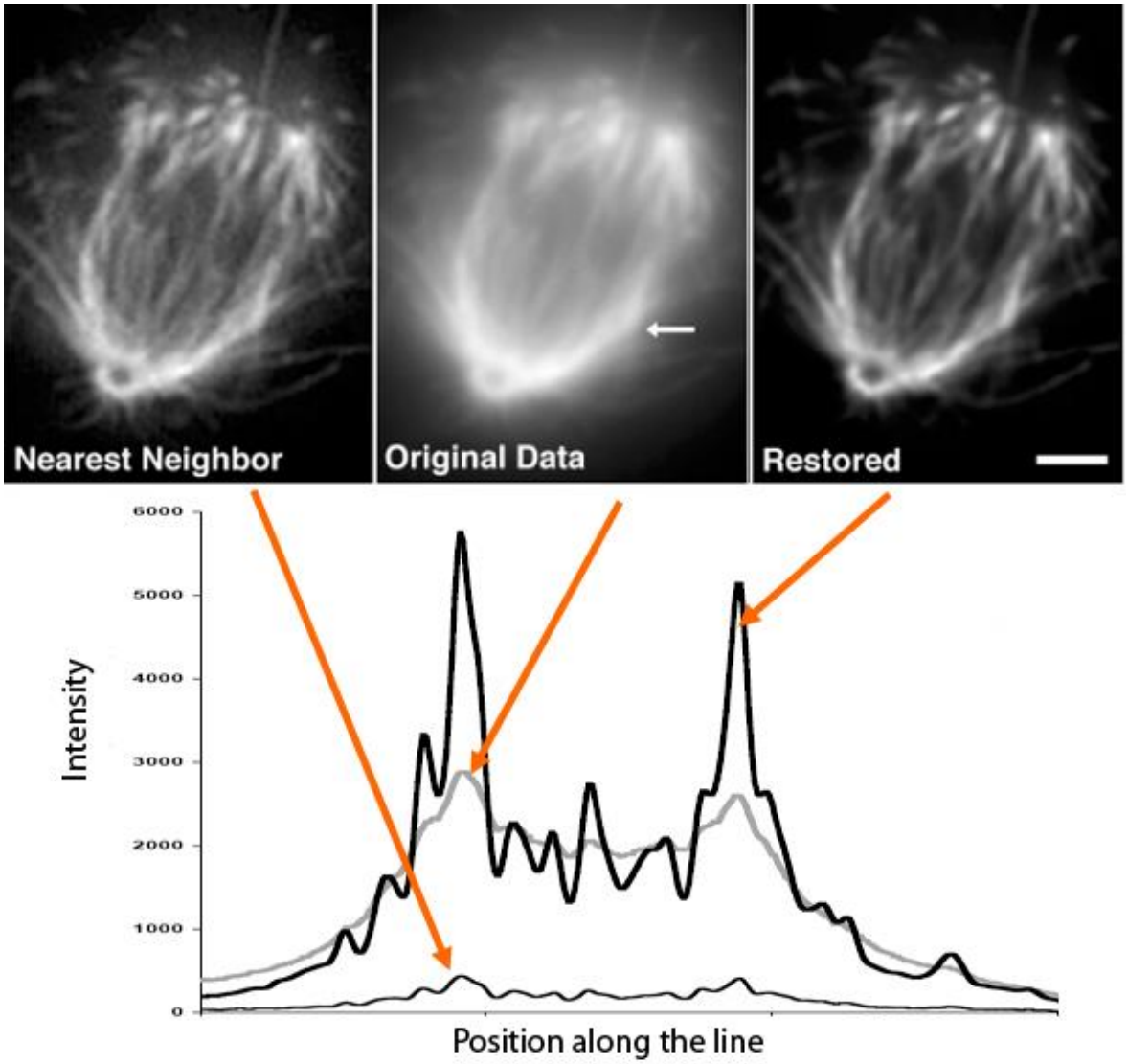
Two types of deconvolution

2. Restoration/3D deconvolution

- Iterative reassignment of photons based on modeled convolution
- Works in 3D (ie considers all the data together)
- More computational intensive, takes a few seconds to minutes
- Conservative and quantitative
- Better able to cope with low SNRs

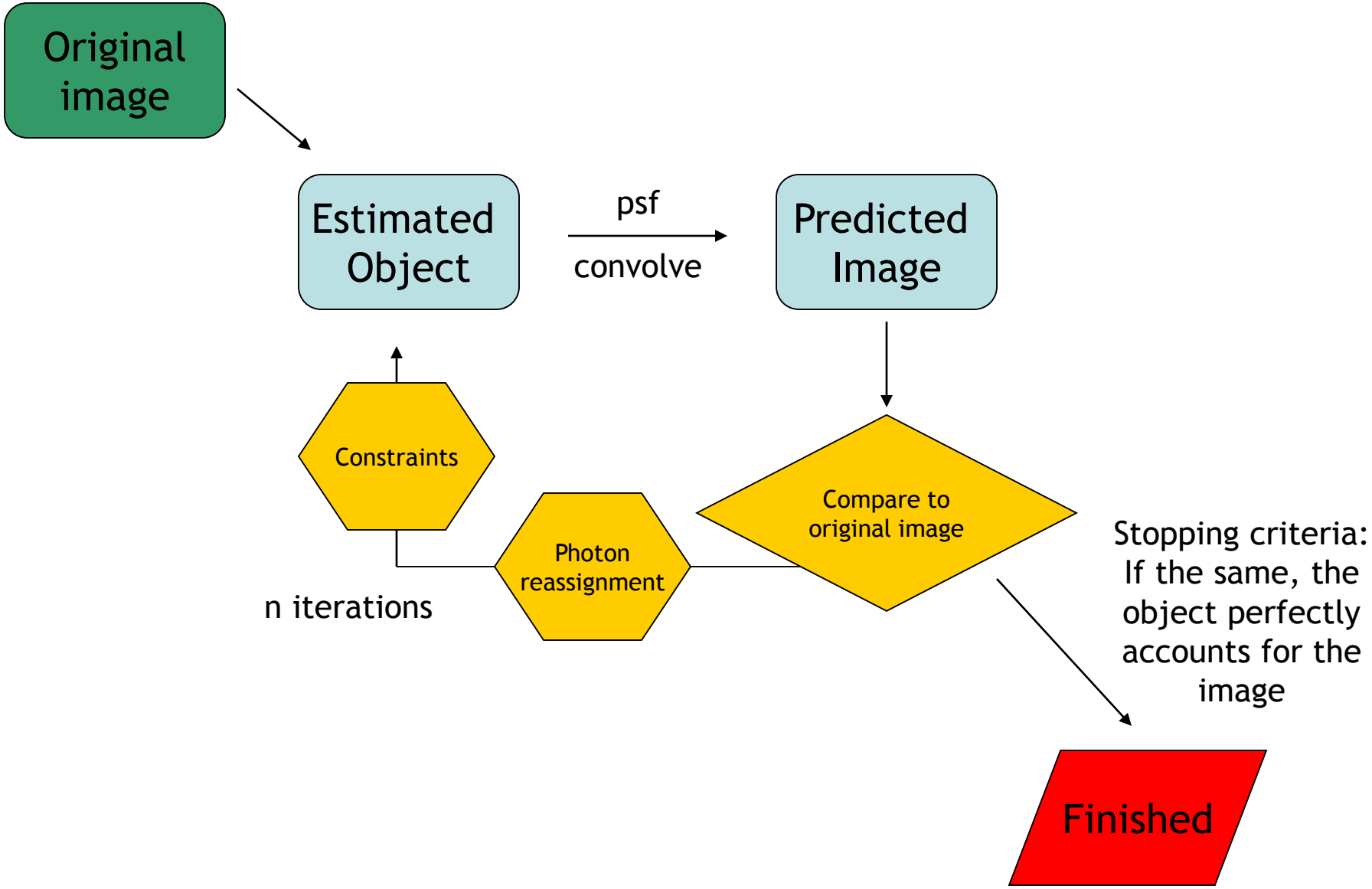


Deblurring v restoration



A Workingperson's Guide to Deconvolution in Light Microscopy

The computational process



Deconvolution software

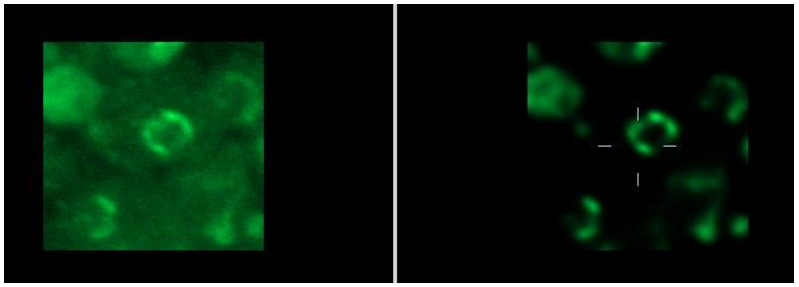
- Various software implement these algorithms with a few tweaks of their own

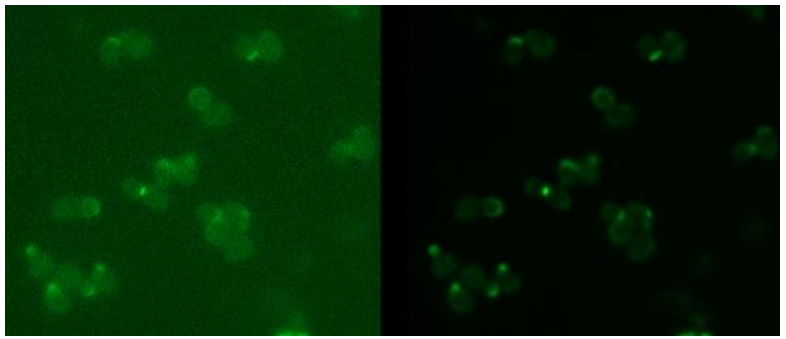
- I don’t know of any powerful open source software so it probably costs money

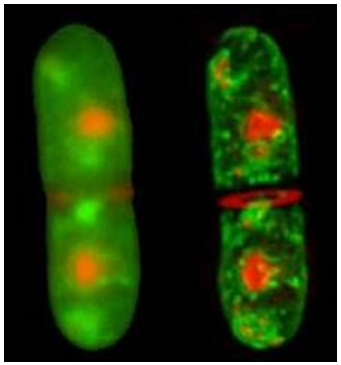
- We have:
  Huygens – Predicted or measured PSF
  SoftWorx – Measured PSF

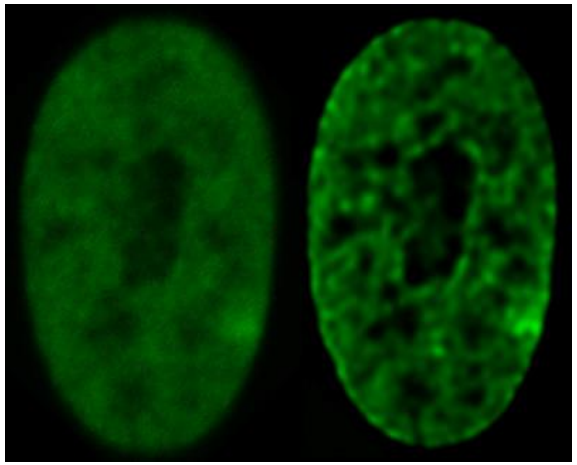
- Need a powerful computer and it takes a while

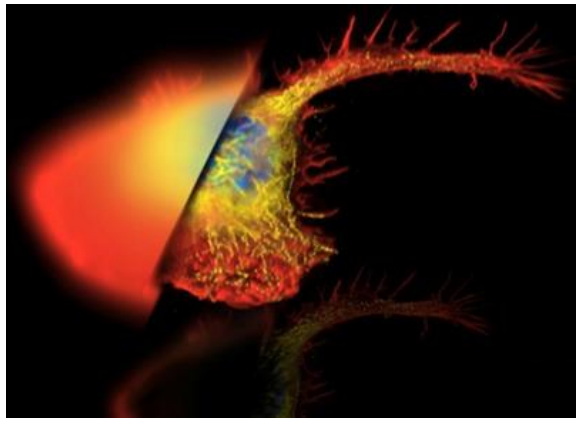
What actually improves during deconvolution?











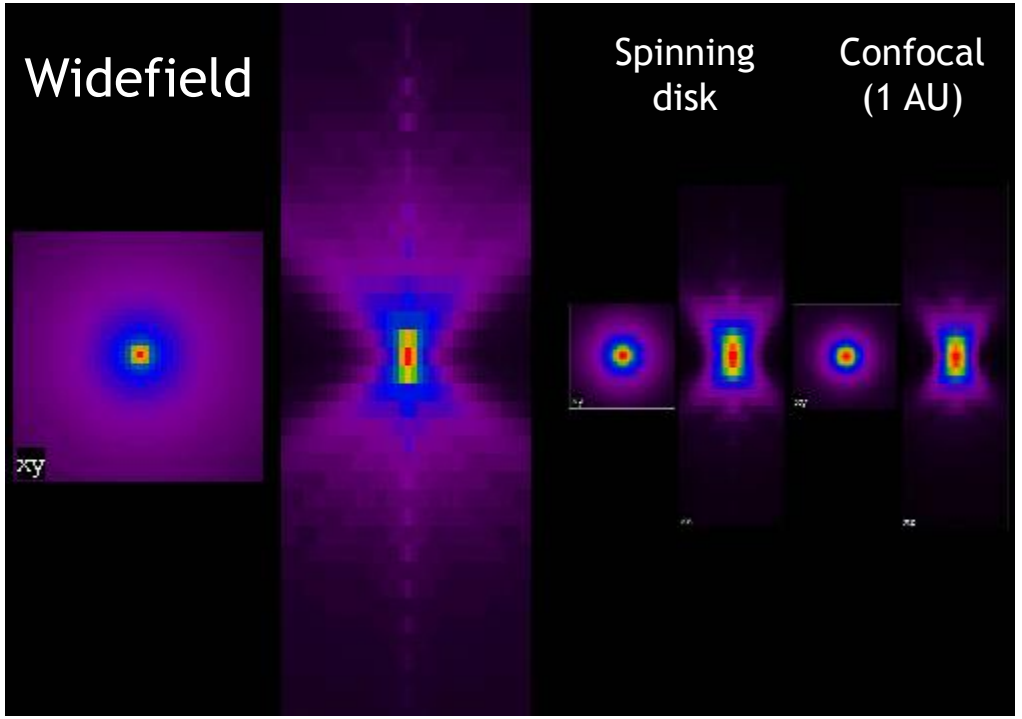
- Photon reassignment: Blur Structure
- Noise . . . So higher SNR

Which modalities does it work with?

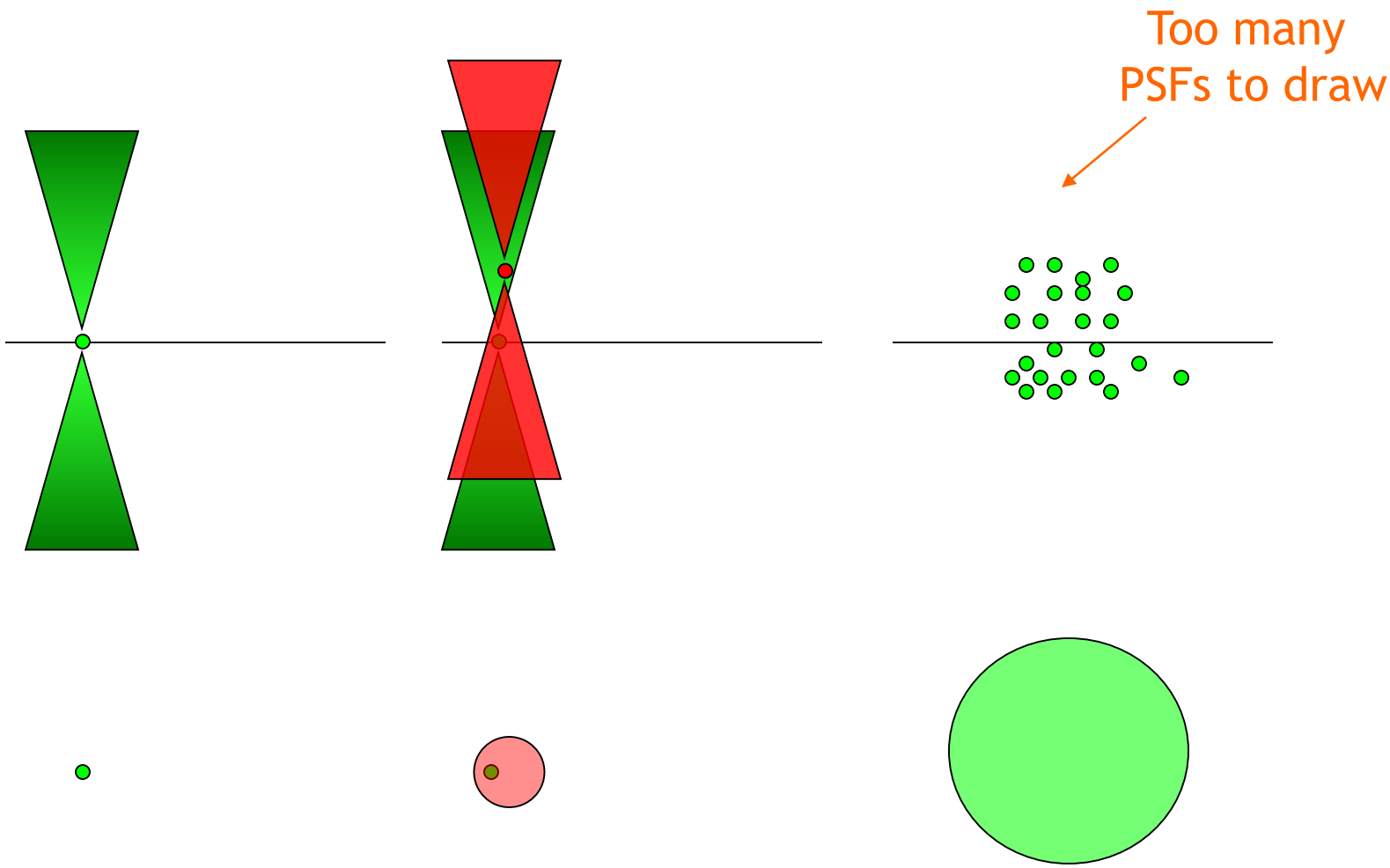
All of them but the relative benefits are different for the different modalities

PSF calculated for 60x/1.4 NA objective Green fluor

= 2 μm



Limited in widefield for thick samples



Summary: What deconvolution is/ is not

Post-processing that works for all (3D) imaging modalities

Fairly computational intensive to calculate properly

It needs good images - Does NOT allow you to take awful images and magically transform them!

Good for live cell imaging: gentle but slightly noisy images + deconvolution = good images with less phototoxicity

It doesn't replace confocal. Anything >30 μm and the blur becomes too much for processing and you need a spatial filter.