Advanced Microscopy Course 2013

Lecture 5:

Basic Image Processing

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* Graeme Ball, Lecture 19: Applied Image Analysis *

Basic Image Processing

- What is a digital image?
- What makes a good image?

    Correct image acquisition

    Signal to Noise

    Resolution and Sampling

- The basics of image processing

    Golden rules of image processing

    Conceptual Hierarchy of Image Processing

        Low Level Processing:
            Display (Figures)
            Filtering
        Mid - Level Processing:
            Segmentation
            Spectral unmixing
        High -Level Processing / **Analysis**:
            Colocalisation
            Tracking
            Statistics

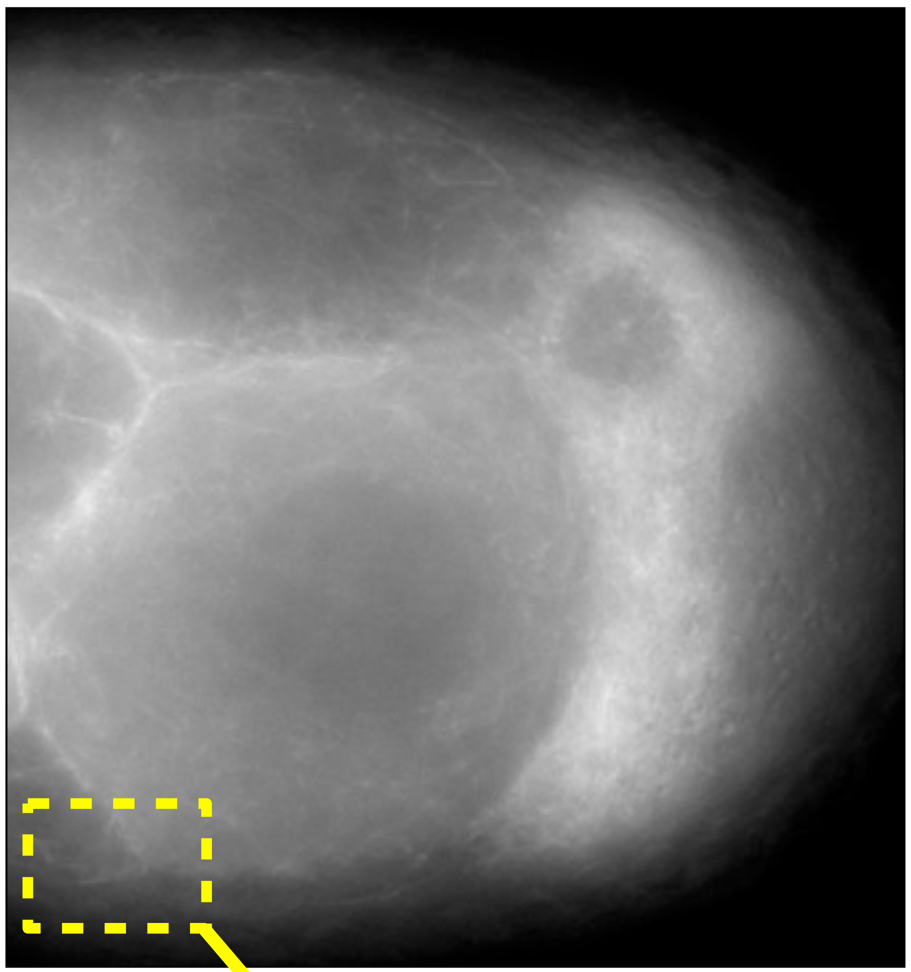
What is a digital image?

What is a digital image?

An image represents the output of the optics and detector of the imaging system

image ≠ object

image = object ⊗ PSF



258	544	1193	1969	2370	2063	1301	613	263
286	562	1173	1893	2251	1959	1241	596	278
313	585	1129	1760	2073	1791	1156	577	300
365	622	1000	1407	1630	1378	930	514	357
386	629	916	1215	1388	1154	819	491	373
401	613	819	1026	1148	946	709	467	372
411	526	613	678	704	614	518	420	328
406	477	511	523	589	458	425	387	307
387	419	420	405	364	340	349	345	284
321	327	307	320	268	260	271	261	245
282	267	243	254	202	203	222	215	217
238	216	193	205	163	165	185	177	186

- **A digital image is a numerical array:**
  elements = pixels or voxels with:
  - defined size (sampling resolution)
  - defined no. of grey levels (bit depth)

- **In addition to "useful" signal there is:**
  - dark signal from the detector
  - autofluorescence (background)
  - statistical noise of photon detection

- **Details are detected within the limitations of:**
  - the imaging optics
  - the sampling rate (pixel size)
  - the statistical noise
  - the sample contrast / detector dynamic range

Image Parameters - what to record (= image metadata)

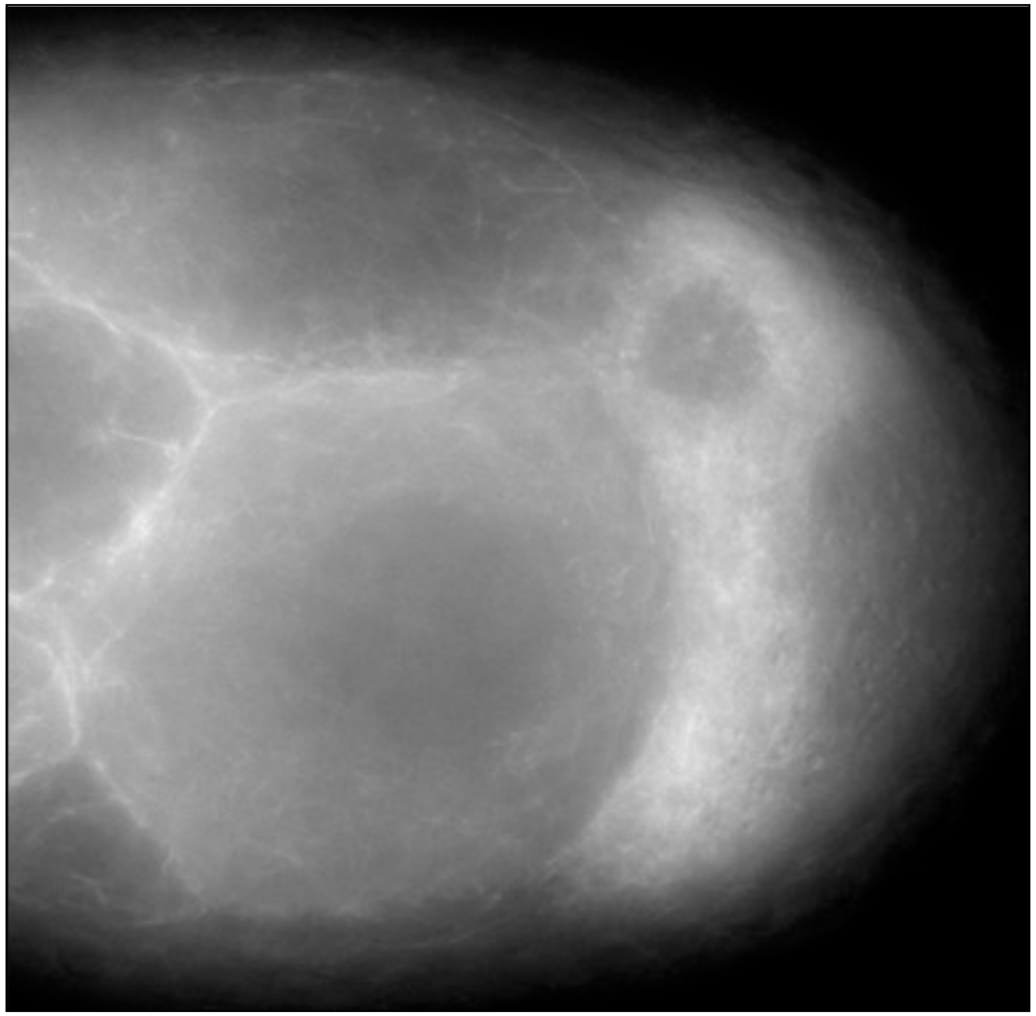
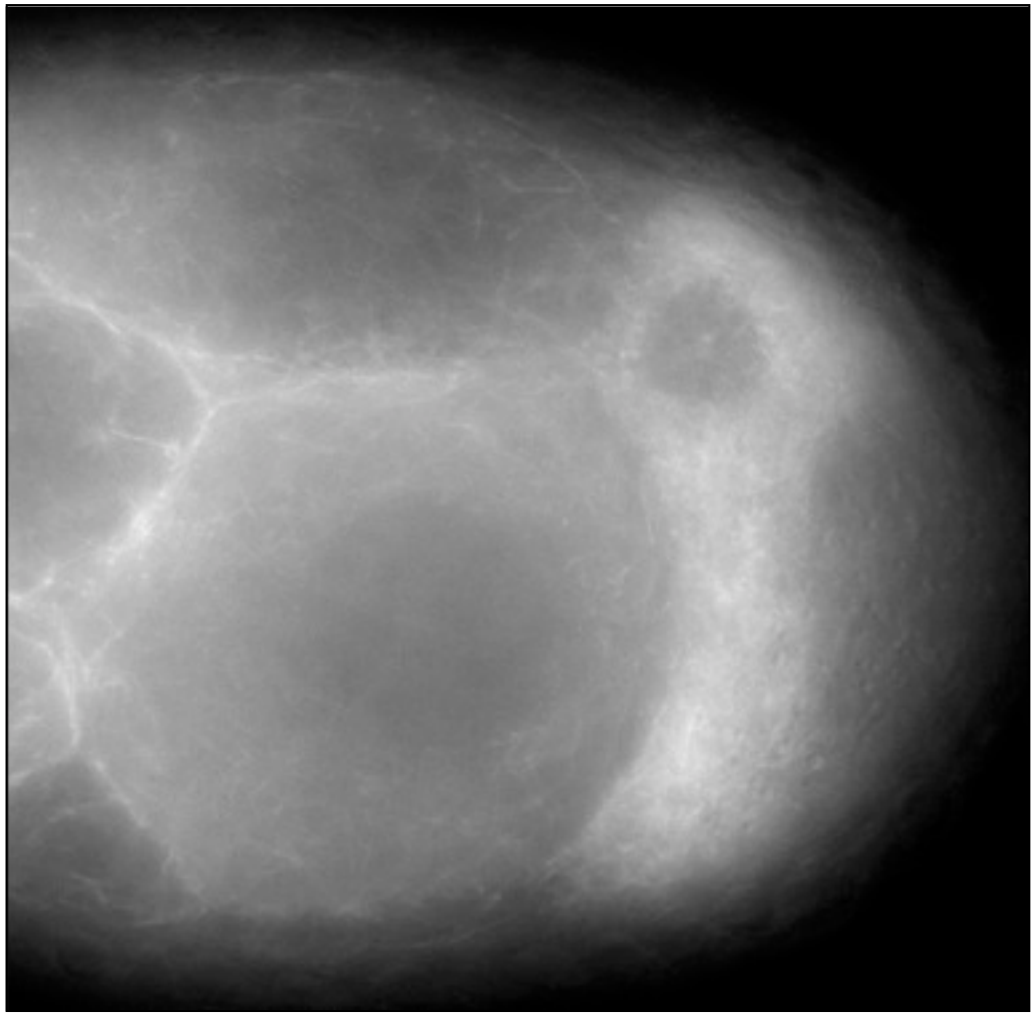


Image Parameters - what to record (= image metadata)



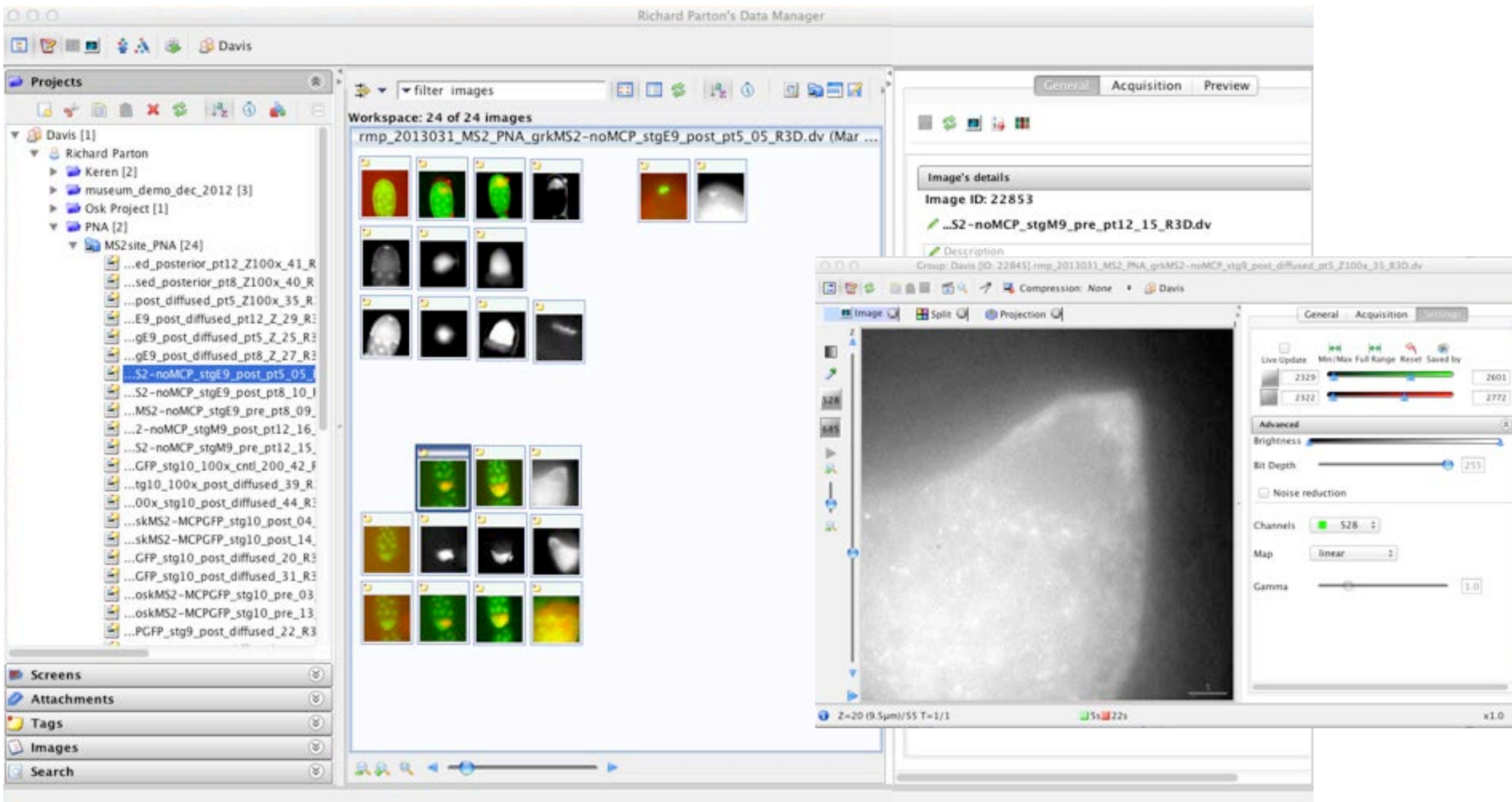
Wide-field fluorescence 490 ex 520 em
X60 1.2 WI
xy 212 nm; 60 (z step 200 nm)
Bin 2x2
250 ms exposure
Contrast stretched to fill 8 bit display
Tau-GFP Oocyte

- **Type of imaging – Wide-field fluorescence**
- **Excitation and Emission wavelengths 490/520**
- **Optics used – x60 NA 1.2 water immersion**
- **Image pixel dimensions – 212x212 (x200) nm**
- **Depth or Dynamic range – 8 bit; 256 greys**
- **Any processing performed**
  - **12 bit to 8 bit conversion**
  - **contrast adjustment**
- **Display parameters - range 0-255, grey scale**
  - **gamma = 1**
- **The Biology - *Drosophila* stage 8 egg chamber**
  - **Tau GFP, labelling microtubules**

OME - Open Microscopy Environment

* Douglas Russell, Lecture 18: Image Management *

- **Purpose: Supporting Metadata Management for Microscopy**
  avoids problems of image formats
  archiving and retrieval
  data sharing



What makes a good image?

Correct Image Acquisition

- **The system must be correctly set up and aligned**
  - PSF verification (beads)

- **The specimen should not cause undue optical aberration**
  - mounting / appropriate optics

- **Avoid underflow and overflow but fill the dynamic range**
  - use a colour LUT
  - beware of auto-intensity scaling

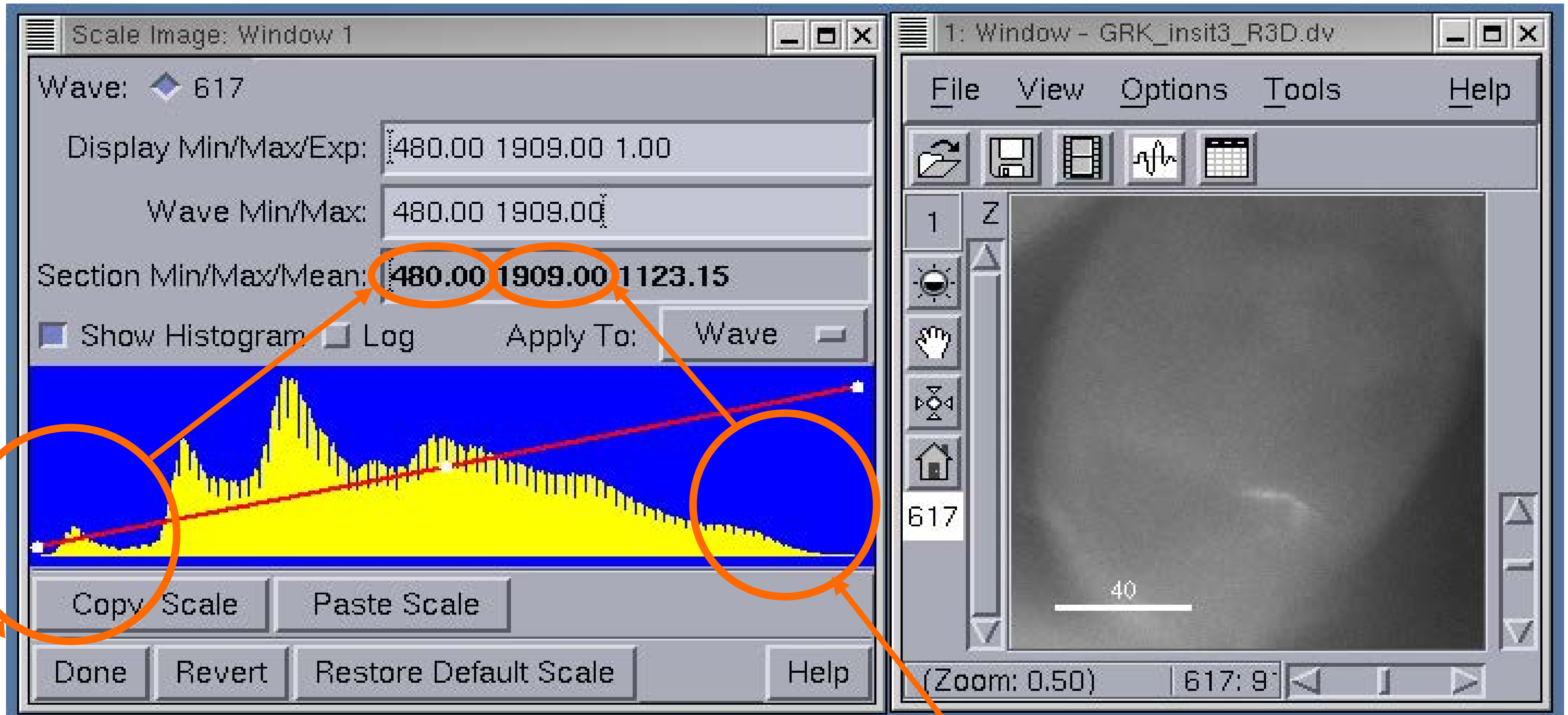
- **Take a dark signal image and/or background**
  – Dark subtraction processing

- **Be aware of XYZ optical resolution of the system and sample appropriately**
  - PSF of the imaging system
  - Pixel (voxel in 3D) size in the image

- **Take care with signal to noise limitations**
  - collect enough light: integrate, average

Correct image acquisition

- Use **histogram analysis** to ensure you collect enough light in the features of interest

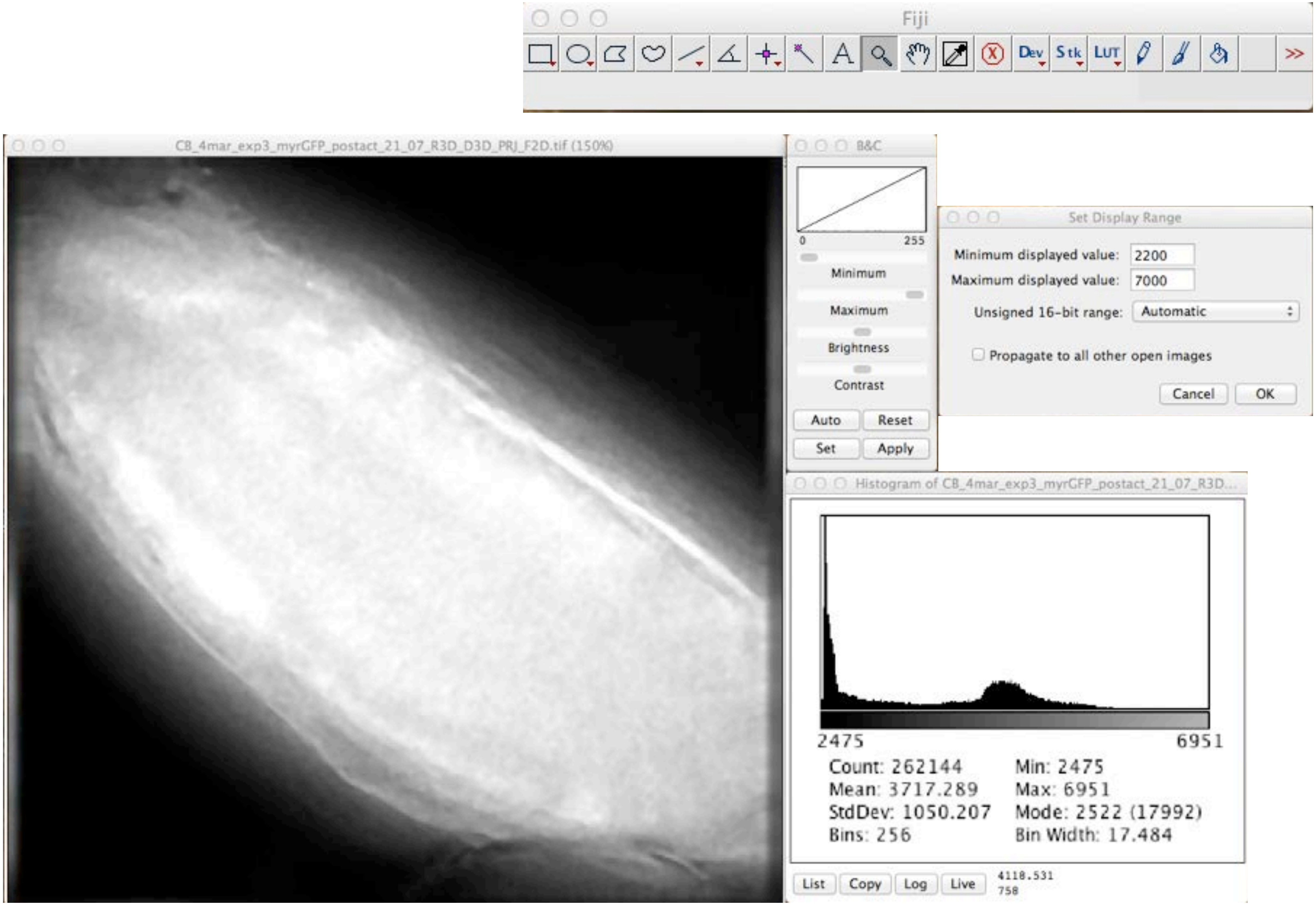


mostly camera offset, dark signal and background

max value should be below detector saturation = avoid saturation

- **NOTE - the brightness of the display is not the best indicator of image signal**
- **Make good use of the dynamic range:8-bit = 255, 12-bit = 4095, 16 bit = 65535**

Correct image acquisition



Noise / Signal to Noise (S/N)

Signal to Noise - definitions:

- One of the most important limitations to image quality and image processing

$$\text{S:N ratio} = \frac{\text{Signal}}{\text{Variation in the signal}}$$

$$\frac{\text{mean}}{\text{S.D.}}$$

- Noise is NOT background, auto-fluorescence or dark signal
- Good image data has a high S:N ratio (>4)
- Fundamental limit = Poisson distributed statistics of photon detection (shot noise)

Poisson distributed variation

$$\text{S:N ratio} = \frac{n}{\sqrt{n}}$$

- Statistics of photon counting dictate the minimum useful signal

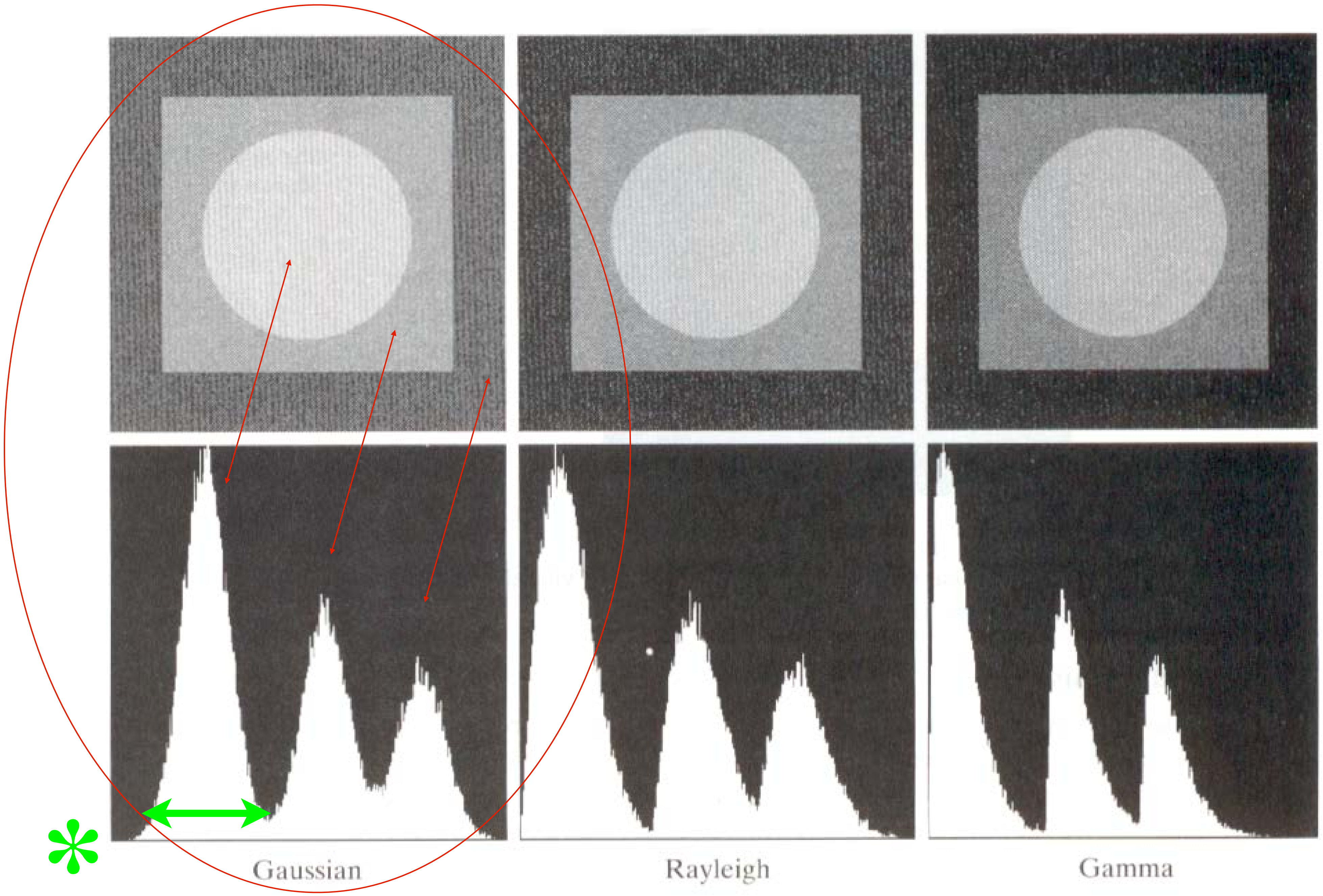
Average signal = 9, S:N ratio = 3
Average signal = 100, S:N ratio = 10
Average signal = 10,000, S:N ratio = 100

A meaningful difference in intensity needs to be at least three times the noise level

- Additional sources of noise from digitisation, detector readout, thermal noise.

Signal to noise
- noise types

Most commonly used noise model for image processing = Gaussian



Gonzales & Woods, 2002. Digital Image Processing 2nd Ed. Prentice-Hall Inc, USA.

How to deal with signal to noise

Acquisition

- **Use sensitive, high dynamic range, low noise detectors: cooled CCD, EMCCD**
- **Count as many photons as possible:**

Bright dyes
Good excitation / emission
Integration time (accumulation or averaging)

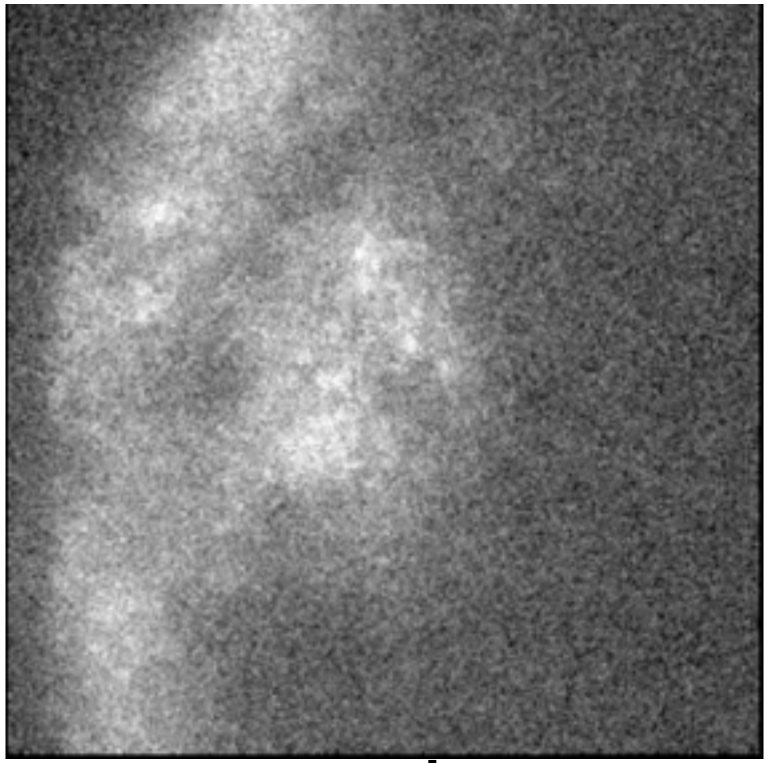
Post Acquisition

- **Image averaging**
- **Noise reduction filtering using a spatial filtering mask – 3x3 median filter**
- **Noise reduction filtering in the frequency domain – Fourier bandpass filter**

Improving signal to noise

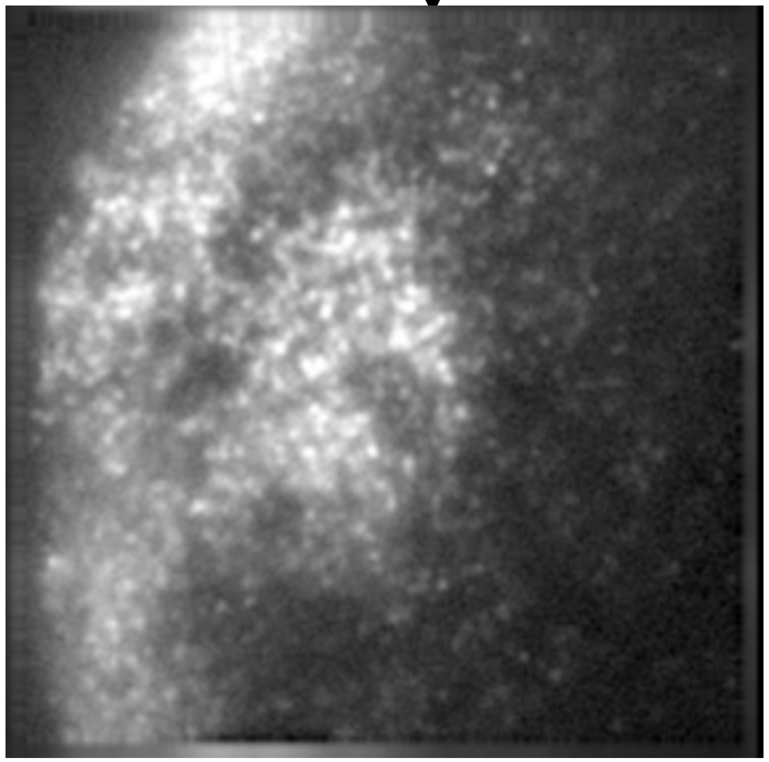
increased signal increases S/N = improved contrast

noisy image
(scaled)



5x integration time

increased number
of photons counted



improved S/N

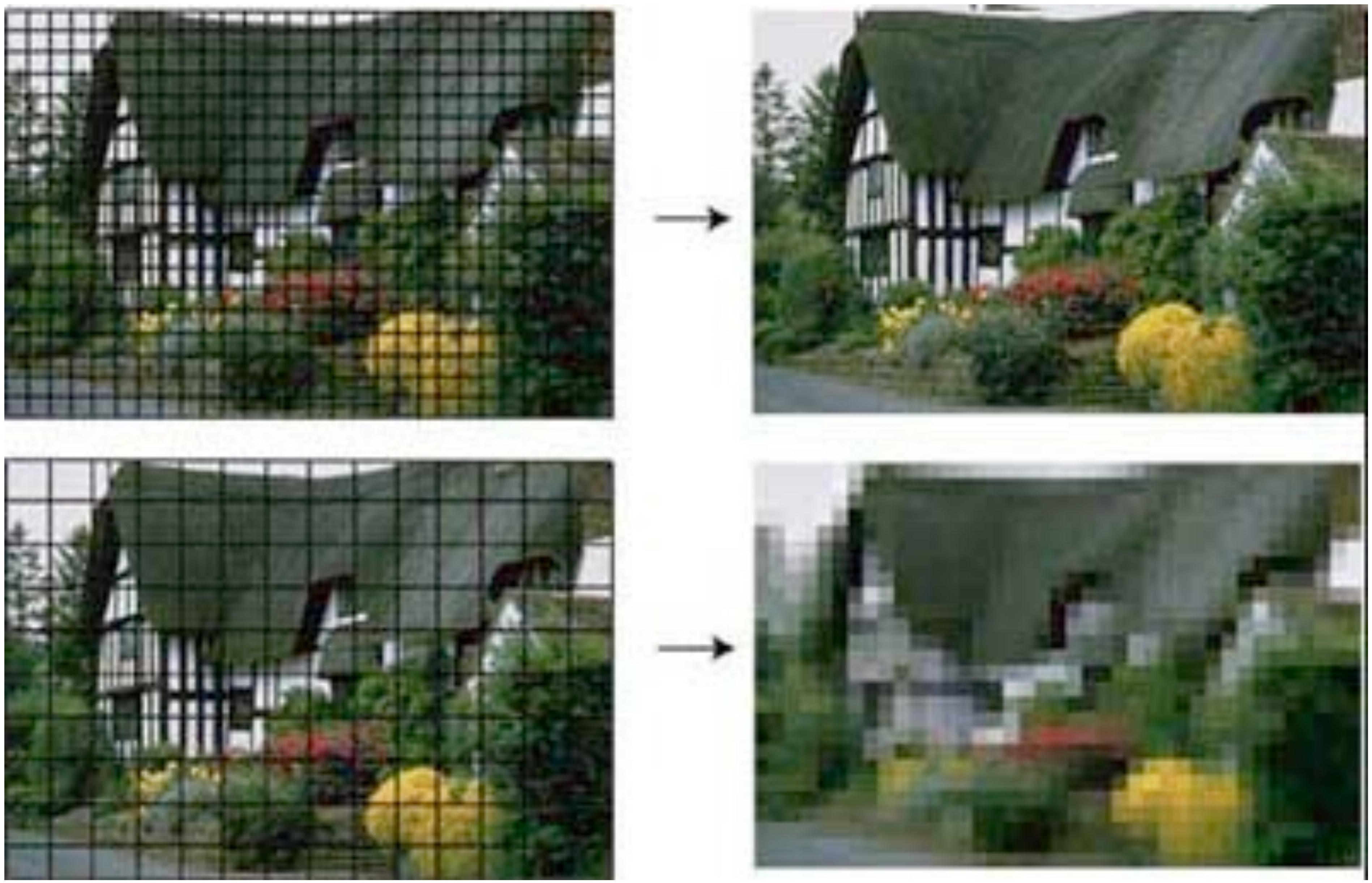
ImageJ

Avoid propagating noise

- **Noise is additive:**
  SO subtracting one noisy image from another propagates noise

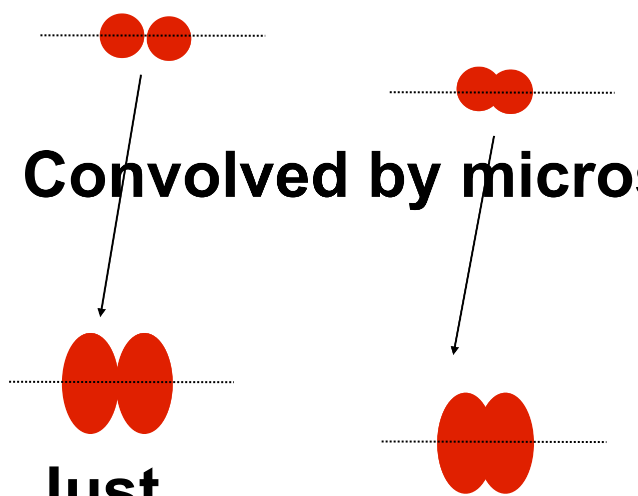
  THEREFORE
  Subtract an AVERAGE signal to avoid noise propagation
  Or
  Where the signal is non-uniform across the field subtract a 4x AVERAGED image to avoid noise propagation

Resolution and Sampling



Optical resolution: The Rayleigh Criterion

Two small objects



Convolved by microscope

Just resolved

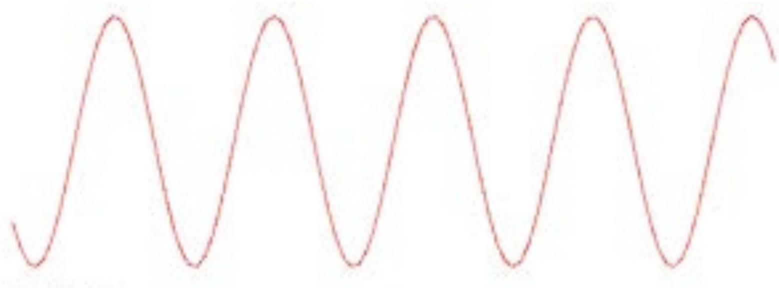
Not resolved
Airy patterns must Overlap by less Than 42% of the Maximal intensities

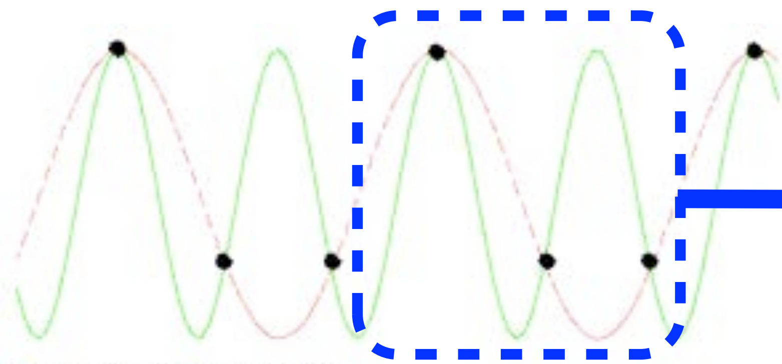
resolution limit $\approx \lambda_{em}/2$

XY resolution ~ 200 nm
Z resolution ~ 500 nm

Resolution: Nyquist sampling theorem

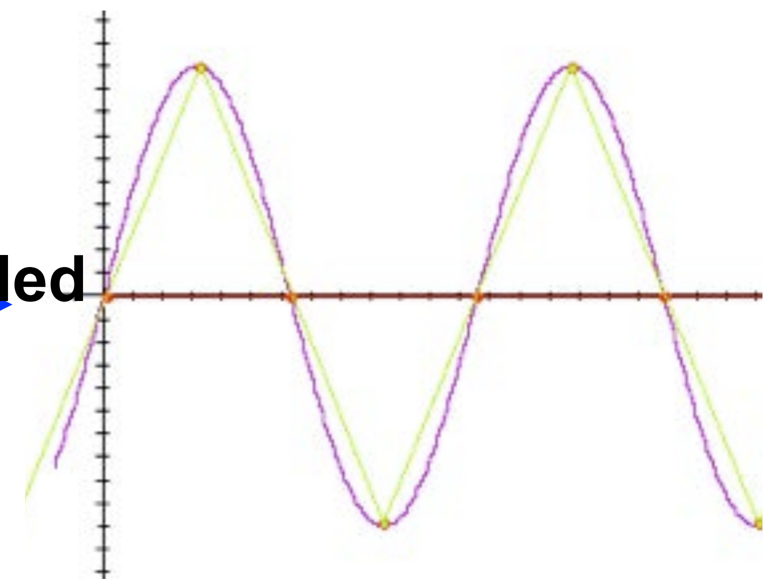
A sine wave





undersampled

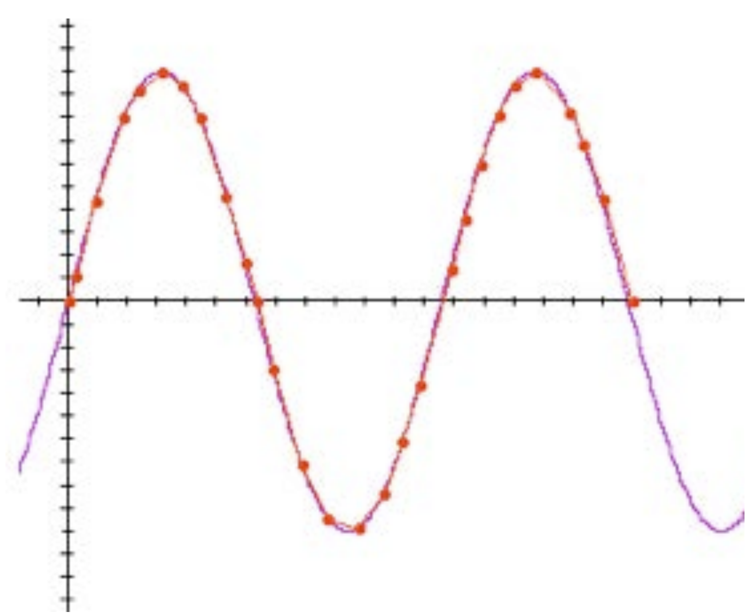
Sampling 1.5 times per cycle



just sampled enough

Sampling 2.0 times per cycle

2.3



over sampled

Sampling many times per cycle

Resolution: sampling

Theoretical Axial Resolution (em 525 nm)	Appropriate Sampling According to Nyquist theorem - at least half the size	Pixel size on Delta Vision
1.4 oil = 229 nm	Nyquist ~ sample at 0.100 um/pixel	x100 = 0.063 um/pixel
1.35 oil = 237 nm	Nyquist – sample at 0.103 um/pixel	x60 = 0.106 um/pixel
1.2 water = 267 nm	Nyquist – sample at 0.116 um/pixel	x40 = 0.158 um/pixel
0.75 air = 427 nm	Nyquist – sample at 0.186 um/pixel	x20 = 0.317 um/pixel

- Undersampling limits the data available

Interactive tutorial – http://www.microscopyu.com/tutorials/java/digitalimaging/pixelcalculator/

Resolution, contrast, noise

- **Noise limits the contrast which limits the details that can be resolved**
  = Noise limits resolution

Resolution, contrast, noise

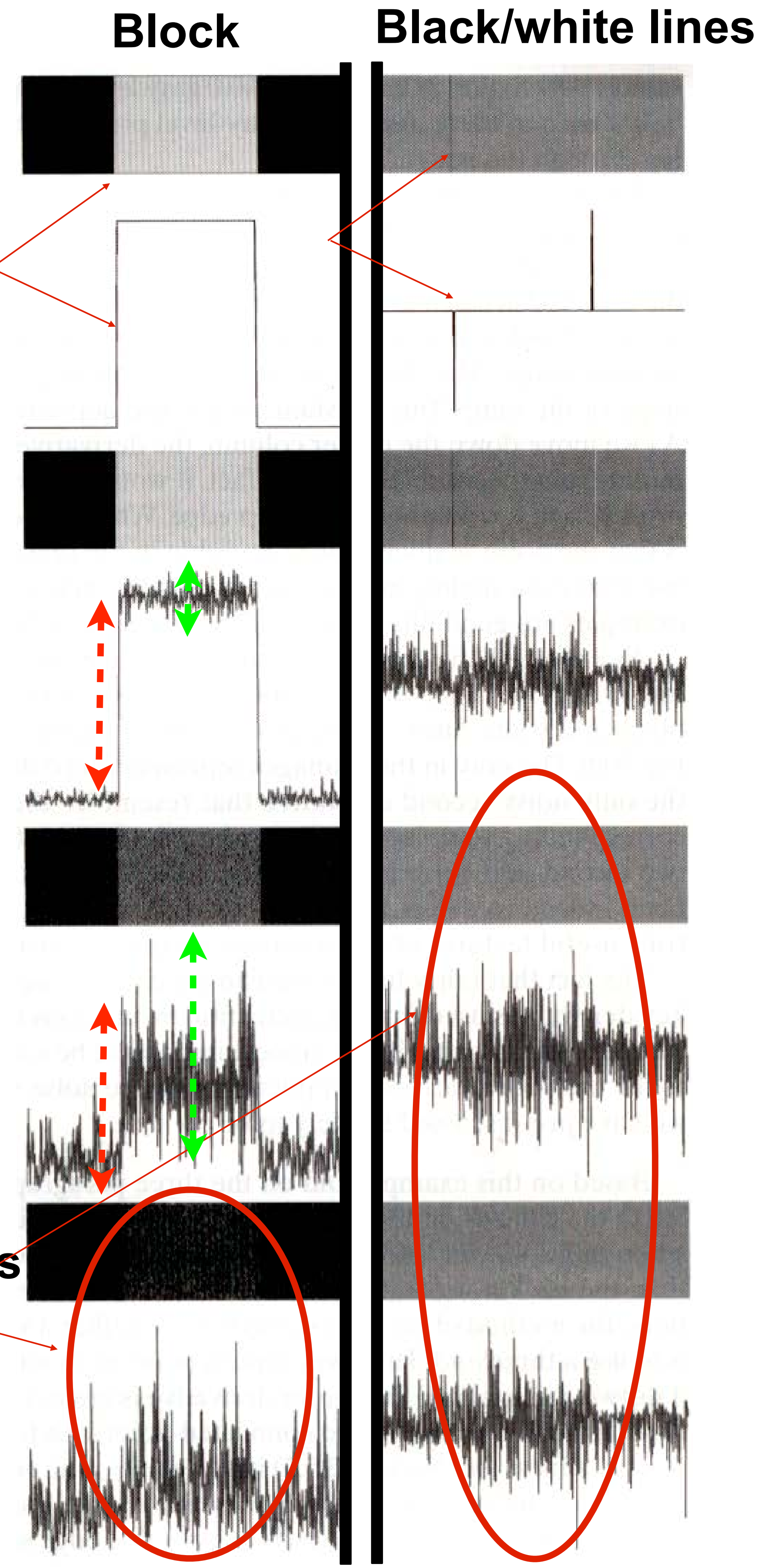
Boundaries and lines easily resolved in the absence of noise

Increasing levels of Gaussian noise

Decreasing S/N

The difference between signal and background must be at least 3X the noise to be detectable

Boundaries and lines no longer resolved

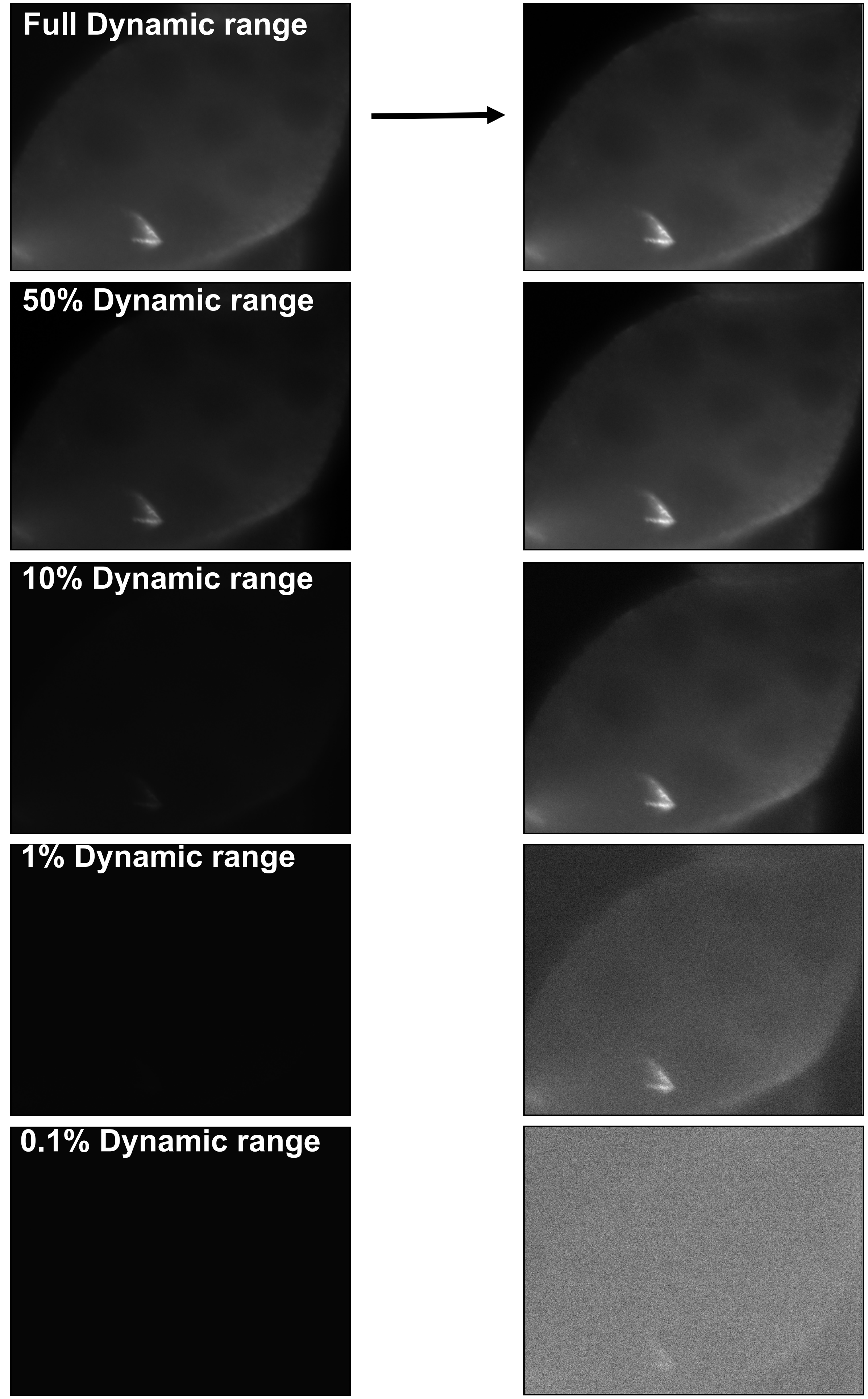


Gonzales & Woods, 2002. Digital Image Processing 2nd Ed. Prentice-Hall Inc, USA.

Resolution
Contrast
noise

Image series collected by decreasing the excitation lamp intensity from 100% to 50%, 10%, 1%, and 0.1%

Scaled 0 - 4095 | **Auto Intensity Scaled display**



Decreasing S/N

Increasingly Noisy looking

Decreasing Image quality

Cannot resolve

The basics of image processing

"I want you to make me the fairest of them all."

Remember what makes a good image

- Good image data has a high S:N ratio (count more photons)
- Correctly sampled to reproduce the optical resolution (pixel = optical resolution/2)
- Avoid aberrations (sample prep / choice of objective / technique)
  - spherical aberration (SA)
  - motion blur
  - bad system alignment
- Correctly annotated (Metadata retained)

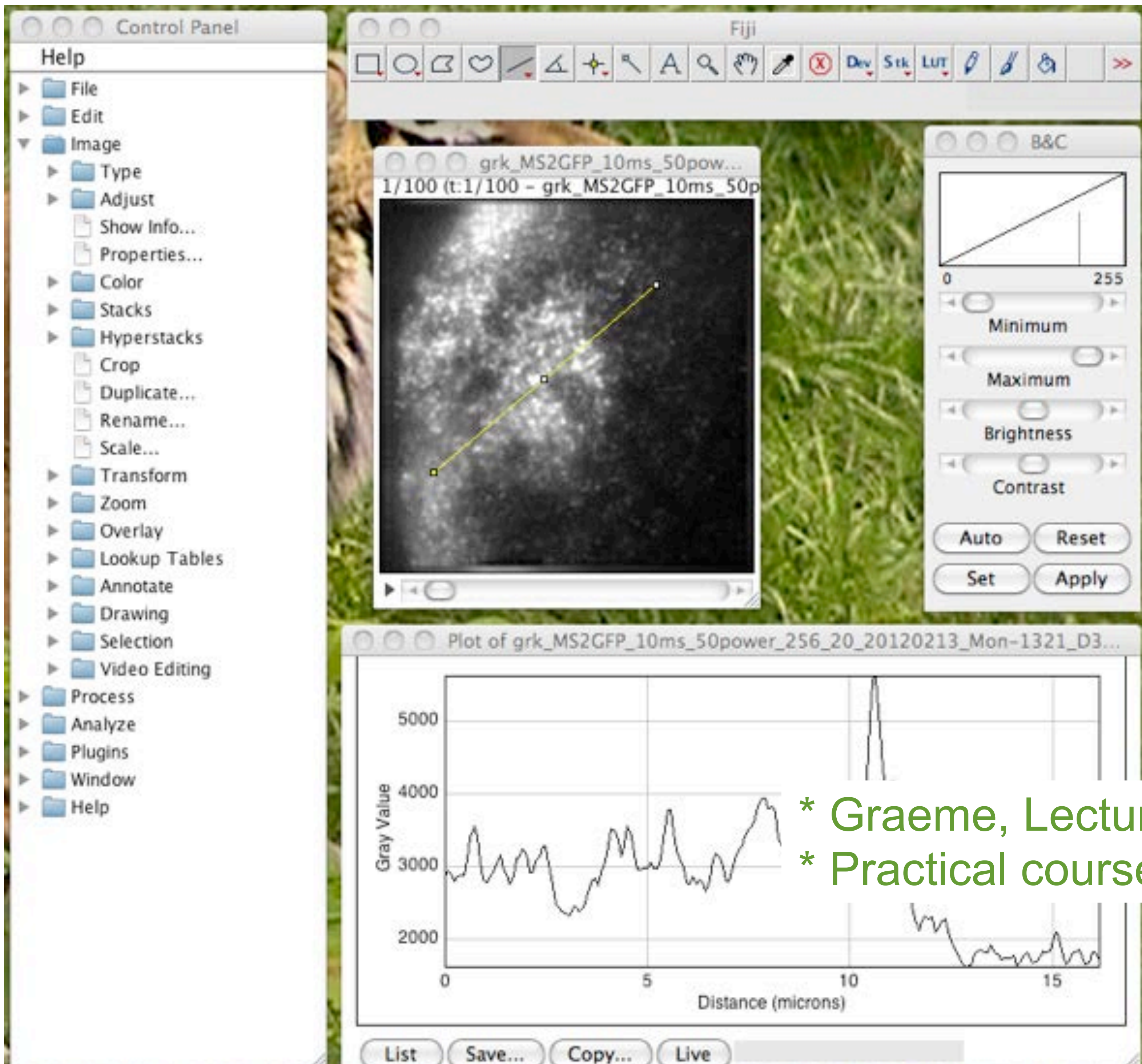
Image Processing is NOT a substitute for a good image

Golden Rules of Image Processing

- Always retain the original data
- Do not corrupt the integrity of the original data through processing:
  Processing should NOT generate data not present in the original image
- Images are arrays of numerical data and should be given appropriate consideration.
- Always record and report all processing steps.

Fiji = Image J http://fiji.sc/wiki/index.php/Fiji (http://rsb.info.nih.gov/ij/)

- Fiji is **FREE** and works on MAC, PC and linux
- Consists of a core program and plugins
- Uses Loci Bioformats to convert between file types



Fiji Is Just ImageJ

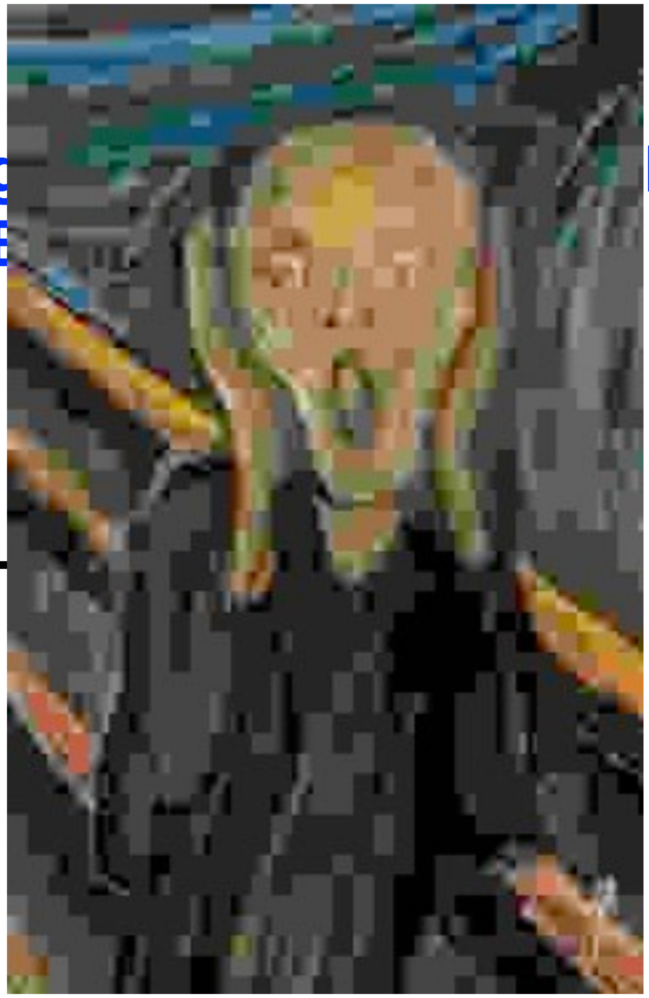
For users, Fiji is easy to install and has an automatic update function, bundles a lot of plugins and offers comprehensive documentation.

* Graeme, Lecture 19: Applied Image Analysis *

* Practical course - Demo of Fiji plugins and macros *

Do not corrupt the integrity of the original data

- Retain your original data in its original file format and original metadata associations
- Consider OME file format for data archiving (lecture 18 - OME, bioformats)
  http://loci.wisc.edu/software/bio-formats
- Ideally use Uncompressed TIF (tagged image file format) for processed data
- AVOID compressed file formats when processing: JPEG, PSD, PDF, compressed TIF…This will cause data corruption and loss
- Most data is collected as single channel grey scale imag h
  Avoid saving primary image data in colour formats (RGB
- Avoid repeated inter-conversions of file formats
- Prepare figures for publication in Adobe Photoshop in T
  and annotate in Adobe Illustrator



Conceptual Hierarchy of image processing

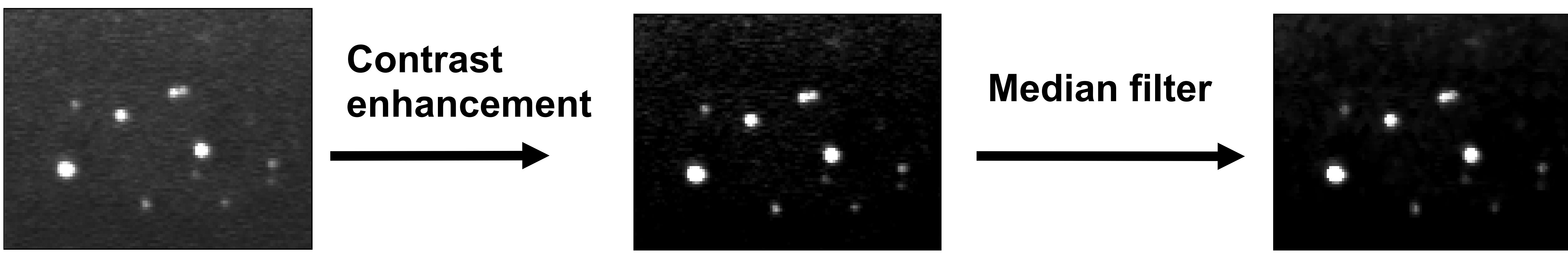
- **Low - level processing = Image enhancement (most common)**
- **Mid - level processing = Features and attributes extracted**
- **High - level processing = Analysis = Interpretation of images**

Gonzales & Woods (2002). Digital Image Processing 2nd Ed. Prentice-Hall Inc, USA.

LOW LEVEL Processing

- Visual enhancement
- Subjective = looks better
- **Input is an image, output is an image**

- Enhancement Filters
- Adjustments for image Display / Making figures



LOW LEVEL Processing - Image Restoration

- "Restoration" = attempting to reverse distortions of the object which arise during image capture

* Flat-field correction - correcting uneven illumination

* Deblurring = Deconvolution, with or without PSF, unsharp mask

* Simple filtering - post acquisition increase in S/N e.g. by averaging

* Graeme, Lecture 19: Applied Image Analysis *

* Normalization - intensity of each time-point scaled to correct bleaching (or flicker)

* Denoising - post acquisition increase in S/N without loss of resolution

* Image registration - alignment of multiple channels for co-localisation analysis

LOW LEVEL Processing - “Background” Correction

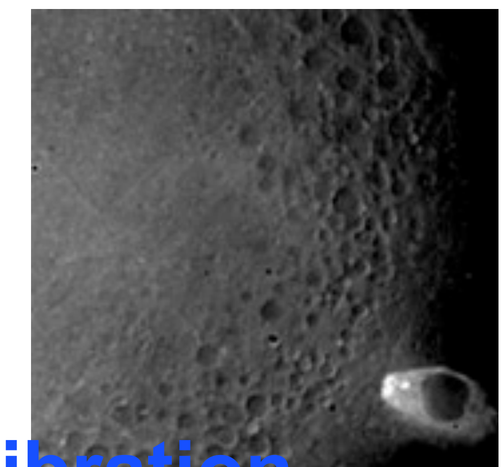
- **Requires dark image, flat field image and detector calibration**

- **Detector offset / background**
  subtract averaged dark value or image

- **Uneven illumination**
  correct using a flat field image

- **Uneven detector response**
  normalise using a detector calibration
  mapping the response of each pixel

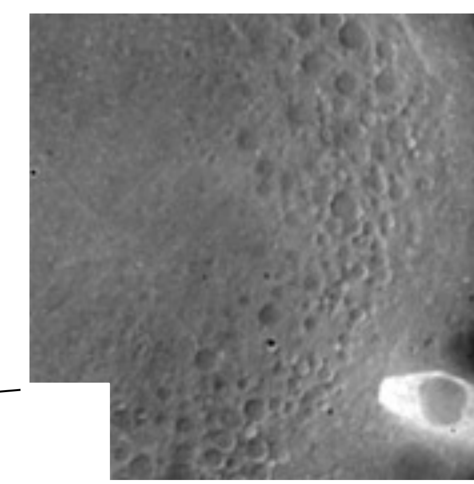
original image



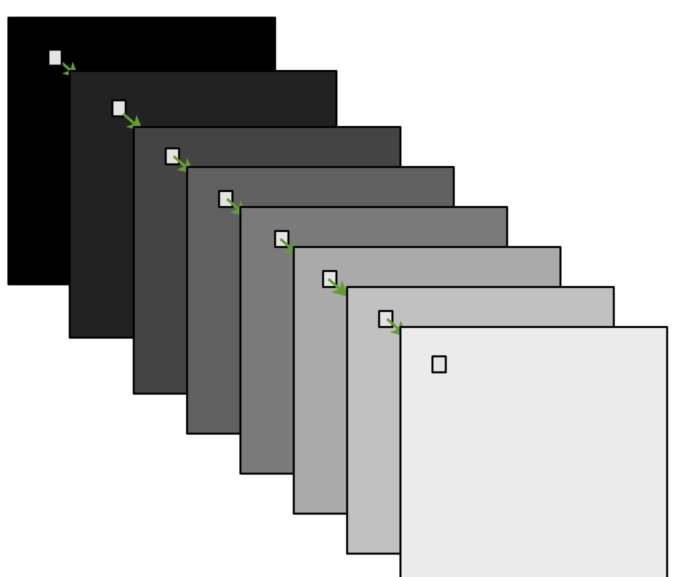
pseudo-flat field

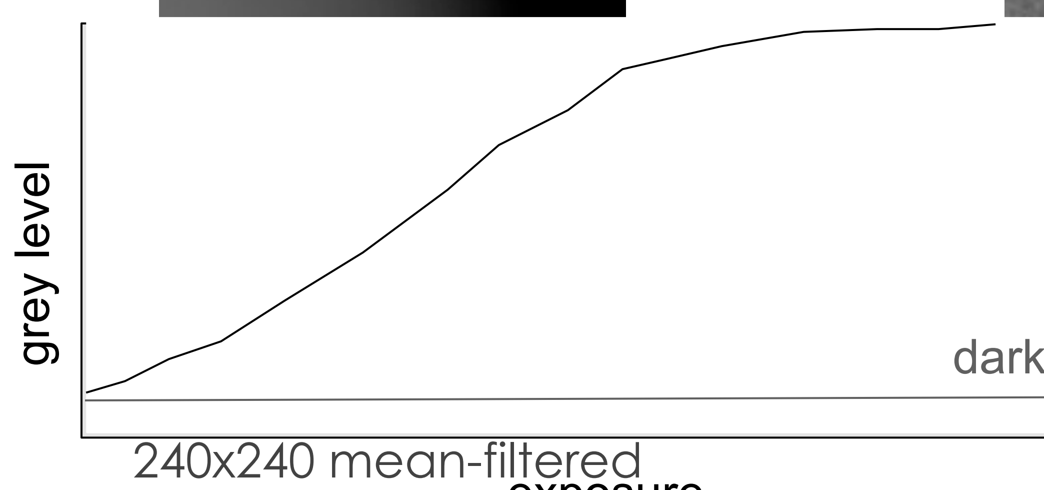
original/mean-filtered * mean value

240x240 mean-filtered



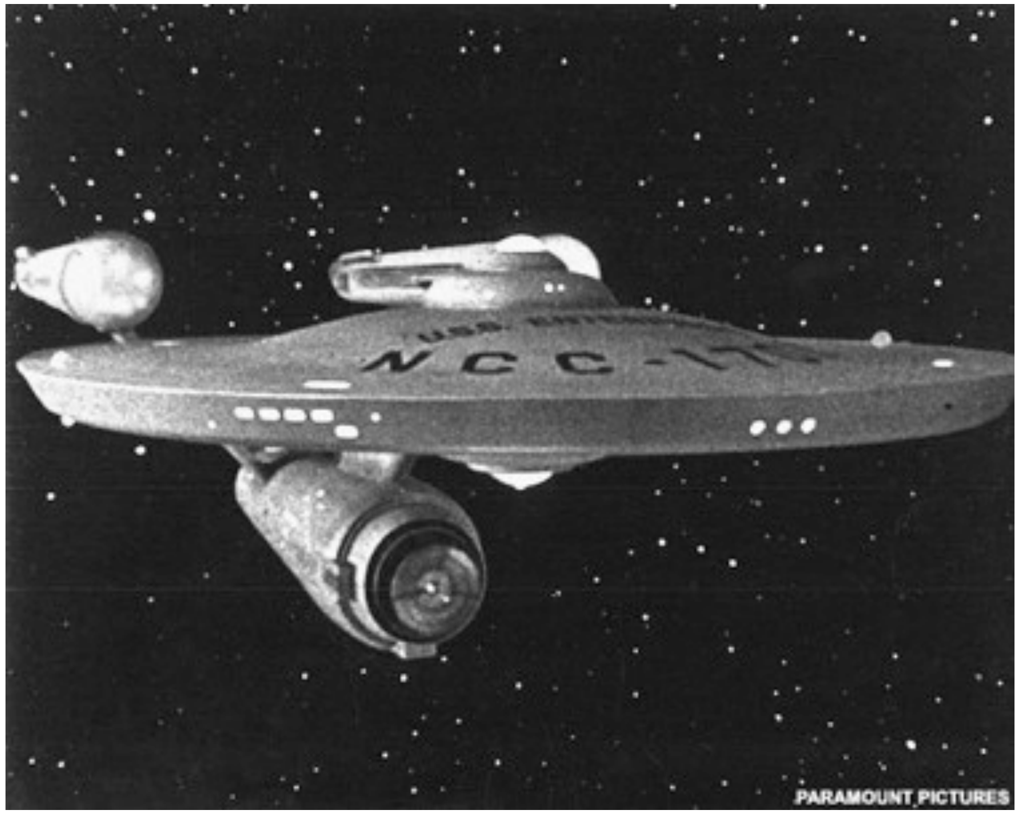
eudo-corrected



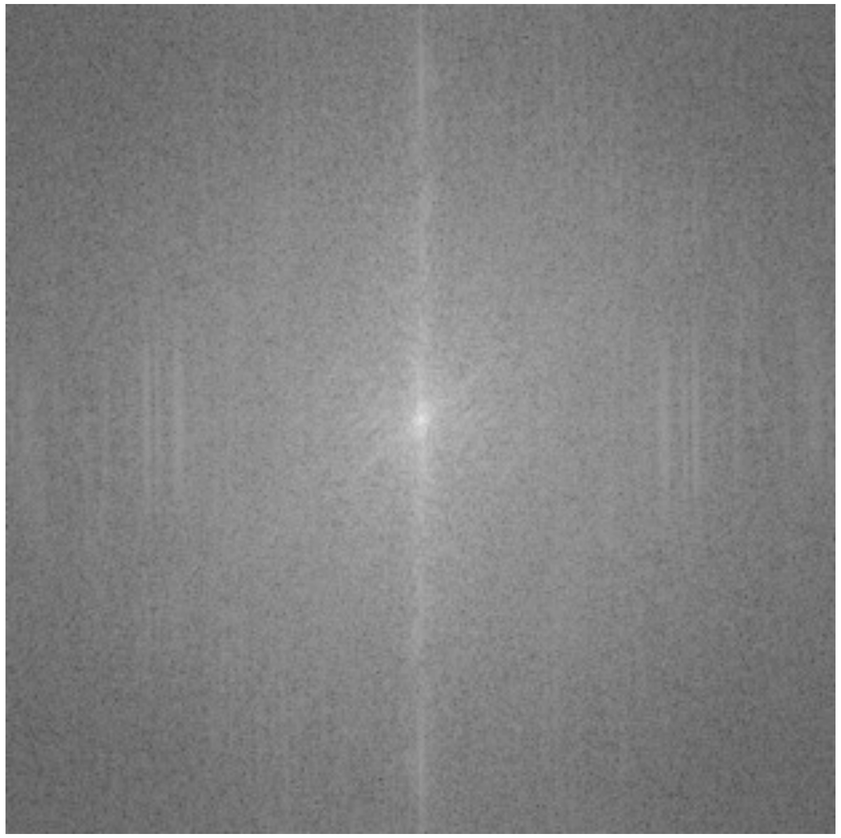


LOW LEVEL Processing - Simple filtering

Processing in
Real Space or Frequency Space



Fourier
Transform
→



Example:

- **Noise reduction filtering** using a spatial filtering mask – **3x3 median filter**
- **Noise reduction filtering** in the frequency domain – **Fourier bandpass filter**

LOW LEVEL Processing - Simple filter, spatial domain

- **Real space - pixel by pixel**

e.g. Noise reduction filtering using a 3x3 median filter

Kernel

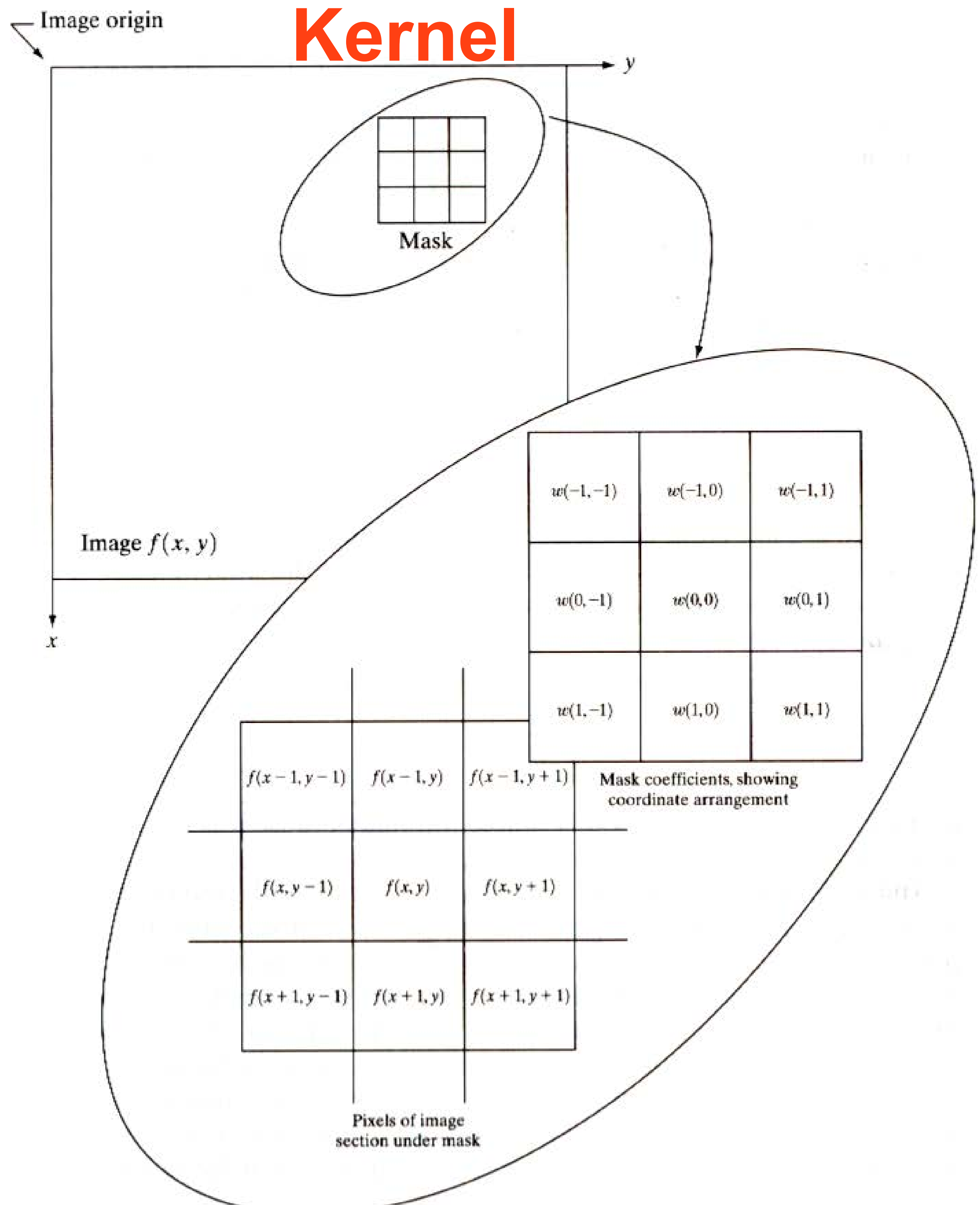


FIGURE 3.32 The mechanics of spatial filtering. The magnified drawing shows a 3×3 mask and the image section directly under it; the image section is shown displaced out from under the mask for ease of readability.

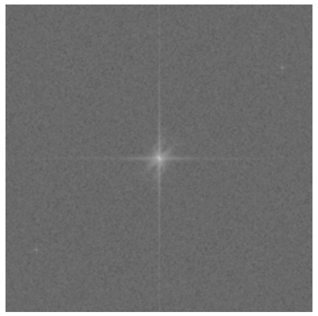
MEDIAN 3x3:
Replaces value of a pixel by the median grey scale value of the ranked values of the 9 neighbourhood pixels.

Gonzales & Woods, 2002. Digital Image Processing 2nd Ed. Prentice-Hall Inc, USA.

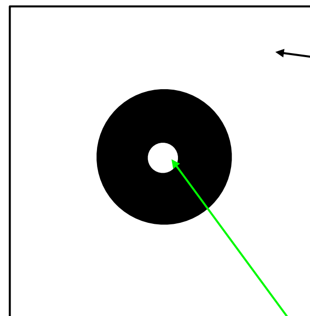
LOW LEVEL Processing - Simple filter, Fourier domain

- **Frequency domain - images converted to Fourier space**

  e.g. Noise reduction using a low pass or band pass filter



x



Fourier "mask" selectively eliminates the highest frequencies = noise and very fine detail.

Can also eliminate large expanses of even intensity - low frequencies

Works on the whole image at once

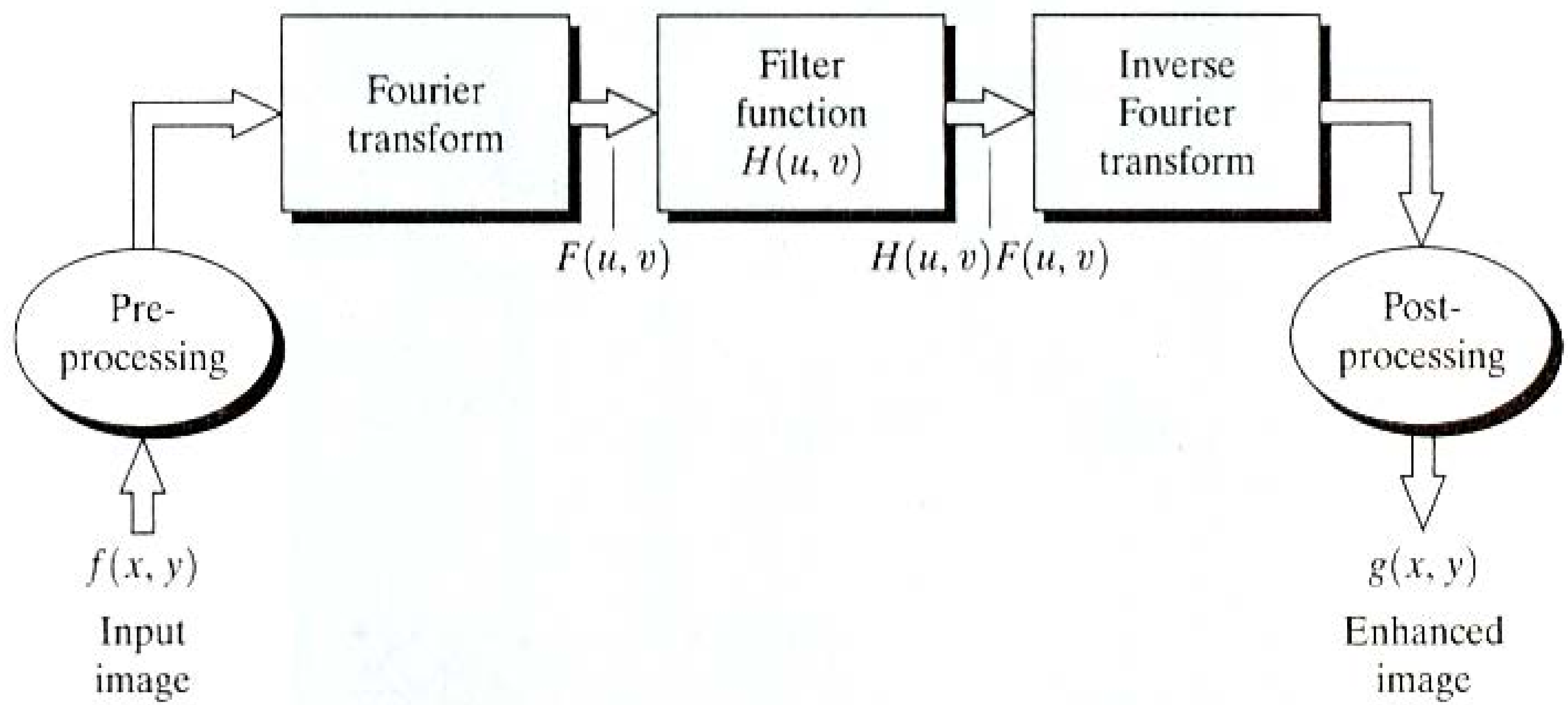


FIGURE 4.5 Basic steps for filtering in the frequency domain.

Gonzales & Woods, 2002. Digital Image Processing 2nd Ed. Prentice-Hall Inc, USA.

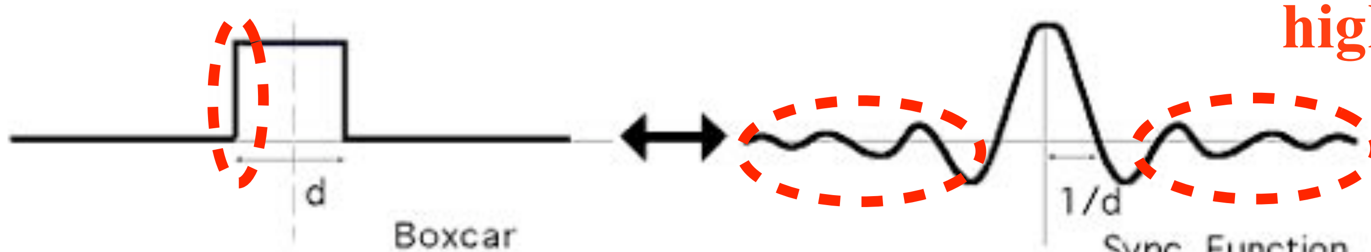
Fourier Space = Frequency space
= Reciprocal space
= K space

Data is broken down into its "frequency" components

real space **reciprocal space**

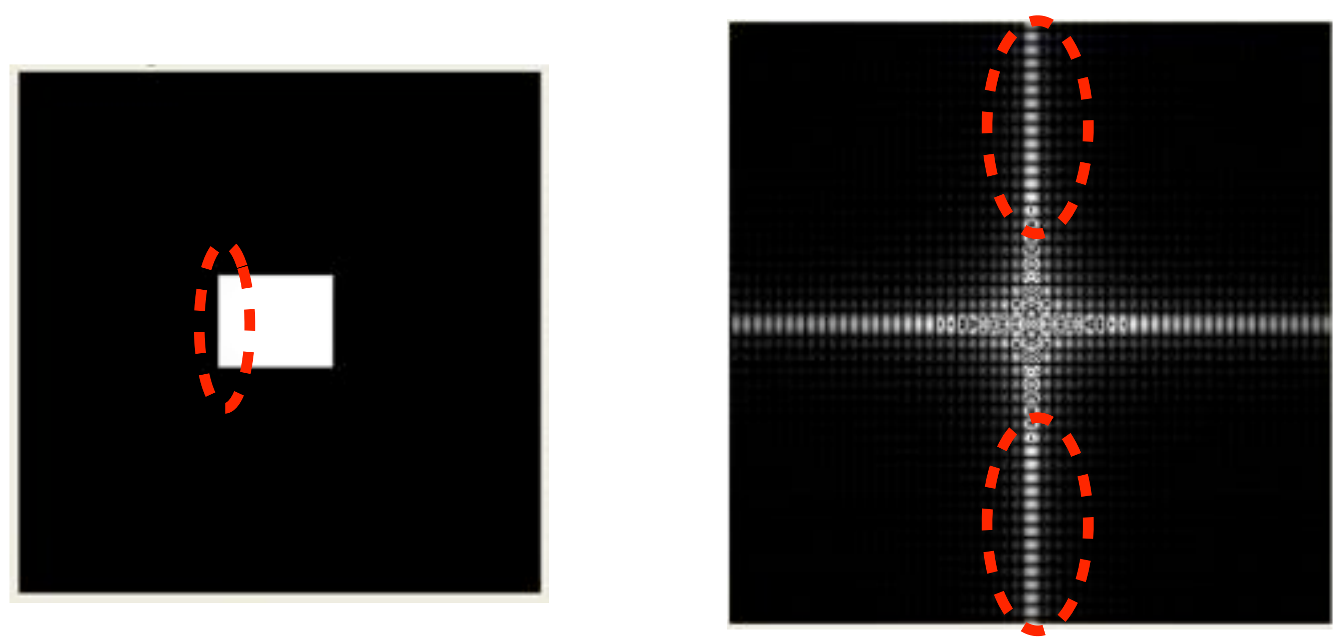
fine detail = high frequencies

1D case



fine detail = high frequencies

2D case



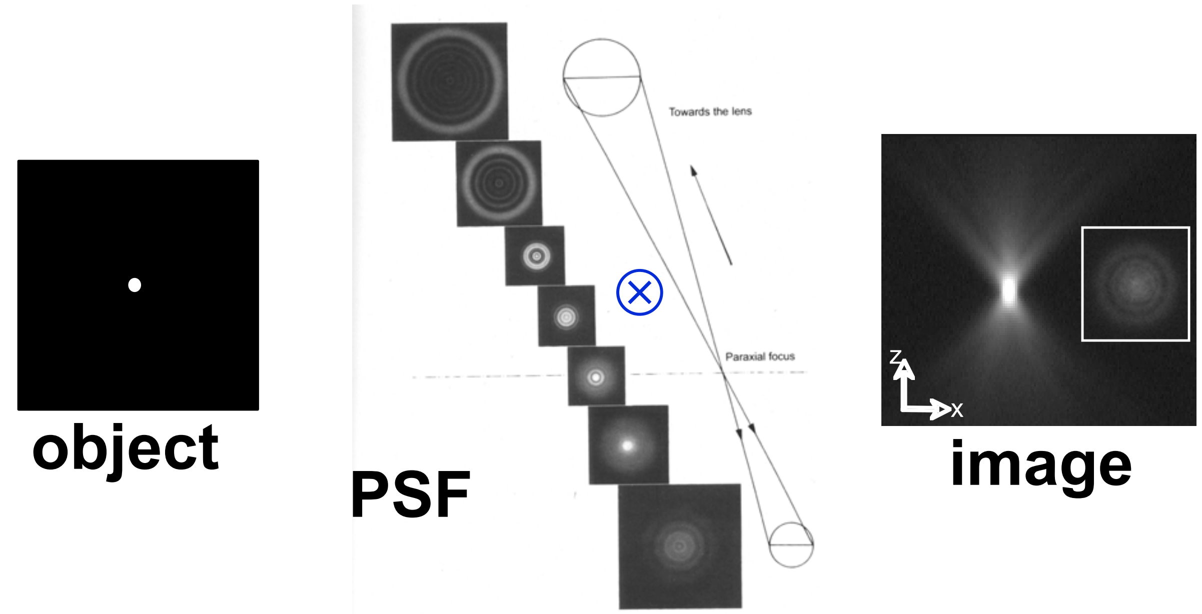
LOW LEVEL Processing - Deconvolution

- **Blur** = out of focus information

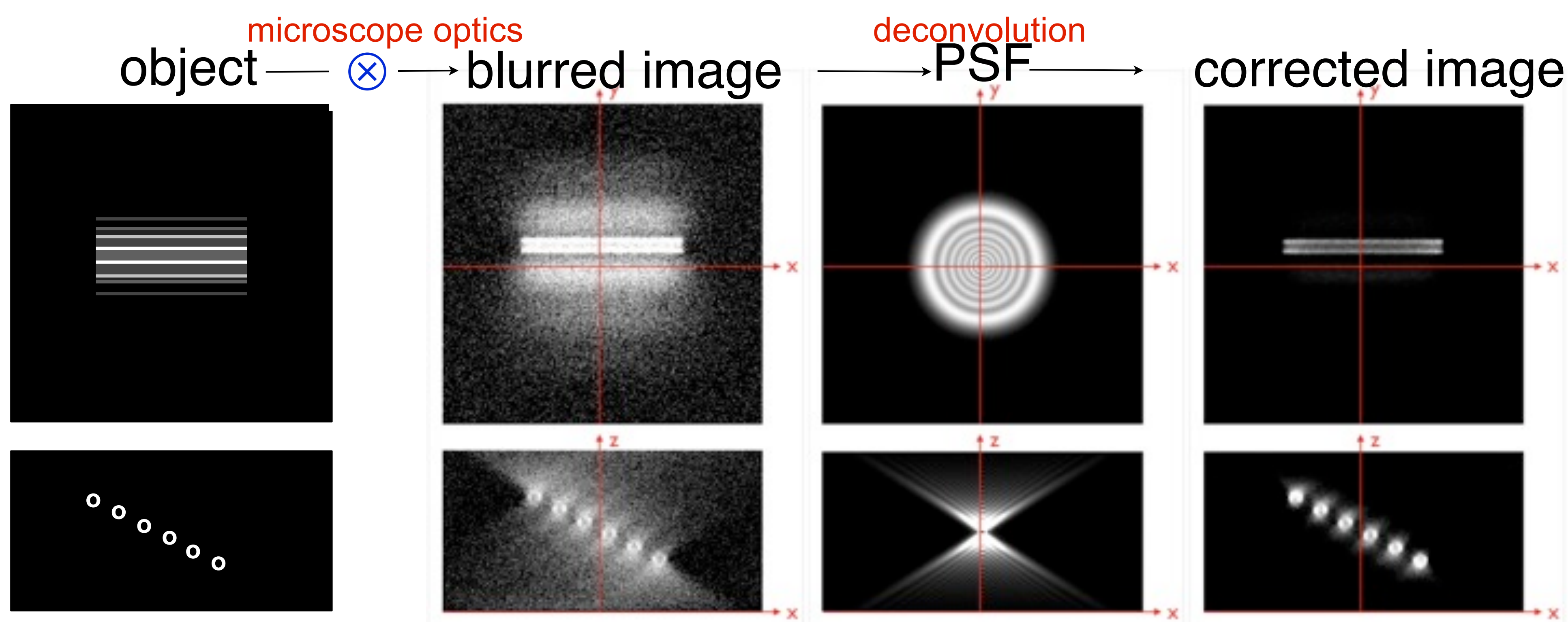
An image represents the output of the optics and detector of the imaging system

image ≠ object

image = object ⊗ PSF



- **Deblurring ≈ Deconvolution = out of focus information removed or reassigned**



reversal of the effects of the PSF

Different classes of deconvolution

- **Deblurring**: nearest neighbours / no neighbours / unsharp mask
  Not true deconvolution, subtractive (throws away light)
  Quick and easy

- **Image restoration**: (inverse filter) constrained iterative algorithms
  True deconvolution, light is re-assigned to its point of origin.
  Can use measured (empirical), theoretical or derived (blind) PSF

The PSF and OTF

Microscope Image	=	object	$\otimes$	PSF

But in Fourier space (or frequency space)

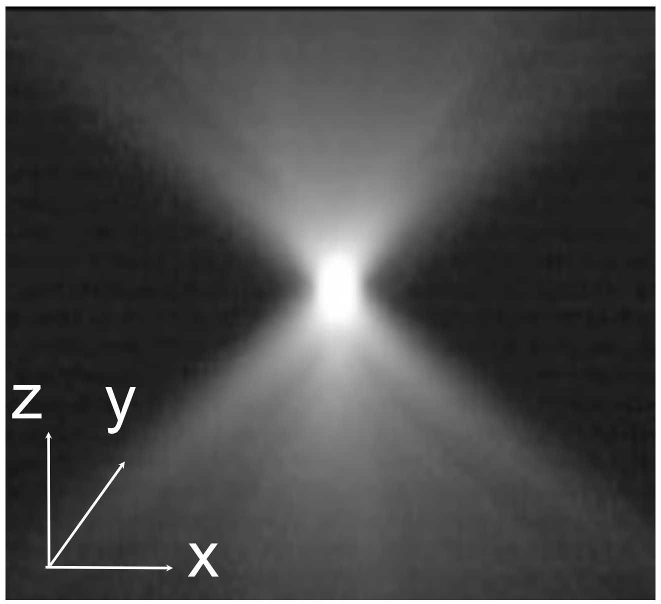
FT microscope Image	=	FT object ("true" image)	X	FT PSF

- **Calculations for deconvolution are done in Fourier space (simpler / faster)**
- **The PSF is Fourier transformed to the OTF (optical transfer function)**
- **The inverse calculation to obtain a "true" image = a linear inverse filter**

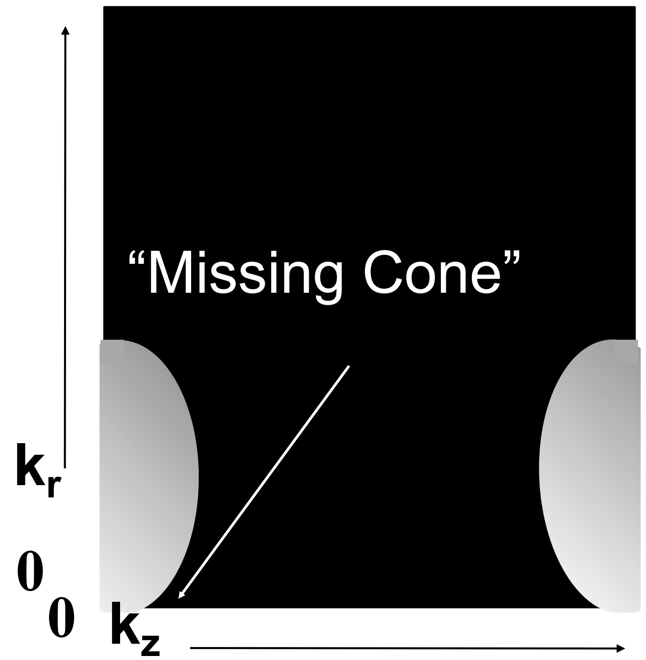
The PSF and OTF

Real space | **Frequency space**

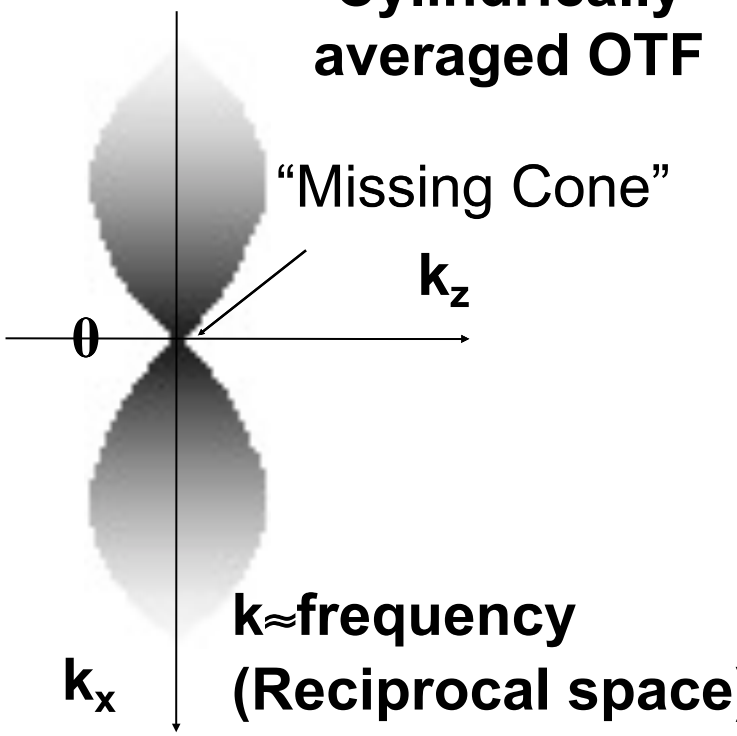
3D - PSF



OTF image (API, softWoRx)



Cylindrically averaged OTF



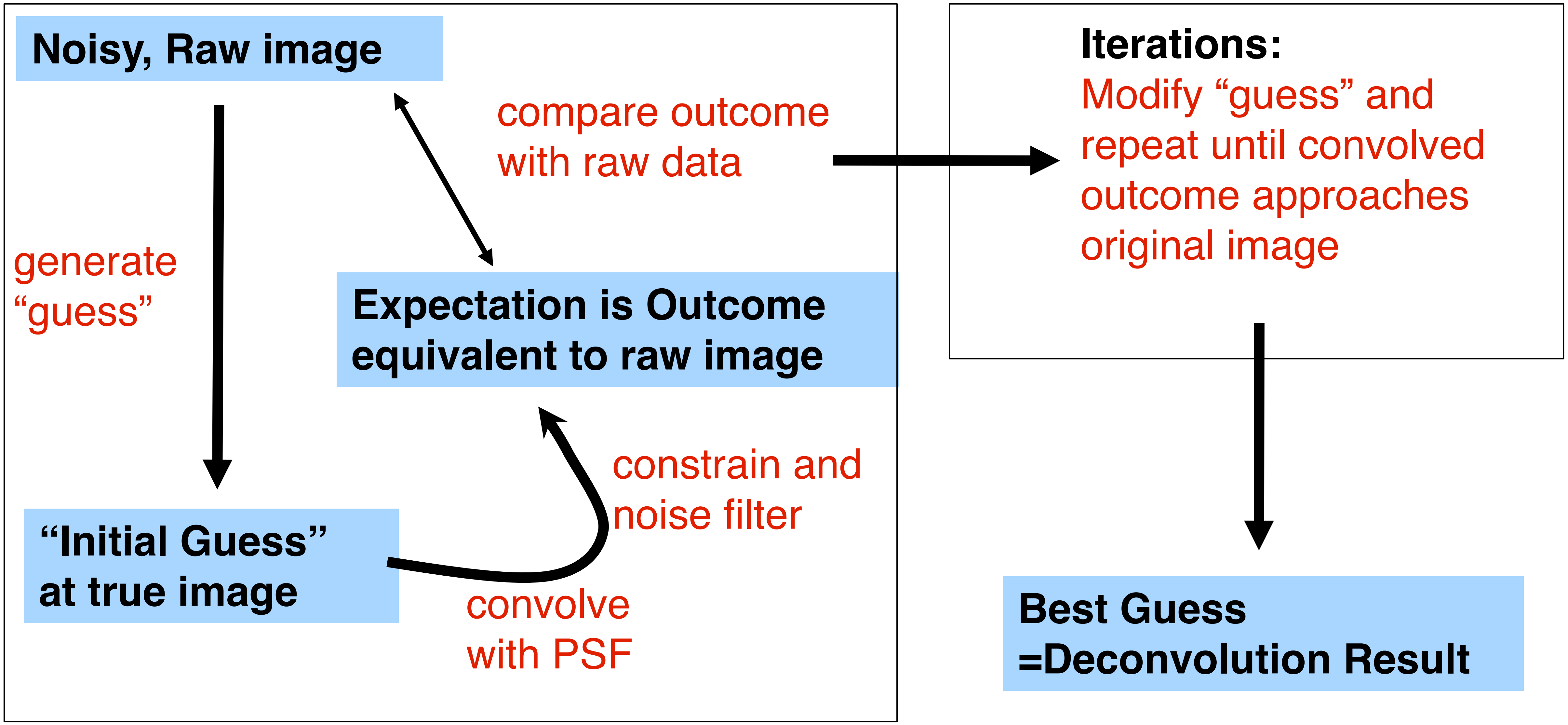
- To simplify deconvolution calculations the OTF is often simplified by assuming radial symmetry for the PSF
- The "missing cone" represents missing frequency information not collected by an objective with a limited NA (not all light can be gathered)

Deconvolution and Noise

~~Microscope Image~~ NOISE ~~ject $\otimes$ PSF~~

- **Much of the complexity of deconvolution is a direct consequence of having to deal with "noise" inherent in image data**

Constrained iterative deconvolution



Data requirements for deconvolution

- Ensure that the imaging system is correctly set up, aligned and calibrated (pixel sizes).

- Reduce aberrations if possible:

  Sample preparation and correction of optics

  Collect more light / average images

  Aim for a high S/N in image data.

- Make sure that images are collected according to the Nyquist sampling criteria (pixel size in XY and Z step).

- Collect sufficient image planes in Z.
  (2D data can be deconvolved but lacks Z information so restoration is limited.)

- Minimise lamp flicker between Z sections.
  (corrected for on the DV system)

- Avoid motion blur from live specimens.
  (short exposure times)

Has deconvolution worked?

- Should look sharper and more contrasted and not excessively noisy
- Should NOT “invent” features not visible in the original data
- Be wary of very small punctate features
- Be wary of “ringing” artefacts - dark circles round structures

LOW LEVEL Processing - Making Figures

LOW LEVEL Processing - Figure Making Guidelines

- Carry out all processing and analysis of images before making figures by using pixel based (raster) programs:
- Handling of images (tif files) for figures should use pixel based (raster) programs

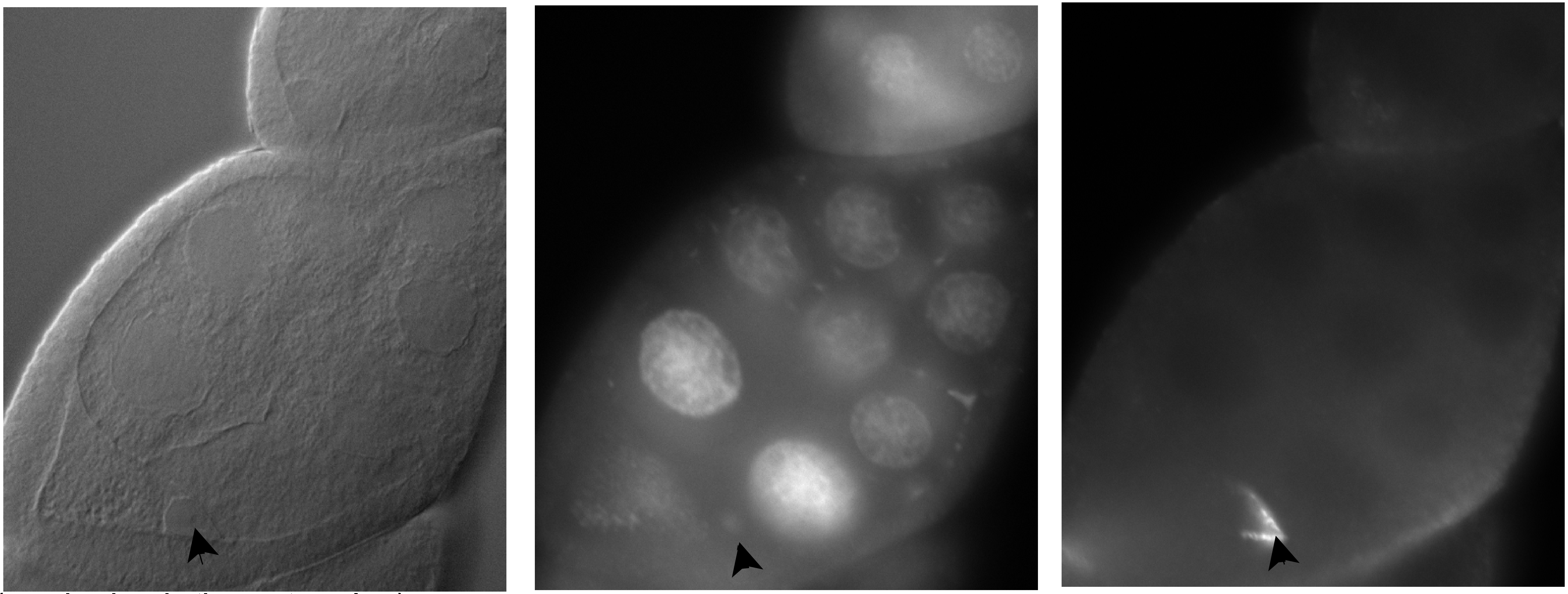
  Arranging multi-panel figures
  Cropping, resizing, rotating

  Before using vector graphics based programs

  Adding lettering, arrows, charts diagrams....
- Do not prepare figures in powerpoint or keynote or use screen capture
- Understand what happens when you resize an image
- Be consistent with processing steps, especially contrasting

LOW LEVEL Processing - Display, Grey Scale

DIC/DAPI/Grk in situ - grey scale images

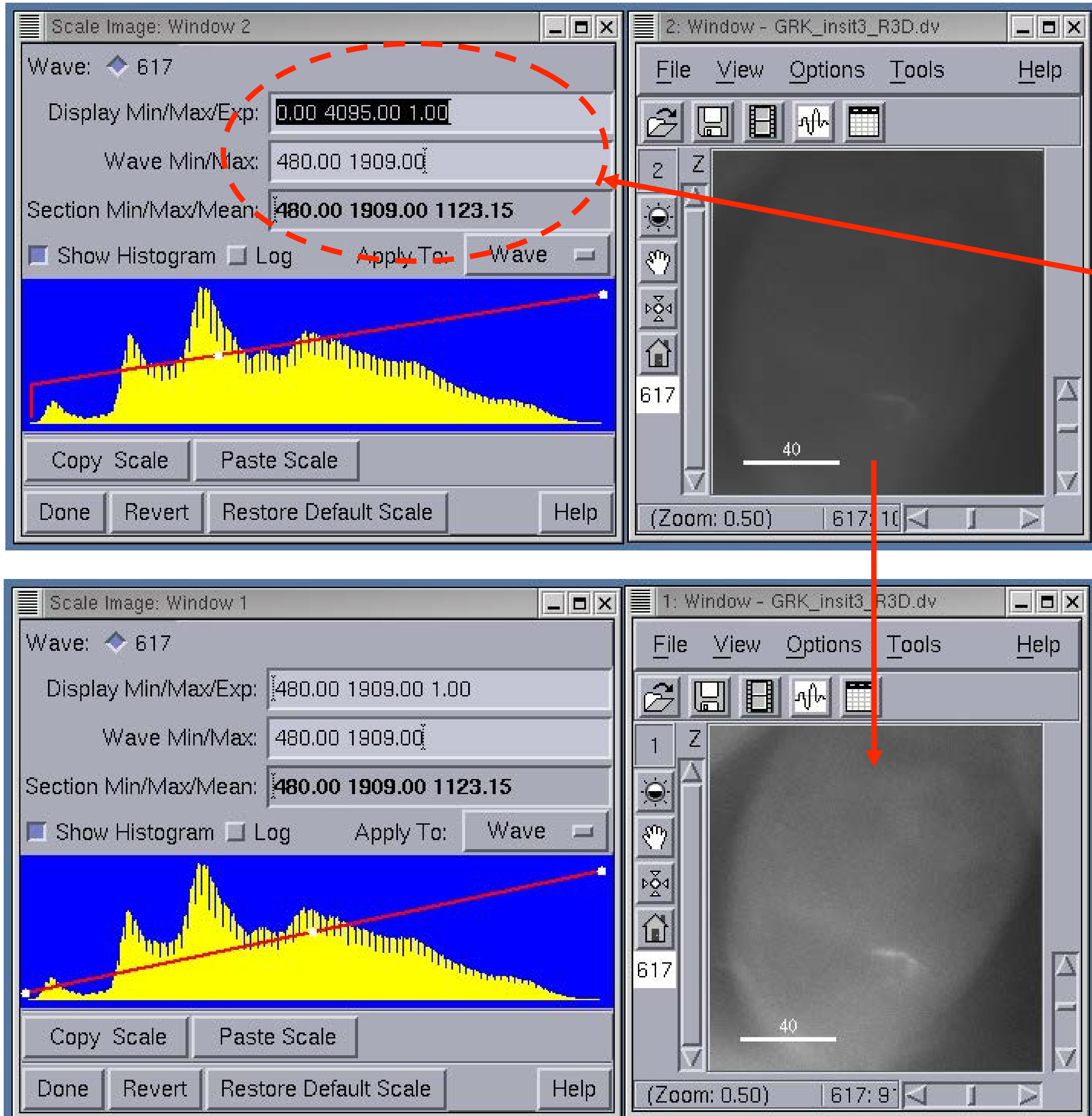


(arrowhead marks the oocyte nucleus)

- For viewing and display use grey scale scale images to see fine detail.

LOW LEVEL Processing - Display, Brightness/Contrast

- **Brightness and contrast - Enhancing details which are too close in grey level to be easily discernible.**



Better to set your contrast by numbers to make the operation more systematic
0-4095 is full range for 12 bit

Auto-intensity scaling by softWoRx scales the display to fill the dynamic range

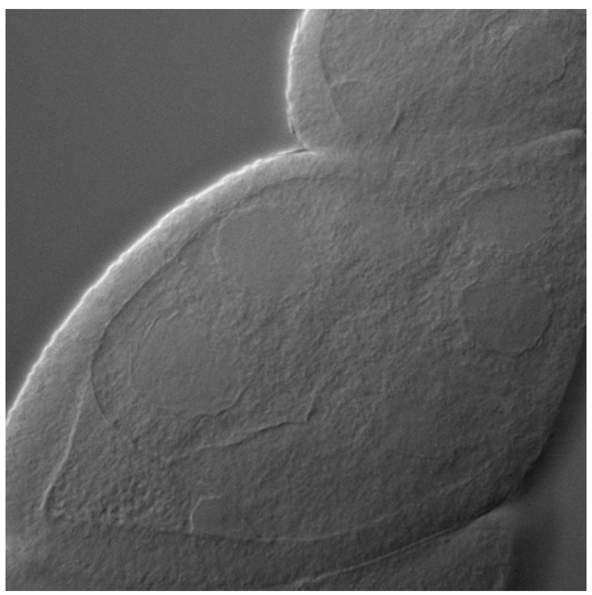
softwoRx; API

LOW LEVEL Processing - Display, Bit Depth (levels)

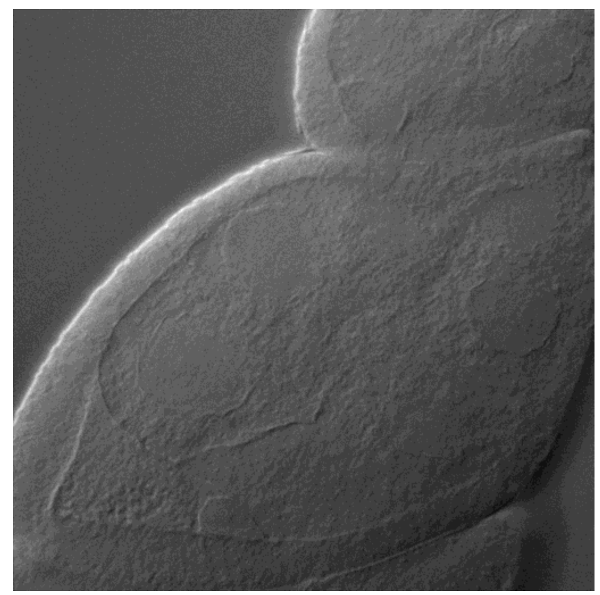
- **“grey levels” = the number of discrete values in an image**
- **Dynamic range = the number of possible grey levels**
- **Eye has limited ability to distinguish grey levels/colours**
  Above 32 grey levels images look smooth - 16 and below grey levels eye perceives objectionable banding = false contours.

Imaging detectors:
8 bit = 2^8 = 256 grey levels
12 bit = 2^{12} = 4096 grey levels
16 bit = 2^{16} = 65536 grey levels

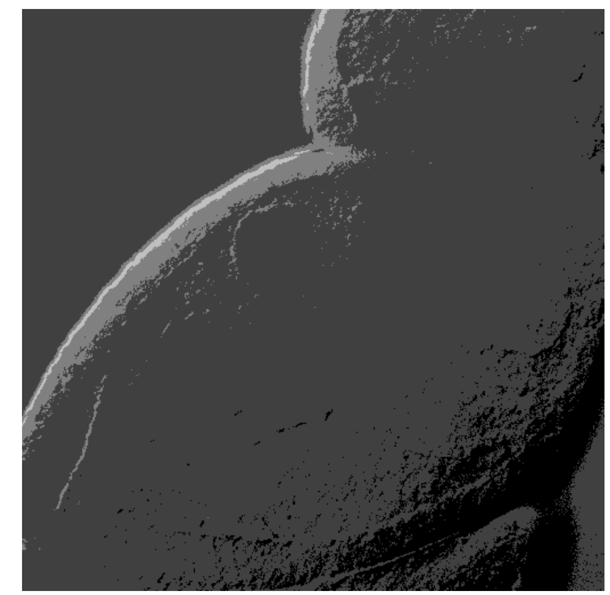
False contouring due to insufficient grey levels ⟶



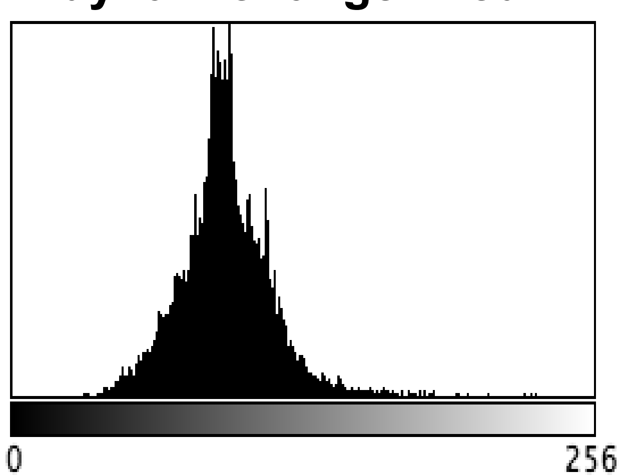
8 bit - 256 greys
dynamic range filled

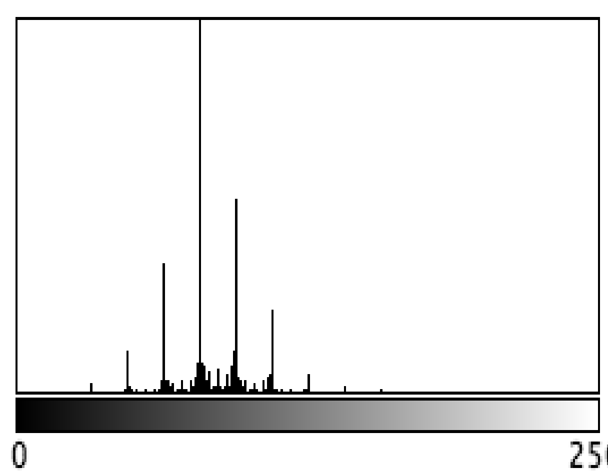


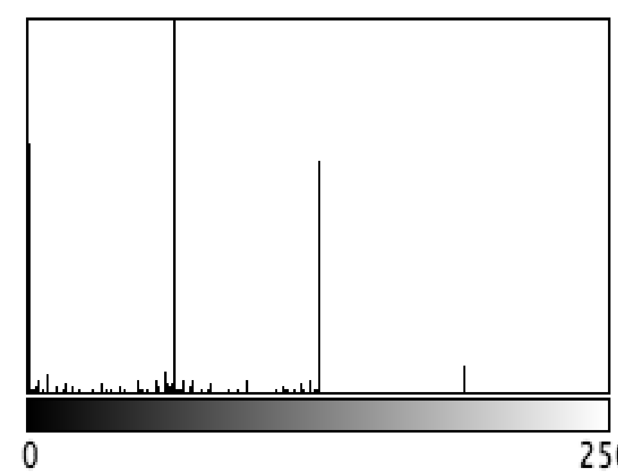
16 greys



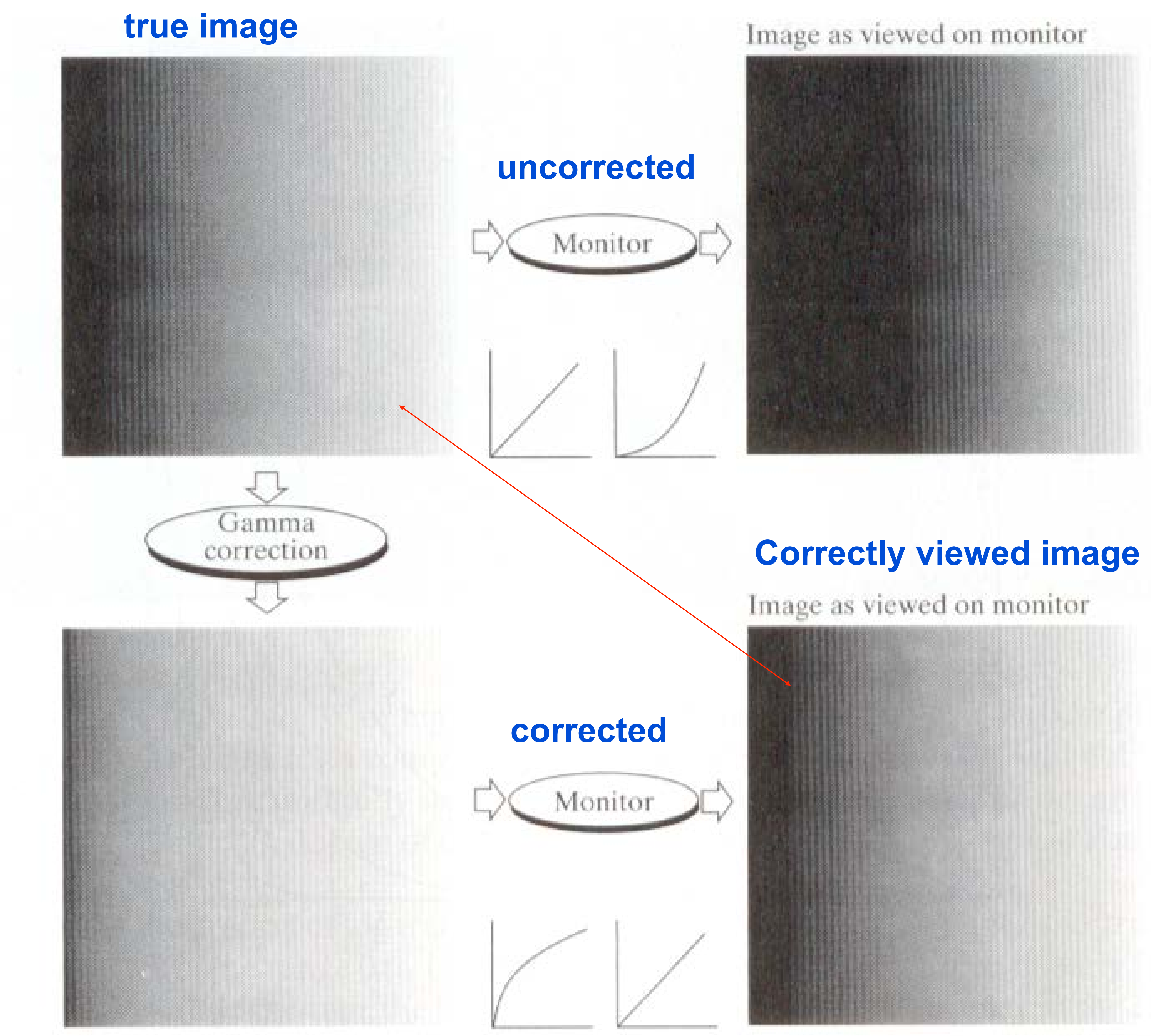
4 greys







LOW LEVEL Processing - Display, Using Gamma

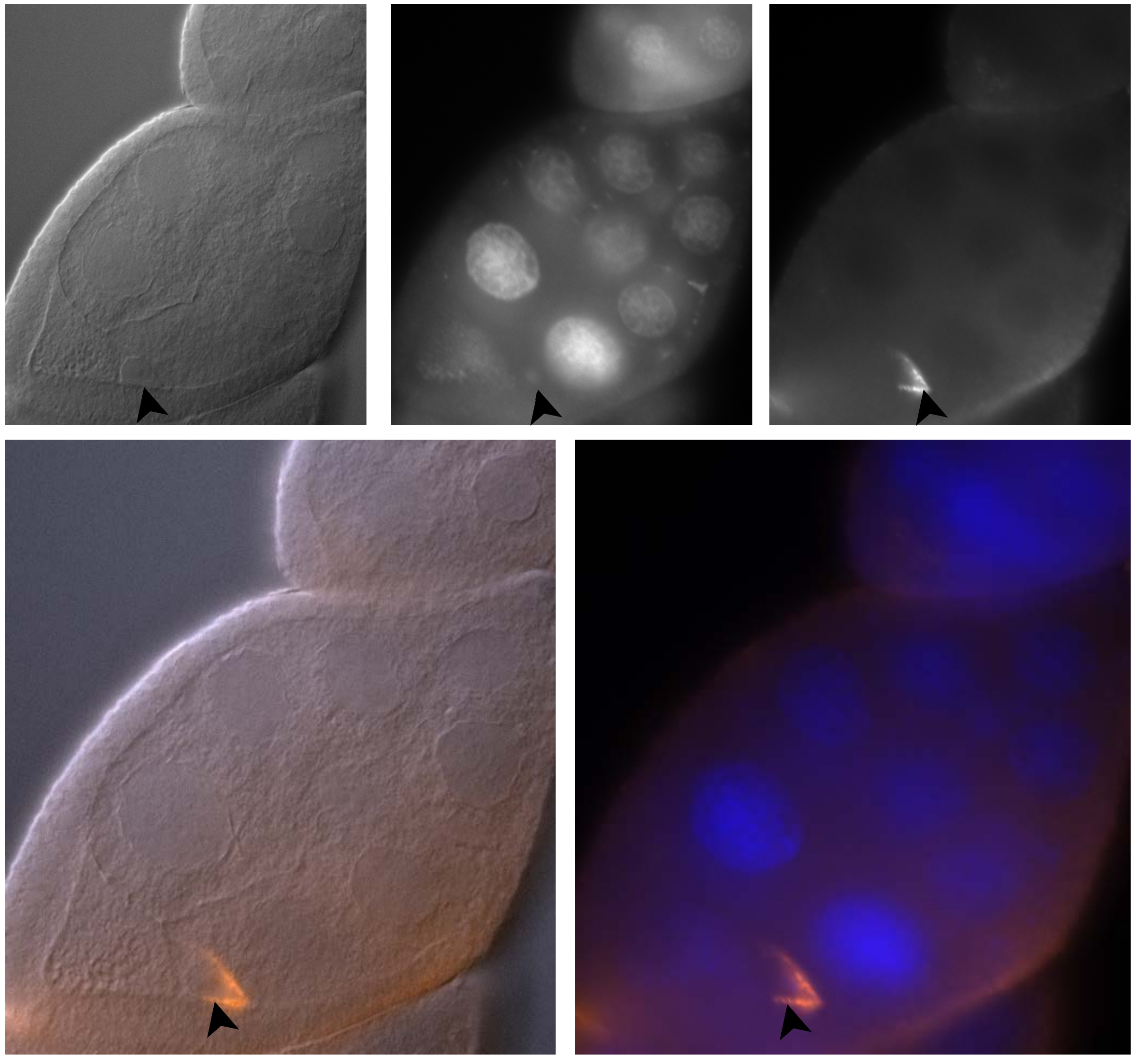


Gonzales & Woods, 2002. Digital Image Processing 2nd Ed. Prentice-Hall Inc, USA.

LOW LEVEL Processing - Display, Colour

DIC/DAPI/Grk in situ - grey scale images, colour blended, additive overlays

- Look-Up Tables (LUTs) map intensity to color

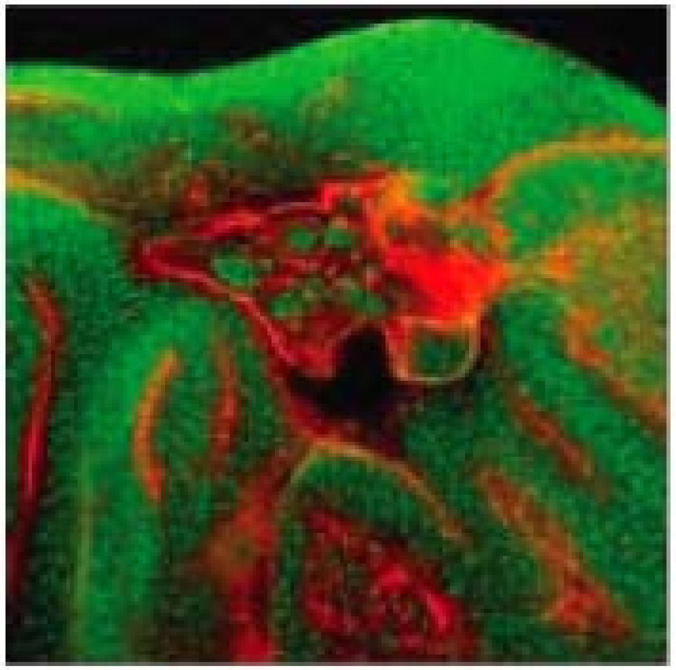


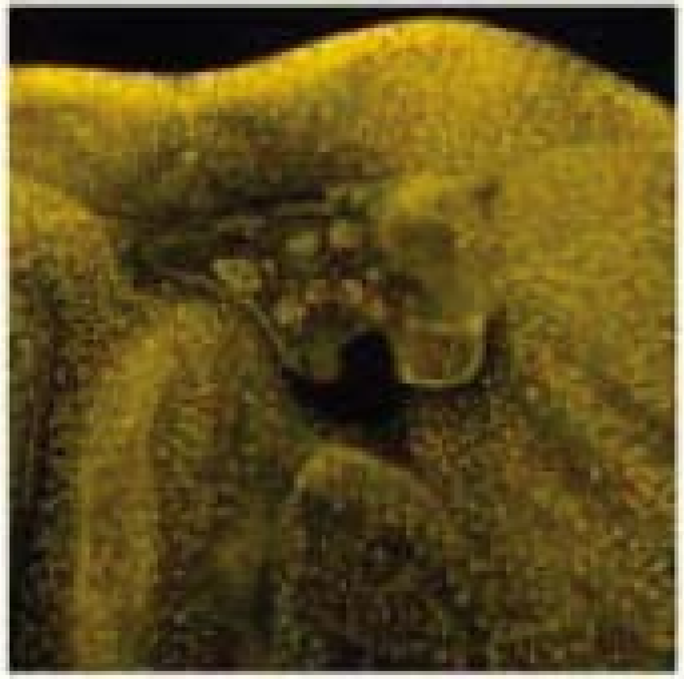
(arrowhead marks the oocyte nucleus)

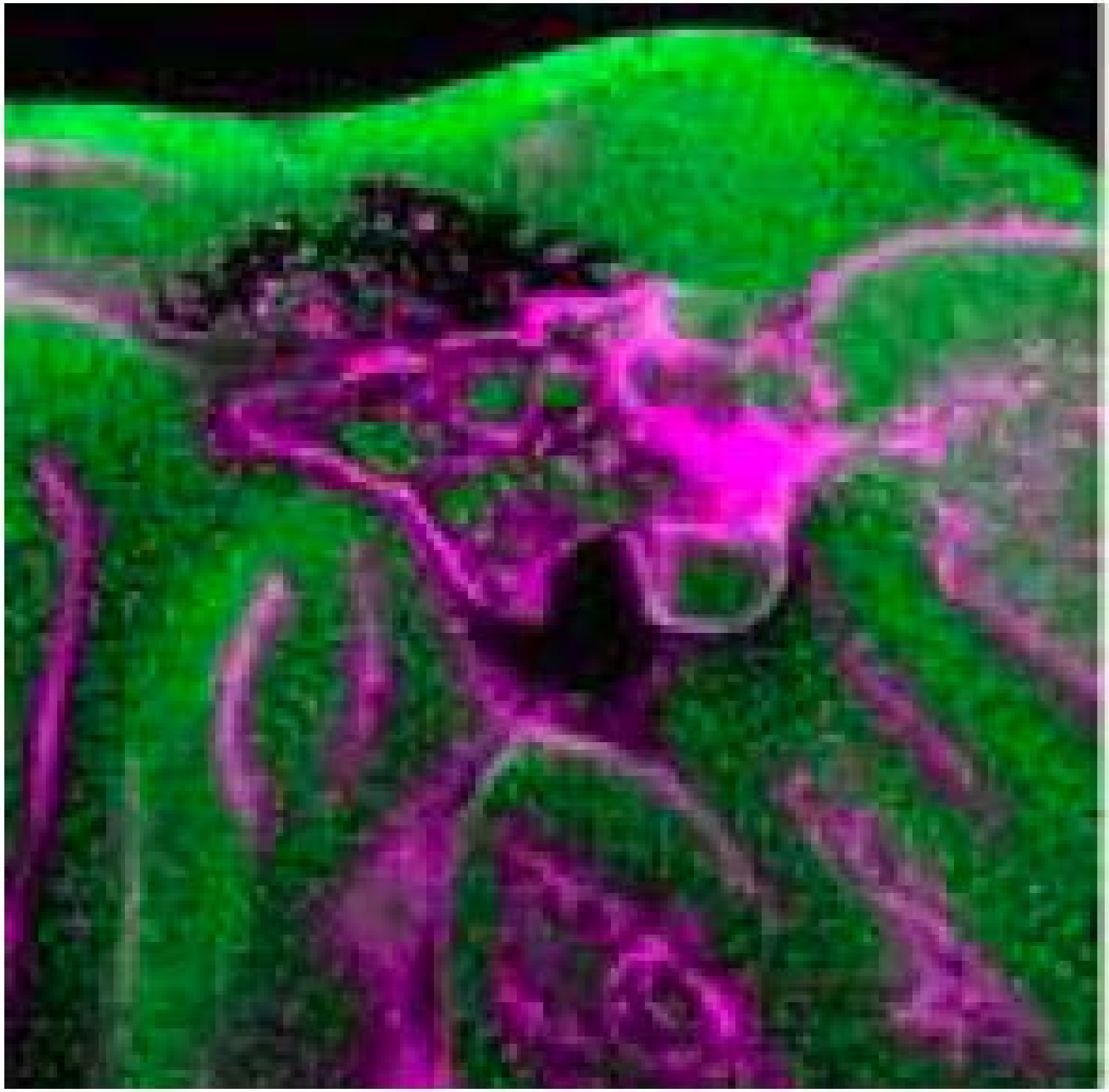
- **Colour should be used for highlighting particular intensity differences / co-localisation.**
- **For publication show greyscale images alongside colour overlays.**

LOW LEVEL Processing - Display, Colour

- Consider colour blind friendly colours: magenta, green







LOW LEVEL Processing - Display, Making Movies

- Movie formats: .avi; .mov; (.mpeg)
- Considerations:

  image quality vs movie size (use of compression).
  speed of play (frames / second).
  speed of play on the computer may be slowed by large movies

- ImageJ

  Will open many file formats and export straight to .avi or .mov.,
  also can open tif image series (image001.tif; image002.tif; etc)
  restack and export to .avi

- Quicktime Pro 7

  Can open tif image series and export to .avi or .mov
  Can interconvert movie formats.
  Has a range of compression options for .mov

Bad Imaging Practices

Do not corrupt the integrity of the original data

Examples of Bad Imaging Practices

Rossner & Yamada (2004). What's in a picture? The temptation of image manipulation. J. Cell Biology 166: 11–15.

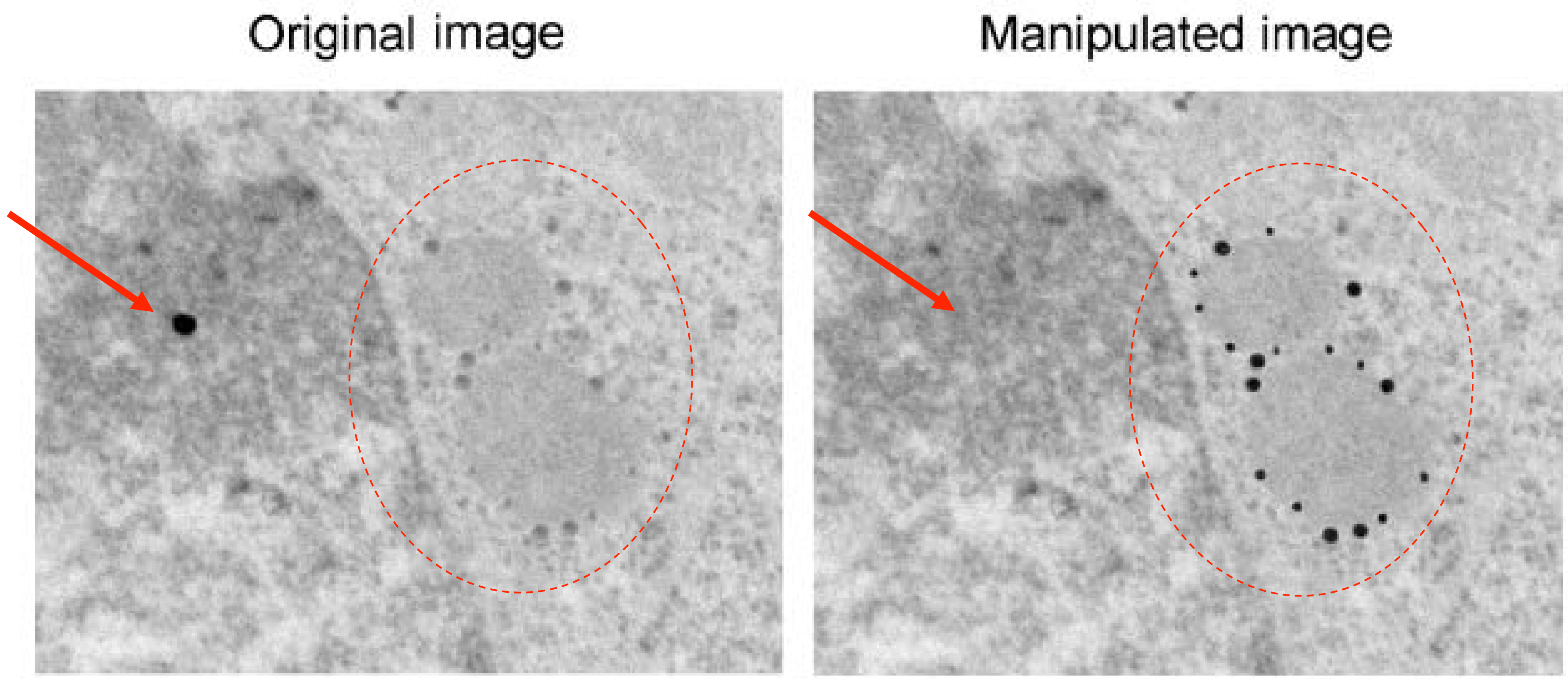
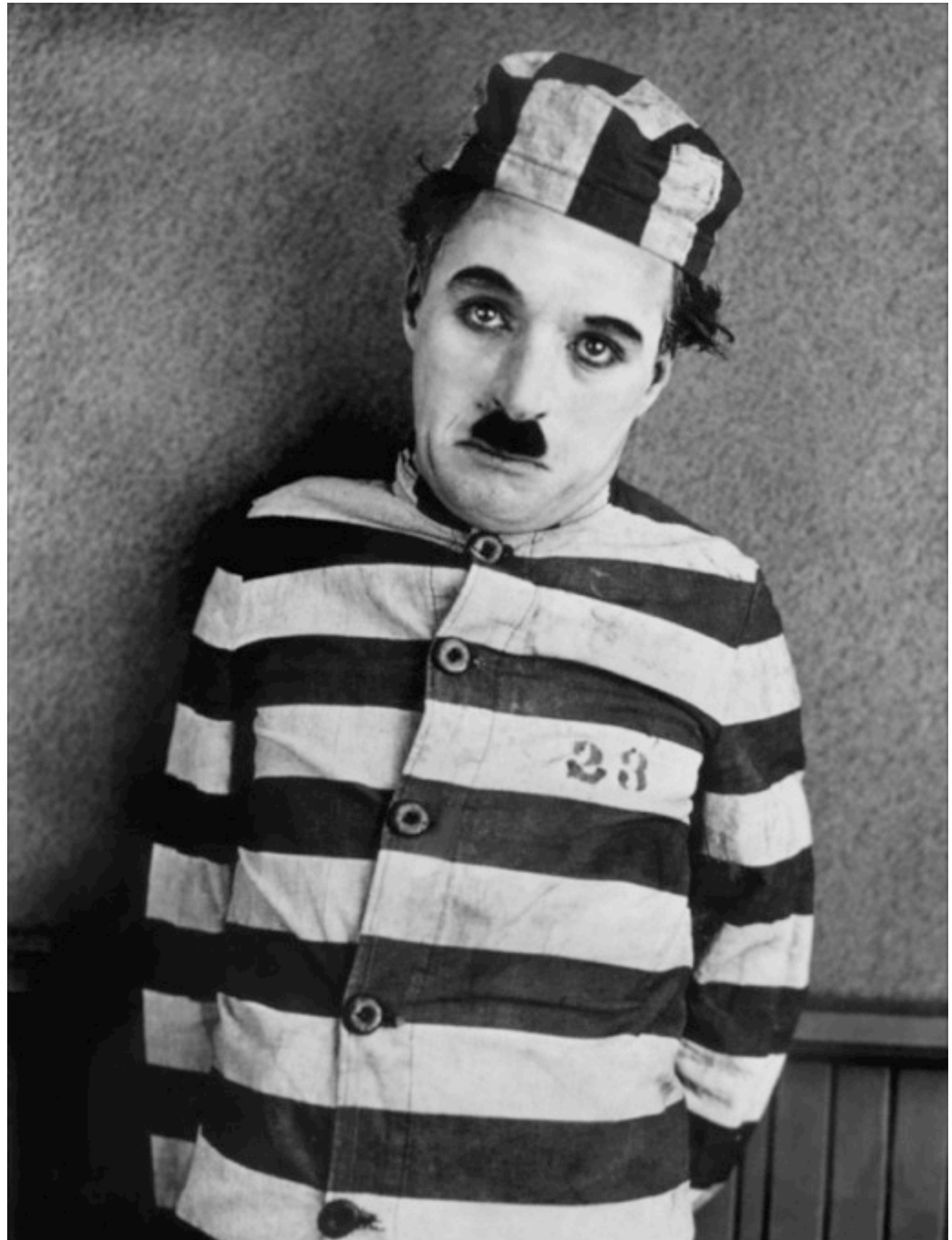


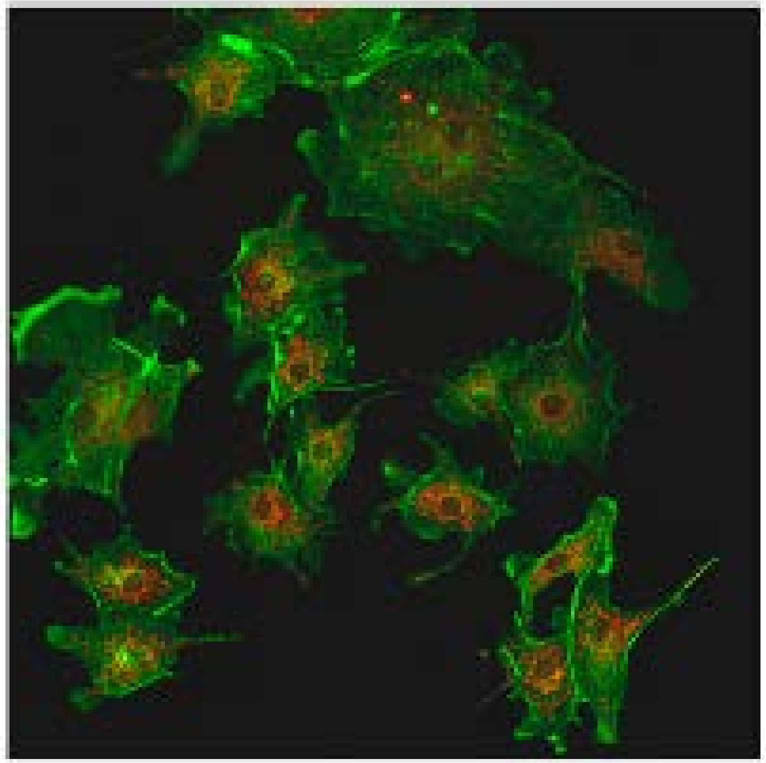
Figure 5. **Misrepresentation of immunogold data.** The gold particles, which were actually present in the original (left), have been enhanced in the manipulated image (right). Note also that the background dot in the original data has been removed in the manipulated image.

- **BAD: manipulated but does not alter interpretation**
- **VERY BAD: Changes interpretation with intention to defraud**
- **Adjustments necessary to reveal a feature ALREADY PRESENT in the original data are acceptable if they can be justified**

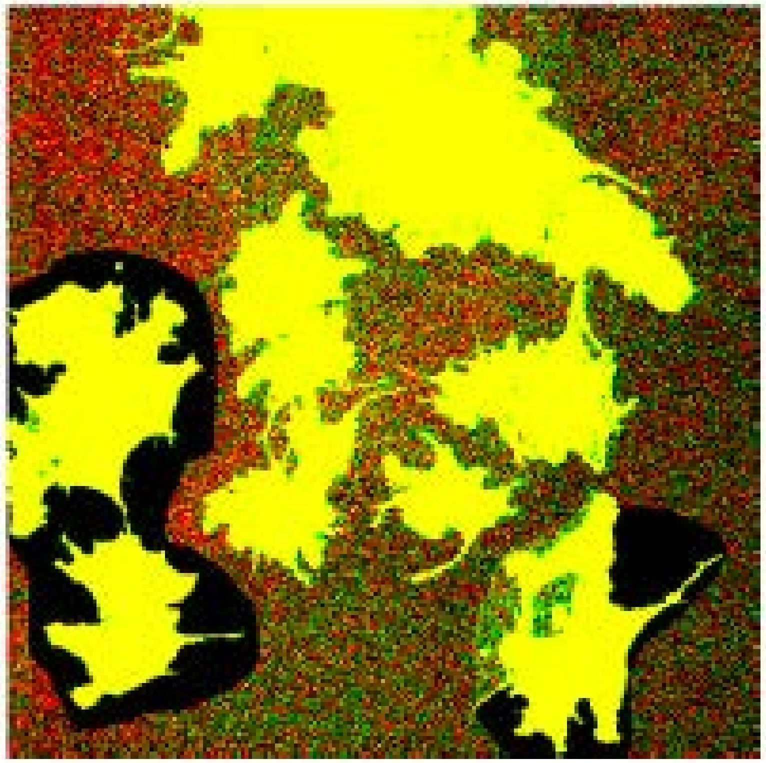
THEY HAVE WAYS OF FINDING OUT WHAT YOU DID!



Manipulated image

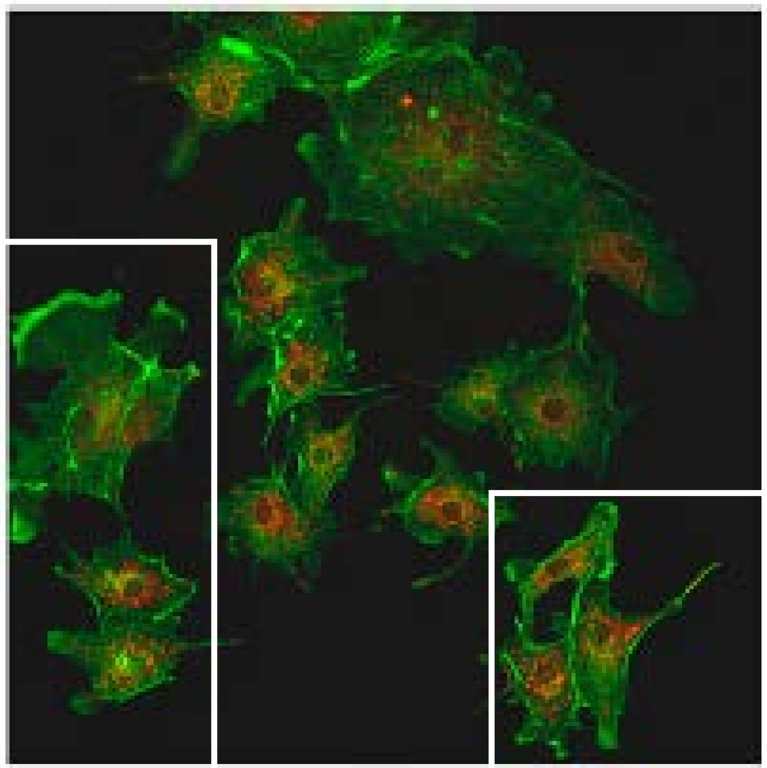


Manipulation revealed by contrast adjustment



- **Miss-representation of cell population within an oberved field**
- **VERY BAD: Changes interpretation with intention to defraud**

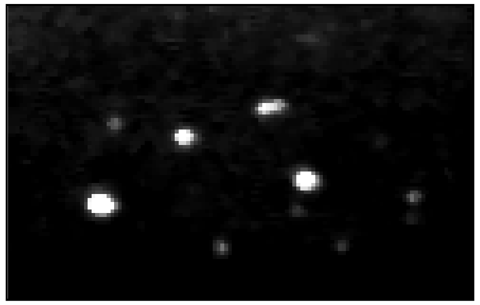
Correct:



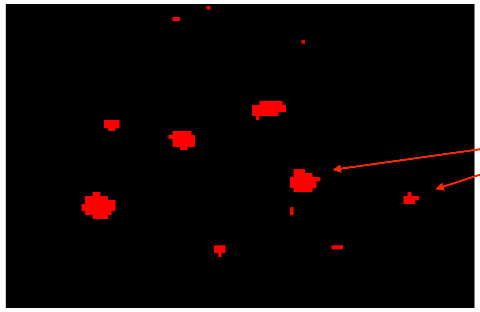
Rossner & Yamada (2004). What's in a picture? The temptation of image manipulation. J. Cell Biology 166: 11–15.

MID LEVEL Processing

- Input is an image, output is an attribute extracted from the image



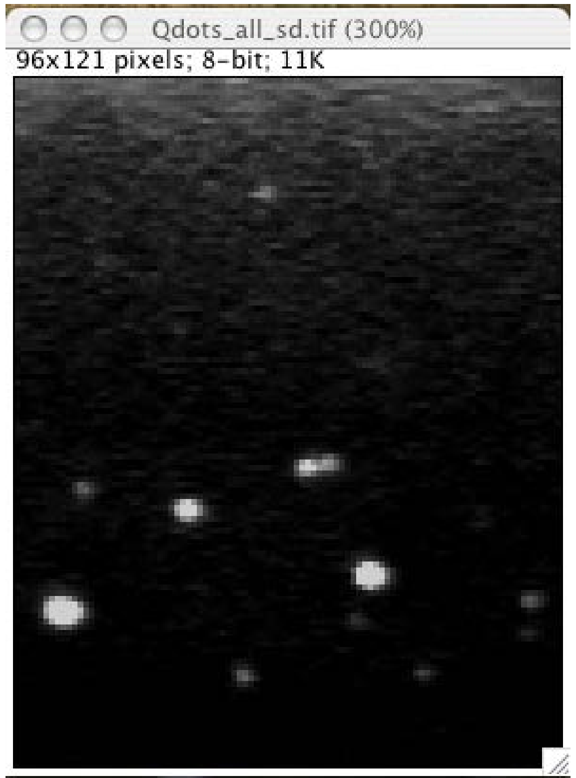
Segmentation



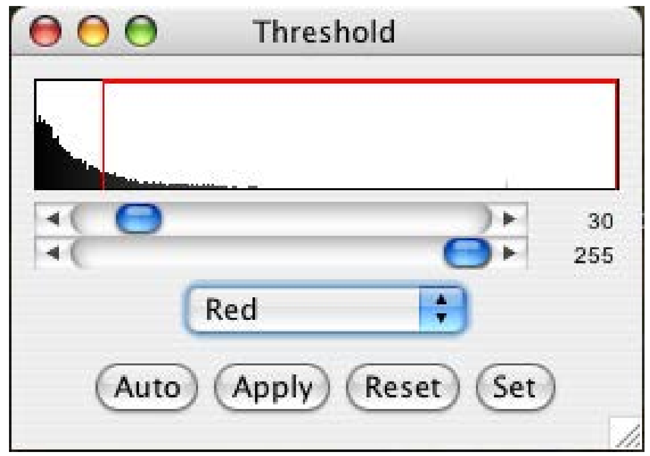
Objects defined

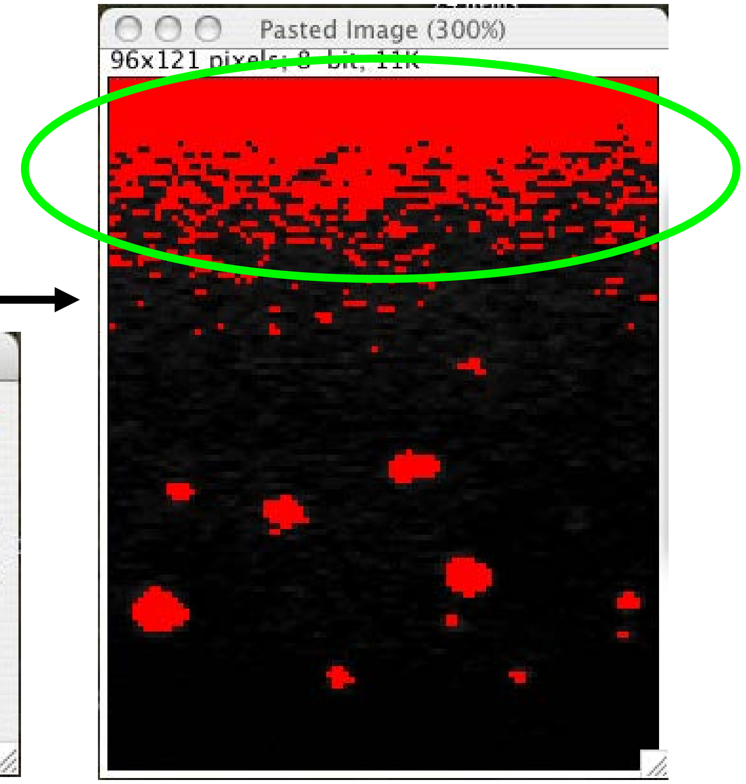
Number of objects
Positions of objects
Size of objects

MID LEVEL Processing - simple segmentation

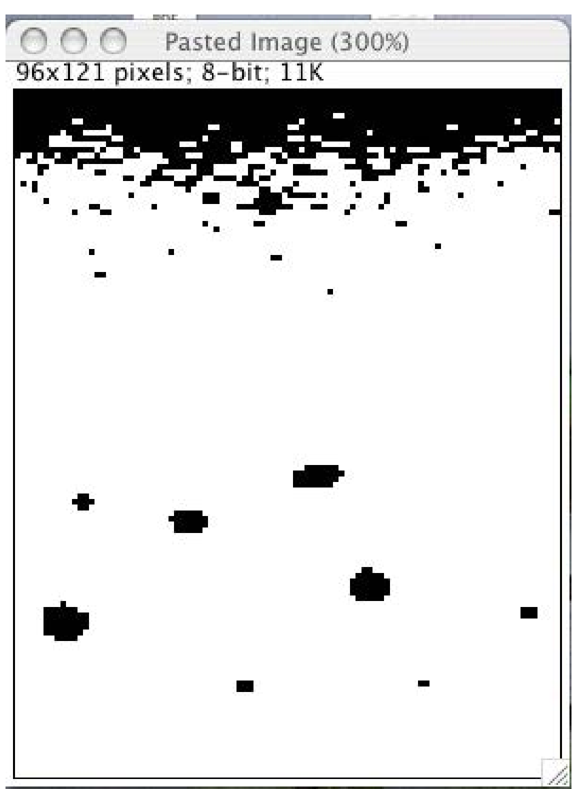


Segment by intensity



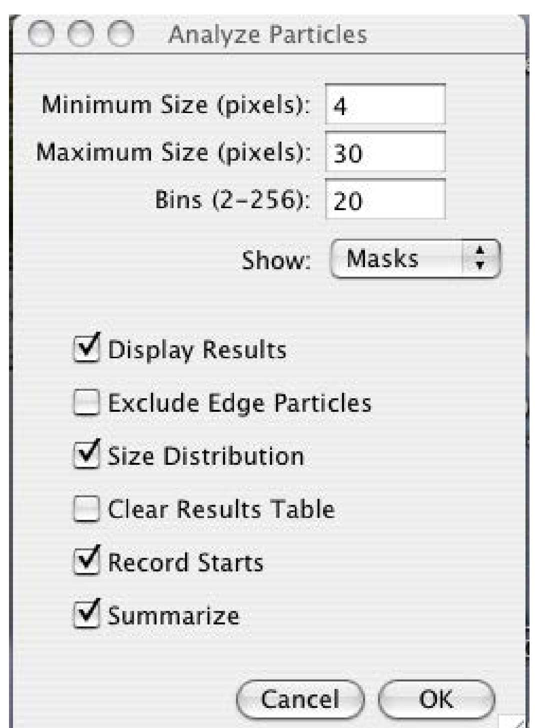


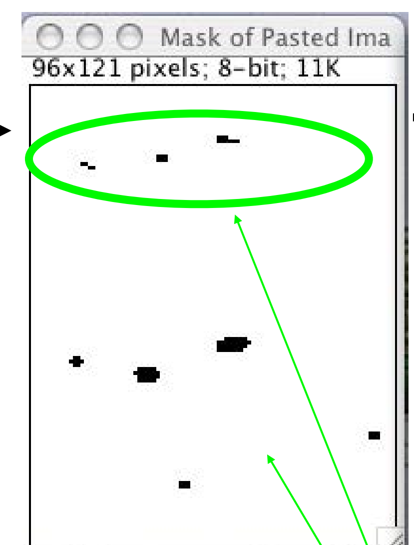
problem region



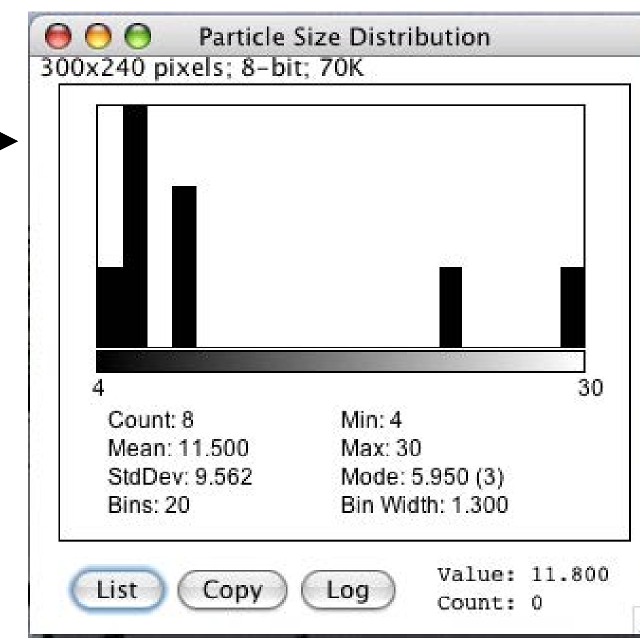
Intensity segmented binary image

Segment again by area size





mask defining regions



attributes

Some particles lost - some artificially created

ImageJ

The human brain is still one of the best segmentation tools

But

Subjective not objective!

Automated segmentation desirable for data quality and sanity

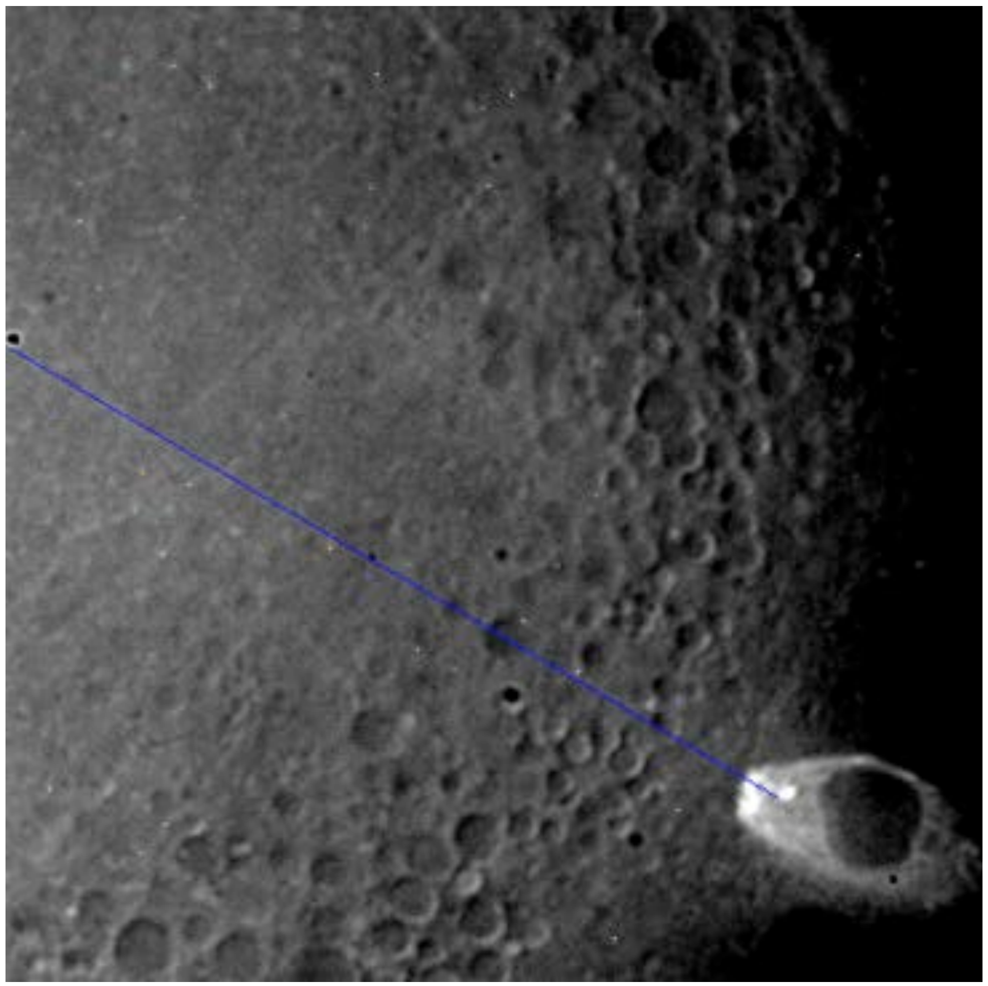
* Graeme, Lecture 19: Applied Image Analysis *

* Practical course - Demo of Fiji plugins and macros *

HIGH LEVEL Processing = Image Analysis

- Outputs are interpretation (making sense)

Automated Particle tracking



Particle velocities

X cent	y cent	dist	Δt	Velocity

Particle trajectories
Particle velocities
Run lengths
Directionality
Net directionality

* Graeme, Lecture 19: Applied Image Analysis *
* Practical course - Demo of Fiji plugins and macros *

END