# Advanced Microscopy Course 2011
## Lecture 5: Basic Image Processing

Richard Parton - Richard.Parton@bioch.ox.ac.uk
Department of Biochemistry
University of Oxford

# 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
      - Filtering
    - Mid - Level Processing:
      - Segmentation
      - Spectral unmixing
    - High -Level Processing:
      - Colocalisation
      - Tracking
      - Statistics

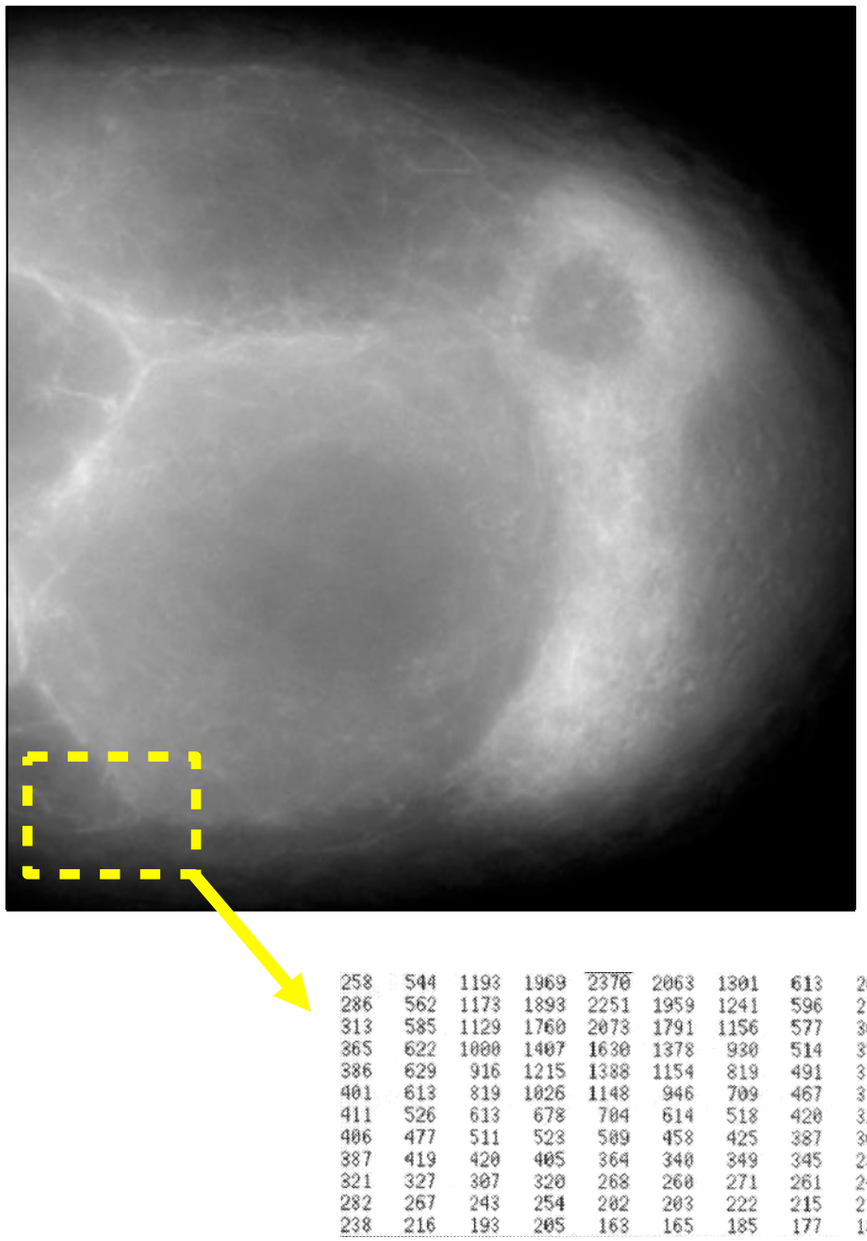
# What is a digital image?

# What is a digital image?

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

**image ≠ object**

**image = object ⊗ PSF**



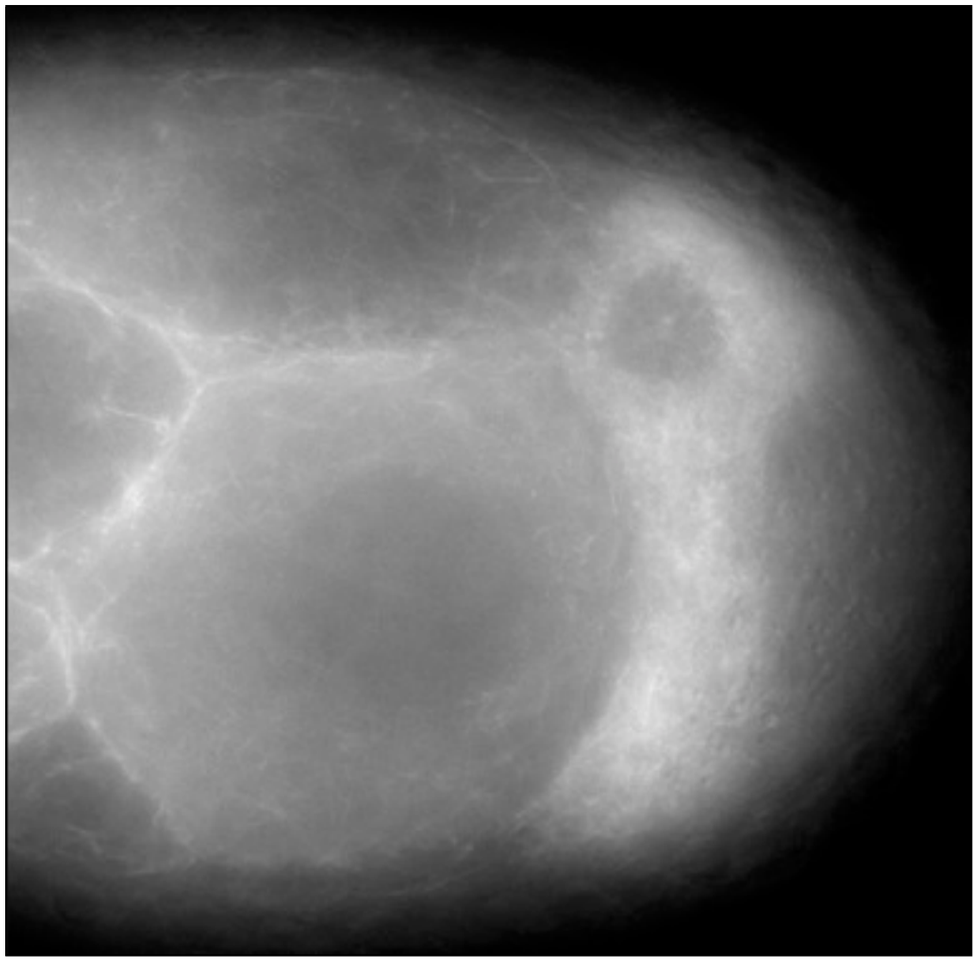
| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 258 | 544 | 1193 | 1969 | 2370 | 2063 | 1301 | 613 | 263 |
| 286 | 562 | 1173 | 1893 | 2251 | 1959 | 1241 | 596 | 278 |
| 313 | 585 | 1129 | 1760 | 2073 | 1791 | 1156 | 577 | 300 |
| 365 | 622 | 1000 | 1407 | 1630 | 1378 | 930 | 514 | 357 |
| 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 | 509 | 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)



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

# Pixel values and photons

- Pixel values relate to photons detected.

- Relating pixel values to photons - requires parameters of the specific detector.

Detector Roper Coolsnap HQ
QE = 62% = only that % of photos is converted to electrons (I.e. detected)
12 bit = 4096 grey levels
Full well depth (6.4 um pixel size) = 12,694 electrons
Pre-Amp gain electrons/ grey scale level = 3.1 (individual camera specific)
Software adds 50 grey scale count offset

-Image signal (300 ms exposure) = 2200 counts (grey level value)

-Dark signal counts (no light) = 150 counts (grey level value) + 50 count offset added

-Signal counts = 2200 - 200 = 2000 3.1 electrons/count = 6200 electrons ~ 6200 photons

- Knowing the number of photons can be important for quantitative image analysis.

# Bit Depth / Digitisation

**Digitisation**

“grey levels” - the number of discrete values in an image

the eye detects 6 to 8 bit

Imaging detectors:

8 bit = $2^8$ = 256 grey levels

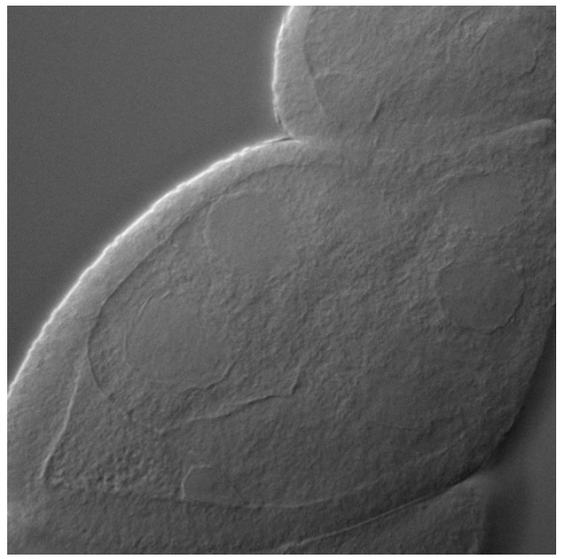
12 bit = $2^{12}$ = 4096 grey levels

16 bit = $2^{16}$ = 65536 grey levels

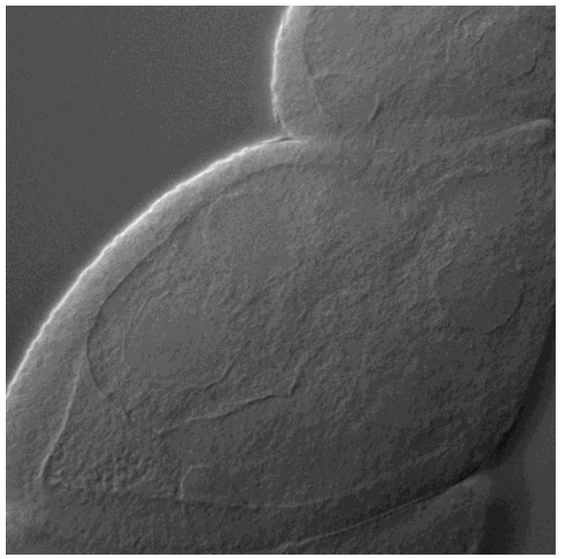
# Bit Depth

- **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.**

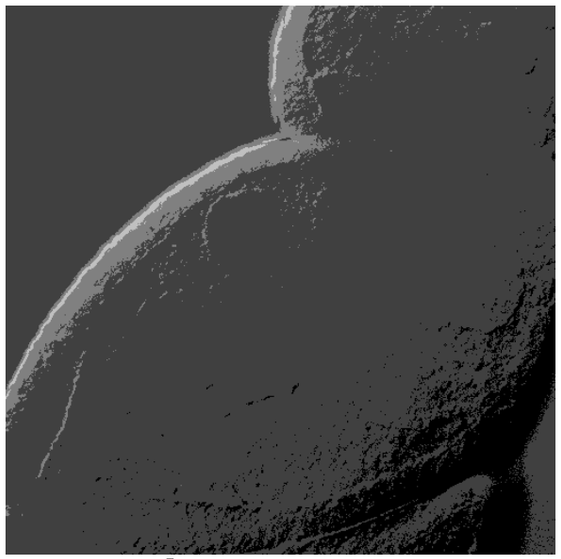
**False contouring due to insufficient grey levels** →



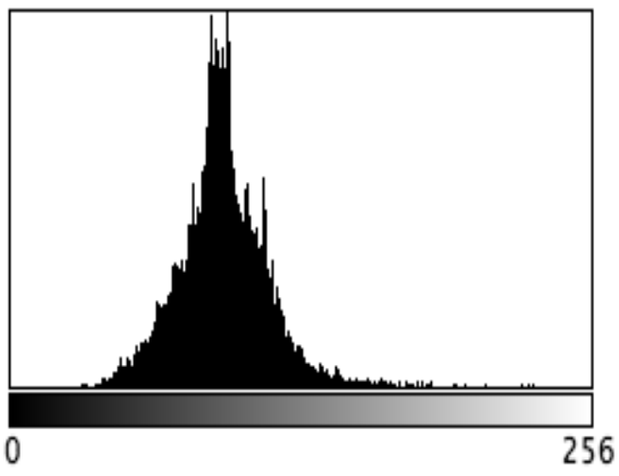
**256 greys**

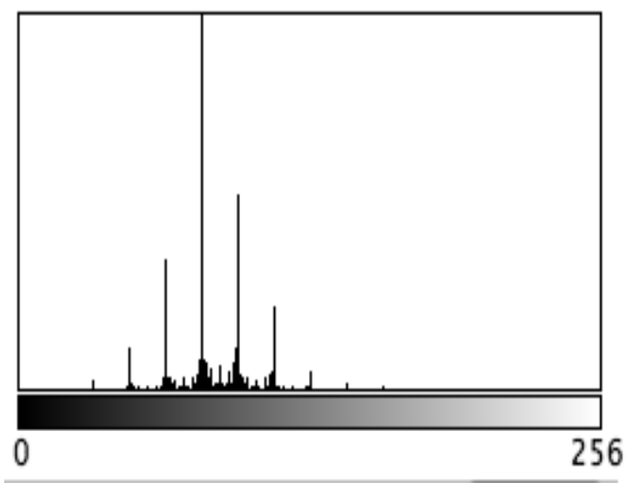


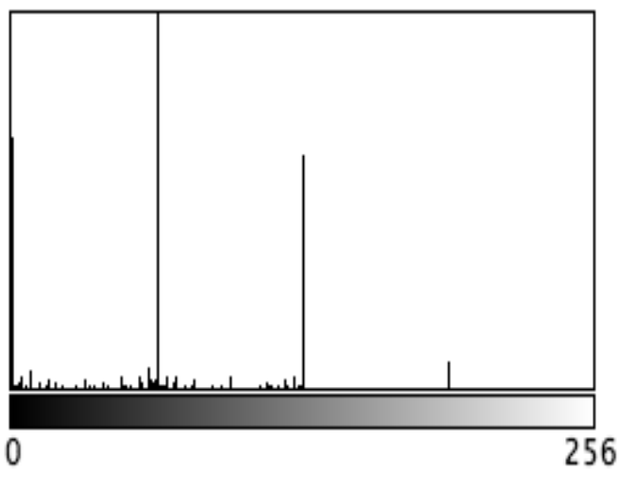
**16 greys**



**4 greys**






http://www.lenswork.com/calibrate.htm#tones

# 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**

# Noise / Signal to Noise (S/N)

# Signal to Noise - definitions:

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

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

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

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

Poisson distributed variation

- Statistics of photon counting dictate the minimum useful signal

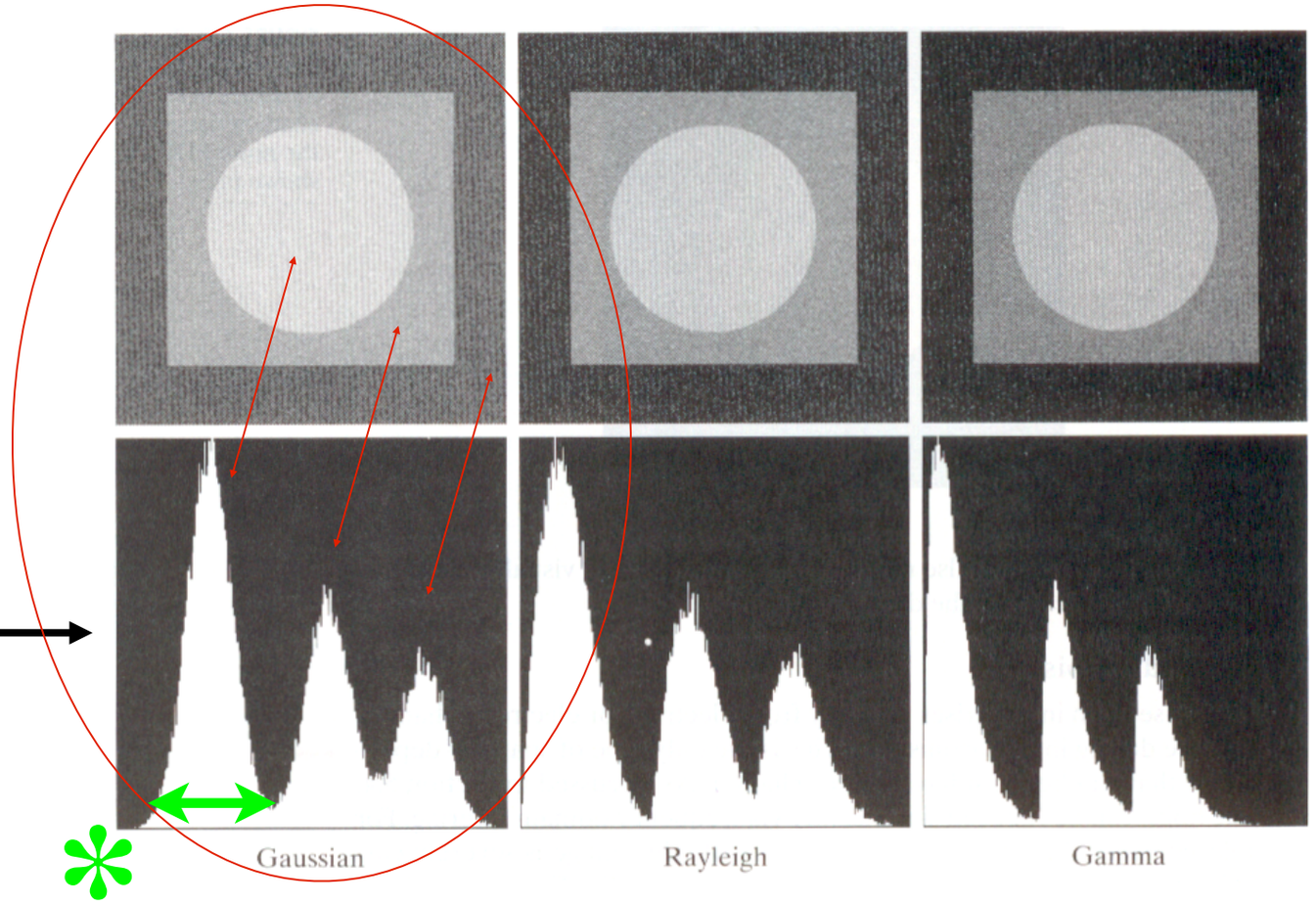
Average signal = 9, S:N ratio = 3
Average signal = 100, S:N ratio = 10
Average signal = 10,000, S:N ratio = 100

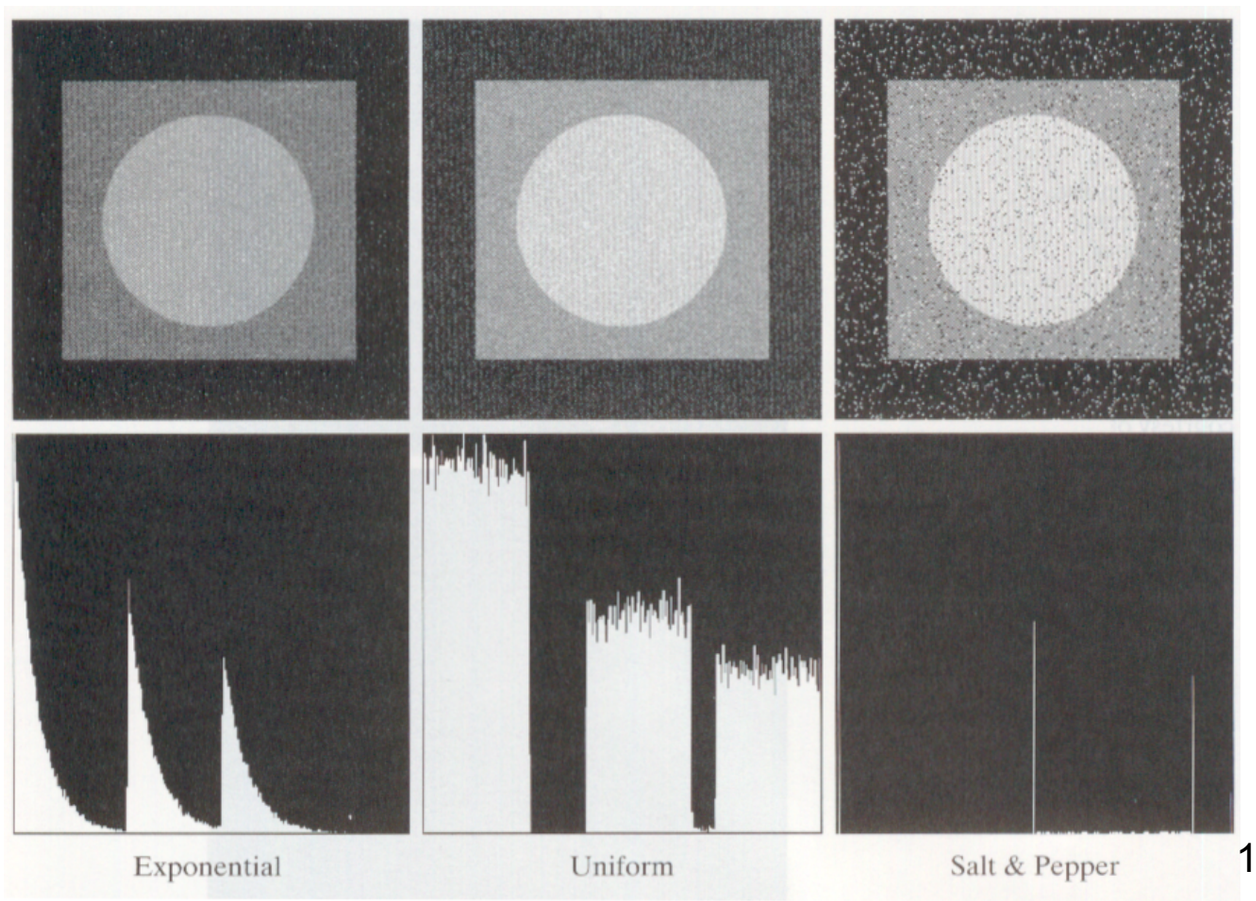
A meaningful difference in intensity needs to be at least three times the noise level

- 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.

# 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**

# 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

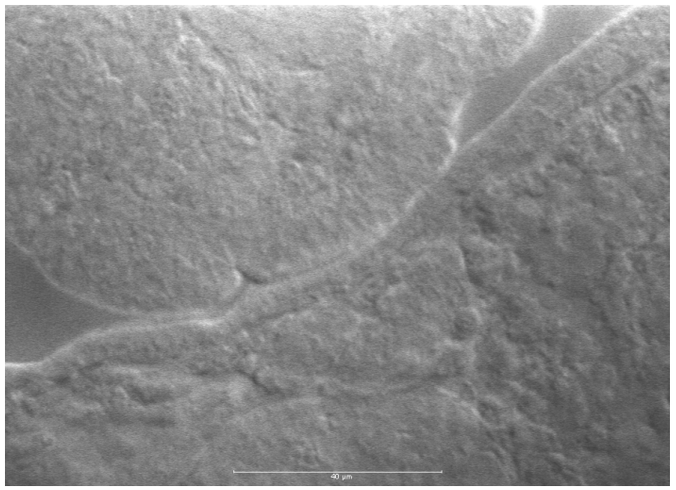


**Averaging increases S/N = improved contrast**

**Effectively increases the number of photons counted**

**Single noisy image**

**Average 5x successive images**



**improved S/N**

**ImageJ**

***LECTURE 13***

# Signal to noise - take home messages

- The definitions of noise components in image data are confusing.

- Noise = VARIATION in signal - you cannot simply subtract a “noise value”.

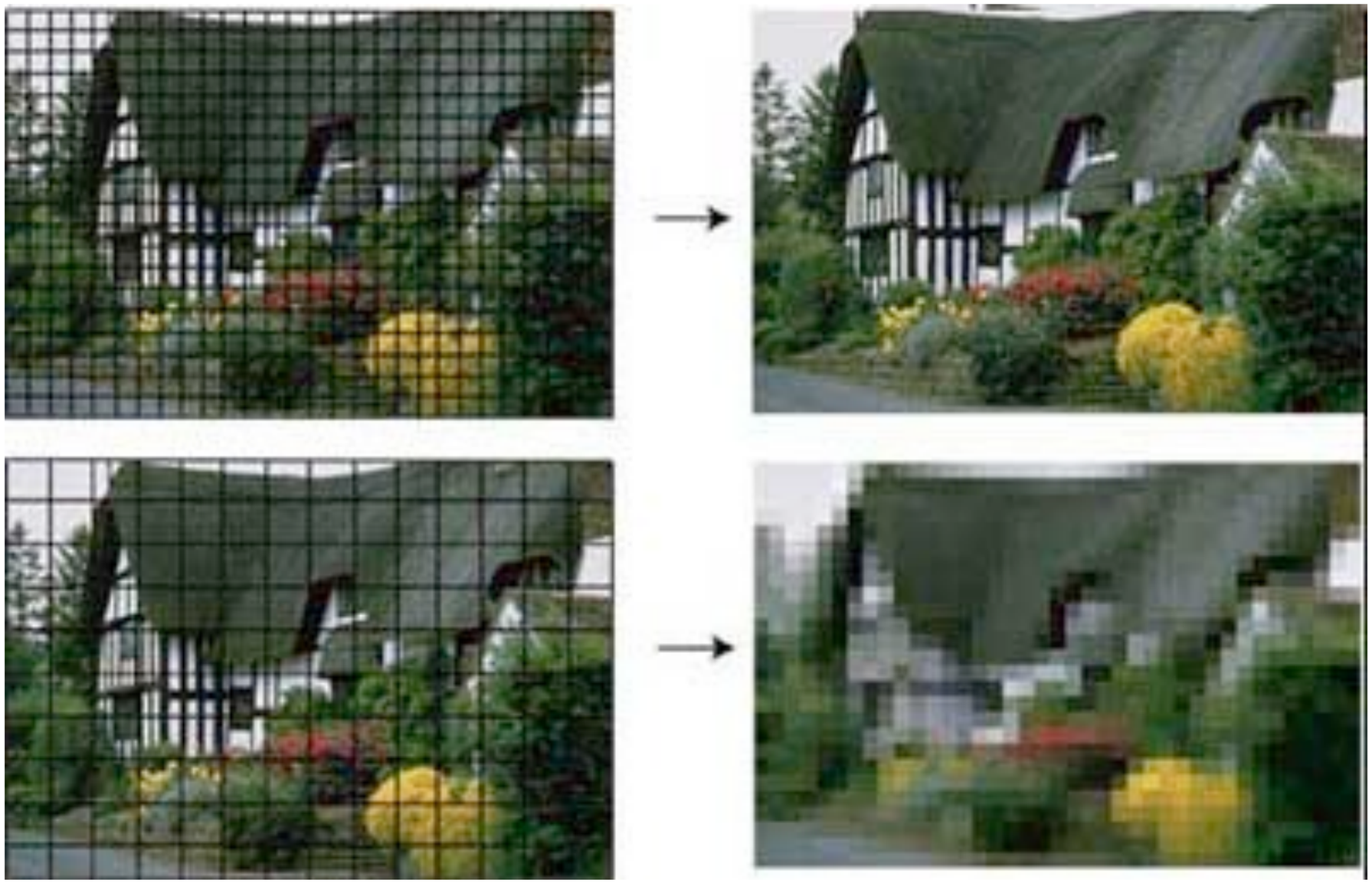
- Noise is NOT dark signal or background but they CONTRIBUTE to image noise.

- Dark signal = generated by camera
  Has an average value component and a noise (variation) component.
  Subtracting a dark offset value does not remove the noise component.

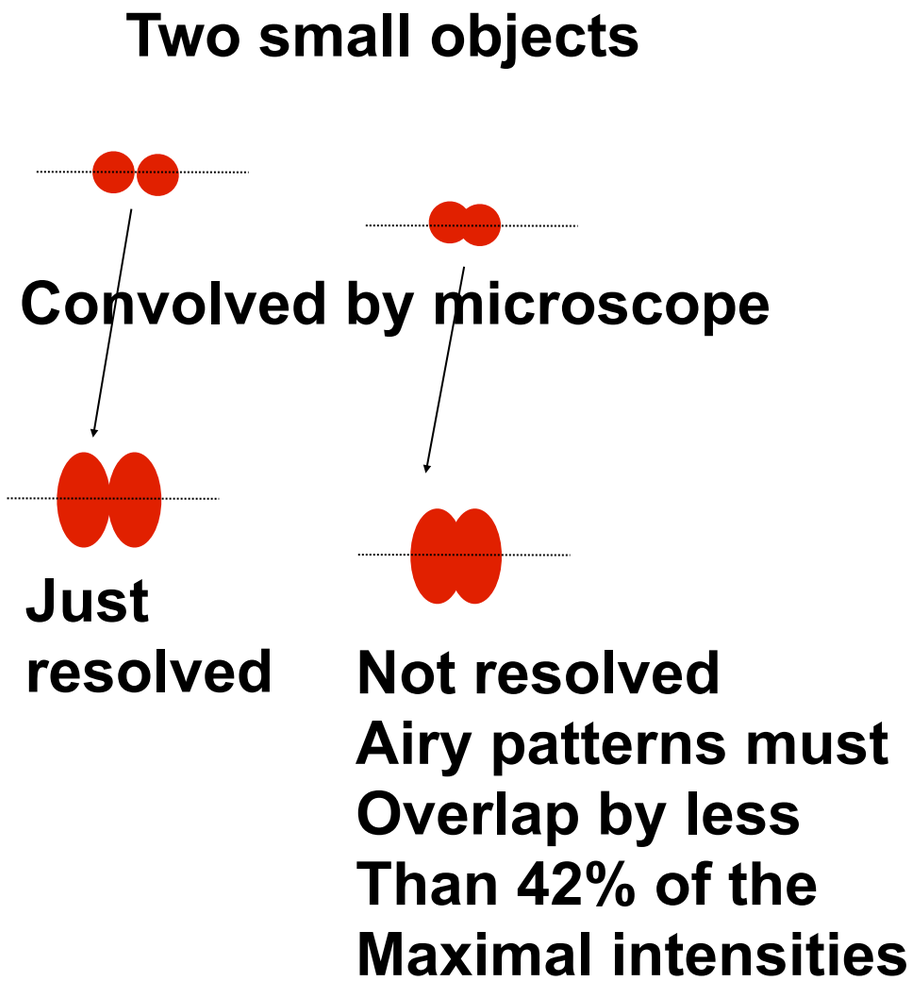
- Background = autofluorescence of sample
  Is a real fluorescence signal and has associated shot noise.
  Subtracting an autofluorescence image does not remove the noise.

- Statistical analysis on intensity should strictly be in relation to photon counts (not pixel values).

# Resolution and Sampling



# Optical resolution: The Rayleigh Criterion

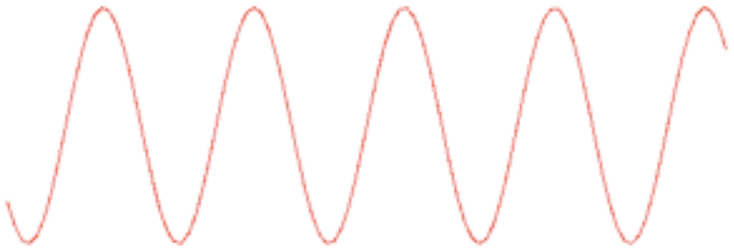


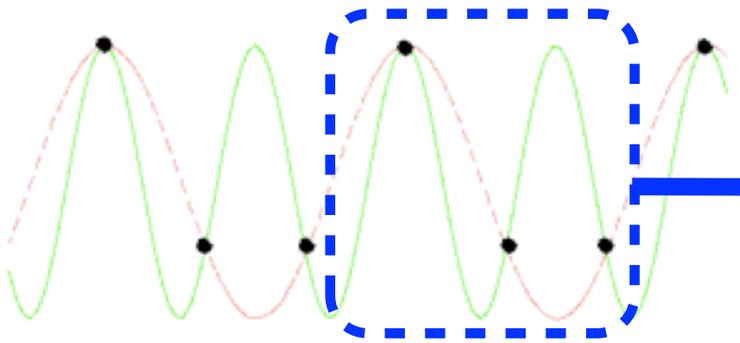

resolution limit $\approx \lambda_{em}/2$

**XY resolution ~ 200 nm**
**Z resolution ~ 500 nm**

# Resolution: Nyquist sampling theorem

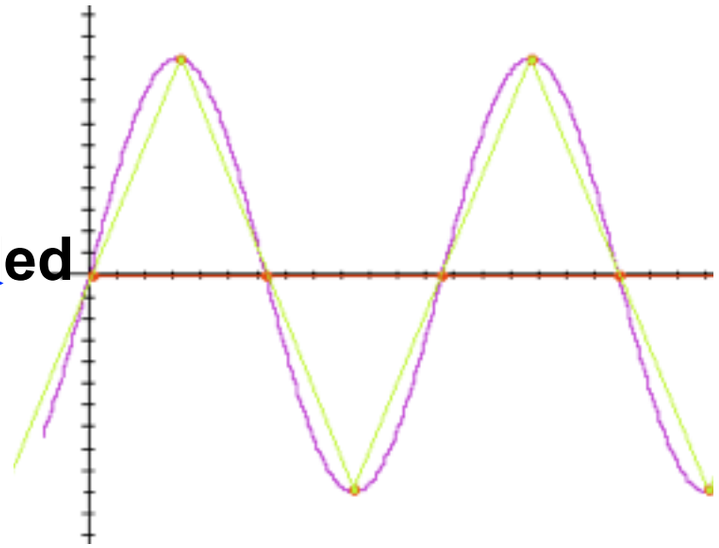
A sine wave





Sampling 1.5 times per cycle

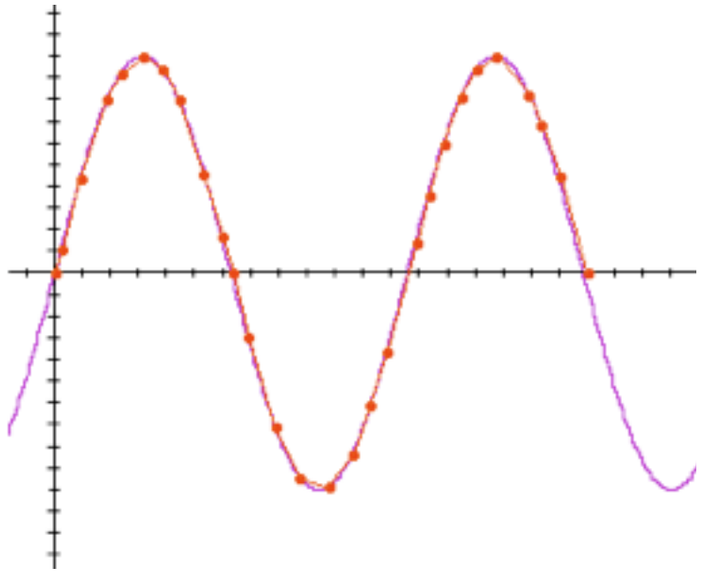
**undersampled**



Sampling 2.0 times per cycle

**just sampled enough**

**2.3**



Sampling many times per cycle

**over sampled**

# 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**

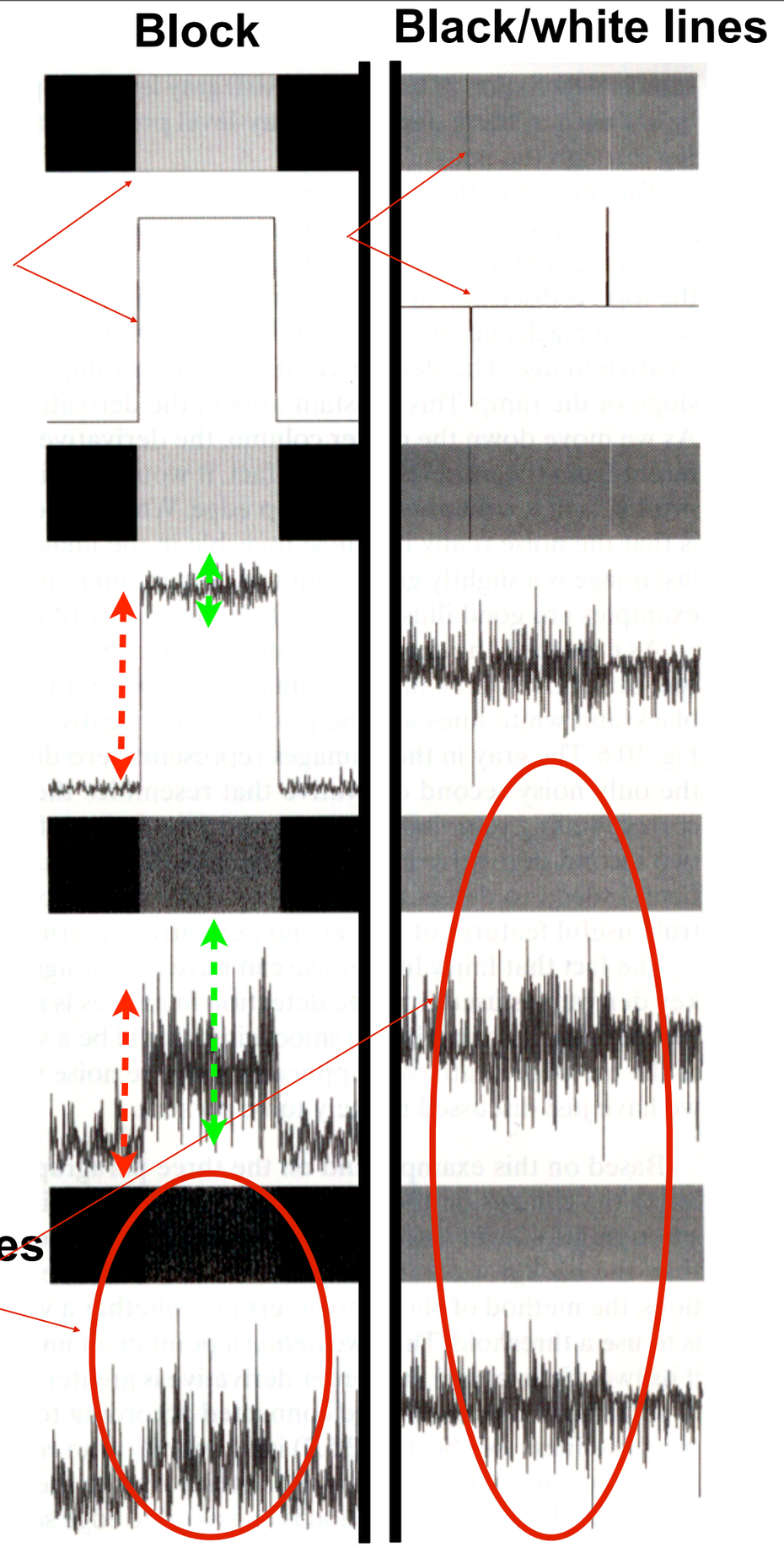
# Resolution, contrast, noise

Boundaries and lines easily resolved in the absence of noise

Increasing levels of Gaussian noise

Decreasing S/N

Boundaries and lines no longer resolved




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

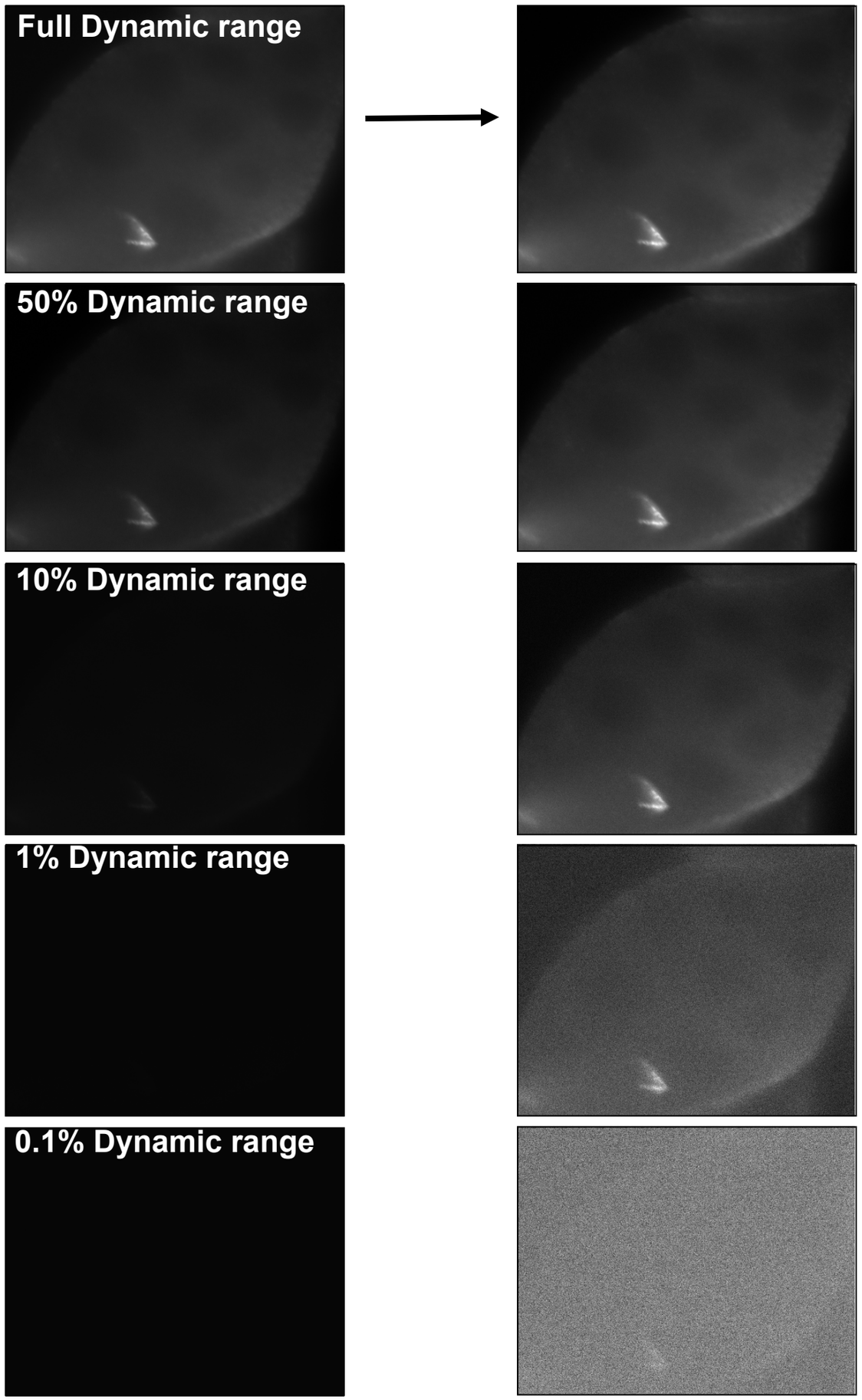
Gonzales & Woods, 2002. Digital Image Processing 2nd Ed. Prentice-Hall Inc, USA.

# 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 = resolution/2)**

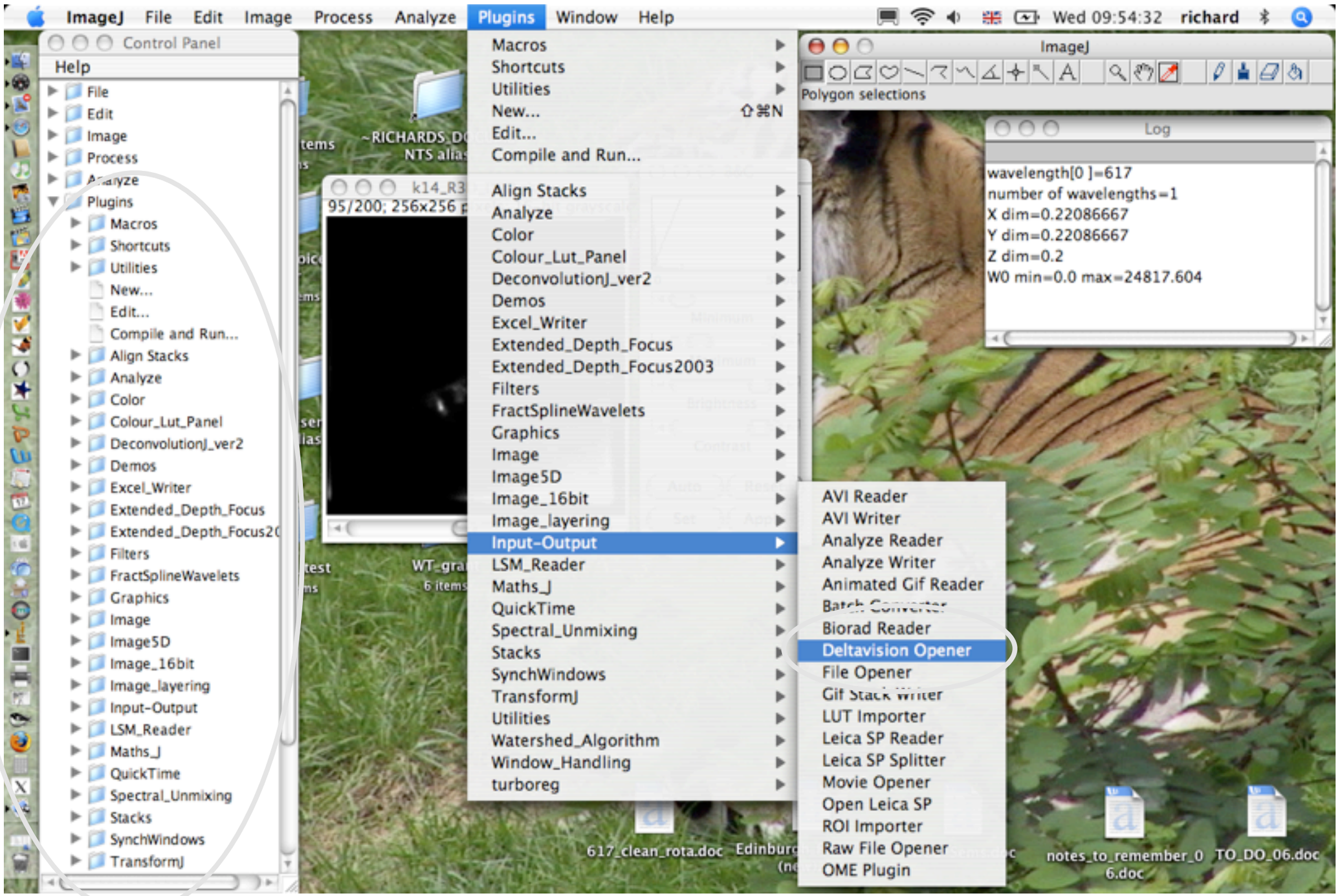
- **Avoid aberrations (sample prep / choice of objective / technique)**
  - **spherical aberration (SA)**
  - **motion blur**
  - **bad system alignment**

- **Correctly annotated (Metadata retained)**

# Image Processing is NOT a substitute for good imaging

# Image J program http://rsb.info.nih.gov/ij/

- Image J is FREE and works on MAC, PC and linux
- Consists of a core program and plugins



# 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.**

# Preserve your data: File Formats

- In preference use Uncompressed TIF (tagged image file format)
- AVOID compressed file formats: JPEG, compressed TIF…
  This will cause data corruption and loss
- Most data is collected as single channel grey scale images at 8 or 16 bit depth
  Avoid saving primary image data in colour formats (RGB)
- Avoid repeated inter-conversions of file formats
- Retain your original data in its original file format and original metadata associations
- Consider OME file format for data archiving
- Prepare figures for publication in Adobe Photoshop in TIFF format and PSD. Arrange and annotate in Adobe Illustrator

# OME - Open Microscopy Environment

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

- **A multi-site collaborative effort among academic laboratories and commercial entities:**
  - **Dundee - Jason Swedlow**
  - **NIA Baltimore - Ilya Goldberg**
  - **Harvard Medical School - Peter Sorger**
  - **Loci - Kevin Eliceiri**

- **All OME formats and software are FREE**

- **Visit** http://www.openmicroscopy.org/

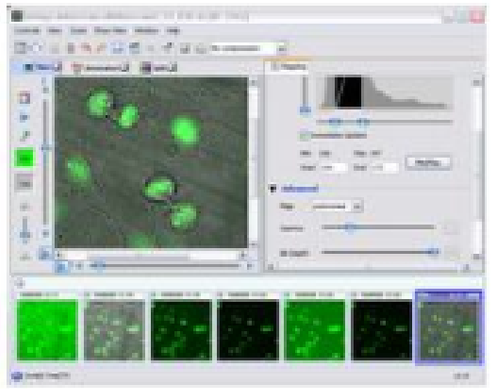
- **Intention to set up an OME server associated with MICRON (Russell Hamilton / Ian Dobbie)**

# OME - Open Microscopy Environment

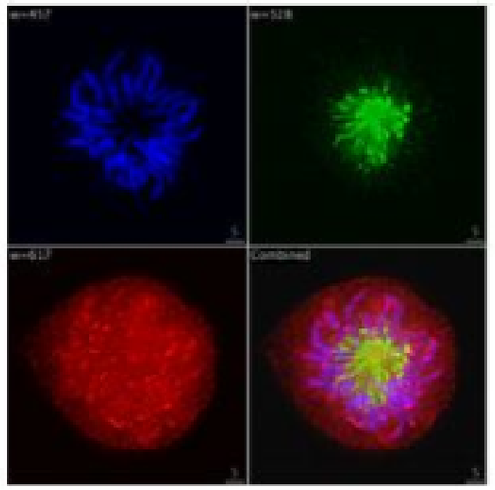
- **OMERO insight provides tools for viewing and managing data in an OMERO server**

http://www.openmicroscopy.org/

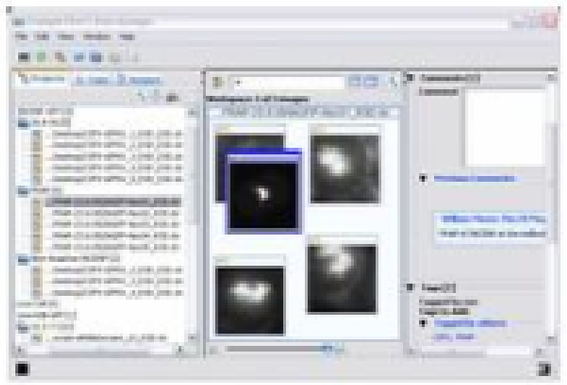
http://www.loci.wise.edu/ome/formats.html



Adjust your images rendering without altering you raw data

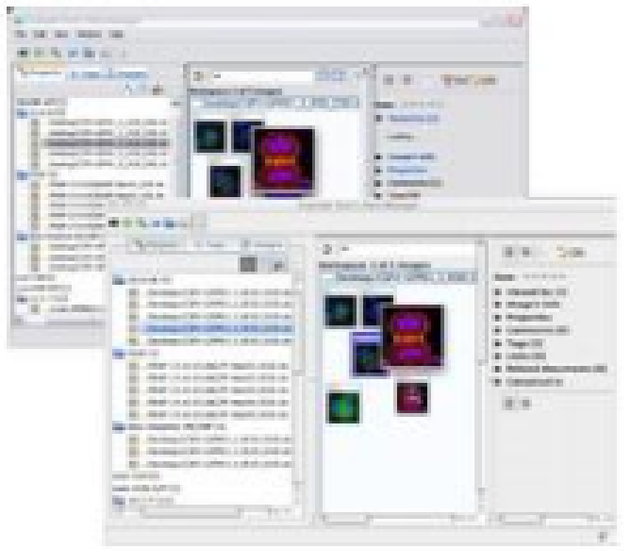


Easily save an annotated figure



Browse your image collections


Select from list of you favourite OMERO servers



A cross-platform solution

# Examples of Bad Imaging Practices

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

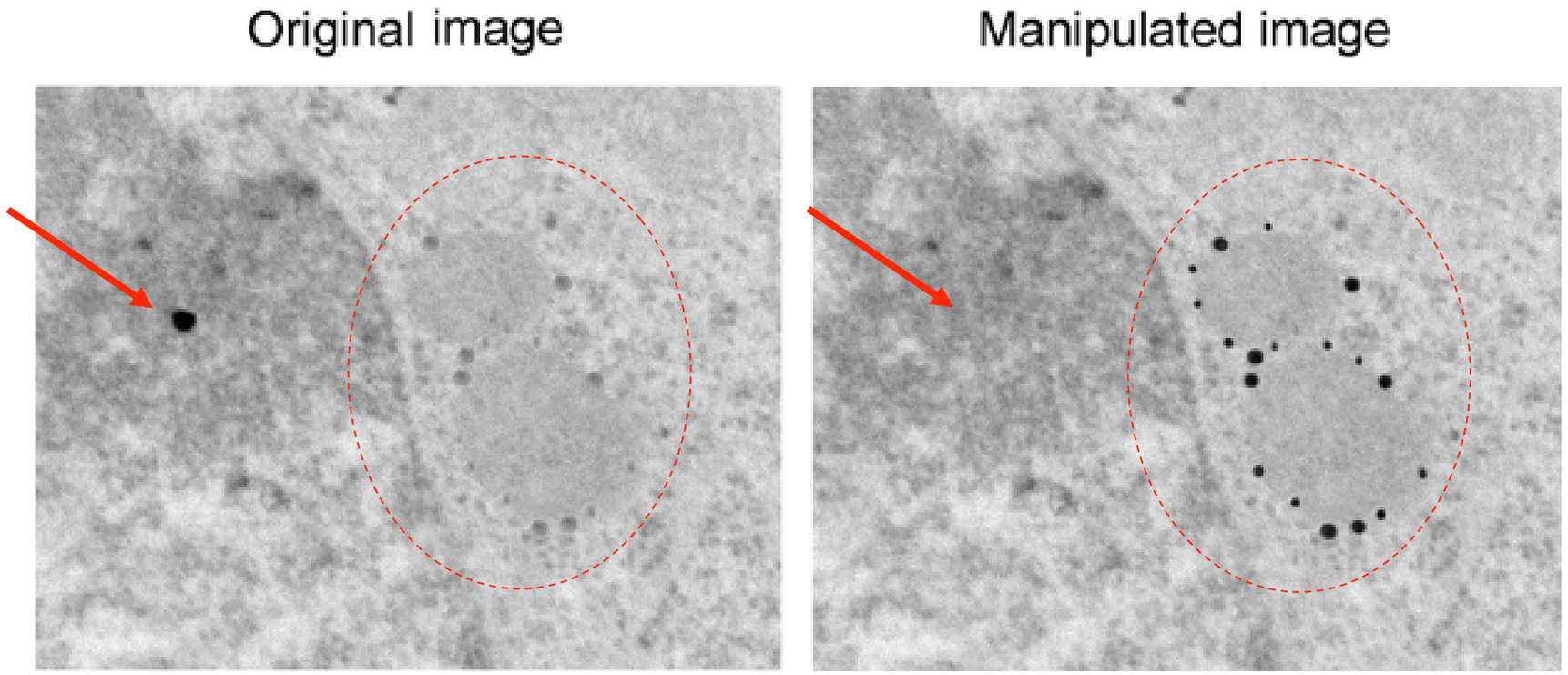
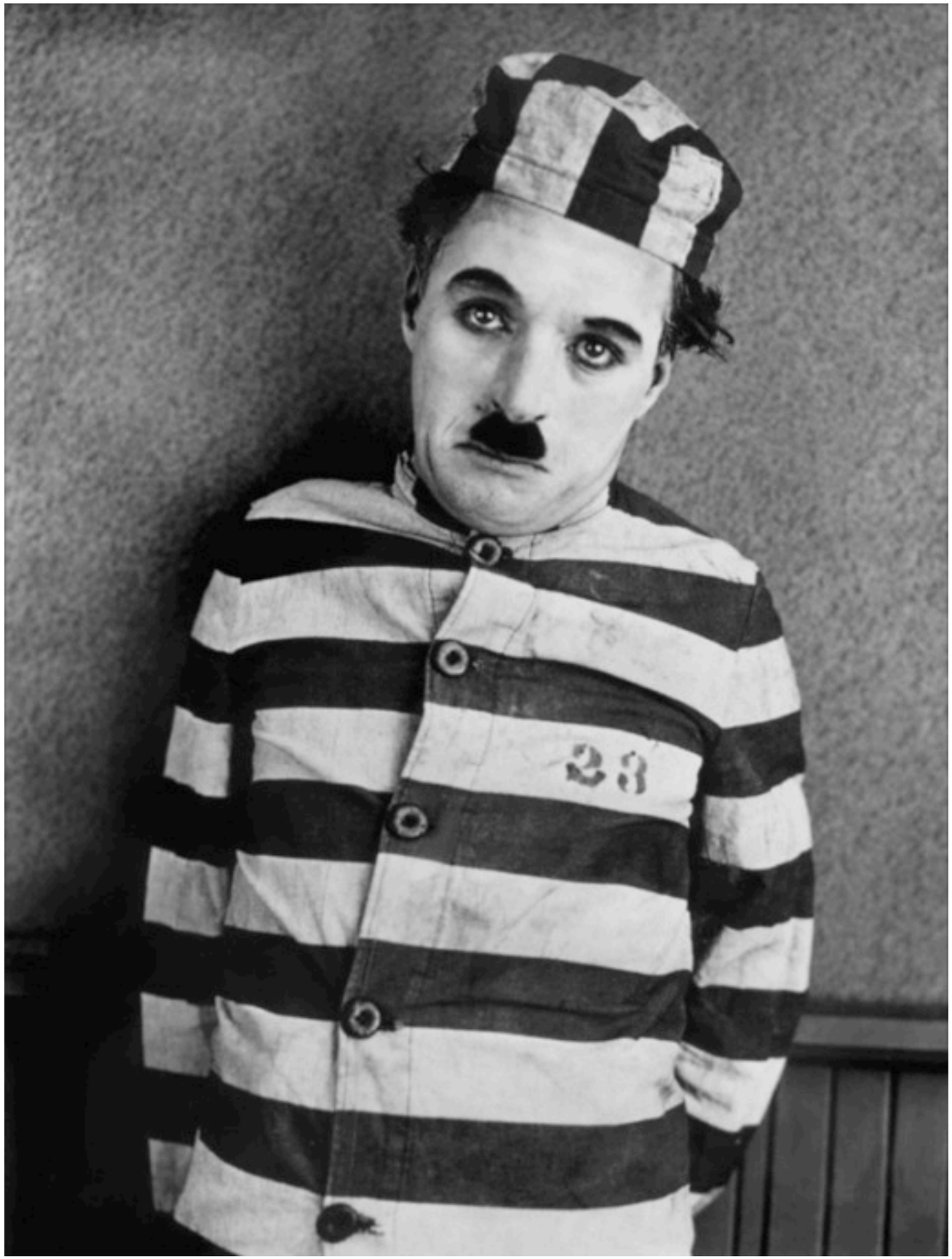


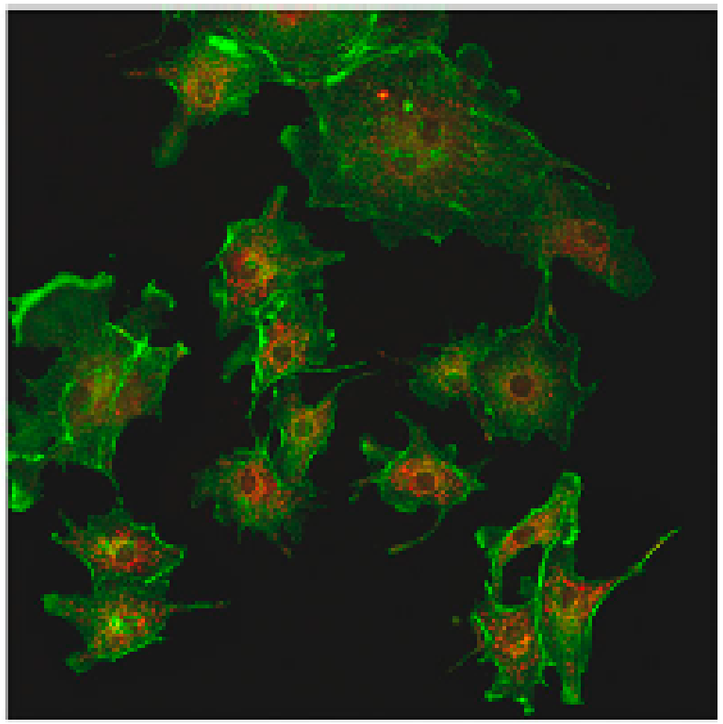

Figure 5. **Misrepresentation of immunogold data.** The gold particles, which were actually present in the original (left), have been enhanced in the manipulated image (right). Note also that the background dot in the original data has been removed in the manipulated image.

- **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!

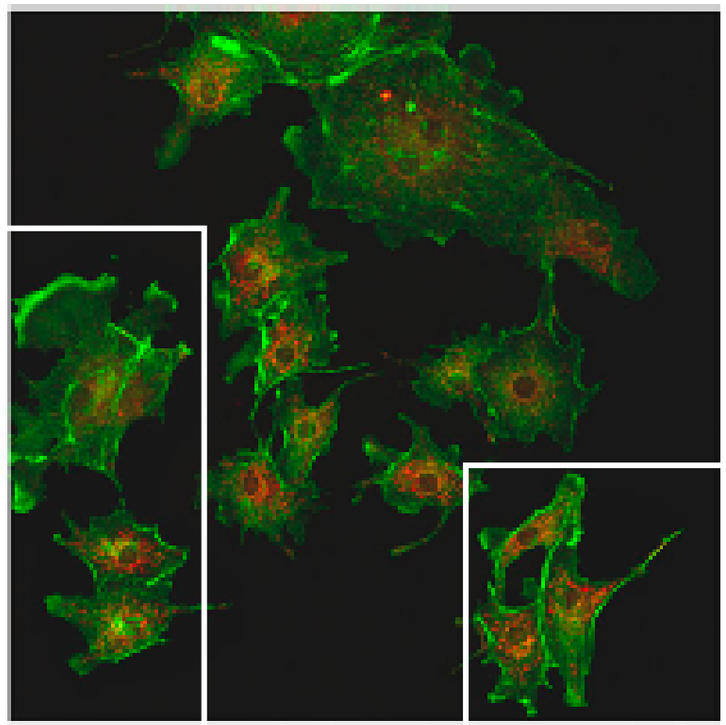


Manipulated image

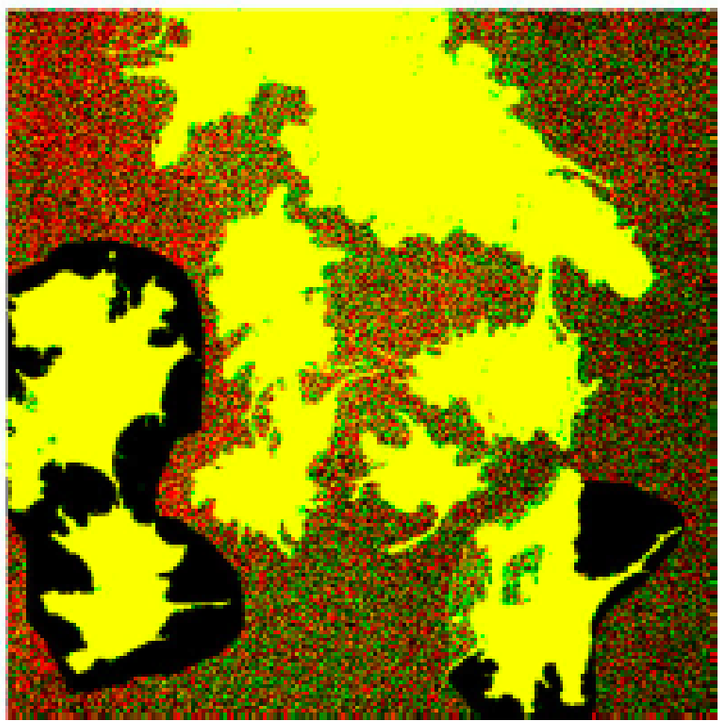


- **Miss-representation of cell population within an oberved field**
- **VERY BAD: Changes interpretation with intention to defraud**

**Correct:**



Manipulation revealed by contrast adjustment



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

# Conceptual Hierarchy of image processing

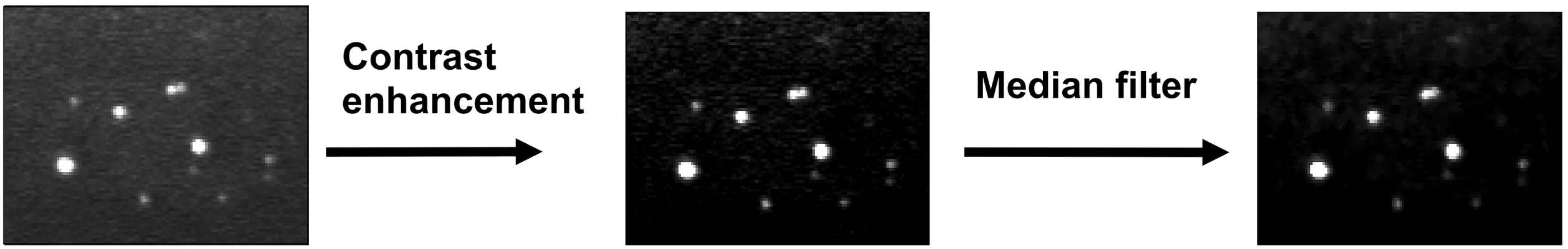
- **Low - level processing = Image enhancement (most common)**
- **Mid - level processing = Features and attributes extracted**
- **High - level processing = Interpretation of images**

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

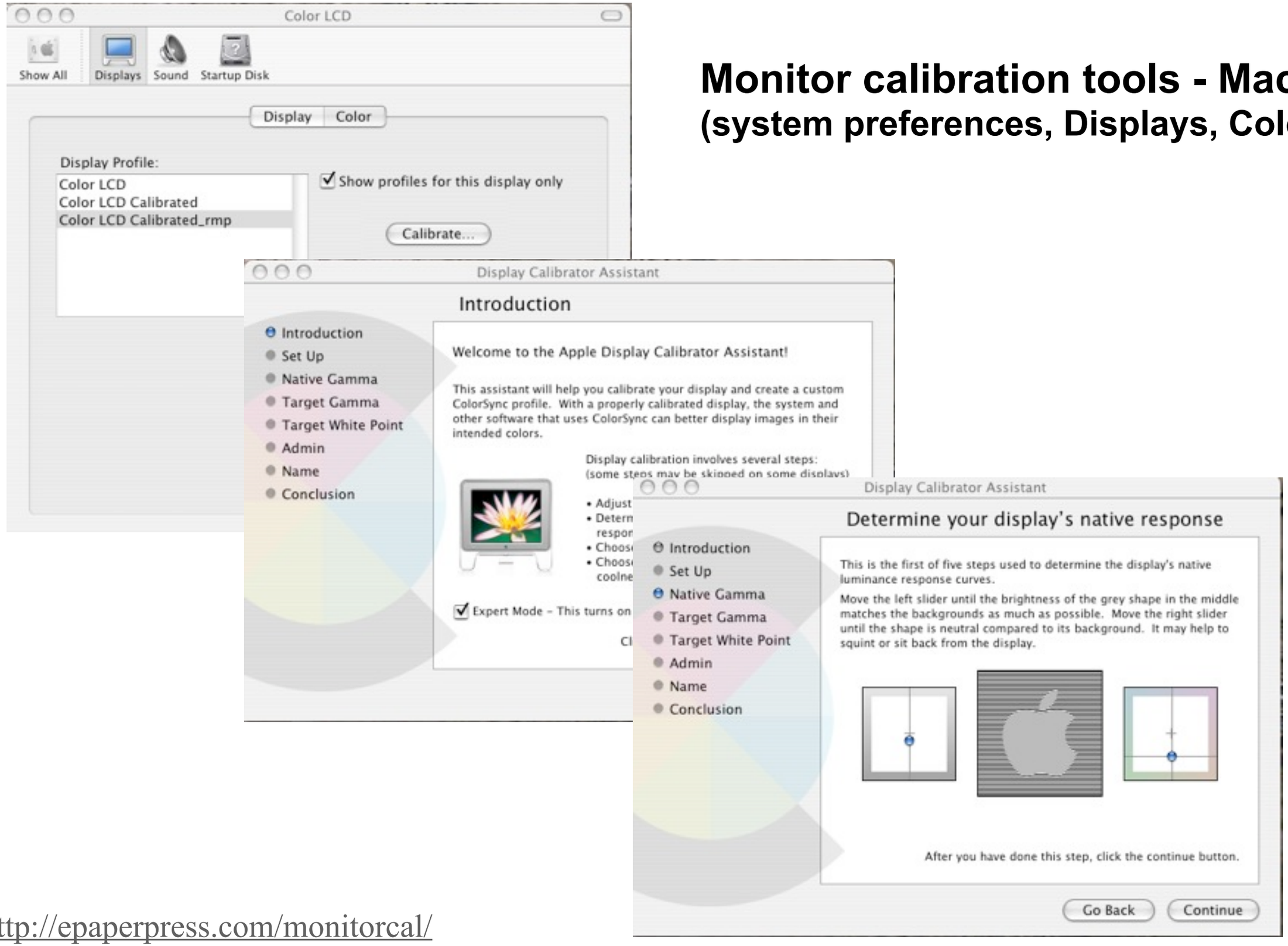
# LOW LEVEL Processing

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

**- Adjustments for image Display / Printing**
**- Enhancement Filters**
**- Deconvolution**



# LOW LEVEL Processing - Display, monitor setup

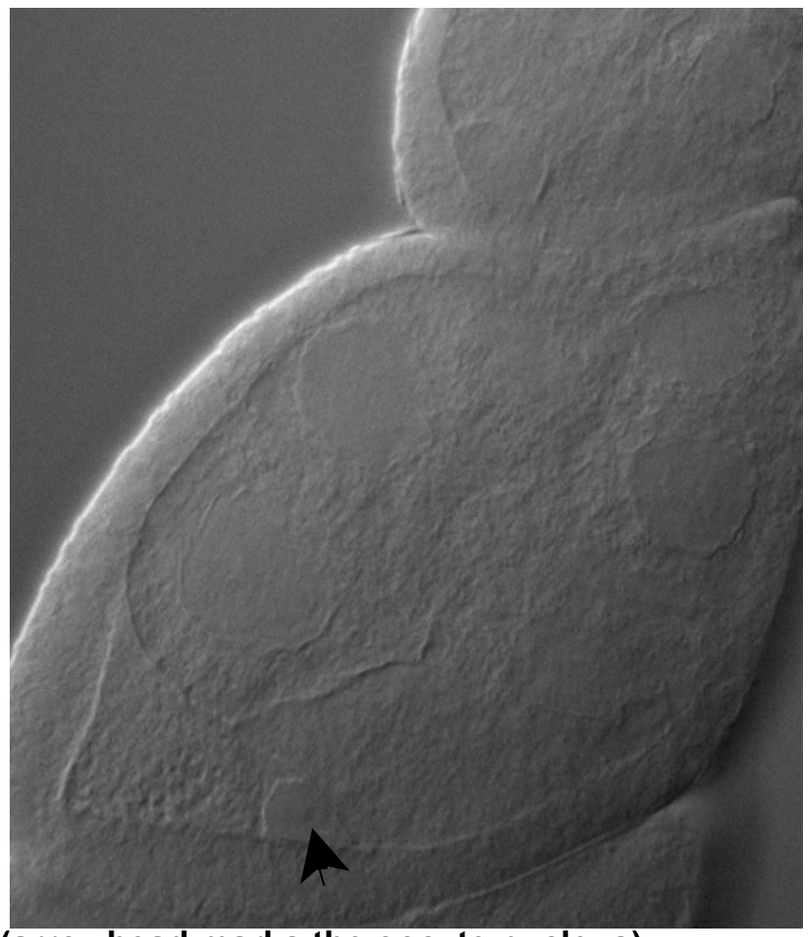


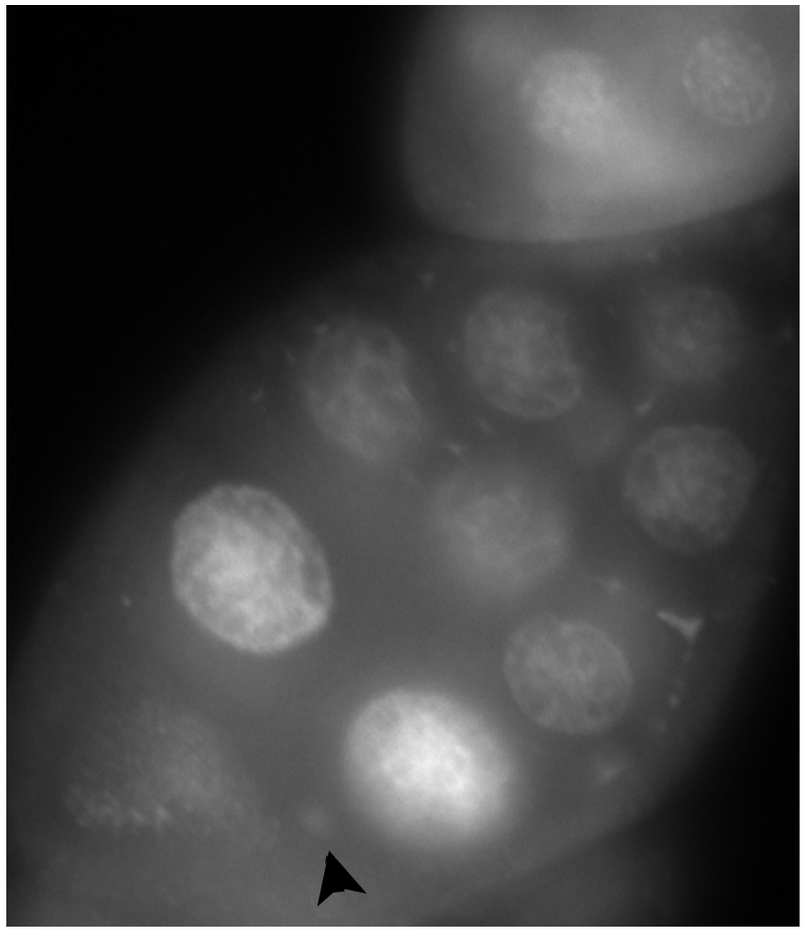

## Monitor calibration tools - Mac OSX
## (system preferences, Displays, Colour)

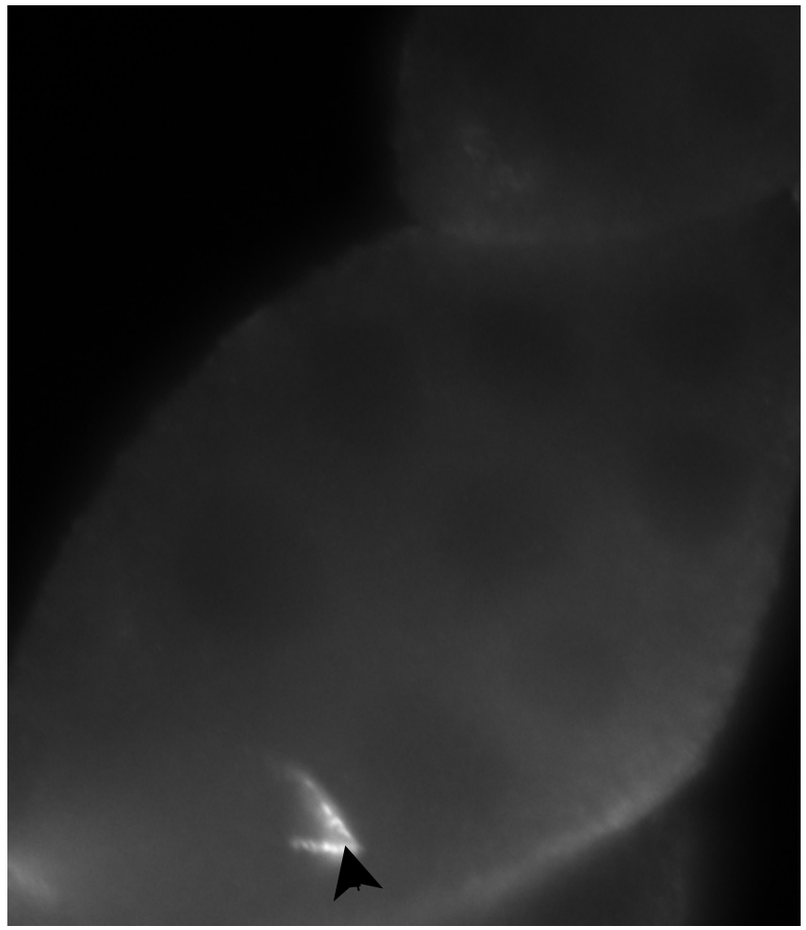
http://epaperpress.com/monitorcal/
http://www.bodoni.co.uk/profiling/colourprofiling.html#monitor
http://www.bodoni.co.uk/profiling/spyder.html

# LOW LEVEL Processing - Display, Grey Scale

## DIC/DAPI/Grk in situ - grey scale images





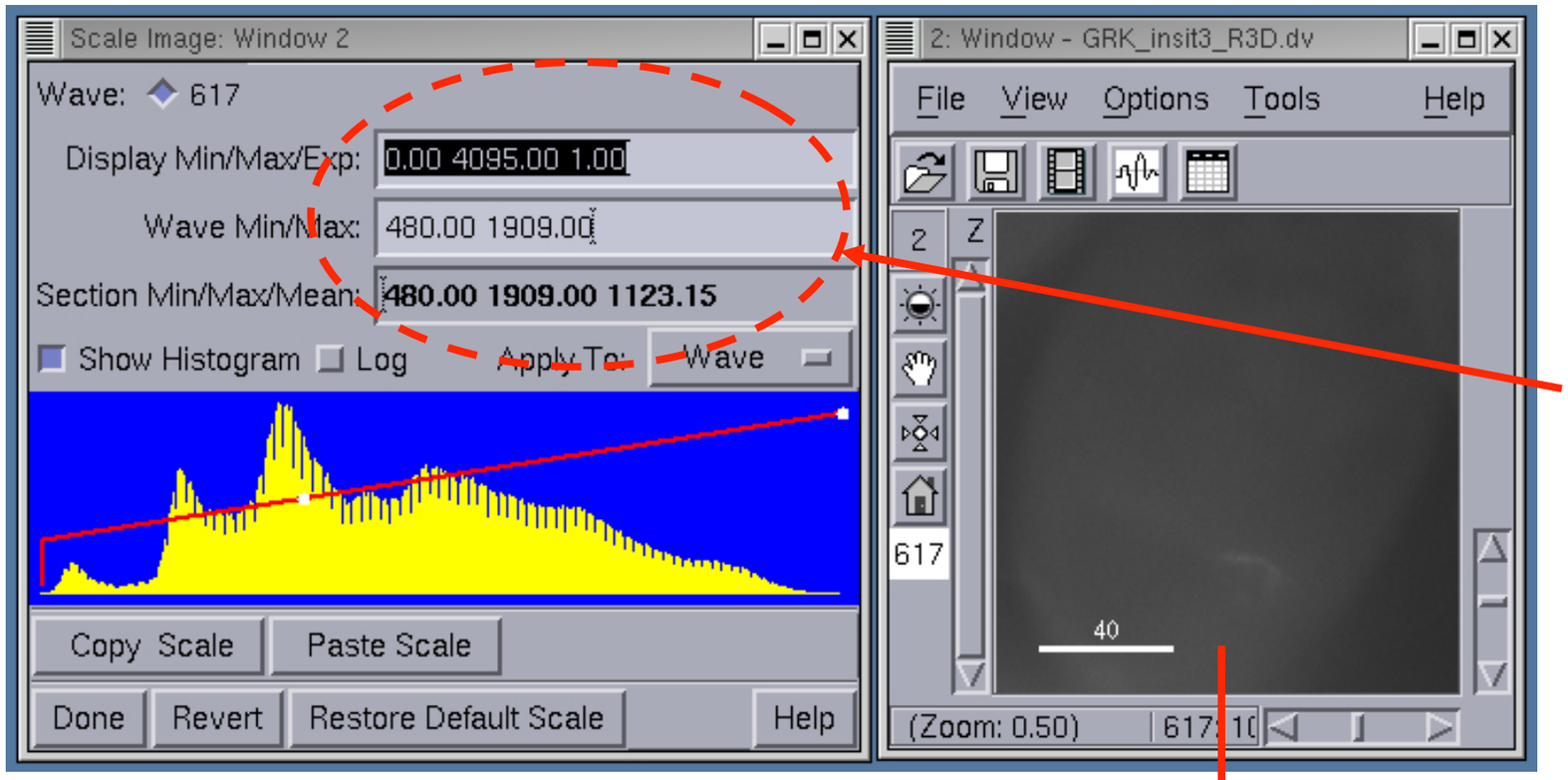


(arrowhead marks the oocyte nucleus)

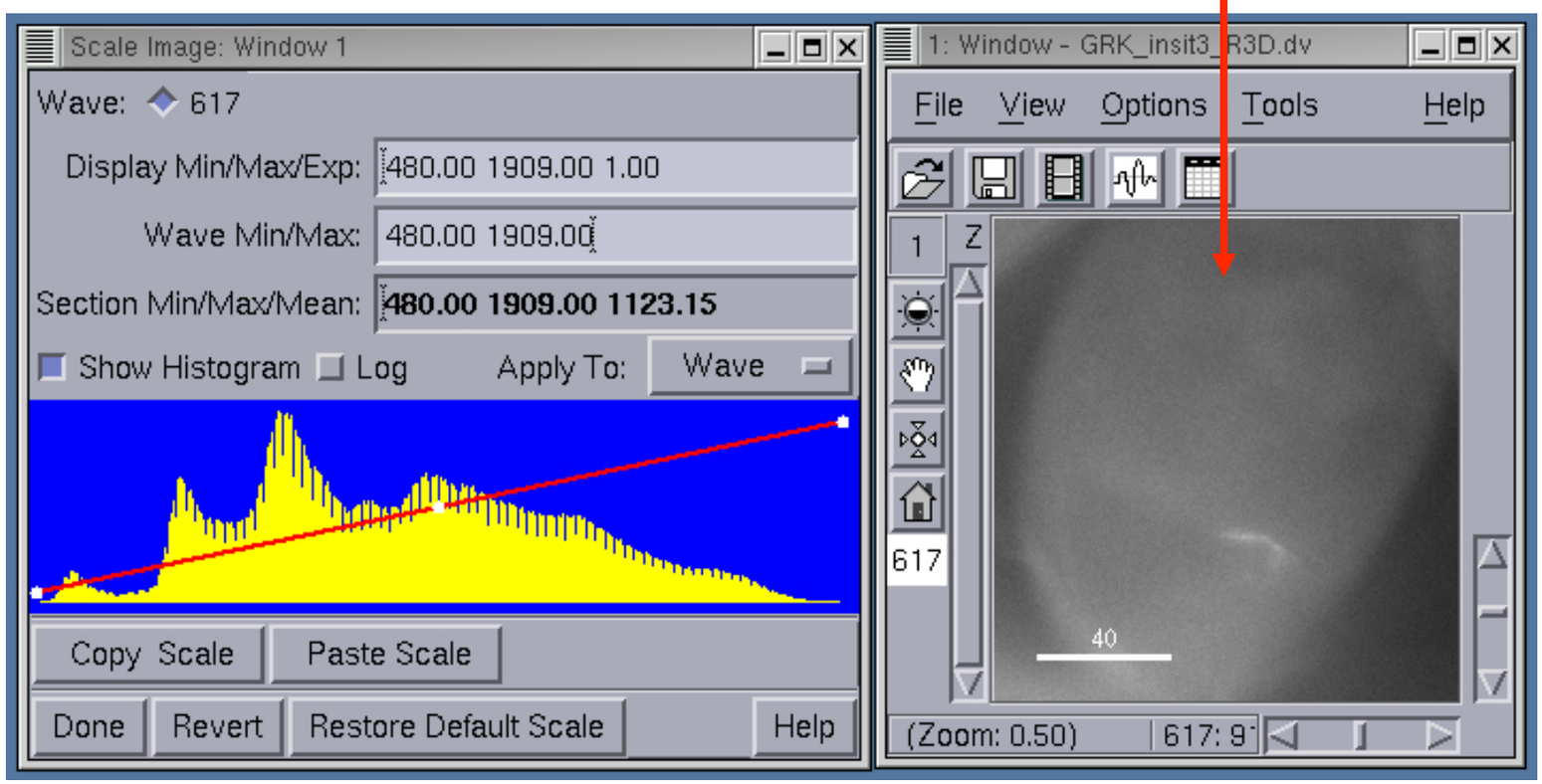
- For viewing and display use grey scale scale images to see fine detail.

# 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**



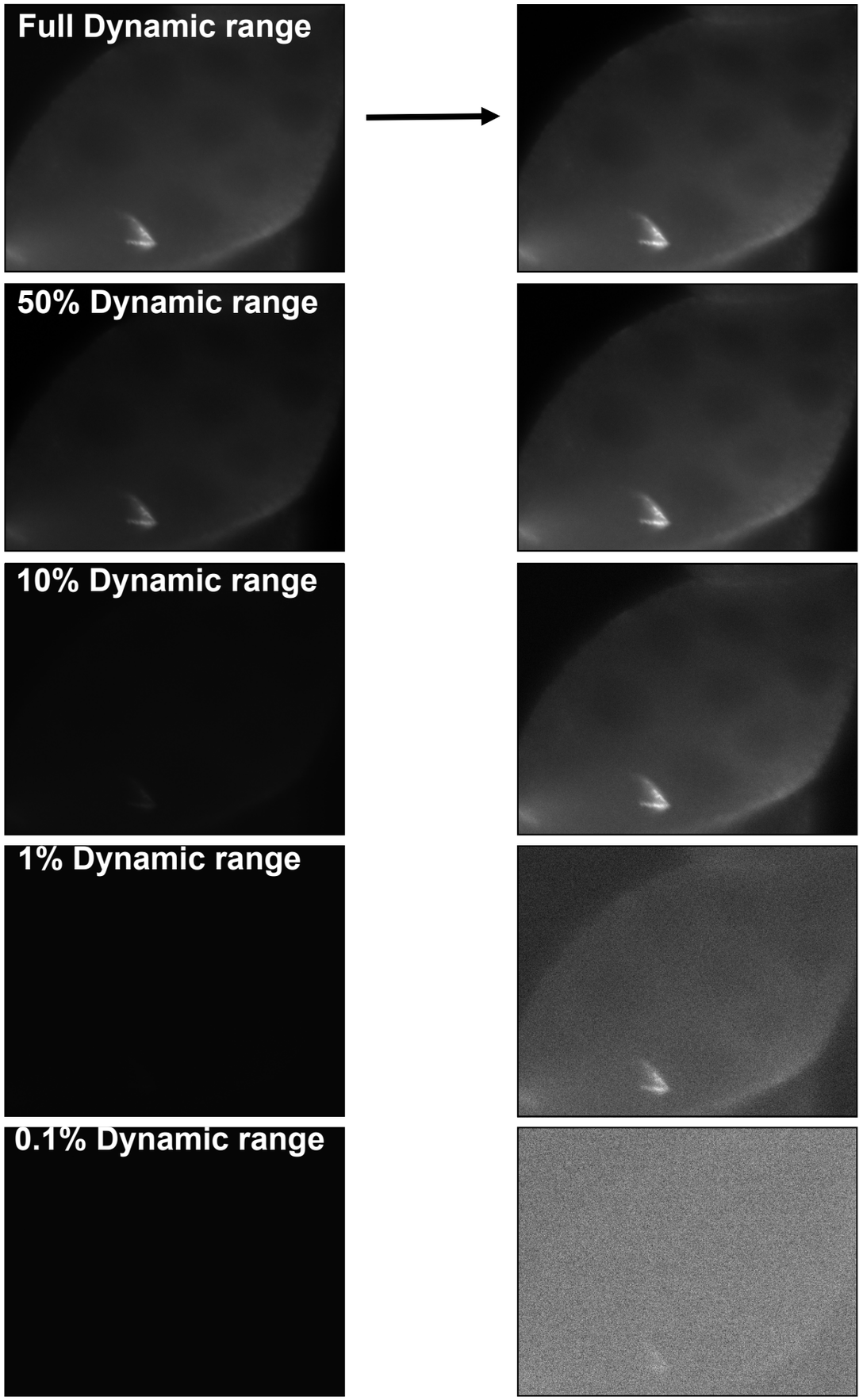

**Auto-intensity scaling by softWoRx scales the display to fill the dynamic range**

# Display Brightness and contrast

**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 signal**

**Increasingly Noisy looking**

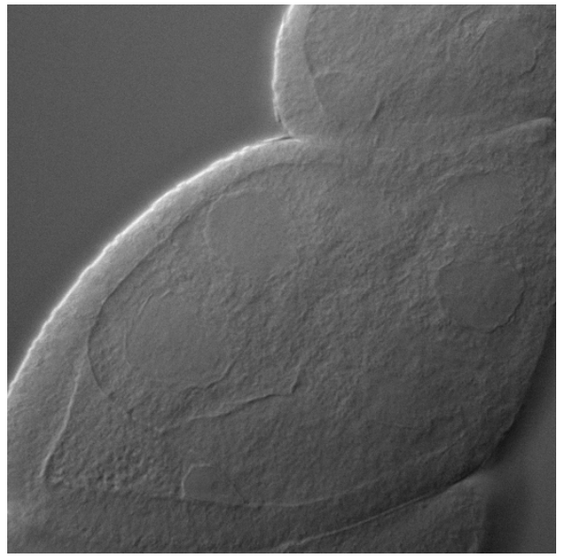
**Decreasing Image quality**

**Cannot resolve**

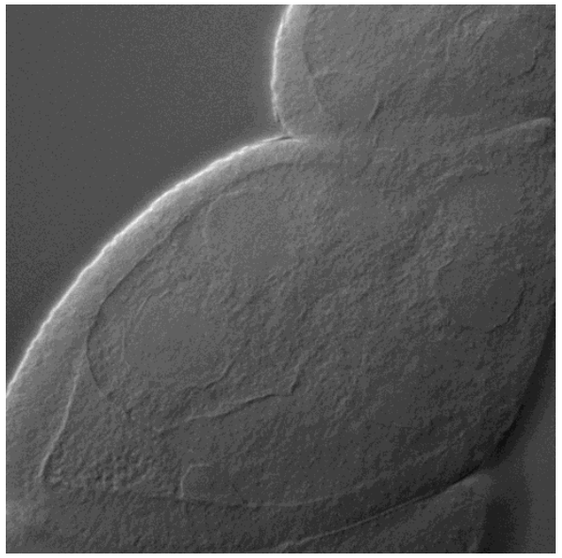
# LOW LEVEL Processing - Display, Bit Depth (levels)

- **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.**

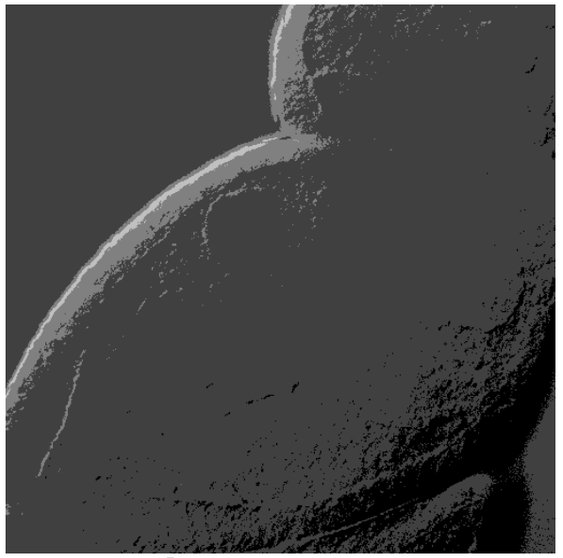
**False contouring due to insufficient grey levels** ⟶



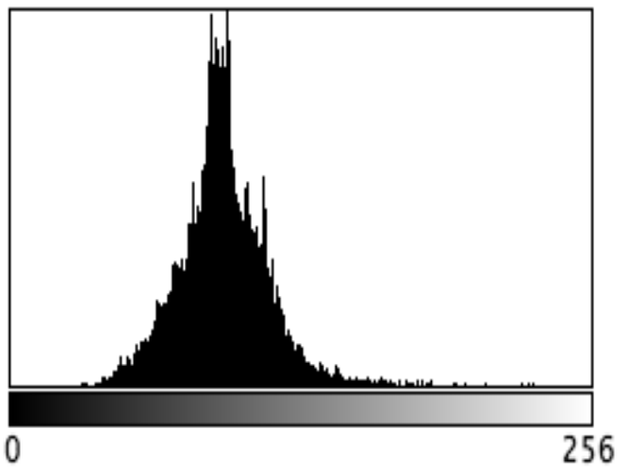
**256 greys**

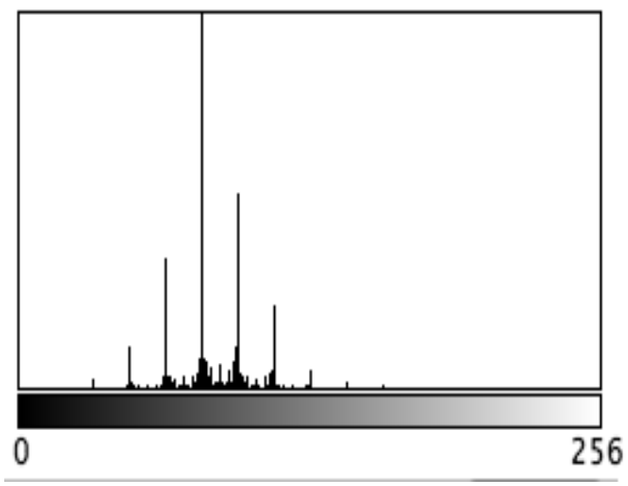


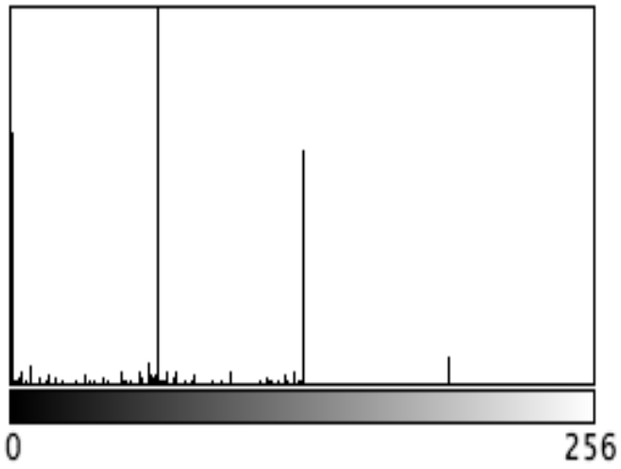
**16 greys**



**4 greys**

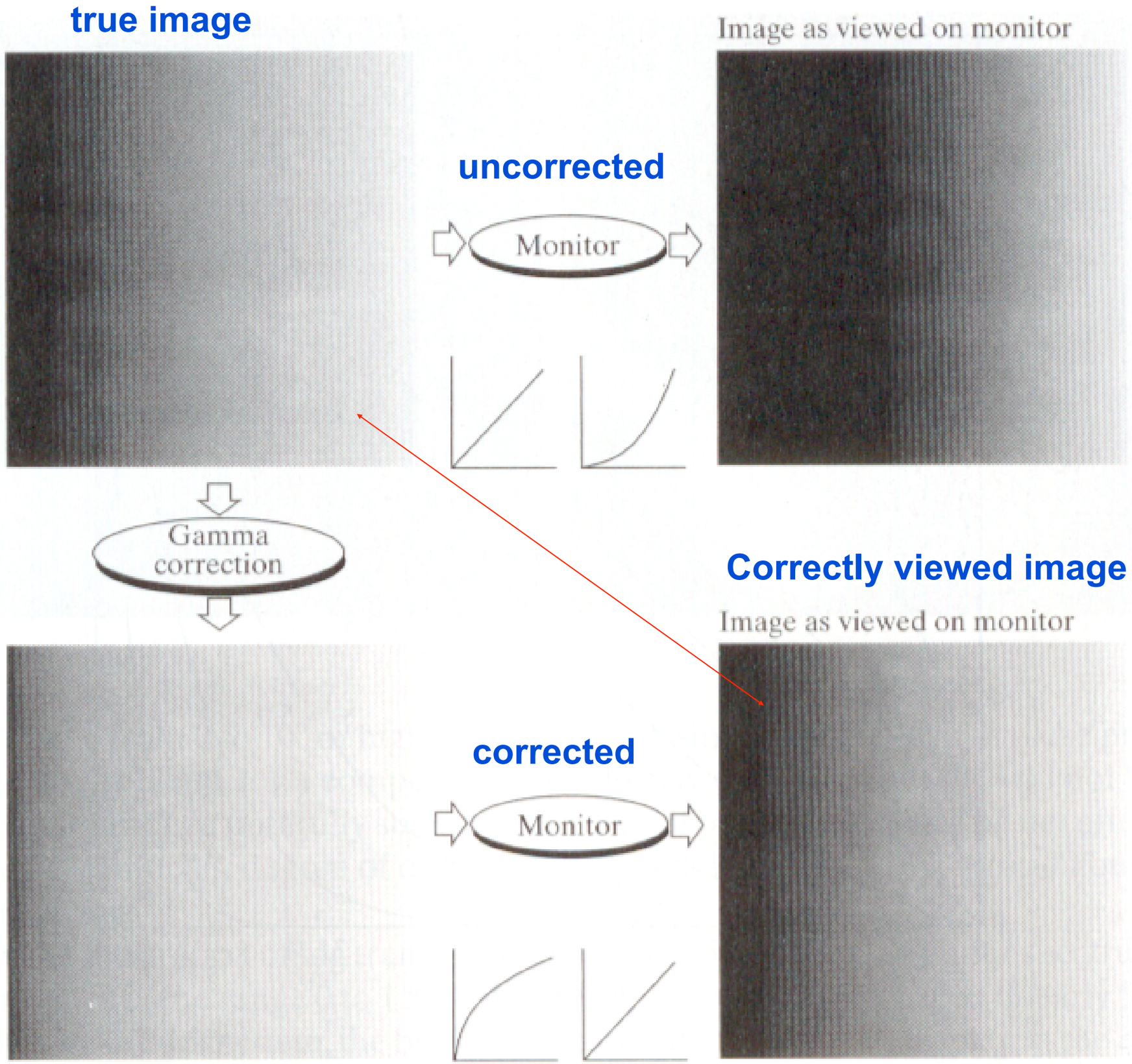



http://www.lenswork.com/calibrate.htm#tones

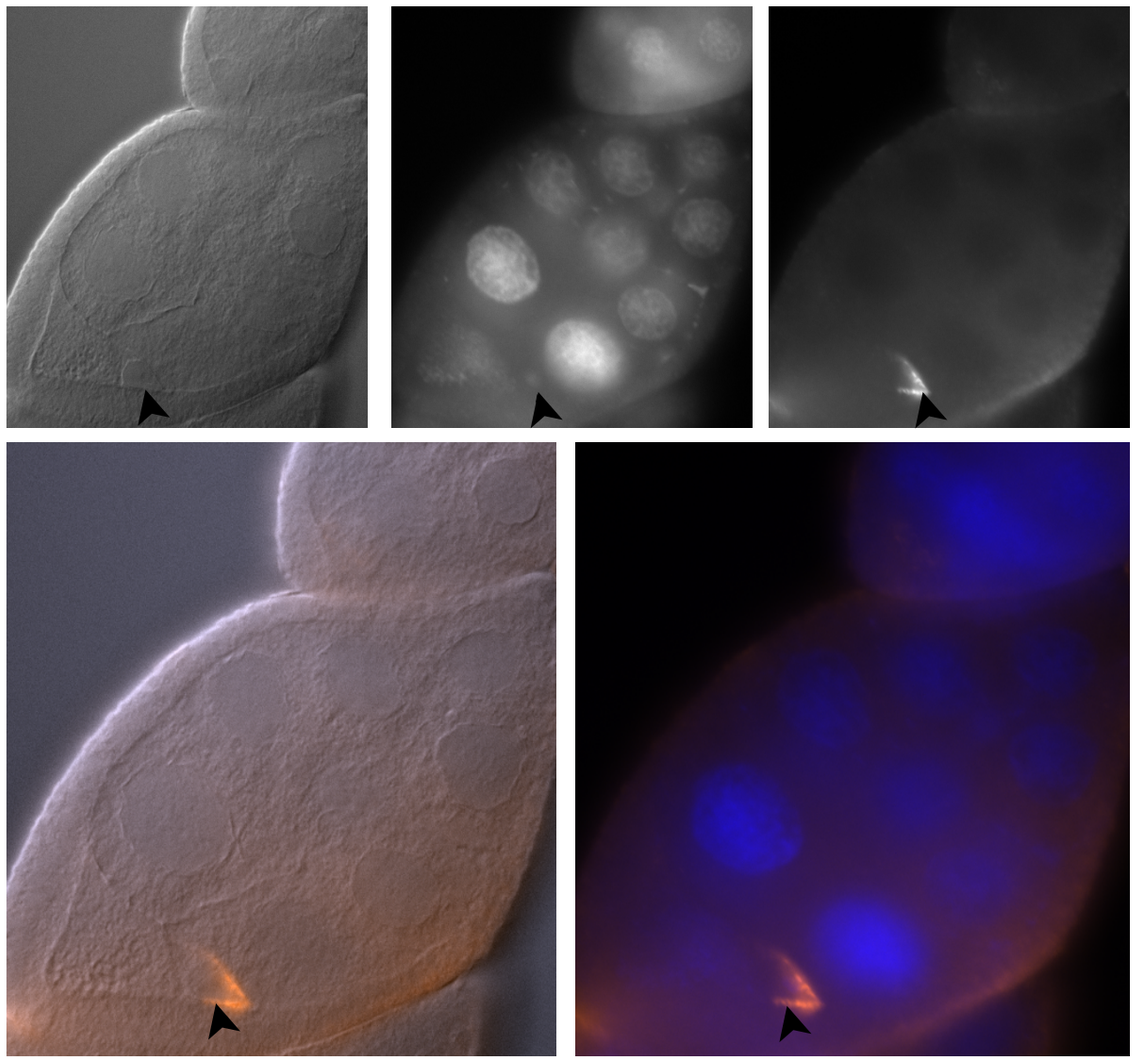
# LOW LEVEL Processing - Display, Using Gamma




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

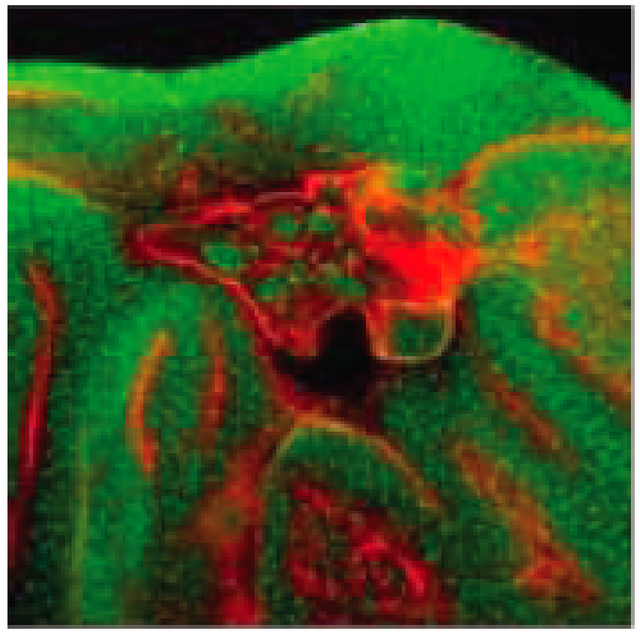


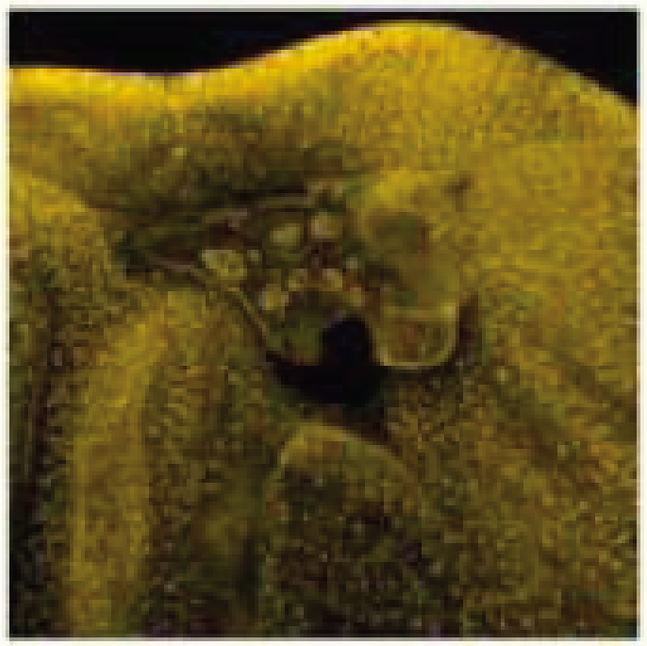
(arrowhead marks the oocyte nucleus)

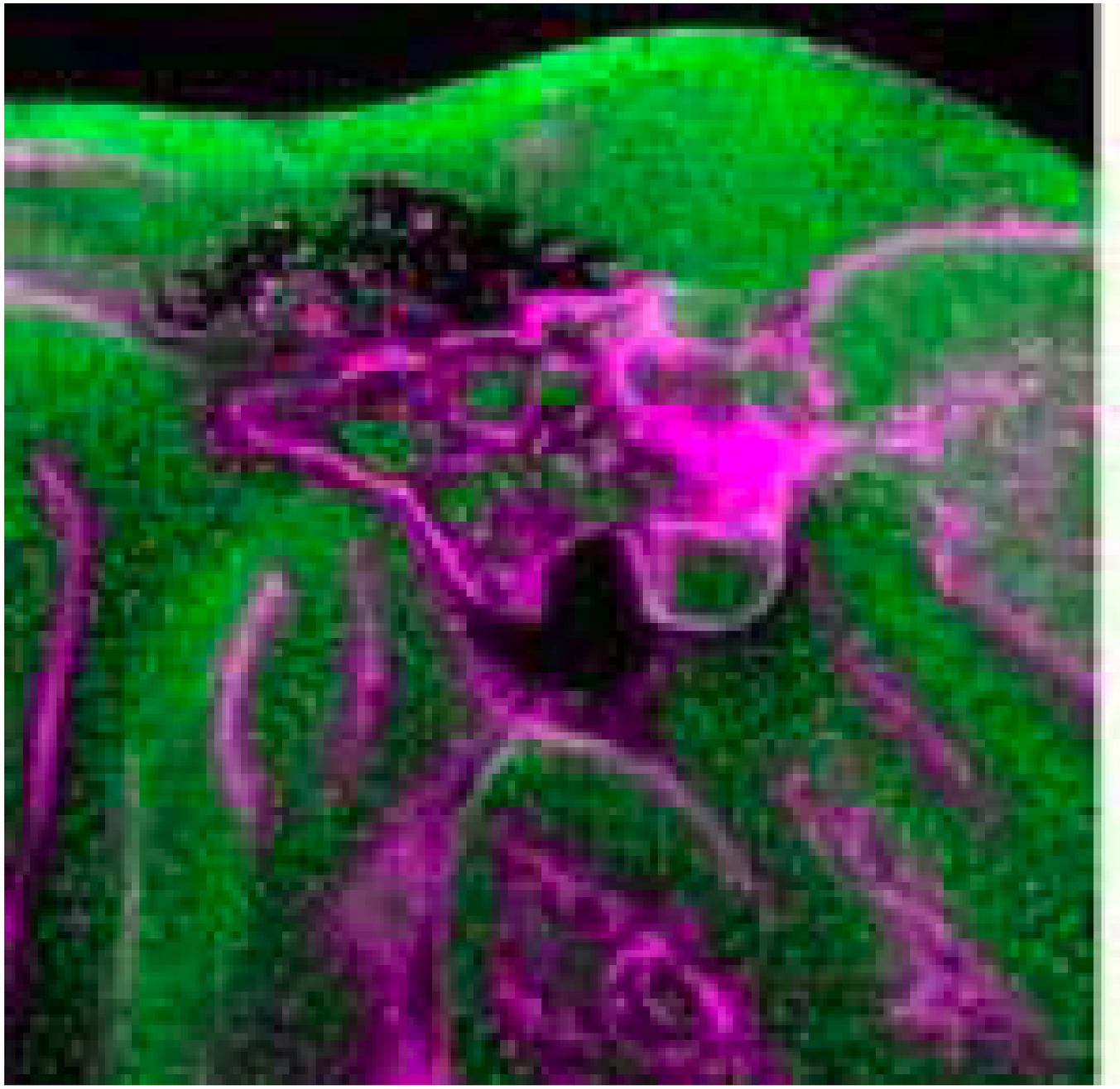
- Colour should be used for highlighting particular intensity differences / co-localisation.
- For publication show greyscale images alongside colour overlays.
- Consider colour-blind friendly colours: green / magenta / white.

# 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

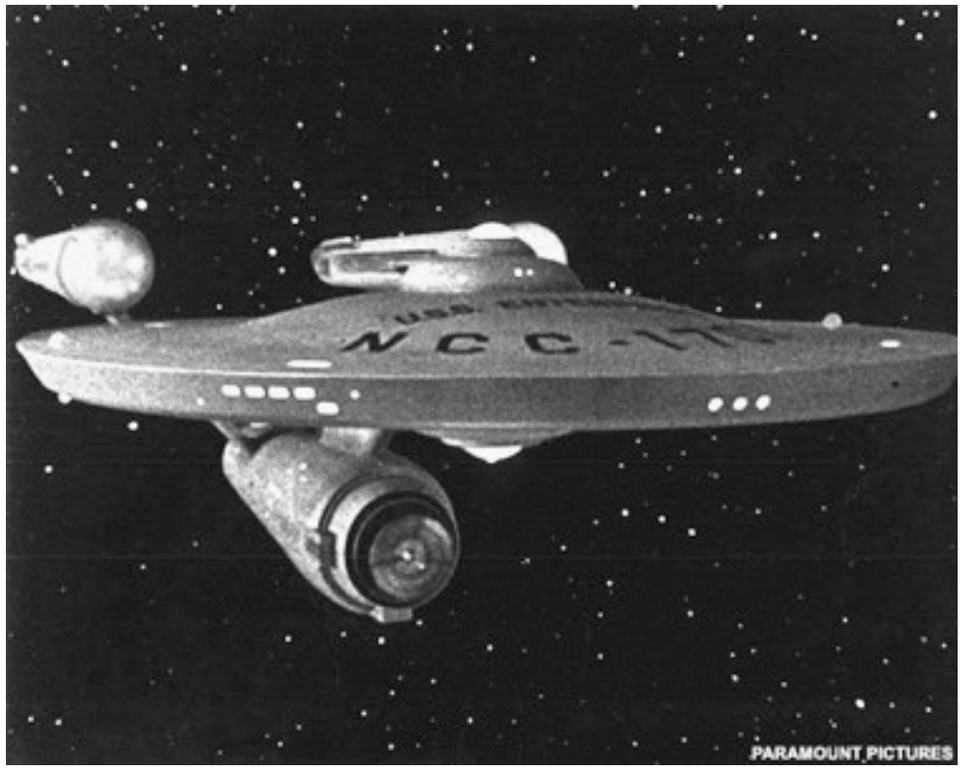
  Will open many file formats and export straight to .avi or .mov.,
  also can open tif image series (image001.tif; image002.tif; etc)
  restack and export to .avi or .mov. Has compression options for .mov

- Quicktime Pro 7

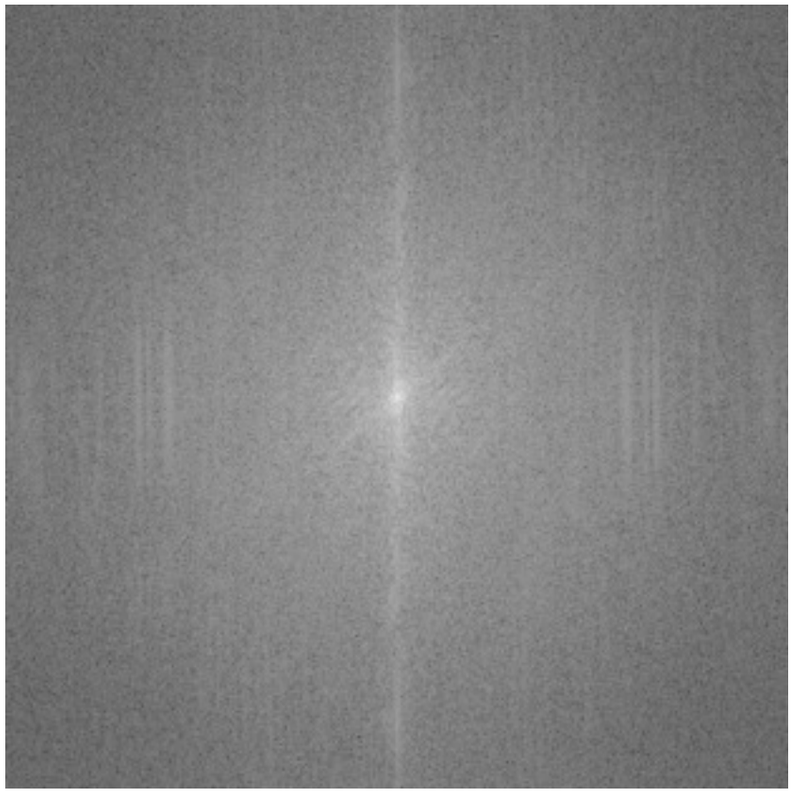
  Can open tif image series and export to .avi or .mov
  Can interconvert movie formats.
  Has a range of compression options for .mov

# 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

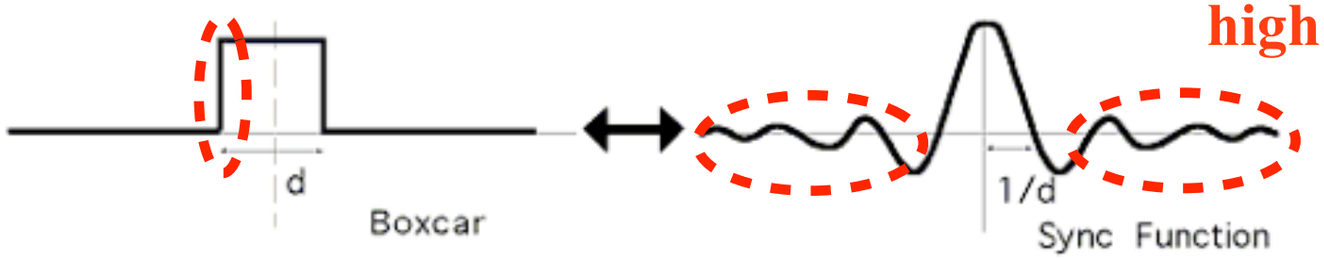
# Fourier Space = Frequency space
## = Reciprocal space
## = K space

**Data is broken down into its "frequency" components**

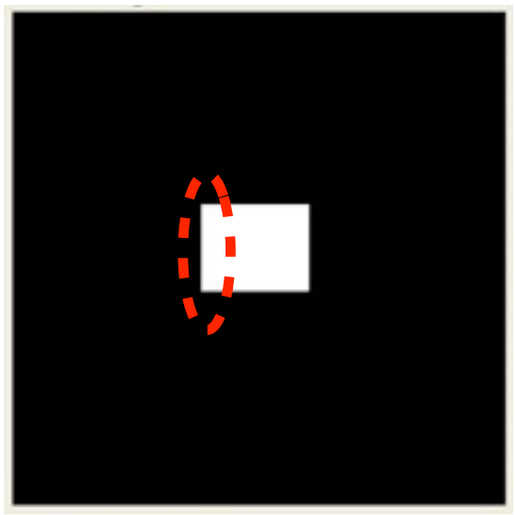
**real space** **reciprocal space**

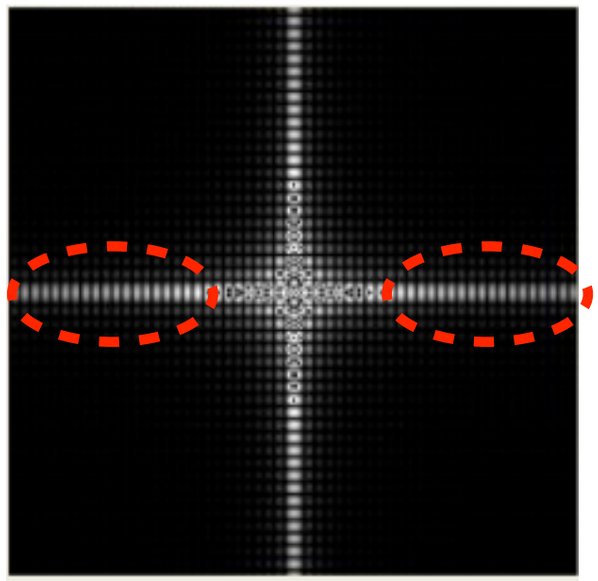
**fine detail = high frequencies**

**1D case**




**2D case**





**fine detail = high frequencies**

# LOW LEVEL Processing - Simple filter, spatial domain

- **Real space - pixel by pixel**

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

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

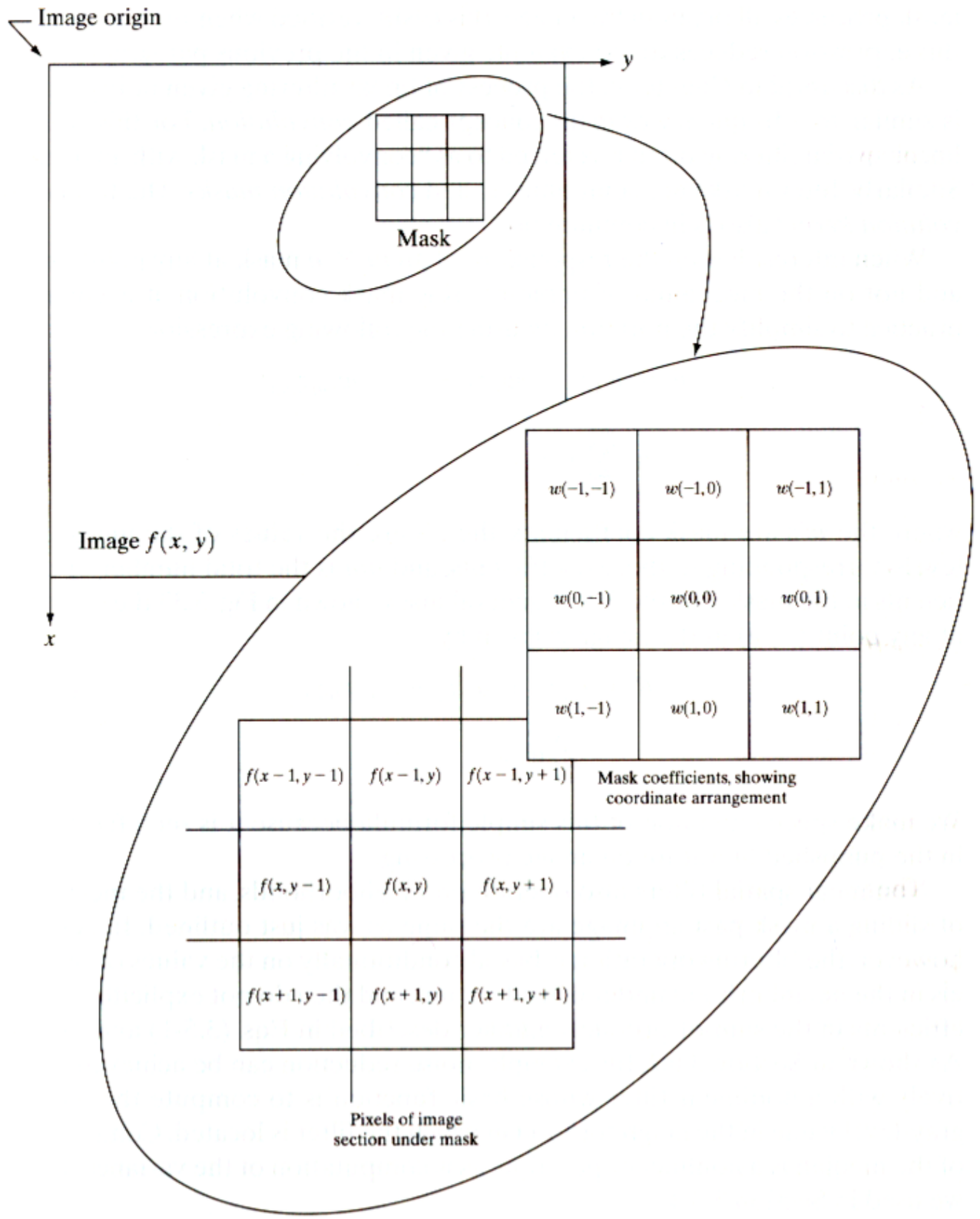
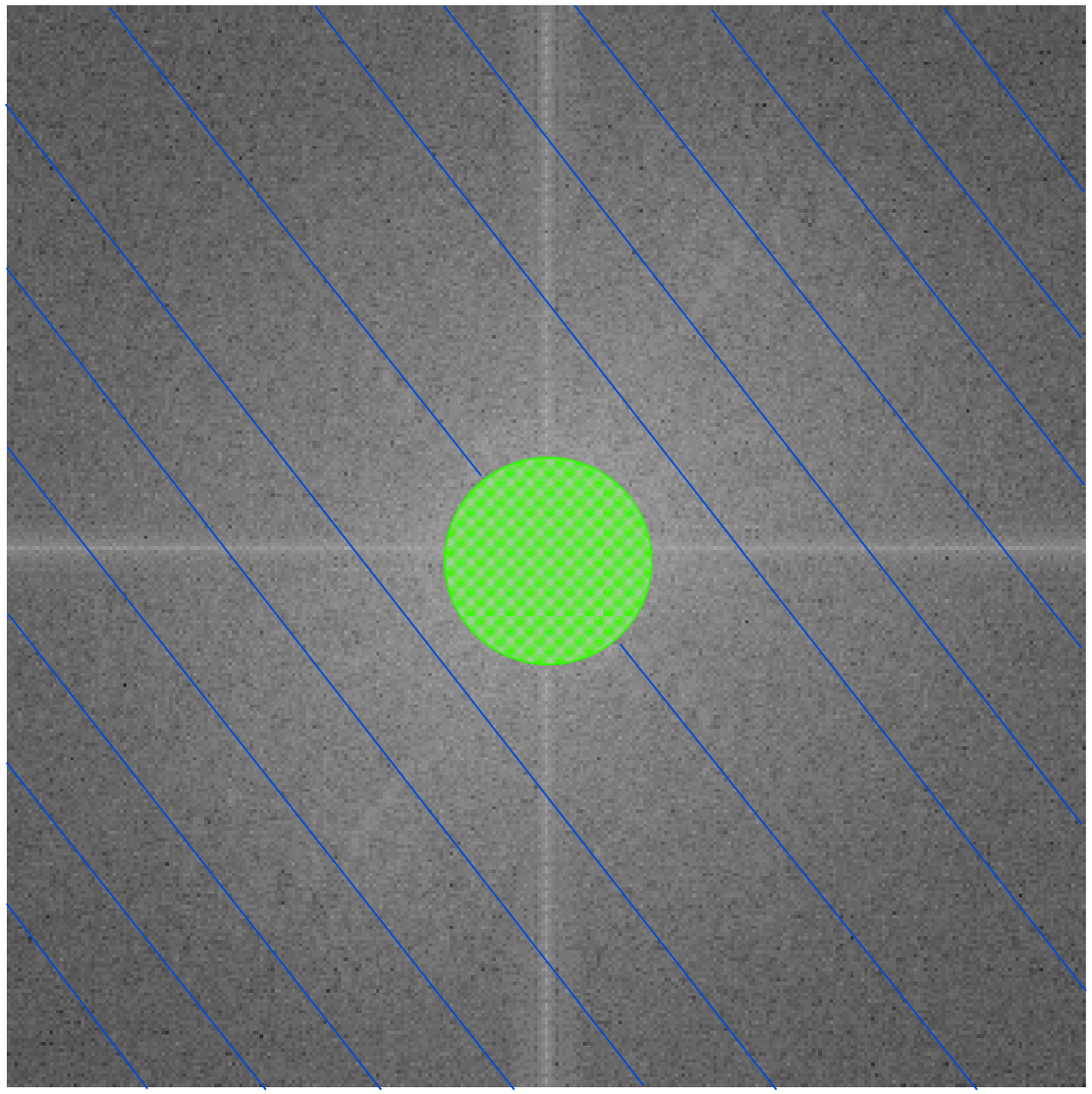



**FIGURE 3.32** The mechanics of spatial filtering. The magnified drawing shows a $3 \times 3$ mask and the image section directly under it; the image section is shown displaced out from under the mask for ease of readability.

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

# LOW LEVEL Processing - Simple filter, Fourier domain

- **Frequency space - all the image at once, computationally easier**
- Requires you to “transform” your image into **Reciprocal** or **Fourier** space
  e.g. in ImageJ: process, FFT (fast Fourier transform)



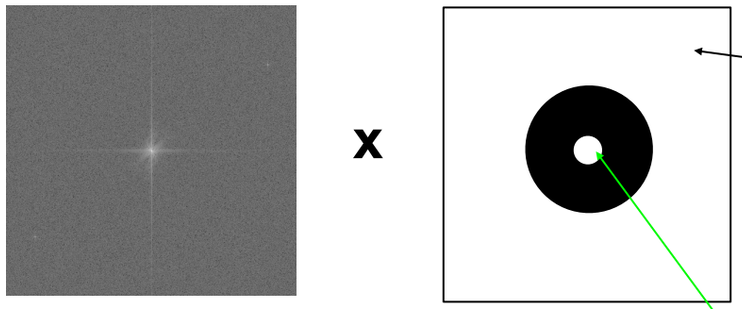
**Low frequencies = coarse detail**

**High frequencies = fine detail and noise**

# 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**

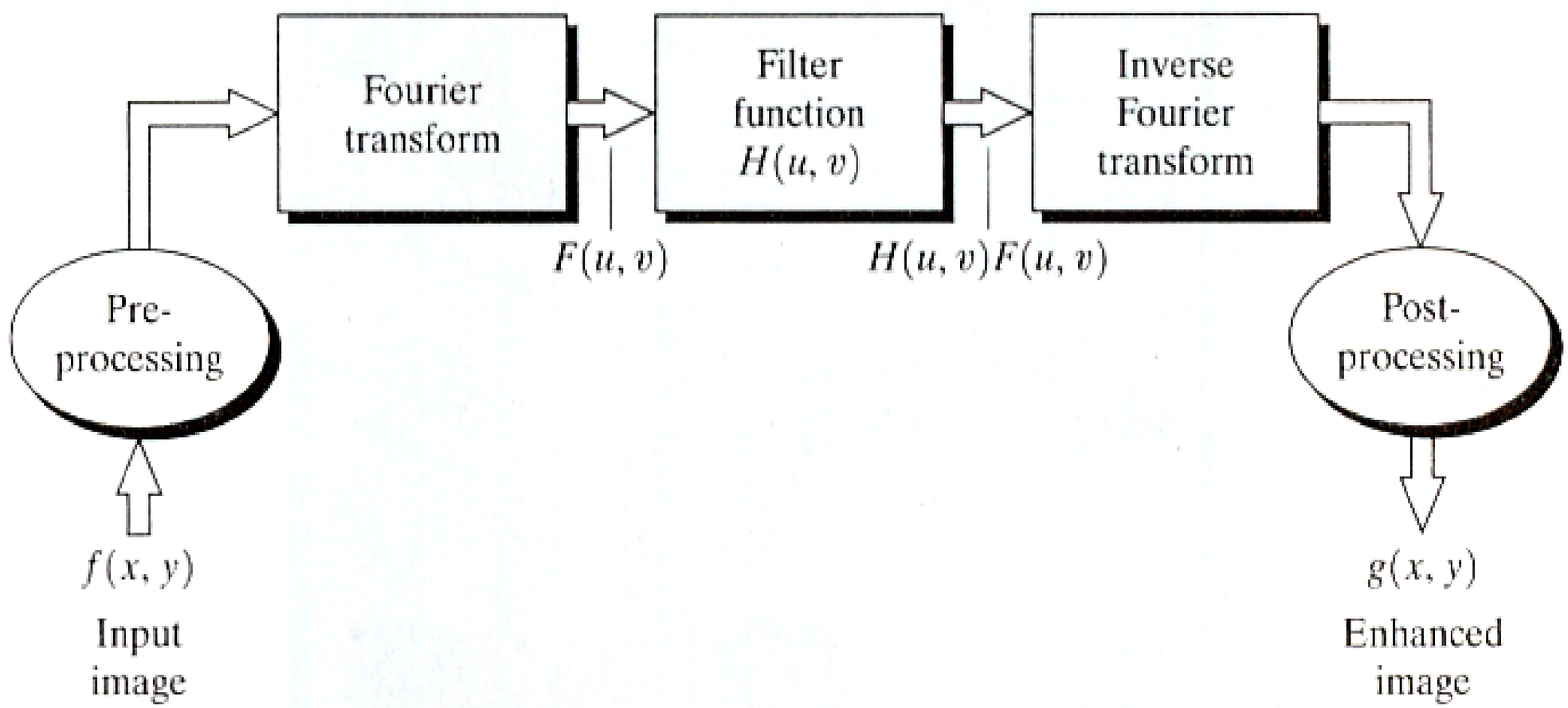
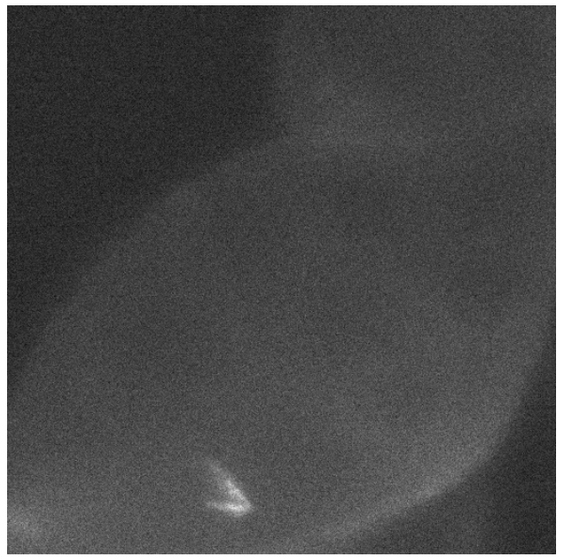



**FIGURE 4.5** Basic steps for filtering in the frequency domain.

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

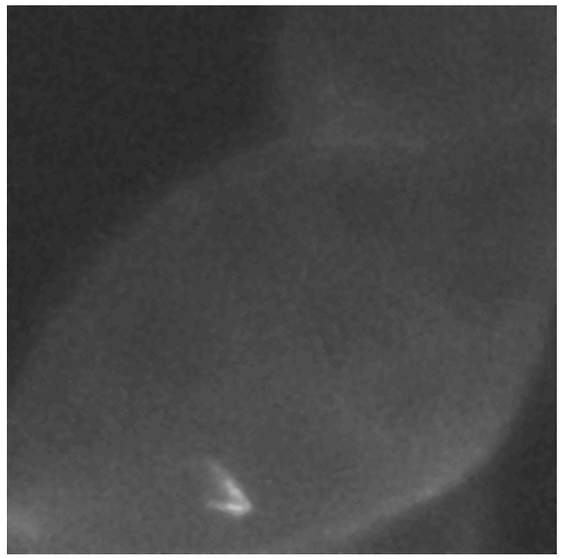
# LOW LEVEL Processing - Simple noise filtering



Noisy image

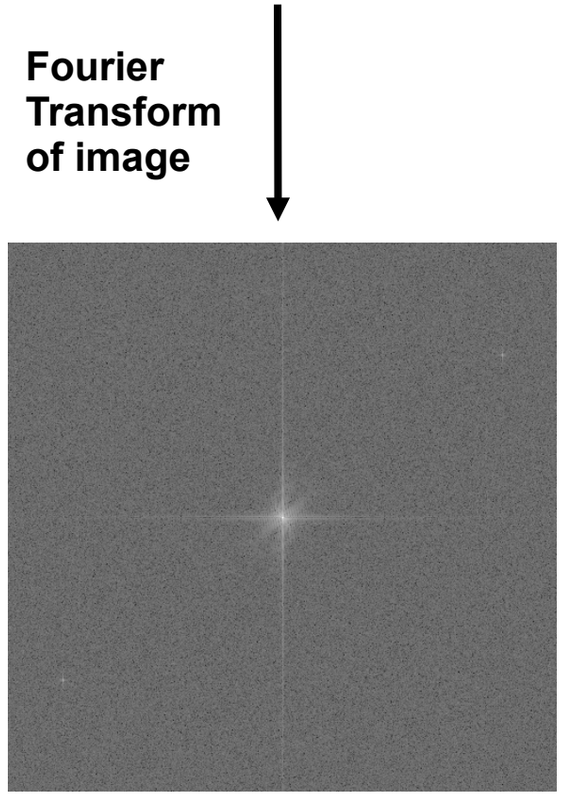
**Spatial domain processing**

3x3 Median
(high pass filter)



Noise reduced
Some false contours

Fourier Transform of image

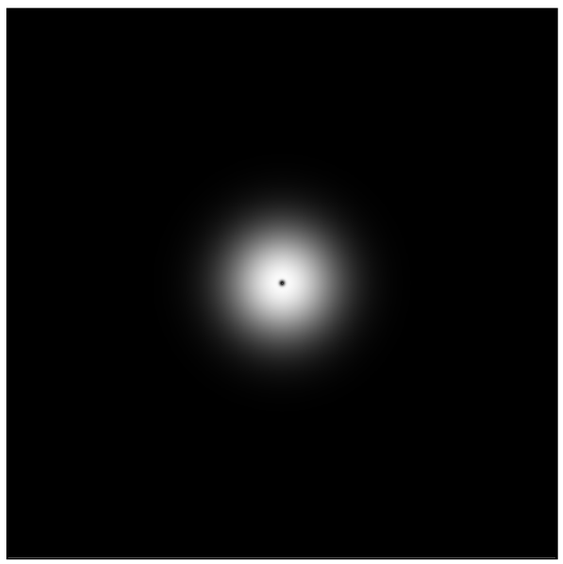


**Frequency domain processing**

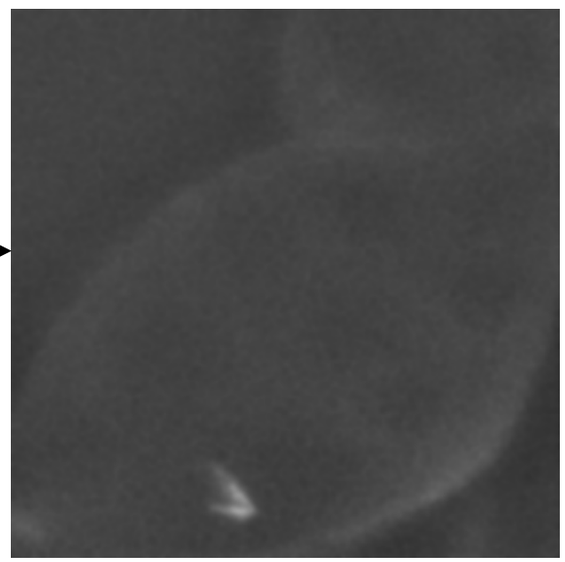
**x**

Multiply FT of image by "mask"

Band pass filter (mask)



inverse Fourier transform



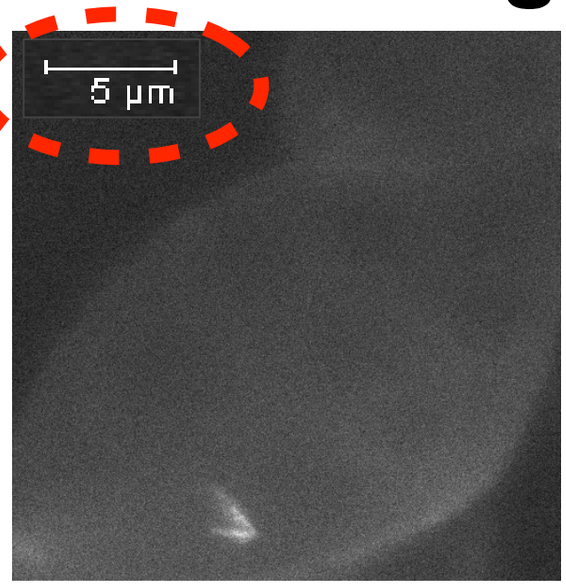
Selected frequencies <u>eliminated</u>
some loss of detail

Noise = high frequency component
Sharp boundries = high frequency component

(ImageJ)

# Noise Filtering

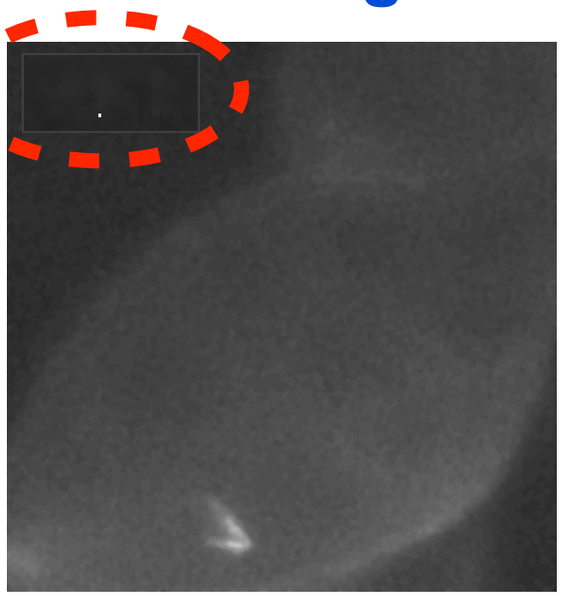
**limited use when resolving fine detail!**




Noisy image

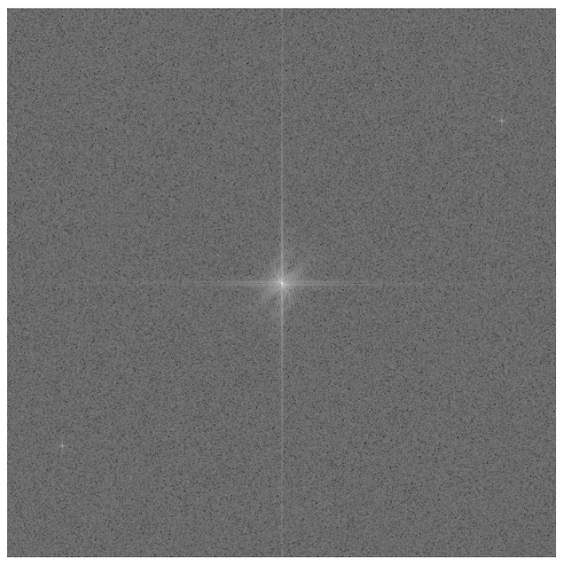
**Spatial domain processing**

**3x3 Median**
**(high pass filter)**



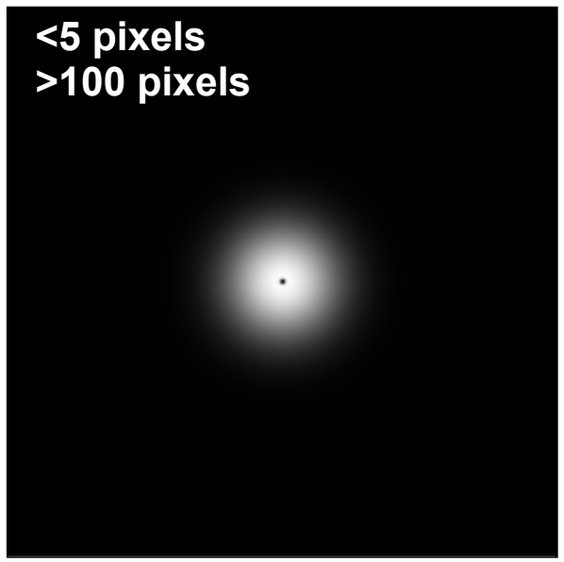
**Noise reduced**
**Some false contours**

**Fourrier transform**

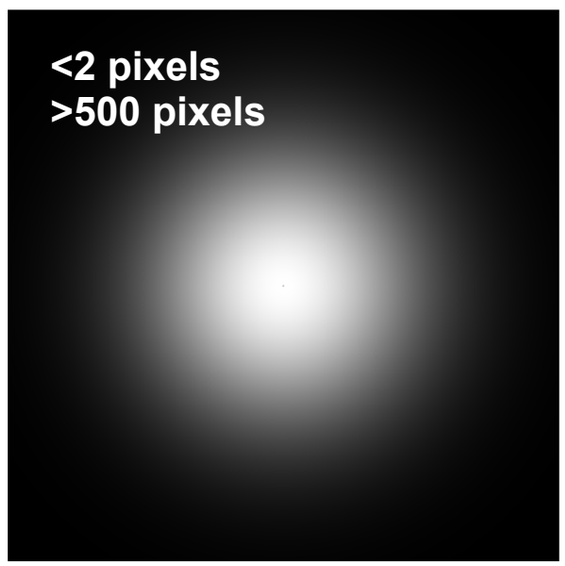


**Frequency domain processing**

**Band pass filters (mask)**



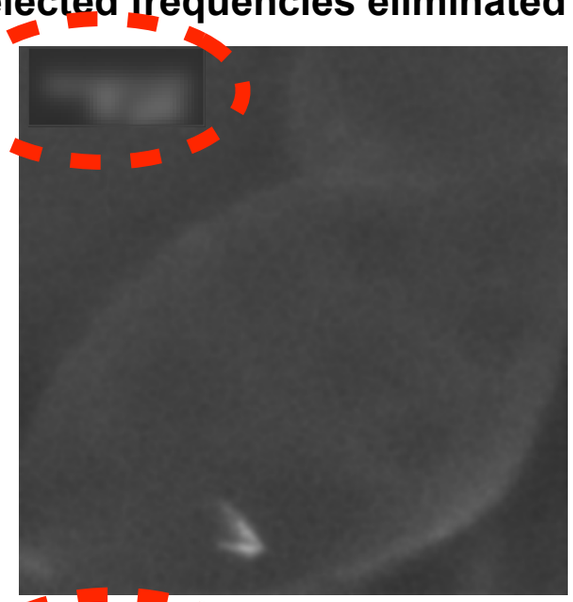

X

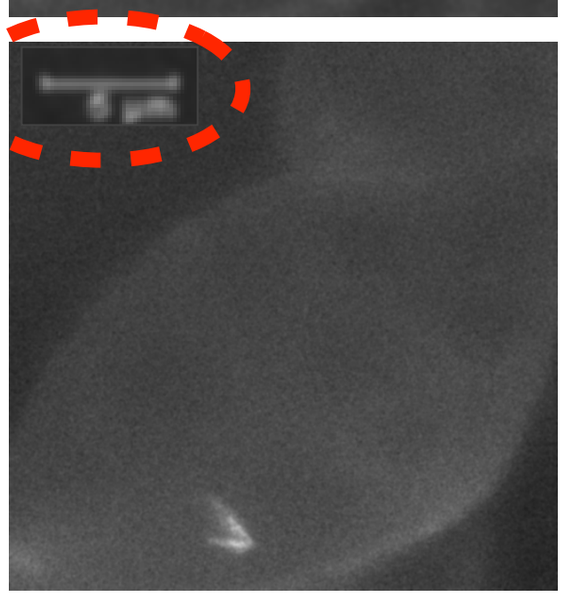



X

**inverse Fourrier transform**

**Selected frequencies eliminated**

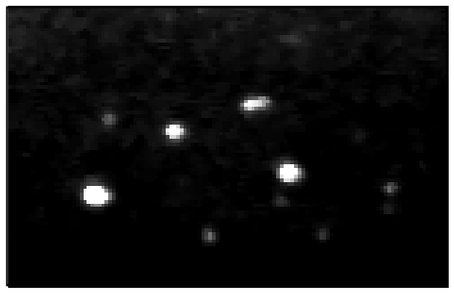




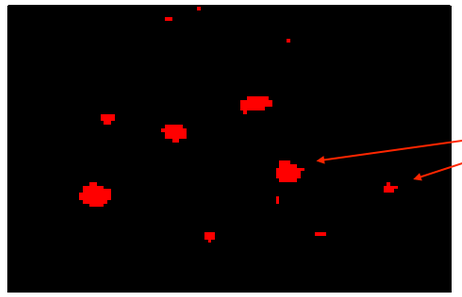
ImageJ

# MID LEVEL Processing

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



**Segmentation**



**Objects defined**

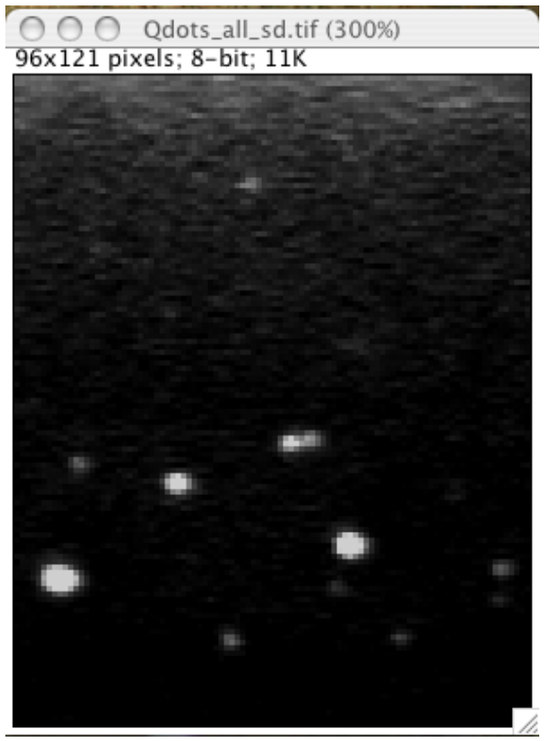
**Number of objects**
**Positions of objects**
**Size of objects**

**Also:**
**- Colocalisation**

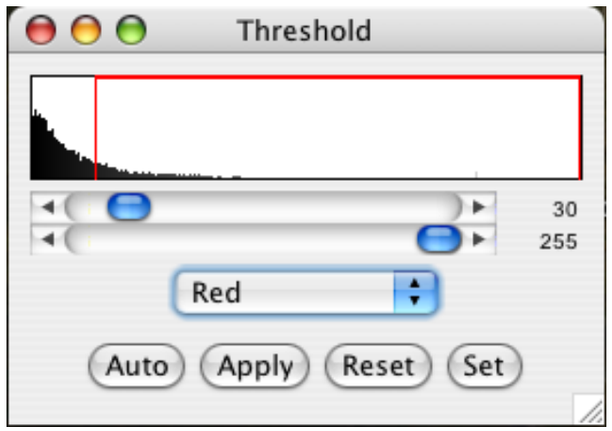
**(See appendices)**

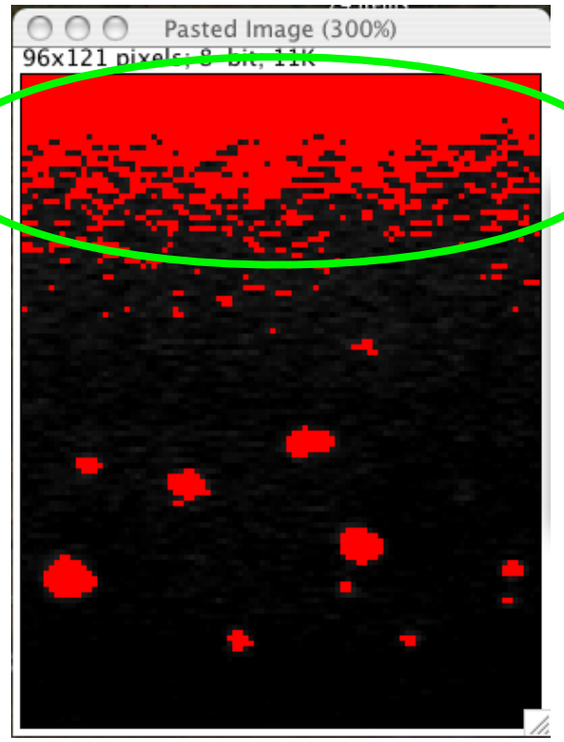
# MID LEVEL Processing - simple segmentation



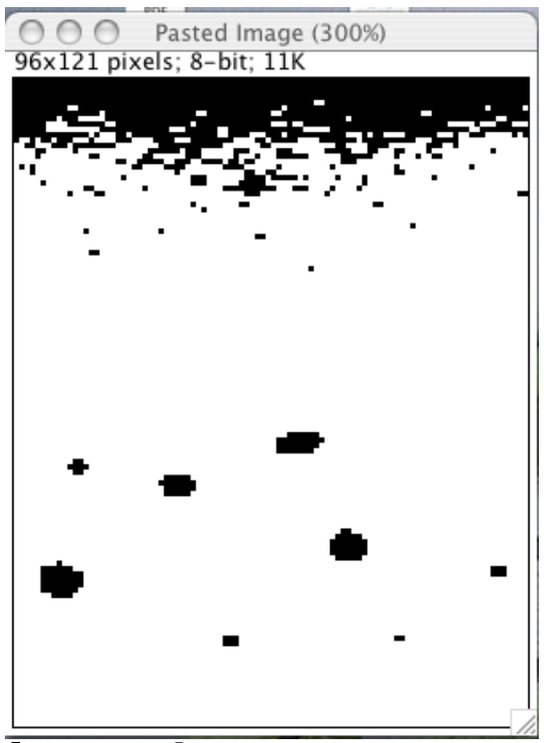



**Segment by intensity**



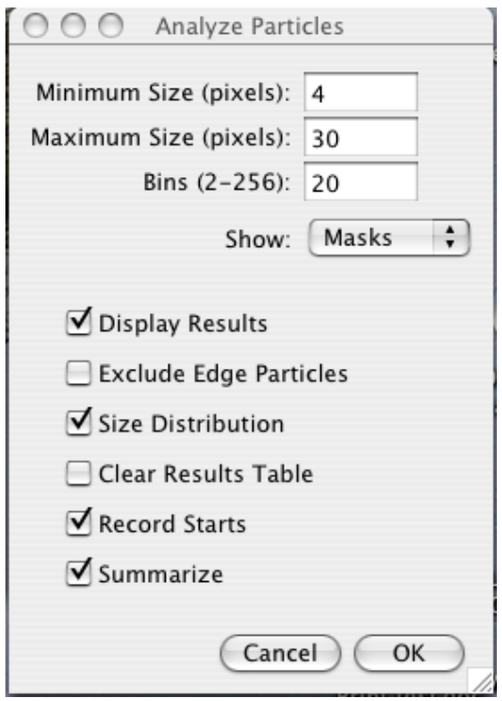



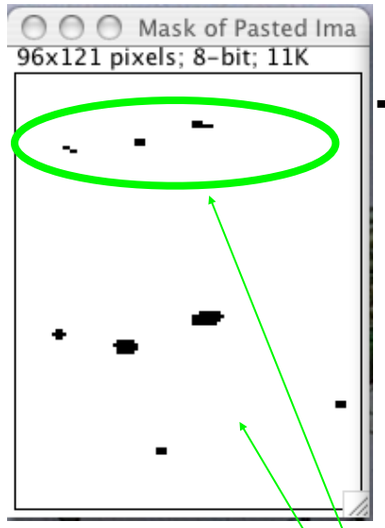

**problem region**



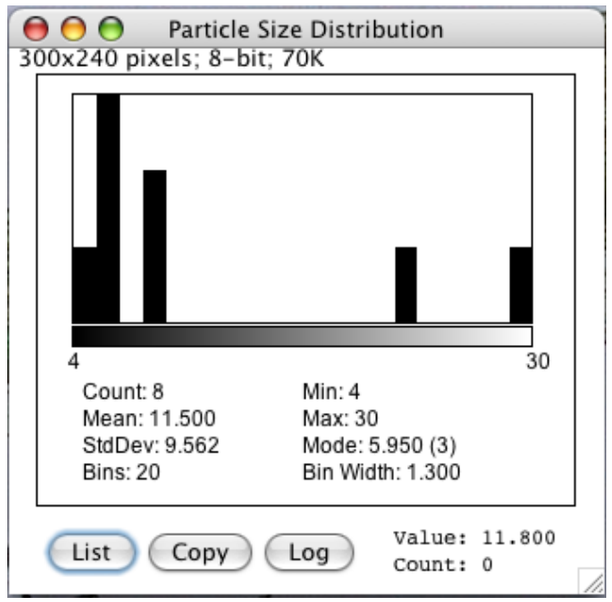

**Intensity segmented binary image**

**Segment again by area size**




**mask defining regions**

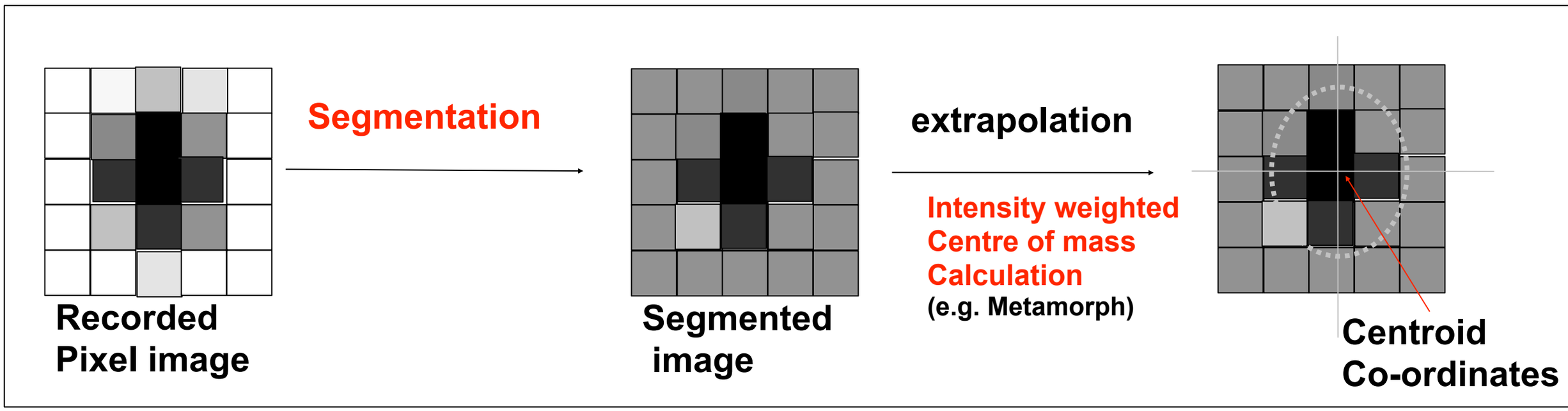



**attributes**

**Some particles lost - some artificially created**

ImageJ

# MID LEVEL Processing - centroid




- **Intensity weighted centre of mass** (takes area and intensities into account)
- Output is centroid XY co-ordinates
- **Precision** is greater than the resolution limit
- Seriously limited by **noise** and **undersampling**

**The human brain is still one of the best segmentation tools**

**But**

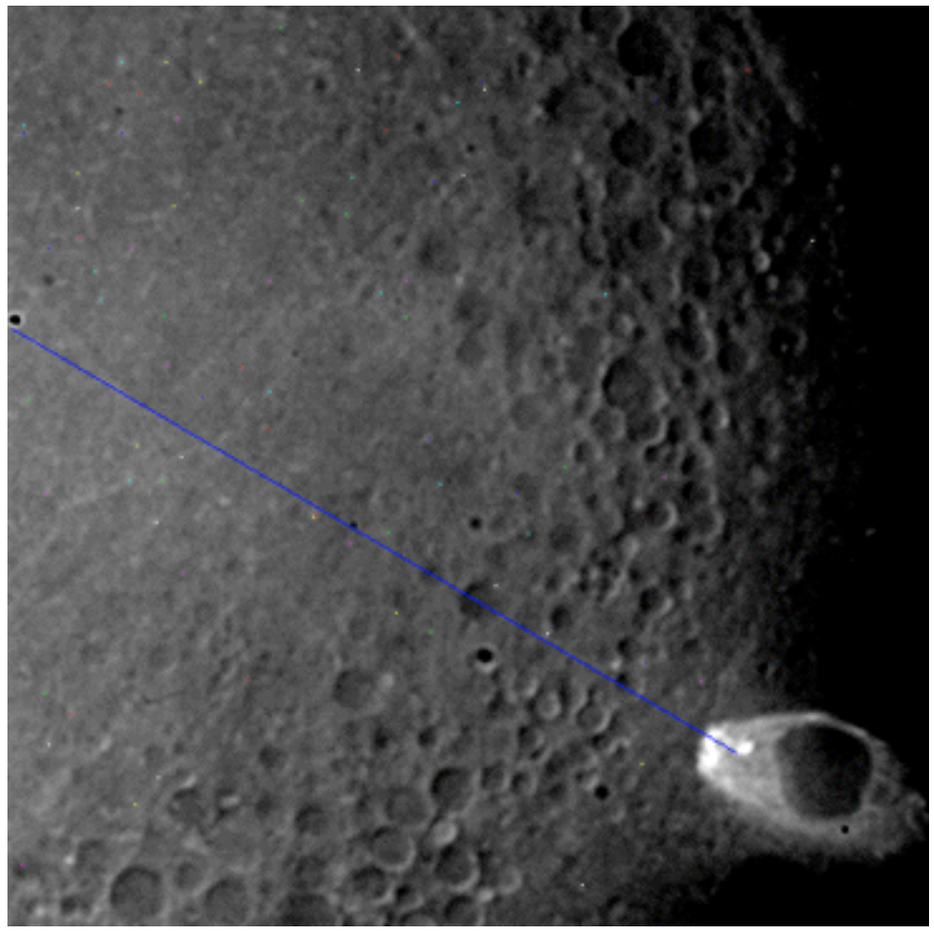
**Subjective not objective!**

**Automated segmentation desirable for data quality and sanity**

# HIGH LEVEL Processing

- **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**

*LECTURE 13*

**Also:**
**- Ratiometric analysis**
**- Quantative analysis and Statistics**

**(See appendices)**

# Statistical analysis

- Image data requires appropriate statistical analysis:
  - Experimental design, n-numbers, true independent repeats, numbers
  - Variation has to be considered within and between images

- May require specialist statistical treatments:
  - Image values are like percentages, constrained maximum value
  - Ratio images - skewed distribution of values
  - Statistics of directionality - "Circular statistics"

# Reference Material

# Useful sources of Information:

- **Confocal List Archive**
  http://listserv.acsu.buffalo.edu/cgi-bin/wa?S1=confocal

- **Articles**
  Rossner & Yamada (2004). What's in a picture? The temptation of image manipulation. J. Cell Biology 166: 11–15.
  Oberholzer M, et al. (1996). Methods in quantitative image analysis. Histochem Cell Biol 105(5): 333-55.
  Swedlow et al., (2002). Measuring tubulin content in Toxoplasma gondii: A comparison of laser-scanning confocal and wide-field fluorescence microscopy. PNAS 99:2014–2019.

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

  Davis, I. (2000). Visualising fluorescence in Drosophila - optimal detection in thick specimens. In Protein Localisation by Fluorescence Microscopy: A Practical Approach, V. J. Allan, ed. (Oxford, OUP), pp. 131-162.

- **General Web Sites**
  http://www.microscopyu.com/articles/digitalimaging/index.html
  http://www.cb.uu.se/presentation/baintro/index_eng.html
  http://www.olympus-europa.com/medical/39_MicroGlossary.cfm = Jargon buster
  http://www.olympus-biosystems.com/downloads/OlympusBioSystems_analySIS_BD.pdf

# Processing software

## Display

- **Adobe Photoshop, MAC/PC**
- **Jasc Paintshop Pro, PC**

## Image processing

- **NIH ImageJ FREE, platform independent**
  http://rsb.info.nih.gov/ij/
  http://www.uhnresearch.ca/facilities/wcif/imagej/ (An ImageJ manual)
  http://www.uhnres.utoronto.ca/facilities/wcif/download.php (more ImagJ stuff)

- **Universal Imaging Metamorph expensive, PC**
  http://www.universal-imaging.com/products/metamorph/image-analysis.cfm

- **Bitplane (Imaris) expensive, PC**
  http://www.bitplane.com/products/imaris/imaris_product.shtml
  http://www.appliedprecision.com/lifescience/DeltaVisionRT.html

- **Intelligent Imaging Innovations Slidebook expensive, PC (incomplete on MAC)**
  http://www.intelligent-imaging.com/slidebook/features.php

- **Appllied precision expensive, Linux (MAC/PC versions = explorer)**
  http://www.appliedprecision.com/lifescience/softworxexplorersuite.html

- **Improvision - Velocity, 3D reconstruction expensive, (PC, MAC)**
  http://www.improvision.com/velocityle

# Additional Topics: further reading

**Segmentation**
- Gonzales & Woods (2002). Digital Image Processing 2nd Ed. Prentice-Hall Inc, USA.
- http://www.mia.uni-saarland.de/Research/Segmentation.html
- http://appsrv.cse.cuhk.edu.hk/~kbxu/research/image_seg.html

**3D reconstruction e.g. Improvision - Velocity**
- http://www.improvision.com/velocityle
- http://biocomp.stanford.edu/3dreconstructuion/software/index.html

**Movement Tracking**
- Tvarusko et al, 2004. Analysing live cell data and tracking Dynamic movements. In Live Cell Imaging a laboratory Manual. Eds RD Goldman and DL spector. CSHL Press, USA. PP 303-326.
- http://www.improvision.com/support/tips/Default.lasso?-database=vipoir.FP3&-response=detail.lasso&recID=9&-search

**Ratio imaging**
- Parton & Read (1999). Calcium and pH imaging in living cells. In Light Microscopy in Biology - a practical approach 2nd Edition. Ed. A.J. Lacey. Oxford University Press.

**Deconvolution**
- Parton & Davis (2006). Lifting the fog: image restoration by deconvolution. In Cell Biology Vol 3. Ed. J.E. Celis. Elsevier Press.
Wallace, W., Schaefer, L.H., Swedlow, J.R. (2001) A workingperson's guide to deconvolution in Light microscopy. BioTechniques *31*, 1076-1097.

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

# Additional Topics: further reading

**Image archiving/ - Open Microscopy Environment** http://www.openmicroscopy.org/
**annotation**

**Co-localisation / spectral unmixing (an expanding area):**

**Often provided with multichannel imaging systems**
http://las.perkinelmer.com/content/ApplicationNotes/006914-MembraneTraffickingUsingUltraVIEWRS.pdf
http://www.boselec.com/products/documents/FLIMFcorrelationforLSMs4-03.pdf
http://www.olympus-biosystems.com/downloads/OlympusBioSystems_Spectral_Unmixing.pdf
http://www.olympus.co.uk/medical/22_FV1000_Key_Features.htm
http://www.olympusfluoview.com/theory/bleedthrough.html
http://www.nyas.org/ebriefreps/ebrief/000321/rr/rr06.pdf
http://www.olympus-biosystems.de/templates_eng/applications/spectral_unmixing.html

**Co-localisation with deconvolved data – better XYZ discrimination, 3D modelling**
Wiegand U.K., et al. (2003). Red, yellow, green go! - a novel tool for microscopic segregation of secretory vesicle pools according to their age Biochem. Soc. Trans. *31*, 851-856.

**Alternative to simple colocalisation is FRET ("molecular ruler")**
GU, et al., (2004) Quantitative fluorescence resonance energy transfer (FRET) measurement with acceptor photobleaching and spectral unmixing. *Journal of Microscopy* **215** (2), 162-173.

# Additional Topics: further reading

**Denoising** http://www.irisa.fr/vista/Themes/Demos/Debruitage/ImageDenoising.html

# Low level Processing: Printing - ICC profiles

(for information)

**DEFINITION**

ICC profile = A file that describes how a particular device reproduces color. The profile defines device gamut in the context of a device-independent color space.International Color Consortium (ICC) = The group established by eight industry vendors (including Adobe Systems) for the purpose of creating, promoting, and encouraging the standardization and evolution of an open, vendor-neutral, cross-platform color management system architecture.

ICC profiles are files that are embedded as part of a document or image. These profiles describe specific information about the image and the output device. These profiles, if used improperly, can cause unwanted color conversions.

ICC profiles are normally embedded by the application saving the PDF, TIFF, or EPS file. To prevent the application you are using from embedding ICC profiles, look closely at the color settings or color management settings for your application. Refer to the application's user manual or online help for more information.

http://www.color.org/ = ICC colour profile standards
http://www.photoexpert.epson.co.uk/UK/EXPERTISE/how_to_icc_page1.htm (for RGB files)
http://www.adobe.com/support/downloads/detail.jsp?ftpID=2347 (downloads of Adobe ICC profiles)
http://www.bodoni.co.uk/profiling/colourprofiling.html#monitor

# Comparing image data

## Absolute requirements:

- Systematic image capture parameters
- Systematic image processing and display
- Do the same to experimental and control samples, ideally collect in the same Imaging session

## Whenever possible:

- Be systematic in both microscope setup and image capture parameters.
- Image controls in the same imaging session as experimental data.
- Do the same processing and display with your controls as you do to your experimental images.
- Use internal standards within the experimental design e.g. keep one probe in common.

- Be very careful directly relating absolute intensity values between image sets, better to compare normalised intensity values. Be aware of the contribution of noise to intensity variation between pixels.
- Positional information is more easily compared.

Swedlow et al., (2002). Measuring tubulin content in Toxoplasma gondii: A comparison of laser-scanning confocal and wide-field fluorescence microscopy. PNAS 99:2014–2019.

# Co-localisation

- Co-localisation analysis
- Spectral unmixing tools
- Ideal case:

  Spectrally distinct markers - no bleed-through / cross talk between channels
  Distinct probe localisation - low background
  Image intensities directly proportional to concentrations of target molecules

  Co-localisation of intensities indicates that:

  Within the limits of the optical resolution - probes occur together
  i.e. there are corresponding sites of local enrichment

  however there is:

  no direct evidence of interaction
  only limited direct evidence of stochiometry

- Co-localisation and Spectral unmixing software helps to deal with non-ideal situations of background and markers which are not spectrally distinct.
- Using correct image capture / processing techniques particularly important so not to misrepresent data
- Require controls imaging the single probes under the same conditions as co-localisation

# More details on co-localisation analysis (edited comments from the confocal list)

Co-localisation – more-or-less subjective, difficult to be strictly objective and quantitative.

Therefore it is important to establish valid and stringent criteria and stick to them throughout your experiments. Then you will have a basis to decide if there is more/less/no co-localization in response to treatment. Using automations/software in a mindless manner will not help

Does the finding make biological sense? That is pre-eminent. Conclusions should be supported by more than one method- one should be able to reproduce protein binding and/or immuno-precipitation of the complex.

Merging an essentially 'green' image field with a 'red' one and showing 'yellow' without any detail. Does not represent co-localisation. Specific details must be evident.

Correlation coefficient

Intensity Correlation Analysis from Li et al. Journal of Neuroscience 24, 4070-4081

Based on position and relative intensities: each pixel from the 8-bit image is represented as a pixel on a graph with the x-axis as, for example, 0-256 grey levels for channel 1 (green) y-axis 0-256 grey levels for channel 2 (red). So, a purely red pixel will be placed adjacent to the y-axis, a purely green pixel next to the x-axis, and anything else plotted in between. The assumption is that a pixel plotted near the diagonal where x and y are equivalent represents a point of colocalisation.