Advanced Microscopy Course 2012
Lecture 5: Basic Image Processing

Lecture 16: Applied Image Processing
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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

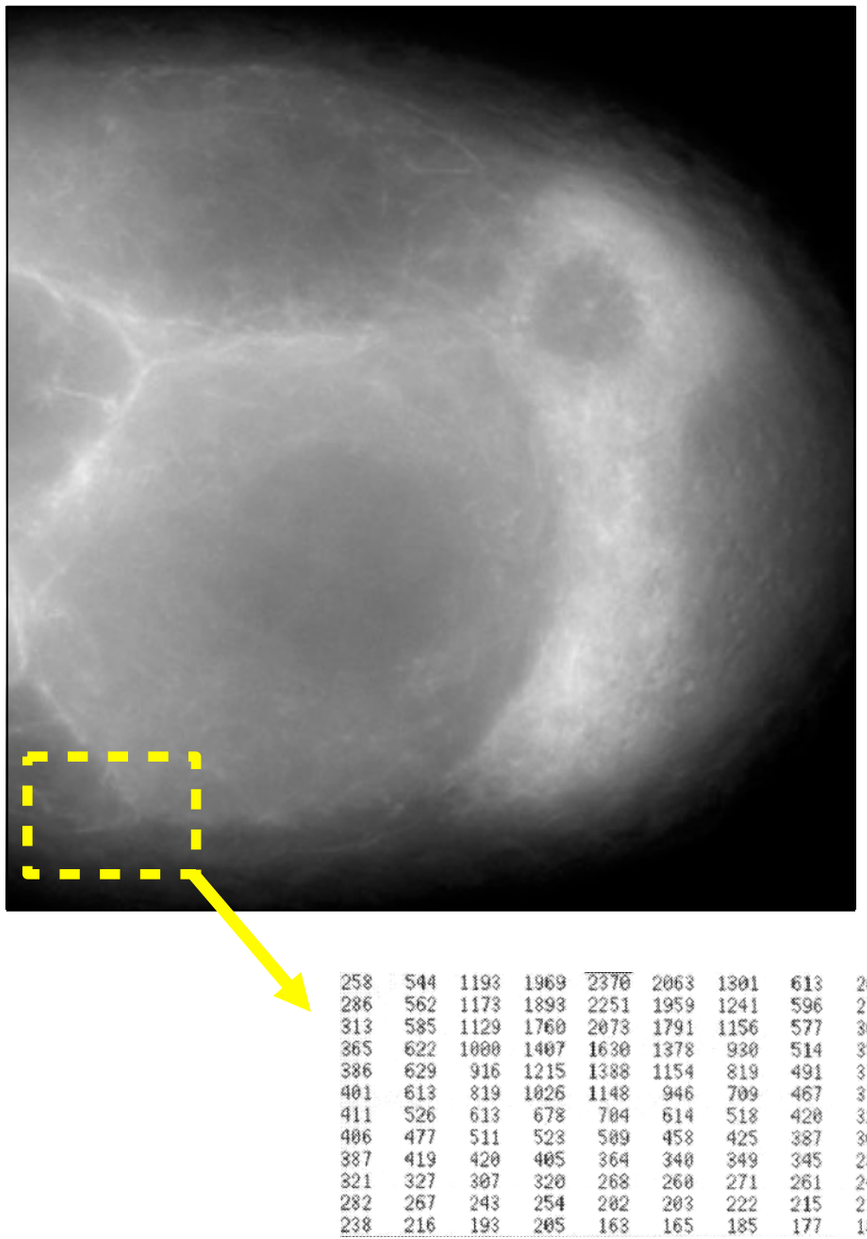
What is a digital image?

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)

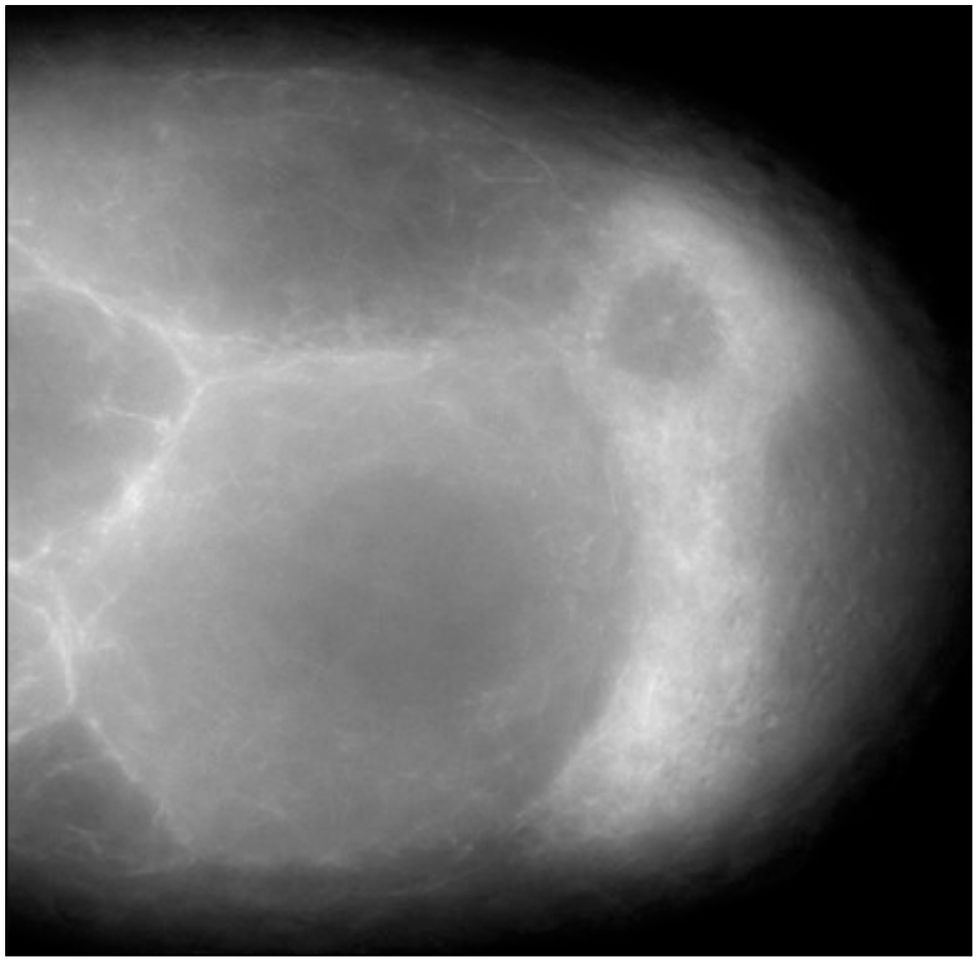
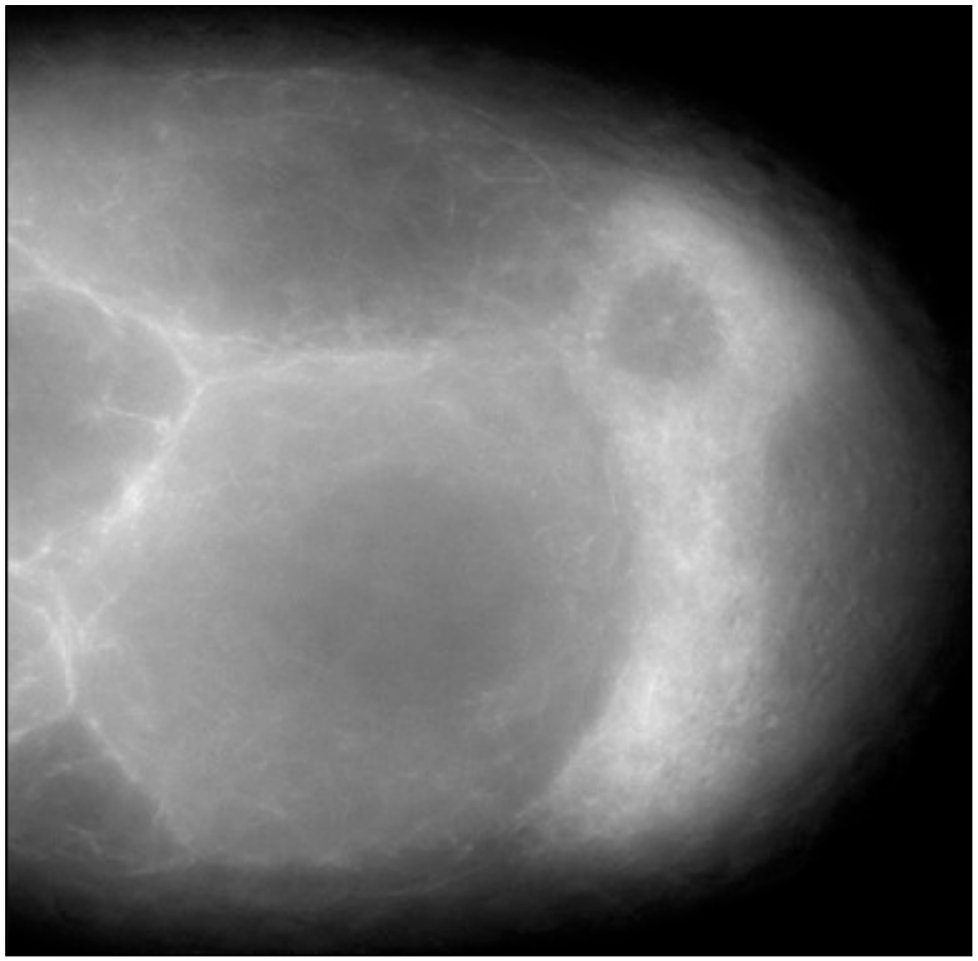


Image Parameters - what to record (= image metadata)



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

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)**

$$\text{Poisson distributed variation} \qquad \text{S:N ratio} = \frac{n}{\sqrt{n}}$$

- **Statistics of photon counting dictate the minimum useful signal**

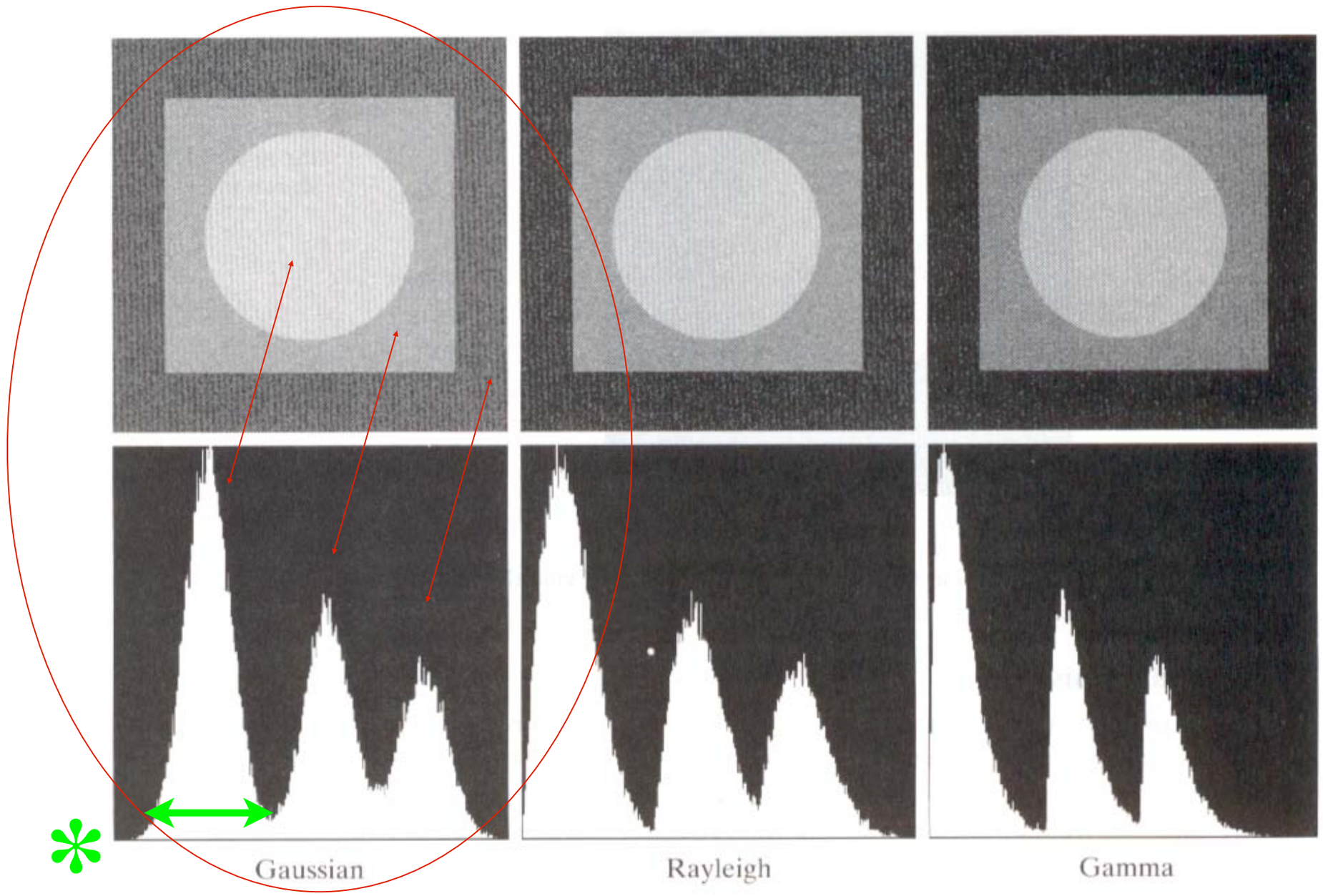
Average signal = 9, S:N ratio = 3
Average signal = 100, S:N ratio = 10
Average signal = 10,000, S:N ratio = 100

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.

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

http://www.dpcorner.com/topics/

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)

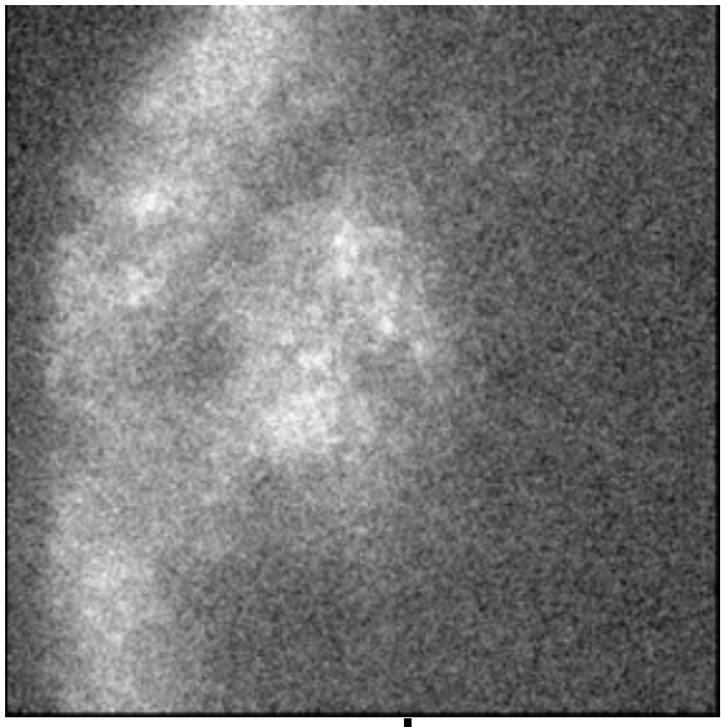
Post Acquisition

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

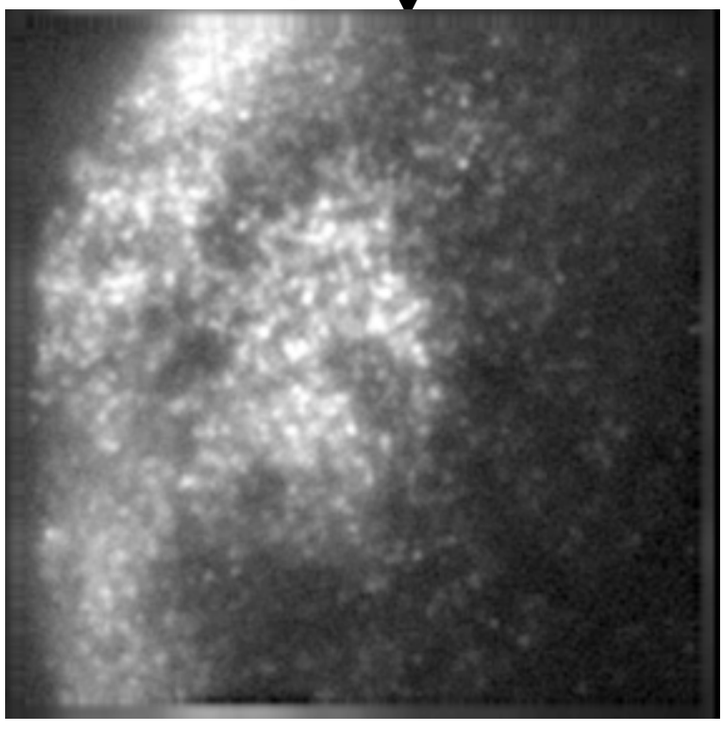
Improving signal to noise

Averaging increases S/N = improved contrast

Single noisy image



Average 5x successive images

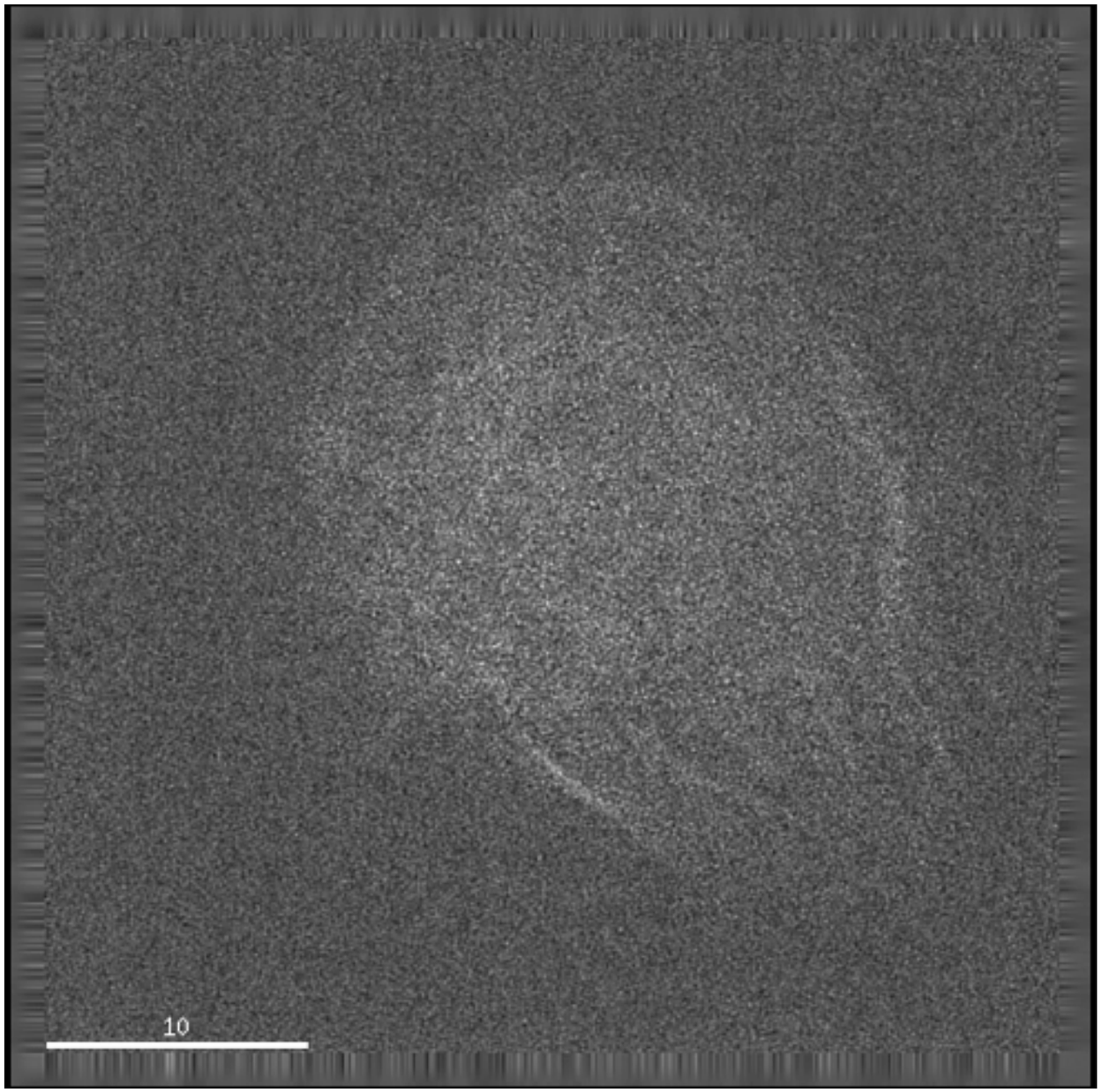


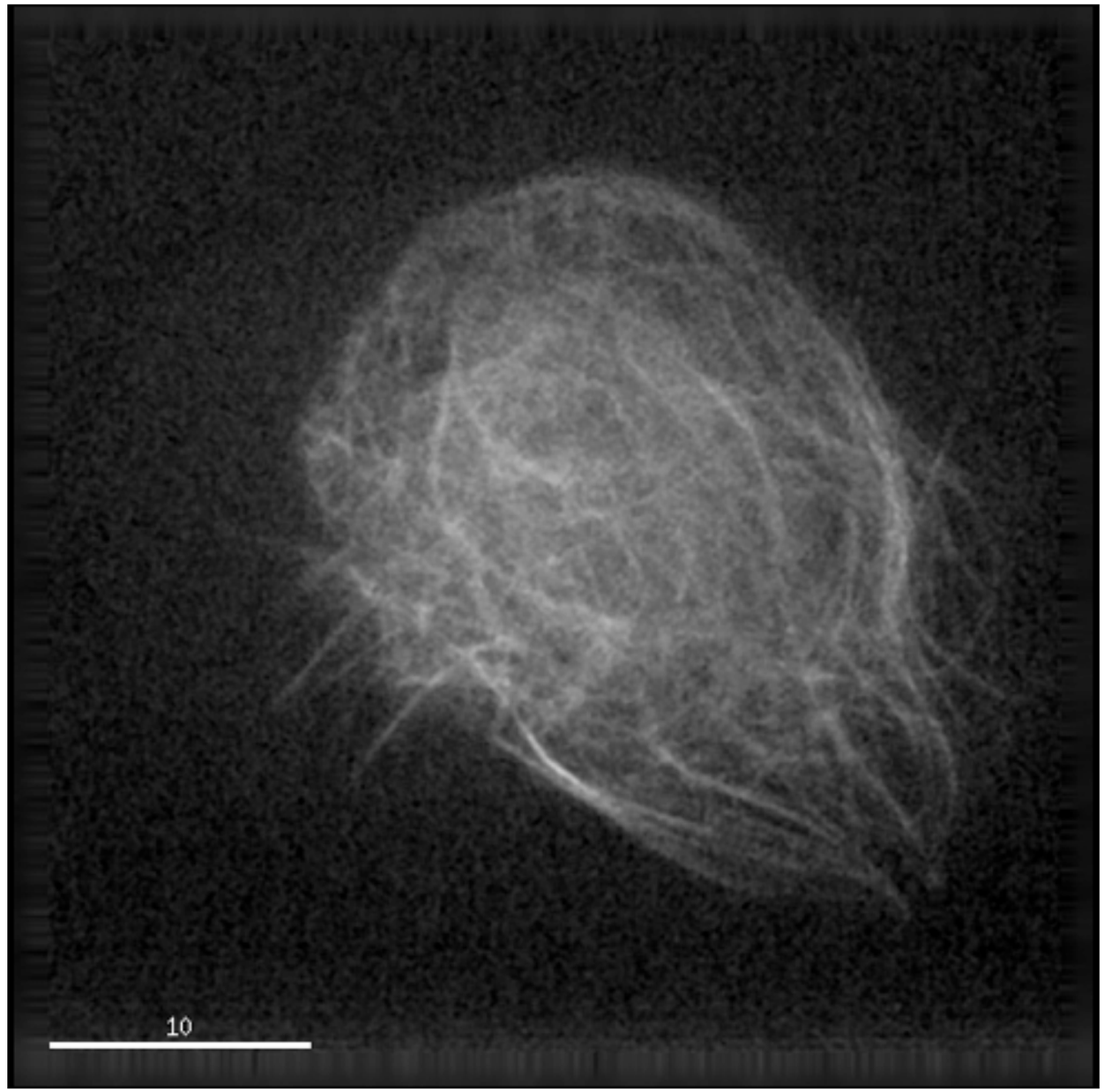
Effectively increases the number of photons counted

improved S/N

ImageJ

Post acquisition denoising





Macrophage: Jupiter-GFP 7Z, 3stacks/s

Jerome Boulanger: SAFIR Denoising software

Integrated into Priism by the John Sedat Group UCSF

J. Boulanger, C. Kervrann, and P. Bouthemy, “Space-time adaptation for patch-based image sequence restoration,” *IEEE Trans. on Pattern Analysis and Machine Intelligence*, vol. 29, no. 6, pp. 1096ñ1102, June 2007

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”.

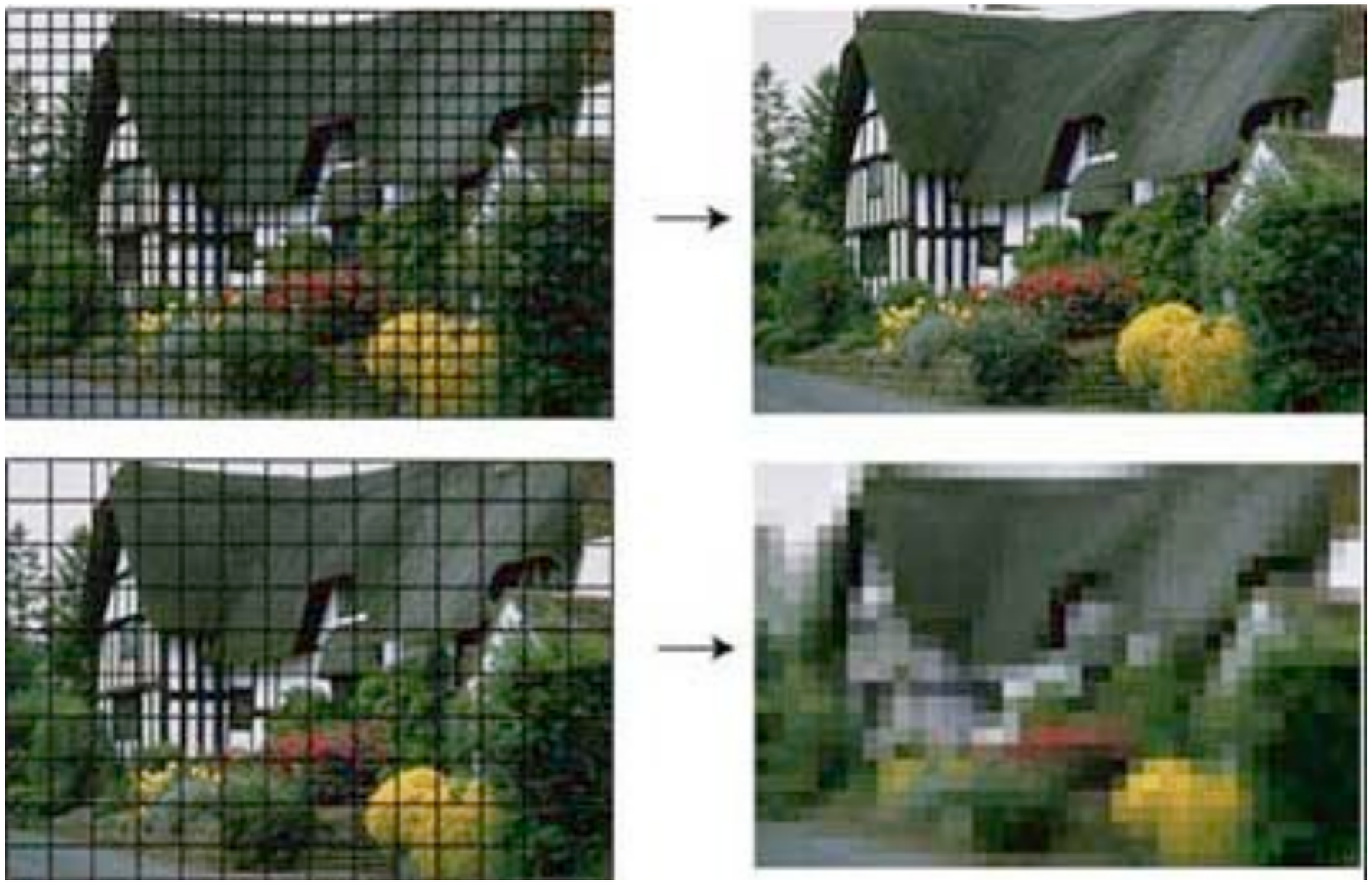
- Noise is NOT dark signal or background but they CONTRIBUTE to image noise.

- 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.

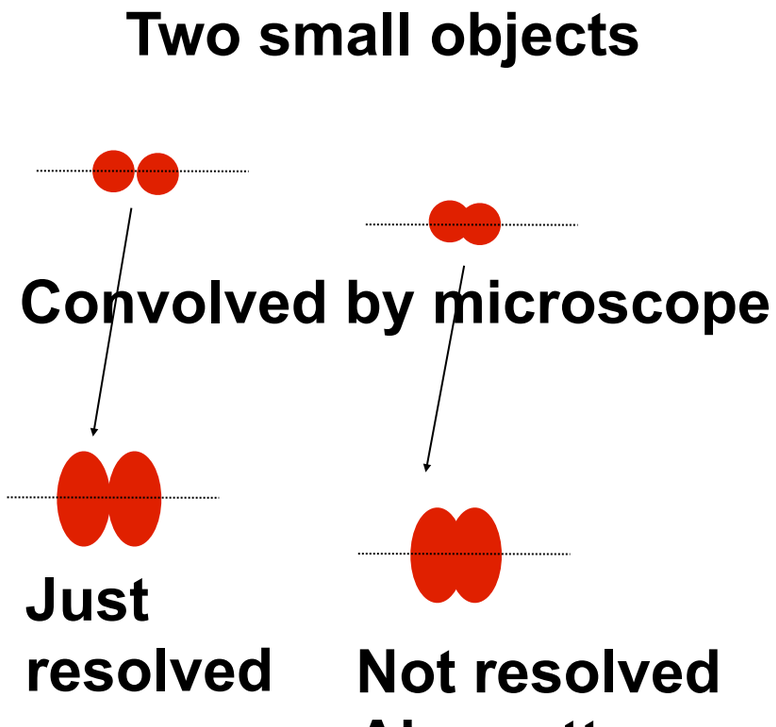
- Background = autofluorescence of sample
  Is a real fluorescence signal and has associated shot noise.
  Subtracting an autofluorescence image does not remove the noise.

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

Resolution and Sampling



Optical resolution: The Rayleigh Criterion

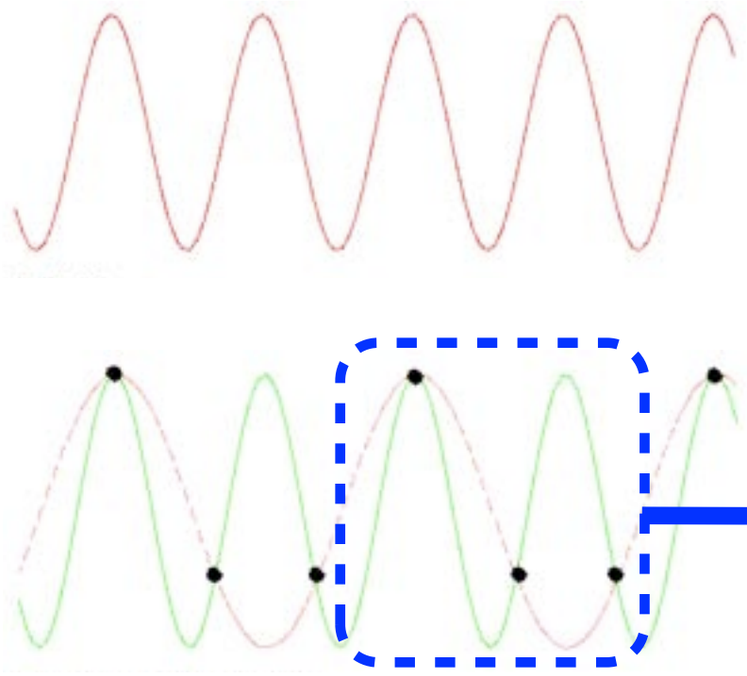


resolution limit $\approx \lambda_{em}/2$

XY resolution ~ 200 nm
Z resolution ~ 500 nm

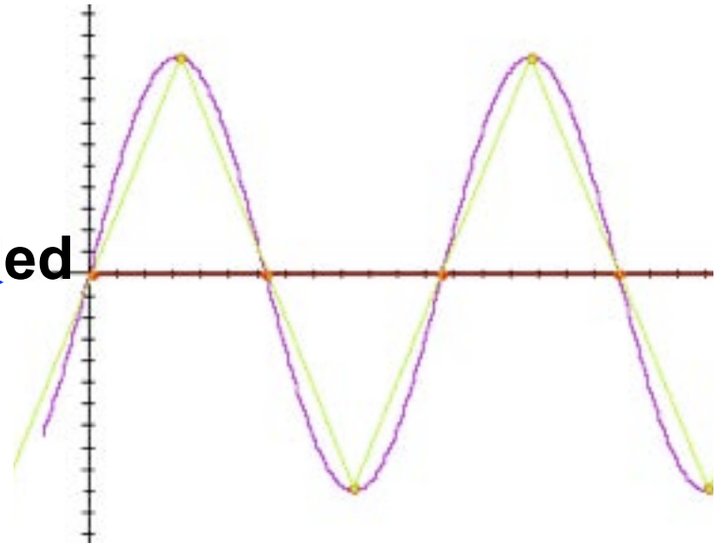
Resolution: Nyquist sampling theorem

A sine wave



Sampling 1.5 times per cycle

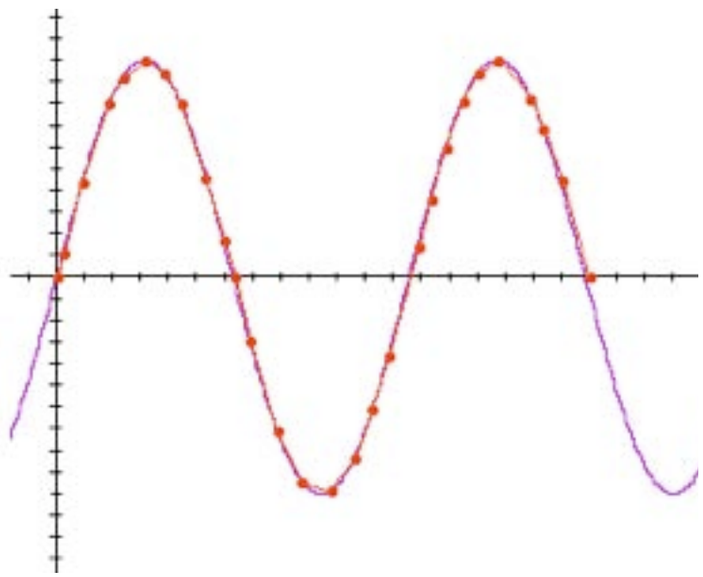
undersampled



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

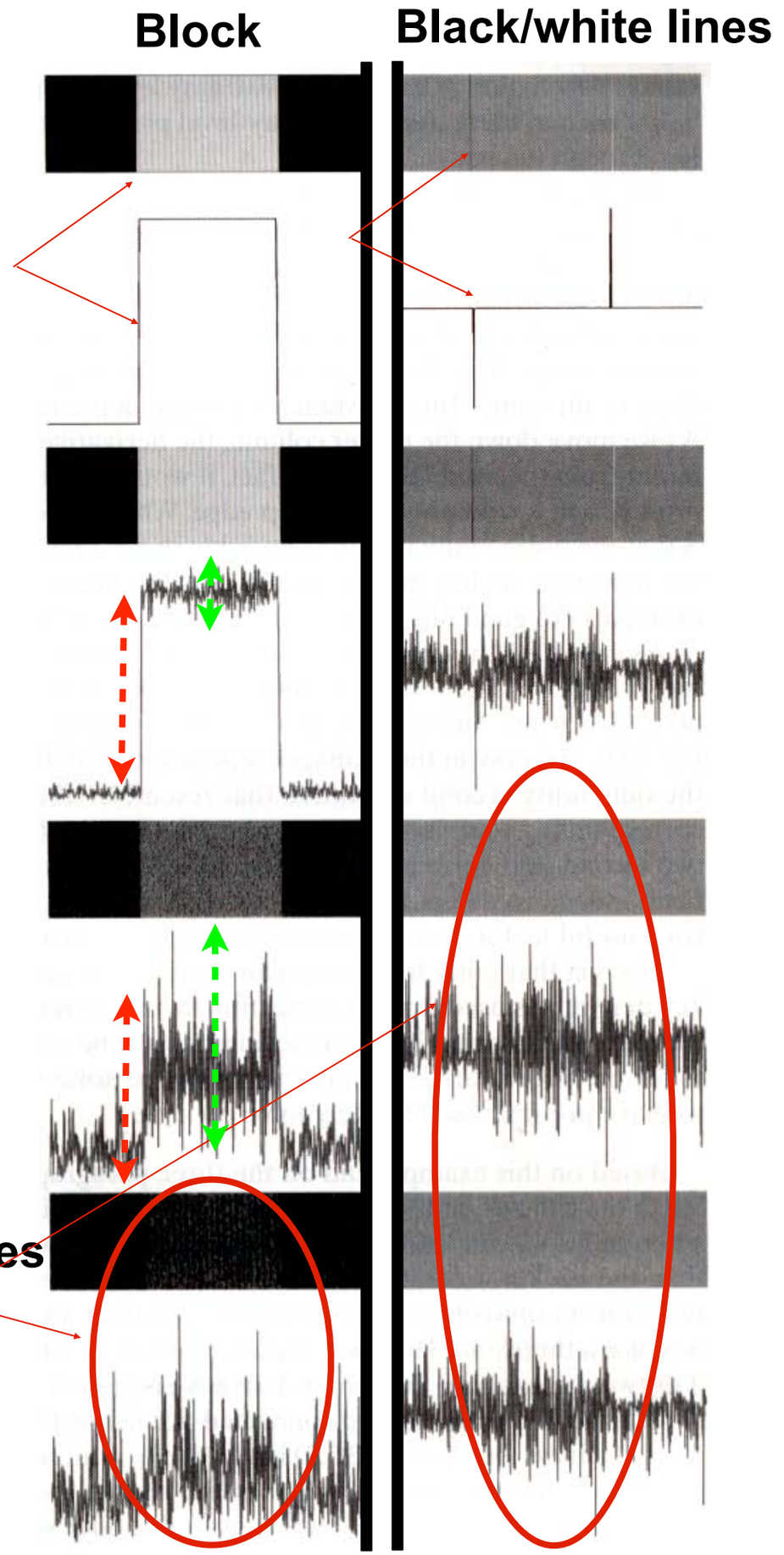
Resolution, contrast, noise

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

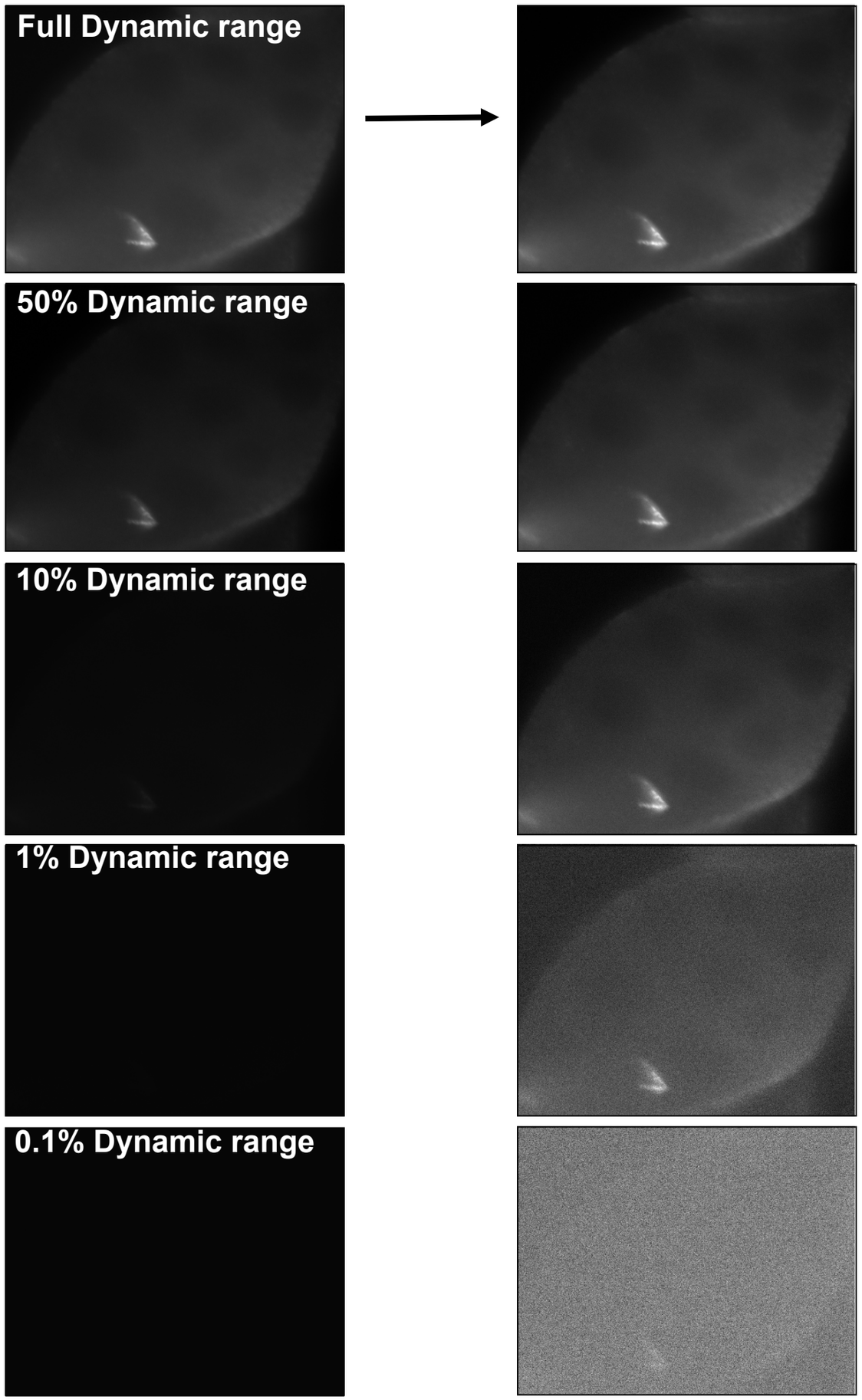
Gonzales & Woods, 2002. Digital Image Processing 2nd Ed. Prentice-Hall Inc, USA.

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)**

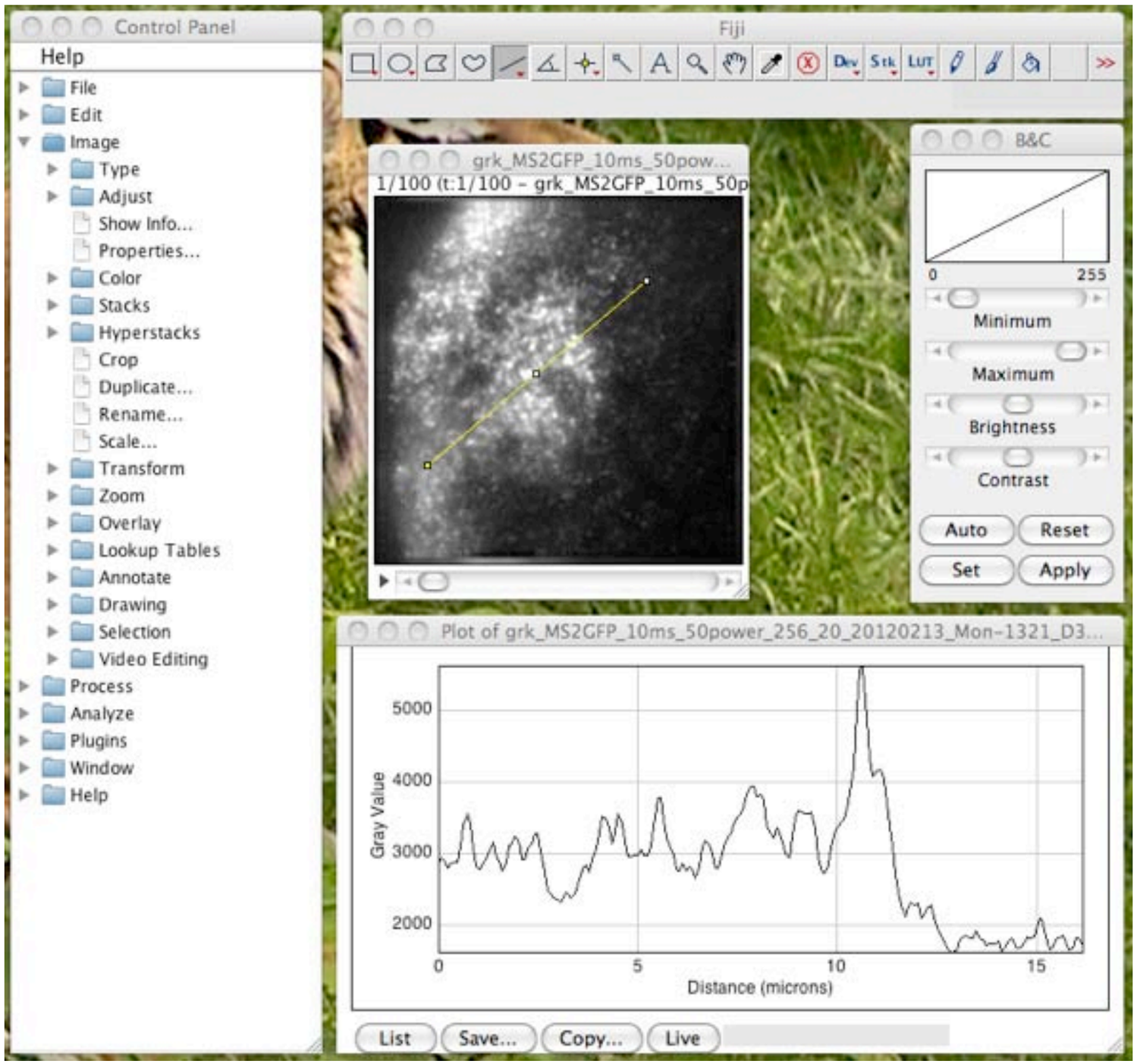
Image Processing is NOT a substitute for a good image

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**
- **Uses Loci Bioformats to convert between file types**

http://loci.wisc.edu/software/bio-formats



Fiji Is Just ImageJ

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

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

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 (lecture 16 - OME, bioformats)**
  http://loci.wisc.edu/software/bio-formats

- **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/

- **We have now set up an OME server associated with MICRON (Graeme Ball / Ian Dobbie)**

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.

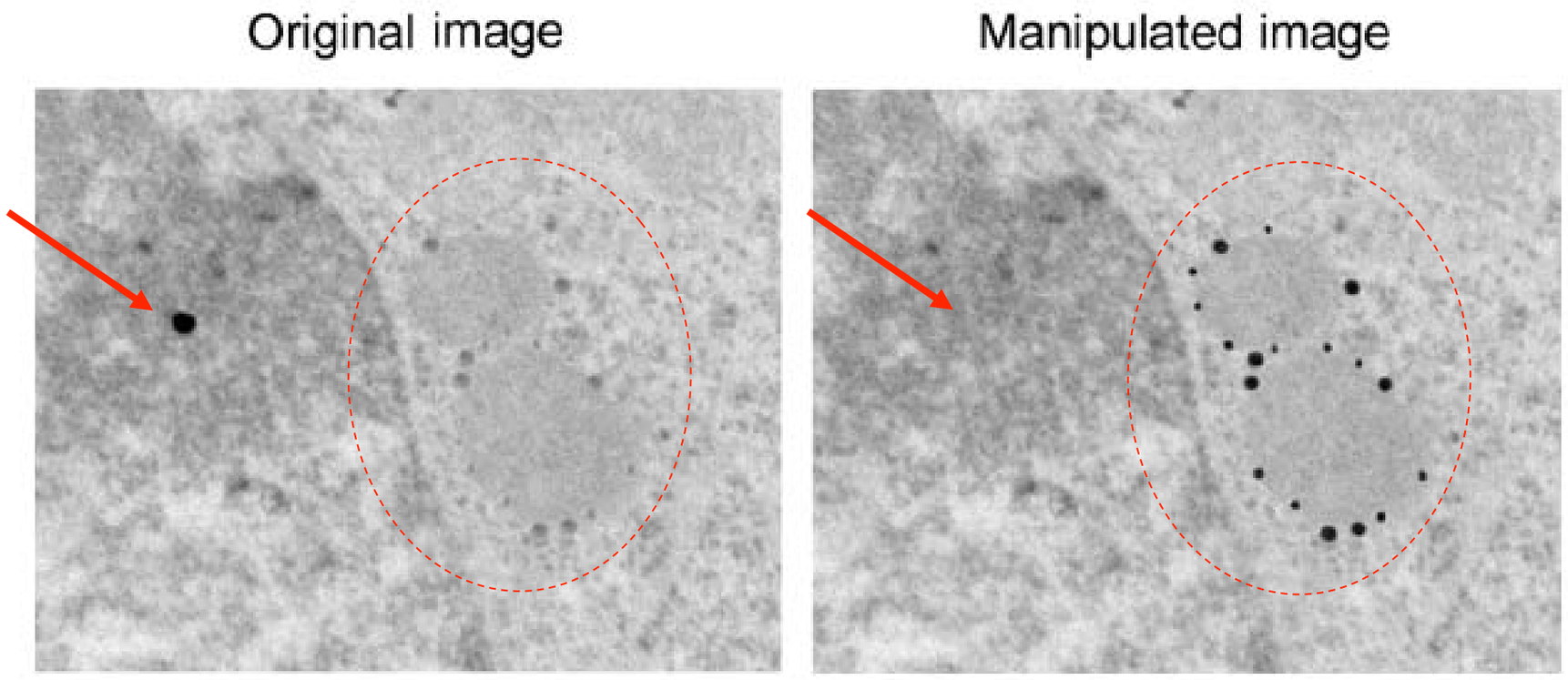
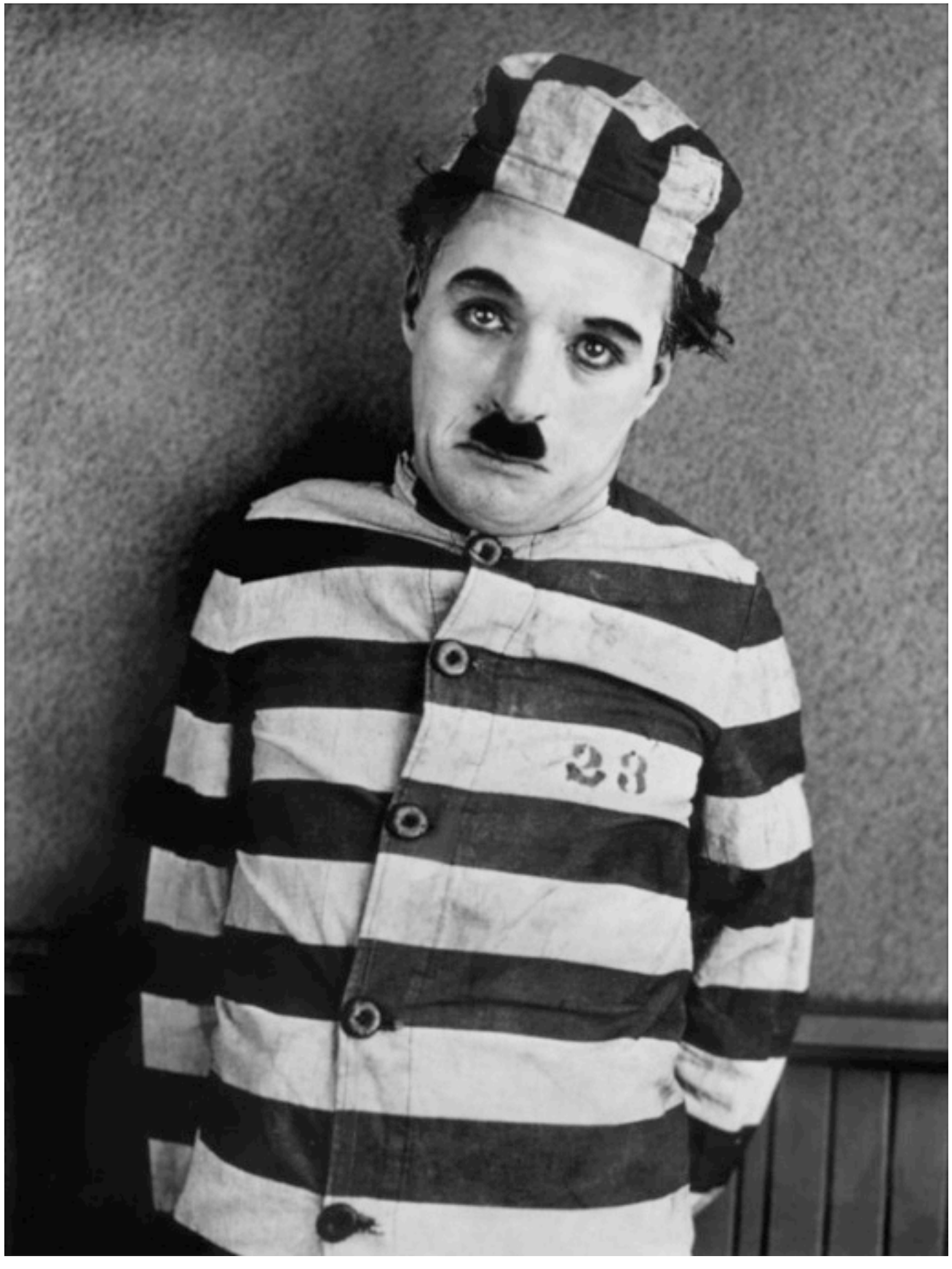


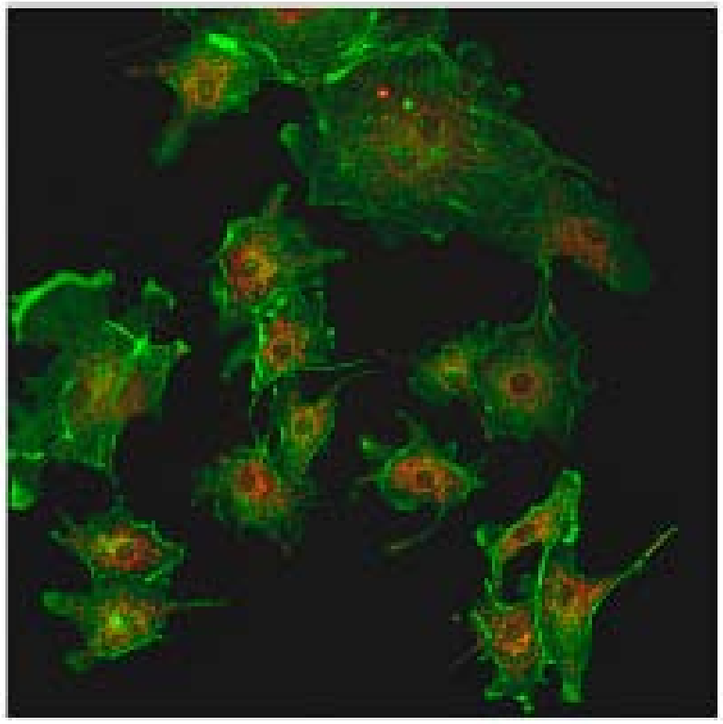
Figure 5. **Misrepresentation of immunogold data.** The gold particles, which were actually present in the original (left), have been enhanced in the manipulated image (right). Note also that the background dot in the original data has been removed in the manipulated image.

- **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!

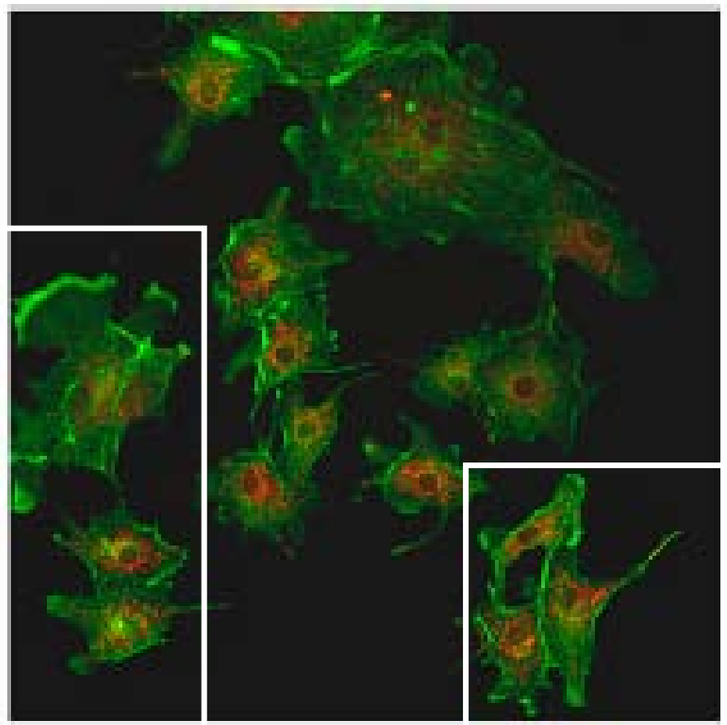


Manipulated image

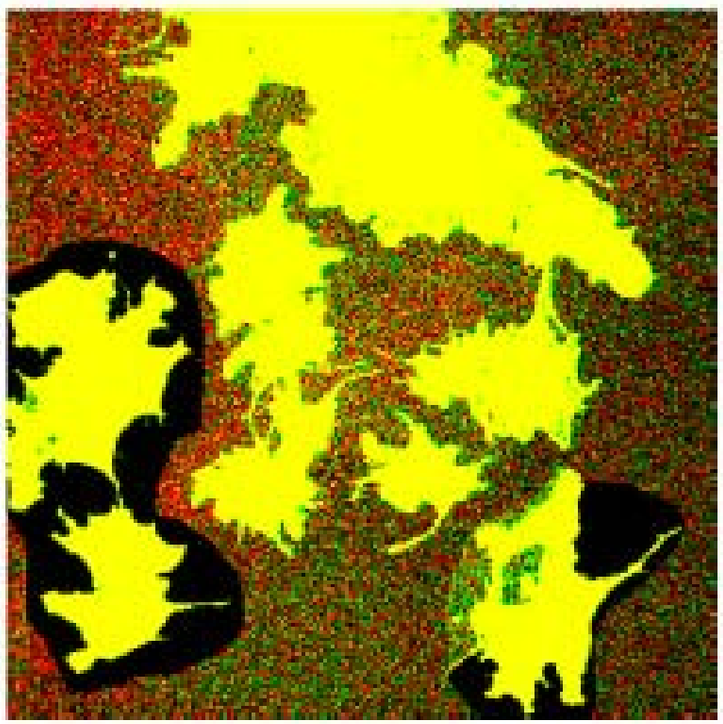


- **Miss-representation of cell population within an oberved field**
- **VERY BAD: Changes interpretation with intention to defraud**

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

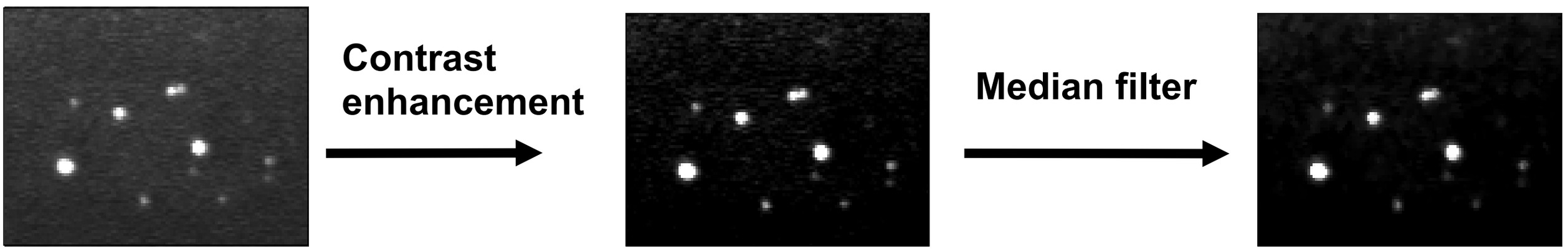
- **Low - level processing = Image enhancement (most common)**
- **Mid - level processing = Features and attributes extracted**
- **High - level processing = Interpretation of images**

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

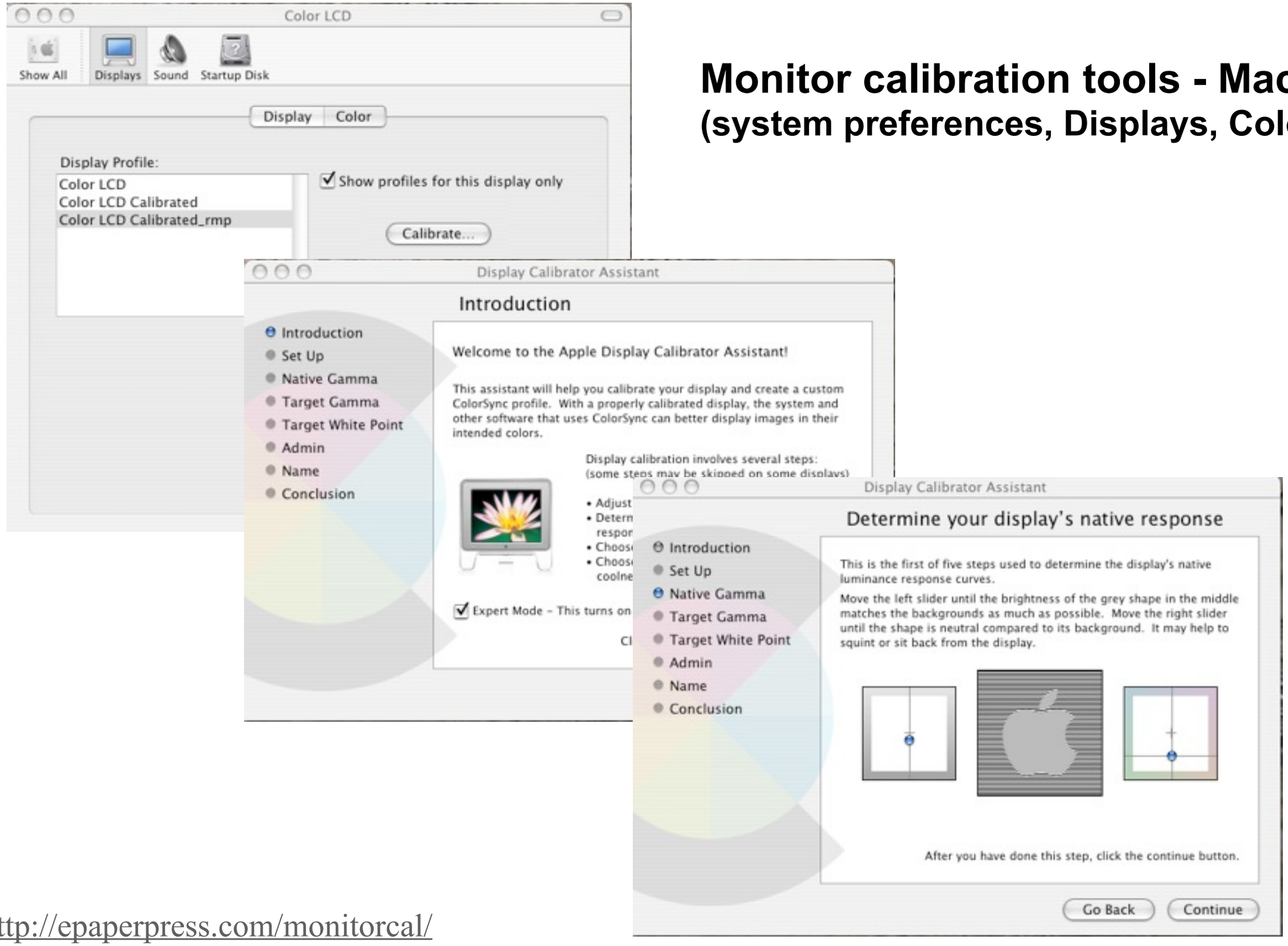
LOW LEVEL Processing

- 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

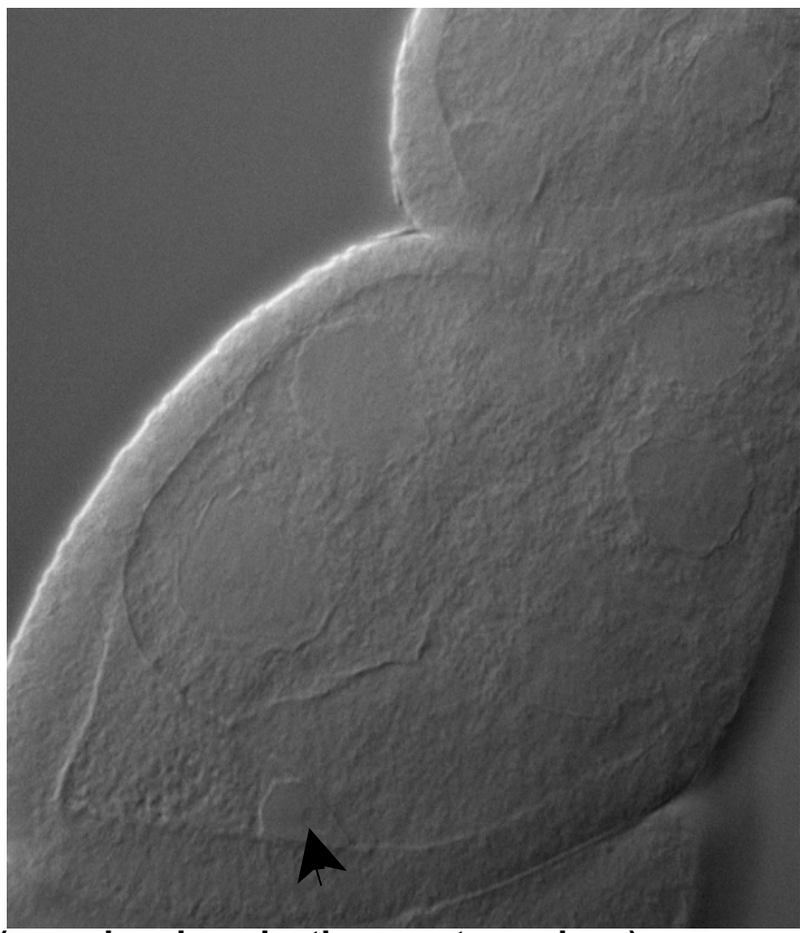


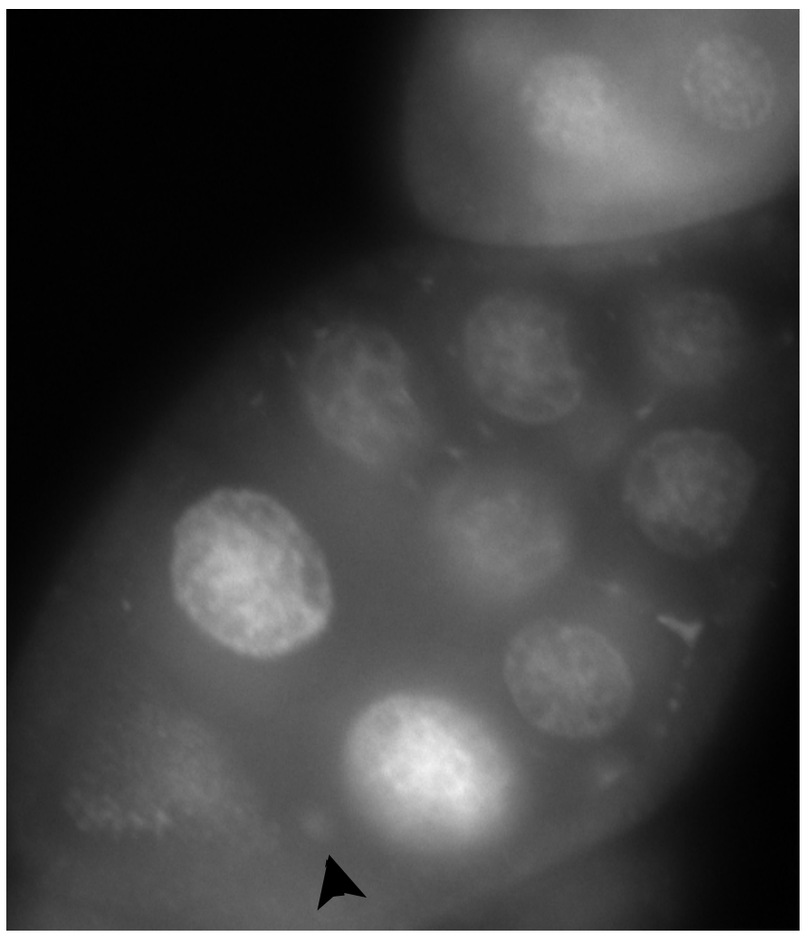
Monitor calibration tools - Mac OSX
(system preferences, Displays, Colour)

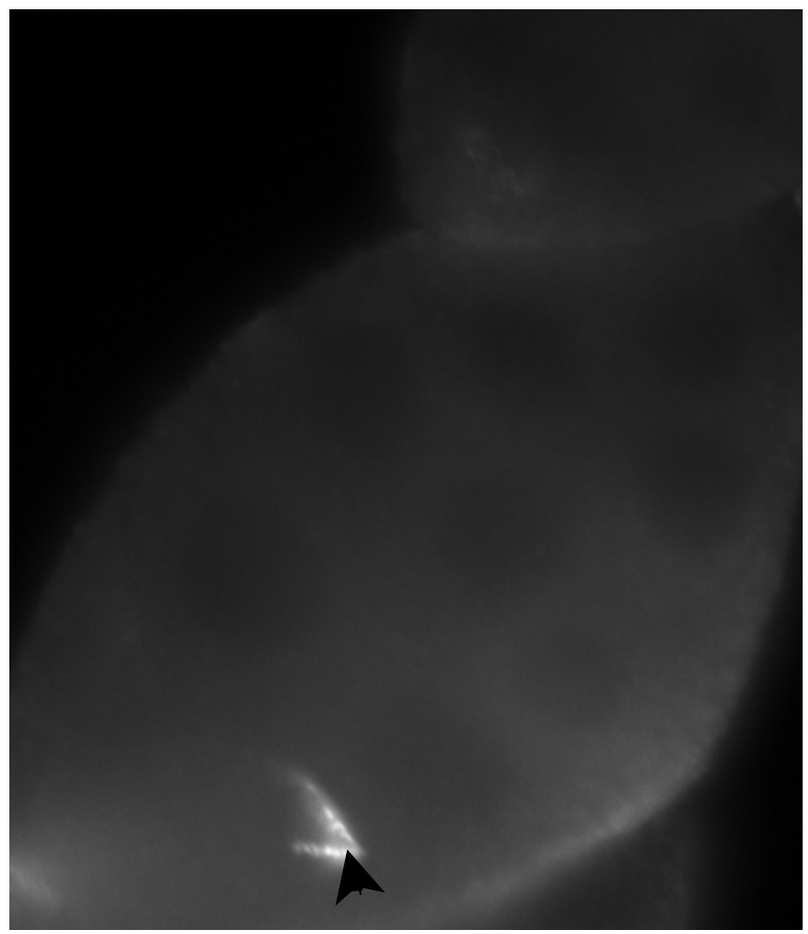
http://epaperpress.com/monitorcal/
http://www.bodoni.co.uk/profiling/colourprofiling.html#monitor
http://www.bodoni.co.uk/profiling/spyder.html

LOW LEVEL Processing - Display, Grey Scale

DIC/DAPI/Grk in situ - grey scale images





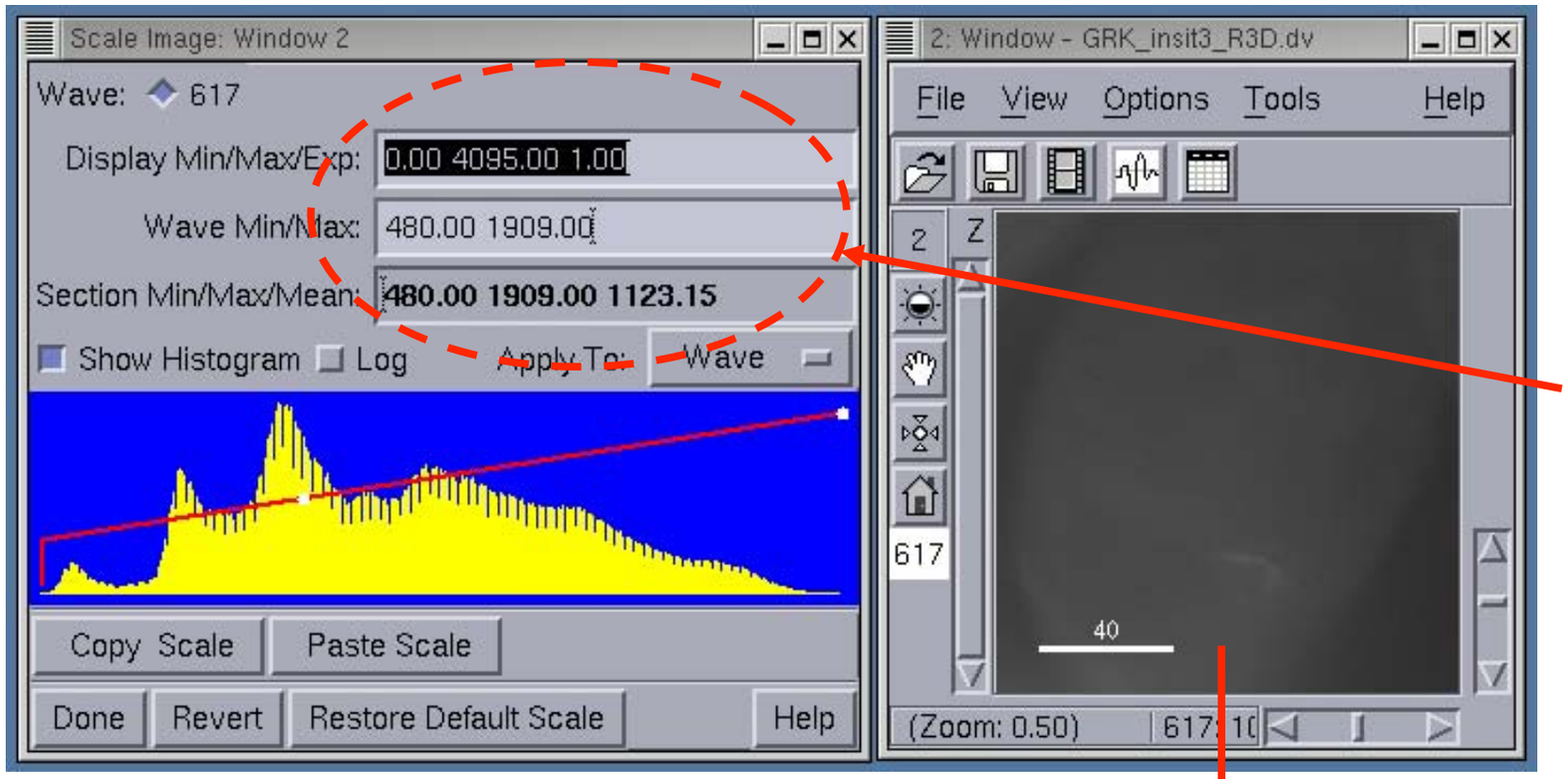


(arrowhead marks the oocyte nucleus)

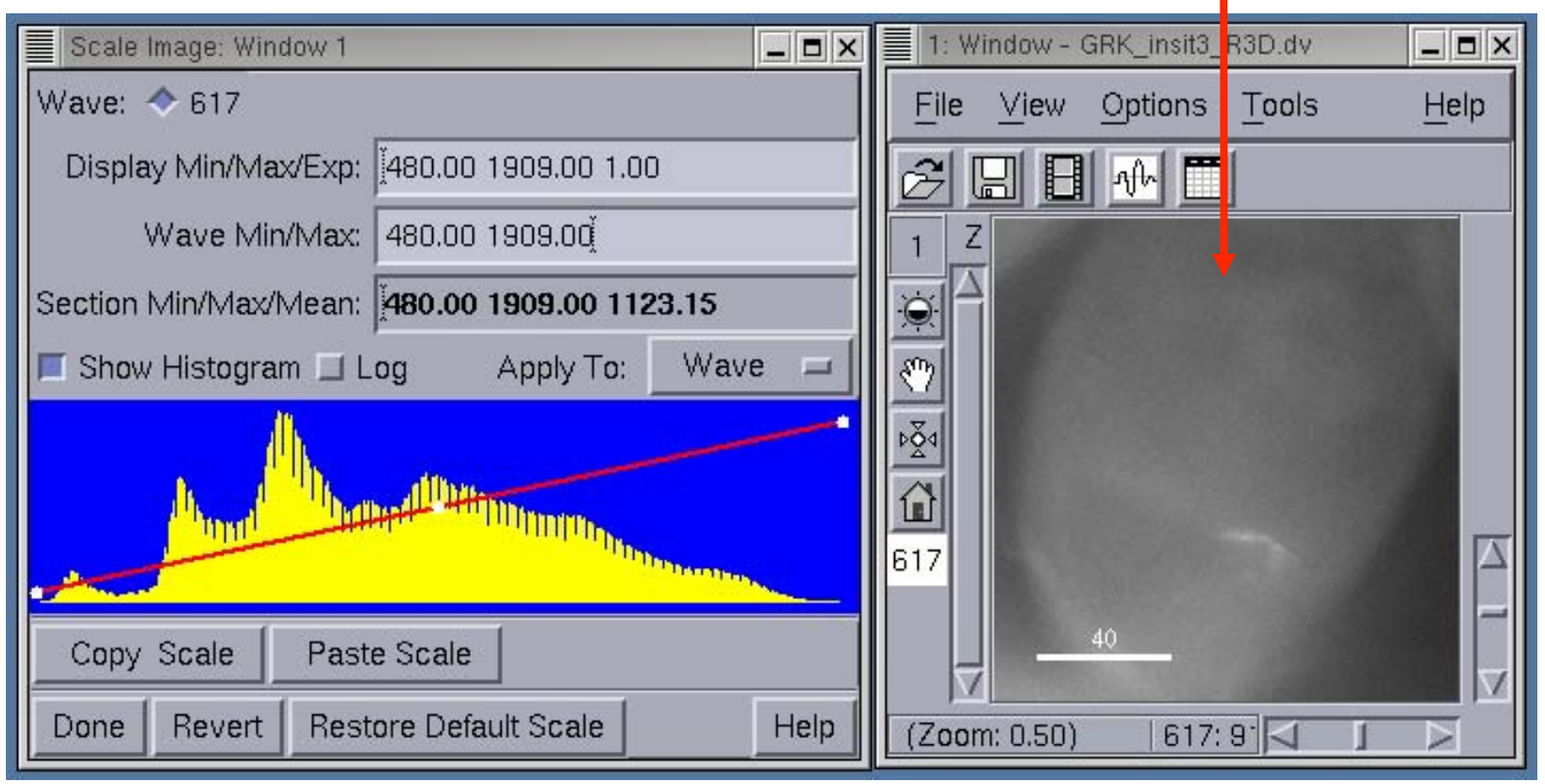
- For viewing and display use grey scale scale images to see fine detail.

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



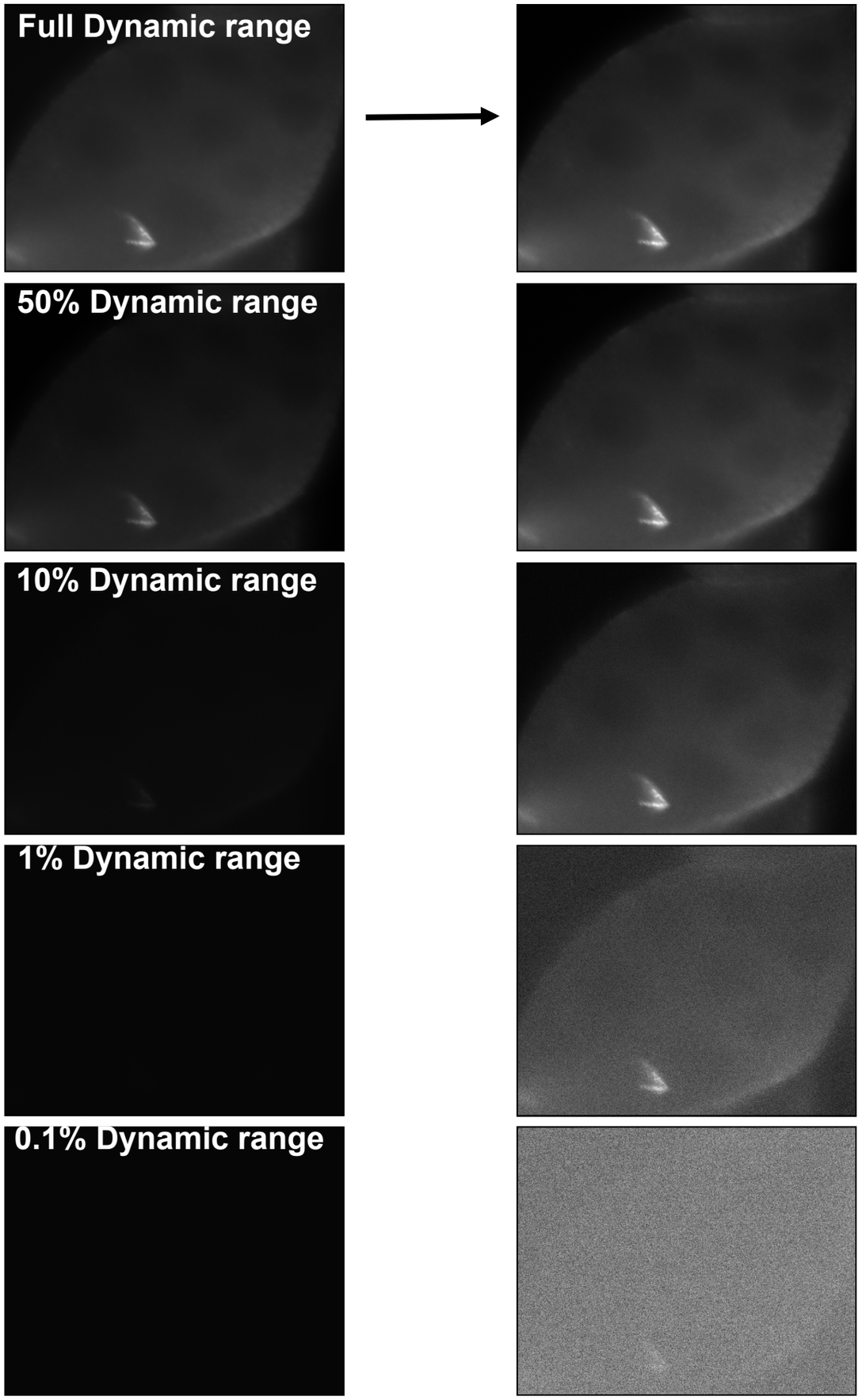
Auto-intensity scaling by softWoRx scales the display to fill the dynamic range

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

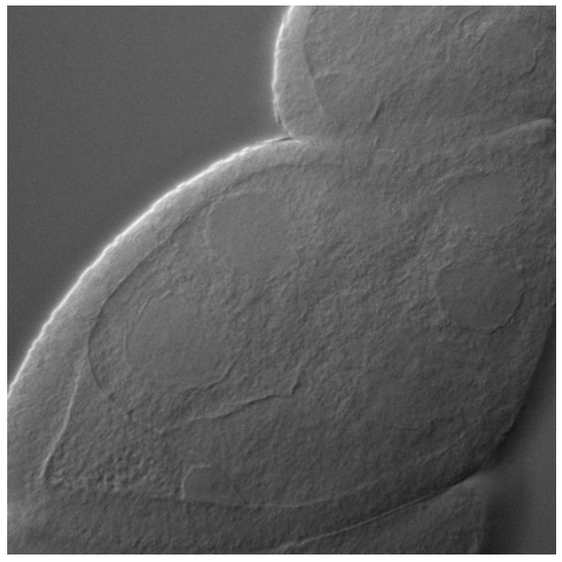
Decreasing Image quality

Cannot resolve

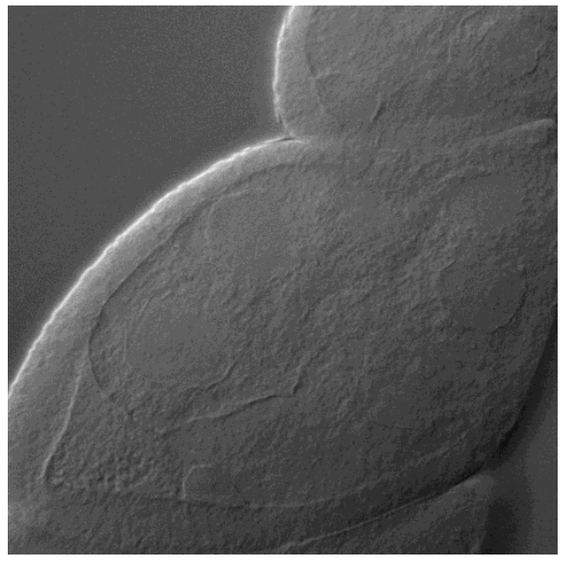
LOW LEVEL Processing - Display, Bit Depth (levels)

- **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.

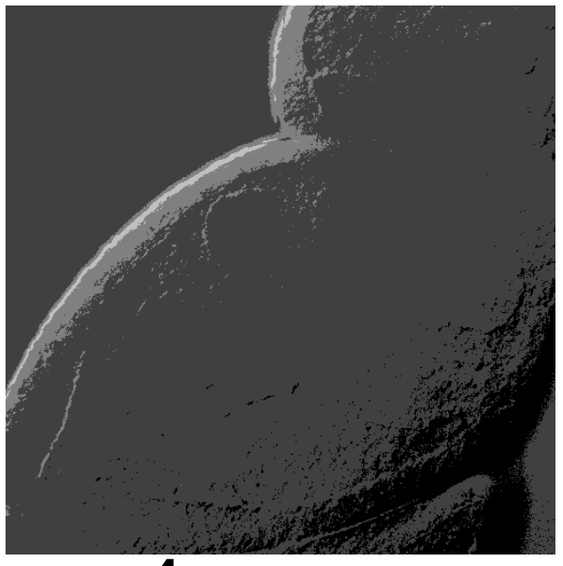
False contouring due to insufficient grey levels ⟶



256 greys



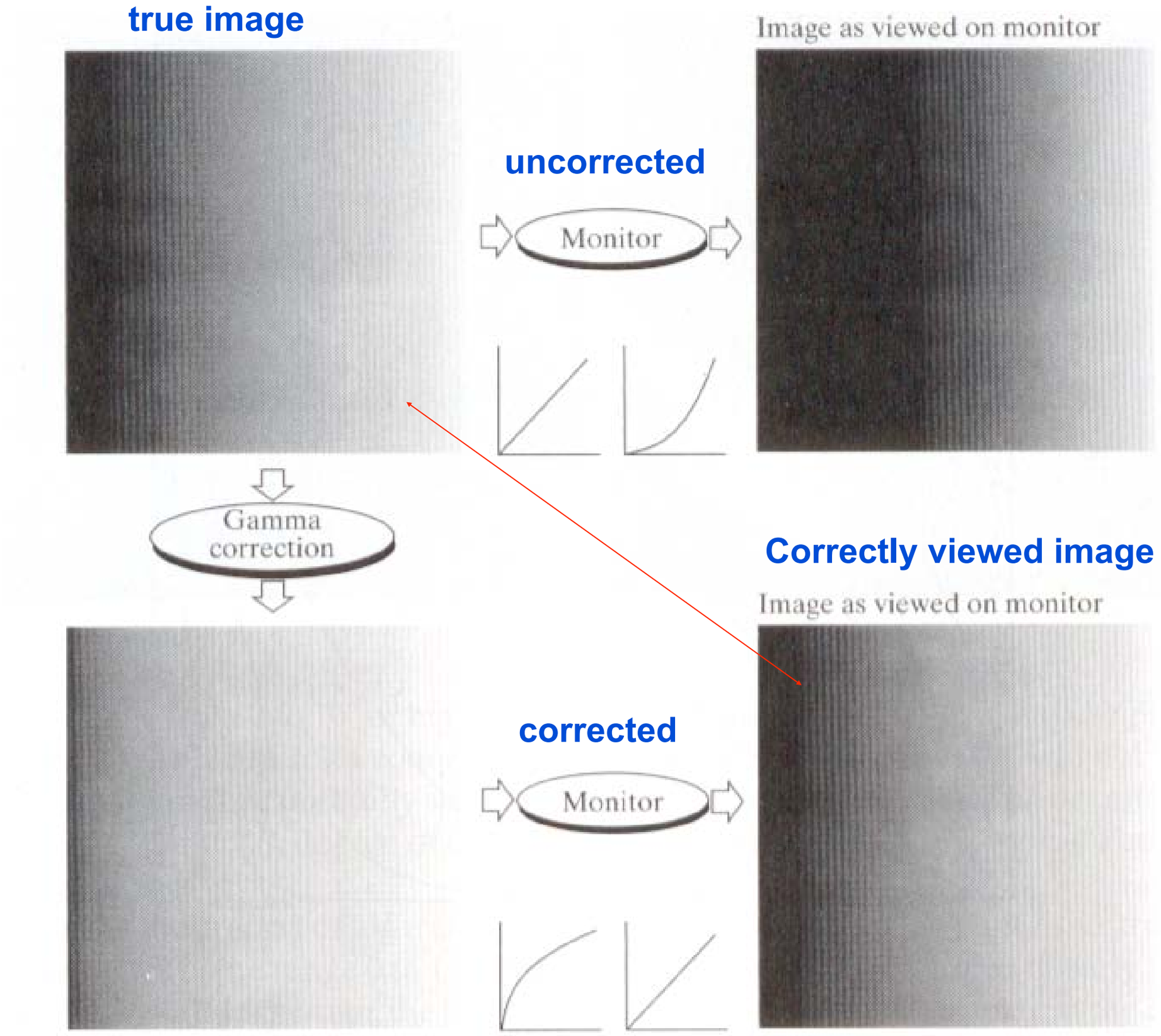
16 greys



4 greys

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

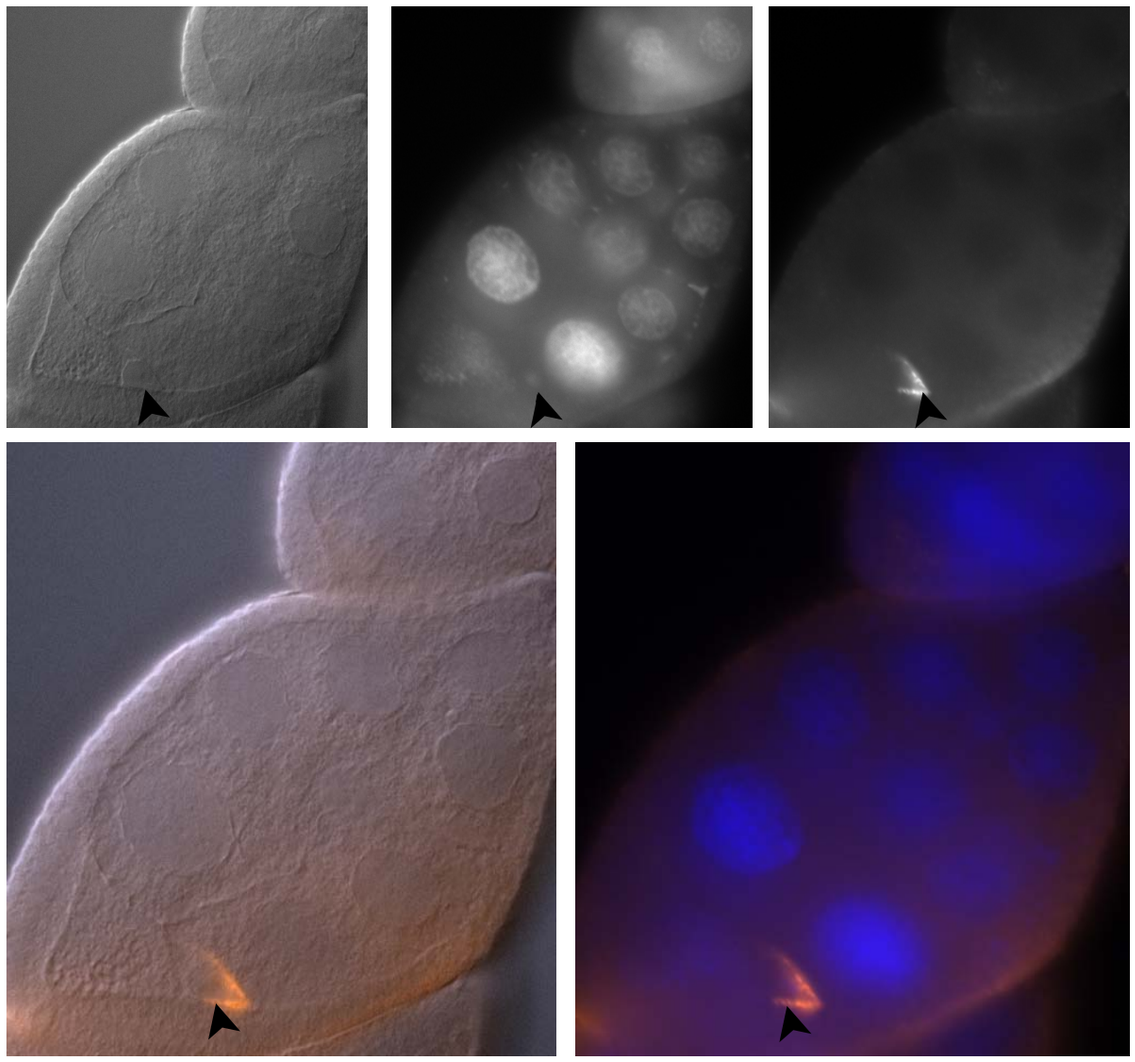
LOW LEVEL Processing - Display, Using Gamma



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

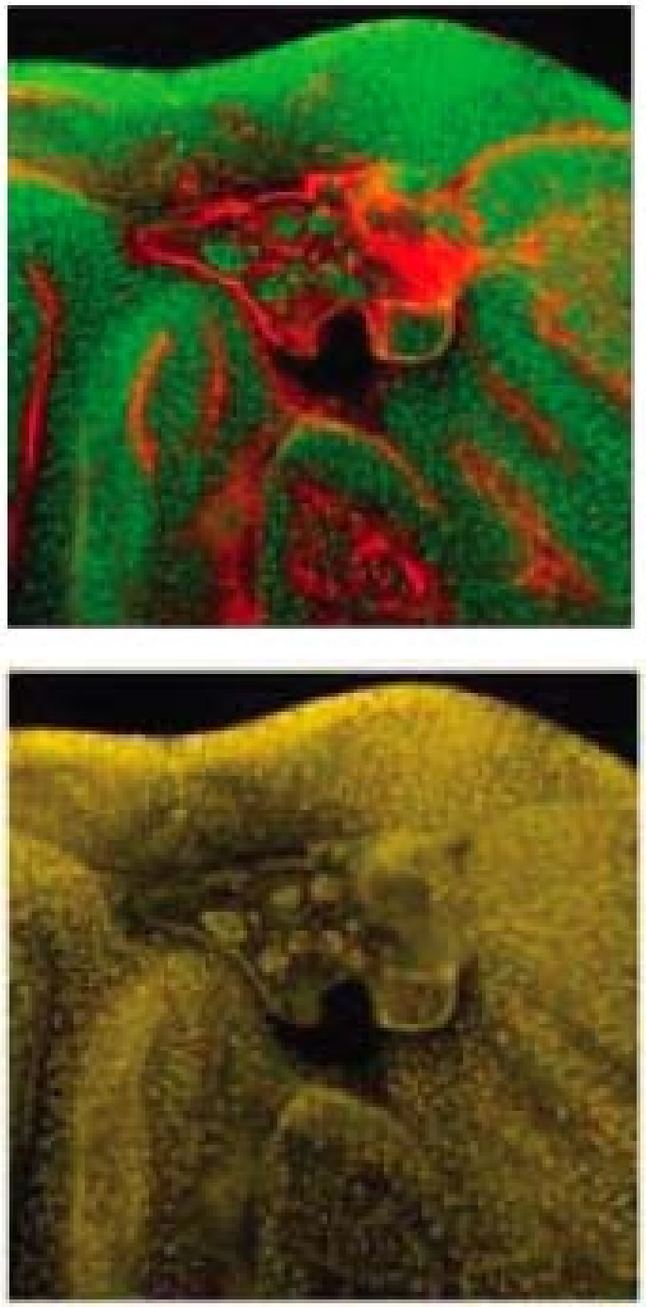


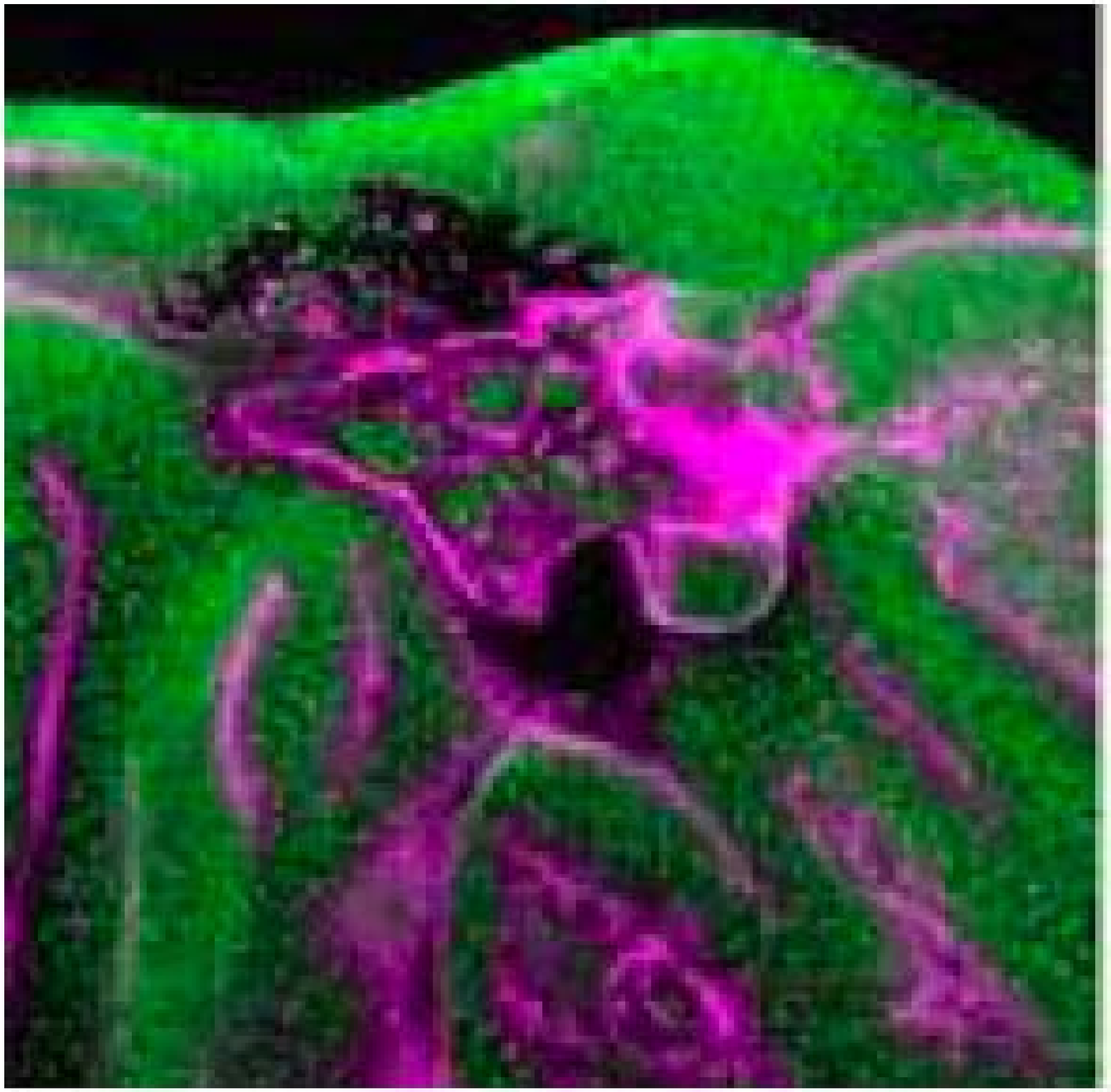
(arrowhead marks the oocyte nucleus)

- 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

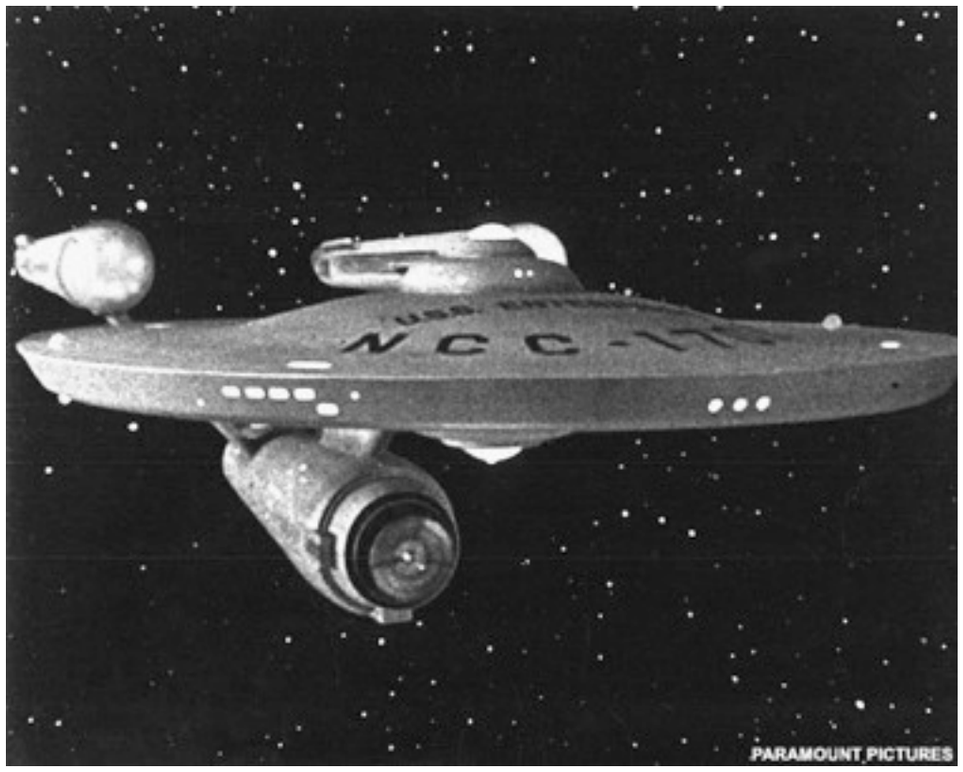
  Will open many file formats and export straight to .avi or .mov.,
  also can open tif image series (image001.tif; image002.tif; etc)
  restack and export to .avi or .mov. Has compression options for .mov

- 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

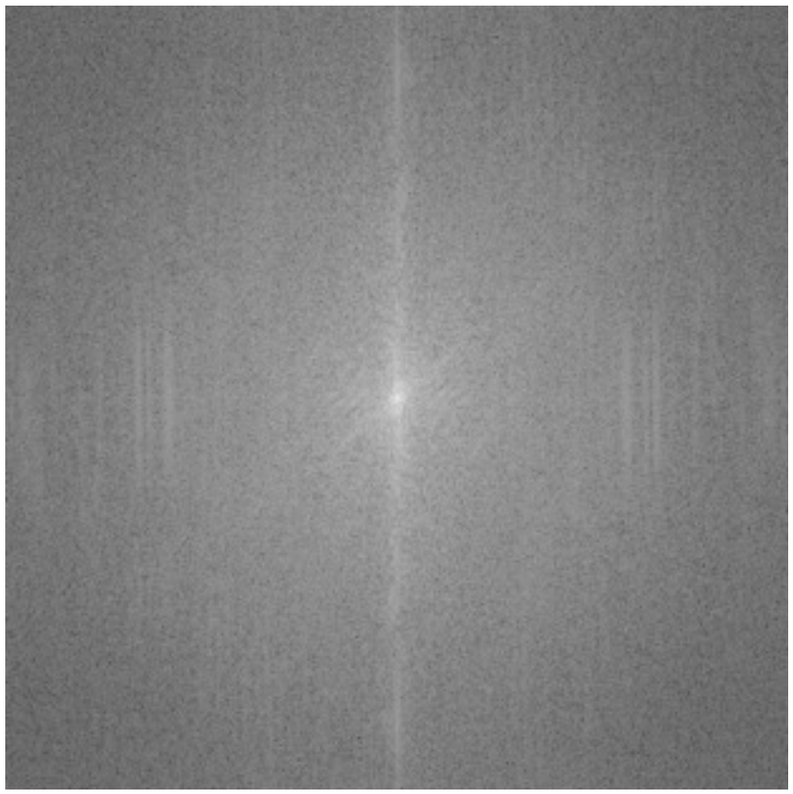
Processing in Real Space



or

Fourier Transform

Frequency Space



Example:

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

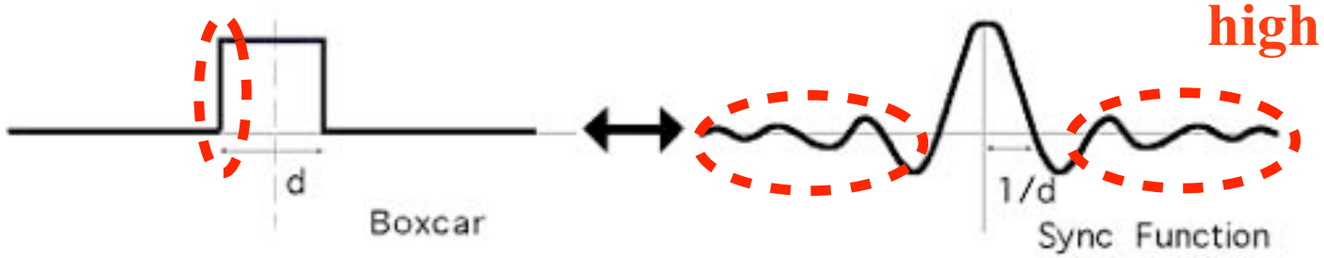
Fourier Space = Frequency space
= Reciprocal space
= K space

Data is broken down into its "frequency" components

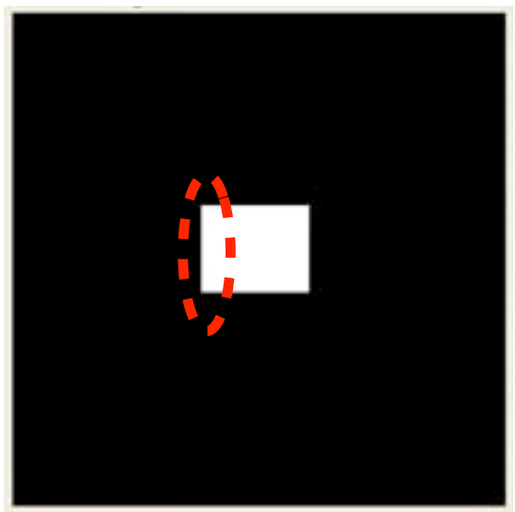
real space **reciprocal space**

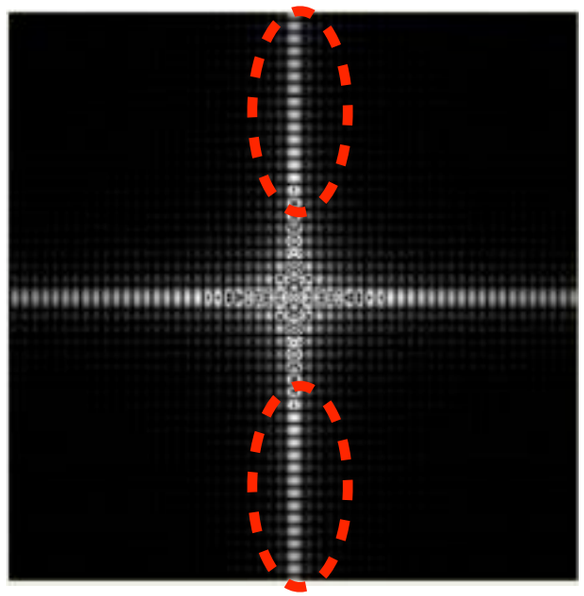
fine detail = high frequencies

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.

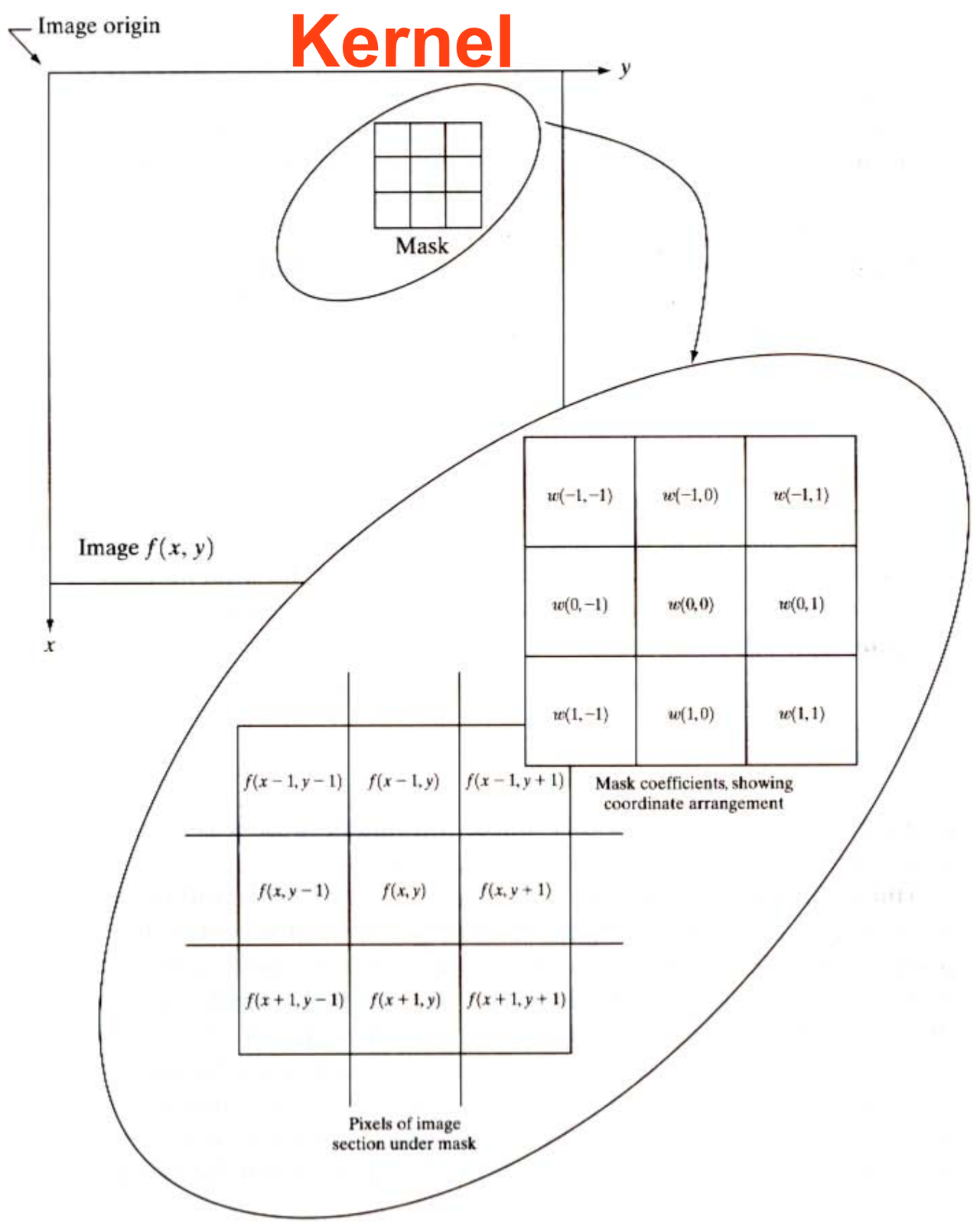
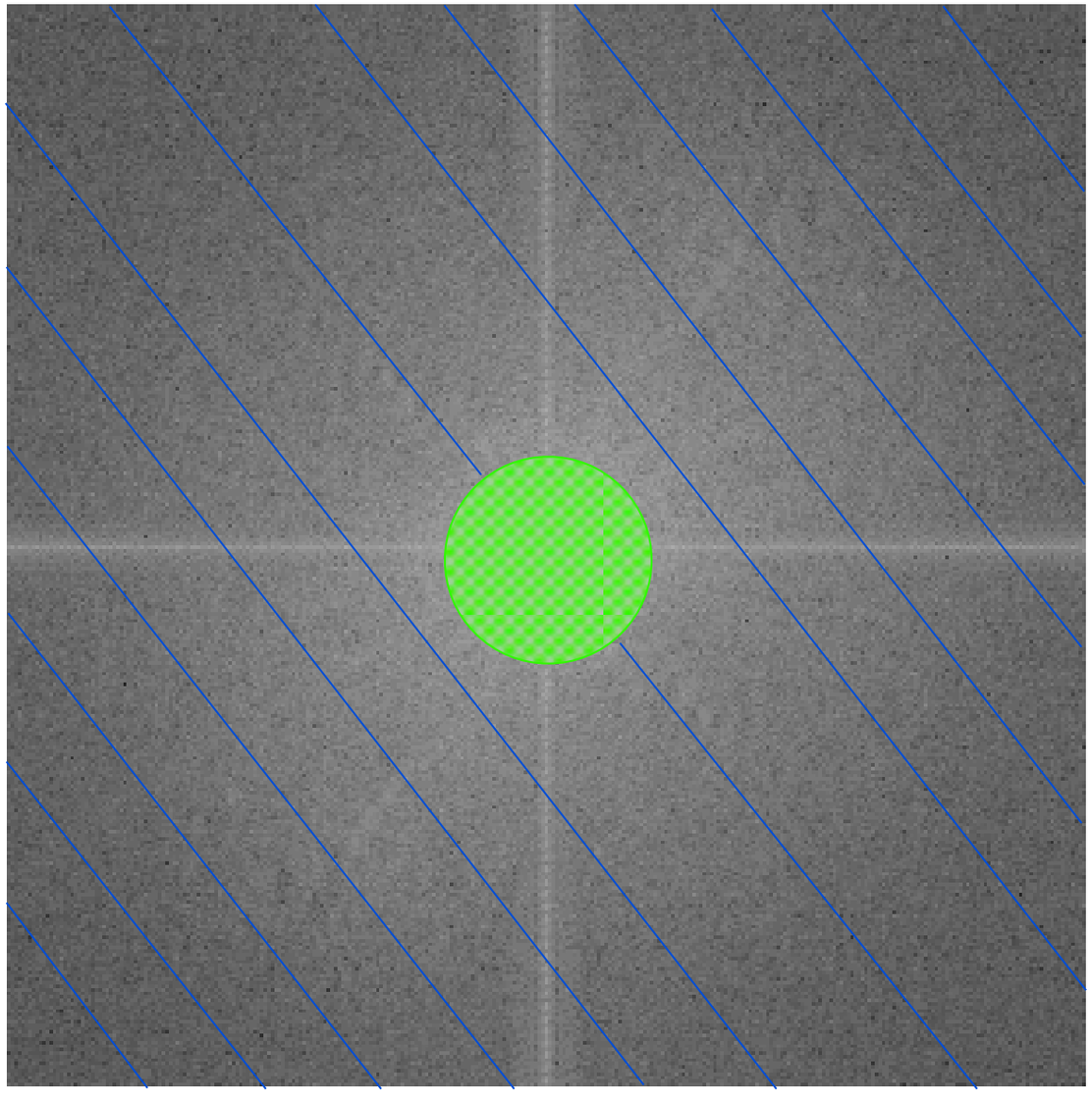


FIGURE 3.32 The mechanics of spatial filtering. The magnified drawing shows a 3×3 mask and the image section directly under it; the image section is shown displaced out from under the mask for ease of readability.

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)



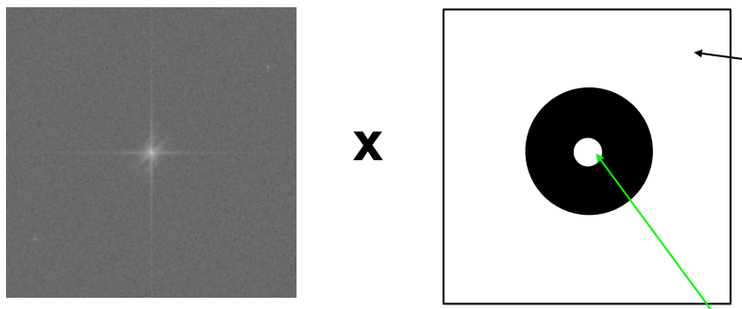
Low frequencies = coarse detail

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

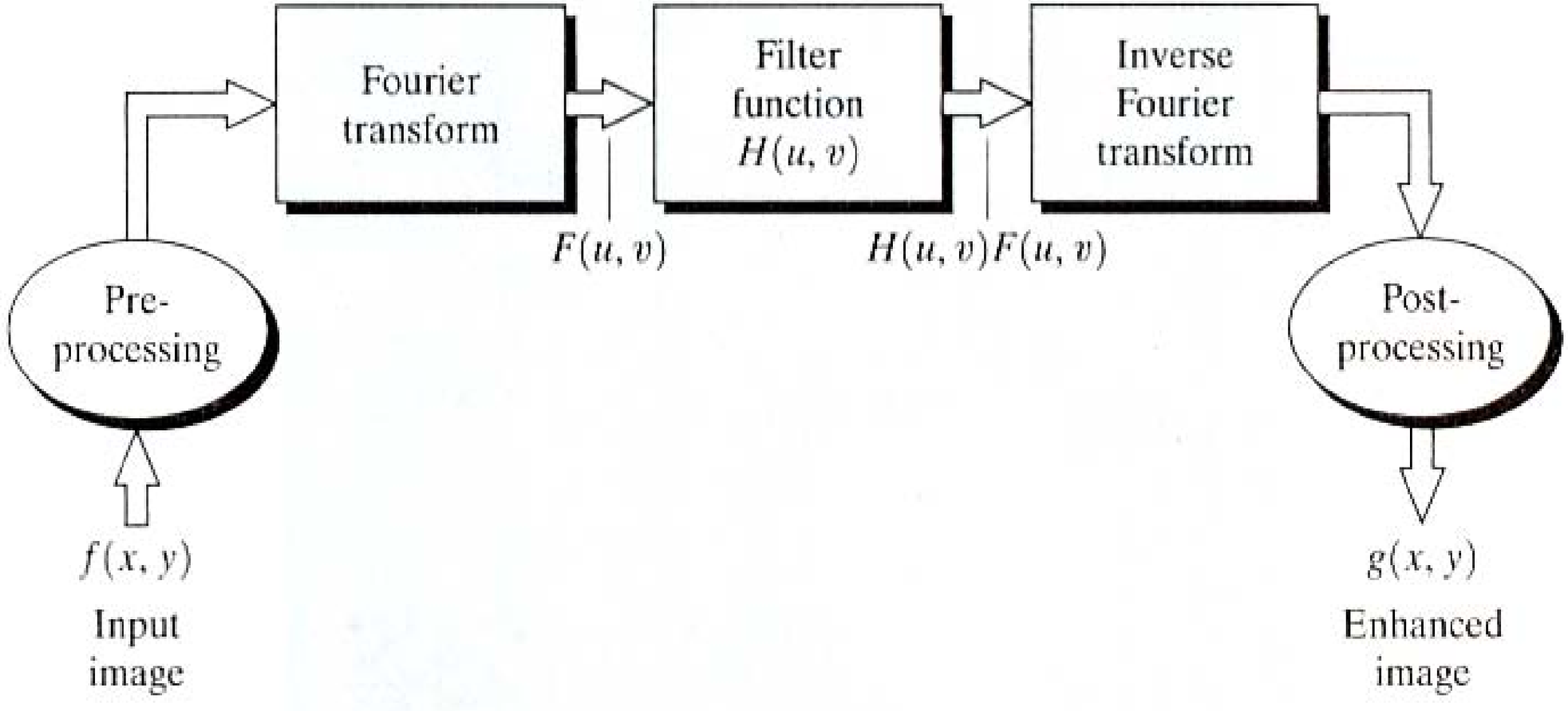
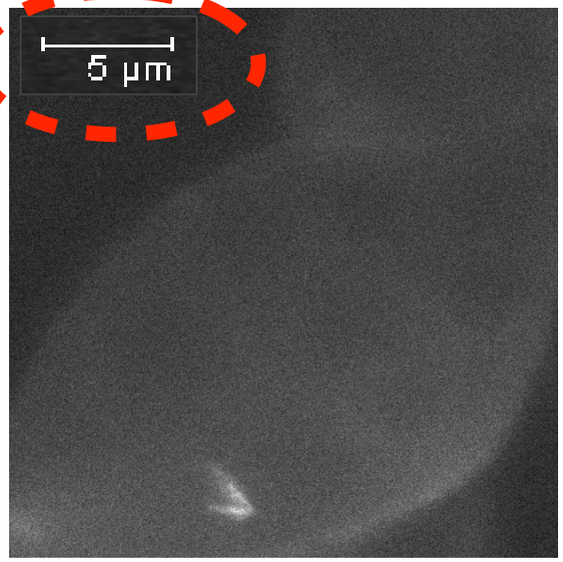


FIGURE 4.5 Basic steps for filtering in the frequency domain.

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

Noise Filtering

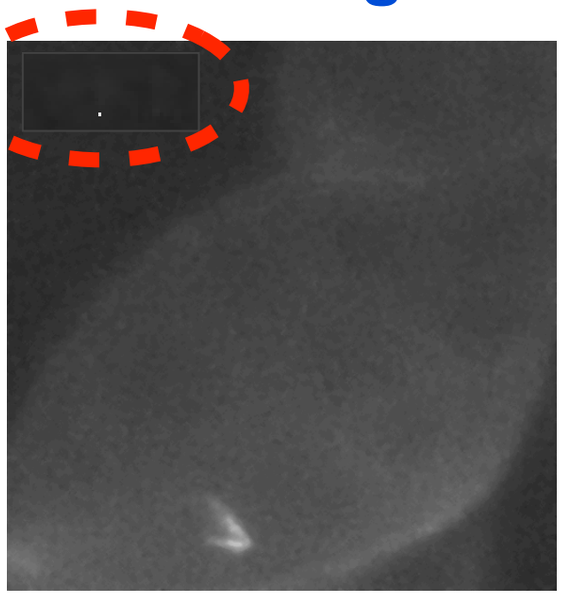
limited use when resolving fine detail!



Noisy image

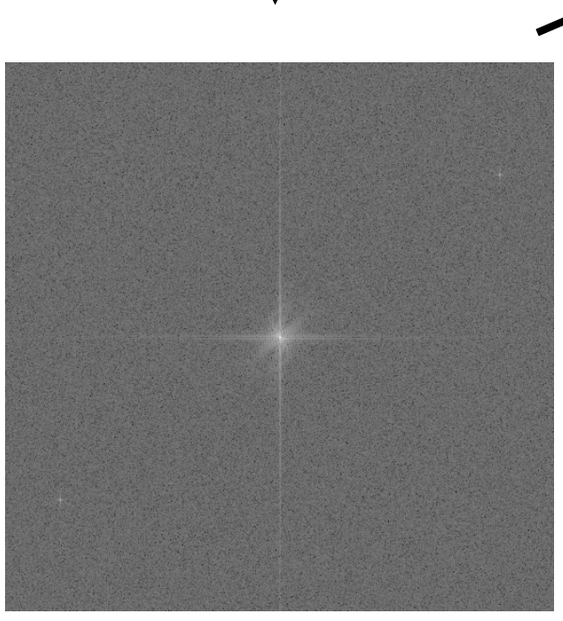
Spatial domain processing

3x3 Median
(high pass filter)



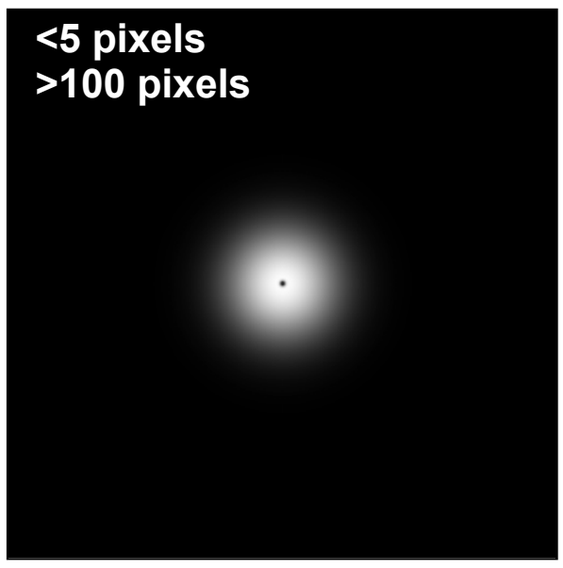
Noise reduced
Some false contours

Fourrier transform

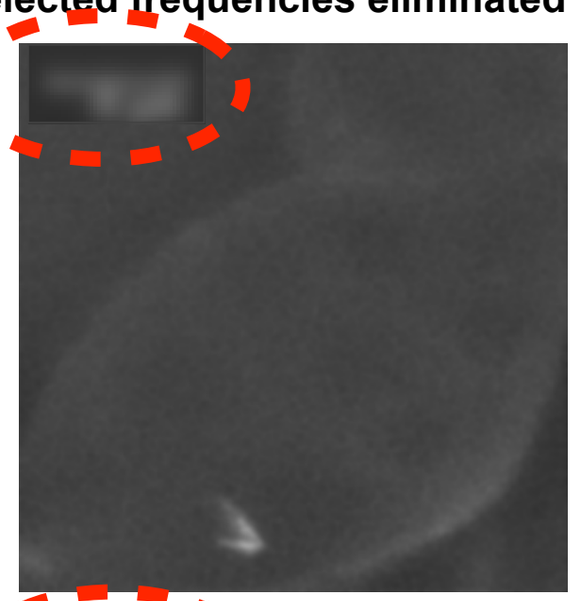


X

Band pass filters (mask)

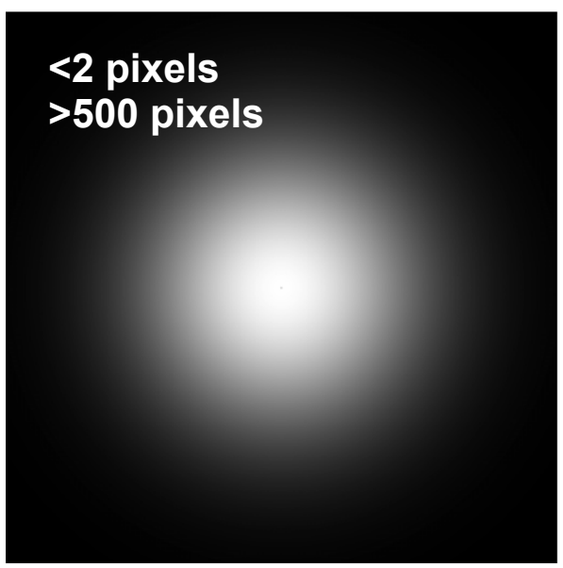


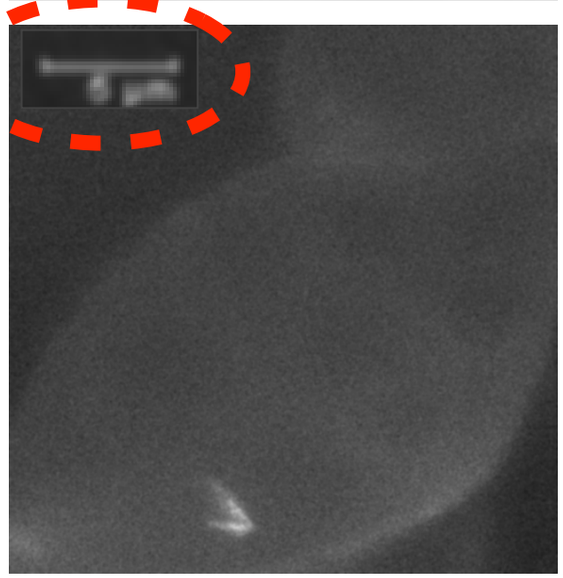
Selected frequencies eliminated



inverse Fourrier transform

X



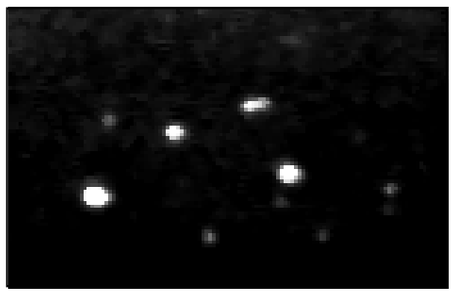


Frequency domain processing

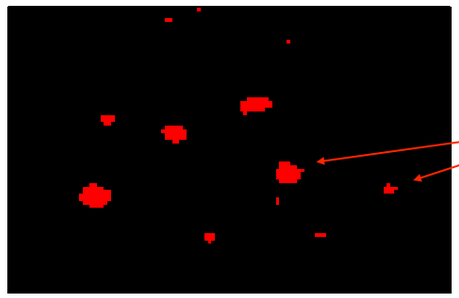
ImageJ

MID LEVEL Processing

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



Segmentation



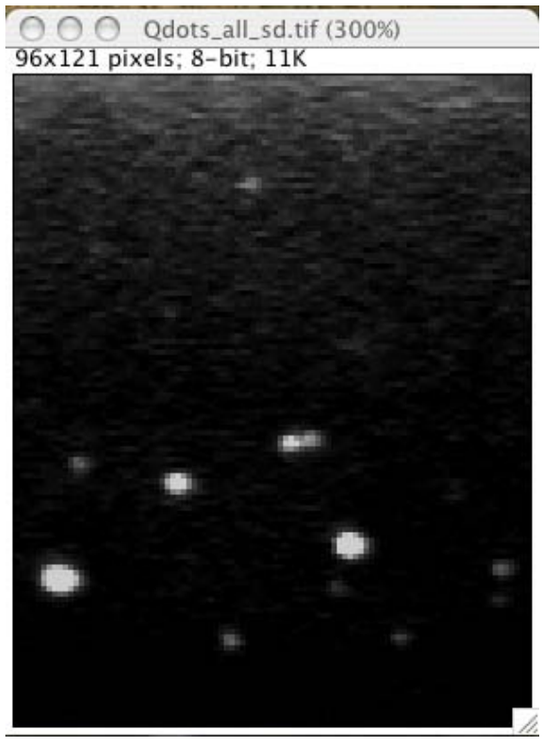
Objects defined

Number of objects
Positions of objects
Size of objects

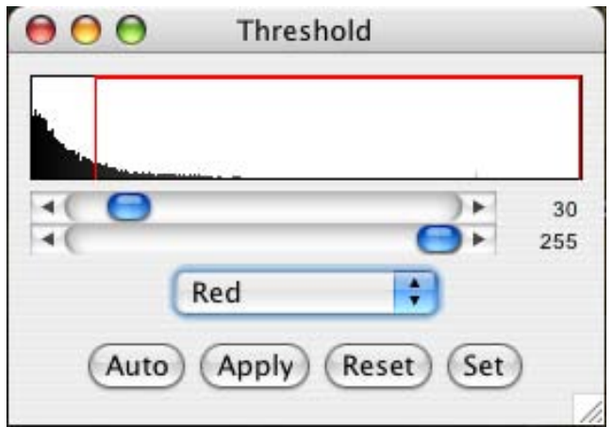
Also:
- Colocalisation

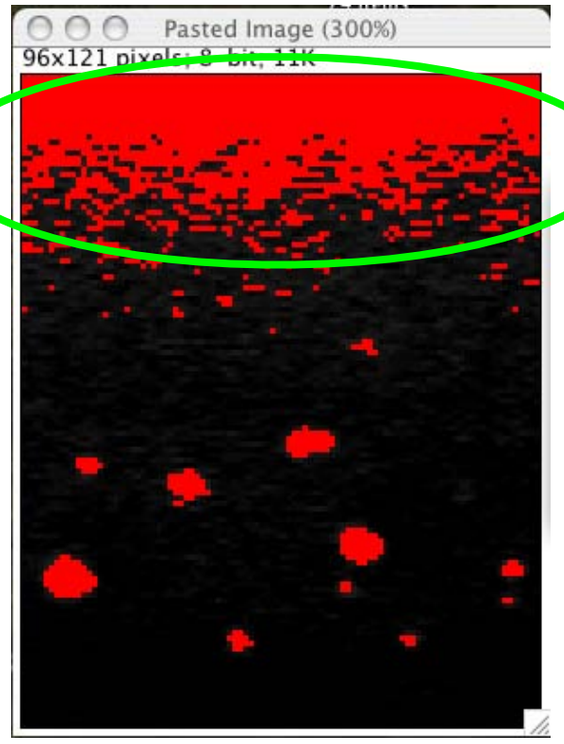
(See appendices)

MID LEVEL Processing - simple segmentation

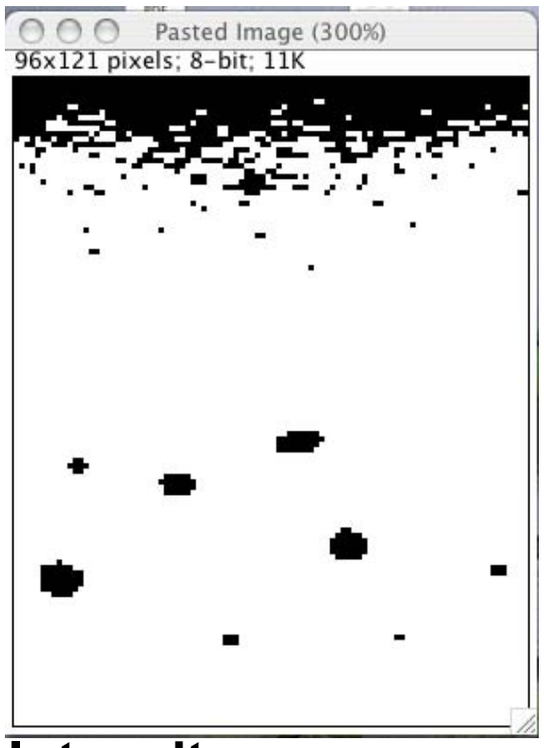


Segment by intensity



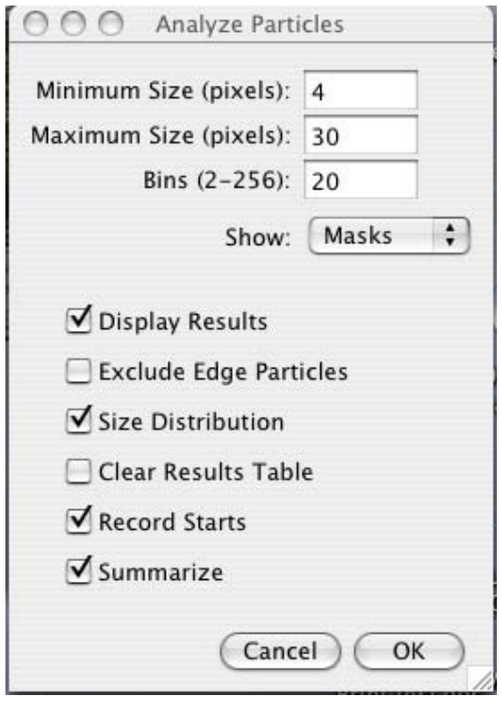


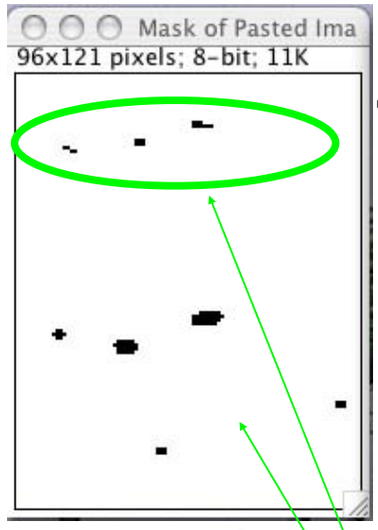
problem region



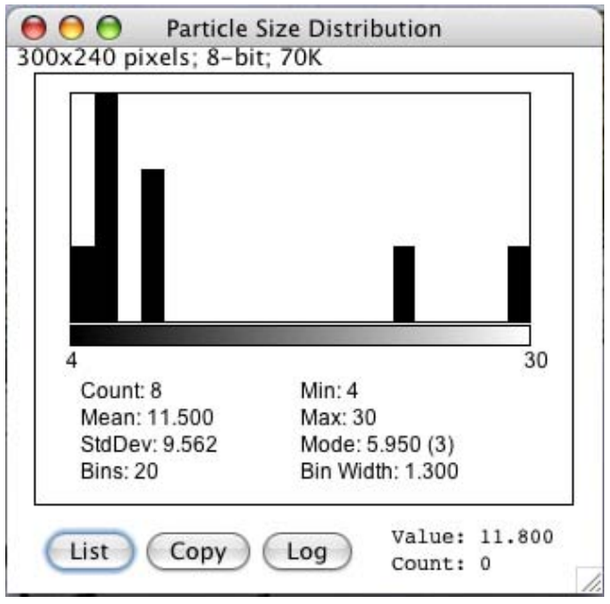
Intensity segmented binary image

Segment again by area size





mask defining regions



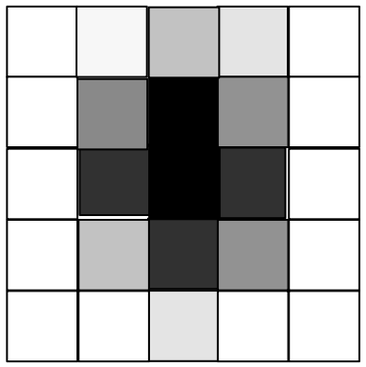
attributes

Some particles lost - some artificially created

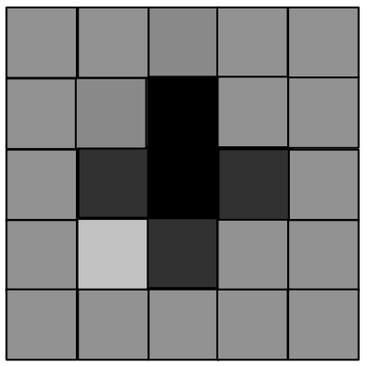
ImageJ

MID LEVEL Processing - centroid

Segmentation

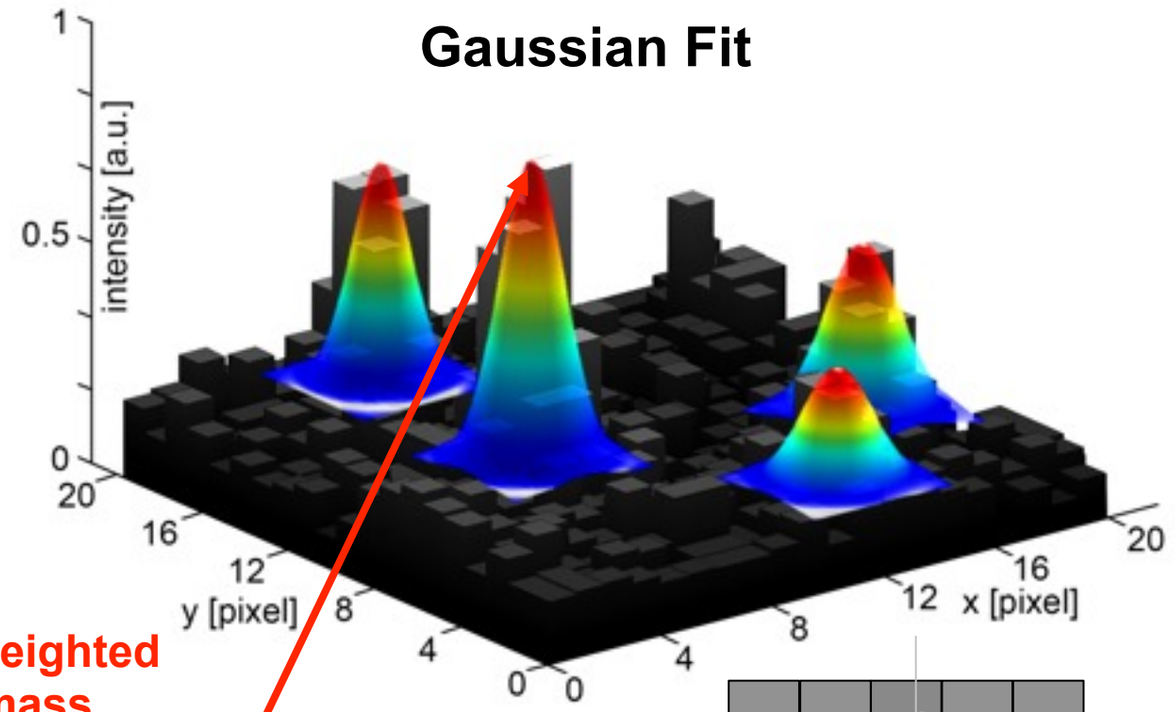


Recorded
Pixel image



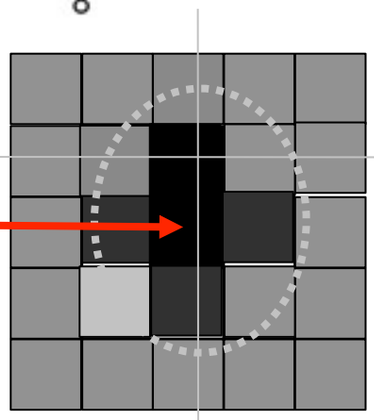
Segmented
image

Gaussian Fit



Intensity weighted
Centre of mass
Calculation

Centroid
Co-ordinates
XY



- 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

The human brain is still one of the best segmentation tools

But

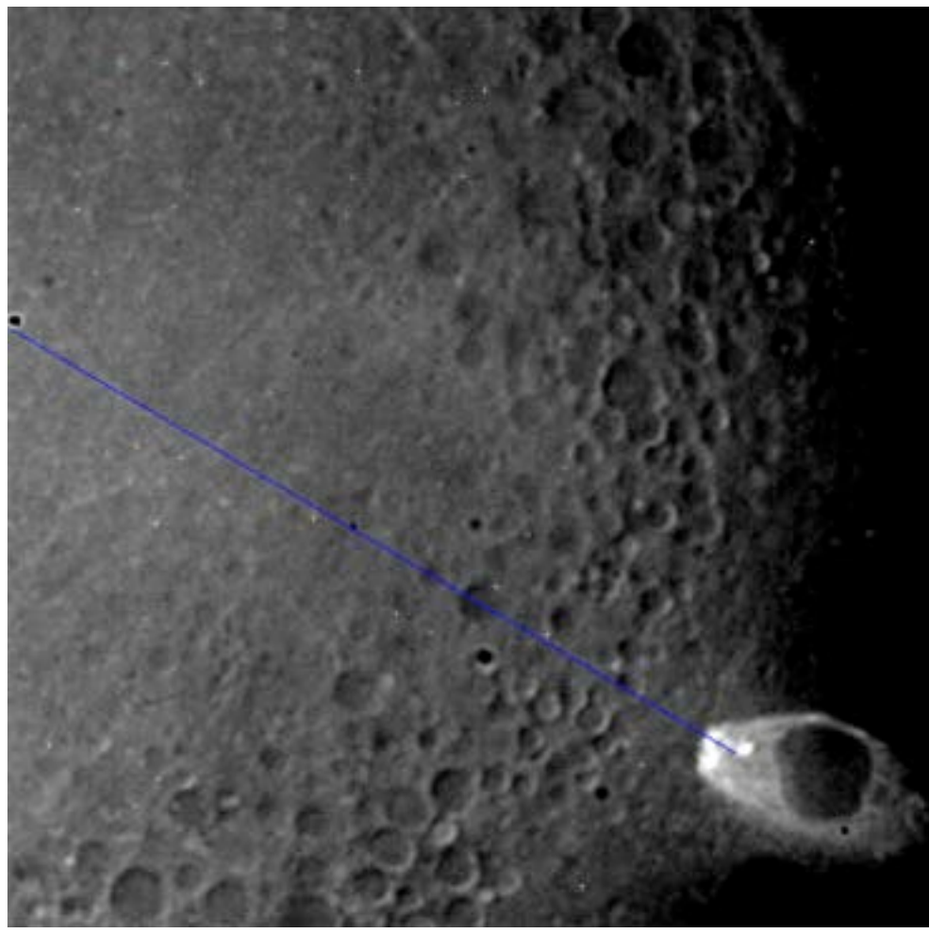
Subjective not objective!

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

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.

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