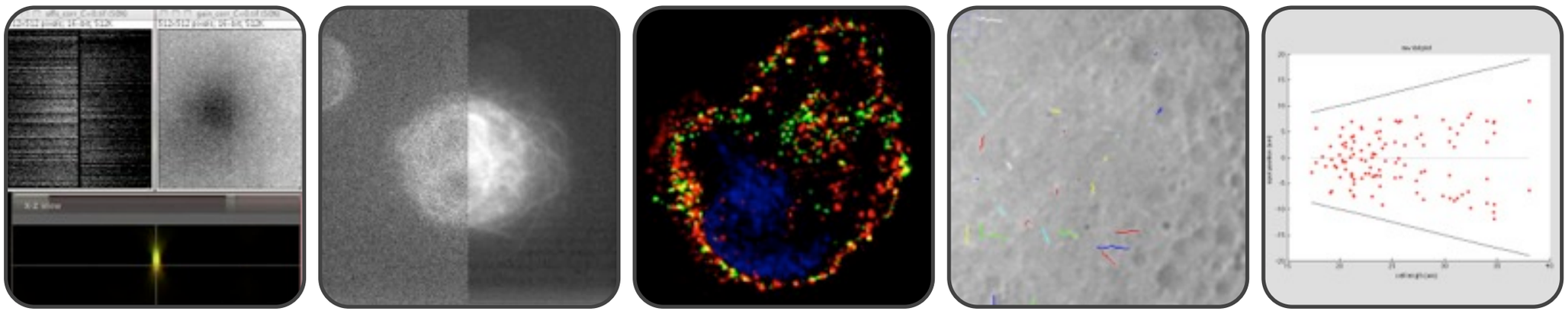
Lecture 19 – Applied Image Analysis

Graeme Ball



graeme.ball@bioch.ox.ac.uk

Three Questions

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1. Why should you be interested in image processing & analysis?

2. What are the key concepts & techniques?

3. How can you apply them most efficiently?

graeme.ball@bioch.ox.ac.uk

1. Science requires data

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Hypothesis testing requires data analysis & statistics

Cell author guidelines:-

"processing may be unavoidable in certain instances and is permitted provided that the final data accurately reflect that of the original"

"any alterations must be clearly stated in the figure legend and in the methods section"

" alterations must be applied to the entire image (e.g., brightness, contrast, color balance)"

" authors will be required to make the original unprocessed data available to the editors"

graeme.ball@bioch.ox.ac.uk

1. Images are data

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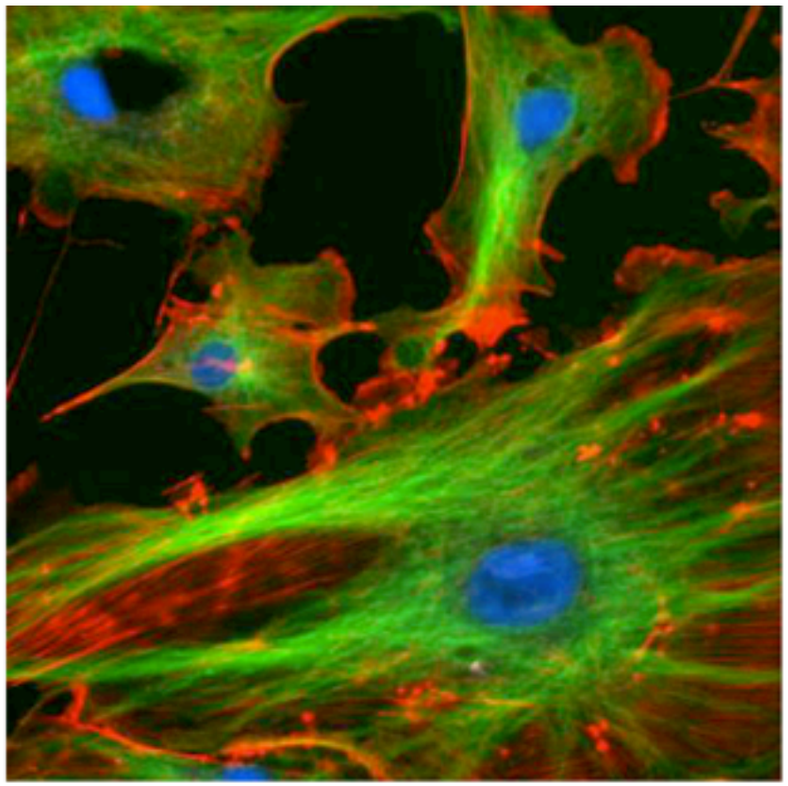
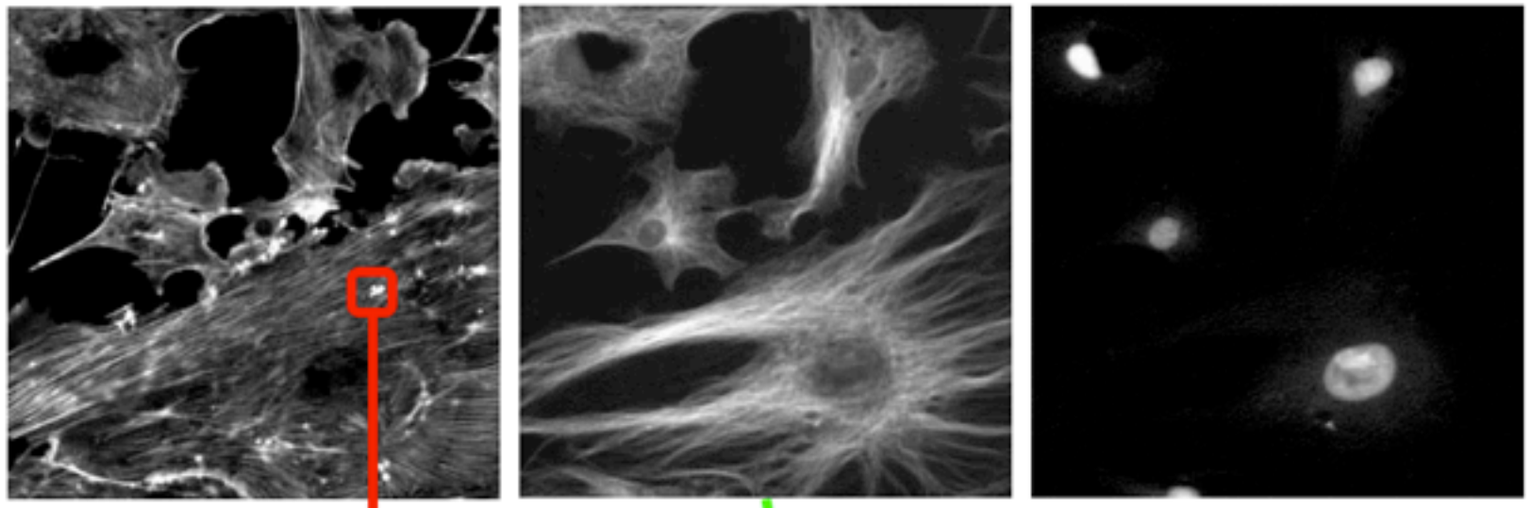
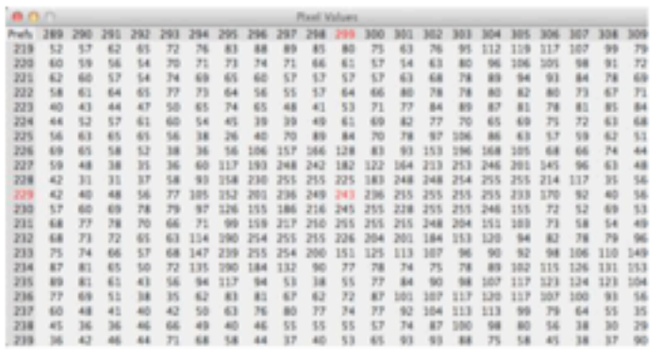
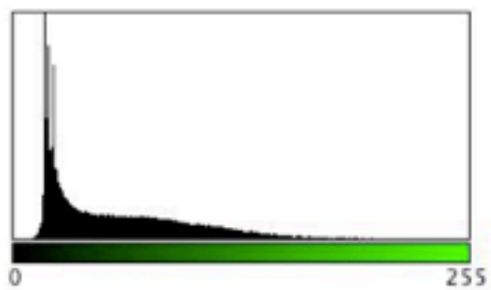


8-bit image (0-255), 3 channels

.. think “data”, not “picture”





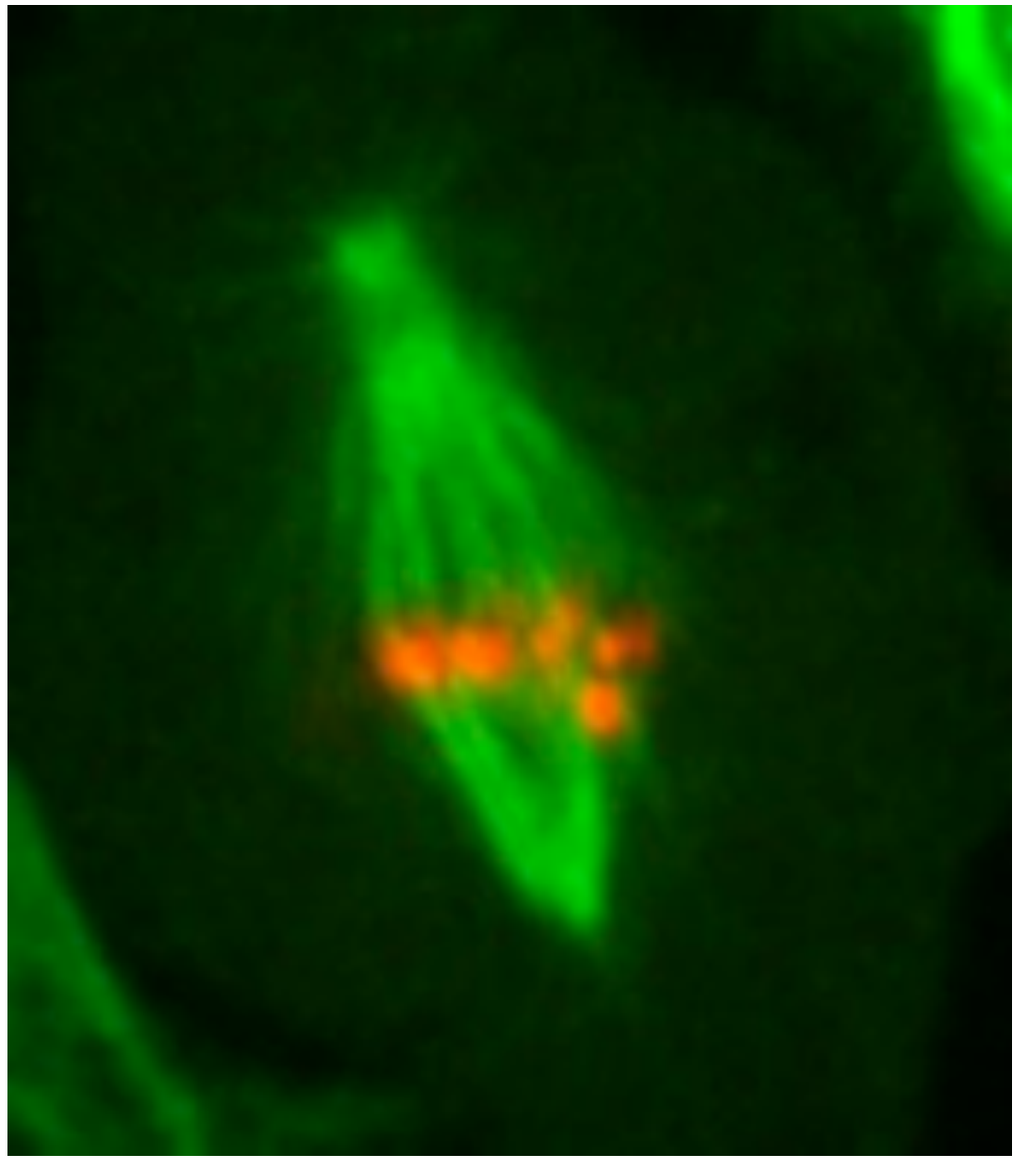


a digital image is:

- data: arrays of carefully calibrated intensity measurements
- metadata: data about the data, which is essential for interpretation

graeme.ball@bioch.ox.ac.uk

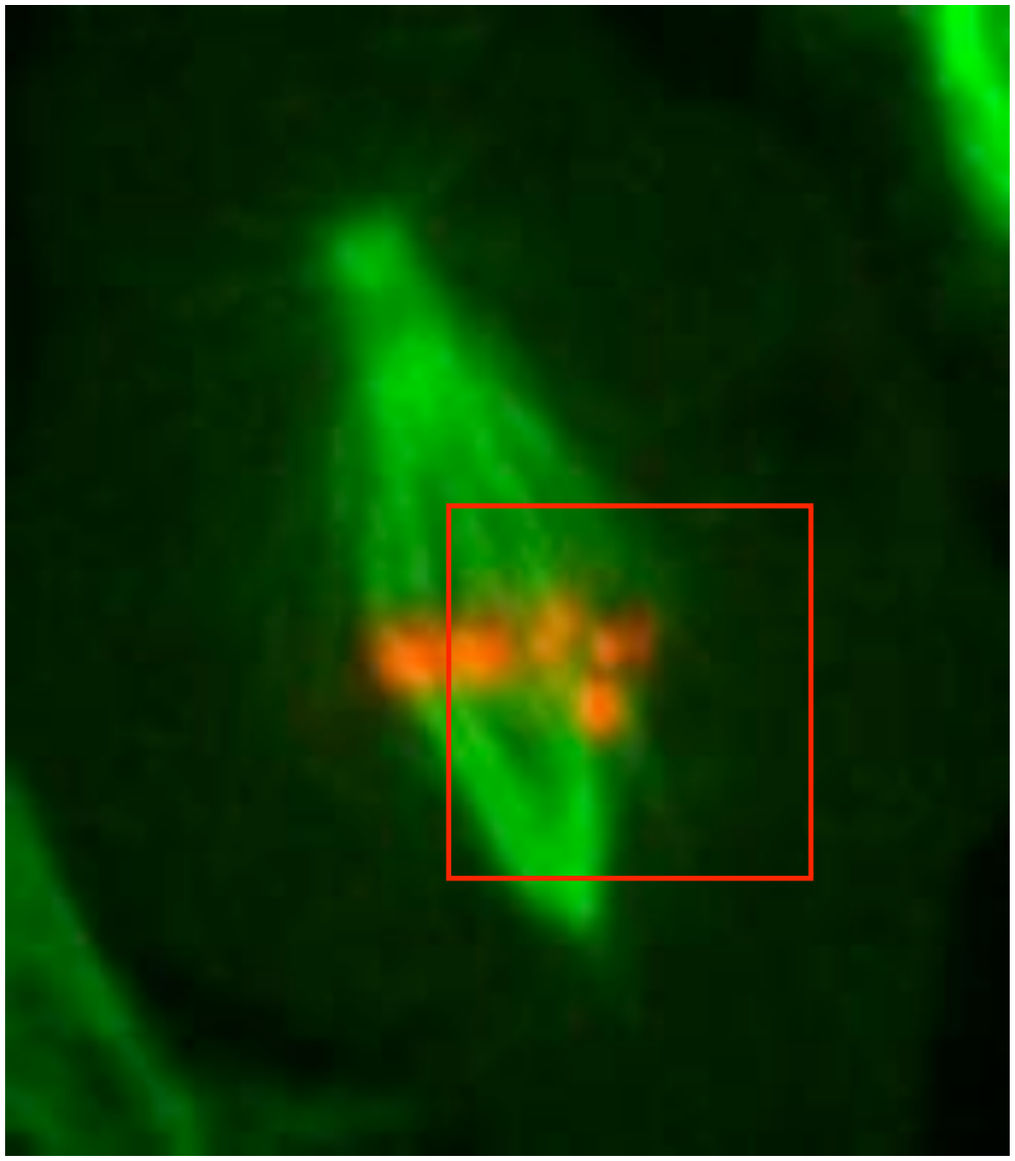
1. JPEGs are (much) less data



5D 16-bit data: 33 MB,

17 million pixels (0-65535)

134 KB per 2D plane



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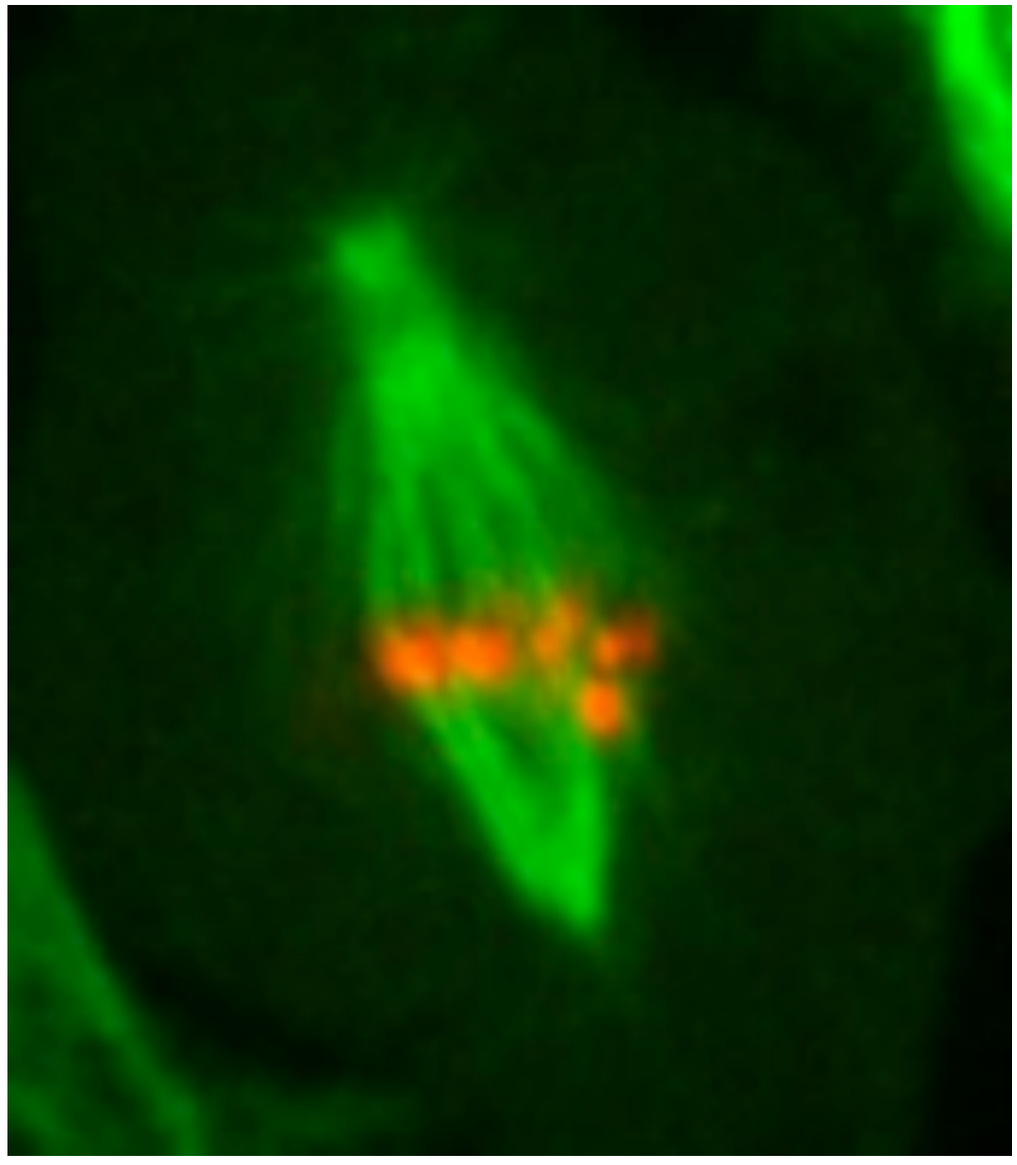

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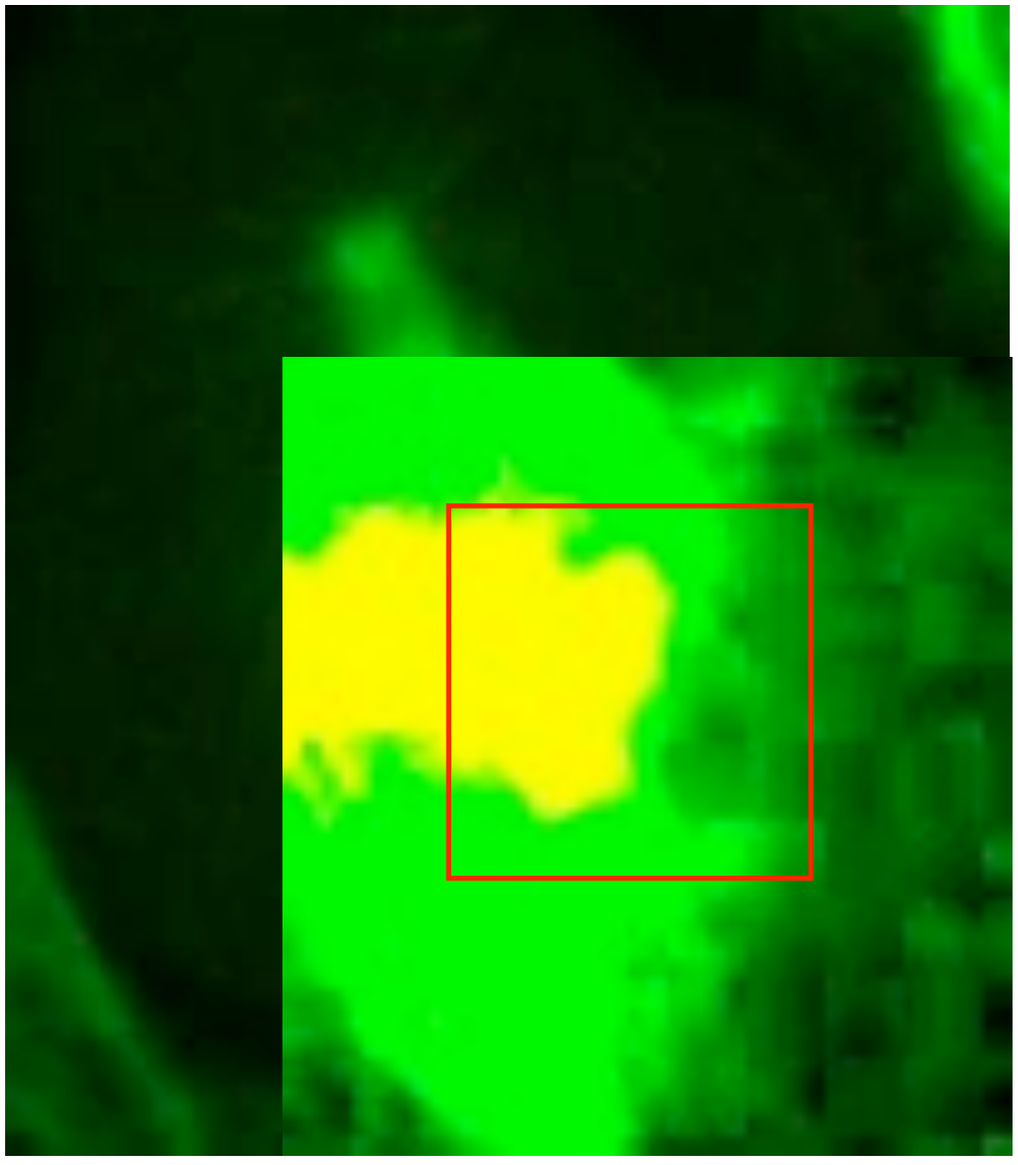

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1. Collect the right data

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What am I trying to measure?

graeme.ball@bioch.ox.ac.uk

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Expression level? ⟹ accurate, calibrated intensities

graeme.ball@bioch.ox.ac.uk

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What am I trying to measure?

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Distance or colocalization? ⇒ contrast-to-noise, resolution, alignment

graeme.ball@bioch.ox.ac.uk

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Dynamics? ⇒ temporal resolution, photostability

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⇒ decide on the instrument (& technique)

graeme.ball@bioch.ox.ac.uk

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Dynamics? ⇒ temporal resolution, photostability

⇒ decide on the instrument (& technique)

⇒ choice of fluorophores

graeme.ball@bioch.ox.ac.uk

2. Image Processing & Analysis

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Image processing

Image restoration – correction, denoising, deblurring

Filtering to enhance features of interest

Segmentation – dividing an image into useful categories (e.g. feature, background)

Image analysis

Identifying and tracking features

Summary statistics: intensity/amount, distribution, speed, colocalization

graeme.ball@bioch.ox.ac.uk

2. Image Restoration

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Image restoration aims to recover the true image

Flat-field correction – uneven illumination (also, pseudo-correction)

Denoising / noise filtering – smoothing, neighborhood filters, non-local

Deblurring – deconvolution with or without PSF, unsharp mask

Image registration – rigid/affine versus elastic – intensity-based or feature-based

Normalization – intensity of each time-point scaled to correct bleaching (& flicker)

graeme.ball@bioch.ox.ac.uk

2. Image Filtering

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Image filtering enhances some features / rejects others

Spatial filters for smoothing & sharpening

Frequency domain filters enhance/suppress features based on size

Histogram operations, e.g. histogram equalization for contrast enhancement

Adaptive filtering equivalent to global filtering after normalization

Time-domain filtering - see temporal median filter in the tracking example

graeme.ball@bioch.ox.ac.uk

2. Image Processing Examples

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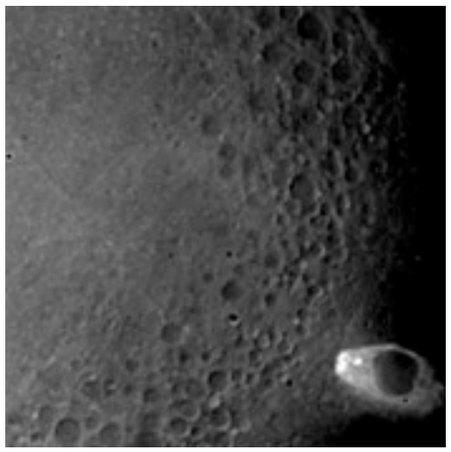
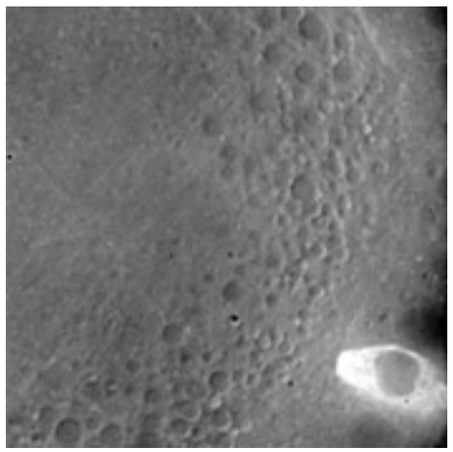
pseudo-flat field

Im = Im-mean * Im/mean-filtered



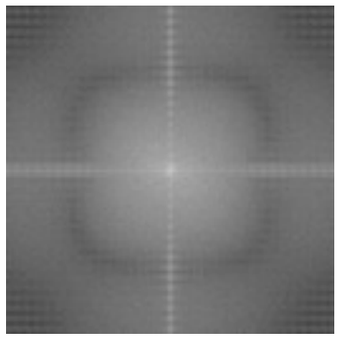
original



pseudo-corrected

240x240 mean-filt

FFT reveals frequencies



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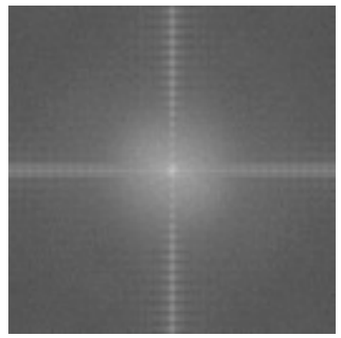
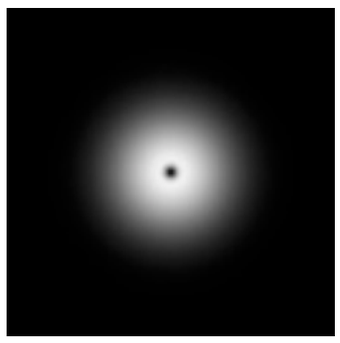
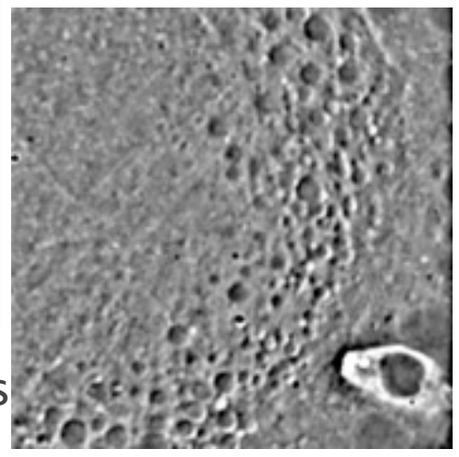


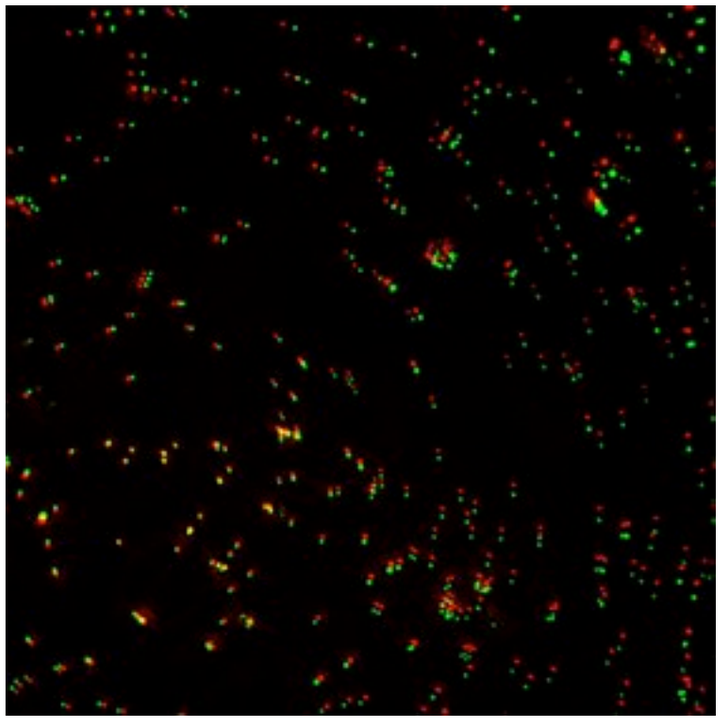
3x3 median



3-25 bandpass



registration can be critical!



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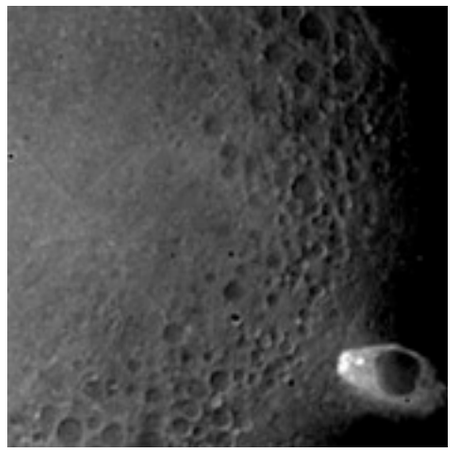
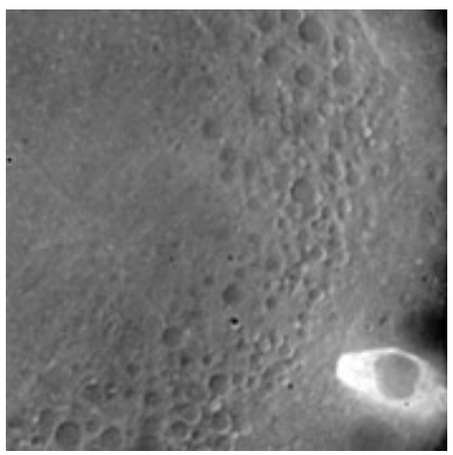


original



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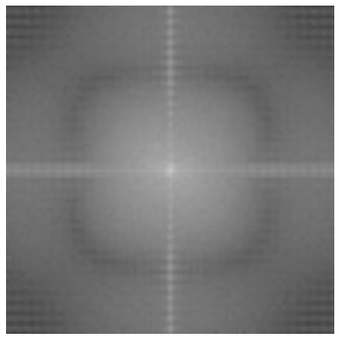

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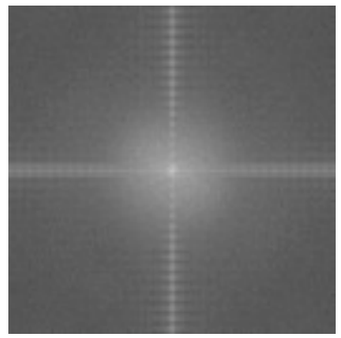

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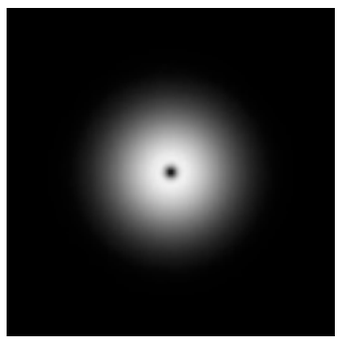

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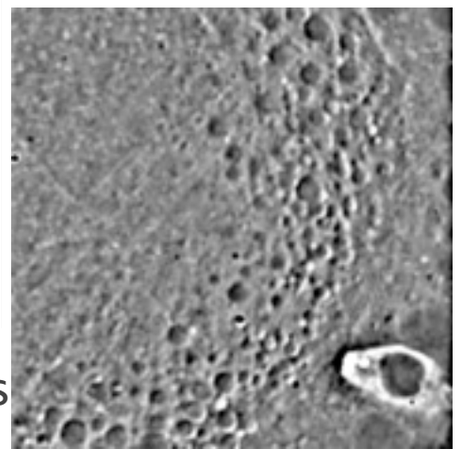

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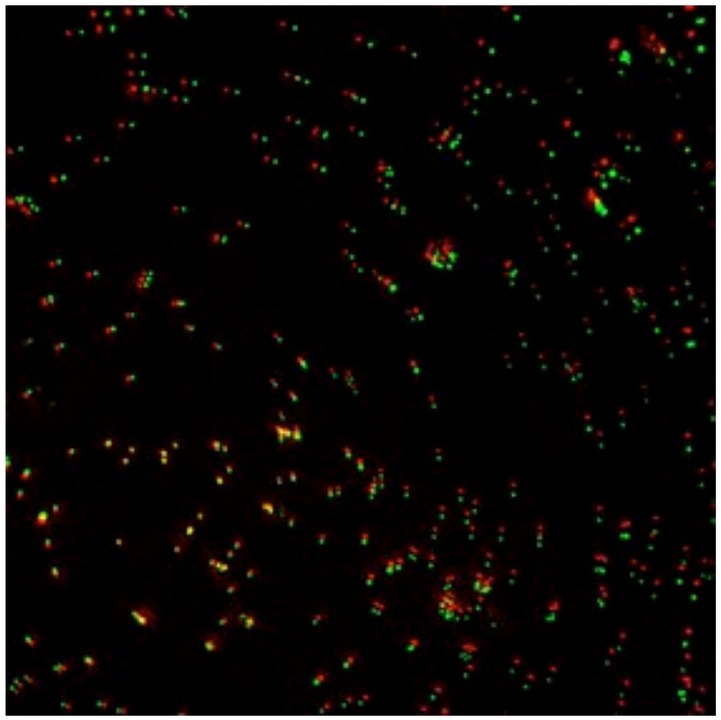

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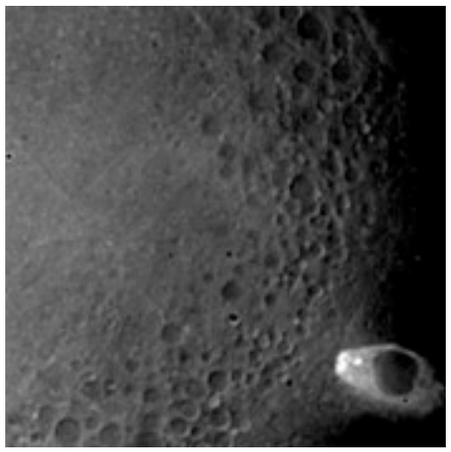

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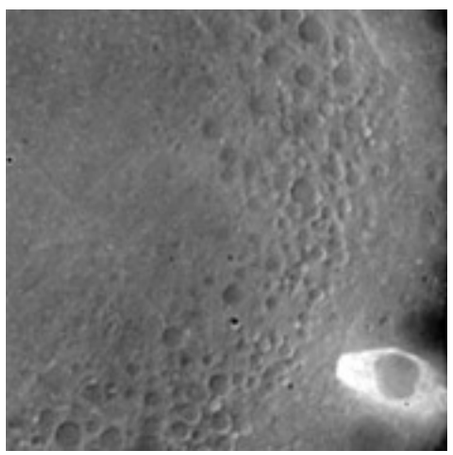

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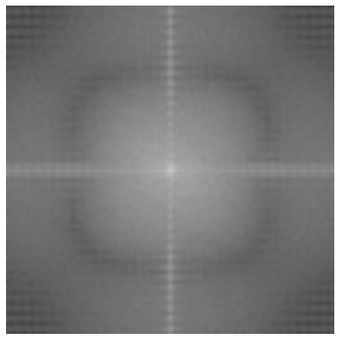

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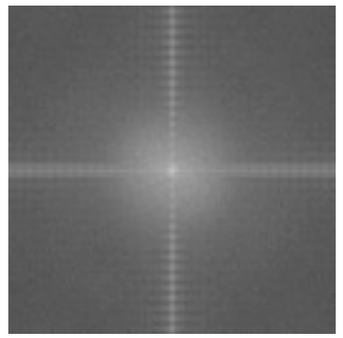

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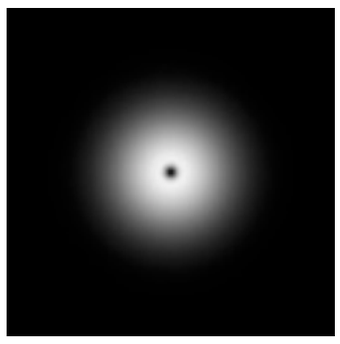

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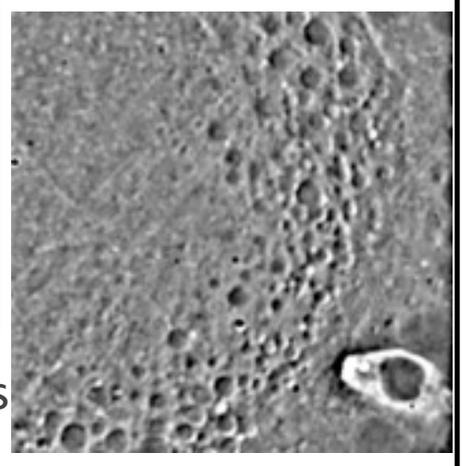

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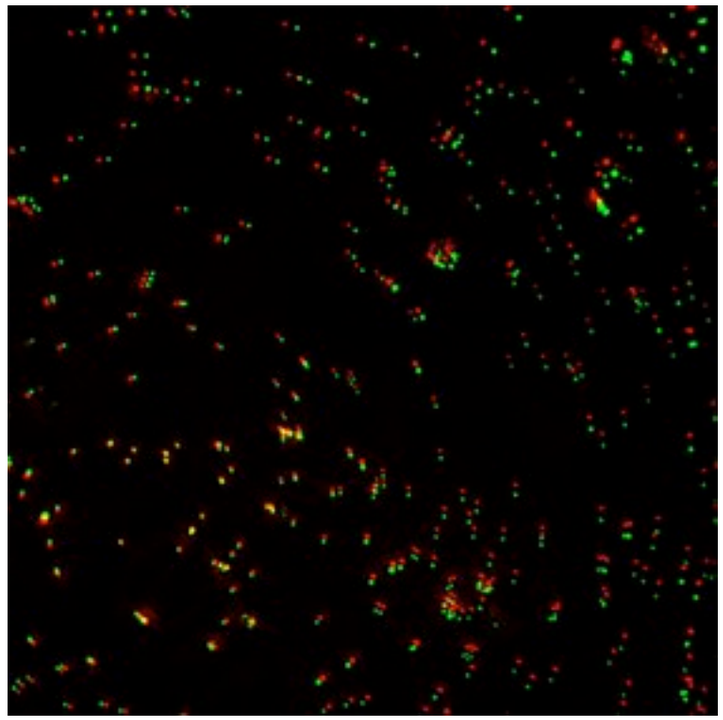

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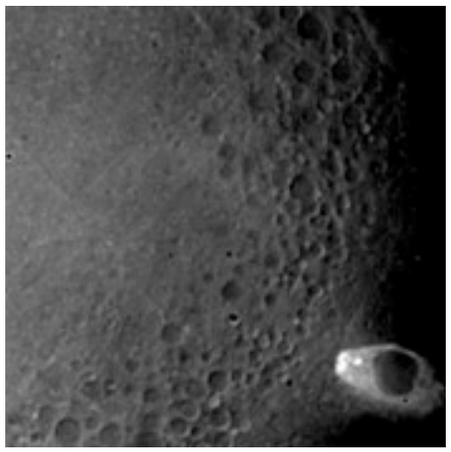

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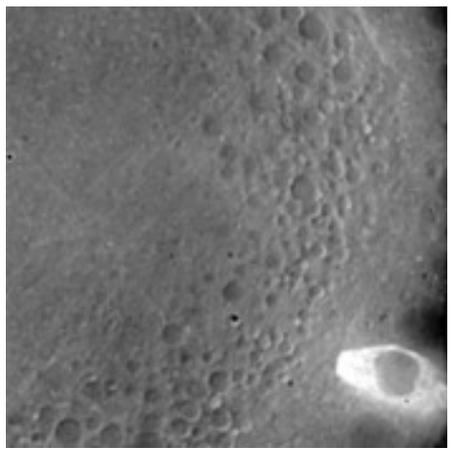

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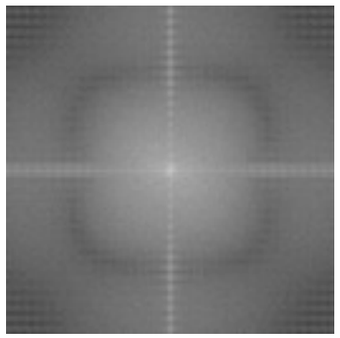

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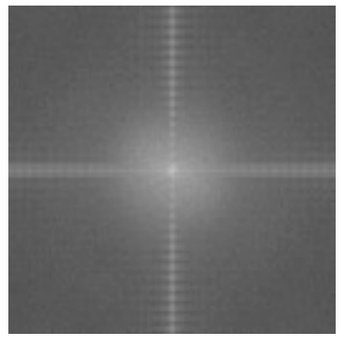

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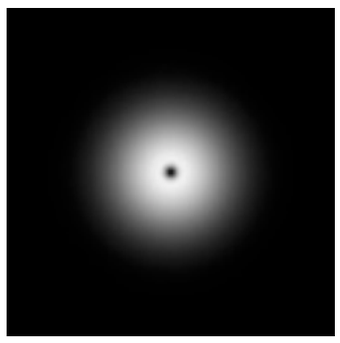

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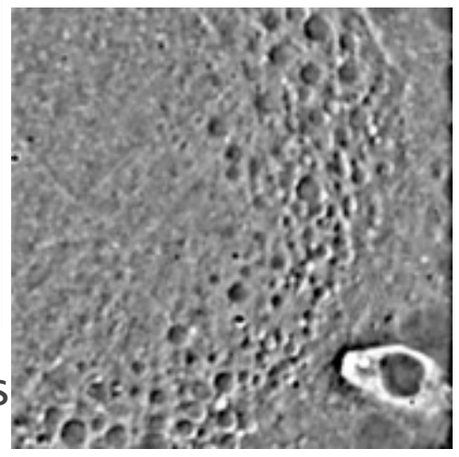

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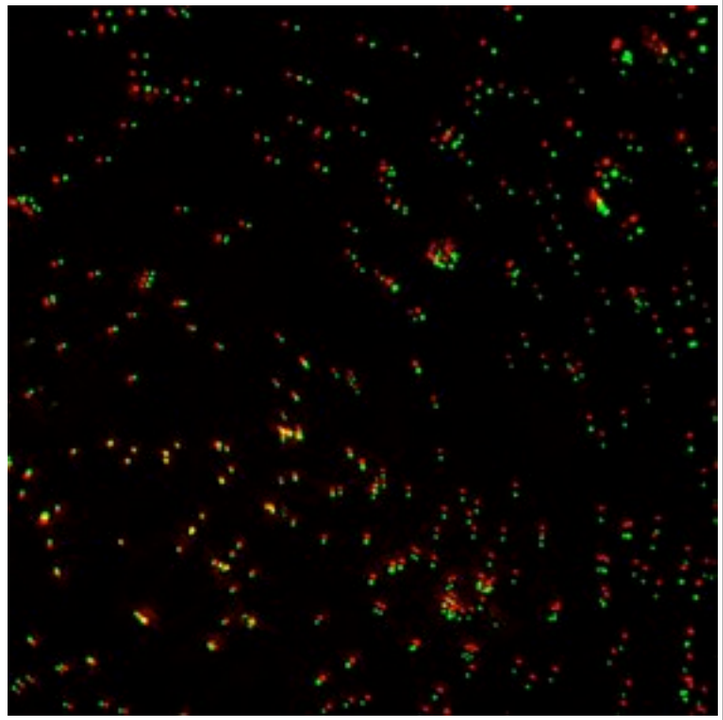

graeme.ball@bioch.ox.ac.uk

Patch-based denoising: 10-100 x less light?

8 ms exposure, 10% 488 Laser power

8 ms exposure, 0.1% 488 Laser power

Macrophage: Jupiter-GFP 7Z, 3stacks/s (Richard Parton)

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

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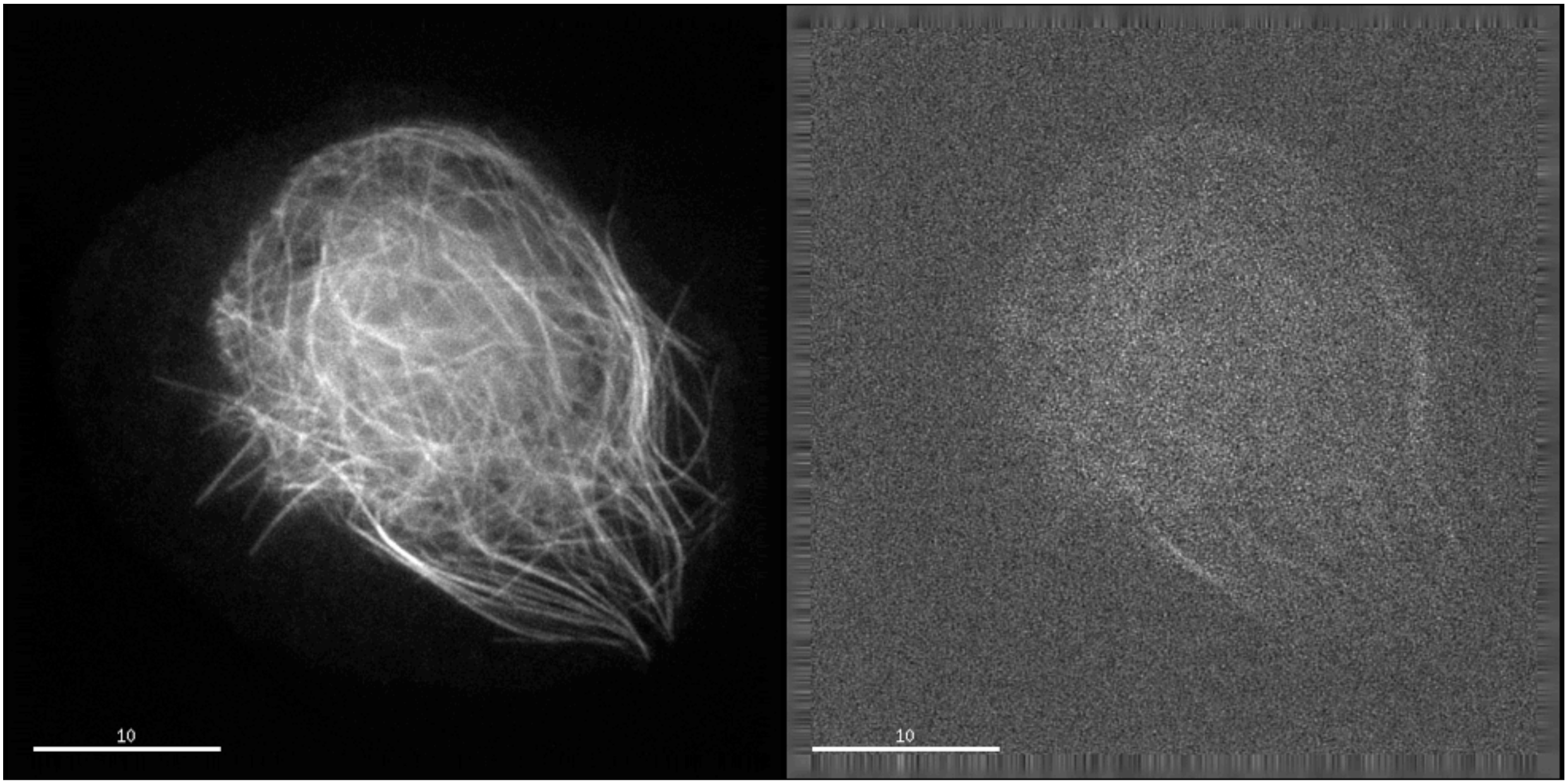

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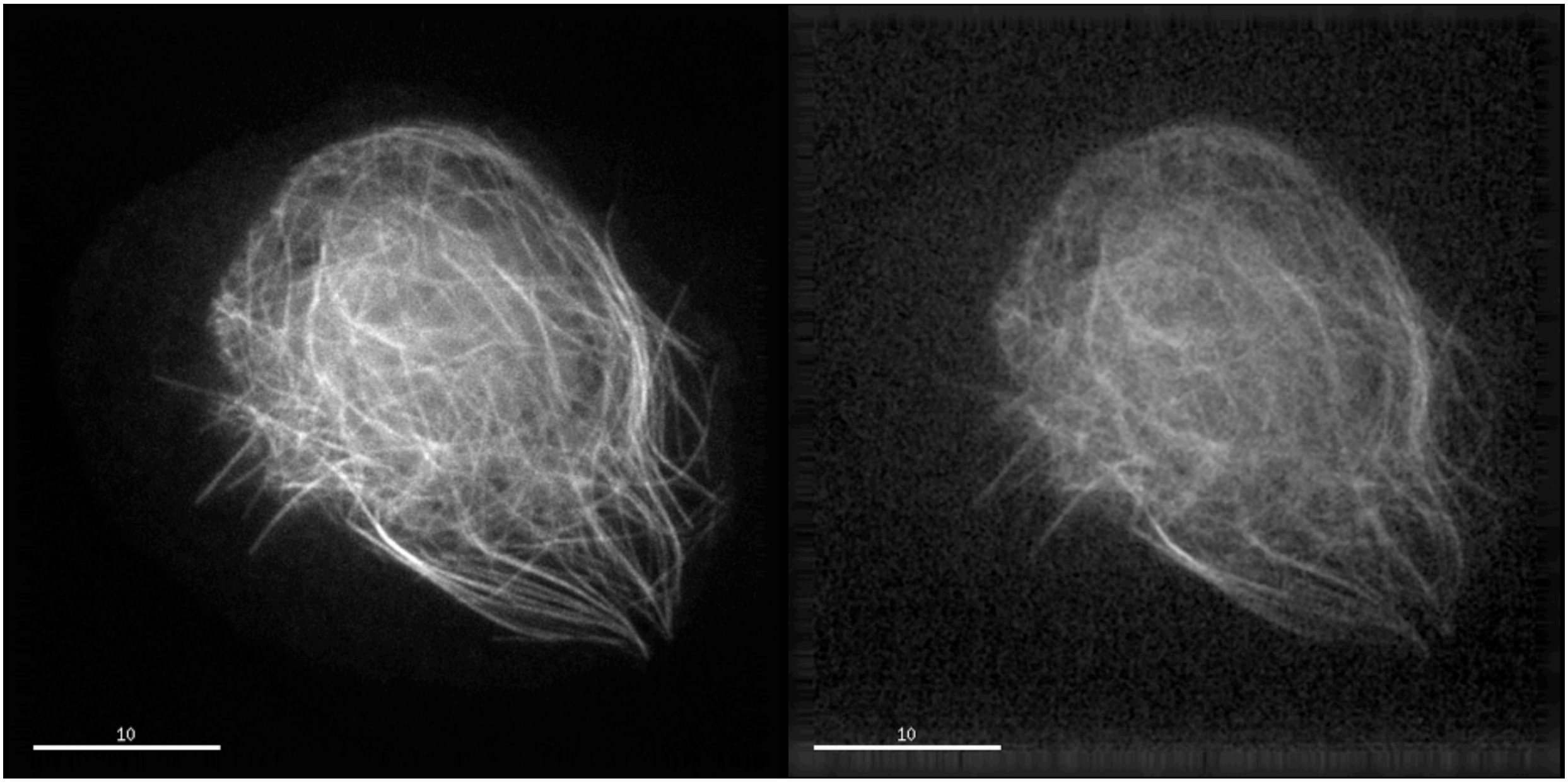

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CLAHE

- Contrast Limited Adaptive Histogram Equalization

graeme.ball@bioch.ox.ac.uk

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2. Image Segmentation

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Segmentation separates an image into subregions

2 common representations: "**binary mask**" images, and Regions Of Interest (**ROI**s)

Simple intensity thresholding – several methods (e.g. Otsu) to estimate threshold

Spot/particle detection – intensity, size and shape

Edge detection (e.g. Sobel) & Morphological image processing* (erosion, dilation)

Watershed calculation, Voronoi diagram, Ultimate eroded points

Machine learning and Manual options

graeme.ball@bioch.ox.ac.uk

2. Binary masks & operations

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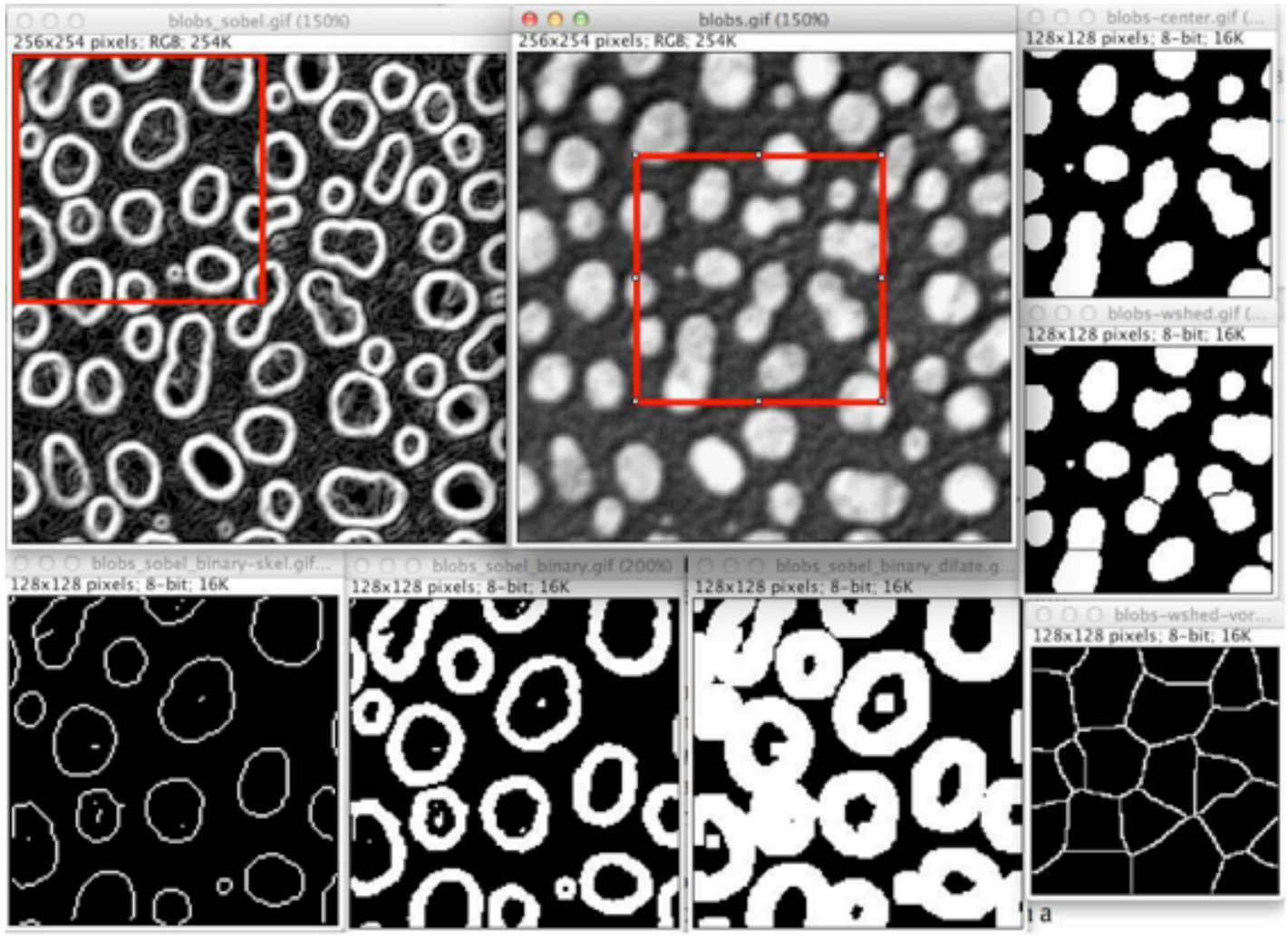
Binary images usually created using a intensity threshold

ImageJ binary images are 8-bit gray, 255 and 0 (instead of 1 and 0)

Binary operations: erode, dilate, skeletonize, watershed, Voronoi



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Edge detection

- "Find edges" is a Sobel operator: detects high gradient at edges

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

Gx

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

Gy

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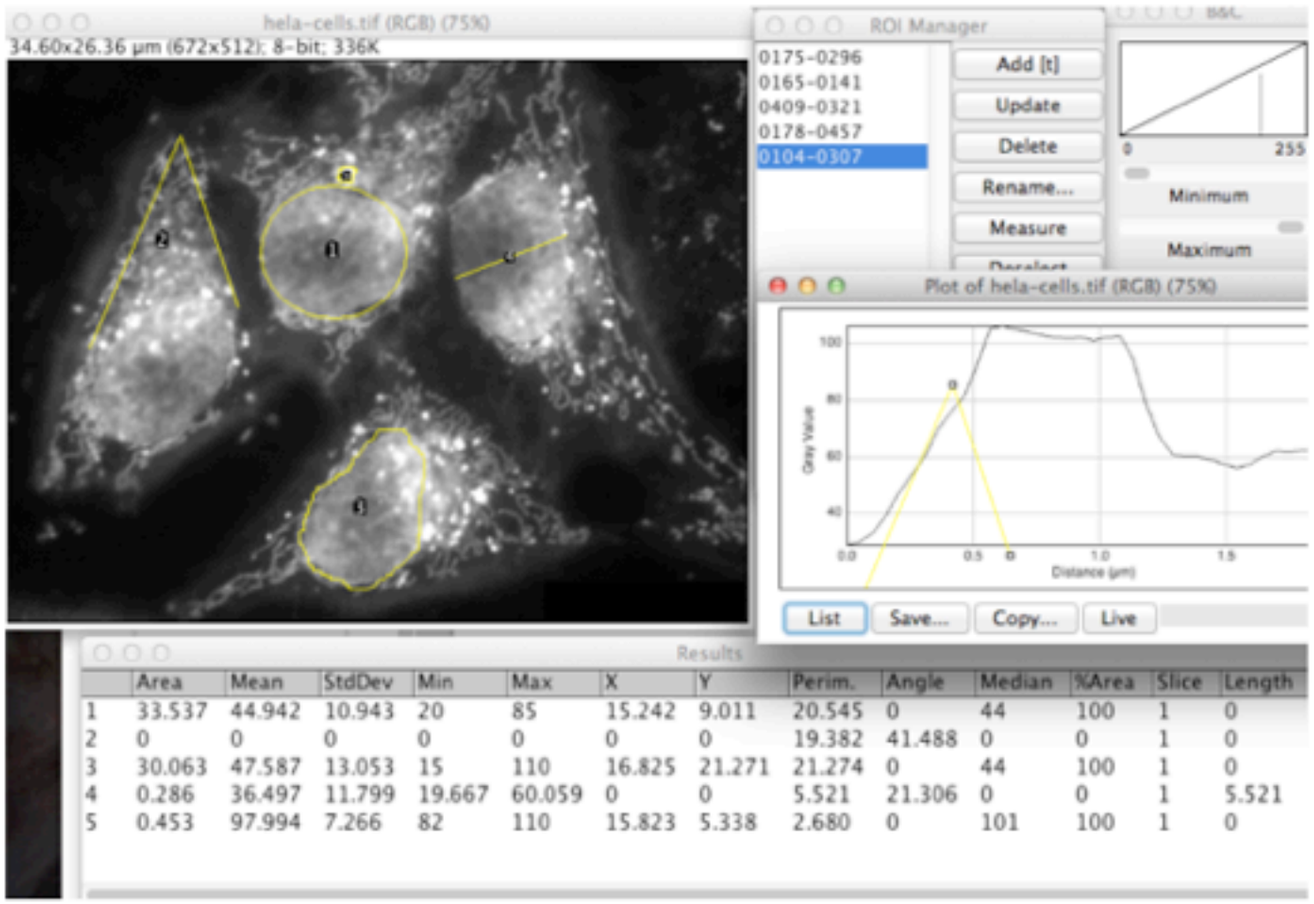
Selections & measurements

- various selection tools: points, lines, enclosed regions, wand tool
- measure distances & angles
- add selection to ROI manager "t"
- *Measure* command (& *Set Measurements*)



	Area	Mean	StdDev	Min	Max	X	Y	Perim.	Angle	Median	%Area	Slice	Length
1	33.537	44.942	10.943	20	85	15.242	9.011	20.545	0	44	100	1	0
2	0	0	0	0	0	0	0	19.382	41.488	0	0	1	0
3	30.063	47.587	13.053	15	110	16.825	21.271	21.274	0	44	100	1	0
4	0.286	36.497	11.799	19.667	60.059	0	0	5.521	21.306	0	0	1	5.521
5	0.453	97.994	7.266	82	110	15.823	5.338	2.680	0	101	100	1	0

graeme.ball@bioch.ox.ac.uk

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2. Fiji/ImageJ tips

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- useful tools that are easy to miss: the wand, ROI manager, brush selection
- understand how to manipulate stacks, hyperstacks and virtual stacks –
  e.g. how to convert, project, reduce, combine; channels tool
- make use of image histogram, “plot profile” and threshold tool
- learn how to “set measurements” and measure
- read the manual: http://rsbweb.nih.gov/ij/docs/user-guide.pdf

graeme.ball@bioch.ox.ac.uk

3. Free tools available

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ImageJ / Fiji – versatile 2D+ image analysis tool with many plugins


Icy, Vaa3D, BioimageXD – 3D image visualization & analysis

KNIME – data analysis workflow for large datasets / batch

CellProfiler – quantitatively measure cell phenotypes in large datasets (+ Worm Toolbox)


Priism/IVE, Priithon & Editor – 2D image processing/analysis (DV/OMX)

OMERO – image repository & visualization

graeme.ball@bioch.ox.ac.uk

3. Commercial Analysis Tools

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Imaris, Amira – 3D visualization & analysis packages

Volocity – 3D visualization & analysis package, spinning disk

SoftWoRx – API Deltavision, deconvolution, SI reconstruction

Metamorph – microscope control, image processing/analysis

Huygens, AutoQuant – Deconvolution software

graeme.ball@bioch.ox.ac.uk

3. 3D visualization & analysis

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- ImageJ/Fiji has 3D visualization & analysis fucntionality:

  3D viewer, Volume Viewer, Image 5D, hyperstacks, orthogonal view,

  3D objects counter

- Imaris, Amira and Volocity are 3D image visualization & analysis packages designed for microscopy (and medical imaging)

- Choice between viewing fluorescence intensity (often MIP) versus generating surface representations of objects

graeme.ball@bioch.ox.ac.uk

3. Imaris movie

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Image of bacterial septa (GFP) and DNA (DAPI), Christian Lesterlin

graeme.ball@bioch.ox.ac.uk

3. Volocity

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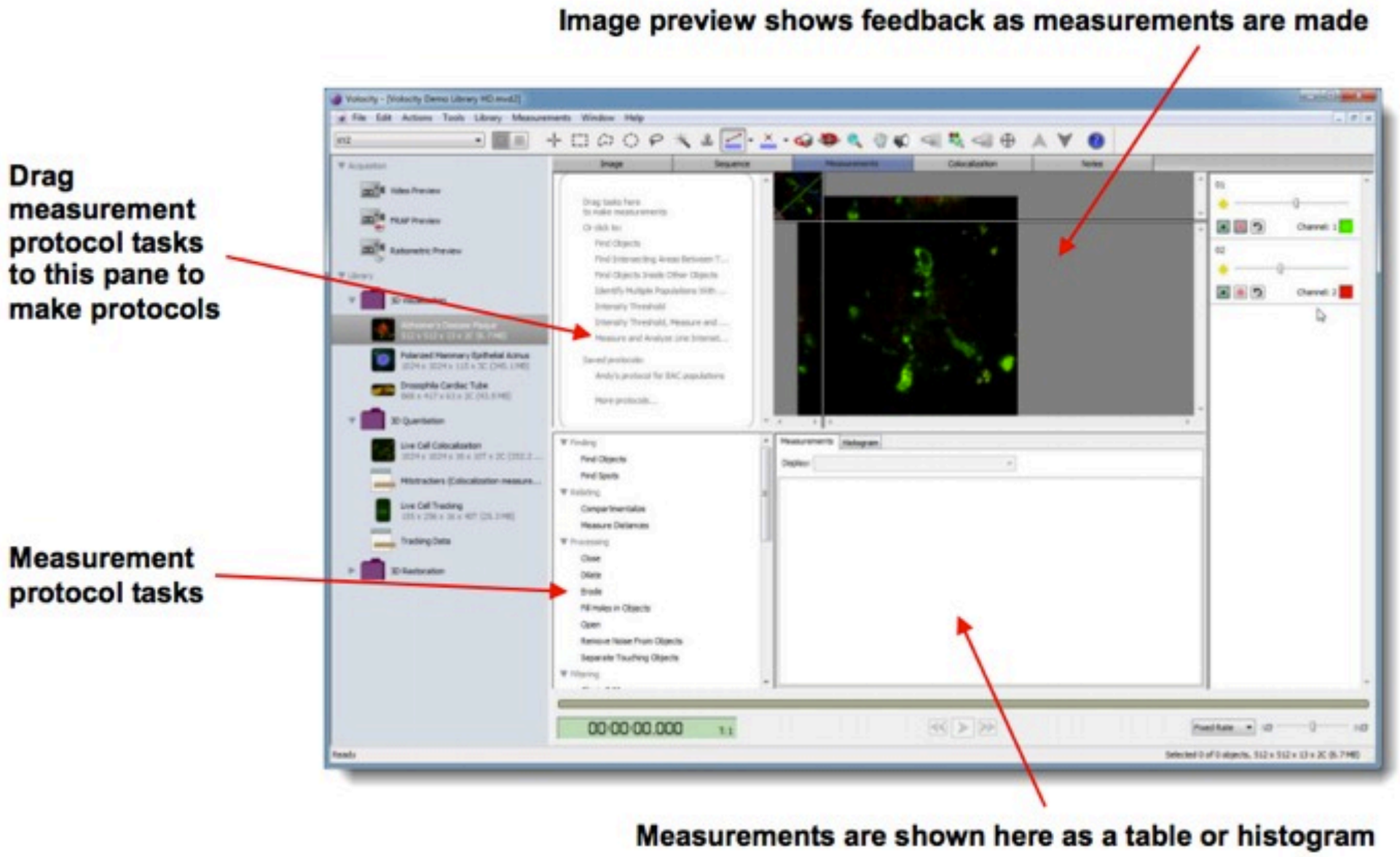
View the Measurements tab. The Measurements View contains all the tools and information needed for selecting objects.



http://www.perkinelmer.com/PDFs/downloads/CreatingMeasurementProtocolVolocitySoftware.pdf

graeme.ball@bioch.ox.ac.uk

3. Measuring Colocalization

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Read Bolte & Cordelieres' 2006 review :-

http://www.ncbi.nlm.nih.gov/pubmed/17210054

- prerequisites: make **very** certain that:-
  1. you do not have bleed-through! (or crosstalk)
  2. your channel alignment is properly calibrated
  3. your images are as noise-free and deblurred as possible

- many colocalization statistics rely on segmenting both channels

  => flat field & meticulous background correction
- use ROIs or masks to **analyze different compartments separately**
- 2 fundamental approaches:
  1. **intensity correlation** scatter plot, Pearson's (PCC)

  1b. Manders coefficients (M1, M2)
  2. **object-based analysis** (distances)

JaCoP
(ImageJ plugin)

graeme.ball@bioch.ox.ac.uk

3. Scatter plots and ICC

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Scatter plot of channel intensities

reveals presence/absence of colocalization:-

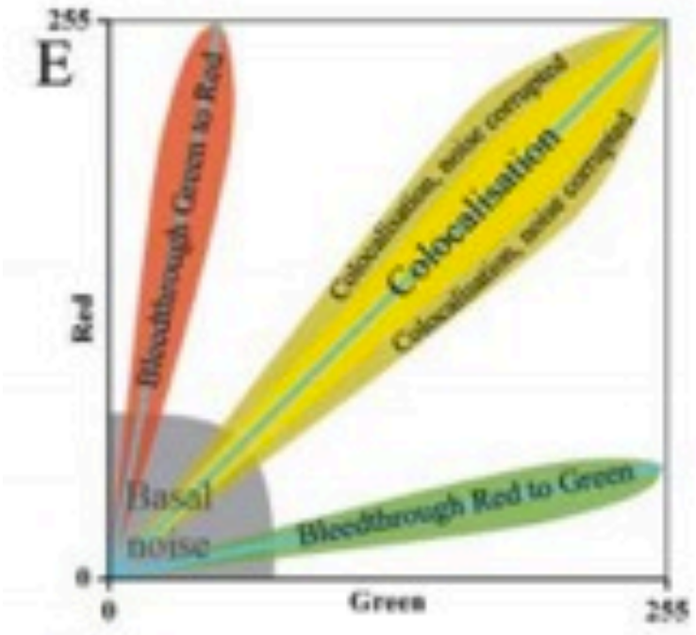

Fig. 5E

(Bolte & Cordelieres)

- Pearson's Correlation Coefficient (**PCC**) of 0.5-1 indicates colocalization
- large difference between M1 and M2 indicates more of one label - NB. if one label is everywhere, colocalization is meaningless!
- unlike PCC, negative values of Li's ICQ indicate mutual exclusion (0 means uncorrelated, 0.5 means colocalization)

Fiji & ImageJ have **Coloc_2** and **JACoP** plugins (JACoP has object-based method)

graeme.ball@bioch.ox.ac.uk

3. Scripts & statistics

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Measurement, automation & statistics

Quantitative results: care, consistency, avoid systematic errors, avoid bias*

Manual analysis vs. Macros vs. customized software tools for automation

In addition to Excel, other useful statistics software: R, MATLAB

* recommend blind analysis to avoid bias

graeme.ball@bioch.ox.ac.uk

3. ImageJ Macros save time

- first, try out some processing/analysis options manually
- turn on the recorder ... “Plugins > Macros > Record”
- you will see a command equivalent to every task you carry out
- paste a sequence of commands into new Macro (Plugins > New > Macro)
- for a description of how Macros work and info about in-built functions, see -

  http://rsbweb.nih.gov/ij/developer/macro/macros.html

  http://rsbweb.nih.gov/ij/developer/macro/functions.html
- result: gbSumMaskedSignal.ijm; for NMJ screen, James Halstead (Davis Lab)

graeme.ball@bioch.ox.ac.uk

3. KNIME for data mining

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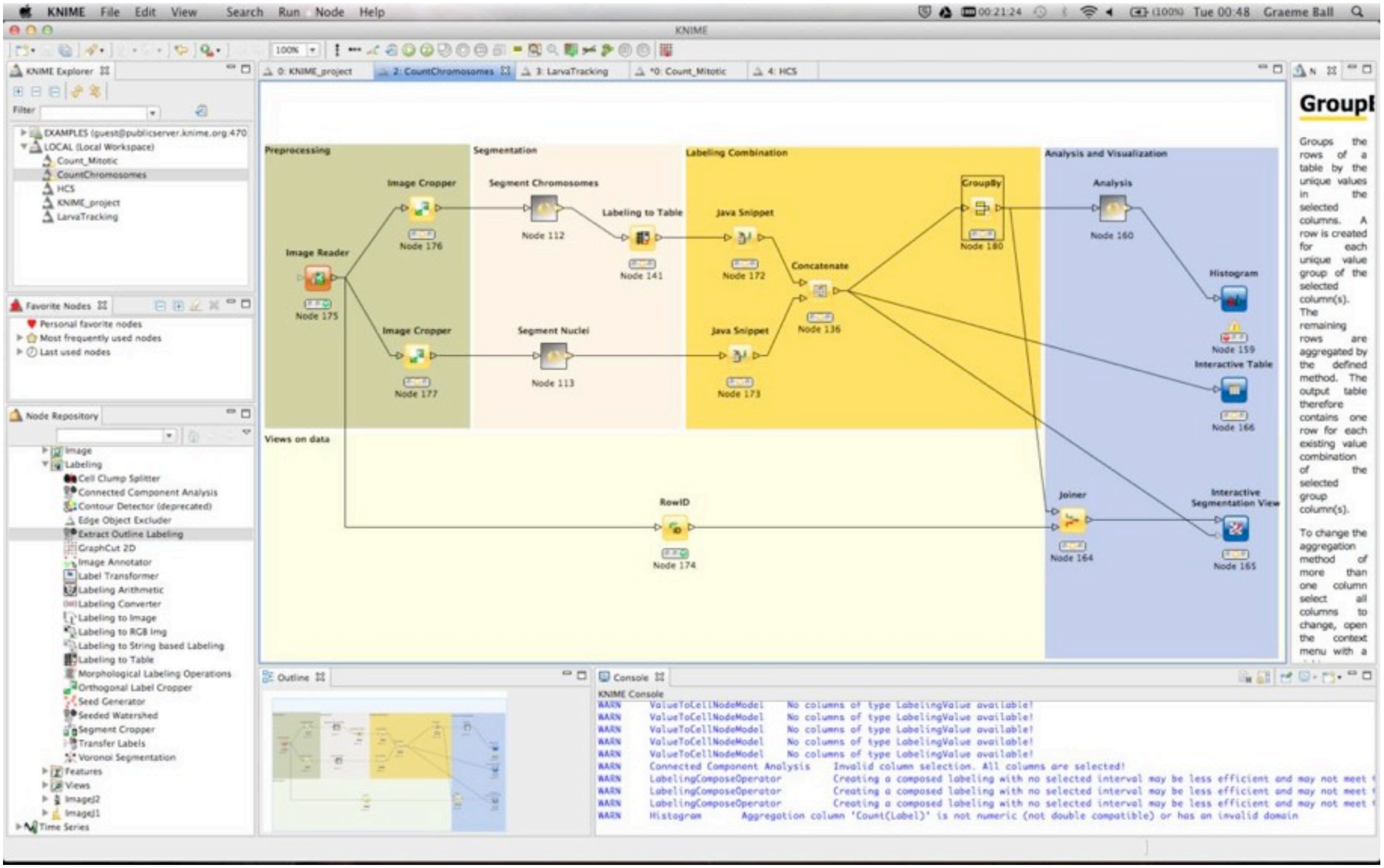

graeme.ball@bioch.ox.ac.uk

3. MATLAB for image processing

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- MATLAB is not free, but many academic institutions have licenses
- Much quicker and easier to prototype new algorithms in MATLAB than e.g. java or C++
- MATLAB is interactive, can use Bioformats to open images, and has an extremely powerful image processing toolbox
- MIJ for ImageJ integration, plus DIPimage image processing library (Delft)

graeme.ball@bioch.ox.ac.uk

3. Tracking in MATLAB

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Custom particle tracker

- Based on Single Particle Tracker from the MOSAIC group (ETH Zurich), which is available as ImageJ plugin and MATLAB code

  I. F. Sbalzarini and P. Koumoutsakos. Feature Point Tracking and Trajectory Analysis for
  Video Imaging in Cell Biology, Journal of Structural Biology 151(2):182-195, 2005.

- Used MATLAB to build up a custom processing and detection scheme

- See: http://www.ncbi.nlm.nih.gov/pubmed/21746854

graeme.ball@bioch.ox.ac.uk

3. Tracking in General

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Tracking

Most common scheme: process, detect/refine, link, correct

Reliable automatic detection is usually the hard part

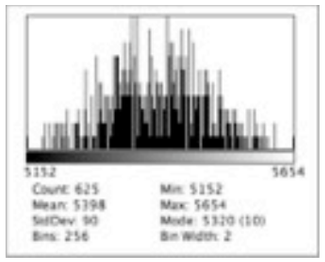
Two essential prerequisites:-

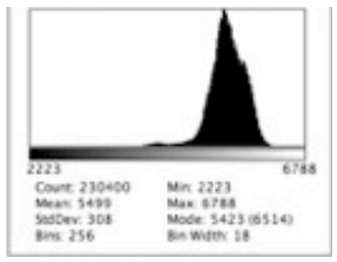
1. contrast-to-noise ratio of >4

2. displacement per. frame less than inter-particle distance

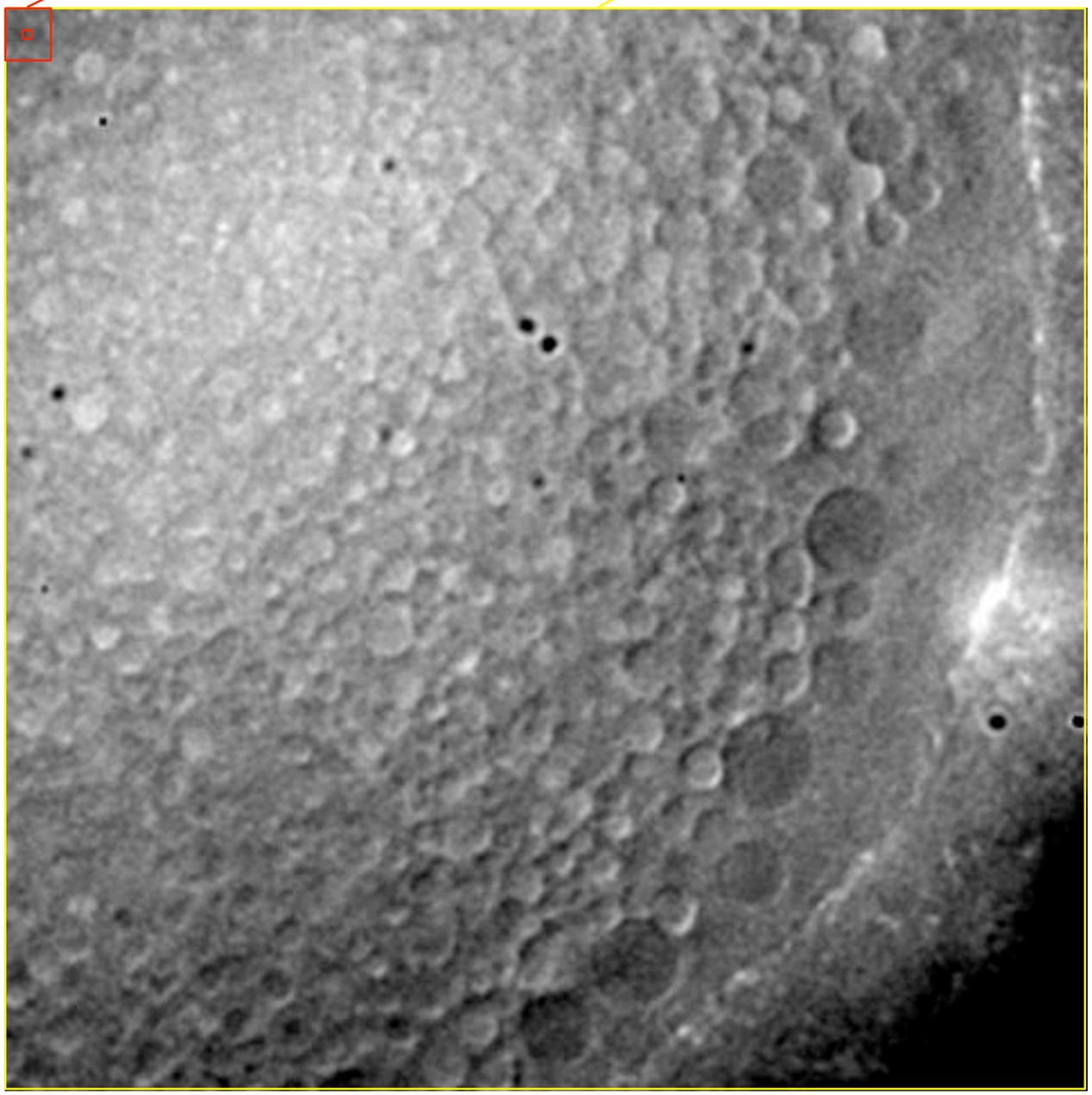
graeme.ball@bioch.ox.ac.uk

1. Image restoration / filtering

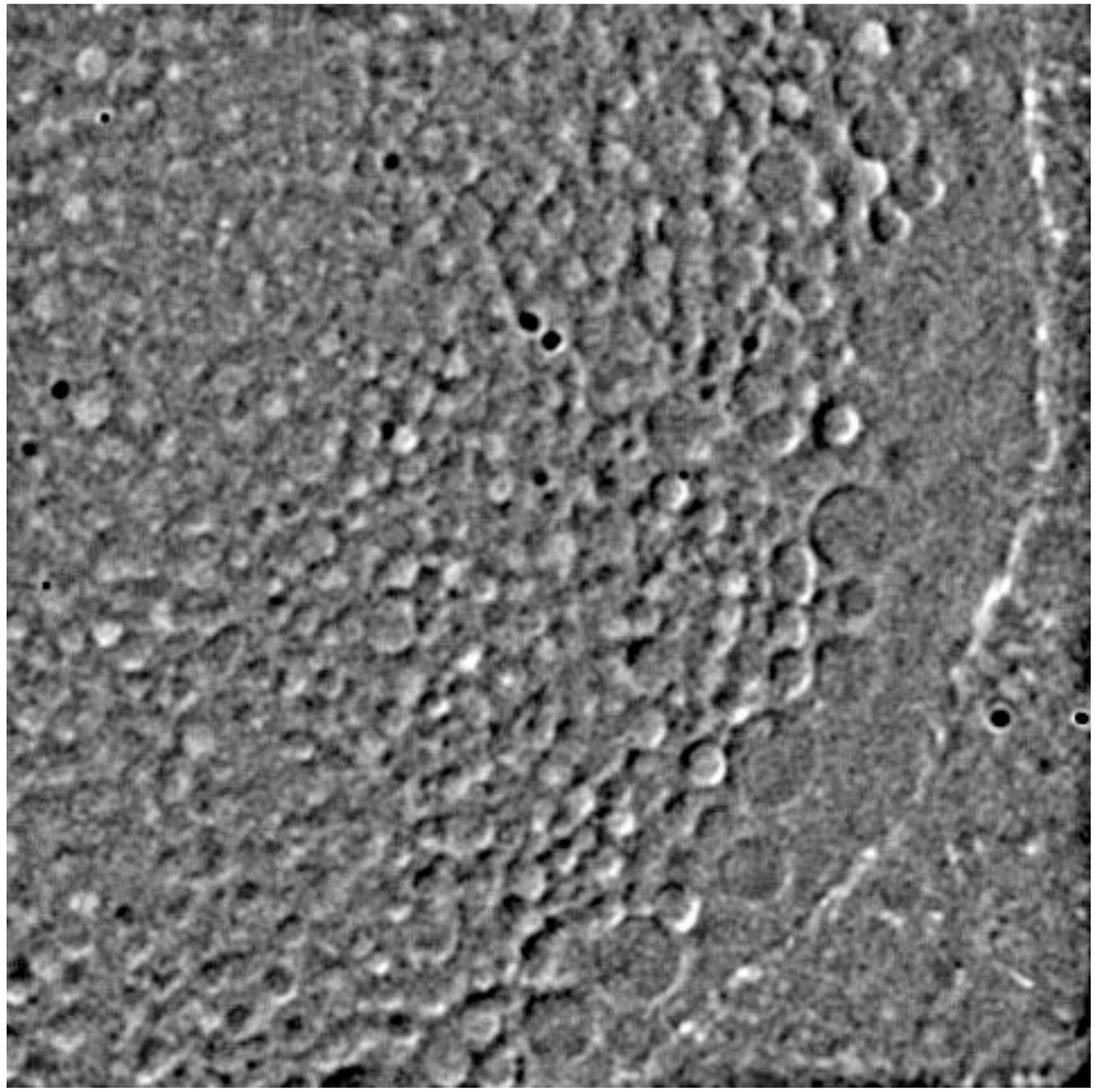




example of a custom intensity transform: scale according to local median



raw data showing uneven illumination



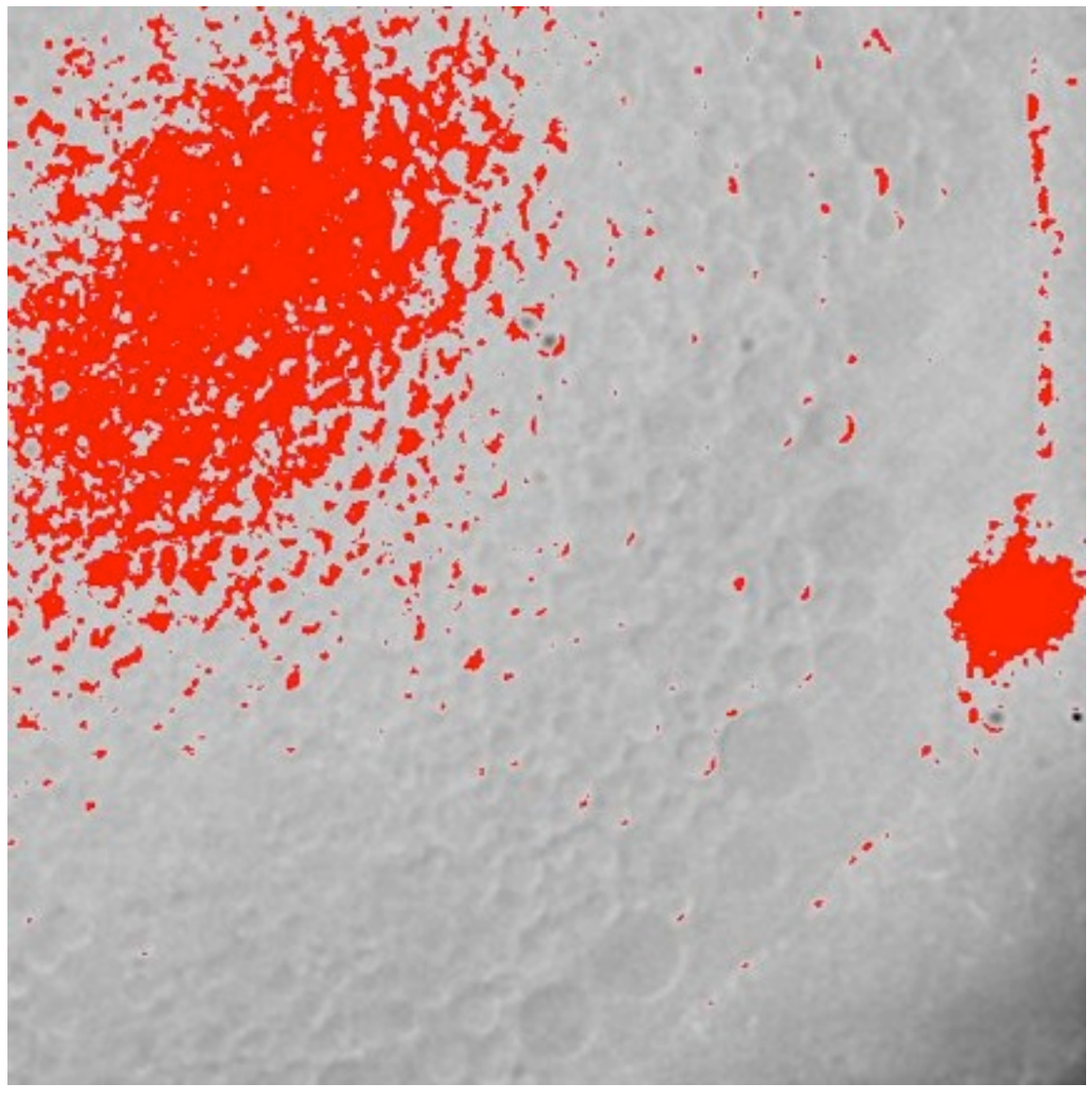
‘normalized’ image

2. Feature extraction

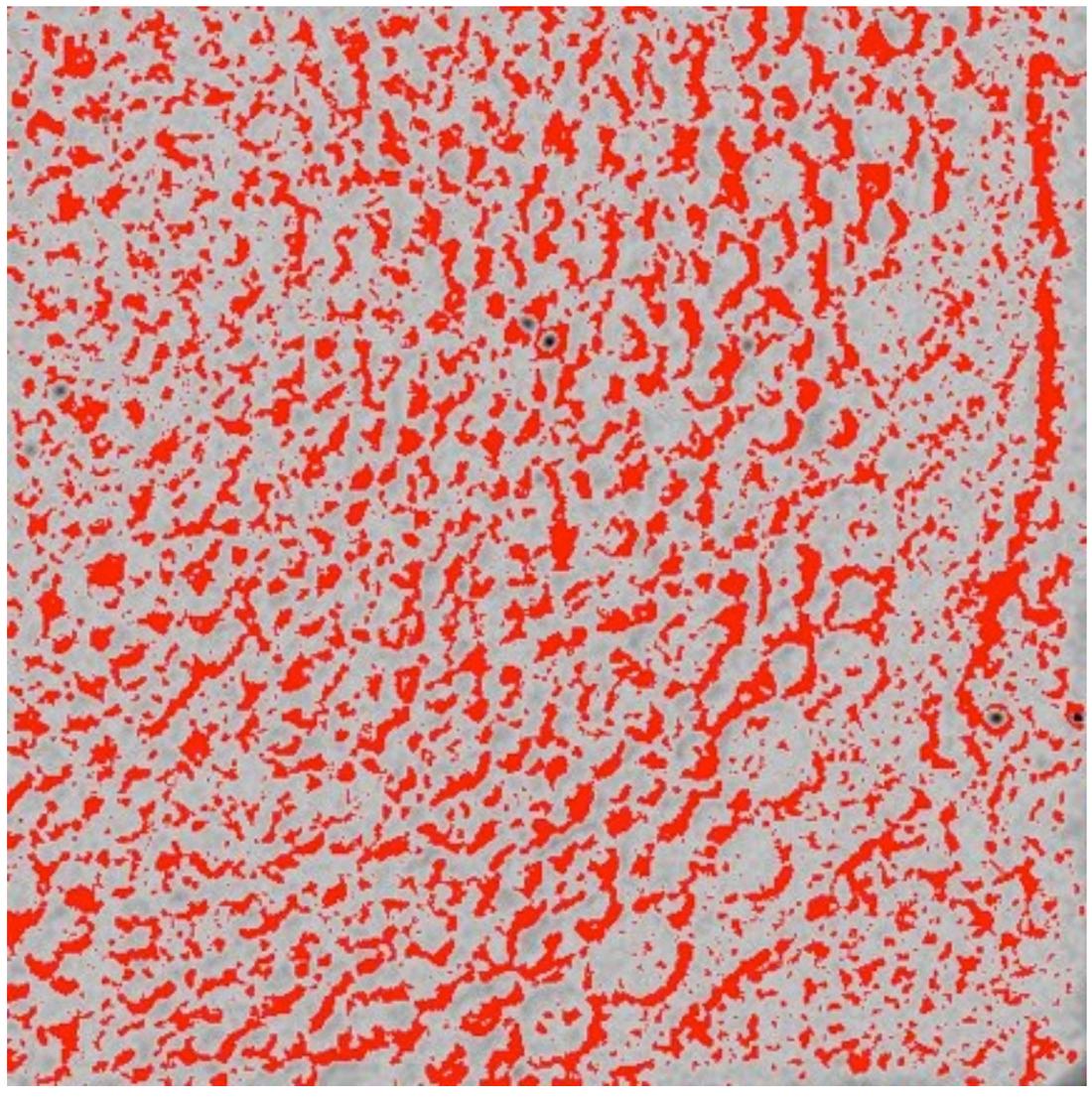
Image segmentation: thresholding

– a global threshold only works if the image is very ‘even’

using raw data



using ‘normalized’ data

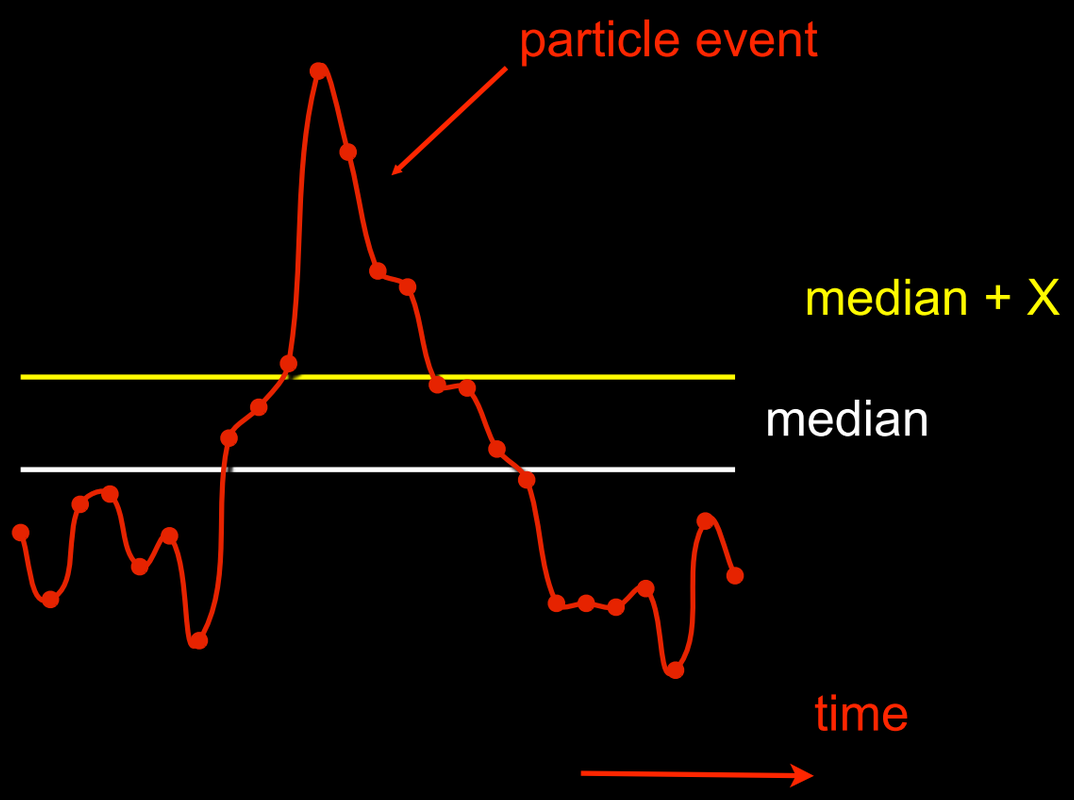


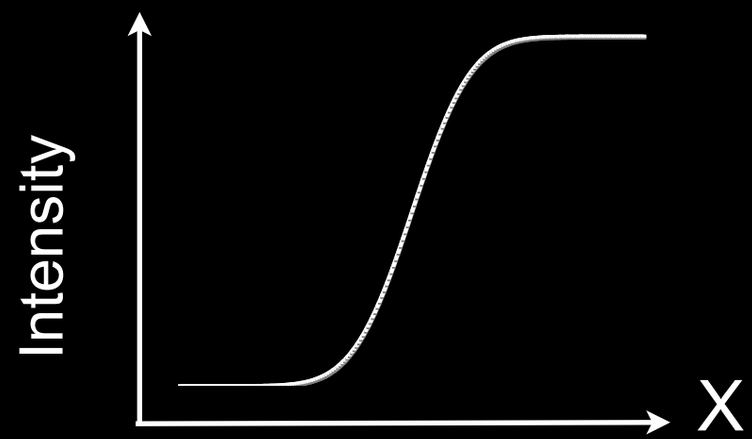
=> ‘adaptive thresholding’, or prior normalization

2. Feature extraction

Image segmentation: identifying ‘foreground’ features

- easy to implement custom filters in MATLAB, like this **temporal median filter** to identify moving foreground





2. Feature extraction

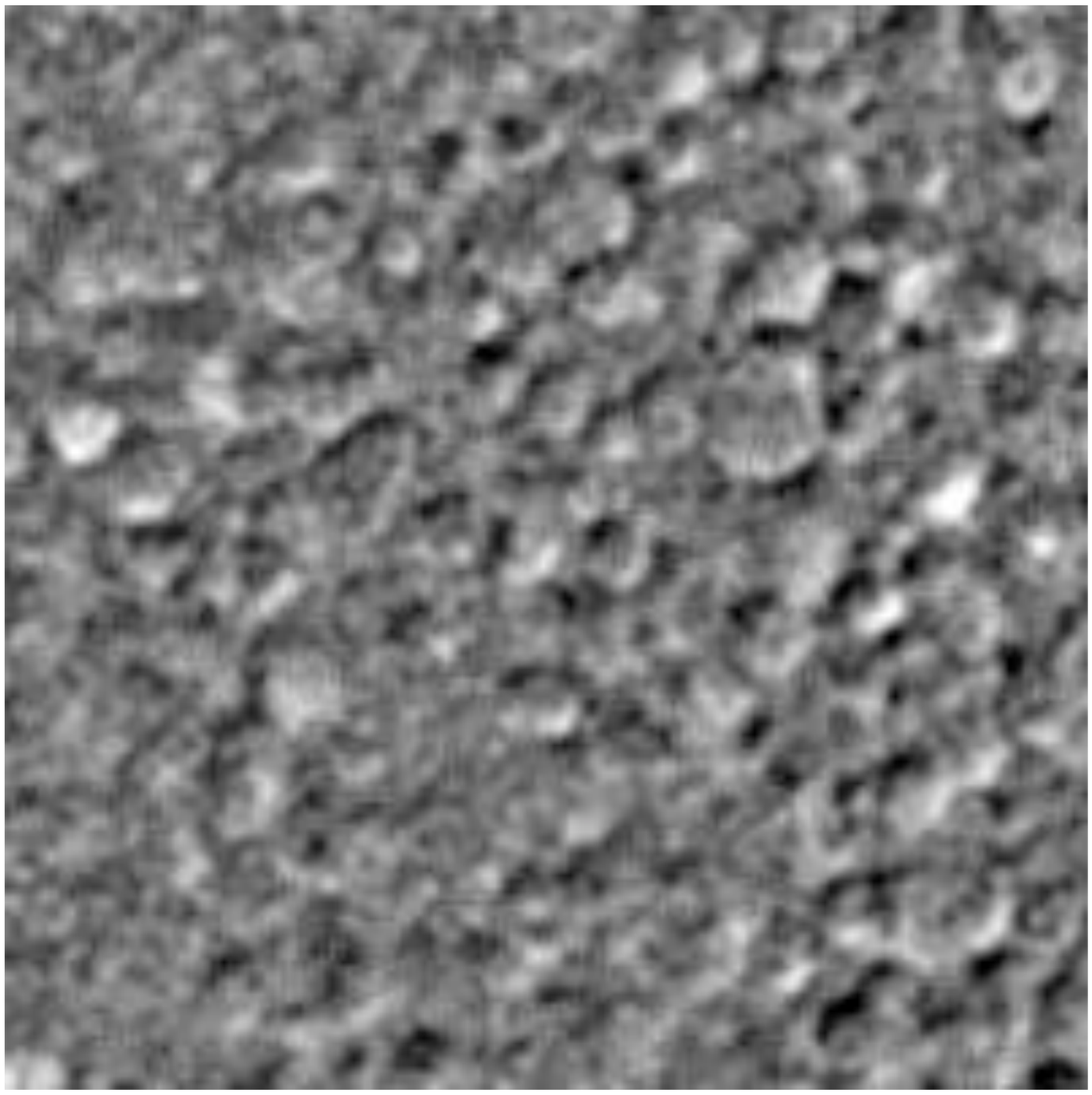
Image segmentation: identifying ‘foreground’ features

200x200 area, normalized

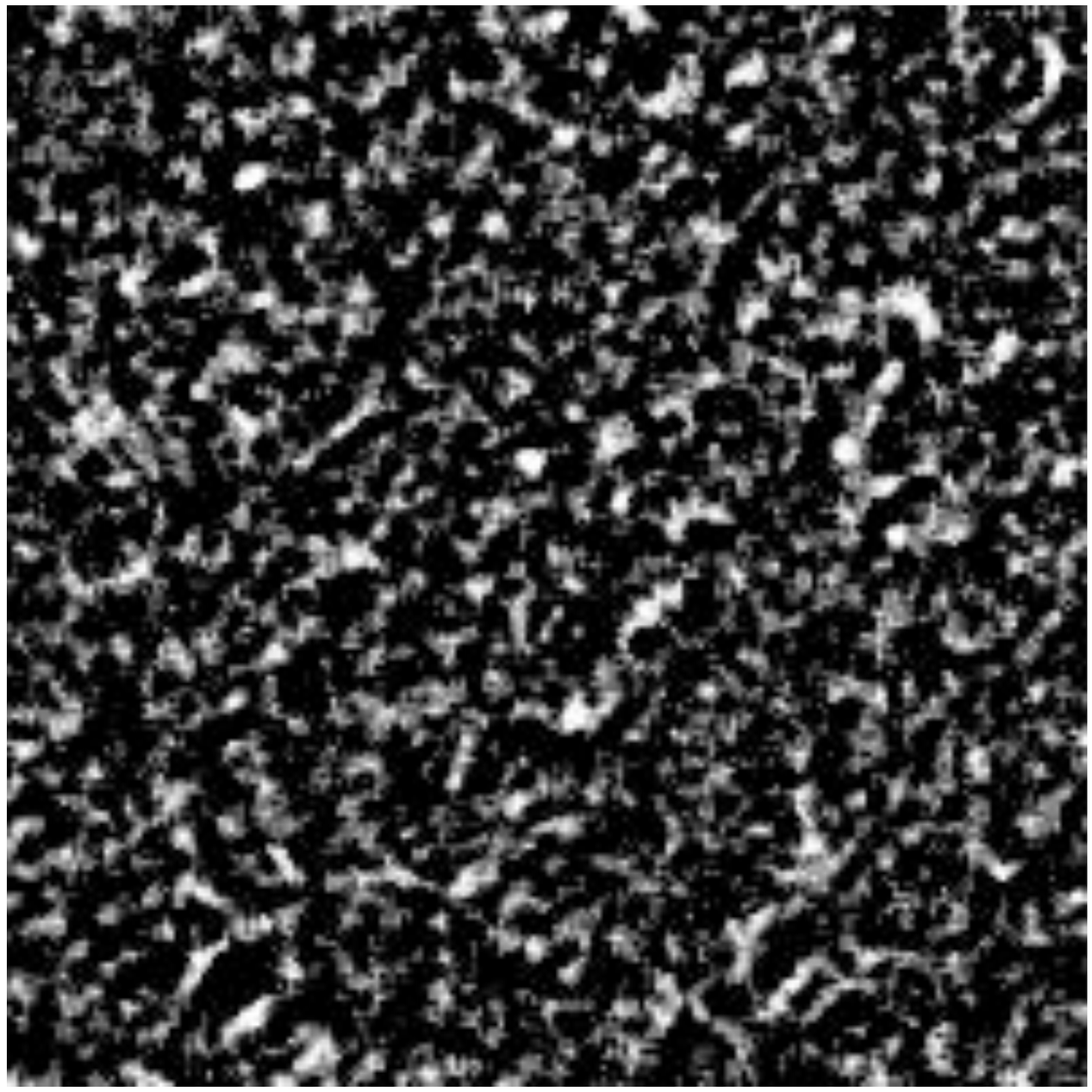
200x200 area, non-background (‘foreground’)

2. Feature extraction

Image segmentation: identifying ‘foreground’ features



200x200 area, normalized



200x200 area, non-background (‘foreground’)

2. Feature extraction

Object recognition

- many tools for point, line & edge detection in MATLAB
- generally work by either:
  - applying a mask to find maxima
    or
  - calculating intensity gradient (steep gradient = edge)

e.g. detection of Haar-like features to find particles

2. Feature extraction

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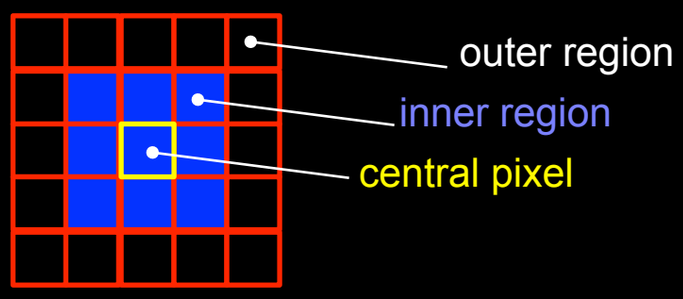
e.g. detection of Haar-like features to find particles

square Haar-like feature



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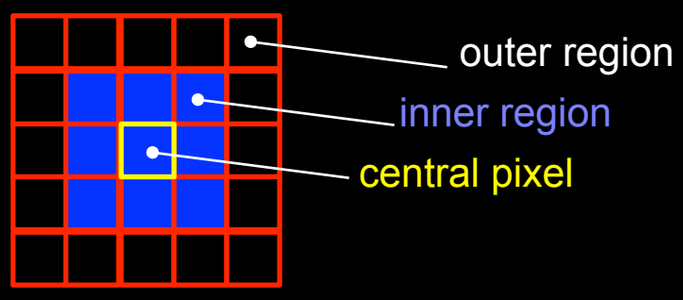
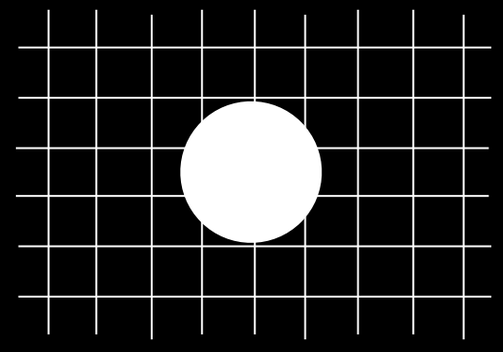
e.g. detection of Haar-like features to find particles

square Haar-like feature





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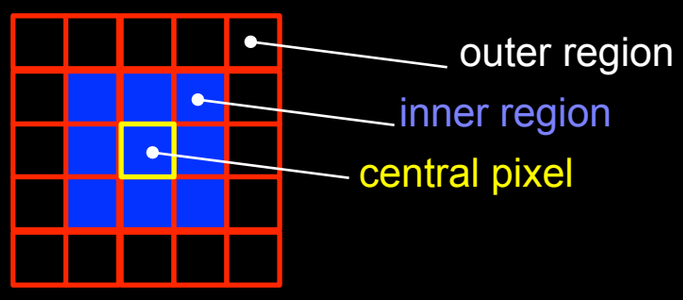

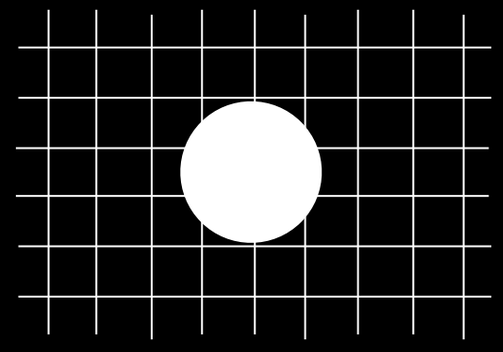

2.4. Tracking in MATLAB

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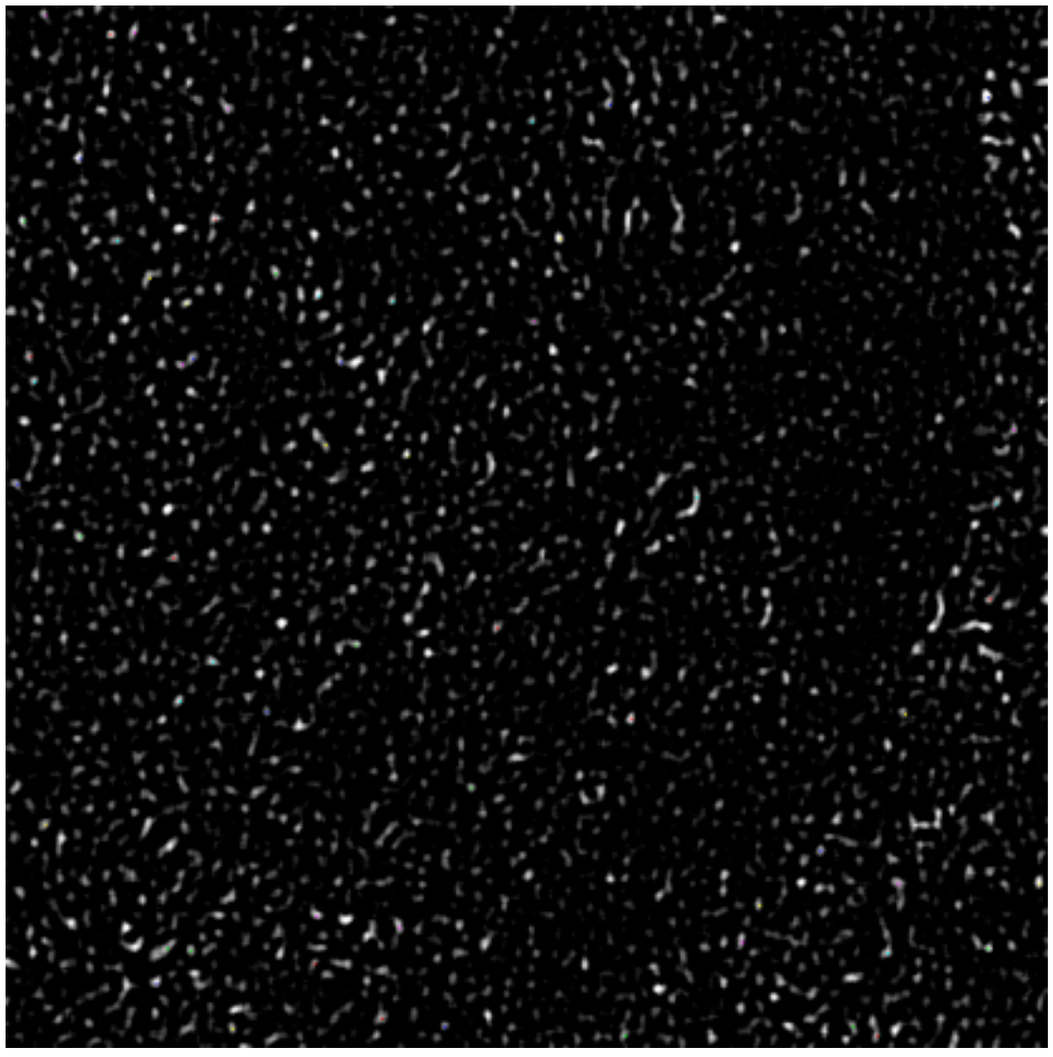
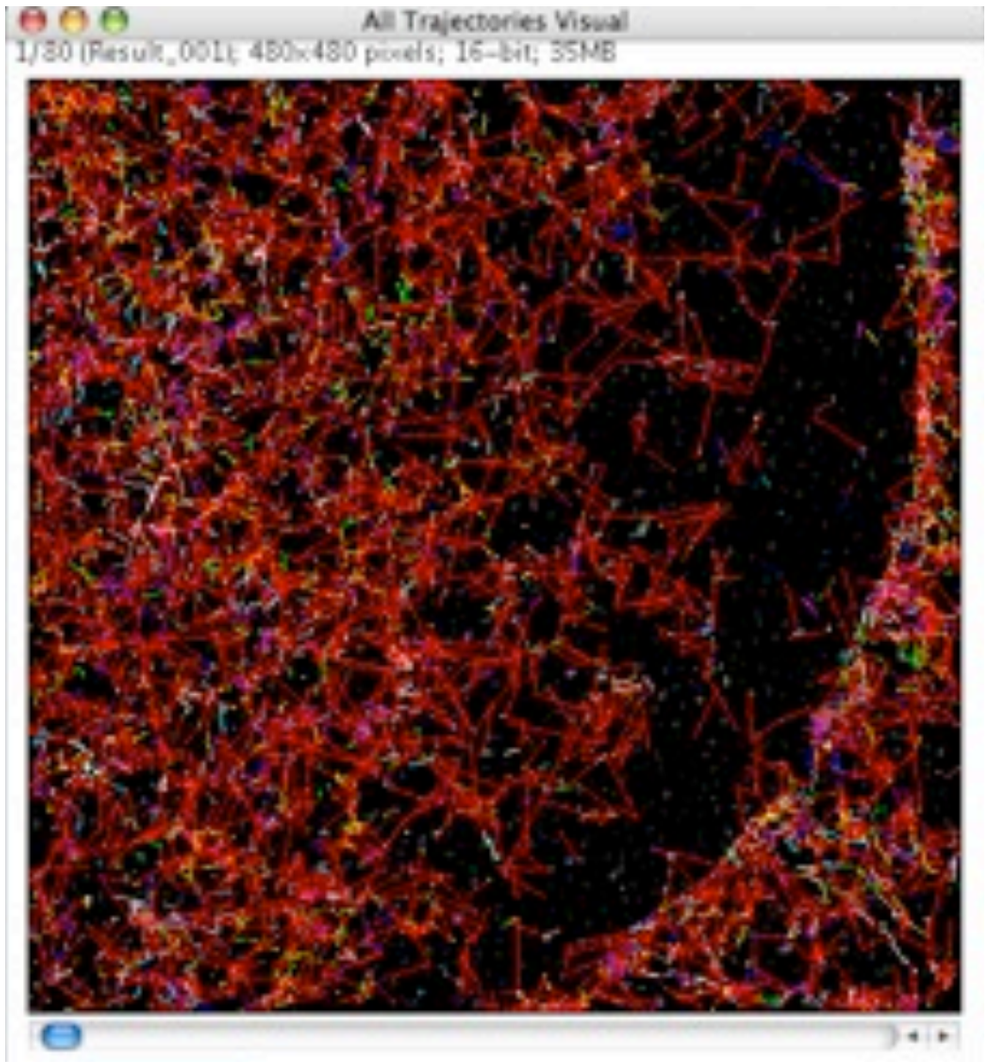
Custom particle tracker



final “particle image” with tracks



MOSAIC imageJ tracker results

graeme.ball@bioch.ox.ac.uk

Summary

- importance of quantitative image analysis
- importance of experimental design – the right data and trade-offs
- overview of processing / analysis concepts
- survey of available software – choosing the right tool (default to Fiji/ImageJ)
- automation: is it necessary? if so, don't be afraid to try / ask for help
- keep data secure, well-organized and annotated
- feedback – problems you are interested in that I haven't covered
- Demo tomorrow – please ask questions

graeme.ball@bioch.ox.ac.uk

3. Summary (7 slides)

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Overview of image processing & analysis

- importance of experimental design & optimization (identify problems early)
- summary of software / choosing the right tool for the job
- processing / analysis tips
- automation: is it necessary? if so, ask / don't be afraid to try
- keep data secure, well-organized and annotated
- feedback – problems you are interested in that I haven't covered
- Demo