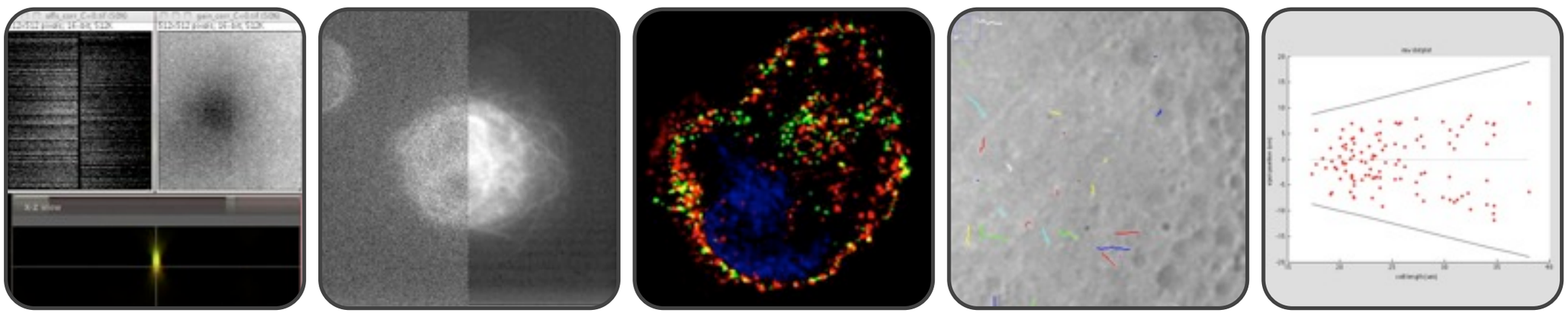
Micron OXFORD
Advanced Bioimaging

Lecture 16 -

Applied Image Analysis

Graeme Ball



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1. Introduction

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Overview of image processing & analysis

1.1. Experimental design

1.2. Image processing: restoration, filtering & segmentation

1.3. Image analysis: measurement, automation, statistics

1.4. Review of image processing & analysis tools

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1.1. Experimental design

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What am I trying to measure?

Expression level? ⇒ accurate, calibrated intensities

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

Dynamics? ⇒ temporal resolution, photostability

⇒ decide on the instrument (& technique)

⇒ **choice of fluorophores**

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1.2. Image Processing

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Image restoration

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

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

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)

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1.2. Image Processing

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Image filtering

Spatial filters for smoothing & sharpening

Frequency domain filters

Adaptive filtering versus transformation + global filtering

Time-domain filtering (see temporal median filter in Section 2, Tracking)

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1.2. Image Processing

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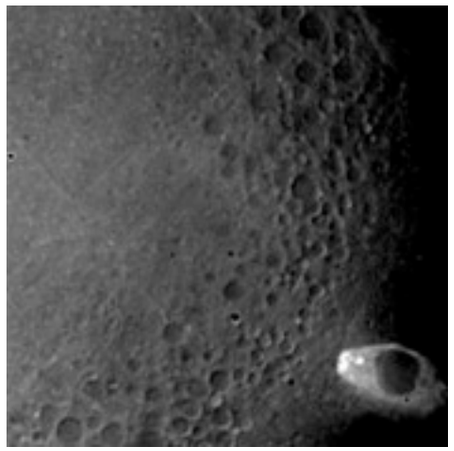
Image restoration & filtering

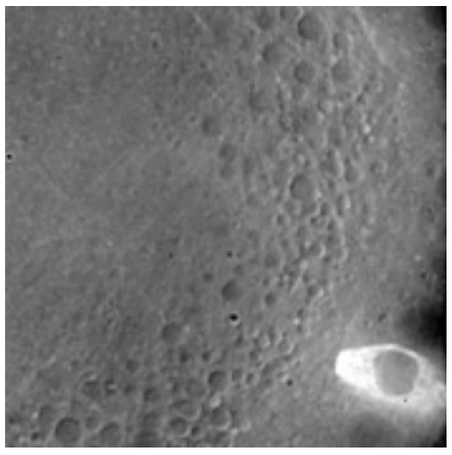
pseudo-flat field

Im = Im-mean * Im/mean-filtered



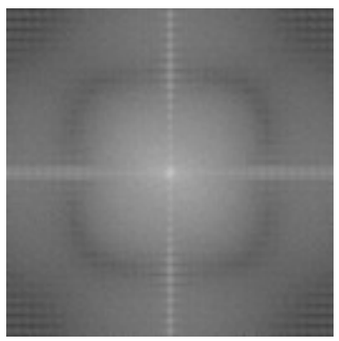
original

240x240 mean-filt

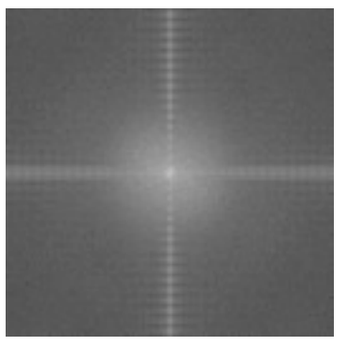


pseudo-corrected

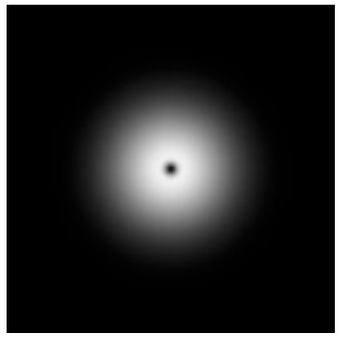
FFT reveals frequencies



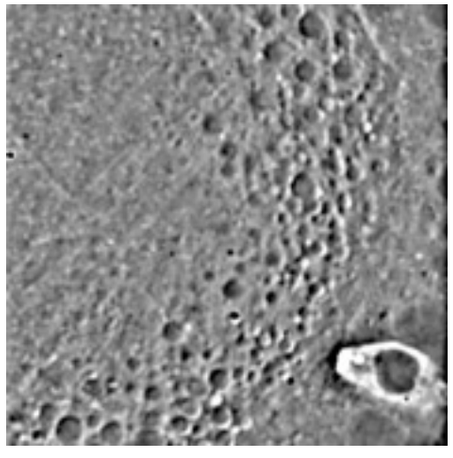
original



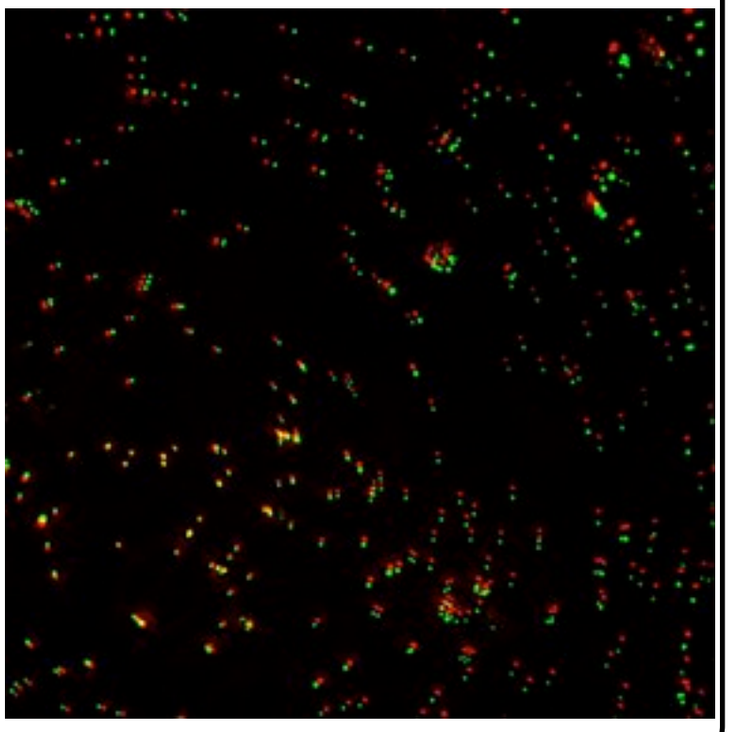
3x3 median



3-25 bandpass



registration can be critical!



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1.2. Image Processing

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Segmentation

Simple intensity thresholding - ROI or binary image

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

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1.2. Image Processing

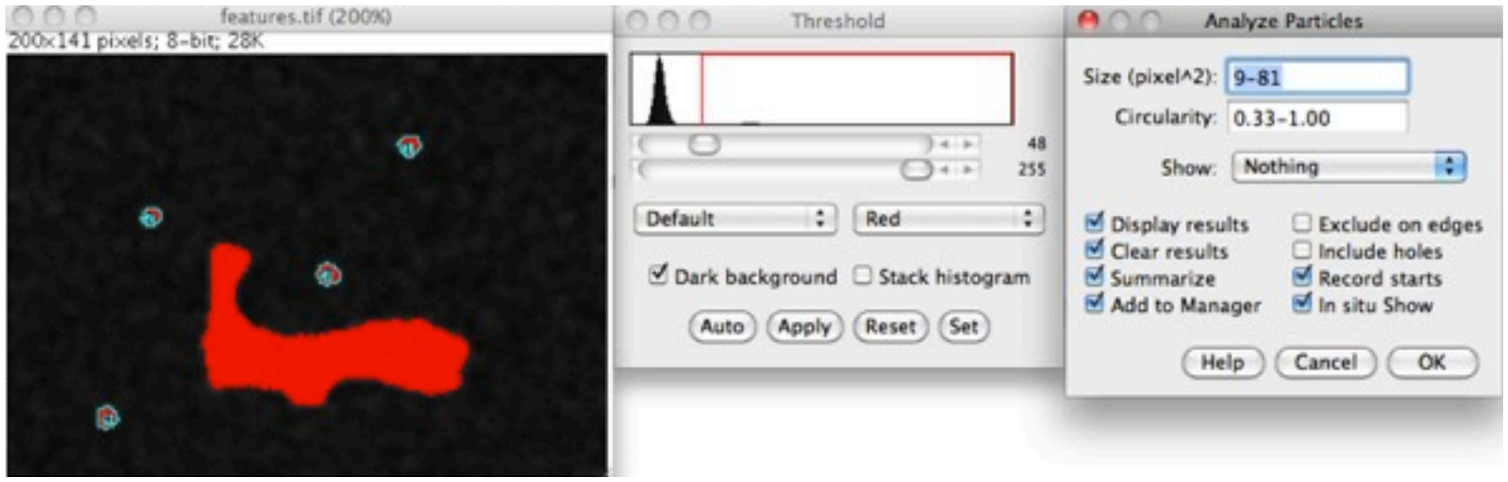


Image > Adjust > Threshold (Set)

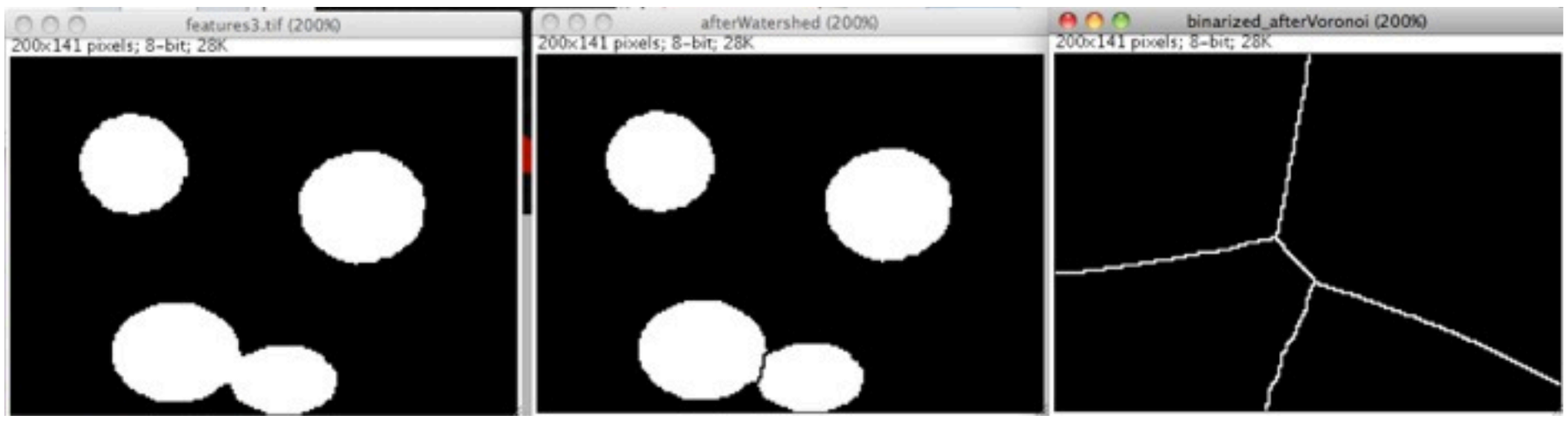
Analyze > Analyze particles

Use: Analyze > Tools > ROI manager

("wand" to select individual ROIs)

Process > Find edges uses Sobel detector

Process > Binary menu contains MIP (erode, dilate etc.)



Binary processing "Watershed" separates touching edges

"Voronoi" finds lines equidistant from feature centres

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1.3. Image Analysis

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

http://microna.bioch.ox.ac.uk/mediawiki/index.php/Fiji_/_ImageJ

1.4. Image Analysis Tools

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Free tools


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

CellProfiler - quantitatively measure cell phenotype (+ Worm Toolbox)

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

OMERO - image repository & visualization

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

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1.4. Image Analysis Tools

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Commerical tools

Volocity - 3D visualization & analysis package, spinning disk

SoftWoRx - API Deltavision, deconvolution, SI reconstruction

MATLAB - custom analysis using Image Processing toolbox

Imaris, Amira - 3D visualization & analysis packages

Metamorph - microscope control, image processing/analysis

Huygens, AutoQuant - Deconvolution software

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2. Image Analysis Examples

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2.1. 3D visualization and analysis options

2.2. 3D visualization & analysis using volocity - colocalization

2.3. Building an analysis Macro in ImageJ/Fiji

2.4. Tracking using a custom MATLAB pipeline

2.5. MicrobeTracker: analyzing dynamic fluorescent foci within cells

2.6. Data management and processing with OMERO

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2.1. 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

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2.2. Volocity

Volocity - 3D visualization & analysis

- produced by Improvision (acquired by PerkinElmer)
- drives PerkinElmer's spinning disk confocal microscopes
- 3D visualization/analysis & movie-making quite good, +FRAP, FRET, coloc.
- slicker than ImageJ, but a lot more expensive! (support though)
- slightly odd to use initially - "libraries" and "measurement tasks/protocols"

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

2.2. Volocity

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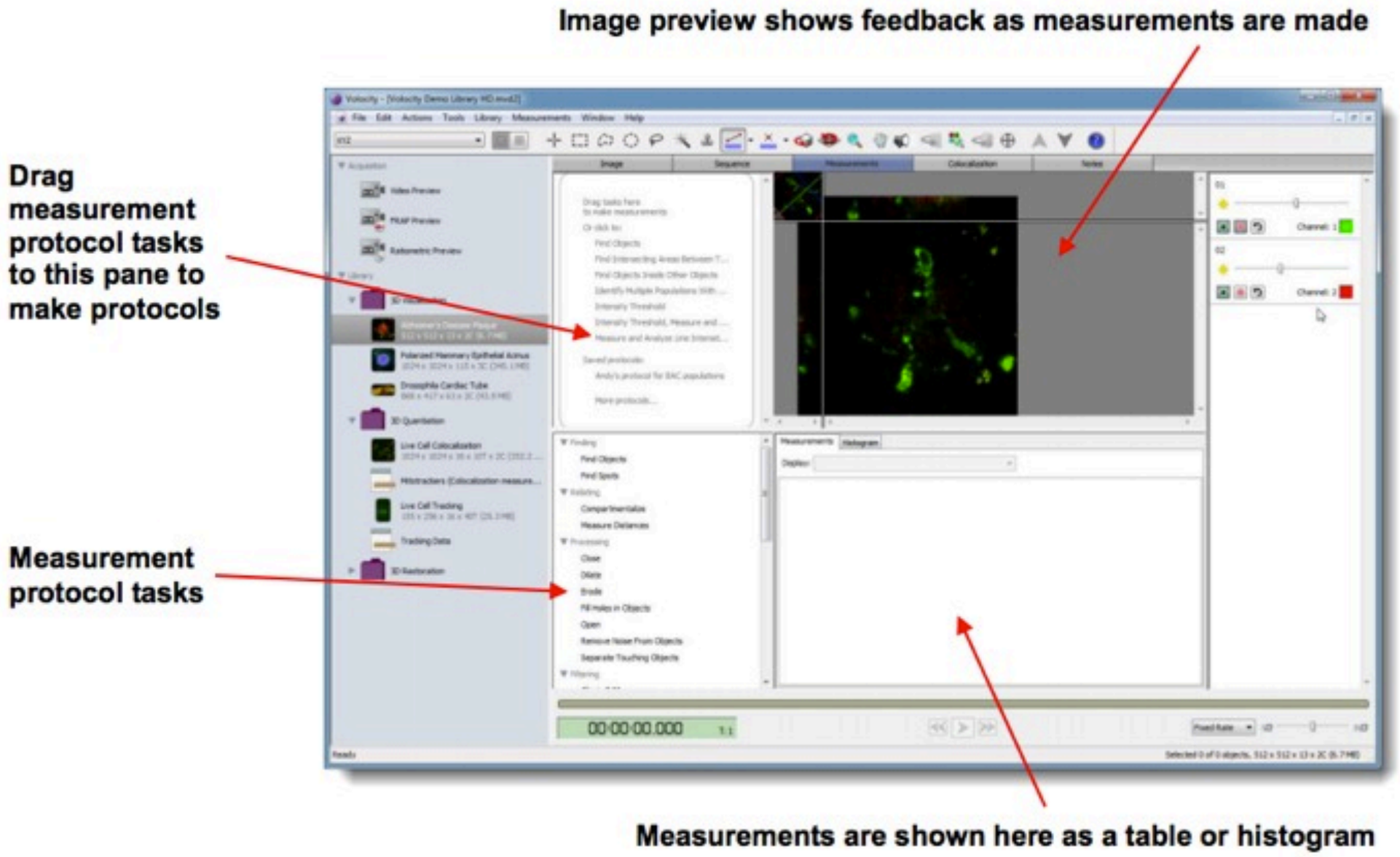
View the Measurements tab. The Measurements View contains all the tools and information needed for selecting objects.



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

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2.2. Volocity - XY Plane view

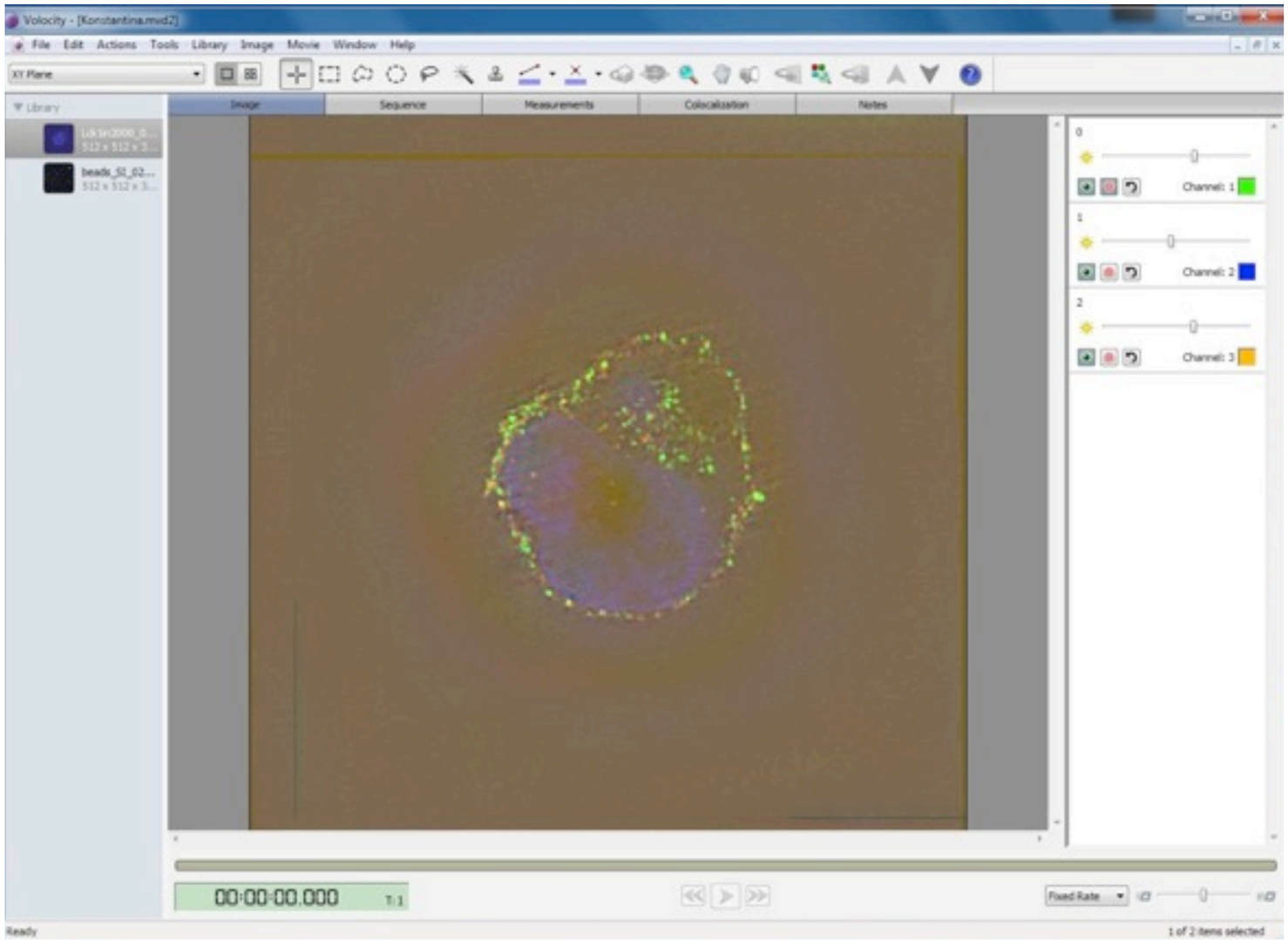

Image of immune cell by Konstantina Nika (Acuto lab, Dunn School)

red/green: antibody staining of (non-)/phosphorylated membrane protein,

blue = DAPI

acquired using OMX V2 (with Eva's help)

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2.2. Volocity - XYZ view

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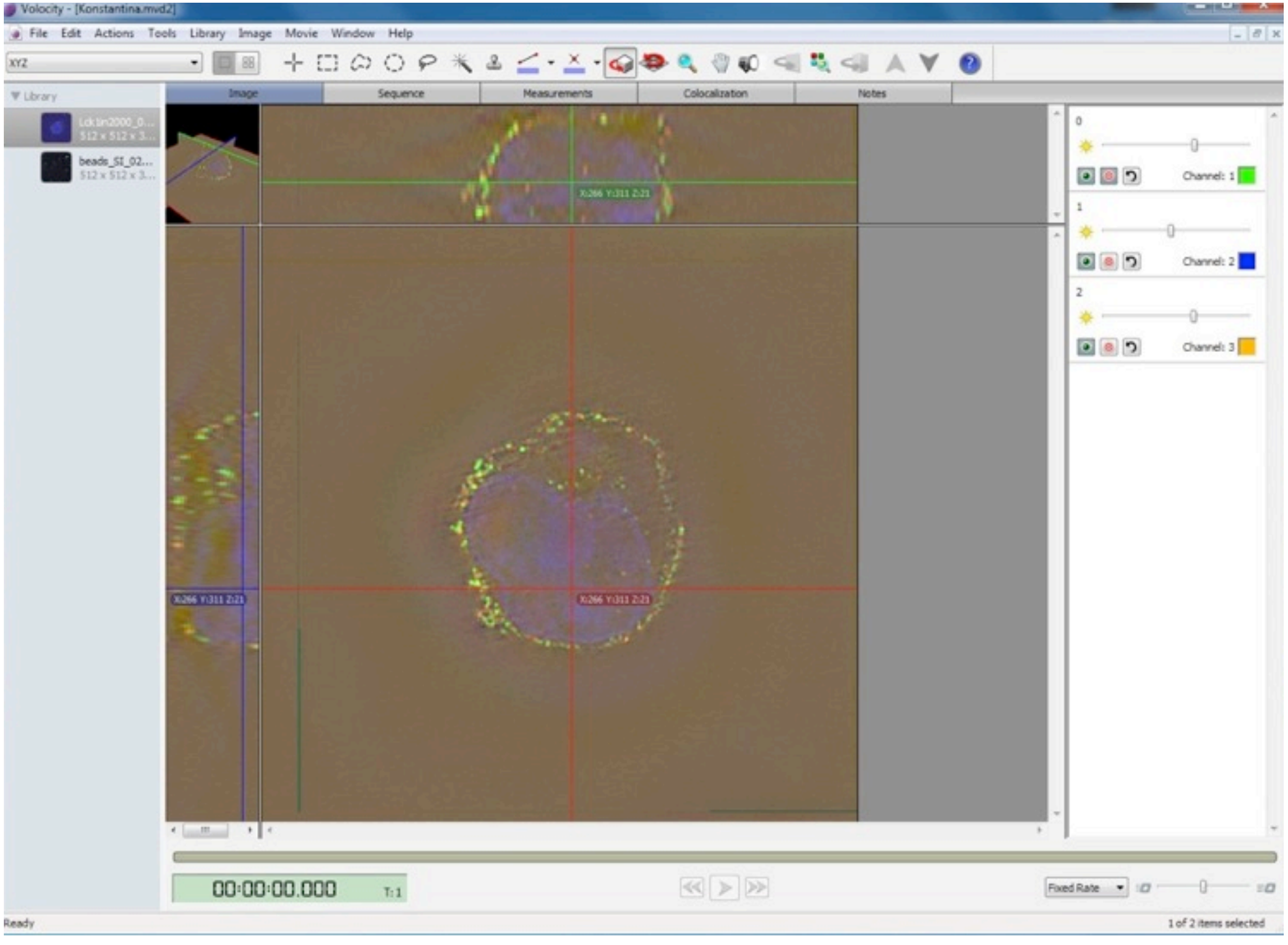

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2.2. Volocity - 3D opacity (MIP)

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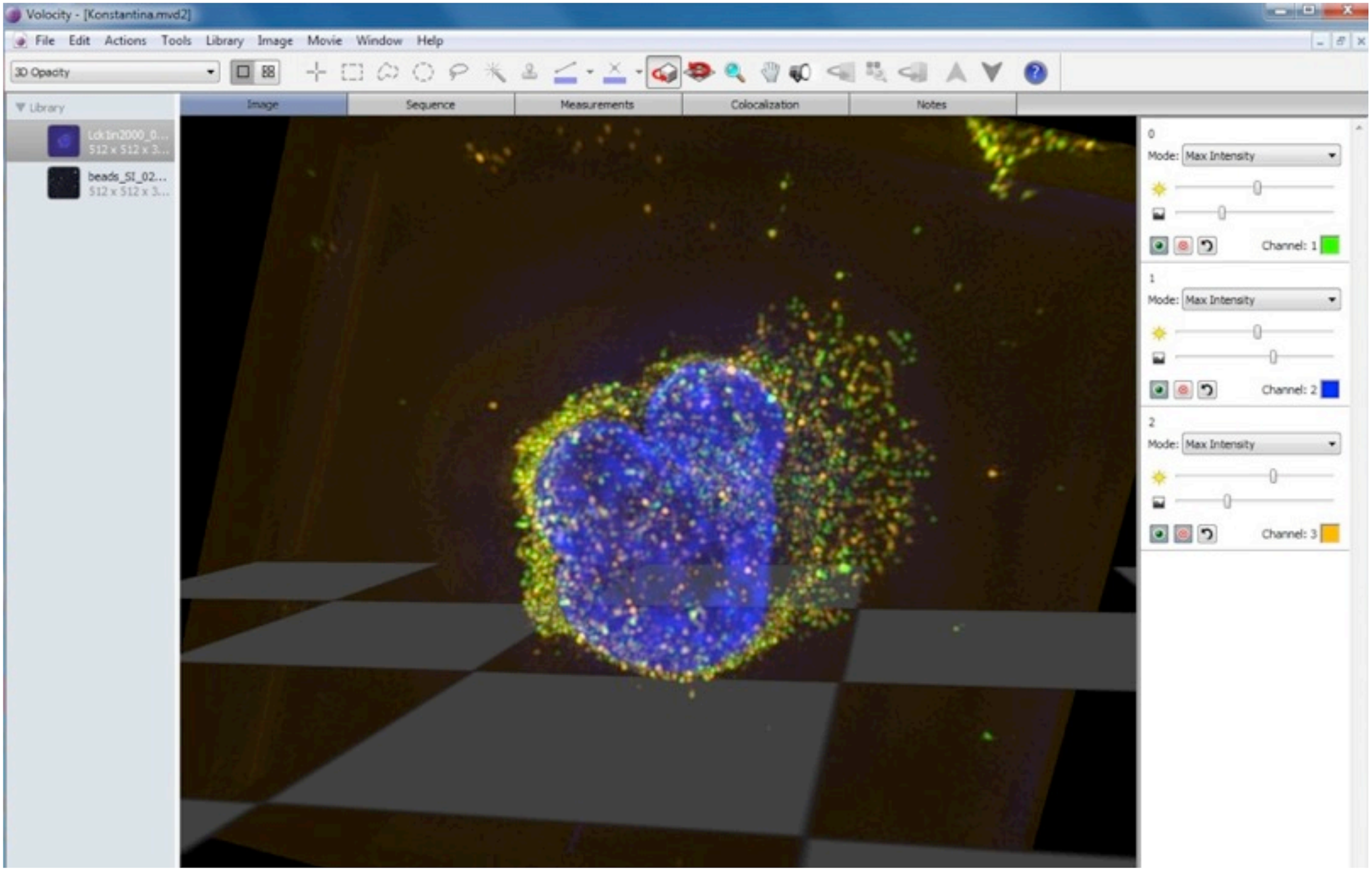

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2.2. Volocity - colocalization

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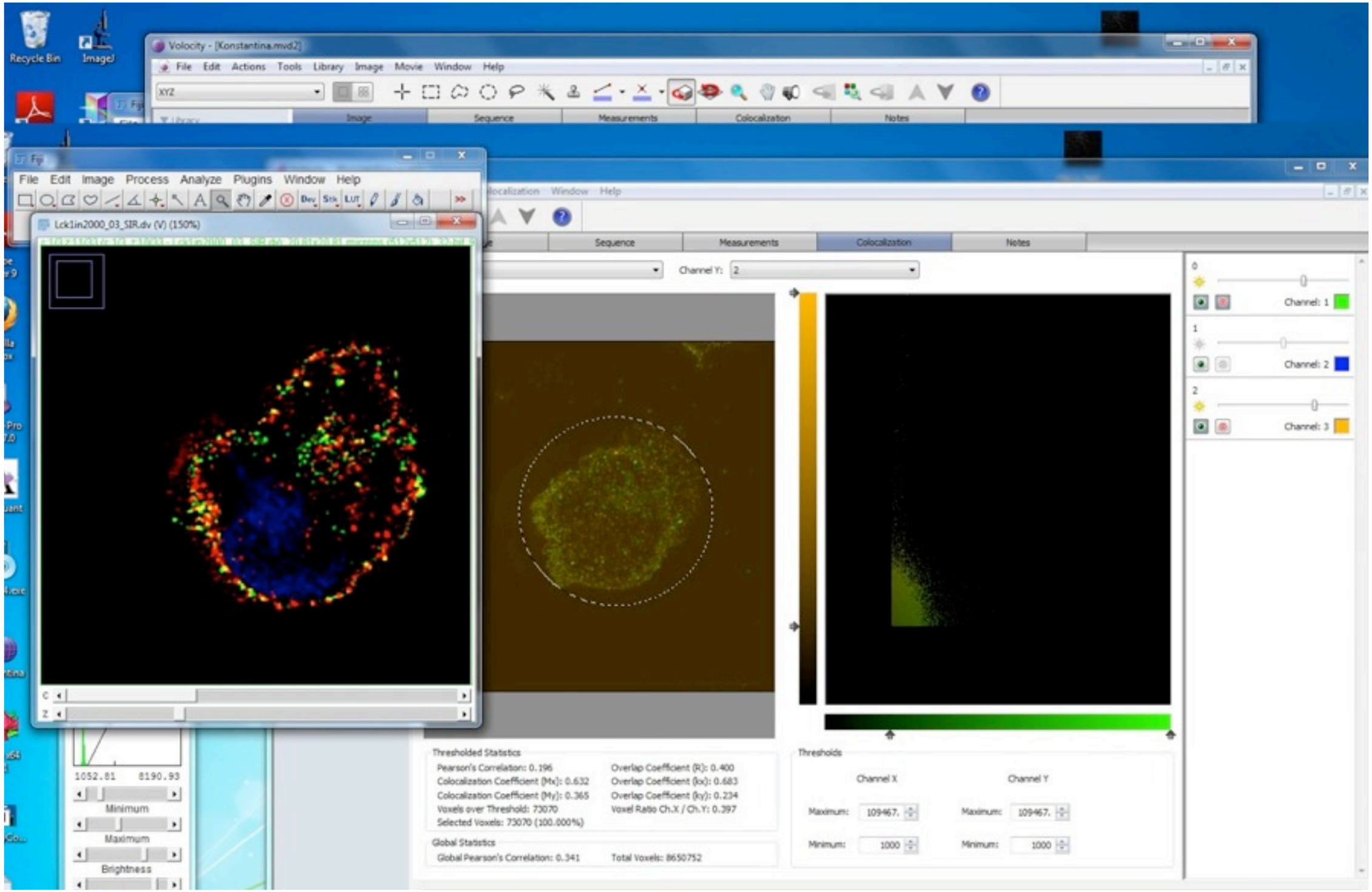

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2.2. Colocalization 101

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- make **very** certain that
  1. you do not have bleed-through!
  2. your channel alignment is properly calibrated
  3. your images are as deblurred as possible
- many colocalization statistics rely on segmenting both channels

  => flat field & meticulous background correction, justifiable ROI & threshold
- read this review:-

  http://www.ncbi.nlm.nih.gov/pubmed/17210054
- 3 fundamental approaches:
  1. intensity correlation (Pearson) / scatter plot
  2. overlap coefficients (Manders: M1, M2)
  3. object-based analysis

JaCoP
(ImageJ plugin)

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2.2. Colocalization 101

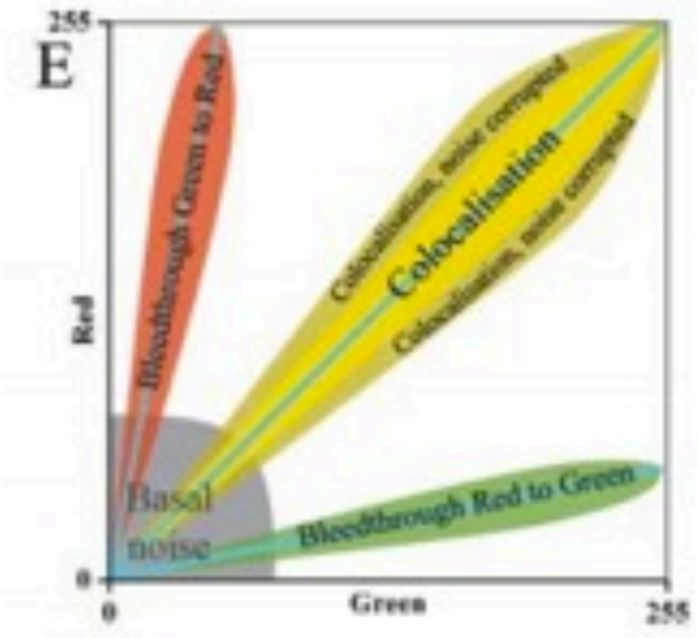
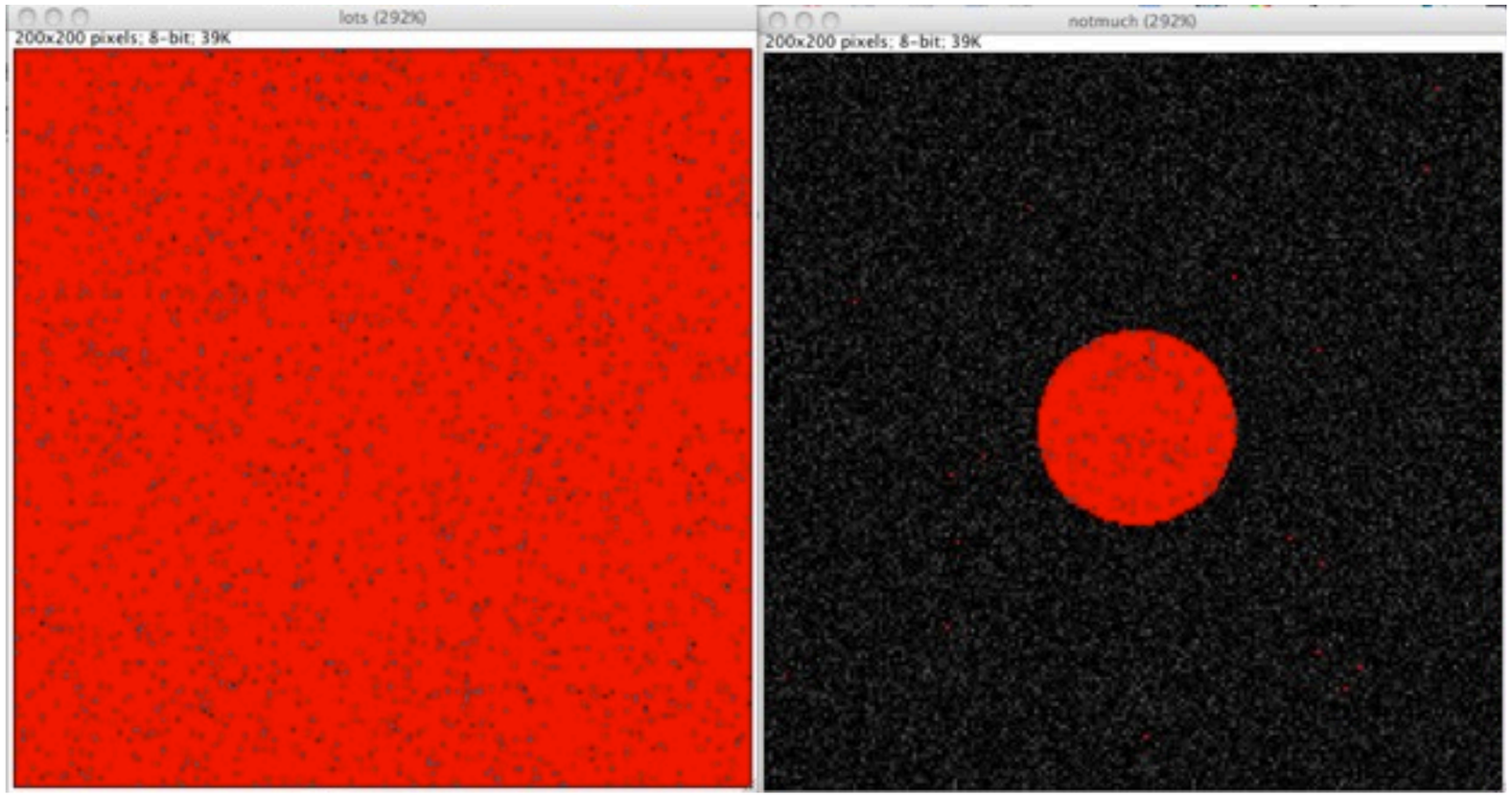


Fig. 5E (Bolte & Cordelieres)



M1=0.053

M2=0.941

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2.3. 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

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2.3. Fiji/ImageJ Macros

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- 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)

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2.4. MATLAB: 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. C++ or java
- MATLAB is interactive, can use Bioformats to open images, and has an extremely powerful image processing toolbox

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2.4. Tracking in MATLAB

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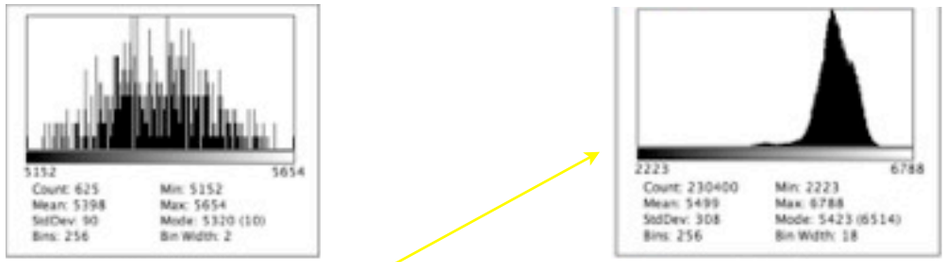
Custom particle tracker

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

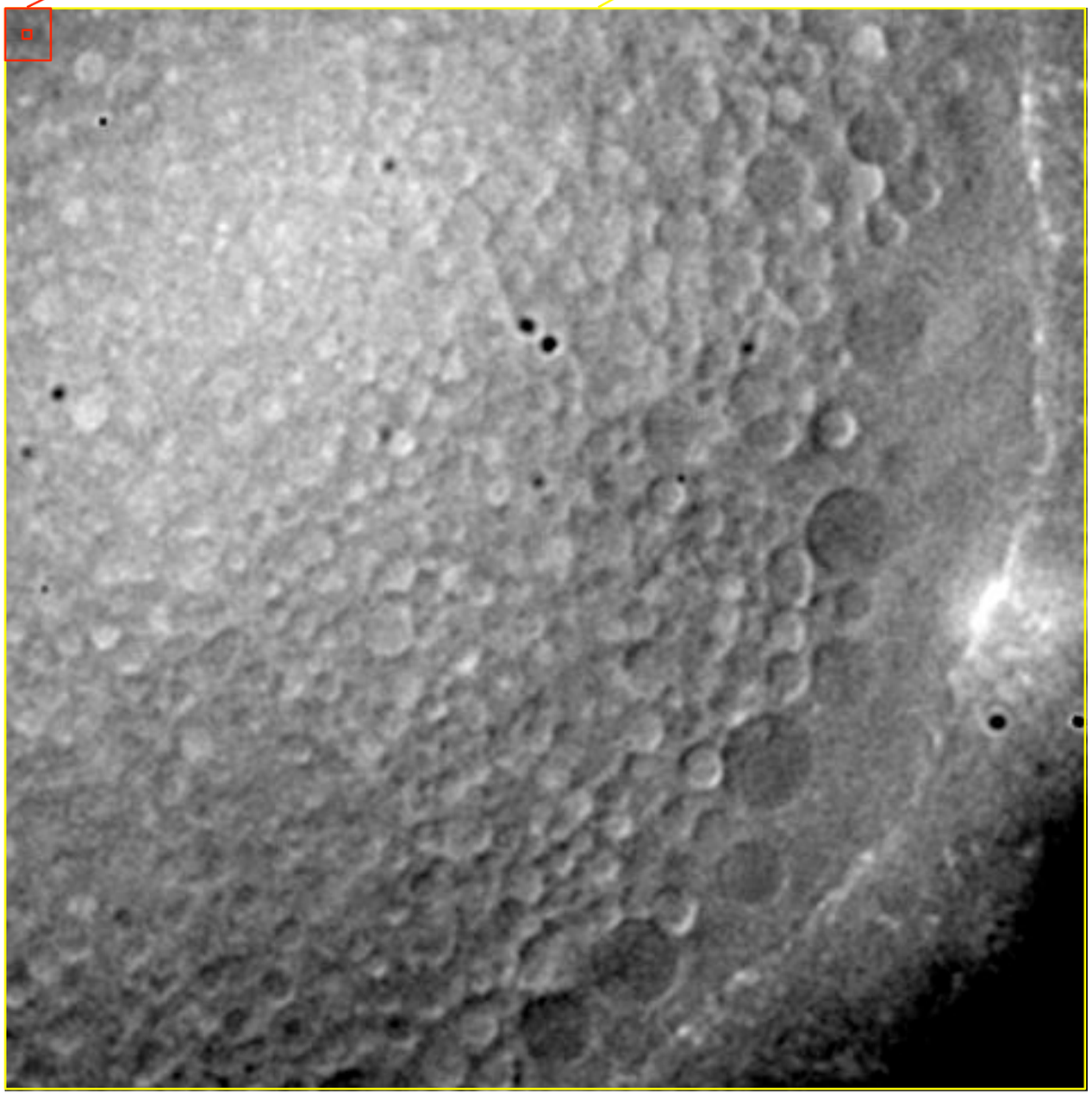
I. F. Sbalzarini and P. Koumoutsakos. Feature Point Tracking and Trajectory Analysis for Video Imaging in Cell Biology, Journal of Structural Biology 151(2):182-195, 2005.

- Used MATLAB to build up a custom processing and detection scheme
- See: http://www.ncbi.nlm.nih.gov/pubmed/21746854

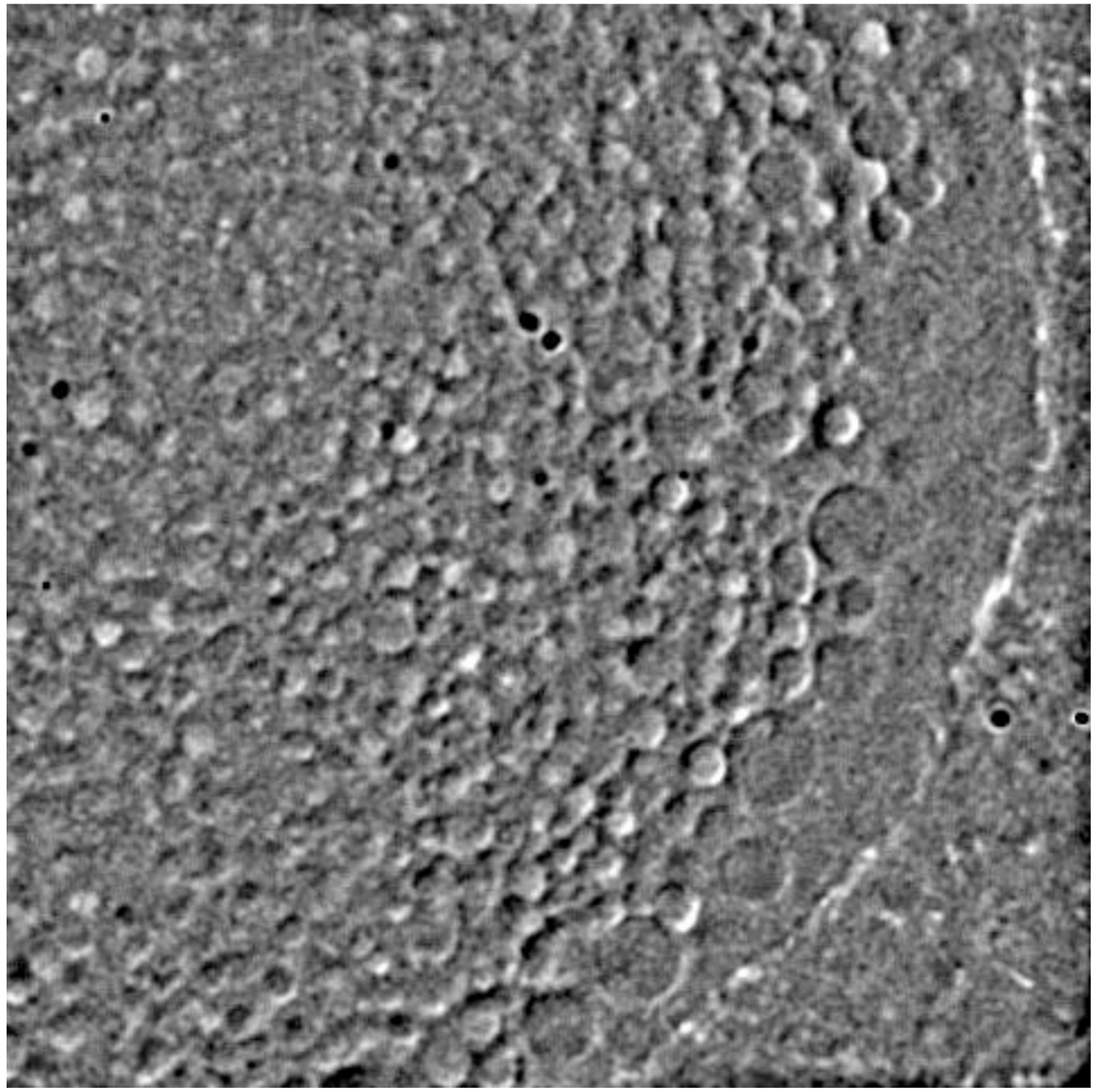
1. Image restoration / filtering



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



raw data showing uneven illumination



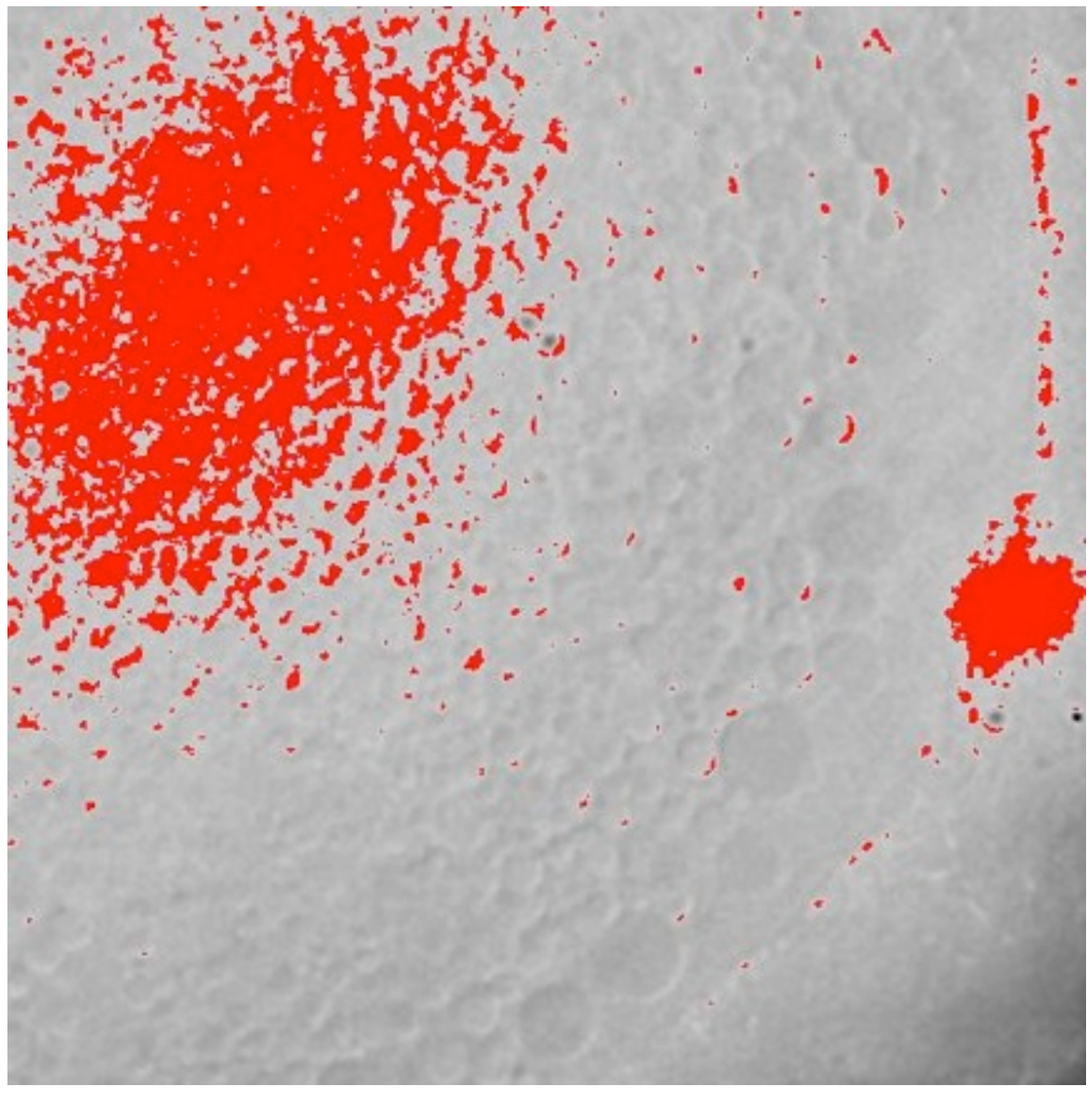
'normalized' image

2. Feature extraction

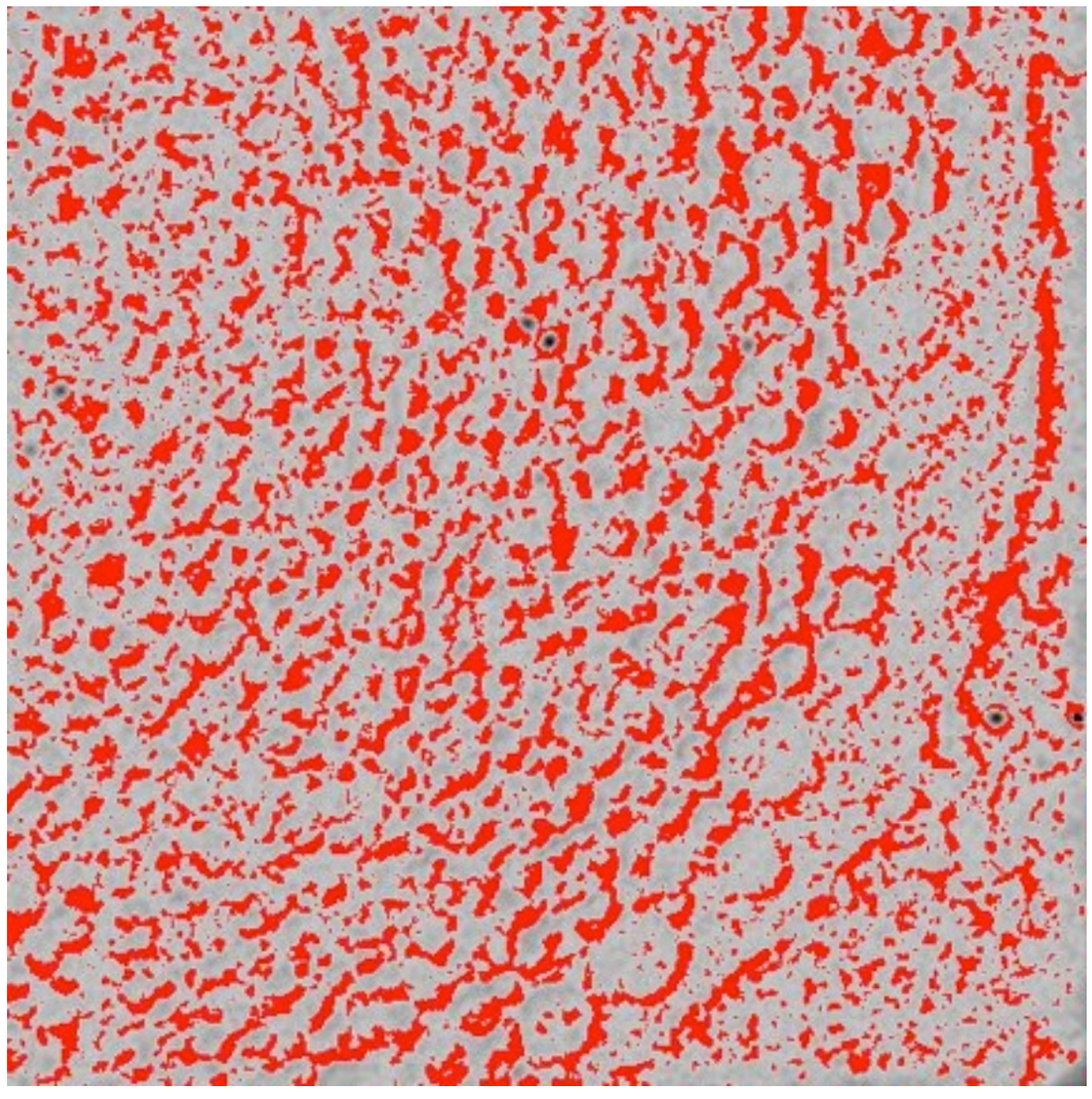
Image segmentation: thresholding

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

using raw data



using ‘normalized’ data



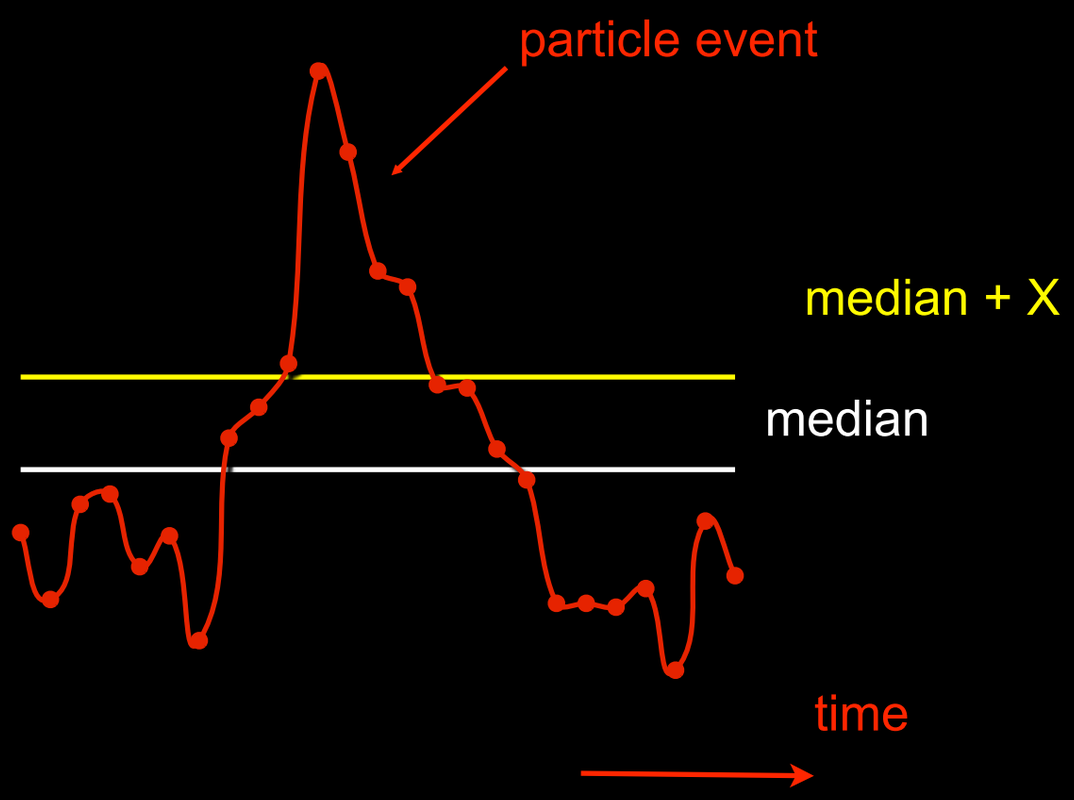
=> ‘adaptive thresholding’, or prior normalization

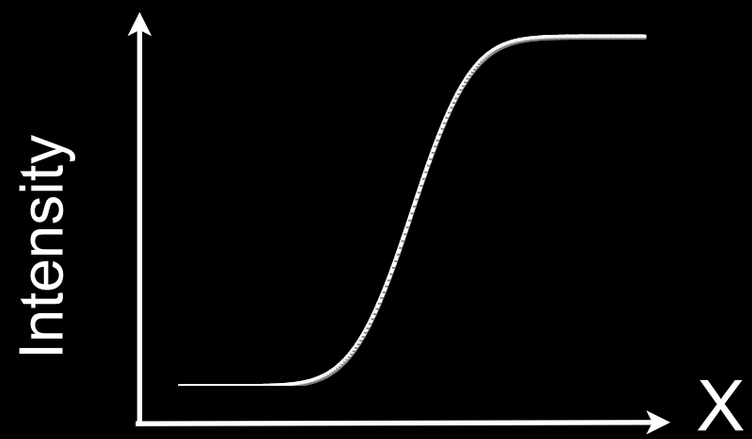
2. Feature extraction

Image segmentation: identifying ‘foreground’ features

- easy to implement custom filters in MATLAB, like this

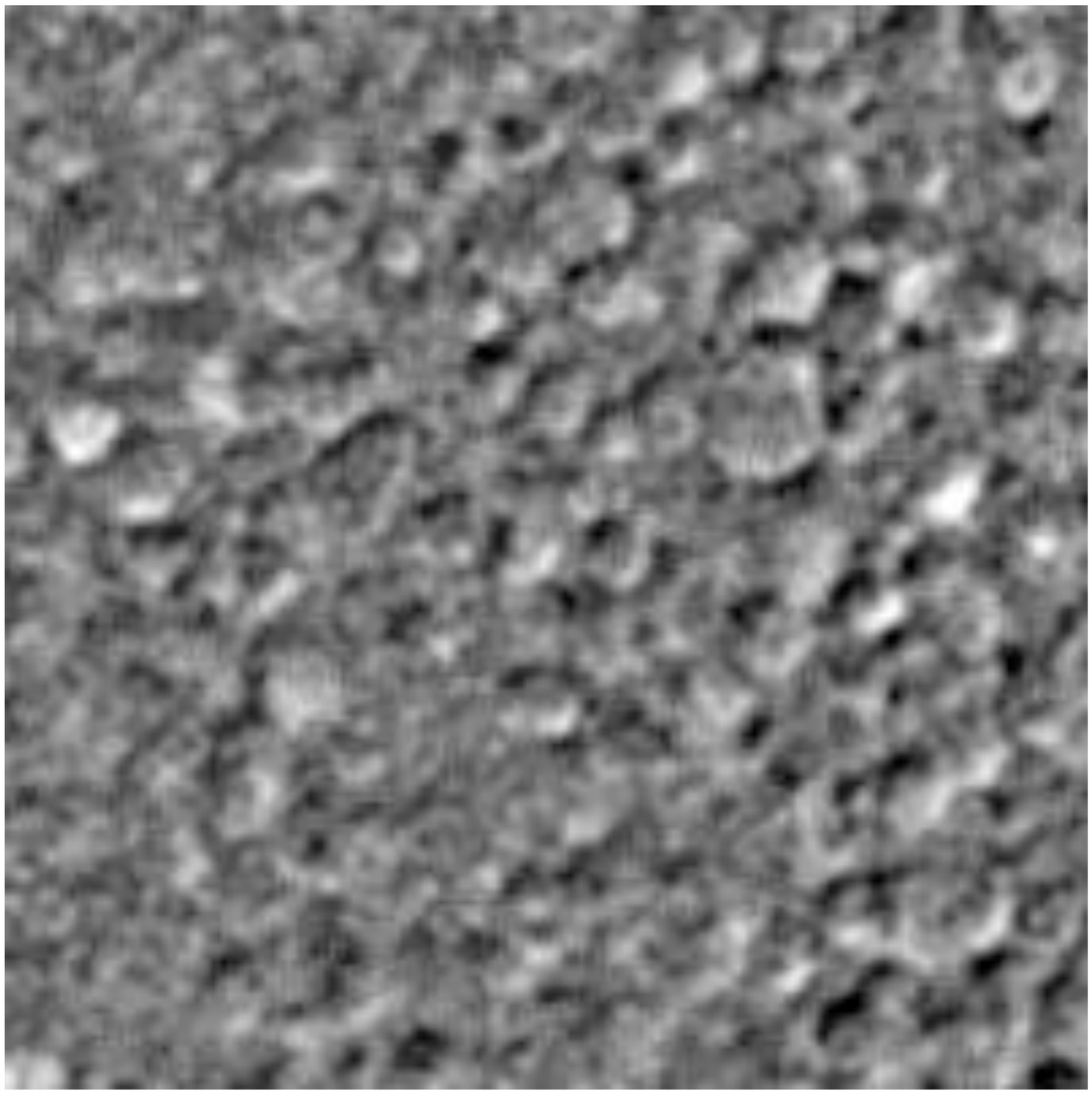
temporal median filter to identify moving foreground



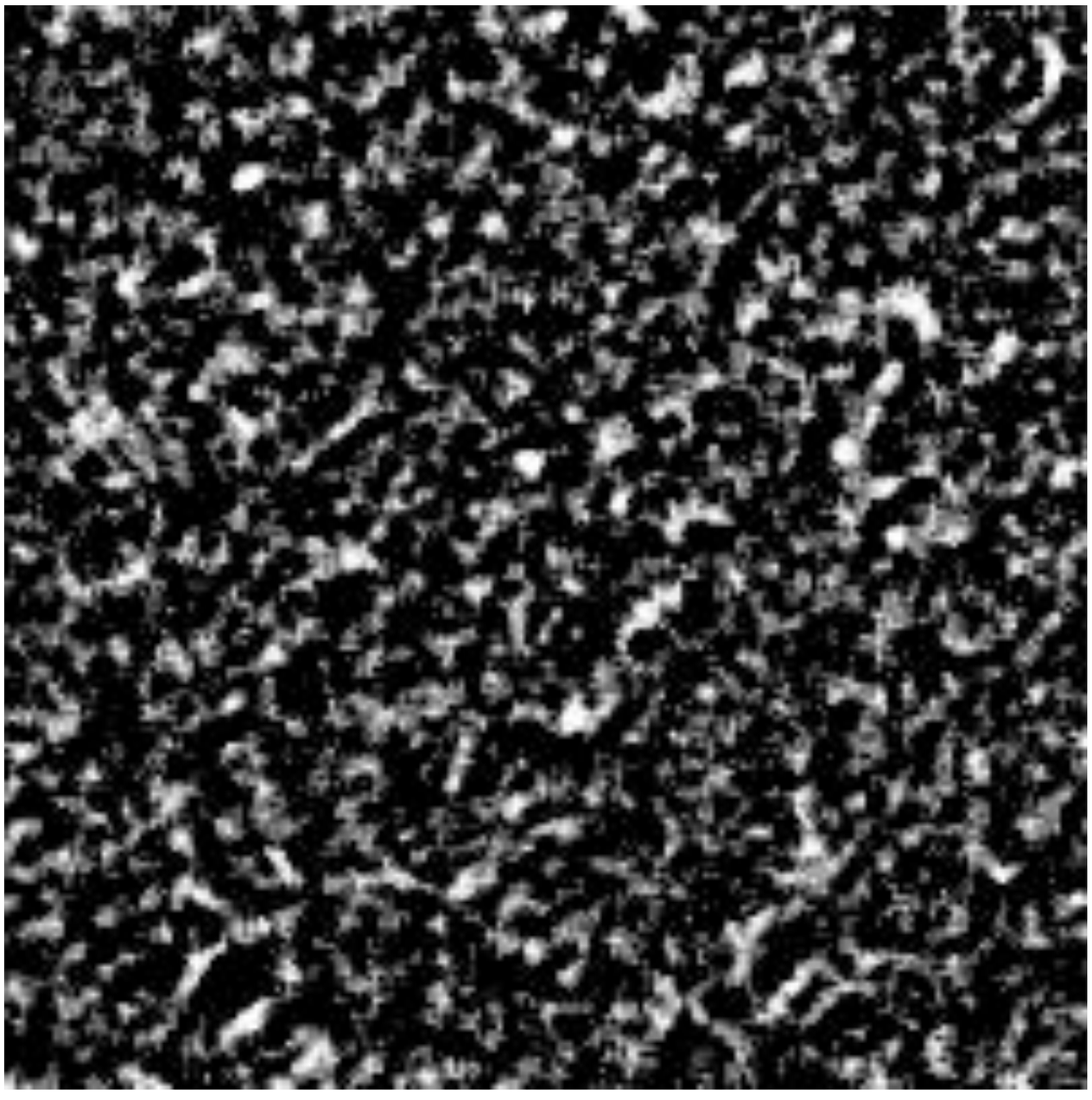


2. Feature extraction

Image segmentation: identifying ‘foreground’ features



200x200 area, normalized



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

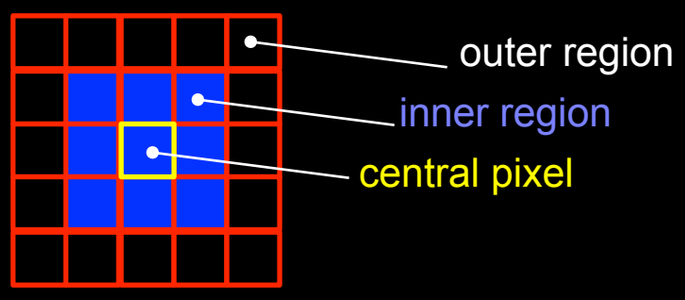
2. Feature extraction

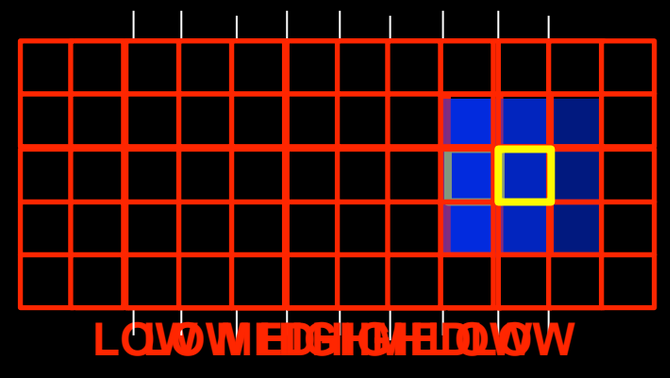
Object recognition

- 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

square Haar-like feature





2.4. Tracking in MATLAB

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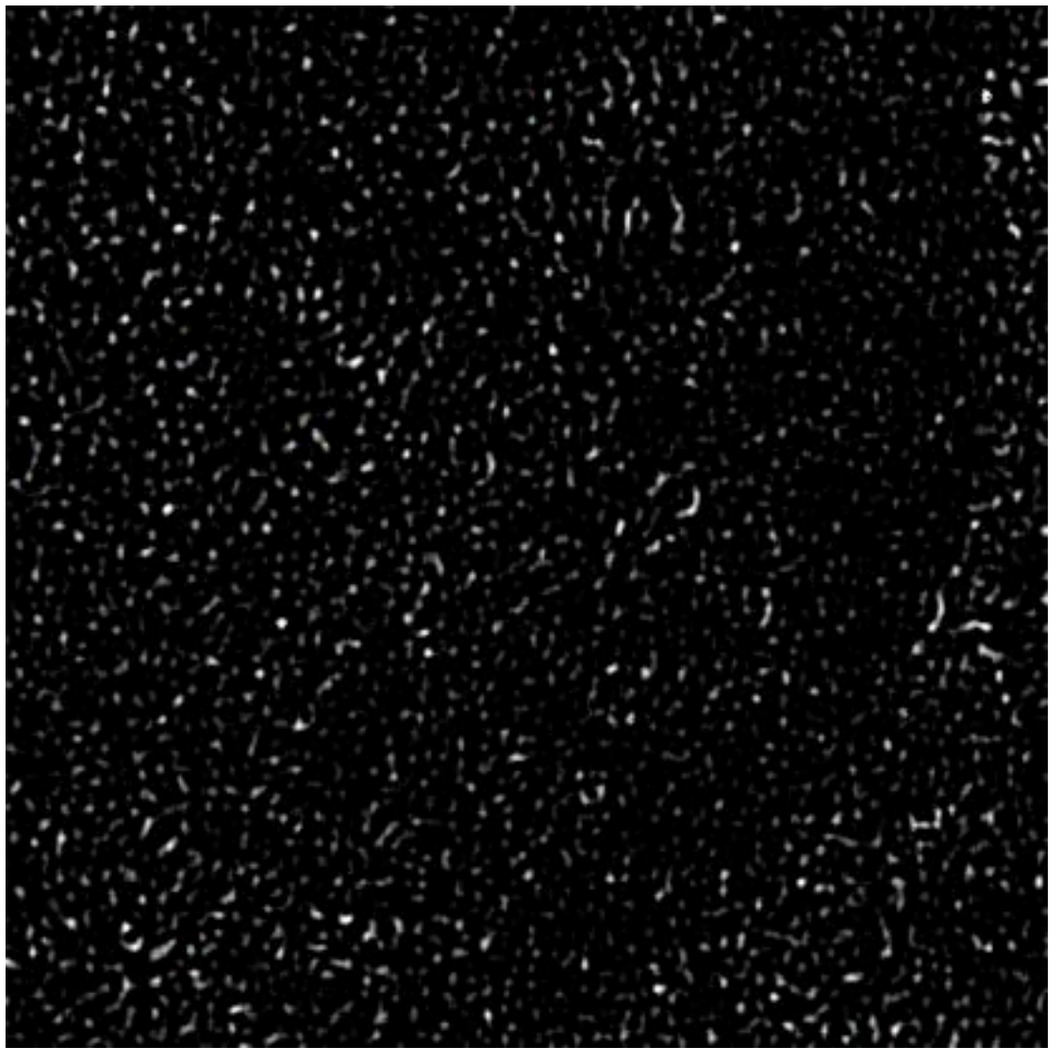
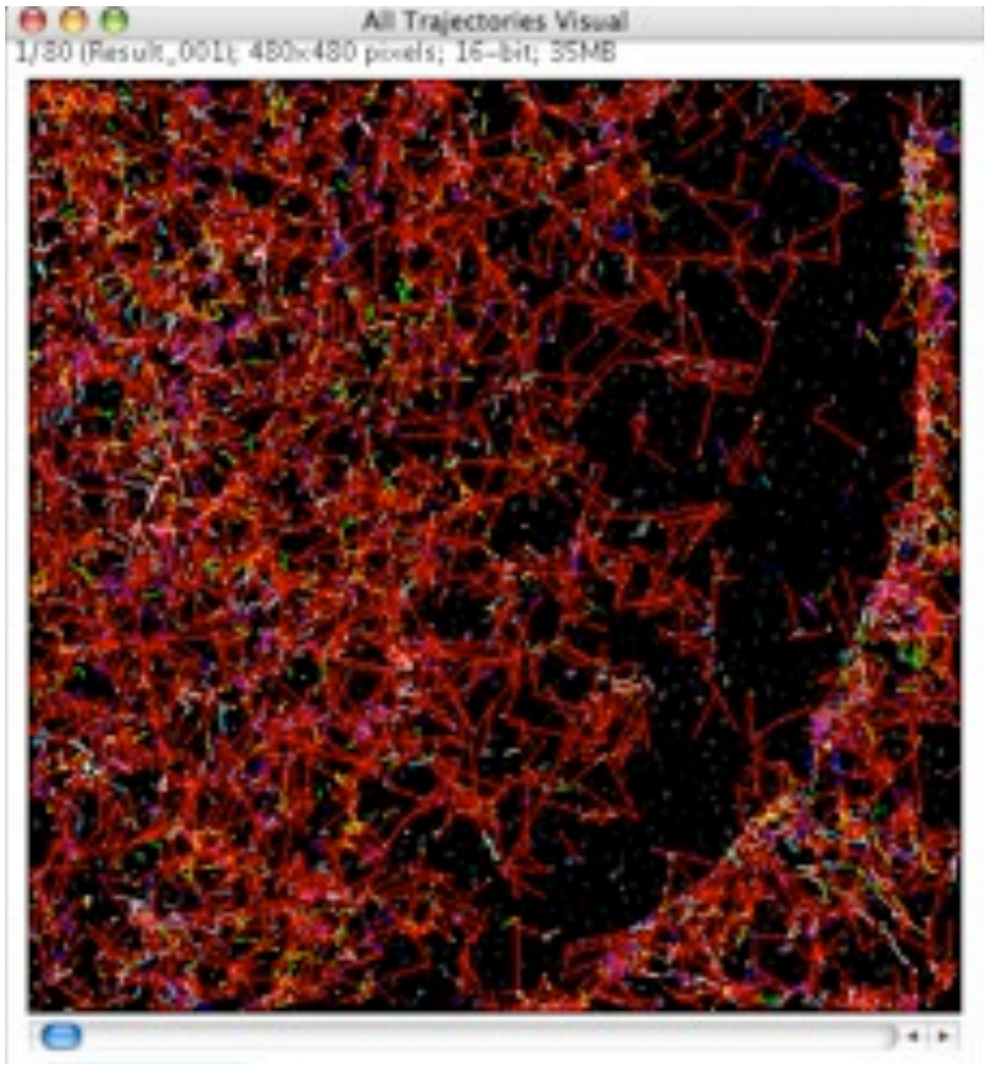
Custom particle tracker



final "particle image" with tracks



MOSAIC imageJ tracker results

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2.4. Tracking in MATLAB

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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

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2.5. MicrobeTracker

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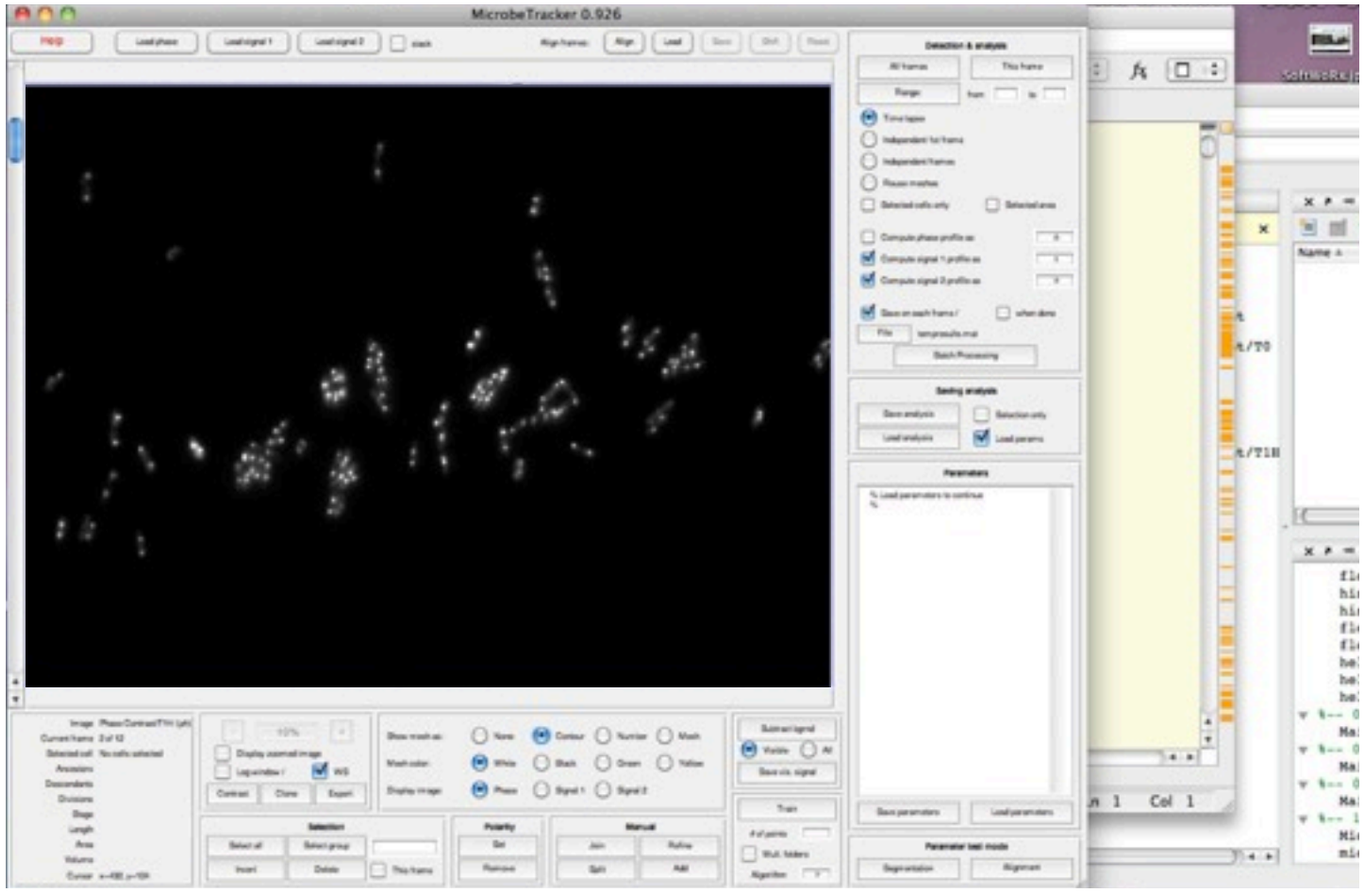
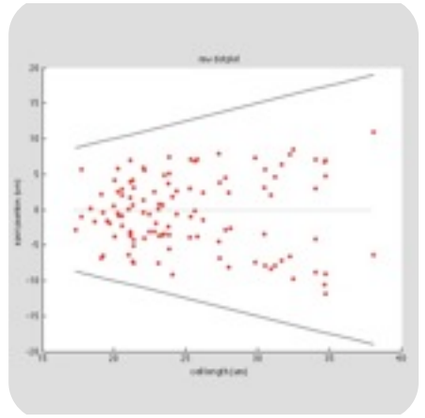
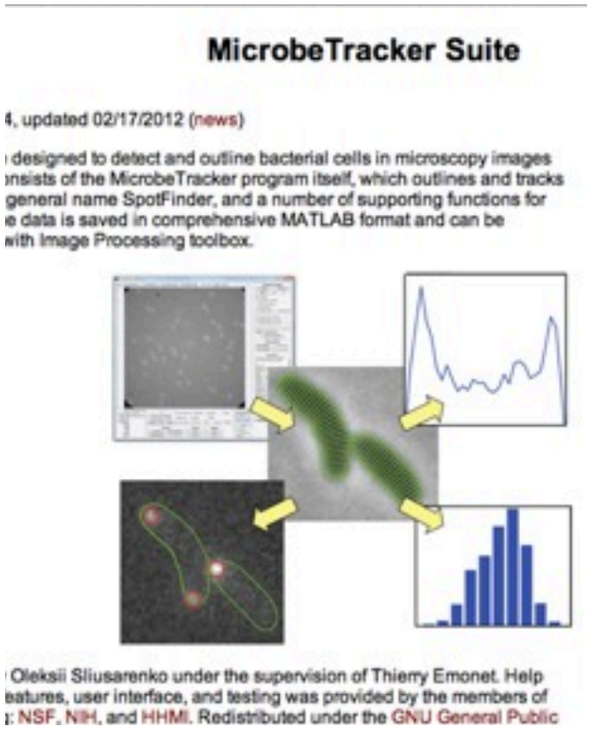
Analysis of fluorescent foci in simple cells

a MATLAB program that can easily be modified or extended







http://emonet.biology.yale.edu/microbetracker/

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2.6. OMERO

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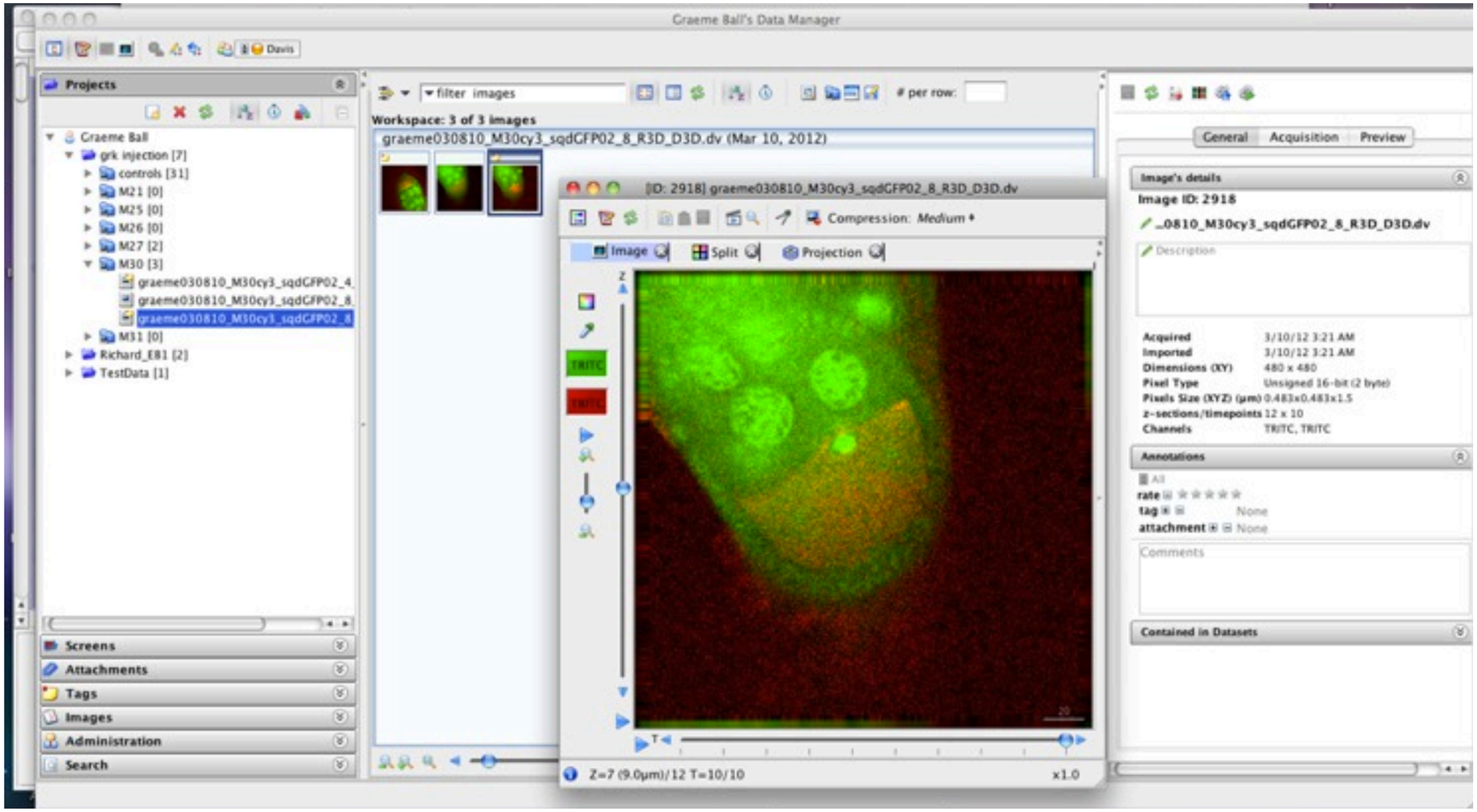
Image repository, viewer + processing/analysis

"OMERO.insight"



& IJ plugin!

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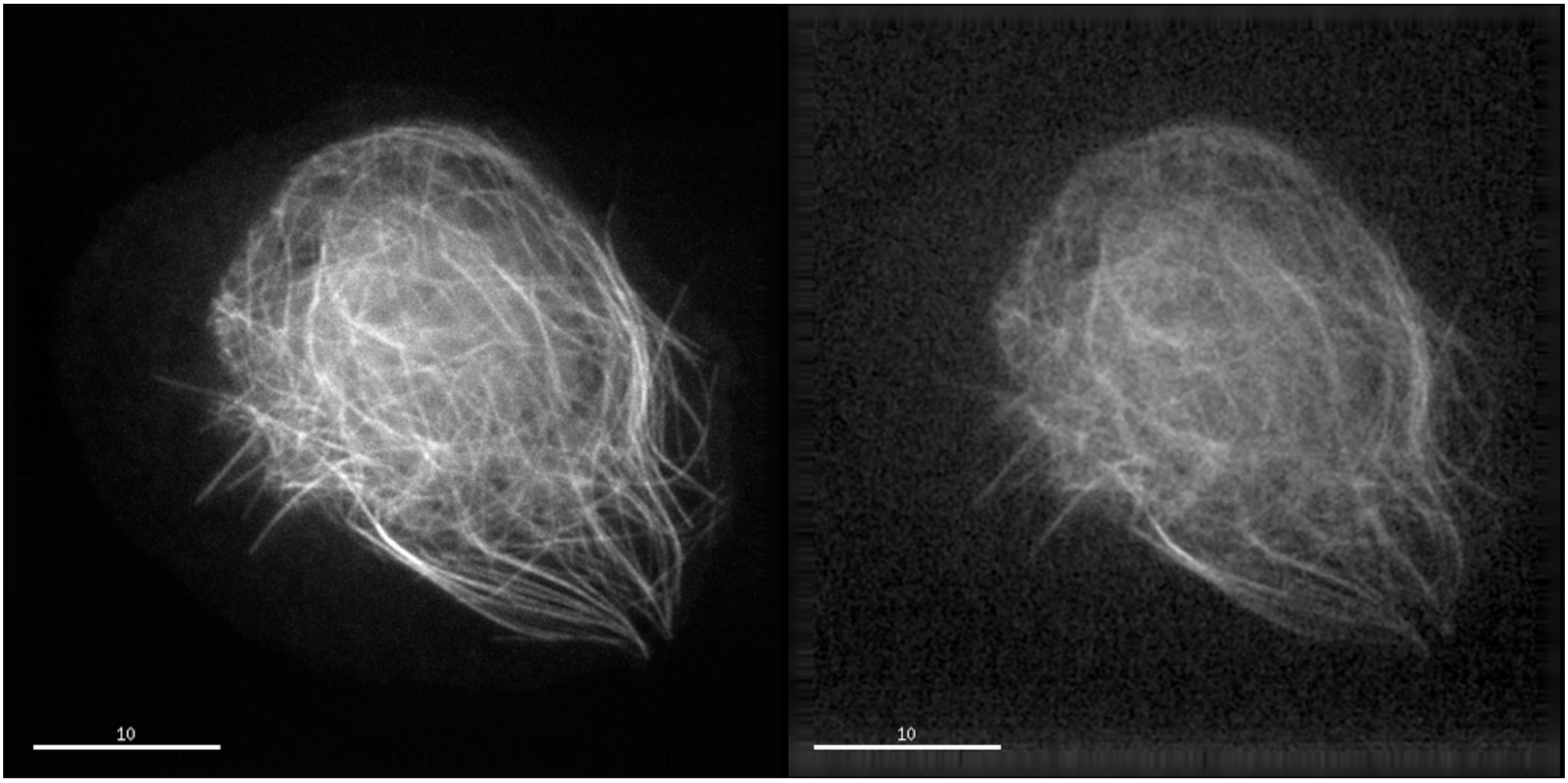
Patch-based denoising: 10-100 x less light?

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

3. Summary

Micron OXFORD Advanced Bioimaging

Overview of image processing & analysis

- importance of experimental design & optimization - identify problems early
- summary of software - choosing the right tool for the job (default to Fiji/ImageJ)
- processing / analysis concepts and 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

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Resources

- ImageJ and Fiji resources
  - MRI ImageJ tutorial: http://www.mri.cnrs.fr/datas/fichiers/articles/60/183.pdf
  - http://rsbweb.nih.gov/ij/
  - http://fiji.sc/wiki/index.php/Fiji
  - McMaster Biophotonics: http://www.macbiophotonics.ca/downloads.htm
- MATLAB demos: http://www.mathworks.co.uk/products/matlab/demos.html
- Digital Image Processing (Gonzalez & Woods), ISBN 013168728X
- Tracking resources
  - MOSAIC group (ETH Zurich): http://www.mosaic.ethz.ch/
  - Danuser lab: http://lccb.hms.harvard.edu/software.html
  - Meijering lab: http://www.imagescience.org/meijering/software/