Presentation and analysis of multidimensional data sets

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ALMF Course in Confocal Microscopy, 2010

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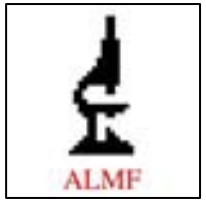

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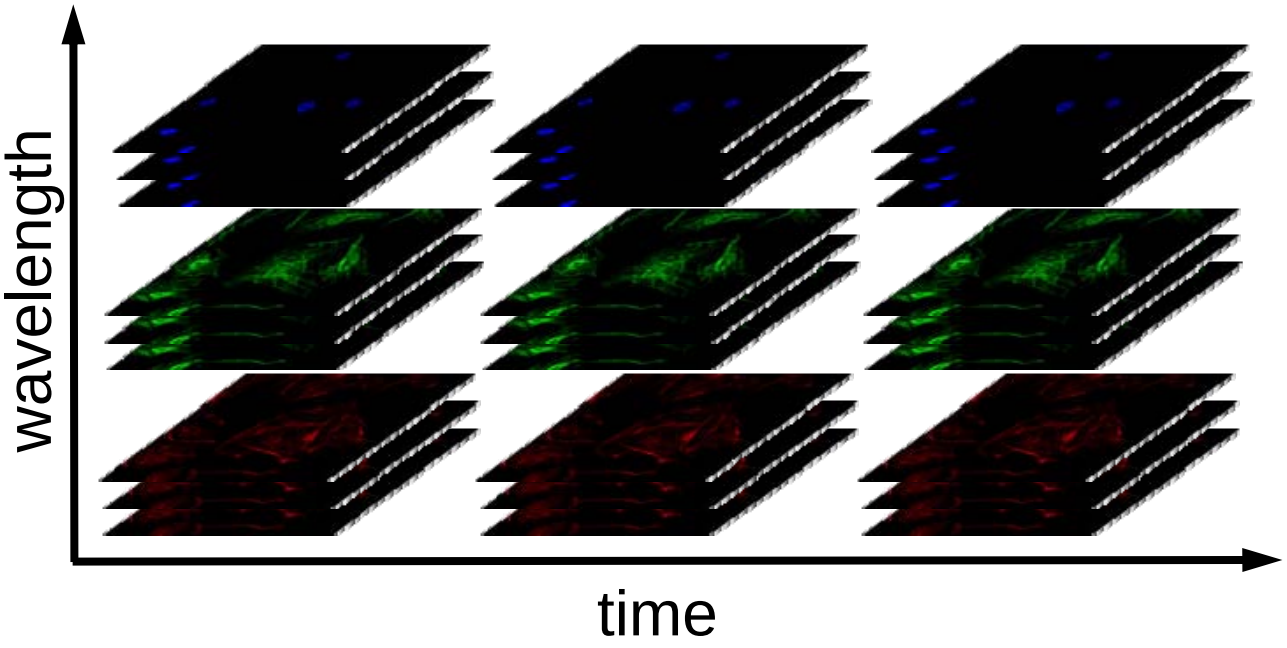
Multidimensional images



- 3D image – width, height, depth (x,y,z)
- Wavelength – multicolour image
- Time – time-lapse image
- Position- multiposition image

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Overview

1. 3D data visualization
2. Time series analysis
3. Colocalization analysis
4. Deconvolution

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1. 3D data visualization

- Data preprocessing
- Projection methods
- Depth color coding
- Rendering methods

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Data preprocessing

- Median (Gaussian) filtering
  - Removing hot pixels, noise
- Background subtraction and flatfield correction
  - Correction for nonuniform illumination, background
- Correction of lamp flickering
  - Polynomial approximation of average intensity in section
- Correction for photobleaching
  - First or second order decay approximation
- Detector calibration
  - CCD pixel sensitivity or non-linearity of PMT and PD
- Image enhancement
  - Contrast stretching, histogram normalization (be careful for quantitative analysys)

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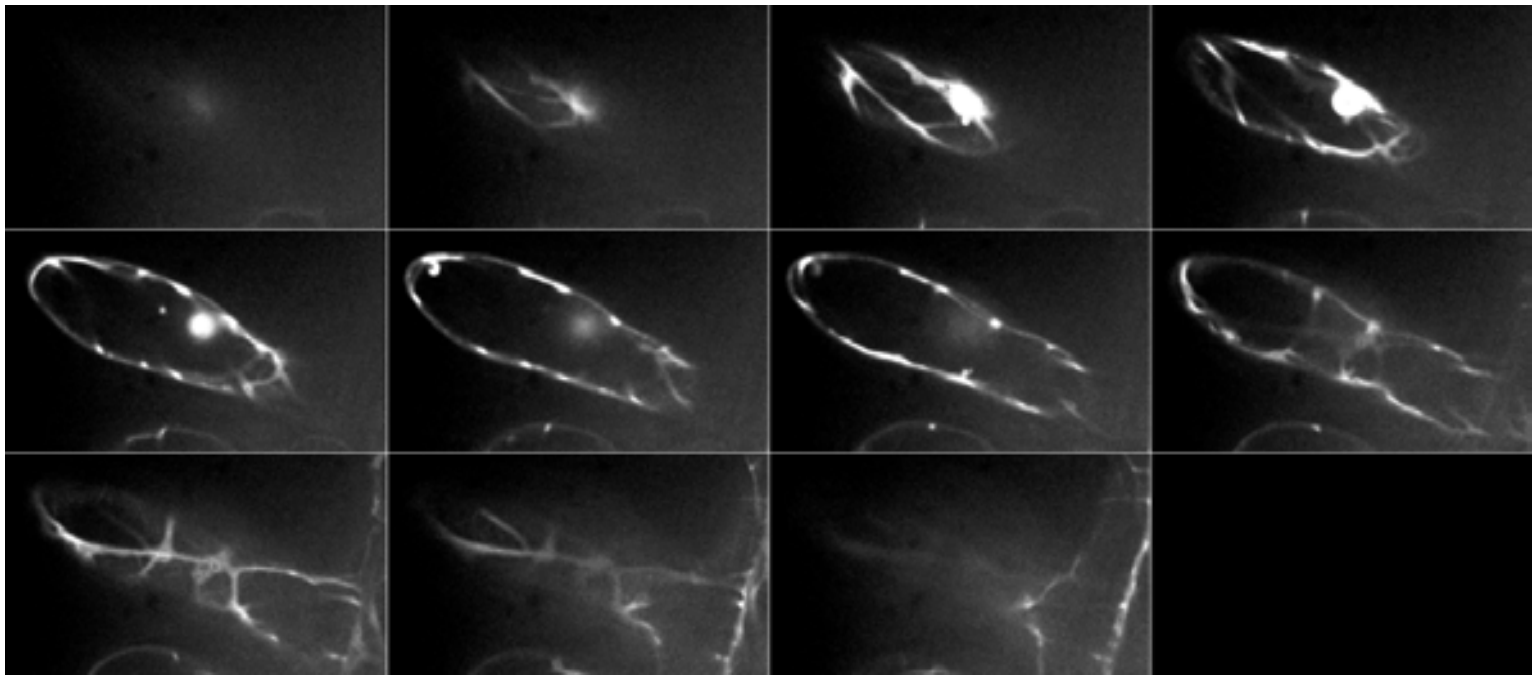
Gallery display of z-slices



Requires no calculations
All sections can be seen simultaneously
Not practical for big stacks (or display a part)

4D dataset: GFP in the cytoplasm of a plant cell (T. Timmers, CNRS/INRA)

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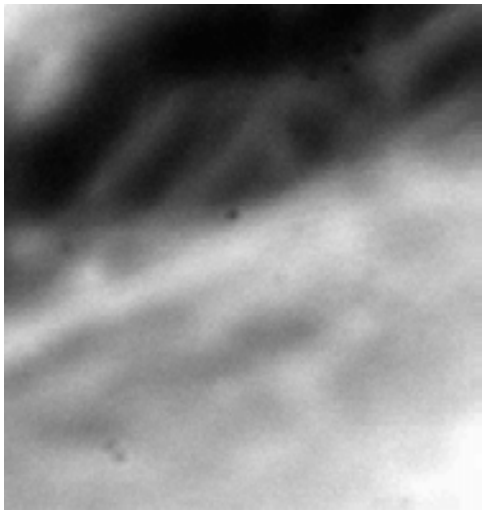
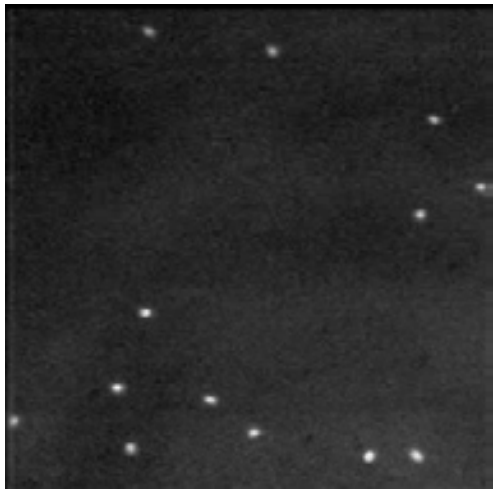
Displaying 3D data as a movie



z-stack



time lapse

Data displayed sequentially
Frame rate can be varied

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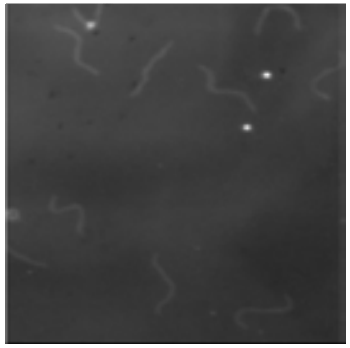
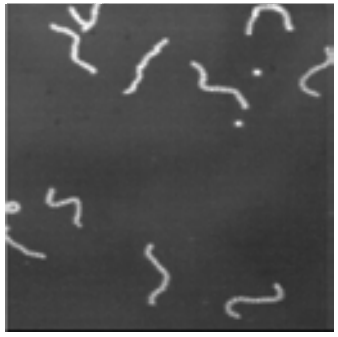
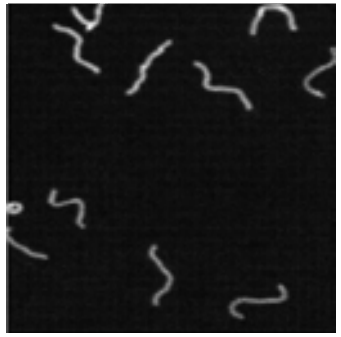
Projections of 3D data



Average



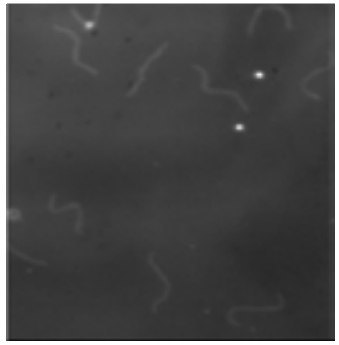
Maximum



Standard deviation

Median

Minimum



Sum

Also can be used for volume rendering

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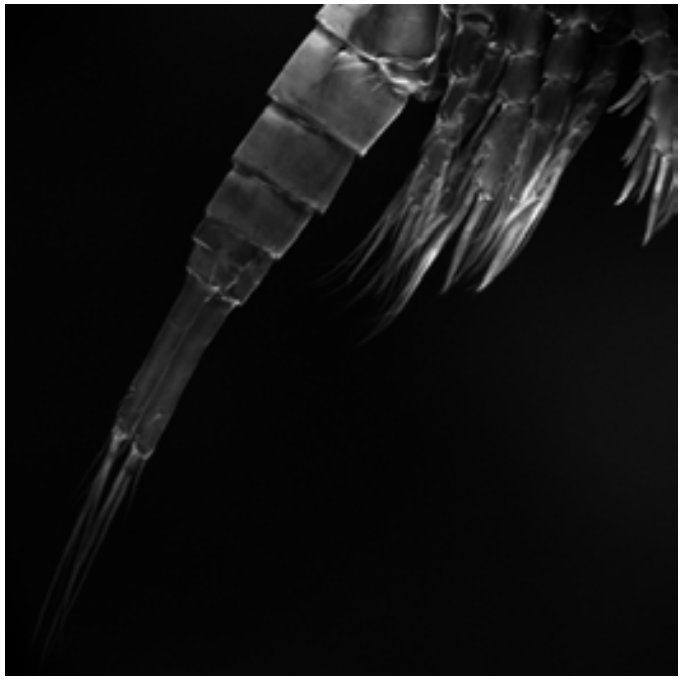
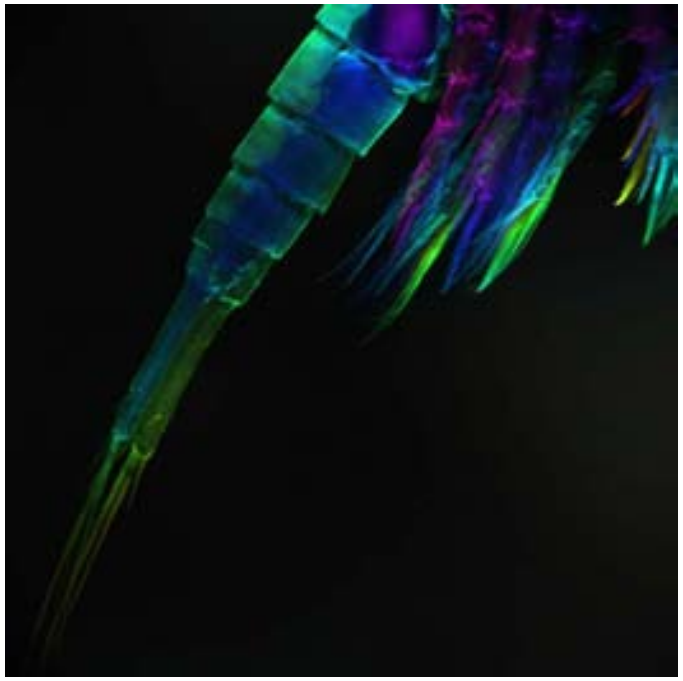
Depth color coding



Standard deviation



Color coded

Every section is coded in a different color according to chosen look up table

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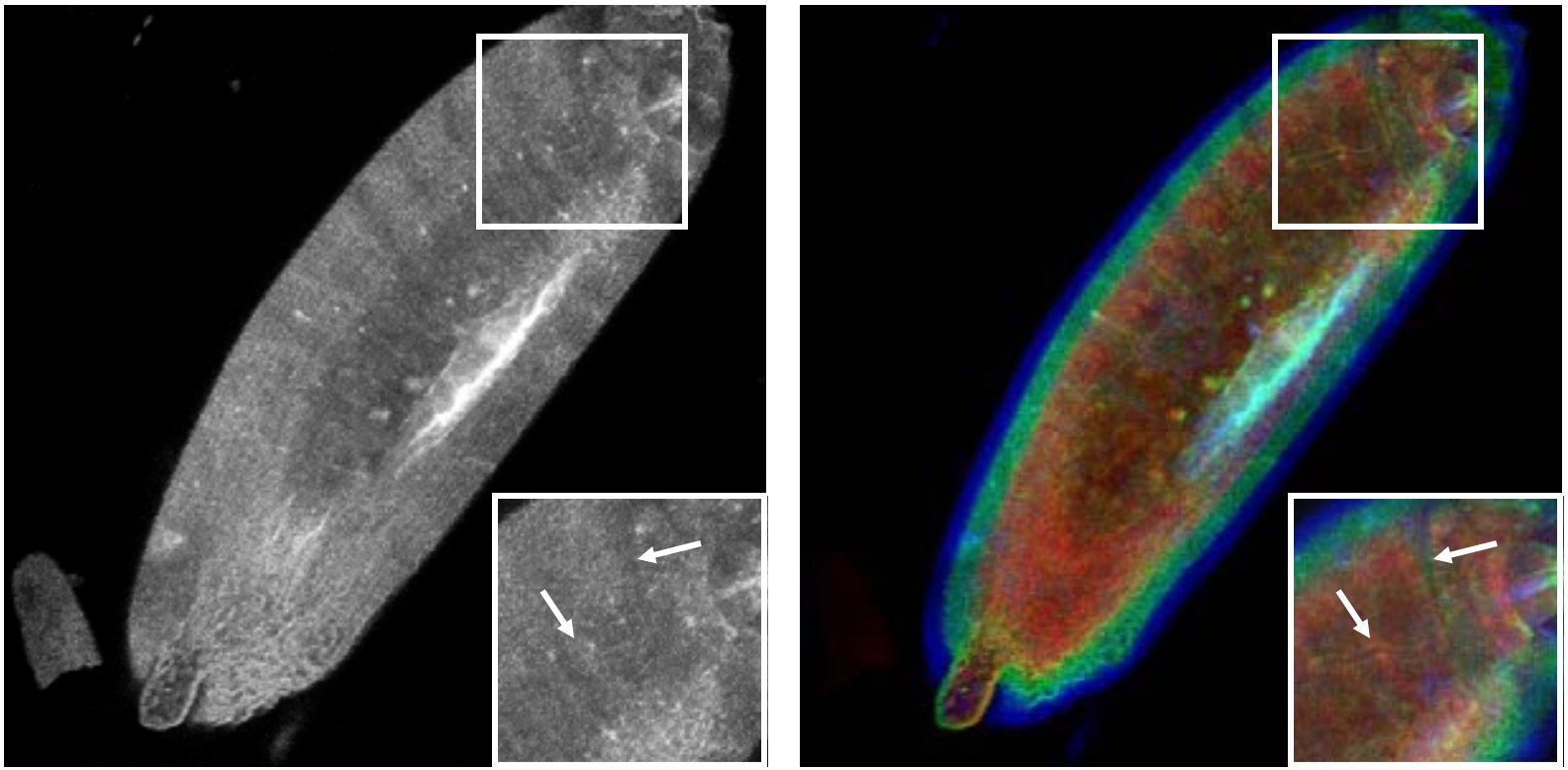
New features can be revealed



Eye is more sensitive to color than to intensity changes

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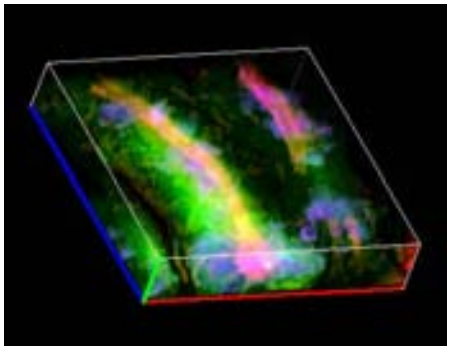
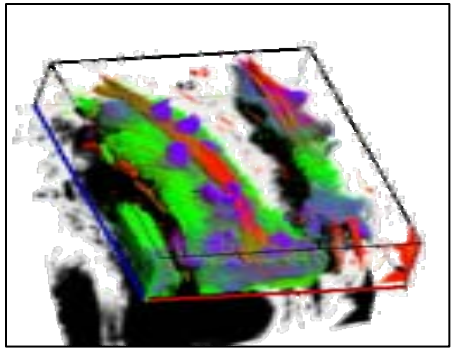
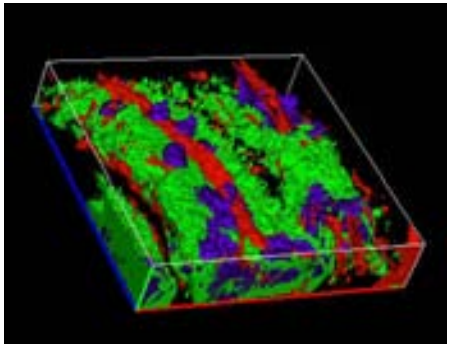
Volume rendering methods



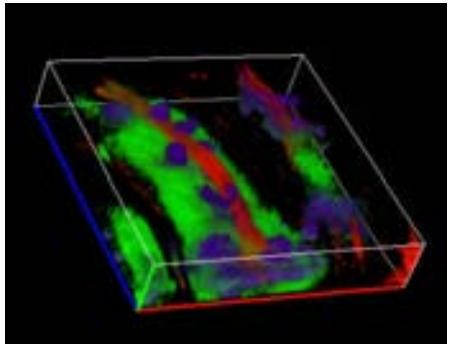
Maximum Projection Mode
Only pixels of the highest intensity along the observation axis are displayed.



Shadow Projection Mode
The image data are illuminated by a virtual light source. The combination of light reflection and opacity creates the impression of structure in space.



Isosurface Mode
The non-transparent surfaces are calculated from the gray values. This results in hard transitions between the various channels.



Transparency Mode
The image data are illuminated from the back with diffuse white light, which results in a transparent appearance.

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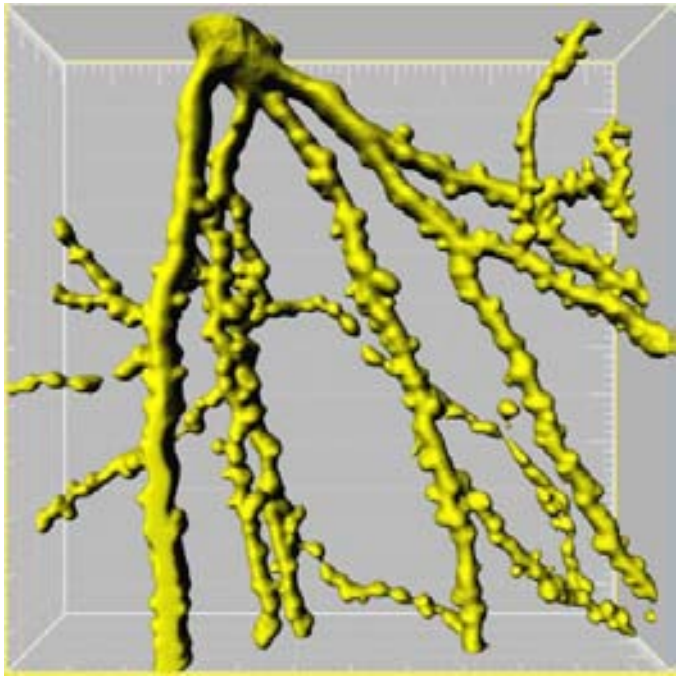
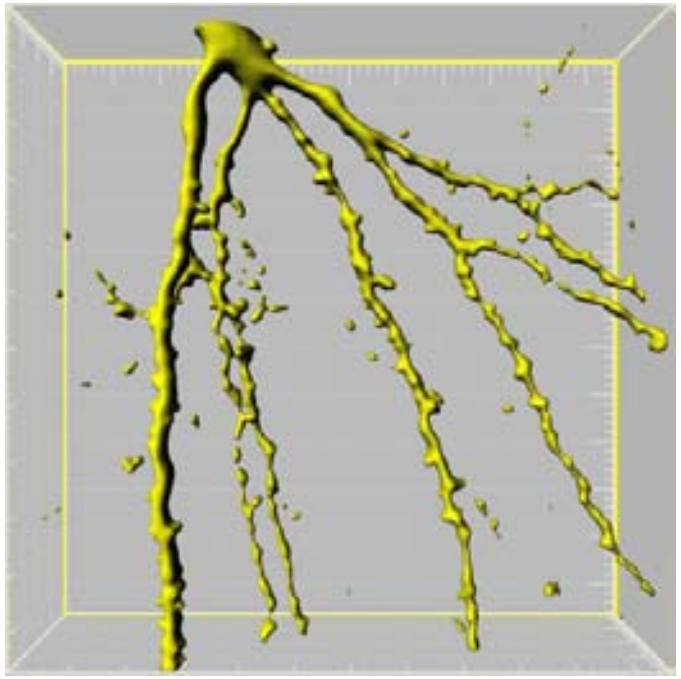
Isosurface rendering



Threshold 10



Threshold 70

Threshold, smoothing, rendering accuracy affect image

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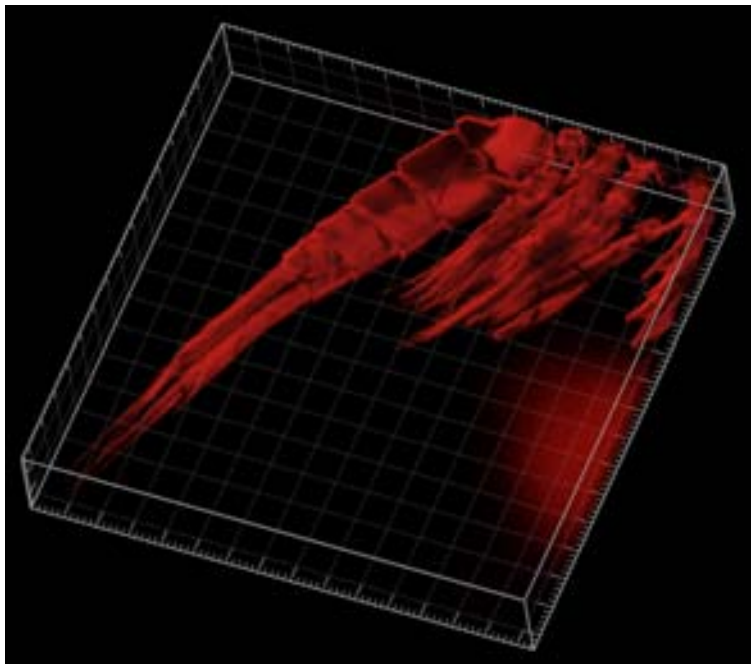
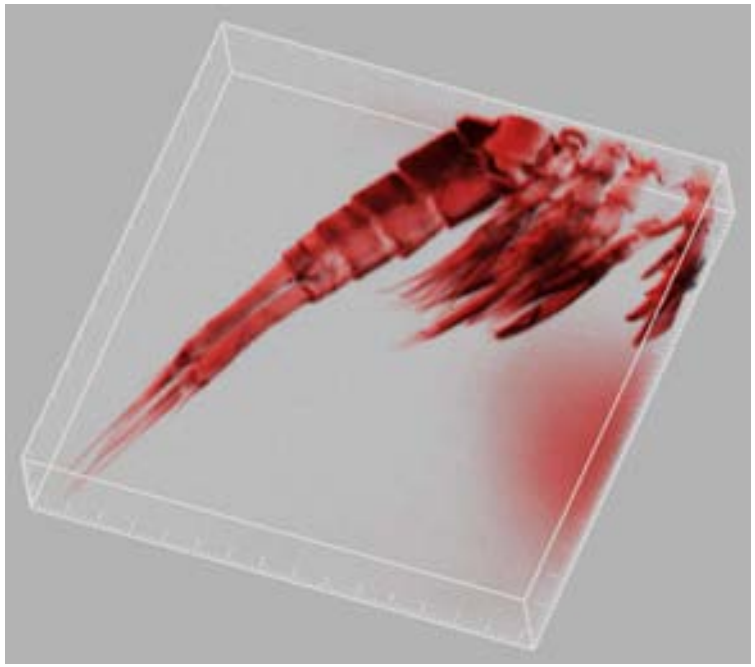
Volume rendering



Maximum Projection



Transparency mode

Opacity, threshold, position of light source affect image

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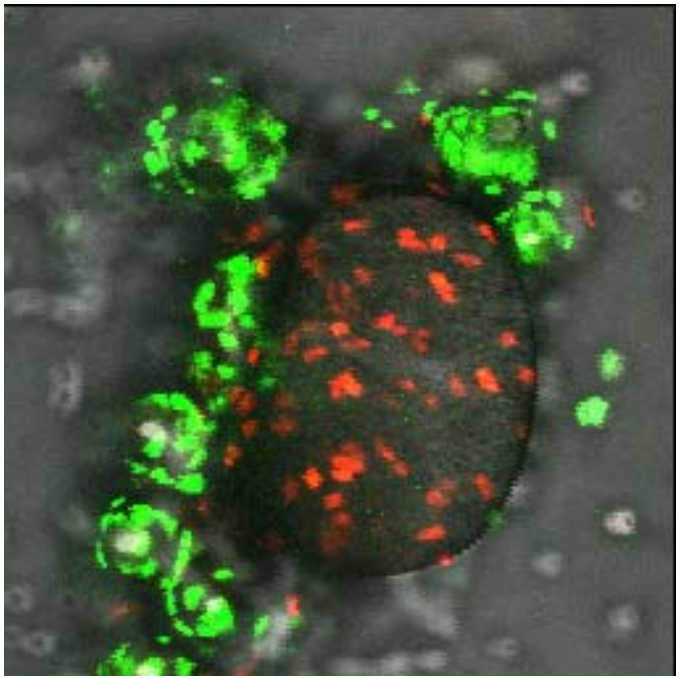
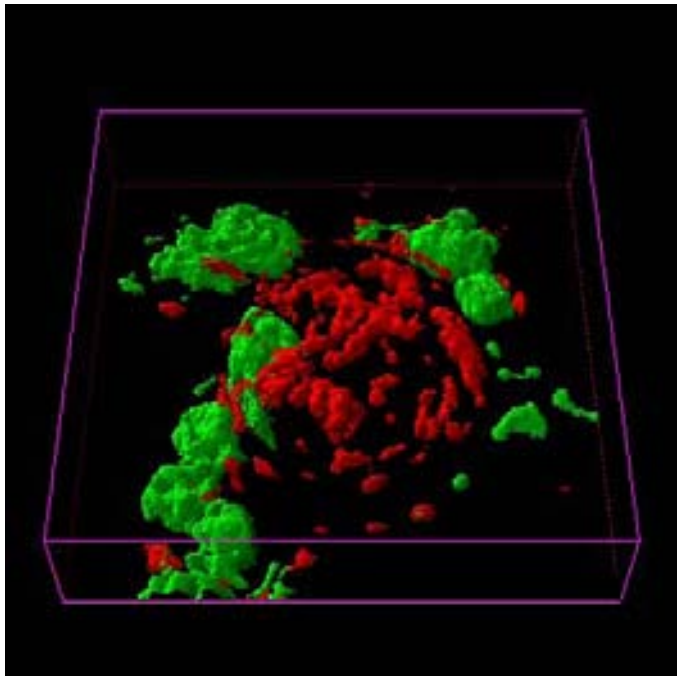
Multicolor 4D imaging





Fluorescence channels can be rendered separately
Merging of transmission and fluorescence channels

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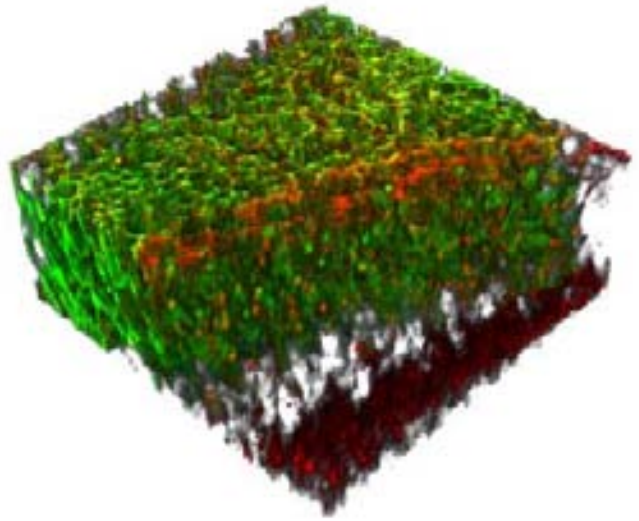
Rotation of rendered image



Drosophila wing disk

Gives better presentation of sample spatial characteristics

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3D data visualization: summary

Ensure correct sampling during data acquisition: both over- and undersampling are counterproductive

Projection methods are very calculation efficient, and give a quick idea about general structure of the specimen

Rendering methods are more calculation intensive, but well represent spatial features of the specimen

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2. Time series analysis

- Optimal imaging conditions
- Kymograph
- Manual particle tracking
- Automatic particle tracking

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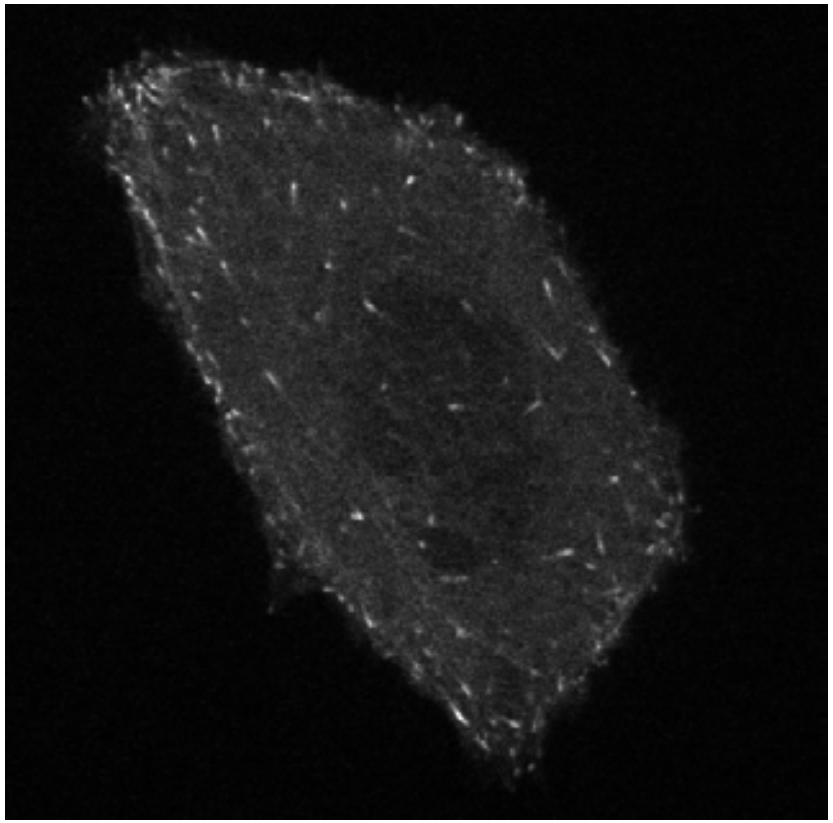
Timelaps measurements



Eb3-GFP in HeLa cell

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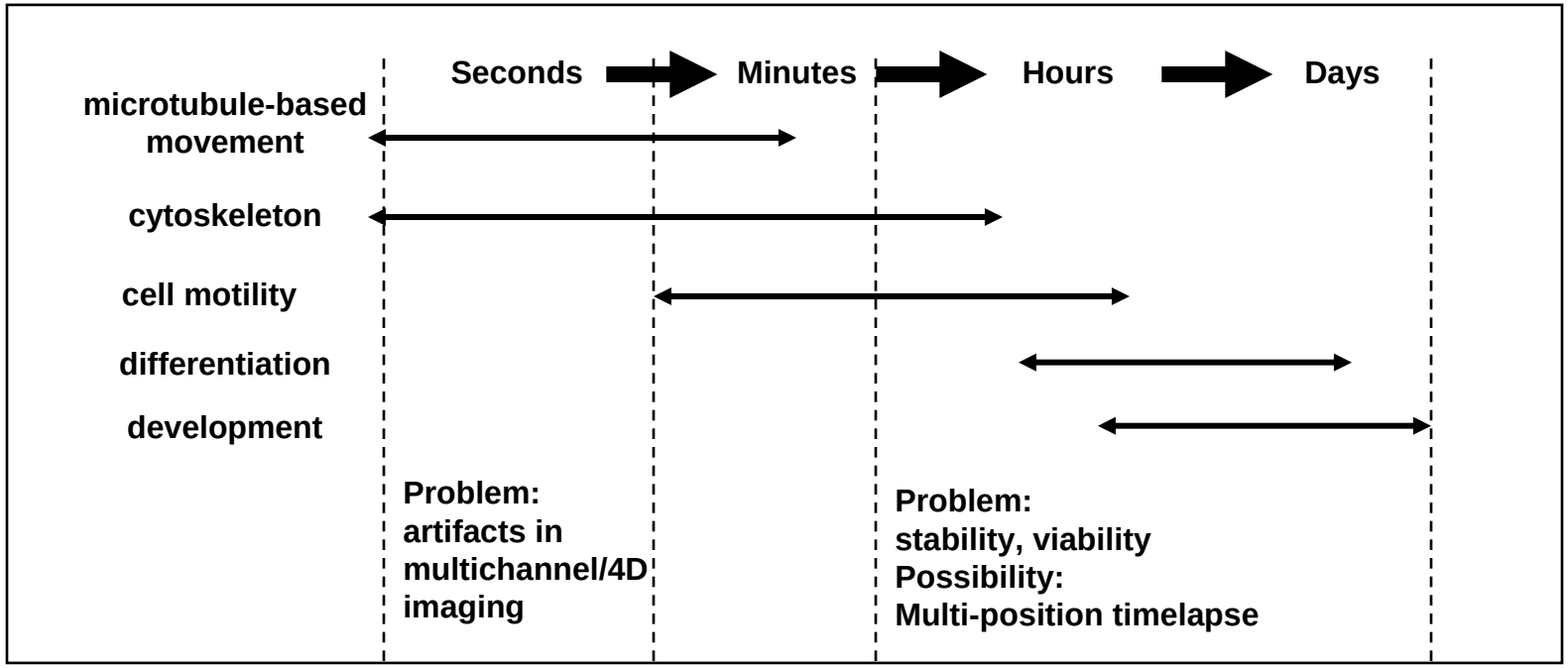
Experimental timescales



Optimal frame rate and length of time lapse are defined by the dynamics of processes in the specimen

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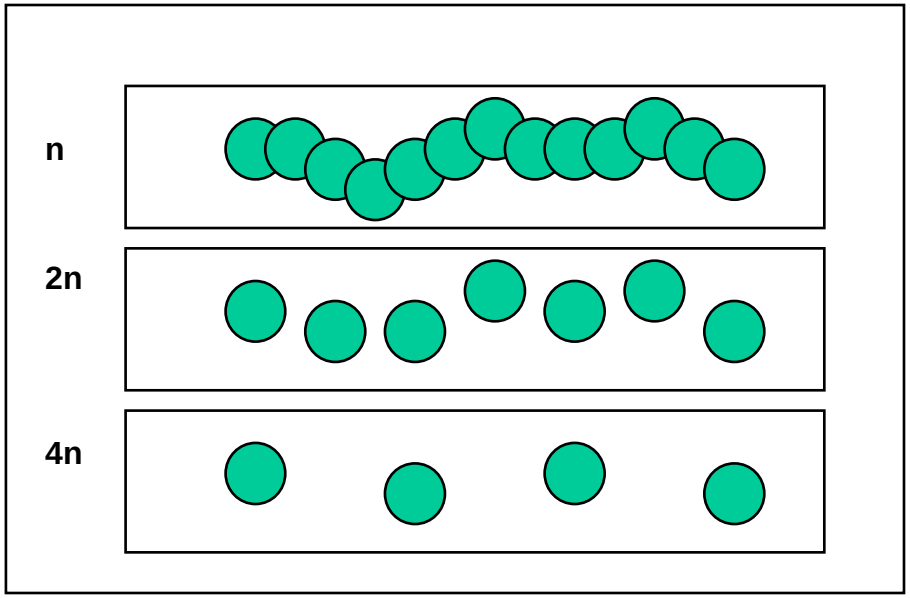
Sampling intervals



Movement between frames should not be too large
Signal to noise ratio decreases for higher frame rates
Density of the objects should be also considered

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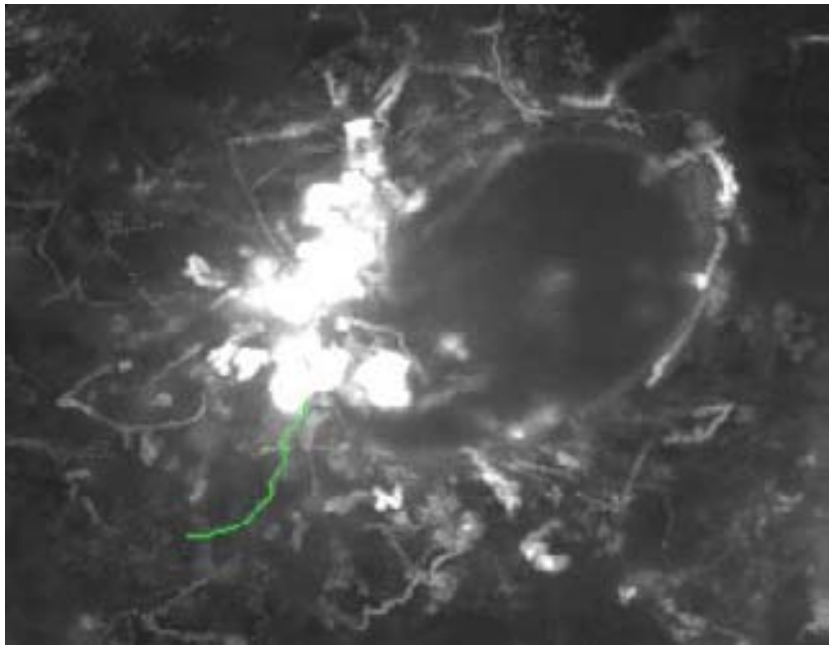
Basic movement analysis



Projection shows a trajectory of moving particles
Data on the intensity along particle trajectory in each frame gives information on particle speed

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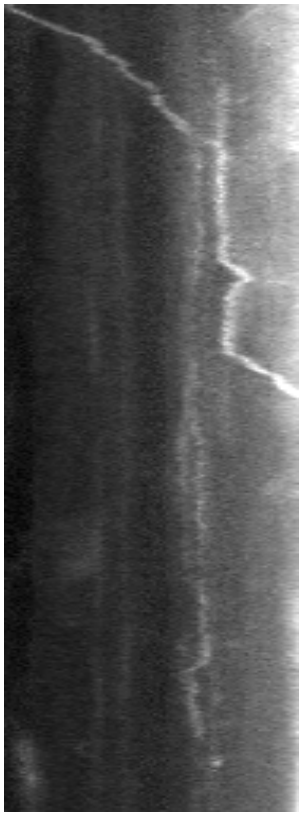
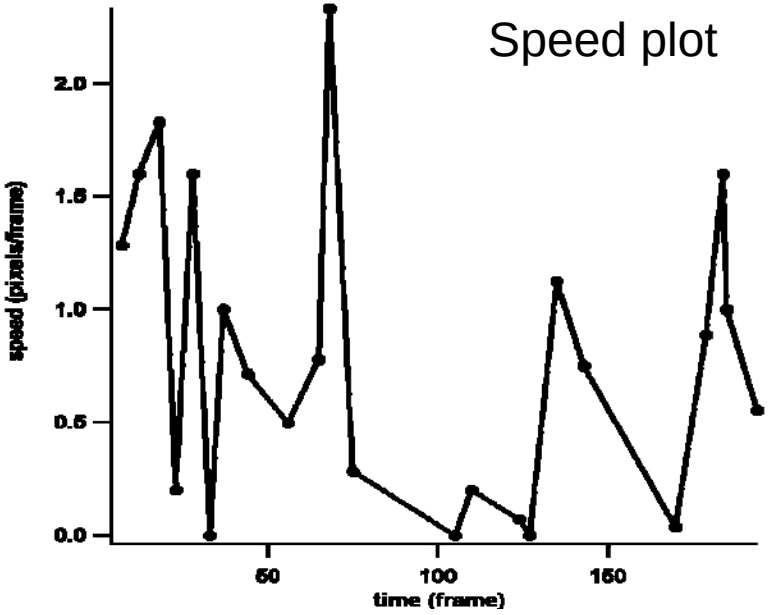
Kymograph (time/space plot)

Time



Space



Speed of the object is calculated from a kymograph. Displacement of the particle should not exceed 2 pixels per frame.

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Manual particle tracking

The position of the object in each frame is marked manually.

Direction of particle movement and speed are calculated based of this data.

Accuracy of the obtained values is not very high. Repeated measurements might be required for higher accuracy.

Works reliably for small objects (vesicles, endosomes, etc.). Object density in the specimen should be low.

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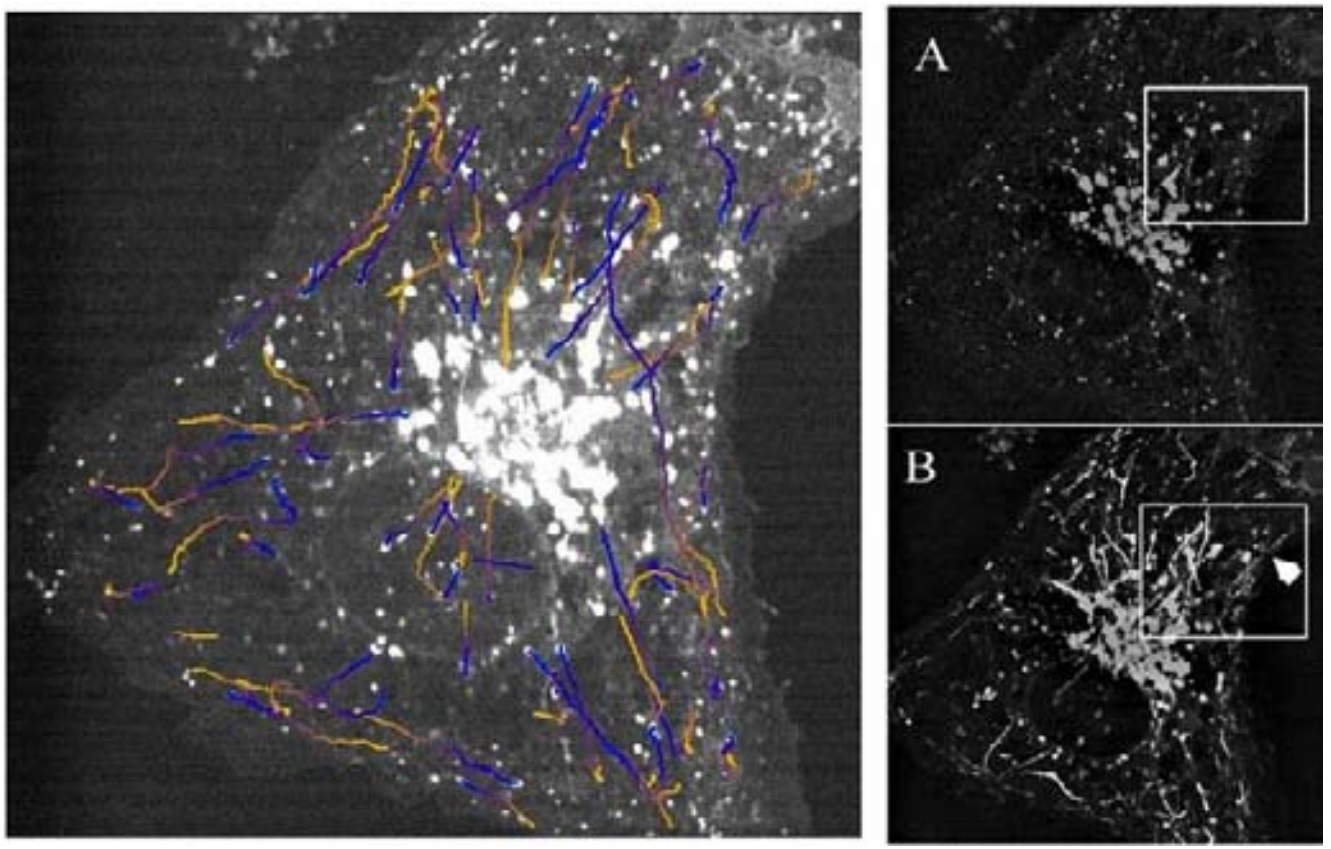
Example of manual particle tracking



Very slow and work intensive procedure

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Automatic particle tracking

Position of the object, its orientation and form change are automatically defined by one of the following methods:

Gaussian fitting method (small particles);
centroid method (small and large particles);
pattern matching method (cells, large organelles);
etc…

Software calculates speed of the objects and statistics data automatically.

Faster and more accurate than manual tracking, but does not always work for dense specimens.

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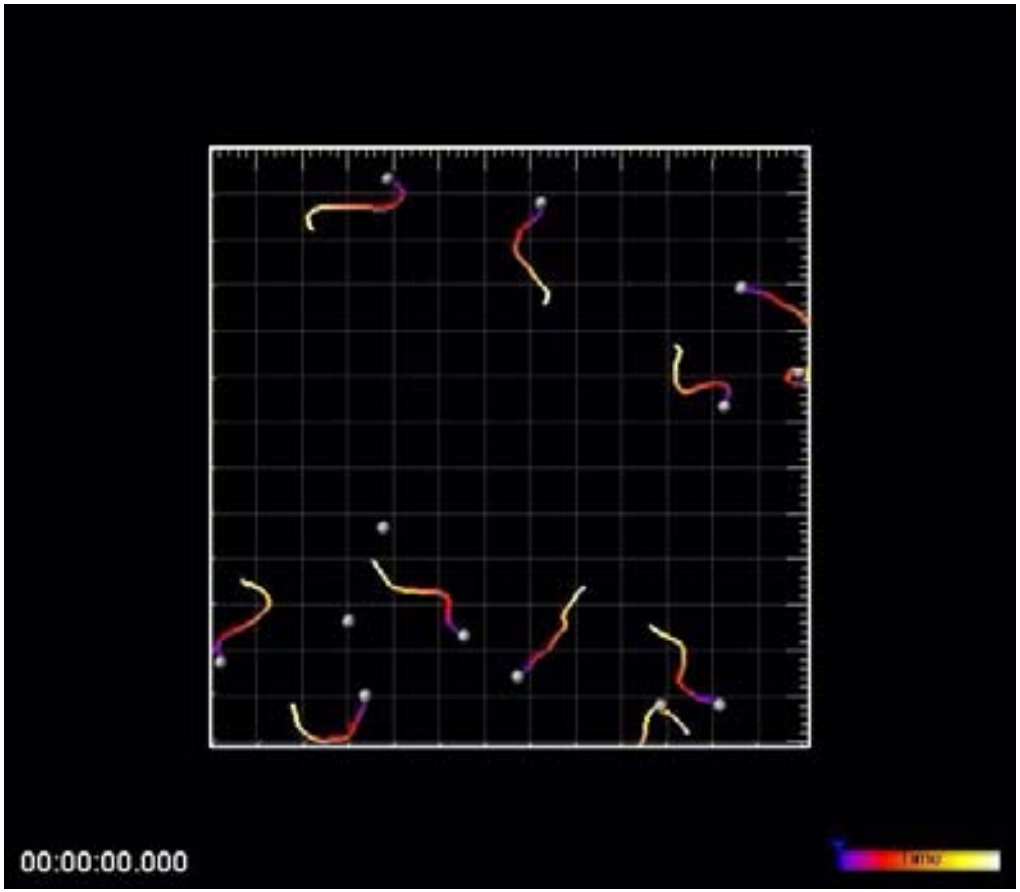
Example of automatic tracking



Trajectories are color coded

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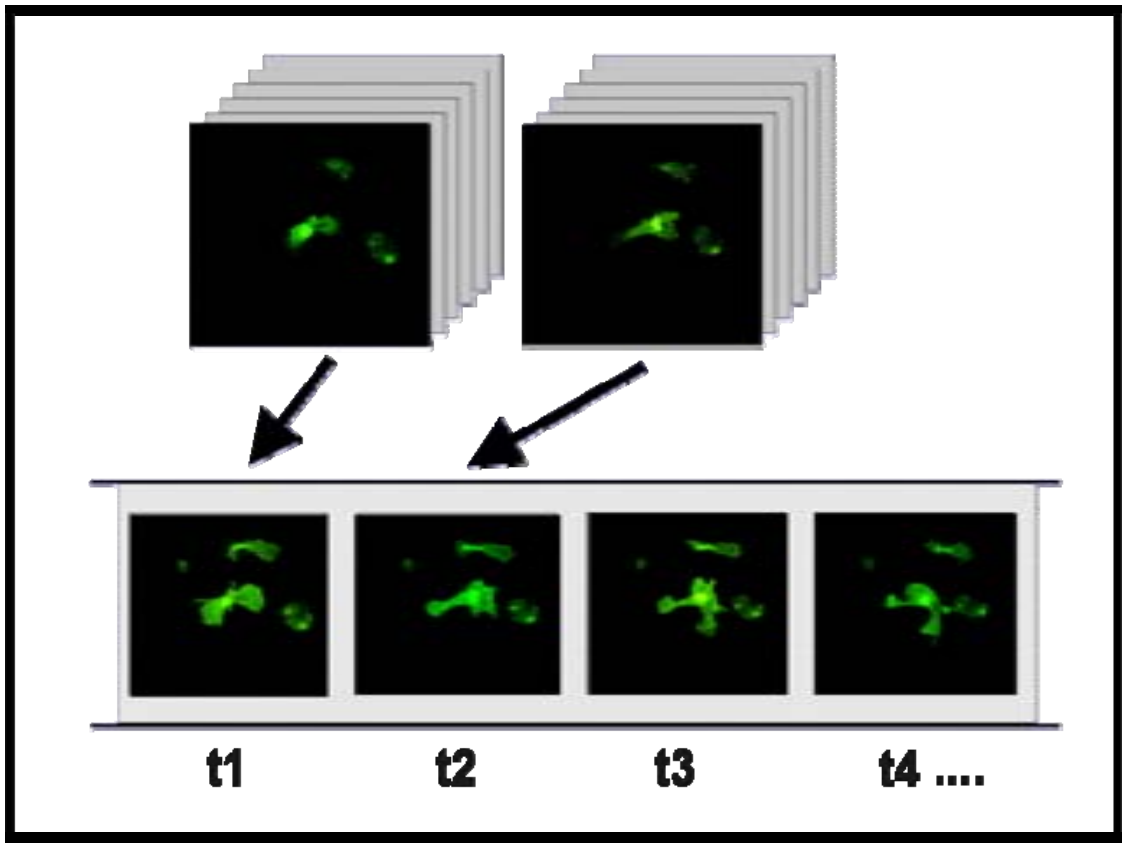
Tracking for 4D datasets



Manual and automatic tracking possible

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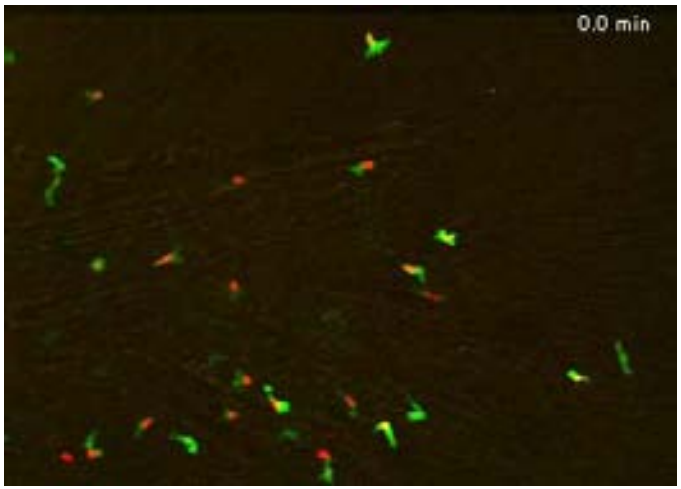
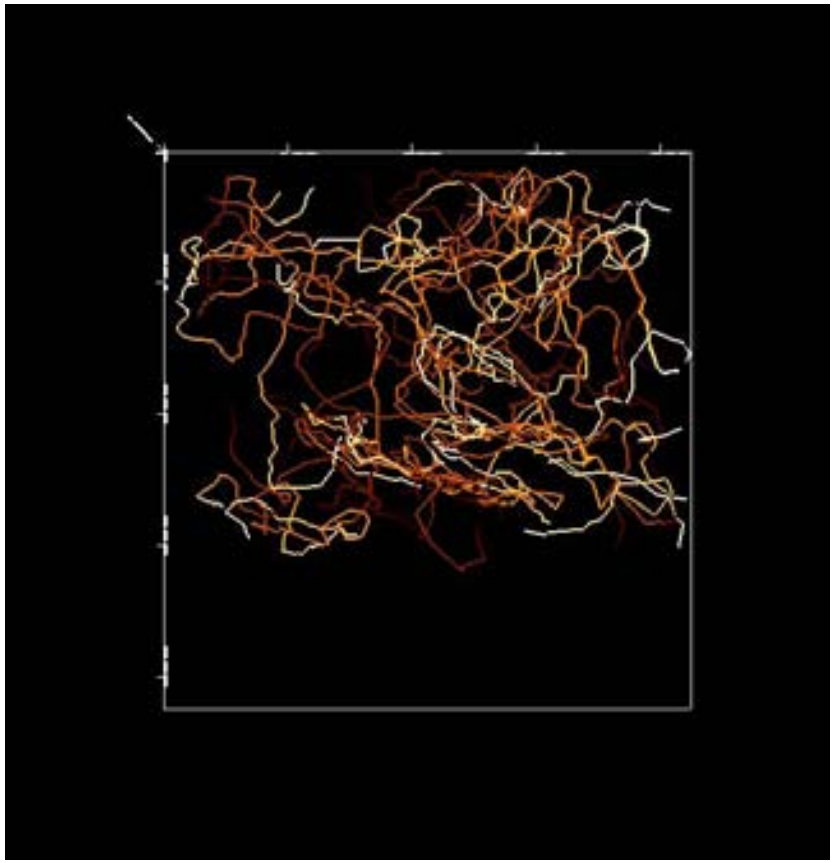
Automatic tracking of objects in 4D



Macrophages in
medaka embryo

Clemens Grabher
and Adam Cliffe



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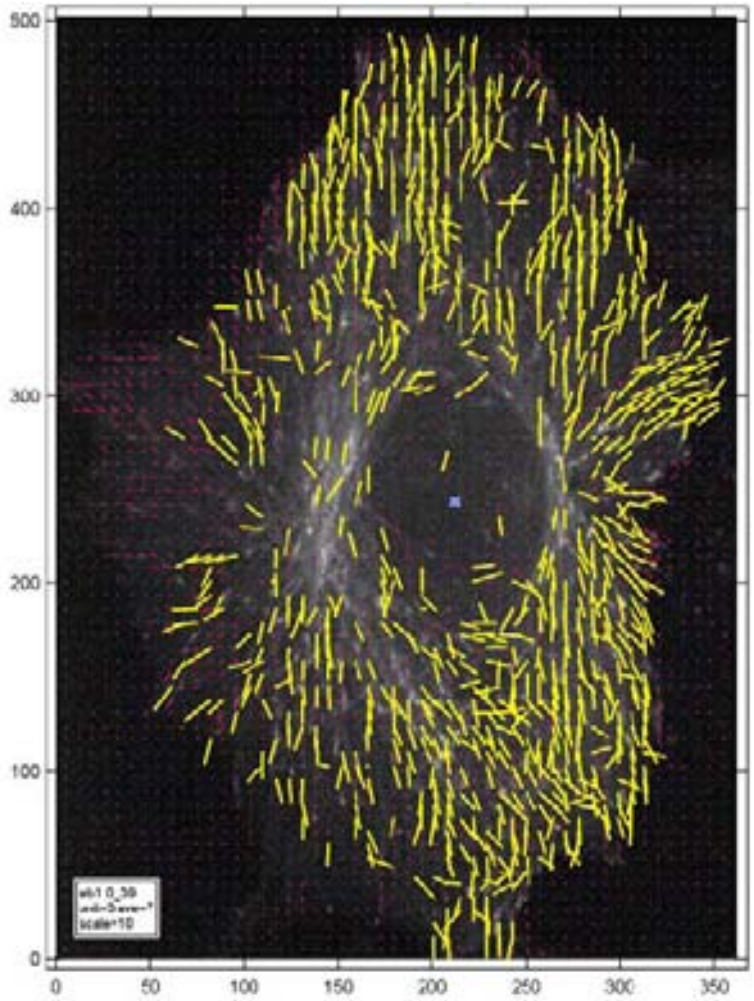
Advanced analysis techniques



Vector field.
Image of Eb1-GFP
in Vero cell.

Kota Miura

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Time series analysis: summary

Frame rate of good time series should correspond the dynamics and signal in the specimen

Kymograph is an useful technique for analyzing speed of the objects in time lapse series

Manual tracking is not very accurate and extremely time consuming, especially for large data sets

Automatic tracking is accurate and time efficient, but strongly depends on algorithm, settings, and the quality of data set

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3. Colocalization analysis

- Optimal image acquisition
- Colocalization scatter plot
- Colocalization coefficients
- Role of threshold adjustment

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Requirements for accurate colocalization

- Low noise level in image
- No bleed through between channels
- Check registration shift between channels
- Reproducible shift can be corrected
- Correct sampling in axial and lateral directions
- Use highly color corrected objectives
- Slide of multicolor beads is a good test sample

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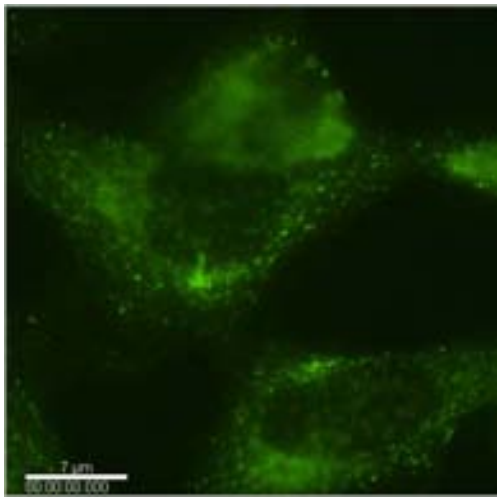
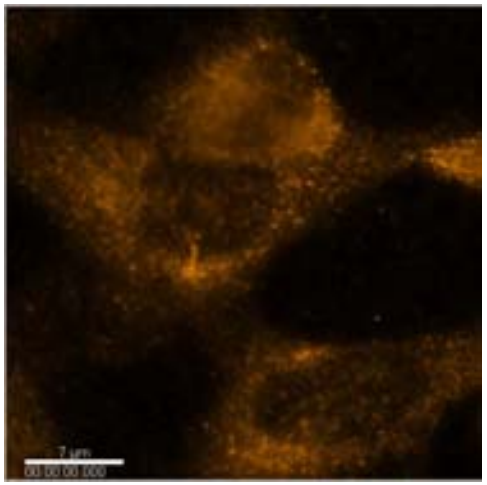
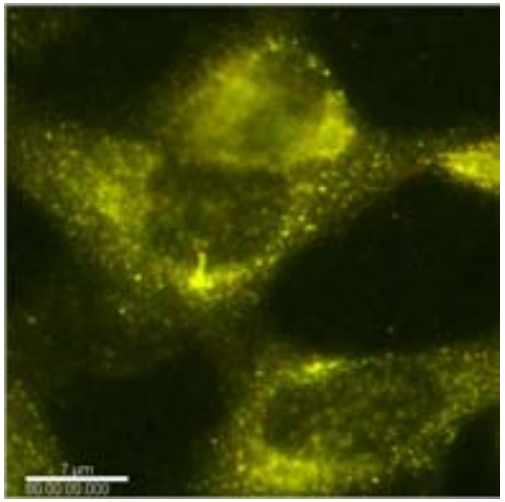
Colocalization by channel merging



green channel



red channel



merged

Colocalized features are yellow
Qualitative and very subjective method

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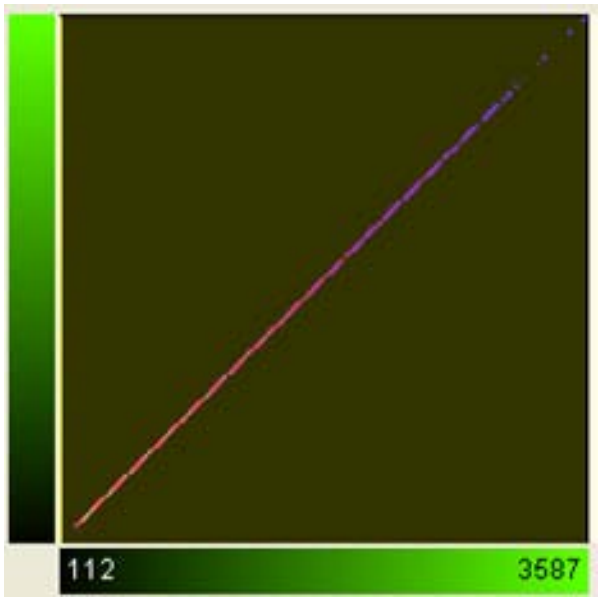
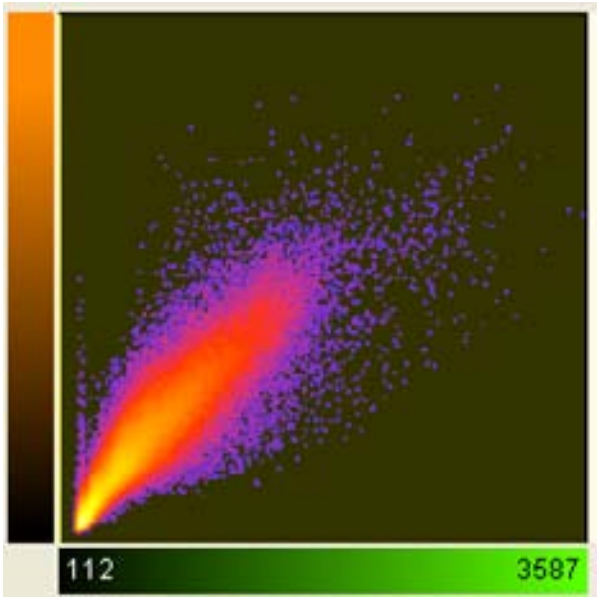
Colocalization scatter plot





Green vs green

Green vs red

Fully colocalized channels give a straight line

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Pearson coefficient (-1 to 1)

$$r_p = \frac{\sum_i (R_i - R_{aver}) \times (G_i - G_{aver})}{\sqrt{\sum_i (R_i - R_{aver})^2 \times \sum_i (G_i - G_{aver})^2}}$$

The correlation coefficient measures the strength of a linear relationship between two variables.

The correlation coefficient is always between -1 and +1. The closer the correlation is to +/-1, the closer to a perfect linear relationship.

- -1.0 to -0.7 strong negative association.
- -0.7 to -0.3 weak negative association.
- -0.3 to +0.3 little or no association.
- +0.3 to +0.7 weak positive association.
- +0.7 to +1.0 strong positive association.

Accounts for similarity of shape but does not consider intensity values

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Overlap coefficients (0 to 1)

$$r = \frac{\sum_i R_i \times G_i}{\sqrt{\sum_i (R_i)^2 \times \sum_i (G_i)^2}}$$

$$k_1 = \frac{\sum_i R_i \times G_i}{\sum_i R_i^2}$$

$$k_2 = \frac{\sum_i R_i \times G_i}{\sum_i G_i^2}$$

Describe differences in intensities between the channels

Relatively insensitive to difference in channel intensity values

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Colocalization coefficients (0 to 1)

$$M_1 = \frac{\sum_i R_{i,coloc}}{\sum_i R_i}$$

$$R_{i,coloc} = R_i \text{ if } G_i > 0 \text{ and } R_{i,coloc} = 0 \text{ if } G_i = 0$$

$$M_2 = \frac{\sum_i G_{i,coloc}}{\sum_i G_i}$$

$$G_{i,coloc} = G_i \text{ if } R_i > 0 \text{ and } G_{i,coloc} = 0 \text{ if } R_i = 0$$

Describe contribution from every channel in the colocalized area

Works also for big difference in channel intensity values

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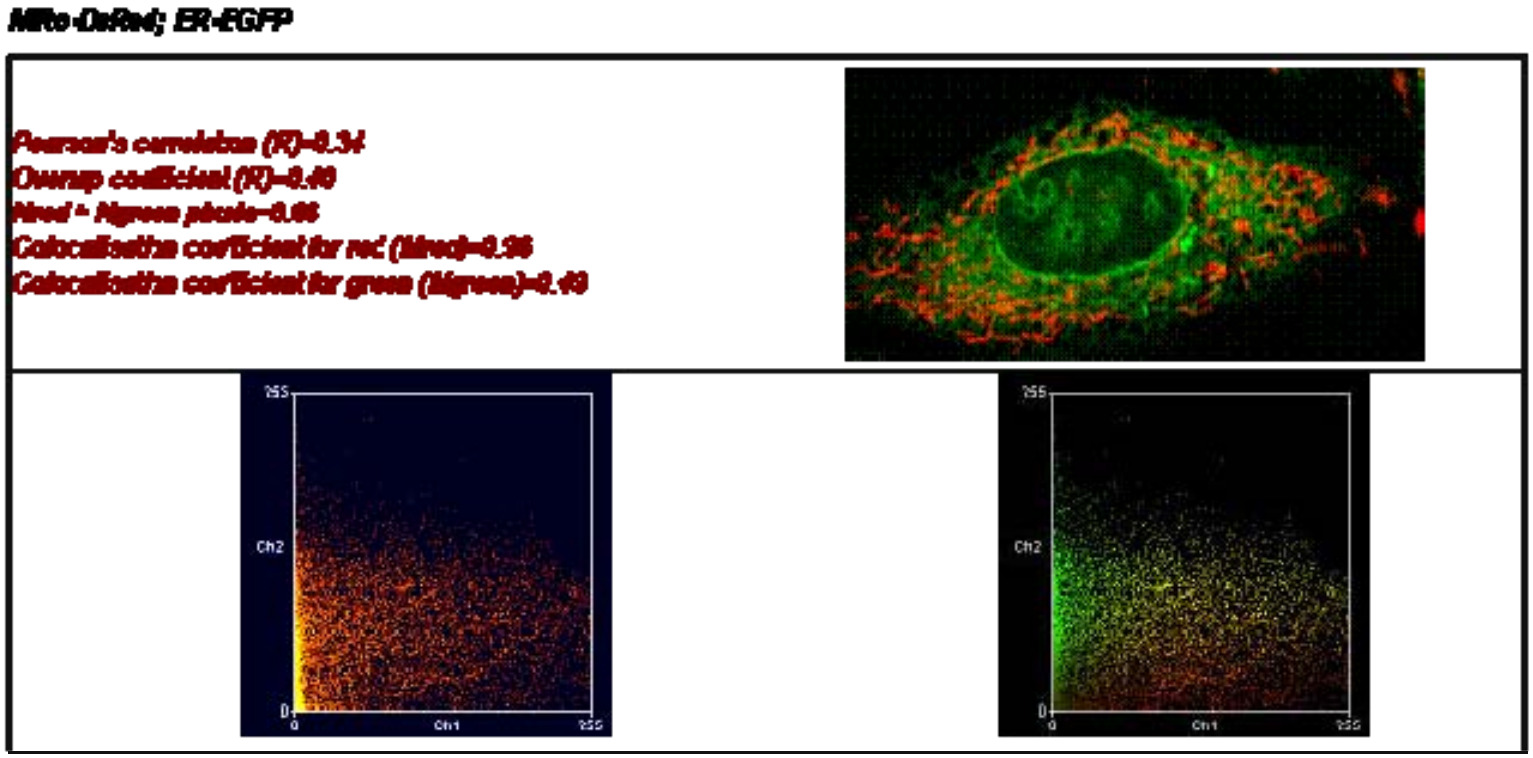
Low degree of colocalization



Scatter graph has no specific form

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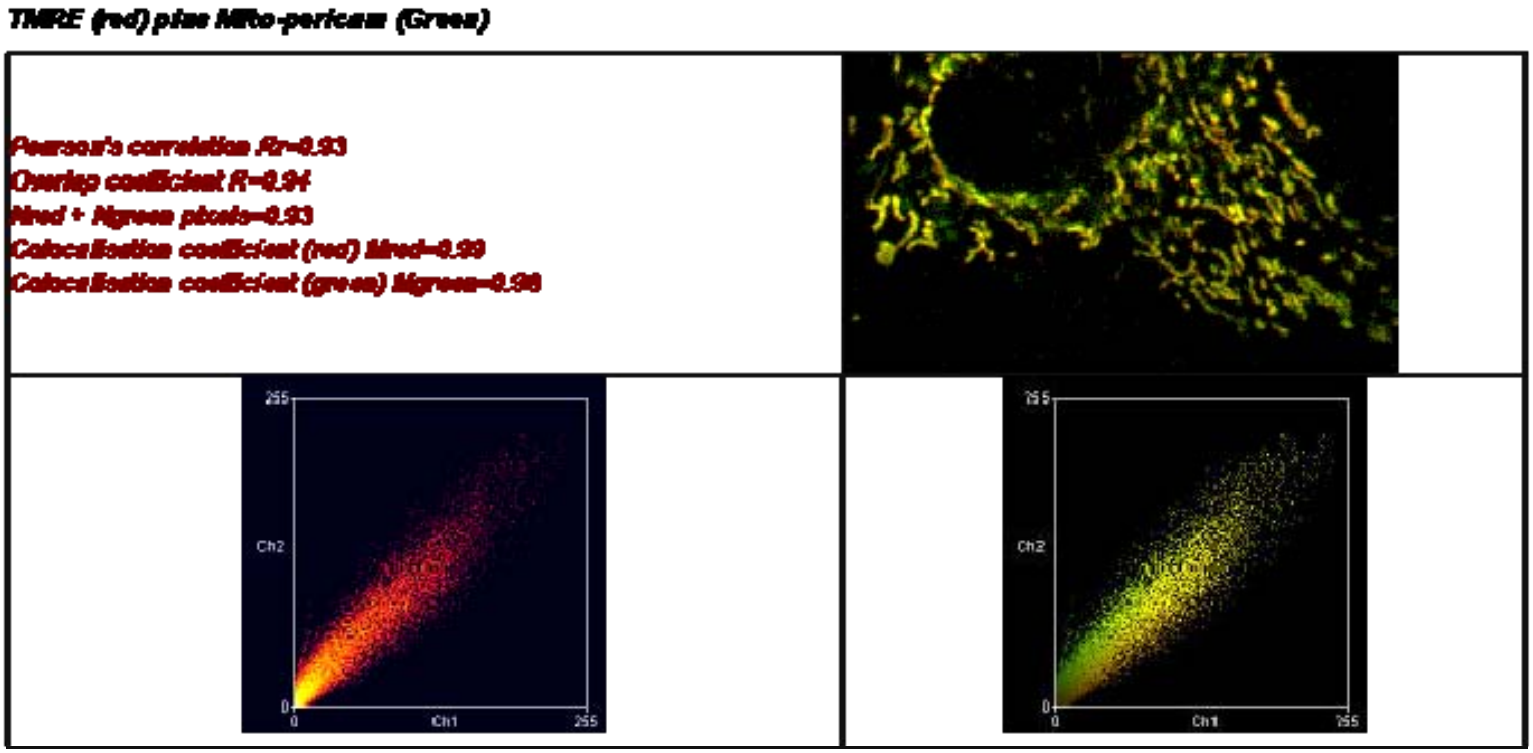
High degree of colocalization



Scatter graph is close to a straight line

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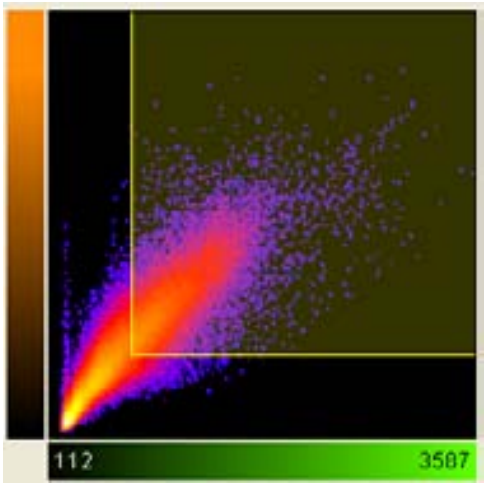
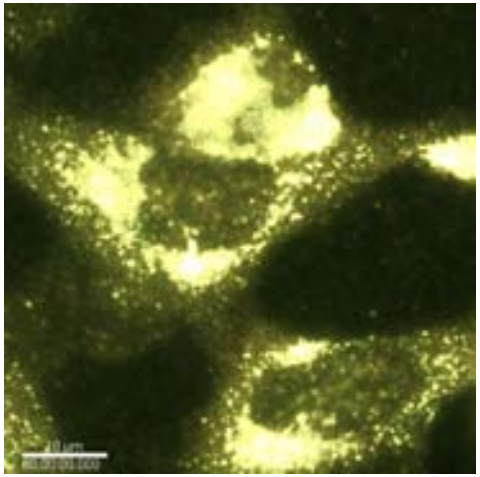
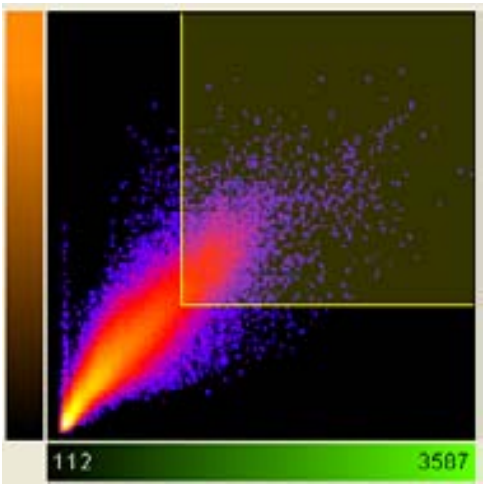
Threshold adjustment

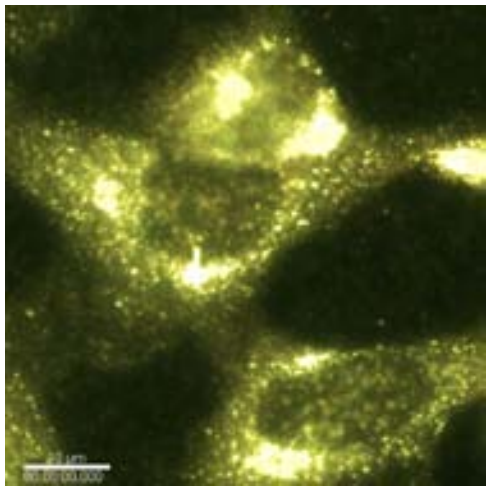


Threshold
800





Threshold
1200



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Results for different thresholds

Parameter	threshold 800	1200
number of colocalized voxels	38743	9964
% of dataset colocalized	14.78	3.80
% of ROI colocalized	14.78	3.80
% of volume A above threshold colocalized	93.68	85.69
% of volume B above threshold colocalized	65.53	55.85
% of material A above threshold colocalized	94.73	86.75
% of material B above threshold colocalized	71.38	59.42
% of ROI material A colocalized	31.81	10.81
% of ROI material B colocalized	31.34	10.35
channel correlation in dataset volume	0.9347	0.9347
channel correlation in ROI volume	0.9347	0.9347
channel correlation in colocalized volume	0.7336	0.5699

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Colocalization: summary

Channel merging is quick but very subjective method of colocalization

Colocalization scatter plot is a good starting point for quantitative analysis

Colocalization coefficients are the quantitative measure of colocalization

Use threshold adjustment for adapting to a signal level in your data set

Deconvolution of data set before the analysis can improve the reliability of the result

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4. Deconvolution

- 2D deconvolution methods
- 3D deconvolution methods
- Deconvolution for widefield
- Deconvolution for confocal

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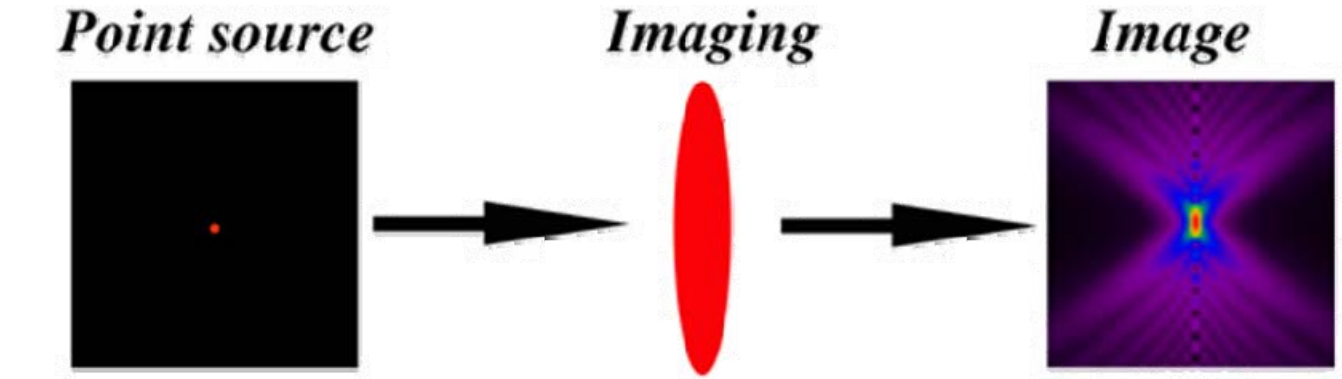
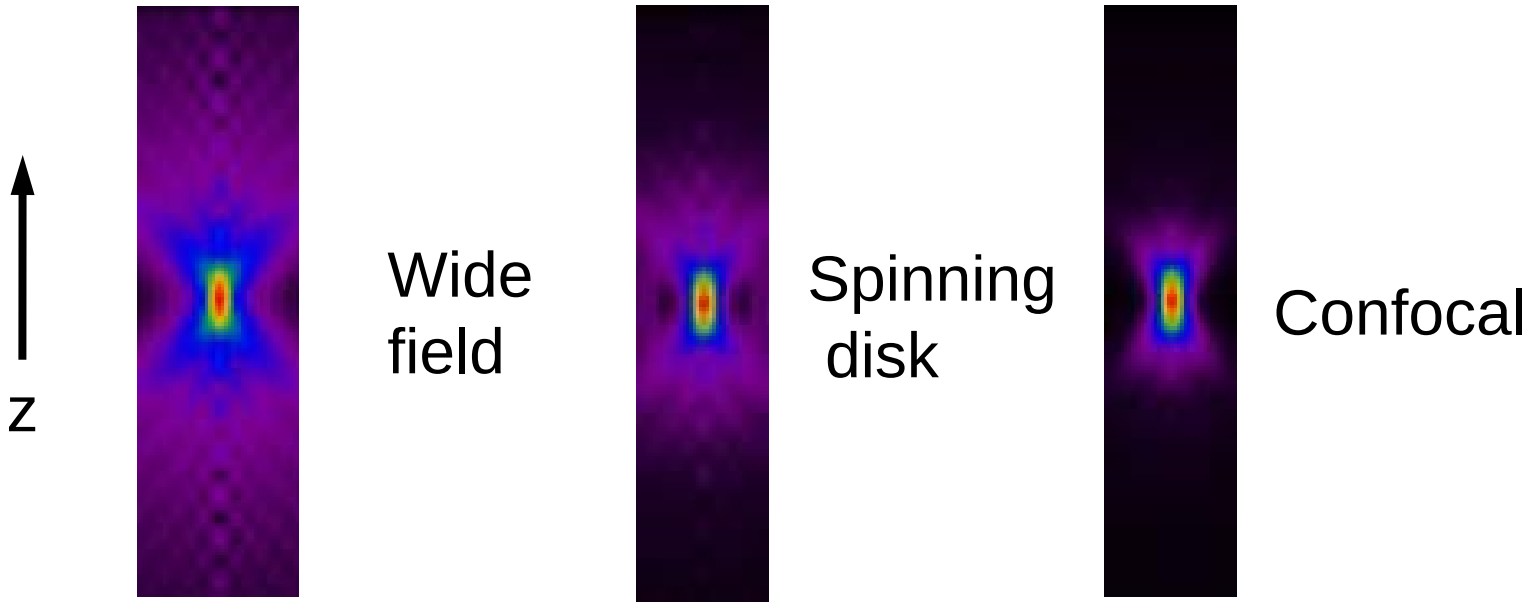
Point spread function (PSF)





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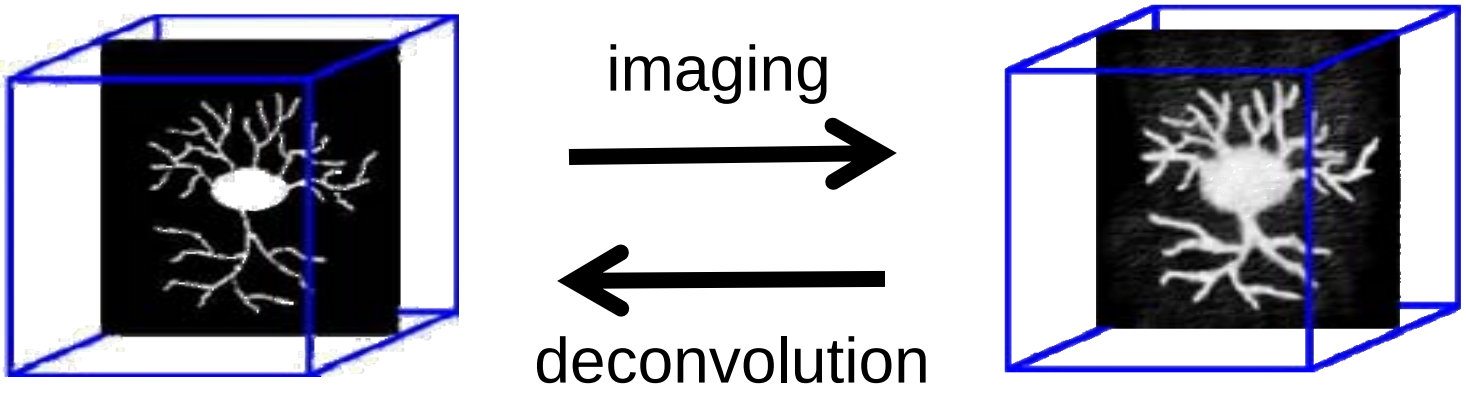
Deconvolution



object image

The imaged object is deconvolved with measured, calculated, or estimated microscope PSF by mathematical means.

The result is the image of the object of better quality.

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Deconvolution methods

- 2D methods (debluring)
  - ➢ Use PSF to estimate blur, which subtracted from image
    - No neighbor
    - Nearest neighbor
    - Inverse (Wiener) filtering

- 3D methods (restoration)
  - ➢ Use imaging equation to estimate object
    - Constrained, iterative deconvolution
    - Blind deconvolution
    - Exhaustive photon reassignment
    - Many others…

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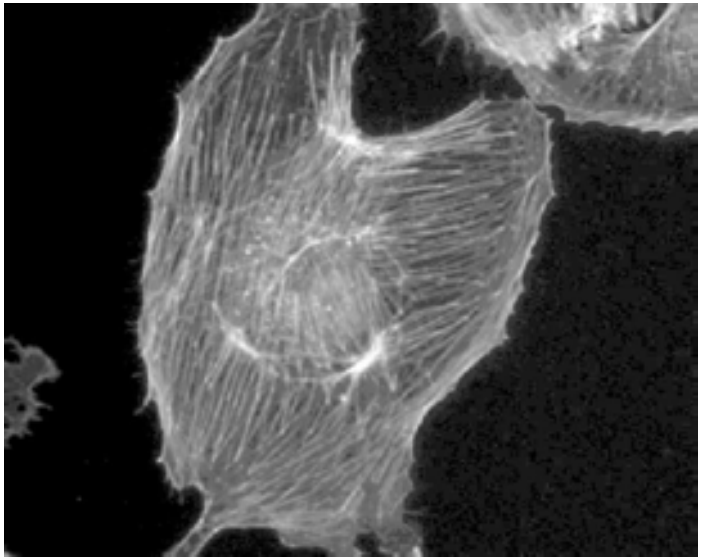
Inverse (Winer) filter

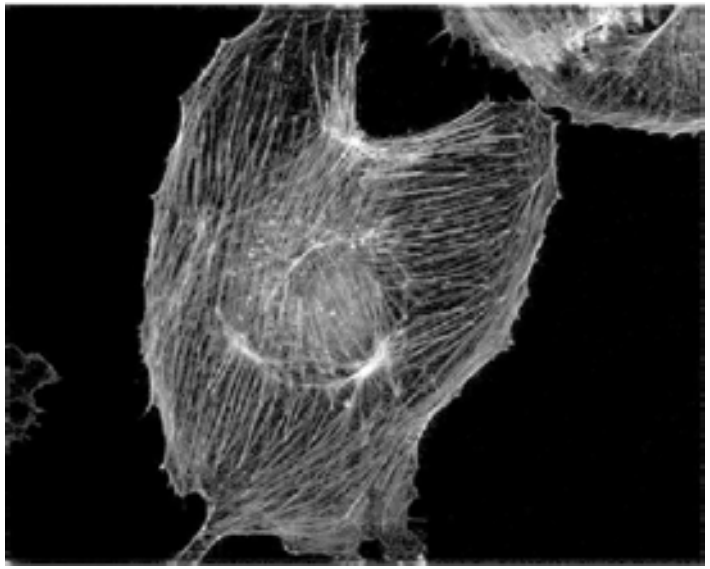
Divide the convolved image by OTF

$G = S_n / (S_n^2 + \alpha)$ Winer filter

$I = OS$

$O = IS / (S^2 + \alpha) = IG$





- Limited by noise amplification
- Possible ringing (-)
- Fast (+)

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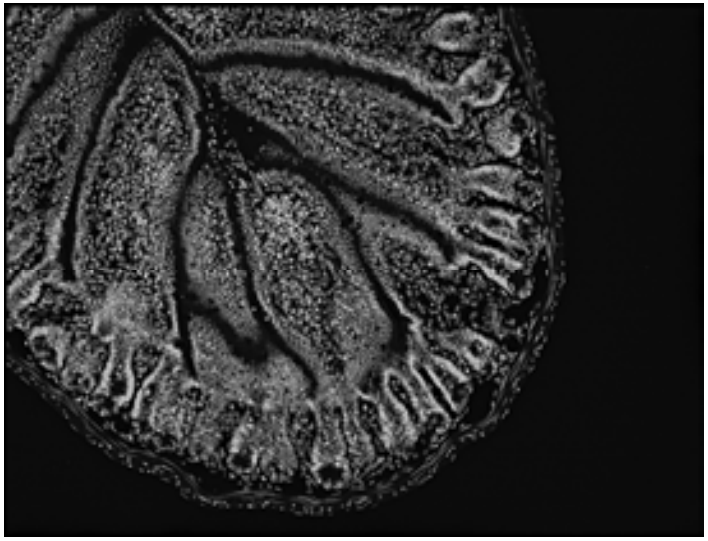
No neighbor

$$I_n = O_n S_n + O_{n+1} S_{n+1} + O_{n-1} S_{n-1}$$

$$I_n = O_n S_n + c(I_{n+1} + I_{n-1}) S_{n-1}$$

$$O_n = (I_n - 2cI_n S_{n-1}) G$$



- Assumptions:
  - Measurements of adjacent planes is not necessary
  - Contribution from adjacent planes is approximated by blurred object $(O_{n+1} = O_{n-1} = I_{n+1} = I_{n-1} = I_n)$
  - OTF is equal in adjacent planes $(S_{n-1} = S_{n+1})$

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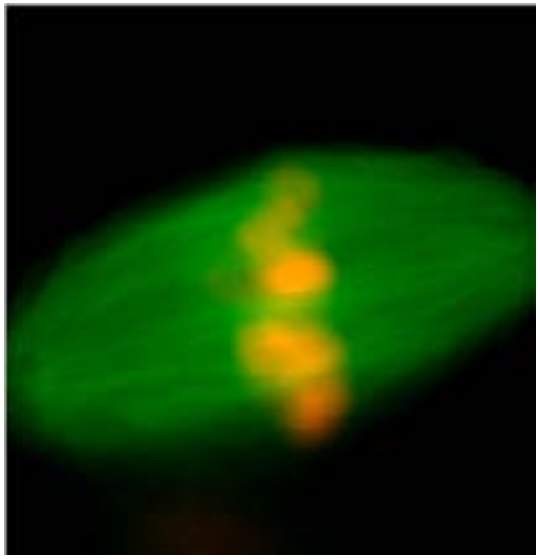
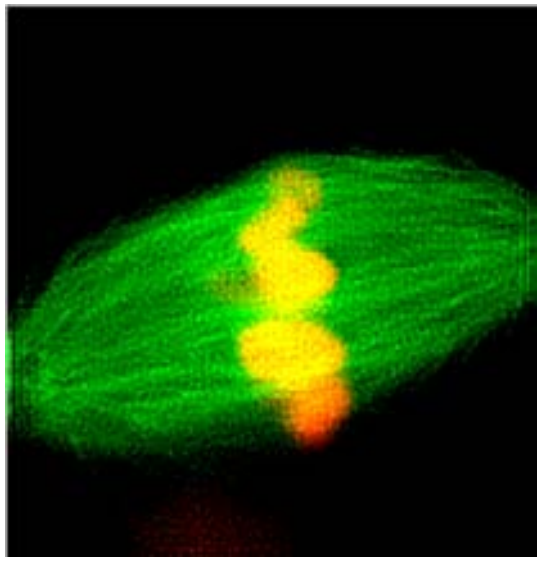
Nearest neighbor

$$I_n = O_n S_n + O_{n+1} S_{n+1} + O_{n-1} S_{n-1}$$

$$I_n = O_n S_n + c(I_{n+1} + I_{n-1}) S_{n-1}$$

$$O_n = (I_n - c(I_{n+1} + I_{n-1}) S_{n-1}) G$$





- Assumptions:
  - Intermediate plane is only blurred by the two adjacent planes
  - Object in the adjacent planes is approximate by the image $(O_{n+1} = I_{n+1}, O_{n-1} = I_{n-1})$
  - OTF is equal in adjacent planes $(S_{n-1} = S_{n+1})$

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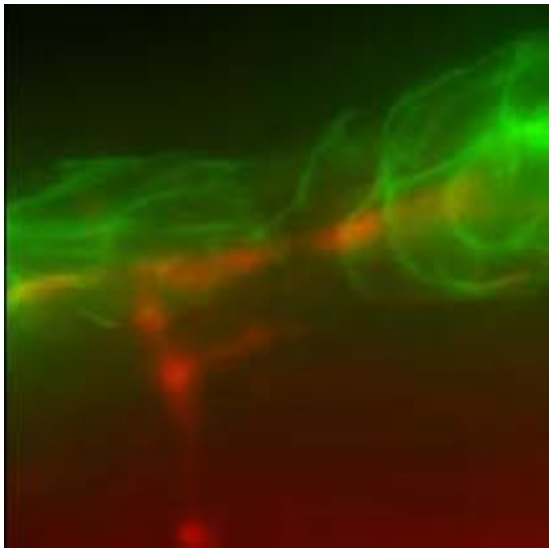
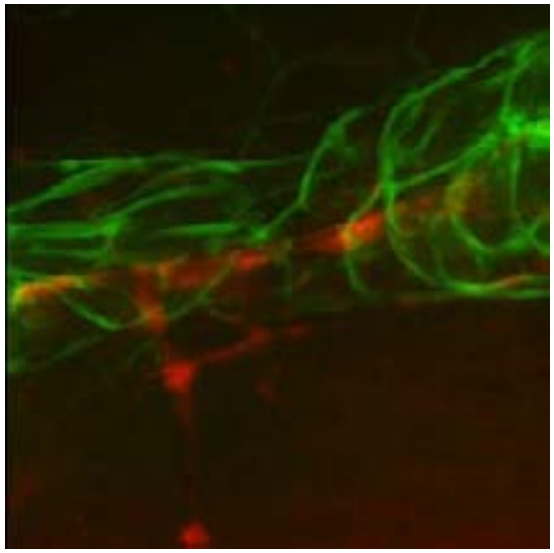
Restoration for wide-field



Wide-field image



Iterative deconvolution

Zebrafish primordium

Delta Vision RT microscope

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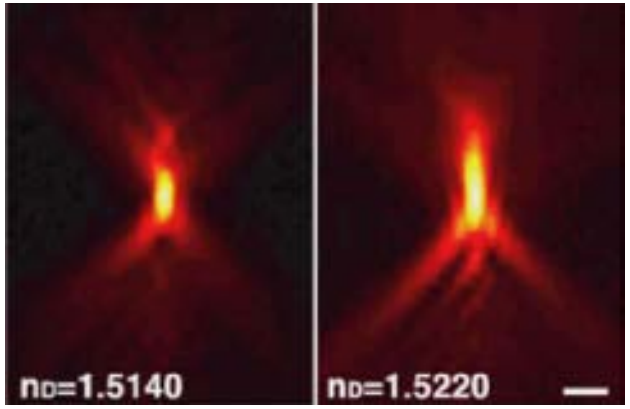
PSF: measured vs. calculated

- Measured
  - Uses sub resolution fluorescence beads (at least 100 nm)
  - Contains all information about aberrations in the system
  - Can take some time to acquire
- Calculated
  - Based on objective NA, wavelength, refraction index, etc.
  - Does not have information about aberrations in the system
  - Very fast



x-z projection of PSF for 100x/NA 1.4 objective measured with 100 nm bead mounted in glycerol (n=1.47) with immersion oil n=1.5140 (left) and n=1.5220 (right).
Mismatch of immersion oil refractive index results in strong spherical aberration.

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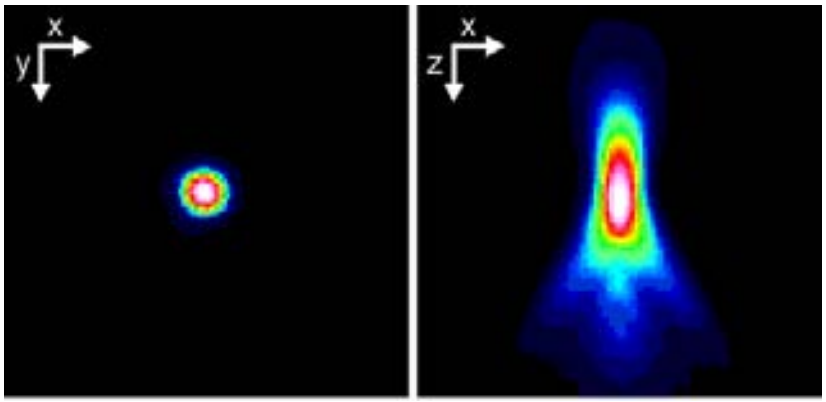
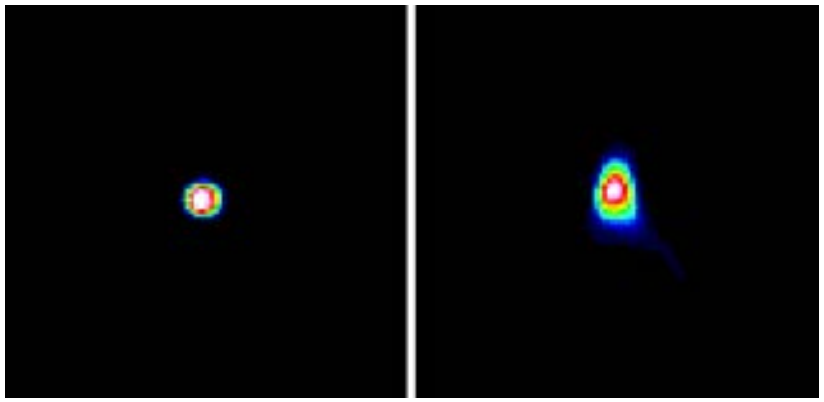
Restoration increases resolution

Maximum liklihood restoration for bead using measured PSF



original image



restored image

- Restoration can significantly increase resolution
- Resolution increase is more pronounced in z-direction
- Resolution increase depends on quality of restoring algorithm
- Sufficient oversampling in x, y and z directions should be assured

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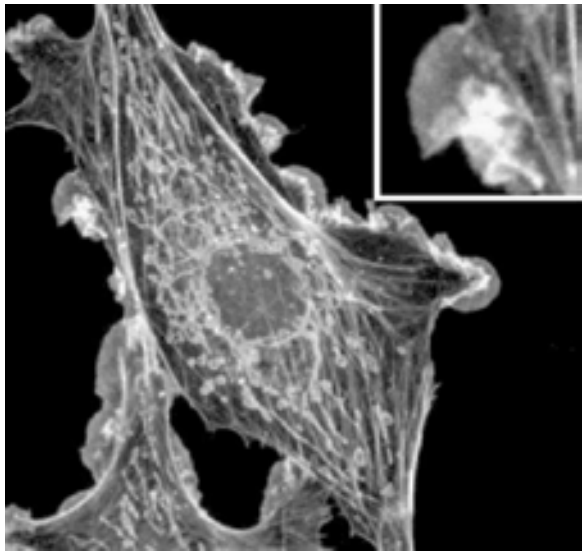
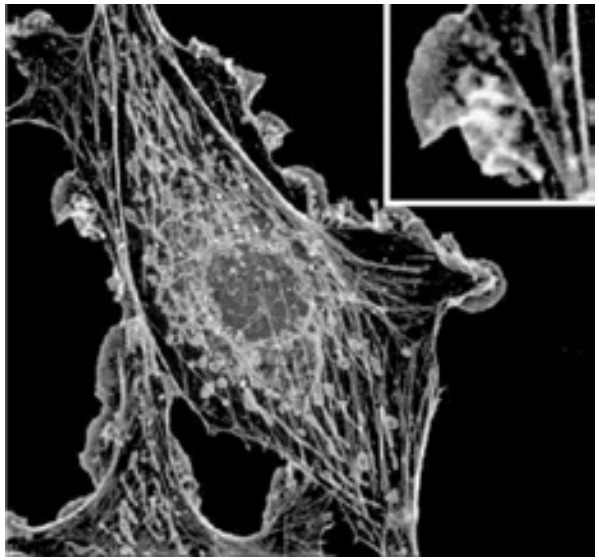
Restoration for confocal



LCSM



restored

Bovine endothelial cell
Deconvolution: maximum likelihood, 15 iterations

- Restoration improves LCSM image quality (+)
- No redistribution of out of focus light (-)
- Resolution mostly enhanced in axial direction (+/-)

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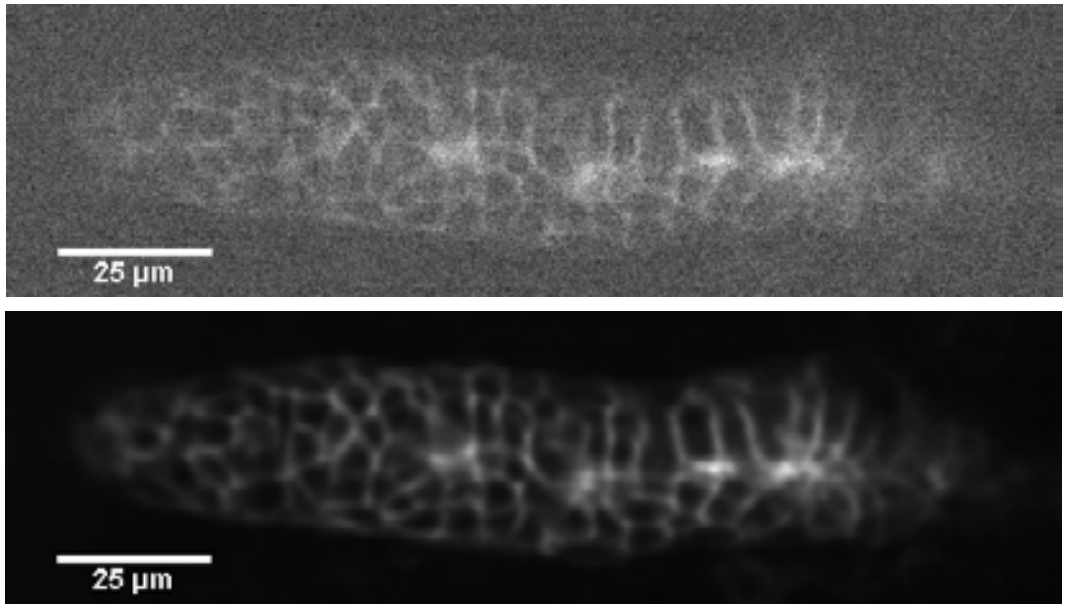
Restoration for spinning disk



Raw data

Restored

Zebrafish primordium
Deconvolution: maximum likelihood, 20 iterations

- Restoration improves image quality (+)
- Redistribution of out of focus light possible (+)
- Resolution mostly enhanced in axial direction (+/-)
- Practical for live cell/organism imaging (++)

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Deblurring vs. restoration

Deblurring

- very fast, runs in real time
- OK for large section spacing
- subtractive method thus loss of intensity
- a two-dimensional method
- can not be used for quantitative analysis
- does not increase resolution

Restoration

- requires 5 to 20 iterations
- correct section spacing necessary
- stable - works with poor SNR
- conservative - no intensity lost or gained
- relatively fast (100 Mb in 3 min)
- can be used for quantitative analysis
- can increase resolution

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Software for deconvolution

Specialized deconvolution packages

- Huygens (Huygens remote manager (HRM))
  - www.svi.nl
- DeltaVision (SoftVoRx)
  - www.appliedprecision.com
- AutoQuant
  - www.aqi.com
- Volocity Restoration
  - www.improvision.com

As a part of image processing software

- MetaMorph
  - www.moleculardevices.com
- Software from microscope manufactures
  - Leica, Nikon, Olympus, Zeiss

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Deconvolution: summary

Optimise imaging condition (illumination, objective, camera, filters, etc.) to get as good original image as possible

For quantification use only data produced by 3D deconvolution methods

Test your data set with several deconvolution algorithms

Do not abuse deconvolution, always compare deconvolved and raw images

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Literature

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