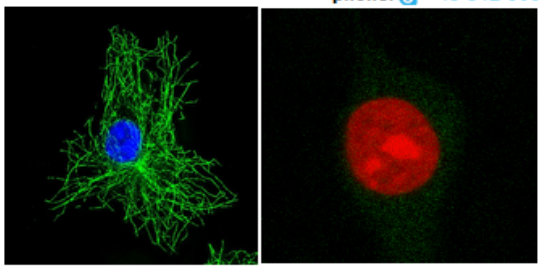
Digital Imaging I: 2nd Part

Martin Offterdinger

Biooptics at MUI

Biooptics/light microscopy

CCB
Division of Neurobiochemistry
Innrain 80-82, 1st floor, room M01.370
A-6020 Innsbruck
phone: +43-512-9003-70287



The Biooptics/microscopy facility of MUI, located at the new CCB (room 01.370), aims at providing university wide access to advanced equipment, training, education and expertise in light microscopy **to scientists at MUI.** We currently offer assisted access to research microscopes and image processing software. Moreover a number of courses is offered within the different PhD training programmes at MUI. The facility is run by the facility manager **Priv.-Doz. Dr. Martin Offterdinger** and supervised by an advisory board. NEW: **gatedSTED microscope is OFFICIALLY offered, introductions can be requested from now on. New prices and rules**! STORM and gSTED secondary antibodies available. Biooptics/flow cytometry can be found here.

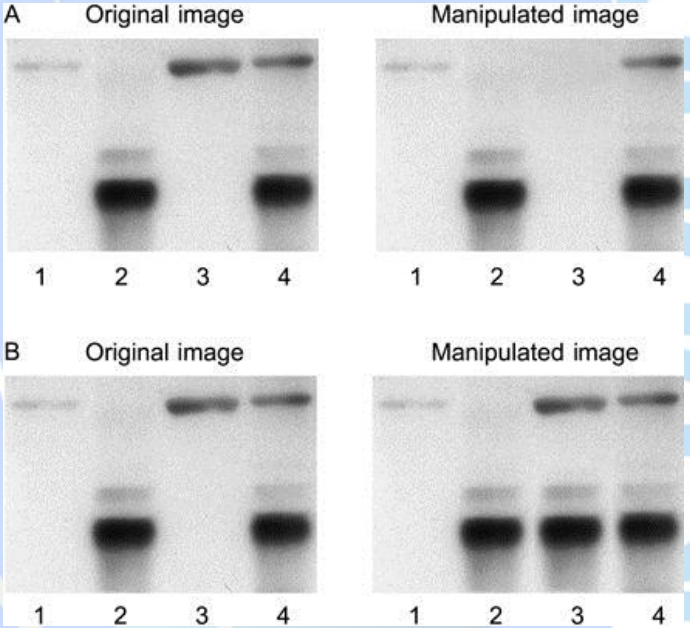
- Microscopes and fees
- Accessing the rooms @CCB
- Software
- Rules
- External users (NEW!!!)
- Booking system **(**Manual**)**
- Data Transfer Policy **(NEW!!!)**
- Protocols
- Superresolution antibodies (gSTED and STORM)
- Mailing Lists: **Do you wish to be informed about down-times, problems etc of your favorite instrument? Make sure to actively register to the relevant mailing lists.**

 Mail an i-master - Letzte Änderung: 29.4.2014, 12:03:01 xims

http://www.i-med.ac.at/neurobiochemistry/neurobiochemistry/Biooptics/Main.html

What MAY we (not) do with digital images?

The Journal of Cell Biology
"No specific feature within an image may be enhanced, obscured, moved, removed, or introduced. The grouping of images from different parts of the same gel, or from different gels, fields, or exposures must be made explicit by the arrangement of the figure (e.g., using dividing lines) and in the text of the figure legend. Adjustments of brightness, contrast, or color balance are acceptable if they are applied to the whole image and as long as they do not obscure or eliminate any information present in the original. Nonlinear adjustments (e.g., changes to gamma settings) must be disclosed in the figure legend."



☺extract relevant scientific information
☺improve visibility in a printed figure
☺fully documented
☺extensive modifications, if justified
☹make data „better“ than they are
☹remove parts, which can‘t be explained
☹undocumented, poorely defined steps

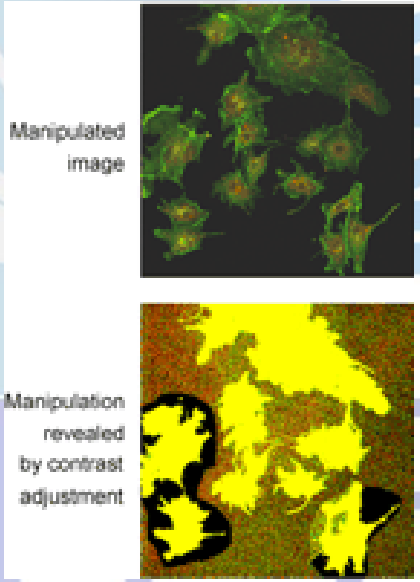
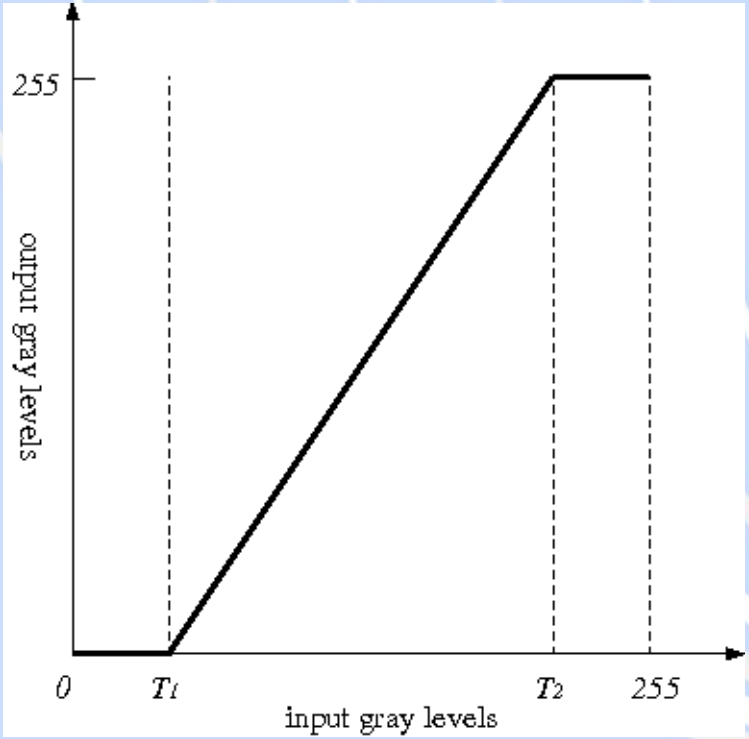
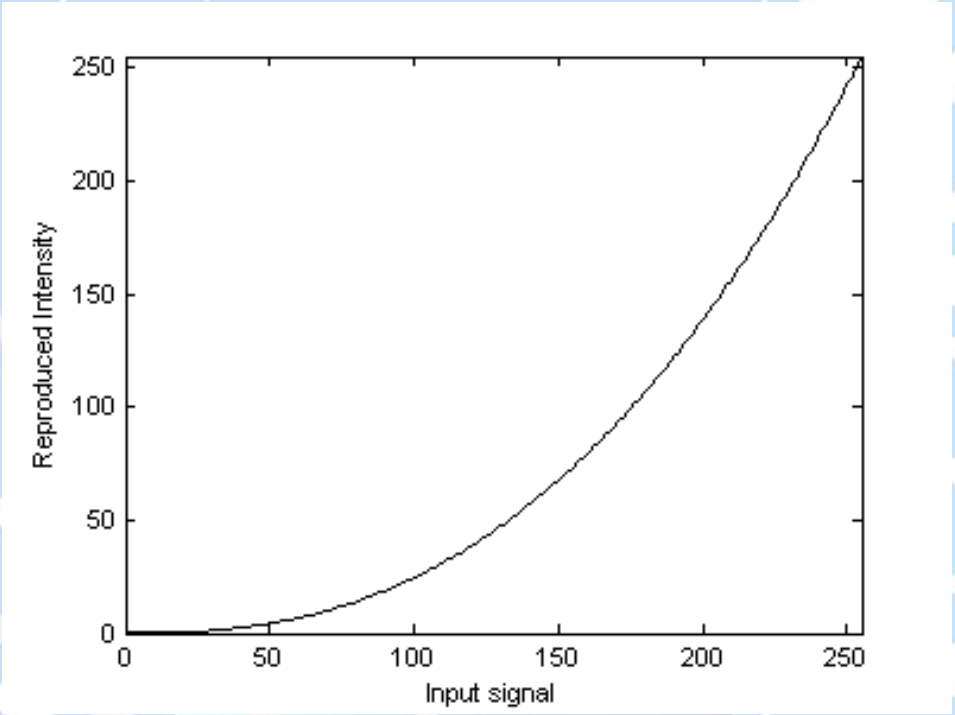


Image scaling, Contrast, Brightness I

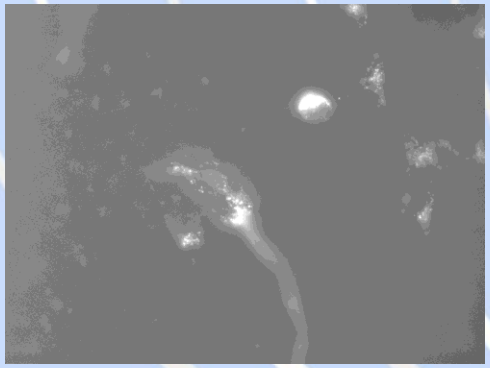




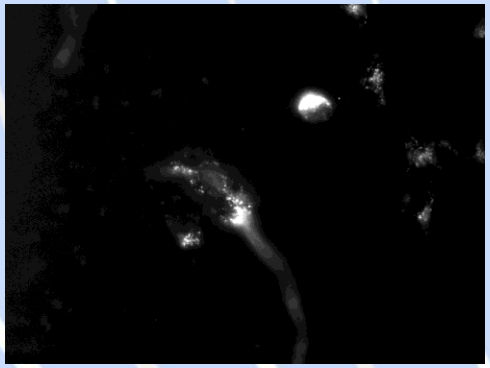
- Linear scaling: input-output gray levels
- Non-linear: „gamma correction“

Image scaling, Contrast, Brightness: DOS and DONTS

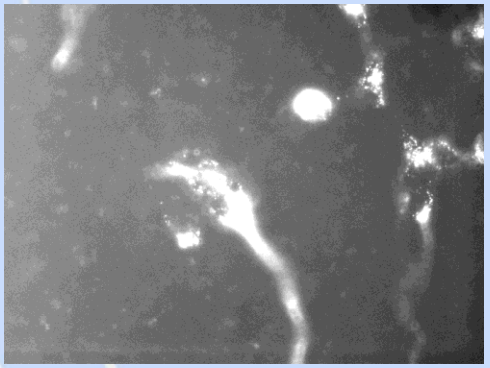
0 to 255

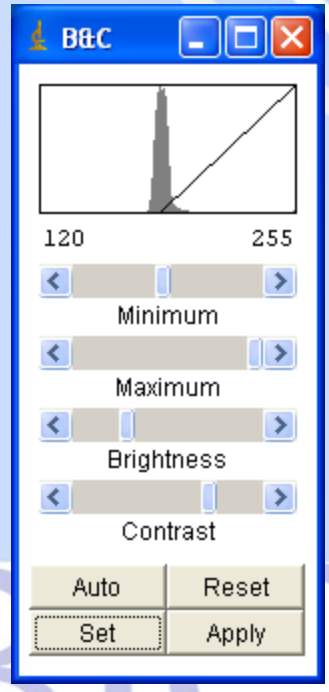


120 to 255



100 to 150

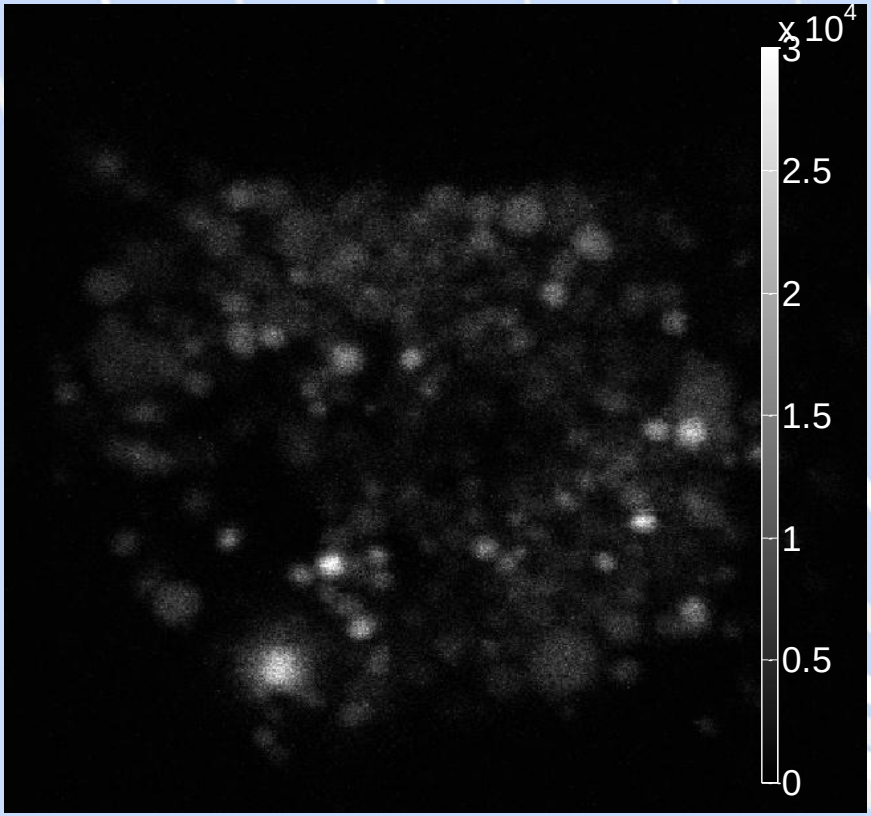


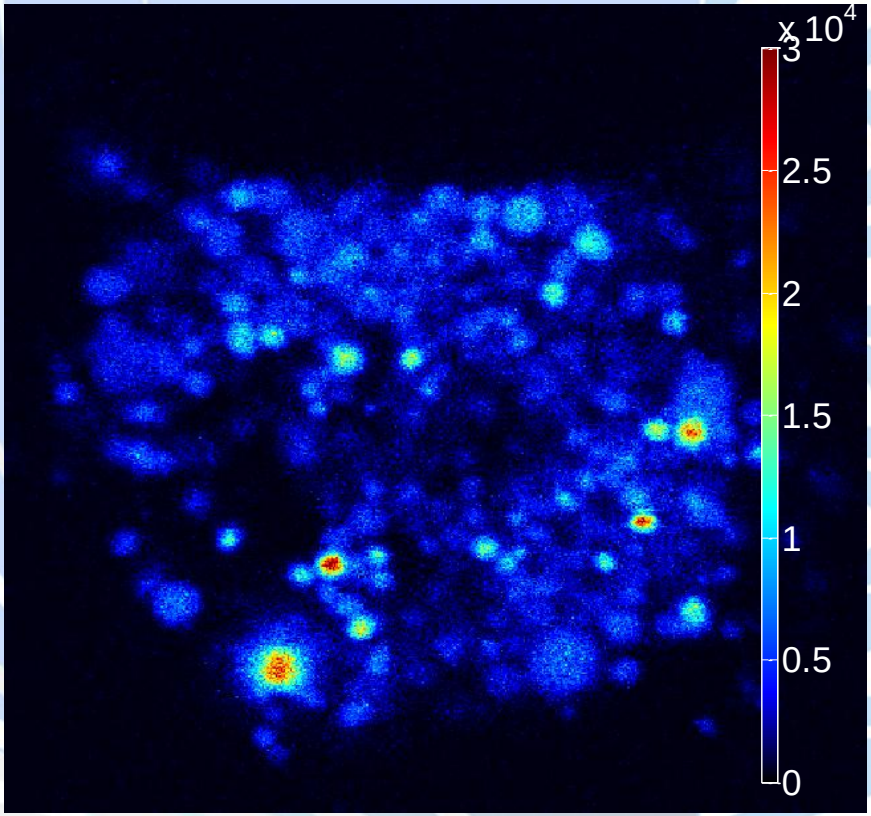


☺scientific software
☺documented changes: protocol!
☺you know what you‘ve done

☹‚photoshopping‘
☹press ‚autoadjust everything‘
☹no idea what happened
☹„..the software did something…“

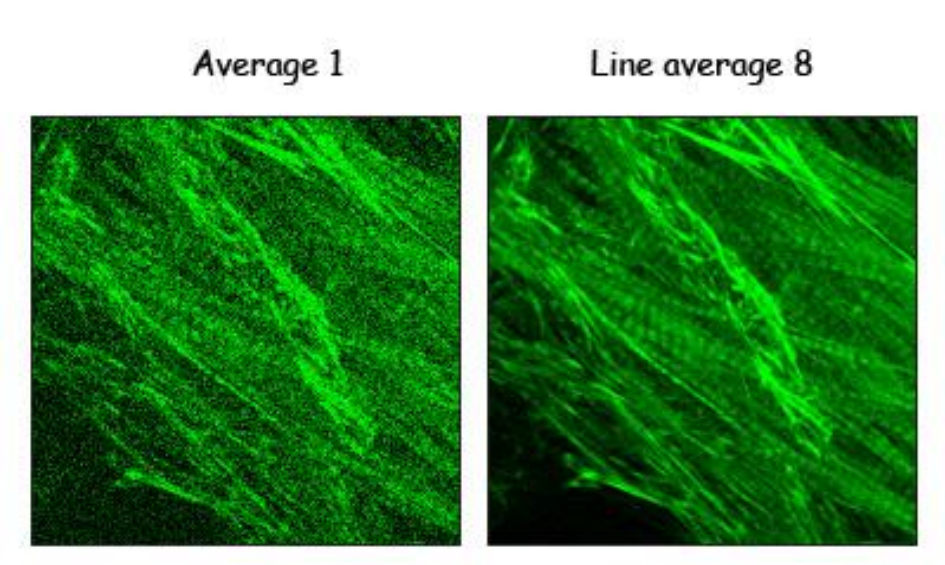
Visualisation: Pseudocolor





pixel intensities = different colors
colormap required
small intensity differences
LUT: „look-up table“: needs to be provided!

Averaging: confocal microscopy



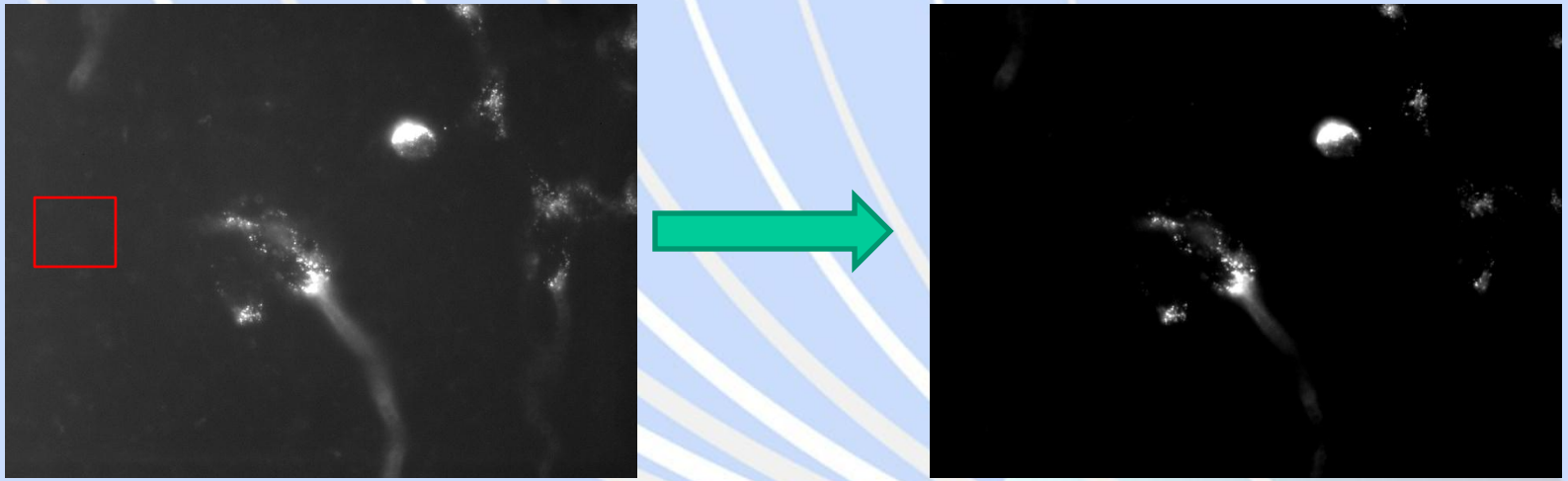
averaging reduces the image noise due to detector noise
line averaging 8: each line scanned 8 times and averaged
localized signals persist, randomly distributed signals (i.e. noise) vanish
„granularity“ of images = due to noise > reduced

- Background, mask, advanced thresholding
- Colocalization
- Deconvolution
- Time series
- 3D images
- Complex thresholding
- Full MATLAB analysis
- Linear unmixing

Background subtraction: fluorescence

ROI: region of interest: drawn in the image

Mean of ROI: subtracted from image

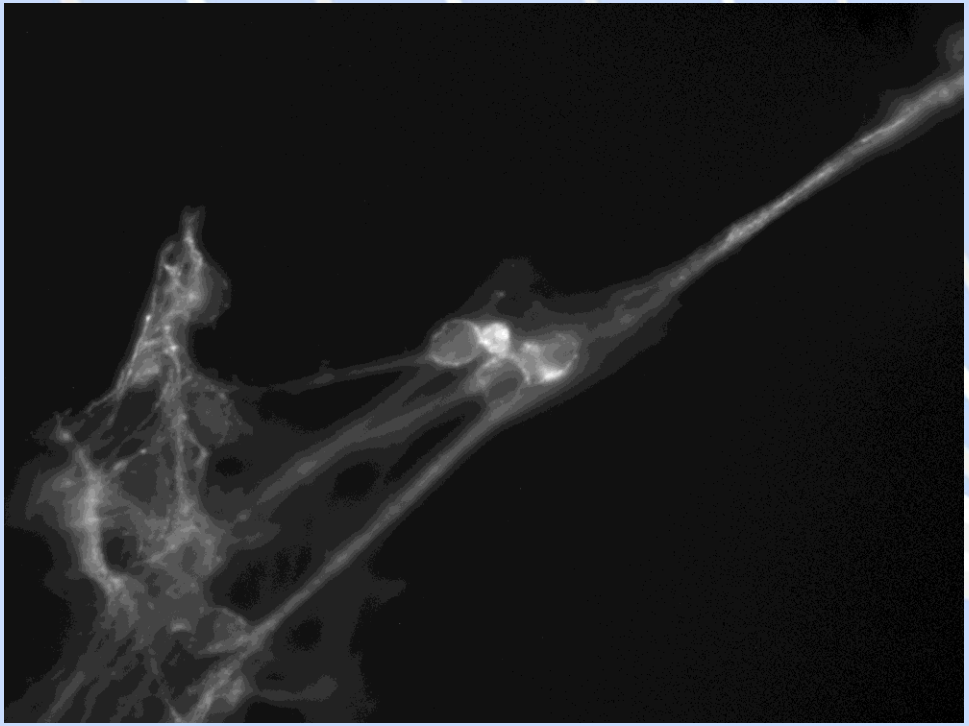


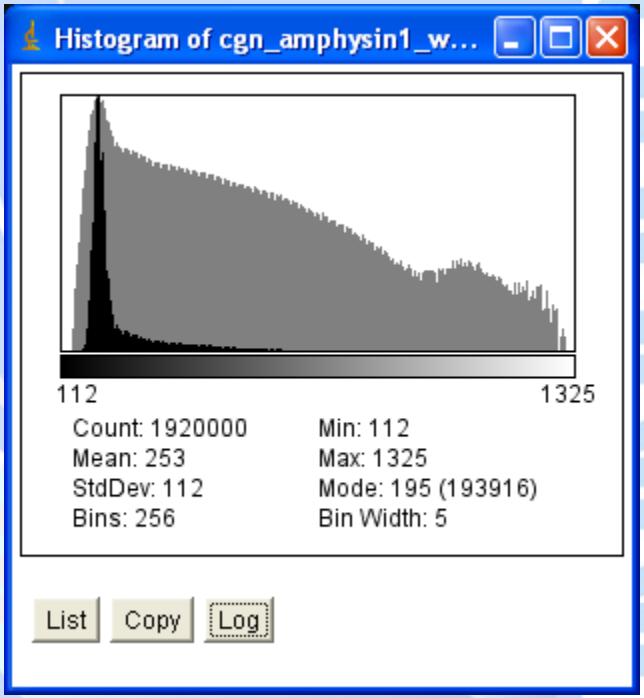
BG subtraction: fluorescence signal consisting of true signal plus background
True signal recovered by BG subtraction

1) Total Signal 200, BG=20 -> Net Signal 180
2) Total Signal 40, BG=20-> Net Signal 20
3) BG Correction: **?5 times higher ->9 times higher signal in 1)**

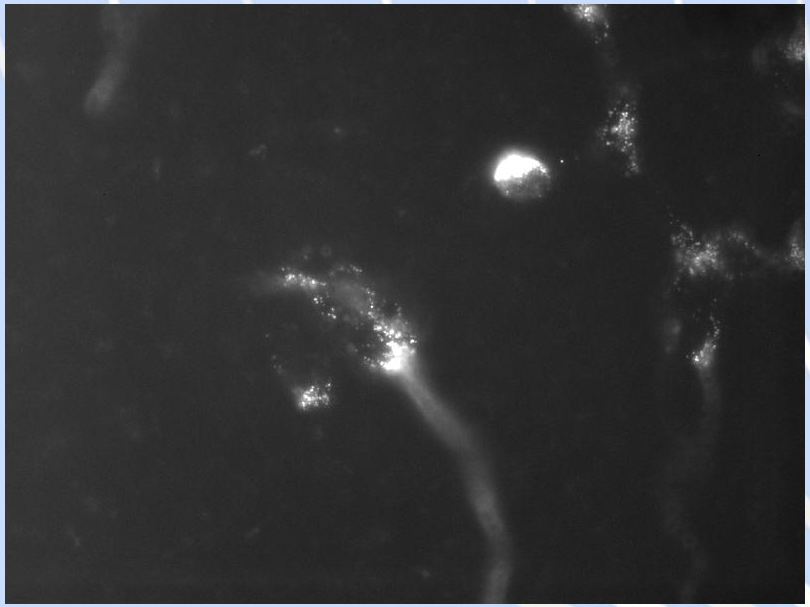
Image histograms (1D)

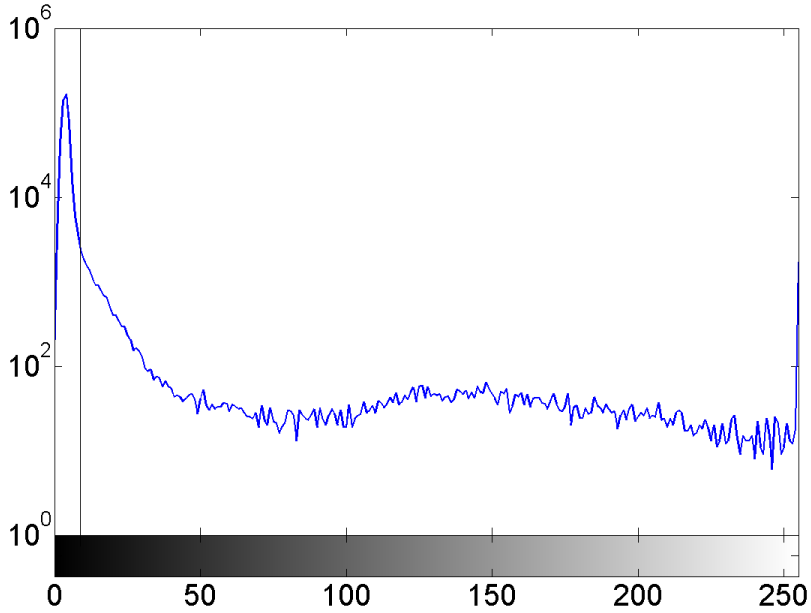
- overview: pixel intensities
- dynamic range





Background subtraction: automatic bg identification



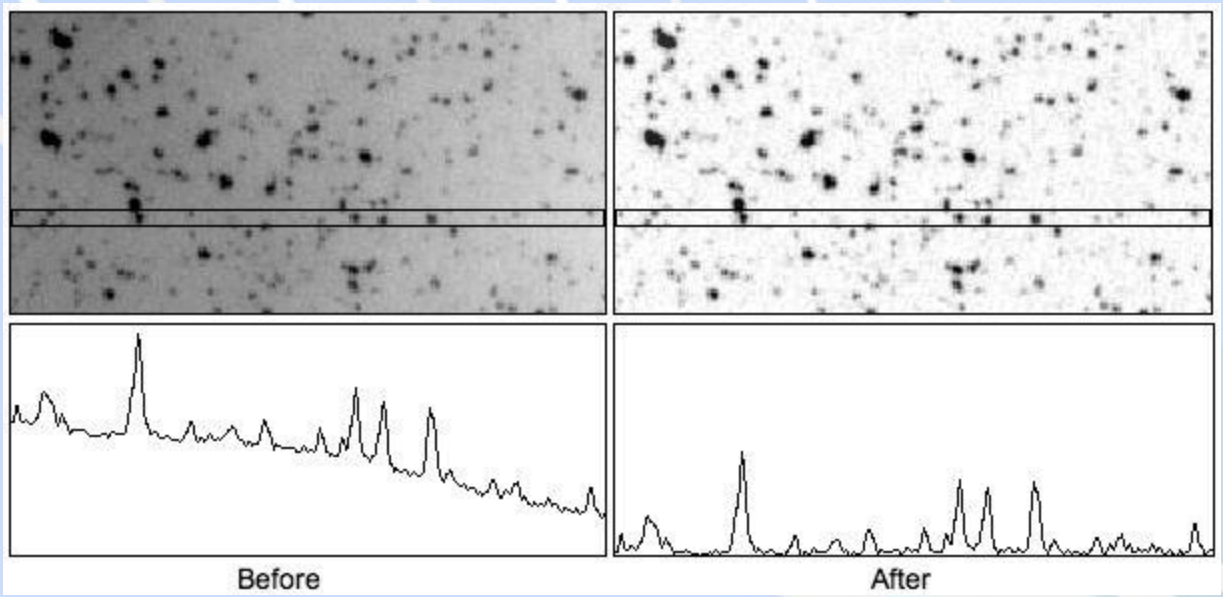


Several automatic methods to identify background

- find peak in histogram
- Otsu‘s method

Dynamic background subtraction: rolling ball algorithm

Ball rolls over the background



Masking, Thresholding, Segmentation

- „Mask“: 1-bit image (1, 0); 1:structures of interest 0: background & irrelevant information
- Multiplication image*mask: remove anything except structures of interest
- Digital Masking: first difficult step in image analysis.
- Getting a good mask is often a challenging multi-step process

Image filters: enhance/remove certain features

a <419x512 uint16>

	1	2	3	4	5	6	7	8	9
1	452	612	579	1154	665	433	1112	1258	1485
2	615	387	417	583	947	785	1331	1687	1884
3	740	439	749	979	3497	2594	1045	1727	1554
4	621	1472	2189	1097	1621	1186	589	729	794
5	1429	701	831	982	443	265	399	772	1081
6	1590	849	1075	1346	394	423	1143	1456	1257
7	1019	690	816	973	400	266	931	1497	945
8	529	376	397	289	156	286	254	183	112
9	614	263	158	172	149	166	112	111	410
10	1244	792	524	440	612	836	463	263	200
11	724	968	1704	809	495	1198	2003	923	424
12	363	1091	3321	1056	225	651	1551	762	111
13	325	930	2863	849	180	506	947	365	93
14	263	274	337	593	478	539	813	324	75
15	800	228	182	541	575	254	254	201	84
16	933	363	328	704	473	215	185	170	182
17	87	303	955	890	496	572	273	272	315
18	3	47	363	391	922	946	345	641	1446
19	146	70	116	516	1596	653	445	633	677
20	303	42	241	969	579	327	422	347	270
21	69	75	776	1902	479	188	412	394	708
22	250	73	253	963	659	365	567	951	1205
23	230	71	32	318	825	491	308	575	1007
24	60	58	31	70	473	494	317	496	635
25	133	123	136	302	709	763	1147	1574	489
26	55	157	229	330	749	986	1369	988	482
27	21	243	311	156	287	577	673	501	504
28	12	765	1340	520	715	848	850	802	2313
29	68	235	243	848	1185	1574	1308	860	2095
30	68	8	22	281	512	598	721	143	209
31	3	0	5	436	841	599	261	179	186

Average (3x3)

1	1	1
1	1	1
1	1	1

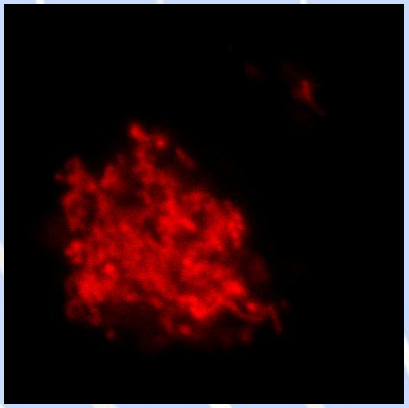
Edge (3x3)

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

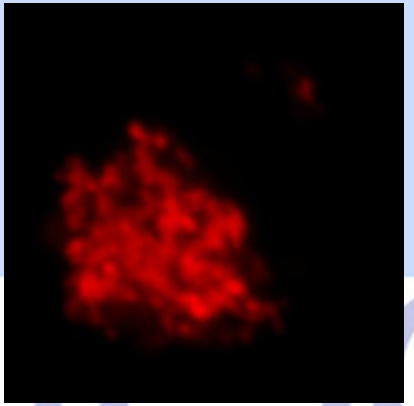
- Multiply: (1*740+1*439+…1*1472+…1*831)/9 > replace 1472 with the result: 860
- Many different image filters exist: median, gaussian, weighted average

Image filters: effects on the image

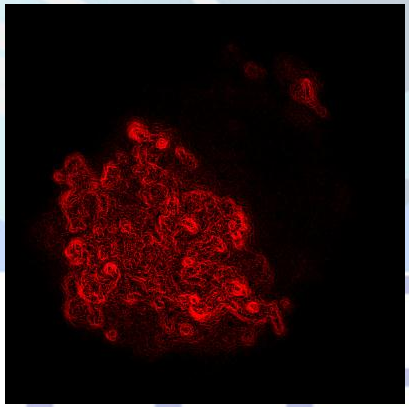
Original



Smooth



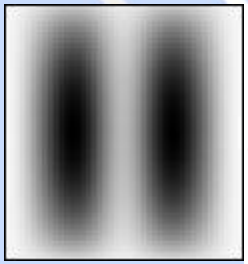
Edge

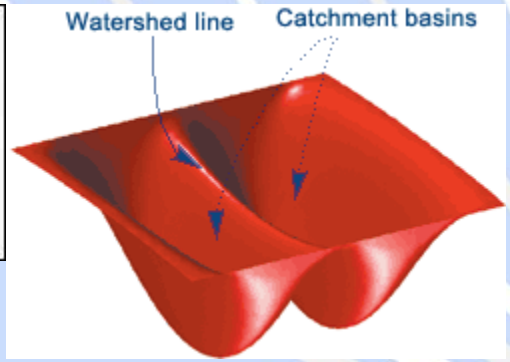


Watershed segmentation

Simple thresholding methods fail if

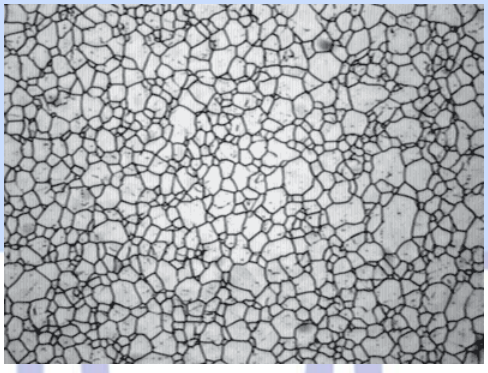
- Objects closely spaced or overlapping
- Idea: objects of interest are basins-watersheds, segmentation occurs along rigdes



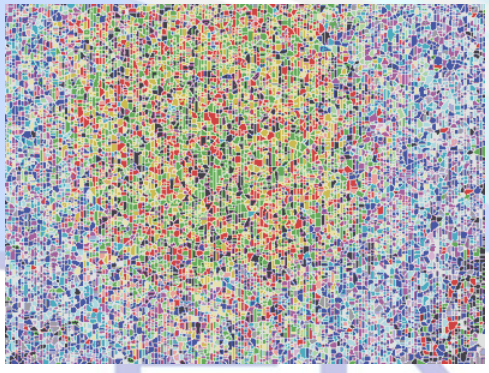


Watershed algorithm:
watershed lines to separate the basins
Requirement: one minimum per basin!

steel grain

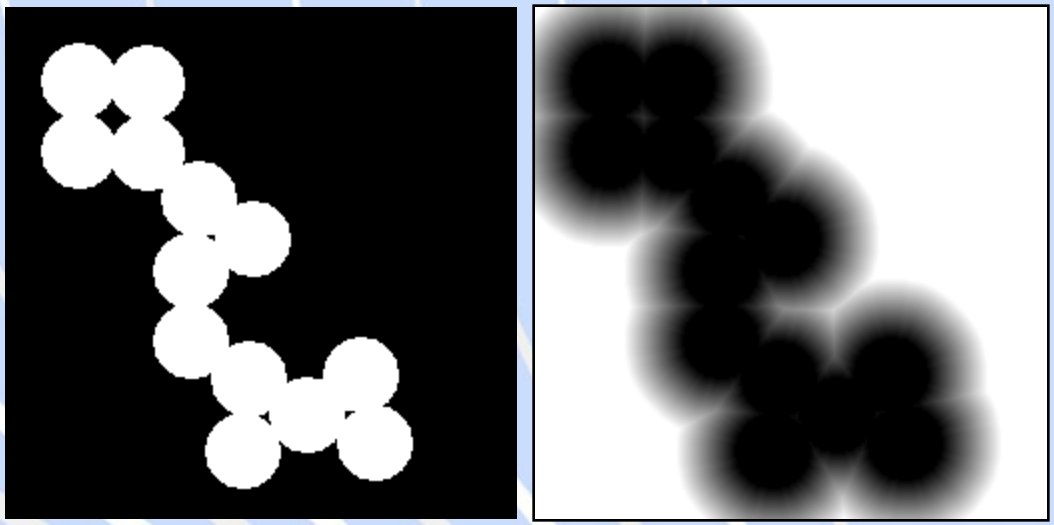


direct watershed



small minima removed

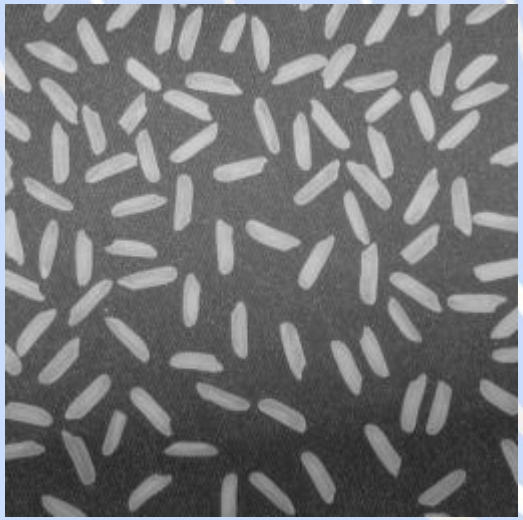
Distance transform

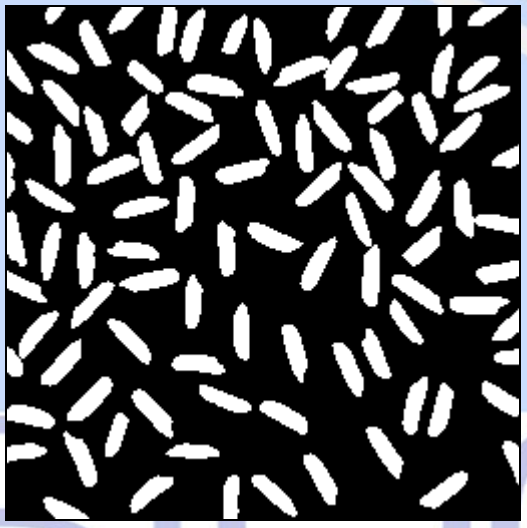


- For each pixel: find distance to nearest non-zero pixel
- Pre-processing for water-shed segmentation

Measurement of geometric properties - blob analysis

Distances, Areas, Perimeters, Diameters…

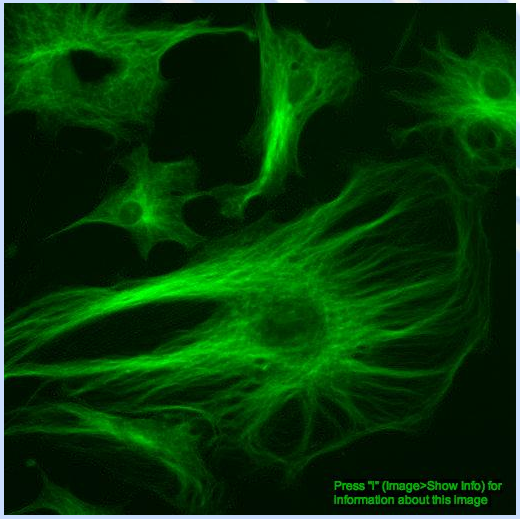


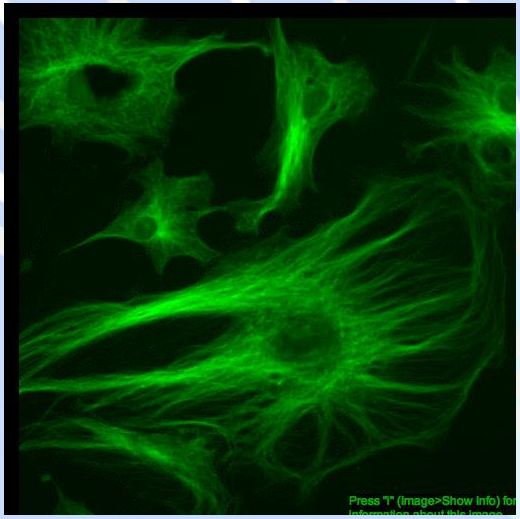


- binary mask
- sum = number of positive pixels= area
- diameter, etc: same principle
- perimeter: number of pixels at the ‚border‘
- magnification: pixelsize in micron…
- count: number of connected pixel groups > labelling (1,2,3,…)

Image registration

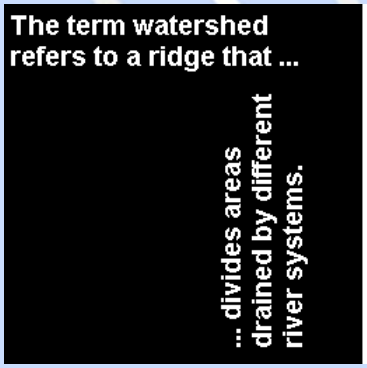
- Image registration: aligning of shifted/rotated/distorted otherwise identical images
- Recover shift and/or rotation angle
- Marker-based or (semi) automated
- Science: ‚rigid-body' model: translated and rotated, not distorted, different size
- Method: Cross-correlation often in frequency domain (fourier transformed images)

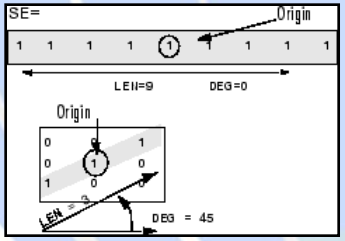


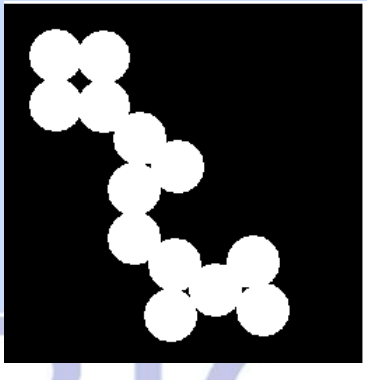


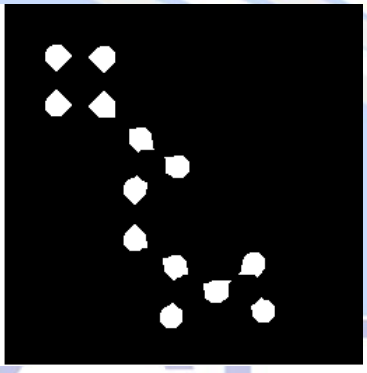
Morphological processing – dilation/erosion

- dilation and erosion: use a structuring element (vertical line, compare filter)
- dilation: prolongs positive pixels: y direction.
- groups of positive pixels prolonged : y direction.
- erosion: removes positive pixels along line



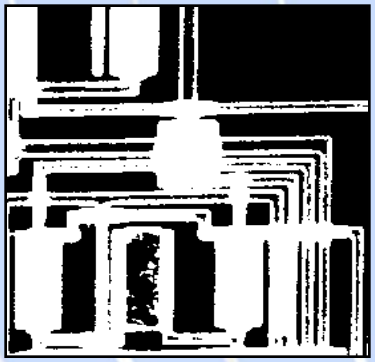






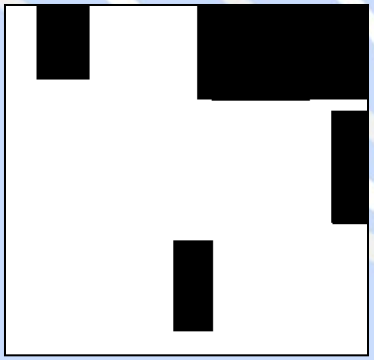
Erosion: disk-shaped structuring element

Morphological processing – opening and closing

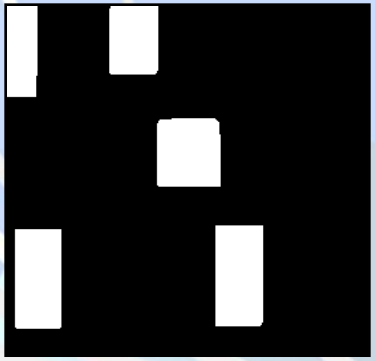


Original BW Image

Closing

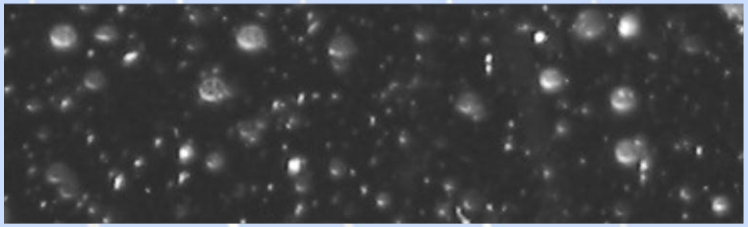


Opening



- (Morphological) **opening**: erosion followed by a dilation, same structuring element
- (Morphological) **closing**: dilation followed by an erosion, same structuring element
- Application: e.g. improve a mask, smoothing,…

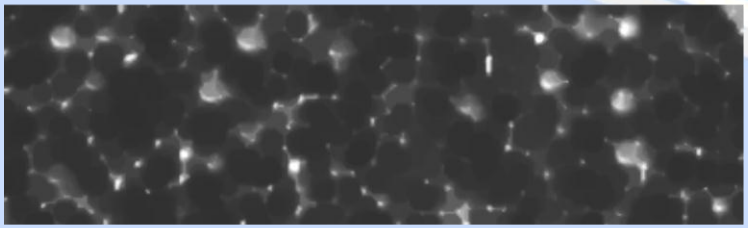
Morphological processing – grayscale images



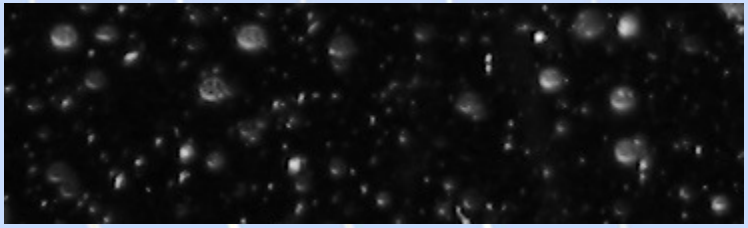
Structuring element: disk, radius 5 pixel

closing

opening



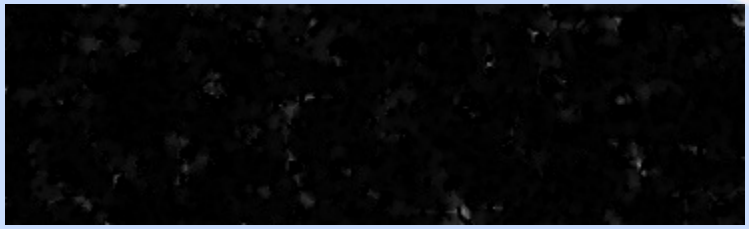
Morphological processing – tophat/bottomhat

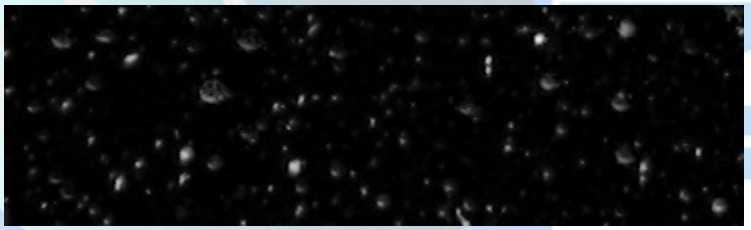


Structuring element: disk, radius 5 pixel

Bottomhat
original-closed image

Tophat
Original-opened image
emphazises small structures





- Background, mask, advanced thresholding
- Colocalization
- Time series
- Deconvolution
- 3D images
- Linear unmixing
- Complex thresholding
- Full MATLAB analysis

Colocalization: applications and limitations

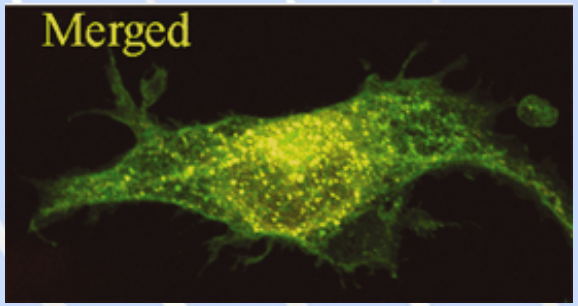
- Are two proteins present at the same place?
- Confocal resolution: 200-250 nm (xy)
- STED: 30-50 nm!!!
- Diameter of protein: 5-20 nm
- Colocalization (confocal) ≠ Interaction: „just same hotel“
- Interaction: FRET, Fluorescence cross correlation spectroscopy (FCCS), fluorescence complementation (BiFC), proximity ligation assay (PLA)…
- **!!!!!Images acquired according to Nyquist!**

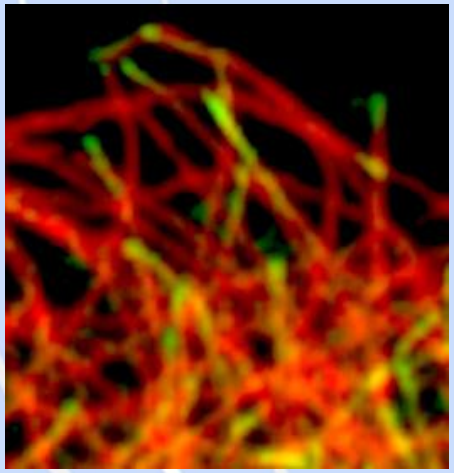
Nyquist-Shannon Theorem

Harry Nyquist (1928):
How to convert any signal from the ‚real world' into an electronic (digital) format in order to recover it after transmission completely? (telephone, radio)
„ … for complete signal reconstruction, the **required** frequency **bandwidth** is proportional to the signaling speed, and that the **minimum bandwidth** is equal **to half the number** of code elements per second. "

"The world around us is analog (i.e. continuous in space-time), but most of the storage equipment digital (i.e. discrete space-time + discrete values). In going from the reality of continuous space-time to stored bits, we perform analog-to-digital conversion. What is the slowest sampling frequency that will let us have faithful representation of the signal that represents the event? Harry Nyquist discovered that for avoiding loss of information during discretization of continous signals, one needs to **sample the value of the signal at least two times** faster than the rate at which the signal changes."

Colocalization - quantitative approaches

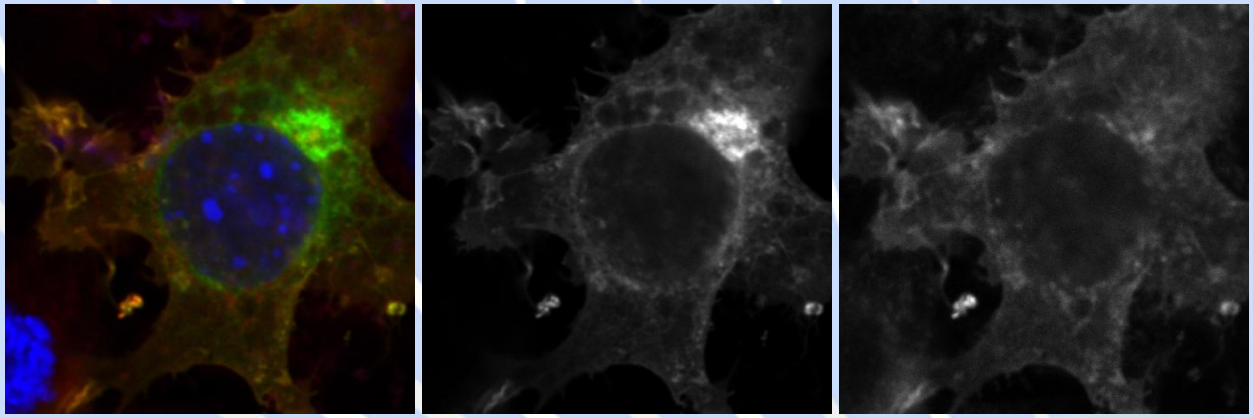


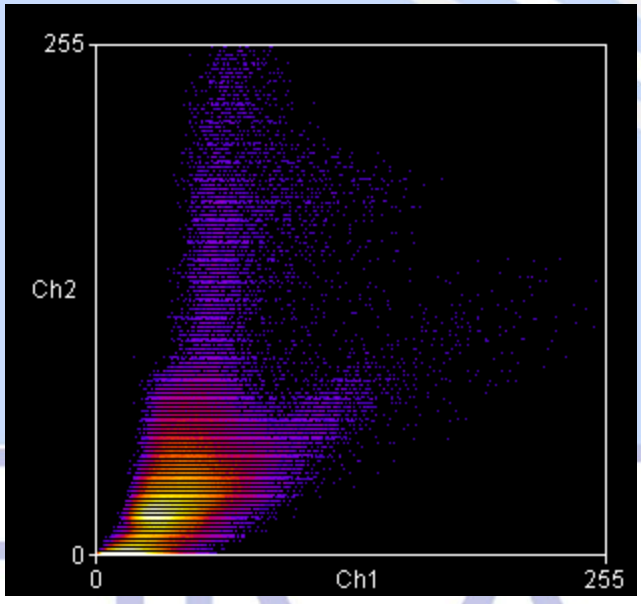


•Qualitative: red-green> yellow: high degrees of colocalization

- Partial colocalizations: microtubule tip binding proteins,…
- Colocalisation coefficients (!)
  - Pearson‘s correlation coefficient
  - Overlap coefficient/s
  - Mander‘s coefficients (!)
  - Intensity correlation analysis
- Statistical relevance: random images

Colocalization - Histograms





- 2D Histograms: Plot green vs red pixel intensity
- Colocalization: along diagonal…

Colocalization – Pearson's correlation coefficient (PCC)

$$R_r = \frac{\sum (R_i - \overline{R}) \times (G_i - \overline{G})}{\sqrt{\sum (R_i - \overline{R})^2 \times \sum (G_i - \overline{G})^2}}$$

For pixel i in the images, R and G are intensities of the red and green channel respectively.

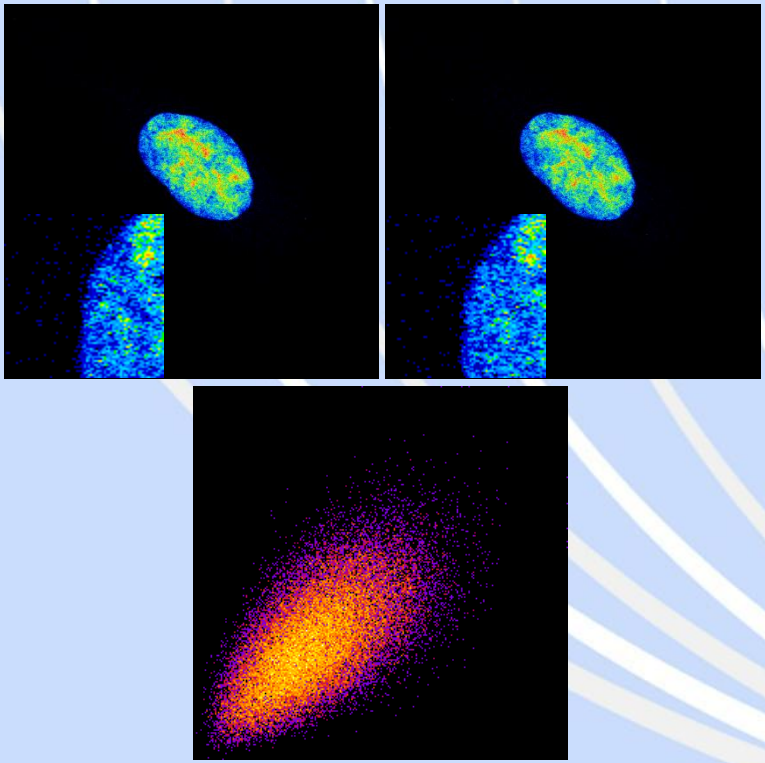
☺Statistical test to compare correlation two properties (not only images!)

•-1 to +1

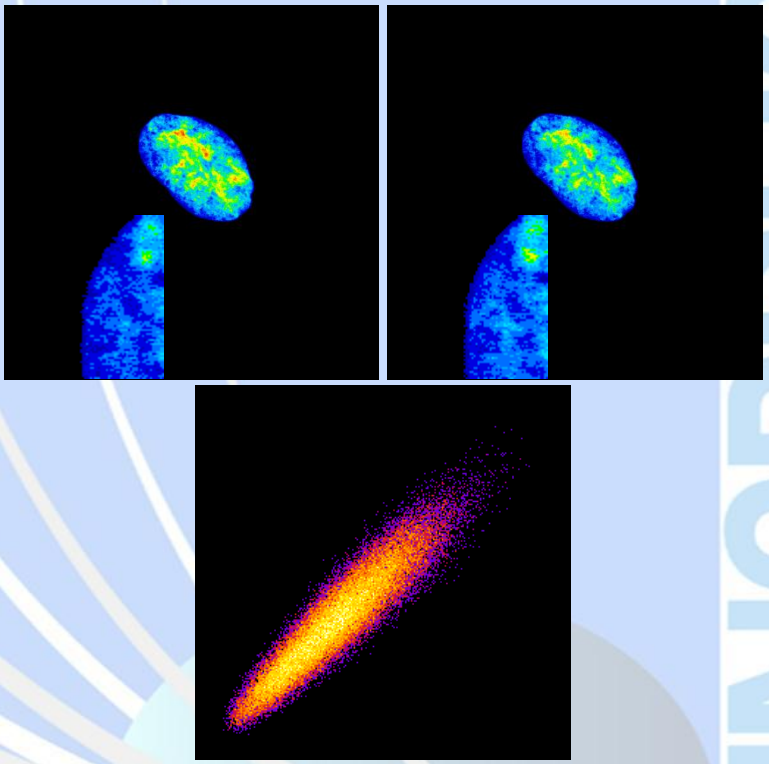
☺ close to +1: reliable colocalisation

☹Negative values and zeros difficult to interpret

Influence of image noise on colocalization analysis



Pearson **: 0.48**



Pearson: **0.92**

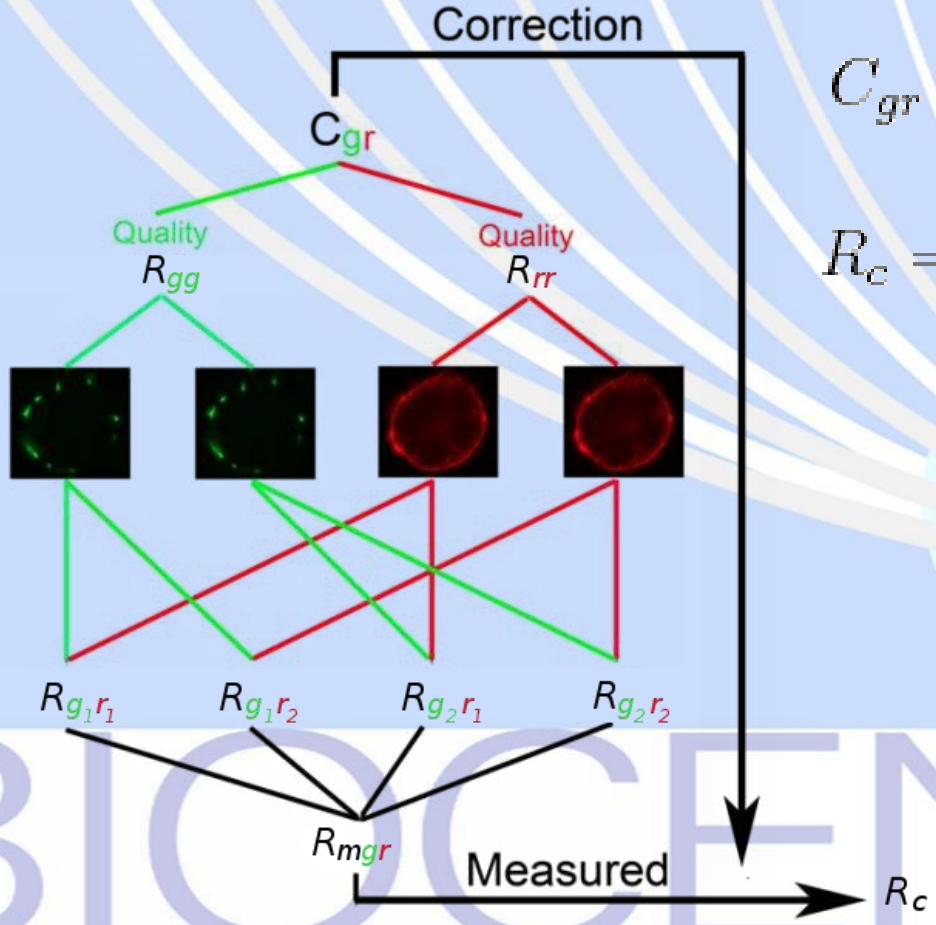
☹same sample, same conditions: **Pearson <1**

☹**same sample, pairwise different conditions: big difference in Pearson**

☹**image noise :crucial influence, underrating coloc**

Replicate-based noise corrected correlation (RBNCC)

☺Same sample: Pearson ≡ 1
☺RBNCC: acquire each fluorescence channel twice!
☺2 channels; 4 images
☺Noise can be corrected, better results



$$C_{gr} = \frac{1}{\sqrt{R_{rr} \cdot R_{gg}}}$$

$$R_c = R_{mgr} \cdot C_{gr}$$

Colocalization – Overlap coefficient

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

☺Easier to understand than Pearson

☺0 <R< +1

☹Valid only if: number red pixels ~ number green pixels

Colocalization – Overlap coefficients red/green (k)

$$k_{red} = \frac{\sum_i (R_i \times G_i)}{(R_i)^2}$$

$$k_{green} = \frac{\sum_i (R_i \times G_i)}{(G_i)^2}$$

☺Problem with equal pixel number solved BUT
☹Absolute fluorescence intensities need to be equal!

Colocalization – Manders‘ colocalization coefficients

$$M_{red} = \frac{\sum_i R_{i,coloc}}{\sum_i R_i} \qquad M_{green} = \frac{\sum_i G_{i,coloc}}{\sum_i G_i}$$

$R_{i,coloc} = Ri$ if $Gi > 0$; $G_{i,coloc} = Gi$ if $Ri > 0$.
i.e. M_{red} is the sum of the intensities of red pixels that have a green component divided by the total sum of red intensities.

☺ absolute fluorescence intensity: not an issue (normalized!)
☺ how well does red channel overlap with green and vice versa (NOT always the same)
☹ background correction very crucial
☹ intensity of second channel not considered:
    ☹bright green/faint red = bright green/bright red

Colocalization – intensity correlation analysis (ICA)

$$PDM = (R_i - \overline{R}) \times (G_i - \overline{G})$$

PDM = Product of the Difference from the Mean for each channel.
For pixel *i* in the image, *R* and *G* are the respective intensities in the red and green channel.

$$ICQ = \left(\frac{N_{+ve}}{N_{total}} \right) - 0.5$$

N_{+ve} = number of positive values for PDM.
N_{total} = total number pixels that do not have a value of zero in each channel.

→ Compare numerator in Pearson's method

☺ generates colocalization images first : product of the differences from the mean (PDM)

☺ 3 cases

- ☺ ICQ < 0: segregation
- ☺ ICQ ~ 0: random (at least one component)
- ☺ ICQ > 0: colocalization

☺ high discriminative power

☺Reference: Li et al., 2004, J. Neuroscience

Colocalization – true or random?

☹ all coefficients (except ICQ) share one problem:

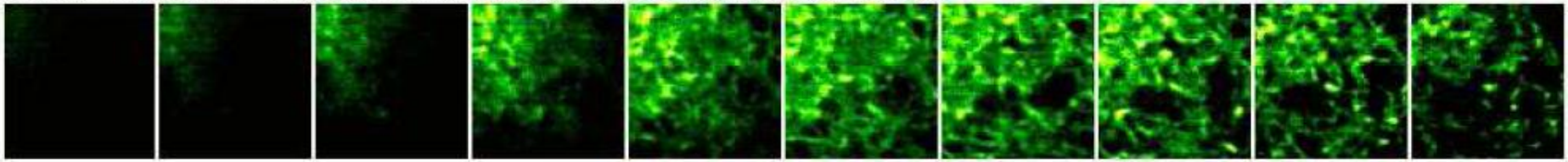
☹Is it true coloc or could it be by chance, only?

☹What does a coloc coeffient really tell us?

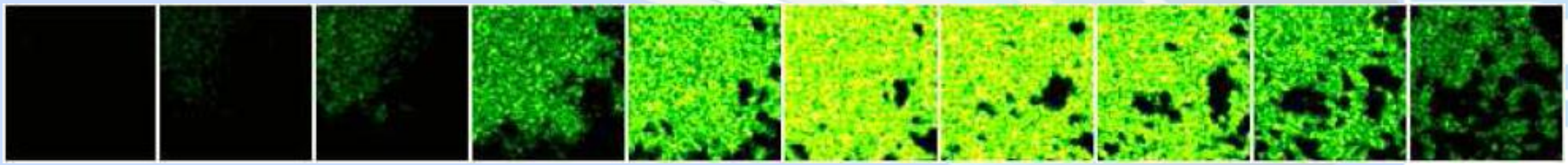
☺ Solution: original images (red vs green) either of the channel has been shifted or randomized. Coloc coefficient both cases.

☺ Rexp>Rrandom: e.g.100 different random images…?

Montage of z-slices from a raw channel 2 image (ER-GFP expressing HeLa cell)



Montage of z-slices from an example randomized image that has been randomized in x, y AND z.



- Background, mask, advanced thresholding
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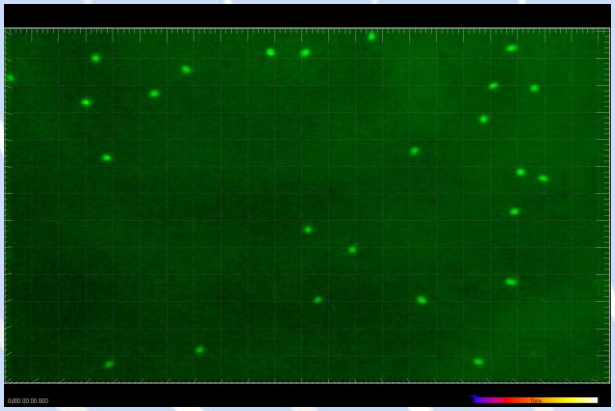
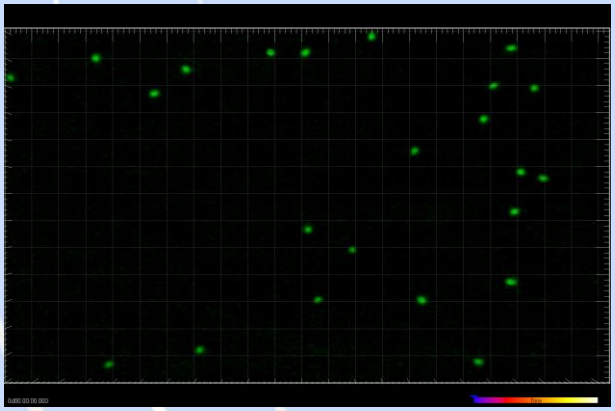
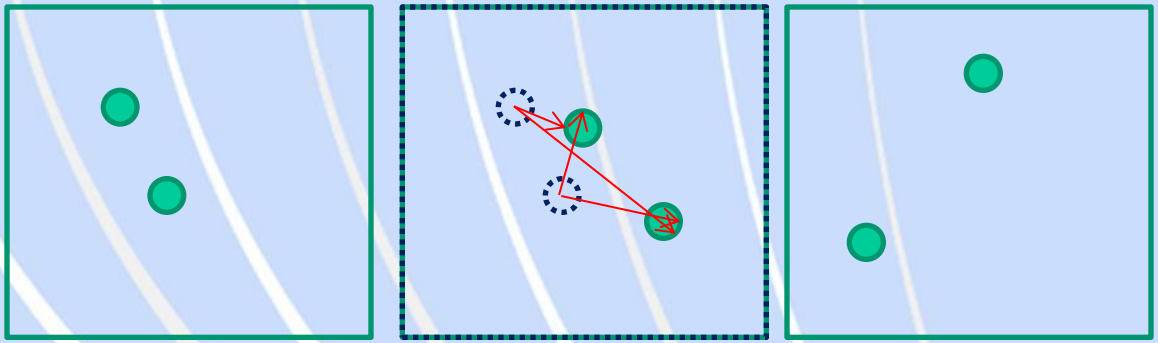
Time series analyses-preprocessing





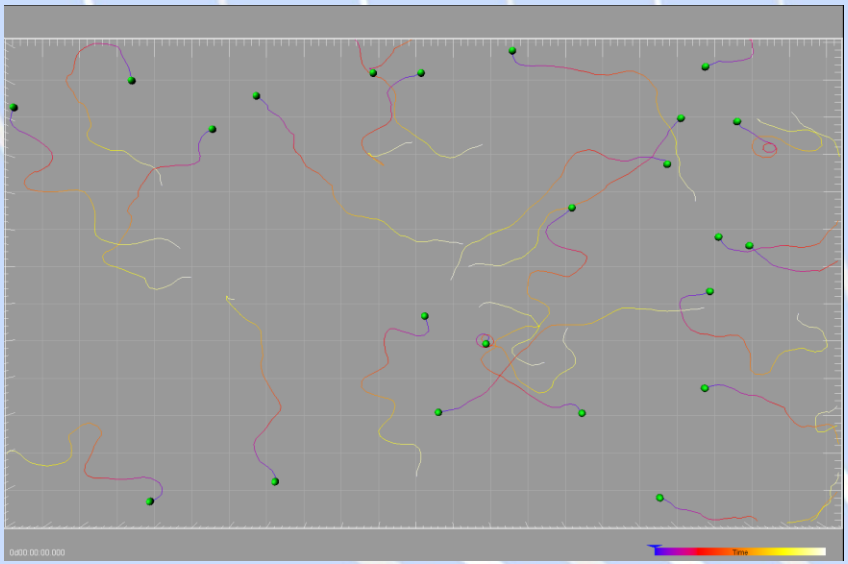
- Subtractive analysis: remove non-moving structures!!
  - Get average over all frames
  - Subtract average from each frame
  - Immobile: high average value/position
  - Mobile: low average value/position
  - IMARIS software!

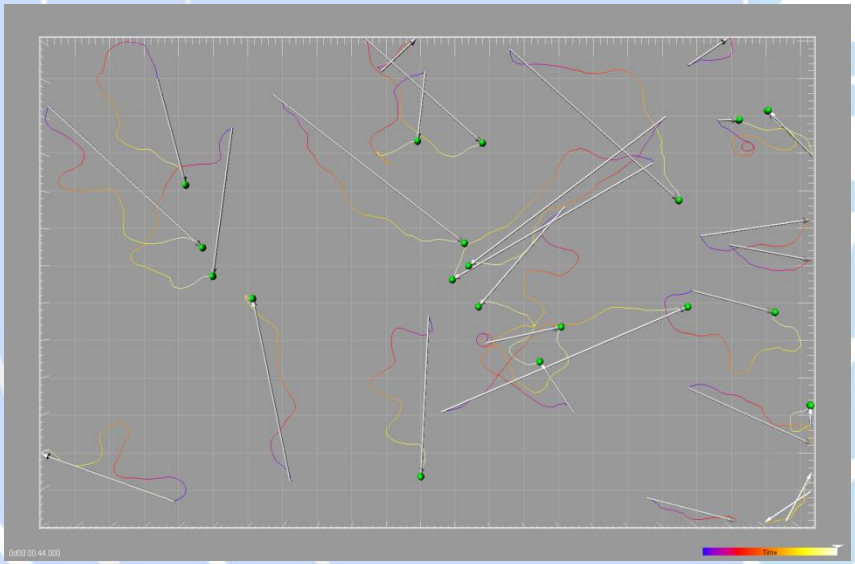
Time series analysis-tracking



- Minimize the sum of all possible vectors (differential equations)
- Assumption: the closest position per particle/successive frames=correct one
- Many particles; impossible too complex
- Only within a certain radius around each particle (additional condition)
- Time intervall small enough to re-identify structures successive planes
- IMARIS

Time series analysis - tracking results



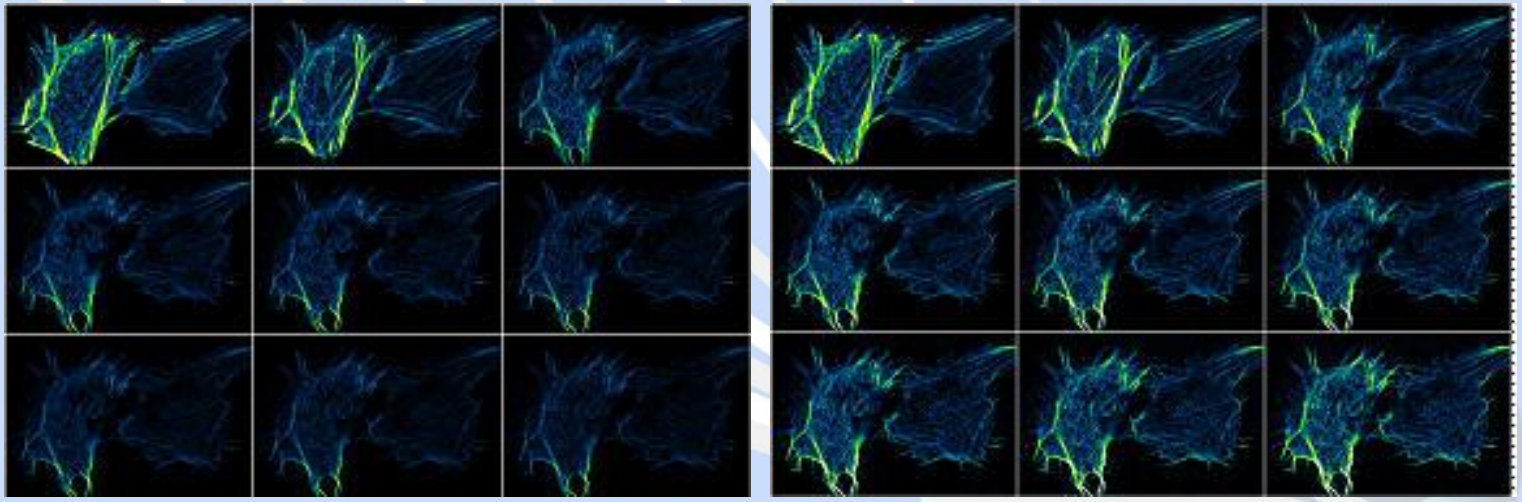


•expected results: speed, distance traveled, total displacement …per object and average

Bleach correction - time series

- Fluorescence microscopy time-course: bleaching, intensity reduced.
- fit with a mono-exponential decay
- k: decay constant, obtained from fit

Corrected intensity = (Intensity at time *t*) / e^{-k*t}



- Background, mask, advanced thresholding
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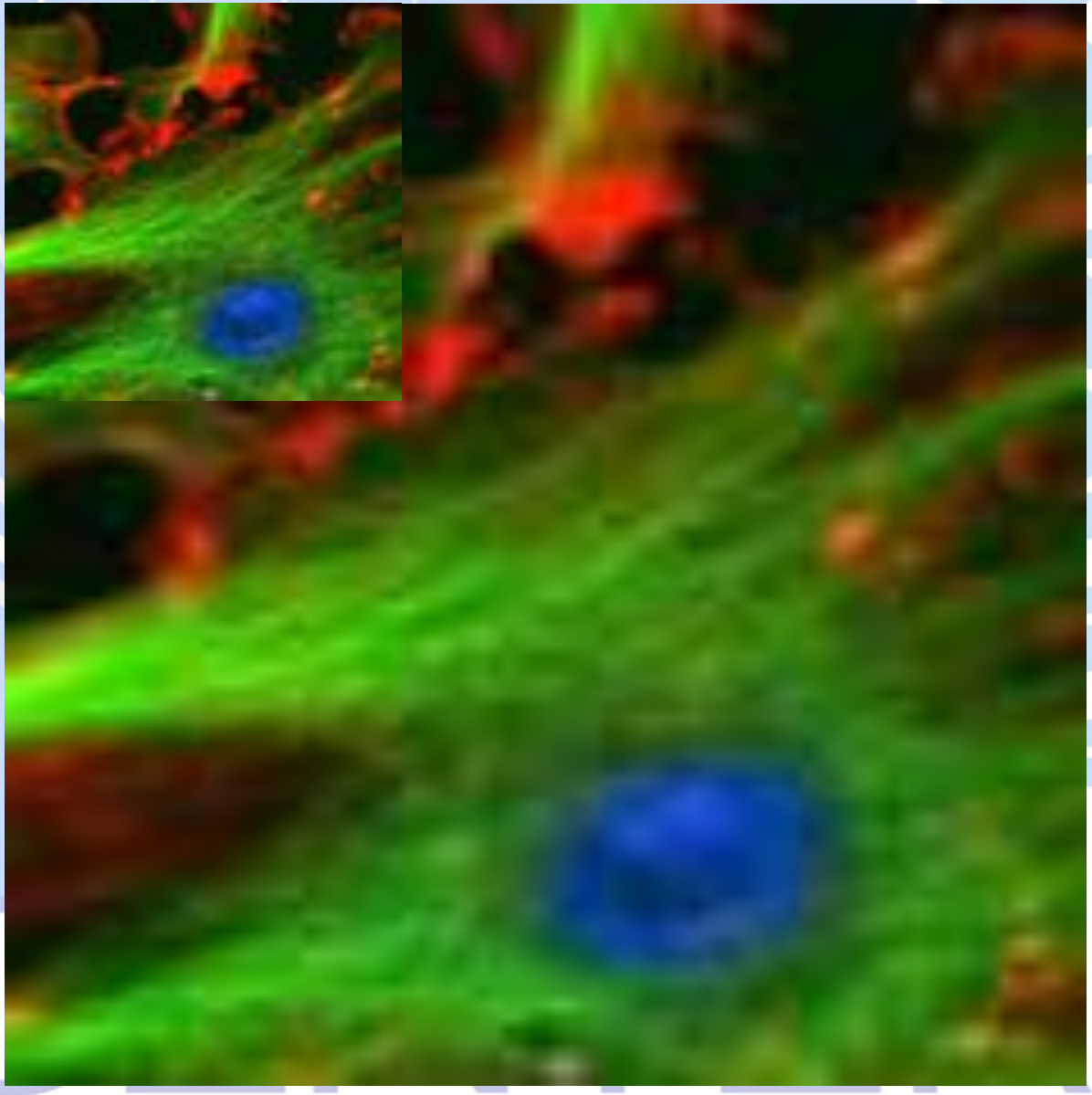
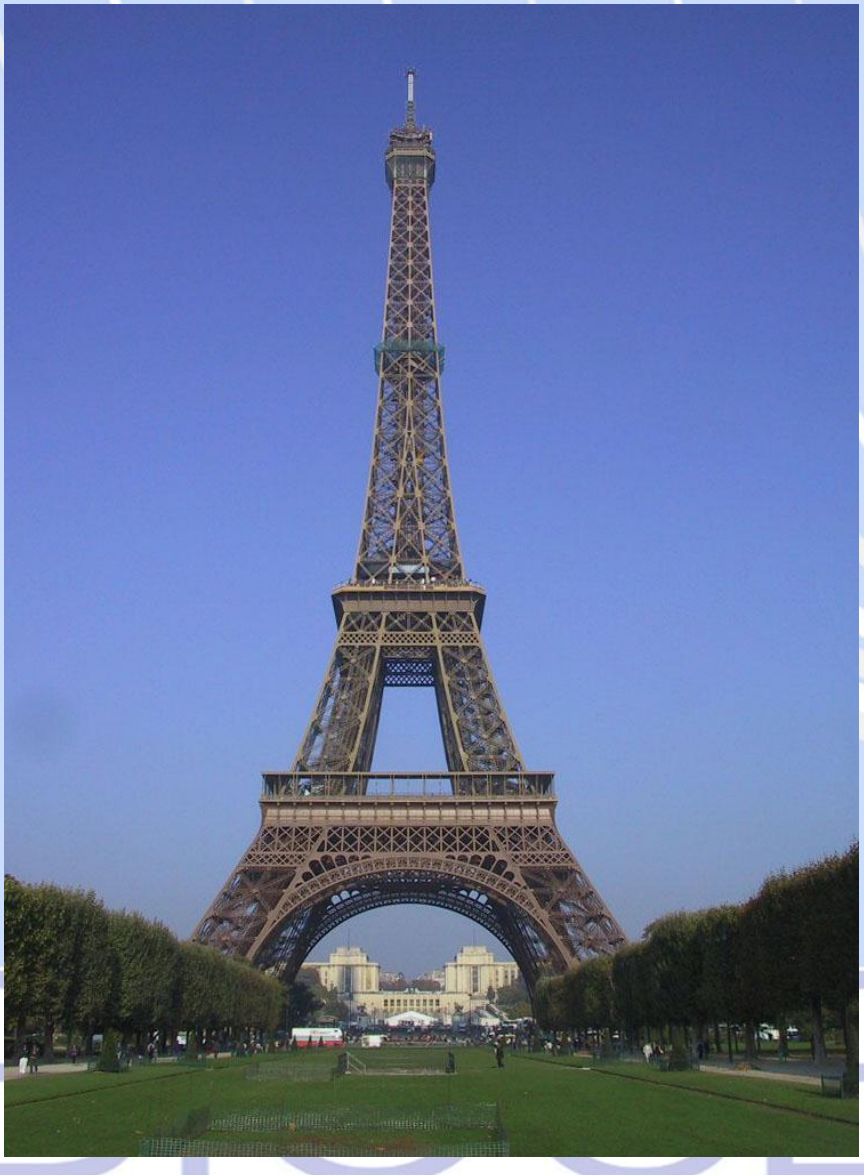
„Empty magnification“

- no add. information
- no increased resolution
- more bleaching
- „just big“
- …avoid it



What is this?



Eiffel tower? NO!

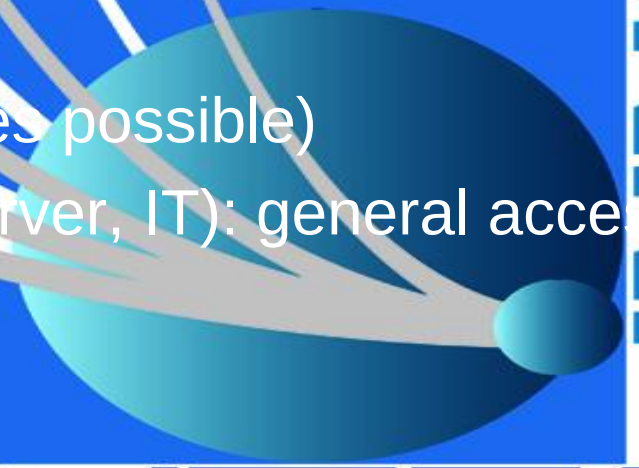
Image of Eiffel tower

… affected by fotography, light, distortions

‚Real' Eiffel tower: different

Deconvolution: Basics

- Image of sample ≠ true sample
- ☹ Microscope optics
  - ☹ lowered resolution
  - ☹ blurring
  - ☹ lowered contrast
- **Point-spread function (psf):** 3D image of ideal point
- PSF^{-1} "Image restoration"
- 3D image stacks (single, t series possible)
- IBK: Huygens Professional (Server, IT): general acce from local PC



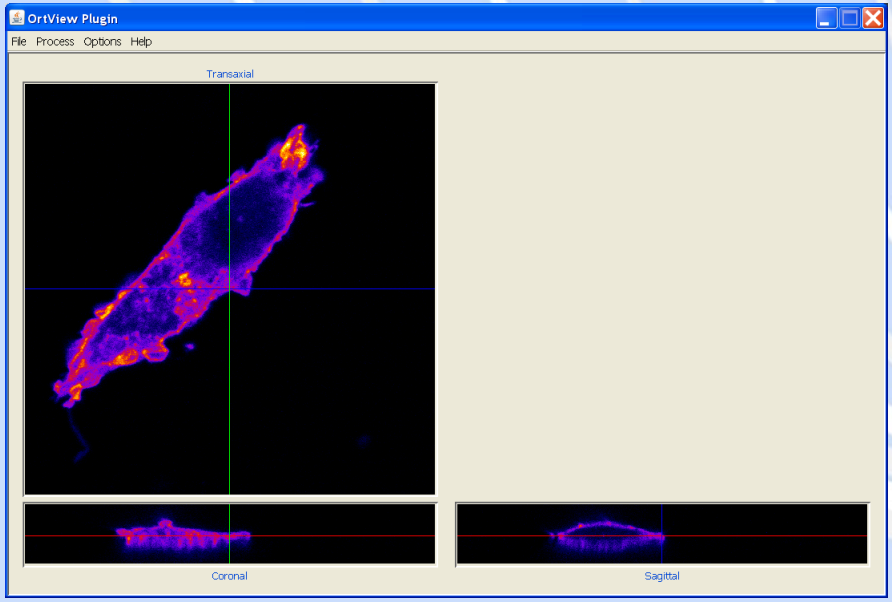
INNSBRUCK

BIOCENTER

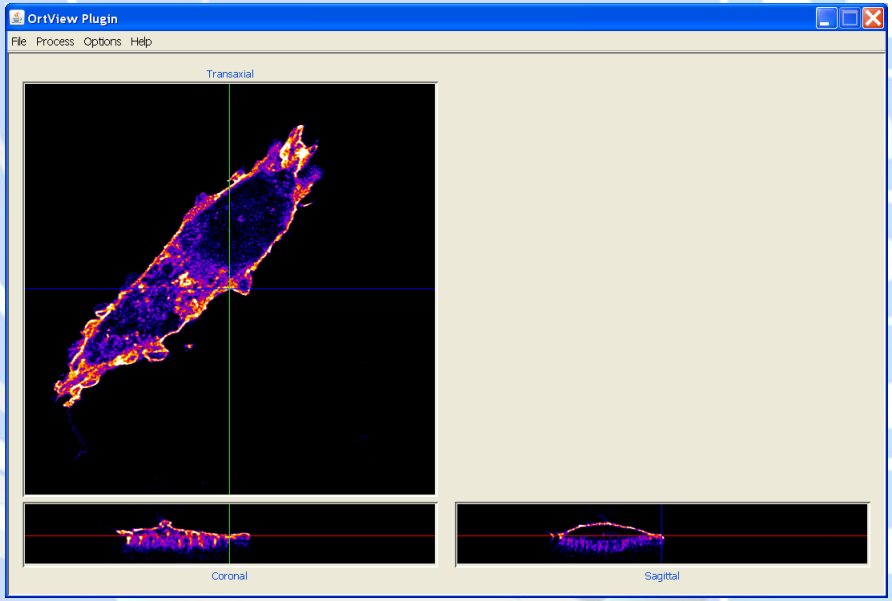
Deconvolution: resolution improvement

- cell expressing membrane receptor
- axial direction ~factor 2-3

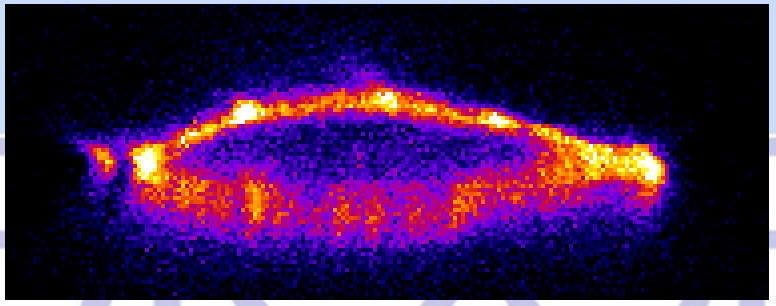
BEFORE

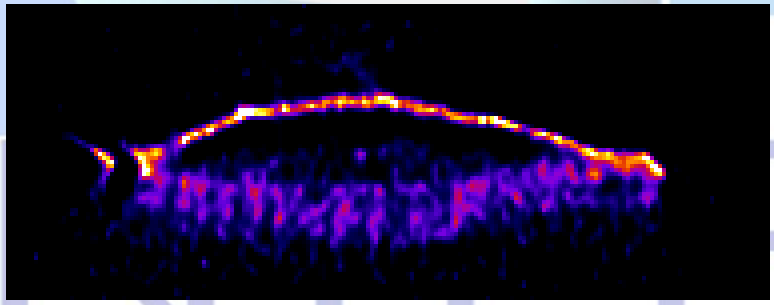


AFTER



yz



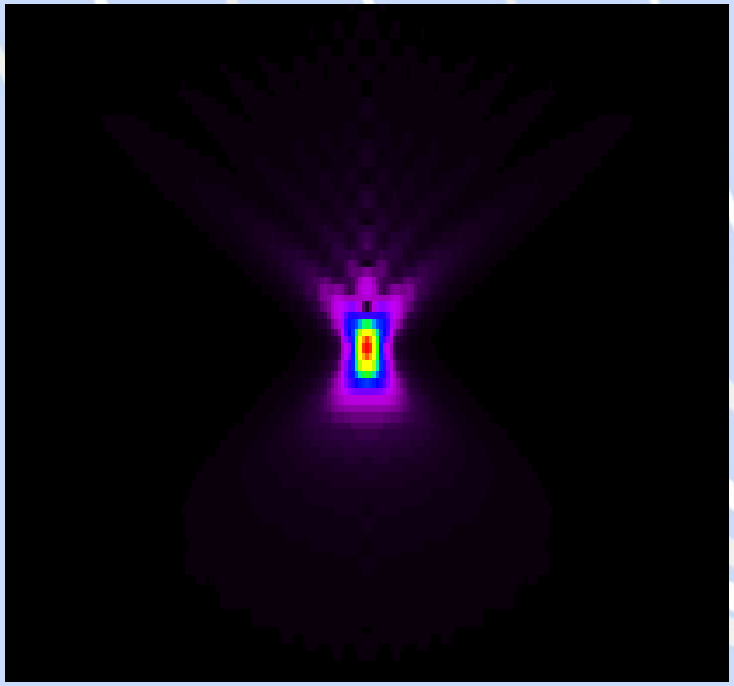


Deconvolution: expected results

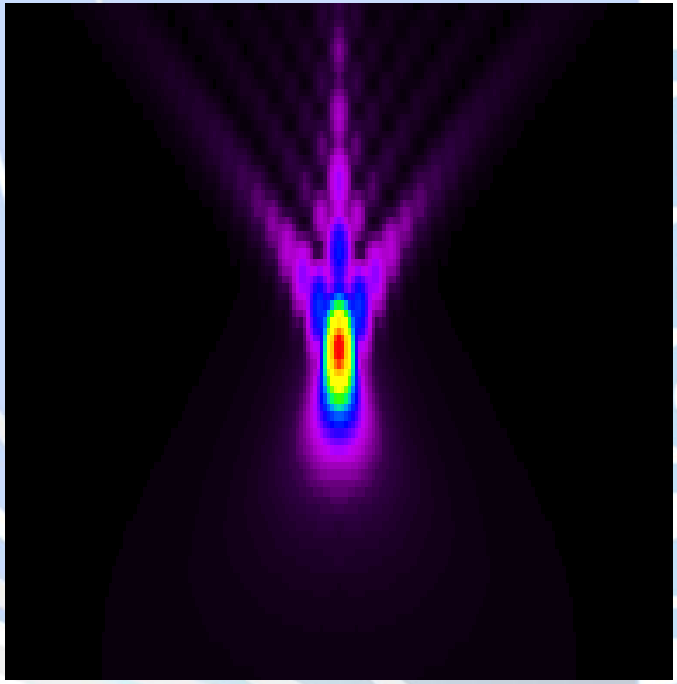
- general resolution improvement (axial!)
- noise removal
- wide-field (background) and confocal data
- 3D reconstructions (movies)

Symetric - Asymetric PSF

Refractive indices match (1.46/1.46)

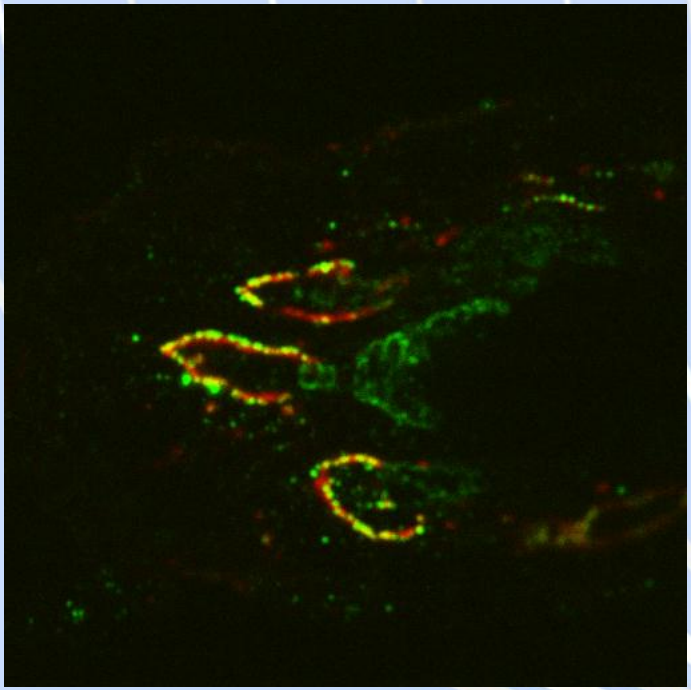


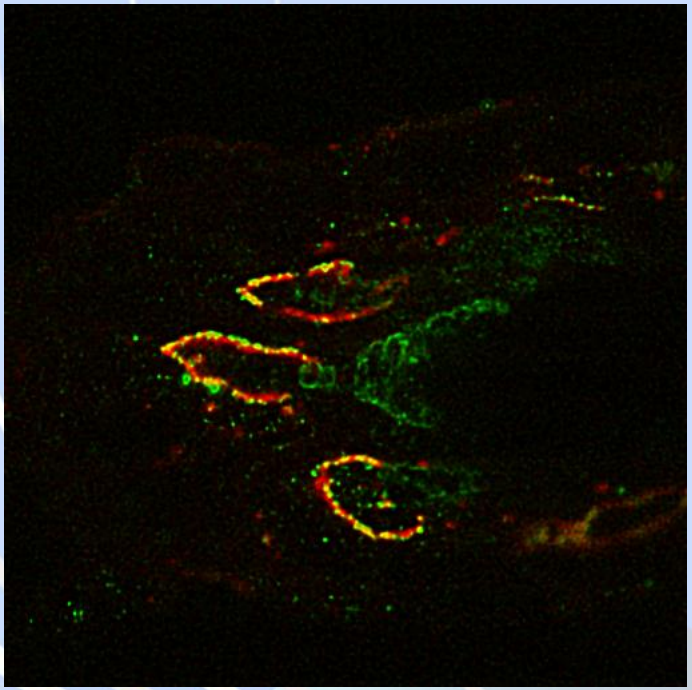
mismatch (1.46/1.33)



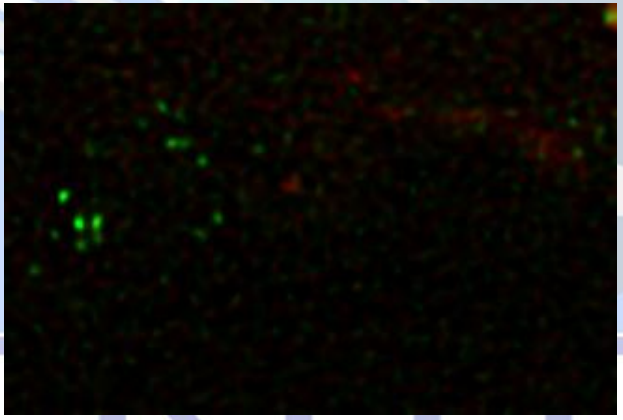
RI mis-match: eg **live cells ~ oil objective (1.33/1.515**)
loss of resolution

Deconvolution - potential problems





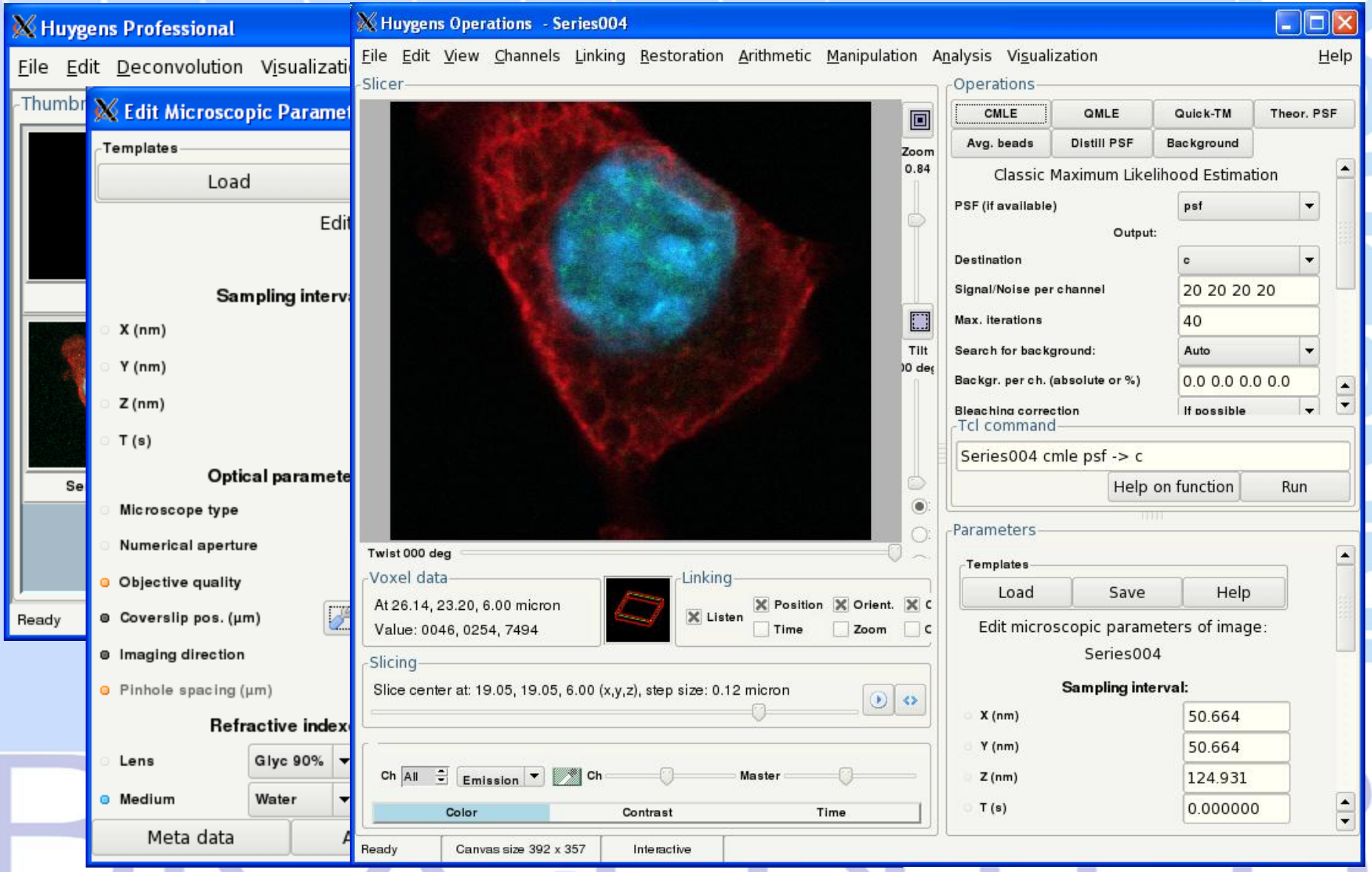
- Over-restoration
- Ringing
- Background amplification



MUI: Huygens

- Deconvolution at MUI
  - Huygens Professional: SERVER based!
  - access for everyone
  - good performance
  - no blocking of local PCs
  - IT department

Deconvolution at MUI: Huygens Professional



Hints for deconvolution with Huygens

- Data aquisition: nyquist sampling
  - xy: pixelsize: typical 40-50nm
  - z: distance between planes: typical 130 nm
- Refractive indices
  - Immersion: oil, water, glycerol
  - Embedding: water (live cells), glycerol,
- Dyes: emission known
- Objective: numerical aperture (1.2; 1.3; 1.4)

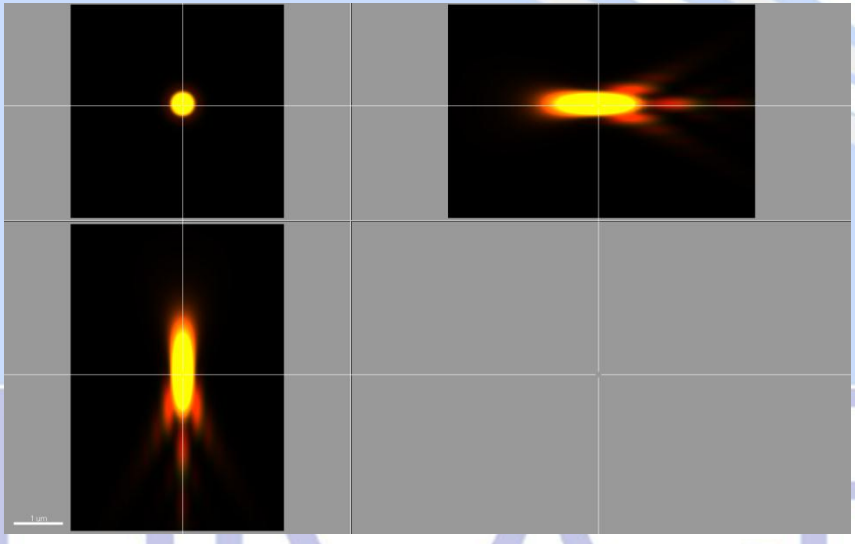
PSF: theoretical vs. experimental

- PSF (point spread function)
  - ideal point is distorted through the optics of the scope.
- Theoretical PSF
  - ideal objective
  - NA, wavelength, refractive index
- Experimental PSF
  - optical parts are NOT ideal, asymetric, errors, etc..
  - fluorescent beads

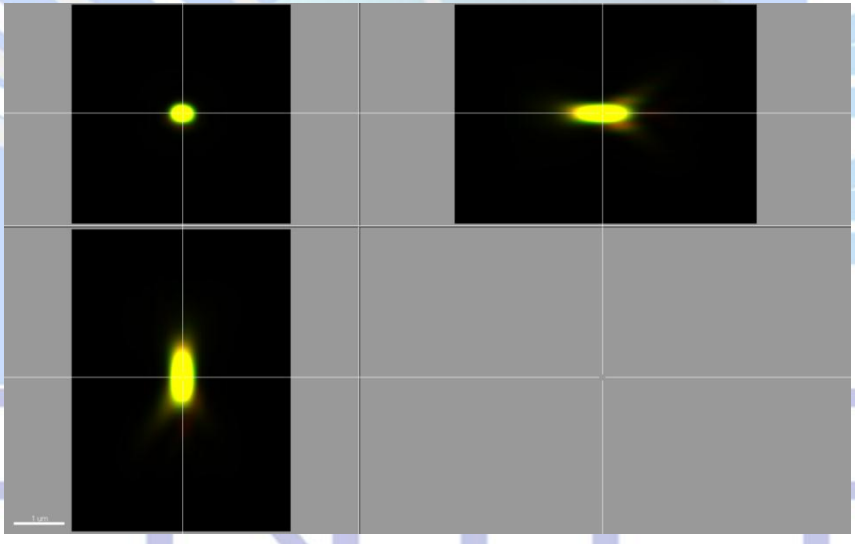
Measuring the PSF

- Beads
  - < 0.2 μm diameter
  - same fluorescence as sample (green, orange-red)
  - same medium
  - same imaging conditions: pixelsize, z-stack, …
  - well-separated
  - for each objective
  - average several beads (Huygens)

Theoretical PSF: green/orange-red



Measured PSF: beads, green, orange-red



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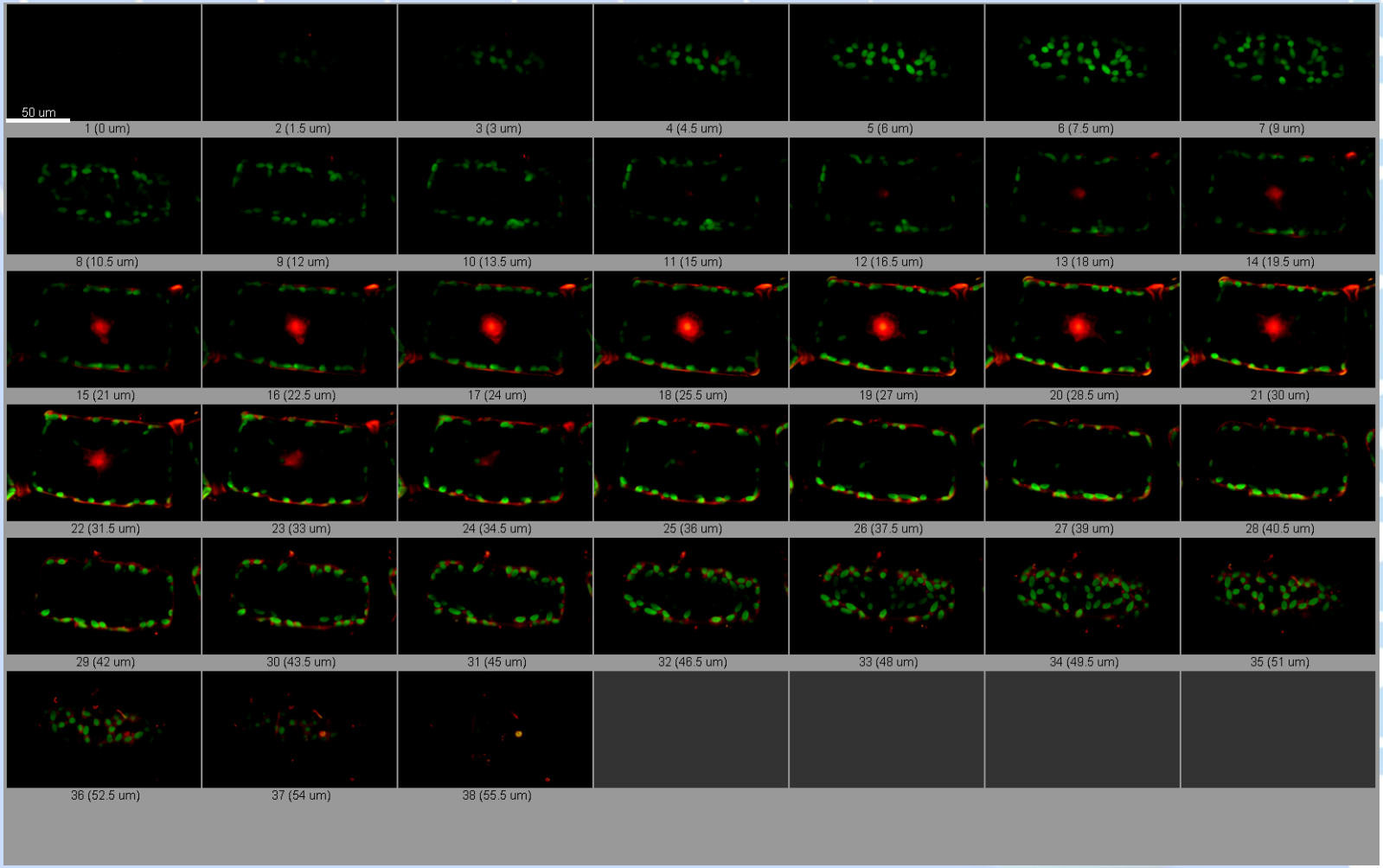
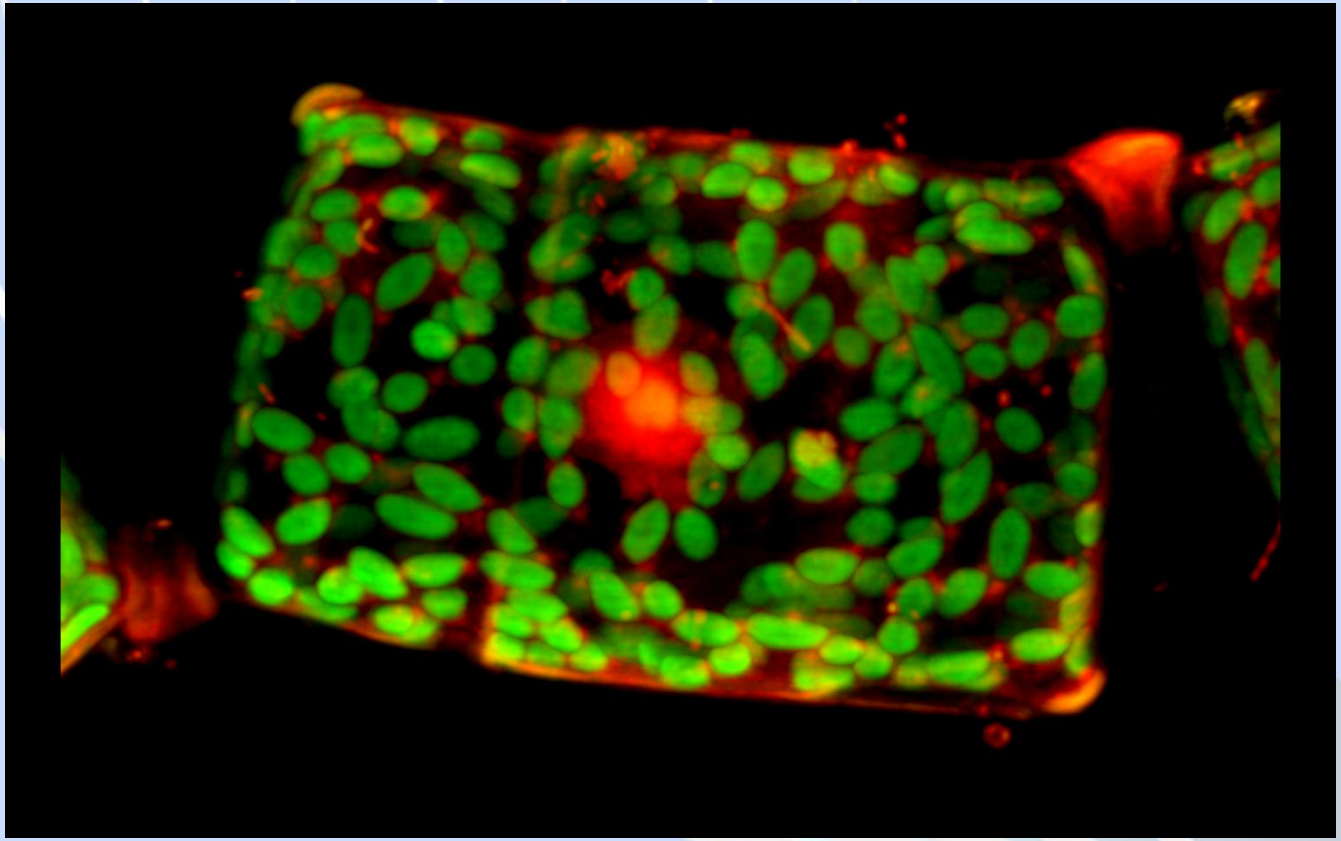
3D analyses – Gallery view



- Confocal image stacks: planes along the optical axis

3D analyses - maximum intensity projection (MIP)

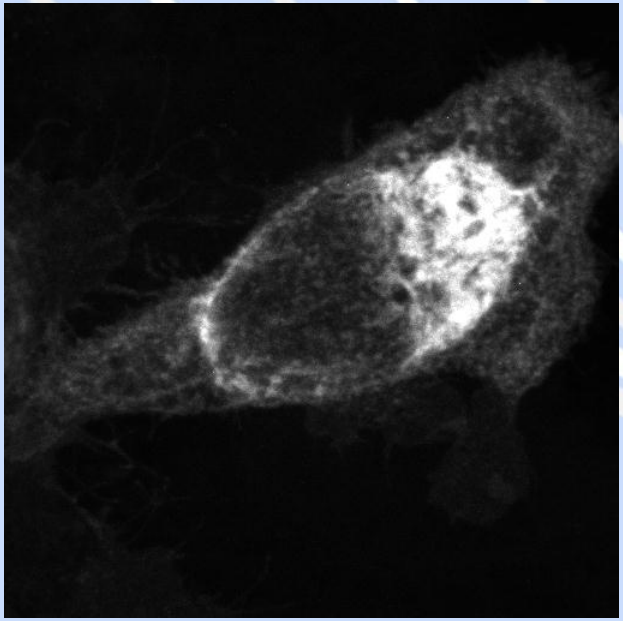


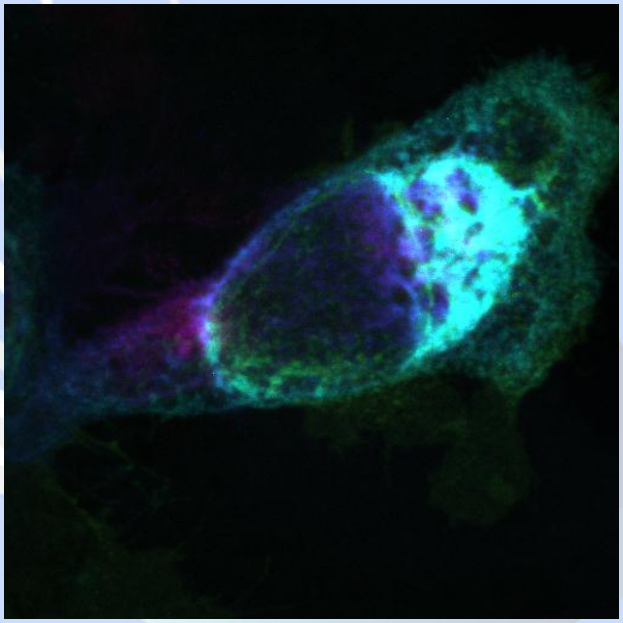
•Project into one xy image plane: maximum intensity along z to form a single xy plane

•Overview but not accurate representation of 3D data

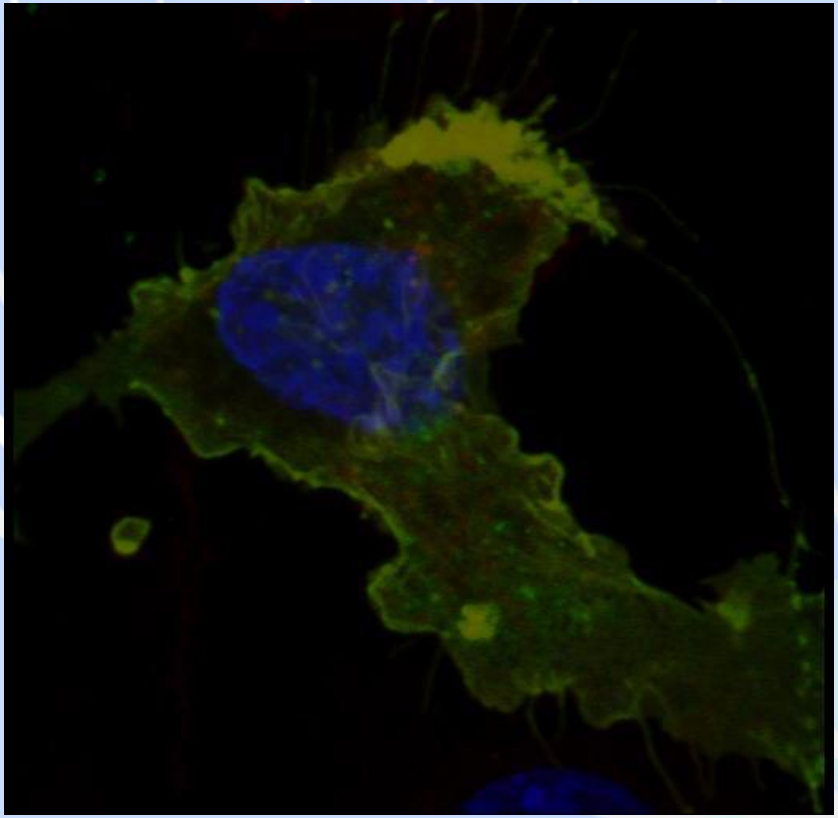
3D analysis: Depth coding

- Use a colormap along the z-axis
- maximum projection
- color : distance from first plane
- only one channel!



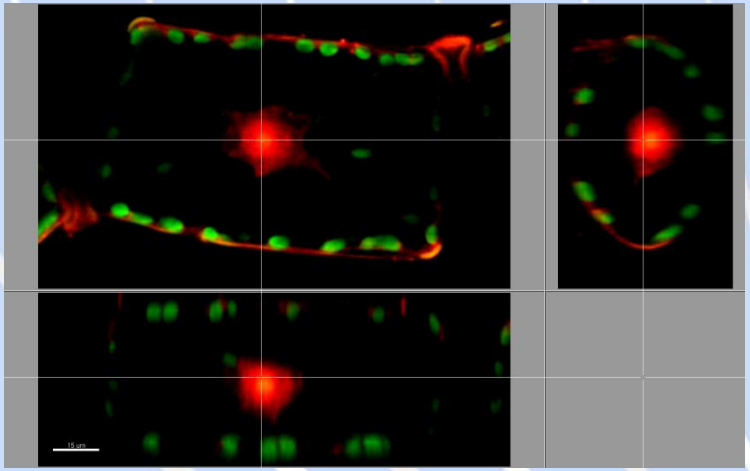


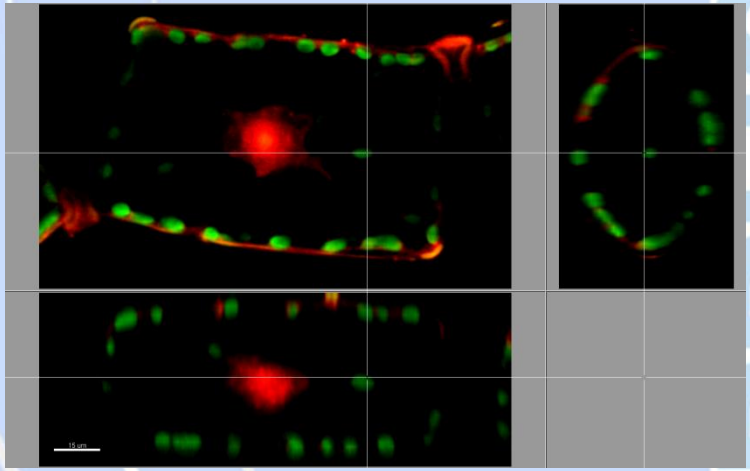
3D analyses – rotations (animated)

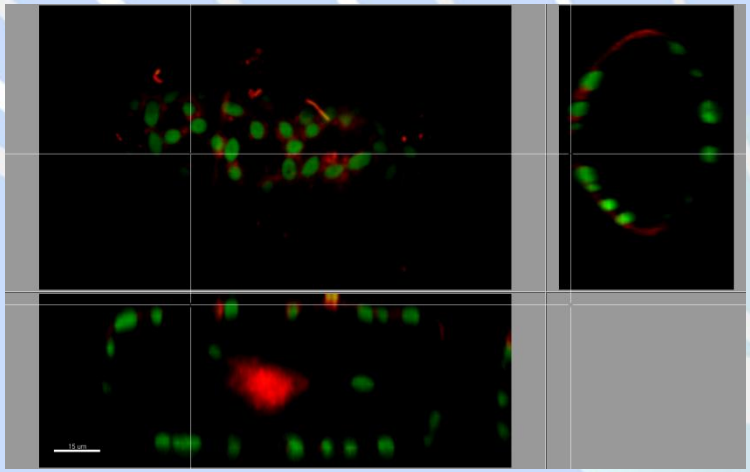


- MIP into several arbitrary planes-“rotation“
- Good representation of 3D data-but not in still images (online only, no print!)

3D analyzes – ortho-slice



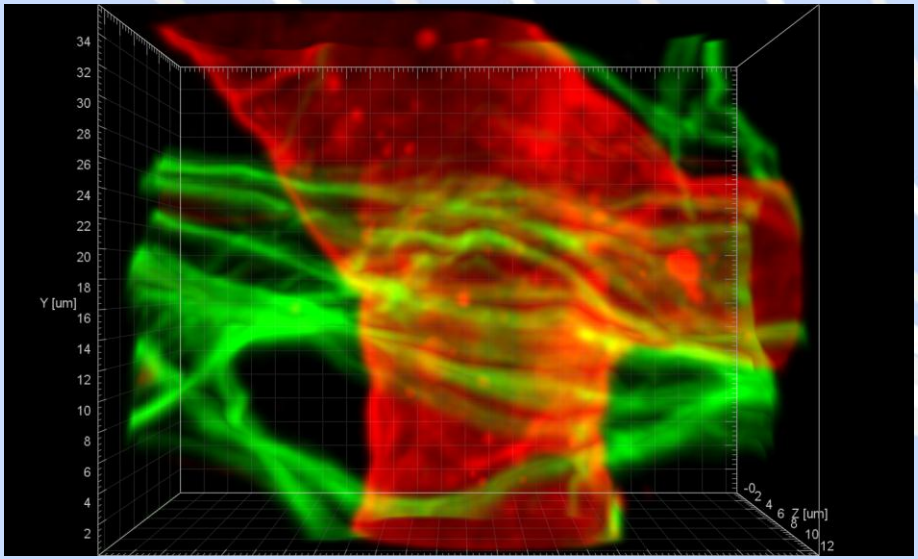


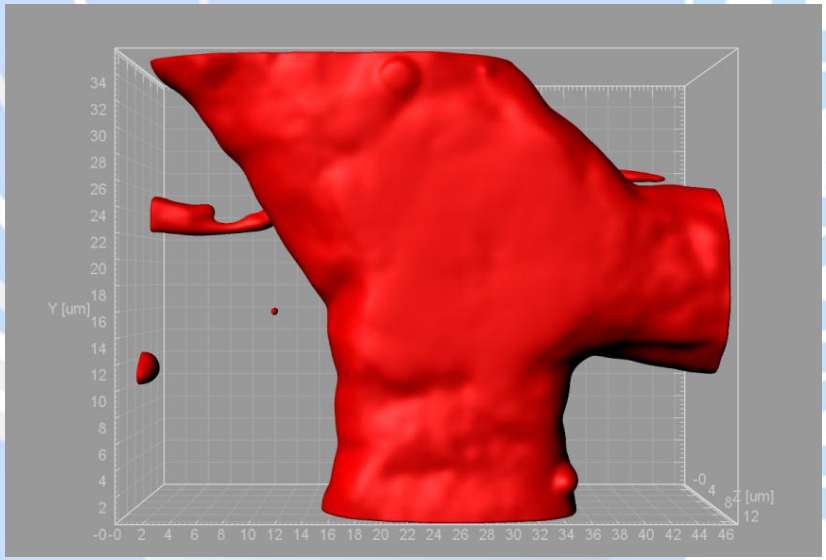


- cut stack with xy, xz, yz plane (orthogonal planes)
- xy-section: top-left, xz: bottom, yz: right

3D analyses - isosurface

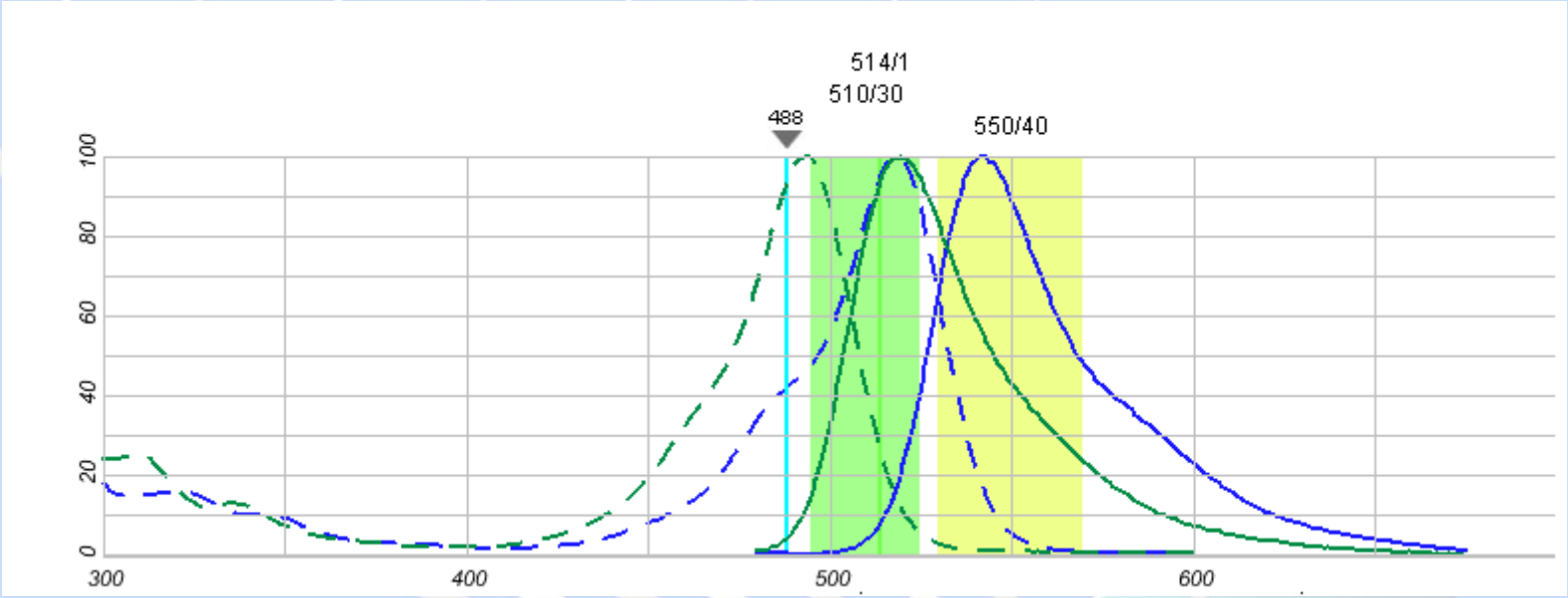
- Isosurfaces: connect voxels with same (iso) intensities with a surface
- Isosurface: “3D mask”
- Applications: analyze within the surface: intensities, distributions
- Surface: area, volume, also tracking, ...





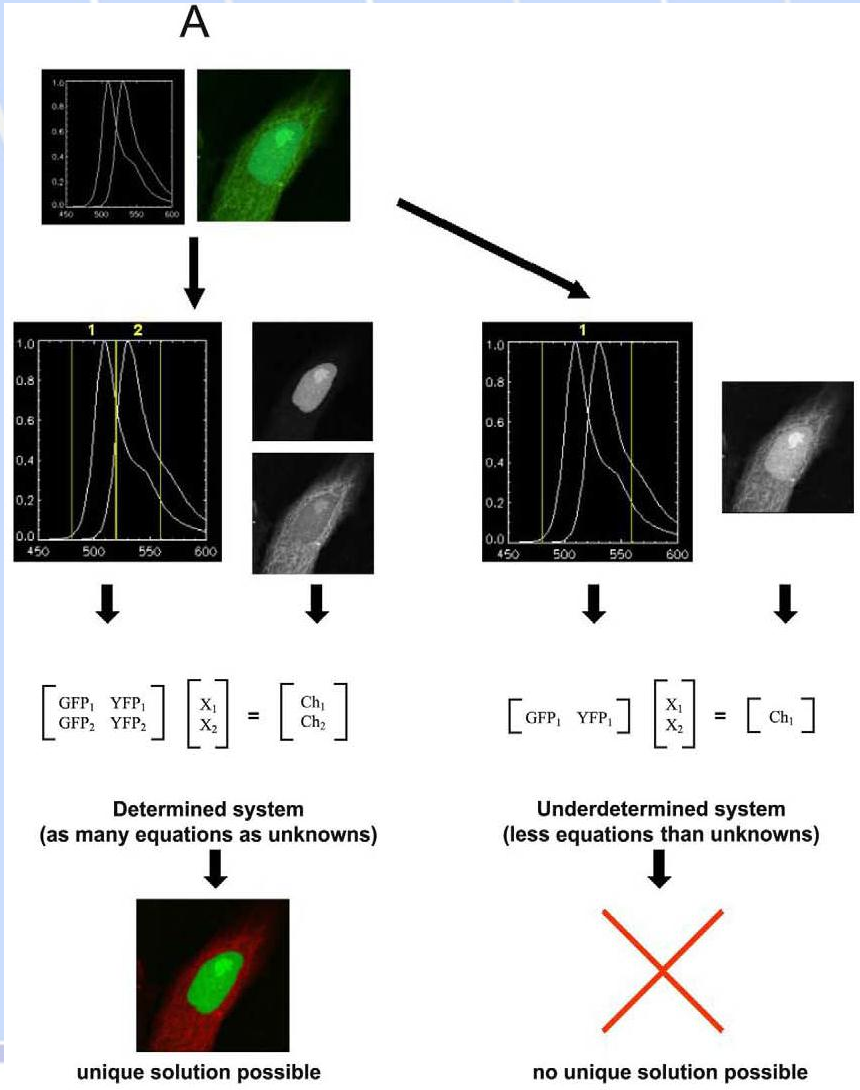
- Background, mask, advanced thresholding
- Colocalization
- Time series
- Deconvolution
- 3D images
- Linear unmixing
- Complex thresholding
- Full MATLAB analysis

Linear unmixing: spectral overlap



Linear unmixing, separation of strongly overlapping dyes using independent measurements of the individual dyes.

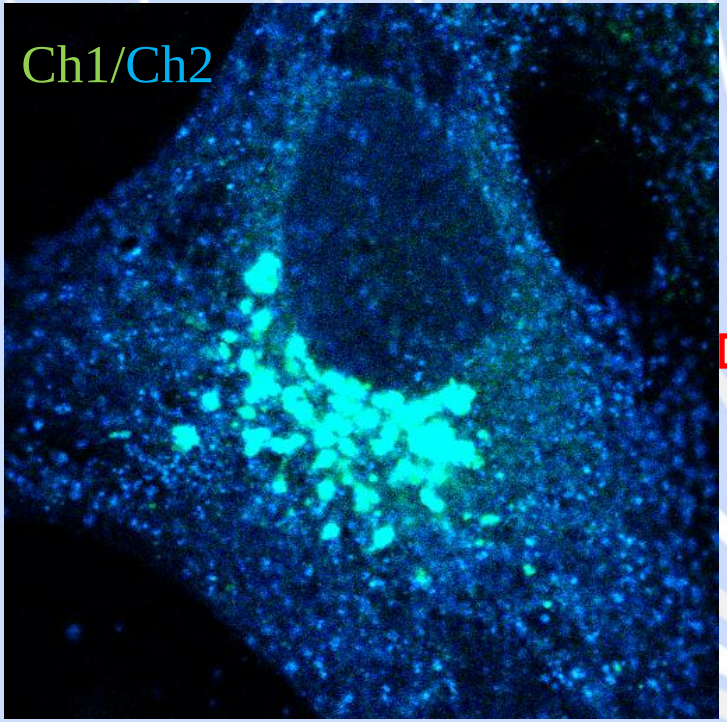
Linear unmixing: theory



- ECFP, EGFP, EYFP
- ECFP/EYFP separation: OK, standard
- ECFP/EGFP or EYFP/EGFP
  - NO, strong overlap
- ‚Dye separation' = linear unmixing

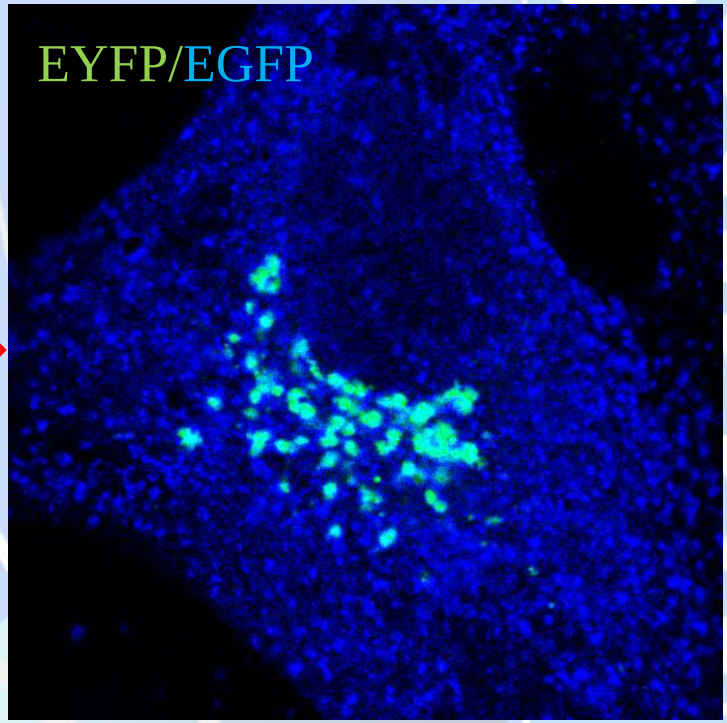
Zimmermann et al., 2003

Linear unmixing: example



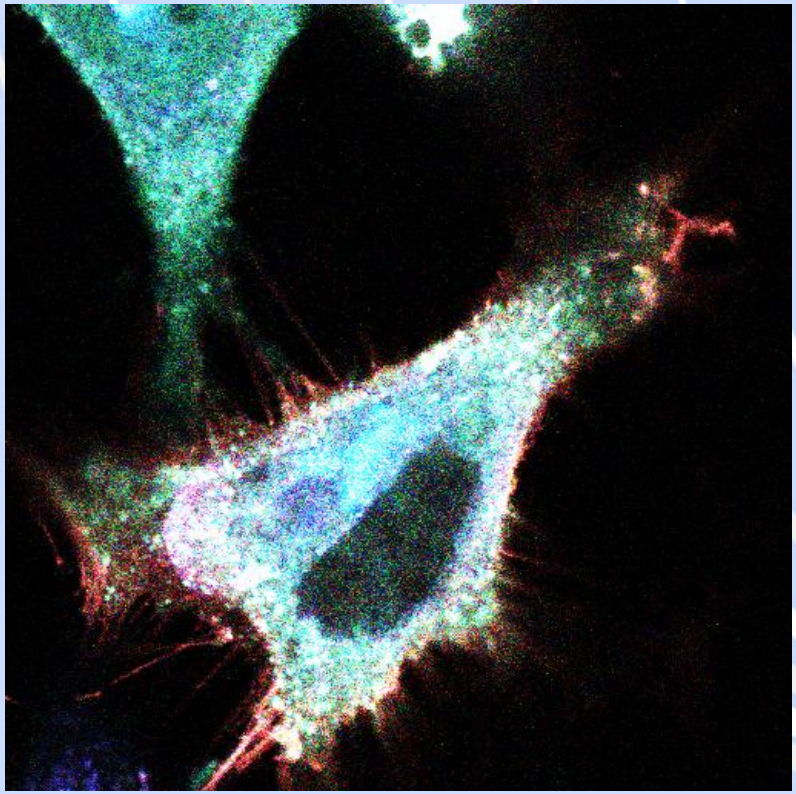
a*EGFP + b*EYFP=CH1
c*EGFP + d*EYFP=CH2

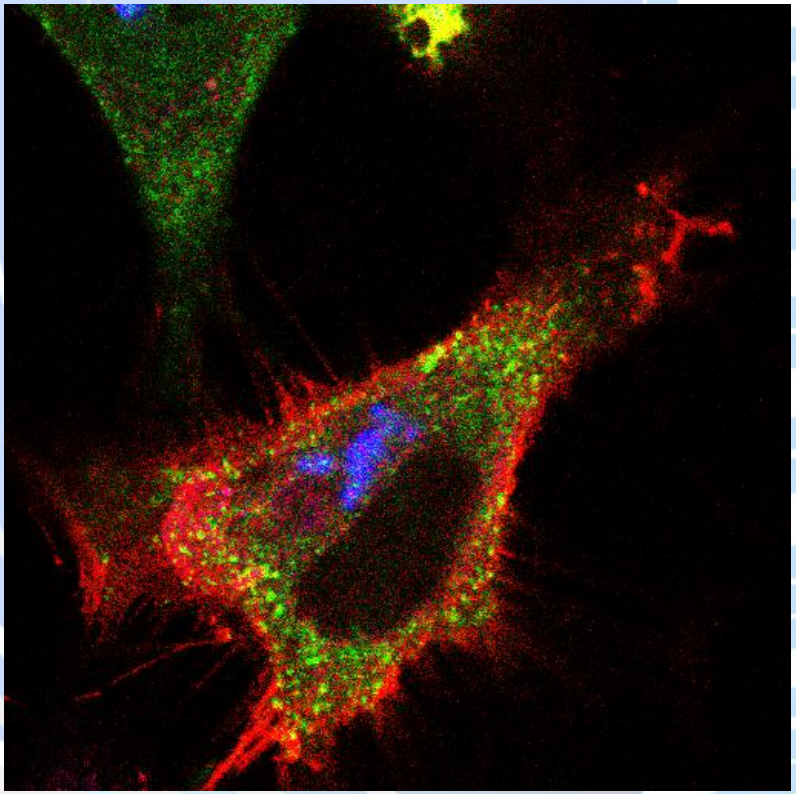
a,b,c,d from calibration



- EYFP-Galactosyltransferase (Golgi), EGFP-light clathrin A
- CH1: 493-521 nm, CH2: 522-584 nm
- 2 calibration samples: EGFP/EYFP only (independent measurement!)
- Reference images, same(!) settings.
- Linear unmixing: „Solve system of linear equations“ … from school!!!
- **CAN BE DONE ON ANY FLUORESCENCE MICROSCOPE!!!**

… 3 colors: ECFP,EGFP and EYFP





•458 nm Laser, CH1: 463-500 nm, CH2: 500-525 nm, CH3: 526-600 nm

•ECFP-EGFR, EGFP-LCA, EYFP-GalT

•3 „equations“ (3 channels) <-->3 dyes, 3 independent calibrations (ECFP, EGFP, EYFP)

Linear unmixing: applications

- More dyes: 3 colors=standard, 4, 5… : unmixing
- Reduce cloning work: EGFP plus EYFP
- **Remove autofluorescence: very efficient…**
  - AF different spectrum than fluorophores!
  - AF treated as a seperate dye
  - AF: intrinsic AF, not for unspecific AB staining
- Large filter range: better sensitivity (gSTED)
- Compare FACS: routine in multi-color FACS („Compensation“)

Linear unmixing: summary

- One channel/dye (…equation)
- One-dye only sample
- No saturation, extra careful acquisition
- Calibration/Acquisition: SAME conditions (detectors, laser power, …)
- **ANY fluorescence microscope**

- Background, mask, advanced thresholding
- Colocalization
- Time series
- Deconvolution
- 3D images
- Linear unmixing
- Complex thresholding
- Full MATLAB analysis

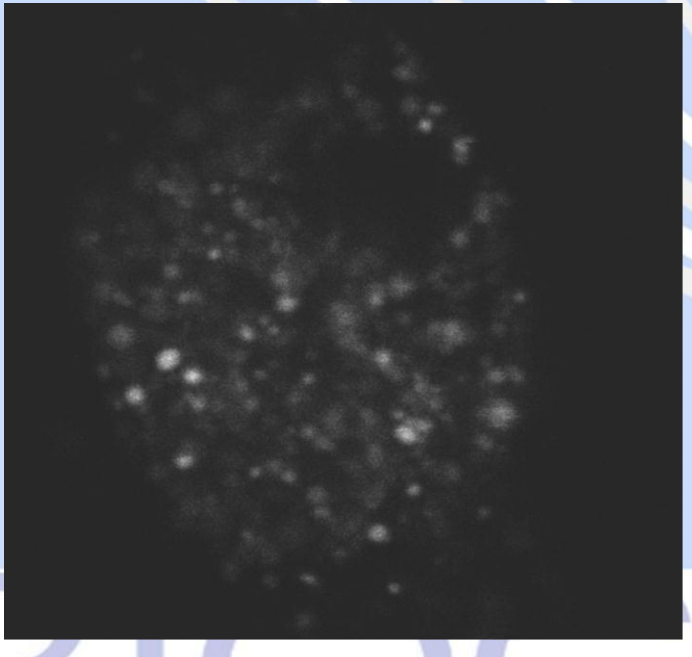
MATLAB based image analysis

- Matlab: campus licence MUI
- General mathematical software
- Image processing toolbox
- Easy to automate>many images
- Easy to combine different steps
- Example: complex tresholding task

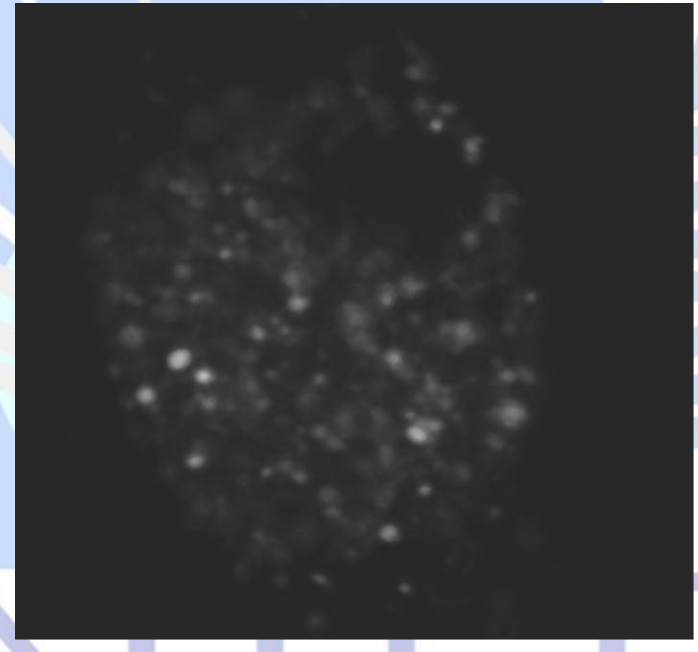
Complex thresholding problem I

primary mouse macrophages
loaded with Dextran-Oregon green 514
Challenge: threshold/mask individual endocytic vesicles

Original

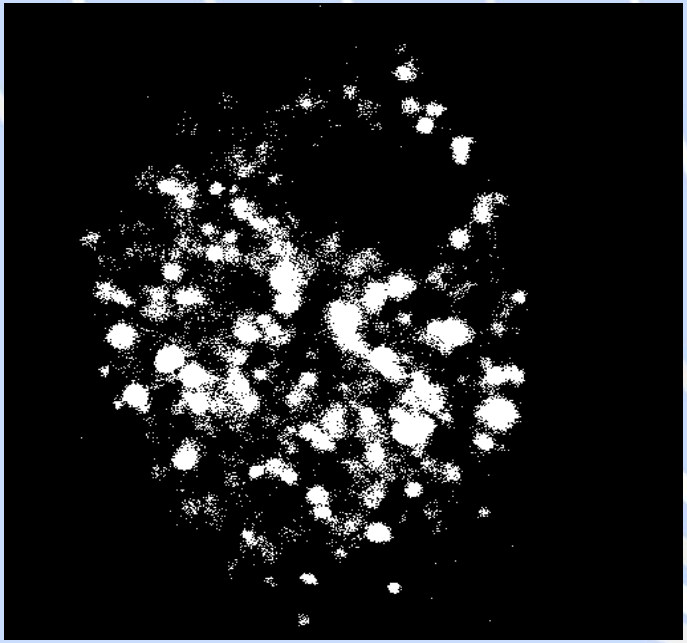


Median filter

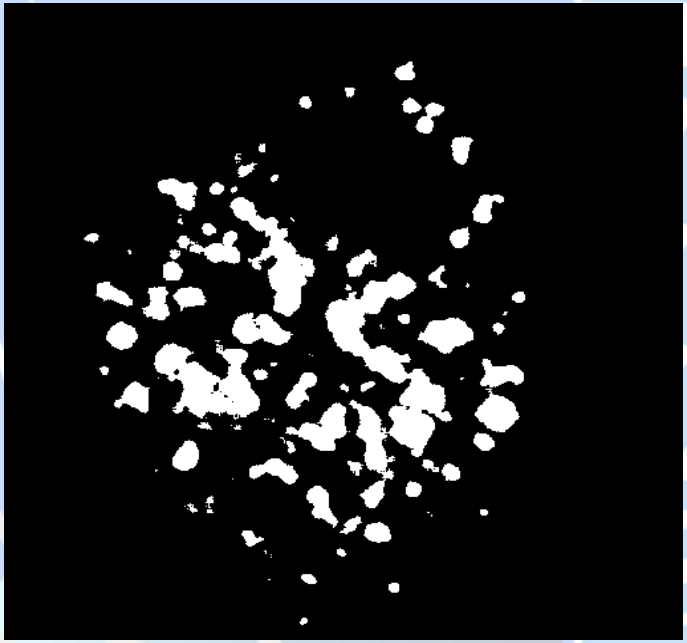


Complex thresholding problem II

Mask from original



Mask from median filter

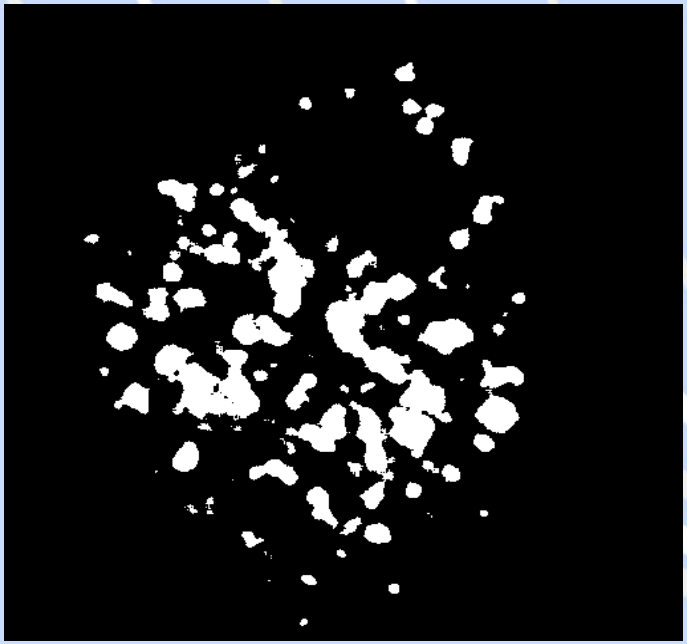


Median filtering: improves but does not solve the problem

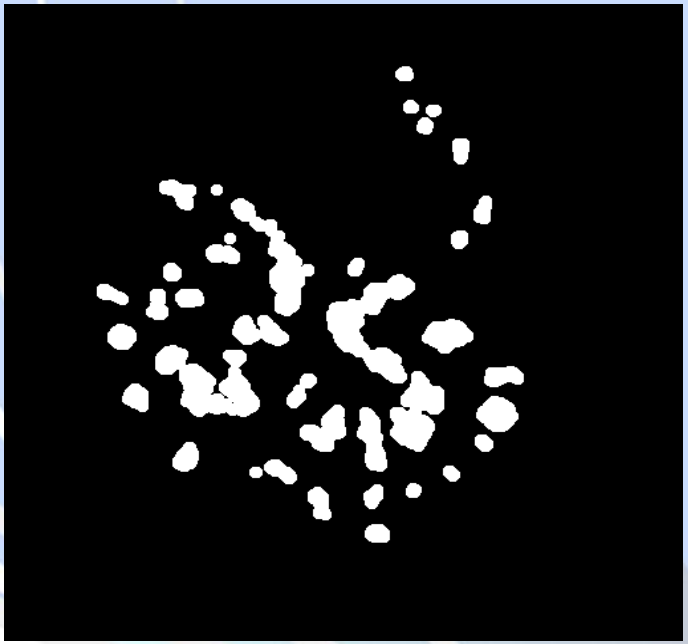
Opening?

Complex thresholding problem III

Mask from median filter



Opened mask, str.: disk, 5 pix



Opening?: separation improved, BUT endosomes lost

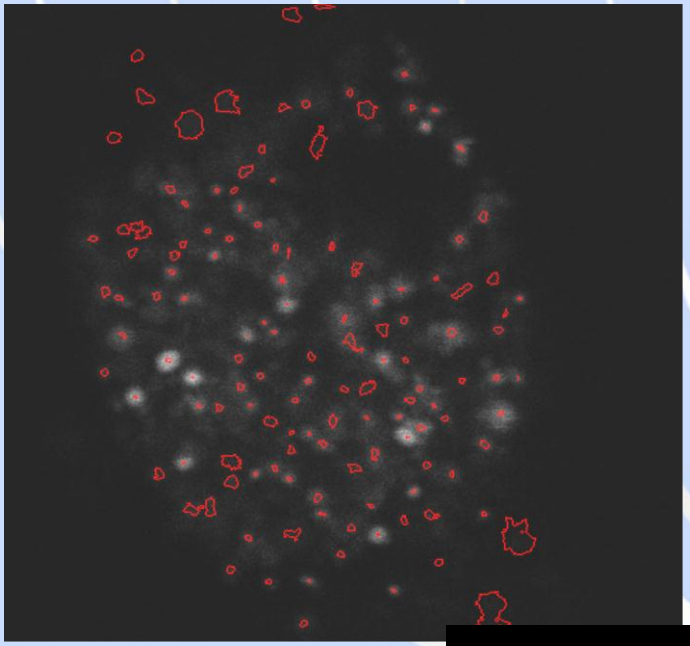
Complex thresholding problem IV - regional maxima

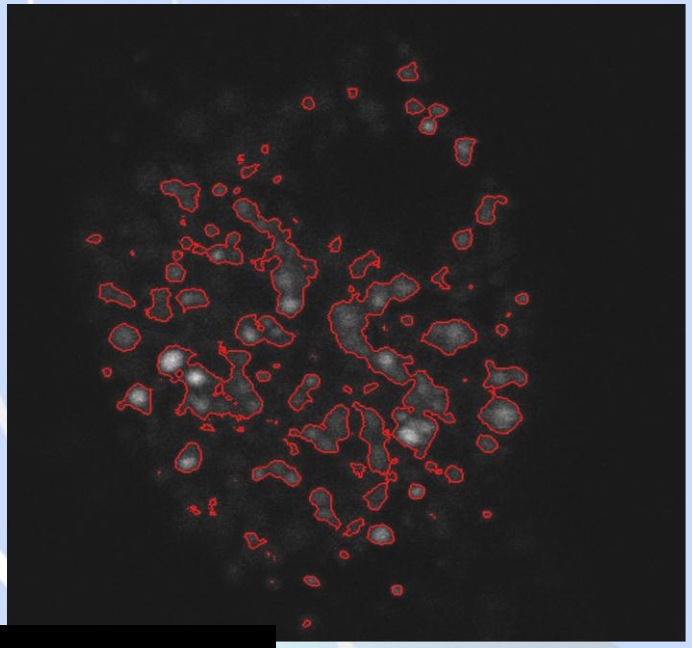
10	10	10	10	10	10	10	10	10	10
10	22	22	22	10	10	44	10	10	10
10	22	22	22	10	10	10	45	10	10
10	22	22	22	10	10	10	10	44	10
10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	33	33	33	10	10
10	10	10	10	10	33	33	33	10	10
10	10	10	10	10	33	33	33	10	10
10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10

0	0	0	0	0	0	0	0	0	0
0	1	1	1	0	0	0	0	0	0
0	1	1	1	0	0	0	1	0	0
0	1	1	1	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	1	1	1	0	0
0	0	0	0	0	1	1	1	0	0
0	0	0	0	0	1	1	1	0	0
0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0

Regional Maxima: a certain difference between local maximum/surrounding pixels
MATLAB: can identify regional maxima: „imextendedmax“

Complex thresholding problem V - regional maxima

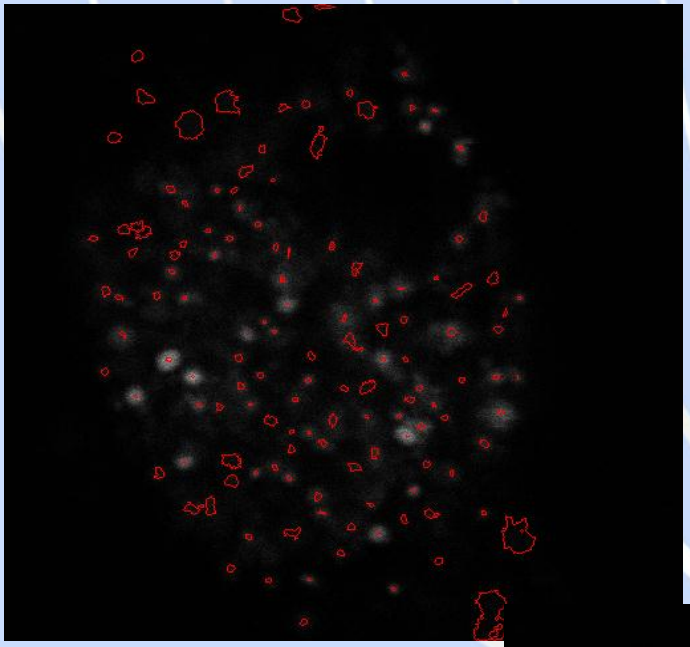


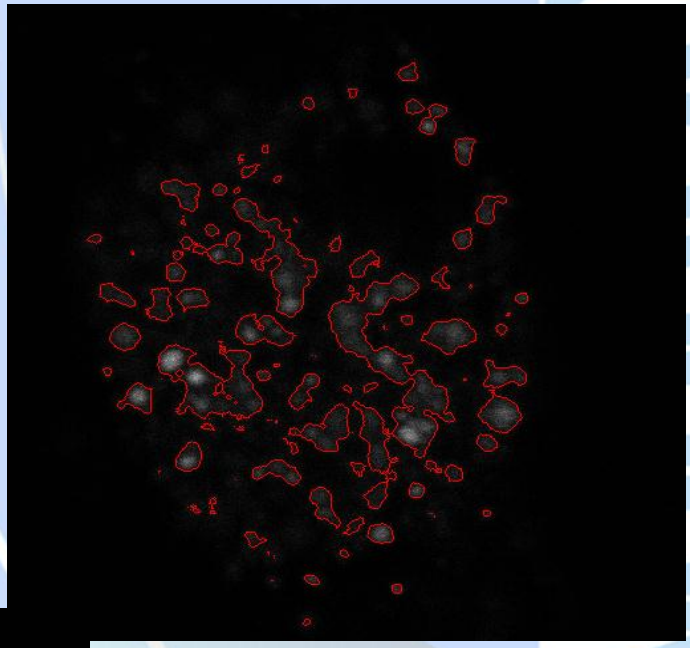


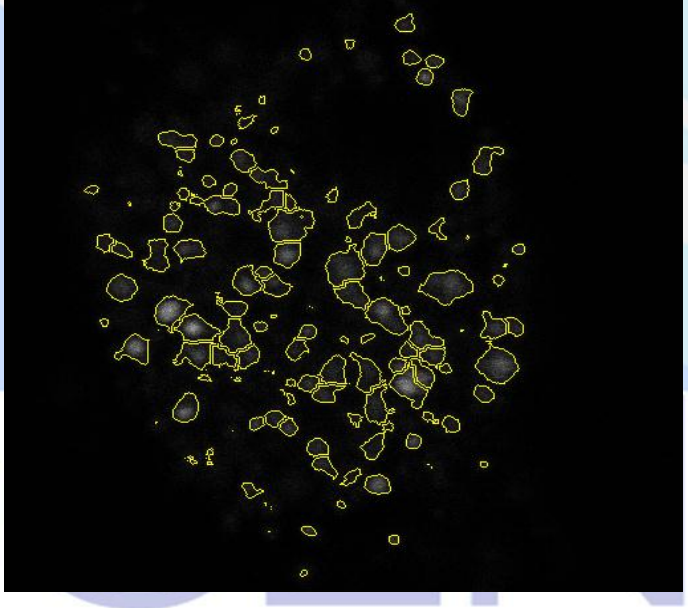
Regional Maxima: certain difference local maximum/surrounding pixels

Combine: regional maxima with absolute thresholding-> watershed!!!!

Complex thresholding problem VI – watershed with maxima







Regional Maxima: certain difference local maximum/surrounding pixels

Watershed mask: improved result

Image normalization

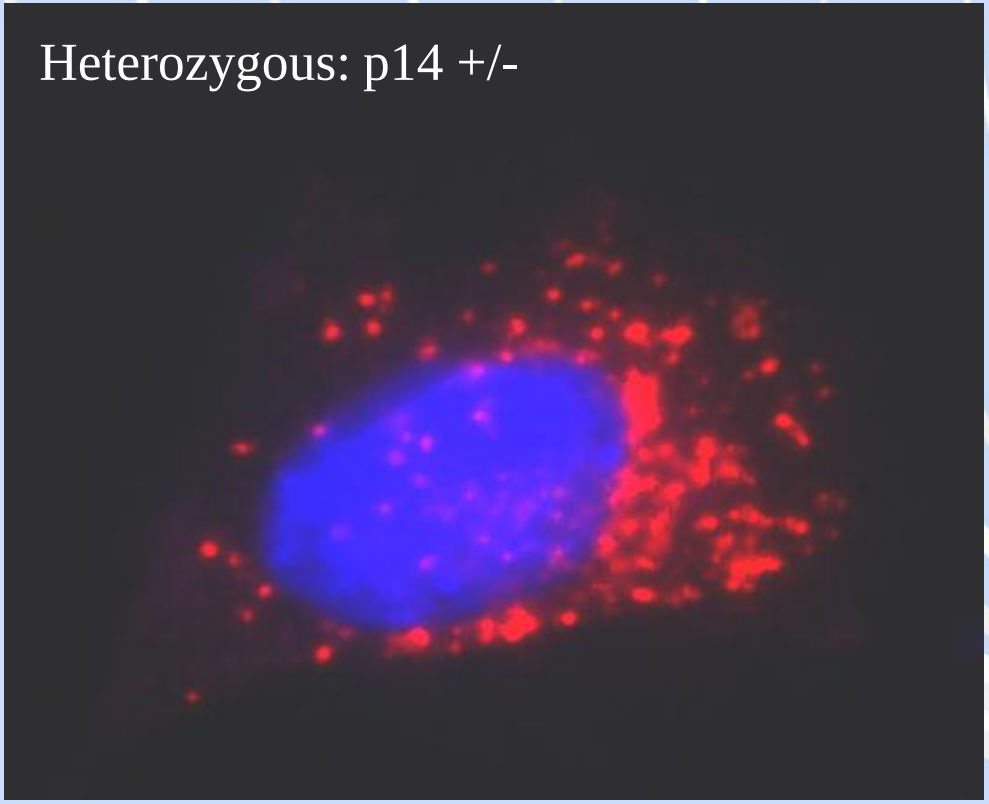
- divide images by maximum value > scale 0 to 1
- compare images of different intensities
- 32-bit floating point

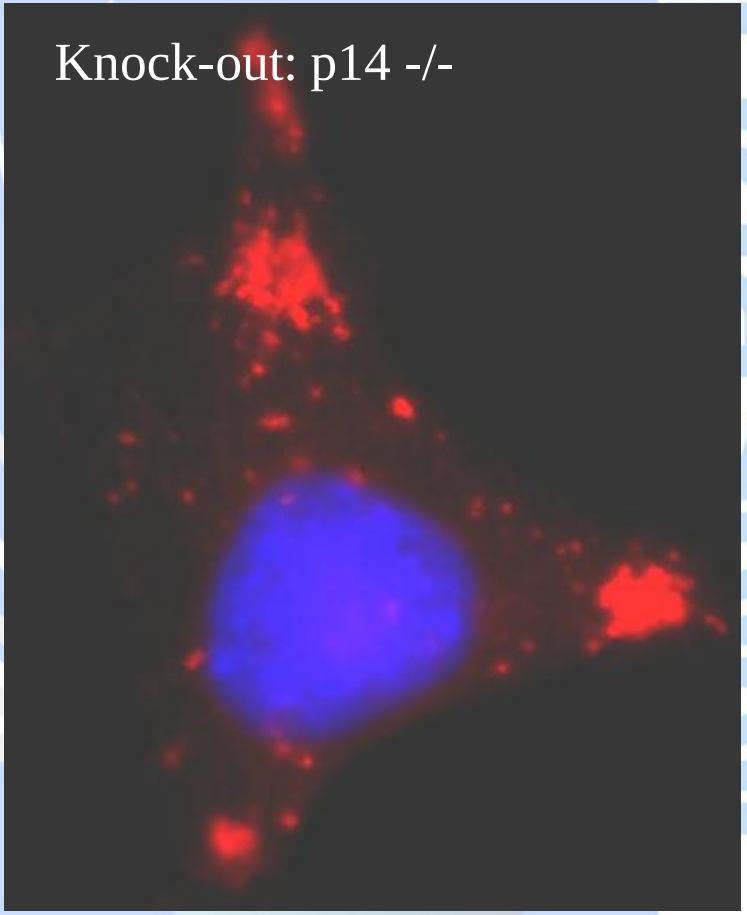
- Background, mask, advanced thresholding
- Colocalization
- Time series
- Deconvolution
- 3D images
- Linear unmixing
- Complex thresholding
- Full MATLAB analysis

Practical example: late endosomes full MATLAB-based analysis

- endocytosis-induced vesicles
- positive for LAMP1
- accumulate at peri-nuclear region
- KO cells transport-defect: late endosomes/cell periphery

“late endosomes: mislocalized in p14 -/- cells”





Referee: “It would have been good to see a quantitative analysis of the late-endosomal mislocalization reported for p14 -/- cells.”

Measuring endosome distributions

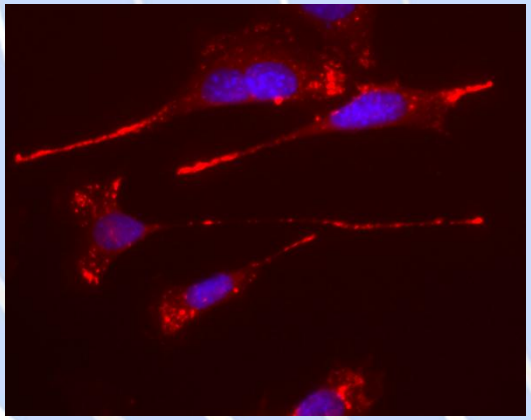
- Print images & measure endosomes with ruler
- Big differences in intensity?
- Closely spaced/touching endosomes?
- Tedious & biased analysis (100s of endosomes/cell…. 1000s to analyze)

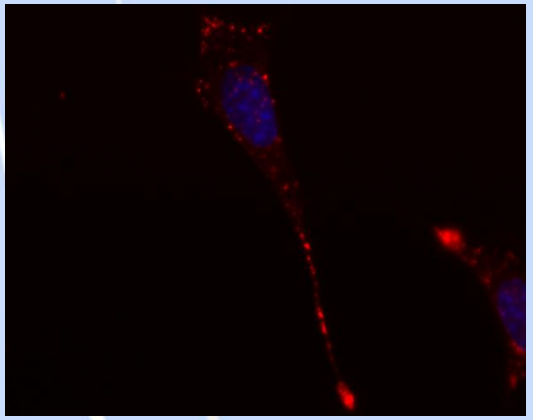
➢ Automatic analysis

Strategy for Quantitation

1. Read and preprocess images
2. Identify cell center
3. Identify endosomes
4. Identify positions
5. Calculate distances
6. Output

Preprocess: one cell/image





- Cut images > one cell/image
- Two complete cells > divide image into 2,…
- Incomplete cell: remove!

1. Read and preprocess images
2. Identify cell center
3. Identify endosomes
4. Identify positions
5. Calculate distances
6. Output

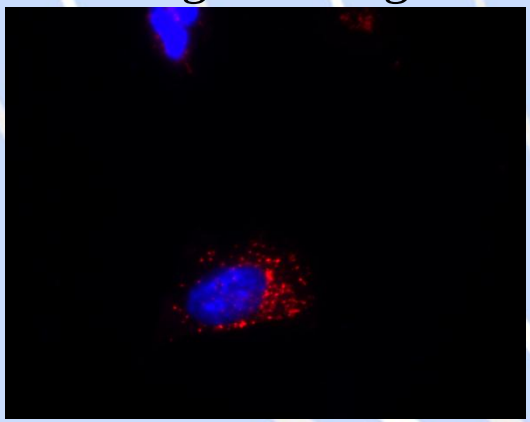
Identify cell center

- "Cell Center = Center of Nucleus (CoN)"
- Basic filtering, segmentation, binary mask
- CoN: Pixel-Coordinates ("x"=lines, "y"=rows)
- Late endosomes are peri-nuclear
- CoN: as reference to measure distribution

1. Read and preprocess images
2. Identify cell center
3. Identify endosomes
4. Identify positions
5. Calculate distances
6. Output

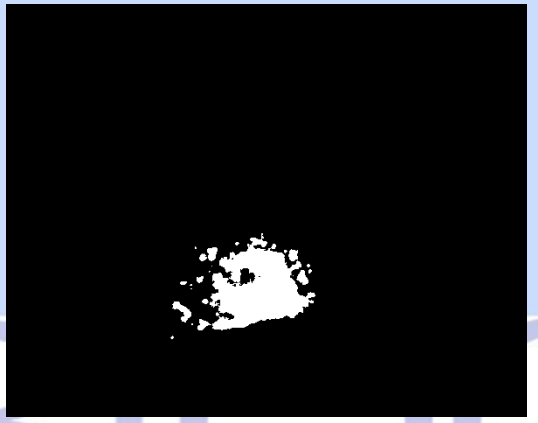
Identify Endosomes: Threshold?

Original Image



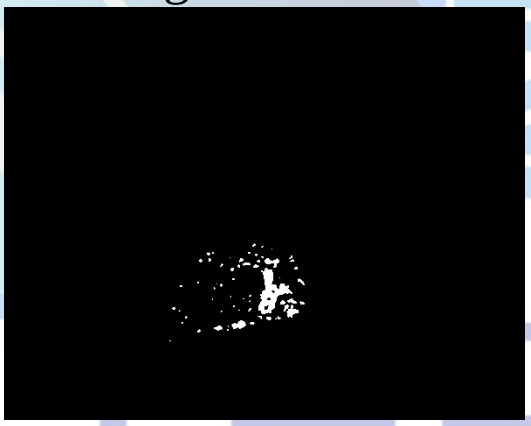
Low threshold = endosomes “fuse”
High threshold = weakly stained endosomes lost

Low…



Medium…

High threshold



Identify endosomes: local maxima

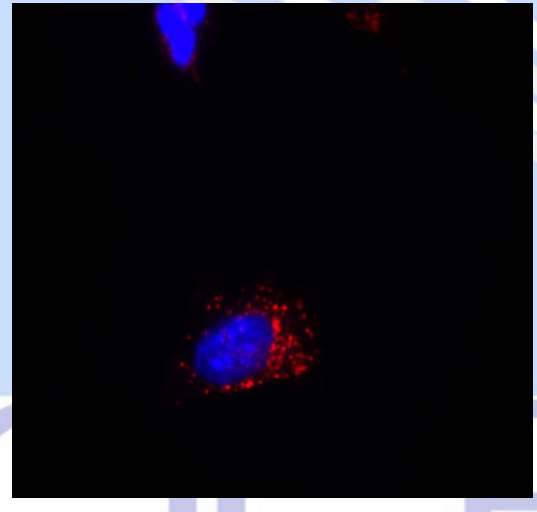
✓local maxima: “pixel that are more intense than the surrounding pixel”

✓“Pixel-value of local maximum > pixel-value of surrounding pixels”

☺Difference can be changed (acc. to image quality)

☹Disadvantage: size not always correct

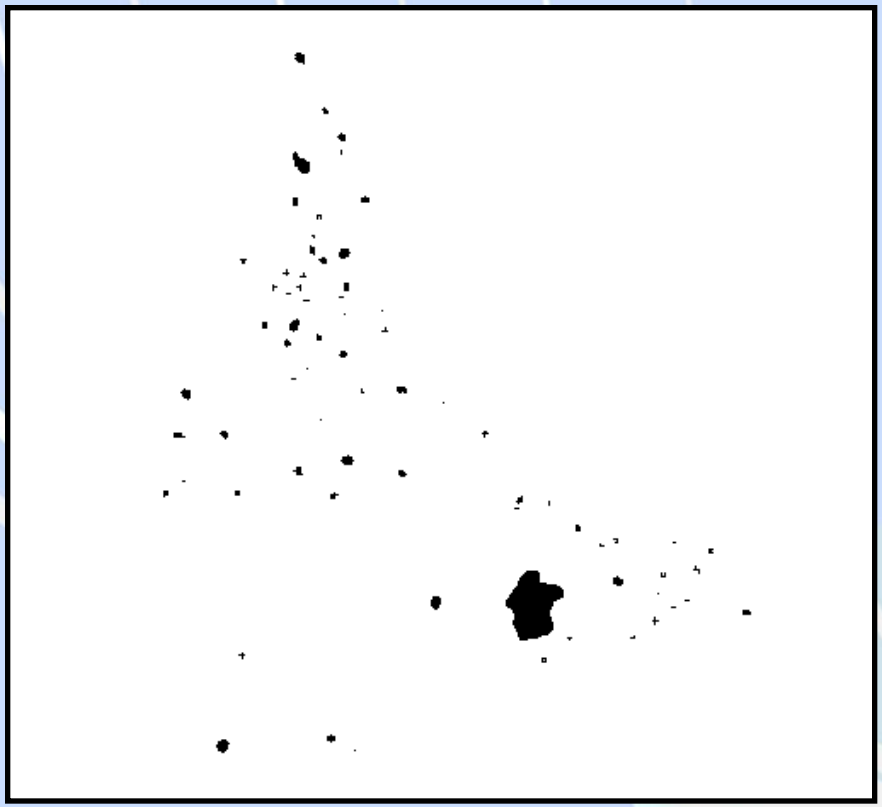
Original Image



Local Maxima

1. Read and preprocess images
2. Identify cell center
3. Identify endosomes
4. Identify positions
5. Calculate distances
6. Output

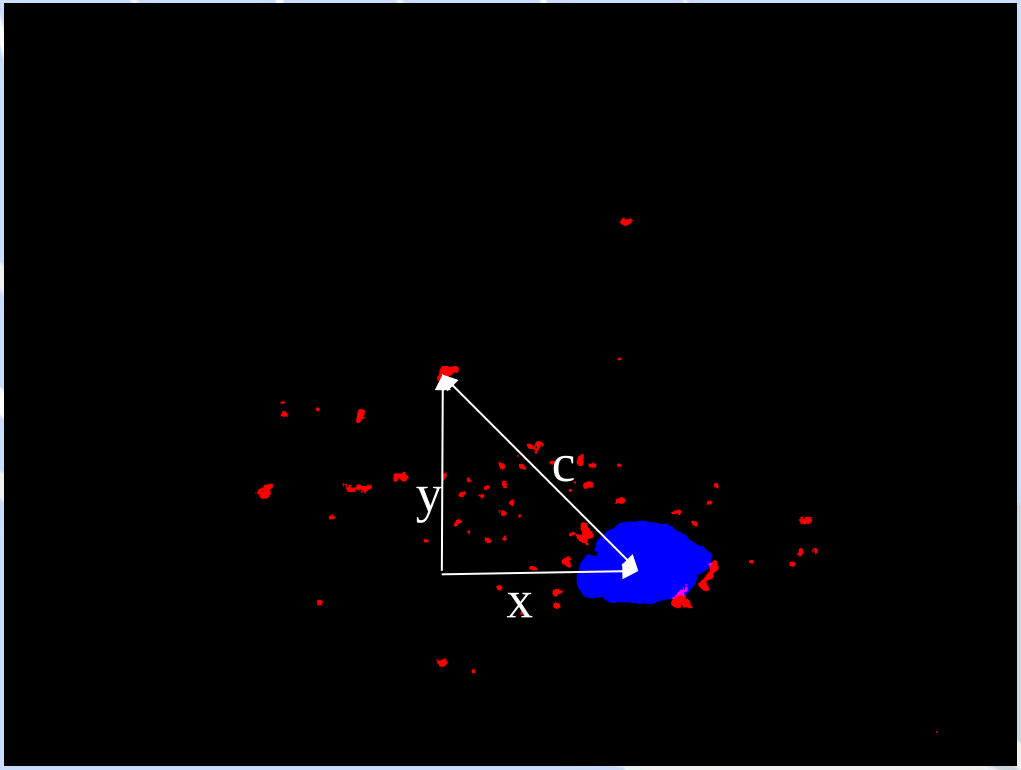
Identify positions



- ✓ Center of endosome
- Get Pixel-Coordinates (“x”=lines, “y”=rows)
- [X,Y] Position for each endosome

1. Read and preprocess images
2. Identify cell center
3. Identify endosomes
4. Identify positions
5. Calculate distances
6. Output

Calculate distances

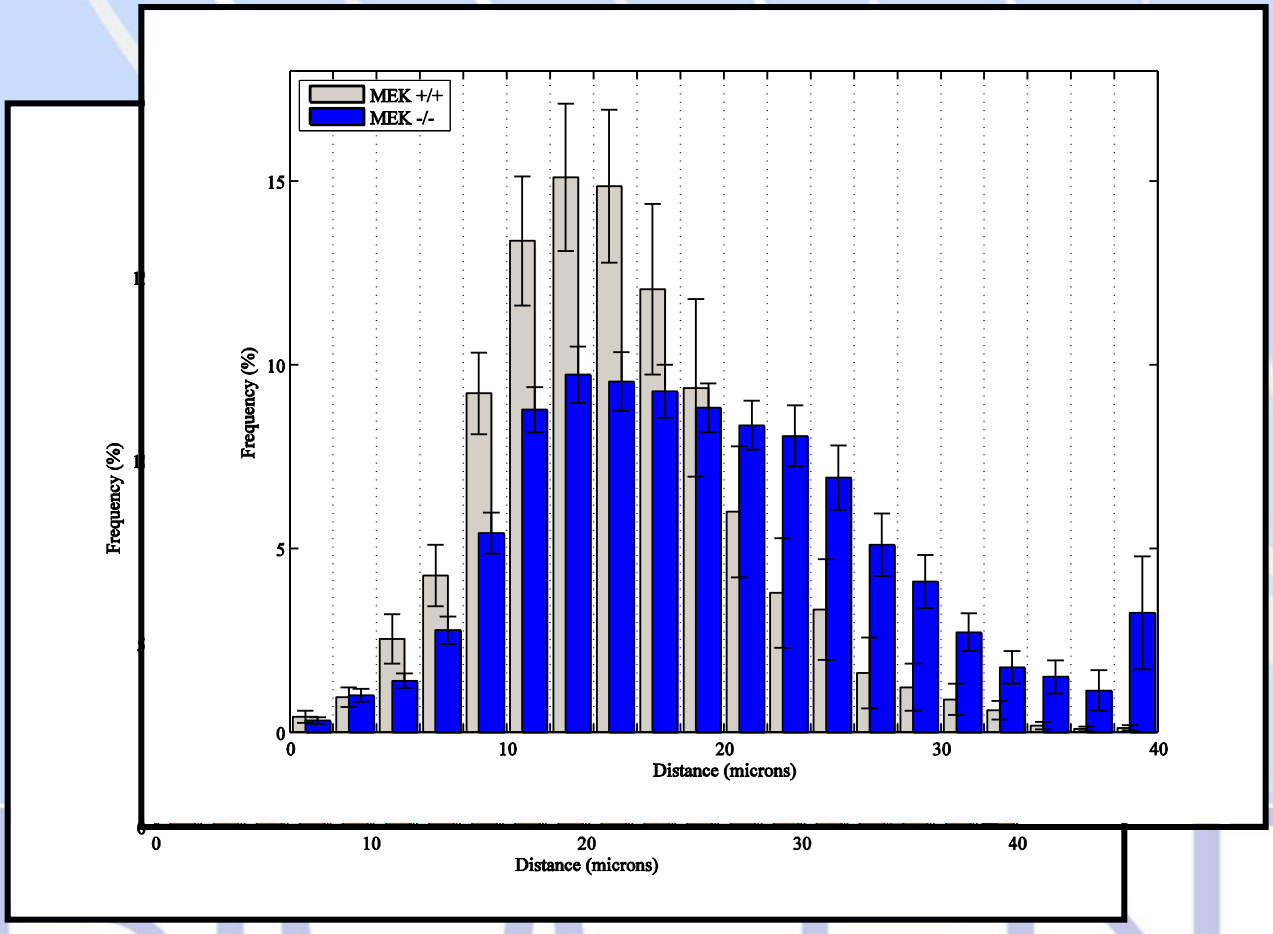


- ✓Distance c in pixel: $x^2 + y^2 = c^2$
- ✓Distance in microns = c*pixelsize (from microscope)

1. Read and preprocess images
2. Identify cell center
3. Identify endosomes
4. Identify positions
5. Calculate distances
6. Output

How many endosomes at each distance?

✓Distance distribution analysis



From: Teis et al.
J Cell Biol,
2006

Statistical significance

- are these distances sig. different?
- chi-square test: groups of properties
- confidence level $p < 0.001$ (as for t-test)
- difference confirmed

Conclusions

- ✓ nuclei as reference points for distance calculation
- ✓ endosomes represent local maxima
- ✓ distance distribution analysis preserves spatial information
- ✓ statistical Analysis: chi-square test

Example: applied IP techniques

Median filtering (nucleus, endosomes)

Mask-intensity threshold: nucleus

Mask-maxima: endosomes

Image-pixel positions: mask_nucleus mask_endosomes

Distances: Pythagoras‘ theorem

Binning

Statistics

Manual/Automated image processing

Manual

- lack of objectivity
- inadequate metrics: publication
- slow, costly
- boredom
- human error
- limited scientific discovery

Automated

- objective results
- reduce boredom
- decrease processing/analysis time
- alleviate human errors
- serendipity

More image processing & analysis

- **SS:**
  - **Microscopy: Basic Course (Neuroscience PhD Program)**
  - **Lecture: ,Lecture Series: Genomics, RNomics**

- **WS**
  - **Digital Imaging II: Applications**
  - **Microscopy: Basic Course (Ageing PhD Program)**
  - **Microscopy: Basic Course (Neuro, PhD)**
  - **Microscopy: Advanced Course (MCBO, FRET, FRAP…)**

3rd Part: Print-Ready Images

- Erich Brenner