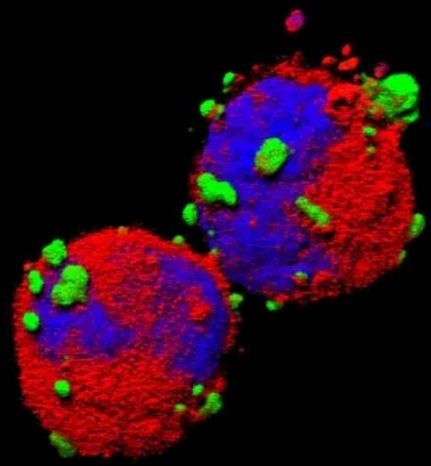
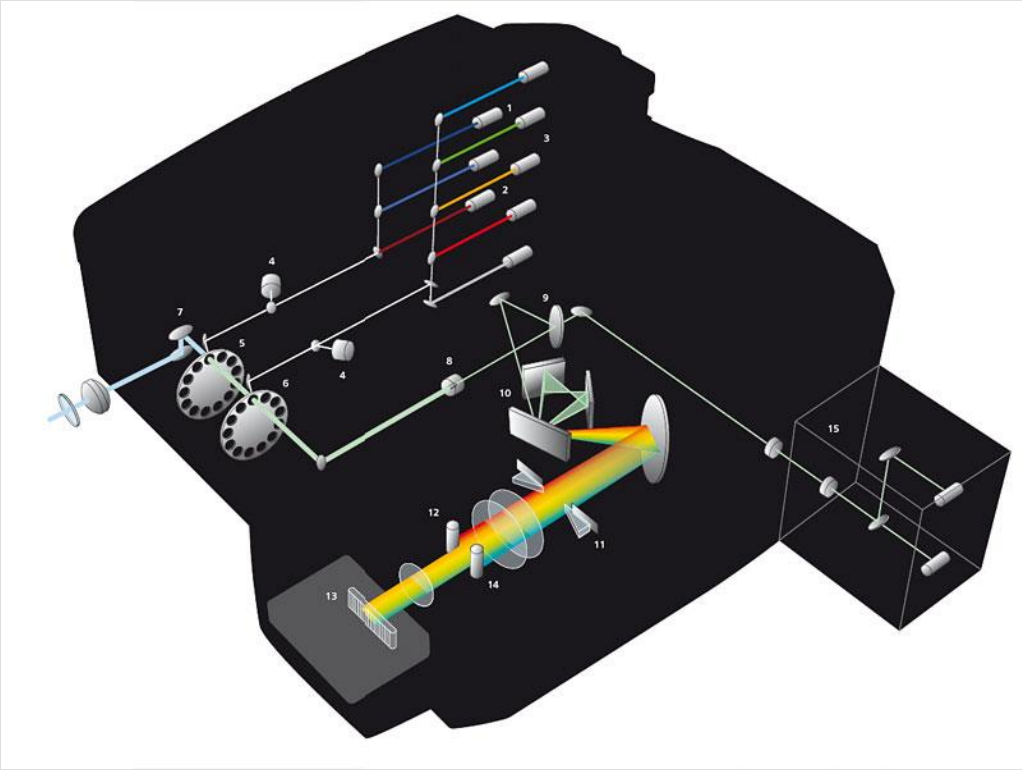
Imaging & analysis with the LSM780 NLO

Discover the secrets beyond the twilight zone

Sven Terclavers



LSM780
System overview



The Scan Module - Core of the LSM 780

1 V/tunable PTC laser ports (405/440, cw/ps; InTune)

2 IR PTC laser port (tunable Ti:Sa)

3 Vis PTC laser ports & Vis AOTF

4 Monitoring diodes

5 InVis TwinGate beamsplitter (upgradable)

6 Vis TwinGate beamsplitter (user exchangeable)

7 Scan mirrors (FOV 20, 6k × 6k)

9 Splitter for external channels

10 Spectral separation and recycling loop

11 Spectral beam guides

12 QUASAR PMT spectral channel # 1

13 QUASAR GaAsP spectral channels # 2–33

14 QUASAR PMT spectral channel # 34

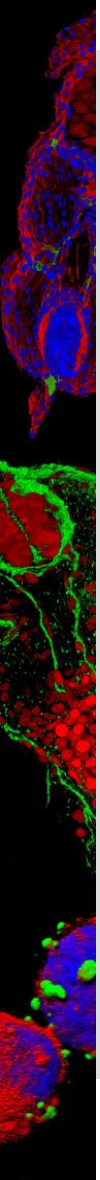
15 Ext. channels (APDs, BiG, FLIM, FCS etc.)

LSM780
System overview

- Multiple laser combinations
  - No dye that cannot be excited
  - Diode-, Gas-, Multiline-, Two-photon lasers
- Recycling loop
  - Capture more than 98% of emission light
- GaAsP detection technology
  - Extreme sensitivity (better S/N ratio than HyD)
  - Enabling regular imaging AND photon counting (thus not only photon counting)
  - Enabling FCS

LSM780
Microscope stand

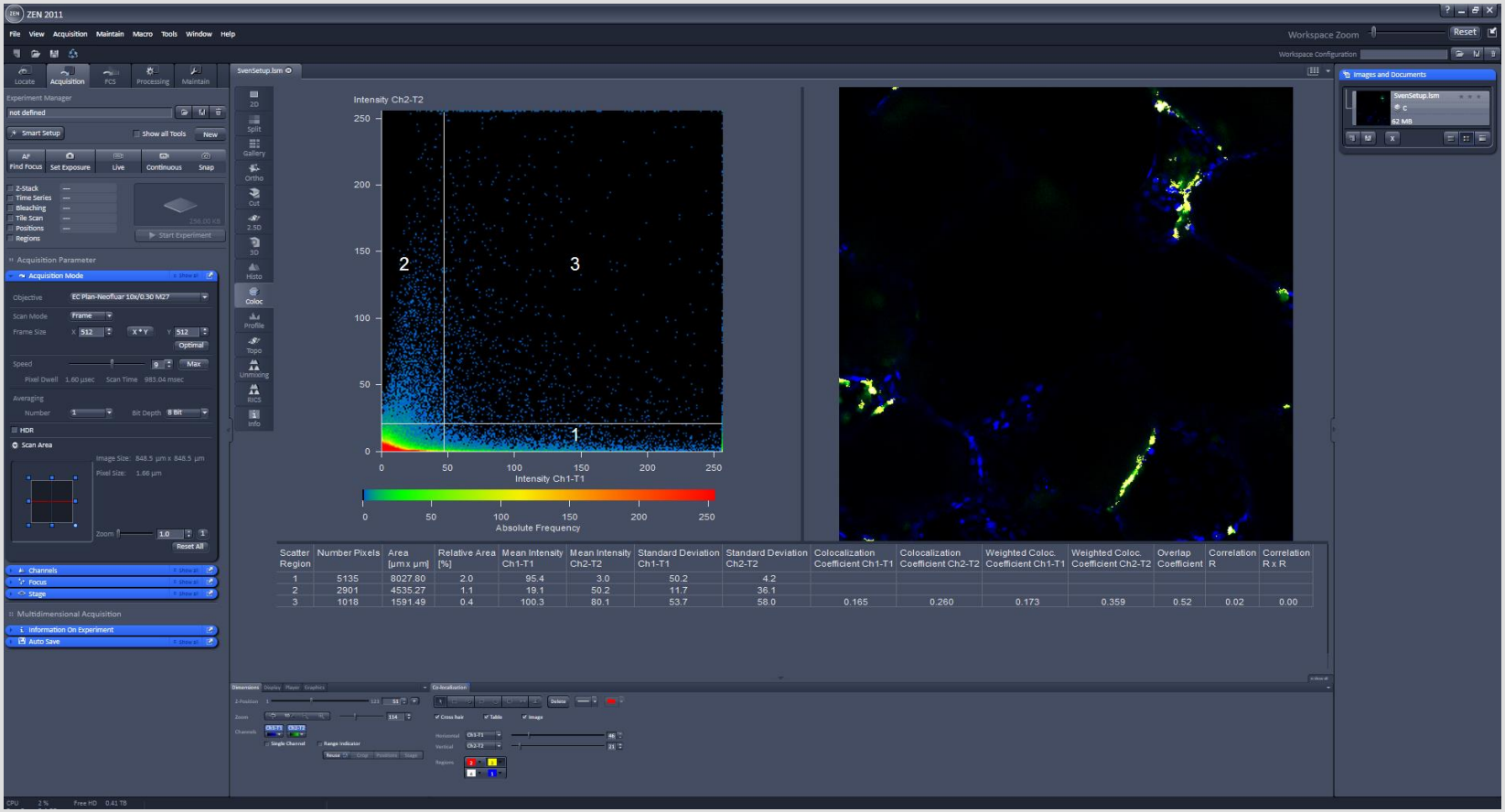
LSM780
Microscope stand



Basis	Imager.Z2	Examiner.Z1	Observer.Z1
Slides	X	x	x
Dishes		X (non-sterile)	X (sterile)
Live animals		X	x
Incubation		x	X
Colocalization	X	X	X
Spectral unmixing	X	X	X
Physiology		X	X
FRAP	x (acquisition)	X	X
FRET	x (acquisition)	x (acquisition)	X
HDR	X		
RICS	x (acquisition)	X	x (acquisition)
Tiles & Positions	X		X
Deconvolution	X	x (acquisition)	x (acquisition)


Visualize

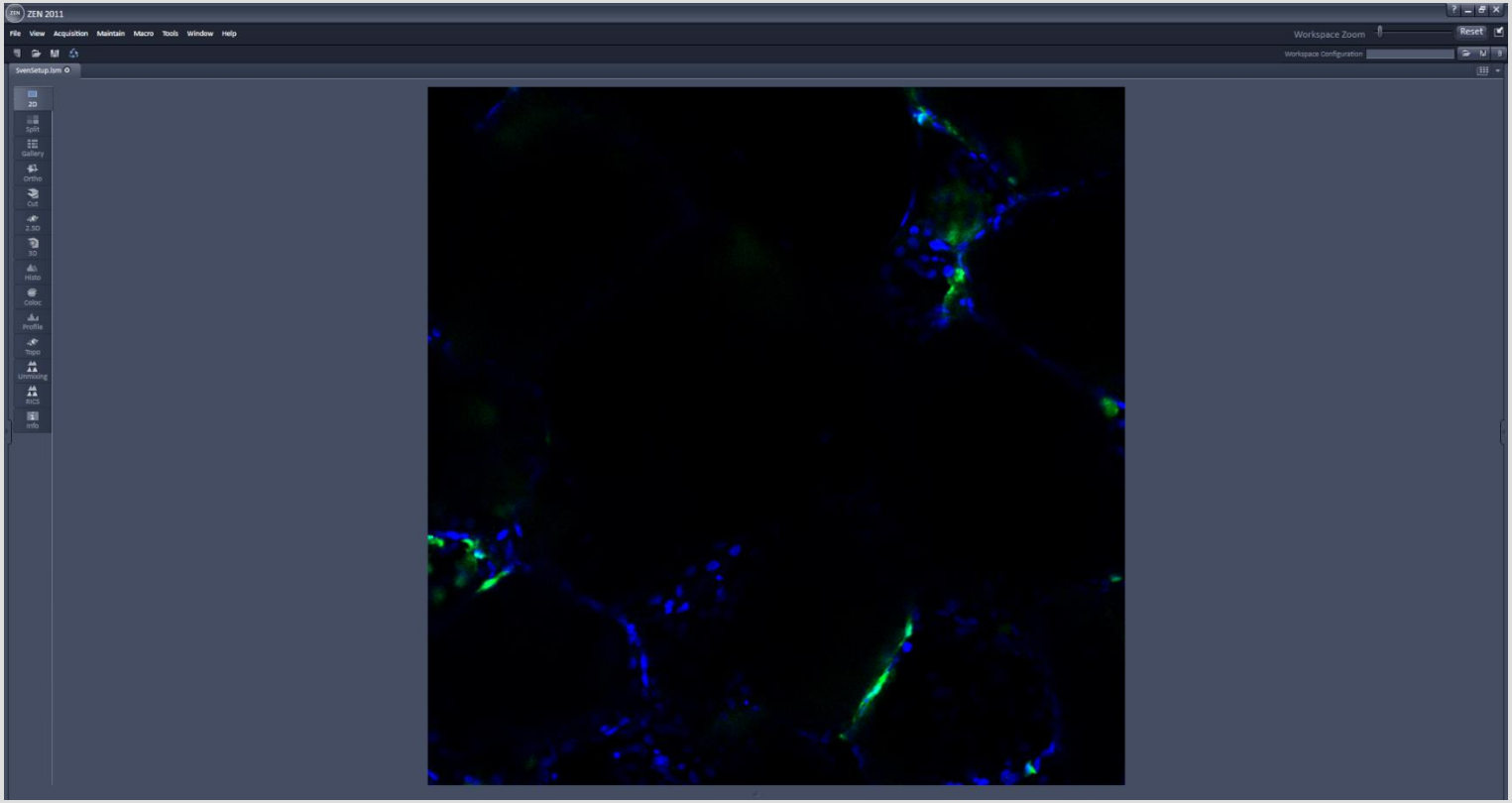
ZEN2012
Visualize and access at ease

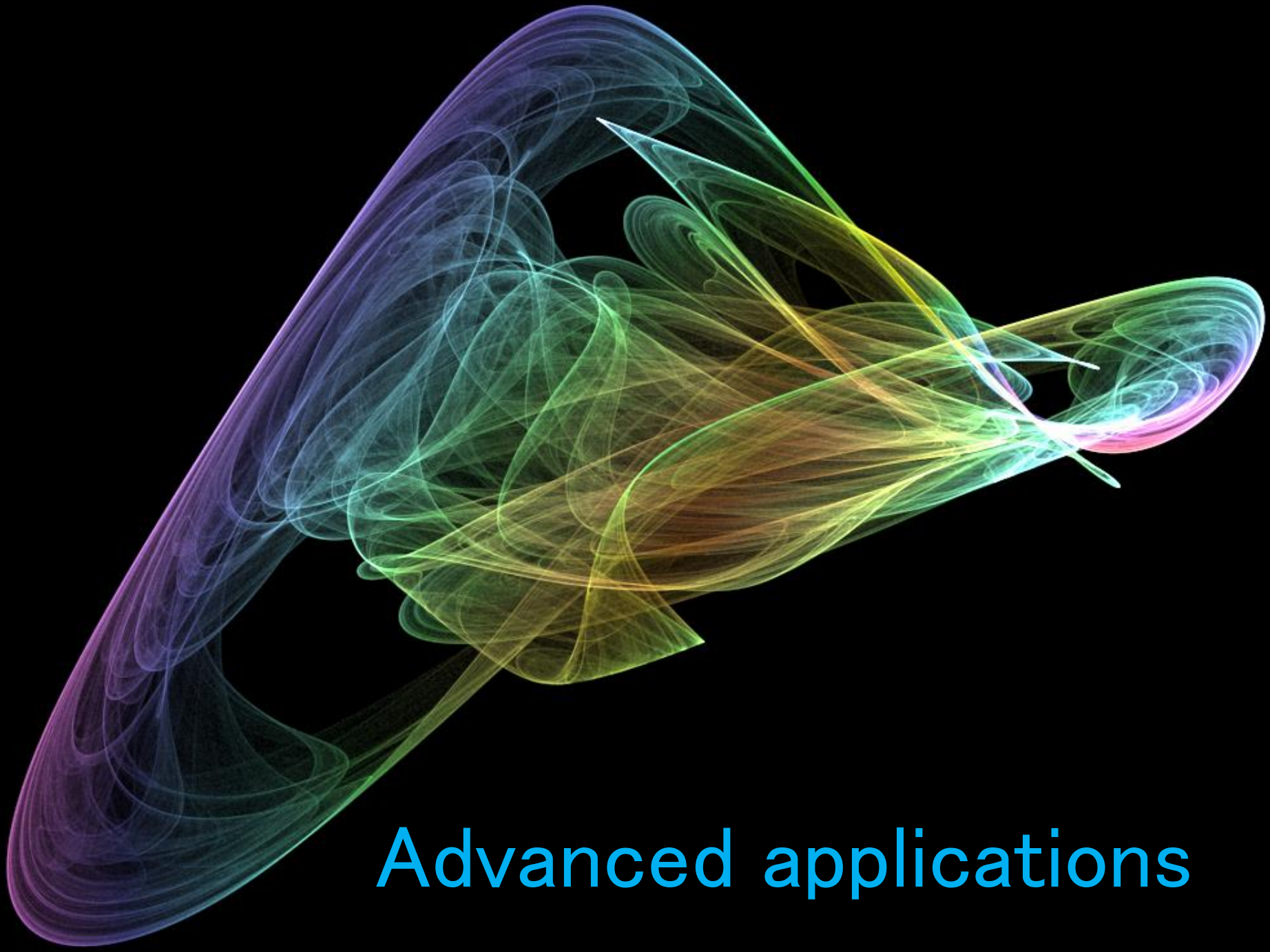


Scatter Region	Number Pixels	Area [µm x µm]	Relative Area [%]	Mean Intensity Ch1-T1	Mean Intensity Ch2-T2	Standard Deviation Ch1-T1	Standard Deviation Ch2-T2	Colocalization Coefficient Ch1-T1	Colocalization Coefficient Ch2-T2	Weighted Coloc. Coefficient Ch1-T1	Weighted Coloc. Coefficient Ch2-T2	Overlap Coefficient	Correlation R	Correlation R x R
1	5135	8027.80	2.0	95.4	3.0	50.2	4.2							
2	2901	4535.27	1.1	19.1	50.2	11.7	36.1							
3	1018	1591.49	0.4	100.3	80.1	53.7	58.0	0.165	0.260	0.173	0.359	0.52	0.02	0.00

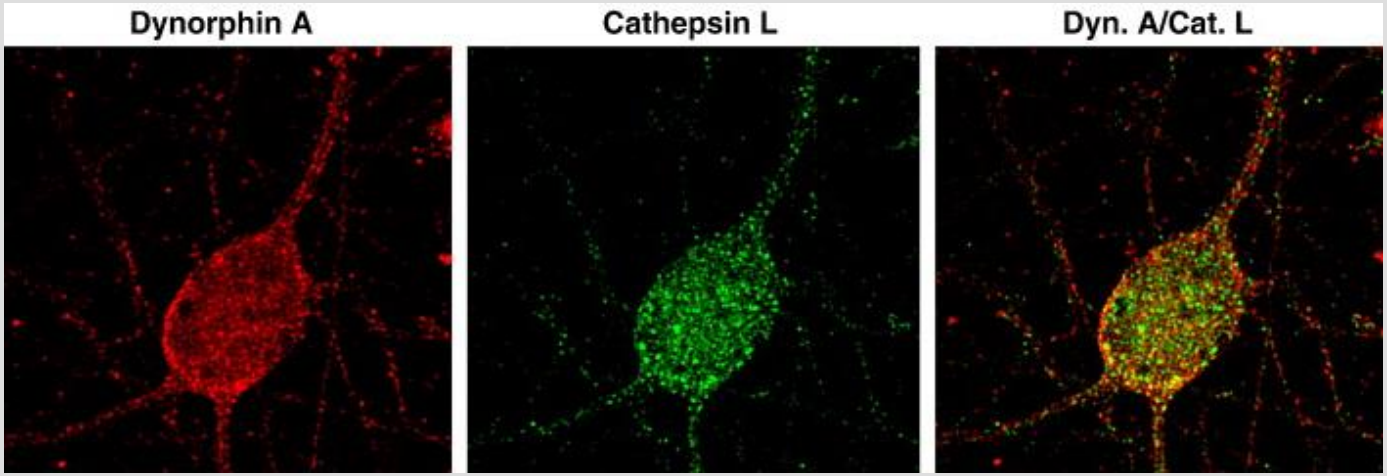
ZEN2012
Visualize and access at ease

- In ZEN 2012, the image is very „ego-centric“
- Configure, scan, image, analyze...all in one GUI, no complexities




Advanced applications

ZEN2012 Applications
Co-localization analysis

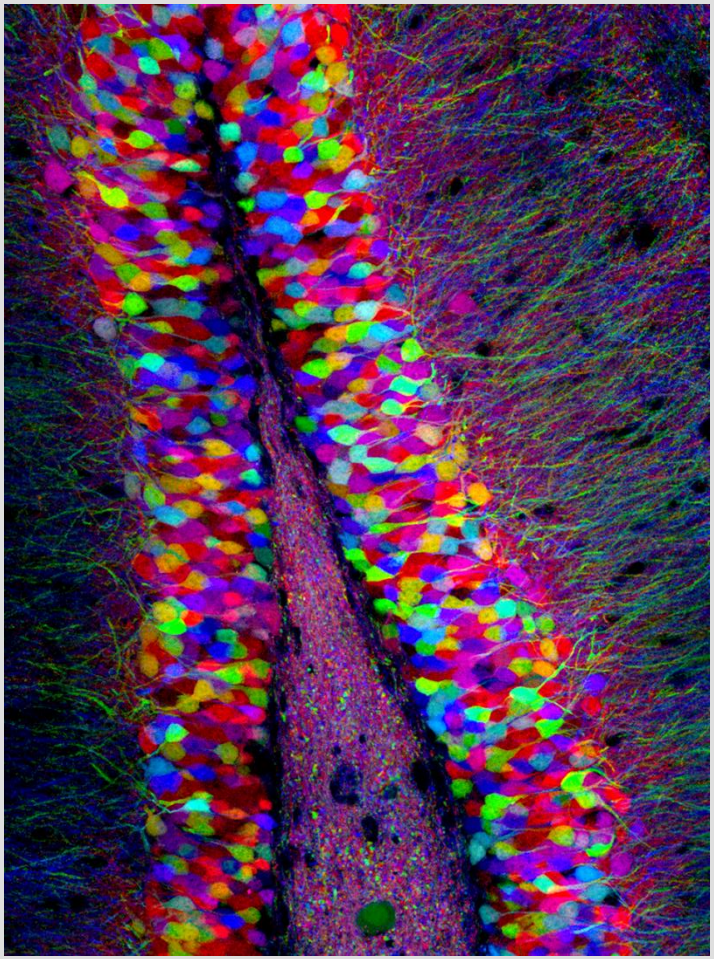


- In colocalization analysis, one observes whether two fluorophores are present in the same volume or not. Yellow pixels are a sum of a red and a green fluorophore. However, it is less straightforward, therefore ratios have to be calculated.

- Analysis helps with determining:
  - Localisation of molecules
  - Dynamical behaviour
  - Molecular interactions

ZEN2012 Applications

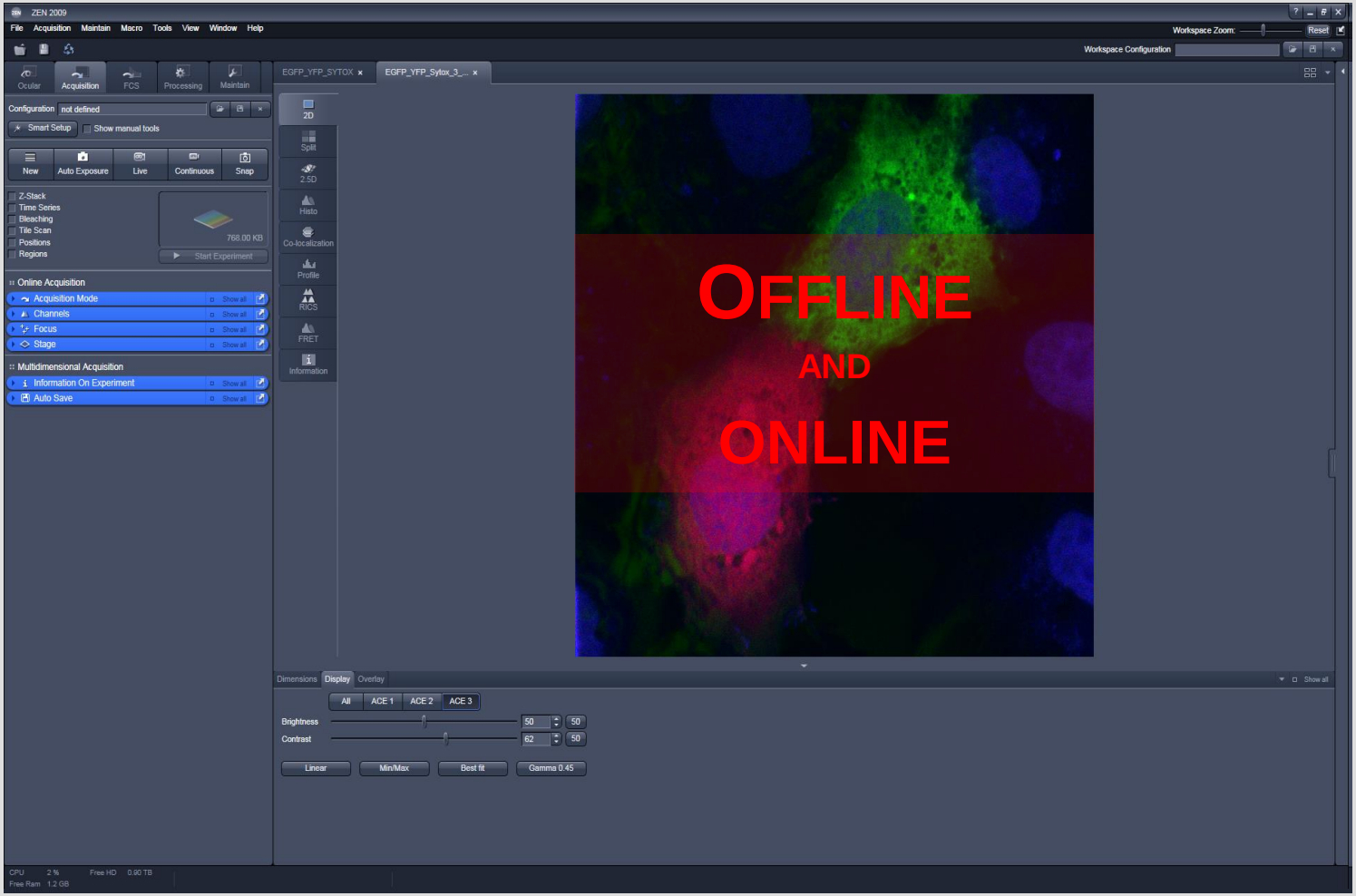
Spectral unmixing

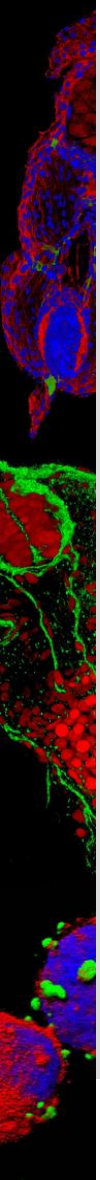


- Using the technique of the “Brainbow” mouse, different subpopulations of neurons emit different colours to allow studying brain connectivity.
- With current available fluorescence filters, it is impossible to seperate all these colours without having bleedthrough. Spectral unmixing – scanning of the whole light spectrum – overcomes this problem.
- Used for:
  - Detection of multiple fluorophores
  - Seperating GFP from YFP-tagged molecules

ZEN2012 Applications

Spectral unmixing





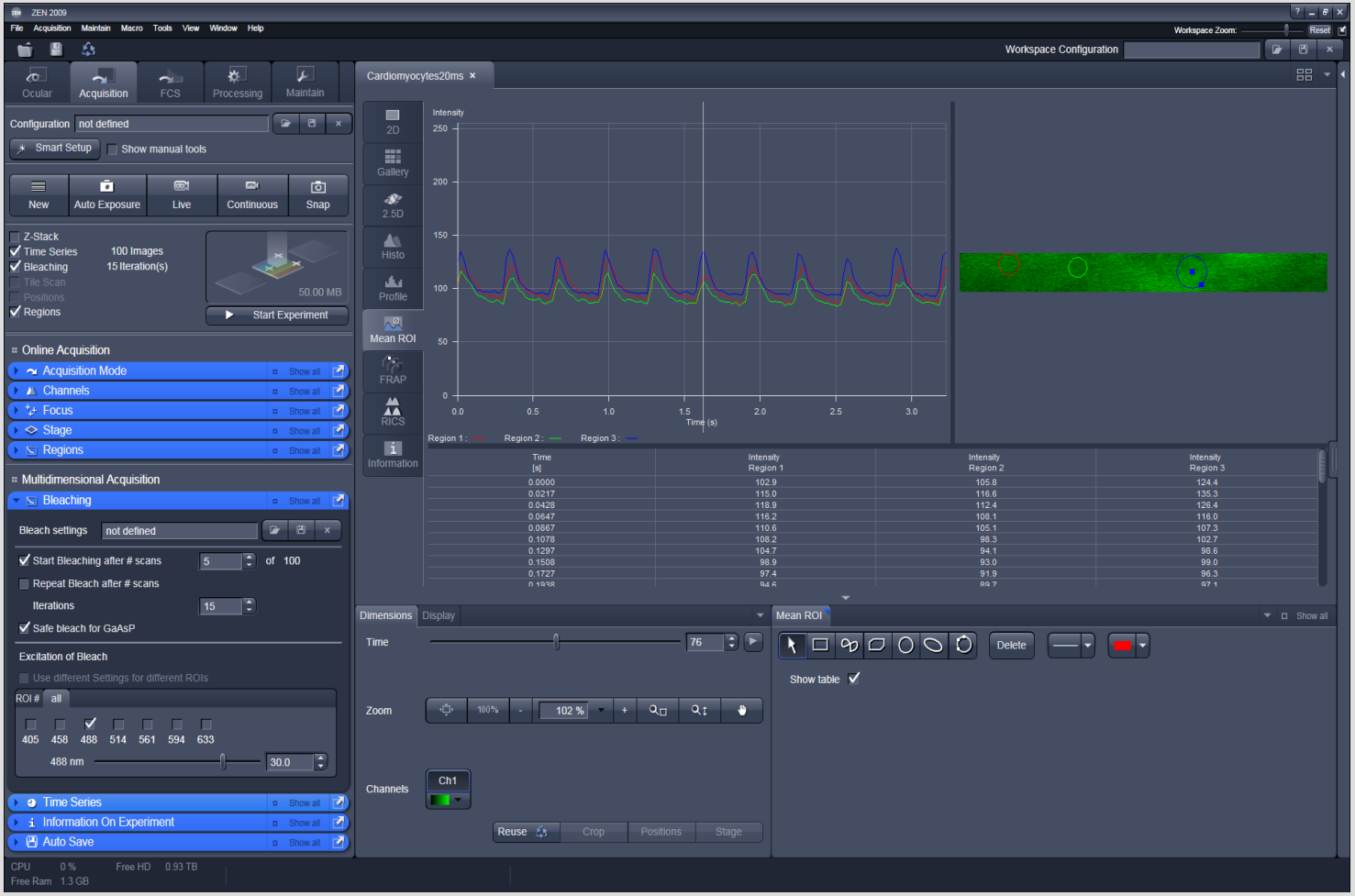
ZEN2012 Applications

Spectral unmixing

- Separate overlapping dyes
- Perform separation offline or online
- Create lambda-scan, define spectra, save in database
- Use database to acquire multiple dyes in one scan while being spectrally separatad
- Advantages:
  - Combine CFP, GFP, YFP etc
  - Improve FRET results
  - Improve co-localization
  - Speed up system
  - Improve RICCS (cross-correlation) analysis

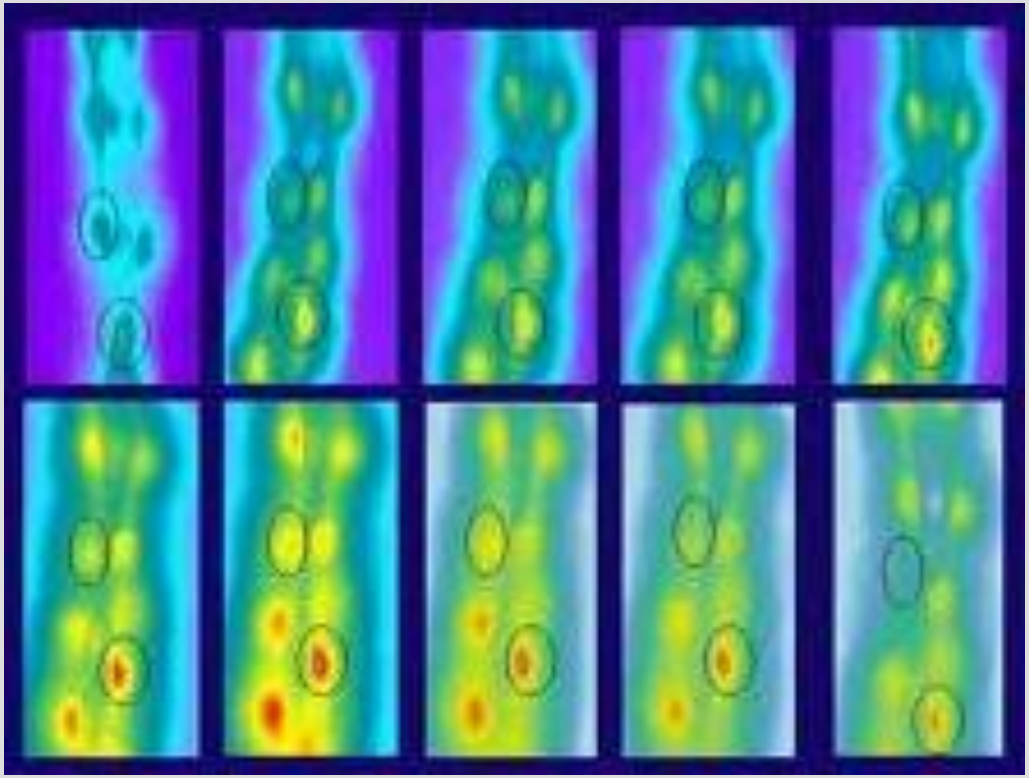
ZEN2012 Applications

Physiology



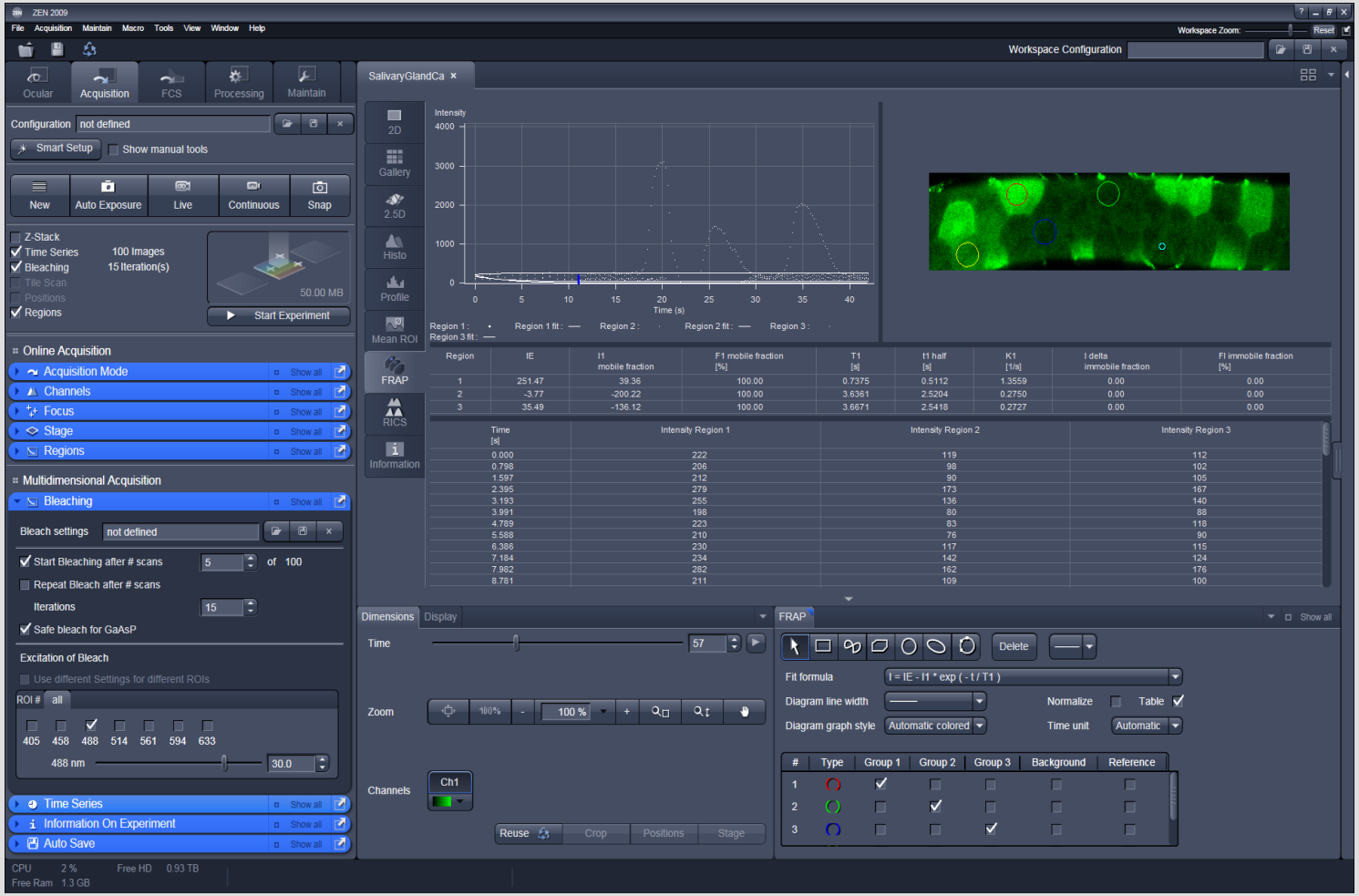
ZEN2012 Applications
Physiology

- Ratiometric imaging by measuring fluorescence intensity
- Used for analysis of:
  - State of calcium presence in cell (bound/free)
  - Intra/extracellular communication
  - Ion channel activity
  - Toxicity



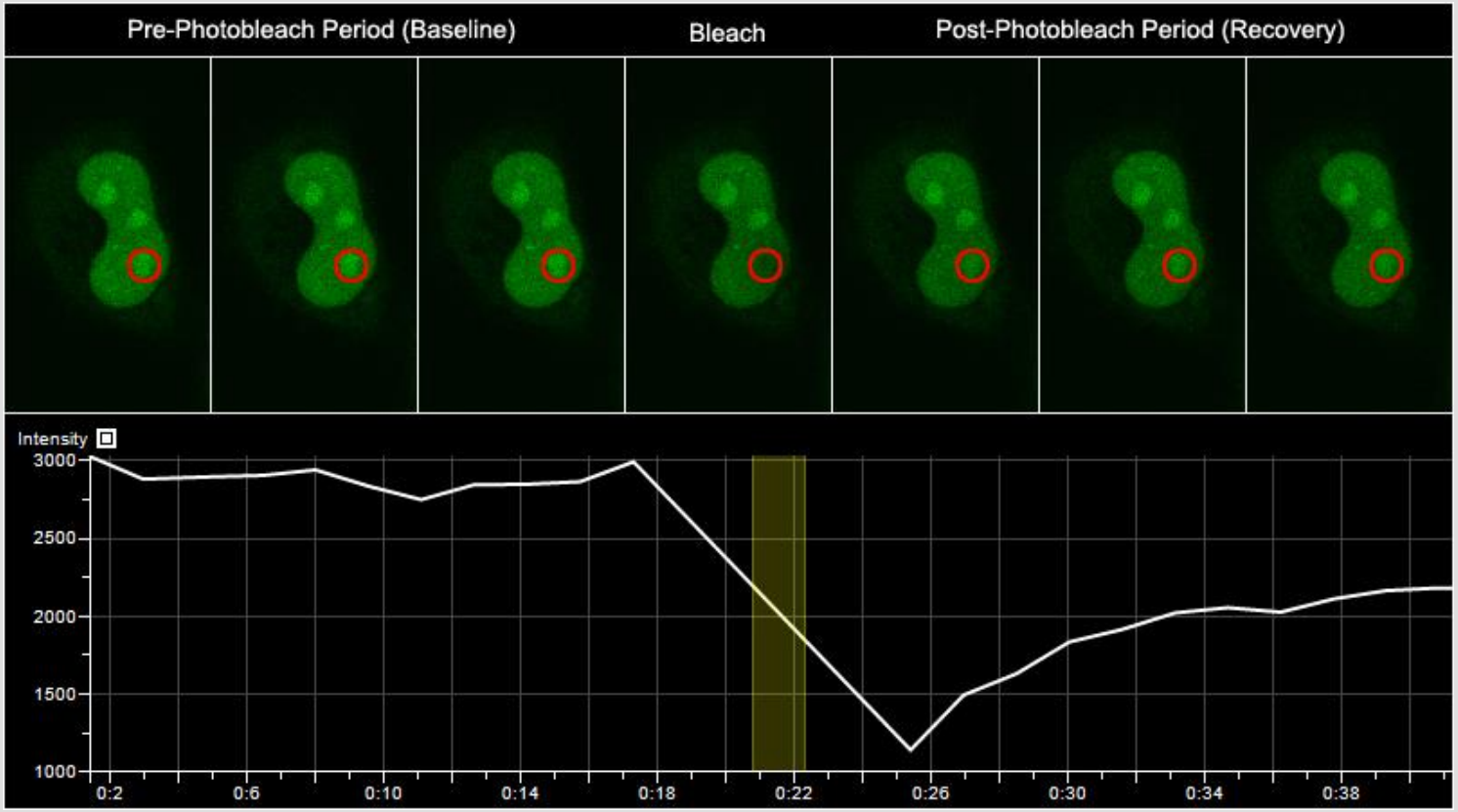
ZEN2012 Applications

FRAP – Fluorescence Recovery After Photobleaching



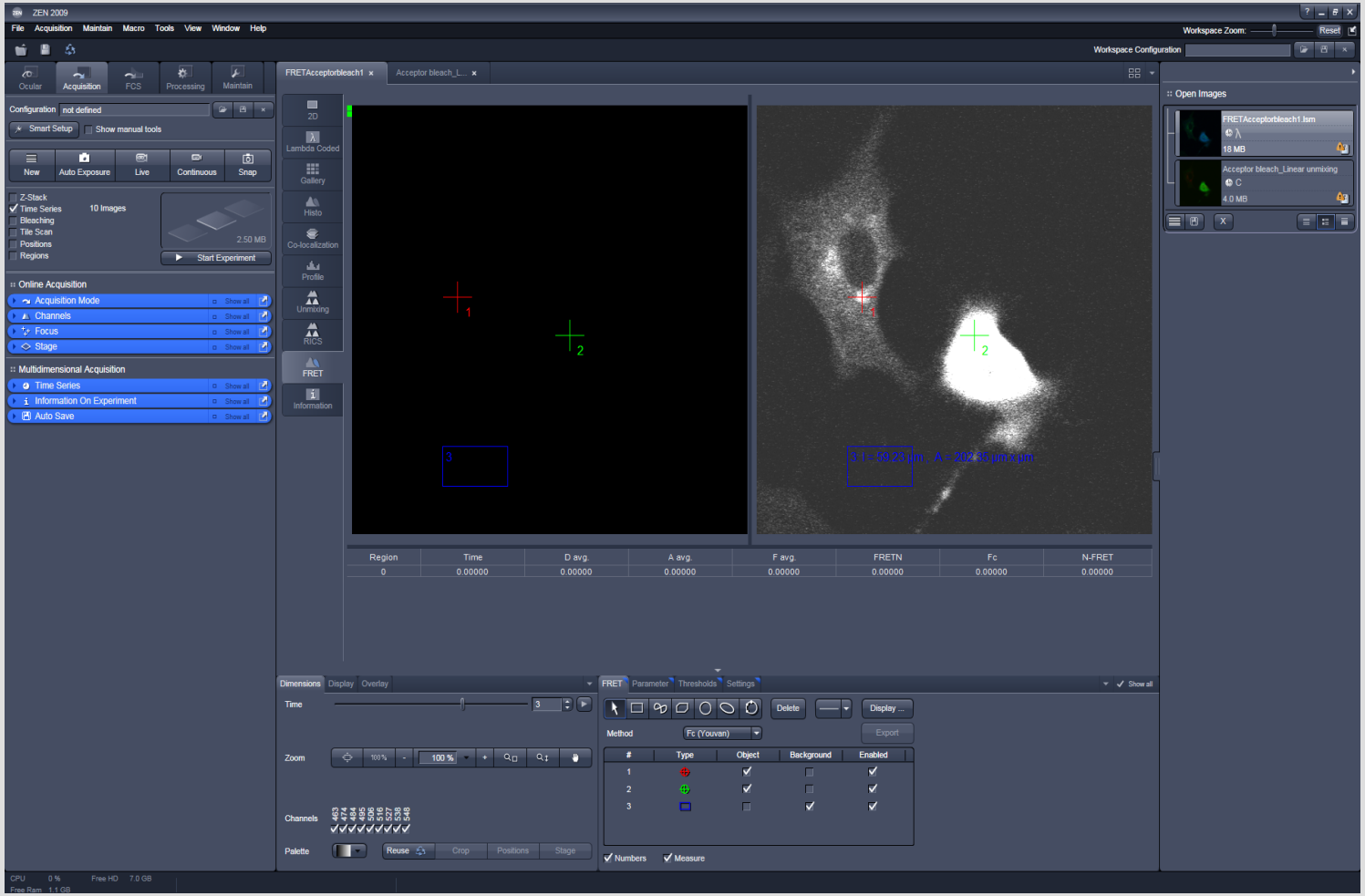
ZEN2012 Applications

FRAP – Fluorescence Recovery After Photobleaching



ZEN2012 Applications

FRET – Fluorescence Resonance Energy Transfer

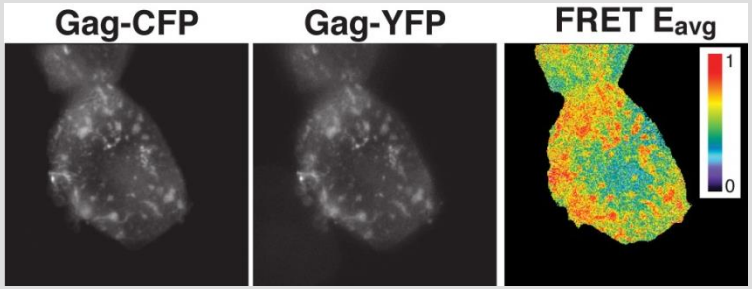


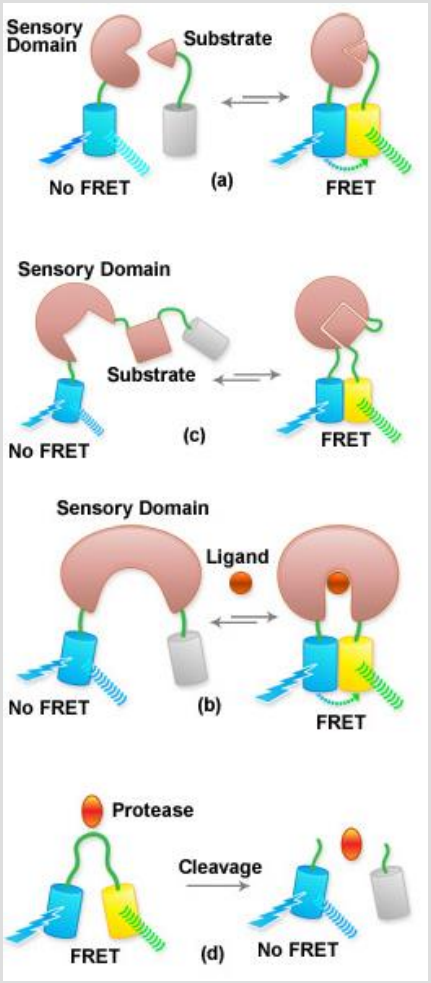
ZEN2012 Applications

FRET – Fluorescence Resonance Energy Transfer

- Used to observe interactions:
  2 differently stained molecules will transfer energy when they are closer than 10nm (interacting), as such that the second fluorophore – which is not excited with light – will start emitting light

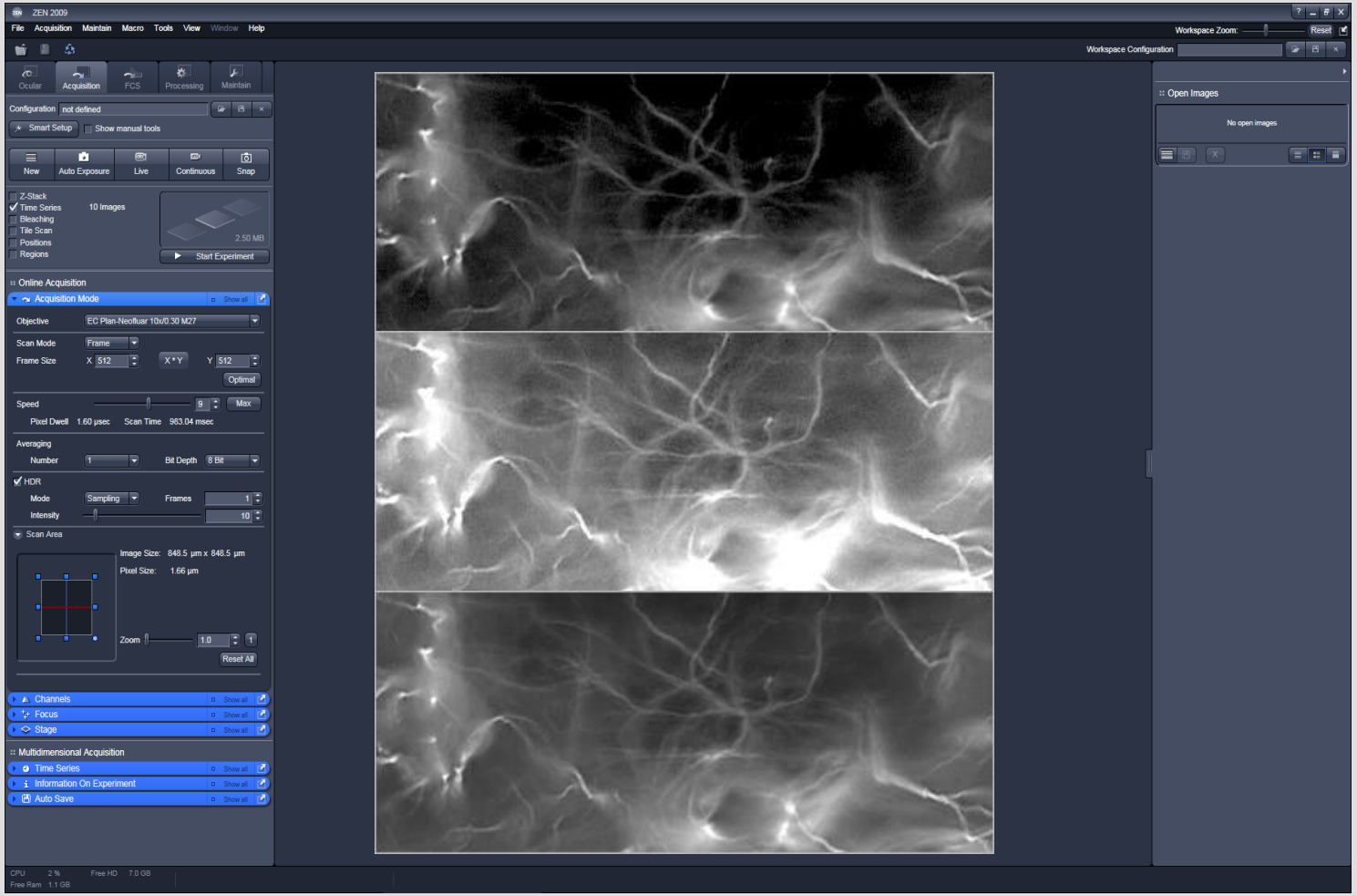
- Analysis of:
  - Receptor binding, antagonist binding,...
  - Structural changes of molecules (protein folding)
  - Ligand binding
  - Enzym activity





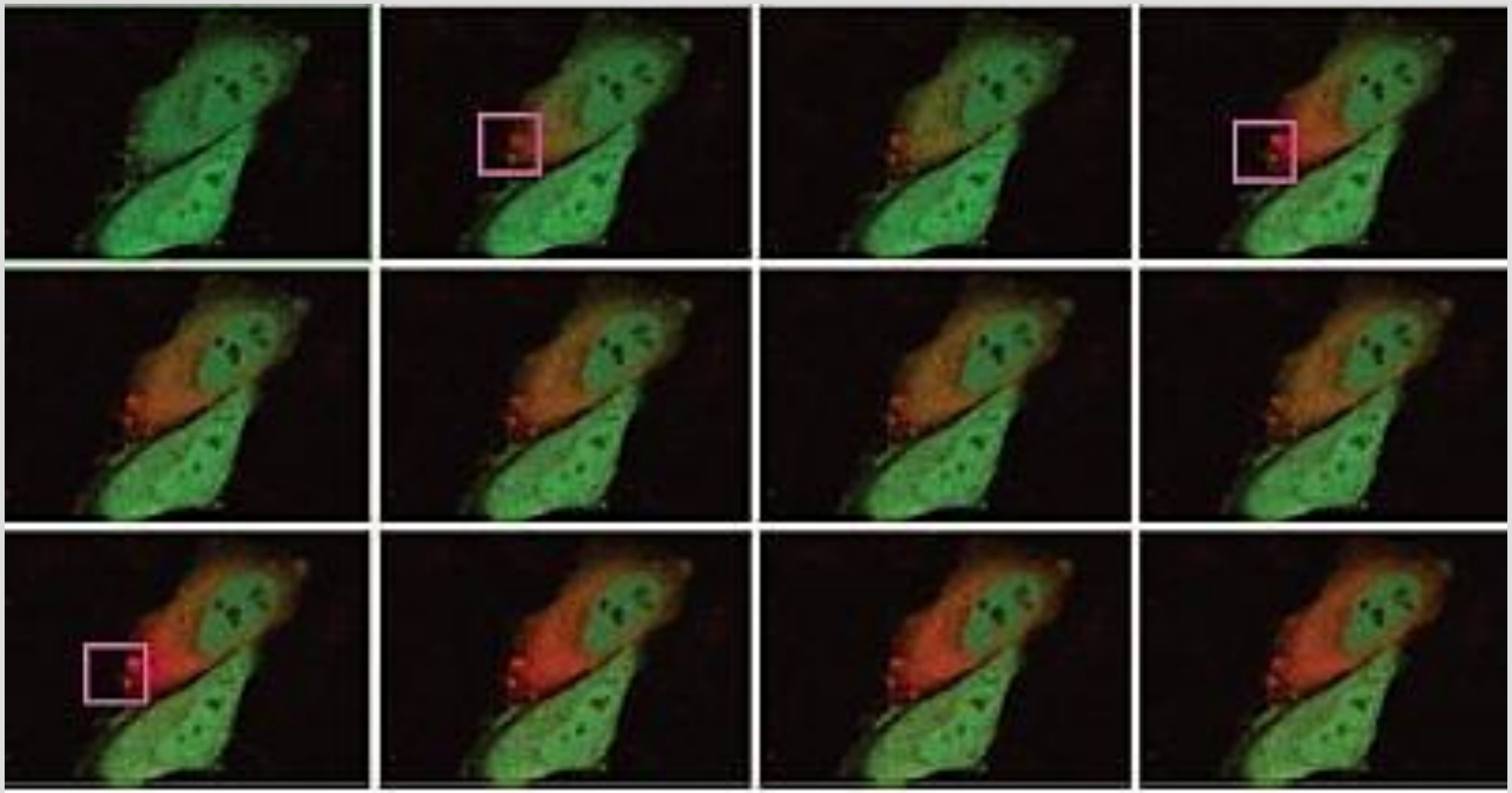
ZEN2012 Applications

HDR – High Dynamic Range



ZEN2012 Applications

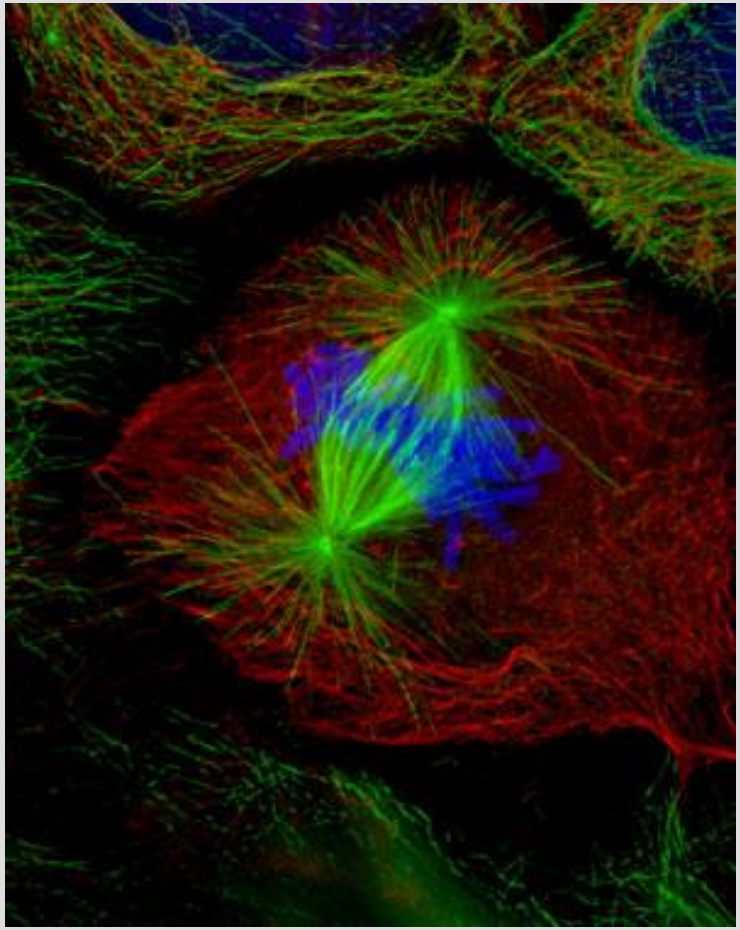
Photoactivation/switching/uncaging



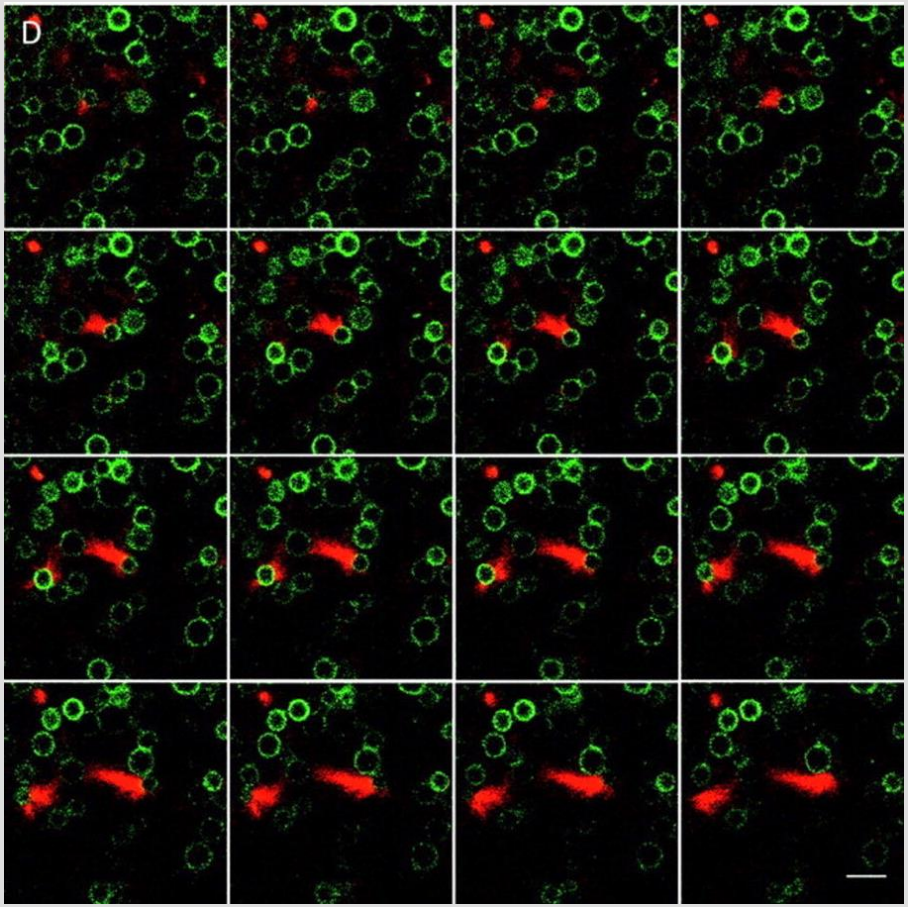

live cell imaging

Live Cell Imaging
Overview

- Heatshock experiments
- Oxygen-dependent experiments
- Extreme photo-sensitive conditions
- Deep tissue imaging with two-photon



Live Cell Imaging
Heatshock experiments

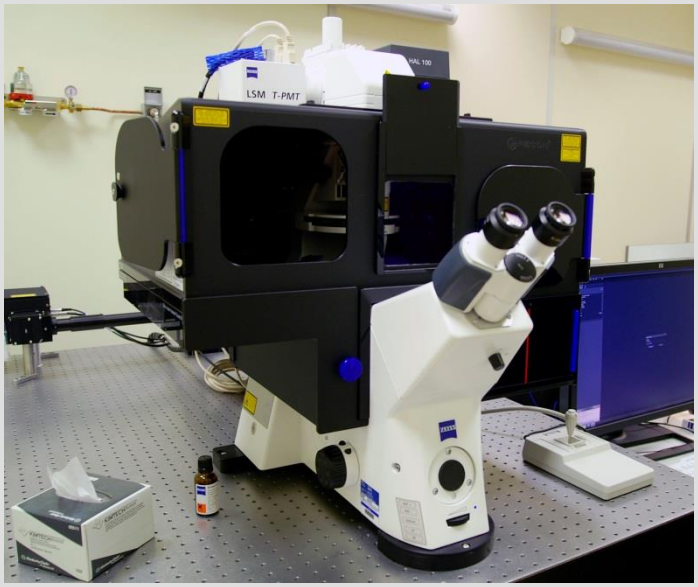


- Activation of genetic constructs for protein expression or enzymatic reactions, initiated by quickly changing t° (mostly 37°C to 45°C and back)
- Genetically engineered
- Heatshock is useful for analysis of:
  - Dynamics analysis
  - Molecular interactions
  - Initiate development
  - Activate protein formation
  - Activate enzyms

Live Cell Imaging

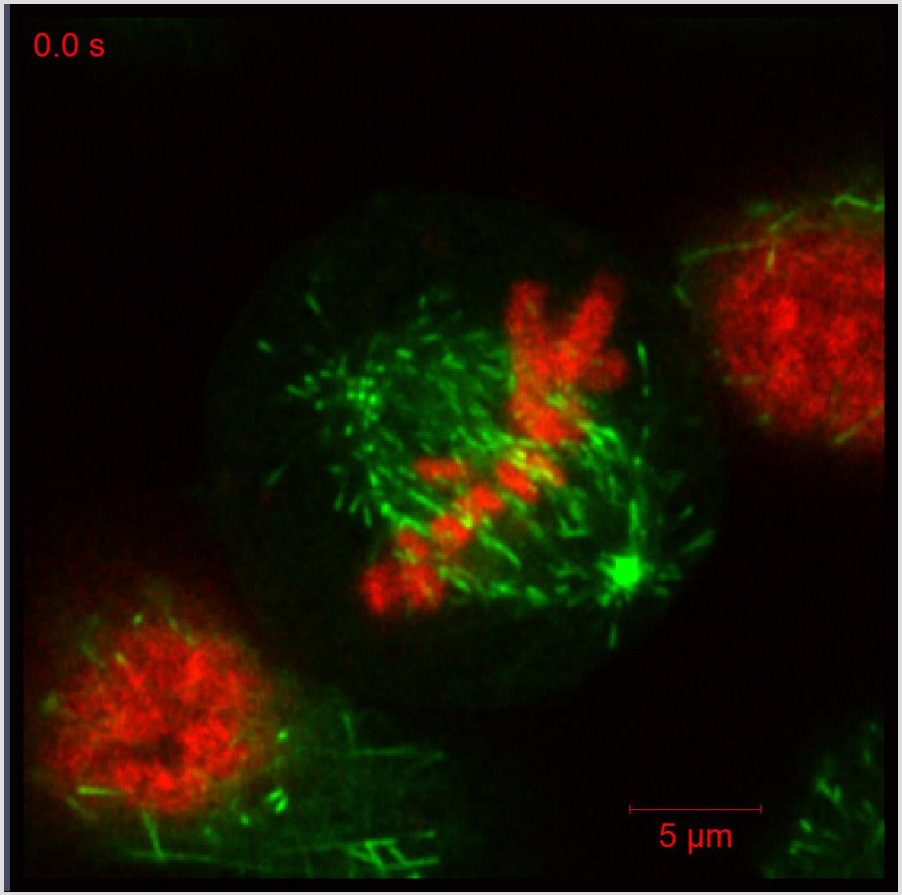
Oxygen dependent experiments

- Special incubators can control not only temperature and CO_2, but also O_2-levels.
- Useful for conditioned imaging:
  - Normoxia
  - Hyperoxia
  - Hypoxia



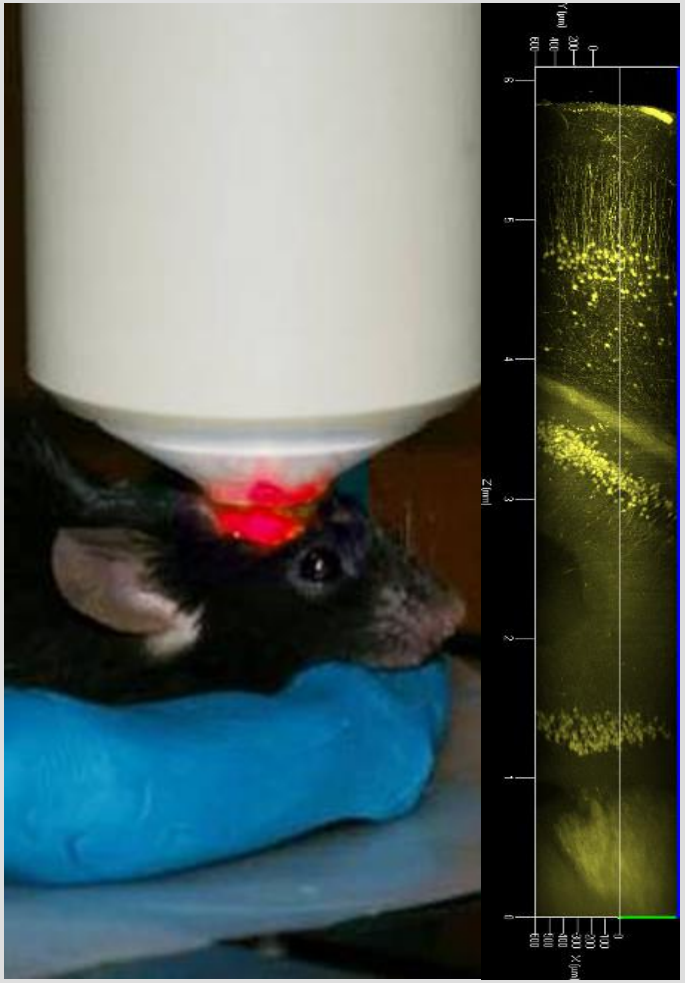
Live Cell Imaging

Extreme photo-sensitive conditions



- Highest sensitivity ever:
  - Use very low laser power
  - Detect extreme weak signals
- Spectral unmixing
- Focus control with Definite Focus
- 2p-laser results in extremely low photo-toxicity & -bleaching

Live Cell Imaging
Deep tissue imaging



- Two-photon imaging, a special type of confocal imaging, allows deep penetration of light
- Special laser is required, many advantages such as less phototoxicity, deep imaging, less photobleaching
- Applicable in many areas:
  - Cranial window (follow up neuronal activity in living mice)
  - 3D reconstruction of thick sections
  - Up to 6mm (!) deep in cleared tissue


Dynamics

FCS

ZEN Applications

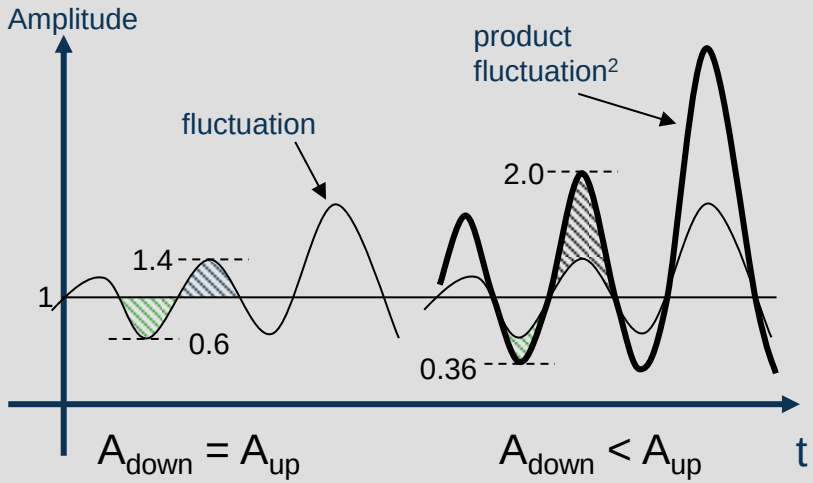
FCS - Fluorescence Correlation Spectroscopy

$$G(\tau) = \frac{\langle \delta I(t) \delta I(t+\tau) \rangle}{\langle I(t) \rangle^2} = \frac{\langle I(t) I(t+\tau) \rangle}{\langle I(t) \rangle^2} - 1$$

$$g^{(2)}(\tau) = 1 + \frac{\gamma}{N} \cdot \left(1 + \frac{T \cdot e^{-t/\tau_T}}{1-T} \right) \cdot \left(\frac{1}{\left(1 + \frac{\tau}{\tau_D} \right) \sqrt{\frac{\tau}{\tau_D} s^2}} \right)$$

$$g^{(2)}(\tau) = 1 + \frac{\gamma}{N} \cdot \left(1 + \frac{T \cdot e^{-t/\tau_T}}{1-T} \right) \cdot \left(\frac{f}{\left(1 + \frac{\tau}{\tau_{D,1}} \right) \sqrt{\frac{\tau}{\tau_{D,1}} s^2}} + \frac{1-f}{\left(1 + \frac{\tau}{\tau_{D,2}} \right) \sqrt{\frac{\tau}{\tau_{D,2}} s^2}} \right)$$

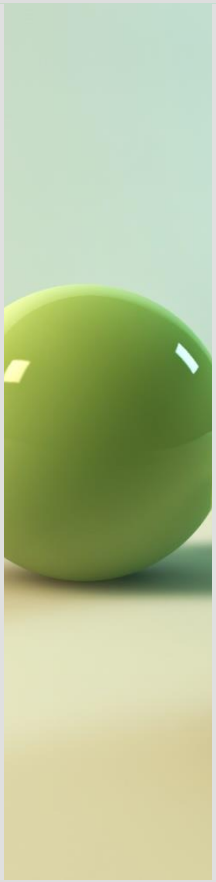
$$g^{(2)}(\tau) = 1 + \frac{\gamma}{N} \cdot \left(1 + \frac{T \cdot e^{-t/\tau_T}}{1-T} \right) \cdot \left(\frac{1}{\left(1 + \frac{\tau}{\tau_D} \right)^{\alpha} \sqrt{\left(\frac{\tau}{\tau_D} \right)^{\alpha} s^2}} \right)$$



ZEN Applications

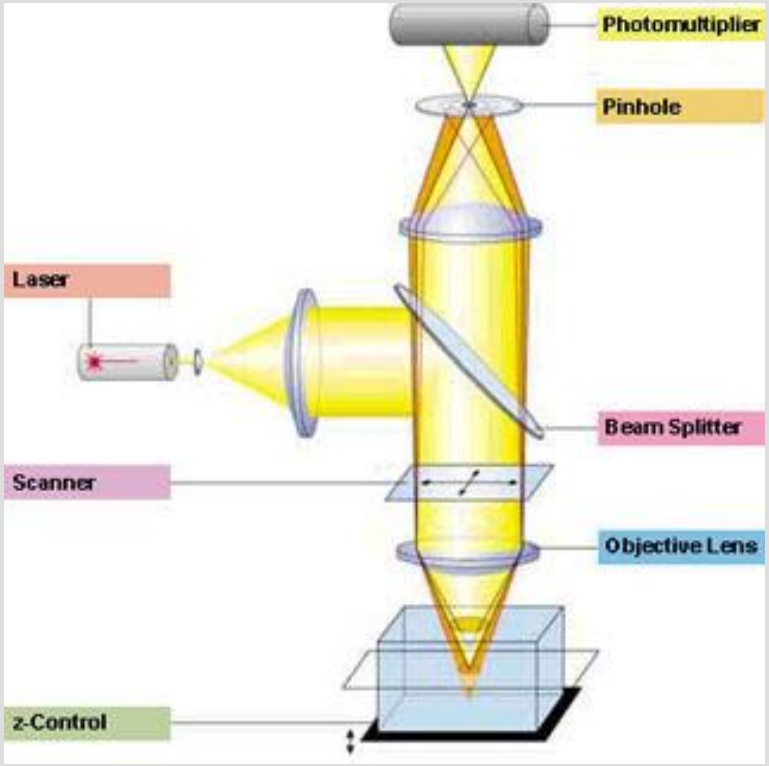
FCS - Fluorescence Correlation Spectroscopy

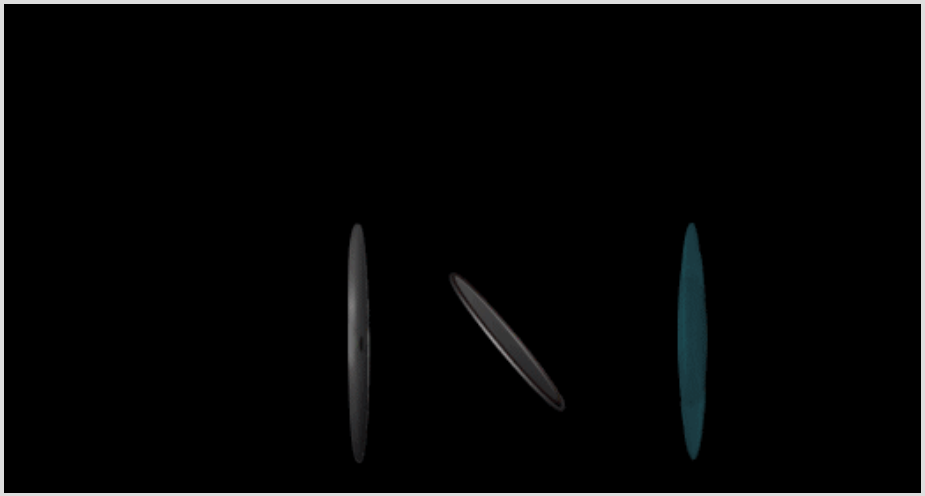
Complex, though easier as it seems. But first the basics...



ZEN Applications

FCS - Fluorescence Correlation Spectroscopy

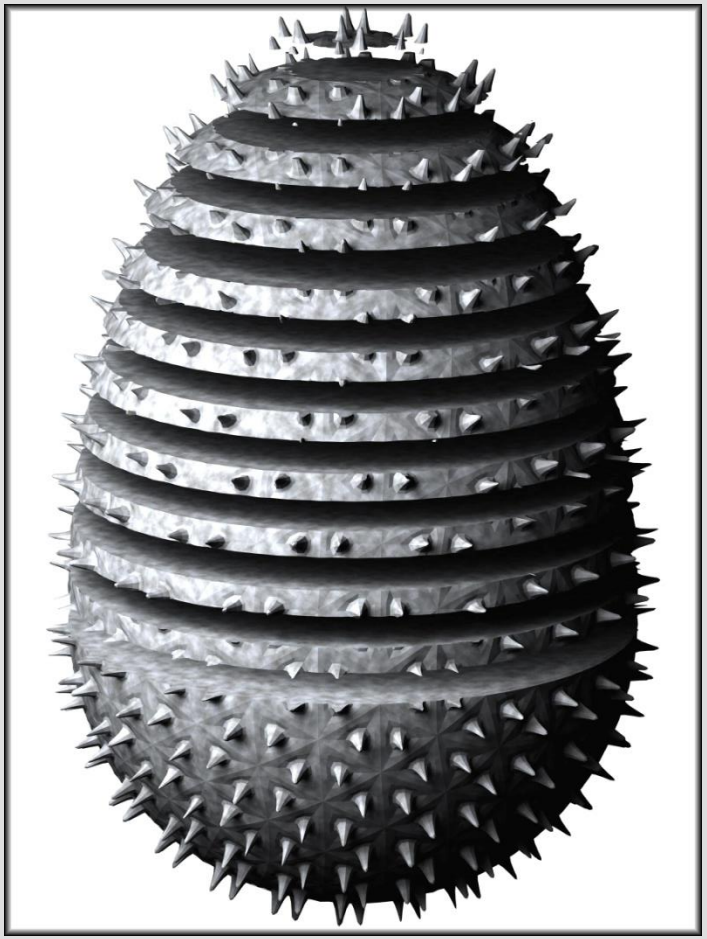




Only the optical section is being observed.

ZEN Applications

FCS - Fluorescence Correlation Spectroscopy



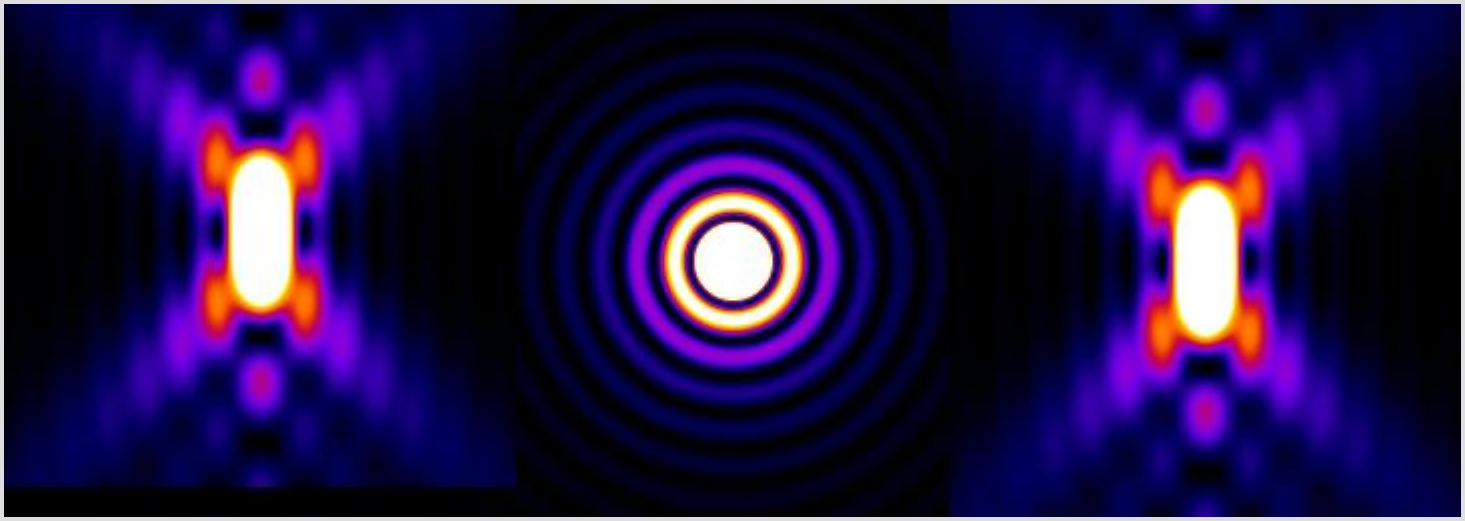
A serie of optical sections can, once combined, recreate the original 3-dimensionale object.

In confocal microsopy one does not acquire a ‚photo' of several pixels at once, however, a scanner scans pixel by pixel and builds up the image as such.

ZEN Applications

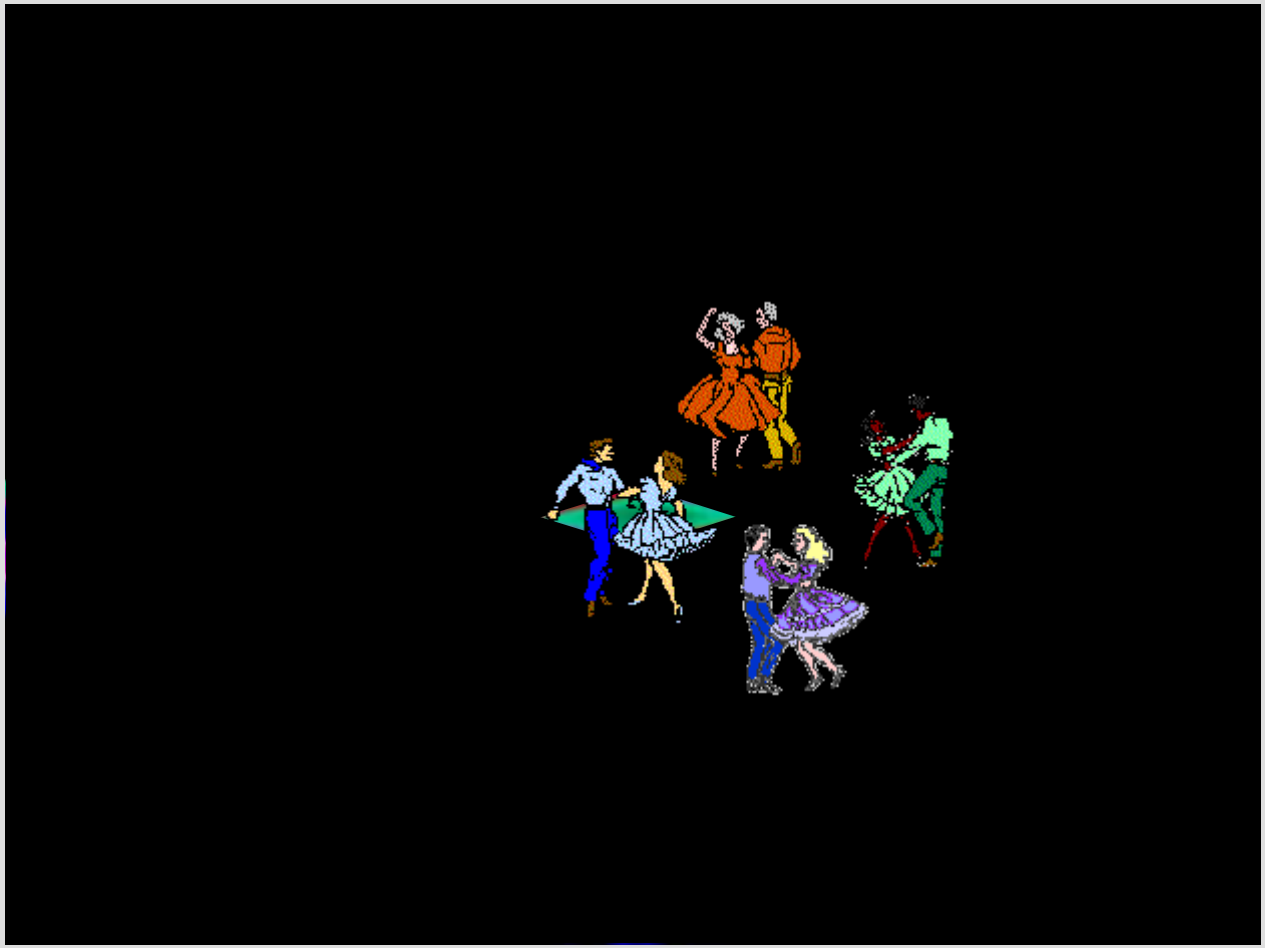
FCS - Fluorescence Correlation Spectroscopy

- In 3D a sphere appears ellipsoid, due to the optical disturbances of the microscope.
- This ellipsoid has a volume which can be calculated.



ZEN Applications

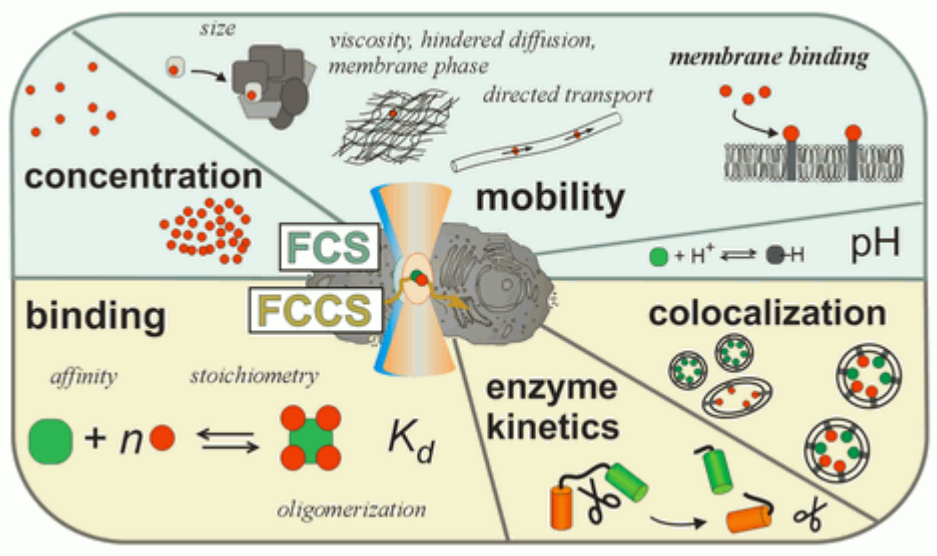
FCS - Fluorescence Correlation Spectroscopy

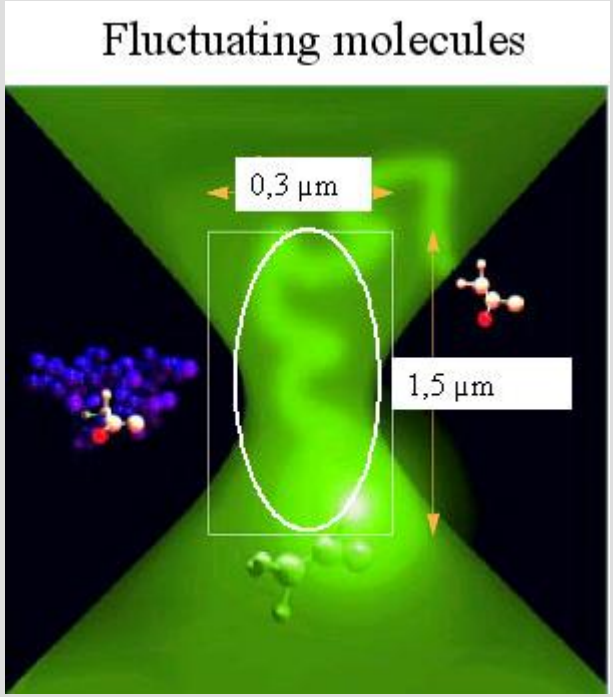


ZEN Applications

FCS - Fluorescence Correlation Spectroscopy

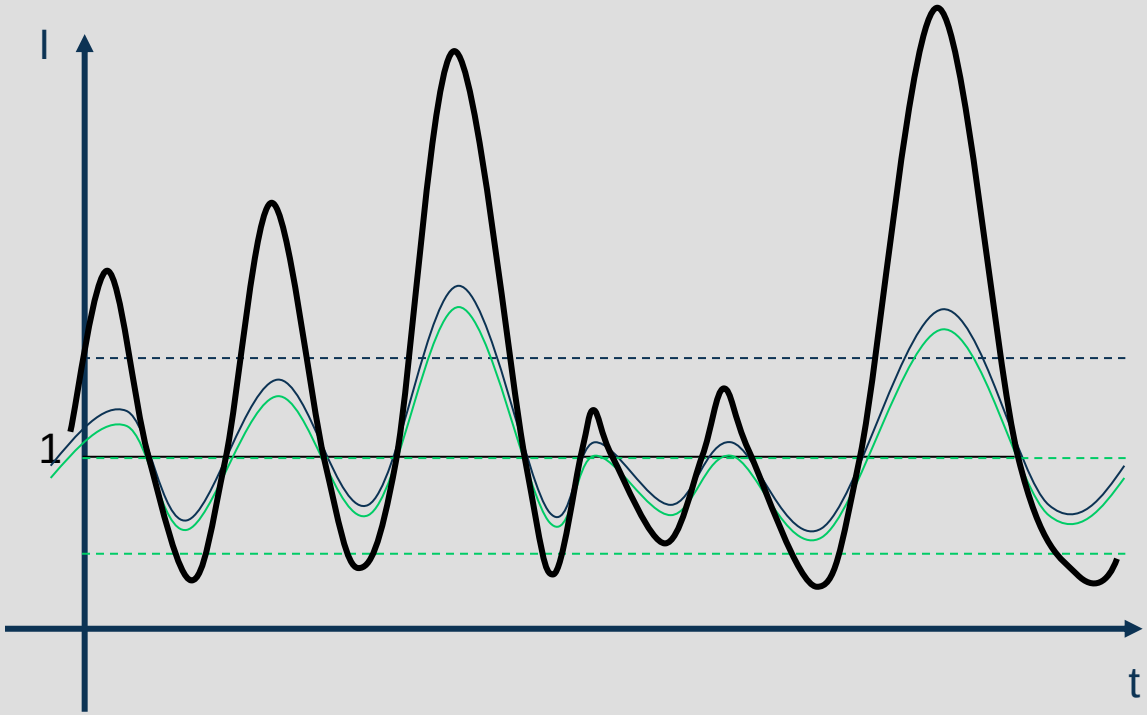
- FCS will thus ‚park' this volume within an object and then define how many, how intense and how fast molecules move through the volume.

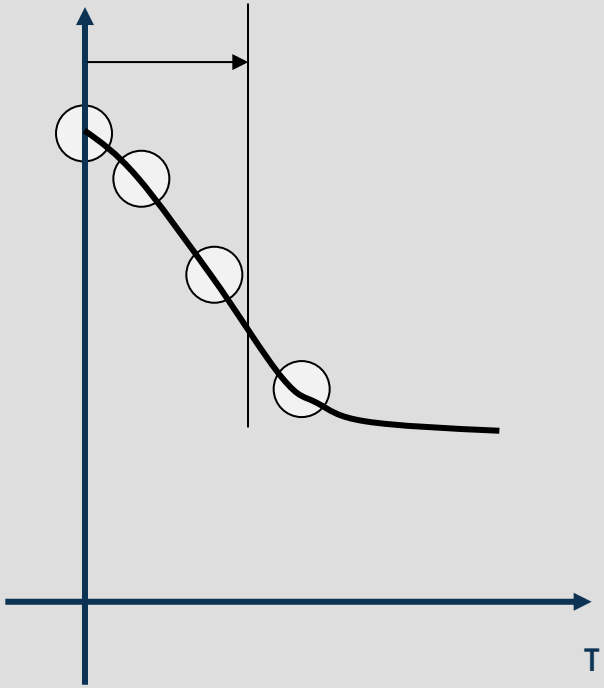




ZEN Applications

FCS - Fluorescence Correlation Spectroscopy

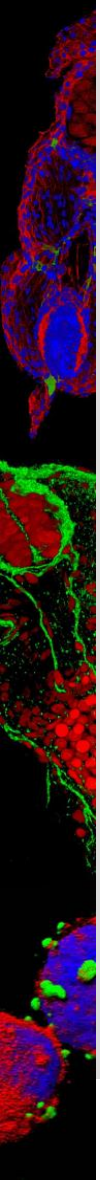


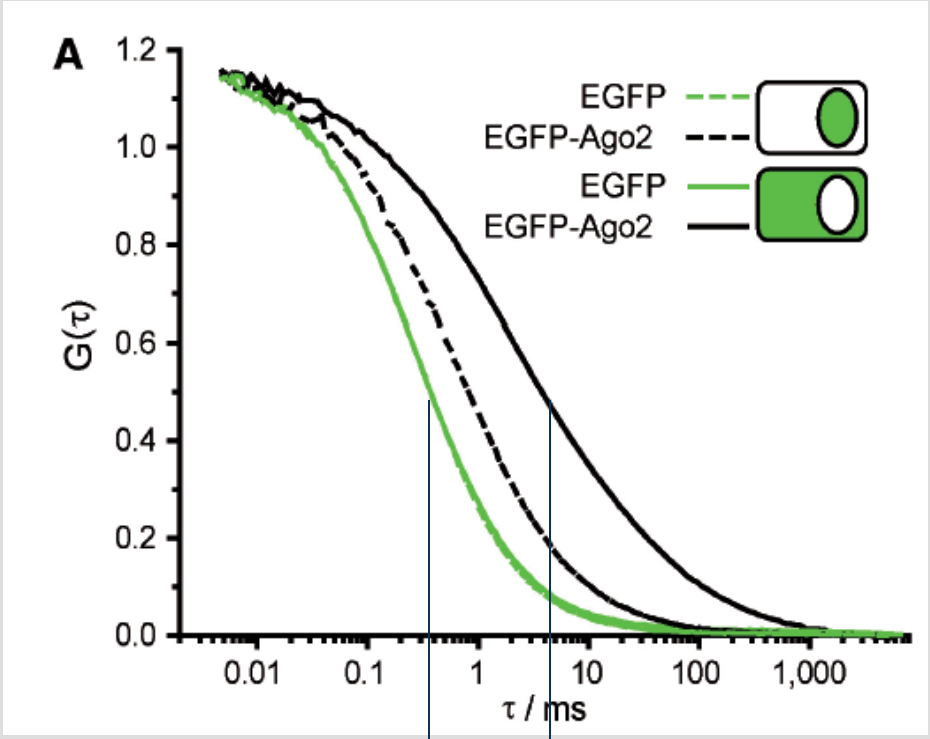


Correlation curve
Slope = speed

ZEN Applications

FCS - Fluorescence Correlation Spectroscopy



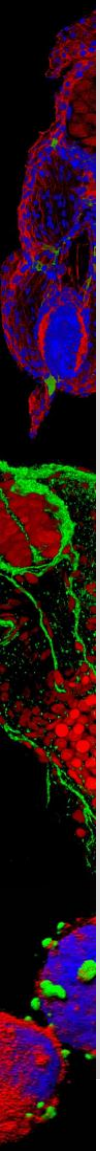


order of mag!

With Ago2: slower

ZEN Applications

FCS - Fluorescence Correlation Spectroscopy



FCS

Single-Molecule Technique

Ultra-sensitive

High detection efficiency

GaAsP

APD

Photon Counting

Statistics

Complicated Maths

Curve-fitting

Photonics

Heterogeneity

Protein-protein interactions

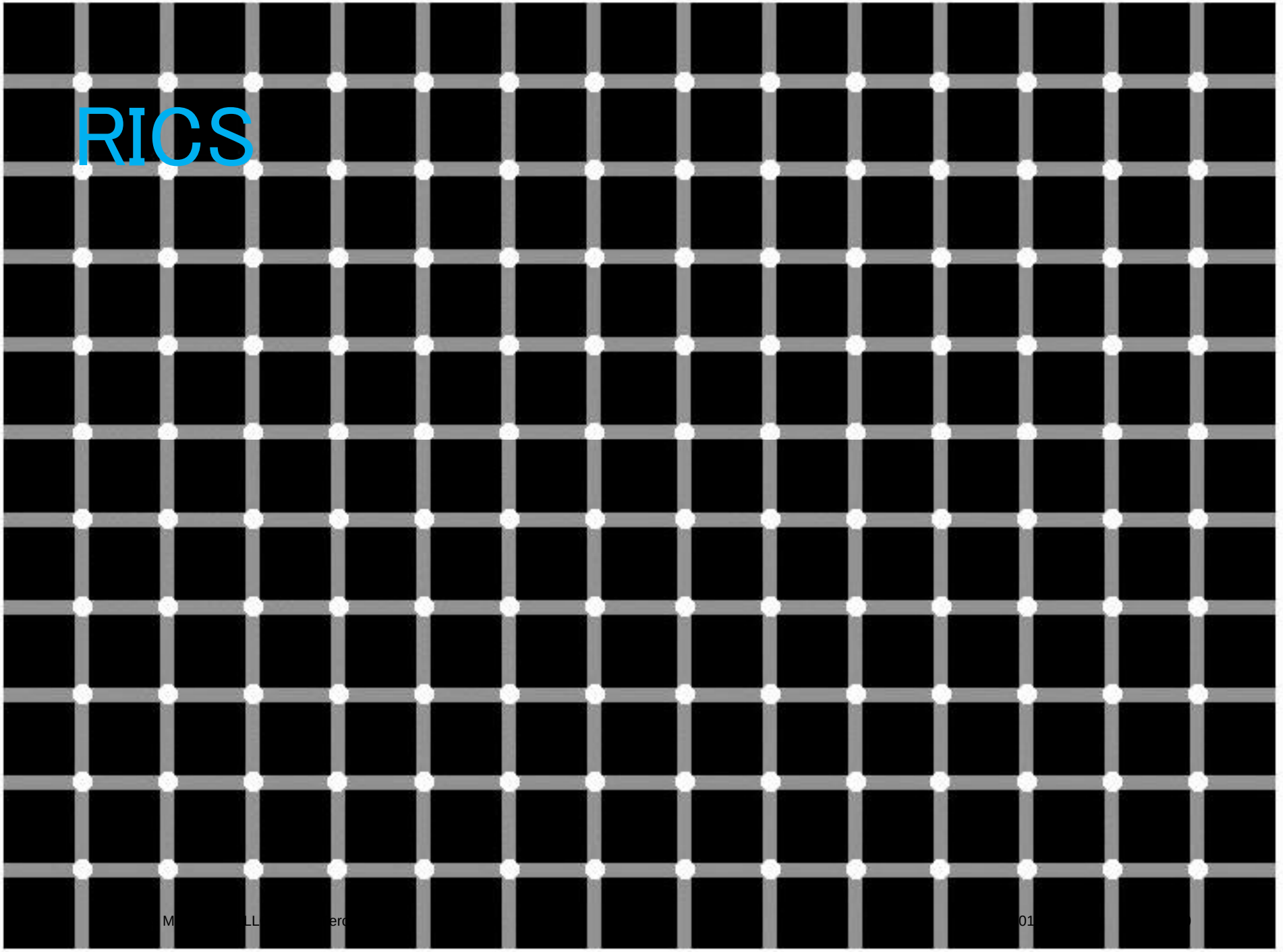
Membrane mobility

Anomalous diffusion

Transport mechanisms

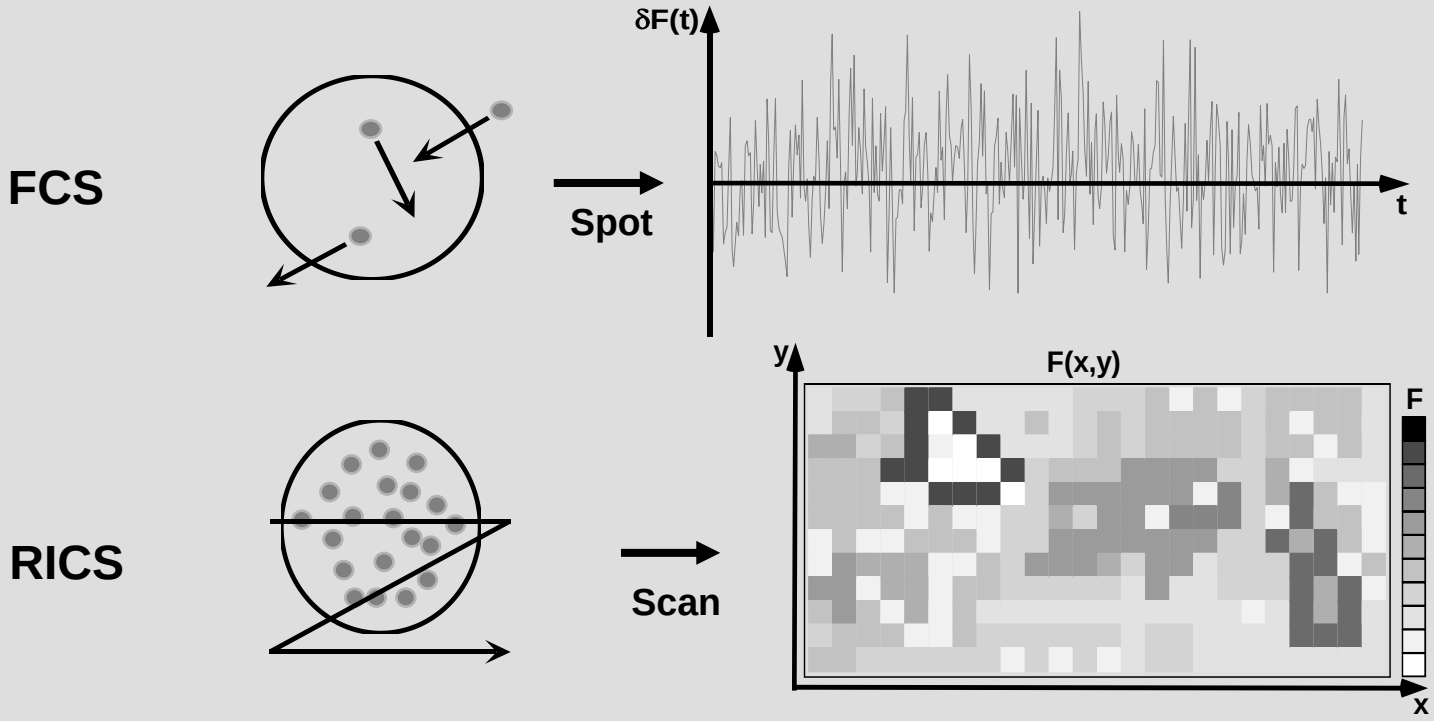
Nano-viscosity

Diffusion Coefficient


RICS

RICS

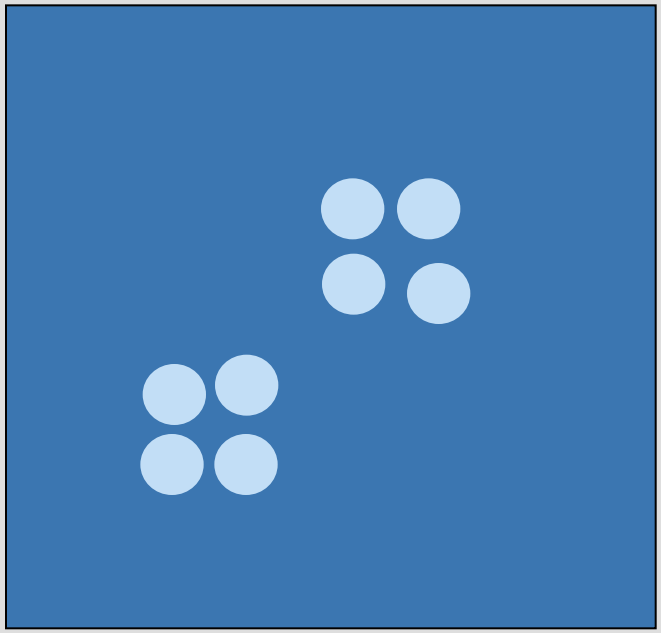
Raster Imaging Correlation Spectroscopy

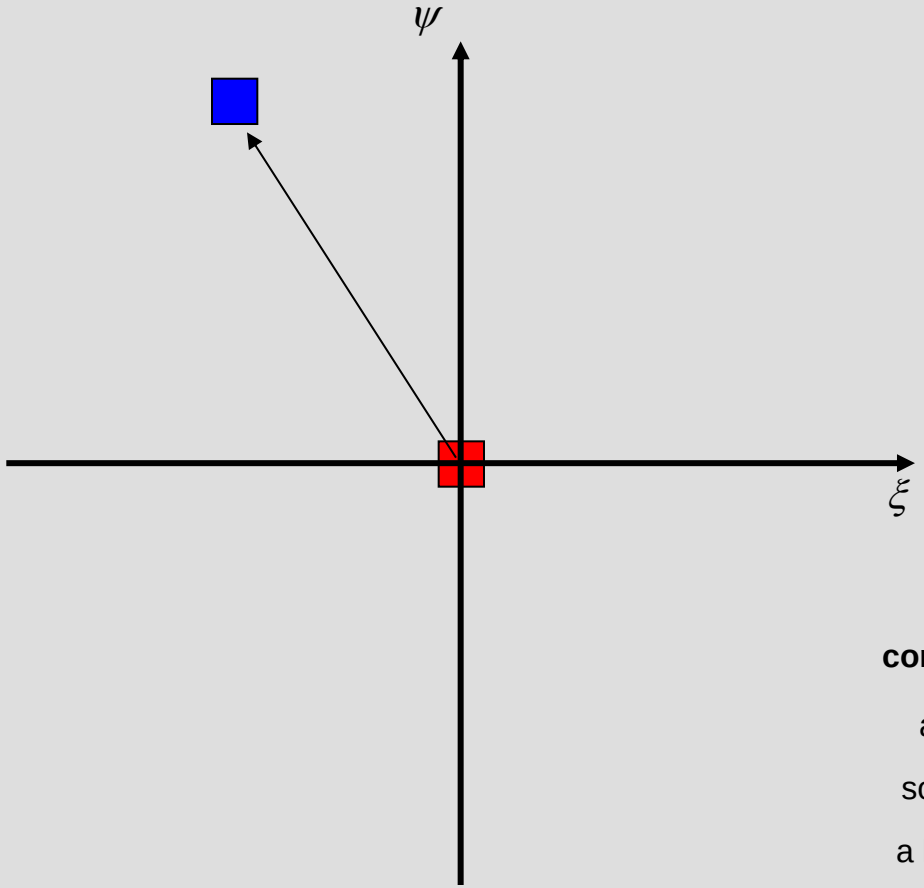


- **RICS is based on the fact that a moving molecule, once imaged on a certain pixel, will most probably be imaged again later on during the same scan on another pixel because of its dynamics.**

RICS

Wat is RICS?





correlation

a lot

some

a little

nothing

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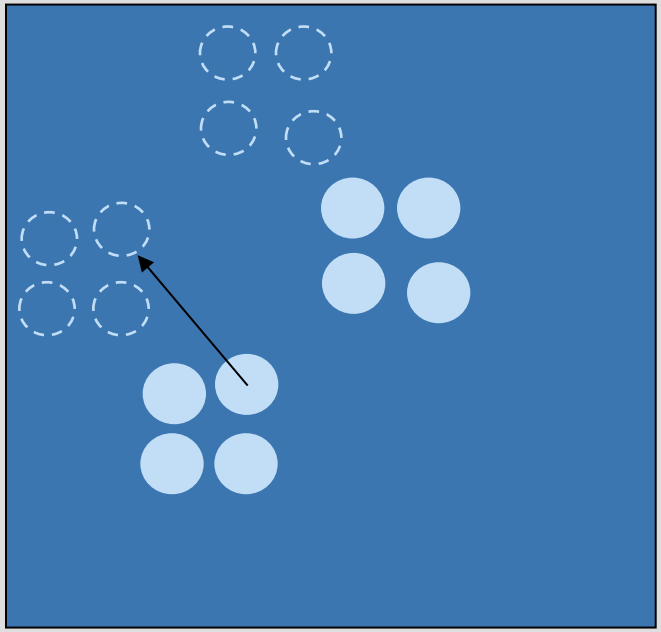

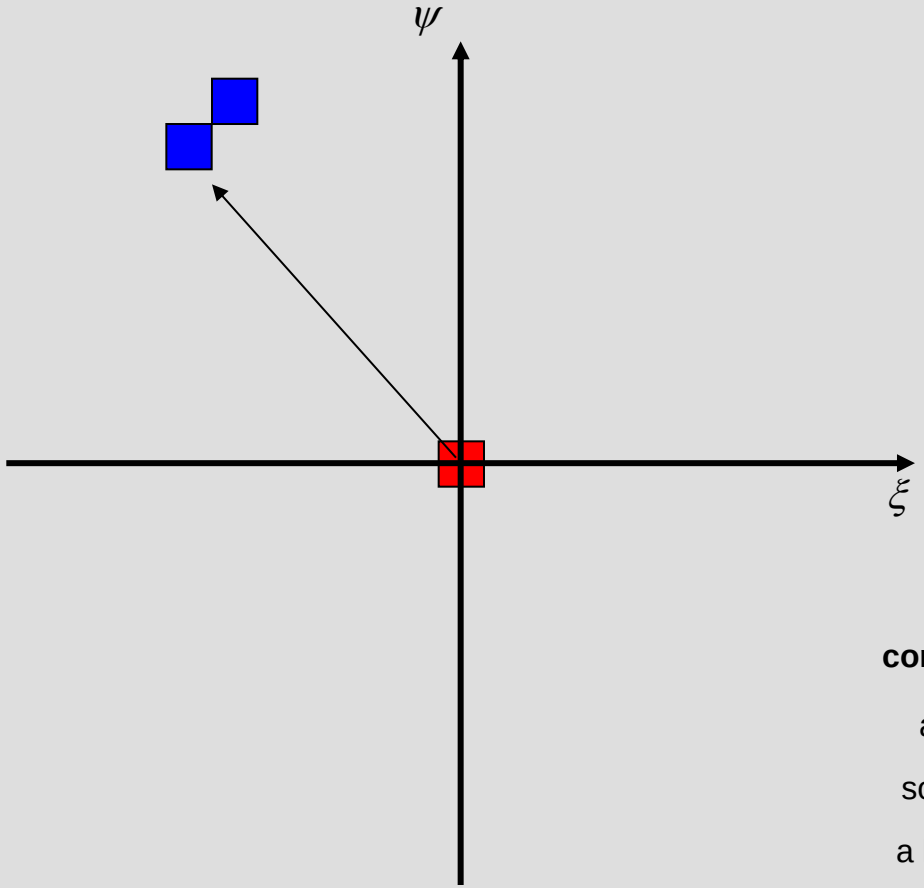

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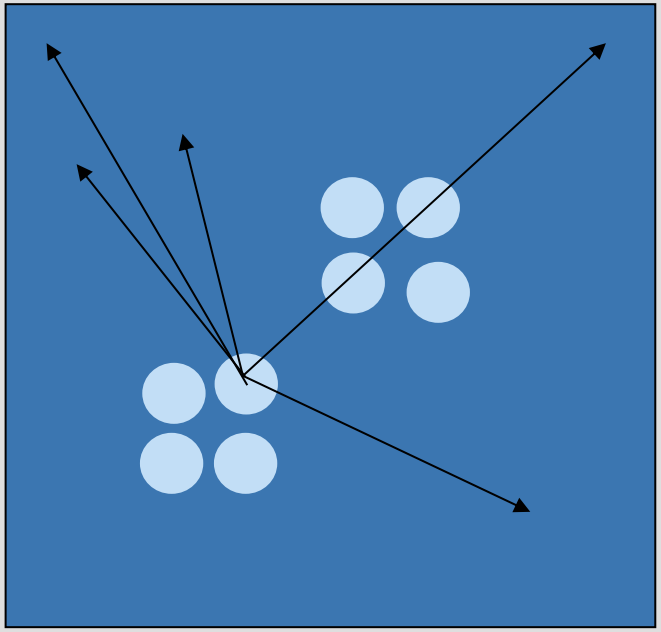

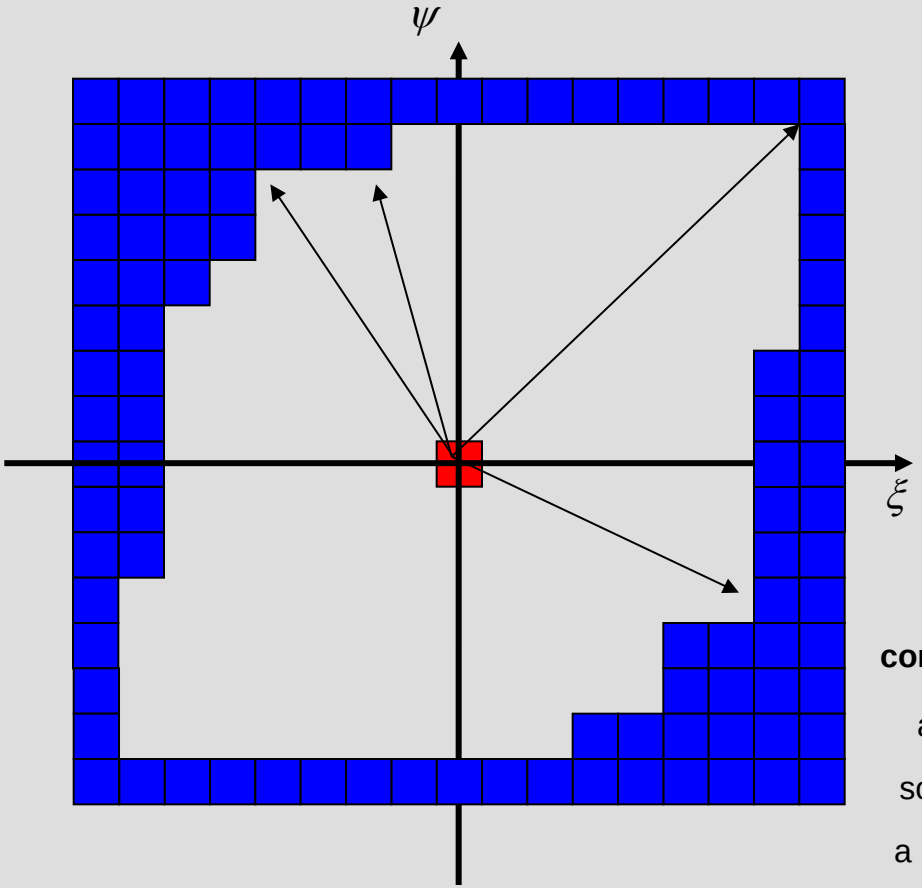

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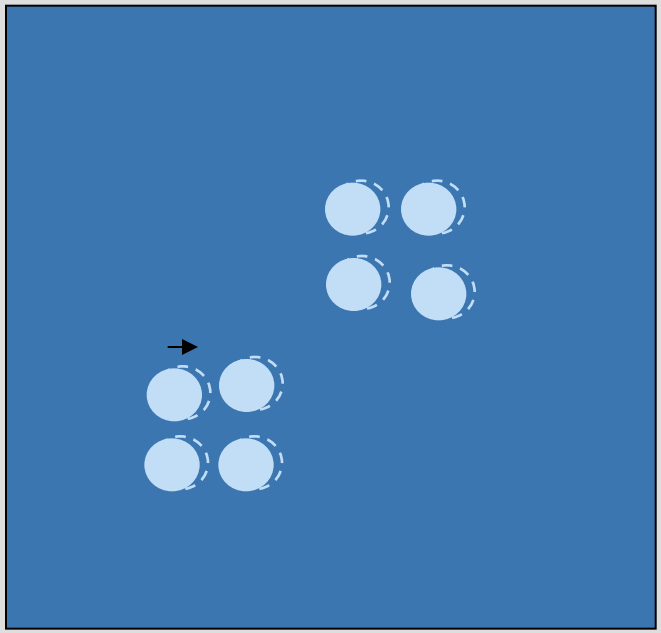

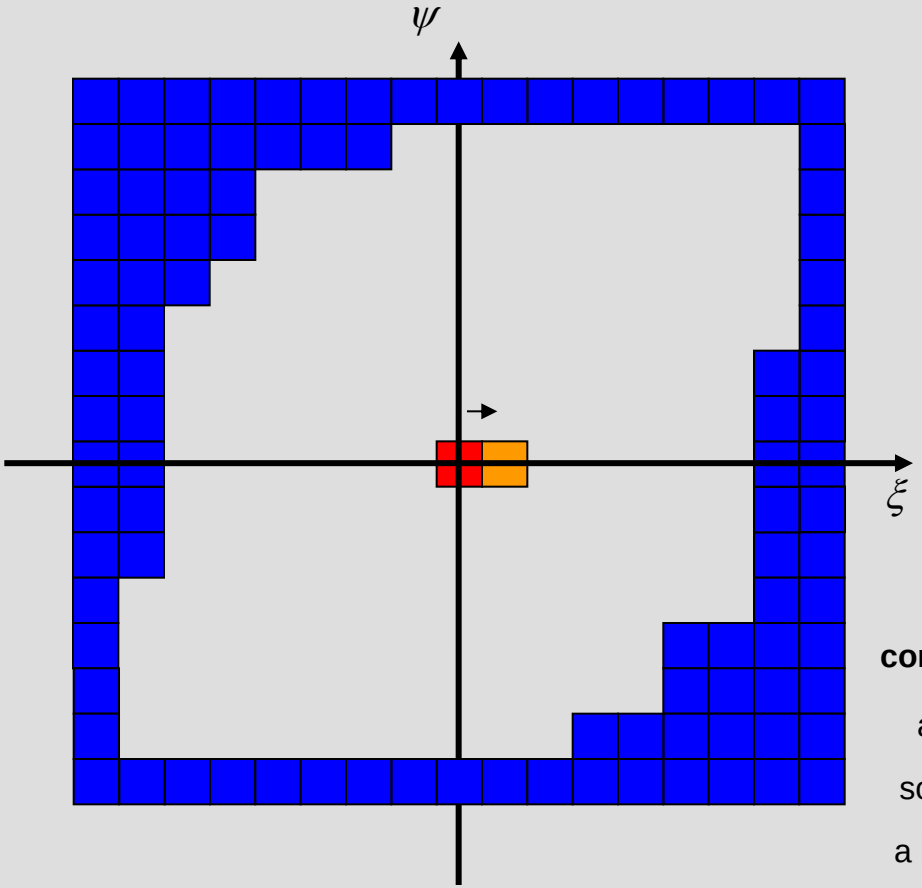

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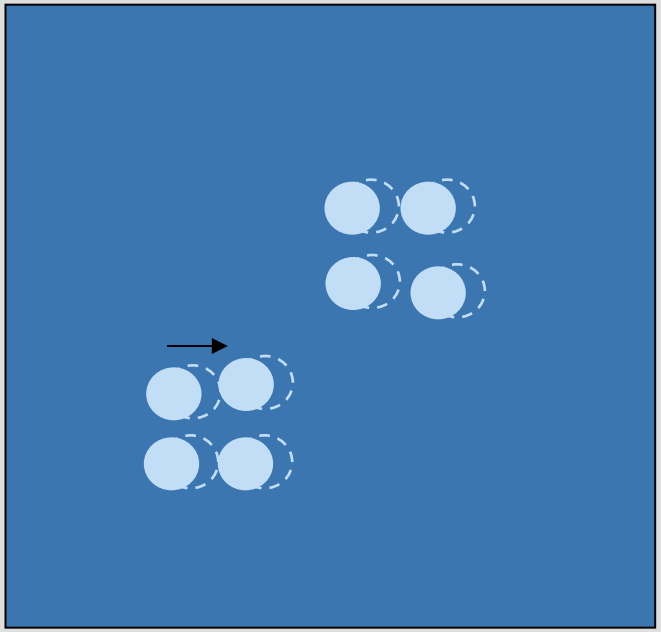

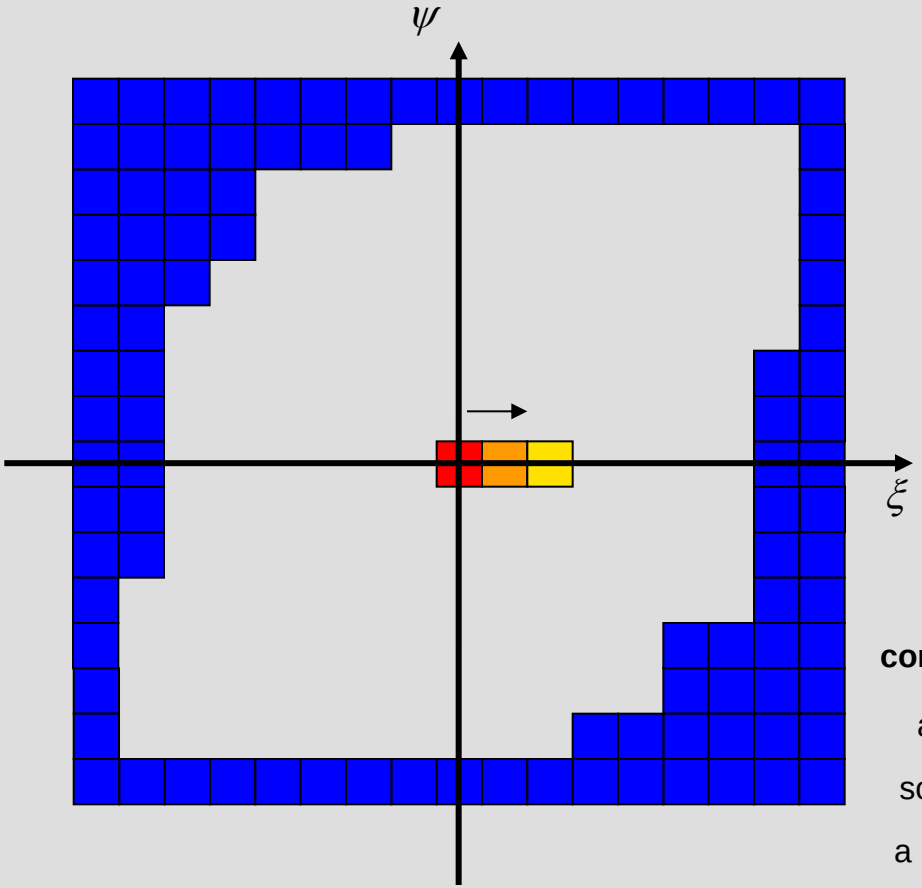

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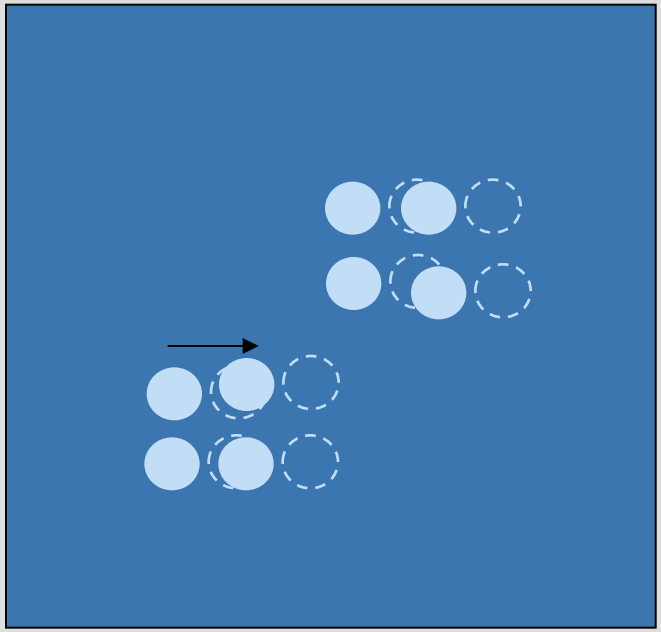

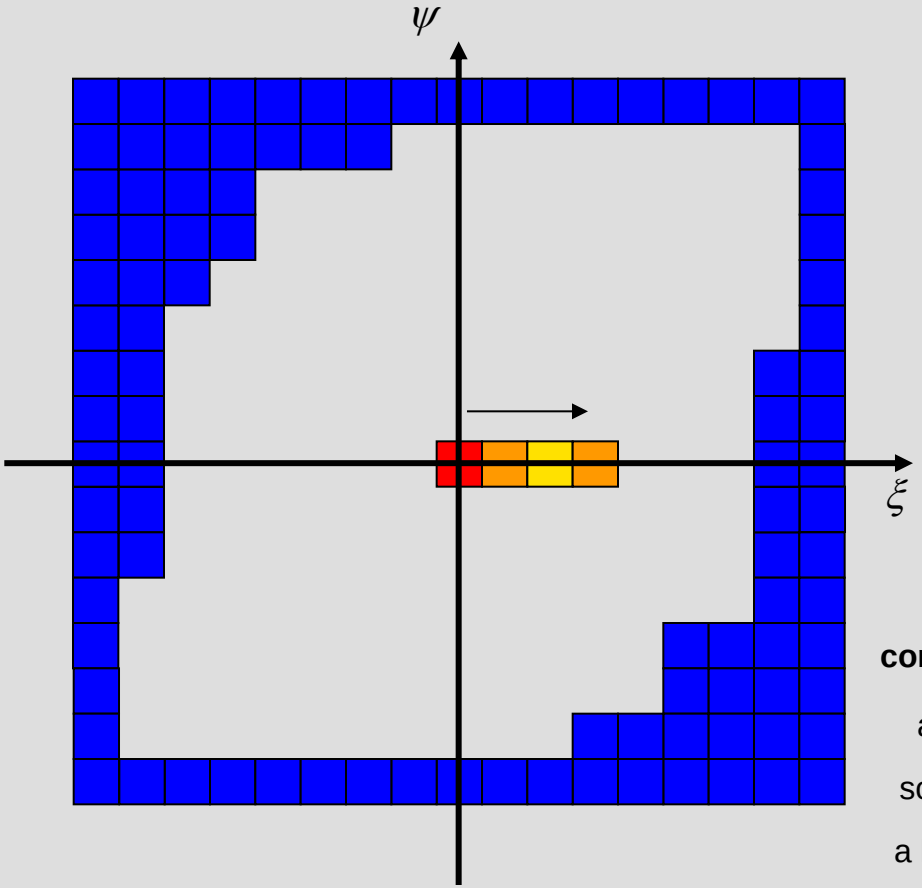

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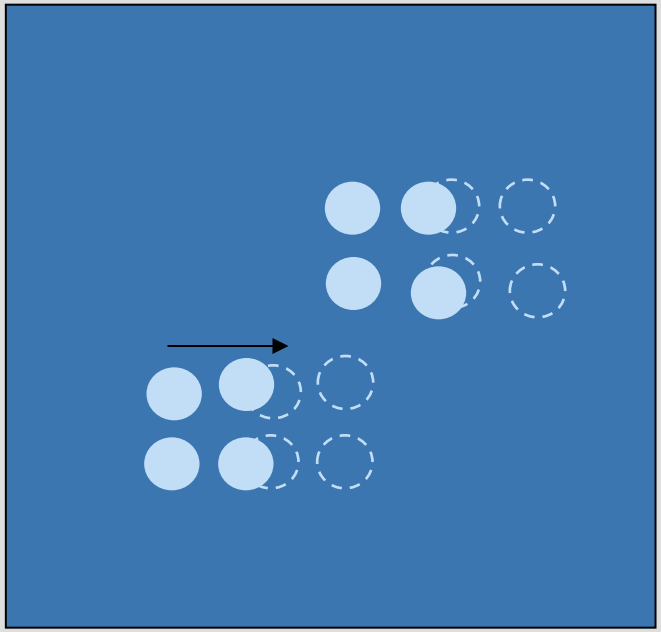

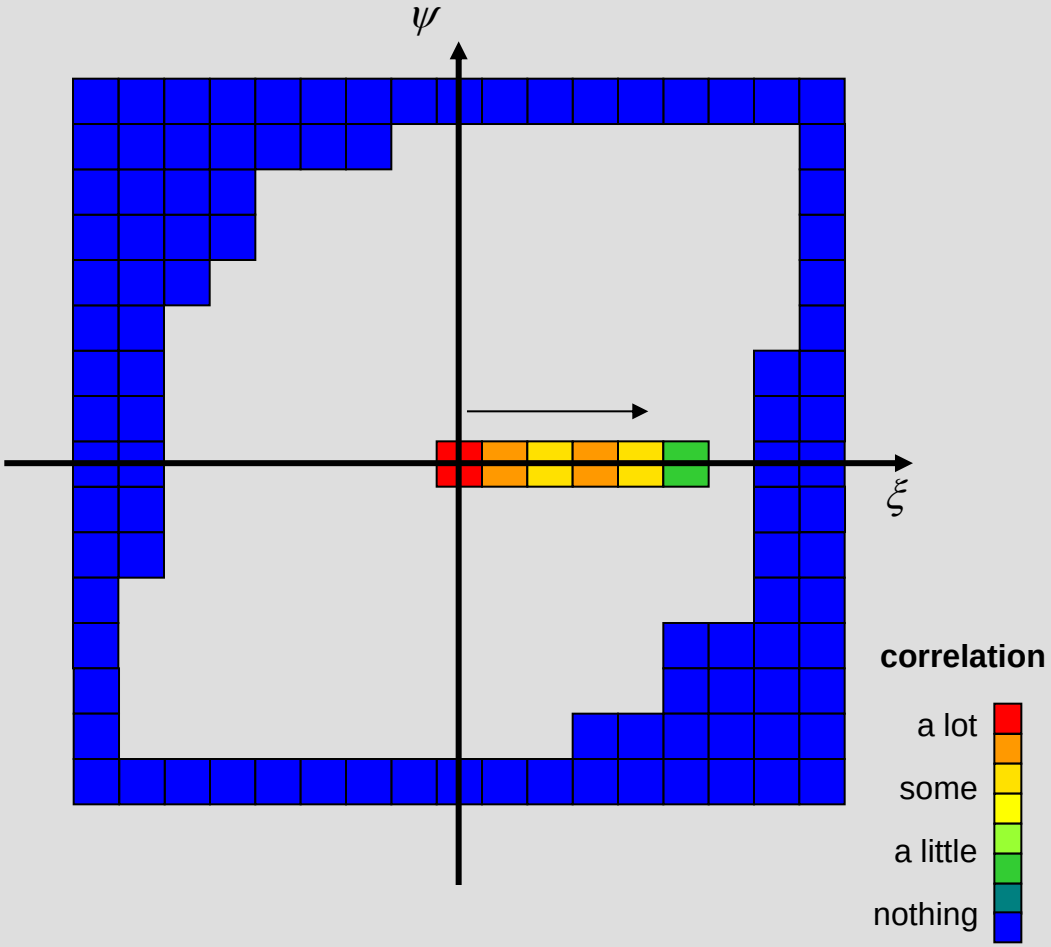

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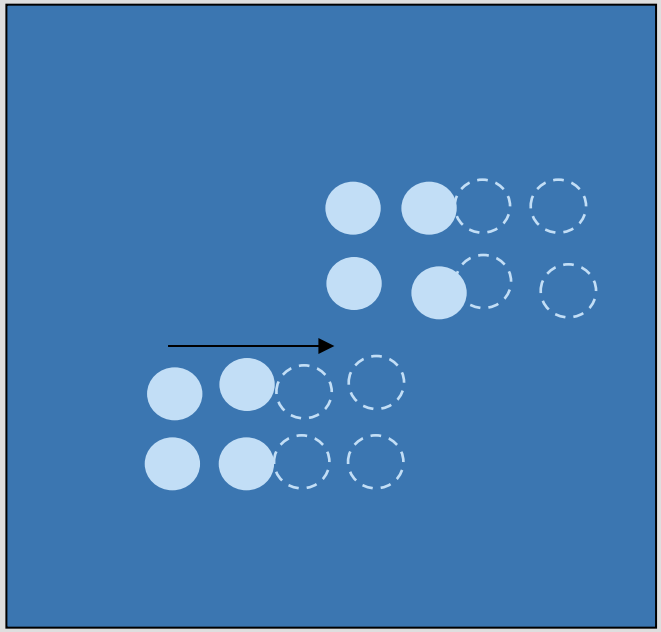

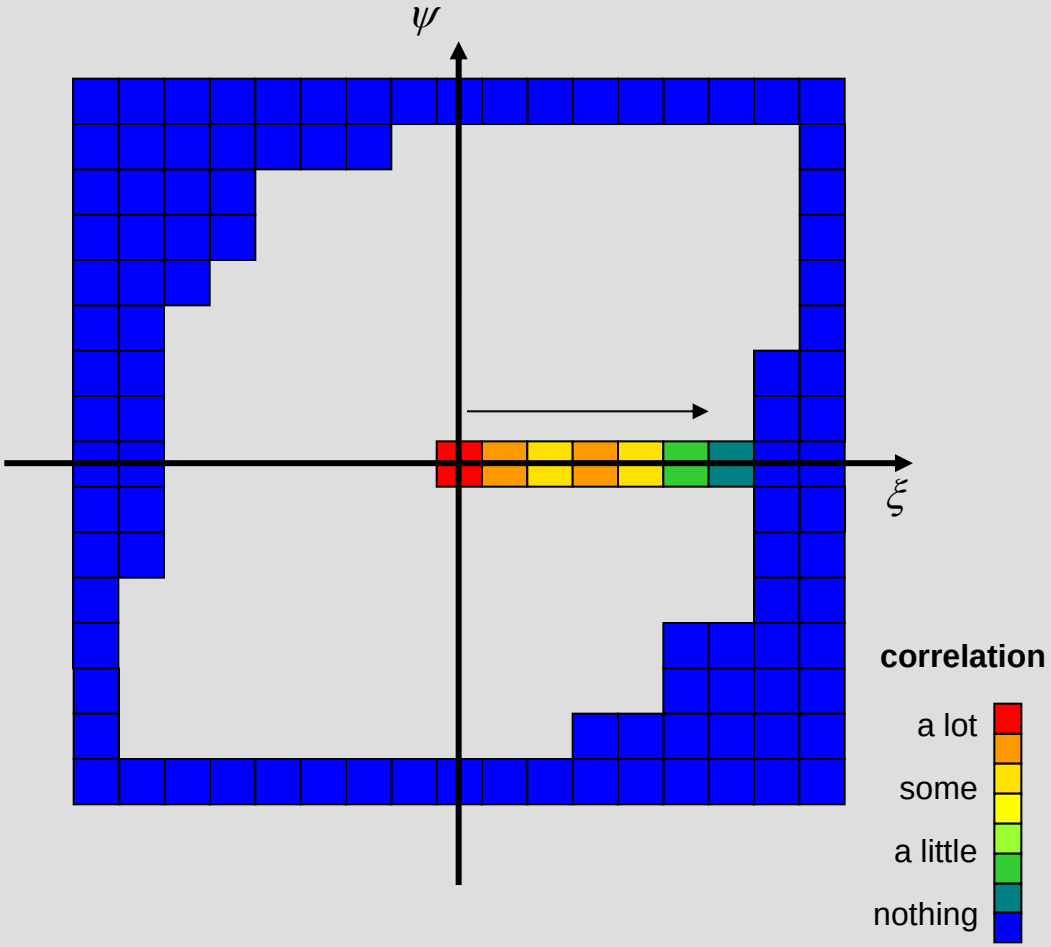

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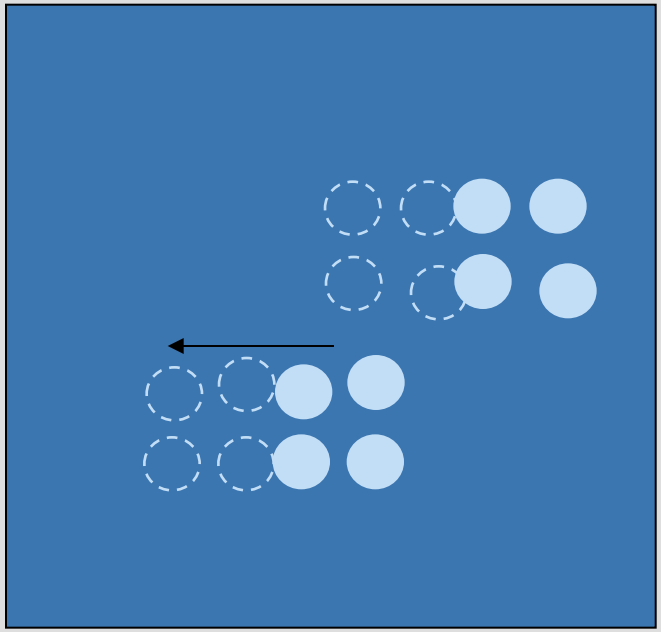
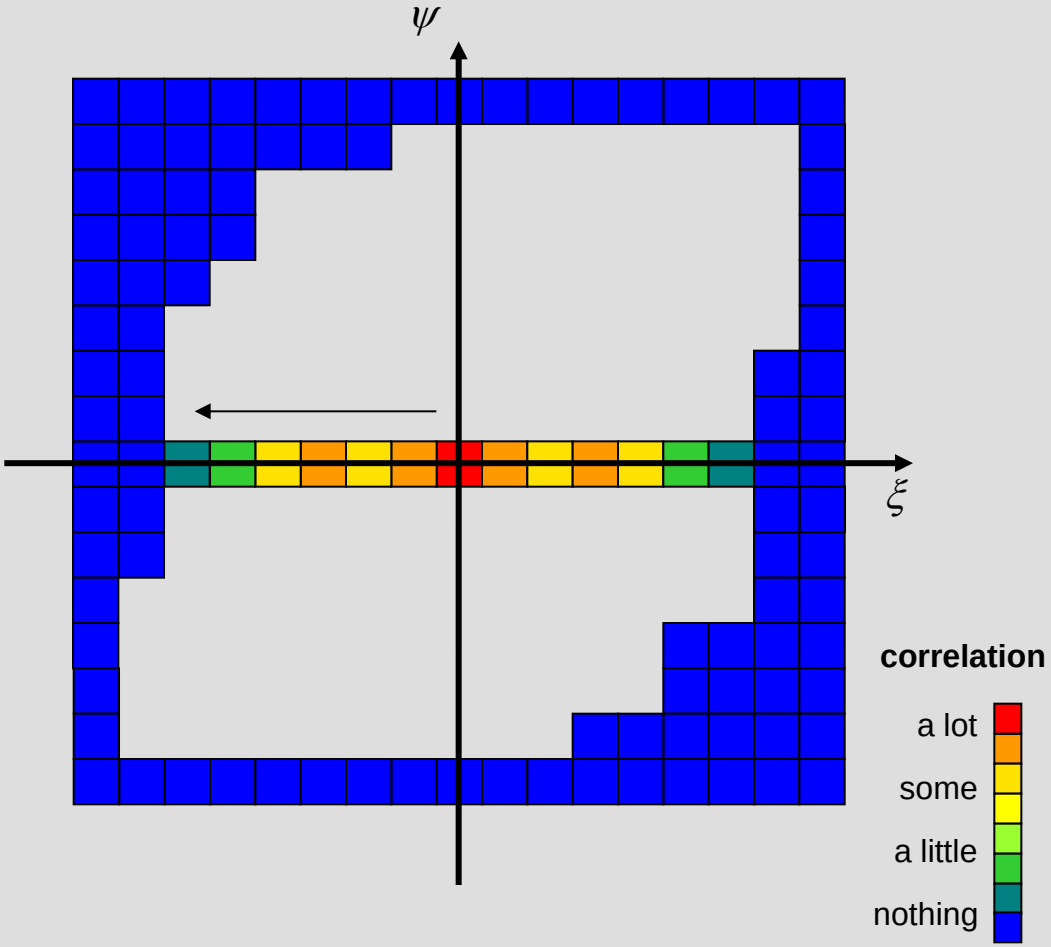
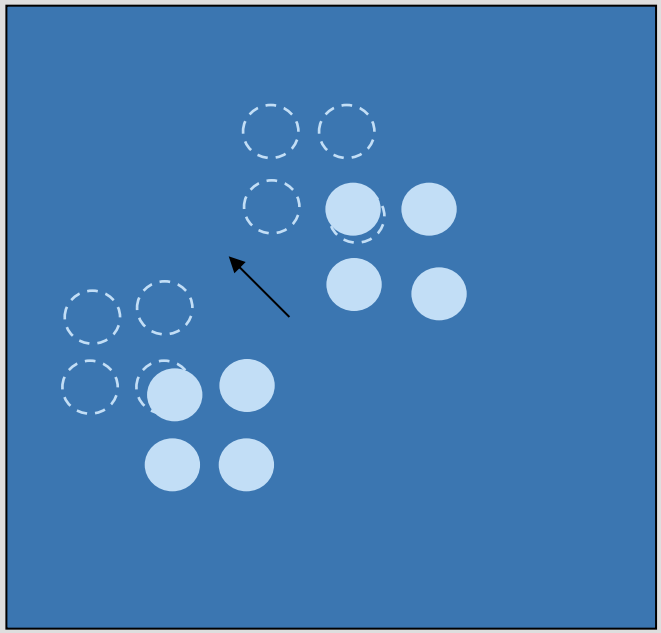
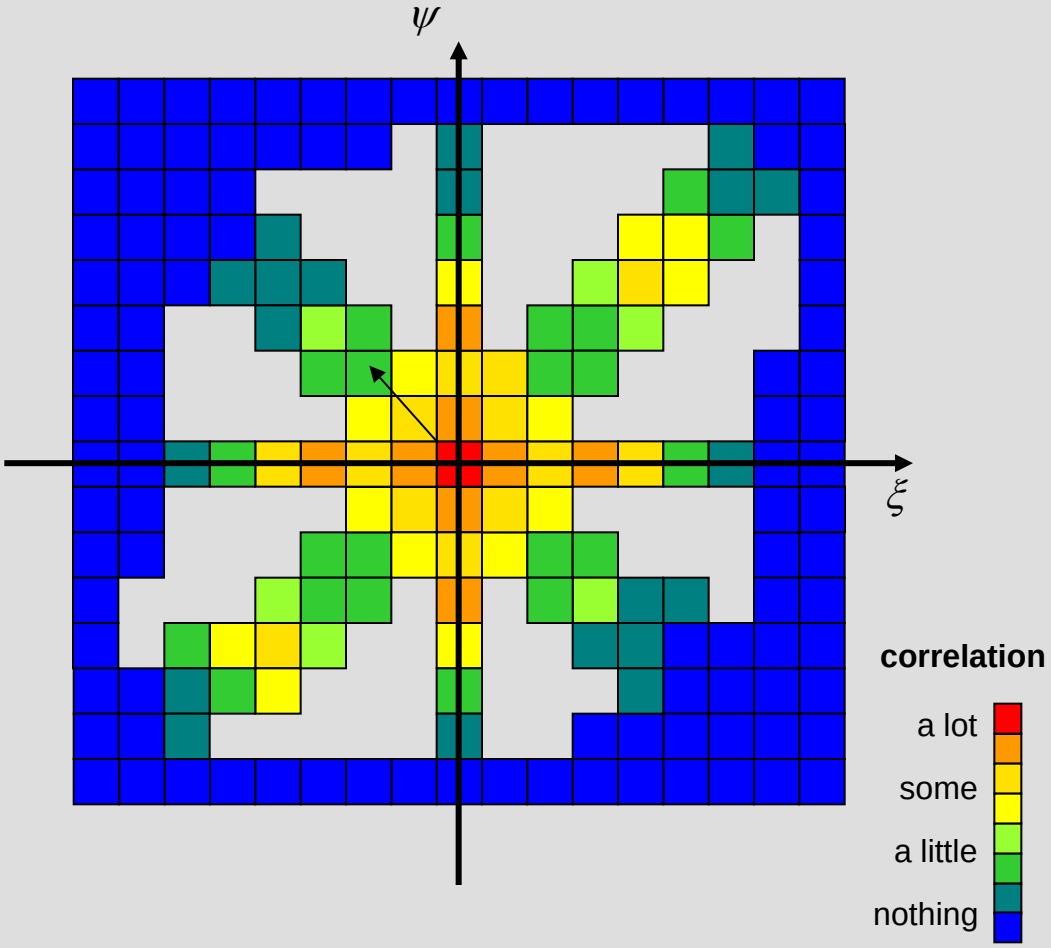




RICS

Het ‘correlogram’





RICS

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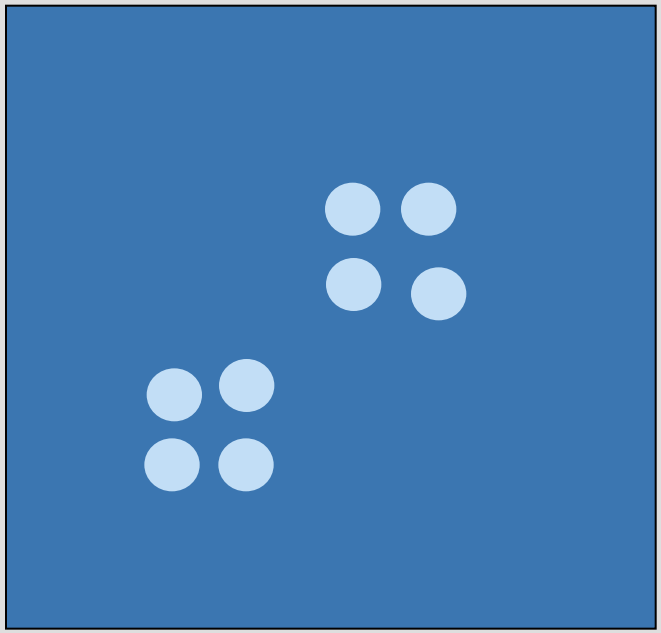
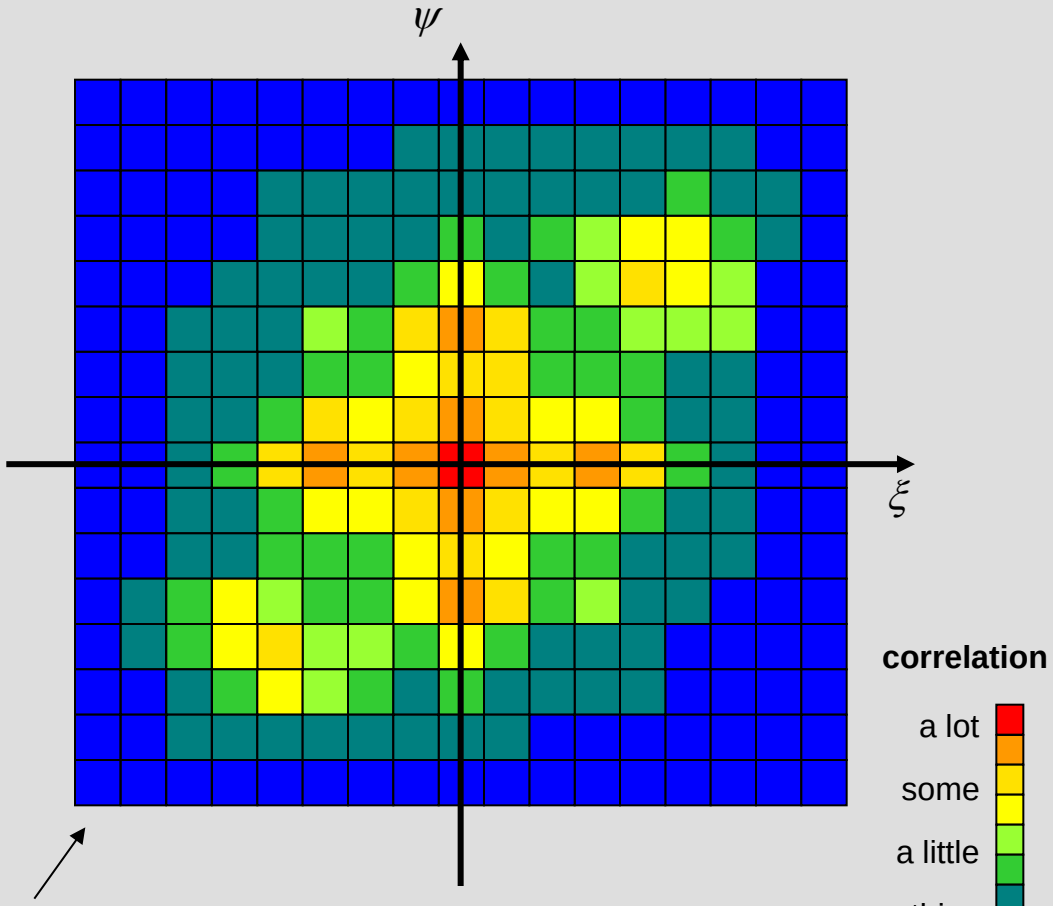
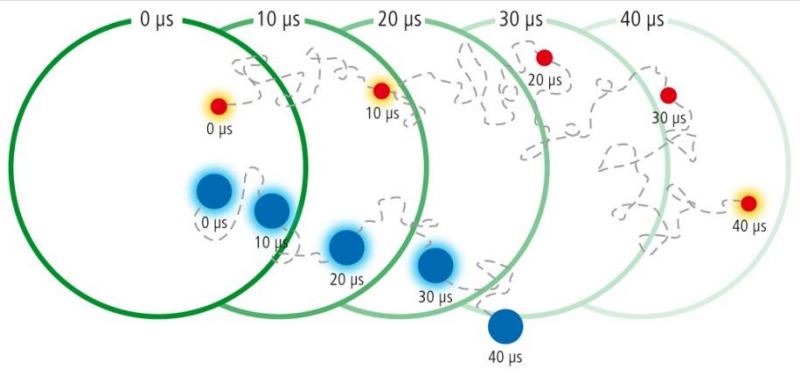




Dit is het resultaat, en bevat heel veel informatie over het beeld!

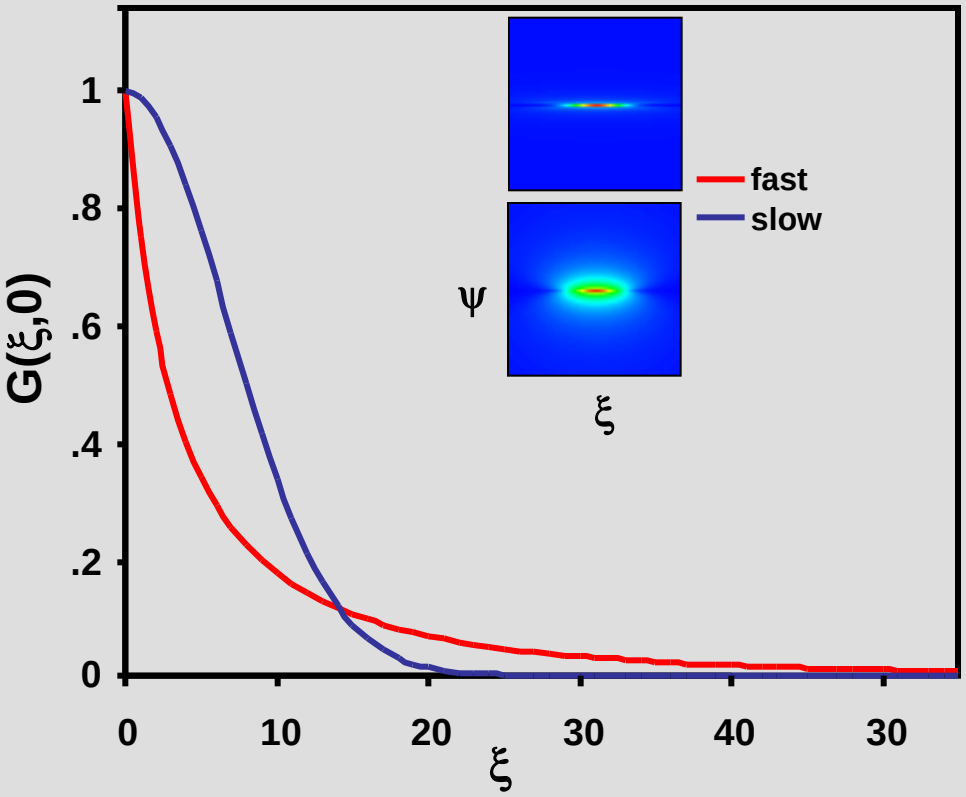
ZEN Applications

RICS - Raster Imaging Correlation Spectroscopy



The spatial correlation describes the probability to see a molecule once detected at location (x,y) also at a later timepoint at a shifted location (x+ξ, y+ψ)

The faster the molecule, the faster the correlation decays at short distances, but the longer it is kept at long distances

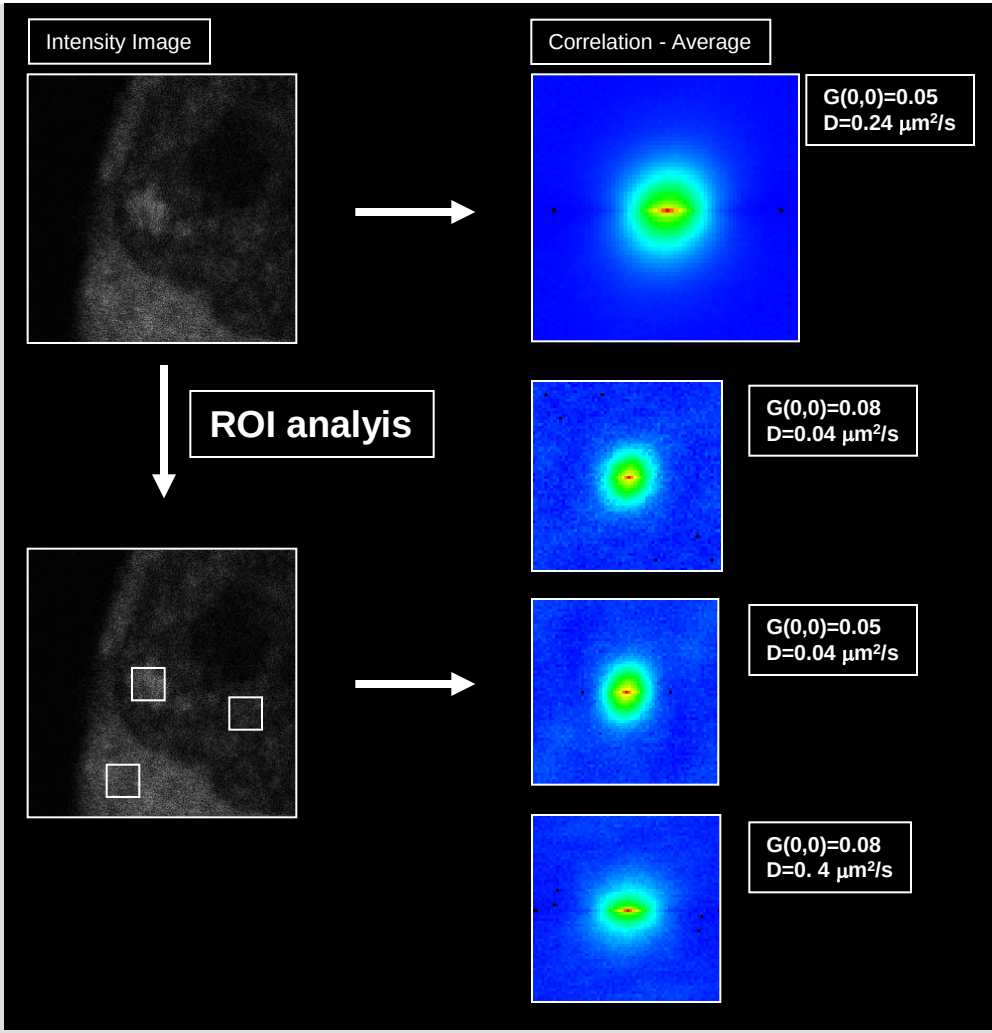


RICS

Example – MS2 RNA complex

Transcript transport

- Record time series
- Compute Correlation
- Fit to appropriate diffusion models
- Compare different regions of interest



Sample : Ute Schmidt and Edouard Bertrand, IGMM – CNRS, Montpellier, France

RICS

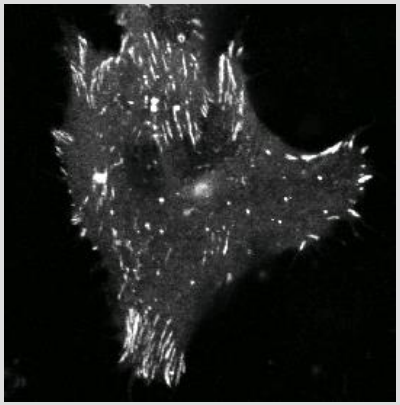
Example – Eos Paxillin

Mobility of Pax between cell adhesion structures

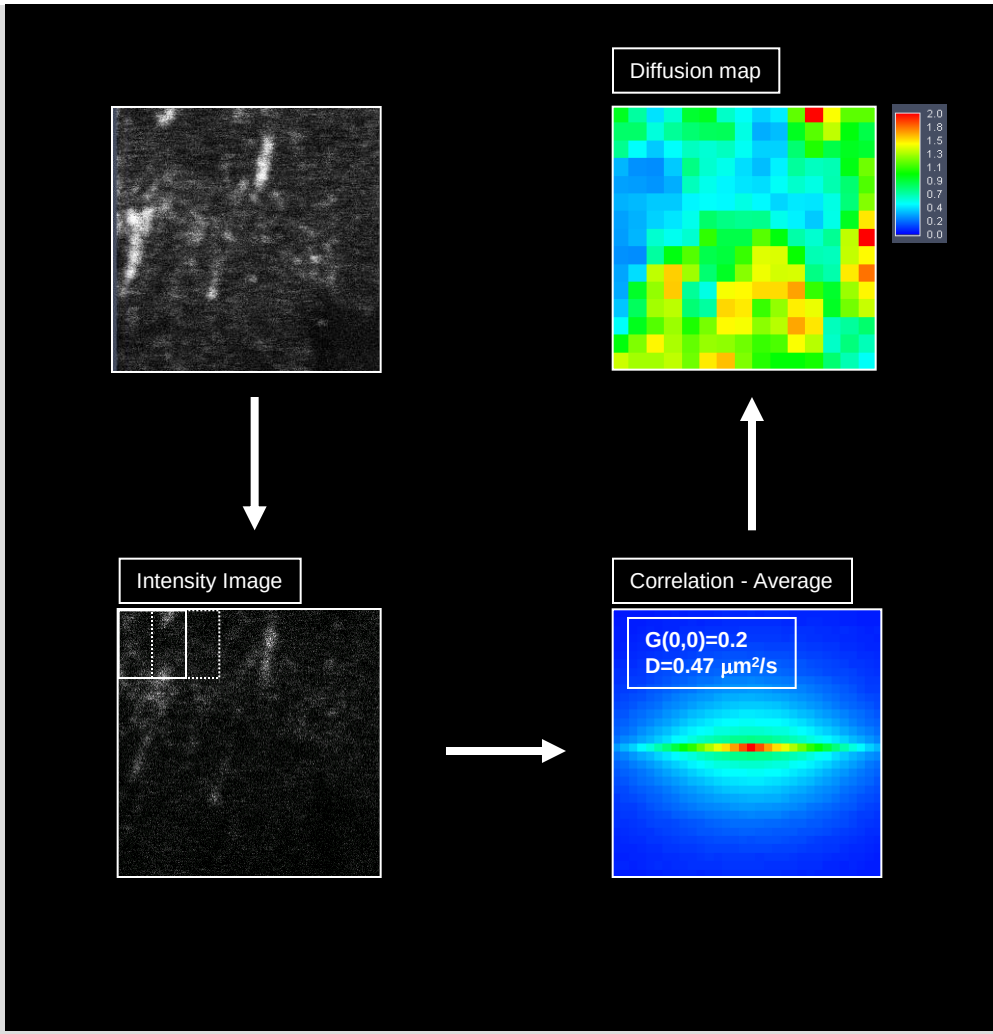
Record time series

Subtract moving average

Compute correlation for overlapping ROIs



Sample: Hari Shroff and Eric Betzig, Janelia Farm Research Campus, Ashburn, VA, USA



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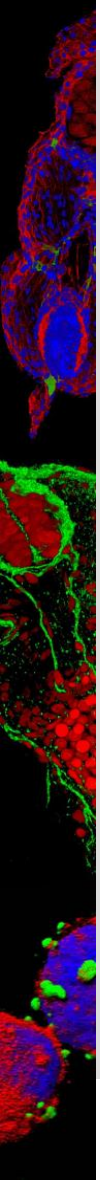


- With 1 scan of e.g. 512*512, there are >250.000 timepoints and positions imaged.
- From this knowledge, dynamical behaviour of molecules can be calculated, just as in FCS
- Within 1 cel, several compartments can be compared with each other (behaviour of a molecule in the nucleus can be different than in the cytoplasm)

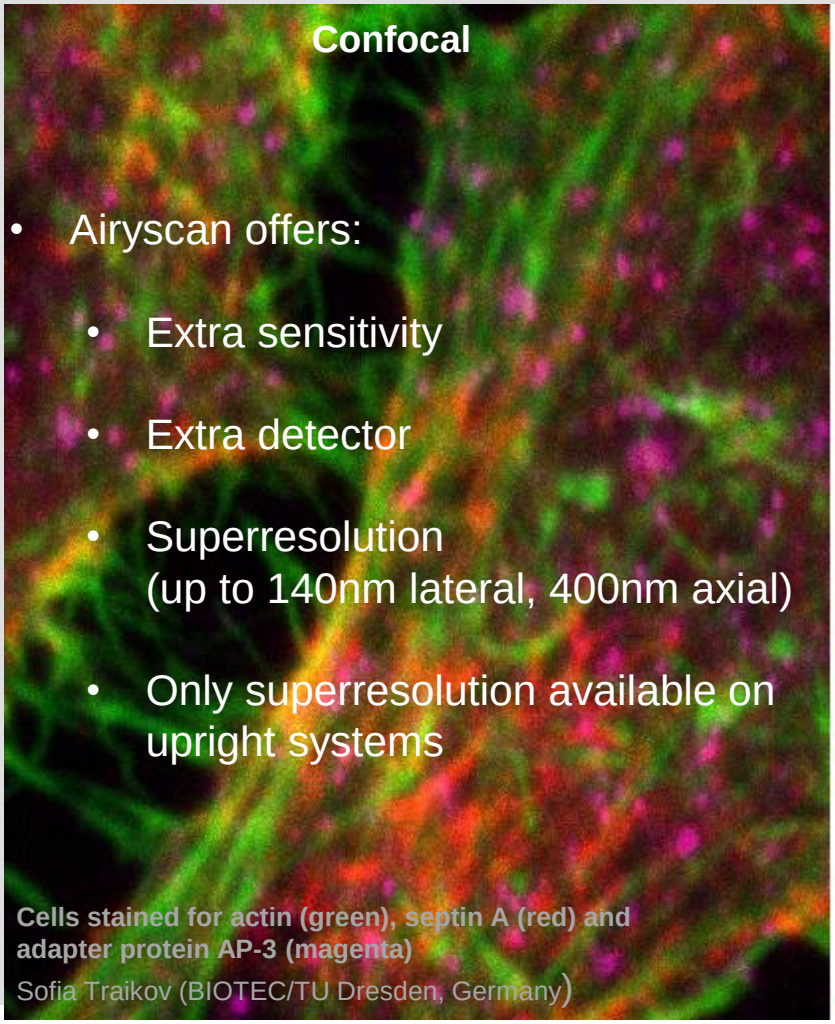
→ FCS data can be extracted without a GaAsP- or APD-detector, though it is less accurate in terms of highly fast dynamics

→ A resonance scanner would visualize the dynamics, but not return any analysis: if one needs to know only the dynamical behaviour of a (set of) molecule(s) (kD, speed, volume), RICS performs better and often even faster.


Explore
New worlds

Upgradable with Airyscan

Upgradable with Airyscan

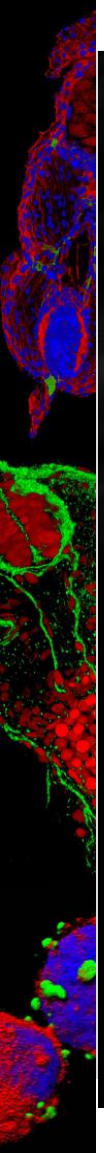


- Airyscan offers:
  - Extra sensitivity
  - Extra detector
  - Superresolution
    (up to 140nm lateral, 400nm axial)
  - Only superresolution available on upright systems

Cells stained for actin (green), septin A (red) and adapter protein AP-3 (magenta)

Sofia Traikov (BIOTEC/TU Dresden, Germany)

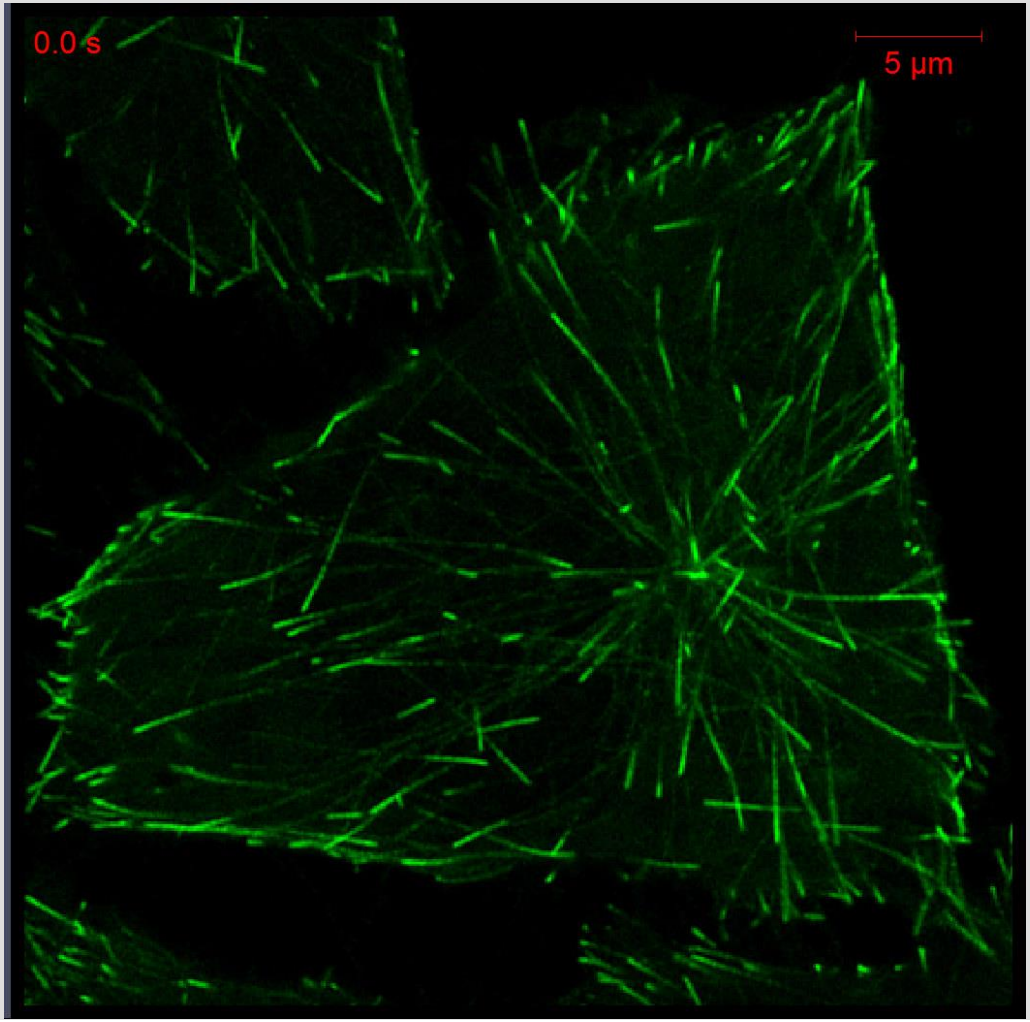
Airyscan reveals more details in your samples by increasing the resolution of LSM 880 up to 1.7-fold



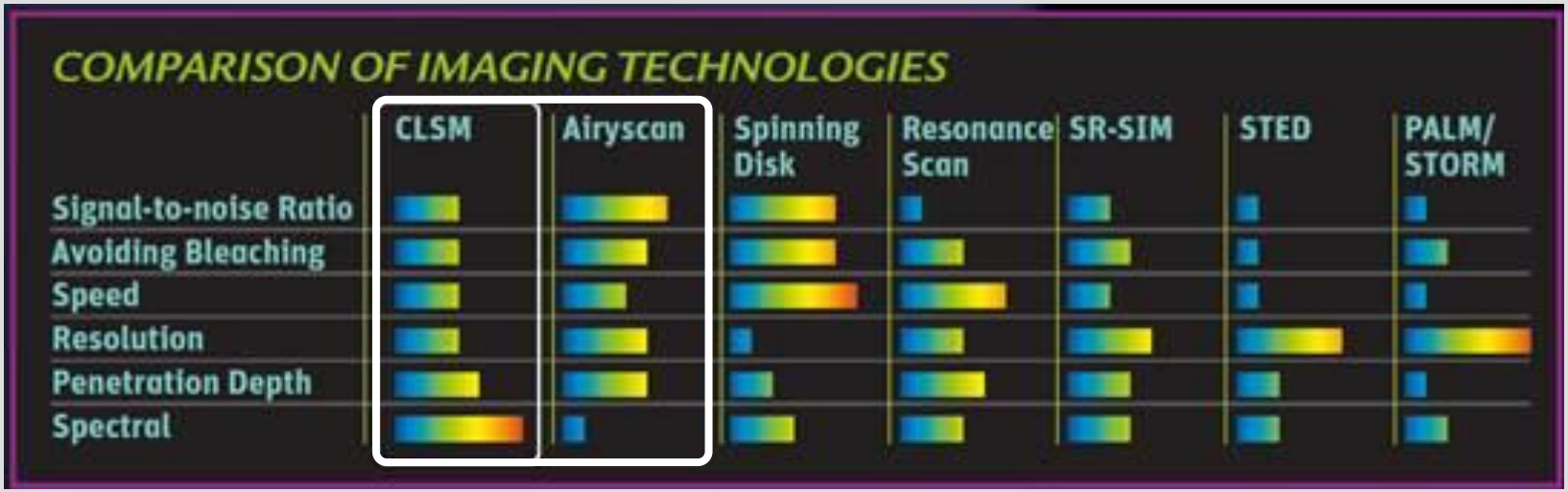
Cultivated mitotic cells stained for tubulin
Peter O'Toole, Ian Morrison (Univ. of York, UK)

LSM 880 – Airyscan: Resolution, SNR, and Flexibility

Intrinsically compatible with live cell imaging



Comparison o imaging technologies



We make it visible.