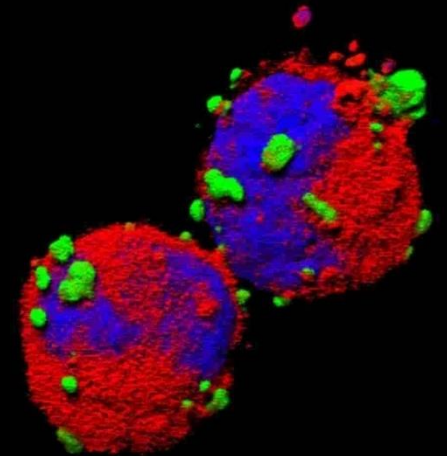


# Imaging & analysis with the LSM780 NLO

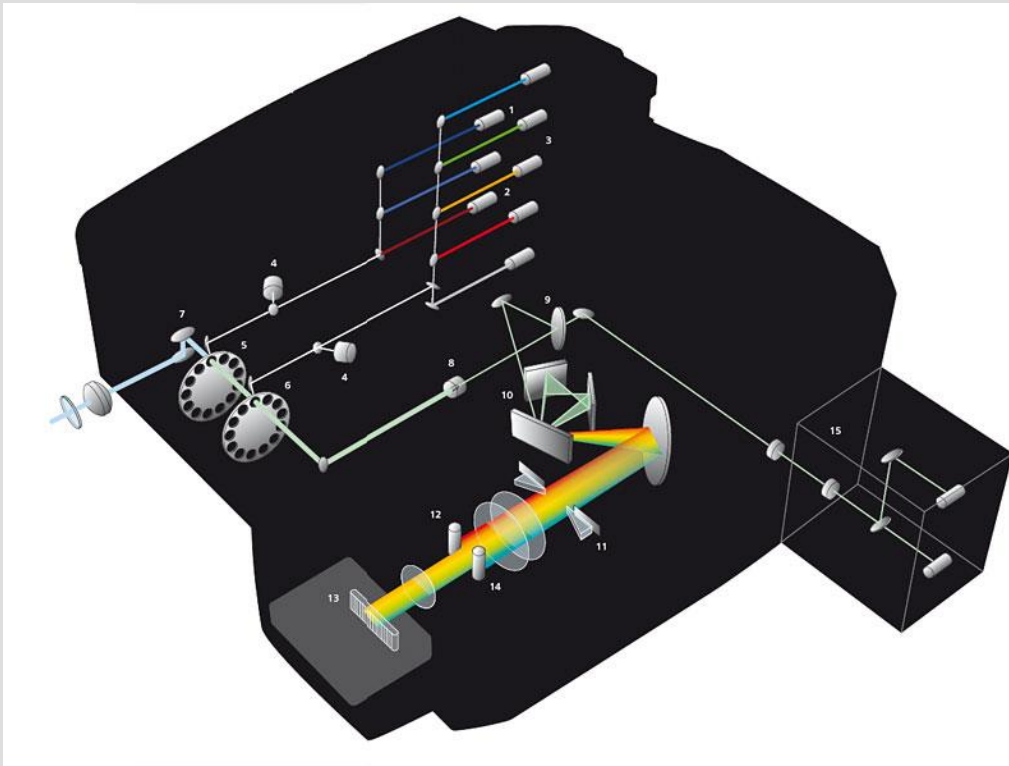
*Discover the secrets beyond the twilight zone*

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 x 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



| <b>Basis</b>      | <b>Imager.Z2</b> | <b>Examiner.Z1</b> | <b>Observer.Z1</b> |
|-------------------|------------------|--------------------|--------------------|
| 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



The screenshot displays the ZEN 2011 software interface. On the left, the 'Acquisition Manager' and 'Acquisition Parameter' panels are visible. The 'Acquisition Mode' is set to 'EC Plan-Neofluar 10x/0.30 M27'. The 'Scan Area' is defined as 848.3 µm x 848.5 µm with a pixel size of 1.66 µm. The 'Channels' panel shows three channels: '1' (blue), '2' (green), and '3' (red).

The central part of the interface features a scatter plot titled 'Intensity Ch2-T2' vs 'Intensity Ch1-T1'. The plot shows a dense distribution of points with a color scale from 0 to 250. A color bar below the plot is labeled 'Absolute Frequency'.

To the right of the scatter plot is a fluorescence image showing the same field of view with three channels (blue, green, red) overlaid. The 'Images and Documents' panel on the far right shows the current workspace configuration.

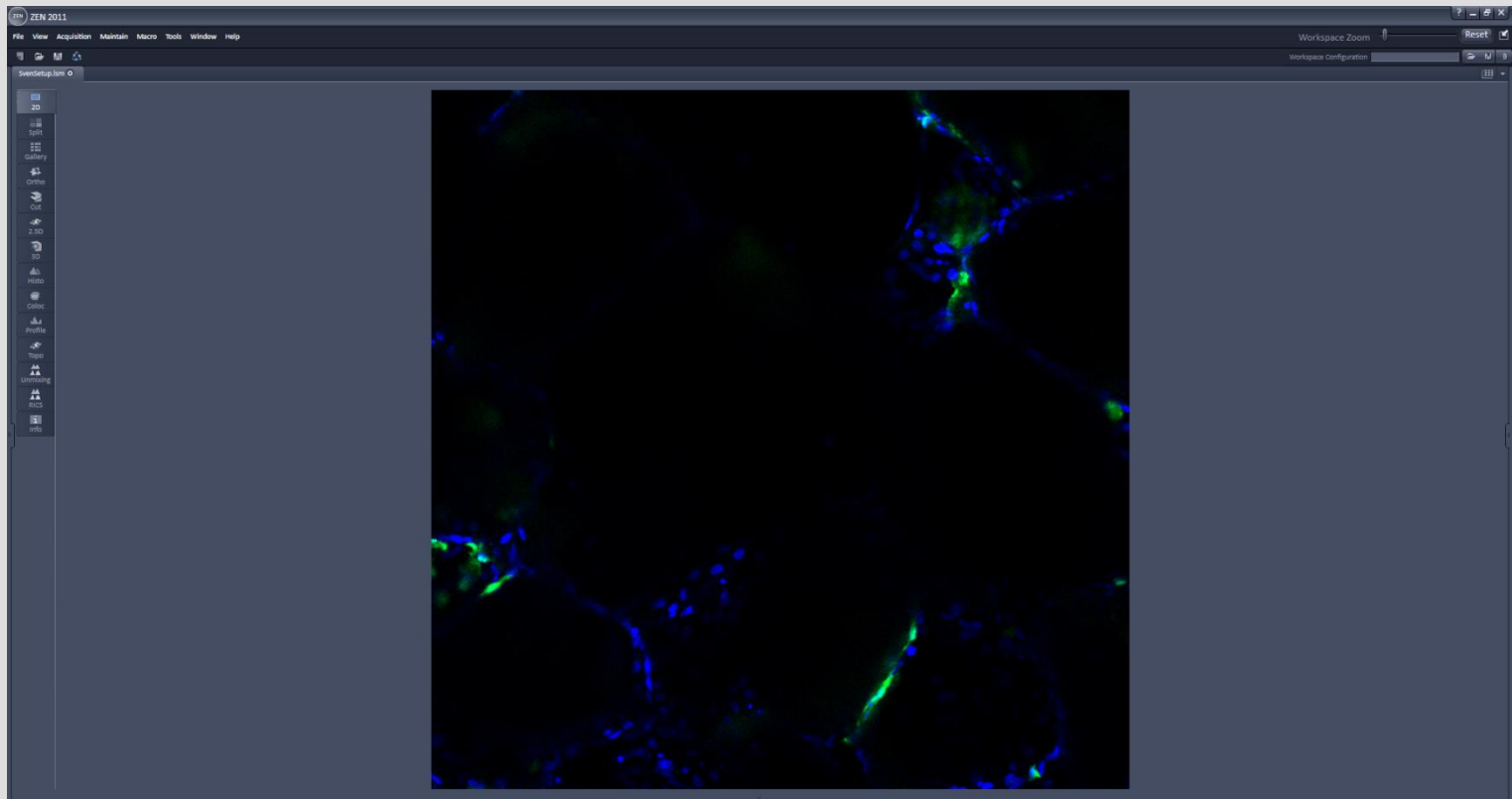
At the bottom, a table provides statistical data for the three channels:

| 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              |

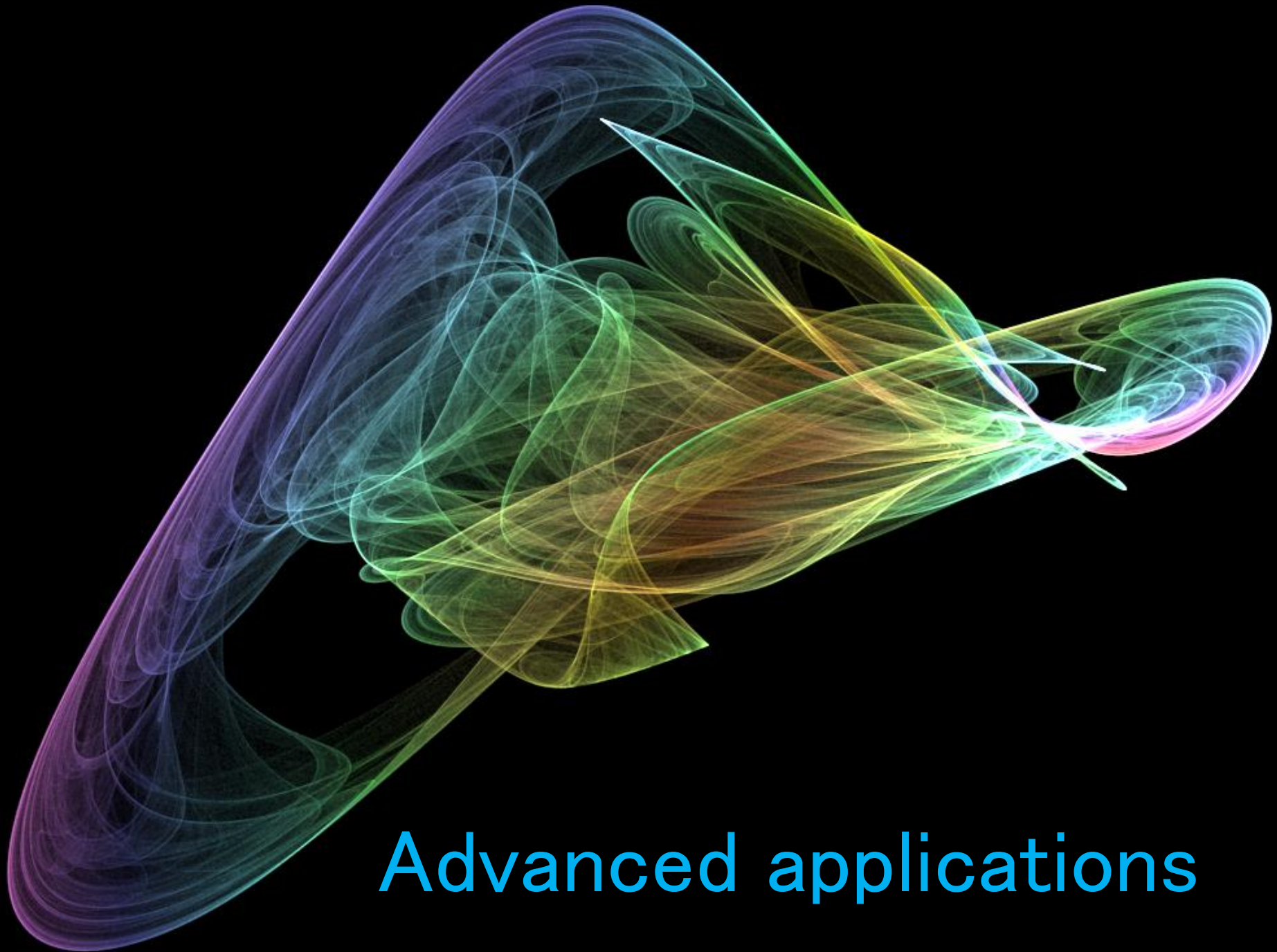
# ZEN2012

Visualize and access at ease

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



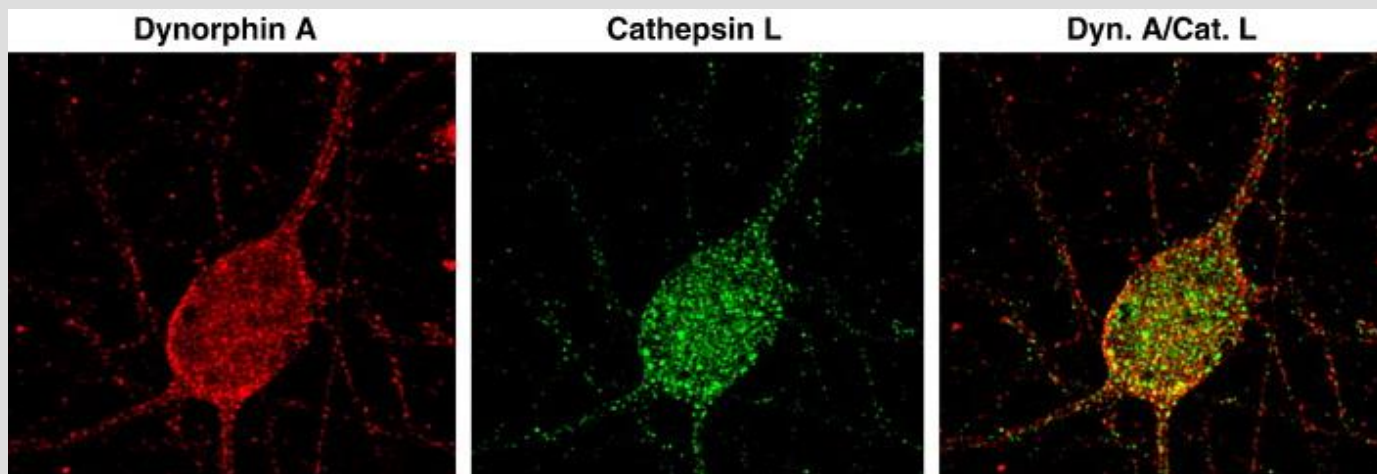




**Advanced applications**

# ZEN2012 Applications

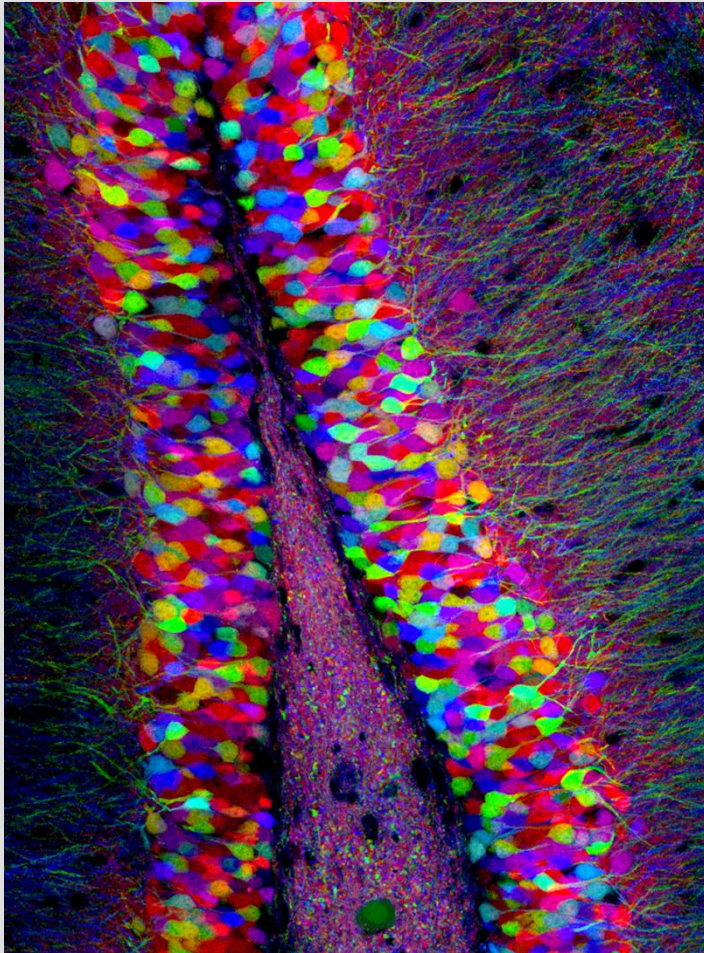
## Co-localization analysis



- 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

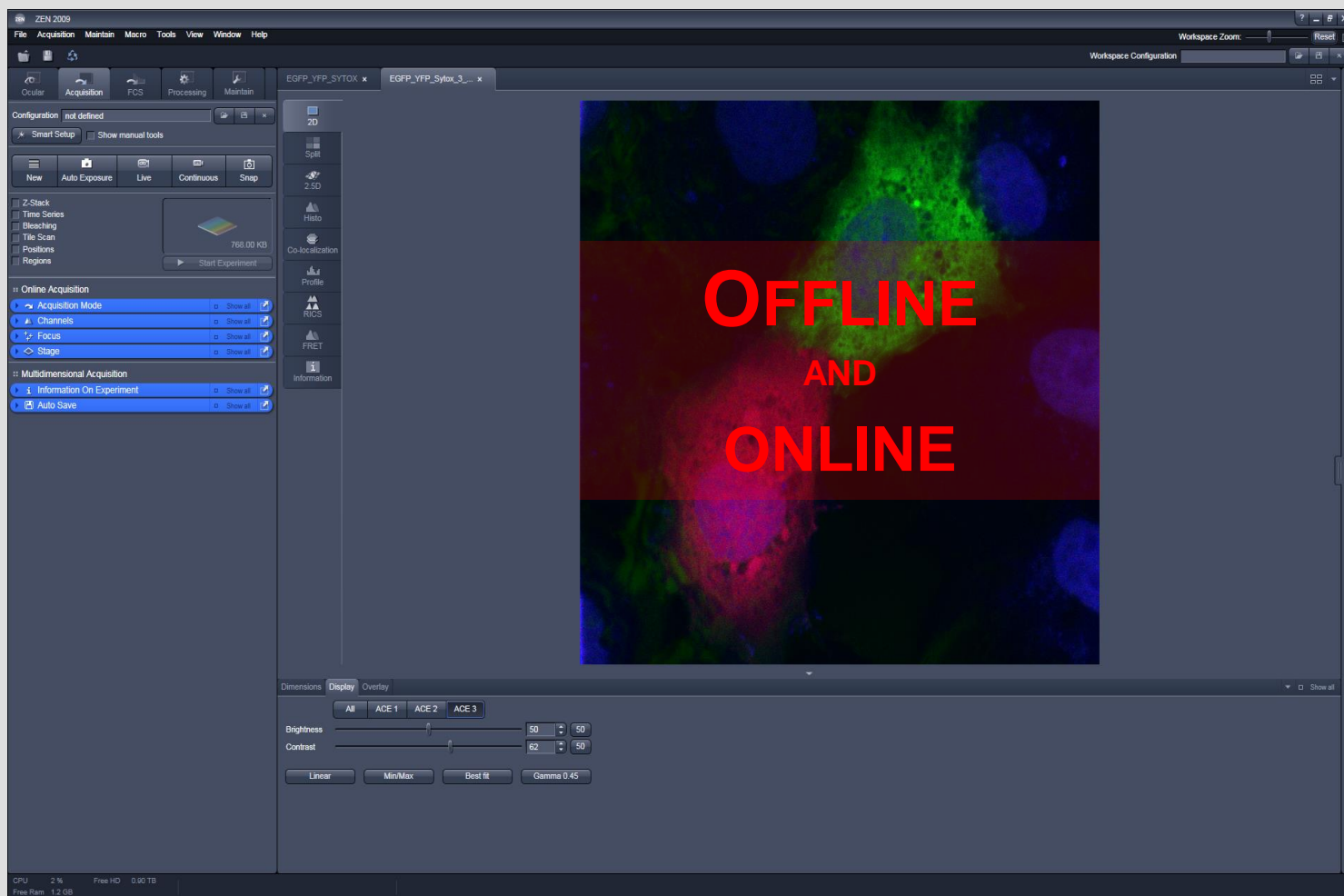


- Using the technique of the “Brainbow” mouse, different subpopulations of neurons emit different colours to allow studying brain connectivity.
- With current available fluorescence filters, it is impossible to separate all these colours without having bleedthrough. Spectral unmixing – scanning of the whole light spectrum – overcomes this problem.
- Used for:
  - Detection of multiple fluorophores
  - Separating 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 separated
- Advantages:
  - Combine CFP, GFP, YFP etc
  - Improve FRET results
  - Improve co-localization
  - Speed up system
  - Improve RICCS (cross-correlation) analysis

# ZEN2012 Applications

## Physiology

**Configuration** not defined

Smart Setup  Show manual tools

New Auto Exposure Live Continuous Snap

Z-Stack  
 Time Series 100 Images  
 Bleaching 15 Iteration(s)  
 Tile Scan  
 Positions  
 Regions

Start Experiment

**Online Acquisition**

- Acquisition Mode  Show all
- Channels  Show all
- Focus  Show all
- Stage  Show all
- Regions  Show all

**Multidimensional Acquisition**

Bleaching  Show all

Bleach settings not defined

Start Bleaching after # scans 5 of 100  
 Repeat Bleach after # scans  
 Iterations 15

Safe bleach for GaASP

Excitation of Bleach

Use different Settings for different ROIs

ROI # all

405 458 488 514 561 594 633

488 nm 30.0

Time Series  Show all  
 Information On Experiment  Show all  
 Auto Save  Show all

CPU 0% Free HD 0.93 TB  
Free Ram 1.3 GB

**Cardiomyocytes20ms**

Intensity

250  
200  
150  
100  
50  
0

0.0 0.5 1.0 1.5 2.0 2.5 3.0

Time (s)

Region 1: (red) Region 2: (green) Region 3: (blue)

| Time [s] | Intensity Region 1 | Intensity Region 2 | Intensity Region 3 |
|----------|--------------------|--------------------|--------------------|
| 0.0000   | 102.9              | 105.8              | 124.4              |
| 0.0217   | 115.0              | 118.6              | 135.3              |
| 0.0428   | 118.9              | 112.4              | 126.4              |
| 0.0647   | 116.2              | 108.1              | 116.0              |
| 0.0867   | 110.6              | 105.1              | 107.3              |
| 0.1078   | 103.2              | 98.3               | 102.7              |
| 0.1297   | 104.7              | 94.1               | 98.6               |
| 0.1508   | 98.9               | 93.0               | 99.0               |
| 0.1727   | 97.4               | 91.9               | 96.3               |
| 0.1948   | 94.4               | 89.7               | 97.1               |

Dimensions Display Mean ROI

Time 76

Zoom 102%

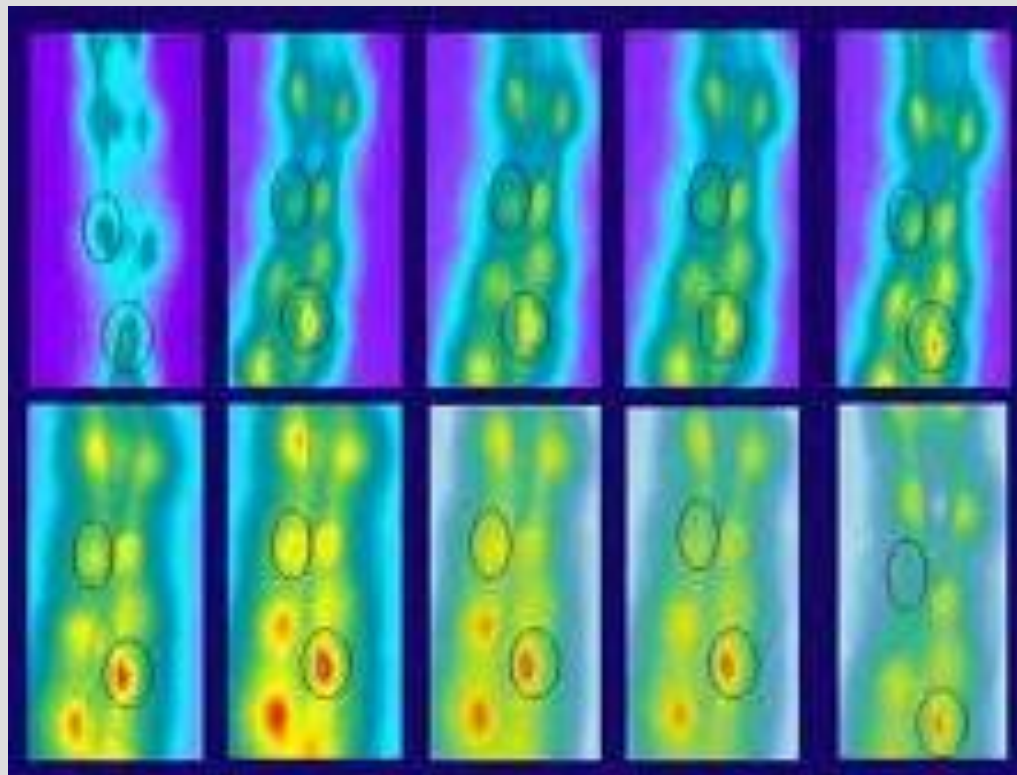
Channels Ch1

Reuse Crop Positions Stage

# 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

The screenshot displays the ZEN 2009 software interface for a FRAP experiment. The main window shows a fluorescence image of a salivary gland with three regions of interest (ROIs) marked in red, green, and blue. A graph plots Intensity (0 to 4000) against Time (0 to 40 seconds), showing a sharp drop in intensity at approximately 10 seconds, followed by a recovery curve. The software interface includes various configuration panels on the left, such as 'Online Acquisition' and 'Multidimensional Acquisition', and a 'Workspace Configuration' panel at the top right. A table below the graph provides detailed FRAP parameters for each region.

| Region | IE     | I1 mobile fraction | F1 mobile fraction [%] | T1 [s] | I1 half [s] | K1 [1/s] | I delta immobile fraction | F1 immobile fraction [%] |
|--------|--------|--------------------|------------------------|--------|-------------|----------|---------------------------|--------------------------|
| 1      | 251.47 | 39.36              | 100.00                 | 0.7375 | 0.5112      | 1.3559   | 0.00                      | 0.00                     |
| 2      | -3.77  | -200.22            | 100.00                 | 3.6361 | 2.5204      | 0.2750   | 0.00                      | 0.00                     |
| 3      | 35.49  | -136.12            | 100.00                 | 3.6671 | 2.5418      | 0.2727   | 0.00                      | 0.00                     |

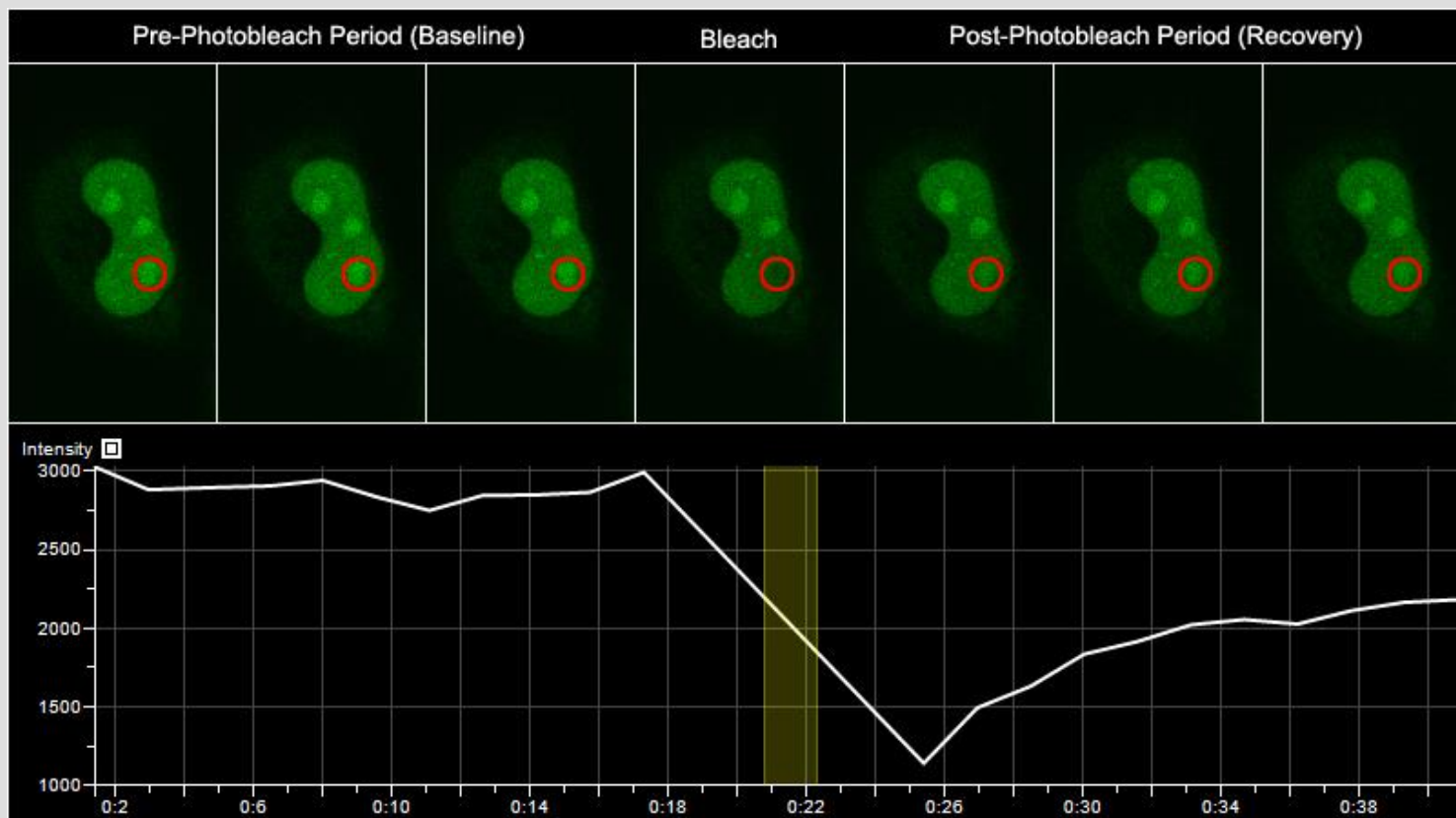
  

| Time [s] | Intensity Region 1 | Intensity Region 2 | Intensity Region 3 |
|----------|--------------------|--------------------|--------------------|
| 0.000    | 222                | 119                | 112                |
| 0.798    | 206                | 98                 | 102                |
| 1.597    | 212                | 90                 | 105                |
| 2.395    | 279                | 173                | 167                |
| 3.193    | 255                | 136                | 140                |
| 3.991    | 198                | 80                 | 88                 |
| 4.789    | 223                | 83                 | 118                |
| 5.588    | 210                | 76                 | 99                 |
| 6.386    | 230                | 117                | 115                |
| 7.184    | 234                | 142                | 124                |
| 7.982    | 282                | 162                | 176                |
| 8.781    | 211                | 109                | 100                |



# ZEN2012 Applications

## FRAP – Fluorescence Recovery After Photobleaching



# ZEN2012 Applications

## FRET – Fluorescence Resonance Energy Transfer

Configuration: not defined

Smart Setup | Show manual tools

New | Auto Exposure | Live | Continuous | Snap

Z-Stack | Time Series (10 Images) | Bleaching | Tile Scan (2.50 MB) | Positions | Regions | Start Experiment

Online Acquisition

- Acquisition Mode
- Channels
- Focus
- Stage

Multidimensional Acquisition

- Time Series
- Information On Experiment
- Auto Save

Workspace Configuration

Open Images

- FRETAcceptorbleach1.lam (18 MB)
- Acceptor bleach\_Linear unmixing (4.0 MB)

Region 0

| Region | Time    | D avg.  | A avg.  | F avg.  | FRETIN  | Fc      | N-FRET  |
|--------|---------|---------|---------|---------|---------|---------|---------|
| 0      | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 |

Dimensions | Display | Overlay

Time: 3

Zoom: 100%

Channels: 4024, 4044, 4050, 4058, 4060, 4062, 4064, 4066, 4068, 4070, 4072, 4074, 4076, 4078, 4080

Palette: Reuse | Crop | Positions | Stage

FRET: Parameter | Thresholds | Settings

Method: Fc (Youvan) | Export

| # | Type | Object                              | Background                          | Enabled                             |
|---|------|-------------------------------------|-------------------------------------|-------------------------------------|
| 1 | +    | <input checked="" type="checkbox"/> | <input type="checkbox"/>            | <input checked="" type="checkbox"/> |
| 2 | +    | <input checked="" type="checkbox"/> | <input type="checkbox"/>            | <input checked="" type="checkbox"/> |
| 3 | +    | <input type="checkbox"/>            | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> |

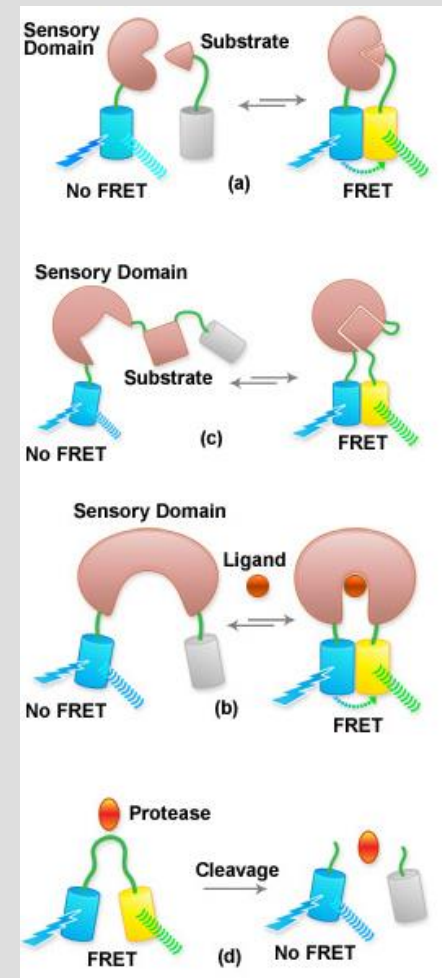
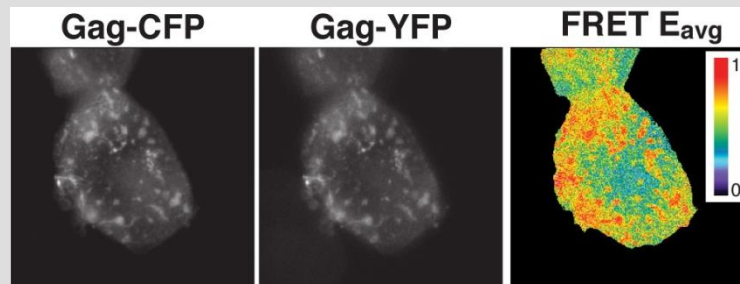
Numbers | Measure

CPU: 0% | Free HD: 7.0 GB | Free Ram: 4.1 GB

# ZEN2012 Applications

## FRET – Fluorescence Resonance Energy Transfer

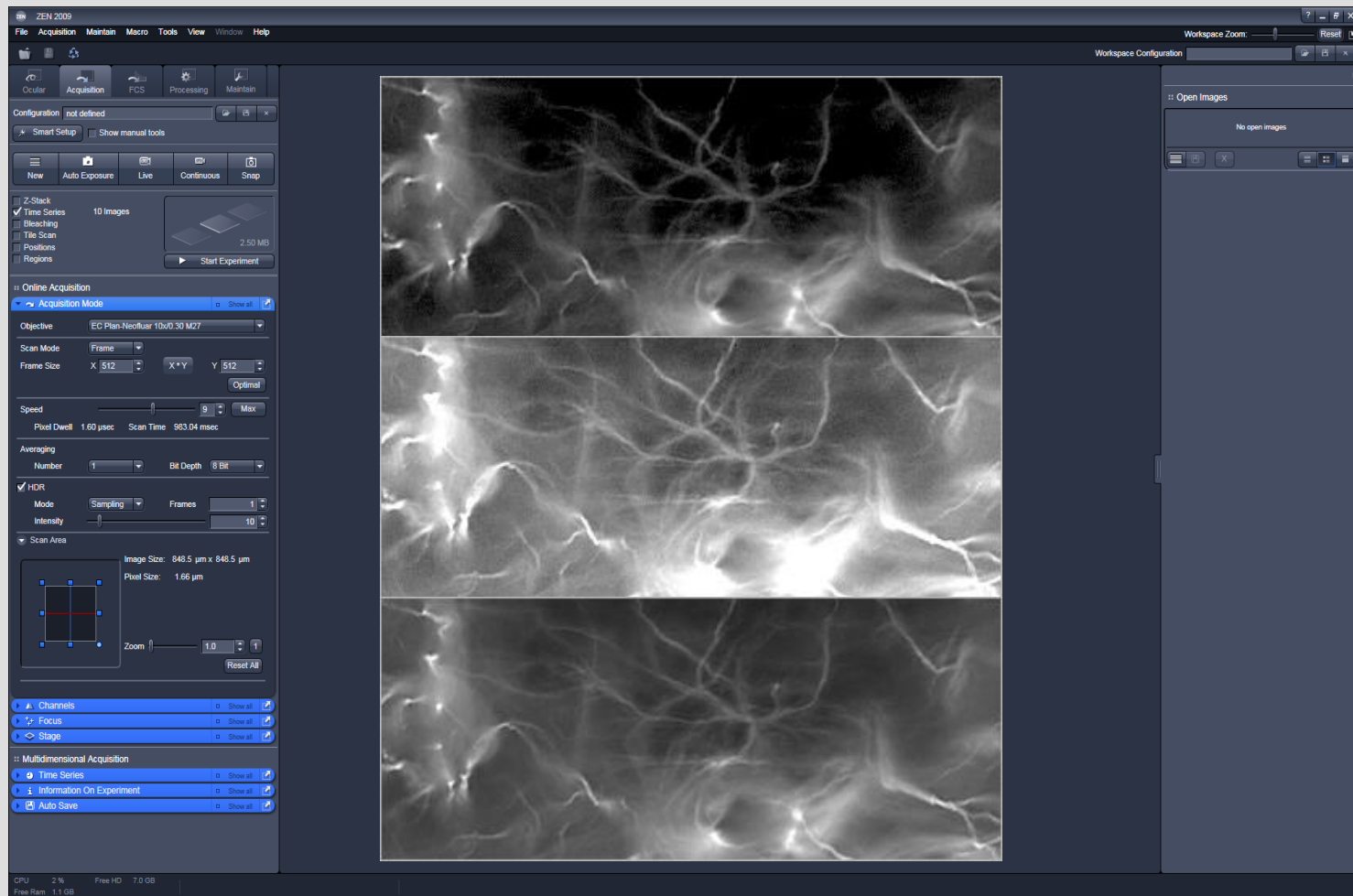
- Used to observe interactions:
  - 2 differently stained molecules will transfer energy when they are closer than 10nm (interacting), as such that the second fluorophore – which is not excited with light – will start emitting light
- Analysis of:
  - Receptor binding, antagonist binding,...
  - Structural changes of molecules (protein folding)
  - Ligand binding
  - Enzym activity





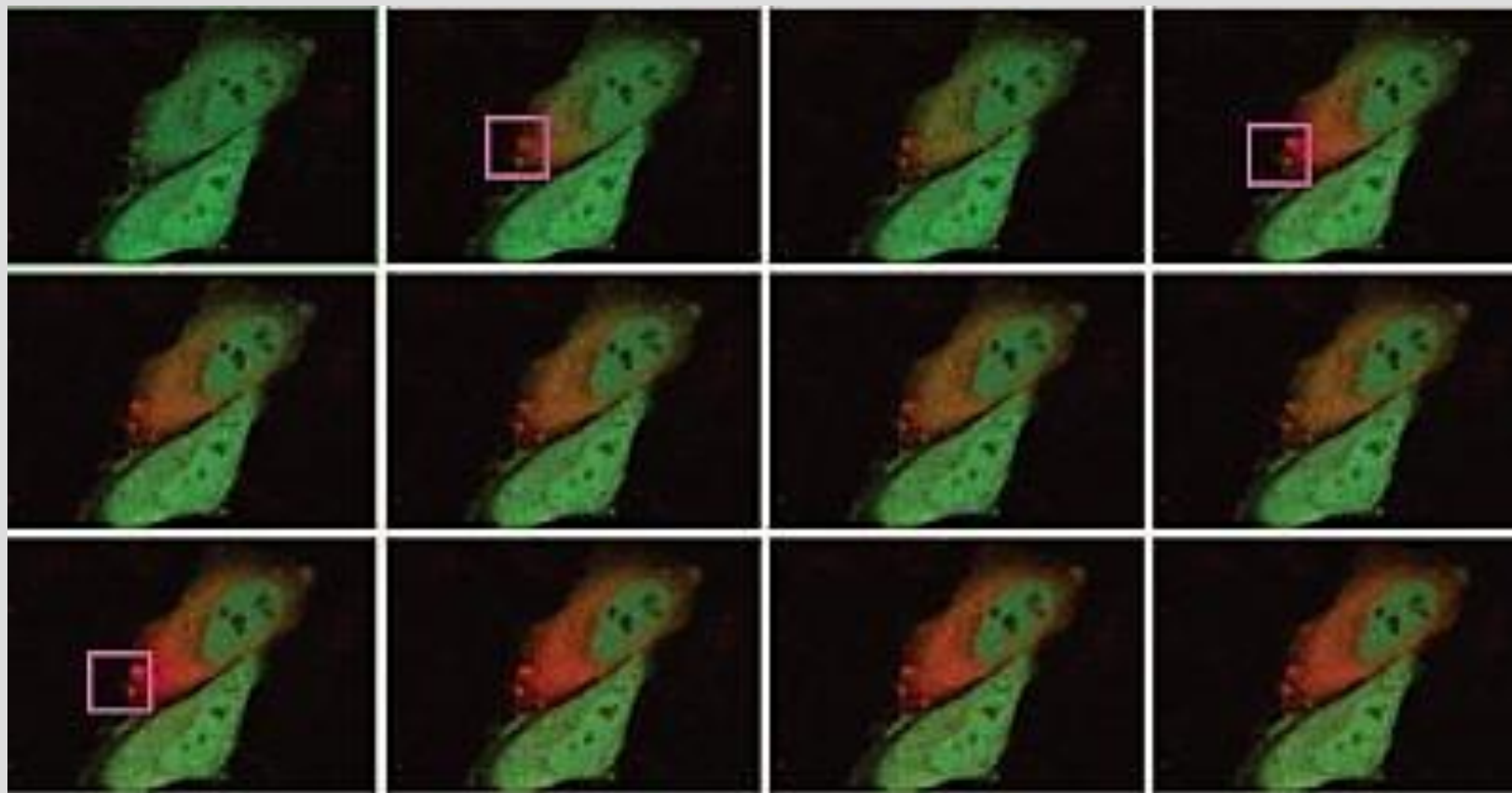
# ZEN2012 Applications

## HDR – High Dynamic Range



# ZEN2012 Applications

## Photoactivation/switching/uncaging





live cell imaging

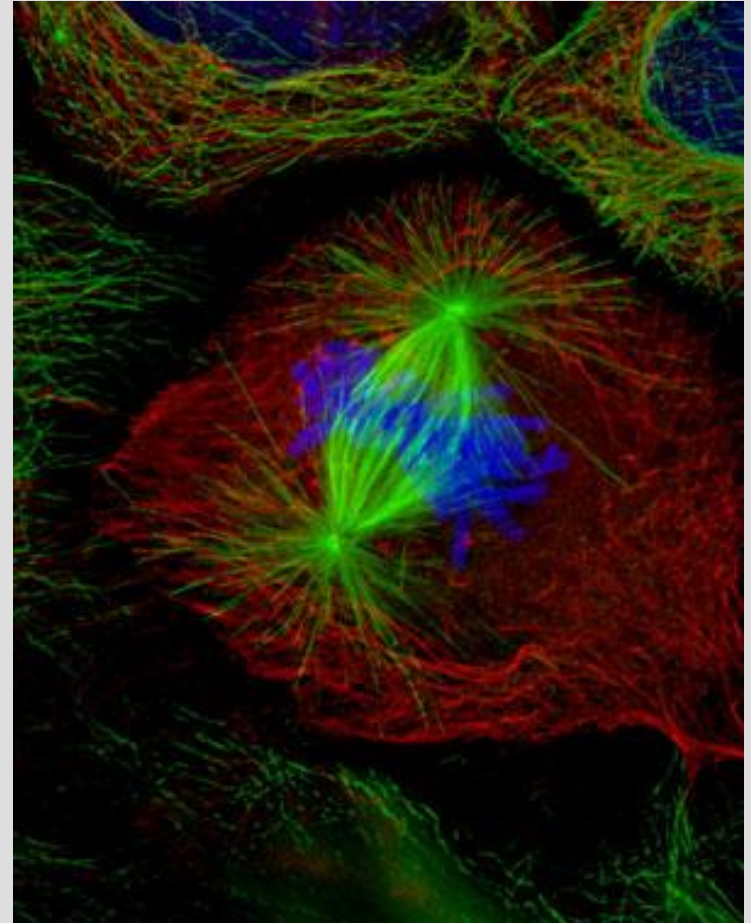
Esther  
Johansson '04

# Live Cell Imaging

## Overview

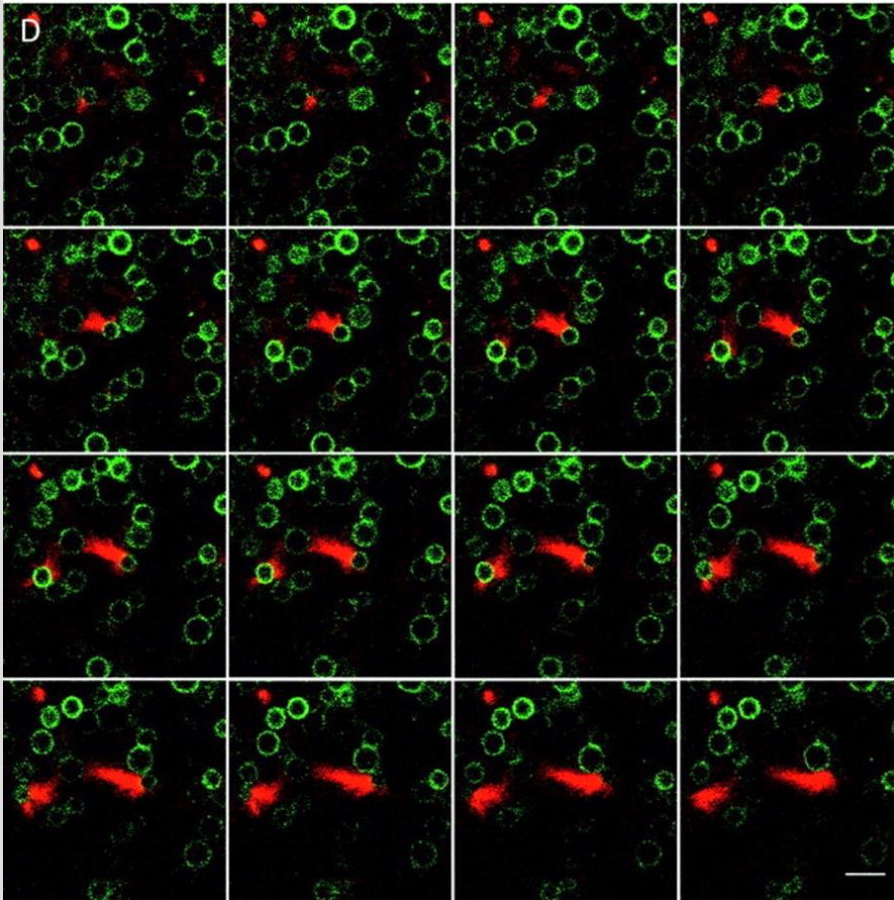


- Heatshock experiments
- Oxygen-dependent experiments
- Extreme photo-sensitive conditions
- Deep tissue imaging with two-photon



# Live Cell Imaging

## *Heatshock experiments*



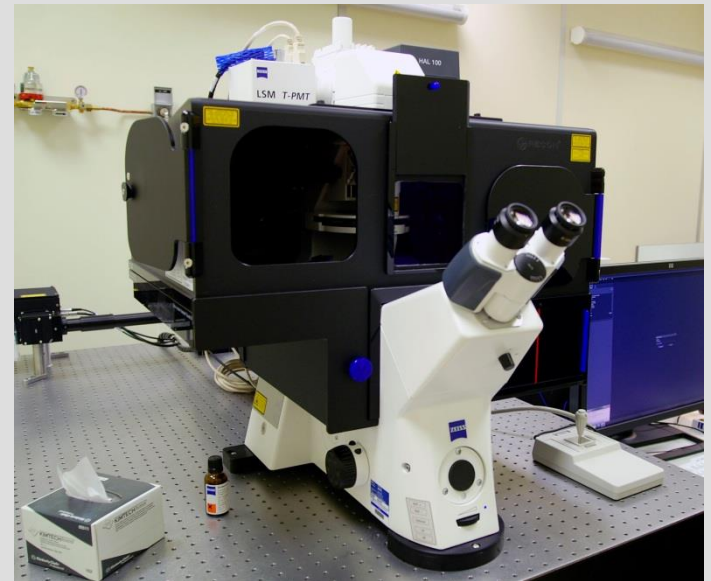
- Activation of genetic constructs for protein expression or enzymatic reactions, initiated by quickly changing  $t^\circ$  (mostly  $37^\circ\text{C}$  to  $45^\circ\text{C}$  and back)
- Genetically engineered
- Heatshock is useful for analysis of:
  - Dynamics analysis
  - Molecular interactions
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



# Live Cell Imaging

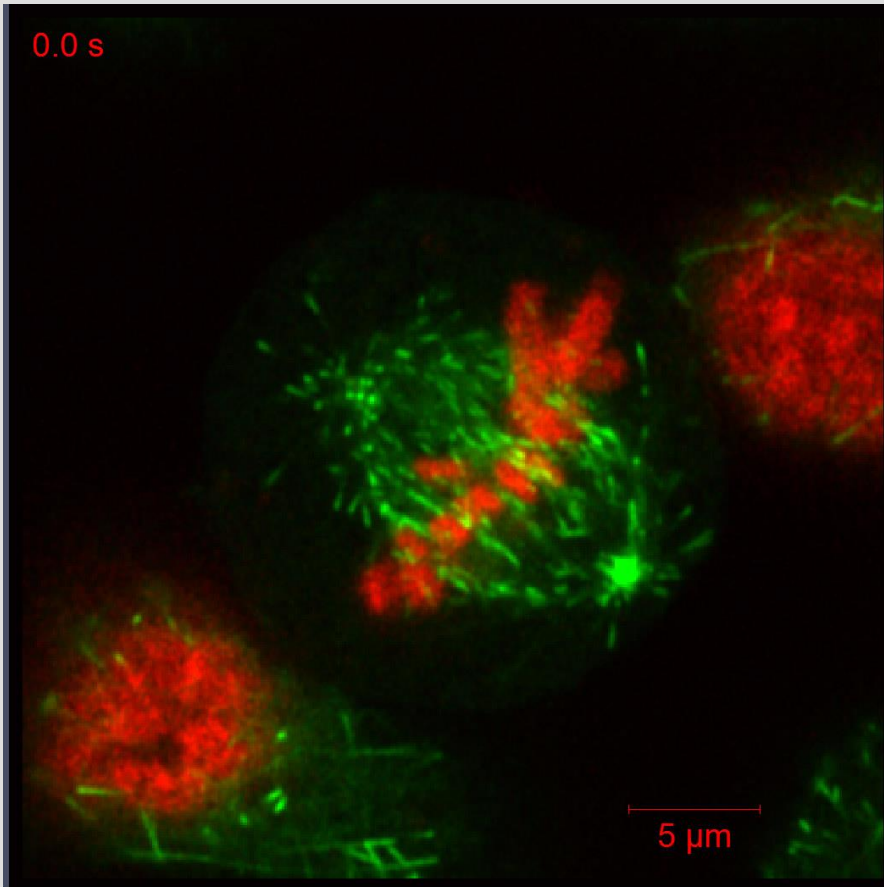
## *Oxygen dependent experiments*

- Special incubators can control not only temperature and CO<sub>2</sub>, but also O<sub>2</sub>-levels.
- Useful for conditioned imaging:
  - Normoxia
  - Hyperoxia
  - Hypoxia



# Live Cell Imaging

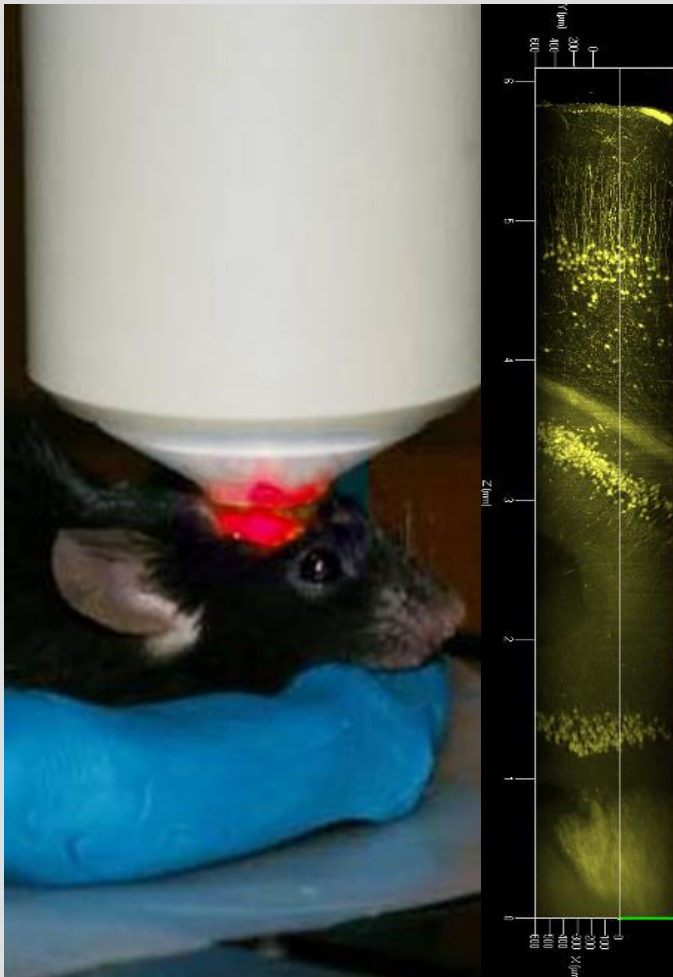
## *Extreme photo-sensitive conditions*



- 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

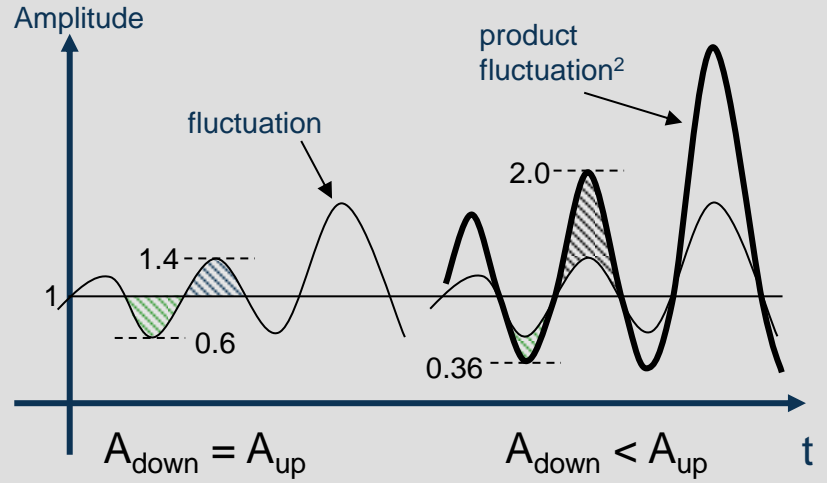
## FCS - Fluorescence Correlation Spectroscopy

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

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

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

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



# ZEN Applications

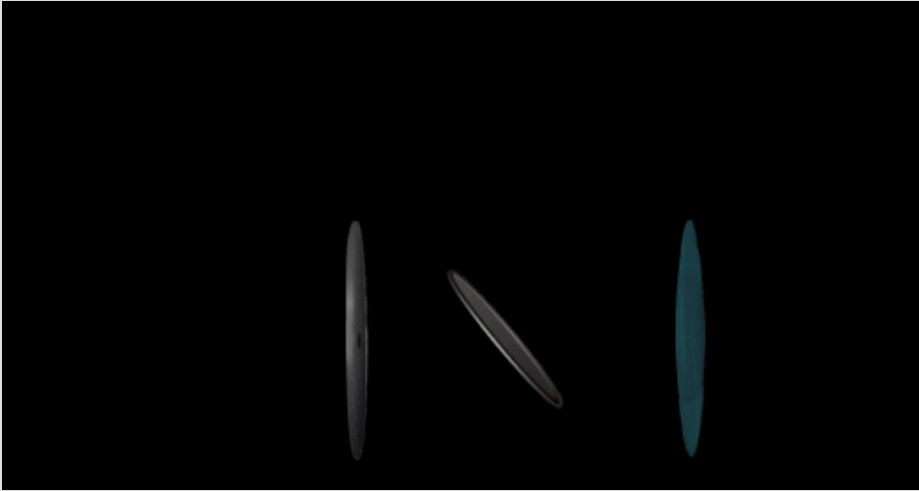
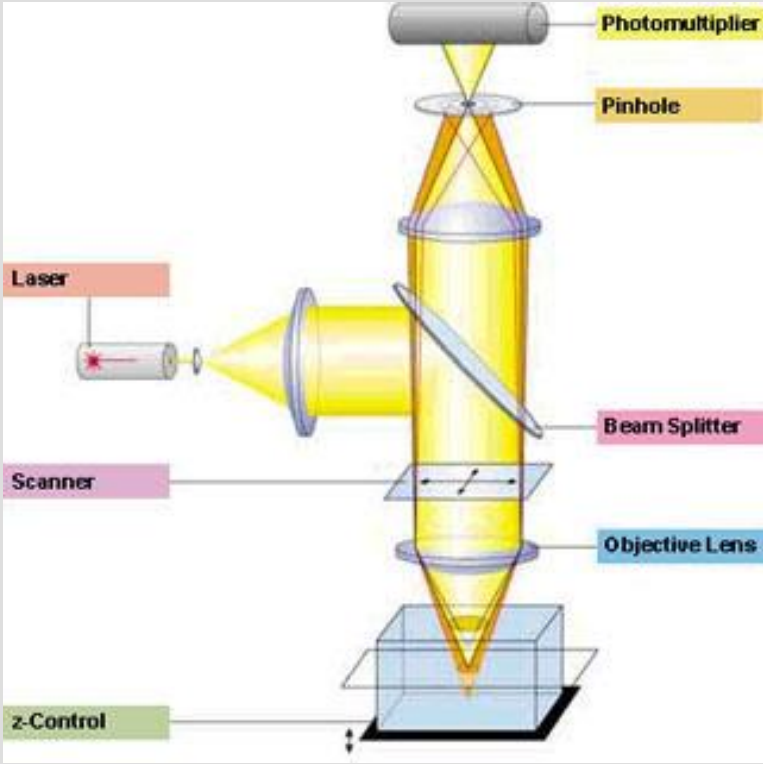
## *FCS - Fluorescence Correlation Spectroscopy*

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



# ZEN Applications

## FCS - Fluorescence Correlation Spectroscopy

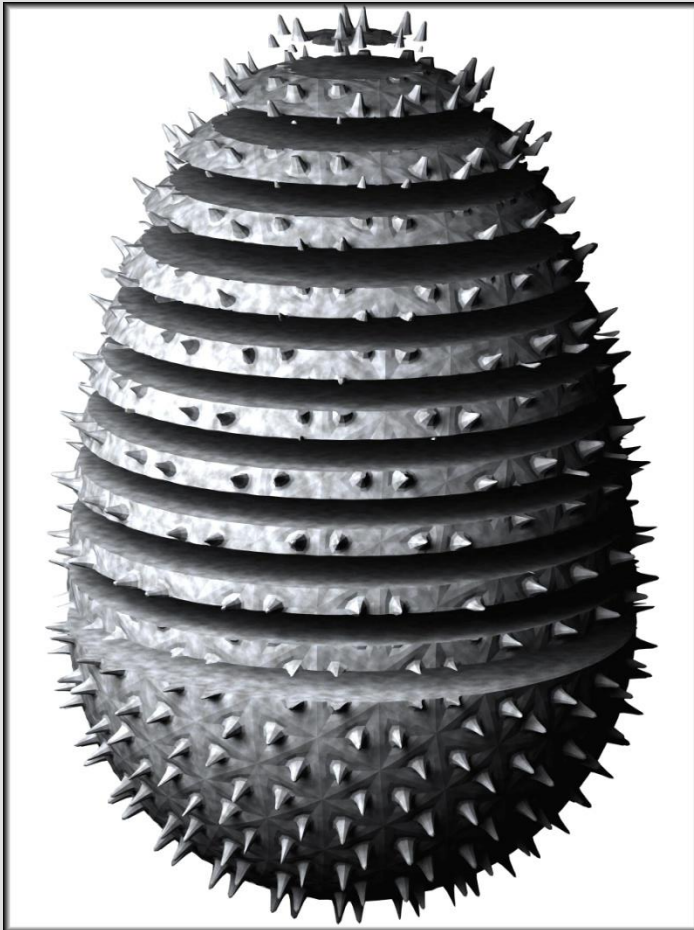


**Only the optical section is being observed.**



# ZEN Applications

## *FCS - Fluorescence Correlation Spectroscopy*



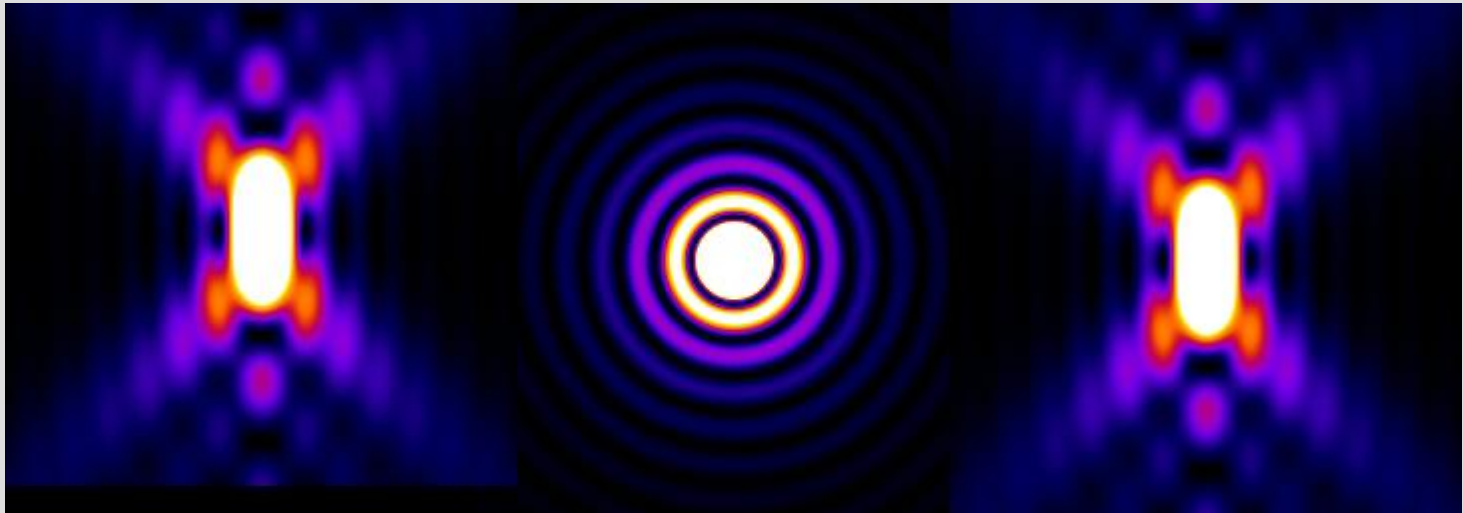
**A serie of optical sections can, once combined, recreate the original 3-dimensionale object.**

**In confocal microscopy 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

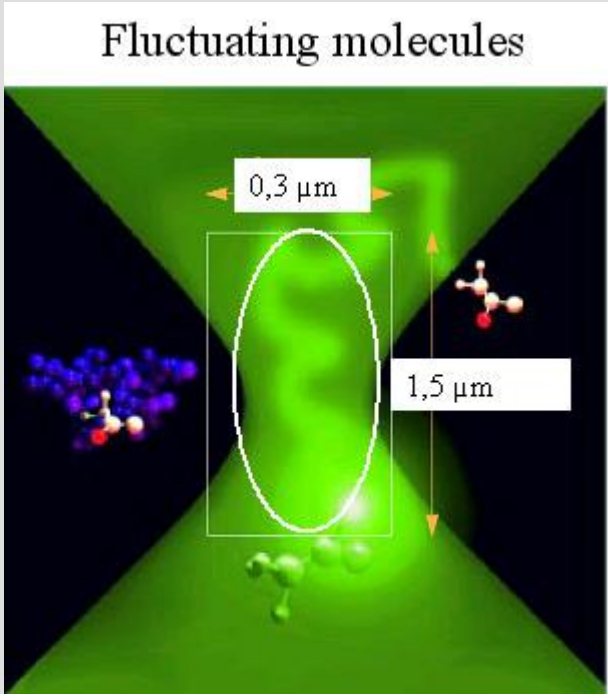
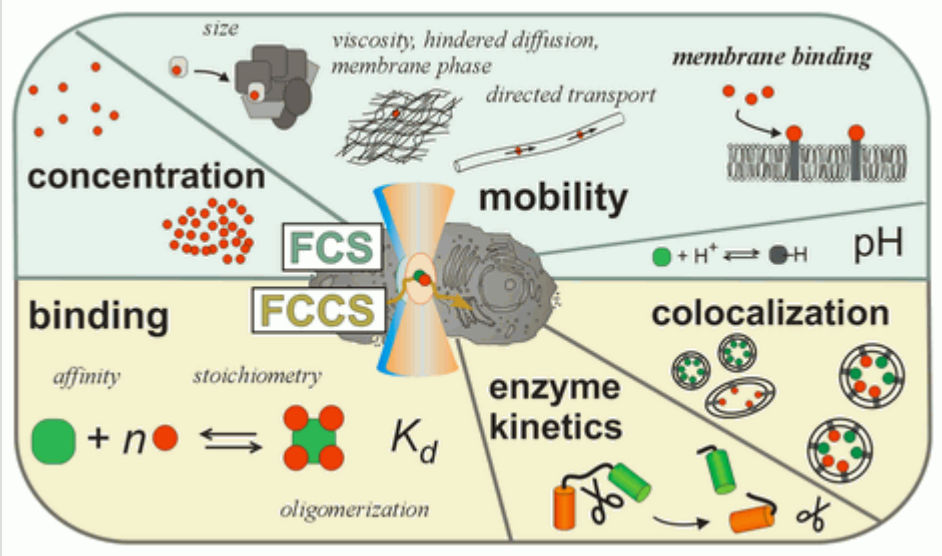
## *FCS - Fluorescence Correlation Spectroscopy*



# ZEN Applications

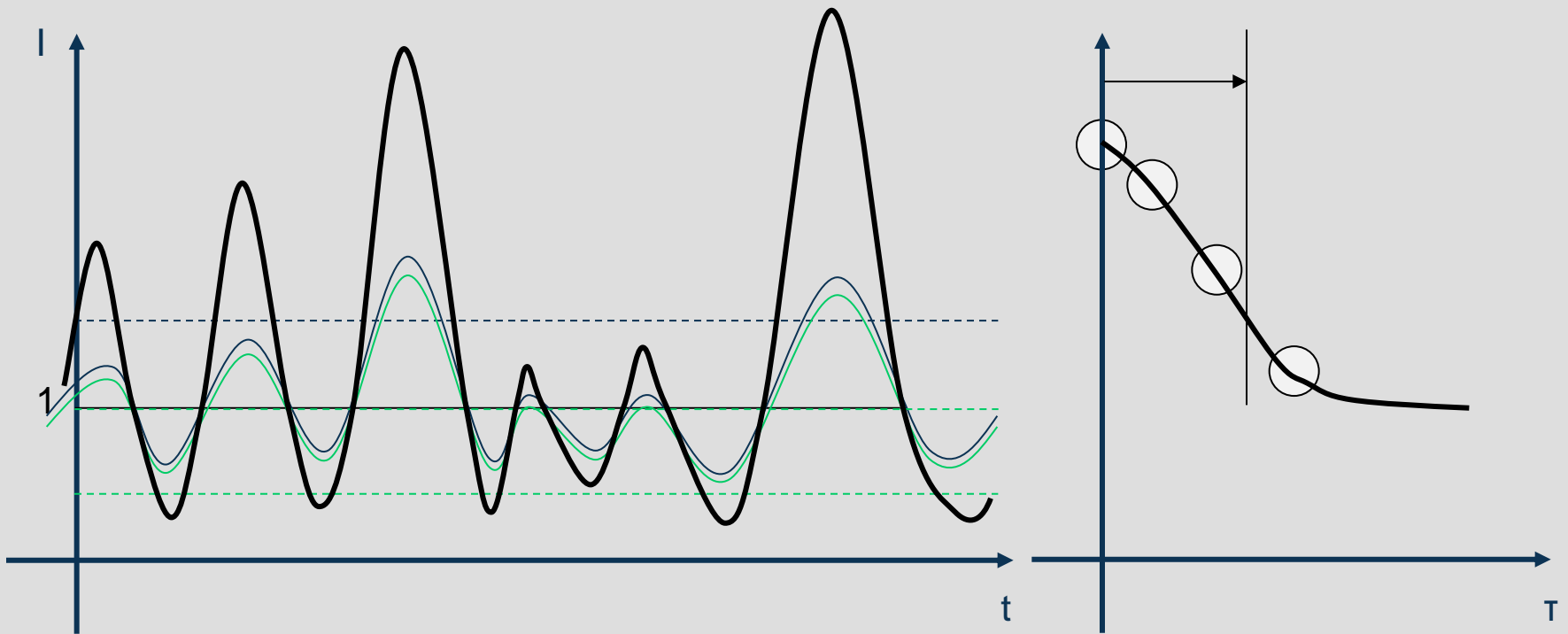
## FCS - Fluorescence Correlation Spectroscopy

- 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

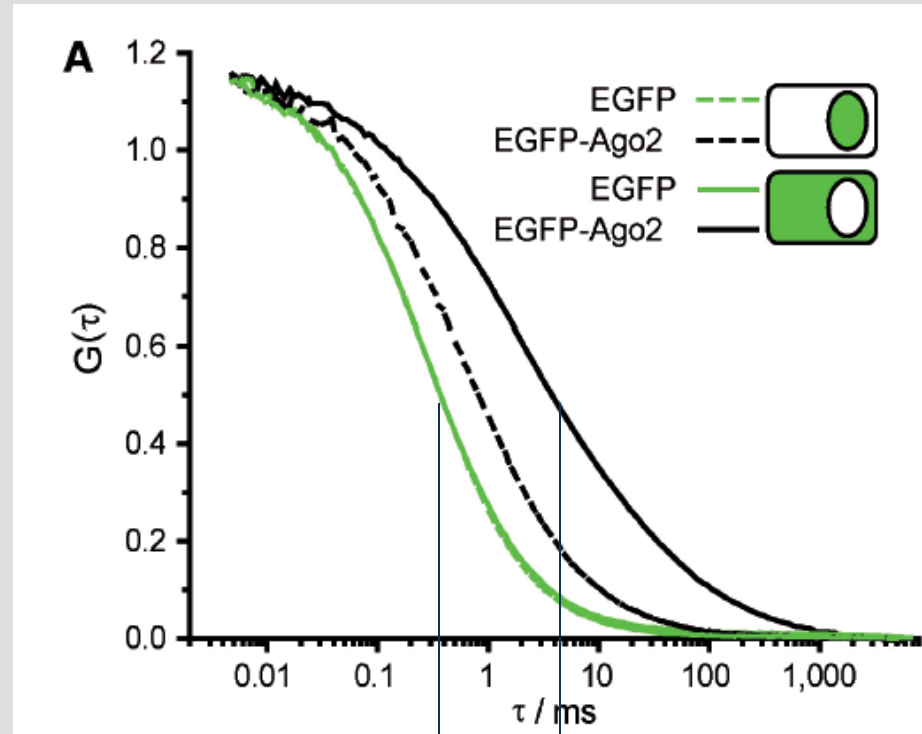
## FCS - Fluorescence Correlation Spectroscopy



Correlation curve  
Slope = speed

# ZEN Applications

## FCS - Fluorescence Correlation Spectroscopy



order of mag!

With Ago2: slower

# ZEN Applications

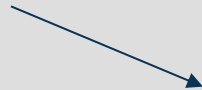
## FCS - Fluorescence Correlation Spectroscopy



**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**

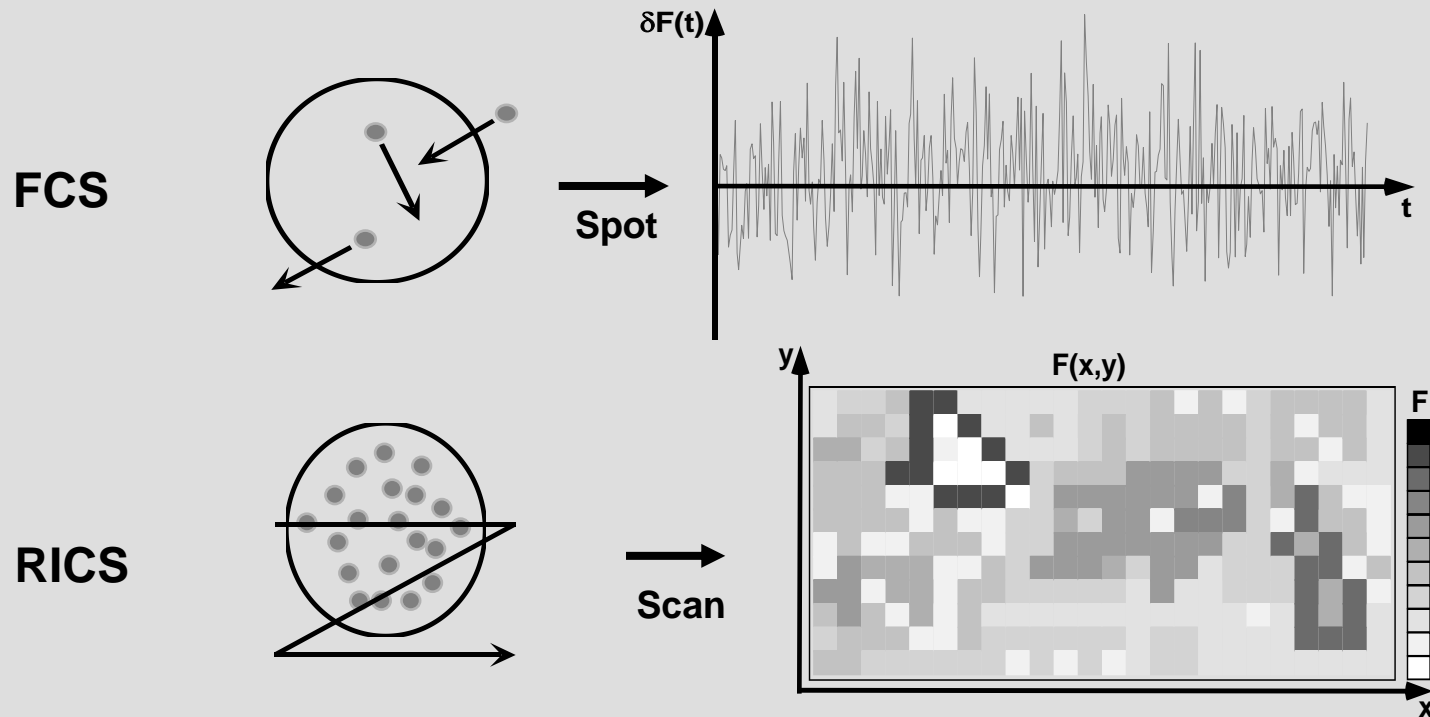
**Diffusion Coefficient**

# RICS



# RICS

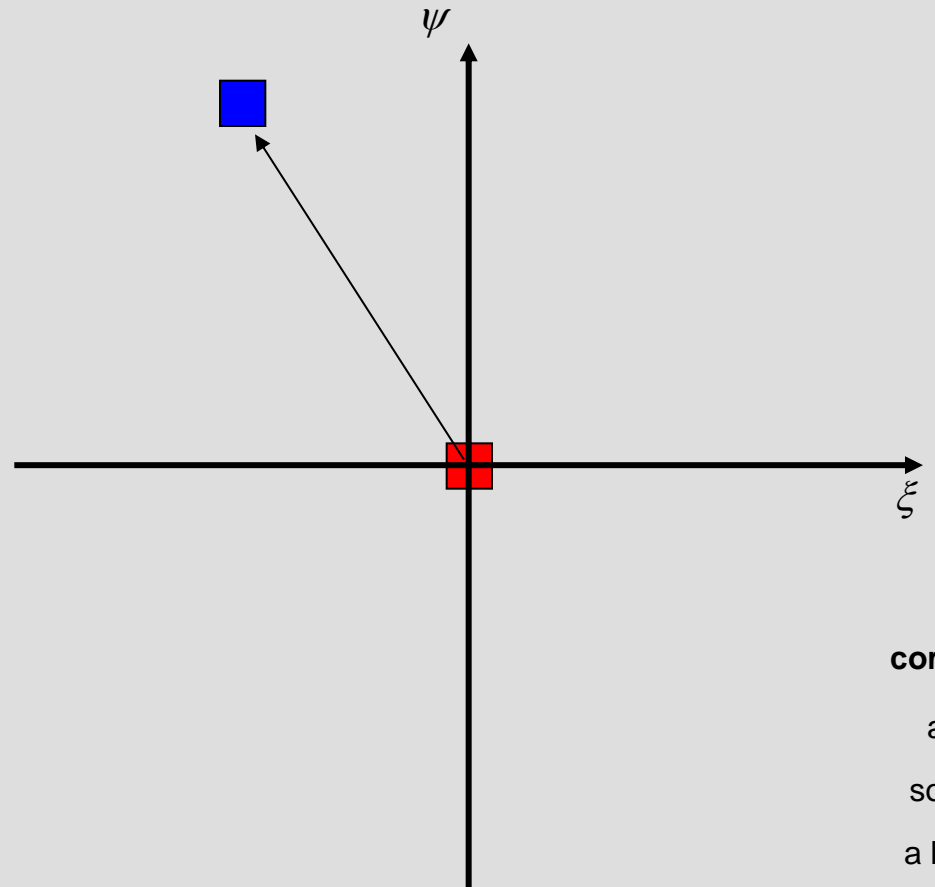
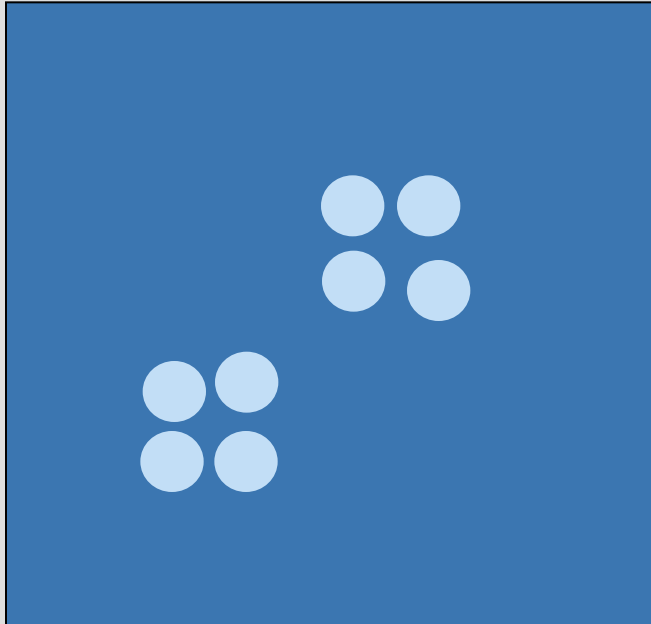
## *Raster Imaging Correlation Spectroscopy*



- 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?

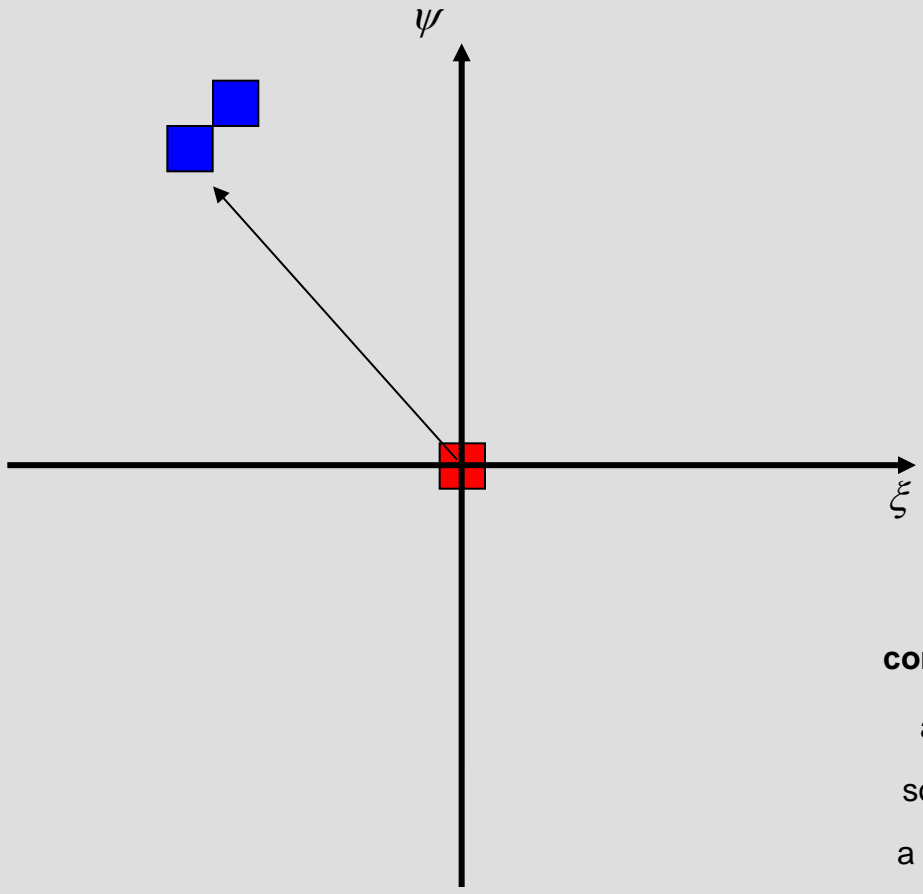
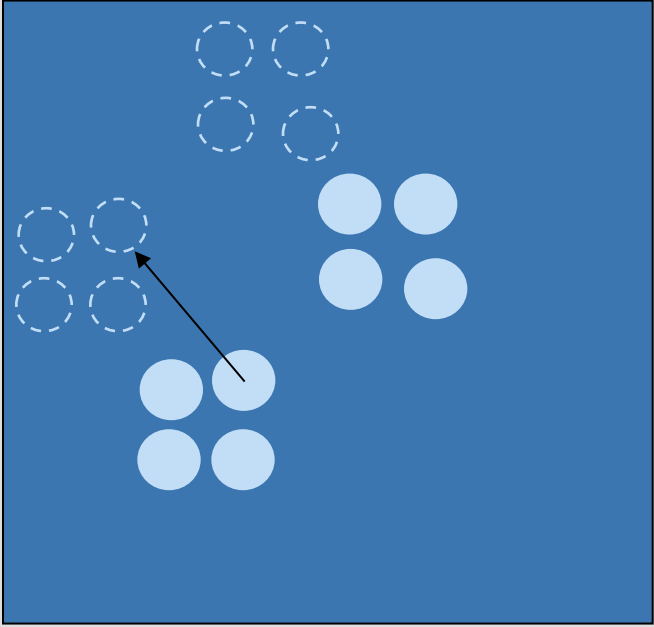


correlation



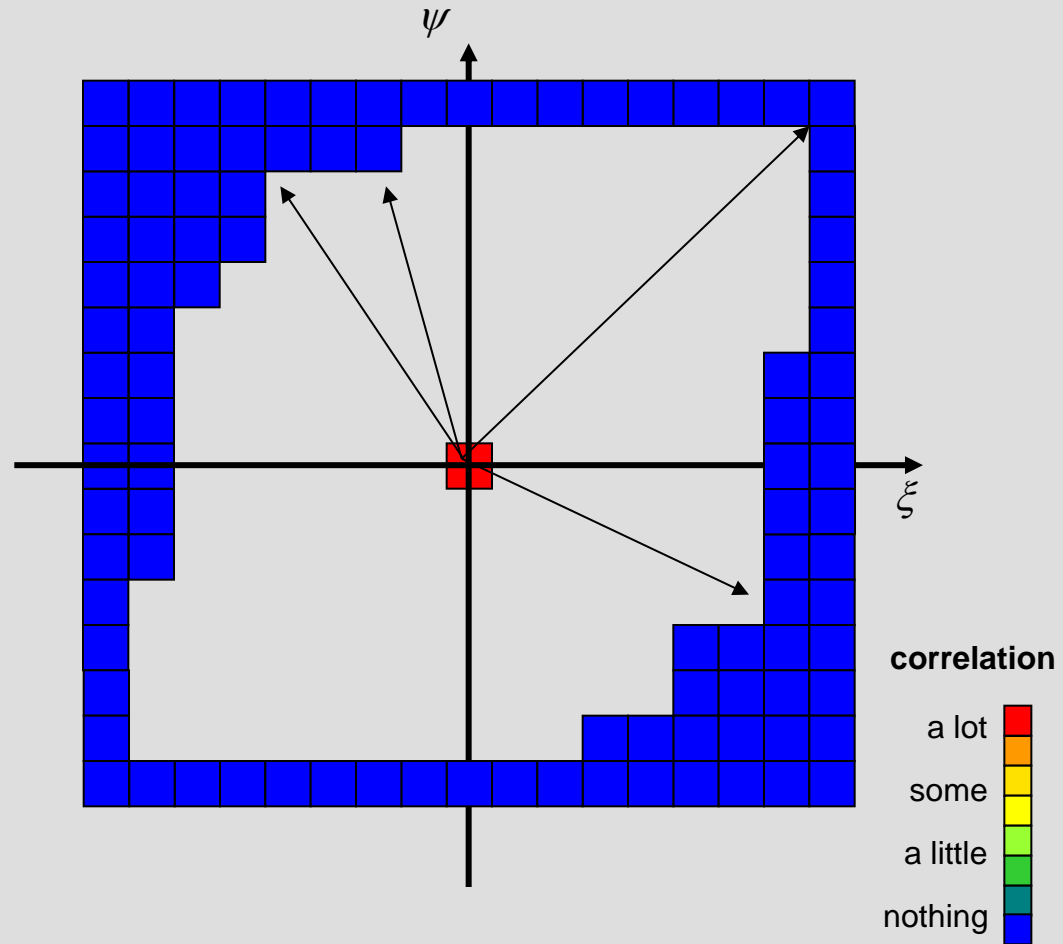
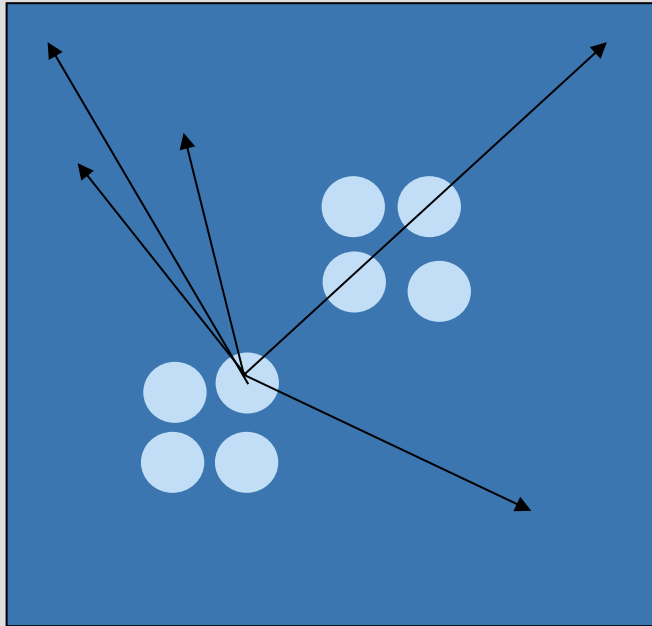
# RICS

Wat is RICS?



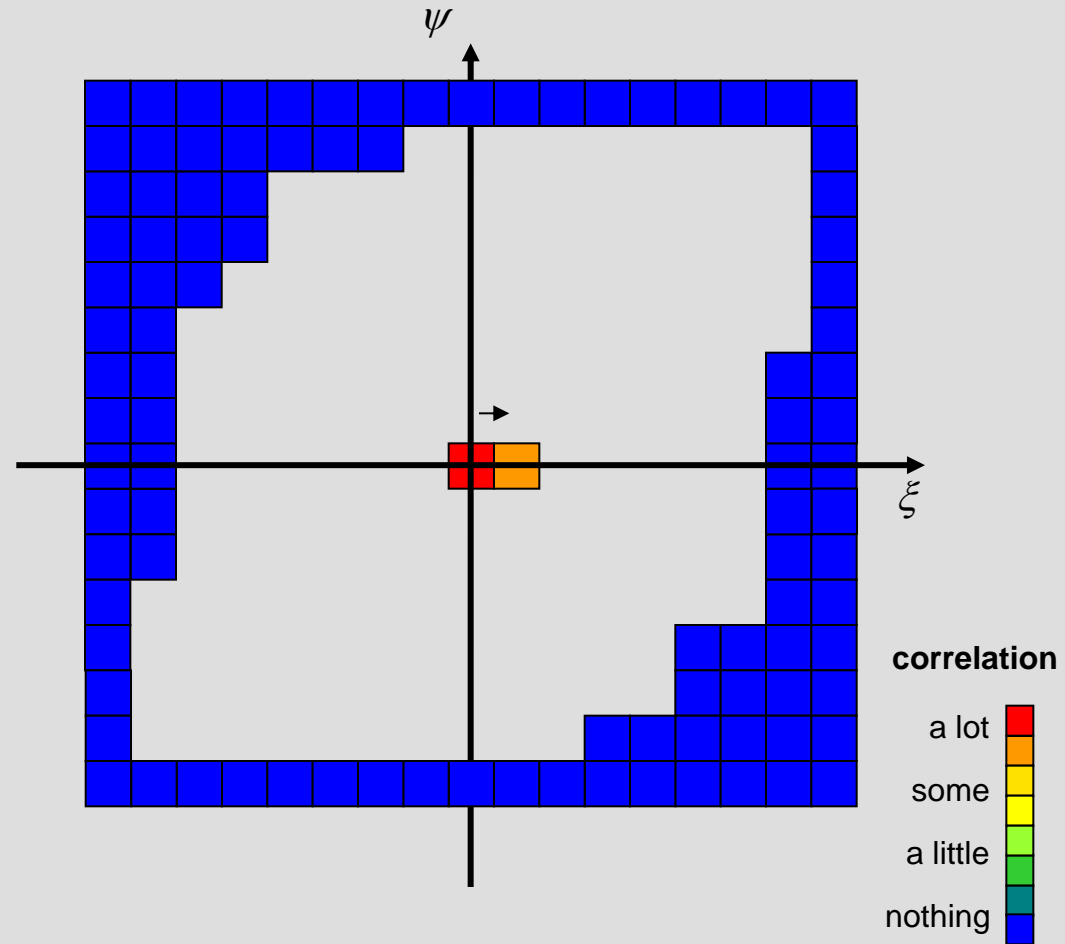
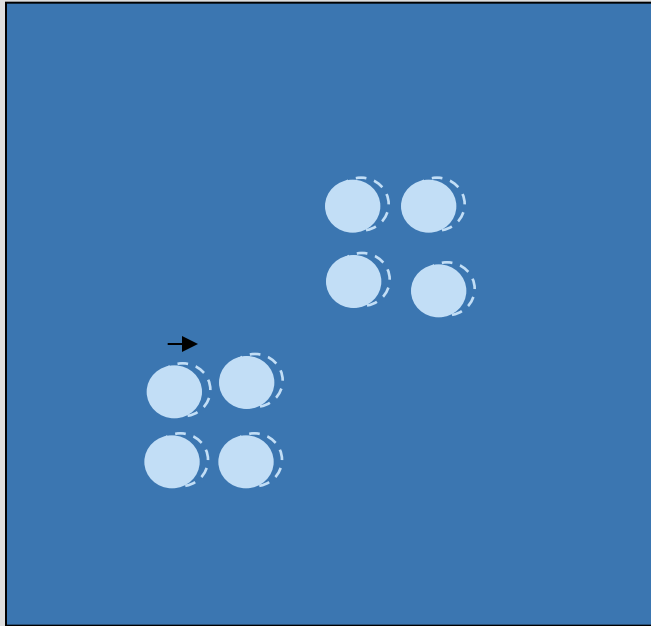
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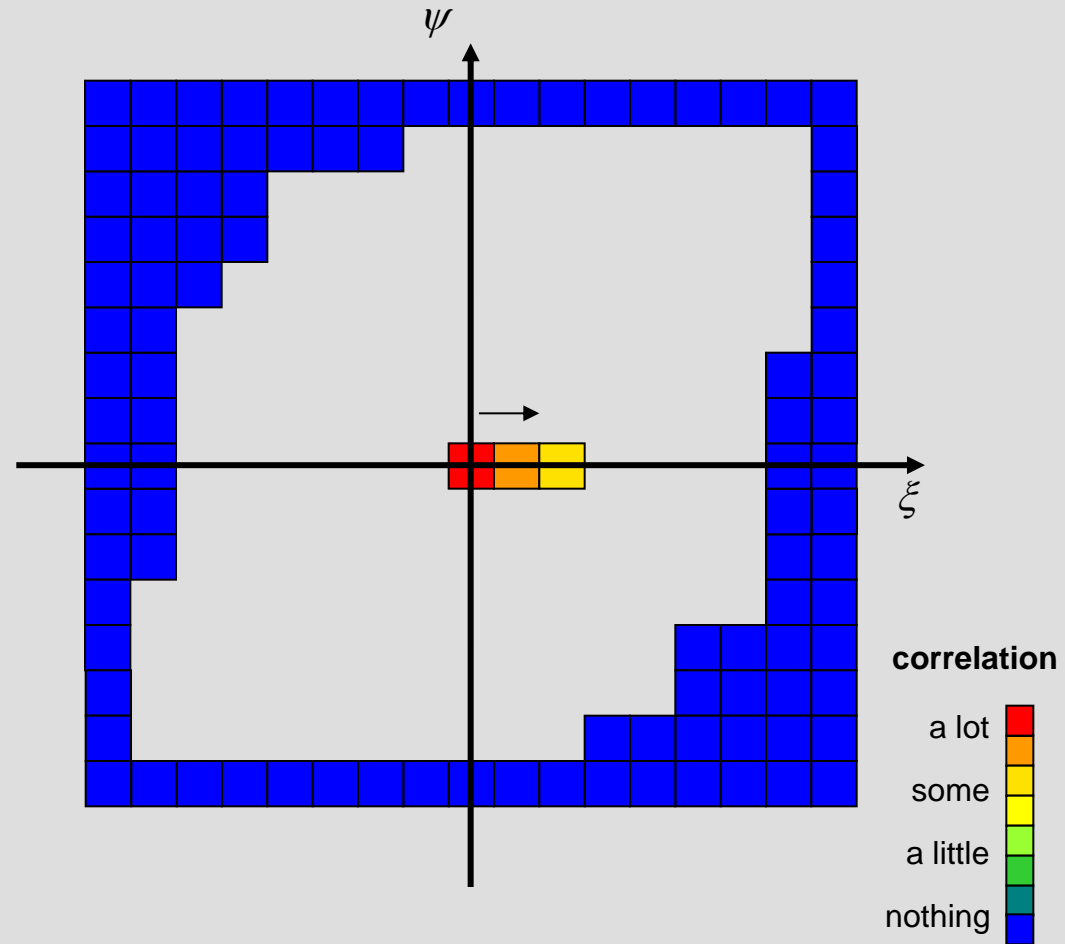
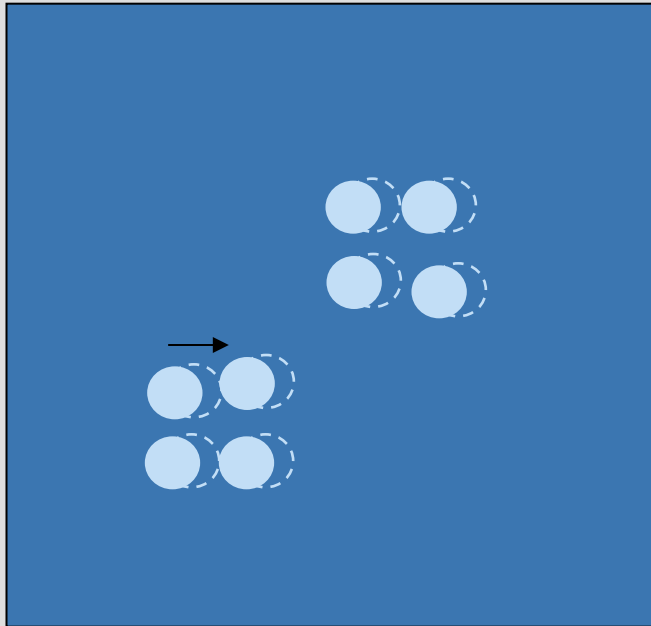
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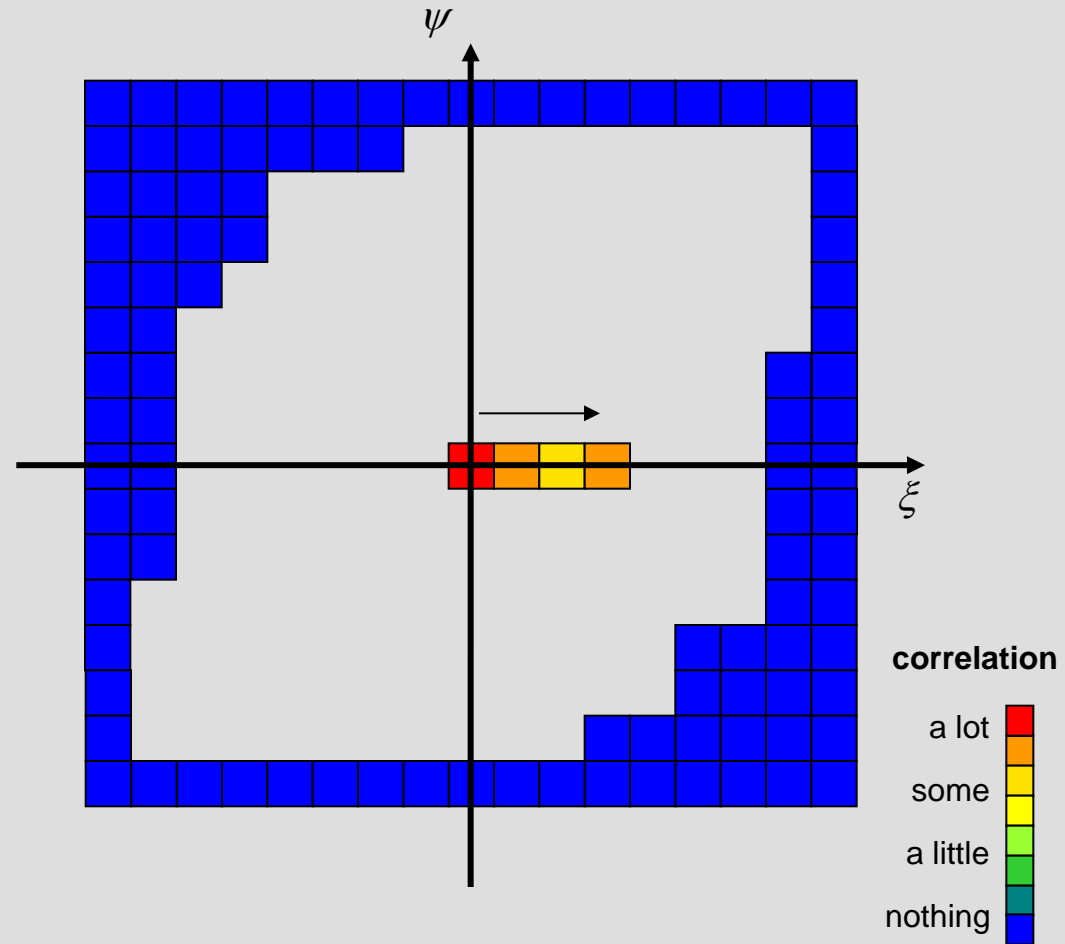
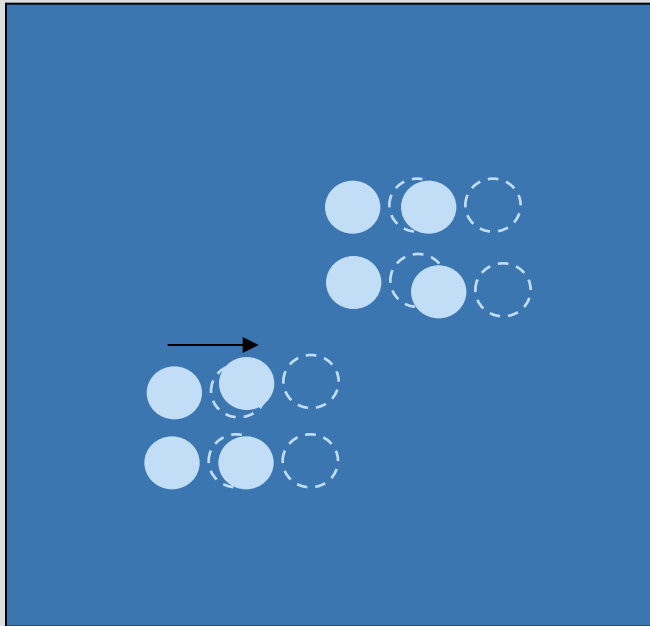
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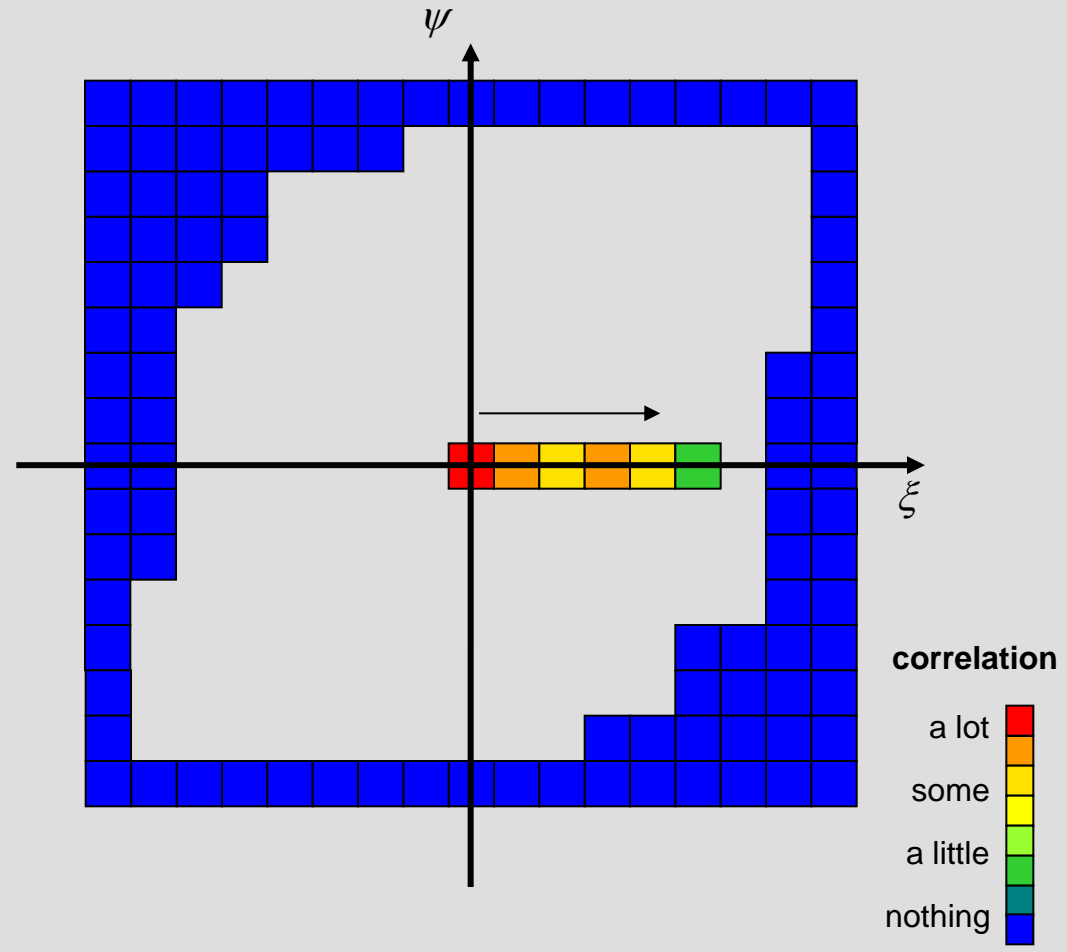
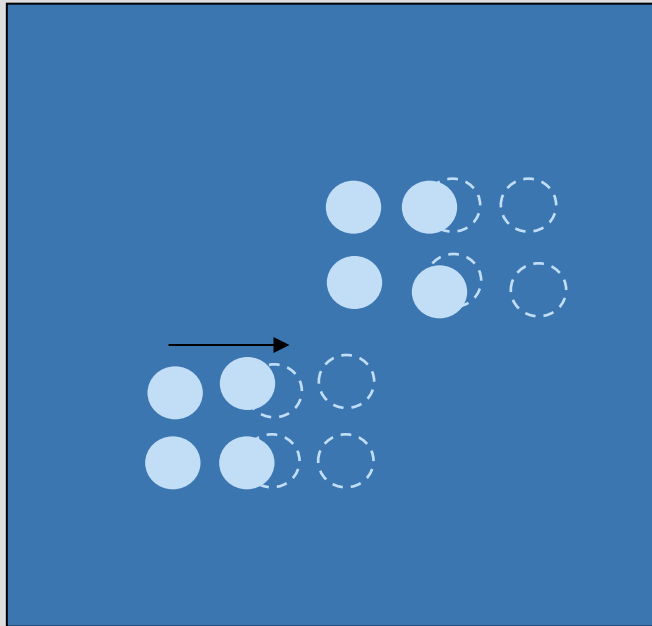
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# RICS

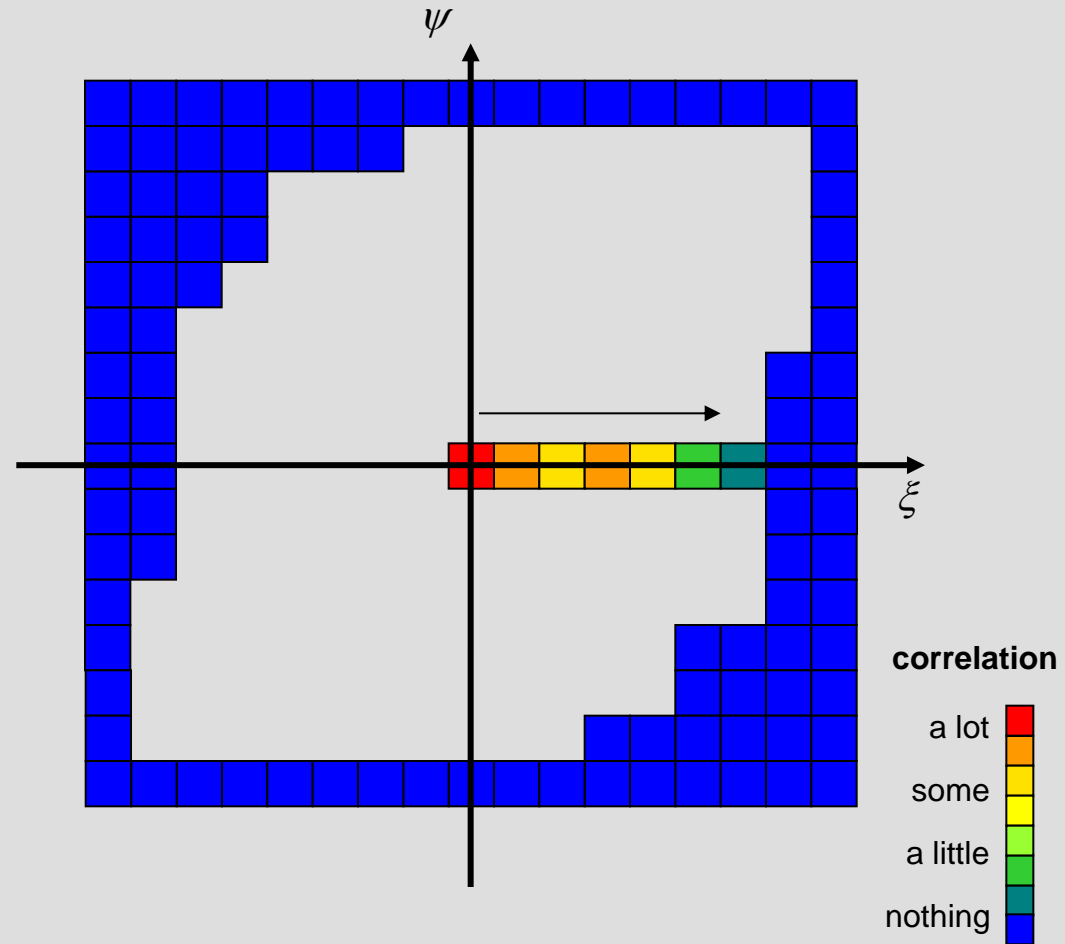
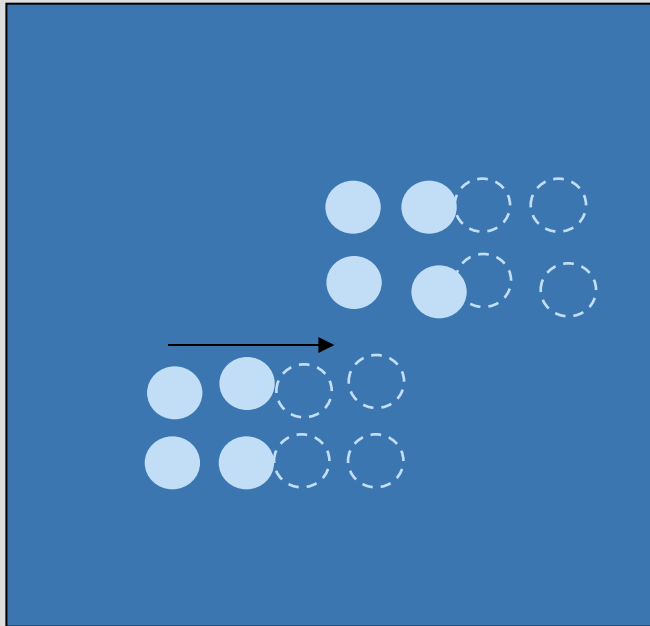
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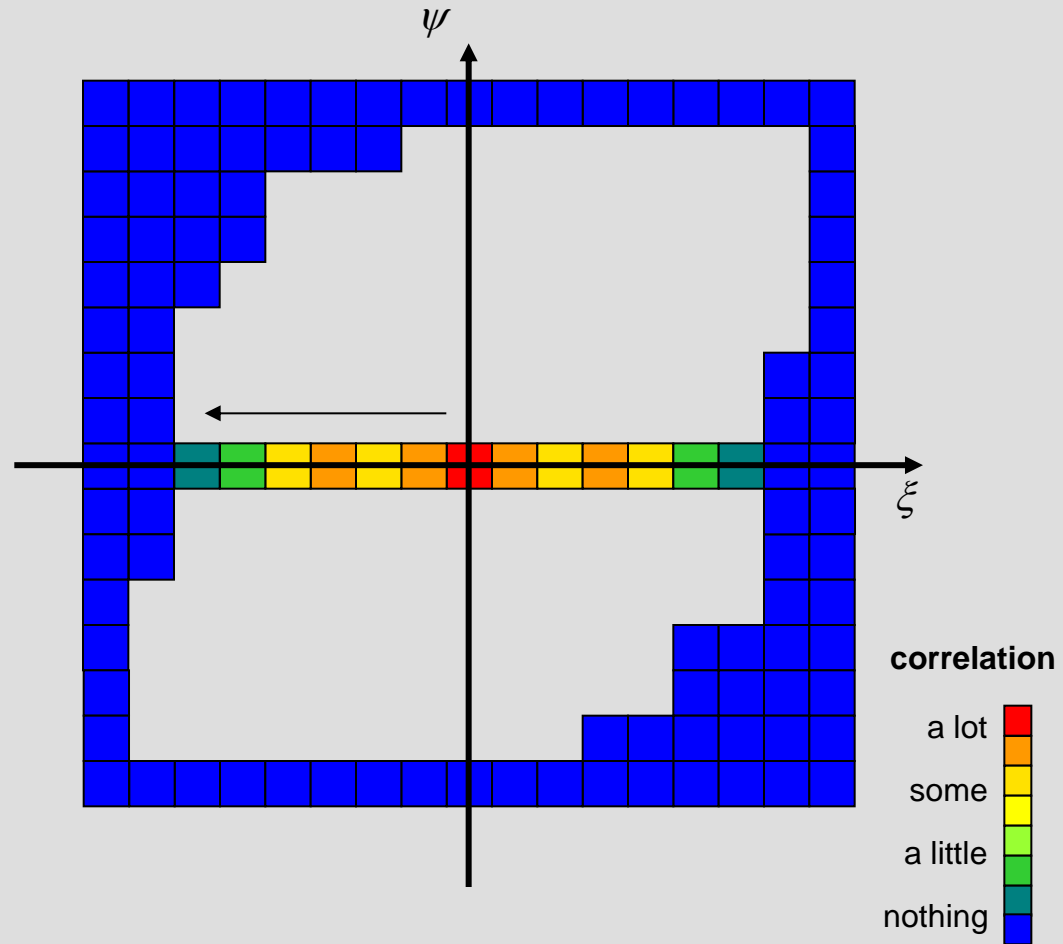
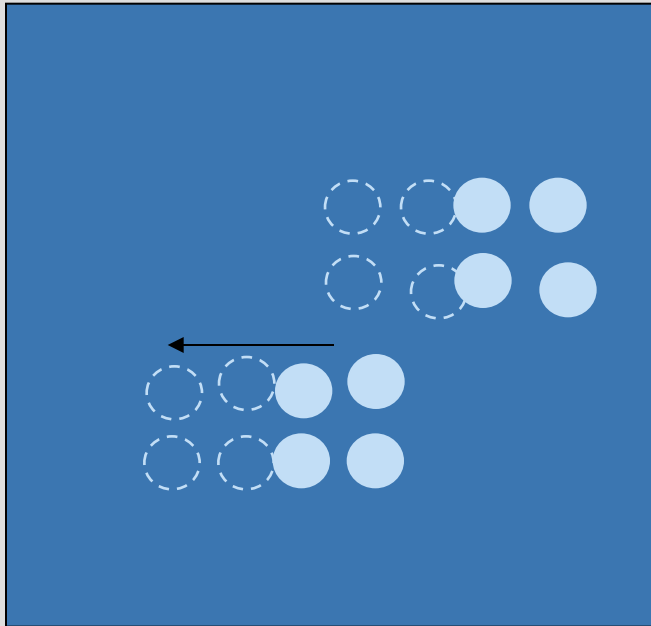
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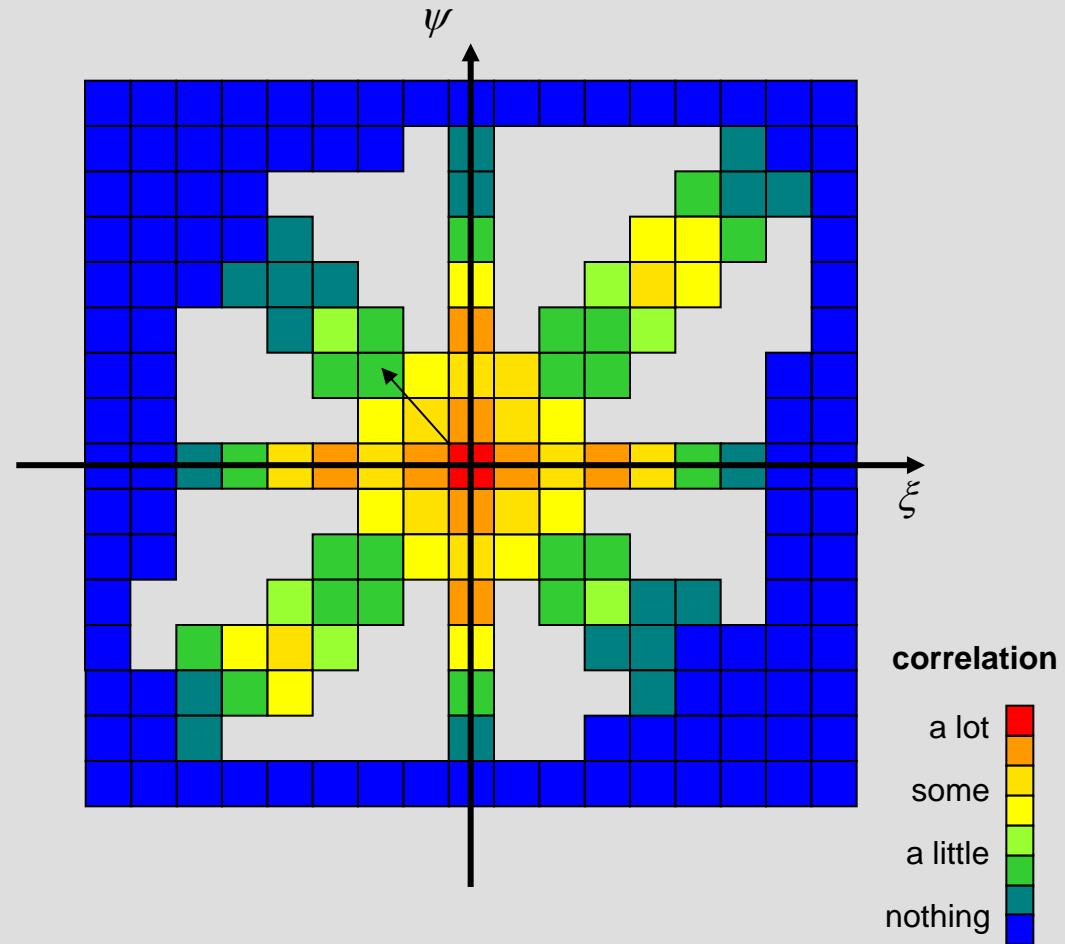
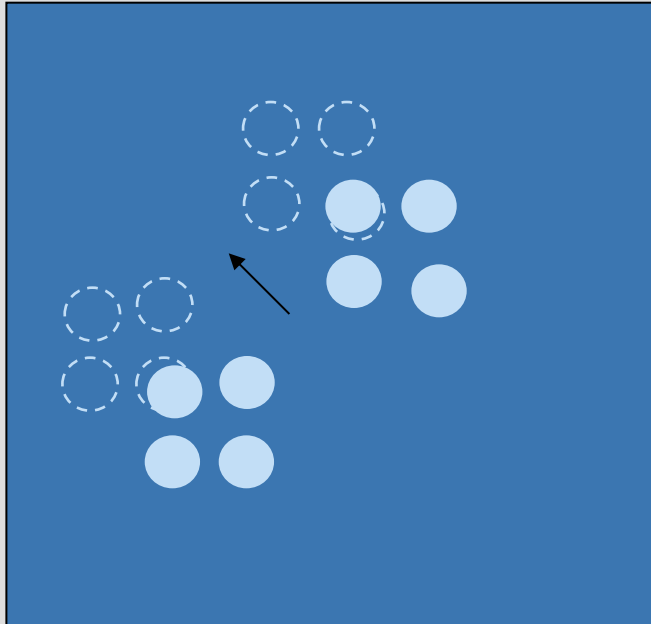
# RICS

Wat is RICS?



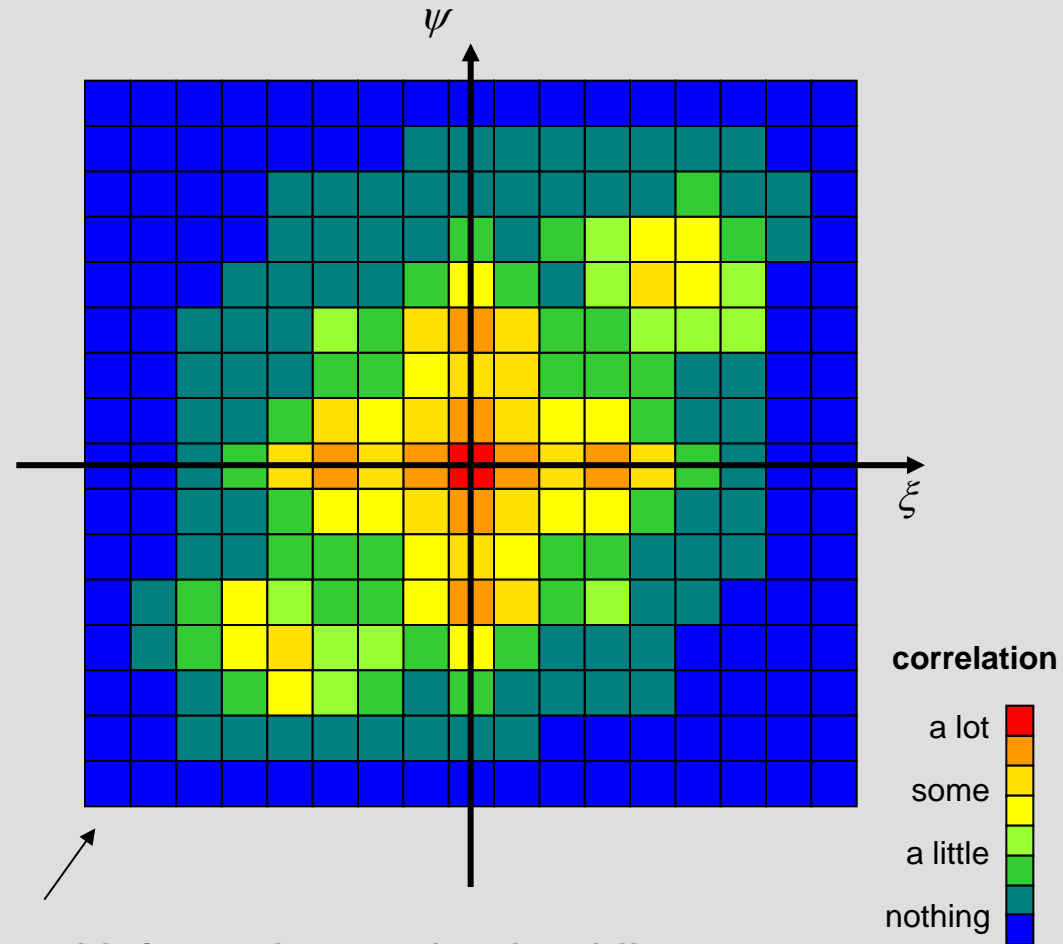
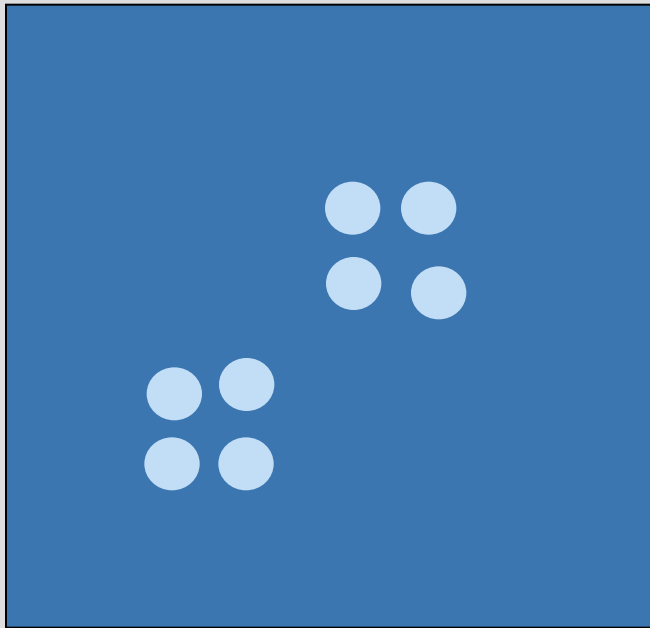
# RICS

Het 'correlogram'



# RICS

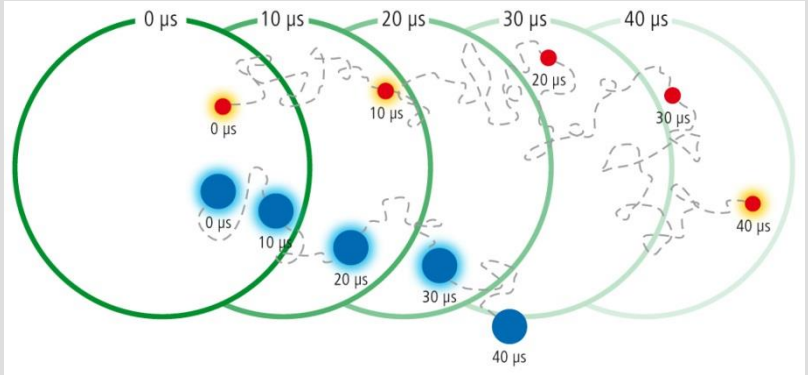
Het 'correlogram'



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

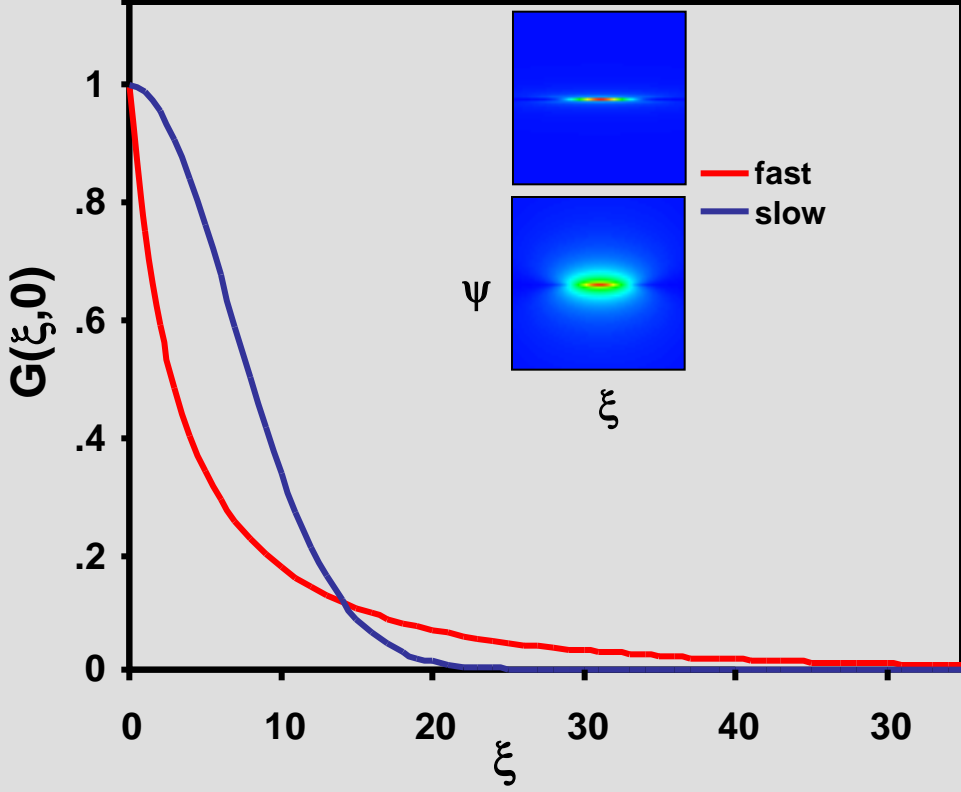
# ZEN Applications

## 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  $(x+\xi, y+\psi)$

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

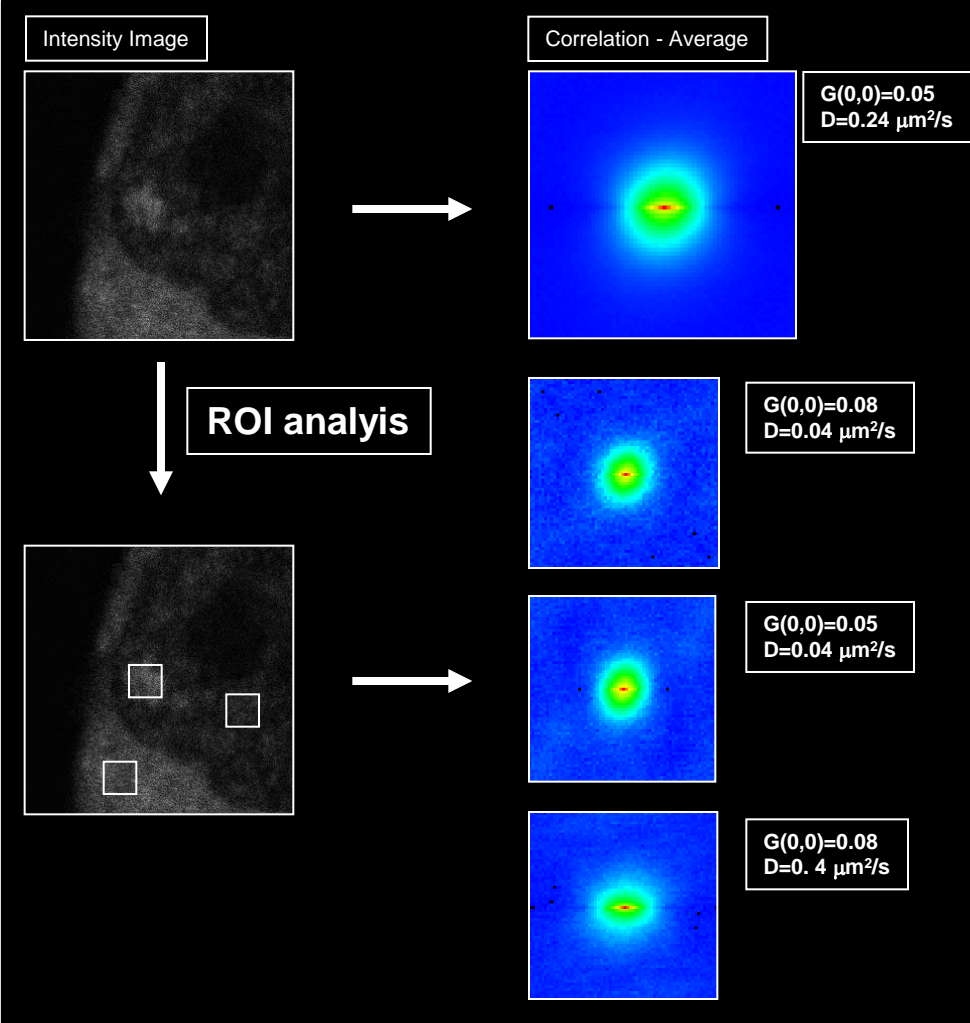


# RICS

## 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

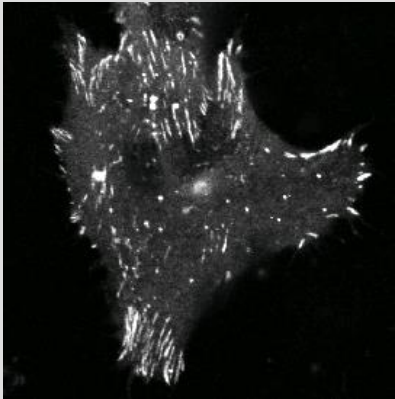
## Example – Eos Paxillin

### Mobility of Pax between cell adhesion structures

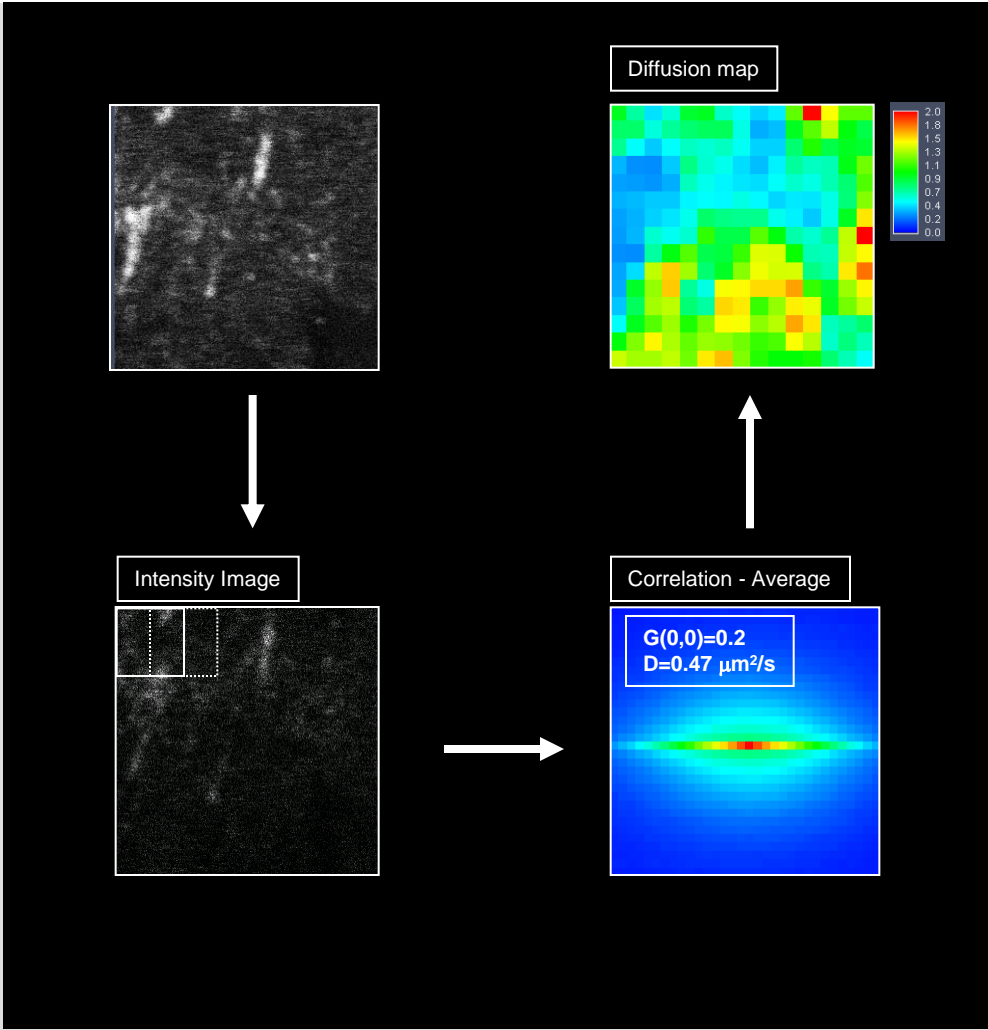
Record time series

Subtract moving average

Compute correlation for overlapping ROIs



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



# ZEN Applications

## *RICS - Raster Imaging Correlation Spectroscopy*

- 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



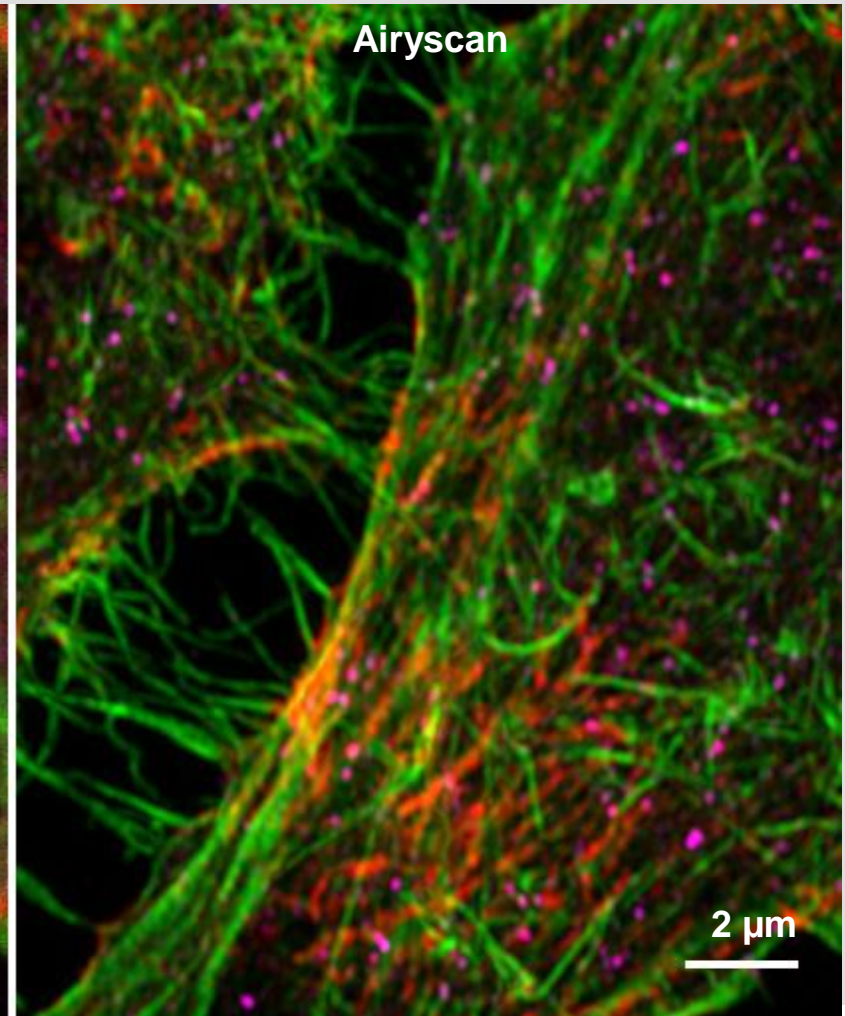
Confocal

- 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





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

Confocal

AiryScan

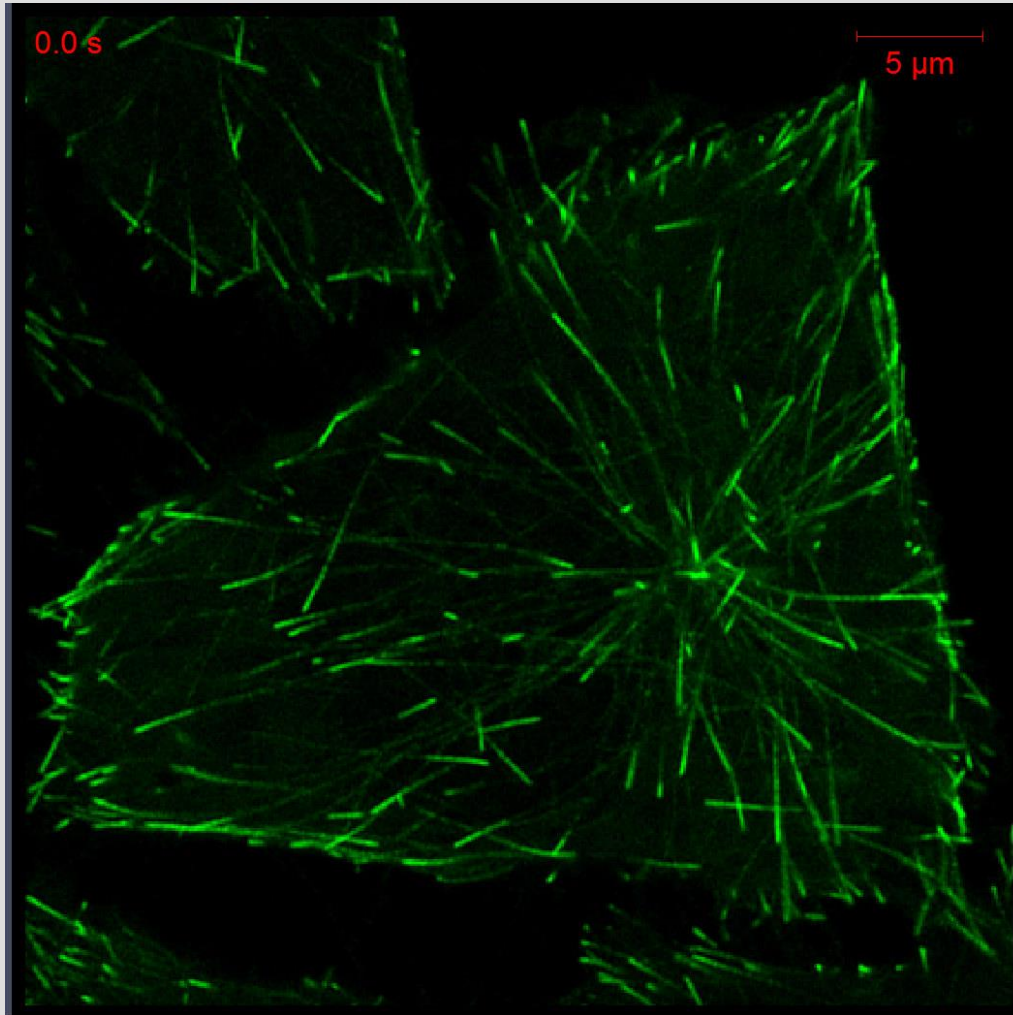
**Cultivated mitotic cells stained for tubulin**  
Peter O'Toole, Ian Morrison (Univ. of York, UK)

2  $\mu$ m

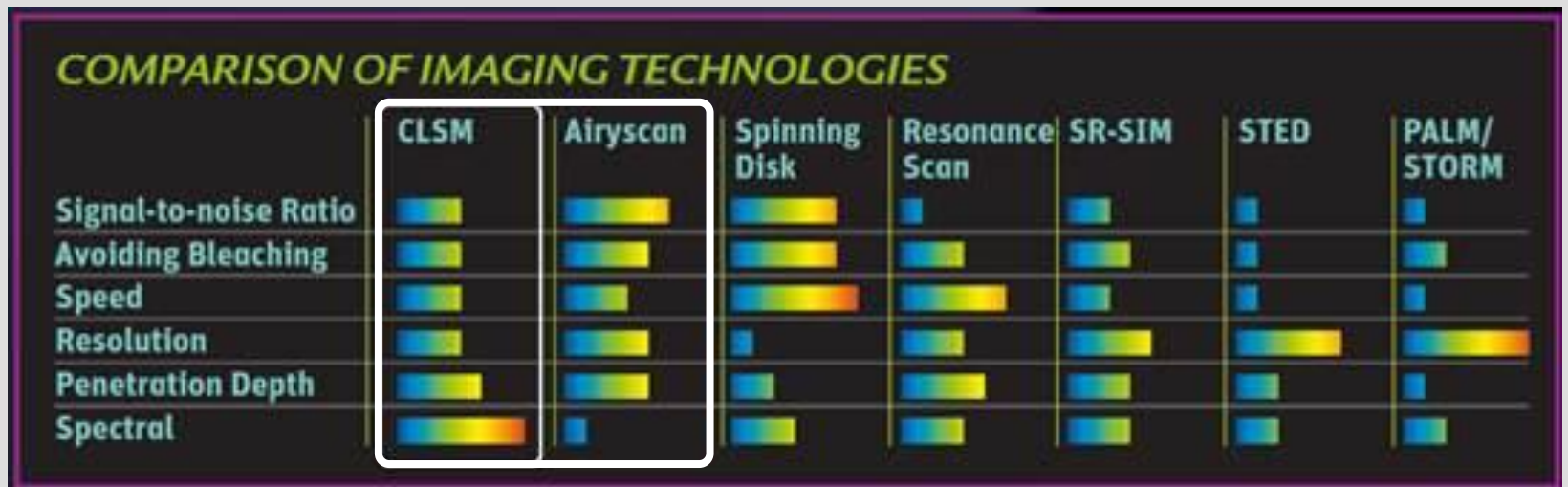


# LSM 880 – Airyscan: Resolution, SNR, and Flexibility

Intrinsically compatible with live cell imaging



# Comparison of imaging technologies





We make it visible.