

# Recent advances in confocal microscopy

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[www.bic.wur.nl](http://www.bic.wur.nl)

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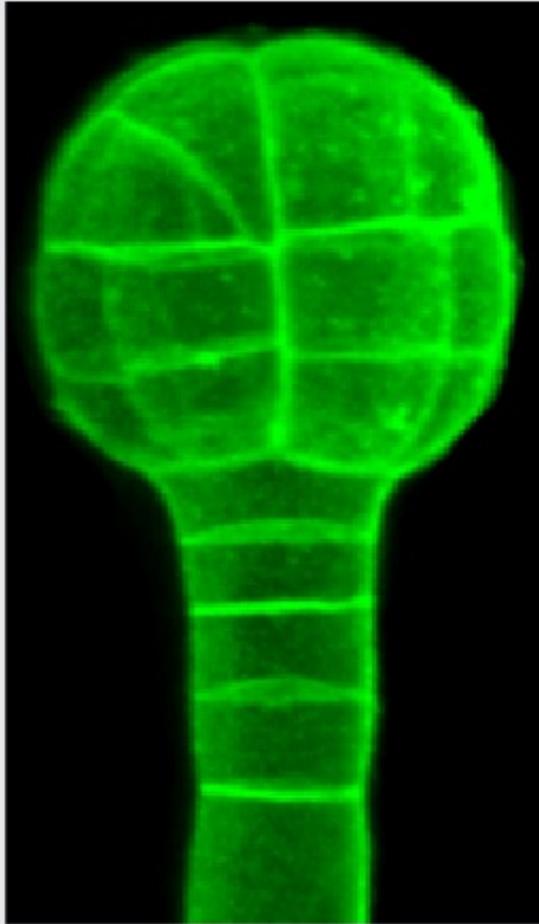


**WISH**



**Wageningen Imaging and Spectroscopy Hub**

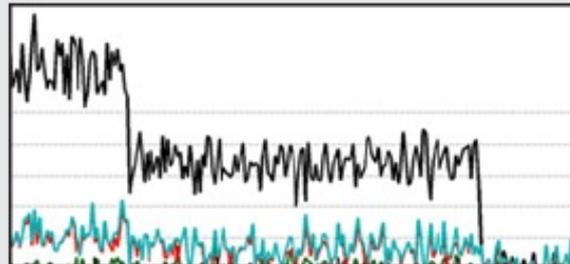
# *Future challenges in Cellular Biochemistry*



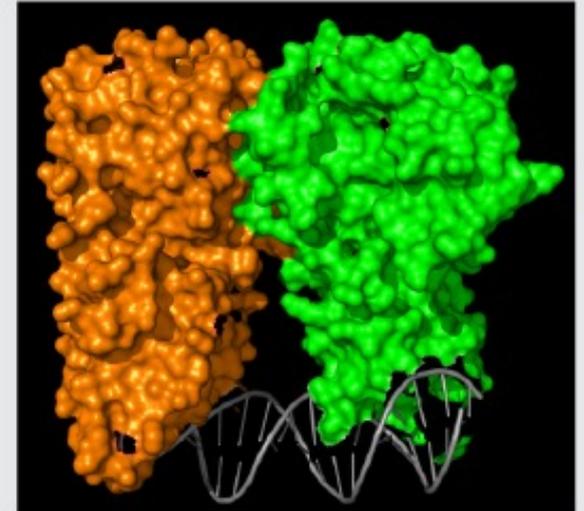
*How do proteins control biological processes?*



*How many/ where?*



*How fast?*



*With whom?*



*Evolution?*

# Quantitative Fluorescence techniques

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- Spectral analysis Fluorimeter
- Fluorescence imaging spinning disk, confocal
- High resolution imaging PALM, Storm
- Dynamics FCS, FRAP
- Biomolecular interactions FRET
  - Fluorescence lifetime/anisotropy analysis

# Multi mode confocal microscope

(Leica SP8)

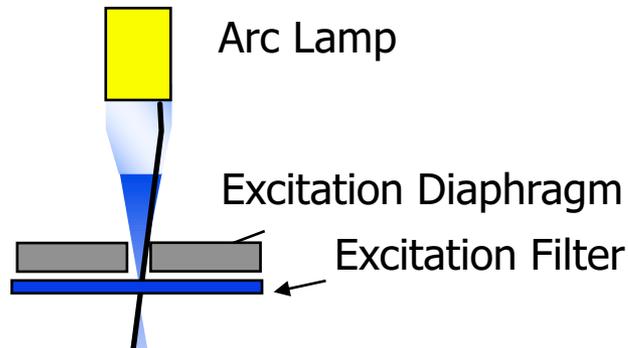
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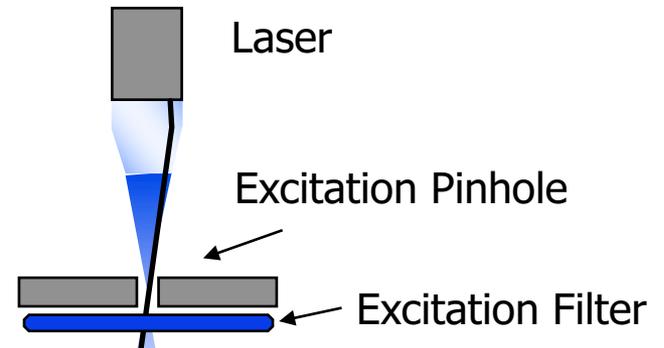
- Point scanning source
  - Selective excitation (lasers)
  - Selective detection (HyD)
  - Improved resolution
    - ✓ pinhole
- User friendly
  - Flexible
  - Sensitive
  - Up-to-date technologies
  - Multi-parameter
    - Spectral
    - Lifetime
    - dynamics

# Confocal principle

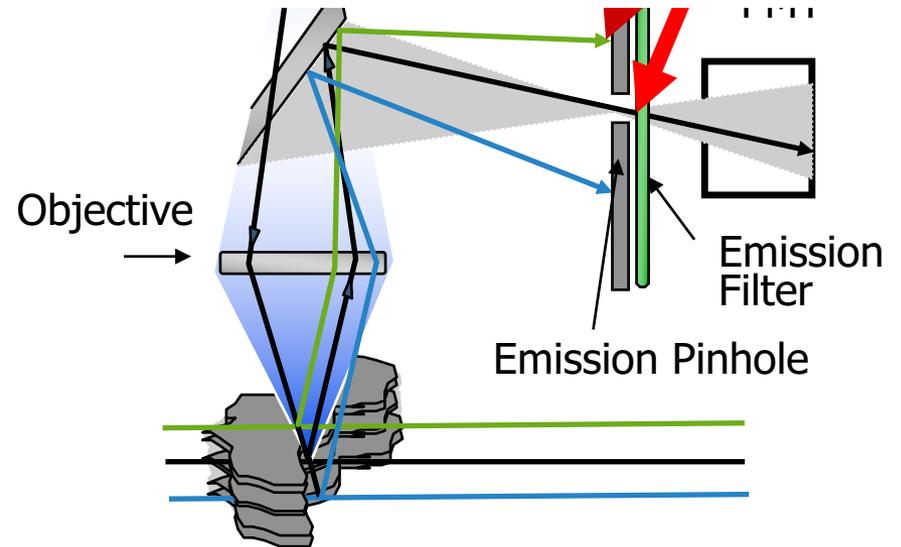
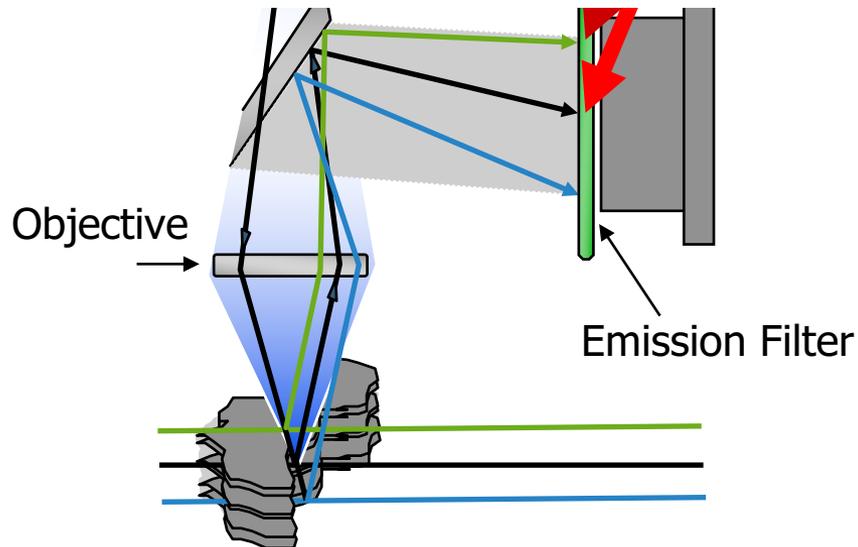
## Widefield Fluorescence Microscope



## Confocal Fluorescence Microscope

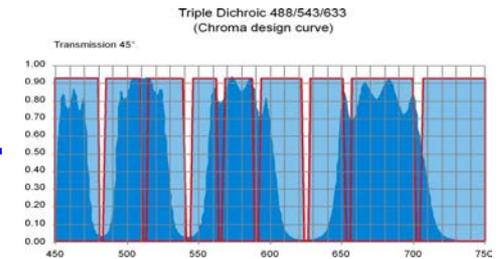
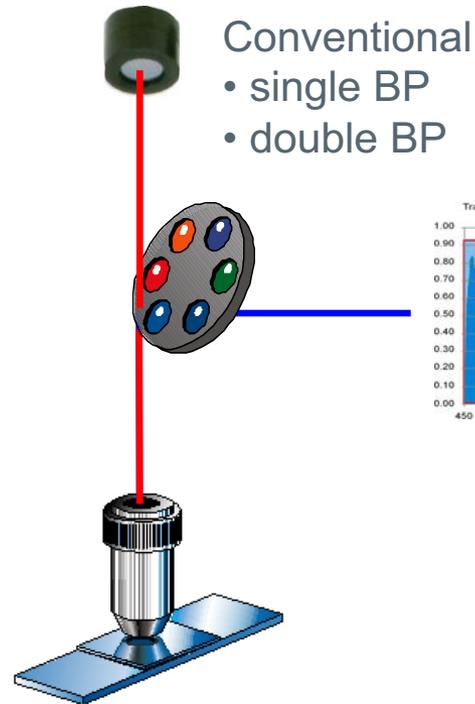
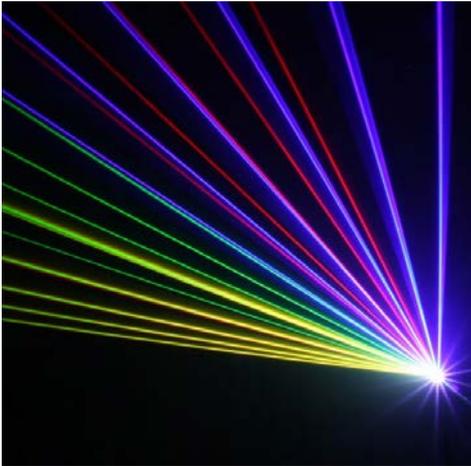


## Blocking out of focus light



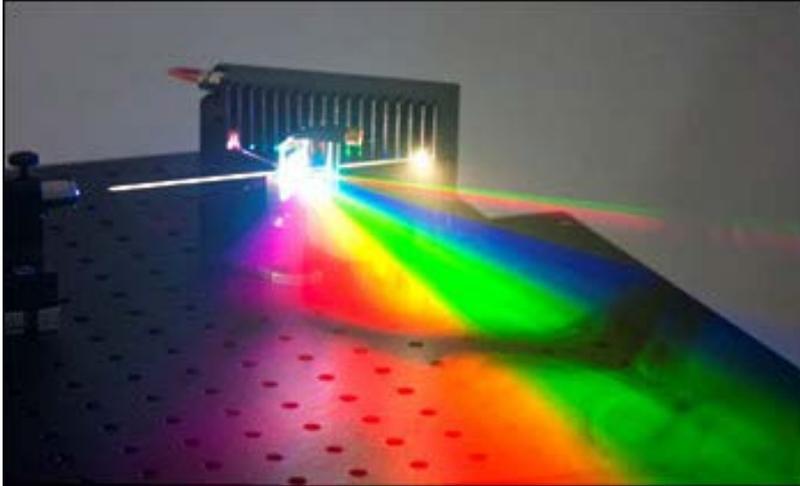


# Excitation of fluorophores

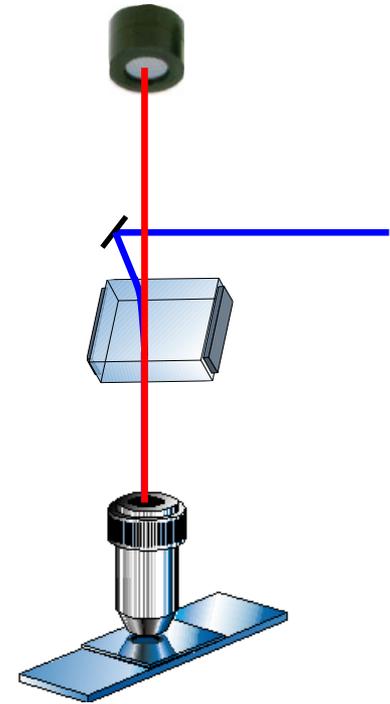


# Supercontinuum (fiber) lasers

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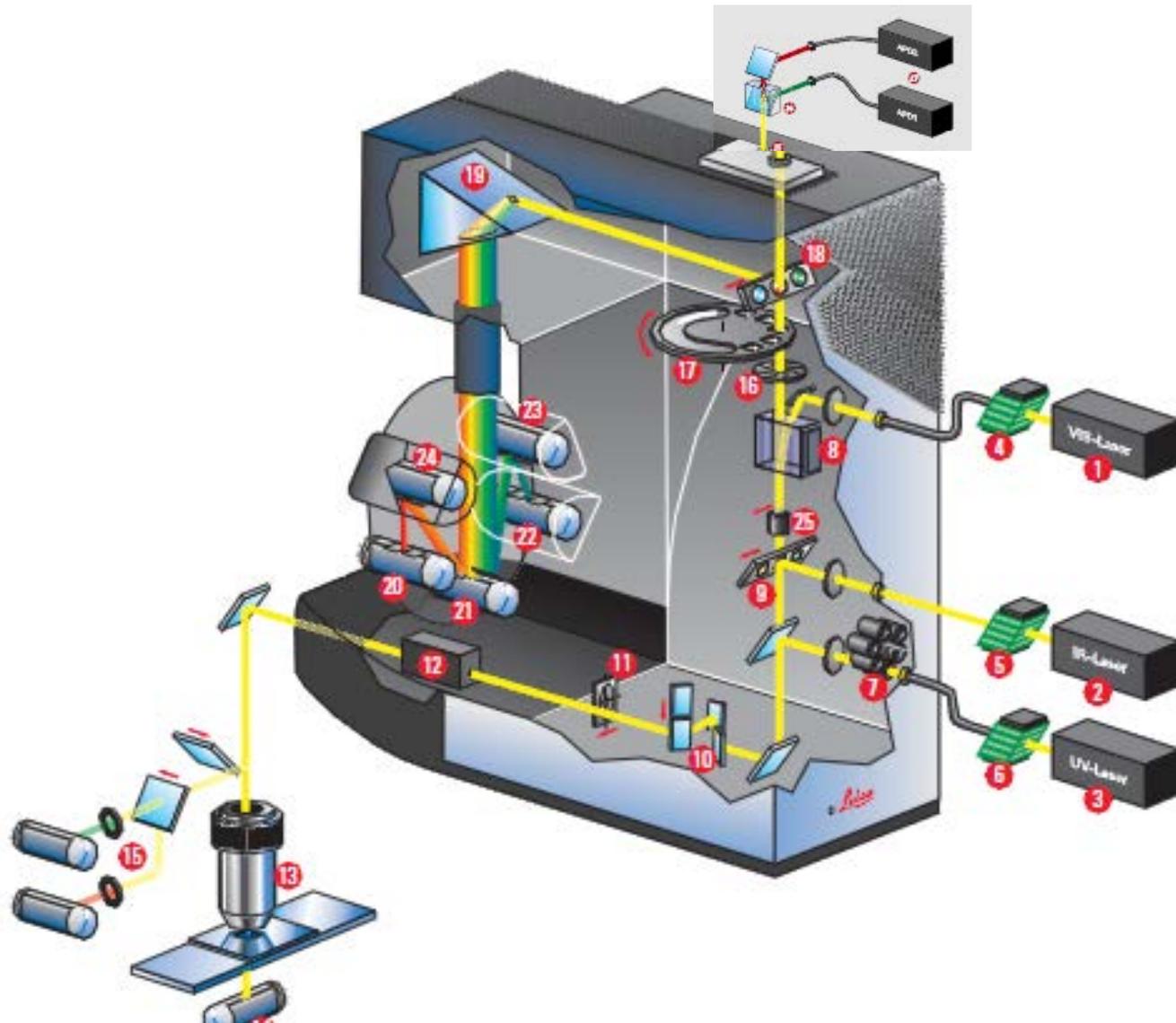


Excitation from 470-670 nm, 8 laser lines simultaneously



AOBS; Acoustic Optical Beam Splitter

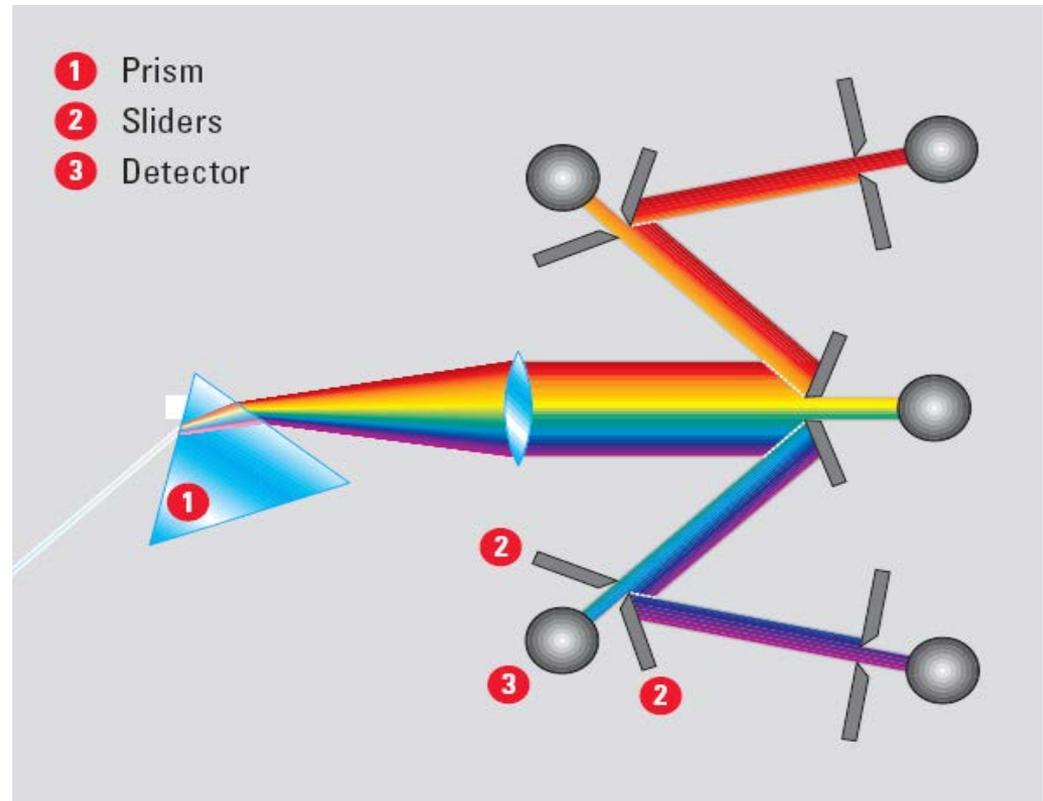
# Leica SP8 concept



# Fluorescence emission

## Spectral detection

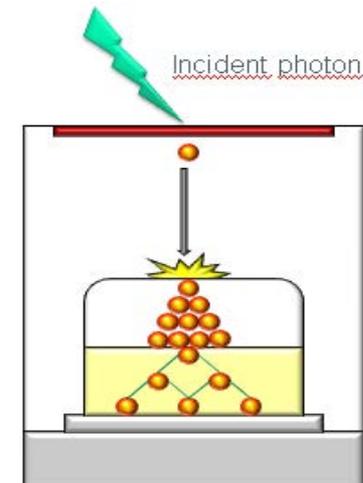
- Low-loss prism optics
- Highly selective
- Flexible colour separation
- Spectral detection
- 5 detectors



# Hybrid detector technology

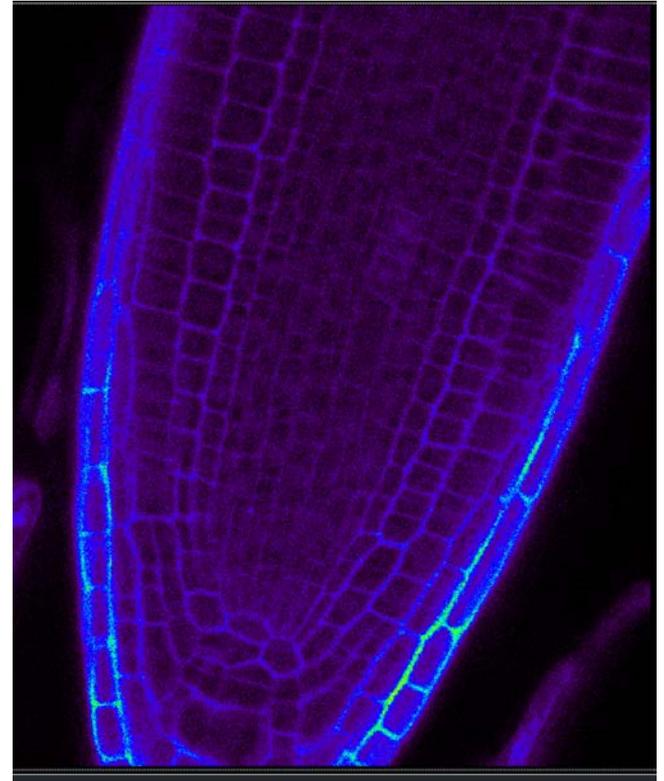
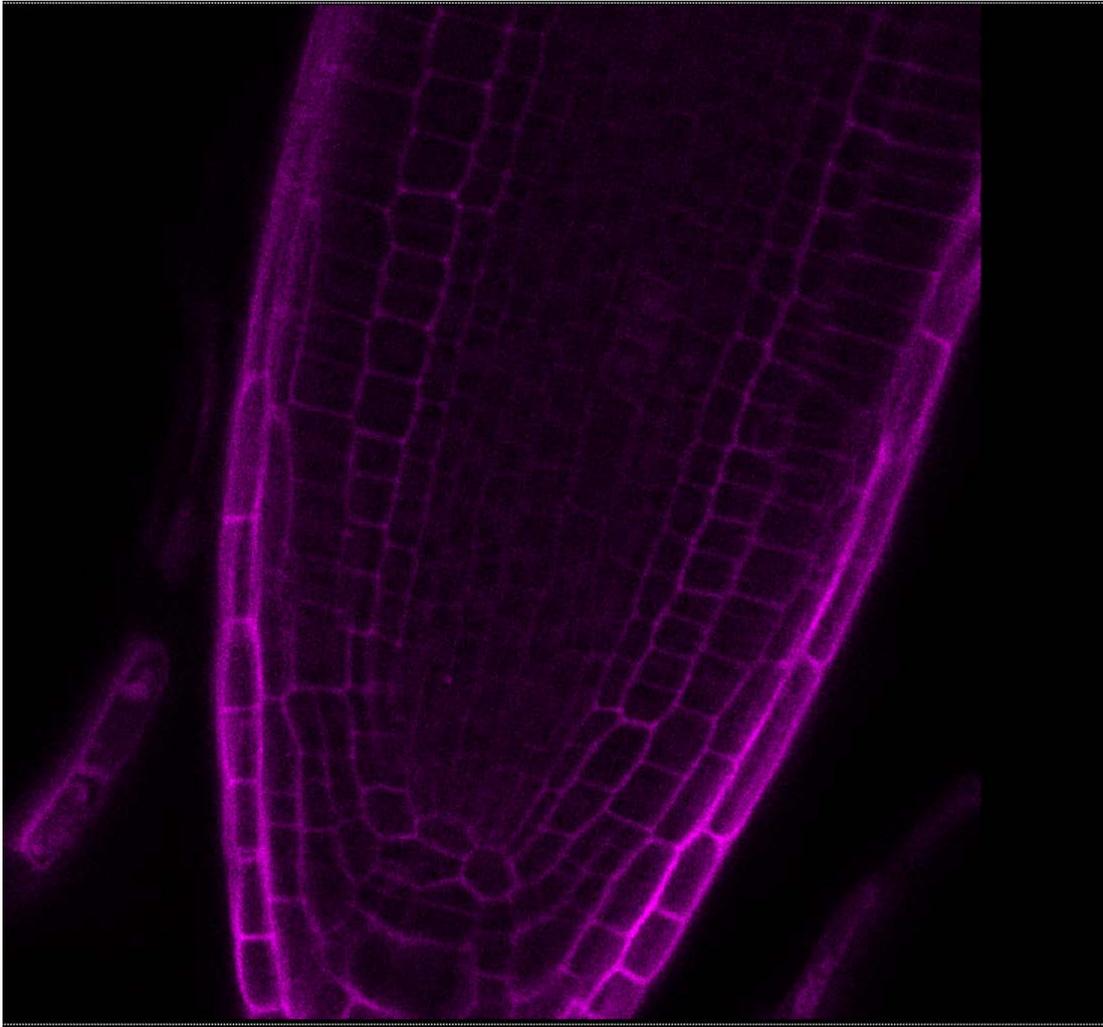
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- HyD stands for hybrid detector
- HyD :
  - half PMT: Large dynamic range
  - half APD: Superior sensitivity
- GaAsP photocathode
  - Gallium-Arsenide-Phosphide
- More advantages:
  - Excellent signal-to-noise
  - Photon-counting option to detect weak signals



# Imaging *Arabidopsis* root

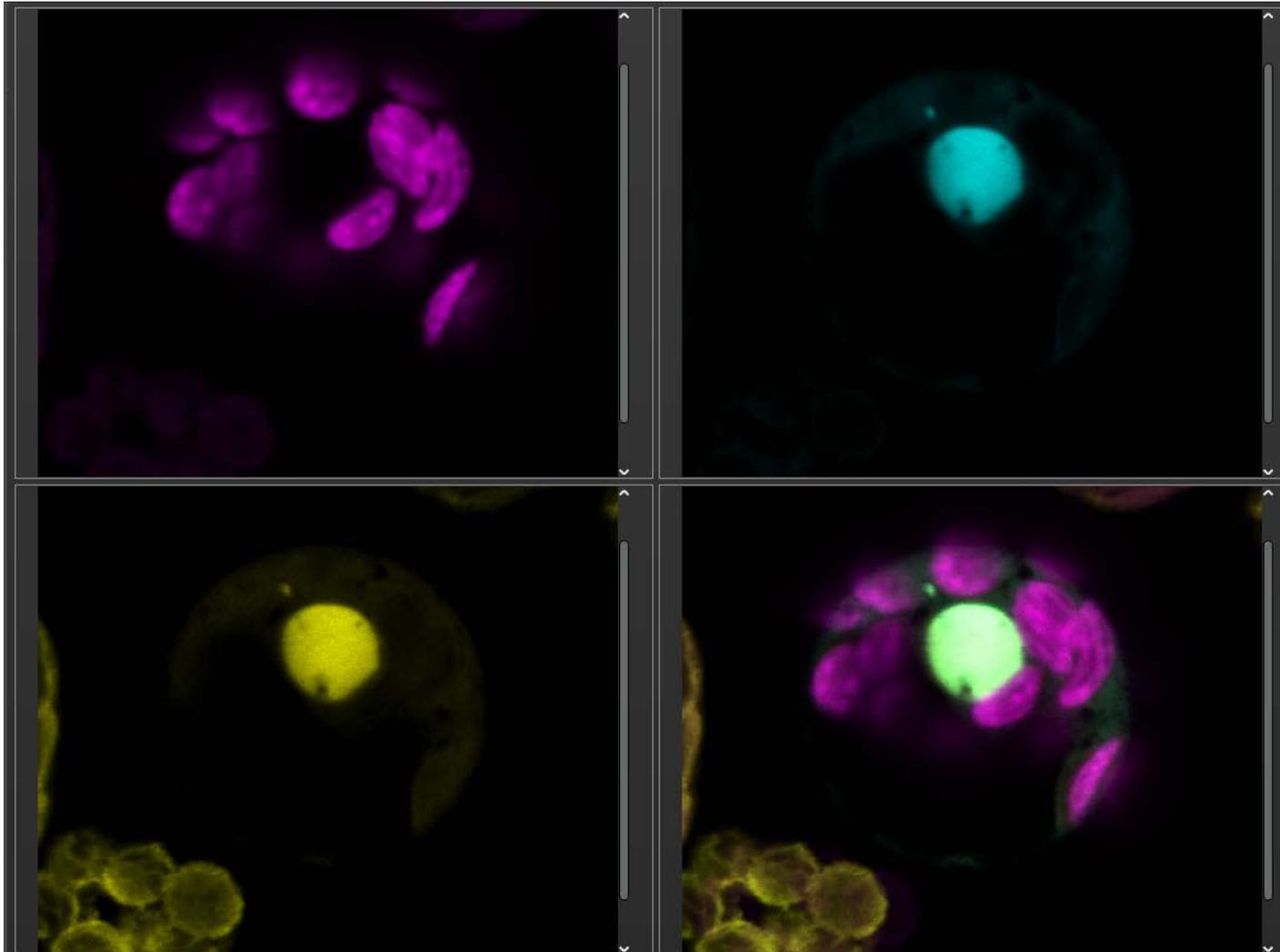
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Arabidopsis root labeled with membrane marker fused to GFP

# Multi-color imaging

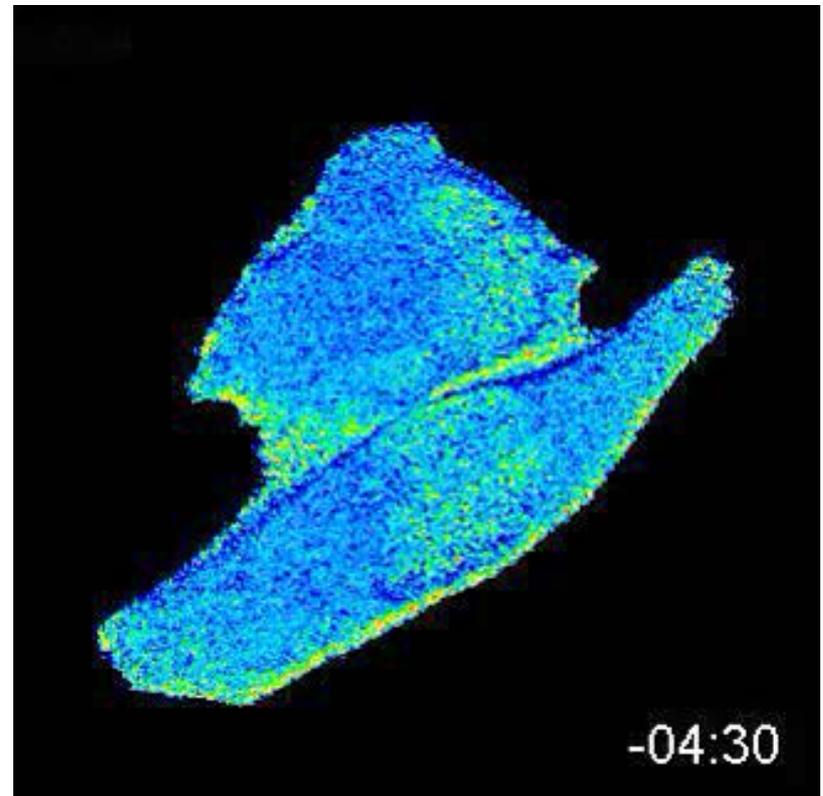
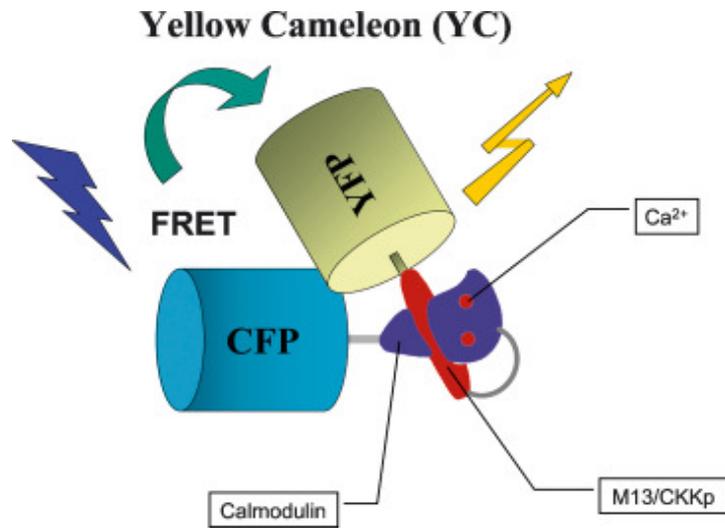
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Transient expression multi-colored transcription factors

# Ratio imaging

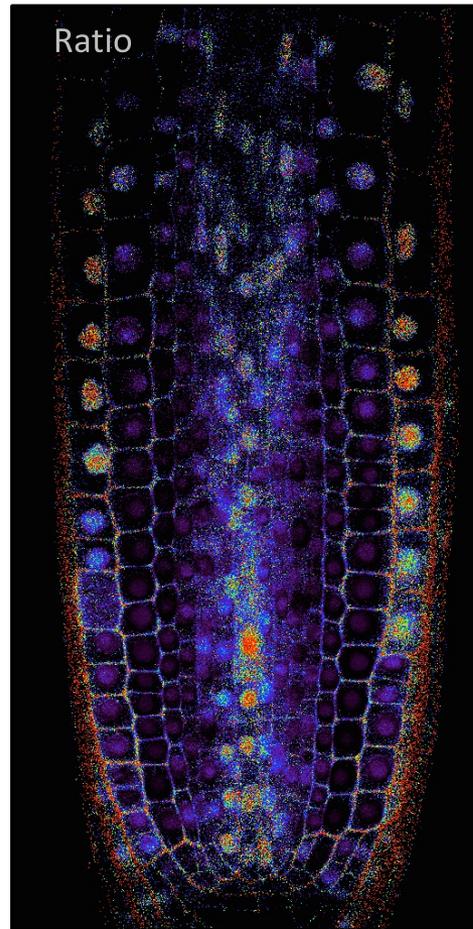
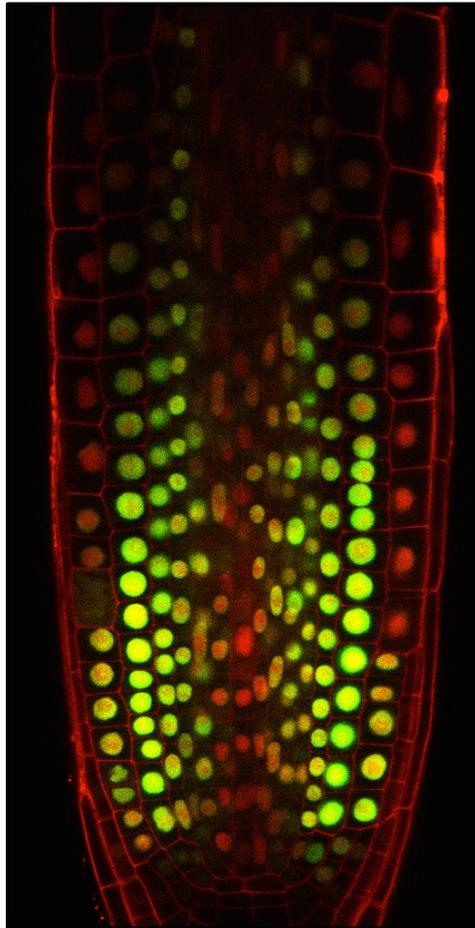
genetically encoded calcium sensor



# Quantifying auxin levels in roots

mDII-ntdTomato

DII-n3VENUS



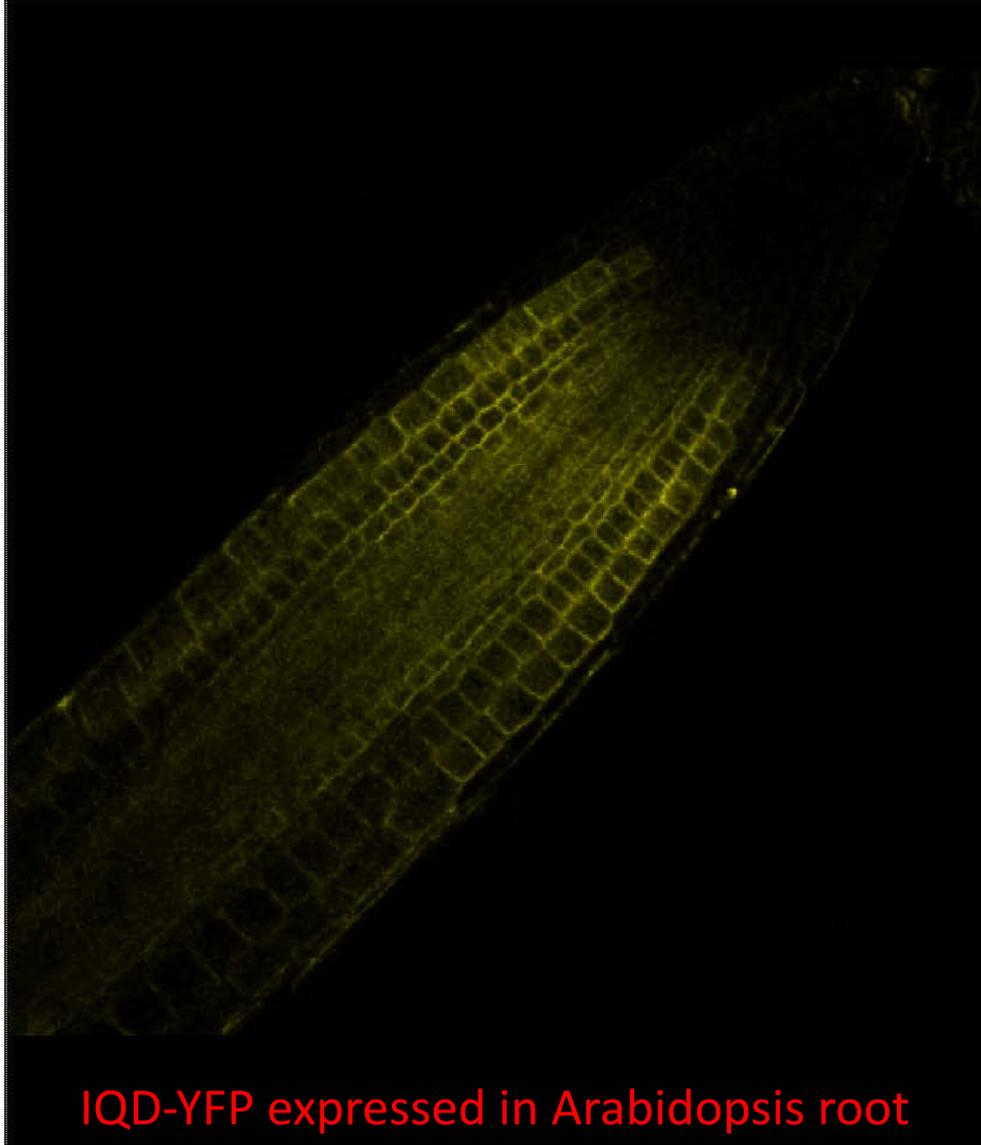
Low



High

# Optical sectioning

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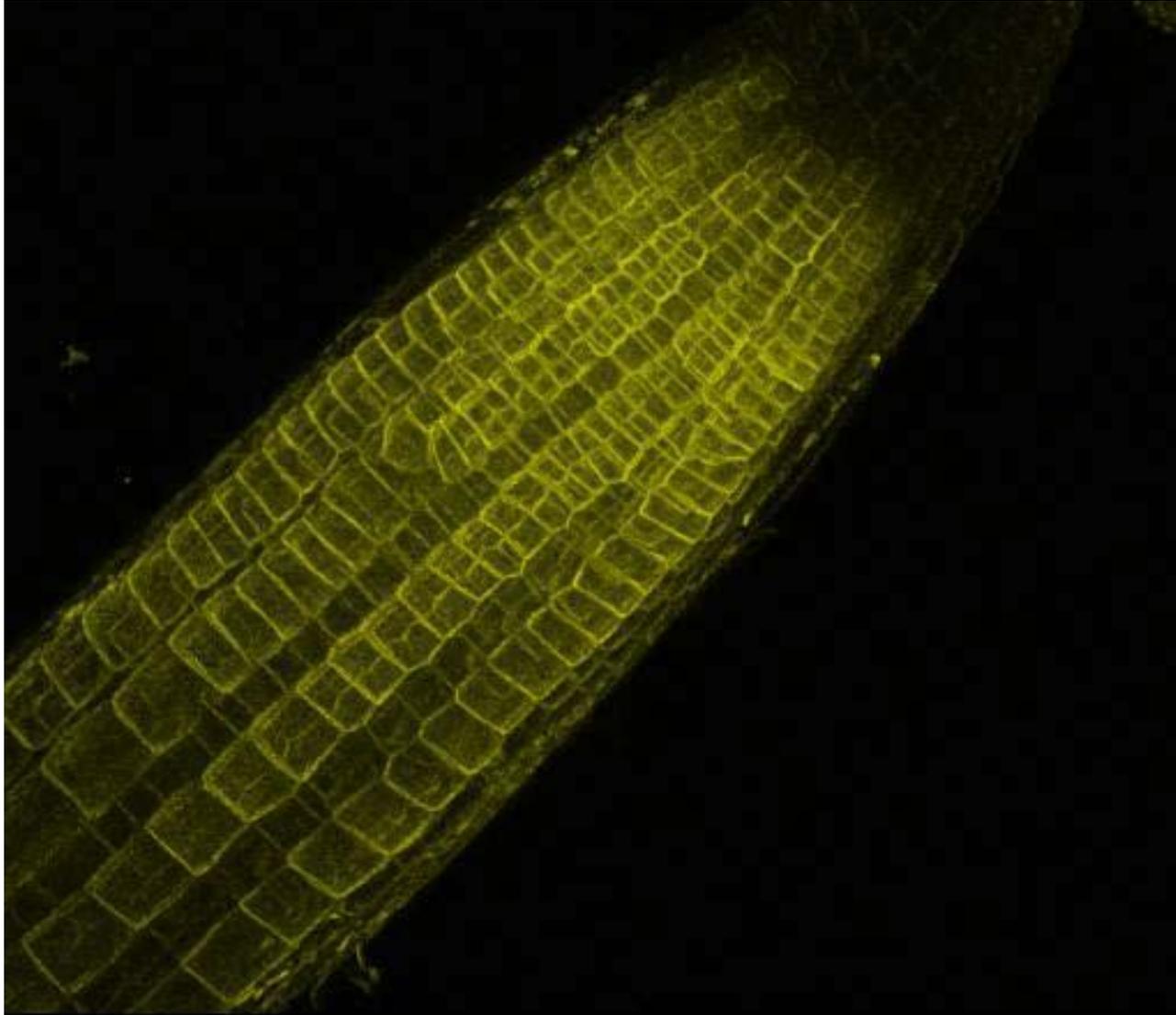


# Optical sectioning of Arabidopsis root



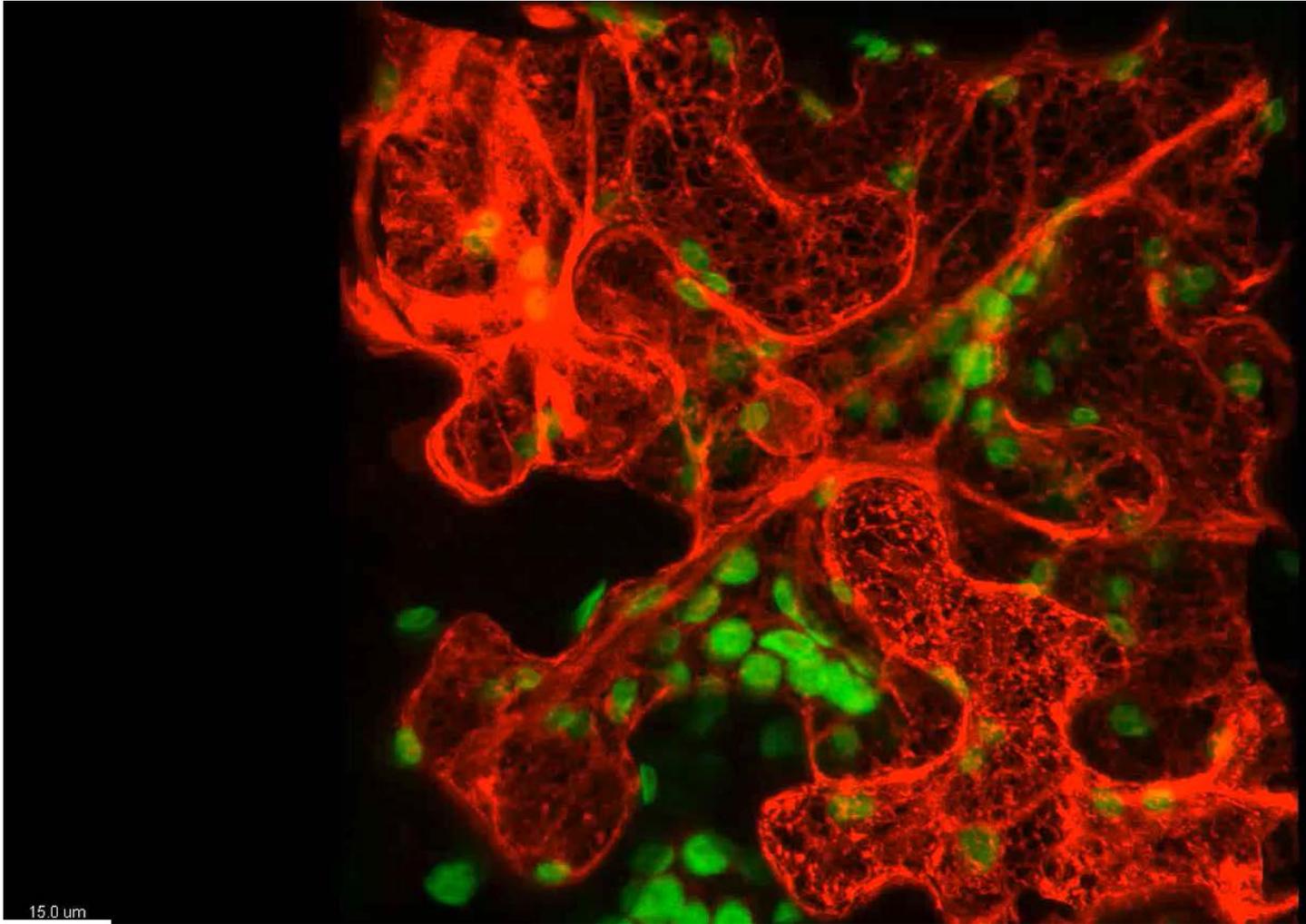
# 3D projection

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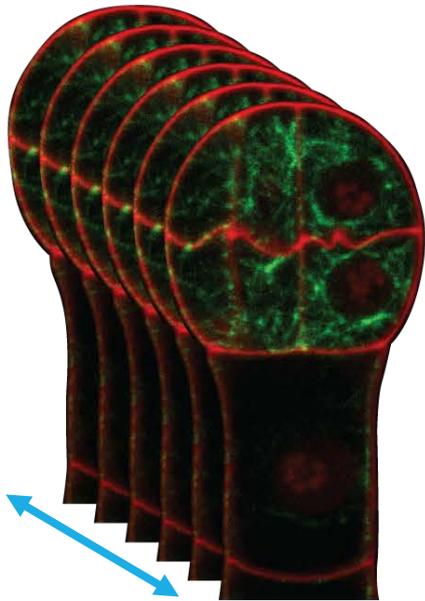


# Endoplasmatisch recticulum

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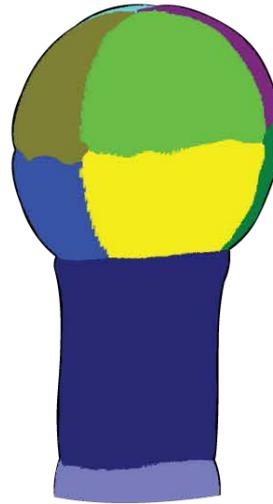
# Quantification of cell volumes



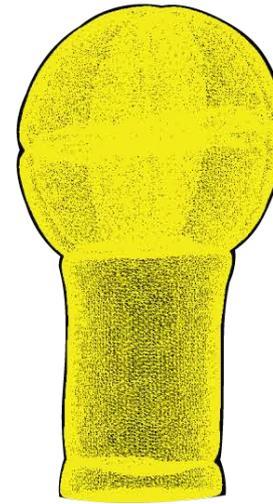
0,2 um stacks  
TUA6-GFP (Microtubule)  
Renaissance stain 2200



3D view



3D Segmentation



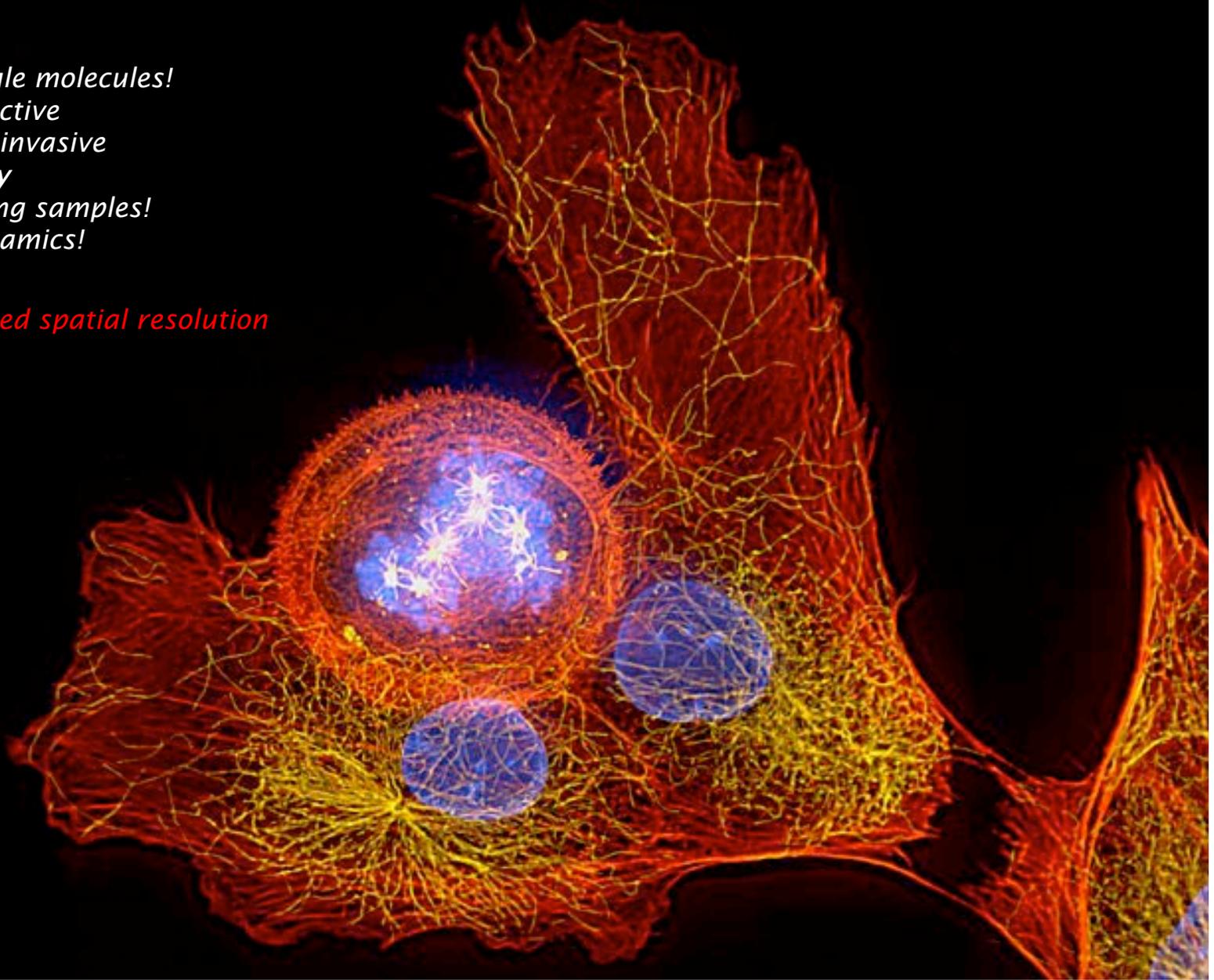
Mesh creation



Signal projection

*Single molecules!*  
*Selective*  
*Noninvasive*  
*Easy*  
*Living samples!*  
*Dynamics!*

*Limited spatial resolution*



# Resolution in confocal microscopy

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Wide field Epi fluorescence microscopy

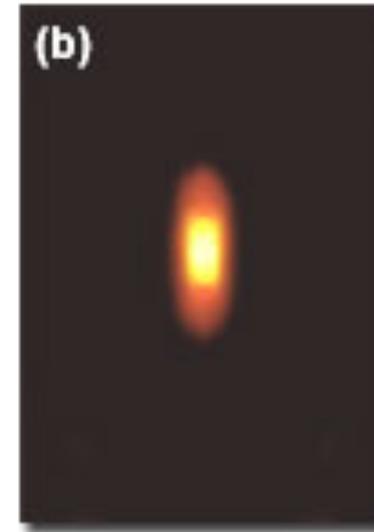
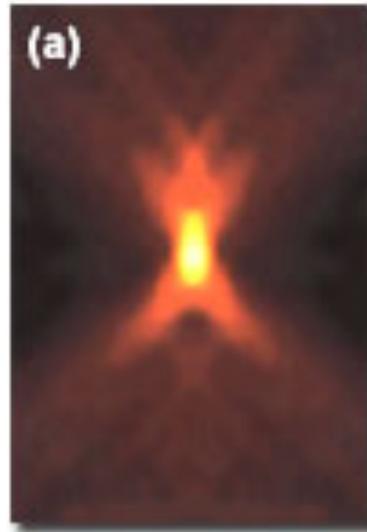
Confocal fluorescence microscopy

Lateral

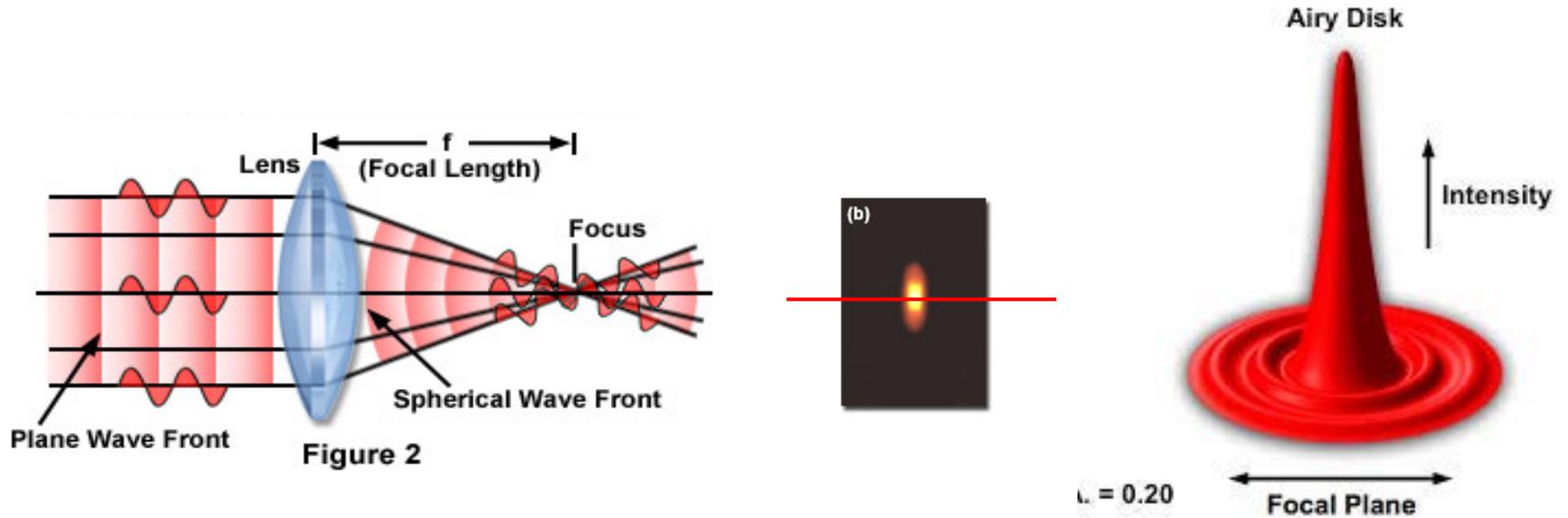
$$R_{\text{lat}} = 0.61\lambda / \text{NA}$$

$$R_{\text{lat}} \approx 0.4\lambda / \text{NA}$$

**Axial** Point Spread  
Function Intensity  
Profiles

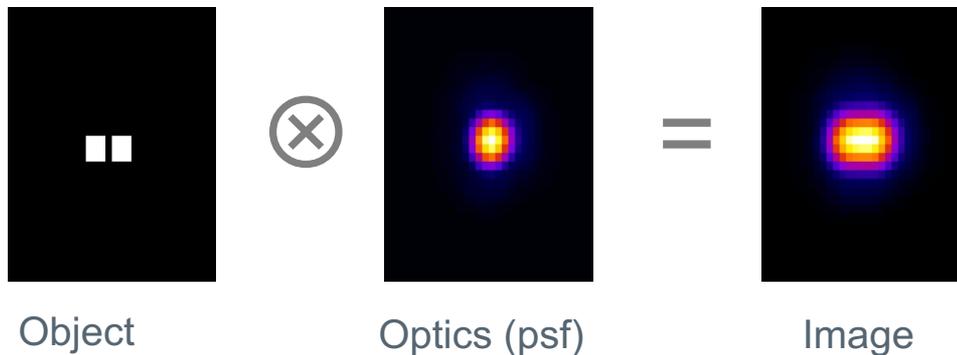


# Spatial resolution



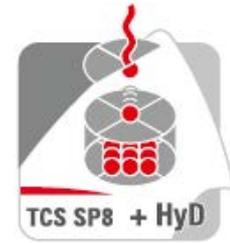
airy pattern formation

## Convolution

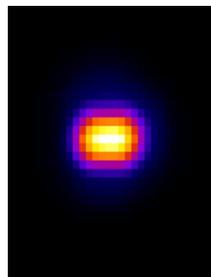


# HyVolution2 - Multicolor confocal superresolution

- Requires SP8 photon efficiency + HyD highly sensitive detection with low noise
- HyD make every photon count – best SNR - and XYZ resolution at pinhole 0.6 AU
- Huygens deconvolution by SVI
- GPU (graphics processing unit) accelerated computing with the CUDA high-performance hardware
- LASX HyVolution Wizard

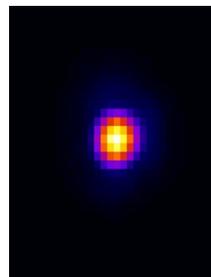


## Deconvolution



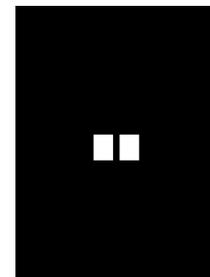
Image

/



Optics

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Restored Image

# HyVolution2

The screenshot displays the HyVolution2 software interface, which is divided into several functional panels. At the top, a navigation bar includes 'Configuration', 'Acquire', 'Deconvolution', 'Process', and 'Quantify'. The 'Acquire' panel is active, showing various acquisition parameters.

**Acquisition Settings:**

- Acquisition Mode: xyz
- HyVolution Settings: Load, Save
- HyVolution Grade: Speed, Resolution
- Deconvolution Settings: Approach: Huygens Essential Auto
- Mounting Medium: Prolong Gold
- Refractive Index: 1.47000
- Strategy: best resolution
- Allow auto-crop of images
- XY: 7352x7352 | 400 Hz | 1.28 | 0.50 AU
- Z-Stack: 76.6µm | 73 Steps

**Internal Settings:**

- Objective: HC PL APO CS 10x/0.40 DRY
- Beamsplitter: TD 488/552/638
- X1-Port: Mirror
- Fluo Turret: Scan-BF

**Channel Configuration:**

Channel	Gain (%)	Offset (%)	Filter
PMT 1	0.0	0.00	None
HyD 2	100.0	1.30	Standard
PMT 3	0.0	0.00	None
HyD 4	100.0	1.30	Standard
PMT 5	0.0	0.00	None

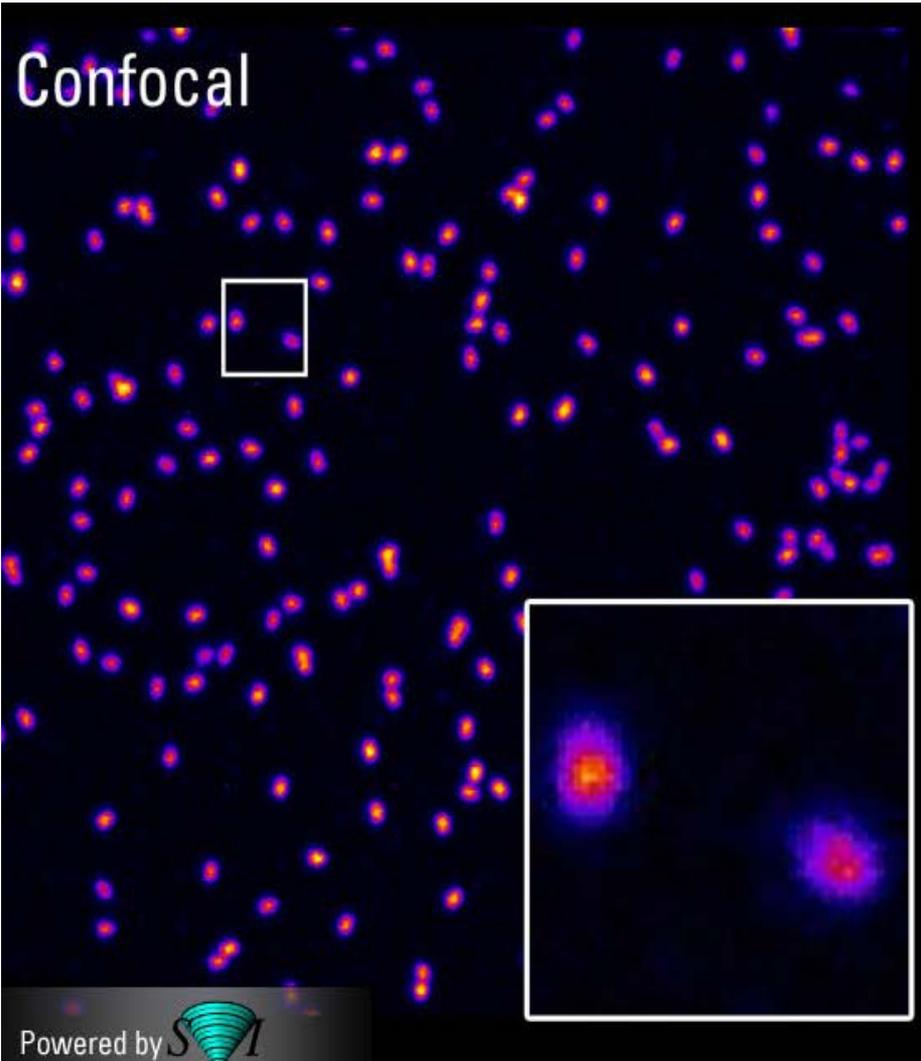
**Acquisition Parameters:**

- Nr. of Steps: 73
- Z-Step Size: 1.06
- System Optimized:
- Z-Compensation: none
- Galvo Flow:
- Travel Range [µm]: 500
- Sequential Scan:

The right side of the interface features a large preview window showing a fluorescence microscopy image of a cell. The image displays green filamentous structures and purple puncta. The preview window includes a toolbar with various tools and a status bar at the bottom right showing 'Line\_Acqu\_16\_HyD x=1632 y=1632 (10.7 MB)' and 'Size 116.25 µm x 116.25 µm'.

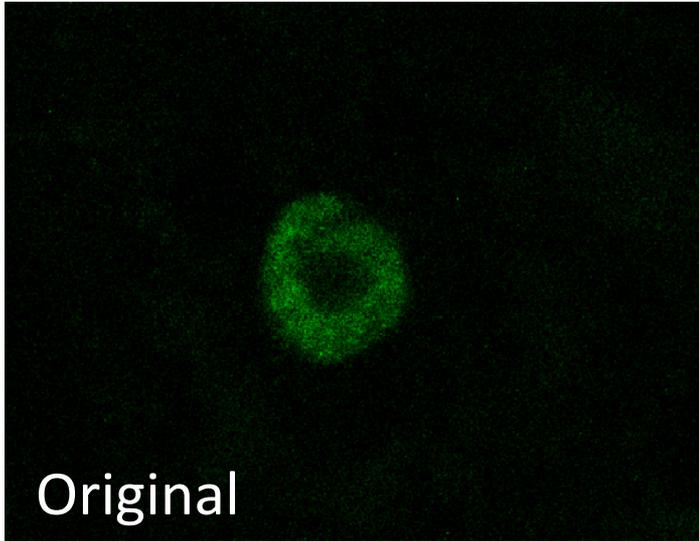
# HyVolution2; spatial resolution 140 nm

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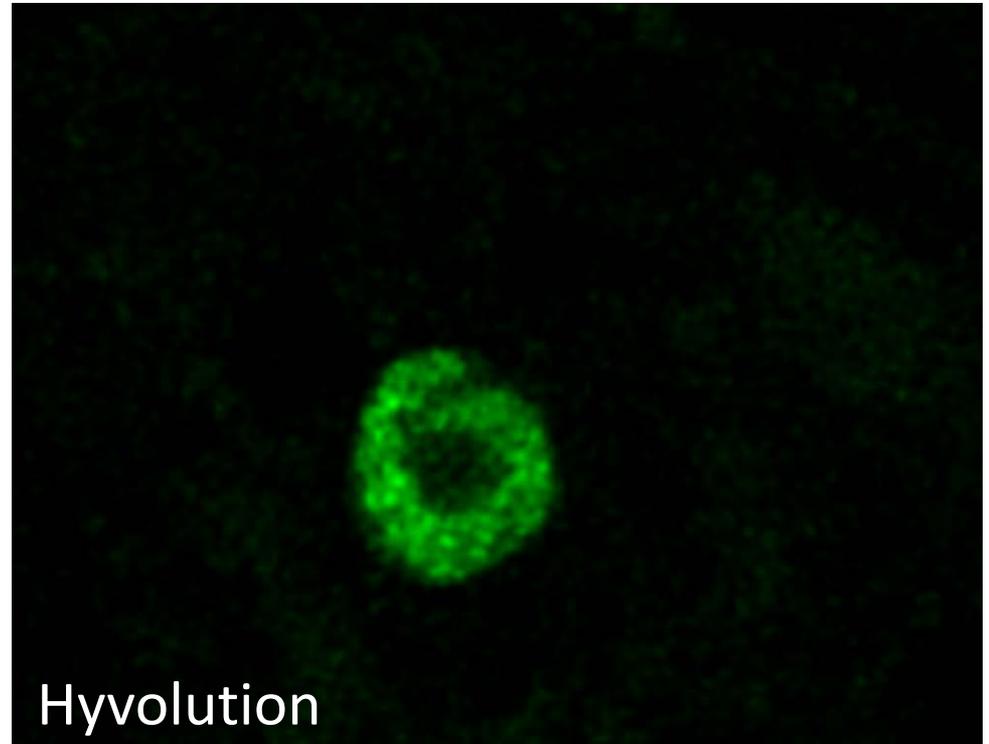
# HyVolution2; examples

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Original

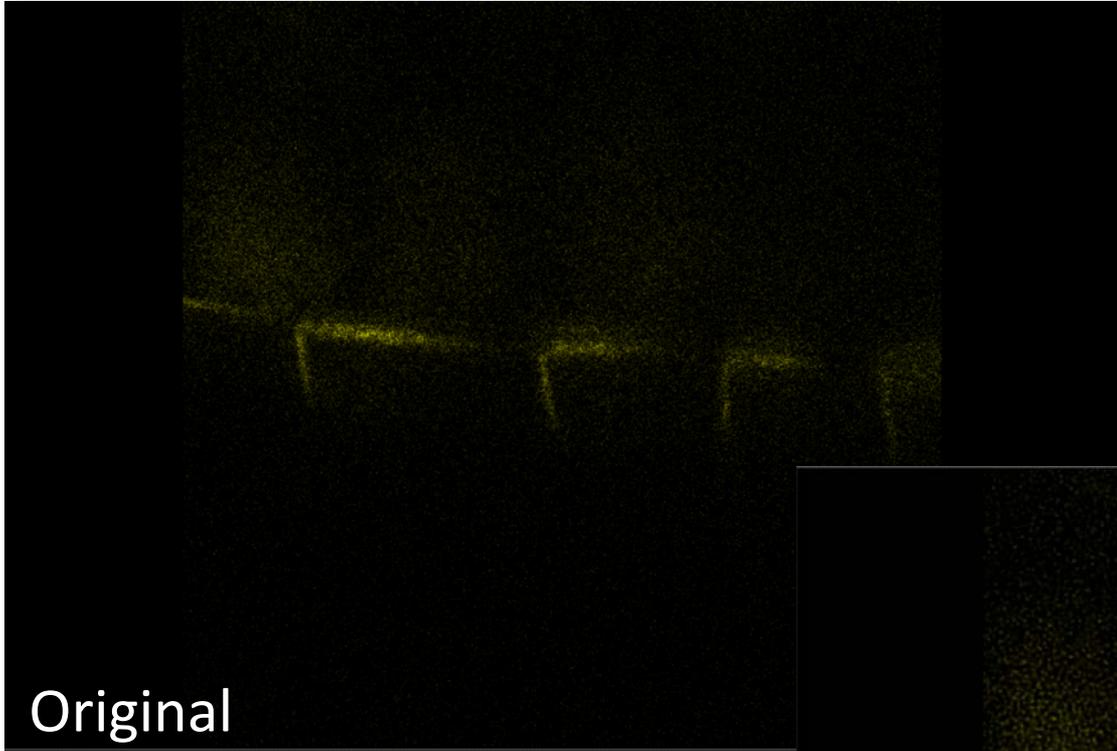
MpARF3-YFP



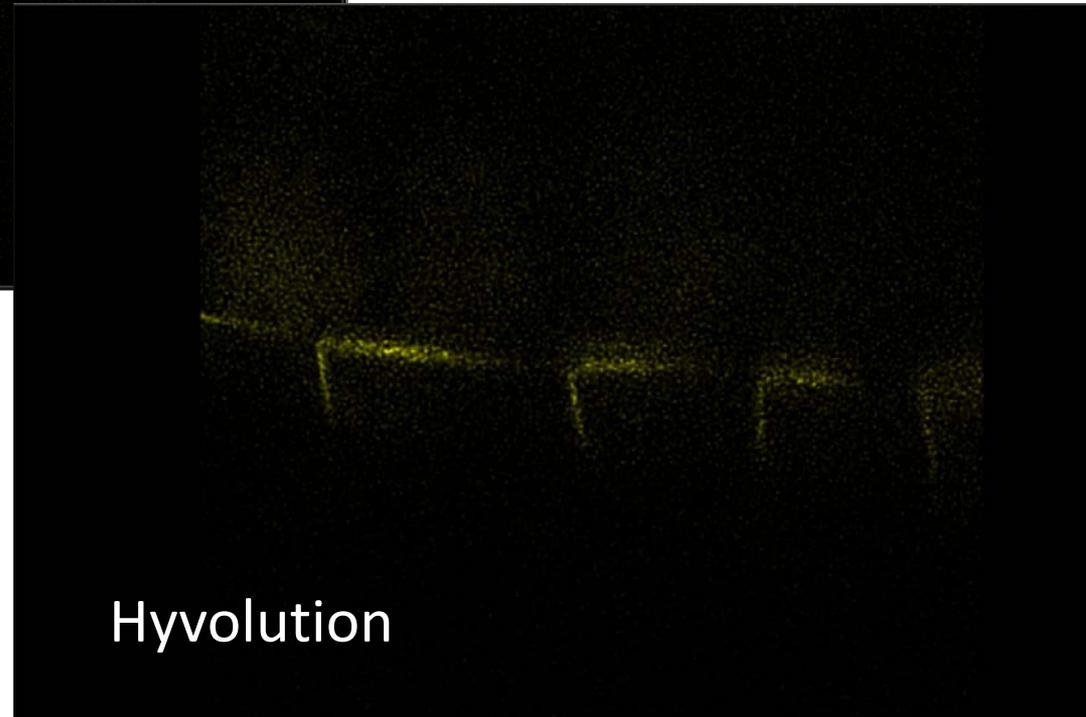
Hyvolution

# Hyvolution2; examples

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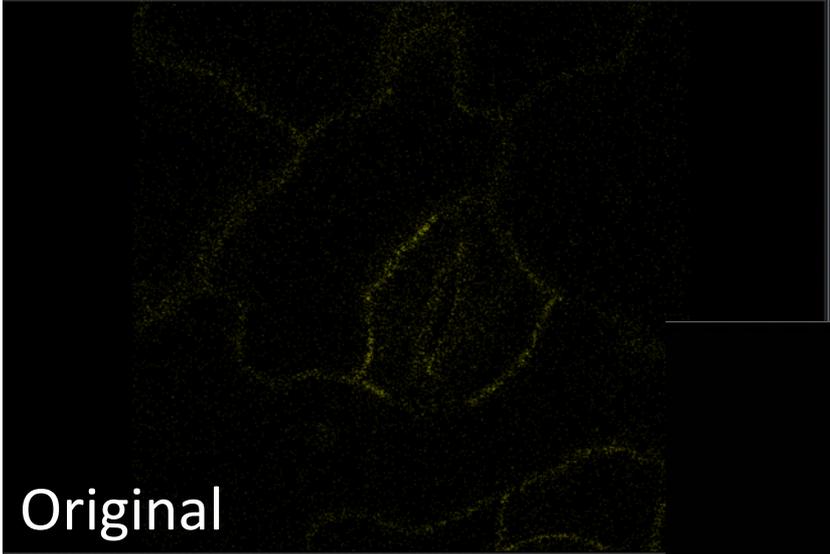
SOK-YFP in roots



# Hyvolution2; examples

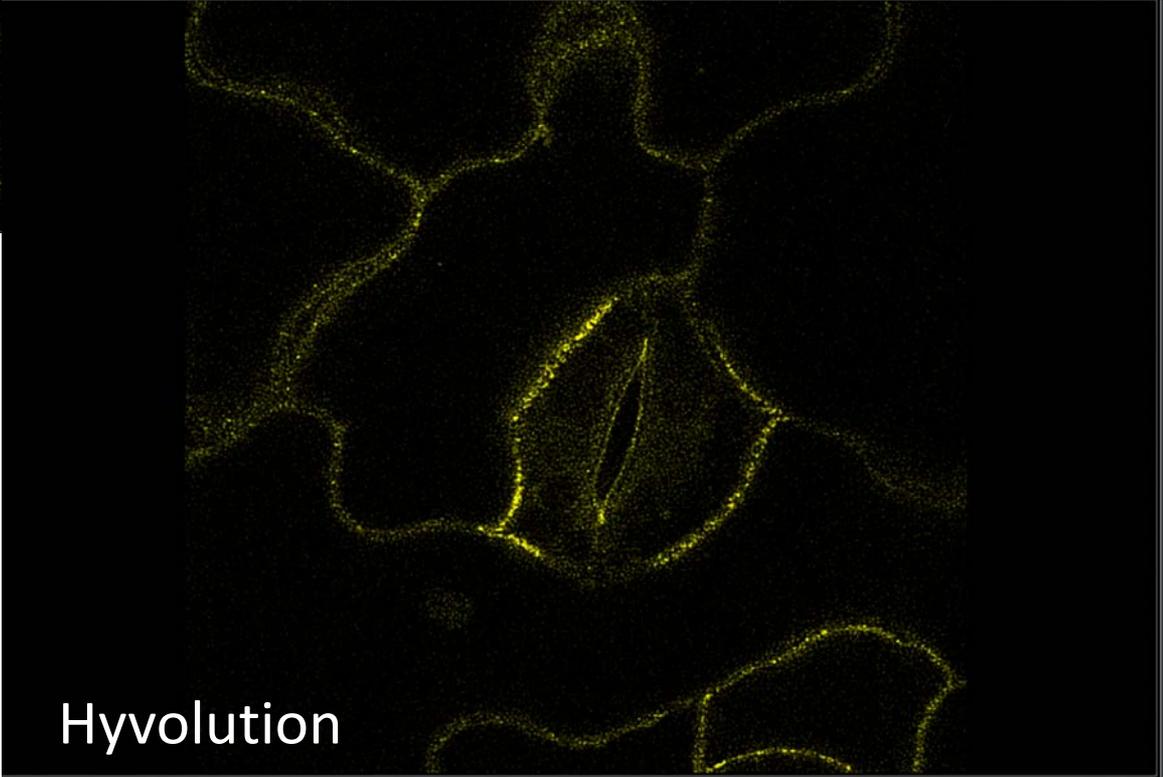
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SOK-YFP in leaves



Original

This image shows a fluorescence microscopy view of a leaf section. The signal is very faint and noisy, with the cell walls appearing as thin, irregular yellow-green lines against a dark background. The overall image quality is low, making it difficult to discern specific cellular structures.



Hyvolution

This image is the same as the 'Original' image but has been processed using Hyvolution. The signal is significantly enhanced, resulting in much brighter and more distinct yellow-green cell walls. The noise is reduced, and the overall contrast is higher, making the cellular structure much clearer and easier to analyze.

# Other features

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- ✓ Time lapse
- ✓ Multi stitching
- ✓ Lambda scan
  
- ✓ Gating system
  
- ✓ Multi mode confocal
- ✓ Fluorescence lifetime imaging
- ✓ Fluorescence (cross) correlation (micro)spectroscopy

# Acknowledgements

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Laboratory of Biochemistry

Adrie Westphal

Bert de Rybel

Maritza van Dop

Che-Yang Liao

Jos Wendrich

Dolf Weijers

Microspectroscopy Research Facility

Arjen Bader

Johannes Hohlbein

Herbert van Amerongen

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# Micro-Spectroscopy

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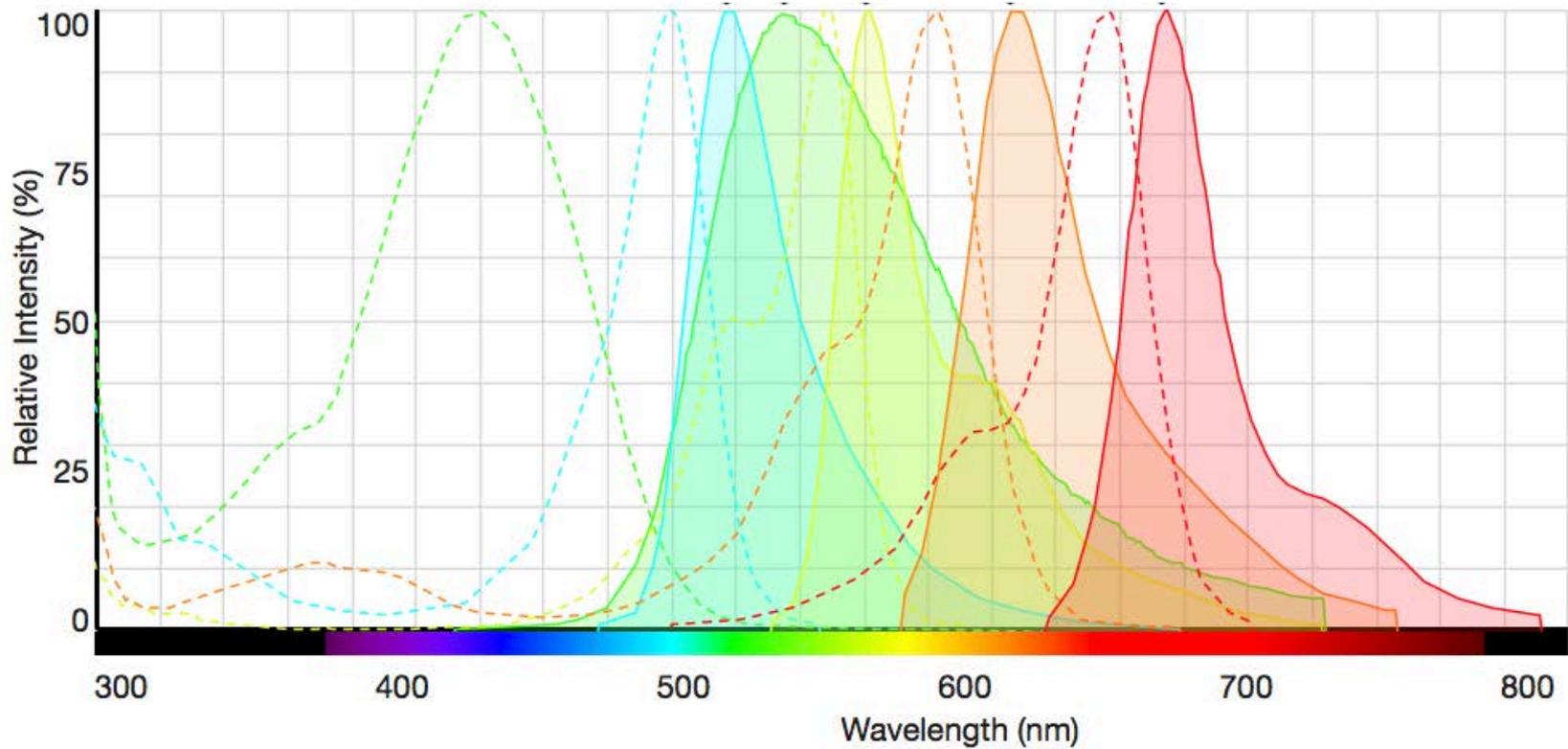
## Application of fluorescence spectroscopy on microscopic objects

### Fluorescence in biological/applied sciences?

- + Sensitive
- + Specific
- + Multiparameter
- + Living cell
- + Dynamics

# Excitation and emission of fluorophores

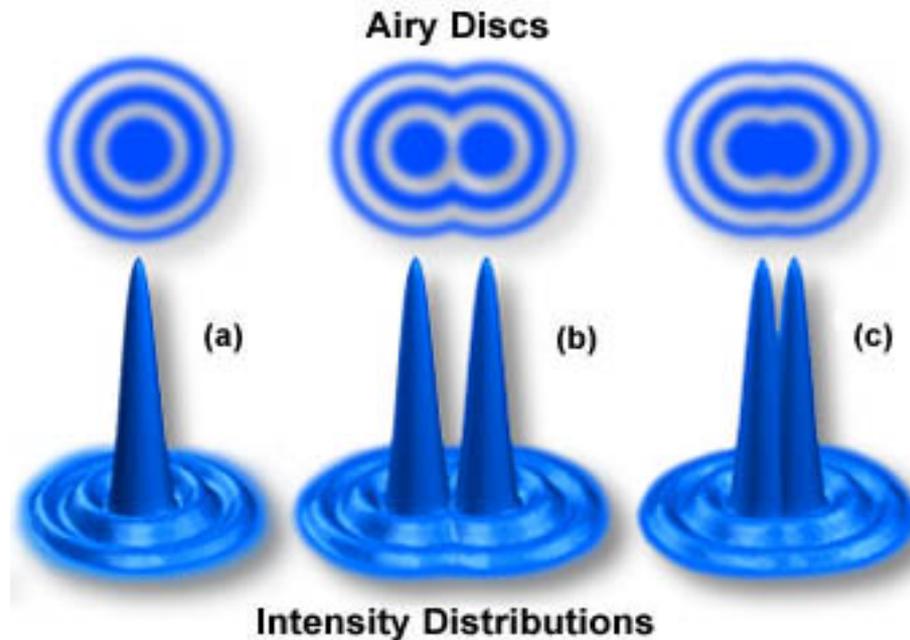
## Fluorescence absorption/emission spectra



# Spatial resolution

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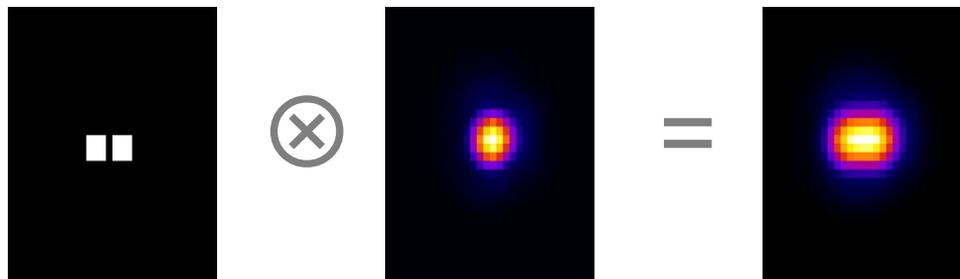
The Rayleigh criterion, defined as the minimum resolvable detail, when the first diffraction minimum of the image of one source point coincides with the maximum of another (C).



# Convolution and Deconvolution

Convolution of 2 objects with PSF

## Convolution



## Deconvolution

