Recent advances in confocal microscopy

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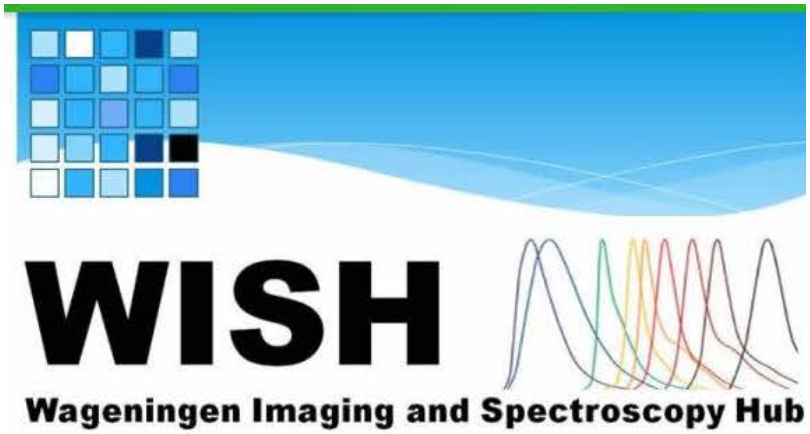
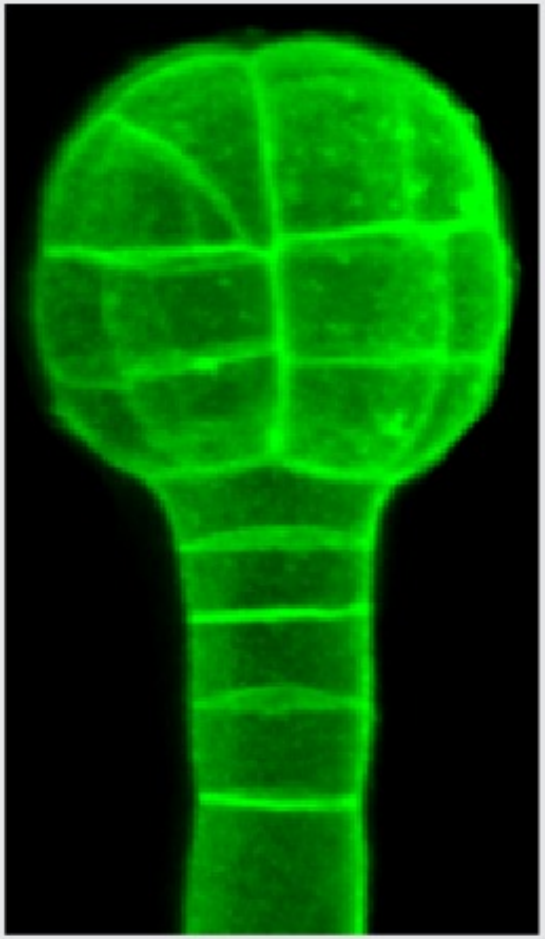
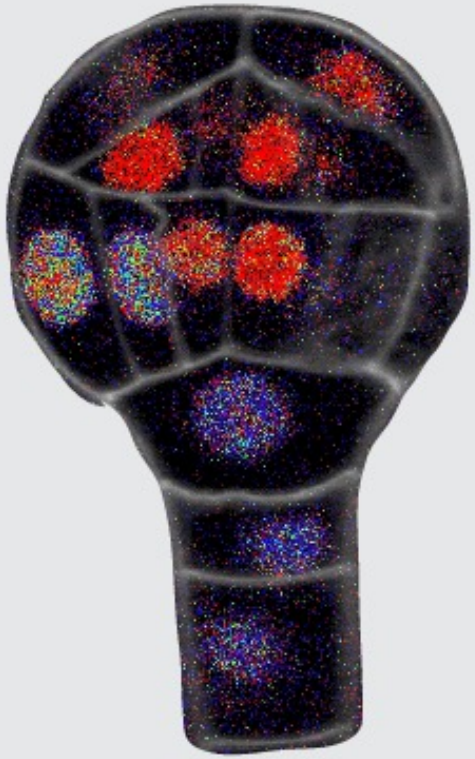


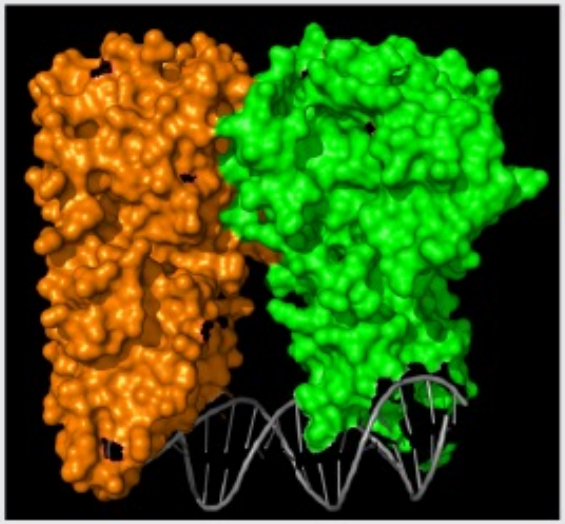
Future challenges in Cellular Biochemistry



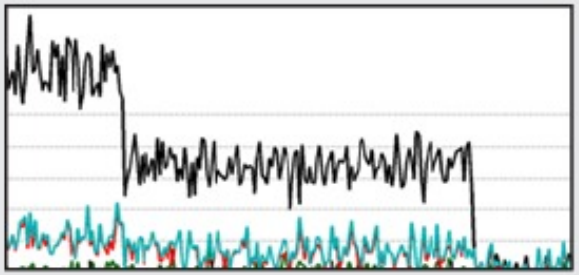
How do proteins control biological processes?



How many/ where?



With whom?



How fast?

Evolution?

Quantitative Fluorescence techniques

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

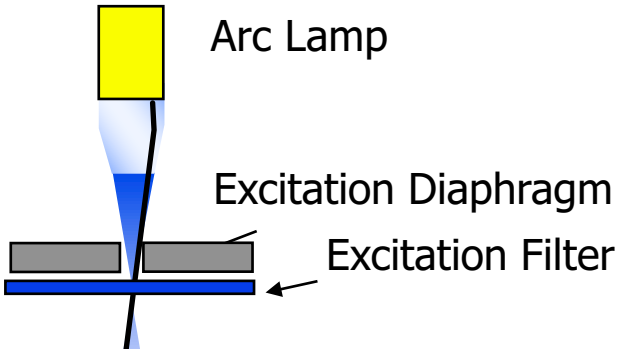
Multi mode confocal microscope
(Leica SP8)

- Point scanning source
- Selective excitation (lasers)
- Selective detection (HyD)
- Improved resolution
  - ✓ pinhole

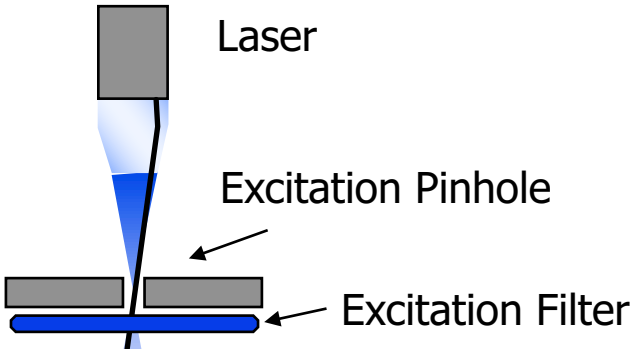
- User friendly
- Flexible
- Sensitive
- Up-to-date technologies
- Multi-parameter
  - Spectral
  - Lifetime
  - dynamics

Confocal principle

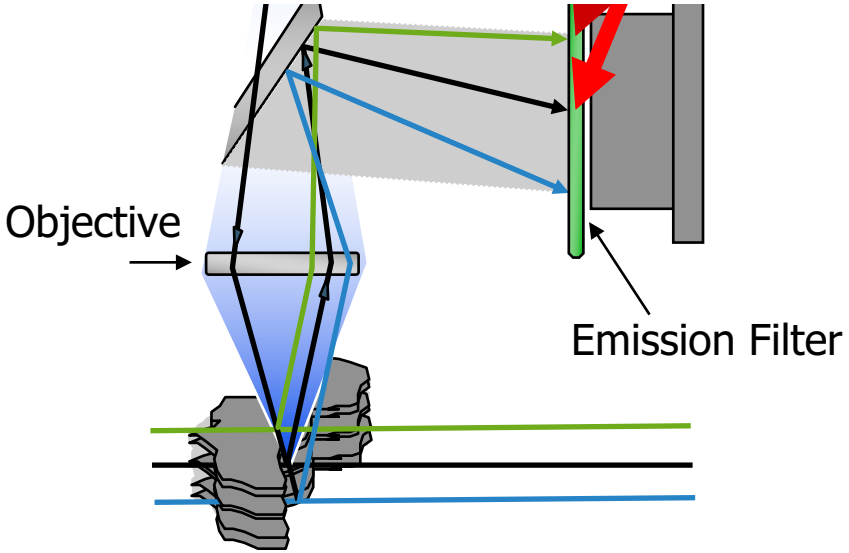
Widefield Fluorescence Microscope

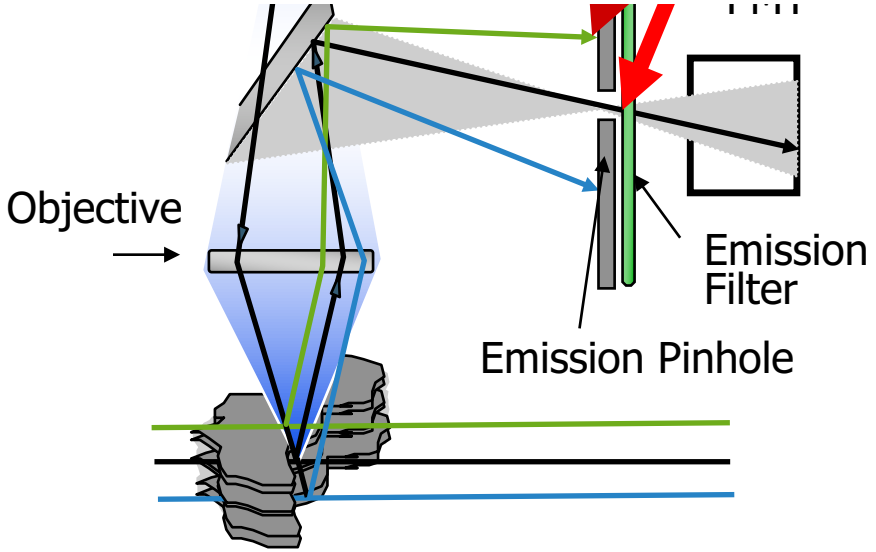


Confocal Fluorescence Microscope

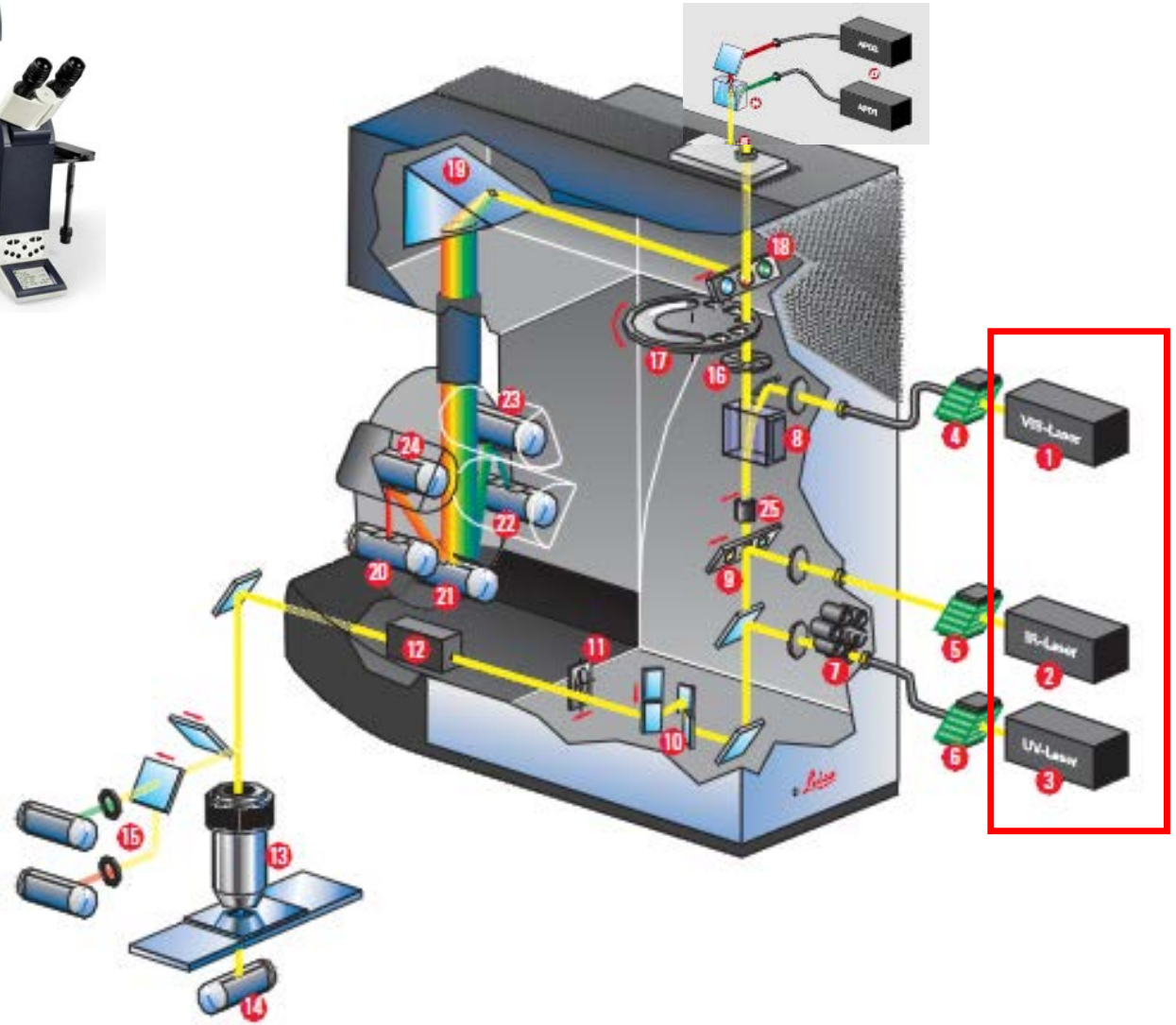


Blocking out of focus light

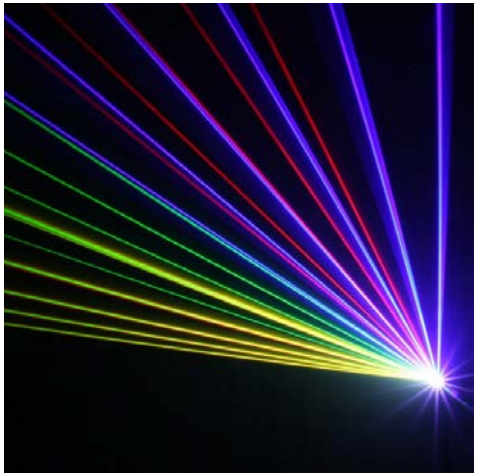


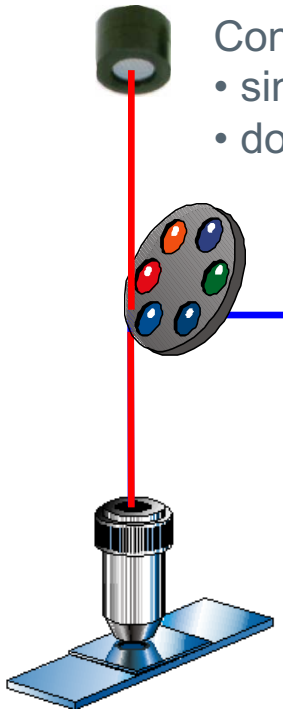


Leica SP8 concept

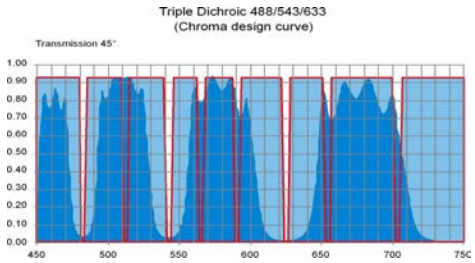


Excitation of fluorophores

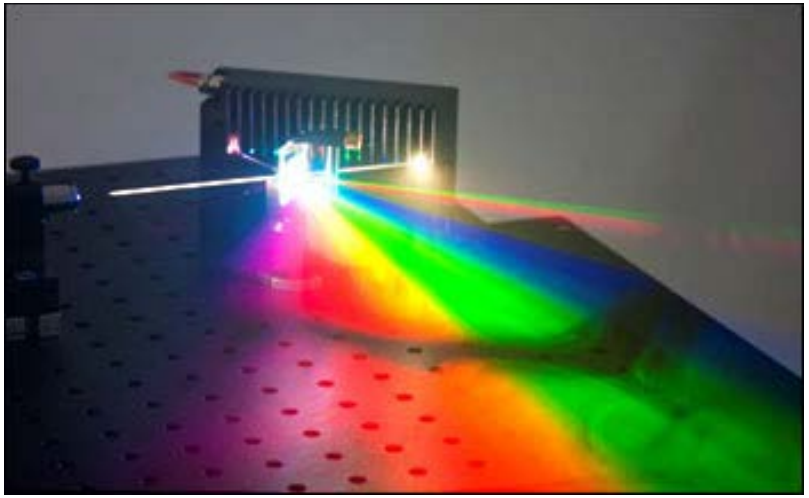


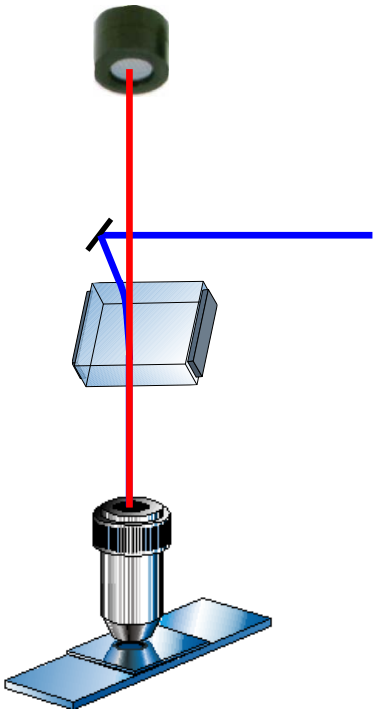


Conventional
- single BP
- double BP



Supercontinuum (fiber) lasers

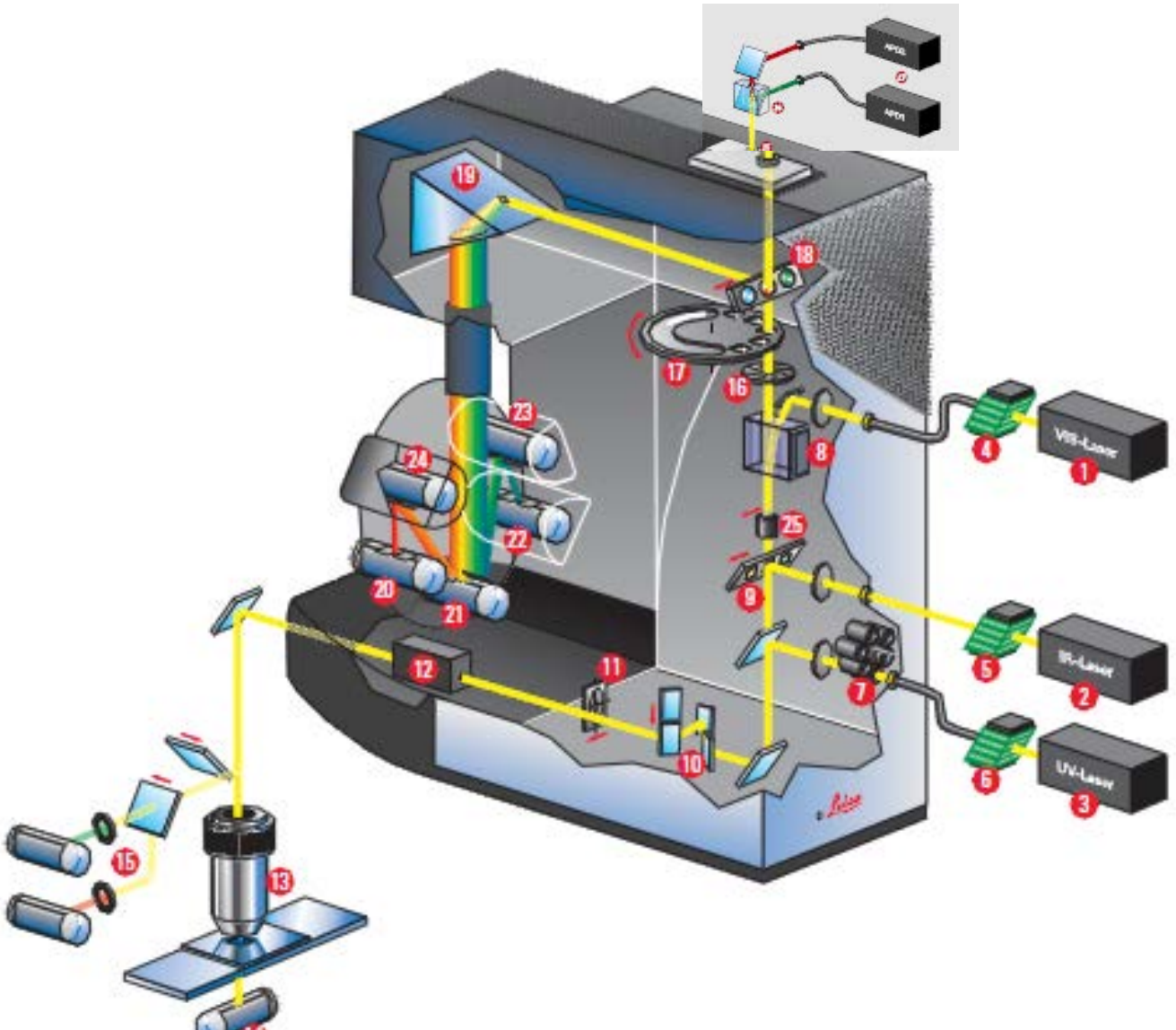




Excitation from 470-670 nm, 8 laser lines simultaneously

AOBS; Acoutic Optical Beam Splitter

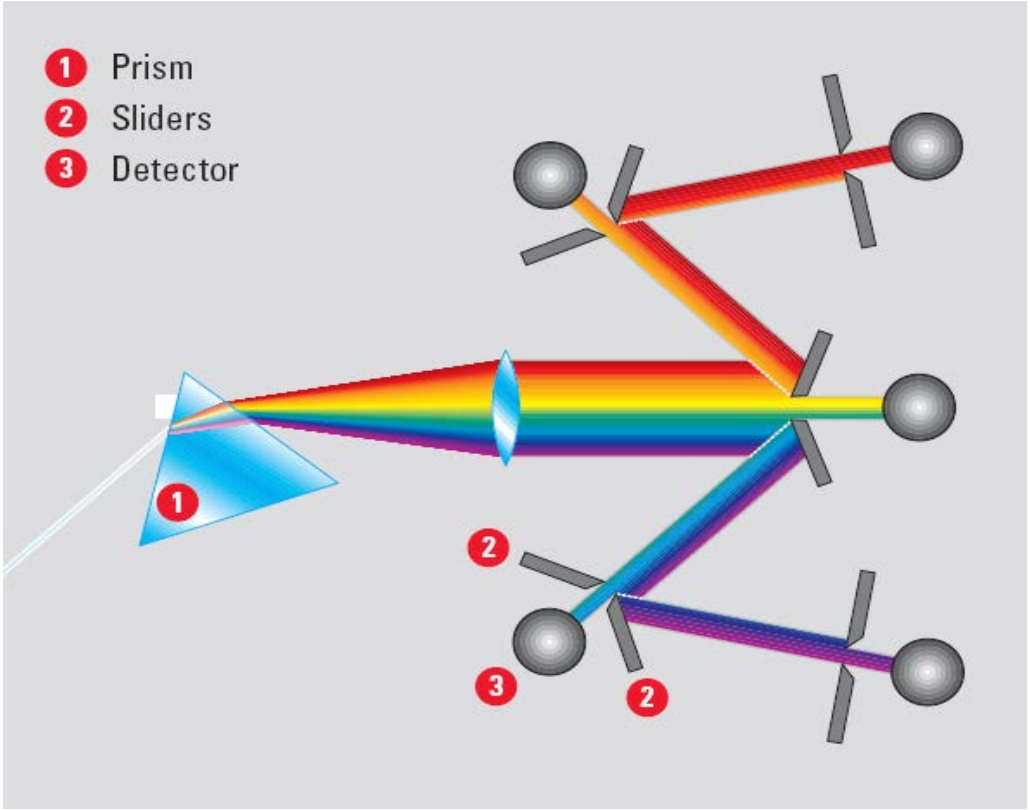
Leica SP8 concept



Fluorescence emission

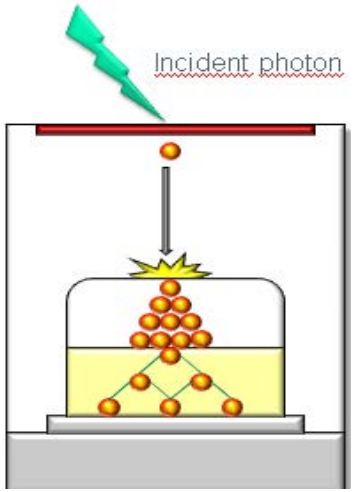
Spectral detection

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

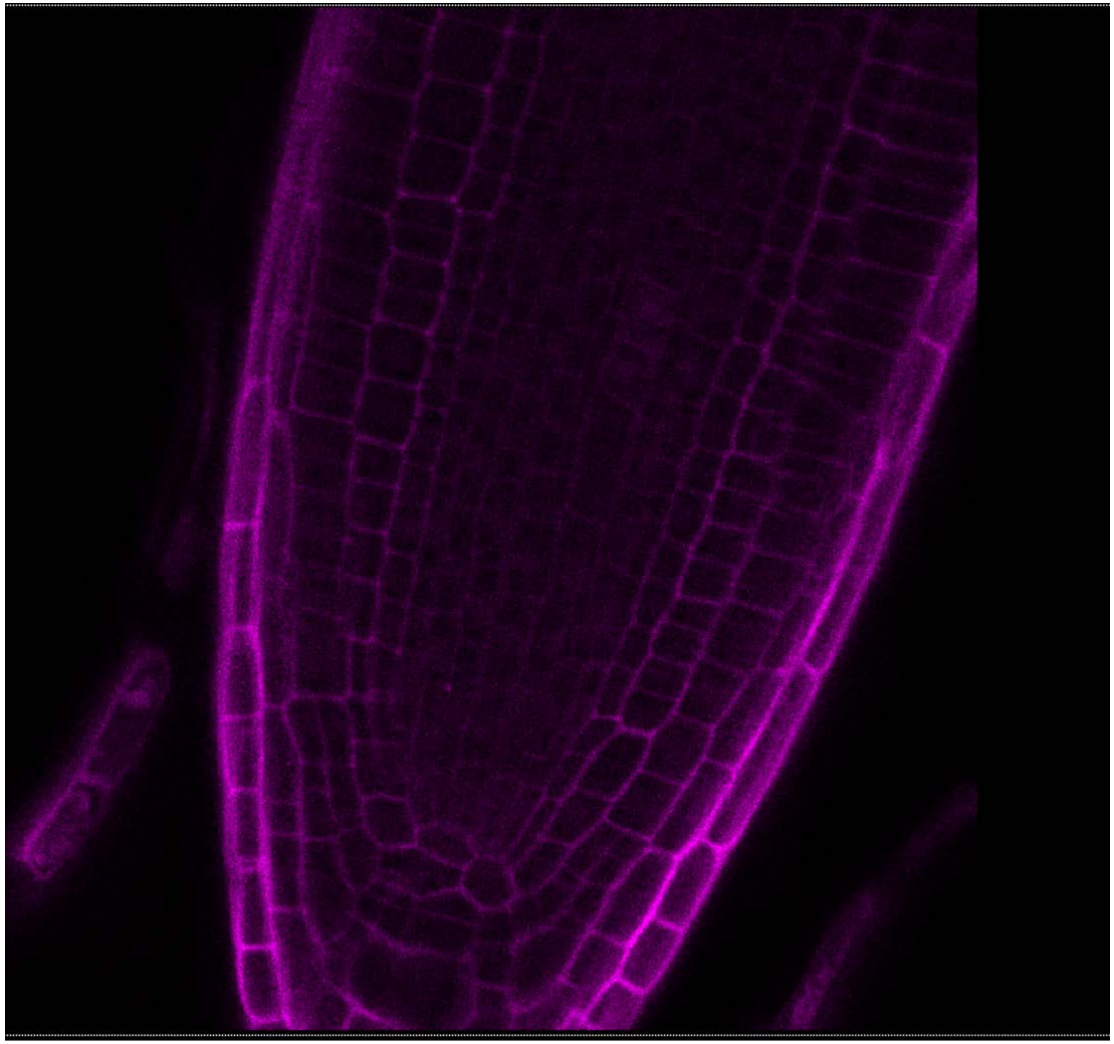


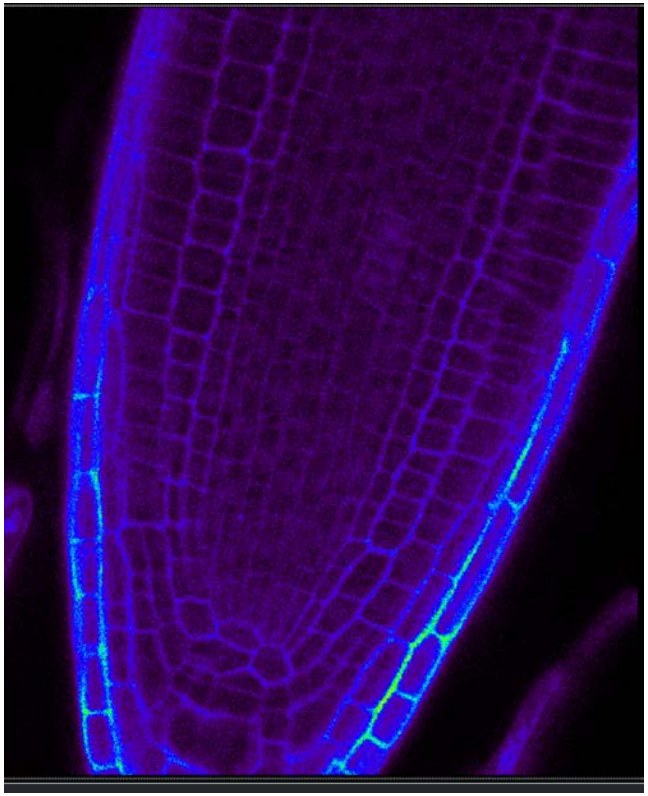
Hybrid detector technology

- 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



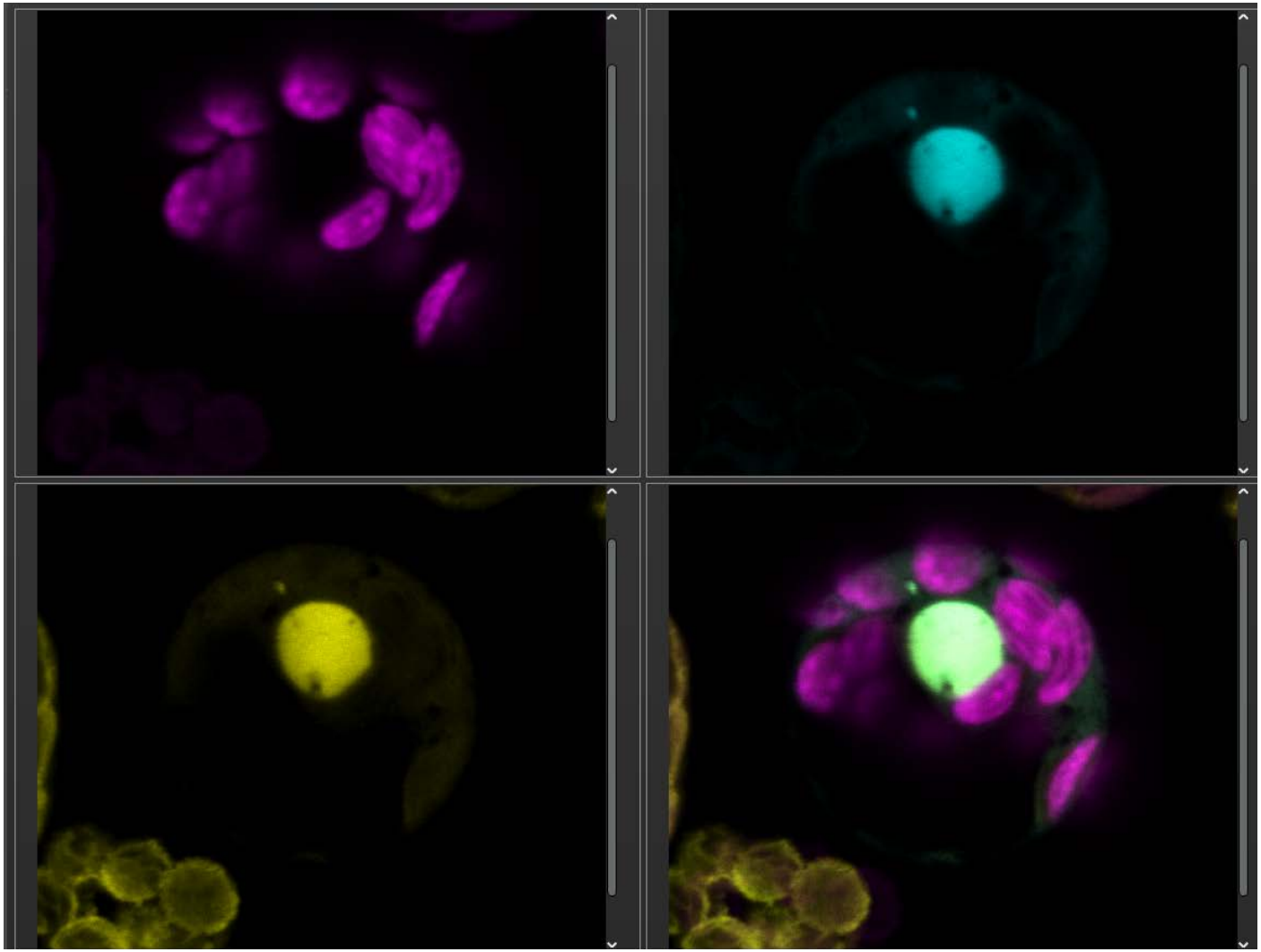
Imaging *Arabidopsis* root





Arabidopsis root labeled with membrane marker fused to GFP

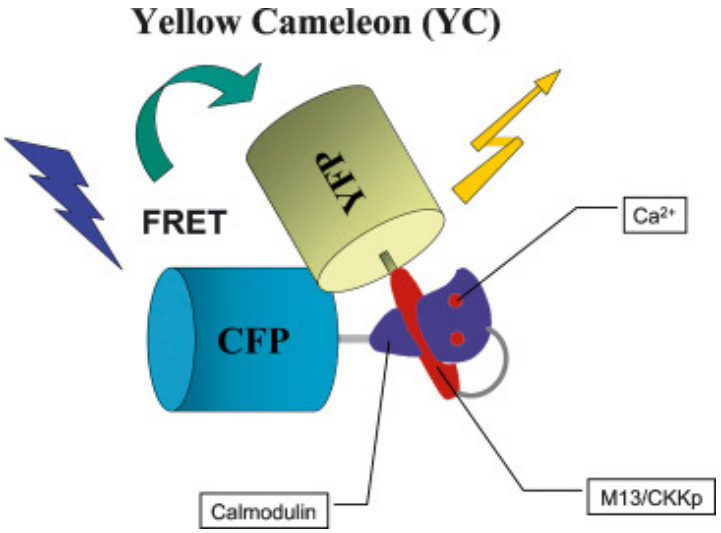
Multi-color imaging

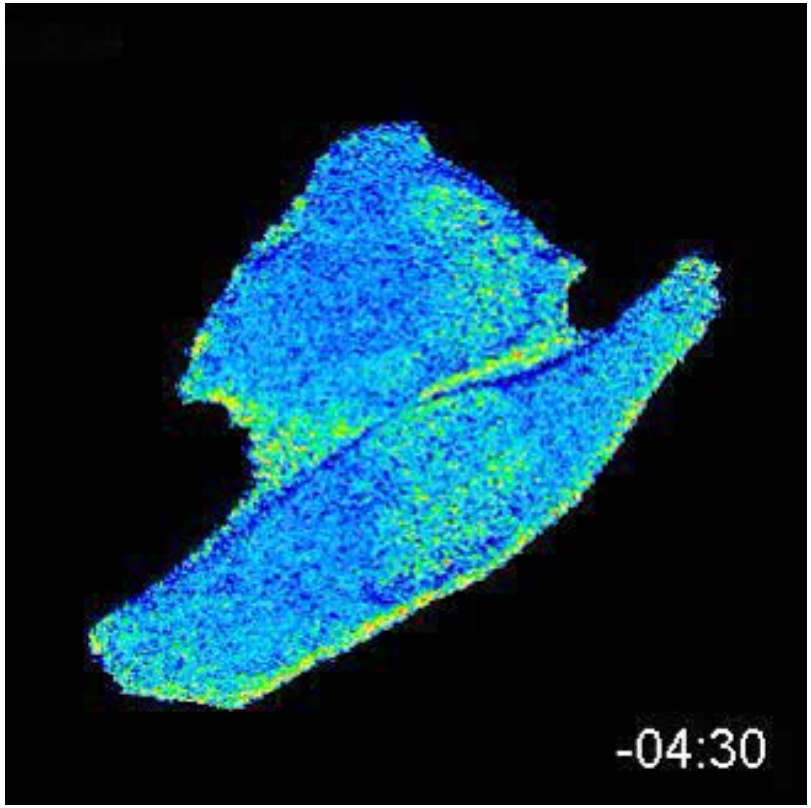


Transient expression multi-colored transcription factors

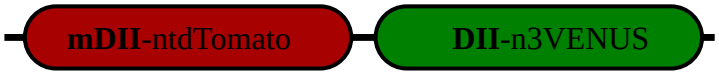
Ratio imaging

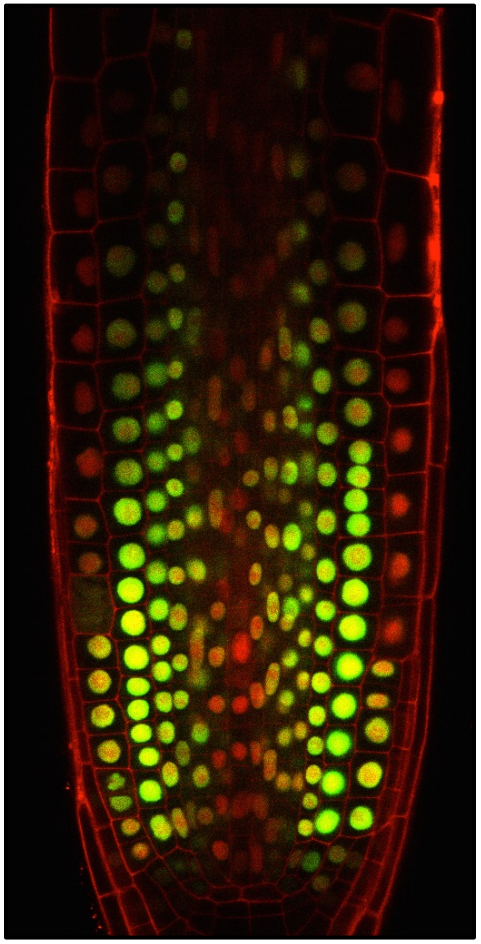
genetically encoded calcium sensor

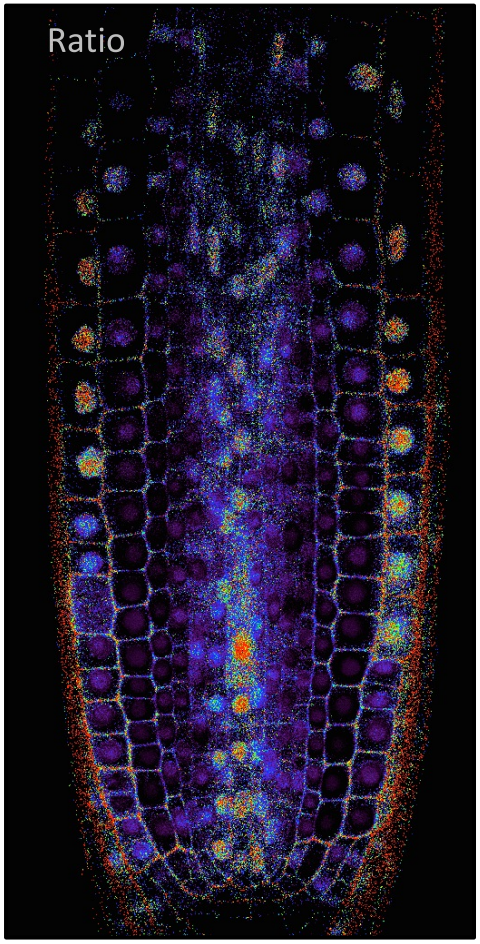




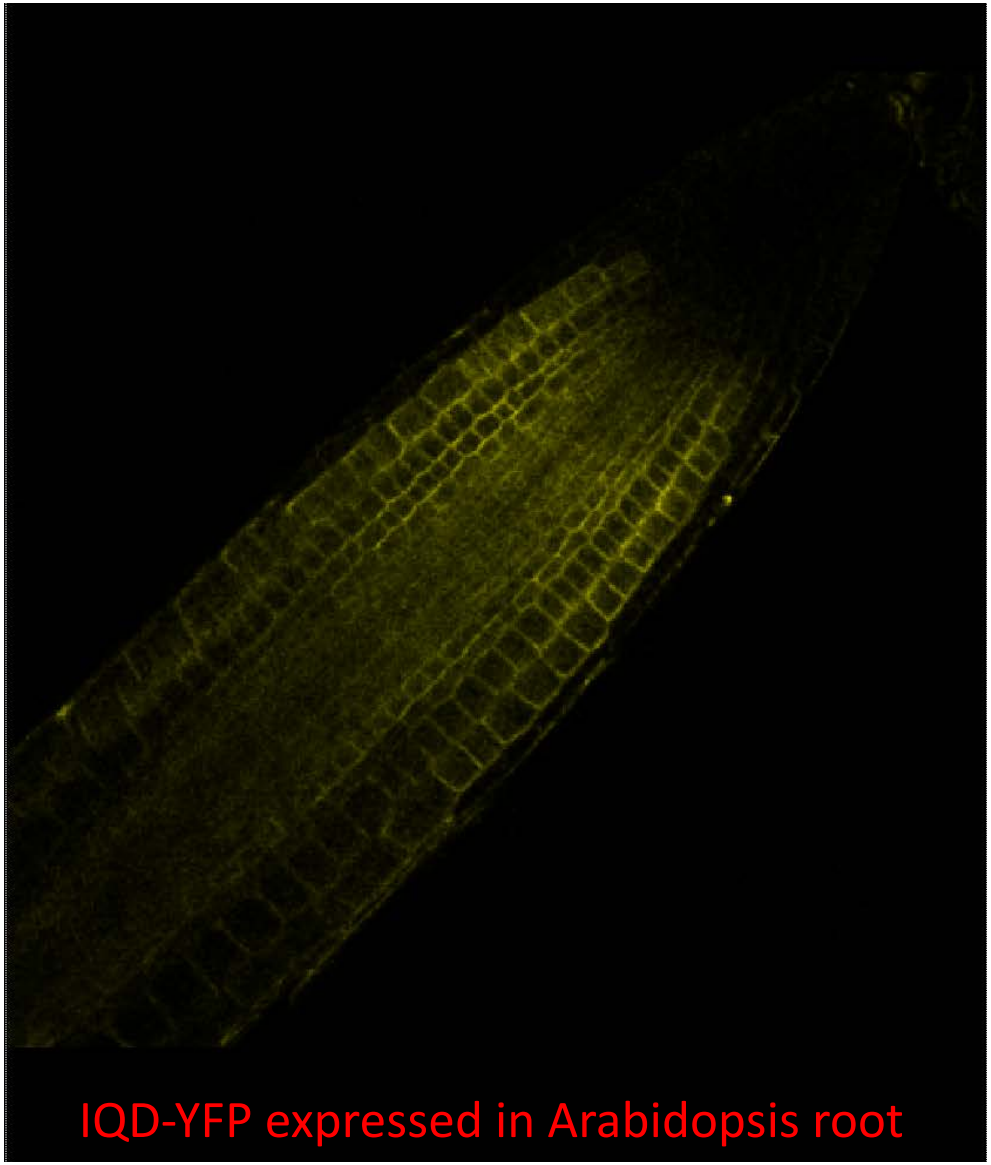
Quantifying auxin levels in roots







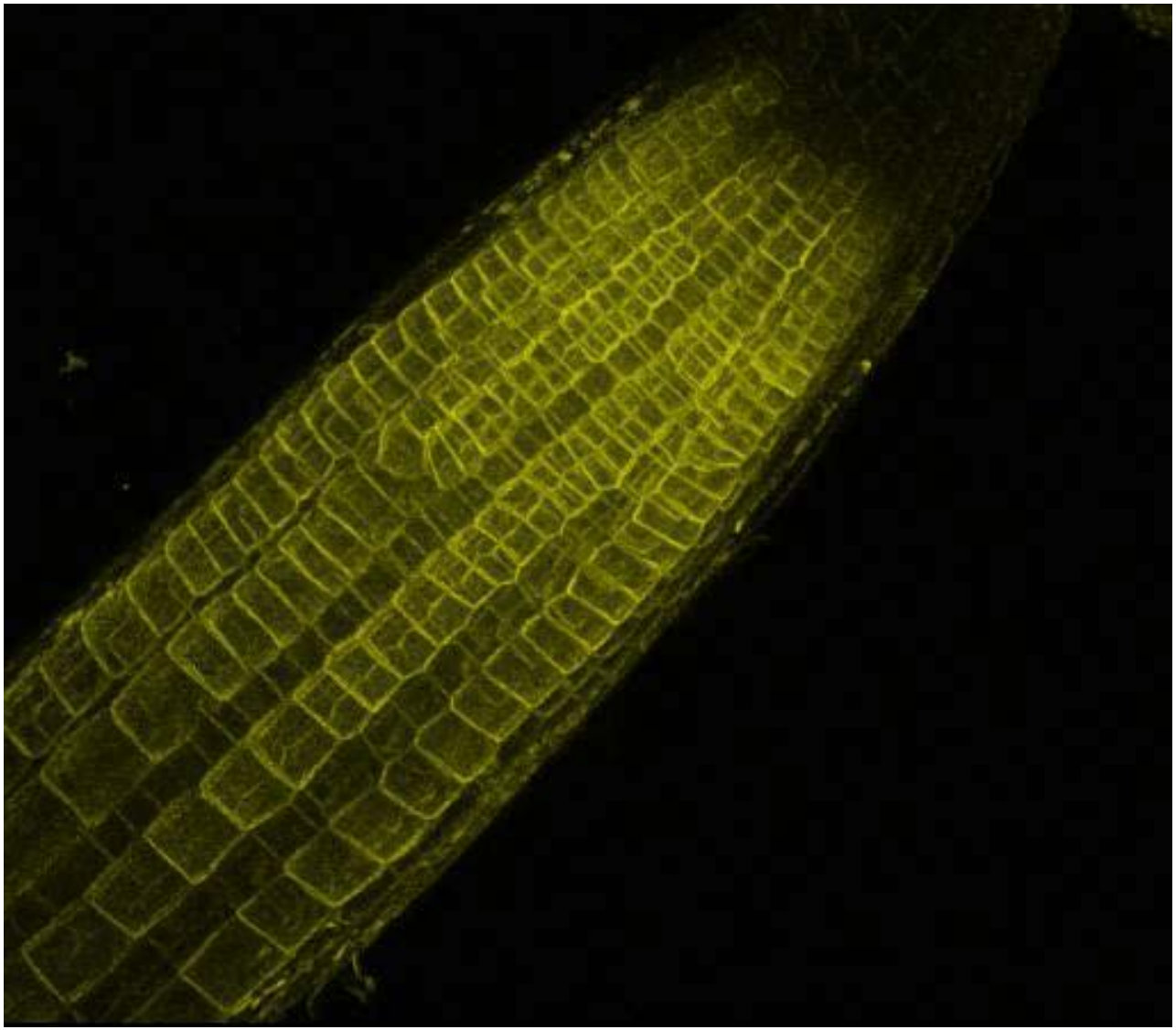
Optical sectioning



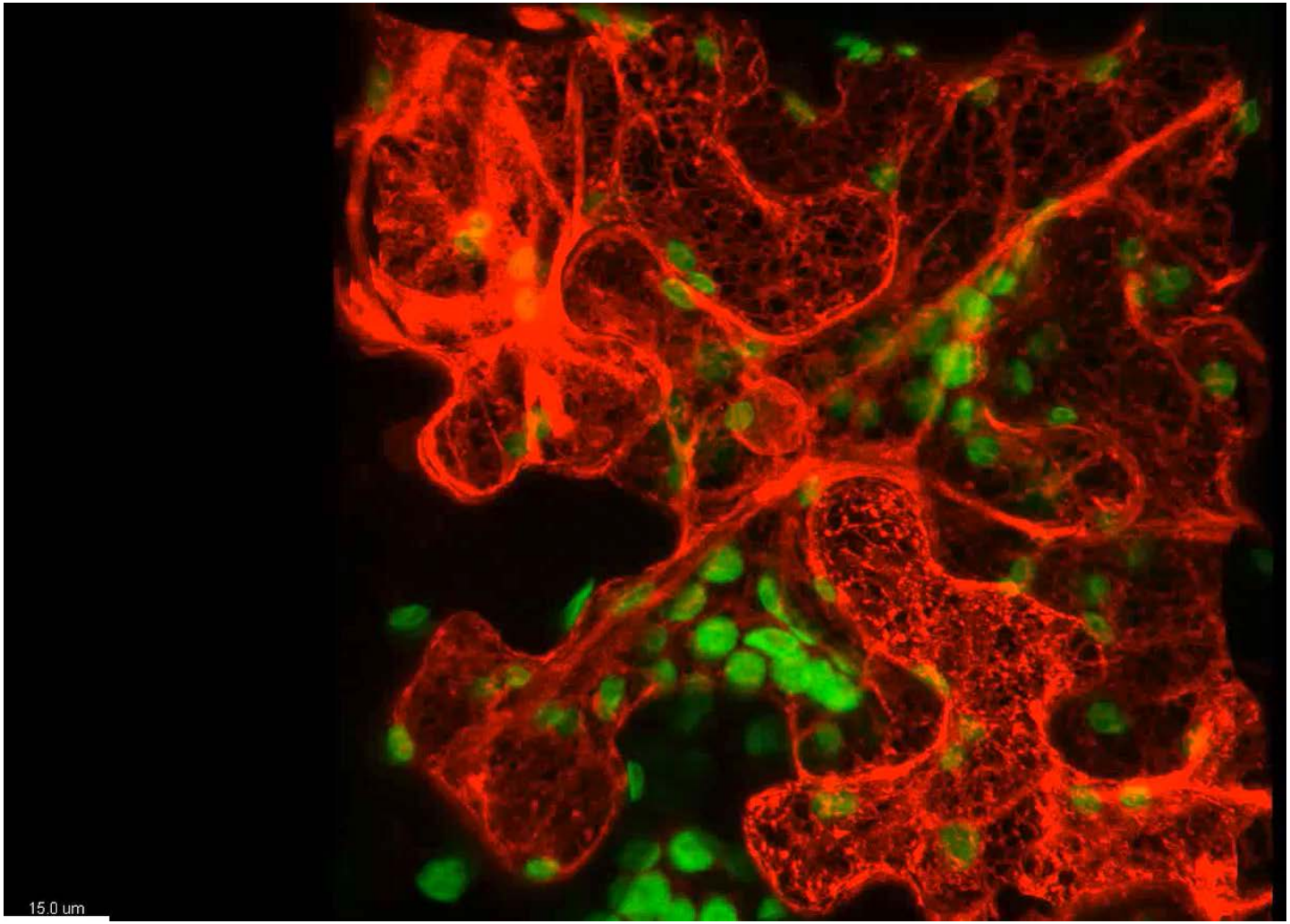
IQD-YFP expressed in Arabidopsis root

Optical sectioning of Arabidopsis root

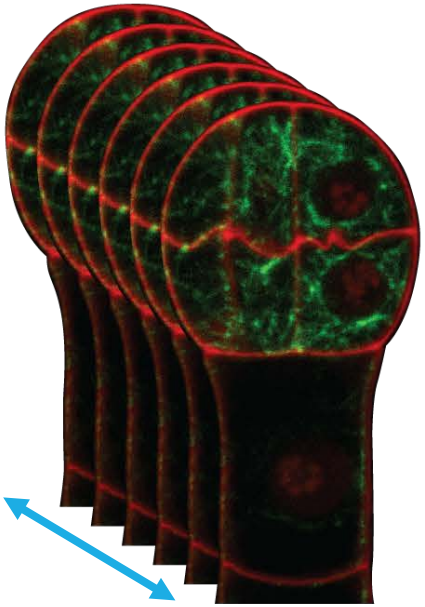
3D projection

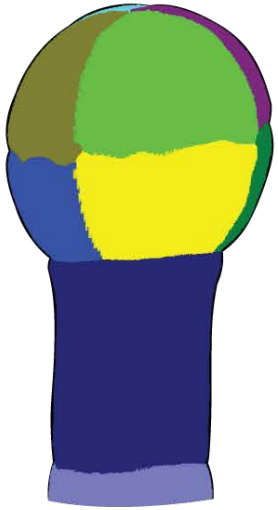


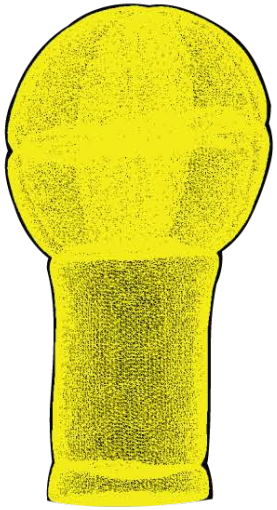
Endoplasmatisch recticulum



Quantification of cell volumes







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

3D view

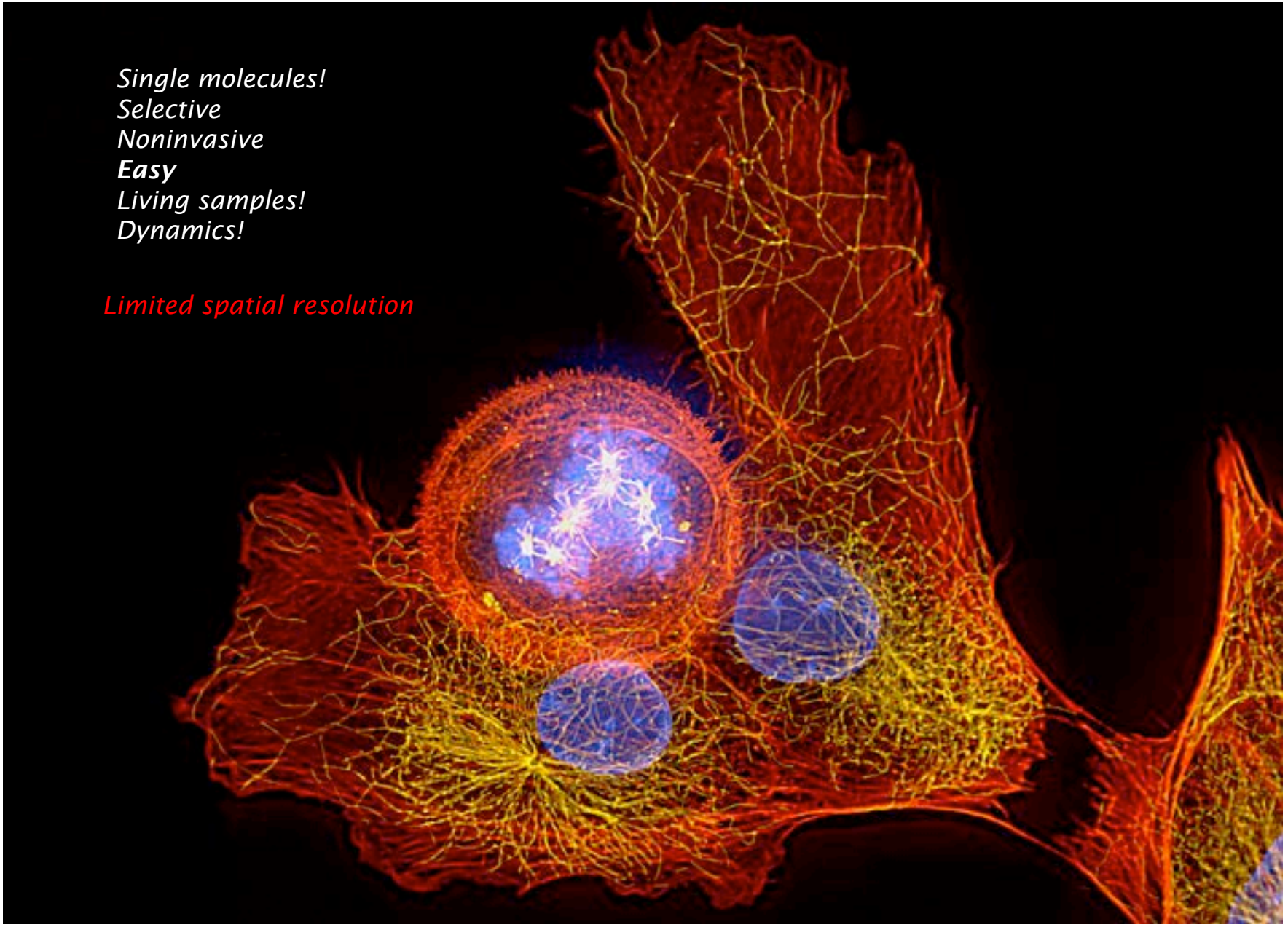
3D Segmentation

Mesh creation

Signal projection

MorphoGraphX: Richard Smith & Pierre Barbier de Reuille

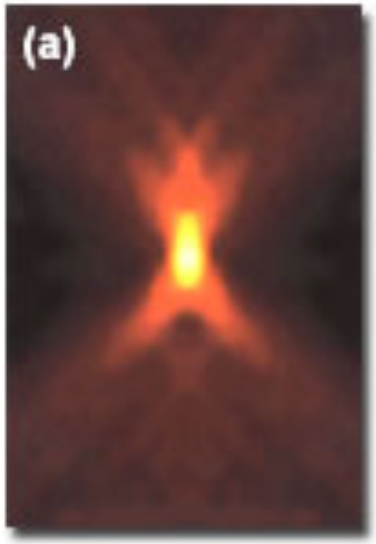
DeRybel, Weijers Science 2014


Single molecules!
Selective
Noninvasive
Easy
Living samples!
Dynamics!
Limited spatial resolution

Resolution in confocal microscopy

	Wide field Epi fluorescence microscopy	Confocal fluorescence microscopy
Lateral	$R_{lat} = 0.61\lambda / NA$	$R_{lat} \approx 0.4\lambda / NA$

Axial Point Spread Function Intensity Profiles



Spatial resolution

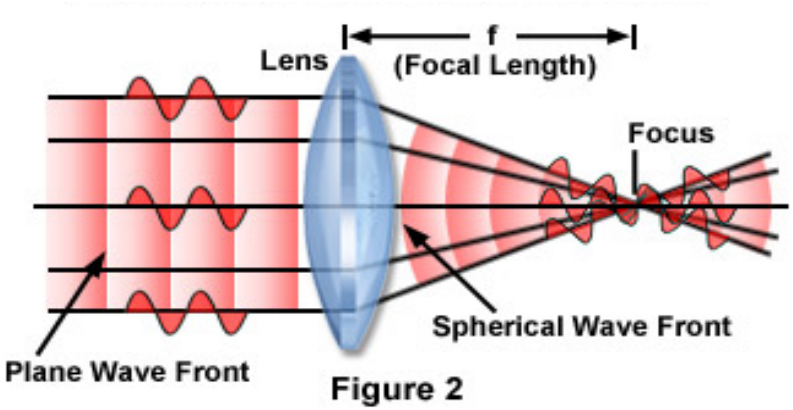
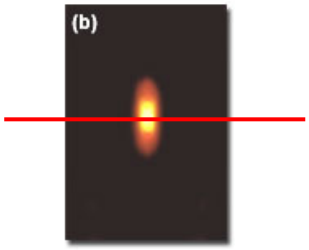
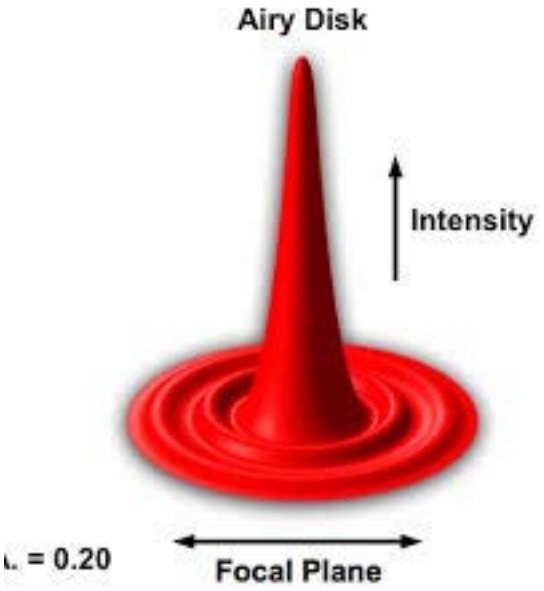


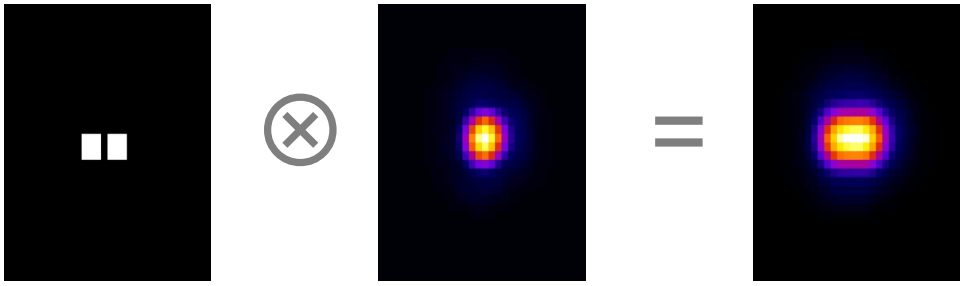
Figure 2





airy pattern formation

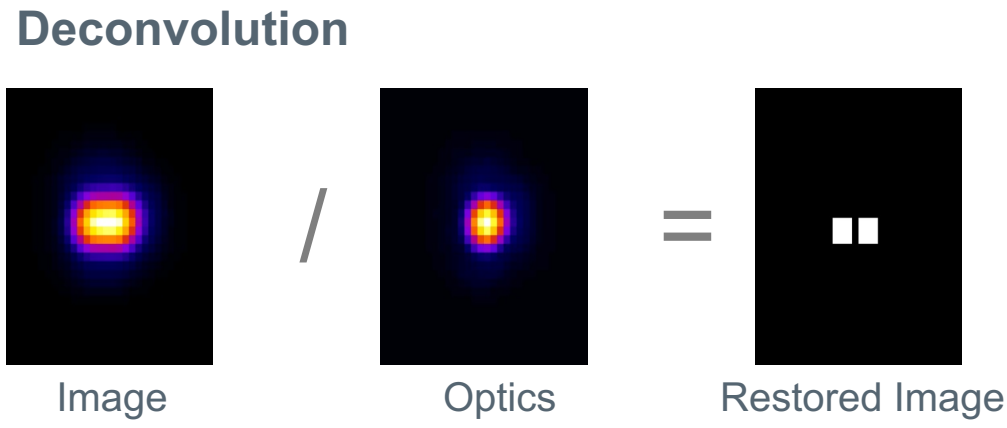
Convolution



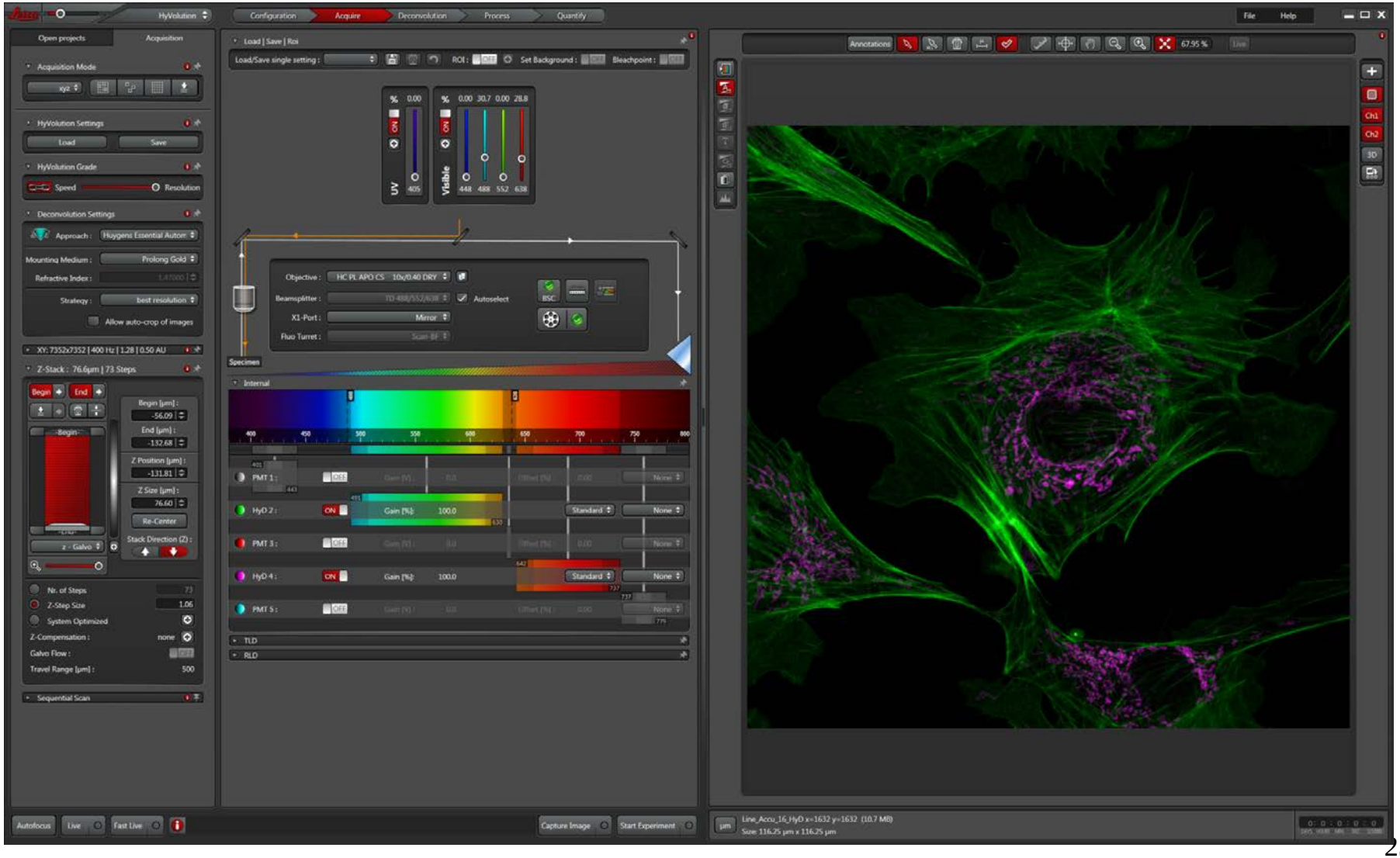
Object Optics (psf) Image

HyVolution2 - Multicolor confocal superresolution

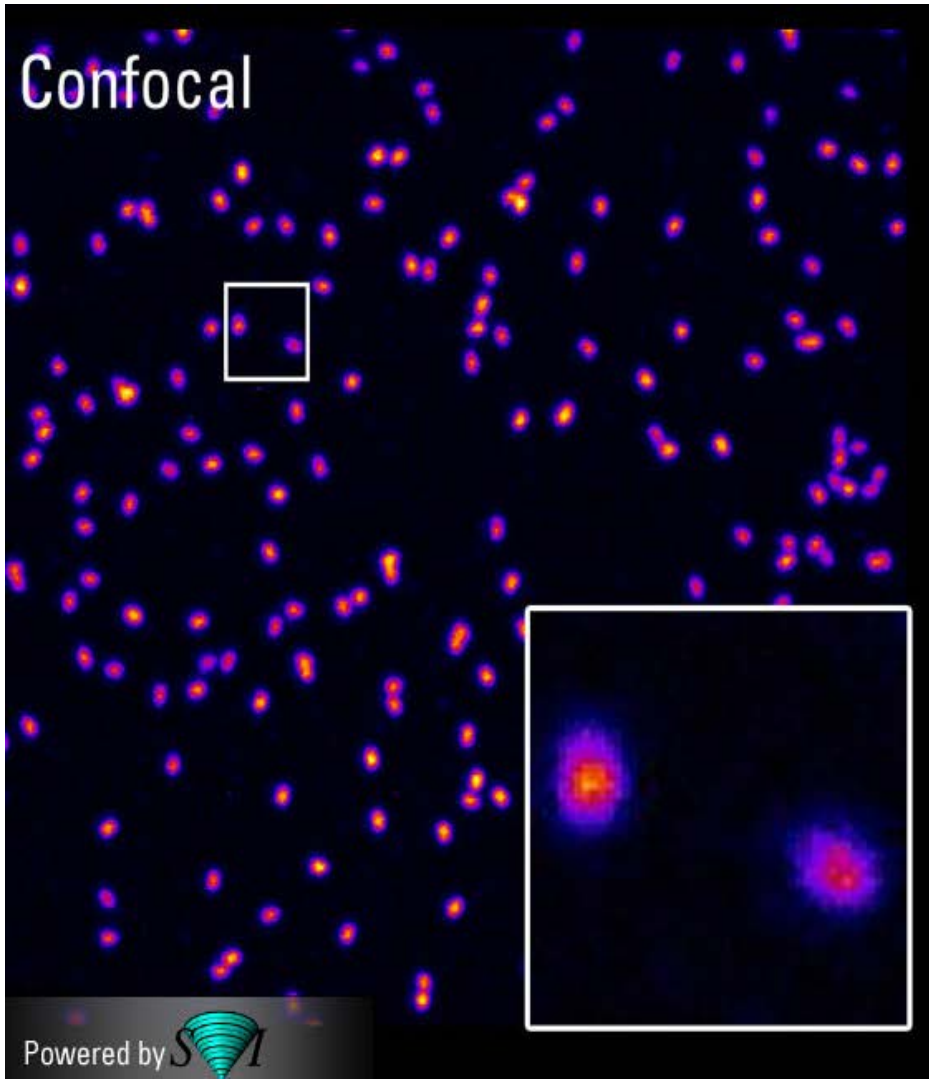
- Requires SP8 photon efficiency + HyD highly sensitive detection with low noise
- HyD make every photon count – best SNR - and XYZ resolution at pinhole 0.6 AU
- Huygens deconvolution by SVI
- GPU (graphics processing unit) accelerated computing with the CUDA high-performance hardware
- LASX HyVolution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



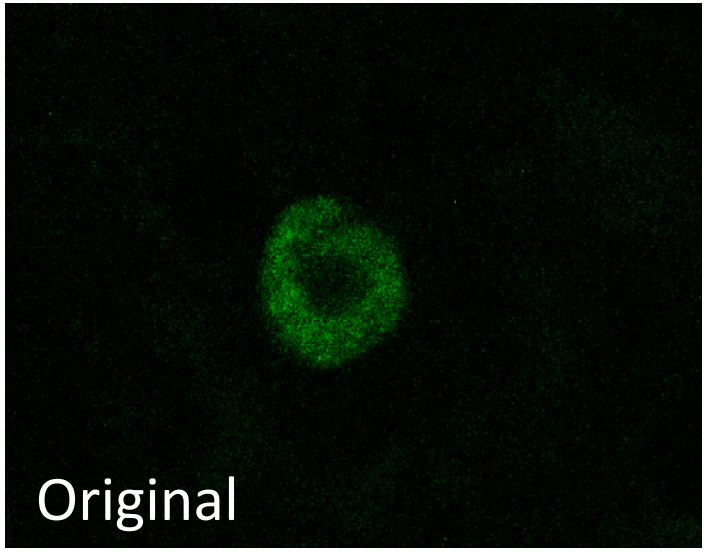
HyVolution2



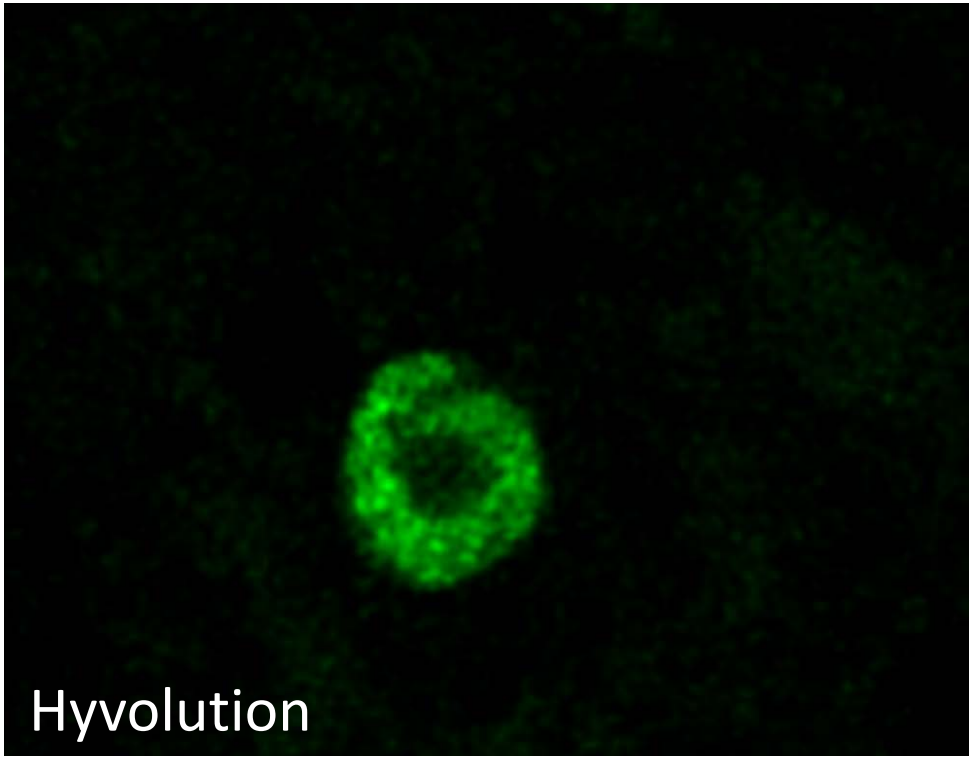
HyVolution2; spatial resolution 140 nm



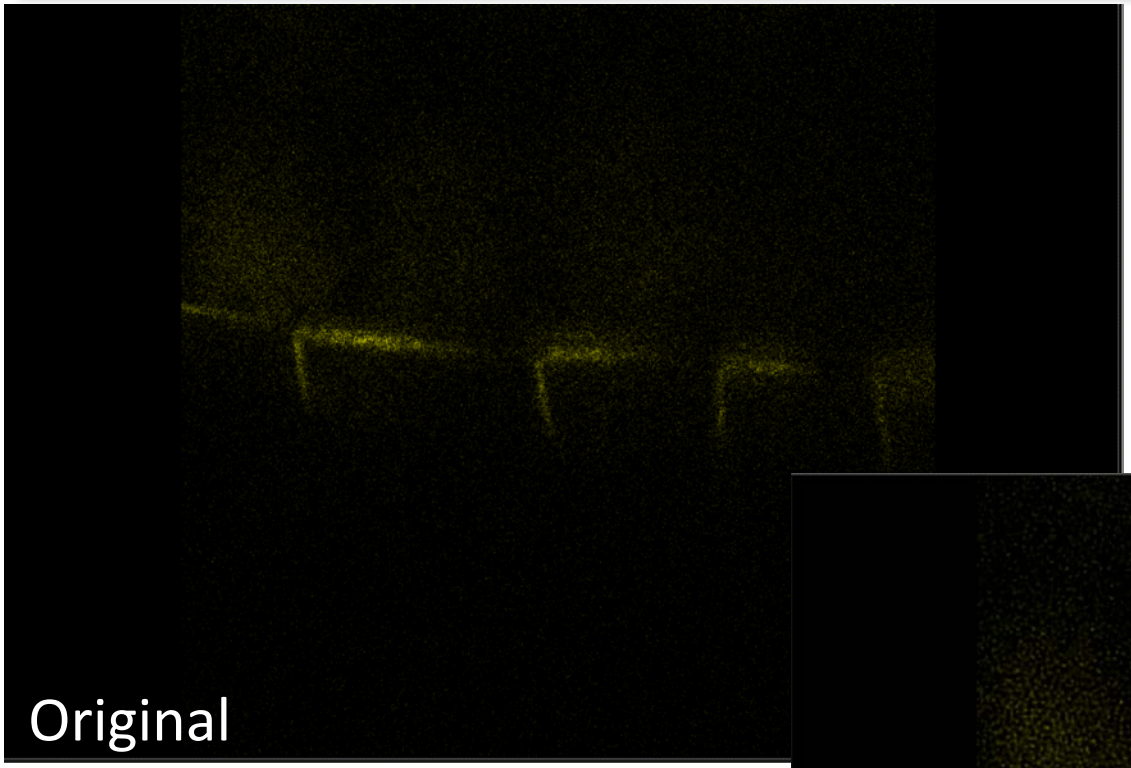
HyVolution2; examples



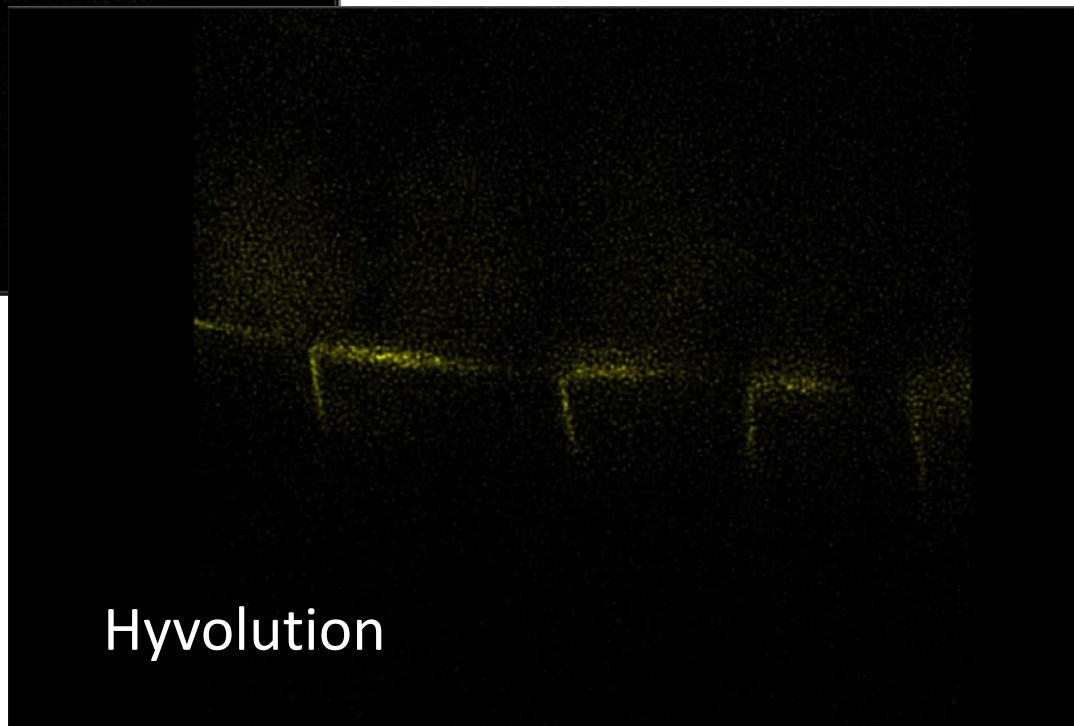
MpARF3-YFP



Hyvolution2; examples

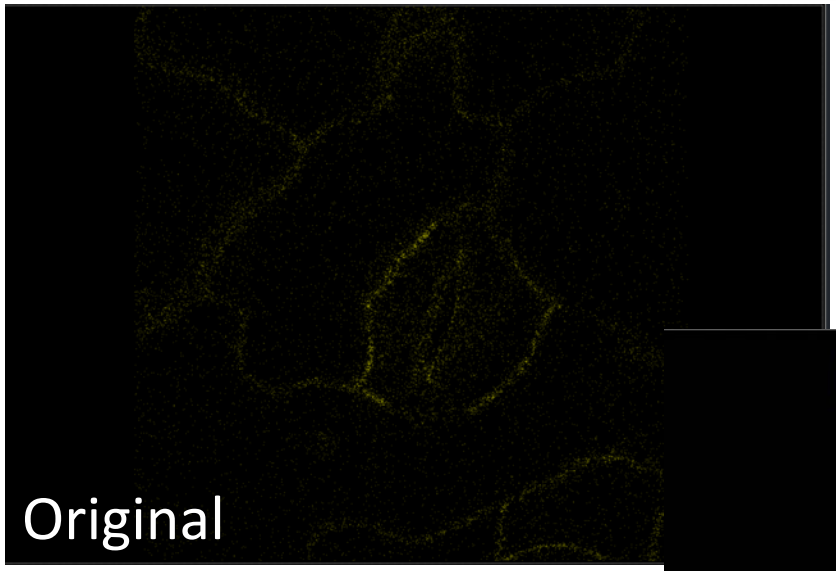


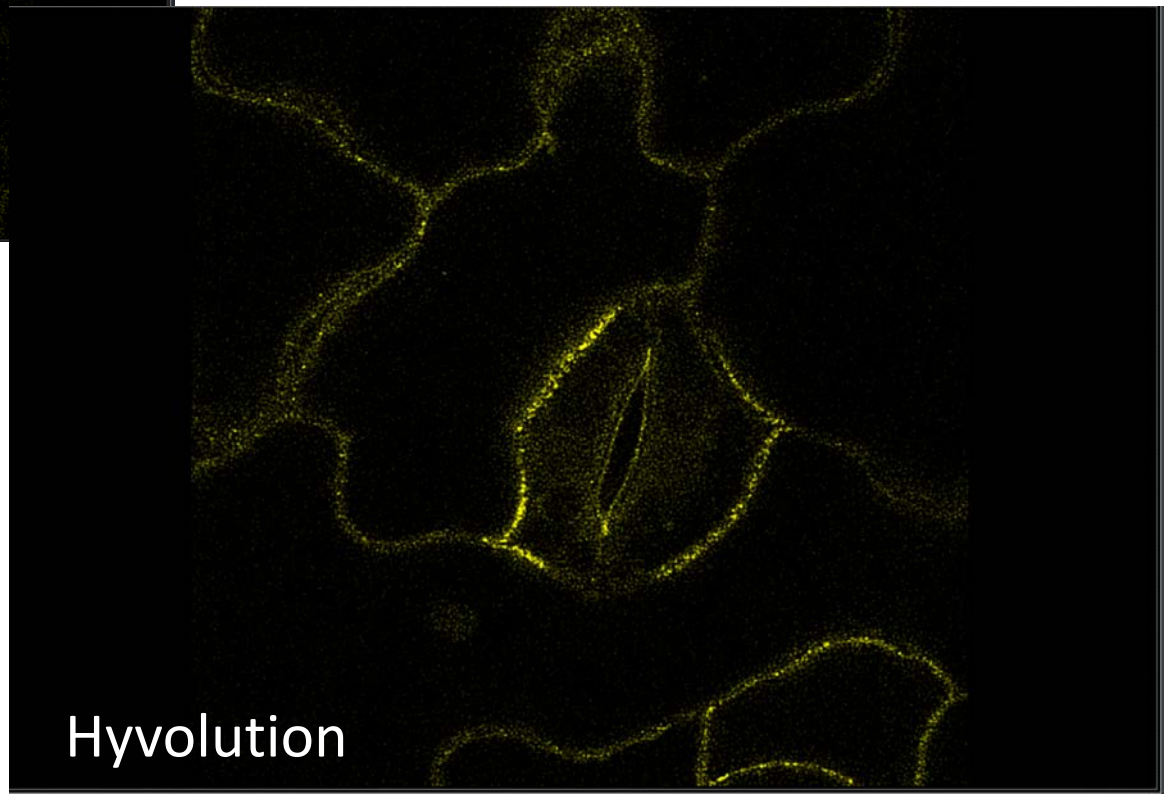
SOK-YFP in roots



Hyvolution2; examples

SOK-YFP in leaves





Other features

- ✓ Time lapse
- ✓ Multi stitching
- ✓ Lambda scan

- ✓ Gating system

- ✓ Multi mode confocal
- ✓ Fluorescence lifetime imaging
- ✓ Fluorescence (cross) correlation (micro)spectroscopy

Acknowledgements

Laboratory of Biochemistry

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Jos Wendrich
Dolf Weijers

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Arjen Bader
Johannes Hohlbein
Herbert van Amerongen

Micro-Spectroscopy

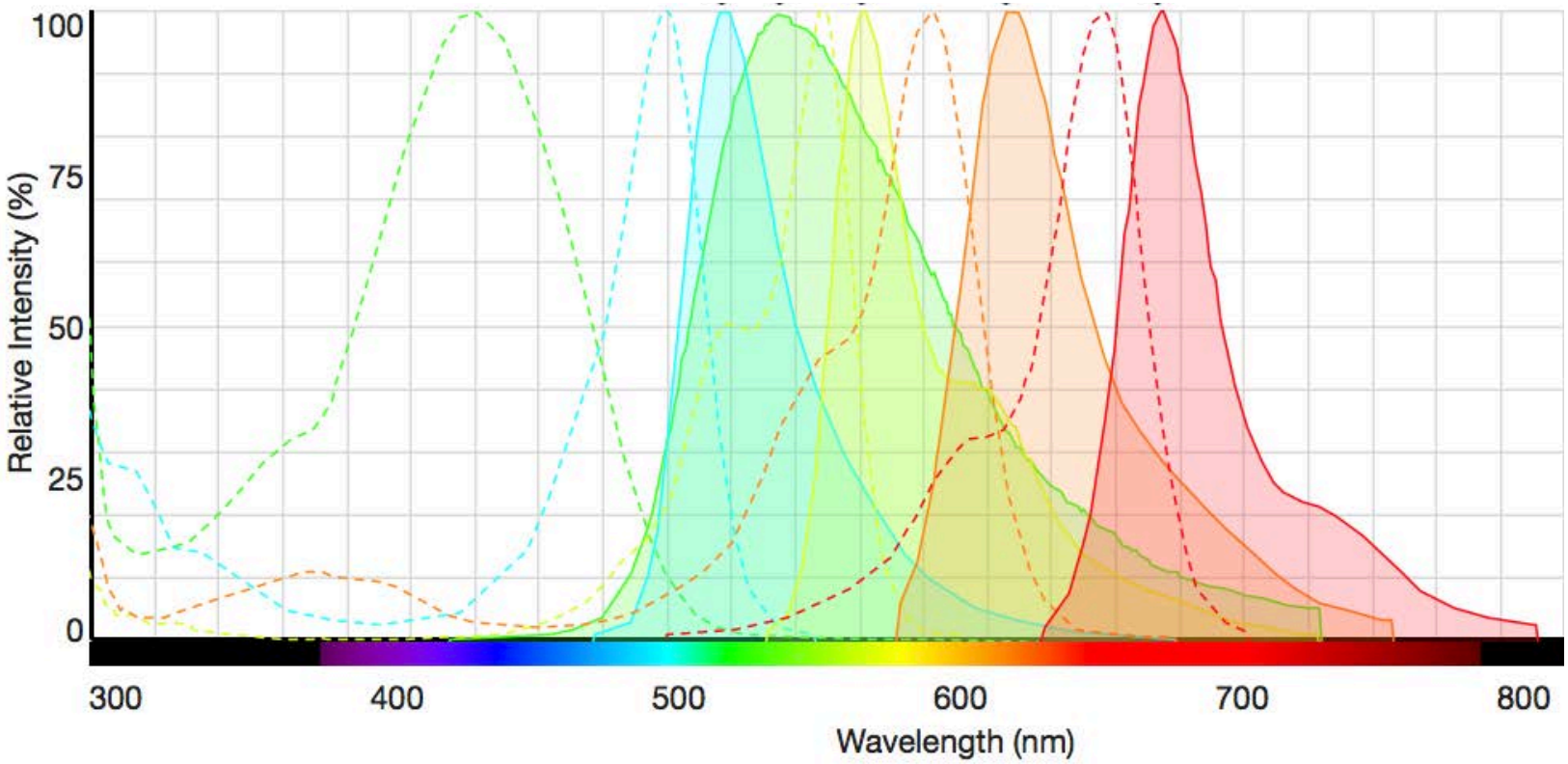
Application of fluorescence spectroscopy on microscopic objects

Fluorescence in biological/applied sciences?

+ Sensitive
+ Specific
+ Multiparameter
+ Living cell
+ Dynamics

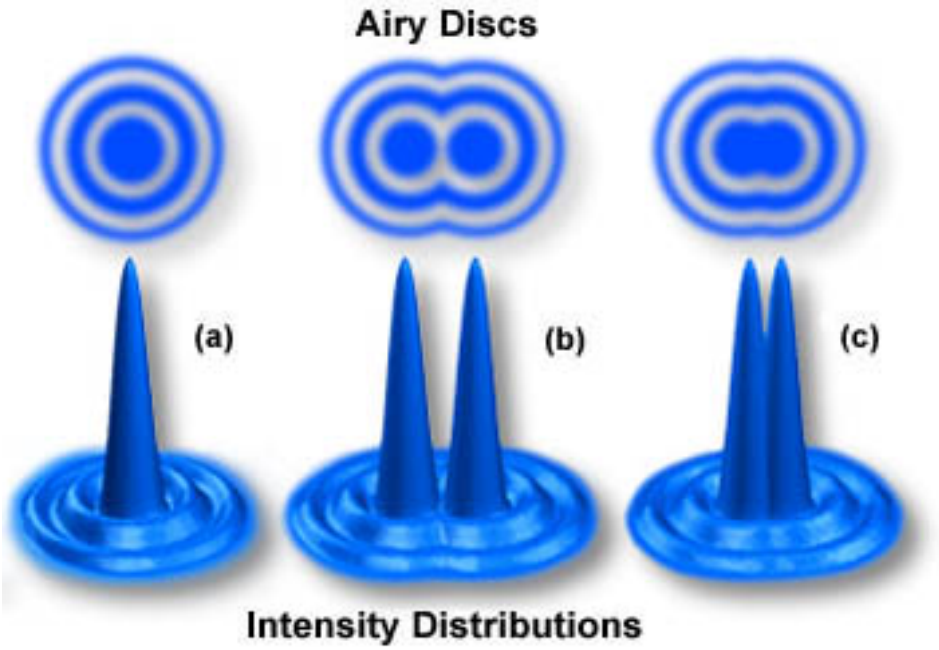
Excitation and emission of fluorophores

Fluorescence absorption/emission spectra



Spatial resolution

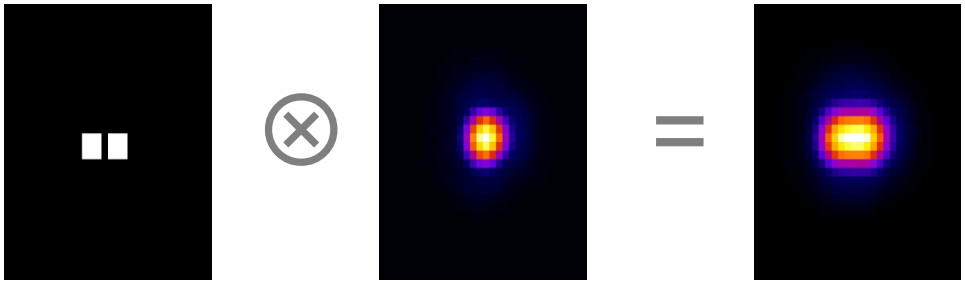
The Rayleigh criterion, defined as the minimum resolvable detail, when the first diffraction minimum of the image of one source point coincides with the maximum of another (C).



Convolution and Deconvolution

Convolution of 2 objects with PSF

Convolution



Deconvolution

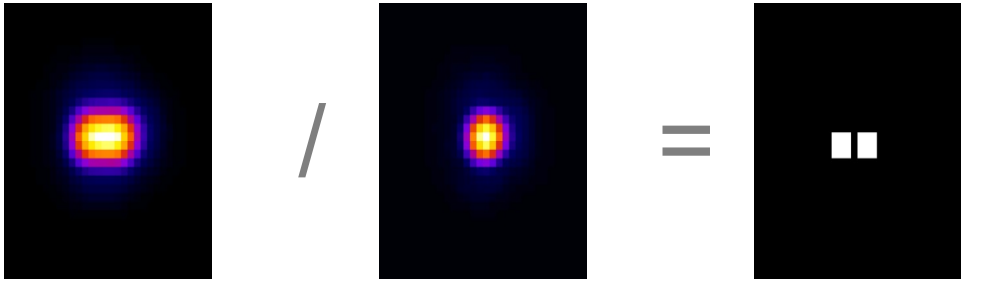


Image Optics Restored Image