CEITEC

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S5015

Light microscopy methods in biology

Lecture 2: Confocal and lightsheet microscopy

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S5015 : Light microscopy methods in biology

Lecture 1: September 30, 2021 at 9:00

History of microscopy, fundamentals of light microscopy. Contrasting techniques in transmitted light microscopy – brightfield, darkfield, phase contrast, DIC.
Widefield fluorescence microscopy - deconvolution, point spread function, point spread function measurements, description.
Detectors in widefield microscopy, filters, light sources.

Lecture 2: October 14, 2021 at 9:00

Confocal microscopy - principles, types of confocal microscopes – scanning, spinning disc, channel vs spectral imaging, linear unmixing.
Lasers, detectors, beam splitters, optical sections. Lightsheet microscopy.

Lecture 3: October 25, 2021 at 9:00

Imaging below diffraction limits – SIM, SMLM, STED

Lecture 4: November 4, 2021 at 9:00

Basics of Image analysis – image types, pixels, voxels, basic filters, image processing, metadata, deconvolution, 3D visualization, objects detection, quantification.

Practical 1: December 14, 2021 at 9:00

Transmitted light microscopy, widefield fluorescence, confocal, Apotome, SIM

Practical 2: December 15, 2021 at 9:00

Sample preparation, single molecule localization microscopy acquisition and analysis, deconvolution.

Widefield fluorescence imaging

+ fast and photo efficient
+ sensitive detectors – sCMOS with 95%QE,
+ variable LED sources
+ easy and accessible technique of imaging
+ relatively cheap instruments

− **blurred images due to out of focus light**

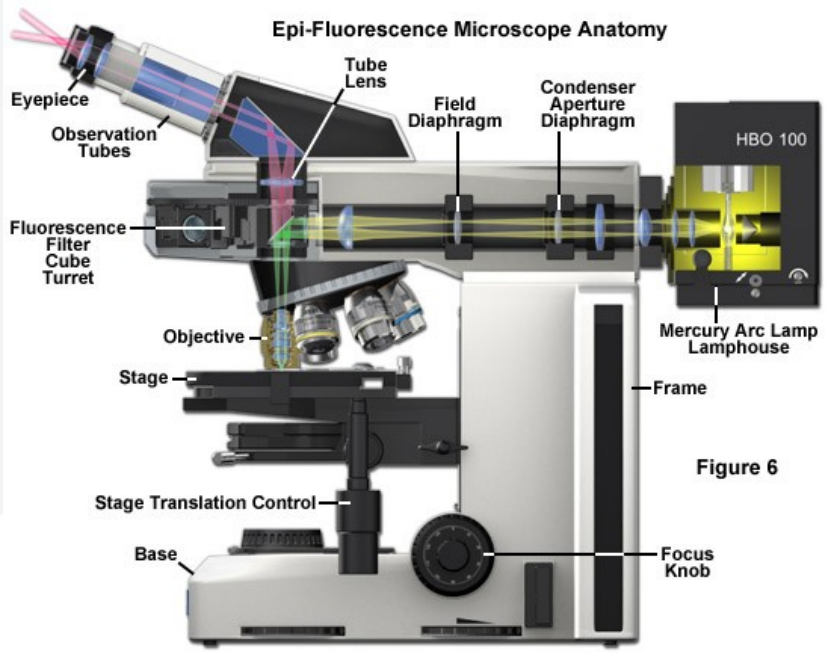
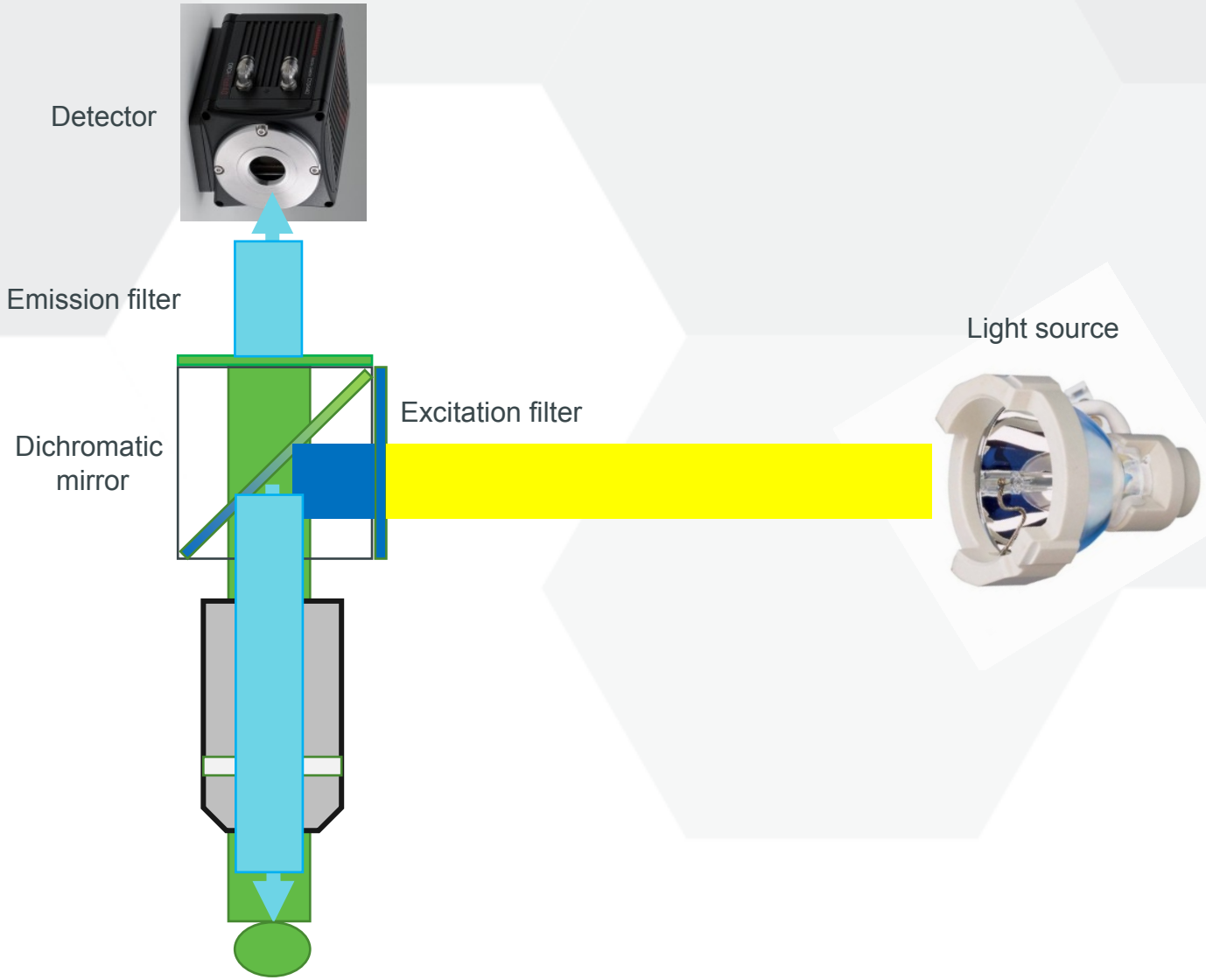
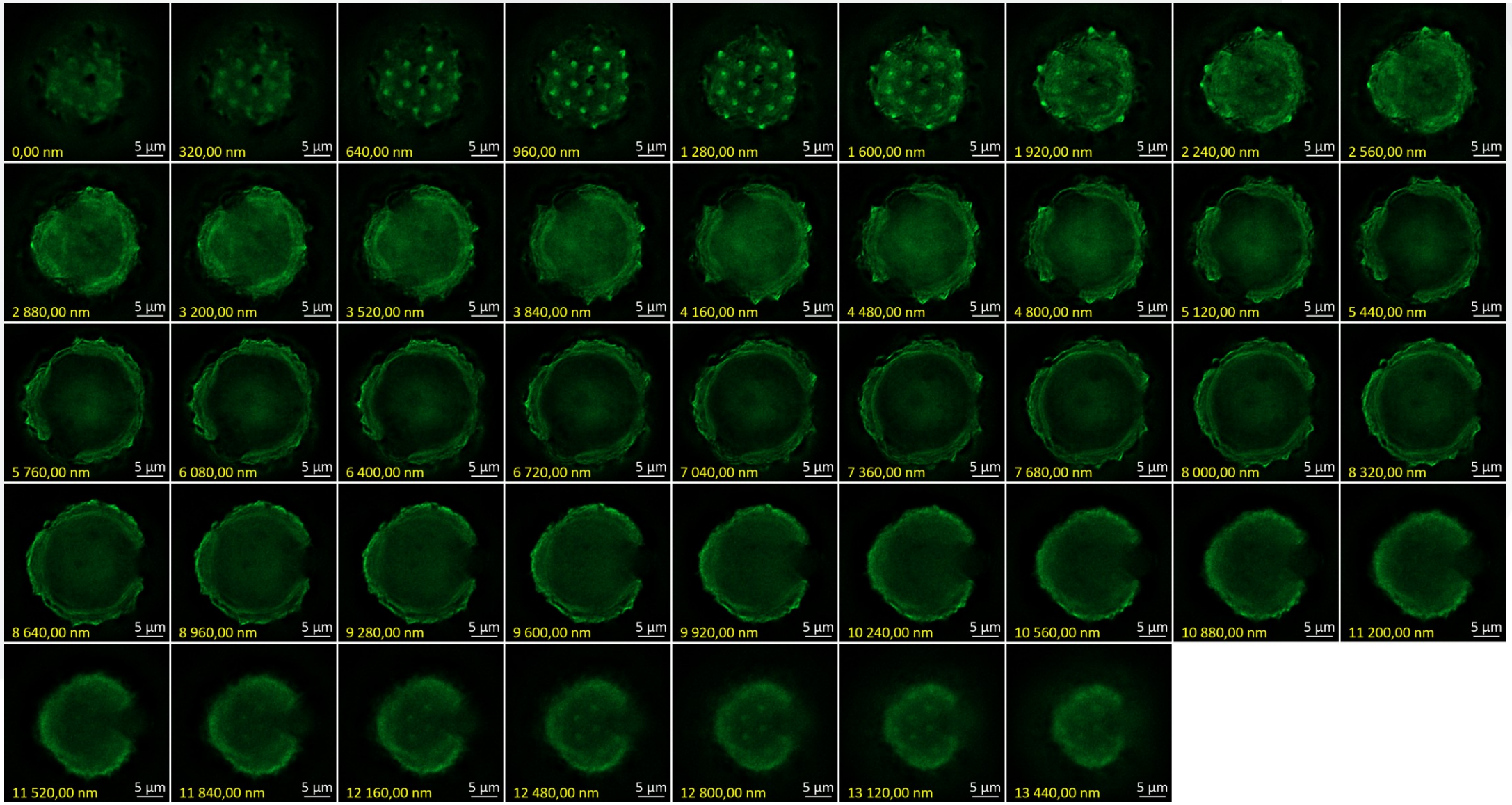


Figure 6



Confocal microscopy

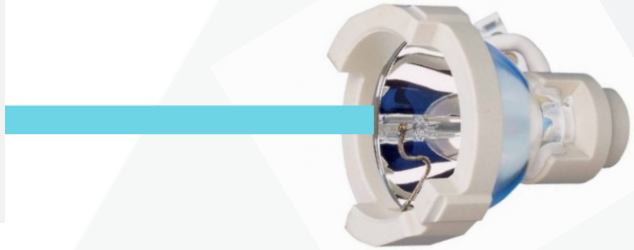


Confocal microscopy

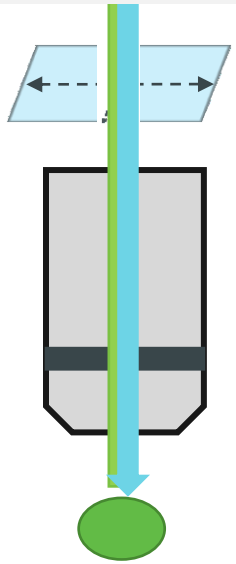
Pinhole

confocal pinhole

- Pinhole physically blocking out of focuse light
- Define optical thickness of scan
- Diameter in Airy units, depends on wavelengths and NA
- Impact resolution
- Multicolor imaging - same opening produce different optical thickness
- Adjustable for each dye in point scanning microscopes
- Spinning disc confocals have fixed pinhole diameter

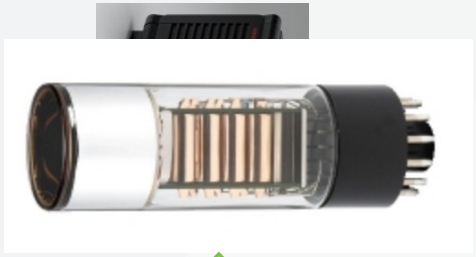


Coherent light source
lasers

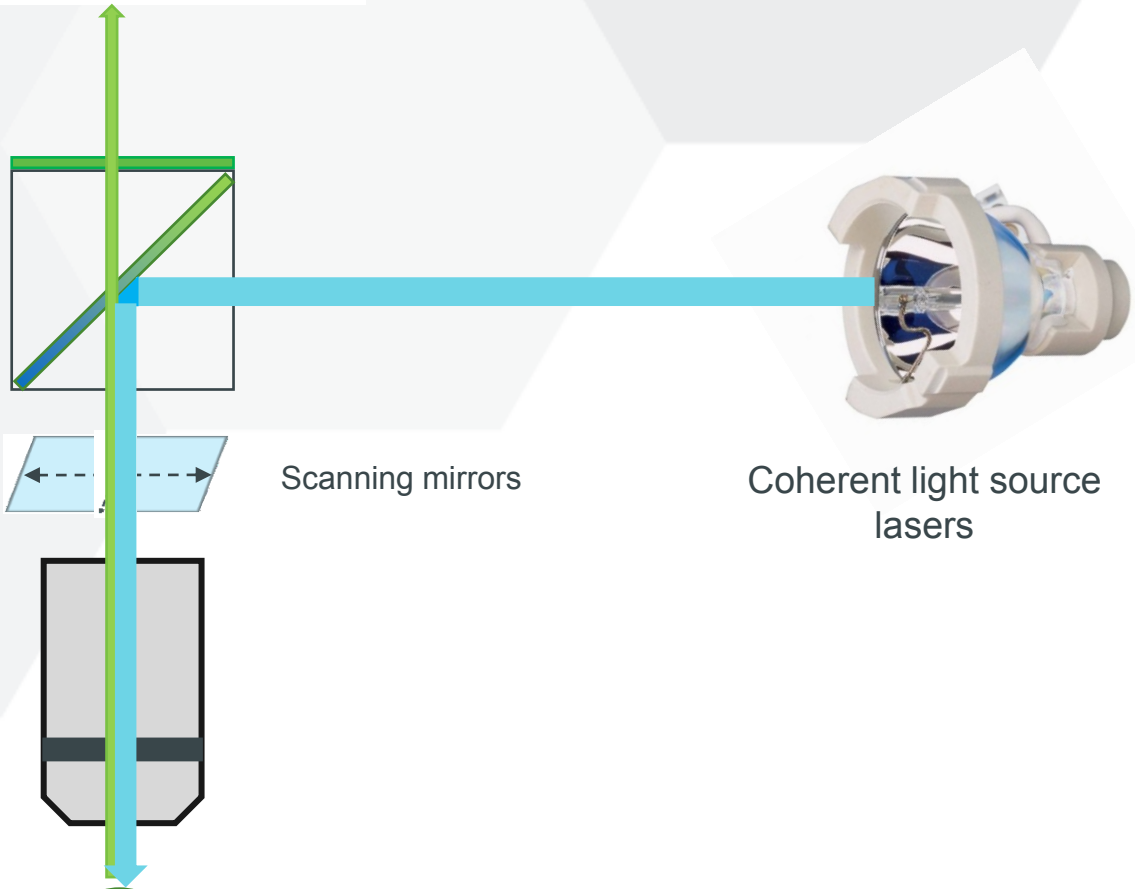


Scanning mirrors

Point scanning confocal microscopy

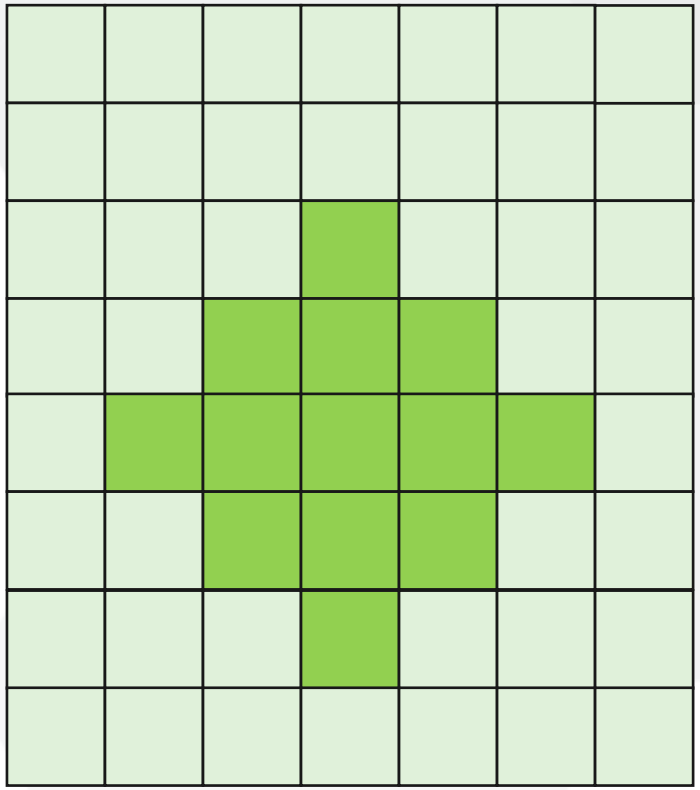


confocal pinhole



Scanning mirrors

Coherent light source
lasers



Nyquist-Shannon criterion

Pixel size should be at least 2.3 times smaller than actual optical resolution of the system.

Spinning disc confocal microscopy

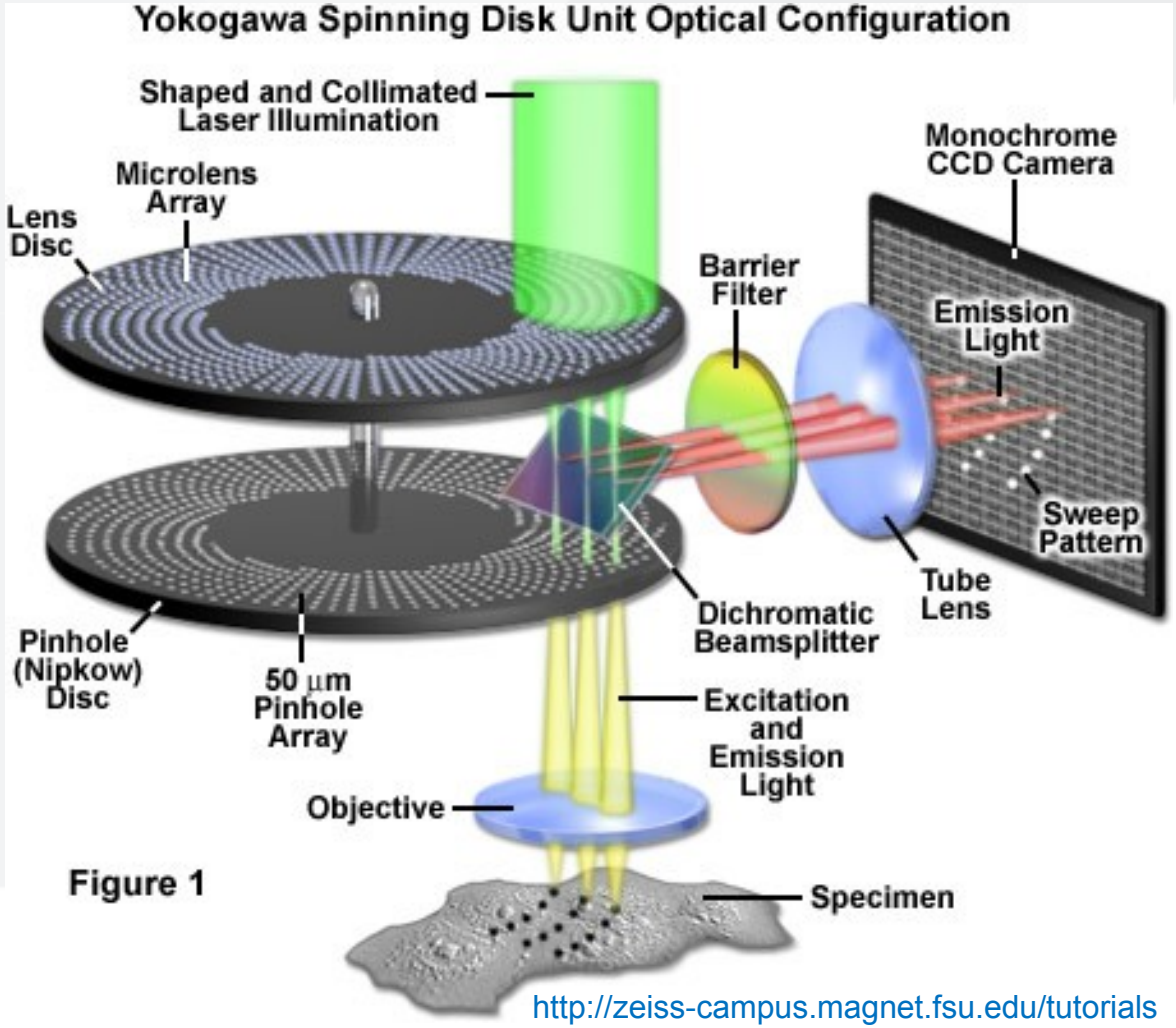


Figure 1

http://zeiss-campus.magnet.fsu.edu/tutorials

+ optical sectioning
+ fast

- pinholes optimized for one objective
- (interchangeable discs)
- sensitive to dust

Resolution

Only slightly increased resolution compare to widefield system
Main advantage – increase contrast and optical sections

Lateral resolution

Conventional microscope

$$d = 0.61 * \frac{\lambda_{emm}}{NA}$$

Confocal microscope
1 AU pinhole

$$d = 0.61 * \frac{\lambda_{exc}}{NA}$$

Confocal microscope
0.25 AU pinhole

$$d = 0.37 * \frac{\lambda_{exc}}{NA}$$

Axial resolution

Conventional microscope

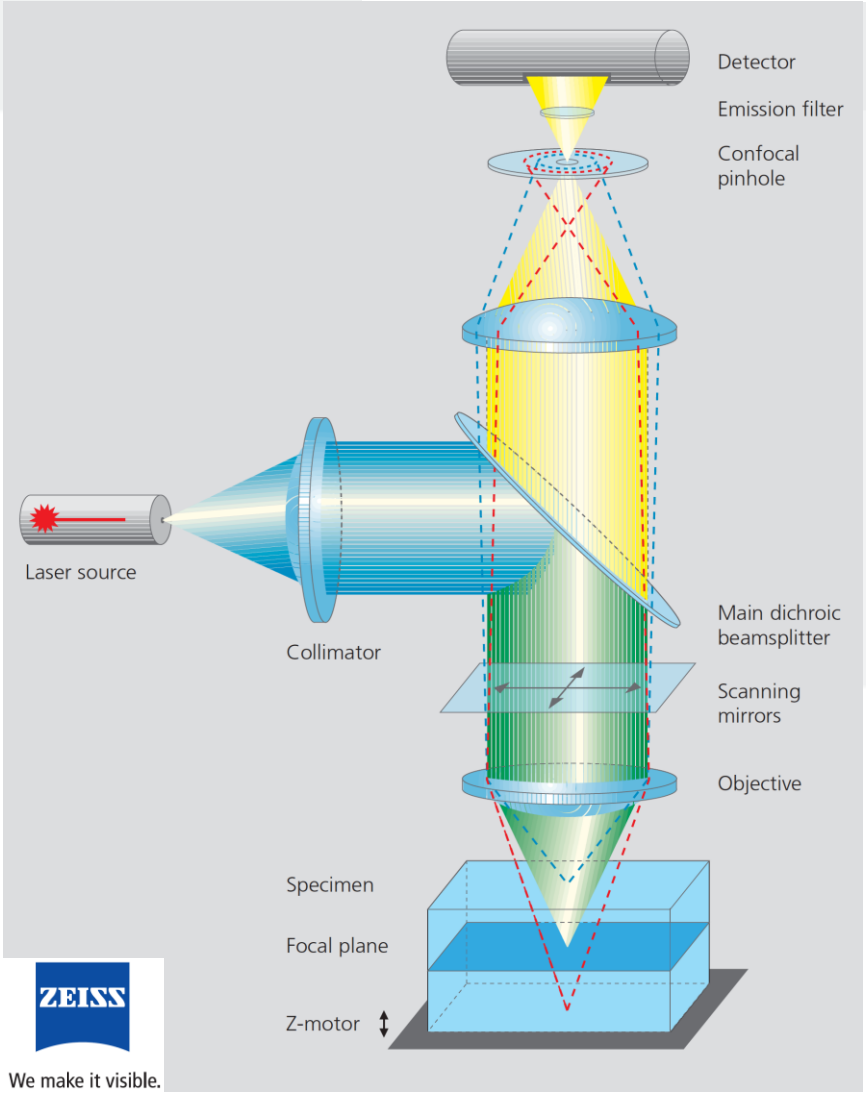
$$d = \frac{n * \lambda_{emm}}{NA^2}$$

Confocal microscope
1 AU pinhole

$$d = \frac{0.88 * \lambda_{exc}}{(n - \sqrt{n^2 - NA^2})}$$

Confocal microscope
0.25 AU pinhole

$$d = \frac{0.64 * \lambda_{exc}}{(n - \sqrt{n^2 - NA^2})}$$



Light source

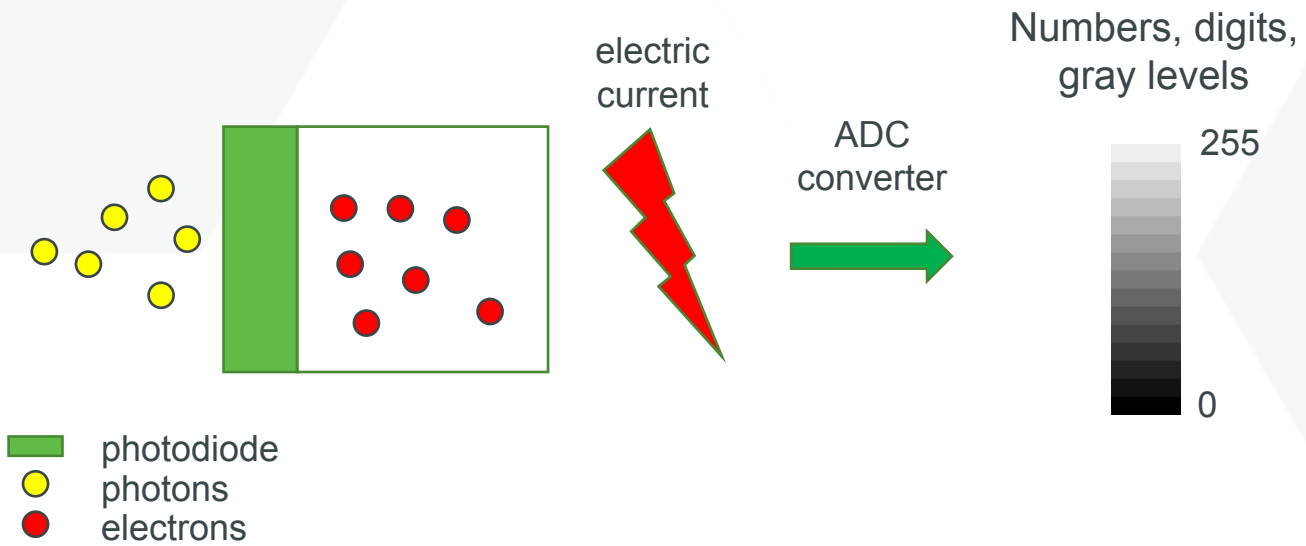
- lasers polarized, coherent, monochromatic, collimated
- Cw – constant wave lasers, mW range
- 405, 488, 561, 640 – standard wavelengths
- 350, 458, 514 and others
- Argon laser source (458, 488, 514) – outdated
- Diode lasers most often
- White laser tunable
- Pulsed lasers, cw lasers
- Lasers are combined together by laser combiner
- Dichromatic beam splitter, or AOBS

Detectors

Capture light emitted from sample ->

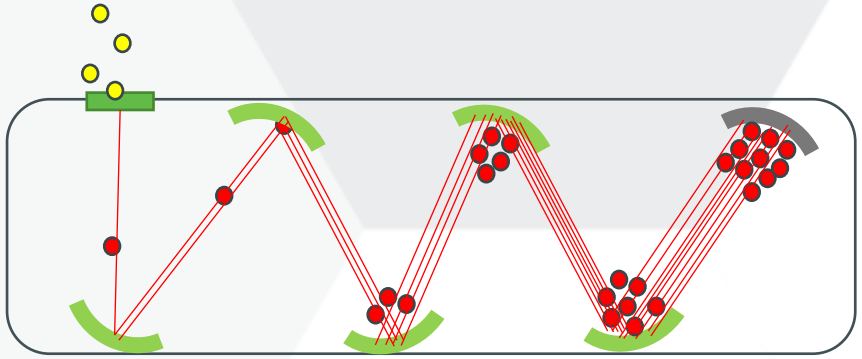
Point scanning confocal microscopes: Photon Multiplying Tube, Avalanche PhotoDiode detectors

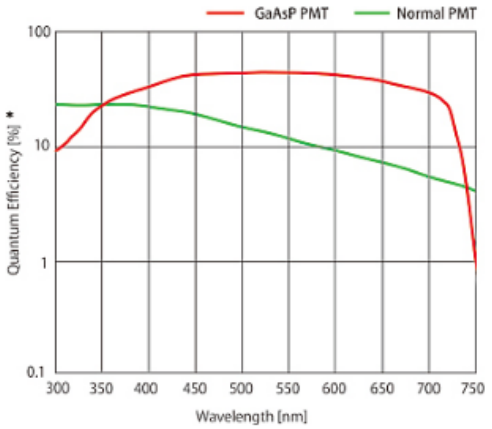
Widefield, spinning disc microscopes: CCD, EM-CCD, sCMOS sensors



Detectors

Photomultiplier tube detector (PMT)





photocathode
photons
electrons

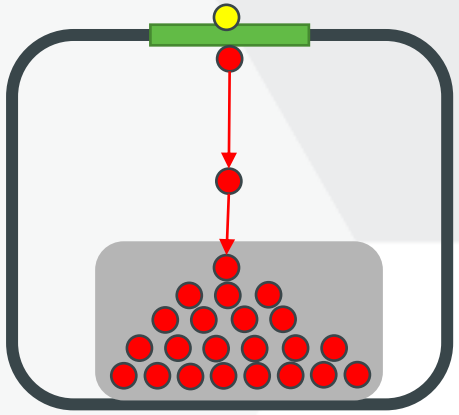
dynode (electrodes)

anode

Photocathode determine QE and spectral sensitivity.
GaAsP (Galium, Arsenid, Phosphid) are more sensitive then standard multialkali PMTs, but are sensitive to overexposure. QE up to 50% in 550 nm.

Detectors

Avalanche photodiode detector (APD) – hybrid detectors (Leica)



Avalanche diode

GaAsP (Galium, Arsenid, Phosphid), but are sensitive to overexposure. QE up to 50% in 550 nm.

GaAsP photocathode
photons
electrons
avalanche photodiode

Detectors

CCD and CMOS devices

CCD – Charge-Coupled Device
Pixels read out by line (interline CCD). Single output node and ADC, lower frame rate.
Consume more electricity.

CMOS – Complementary Metal-Oxide-Semiconductor
Faster read out then CCD, each pixels read out individually. Each row has its own output node with ADC.
More noise compare to CCD, but faster fps. Major improvements in last years.

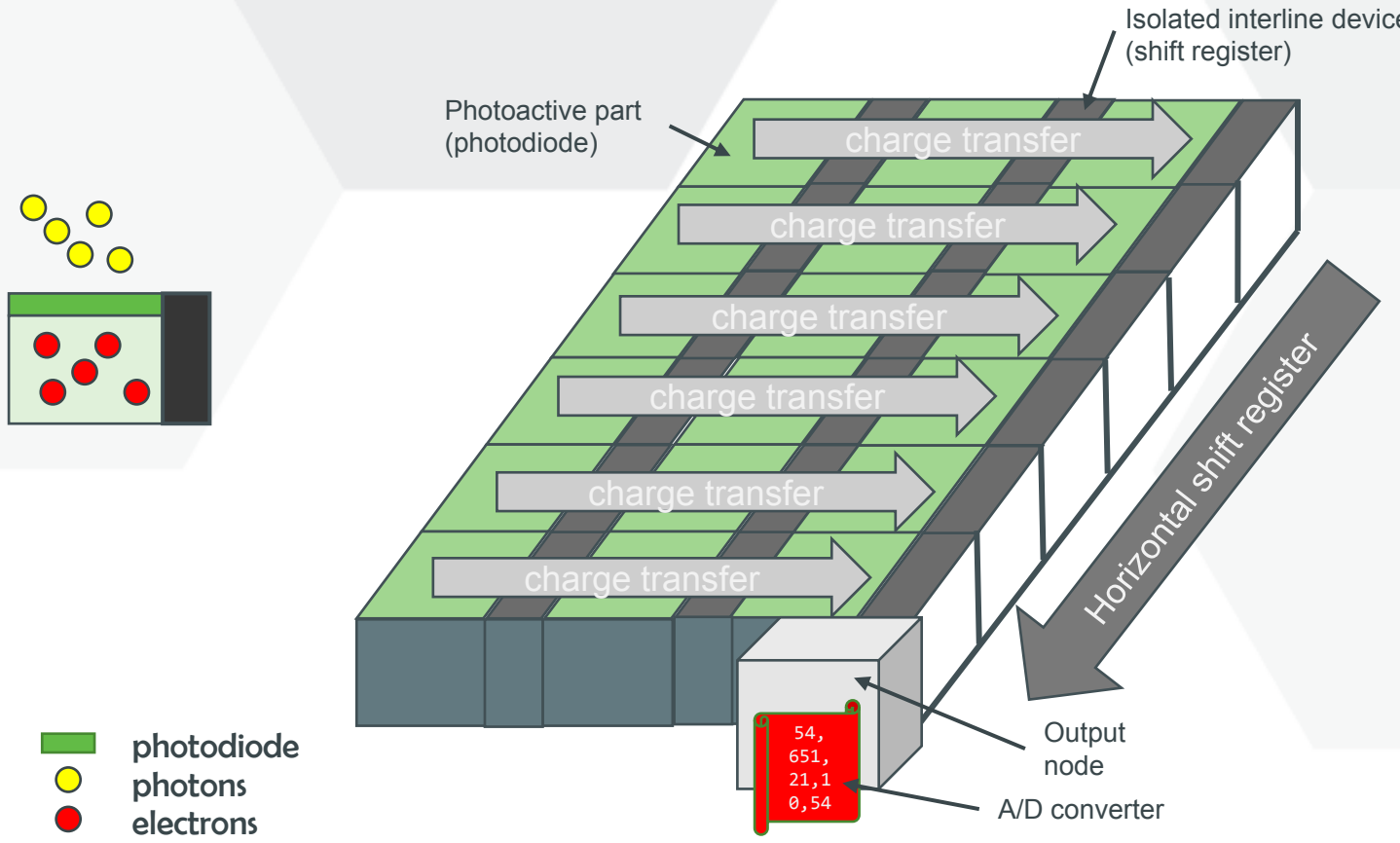
Photoactive part releasing electrons and generating charge, which is converted into a voltage.
Analog signal (voltage) is converted to discrete signal (numbers).
Array of multiple units (pixels).

HAMAMATSU
PHOTON IS OUR BUSINESS

Detectors

CCD sensor

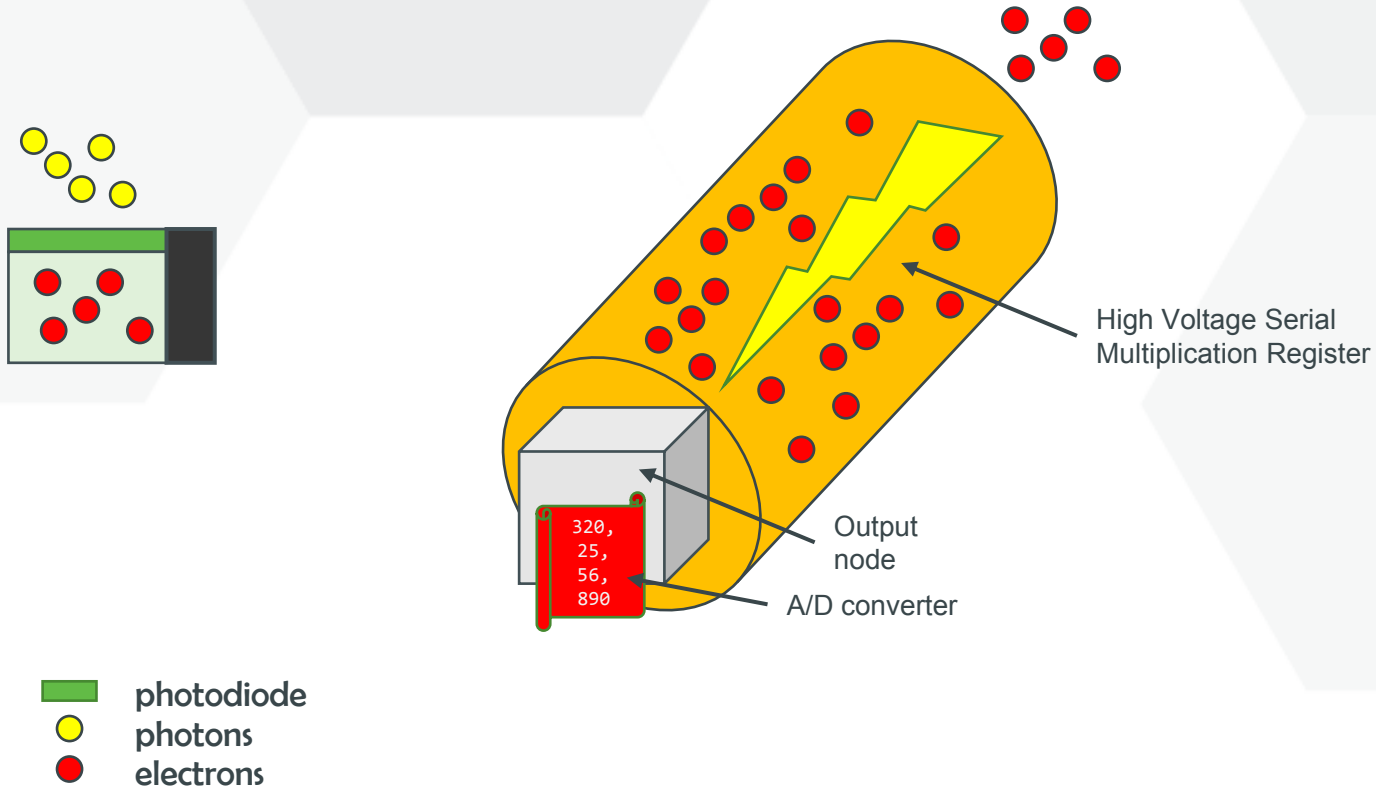
Pixel charge fill to neighborhood pixels horizontally and vertically (interline CCD)



Detectors

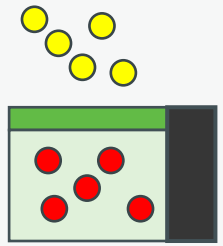
EM-CCD sensor

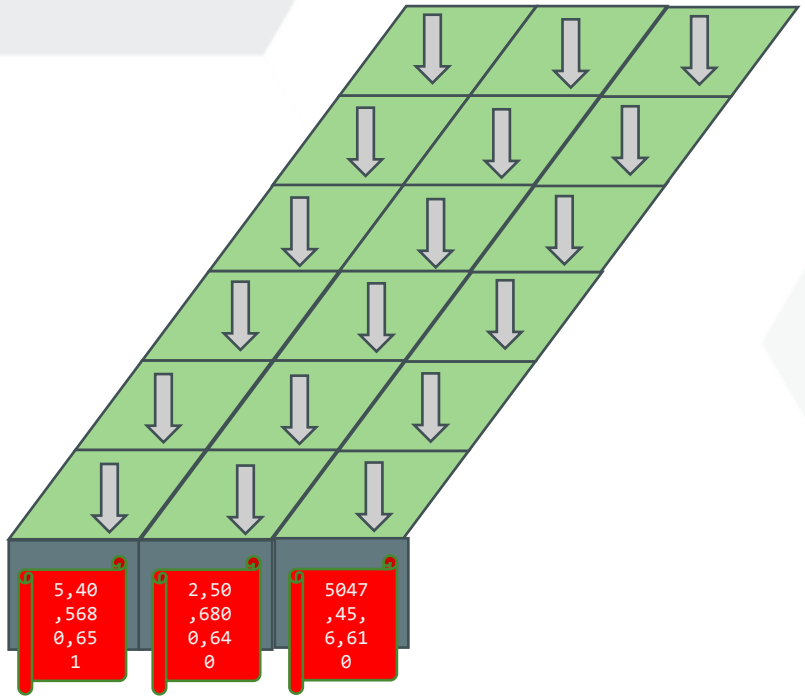
Electron multiplying charge-coupled device – on-chip amplification
Advantage - Extremely sensitive, near 100% QE
Disadvantage – low pixel number, big pixel size



Detectors

CMOS sensor





photodiode
photons
electrons

NOISE

Hamamtsu ORCA Fusion

Photon (shot) noise – most important part of the noise, caused by quantum nature of light (photons).

– follow Poisson distribution, SD = $\sqrt{photon\ count}$

Dark current noise – randomly generated electrons without photons (thermally generated electrons). Depends on temperature.

Read noise – several sources, errors during quantification of electrons to current (voltage) and subsequent analog-digital (A/D) conversion, higher at faster pixel read-out rates.

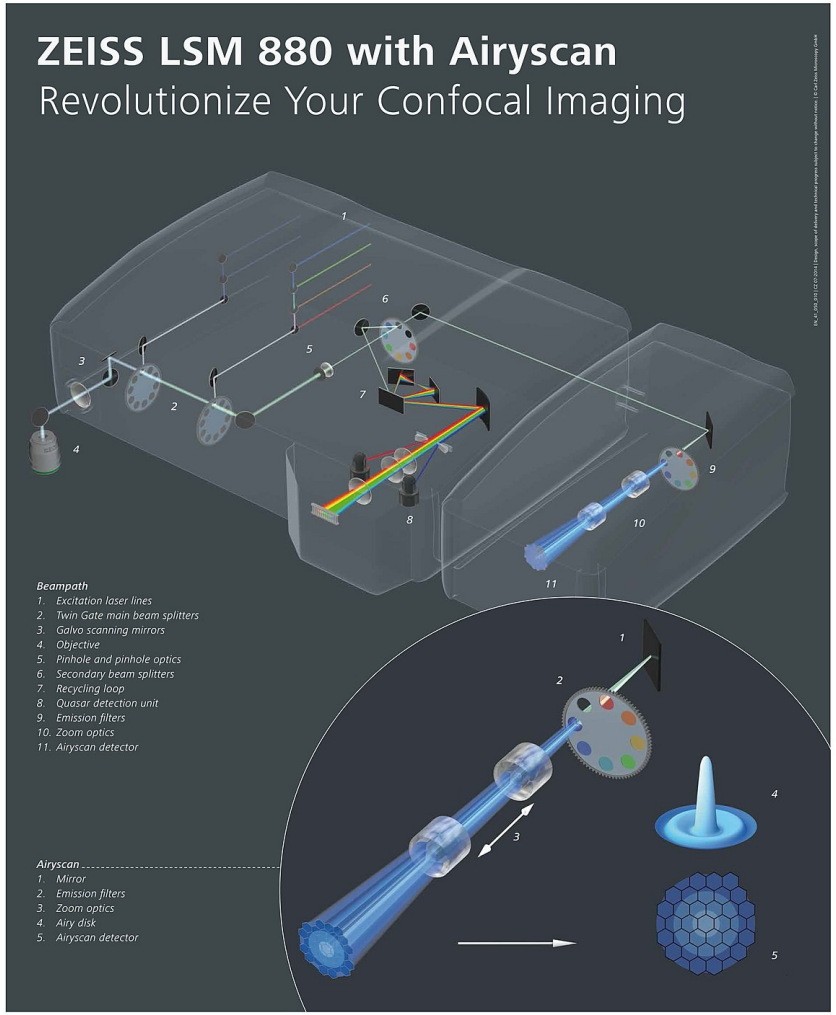
Fast scan: 1.4 electrons rms *1
Standard scan: 1.0 electrons rms *1
Ultra-quiet scan: 0.7 electrons rms *1

$$\text{Noise} = \sqrt{(photon\ noise)^2+(dark\ noise)^2+(read\ noise)^2}$$

Confocal image acquisition

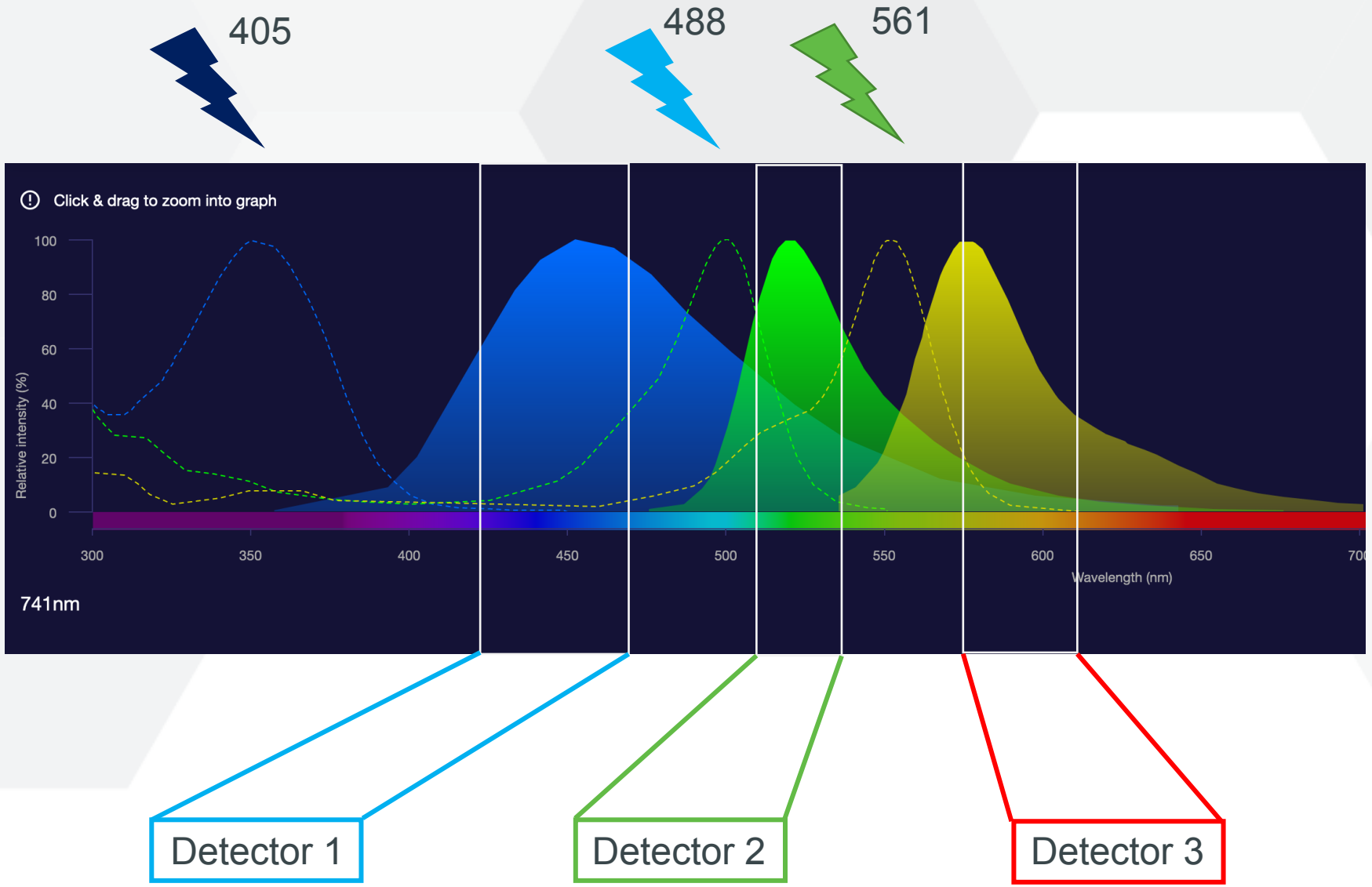
Confocal microscope have usually multiple detectors for fluorescence and one detector for transmitted light.
Based on fluorochromes we can acquire multicolor image in different ways

- Sequential scanning with interchangeable hardware setting between tracks
- Sequential scanning without interchangeable setting hardware between tracks
- Simultaneouse scanning
- Lambda scanning



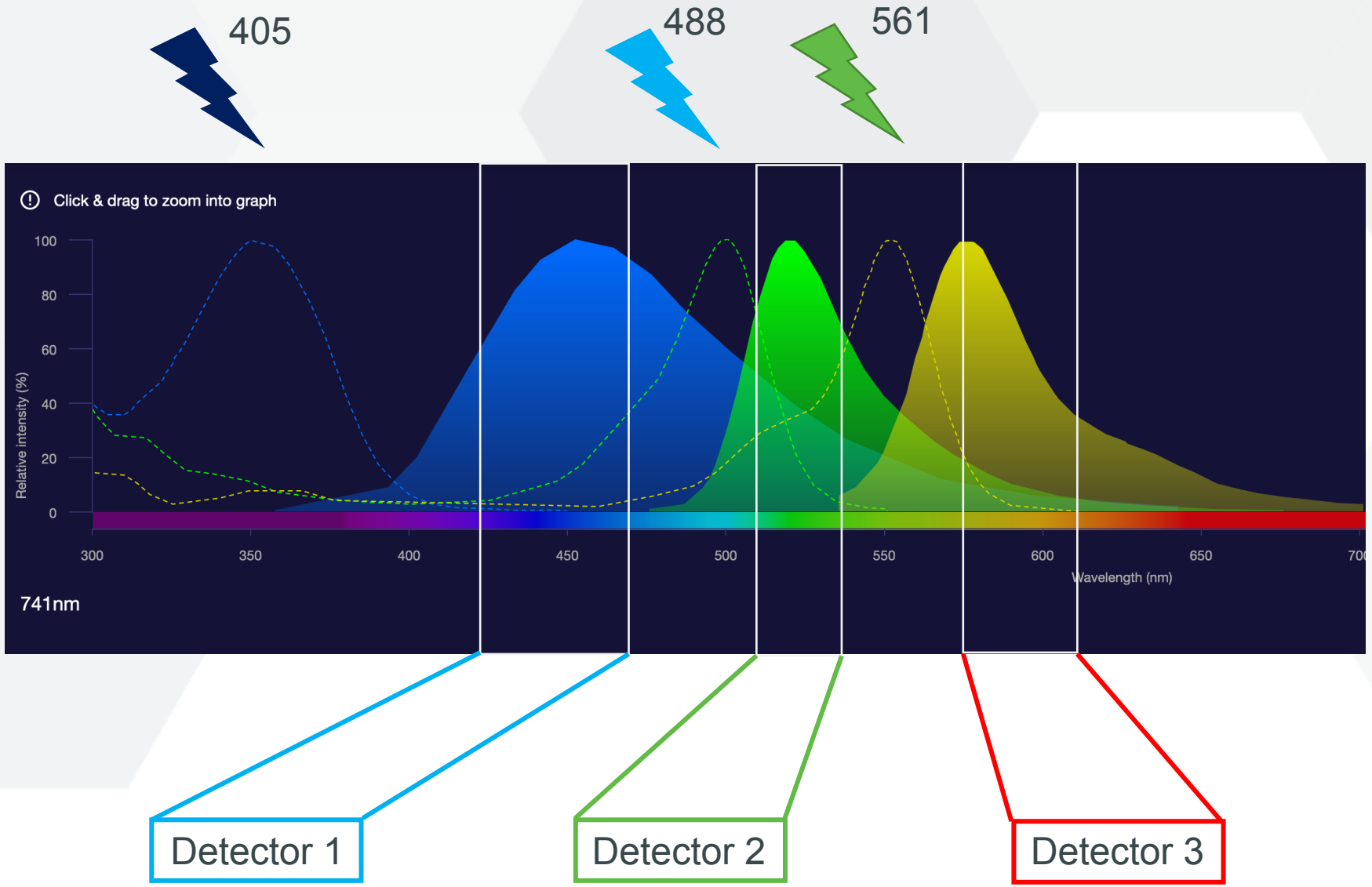
Channel imaging

Sequential acquisition



Channel imaging

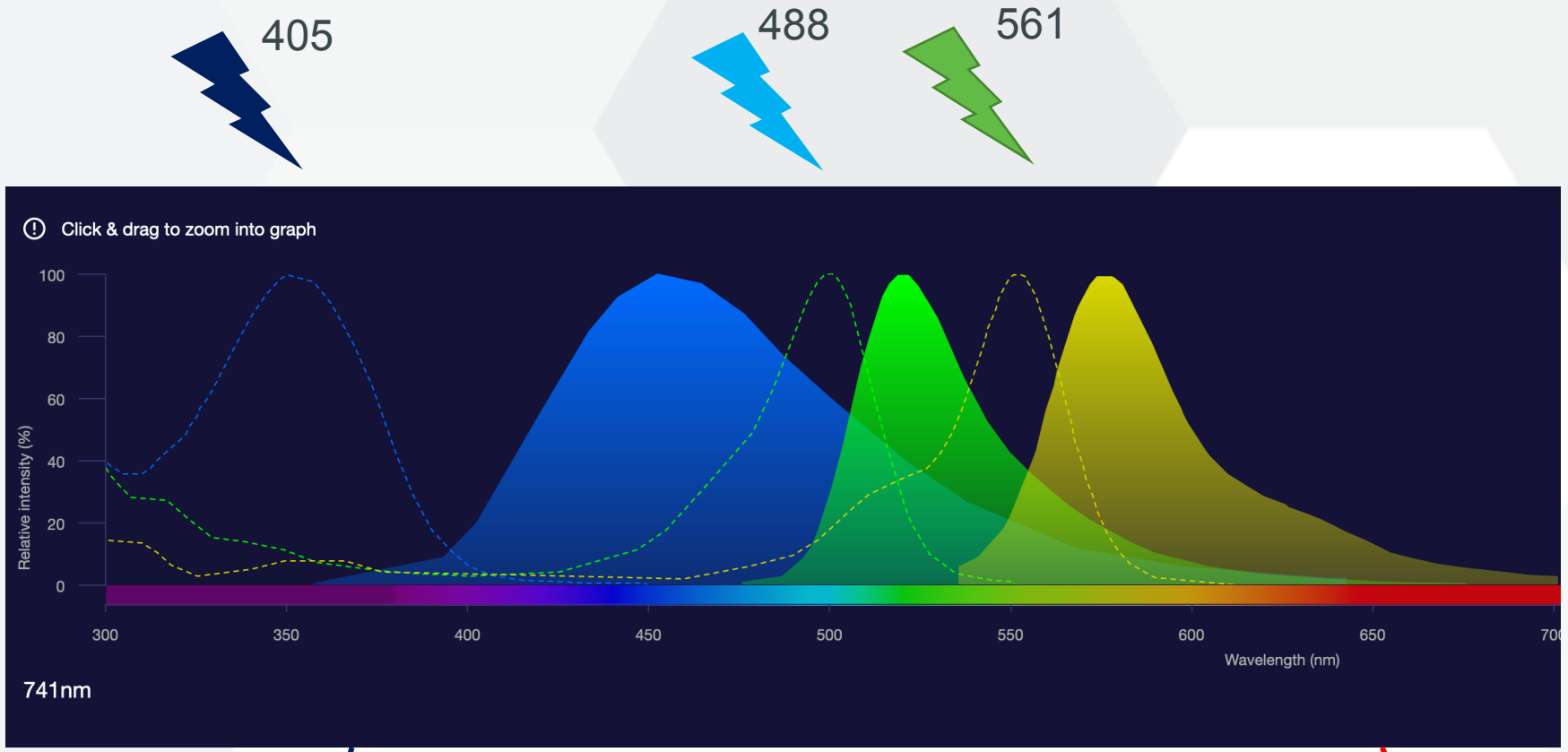
Simultaneous acquisition



+ speed

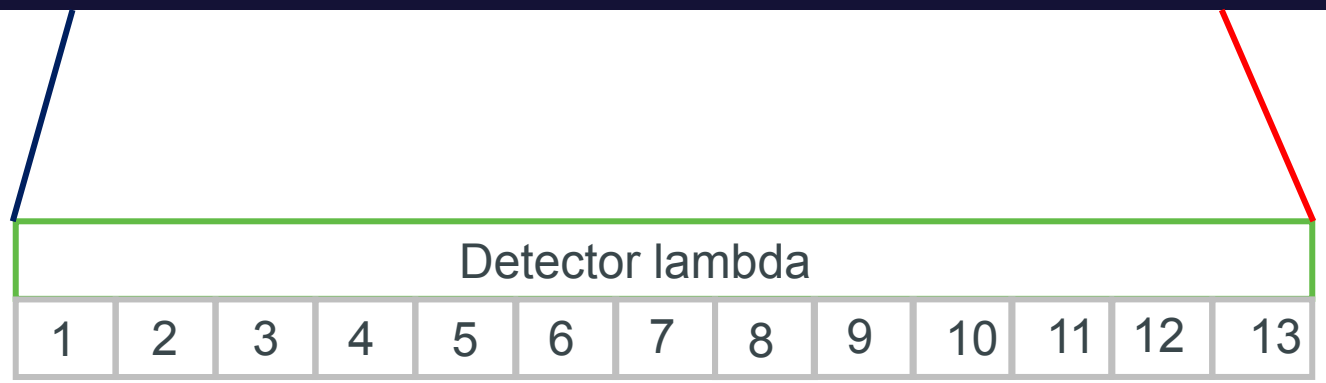
- crossexcitation and bleedthrough

Lambda imaging



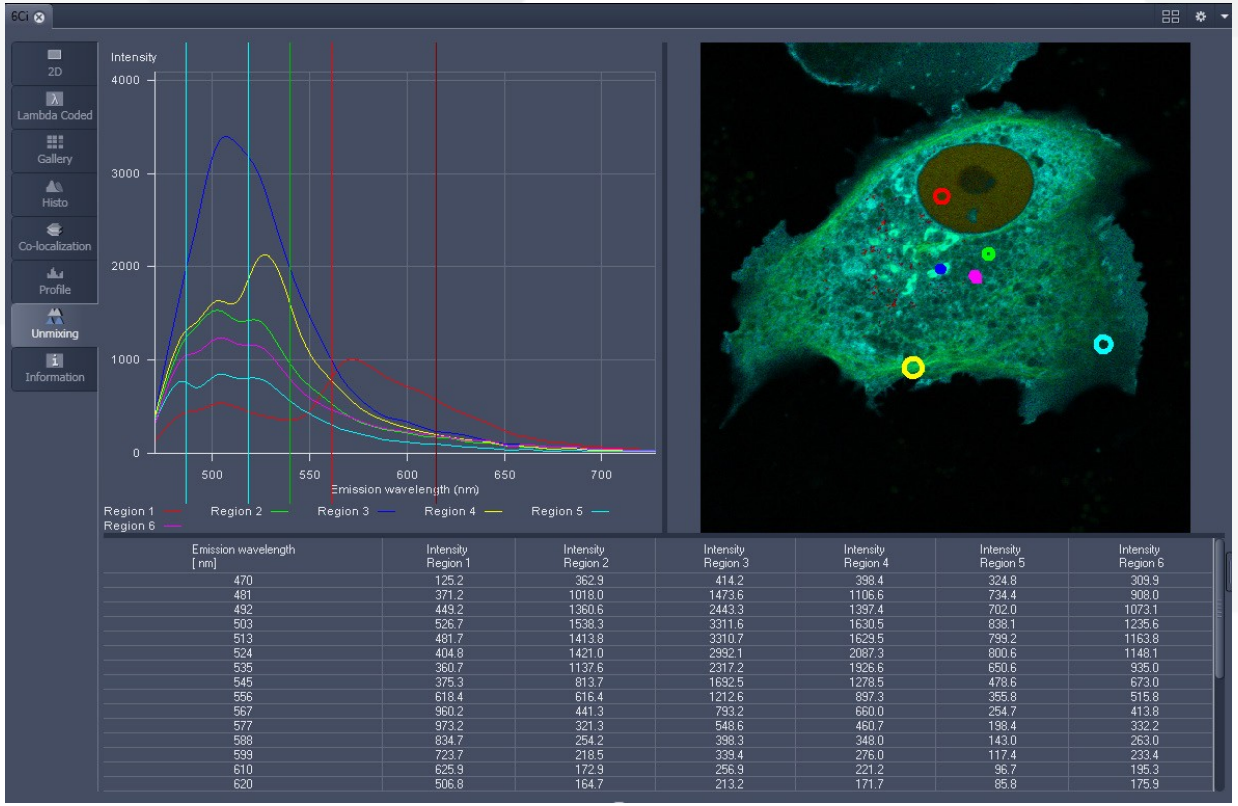
+ speed
+ separation of similar spectras

- Mathematical separation
- of fluorochromes



Linear unmixing

Acquire lambda scan and define spectra of each fluorophore separately
Spectra saved in databes
Using the same setings acquire mixture of fluorophores
Different algorhitms to separate lambda scan to individual fluorophores – multichannel image
Defined spectra or unknown spectra



Emission wavelength [nm]	Intensity Region 1	Intensity Region 2	Intensity Region 3	Intensity Region 4	Intensity Region 5	Intensity Region 6
470	125.2	362.9	414.2	398.4	324.8	309.9
481	371.2	1018.0	1473.6	1106.6	734.4	908.0
492	449.2	1360.6	2443.3	1397.4	702.0	1073.1
503	526.7	1538.3	3311.6	1630.5	838.1	1235.6
513	481.7	1413.8	3310.7	1629.5	799.2	1163.8
524	404.8	1421.0	2992.1	2087.3	800.6	1148.1
535	360.7	1137.6	2317.2	1926.6	650.6	935.0
545	375.3	813.7	1692.5	1278.5	478.6	673.0
556	618.4	616.4	1212.6	897.3	355.8	515.8
567	960.2	441.3	793.2	660.0	254.7	413.8
577	973.2	321.3	548.6	460.7	198.4	332.2
588	834.7	254.2	398.3	348.0	143.0	263.0
599	723.7	218.5	339.4	276.0	117.4	233.4
610	625.9	172.9	256.9	221.2	96.7	195.3
620	506.8	164.7	213.2	171.7	85.8	175.9

Parameters of confocal imaging

Pinhole diameter: ↓ diameter = ↓ light = ↓ optical thickness and ↑ lateral resolution

Detector gain: ↑ gain = ↑ noise = ↓ laser power

Pixel dwell time (sec): time of measurement of each pixel

Uni-direction or bi-directional scan

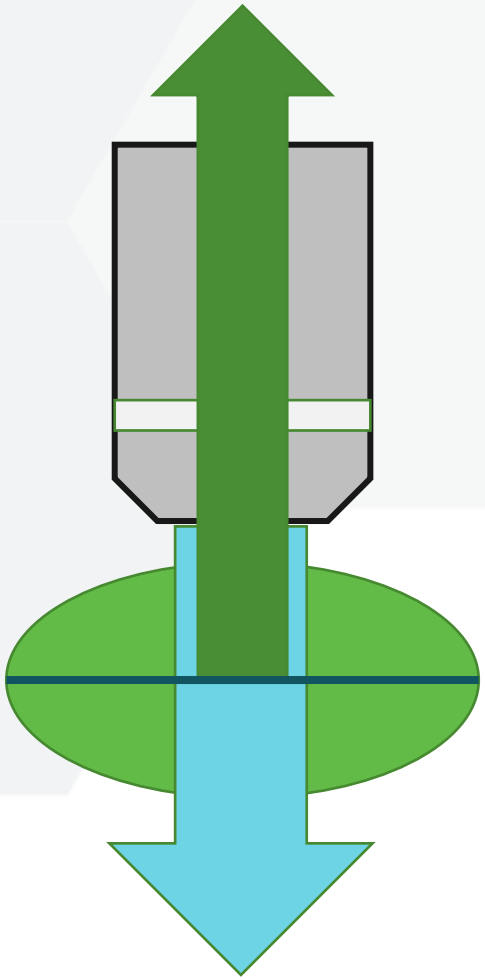
Number of pixels: size an numbers freely adjustable in point scanning systems

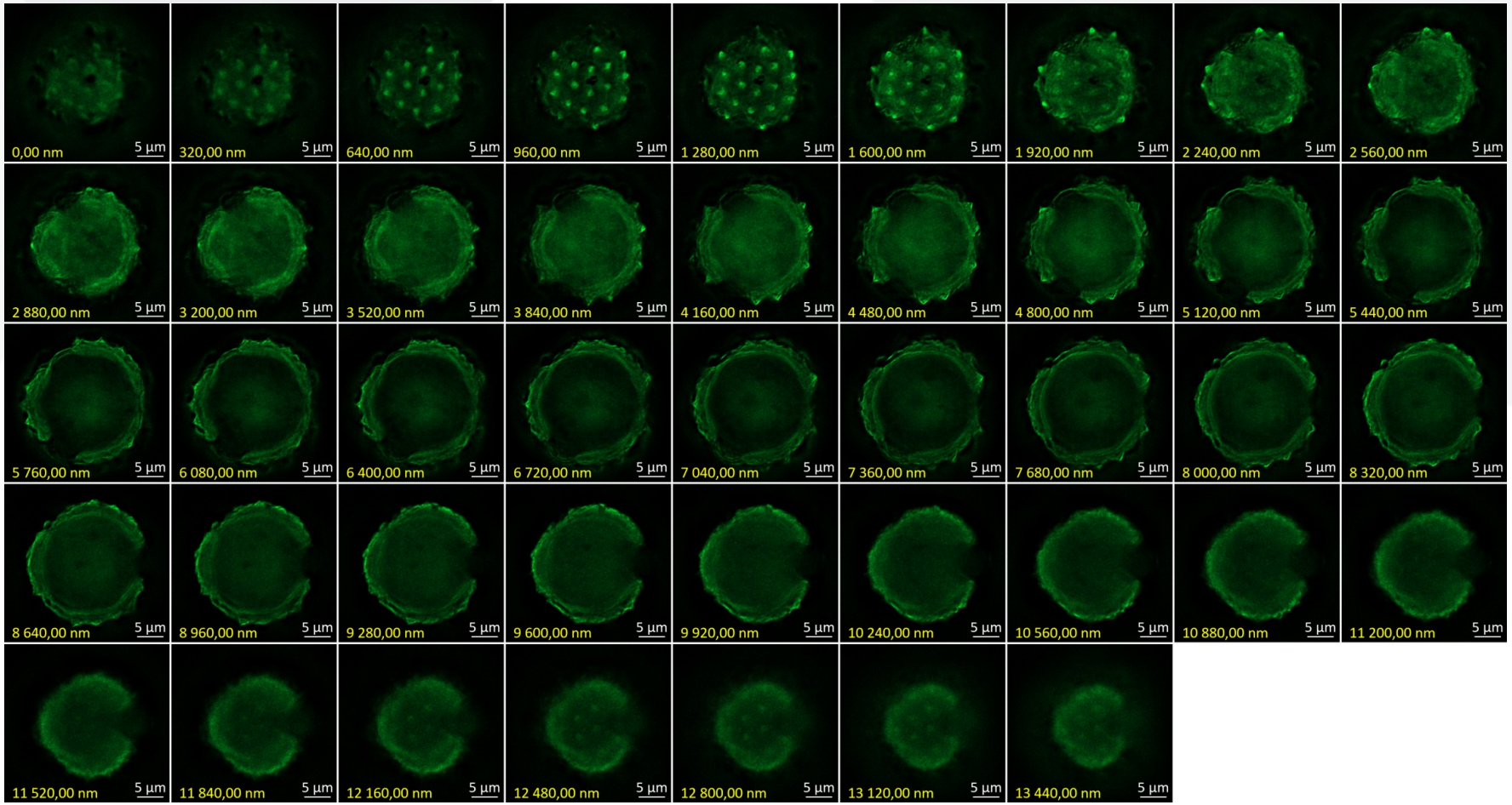
Bit depth: adjustable

Averaging: measurement of one pixel several time

Limitations of confocal microscope

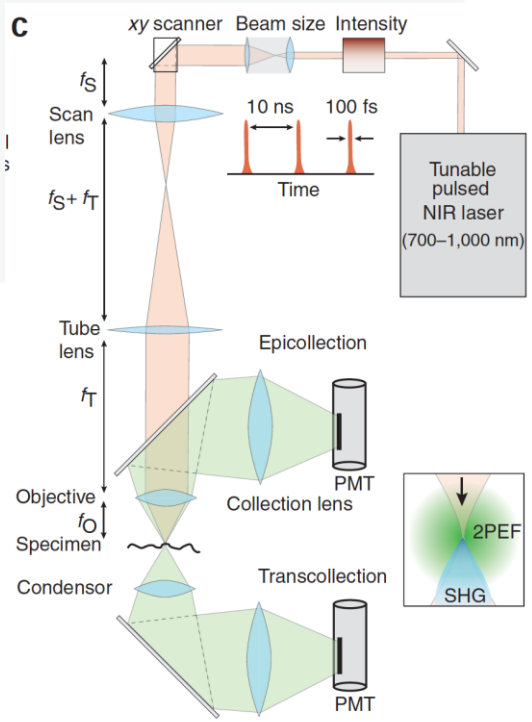
- speed, phototoxicity, photobleaching, penetration depth





Two photon confocal microscopy

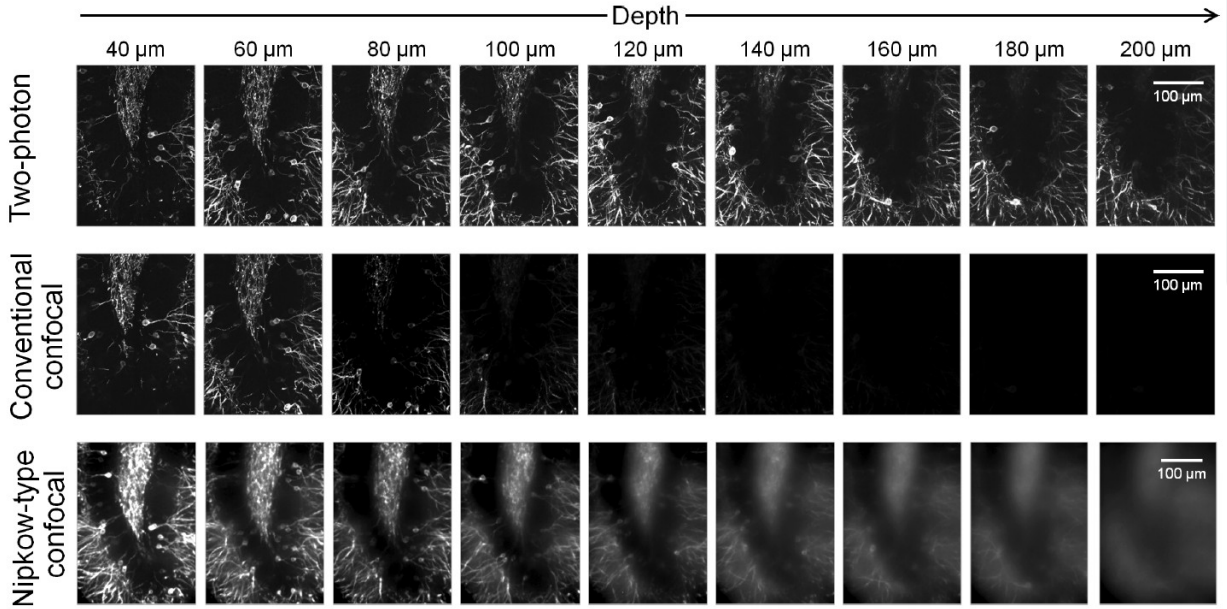
Excitation with longer wavelength laser, than emmision
Using near infrared femtoseconds pulsed laser
Longer wavelength = lower energy, two photons required for exciation
Near IR laser penetrates deeper in tissue, less scattering
Confocality assured by excitation point spread function – no pinhole
Superior to standard one photon confocal microscopes for deep tissue imaging



Deep tissue two-photon microscopy

Fritjof Helmchen & Winfried Denk

Nature Methods 2, 932–940 (2005) | Cite this article



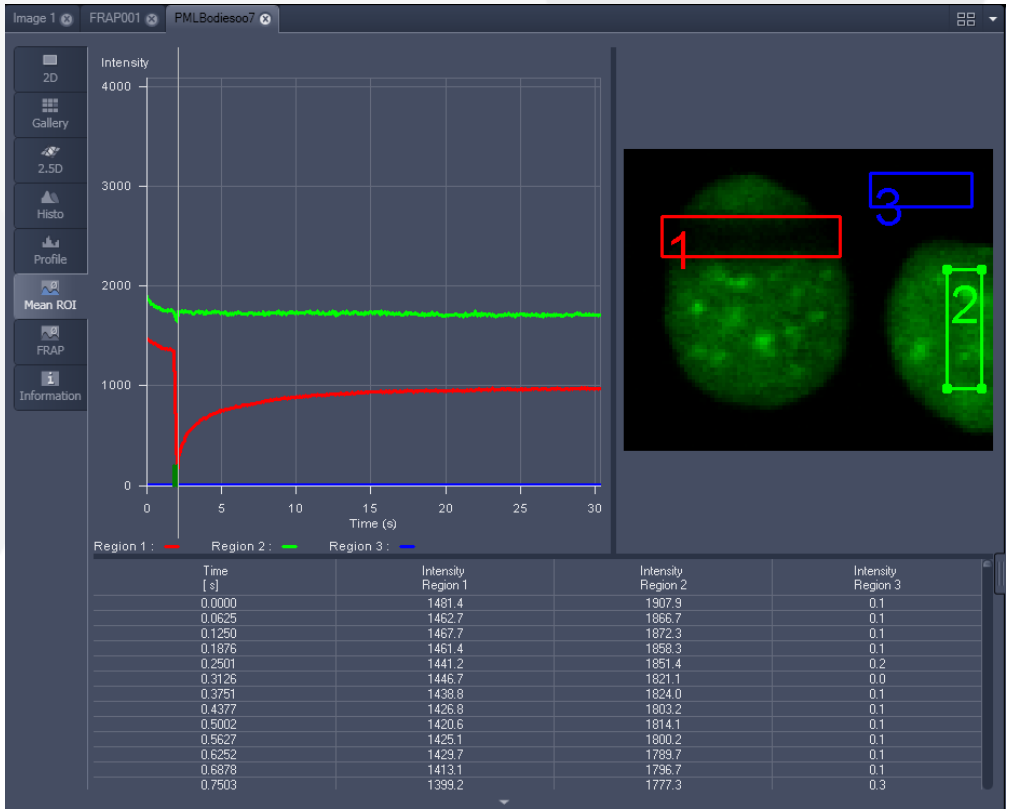
NIPKOW CONFOCAL IMAGING FROM DEEP BRAIN TISSUES

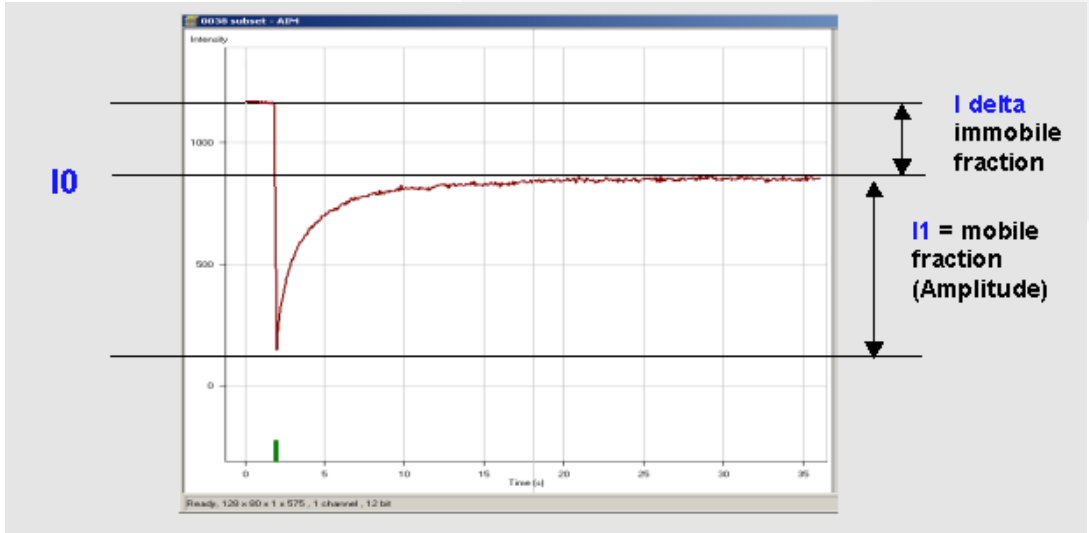
YUJI TAKAHARA*, NORIO MATSUKI* and YUJI IKEGAYA*,†,‡

Journal of Integrative Neuroscience, Vol. 10, No. 1 (2011) 121–129

Fluorescence recovery after photobleaching - FRAP

Methods to study the mobility of fluorescently labeled molecules inside cells
Photobleach selected region and measure restoration of signal intensity over time
Active transport or diffusion





Fluorescent lifetime imaging - FLIM

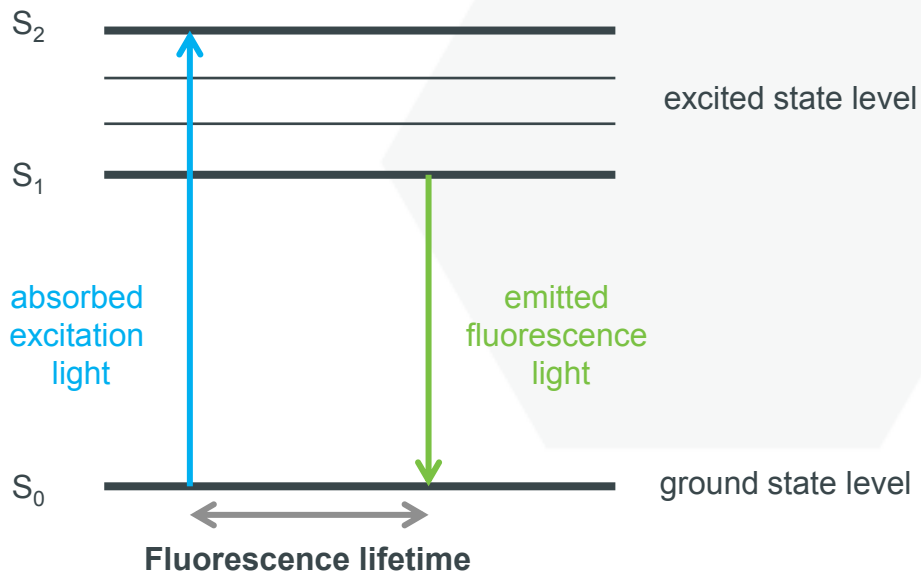
Lifetime = time fluorophor remains in excited state (before starting emmiting photons)
Each molecule has different lifetime – picoseconds to nanoseconds
Time between excitation and arrival of photon to detector – transition to ground state
Sensitive to local environment – pH, temperature, ion concentration

Requirements
Pulsed laser – femtoseconds lasers
Single photon detectors – single photon avalanche diode, PMT, hybrid detectors
Special timing electronics for data registration

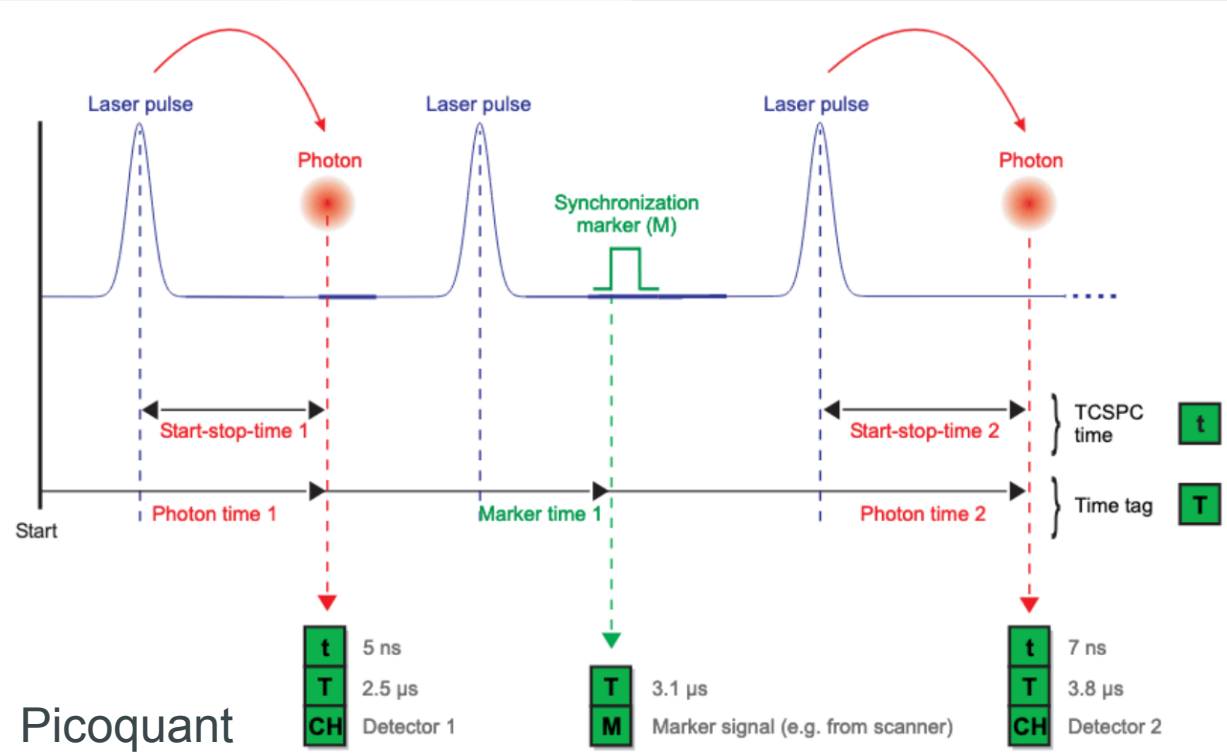
Environmental sensing – pH, ion concentration, protein interactions

Multiple fluorophores separation

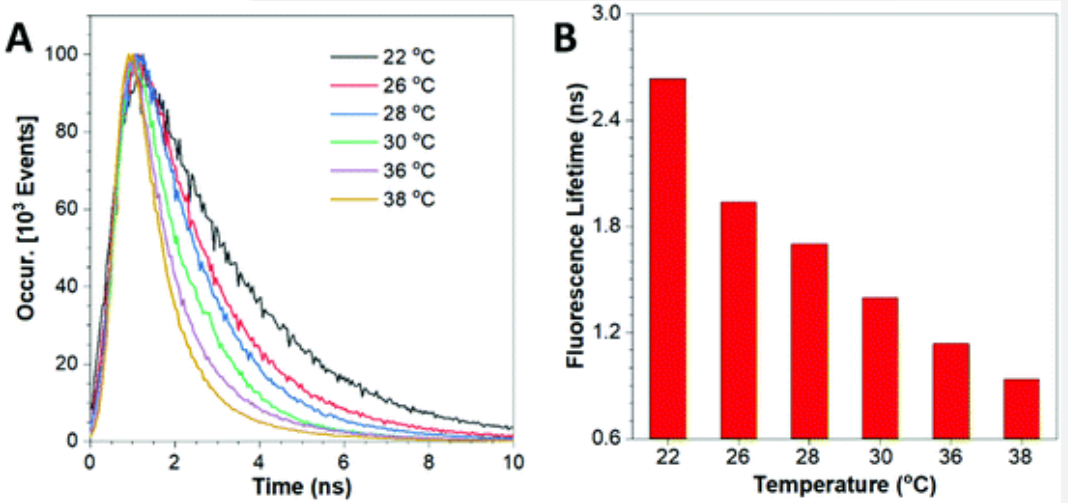
Conformational changes



Fluorescent lifetime imaging - FLIM



Picoquant



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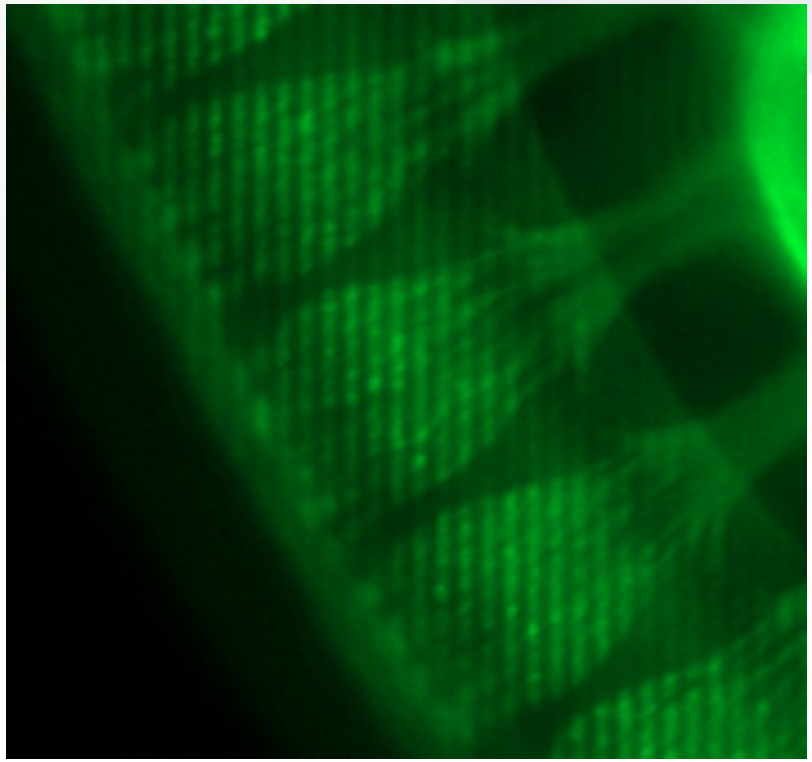
rsc.li/nanoscale-horizons

A simple yet effective AIE-based fluorescent nano-thermometer for temperature mapping in living cells using fluorescence lifetime imaging microscopy†

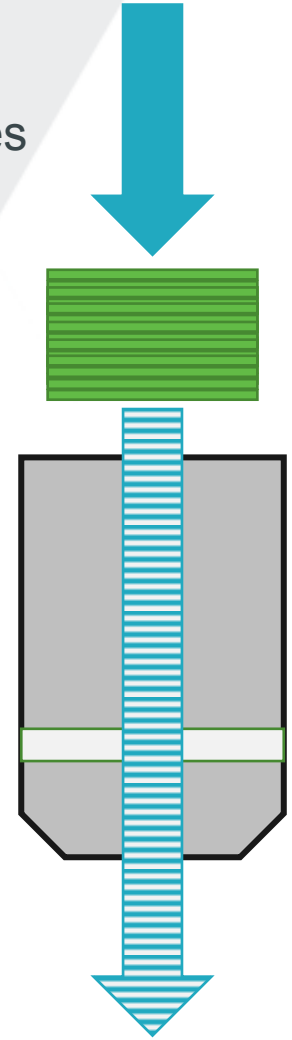
Hui Gao,[ab] Chuen Kam,[a] Tsu Yu Chou,[a] Ming-Yu Wu,[ac] Xin Zhao[cd] and Sijie Chen[*a]

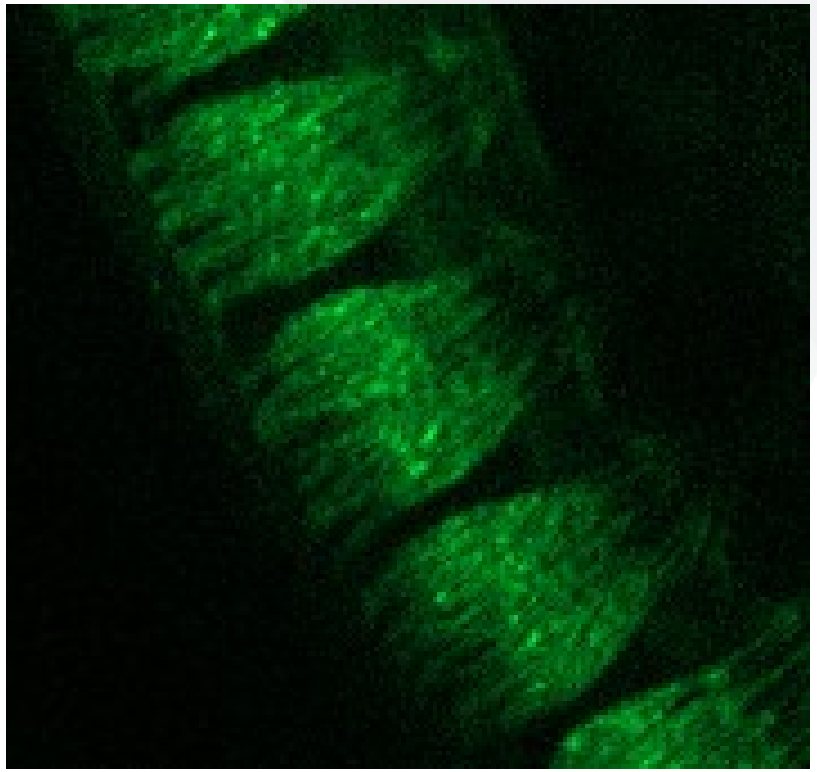
SIM Apotome for optical sectioning

Illumination of sample with a known spatially structured pattern
Grid structure is projected on focal plane of sample
Grid is moving in one direction only, several images acquired with different grid position
Different grid structures – magnification and wavelength
Final image calculated from several phase shifted images



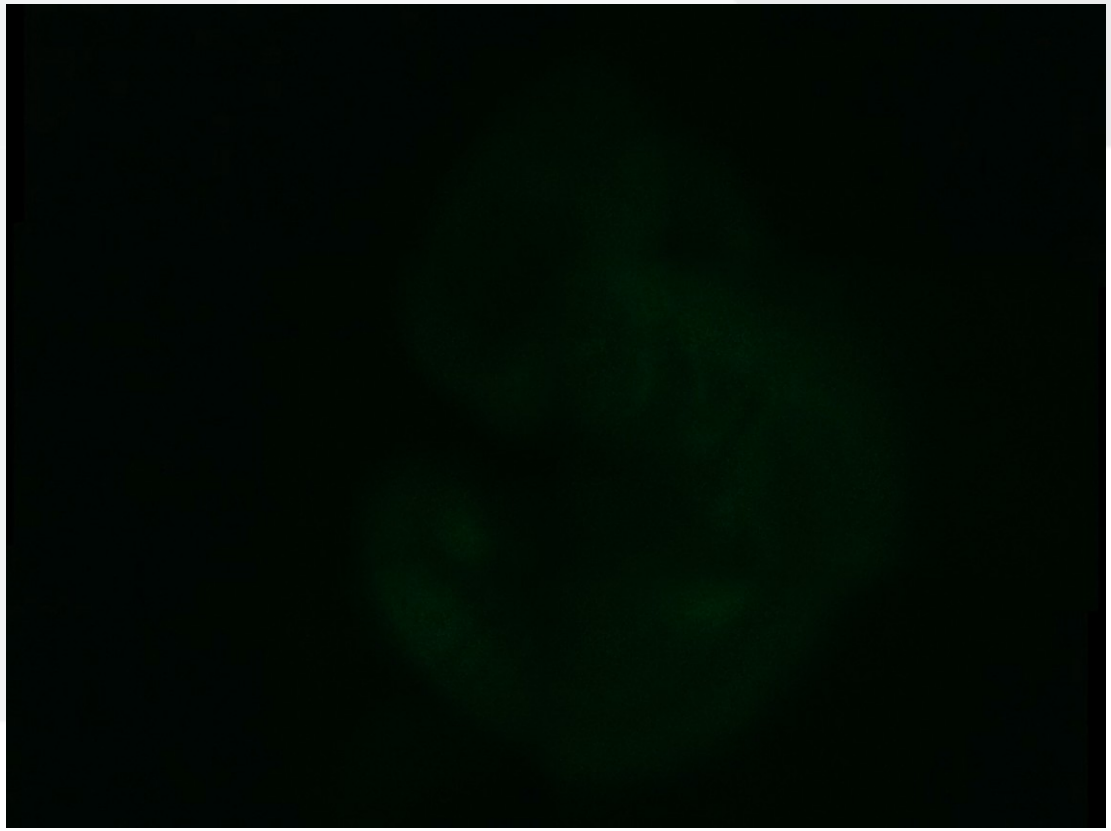
Nela Jandová, Marcela Buchtová, ÚŽFG AVČR Brno

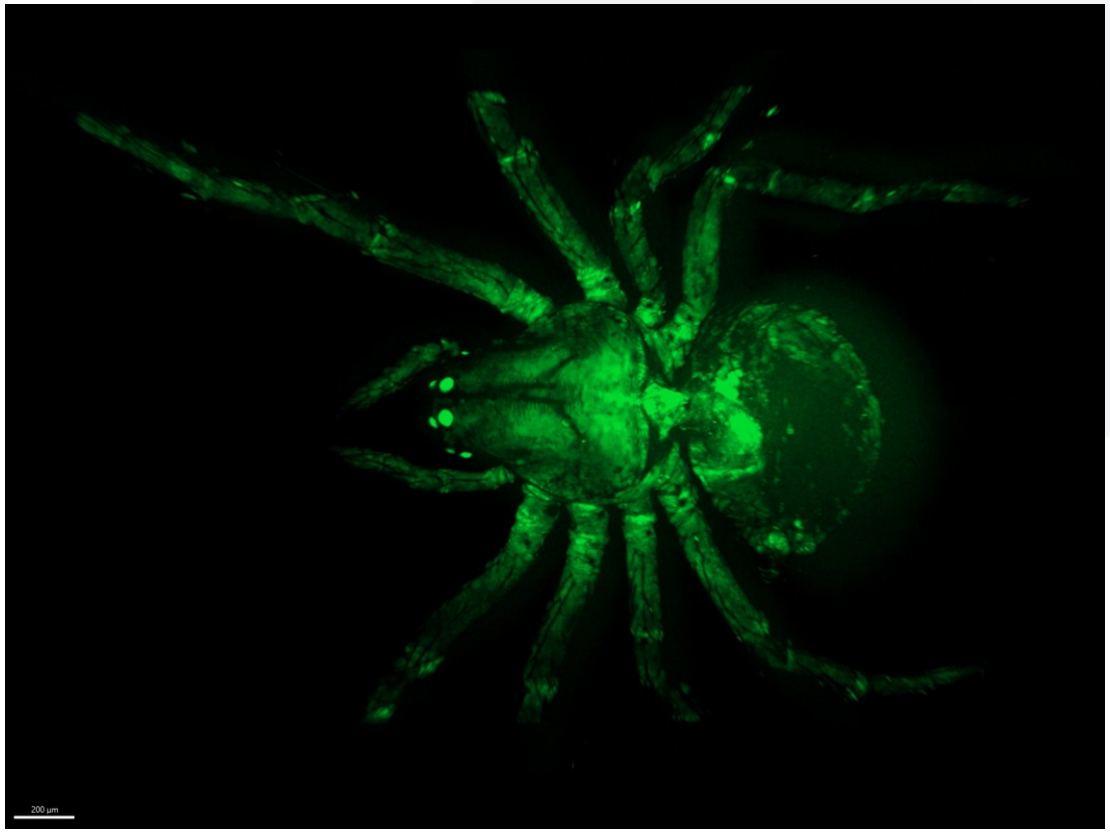




SIM Apotome for optical sectioning

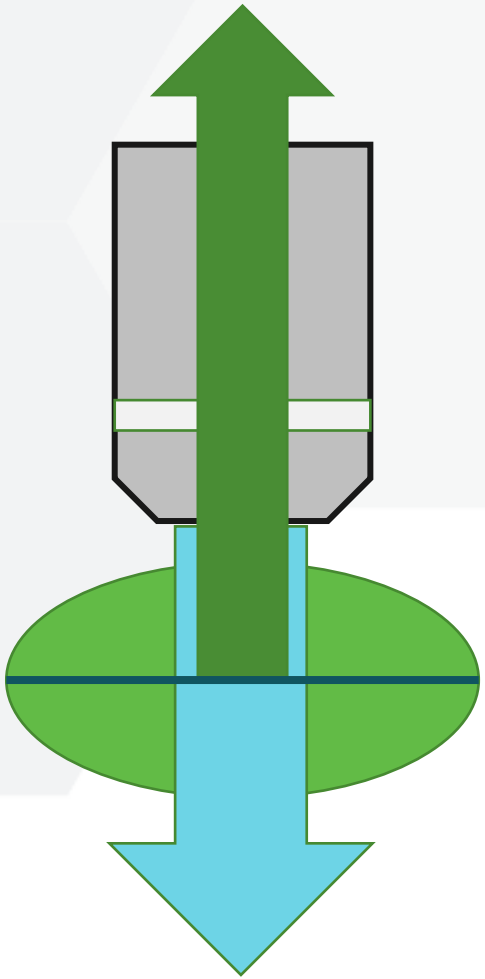
Economic solution compare to confocal microscope and faster. Not suitable for all samples.
Same sample preparation as for widefiled, or confocal microscopy.





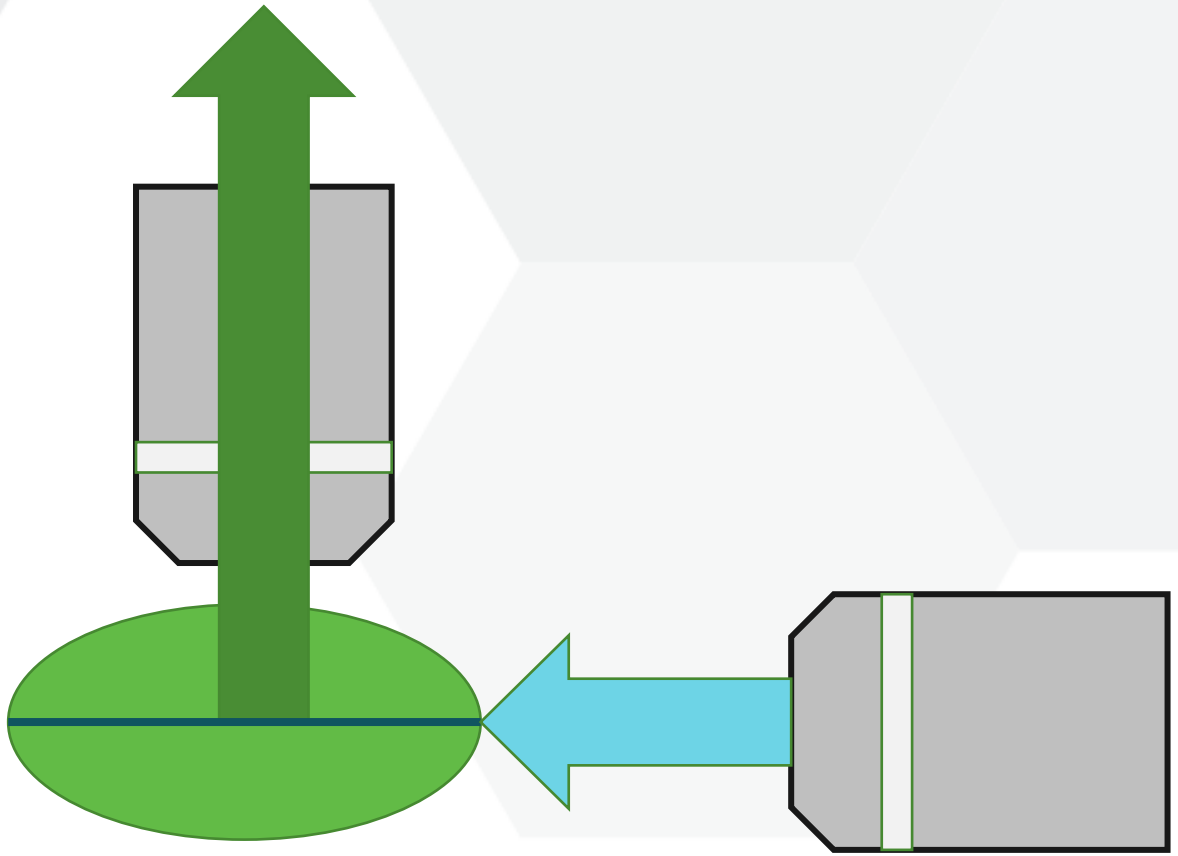
Nela Jandová, Marcela Buchtová, ÚŽFG AVČR Brno

Other optical sectioning methods



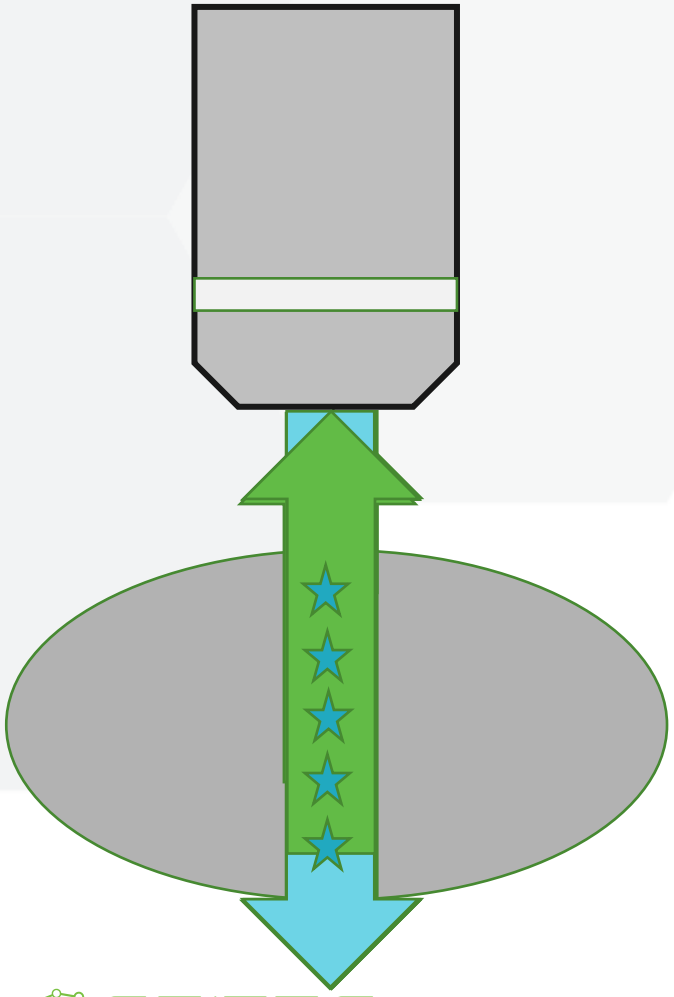
+ optical sectioning
+ minimal photobleaching
+ fast

- Sample preparation
- Image analysis and storage

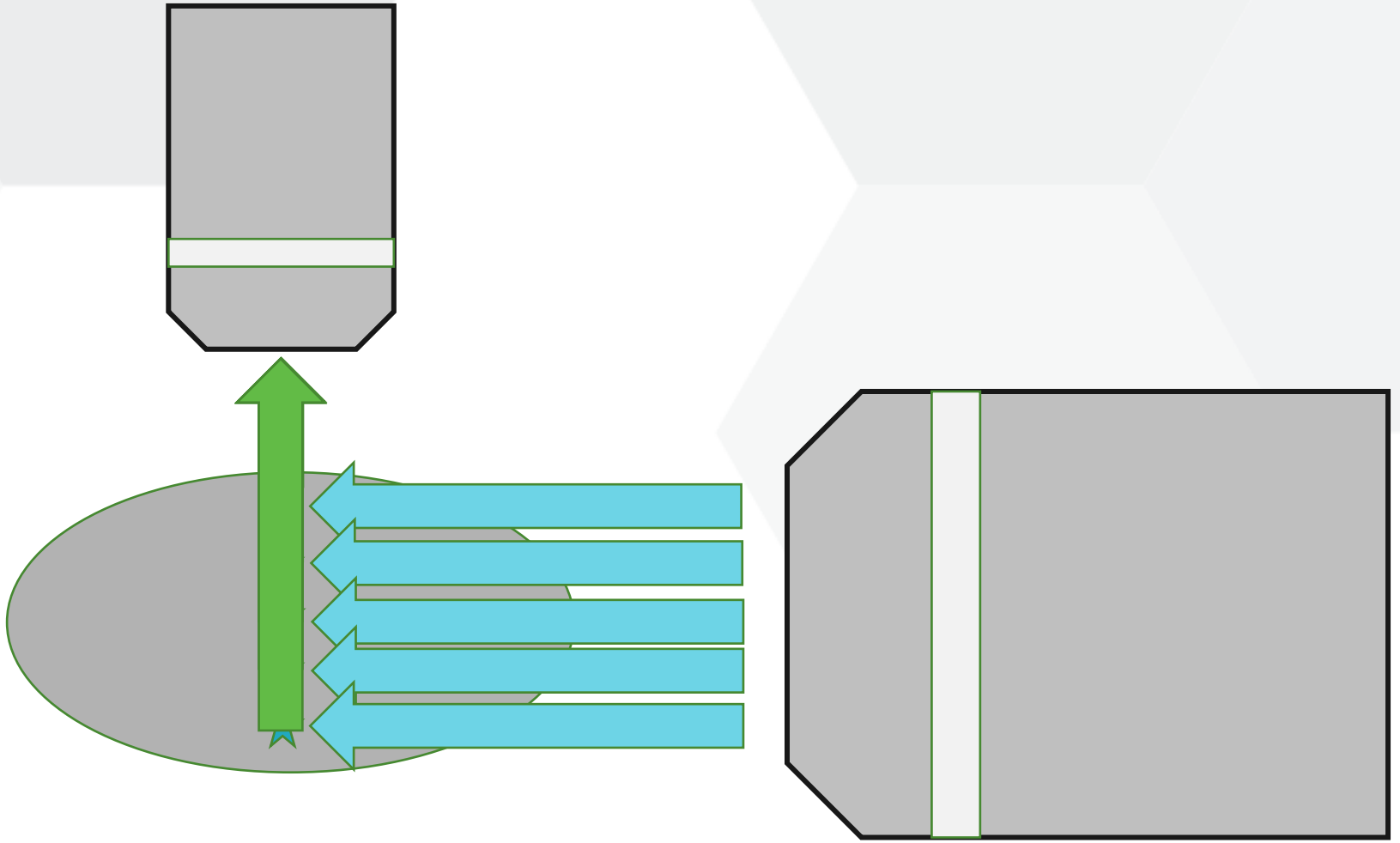


Z sectioning and photobleaching

all planes are excited, also out of focus.

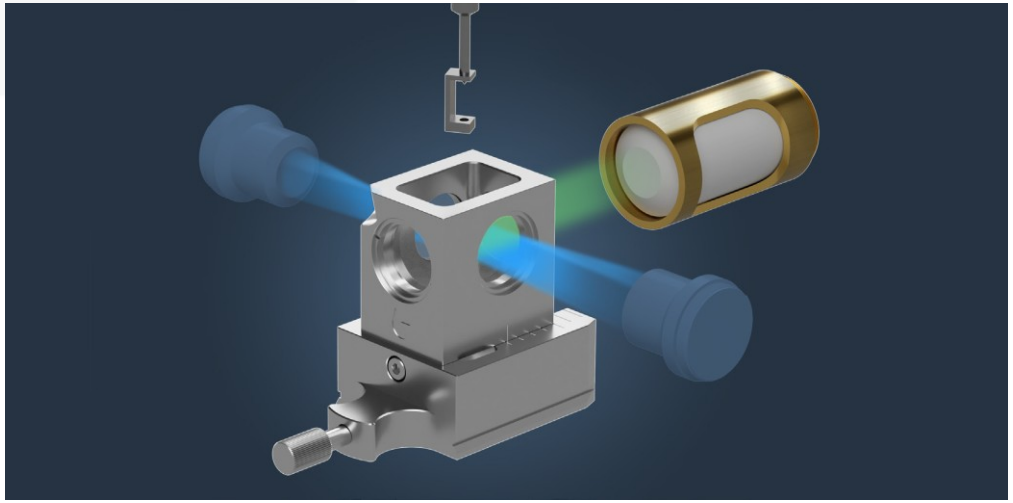


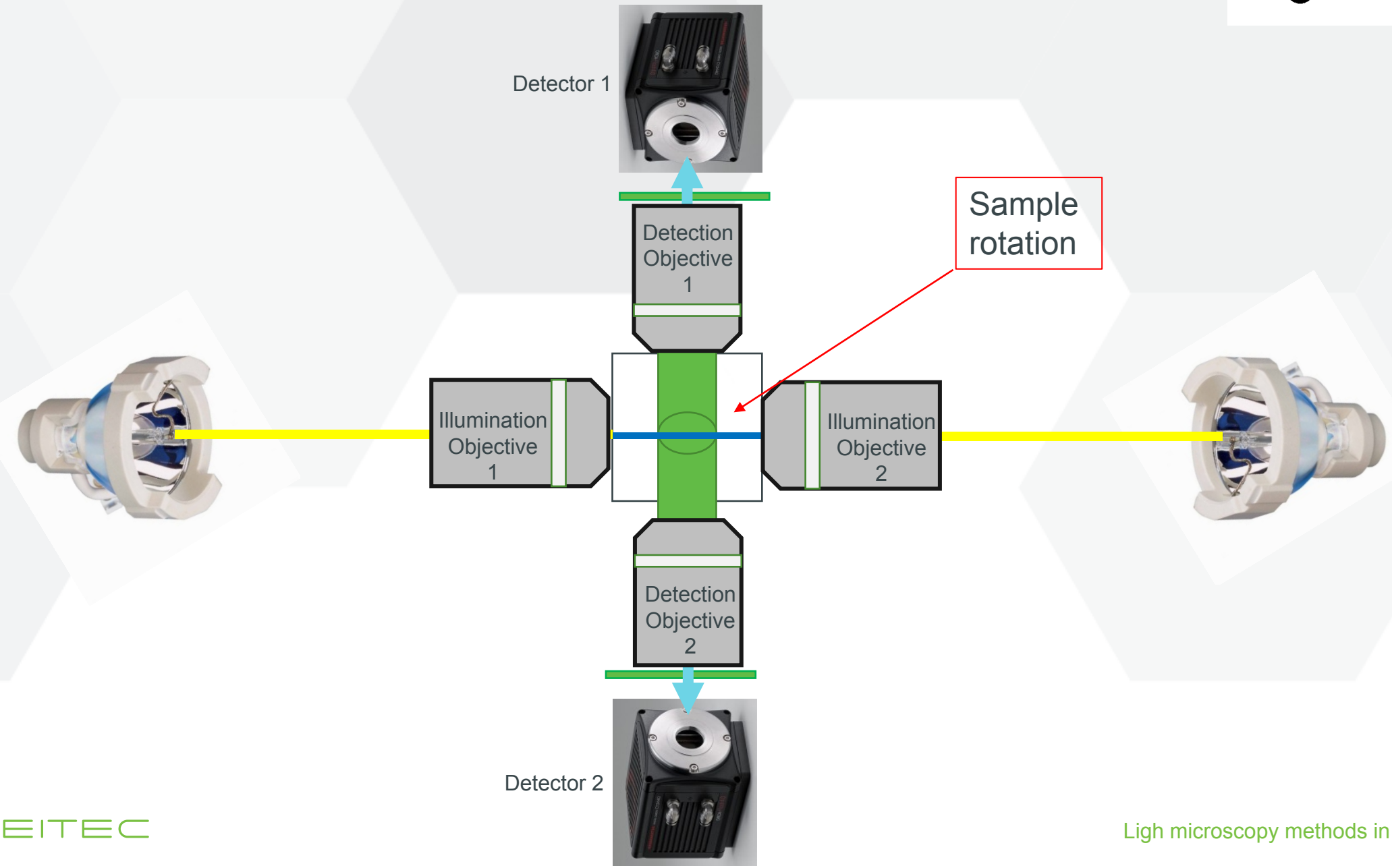
Lightsheet illuminates only planes in focus, much more gentle to sample



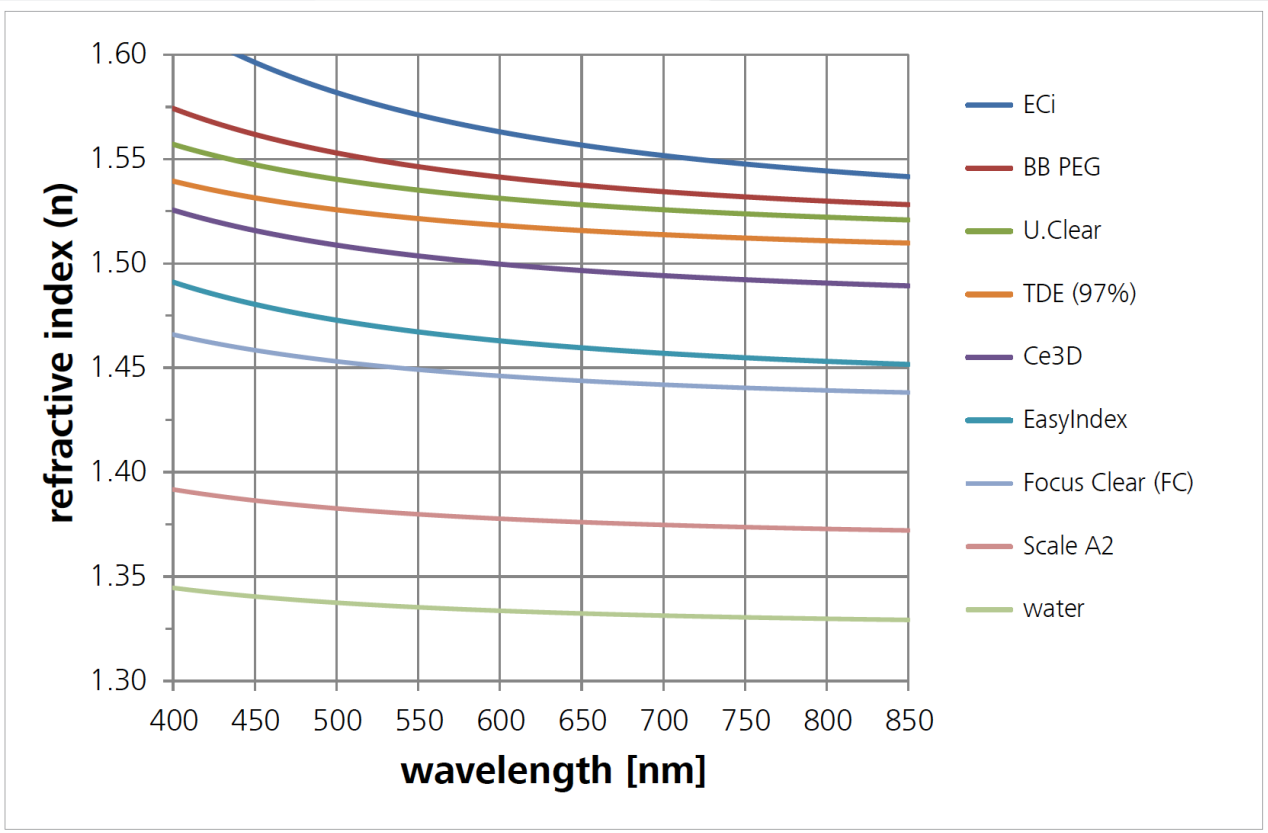
Gaussian beam lightsheet microscopes

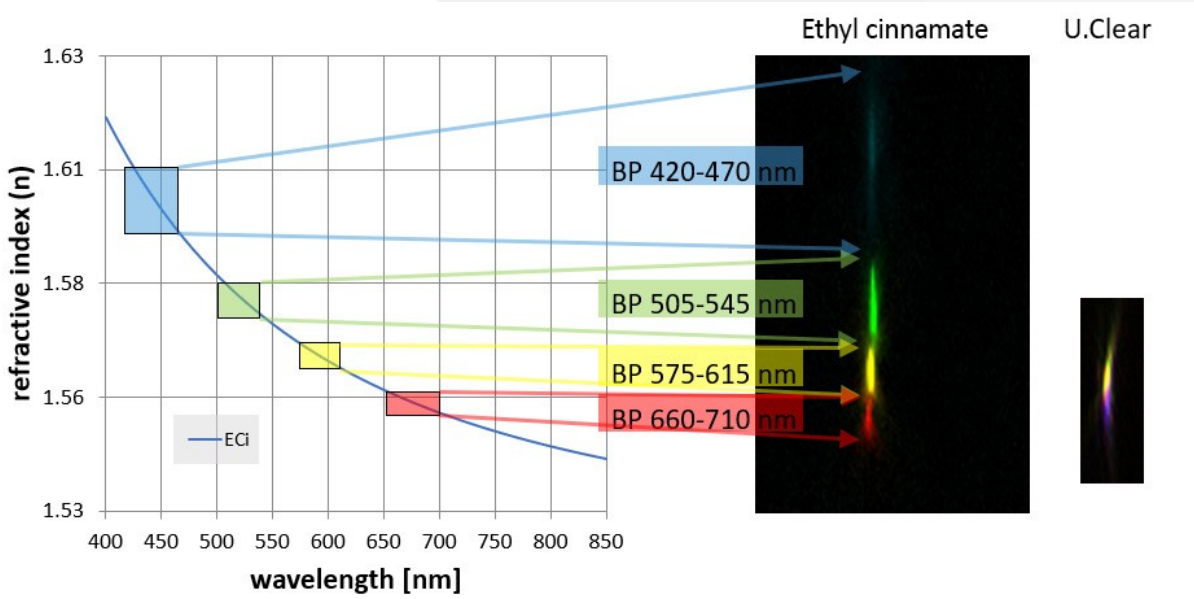
Gaussian beam – diffraction limited
Multiple optical setups possible and available
Optical thickness defined by thickness of lightsheet
Multiple excitation objectives and detection objectives
Imaging of large specimens – water, clearing objectives
Major drawback – data processing



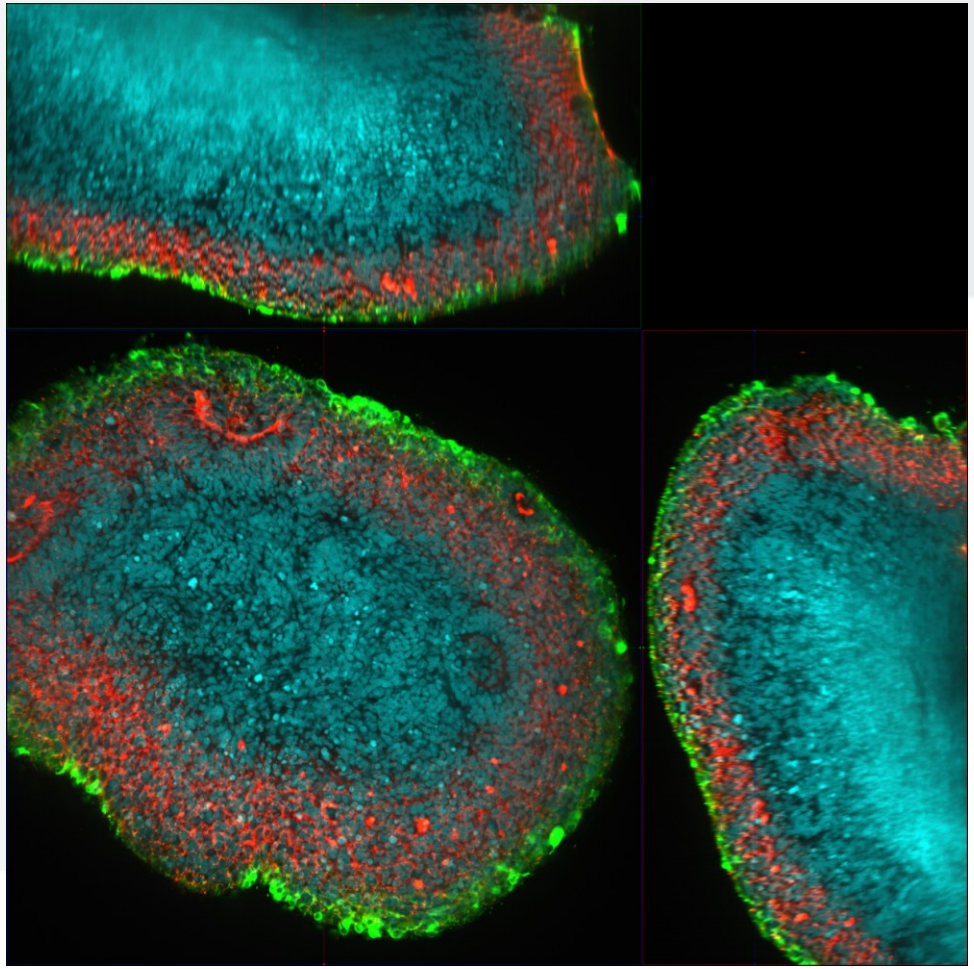

Detector 1
Detection Objective 1
Sample rotation
Illumination Objective 1
Illumination Objective 2
Detection Objective 2
Detector 2

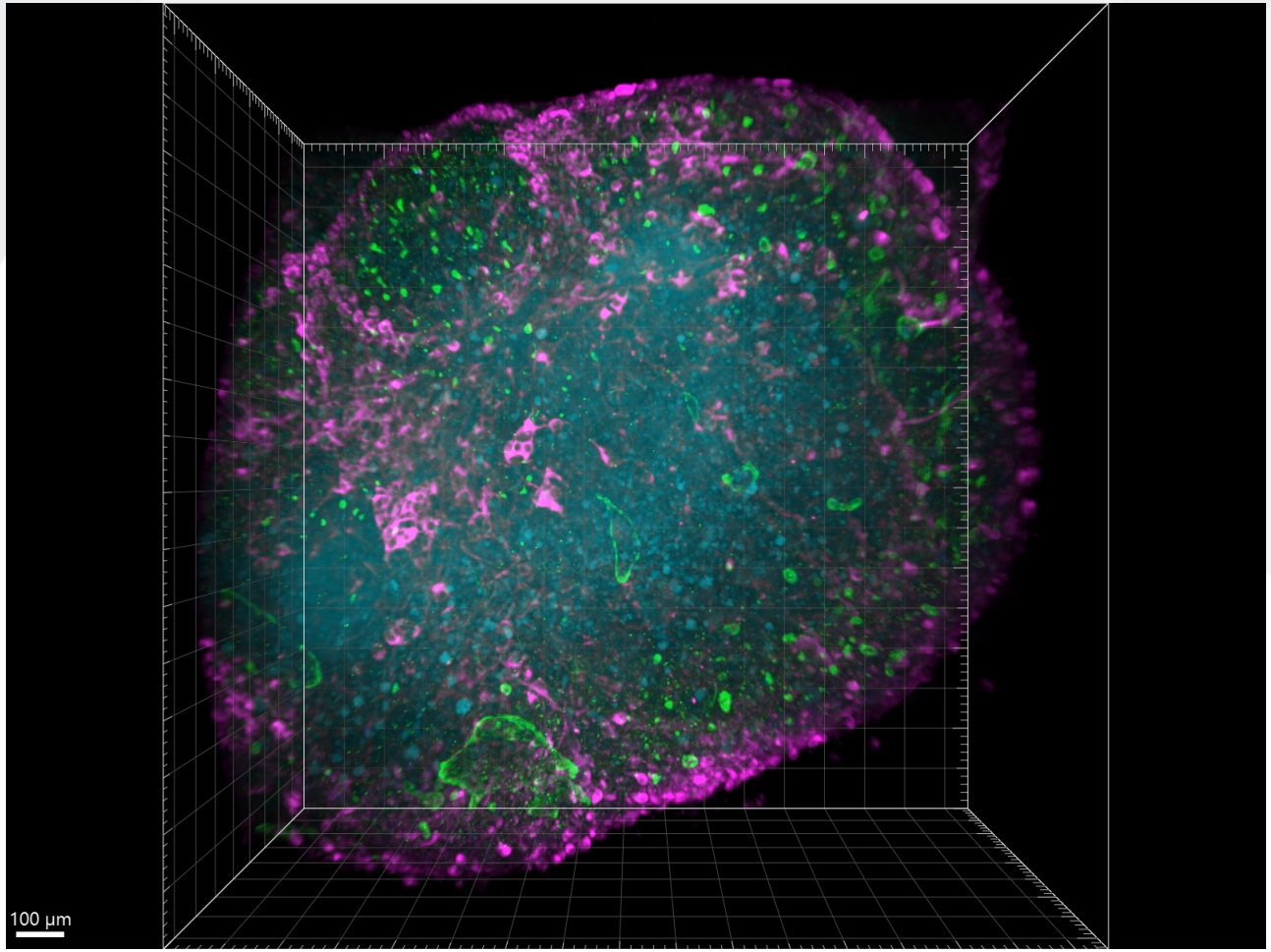
Refractive index match importance





Gaussian beam lightsheet microscopes





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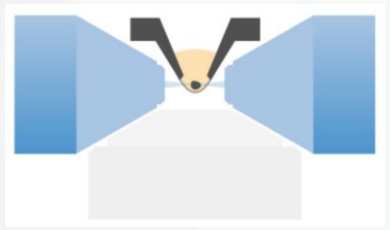
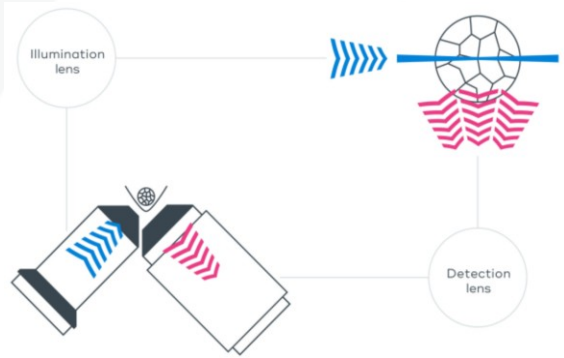
Alternative lightsheet geometry

Example of lighsheet for live organoid imaging
Similar geometry to inverted microscope, special culture dishes
Samples in liquid media, not need to special mounting into agar
Easy multi positions imaging



Viventis Microscopy



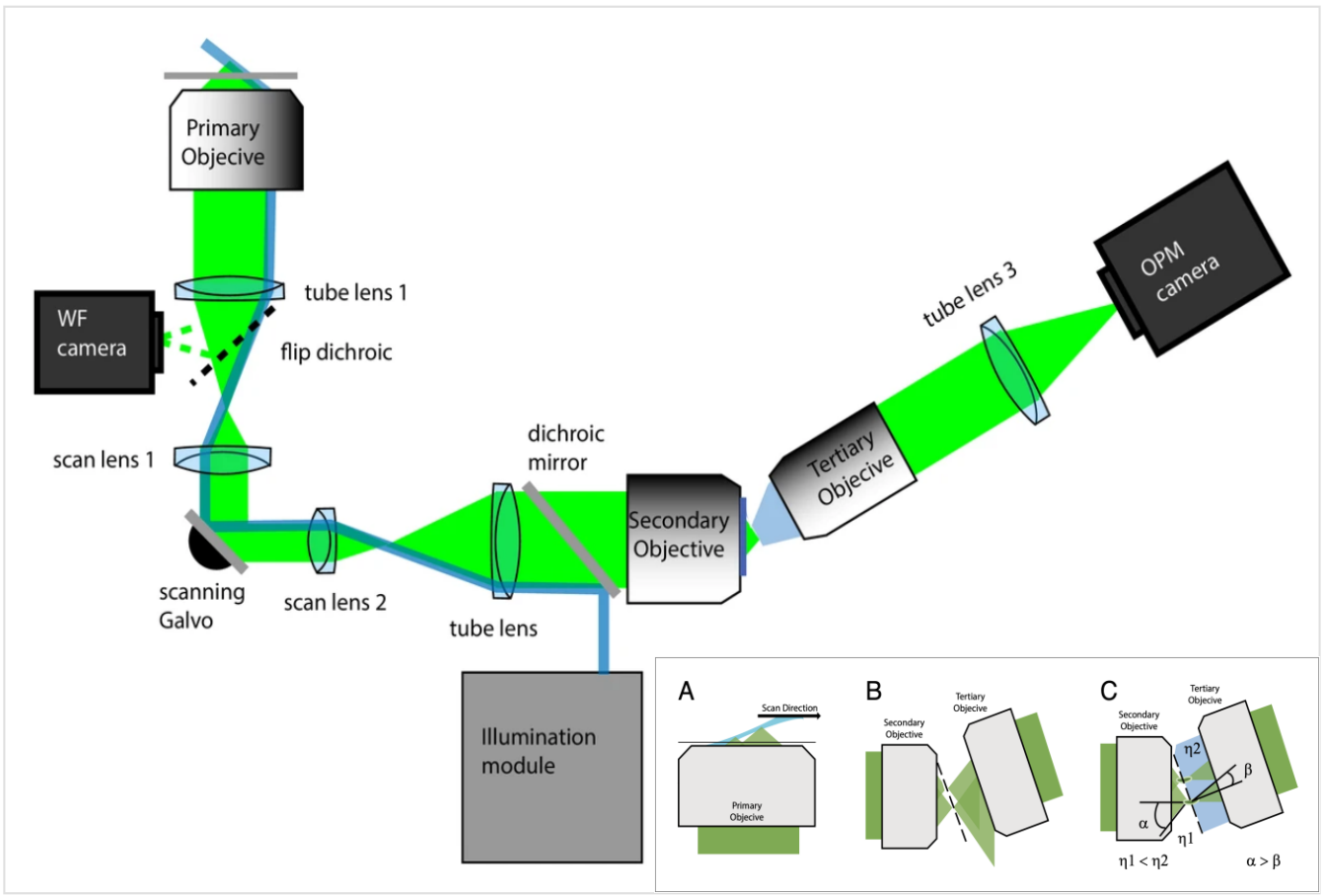
BRUKER

3i

Single objective lightsheet microscopes

Oblique plane microscope

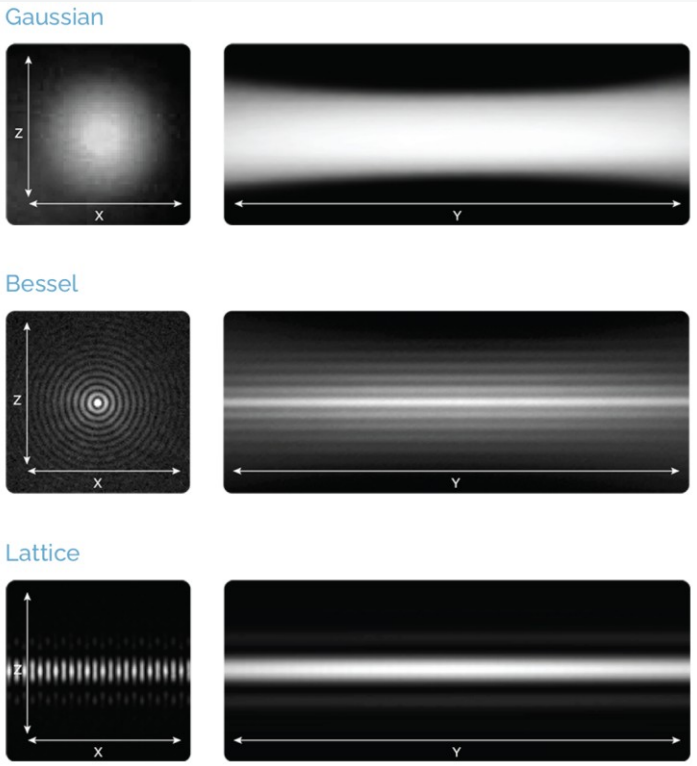
Resolution comparable to lattice light sheet

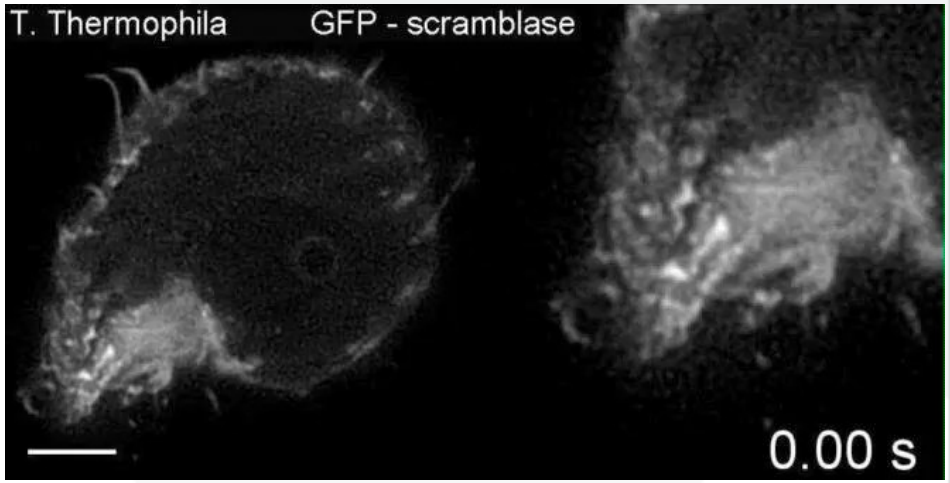


Sapoznik *et al.* eLife 2020;9:e57681. DOI: https://doi.org/10.7554/eLife.57681

Lattice light sheet

Most suitable for fast and gentle super-resolution live cell imaging in 3D
Developped initially by E. Betzig
Using non diffracting Bessel beam instead of classical Gaussian beam
Non-diffracting light sheet formed by interference patterns





Protozoa Tetrahymena thermophila

Lattice light-sheet microscopy: Imaging molecules to embryos at high spatiotemporal resolution

Bi-Chang Chen[1,*,†], Wesley R. Legant[1,*], Kai Wang[1,*], Lin Shao[1], Daniel E. Milkie[2], Michael W. Davidson[3], Chris Janetopoulos[4], Xufeng S. Wu[5], John A. Hammer III[5], Zhe Liu[1], Brian P. English[1], Yuko Mimori-Kiyosue[6], Daniel P. Romero[7], Alex T. Ritter[8,9], Jennifer Lippincott-Schwartz[8], Lillian Fritz-Laylin[10], R. Dyche Mullins[10], Diana M. Mitchell[11,‡], Joshua N. Bembenek[11], Anne-Cecile Reymann[12,13,§], Ralph Böhme[12,13], Stephan W. Grill[12,13,§], Jennifer T. Wang[14], Geraldine Seydoux[14], U. Serdar Tulu[15], Daniel P. Kiehart[15], Eric Betzig[1,‖]

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DOI: 10.1126/science.1257998

[redacted]

Thank you for your attention

Lecture 3: October 25, 2021 at 9:00

Imaging below diffraction limits – SIM, SMLM, STED