WIMM Methods & Techniques Course 2019

Confocal Microscopy

Christoffer Lagerholm
Facility Manager
Wolfson Imaging Centre

Wolfson Imaging Centre Oxford

https://www.imm.ox.ac.uk/research/facilities/wolfson-imaging-centre

- **Microscope Equipment**

WIDE-FIELD Microscopes

1) DeltaVision Elite Live Cell Imaging System
2) Leica DMi8

CONFOCAL Microscopes

1) Zeiss 780 Inverted Confocal Microscope
2) Zeiss 880 Inverted Confocal Microscope with Airyscan detector
3) Zeiss 780 Upright Confocal Multiphoton Microscope
4) Zeiss Cell Observer Spinning Disc Confocal
5) Leica SPEII Inverted Confocal Microscope

SUPER-RESOLUTION Microscopes

1) Leica SP8 STED3X Microscope
2) Olympus IX83 TIRF Microscope

Image Analysis and Image Storage

1) Huygens
2) Imaris
3) OMERO 100 TB File Server

Wolfson Imaging Centre Staff

- Facility Manager
- 2 Assistant Managers – Dr. Jana Koth & Dr. Silvia Galiani
- Image Analyst – Dr. Ulrike Schulze

Staff Duties

- Training (and managing) users,
- System maintenance and validation
- Implementation of new methods

Microscope Users

~ 65-70 users/month

~ > 1000+ hrs/month

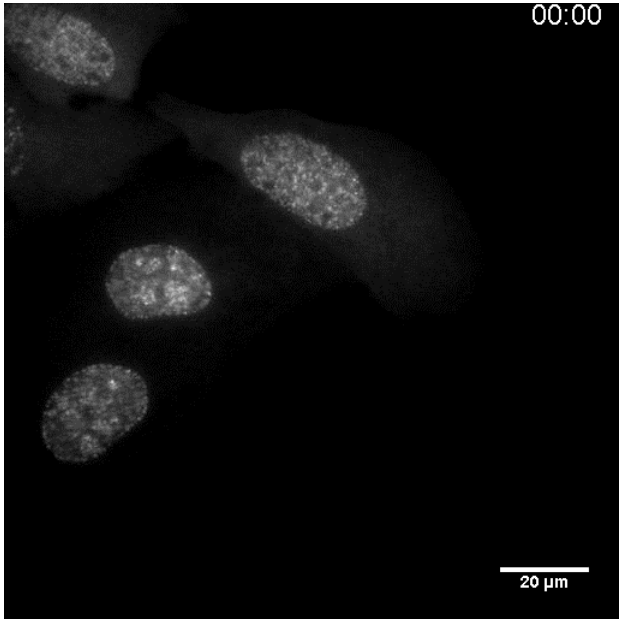
Outline

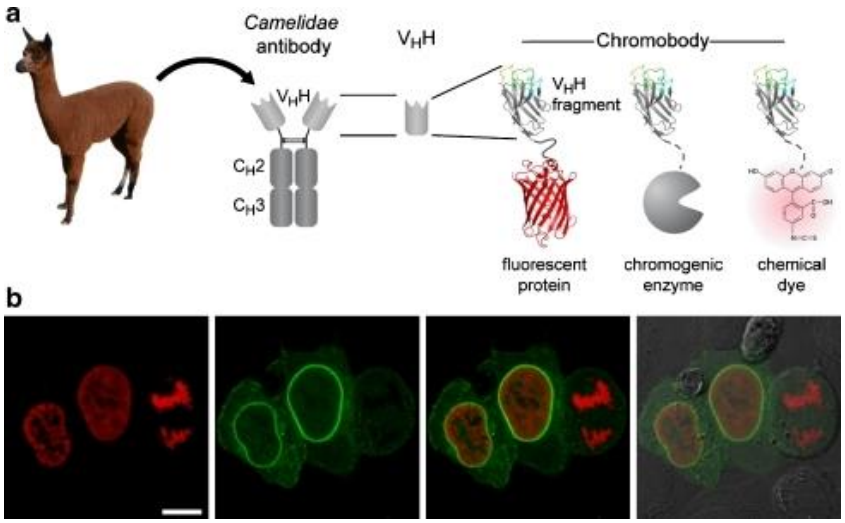
- **What can we learn from light microscopy?**
- **Why so many microscopes?**
- **Confocal microscopy**

What can we learn from light microscopy?

Example 1: Cell cycle dynamics

U2OS cells expressing PCNA-Chromobody-RFP imaged on Widefield/TIRF microscope at 24 z-planes/h

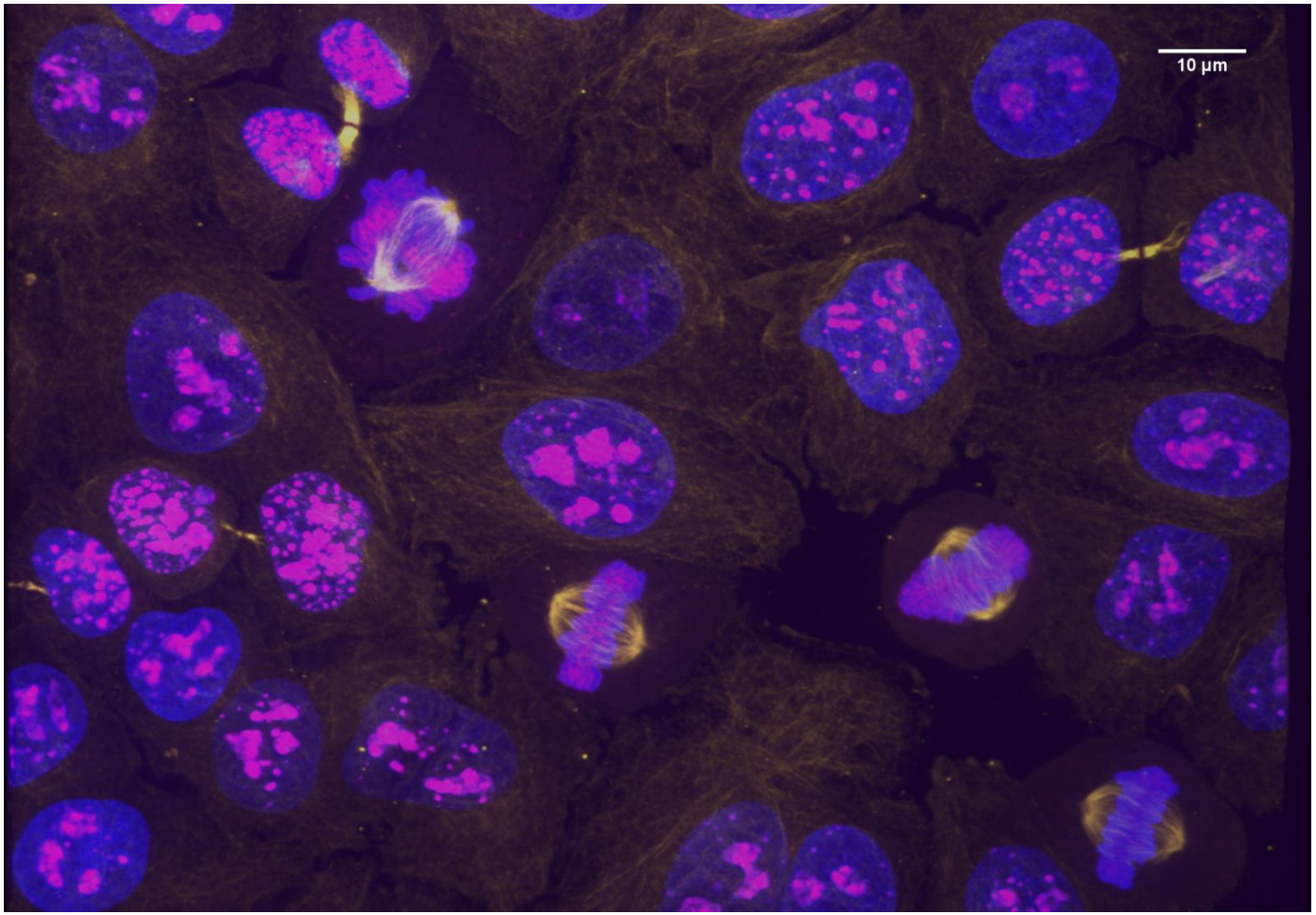




Chromotek
https://www.chromotek.com/

Example 2: Cell cycle dynamics

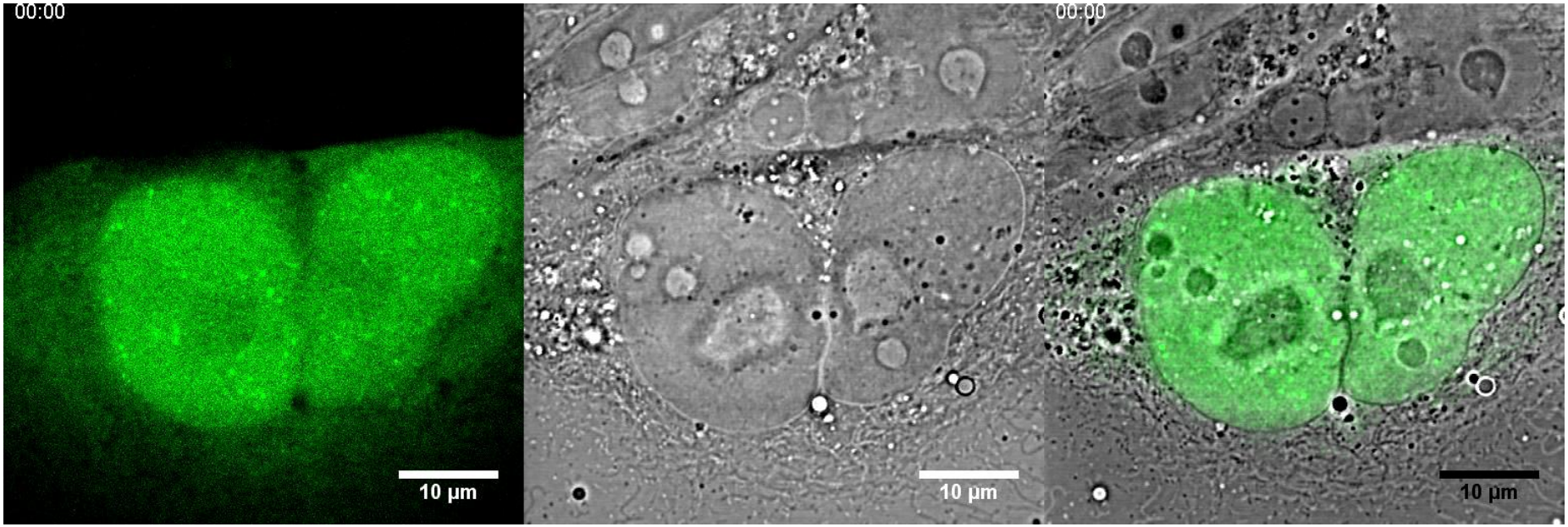
Fixed time-point; HeLa cells



3D Immunohistochemistry of fixed HeLa cells expressing H2B-GFP (blue) and stained with antibodies for proliferation marker Ki67 (magenta) and tyrosine –α-tubulin (yellow) (Max Intensity projection of z-stack (112 slices); FOV ~150 µm x 100 µm)

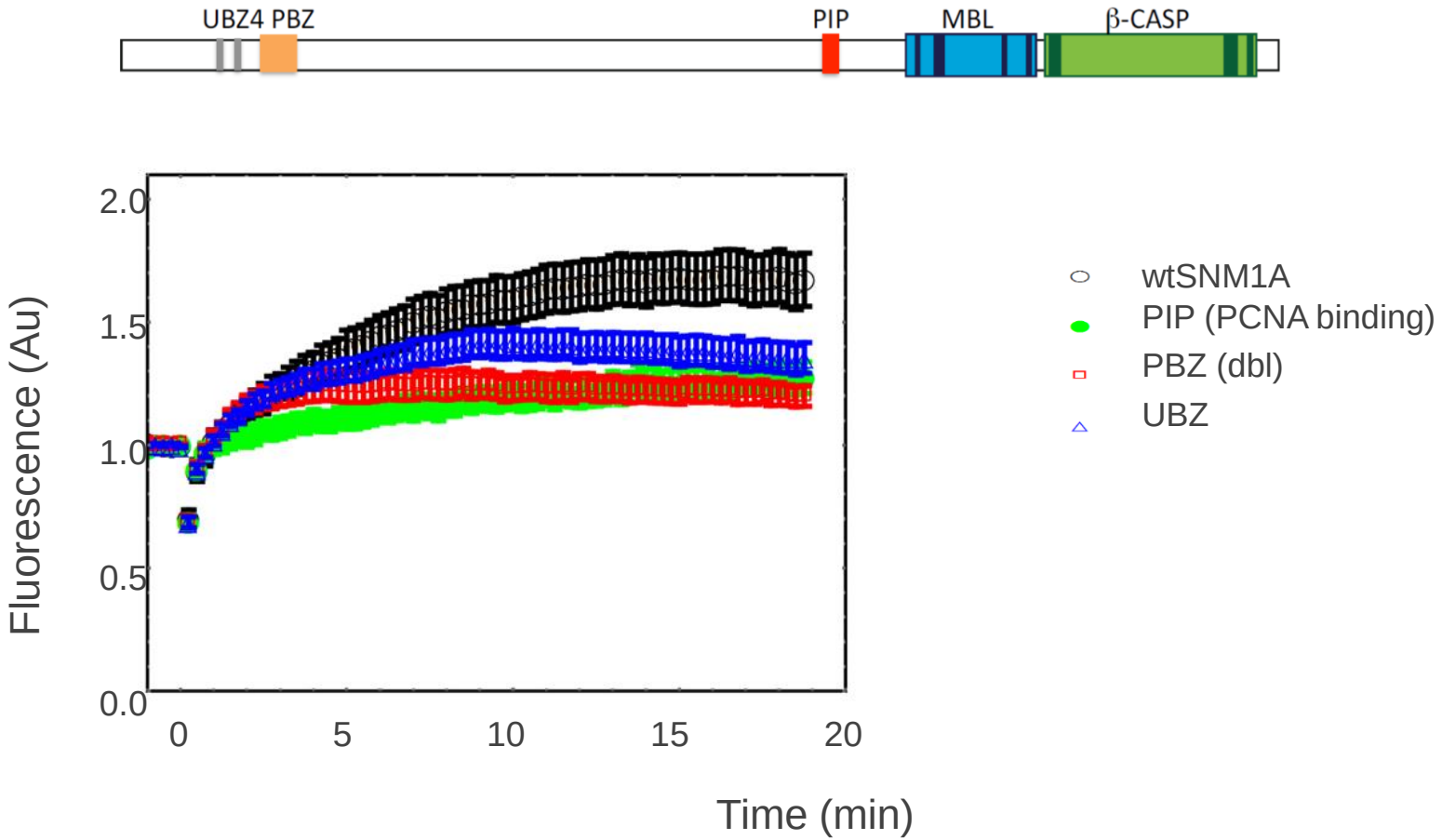
Example 3: Quantitating kinetics and relevance of protein motifs upon DNA damage response.

Induction of DNA damage with 405 nm laser



Characterization of role of *SNM1A*, a 5′–3′ exonuclease, in DNA damage response (Lonnie Swift, Ghadir Almuhaini, Christoffer Lagerholm, Peter McHugh)

Example 3: Relevance of protein motifs upon DNA damage response.



Characterization of role of *SNM1A*, a 5′–3′ exonuclease, in DNA damage response (Lonnie Swift, Ghadir Almuhaini, Christoffer Lagerholm, Peter McHugh)

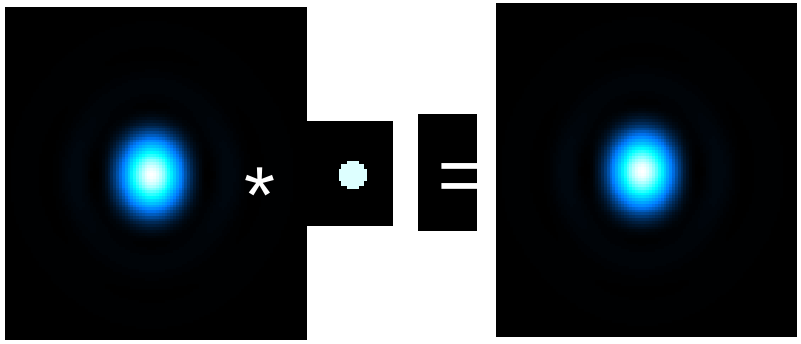
Why so many different types of microscopes?

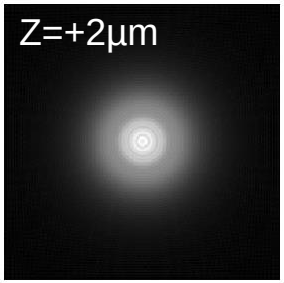
Image Formation

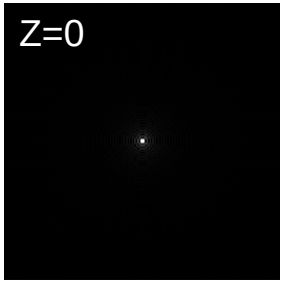
Convolution of microscope optics (PSF) with object = Airy Pattern

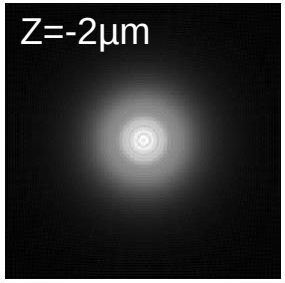
Point Spread Function (PSF)

Object

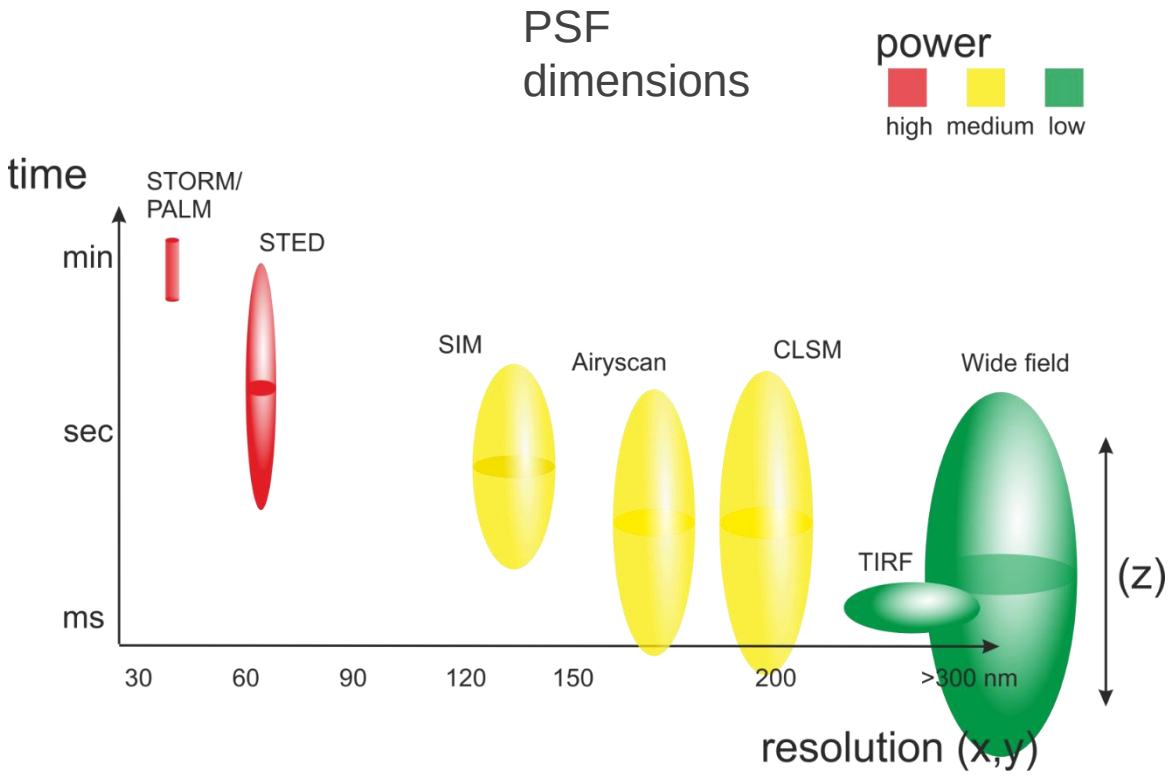






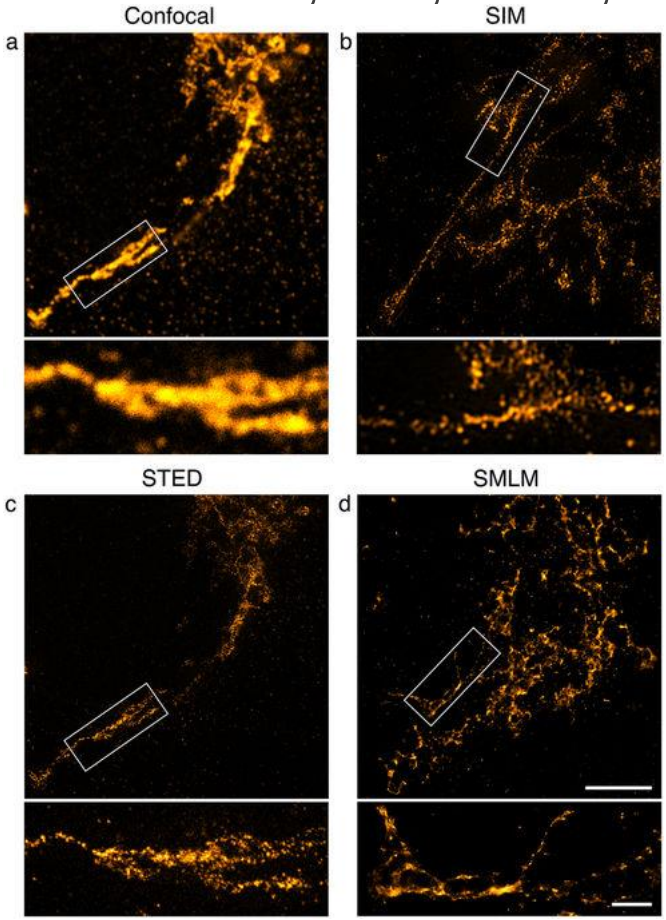


Different microscopes have different PSFs



Modified from Schermelleh, L. et al. *J. Cell Biol.* **2010**, *190*, 165–175.

Image Comparison of Confocal, SIM, STED, and dSTORM

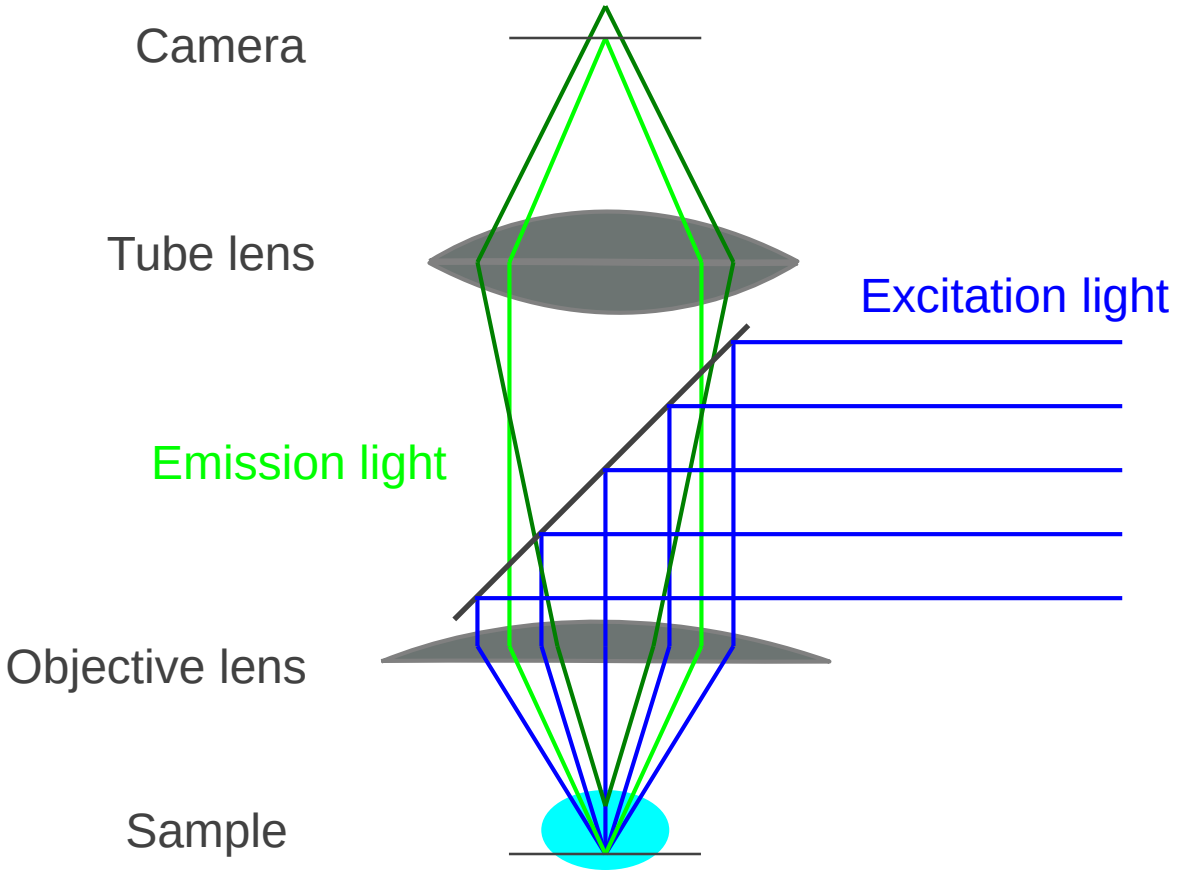


Trans-golgi network in COS7 cells
(TGN46 (Primary+Alexa488 Secondary IgG))

Imaging cellular structures in super-resolution with SIM, STED and Localisation Microscopy: A practical comparison. E Wegel, A Göhler, BC Lagerholm, A Wainman, S Uphoff, R Kaufmann, IM Dobbie. (2016) Scientific Reports 6, DOI: 10.1038/srep27290

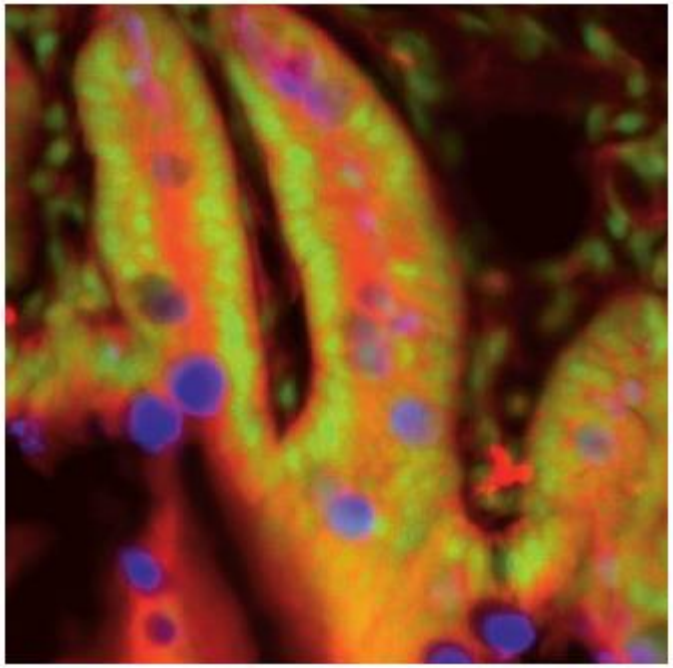
(Laser-scanning) Confocal Microscopy

Conventional Wide-field Microscopy



Problem – fluorescence is emitted along entire illuminated cone, not just at focus

Wide-field Microscopy

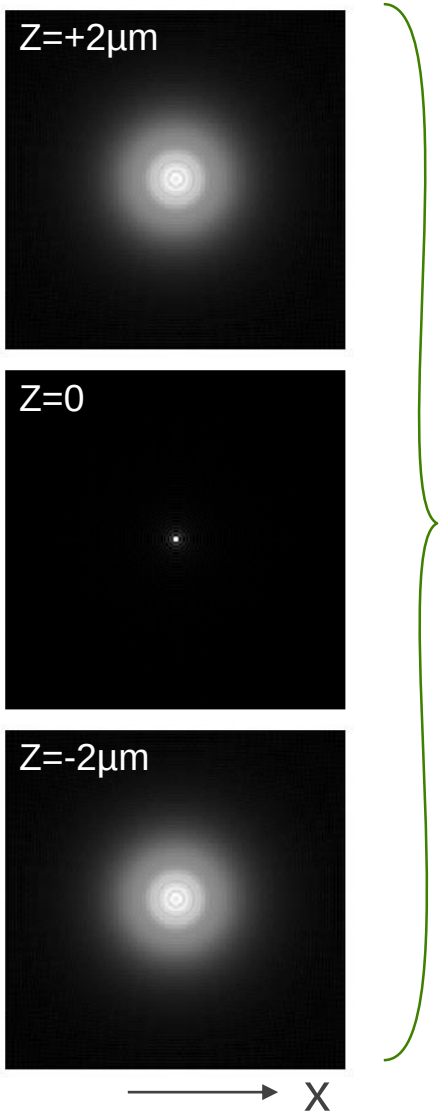


Triple-labeled cell aggregate in mouse intestine section
(Carl Zeiss. Confocal Laser Scanning Microscopy)

The Point Spread Function (PSF)

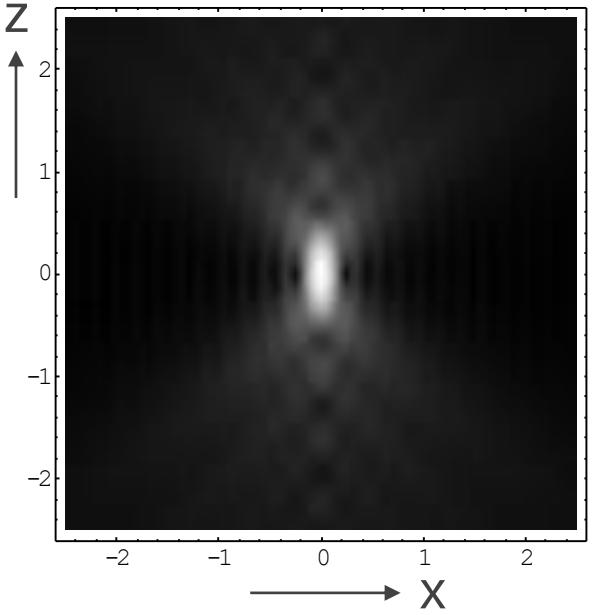
2D PSF for different defocus

The image of a point object

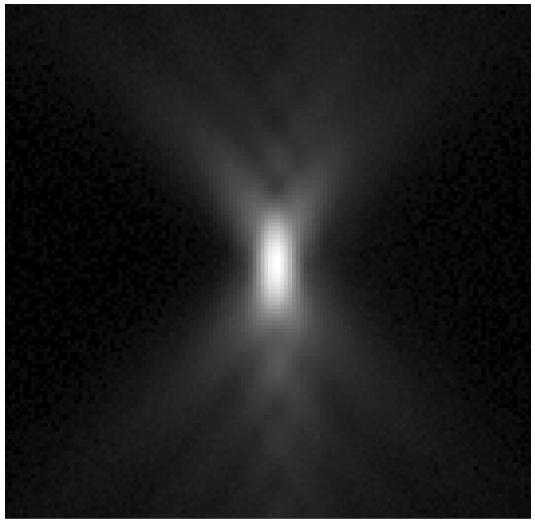


3D PSF

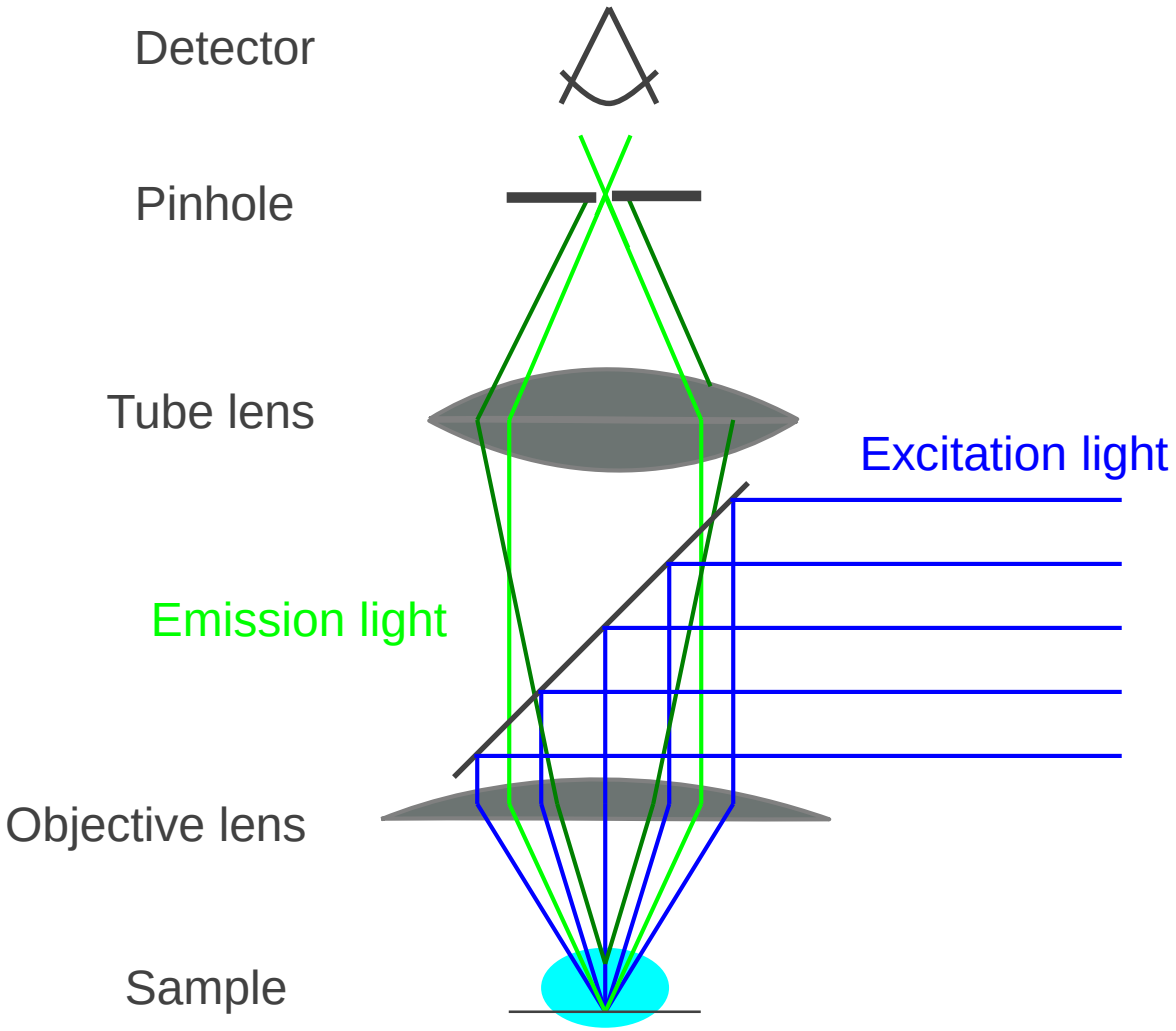
Calculated



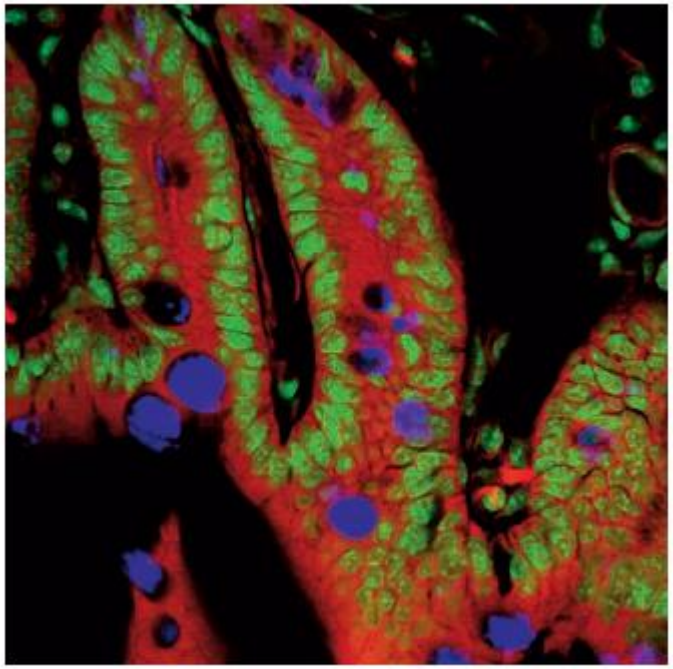
Measured



The confocal microscope

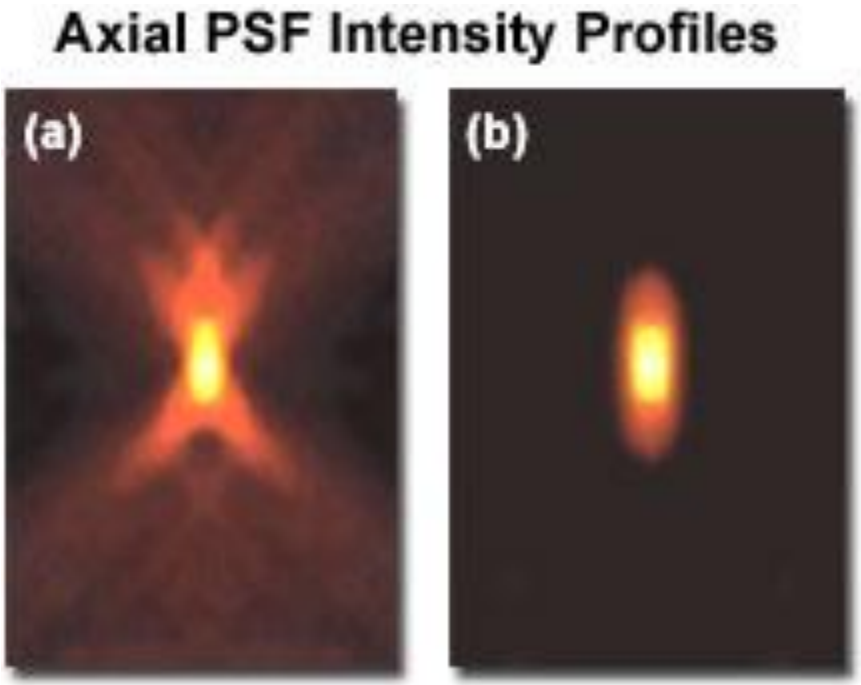


Laser Scanning Confocal Microscopy



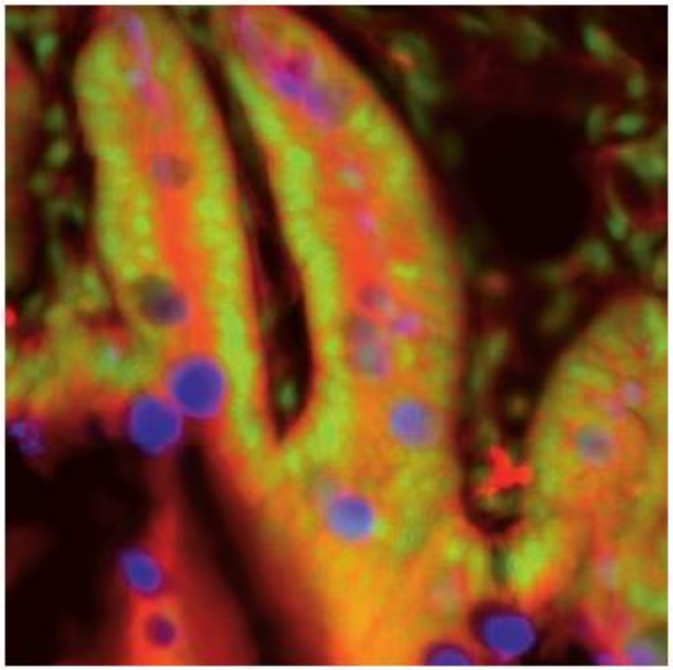
Triple-labeled cell aggregate in mouse intestine section
(Carl Zeiss. Confocal Laser Scanning Microscopy)

What do you get?

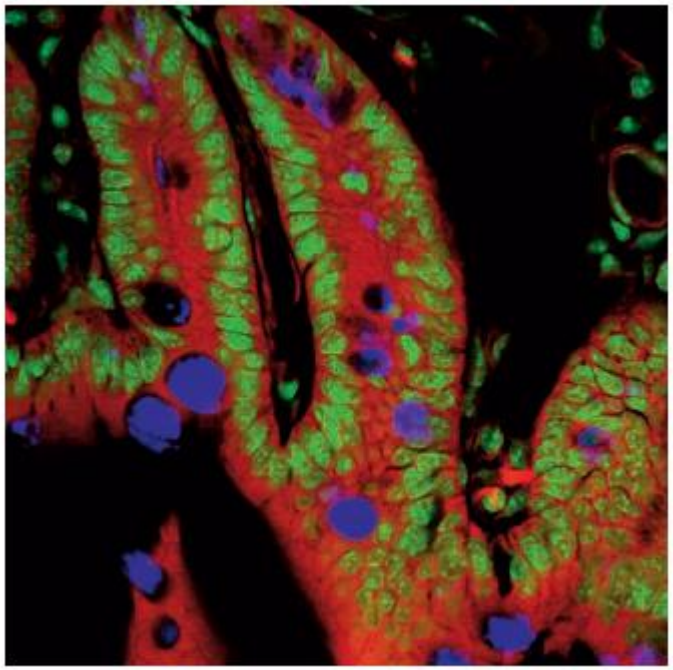


Wide-field = No pinhole Confocal = Pinhole

Wide-field Microscopy



Laser Scanning Confocal Microscopy

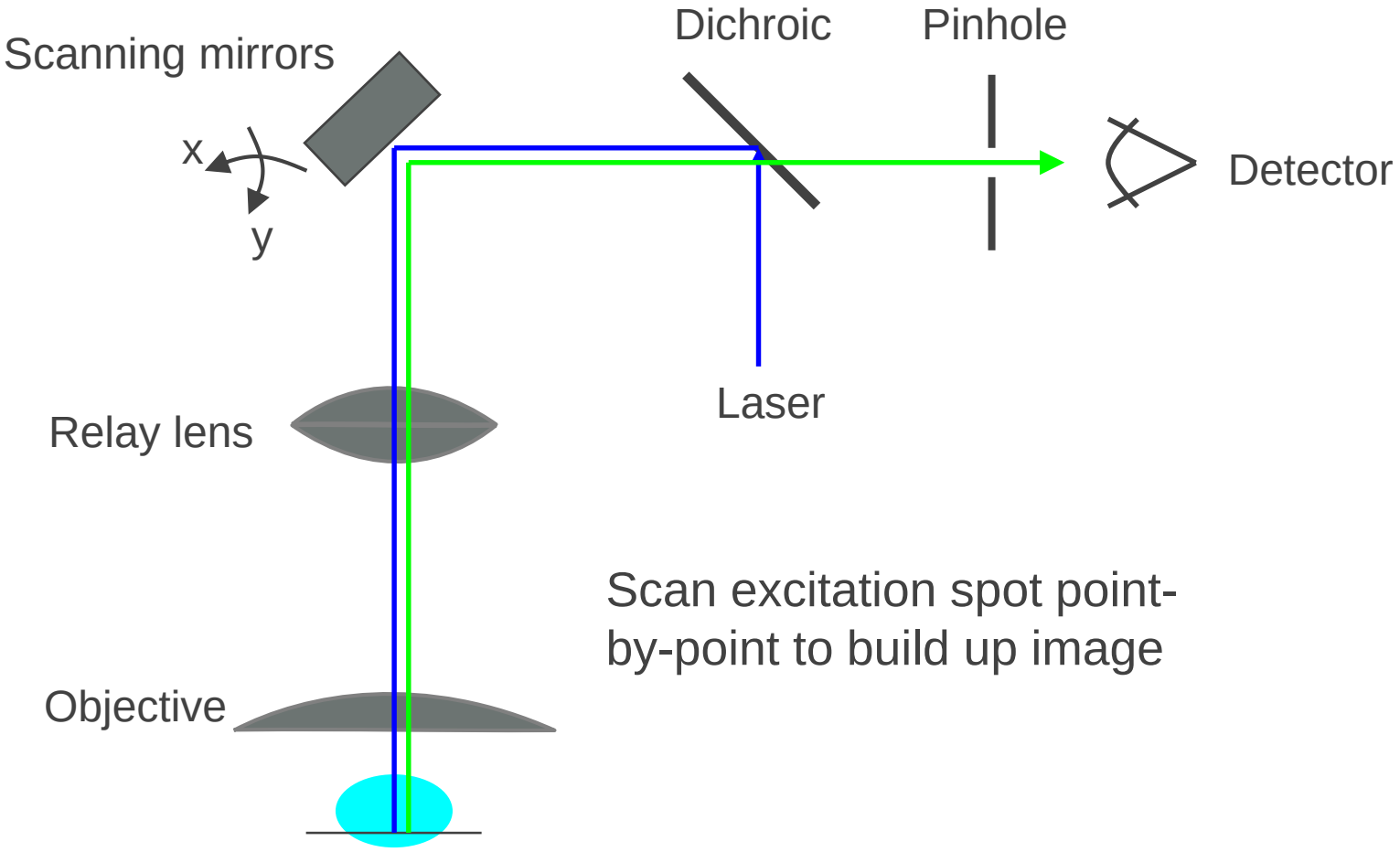


Triple-labeled cell aggregate in mouse intestine section
(Carl Zeiss. Confocal Laser Scanning Microscopy)

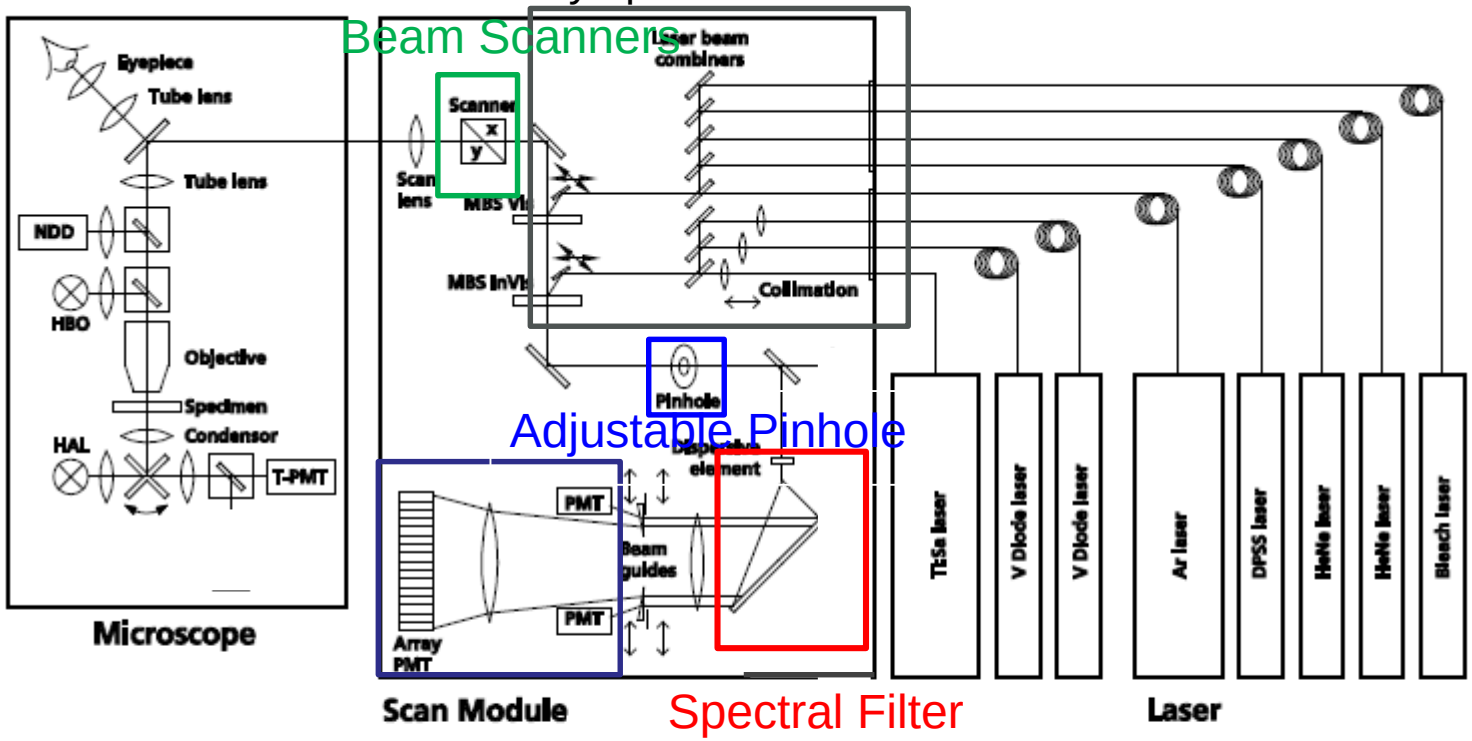
Laser-scanning Confocal Microscope Componets

1) Microscope (Inverted or Upright)
2) Lasers (Fluorescence Excitation)
3) Confocal Scan Module
    1) Relay optics (for Collimation)
    2) Dichroic mirrors (Reflect lasers to sample & Reflect fluorescence emission to detectors)
    1) Scanning mirrors (Enables multi-point imaging)
    2) Adjustable Pinhole(s) (Remove out-of-focus component)
    3) Spectral filters (Enables selection of spectral window to send to detectors)
    4) Single Point Detectors (PMTs, APDs)
4) Electronics (lots)
5) Software!

Laser-scanning Confocal optical path

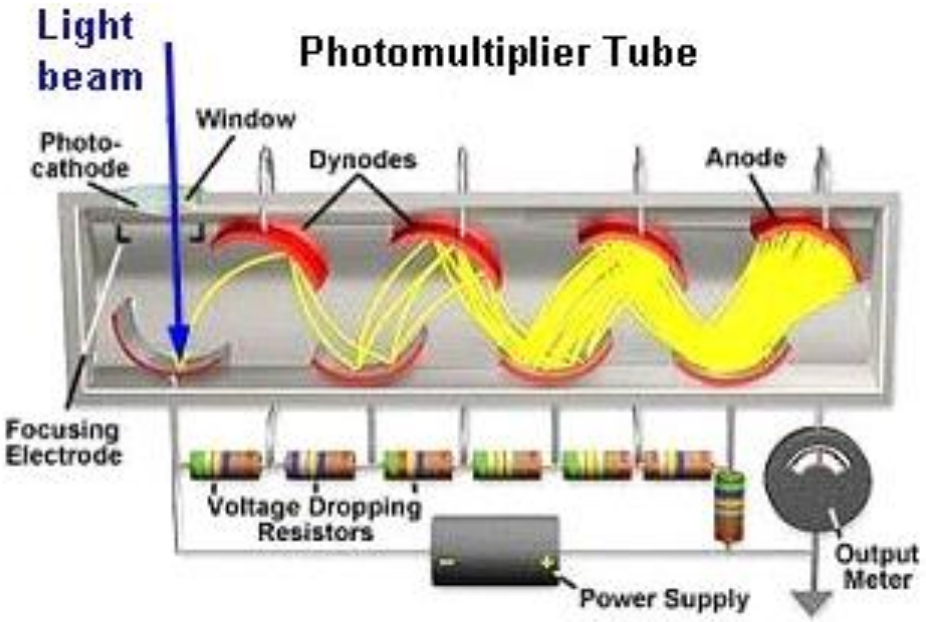


2.3 Optical Diagram of the LSM 710 / LSM 780 (Schematic)



Point Detectors - PMTs

- Must be fast – confocal beam spends only a few μs on each pixel
  - Photomultiplier tubes



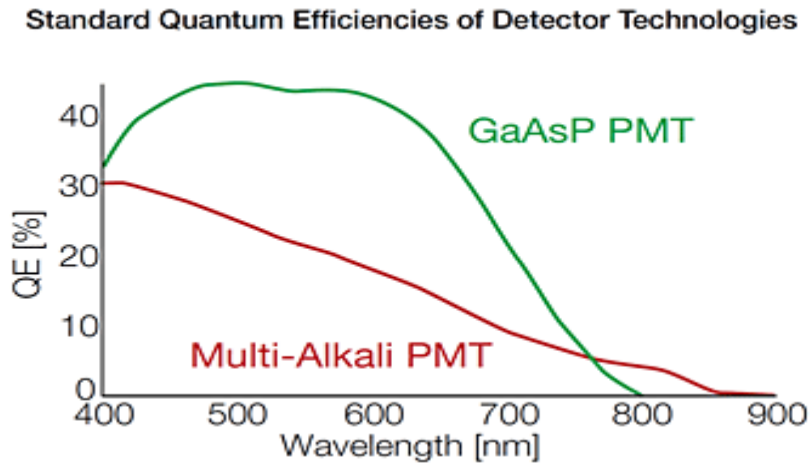
Pulse width for single photon
~ 10-100ns

Very linear

Very high gain

Low read noise

Point Detectors - New vs. Old PMTs



Practical Laser-scanning Confocal Microscopy

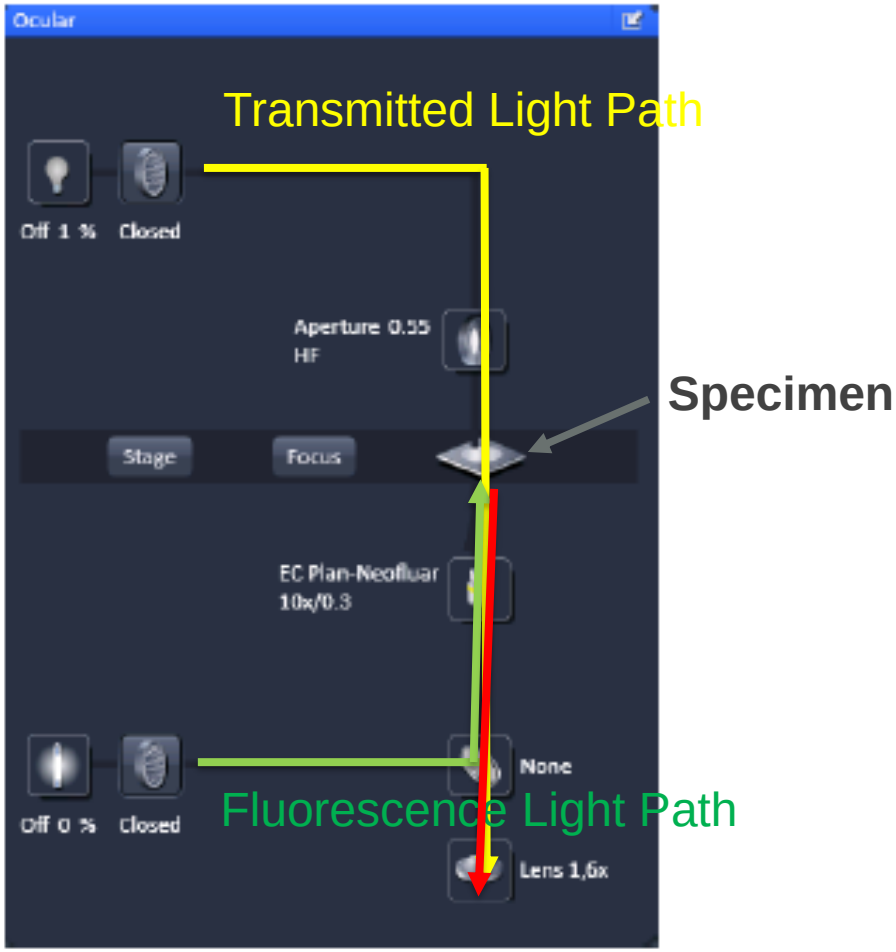
Zeiss 780 Inverted Confocal Microscope

- The Zeiss 780 Inverted Confocal is a conventional laser-scanning confocal microscope that has been optimized for high-sensitivity, multi-color 3D imaging.

- This system is equipped with lasers (405, 458, 488, 514, 561, 594, and 633 nm) and detectors for imaging in the visible spectrum ($400 < \lambda < 700$ nm) .

Zeiss Confocals – Zeiss Zen Software

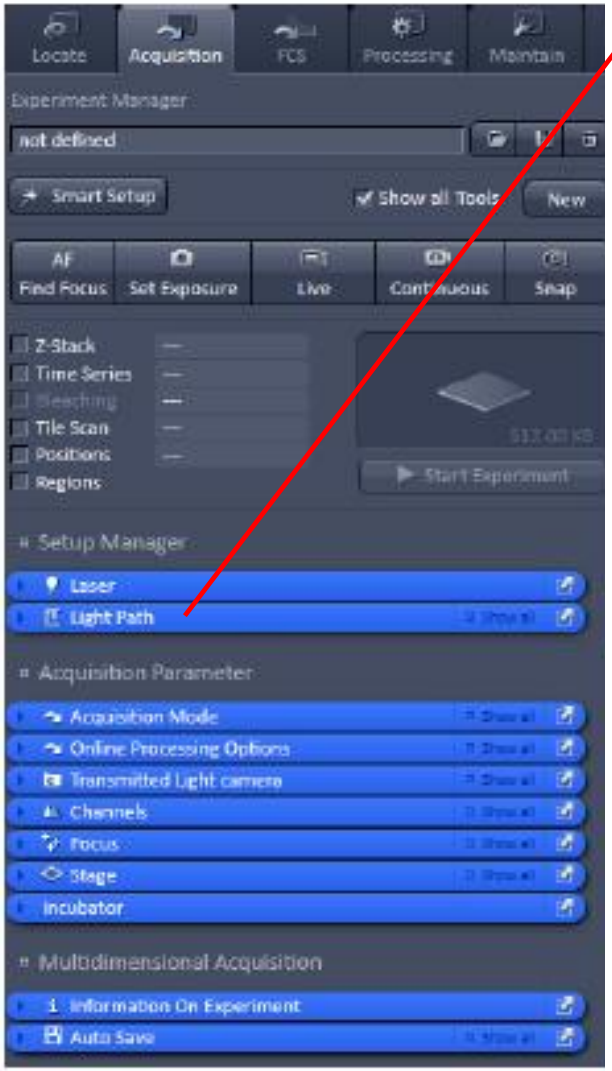
Locate / Ocular Mode – Widefield (for focusing on specimen and to select are of interest)

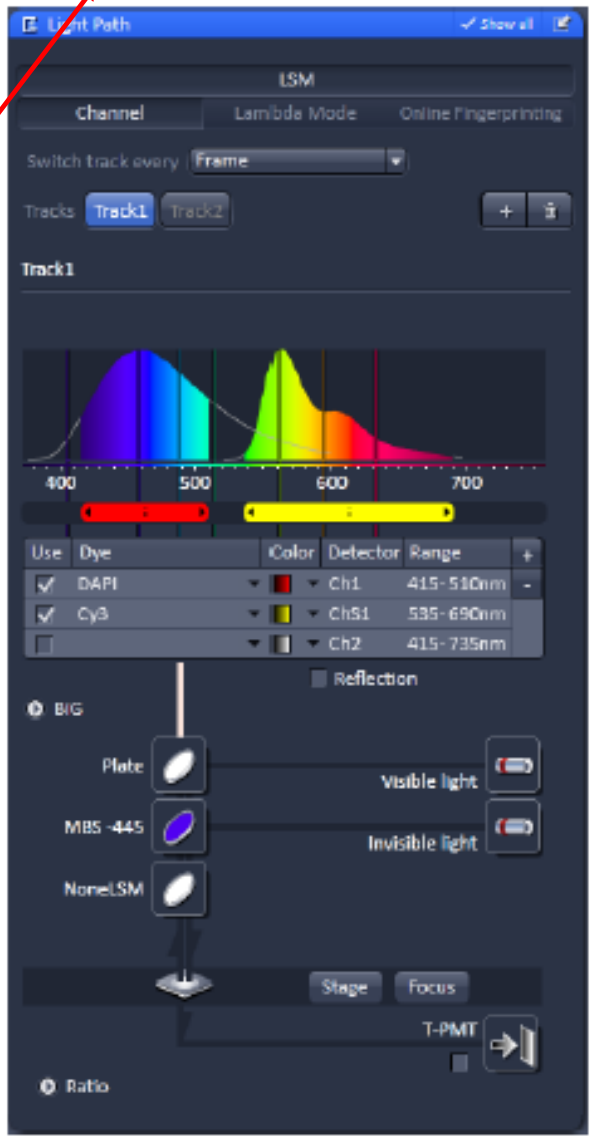


Inverted Microscope

Confocal Mode - Acquisition

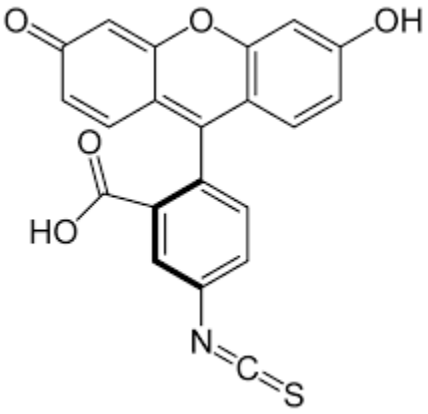
Light Path – Zeiss 780



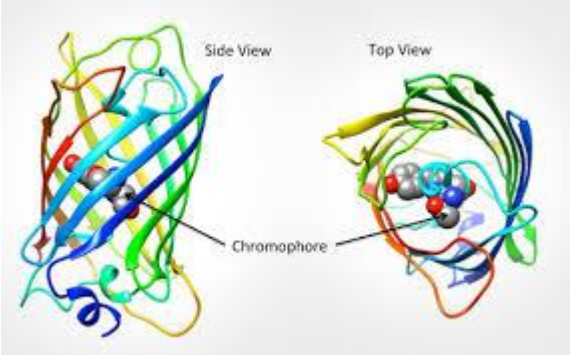


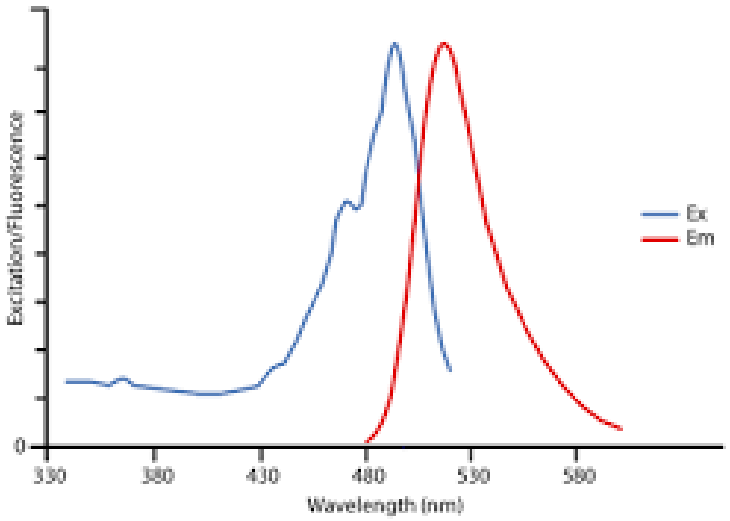
Designing the light path in confocal microscopy

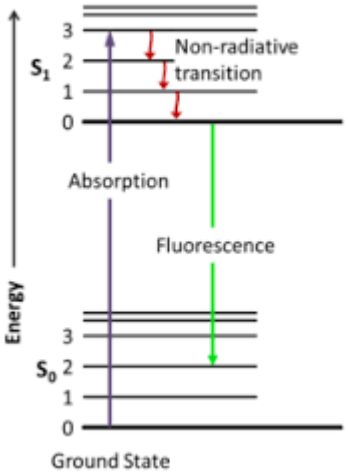
Fluorescein



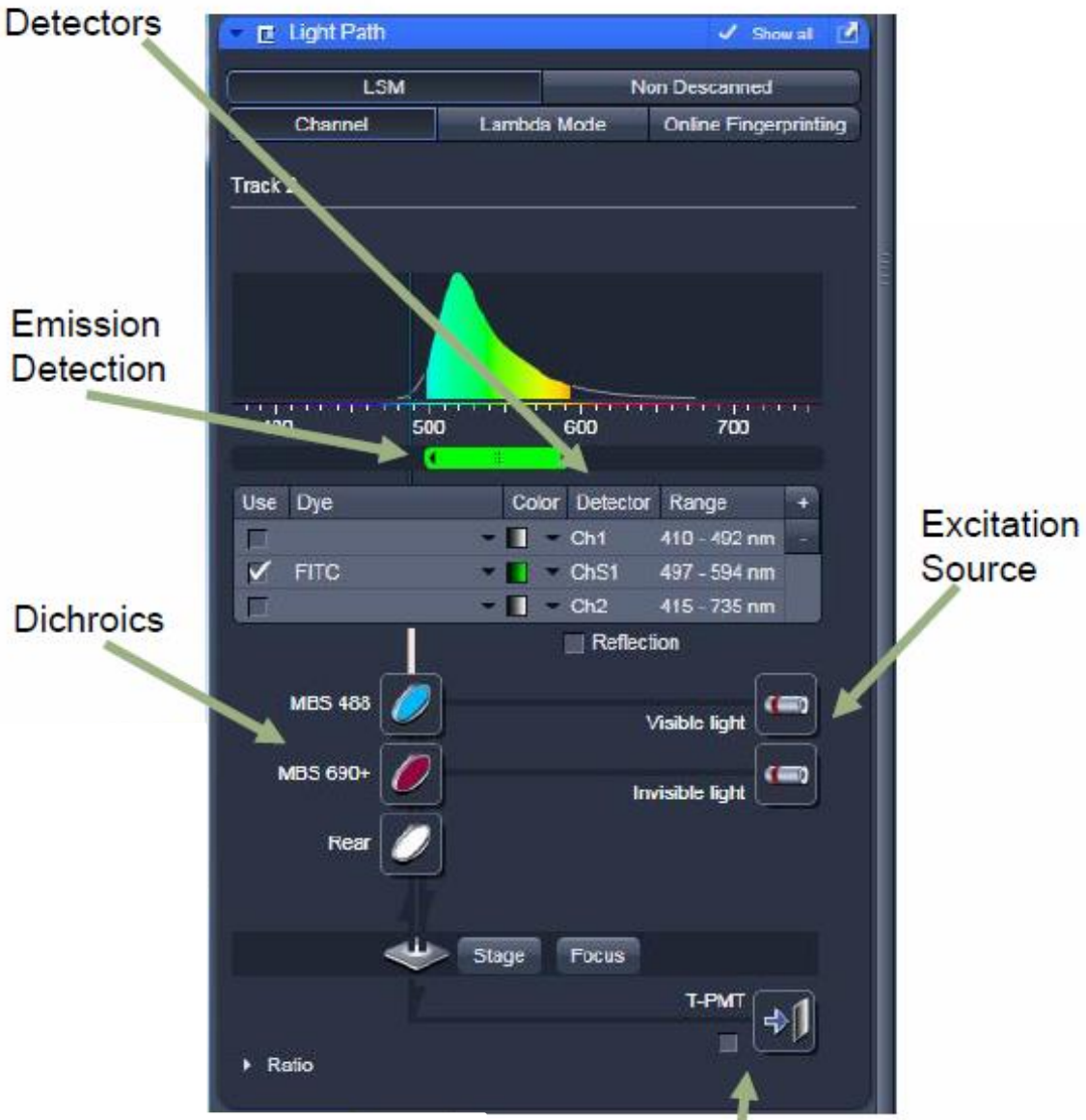
Green Fluorescent Protein







Single Imaging Track



Multiple Imaging Tracks

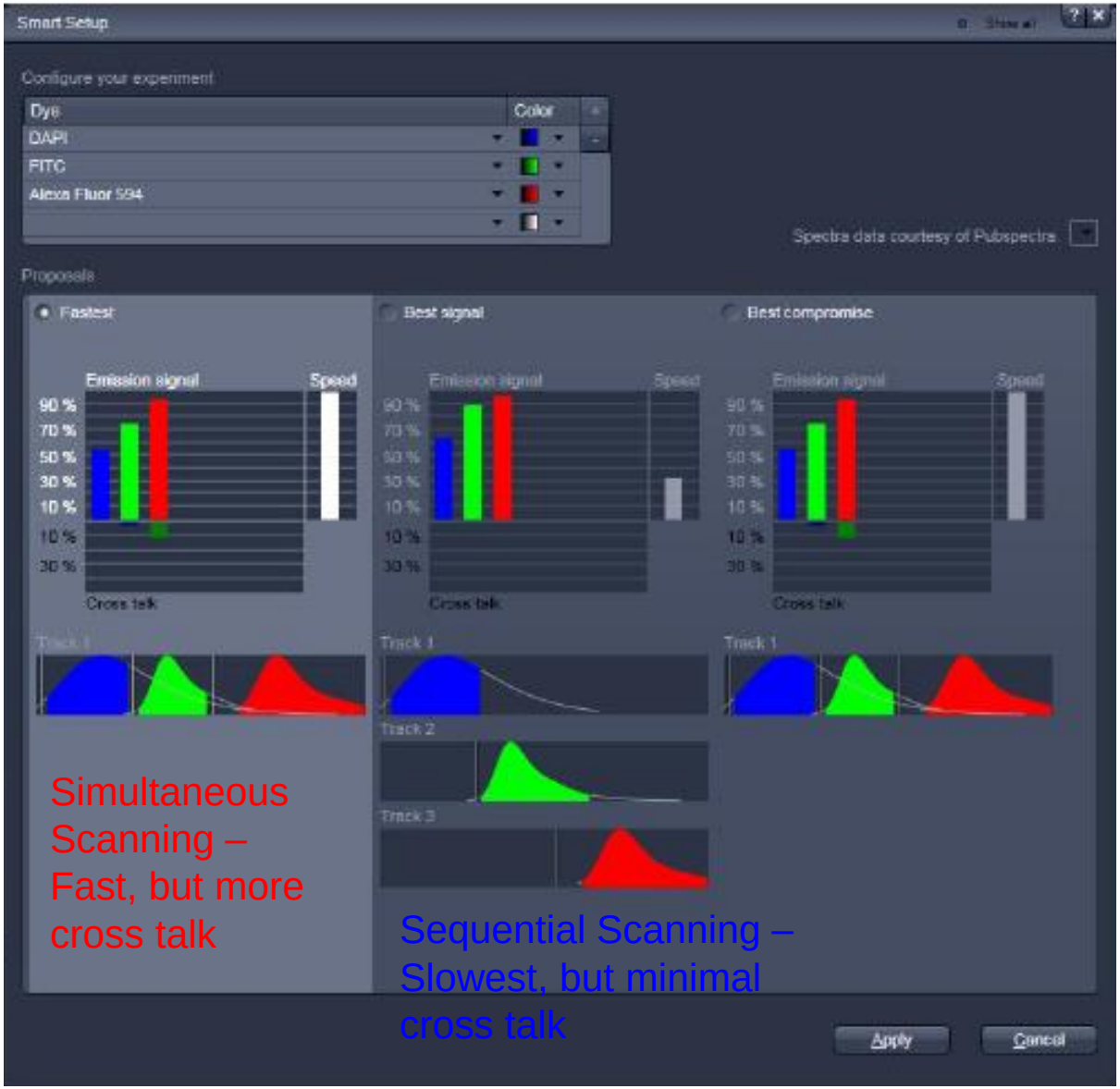
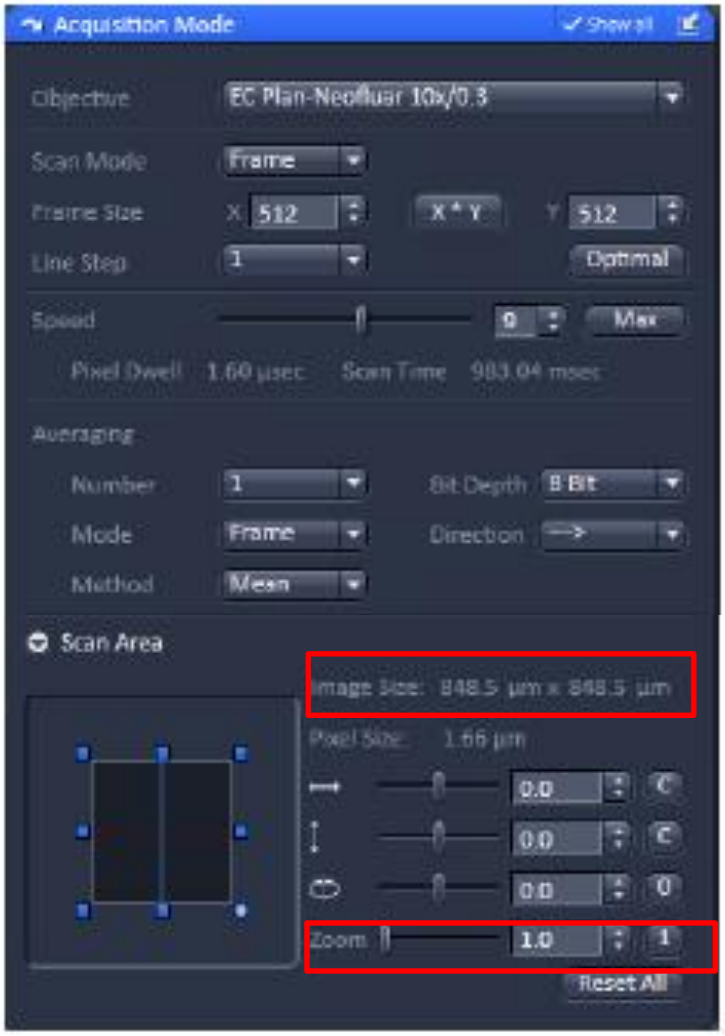


Image optimization on a Confocal Microscope – Image size, Scan speed, Bit depth, Scan Direction, Zoom, Averaging

1. Scan Mode (Line/Frame/Spot)
2. Image Size
3. Scan speed (Pixel Dwell Time)
4. Bit depth (Gray Levels, 12-bit=2^{12}=4096)
5. Scan Direction (Mono/Bi-directional)
6. Magnification Zoom *****
7. Averaging

Image optimization on a Confocal Microscope – Optical Zoom



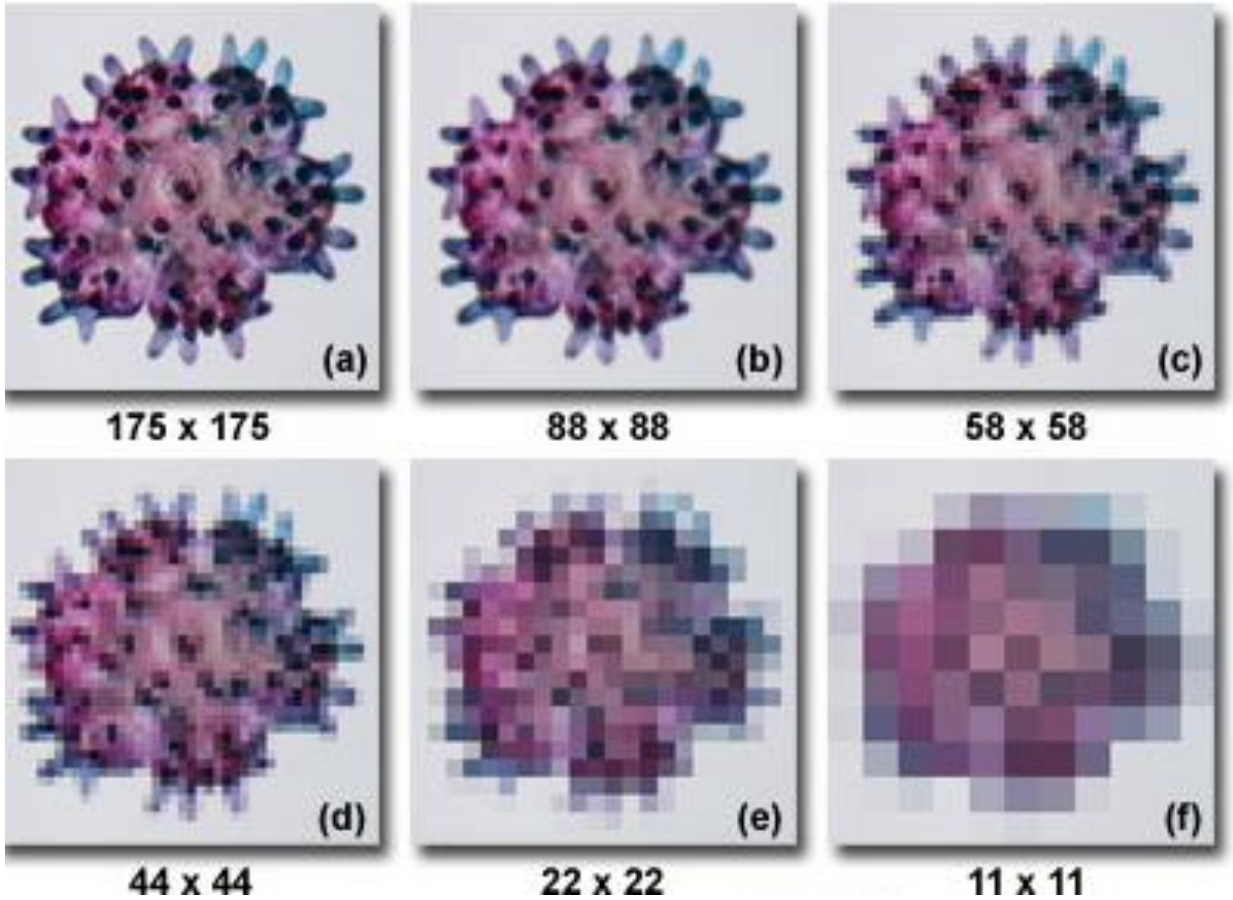
Optimal Sampling ➡ Pixel Size in Image = $\frac{\text{Resolution}}{3}$

Magnification Zoom

6. Magnification Zoom

Nyquist sampling theorem – lateral

- the size of the pixel should be 2-3x smaller than the lateral optical resolution to realize maximum optical resolution



Consequences of not sampling at Nyquist

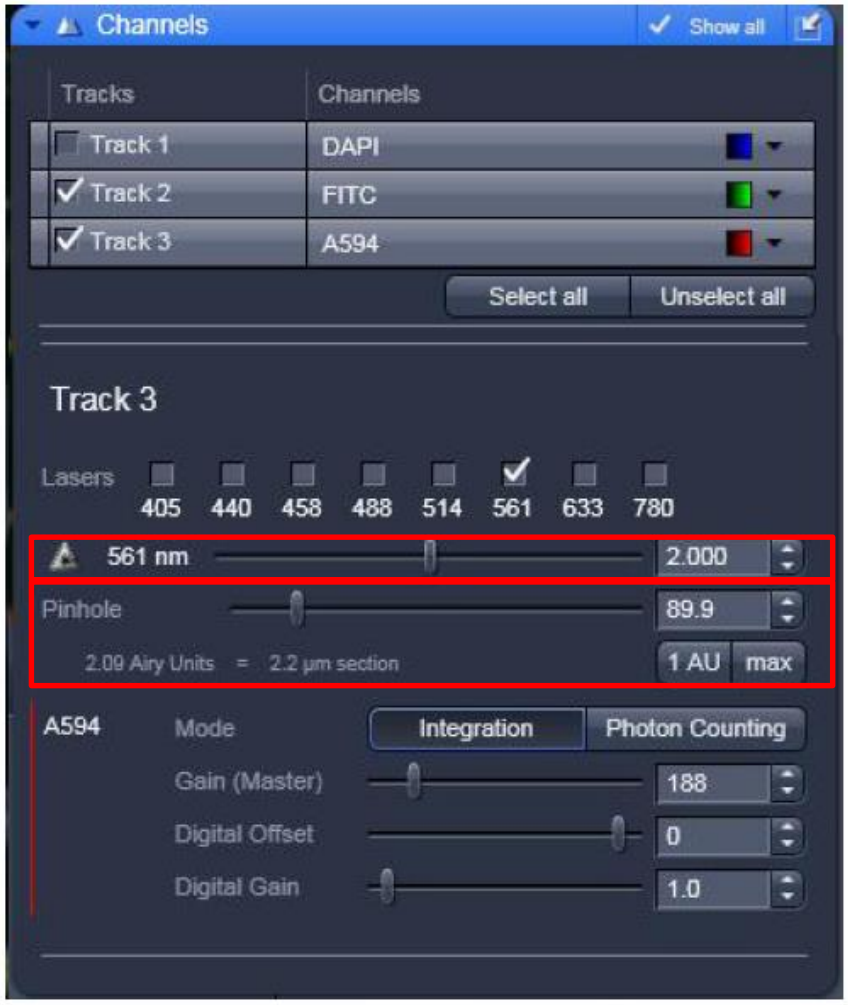
Oversampling

- pixels small compared to the optical resolution
- specimen needlessly exposed to light
- image needlessly large

Undersampling

- degraded spatial resolution
- photobleaching reduced
- image artefacts (eg. aliasing)

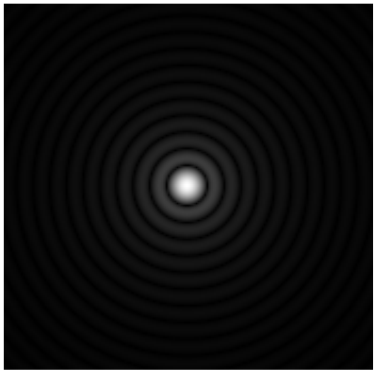
Image optimization on a Confocal Microscope – Laser Power, Pin hole size, Gain (Master), Digital Offset



1. Laser Power
2. Pinhole

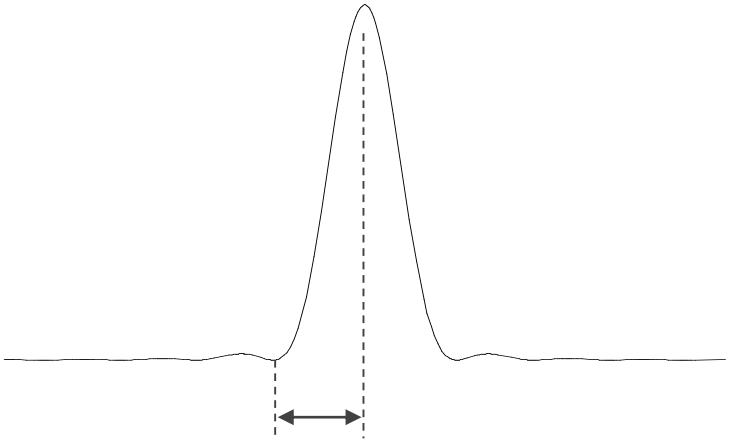
Adjustment of pin hole size for image optimization

Resolution is limited by the point-spread function



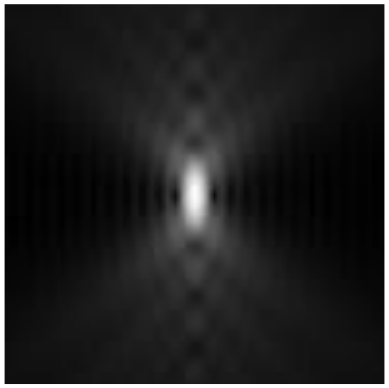
Y

X



Airy disk radius

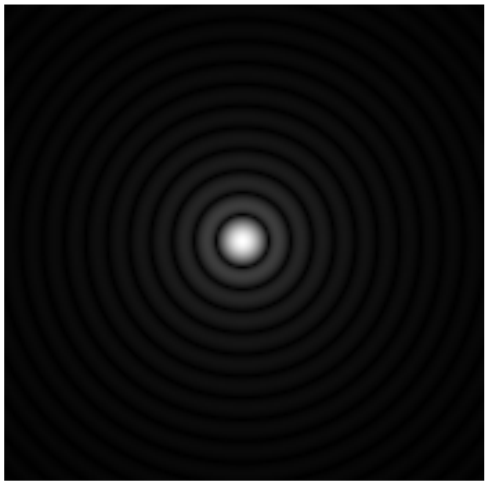
≈ 0.61 λ /*NA*



Z

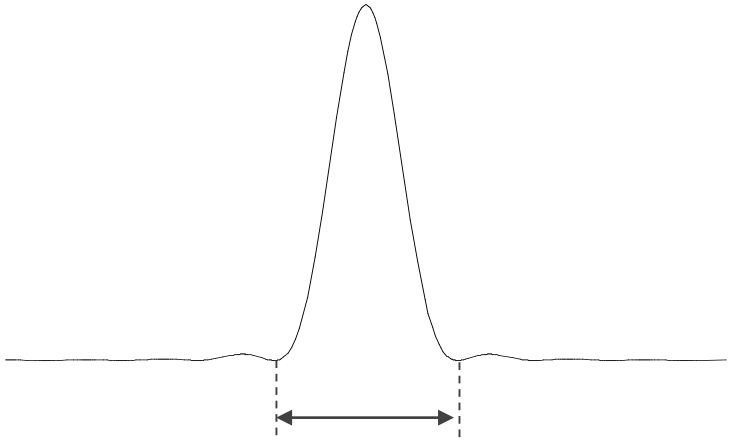
How big should your pinhole be?

Want pinhole to pass entire Airy disk



Y

X

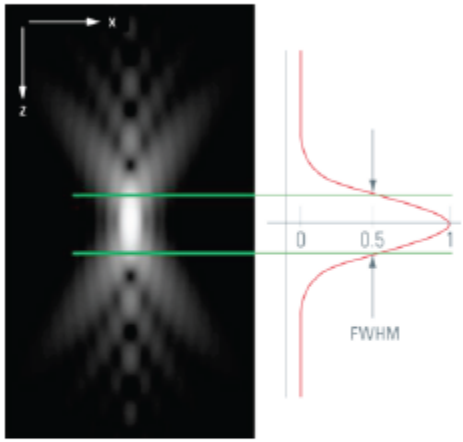


Airy disk diameter
≈ 1.22 λ /*NA*

Width of point spread function at pinhole:
Airy disk diameter × magnification of lens

How big should your pinhole be?

- Width of point spread function at pinhole =
  Airy disk diameter × magnification of lens = 1 Airy unit
  = resolution of lens × magnification of lens × 2
  - 100x / 1.4 NA: resolution = 220nm, so 1 Airy unit = 44 μm
  - 40x / 1.3 NA: resolution = 235nm, so 1 Airy unit = 19 μm
  - 20x / 0.75 NA: resolution = 407nm, so 1 Airy unit = 16 μm
  - 10x / 0.45 NA: resolution = 678nm, so 1 Airy unit = 14 μm



FWHM=Full Width Half-Maximum

Shorter the wavelength the thinner the optical section

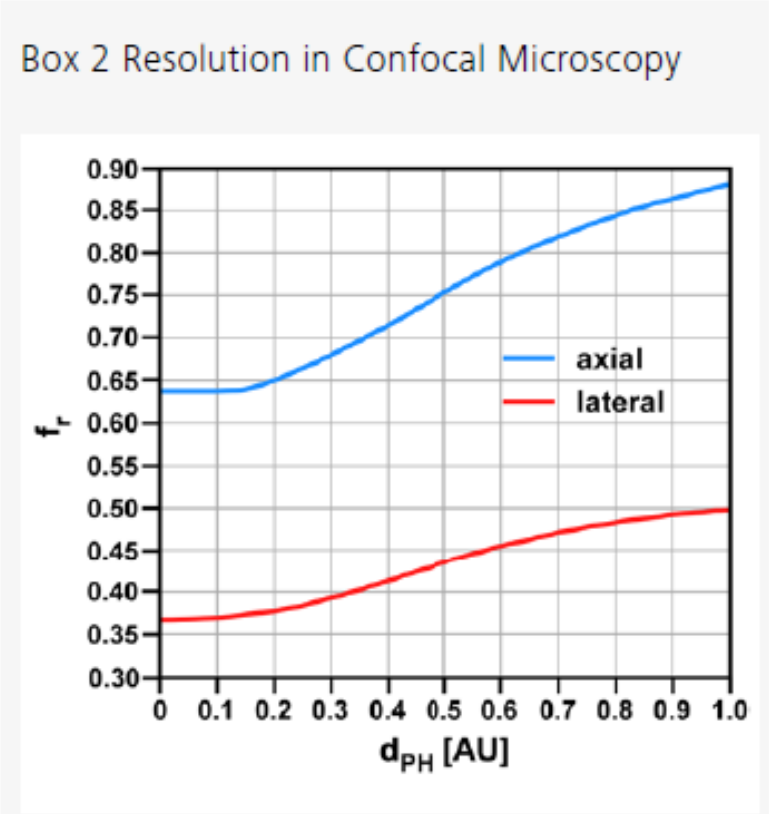
$$FWHM_{tot,axial} = \frac{0.64\,\overline{\lambda}}{\left(n - \sqrt{n^2 - NA^2}\right)}$$

Diameter of the pinhole
Larger pinhole thicker section

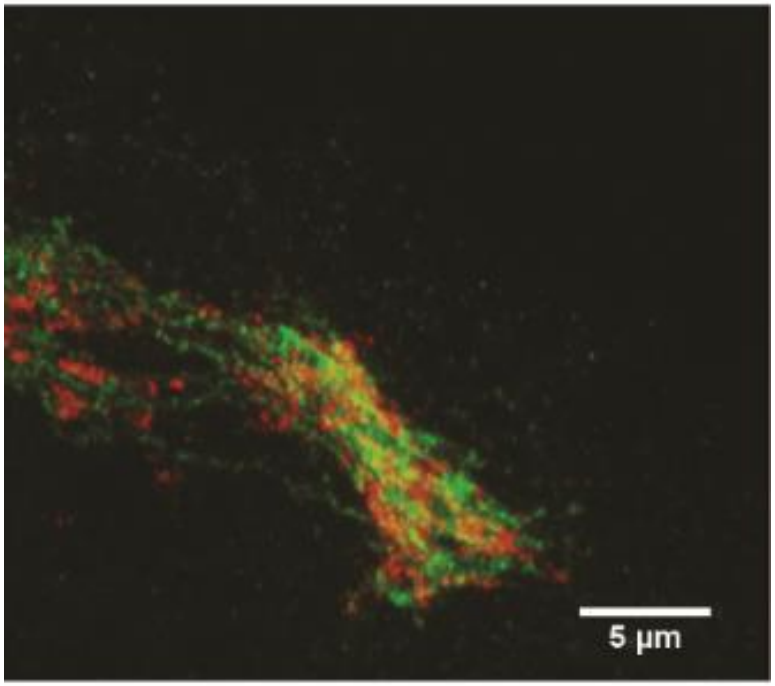
The higher the NA.
the thinner the section

Weak signal > open pinhole > more light but thicker section

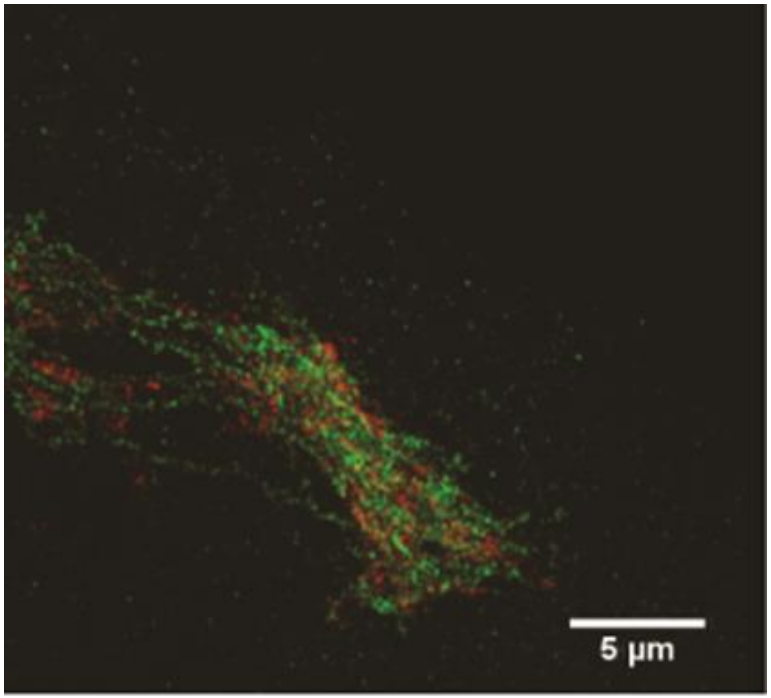
But beware – Resolution of confocal is improved with smaller pinhole setting – Airyscan/Lightening



Confocal (pinhole=1AU)

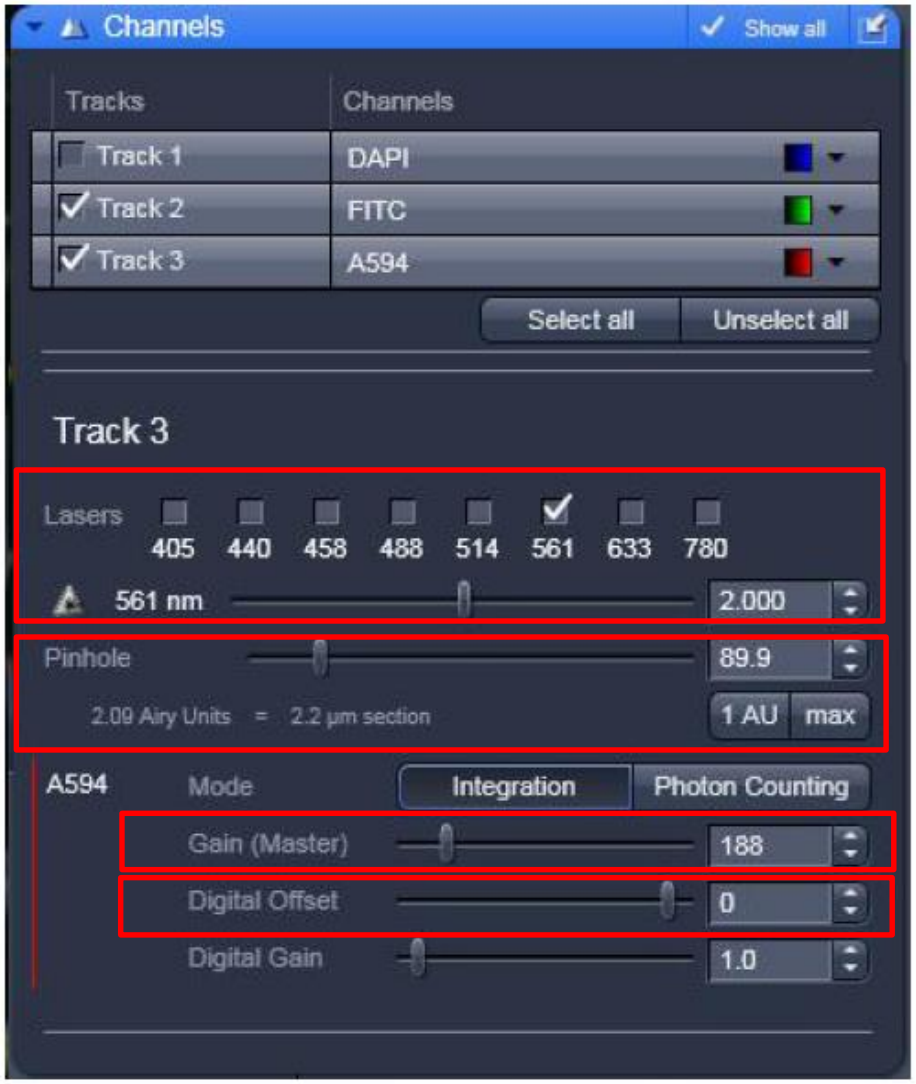


Airyscan (pinhole=0.2 AU)



Dr. Ian Dobbie, Antonia Goehler, Alan Wainman, Rainer Kauffman, Micron, Biochemistry, U. of Oxford
Dr. Eva Wegel, John Innes Centre (previously Micron, Biochemistry, U. of Oxford)

Image optimization on a Confocal Microscope – Laser Power, Pin hole size, Gain (Master), Digital Offset



1. Laser Power

2. Pinhole

3. Gain (Master)
4. Digital Offset

Adjustment of detector gain/offset for image optimization

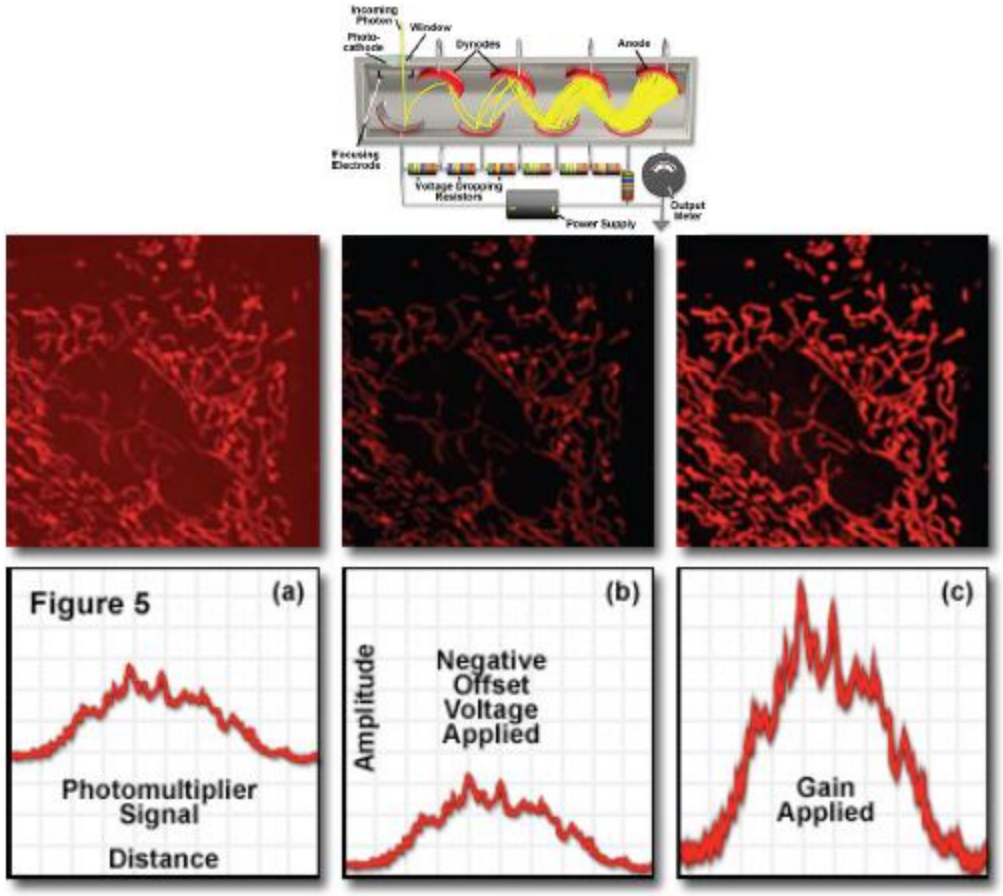
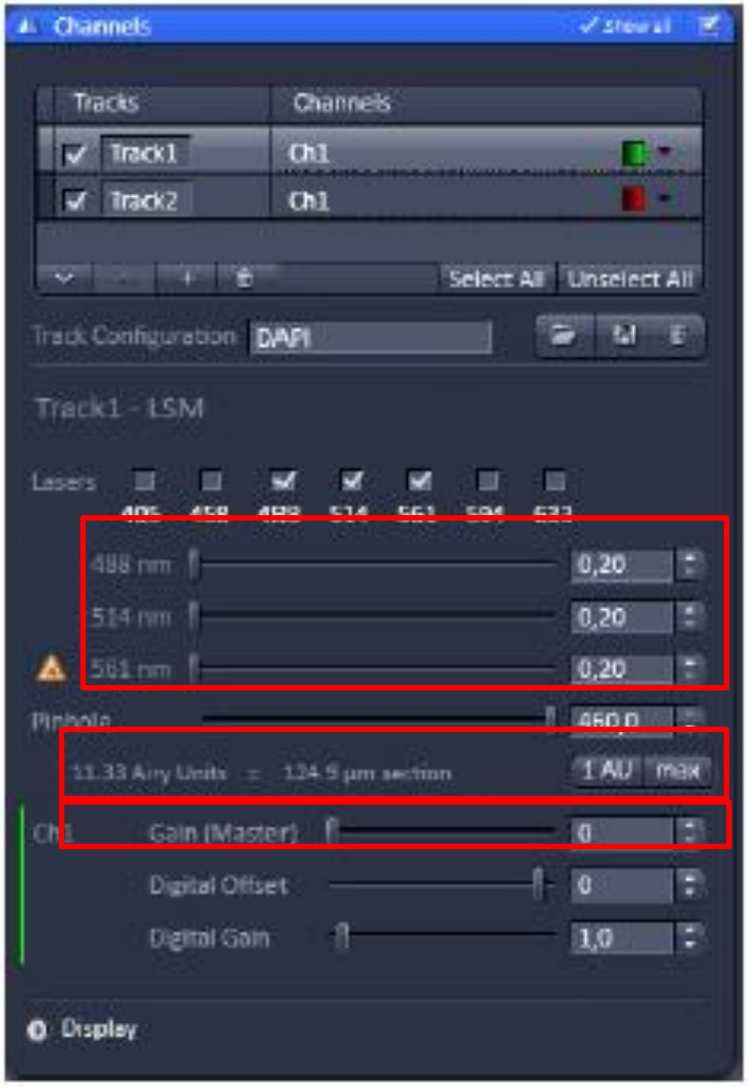
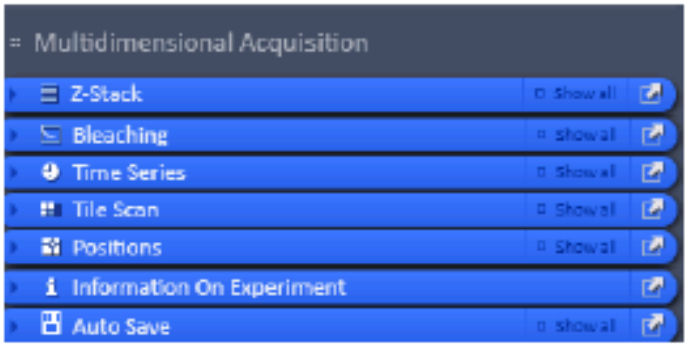


Image optimization on a Confocal Microscope – Image Brightness

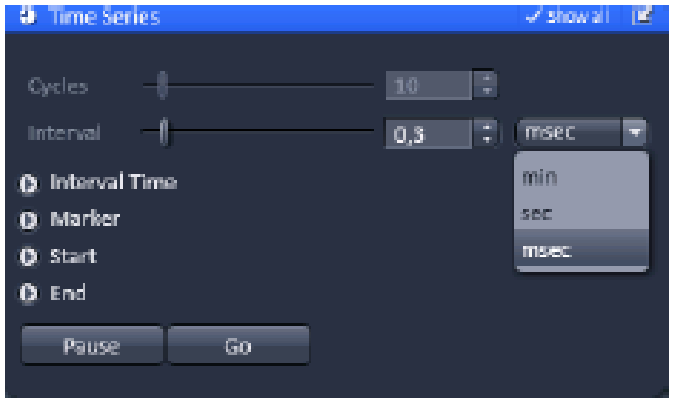
Scan speed, Zoom, Laser Power, Pinhole size, Gain (Master)



Multi-dimensional Acquisition

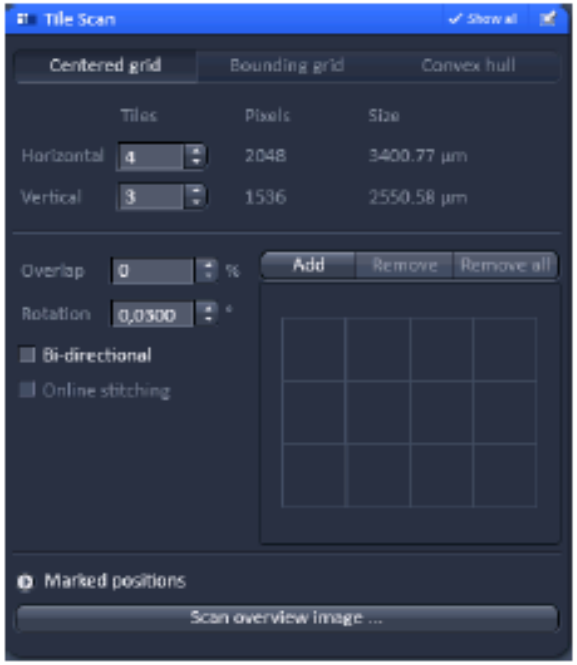


Time-lapse

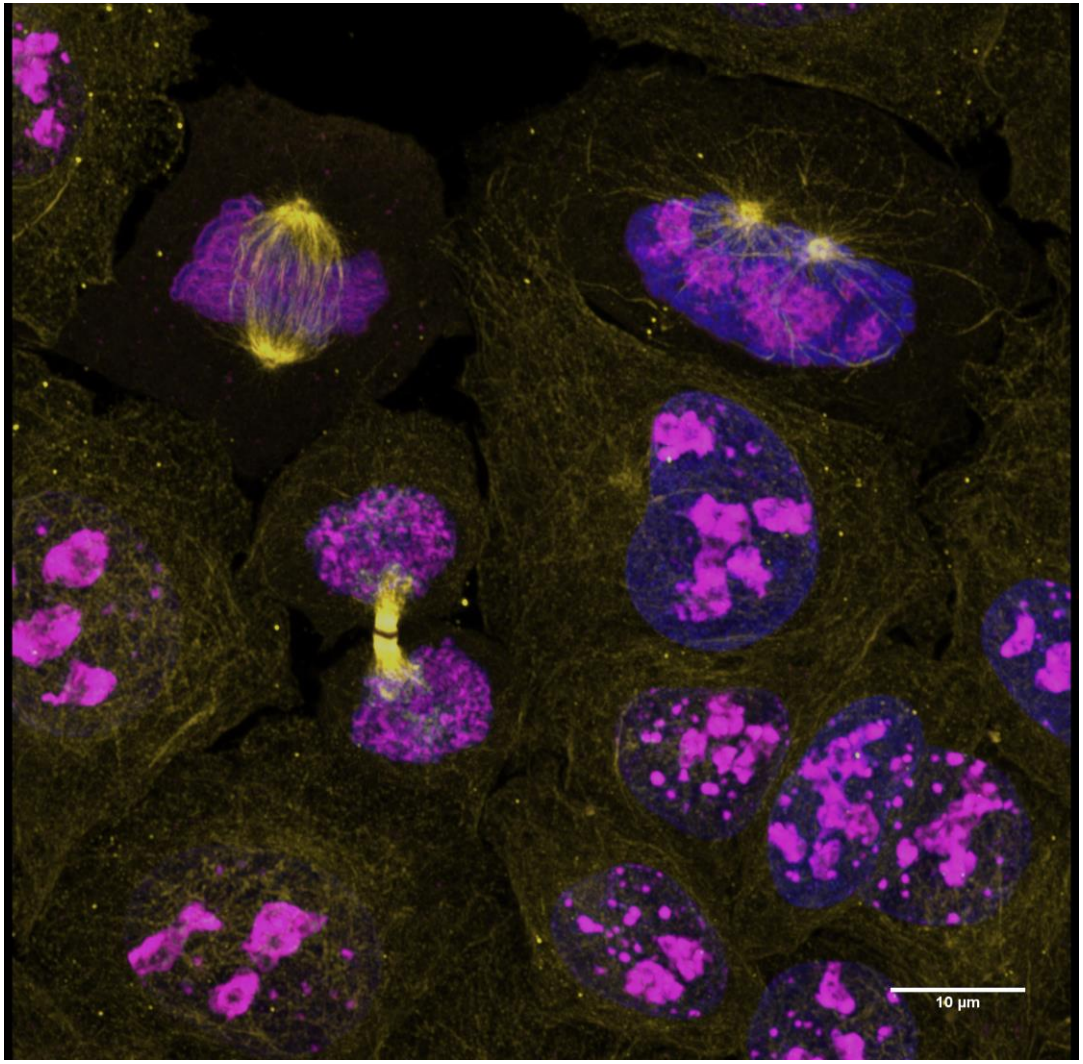


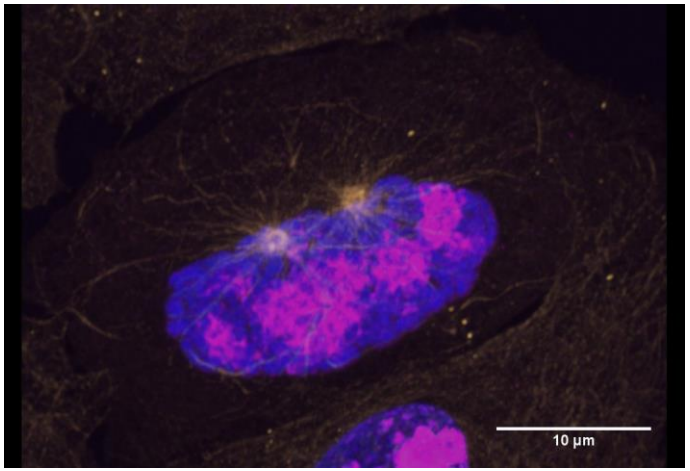
Z-stack

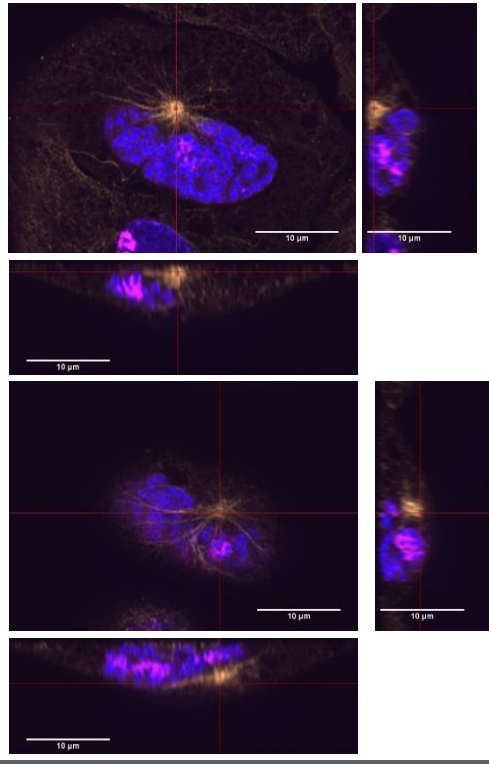
Tile-scan



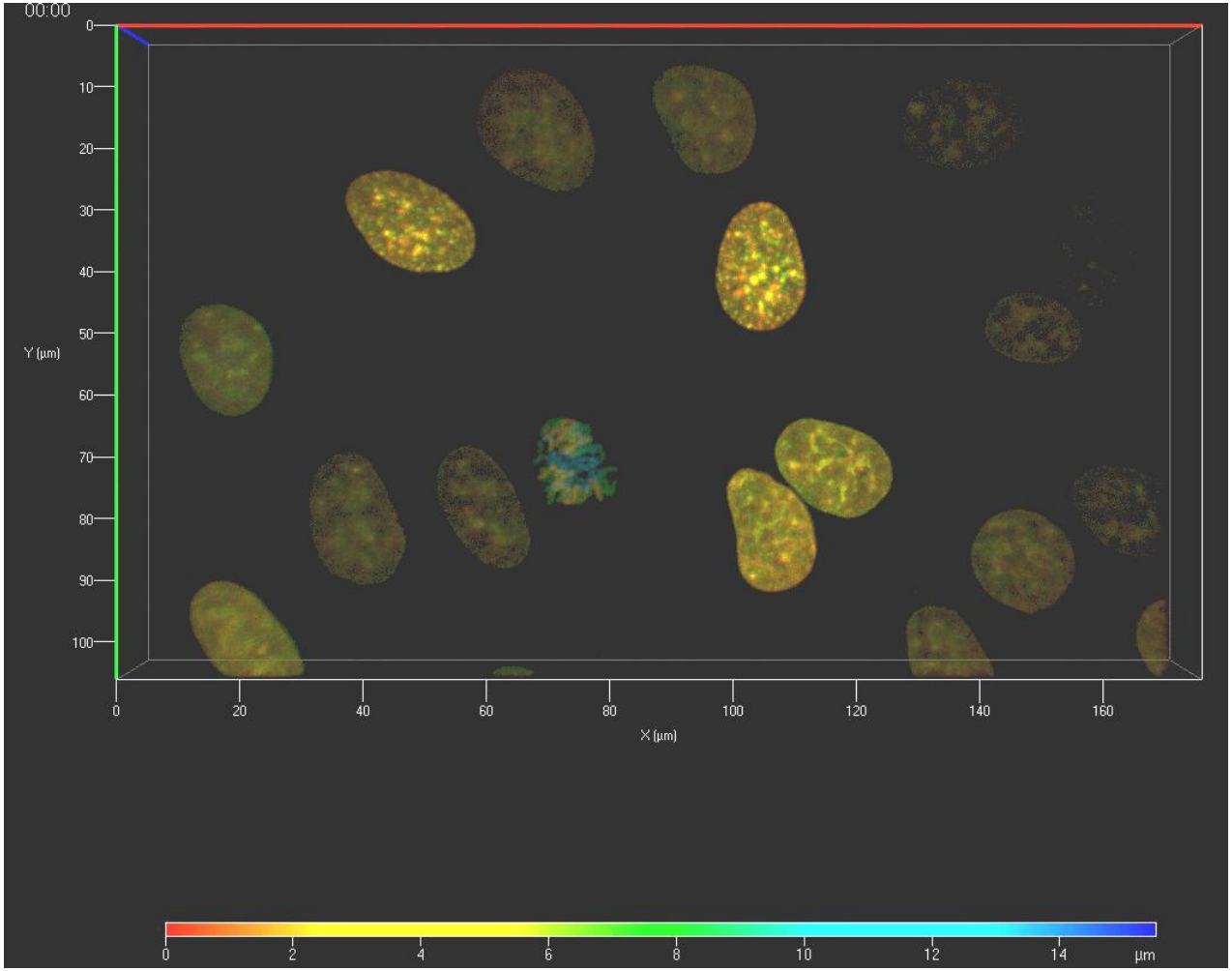
- **Advanced confocal microscopy**
  - **Spinning Disc (Fast Confocal at fixed pinhole size)**
  - **Multi-photon (Deep imaging)**
  - **Airyscan (Confocal with 0.2AU pinhole)**
  - **FCS/FRAP/IRM/DNA damage.....**


10 µm

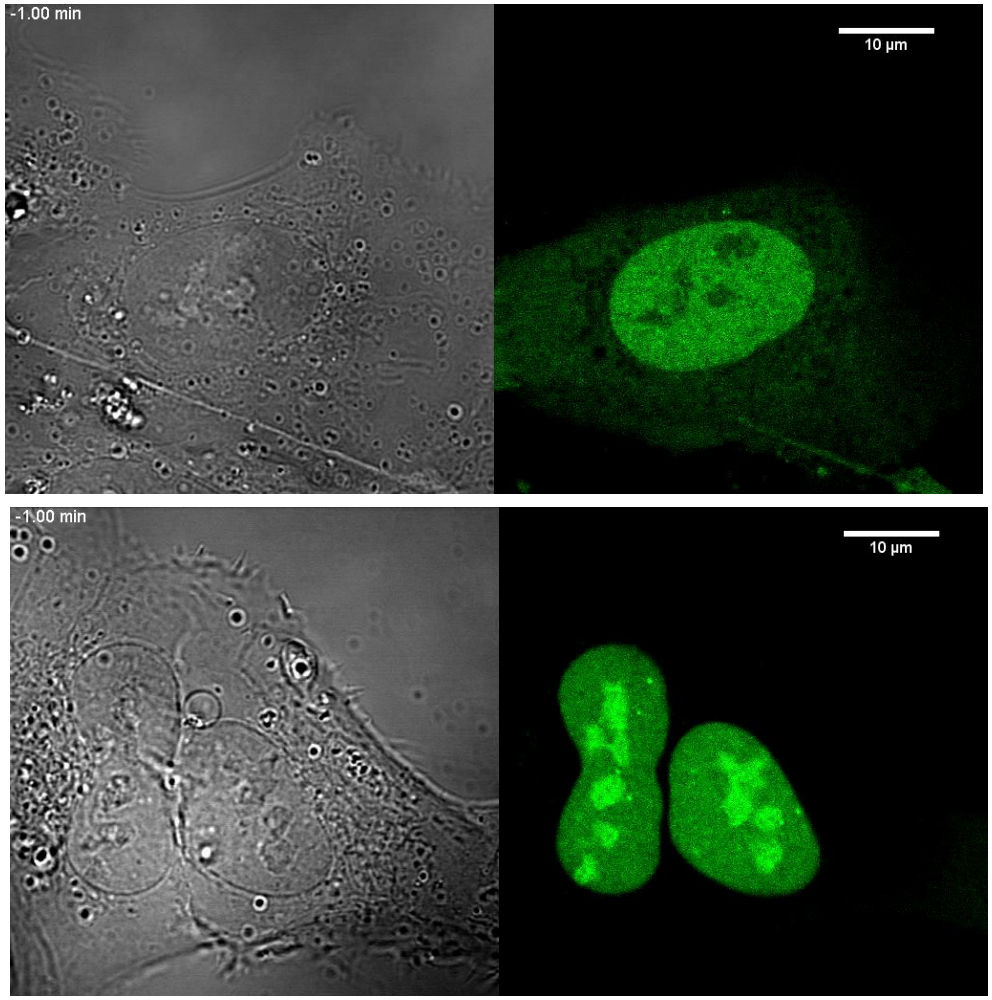

10 µm



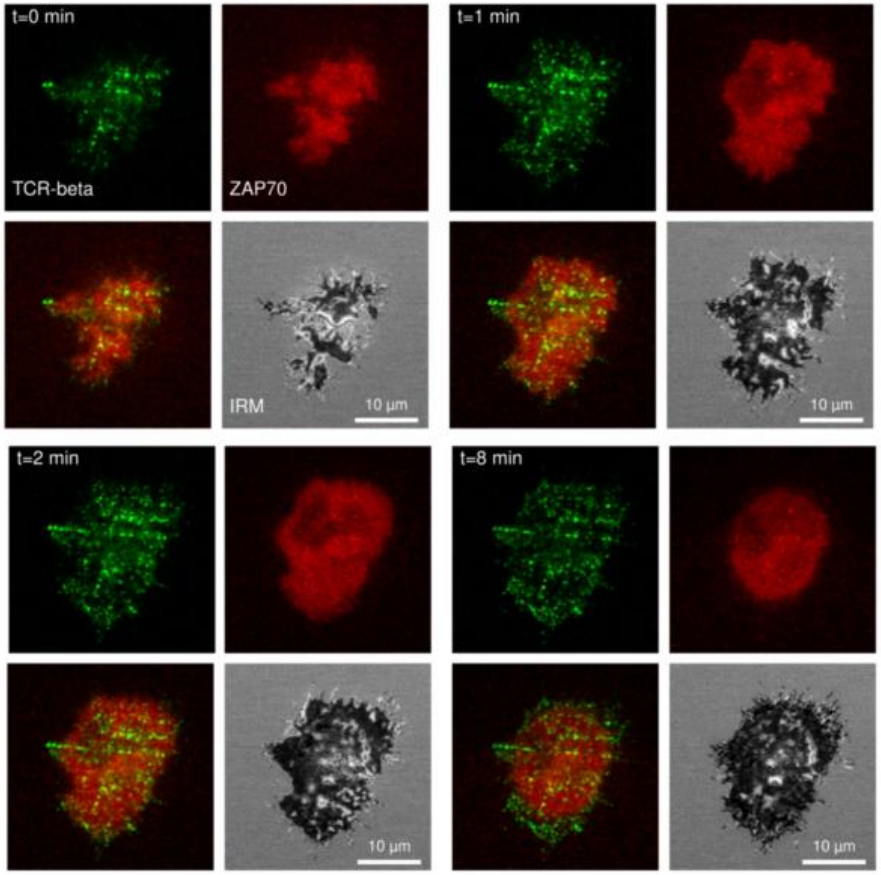
Example 4: Another Cell cycle example; HeLa cells expressing Histone 2B-GFP imaged on spinning disc microscope



DNA damage induced with 405 nm laser

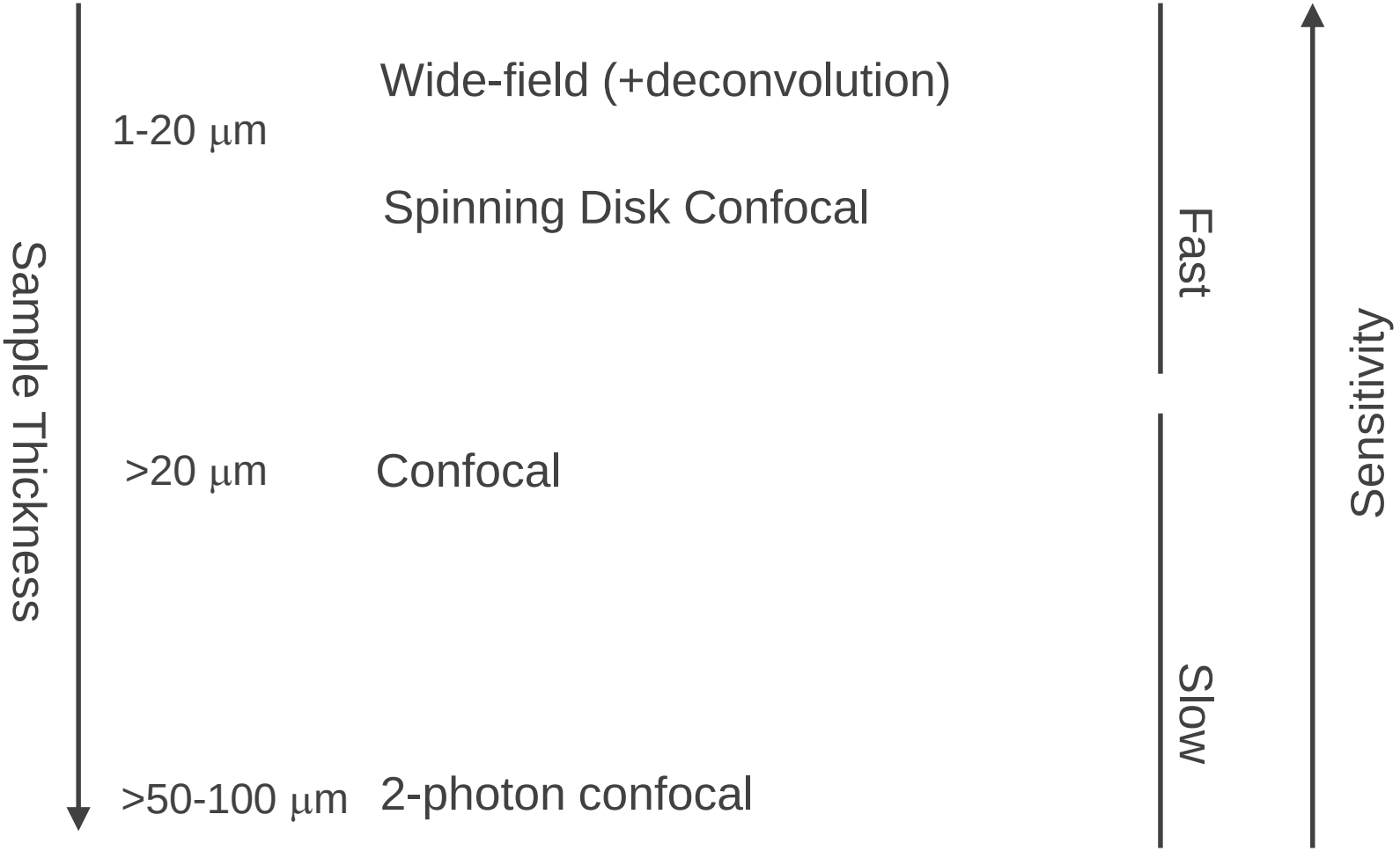


Characterization of role of *SNM1A*, a 5’–3’ exonuclease, in DNA damage response (Lonnie Swift, Ghadir Almuhaini, Christoffer Lagerholm, Peter McHugh)



Spatio-temporal dynamics of T-cell activation. Selected time-points from a time-lapse sequence following Jurkat T-cell activation on OKT3 antibody-coated glass coverslips showing the organization of TCR-β (green) and ZAP70 (red) as a function of the size of the contact formed with the coverslip (simultaneously visualized by interference reflection microscopy). The data shows how rapidly ZAP70 is recruited to the contact, and how quickly the TCR begins to form clusters. However, the experiment also emphasizes that these processes are uncorrelated, at least early on, contrary to accepted belief. The image was generated by Christoffer Lagerholm, Mafalda Da Cunha Santos, Corinna Kulicke and Simon Davis.

Which imaging technique should I use?



Useful online links

- Zeiss – Microscopy from the very beginning

http://zeiss-campus.magnet.fsu.edu/index.html

- Molecular Expressions homepage

http://micro.magnet.fsu.edu/

- *Alison J. North.* **Seeing is believing? A beginners' guide to practical pitfalls in image acquisition.** *JCB Volume 172(1):9-18 January 2, 2006*