

Lasers and Microscopy

Yaron Silberberg

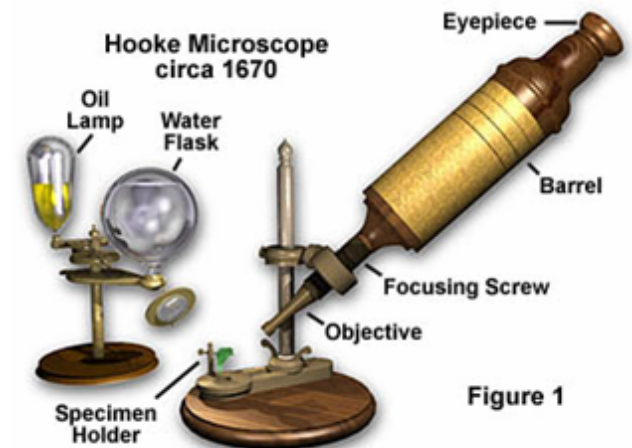
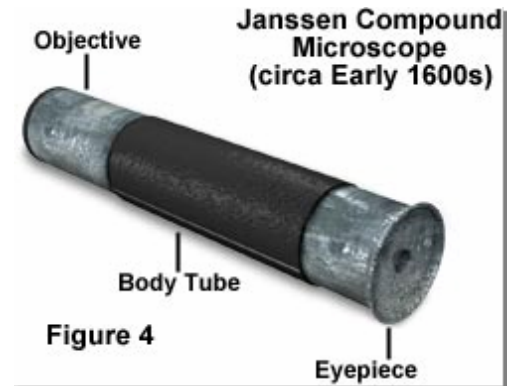
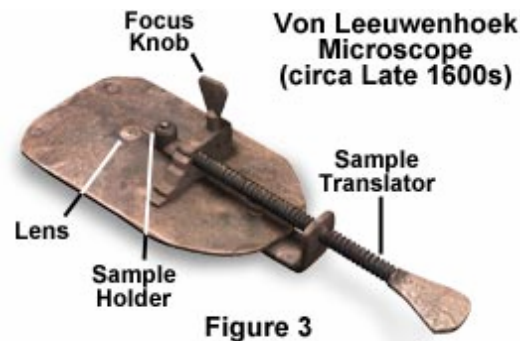
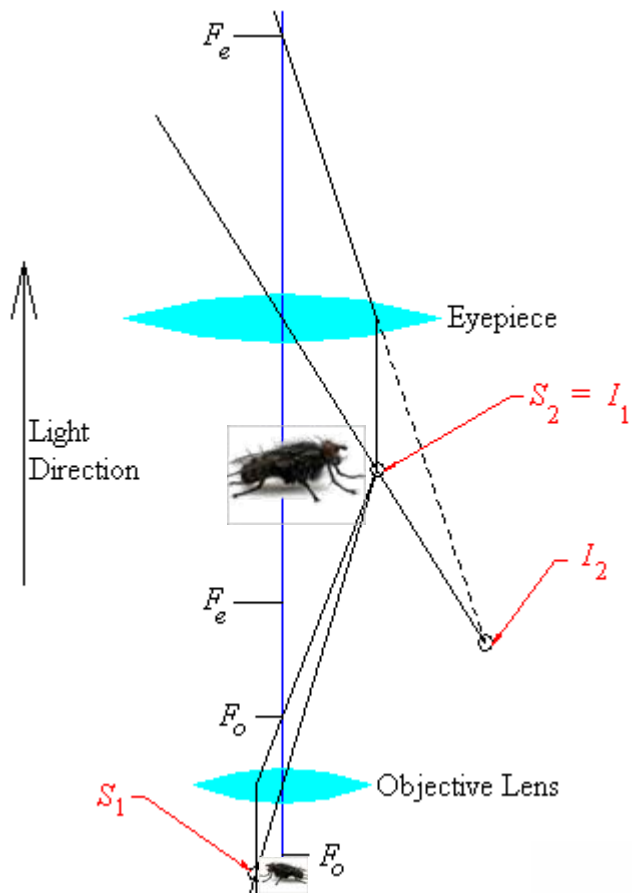
Physics of Complex Systems

*Weizmann Institute of Science
Rehovot, Israel*

<http://www.weizmann.ac.il/home/feyaron/>

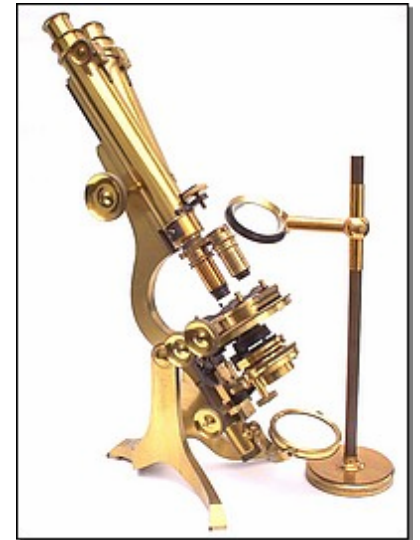


The compound microscope



Lasers and Microscopy

1. Brief history of microscopy
2. Lasers meet Microscopy – Confocal Microscopy
3. Nonlinear microscopy
4. Breaking the resolution limit

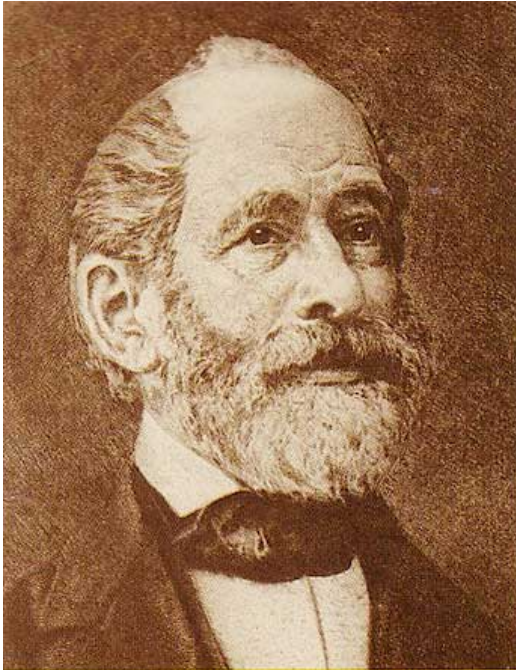


Microscope craftsmanship



Late 19th century brass microscope

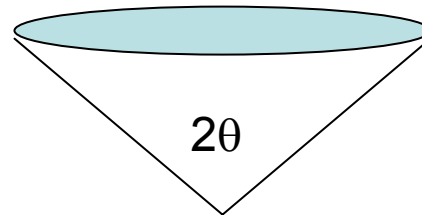
Carl Zeiss and Ernst Abbe – the first high-tech company?



Carl Zeiss (1816–1888)

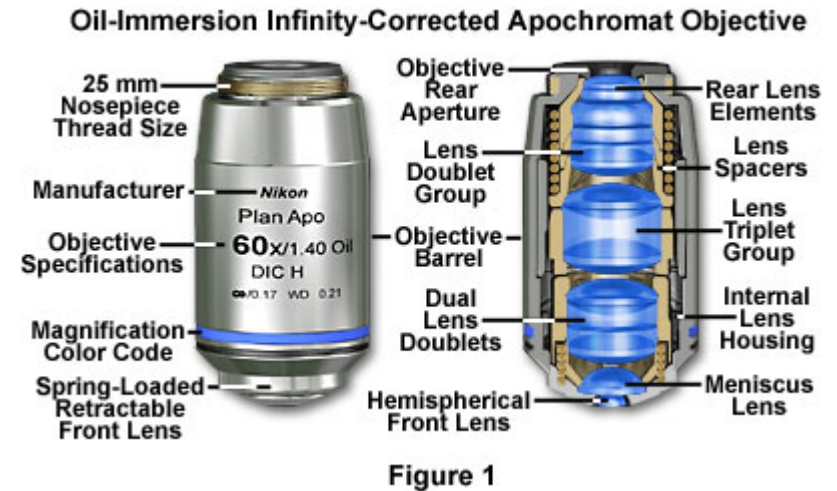
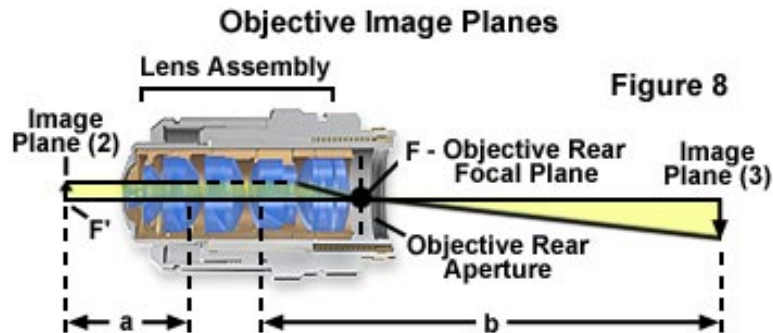
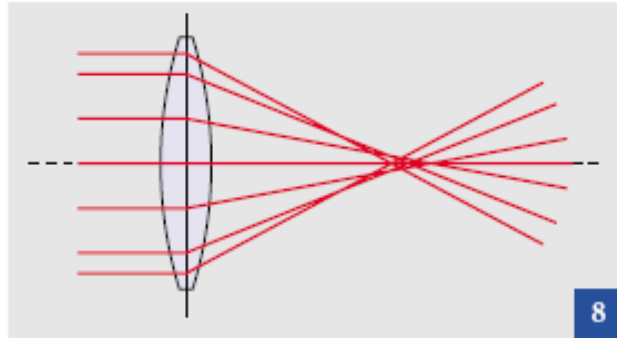
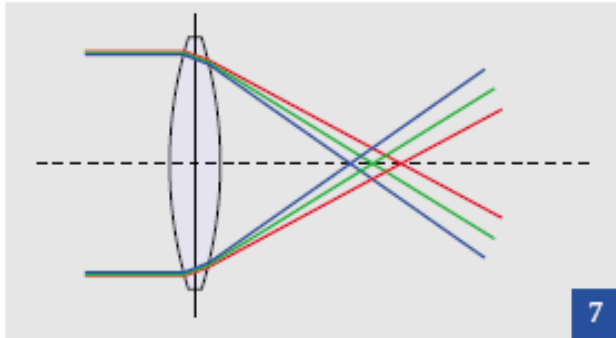


Ernst Abbe (1840–1905)



$$d = \frac{\lambda}{2n \sin(\theta)}$$

Reaching the diffraction limit



Zeiss and Schott

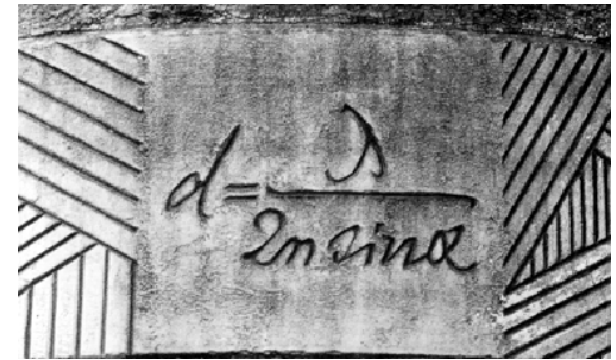


Otto Schott (1851-1935)

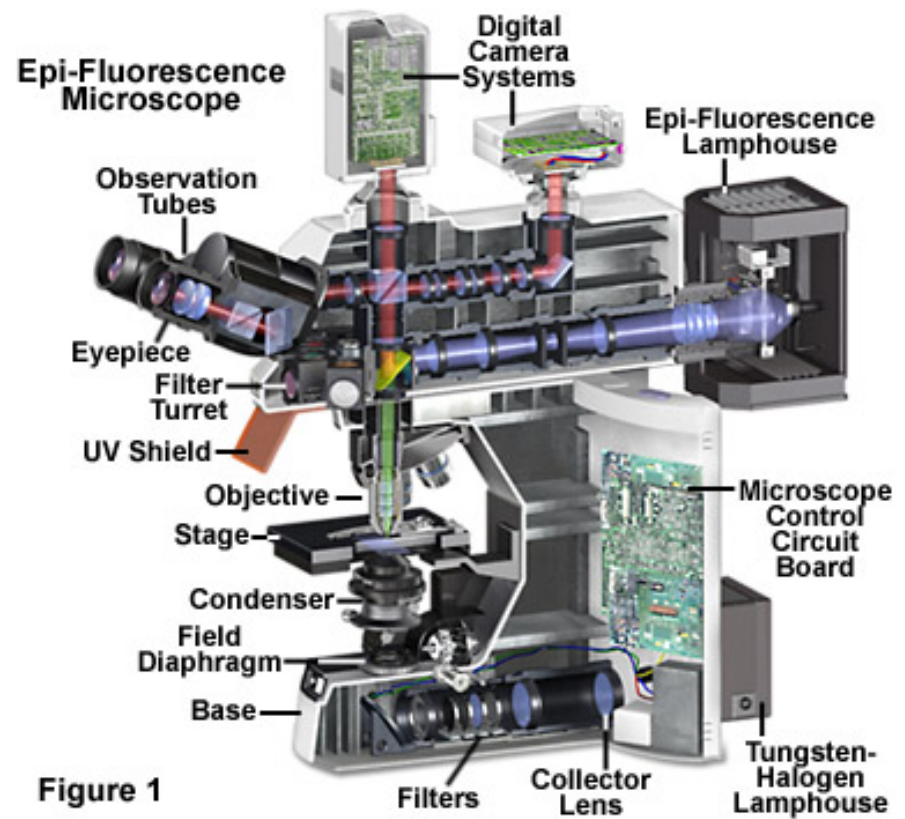
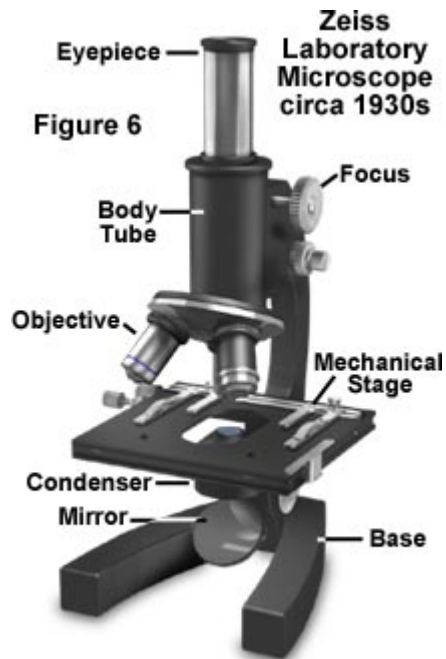
Microscopy development brought the need for advanced optical glasses



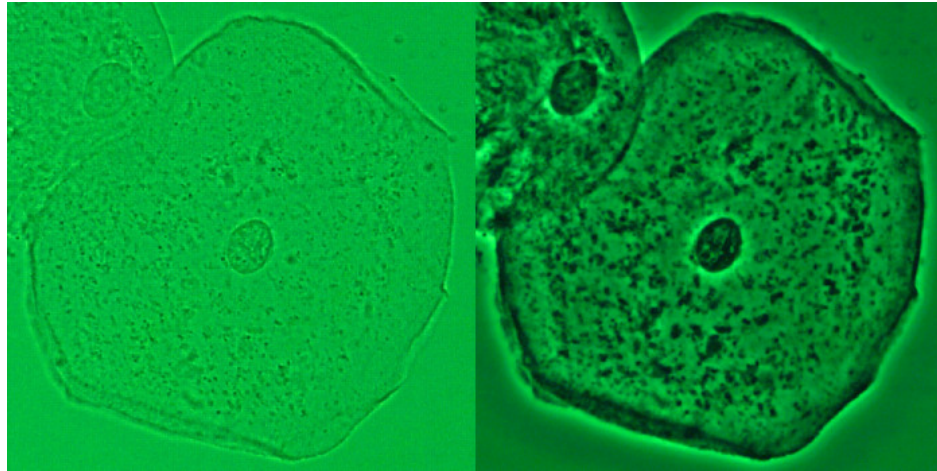
Abbe Criterion



Optical Microscopy 1900-2000



Fritz Zernike and Phase Microscopy



Nobel Prize in Physics, 1953

Fluorescence Microscopy

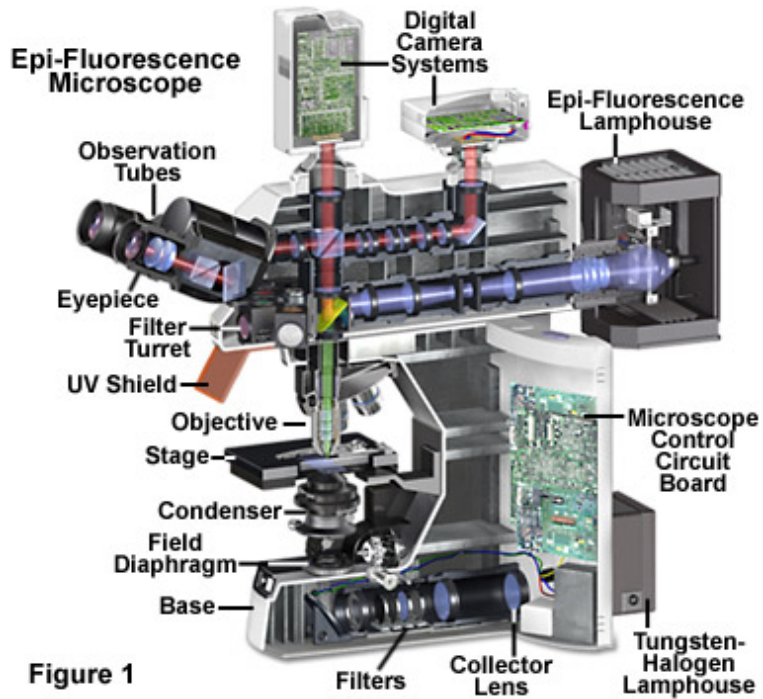
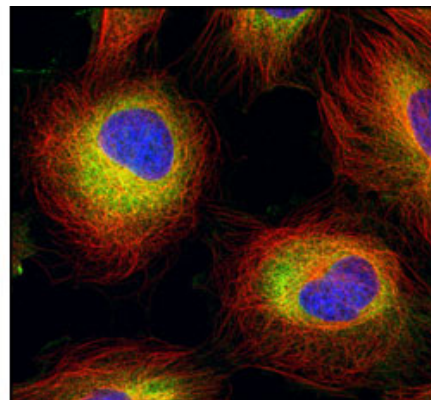
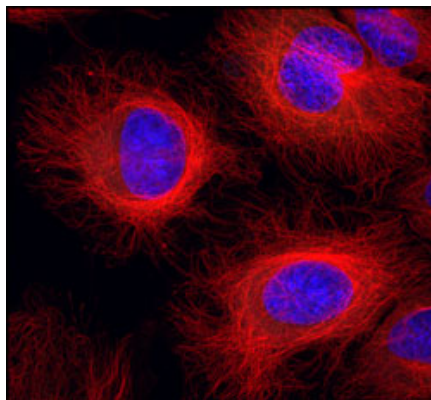
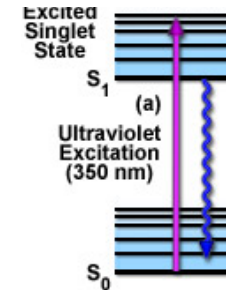
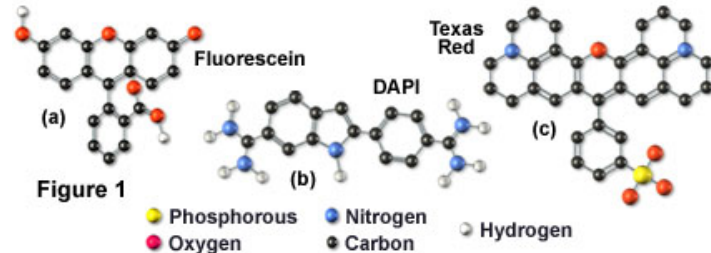
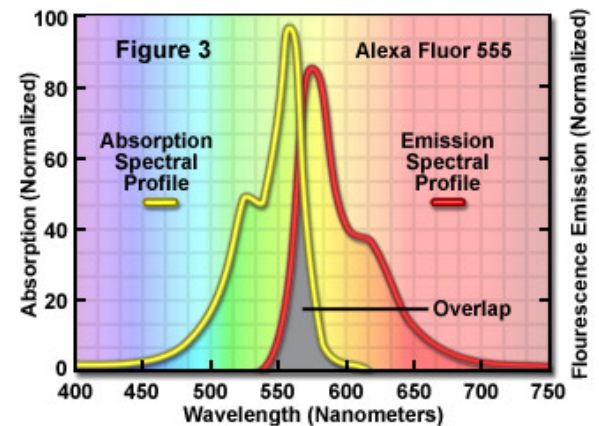


Figure 1

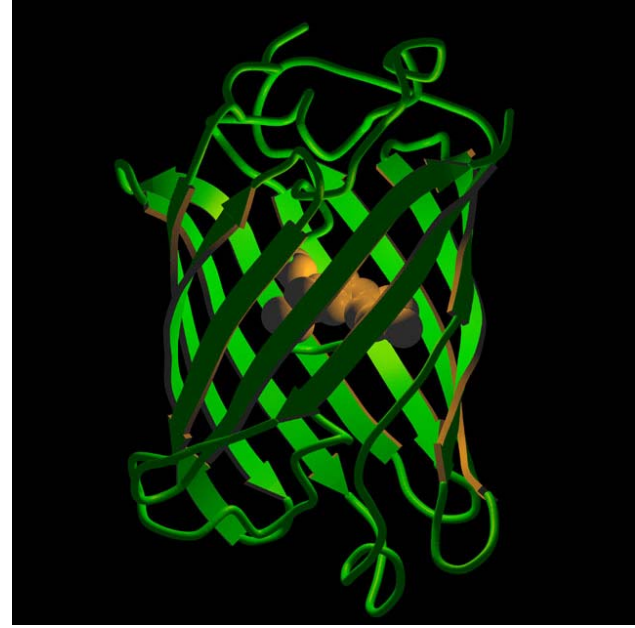
Common Fluorophores in Widefield and Confocal Microscopy



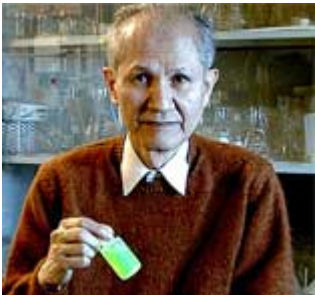
Fluorophore Absorption and Emission Profiles



Fluorescence Proteins

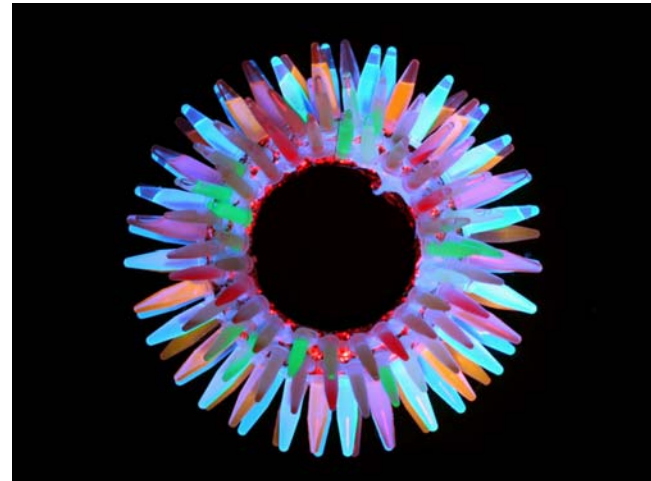


Nobel Prize in Chemistry 2008



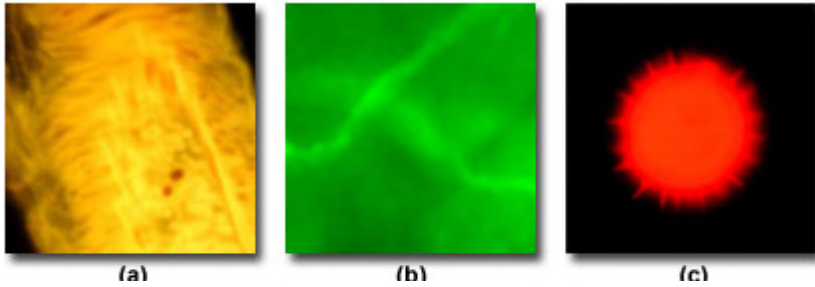
Osamu Shimomura

Roger Tsien



The third dimension

Confocal and Widefield Fluorescence Microscopy



Pollen Grain Serial Optical Sections by Confocal Microscopy

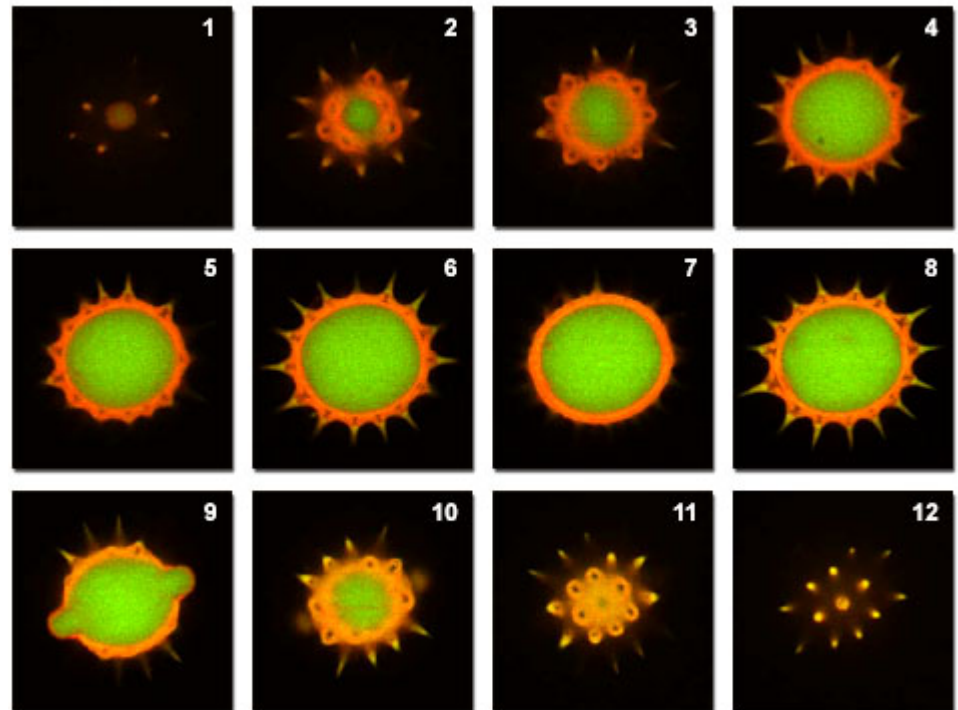
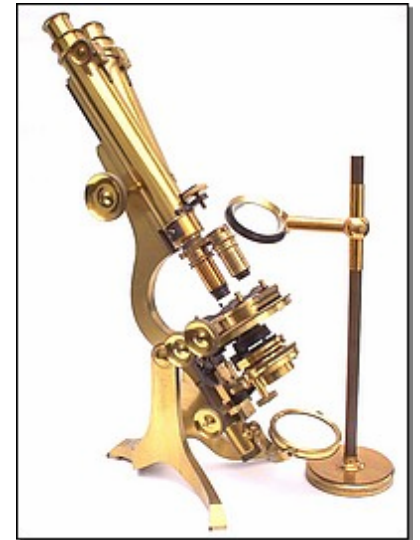


Figure 6

Lasers and Microscopy

1. Brief history of microscopy
2. Lasers meet Microscopy – Confocal Microscopy
3. Nonlinear microscopy
4. Breaking the resolution limit



Confocal Microscopy – Scanning Laser Microscopy

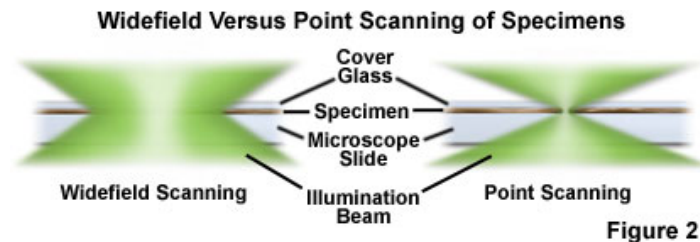
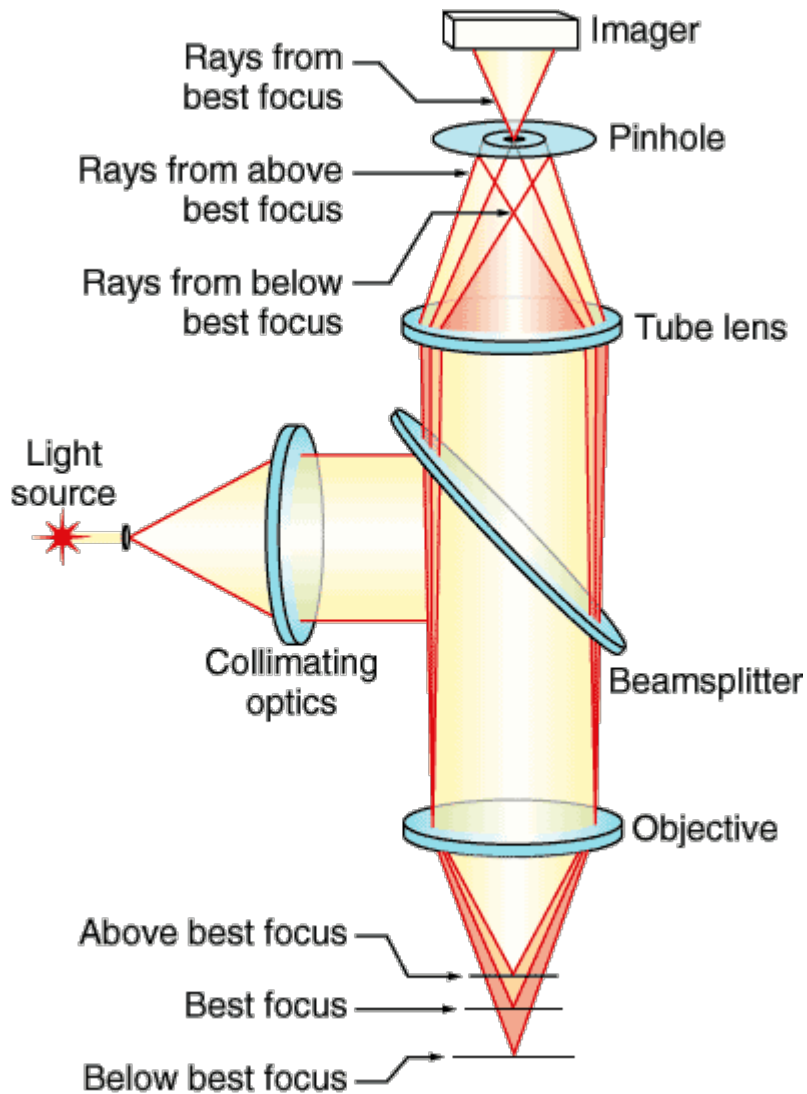


Figure 2

Confocal Microscopy – Scanning Laser Microscopy



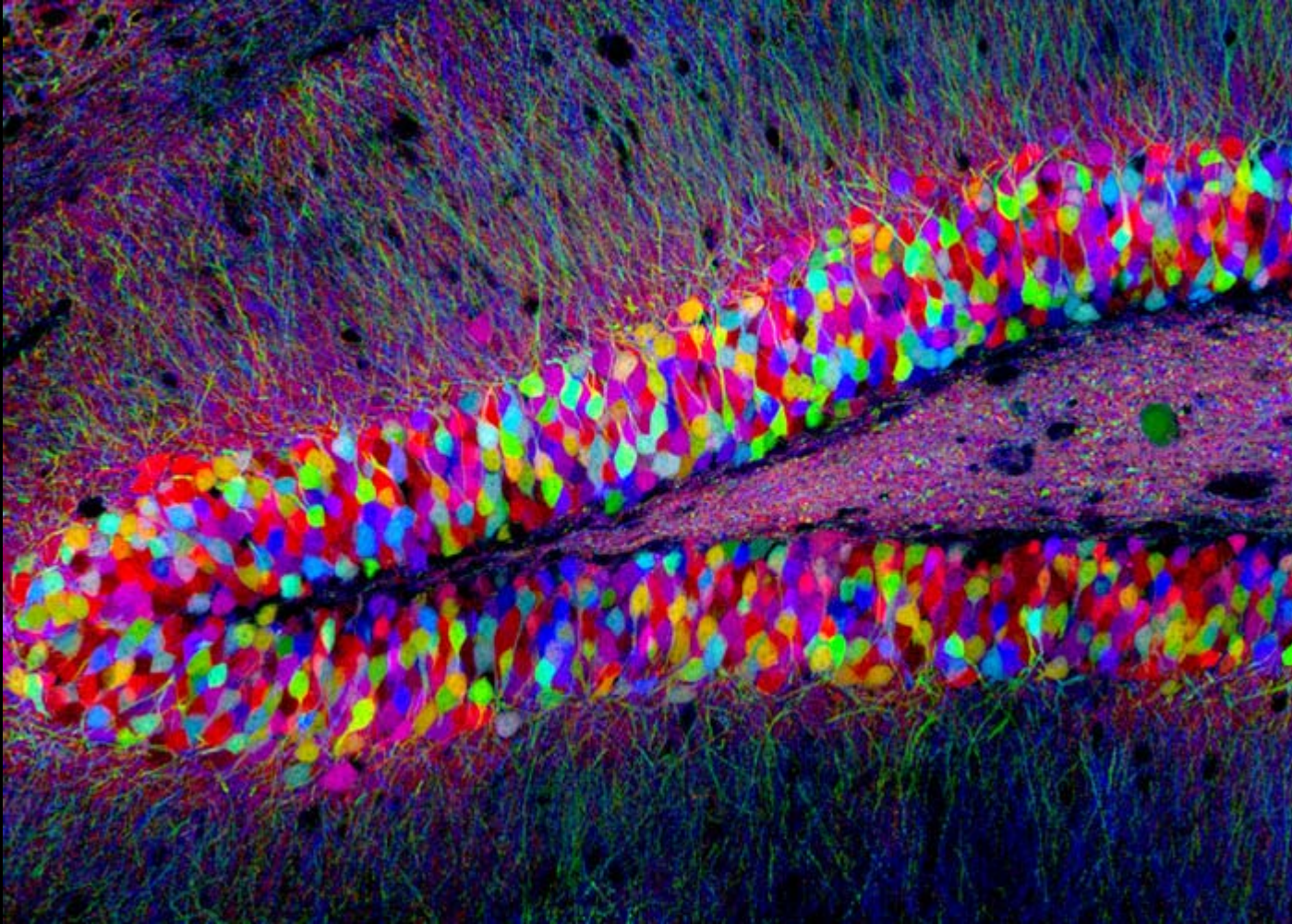
Optical sectioning
3 d imaging
Digital images
Multi exposures

Confocal Microscopy



High Resolution Imaging Facility - University of Alabama at Birmingham - Birmingham, Alabama, United States
Specimen: *Convallaria majalis* (Lily of the Valley) (1300x)
Technique: Confocal

Confocal Microscopy

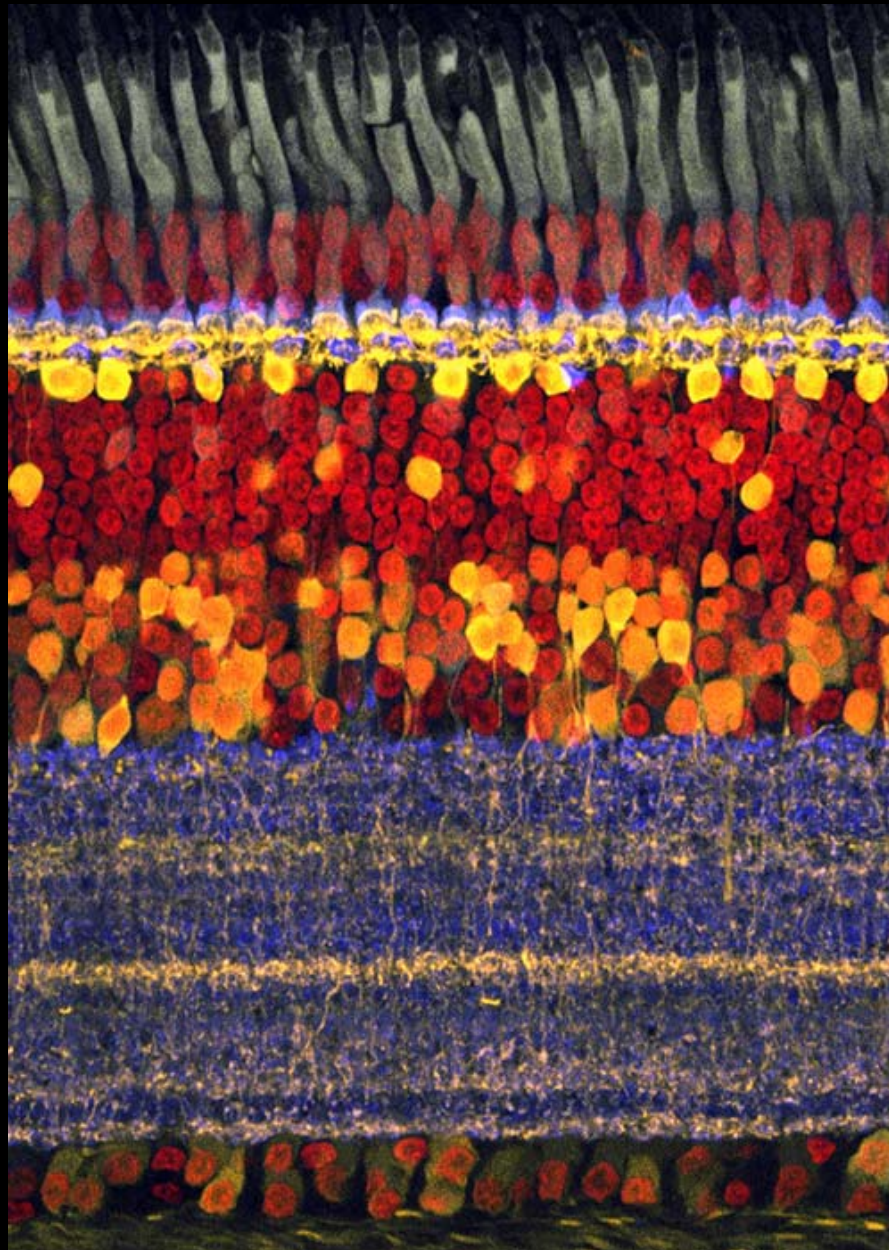


Department of Molecular and Cellular Biology - Harvard University - Cambridge, Massachusetts, United States

Specimen: "Brainbow" transgenic mouse hippocampus (40x)

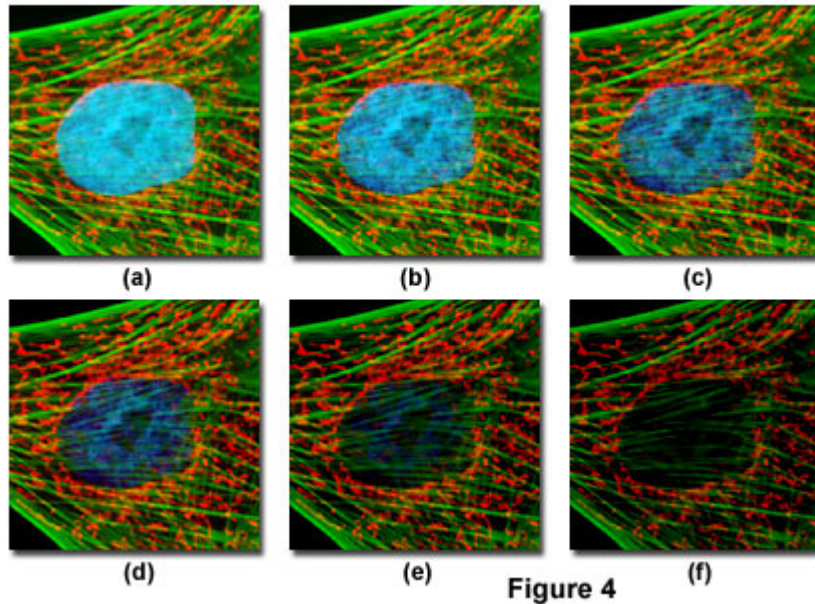
Technique: Confocal

Confocal Microscopy



Some issues in confocal microscopy

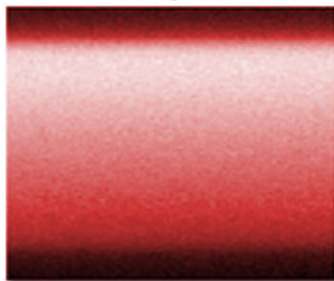
Photobleaching Rates in Multiply Stained Specimens



Photobleaching:

Many fluorescent markers fade under illumination

X-Z Scan



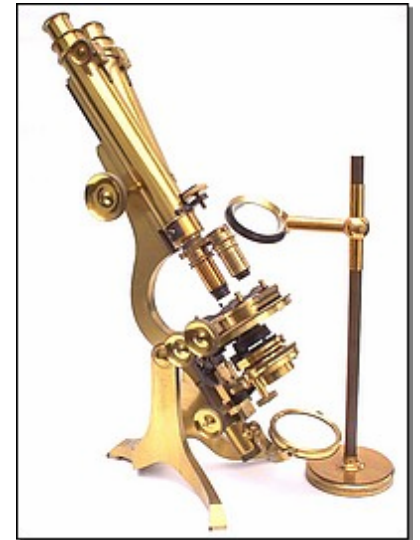
Scattering in thick samples:

Deep layers are hard to view

Need for staining

Lasers and Microscopy

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3. **Nonlinear microscopy**
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Two-Photon Microscopy

Two-Photon Jablonski Energy Diagram

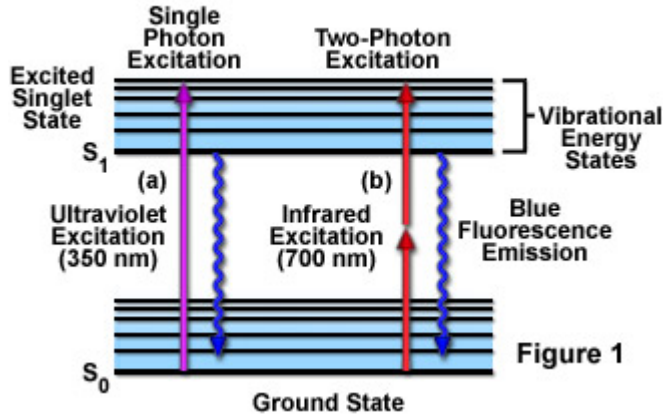


Figure 1

Regular absorption:

$$I_{fl} \propto \alpha \cdot I_{ex}$$

2-Photon absorption:

$$I_{fl} \propto \beta \cdot I_{ex}^2$$

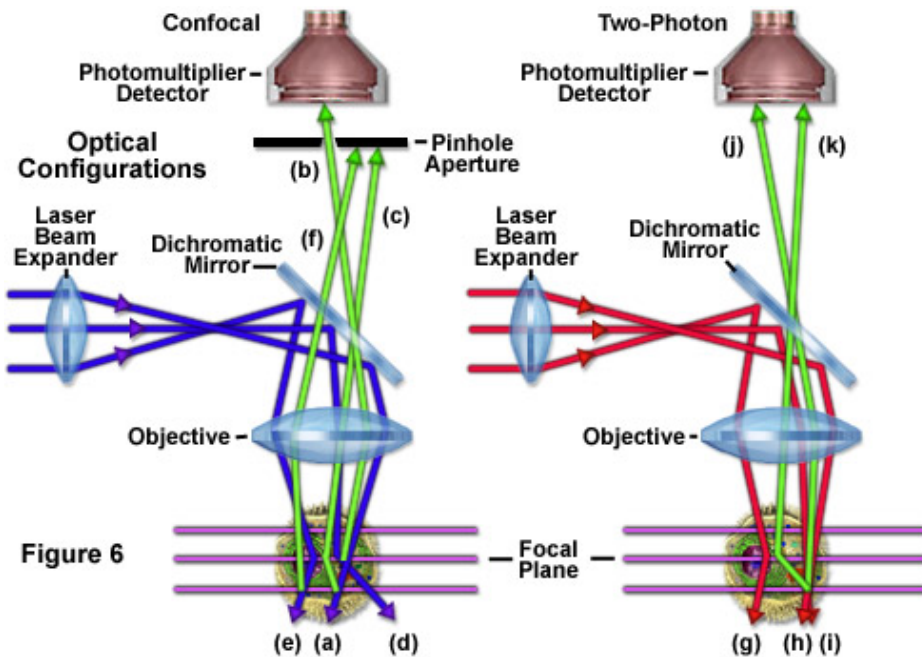
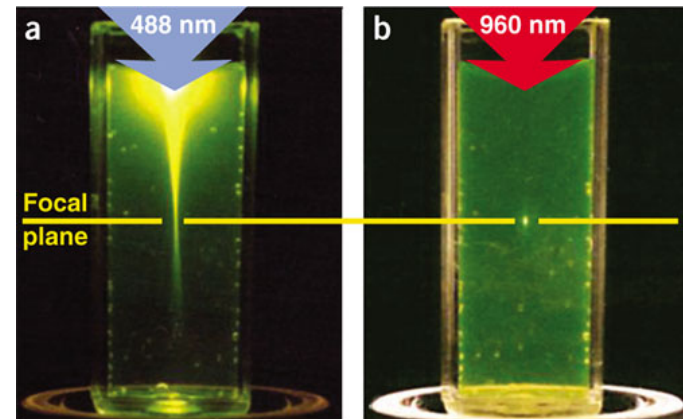


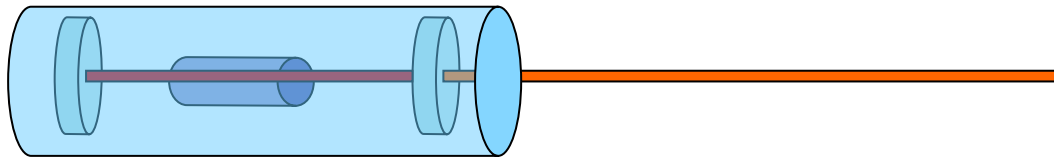
Figure 6

W W Webb, 1990

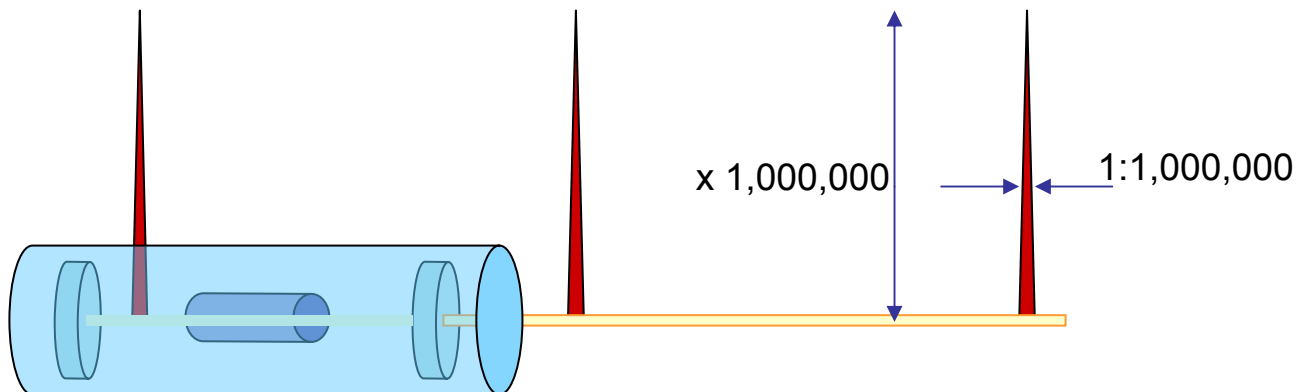


Femtosecond lasers

Continuous wave laser

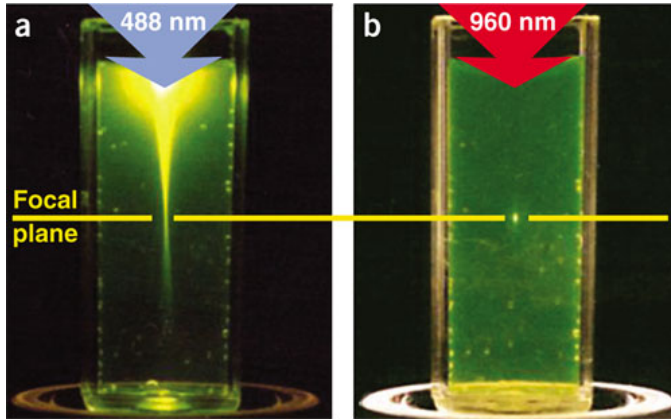


Pulsed laser



Similar average power, much higher peak powers

Two-Photon Microscopy



Absorption only at focal point:

Reduced photobleaching

No need for filtering

Two-Photon Jablonski Energy Diagram

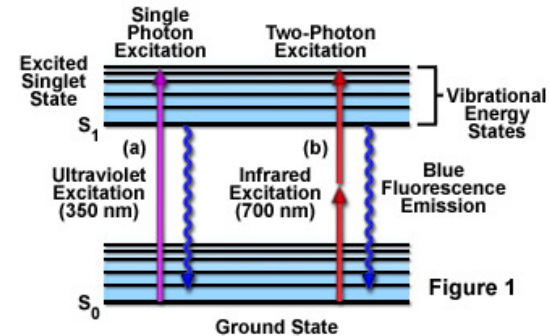
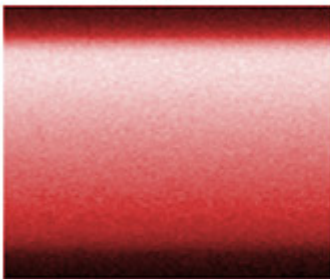


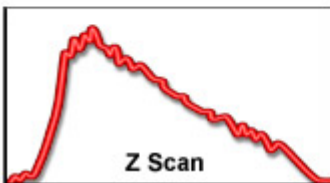
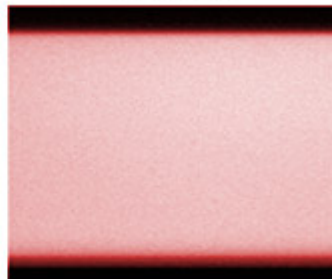
Figure 1

Single and Two-Photon Scanning Profiles

One-Photon Excitation
X-Z Scan



Two-Photon Excitation
X-Z Scan



Z Scan

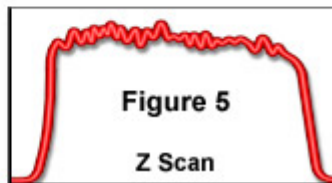


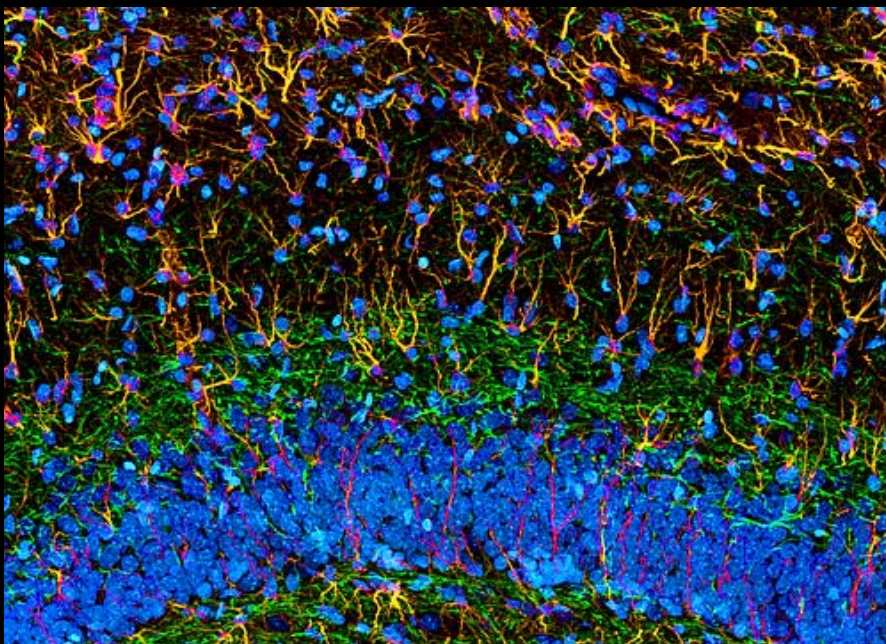
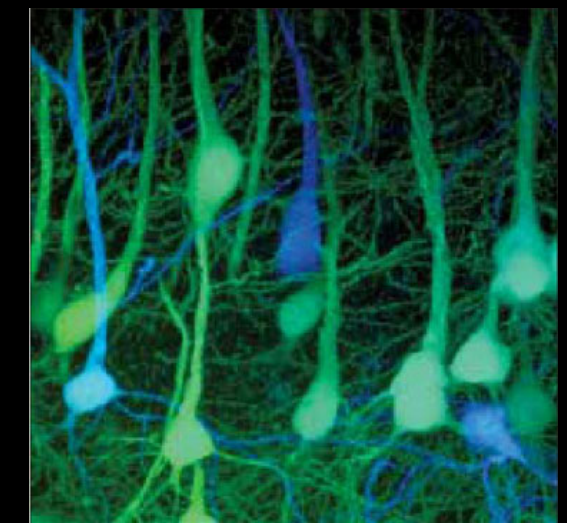
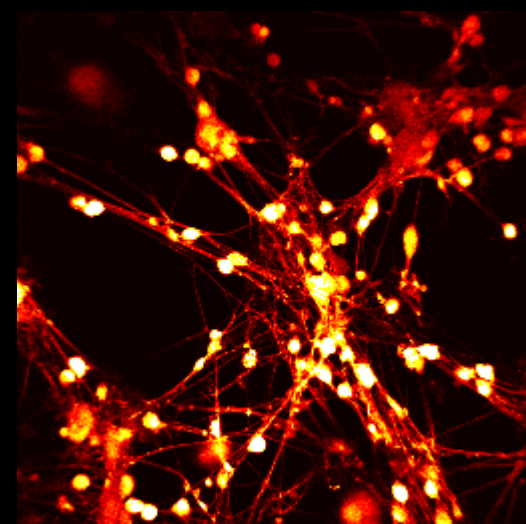
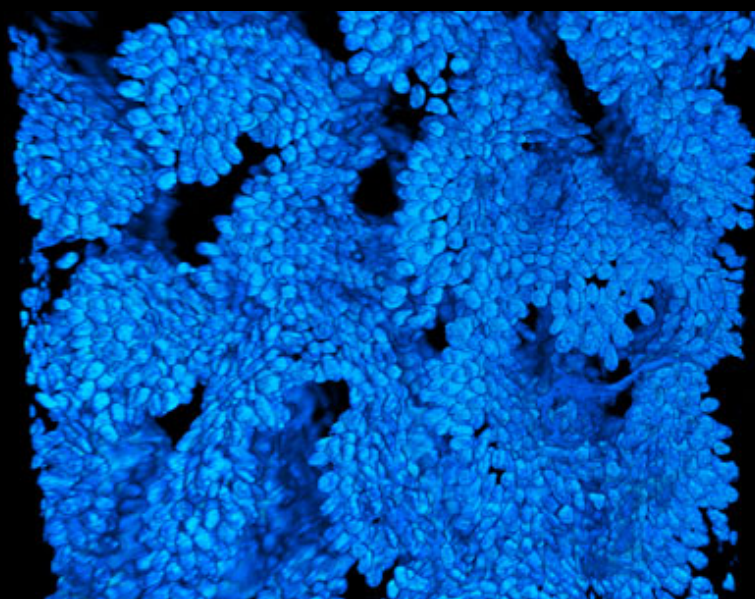
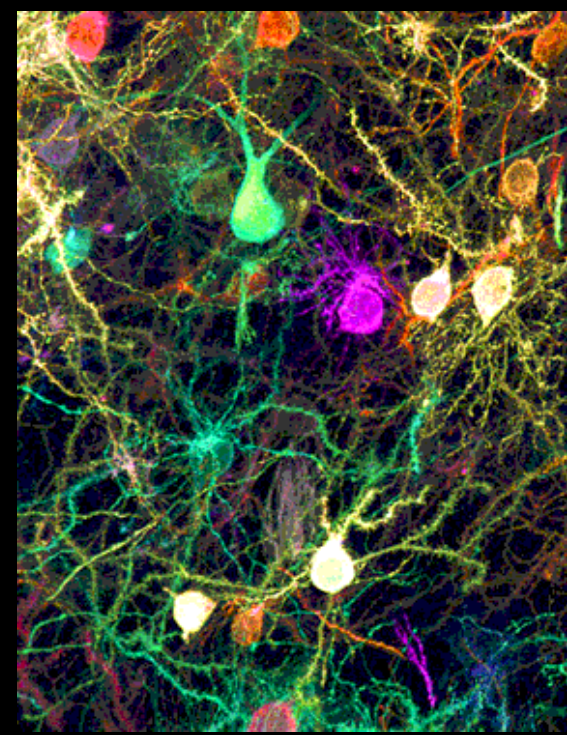
Figure 5

Z Scan

Longer excitation wavelength:

Deeper penetration in thick specimens

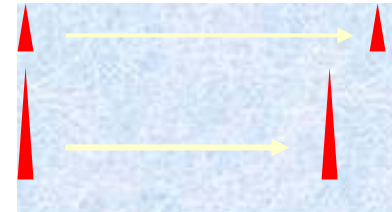
Two-Photon Microscopy in Neurobiology



Nonlinear Optics – the Strange Optics of Intense Light

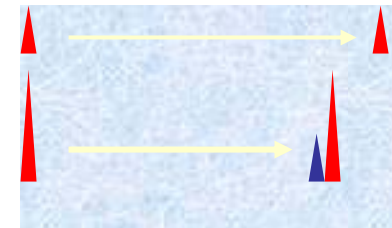
Intense light travels slower

Index of refraction increases at high light intensities (Kerr effect)

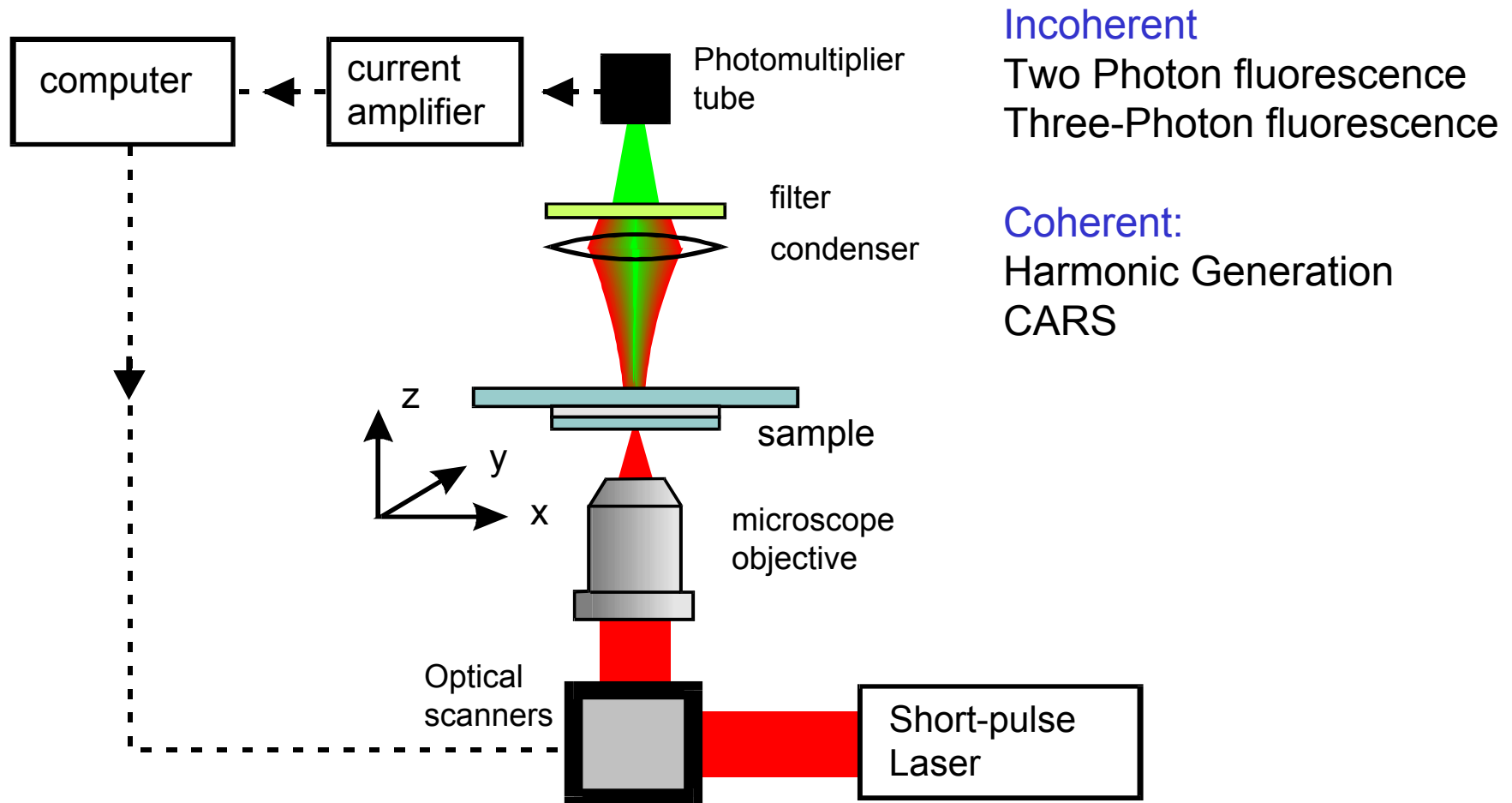


Intense light may generate new colors

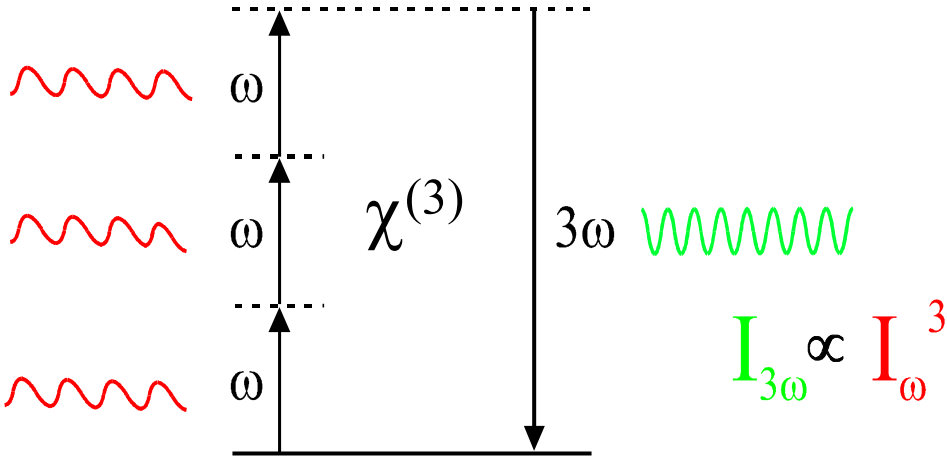
Small amount of light at new frequencies could be generated.



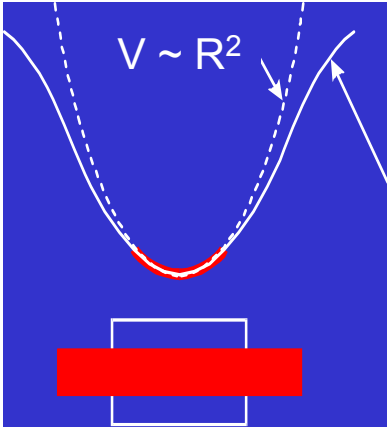
Nonlinear Laser Scanning Microscopy



Third Harmonic Generation

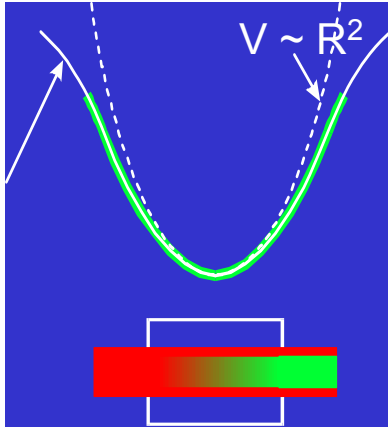


Low intensity



Linear optics

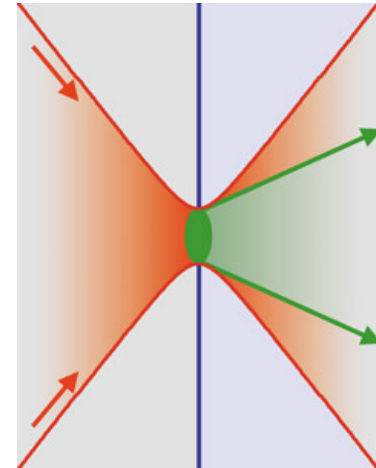
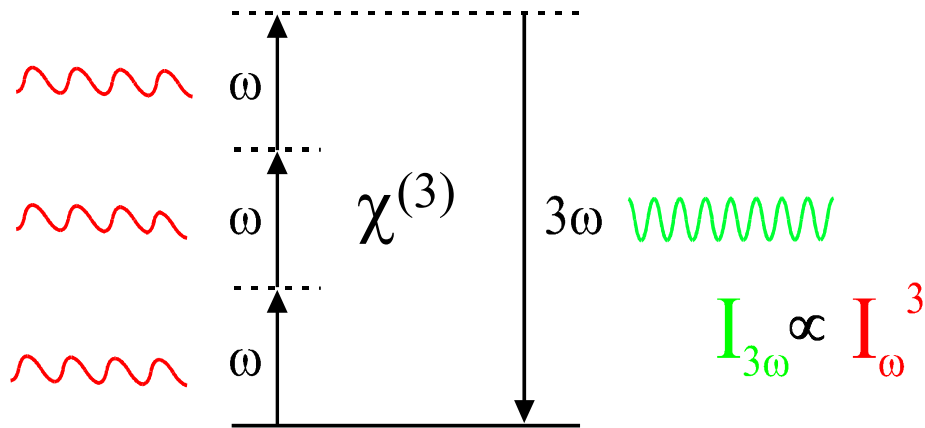
High intensity



Non-linear optics

Electron potential (V)

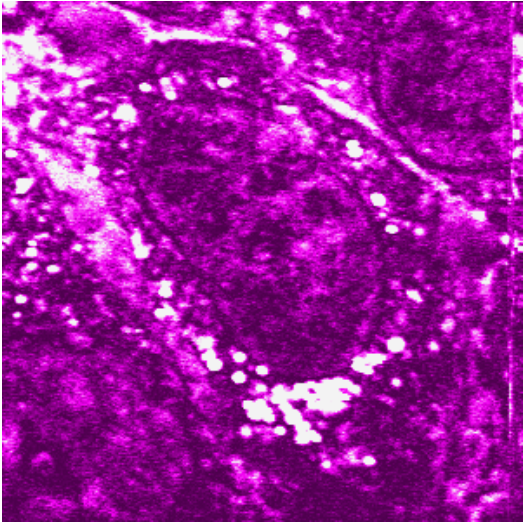
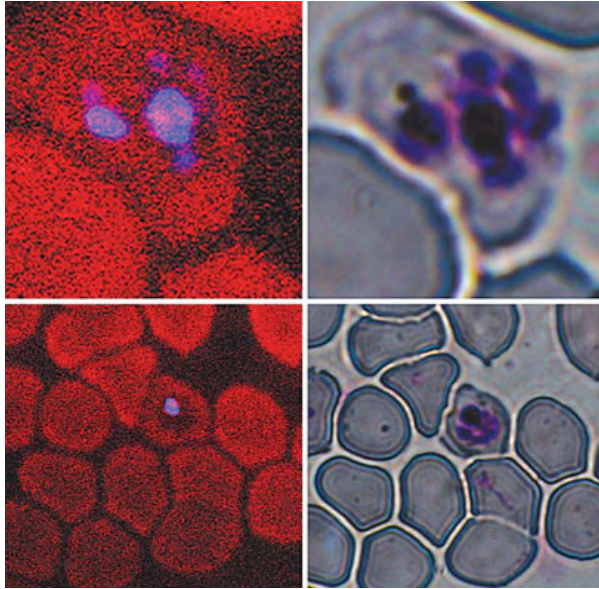
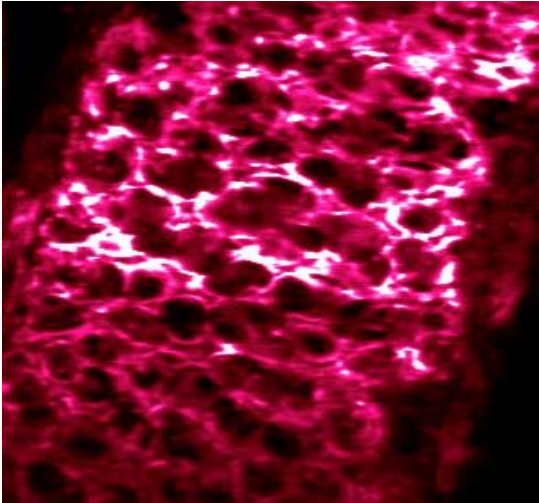
Third Harmonic Generation



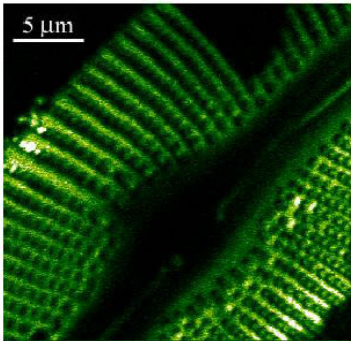
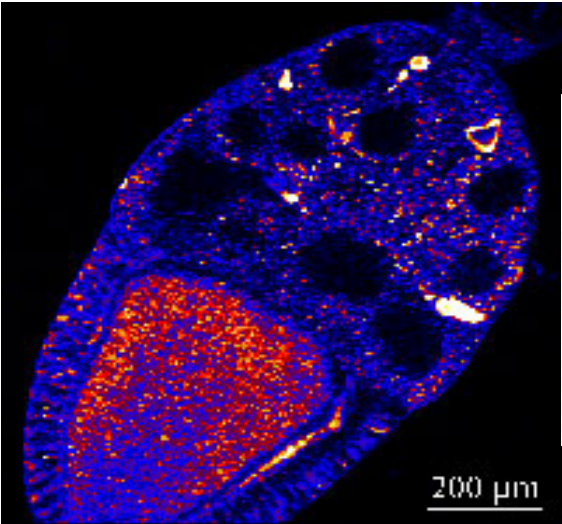
Universal process
General structural imaging
Coherent process

Third Harmonic Generation microscopy

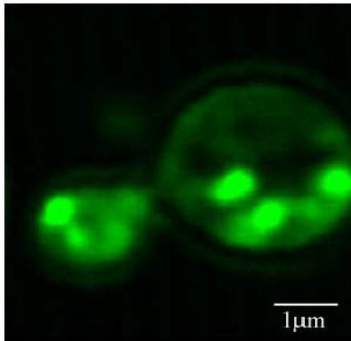
Mouse bone



Drosophila ovary



fossil

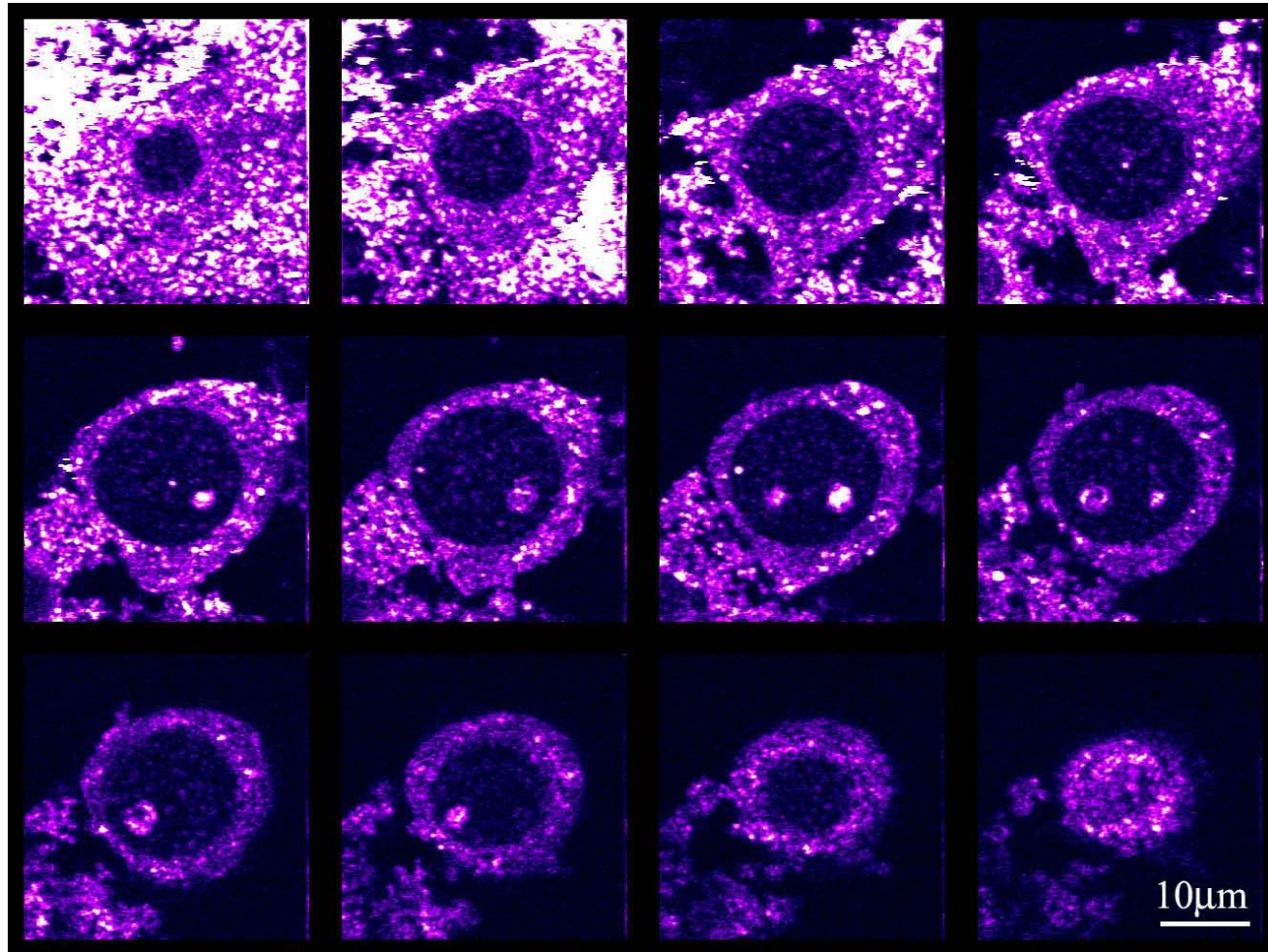


Xenopus embryo



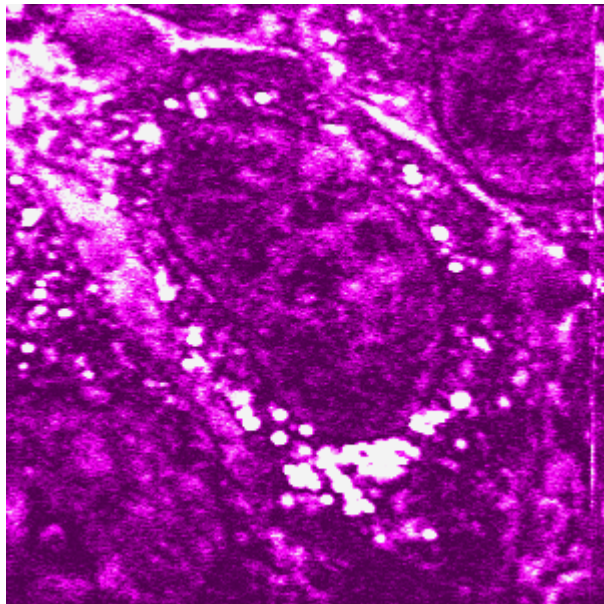
THG of live specimens

Optical sections of a neuron by THG



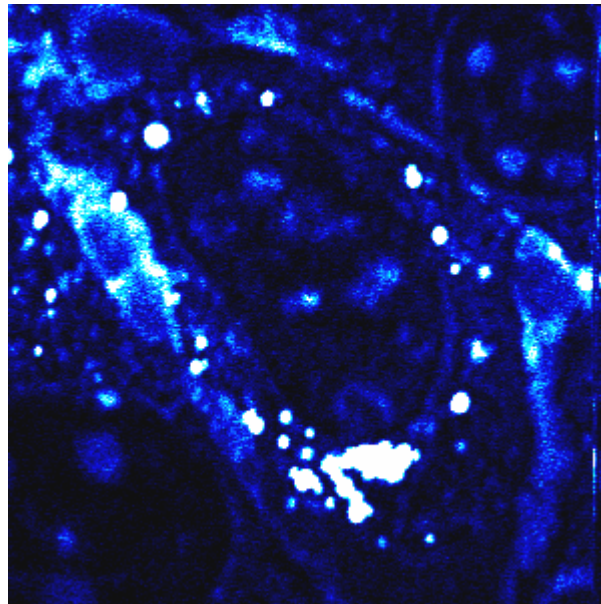
Sections separated by 1 μ m

Third Harmonic Generation microscopy



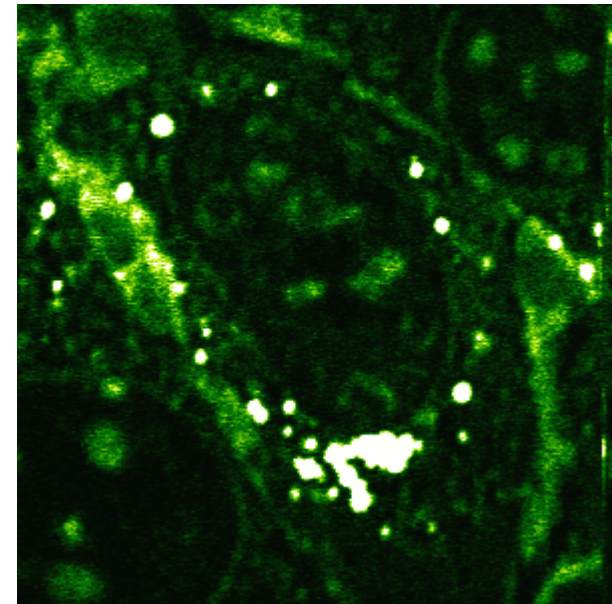
$$\lambda_{\omega} = 0.81 \mu\text{m}$$
$$\lambda_{3\omega} = 0.27 \mu\text{m}$$

Higher resolution



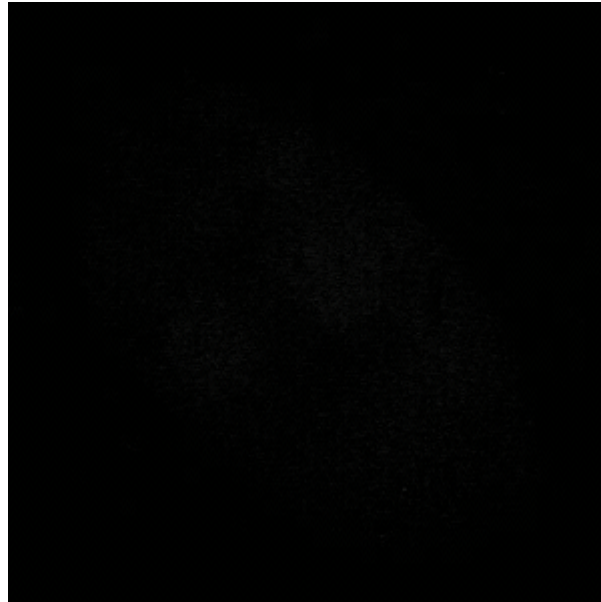
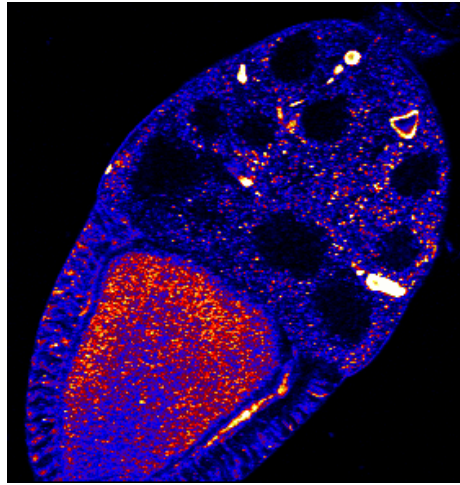
$$\lambda_{\omega} = 1.24 \mu\text{m}$$
$$\lambda_{3\omega} = 0.43 \mu\text{m}$$

Deeper penetration



$$\lambda_{\omega} = 1.5 \mu\text{m}$$
$$\lambda_{3\omega} = 0.5 \mu\text{m}$$

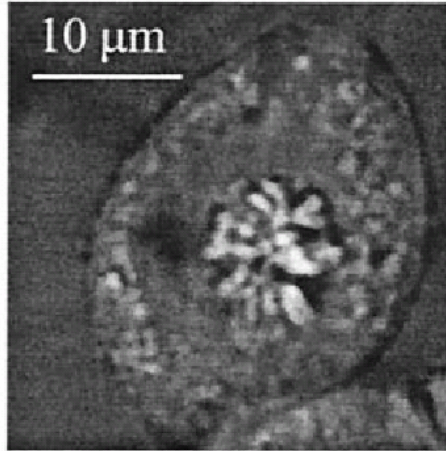
Third Harmonic Generation microscopy



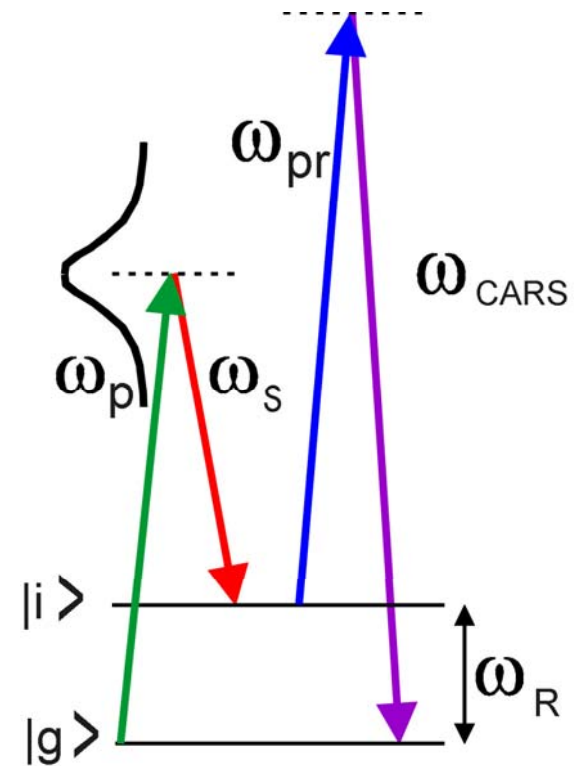
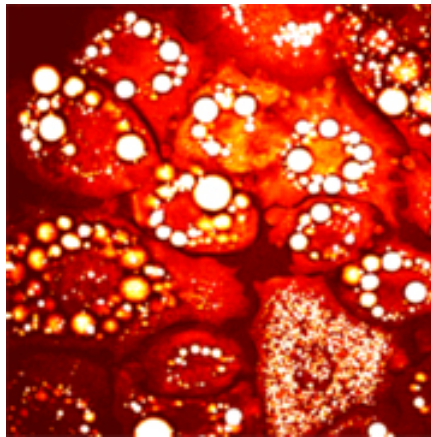
A fly through
a fly egg

The dream of molecular imaging

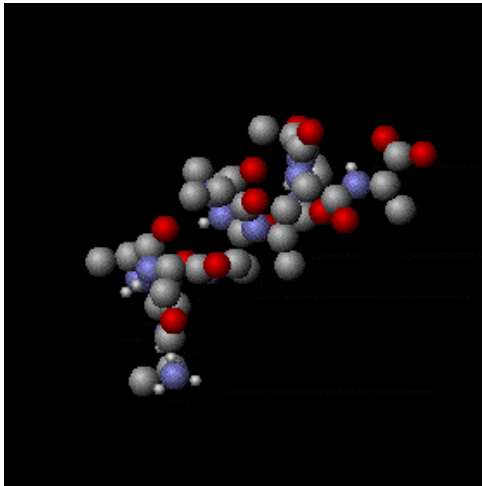
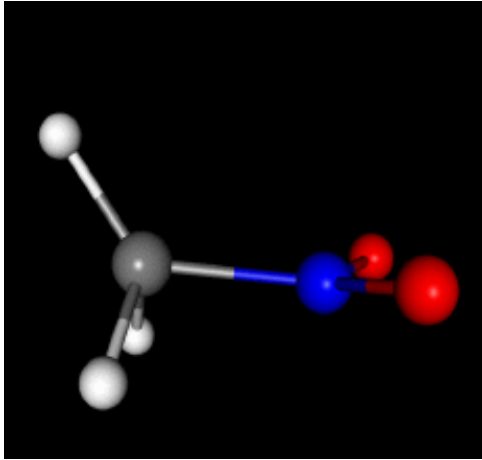
CARS Image tuned to DNA backbone vibration at 1090 cm^{-1} in mitosis



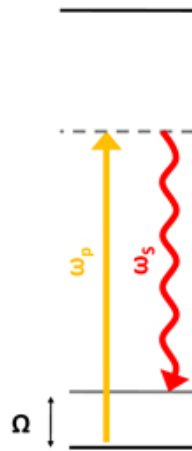
CARS image of **fibroblast cells** that are stimulated to synthesize lipids. The lipid droplets are visualized with CARS tuned to the C-H vibration at 2845 cm^{-1} .



Raman and CARS microscopy



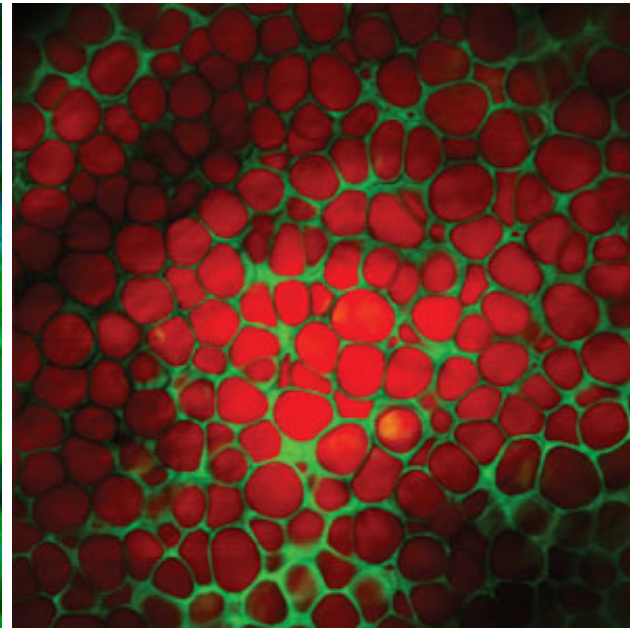
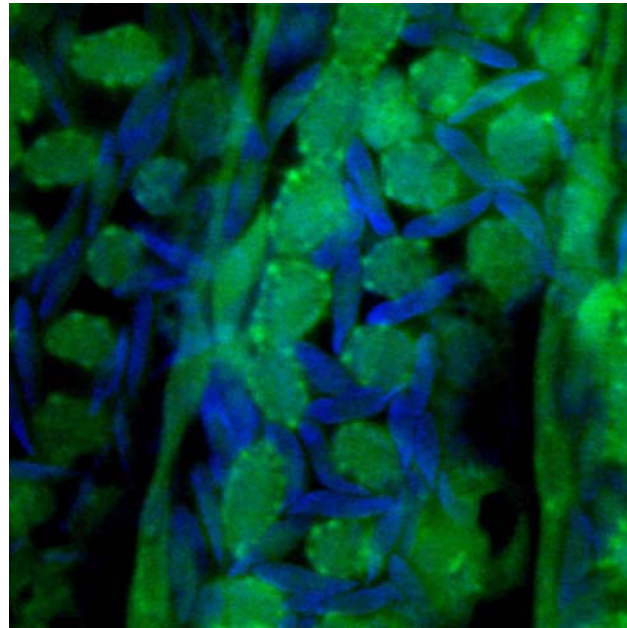
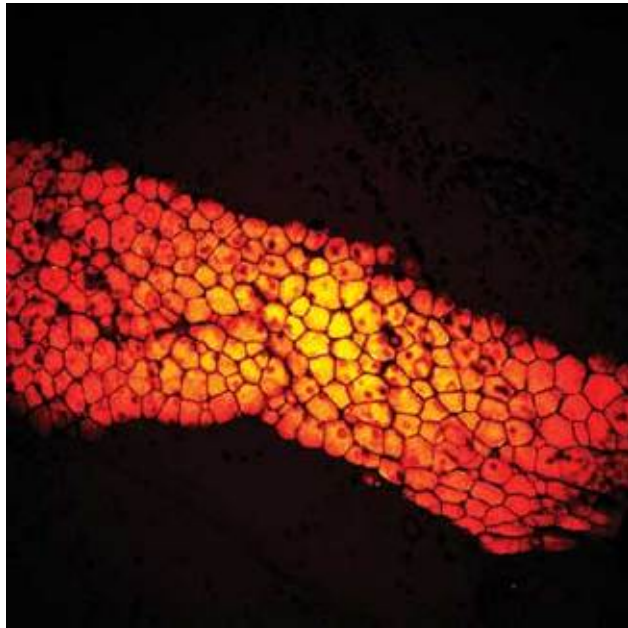
a) Spontaneous Raman



Sir Chandrasekhara Venkata Raman, FRS

Nobel Prize 1930

CARS Images

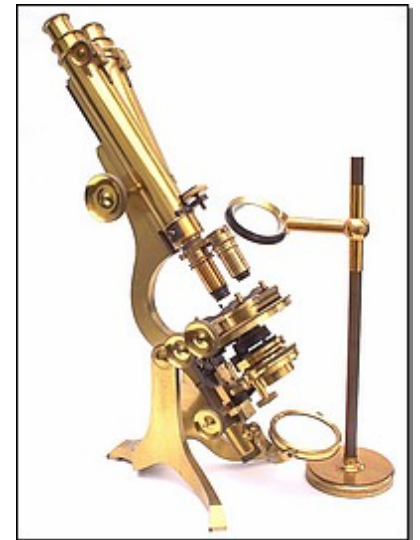


Nonlinear Microscopy

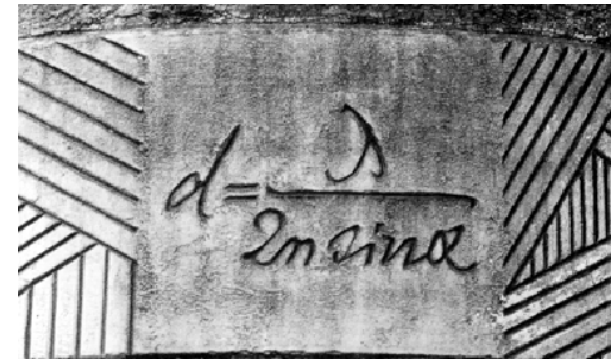
1. Optical sectioning (all)
2. Reduced photobleaching (TPFE)
3. New contrast mechanisms, no labeling, live specimens (SHG, THG, CARS..)
4. Reduced scattering, deep imaging (TPFE, SHG, THG)
5. Molecular imaging (CARS)
6. Enhanced resolution (STED)

Lasers and Microscopy

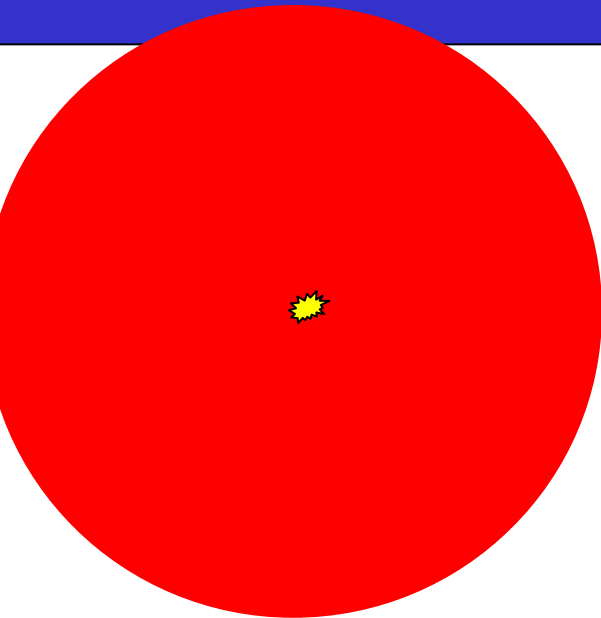
1. Brief history of microscopy
2. Lasers meet Microscopy – Confocal Microscopy
3. Nonlinear microscopy
4. **Breaking the resolution limit**



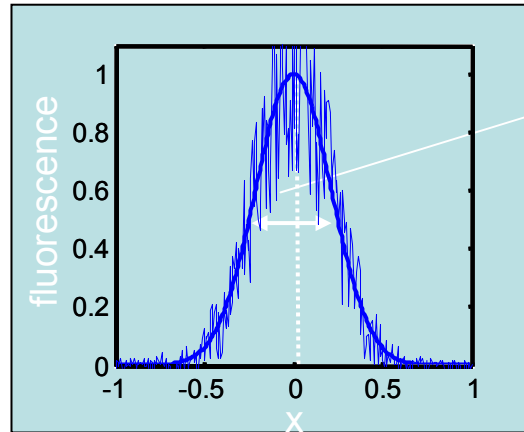
Abbe Criterion



Single molecule imaging



Single molecule PSF

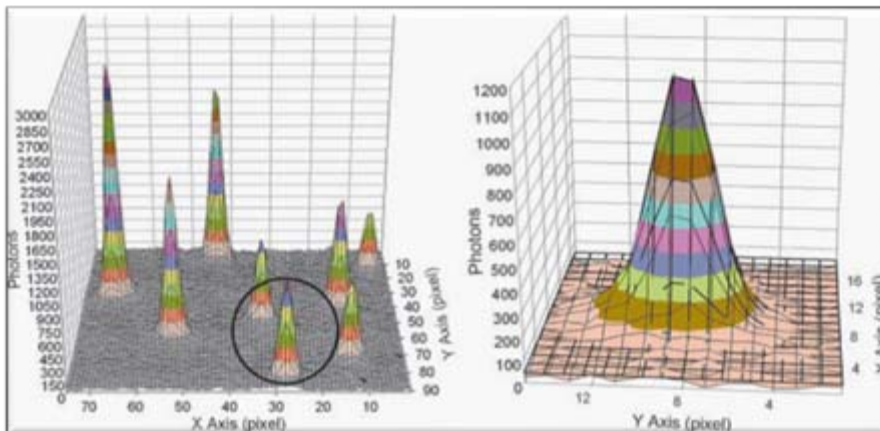


$$s \approx \frac{\lambda}{2NA} \approx 200 \text{ nm}$$

$$\bar{x} = ?$$

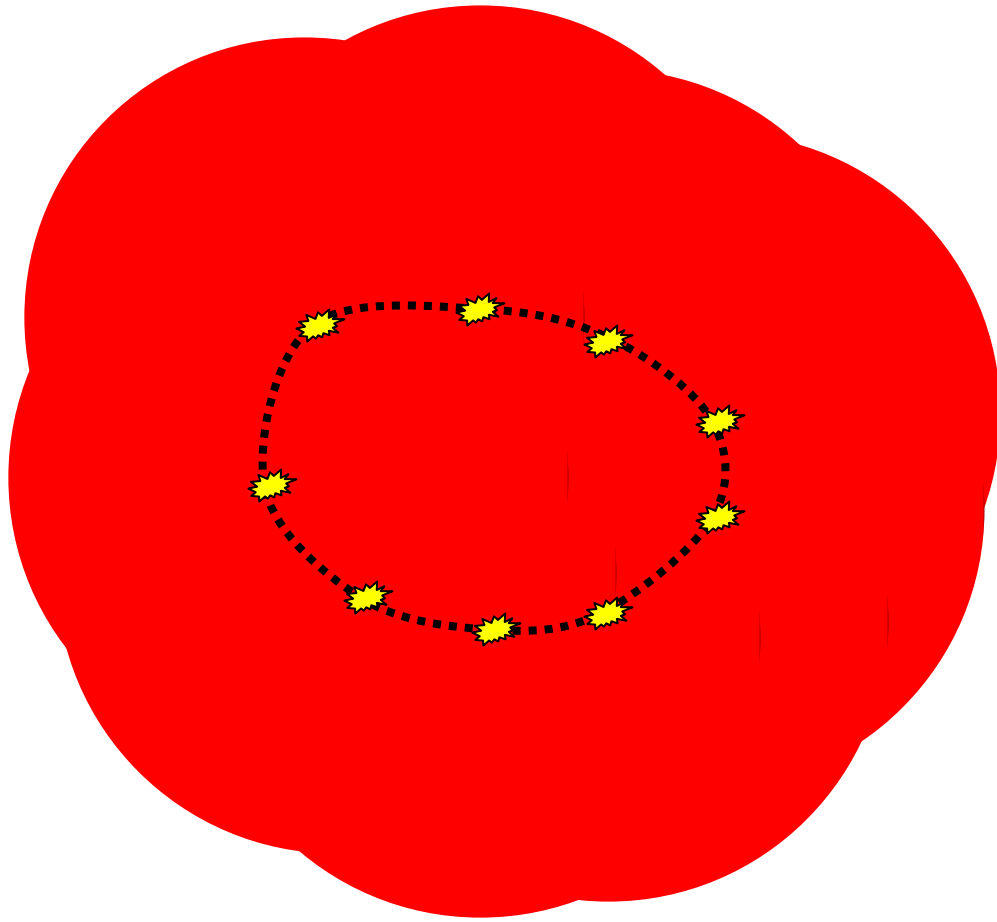
$$\sigma_x \approx \frac{s}{\sqrt{N}}$$

$$N \approx 10^4 \quad \sigma_x \approx 2 \text{ nm}$$

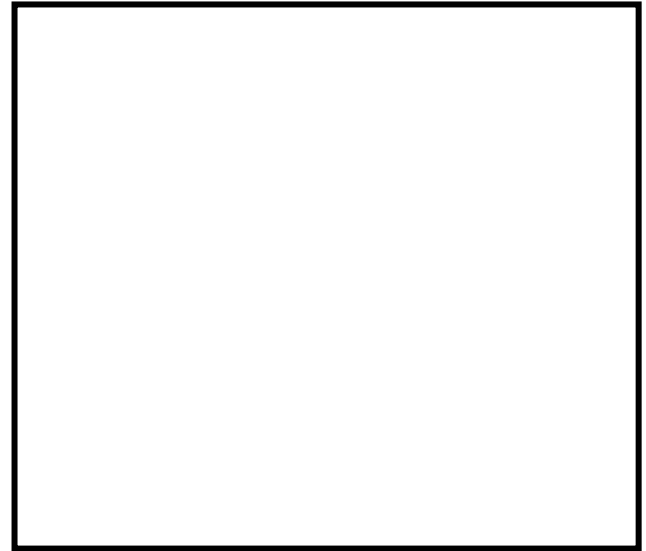
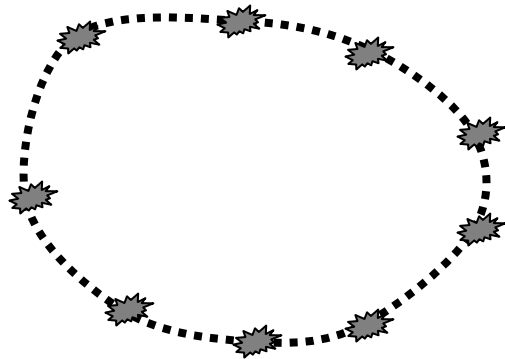


Yildiz *et al.*, Science (2003)

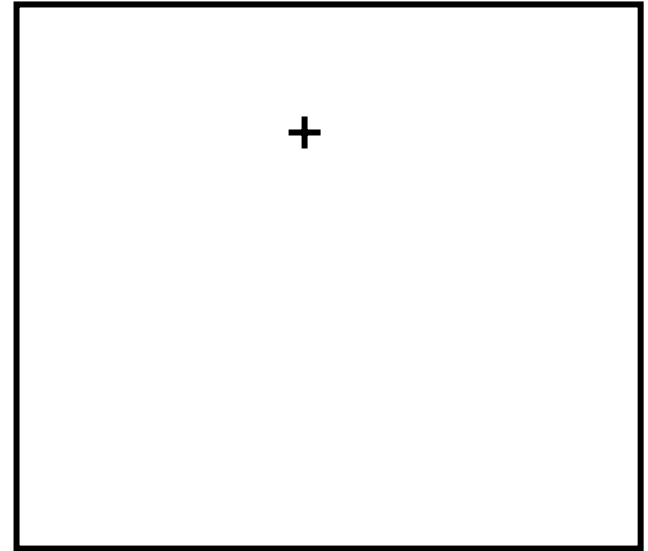
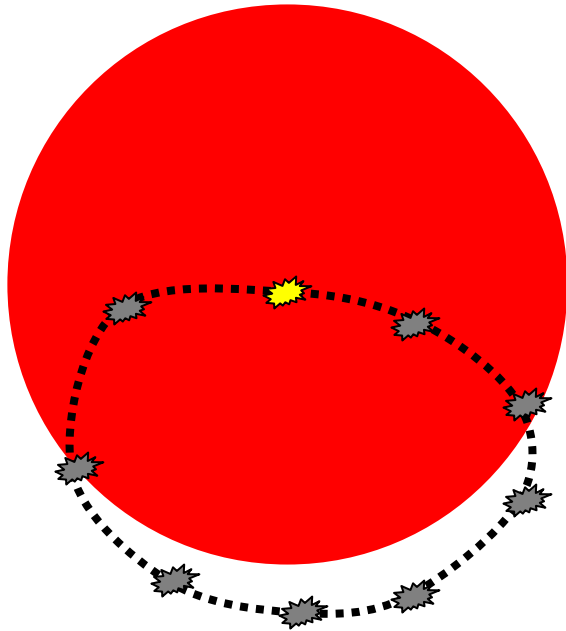
Imaging with molecular resolution



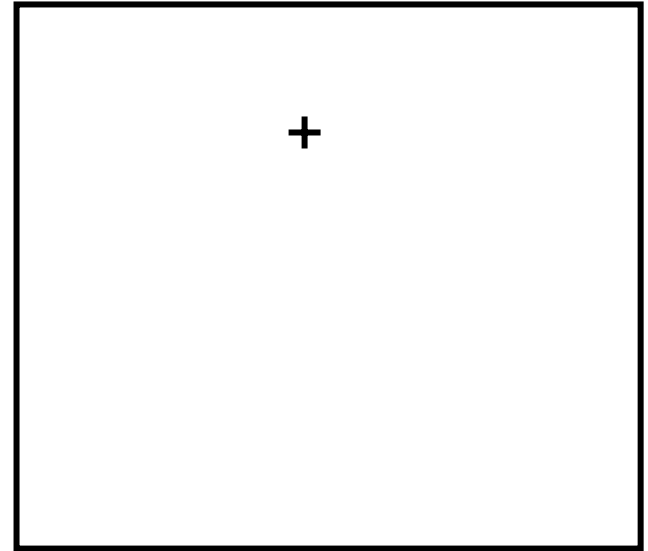
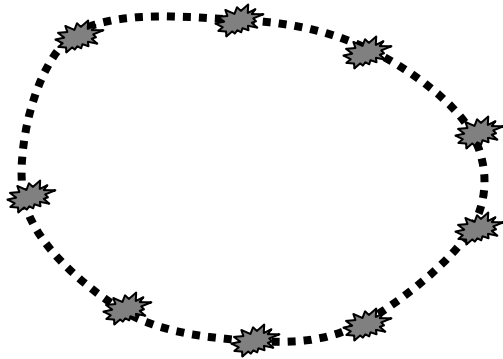
Imaging with molecular resolution



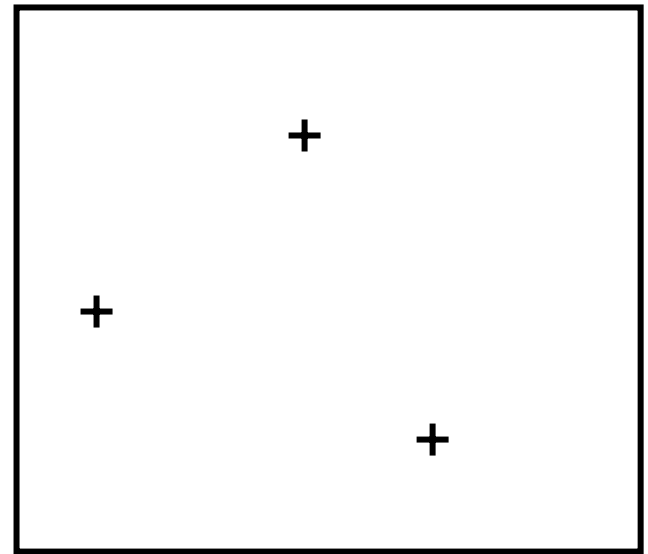
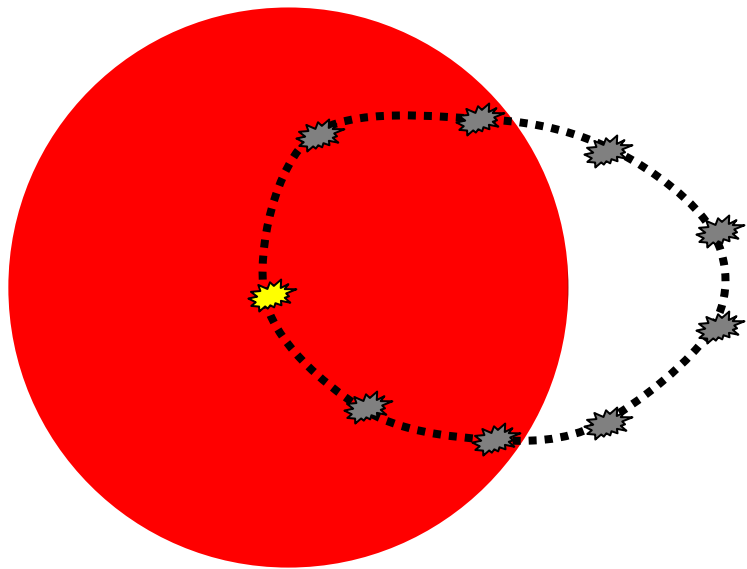
Imaging with molecular resolution



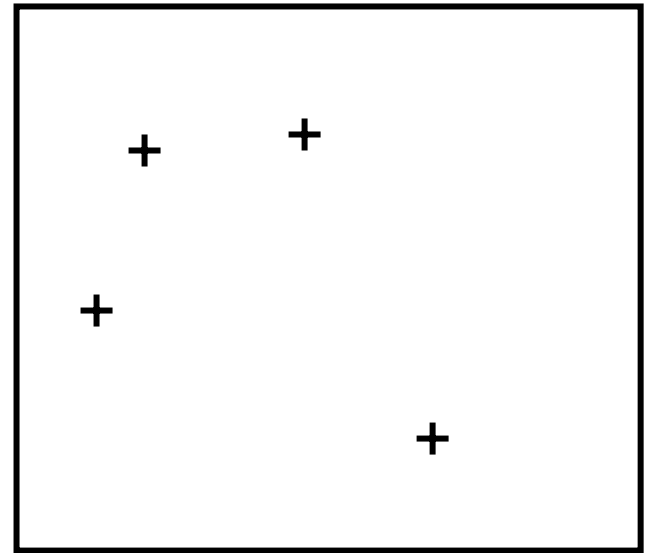
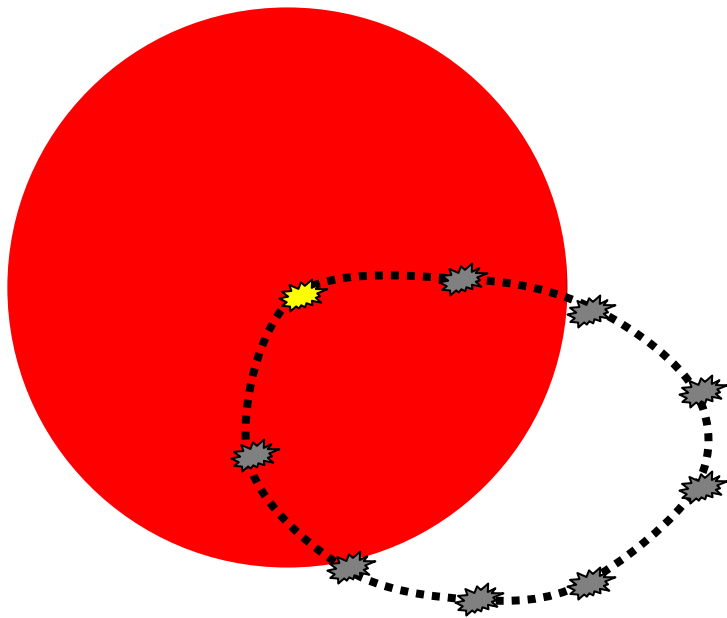
Imaging with molecular resolution



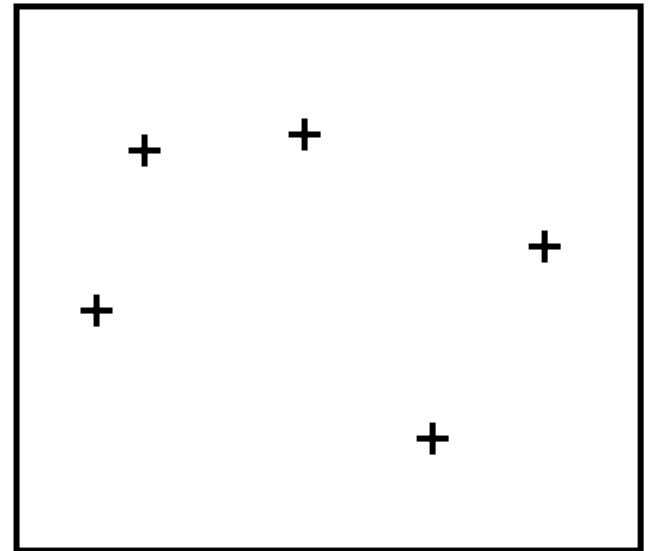
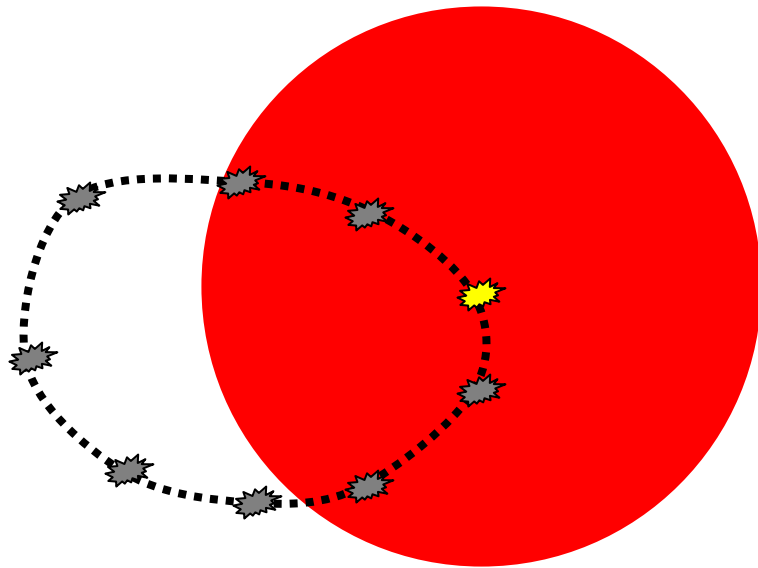
Imaging with molecular resolution



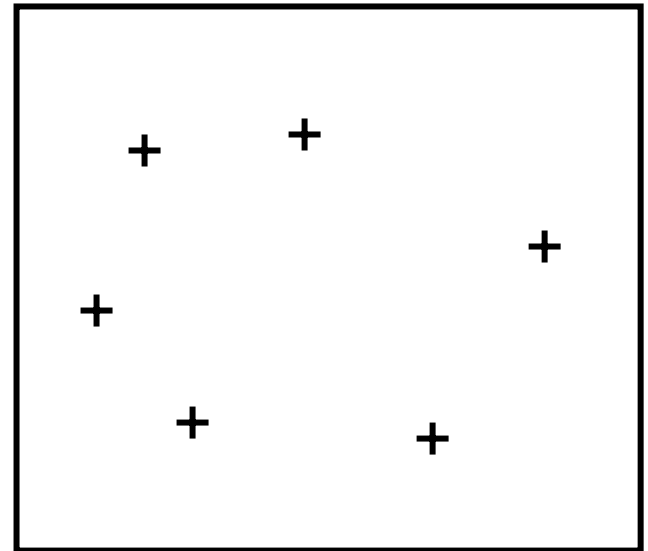
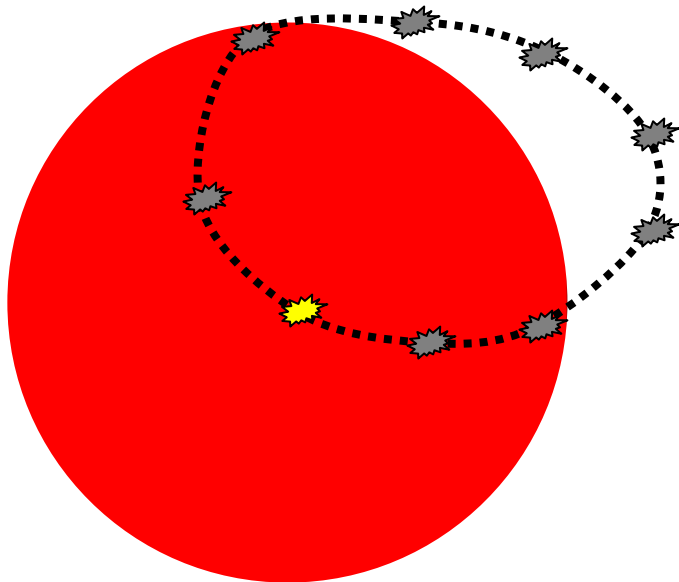
Imaging with molecular resolution



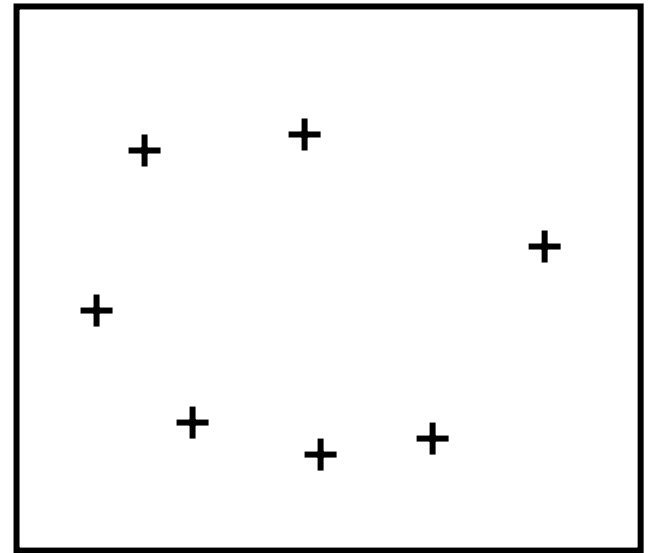
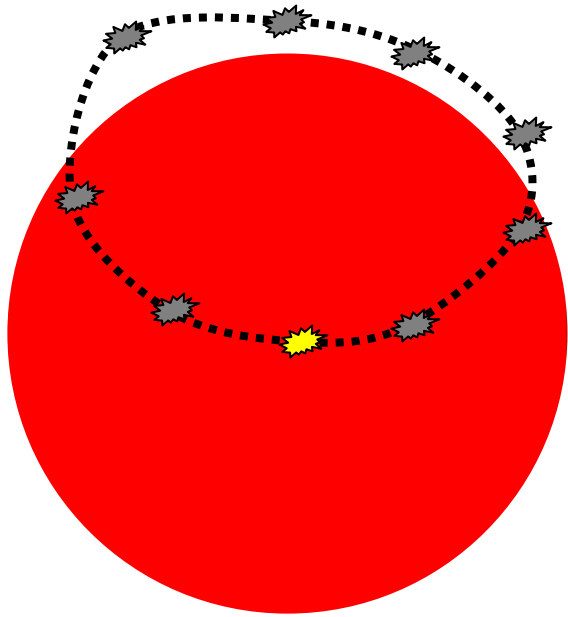
Imaging with molecular resolution



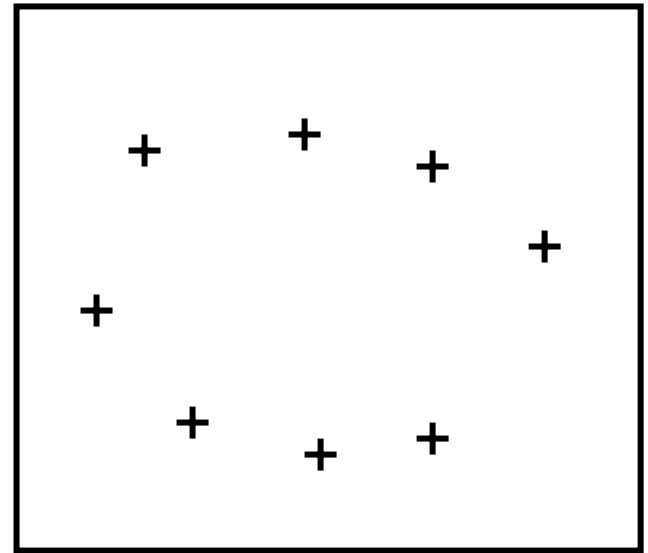
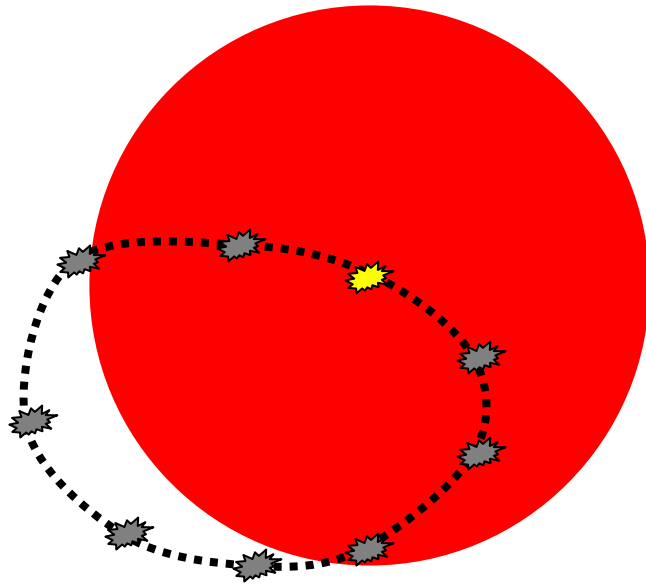
Imaging with molecular resolution



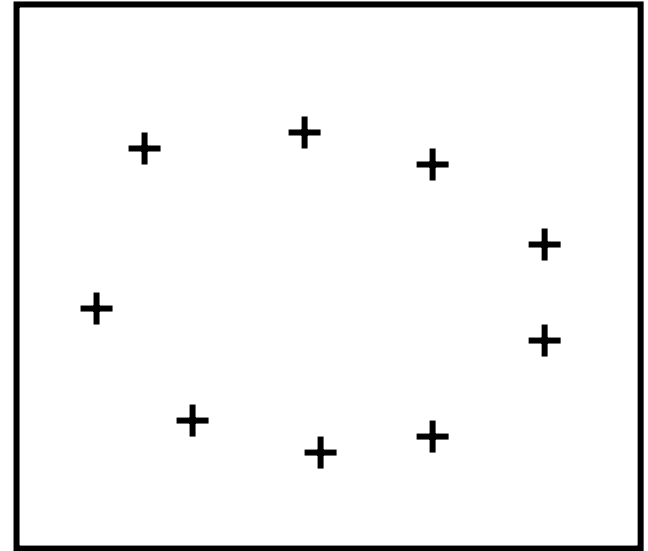
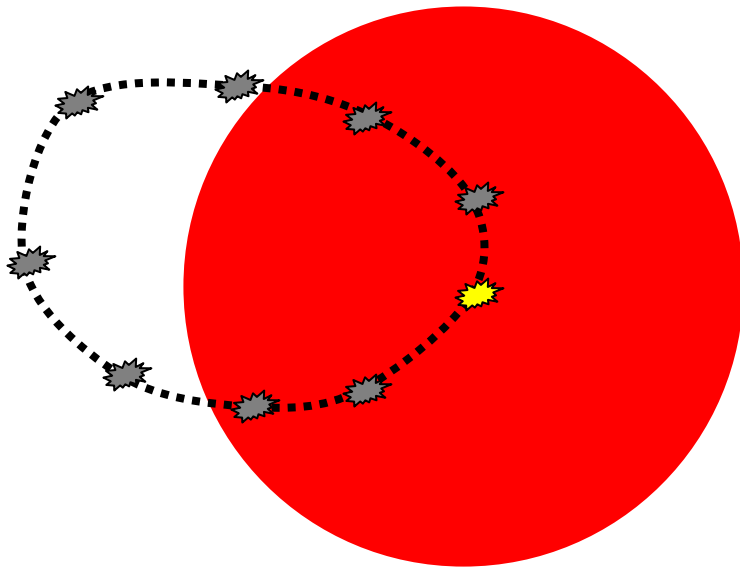
Imaging with molecular resolution



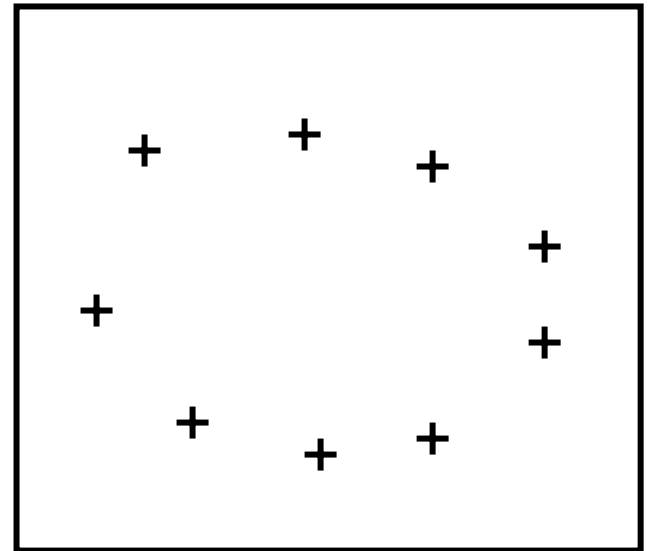
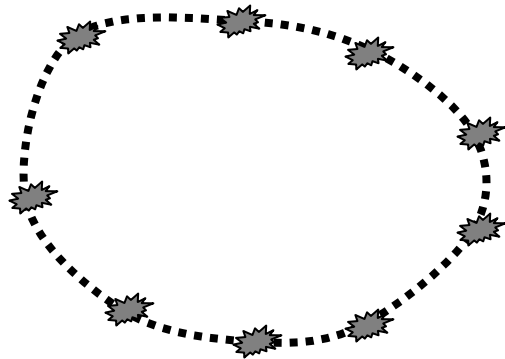
Imaging with molecular resolution



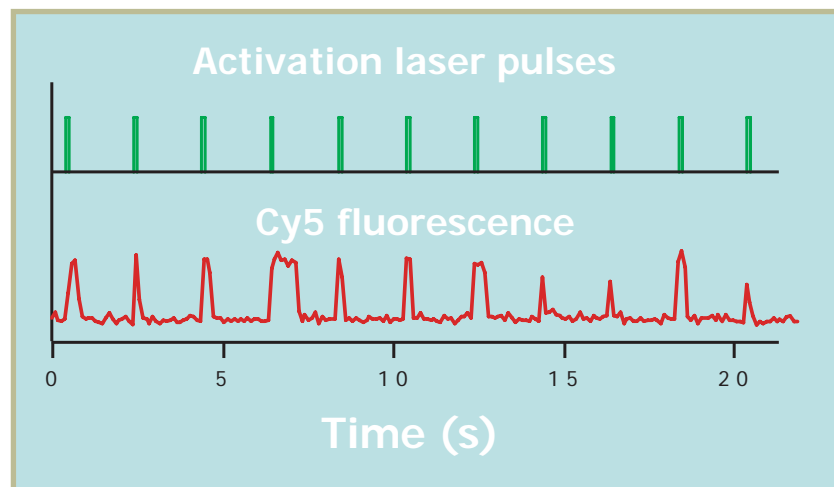
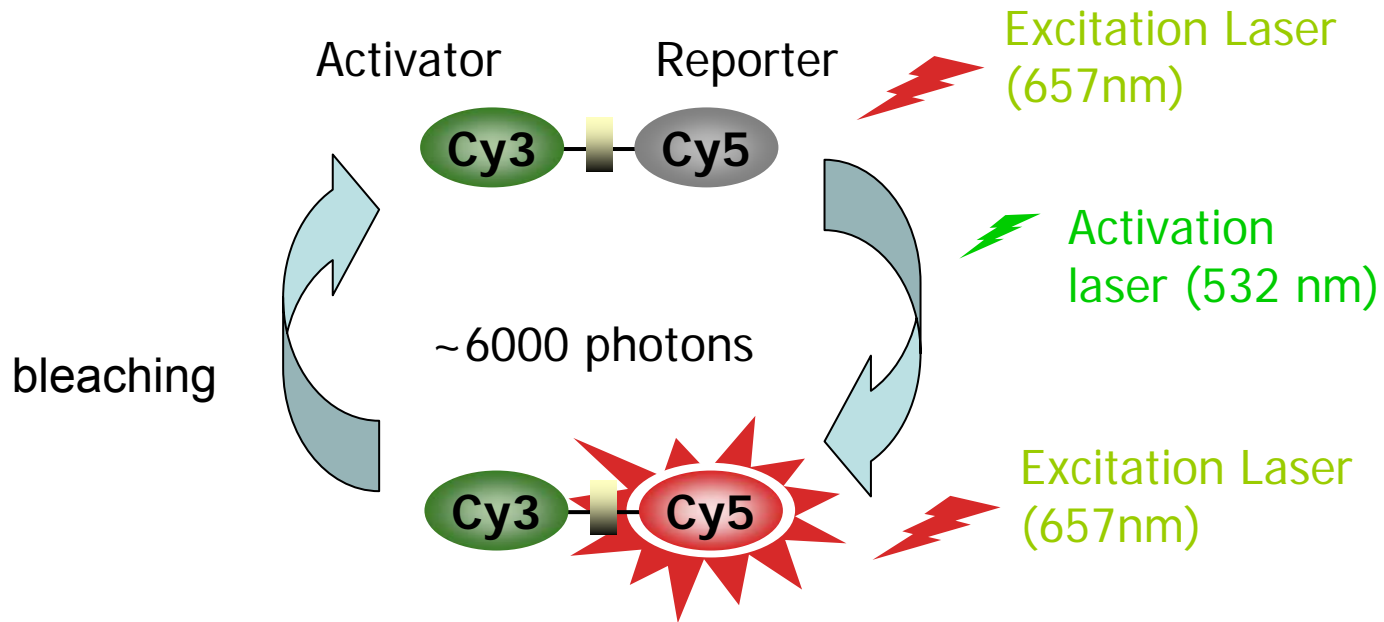
Imaging with molecular resolution



Imaging with molecular resolution

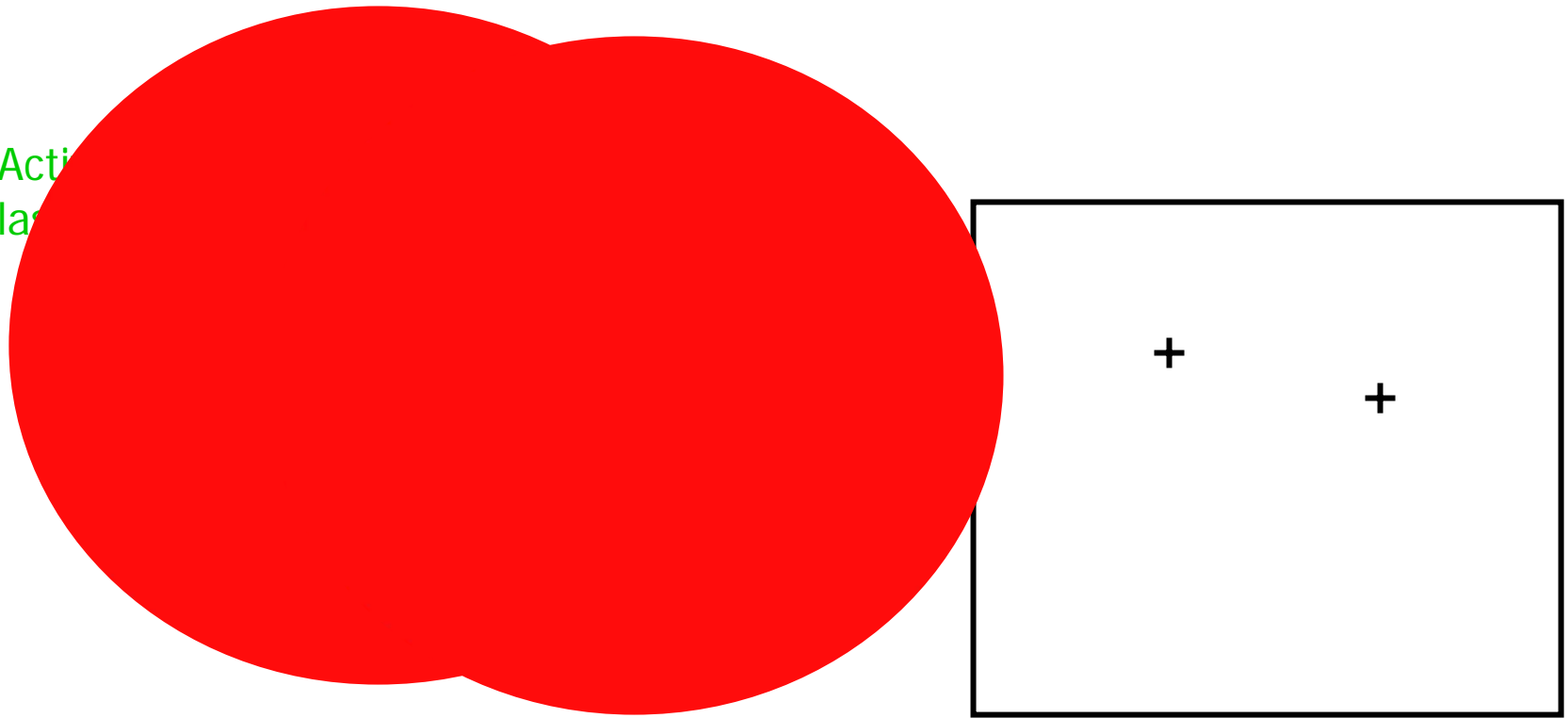


Imaging with molecular resolution

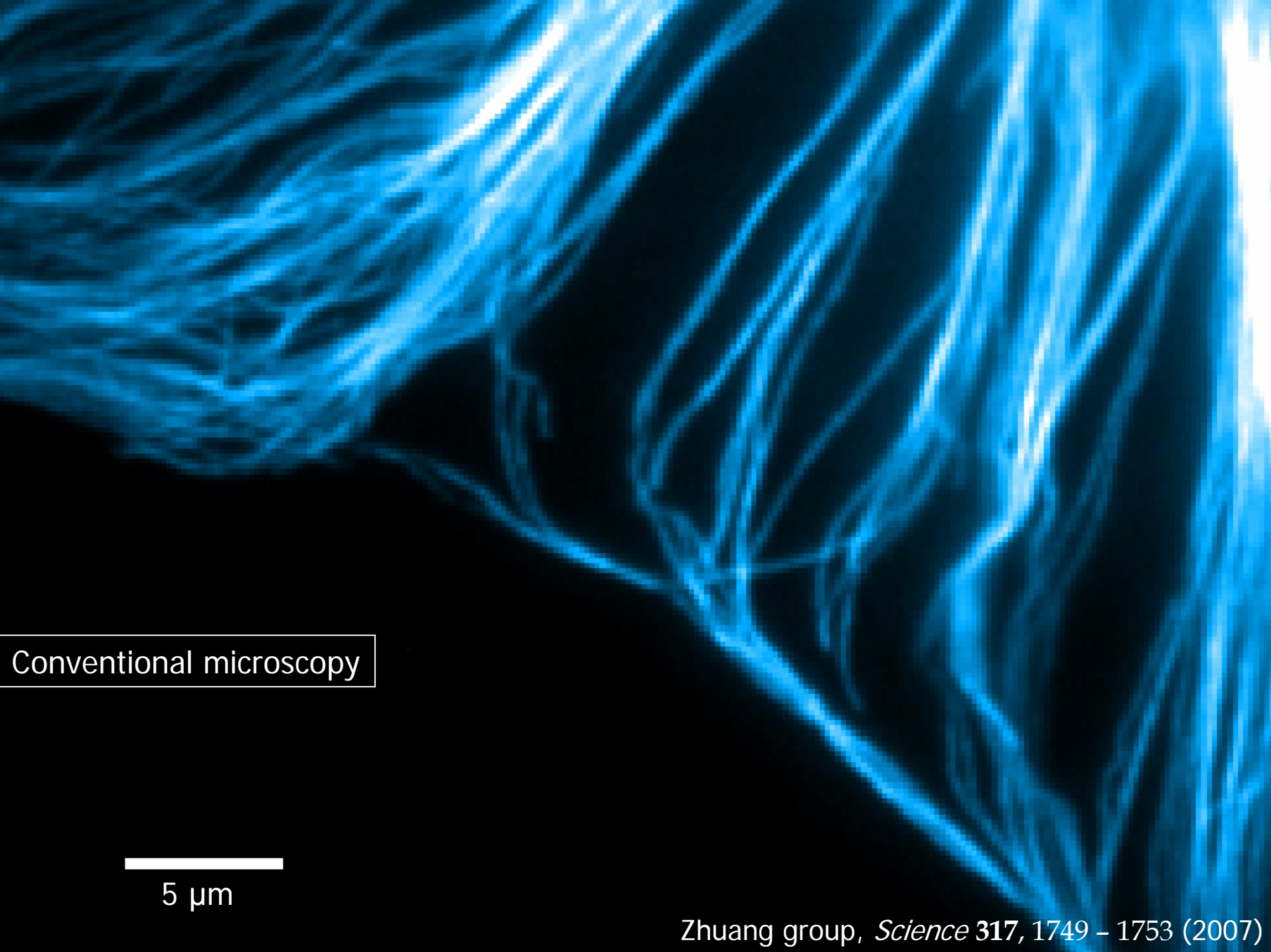


Imaging with molecular resolution

Acti
lar



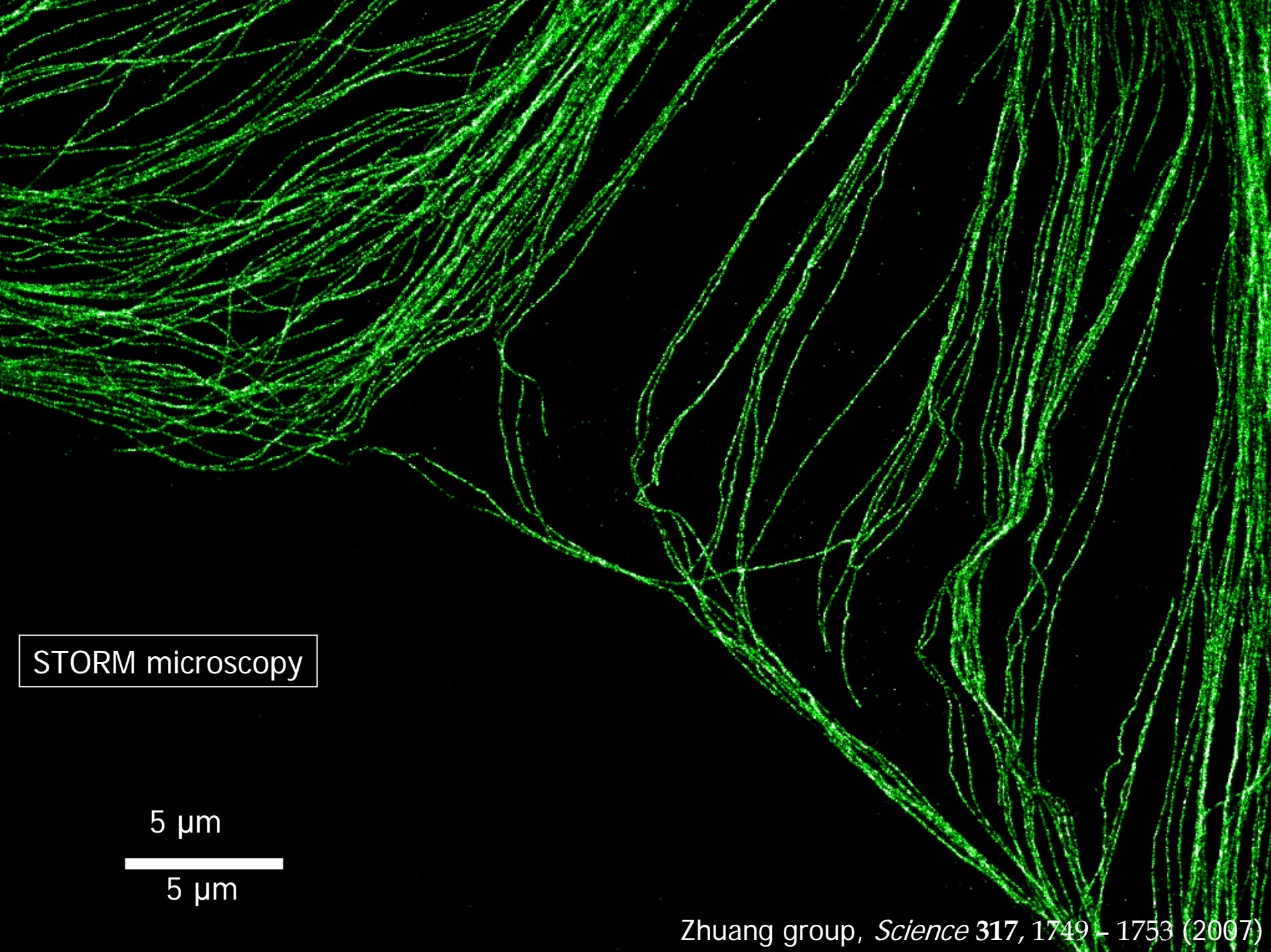
PALM, Betzig & Hess
STORM, Zhuang



Conventional microscopy



5 μm

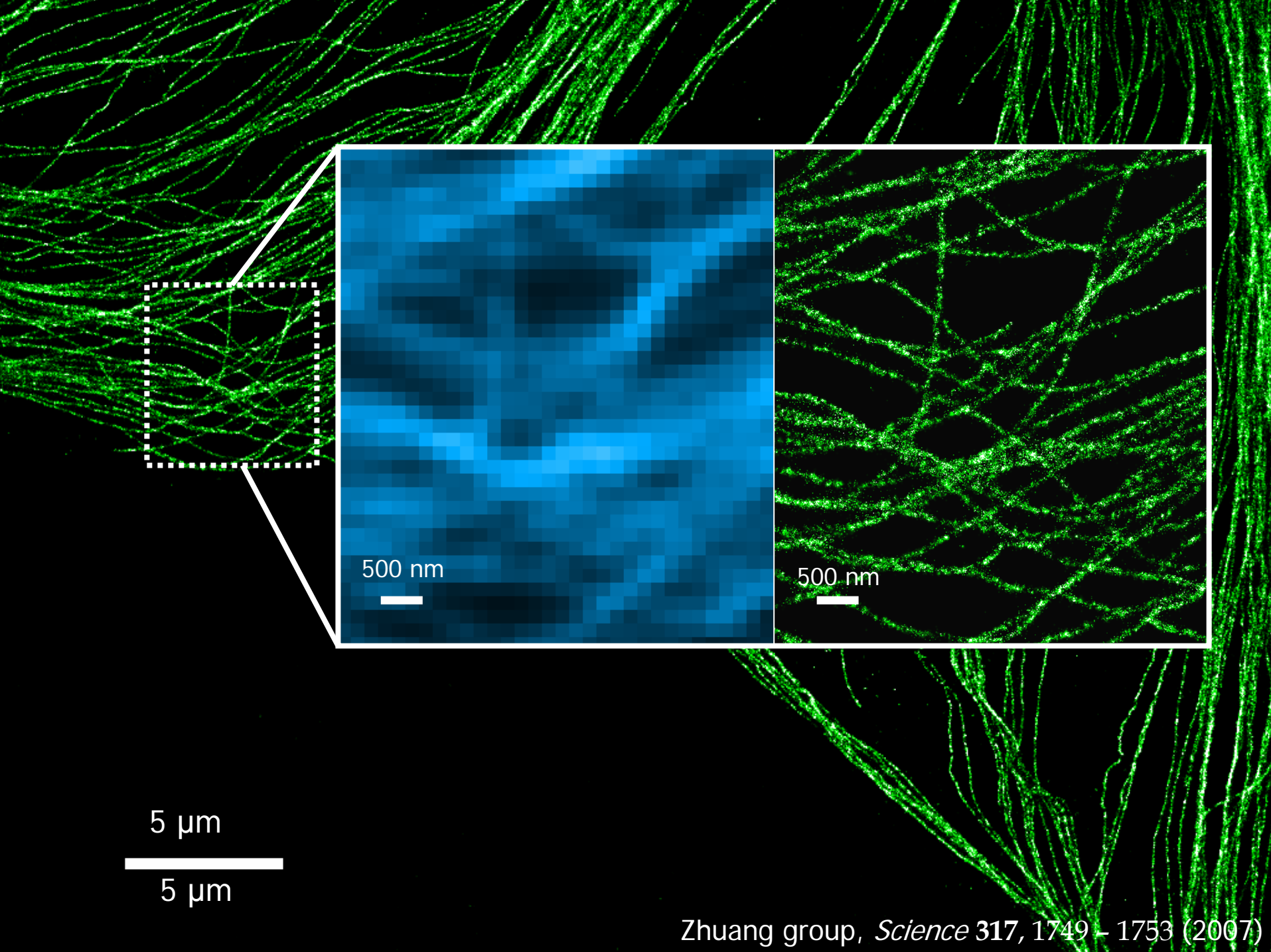


STORM microscopy

5 μm



5 μm

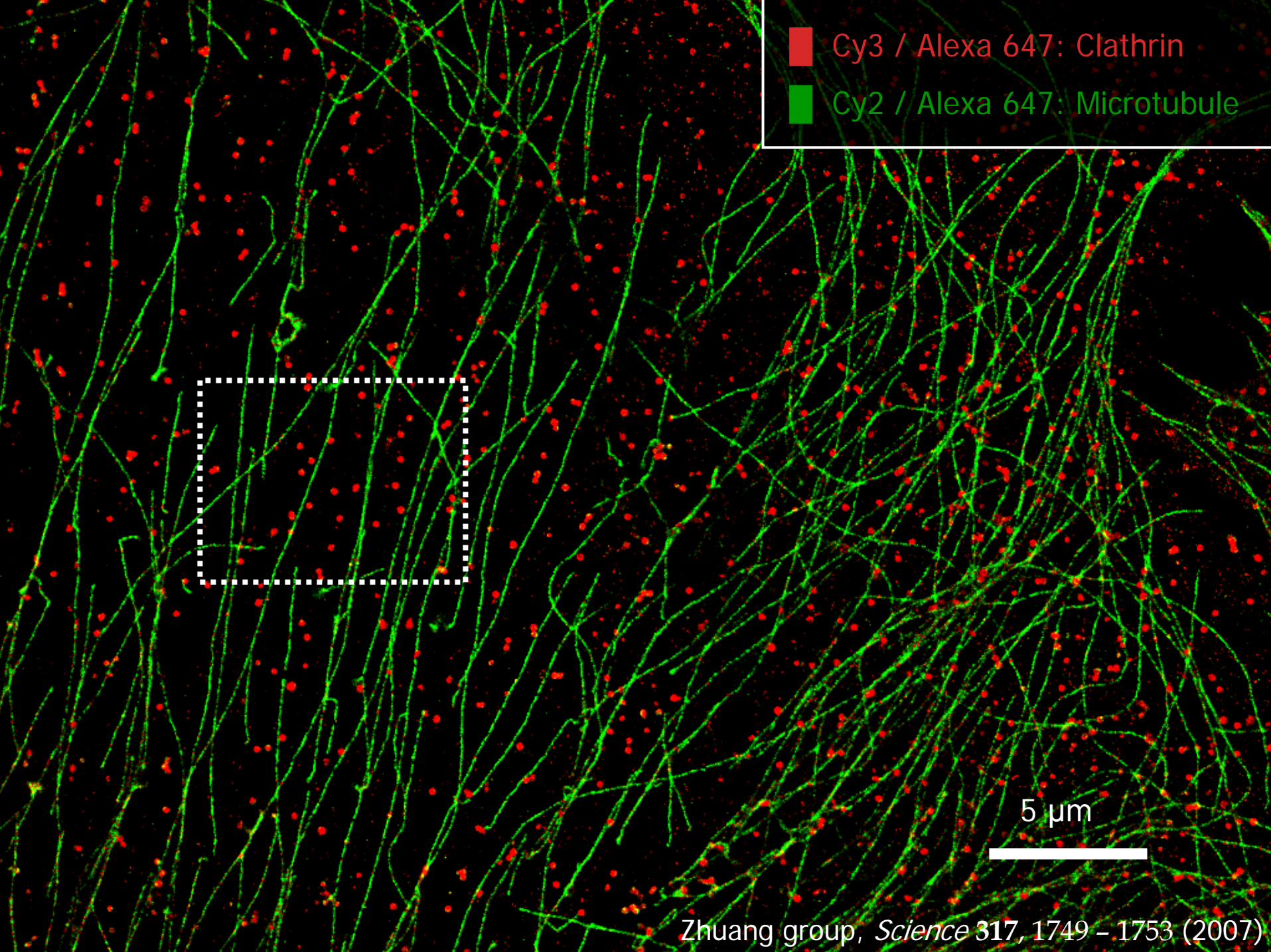


5 μm

5 μm

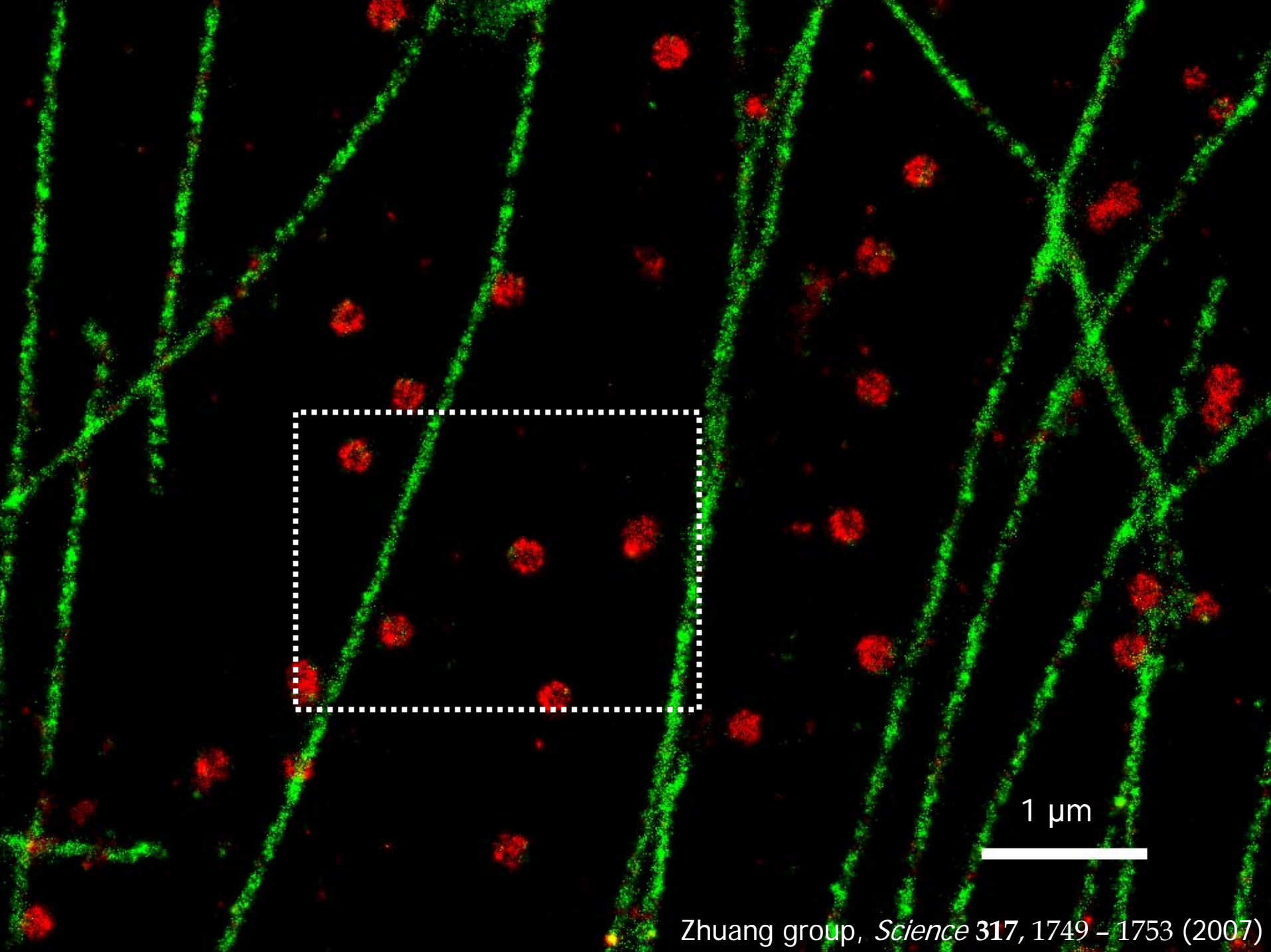
500 nm

500 nm

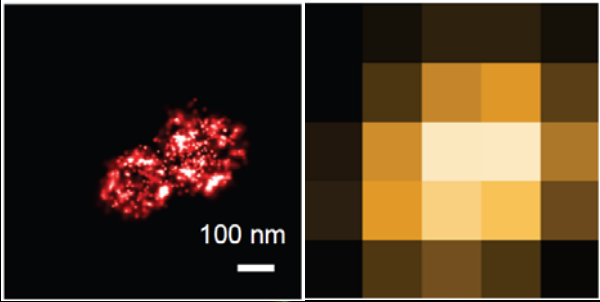
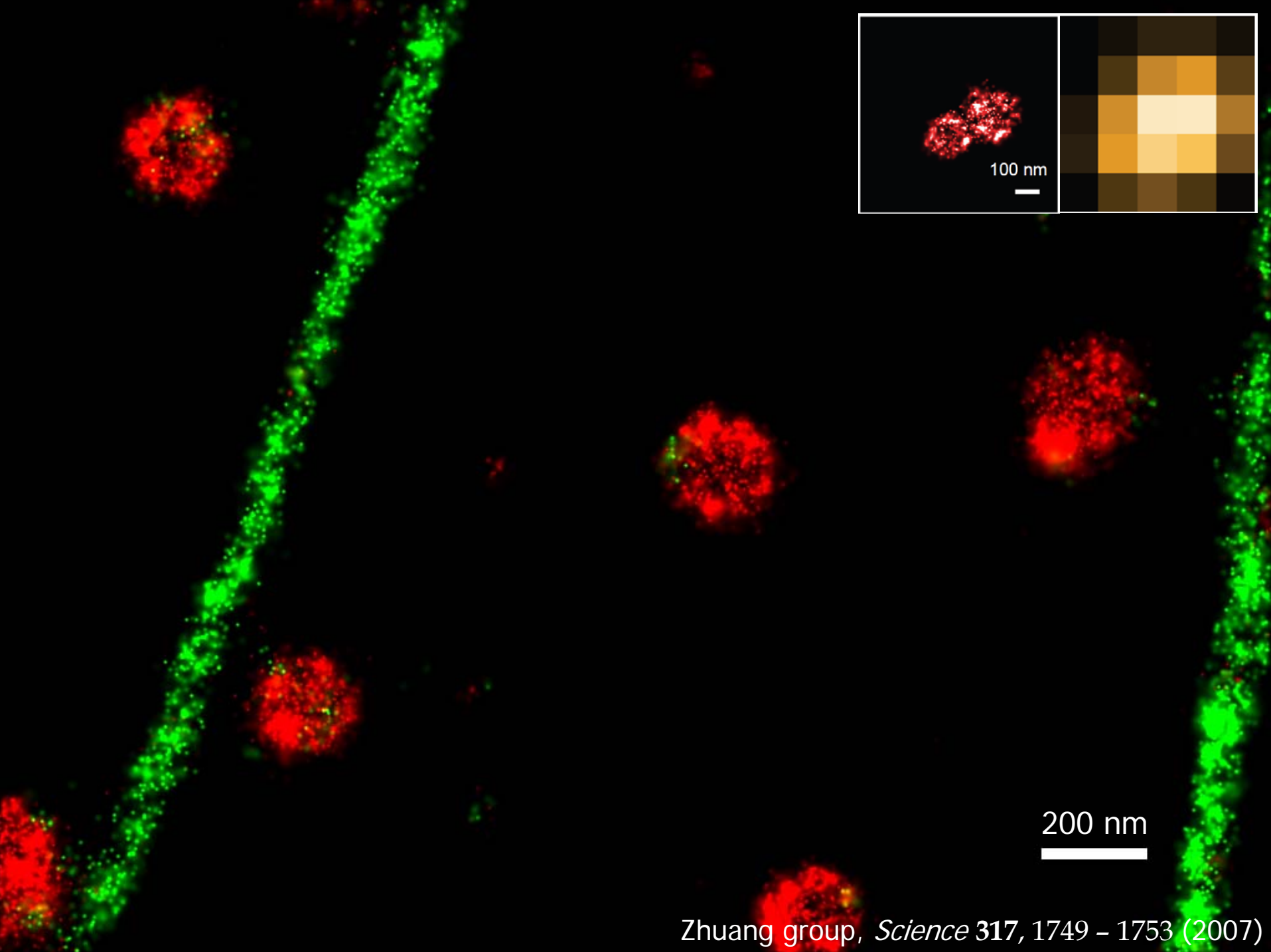


■ Cy3 / Alexa 647: Clathrin
■ Cy2 / Alexa 647: Microtubule

5 μm

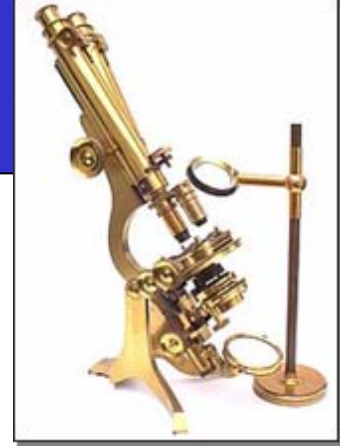


1 μm



Zhuang group, *Science* 317, 1749 – 1753 (2007)

Lasers and Microscopy



Brief history of microscopy

Microscopy has always been a driving force in optics

Lasers meet Microscopy

LSM – bread-and-butter of modern biological microscopy

Nonlinear microscopy

Nonlinear optics brings new contrast mechanisms

Breaking the resolution limit

PALM/STORM microscopy w molecular resolution

Microscopy resources

Web resources on microscopy:

<http://www.microscopyu.com/>

by Nikon

<http://www.olympusmicro.com/>

by Olympus

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

by Molecular Expressions

<http://zhuang.harvard.edu/storm.html>

on STORM microscopy

<http://www.weizmann.ac.il/home/feyaron/>

on THG microscopy

<http://bernstein.harvard.edu/research/cars.html>

on CARS microscopy

<http://www.drbio.cornell.edu/>

Cornell imaging center

<http://www.antique-microscopes.com/>

Antique microscopes

