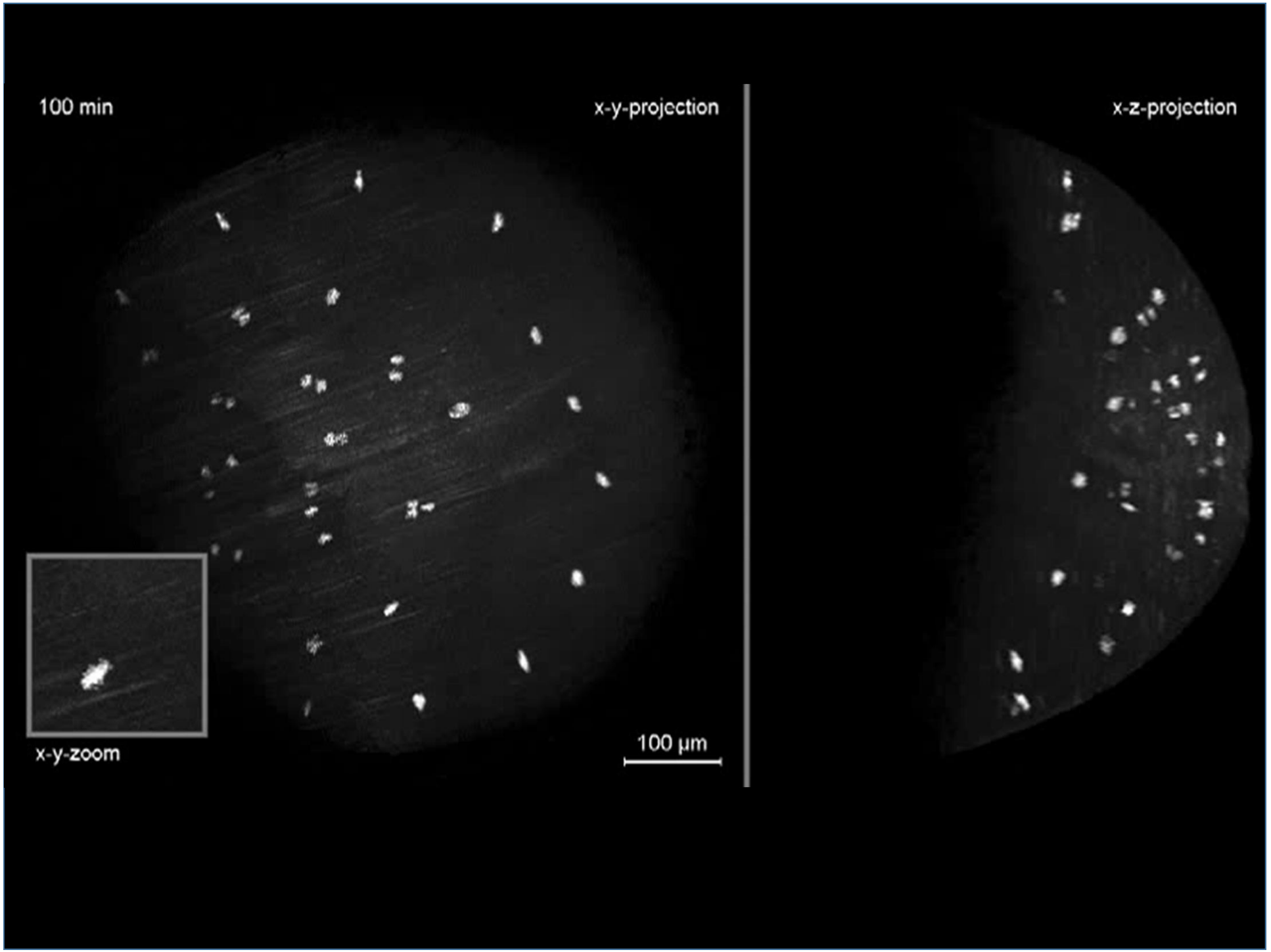
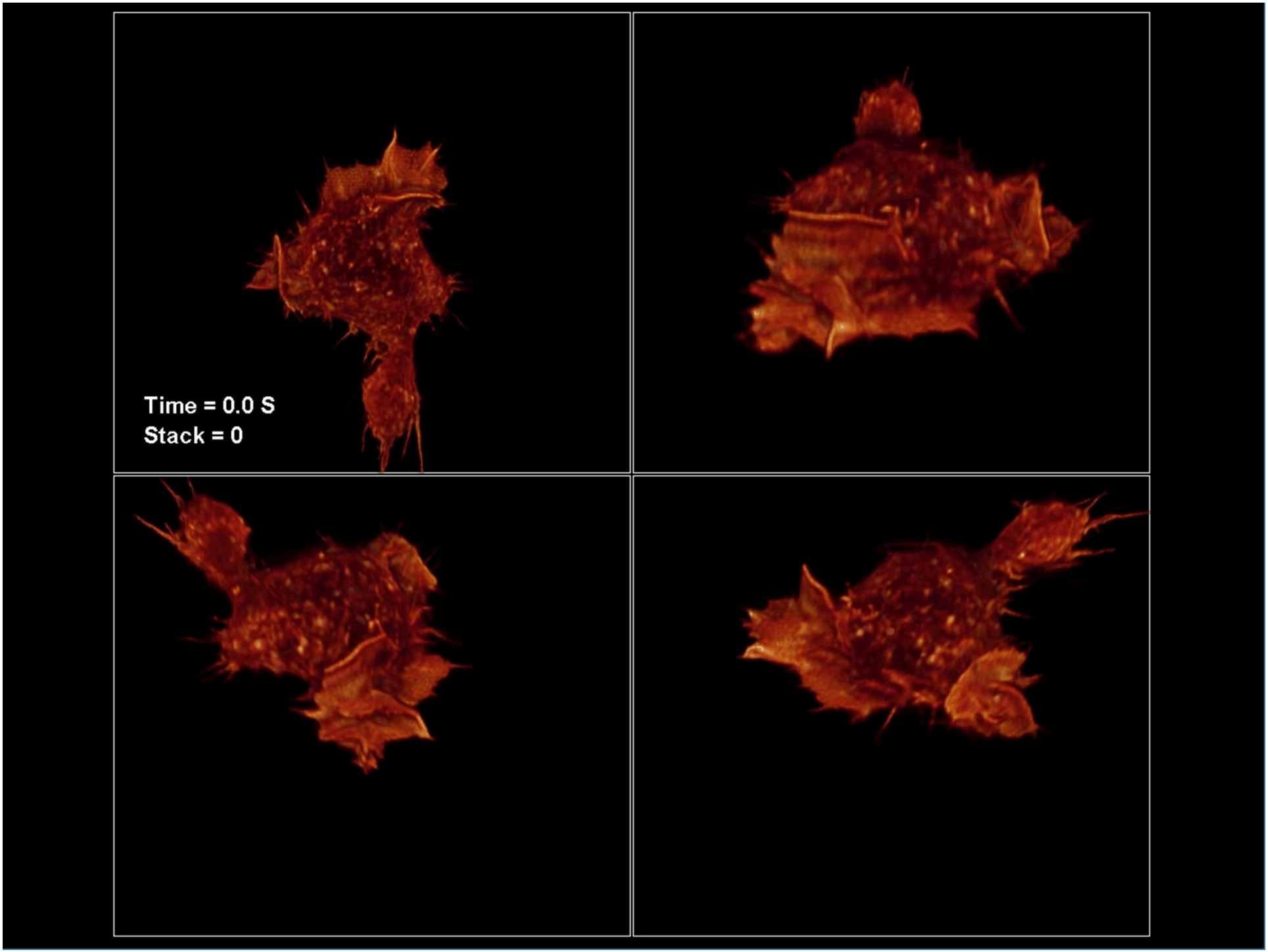
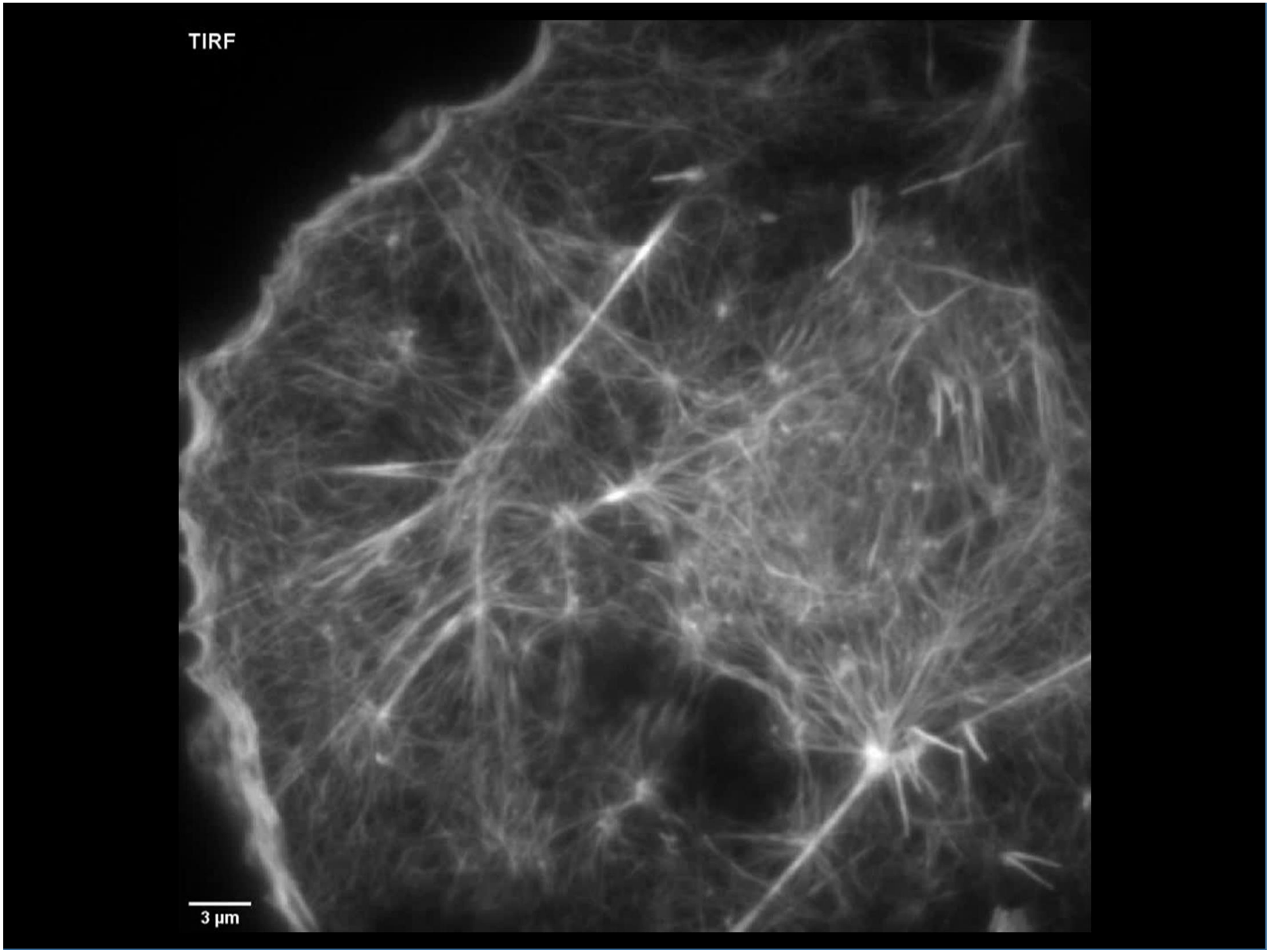
Optical Imaging for Biological Sciences

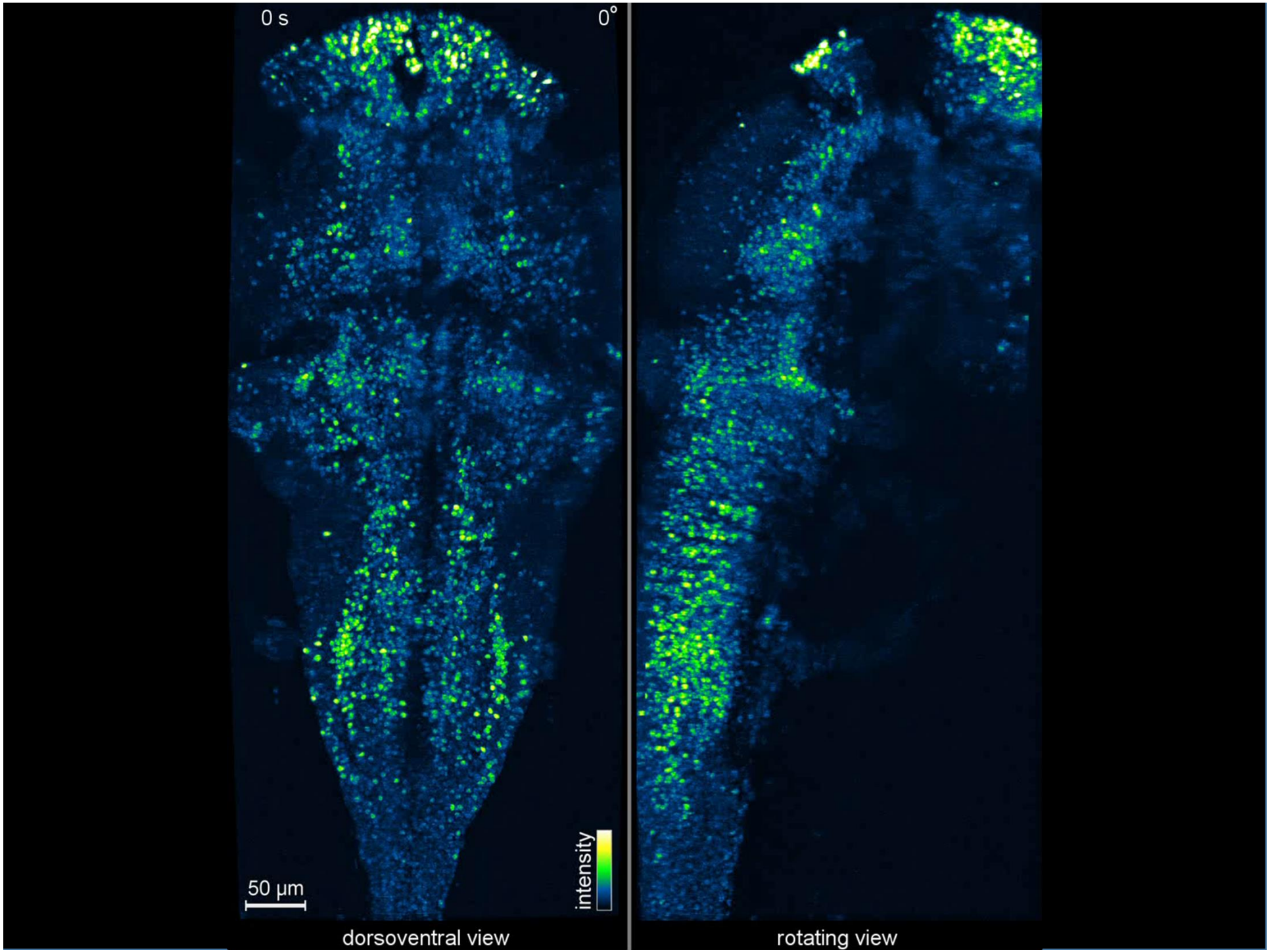
Kai Wang

2020


100 min
x-y-projection
x-z-projection
x-y-zoom
100 µm


Time = 0.0 S
Stack = 0


TIRF
3 µm


0 s
0°
50 µm
intensity
dorsoventral view
rotating view

Outline

About the light and imaging

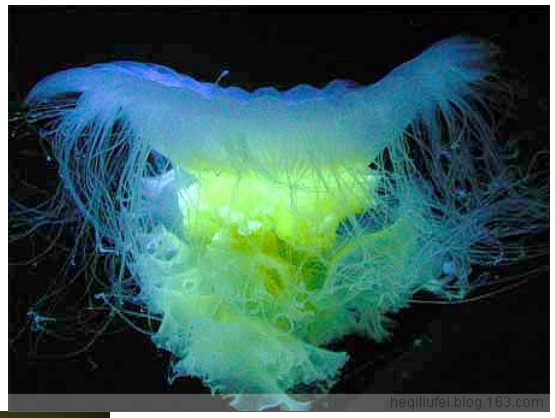
Optical imaging

- Basics
- Optical Neuroimaging

About the light

Energy

& Signal

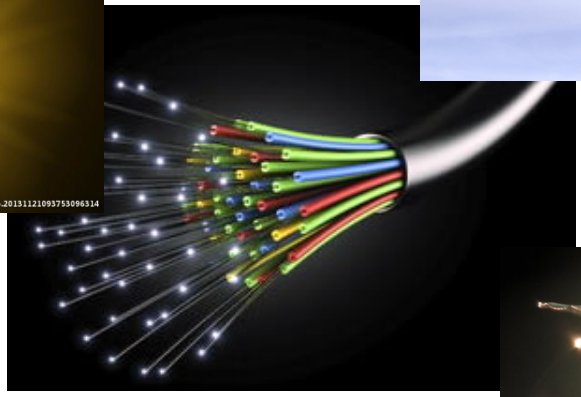


About the light

firework

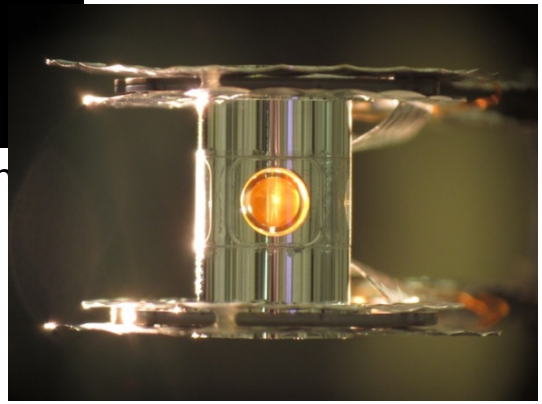
illumination

Laser weapon



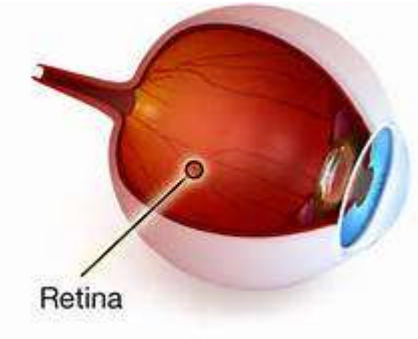
Optical communication

display

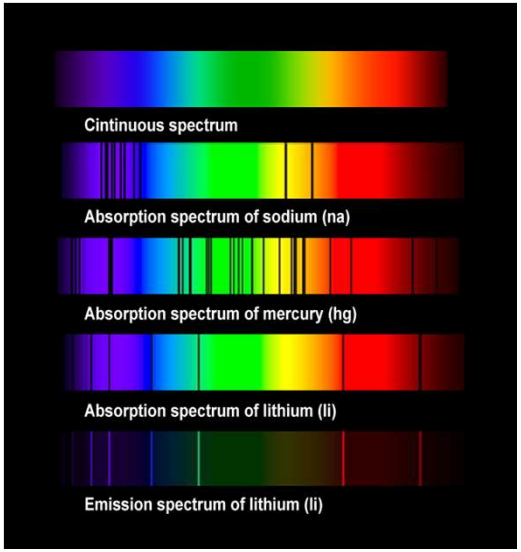


Laser induced nuclear fusion

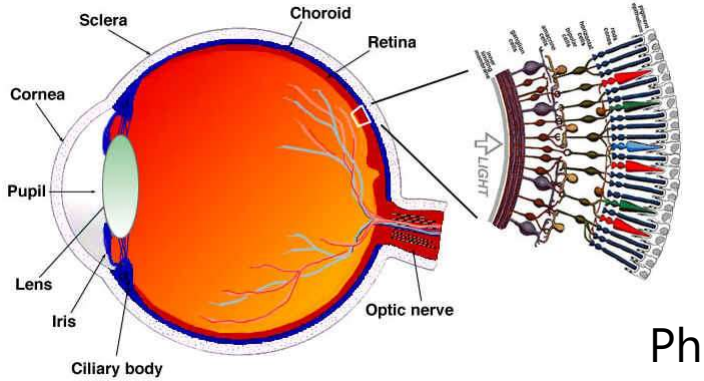
About the light



Colors &
Contrast



Absorption spectrum

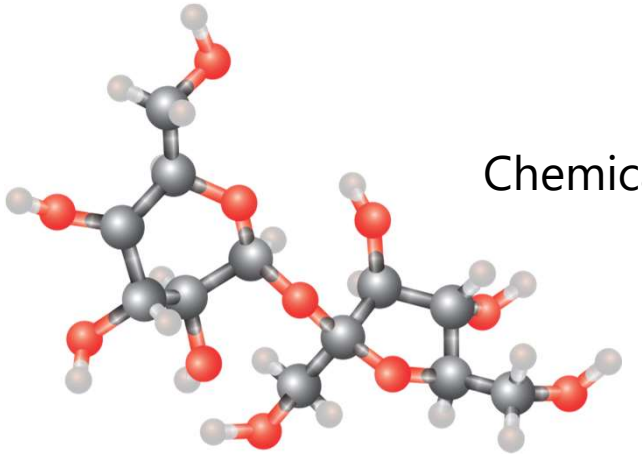


Photoreceptor

About the light

Why light is so important and everywhere in our lives?

Light is electromagnetic wave and can mediate electromagnetic interaction, which is one of the four fundamental interactions we know so far.



Chemical bond and reaction

About the light

Is there any other better means?

No!

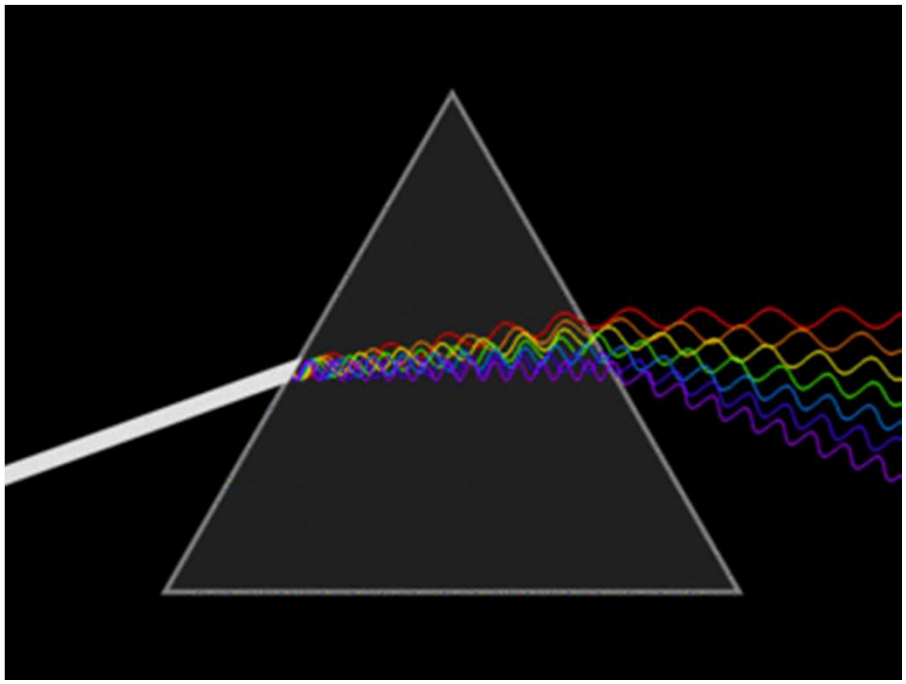
Stick to light, there is no other way out!!!

About the light

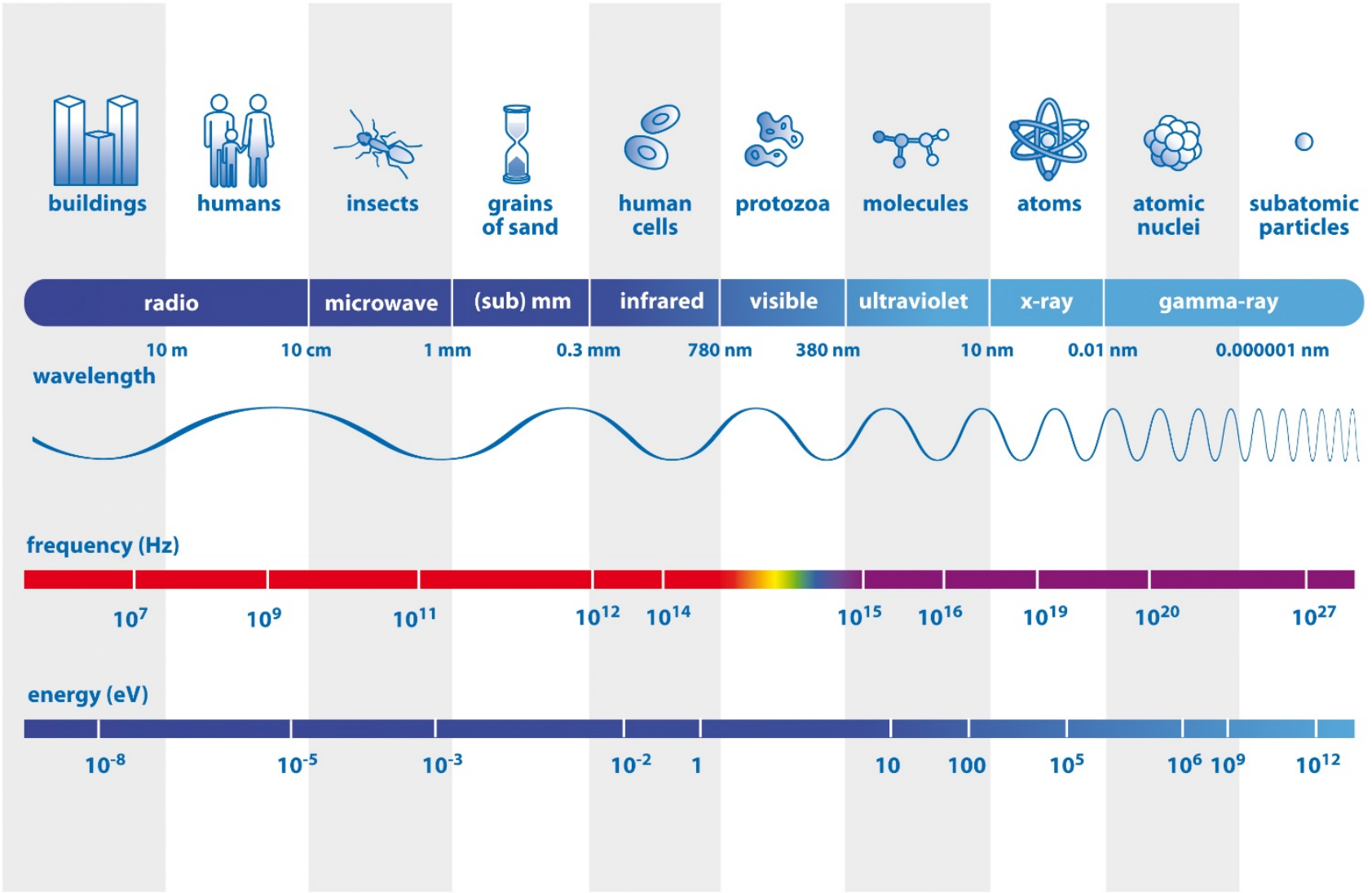
Physical Properties of Light

- Wavelength
- Photon energy

$$E = \hbar\nu$$

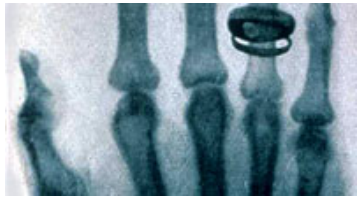


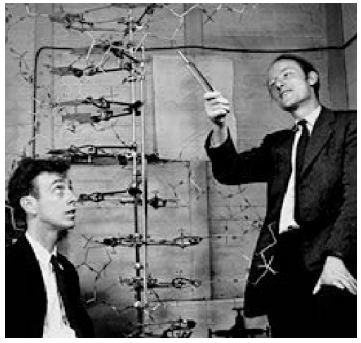
About the light



X Ray

First Nobel Prize in Physics





1900
1901
W. C. Röntgen, in physics, for the discovery of X-rays.

1910
1914
M. von Laue, in physics, for the discovery of X-rays by crystals.

1915
W. H. Bragg and W. L. Bragg, in physics, for the determination of crystal structures using X-rays.

1920
1917
C. G. Barkla, in physics, for the discovery of the characteristic X-ray radiation of the elements.

1930
1924
M. Siegbahn, in physics, for discoveries in the field of X-ray spectroscopy.

1940
1927
A. H. Compton, in physics, for revealing the particle nature of X-rays in scattering experiments on electrons.

1936
P. Debye, in chemistry, for determining molecular structures by X-ray diffraction in gases.

1950

1960
1962
M. F. Perutz and J. C. Kendrew, in chemistry, for determining the structure of hemoglobin and myoglobin.

1970
1962 F. Crick, J. Watson and M. Wilkins, in medicine, for their discoveries concerning the molecular structure of nucleic acids and its significance for information transfer in living material.

1980
1964
D. Crowfoot Hodgkin, in chemistry, for the determination of the structure of penicillin and other important biochemical substances.

1990
1976
W. N. Lipscomb, in chemistry, for the determination of boranes.

1979
A. M. Cormack and G. N. Hounsfield, in medicine, for the development of computerized tomography.

1981
M. Siegbahn, in physics, for developing high resolution electron spectroscopy.

1985
H. A. Hauptman and J. Karle, in chemistry, for the development of direct methods for X-ray crystallographic structure determination.

1988
J. Deisenhofer, R. Huber and H. Michel, in chemistry, for the determination of protein structures crucial to photosynthesis.


The Nobel Prize in Physics 1901 - Wilhelm Conrad Röntgen »


The Nobel Prize in Physics 1914 - Max von Laue »


The Nobel Prize in Physics 1915 - Sir William Henry Bragg »


The Nobel Prize in Physics 1915 - William Lawrence Bragg »


The Nobel Prize in Physics 1917 - Charles Glover Barkla »


The Nobel Prize in Physics 1924 - Karl Manne Georg Siegbahn »


The Nobel Prize in Physics 1927 - Arthur Holly Compton »


The Nobel Prize in Chemistry 1936 - Petrus (Peter) Josephus Wilhelmus Debye »


The Nobel Prize in Chemistry 1962 - Max Ferdinand Perutz »


The Nobel Prize in Chemistry 1962 - John Cowdery Kendrew »


The Nobel Prize in Physiology or Medicine 1962 - Francis Harry Compton Crick »


The Nobel Prize in Physiology or Medicine 1962 - James Dewey Watson »


The Nobel Prize in Physiology or Medicine 1962 - Maurice Hugh Frederick Wilkins »


The Nobel Prize in Chemistry 1964 - Dorothy Crowfoot Hodgkin »


The Nobel Prize in Chemistry 1976 - William N. Lipscomb »


The Nobel Prize in Physiology or Medicine 1979 - Allan M. Cormack »


The Nobel Prize in Physiology or Medicine 1979 - Godfrey N. Hounsfield »


The Nobel Prize in Physics 1981 - Kai M. Siegbahn »


The Nobel Prize in Chemistry 1985 - Herbert A. Hauptman »


The Nobel Prize in Chemistry 1985 - Jerome Karle »

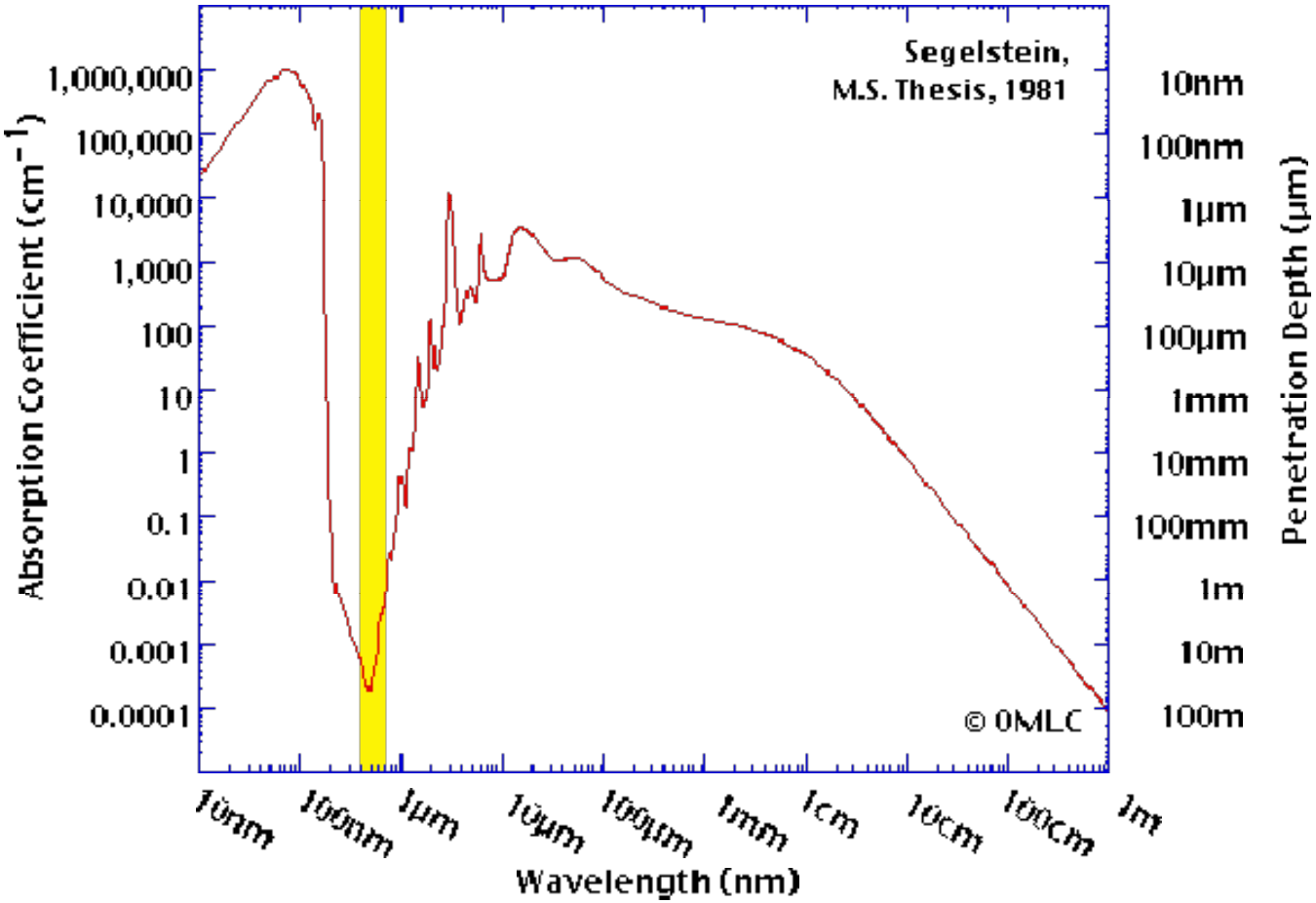

The Nobel Prize in Chemistry 1988 - Johann Deisenhofer »


The Nobel Prize in Chemistry 1988 - Robert Huber »


The Nobel Prize in Chemistry 1988 - Hartmut Michel »

Visible light can penetrate deep into water

Water absorption spectrum



Visible light has proper energy to interact moderately with materials

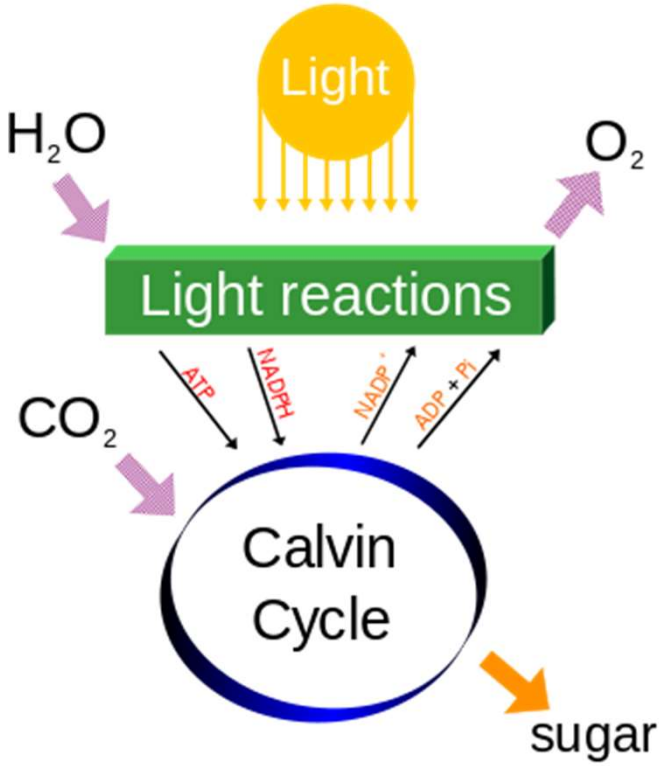
Bond	Bond	Bond-dissociation energy at 298 K			Comment
		(kcal/mol)	(kJ/mol)	(eV)	
C - C	C - C bond	83 - 85	347 - 356	3.60 - 3.69	Strong, but weaker than C - H bonds
Cl - Cl	Chlorine	58	242	2.51	Indicated by the yellowish colour of this gas
Br - Br	Bromine	46	192	1.99	Indicated by the brownish colour of Br_2 Source of the $Br^{\bullet}$ radical
I - I	Iodine	36	151	1.57	Indicated by the purplish colour of I_2 Source of the $I^{\bullet}$ radical
H - H	Hydrogen	104	436	4.52	Strong, nonpolarizable bond Cleaved only by metals and by strong oxidants
O - H	Hydroxyl	110	460	4.77	Slightly stronger than C - H bonds
O=O	Oxygen	119	498	5.15	Stronger than single bonds Weaker than many other double bonds
N≡N	Nitrogen	226	945	9.79	One of the strongest bonds Large activation energy in production of ammonia

Visible light has proper energy to interact moderately with materials

Bond	Bond	Bond-dissociation energy at 298 K		Comment
		(kcal/mol)	(kJ/mol)	
H_3C-H	Methyl C-H bond	105	439	One of the strongest aliphatic C-H bonds
C_2H_5-H	Ethyl C-H bond	101	423	Slightly weaker than H_3C-H
$(CH_3)_3C-H$	Tertiary C-H bond	96.5	404	Tertiary radicals are stabilized
CH_2CH-H	Vinyl C-H bond	111	464	Vinyl radicals are rare
HC_2-H	acetylenic C-H bond	133	556	Acetylenic radicals are very rare
C_6H_5-H	Phenyl C-H bond	113	473	Comparable to vinyl radical, rare
CH_2CHCH_2-H	Allylic C-H bond	89	372	Such bonds show enhanced reactivity
$C_6H_5CH_2-H$	Benzylic C-H bond	90	377	Akin to allylic C-H bonds Such bonds show enhanced reactivity
H_3C-CH_3	Alkane C-C bond	83-85	347-356	Much weaker than a C-H bond
$H_2C=CH_2$	Alkene C=C bond	146-151	611-632	About 2× stronger than a C-C single bond
$HC\equiv CH$	Alkyne C≡C triple bond	200	837	About 2.5× stronger than a C-C single bond

96.485 kJ/mol ≡ 1 eV

Photosynthesis



$6CO_2$ Carbon dioxide + $6H_2O$ Water $\xrightarrow{\text{Light}}$ $C_6H_{12}O_6$ Sugar + $6O_2$ Oxygen

From wiki

Visible light

Visible light is bio-compatible.

Use visible light for in *vivo* biological studies!

Optical imaging

Why imaging?

Seeing is believing!

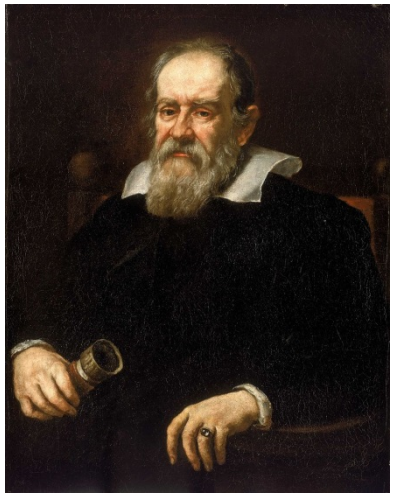
Why imaging?

What's is understanding?

Subjective	The world is 3D. The world is physically there. This is how the world works, despite what you think
Objective	Our understanding is built on our collected information and experience, based on which we can predict.

Vision is our major sense to collect information, so seeing leads to understanding!!!

Early times of optical imaging



Galileo Galilei 1564 – 1642

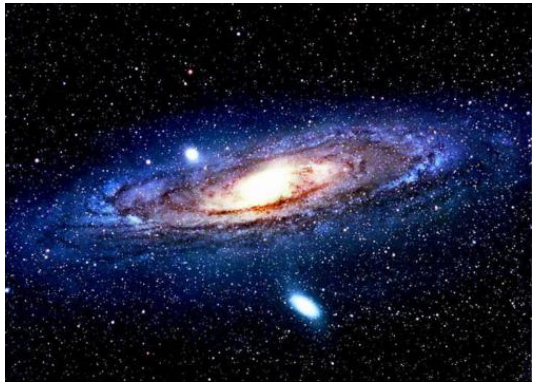
- "Father of observational astronomy"
- "Father of modern physics"
- "Father of scientific method"
- "Father of science"
- "Father of modern science", by Albert Einstein

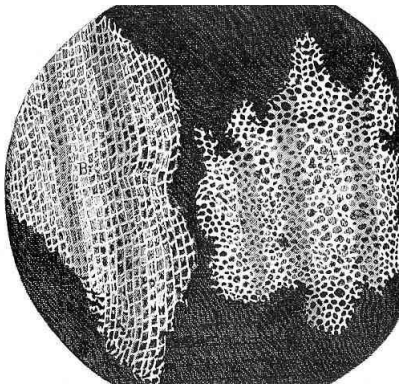
"In 1609, Galileo was, along with Englishman Thomas Harriot and others, among the first to use a refracting telescope as an instrument to observe stars, planets or moons."

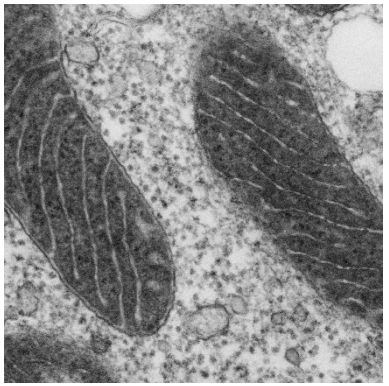
"In 1610, he used a telescope at close range to magnify the parts of insects."

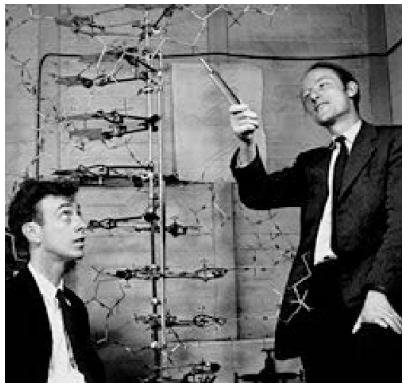
Seeing is believing

X ray









10^{26}m

10^{-6}m

10^{-9}m

10^{-11}m

Outline

About the light and imaging

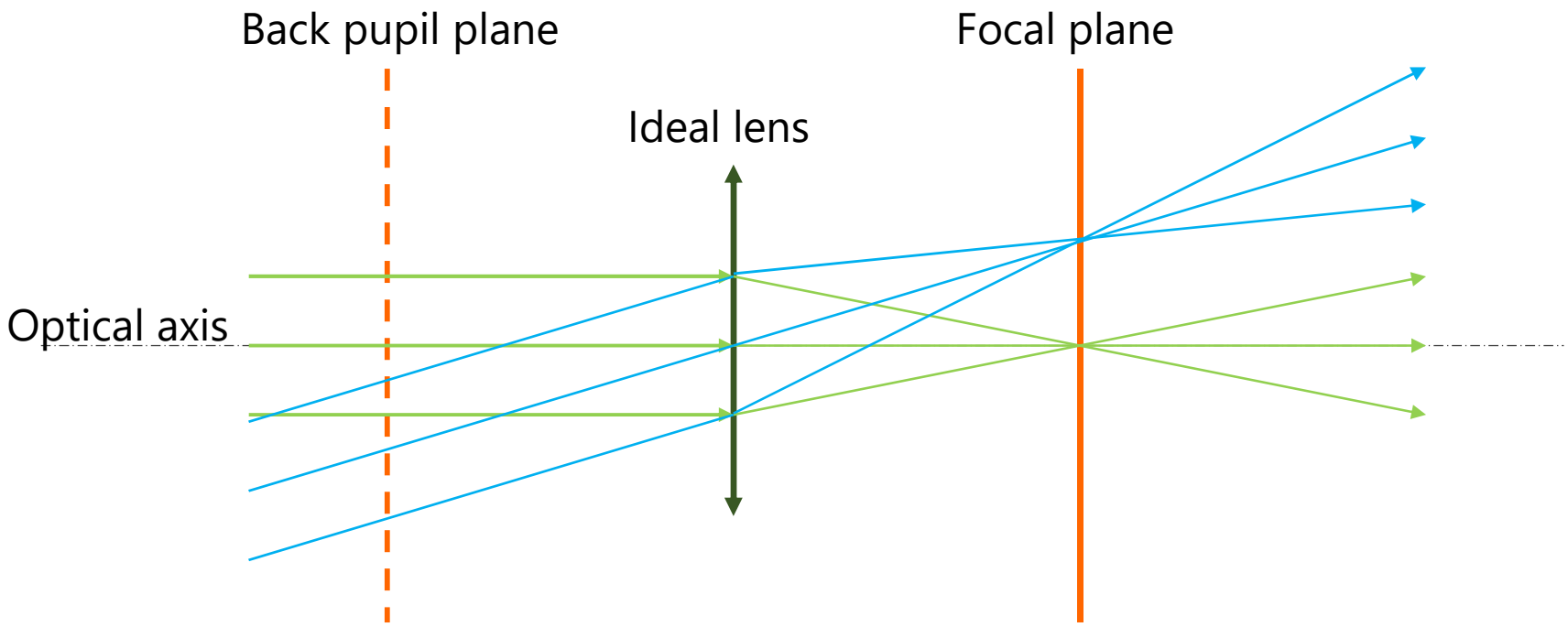
Optical imaging

- Basics
- Optical Neuroimaging

Basics of optical imaging

- Light Ray Model
- Light wave model
- Frequency domain model

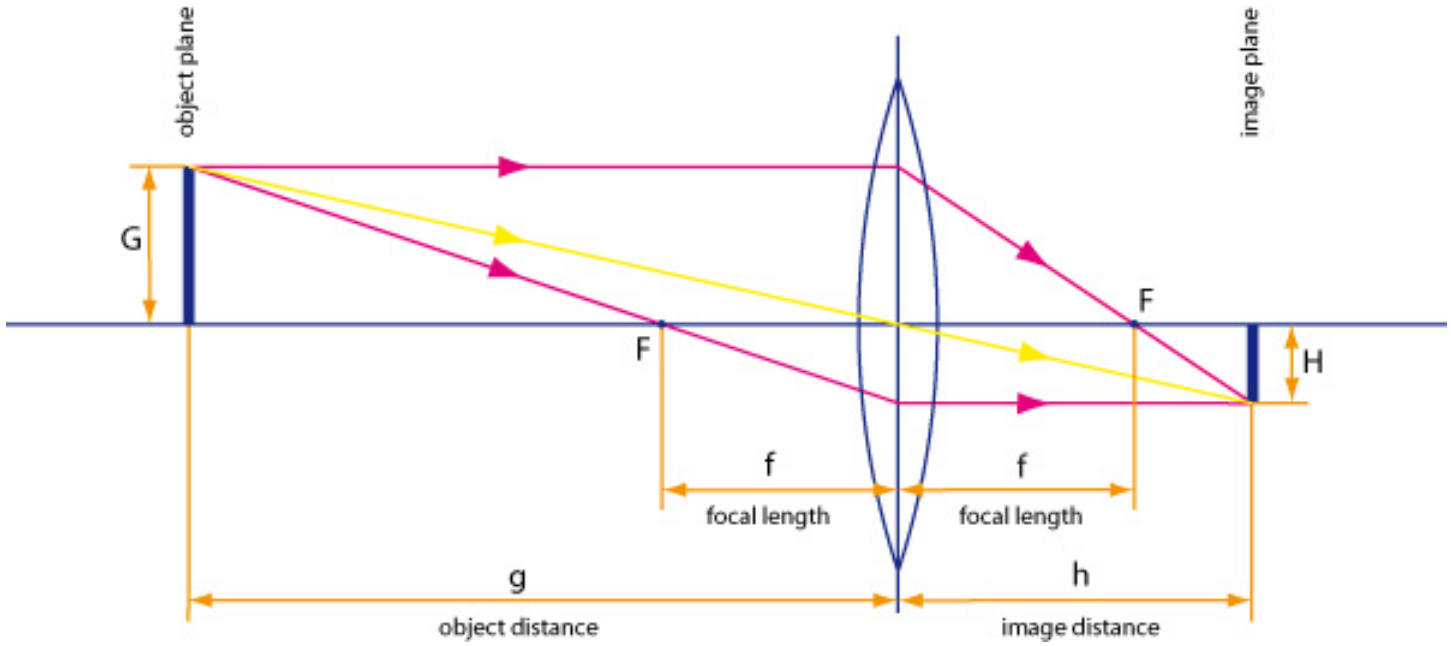
Light ray model of an ideal lens



Rule 1: Light ray will not be deflected when passing through the center of lens

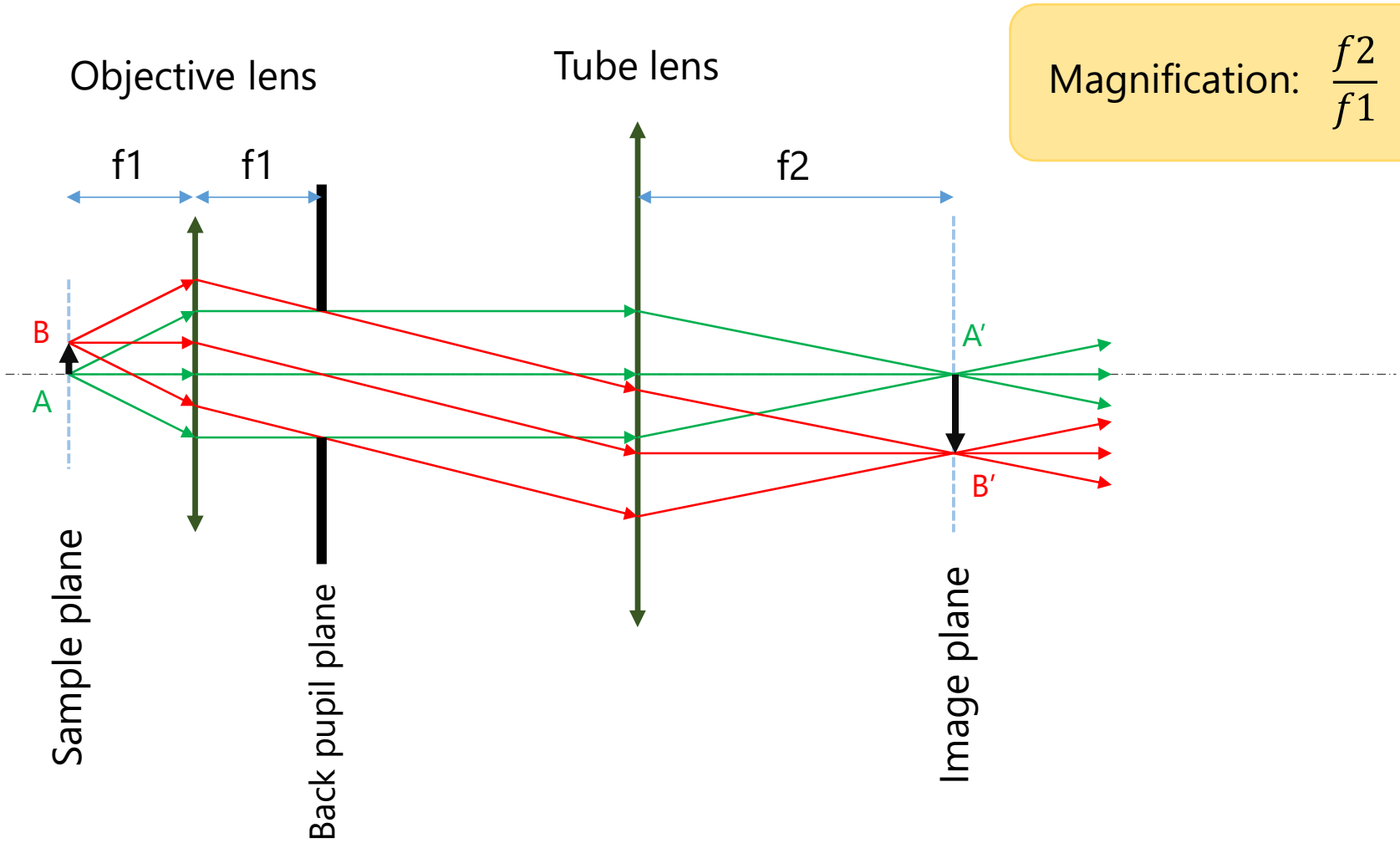
Rule 2: Parallel light rays will be focused into a single spot on focal plane

Imaging system: Light ray model



$$\frac{1}{d_o} + \frac{1}{d_i} = \frac{1}{f}$$

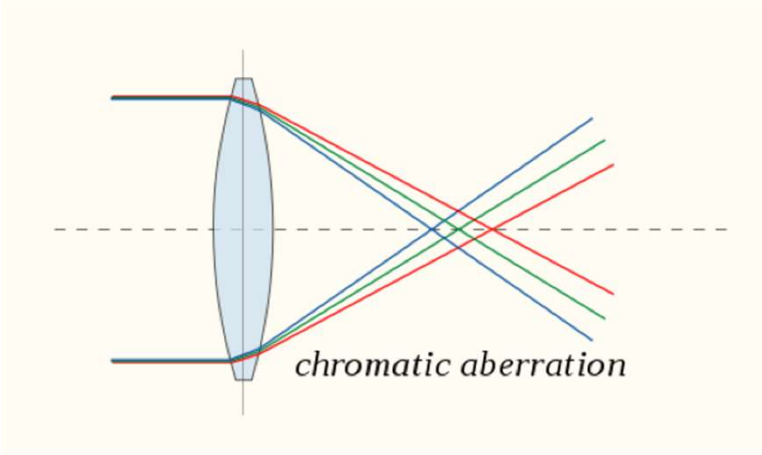
Infinity corrected imaging system

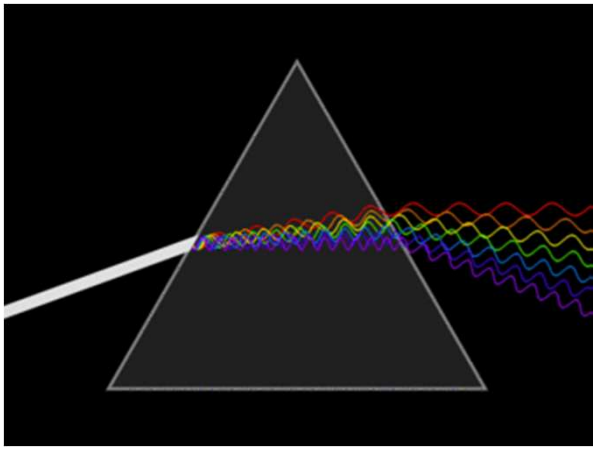


Aberrations

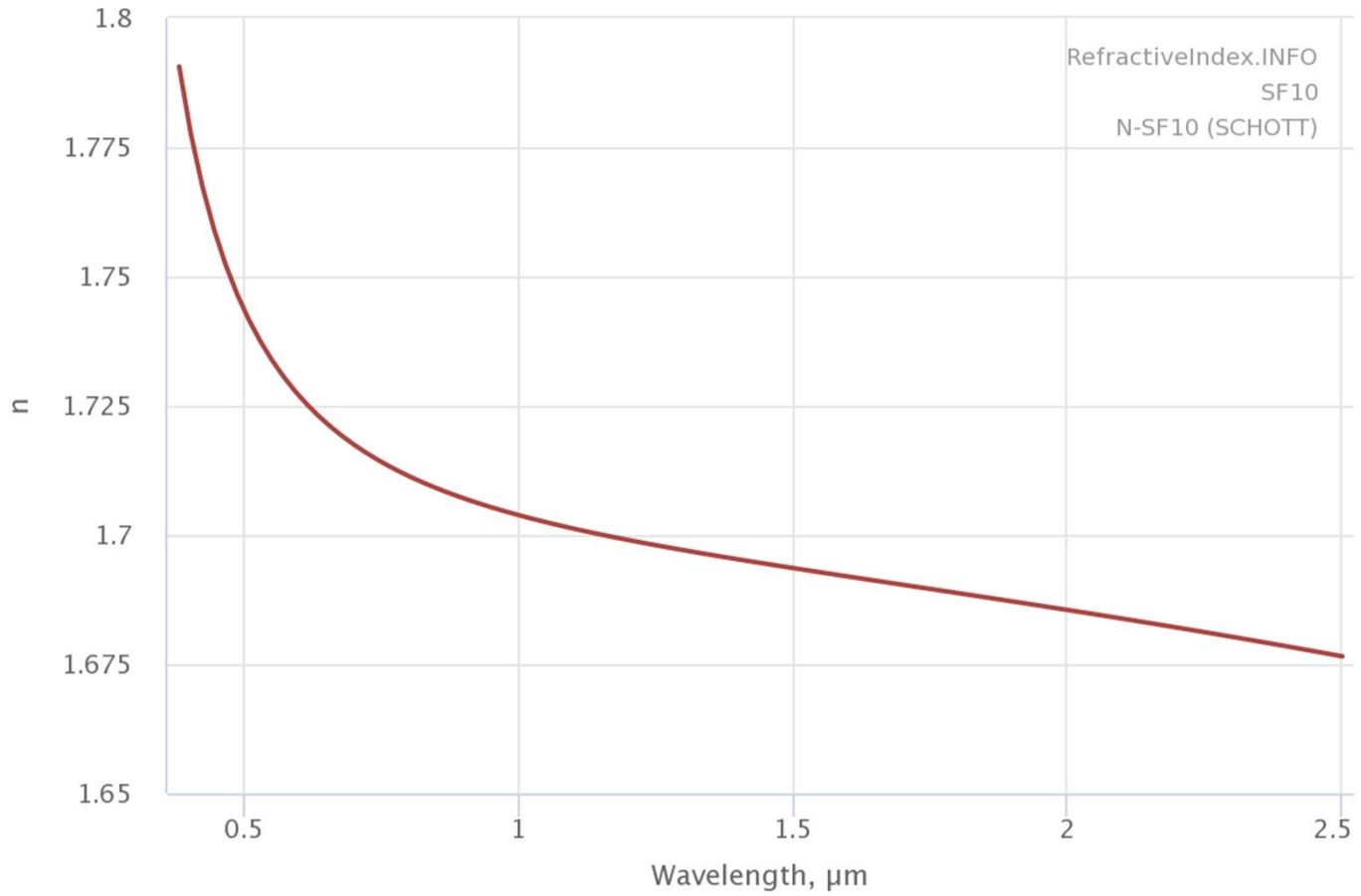
- Chromatic aberrations
- Spherical aberrations
- Comma
- Astigmatism

Chromatic aberration

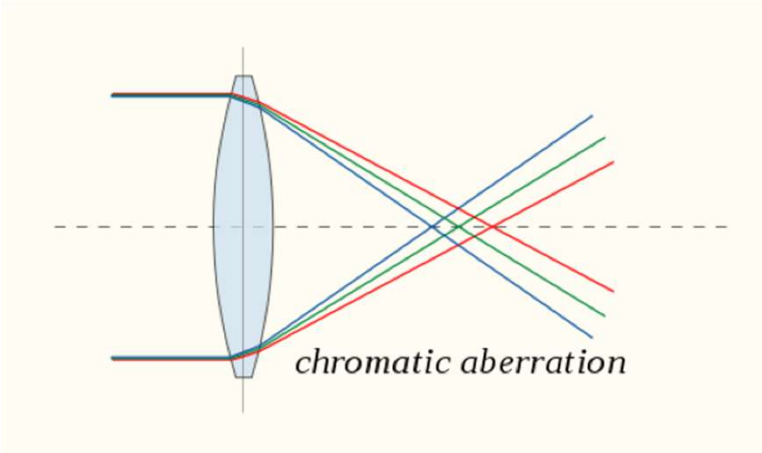


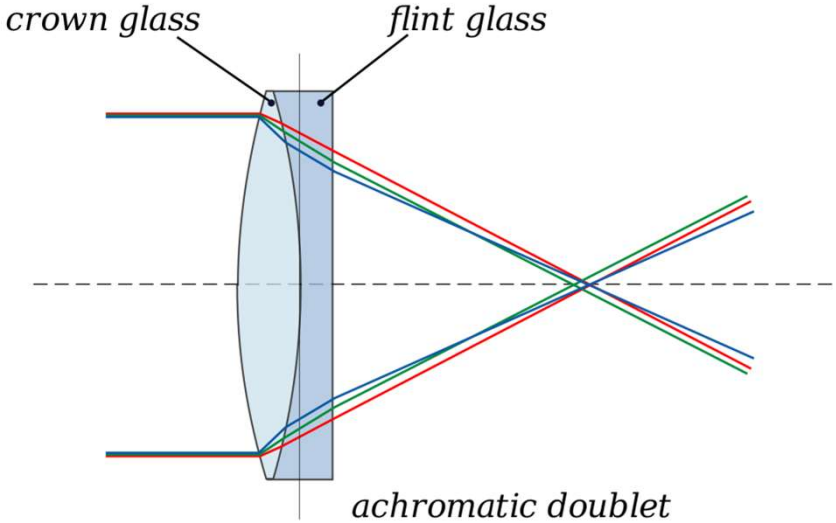


Dispersion


RefractiveIndex.INFO
SF10
N-SF10 (SCHOTT)
1.8
1.775
1.75
1.725
1.7
1.675
1.65
n
0.5
1
1.5
2
2.5
Wavelength, µm

Achromatic





1590 - Hans & Zacharias Janssen of Middleburg, Holland manufactured the first compound microscopes.

Around the year 1733, a barrister names **Chester More Hall** observed that flint glass (newly made glass) dispersed colors much more than “crown glass” (older glass). He managed to build the first achromatic objective, consisting of a combination of a convex Crown glass and a concave Flint glass. Hall tried to keep this a secret by having one type of glass manufactured by one company and the other by another company.

Objectives' parameters

Correction spec:

Magnification:

Numerical Aperture (NA):

Working Distance (WD):

Immersion medium:

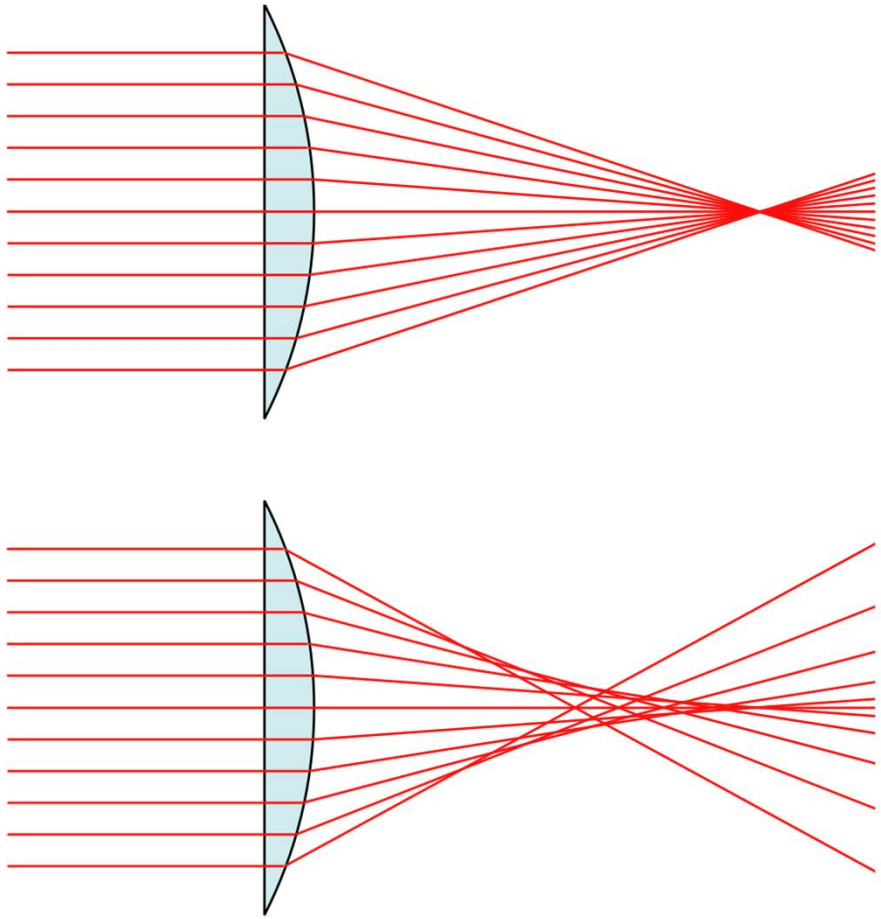
Figure 1

Objective Correction for Optical Aberration

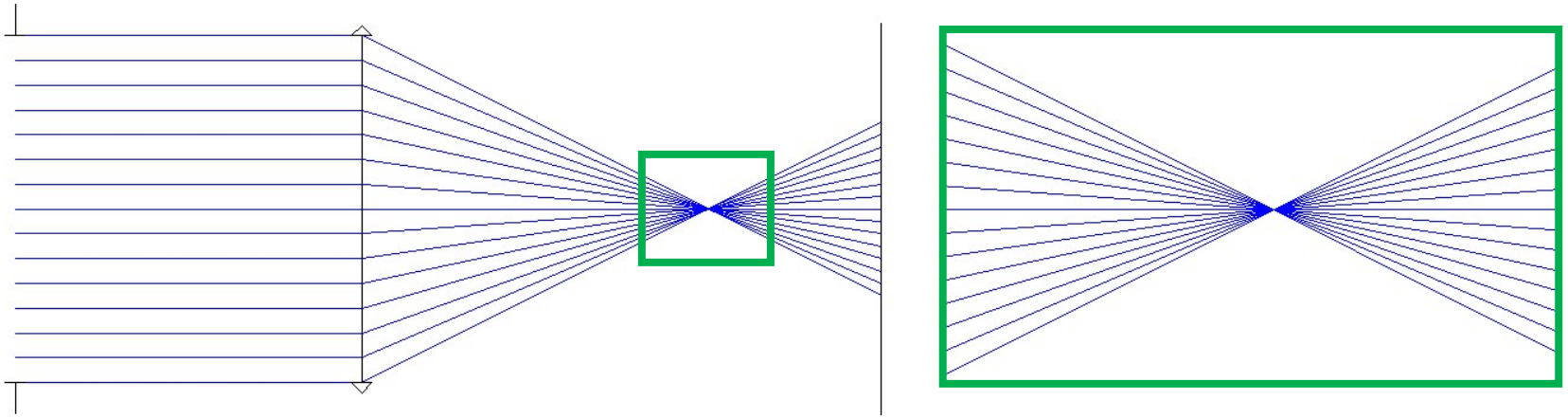
Objective Type	Spherical Aberration	Chromatic Aberration	Field Curvature
Achromat	1 Color	2 Colors	No
Plan Achromat	1 Color	2 Colors	Yes
Fluorite	2-3 Colors	2-3 Colors	No
Plan Fluorite	3-4 Colors	2-4 Colors	Yes
Plan Apochromat	3-4 Colors	4-5 Colors	Yes

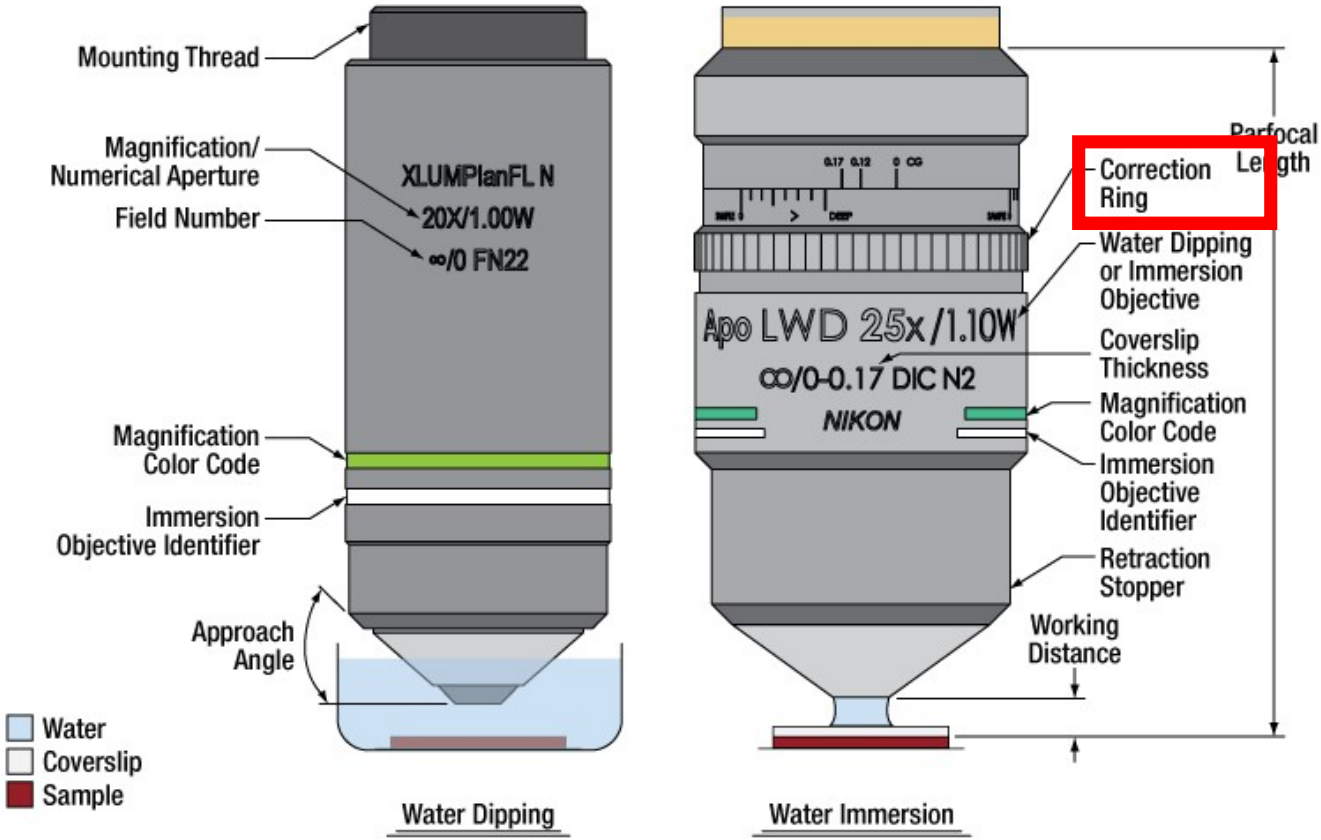
Spherical Aberrations

Spherical lens are not perfect lens



Cover glass and correction ring




Mounting Thread
Magnification/
Numerical Aperture
Field Number
XLUMPlanFL N
20X/1.00W
∞/0 FN22
Magnification
Color Code
Immersion
Objective Identifier
Approach
Angle
Water
Coverslip
Sample
Water Dipping
Apo LWD 25x/1.10W
∞/0-0.17 DIC N2
NIKON
Correction
Ring
Parfocal
Length
Water Dipping
or Immersion
Objective
Coverslip
Thickness
Magnification
Color Code
Immersion
Objective
Identifier
Retraction
Stopper
Working
Distance
Water Immersion

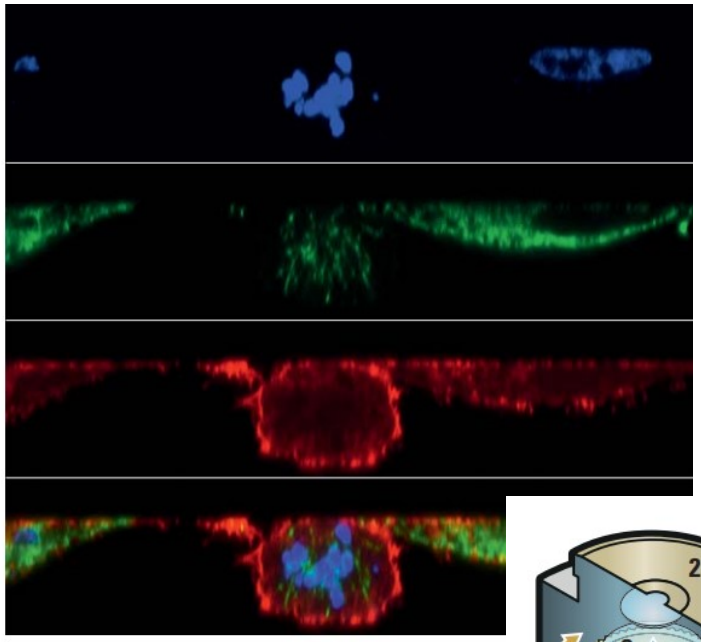
Cover glass specs

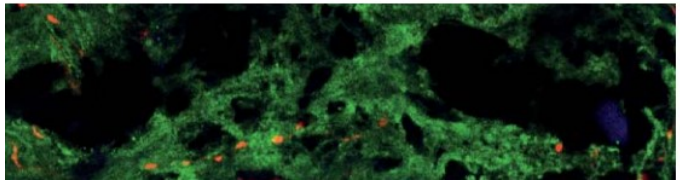
Number	Thickness	Tolerance
#0	0.10 ± 0.02 mm	0.08-0.12 mm (80-120 µm)
#1	0.15 ± 0.02 mm	0.13-0.17 mm (130-170 µm)
#1.5 High Tolerance	0.17 ± 0.01 mm	0.16-0.18 mm (160-180 µm)
#1.5	0.17 ± 0.02 mm	0.15-0.19 mm (150-190 µm)
#2	0.22 ± 0.02 0 mm	0.19-0.23 mm (190-230 µm)

Tolerance for circular coverglass is nominally ±0.20 mm up to ±0.30 mm, (rarely) ±0.50 mm

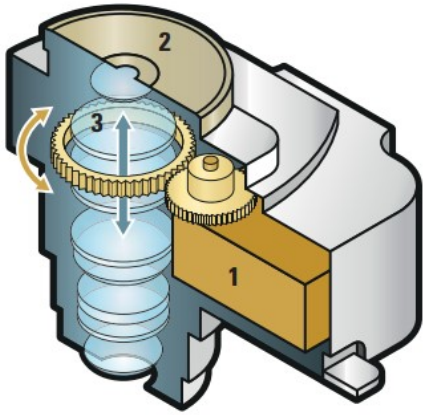
Tolerance for square and rectuangular coverglass is nominally ±0.20 up to ±0.30 mm

Motorized correction collar for in vivo imaging

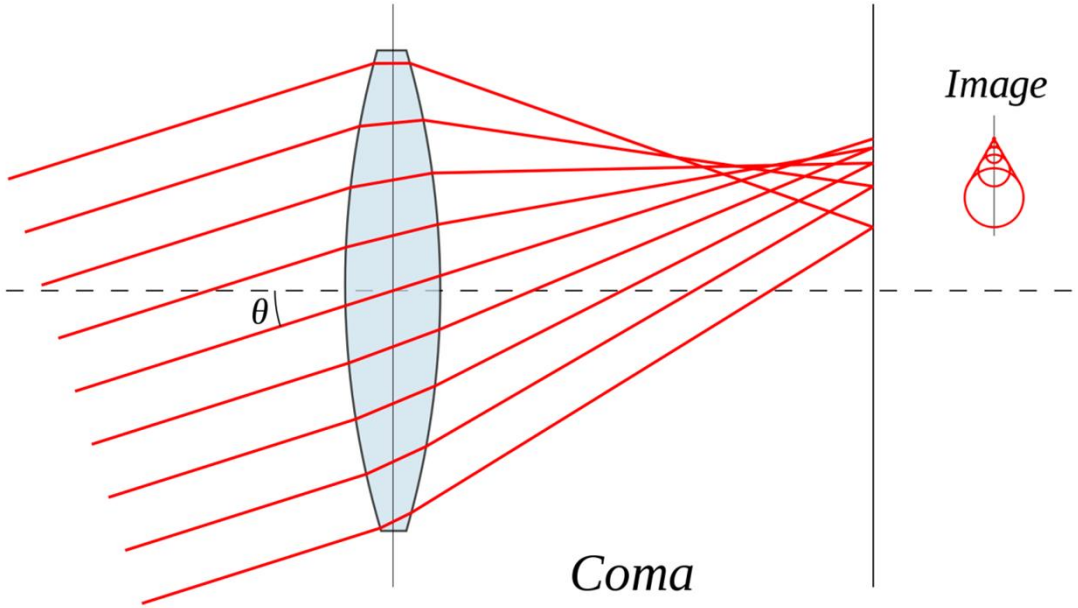


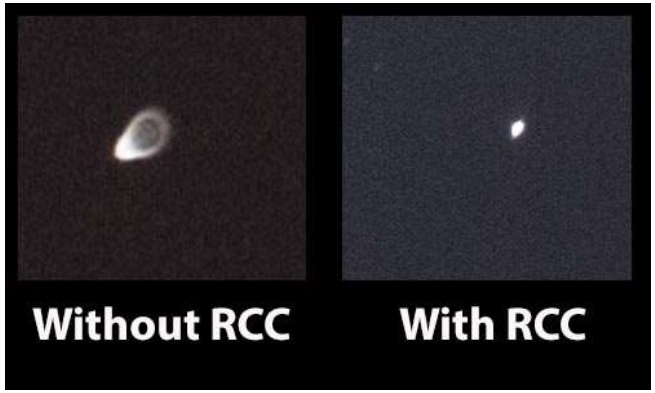


Leica motCORR™
Objectives

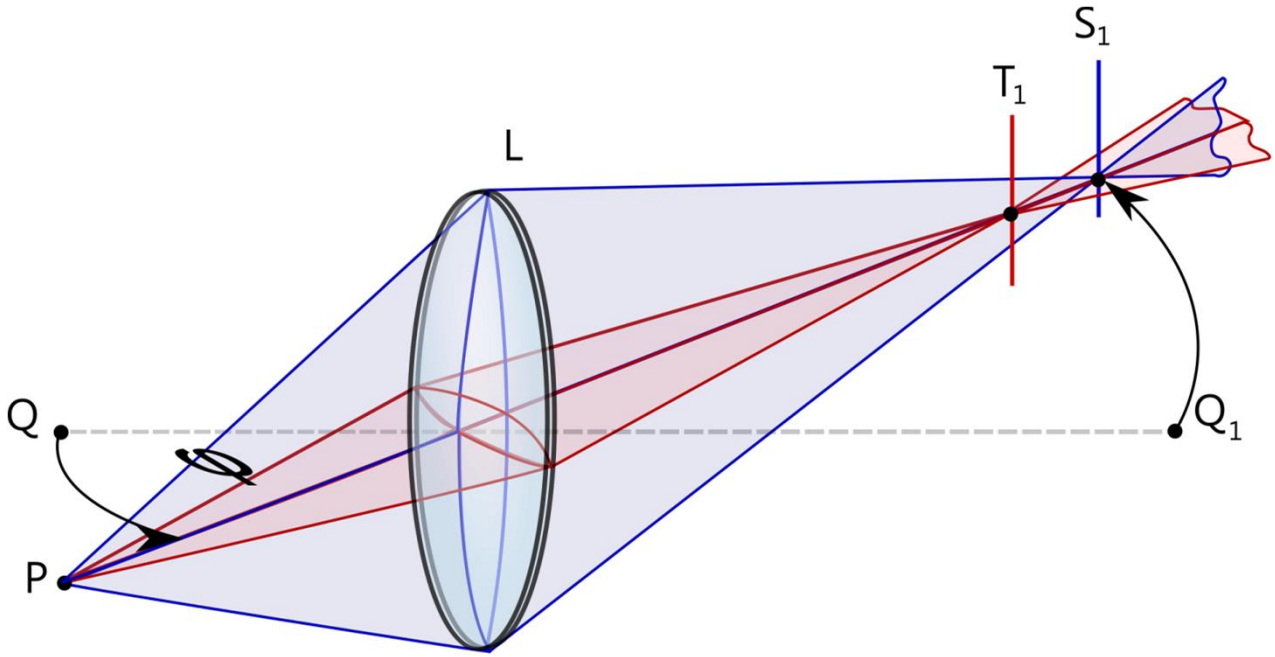


Coma Aberration



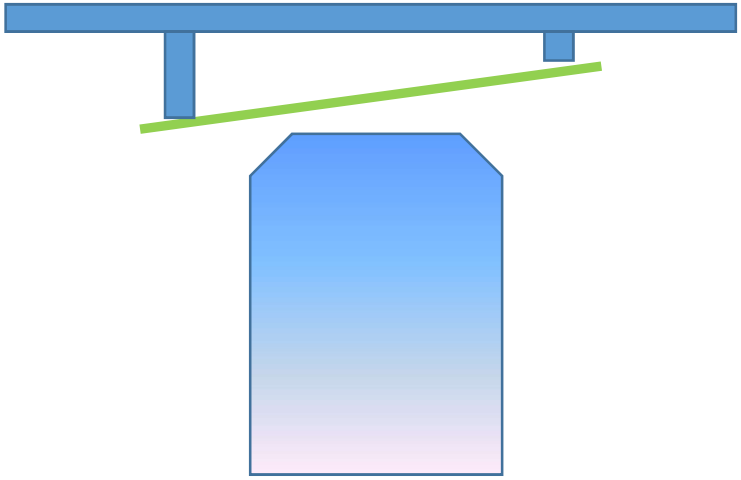


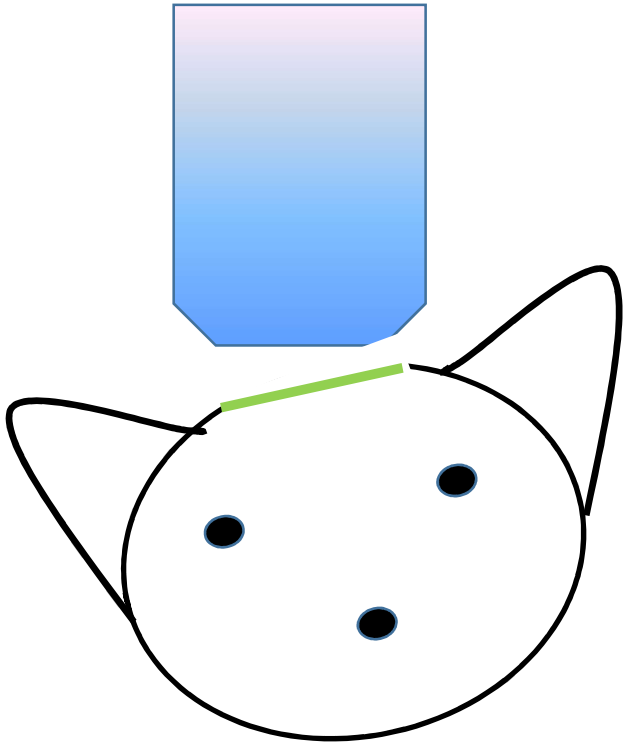
Astigmatism



Coma & Astigmatism

Optical interfaces should be perpendicular to the optical axis of objective





Objectives' parameters

Correction spec:

Magnification:

Numerical Aperture (NA):

Working Distance (WD):

Immersion medium:

Figure 1

Objective Correction for Optical Aberration

Objective Type	Spherical Aberration	Chromatic Aberration	Field Curvature
Achromat	1 Color	2 Colors	No
Plan Achromat	1 Color	2 Colors	Yes
Fluorite	2-3 Colors	2-3 Colors	No
Plan Fluorite	3-4 Colors	2-4 Colors	Yes
Plan Apochromat	3-4 Colors	4-5 Colors	Yes

Objectives' parameters

Microscope Optical Train Components

Manufacturer	Tube Lens Focal Length (Millimeters)	Parfocal Distance (Millimeters)	Thread Type
Leica	200	45	M25
Nikon	200	60	M25
Olympus	180	45	RMS
Zeiss	165	45	RMS

Example:

100x objectives of different brands have different focal length:

Leica & Nikon Objectives: 200/100=2 mm

Olympus Objective: 180/100 = 1.8 mm

Zeiss Objective: 165/100 =1.65 mm

Summary of light ray model

- Lens focuses parallel light rays of different directions into spots at different positions on the focal plane.
- Modern imaging system (infinity corrected imaging system) consists of two lens. Light from a point source is converted into plane wave, then back to a spot.
- The magnification of the imaging system can be calculated as:

$$M = \frac{f_{tube\ lens}}{f_{objective\ lens}}$$

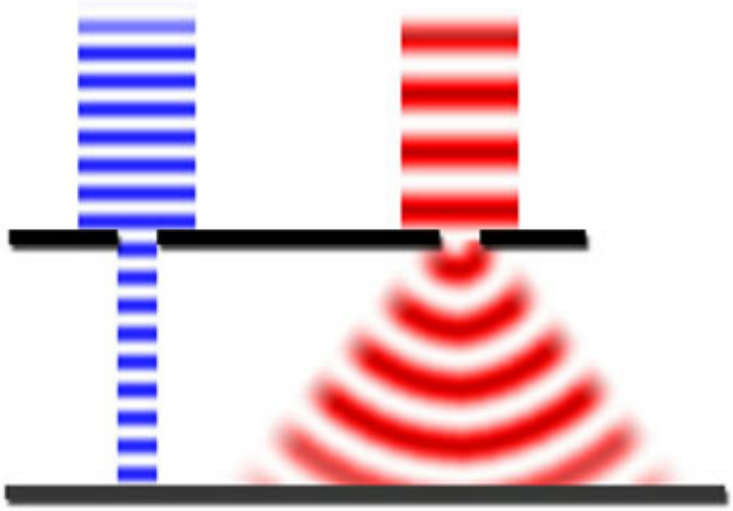
Basics of optical imaging

- Light Ray Model
- Light wave model
- Frequency domain model

Wave nature of light

Water ripple

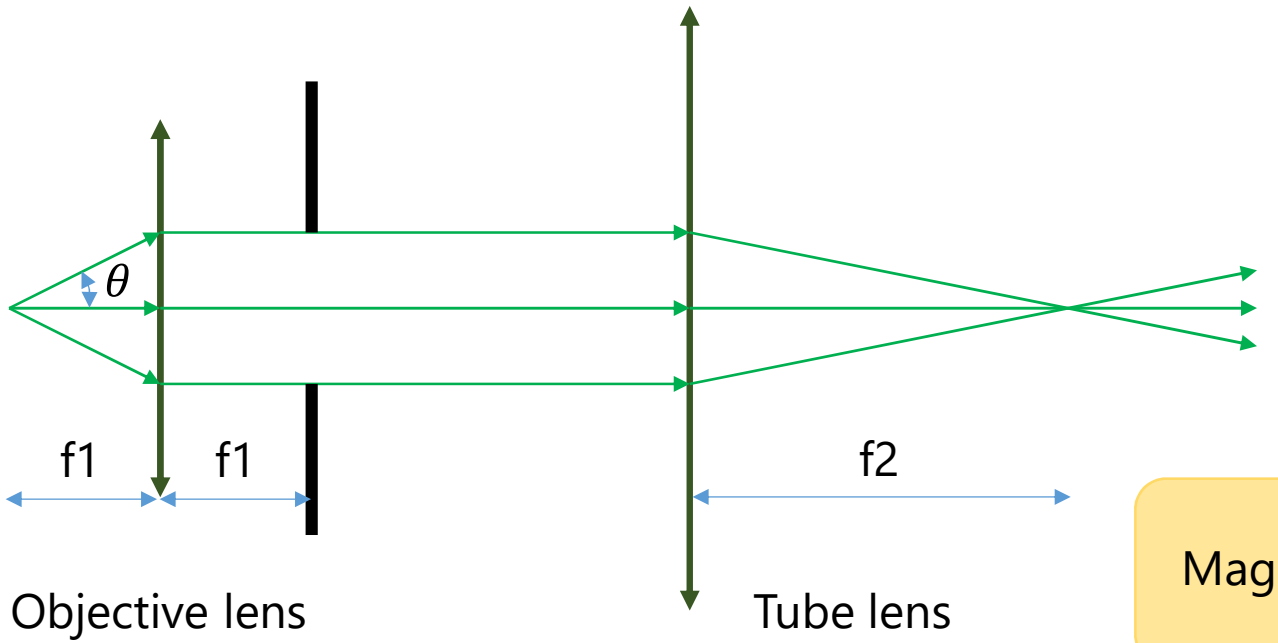
diffraction



Light wave model of optical imaging system

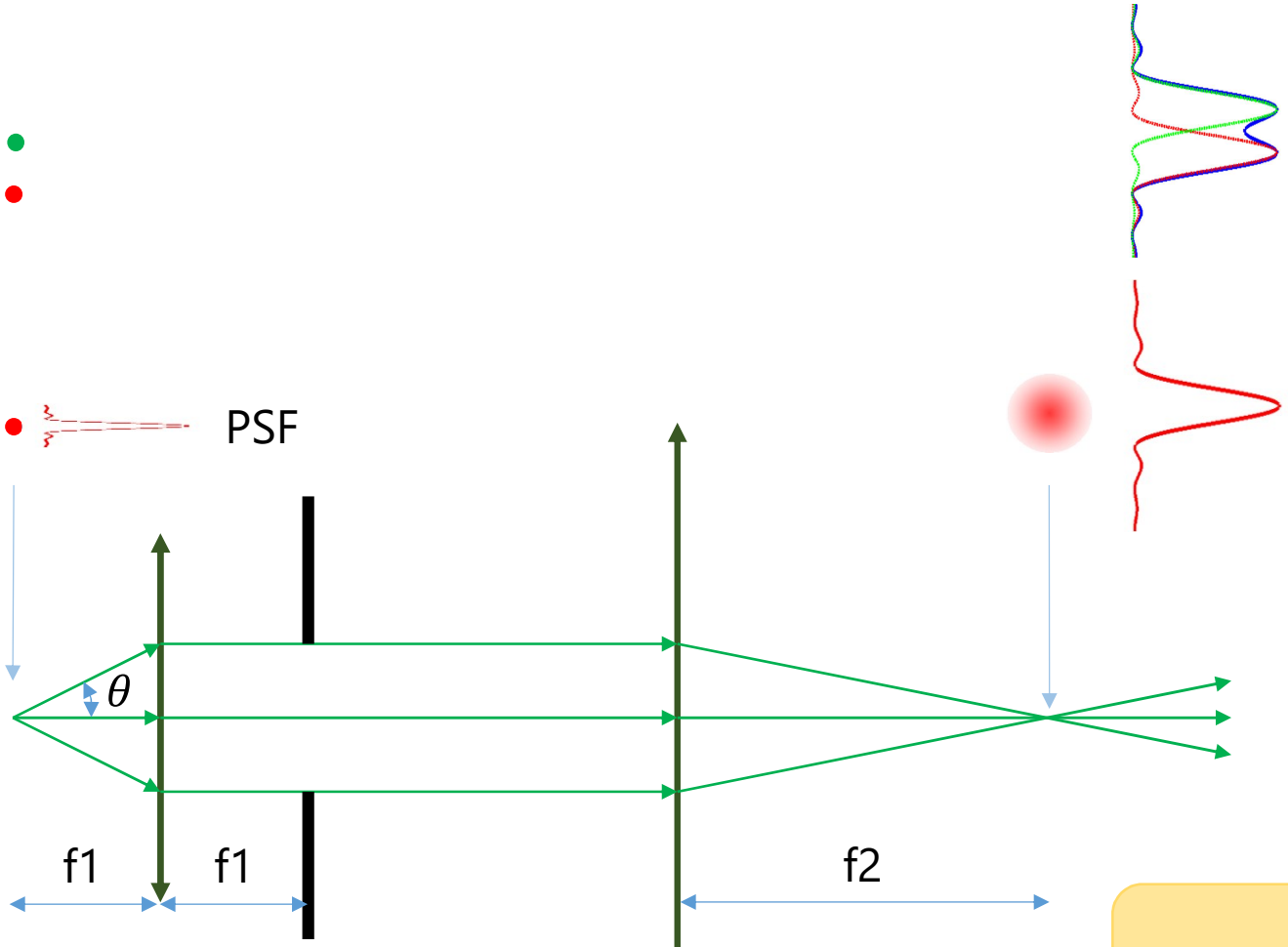
What's the resolution limit of the optical imaging system?

Point spread function (PSF):



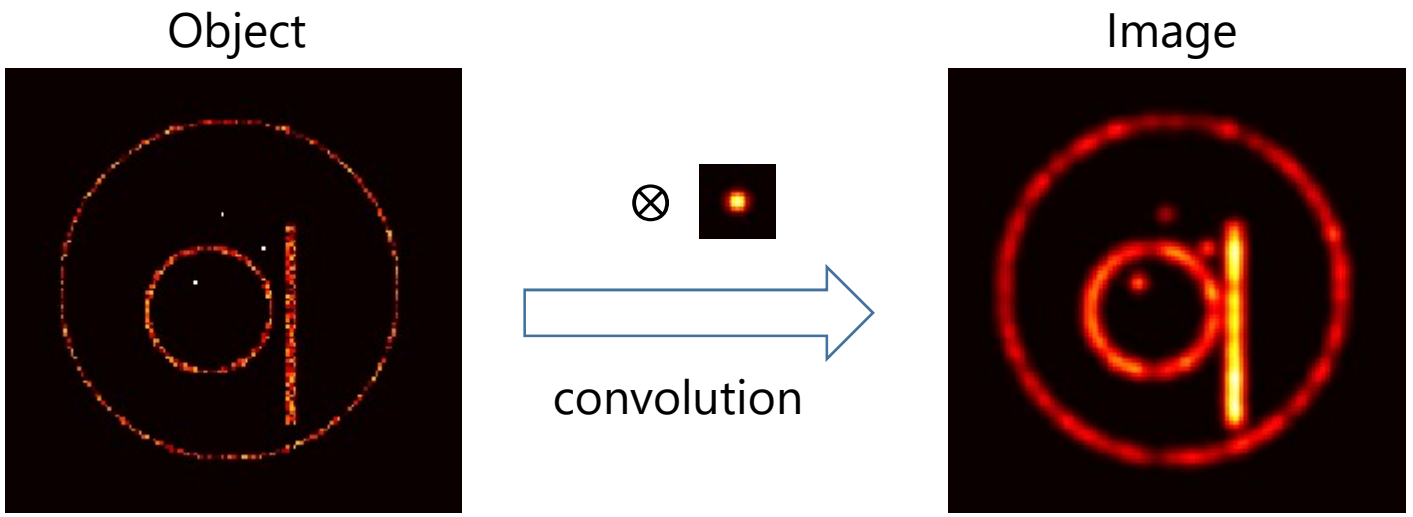
Magnification: $\frac{f2}{f1}$

Light wave model of optical imaging system

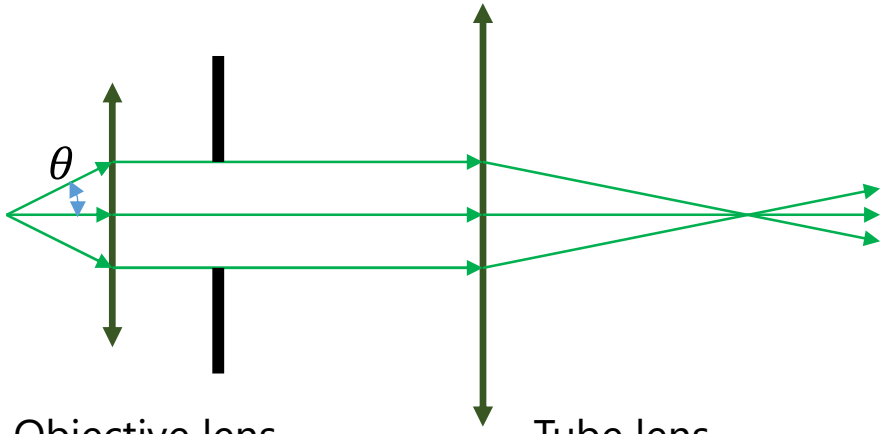


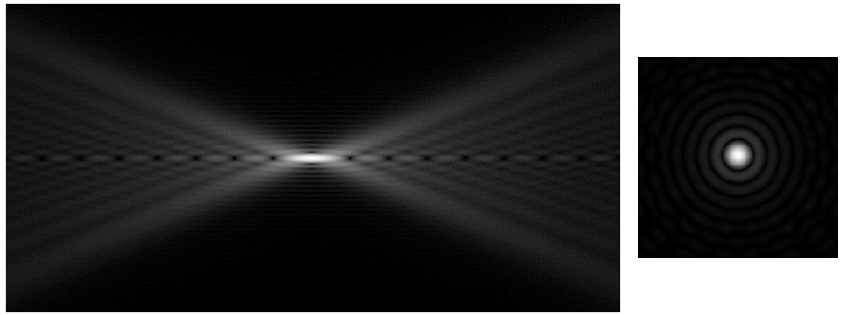
Magnification: $\frac{f2}{f1}$

Optical imaging system is linear & spatial invariant



Point spread function





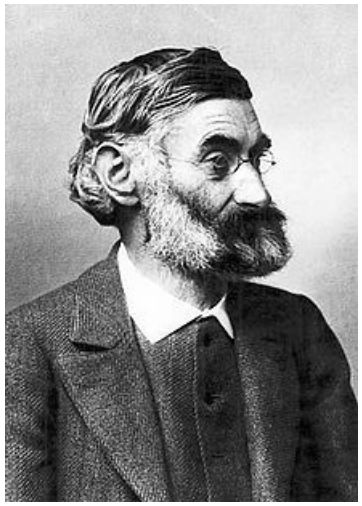
Fraunhofer diffraction pattern of a circular aperture (Airy disk)

$$I(x) \sim \left(\frac{J_1(kx)}{kx}\right)^2 \qquad k = \frac{2\pi}{\lambda} n sin\theta = \frac{2\pi}{\lambda} NA$$

$NA = nsin\theta$

Numerical Aperture

Point spread function & Resolution



Ernst Karl Abbe

Resolution: $d = \frac{\lambda}{2nsin\theta} = \frac{\lambda}{2 * NA}$

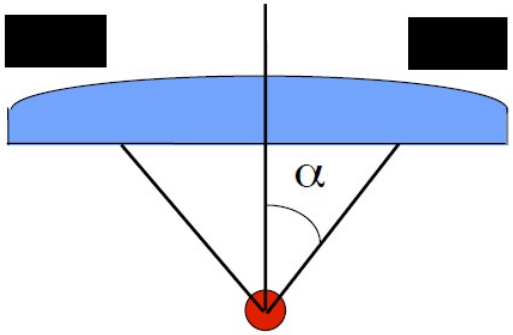
Numerical Aperture: $NA = nsin\theta$

Numerical aperture

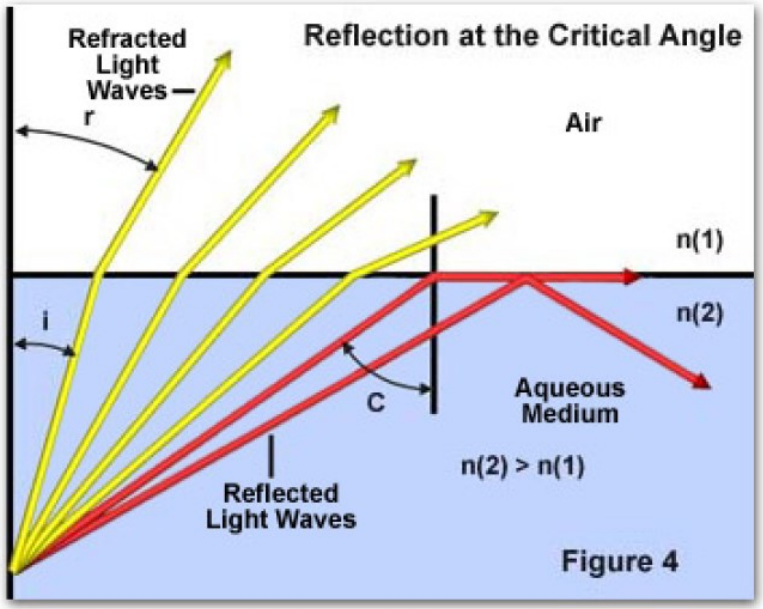
Rule of thumb

NA of lens cannot exceed the refractive index of the medium

Air 1
Water 1.3
Oil 1.515-1.534

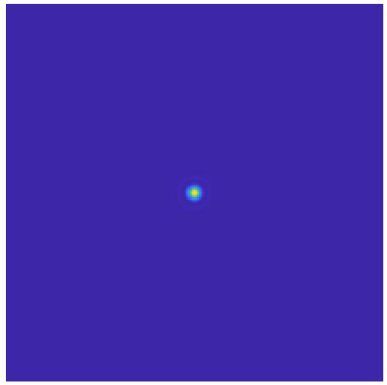


NA = R.I. sin α

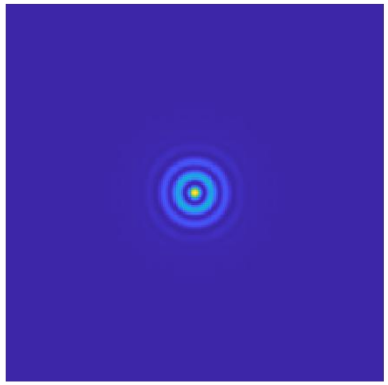


Aberrated PSFs

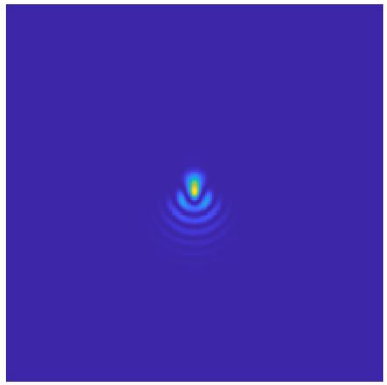
Diffraction limited



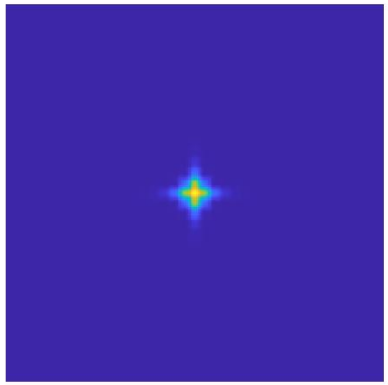
Spherical Aberration



Coma

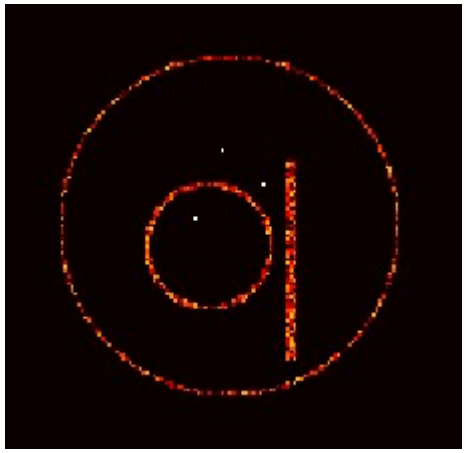


Astigmatism



Optical imaging system is linear & spatial invariant

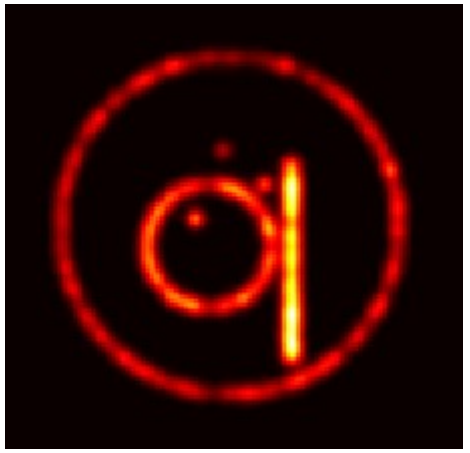
Object

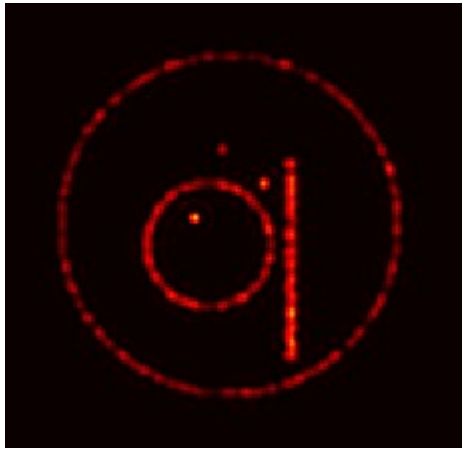


⊗

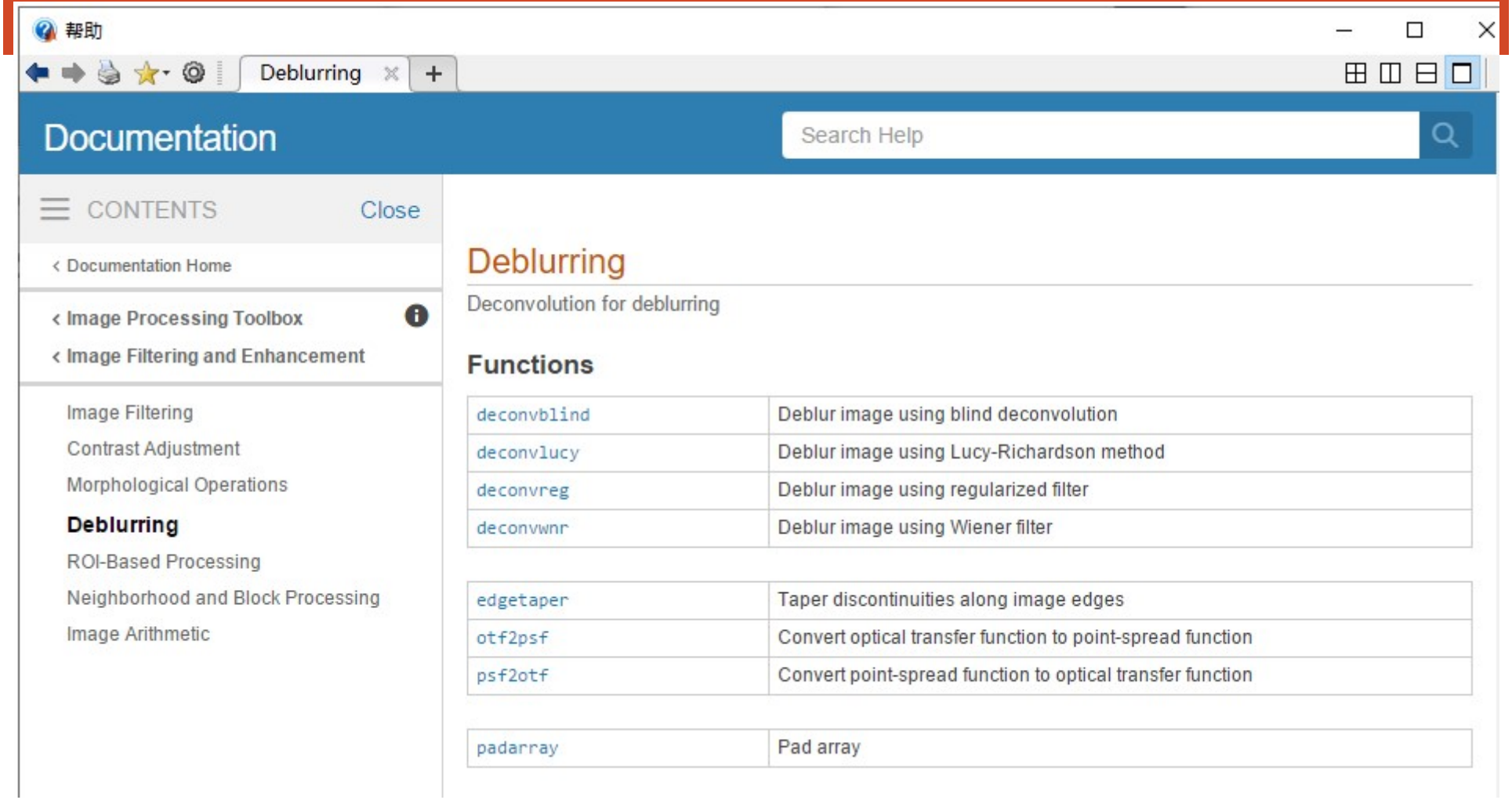
convolution

Image





deconvolution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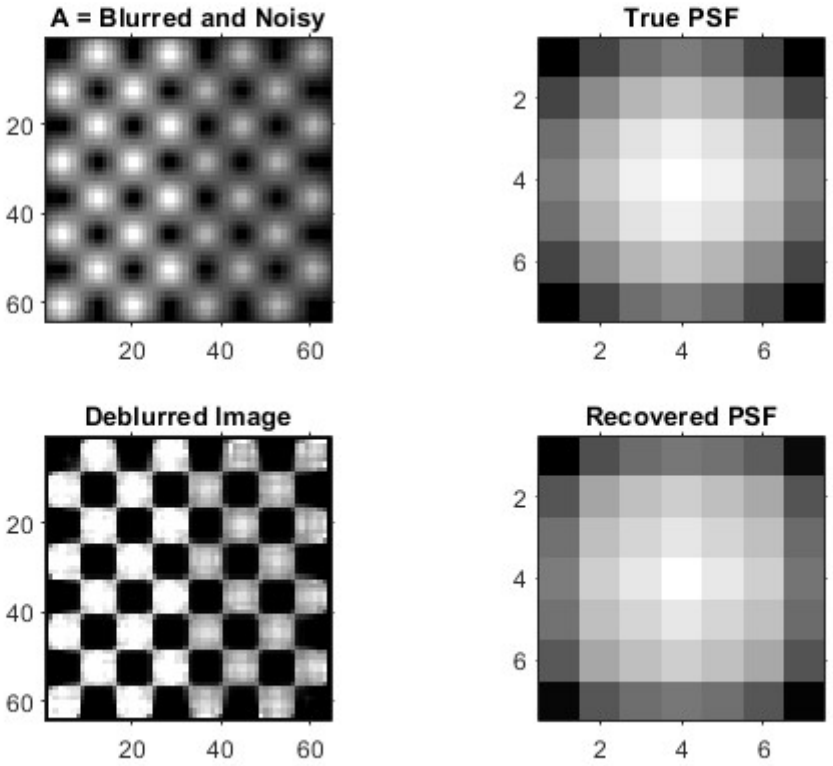
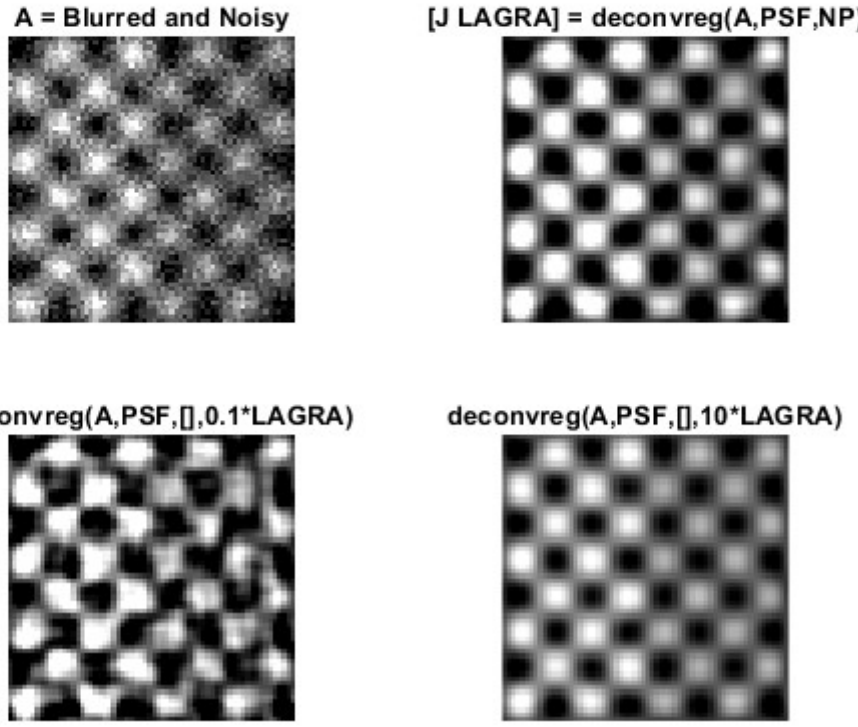
Neighborhood and Block Processing
Image Arithmetic
Deblurring
Deconvolution for deblurring
Functions
deconvblind
Deblur image using blind deconvolution
deconvlucy
Deblur image using Lucy-Richardson method
deconvreg
Deblur image using regularized filter
deconvwnr
Deblur image using Wiener filter
edgetaper
Taper discontinuities along image edges
otf2psf
Convert optical transfer function to point-spread function
psf2otf
Convert point-spread function to optical transfer function
padarray
Pad array

```
subplot(221);imshow(BlurredNoisy);
title('A = Blurred and Noisy');
subplot(222);imshow(PSF,[]);
title('True PSF');
subplot(223);imshow(J);
title('Deblurred Image');
subplot(224);imshow(P,[]);
title('Recovered PSF');
```



```
subplot(221); imshow(BlurredNoisy);
title('A = Blurred and Noisy');
subplot(222); imshow(J);
title('[J LAGRA] = deconvreg(A,PSF,NP)');
subplot(223); imshow(deconvreg(BlurredNoisy,PSF,[],LAGRA/10));
title('deconvreg(A,PSF,[],0.1*LAGRA)');
subplot(224); imshow(deconvreg(BlurredNoisy,PSF,[],LAGRA*10));
title('deconvreg(A,PSF,[],10*LAGRA)');
```



Point Spread Function & Resolution

Example:

An Olympus Plan Apochromat, 60X, NA 1.27, water objective is mistakenly installed in a Nikon microscope, please calculate the magnification and expected resolution when imaging a GFP labelled cell.

Olympus tube lens focal length: 180 mm
Nikon tube lens focal length: 200 mm
Emission wavelength of GFP: 510 nm

Olympus objective focal length: 180 mm/60 =3 mm
Magnification: 200 mm/3 mm = 66.7

Resolution: $\frac{\lambda}{2NA} = \frac{510\ nm}{2*1.27} = 200.8\ nm$

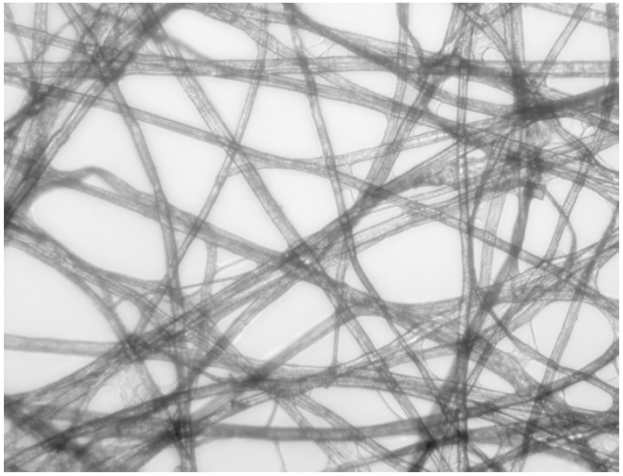
Optical contrast

Without contrast, resolution is nothing

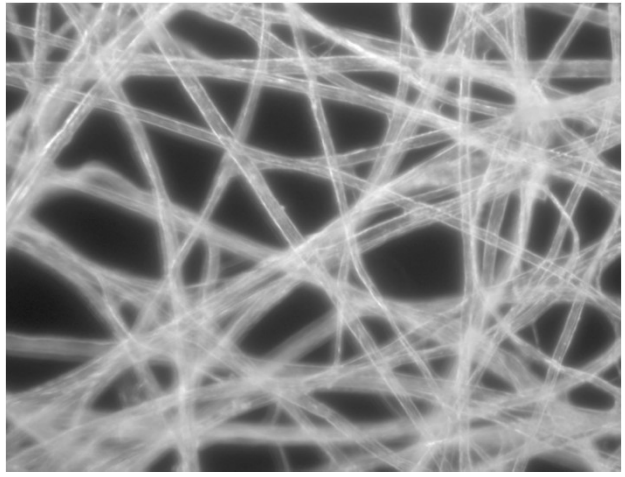
- Intrinsic imaging
  - Absorption
  - Scattering
  - Refractive index change
- Staining\labeling
  - Staining for wide field imaging
  - Fluorescent staining\labeling for fluorescence imaging

Intrinsic imaging

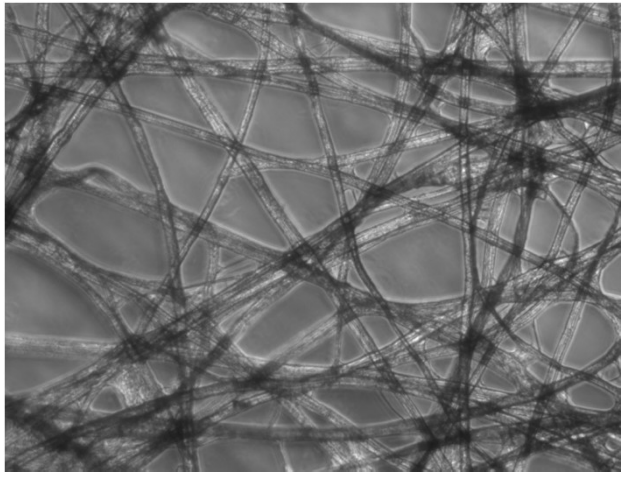
Bright Field



Dark Field

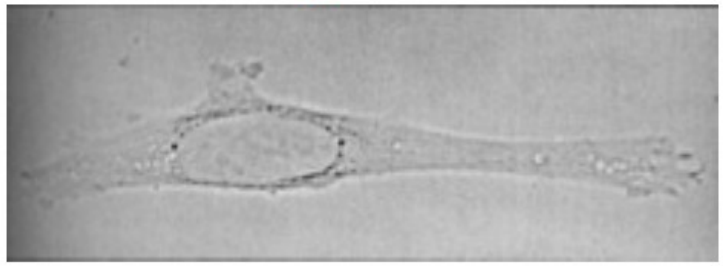


Phase Contrast/ Different Interference Contrast (DIC)

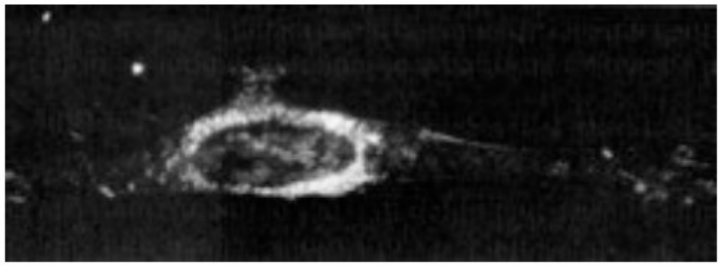


Phase imaging

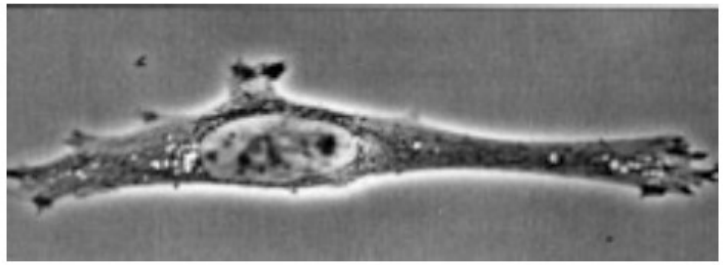
Brightfield



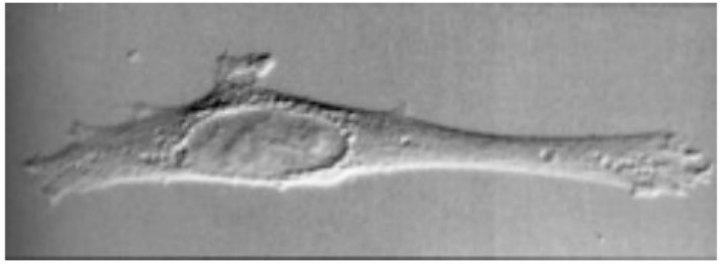
Darkfield



Phase contrast

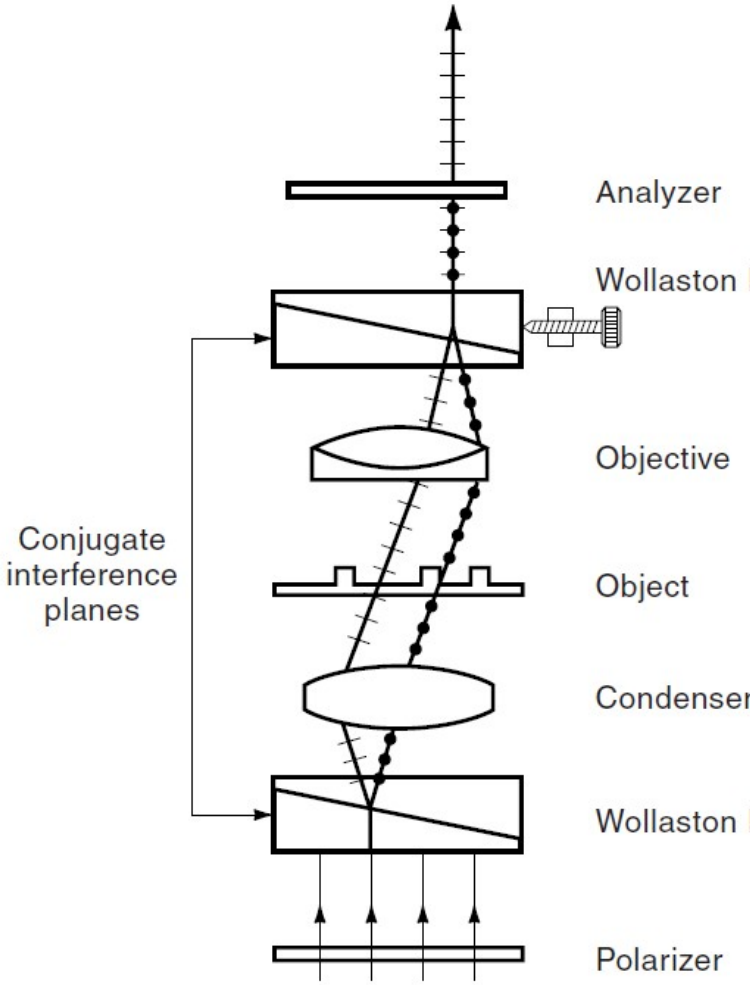


Differential interference contrast



Frits Zernike, Nobel prize 1953

DIC microscope



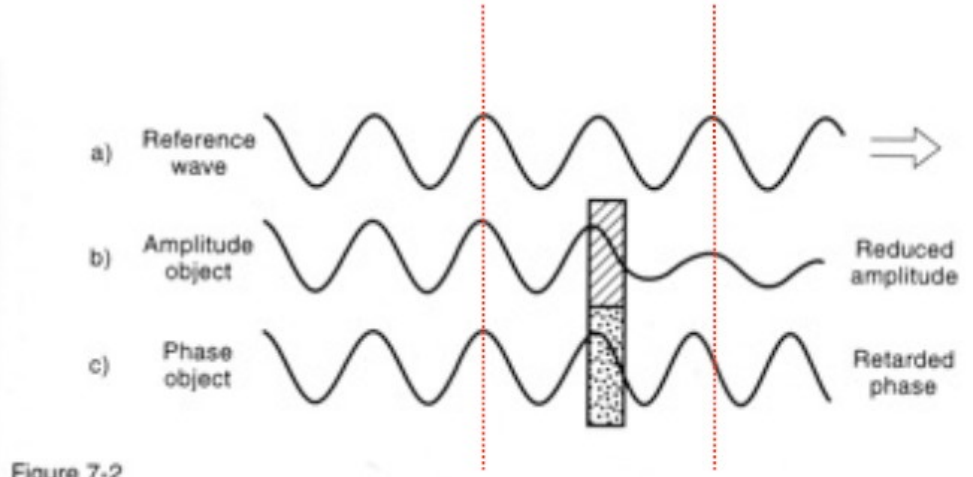
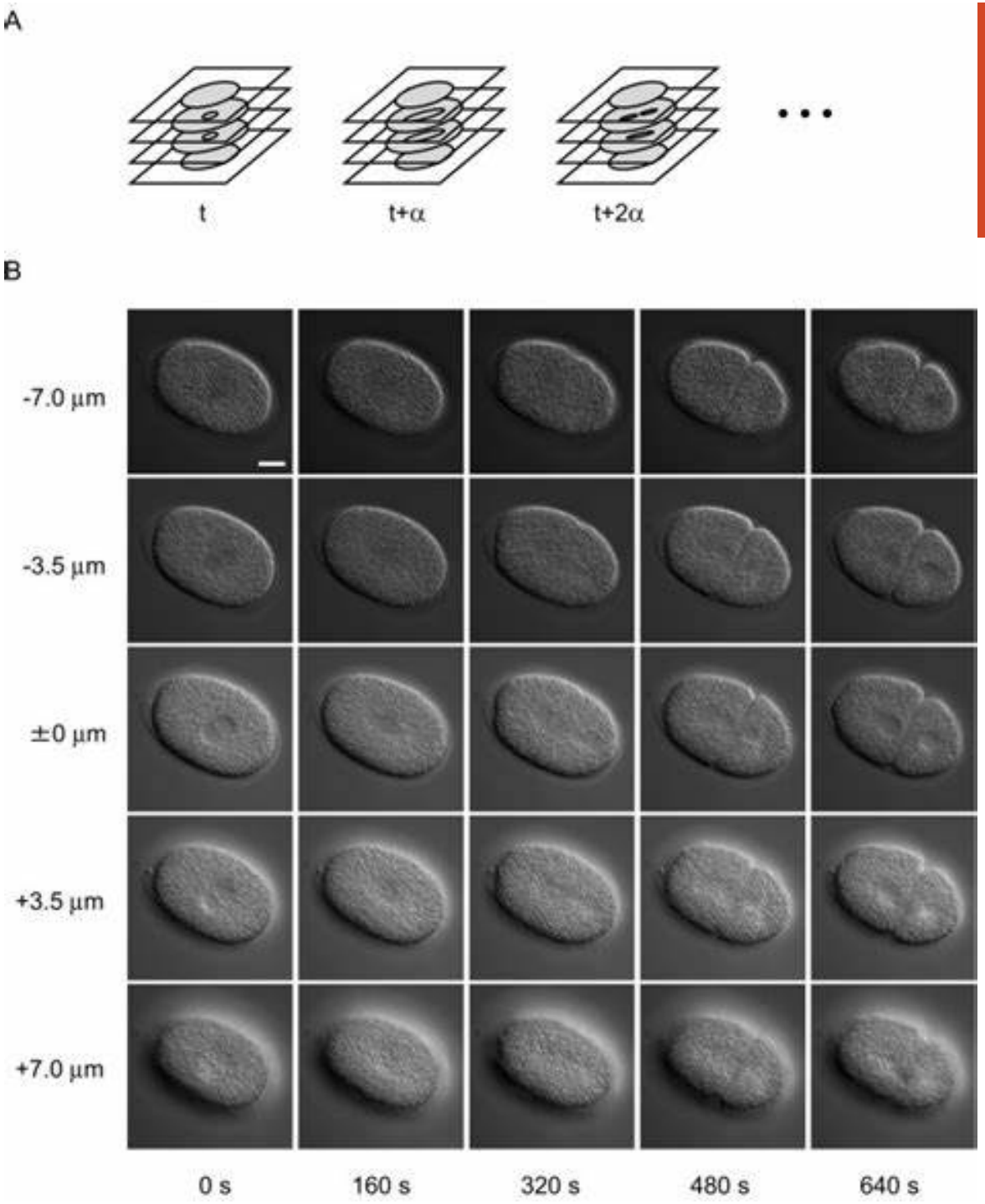


Figure 7-2

Effects of amplitude and phase objects on the waveform of light. (a) Reference ray with characteristic amplitude, wavelength, and phase. (b) A pure amplitude object absorbs energy and reduces the amplitude, but does not alter the phase, of an emergent ray. (c) A pure phase object alters velocity and shifts the phase, but not the amplitude, of an emergent ray.



DIC can do 3D imaging

Marriage with ML

Rivenson et al. *Light: Science & Applications* (2019)8:23
https://doi.org/10.1038/s41377-019-0129-y

Official journal of the CIOMP 2047-7538
www.nature.com/lsa

ARTICLE Open Access

PhaseStain: the digital staining of label-free quantitative phase microscopy images using deep learning

Yair Rivenson[1,2,3], Tairan Liu[1,2,3], Zhensong Wei[1,2,3], Yibo Zhang[1,2,3], Kevin de Haan[1,2,3] and Aydogan Ozcan[1,2,3,4]

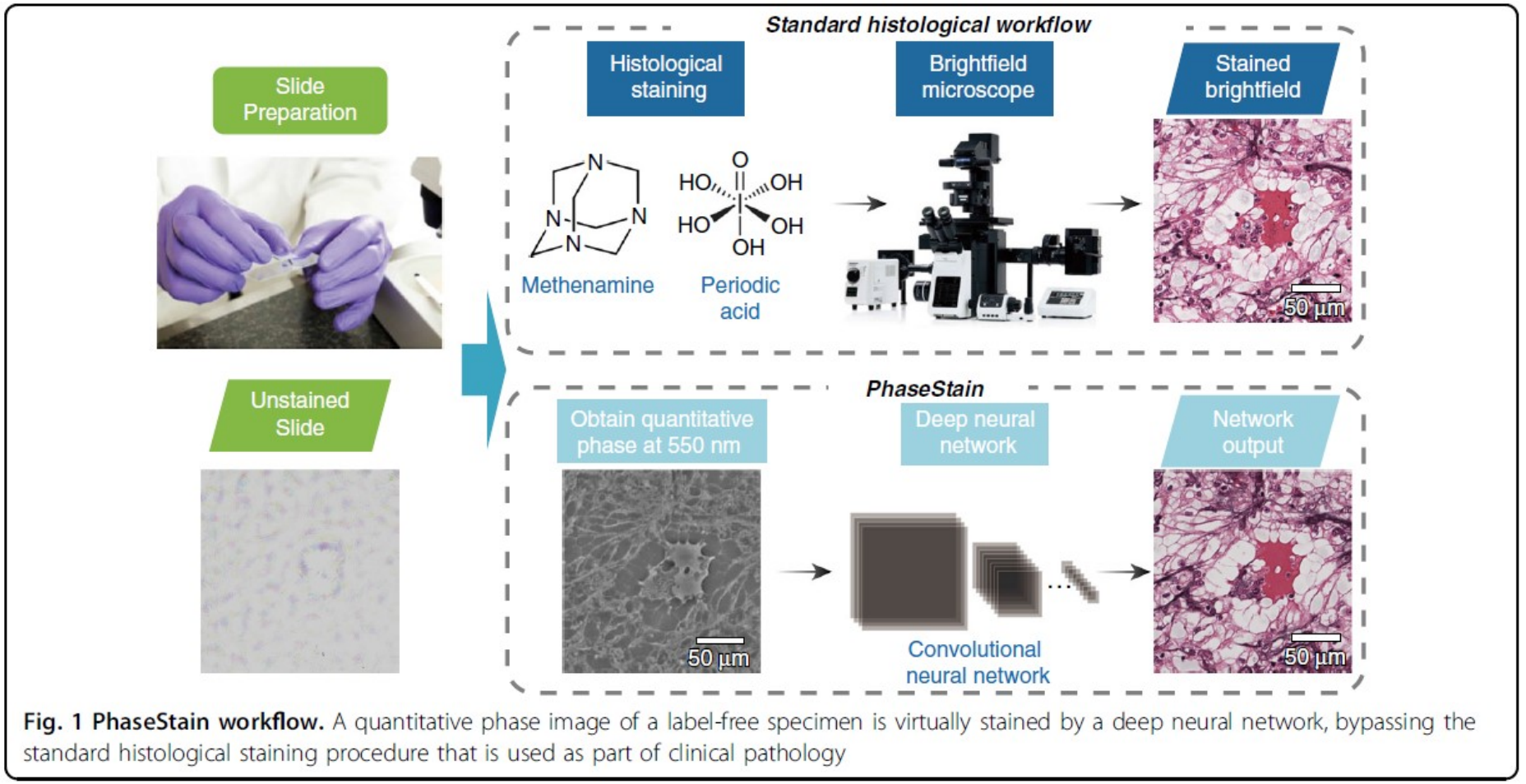
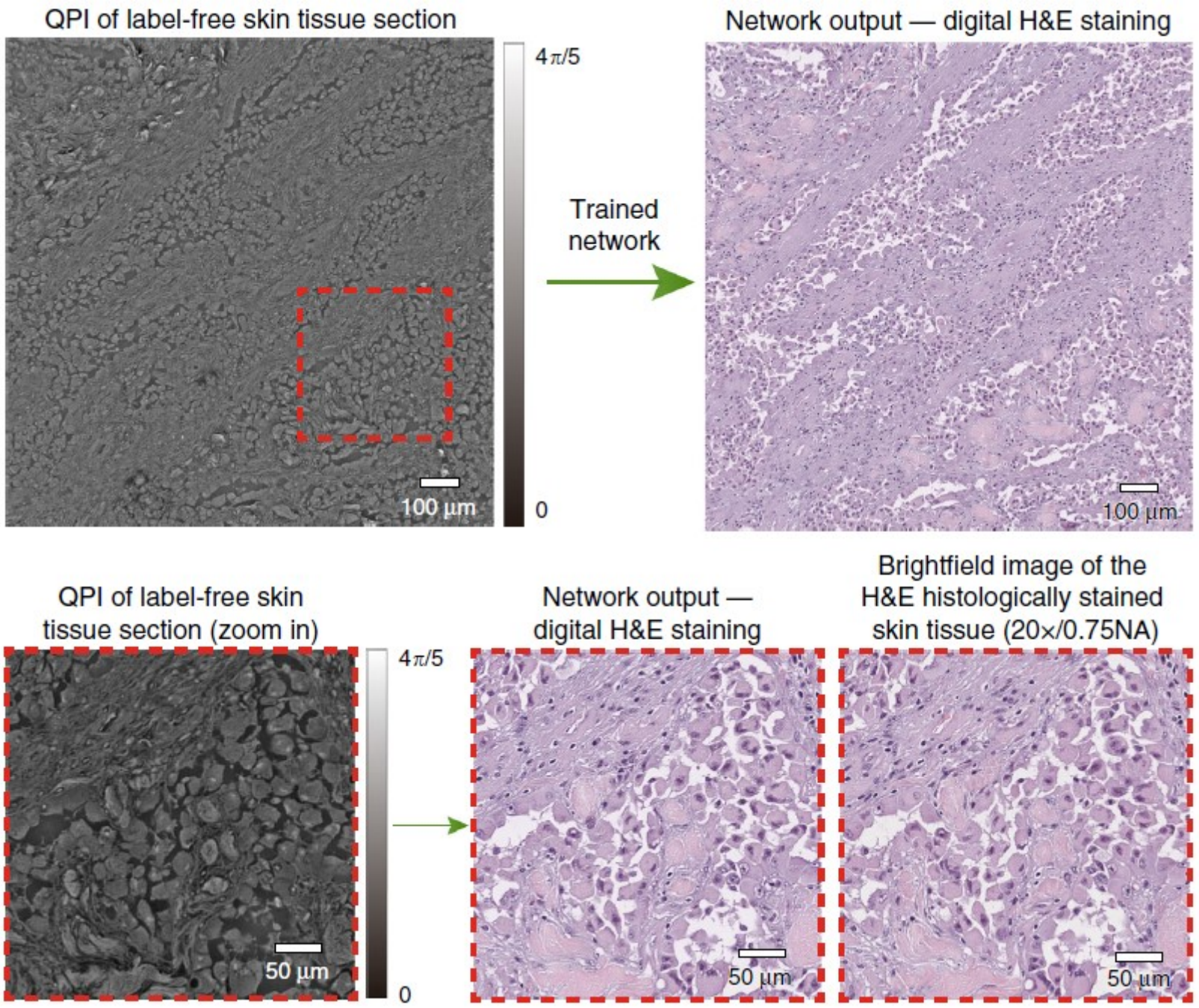
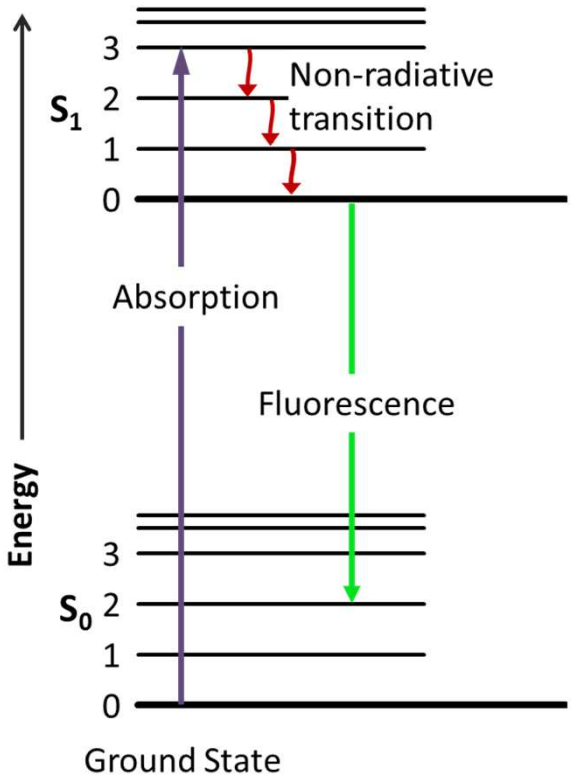


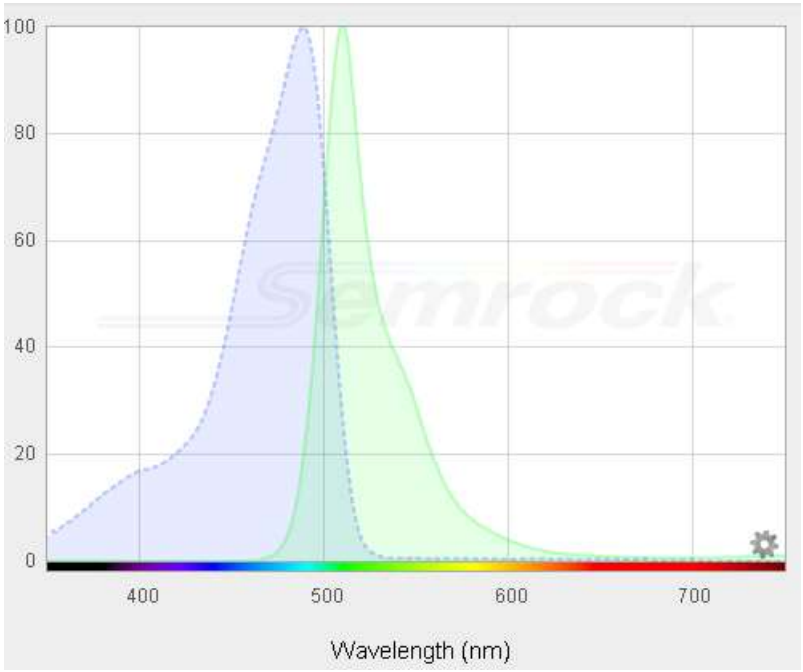
Fig. 1 PhaseStain workflow. A quantitative phase image of a label-free specimen is virtually stained by a deep neural network, bypassing the standard histological staining procedure that is used as part of clinical pathology


QPI of label-free skin tissue section
4π/5
0
100 µm
Trained network
Network output — digital H&E staining
100 µm
QPI of label-free skin tissue section (zoom in)
4π/5
0
50 µm
Network output — digital H&E staining
50 µm
Brightfield image of the H&E histologically stained skin tissue (20×/0.75NA)
50 µm

Fluorescent indicator



EGFP

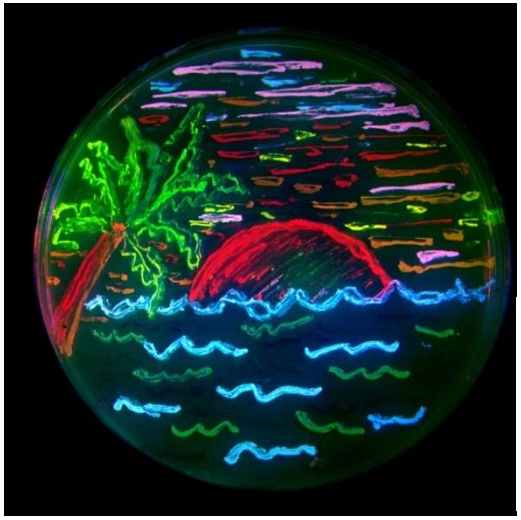


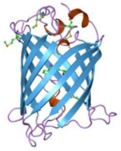
- Single molecule sensitivity

Fluorescent indicator

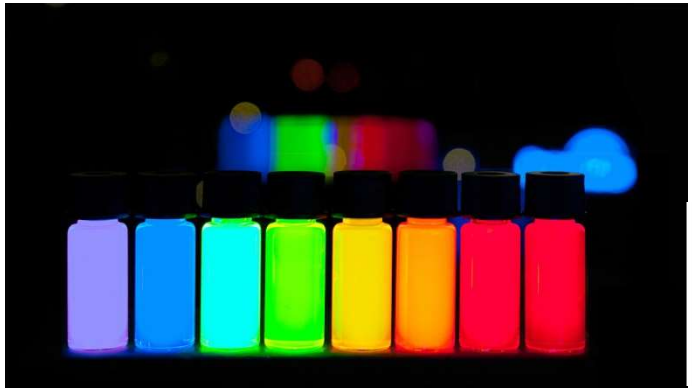
Dye

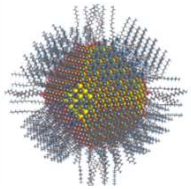
Protein



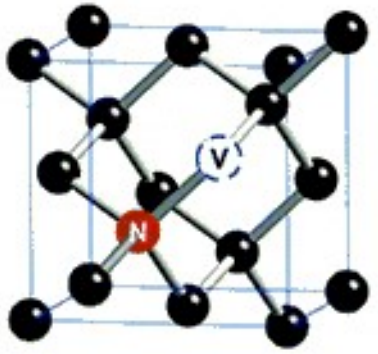


Quantum dot

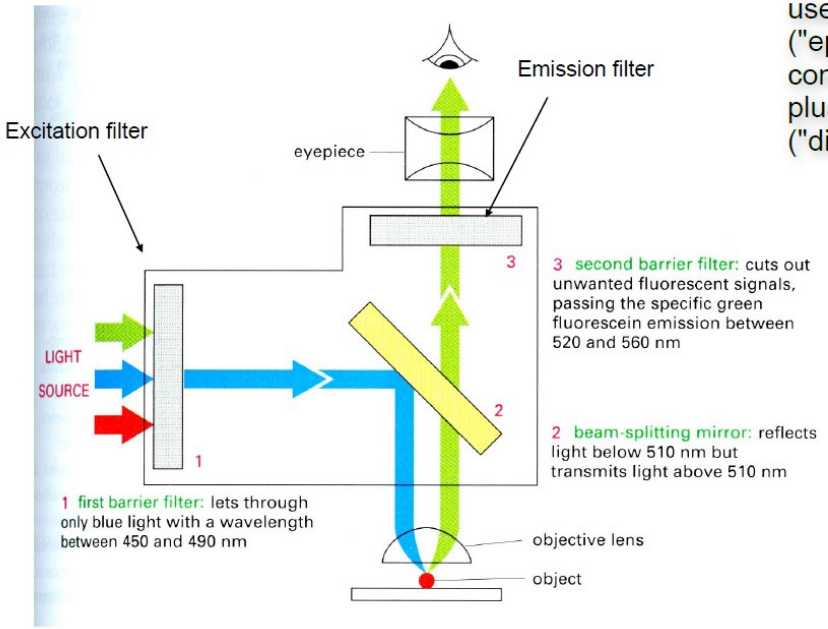




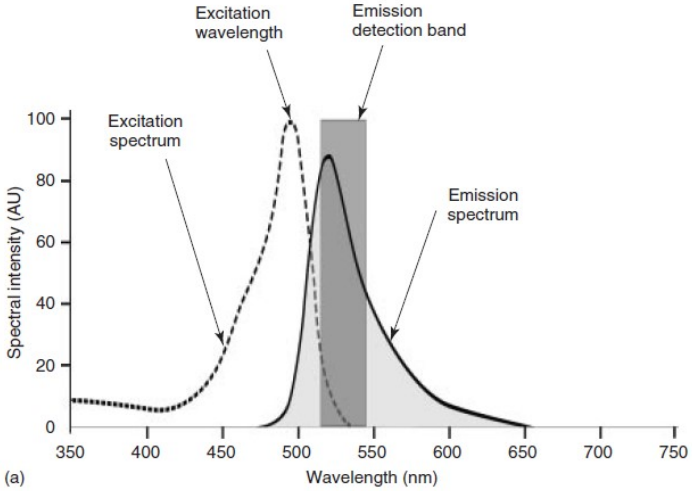
Nano Diamond

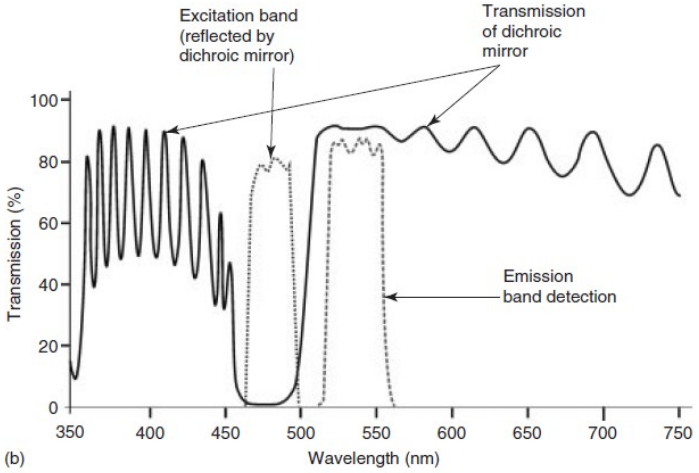


Epifluorescence microscope design

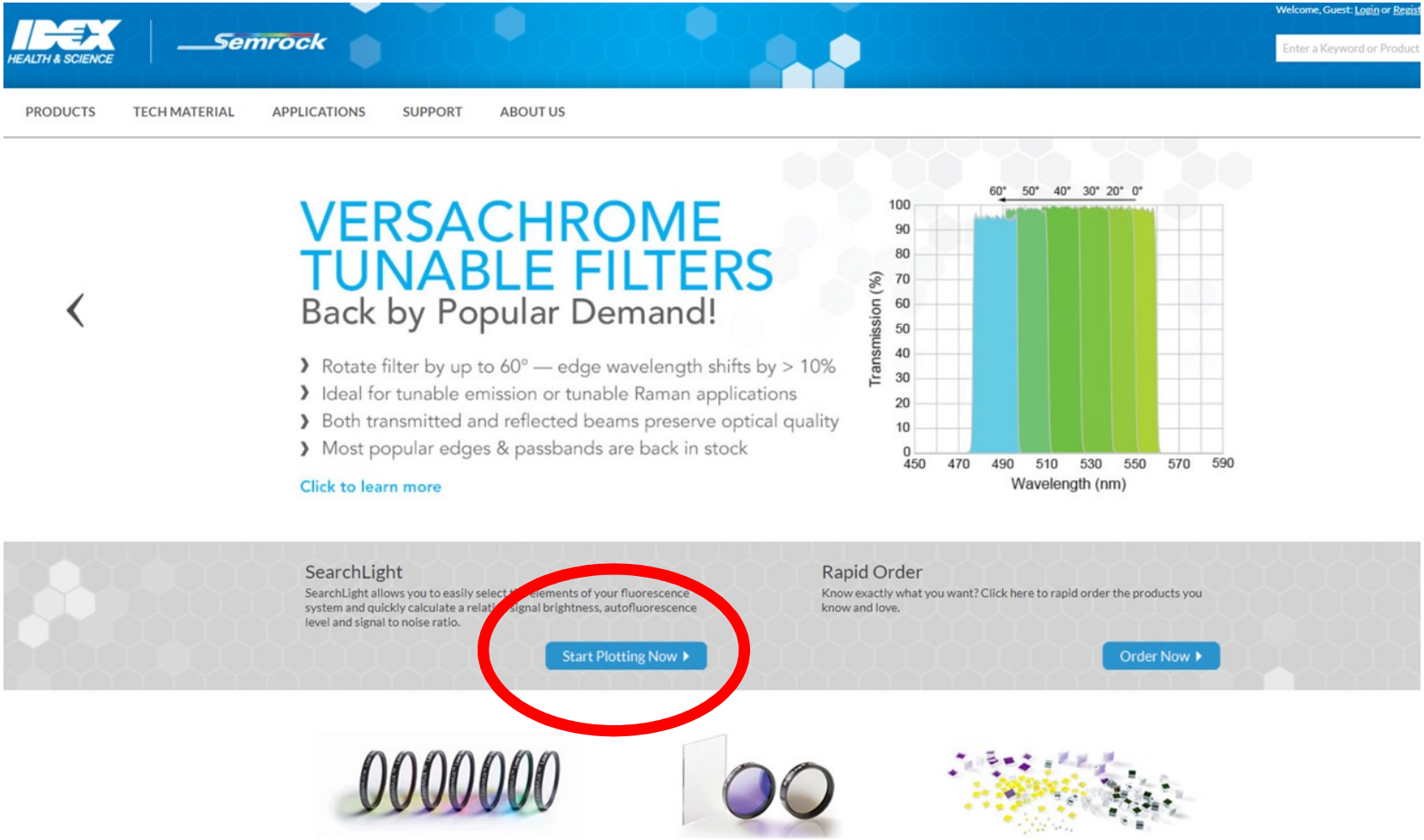


Epifluorescence microscopy uses illumination from above ("epi-") and a special cube containing two coloured filters plus a special beam-splitting ("dichroic") mirror

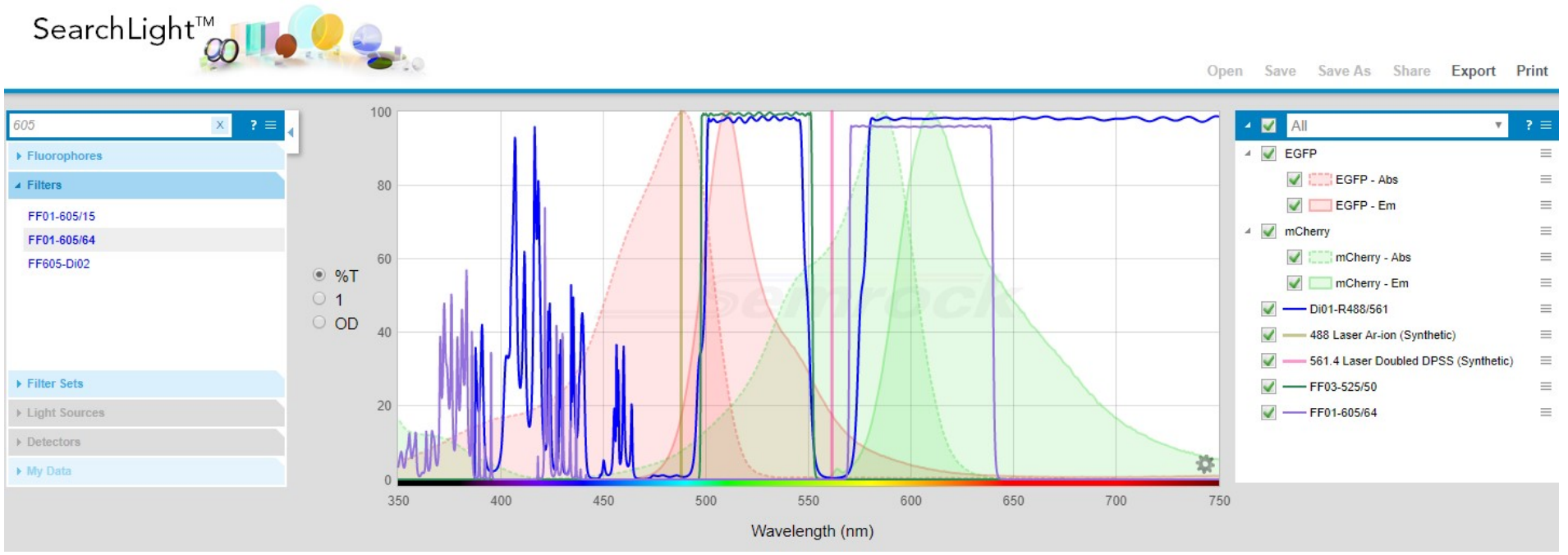




https://www.semrock.com/



Choose right filter sets



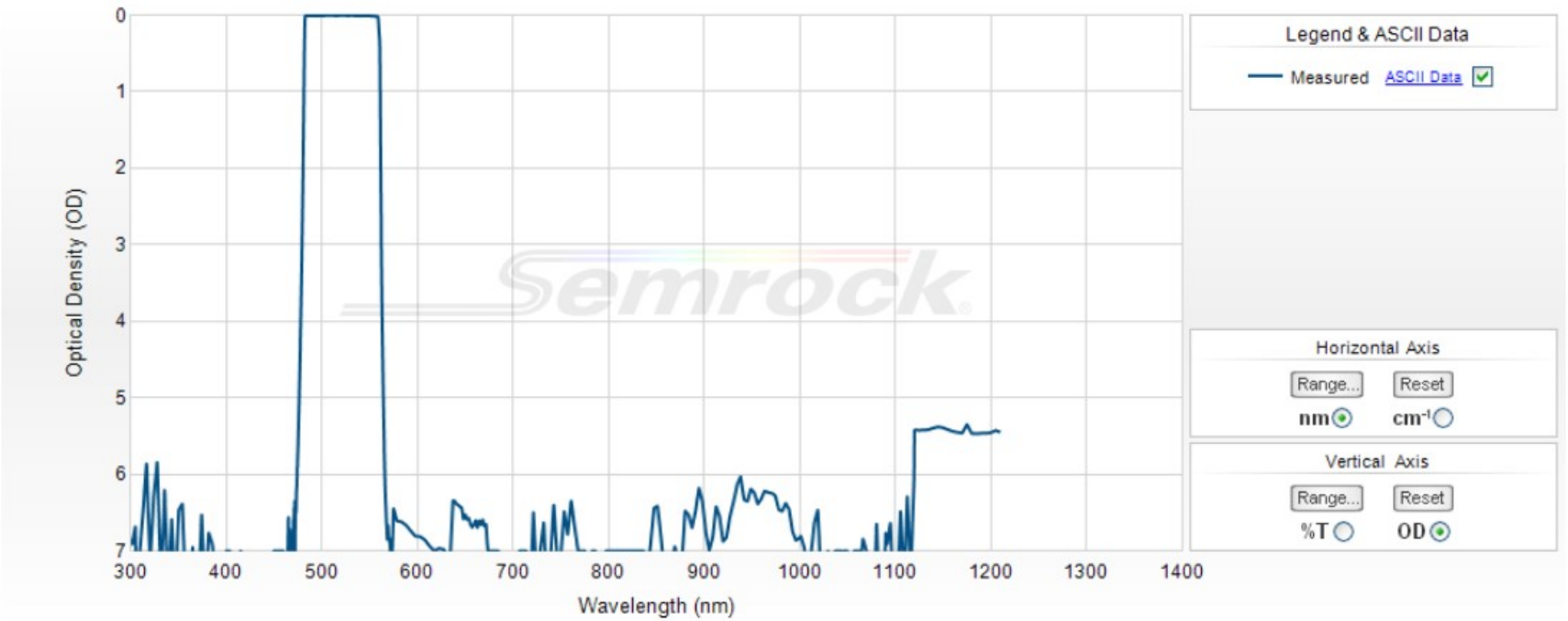
520/70 nm BrightLine® Two-photon emission filter

Part Number: FF01-520/70-25

Left-click & drag to zoom. Right-click to save, print or reset

Click for MyLight Tool

PDF Spec Sheet



- Your filter spectrum may differ slightly from the typical spectrum above, but is certified to meet the optical specifications noted below.
- Note that the change in blocking at 1120 nm is due to a change in the detector on the measurement equipment; the filter meets all optical specifications noted below.

Description and Pricing | Specifications | Technical Info

520/70 nm BrightLine® Two-photon emission filter
Ultrahigh-performance multiphoton fluorescence filters that accommodate a wide range of fluorescence dyes. Designed to be a fixed component in any multiphoton or nonlinear (harmonic-generation) microscope, so that when desired individual, narrower bandpass filters can be added without having to worry about the near-IR blocking of these filters. The transmission bands of these short-pass emitters are so wide, they appear clear at normal incidence.

We use fluorescence because of its sensitivity

Mountain by day

Small signal
High background

like absorbance

Same mountain by night

Small signal
Low background

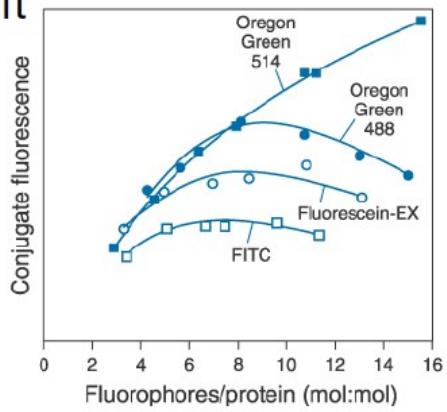
like fluorescence

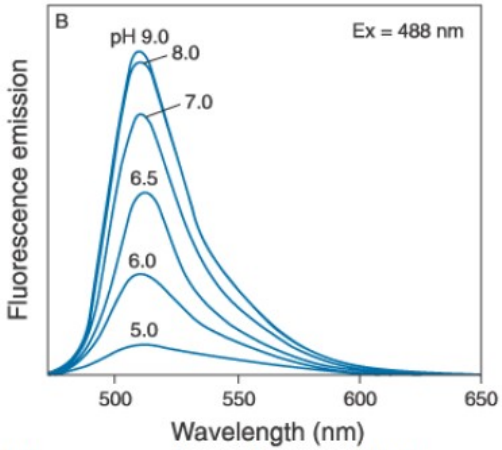
How good is a fluorophore?

1. Excitation and emission appropriate
   - background worse in UV + with small Stokes shift
   - good match to filters on your microscope
   - look at other fluorophores at same time
2. Bright
   - see small numbers of fluorophores,
   - low self-quenching, high QY and absorbance
3. Stable to photobleaching
   - exciting light damages fluorophore
4. Non-toxic
5. Environment-insensitive (especially to pH)
6. Little non-specific binding
7. Small
8. Little blinking

(9. Cost)

Green dye self-quenching

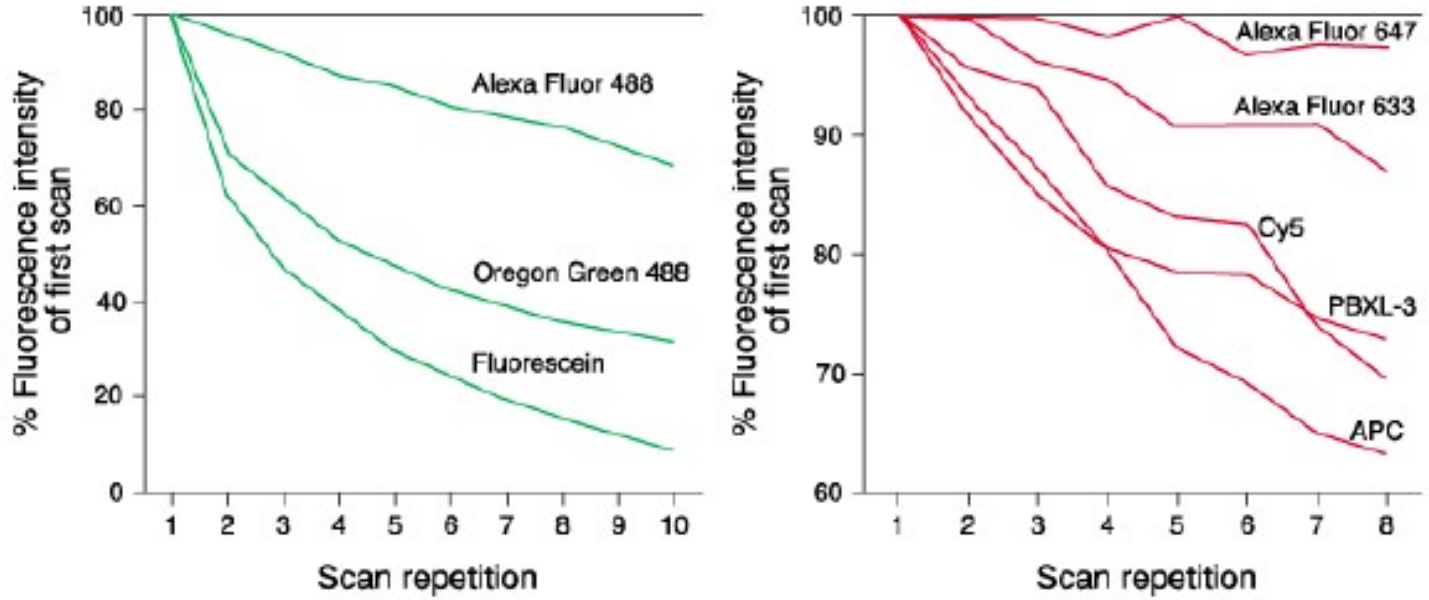




Fluorescein pH sensitivity

Photo stability

Laser-scanning cytometry
EL4 cells
biotin-anti-CD44 + streptavidin conjugates



Fluorescein is the commonest dye but has poor photostability.

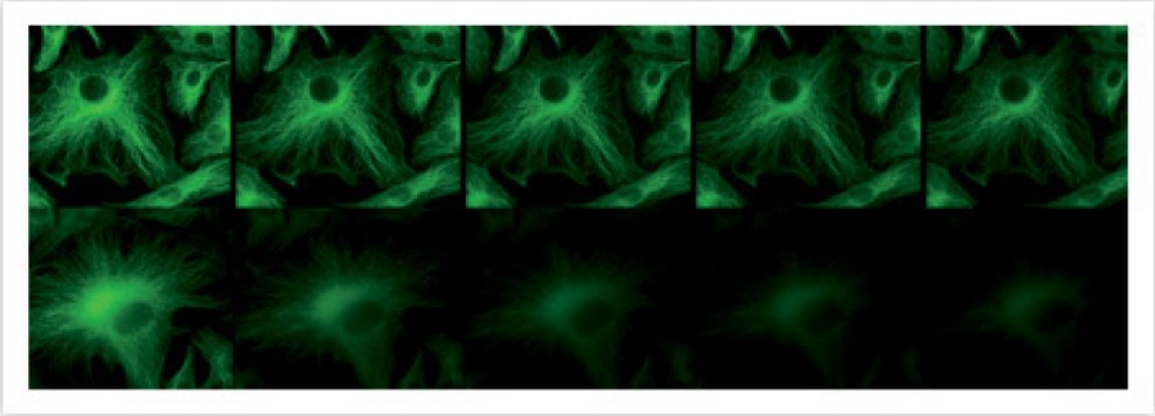
Scavenge and prevent reactive oxygen species from forming.

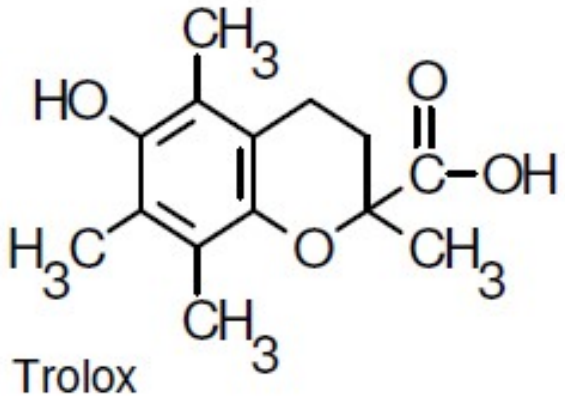
For fixed cells:

Home made: 0.3% p-phenylene-diamine (Sigma)
or Propyl Gallate
Vectashield: Proprietary, very effective all round, affects psf
Dabco
Prolong Gold®

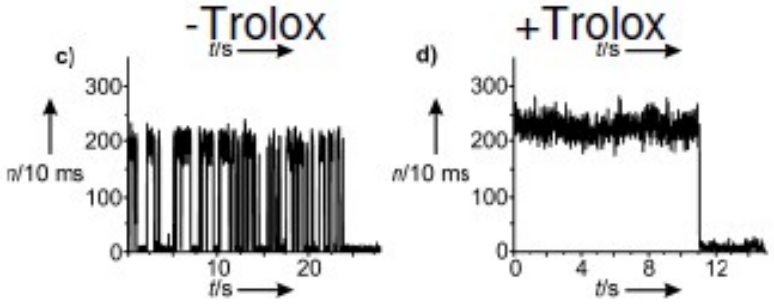
+ Prolong Gold

Untreated





Trolox

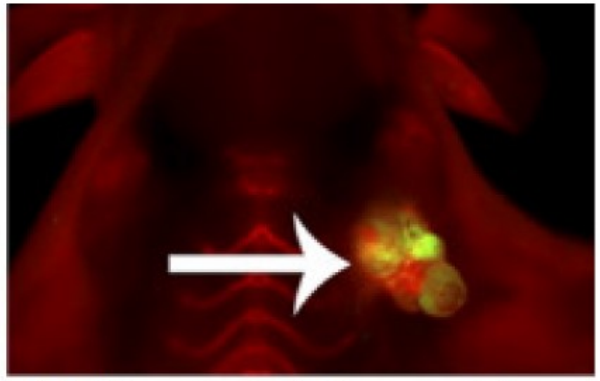


Blinking of single molecule of Atto647N on DNA, Vogelsang Tinnefeld Ang Chem 2008

- Trolox is an antioxidant that can reduce bleaching
  compatible with live specimens
  water-soluble
  working conc. ~100 µM
- Ascorbic acid is an alternative antioxidant
- Depleting oxygen (especially used for some single molecule experiments) with Glucose Oxidase and Catalase greatly reduces bleaching.
- Can stop not only bleaching but also blinking

Why use small molecule rather than genetically-encoded probes?

1. No need to transfect
   - hard for some organisms and primary cells
   - easier to titrate
   - potential clinical application- e.g. image-guided surgery



MMP-activated Cy5 peptide labels tumour (RY Tsien 2010)

2. Probes often brighter, with bigger signal to noise
   - struggle to make GFP-based calcium reporter as good as fura-like dyes
3. Probes with entirely different fluorescent properties
   - QD photostability, probes with long fluorescence lifetimes, photouncaging
4. Smaller
   - e.g. calcium conc. right next to pore of ion channel

functional contrast in fluorescence imaging

- Functional sensors: calcium, voltage, pH, temperature .....

  Very active and continuously developing field

- Fluorescence Recovery After Photobleaching (FRAP)

  Measure diffusion process

- Fluorescence Correlation Spectroscopy (FCS)

  Measure diffusion parameters and fluorophore concentrations

- Fluorescence (Förster) Resonance Energy Transfer (FRET)

  Measure distance in nanometer range

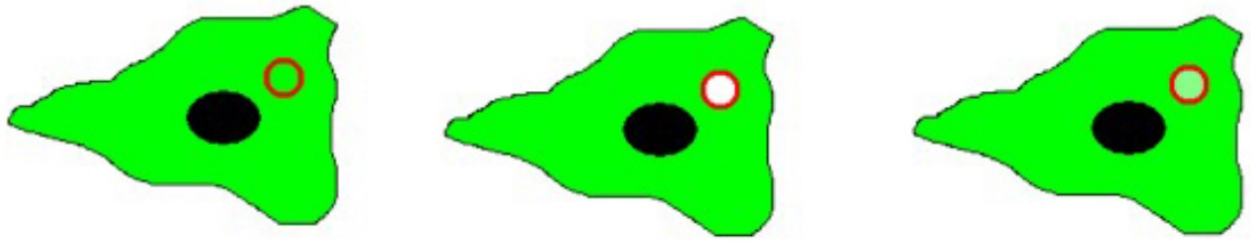
- Fluorescence Lifetime Imaging (FLIM)

Fluorescence Recovery After Photobleaching (FRAP)

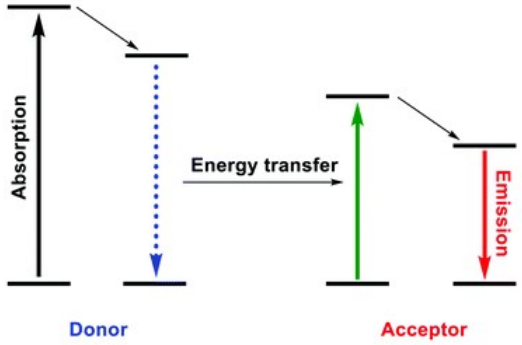
A region is rapidly bleached and the rate at which fluorescence fills the bleach region is determined by the diffusion of unbleached molecules.

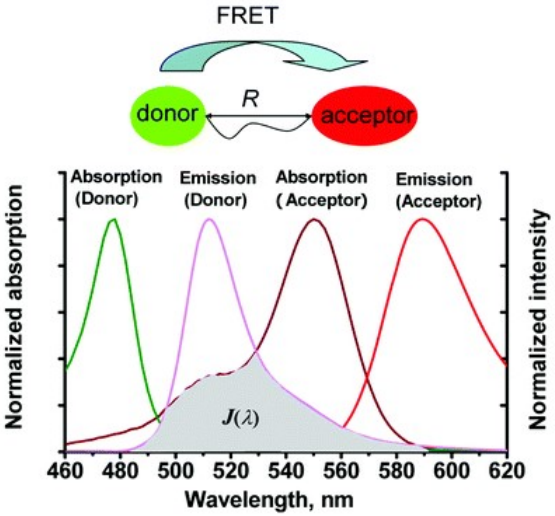
Small objects – fast diffusion

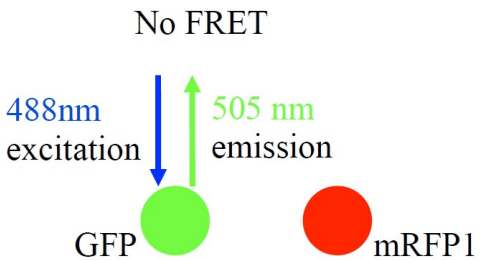
Large objects – slow diffusion

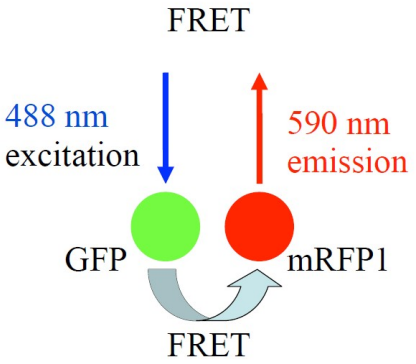


FRET


Absorption
Energy transfer
Emission
Donor
Acceptor

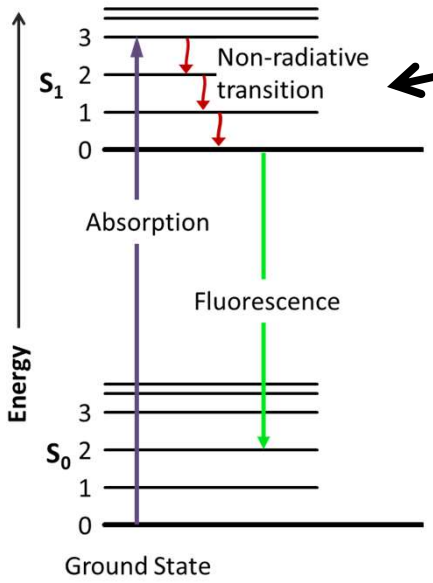

FRET
donor
R
acceptor
Absorption (Donor)
Emission (Donor)
Absorption (Acceptor)
Emission (Acceptor)
Normalized absorption
Normalized intensity
J(λ)
460 480 500 520 540 560 580 600 620
Wavelength, nm


No FRET
488nm excitation
505 nm emission
GFP
mRFP1


FRET
488 nm excitation
590 nm emission
GFP
mRFP1
FRET

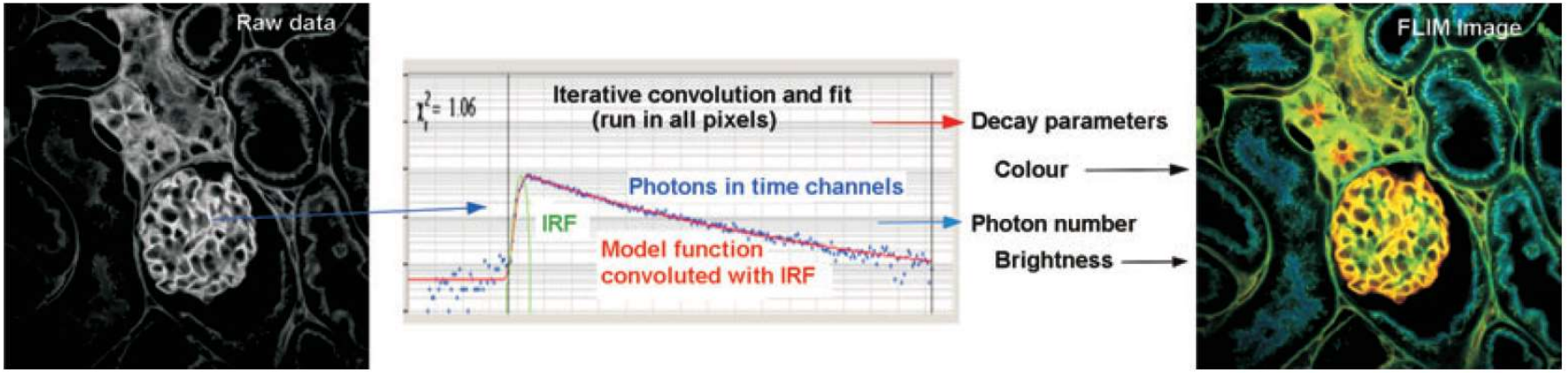

λex = 403 nm
λem = 454 nm
FRET off
ATP + H+
(~ pH 5)
λex = 403 nm
FRET on
λem = 557 nm
23

FLIM



Life time, ~ns, sensitive to environment

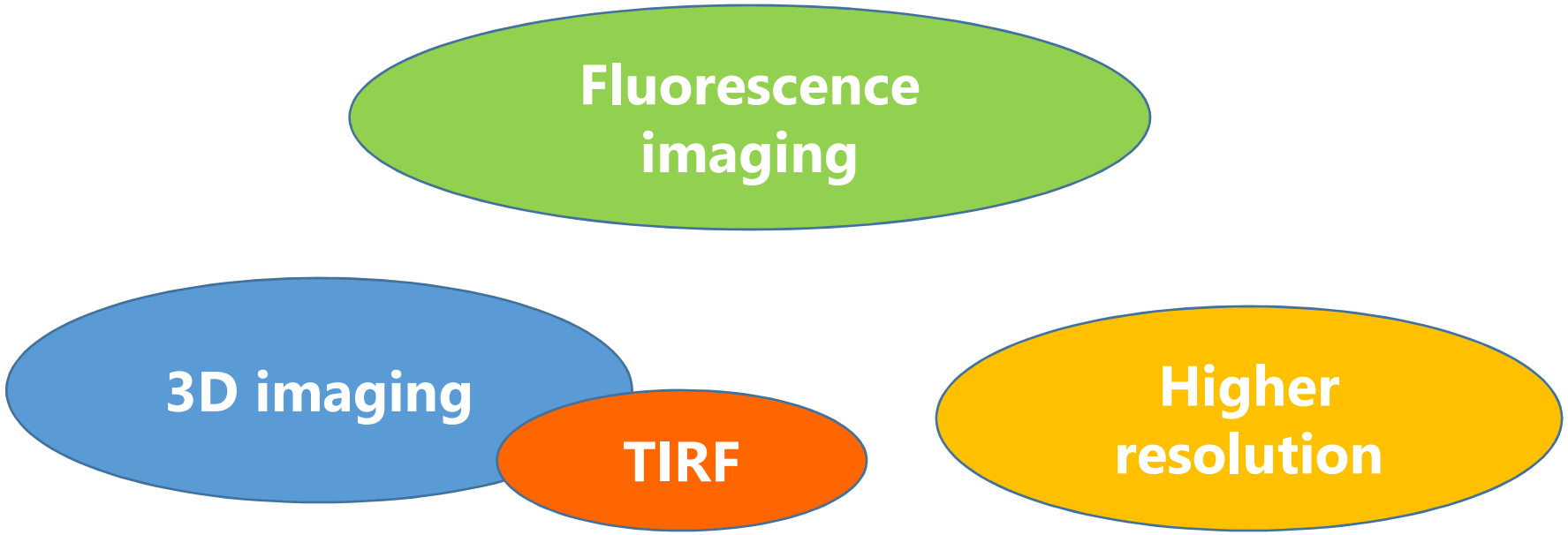
- Quenching by various ions: Ca, Cl, oxygen, pH ..
- FRET
- Aggregation
- Local viscosity
- Proximity to metal surfaces
- .....



Steps for fluorescence imaging

1. Label your sample with high quality
2. Choose right imaging technique
3. Image correctly
4. Apply proper post processing

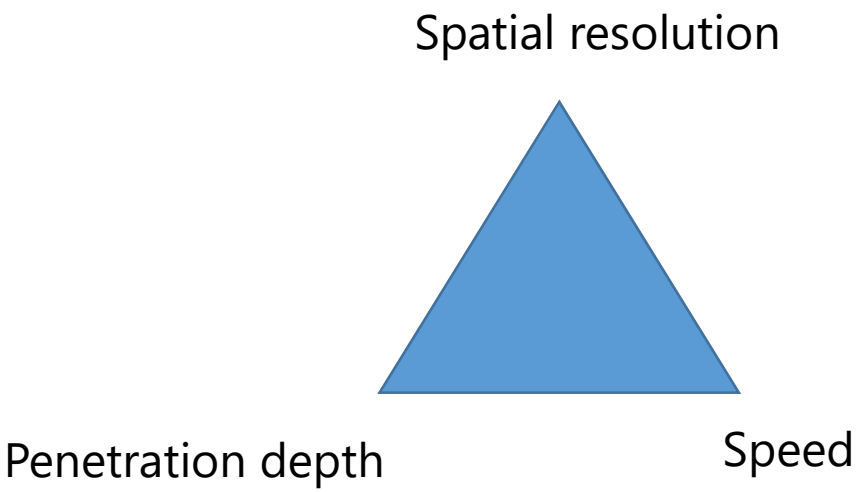
Hot topics in optical imaging



- Confocal
- Multiphoton
- Light sheet
- Light field
- Sectioning SIM

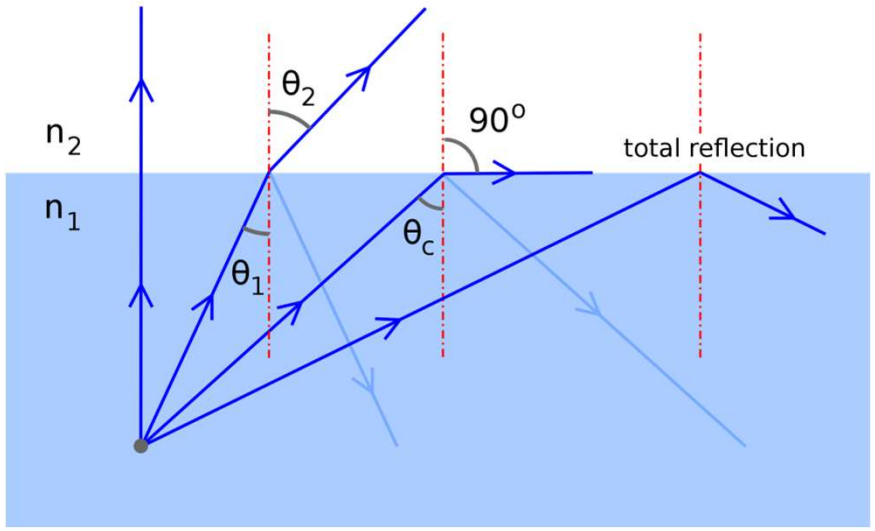
- PALM/STORM
- STED
- Super-resolution SIM
- Expansion microscope

Problems in optical imaging

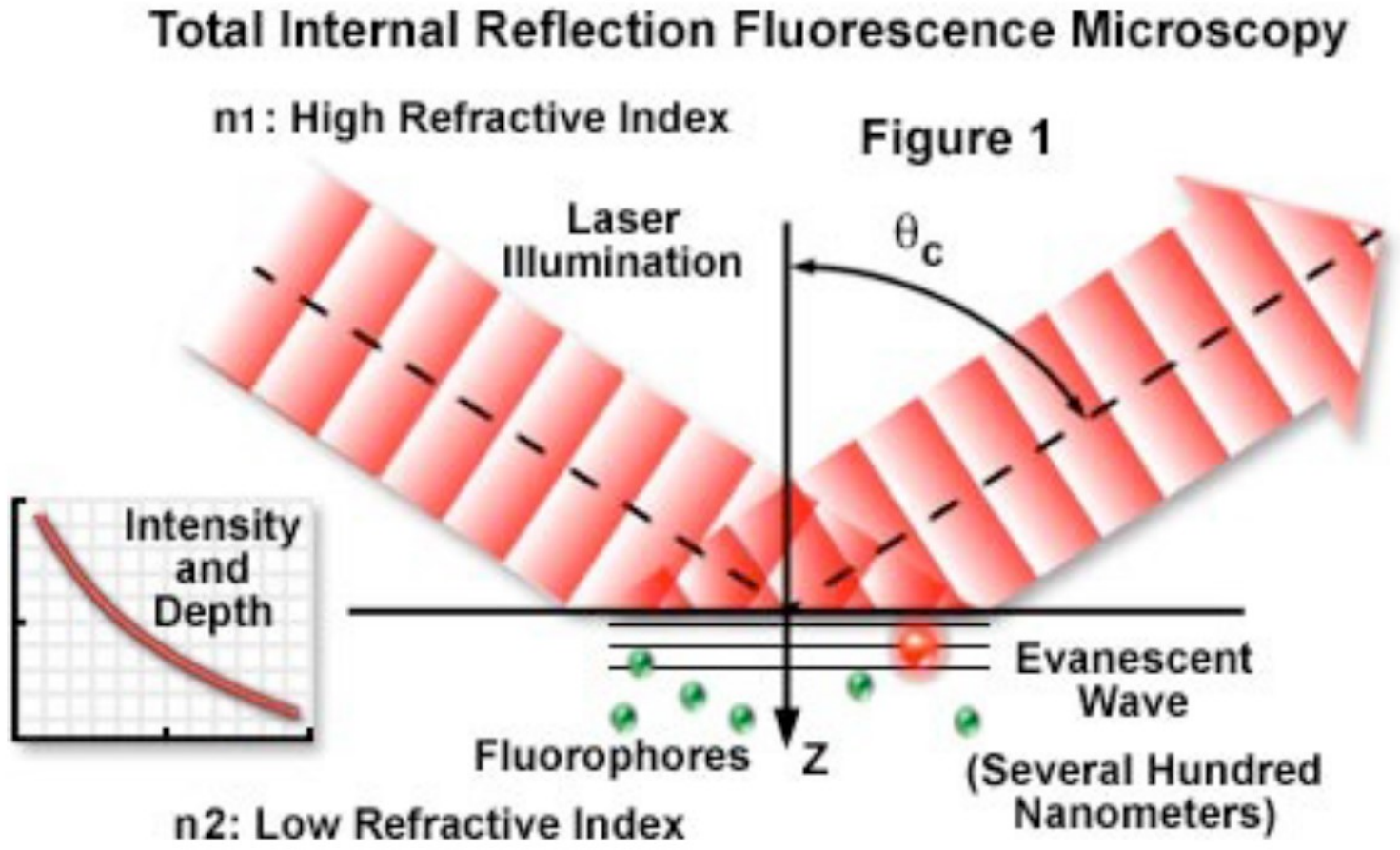


- Diffraction limit
- Background noise
- Limited numbers of photons
- Tissue aberrations
- Tissue scattering

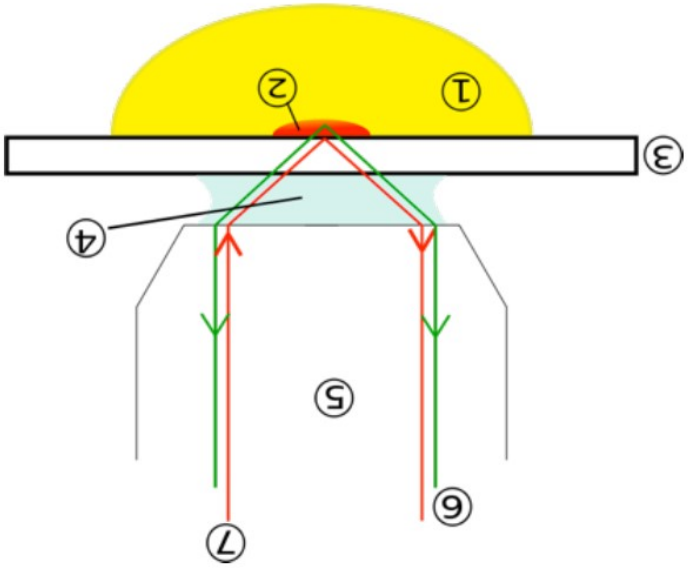
Total Interal Reflection Fluorescent Microscopy (TIRF)



倏逝波 Evanescent field



Single objective TIRF

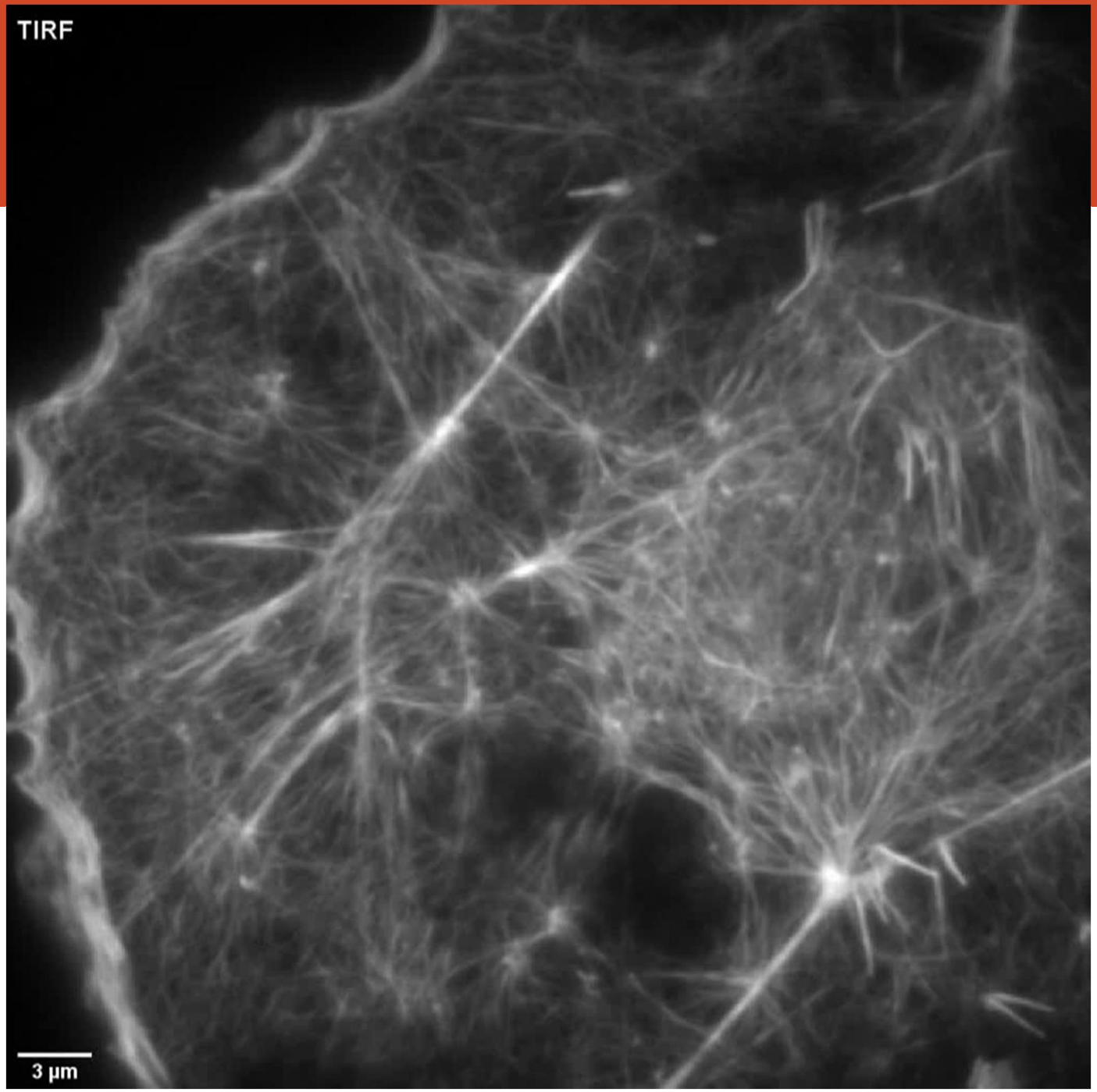


Objective based TIRF

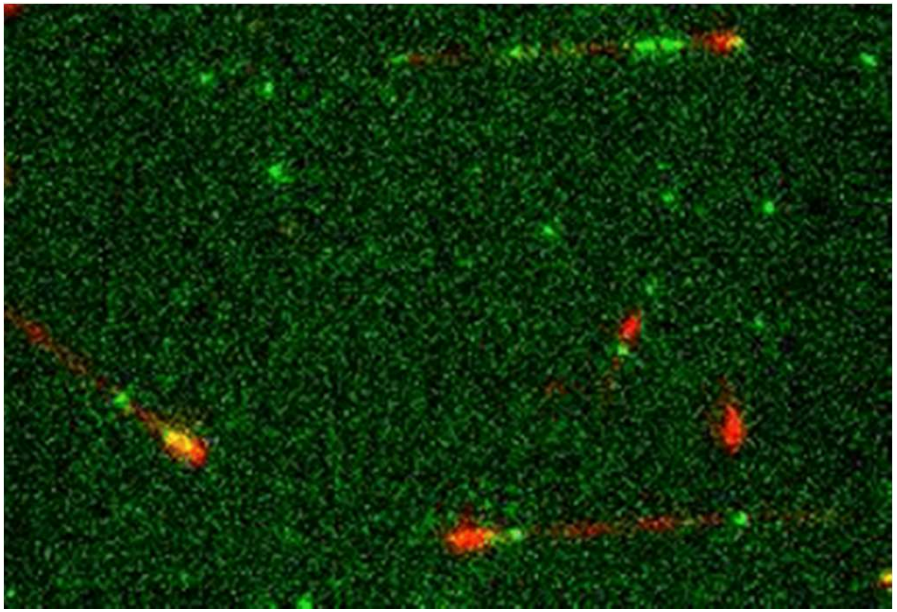
Oil immersion
NA>1.33

Very low background and crisp image

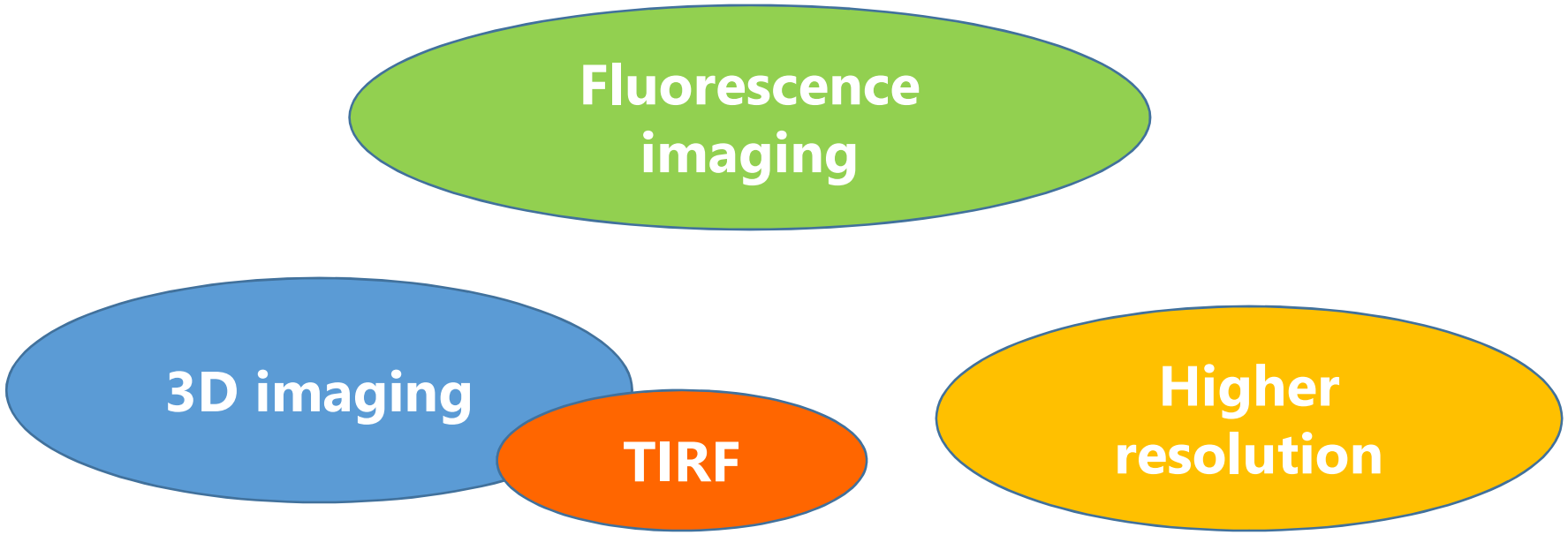
Best for near cover glass


TIRF
3 µm

Due to extremely low background, nearly all the single molecule imaging experiments were done using TIRF



Hot topics in optical imaging



- Confocal
- Multiphoton
- Light sheet
- Light field
- Sectioning SIM

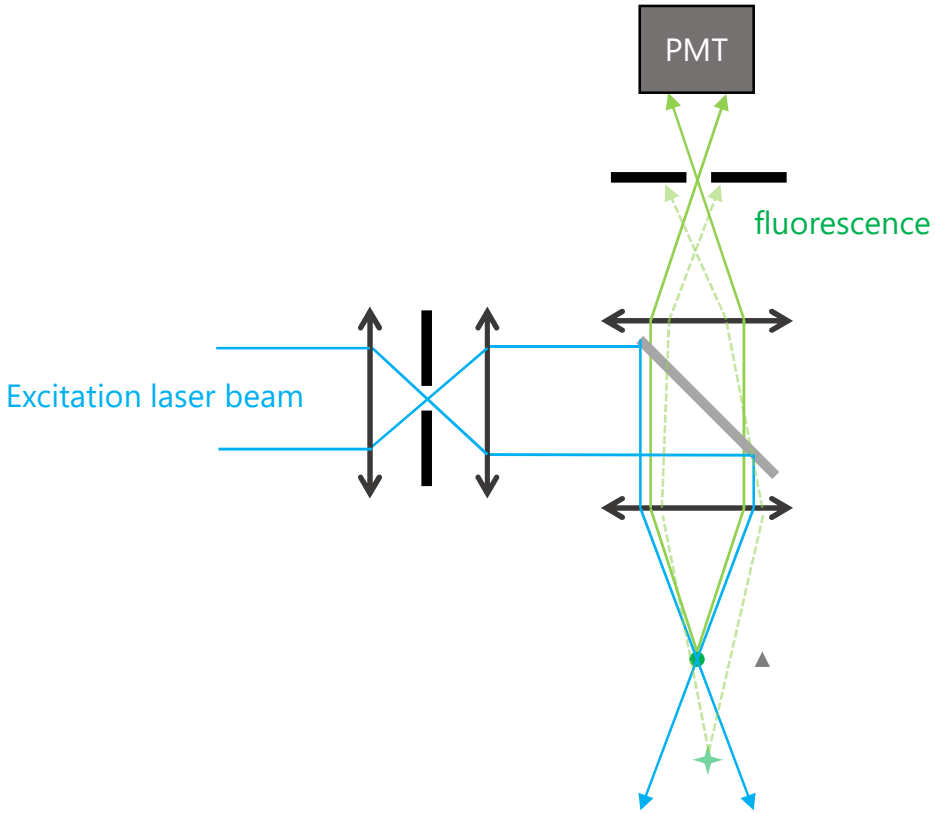
- PALM/STORM
- STED
- Super-resolution SIM
- Expansion microscope

3D Imaging

- Confocal microscope
- Two photon microscope
- Light sheet microscope
- Light field microscope

Confocal (Laser) Scanning Microscope

- Confocal: Two pinholes co-aligned (conjugated) to each other
- Scanning: Scan a focused laser spot to get intensities pixel-by-pixels



Confocal and Widefield Fluorescence Microscopy

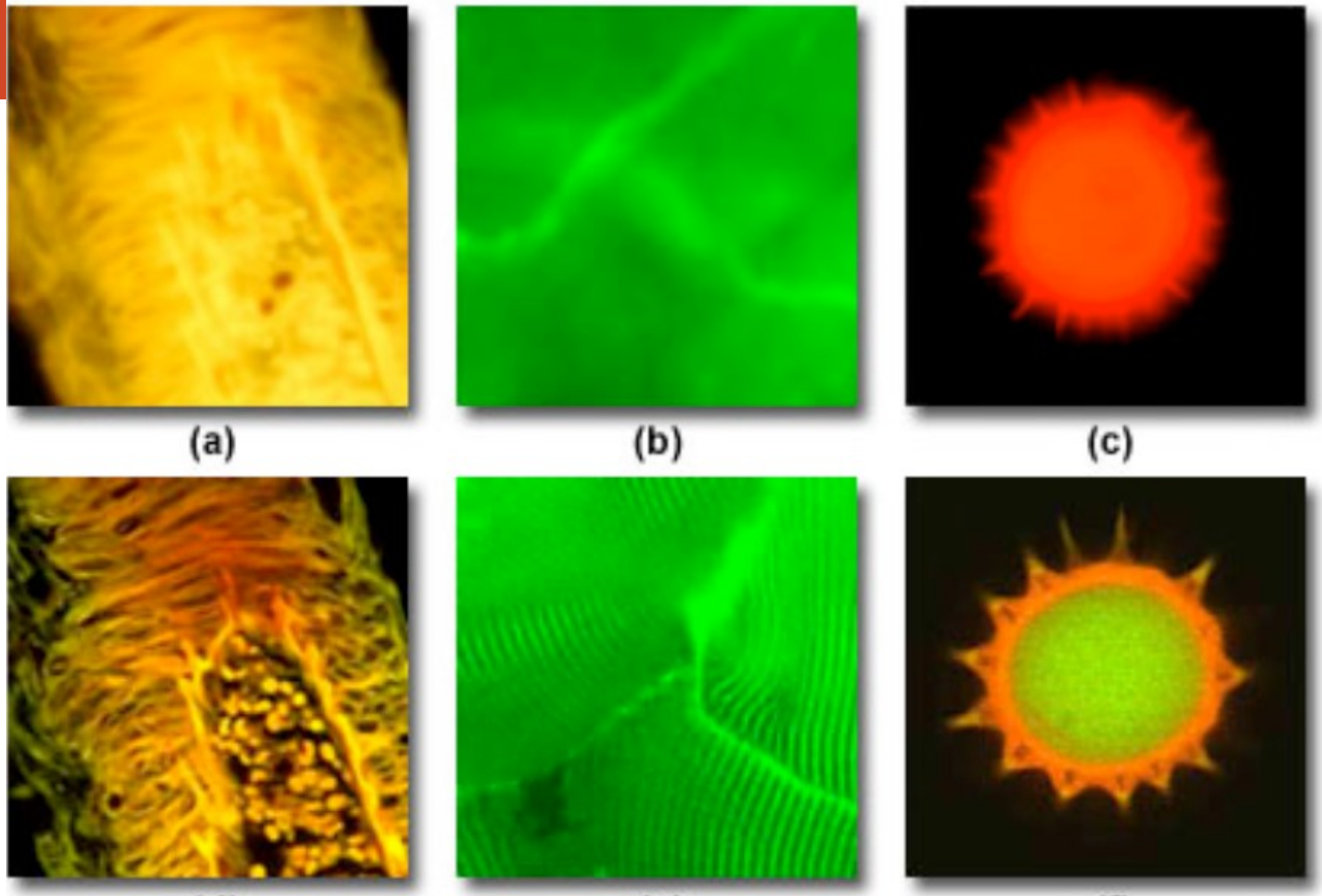
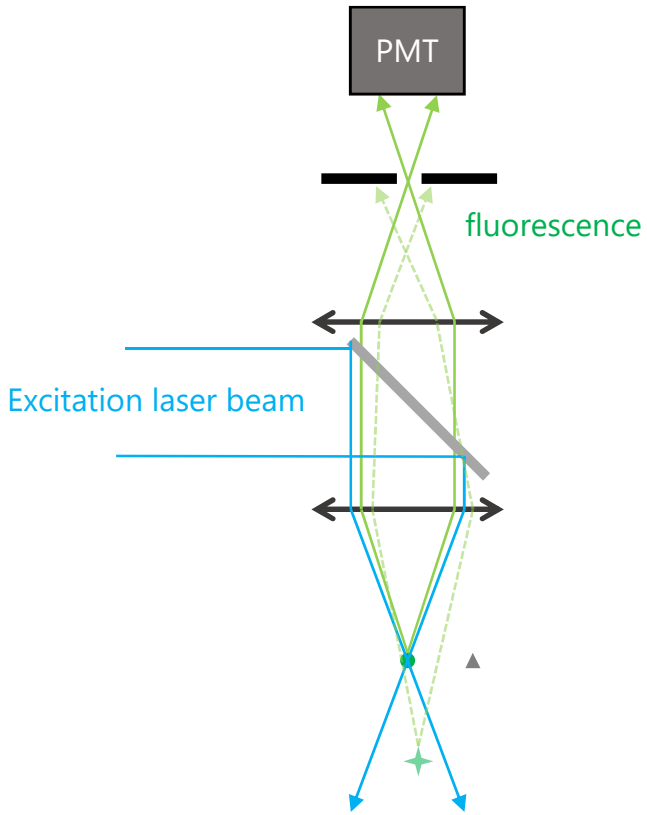


Figure 1

Confocal Scanning Microscope

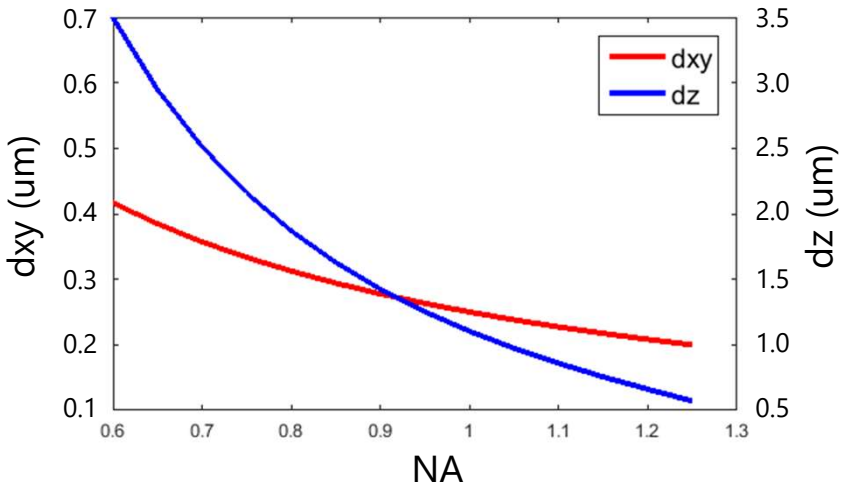
In 1940 Hans Goldmann, ophthalmologist in Bern, Switzerland, developed a slit lamp system to document eye examinations. This system is considered by some later authors as the first confocal optical system.



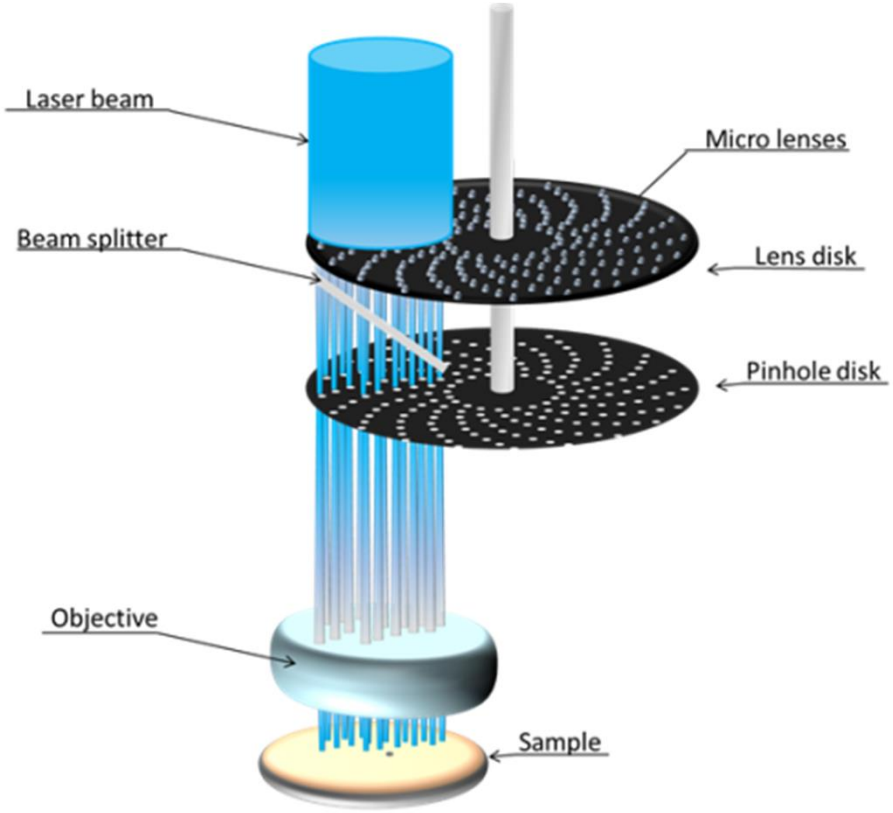
Resolution: $d_{xy} = \frac{\lambda}{2 * NA}$

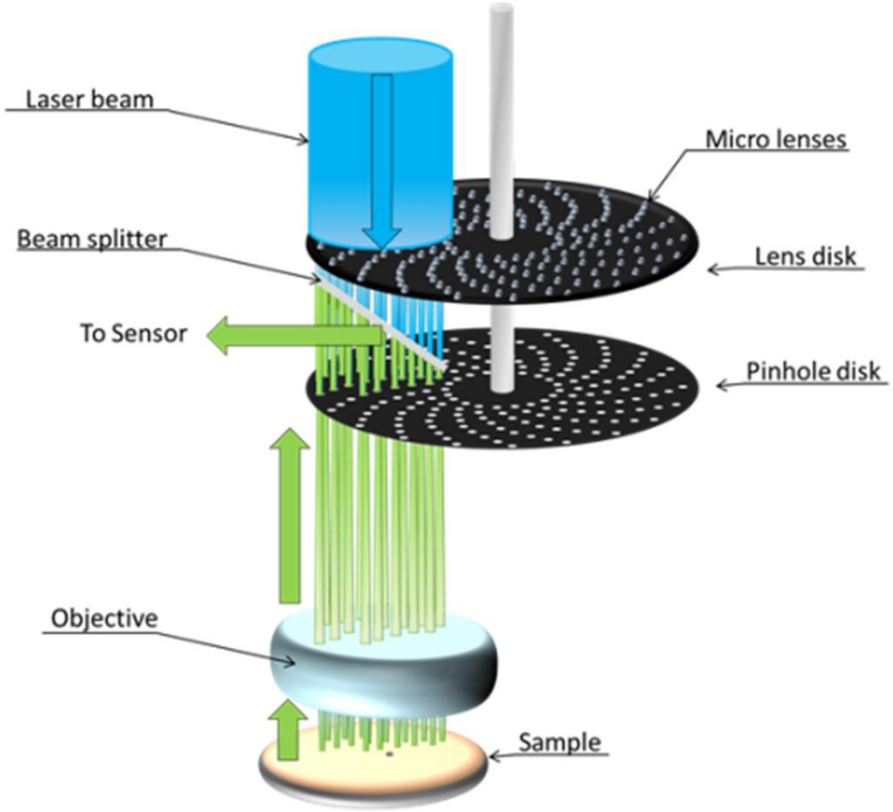
$$d_z = \frac{\lambda}{n - \sqrt{n^2 - NA^2}}$$

Imaging resolution of GFP using water objective of different NA



Spinning disk microscopy





Photon reassignment microscopy
Imaging shift microscopy

Conventional microscope

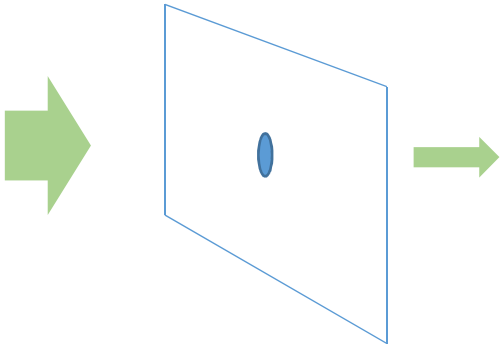
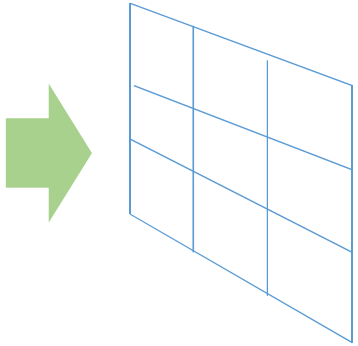


Photo detector

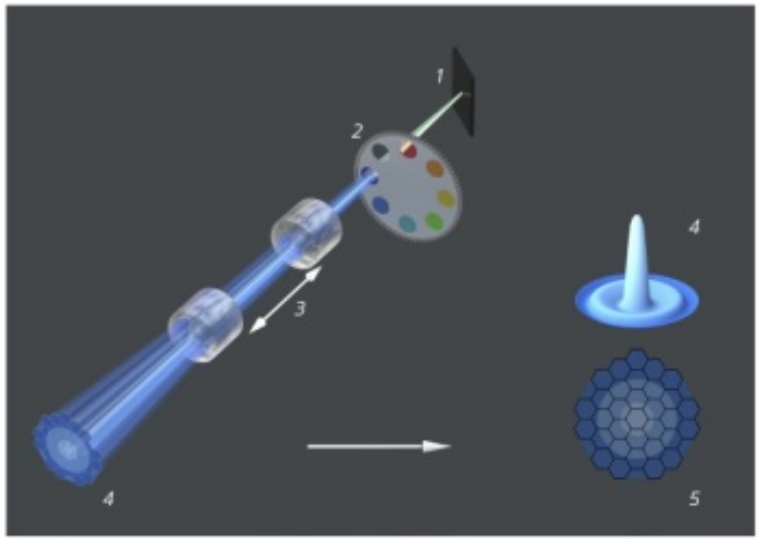
Photo re-assignment



Multi-pixel photo detector

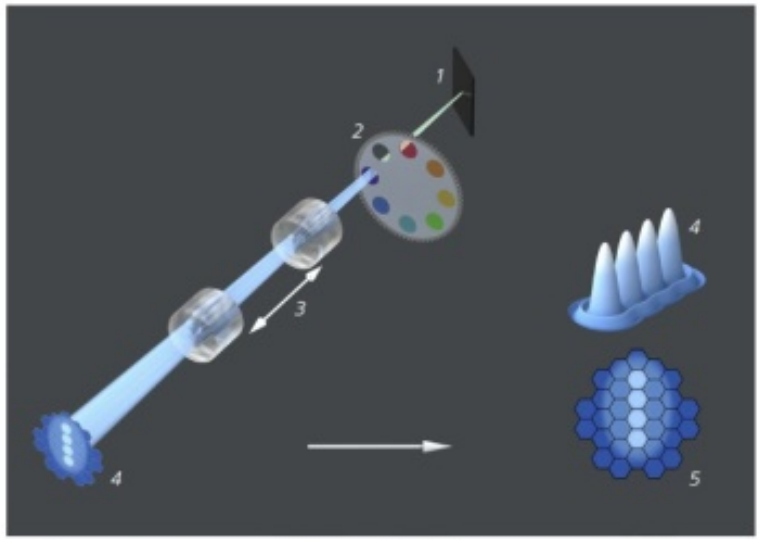
Best for samples with weak signals

Zeiss Airyscan



1. Mirror
2. Emission filters
3. Zoom optics
4. Airy disk
5. Airyscan detector

Figure 1 Beampath Airyscan



1. Mirror
2. Emission filters
3. Zoom optics
4. Airy disk
5. Airyscan detector

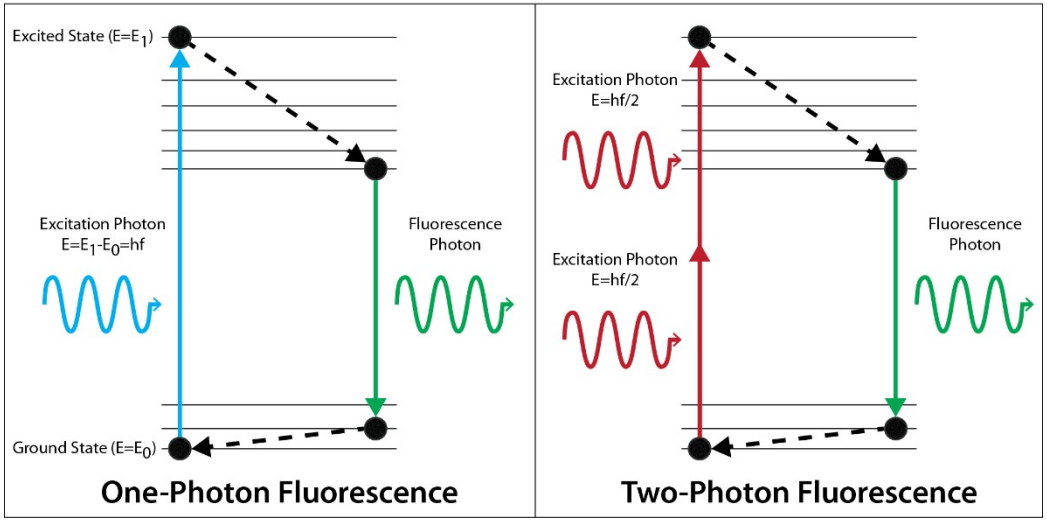
Figure 2 Airyscan Fast mode

Two photon microscope

Theory of two photon absorption:

Maria Goeppert-Mayer

1 GM = 10e-50 cm4 s/photon



Wide field: 1e4 W/m^2 1e16 W/m^2

Two photon microscope

Two-Photon Laser Scanning Fluorescence Microscopy

WINFRIED DENK,* JAMES H. STRICKLER, WATT W. WEBB

Science, Vol. 248, Issue 4951, pp. 73-76 (1990)

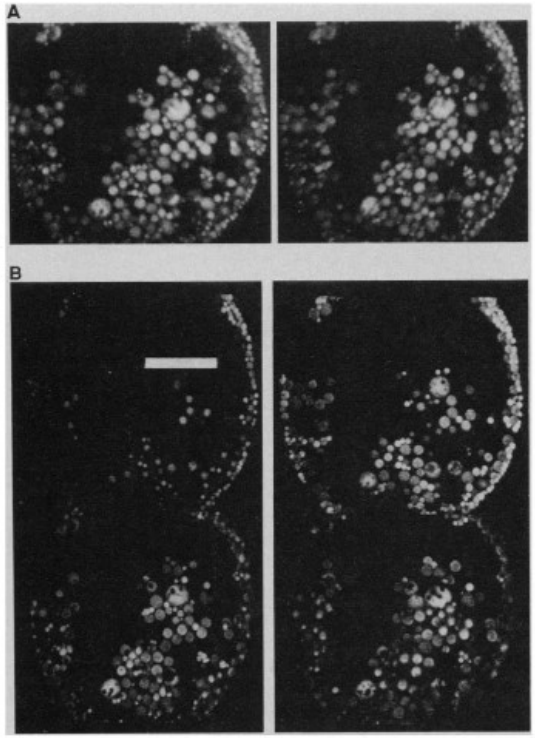
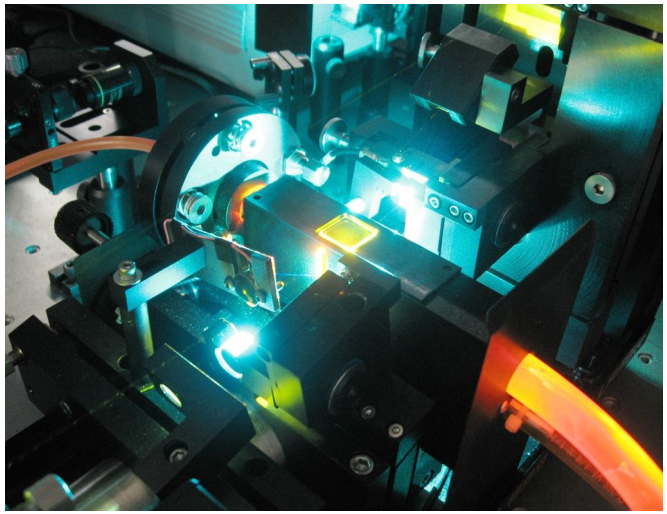


Fig. 1. (**A**) A stereo image pair is synthesized from a stack of six cross sections (*xy* sections) with an axial (*z*) increment of 3 μm. Blue (380 nm ≤ λ ≤ 445 nm) fluorescence excited by two-photon (630 nm) absorption was detected to record these images of a cluster of fluorescent beads with an LSM but with its confocal pinhole fully opened. The latex beads are volume-stained with the dye Coumarin 138 and have their measured absorption and emission maxima at 365 and 415 nm, respectively. The data comprise ten averages for each section with no background subtraction or image enhancement. The total time to acquire the data was less than 2 min. (**B**) The topmost four of the images, *xy* sections, used to synthesize the stereo pair in (A). Scale bar, 50 μm.

Femto second dye laser

80MHz, 100fs pulse width

→ peak power is 125000 times of the average power

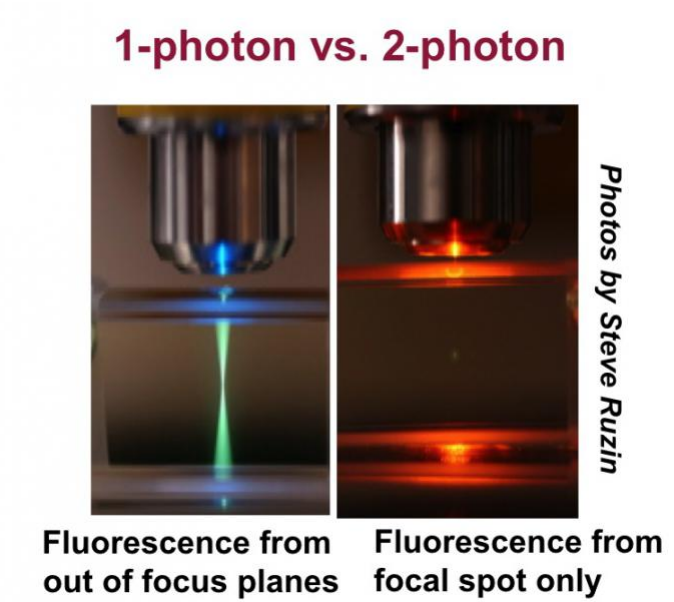


Laser source

Ti: Sapphire Laser

Coherent Chameleon familiy

Two photon microscope



	1-photon	2-photon
Thin sample	Low laser intensity Higher resolution	
Thick sample		Less scattering Low photo damage Low photo bleaching

Problem: speed!!!!!!

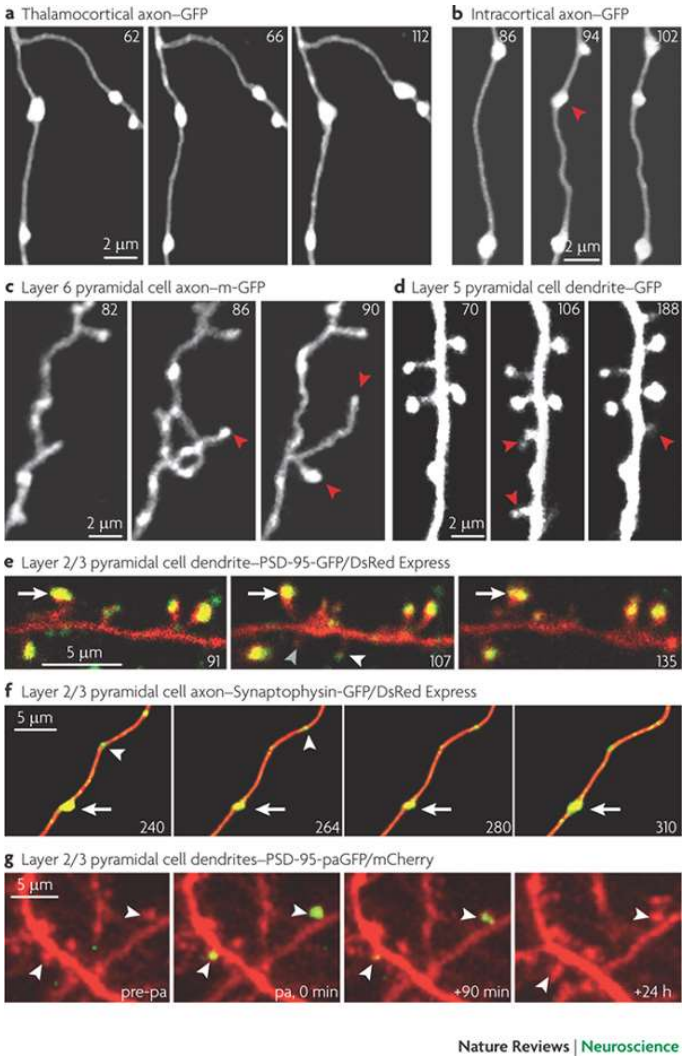
Two photon microscope

Brain Prize 2015

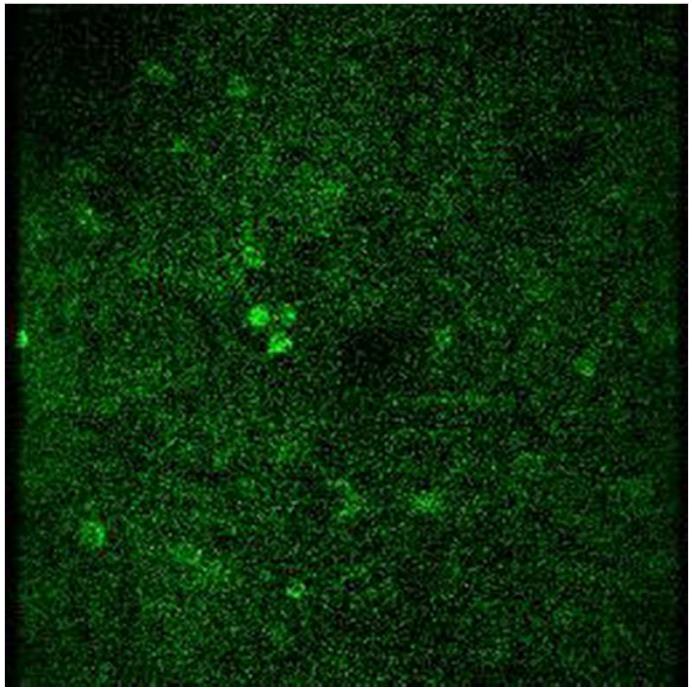
“invention, refinement and use of two-photon microscopy to provide detailed, dynamic images of activity in individual nerve cells, dendrites and synapses, thereby transforming the study of development, plasticity and functional circuitry of the brain.”

Time lapse two photon imaging of neuron plasticity

Behavioral Experiments



Two photon functional imaging



Light sheet microscope & whole brain imaging

Richard Adolf Zsigmondy

Nobel Prize in chemistry in 1925

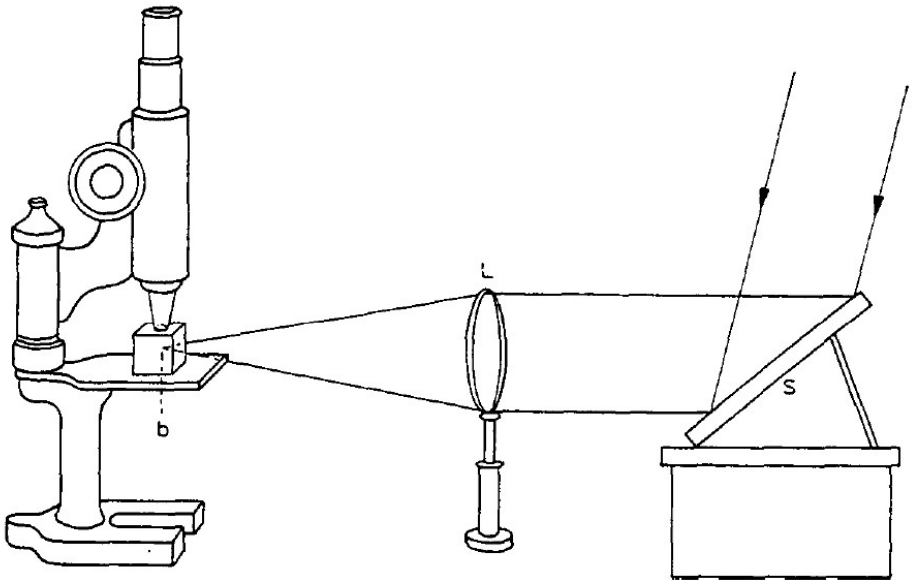
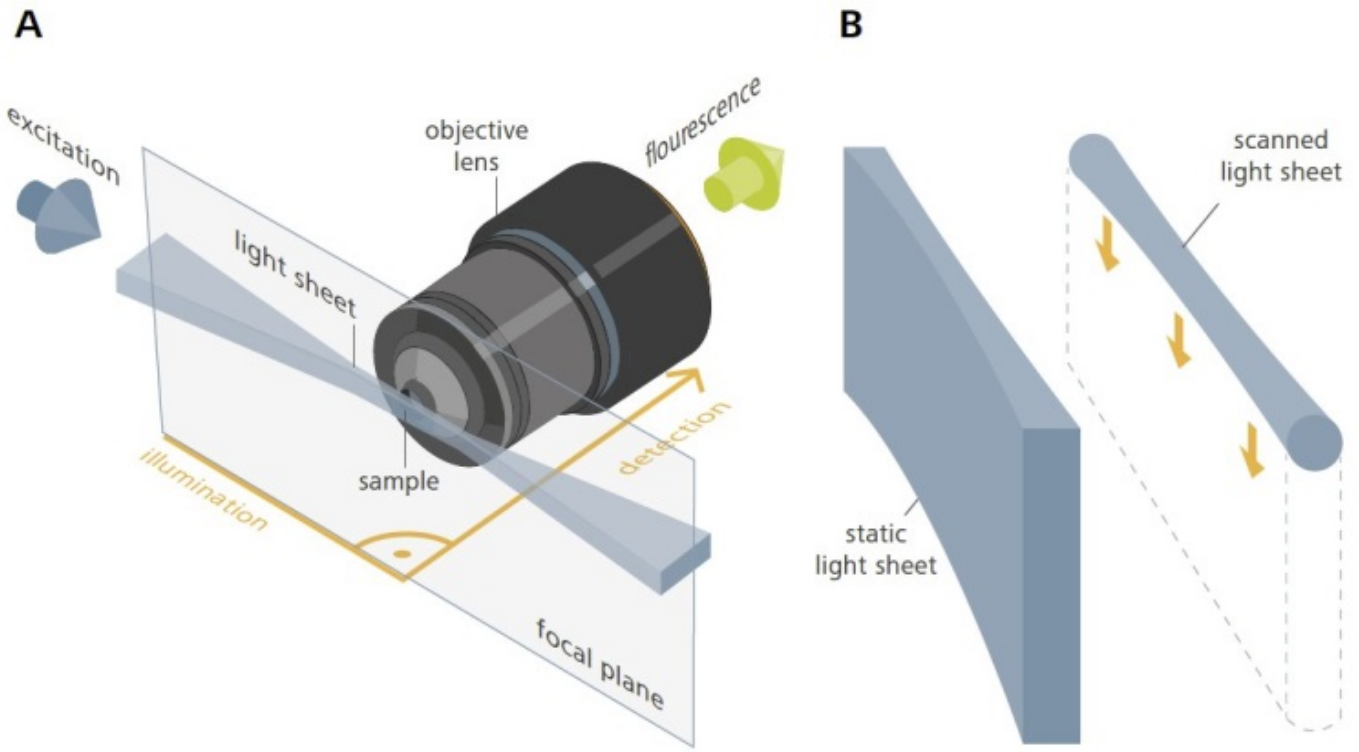


Fig. 1. The first arrangement for making ultramicroscopic particles visible.

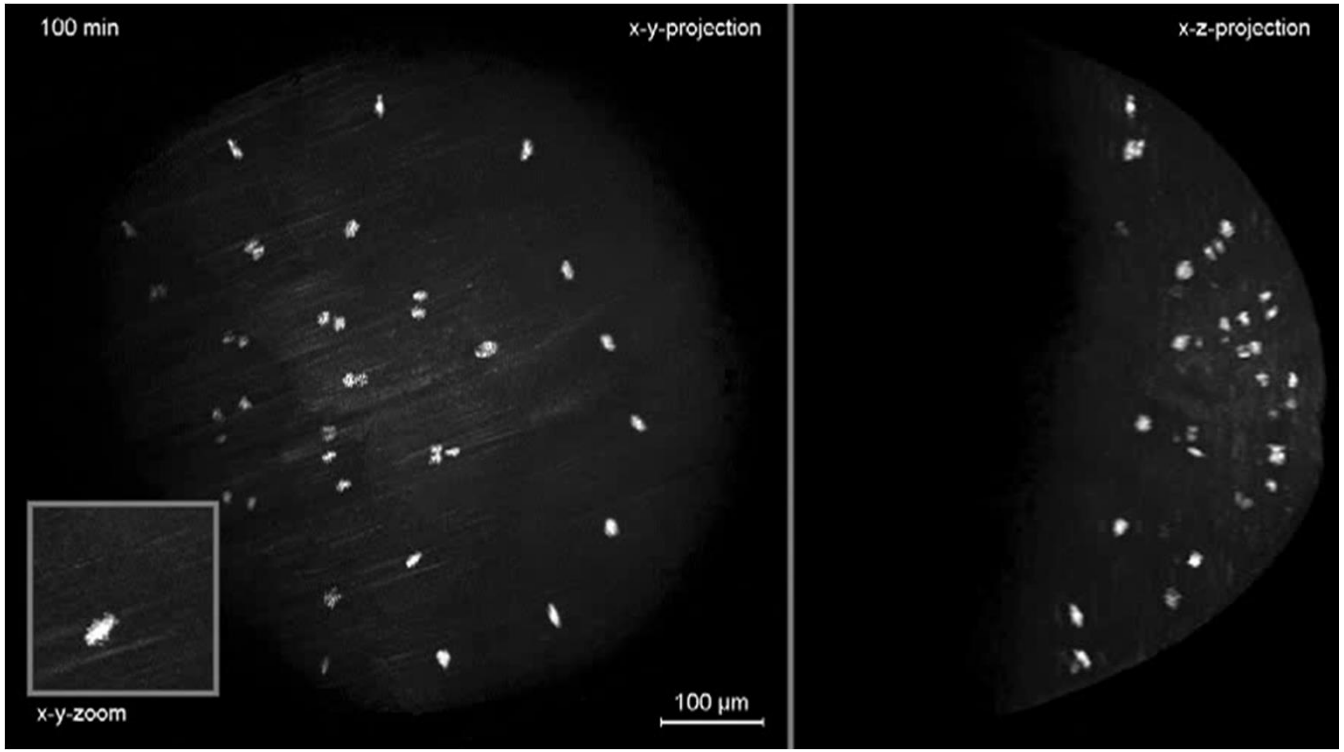
Light sheet microscope



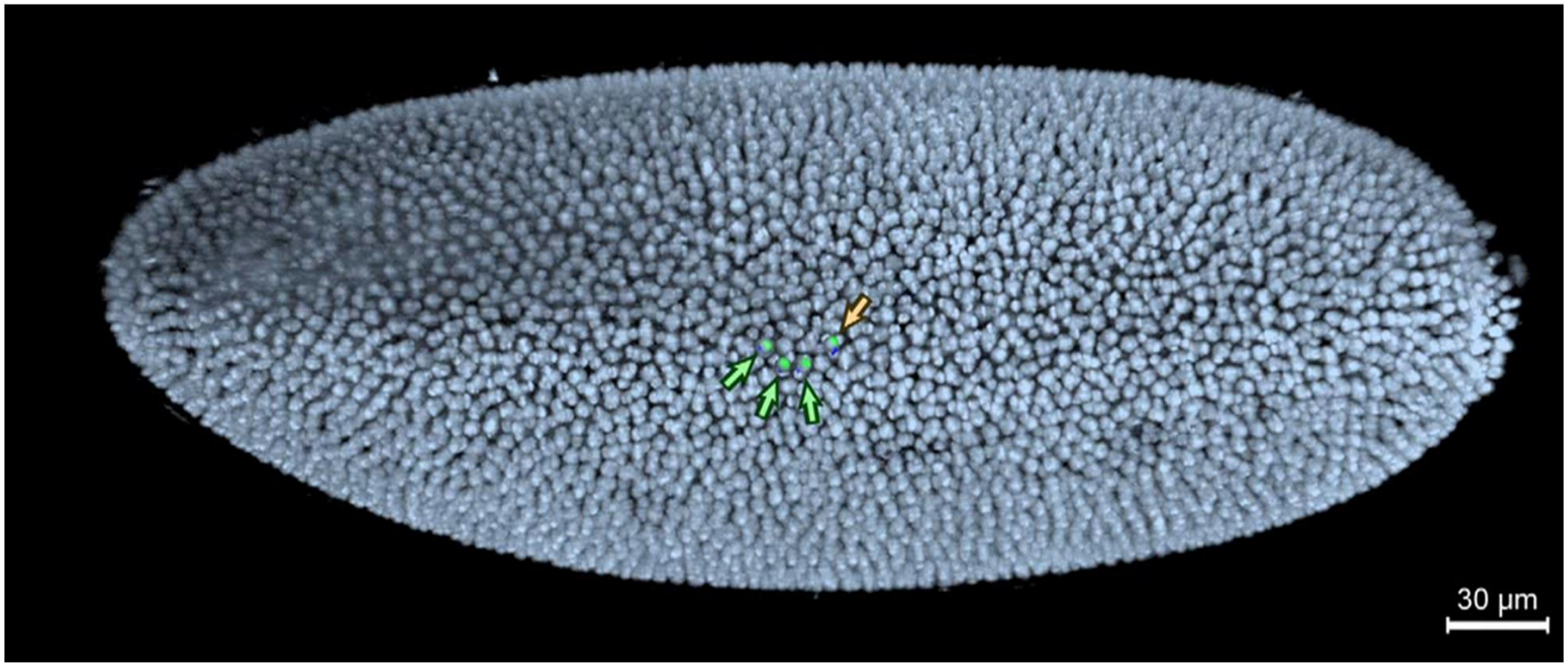
Light sheet microscope

Reconstruction of Zebrafish Early Embryonic Development by Scanned Light Sheet Microscopy

Philipp J. Keller,[1,2]* Annette D. Schmidt,[2] Joachim Wittbrodt,[1,2,3,4]* Ernst H.K. Stelzer[1]



Light sheet microscope for development biology

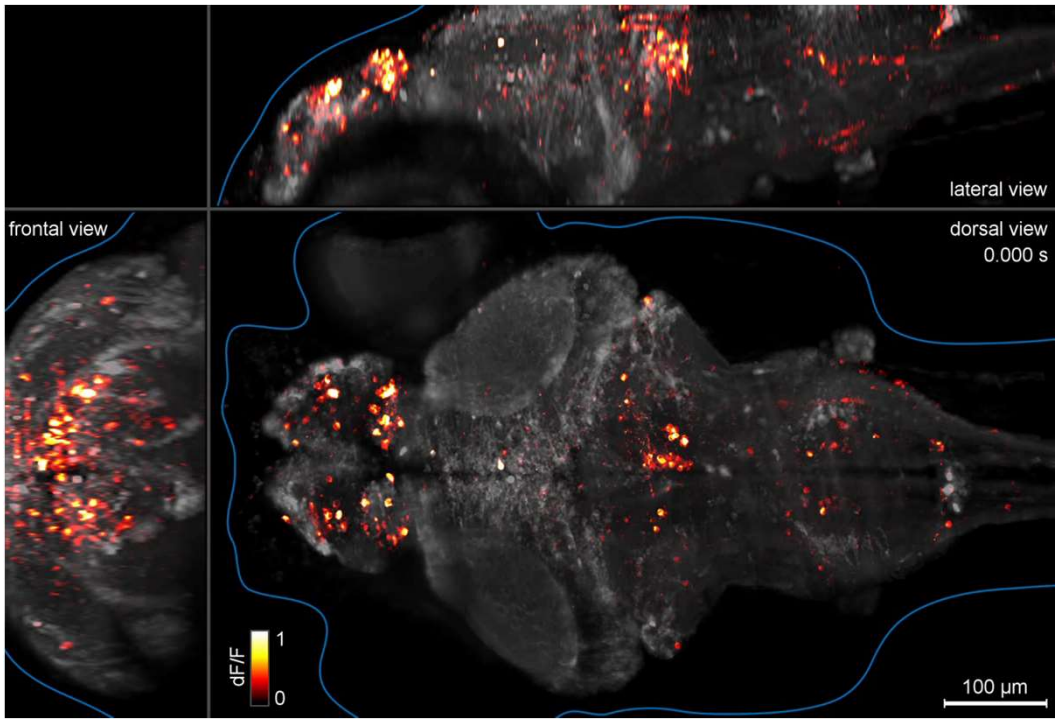


Tomer, R. et al. Nature Methods 9, 755-763 (2012)

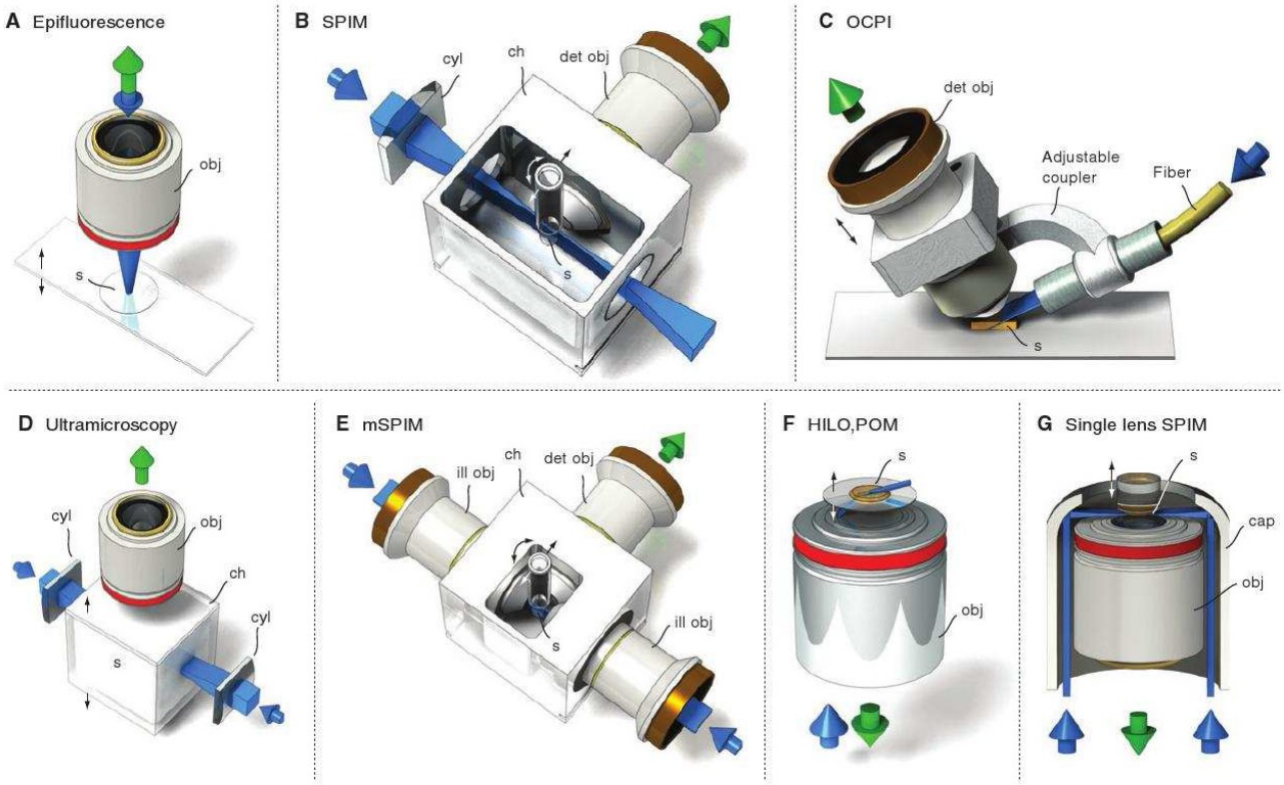
Light sheet microscope

Whole-brain functional imaging at cellular resolution using light-sheet microscopy

Misha B Ahrens[1], Michael B Orger[2], Drew N Robson[3], Jennifer M Li[3] & Philipp J Keller[1]



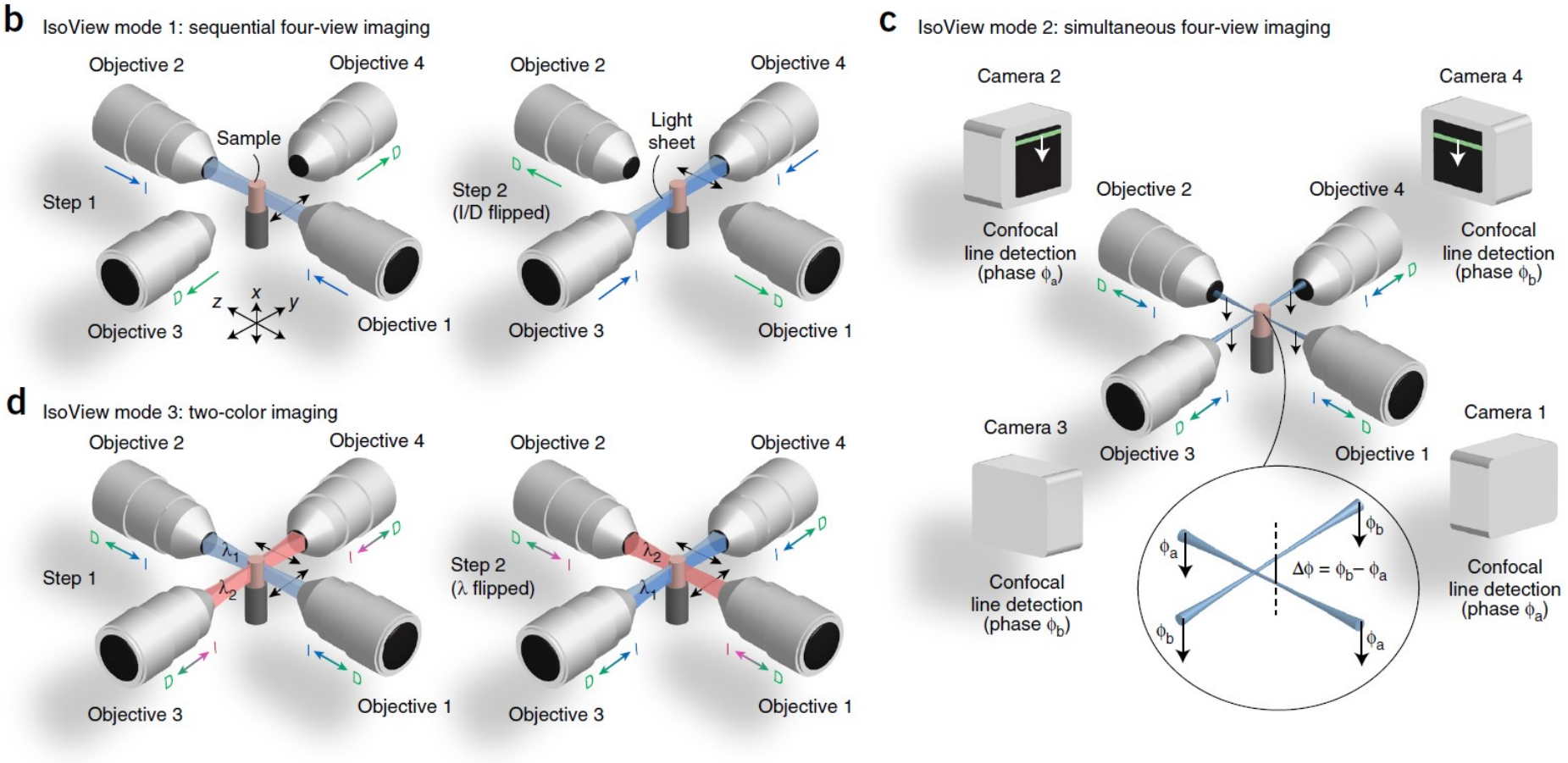
Light sheet microscope

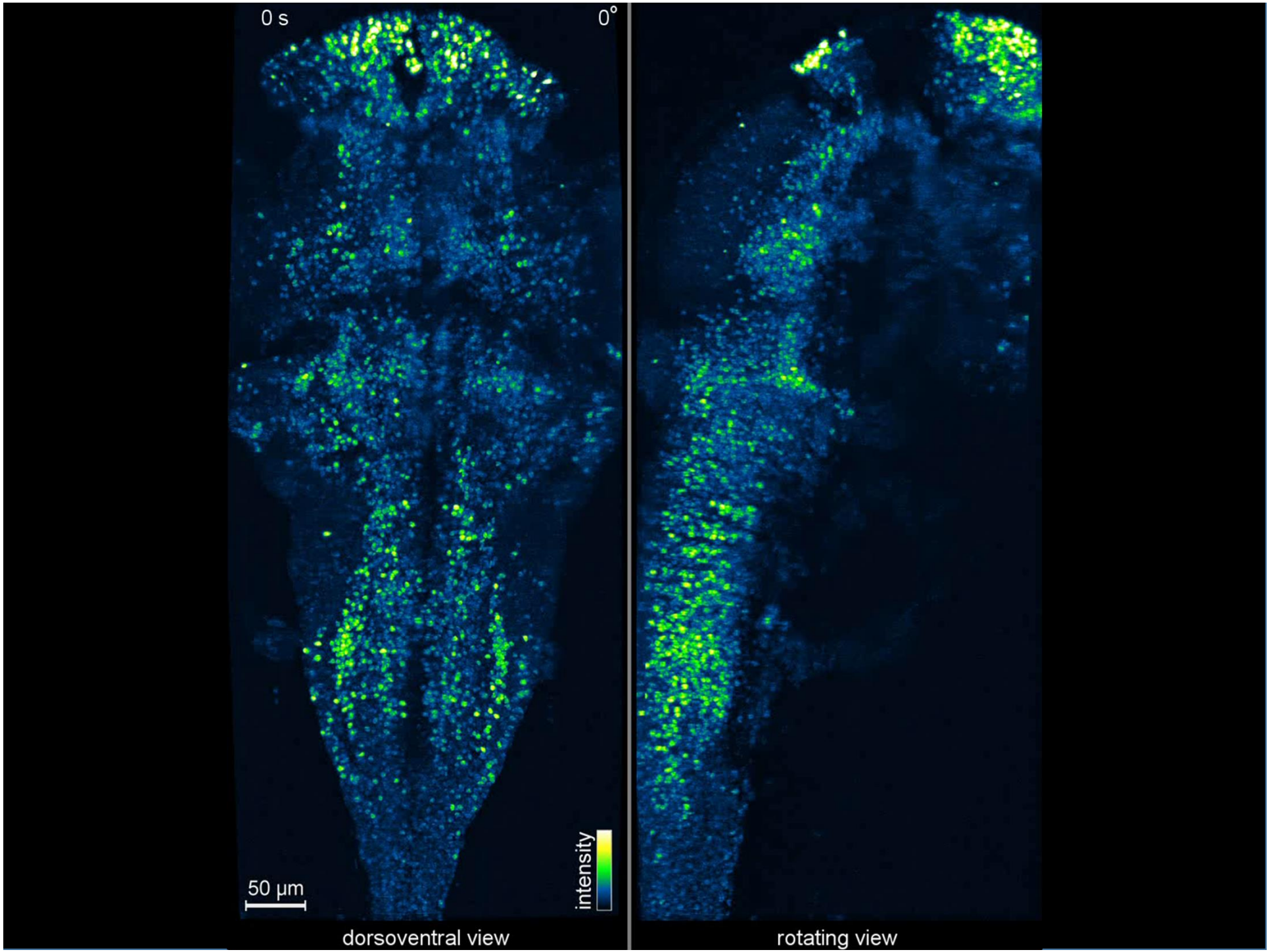


Light sheet microscope

Whole-animal functional and developmental imaging with isotropic spatial resolution

Raghav K Chhetri, Fernando Amat, Yinan Wan, Burkhard Höckendorf, William C Lemon & Philipp J Keller

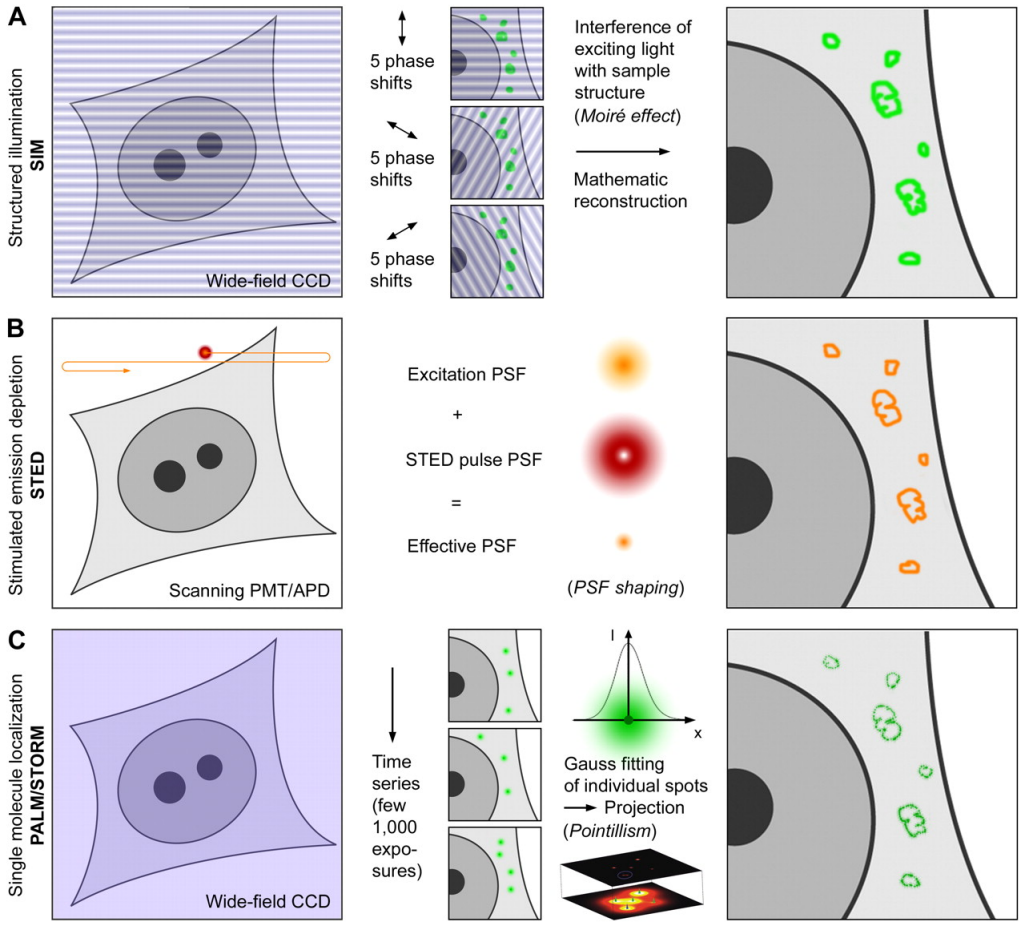



0 s
0°
50 µm
intensity
dorsoventral view
rotating view

Super resolution microscope

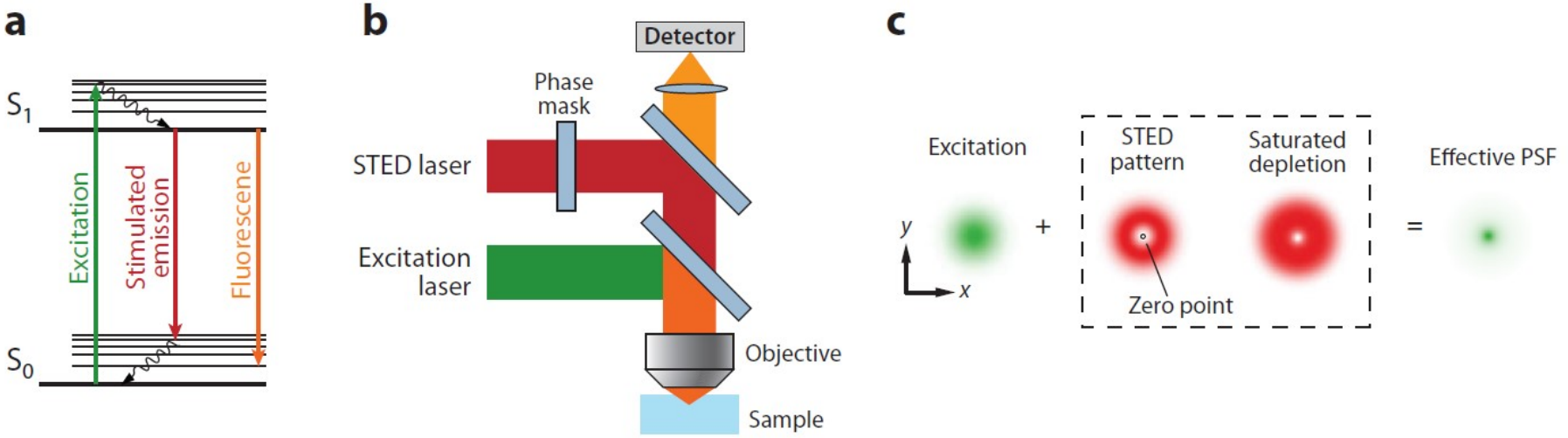
- STED Microscope
- Localization Microscope: PALM/STORM
- Structured Illumination Microscope (SIM)

Super-resolution microscope

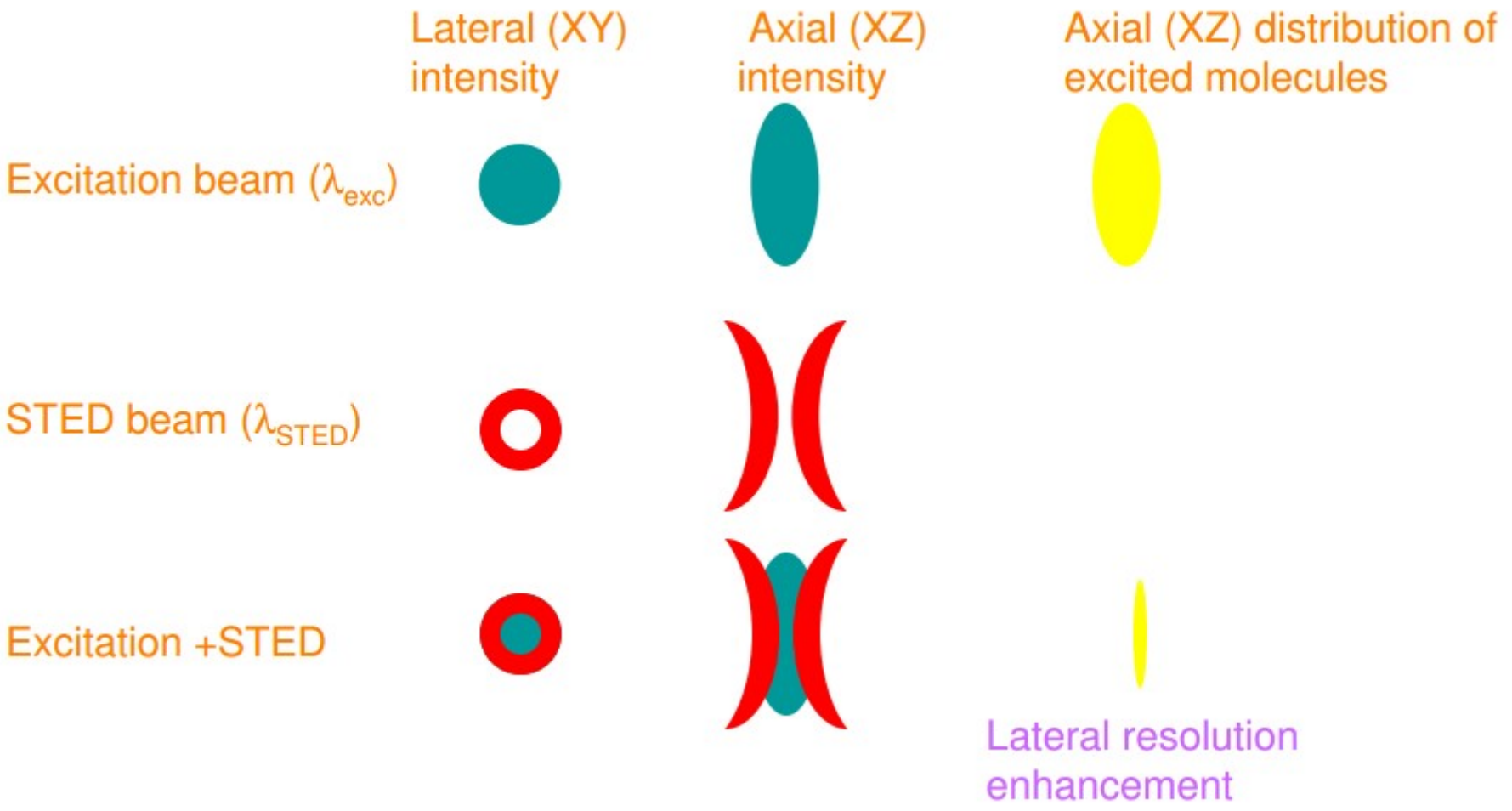


Lothar Schermelleh et al. J Cell Biol 2010;190:165-175

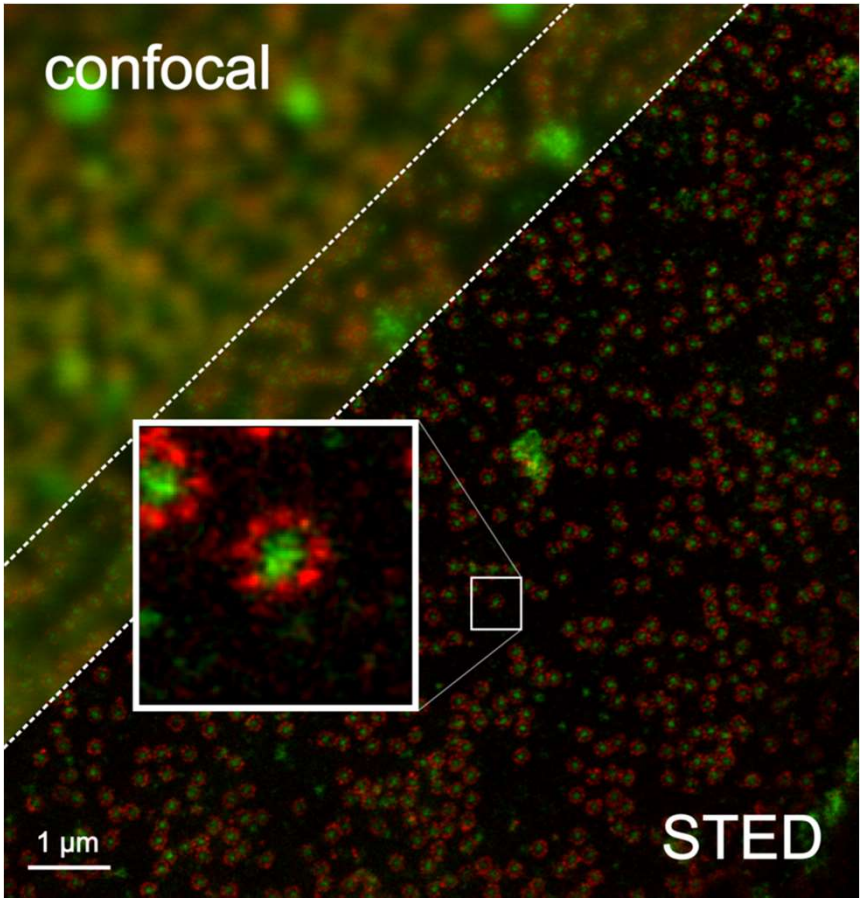
STimulated Emission Depletion (STED) Microscopy



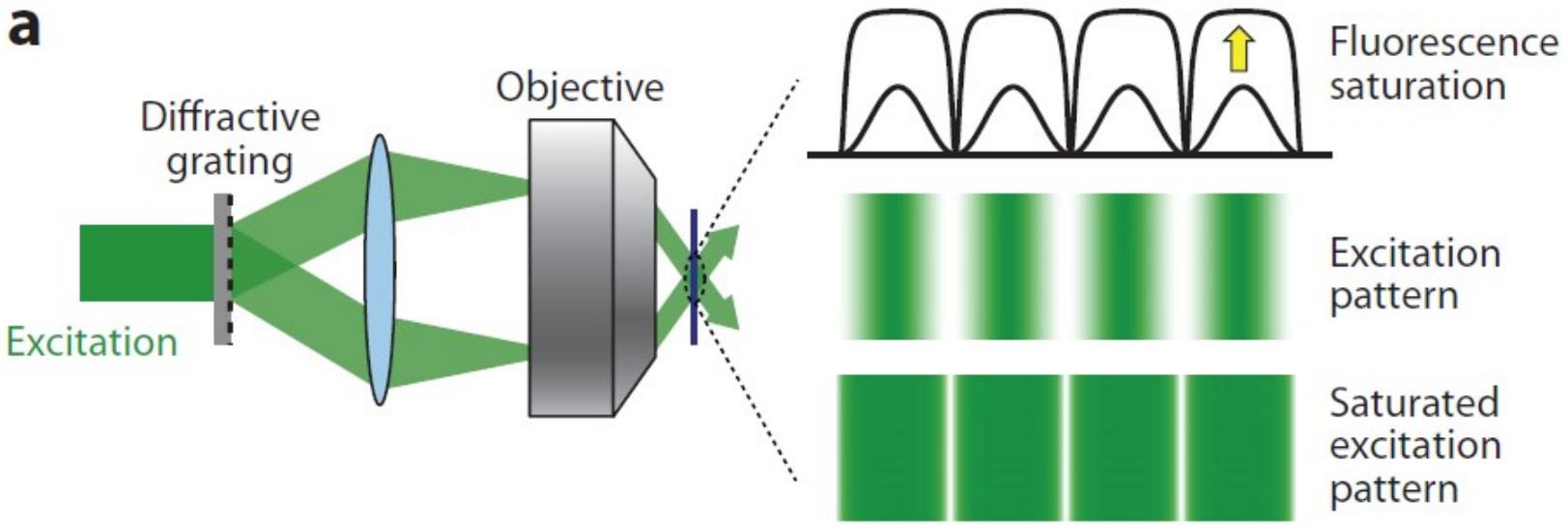
STimulated Emission Depletion (STED) Microscopy



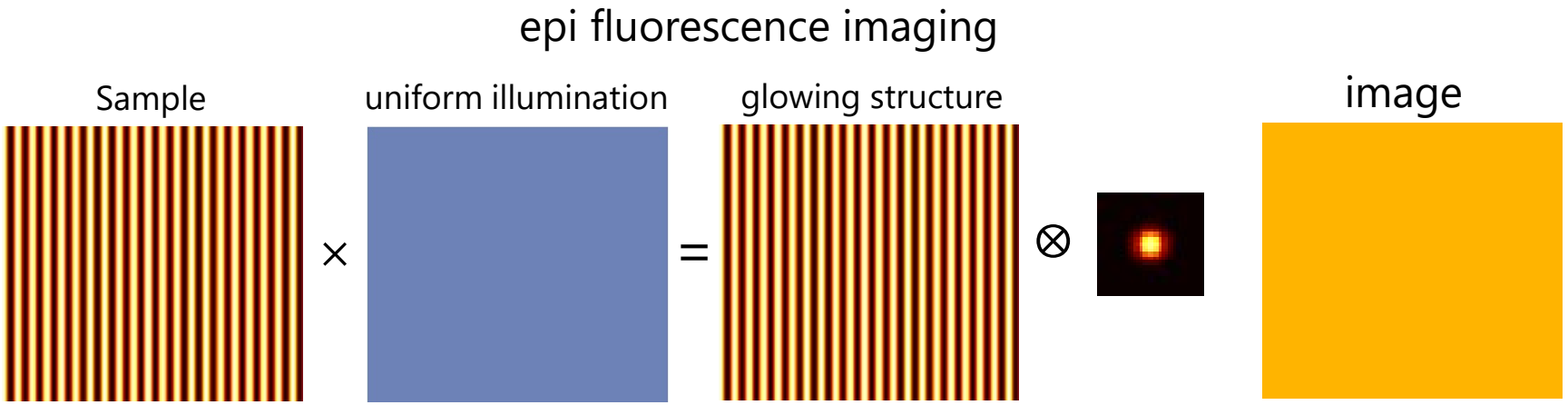
STimulated Emission Depletion (STED) Microscopy



Structured Illumination Microscope (SIM)



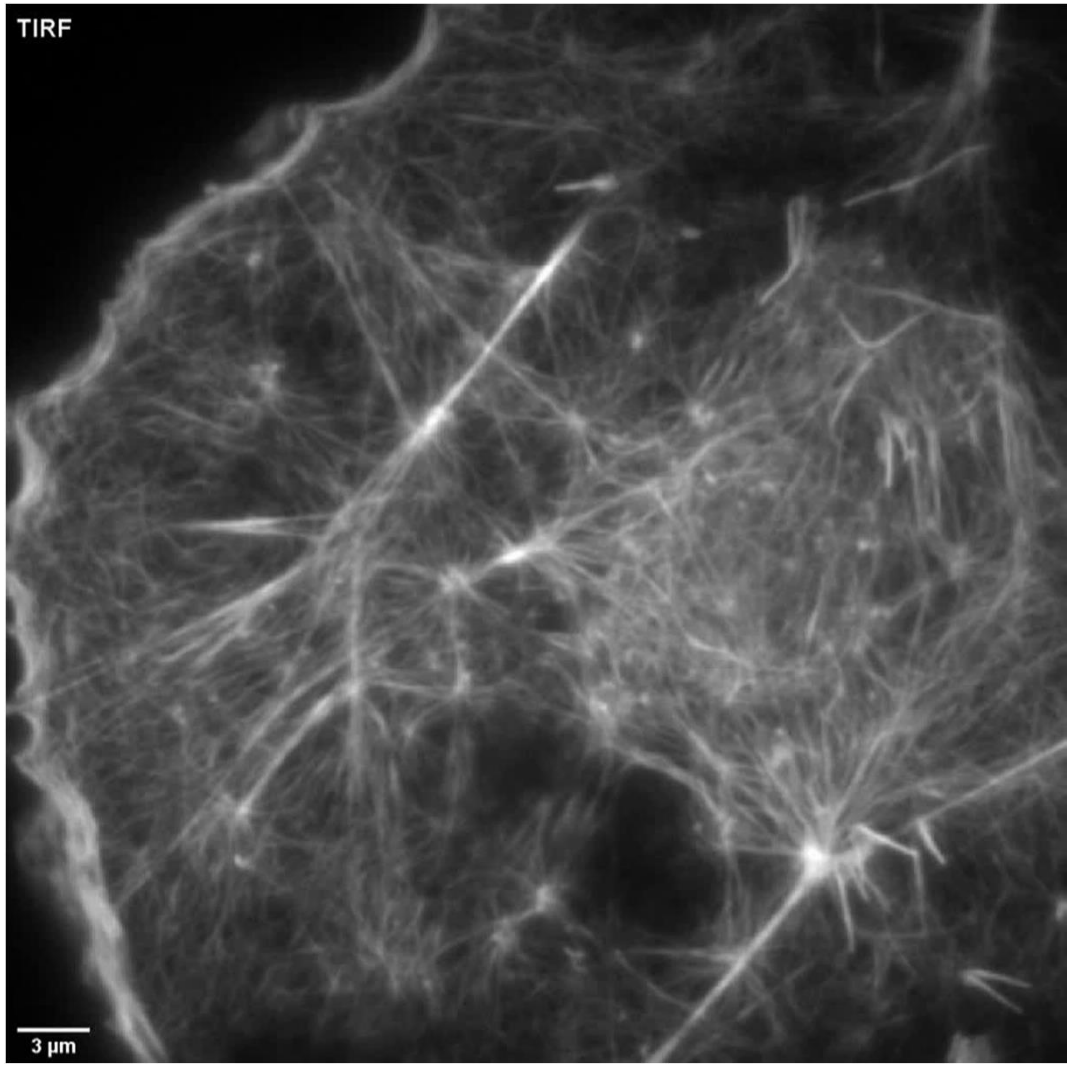
Structured Illumination Microscope (SIM)



Structured Illumination Microscope (SIM)

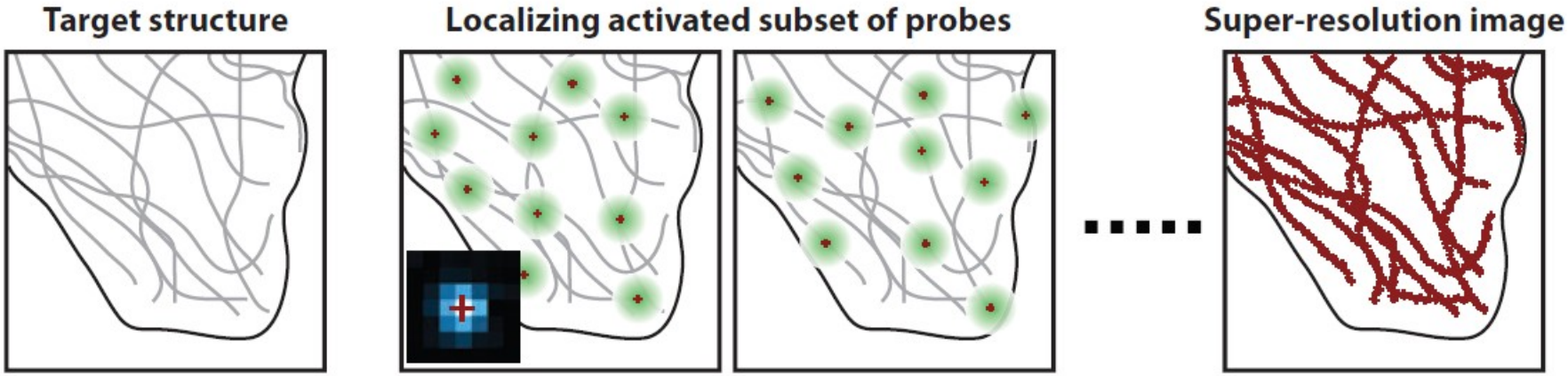
Live imaging demonstrated

	3D resolution		2D x-y resolution
	x-y	z	
Wide field	200 nm	600 nm	170 nm
Linear SIM	100 nm	300 nm	90 nm
1st nonlinear SIM	-	-	60 nm
2nd nonlinear SIM	-	-	45 nm



D. Li et a. *Science*, 2015; 349 (6251)

Localization microscope: PALM/STORM



Resolution is limited by labeling density and photon budget of each fluorophore

~10,000 images are required to reconstruct one image

Localization microscope: PALM/STORM

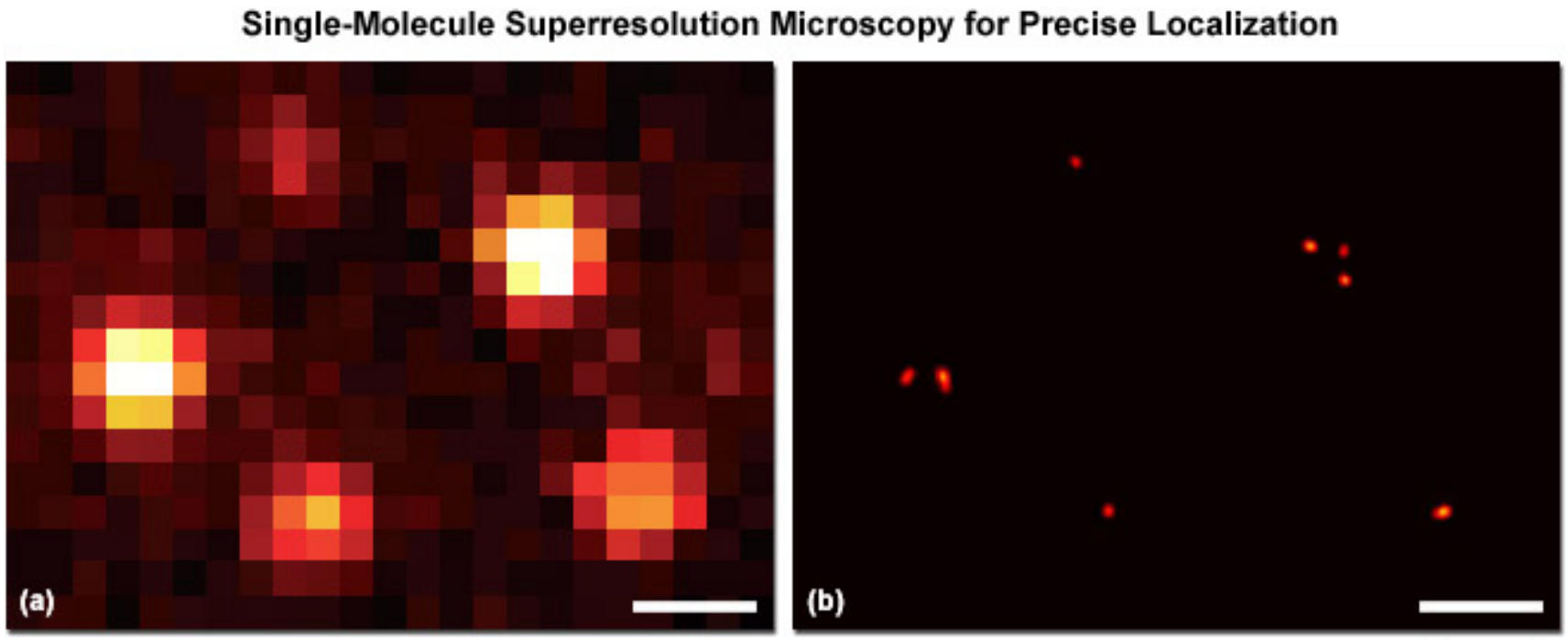
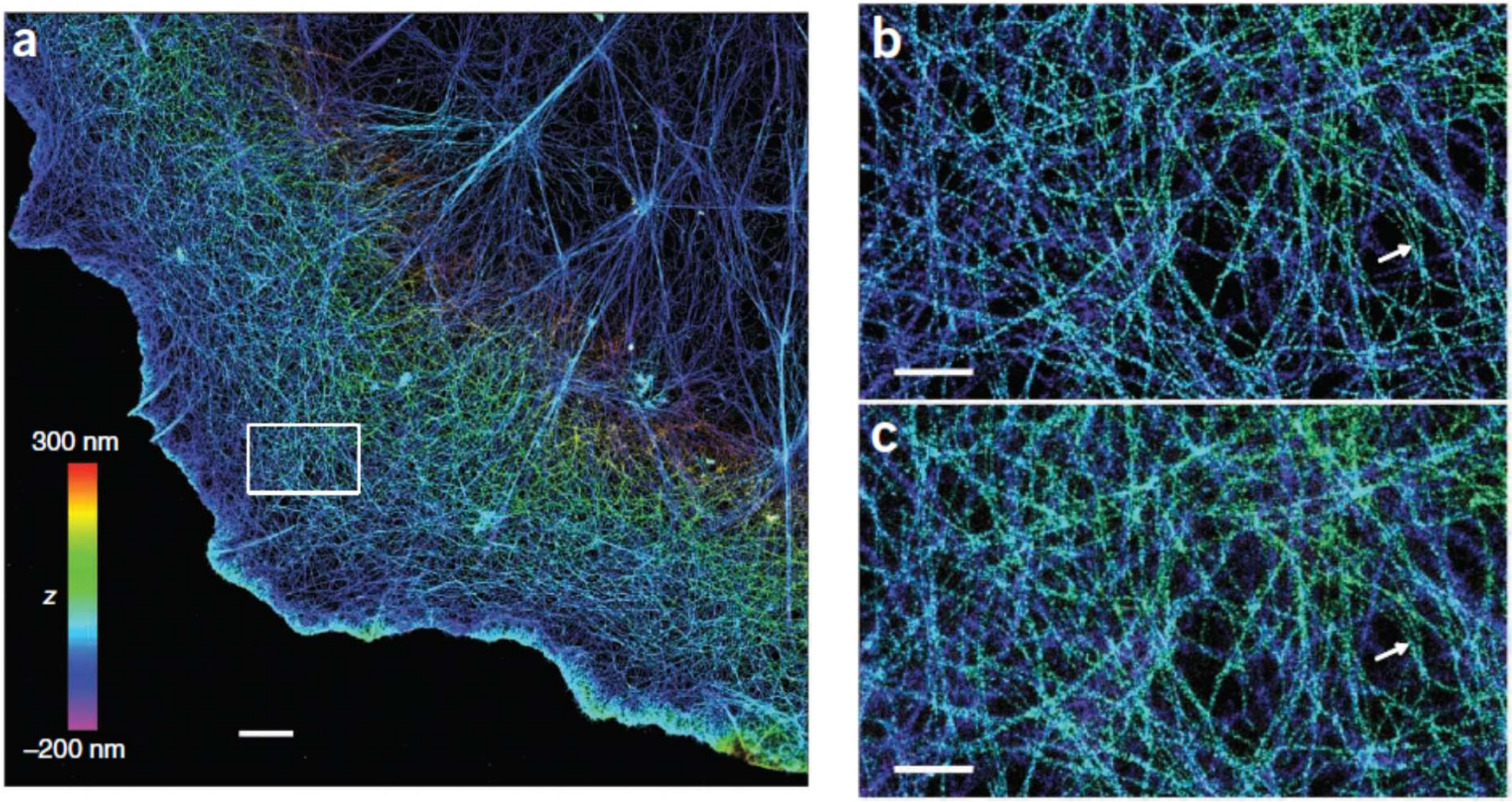


Figure 1

3D STORM



Ke Xu et al. Nature Methods 185-188 (2012)

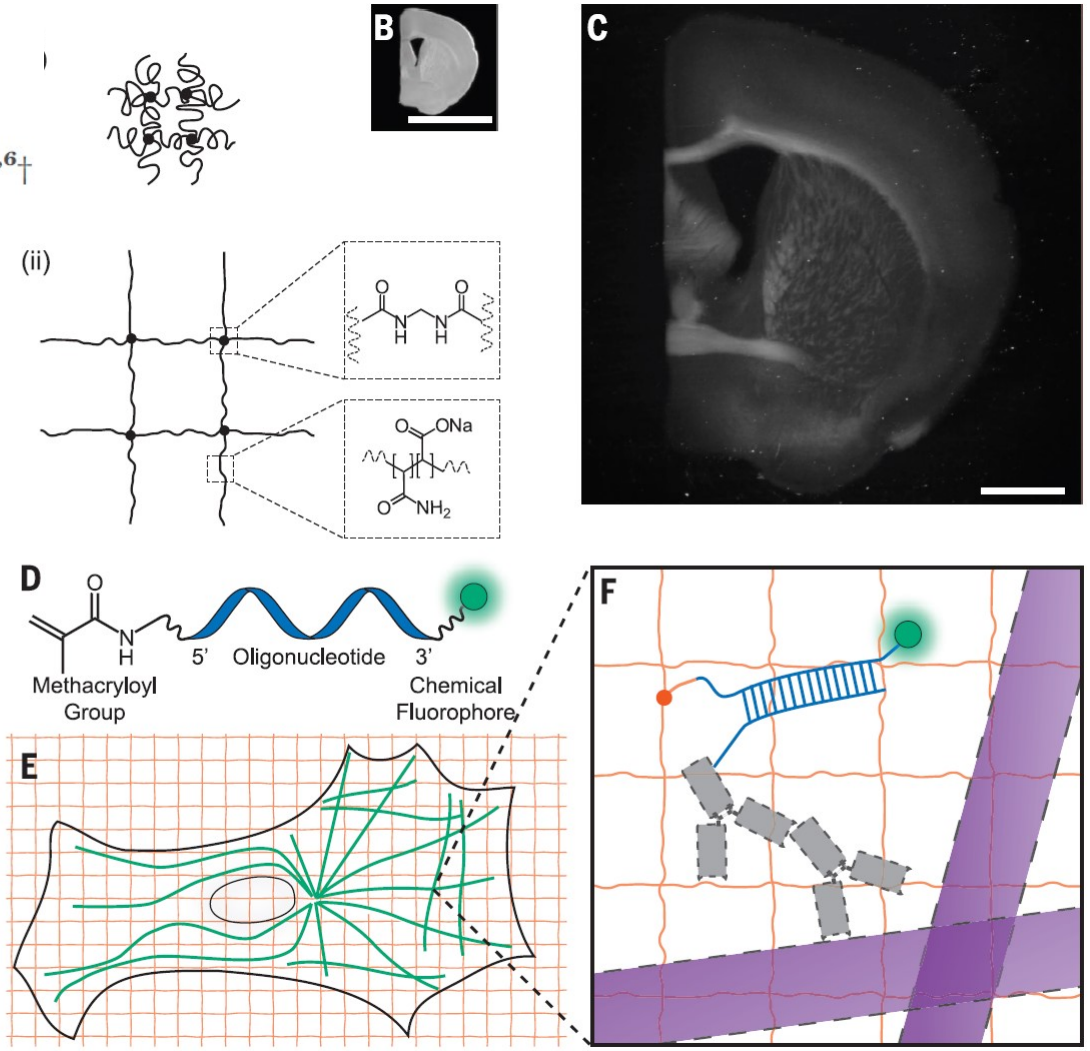
Expansion microscope

OPTICAL IMAGING

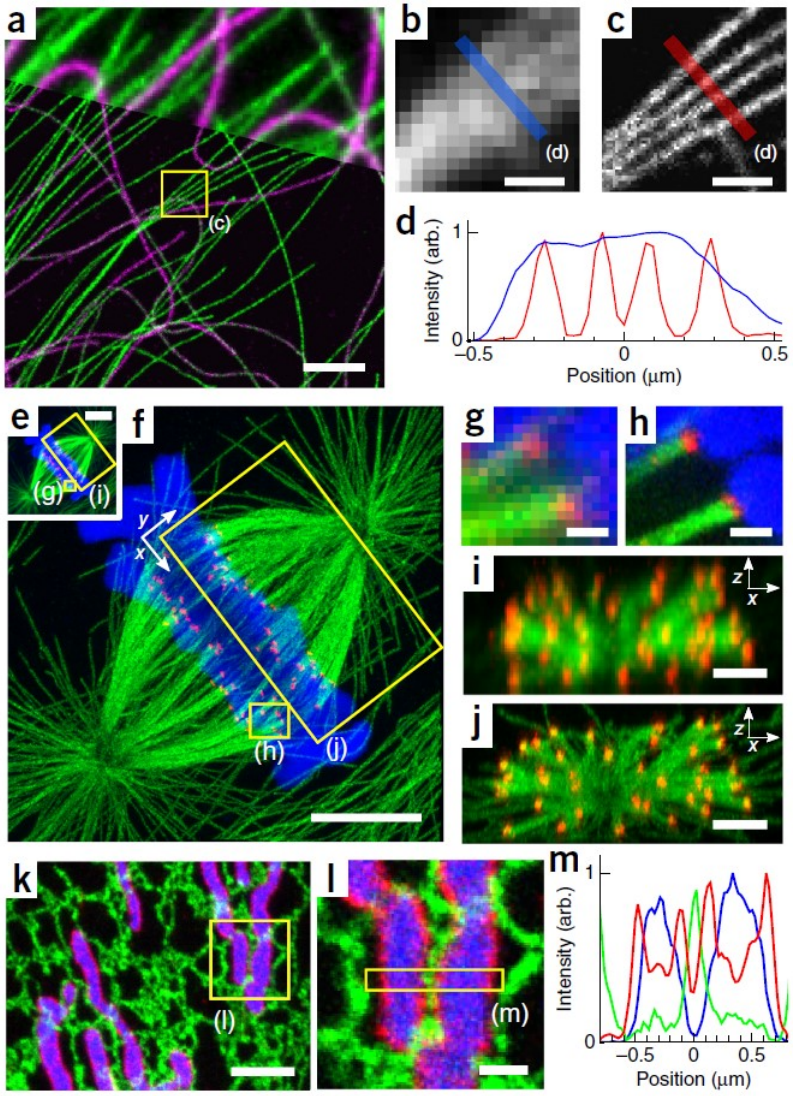
Expansion microscopy

Fei Chen,[1*] Paul W. Tillberg,[2*] Edward S. Boyden[1,3,4,5,6†]

illumination. (**D**) Schematic of label that can be anchored to the gel at site of a biomolecule. (**E**) Schematic of microtubules (green) and polymer network (orange). (**F**) The label of (D), hybridized to the oligo-bearing secondary antibody top (top gray shape) bound via the primary (bottom gray shape) to microtubules (purple), is incorporated into the gel (orange lines) via the methacryloyl group (orange dot) and remains after proteolysis (dotted lines). Scale bars, (B) and (C) 5 mm. Schematics are not to scale.



Expansion microscope



Expansion microscopy with conventional antibodies and fluorescent proteins

Tyler J Chozinski[1,4], Aaron R Halpern[1,4], Haruhisa Okawa[2], Hyeon-Jin Kim[1], Grant J Tremel[1], Rachel O L Wong[2] & Joshua C Vaughan[1,3]

nature biotechnology

Protein-retention expansion microscopy of cells and tissues labeled using standard fluorescent proteins and antibodies

Paul W Tillberg[1,2,10], Fei Chen[2,3,10], Kiryl D Piatkevich[2], Yongxin Zhao[2], Chih-Chieh (Jay) Yu[2,3], Brian P English[4], Linyi Gao[3], Anthony Martorell[5], Ho-Jun Suk[2,6], Fumiaki Yoshida[7,8], Ellen M DeGennaro[5,8], Douglas H Roossien[9], Guanyu Gong[3], Uthpala Seneviratne[3], Steven R Tannenbaum[3], Robert Desimone[5,8], Dawen Cai[9] & Edward S Boyden[2,3,5,8]

Nanoscale imaging of RNA with expansion microscopy

Fei Chen[1–3,10], Asmamaw T Wassie[1–3,10], Allison J Cote[4], Anubhav Sinha[5], Shahar Alon[2,3], Shoh Asano[2,3], Evan R Daugharthy[6,7], Jae-Byum Chang[2,3], Adam Marblestone[2,3], George M Church[6,8], Arjun Raj[4] & Edward S Boyden[1–3,9]