UNIVERSITÉ DE FRIBOURG
UNIVERSITÄT FREIBURG

University of Fribourg, Switzerland March 14, 2024

Advanced Fluorescence Imaging

Prof. Dr. Fernando D. Stefani

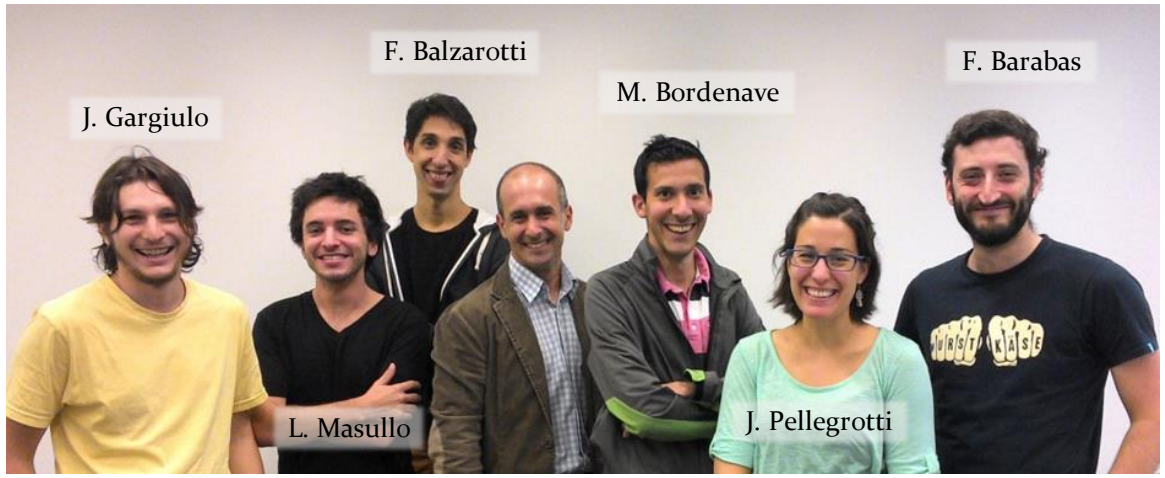
CIBION
CONICET

https://stefani-lab.ar/

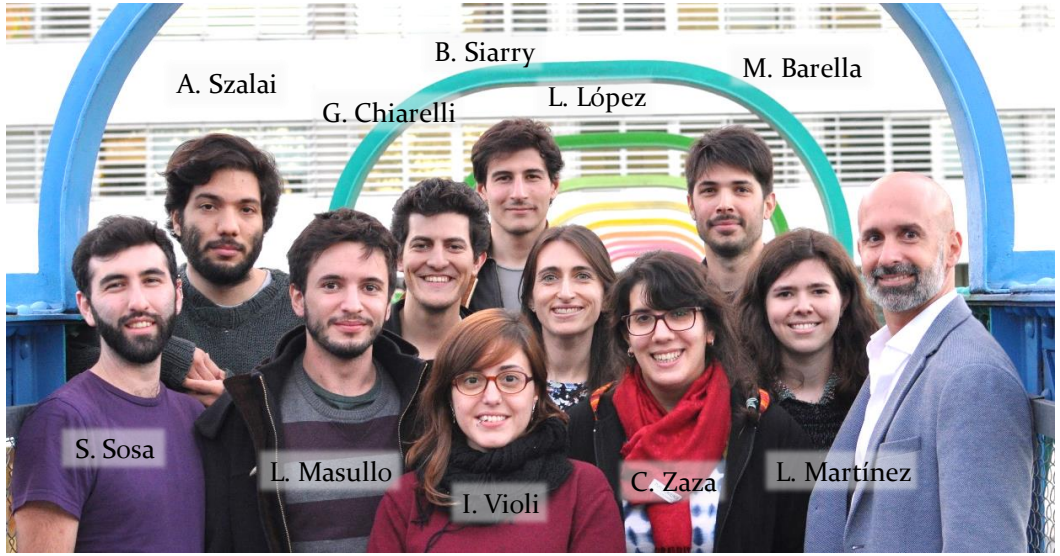
https://github.com/Stefani-Lab


Center for Bionanoscience Research
(CIBION)
Buenos Aires, Argentina

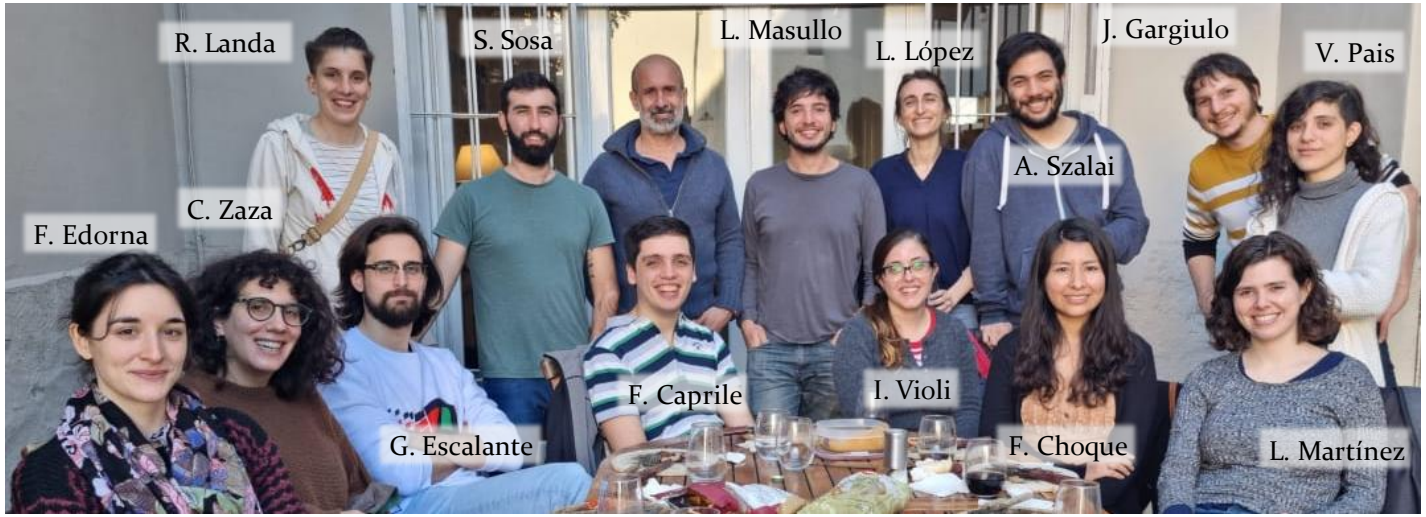
2015



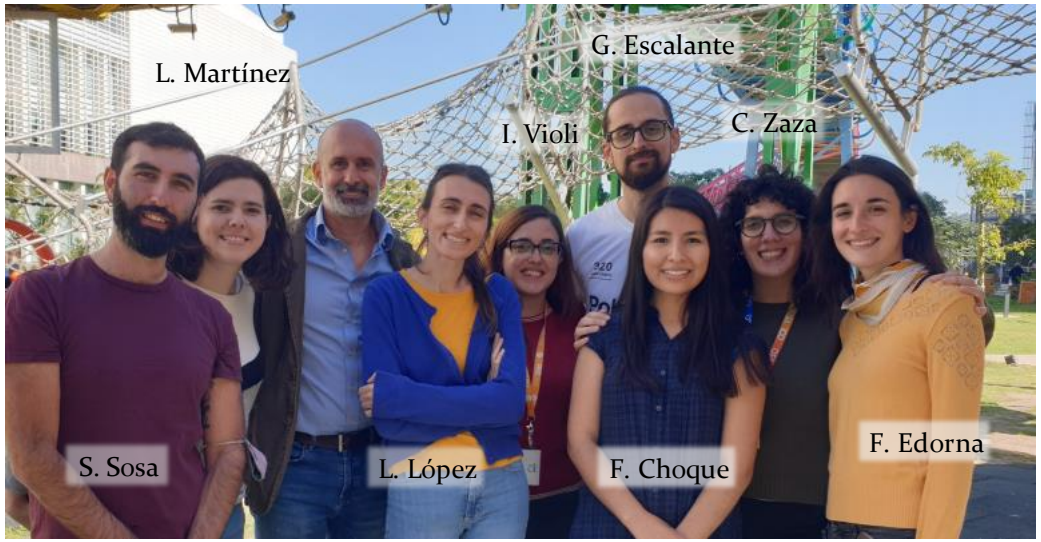
2018



2021



2022



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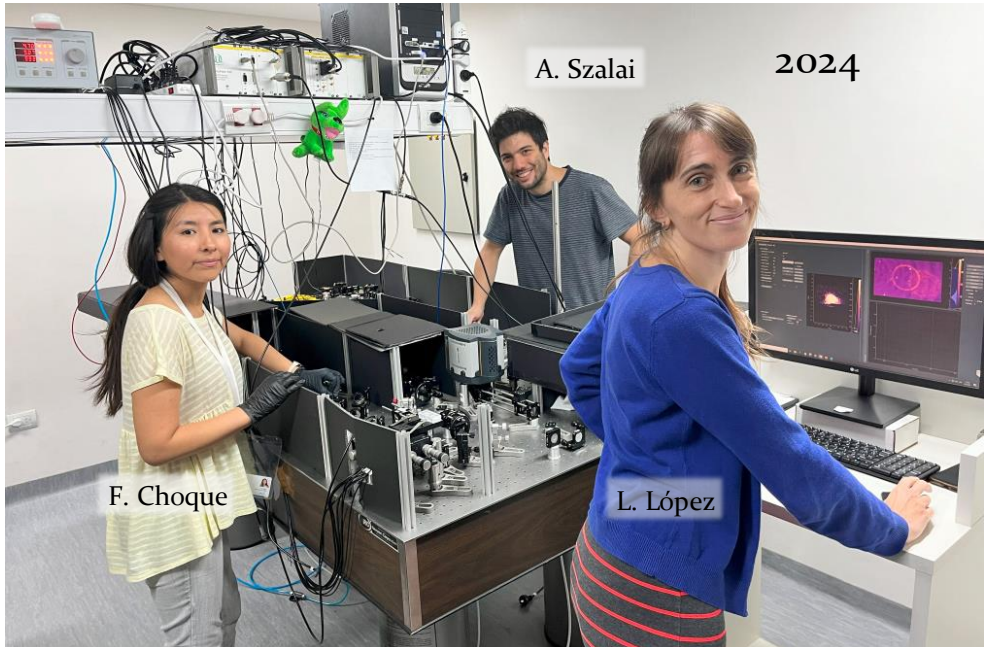
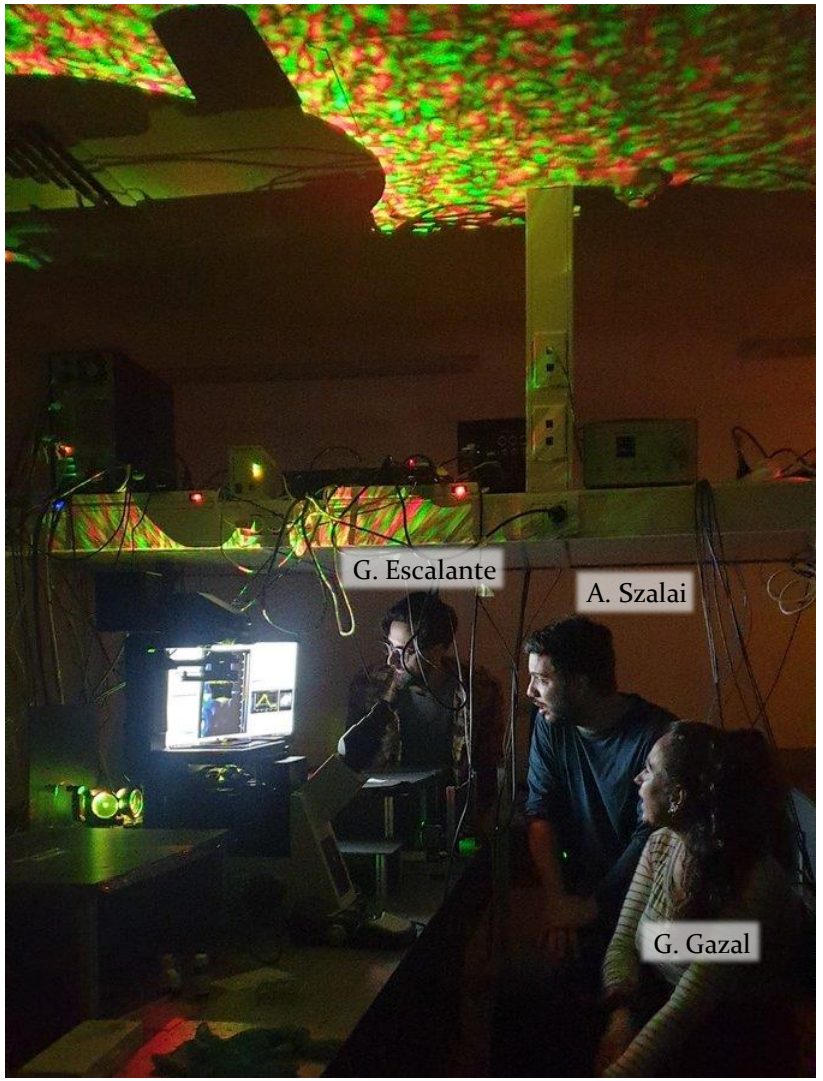
2023 / 2024





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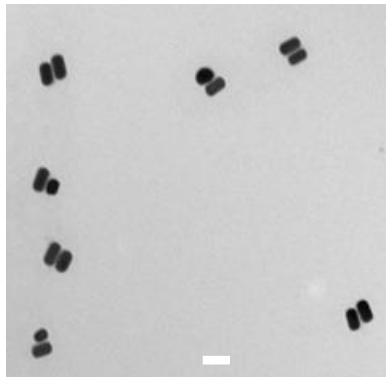
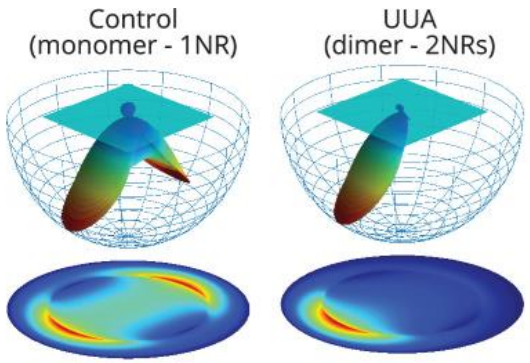
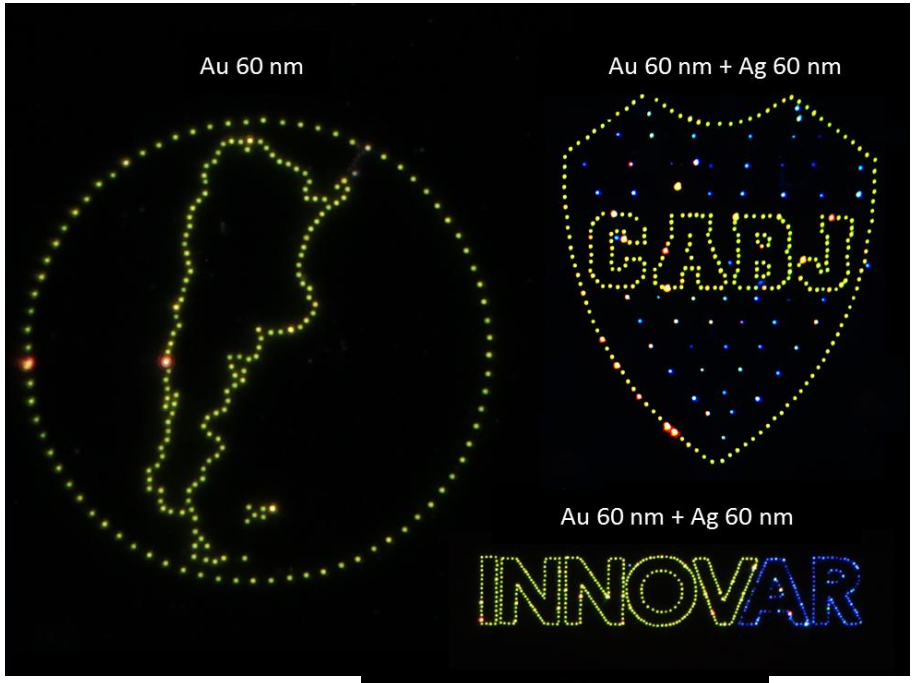
DNA nanophotonics

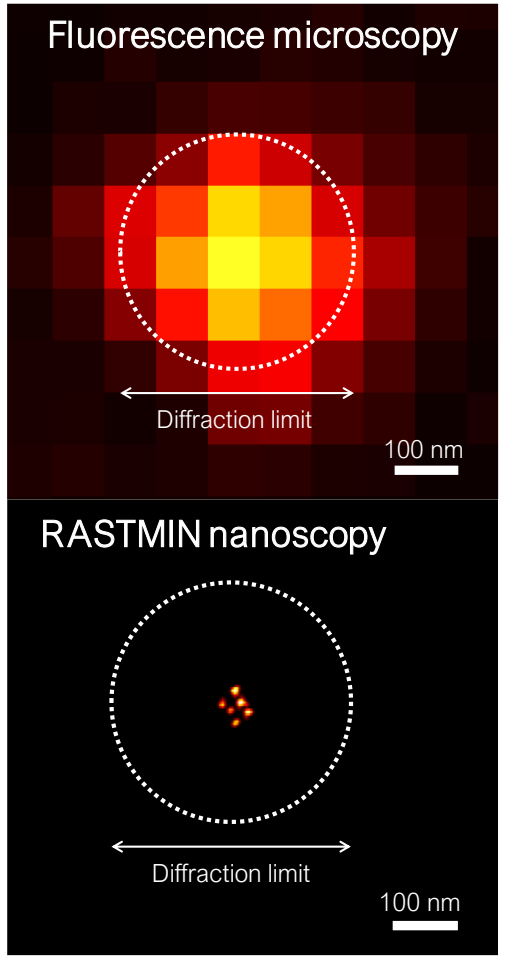




Optical manipulation of NPs



Superresolution microscopy



Acknowledgements

MPI-bpc Göttingen
Stefan W. Hell
Tom Jovin

INIMEC Córdoba
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Nicolás Unsain
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FIL Buenos Aires
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Monash Uni Melbourne
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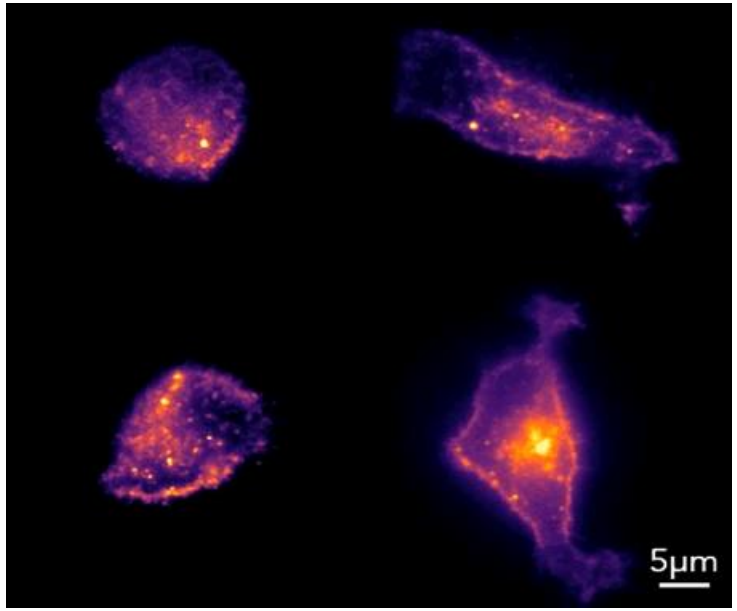
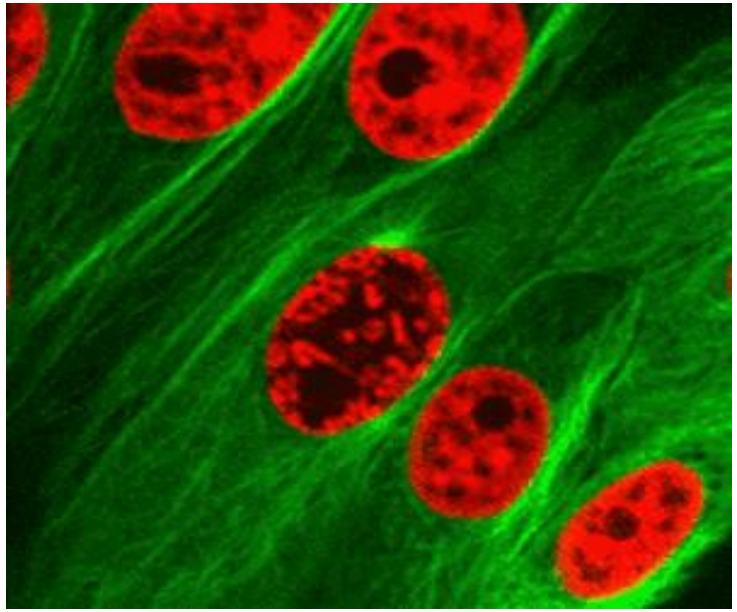
Fluorescence Microscopy

Fluorescence Microscopy

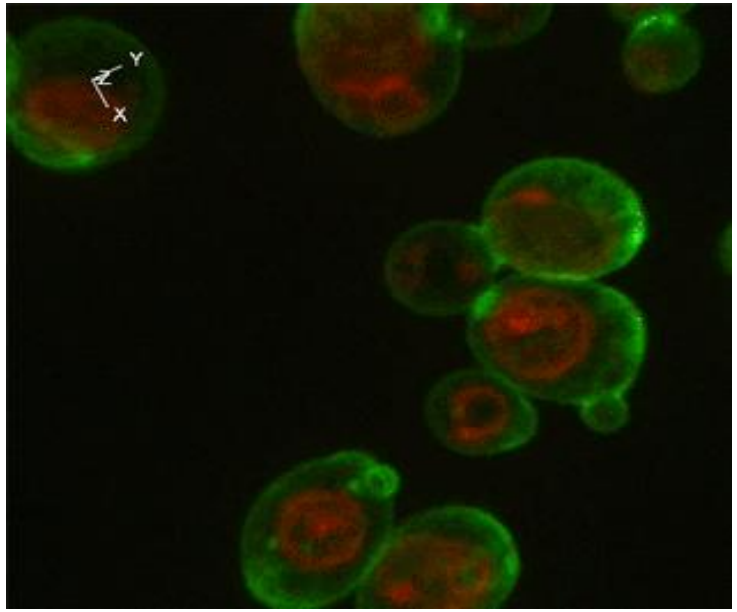
High specificity and sensitivity



Low invasiveness

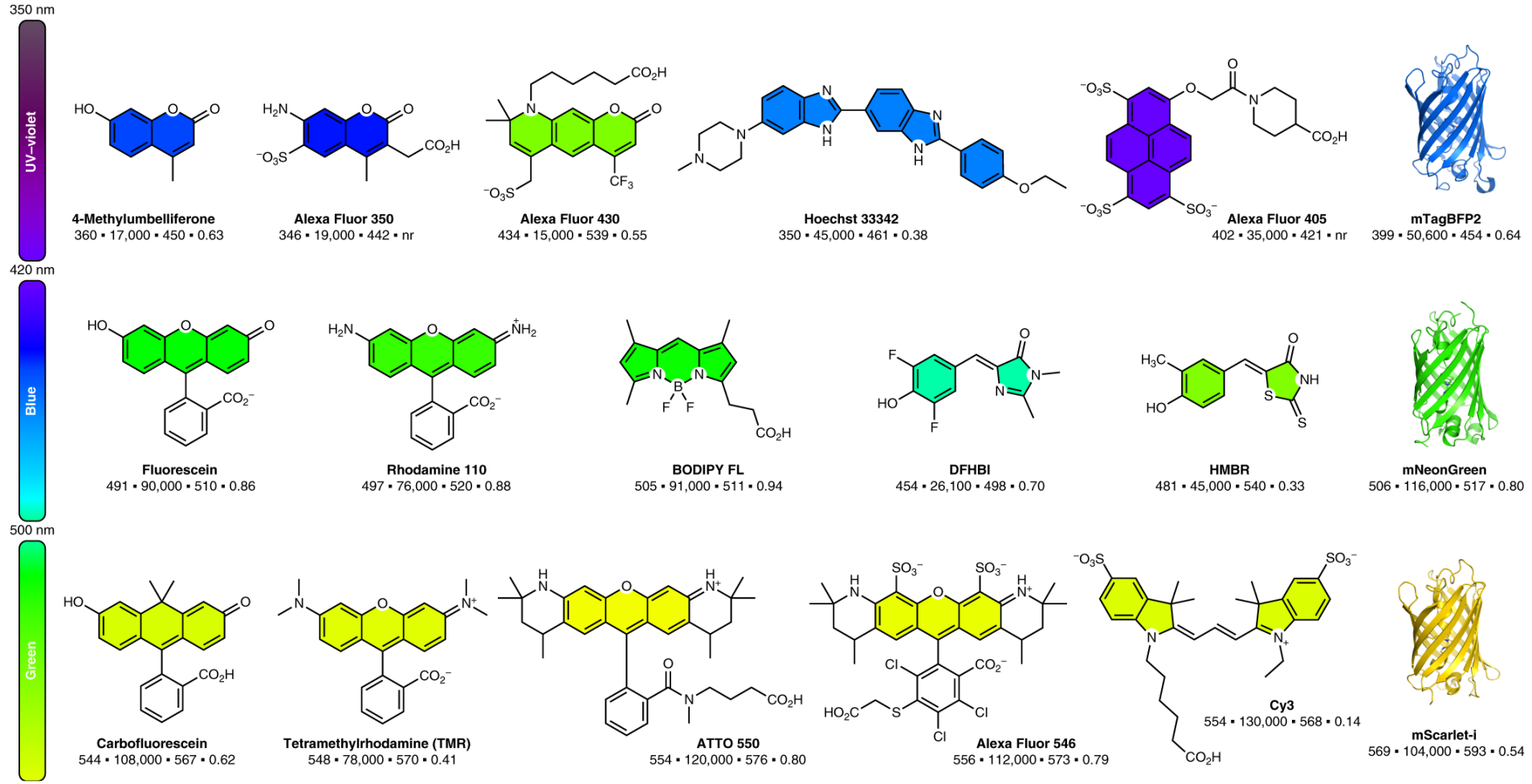


3D imaging



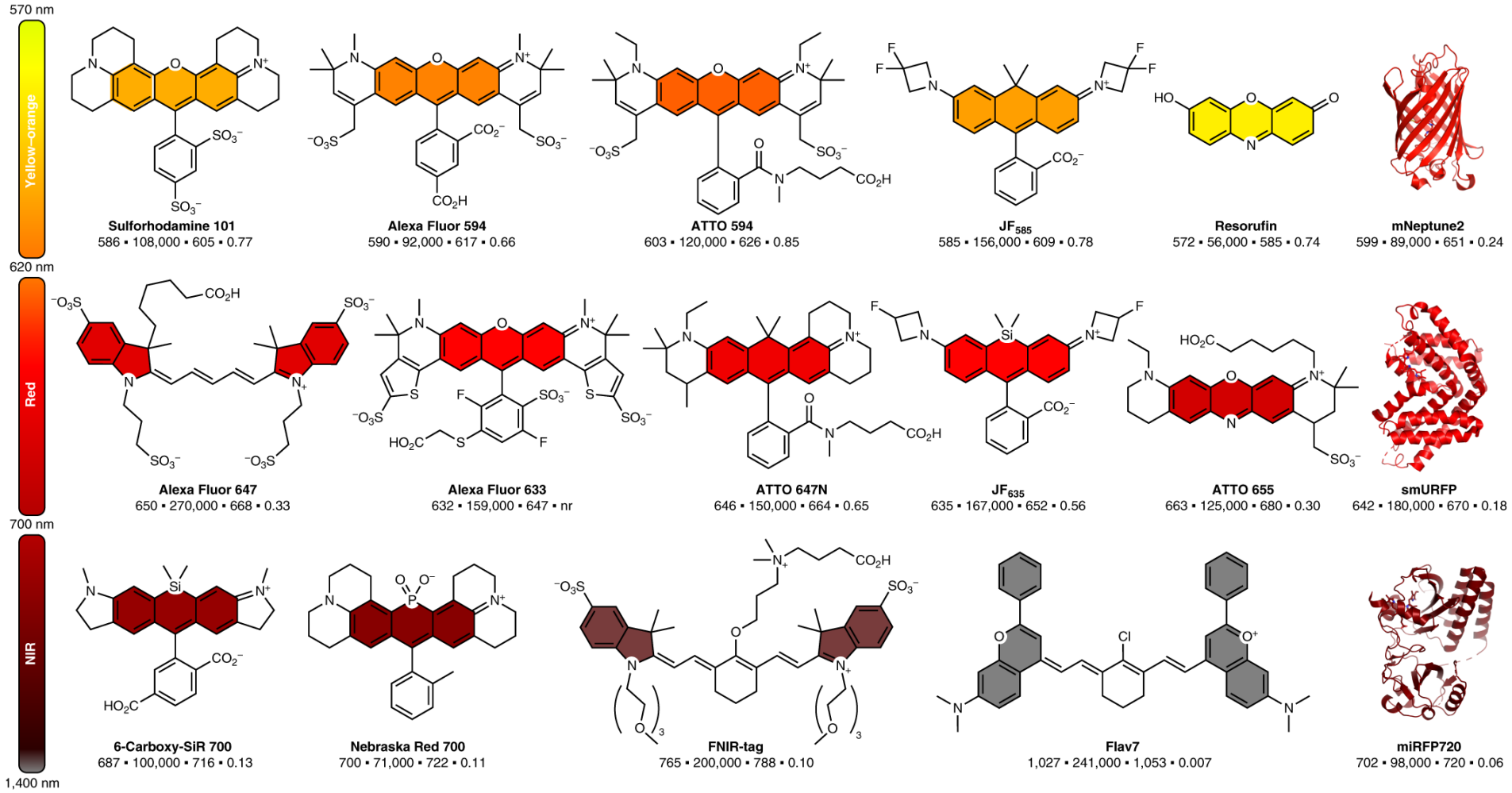
1. **Fluorescent markers**: molecules (fluorophores), fluorescent proteins, quantum dots, nanoparticles,…
2. **Labeling strategy**: antibodies, nanobodies, transfection, drug-like fluorescent molecules, etc…
3. **Imaging method**: wide-field, scanning (1-photon, 2-photon), light-sheet
4. **Signal:** intensity, lifetime, FRET, polarization, …

Fluorescent Molecules (Fluorophores)



J. B. Grimm & L. Davis, *Nature Methods* 19 (2022) 149–158

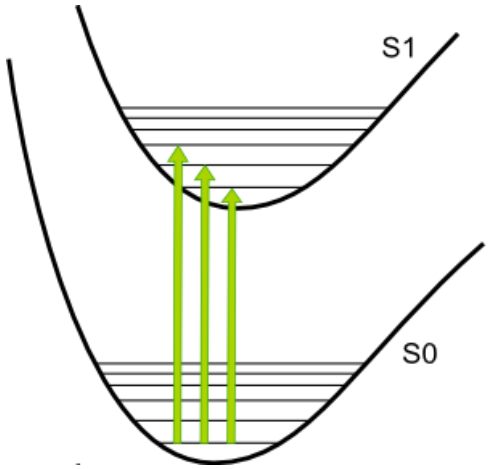
Fluorescent Molecules (Fluorophores)

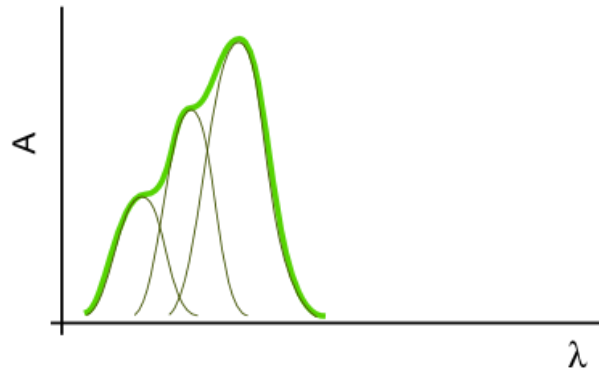


J. B. Grimm & L. Davis, *Nature Methods* 19 (2022) 149–158

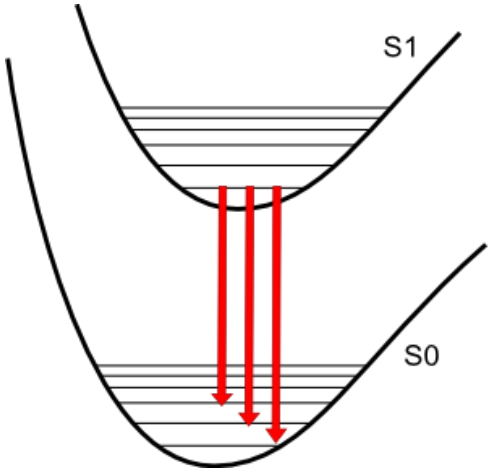
Fluorescent Molecules (Fluorophores)

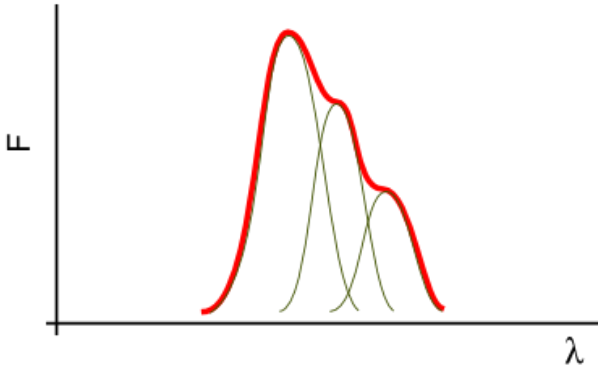
Absorption



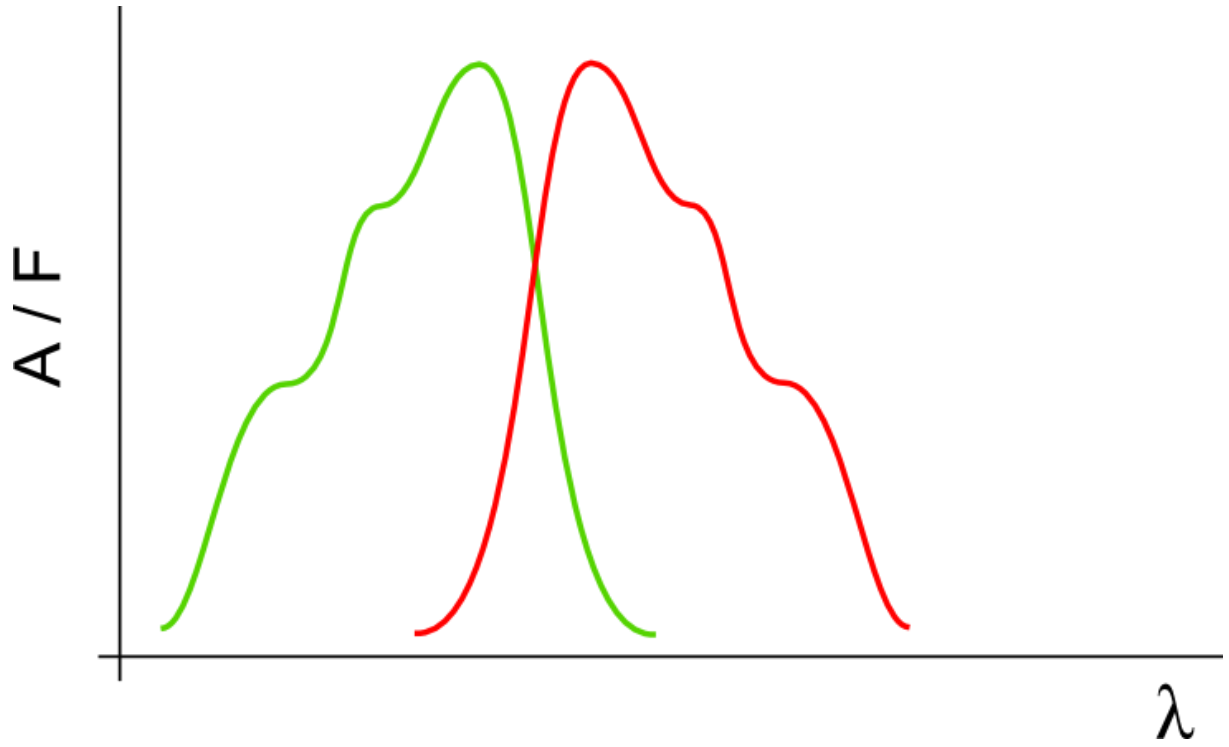


Emission

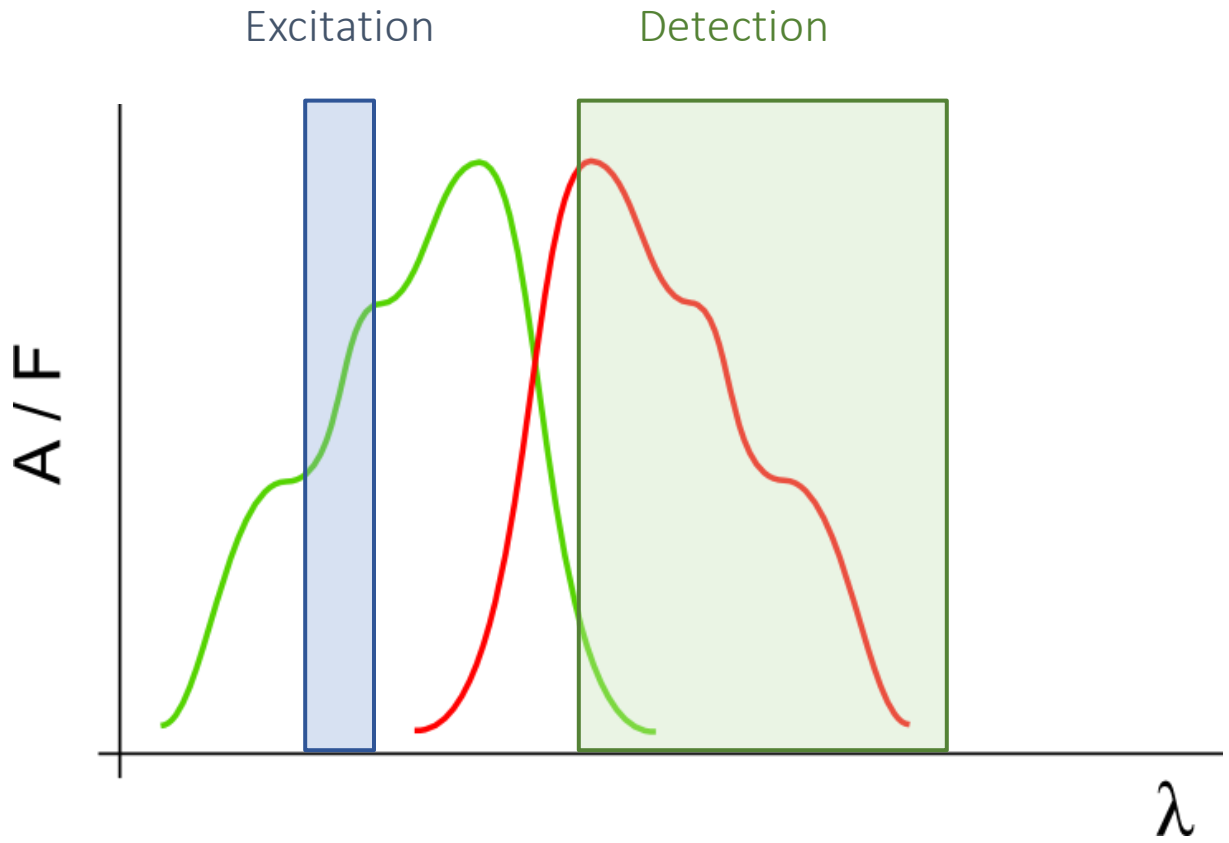


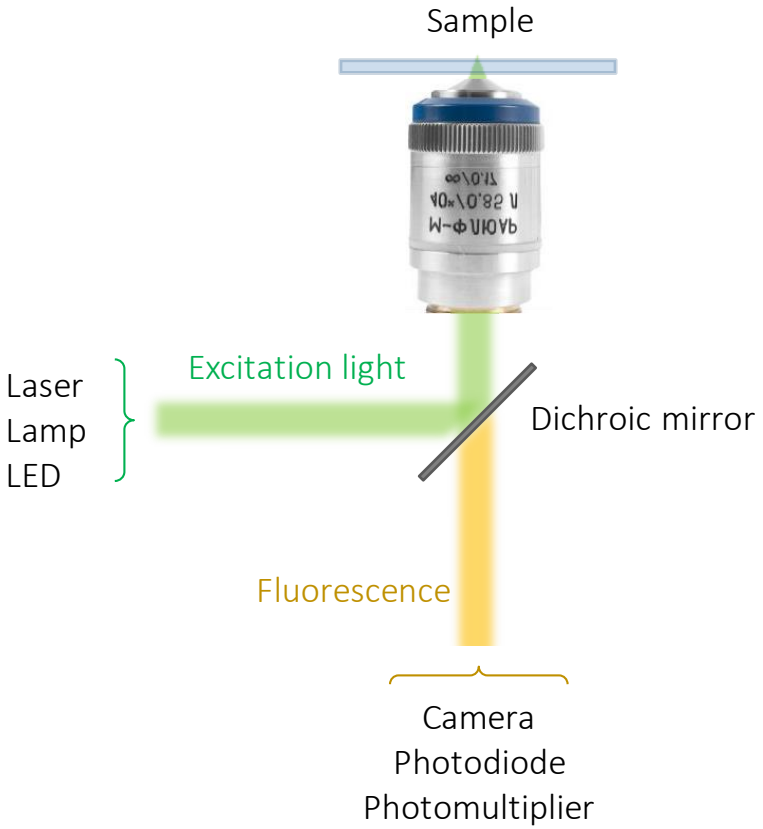


Fluorophores

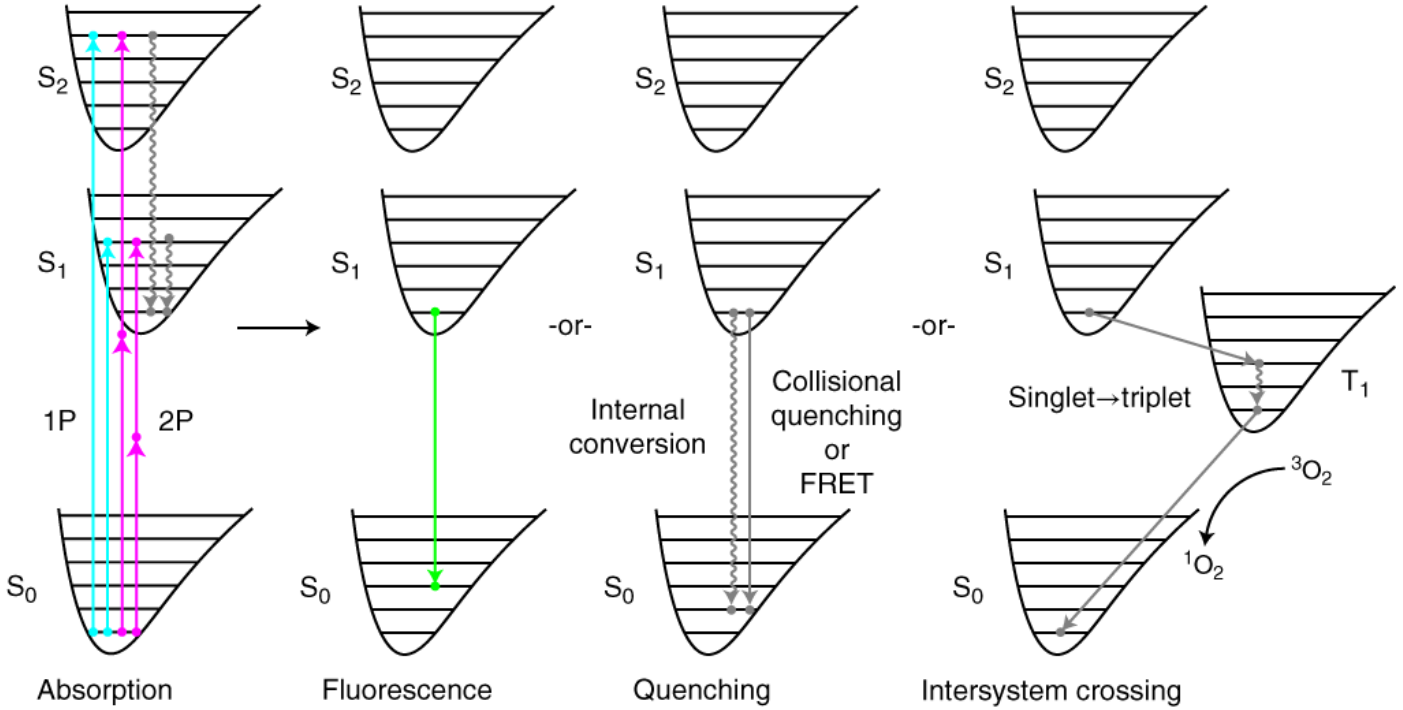


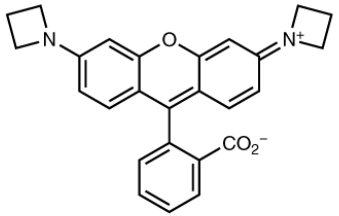
Fluorescence Microscopy



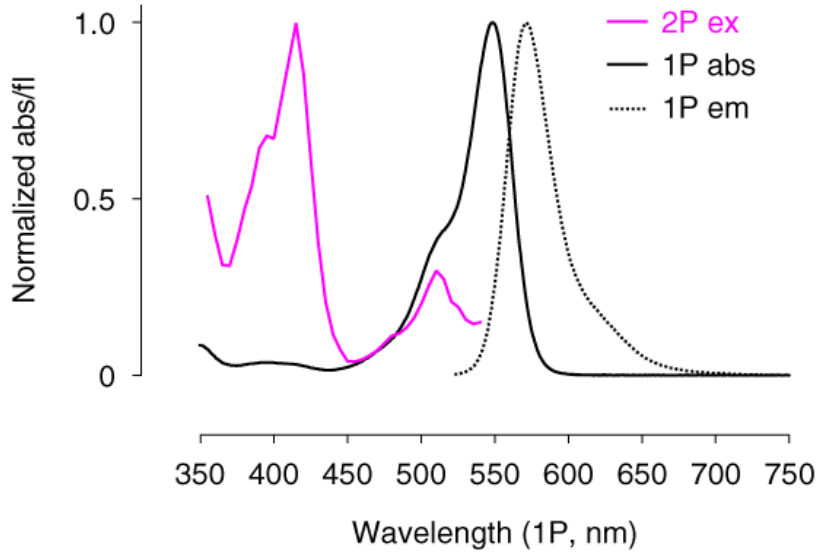


Fluorophores



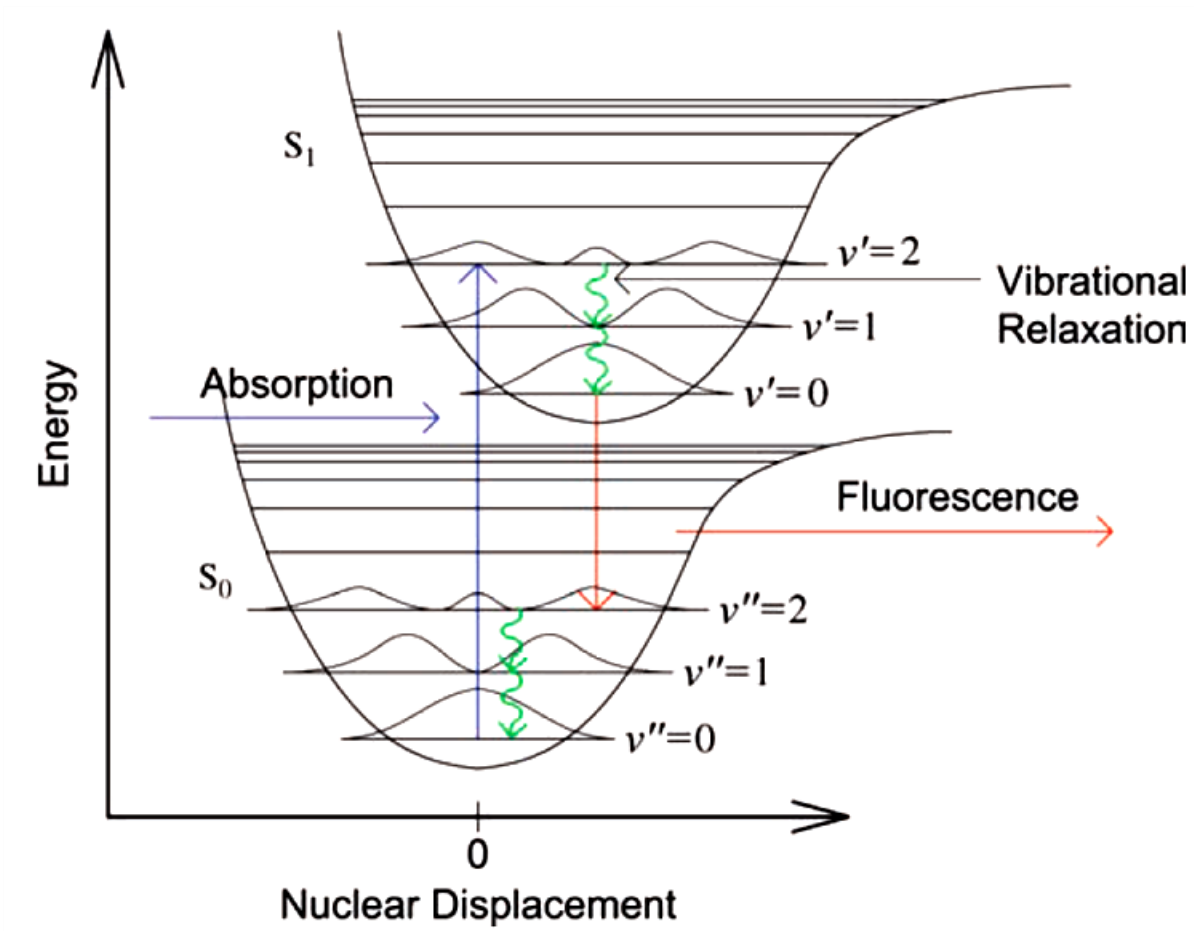


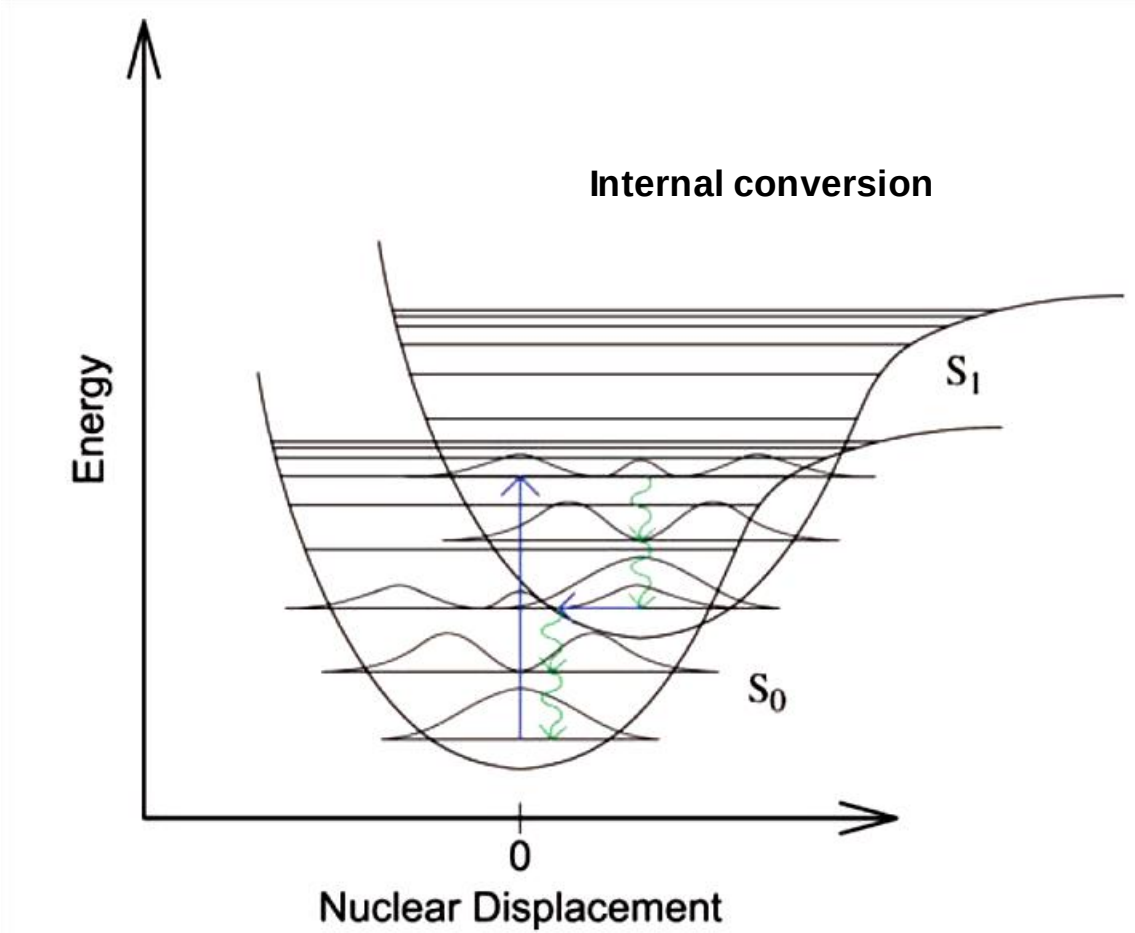
JF_{549}
549 ▪ 101,000 ▪ 571 ▪ 0.88



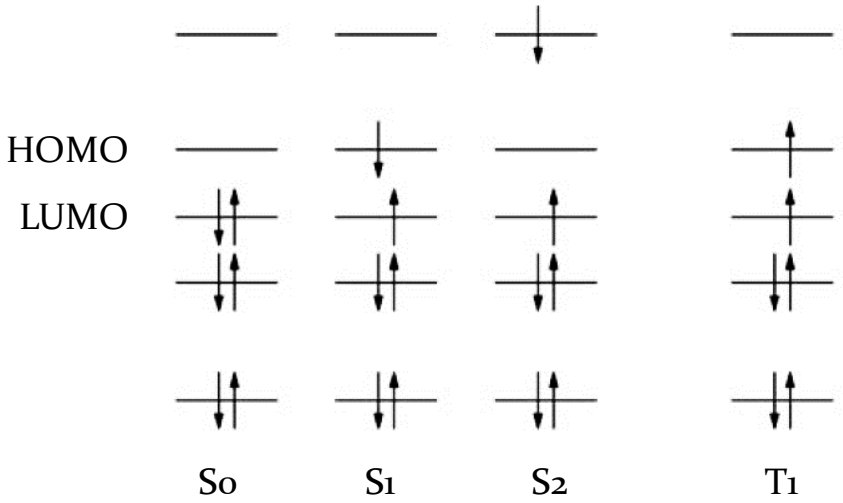
J. B. Grimm & L. Davis, *Nature Methods* 19 (2022) 149–158

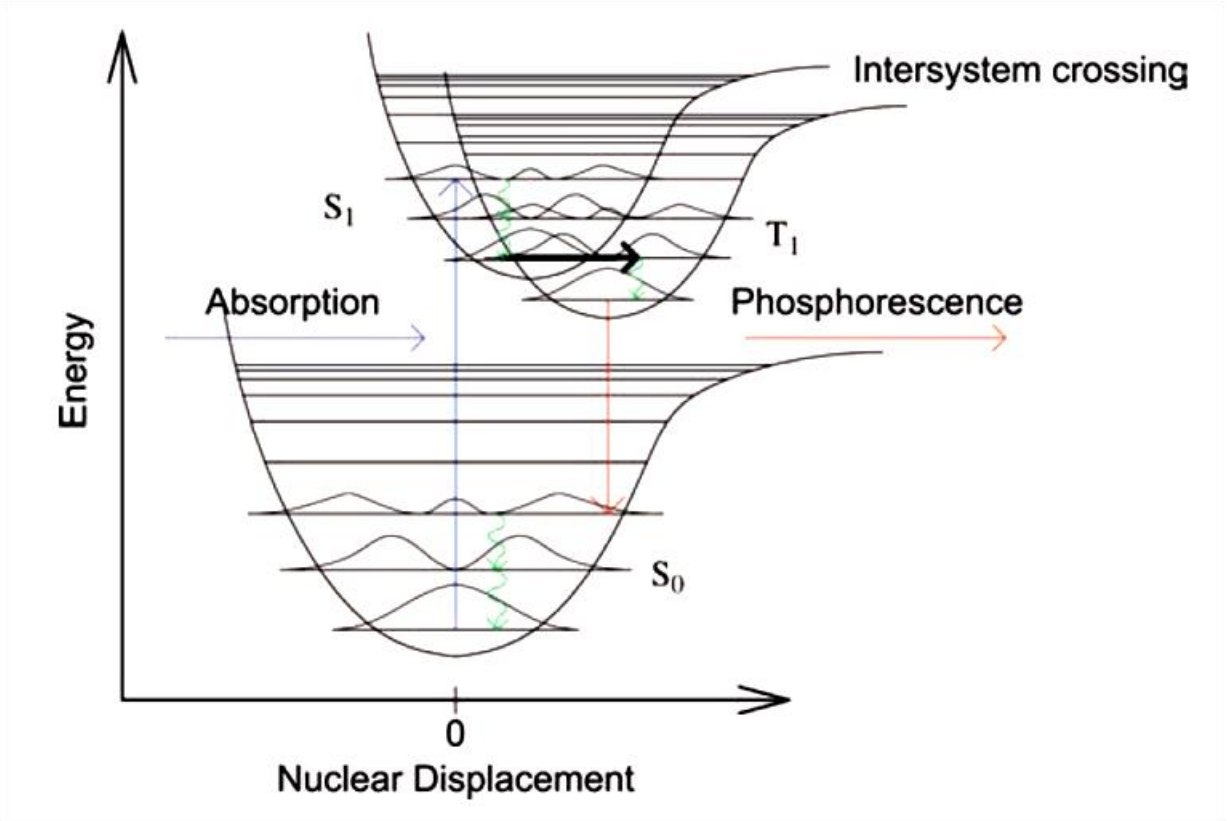
The fate of excited states



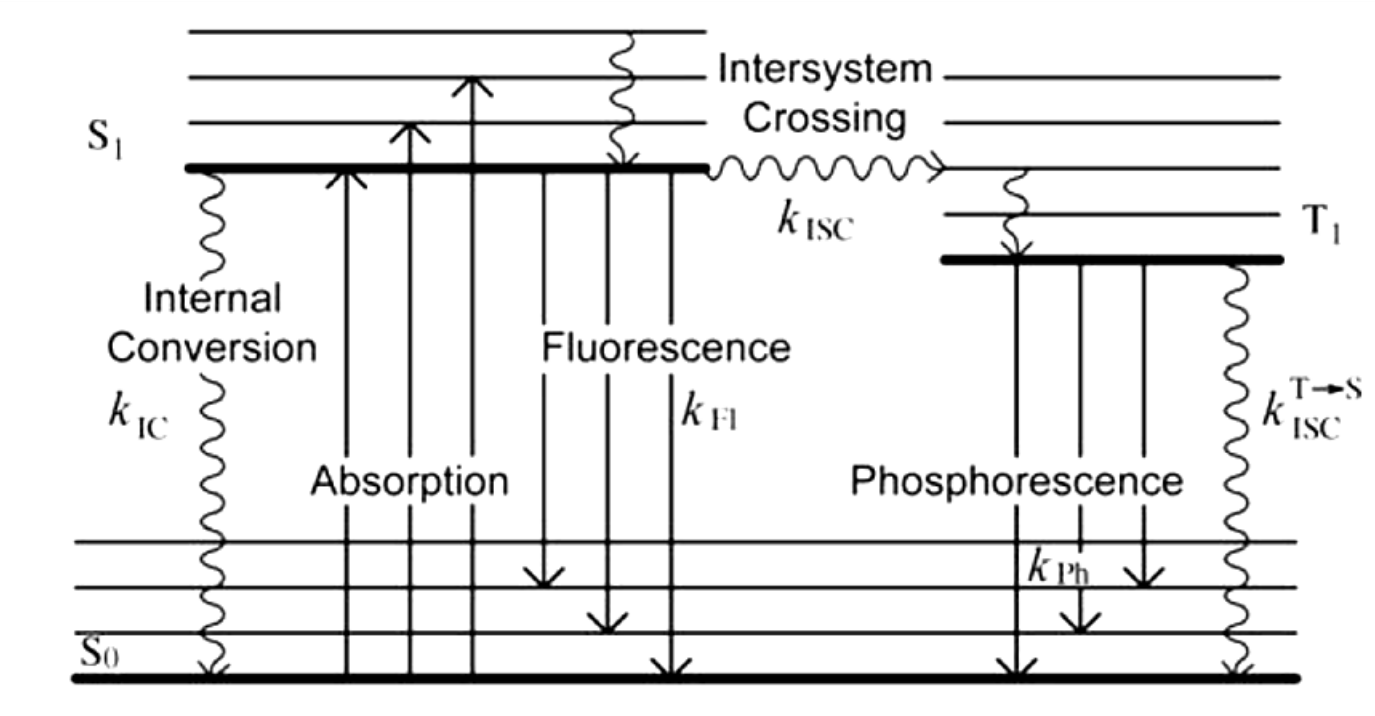


The fate of excited states

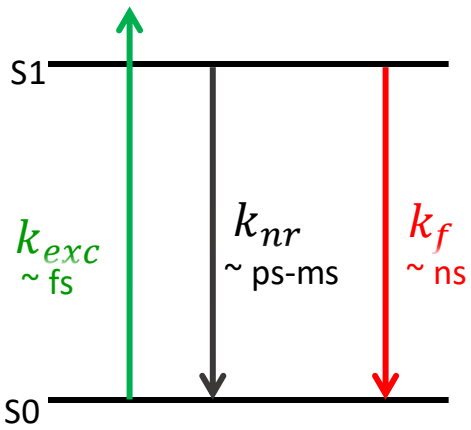




Jiablonski diagram



Fluorescence rates

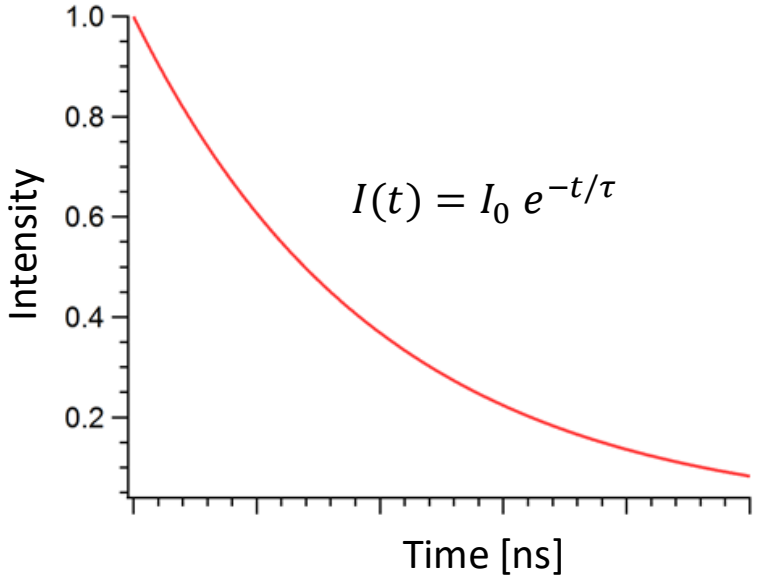


Fluorescence quantum yield

$$\phi_f = \frac{k_r}{k_r + k_{nr}}$$

ISC quantum yield

$$\phi_{ISC} = \frac{k_{ISC}}{k_{fl} + k_{IC} + k_{ISC}}$$

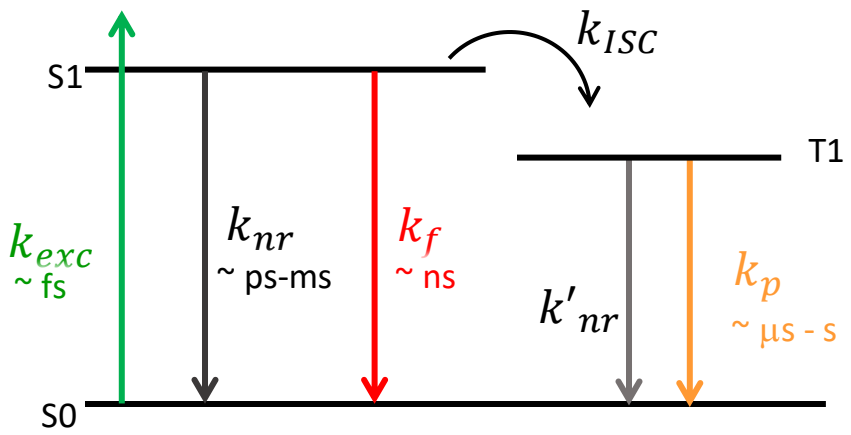


$$I(t) = I_0\, e^{-t/\tau}$$

Fluorescence lifetime

$$\tau = \frac{1}{k_r + k_{nr}}$$

Fluorescence blinking



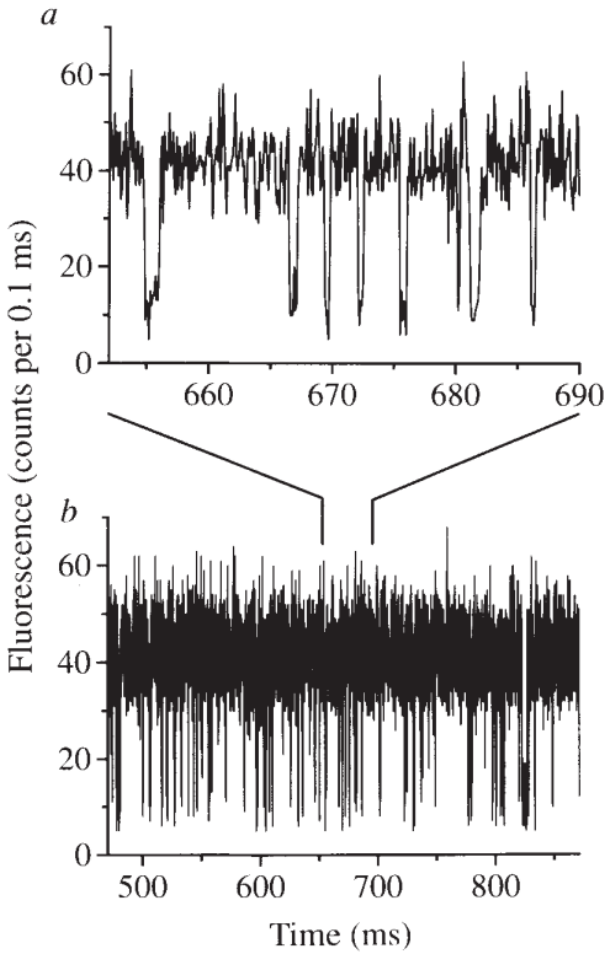
NATURE · VOL 373 · 12 JANUARY 1995

Direct spectroscopic observation of quantum jumps of a single molecule

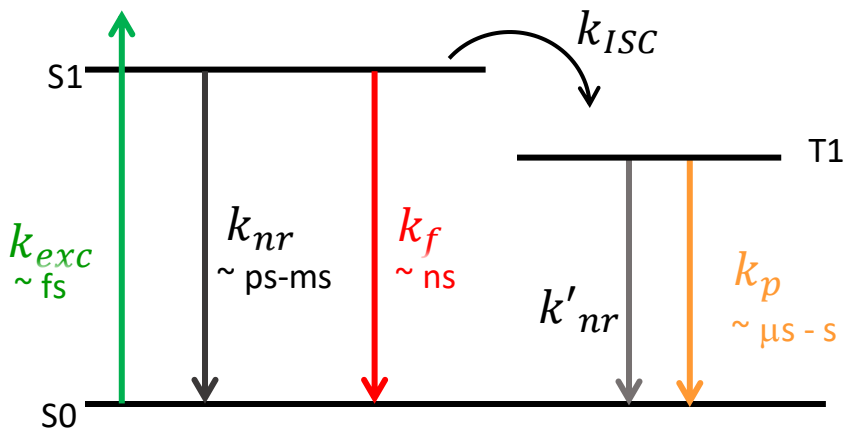
Th. Basché, S. Kummer & C. Bräuchle

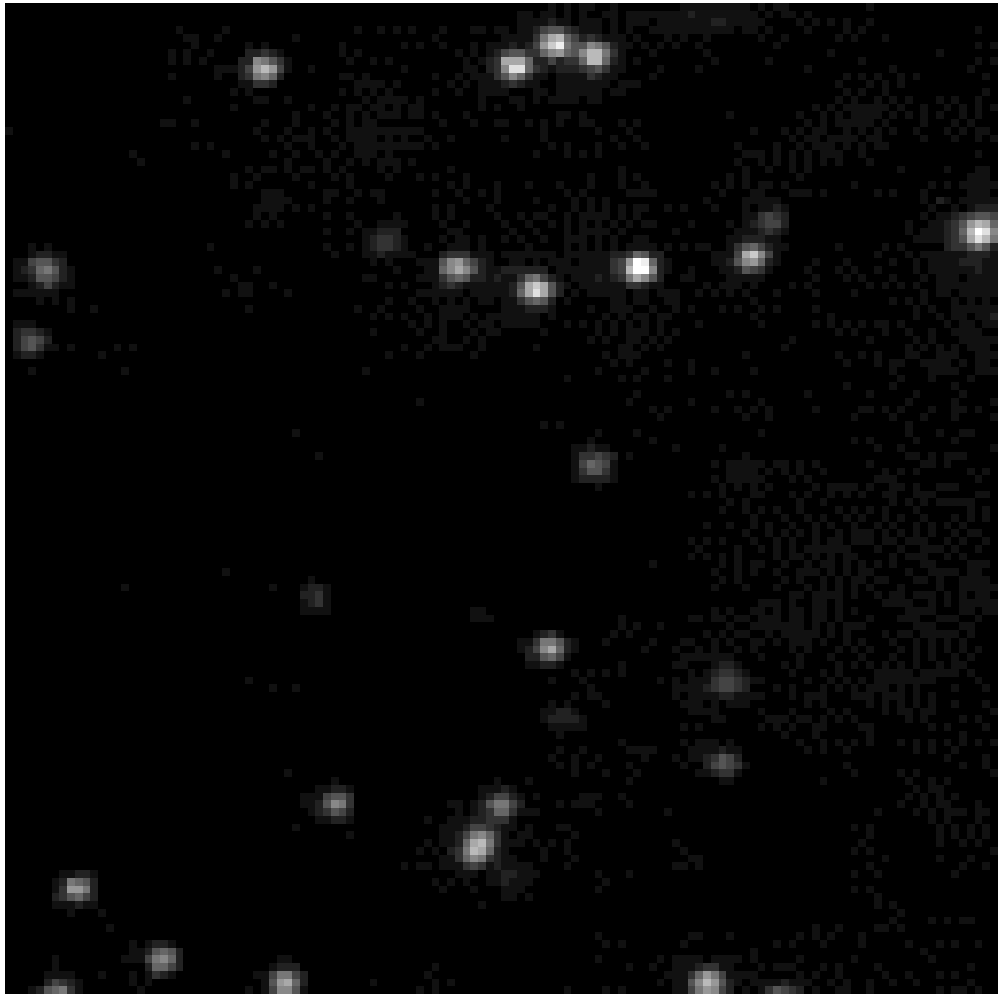
Institut für Physikalische Chemie, Universität München, Sophienstrasse 11, 80333 München, Germany

Vacuum, T= 1.4 K



Fluorescence blinking





Fluorophores

Emission Peak 375-425nm	EX	EM
Calcein Blue	375	420
Cascade Blue®	400	420
DIDS	341	414
Fast Blue	365	420
Fluoro-Gold™ (low pH)	323	408
Indo-1 (high calcium)	330	401
LysoSensor™ Blue (pH 5)	374	424
Mag-Indo-1	330	417
Pyrene	345	378

Emission Peak 425-455nm	EX	EM
4-Methylumbelliferone	360	449
7-Hydroxy-4-methylcoumarin (pH 9)	360	449
7-Amino-4-methylcoumarin	351	430
Alexa Fluor® 350	347	442
AMCA/AMCA-X	345	445
ANS	372	455
BFP (Blue Fluorescent Protein)	382	448
Calcofluor® White	350	440
Hoechst 33342 & 33258	352	449
Pacific Blue™	410	455
SITS	337	436
SpectrumBlue®	400	450

Emission Peak 455-490nm	EX	EM
1,5-IAEDANS	336	482
AmCyan	454	488
BOBO™-1	462	481
BO-PRO™-1	462	481
CFP (Cyan Fluorescent Protein)	434	477
Chromomycin A3	450	470
CPM	385	471
DAPI	358	461
Dansylchloride	380	475
Marina Blue®	365	460
POPO™-1	434	456
PO-PRO™	434	456
SpectrumAqua®	433	480
SYTO® 45	452	484
SYTOX® Blue	445	470

Emission Peak 490-510nm	EX	EM
Aniline Blue	370	509
Cy2®	489	506
DiO (DiOC(18) (3)	484	501
Fura-2 (high calcium)	335	505
GFP (enhanced)	488	508
GFP (sapphire)	395	508
LysoSensor™ Green (pH 5)	442	505
Mag-Fura-2	330	491
PBFI	334	504
Quinacrine Mustard	423	503
SYTO® 13	488	509
YO-PRO®-1	491	509
YOYO®-1	491	509
ZsGreen	492	505

Emission Peak 510-545nm	EX	EM
5-Carboxyfluorescein (5-FAM)	492	518
Acridine orange (+DNA)	500	526
Alexa Fluor® 430	434	540
Alexa Fluor® 488	495	519
BCECF (high pH)	503	528
BODIPY® FL	505	513
BTC	401/464	529
Calcein	494	517
Calcium Green-1™	506	531
Cascade Yellow™	402	545
CCF2 (GeneBLAzer™)	402	520
CI-NERF (low pH)	504	540
Dansyl cadaverine	335	518
DM-NERF (4.5-6.5 pH)	510	536
ELF®-97 alcohol	345	530
Eosin	524	544
Fluo-3	506	526
Fluo-4	494	516
Fluorescein (FITC)	494	518
Fluoro-Jade	475	525
FluorX®	494	519
Lucifer Yellow	428	536
LysoSensor™ YellowBlue (pH 4.2)	384	540
LysoTracker® Green	504	511
LysoTracker® Yellow	465	535
Magnesium Green™	506	531
Mithramycin	395	535
MitoTracker® Green	490	516
NBD (amine)	465	535
Oregon Green® 488	496	524
Oregon Green® 500	503	522
Oregon Green® 514	511	530
Qdot™ 525	UV	525
Rhodamine 110	496	520
Rhodamine 123	507	529
Rhodamine Green™	502	527
SBFI	334	525
Sodium Green™	507	535
SpectrumGreen®	497	524
SYTO® 11	508	527
SYTOX® Green	504	523
TO-PRO®-1	514	533
TOTO®-1	514	533
YFP (Yellow Fluorescent Protein)	513	527
ZsYellow	528	540

Emission Peak 545-570nm	EX	EM
TAMRA	542	568
Rhodamine 6G	525	555
6-JOE	525	555
Alexa Fluor® 532	531	554
Alexa Fluor® 555	553	568
ATTO-TAG™ CBQCA	465	560
Auramine O-Feulgen	460	550
BODIPY® 530/550	533	550
BODIPY® 558/568	558	568
DiI (DiIC(18)(3))	549	565
Erythrosin	529	554
Fluoro-Gold™ (high pH)	368	565
JOJO™-1	529	545
JO-PRO™-1	529	545
PKH26	551	567
Qdot™ 565	UV	565
Rhodamine Phalloidin	542	565
SNAFL®-2	525	546
Spectrum Gold®	530	555

Emission Peak 570-585nm	EX	EM
Alexa Fluor® 546	556	573
BODIPY® 564/570	564	570
BODIPY® TMR	542	574
Calcium Orange™	549	576
CryptoLight CF-3™	545	576
CryptoLight CF-4™	555	580
Cy3®	550	570
Dapoxyl®	373	574
LOLO™-1	565	579
LO-PRO™-1	565	579
MitoTracker® Orange	551	576
POPO™-3	534	572
PO-PRO™-3	534	572
PyMPO	415	570
Pyronin Y	555	580
Qdot™ 585	UV	585
R-PE	488	575
Rhod-2	550	571
R-phycoerythrin (PE)	565	575
SYTOX® Orange	547	570

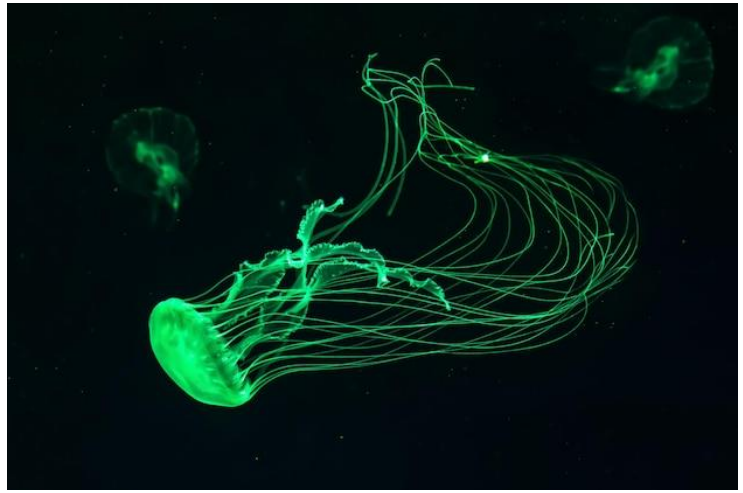
Emission Peak 585-595nm	EX	EM
ATTO-TAG™ FQ	486	591
BODIPY® 581/591	582	590
DsRed2 (Red Fluorescent Protein)	558	583
Lissamine rhodamine B	570	590
LysoTracker® Red	577	592
Resorufin	570	585
Rhodamine B	555	580
Rhodamine Red™	570	590
SpectrumOrange®	559	588
Tetramethylrhodamine (TRITC)	555	580

Emission Peak 595-605nm	EX	EM
5-ROX (carboxy-X-rhodamine)	574	602
Alexa Fluor® 568	579	604
AsRed	577	597
BOBO™-3	570	604
BO-PRO™-3	570	604
CryptoLight CF-5	566	597
Cy3.5®	581	596
Di-4 ANEPPS	488	605
Ethidium bromide	518	605
FM® 1-43	479	598
MitoTracker® Red	578	599
Qdot™ 605	UV	605
X-Rhodamine (XRITC)	580	605

Emission Peak 605-655nm	EX	EM
7-Aminoactinomycin D (7-AAD)	546	647
Acridine orange (+RNA)	460	650
Alexa Fluor® 594	591	618
Alexa Fluor® 633	632	647
BODIPY® 630/650-X	630	650
BODIPY® TR-X	589	617
Calcium Crimson™	590	615
C-phycocyanin	620	648
CryptoLight™ CF-1	575	641
CryptoLight™ CF-2	585	658
CryptoLight™ CF-6	566	615
CTC Formazan	450	630
Di-8 ANEPPS	468	635
DiA (4-Di-16-ASP)	491	613
Ethidium homodimer-1 (EthD-1)	528	617
Europium (III) Chloride	337	613
Fura Red™ (high calcium)	436	637
HcRed	591	613
Nile Red	549	628
PE-Alexa Fluor® 610	488	628
PE-Texas Red®	488	615
Propidium Iodide	536	617
Qdot™ 655	UV	655
RH 414	500	635
R-phycocyanin	618	642
SNAFL®-1 (high pH)	576	635
SNARF®-1 (high pH)	575	635
SpectrumRed®	587	612
SYTO® 17	621	634
Texas Red® / Texas Red®-X	595	615
YO-PRO®-3	612	631
YOYO®-3	612	631

Emission Peak 655-800nm	EX	EM
5-Carboxynaphthofluorescein	598	668
Alexa Fluor® 647	653	669
Alexa Fluor® 660	663	690
Alexa Fluor® 680	679	702
Alexa Fluor® 700	702	723
Alexa Fluor® 750	749	775
Allophycocyanin (APC)	650	660
APC	633/635	660
APC-Cy5.5	633/635	694
APC-Cy7	633/635	767
BODIPY® 650/665-X	650	665
Cy5®	649	670
Cy5.5®	675	694
Cy7®	743	767
DiD (DiIC (18)(5))	644	665
DiR (DiIC(18)(7))	750	779
PE-Alexa Fluor® 647	633/635	668
PE-Cy5	633/635	670
PE-Cy5.5	633/635	694
PE-Cy7	633/635	767
PerCP	488	675
PerCP/Cy5.5	488	710
SensiLight™ P1	545	666
SensiLight™ P3	614	662
SpectrumFRed®	655	675
TO-PRO®-3	642	660
TO-PRO®-5	748	768
TOTO®-3	642	660

Fluorescent proteins



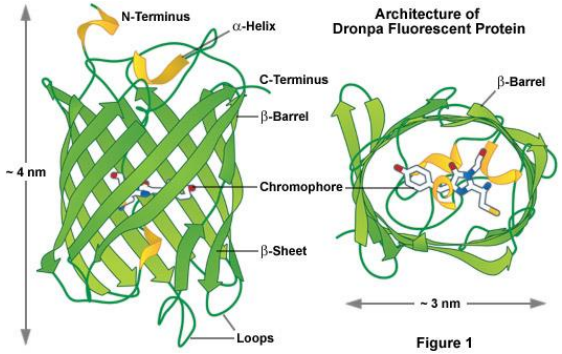
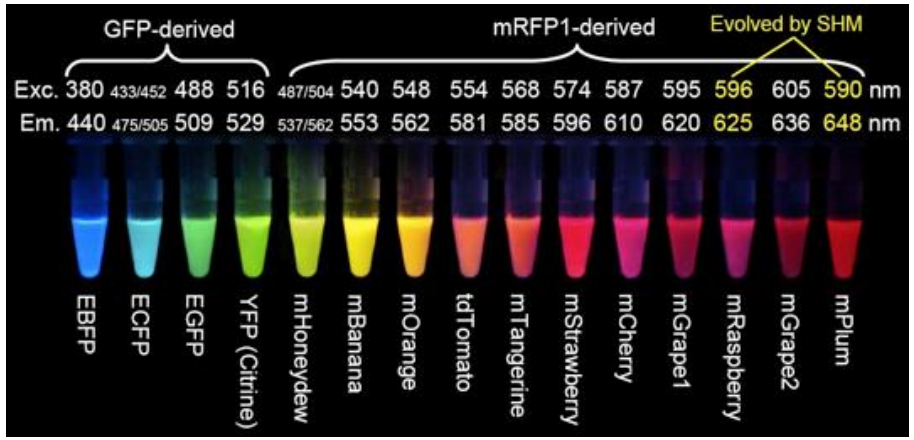
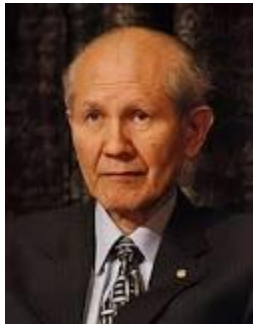


Figure 1



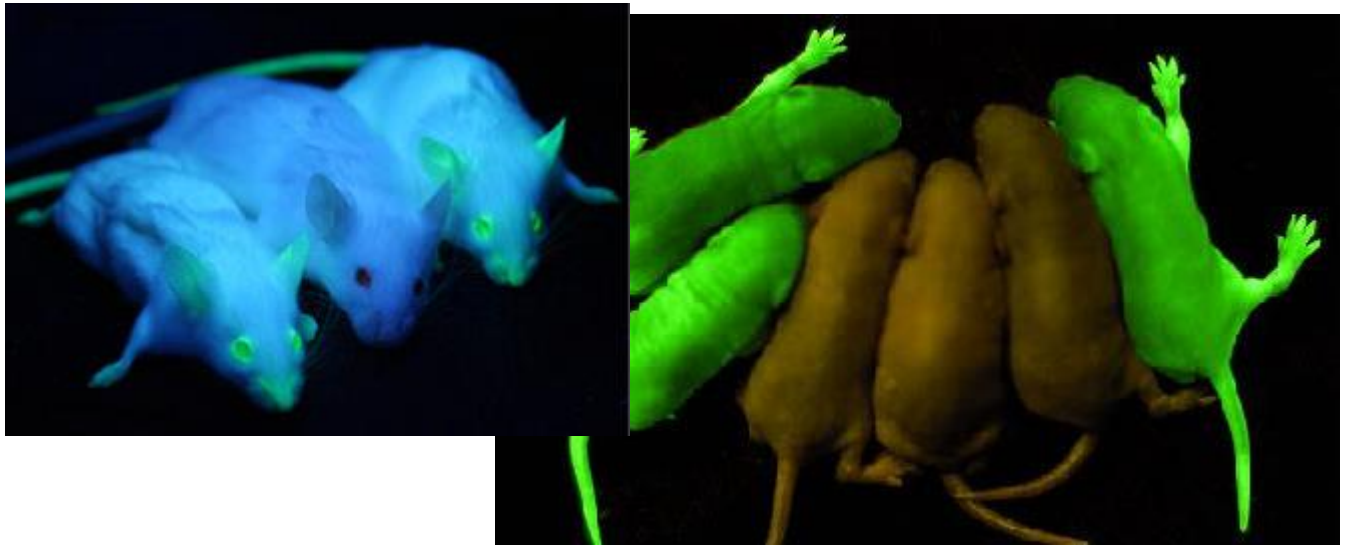
Nobel Prize in Chemistry 2008



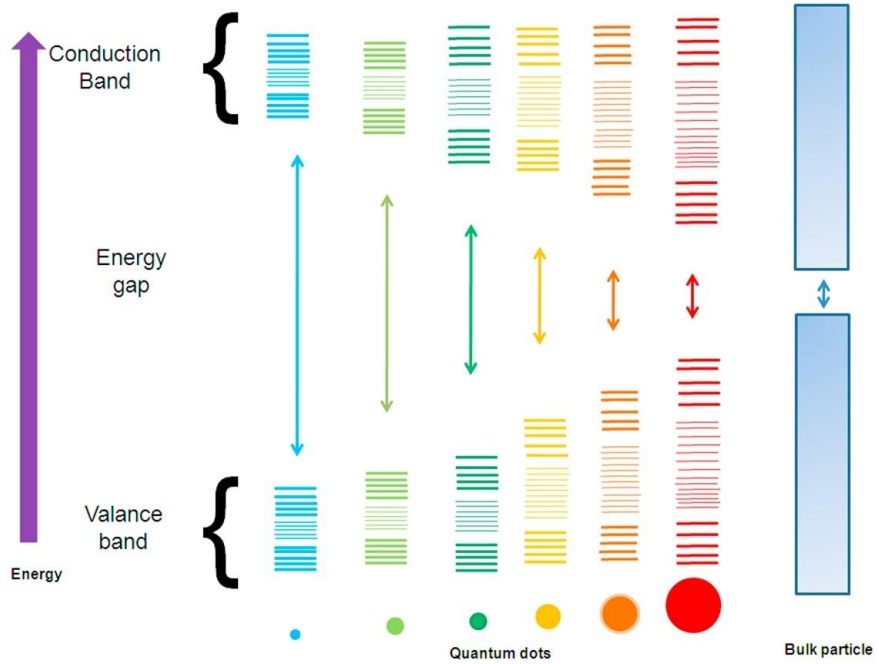
Osamu Shimomura

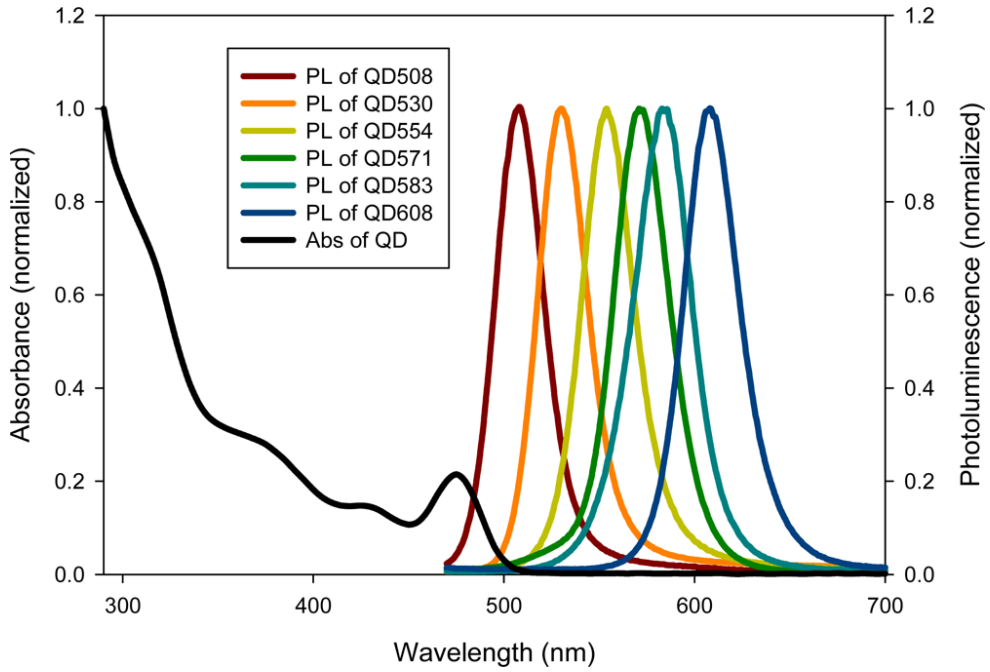
Martin Chalfie

Roger Y. Tsien



Quantum dots

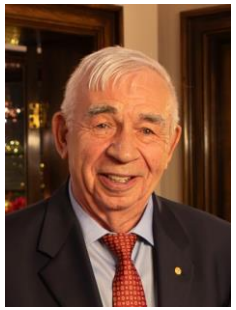




Nobel Prize in Chemistry 2023

Moungi G. Bawendi

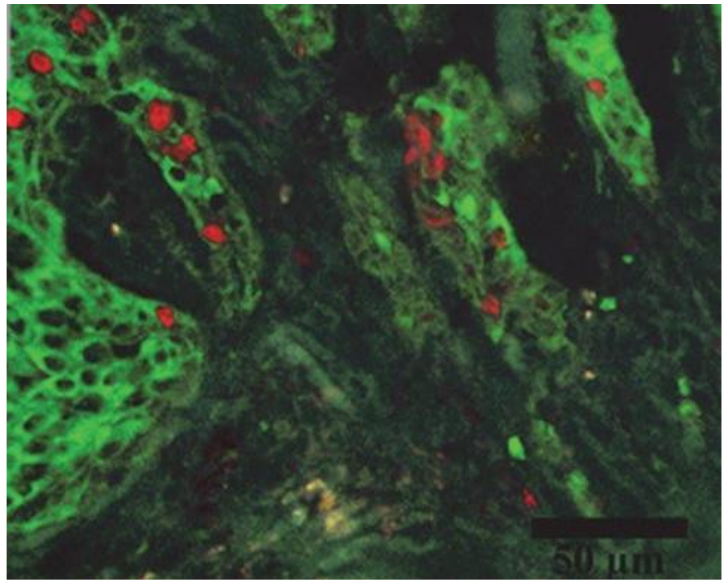
Louis E. Brus



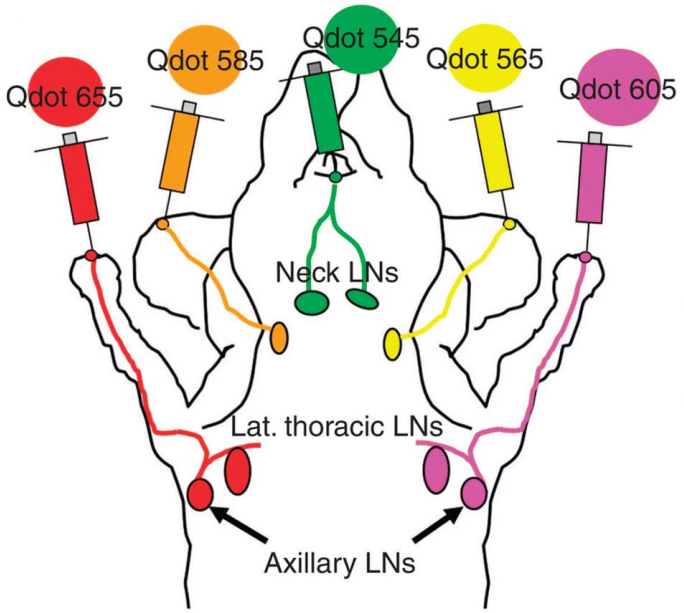
Aleksey Yekimov

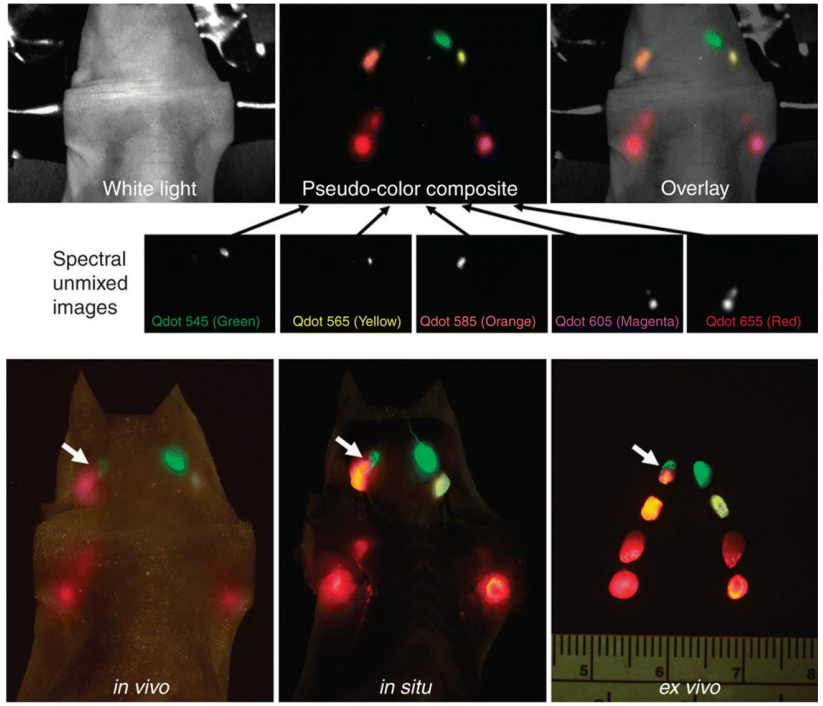
V.G. Reshma, P.V. Mohanan, Journal of Luminescence 205 (2019) 287-298

Quantum dots



Ki67 (605-QD) and CK (525-QD) in breast cancer specimens excited by UV light





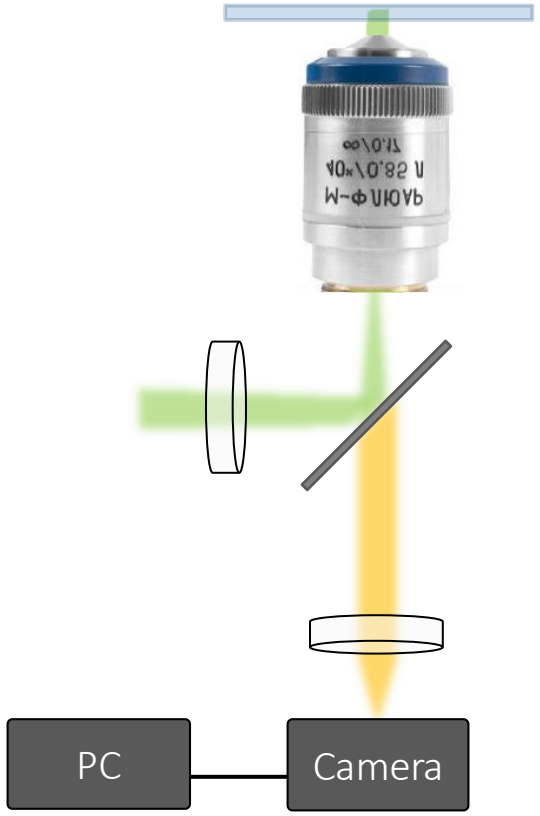
Wang et al. Quantum Dot-Based Simultaneous Multicolor Imaging *Molecular Imaging and Biology* 22 (2020) 820 - 831

Labelling strategies

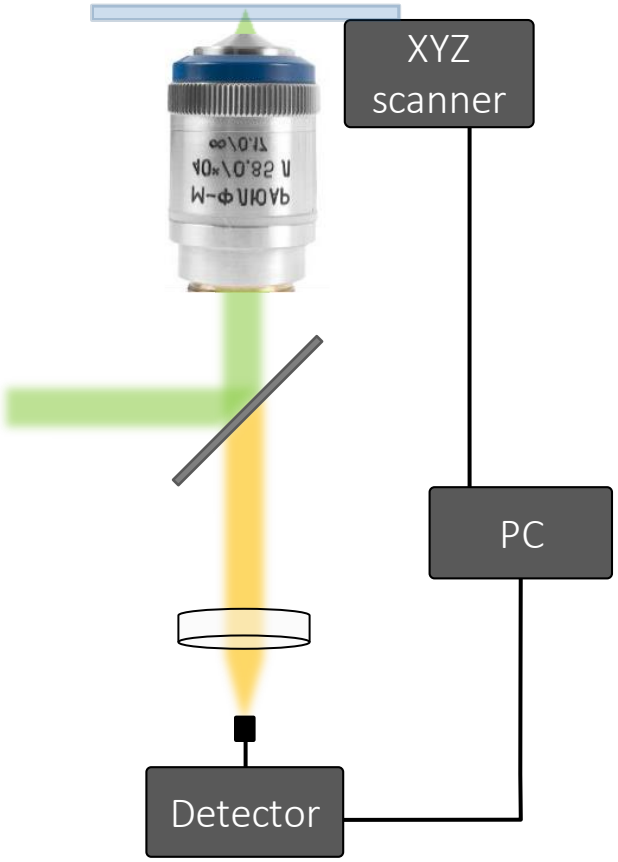
- Genetically encoded fluorophores (fluorescent proteins)
- Genetically encoded tags (Halo-tag, SNAP-tag, …) + synthetic ligand
- Immunolabeling: antibodies, directly labeled, primary-secondary, Fab fragments, nanobodies
- High affinity small fluorescent molecules (drug-like, DNA intercalators, etc.)
- DNA, RNA hybridization
- …

Fluorescence microscopy modalities

Wide-field
Fluorescence Microscope

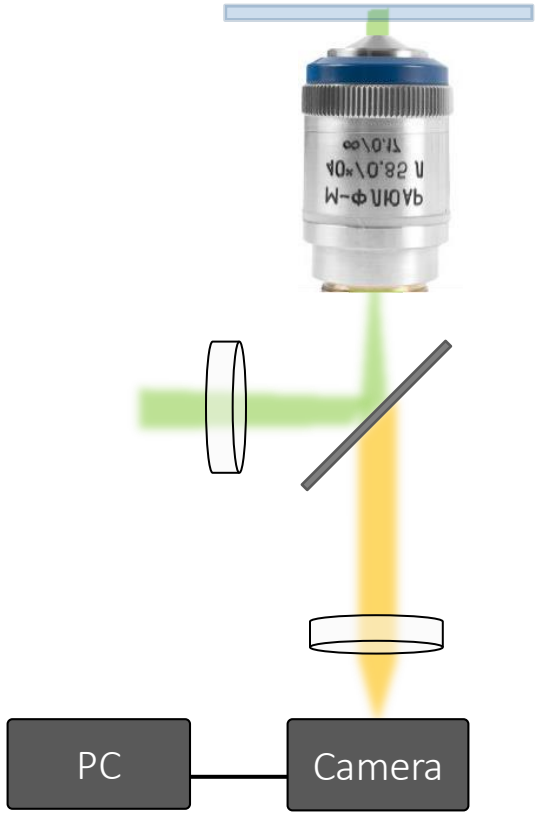


Sample Scanning (Confocal)
Fluorescence Microscope

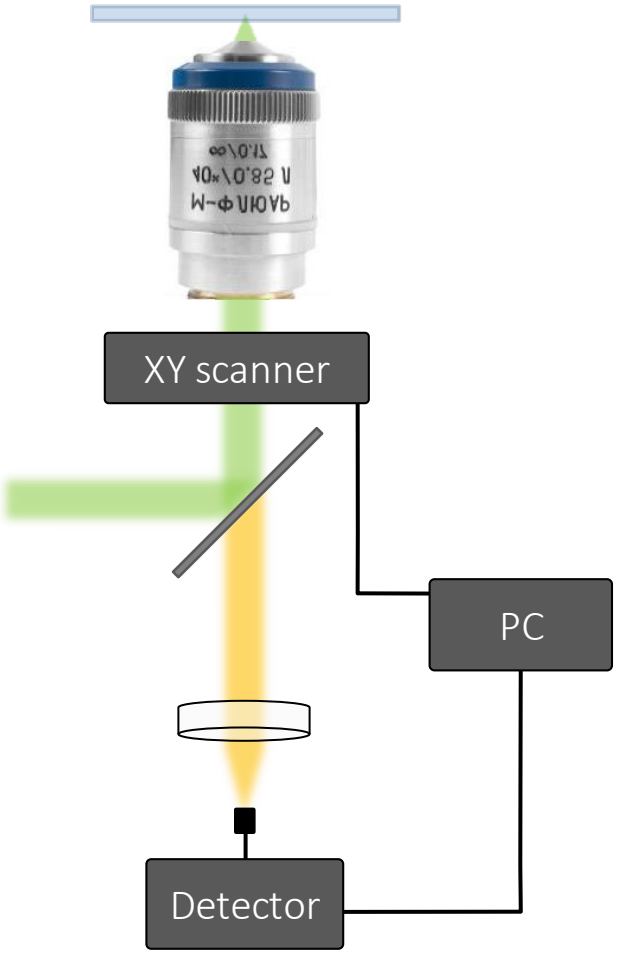


Fluorescence microscopy modalities

Wide-field
Fluorescence Microscope

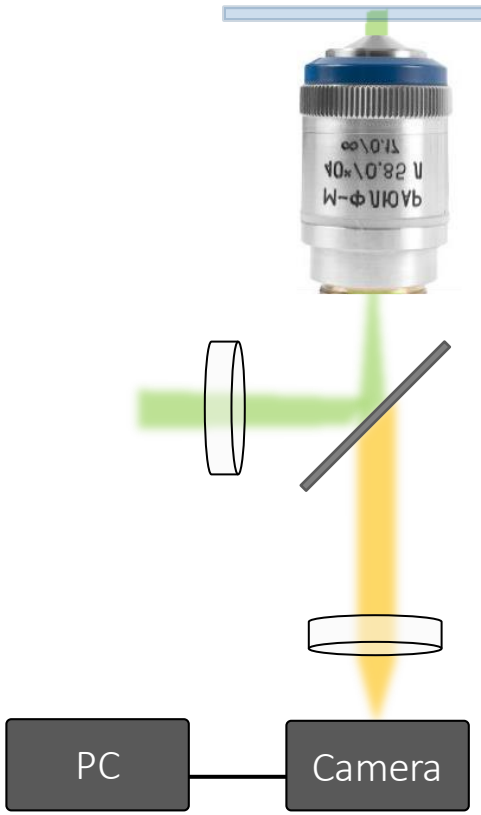


Beam Scanning
(Confocal) Fluorescence Microscope



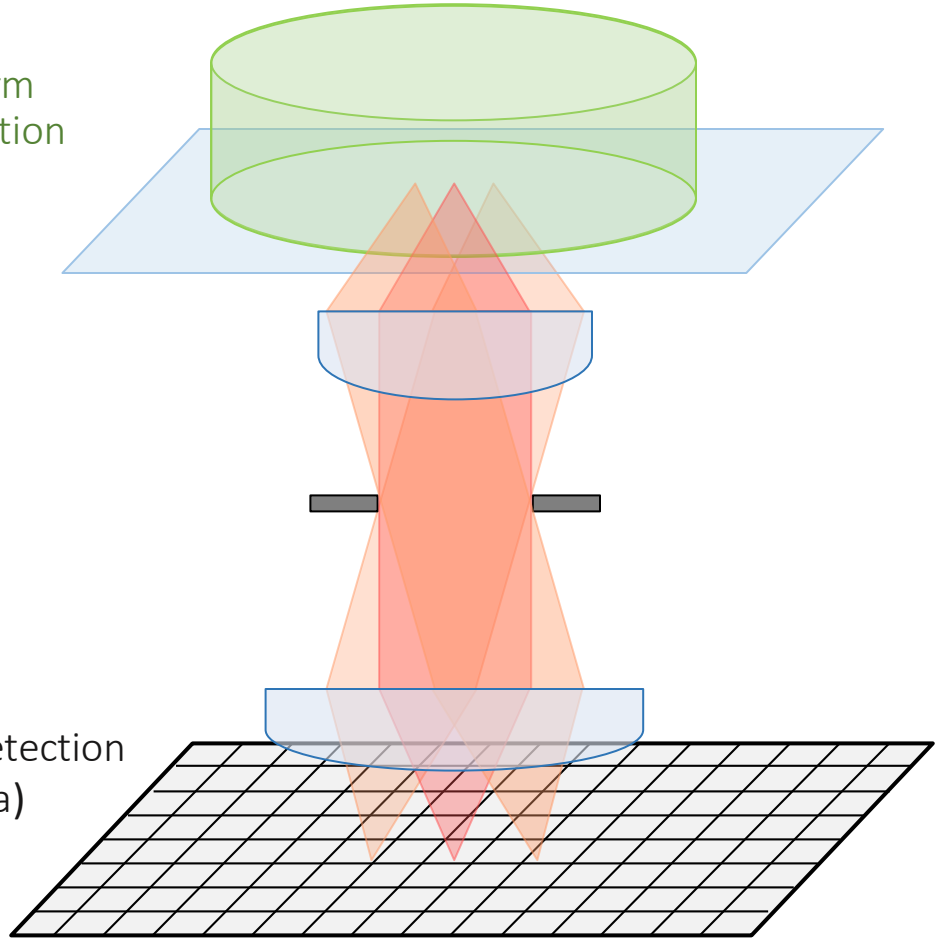
Wide-field fluorescence microscopy

Wide-field
Fluorescence Microscope

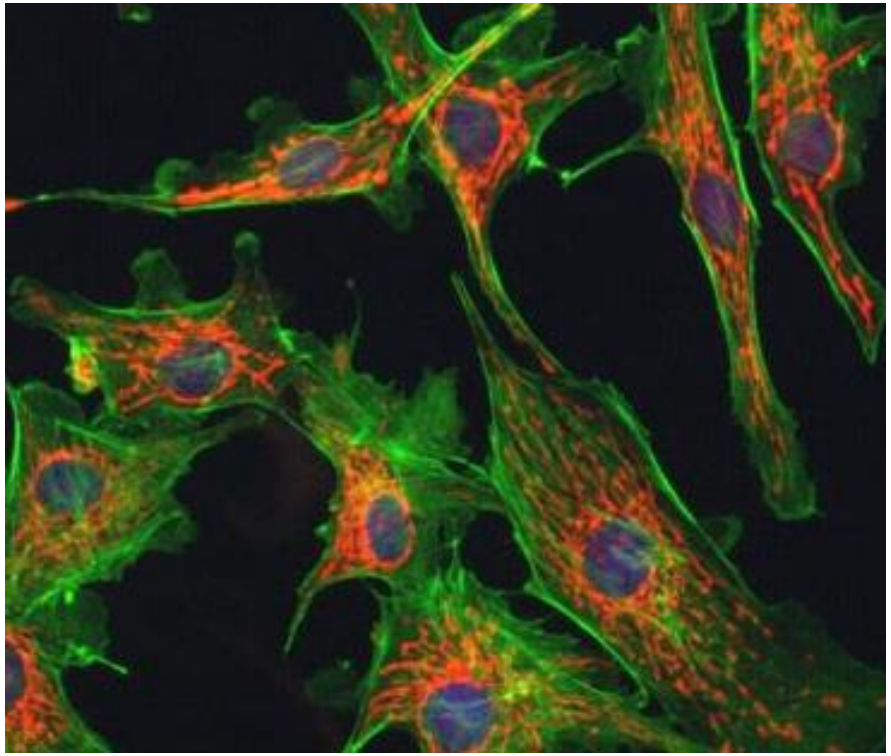


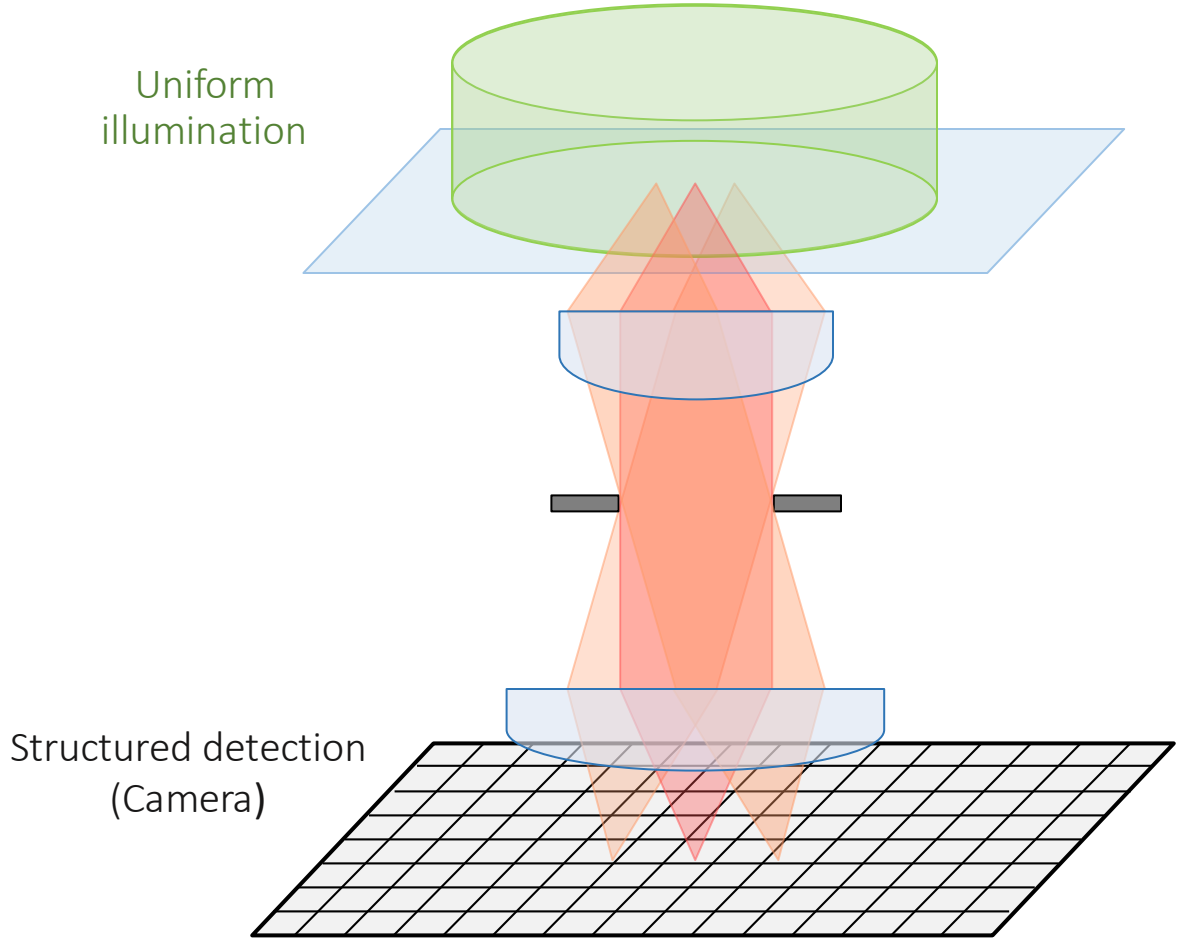
Uniform
illumination

Structured detection
(Camera)

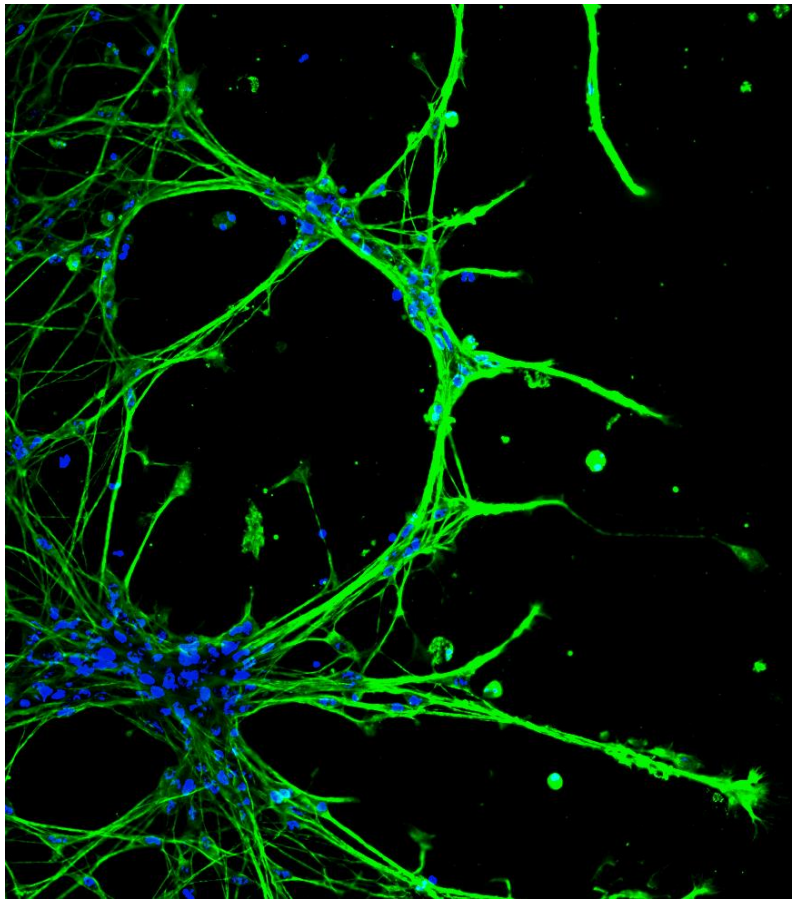


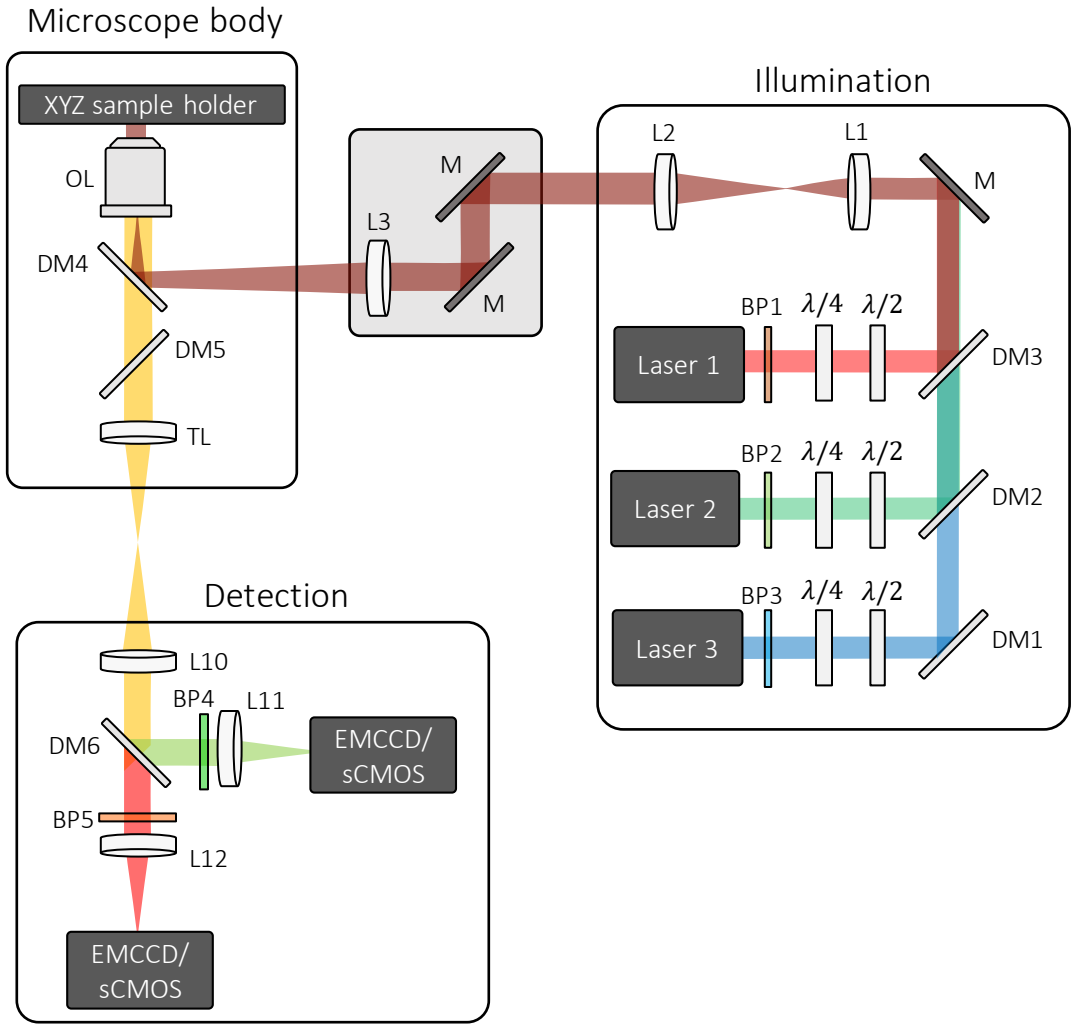
Wide-field fluorescence microscopy



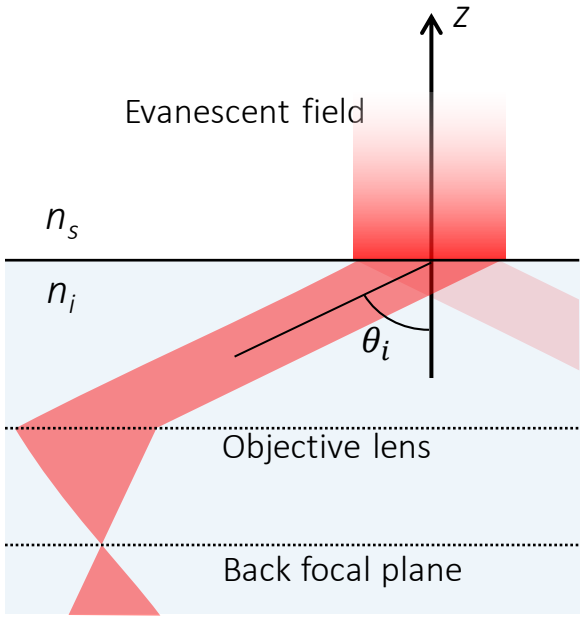


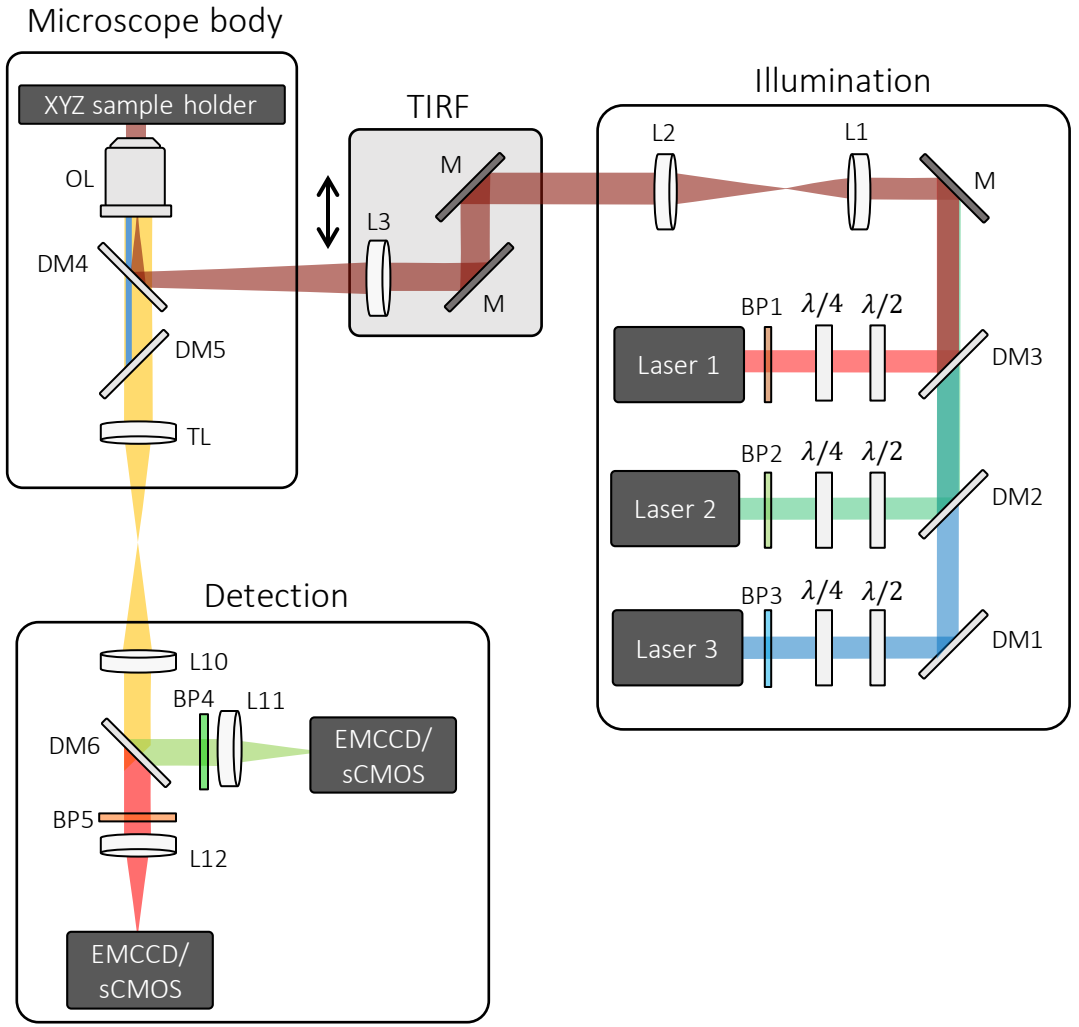
Wide-field fluorescence microscopy





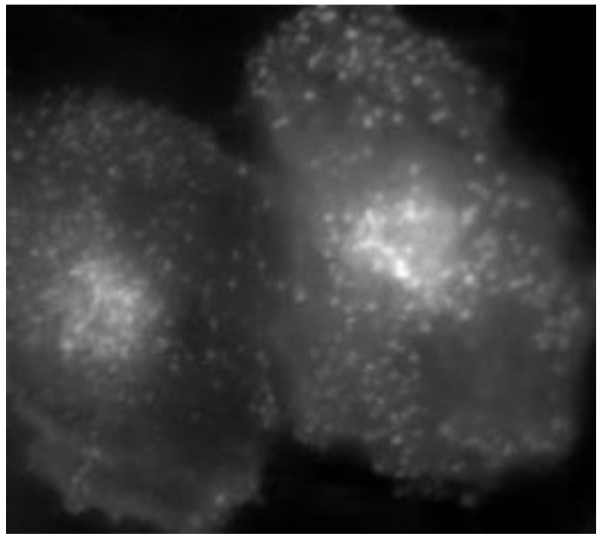
Total Internal Reflection Fluorescence (TIRF) microscopy



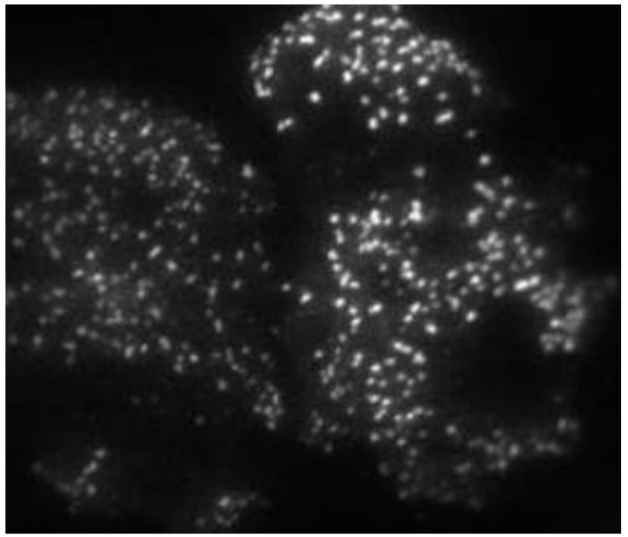


Total Internal Reflection Fluorescence (TIRF) microscopy

Epifluorescence

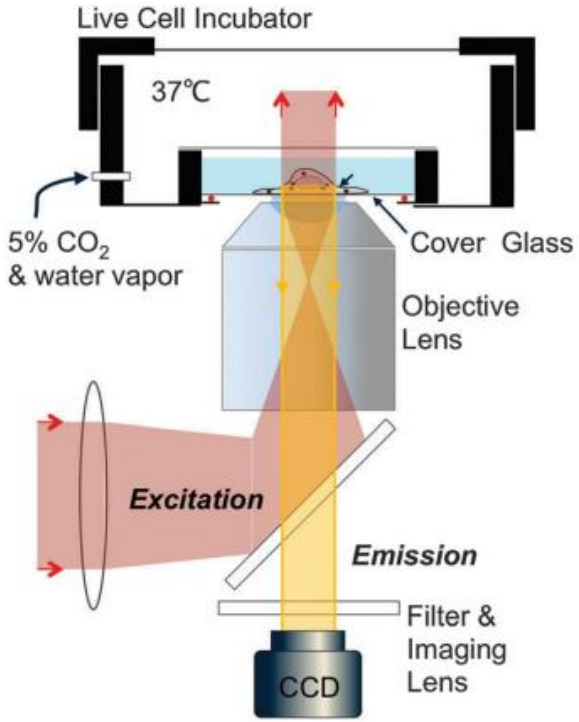


TIRF

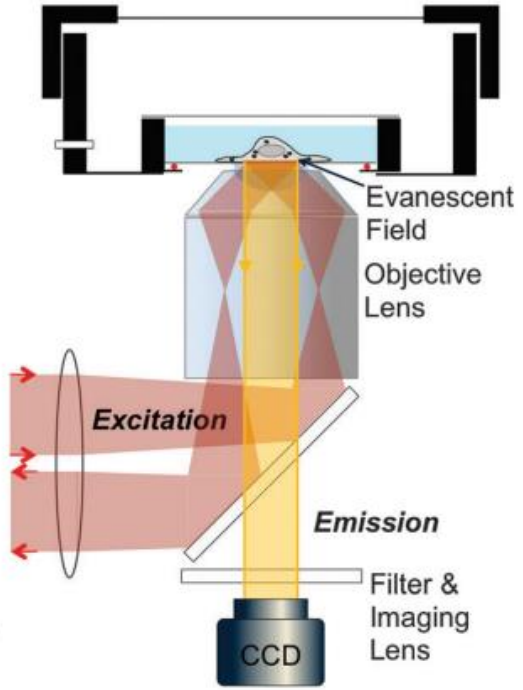


Wide-field fluorescence microscopy

Epifluorescence

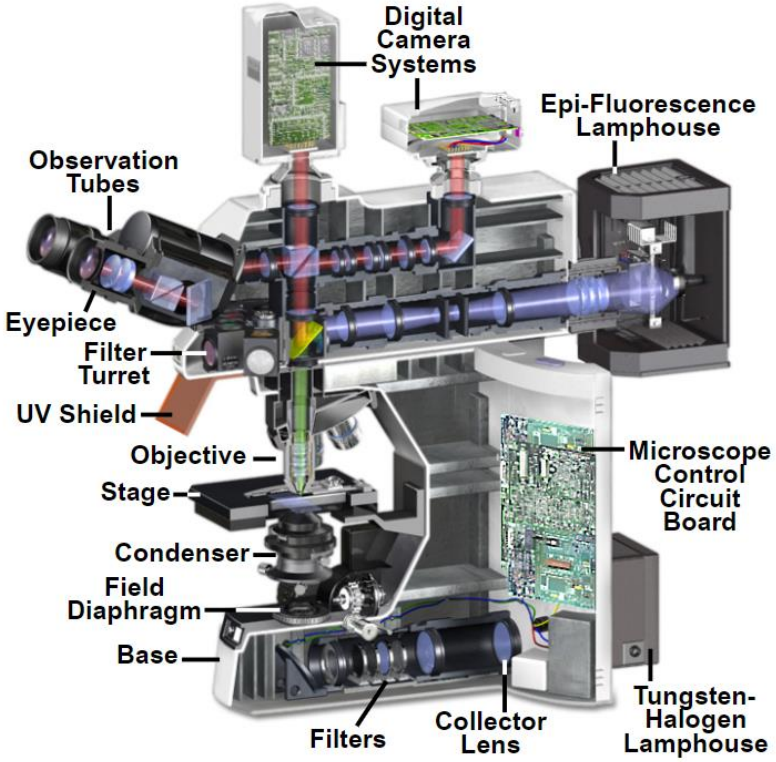


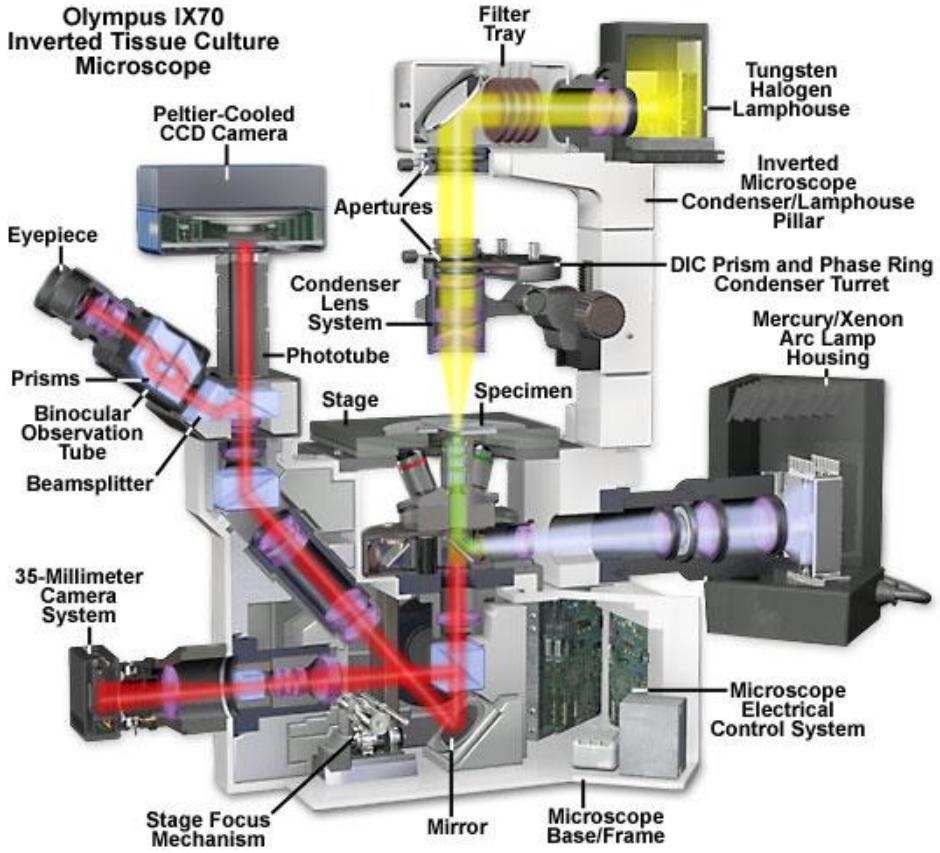
TIRF



Park et l. *Chem. Soc. Rev*. 44 (2015) 1302--1317

Wide-field fluorescence microscopy

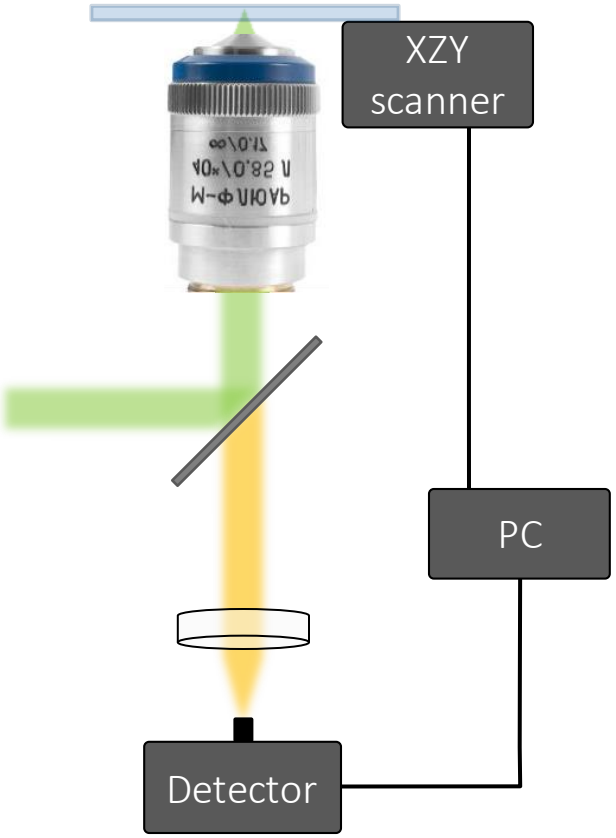


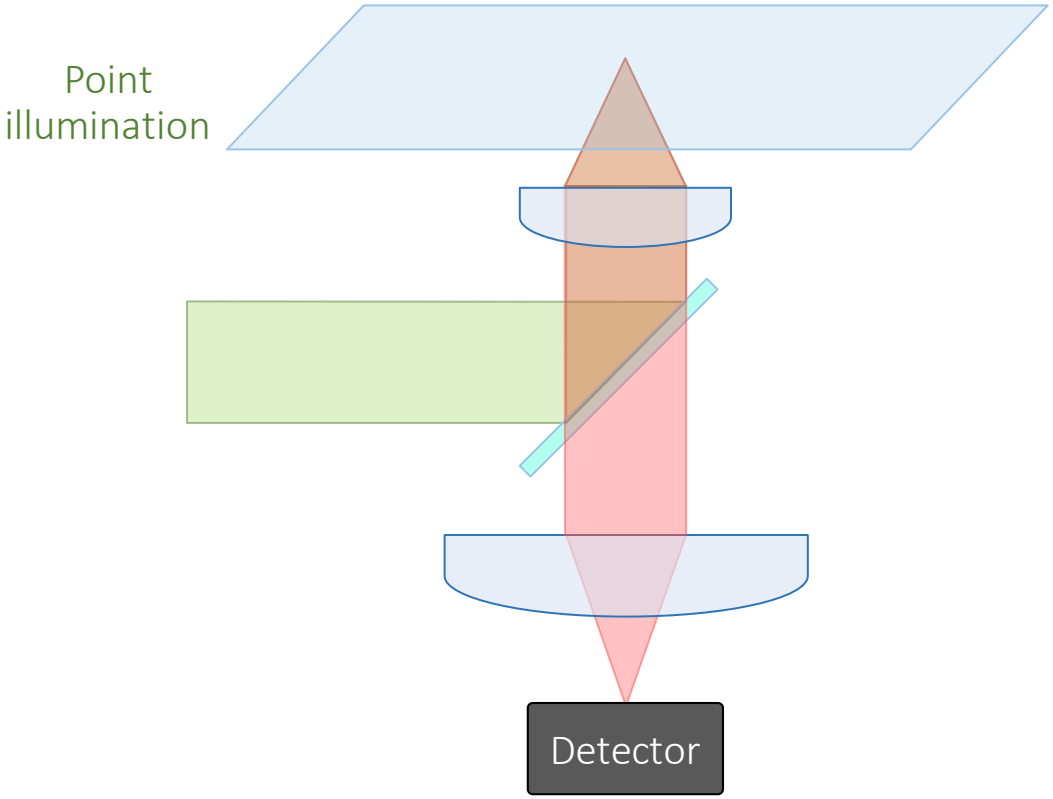


Wide-field fluorescence microscopy

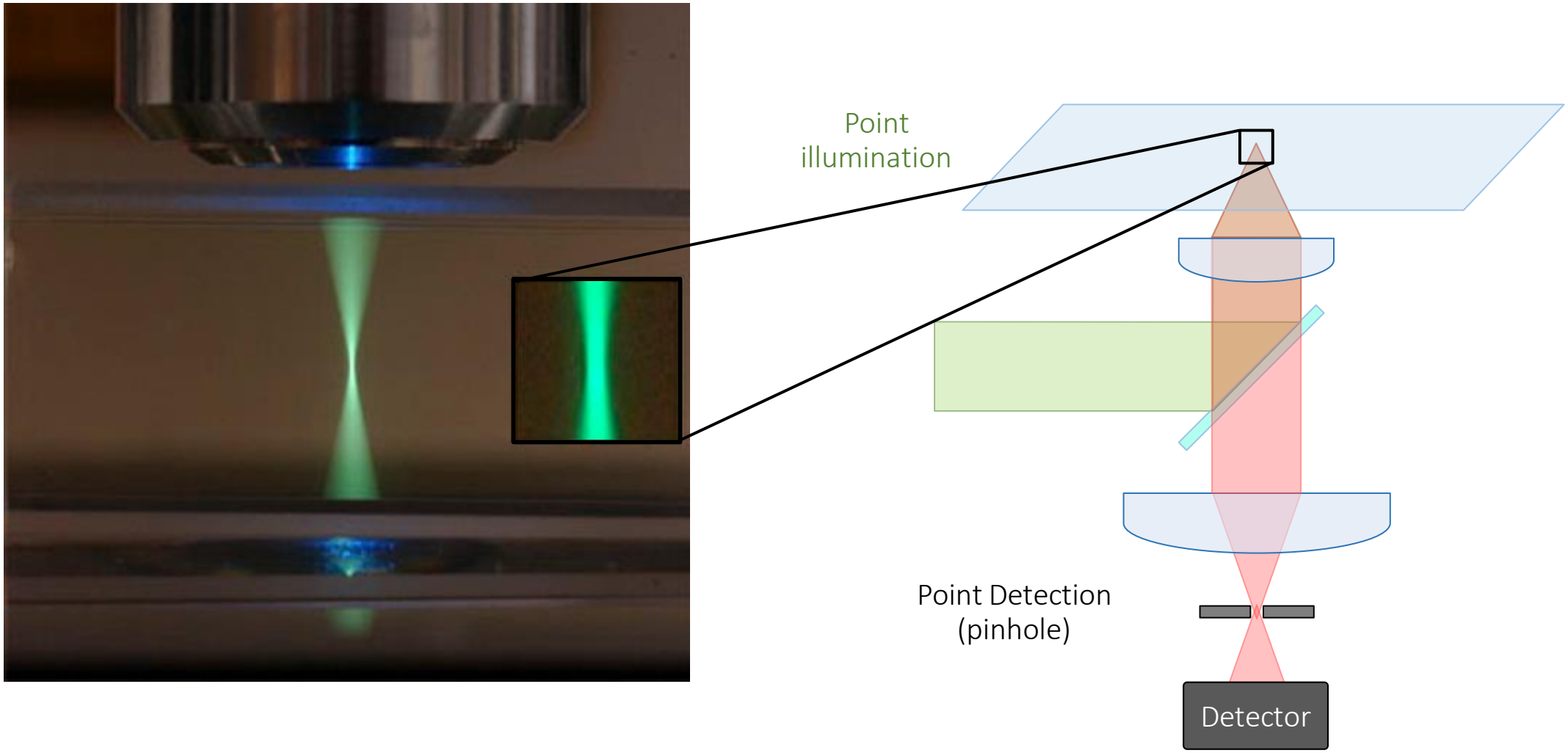
Scanning fluorescence microscopy

Scanning
Fluorescence Microscope



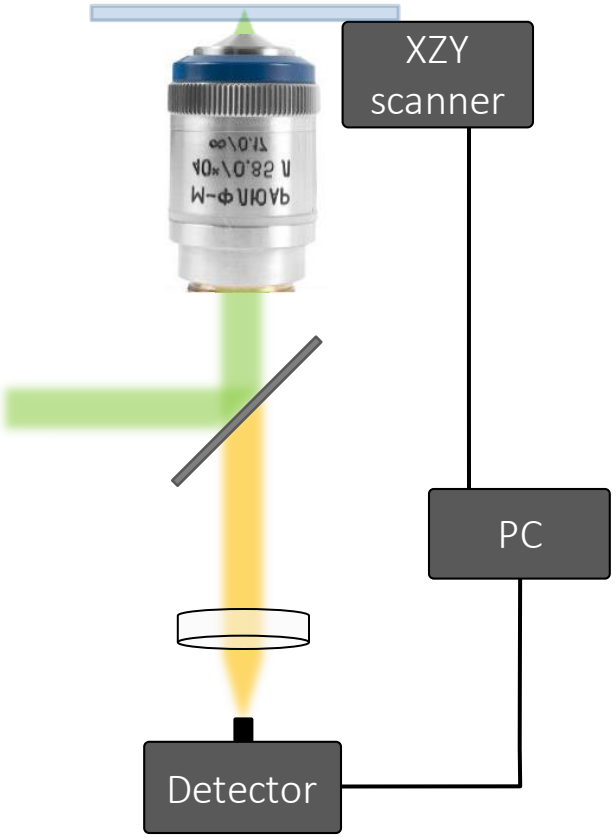


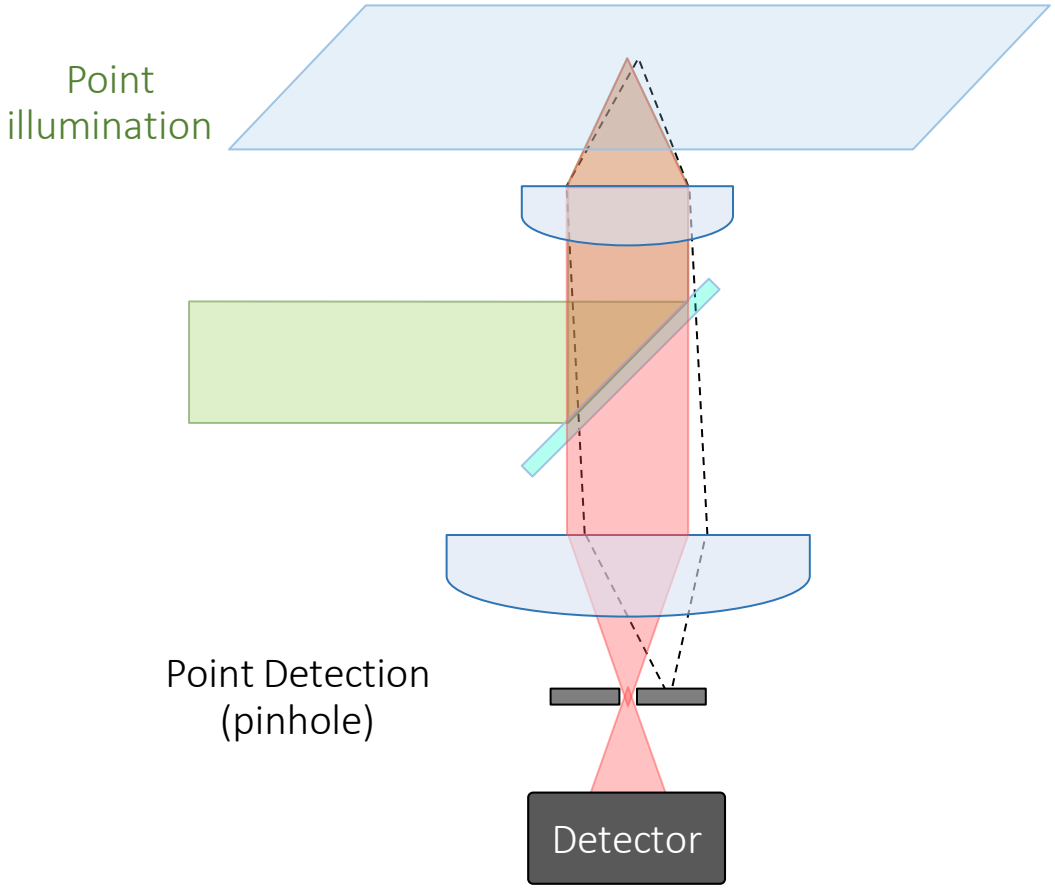
Scanning confocal fluorescence microscopy



Scanning confocal fluorescence microscopy

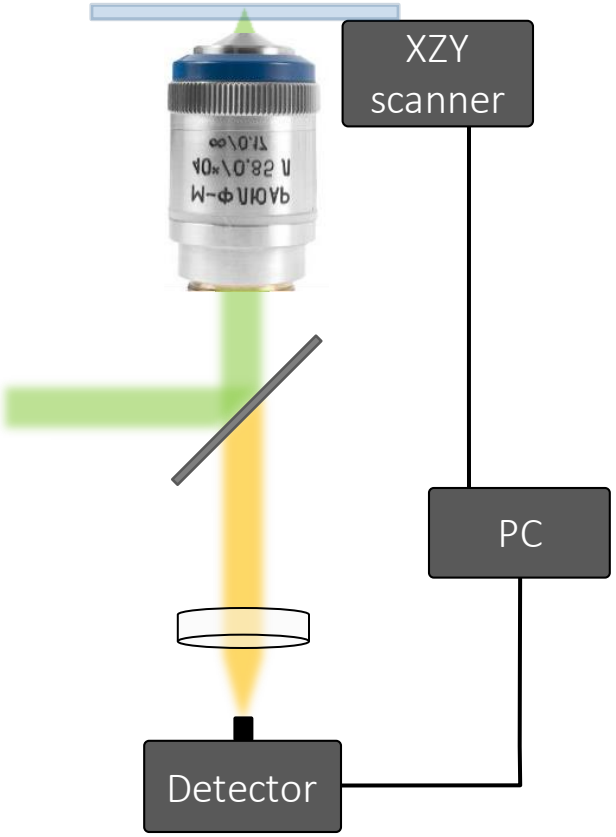
Scanning
Fluorescence Microscope

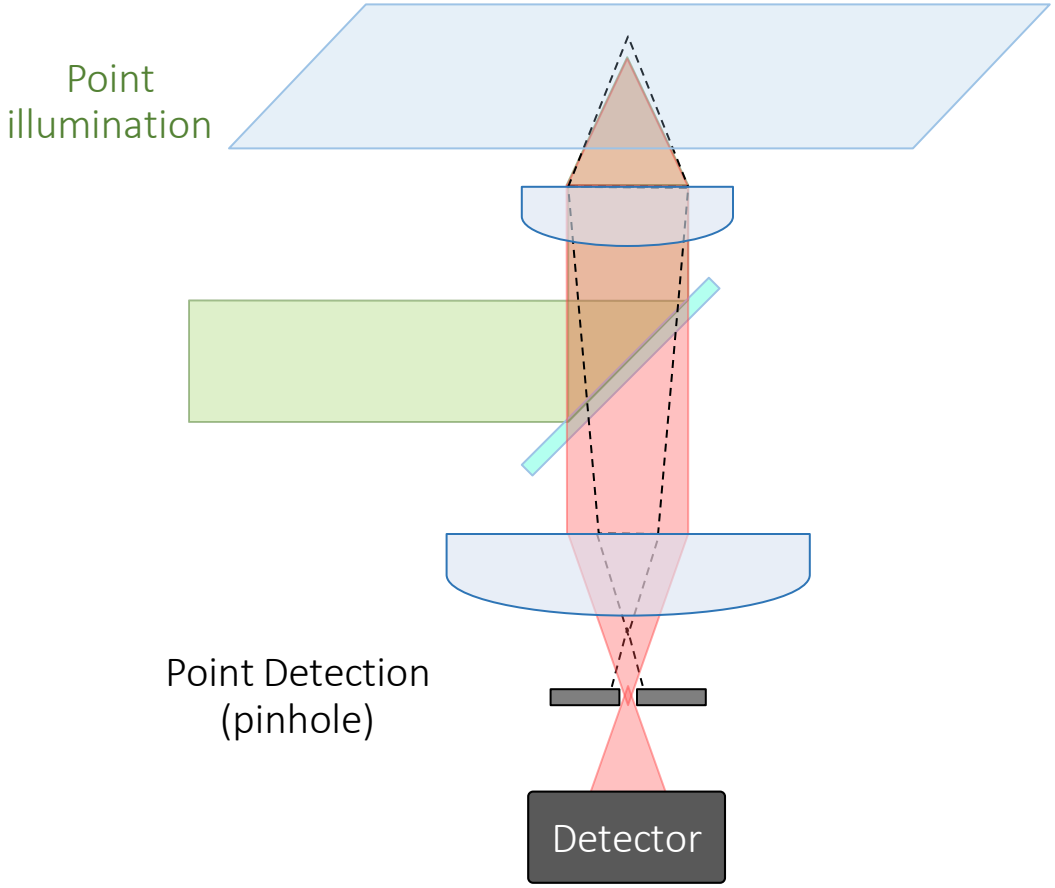




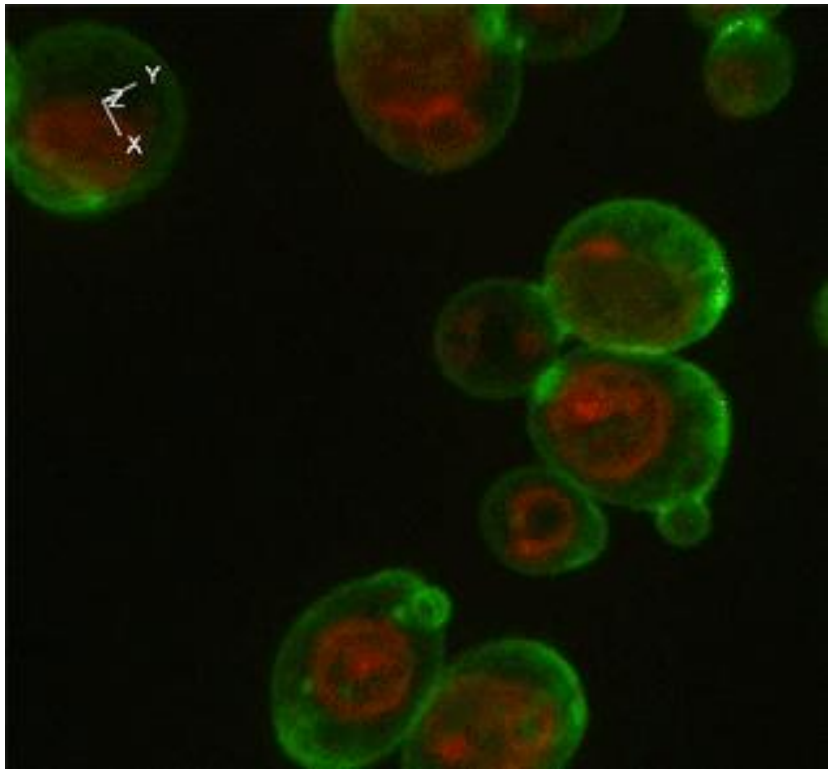
Scanning confocal fluorescence microscopy

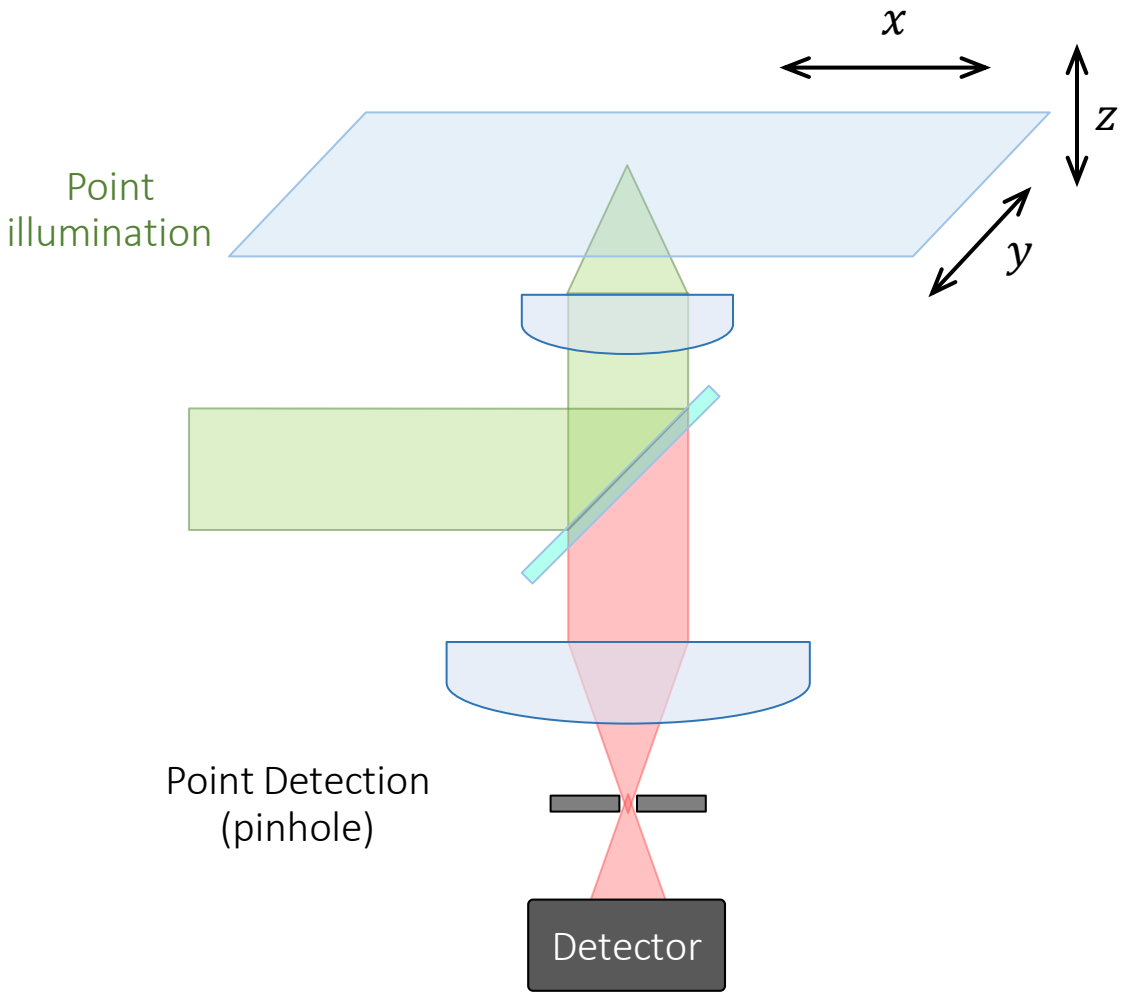
Scanning
Fluorescence Microscope



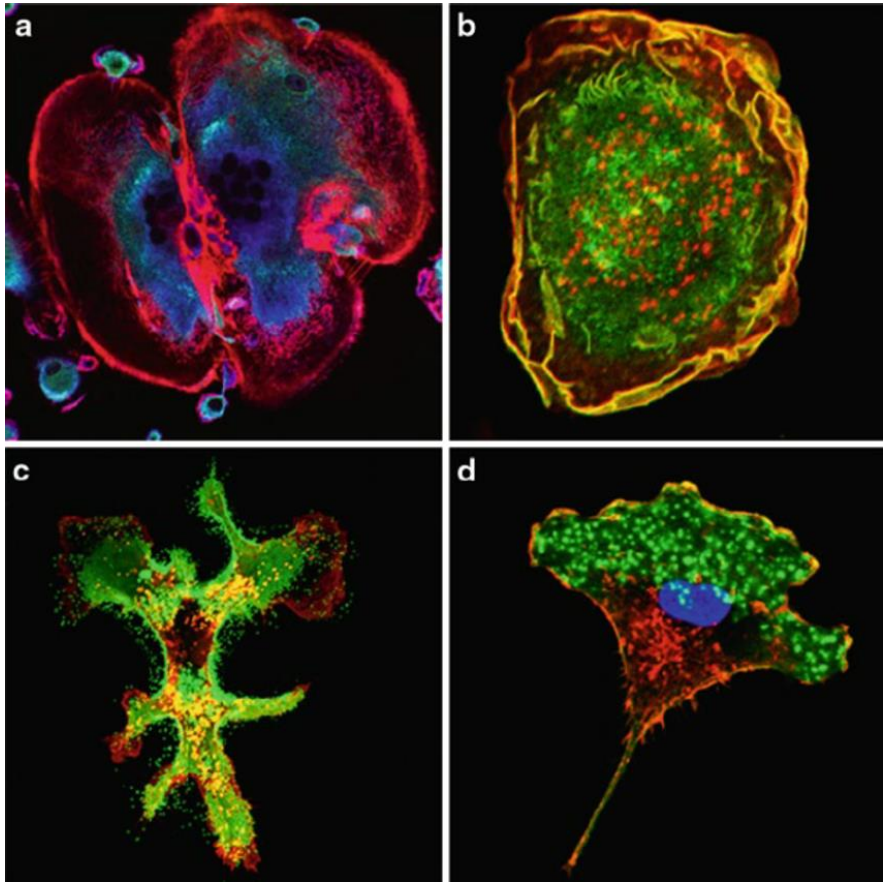


Scanning confocal fluorescence microscopy

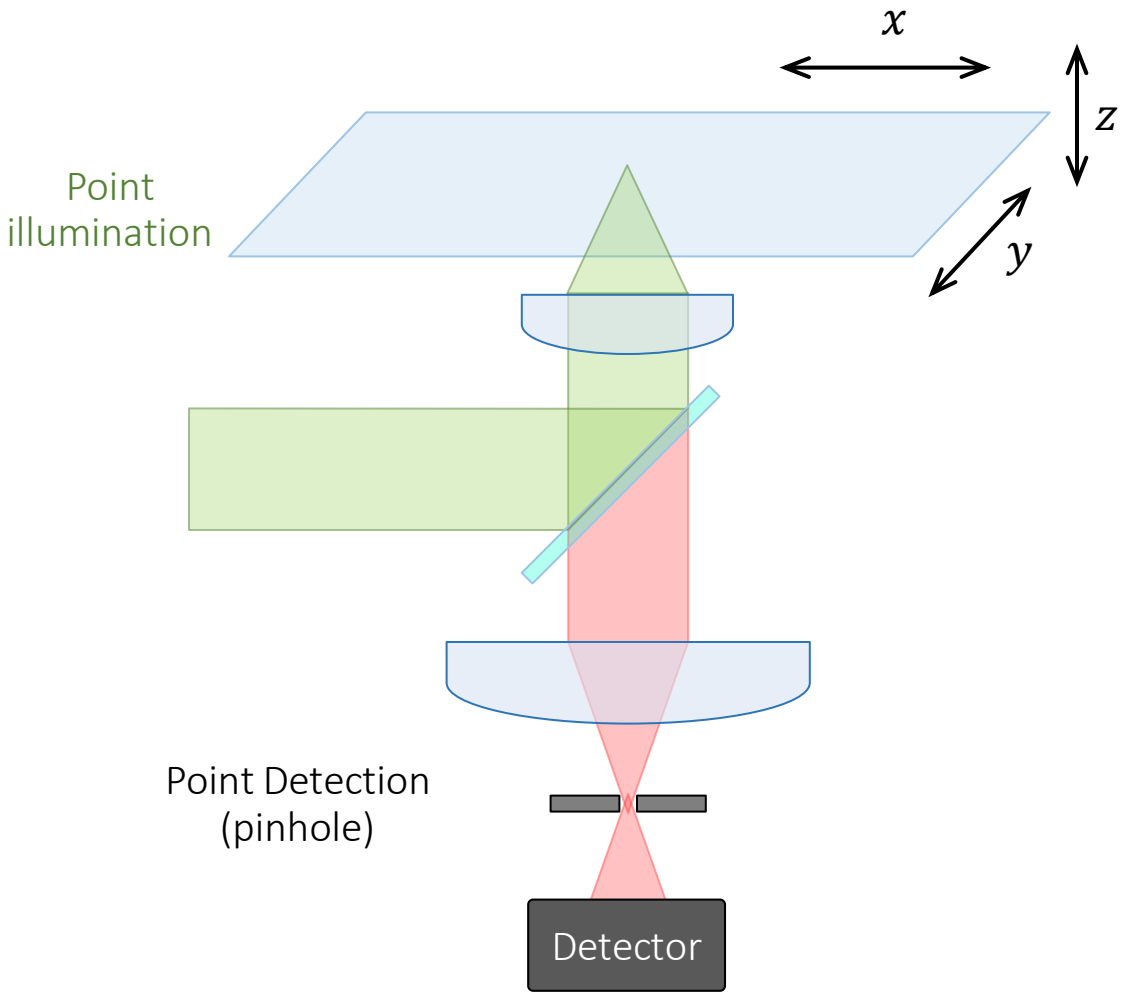




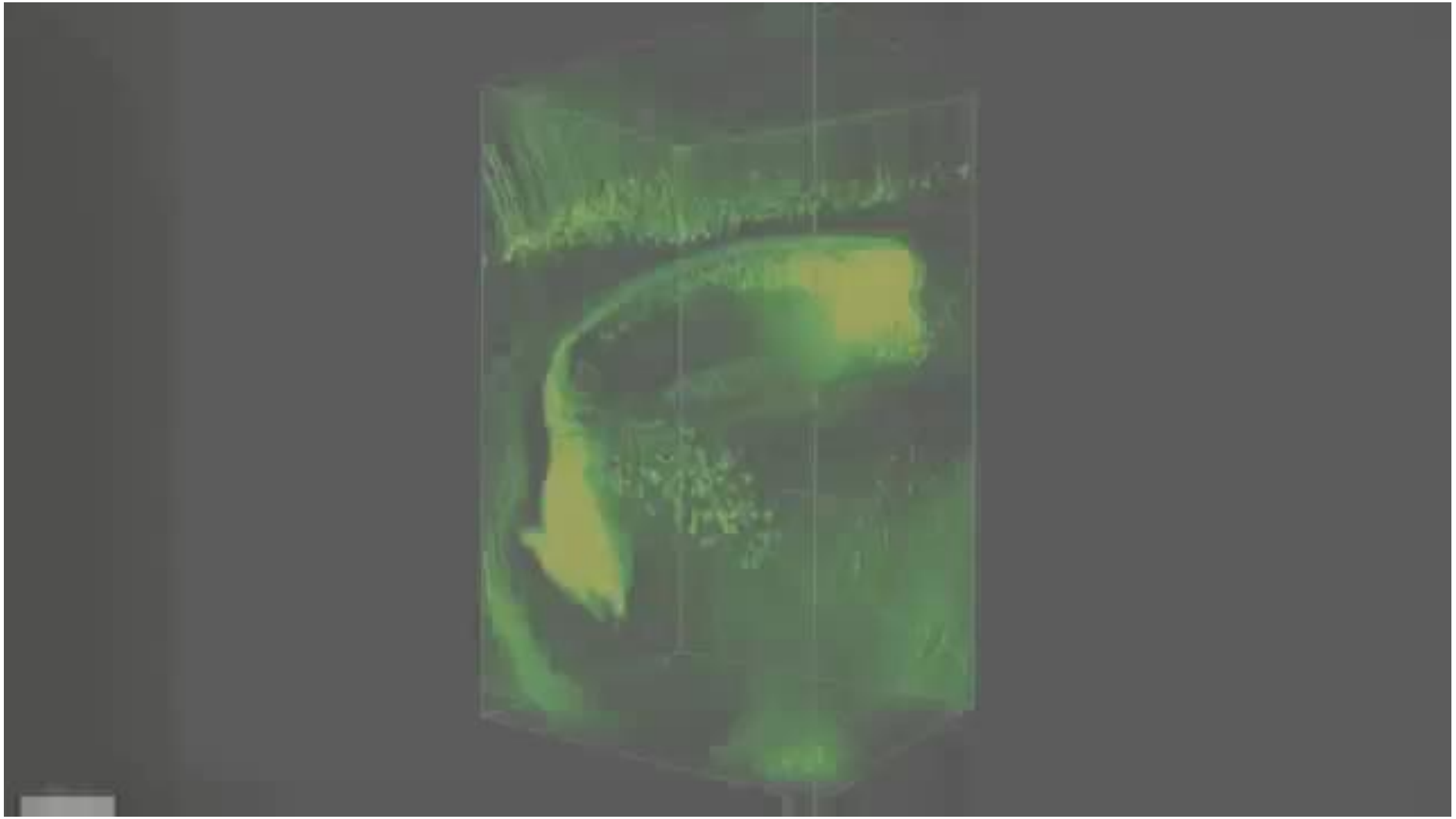
Scanning confocal fluorescence microscopy



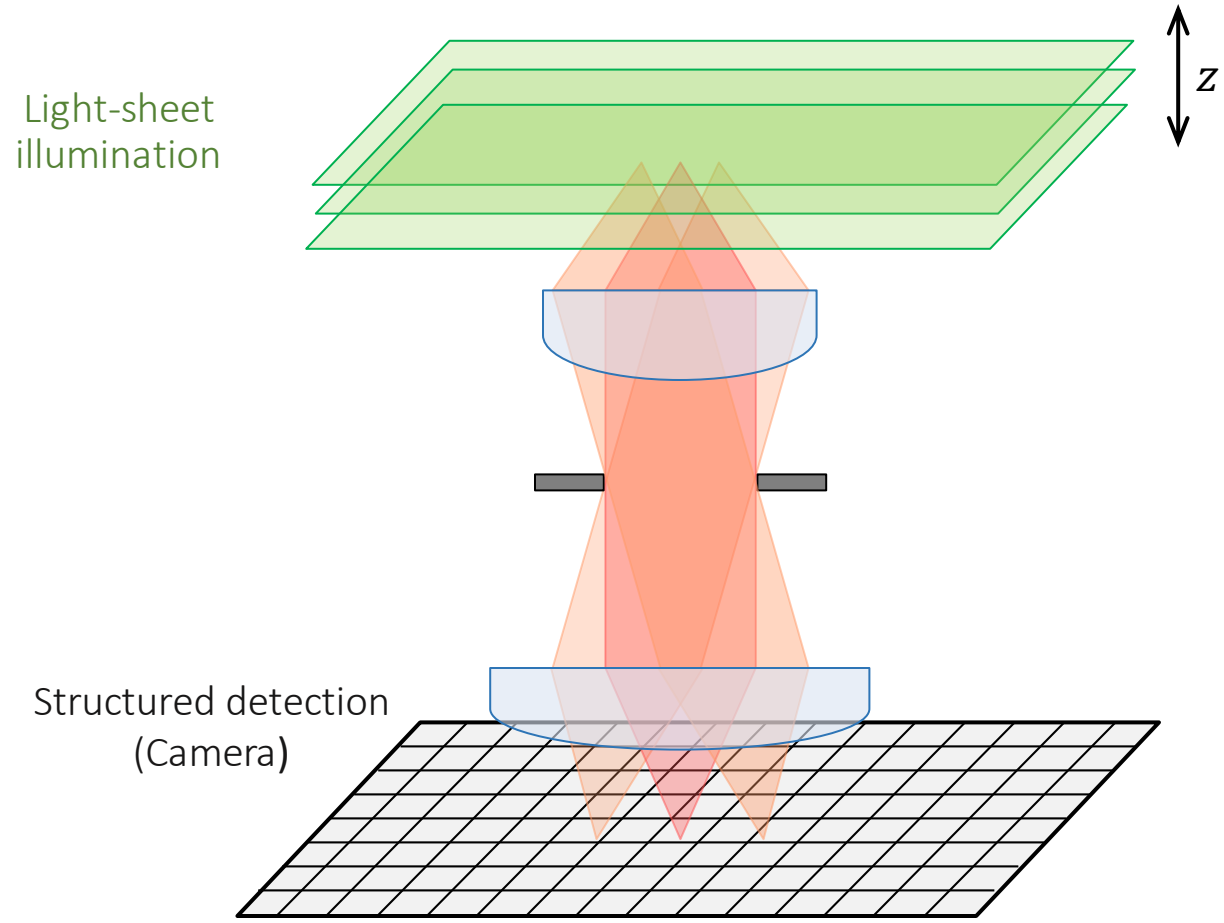
O. Hanrahan, J. Harris, & C. Egan.
Advanced Microscopy: Laser Scanning Confocal Microscopy.
Methods in molecular biology 784 (2011) 169-80



Scanning confocal fluorescence microscopy

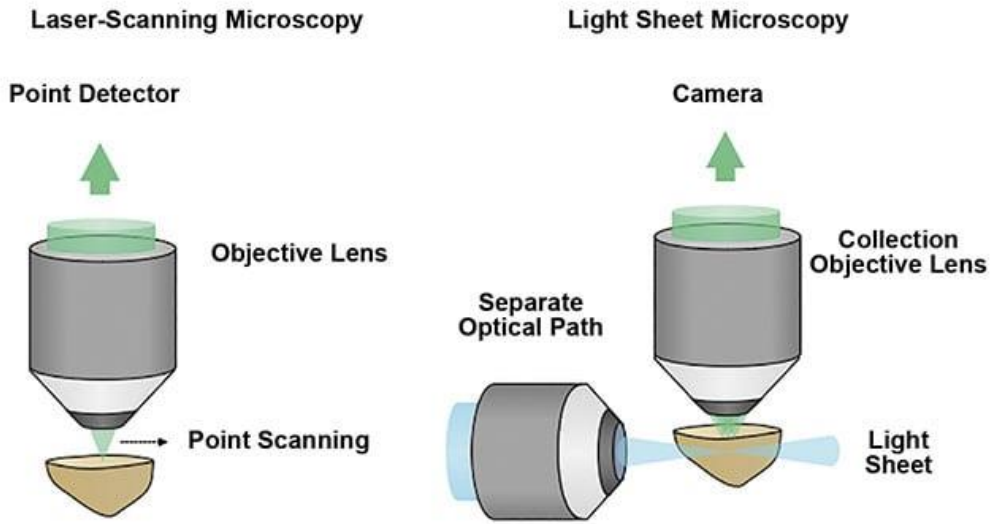


Light-sheet fluorescence microscopy

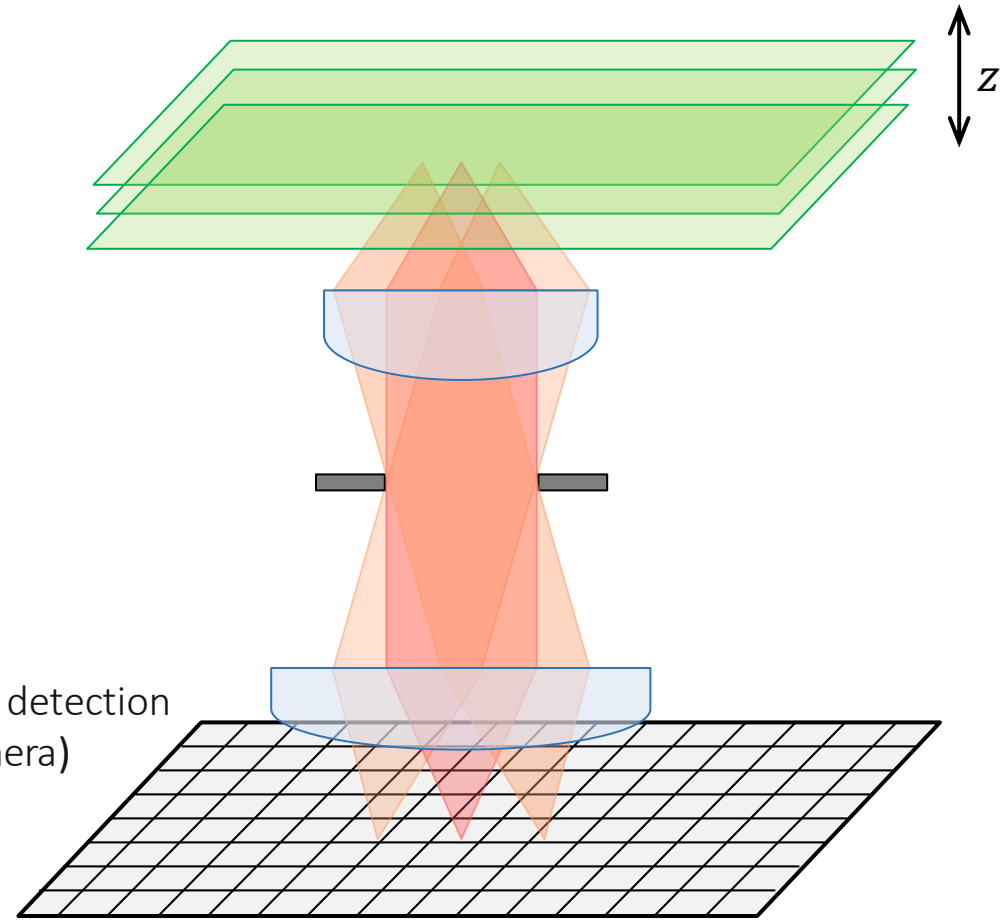


- High throuput
- Lower exposure/less photobleaching

Light-sheet fluorescence microscopy



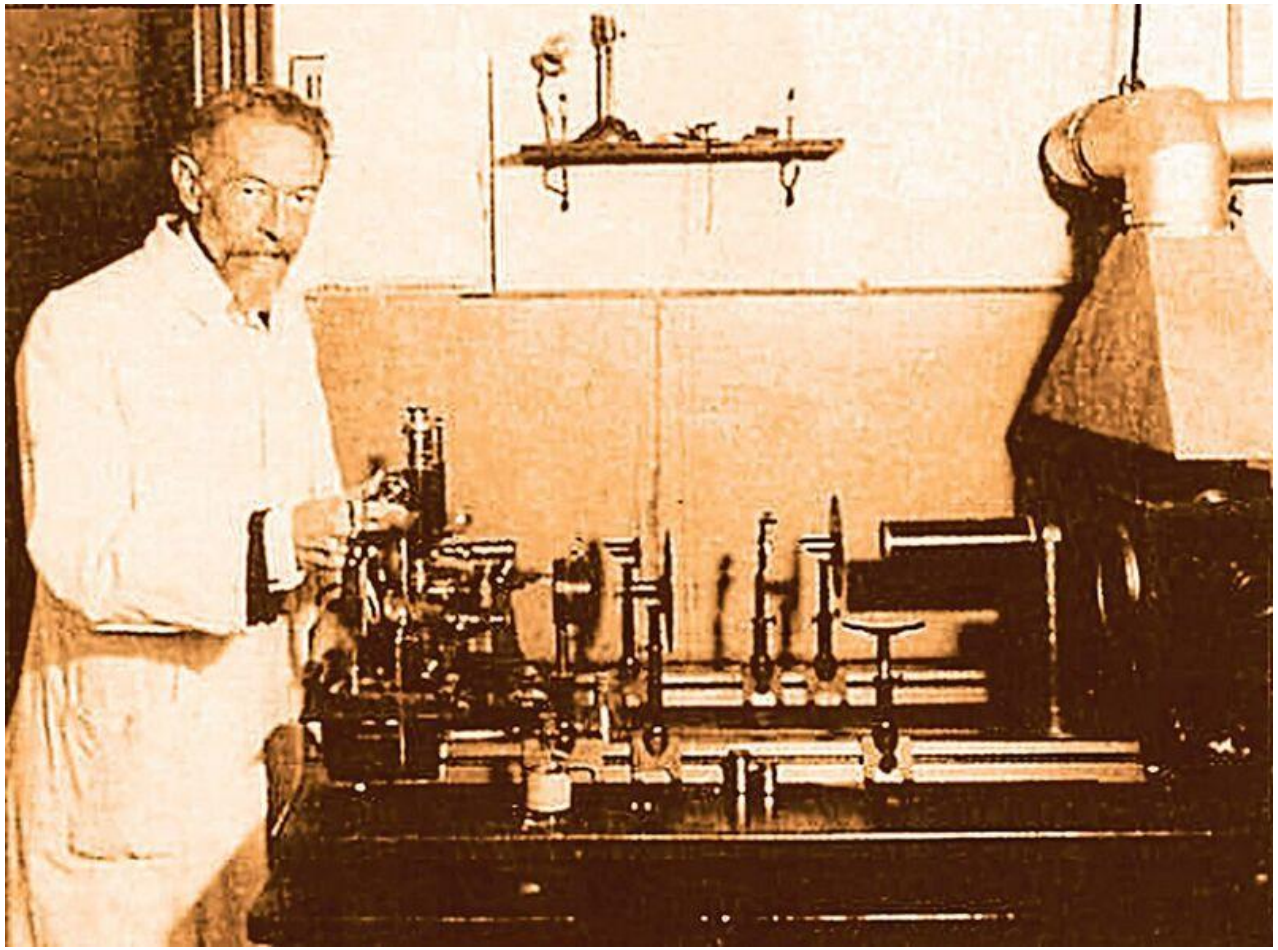
Light-sheet illumination

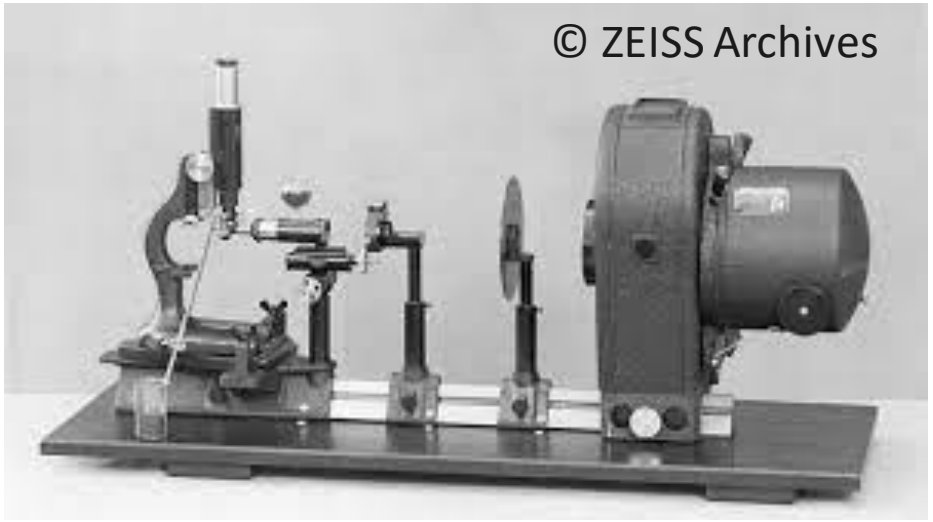


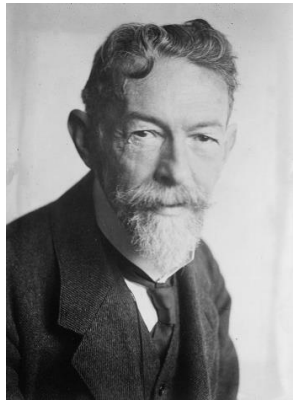
Structured detection (Camera)

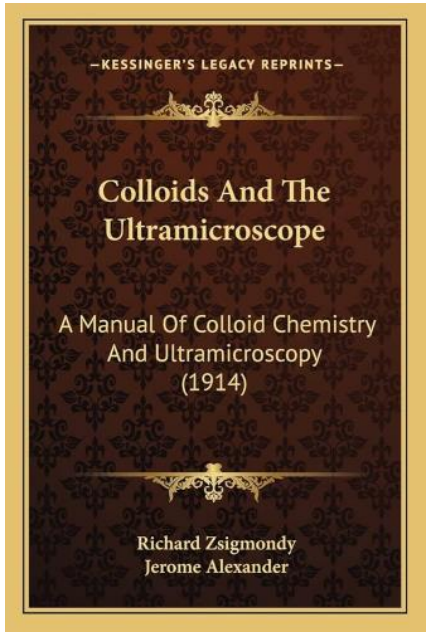
- High throuput
- Lower exposure/less photobleaching

The “Ultramicroscope” 1903





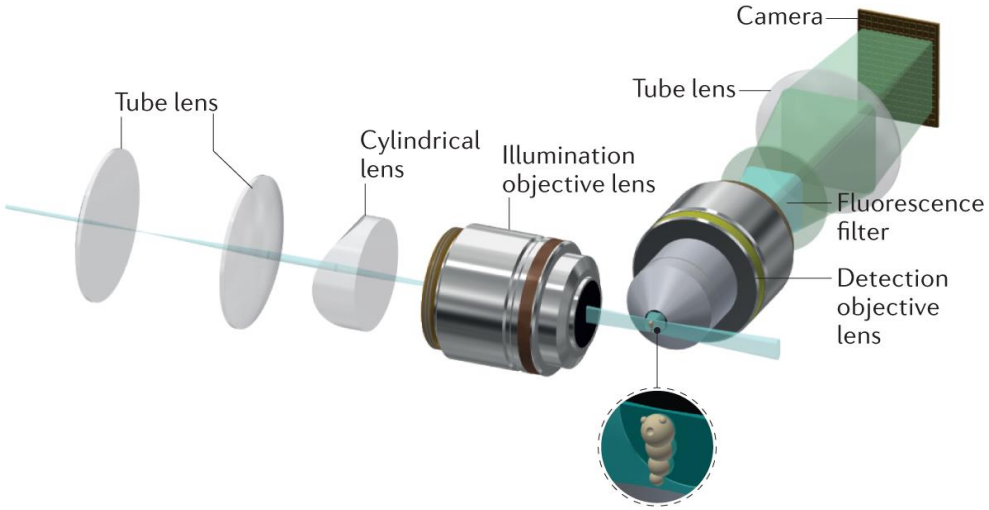


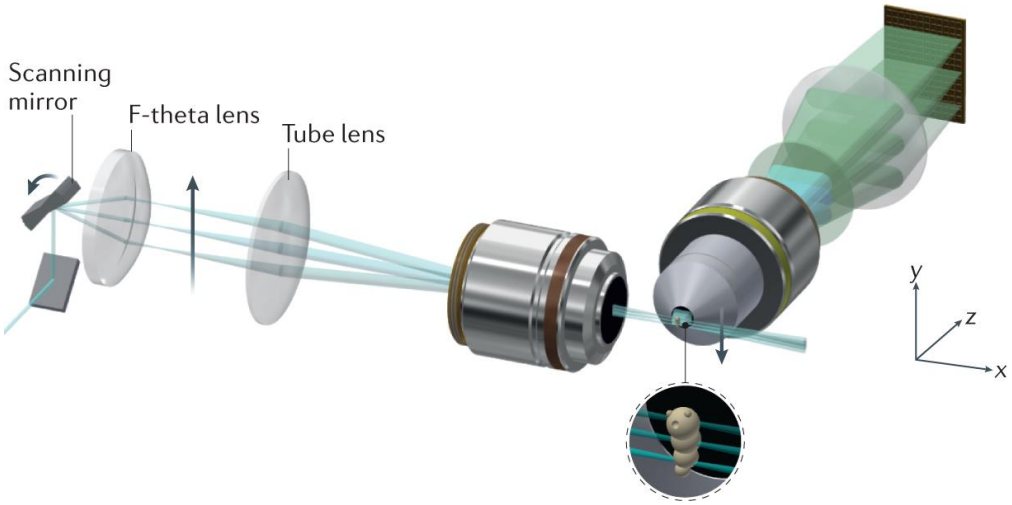


Richard Zsigmondy

Nobel Prize in Chemistry 1925

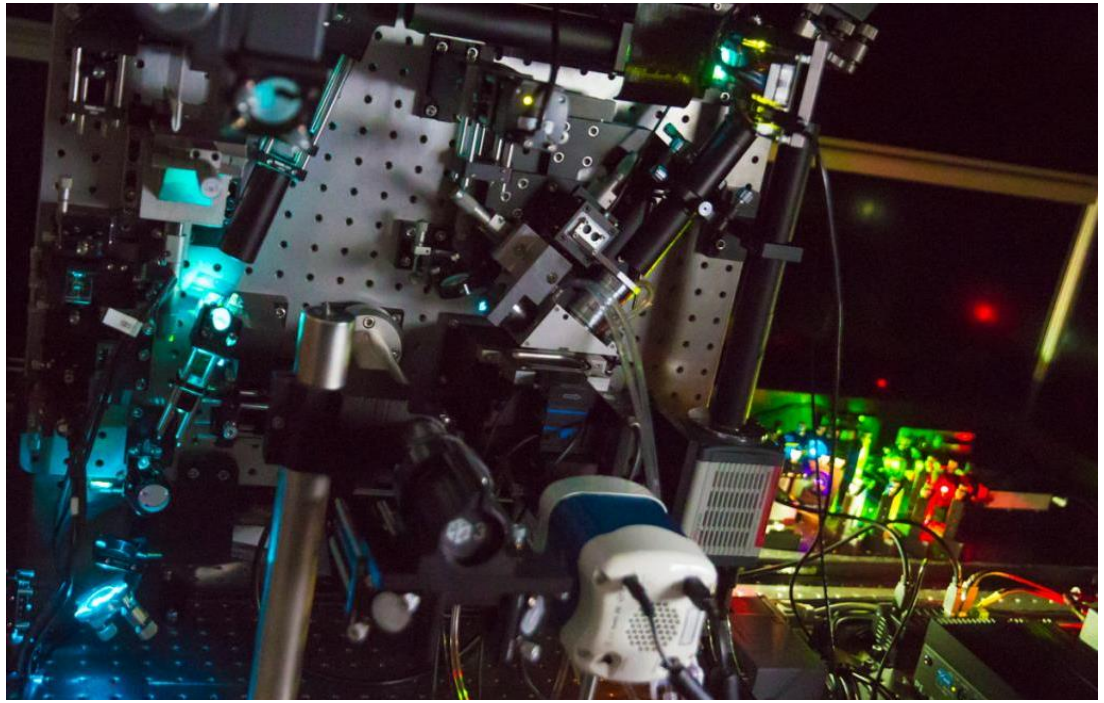
Light-sheet fluorescence microscopy





Stelzer, E.H.K., Strobl, F., Chang, BJ. *et al.* Light sheet fluorescence microscopy. *Nat Rev Methods Primers* 1 (2021) 73

Light-sheet fluorescence microscopy



https://huiskenlab.com/flamingo/

PROF. DR. JAN HUISKEN
Georg-August-University Göttingen

Light-sheet fluorescence microscopy

LUXENDO
A BRUKER COMPANY

BRUKER

Drosophila Embryo Development

What is it?

Transgenic line expressing His2Av-mCherry as fluorescent nuclear reporter. The fruit fly embryo was imaged for almost one complete day (4 x 200 slices every 30 seconds).

Courtesy of:

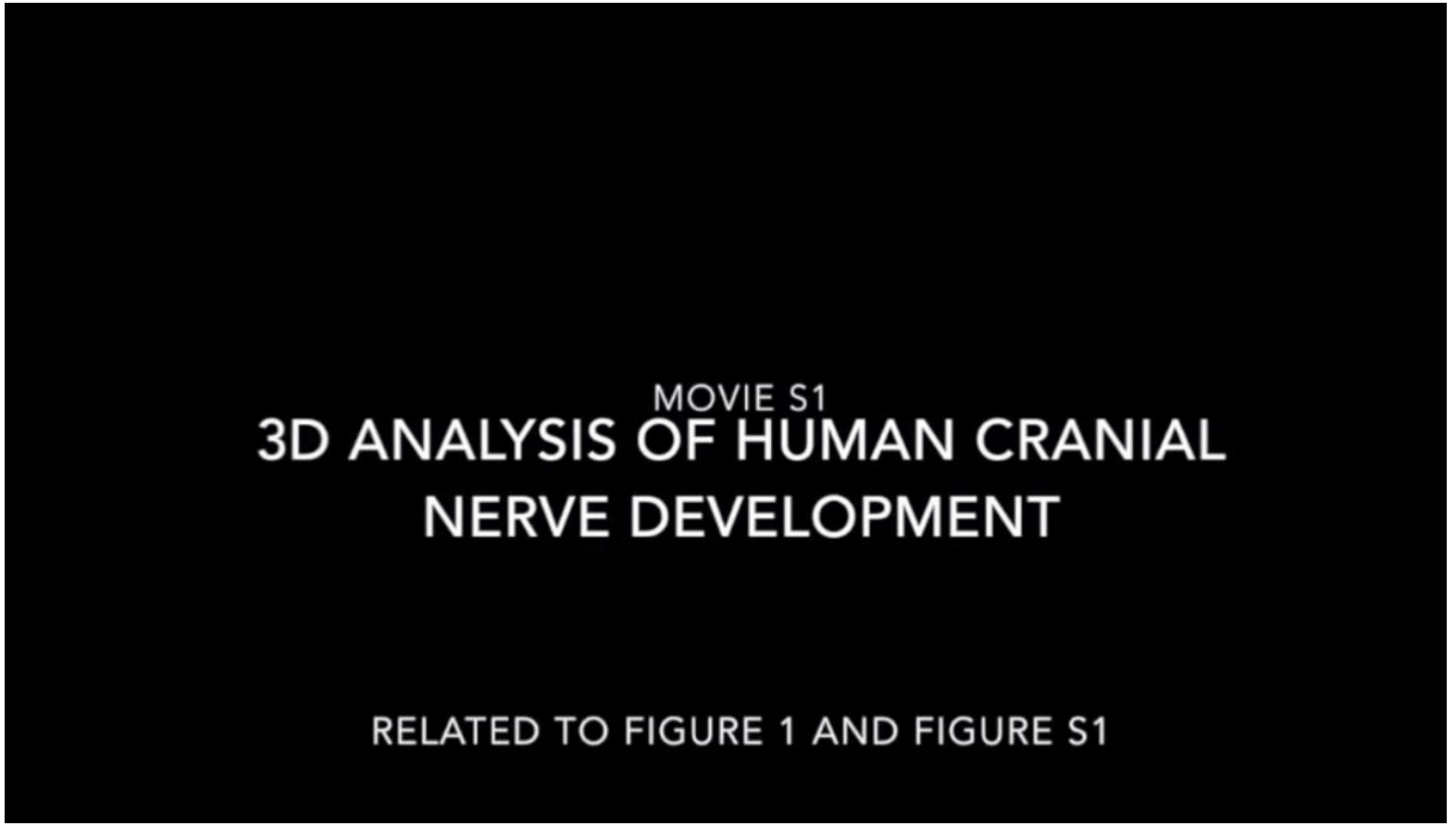
Lars Hufnagel
European Molecular Biology Laboratory (EMBL)
Heidelberg, Germany

Transgenic line expressing His2Av-mCherry as fluorescent nuclear reporter
1 day imaging (4 × 200 slices every 30 seconds)

Light-sheet fluorescence microscopy

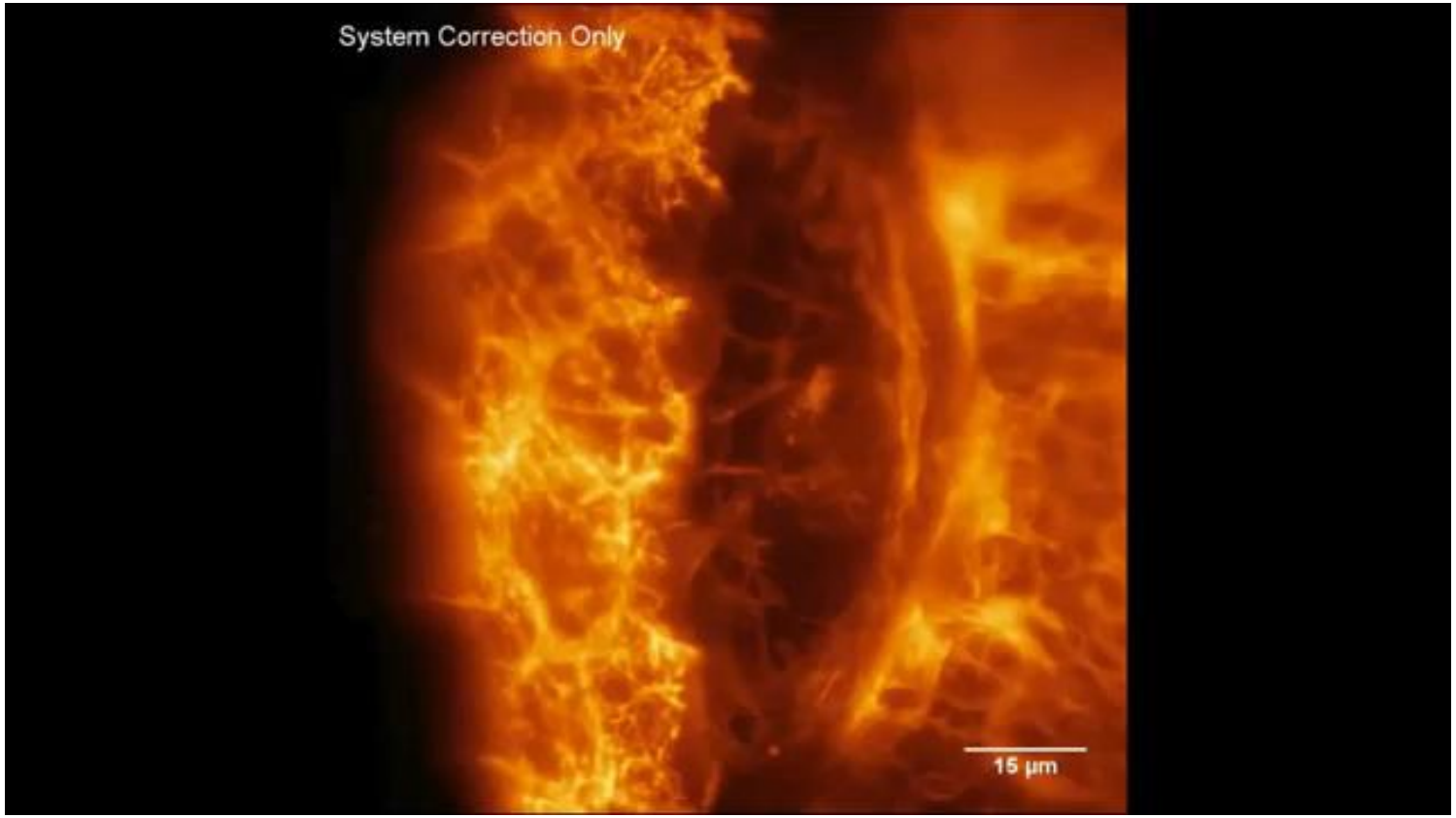
Zebrafish imaged 16 hours every 10min

Light-sheet fluorescence microscopy



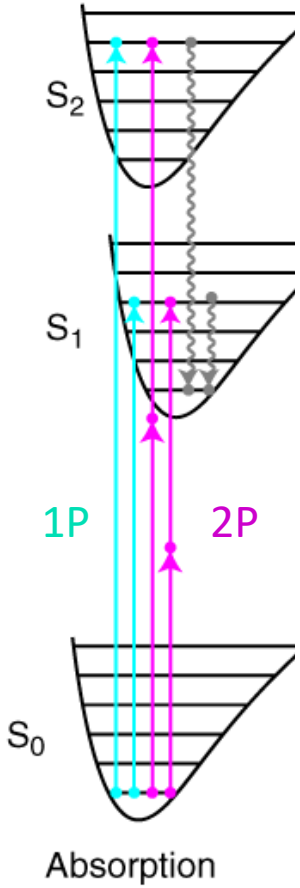
Human cranial nerve development
M. Belle et al. *Cell* 169 (2017) 161-173

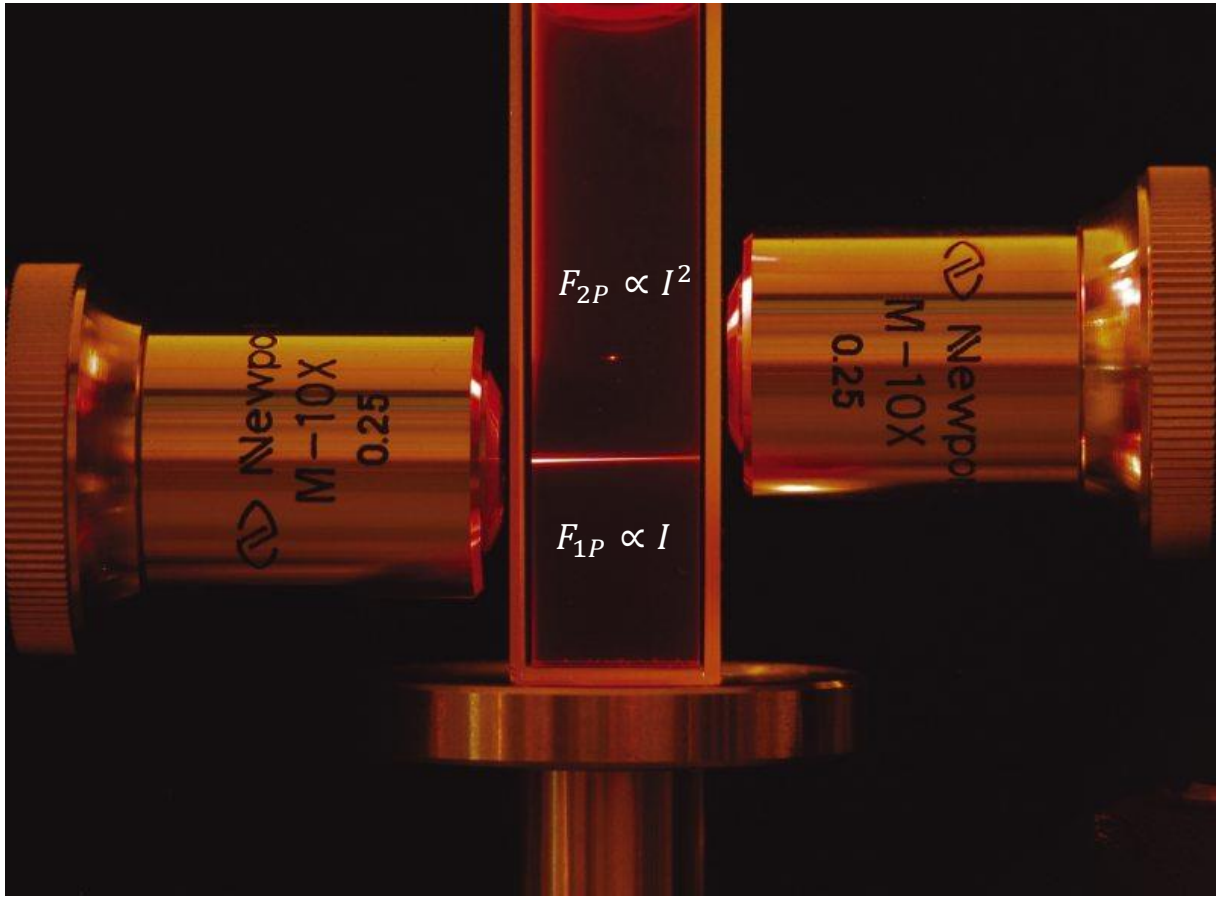
Light-sheet fluorescence microscopy



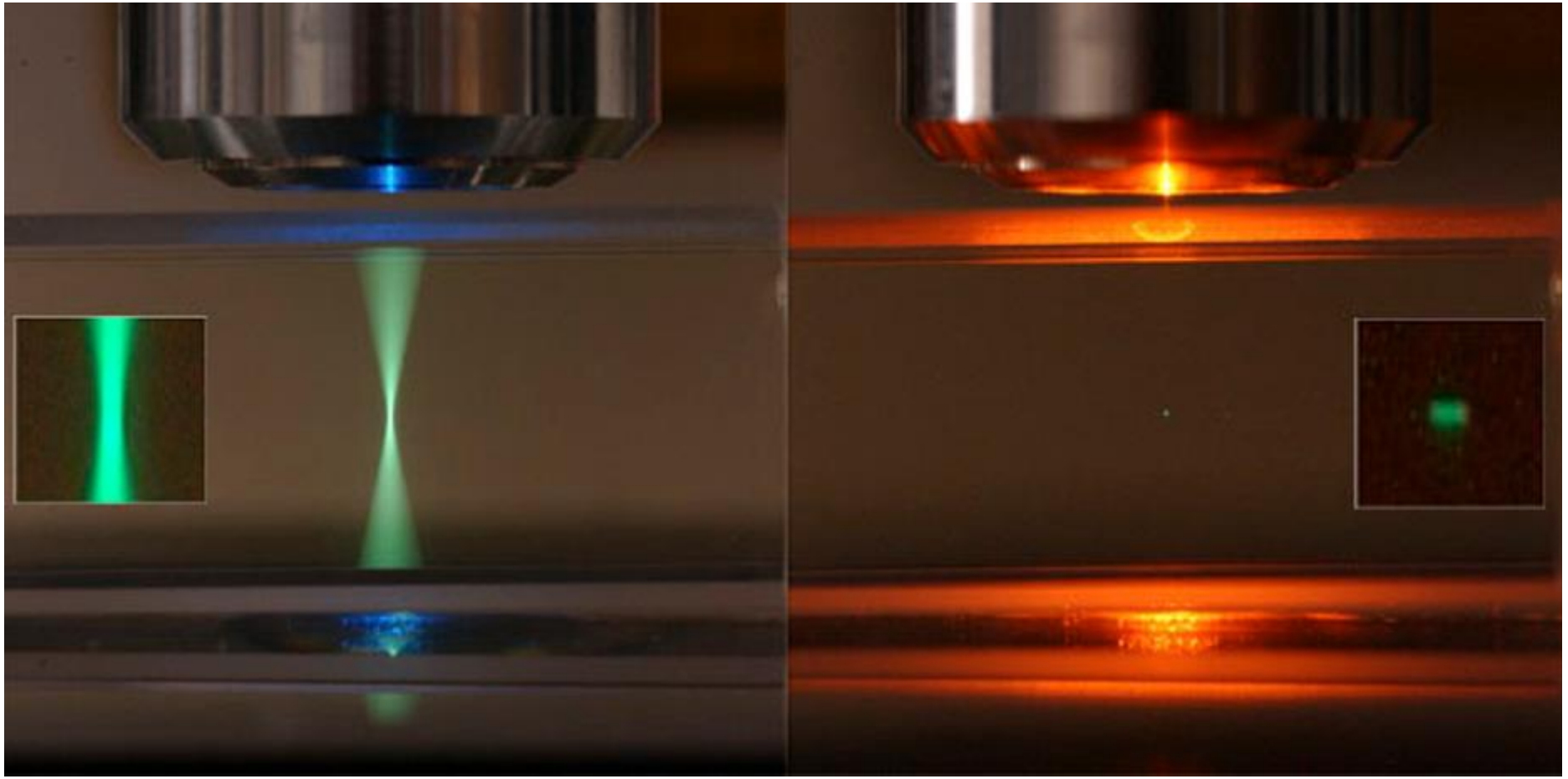
https://www.hhmi.org/news/new-microscope-captures-detailed-3-d-movies-cells-deep-within-living-systems
Liu et al., *Science* 360, eaaq1392 (2018)

Two-photon fluorescence microscopy

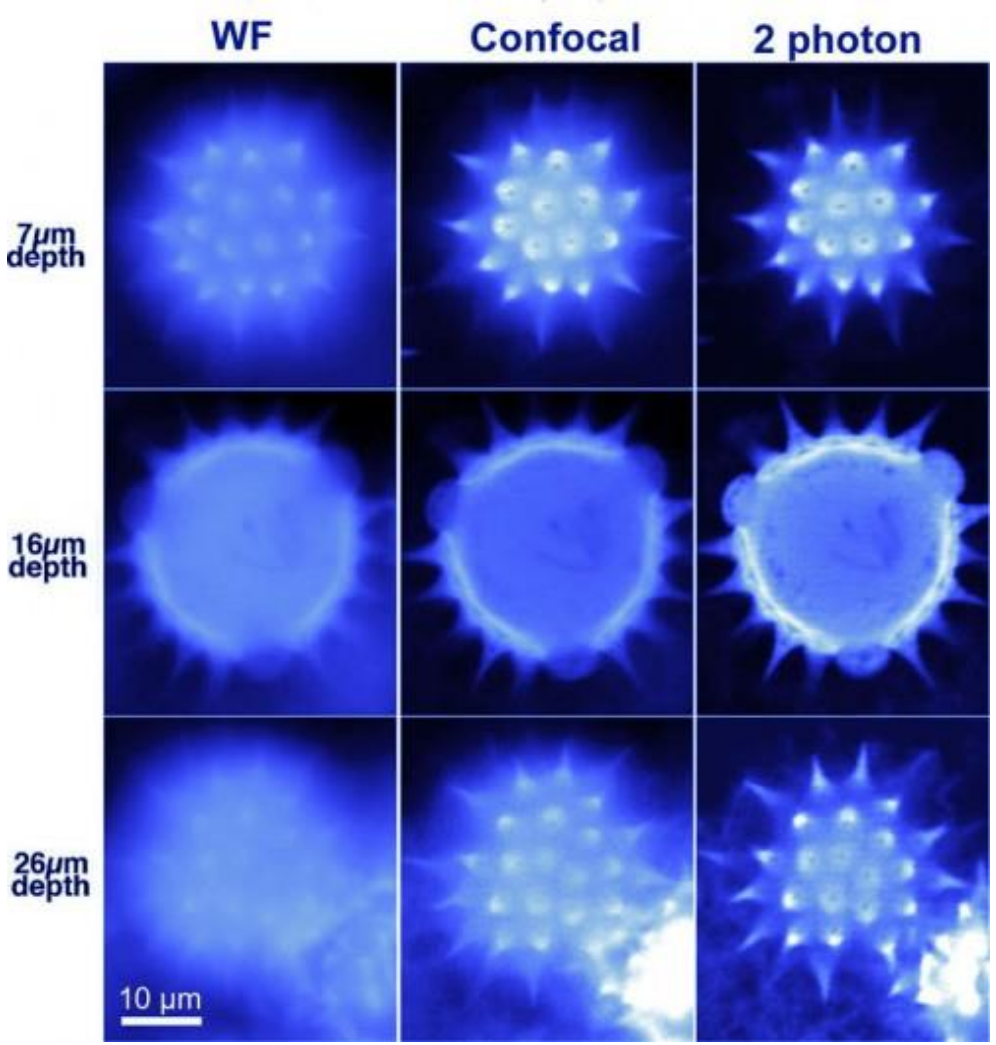




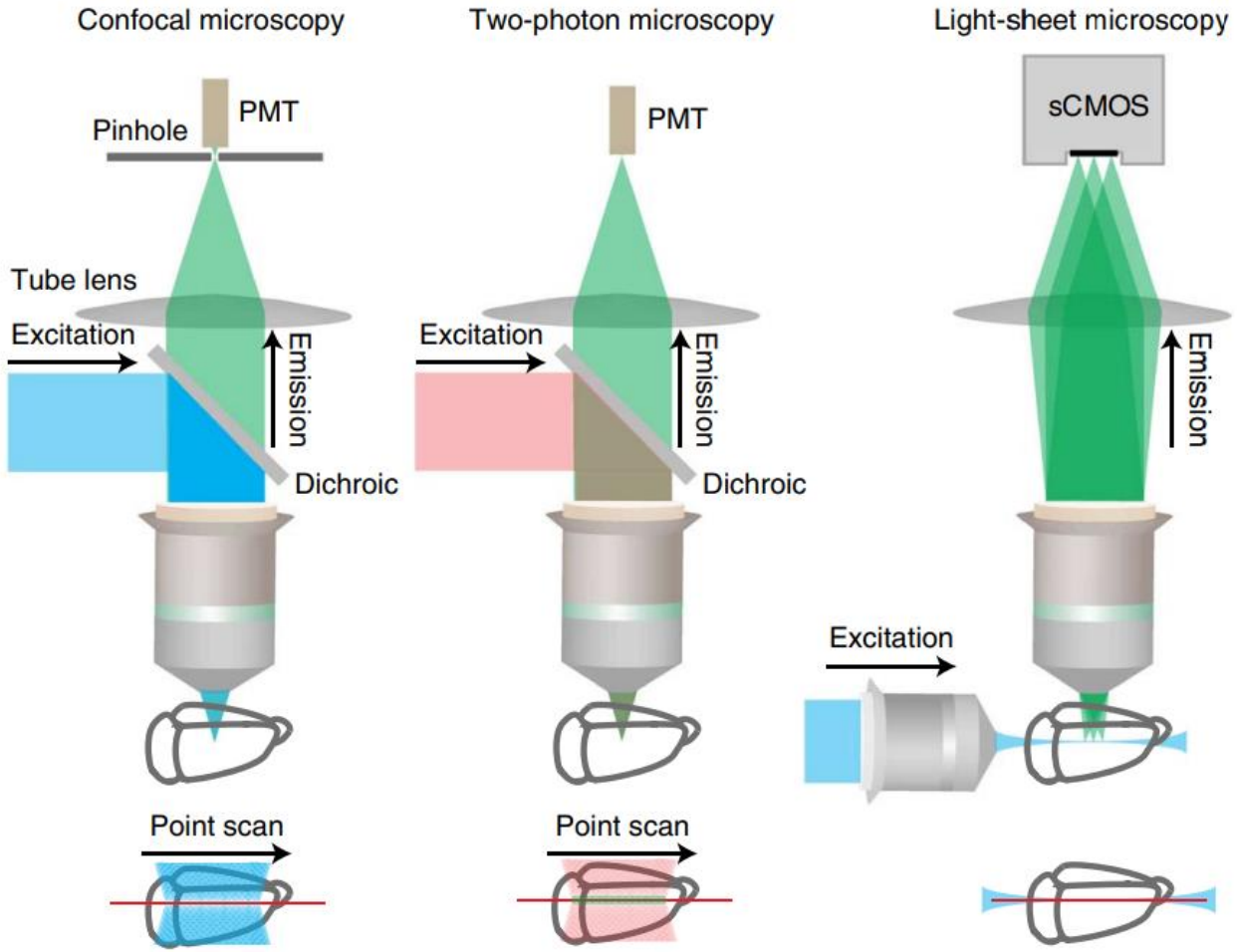
Two-photon fluorescence microscopy



Two-photon fluorescence microscopy

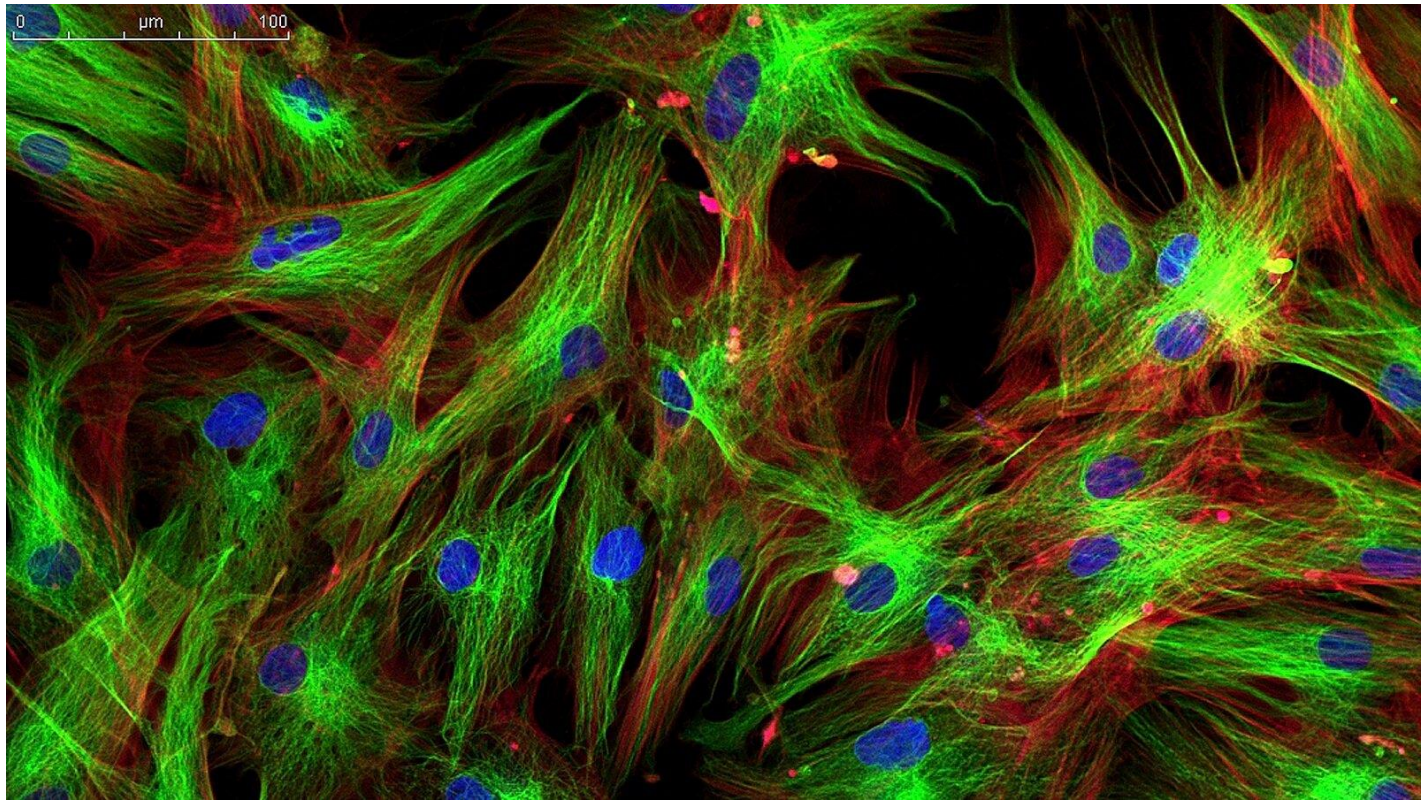


Confocal, two-photon, and light-sheet fluorescence microscopy



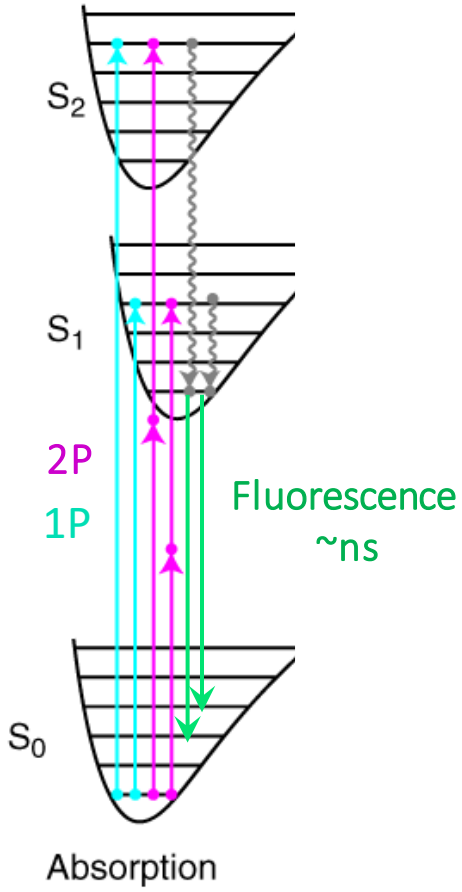
Tomer et al. "Advanced CLARITY for rapid and high-resolution imaging of intact tissues"
Nature Protocols 1682 9 (2014)

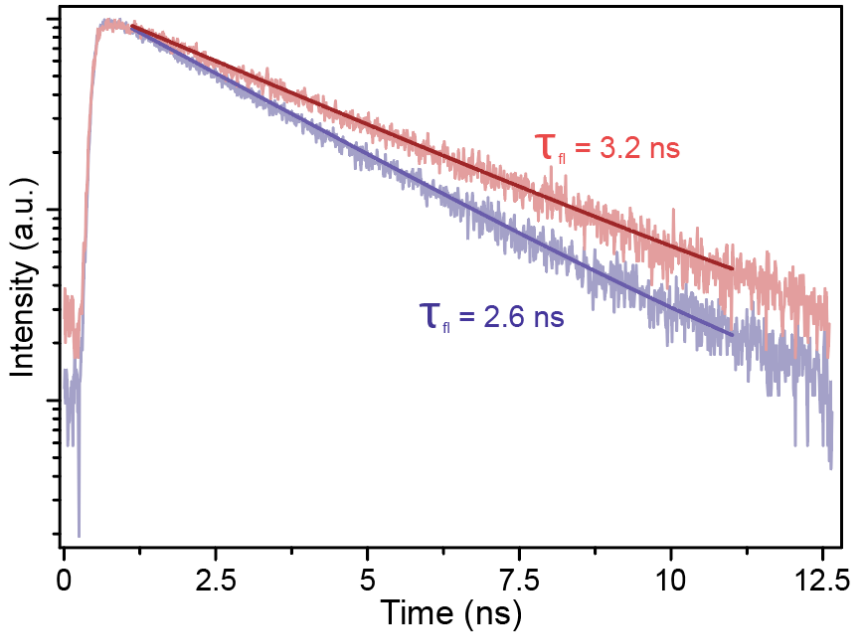
Fluorescence signals: intensity



- Spatial distribution
- Identification – presence/absence
- Morphology
- (Quantitative abundance)

Fluorescence signals: lifetime

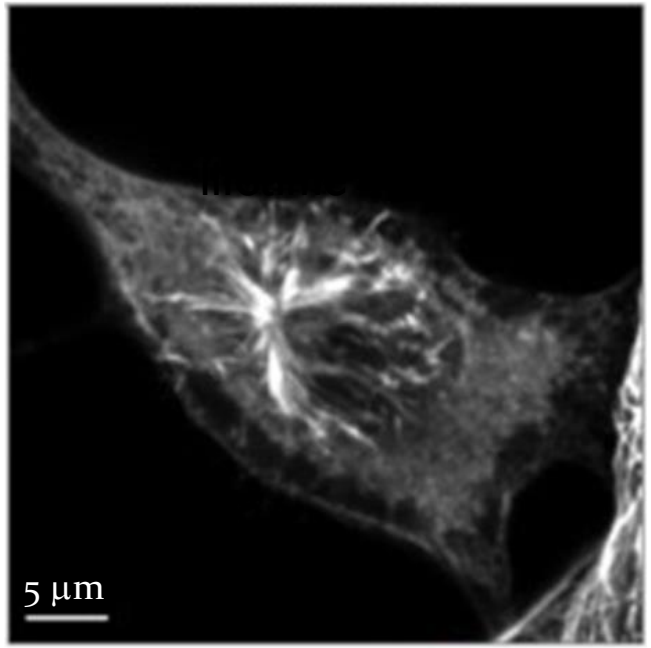


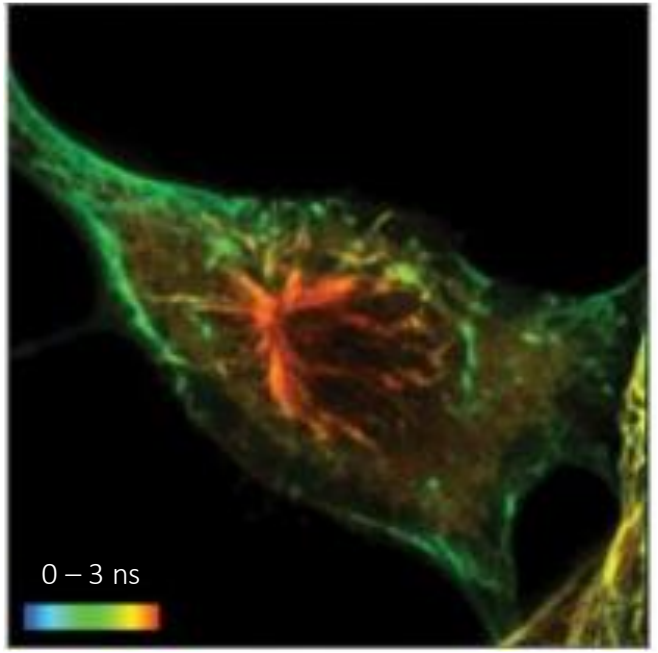


- Molecular environment (conformational changes, association)
- Chemical environment (pH, polarity, etc.)
- Optical environment (PMD)
- Temperature
- Energy transfer
- …

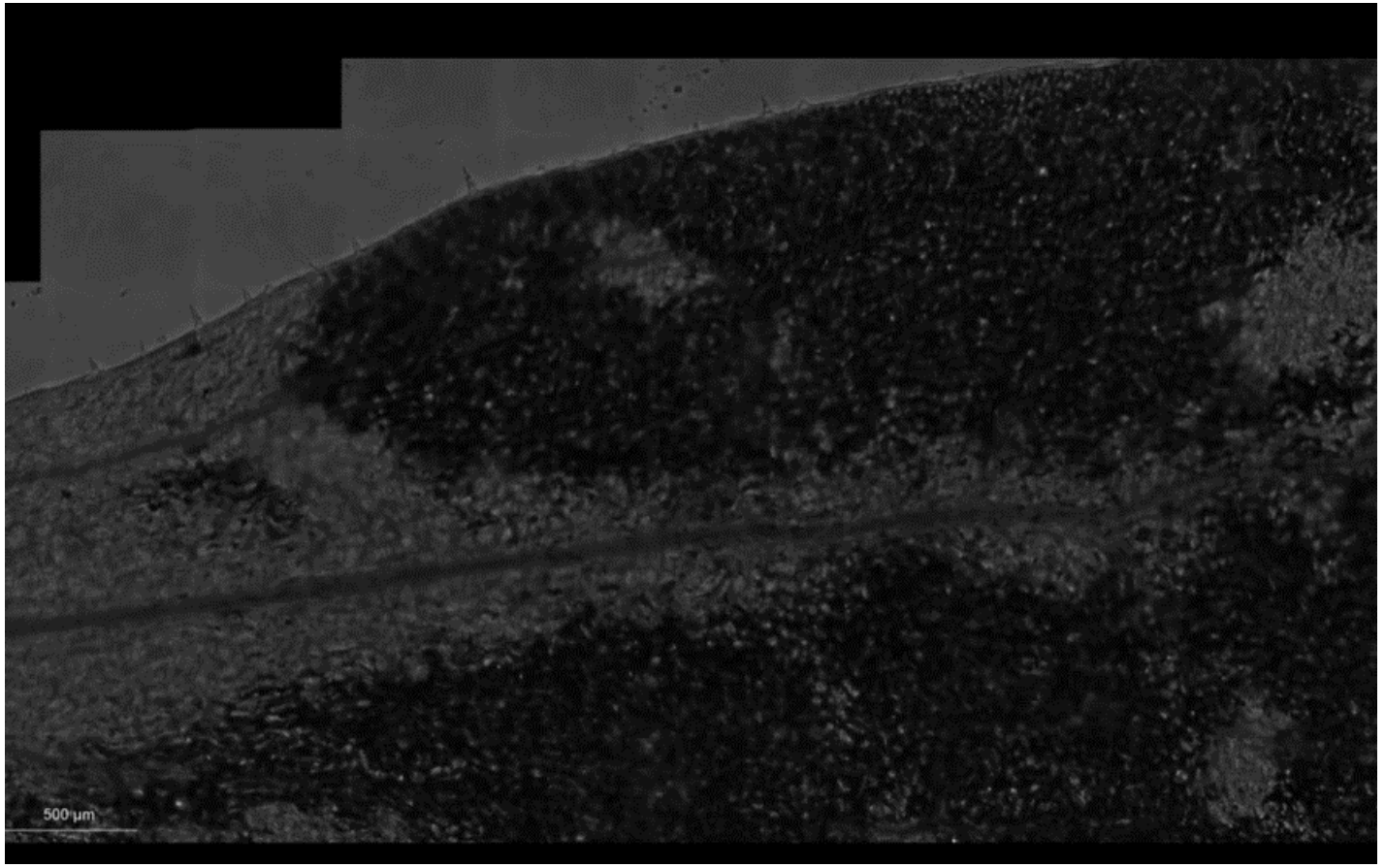
Fluorescence Lifetime Imaging (FLIM)

- Pulsed or modulated light sources
- Time resolved detection
- Sensor marker





Fluorescence Lifetime Imaging (FLIM)

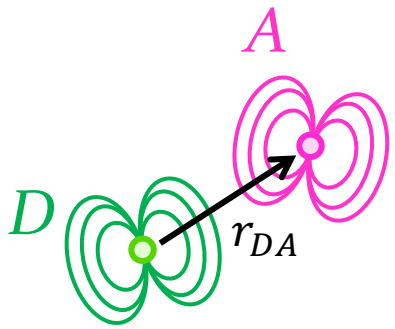


Transmission image shows the leaf structure

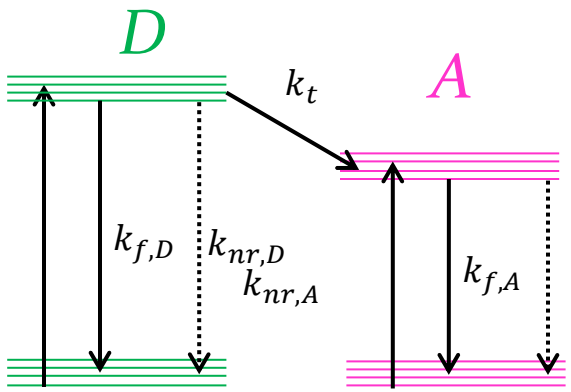
The endogenous chloroplast fluorescence is shown in green.

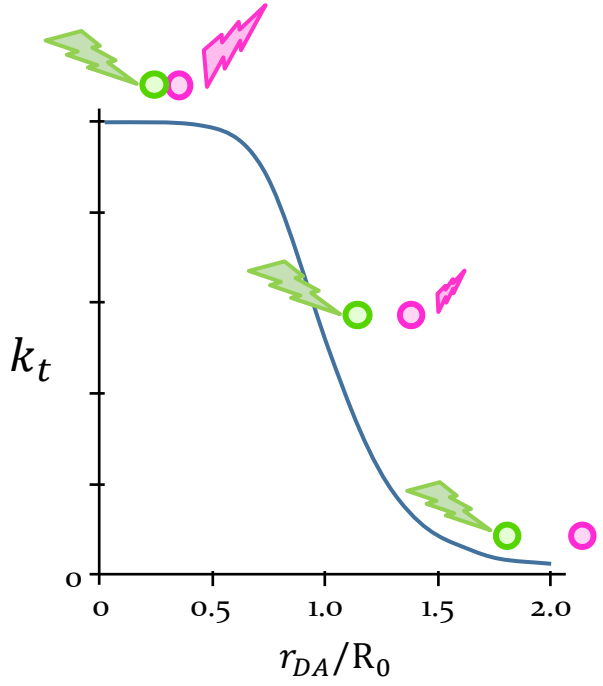
Fluorescence lifetime imaging reveals oxidative potential for each chloroplast.

Fluorescence signals: Förster Resonance Energy Transfer (FRET)



$$k_t = \frac{1}{\tau_D}\left[\frac{R_0}{r_{DA}}\right]^6 \qquad R_0^6 = \frac{J\kappa^2}{n^4}\phi_D$$





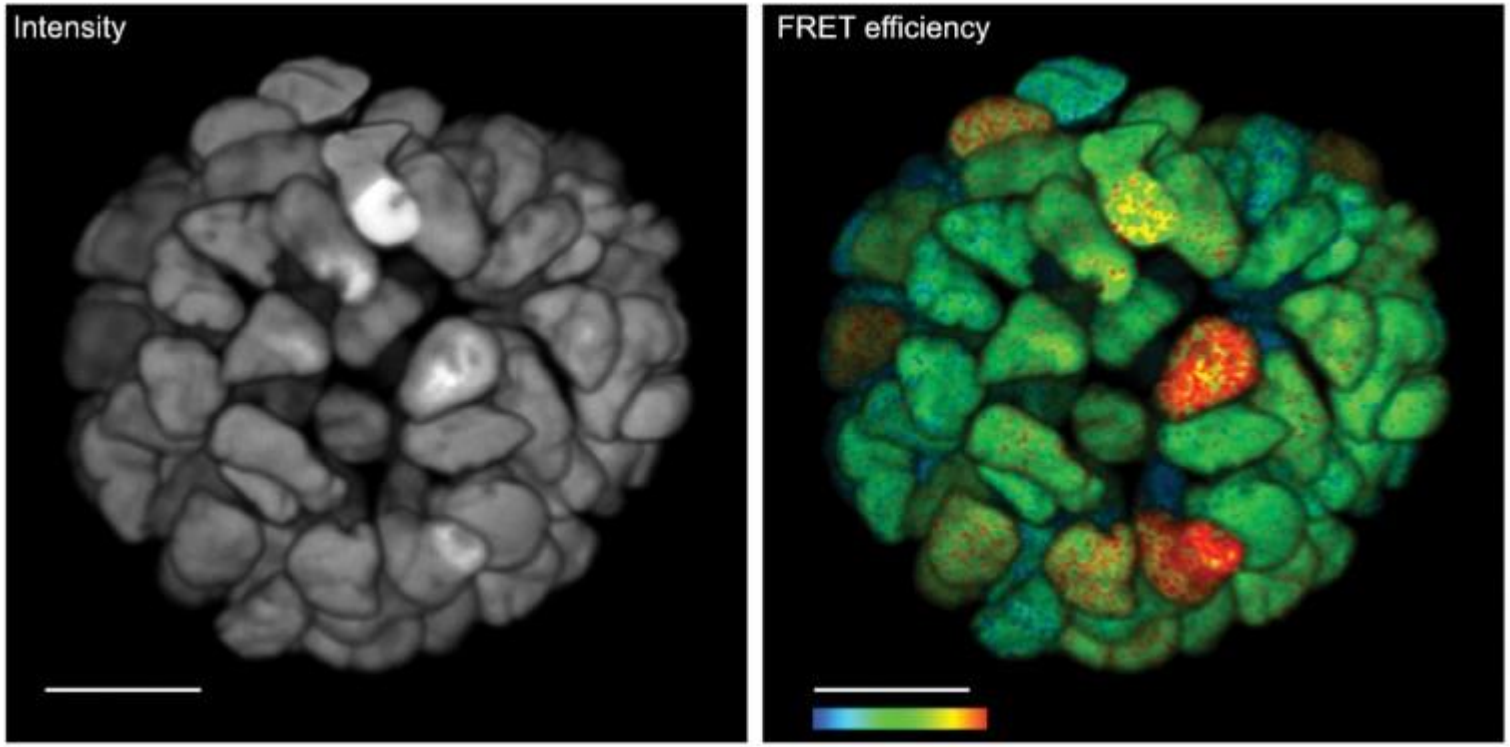
FRET imaging

FRET measurements

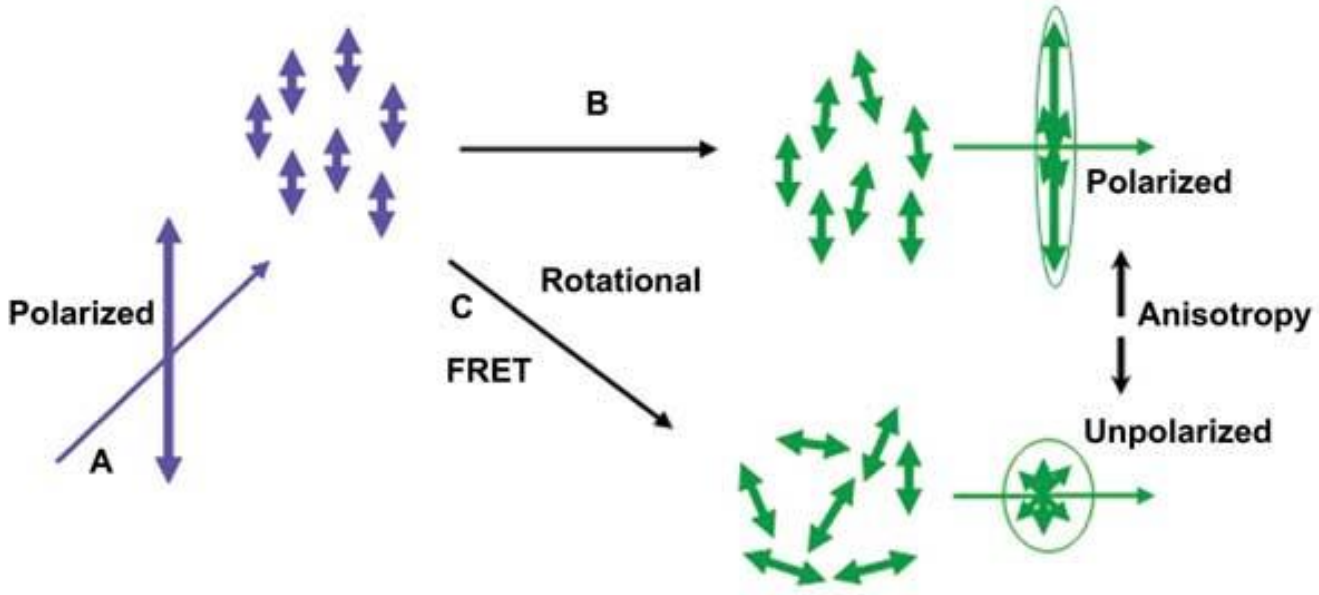
- Intensity
- Lifetime

FRET information

- Molecular interactions
- Conformational changes



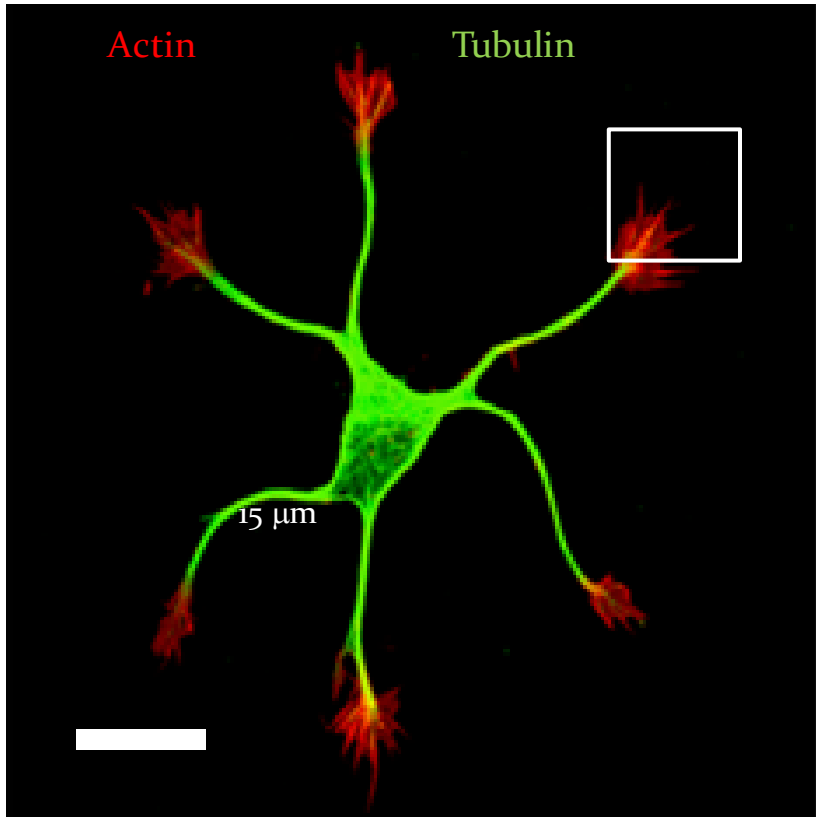
Fluorescence signals: anisotropy



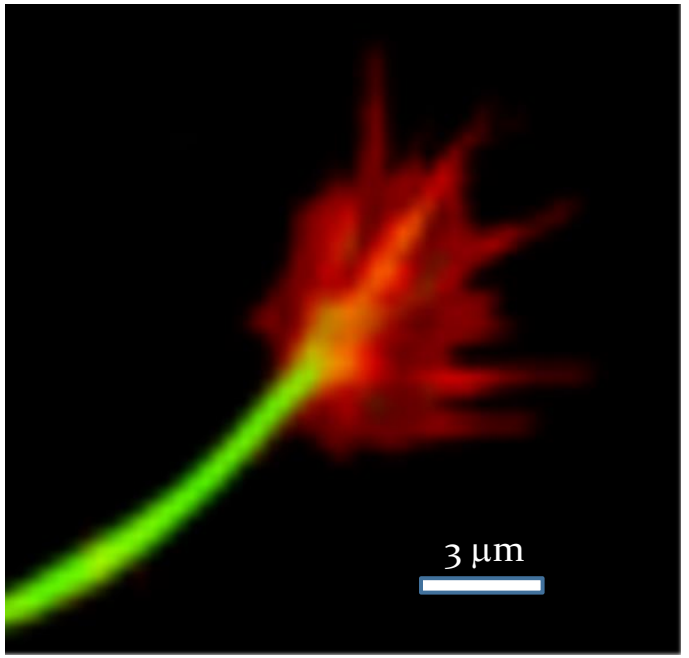
- Polarized excitation
- Polarized detection

- Molecular environment (viscosity)
- Molecular association
- Energy transfer
- …

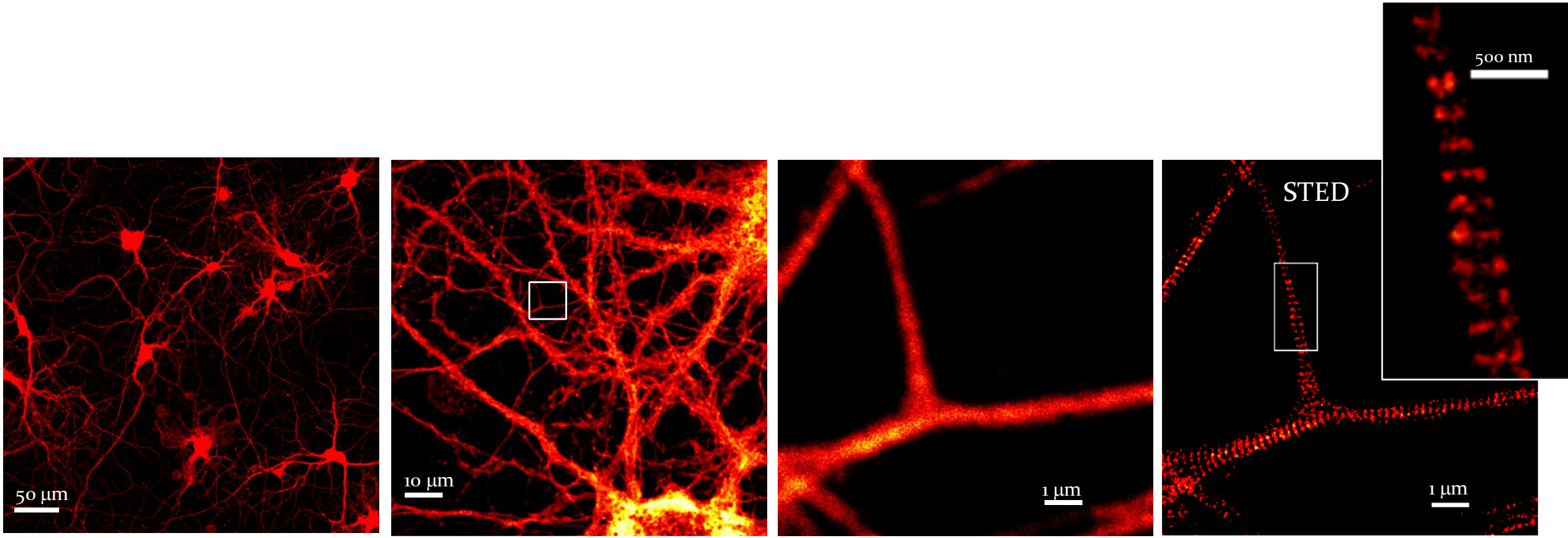
Diffraction-limited spatial resolution



Diffraction-limited imaging



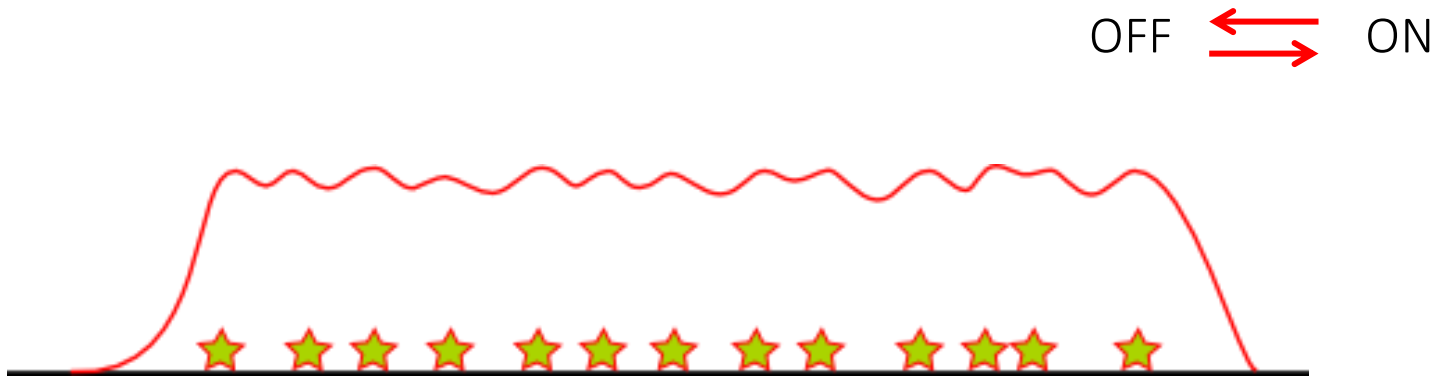
Super-resolution fluorescence microscopy



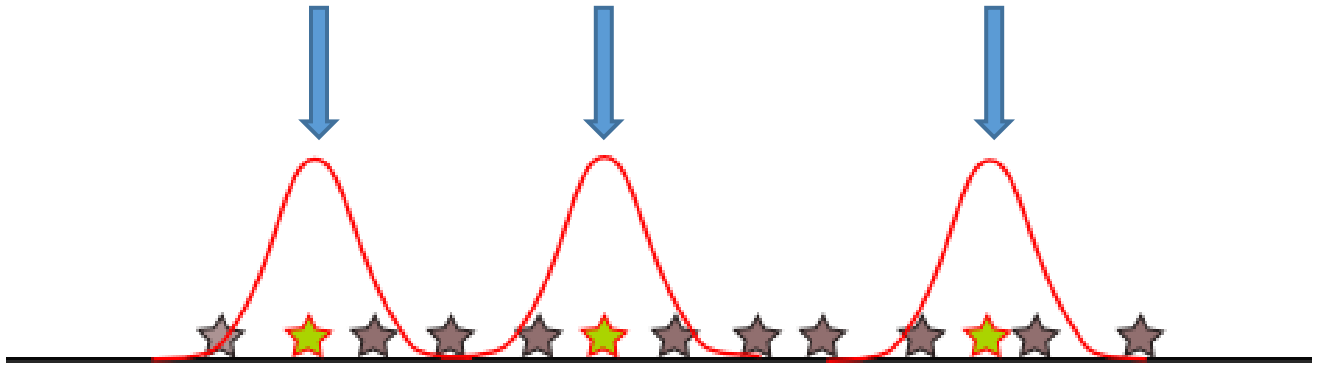
Barabas, F.M., *et al.* Automated quantification of protein periodic nanostructures in fluorescence nanoscopy images: abundance and regularity of neuronal spectrin membrane-associated skeleton.
Sci Rep 7 (2017) 16029

Fluorescence Nanoscopy

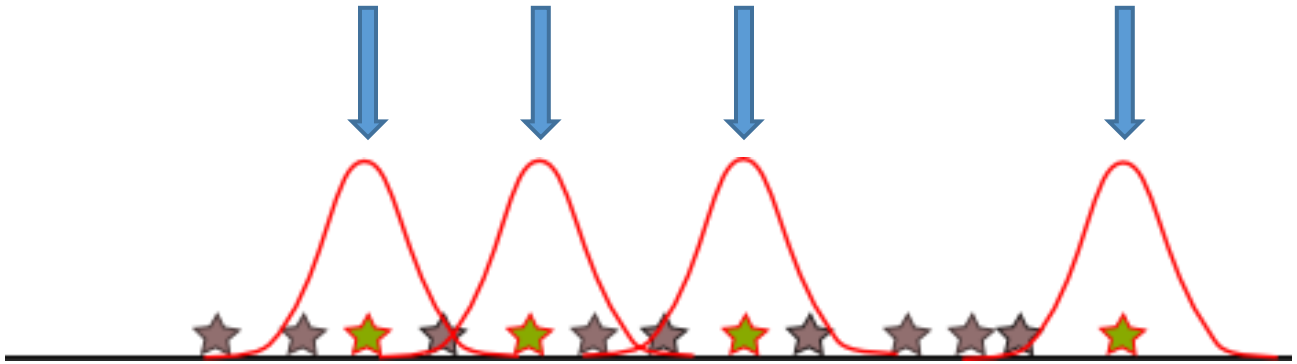
Single-Molecule Localization Microscopy (SMLM)



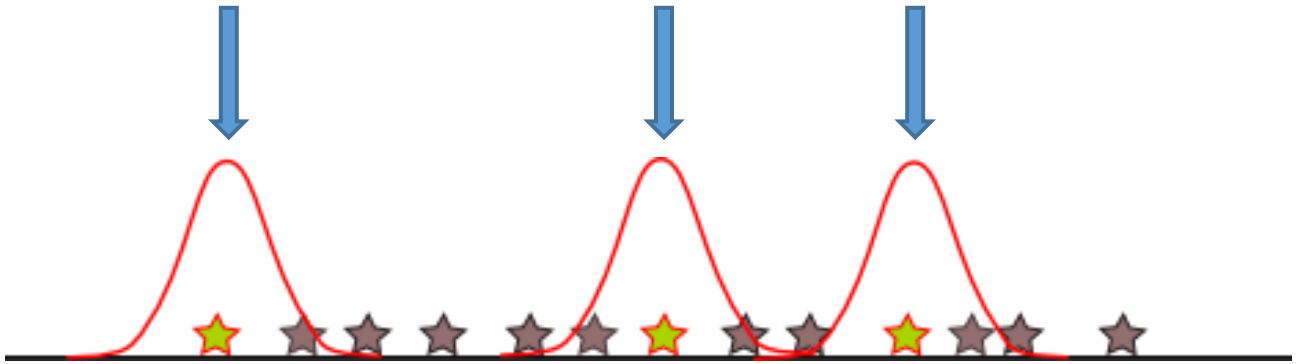
Single-Molecule Localization Microscopy (SMLM)



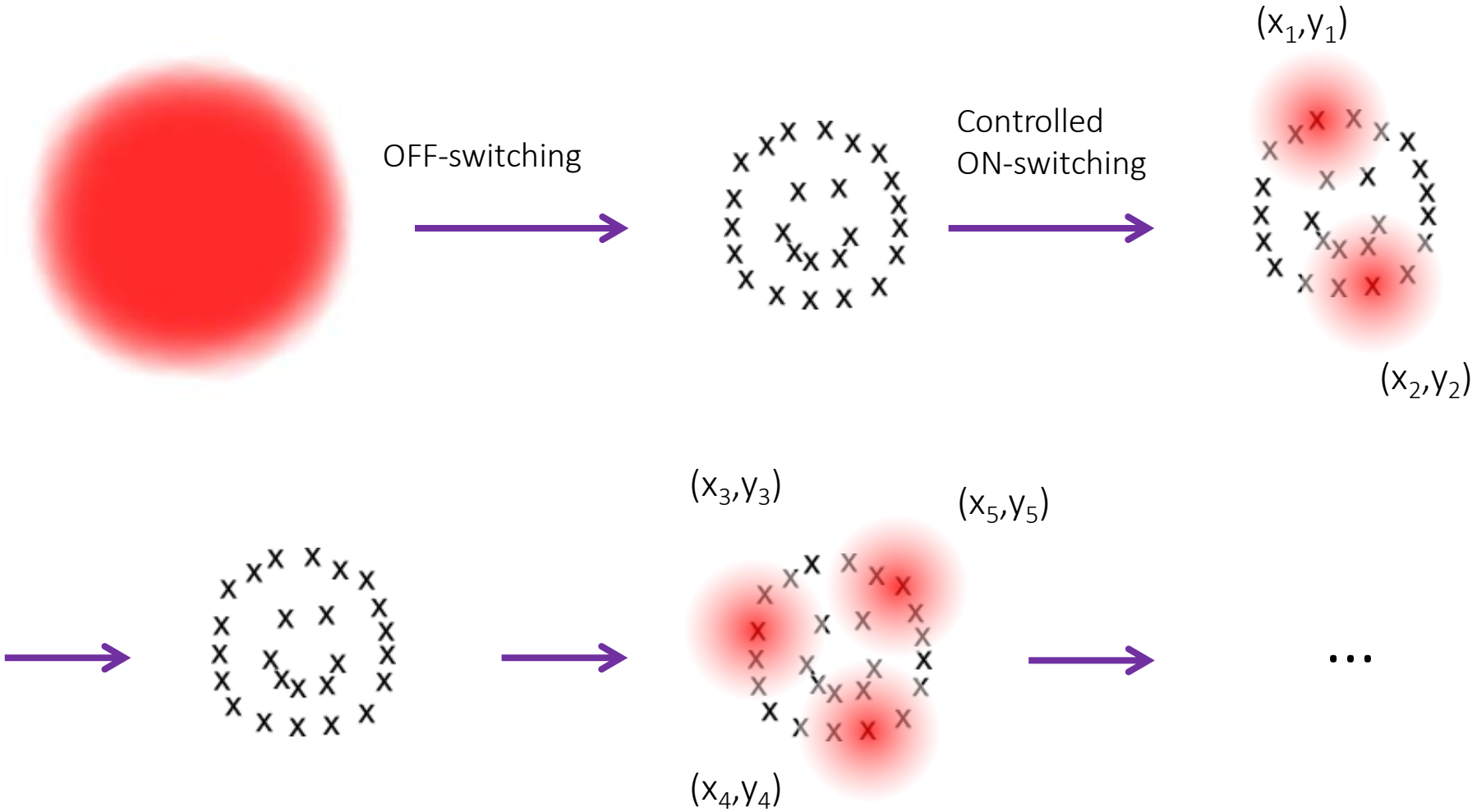
Single-Molecule Localization Microscopy (SMLM)



Single-Molecule Localization Microscopy (SMLM)



Single-Molecule Localization Microscopy (SMLM)



Single-Molecule Localization Microscopy (SMLM)

(x_1,y_1)

(x_2,y_2)

(x_3,y_3)

(x_4,y_4)

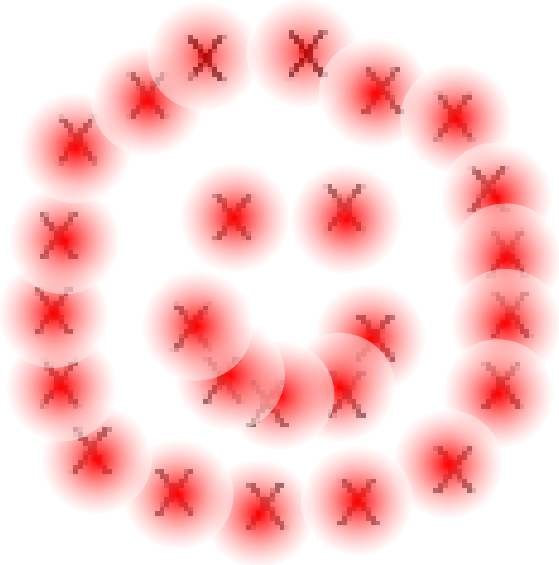
(x_5,y_5)

(x_6,y_6)

(x_7,y_7)

(x_8,y_8)

...

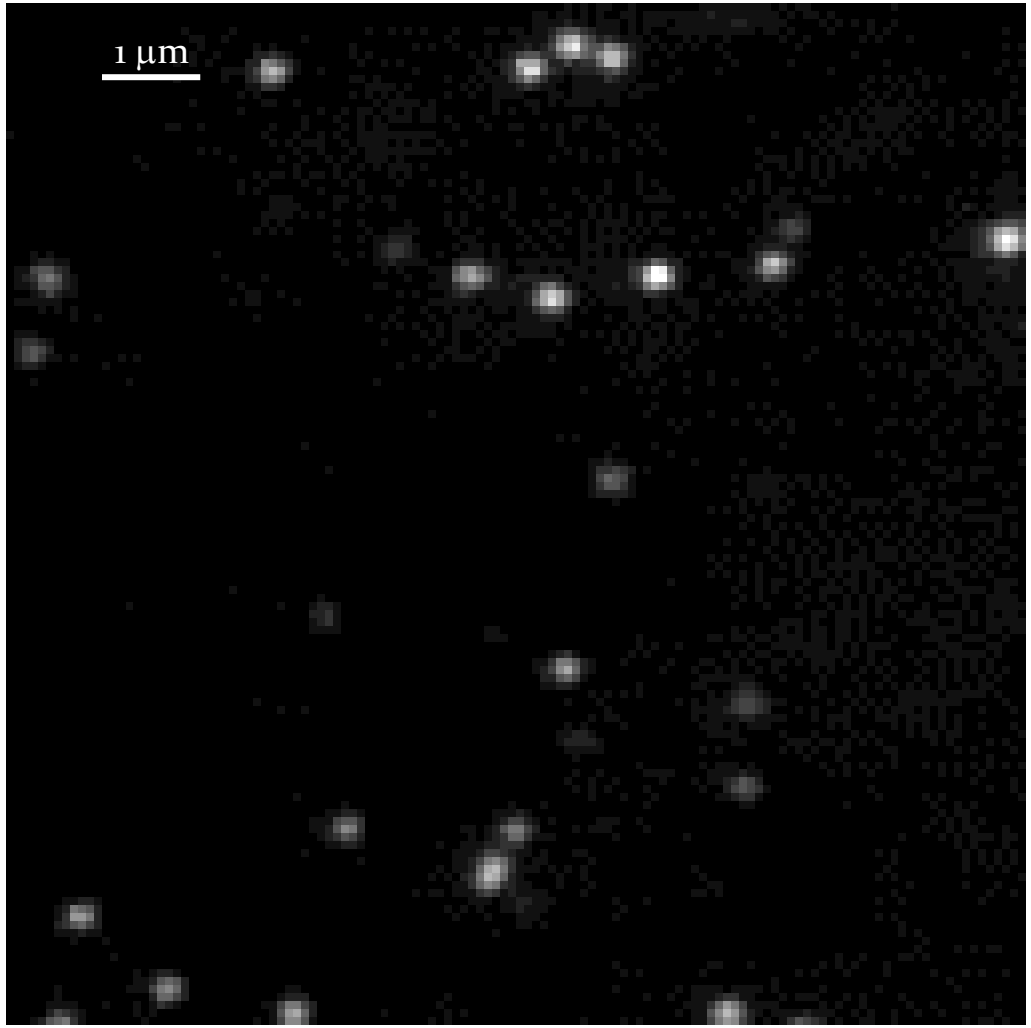


STORM – Stochastic Optical Reconstruction Microscopy

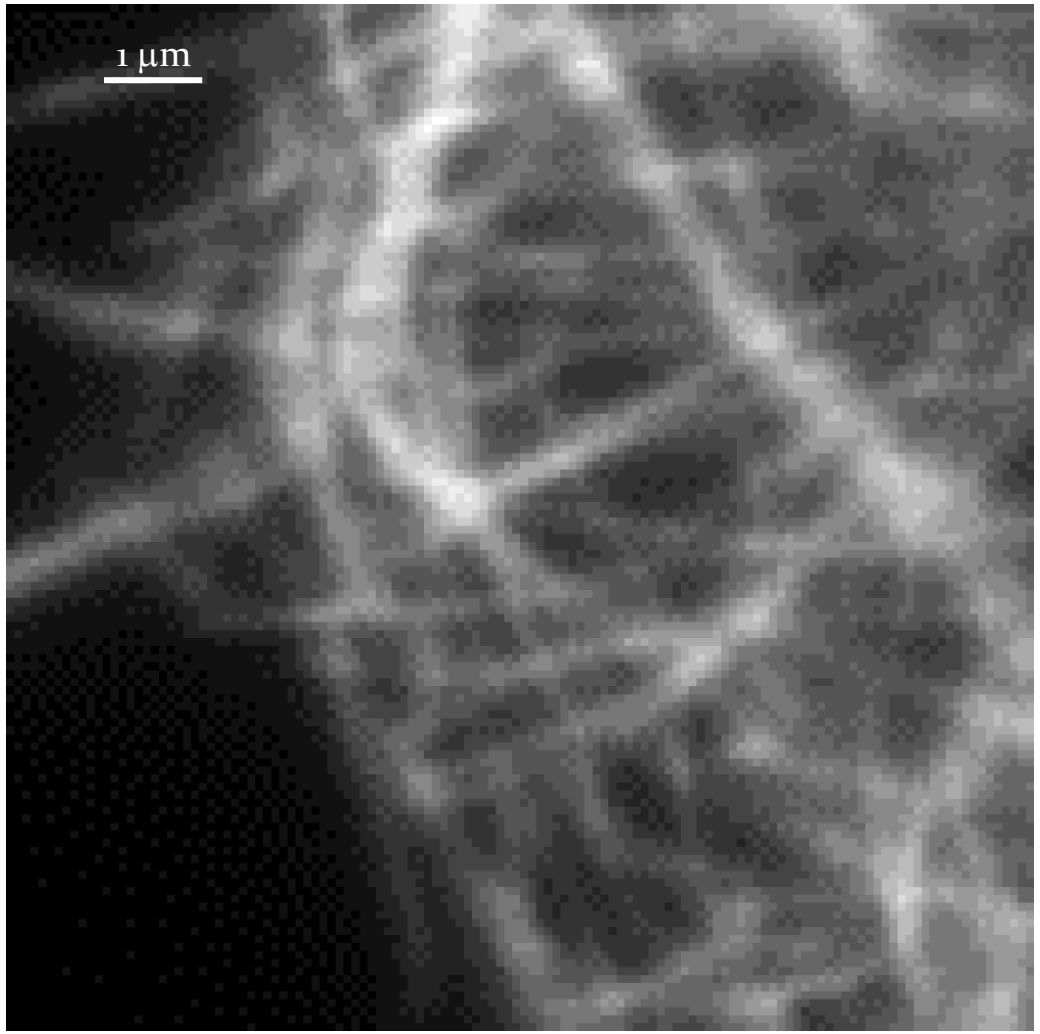
PALM – Photo-Activated Localization Microscopy

PAINT – Points Accumulation for Imaging in Nanoscale Topography

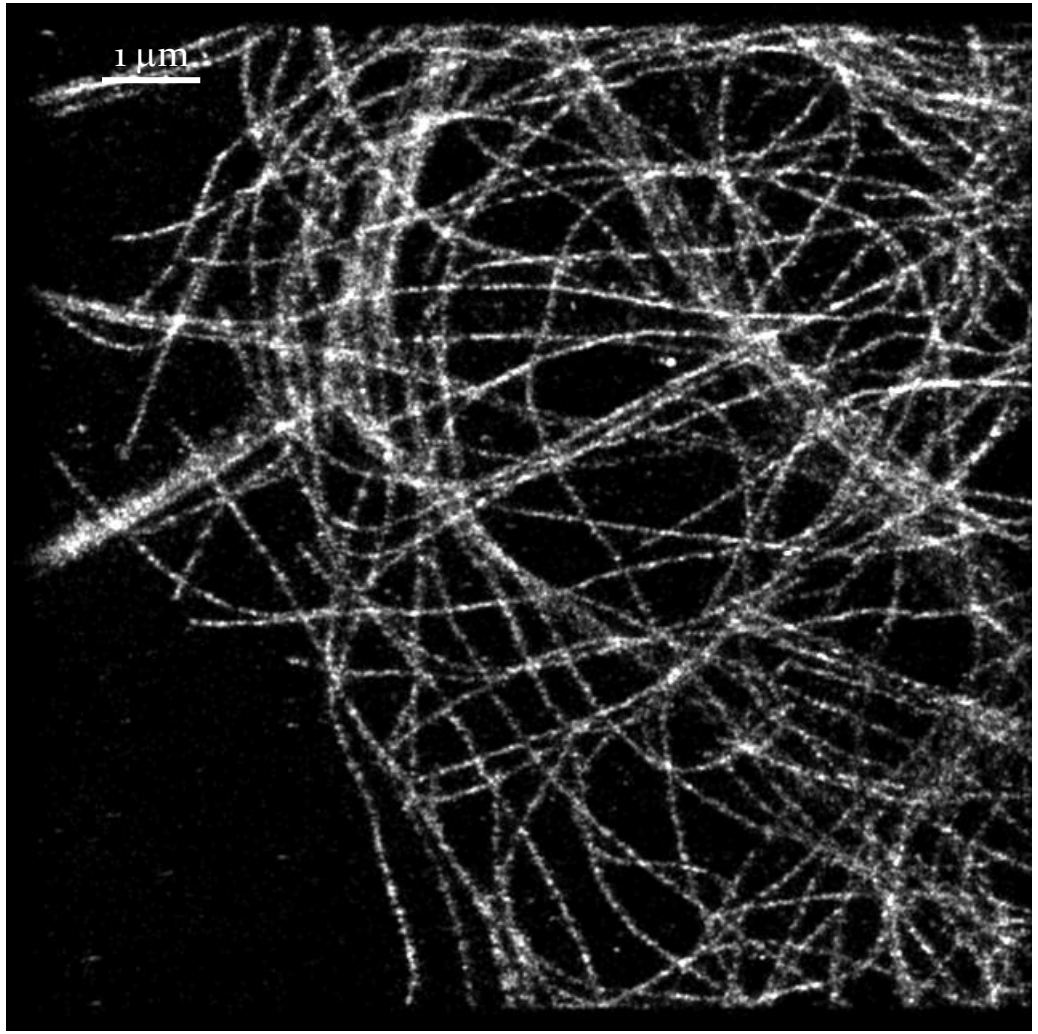
Single-Molecule Localization Microscopy (SMLM)



Single-Molecule Localization Microscopy (SMLM)



Single-Molecule Localization Microscopy (SMLM)



Federico Barabas – PhD thesis

Single-Molecule Localization Microscopy (SMLM)

Let’s go to the lab and see how it is done