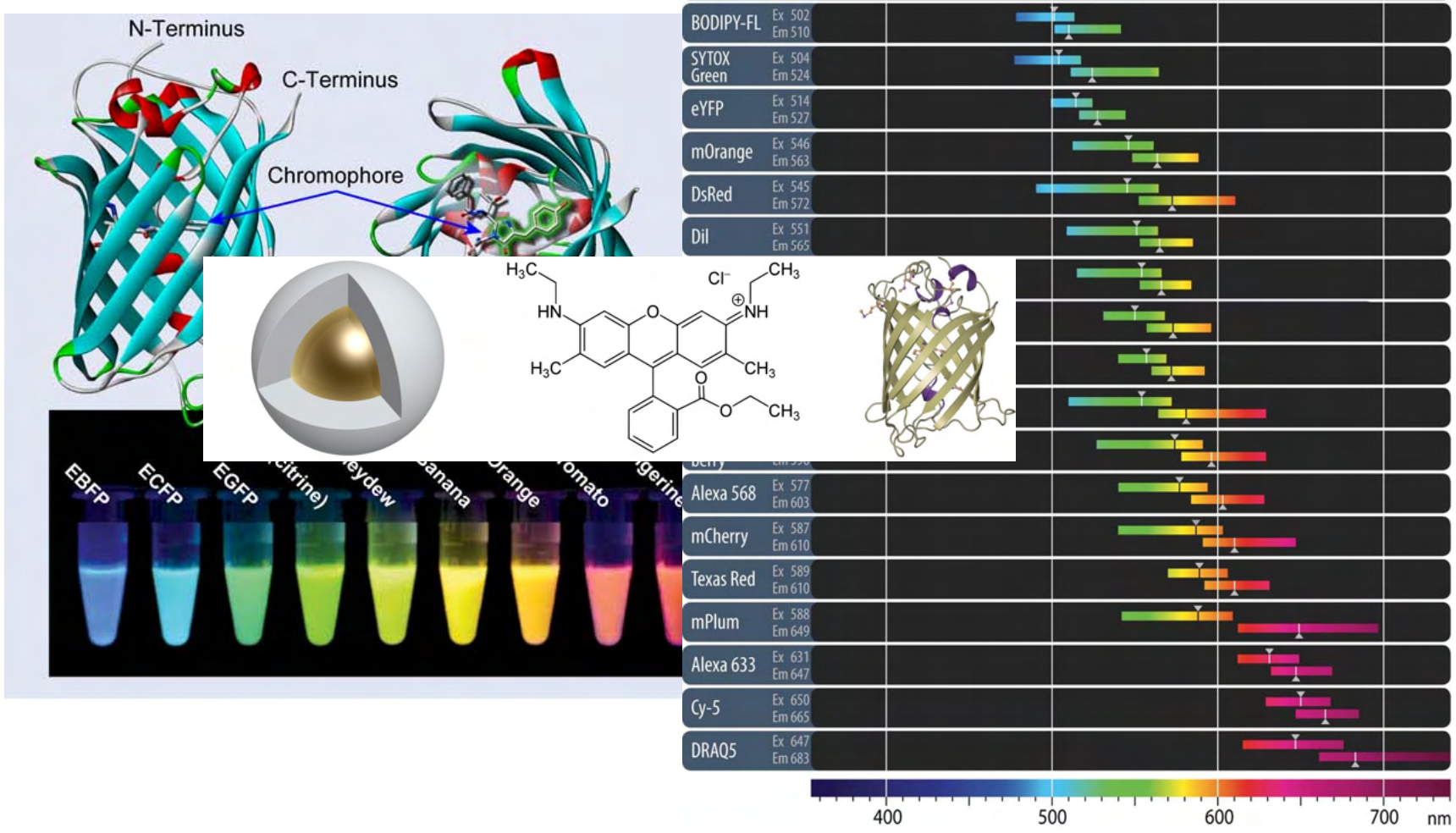
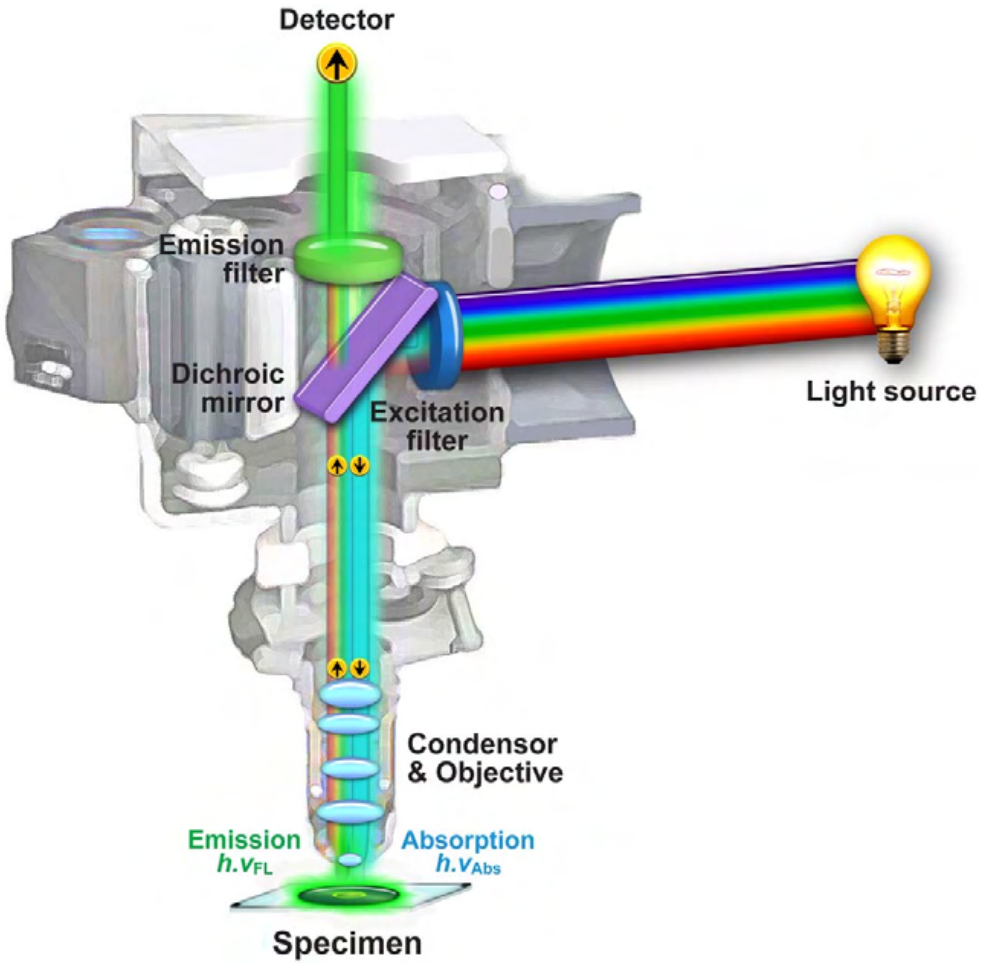
Advanced Fluorescence Microscopy Techniques

Fluorescent proteins

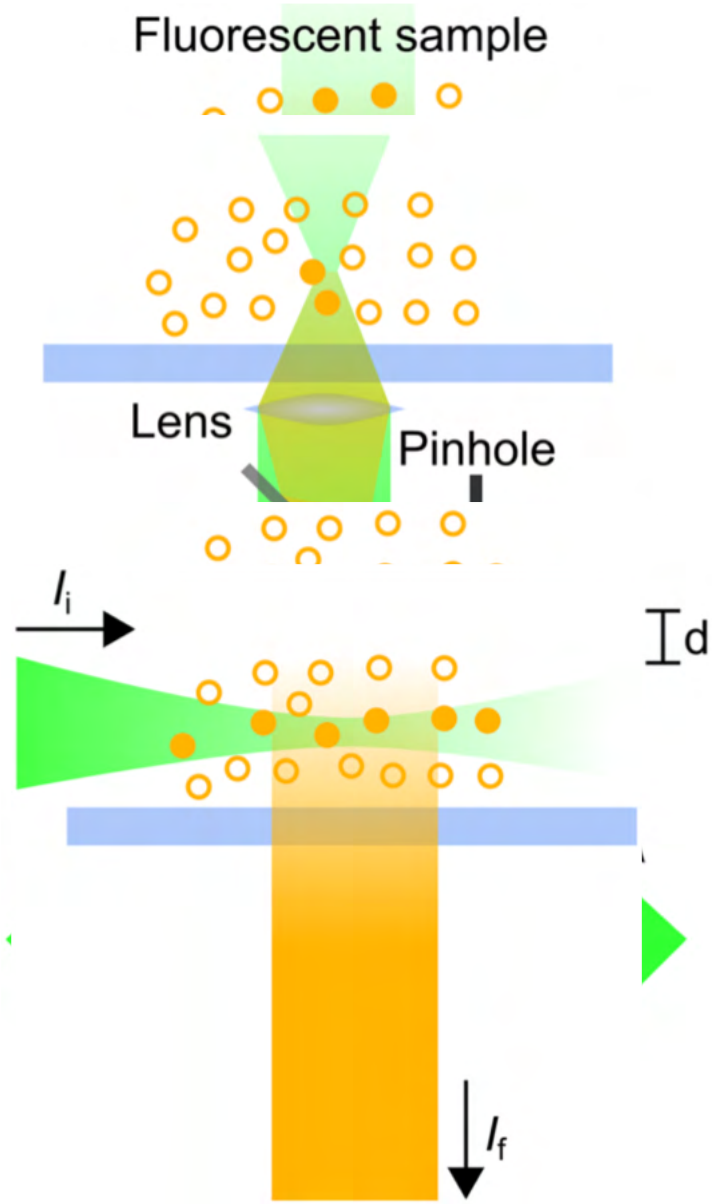


Epi fluorescence microscopy

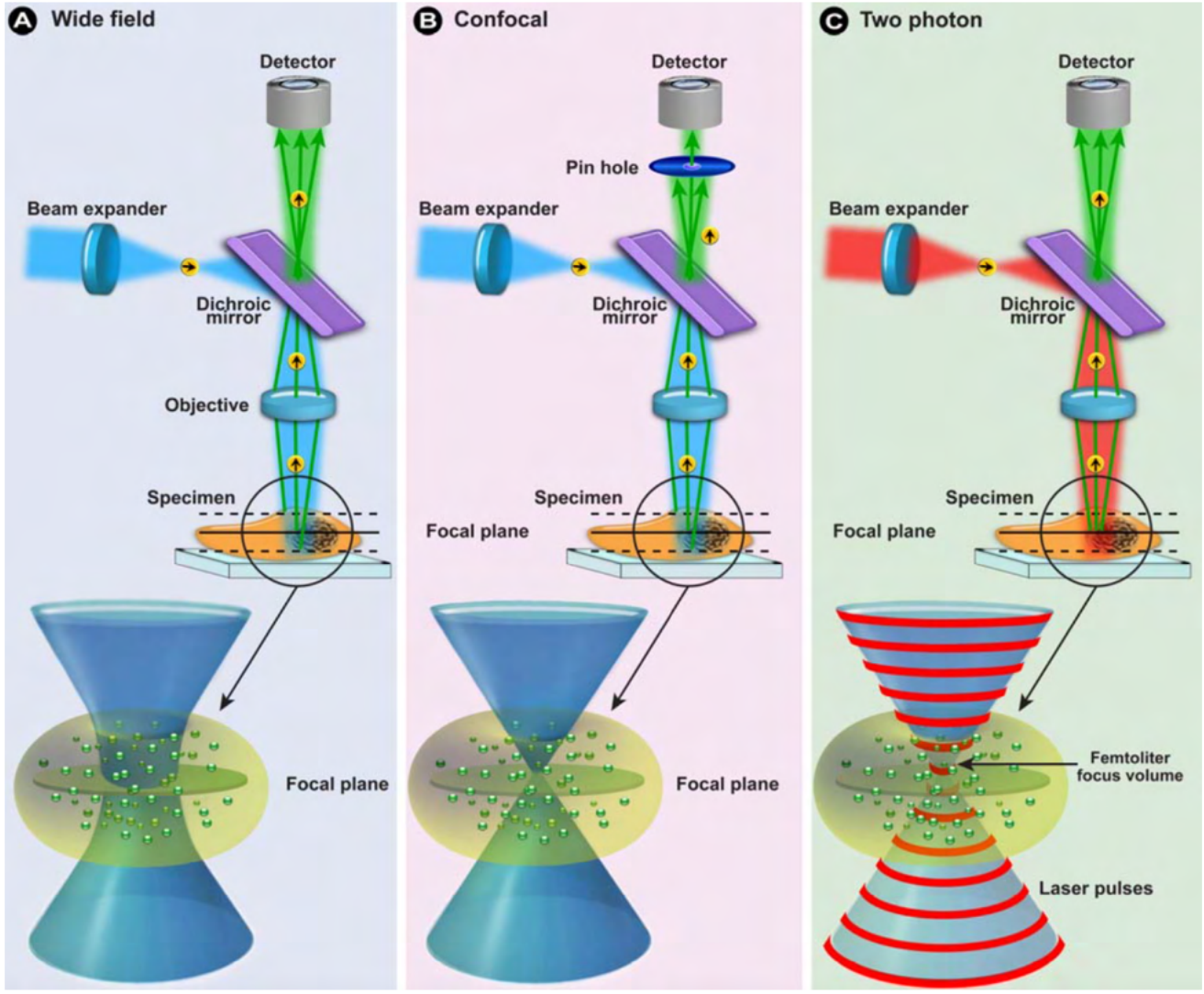


Fluorescence microscopy

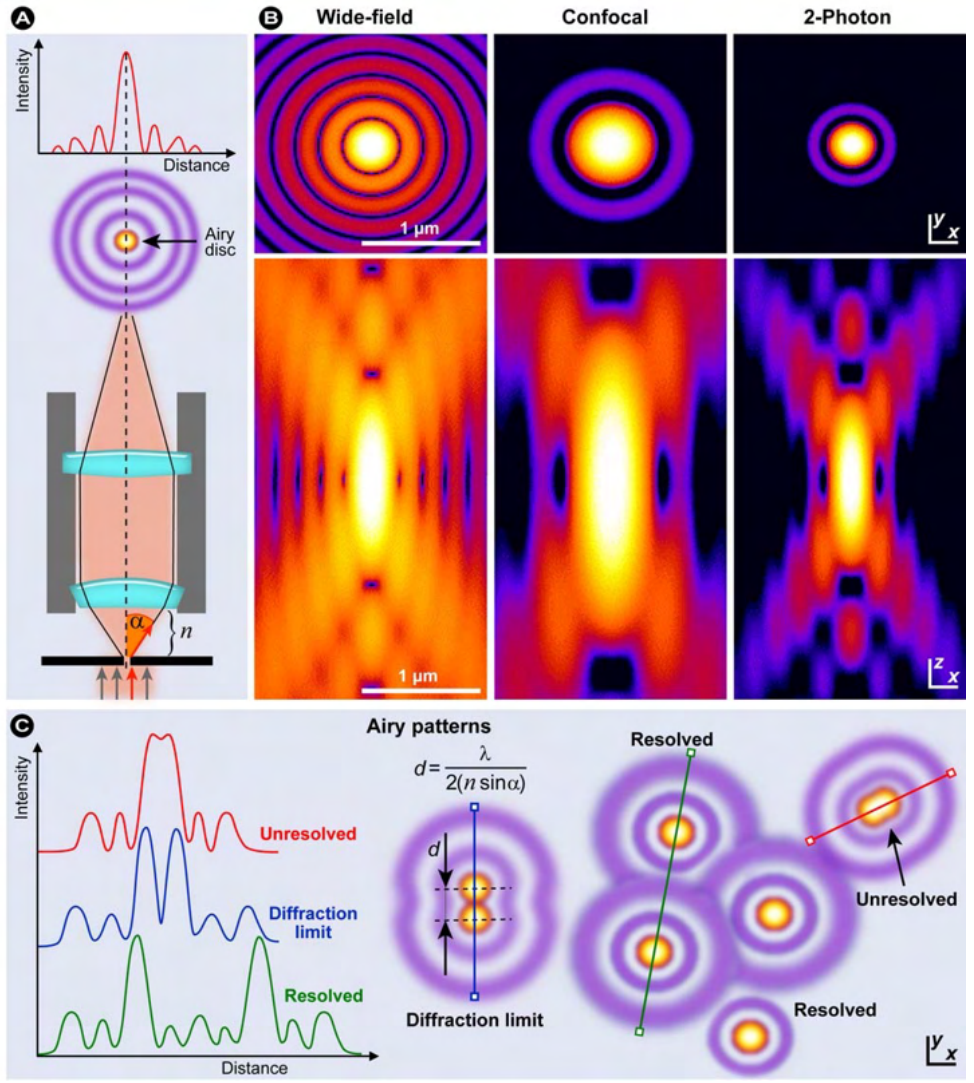
- Fluorescent Widefield Microscopy
- Laser Scanning Confocal Microscopy
- Spinning Disk Confocal Microscopy
- Two-Photon Microscopy
- Total Internal Reflection Microscopy (TIRF)
- Light Sheet Microscopy
- Super Resolution Microscopy



Confocal Laser Scanning Microscopy



Optical resolution



Diffraction limited microscopy

Type	Imaging mode	Technique	Principle of operation	Spatial / temporal resolutions**	Compatible fluorescent label(s)*	Limitations	Compatible samples	Applications
Diffraction-limited	Wide field	**TIRF-M**	Spatial confinement of emission by exciting at an angle above the critical angle	200 nm / 2 ms	All	Diffraction-limited resolution	Plasma membrane and substrate supported artificial lipid bilayers	Imaging and tracking of single protein molecules in cellular and reconstituted membranes
		LSFM	Excitation using a periodic structure of light sheets followed by orthogonal collection of emission to construct an image	200 nm / 2 ms	All	Diffraction-limited resolution	*Drosophila melanogaster*, HeLa, *T. thermophile*, HL-60, and, *Caenorhabditis elegans* cells	Imaging binding kinetics of transcription factors, organization of nuclear lamins, microtubule movement during mitosis, and, Polymerase organization
	Scanning	**Confocal**	Spatial confinement of excitation / emission to a small volume followed by lateral sample / laser scanning to construct an image	200 nm / 5 ms for 512 pixels × 512 pixels	All	Diffraction-limited resolution and Slow acquisition rates		

Other fluorescence microscopes

- Photoactivated Localization Microscopy (PALM)
- Stochastic Optical Reconstruction Microscopy (STORM)
- Ground State Depletion followed by Individual return Microscopy (GSDIM)
- Stimulated Emission by Depletion (STED) microscopy
- Structured Illumination Microscopy (SIM)
- Binding-Activation Localization Microscopy (BALM)
- Scanning Near field Optical Microscopy (SNOM)

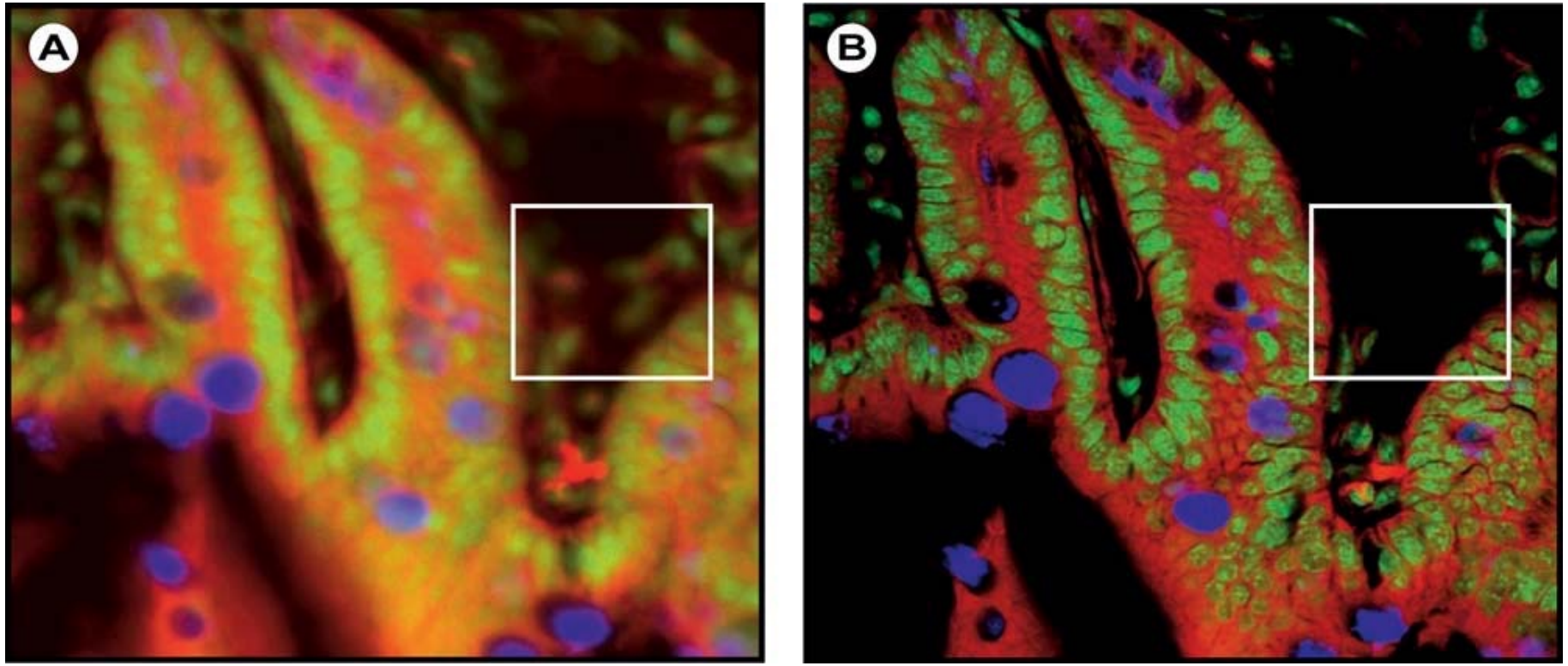
Higher resolution in microscopy

Type	Imaging mode	Technique	Principle of operation	Spatial / temporal resolutions**	Compatible fluorescent label(s)*	Limitations	Compatible samples	Applications
		PALM	Stochastic (random) activation of different subsets of spatially distinct fluorophores followed by localization and photobleaching to construct a super-resolved image	20 nm / 1 min for 15 μm^2	STORM-compatible fluorescent dyes	Slow acquisition rates	CHO, NIH 3T3, COS-7, FoLu and HFF-1 cells	Mapping bacterial Chemo taxis networks, imaging the interaction of adhesion complexes, dynamic clustering of Hemagglutinin, organization of gene clusters, and, bacterial cell division.
		STORM	Stochastic switching of different subsets of spatially distinct fluorophores followed by localization to construct a super-resolved image	9 nm / 25 min for 1500 μm^2	STORM-compatible fluorescent dyes Compatible with ZnS-coated CdSe Qdot705	Slow acquisition rates	IMR90, Ganglion, *Drosophila*, BSC-1, Cos-7, Sperm Flagella and IgG1 cells and *in vitro*	Genetic profiling *in vivo*, imaging chromosomal organization *in vivo*, periodic cytoskeleton organization, viral entry, coating and budding, and, endocytosis
		GSDIM	Ground state depletion followed by stochastic return of single molecules and localization to achieve super resolution imaging	30 nm / 30 min for 50 μm^2	Rhodamine 6G, ATTO 532, ATTO 565, EGFP, EYFP, Citrine and PhiYFP	High laser powers and slow acquisition rates	MDCK and PtK2 cells	Imaging Epithelial morphogenesis

Super Resolution Microscopy

Type	Imaging mode	Technique	Principle of operation	Spatial / temporal resolutions**	Compatible fluorescent label(s)*	Limitations	Compatible samples	Applications
Super resolution	Wide field	**SIM**	Illumination using a structured (periodic) pattern followed by sample or pattern rotation to achieve super resolution imaging	50 nm / 5 s for 225 μm^2	All	High computational demand	*Drosophila melanogaster* S2, HeLa, U2OS, and, RPE cells	Imaging mitochondria, Clathrin-coated vesicles, and the actin cytoskeleton
		BALM	Dye activation upon binding to substrate followed by fluorescence localization to construct a super-resolved image	14 nm / 10 min for 100 μm^2	YOYO-1, PicoGreen and LCO-pFTAA	Slow acquisition rates	*E. Coli* and *in vitro*	Imaging the organization of bacterial chromosomes and the structure of amyloid fibrils
	Scanning	**STED**	Stimulated emission of fluorophores by depletion followed by collection of spontaneous emission and scanning to construct a super-resolved image	20 nm at 3.8 mW / 1 ms for 10 μm^2	STED-compatible fluorescent dyes Compatible with ZnS-coated CdSe Qdot705	High laser powers and slow acquisition rates	Cardiac myocytes, *Rattus norvegicus*, U2OS, NRK fibroblasts, IA32 MEFs, *Drosophila*, HEK293 and PtK2 cells and substrate supported lipid bilayers	Mapping the dynamic organization of cellular membranes, imaging receptors' clustering during synapsis, and, recruitment and sorting of signaling proteins
		SNOM	Excitation, or collection of emission, in the near field (close to the sample) to achieve super resolution	10 nm / 100 s for 65 μm^2	All	Slow acquisition rates	Plasma, and nuclear, membranes of *Xenopus laevis*, T lymphoma, and, dendritic cells	Imaging the organization of cellular membranes, clustering of membrane receptors and transport dynamics at nuclear pores

Confocal Laser Scanning Microscopy



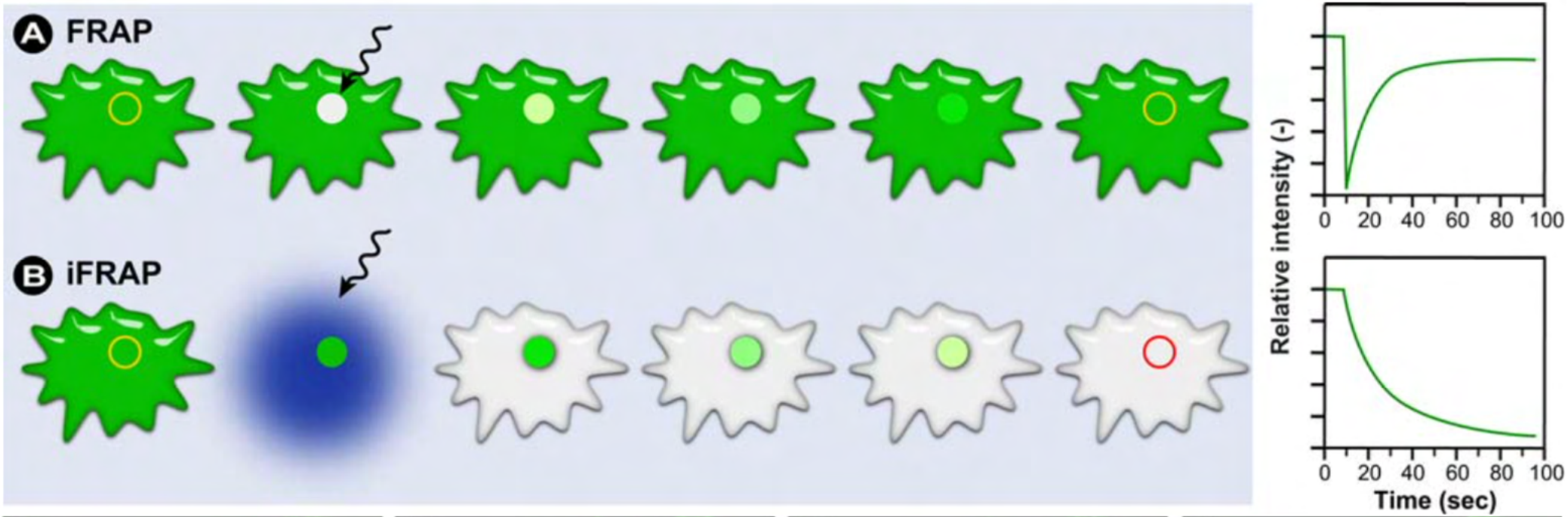
Techniques for Confocal Microscopy

- *Fluorescence Recovery after Photobleaching (FRAP)*
- *Fluorescence Loss in Photobleaching (FLIP)*
- *Fluorescence Localization after Photobleaching (FLAP)*
- *Förster Resonance Energy Transfer (FRET)*
  - *Sensitized Emission*
  - *Acceptor Photobleaching*
- *Fluorescence Lifetime Imaging Microscopy (FLIM)*
  - *FLIM-FRET*
- *Bimolecular Fluorescence Complementation (BiFC)*

FRAP

- Protein movement and diffusion (diffusional speed).
- Compartmentalization and connections between intracellular compartments.
- The speed of protein exchange between compartments (exchange speed).
- Binding characteristics between proteins.
- Immobilization of proteins that bind to large structures, e.g., DNA, nuclear envelope, membranes, cytoskeletal elements, etc.

FRAP

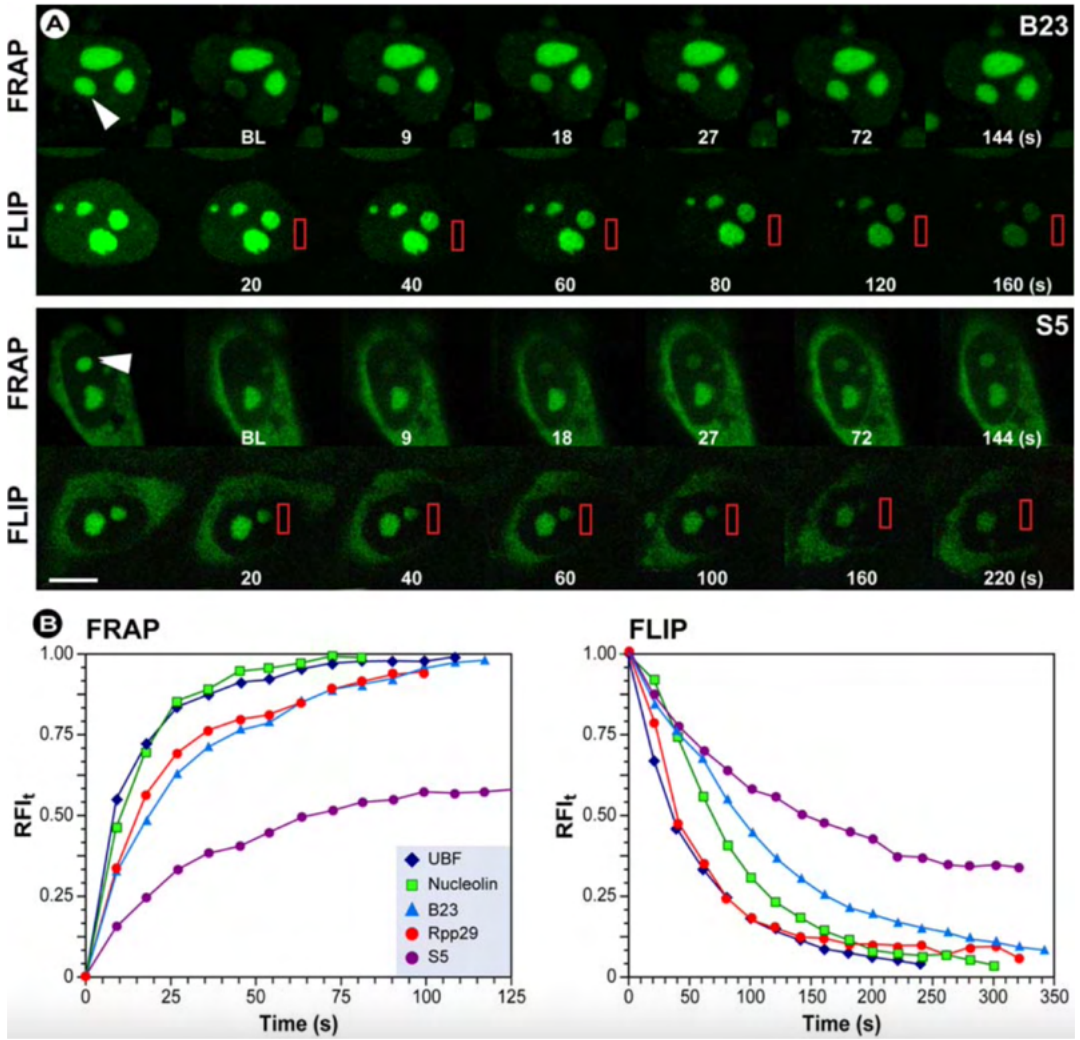

A FRAP
B iFRAP
Relative intensity (-)
0 20 40 60 80 100
0 20 40 60 80 100
Time (sec)
A
B
ROI
C
D
100 µm
Pre-bleach
Bleaching ROI
Post-bleach
Fluorescence recovery

FLIP

Repetitive bleaching of the ROI, thereby preventing recovery of fluorescence in that region.

- Connectivity between different compartments in the cell.
- The mobility of a protein within the whole compartment.

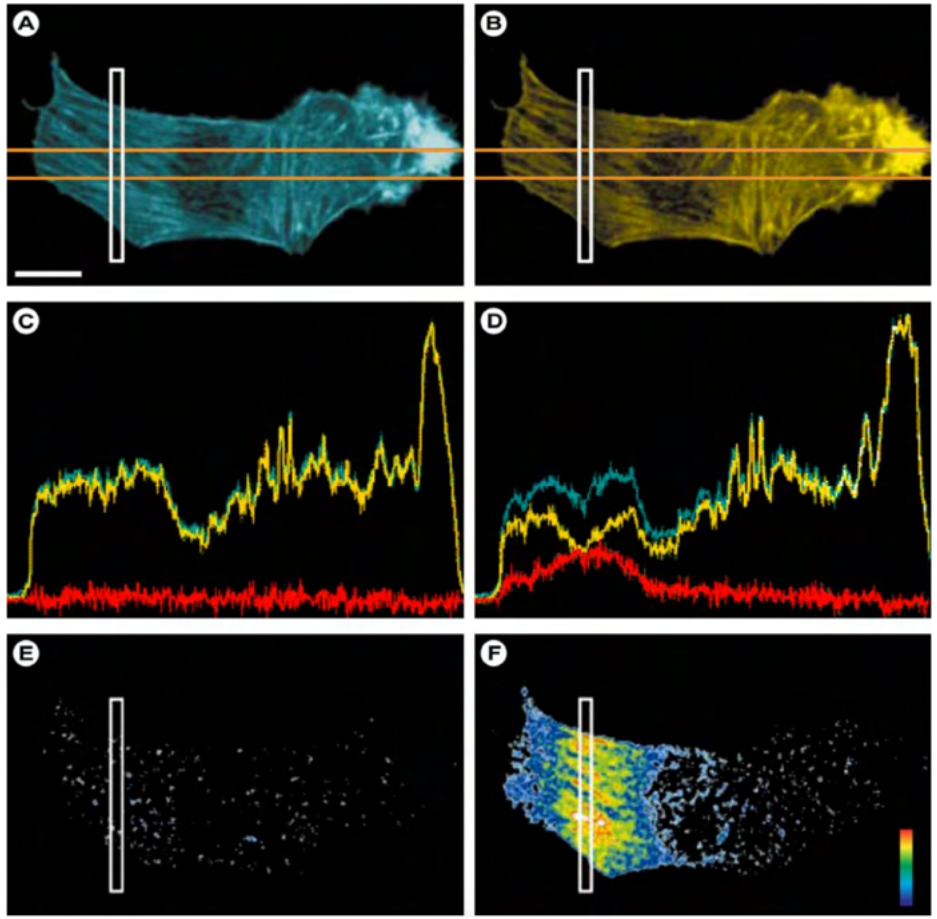
FLIP


A
B23
FRAP
BL
9
18
27
72
144 (s)
FLIP
20
40
60
80
120
160 (s)
S5
FRAP
BL
9
18
27
72
144 (s)
FLIP
20
40
60
100
160
220 (s)
B
FRAP
1.00
0.75
0.50
0.25
0
RFIt
0
25
50
75
100
125
Time (s)
UBF
Nucleolin
B23
Rpp29
S5
FLIP
1.00
0.75
0.50
0.25
0
RFIt
0
50
100
150
200
250
300
350
Time (s)

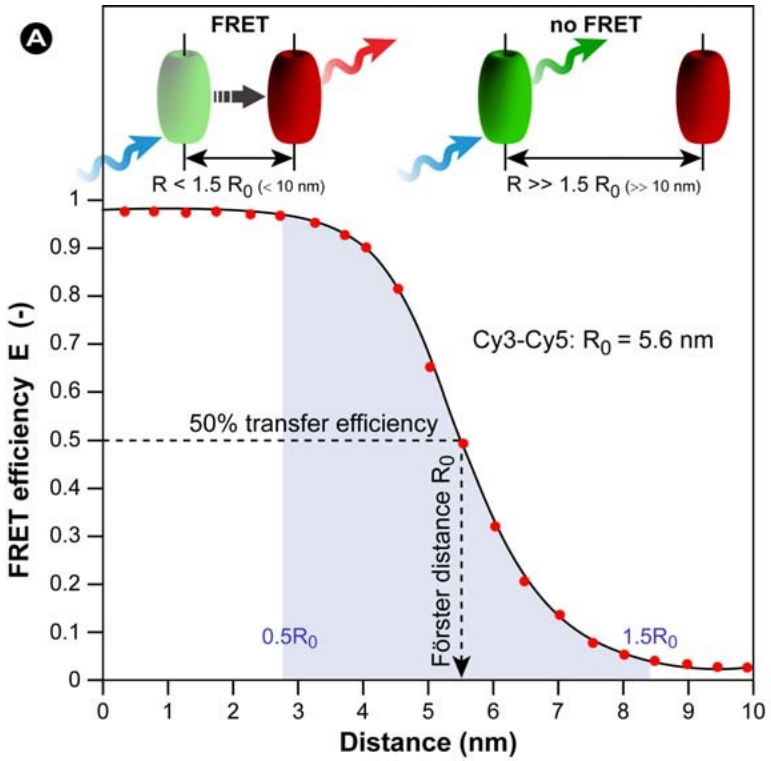
FLAP

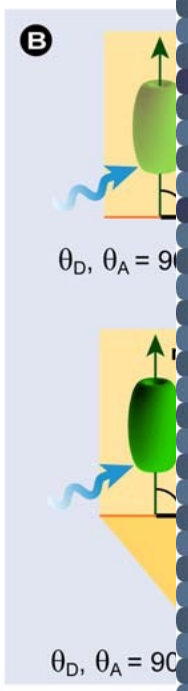
- Allows detecting and tracking of sub-populations that move rapidly and have short residence times.

FLAP



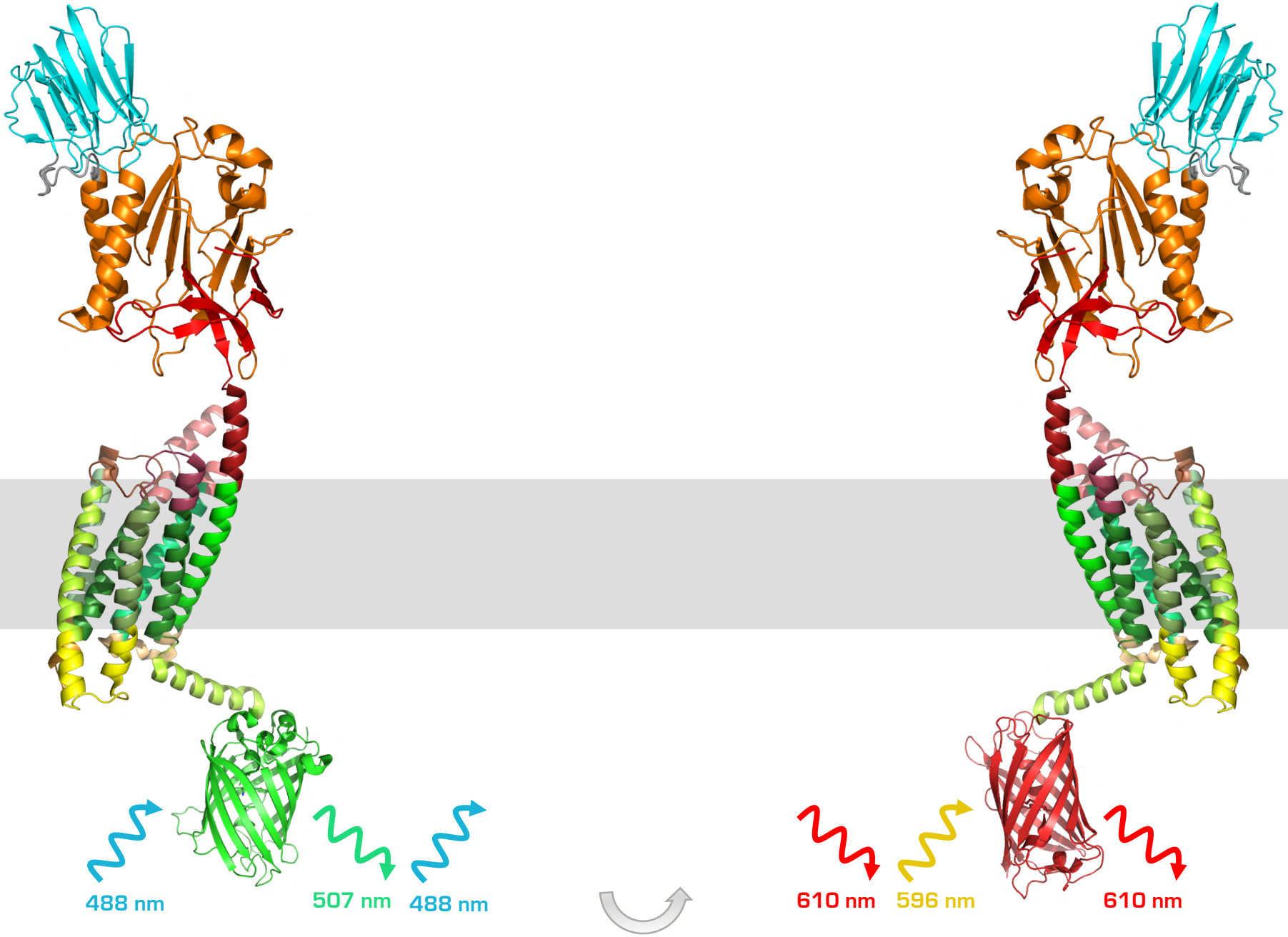
FRET





Donor	Acceptor	Donor Excitation λ_{max} (nm)	Acceptor Emission λ_{max} (nm)	Donor QY (–)	Acceptor ε ($M^{-1}cm^{-1}$)	Förster Distance (nm)
Fluorescent protein FRET pairs						
BFP	DsRFP	380	586	0.18	72,500	3.1–3.3
EBFP2	mEGFP	383	507	0.56	57,500	4.8
CFP	GFP	433	509	0.40	21,000	4.7–4.9
CFP	YFP	433	526	0.40	77,000	~ 5.0
Cerulean	YFP	440	526	0.62	77,000	–
ECFP	EYFP	440	527	0.40	83,400	4.9
Cerulean	Venus	440	528	0.62	92,200	5.4
MiCy	mKO	472	559	0.90	51,600	5.3
GFP	YFP	475	526	0.77	77,000	5.5–5.7
GFP	mRFP	475	579	0.77	50,000	~ 4.7
CyPet	YPet	477	530	0.51	104,000	5.1
TFP1	mVenus	492	528	0.85	92,200	5.1
EGFP	mCherry	507	510	0.60	72,000	5.1
Venus	mCherry	528	610	0.57	72,000	5.7
Venus	tdTomato	528	581	0.57	138,000	5.9
Venus	mPlum	528	649	0.57	41,000	5.2
Fluorescent protein-dye FRET pairs						
EGFP	Alexa Fluor 555	484	568	0.6	155,000	6.3
EGFP	Alexa Fluor 546	484	573	0.6	112,000	5.7
EGFP	Alexa Fluor 594	484	618	0.6	92,000	5.3
EGFP	Alexa Fluor 568	484	603	0.6	88,000	5.4
Dye and dye-biofluorochrome FRET pairs						
Cy2	Cy3	489	570	> 0.12	150,000	5.0–6.0
Cy3	Cy5	550	670	> 0.15	250,000	>5.0
Cy5	Cy5.5	649	694	> 0.28	250,000	>8.0
Fluorescein	Tetramethylrhodamine	487 [1]	574	0.93 [2]	~ 87,000	4.9–5.5
FITC	TRITC	494	572	0.92	100,000	5.4
Phycoerythrin	APC [3]	(546), 565	660	0.98	700,000	4.0–11.0
Europium	APC	340	660	–	700,000	9.0
Tryptophan	Dansyl	280	525	0.01–0.35 [4]	4050	2.1
Dansyl	FITC	335	519	< 0.035 [5]	77,000	3.3–4.1
Dansyl	Octadecylrhodamine	335	625	< 0.035	106,000	4.3
Europium	Cy5	340	670	–	250,000	7.0
Atto 488	Atto 647N	501	670	0.8	150,000	5.1
Atto 488	Atto 590	501	621	0.8	120,000	6.0
Atto 550	Atto 647N	554	670	0.8	150,000	6.5
Atto 550	Atto 655	554	684	0.8	125,000	6.4
Atto 590	Atto 655	594	684	0.8	125,000	7.3
Alexa 405	Alexa 430	401	541	–	16,000	–
Alexa 488	Alexa 514	495	542	0.92	80,000	–
Alexa 488	Alexa 532	495	554	0.92	81,000	–
Alexa 488	Alexa 546	495	573	0.92	104,000	6.4
Alexa 488	Alexa 610	495	628	0.92	138,000	–
Alexa 647	Alexa 680	650	702	0.33	184,000	–
Alexa 647	Alexa 700	650	723	0.33	192,000	–
Alexa 647	Alexa 750	650	780	0.33	240,000	–
Non-fluorescent acceptor pairs						
Rhodamine 6G	Malachite Green (Abs 628 nm)	526	NF	0.95	76,000	6.1
Alexa 488	QSY 35 (Abs 475 nm)	495	NF	0.92	23,000	4.4
Alexa 488	Dabcyl (Abs 453 nm)	495	NF	0.92	32,000	4.9
Alexa 647	QSY 21 (Abs 661 nm)	650	NF	0.33	90,000	6.9

FRET



BiFC