Introduction to fluorescent probes

Cranfill et al. Quantitative assessment of fluorescent proteins. Nat. Methods, 2016

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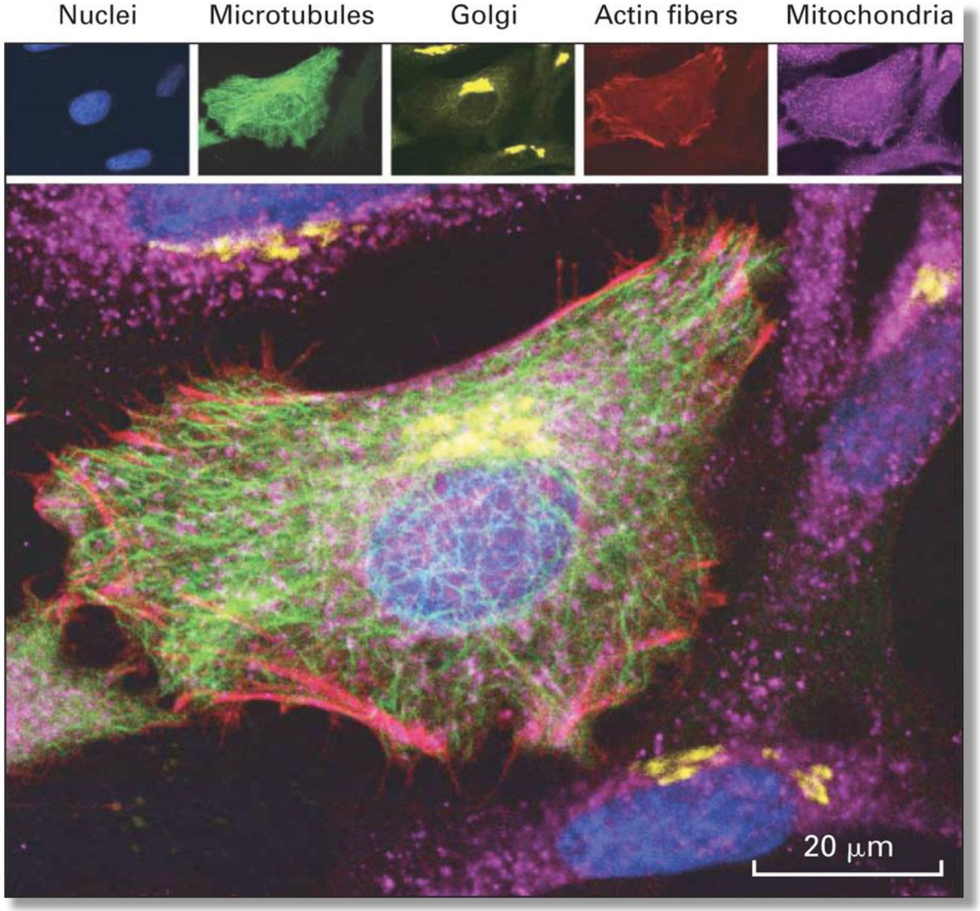
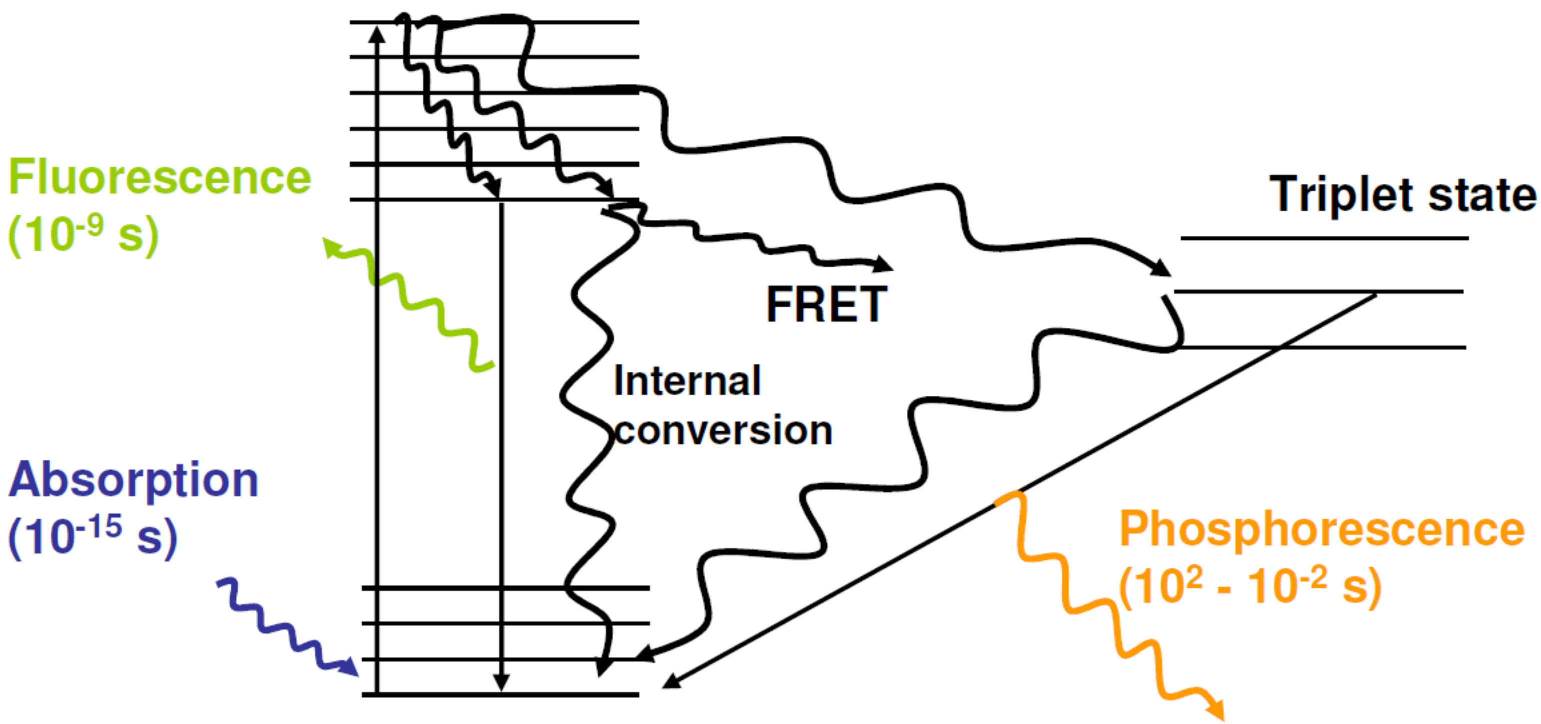
Why is fluorescence microscopy so popular ?

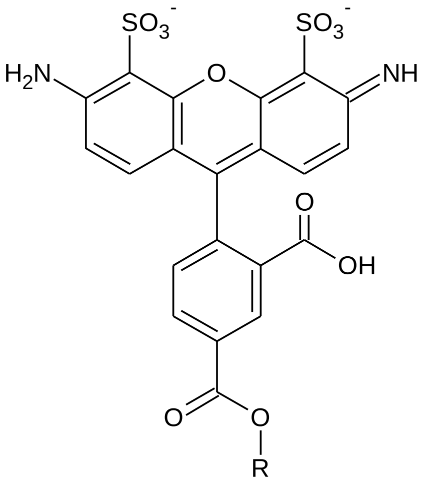


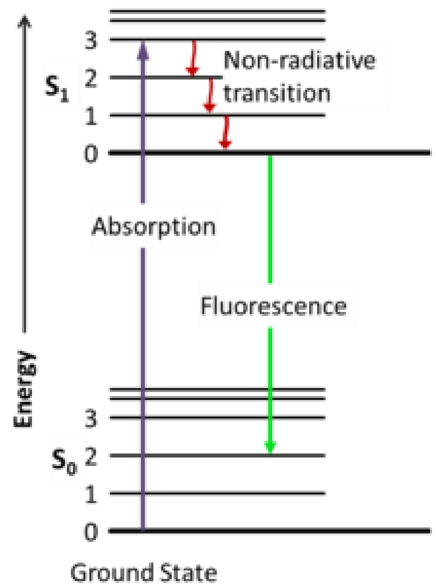
- high contrast and sensitivity
- Spatial resolution from few mm down to nm
- alot of colors
- Small and versatile labels (
- Works in intact and living cells

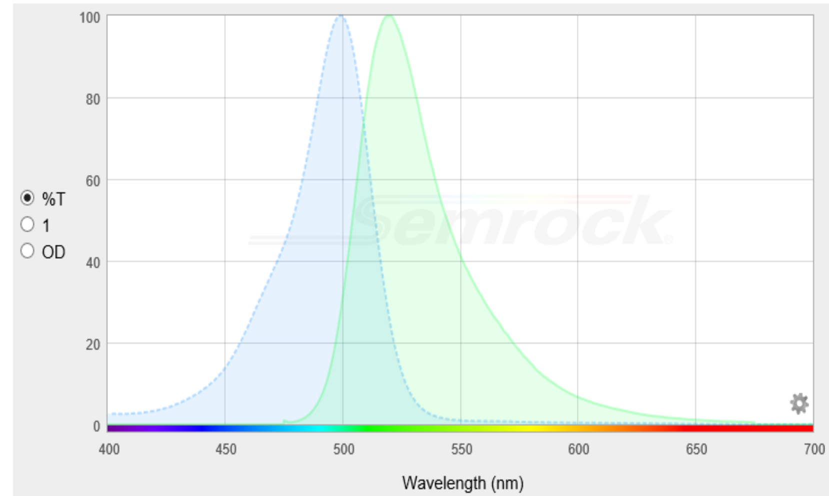


Fluorescence
(10-9 s)
Absorption
(10-15 s)
FRET
Internal conversion
Triplet state
Phosphorescence
(102 - 10-2 s)

What is a fluorophor ?



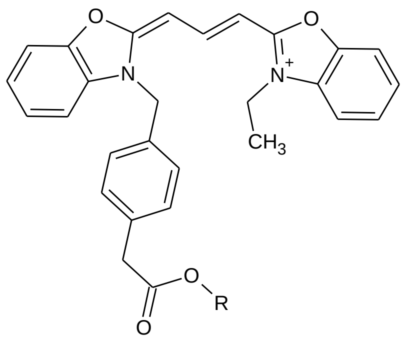




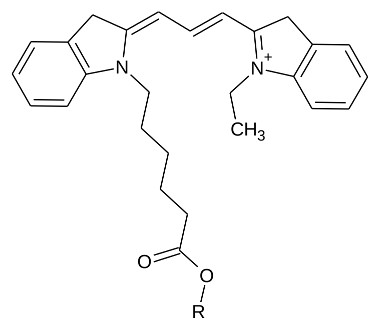
Alexa Fluor 488 (Rhodamine dye)

- Exc. 495nm , Em 519nm Stokes shift = 24 nm
- Absorption Coefficient: ε = 73000 **L·mol^{-1}·cm^{-1}** 7(Beer Lambert law)
- Quantum yield: Φ = 0.92
- Brightness (Φ * ε/1000 = 67)
- Photostability
- pKa

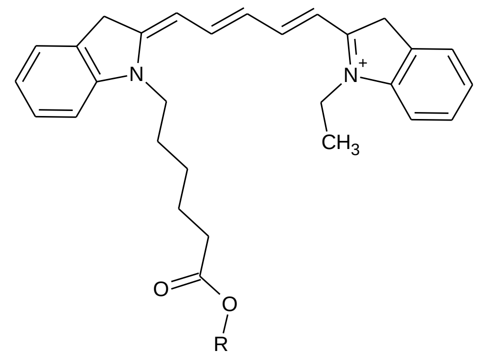
larger conjugated system have the lower the abs energy



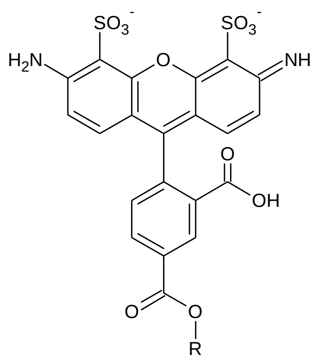
Cy2
489/506nm
QY = 0.15



Cy3
550/570nm



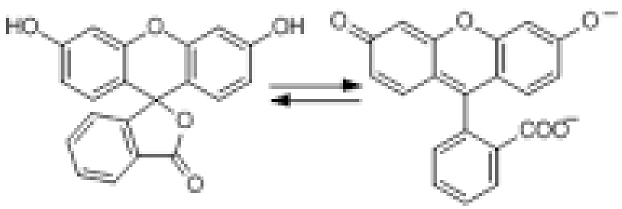
Cy5
550/570nm



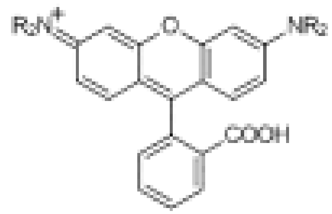
Alexa 488
495/519nm
QY = 0.8

Rigid conjugated system have high Qunatum yields

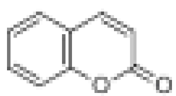
Organic fluorophors



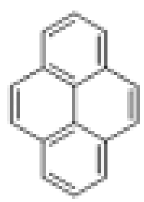
Fluorescein Type



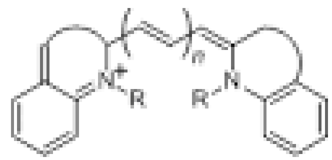
Rhodamine Type



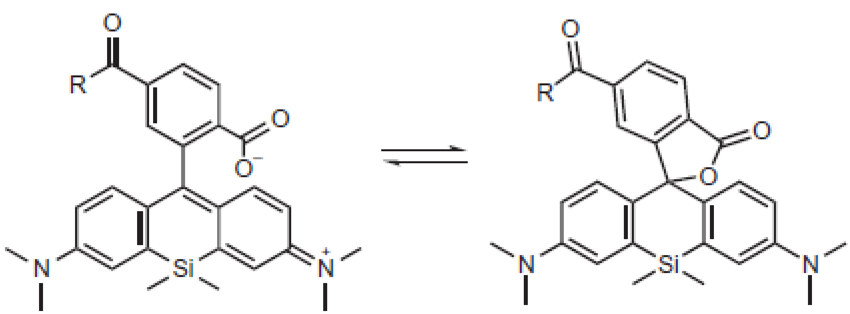
Coumarin Type



Pyrene Type



Cyanine Type

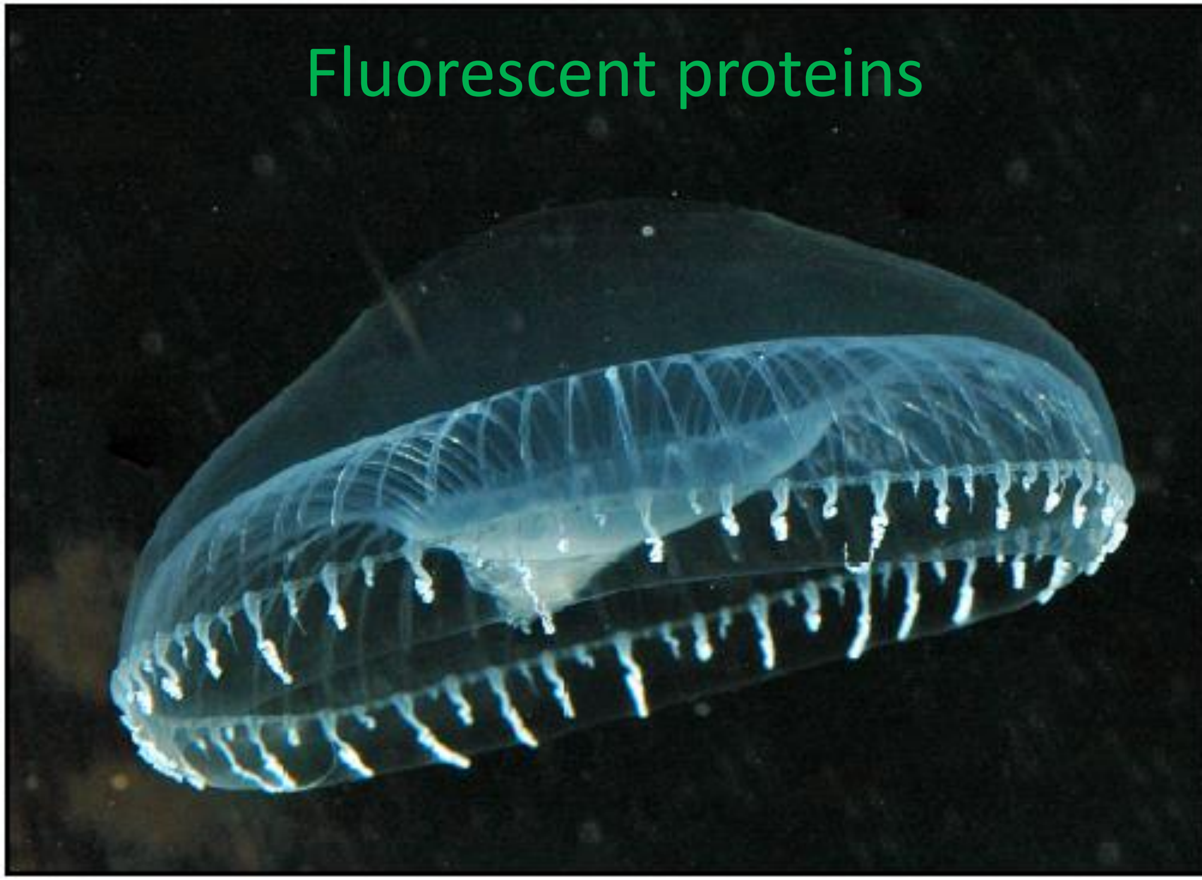


SiRhodamine   Lukinavicius et al. 2013

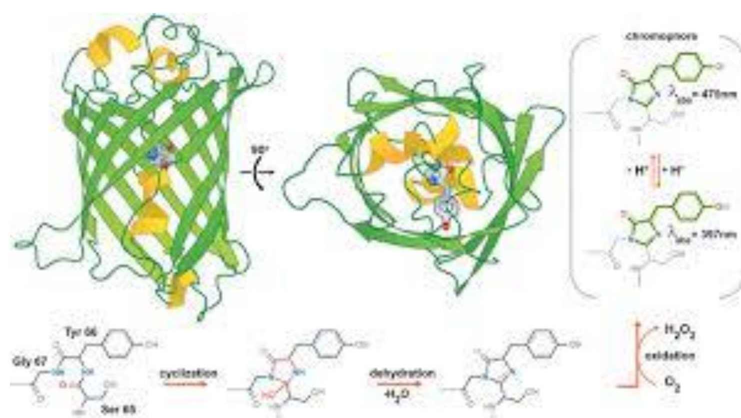
Dye	Excitation maximum (nm)[a]	Emission maximum (nm)[a]	Extinction $(M^{-1}\ cm^{-1})^{b}$	Quantum yield[c]	Brigthness
Blue-absorbing					
* Atto 488	501	523	90,000	0.8	72
* Alexa Fluor 488	495	519	71,000	0.92	65
Atto 520	516	538	110,000	0.9	
Fluorescein	494	518	70,000	0.79	
FITC	494	518	70,000	0.8	
Cy2	489	506	150,000	0.12	
Yellow-absorbing					
Cy3B	559	570	130,000	0.67	
Alexa Fluor 568	578	603	91,300	0.69	
TAMRA	546	575	90,430	0.2	
Cy3	550	570	150,000	0.15	
Cy3.5	581	596	150,000	0.15	
* Atto 565	563	592	120,000	0.9	108
Red-absorbing					
* Alexa Fluor 647	650	665	239,000	0.33	78
Cy5	649	670	250,000	0.28	
Atto 647	645	669	120,000	0.2	
* Atto 647N	644	669	150,000	0.65	97
Dyomics 654	654	675	220,000	–	
Atto 655	663	684	125,000	0.3	
Atto 680	680	700	125,000	0.3	
Cy5.5	675	694	250,000	0.28	
NIR-absorbing					
DyLight 750	752	778	220,000	–	
Cy7	747	776	200,000	0.28	
Alexa Fluor 750	749	775	240,000	0.12	
Atto 740	740	764	120,000	0.1	
Alexa Fluor 790	785	810	260,000	–	
IRDye 800 CW	778	794	240,000	–	

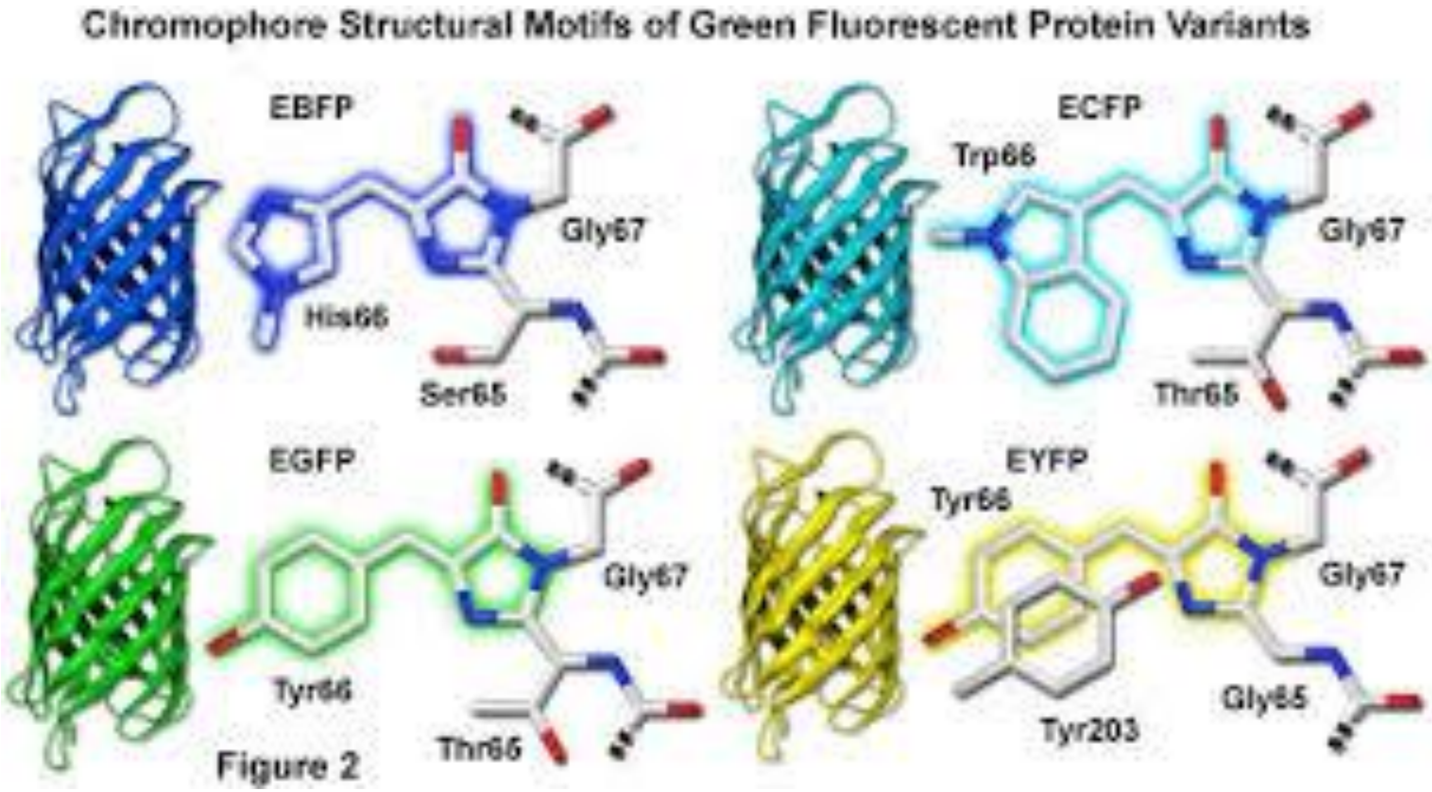
Dempey et al 2011

Fluorescent proteins



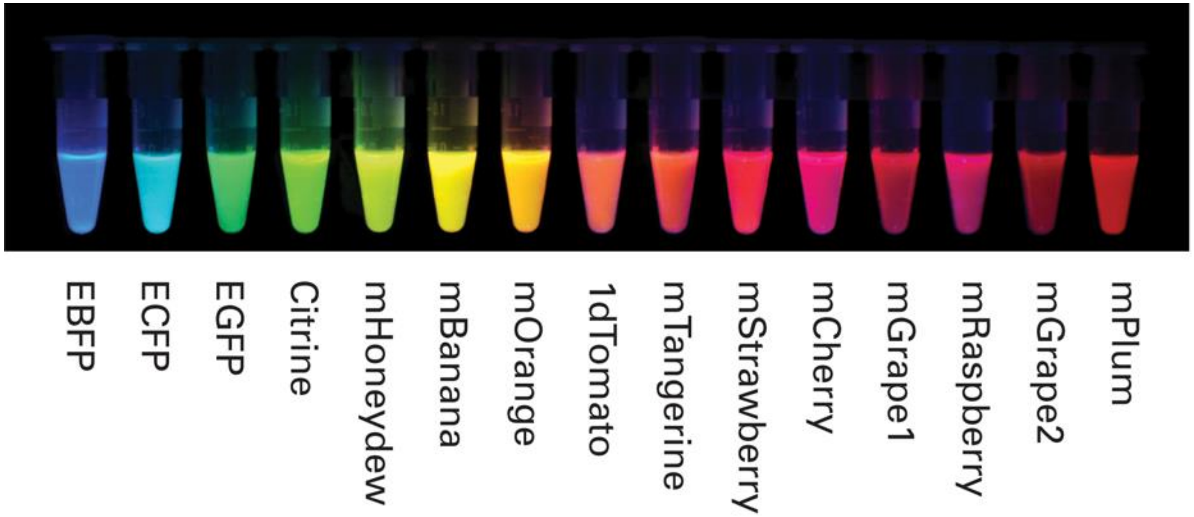
- Isolated & Cloned from bioluminiscent organisms
- 25kDa
- 11 beta-barels and 1 helix

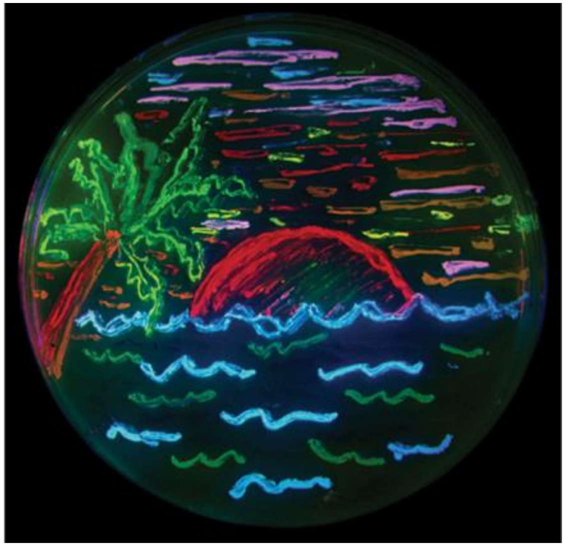




larger conjugated system have the lower the abs energy

Fluorescent proteins





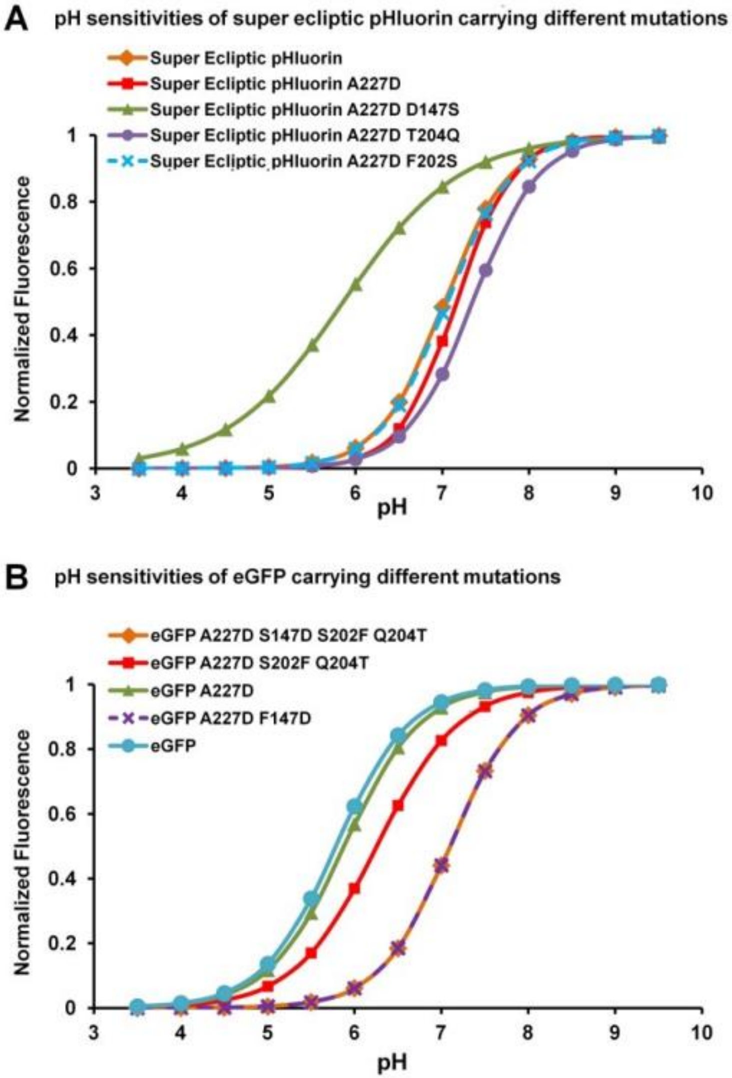
- Absorbance and Emission Spectra/Maxima
- Excitation coefficient (ϵ in 10^3 Mol^{-1} cm^{-1})
- Quantum yield (QY)
- Brightness (10^{-3} QY * ϵ), e.g. 38 for eGFP and 67 for AF488
- pKa
- Lifetime (ns)
- Photostability
- Maturation half life, e.g. sfGFP and mVenus
- oligomerisation state
- Photochromic effects, e.g photoswitching of mEos

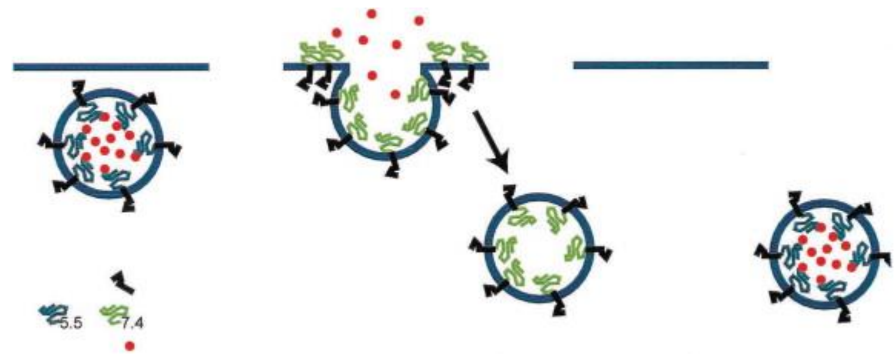
Table 1 | Characteristics of the mScarlet variants and reference RFPs

	Spectroscopic characteristics						Brightness		Photostability			Maturation	
	Abs max[a] (nm)	Em max[b] (nm)	ε[c] (10^3 M^{-1} cm^{-1})	QY[d] (–)	$\bar{\tau}$[e] (ns)	p*K*a[f] (–)	εQY[g] (10^3 M^{-1} cm^{-1})	In cells[h] (%)	WF $t_{1/2}$[i] (s)	Csd $t_{1/2}$[j] (s)	Ph[k] (%)	Accum in cells[l] (%)	Δt_{mat}[m] (h)
mScarlet	569	594	100	0.70	3.9	5.3	71	313	277	161	<1	89	2.9
mScarlet-I	569	593	104	0.54	3.1	5.4	57	363	225	190	3	129	0.6
mScarlet-H	551	592	74	0.20	1.3	4.8	15	75	574	368	<1	99	4.4
mRuby3	-	-	-	-	-	-	-	155	*	*	42	54	9.3
mRuby2	559	594	125	0.45	2.5	-	57	14	*	*	19	5	8.9
mKate2	587	631	63	0.39	2.5	-	25	69	390	169	<1	56	1.2
TagRFP-T	556	585	110	0.48	2.3	-	53	19	*	*	16	7	2.5
mApple	568	593	82	0.47	2.9	-	38	245	*	*	48	129	0.7
mCherry	586	610	88	0.23	1.5	-	20	100	376	300	<1	100	0.7
dTomato	555	582	90	0.69	3.4	-	62	254	494	337	<1	82	2.0

[a]Absorbance maximum. [b]Emission maximum. [c]Extinction coefficient at maximum absorbance. [d]Quantum yield relative to dTomato[7]. [e]Average fluorescence lifetime weighed by amplitude. [f]Apparent pK_a value. [g]Calculated brightness, product of extinction coefficient and quantum yield. [h]Brightness in mammalian cells normalized to mCherry. [i,j]Time in seconds to reduce emission rate from 1,000 to 500 photons s^{-1} $molecule^{-1}$ under widefield[i] and confocal spinning disk[j] conditions in mammalian cells. [k]Photochromic amplitude. [l]Accumulation in cells normalized to mCherry. [m]Apparent delay time of maturation relative to mTurquoise2 in mammalian cells. *, not applicable due to photochromic behavior. -, not determined. For calculation of mRuby3 accumulation in cells[l] the published calculated brightness[12] was used; all other values were obtained in this study.

pH sensitivity

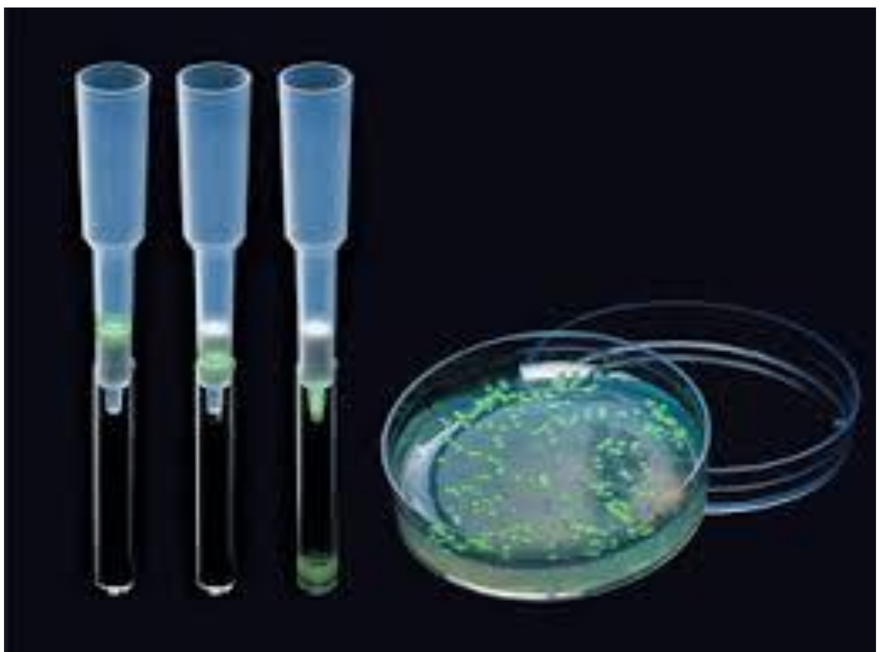


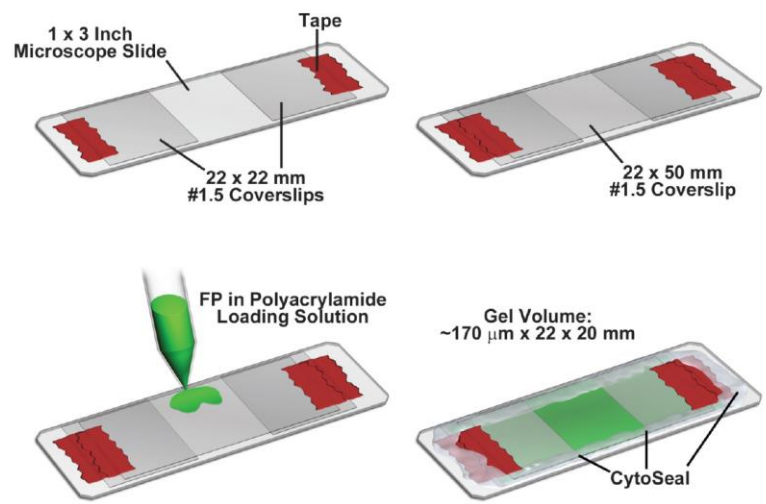


Quantitative assessment of fluorescent proteins

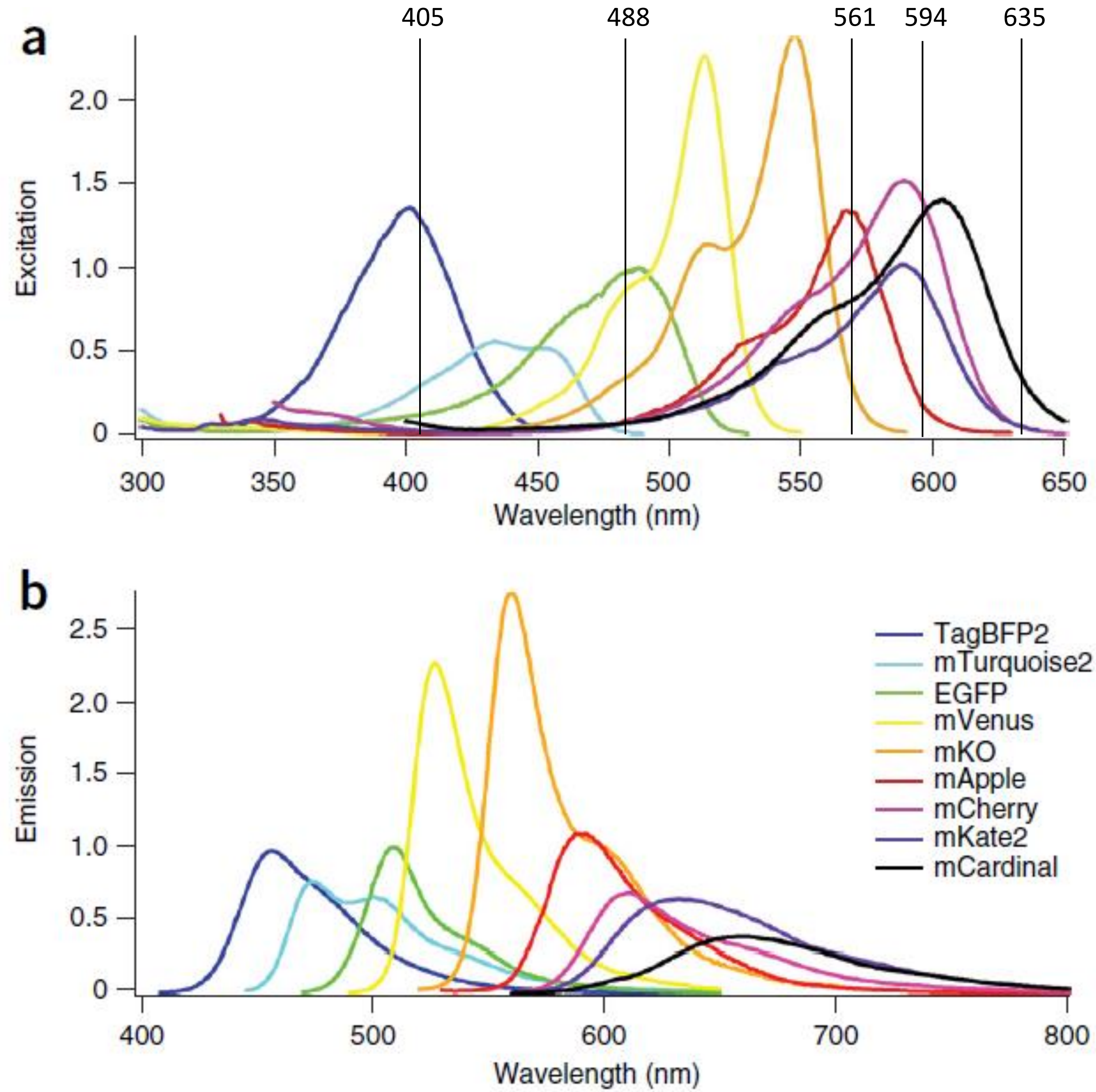
Paula J Cranfill[1,2,5], Brittney R Sell[1,5], Michelle A Baird[1,5], John R Allen[1,5], Zeno Lavagnino[2,3], H Martijn de Gruiter[4], Gert-Jan Kremers[4], Michael W Davidson[1,6], Alessandro Ustione[2,3] & David W Piston[2,3]

- Purification of HIS-tagged fluorescent proteins
- Protein conc. against BSA standard
- Measure spectra, Abs, Exc, QY,
- Embed fluorescent proteins in polyacryl gel to measure photobleaching in confocal and widefield microscopy
- OSER test for protein aggregation

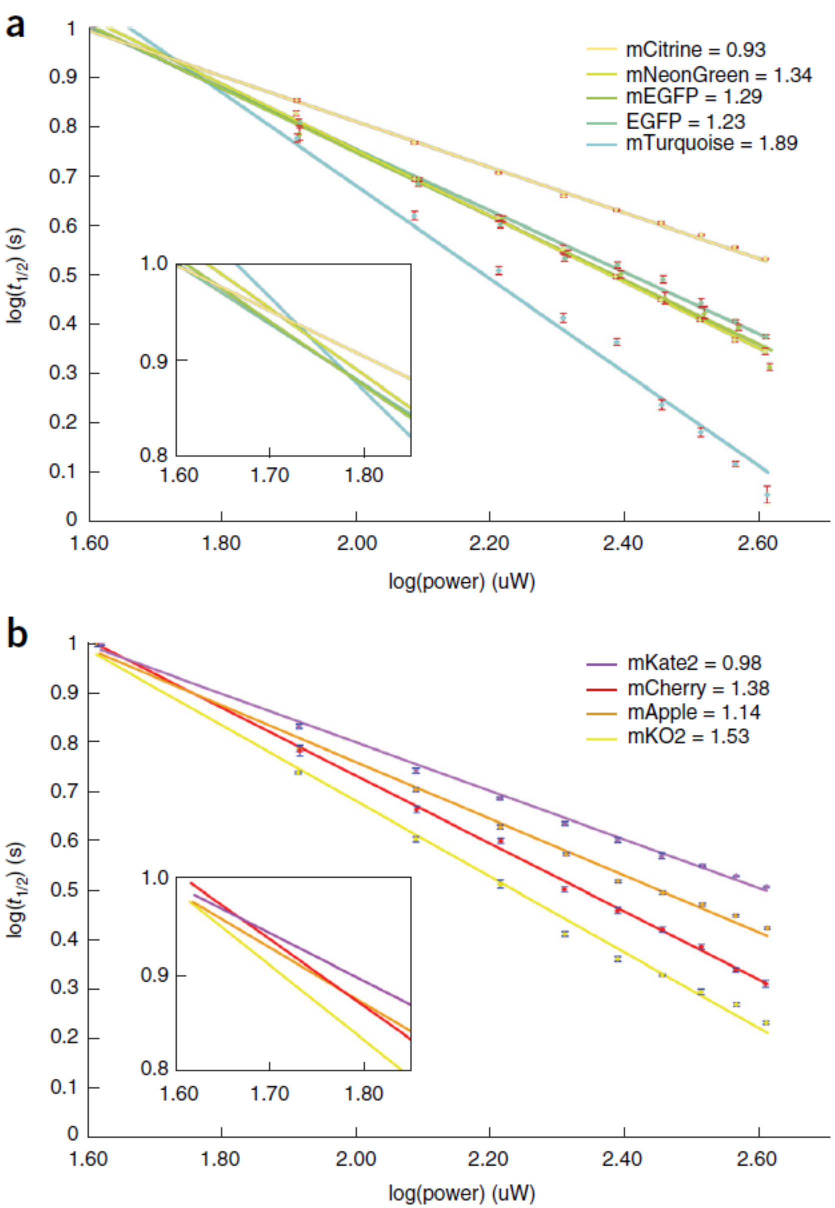




Absorbance and Emission Spectra



Bleaching rates for fluorescent proteins are supra-linear



$$\log(F) = -\alpha \log(P) + c,$$

$$k_{\text{bleach}} = bI^{\alpha},$$

- α= 1.07 : 2.4 x more bleaching with confocal
- α= 1.07 : 10 x more bleaching with confocal
- α= 1.35 : 100 x more bleaching with confocal

FP	in vitro	in cells (60 vs 140uW)
mCerulean	1.12	1.09
mEGFP	1.29	1.19
mVenus	1.13	1.14
mCherry	1.38	1.41

- Bleaching depends on environment
- Less bleaching with Spinning Disk Confocal, TIRF, light sheet or SIM

OSER assay shows oligomerisation of fluorescent proteins

Figure 3 | Wide-field fluorescence images of FP–CytERM fusion proteins expressed in live cells. (**a–l**) CytERM-fused mEGFP (A206K) (**a**), mCherry (**b**), mCitrine (A206K) (**c**), mOrange2 (**d**), mNeonGreen (**e**), mKate2 (**f**), EGFP (A206) (**g**), mKO2 (**h**), mTagBFP2 (**i**), mTagRFP-T (**j**), Citrine (A206) (**k**) and DsRed2 (**l**) expressed in HeLa cells. Scale bars, 10 μm. All images are representative of at least 7,000 cells analyzed for each FP.

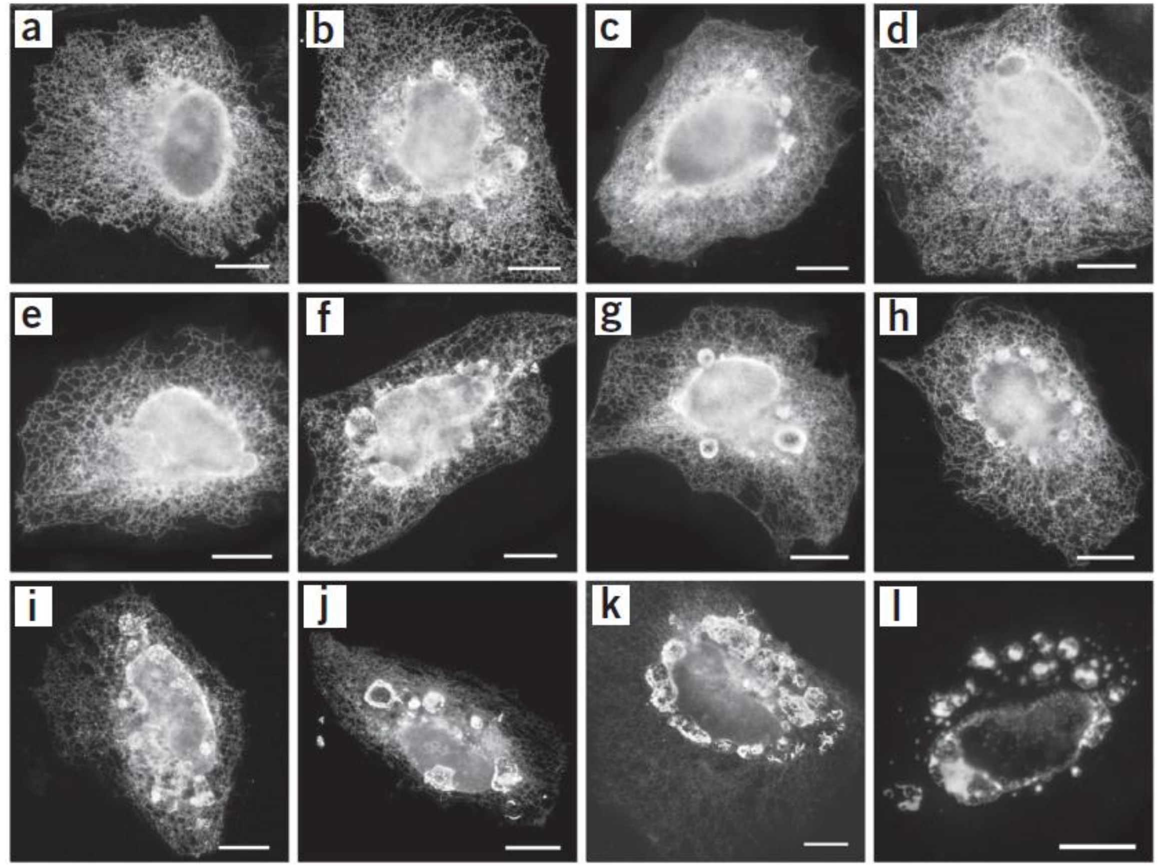


Table 1 | Percentage of cells scored without visible OSER whorls as a function of FP–CytERM fusion protein, ranked from most monomeric (100%) to most strongly oligomeric (0%)

FP	Normal cells (%)	s.d. (%)
mEGFP (L221K)	98.8	1.2
mEGFP (A206K)	98.1	1.6
mEmerald (A206K)	96.6	1.1
mRFP1	95.8	1.1
mT-Sapphire (A206K)	95.5	0.6
mApple	95.3	1.7
mPapaya	95.1	1.1
mCherry	95.0	0.8
CyPet	94.0	2.4
mKate2.5	93.9	1.7
mCitrine (A206K)	93.8	2.6
mTurquoise2 (A206K)	93.8	1.0
mTurquoise (A206K)	93.3	1.2
mRuby	93.1	2.1

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Fluorescent Protein Properties

	Class	Protein	Excitation (nm)		Emission (nm)		Fluorescence Quantum Yield			Extinction Coefficient ($M^{-1}cm^{-1}$)			Brightness (x 10^{3} $M^{-1}cm^{-1}$)		pK_a			Reference
			Literature	Our Data	Literature	Our Data	Literature	Our Data	s.d.	Literature	Our Data	s.d.	Literature	Our Data	Literature	Our Data	s.d.	
BFP2	Blue	EBFP2	383	386	448	448	0.56	0.53	0.01	32,000	39,000	725	17.92	20.67	5.3	4.4	0.07	43
		mTagBFP2	399	400	454	454	0.64	0.48	0.01	50,600	76,000	4,000	32.38	36.48	2.7	2.4	0.02	44
	Cyan	mTurquoise	434	434	474	474	0.84	0.84	0.02	34,000	31,000	400	28.56	26.04	4.5	3.5	0.02	45
mTrq2		mTurquoise2	434	434	474	473	0.93	0.92	0.03	30,000	31,000	300	27.90	28.52	3.1	3.6	0.01	46
		mCerulean	434	434	475	475	0.49	0.51	0.02	33,000	28,000	1,100	16.17	14.28	4.5	3.9	0.12	47
		mCerulean3	433	433	475	475	0.80	0.80	0.01	40,000	29,000	730	32.00	23.20	3.2	3.4	0.01	48
		mTFP1	462	467	492	492	0.85	0.85	0.02	64,000	53,000	1,000	54.40	45.05	4.3	4.3	0.12	49
	UV-Excitable Green	mT-Sapphire	399	396	511	509	0.60	0.59	0.00	44,000	34,000	1,100	26.40	20.06	4.9	4.8	0.05	50
	Green	EGFP	488	488	507	508	0.60	0.67	0.02	56,000	56,000	1,300	33.60	37.52	6.0	6.1	0.25	12
eGFP		mEGFP	NA	489	NA	508	NA	0.74	0.01	NA	62,000	1,550	0.001	45.88	6.0	5.8	0.14	17
		Emerald	484	483	509	509	0.68	0.75	0.01	57,500	62,000	1,150	39.10	46.50	6.0	4.6	0.02	51
		mEmerald	NA	483	NA	510	NA	0.79	0.01	NA	62,000	1,500	0.001	48.98	6.0	4.7	0.16	17
		sfGFP	485	487	507	509	0.65	0.72	0.01	83,300	53,000	1,750	54.15	38.16	5.5	5.8	0.09	14
	Yellow-Green	mPapaya	NA	528	NA	540	NA	0.74	0.02	NA	62,000	1,600	0.00	45.88	NA	6.6	0.02	6
		YPet	517	517	530	527	0.77	0.76	0.01	104,000	132,000	1,950	80.08	100.32	5.6	5.5	0.01	52
		Citrine	516	515	529	526	0.76	0.70	0.01	77,000	117,000	2,000	58.52	81.90	5.7	5.4	0.08	15
		mCitrine	NA	515	NA	528	NA	0.74	0.01	NA	120,000	2,600	0.001	88.80	5.7	5.6	0.13	17
		Venus	515	515	527	526	0.63	0.65	0.01	110,000	126,000	2,000	69.30	81.90	6.0	5.6	0.05	36
mVenus		mVenus	515	515	527	528	0.64	0.67	0.01	105,000	127,000	3,750	67.20	85.09	6.0	5.4	0.08	36
		Topaz	514	515	527	527	0.57	0.71	0.02	94,500	113,000	4,000	53.87	80.23	NA	6.3	0.12	51
		mTopaz	NA	515	NA	527	NA	0.68	0.02	NA	108,000	1,900	0.001	73.44	NA	5.9	0.16	17
		Clover	505	505	515	517	0.76	0.88	0.02	111,000	105,000	2,500	84.36	92.40	6.2	5.9	0.08	53
		mClover	NA	505	NA	516	NA	0.84	0.01	NA	105,000	1,800	0.001	88.20	NA	5.9	0.06	53
		mNeonGreen	506	504	517	517	0.80	0.80	0.01	116,000	113,000	1,900	92.80	90.40	5.7	5.4	0.01	54
	Orange	mOrange	548	548	562	563	0.69	0.64	0.02	71,000	112,000	7,750	48.99	71.68	6.5	6.3	0.10	6
		mOrange2	549	550	565	564	0.60	0.56	0.02	58,000	73,000	800	34.80	40.88	6.5	6.5	0.14	18
mKO		mKO	548	547	559	560	0.60	0.77	0.02	51,600	134,000	4,700	30.96	103.18	5.0	4.9	0.15	55
		mKO2	551	551	565	565	0.57	0.71	0.02	63,800	105,000	3,100	36.37	74.55	5.5	5.5	0.13	56
	Orange-Red	tdTomato	554	555	581	581	0.69	0.55	0.02	138,000	92,000	7,400	95.22	50.60	4.7	4.5	0.05	6
		TagRFP	555	556	584	581	0.48	0.33	0.02	100,000	130,000	4,100	48.00	42.90	3.1	3.0	0.15	57
		TagRFP-T	555	557	584	583	0.41	0.32	0.01	81,000	106,000	6,000	33.21	33.92	4.6	4.3	0.12	18
		DsRed2	563	561	582	583	0.55	0.53	0.02	43,800	77,000	690	24.09	40.81	NA	4.2	0.12	4, 58
	Red	mRuby	558	558	605	587	0.35	0.38	0.01	112,000	109,000	1,800	39.20	41.42	4.4	4.4	0.05	59
mApple		mRuby2	559	559	600	590	0.38	0.37	0.01	113,000	107,000	2,800	42.94	39.59	5.3	4.4	0.05	53
		mApple	568	569	592	591	0.49	0.46	0.02	75,000	75,000	1,000	36.75	34.50	6.5	6.5	0.09	18
mCherry		mRFP1	584	586	607	609	0.25	0.35	0.01	50,000	55,000	1,500	12.50	19.25	4.5	3.8	0.20	60
		mCherry	587	586	610	610	0.22	0.30	0.01	72,000	85,000	2,000	15.84	25.50	< 4.5	3.8	0.11	6
		FusionRed	580	577	608	604	0.19	0.30	0.01	83,000	85,000	1,800	15.77	25.50	4.6	4.2	0.01	61
	Far-Red	mKate2	588	587	633	623	0.40	0.42	0.02	62,500	57,500	600	25.00	24.15	5.4	5.5	0.05	62
mCardial		mNeptune	600	599	650	640	0.20	0.23	0.01	57,500	55,000	1,300	11.50	12.65	5.4	5.3	0.04	63
		mCardinal	604	603	659	651	0.19	0.18	0.00	87,000	79,000	1,550	16.53	14.22	NA	5.3	0.12	64
		mPlum	590	588	649	645	0.10	0.13	0.01	41,000	80,000	1,100	4.10	10.40	< 4.5	4.6	0.05	65

BFP2

mTrq2

eGFP

mVenus

mKO

mApple

mCherry

mCardinal

Class	Protein	Filter Set (WF)	Laser (Confocal)	LED (WF)	Photostability (α = slope of logBleachPower vs logt1/2) Confocal		Widefield Metal Halide		Widefield LED		t(1/2) 80 uW	t(1/2) 200 uW	Addgene Plasmid #
					α	s.d.	α	s.d.	α	s.d.			
Blue	EBFP2	DAPI	405	380	1.73	0.01	1.76	0.06	1.68	0.05	15.31±0.26	2.99±0.08	54542
	mTagBFP2	DAPI	405	380	0.77	0.01	0.72	0.02	0.83	0.02	6.21±0.07	3.1±0.02	54572
Cyan	mTurquoise	CFP	458	455	1.89	0.01	1.53	0.01	1.42	0.00	391.51±6.36	73.52±0.85	55584**
	mTurquoise2	CFP	458	455	1.92	0.01	1.68	0.01	1.60	0.01	71.71±1.28	14.01±0.39	54844
	mCerulean	CFP	458	455	1.12	0.01	1.25	0.01	1.28	0.02	74.63±2.31	32.54±0.53	54666
	mCerulean3	CFP	458	455	1.56	0.01	1.31	0.01	1.13	0.00	76.83±1.38	18.43±0.23	54730**
	mTFP1	CFP	458	455	1.36	0.01	1.47	0.01	1.28	0.01	72.34±1.41	14.03±0.50	54553
UV-Excitable Green	mT-Sapphire	Sapphire	405	380	1.16	0.01	1.71	0.01	1.61	0.02	5.99±0.07	1.88±0.05	54571
Green	EGFP	GFP	488	470	1.23	0.01	1.07	0.01	1.15	0.01	179.21±2.09	50.69±0.56	54762
	mEGFP	GFP	488	470	1.29	0.01	0.89	0.03	1.04	0.01	159.66±3.88	52.47±0.68	54622
	Emerald	GFP	488	470	1.03	0.01	0.98	0.03	0.86	0.02	79.36±0.69	37.74±0.48	54776
	mEmerald	GFP	488	470	1.02	0.01	0.98	0.01	0.92	0.02	80.75±0.65	32.02±0.34	54220
	sfGFP	GFP	488	470	1.12	0.01	1.02	0.01	0.99	0.00	208.26±5.28	67.63±0.51	54519
Yellow-Green	mPapaya	YFP	514	505	1.01	0.02	0.97	0.01	0.73	0.02			54838
	YPet	YFP	514	505	1.07	0.00	1.24	0.01	1.15	0.00	30.83±0.41	11.15±0.10	54860
	Citrine	YFP	514	505	1.03	0.00	1.04	0.01	1.14	0.01			54772
	mCitrine	YFP	514	505	0.93	0.00	1.02	0.01	1.07	0.00	15.67±0.09	6.46±0.03	54723
	Venus	YFP	514	505	1.15	0.00	1.18	0.01	1.21	0.01			54859
	mVenus	YFP	514	505	1.13	0.00	1.17	0.01	1.27	0.00	26.46±0.11	9.37±0.05	54845
	Topaz	YFP	514	505	1.04	0.00	1.07	0.01	1.16	0.00	27.53±0.21	10.70±0.08	54623
	mTopaz	YFP	514	505	1.04	0.00	1.05	0.01	1.18	0.00	28.08±0.17	9.89±0.10	54841
	Clover	YFP	514	505	1.07	0.00	1.00	0.01	1.11	0.00	61.83±0.18	23.71±0.13	54575
	mClover	YFP	514	505	1.12	0.00	0.96	0.01	1.15	0.00	53.22±0.20	19.20±0.05	54805
	mNeonGreen	YFP	514	505	1.34	0.00	0.98	0.01	1.12	0.01	197.22±2.80	32.88±0.67	Allele Biotechnology
Orange	mOrange	TRITC	561	530	1.01	0.00	1.14	0.03	1.00	0.01	13.69±0.28	5.60±0.01	54751
	mOrange2	TRITC	561	530	1.36	0.00	1.08	0.01	1.10	0.01	353.61±5.94	83.63±0.74	54531
	mKO	TRITC	561	530	1.67	0.01	1.52	0.01	1.22	0.01	531.84±11.11	108.86±1.03	54735
	mKO2	TRITC	561	530	1.53	0.00	1.10	0.01	0.96	0.01	51.22±0.28	11.38±0.12	54555
Orange-Red	tdTomato	TRITC	561	530	1.77	0.01	1.73	0.01	1.60	0.01	31.81±0.30	6.08±0.04	54856
	TagRFP	TRITC/mCherry*	561	530	0.96	0.01	1.00	0.01	0.82	0.03	29.02±1.42	13.53±0.10	Evrogen
	TagRFP-T	TRITC	561	530	1.33	0.01	1.04	0.03	1.20	0.01	84.74±1.97	27.10±0.73	42635**
	DsRed2	TRITC	561	530	0.65	0.00	1.44	0.03	1.54	0.01	2.70±0.01	1.55±0.00	54608
Red	mRuby	TRITC	561	590	1.17	0.01	1.04	0.01	1.07	0.01	40.69±0.47	14.38±0.16	54763
	mRuby2	TRITC	561	590	1.23	0.00	1.11	0.01	0.94	0.02	44.19±0.57	13.69±0.04	54771
	mApple	TRITC	561	590	1.14	0.00	1.00	0.01	0.95	0.01	75.92±1.12	28.33±0.12	54536
	mRFP1	mCherry	594	590	1.08	0.00	1.10	0.02	1.11	0.01	26.30±0.16	9.44±0.08	54667
	mCherry	mCherry	594	590	1.38	0.01	1.15	0.01	1.09	0.01	318.94±7.64	87.97±0.86	54630
	FusionRed	mCherry	594	590	1.06	0.00	1.08	0.02	1.08	0.01	7.54±0.03	2.88±0.01	54677
Far-Red	mKate2	mCherry/Cy5*	594	590	0.98	0.00	1.21	0.01	1.17	0.00	51.61±0.57	20.88±0.16	Evrogen
	mNeptune	Cy5	594	590	1.22	0.01	1.04	0.01	0.96	0.02	150.81±2.78	42.93±0.87	54714
	mCardinal	Cy5	633	590	1.39	0.01	1.00	0.01	0.96	0.02	539.36±14.24	153.48±2.11	54800
	mPlum	Cy5	594	590	1.55	0.01	1.07	0.02	1.03	0.01	388.63±9.79	95.90±2.58	54564

Characterization and development of photoactivatable fluorescent proteins for single-molecule–based superresolution imaging

Siyuan Wang[a], Jeffrey R. Moffitt[a], Graham T. Dempsey[a], X. Sunney Xie[a], and Xiaowei Zhuang[a,b,c,1]

Departments of [a]Chemistry and Chemical Biology and [b]Physics, Harvard University, [c]Howard Hughes Medical Institute, Cambridge, MA 02138

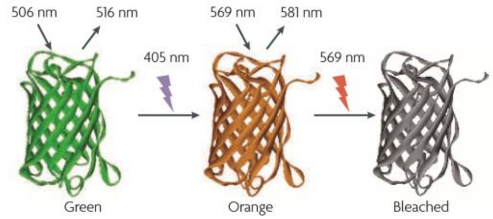


Table 1. Properties of PAFPs

PAFP	Preactivation/postactivation emission wavelength, nm*	Photon no.	On–off switching rate ratio	ClpP clustering†	No. of localizations per cell‡	Maturation time, min§
Dendra2	507/573	686	4.2×10^{-6}	–	1,810	38
mEos2	519/584	745	2.9×10^{-6}	+	1,290	340
mEos3.2	516/580	809	2.6×10^{-6}	–	1,950	330
tdEos	516/581	774	3.2×10^{-6}	–	1,800	330
mKikGR	515/591	599	4.1×10^{-6}	+	3,800	31
PAmCherry	—/595	706	7.8×10^{-6}	+	4,200	61
PAtagRFP	—/595	906	5.7×10^{-6}	–	760	200
mMaple	505/583	798	1.9×10^{-6}	+	24,000	48
mMaple2	506/582	783	1.0×10^{-6}	+	21,000	62
mMaple3	506/583	675	6.2×10^{-7}	–	12,300	49
PAGFP	—/517	313	1.3×10^{-3}	–		<10
PSCFP2	468/511	223	8.1×10^{-6}	+		
Dronpa	—/517	262	5.8×10^{-4}	–		25
mGeosM	—/514	248	4.9×10^{-4}	+		<10

A 405-nm laser was used for photoactivation. The photon number and on–off switching rate ratio were measured in live BS-C-1 cells. The ClpP clustering, number of localizations per cell, and maturation time were measured in live *E. coli* cells.

*The mMaple2 and mMaple3 emission wavelengths were measured in this work with purified proteins. The other wavelengths are cited from refs. 4 and 16.

†The "+" indicates that ClpP-PAFP exhibits clustered distributions in at least a subset of cells, whereas "–" indicates that ClpP-PAFP does not exhibit clustered distributions in any cells. The results on Dendra2, Dronpa, and mEos2 are consistent with a previous report (26).

‡The number of HU-PAFP localizations per *E. coli* cell.

§Maturation time is defined as the half-life of the immature state.

Wang & Zhuang 2013

Evaluation of fluorophores for optimal performance in localization-based super-resolution imaging

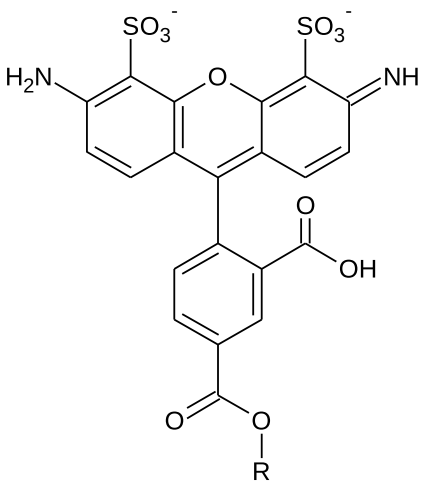
Graham T Dempsey[1,6], Joshua C Vaughan[2,3,6], Kok Hao Chen[3,6], Mark Bates[4] & Xiaowei Zhuang[2,3,5]

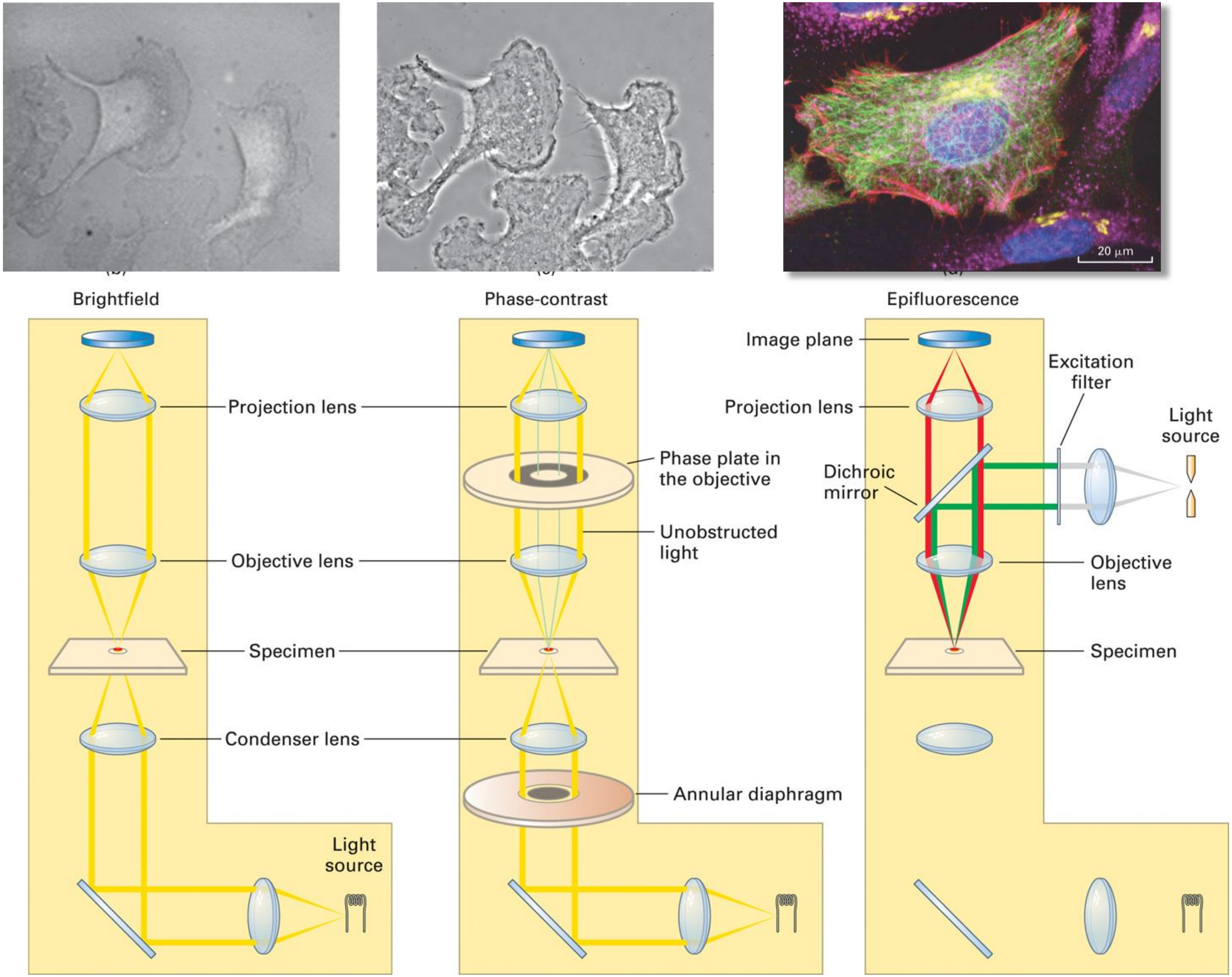
Table 1 | Summary of switching properties of the 26 dyes tested in this study

Dye	Excitation maximum (nm)[a]	Emission maximum (nm)[a]	Extinction (M^{-1} cm^{-1})[b]	Quantum yield[c]	Detected photons per switching event: MEA	Detected photons per switching event: βME	Equilibrium on-off duty cycle (400–600 s): MEA	Equilibrium on-off duty cycle (400–600 s): βME	Survival fraction after illumination for 400 s: MEA	Survival fraction after illumination for 400 s: βME	Number of switching cycles (mean: MEA	Number of switching cycles (mean: βME
Blue-absorbing												
Atto 488	501	523	90,000	0.8	1,341	1,110	0.00065	0.0022	0.98	0.99	11	49
Alexa Fluor 488	495	519	71,000	0.92	1,193	427	0.00055	0.0017	0.94	1	16	139
Atto 520	516	538	110,000	0.9	1,231	868	0.0015	0.00061	0.92	0.86	9	17
Fluorescein	494	518	70,000	0.79	1,493	776	0.00032	0.00034	0.51	0.83	4	15
FITC	494	518	70,000	0.8	639	1,086	0.00041	0.00031	0.75	0.9	17	16
Cy2	489	506	150,000	0.12	6,241	4,583	0.00012	0.00045	0.12	0.19	0.4	0.7
Yellow-absorbing												
Cy3B	559	570	130,000	0.67	1,365	2,057	0.0003	0.0004	1	0.89	8	5
Alexa Fluor 568	578	603	91,300	0.69	2,826	1,686	0.00058	0.0027	0.58	0.99	7	52
TAMRA	546	575	90,430	0.2	4,884	2,025	0.0017	0.0049	0.85	0.99	10	59
Cy3	550	570	150,000	0.15	11,022	8,158	0.0001	0.0003	0.17	0.55	0.5	1.6
Cy3.5	581	596	150,000	0.15	4,968	8,028	0.0017	0.0005	0.89	0.61	5.7	3.3
Atto 565	563	592	120,000	0.9	19,714	13,294	0.00058	0.00037	0.17	0.26	4	5
Red-absorbing												
Alexa Fluor 647	650	665	239,000	0.33	3,823	5,202	0.0005	0.0012	0.83	0.73	14	26
Cy5	649	670	250,000	0.28	4,254	5,873	0.0004	0.0007	0.75	0.83	10	17
Atto 647	645	669	120,000	0.2	1,526	944	0.0021	0.0016	0.46	0.84	10	24
Atto 647N	644	669	150,000	0.65	3,254	4,433	0.0012	0.0035	0.24	0.65	9	39
Dyomics 654	654	675	220,000	–	3,653	3,014	0.0011	0.0018	0.79	0.64	20	19
Atto 655	663	684	125,000	0.3	1,105	657	0.0006	0.0011	0.65	0.78	17	22
Atto 680	680	700	125,000	0.3	1,656	987	0.0019	0.0024	0.65	0.91	8	27
Cy5.5	675	694	250,000	0.28	5,831	6,337	0.0069	0.0073	0.87	0.85	16	25
NIR-absorbing												
DyLight 750	752	778	220,000	–	712	749	0.0006	0.0002	0.55	0.58	5	6
Cy7	747	776	200,000	0.28	852	997	0.0003	0.0004	0.48	0.49	5	2.6
Alexa Fluor 750	749	775	240,000	0.12	437	703	0.00006	0.0001	0.36	0.68	1.5	6
Atto 740	740	764	120,000	0.1	779	463	0.00047	0.0014	0.31	0.96	3	14
Alexa Fluor 790	785	810	260,000	–	591	740	0.00049	0.0014	0.54	0.62	5	2.7
IRDye 800 CW	778	794	240,000	–	2,753	2,540	0.0018	0.038	0.6	1	3	127

Excitation wavelength, dichroic mirrors and emission filters used for characterization and imaging for each spectral range were 488 nm, T495LP (Chroma) and ET535/50m (Chroma) for blue-absorbing dyes; 561 nm, Di01-R561 (Semrock) and FF01-617/73-25 (Semrock) for yellow-absorbing dyes; 647 nm, Z660DCXRU (Chroma) and ET700/75m (Chroma) for red-absorbing dyes; 752 nm, Q770DCXR (Chroma) and HQ800/60m (Chroma) for NIR-absorbing dyes, respectively. Dye-switching properties are reported in the presence of GLOX and 10 mM MEA as well as GLOX and 140 mM βME.
[a]Excitation and emission peak wavelengths from dye spectra. [b]Extinction coefficients from the dye manufacturers. [c]Quantum yields from either the dye manufacturer when known or from the McNamara 2007 fluorophore data tables. –, quantum yield values not available from dye manufacturer or McNamara data tables.

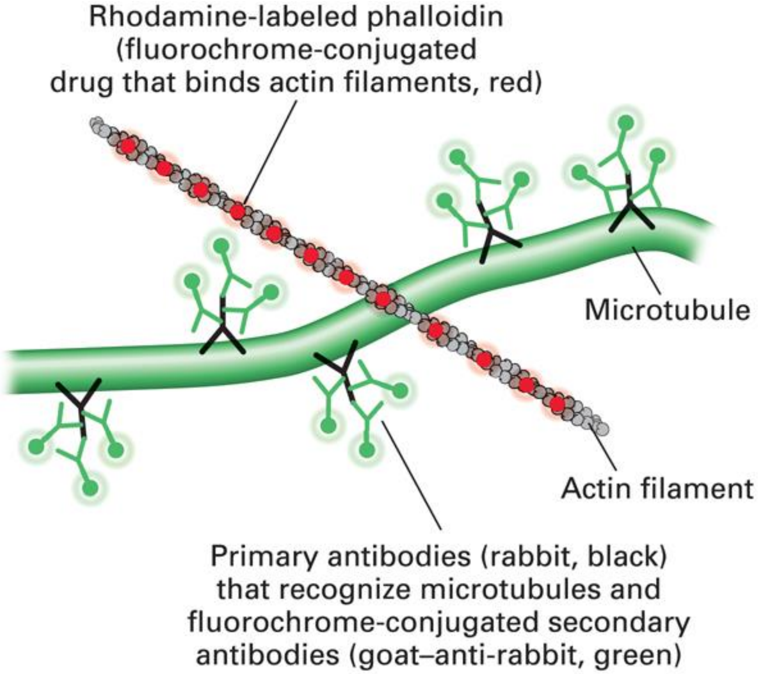
What is fluorescence ?

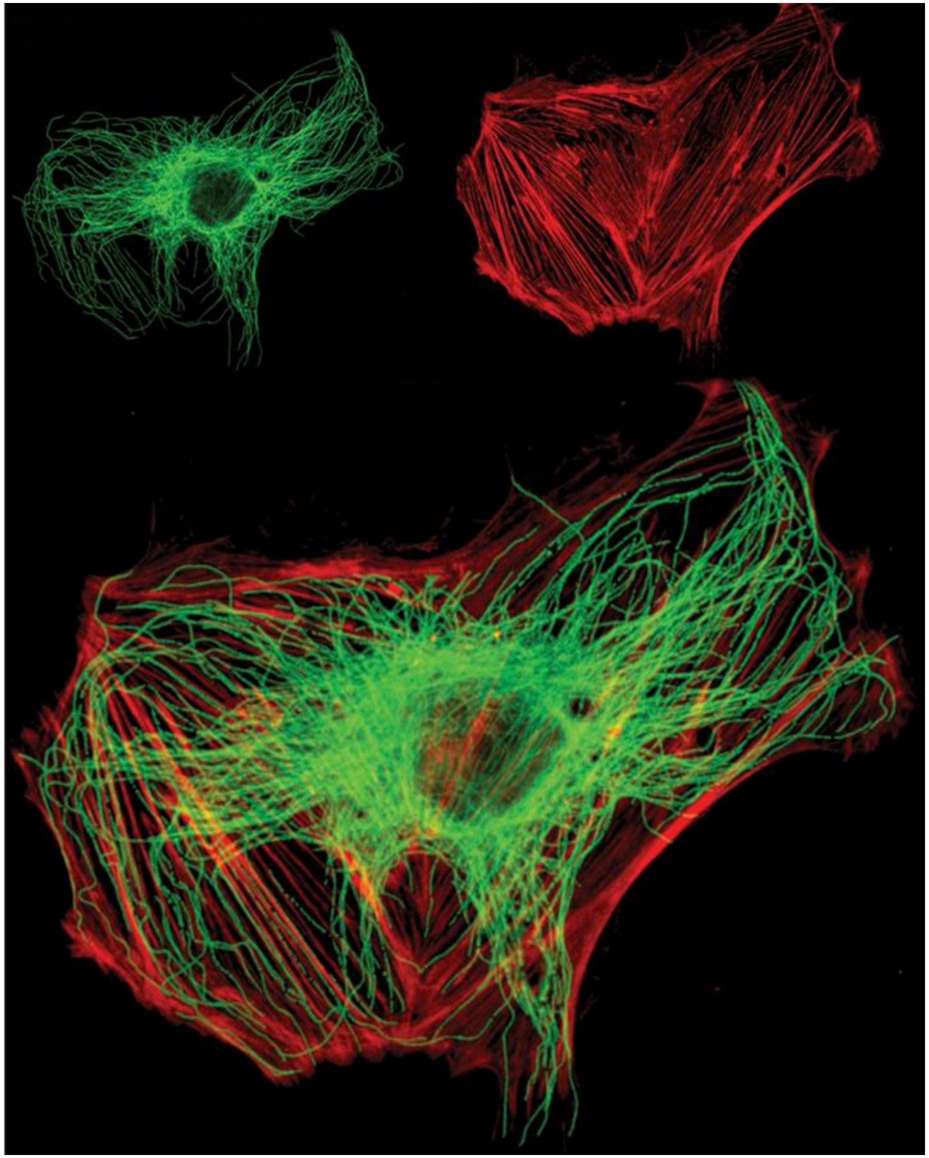




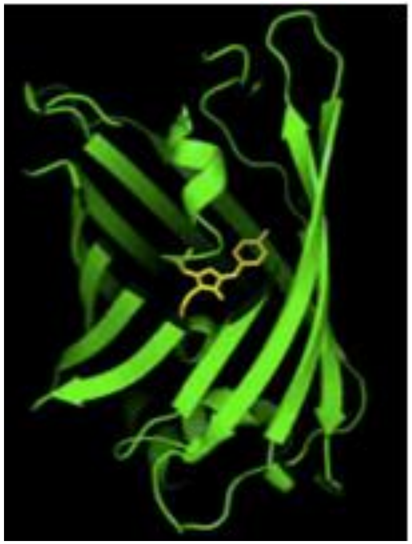
20 μm
Brightfield
Phase-contrast
Epifluorescence
Image plane
Projection lens
Excitation filter
Light source
Phase plate in the objective
Dichroic mirror
Unobstructed light
Objective lens
Specimen
Condenser lens
Annular diaphragm
Light source

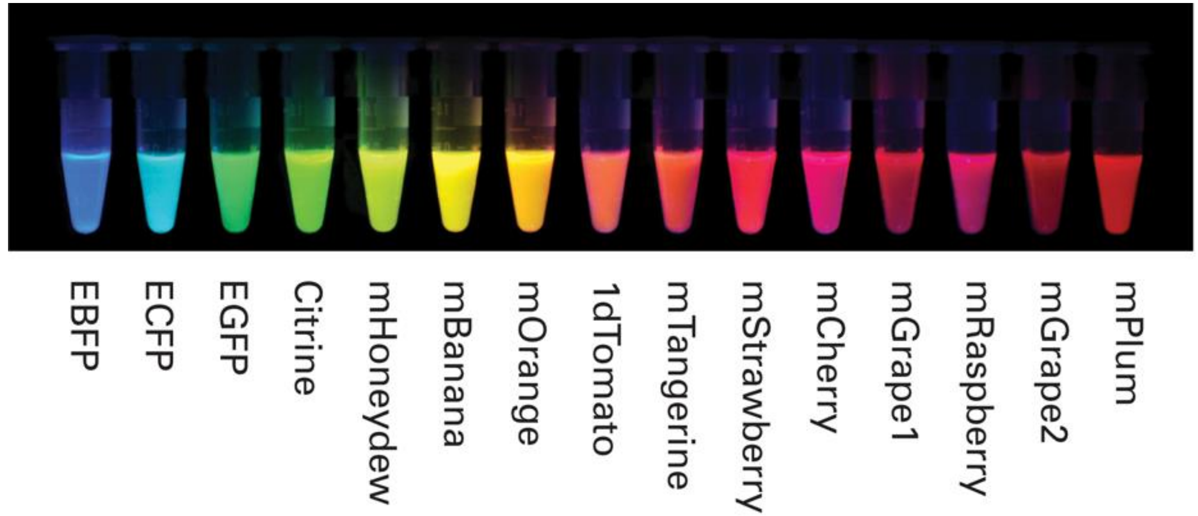
Fluorophors enable specific staining of cellular structures

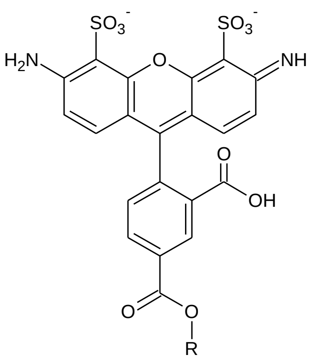




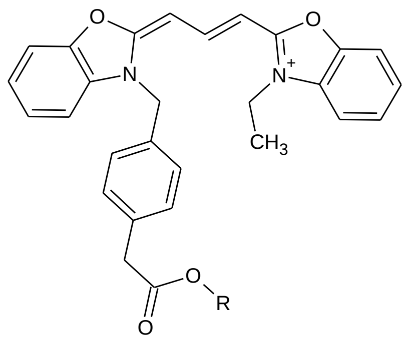
Fluorophors can be genetically encoded or organic dyes



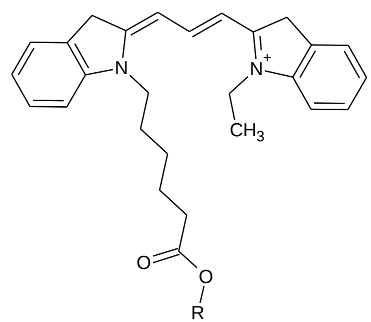




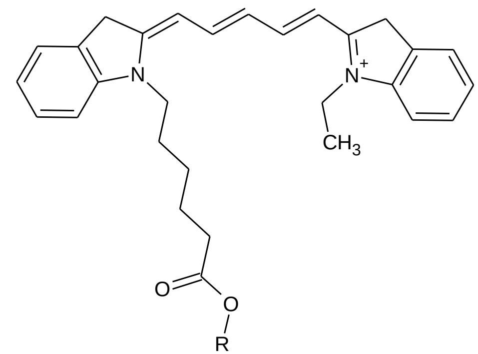
Alexa 488



Cy2



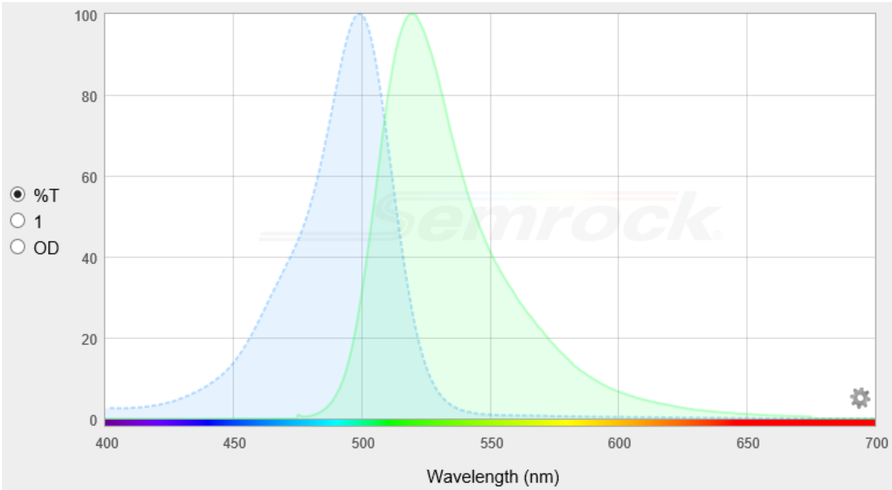
Cy3



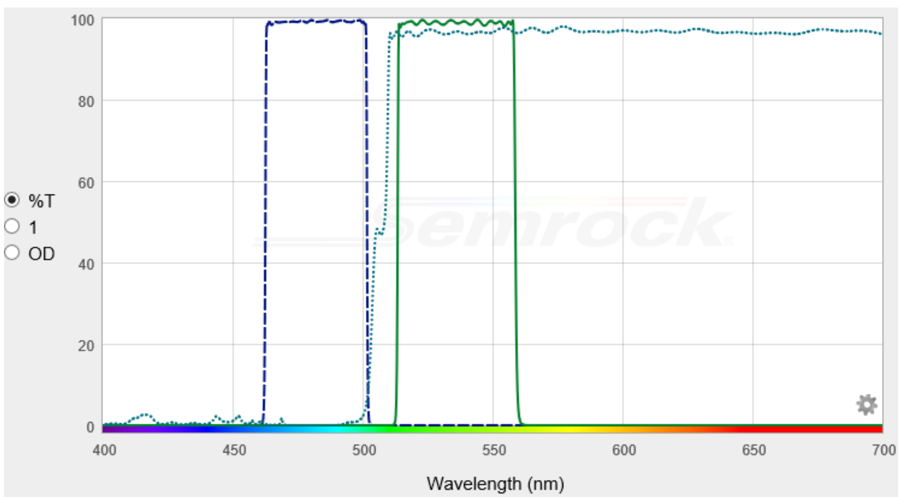
Cy5

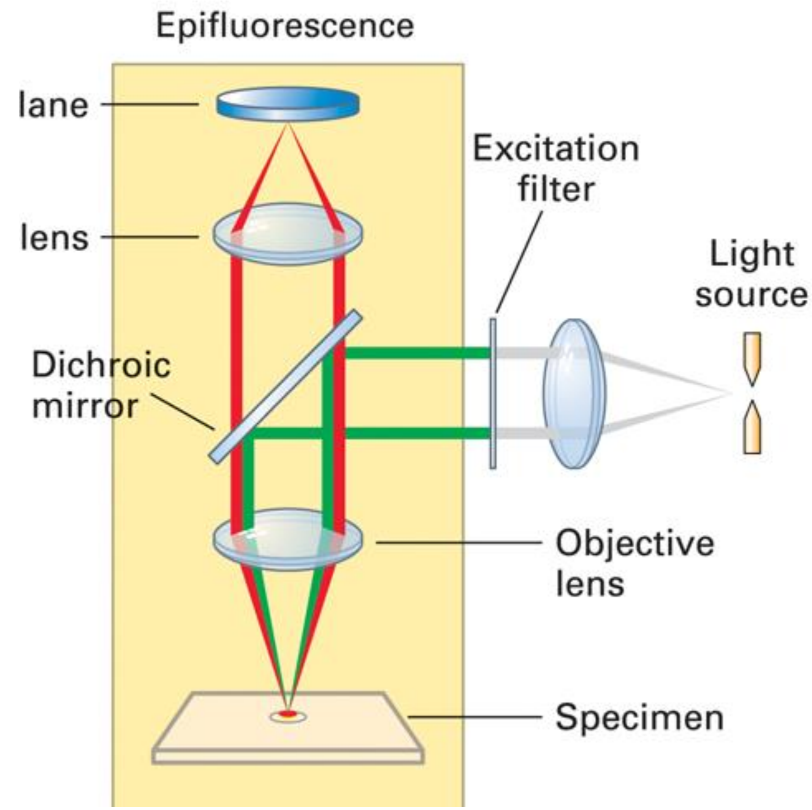
What else do we need for Imaging ?

Fluorophor (AF 488)



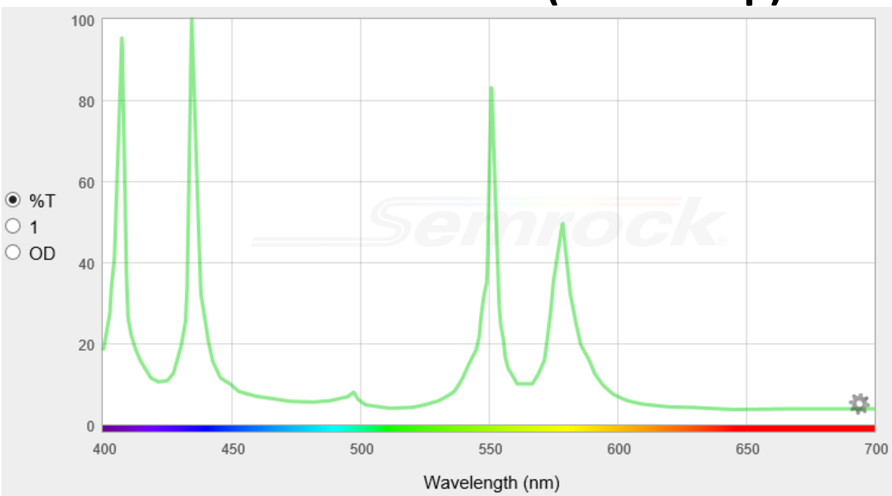
Filer Cube



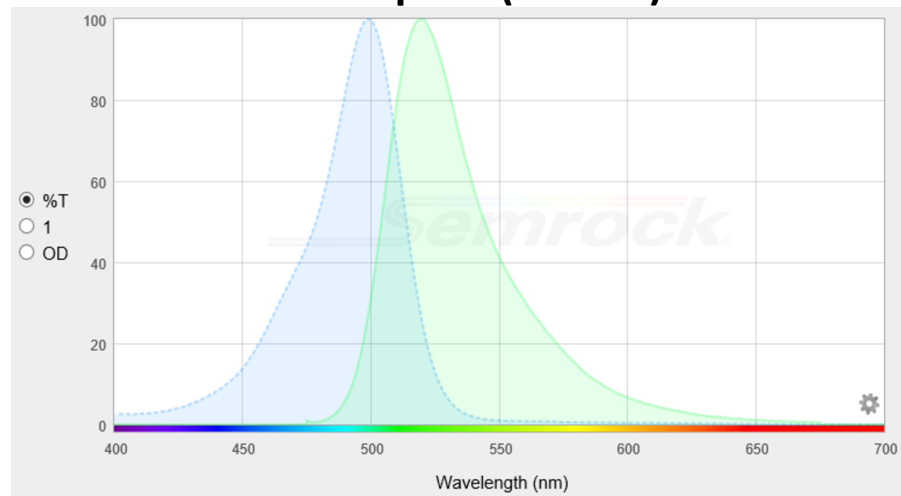


What else do we need for Imaging ?

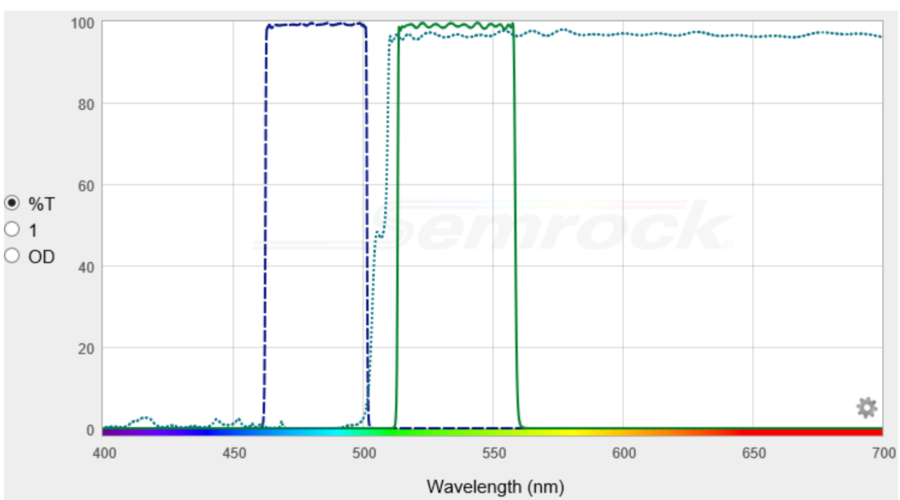
Excitation source (HBO Lamp)



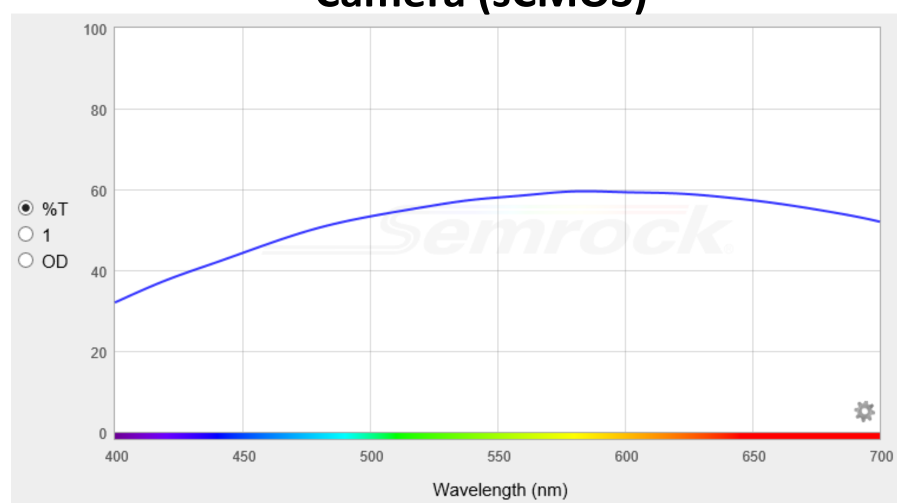
Fluorophor (AF 488)



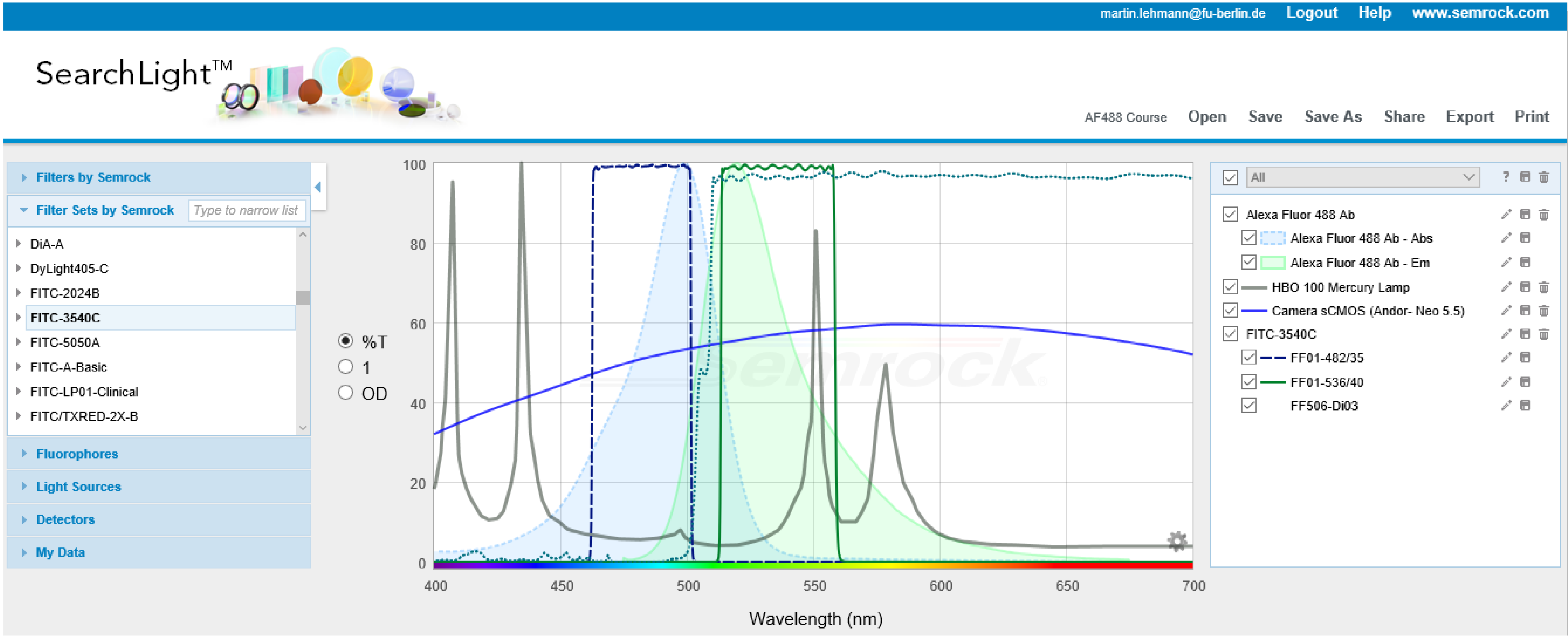
Filer Cube



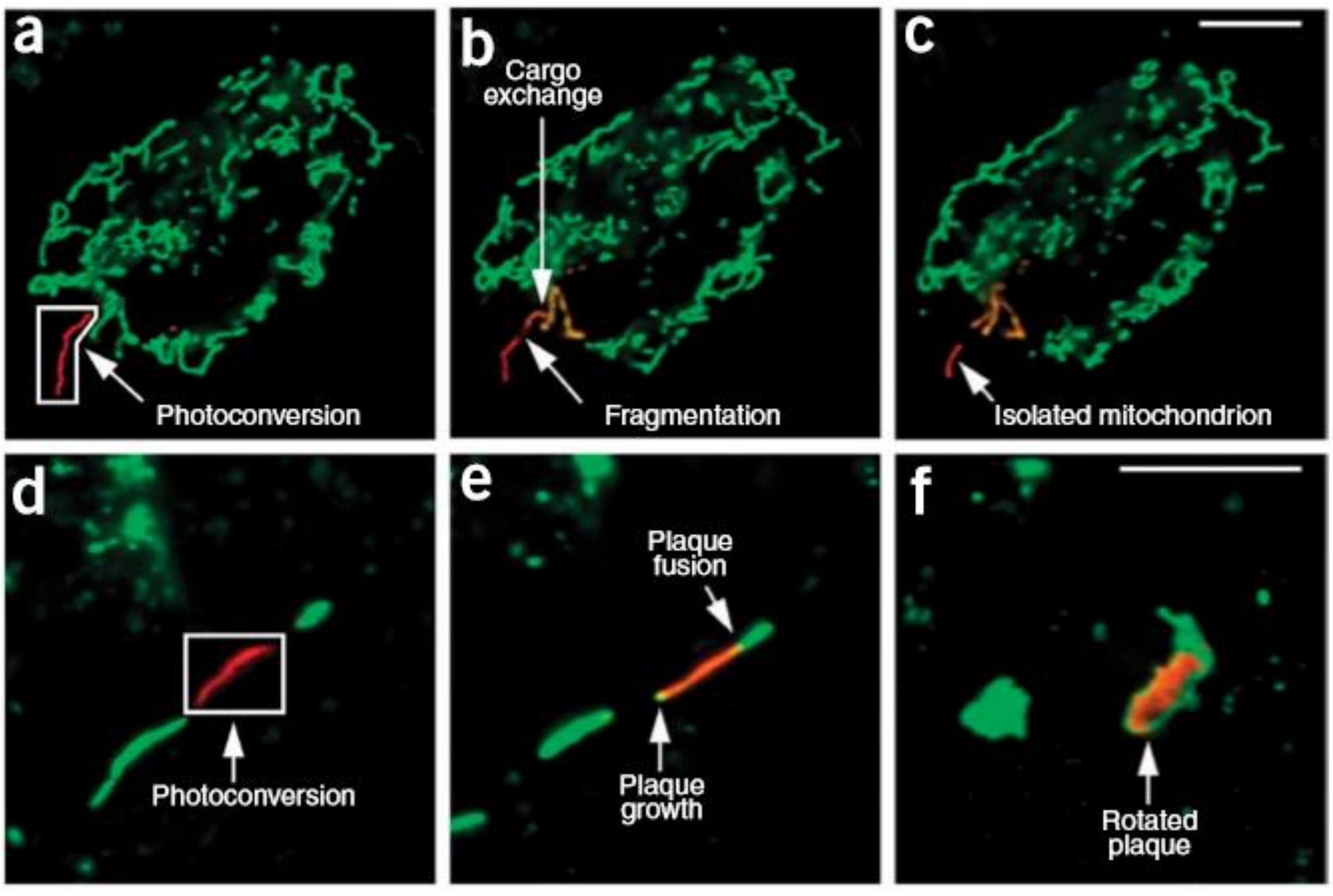
Camera (sCMOS)



http://searchlight.semrock.com/



Pulse-Chase Microscopy with mEos2



McKinney 2009

Wrap-up

1. Carefully choose right label & Microscope
2. What size do the structures have that I need to visualize?
3. What other cellular structures needs to be labelled for reference?
4. How thick are my samples ?
5. Are the structures observed in living and fixed cells ?
6. Are overexpressed proteins still functional and able to report a true picture?
7. Are my living cells still happy after imaging ?
8. Do I need 3D or temporal information or both ?
9. Should I automate image aquisition and analysis?

What is the resolution of a 60 x 1.5 NA Objective at 600nm ?
Which Emission Filter would you choose for eGFP? 520/40 , 605/70 or 440/40
How can one remove out of focus light ?
What Super-resolution Microscope has the highest resolution?

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Leibniz-Gemeinschaft

Andre Lampe

Gregor Lichtner

Georgi Tadeus

FUTURE PROJECTS:

- **Correlative Light and Electron Microscopy**
- **3D and live-cell imaging of cellular structures**
- **Set-up Single particle tracking**
- **Optimize STORM and STED microscopy**
- **Quantitative Imaging and automated Image Analysis**

If Interested in Lab rotation, master or PhD ? Please contact Volker Haucke, Tanja Maritzen Michael Krauss or Martin Lehmann