

Suitable dyes for Multi-Photon

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This table provides an overview of the excitation spectra obtained with different dyes. The data are based on empirical measurements; they may slightly vary with specimen treatment. (See also: Xu, et al. (1996), PNAS, 93, 10763)

Fluorochrome	Absorption	Emission
Alexa Fluor 350	720-800	440
Alexa Fluor 488	720-800	515
Alexa Fluor 546	720-840	569
Alexa Fluor 568	720-840	596
Alexa Fluor 594	720-850	610
Alexa Fluor 633	720-900	647
AMCA	780-800	444
bis-MSB	680-750	420
Bodipy	900-950	512
Calcium Crimson	900	615
Calcium green	780-850	531
Cascade Blue	750-800	420
Coumarin 307	780-800	530
CY2	780-800	506
CY3	780	565, 615
CY5	780-820	670
Dansyl Hydrazine	700-750	440
DAPI, Hoechst	700-820	455, 478
DiA	800-860	580
DID	780-820	670
DiO	780-830	510
eCFP	800-900	476
eGFP	820-950	509
eYFP	860-950	532
Fluorescein	780 - 820	519
Indo-1 free	690-720	490
Indo-1 Ca ²⁺	690-720	400
Lucifer Yellow	860-890	533
Mito Tracker red	750-840	600
Nile Red	810	640
Oregon Green	780-860	526
Propidium Iodide (PI)	820-850	617
Rhodamin B	800-840	600
Rhodamine 123	780-860	550
Sytox Green	740-760 or 880-940	524
TRITC	800-840	572



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Fluorochrome excitation with one-photon and two-photon systems.

Some of the information provided (e.g. 2P exc with “>” and “<” marks) is from Leica. This information is from a Leica system with a SpectraPhysics laser. Other info is empirical on our Leica SP2 with a Coherent Mira 9000 system. If you have more fluorochromes to add or wish to modify the existing data, please contact us with the dye name and any synonyms, and other information that will make this chart more useful to all users.

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Application	Dye	Solvents	1P exc	2P exc	UV exc	Max em
Cell wall/ fibers	Aniline blue	water	500	790	607-610	
	Calcofluor	water	440/500-520	780>820	340-400	400-440
(Polysciences)	FungiFluor (cellufluor)	water	350-400	780	250-400	350-400
Nucleic acid stains	DAPI, Hoechst	Water	350/470	700, 780>820	345	455, 478
	Feulgen	Water, ethanol	480/560	780>820	460	550
	Propidium Iodide (PI)	Water	540		305, 536	617
	Acridine orange		467		503	530, 640
	Ethidium Bromide	Water	488		493	620
(Molecular Probes)	SYTO 13				488	509
	SYTO 85				579	599
	YOYO-1				491	508
Protein conjugates	FITC	Ethanol, Water	490/525	780>820	495	519
	TRITC (rhodamine)		541/572	800-840	547	572
	Rhodamine B			840		600
	Texas red		596/620	780	589	615
(Jackson Imm. Res.)	CY2	water	489/506	780>800	489	506
	CY3	water	550/570	780	512, 552	565, 615
	CY5	water	649/670	780<820	625-650	670
	AMCA (7 amino-4 methylcoumarin-3-acetate)		431/498	780	347	444
(Molecular Probes)	Alexa 488				495	519
	Alexa 532				531	554
	Alexa 546				556	573
	Alexa 568				578	603
	Alexa 594				590	617
Gene expression (ClonTech)	BFP		395/509	780>820	382	448
	CFP		434/477	780>840	430	476
	GFP		488/507	800-850	498	516
	E GFP		490	900-950	498	516
	YFP		514/527	860<900	520	532
	DsRed1 & 2 (redFP)				558	583
Mitochondria (Molecular Probes)	Rhodamine 123		507/529	780-860	480	550
	Mito Tracker red	DMSO			579	599
	MitoTracker red 594				598	630
	MitoTracker Green FM				490	516

Lipids	Nile Red	ethanol		810	559	640
Calcium	Calcium green		488/530	780>820	506	531
	Ca Green/ Texas Red		488/530, 596/620	780		
	Yellow Chameleon		464/527	780>820		
	Indo1-free			700		490
	Indo1-Ca ⁺⁺			590		405
	Fura-2 free			700		362
	Fura-2 Ca ⁺⁺			?		?
Viability, esterases (PolySciences)	Fluorescein Diacetate	acetone	495/520	780>820	490-512	515-535
	CTC (viab – bacteria)			720-740	450	630
Neuronal tracer	DID			780		
	Lucifer Yellow CH	EtOH (water)			488 (428)	531
Lysosomes	Lysotracker (Mol. Probes)	MeOH, DMSO			577	590
Membrane Probes	DiI			700	561	565
	Filipin				380	510
triple probe (on Leica/Coherent system)	Dapi, FITC, and Rhodamine			740		see above

Table 3.3.1a

Extrinsic Fluorophores	λ (nm)	$\eta\delta_2$	δ_2
Bis-MSB	691/700	6.0 ± 1.8	6.3 ± 1.8
Bodypy	920	17 ± 4.9	–
Calcium Green	740 – 990	–	~ 80
Calcofluor	780/820	–	–
Cascade blue	750 – 800	2.1 ± 0.6	~ 3
Coumarin 307	776, 700 – 800	19 ± 5.5	~ 20
CY2	780/800	–	–
CY3	780	–	–
CY5	780/820	–	–
DAPI (free)	700/720	0.16 ± 0.05	~ 3.5*
Dansyl	700	1	–
Dansyl Hydrazine	700	0.72 ± 0.2	–
Dil	700	95 ± 28	–
Filipin	720	–	–
FITC	740 – 820	–	~ 20 – 38*
Fluorescein (pH ~ 11)	780	–	38 ± 9.7
Fura-2 (free)	700	11	–
Fura-2 (high Ca)	700	12	–
Hoechst	780/820	–	–
Indo-1 (free)	700	4.5 ± 1.3	12 ± 4
Indo-1 (high Ca)	590/700	1.2 ± 0.4	2.1 ± 0.6
Lucifer Yellow	840 – 860	0.95 ± 0.3	~ 2
Nile Red	810	–	–
Oregon Green Bapta 1	800	–	–
Rhodamine B	840	–	210 ± 55
Rhodamine 123	780 – 860	–	–
Syto 13	810	–	–
Texas red	780	–	–
Triple probe (Dapi, FITC, and Rhodamine)	720/740	–	–
TRITC (rhodamine)	800 – 840	–	–

Table 3.3.1b

Intrinsic Emitters	λ (nm)	δ_2
GFP wt	800 – 850	~ 6
GFP S65T	~ 960	~ 7
BFP	780/820	–
CFP	780/840	–
YFP	860/900	–
EGFP	940 – 1000	~ 250
DsRed-Coral Red	960 – 990	~ 20 – 110
Citrine- Coral yellow	950	~ 70
Phycoerythrin	1064	~ 300
Flavins	~ 700 – 730	~ 0.1 – 0.8
NADH	690 – 730	~ 0.02 – 0.09
Retinol	700 – 830	~ 0.07
Pyridoxine	690 – 710	~ 0.008
Folic acid	700 – 770	~ 0.007
Lipofuscin	700 – 850	–
Collagen, Elastin	700 – 740	–
Qdts	700 – 1000	~ 2000 – 47000

Blue/Cyan Dyes

Dye	Excitation
Alexa 350	780-800 nm
Hoechst	780-800 nm 900-1100 nm
DAPI	780-800 nm 900-1100 nm
CFP	800-900 nm

Green Dyes

Dye	Excitation
Oregon Green	800-860 nm
Alexa 488	800-830 nm
eGFP	920-990 nm
BODIPY	900-950 nm
FITC	750-800 nm
DiO	780-830 nm

Yellow/Orange Dyes

Dye	Excitation
YFP	890-950 nm
DiA	800-860 nm

Red Dyes

Dye	Excitation
DiI	830-920 nm
Rhodamine B	800-860 nm
Alexa 568	780-840 nm

Fluorophore Name (Abbreviation)	Excitation Wavelength (Nanometers)
(BM) <i>p</i> -bis (<i>o</i> -methylstyryl) benzene	691
(CB) Cascade Blue hydrazide trisodium salt	750
(YL) Lucifer Yellow CH ammonium salt	860
(BD - Bodipy) 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a, 4a-diazaindacene-2,6-disulfonic acid disodium salt	920
(DP - DAPI not DNA bound) 4',6-diamidino-2-phenylindole dihydrochloride	700
(DN - Dansyl) 5-dimethylaminonaphthalene-1-sulfonyl hydrazine	700
(PY) 1,2-bis-(1-pyrenedecanoyl)- <i>sn</i> -glycero-3-phosphocholine	700
(CM) coumarin 307	776
(IC) indo-1 with Ca ⁺⁺	700
(IF) indo-1 without Ca ⁺⁺	700
(FC) fura-2 with Ca ⁺⁺	700
(FF) fura-2 without Ca ⁺⁺	720
(CG) Calcium Green-1 with Ca ⁺⁺	725
(CO) Calcium Orange with Ca ⁺⁺	800
(CC) Calcium Crimson with Ca ⁺⁺	850
(F3) fluo-3 with Ca ⁺⁺	800

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Table 1. Fluorophores and Chromophores for Two-Photon Excitation

Fluorophores/Chromophores	Φ^a (GM)	2PE ^b (nm)	Em. (nm)	Note	References
Calcium indicators					
Fluo -3, -4, -5F, 4FF et al.		810 ^c	520–530		(Yasuda et al., 2004)
Oregon Green BAPTA -1, -2 et al.		810 ^c	520		(Yasuda et al., 2004)
Calcium green-1 + Ca ²⁺ ; Calcium green-1 – Ca ²⁺	30, 2	820	530		(Xu and Webb, 1996; Xu et al., 1996)
Fura-2 + Ca ²⁺ ; Fura-2 – Ca ²⁺	6, 0.2	800	505		(Wokosin et al., 2004)
Indo-1 + Ca ²⁺ ; Indo-1 – Ca ²⁺	3.5, 1.5	700	400		(Xu and Webb, 1996; Xu et al., 1996)
Quantum dots					
Quantum dots	up to 47,000	broad	variable		(Larson et al., 2003)
Fluorescent proteins					
eCFP	100–200	800–900	505		(Zipfel et al., 2003)
eGFP	100–200	900–1000	510		(Zipfel et al., 2003)
eYFP	100–200	930–1000	530		(Zipfel et al., 2003)
mRFP, mCherry		1030 ^c	610	Ytterbium-doped laser	(Campbell et al., 2002; Shaner et al., 2004)
Photoswitchable fluorescent proteins (see also Lukyanov et al., 2005)					
paGFP		750 ^g	515		(Patterson and Lippincott-Schwartz, 2002; Schneider et al., 2005)
Kaede		730 ^d	520 → 580	green to red; tetramer	(Ando et al., 2002)
KFP1		1120 ^d	600	tetramer	(Chudakov et al., 2003)
Dronpa		780 ^{d,e} , 1010 ^{d,f}	520	reversible	(Ando et al., 2004; Habuchi et al., 2005)
psCFP		800 ^d	470 → 510	cyan to green	(Chudakov et al., 2004)
PA-mRFP		760 ^d	605		(Verkhusha and Sorkin, 2005)
KikGR		760 ^c	520 → 590	green to red; tetramer	(Tsutsui et al., 2005)
Dendra		960 ^d	505 → 575	green to red	(Gurskaya et al., 2006)
mEosFP		780 ^d	520 → 580	green to red	(Wiedenmann et al., 2004)
Caged glutamate					
MNI-glutamate	0.06	730			(Matsuzaki et al., 2001)
Caged calcium					
DM-nitrophen	0.013	730		K_d : 2 nM ^h , 1.5 mM ⁱ	(Brown et al., 1999; Momotake et al., 2006)
Azid-1	1.4	700		K_d : 230 nM ^h , 0.12 mM ⁱ	(Brown et al., 1999; Momotake et al., 2006)
NDBF-EGTA	0.6	710		K_d : 14 nM ^h , 1 mM ⁱ	(Momotake et al., 2006)

^a Two-photon cross-section, if known.

^b Wavelength corresponding to the measured two-photon cross-section, unless indicated otherwise.

^c Wavelength typically used for two-photon excitation.

^d Wavelength corresponding to twice the peak of one-photon absorption. For fluorescent proteins, this is typically a good estimate of the location of the peak of the two-photon cross-section (Zipfel et al., 2003).

^e Photoactivation wavelength.

^f Photoquenching wavelength.

^g Although its absorption maximum is at 750 nm, we recommend longer wavelength to photo-activate paGFP (e.g., 810 nm). To image paGFP without photoactivating, we use 990 nm (Ti:Sapphire) or 1030 nm (Ytterbium-doped laser).

^h Affinity for calcium before photolysis.

ⁱ Affinity for calcium after photolysis.

microscopy. 2PE microscopy requires large photosensitive areas (millimeters) (Oheim et al., 2001), which precludes the use of avalanche photodiodes. Other important factors include the quantum efficiency (QE), gain, absorption spectra, dark noise, and the acceptance angle of the detector. Photomultiplier tubes (PMTs) are best for most applications. The recently developed GaAsP photocathode PMTs (Hamamatsu, H7422P) have improved QE, and short transit time spreads (jitters) which make them useful general purpose detectors, including for fluorescence lifetime imaging (see below). However, their small acceptance angles may lead to signal losses when combined with high-NA/low-magnification objectives.

Software

2PE microscope hardware can be assembled mostly with off-the-shelf components. An important component is suitable software to control the microscope and acquire data. Flexible and user-friendly open-source

custom software tools are freely available (Pologru et al., 2003; Tsai et al., 2002; Nguyen et al., 2006).

Fluorophores and Chromophores

The photophysical properties of fluorescent probes are critical for experimental design (Table 1). It is difficult to predict 2PE spectra from the one-photon spectra, because different quantum mechanical selection rules apply. The 2PE spectra of several useful fluorescent molecules have been measured (Albota et al., 1998a, 1998b; Xu and Webb, 1996; Xu et al., 1996; Bestvater et al., 2002; Spiess et al., 2005; Fisher et al., 2005; Shear et al., 1997; Wokosin et al., 2004). 2PE cross-sections are expressed in units of Göppert-Mayer (GM $\equiv 10^{-50}$ cm⁴ s). Excellent fluorophores, such as rhodamine B, have cross-sections larger than 100 GM (Xu and Webb, 1996). Bright one-photon fluorophores typically make good two-photon fluorophores, but exceptions have been reported (Xu and Webb, 1996). Compared