Fluorescence in Flow Cytometry

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EMBL, Heidelberg

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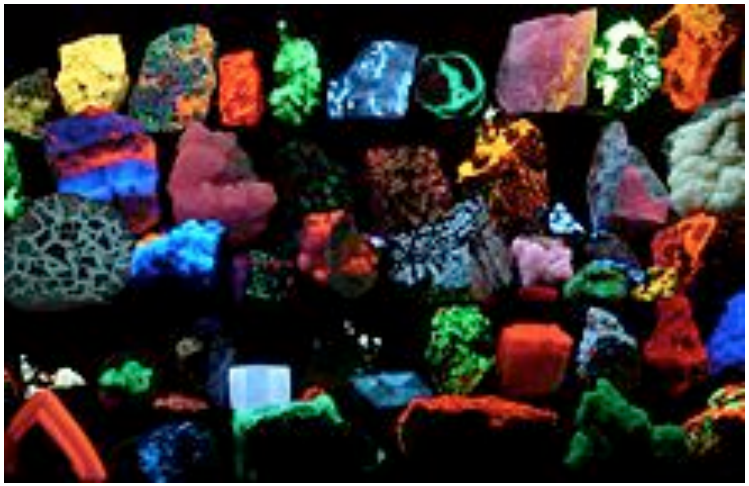
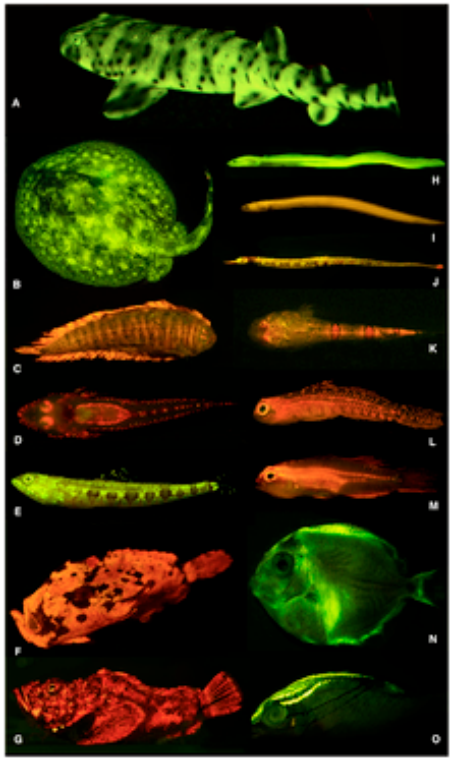
Fluorescence is everywhere!

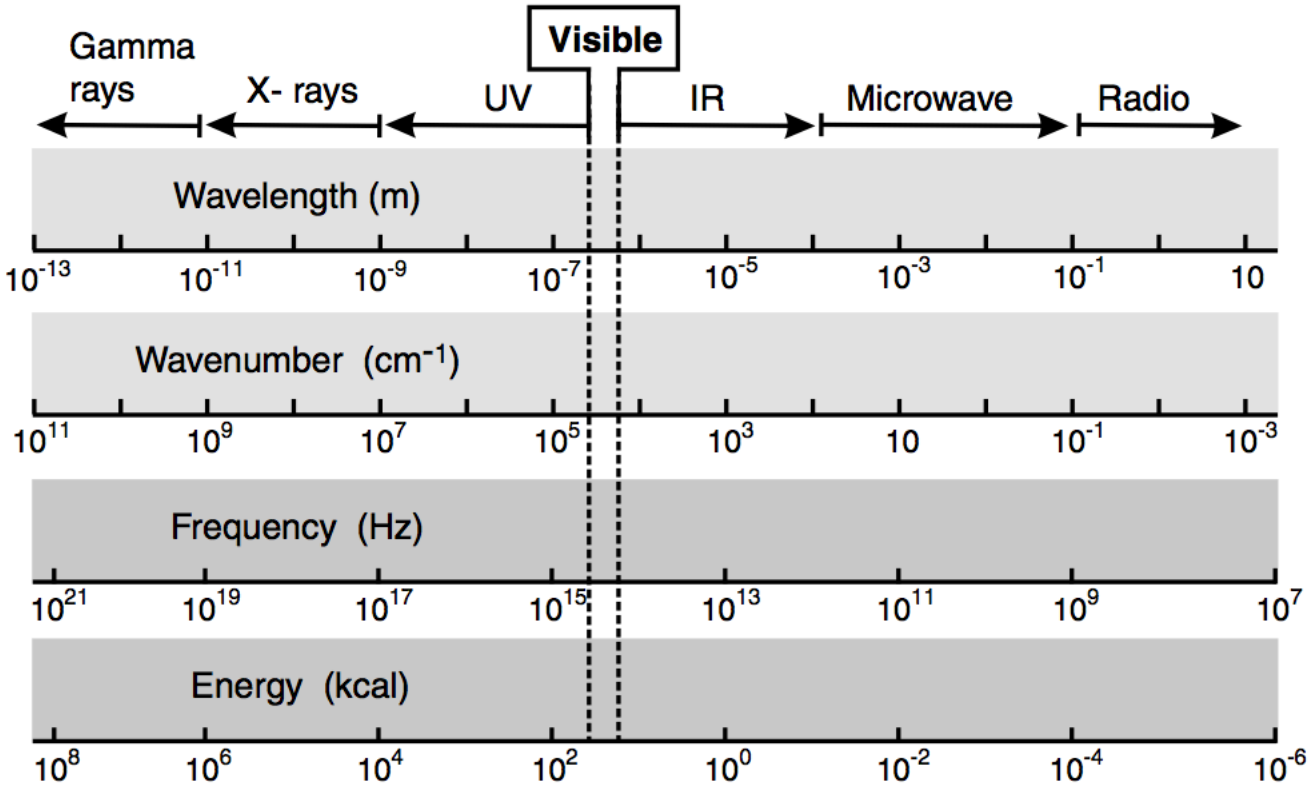


In nature…



In biotech…

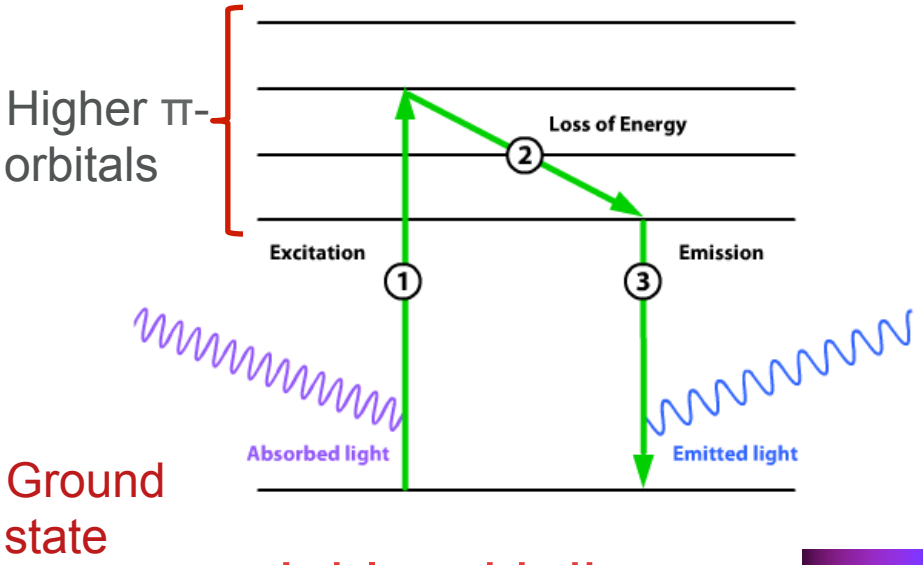
How does Fluorescence work



Wiley, Principles of spectroscopy

How does Fluorescence work

Fluorescence in a nutshell

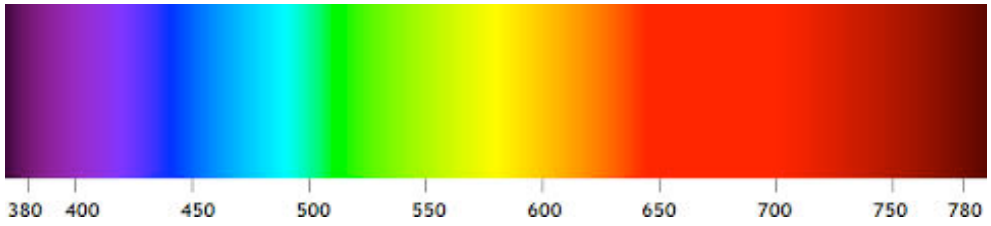


$S_0 + hv_{ex} \rightarrow S_1$

$S1 \rightarrow S0 + hv_{em} + hv_{heat}$

Jablonski diagram

(image source Wikipedia)



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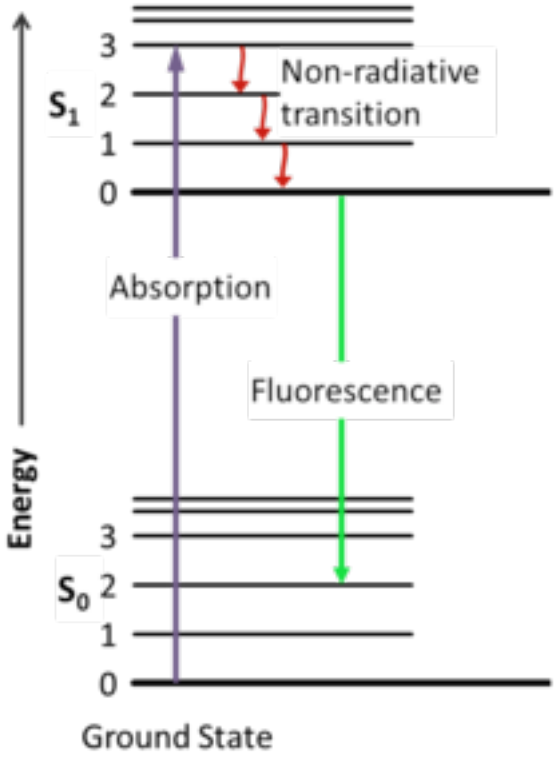
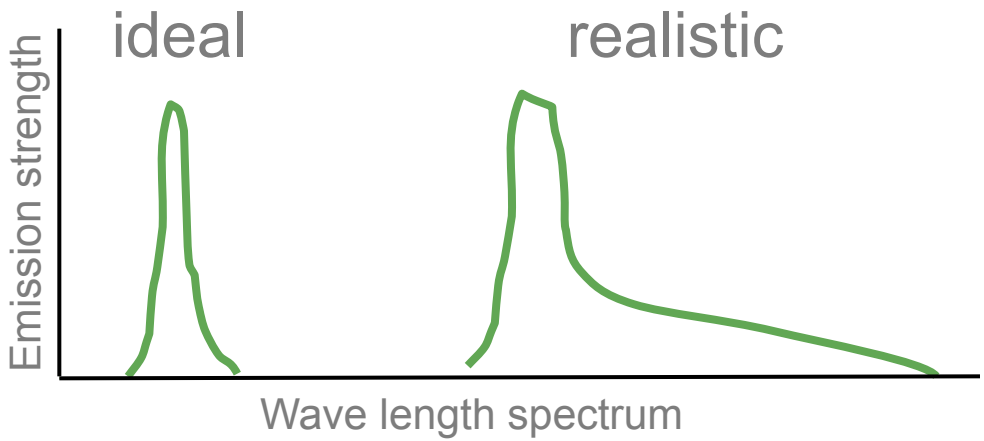
How does Fluorescence Work



Jablonski diagram

(image source Wikipedia)

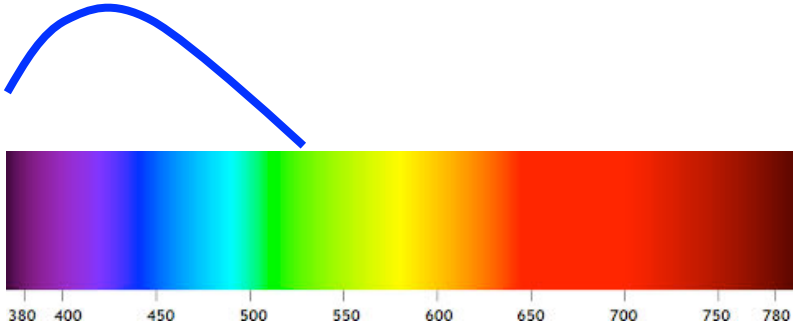
→ Depending on the amount of heat dissipation and molecule movements, the emission is never homogeneous!



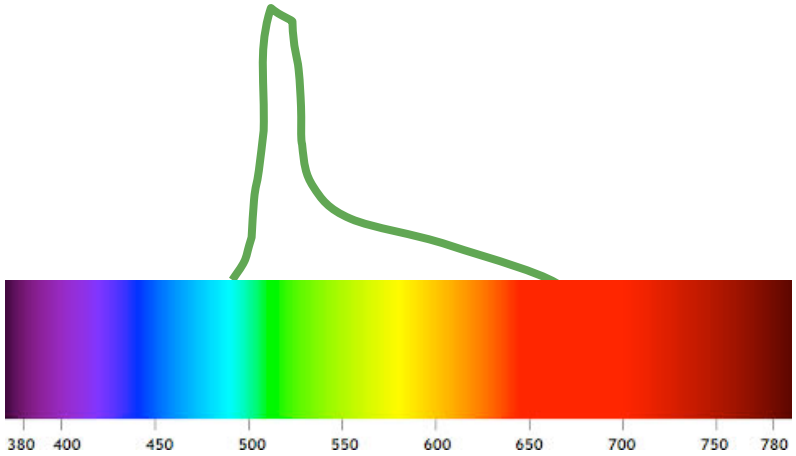
How does Fluorescence Work

→ Depending on the amount of heat dissipation and molecule movements, the emission is never homogeneous!

→ We see a ‘distinct’ fluorescence color, because this is the most abundant fluorescence emitted from the respective molecule



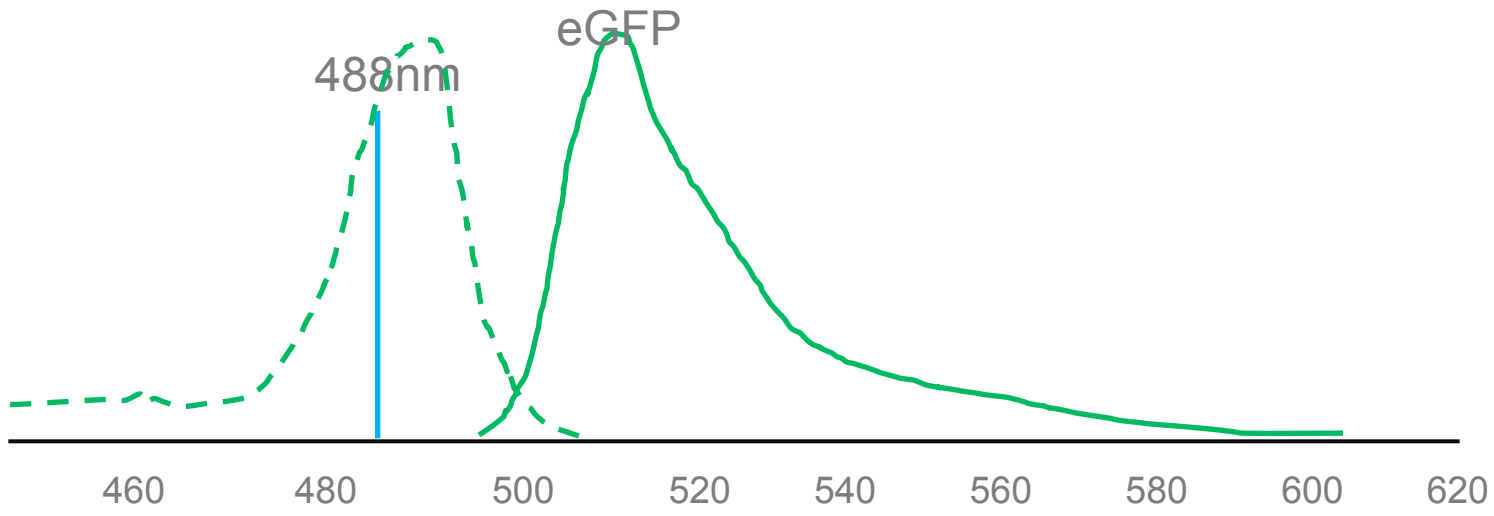
DAPI



FITC

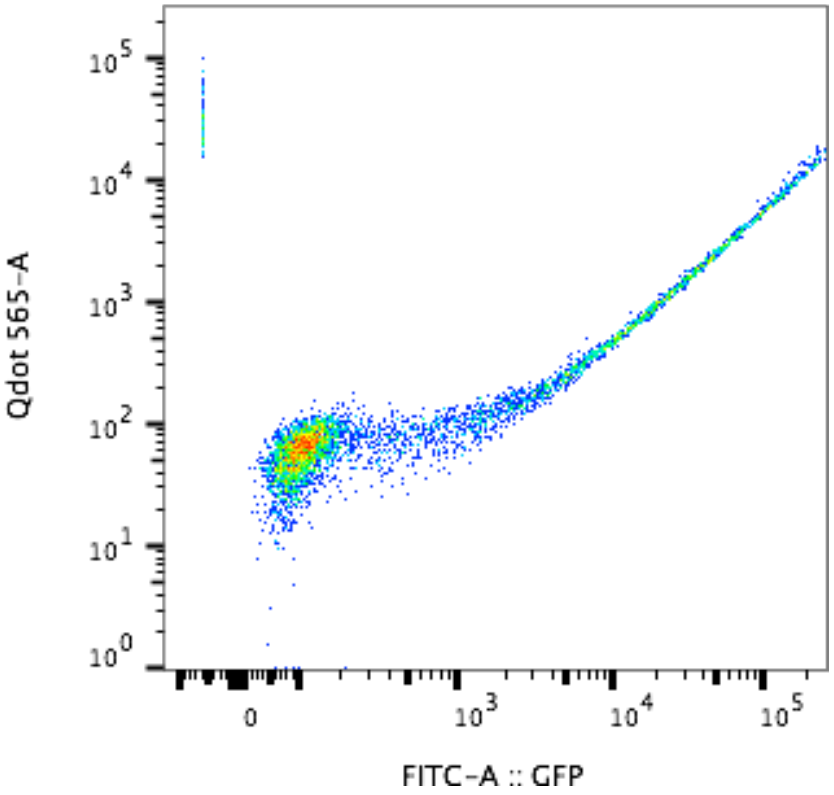
How does Fluorescence work

→ As the emission of fluorescence is commonly biased but stochastic, how does the excitation side behave?



Mirror-Effect: Excitation and emission behave as if they are mirrored!

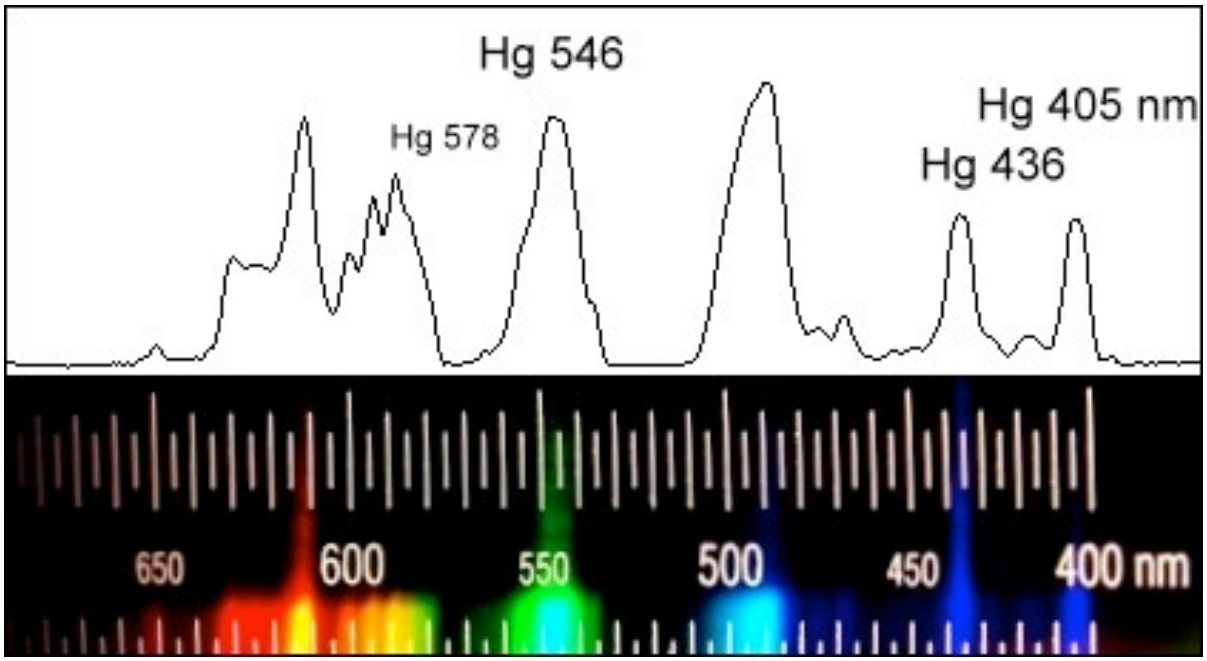
How does Fluorescence work



Mirror-Effect: Excitation and emission behave as if they are mirrored!

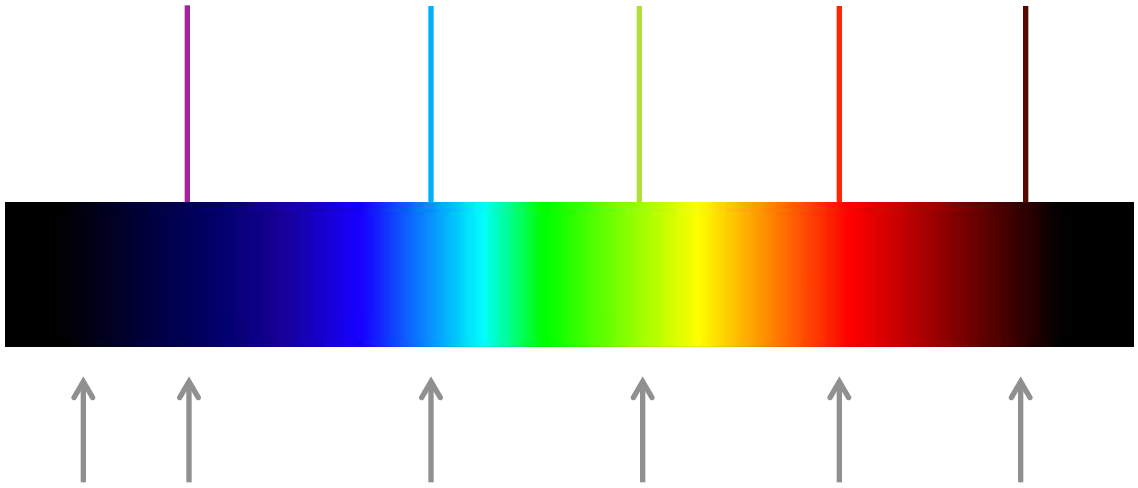
How does Fluorescence work

Hg low pressure lamps are commonly used in simple microscopes, but they would only be mildly suitable for cytometers: too dim, to broad in their spectrum, slightly suboptimal wave lengths.



How does Fluorescence work

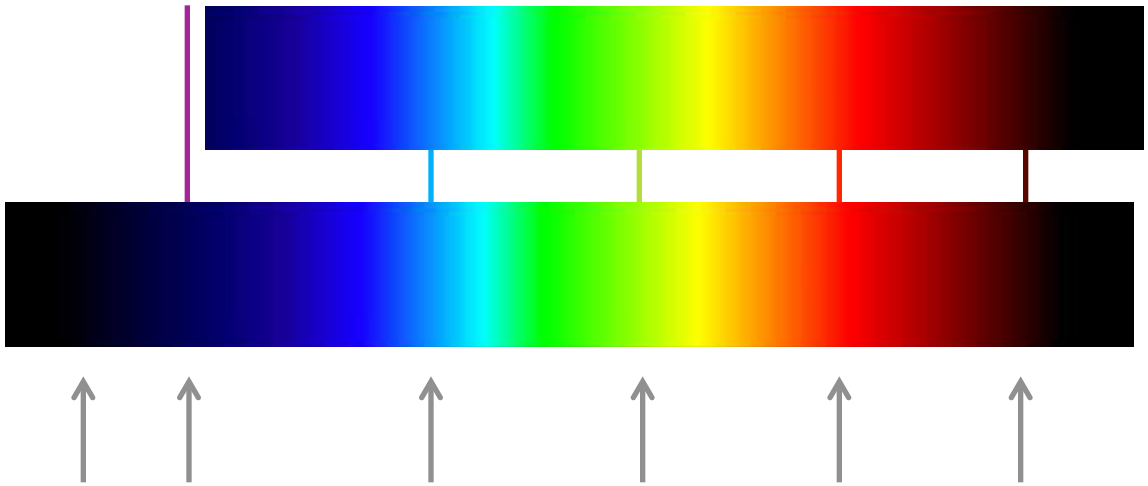
To generate specificity and efficiency of excitation one uses lasers of different wave lengths



Typical laser wave lengths	355	405	488	561	640	780 nm

How does Fluorescence work

As the fluorescence usually* has a longer wavelength than the excitation, and this limits the choice of dyes that can be used.



Typical laser wave lengths

355 405 488 561 640 780 nm

How does Fluorescence work

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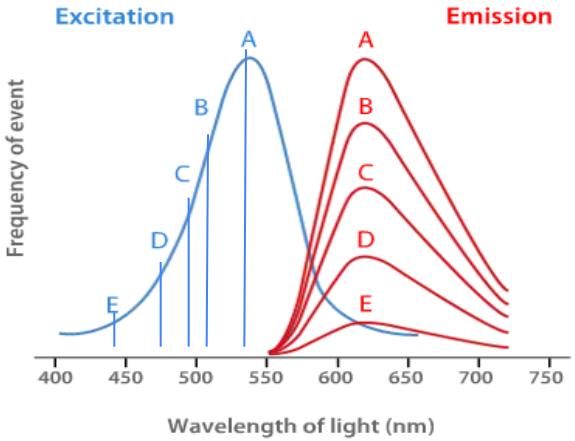
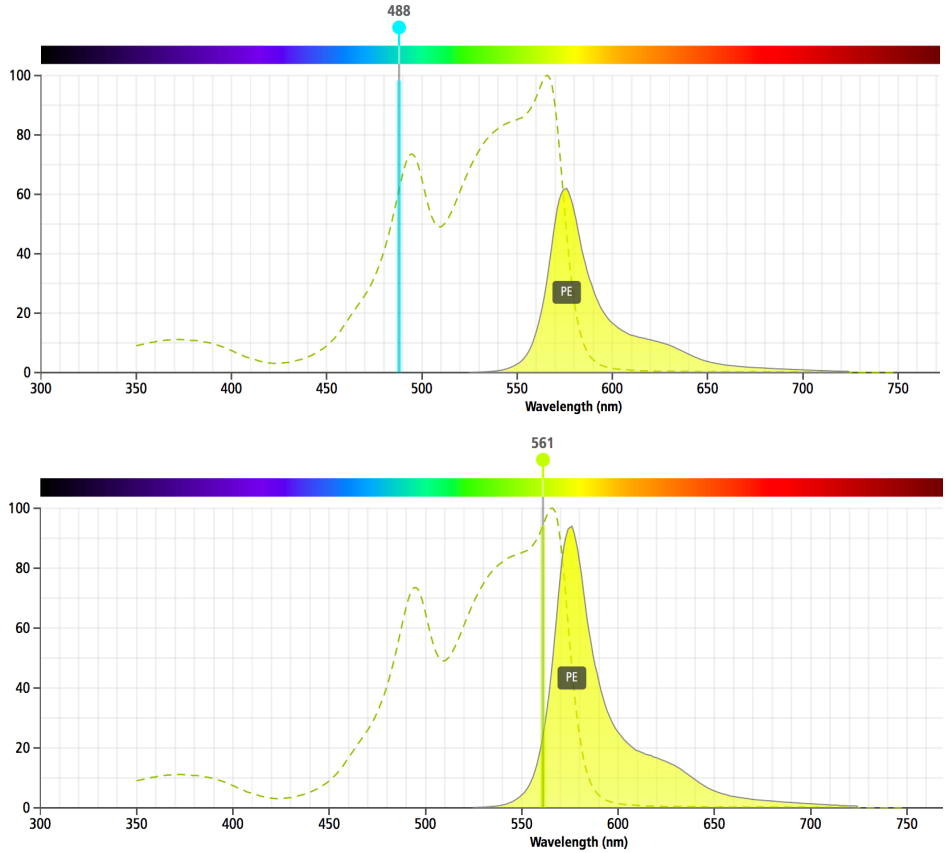
Typical laser wave lengths

355 405 488 561 640 780 nm

How does Fluorescence work

The intensity of Fluorescence is coupled to the maximum excitation, but its emission spectrum will never change no matter the excitation used!





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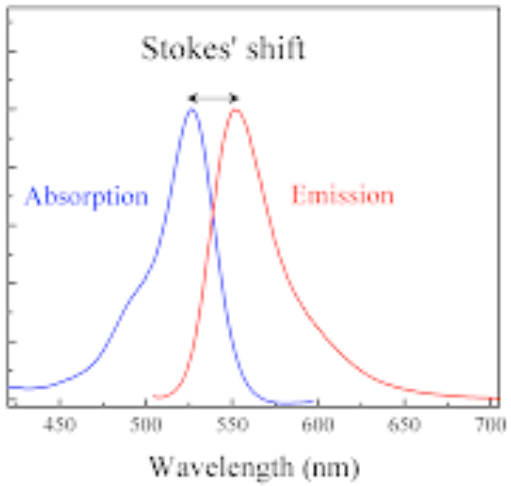
How does Fluorescence work

The distance in nm wave length between the excitation and emission maxima is called “Stoke’s Shift”.



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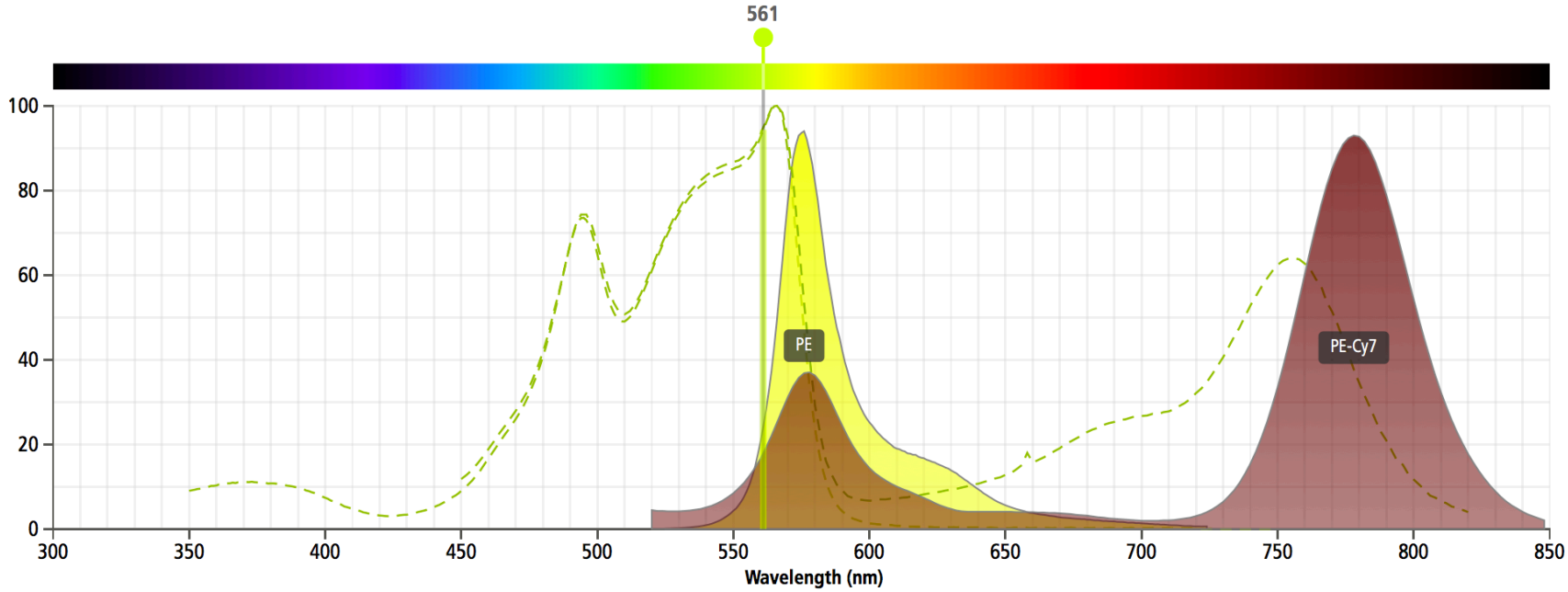
How does Fluorescence work

The “Stoke’s Shift” can be increased by utilizing suitable FRET pairs which are known in Flow Cytometry as “Tandem Dyes”.



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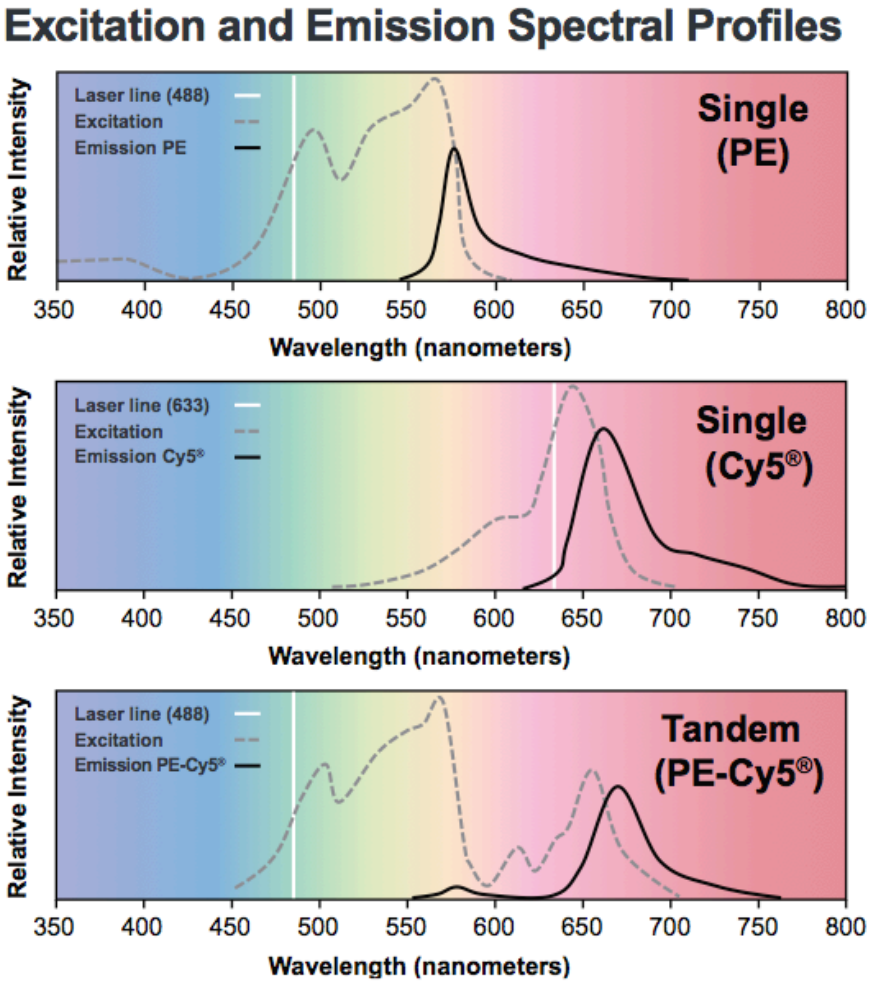
How does Fluorescence work



Abcam website

How does Fluorescence work

Classical tandem dyes:

PerCP-Cy5 and PerCP-Cy5.5
PE-CF594 (PE-TexasRed)
PE-Cy5 and PE-Cy5.5
PE-Cy7
APC-R700
APC-Cy7 and APC-H7

BV605, BV655, BV711 and BV786
BUV737 and BUV800

Abcam website

How does Fluorescence work

The brightness of a fluorochrome is based on its ability to pick up photons and to convert them into fluorescence: Quantum Efficiency/Quantum Yield

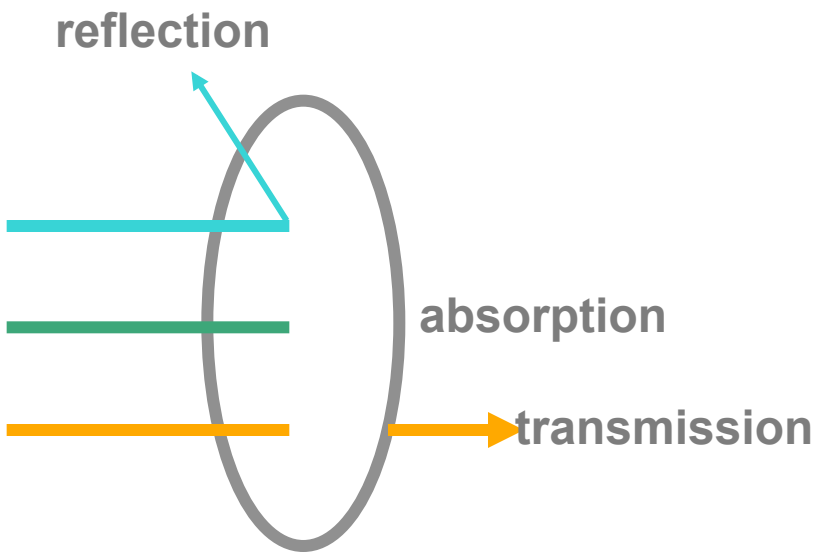
So we can formulate that dyes differ on their ability to create fluorescence: dim, mid, and bright dyes!

How do we utilize fluorescence

…in cytometers we „pick“ the maximum of the fluorescence peaks with filters to limit the light that hits the detector and to create specificity.

Optical filters modify the spectral distribution of the light scatter and fluorescence signals on their way to the detectors

An optical filter can either reflect, absorb or transmit photons

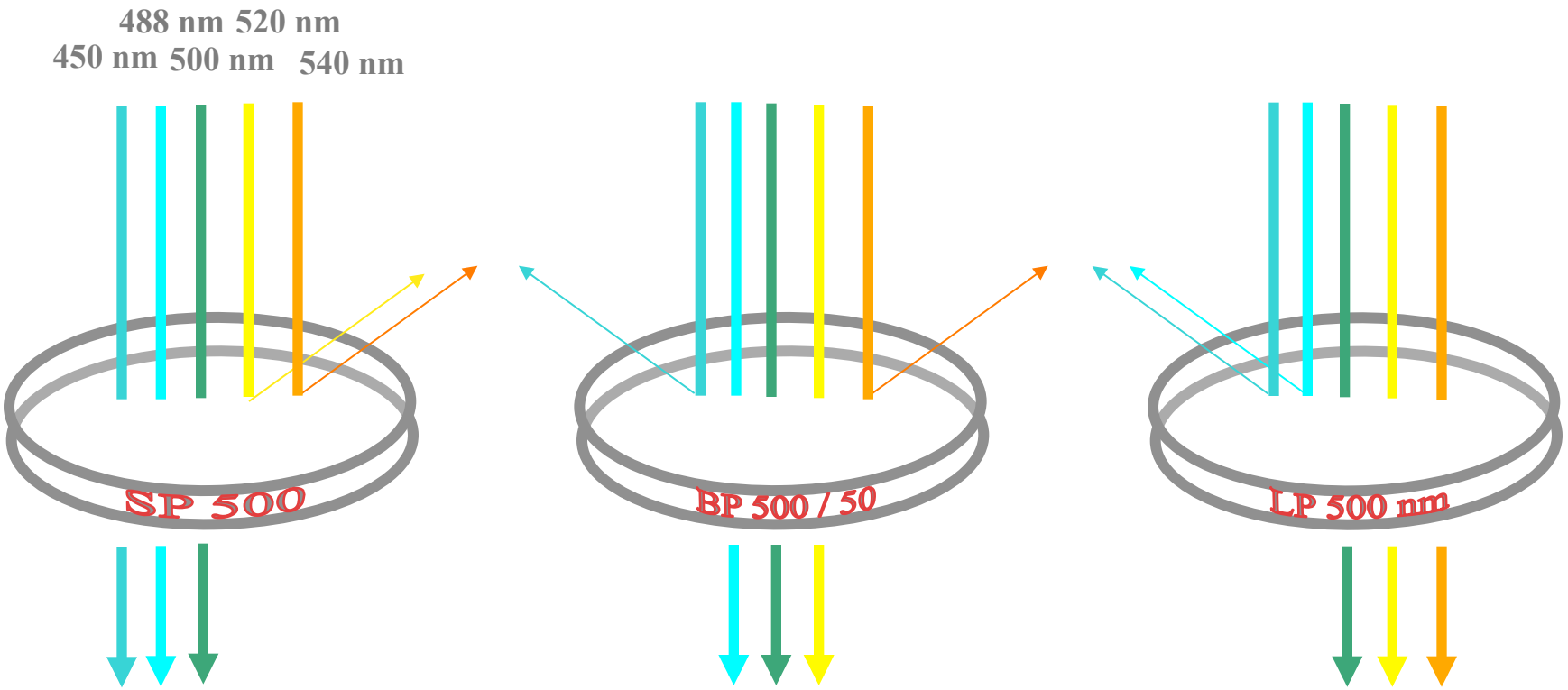


How do we utilize fluorescence

Short Pass

Band Pass

Long Pass



BP 500/ 50 = 475 - 525 nm

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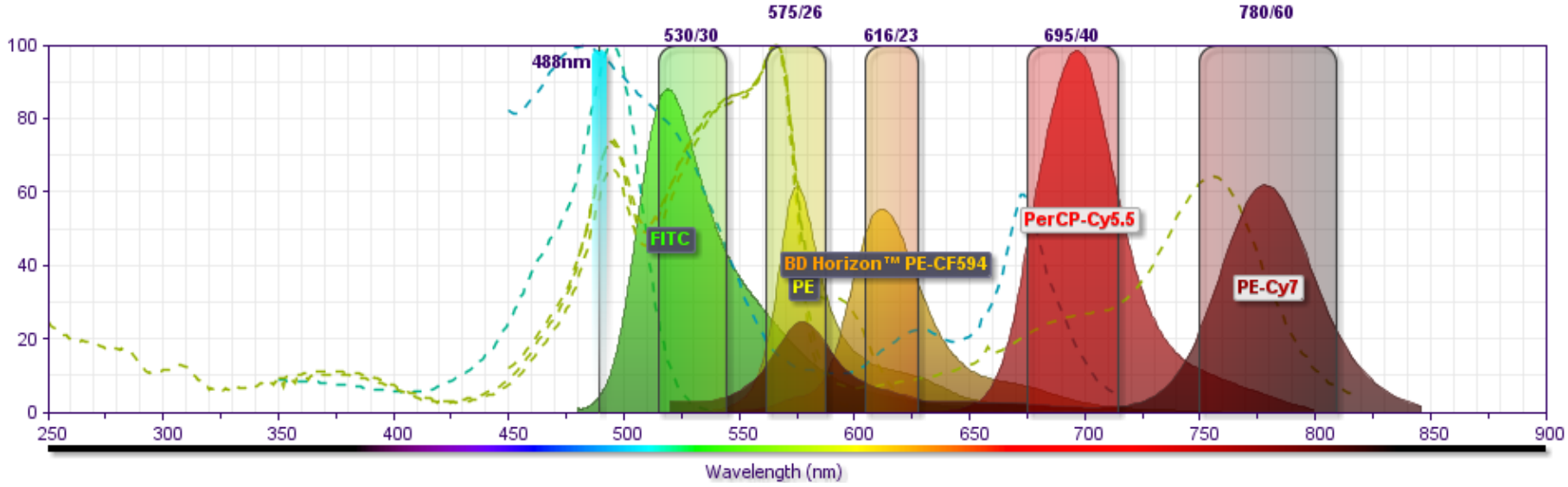

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How do we utilize fluorescence

Functional dyes:

DNA binding dyes (DAPI Hoechst PI 7AAD SybrGreen, etc)

Mitochondrial dyes (Mitotracker, JC-1, etc.)

Calcium flux dyes (Indo-1, etc)

…many, many more!

Fluorescent proteins:

Blue (BFP, CFP)

Green (GFP, YFP, Venus, Citrulin)

Orange (RFP, mCherry, dTomato)

Red (iRFP, etc…)

Reporter cell lines and protein tags…

Labeling Dyes:

“Proteins”: PE, APC, PerCP

Classic small molecules: Alexa’s, Daylight’s, FITC

Semiconductors: Qdots or nano crystals

Organic arrays: Brilliant (ultra) Violets

How do we utilize fluorescence

V450 V500 FITC PE PE-CF594 PerCP APC PerCP-Cy5.5 AF700 PE-Cy7 APC-Cy7

BV421 BV510 BV570 BV605 BV655 BV711 BV786

BUV393 BUV496 BUV563 BUV661 BUV737 BUV805

AFs →

Dylights →

Qdots →

How do we utilize fluoresence

Whenever you think about setting up a FACS experiment or you want to explore options, use a spectrum viewer tool

https://www.bdbiosciences.com/sg/research/multicolor/spectrum_viewer/index.jsp

https://www.thermofisher.com/de/de/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html

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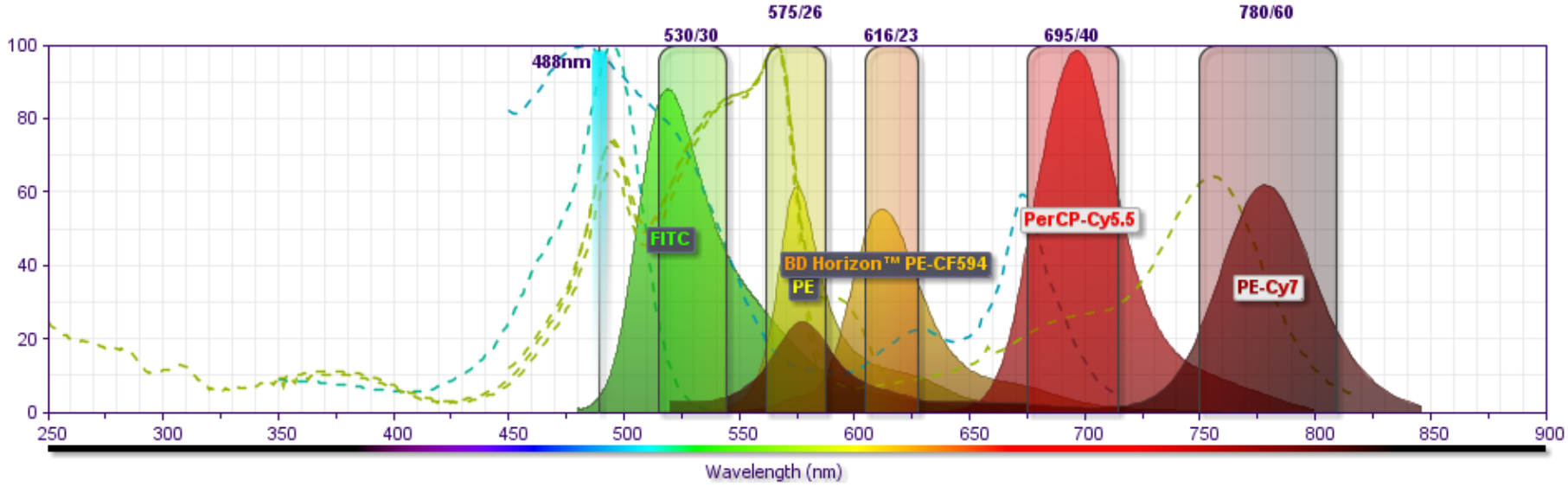

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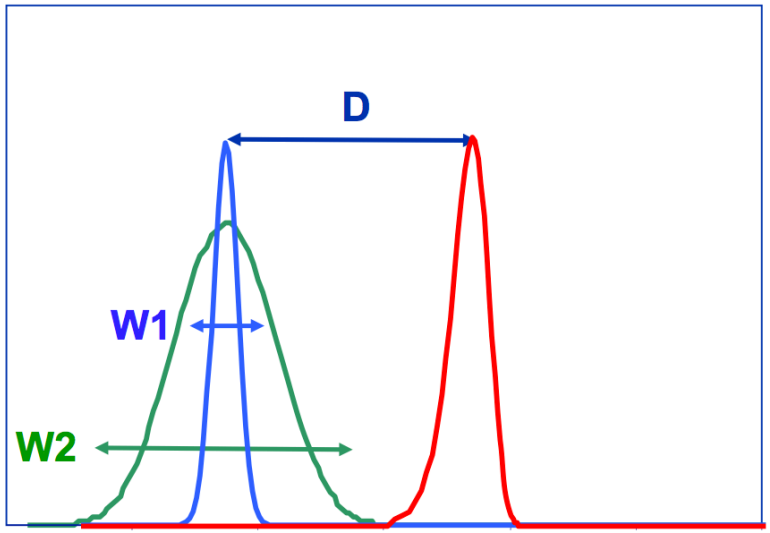
How do we match dyes and epitope

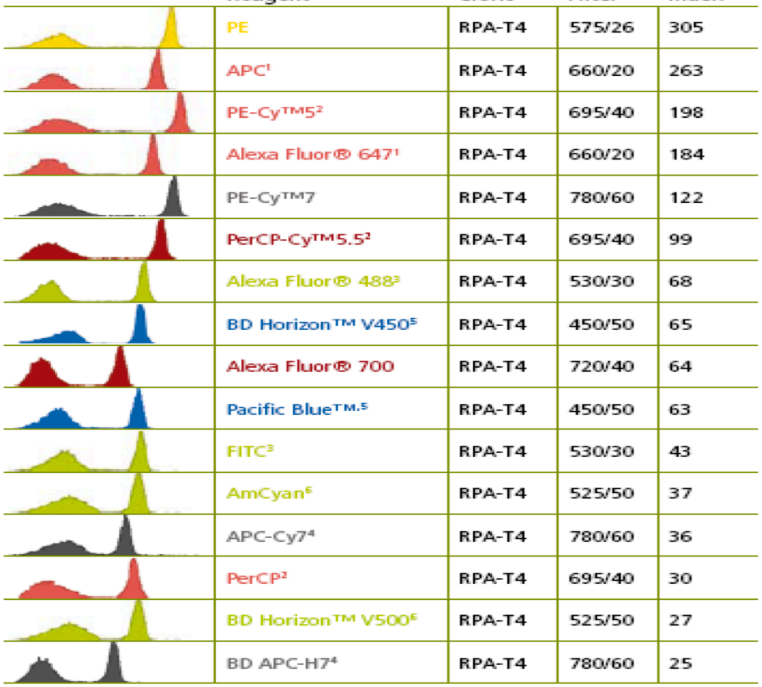


$$\text{Stain Index (SI)} = {}^{D}/_{W}$$

	Reagent	Clone	Filter	Stain Index
	PE	RPA-T4	575/26	305
	APC[1]	RPA-T4	660/20	263
	PE-Cy™5[2]	RPA-T4	695/40	198
	Alexa Fluor® 647[1]	RPA-T4	660/20	184
	PE-Cy™7	RPA-T4	780/60	122
	PerCP-Cy™5.5[2]	RPA-T4	695/40	99
	Alexa Fluor® 488[3]	RPA-T4	530/30	68
	BD Horizon™ V450[5]	RPA-T4	450/50	65
	Alexa Fluor® 700	RPA-T4	720/40	64
	Pacific Blue™.[5]	RPA-T4	450/50	63
	FITC[3]	RPA-T4	530/30	43
	AmCyan[6]	RPA-T4	525/50	37
	APC-Cy7[4]	RPA-T4	780/60	36
	PerCP[2]	RPA-T4	695/40	30
	BD Horizon™ V500[6]	RPA-T4	525/50	27
	BD APC-H7[4]	RPA-T4	780/60	25

How do we match dyes and epitope

Chromophore brightness



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+

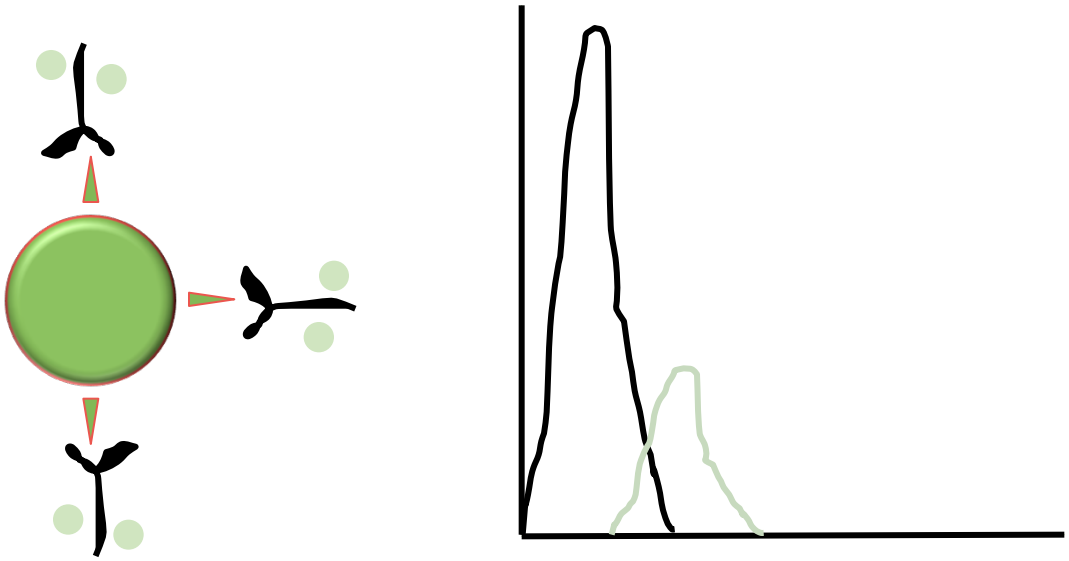
Antigen density

Bright dyes on lowly expressed markers!

Dim dyes on highly expressed markers!

How do we utilize fluorescence

Rule of thumb: Bright dyes go on lowly expressed epitopes



How do we utilize fluorescence

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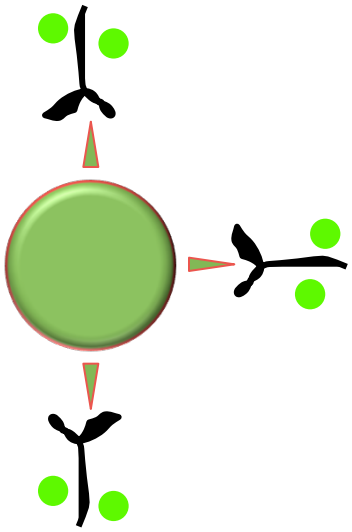
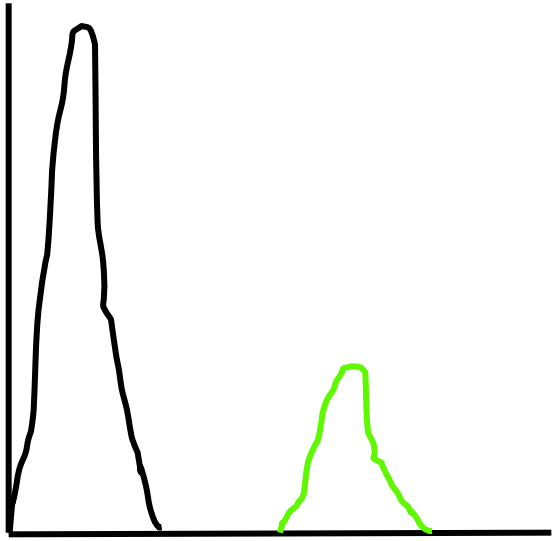
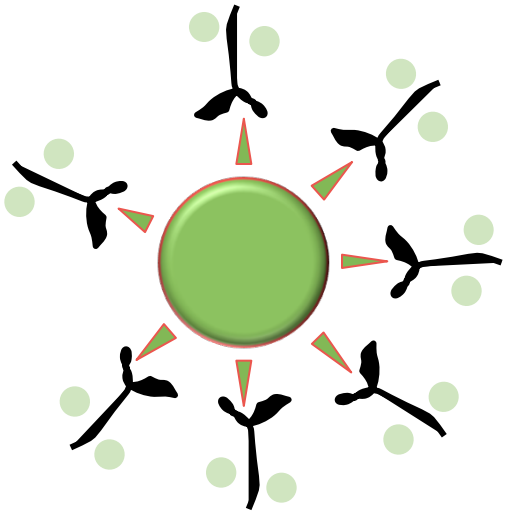
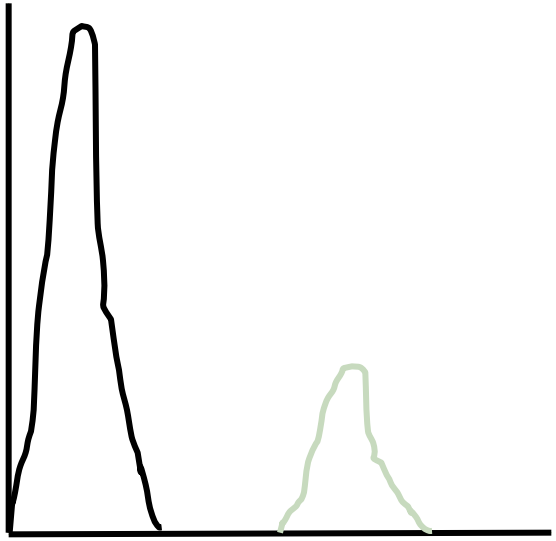




How do we utilize fluorescence

Rule of thumb: Dim dyes go on highly expressed epitopes





How to approach fluorescence

How many colors will I need for my experiment?

What colors are available to me (limitation on Abs)

What instrument do I have?

Will I have to use a fluorescent protein?

Can I CRISPR a HA- or Flag-Tag into my cells instead if I have a membrane protein?

Primary coupled Abs are usually better for FACS, but sometimes you need to amplify your signal.


Questions…please hit me with’em