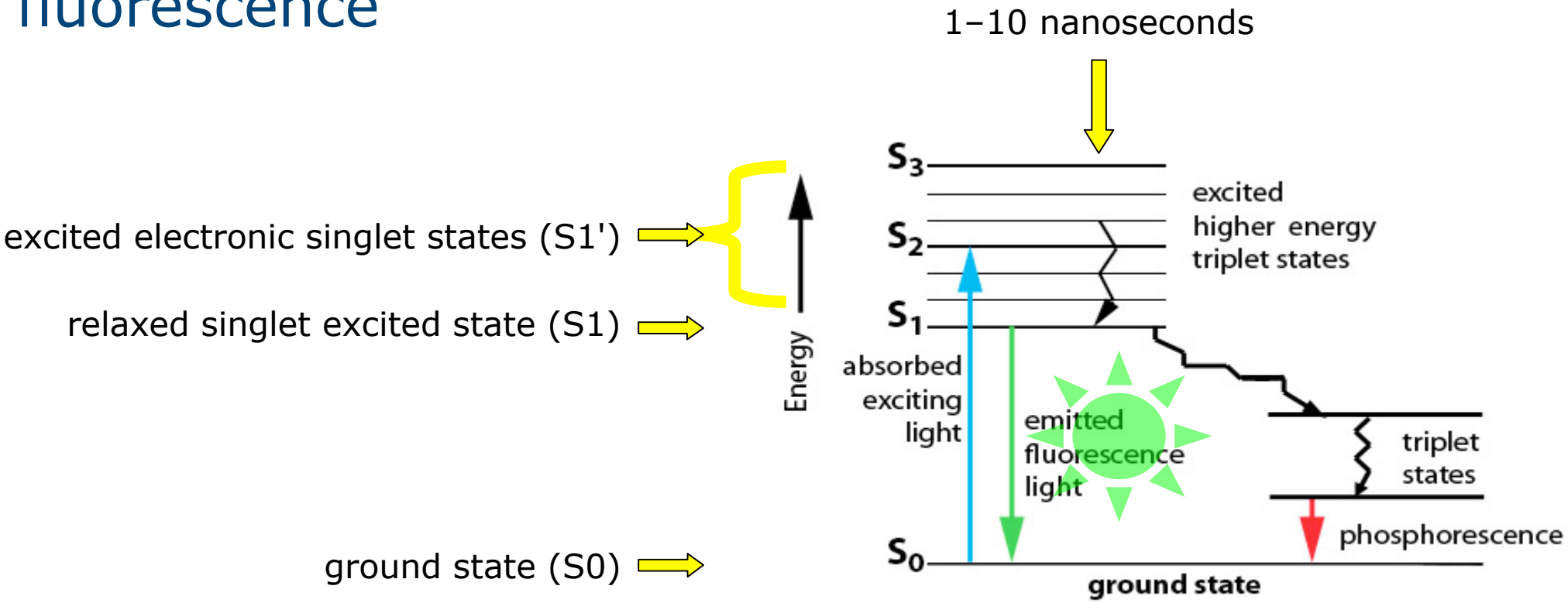
fluorescence microscopy

- what is fluorescence?
- fluorescence excitation and emission
- Stokes' shift and emission intensity
- light source
- epi-illumination
- filter cubes and filter types
- filter sets
- fluorescence in confocal systems
- 2-photon fluorescence
- into the future

Dave Johnston
Biomedical Imagng Unit

fFundamentals of fluorescence (1) fluorescence



energy is absorbed to boost electron to higher shell (S0 to S1')

some energy is lost when electron drops to the relaxed singlet excited state of higher shell (S1' to S1)

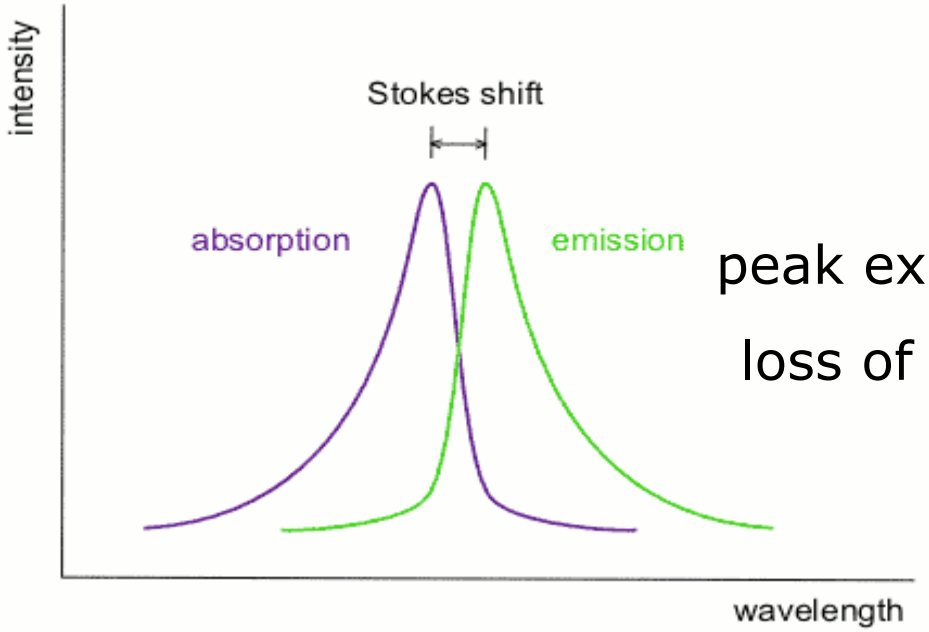
remaining energy is released as fluorescence when electron drops back to ground state (S1 to S0)

University of Victoria

fundamentals of fluorescence (2) Stokes' shift

energy loss when electron drops to the relaxed singlet excited state of higher shell means fluorescence emission is lower energy than excitation

lower energy = longer wavelength

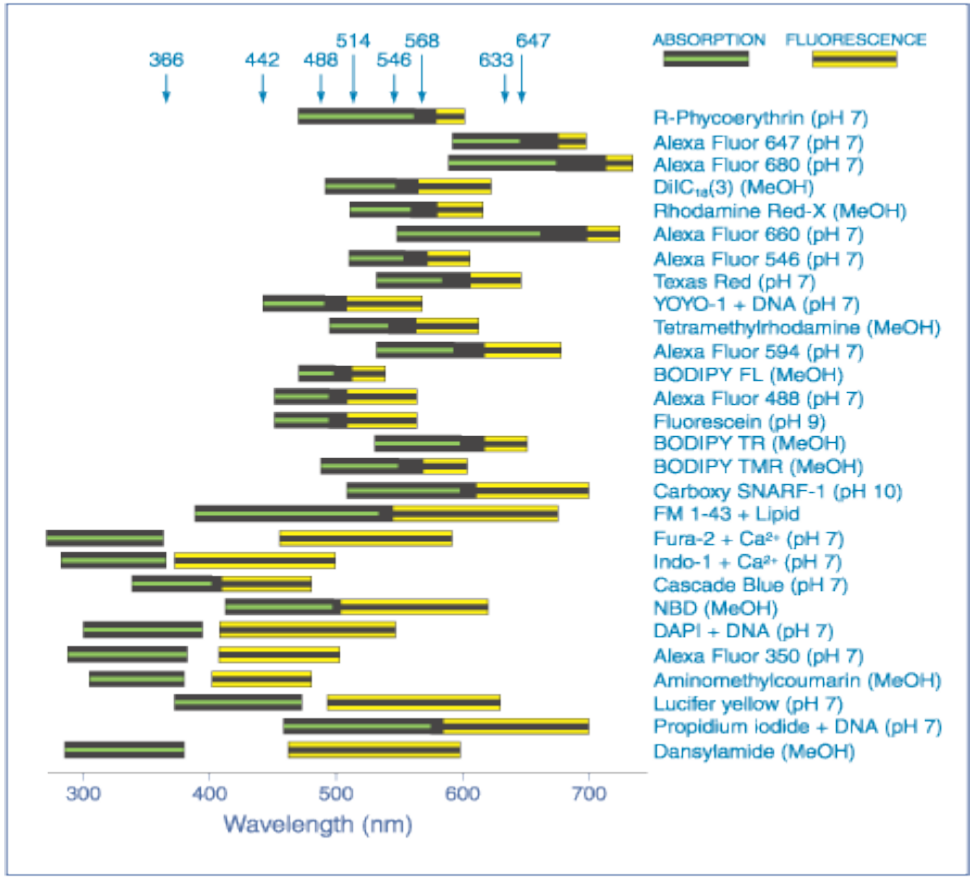


peak excitation – peak emission = Stokes' shift

loss of photon energy (= longer wavelength)

Fluorescence

fundamentals of fluorescence (3) Stokes' shift



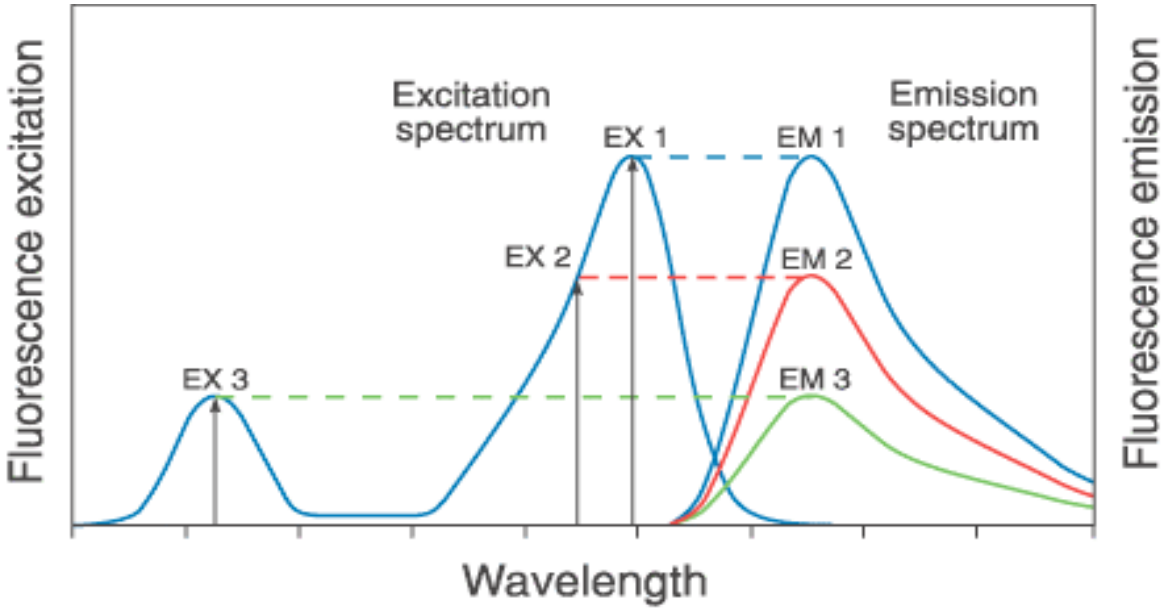
absorption and emission spectra are specific to each fluorescent molecule

fundamentals of fluorescence (4)

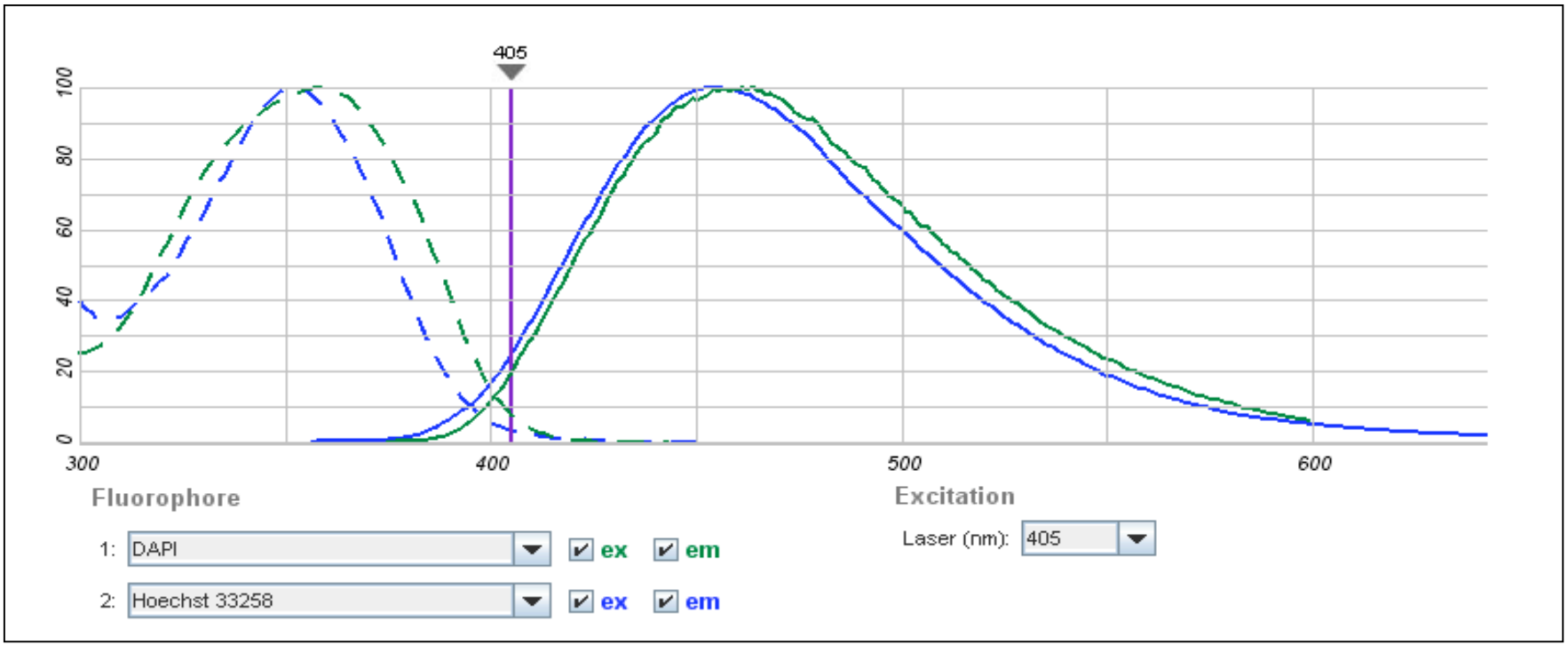
excitation and emission

Excitation of a fluorophore at different wavelengths **does not change the emission profile**

but it **does produce variations in fluorescence emission intensity** that correspond to the relative amplitude of the excitation spectrum at the wavelength concerned



why DAPI and Hoechst are poor on the SP5 Confocal

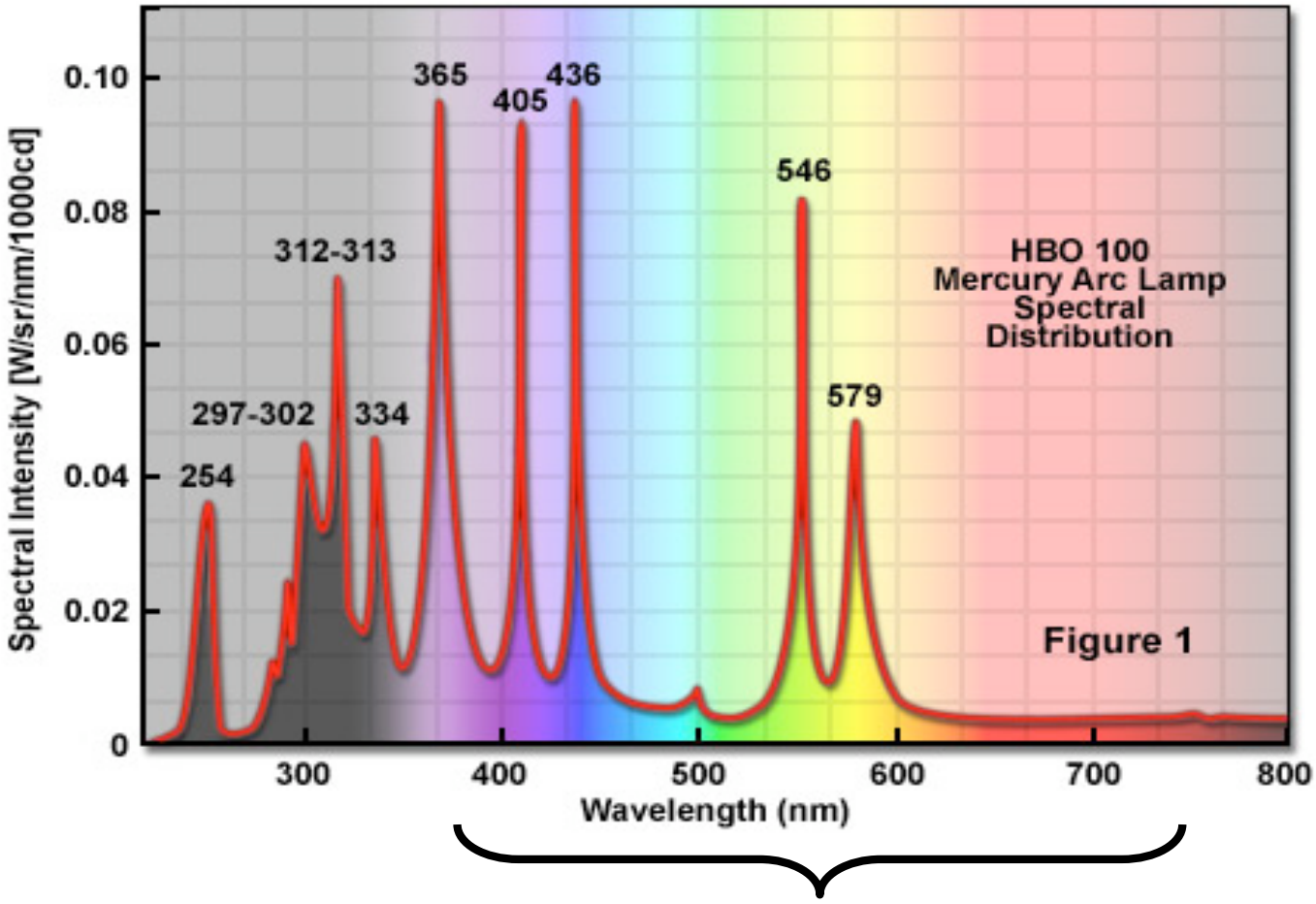


405 nm laser excitation at far RHS of excitation spectrum means very inefficient excitation (3 - 7%) and consequently very faint emission

broad fluorescence spectrum means you rarely monitor the whole emission (25%)

monitored emission may only be 1% as bright as staining seen down eyepiece

HBO100 mercury bulb spectrum



visible range 380-750nm

http://zeiss-campus.magnet.fsu.edu/articles/lightsources/mercuryarc.html

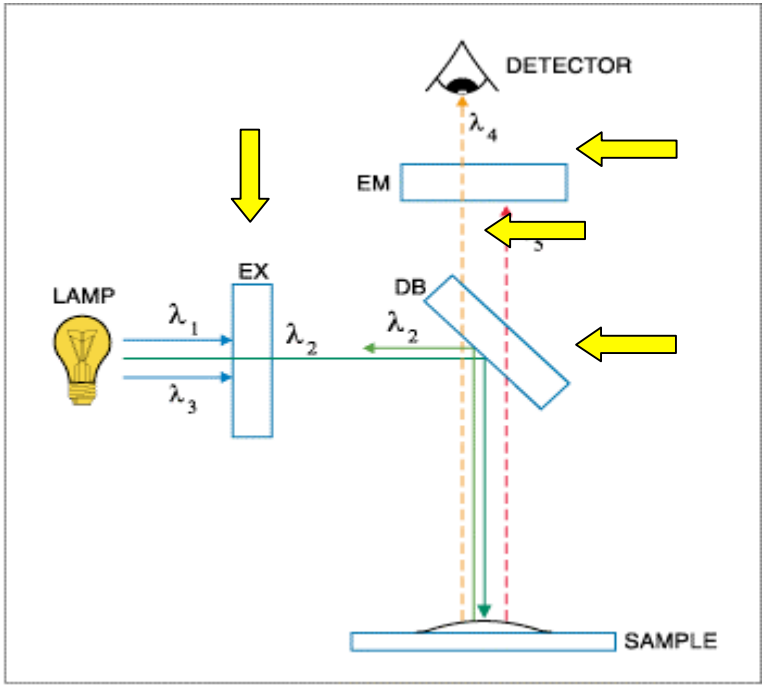
epifluorescence

the desired excitation wavelength (λ2) is selected from the spectral output of the lamp by an excitation filter (EX)

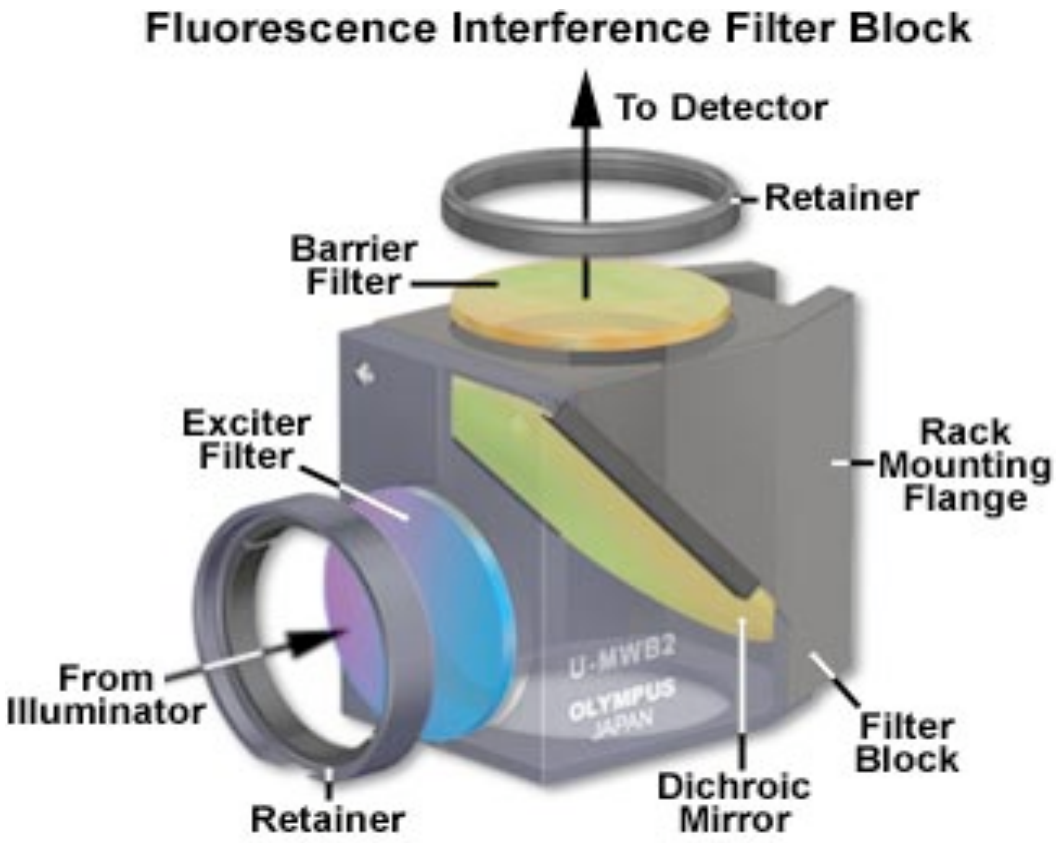
and directed to the sample via a dichroic beamsplitter (DB)

the beamsplitter separates emitted fluorescence (---) from back scattered excitation light (—)

the emission filter (EM) selectively transmits a portion of the sample's fluorescence emission (λ4) for detection and blocks other emission components (λ5).

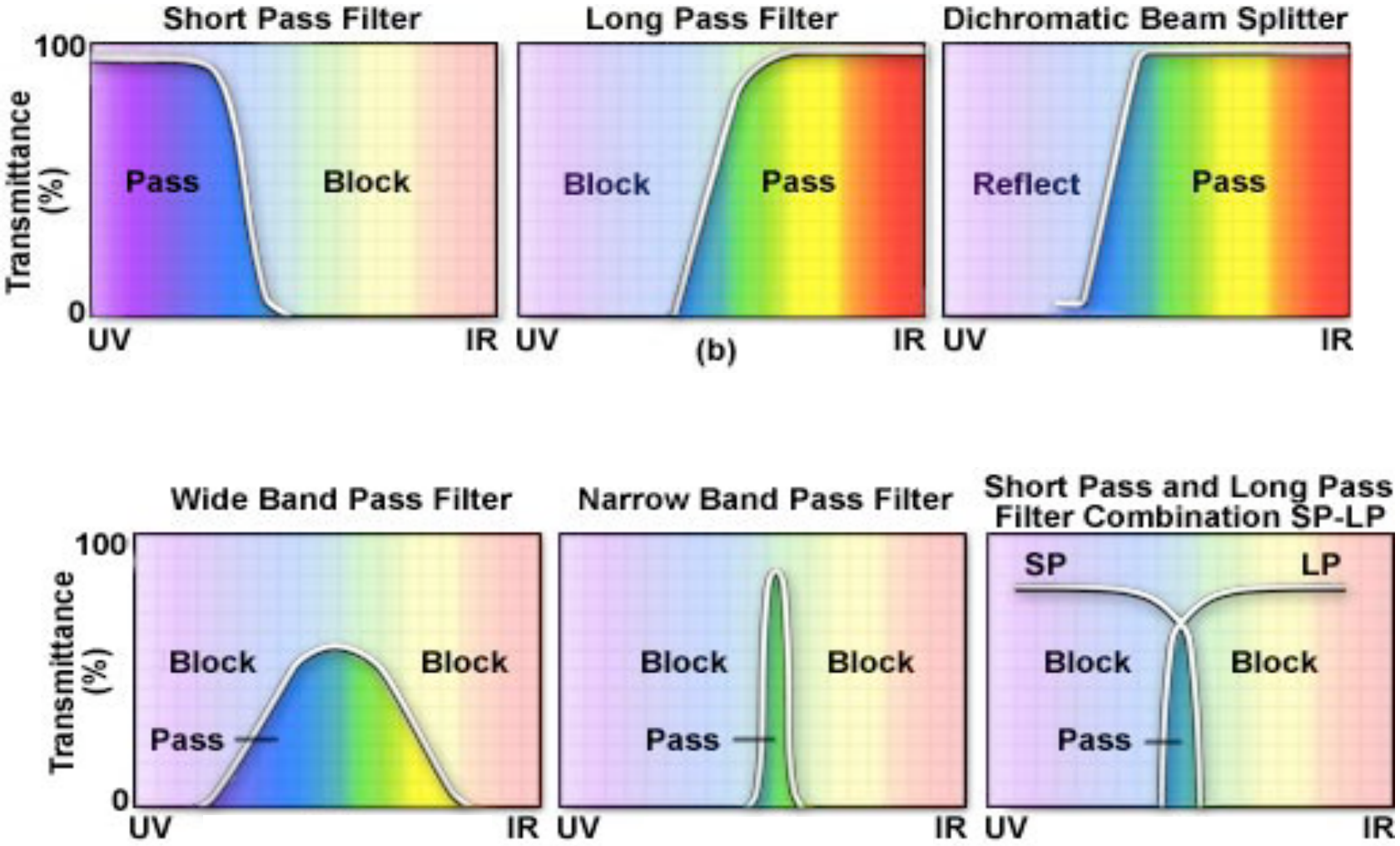


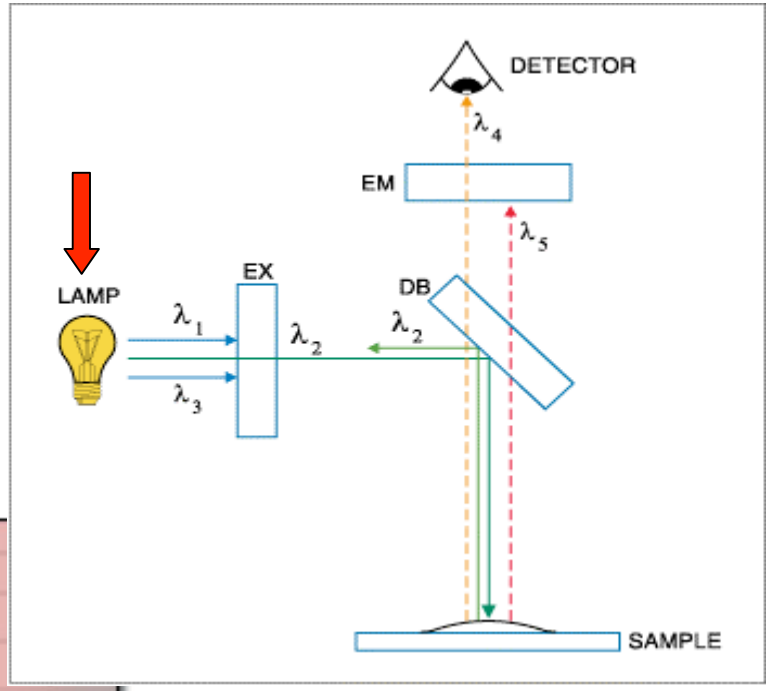
fluorescence filter block



block is specific to microscope and to flurochrome

filter types




DETECTOR
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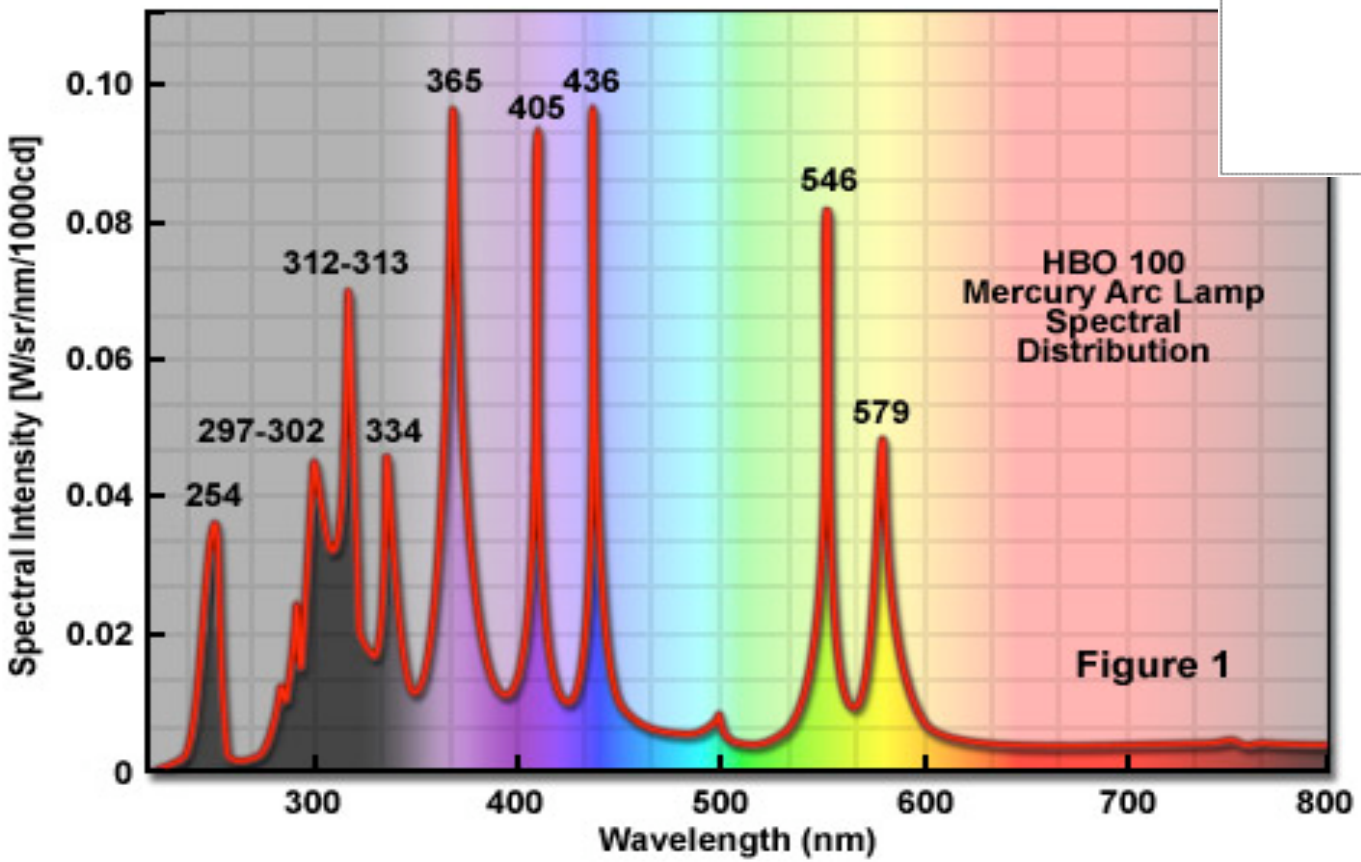
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Wavelength (nm)
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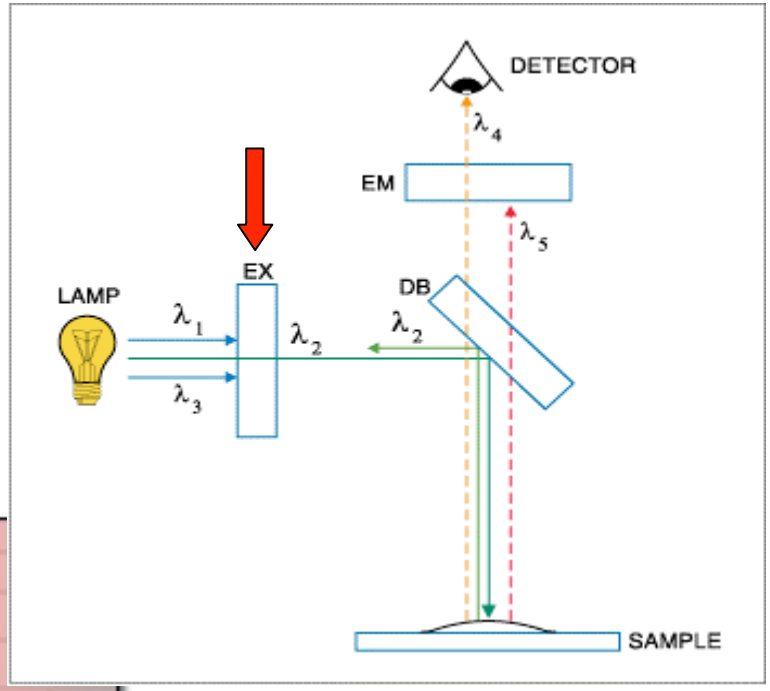
Mercury Arc Lamp
Spectral
Distribution
Figure 1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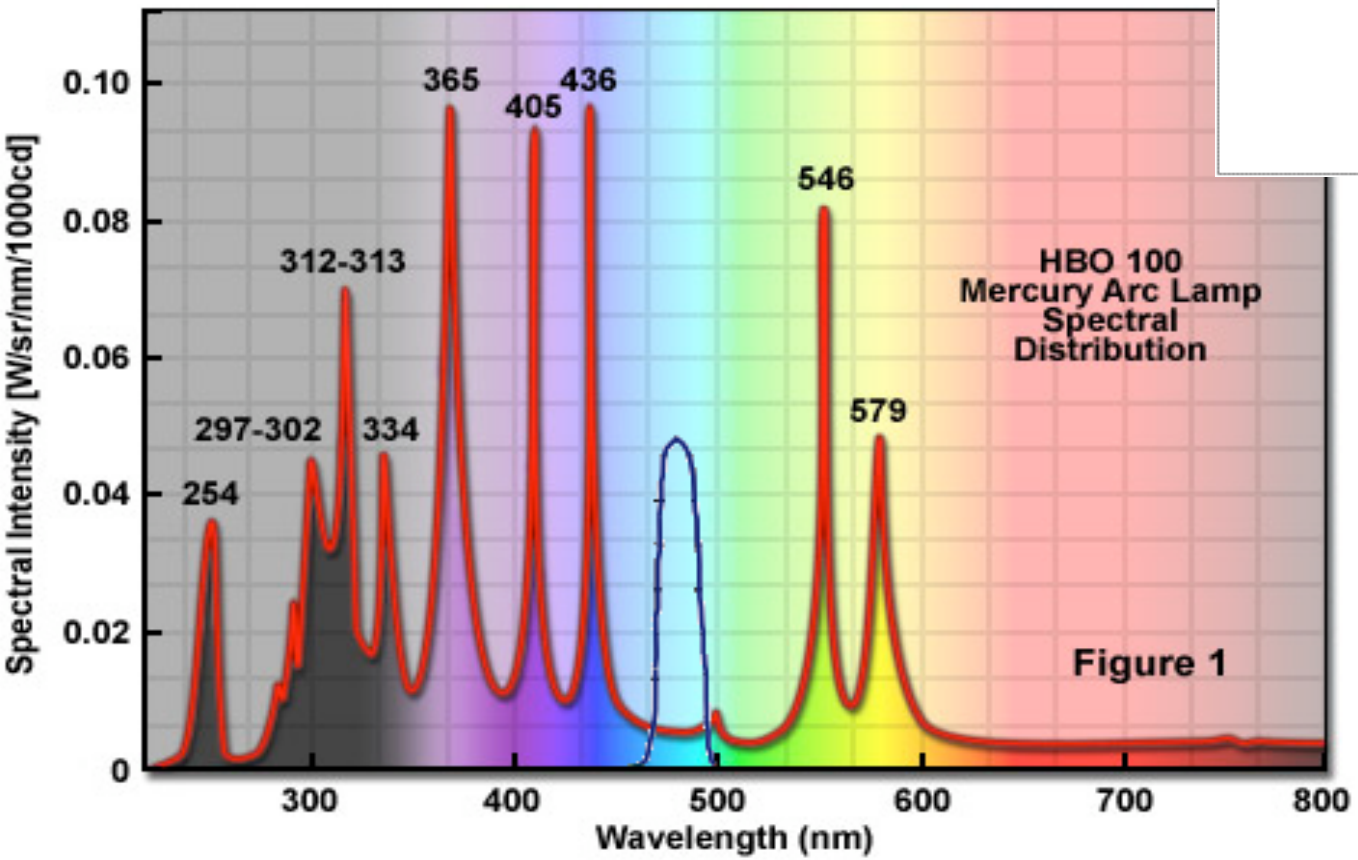
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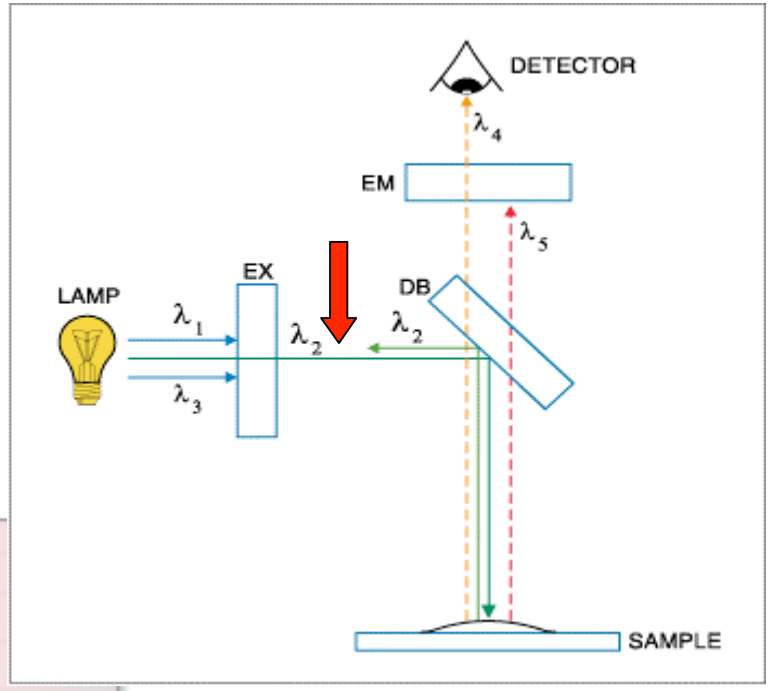
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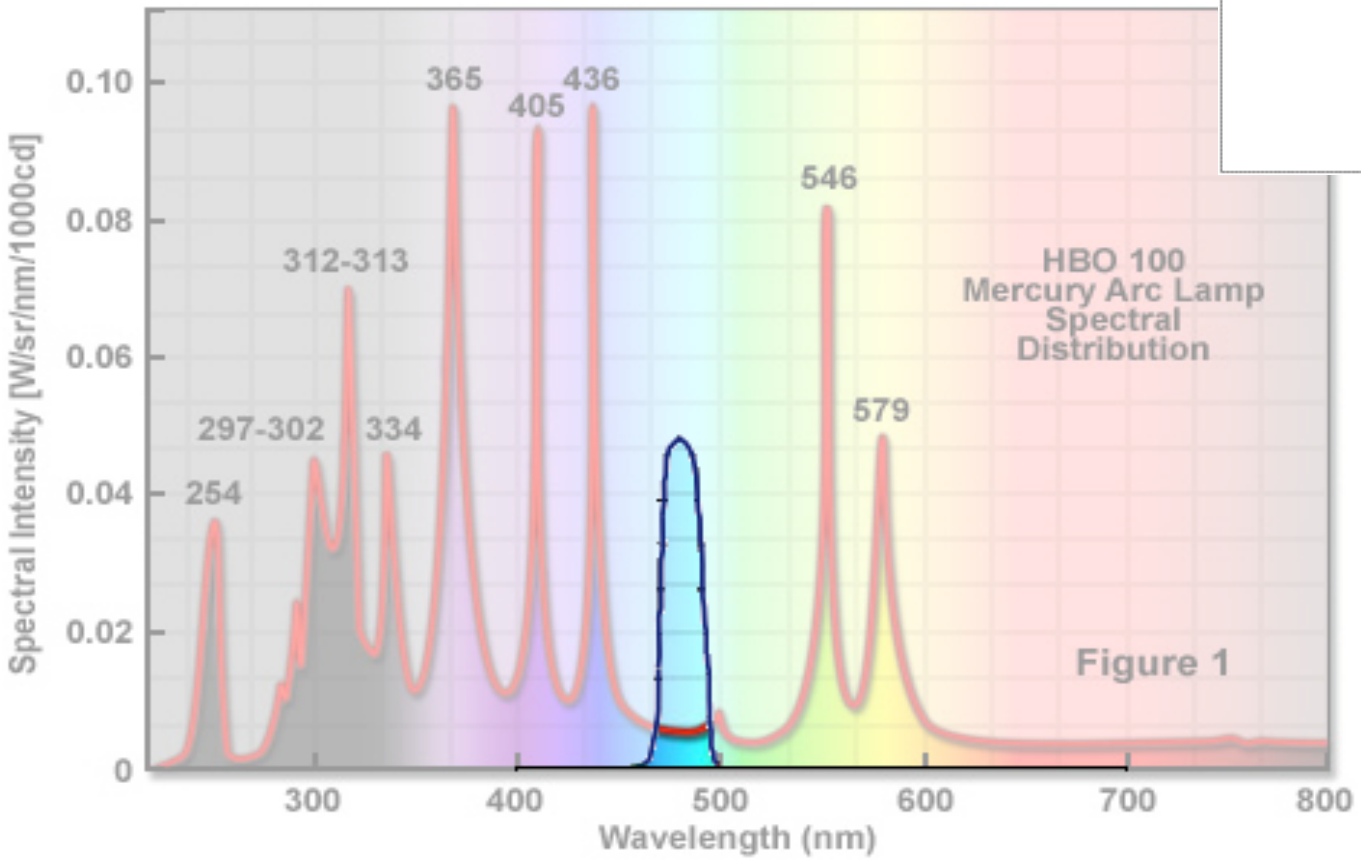
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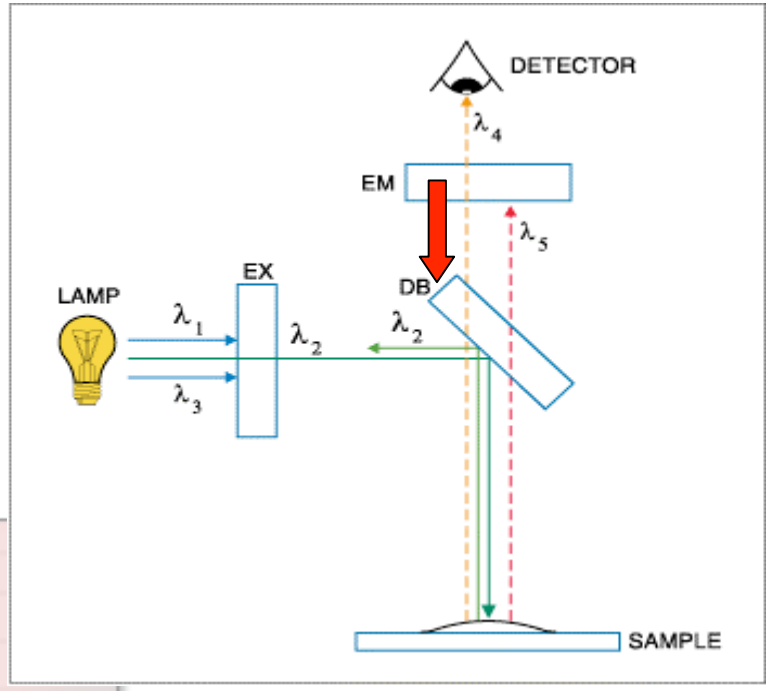
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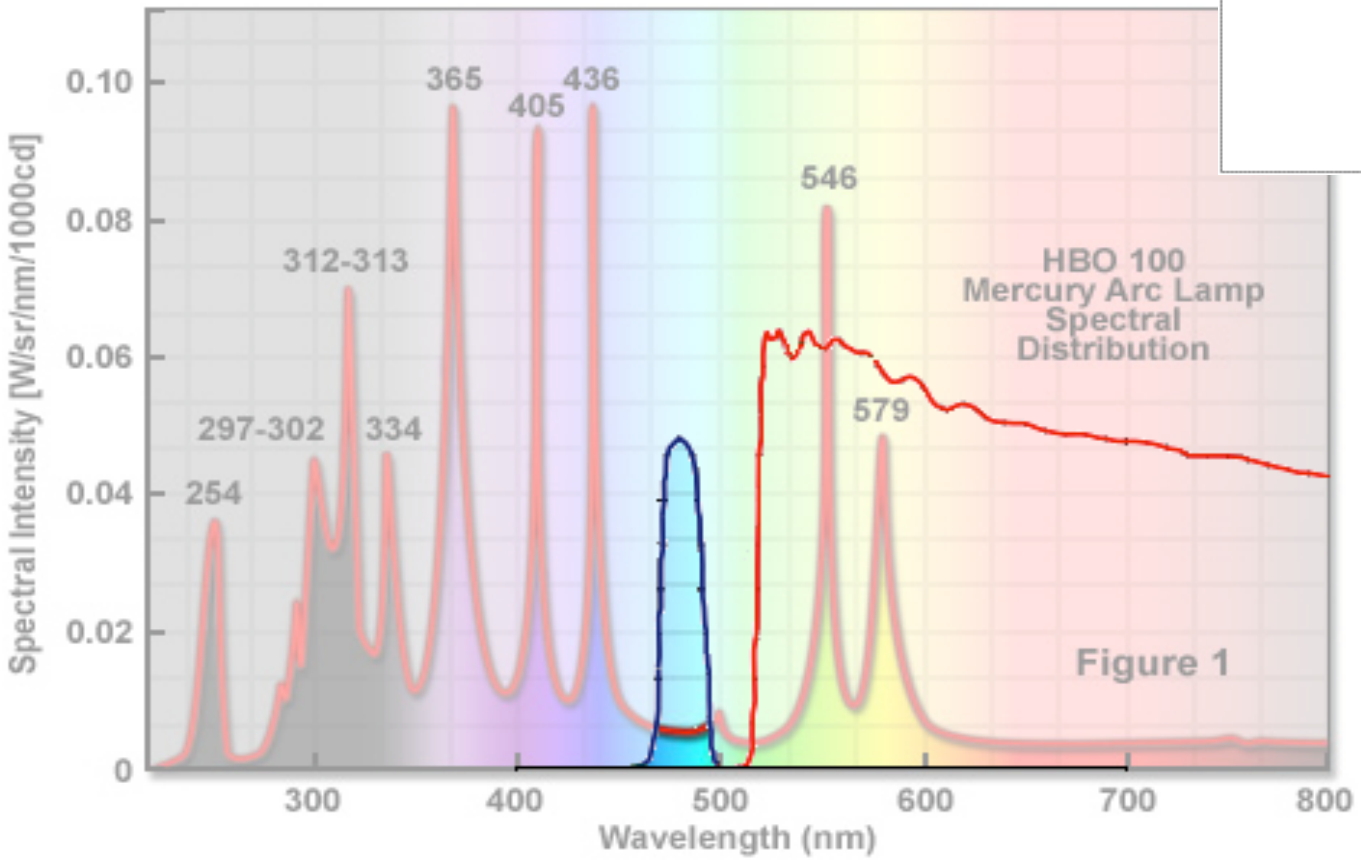
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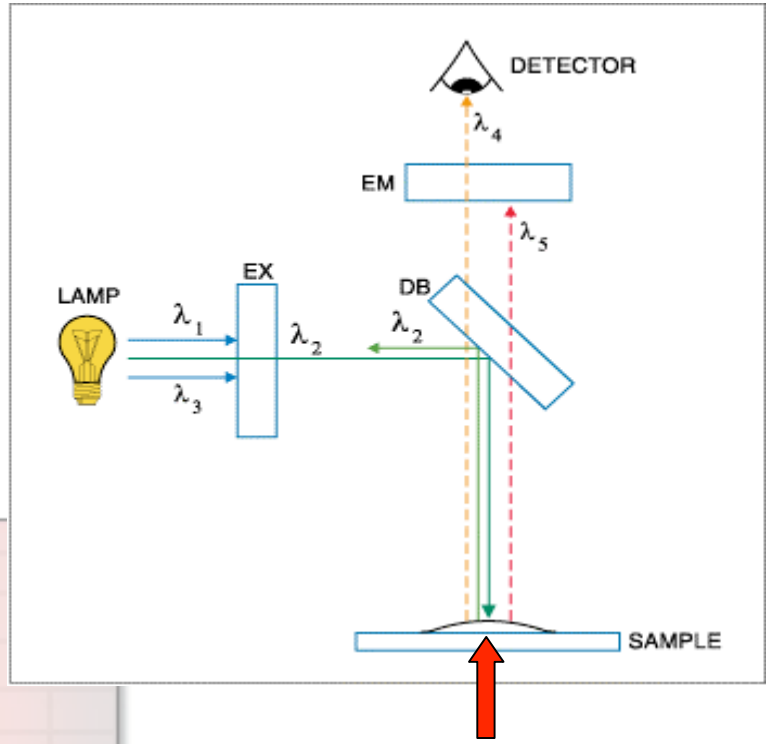
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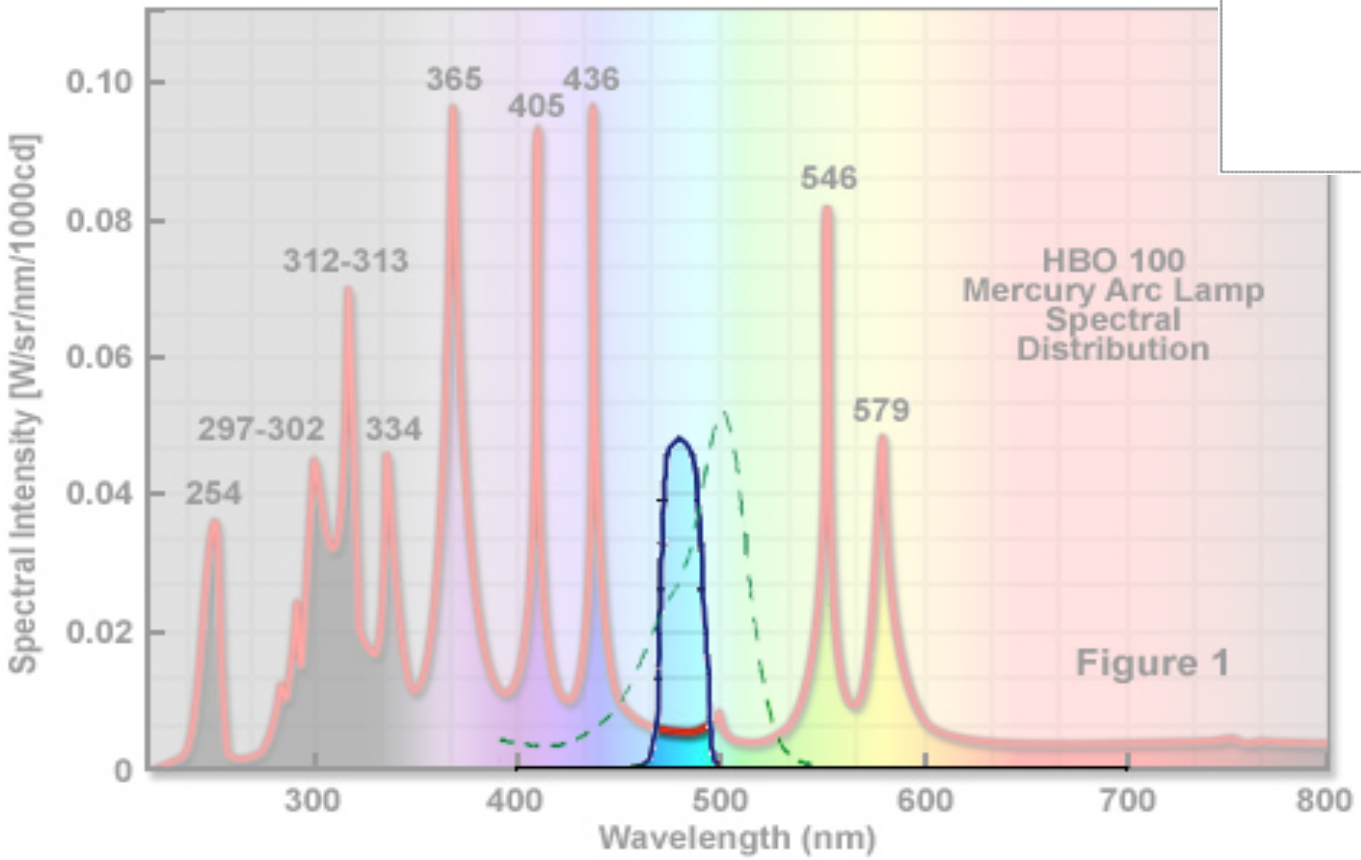
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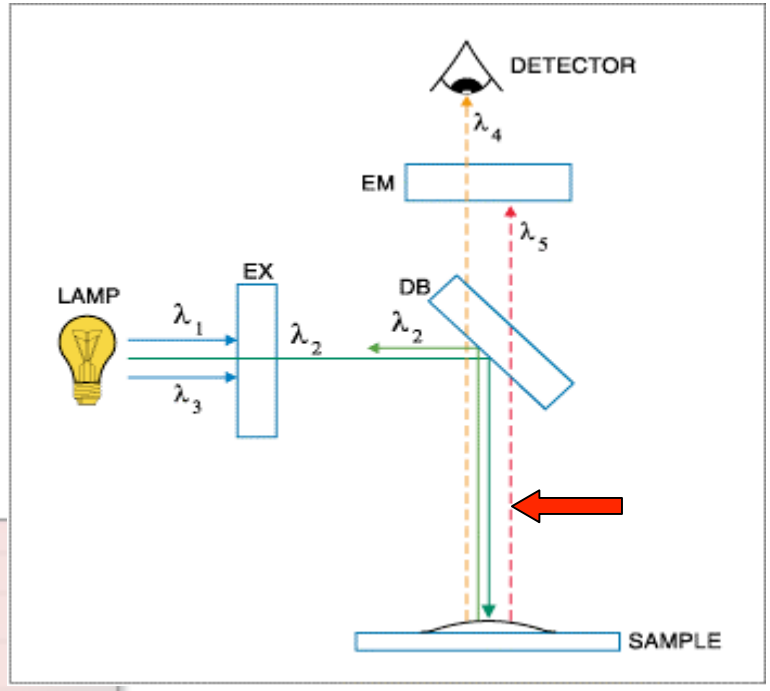
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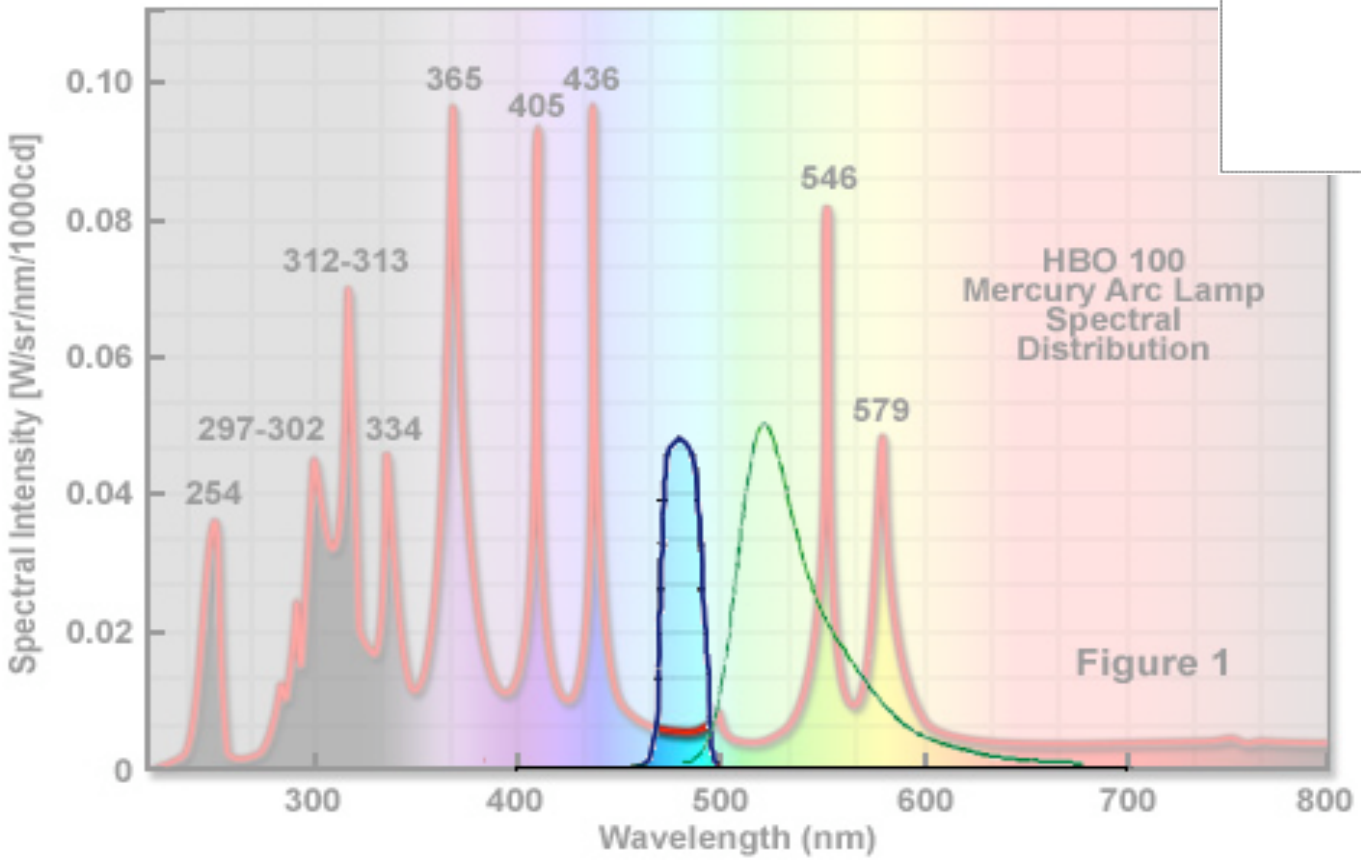
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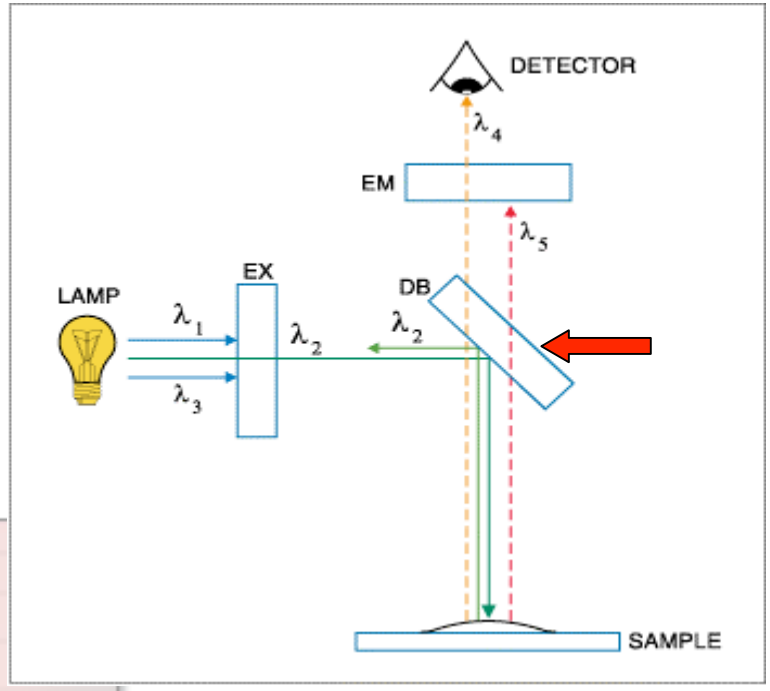
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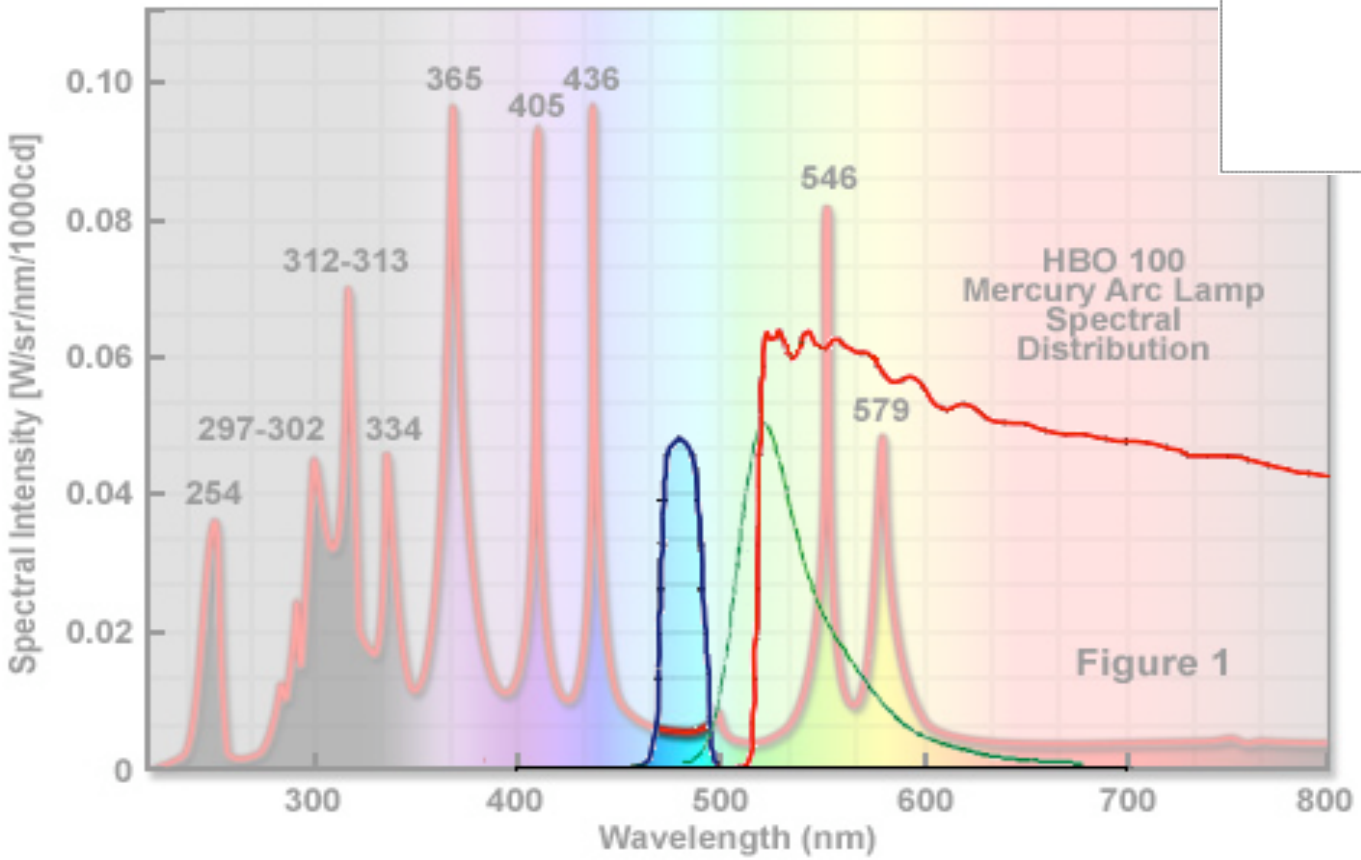
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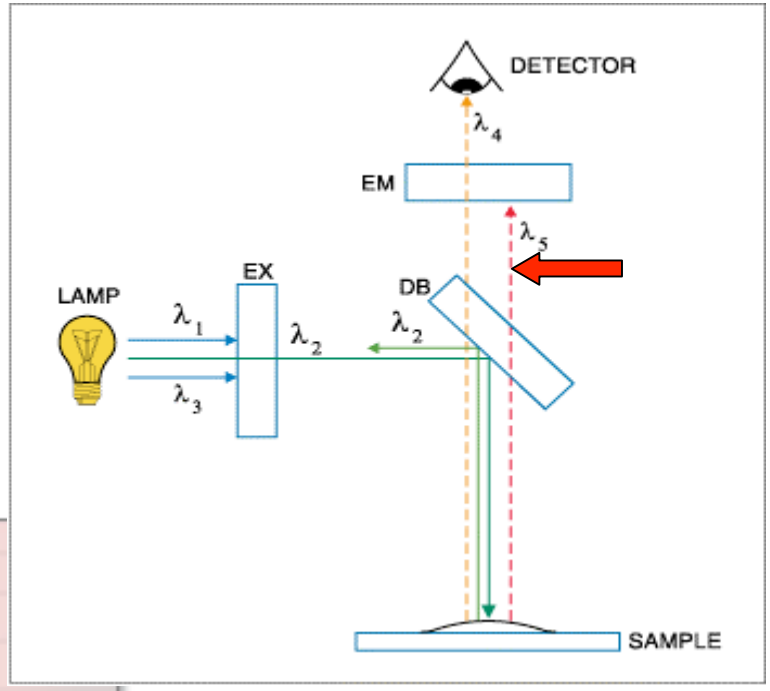
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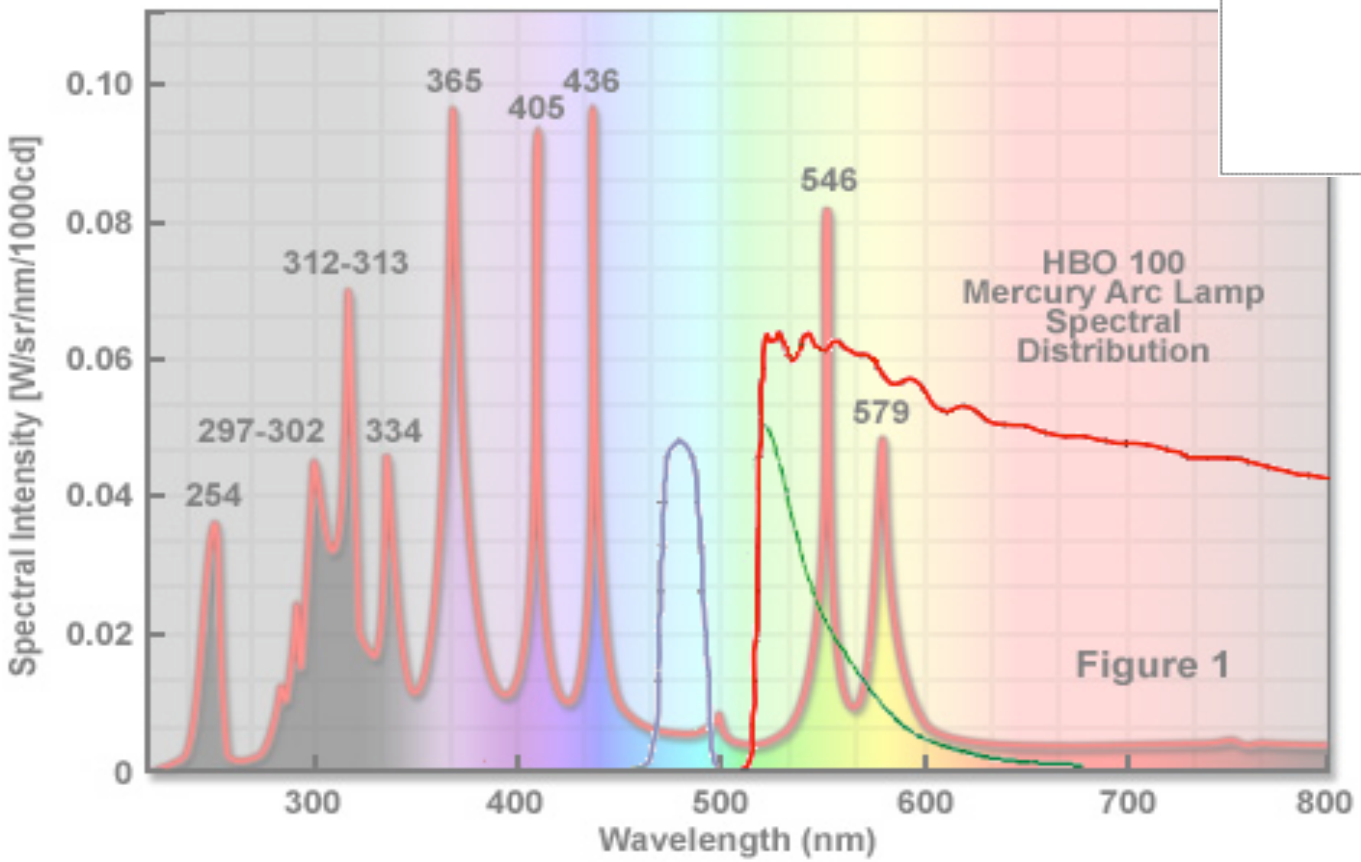
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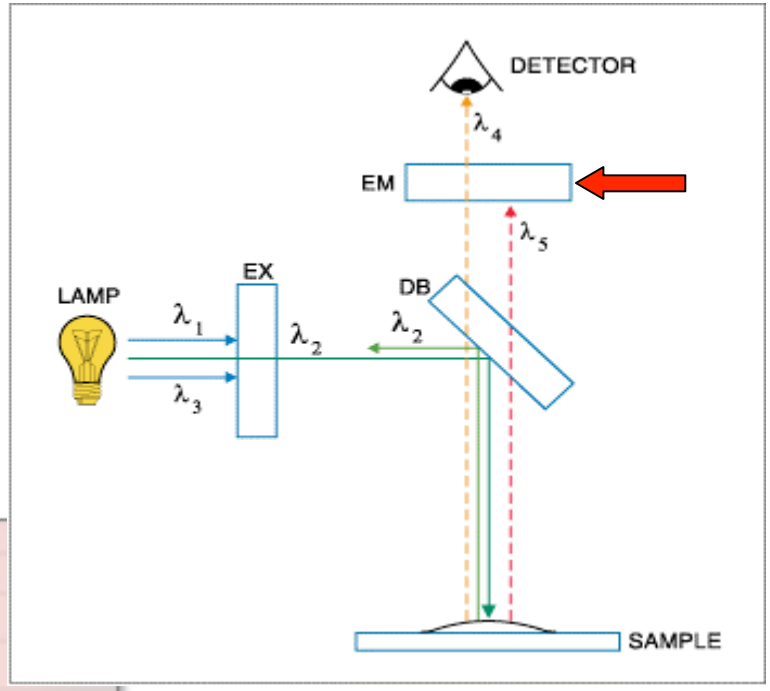
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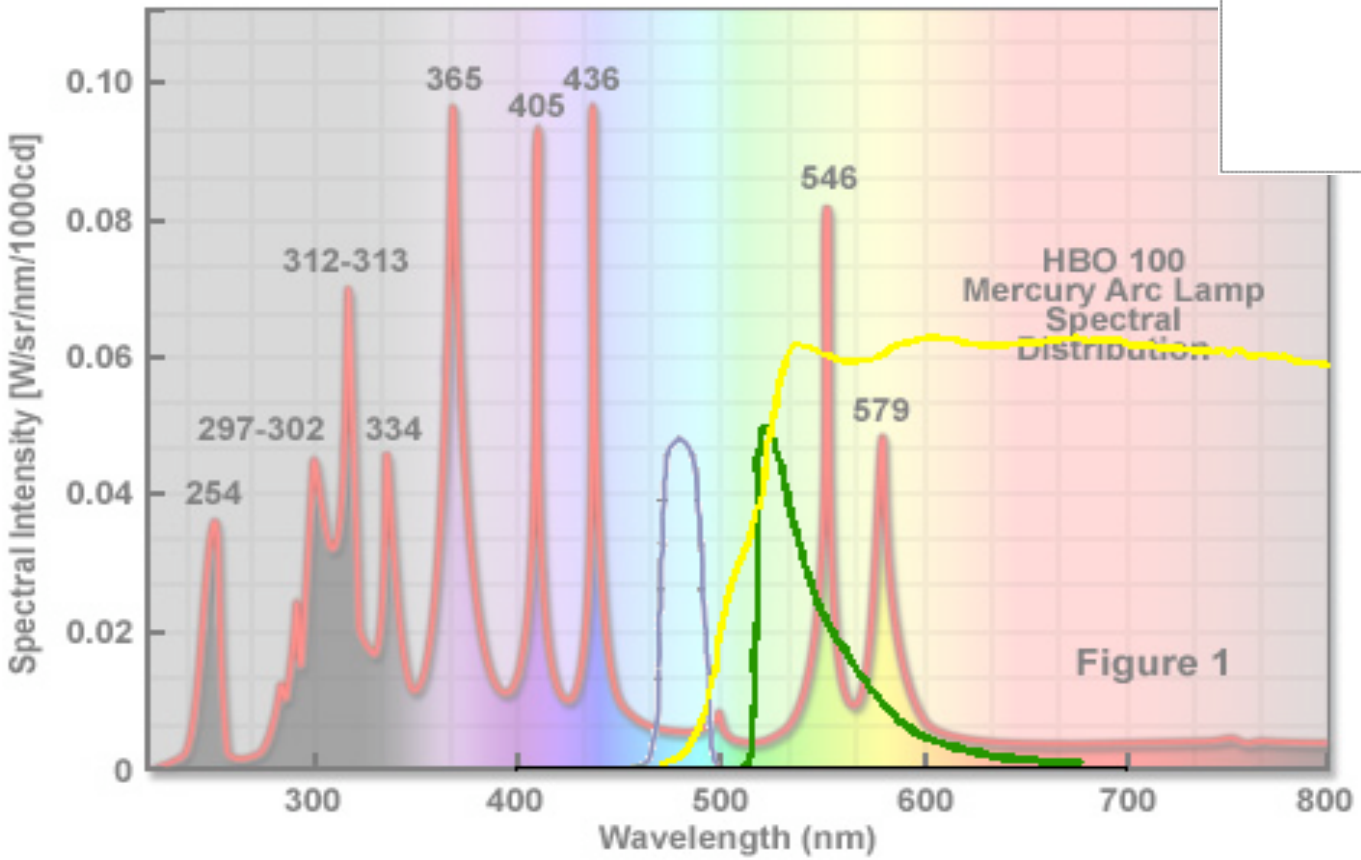
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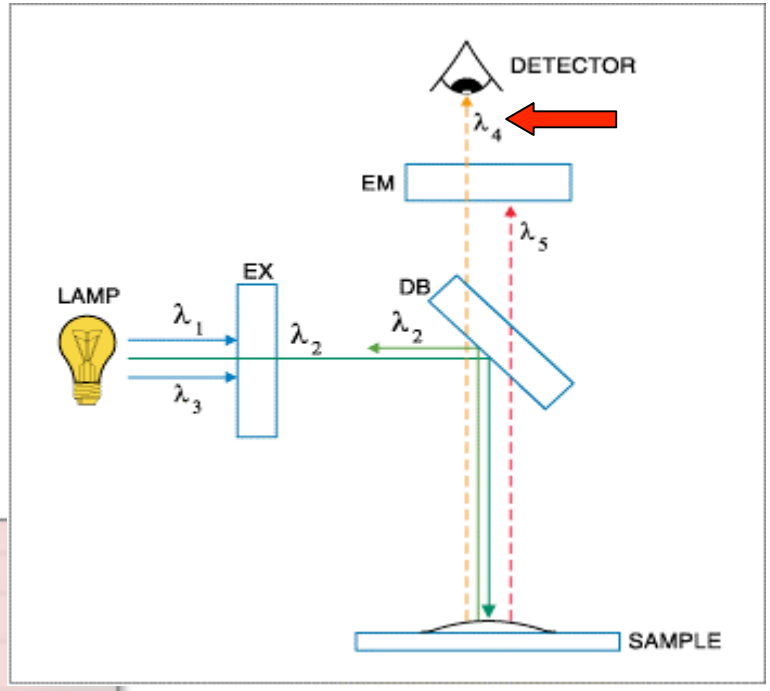
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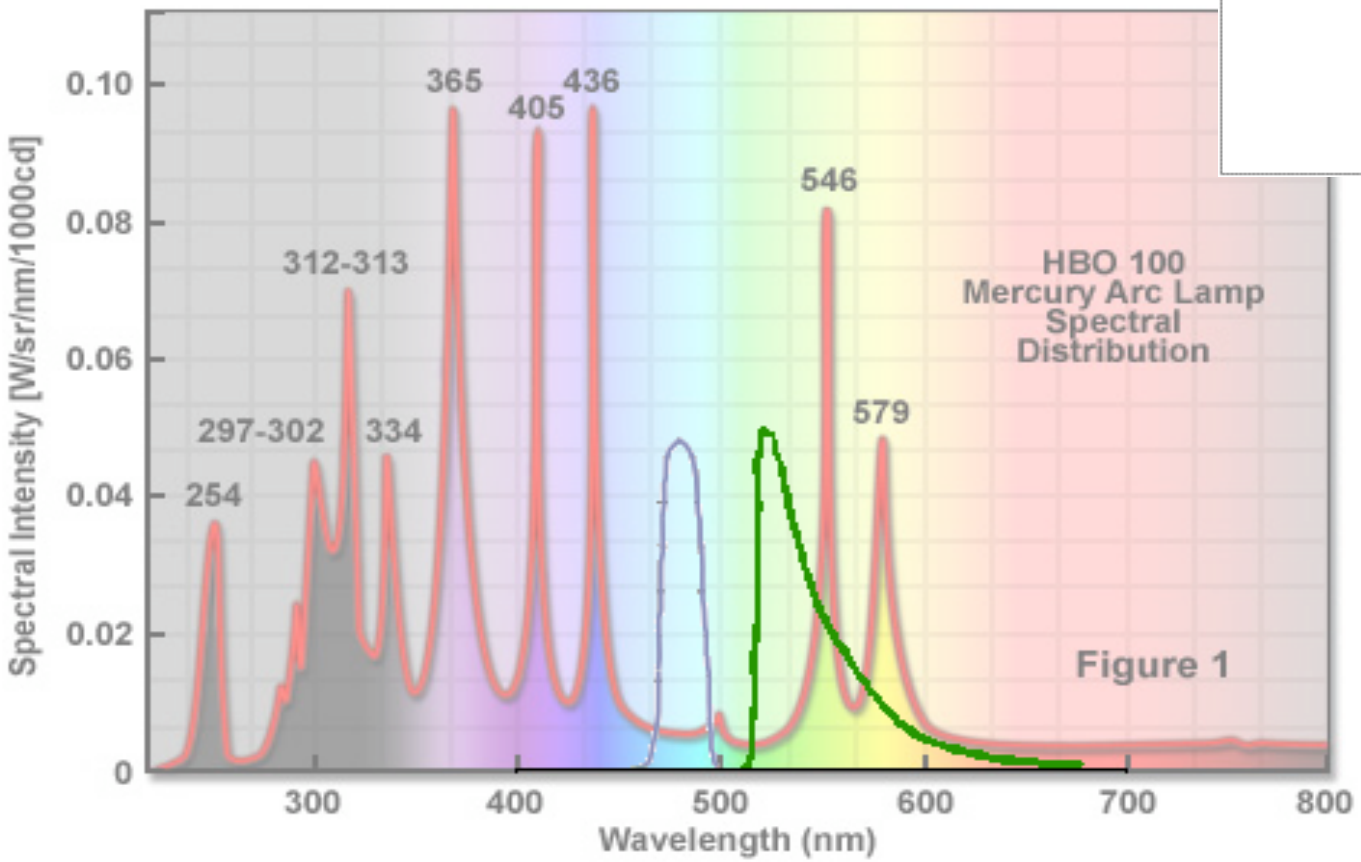
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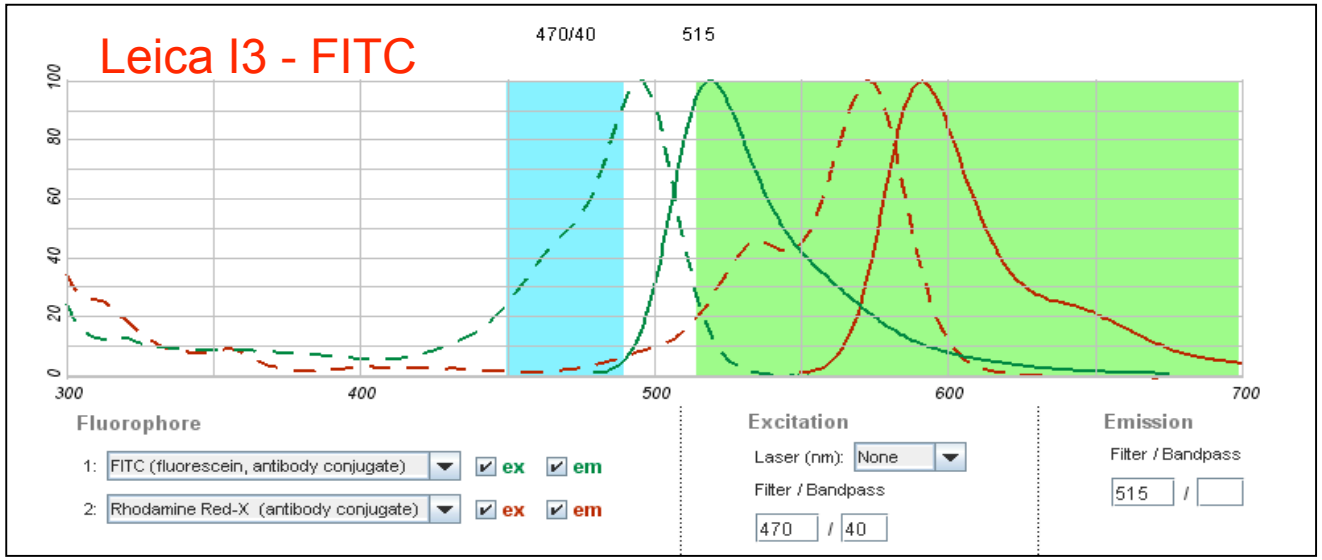
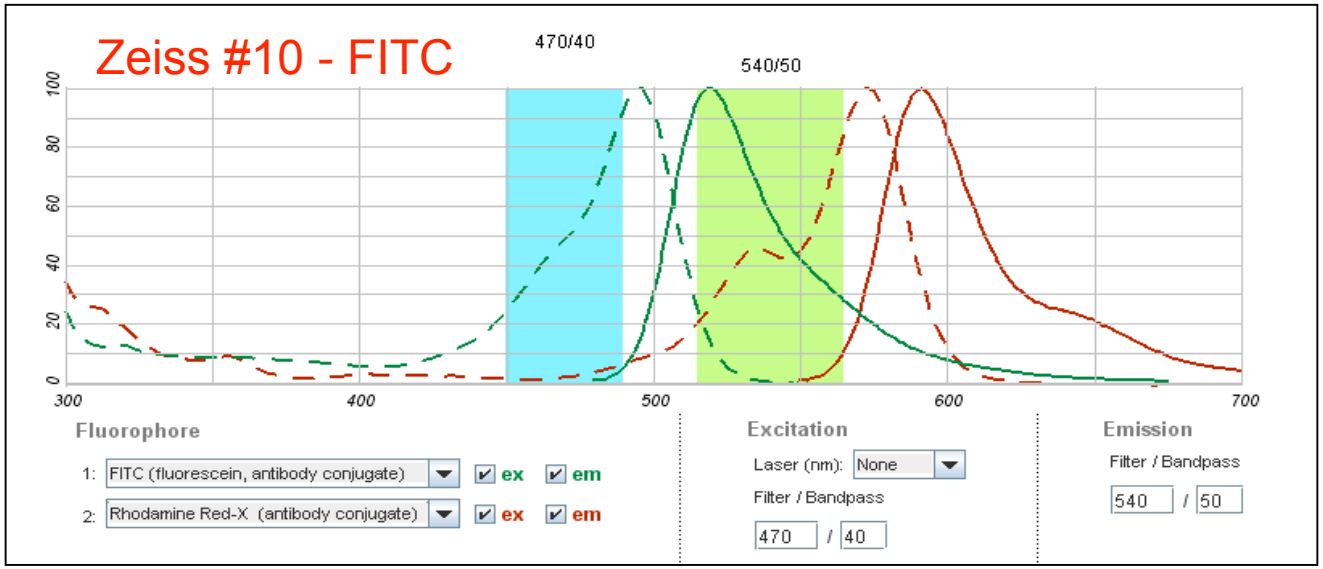
filter sets on BIU and HRU microscopes

Type	Microscope	Filter Set	Excitation	Dichroic	Emission
"UV"	BIU Olympus IX81	U-MNU2	360-370	400	lp420
	BIU Leica SP2	-	-	-	-
	BIU Leica SP5	A	340-380	400	470/40
	SW Zeiss Axioskop	02	G365	395	lp420
	PL Leica DMRBE	A	340-380	400	470/40
"Blue"	BIU Olympus IX81	U-MWBV2*	400-440	455	lp474
	BIU Olympus IX81	U-MWB2	450-480	500	lp515
	BIU Leica SP2	I3	450-490	510	lp515
	BIU Leica SP5	I3	450-490	510	lp515
	SW Zeiss Axioskop	10	450-490	510	bp515-565
	PL Leica DMRBE	I3	450-490	510	lp515
"Green"	BIU Olympus IX81	U-MNG2	530-550	570	lp590
	BIU Leica SP2	N2.1	515-560	580	lp590
	BIU Leica SP5	N2.1	515-560	580	lp590
	SW Zeiss Axioskop	15	546/12	580	lp590
	PL Leica DMRBE	N2.1	515-560	580	lp590

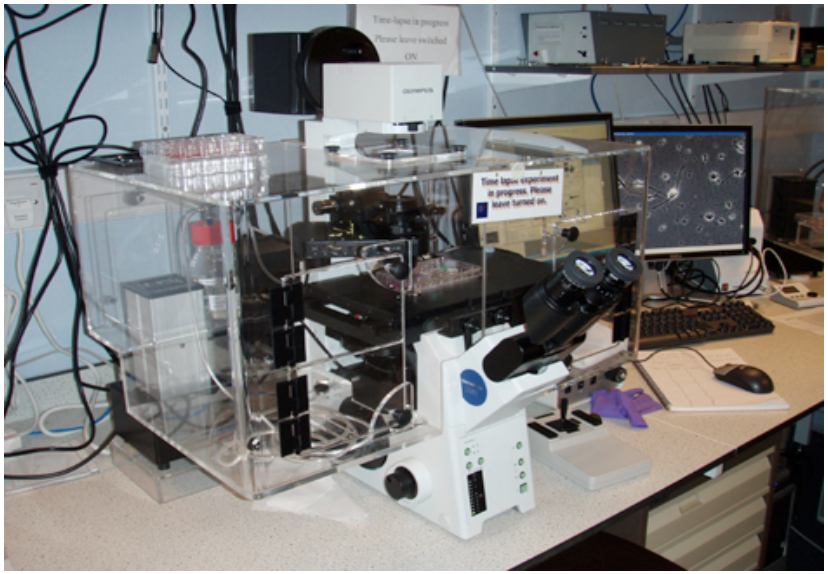
filter sets on BIU and HRU microscopes



Zeiss #3 has a bandpass emision filter to minimise bleed through of rhodamine signal excited by FITC excitation wavelengths

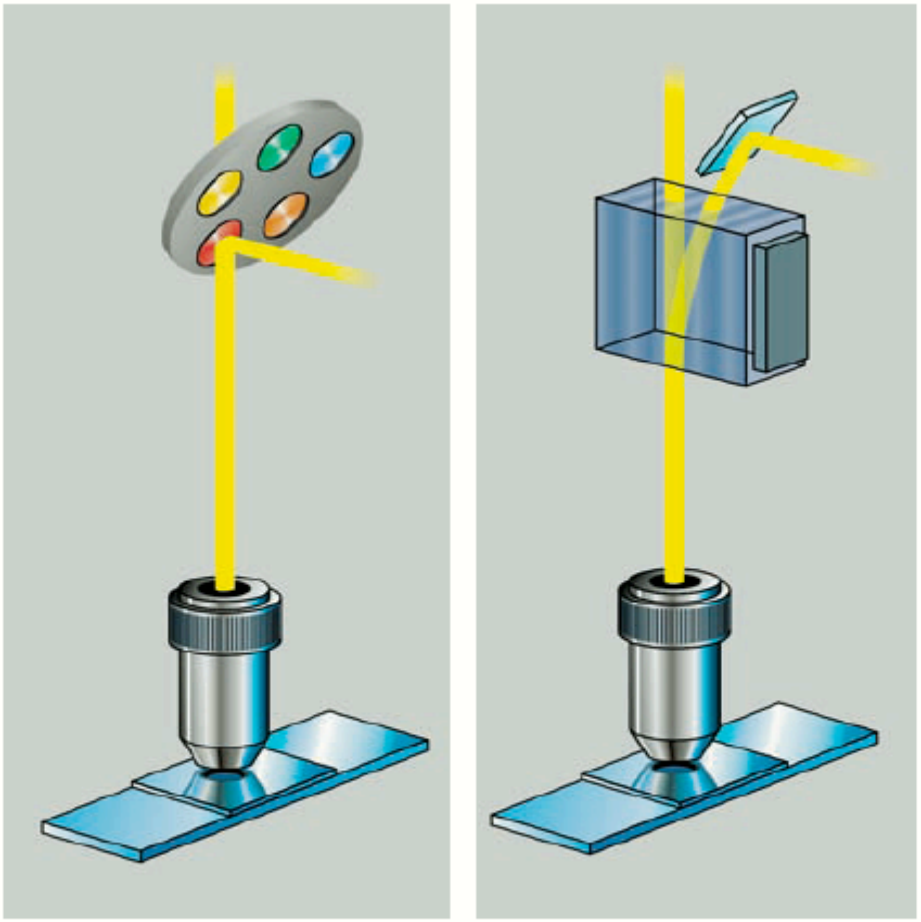


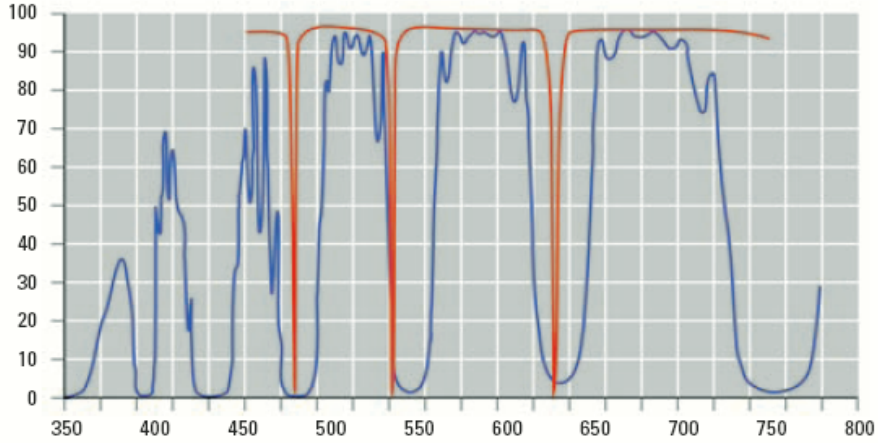
Olympus IX81



multilocation
FITC/TRITC/UV/Phase
timelapse
microscopy

Leica confocal AOBS





AOBS tuneable crystals in Leica confocal microscopes gives a much cleaner signal cut off than standard optical filters

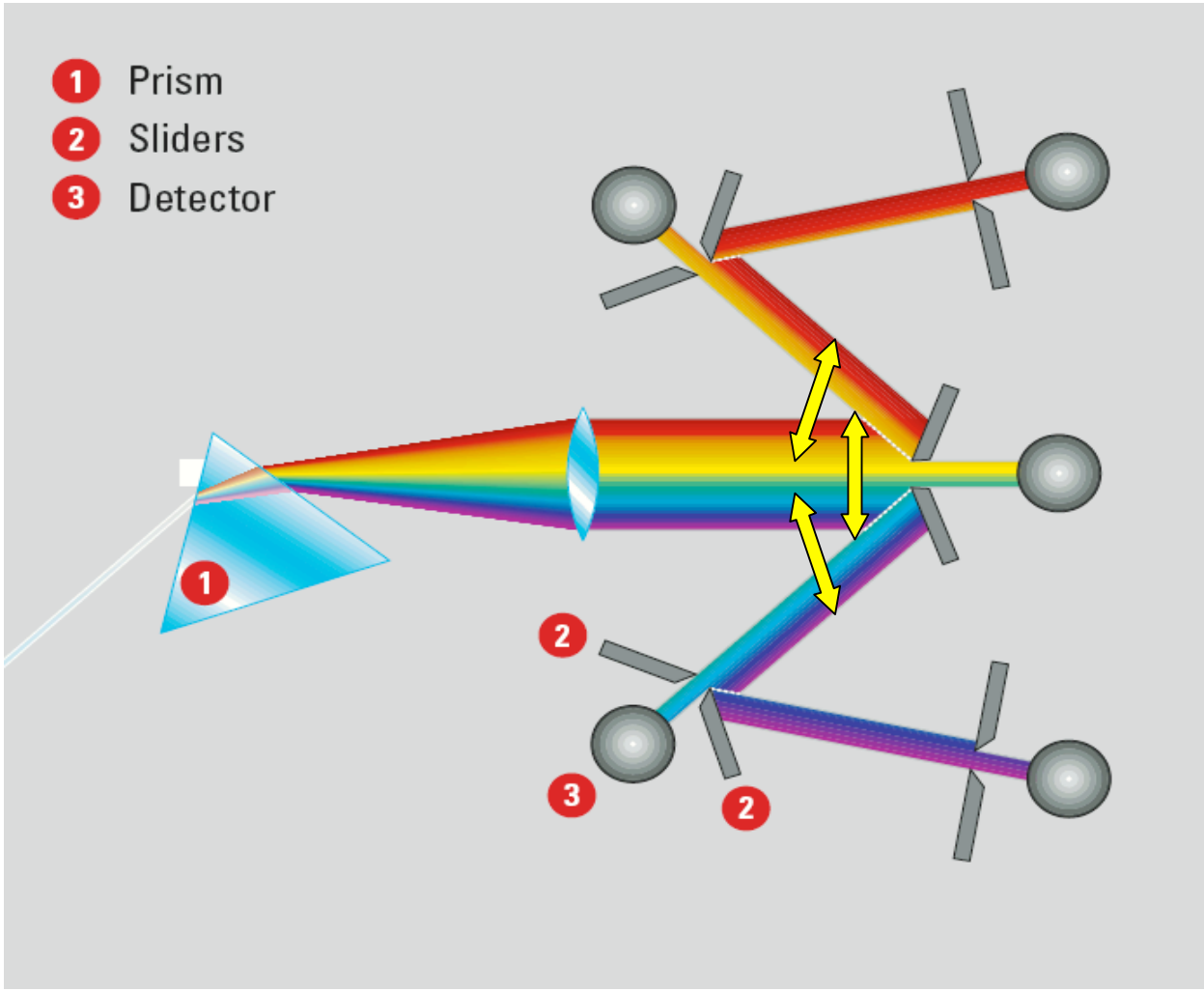
Leica confocal detector

fluorescence is focussed into a parallel "rainbow" light path

a slit edged with mirrors can be moved across the light path and opened or closed to specify which wavelengths reach the detector behind the slit

other wavelengths are deflected by the mirrors towards other detector /mirror / slit units

our SP5 has 4 detectors



Leica SP5

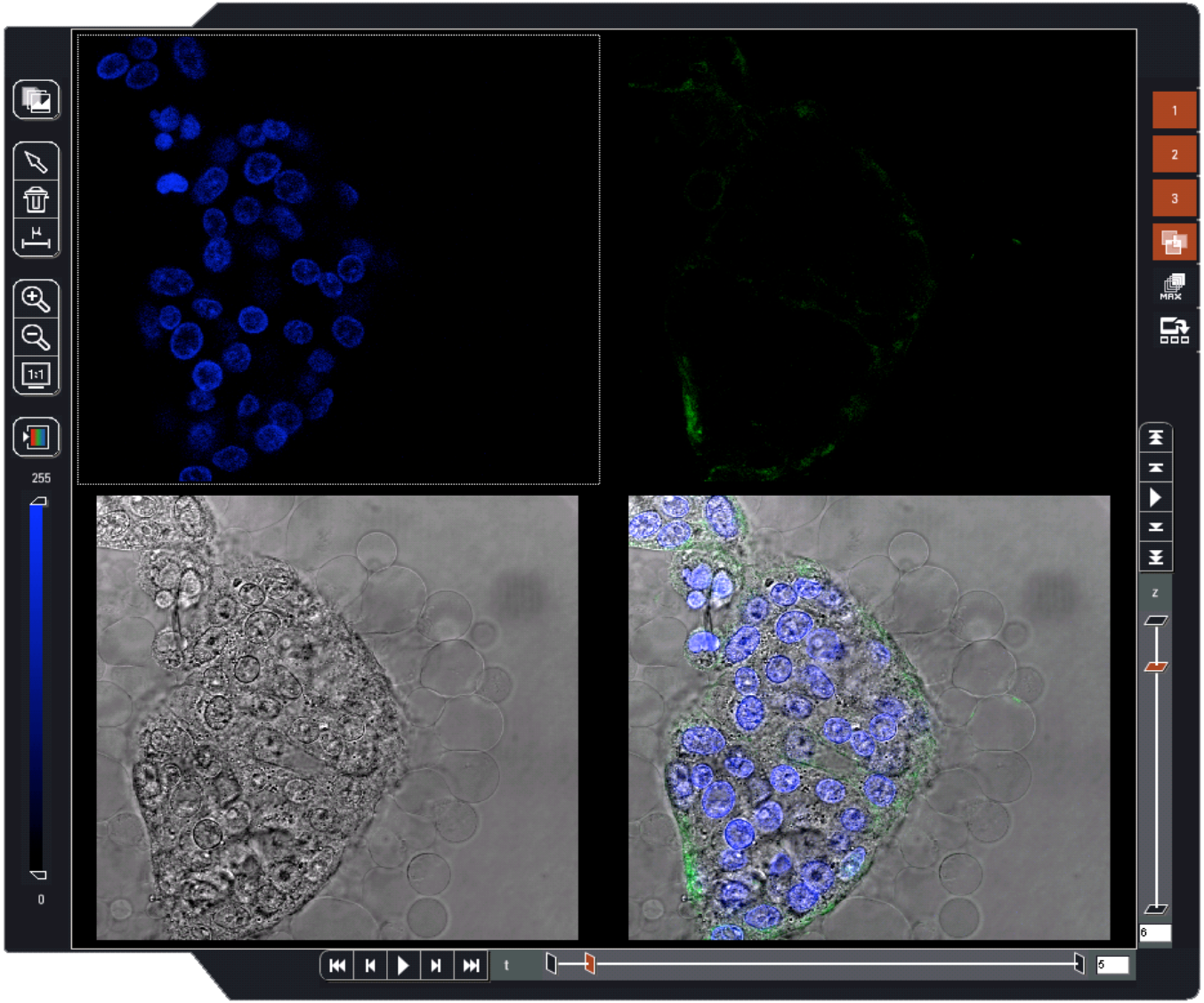
Leica DM1600 automated, inverted stand

heated chamber

5% CO2 in air

resonant scanner

Leica SP5



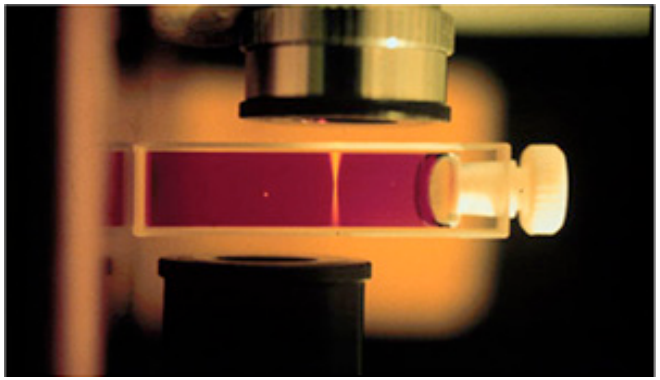
multilocation
multichannel
sequential
multifocus
timelapse
confocal
microscopy

multiphoton fluorescence microscopy

pulsed laser light

2 photons must arrive simultaneously at the focal point to excite fluorophore

summed energies result in emission of a shorter wavelength



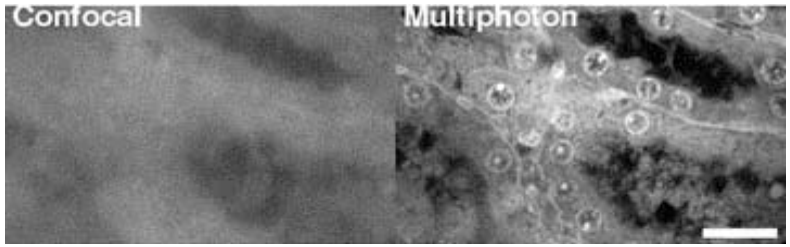
long wavelength

low energy excitation is suited to live cell imaging

less scatter

good depth penetration

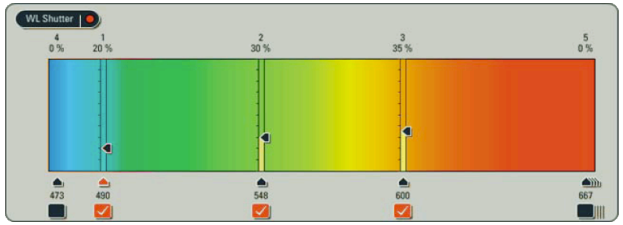
superior resolution

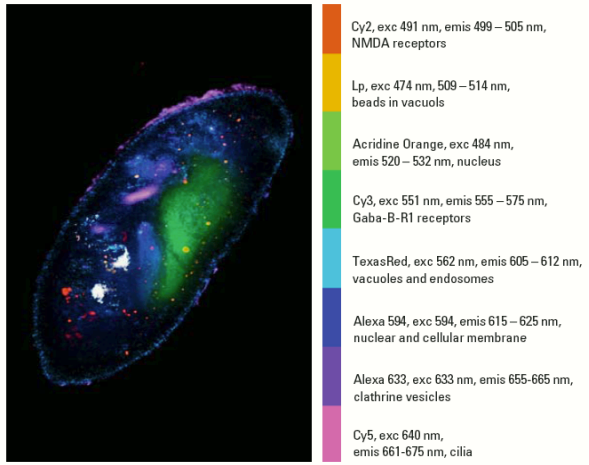


new advances

Leica SP5 X white continuum laser

for confocal systems providing multiple and any wavelengths with nm resolution from a single laser





Cy2, exc 491 nm, emis 499 – 505 nm, NMDA receptors

Lp, exc 474 nm, 509 – 514 nm, beads in vacuols

Acridine Orange, exc 484 nm, emis 520 – 532 nm, nucleus

Cy3, exc 551 nm, emis 555 – 575 nm, Gaba-B-R1 receptors

TexasRed, exc 562 nm, emis 605 – 612 nm, vacuoles and endosomes

Alexa 594, exc 594, emis 615 – 625 nm, nuclear and cellular membrane

Alexa 633, exc 633 nm, emis 655-665 nm, clathrine vesicles

Cy5, exc 640 nm, emis 661-675 nm, cilia

Leica FLUO LED illumination

bright and long lasting excitation spectra from tuned LED units with touchscreen brightness control