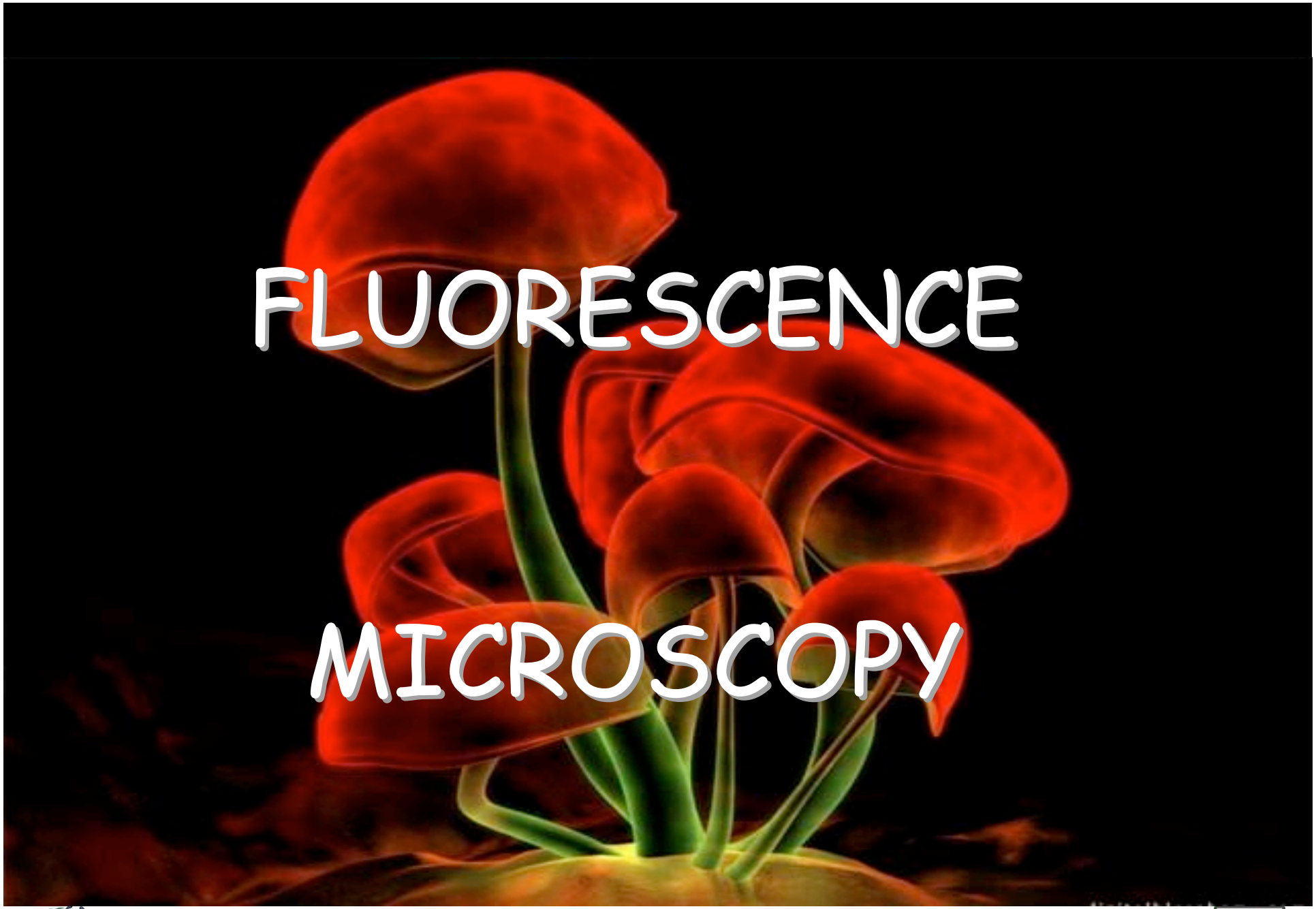
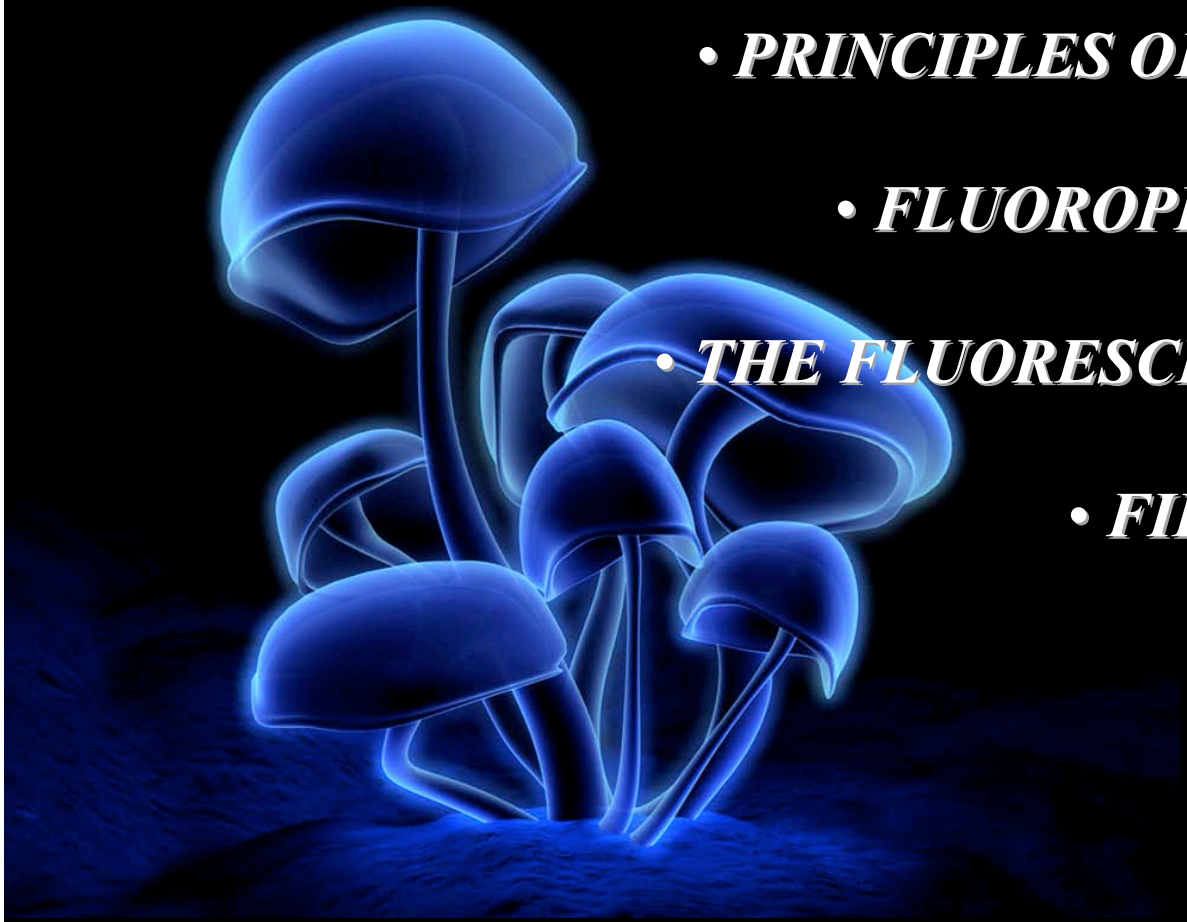
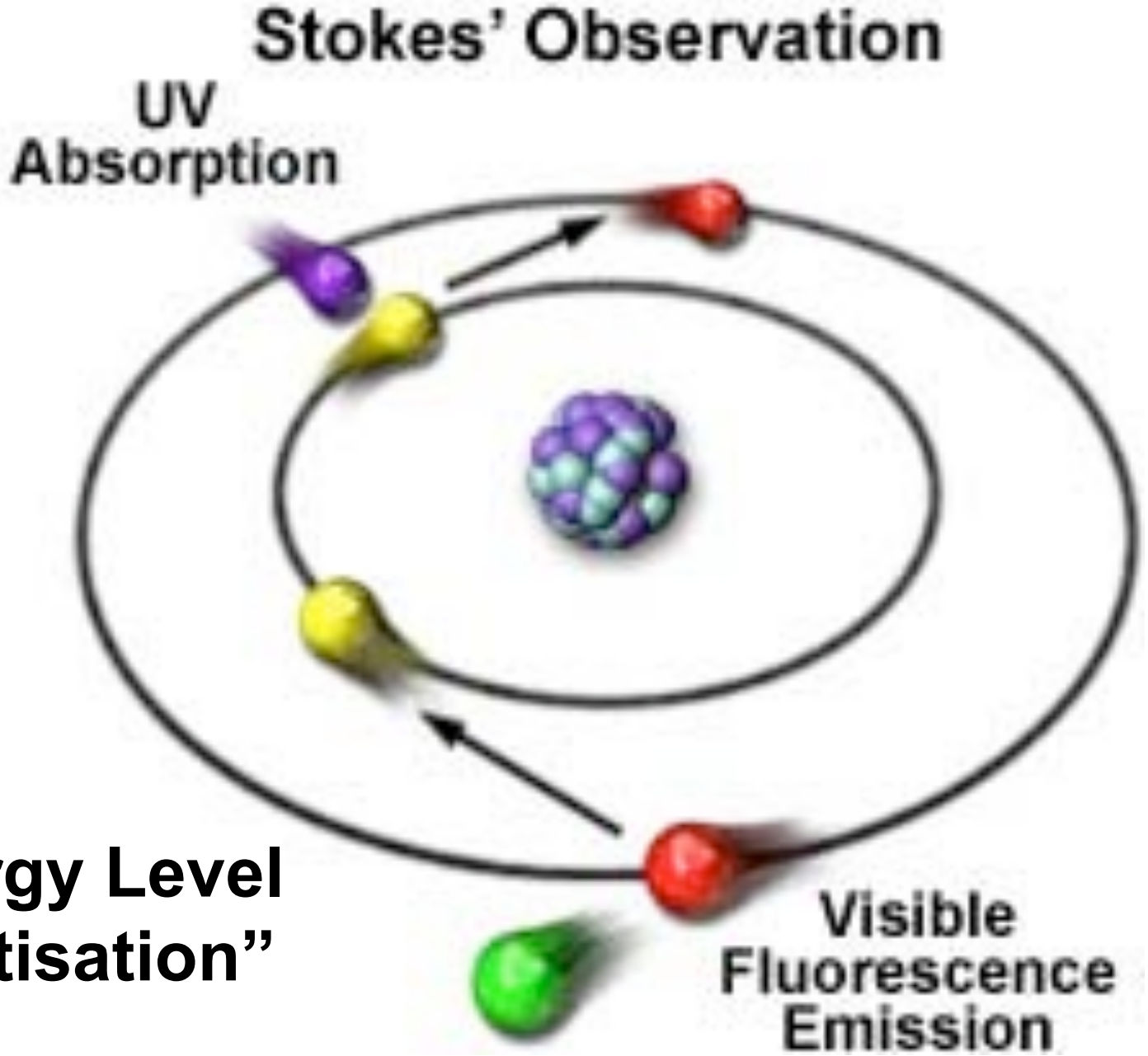

FLUORESCENCE
MICROSCOPY

TOPICS



- *PRINCIPLES OF FLUORESCENCE*
- *FLUOROPHORES / DYES*
- *THE FLUORESCENCE MICROSCOPE*
- *FILTERS*



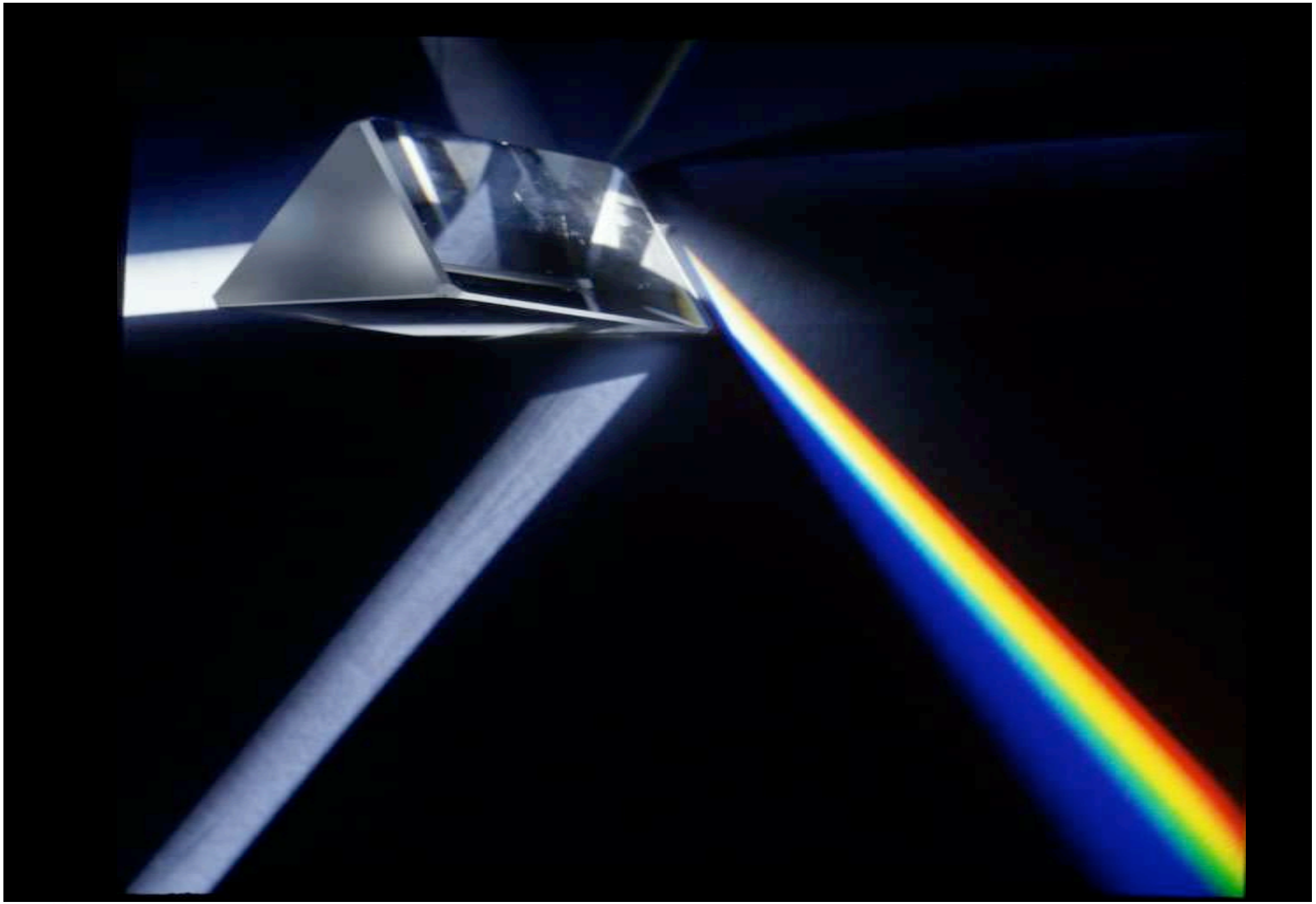
"Energy Level Quantisation"

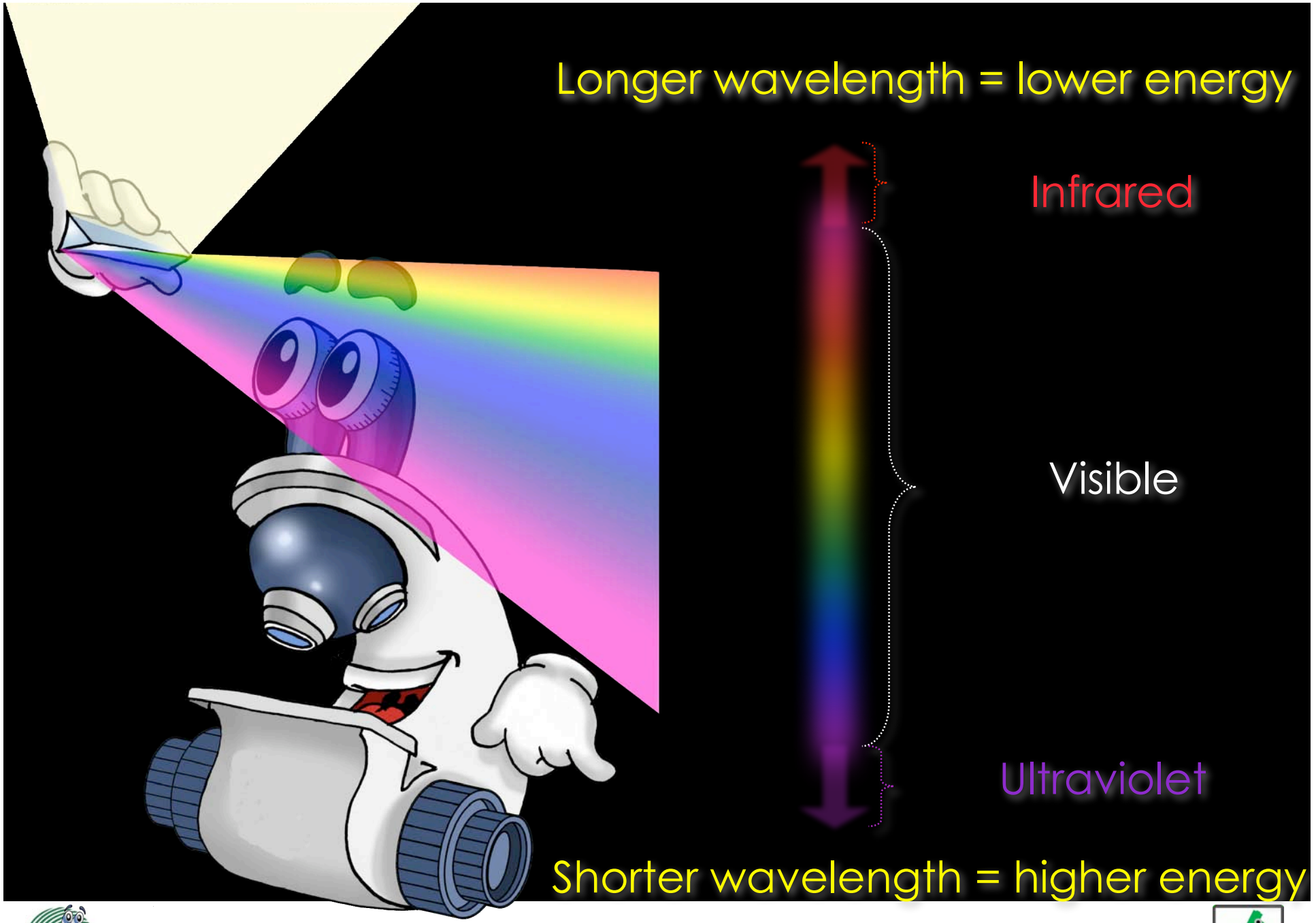
Processes leading to fluorescence in fluorescent dyes

1. **Absorption of a photon by a Dye Molecule**

2. **Dye Molecule relaxes**

3. **Dye Molecule emits a photon**

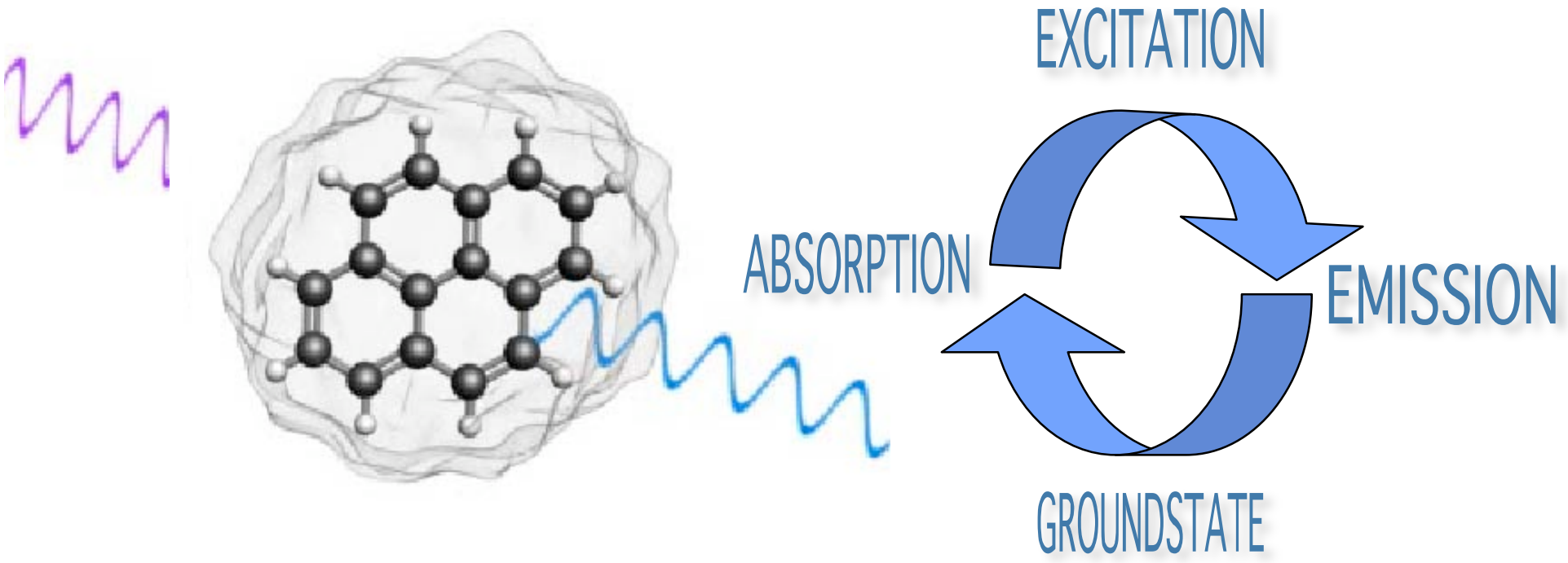



Longer wavelength = lower energy
Infrared
Visible
Ultraviolet
Shorter wavelength = higher energy

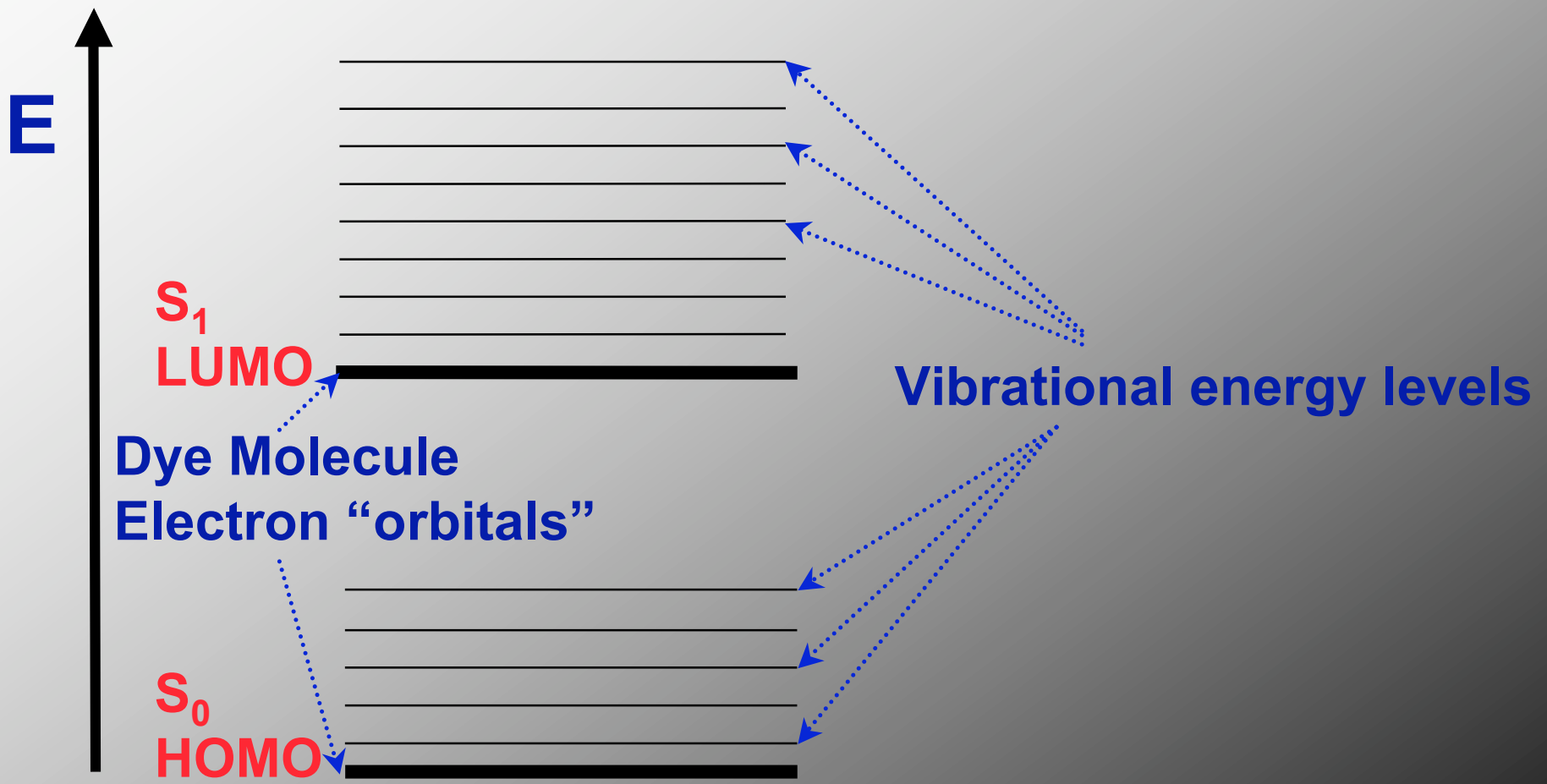
ABSORPTION OF LIGHT

All molecules absorb light
different molecules - different wavelengths

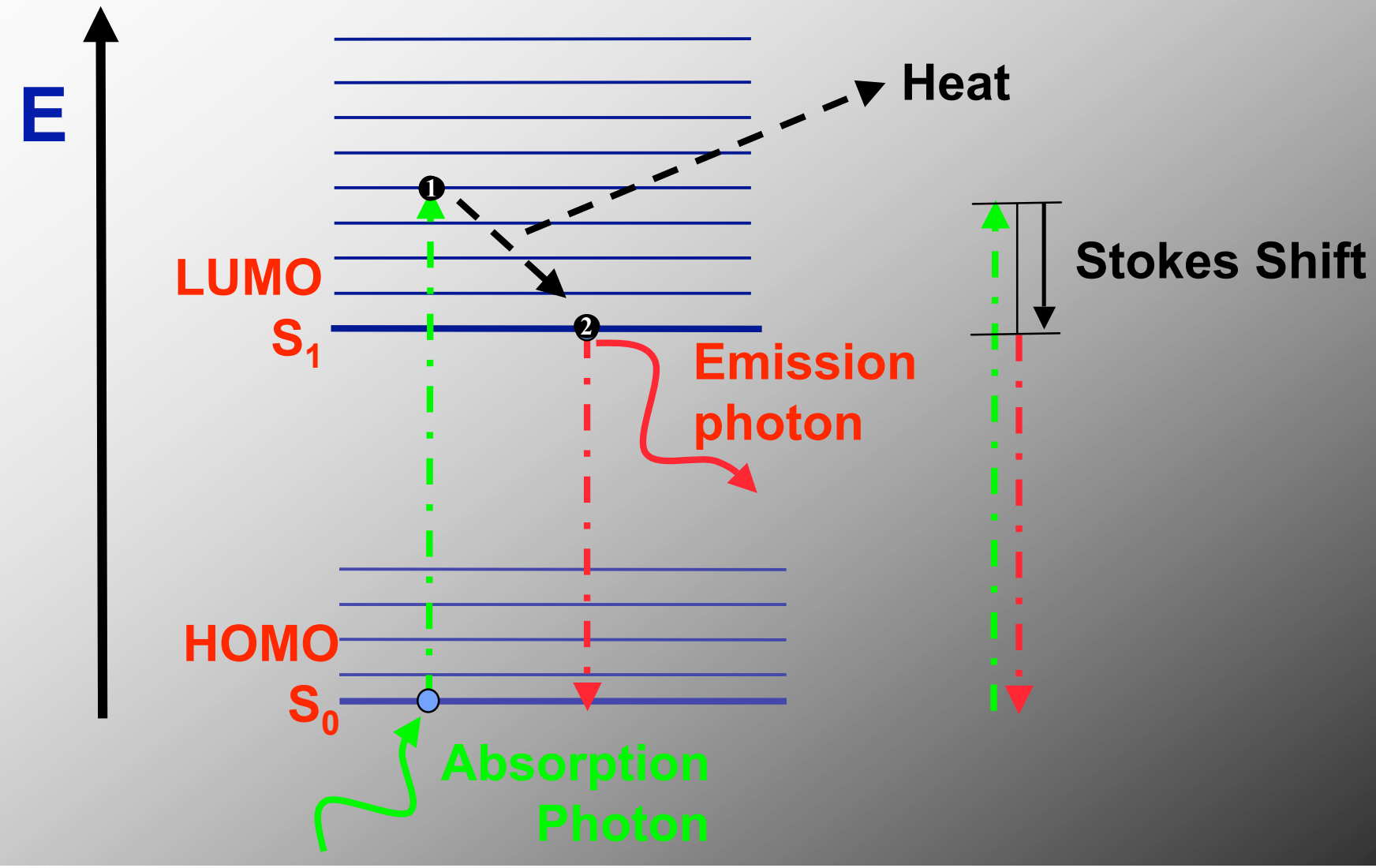
- Absorption of microwaves causes molecular rotations,
- Absorption in the infra red causes molecular bond vibrations,
- Absorption of UV/visible light causes electrons to jump to higher energy “orbitals”.


EXCITATION
ABSORPTION
EMISSION
GROUNDSTATE

Energy Level Diagram

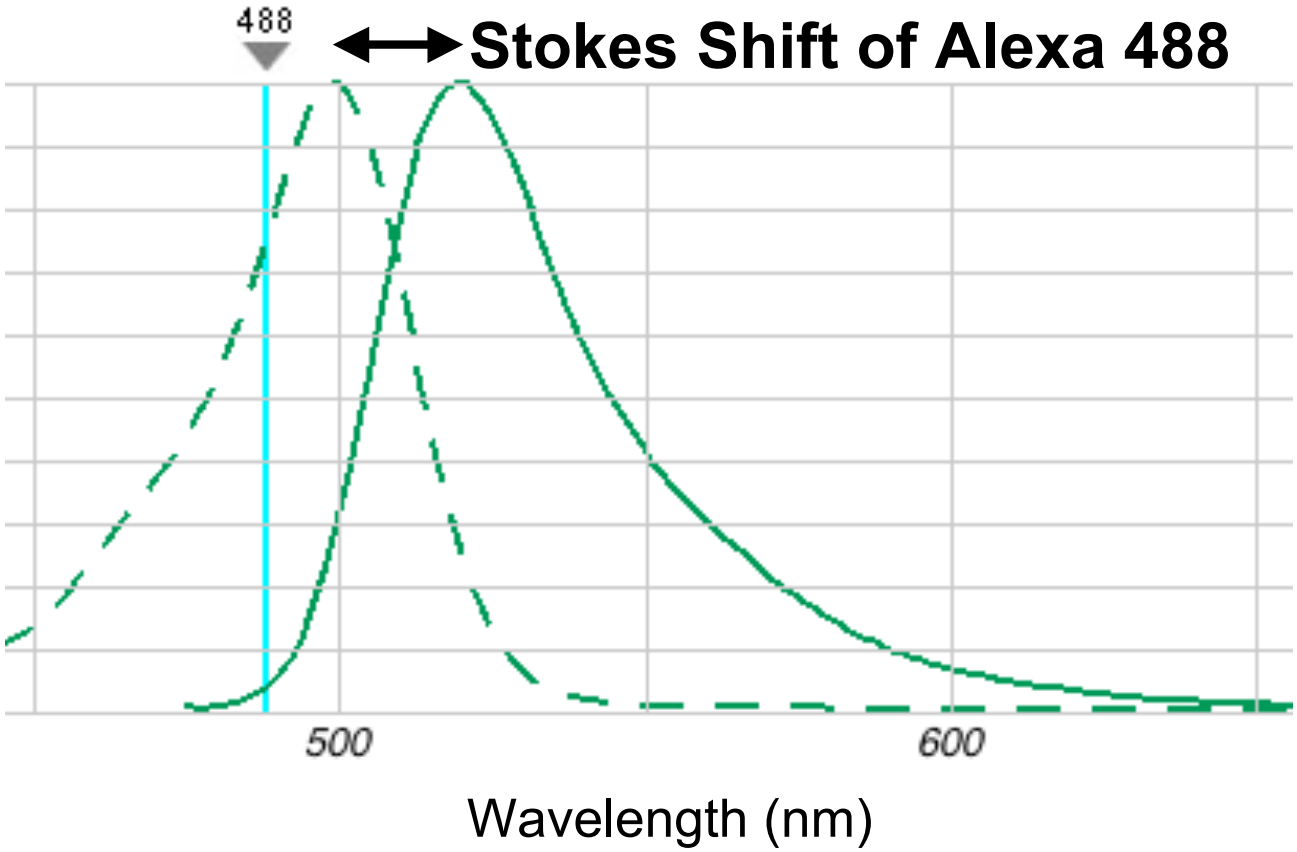


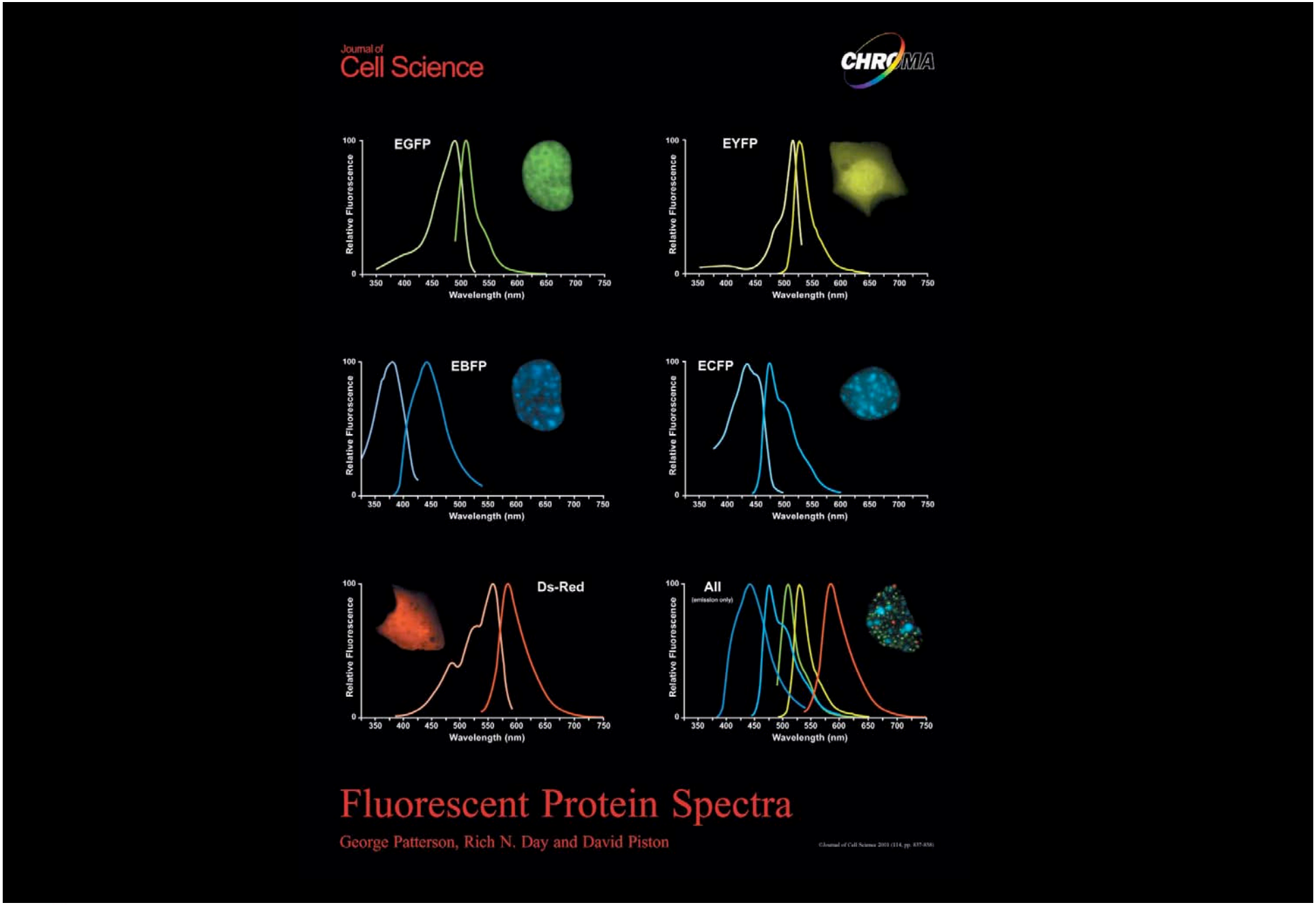
Fluorescence Absorption / Emission



Emission has lower energy ⇨ **Longer wavelength**

Absorption = Excitation Emission = Fluorescence

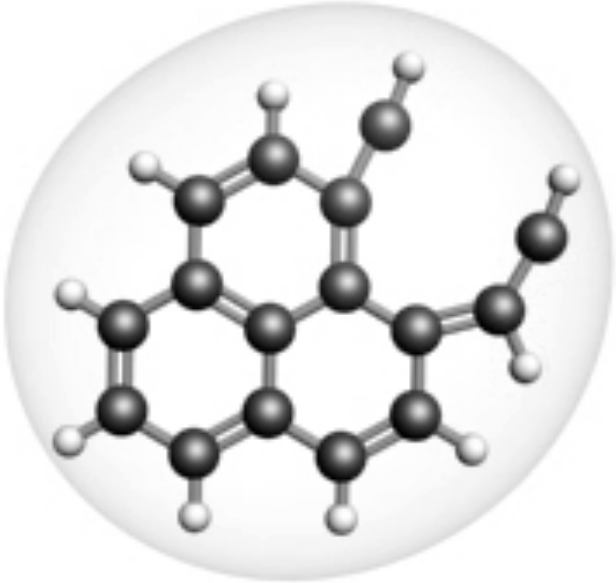


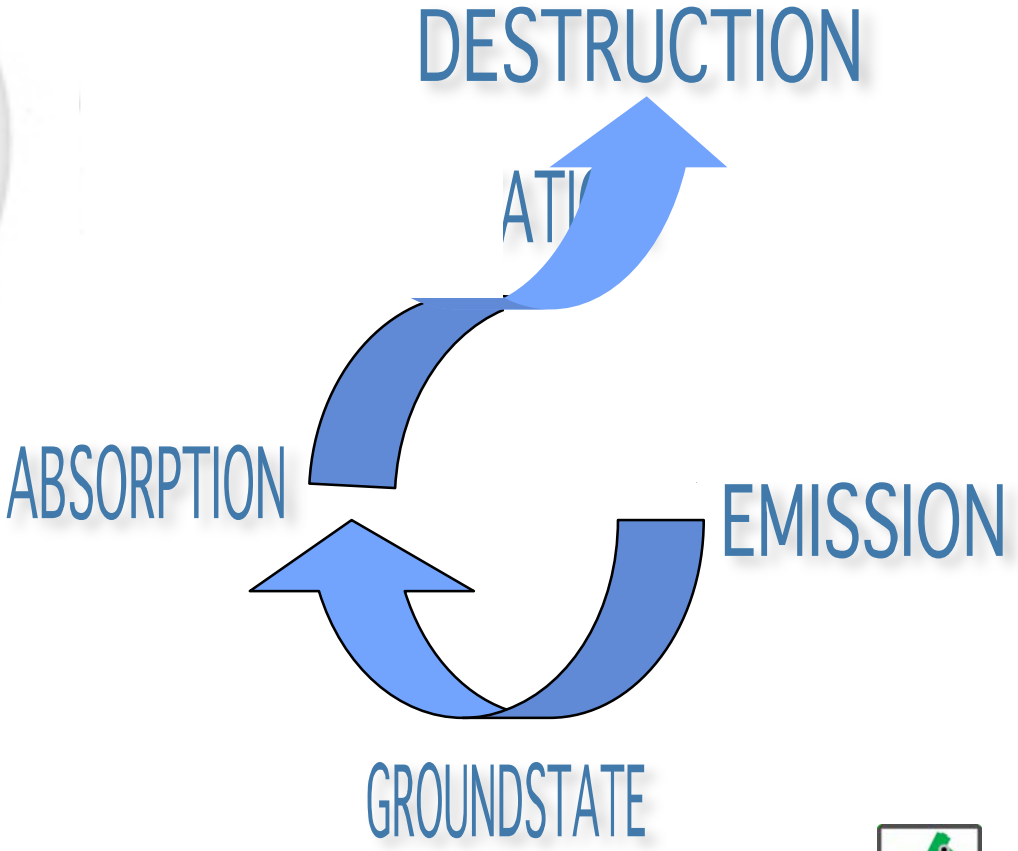

Journal of
Cell Science
CHROMA
EGFP
EYFP
EBFP
ECFP
Ds-Red
All
(emission only)
Relative Fluorescence
Wavelength (nm)
100
0
350
400
450
500
550
600
650
700
750
Fluorescent Protein Spectra
George Patterson, Rich N. Day and David Piston

1st TAKE HOME MESSAGE.....

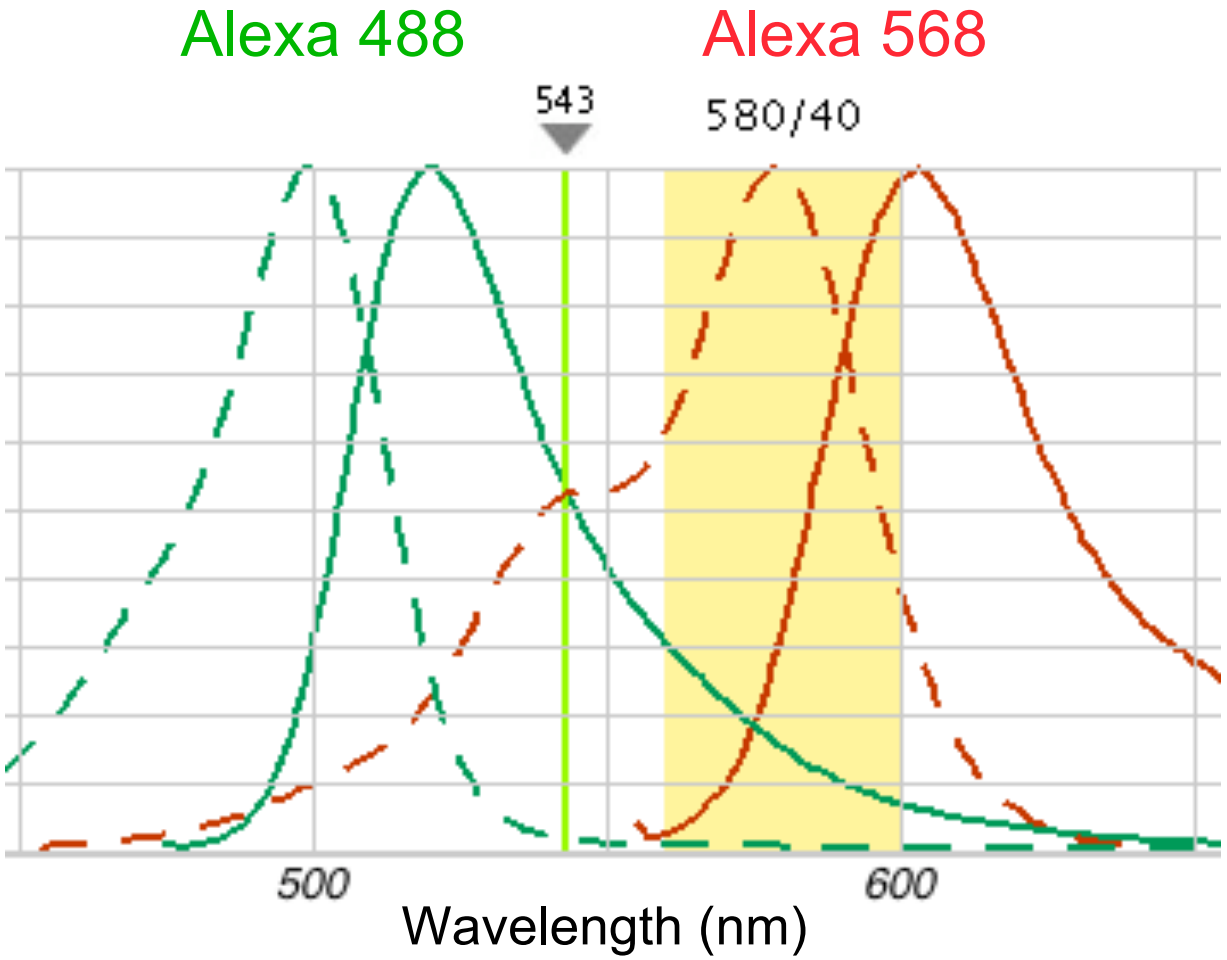
KNOW YOUR FLUOROPHORE!!!!

PHOTOBLEACHING

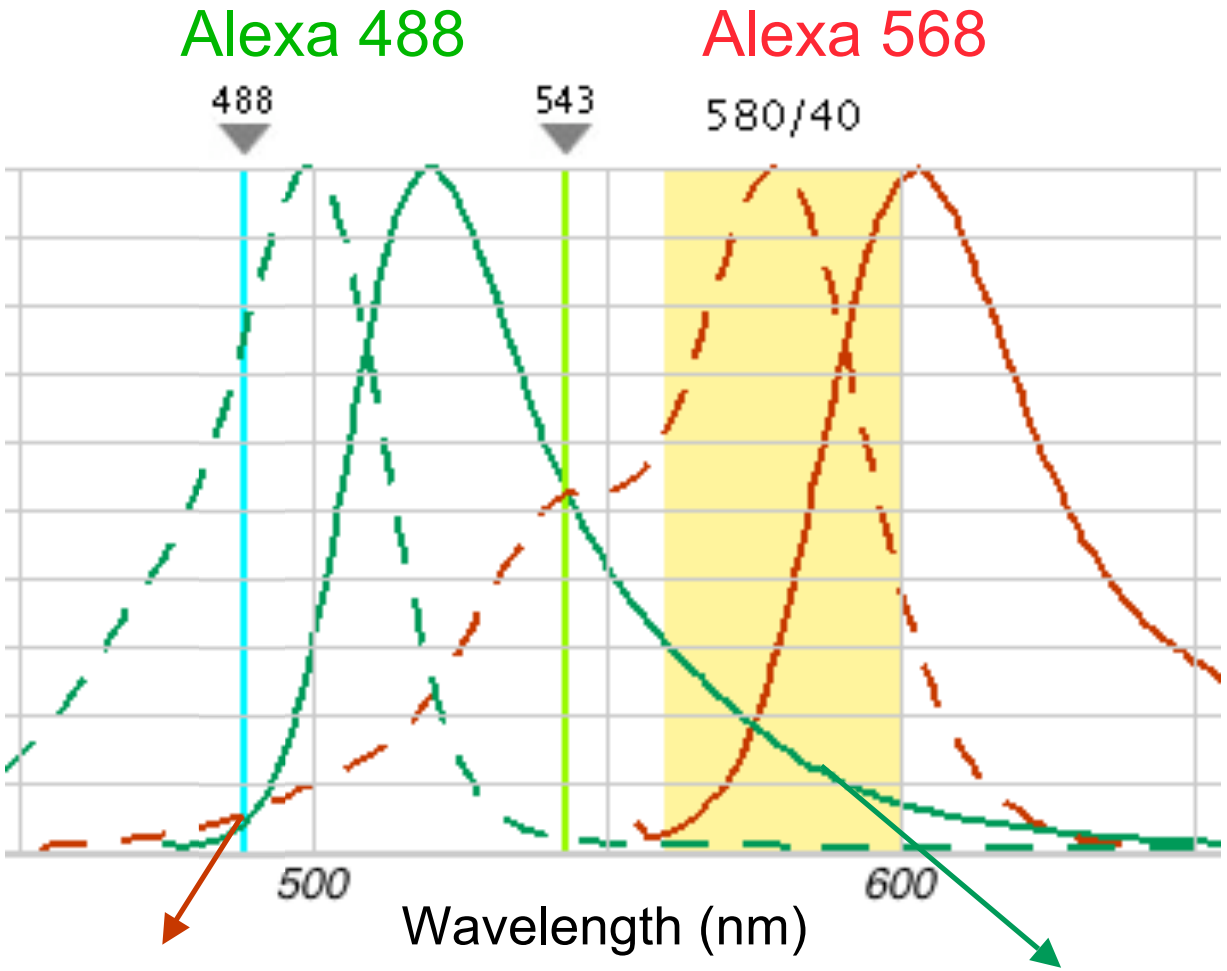


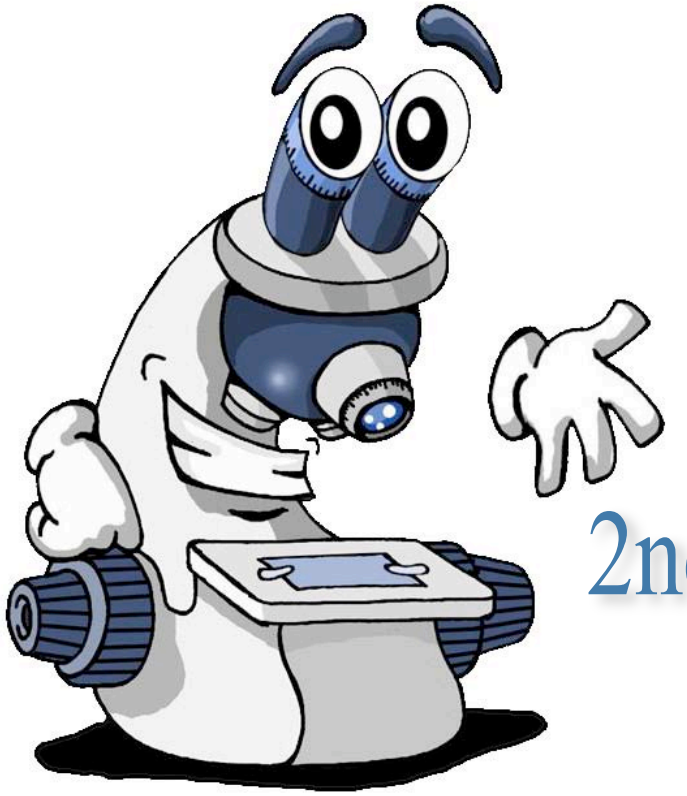


Multiple colour/dye imaging…



Beware ! Crosstalk and Bleed Through

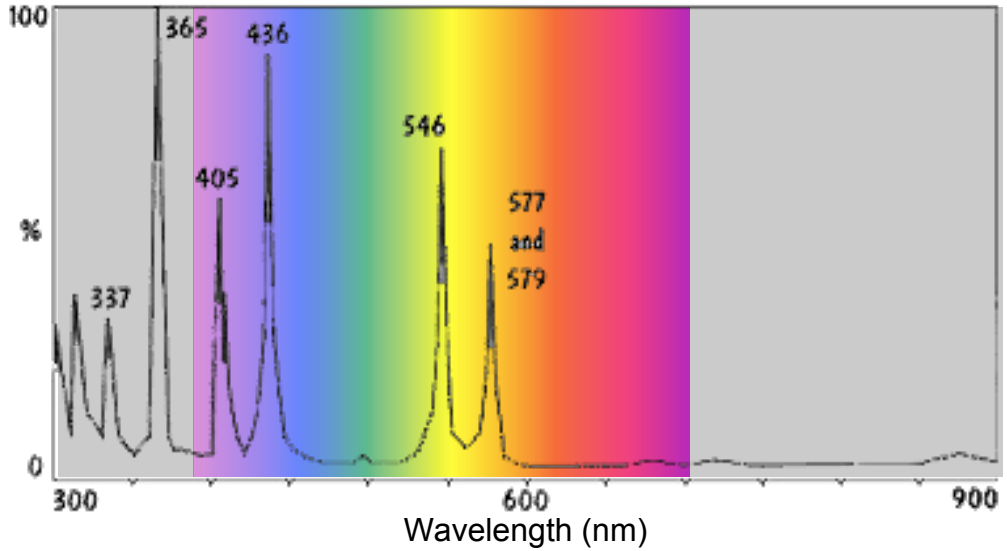




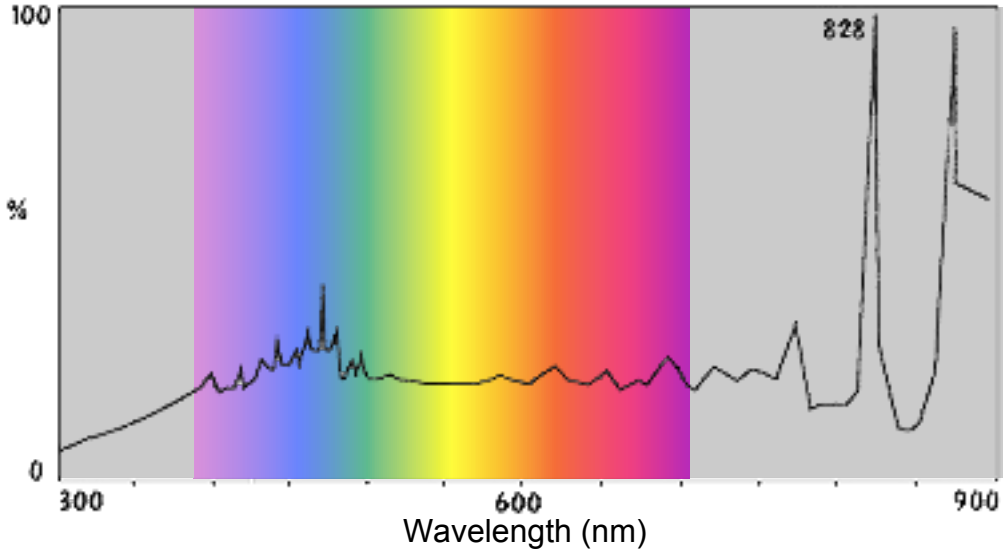
2nd TAKE HOME MESSAGE.....
KNOW YOUR MICROSCOPE!!!!


NO LIGHT.....

Spectrum of a Mercury Lamp

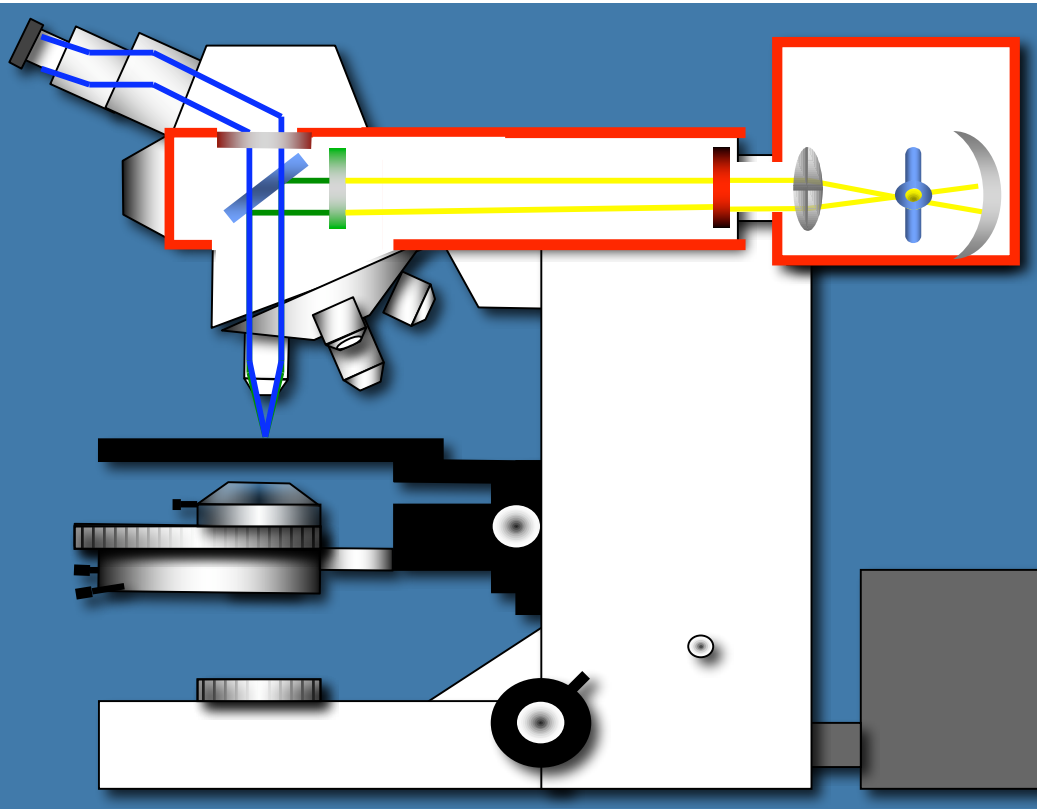


Spectrum of a Xenon Lamp



Light sources for fluorescence

(Modified from: http://www.cairn-research.co.uk)



Mercury Lamp

Mirror

Collimating Lens

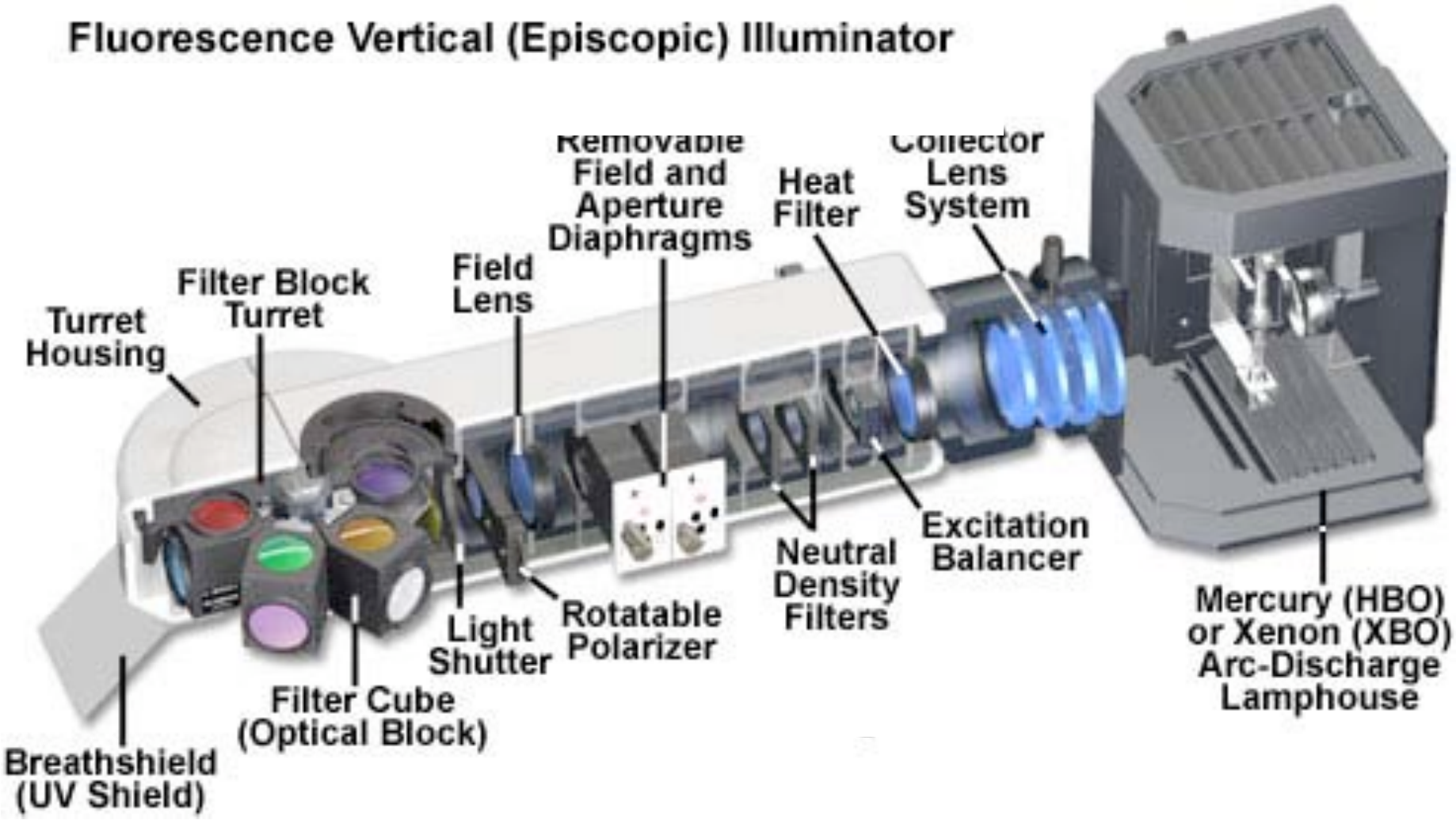
Heat Filter

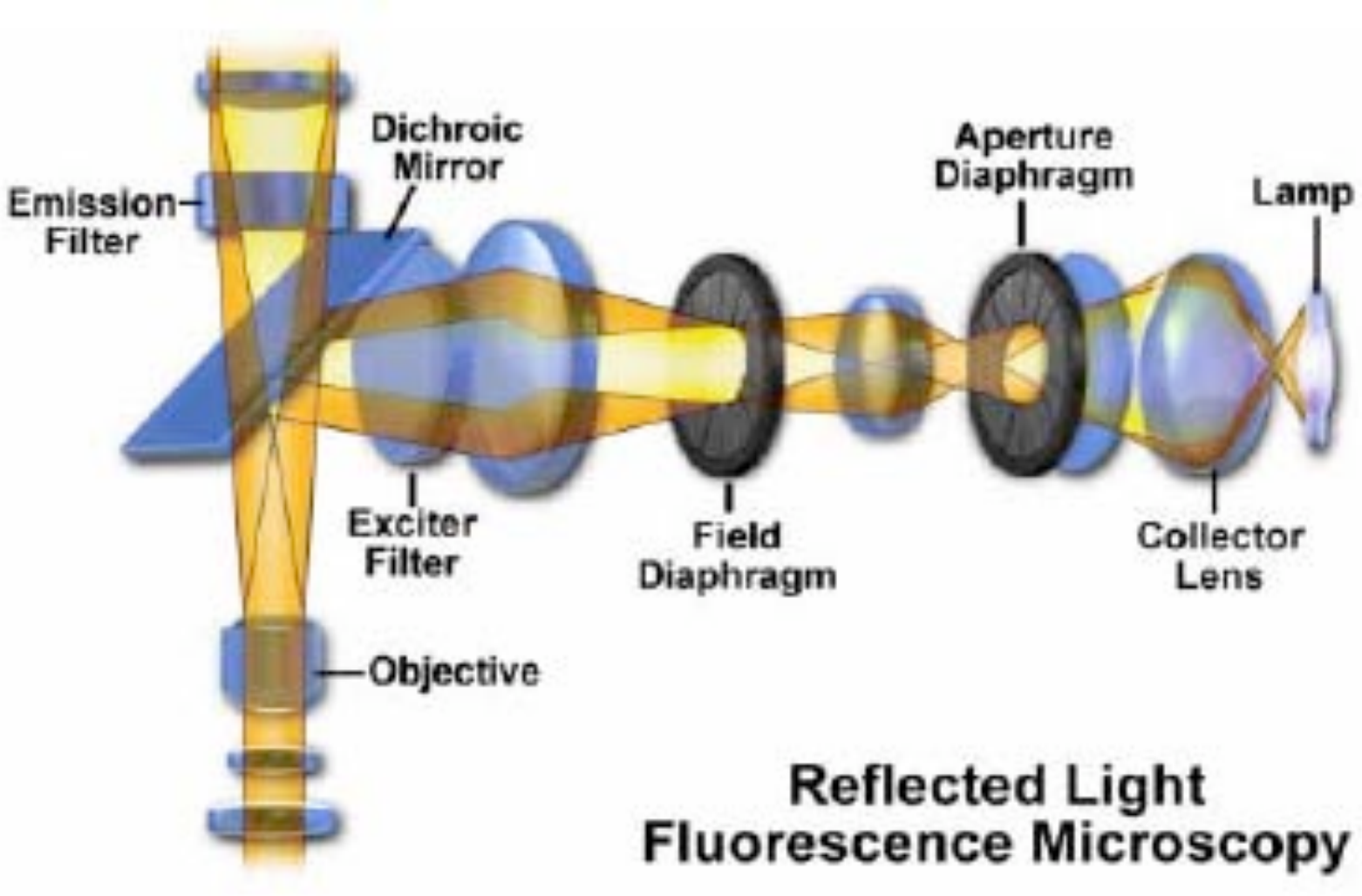
Excitation Filter

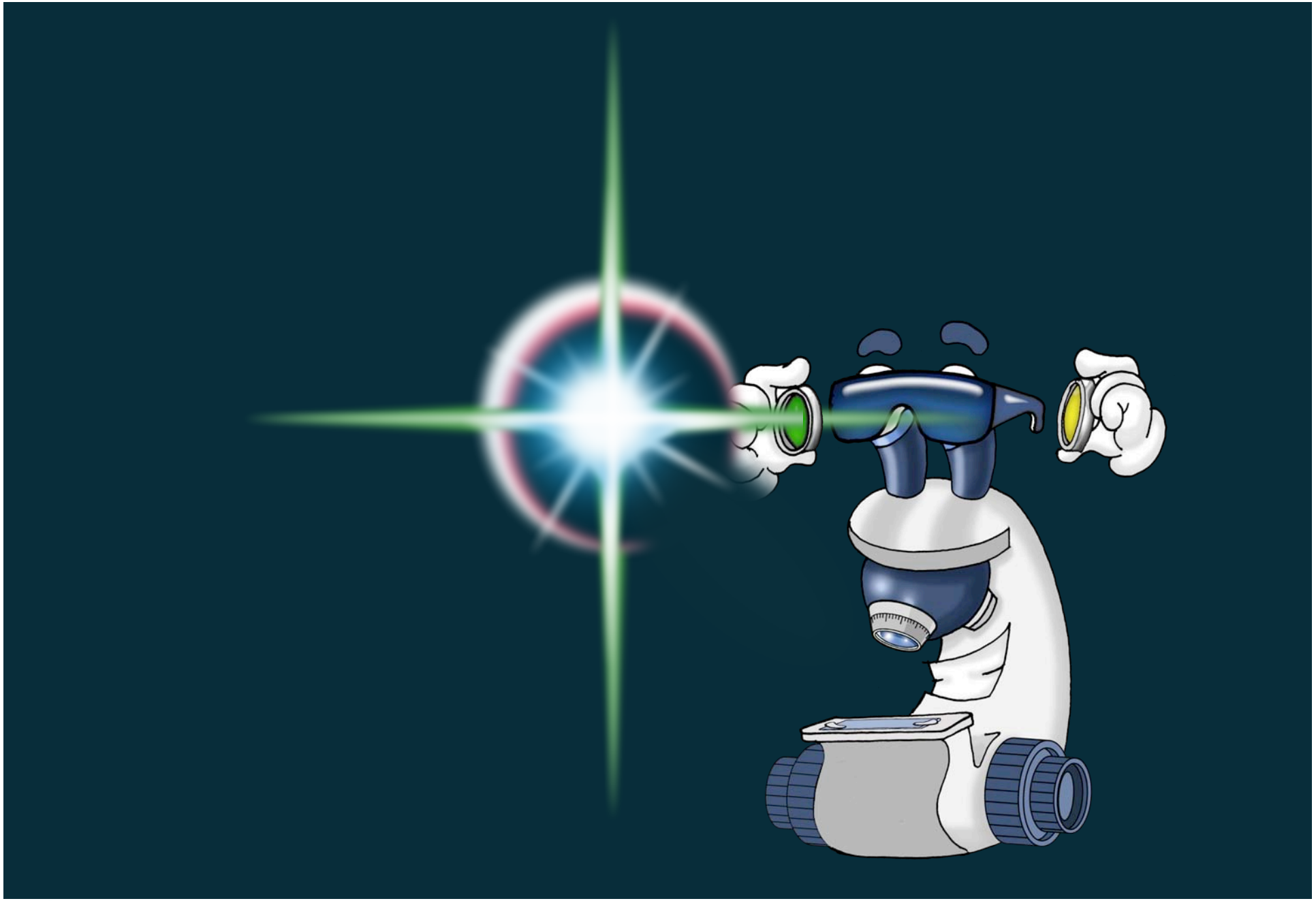
Dichromatic Mirror

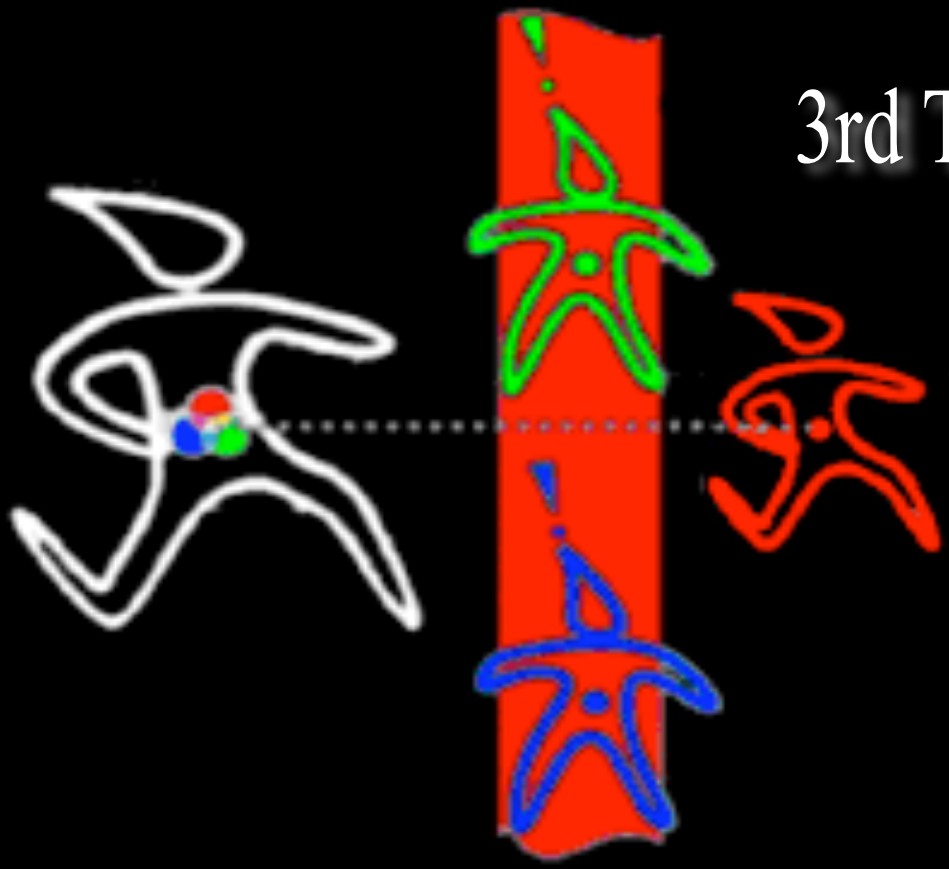
Emission Filter

Fluorescence Vertical (Episcopic) Illuminator



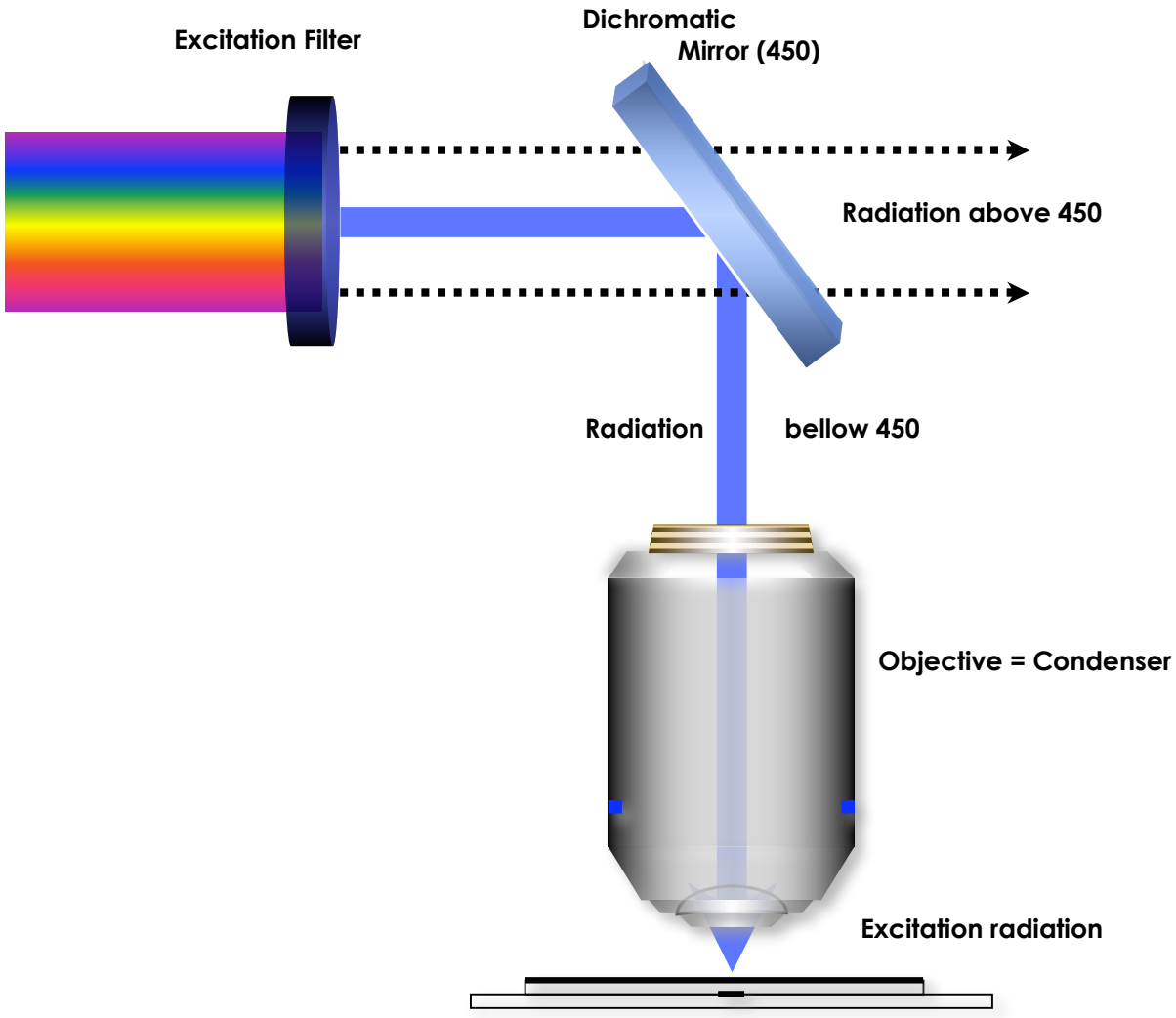

Dichroic
Mirror
Emission
Filter
Aperture
Diaphragm
Lamp
Exciter
Filter
Field
Diaphragm
Collector
Lens
Objective
Reflected Light
Fluorescence Microscopy

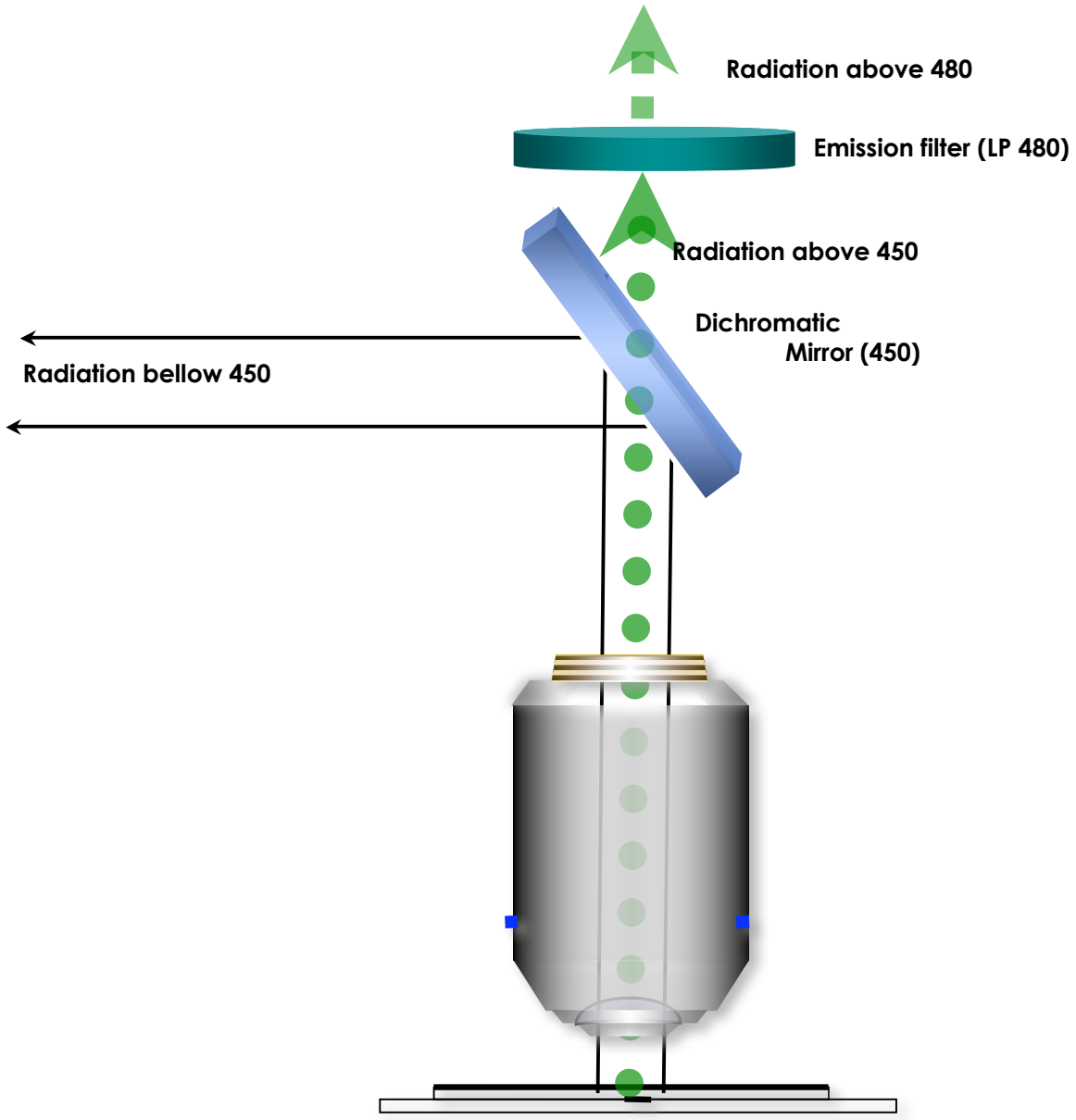


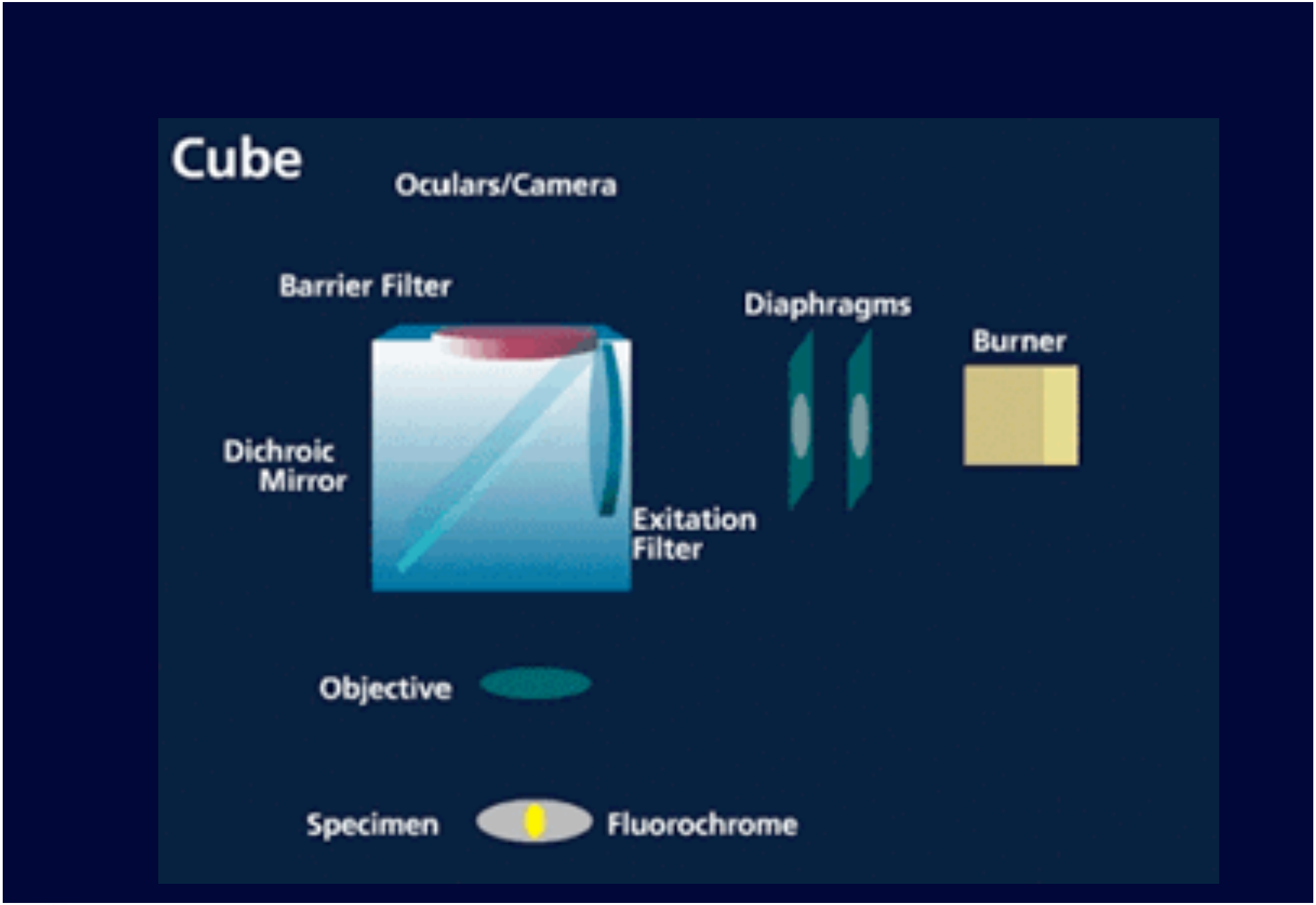


3rd TAKE HOME MESSAGE.....
KNOW YOUR FILTER SYSTEM!!!!

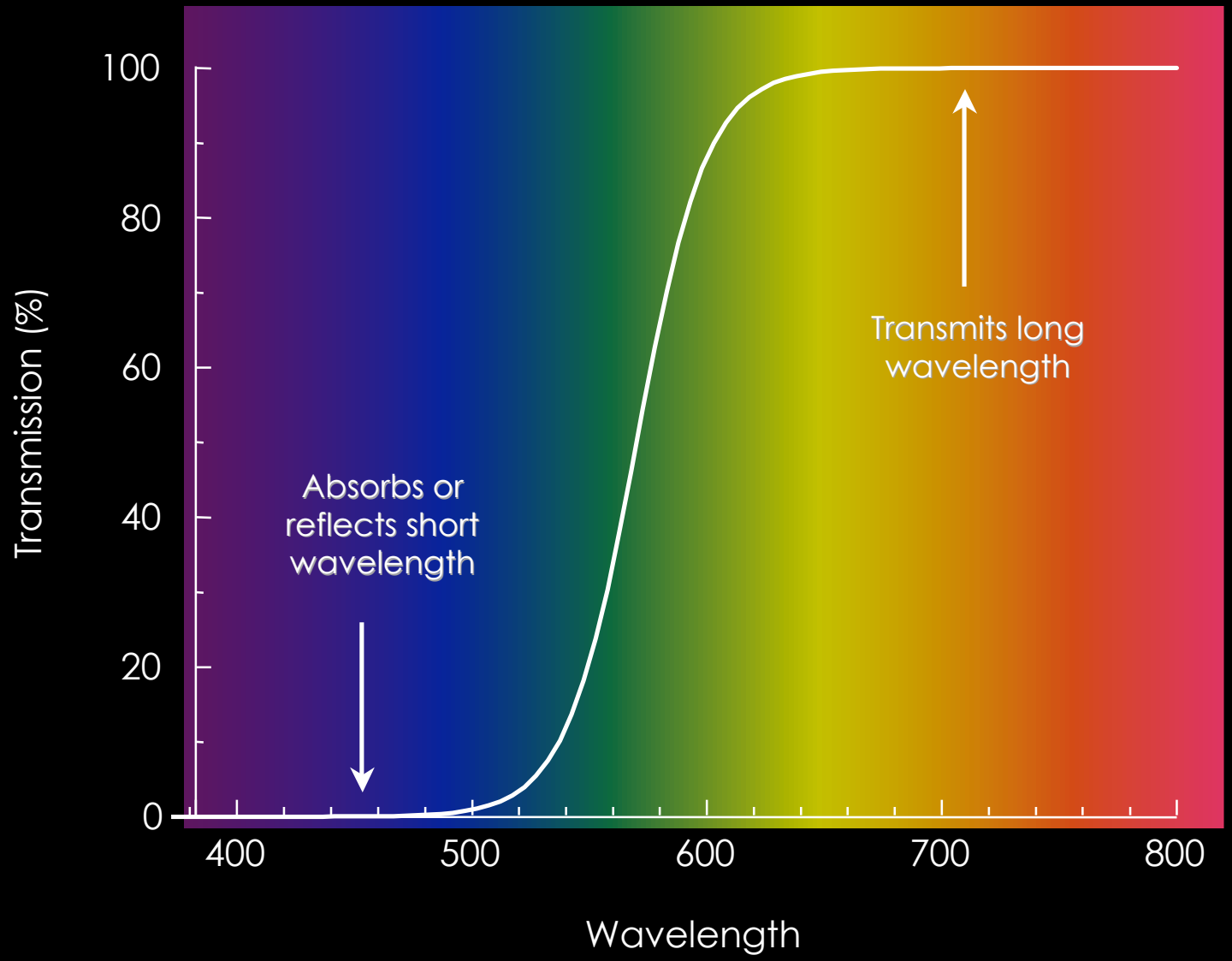

U-MNUA2
OLYMPUS


Excitation Filter
Dichromatic
Mirror (450)
Radiation above 450
Radiation
bellow 450
Objective = Condenser
Excitation radiation

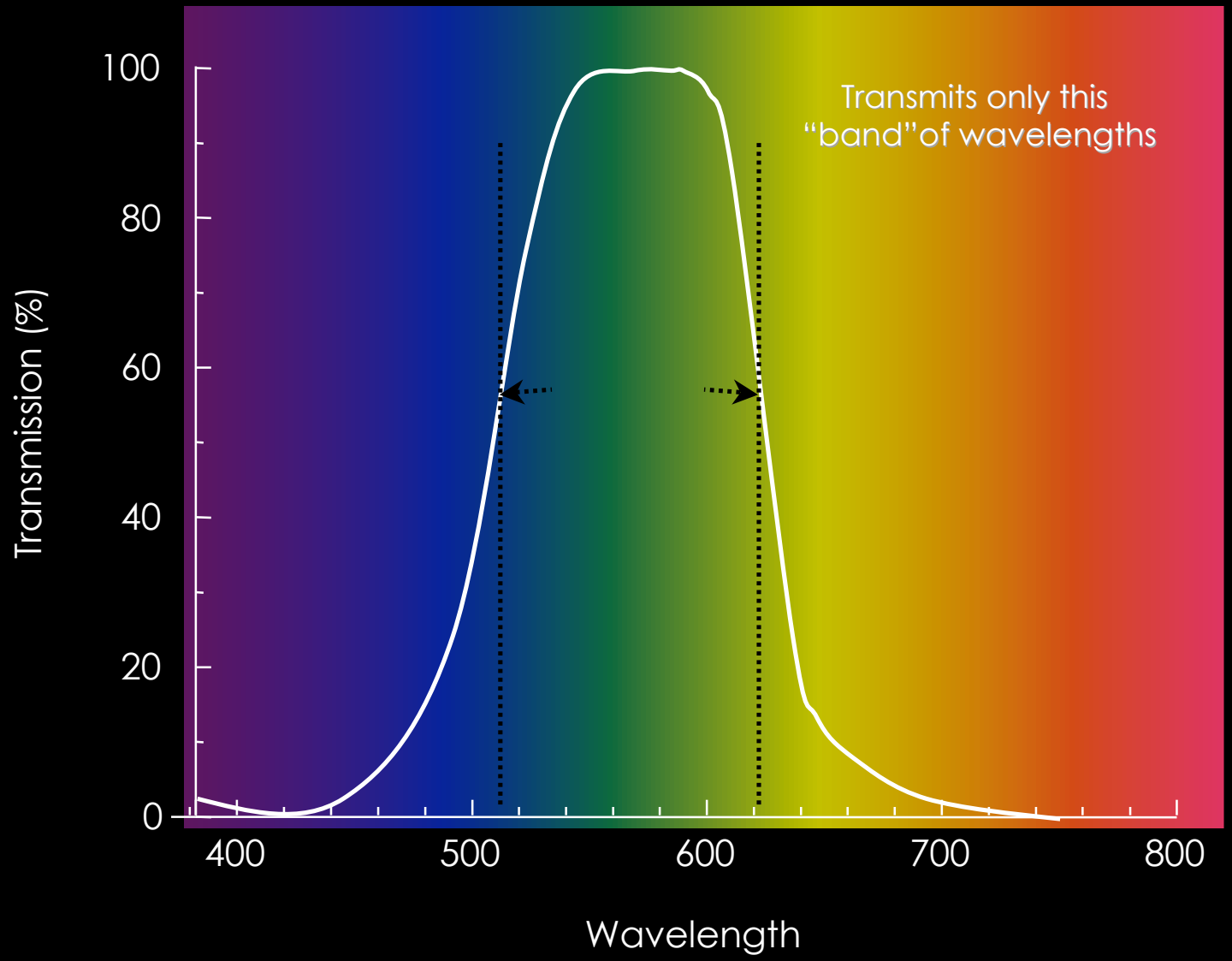

Radiation above 480
Emission filter (LP 480)
Radiation above 450
Dichromatic
Mirror (450)
Radiation bellow 450


Cube
Oculars/Camera
Barrier Filter
Diaphragms
Burner
Dichroic Mirror
Exitation Filter
Objective
Specimen
Fluorochrome

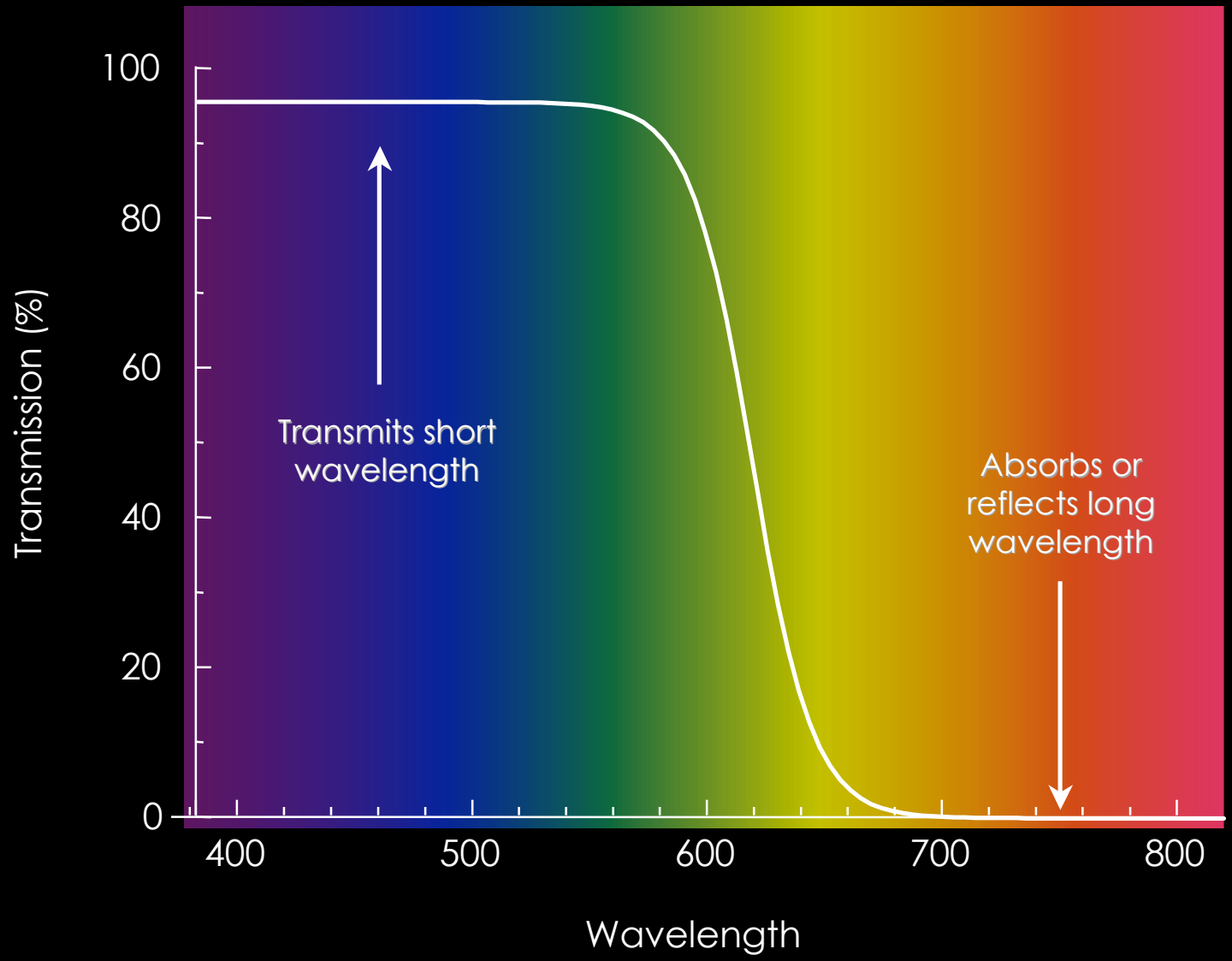
Long Wave Pass Filter (LWP)

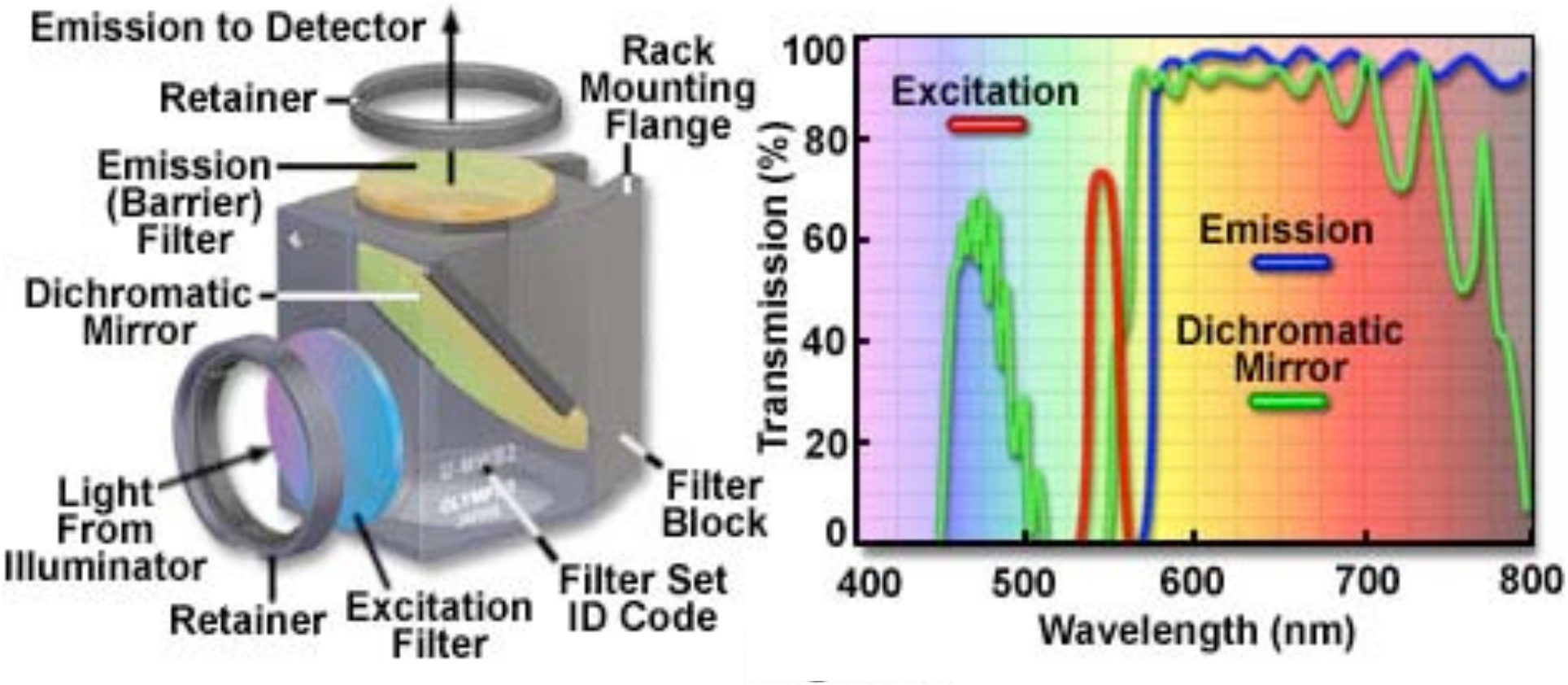


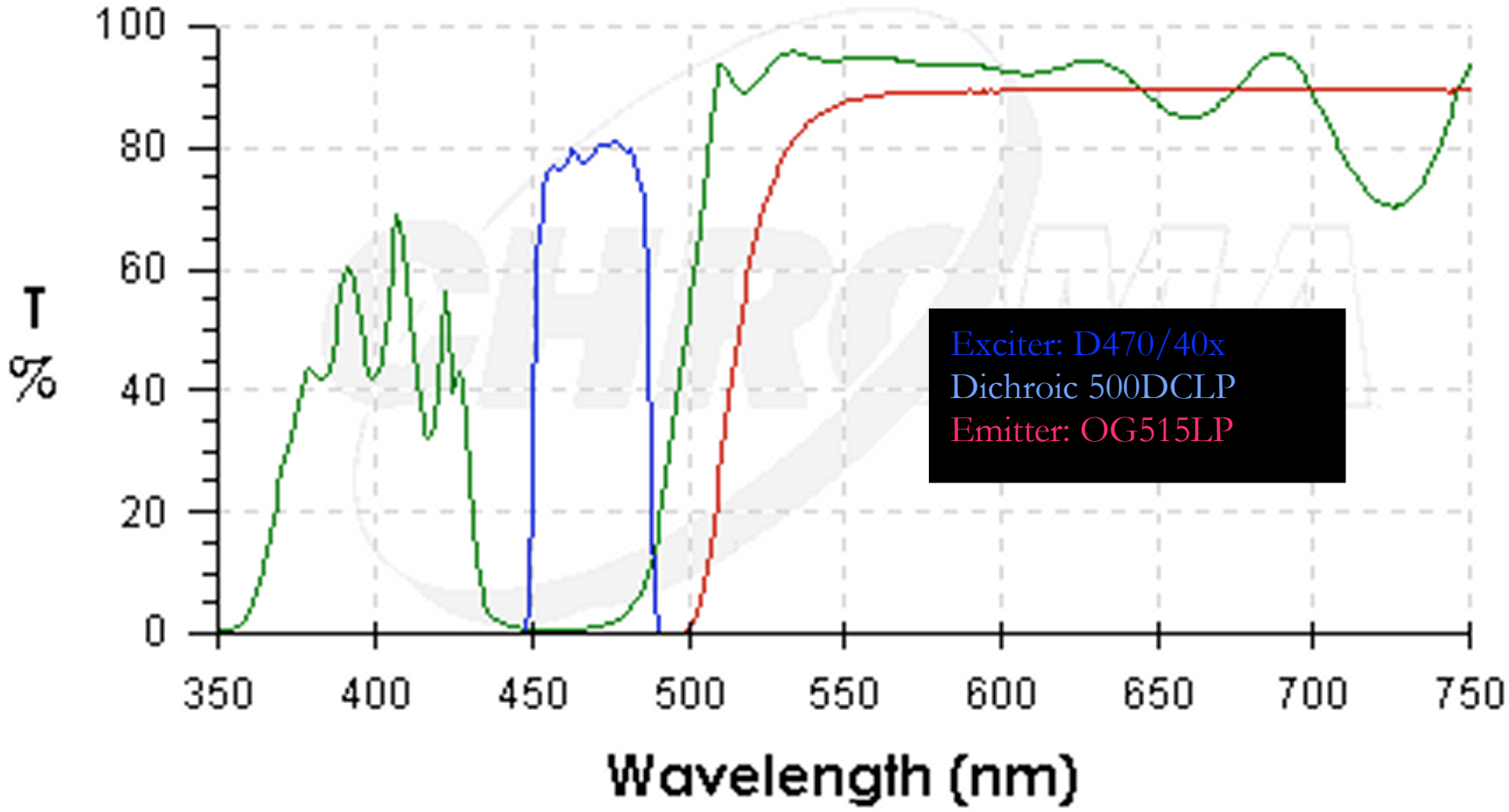
Bandpass Filter

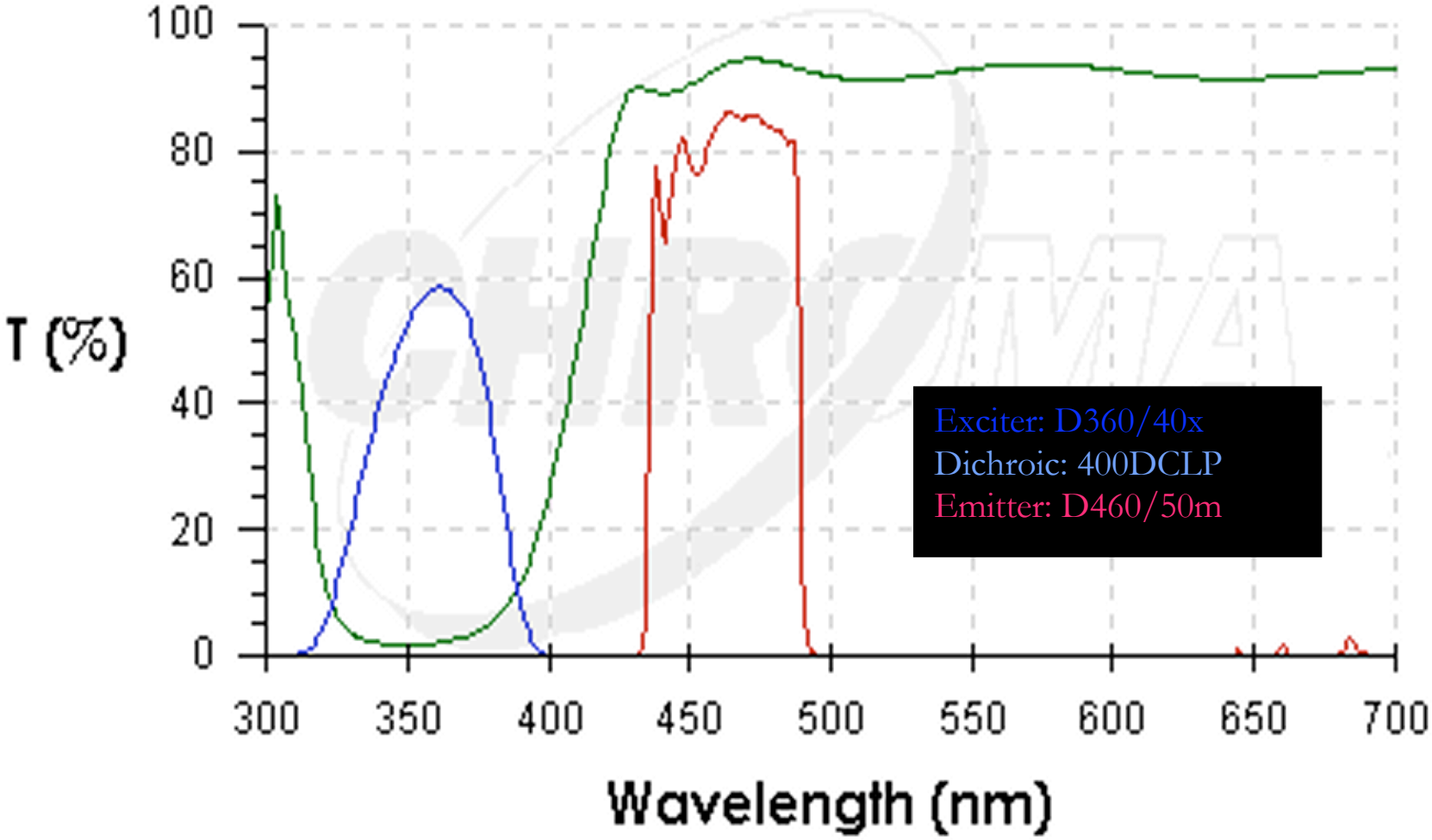


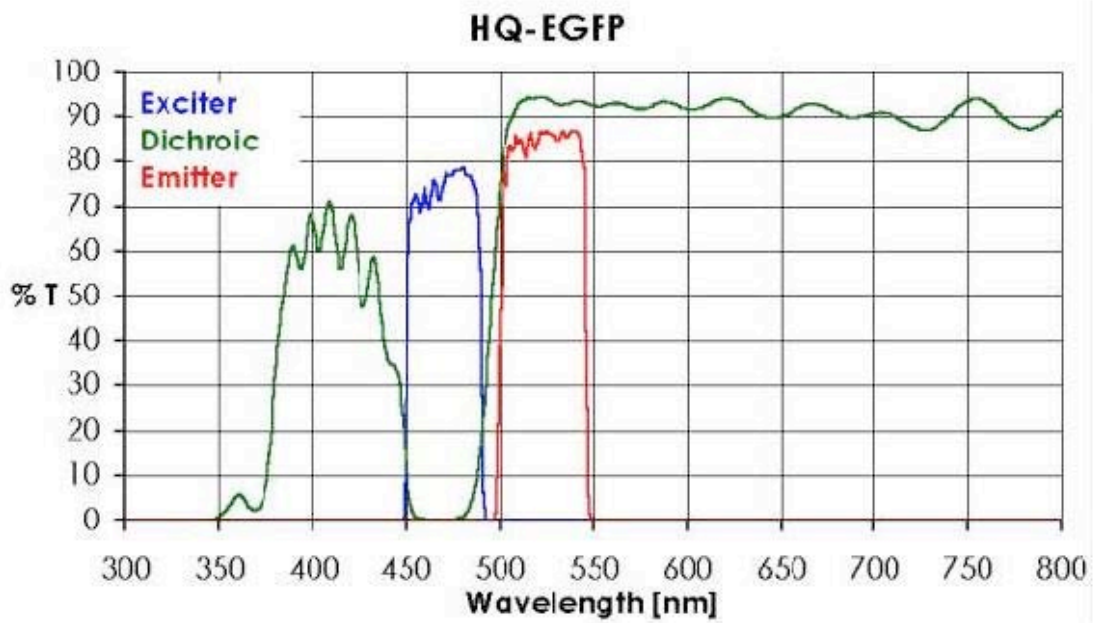
Short Wave Pass Filter (SWP)



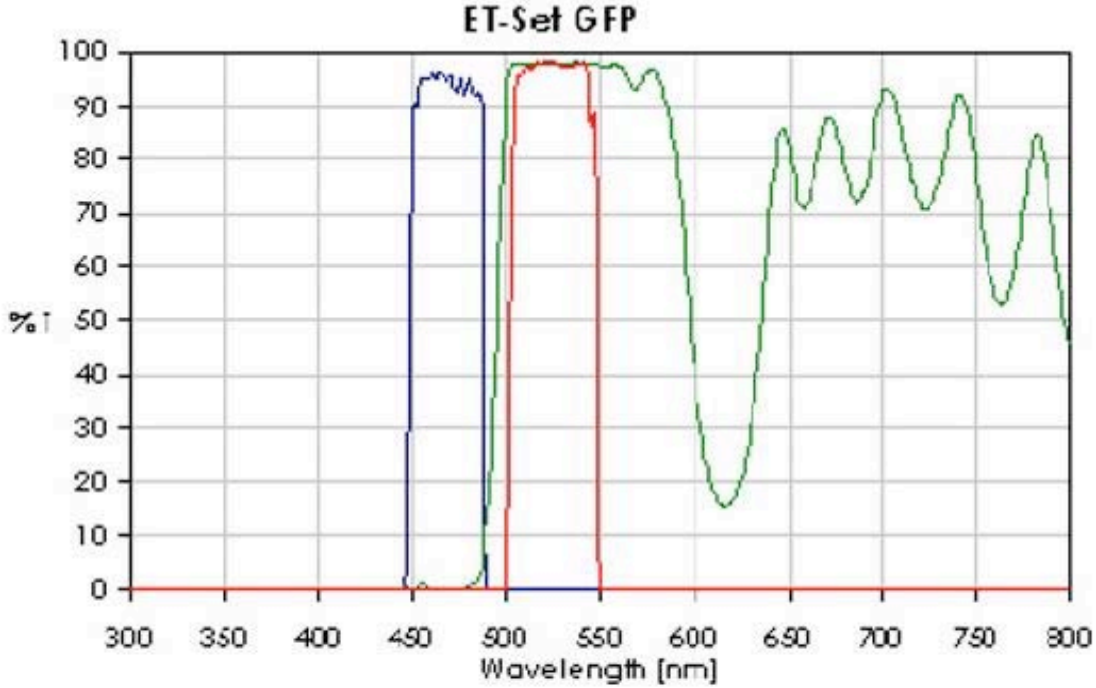

Emission to Detector
Retainer
Rack Mounting Flange
Emission (Barrier) Filter
Dichromatic Mirror
Light From Illuminator
Retainer
Excitation Filter
Filter Set ID Code
Filter Block
Transmission (%)
100
80
60
40
20
0
Excitation
Emission
Dichromatic Mirror
400
500
600
700
800
Wavelength (nm)


100
80
60
40
20
0
T
%
350
400
450
500
550
600
650
700
750
Wavelength (nm)
Exciter: D470/40x
Dichroic 500DCLP
Emitter: OG515LP


100
80
60
40
20
0
T (%)
300
350
400
450
500
550
600
650
700
Wavelength (nm)
Exciter: D360/40x
Dichroic: 400DCLP
Emitter: D460/50m

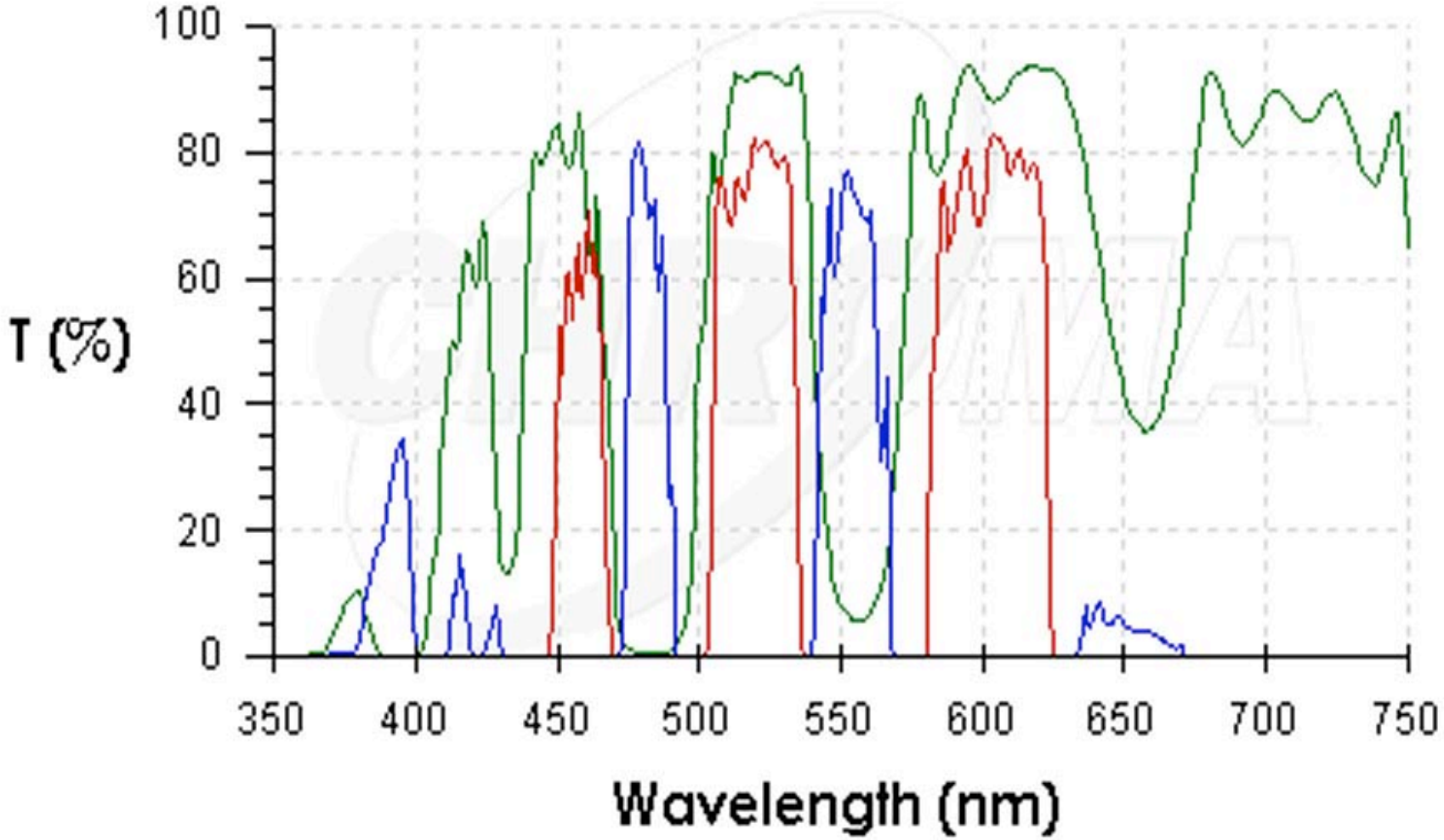


X: 470/40
M: 525/50
BS: 495 LP

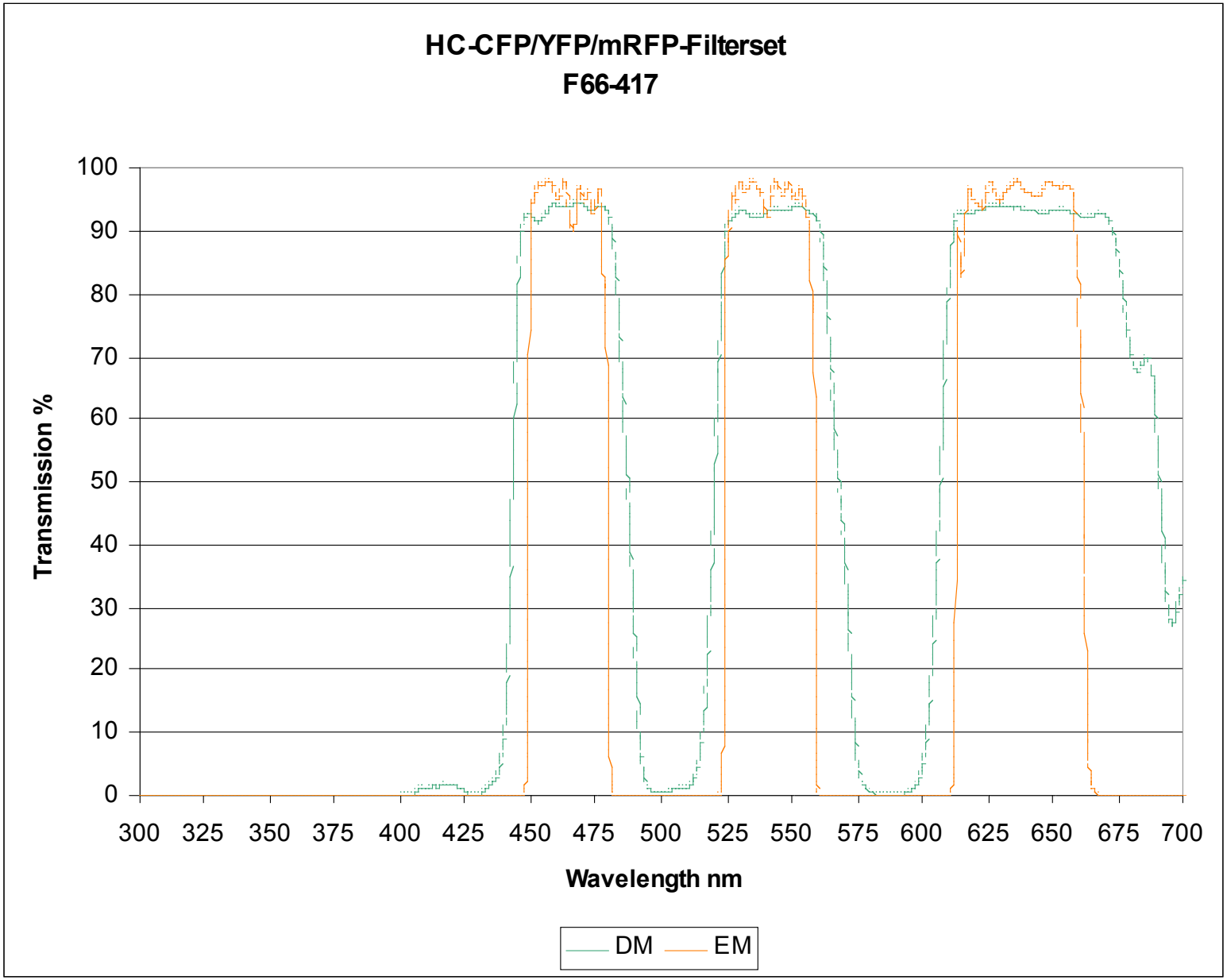


X: 470/40
M: 525/50
BS: 495 LP

(http://www.ahf.de)



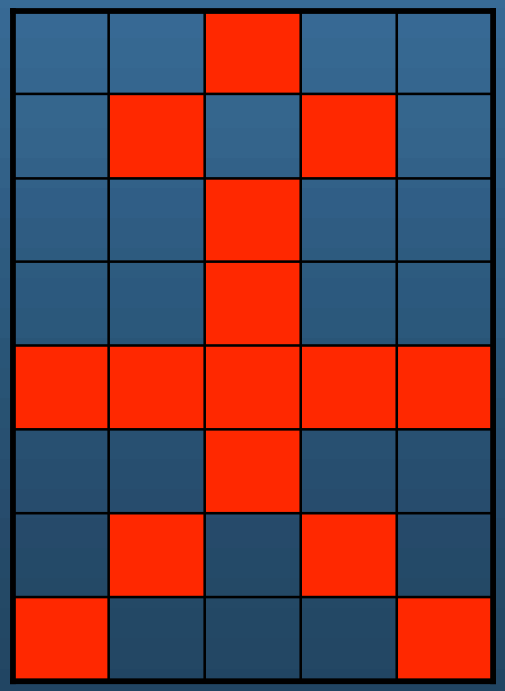
Exciter: 61000v2x Dichroic 61000v2bs Emitter 61000v2m


HC-CFP/YFP/mRFP-Filterset
F66-417
Transmission %
100
90
80
70
60
50
40
30
20
10
0
300 325 350 375 400 425 450 475 500 525 550 575 600 625 650 675 700
Wavelength nm
DM
EM

What is an Image anyway..?

An image is a representation of reality (not real)

- Image of a point is not a point (Point Spread Function)
- Pixelated by detector (CCD or point scanner)



A digital image of ???

Image Analysis (Brain or Computer)

A stick man?
How do I know?
How can computer know? - algorithm?

What is an Image anyway..?

Images contain information (not just pretty pictures)

- Manipulate Image = Changed Info
  (Brightness / Contrast - Extreme Caution!!!)
- Image data can be quantified / measured / analysed
- You cant add lost info back.
- Meta data (What, Where, When, How)

A digital image
How many objects?
How "bright" is it?
How big is it?
What is it?
etc.

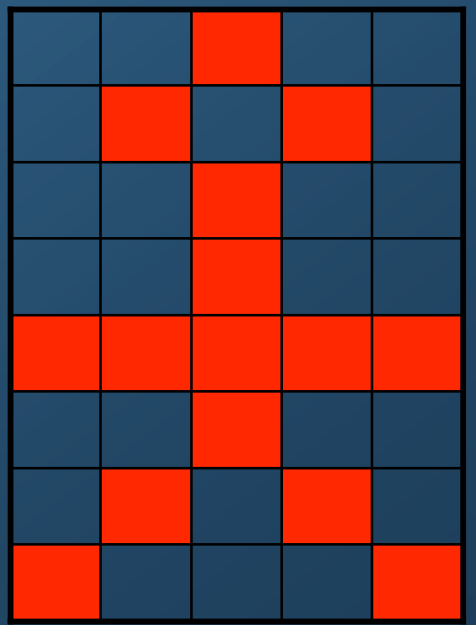
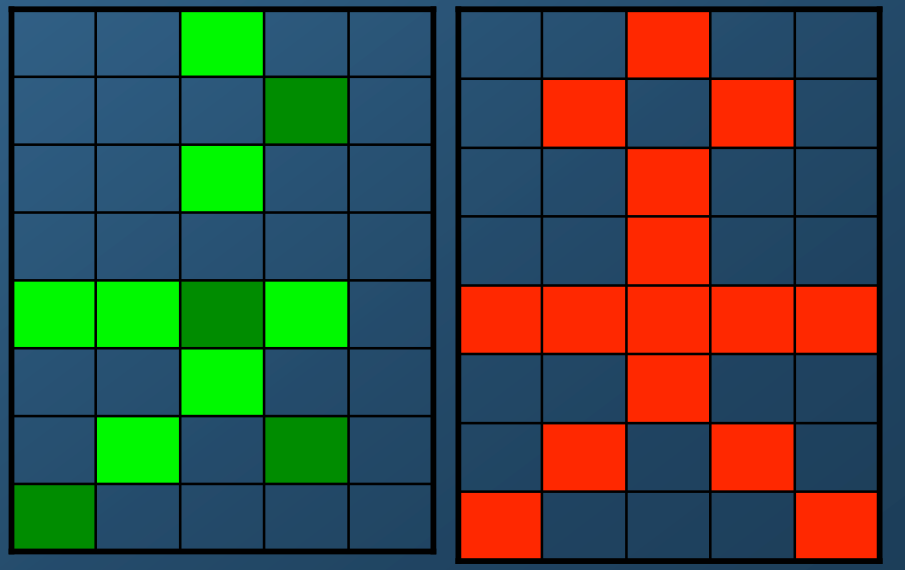


Image Data? What is it?

- Intensity is related to what? Something physical?
  - Dye concentration Or is it? Why not?
    - Noisy Images? Averaging? Pixel Time?
- Comparison of 2 colours/dyes - Biology / BioChemistry / Interaction ?
- Shapes, Movement, Structure?

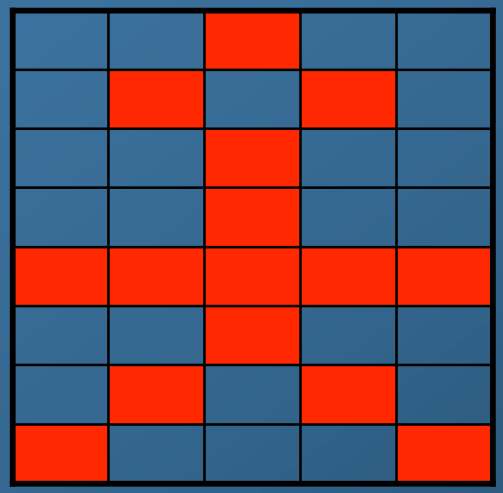
A digital image
With 2 channels / colours

What can you say here?



Photographer or Spectroscopist?

- We can show you how to take pretty pictures (Art)
- We can teach you to get useful information (Science)
- You have to choose which you want to be! (Both is OK)



This

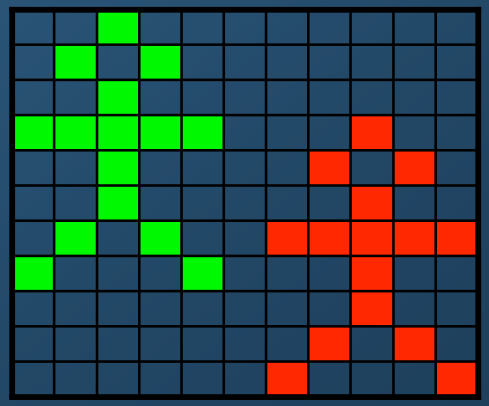
Is simply a way to "Visualise"

This

0	0	1	0	0
0	1	0	1	0
0	0	1	0	0
0	0	1	0	0
1	1	1	1	1
0	0	1	0	0
0	1	0	1	0
1	0	0	0	1

Quantitative Image Analysis?
…what does that mean?

- Pretty pictures are great for journal covers...
- Movies are great for visual presentation of images...
- Interactive 3D visualisation, data exploration...
- But for meaningful biological conclusions...
  - Scientists need numerical results from image data
  - Need to measure many objects
  - Need statistics from many images
  - Computers become useful!



Quantitative Microscopy - First Think...

- Choosing experimental and image processing methods:
  - What BIOLOGY am I trying to see or measure?
  - Do I need 3D information? Resolution? Object size?
  - Choose / Optimise microscope system to use!
  - Statistics? How many images / data points / experiments?
  - **Controls!!!**

- Signals within the range of the detector?
  - Your eyes lie! You can't see low intensities close to black! Use Range Indicator / HiLo / OU and spectrum CLUTs
  - Adjust so brightest part is within detector range.
  - Remember to check z dir. also.
  - Don't over expose the image! Why not? Lost Info!

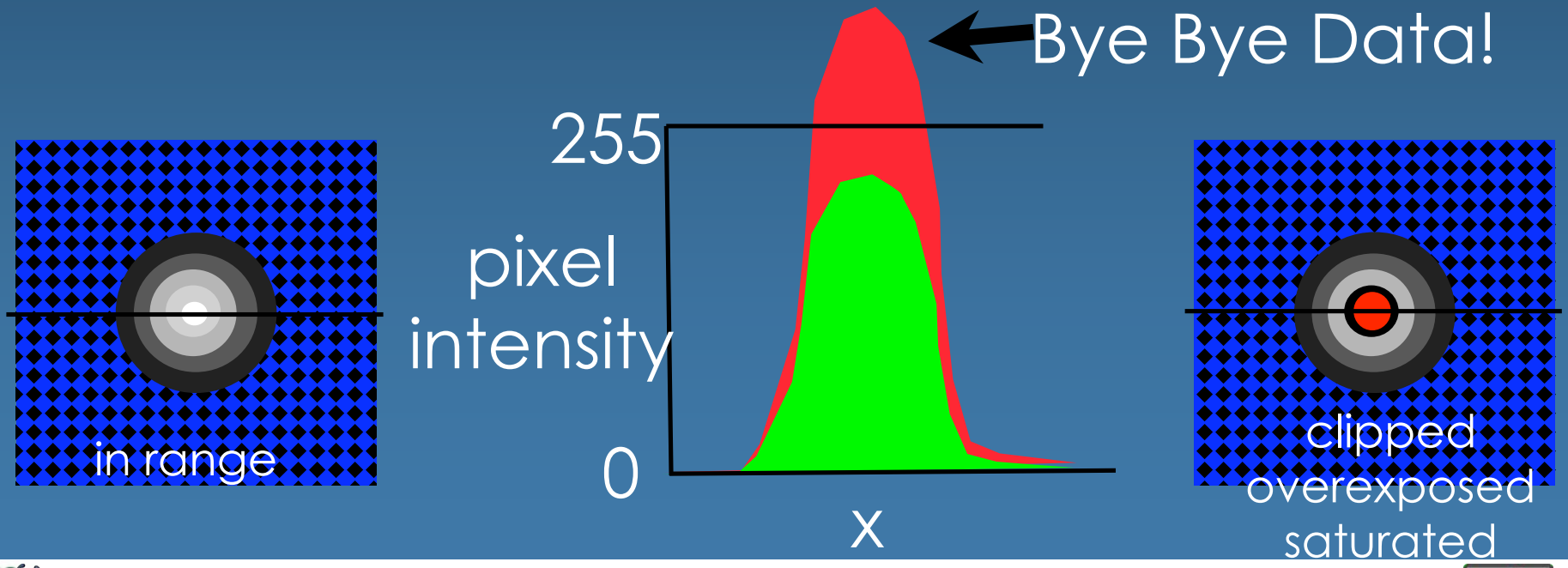
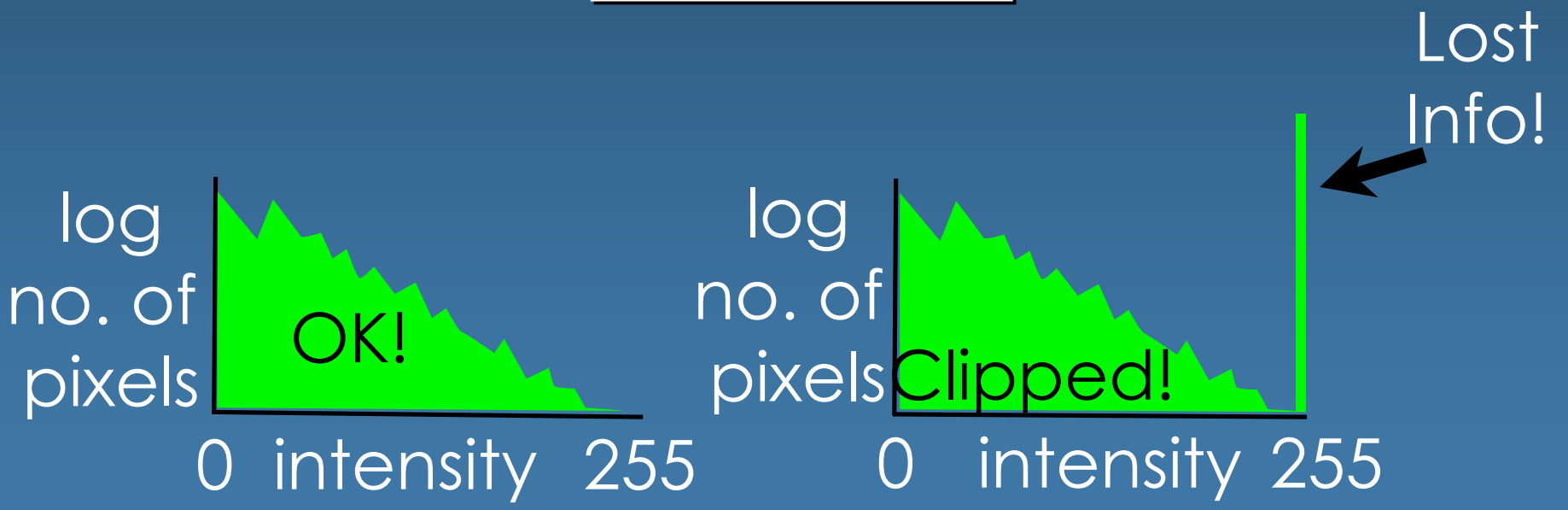


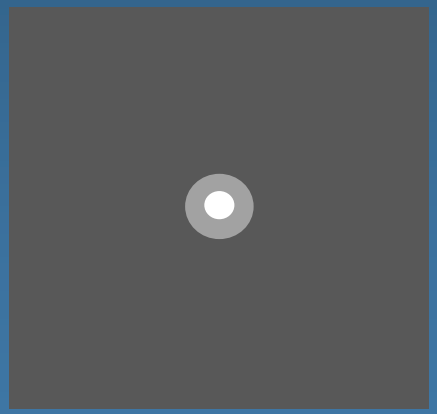
Image Histograms are your friends!

Use them!

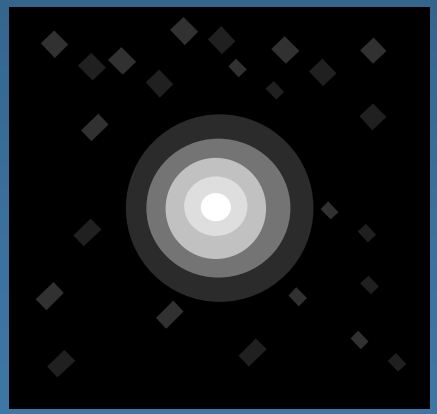


- Signal within the range of detector?
  - Offset / Zero Background - Set properly.
  - Why? "background" » zero, but keep low intensity info
    - What is "Background"? You decide!
  - Range indicator / HiLo CLUT

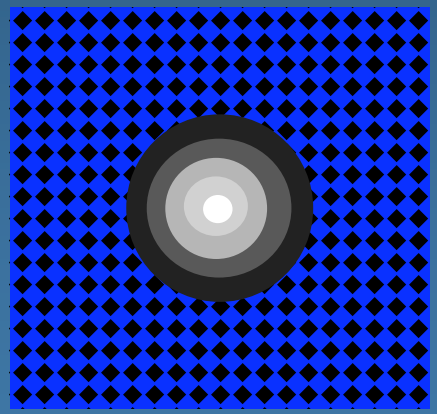
    background black and blue ~50:50
  - (0 = Blue, 1 = Black, 254 = White, 255 = Red)



too high



too low

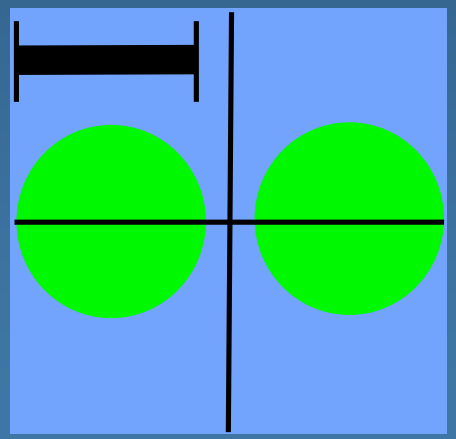


correct

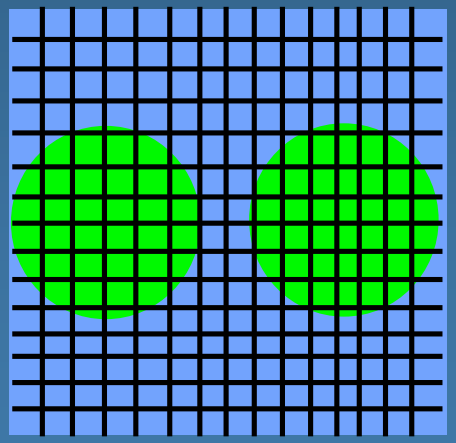
Pixel Size / Resolution

- "Correct" image size? (64x64, 512x512, 2048x2048)?
  - Get all information microscope can resolve, but files not too big
  - Proper spatial sampling (Nyquist sampling theory)
  - 2.3-3 pixels over optical resolution distance. (x, y and z)
  - Adjust zoom and image size.
  - Auto Pinhole or 1 Airy unit

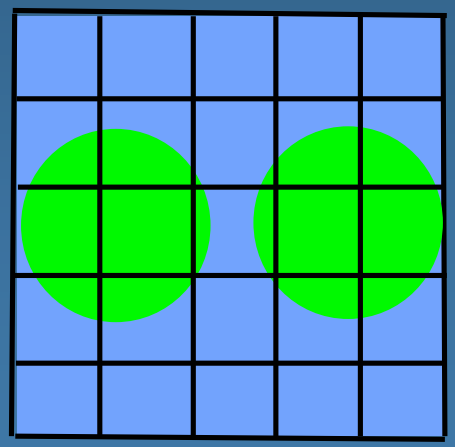
1 Airy unit



under sampled



over sampled

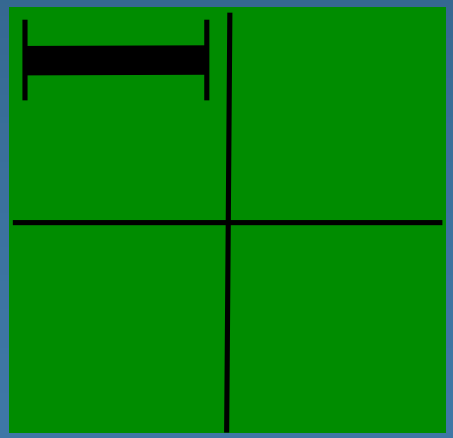


correct sampling

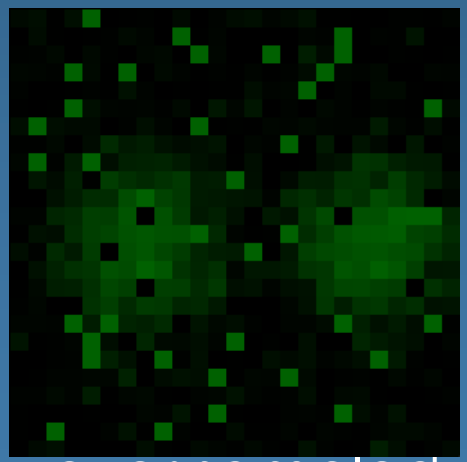
Pixel Size / Resolution

- "Correct" image size? (64x64, 512x512, 2048x2048)?
  - Get all information microscope can resolve, but files not too big
  - Proper spatial sampling (Nyquist sampling theory)
  - 2.3-3 pixels over optical resolution distance. (x, y and z)
  - Adjust zoom and image size.
  - Auto Pinhole or 1 Airy unit

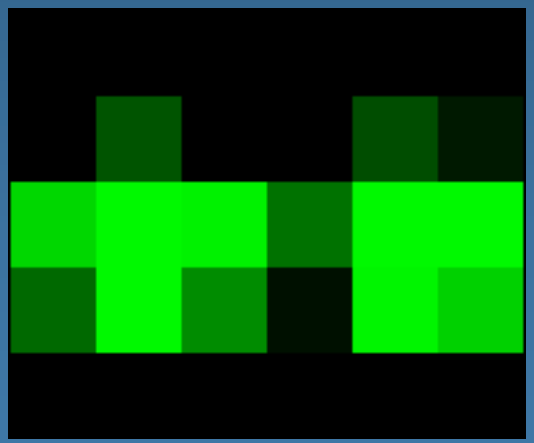
1 Airy unit



under sampled



over sampled



correct sampling