Principles of Fluorescence

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Resources courtesy from Dr.Santiponse Chatchavalvanich

CLO

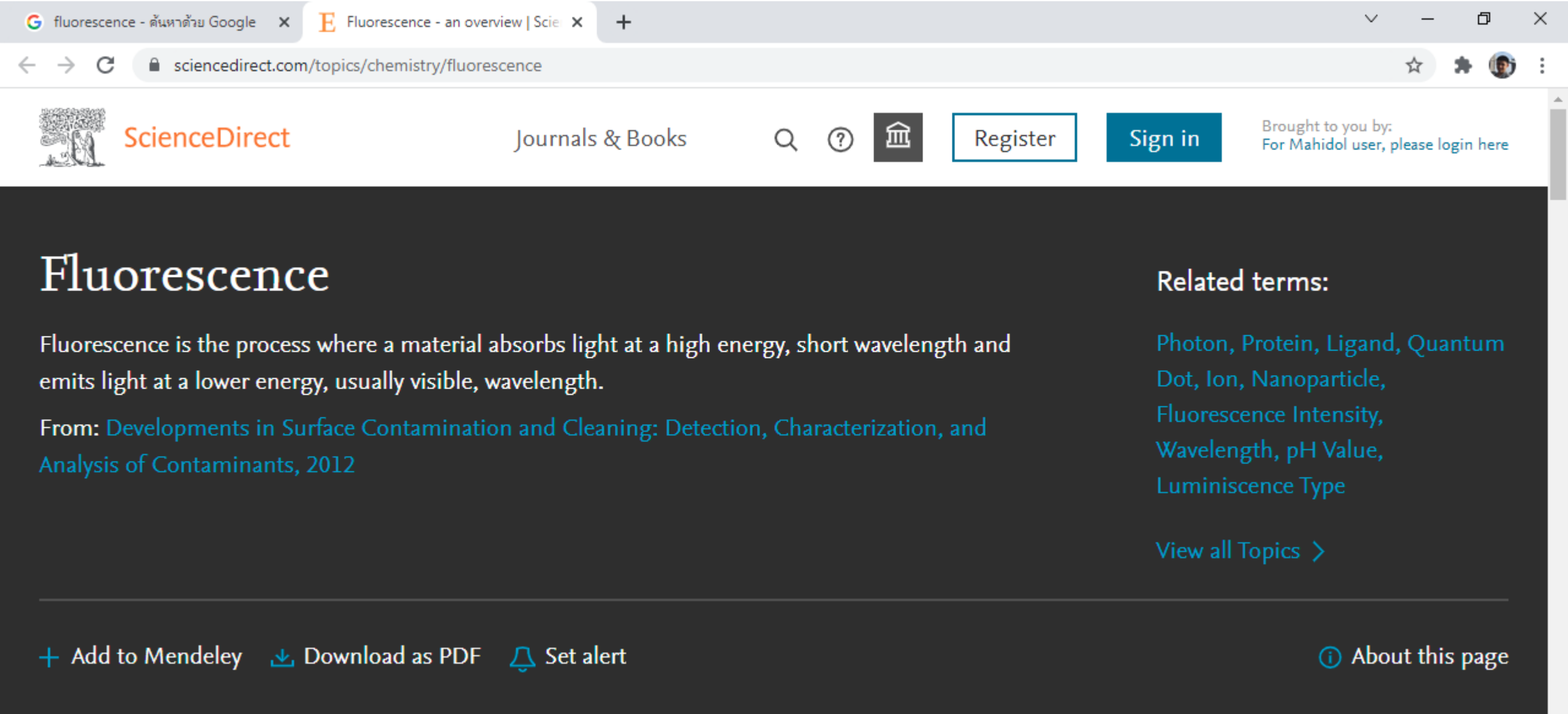
1. Explain the following aspects of experimental methods used in physiological research: Principles, Methods, Interpretation, Advantages and Disadvantages
2. Apply the knowledge to choose appropriate methods in experimental designs and appraise the choice of methods employed in literatures
3. Participate in discussion of designing or appraising physiological experiments in literatures

Objectives

- Describe principles of fluorescence
- Select appropriate fluorochromes
- Select suitable fluorescent applications

Mahidol University
Faculty of Medicine
Siriraj Hospital

What is fluorescence?

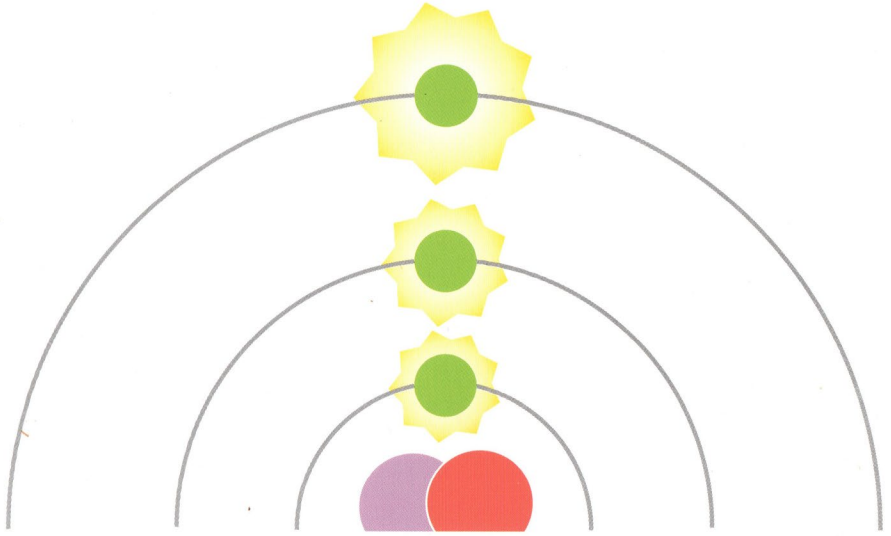


Quantum physics

All balls are made of atoms.

Electron energy level

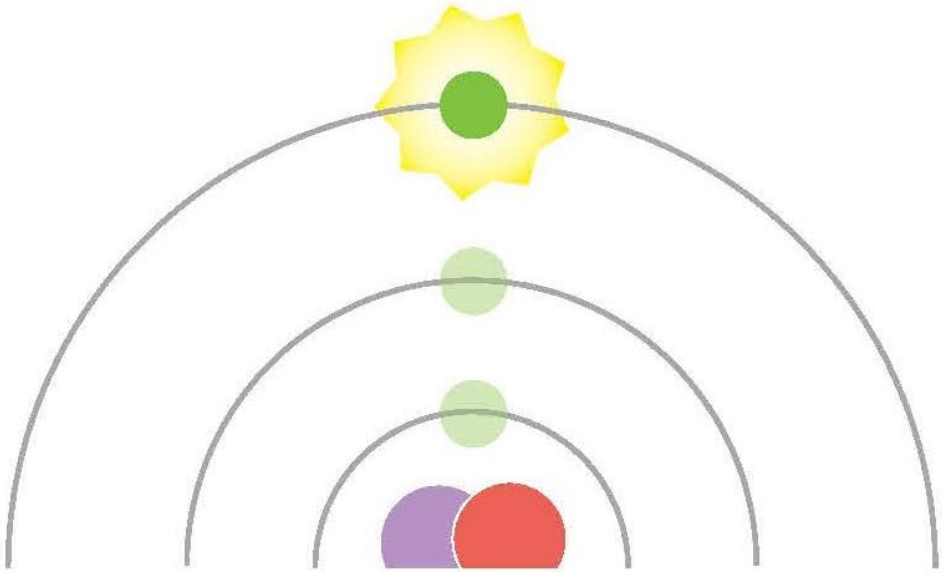
- At atomic level, electrons move around the nucleus in orbits/shells



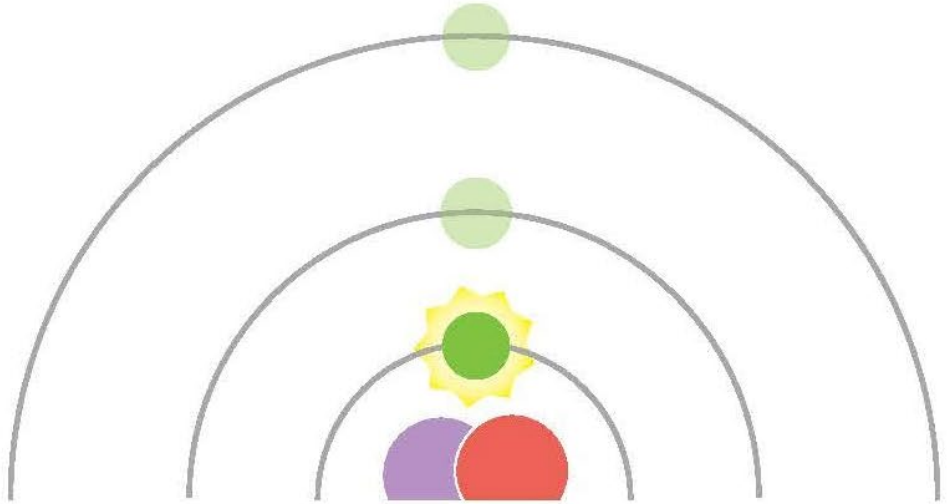
All electrons have energy.

Electron energy level

- Each orbit has its own specific energy level



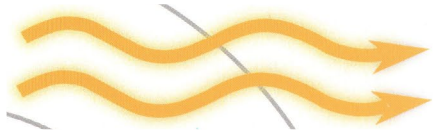
This electron has the most energy.



This electron has the least energy.

Electron energy level

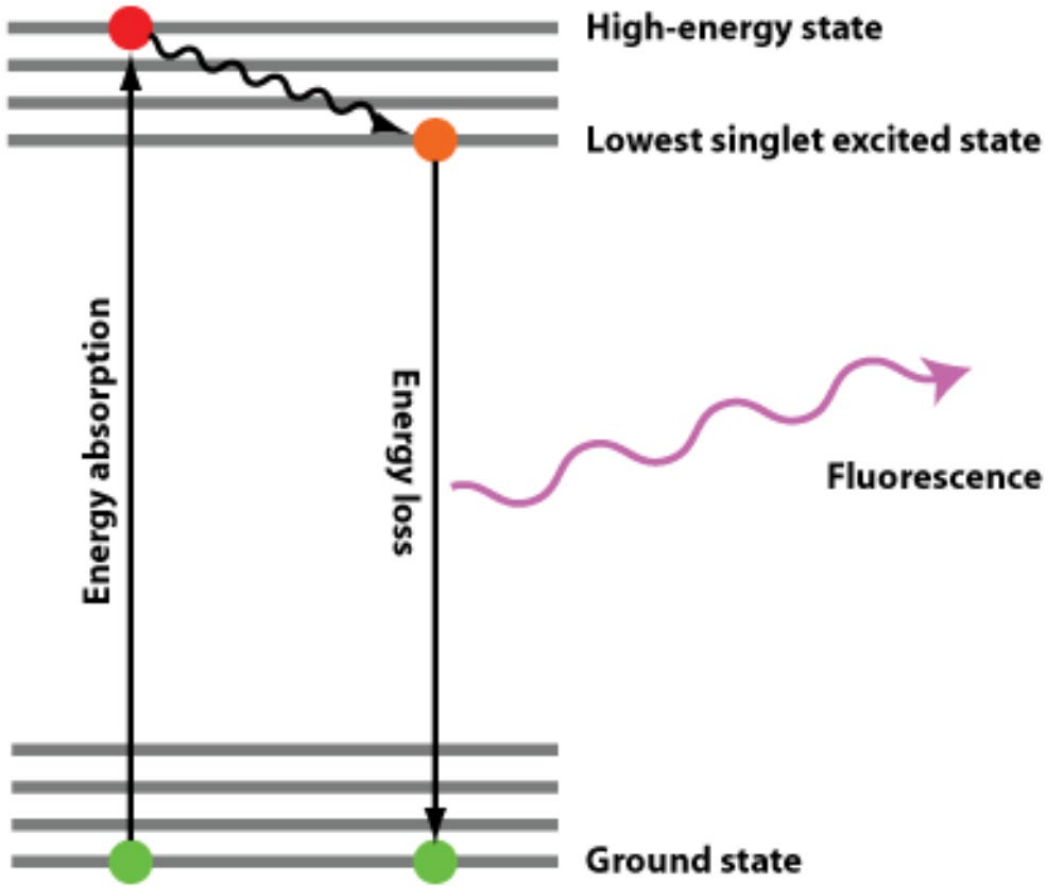
- Electrons can move between orbits by receiving or releasing energy

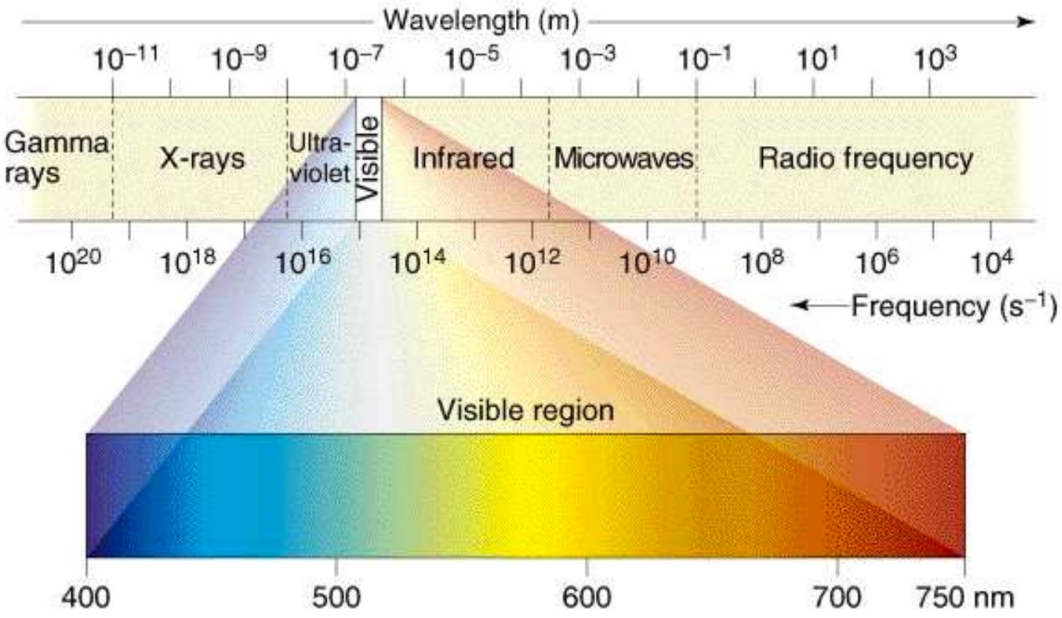


This amount of energy is a quantum.

What is fluorescence?

= Energy output emission from electrons movement

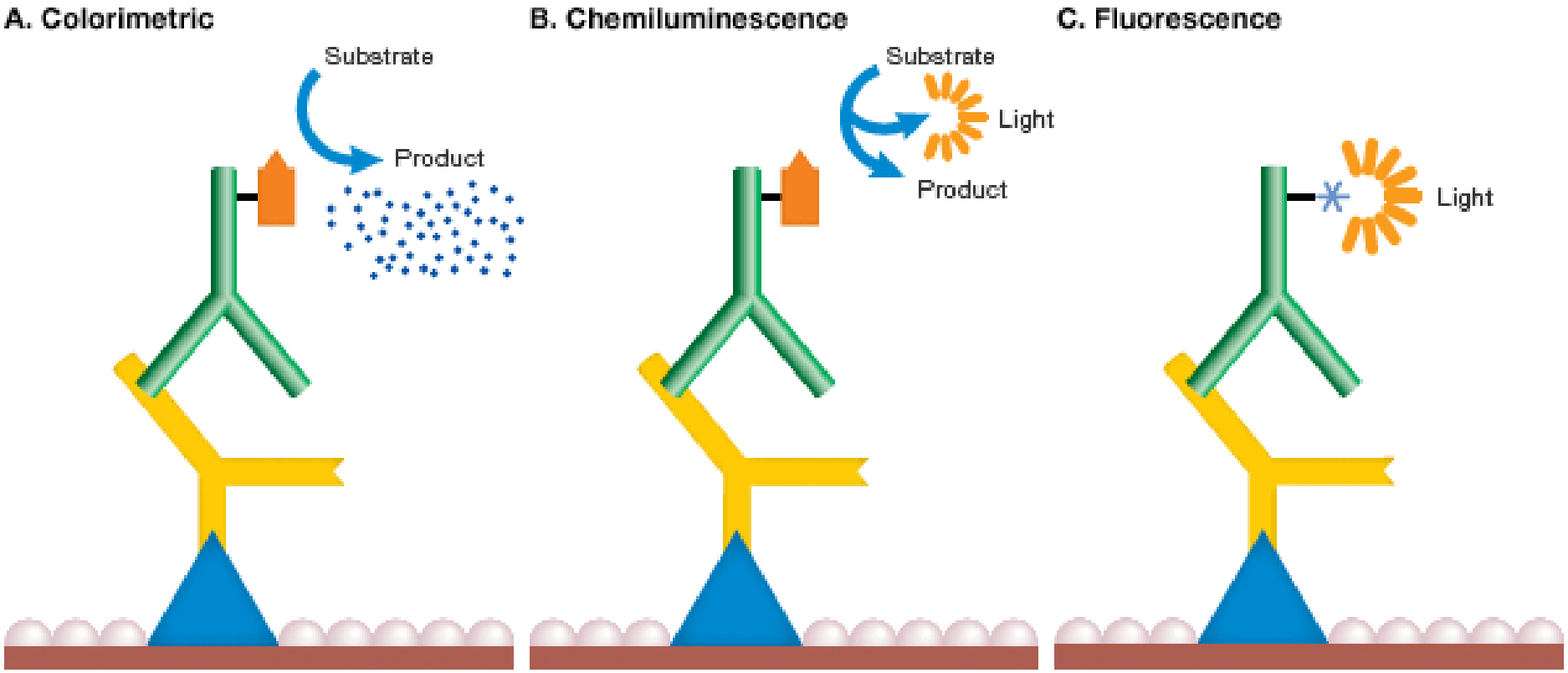




Why fluorescence?

1. Light-based detection system
   - Machine for light detection is well developed
     - CCD camera
     - Filters
   - Examples
     - Colorimetric (Color absorbance)
     - Luminescence
     - Fluorescence
2. Specificity

Light-based detection method



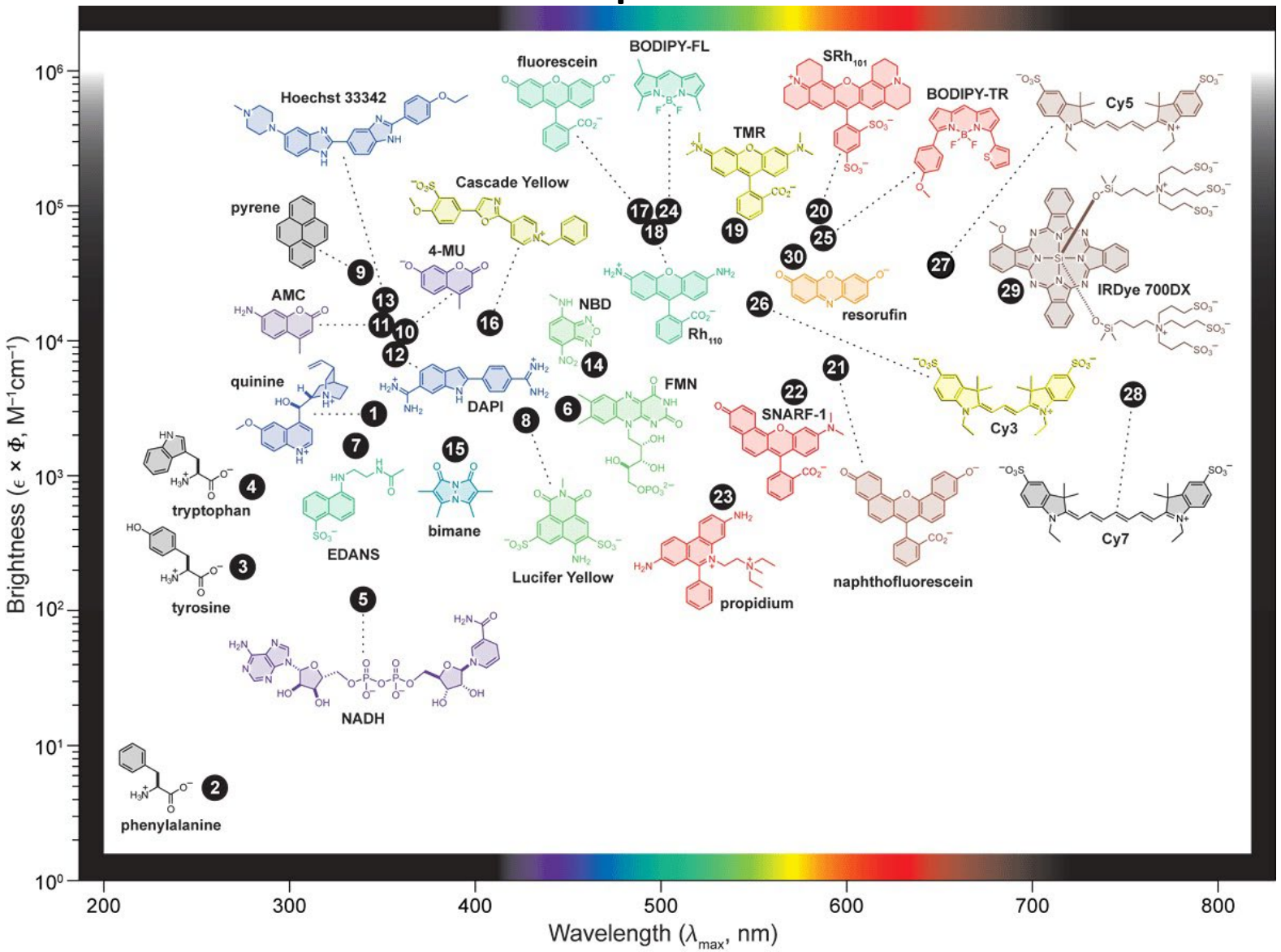
https://www.bio-rad.com/en-th/applications-technologies/detection-methods?ID=LUSQ6KKG4

Fluorochromes/Fluorophores

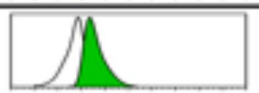
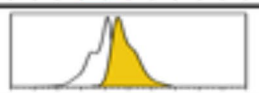
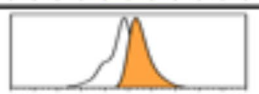
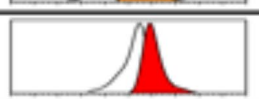
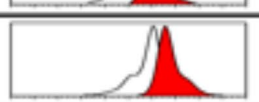
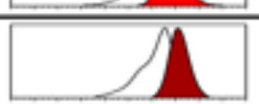
- Materials with fluorescent property

- Proteins
  - GFP, YFP, CFP, RFP, Td-Tomato, …
- Non-proteins
  - Fluorescein, Rhodamine, Texas Red, Alexa Fluoro, …

Fluorochromes/Fluorophores



Fluorochomes/Fluorophores

Spectral Properties of DyLight Fluorescent Dyes					
Emission	Fluor	Ex/Em†	See Spectra	ε††	Spectrally Similar Dyes
Blue	DyLight 350	353/432		15K	Alexa Fluor® 350, AMCA
Blue	DyLight 405	400/420		30K	Alexa Fluor 405, Cascade Blue®
Green	DyLight 488	493/518		70K	Alexa Fluor 488, fluoresœin, FITC
Yellow	DyLight 550	562/576		150K	Alexa Fluor 546, Alexa Fluor 555, Cy3®, TRITC
Red	DyLight 594	593/618		80K	Alexa Fluor 594, Texas Red®
Red	DyLight 633	638/658		170K	Alexa Fluor 633
Red	DyLight 650	652/672		250K	Alexa Fluor 647, Cy5®
Near IR	DyLight 680	692/712		140K	Alexa Fluor 680, Cy5.5®
Near IR	DyLight 750	752/778		210K	Alexa Fluor 750
Infrared	DyLight 800	777/794		270K	IRDye® 800

†Excitation and emission maxima in nanometers (+/- 4nm) in phosphate-buffered saline (PBS)
††Molar extinction coefficient (M^{-1} cm^{-1}) at the absorption maximum

DAPI, Hoechst

Pacific Blue

AF488, GFP, FITC, PE

AF555, Cy3

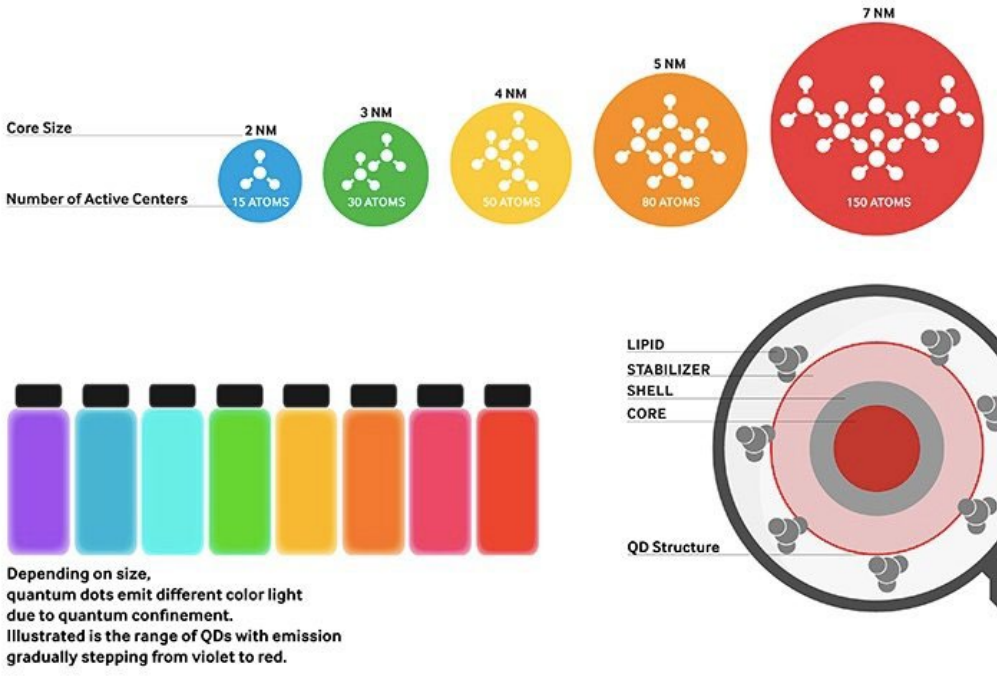
Texas Red

APC, Cy5

Cy5.5

Cy7

Quantum dots

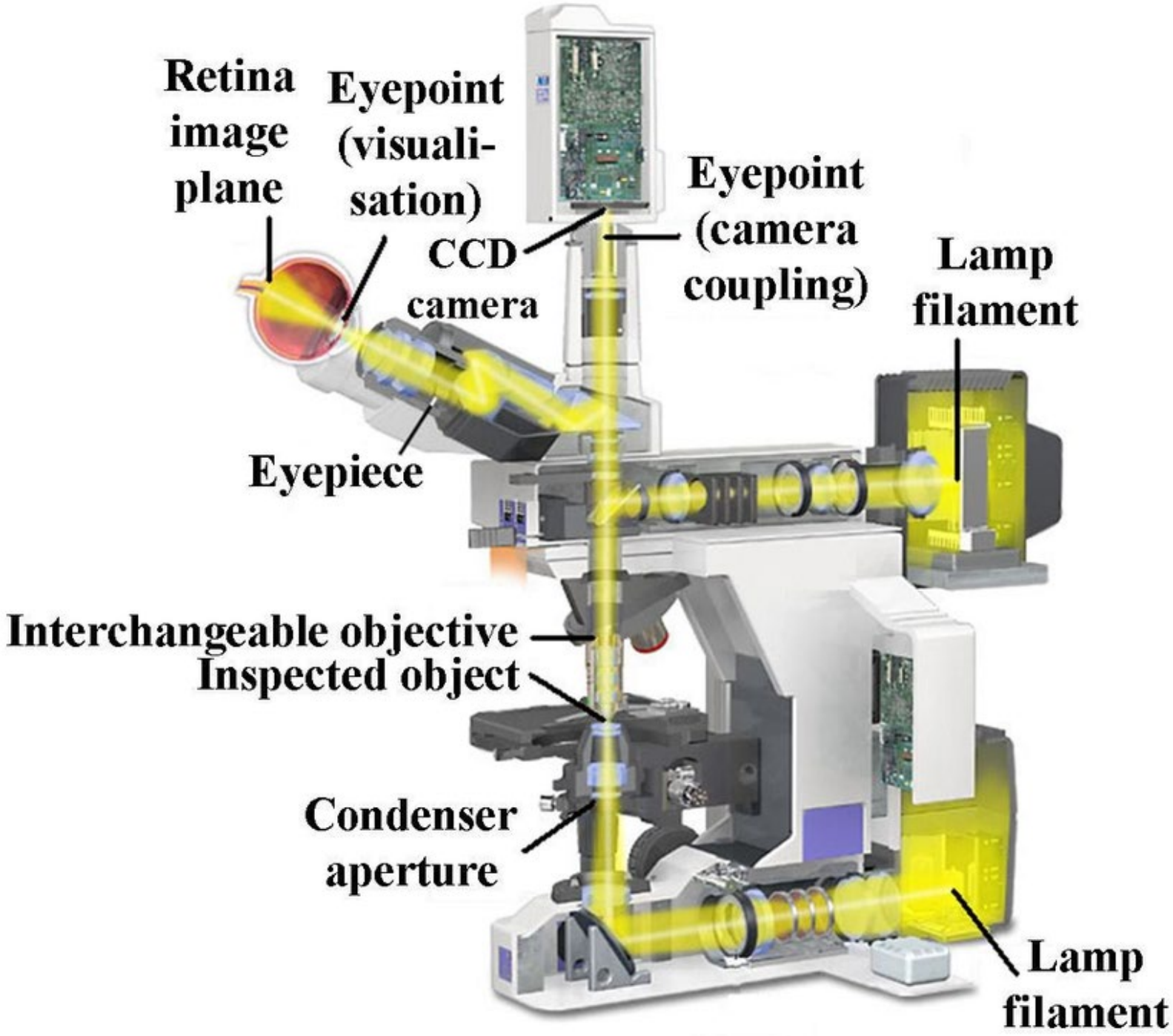


Depending on size,
quantum dots emit different color light
due to quantum confinement.
Illustrated is the range of QDs with emission
gradually stepping from violet to red.

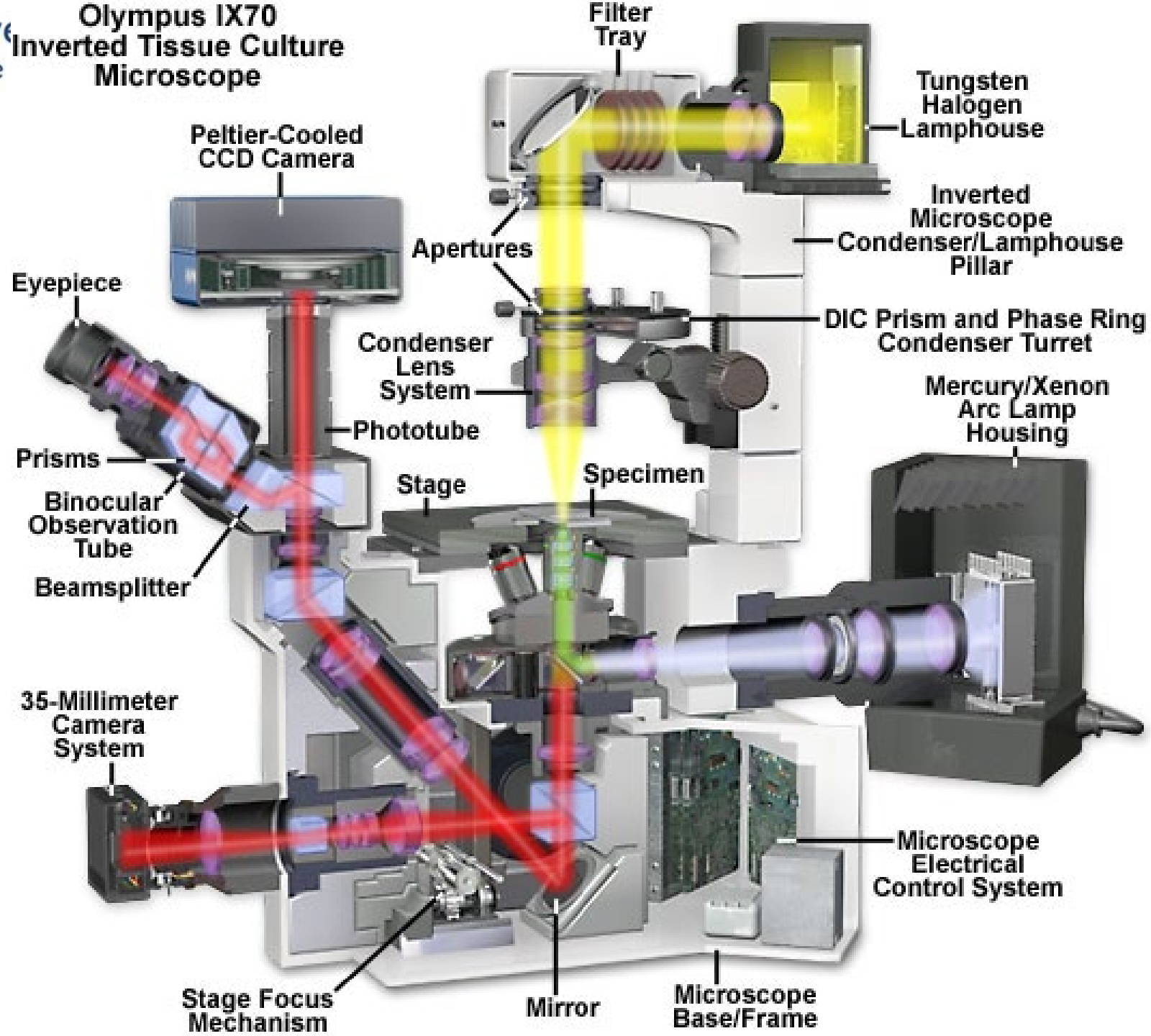
What should we do if we want to work with fluorescence?

Example:

- Protein detection in tissue samples using fluorescence dyes
- Immunohistochemistry / Immunofluorescent staining
  - (Lecture 12: Principles of histological and imaging studies)
- Solution:
  - Microscope → Fluorescent microscope


Retina image plane
Eyepoint (visualisation)
CCD camera
Eyepoint (camera coupling)
Lamp filament
Eyepiece
Interchangeable objective
Inspected object
Condenser aperture
Lamp filament

Olympus IX70 Inverted Tissue Culture Microscope



Example

- Protein detection in tissue samples using fluorescence dyes

- Fluorescent microscope
  - Which fluorophore should be used?
  - Check the microscope specs!!

Olympus IX70 Inverted Phase Contrast DIC Fluorescence Microscope

Olympus | ☆ ☆ ☆ ☆ ☆ Write a review

SKU: IX70-DIC

$14,750.00

Qty 1

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Description

This Olympus IX70 microscope is capable of phase contrast, DIC, and fluorescence. It comes with an ergonomic tilting binocular head with 10x eyepieces. The microscope has a front and side photo port for adding a camera. The 0.5 N.A. condenser has DP10 and DP20 DIC prisms and Ph1, PhC phase annuli installed with the IX-LWPO polarizer. The 6 position nosepiece has UPlanFLN 4x PhL, UPlanFL 10x, CPlan 10x PhC, LCPlanFL 20x Ph1 w/ CAP P 1.1, and LCPlanFL 20x w/ CAP P 1.1.. The 5 position fluorescence turret contains WIBA, and NU fluorescence filters. Also included is the IX-AN analyzer and U-DICT DIC prism. The microscope has a built in 1.0x / 1.5x mag changer. This refurbished microscope can be fully customized and comes fully serviced with a warranty.

SPECIFICATIONS	
Head:	Ergo Tilting Binocular Front & Side Photo Port

Head:	Front & Side Photo Port
Eyepieces:	10x Eyepieces
Nosepiece:	6 Position
Objectives:	UPlanFLN 4x PhL UPlanFL 10x CPlan 10x PhC LCPlanFL 20x Ph1 - CAP-P 1.1 LCPlanFL 20x - CAP-P 1.1
Stage:	XY Mechanical
Condenser:	0.55 N.A. w/ IX-LWPO DP10, DP20, Ph1, Phc
Fluorescence	Mercury Lamphouse & Power Supply 5 Position Fluorescence Turret WIBA, NU Filters

Example

- Protein detection in tissue samples using fluorescence dyes

- Fluorescent microscope
  - Which fluorophore should be used?
  - ~~Check the microscope specs!!~~
  - Fluorescent system(s) equipped with the microscope

- 1x C-mount adapter

Camera:

- Bestscope 8.3MP color CMOS camera

Computer & Software:

- Dell laptop computer with Windows 10
- ImageJ analysis software
- Camera software

Accessories:

- Necessary power and communication cables

This configuration includes any 3 of these new filter sets.

- New filter set - DAPI (EX: 365/28nm, DM: 405nm, EM: 445/50nm) (Dye compatibility: DAPI/Hoechst/AlexaFluor 350)
- New filter set - EGFP/FITC/Cy2 (EX: 475/30nm, DM: 500nm, EM: 530/40nm) (Dye compatibility: EGFP/FITC/Cy2/AlexaFluor 488)
- New filter set - TRITC/Cy3/Dsred (EX: 545/20nm, DM: 562nm, EM: 605/60nm) (Dye compatibility: TRITC/Cy3/RFP/Rhodamine/AlexaFluor 546)
- New filter set - Texas Red (EX: 560/20nm, DM: 585nm, EM: 630/40nm) (Dye compatibility: Texas Red/mCherry/AlexaFluor 594)
- New filter set - Cy5 (May require an upgraded light source) (EX: 630/50nm, DM: 662nm, EM: 695/50nm) (Dye compatibility: Cy5/AlexaFluor 647/Draq5)

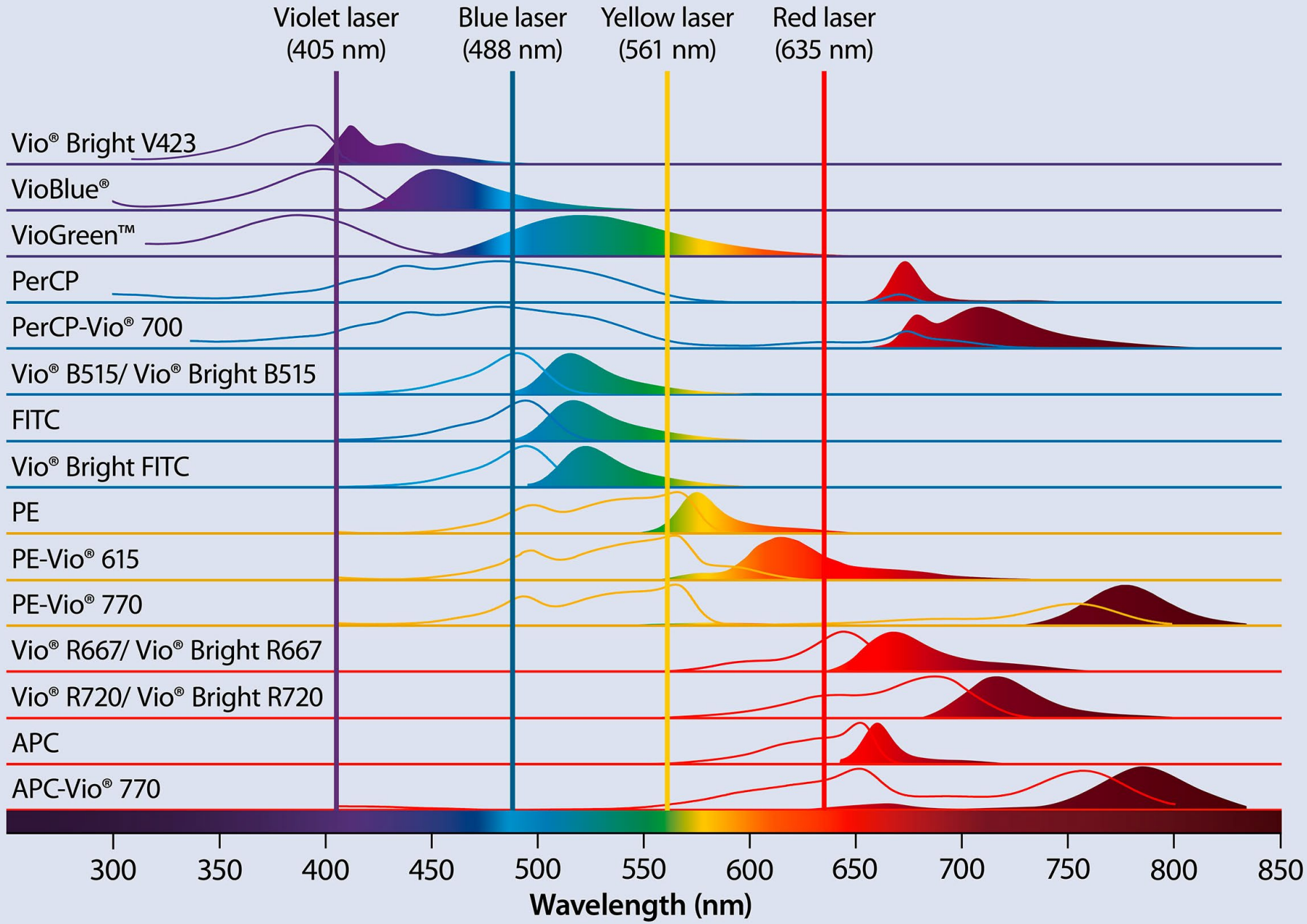
This microscope's imaging capabilities:

Imaging software highlights:

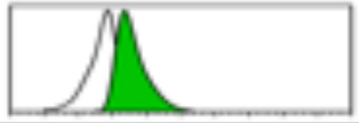
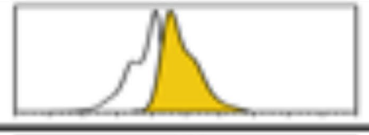
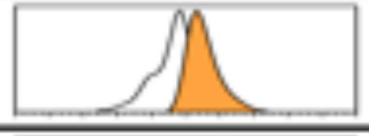
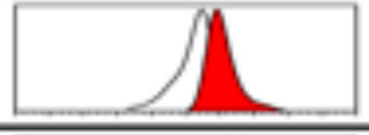
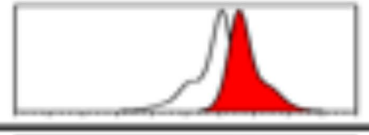
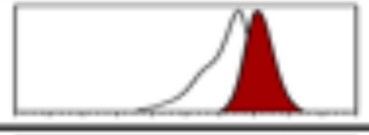
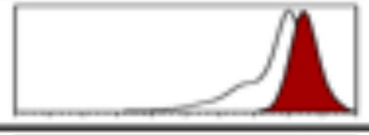
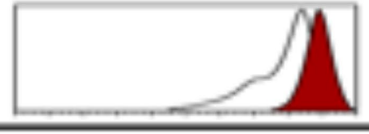
- Ability to take still images and live videos of your samples
- Display, annotate, edit, calibrate, measure, analyze, process, print, and save raster
- Variable linear measurements such as line segment, radius values, and others

We can customize this microscope to fit your requirements:

Fluorescence spectra overview



https://www.miltenyibiotec.com/LU-en/products/macs-antibodies/vio-dye-fluorophores.html

Emission	Fluor	Ex/Em†	See Spectra
Blue	DyLight 350	353/432	
Blue	DyLight 405	400/420	
Green	DyLight 488	493/518	
Yellow	DyLight 550	562/576	
Red	DyLight 594	593/618	
Red	DyLight 633	638/658	
Red	DyLight 650	652/672	
Near IR	DyLight 680	692/712	
Near IR	DyLight 750	752/778	
Infrared	DyLight 800	777/794	

Laser Line	Laser Type
~ 405 nm	Diode
~ 440 nm	Diode
473 nm	Doubled DPSS
488.0 nm	Ar-ion gas
~ 488 nm	Doubled OPS
514.5 nm	Ar-ion gas
515.0 nm	Doubled DPSS
561.4 nm	Doubled DPSS
568.2 nm	Kr-ion gas
593.5 nm	Doubled DPSS
632.8 nm	HeNe gas
~ 635 nm	Diode
647.1 nm	Kr-ion gas

Example

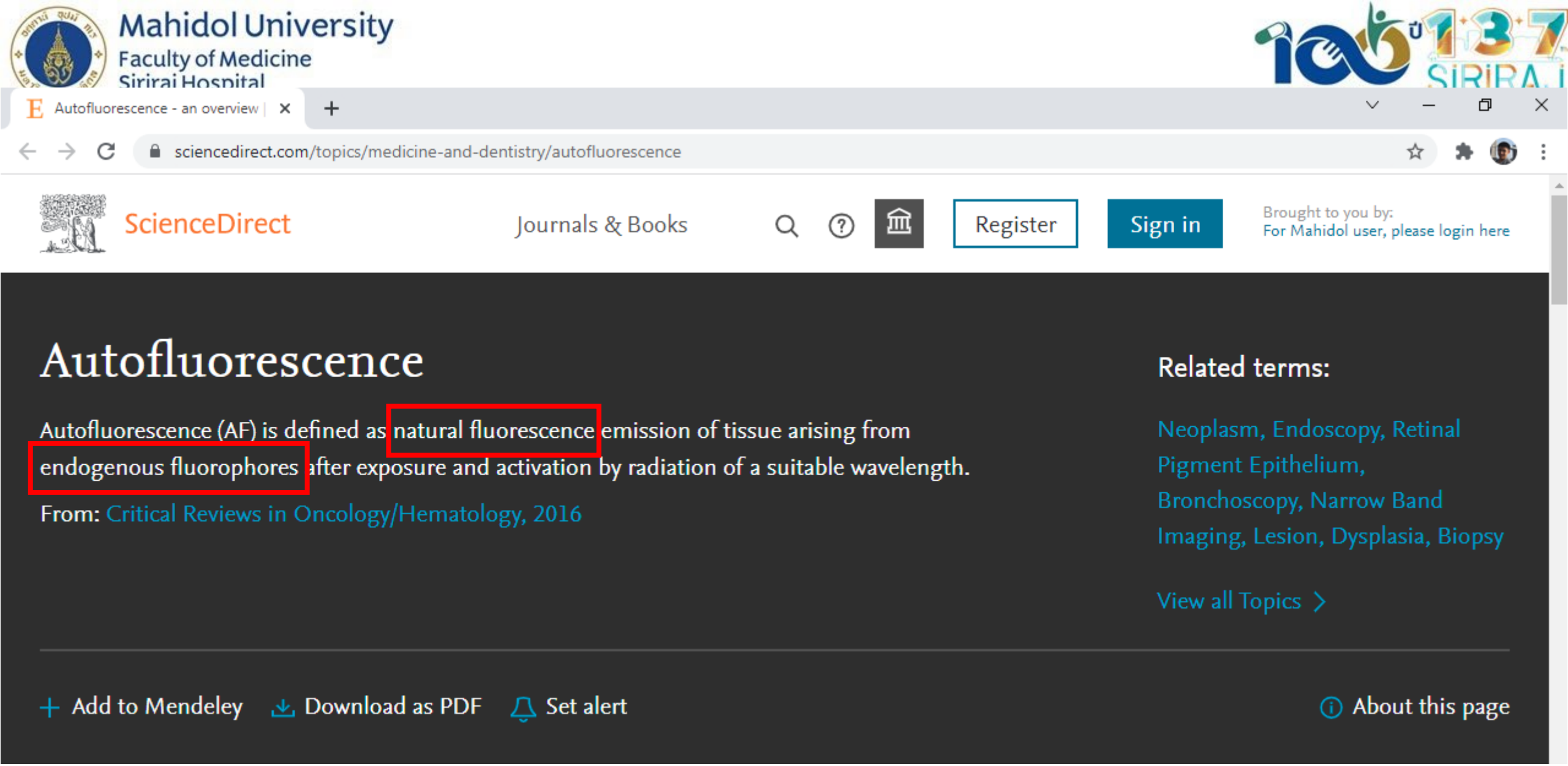
- Protein detection in tissue samples using fluorescence dyes

- Fluorescent microscope
  - Which fluorophore should be used?
  - Fluorescent system(s) equipped with the microscope
    - Choose fluorophores that their spectra are compatible with the microscope

What should be concerned when working with fluorescence?

2 most common problems

1. Autofluorescence
2. Bleaching / Fading


Autofluorescence - an overview
sciencedirect.com/topics/medicine-and-dentistry/autofluorescence
ScienceDirect
Journals & Books
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Autofluorescence
Autofluorescence (AF) is defined as natural fluorescence emission of tissue arising from endogenous fluorophores after exposure and activation by radiation of a suitable wavelength.
From: Critical Reviews in Oncology/Hematology, 2016
Related terms:
Neoplasm, Endoscopy, Retinal Pigment Epithelium, Bronchoscopy, Narrow Band Imaging, Lesion, Dysplasia, Biopsy
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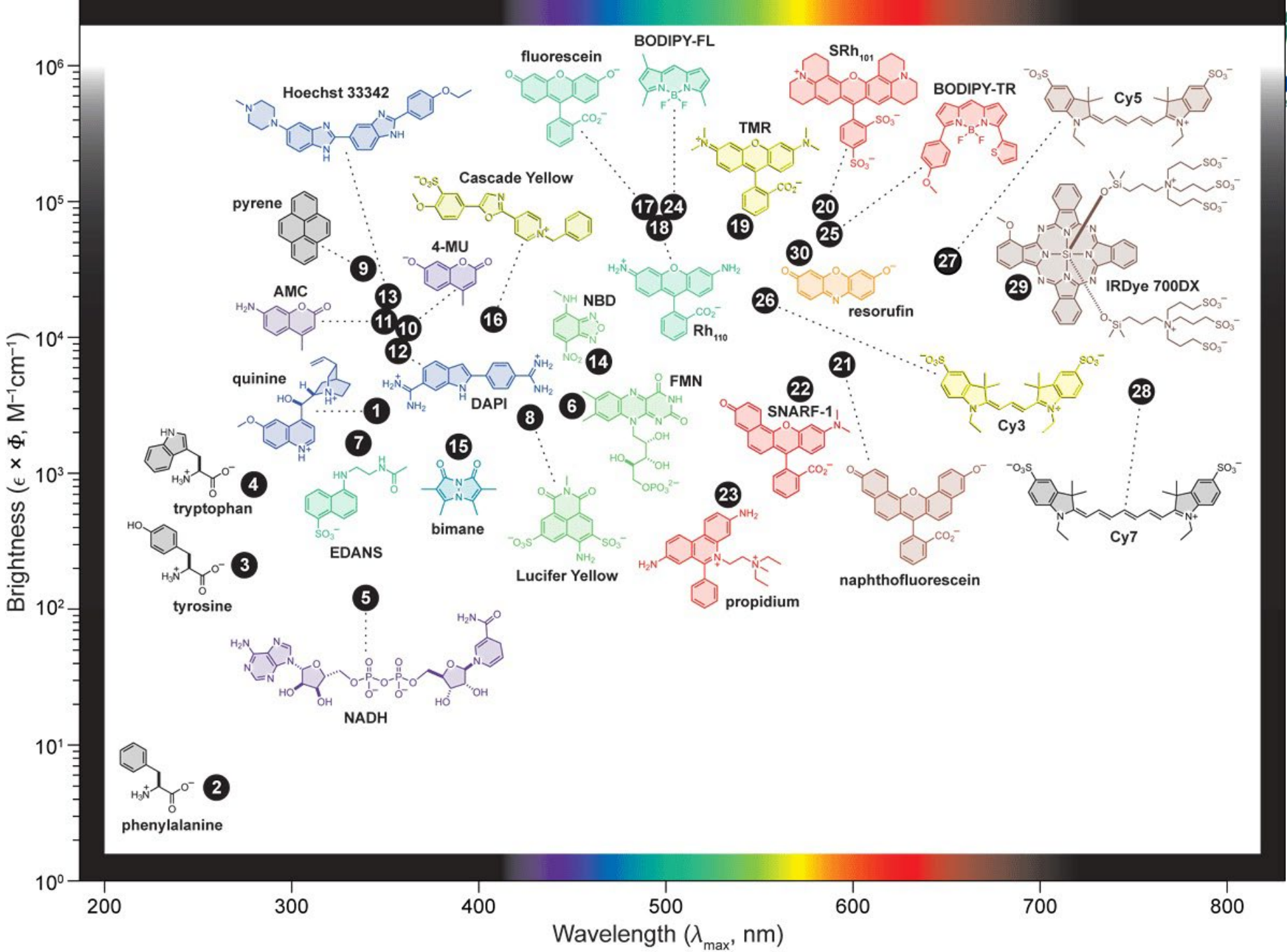
Brightness (ε × Φ, M⁻¹cm⁻¹)
Wavelength (λmax, nm)
10⁰
10¹
10²
10³
10⁴
10⁵
10⁶
200
300
400
500
600
700
800
Hoechst 33342
fluorescein
BODIPY-FL
SRh101
BODIPY-TR
Cy5
TMR
Cascade Yellow
pyrene
4-MU
AMC
NBD
Rh110
resorufin
IRDye 700DX
quinine
DAPI
FMN
SNARF-1
Cy3
tryptophan
EDANS
bimane
Lucifer Yellow
propidium
naphthofluorescein
Cy7
tyrosine
NADH
phenylalanine
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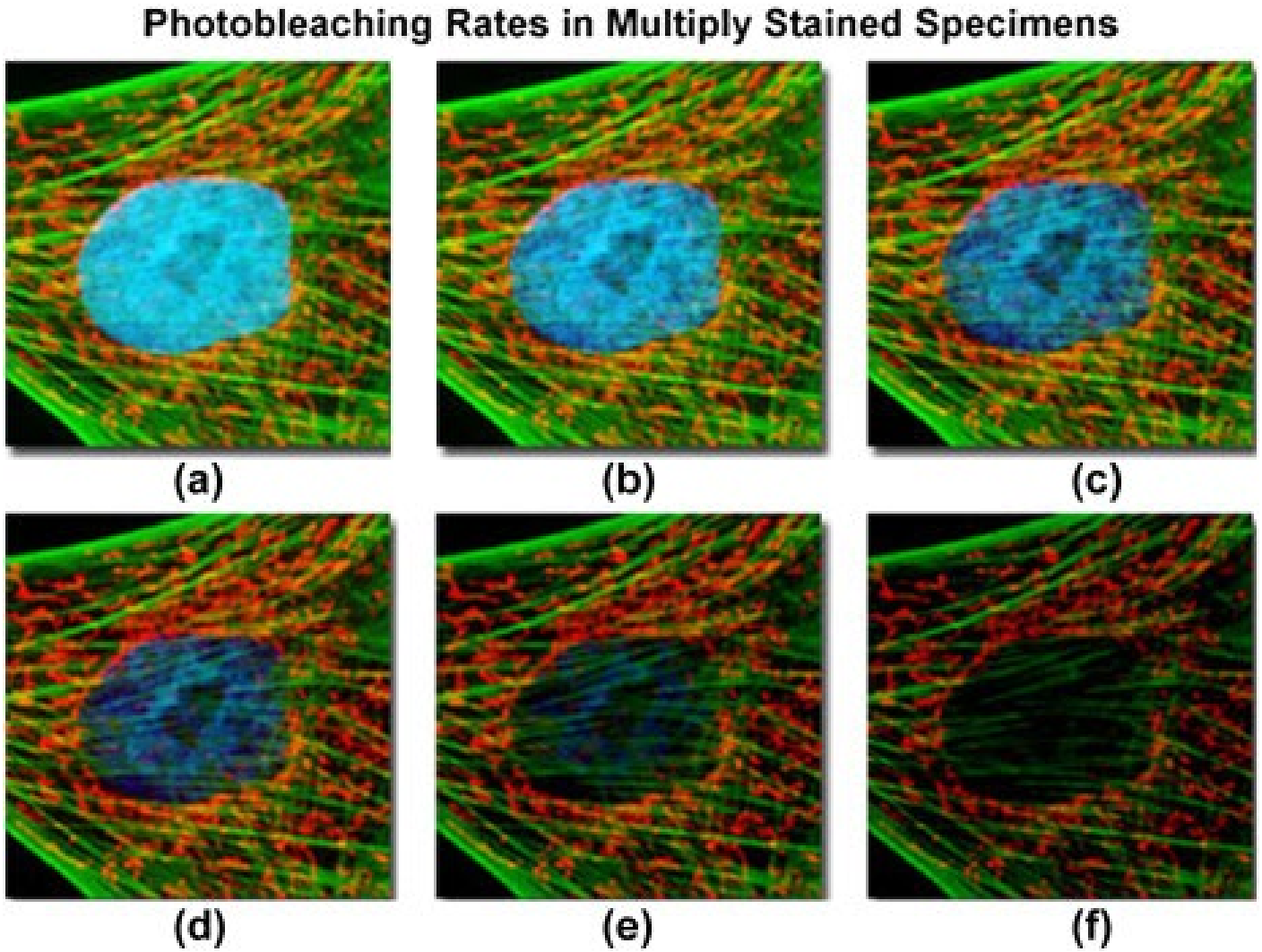
Bleaching/Fading



Images taken at 2 minutes interval

Applications

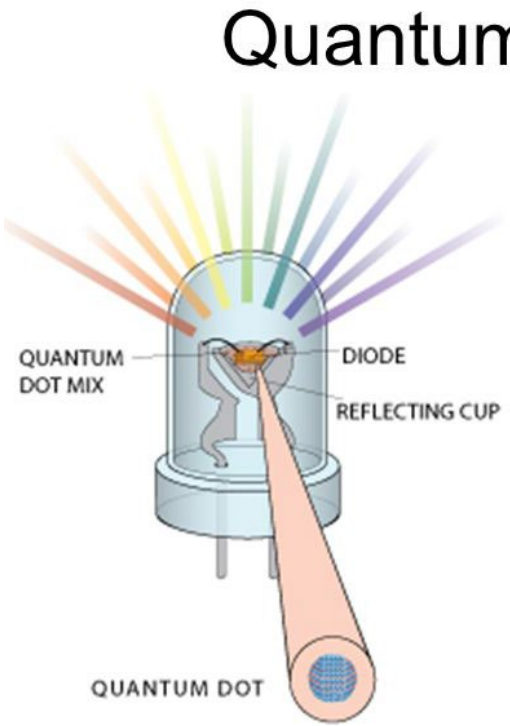
Have you used/seen fluorescence in any experiments?

What did you use/see?

Lighting

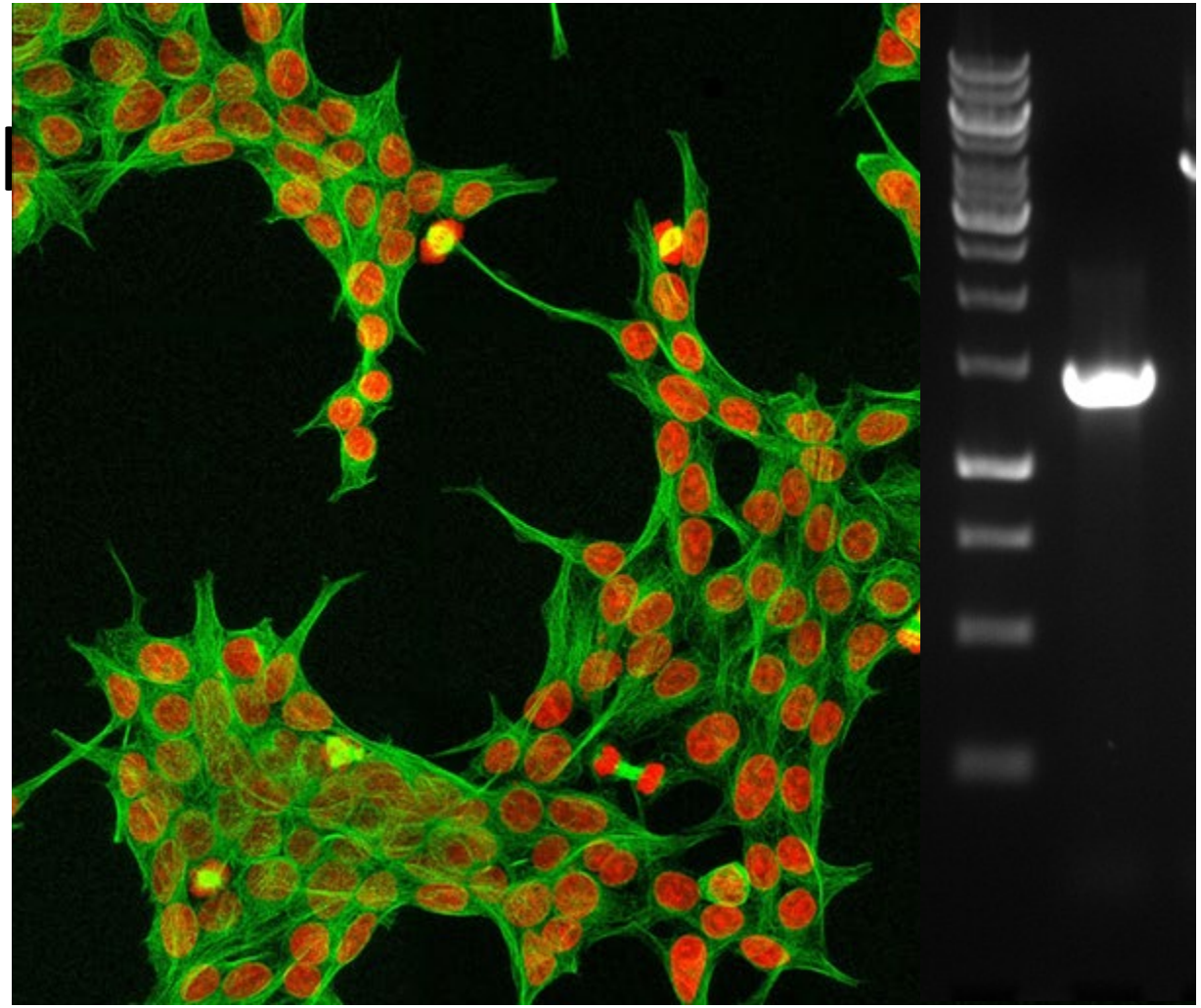
Fluorescent lamp/light bulb

Quantum dots

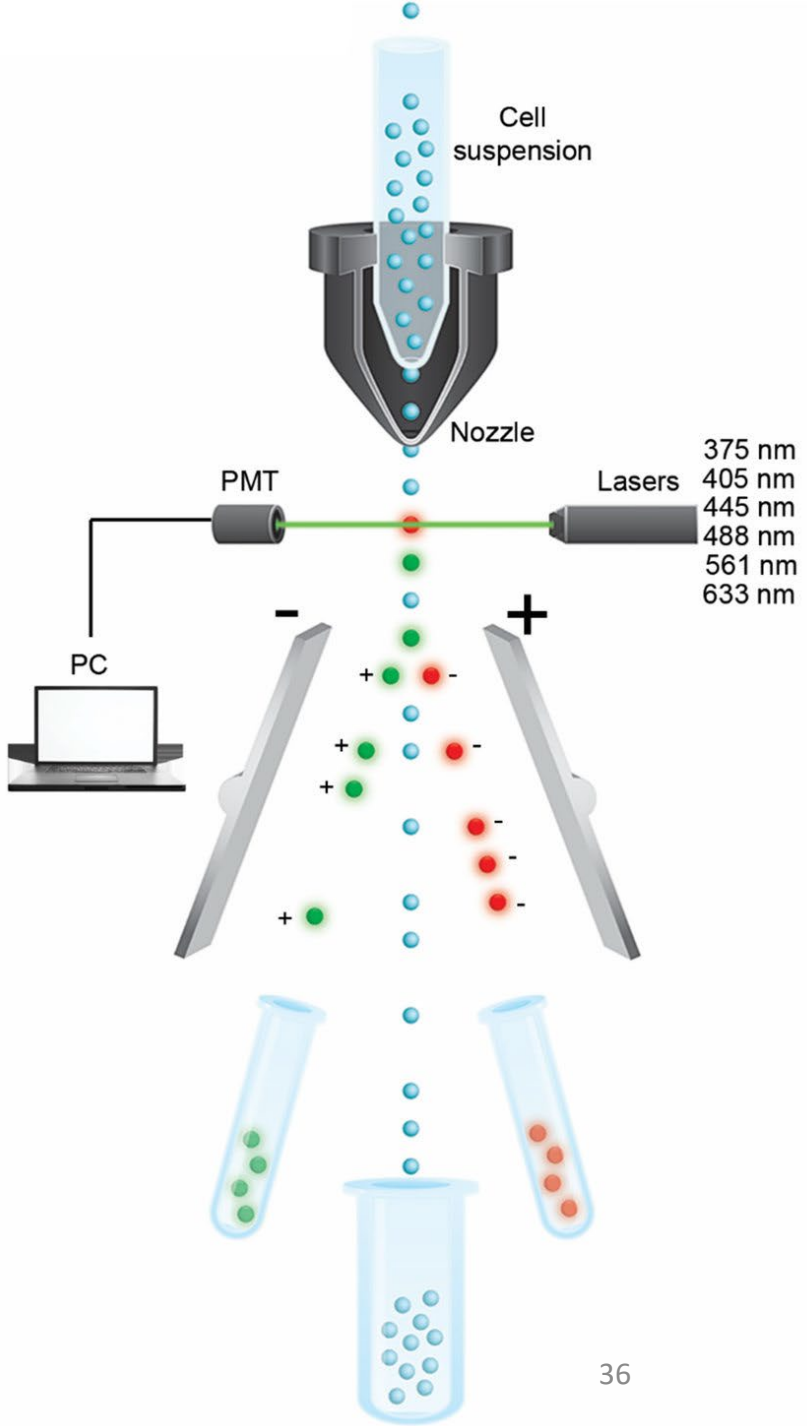


Biomedical research

- Direct I



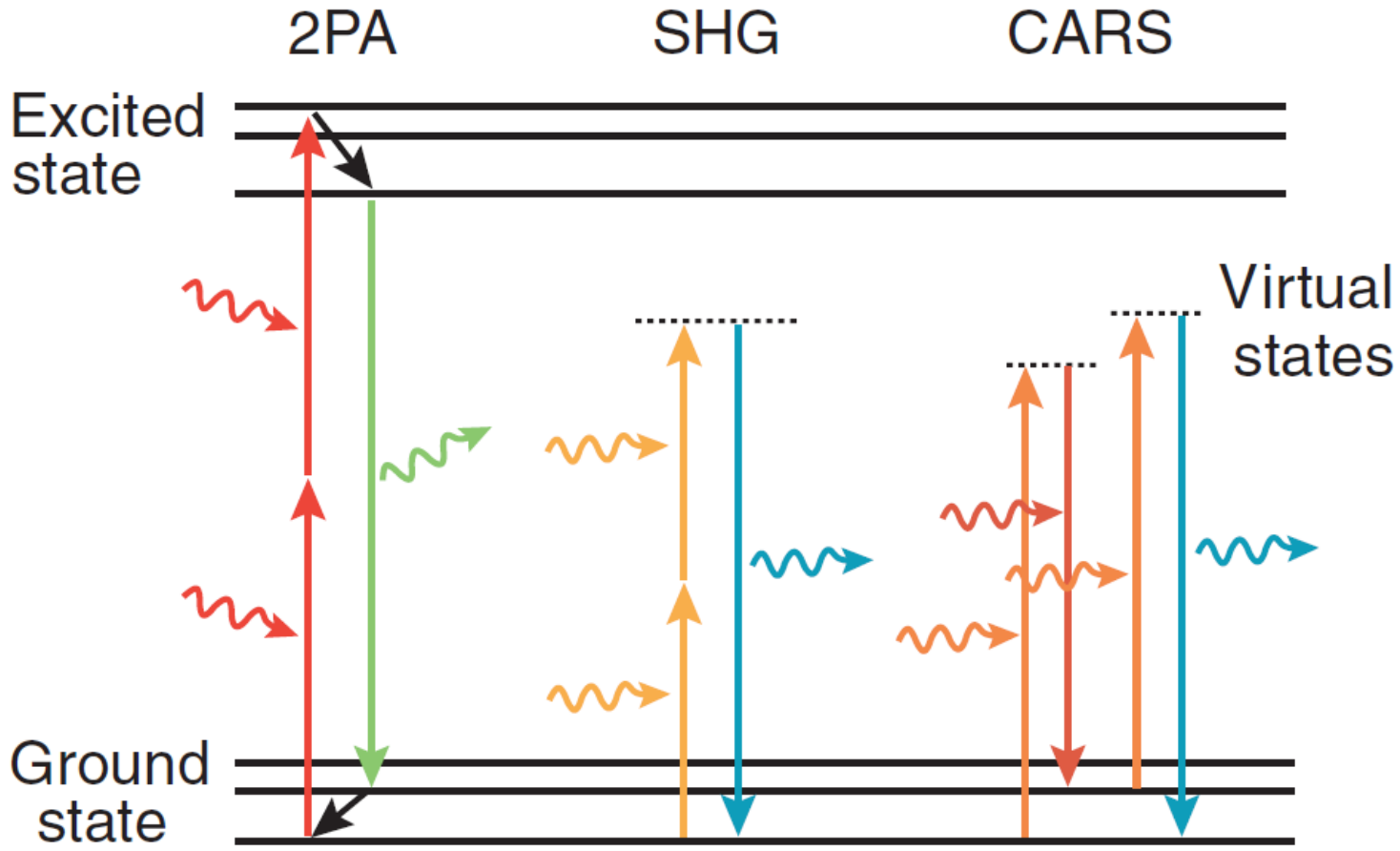
Staining



Flow cytometry/FACS – Fluorescent Activated Cell Sorting

Biomedical research

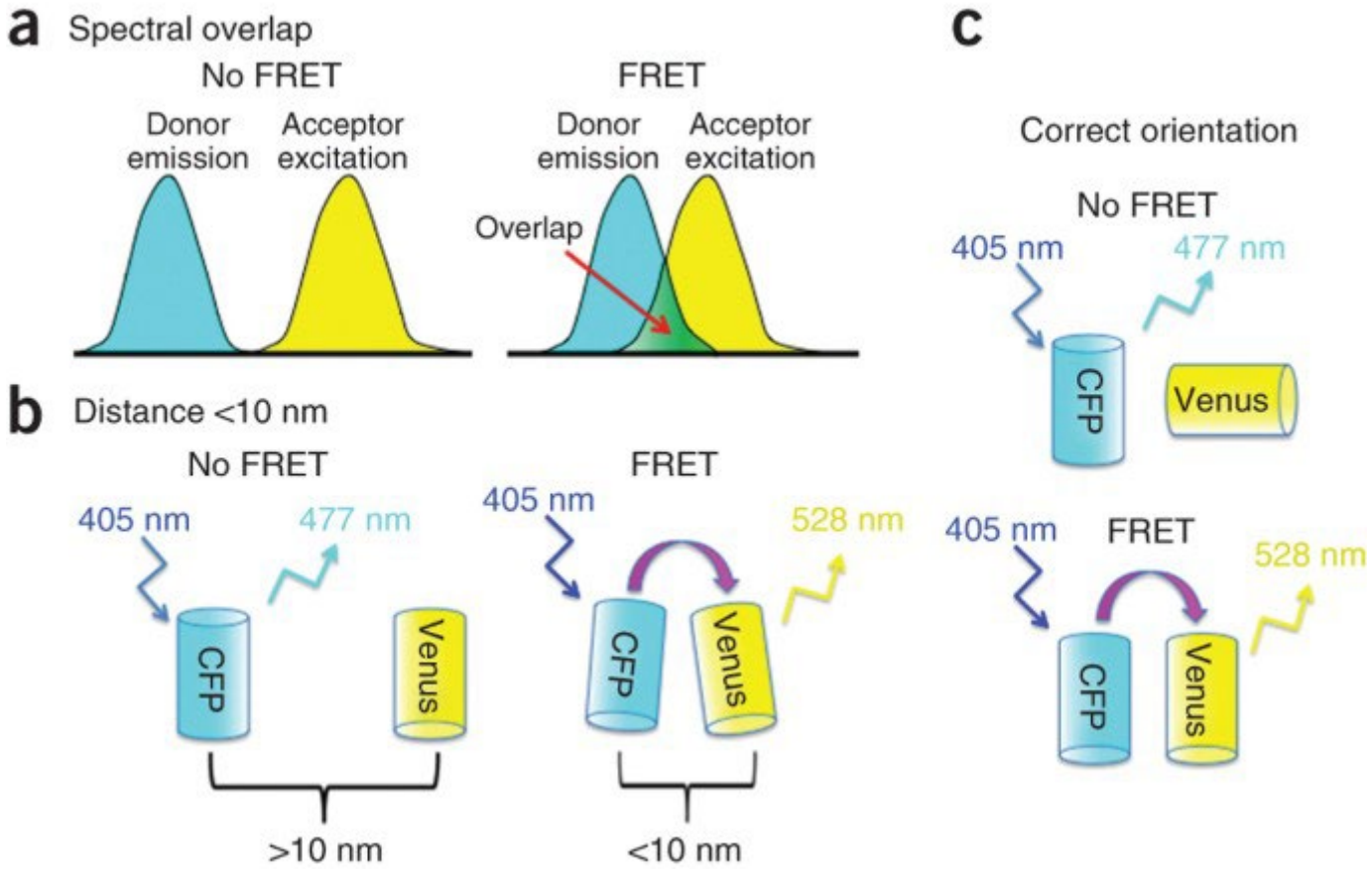
- Multiphoton microscopy



Helmchen & Denk, 2005

Biomedical research

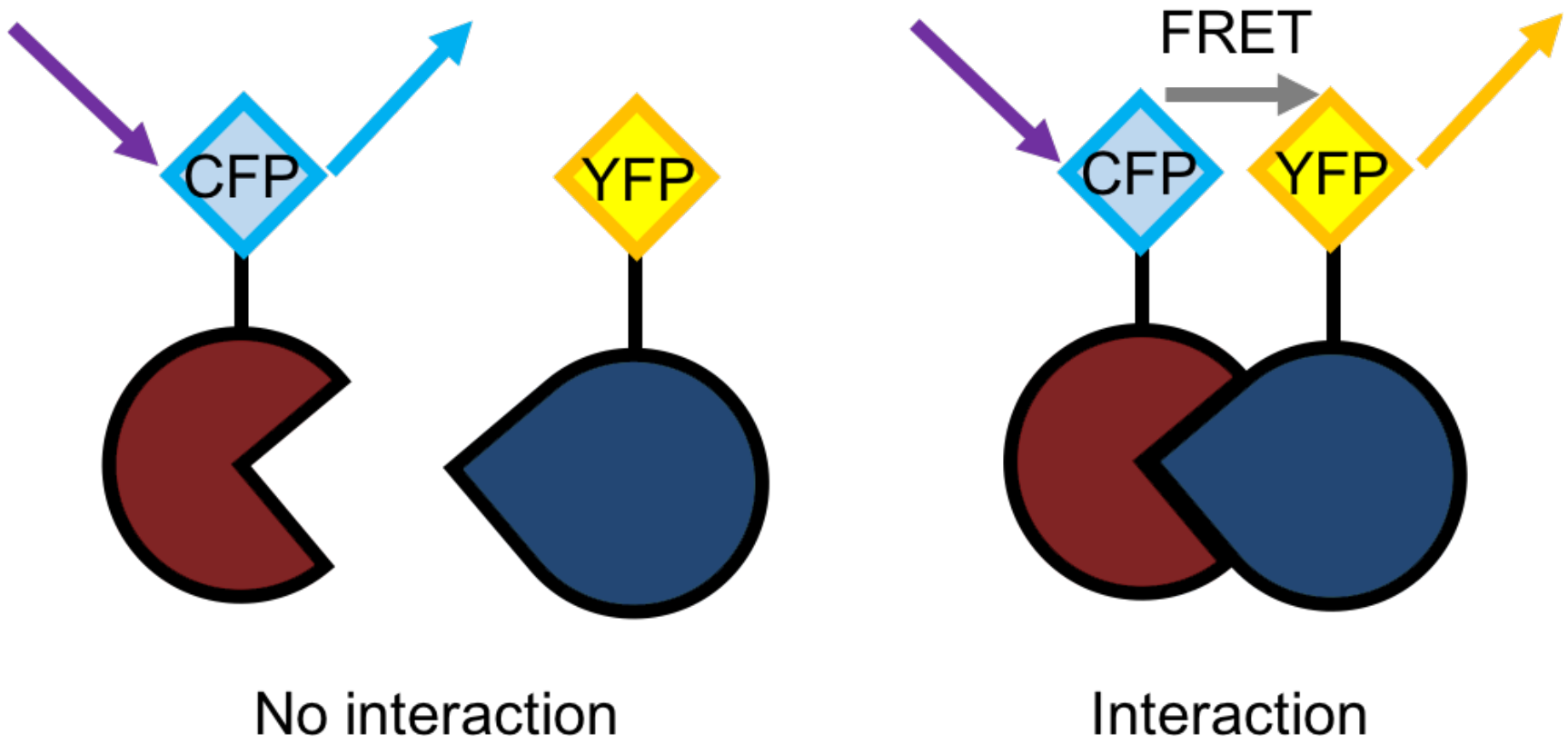
- FRET: Fluorescent Resonance Energy Transfer



Broussard et al, 2013

Biomedical research

- FRET: Fluorescent Resonance Energy Transfer



https://www.creative-biostructure.com/coronavirus/fluorescence-resonance-energy-transfer-fret-assay-p37.htm

Questions?

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