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# Optical spectroscopy and biosensors for investigation of biomolecules and their interactions

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# Fluorescence Spectroscopy I

# Content

- Jablonski diagram, fluorophore key characteristics
- Angular distribution of emission
- Conjugation of fluorophores with biomolecules
- Fluorescence energy transfer (FRET)
- Optical configuration used for the excitation and collection of fluorescence light in bioassays
- Fluorescence correlation spectroscopy
- Fluorescence anisotropy

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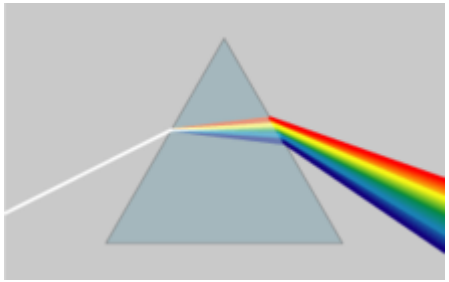
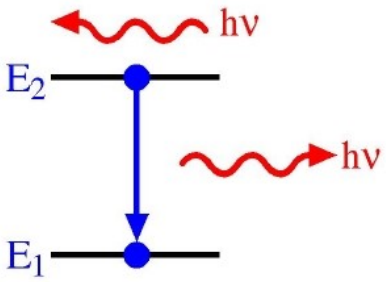
# Refractive Index

Parameter that describes interaction of light with matter composed of elements (e.g. atoms) that are << $\lambda$ and exhibit polarizability. By averaging over many atoms that are be polarized by the oscillating electric field.

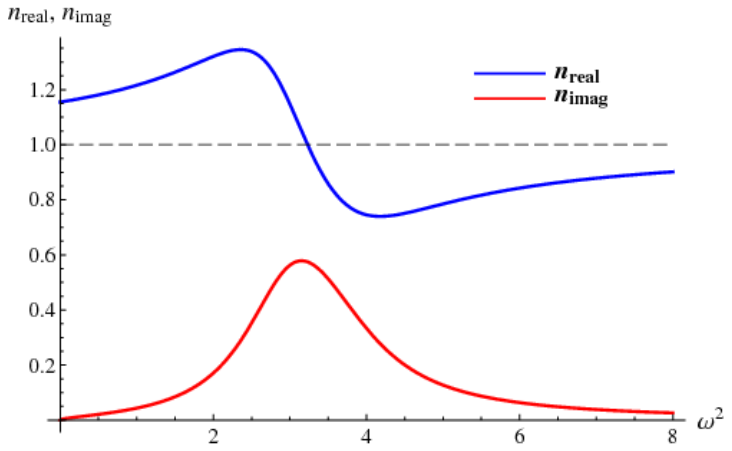
~

Real part – refraction






Imaginary part - absorption




For more details, see Lorenz Lorenz or Clausius-Mossotti theories.

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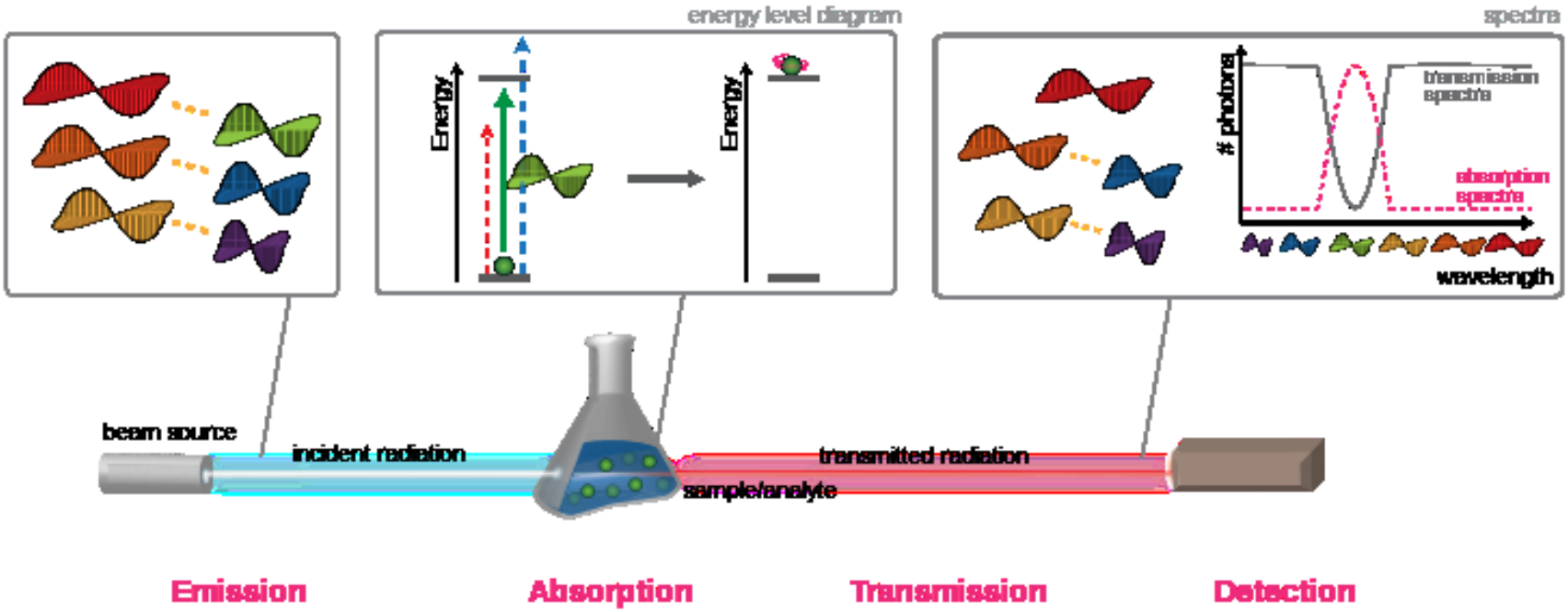
# Absorption of Molecules

Absorption of light by molecules is accompanied with their transition from a ground state to excited state (followed by a relaxation). It typically occurs at distinct energies leading to specific bands in absorption spectrum.




*E.g.* is routinely used for measuring total concentration of proteins – UV absorption spectroscopy.

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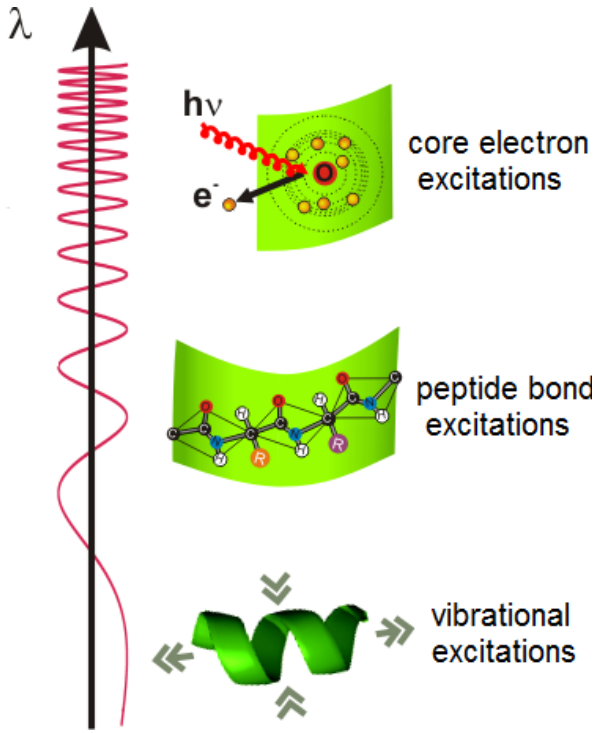
# Absorption of Molecules - Wavelength Spectrum




Electronic lines correspond to a change in the electronic state of an atom or molecule. Typically UV-Vis.

Vibrational lines correspond to changes in the vibrational state of the molecule and are typically found in the infrared region.

Rotational lines, for instance, occur when the rotational state of a molecule is changed. Rotational lines are typically found in the microwave spectral region.

Combination of above can lead to rather complex spectra.

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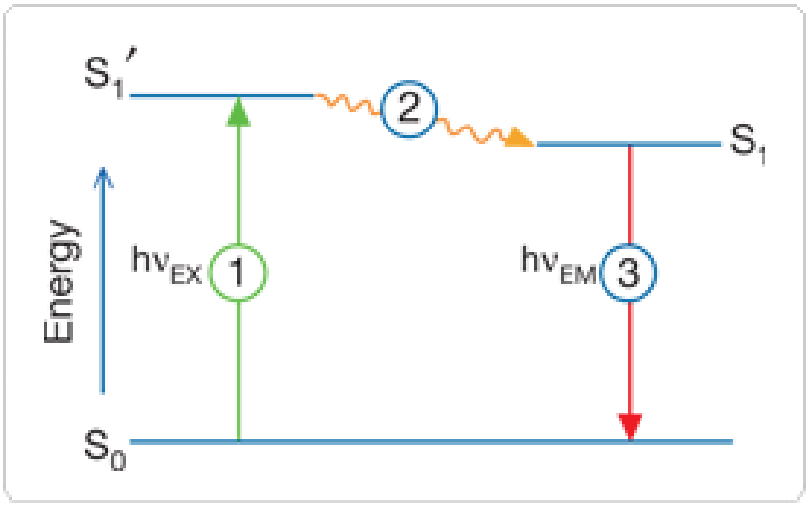
# Jablonski Diagram




(1) **Photo-excitation:** from the ground electronic state $S_0$ creates excited states $S_1$, ($S_2$, …, $S_n$)

(2) **Internal conversion:** Molecules rapidly ($10^{-14}$ to $10^{-11}$ s) relax to the lowest vibrational level of $S_1$.

(3) **Returning** to its ground state $S_0$.

As the energy $hv_{ex}$ is higher than that emitted $hv_{em}$ – Stokes shift occurs.

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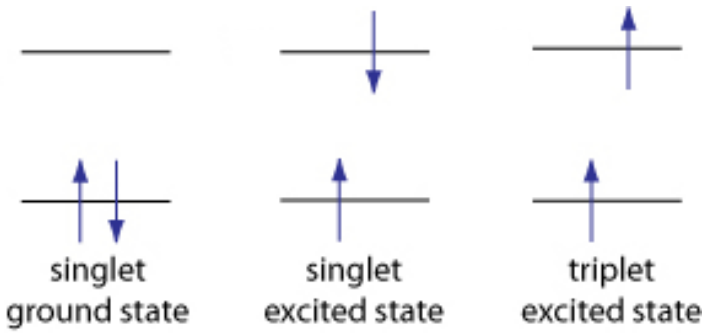
# Fluorescence

Process in which a fluorophore (e.g. a dye molecules, quantum dot) absorbs a photon and re-emits it at a higher wavelength. Form of luminescence:

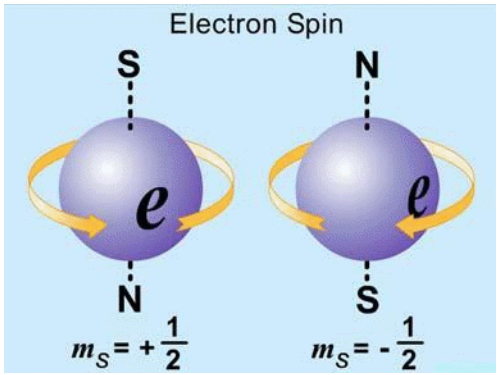
Luminescence

**Fluorescence:** Lifetime from $<10^{-10}$ to $10^{-7}$ s (from singlet state).

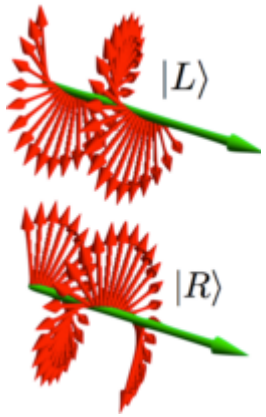
**Phosphorescence:** Lifetime from $10^{-5}$ to $>10^{+3}$ s from (triplet excited state).



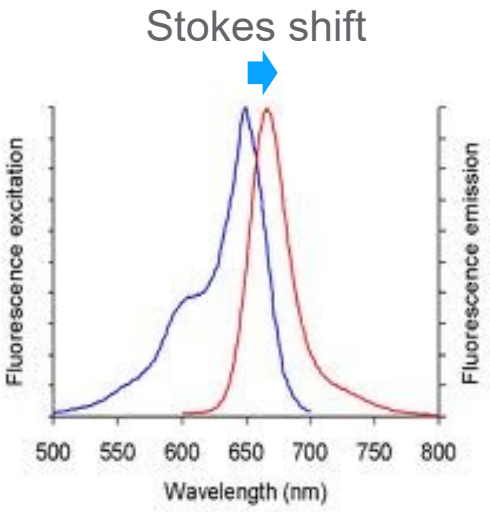

Electron spin 1/2



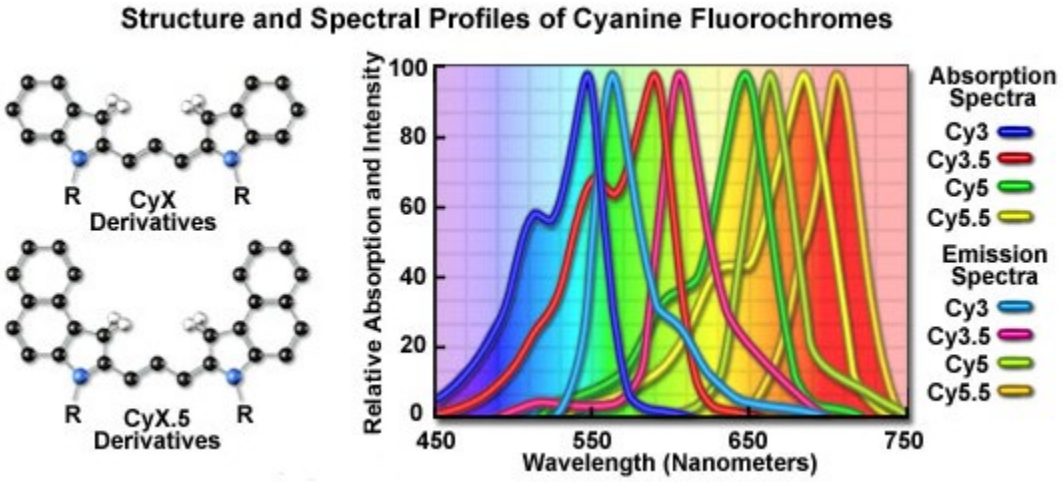

Photon spin 1



# Fluorescence Emission Spectra




Abs. and Emission spectrum of Cy5 dye




http://www.olympusmicro.com

# Fluorophore Quantum Yield

**Quantum yield / quantum efficiency:** Ratio of number of emitted vs. absorbed photons, Φ/QE.

QE is between 0 (non-fluorescent molecule) and 1 (perfectly emitting molecule). QE is limited by the competing transitions to the ground state that are not radiative.

Table 1 | Common fluorescent dyes; their associated wavelengths of absorption (excitation) and emission, and colours

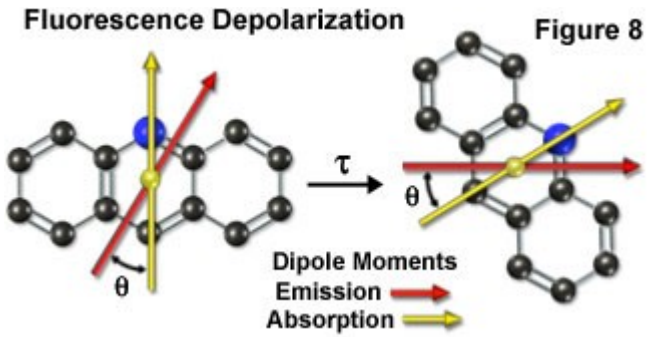
| Name | $\lambda_{max}$ / nm (absorption) | $\lambda_{max}$ / nm (emission) | Colour | $E$ at $\lambda_{max}$ | Φ | τ / ns |
|---|---|---|---|---|---|---|
| Cyanine-3 | 550 | 570 | Dark pink | 136 000 | 0.15 | - |
| Cyanine-3.5 | 591 | 604 | - | 116 000 | 0.15 | < 0.3 |
| Cyanine-3b | 558 | 572 | - | 130 000 | 0.67 | 2.8 |
| Cyanine-5 | 649 | 670 | Blue | 250 000 | 0.3 | - |
| Cyanine-5.5 | 675 | 695 | Blue | 209,000 | 0.3 | - |

https://www.atdbio.com/content/32/Cyanine-dyes

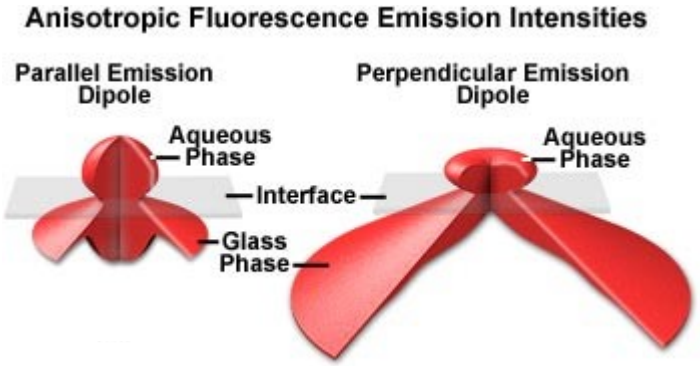
# Absorption and Emission Dipoles

**Molar extinction coefficient**: Describes the strength of the absorption $[\varepsilon_{max}]=M^{-1}cm^{-1}$.

Absorption and emission of electromagnetic field can be treated by using respective **dipoles** (giving rise to angular dependence of the effects).



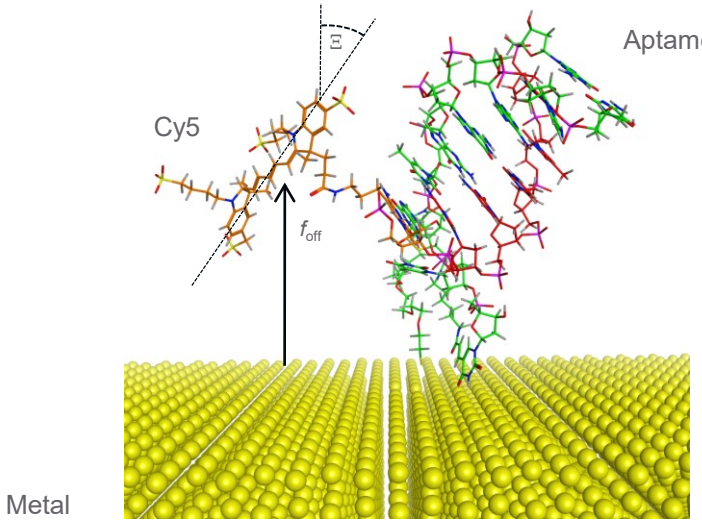

https://www.olympus-lifescience.com/en/microscope-resource/primer/techniques/fluorescence/fluorescenceintro/

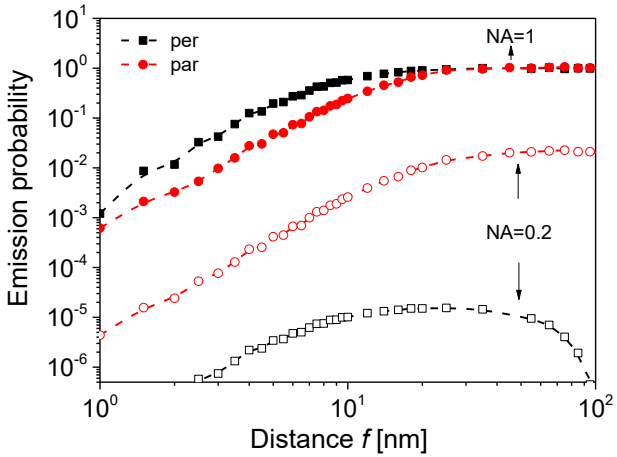



https://www.olympus-lifescience.com/de/microscope-resource/primer/techniques/fluorescence/tirf/tirfintro/

# Quenching

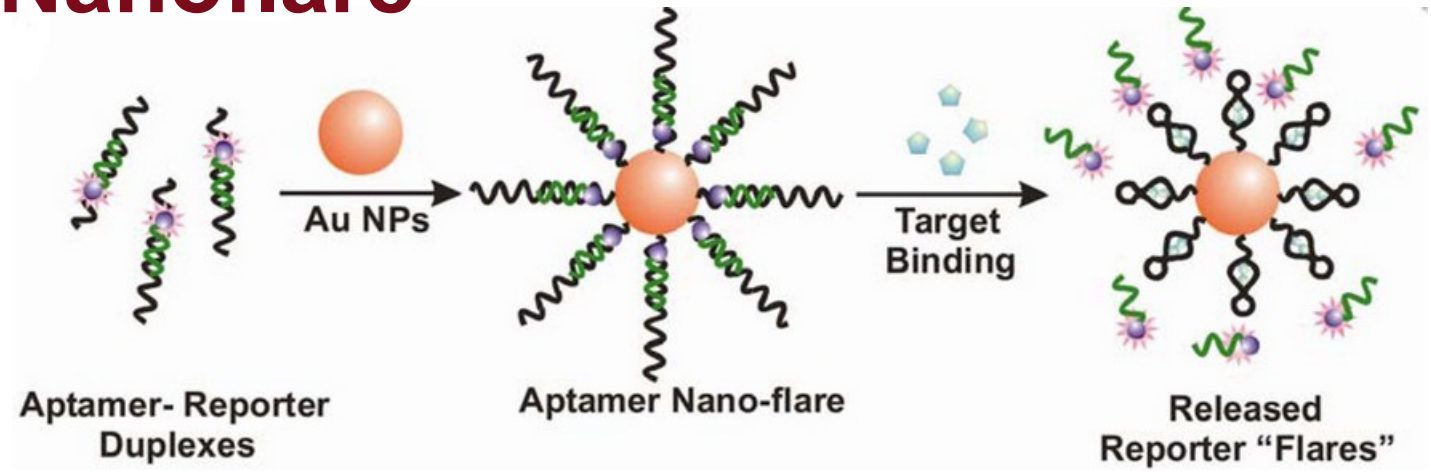
**Quenching:** Loss of fluorescence signal, interactions between the fluorophore and the local molecular environment (collisions), including other fluorophores (e.g., fluorescence resonant energy transfer FRET, non-radiative channels in proximity to metal…).




Khulan Sergelen, Stefan Fossati, Aysegül Turupcu, Chris Oostenbrink, Bo Liedberg, Wolfgang Knoll, and Jakub Dostálek, Plasmon field-enhanced fluorescence energy transfer for hairpin aptamer assay readout, ACS Sensors, 2017, 2 (7), 916-923.

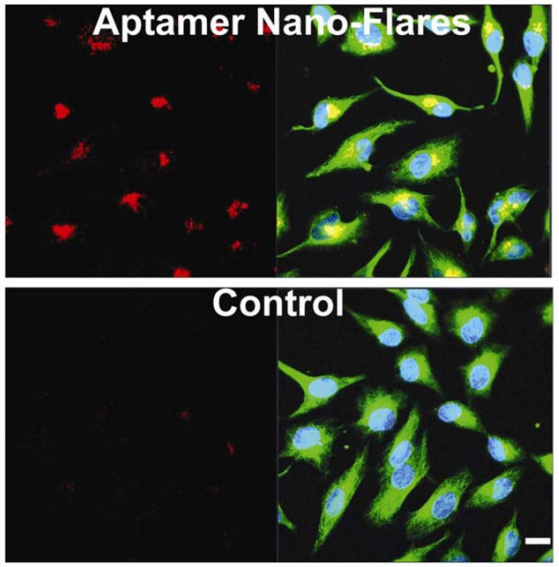
# Nanoflare




FRET – metallic NP serves as a donor and quenches the fluorescence

Spherical DNA – allows for simple cellular uptake

Aptamer – oligonucleotide designed for specific capture of molecular analytes (ATP in this case)




**Mirkin lab:** doi:10.1021/nl901517b

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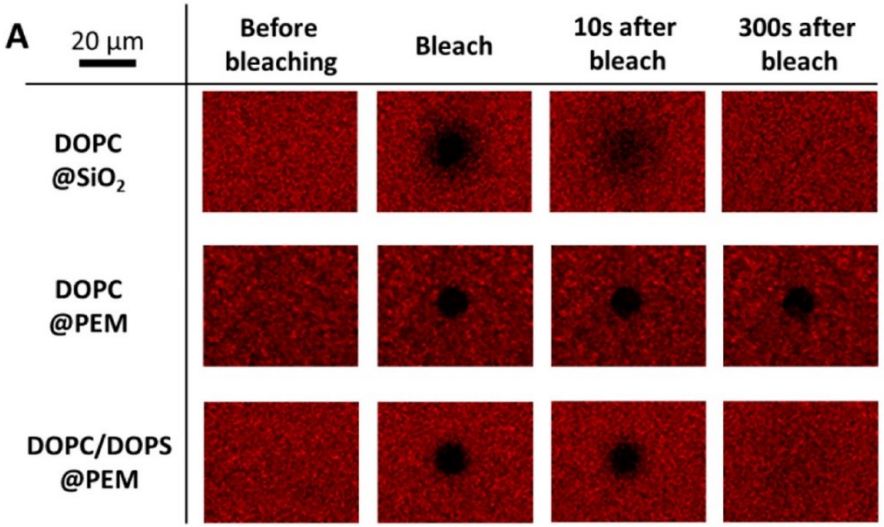
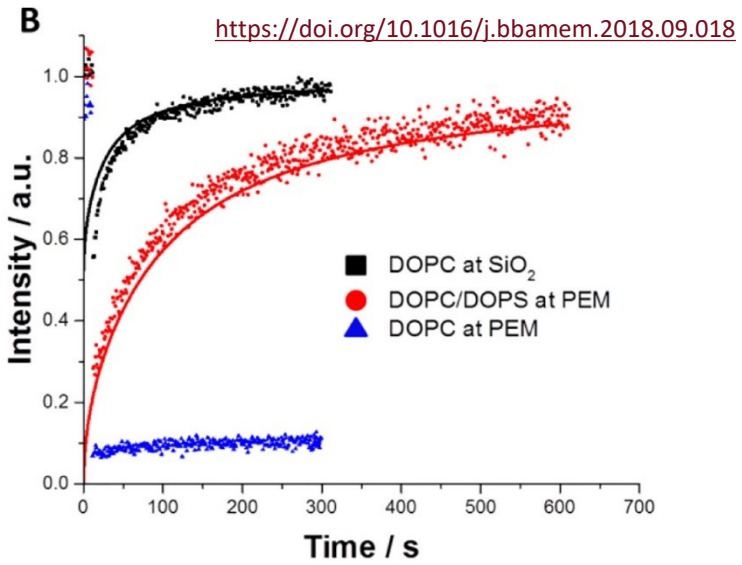
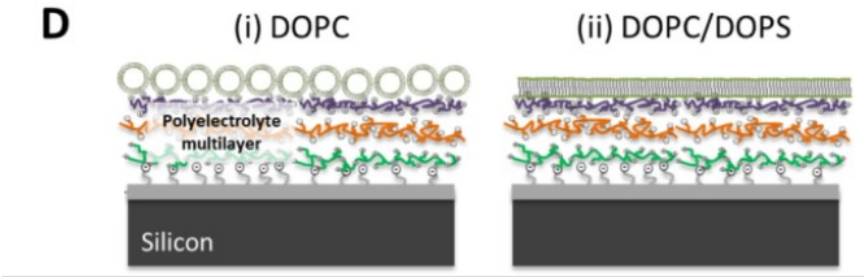
# Photo-bleaching

**Photo-bleaching:** Only certain number of absorption / emission is possible before destruction of the excited fluorophores occurs.




Example: investigation of fluid lipid membranes by local photo-bleaching.

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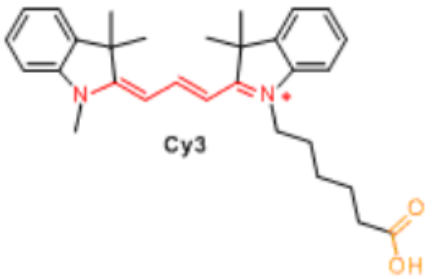
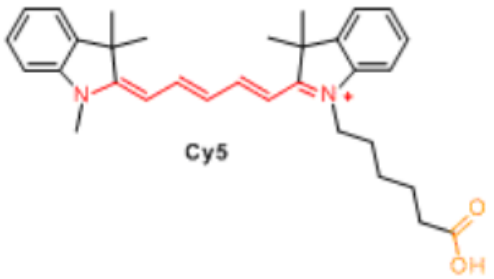
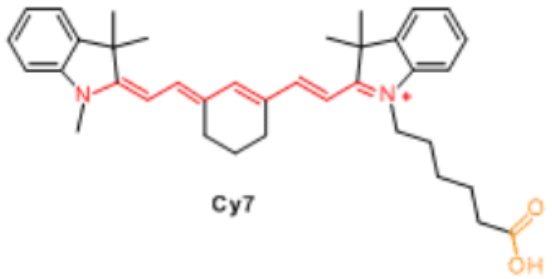
# Organic Dyes

A large variety of synthetic organic fluorophores are available. Typically charged in order to promote solubility.



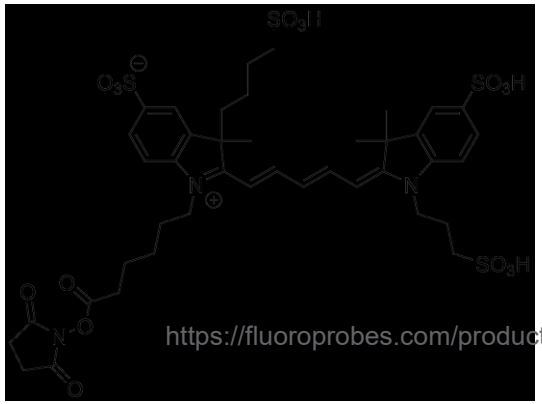




https://www.lumiprobe.com/tech/cyanine-dyes

Cyanine dyes (+1)

Alexa Fluor 647 (-3)

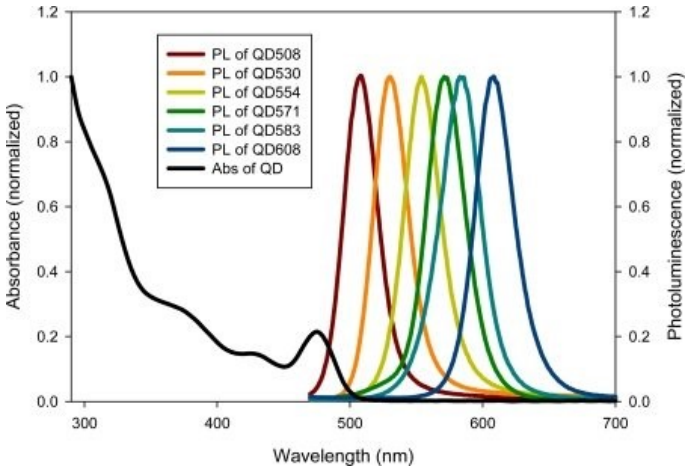



https://fluoroprobes.com/product/alexa-fluor-647-nhs-ester/

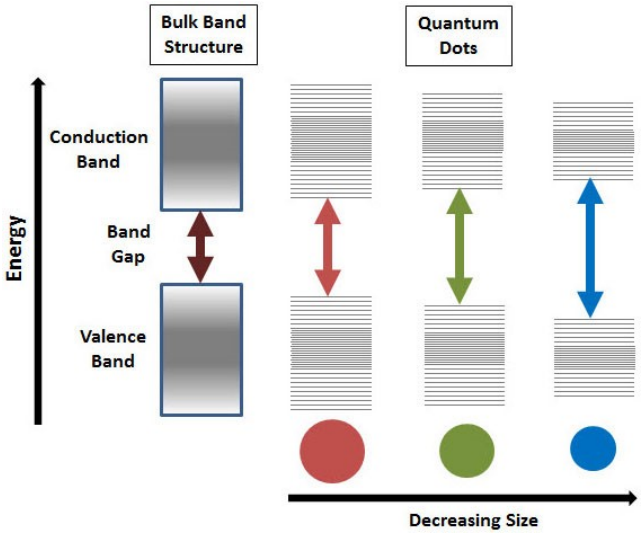
# Quantum Dots

Nanometer-sized semiconductor particles that exhibit narrow tunable emission bands and capped with biocompatible coatings.

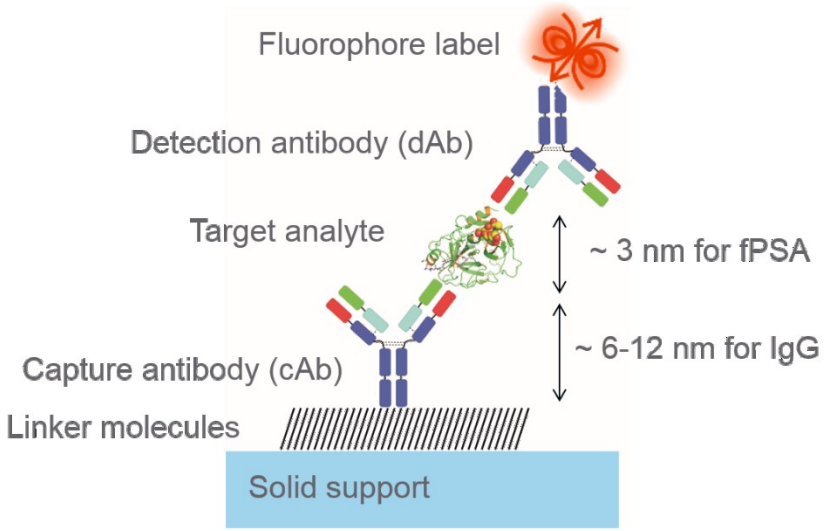
https://www.sigmaaldrich.com/technical-documents/articles/materials-science/nanomaterials/quantum-dots.html

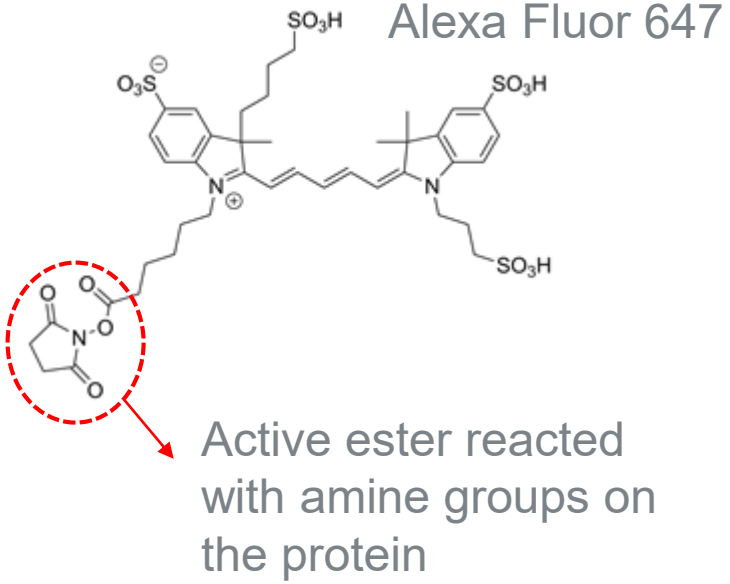



- Cannot be bleached as organic dyes.
- Effect of photo-blinking may complicate their use.
- Become established materials in a broad field of applications including labeling biomolecules



# Conjugation with Biomolecules




Active ester reacted with amine groups on the protein

- Often amine coupling is used and there are many commercial kits available.
- The number of fluorophore attached per e.g. IgG molecule is adjusted (3-5) to balance between decreased IgG activity, self-quenching.

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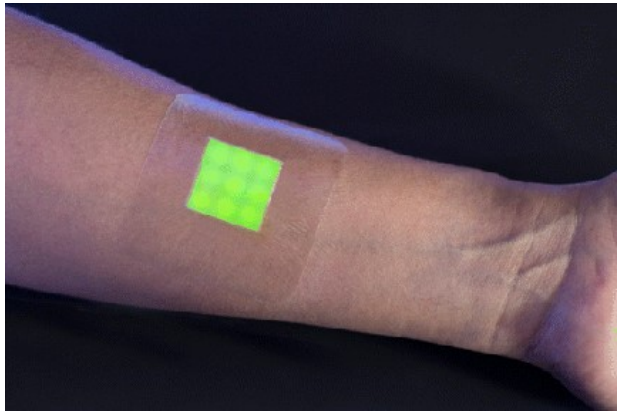
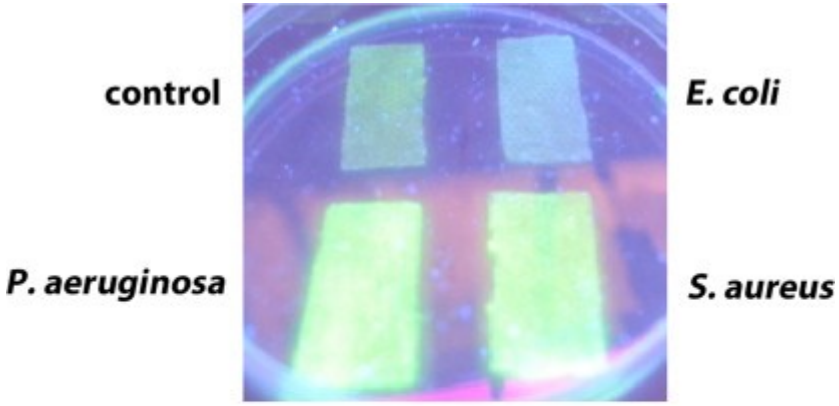
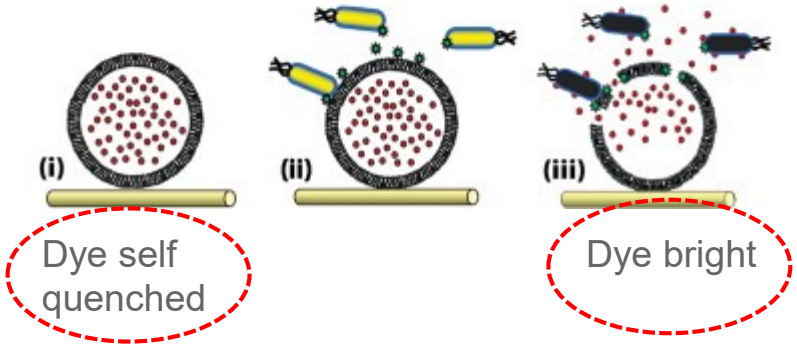
# Smart Wound Dressing



Biosensors embedded in wound dressings to monitor bacterial infections. Possible incorporation of triggered release of a drug.




Fluorescent dye loaded to lipid vesicle, toxic bacteria destroy the lipid bilayer wall and leaches the dye reporter.

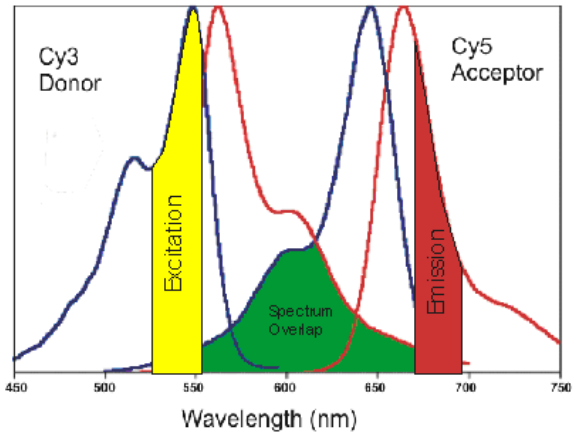
Toby Jenkins laboratory - 10.1021/acsami.5b07372

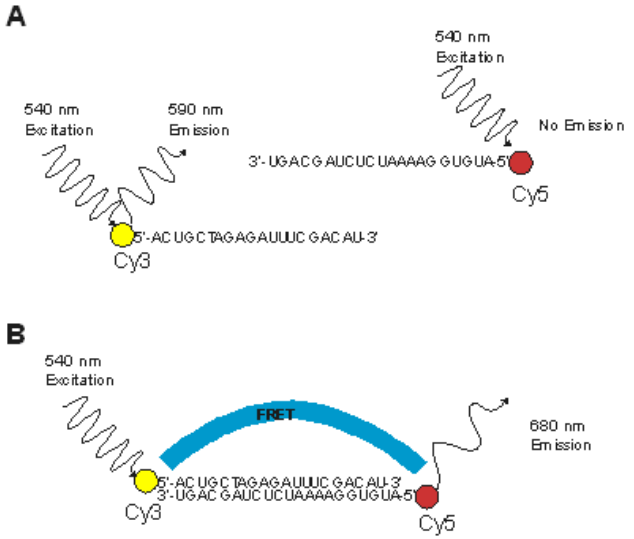
# Fluorescence Resonant Energy Transfer - FRET

**Förster / fluorescence resonant energy transfer: dipole-dipole coupling of two fluorophores which changes the emission spectrum. Efficient at small distances, typically $r < 10$ nm.**

$$E = \frac{1}{1 + (r/R_0)^6}$$

$R_0$ is Förster distance at which $E$=50%

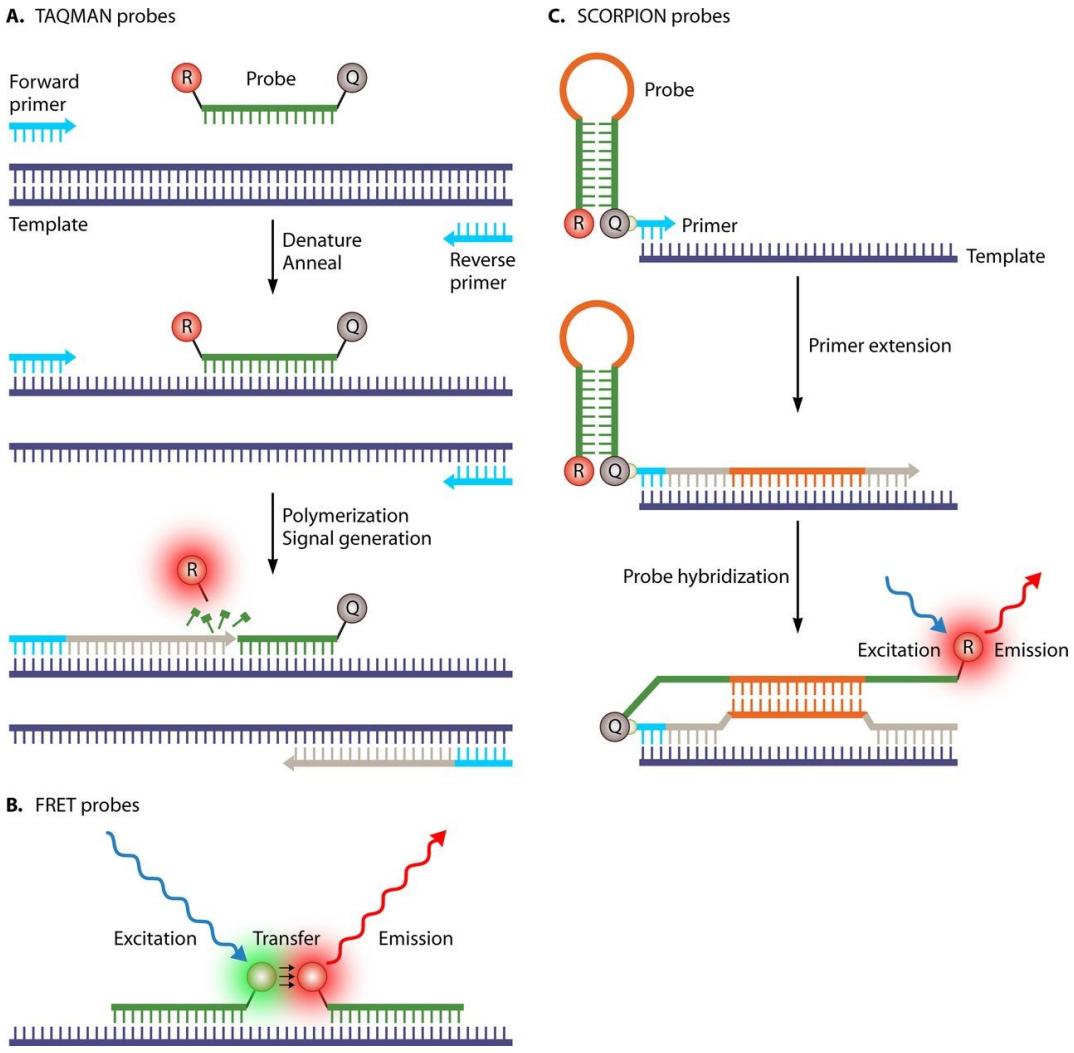



Spectral overlap of absoprtion and emission bands of donor and acceptor chromophores

Applications: conformation changes studies, immunoassays, DNA hybridization.

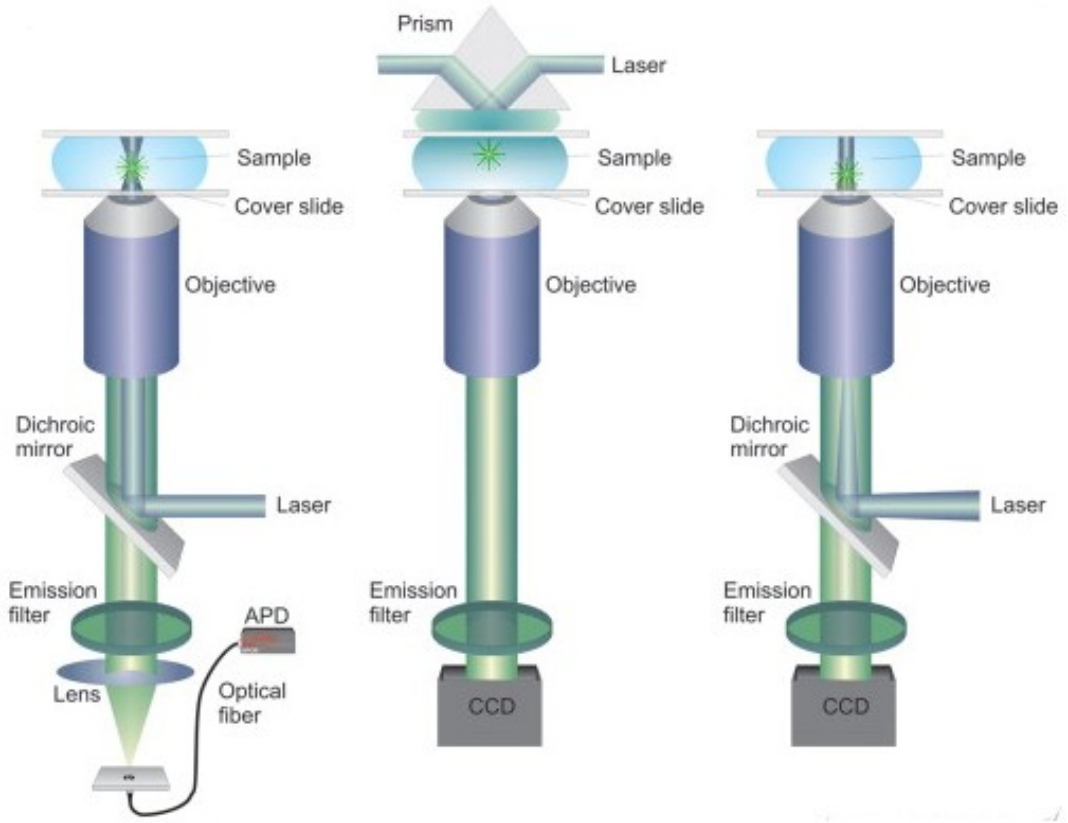
# Example – Probes for Real Time PCR




- Probes are loaded to a droplet together with sample and PCR reagents
- Without the analyte they are dark and when the target analyte is present, are turned to bright.
- Based on fluorescence and FRET or quenching.

# Fluorescence Detection

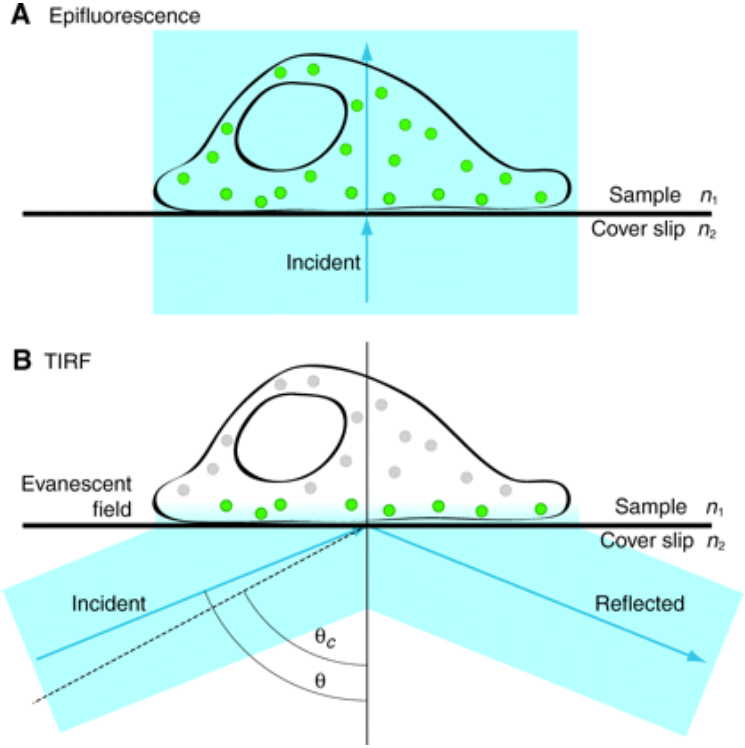
Most common configuration are: confocal fluorescence (left), total internal reflection - TIRF (middle) and epi-fluorescence (right).

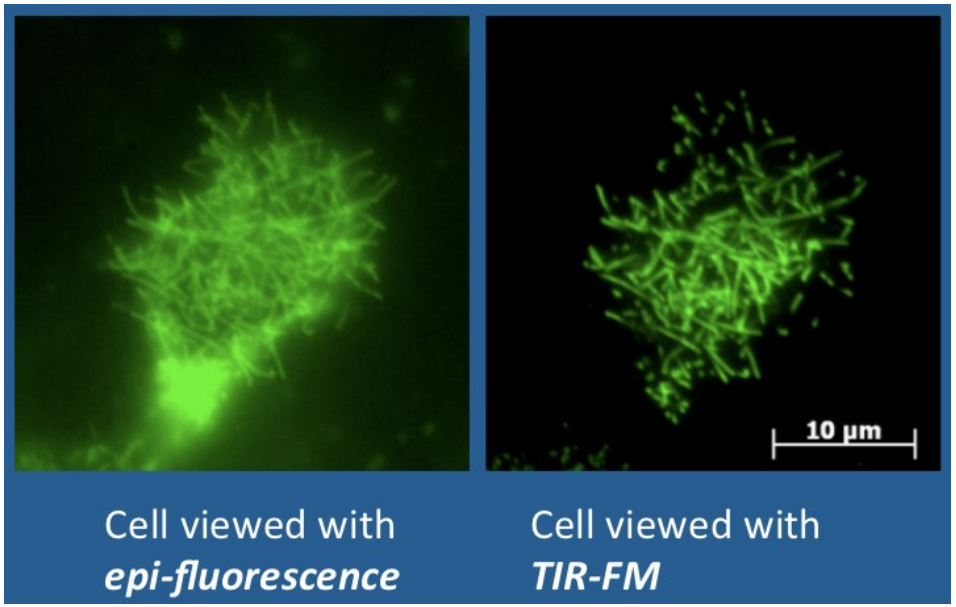



Current Opinion in Structural Biology Volume 14, Issue 5, October 2004, Pages 531–540

# Total Internal Reflection Fluorescence (TIRF)

The excitation via evanescent field of totally internally reflected wave allows for the observation of fluorescence signal from close proximity (<1 µm) to the surface and decreasing background.




http://lightmicroscopy.ucdenver.edu

Mattheyses A L et al. J Cell Sci 2010;123:3621-3628

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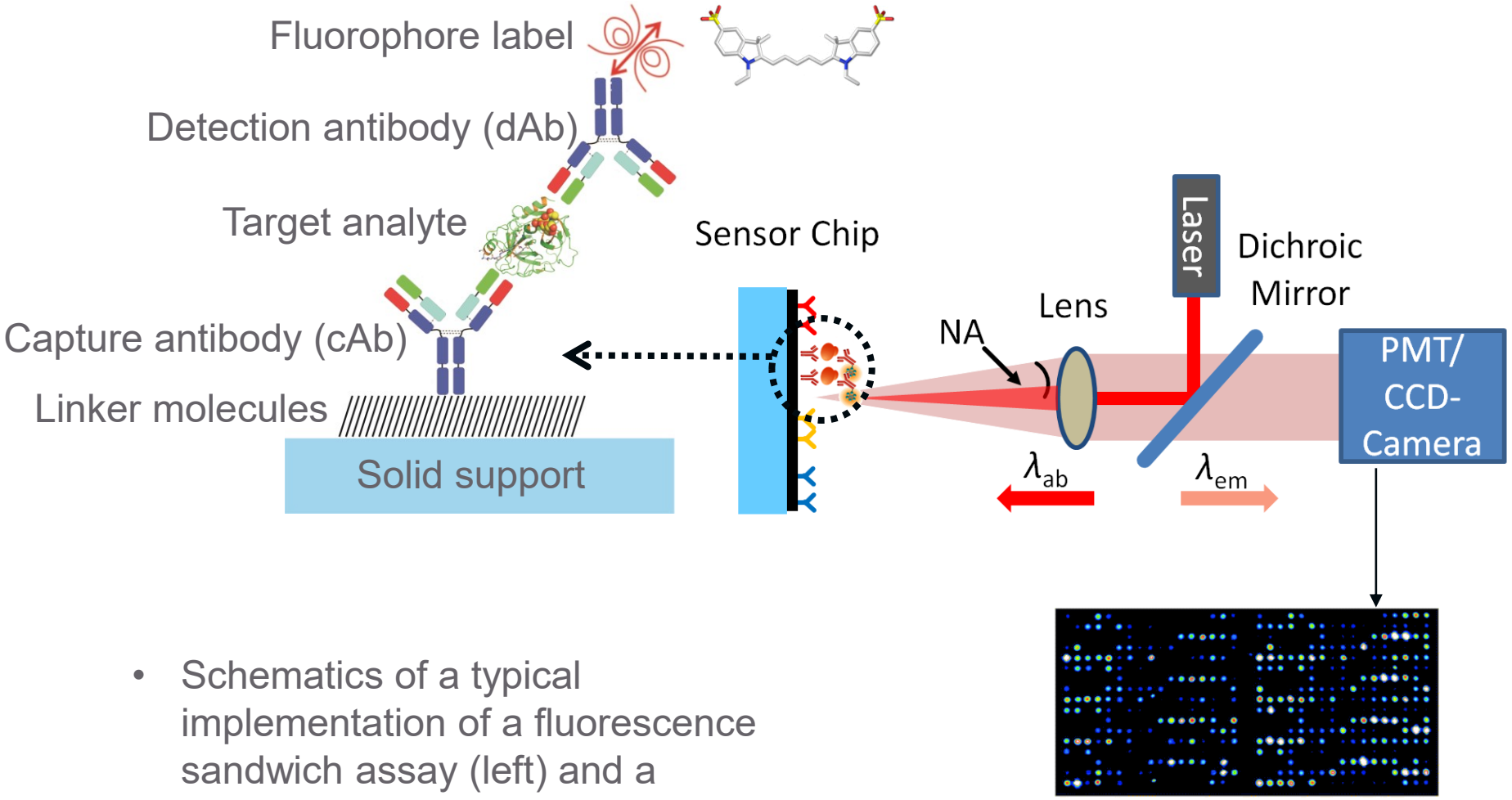
# Fluorescence Immunoassays

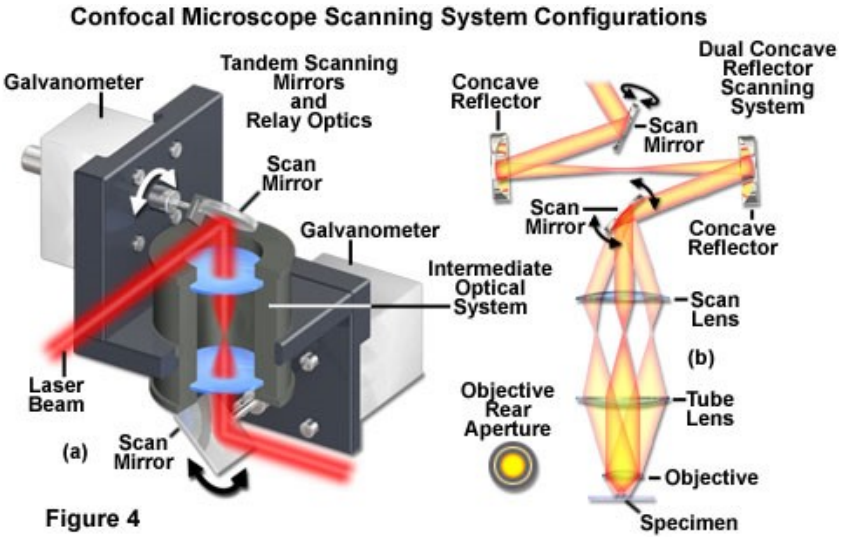


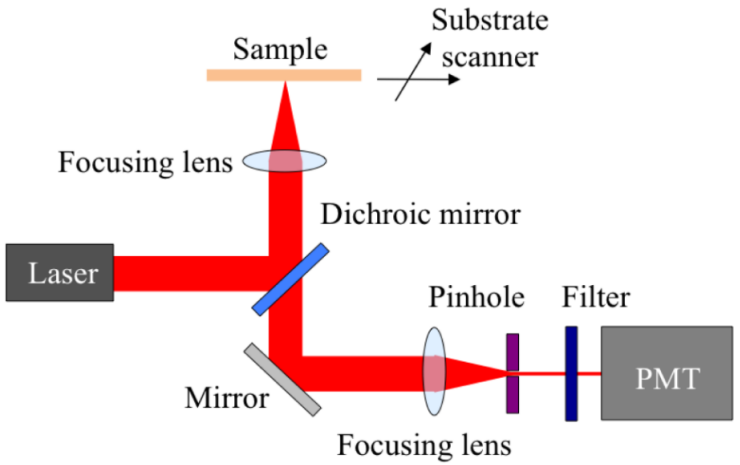

- Schematics of a typical implementation of a fluorescence sandwich assay (left) and a respective reader (right).

# Fluorescence Scanners

https://www.olympus-lifescience.com/fr/microscope-resource/primer/techniques/confocal/confocalscanningsystems/

https://doi.org/10.3390/s130505561

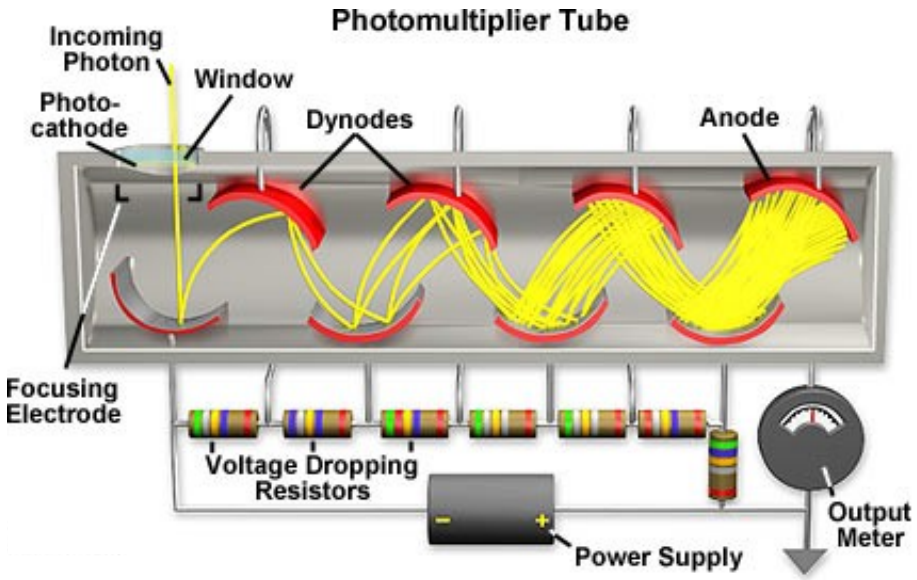



A) Scanning the probing beam across the specimen.

B) Scanning the substrate and keeping the optics fixed.

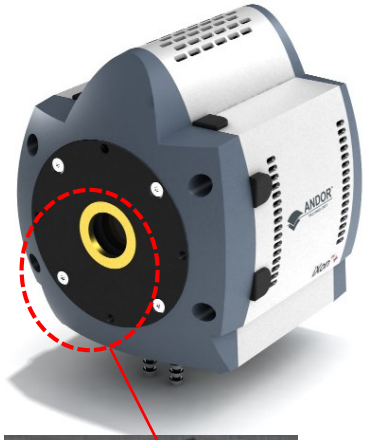
# Photomultiplier

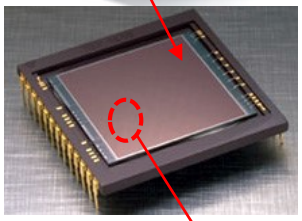


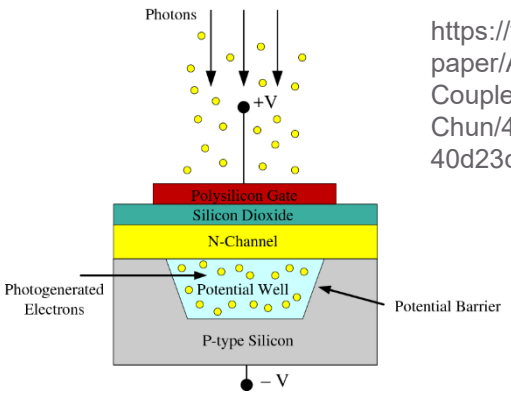

- Detector designed for the measurement of low intensity of light.
- Allows for counting of individual photons (e.g. counts per second – cps, fluorescence rate in Hz)

# Charged Coupled Device - CCD

- Allows for acquisition of the whole image without scanning the beam or the sample.
- Sensitivity can reach single photon counting for each pixel.
- Recently, there are available (comparable) electron multiplying charge-coupled device (EMCCD) or complementary metal oxide semiconductor (CMOS)

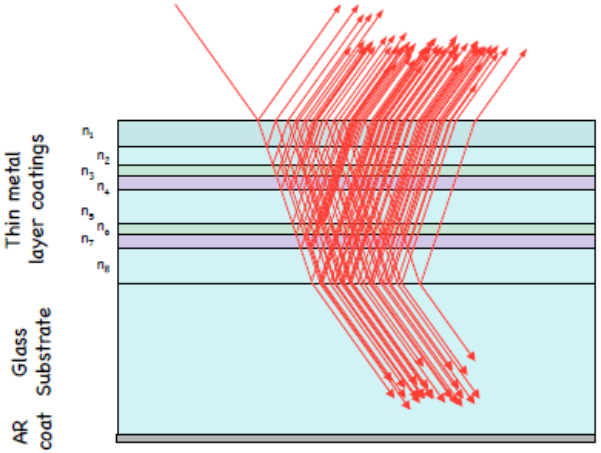


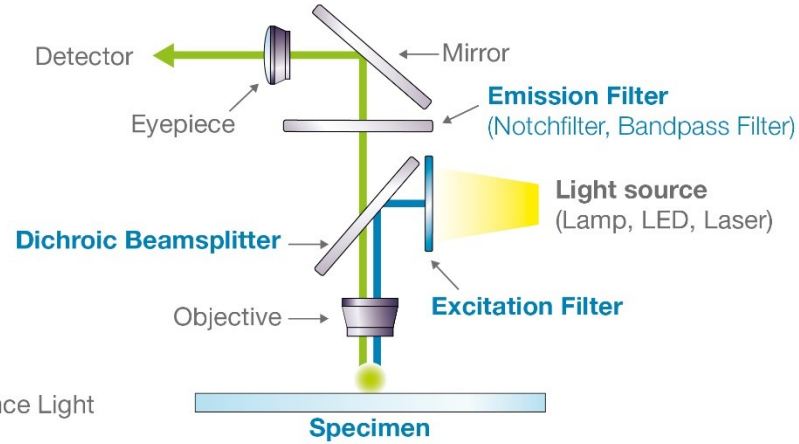



https://www.semanticscholar.org/paper/A-High-Voltage-Charge-Coupled-Device-(CCD)-ASIC-for-Chun/4bceb2f351025c1f4688f56640d23cc0493654e5

# Fluorescence Filters








- Sensitive fluorescence measurement relies heavily on optical filters to spectrally filter the excitation and emission light:

- Laser band pass filter – transmission of only $\lambda_{ex}$
- Emission band pass filter – transmission of only $\lambda_{em}$
- Notch filter – used to stop $\lambda_{ex}$
- Dichroic mirror – high reflectivity for λex and high transmission for $\lambda_{em}$ at tilted angle.

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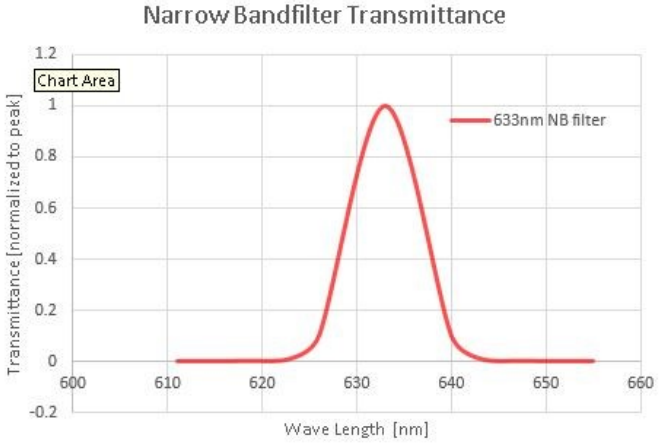
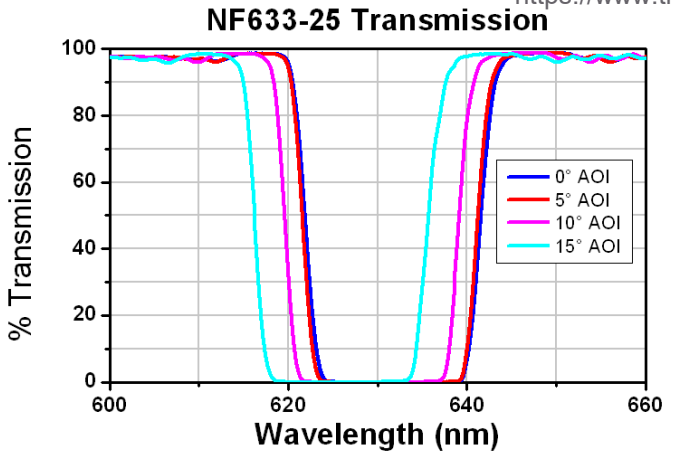
# Fluorescence Filters (Cy5)

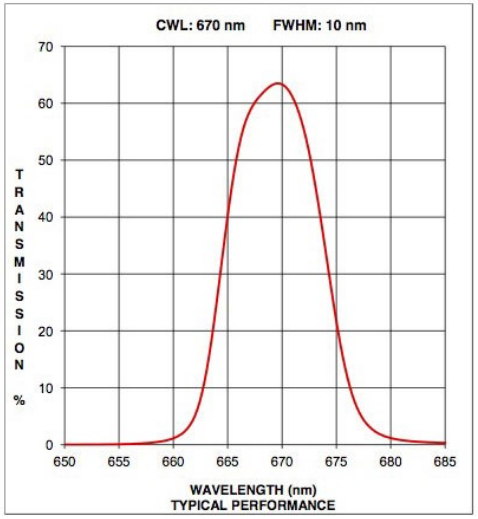
Laser band pass filter




https://www.thorlabs.com/




Emission band pass filter




- Filters needs to be selected with respect the used fluorophore and the excitation wavelength.
- Due to the fact that are based on interference, they are sensitive to angle of incidence.
- The suppression of transmission is quantified by optical density (3-7 orders of magnitude):

$$\mathrm{OD} = \log_{10}\left(\frac{1}{T}\right), \text{ or } \; T = 10^{-\mathrm{OD}}$$

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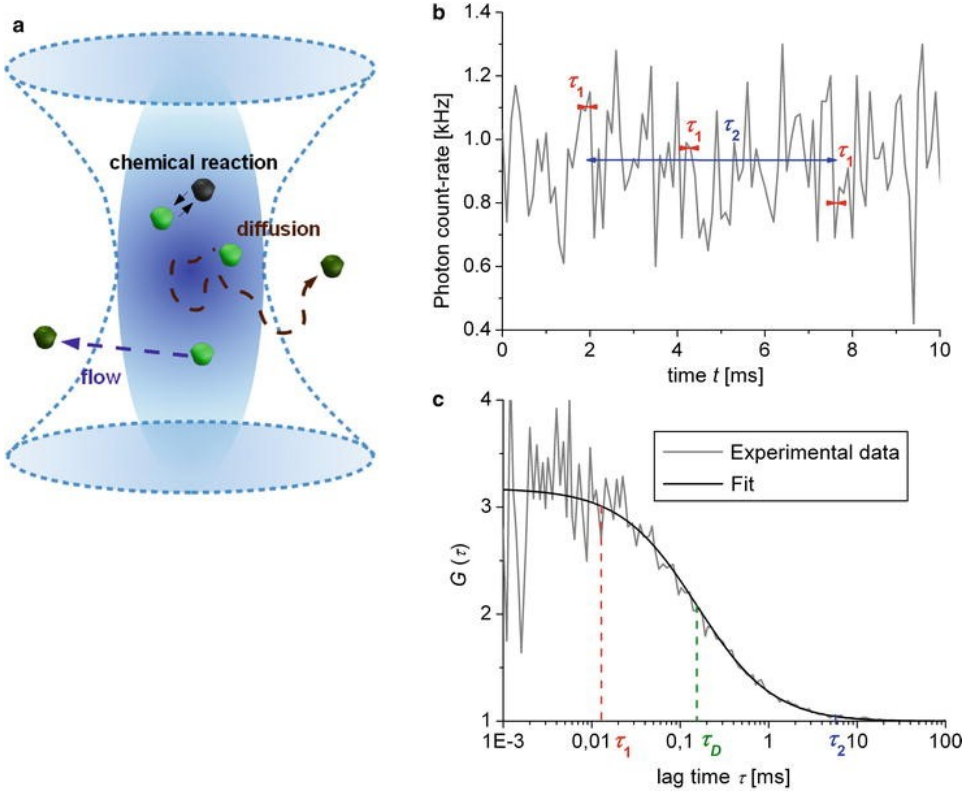
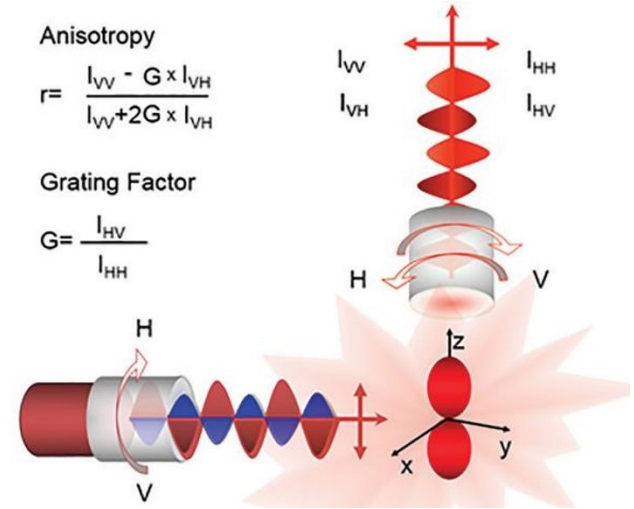
# Fluorescence Correlation Spectroscopy

https://doi.org/10.1007/978-3-642-16712-6_560




- Allows for the measurements of molecules diffusing in and out of the focal volume of the excitation beam.
- The autocorrelation function enables determining of diffusion coefficient, hydrodynamic radius, reaction rates....

# Fluorescence Anisotropy

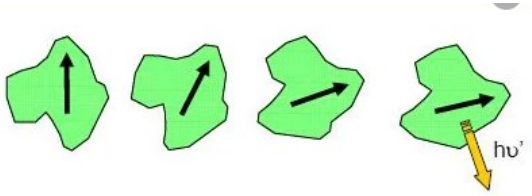



https://www.horiba.com/

- Rotational correlation time: $\phi_r = \frac{\eta V}{k_B T}$

  (viscosity ($\eta$), temperature (T), Boltzmann constant ($k_B$) and volume ($V$) of the nanoparticle)

- Tumbling rate (for spherical object) $r(t) = r_0 \exp\left(-\frac{t}{\phi_r}\right)$




Fluorescence anisotropy can be used to measure the binding constants and kinetics of reactions that cause a change in the rotational time of the molecules.