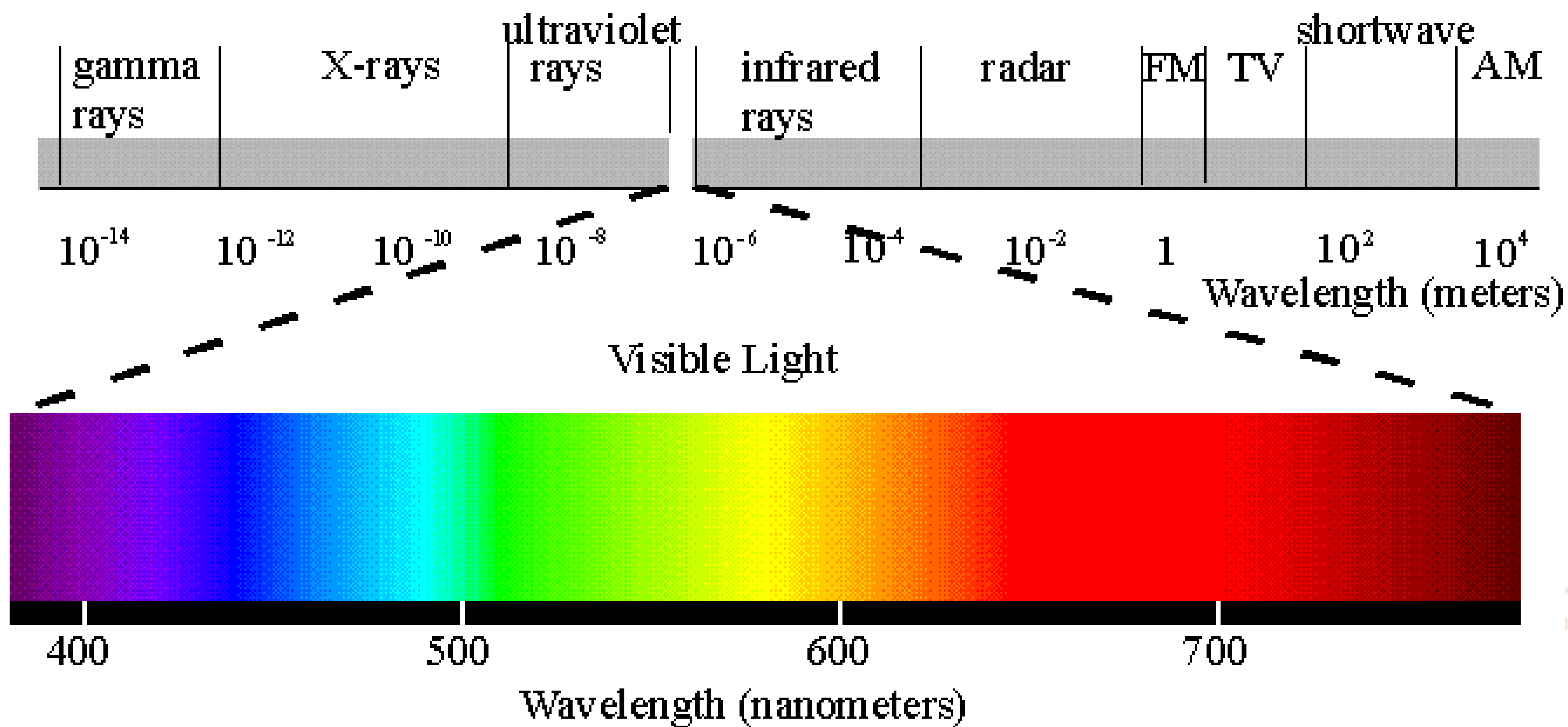
Spectroscopic and related methods

S2004
Methods for characterization of biomolecular interactions – classical versus modern

Mgr. Josef Houser, Ph.D.
houser@mail.muni.cz

Spectrum of light



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Spectroscopic methods

- **UV/Vis spectroscopy**
- **Fluorescence spectroscopy**

    + Fluorescence resonance energy transfer (FRET)
- **Circular dichroism** (CD)
- Static and Dynamic **light scattering** (SLS, DLS)
- Fourier transformed **infrared spectroscopy** (FTIR)

    + ATR-FTIR – attenuated total reflectance
- Surface enhanced **Raman spectroscopy** (SERS)
- Nuclear magnetic resonance (NMR)
- Surface plasmon resonance (SPR)
- Micro-scale thermophoresis (MST)

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UV/VIS absorption

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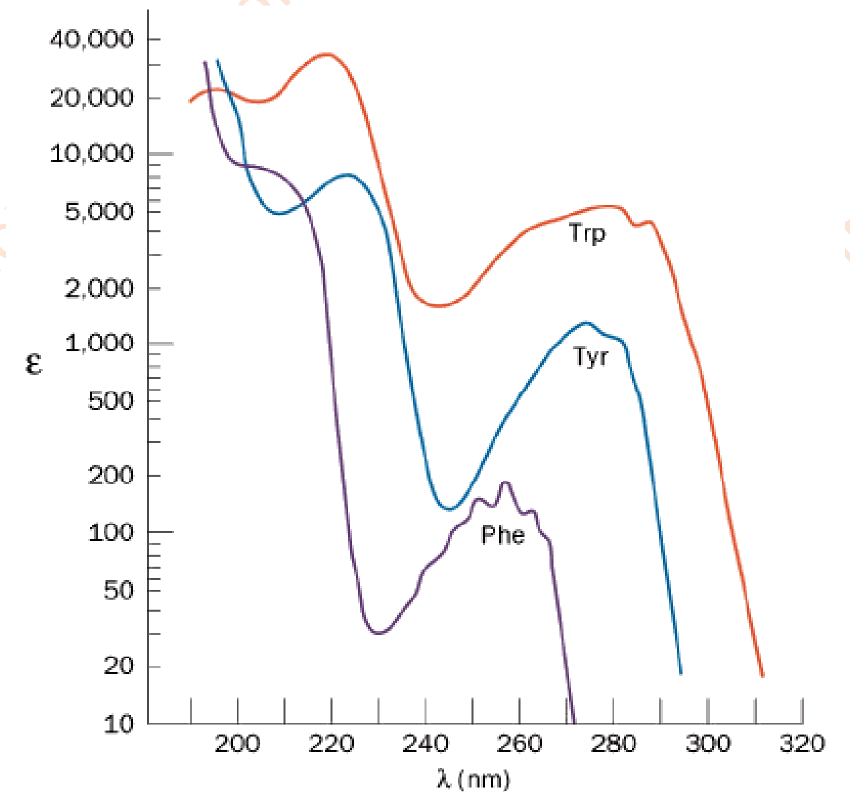
UV/VIS spectroscopy

Wavelegths: UV 180 – 350 nm (160 – 380 nm)
VIS 380 – 750 nm

Absorption spectroscopy – absorption of light of given wavelength

Proteins – 250-300 nm – aromatic AA
<220 – peptide bond
>300 nm – colourful proteins
Nucleic acids – 240-300 nm



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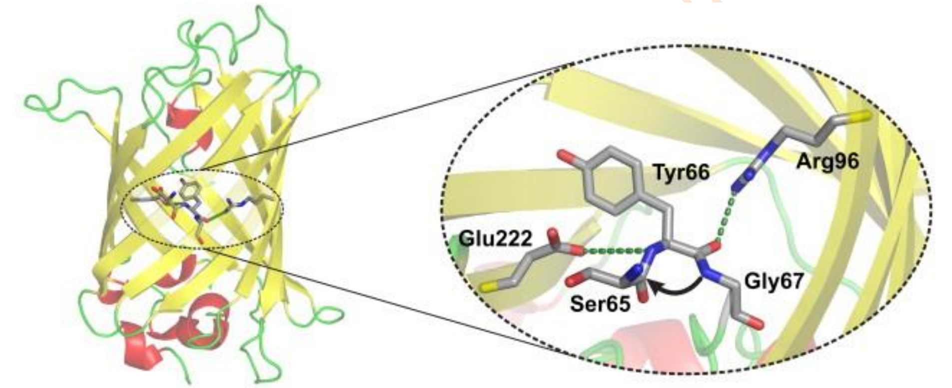
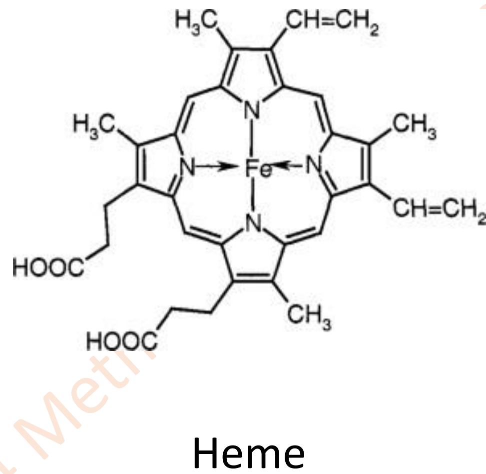
UV/VIS spectroscopy

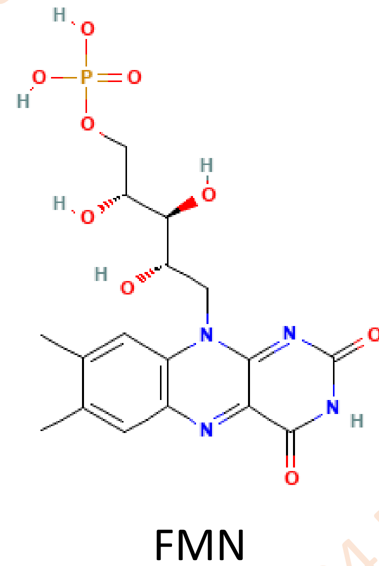
Coloured proteins:

- Special **amino acid arrangement – e.g. GFP**
- **Prosthetic groups** – heme, flavin,...

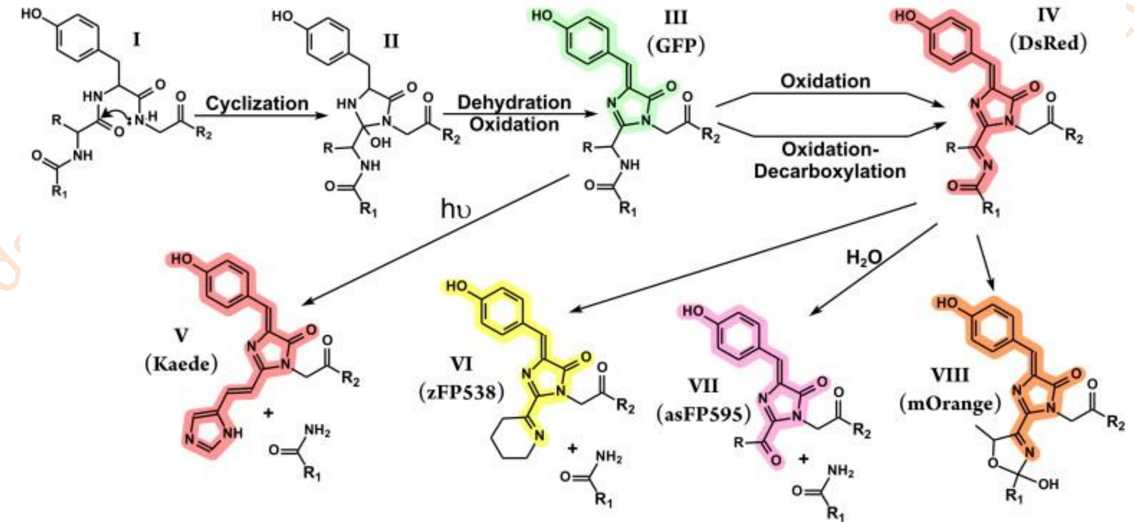




Heme



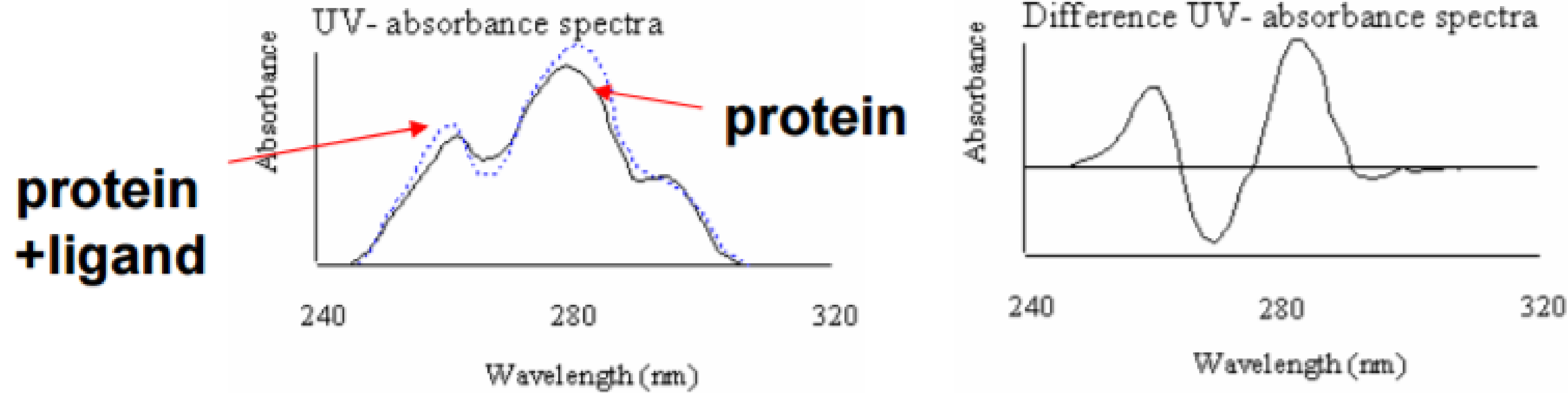
FMN



Pakhomov 2008, Cell

UV/VIS spectroscopy

Analysis of absorption spectra with and without ligand
Binding in Trp proximity distinguishable



Mainly for relatively low concentrations (nonlinearity at high OD)

Interaction of protein at high density (Ab drugs) – based on non-ideality in spectra measured

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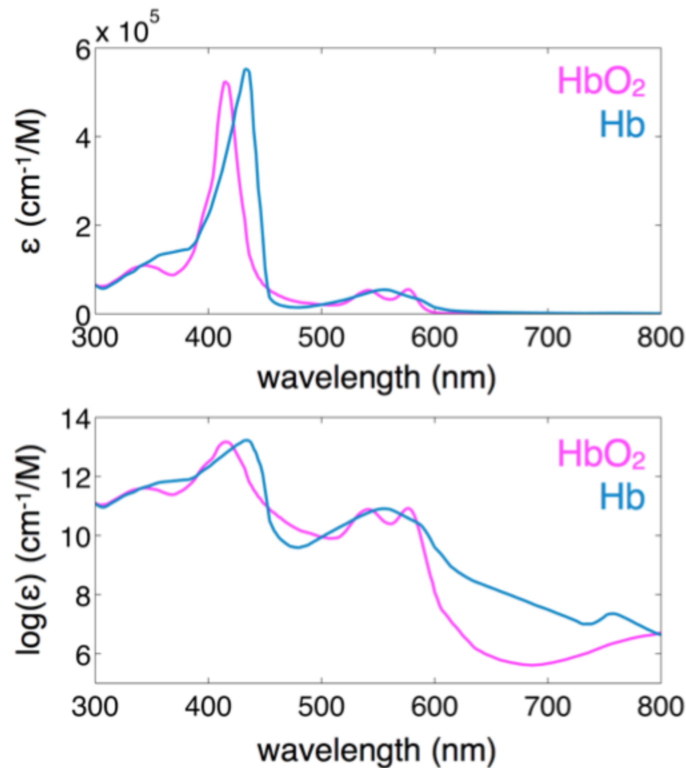
UV/VIS spectroscopy

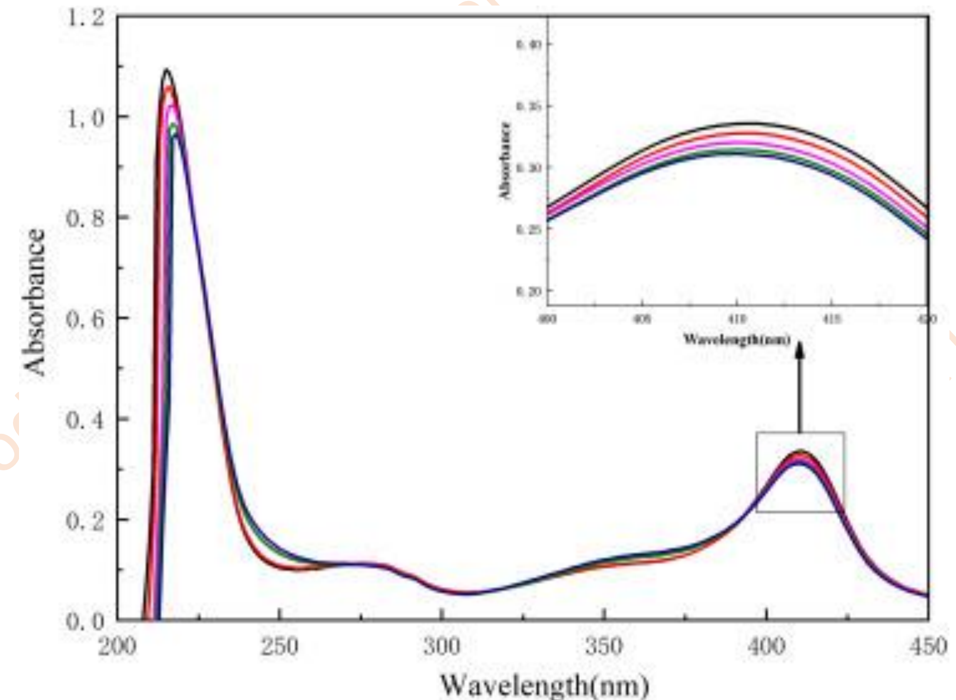
Hemoglobin – interaction with O_2, drugs/inhibitors

Spectrum changes higher for chromophore proximity



chegg.com

Hemoglobin – levamlodipine



Xu 2019, Spectrochimica Acta A

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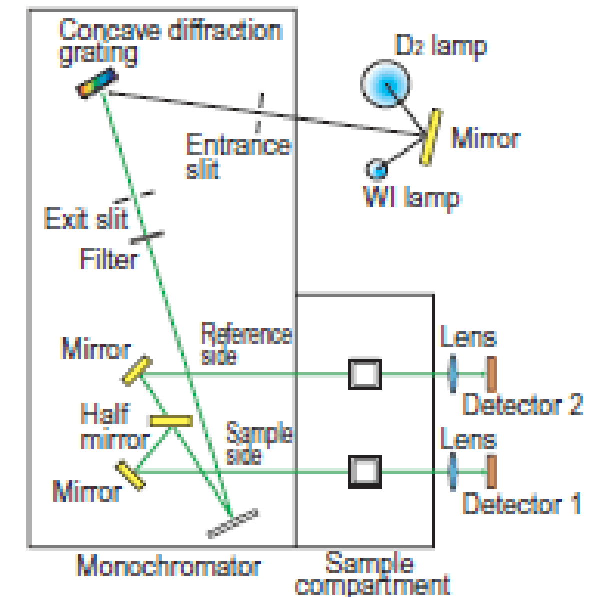
UV/VIS spectra measurement

Spectrometers

- Various wavelength range
- Single / Dual beam

Cuvettes

- Optical glass (VIS) or Quartz (UV)
- Fixed path length (0.01 – 10 mm)
- Demountable cuvettes – lower accuracy



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Fluorescence

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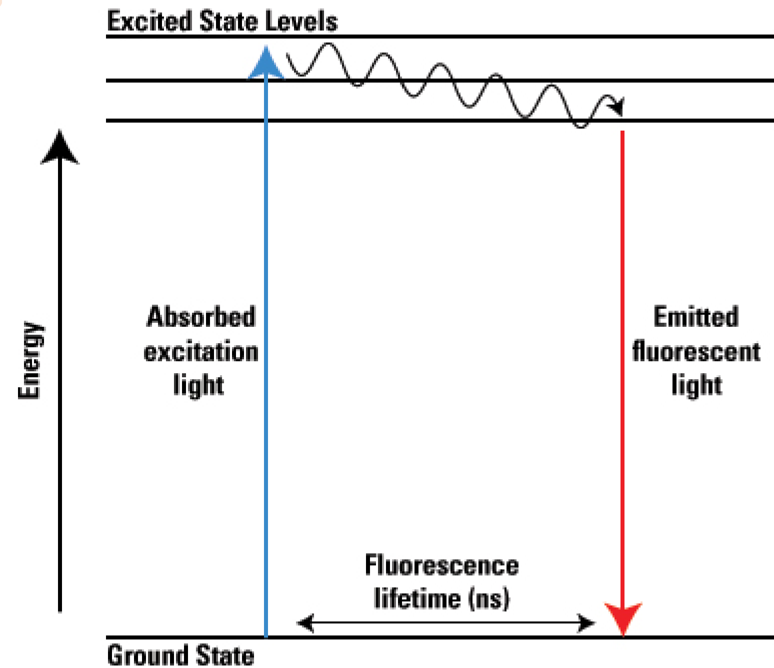
Fluorescence spectroscopy

Fluorescence

- Excitation – higher energy ~ shorter wavelength
- Emission – lower energy ~ longer wavelength (red shift)



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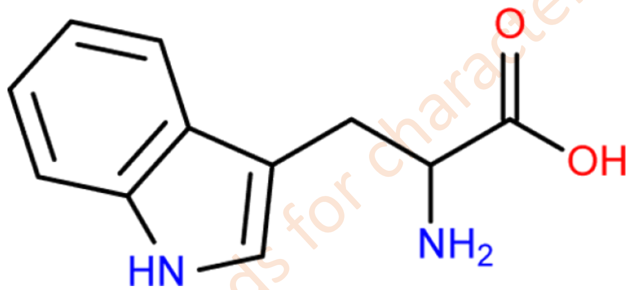
Fluorescence spectroscopy

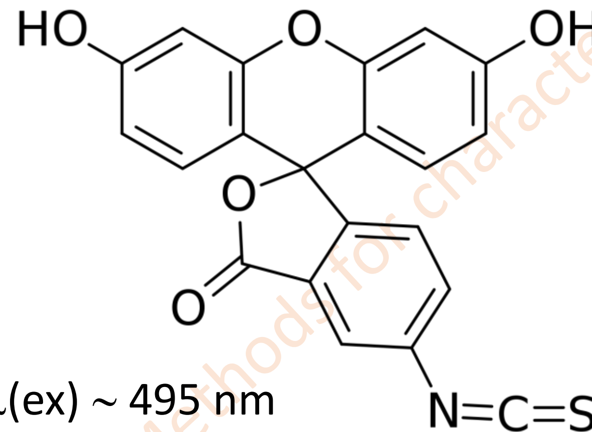
Fluorophores

- Dyes
- Fluorescent proteins (GFP, YFP,…)
- **Tryptophan** – intrinsic fluorescence



Tryptofan

λ(ex) ~ 280 nm
λ(em) ~ 350 nm



λ(ex) ~ 495 nm
λ(em) ~ 519 nm

FITC (fluorescein isothiocyanate)

Dye Name	Excitation Max, nm	Emission Max, nm	Color
Alexa 350	346	442	Blue
Pacific Blue	416	451	
Marina Blue	362	459	Blue-Green
Acridine	362	462	
Edans	336	468	
Coumarin	432	472	
BODIPY 493/503	493	503	
Cy2	489	506	Green
BODIPY FL-X	504	510	
DANSYL	335	518	
Alexa 488	495	519	
FAM	495	520	
Oregon Green	500	520	
Rhodamine Green-X	503	528	
NBD-X	466	535	
TET	521	536	
Alexa 430	434	541	Yellow-Green
BODIPY R6G-X	529	547	
JOE	520	548	
Yakima Yellow	531	549	
Alexa 532	532	554	
VIC	538	554	
HEX	535	556	
R6G	524	557	Yellow
Alexa 555	555	565	
BODIPY 564/570	563	569	
BODIPY TMR-X	544	570	
Cy3	550	570	
Alexa 546	556	573	
TAMRA	555	576	
Rhodamine Red-X	560	580	
BODIPY 581/591	581	591	
Redmond Red	579	595	
Cy3.5	581	596	Yellow-Orange
ROX	575	602	
Alexa 568	578	603	
Cal Red	583	603	Orange
BODIPY TR-X	588	616	Orange-Red
Alexa 594	590	617	
BODIPY 630/650-X	625	640	
LC Red 640	625	640	
Alexa 633	632	647	
BODIPY 650/665-X	646	660	
Alexa 647	650	665	
Cy5	649	670	Red
Alexa 660	663	690	
Cy5.5	675	694	
Alexa 680	679	702	
LC Red 705	689	705	
Alexa 700	702	723	
Alexa 750	749	775	Far Red

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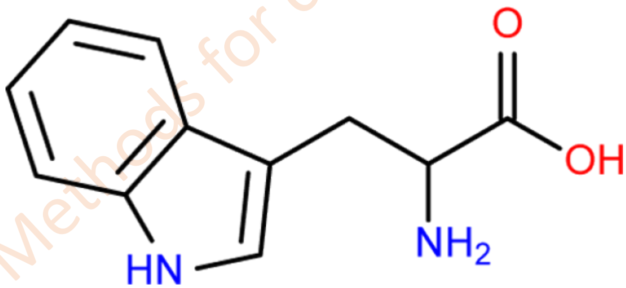
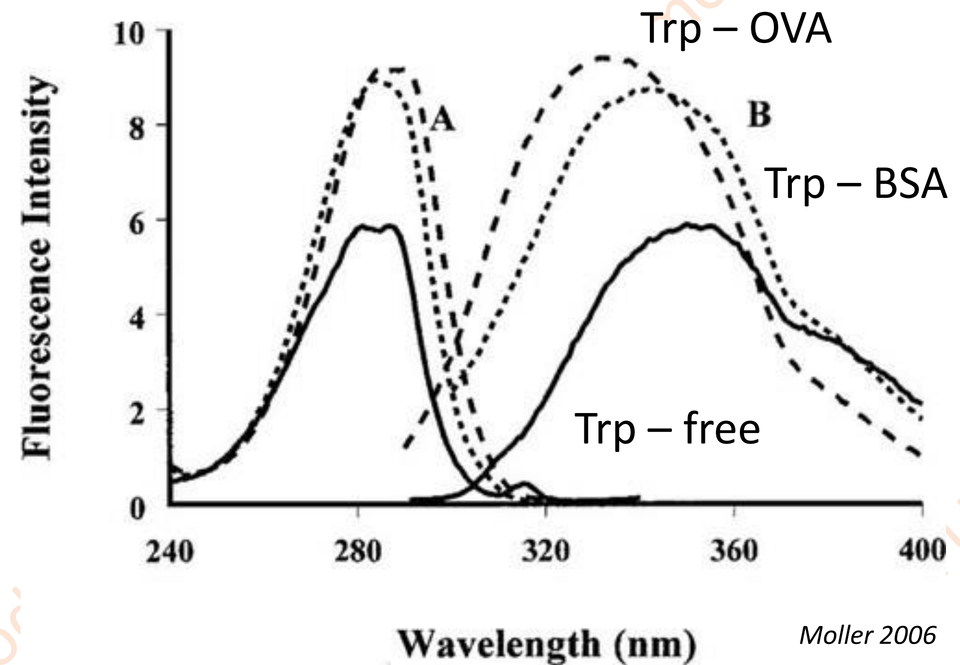
Fluorescence spectroscopy

Influence on fluorescence

- Environment polarity
- Solvent viscosity
- Probe conformational changes
- pH (protonation)
- …



λ(ex) ~ 280 nm
λ(em) ~ 350 nm



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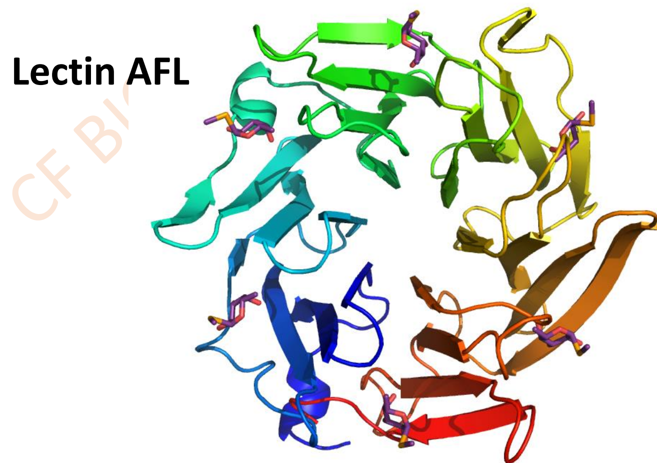
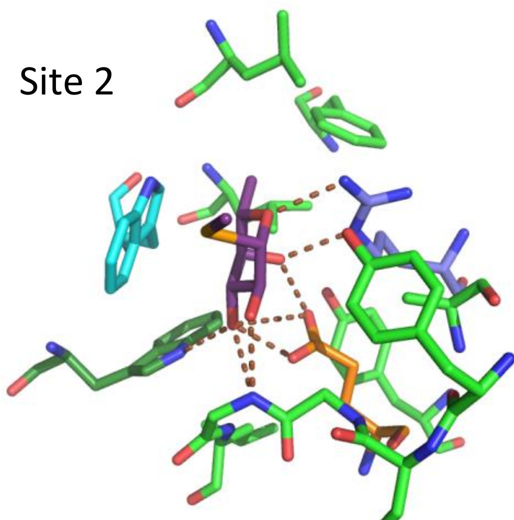
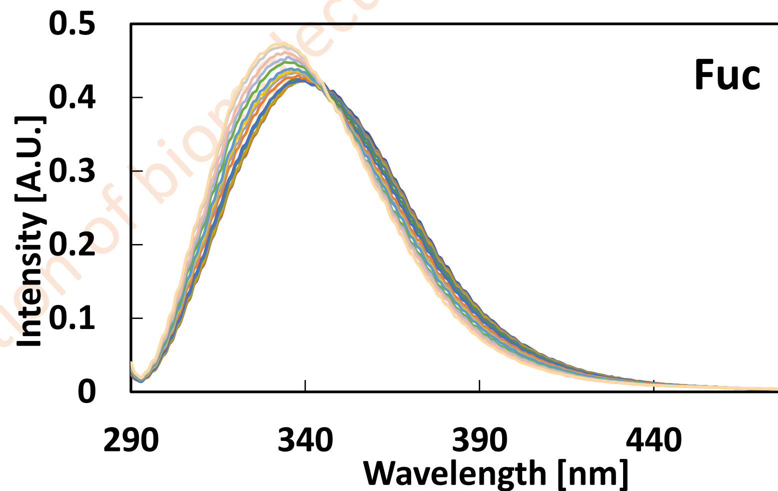
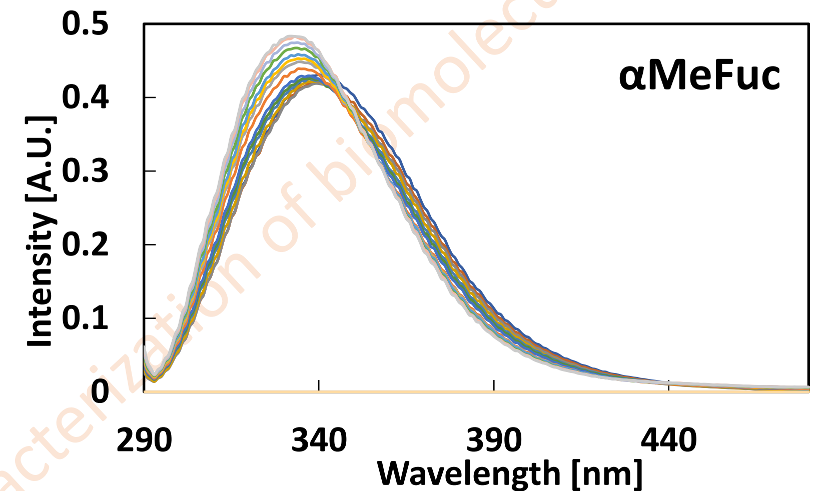
Fluorescence spectroscopy

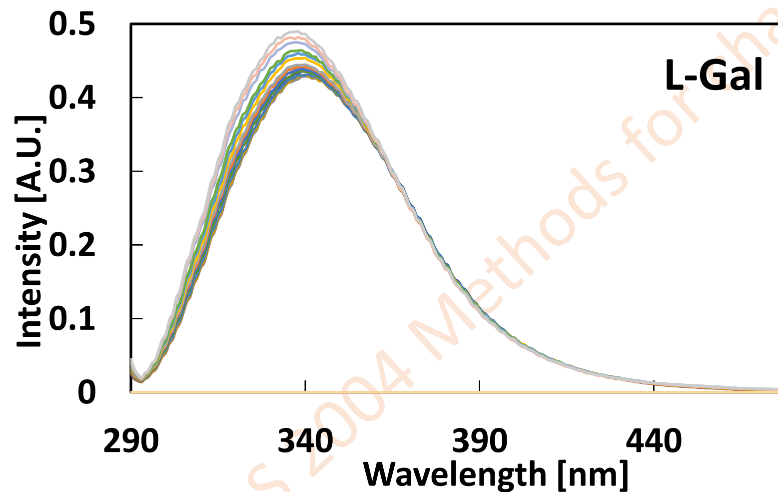
- Change of fluorescence upon ligand binding

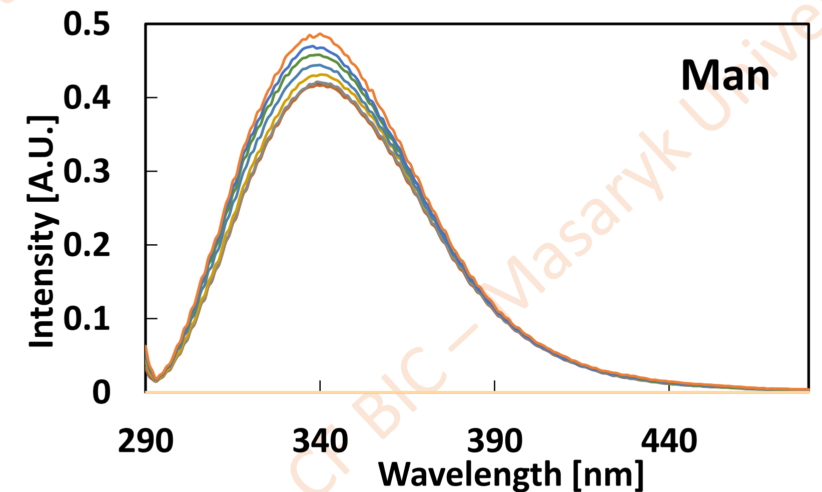












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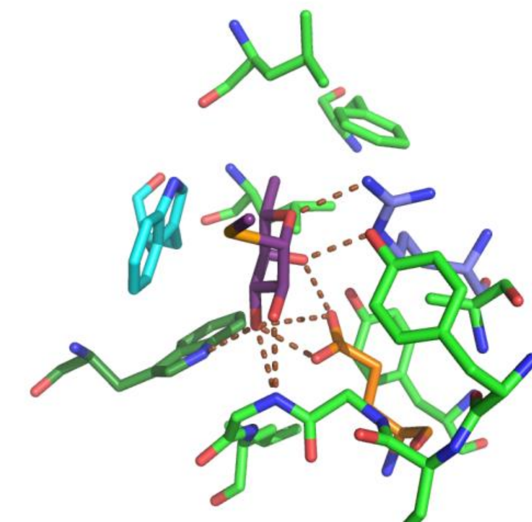
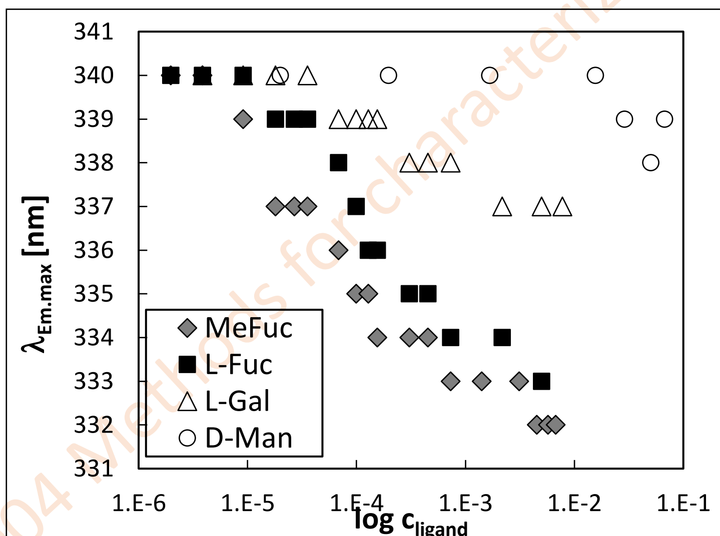
Fluorescence spectroscopy

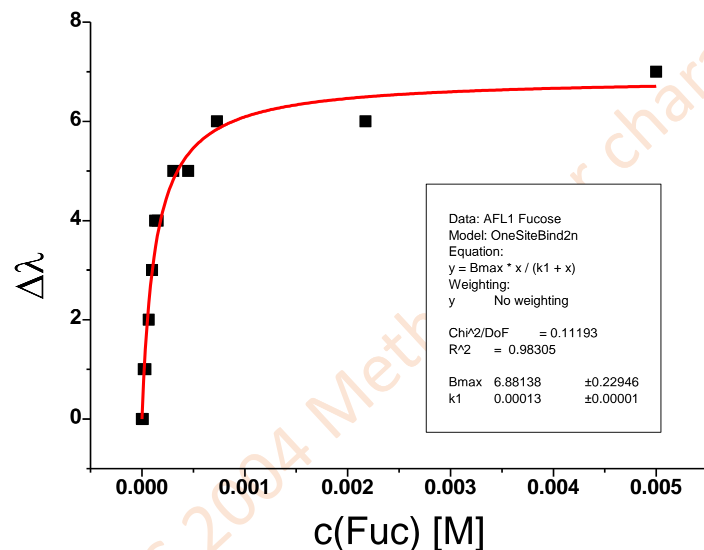


Properties to analyze:

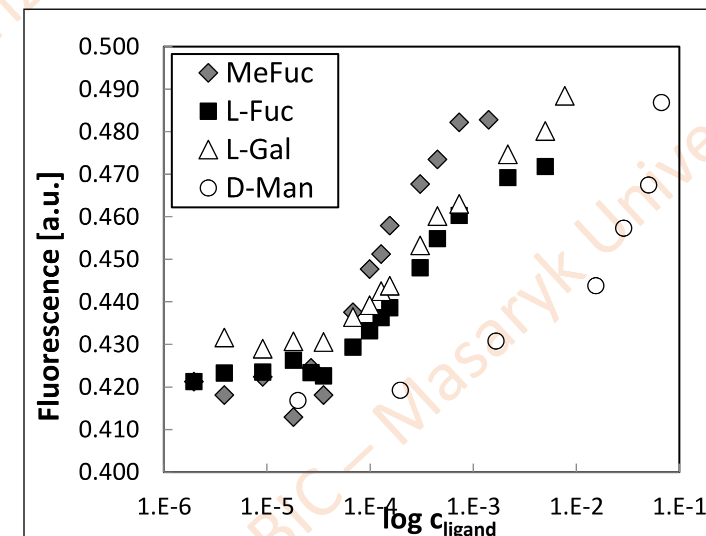
- Excitation/emission maximum
- Quantum yield – fluorescence intensity

λ(max)





Total fluorescence (quantum yield)



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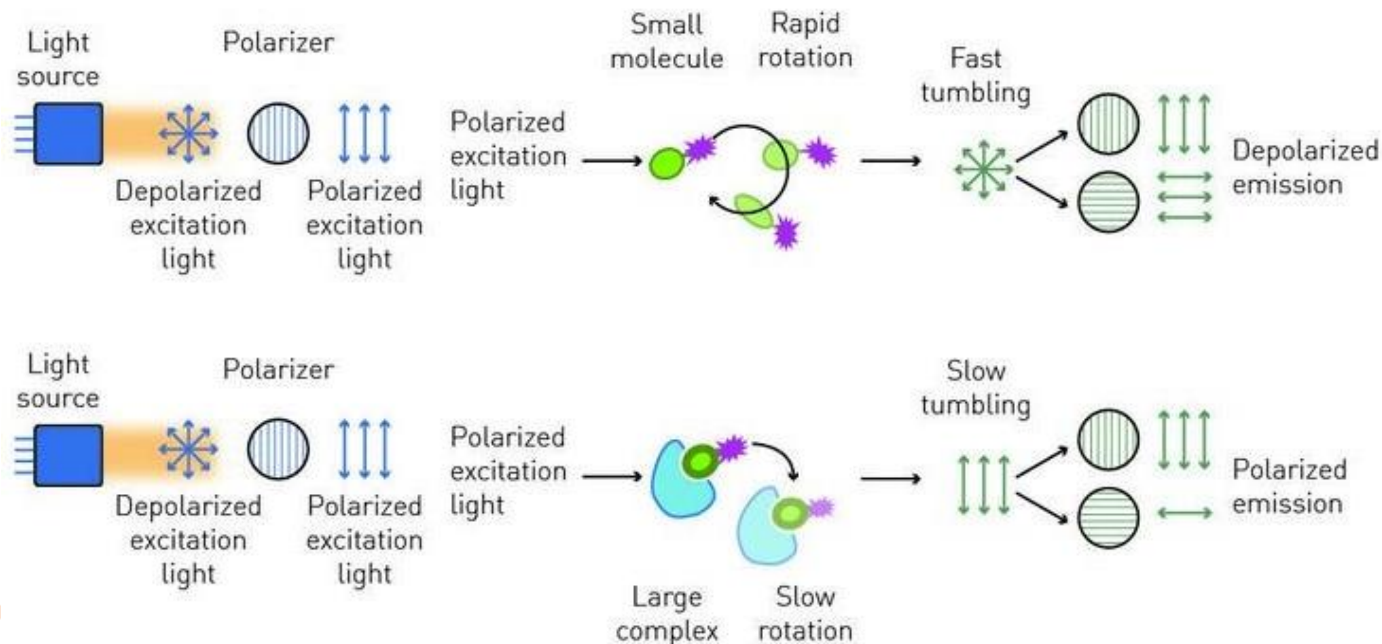
Fluorescence anisotropy

= fluorescence polarization

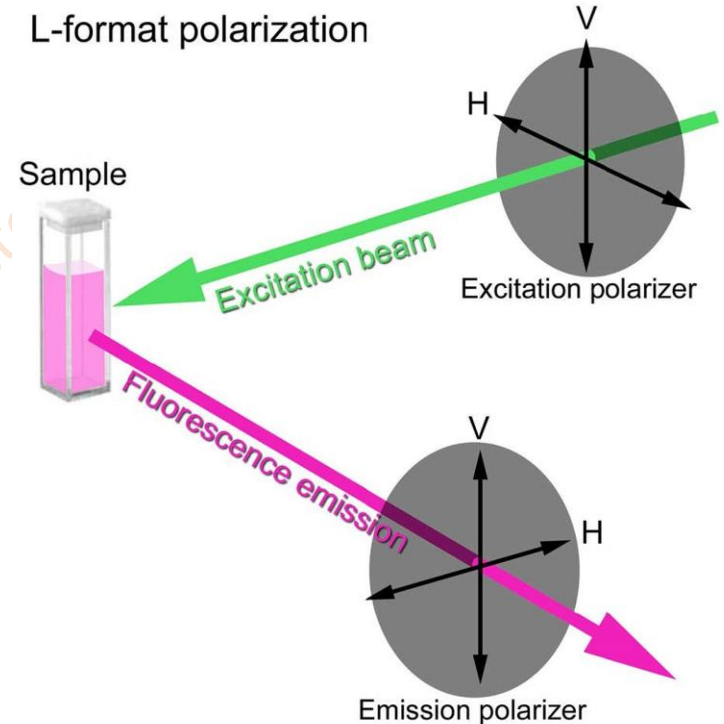
- Change of fluorescence polarization upon interaction
- Faster for smaller particles
- Influenced by viscosity and temperature

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$

VV denotes vertical excitation, vertical emission
VH denotes vertical excitation, horizontal emission



BMG Labtech



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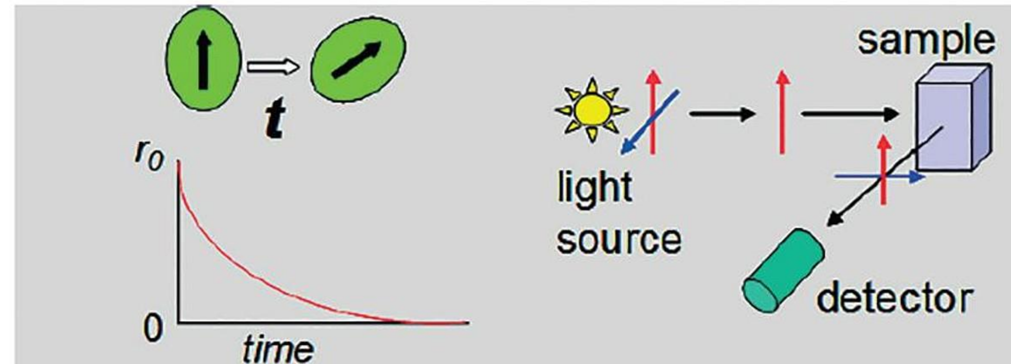
Fluorescence anisotropy

- Two ways of analysis:
  - Steady state – various ligand conc.
  - Anisotropy decay over time
- Fit of data by binding curve
- Free vs. bound ligand – FA difference



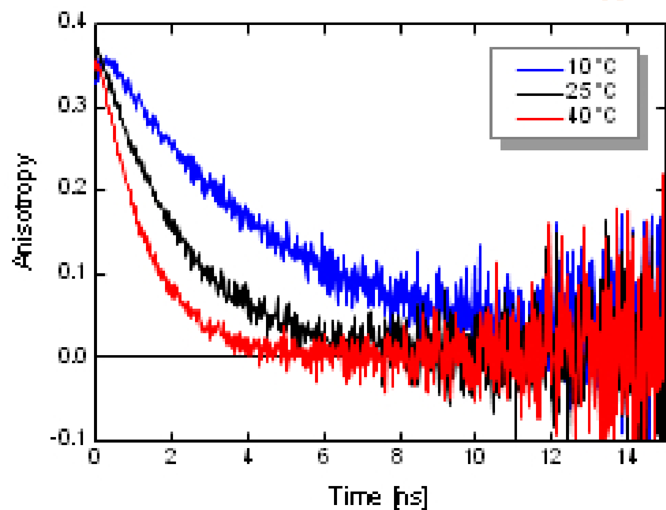
steady state

$$r = [I_{||} - I_{\perp}] / [I_{||} + 2I_{\perp}]$$

time-resolved

$$r(t) = r_\infty + (r_0 - r_\infty) \exp(-t/\tau_R)$$

$$\tau_R = V\eta / kT \qquad 2\left(\frac{r_\infty}{r_0}\right)^{1/2} = \cos^2\theta + \cos\theta$$



Europe PMC Funders Group
Author Manuscript
Nat Protoc. Author manuscript; available in PMC 2011 September 03.

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Nat Protoc. 2011 March ; 6(3): 365–387. doi:10.1038/nprot.2011.305.

Analysis of protein-ligand interactions by fluorescence polarization

Ana M. Rossi and **Colin W. Taylor**
Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1PD, UK.

Abstract

Quantification of the associations between biomolecules is required both to predict and understand the interactions that underpin all biological activity. Fluorescence polarization (FP) provides a non-disruptive means of measuring the association of a fluorescent ligand with a larger molecule. We describe an FP assay in which binding of fluorescein-labelled inositol 1,4,5-trisphosphate (IP_3) to N-terminal fragments of IP_3 receptors can be characterised at different temperatures and in competition with other ligands. The assay allows the standard Gibbs free energy (ΔG°), enthalpy (ΔH°) and entropy (ΔS°) changes of ligand binding to be determined. The method is applicable to any purified ligand-binding site for which an appropriate fluorescent ligand is available. FP can be used to measure low-affinity interactions in real-time without use of radioactive materials, it is non-destructive, and with appropriate care it can resolve ΔH° and ΔS°. The first part of the protocol, protein preparation, may take several weeks, while the FP measurements, once they have

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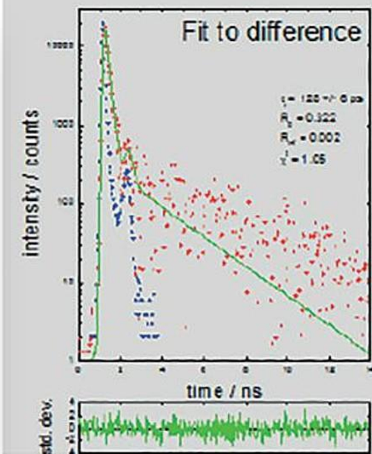

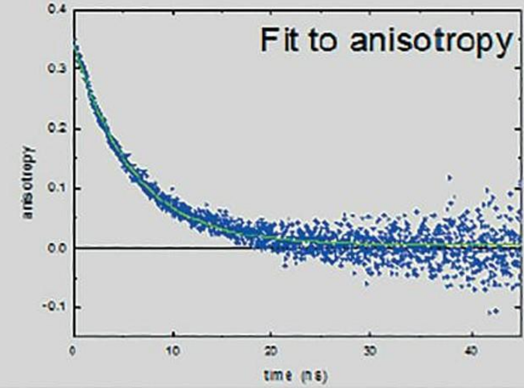

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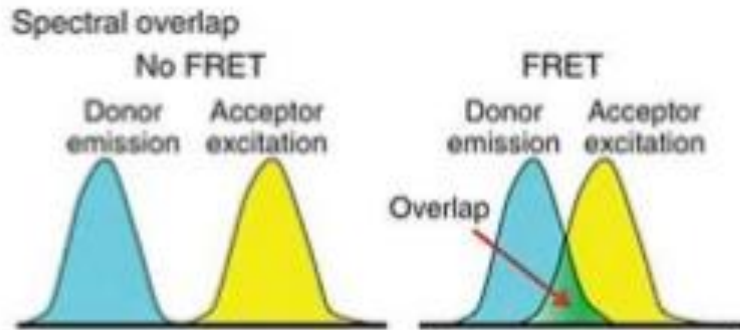
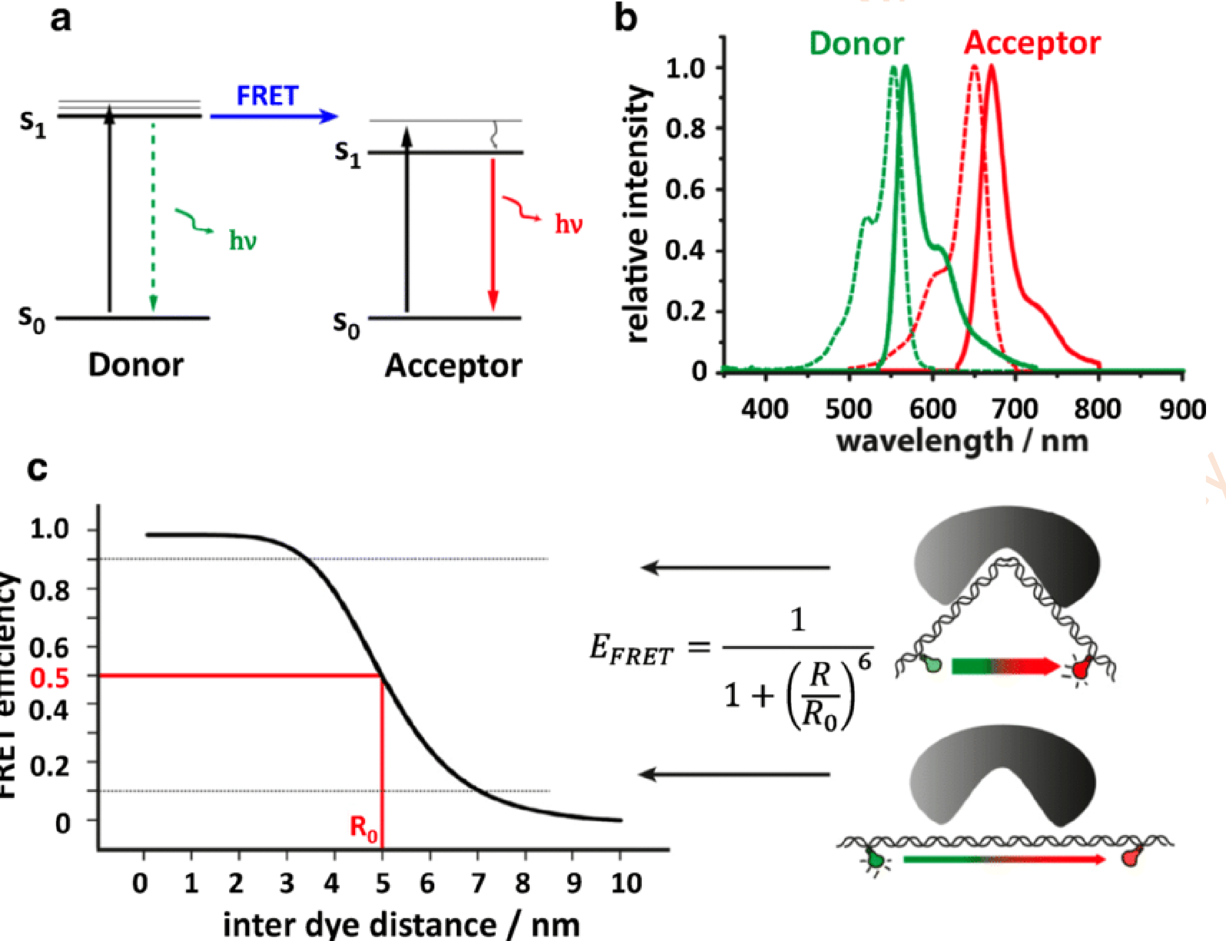
FRET (Fluorescence resonance energy transfer)

Two fluorescent dyes

- Dye 1 excited by specific wavelength
- Dye 1 emission is able to excite Dye 2, when close
- Dye 2 emission is measured



From: Broussard et al. 2013; *nature protocols*



Pehlivan 2019, Microchimica Acta

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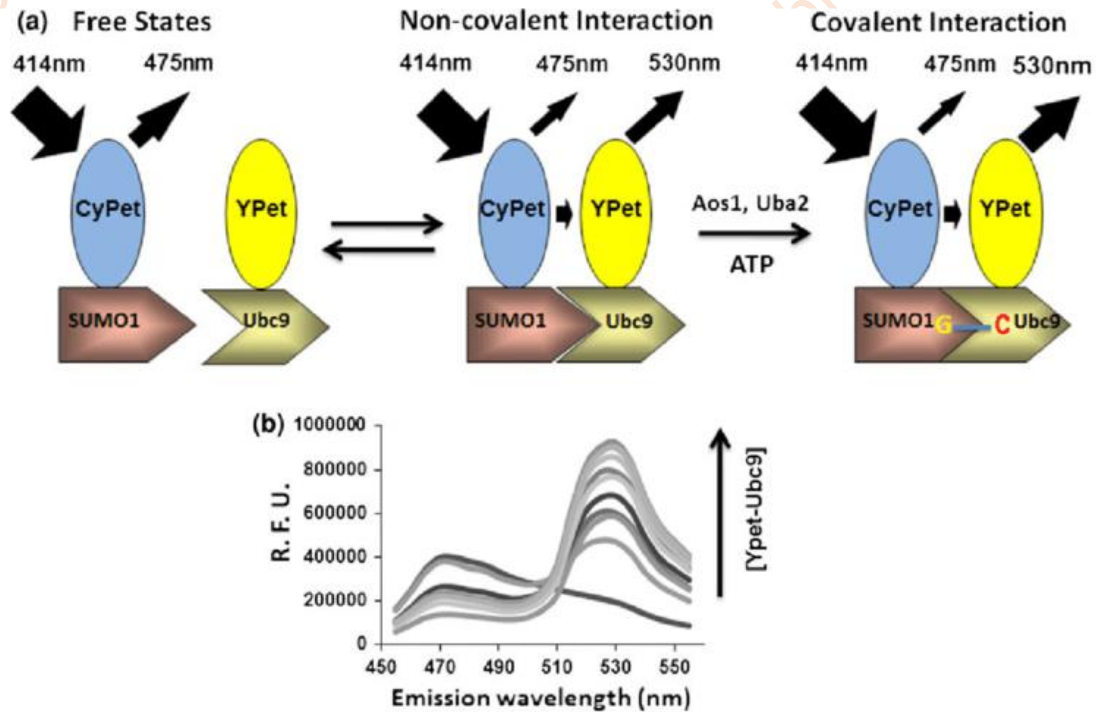
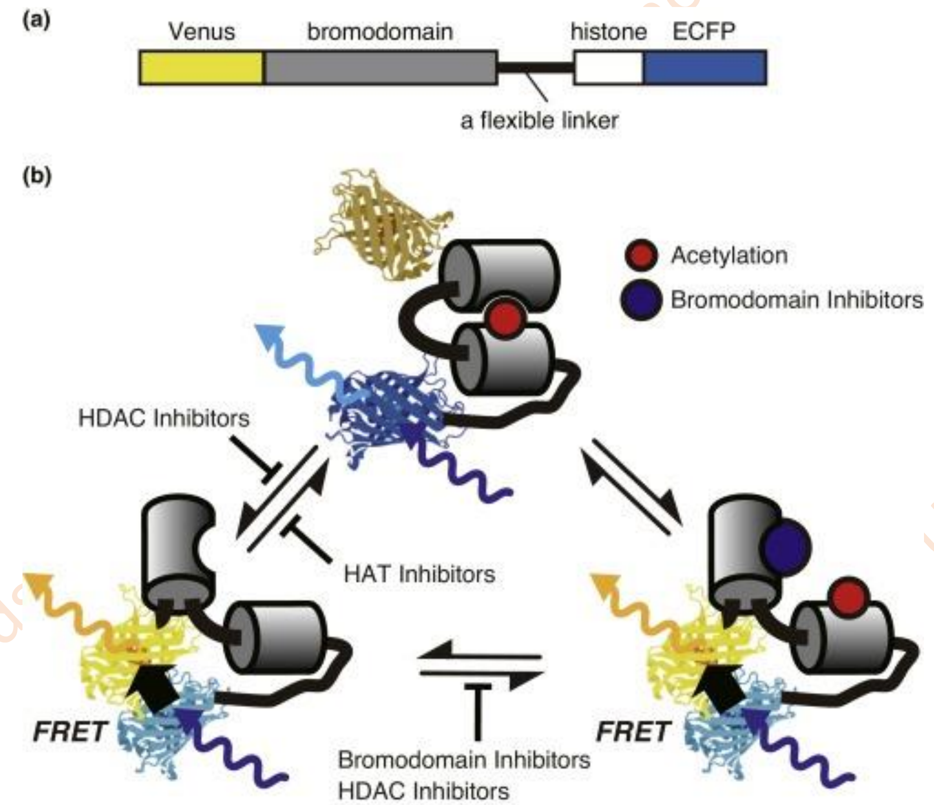
FRET (Fluorescence resonance energy transfer)

Example: FRET-based detection of SUMO1 and its E2 ligase, Ubc9, interaction.



Song 2010, Annals of Biomedical Engineering

Example: Tracking of bromodomain/ histone interactions in cells



Sasaki & Yoshida 2016, Drug Discovery Today: Technologies

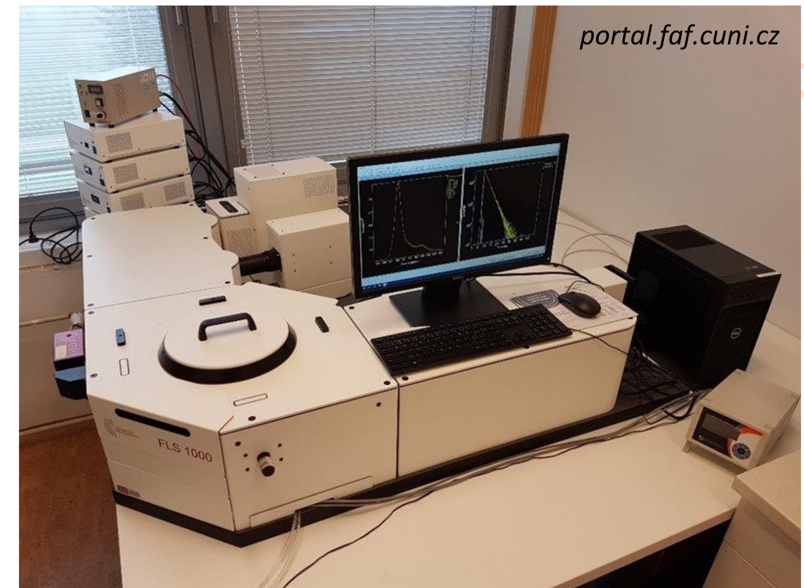
Fluorescence measurement

Fluorimeters – dedicated spectrometers
Monochromator/filters
Excitation and emission spectra
90° fluorescence measured

Cuvettes
Quartz or optical glass

Coupled to **imaging** – fluorescence microscopy

Labeling
intrinsic, chemical
in situ, co-expression



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Circular dichroism

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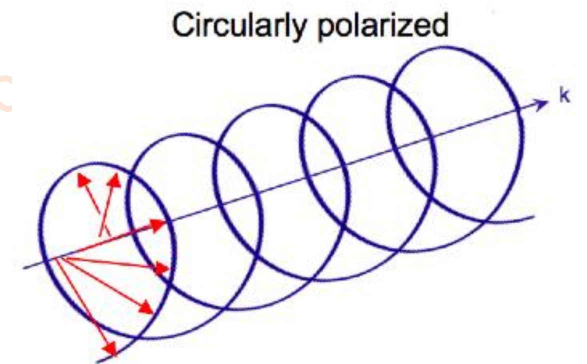
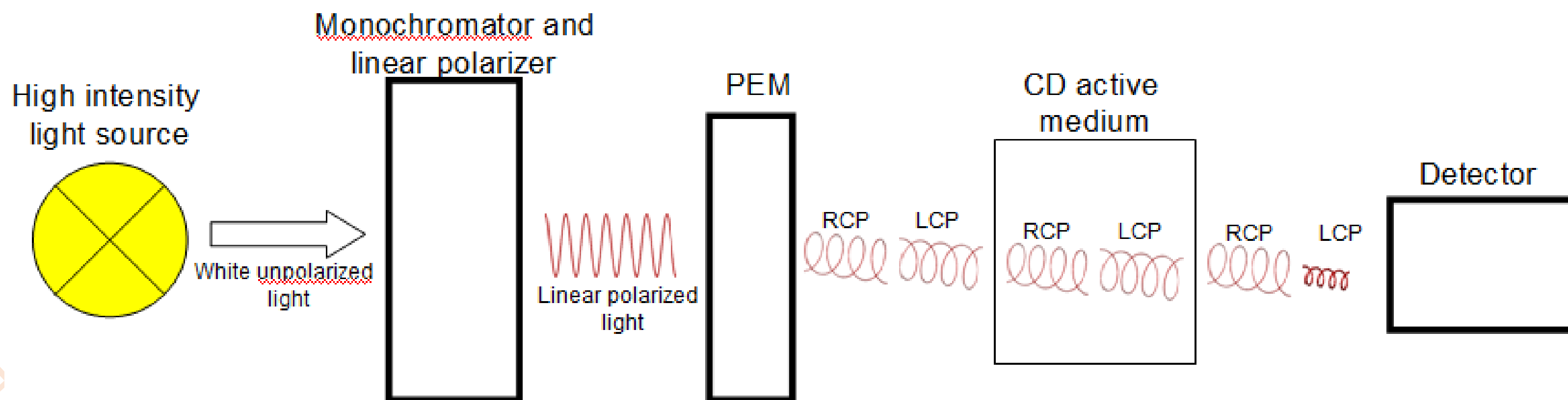
Circular dichroism spectroscopy (CD)

- CD is the difference in absorption of left and right circularly polarized light
- Proteins and nucleic acids are chiral = CD active

$$\Delta A = A_L - A_R = \Delta\varepsilon cl = (\varepsilon_L - \varepsilon_R)cl$$





chem.libretexts.org

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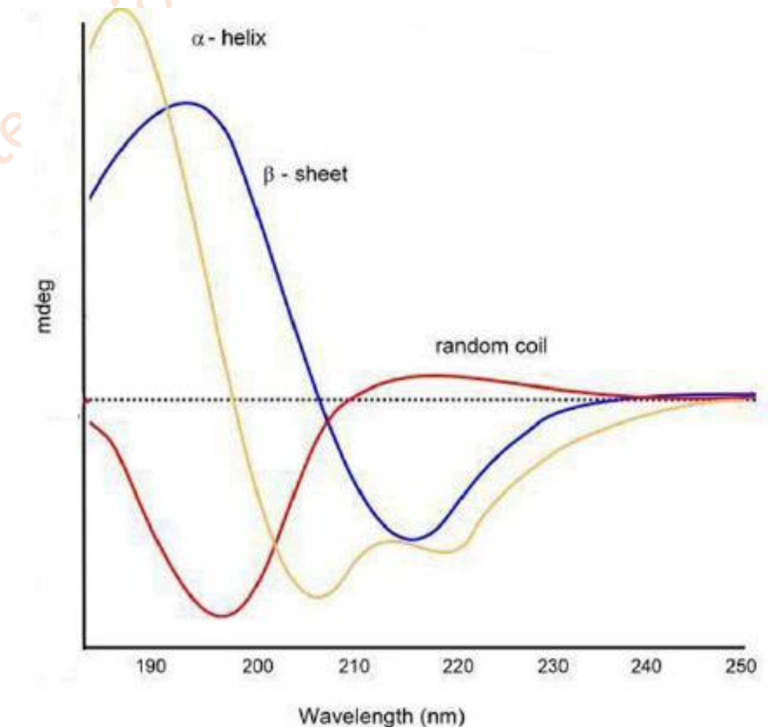
Characterization of biomolecular interactions

CD spectroscopy

- Frequently used to determine 2D structure
- Specific absorption curves
- **Induced circular dichroism** – caused by interaction between chiral and achiral compound
- **Differential CD spectra** analyzed



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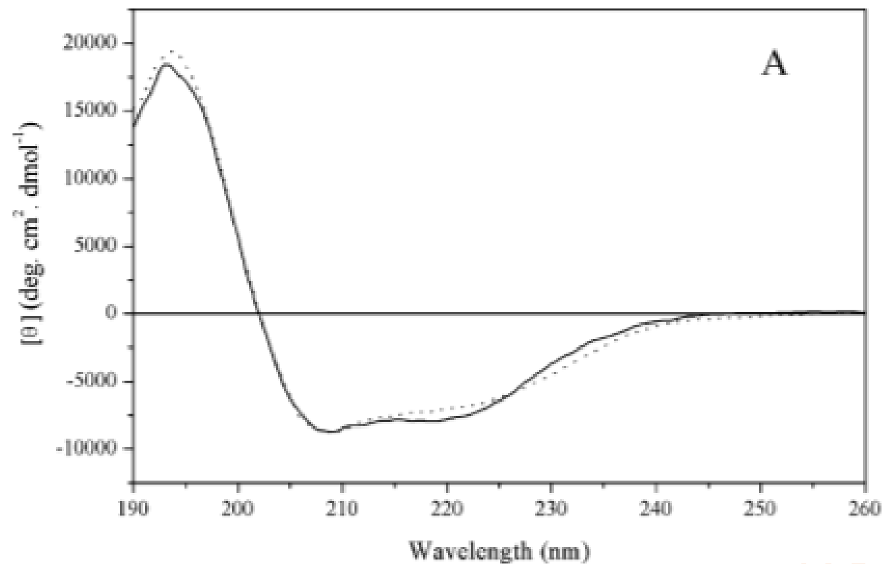
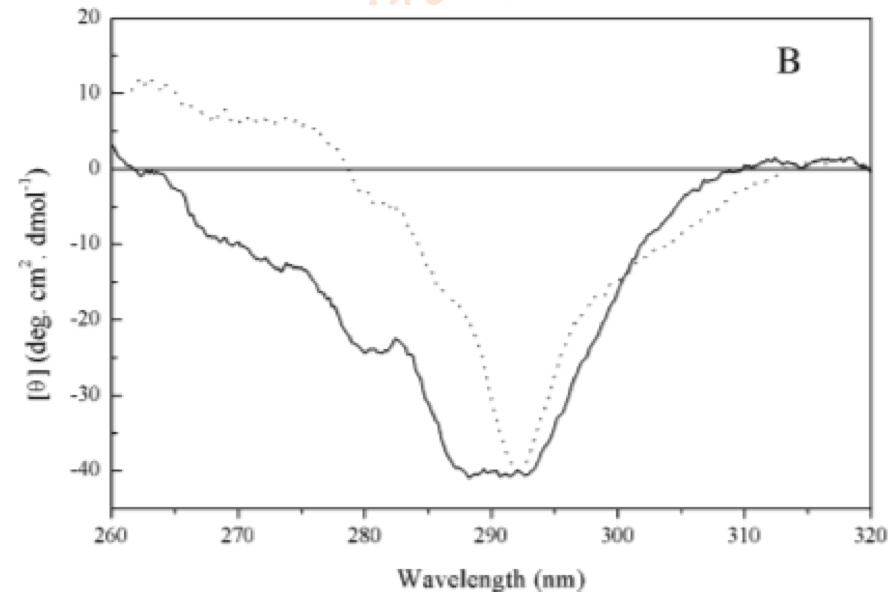
CD spectroscopy

- Full/Partial **folding/unfolding** of protein **upon interaction – IDPs**
- Sometimes even minor changes can be detected

Example: Molybdate-sensing protein ModE in absence (solid) and presence (dotted) of molybdate





S.M. Kelly et al. / Biochimica et Biophysica Acta 1751 (2005) 119 – 139

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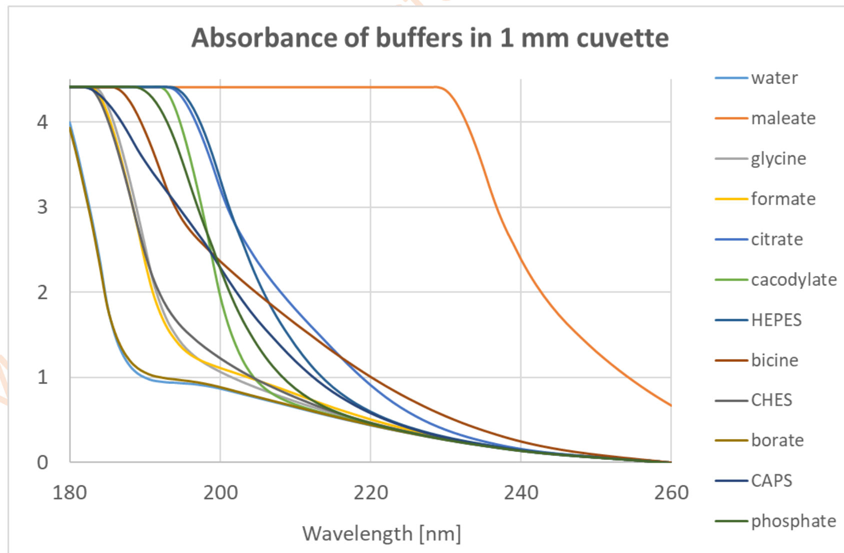
CD measurement

- Dedicated CD spectrometers
- Mostly UV region
  - Cuvettes from quartz
  - Buffer absorption
  - Sample purity importance



J-815 (Jasco)

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Light scattering

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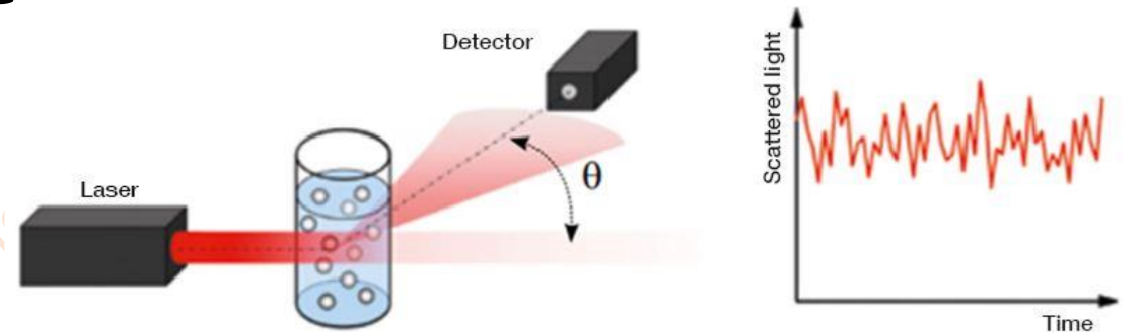
Light scattering

- Light scattering depends on size of particles in solution
- **Static light scattering**
    - **Intensity** of scattered light
    - Proportional to **molecular mass**
- **Dynamic light scattering**
    - **Time fluctuations** of scattered light
    - Proportional to **molecular size**



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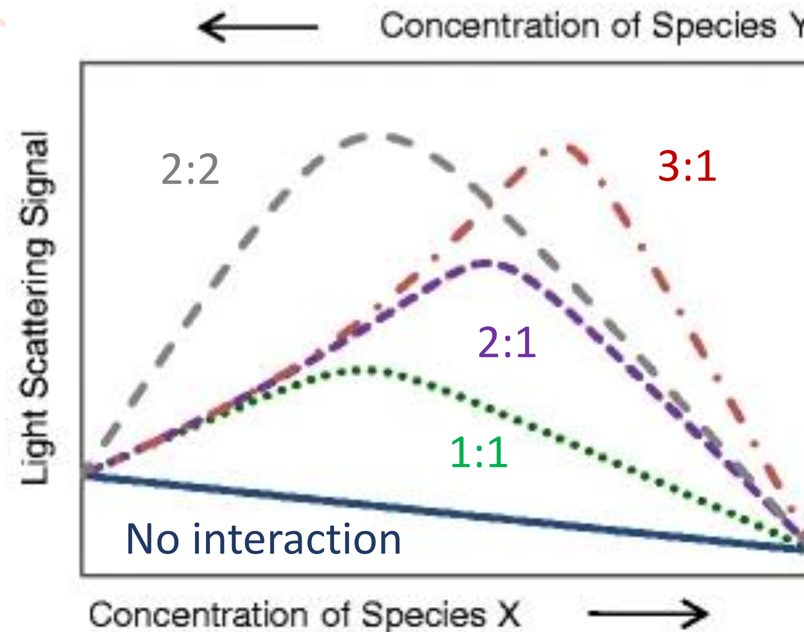
Static light scattering (SLS)

- Multi-angle light scattering (**MALS**)
- Analysis of set of samples with various composition – **concentration gradient** (CG-MALS)
- **Separation** of individual species coupled to LS – SEC-MALS, FFF-MALS



Some 2013, Biophys Rev

Theoretical SLS signal for different protein Y – protein X ratios.

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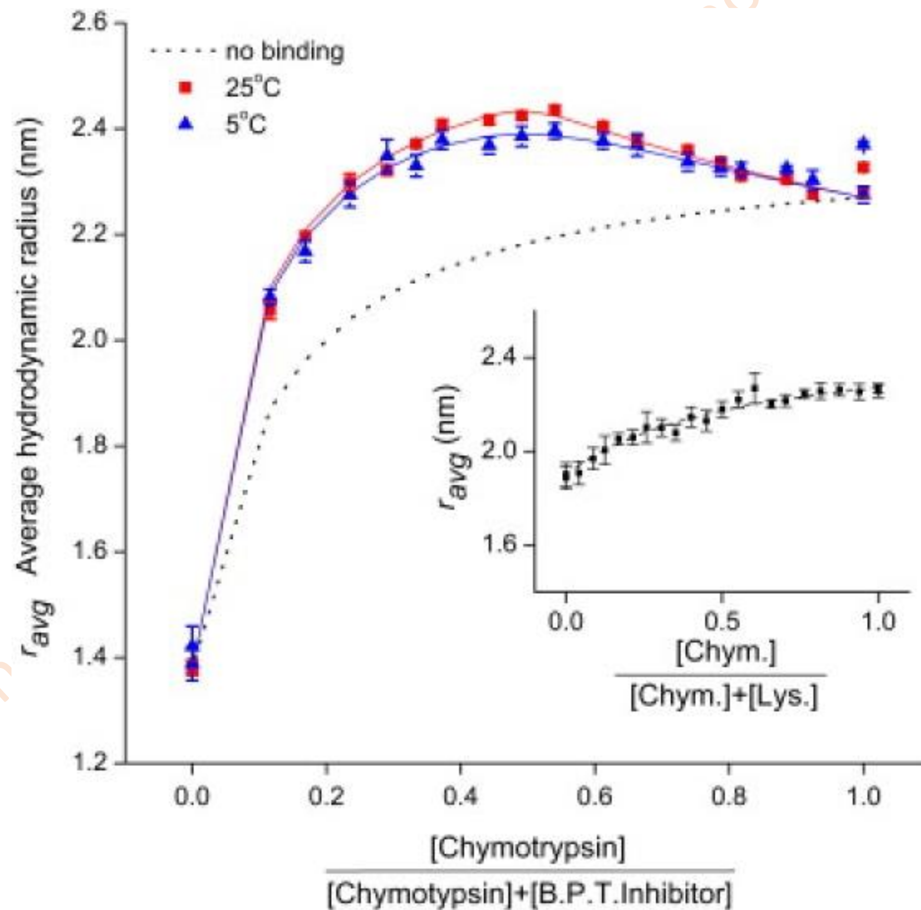
Dynamic light scattering (DLS)

- Low resolution
- Protein-protein or protein-NA interactions



Biophysical Journal Volume 98 January 2010 297–304

Free-Solution, Label-Free Protein-Protein Interactions Characterized by Dynamic Light Scattering

Amy D. Hanlon,* Michael I. Larkin,* and Ryan M. Reddick
Department of Research and Development, Wyatt Technology, Santa Barbara, California

1:1 α-chymotrypsin/bovine pancreatic trypsin inhibitor interaction. The profile expected for no association is shown by the dotted line. (Inset) Negative control of α-chymotrypsin and lysozyme.

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Infrared and Raman spectroscopy

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FTIR (Fourier transformed infrared spectroscopy)

All biomolecules absorb in infra red region – vibration of chemical bonds:

Wavelength λ = app. 1 – 50 µm
Wavenumber $\tilde{\nu}$ = 10 000 cm^{-1} – 200 cm^{-1}

Strong absorption by water

Analysis of IR spectra of free components and the complex

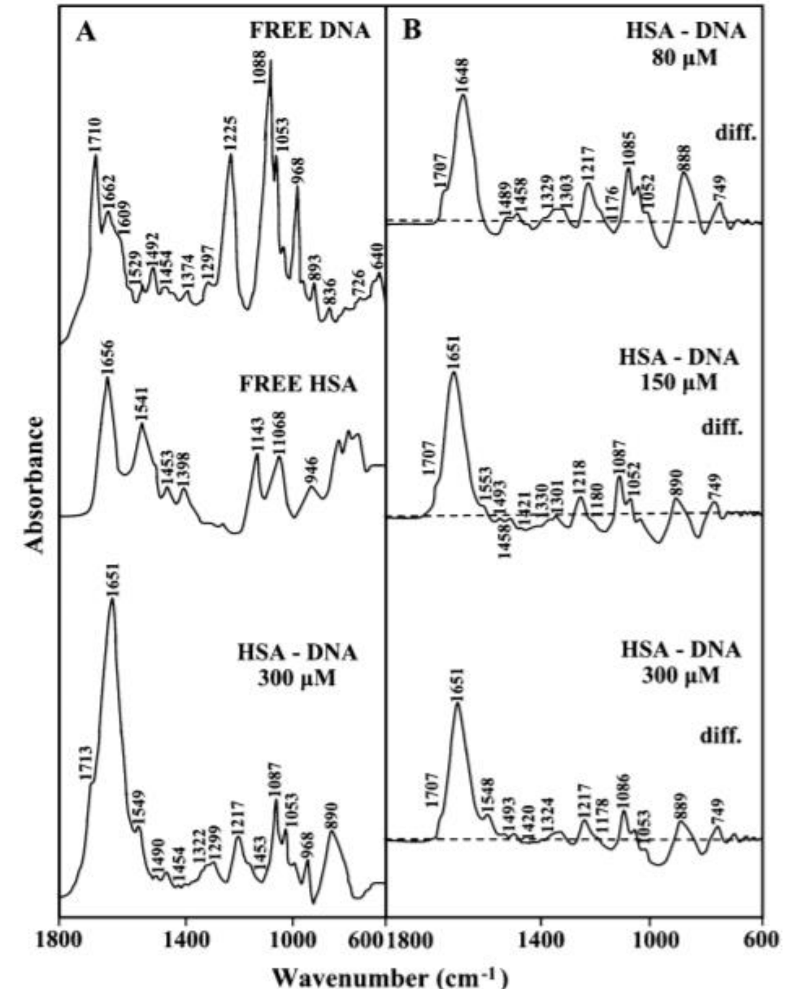
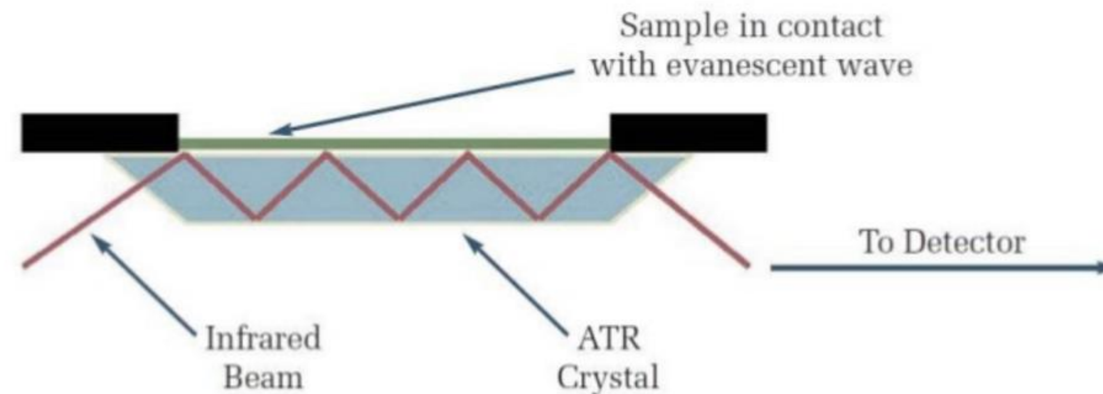


Fig. 2. FTIR spectra (A) and difference spectra [(DNA solution + protein solution) − (DNA solution)] (B) in the region of 1800–1500 cm^{-1} for the free DNA and human serum albumin (HSA) and their complexes in aqueous solution at physiological pH with various protein concentrations.

ATR-FTIR (Attenuated total reflectance FTIR)

- Molecule of interest is present **near the sensor surface**
- Signal of water is highly reduced
- **Higher sensitivity**, **lower concentration** needed
- Surface bound receptor – ligand is detected only upon binding

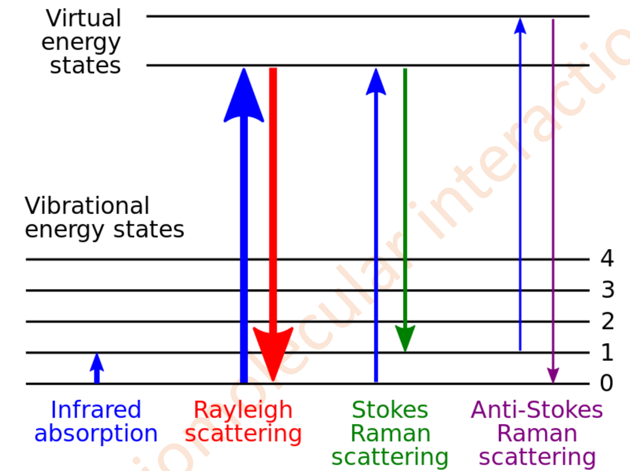


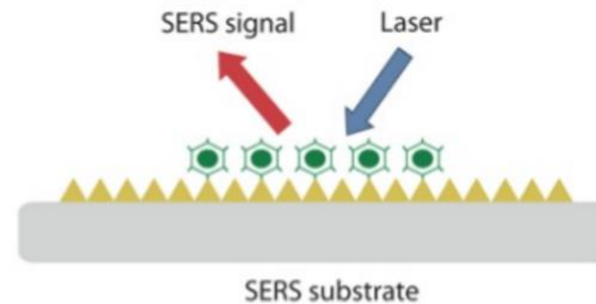
From:

Kazarian, Sergei G., and KL Andrew Chan. "ATR-FTIR spectroscopic imaging: recent advances and applications to biological systems." Analyst 138.7 (2013): 1940-1951.

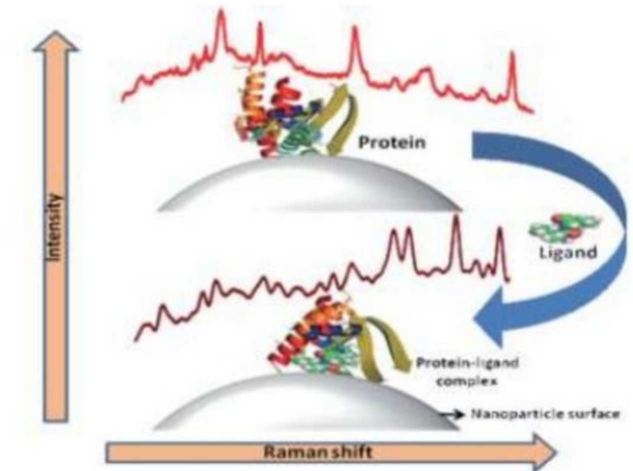
SERS (Surface enhanced Raman spectroscopy)

- **Raman spectroscopy** – analysis of vibrational/ rotational/etc. states in system through scattered light
- Very weak
- Strong enhancement (10^2 – 10^{14}) by adsorption on surface of metal or semiconductor





From: www.semrock.com



From: Siddhanta et al. 2012; *Nanomaterials and Nanotechnology*

Siddhanta, Soumik, and Chandrabhas Narayana. "Surface Enhanced Raman Spectroscopy of Proteins: Implications in Drug Designing." *Nanomaterials and Nanotechnology* 2.1 (2012): 1-13.

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Raman vs. Infrared spectroscopy

Raman	IR
Due to the scattering of light by the vibrating molecules.	Result of absorption/reflectance of light by vibrating molecules.
The vibration is Raman active if it causes a change in polarisability.	The vibration is IR active if there is a change in dipole moment during the vibration.
Many distinguishable peaks with high intensities	Few distinguishable peaks with weak intensities (even in ATR-FTIR)
Water can be used as a solvent.	Water cannot be used due to its intense absorption (not for ATR).
Sample preparation is not very elaborate sample can be almost in any state.	Sample preparation is elaborate Gaseous samples can rarely be used.
Cost of instrumentation is very high	Comparatively inexpensive.

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Light as a tool

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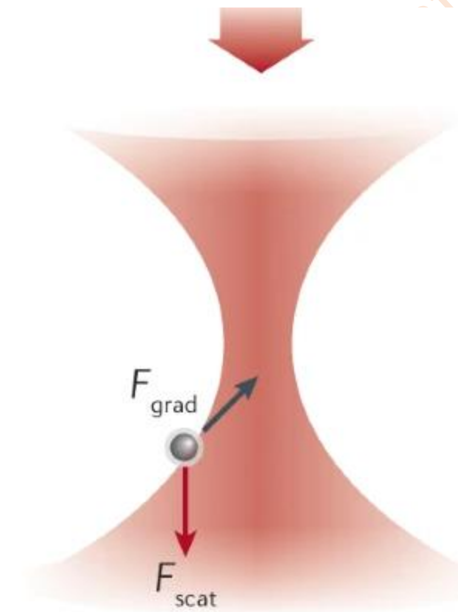
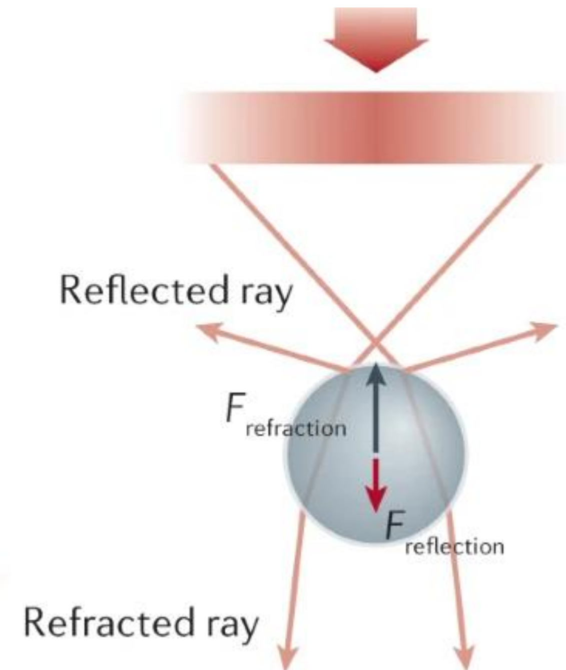
Optical tweezers

- Manipulation of molecules/microscopic objects by light

- Focused laser beam
- Combined with microscope
- Objects up to micrometer size – living cells

Tweez250 (Quantum Design)





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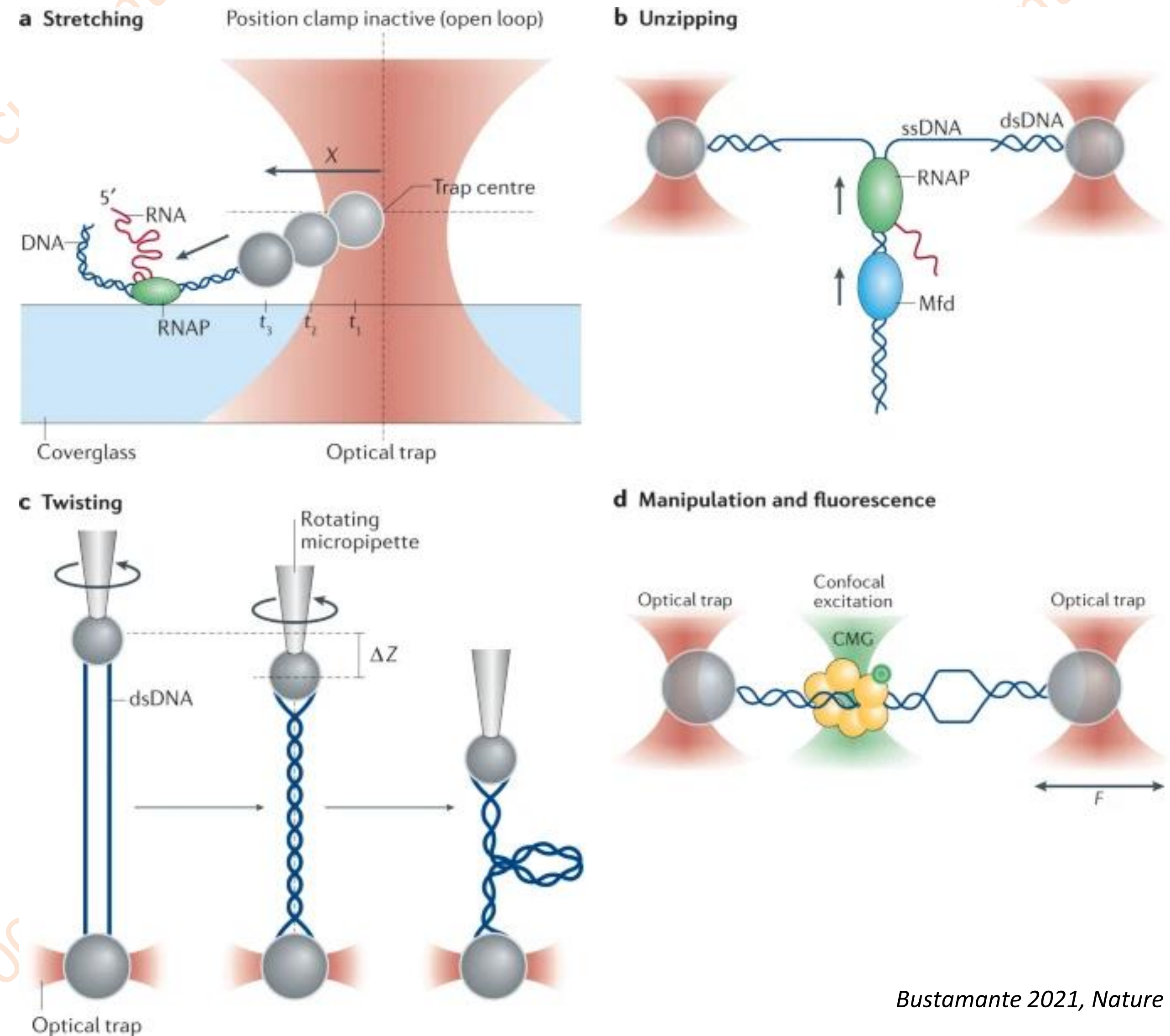
Optical tweezers – applications

Systems:
- Protein-protein
- Protein-NA
- Nanoparticles
- Cells

Features:
- Protein folding
- DNA stability
- Interactions
- Binding forces



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Spectroscopic and related methods

- Various use of light – **absorbance**, **fluorescence**, **scattering**
- Broad range of **wavelengths**
- **Intrinsic** properties vs. specific **labeling**
- Level of description of interaction
  - **Detection**
  - **Quantification** – K_D, K_A
  - Detailed **description** – interaction forces

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Biomolecular **I**nteraction and **C**rystallization Core Facility

bic@ceitec.cz

bic.ceitec.cz

MUNI

Josef Houser

- +420 549 492 527
- josef.houser@ceitec.cz

CF Head: Michaela Wimmerová

- +420 549 498 166
- michaela.wimmerova@ceitec.cz

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