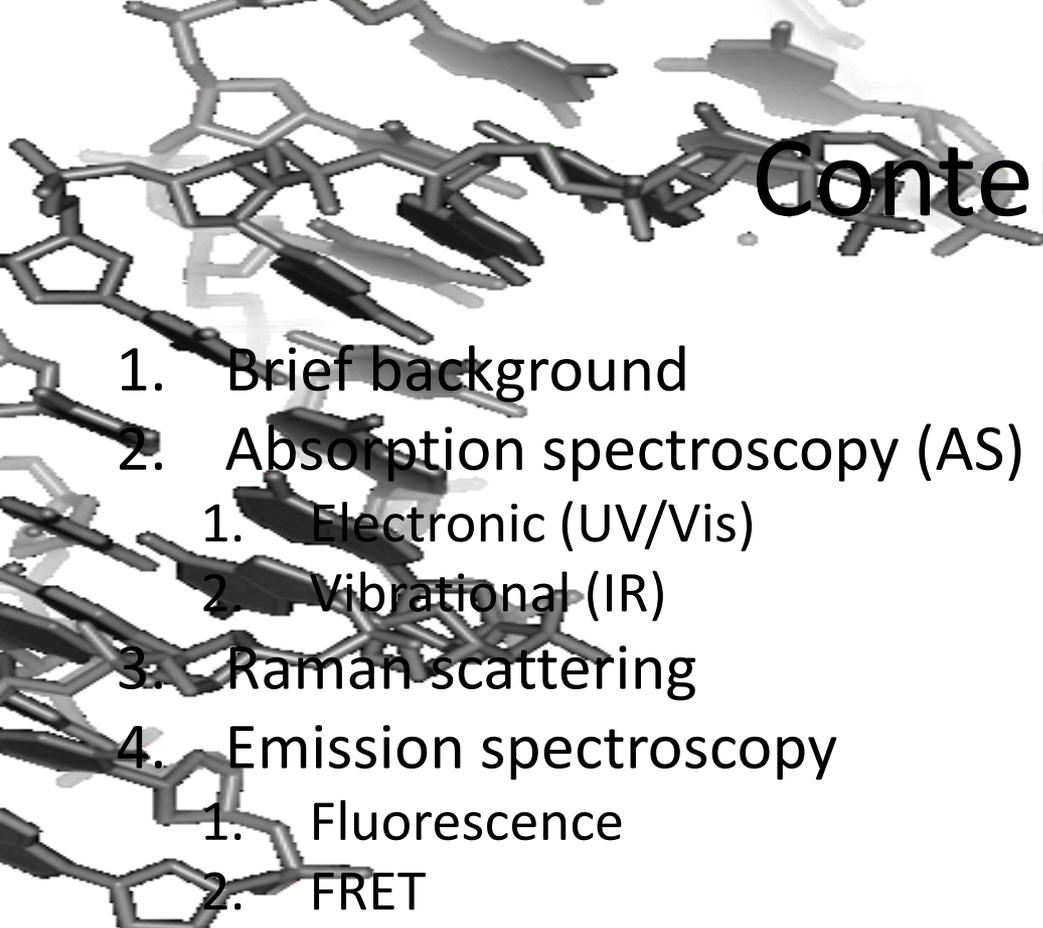


Optical spectroscopic methods

Daniel Renčiuk

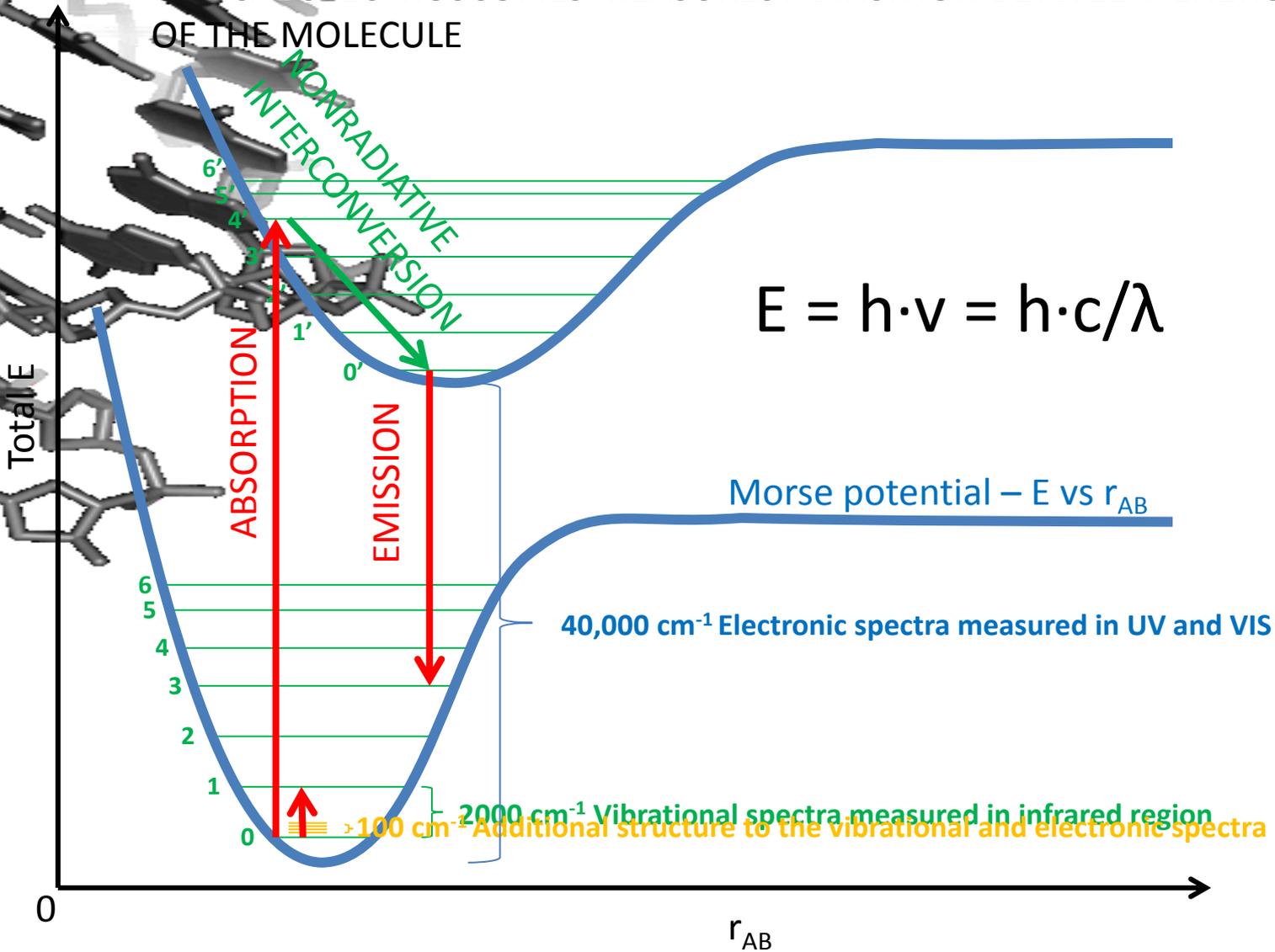


Contents

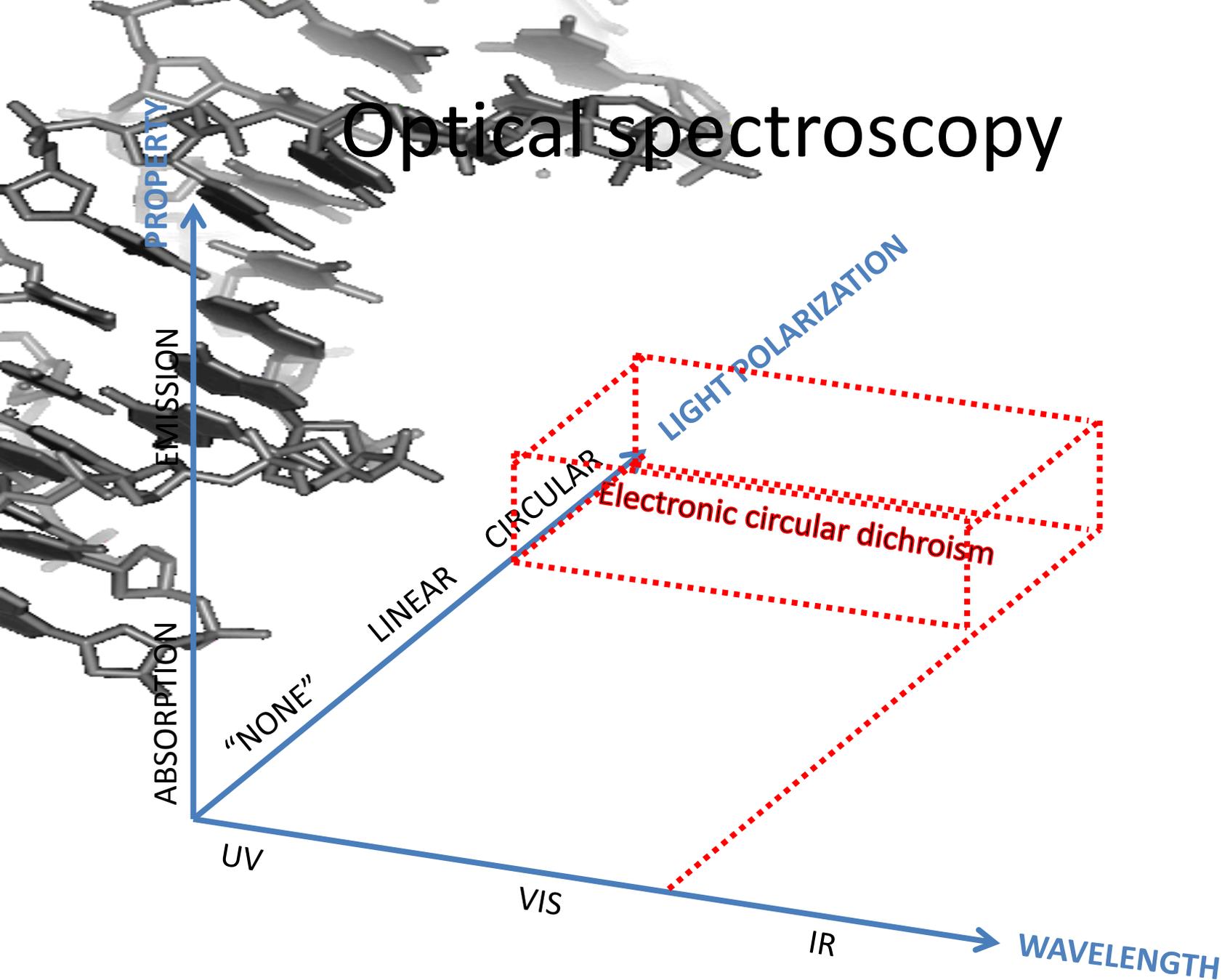
1. Brief background
2. Absorption spectroscopy (AS)
 1. Electronic (UV/Vis)
 2. Vibrational (IR)
3. Raman scattering
4. Emission spectroscopy
 1. Fluorescence
 2. FRET
 3. Fluorescence polarisation / anisotropy
5. Chiroptical methods
 1. Linear dichroism
 2. Circular dichroism

Transitions

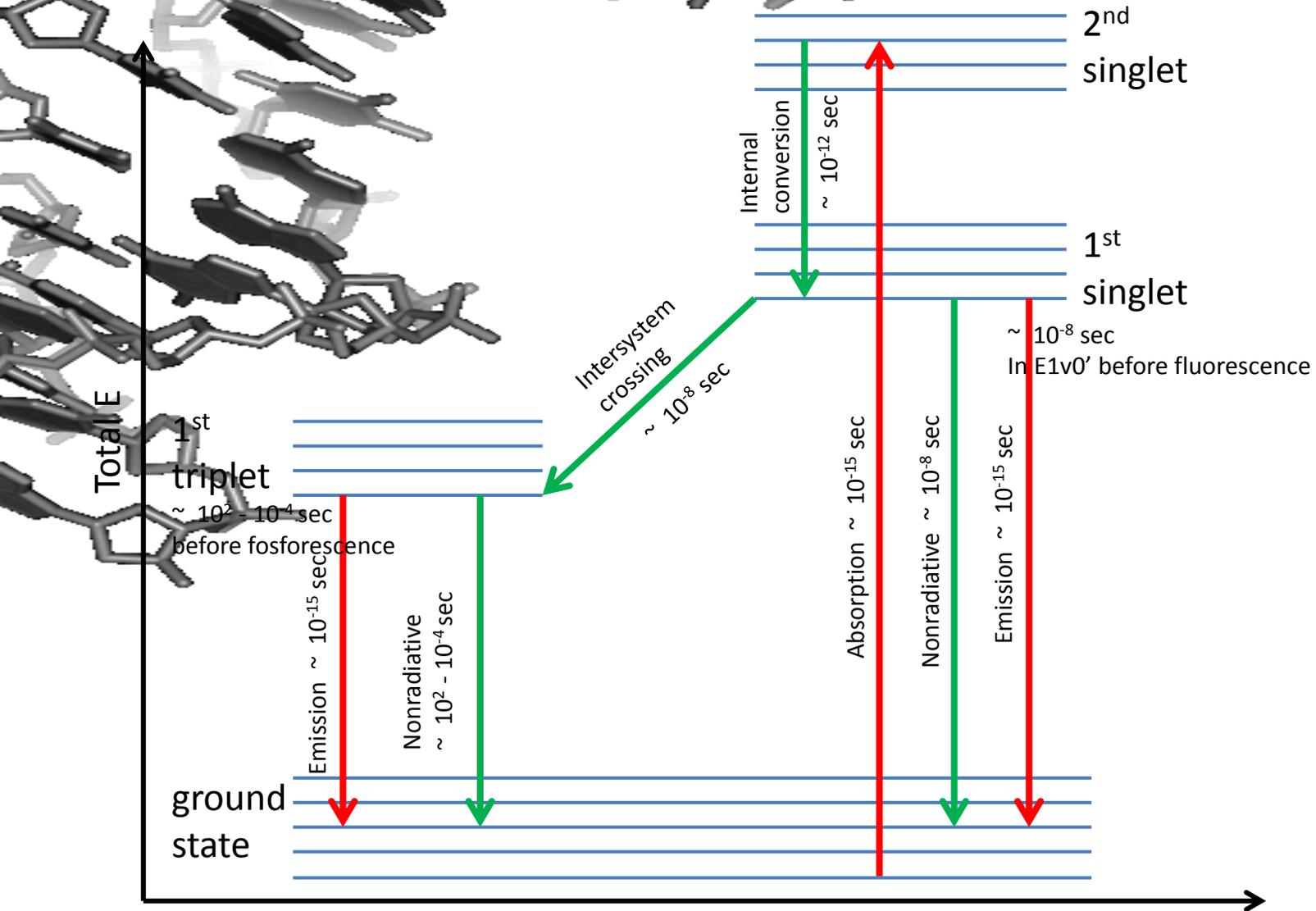
OPTICAL SPECTROSCOPIES MEASURE TRANSITION BETWEEN ENERGY STATES OF THE MOLECULE



Optical spectroscopy



Transition times



Background – Franck-Condon principle

- Transition to an excited electronic state can be to any of the vibrational level
- Vibrational transitions are very slow, compared to electronic transitions
- Certain vertical transitions corresponding to no nuclear displacement during an electronic transition have the highest probability (**Franck-Condon principle**)
- Absorption band has the vibronic structure - one E0-E1 transition is a superposition of several transitions v_0-v_n' characterized by different energy and probability intensity

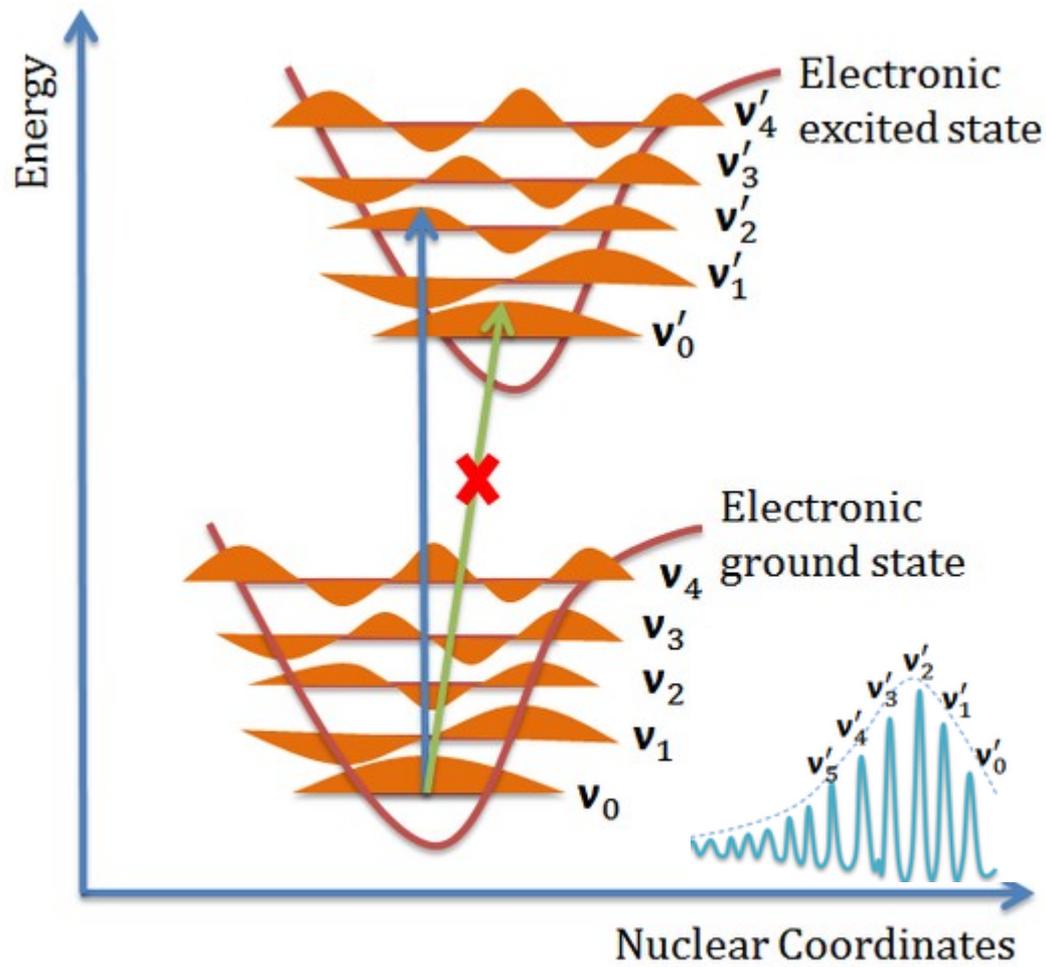
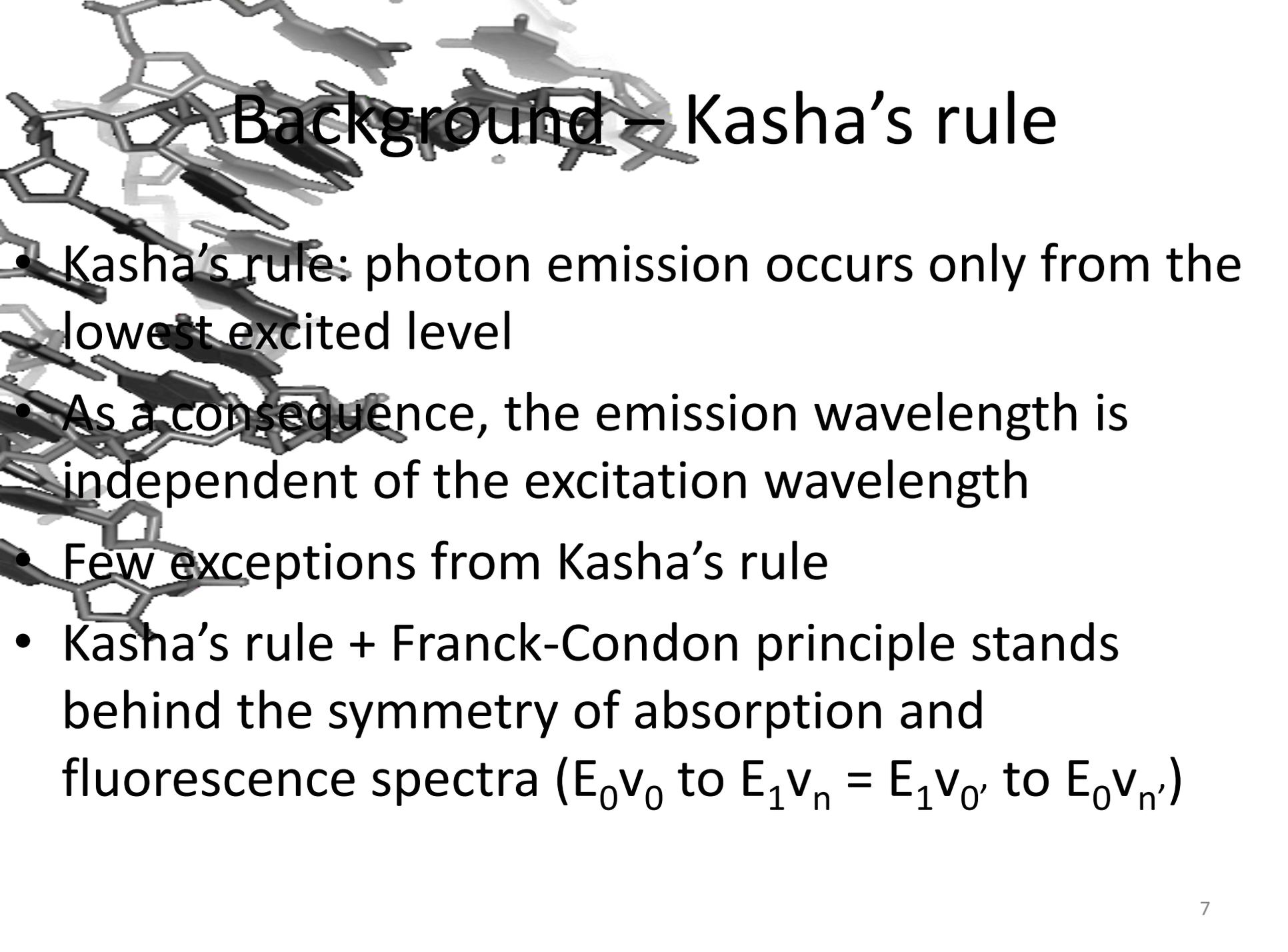


Fig. 2 Franck-Condon energy diagram

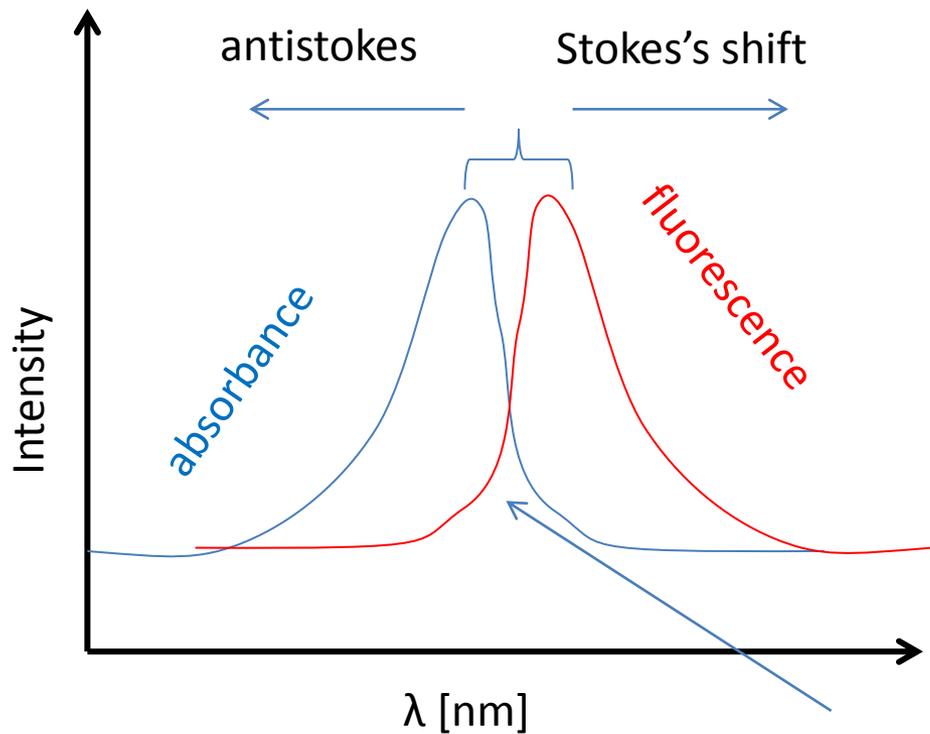
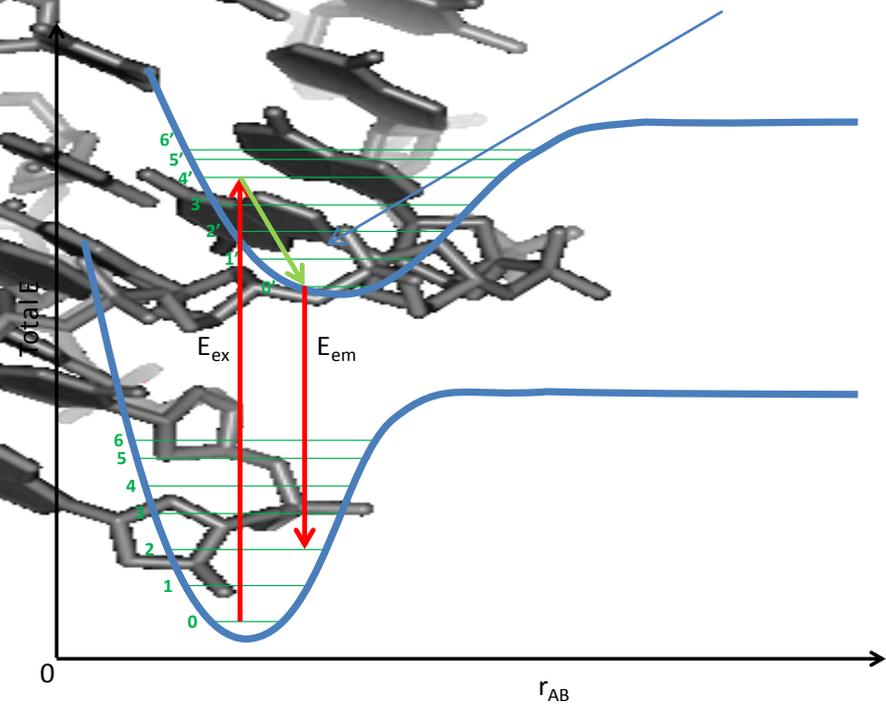


Background – Kasha's rule

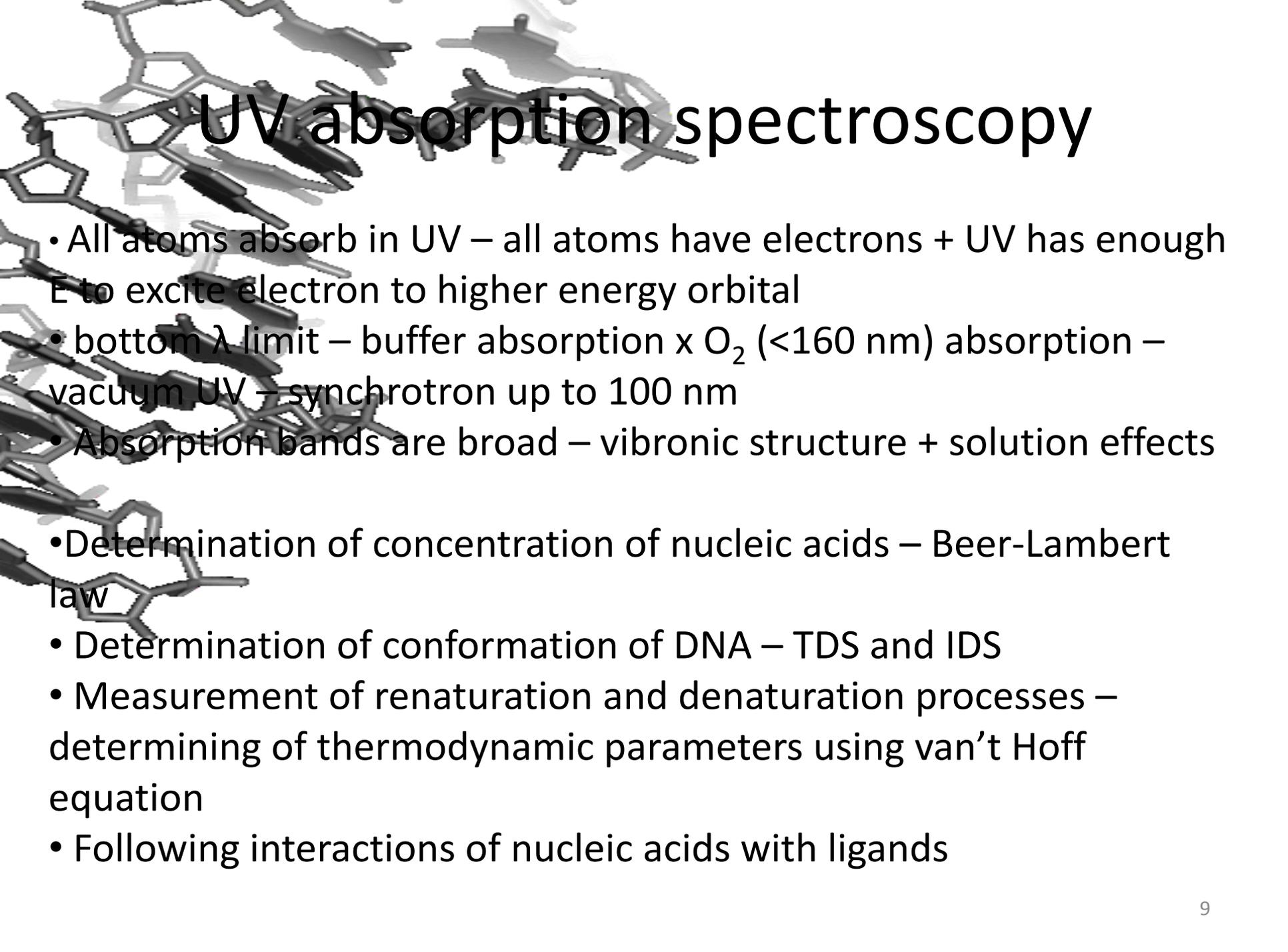
- Kasha's rule: photon emission occurs only from the lowest excited level
- As a consequence, the emission wavelength is independent of the excitation wavelength
- Few exceptions from Kasha's rule
- Kasha's rule + Franck-Condon principle stands behind the symmetry of absorption and fluorescence spectra (E_0v_0 to $E_1v_n = E_1v_0'$ to $E_0v_{n'}$)

Background - Stokes and antistokes shift

KASHA'S RULE



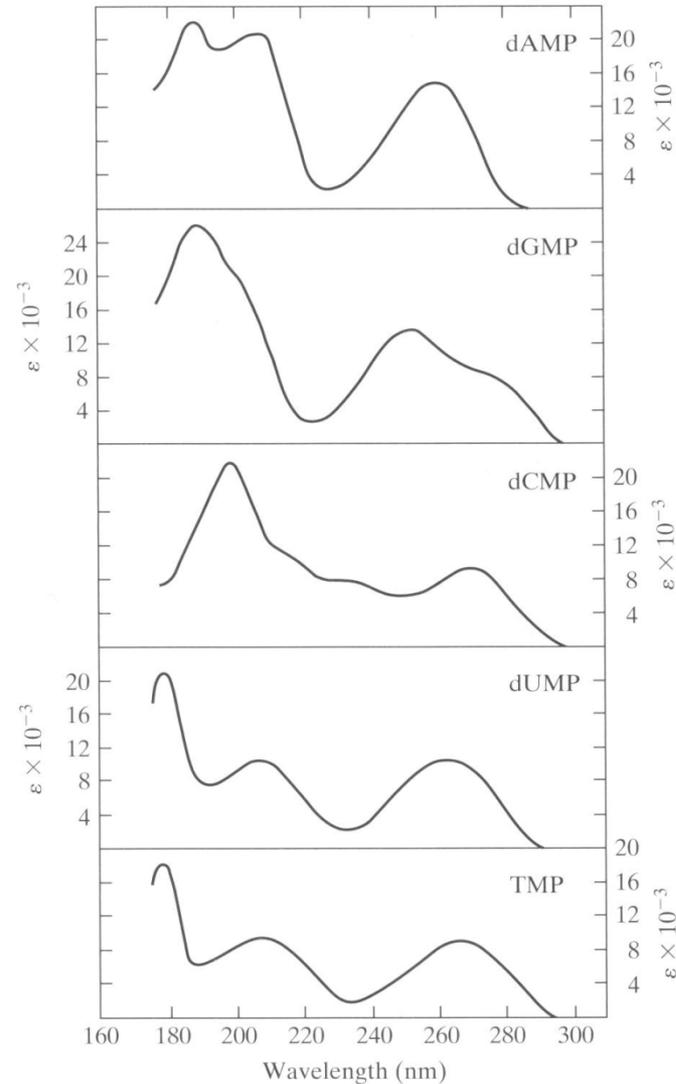
$$E_{\text{ex}} > E_{\text{em}} \Rightarrow \nu_{\text{ex}} > \nu_{\text{em}} \Rightarrow \lambda_{\text{ex}} < \lambda_{\text{em}}$$



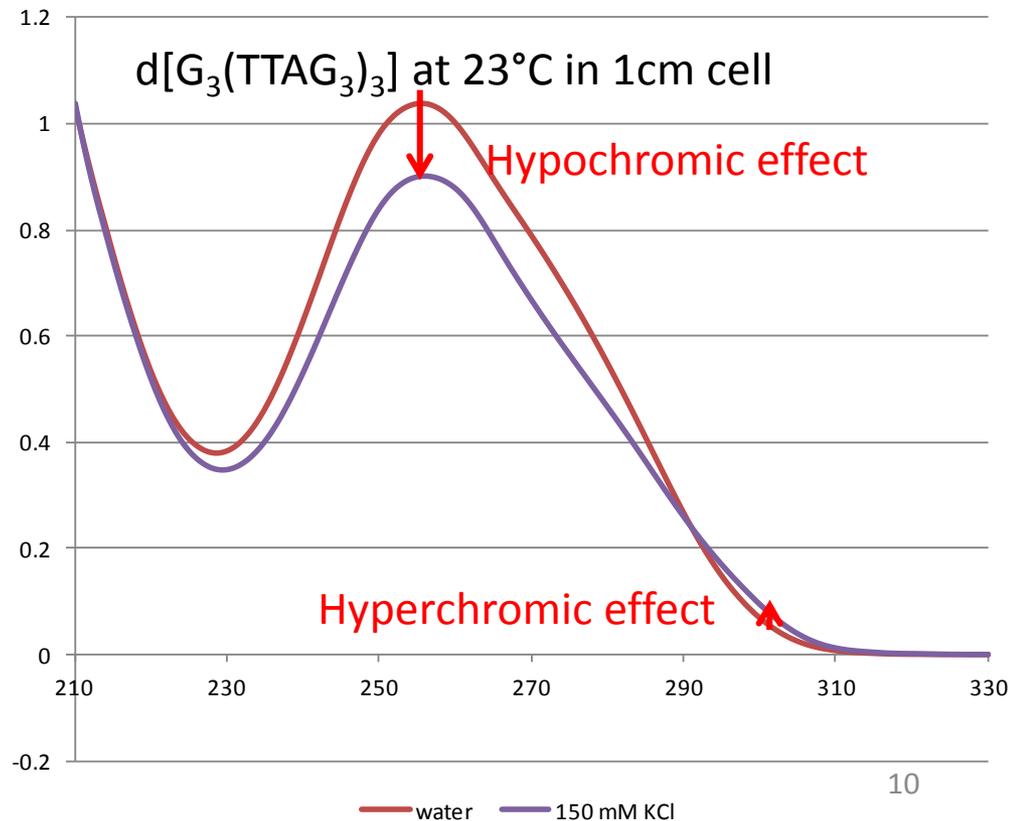
UV absorption spectroscopy

- All atoms absorb in UV – all atoms have electrons + UV has enough E to excite electron to higher energy orbital
- bottom λ limit – buffer absorption x O_2 (<160 nm) absorption – vacuum UV – synchrotron up to 100 nm
- Absorption bands are broad – vibronic structure + solution effects
- Determination of concentration of nucleic acids – Beer-Lambert law
- Determination of conformation of DNA – TDS and IDS
- Measurement of renaturation and denaturation processes – determining of thermodynamic parameters using van't Hoff equation
- Following interactions of nucleic acids with ligands

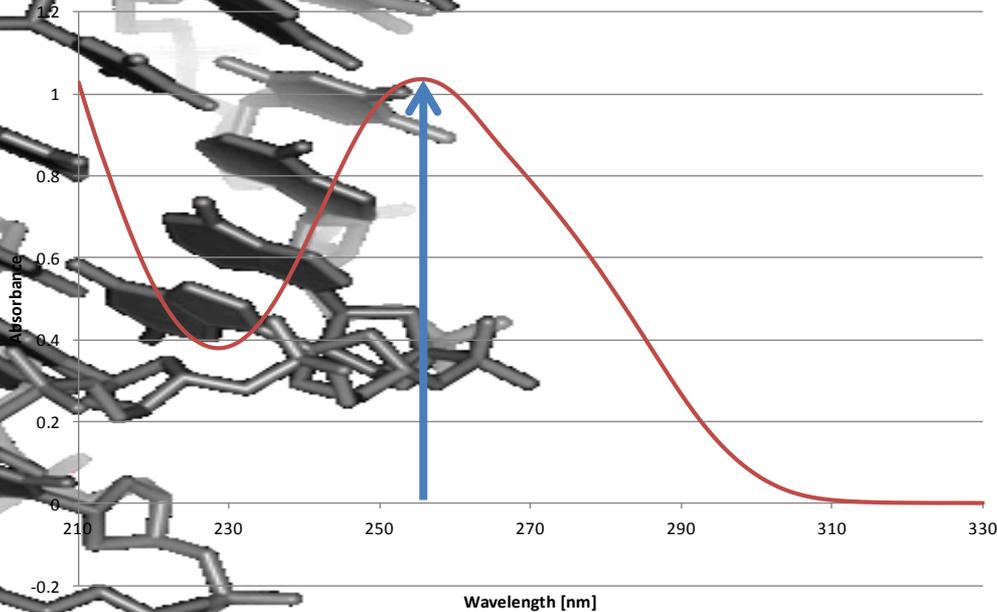
UV absorption



- final NA spectrum is based on contributions of individual monomers in primary sequence + contributions of their interactions
- spectrum different for structured and non-structured NA (hypochromism around 260 nm after folding)



UV absorption – NA concentration



Beer-Lambert law
 $A = c \cdot \epsilon \cdot l = \log_{10} I_0 / I$

$$I = I_0 \cdot 10^{-c \cdot \epsilon \cdot l}$$

I_0 – incident light

I – output light

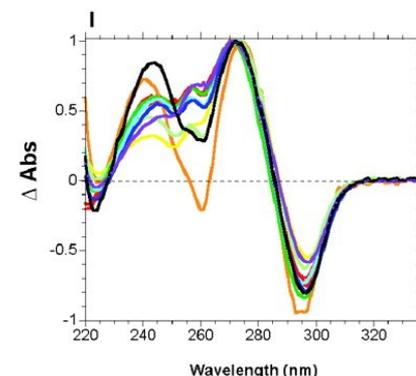
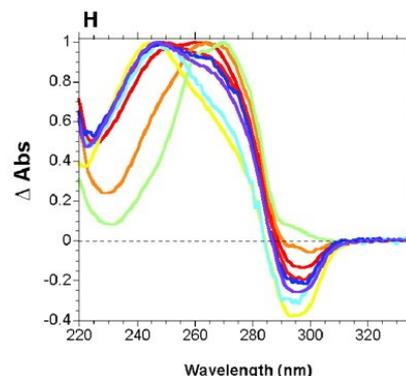
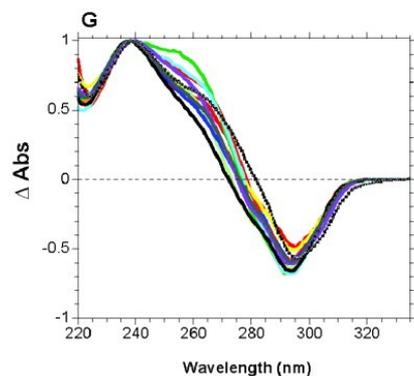
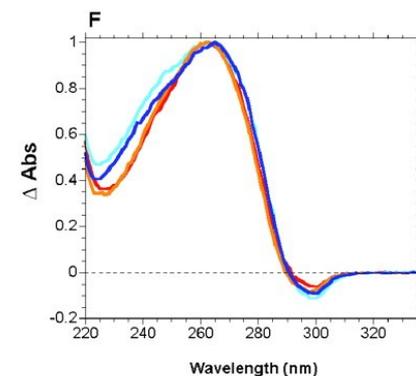
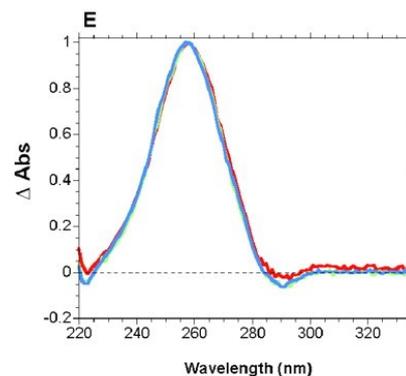
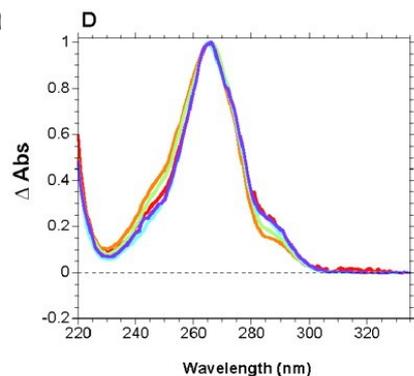
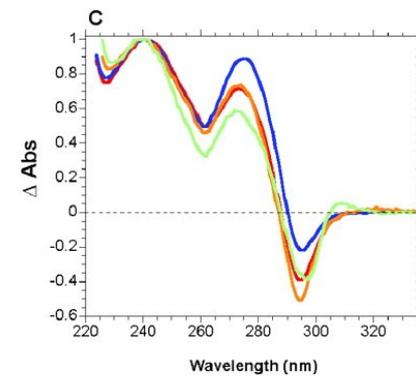
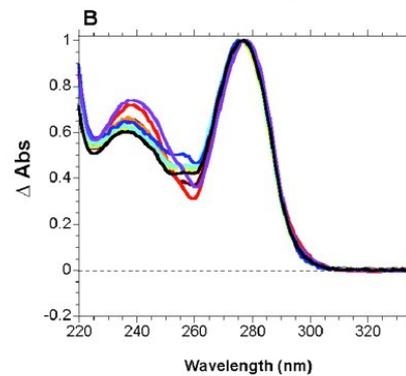
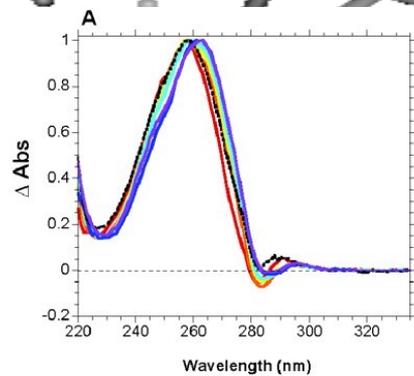
Light intensity decreases exponentially when passing through sample thus absorbance (as log) increases linearly – 2x sample concentration or pathlength = 2x absorbance but 10x less light
Optimal absorbance 0.6-0.8

ϵ – molar absorption coefficient - specific for each NA primary sequence

- calculated – sum of ϵ of dimers – sum of ϵ of monomers (Gray et al., 1995, Methods Enzymol)
- analytically determined – amount of phosphorus vs absorbance

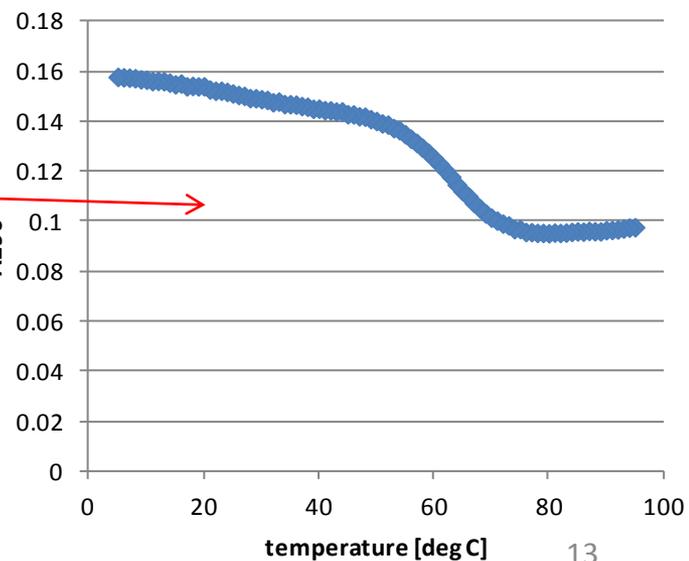
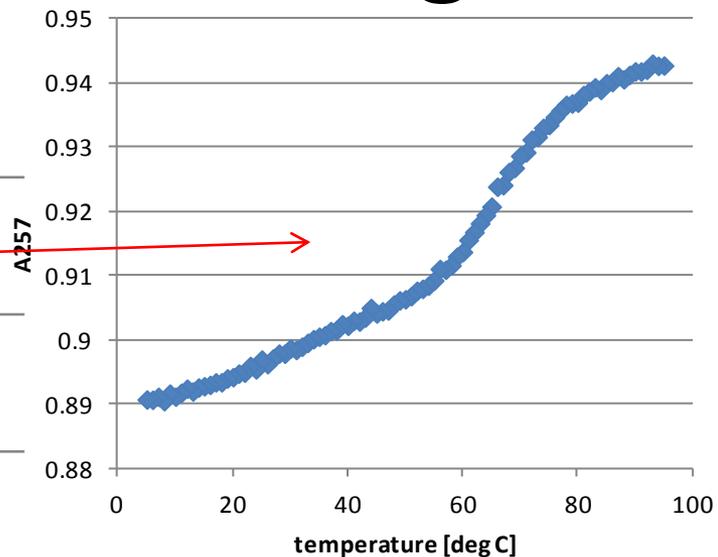
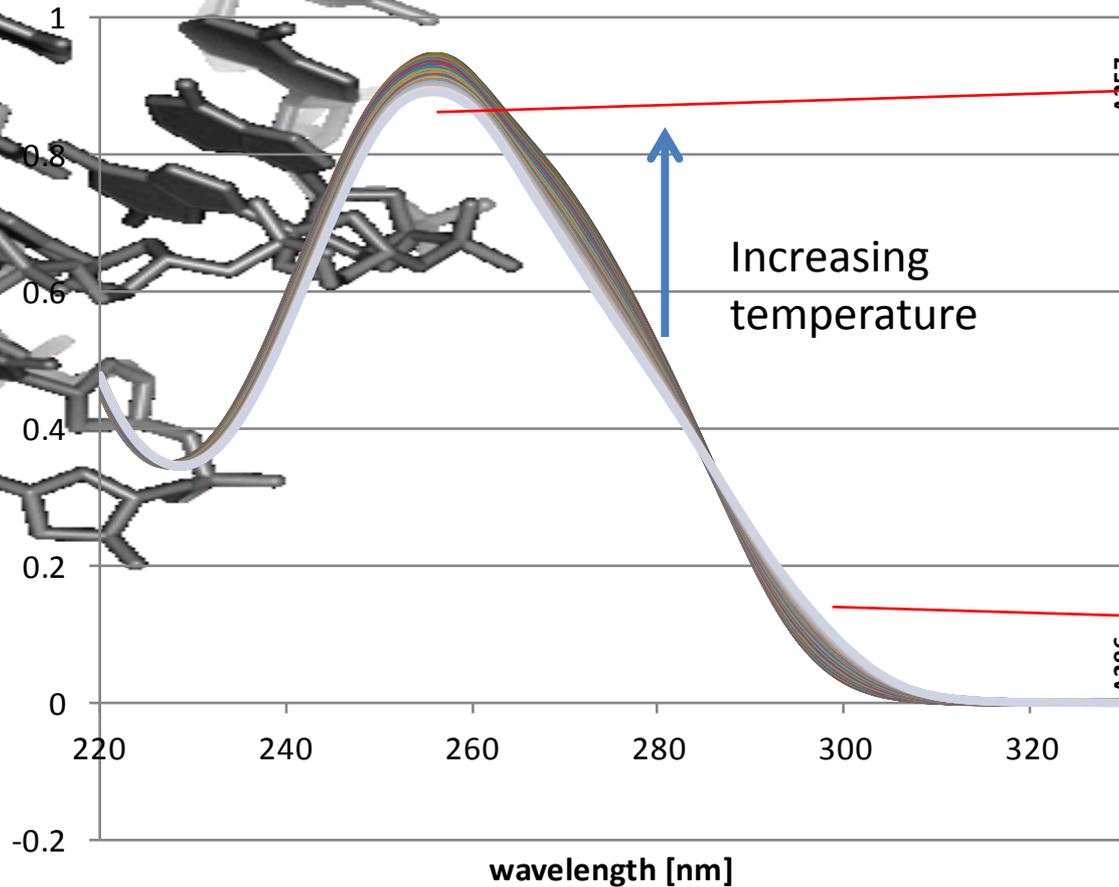
UV Absorbance – TDS (or IDS)

Normalized differential absorbance signatures: (A) DNA self-complementary duplexes, 100% AT; (B) DNA self-complementary duplexes 100% GC; (C) Z-DNA; (D) Parallel-stranded DNA; (E) GA DNA duplexes; (F) Hoogsteen DNA duplexes; (G) i-DNA; (H) Pyrimidine triplexes; (I) DNA G-quadruplexes in Na⁺.



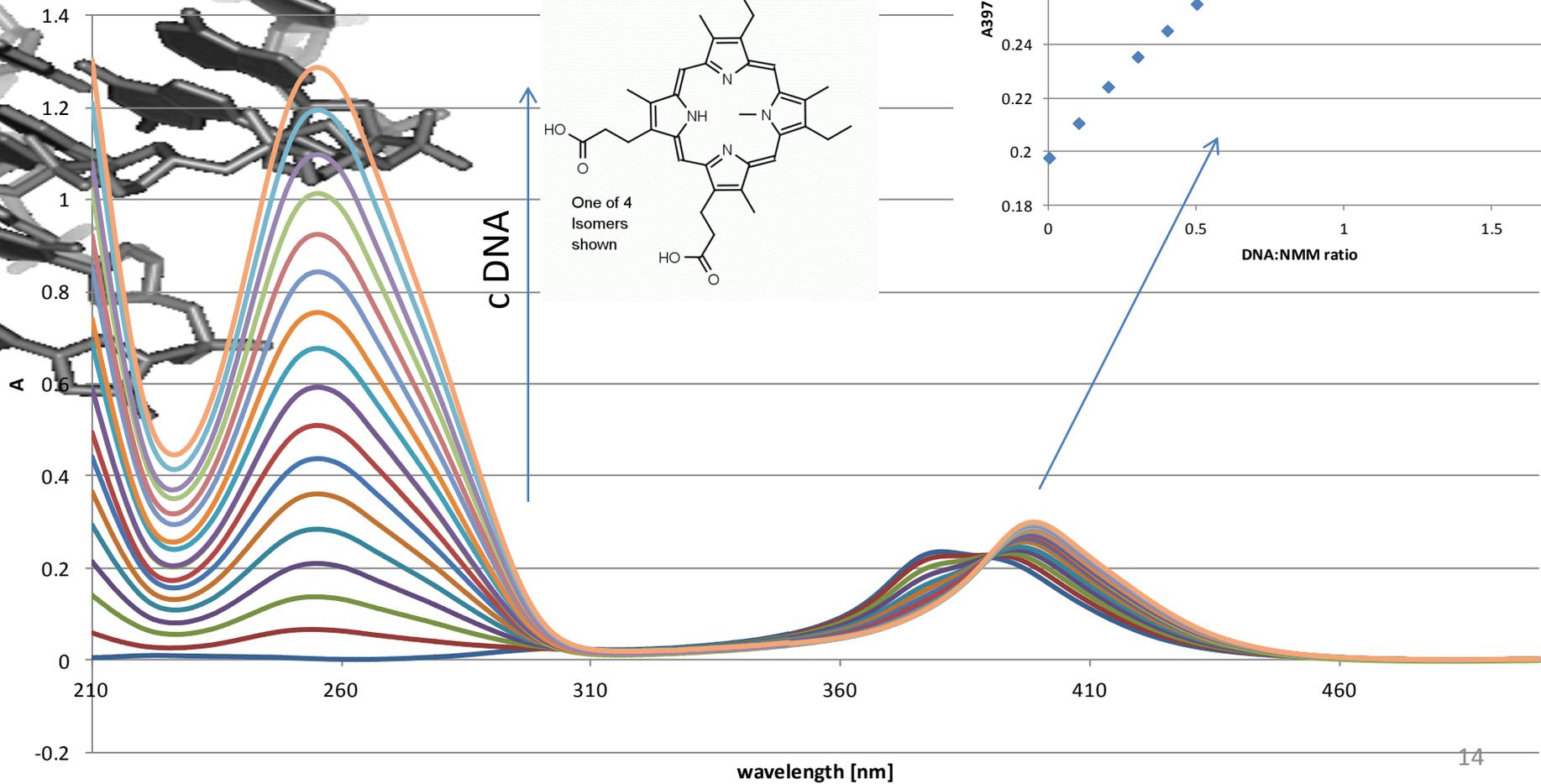
UV Absorbance – NA melting

Guanine quadruplex $G_3(TTAG_3)_3$ in 150 mM Na



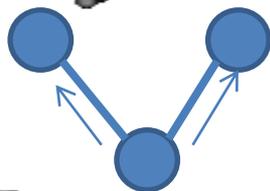
UV Absorbance – ligand interaction

NMM ligand titrated by guanine quadruplex

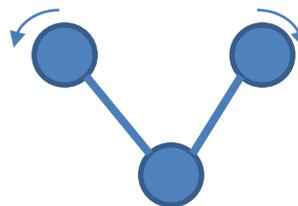


IR absorption

- Measures the energies of vibration of atomic nuclei in the molecule
- Each molecule has $3n-6$ internal degrees of freedom (n =number of atoms in molecule)
- specific absorption bands for various chemical groups



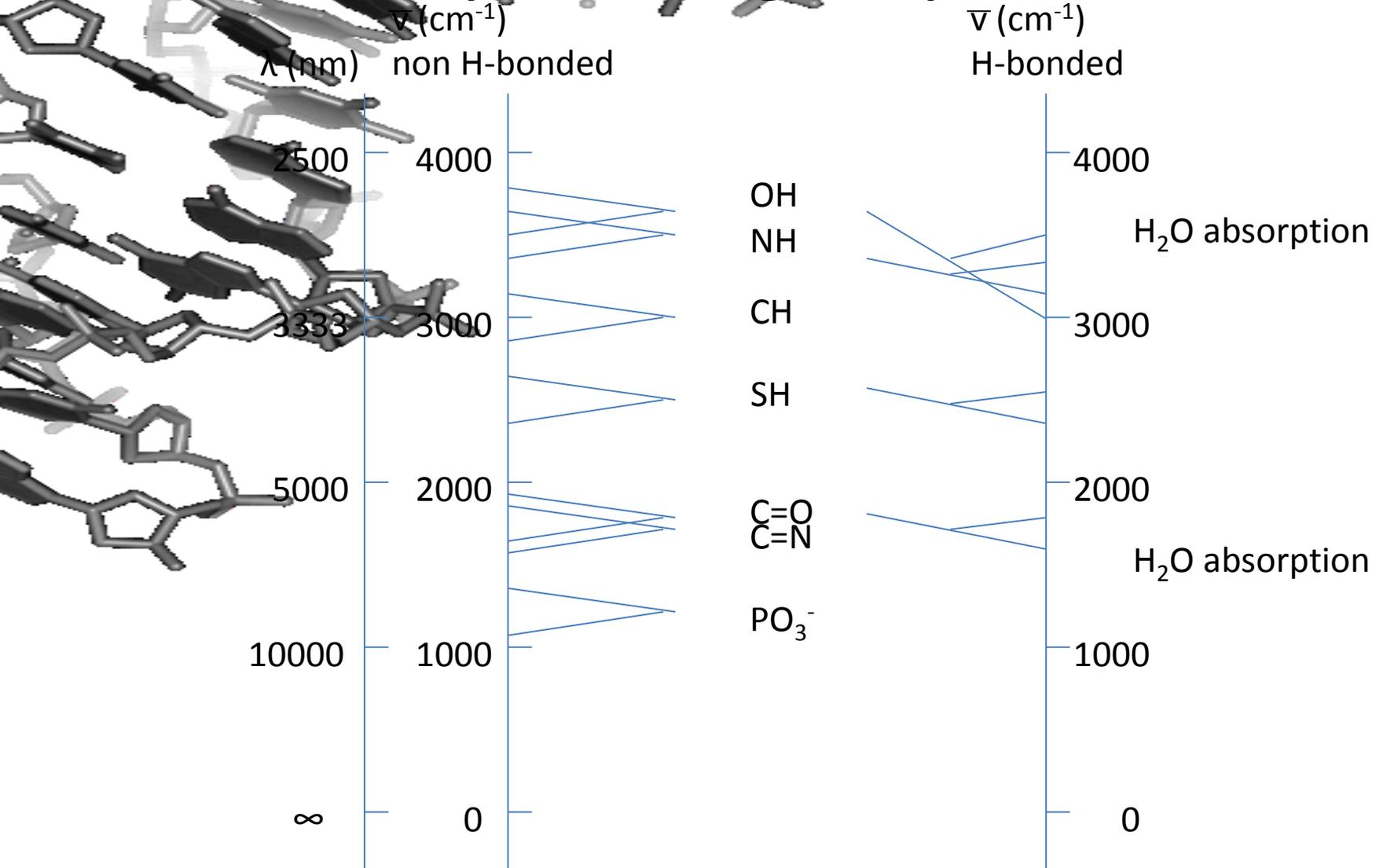
stretching



In-plane bending

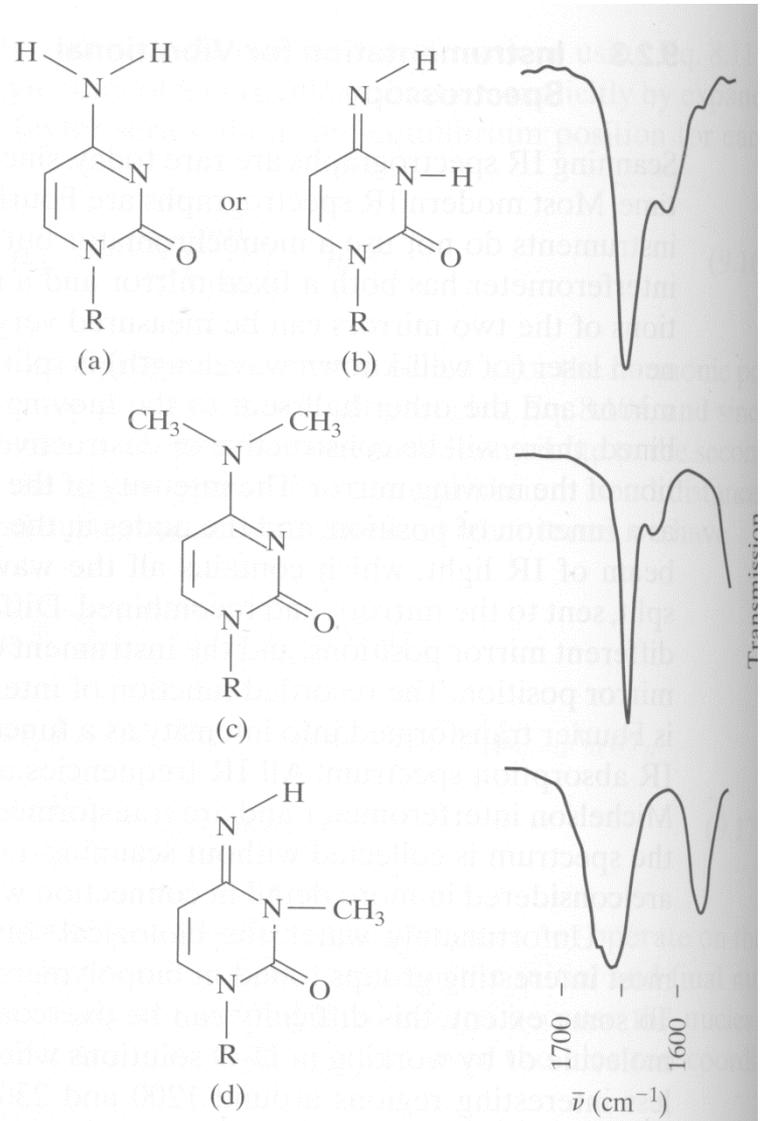
- modern IR spectrophotometers are Fourier transform instruments – Michelson interferometer + FT transformation of intensity to frequency – all frequencies taken simultaneously
- water absorption in interesting IR regions – D_2O , films

IR absorption – group vibrations



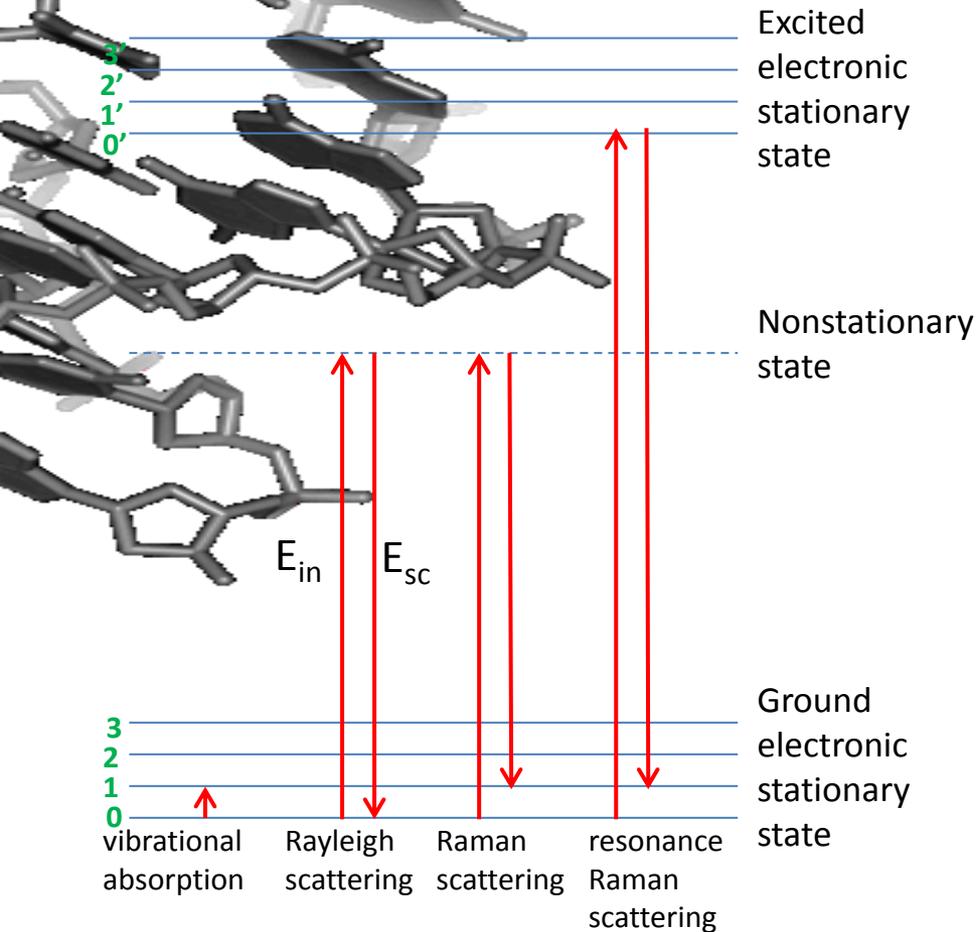
IR absorption – Miles experiment

IR spectra in the 1750 to 1550 cm^{-1} region for two nontautomerizing methyl derivatives (c) and (d), and cytidine, now known to be in the first tautomeric form shown (a).



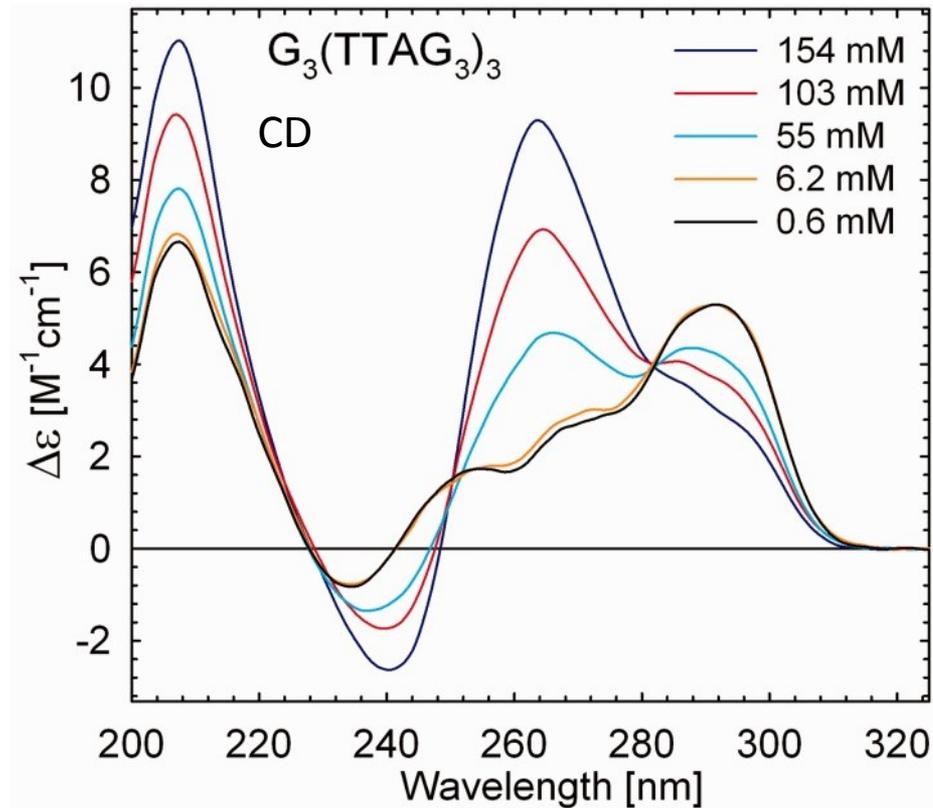
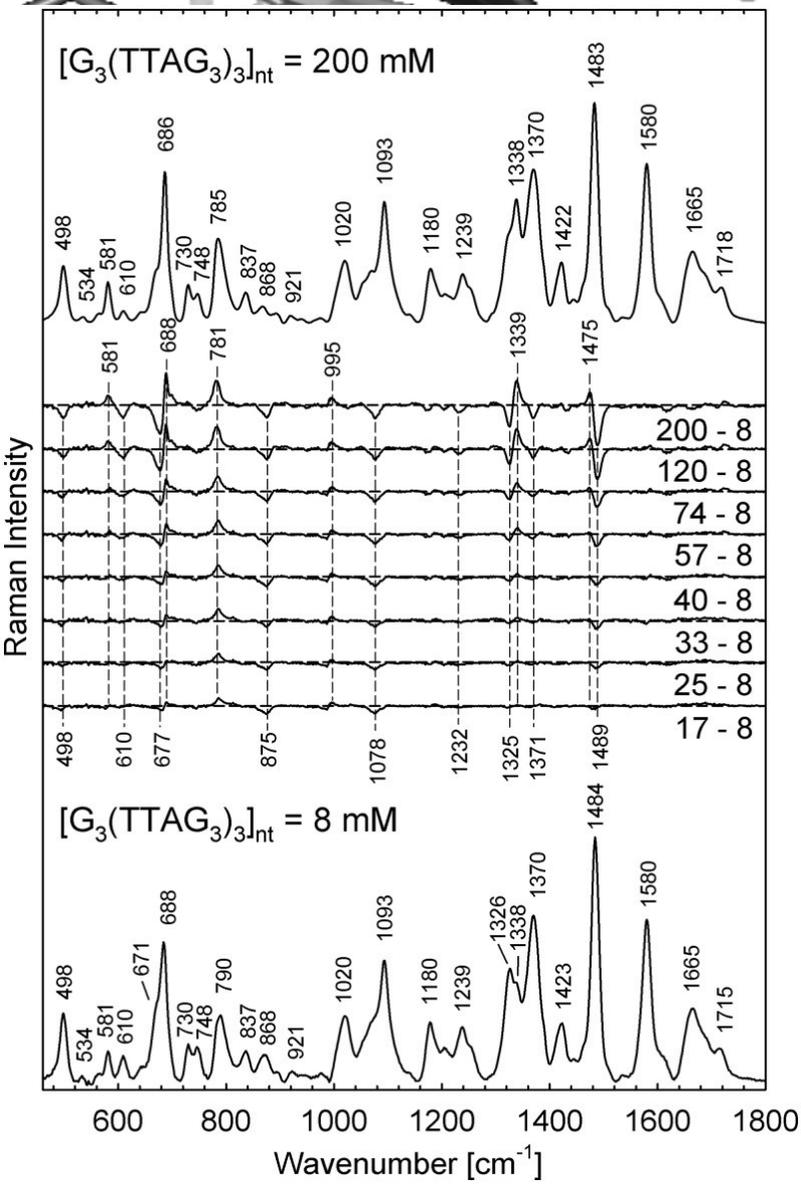
Raman spectroscopy

Band position: $\nu_{01} = (E_{in} - E_{sc})/hc$



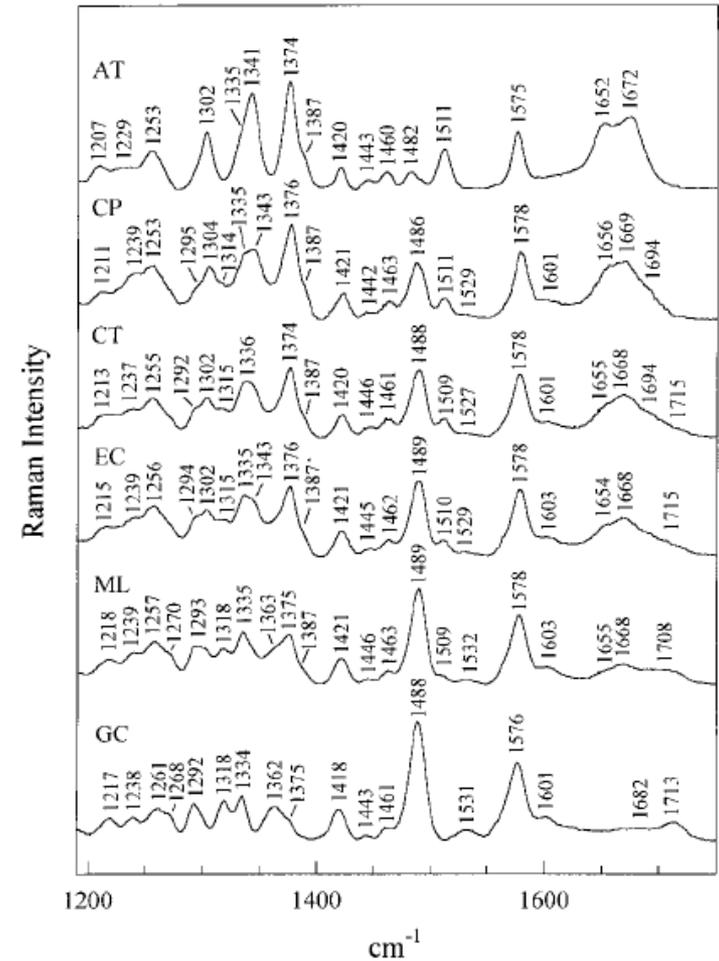
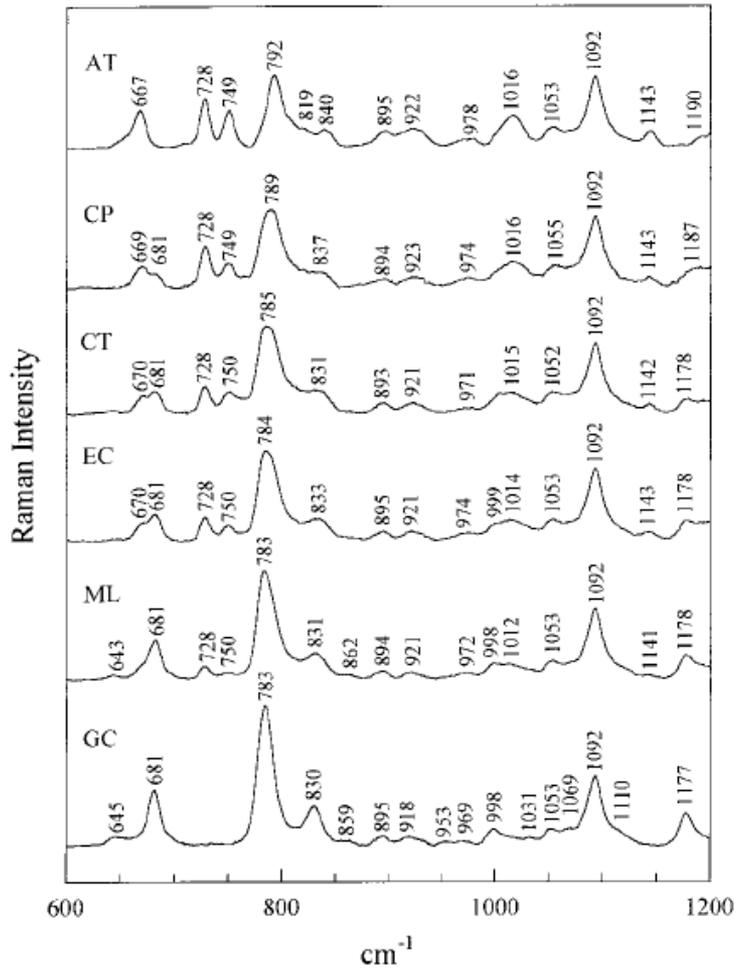
- when used light with $E < E_0 - E_1$ – scattering
- in most cases $E_{in} = E_{sc}$ – Rayleigh scattering
- sometimes $E_{in} \neq E_{sc}$ – Raman scattering
 - $E_{in} > E_{sc}$ – Stokes
 - $E_{in} < E_{sc}$ – anti-Stokes
- Raman photon incidence around 10^{-8}
- Raman band position: $\nu_{01} = (E_{in} - E_{sc})/hc$
- complementary to vibrational absorption – the same transition (0-1)
 - Raman – visible photon
 - vibrational – IR photon
 - some vib. transitions detected differently
- nonstationary states are not quantized => any UV/Vis source may be used
- practically lasers – intense monochromatic light
- scattered light split by monochromator

Raman spectroscopy



Raman spectra of $G_3(TTAG_3)_3$ in 200 mM K^+ (30 mM of PBS, pH 6.8, $t = 5^\circ\text{C}$) at the nucleoside concentrations of 8 mM (bottom trace) and 200 mM (top trace). Intermediate traces show the differences between the spectra at indicated concentration and that of the lowest one

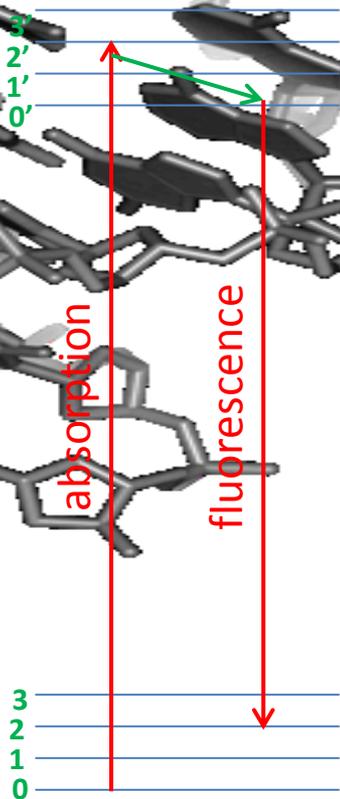
Raman spectroscopy



poly(dA-dT) · poly(dA-dT) (0% G+C), *C. perfringens* DNA (27% G+C), calf thymus DNA (42% G+C), *E. coli* DNA (50% G+C), *M. luteus* DNA (72% G+C), and poly(dG-dC) · poly(dG-dC) (100% G+C).

Fluorescence in nucleic acids

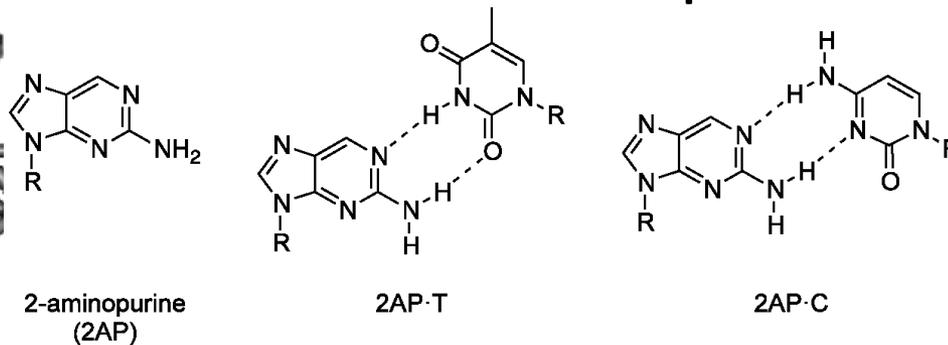
- Spontaneous emission of the photon followed by transition to electronic ground state (any vibrational state – Franck-Condon)
- Emission always from the vibrational ground state of the electronic excited state (Kasha's rule)
- fluorescence itself very fast (10^{-15} s), but some time takes nonradiative conversion to v_0'



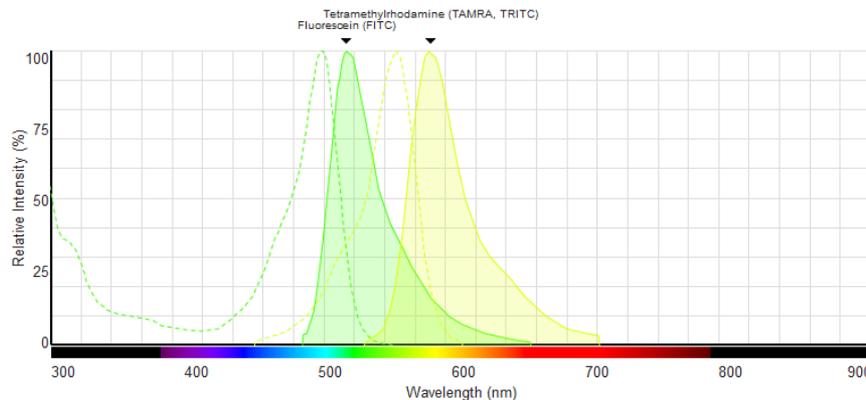
Fluorescence in nucleic acids

- Very low intrinsic fluorescence, thus:

1. Fluorescent base – 2-aminopurine



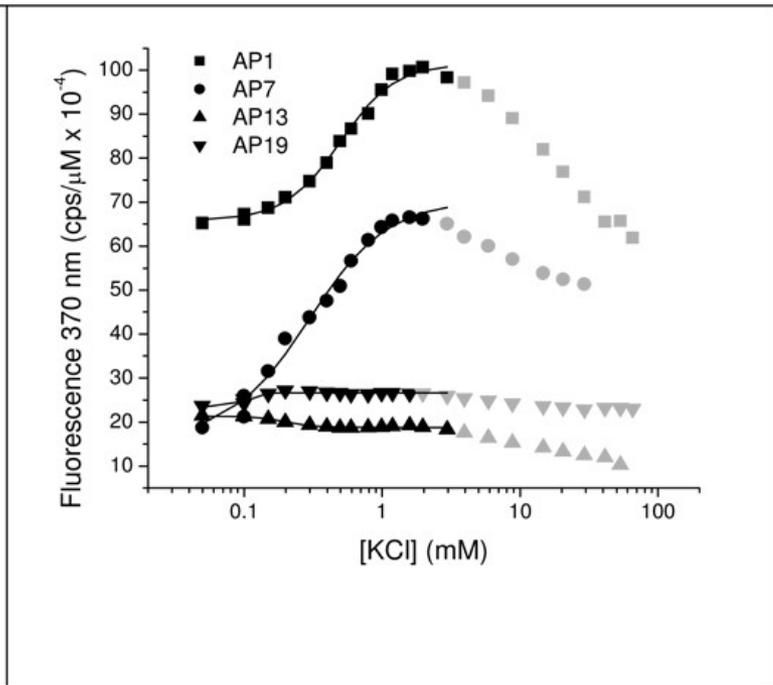
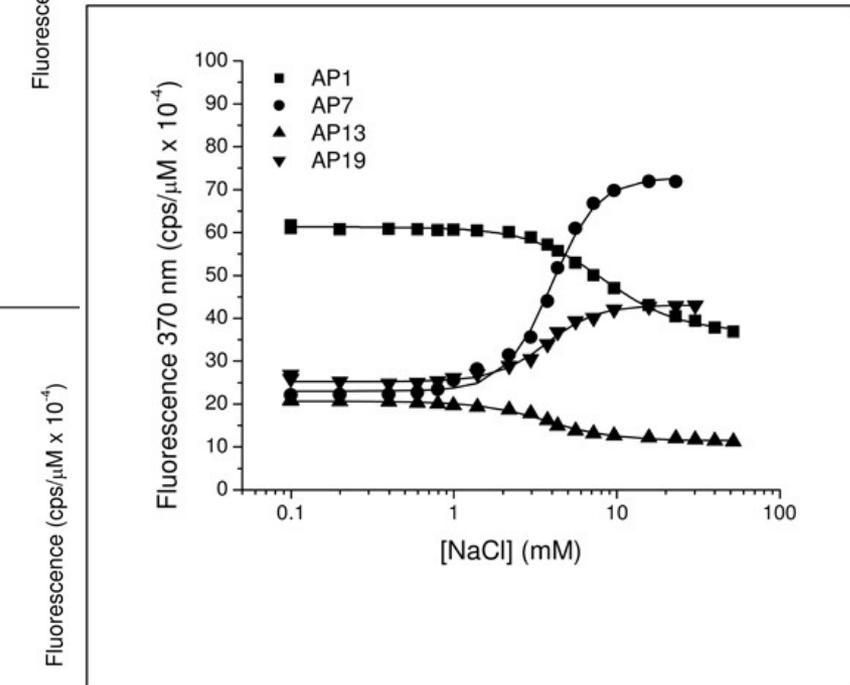
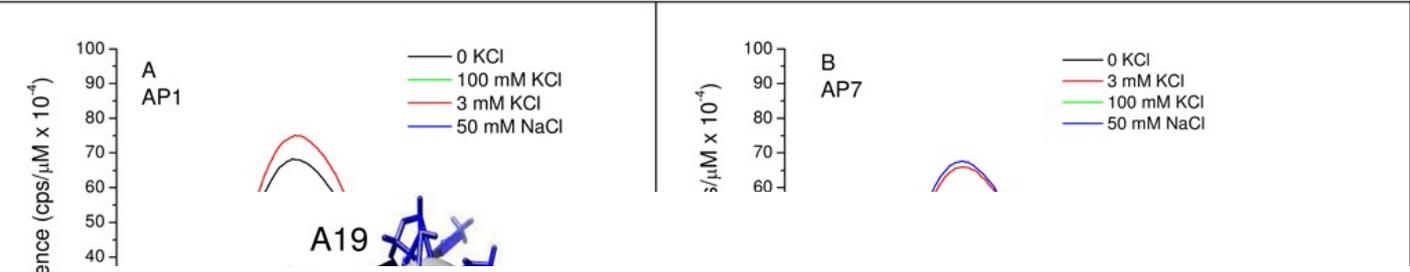
2. Fluorescent labels – FITC, TAMRA, ...



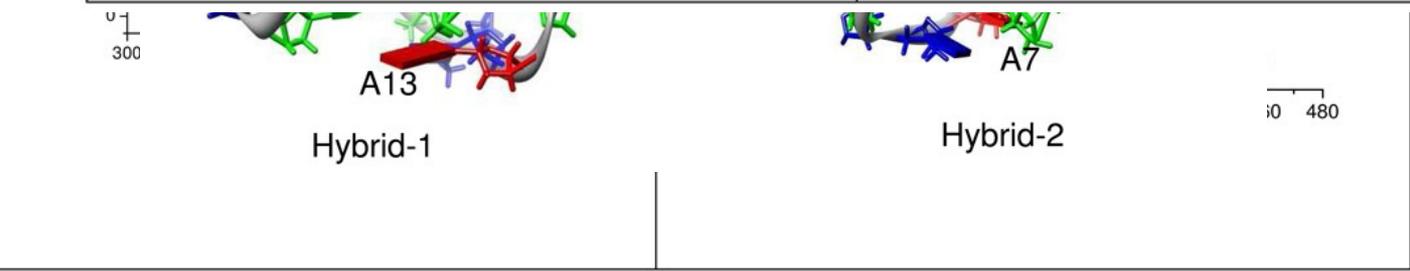
ThermoFisher Scientific
Fluorescence spectra viewer

3. Fluorescent ligand – EtBr, porphyrins, ...

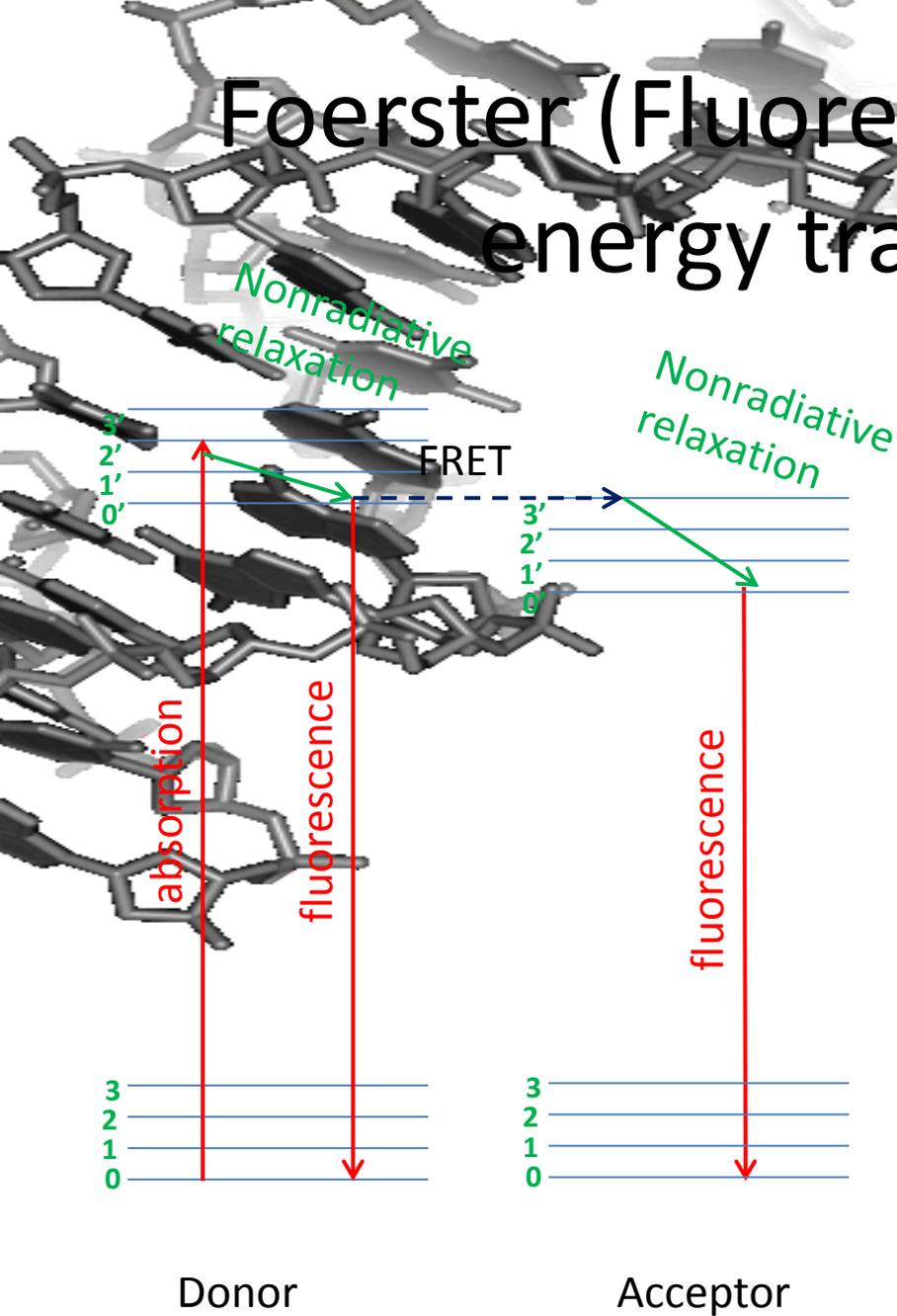
Fluorescence – guanine quadruplex



K⁺-
 ent
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 derivatives
 as a
 of cation
 concentration.

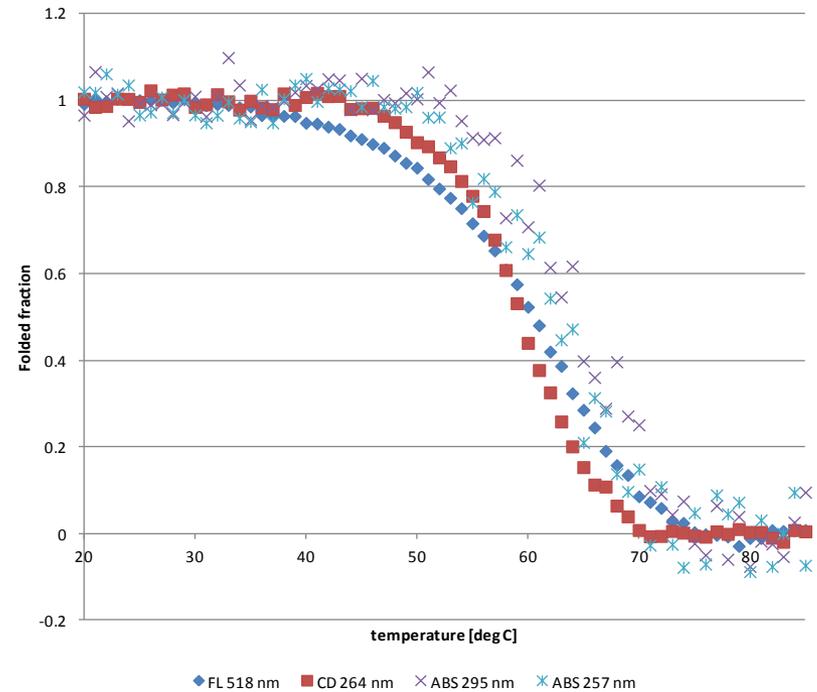
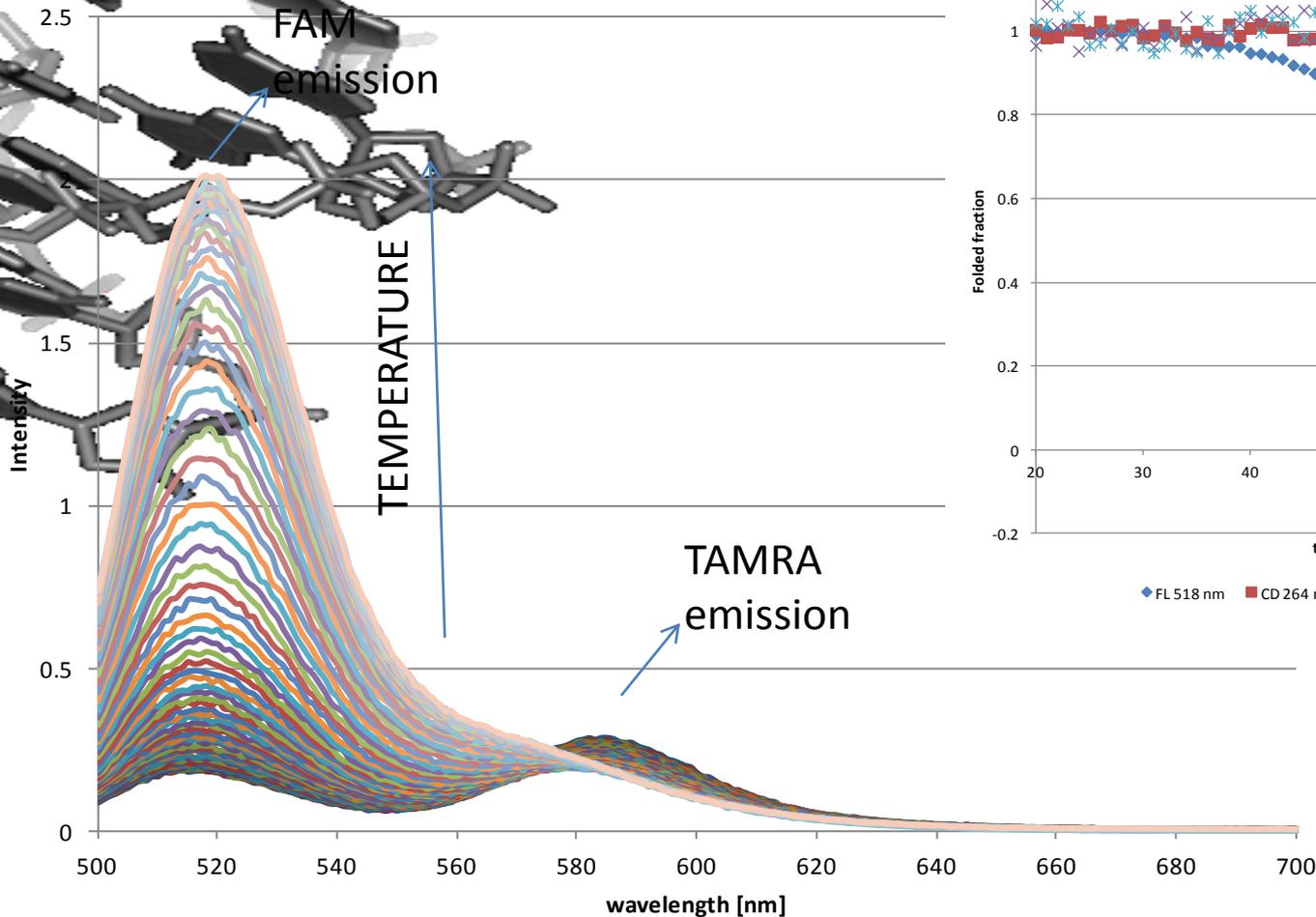


Foerster (Fluorescence) resonance energy transfer (FRET)

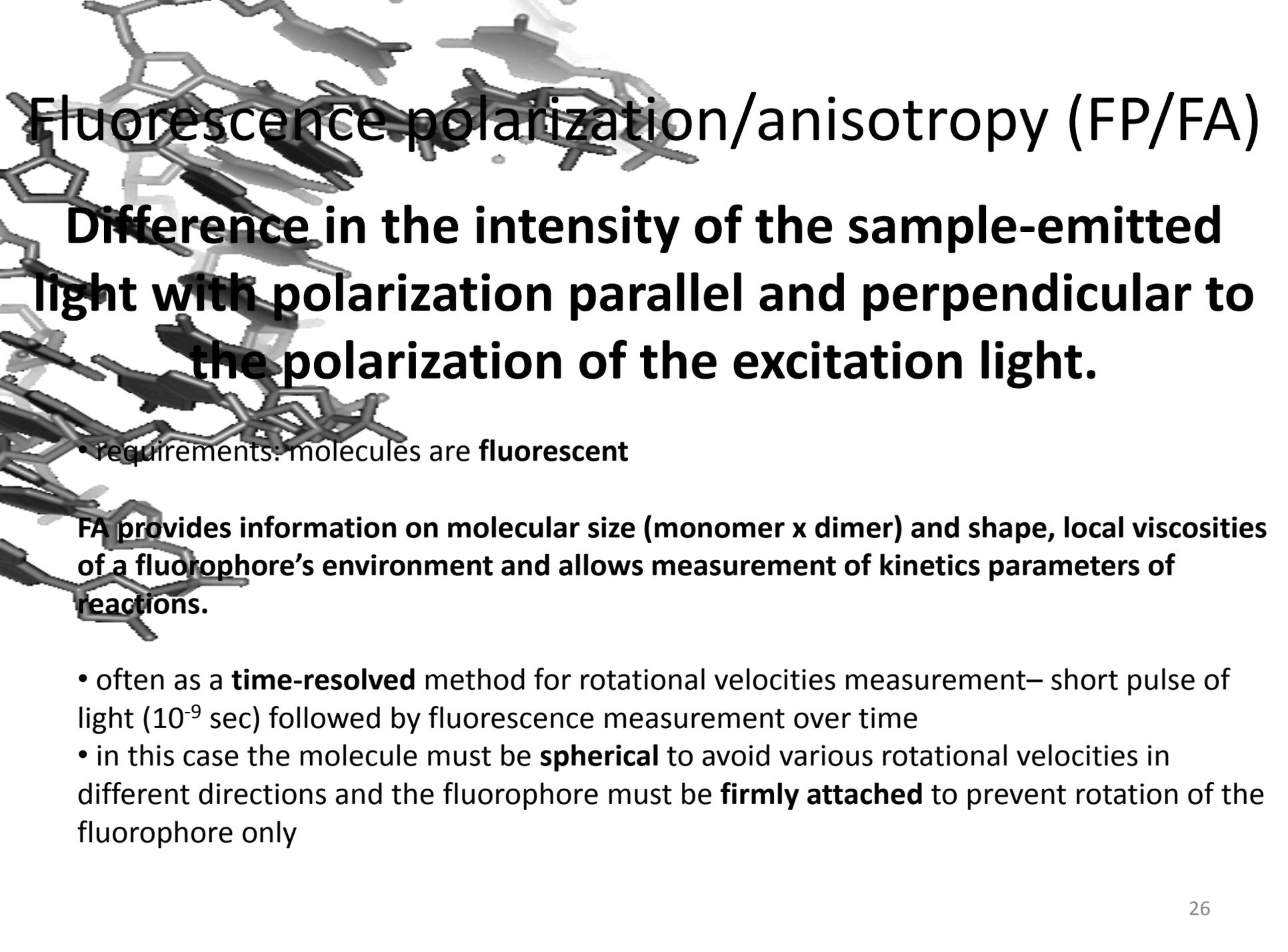


- FRET might occur when the emission band of the donor overlaps with the excitation band of the acceptor and the molecules are close enough.
- FRET range 1-10 nm
- Various FRET pairs, characterized by R_0 (distance where FRET is 50% for this pair)
- FRET efficiency $E = 1 / (1 + r / R_0)^6$

Foerster (Fluorescence) resonance energy transfer (FRET)



FAM – GQ – TAMRA
150 mM K
Excitation: 480 nm



Fluorescence polarization/anisotropy (FP/FA)

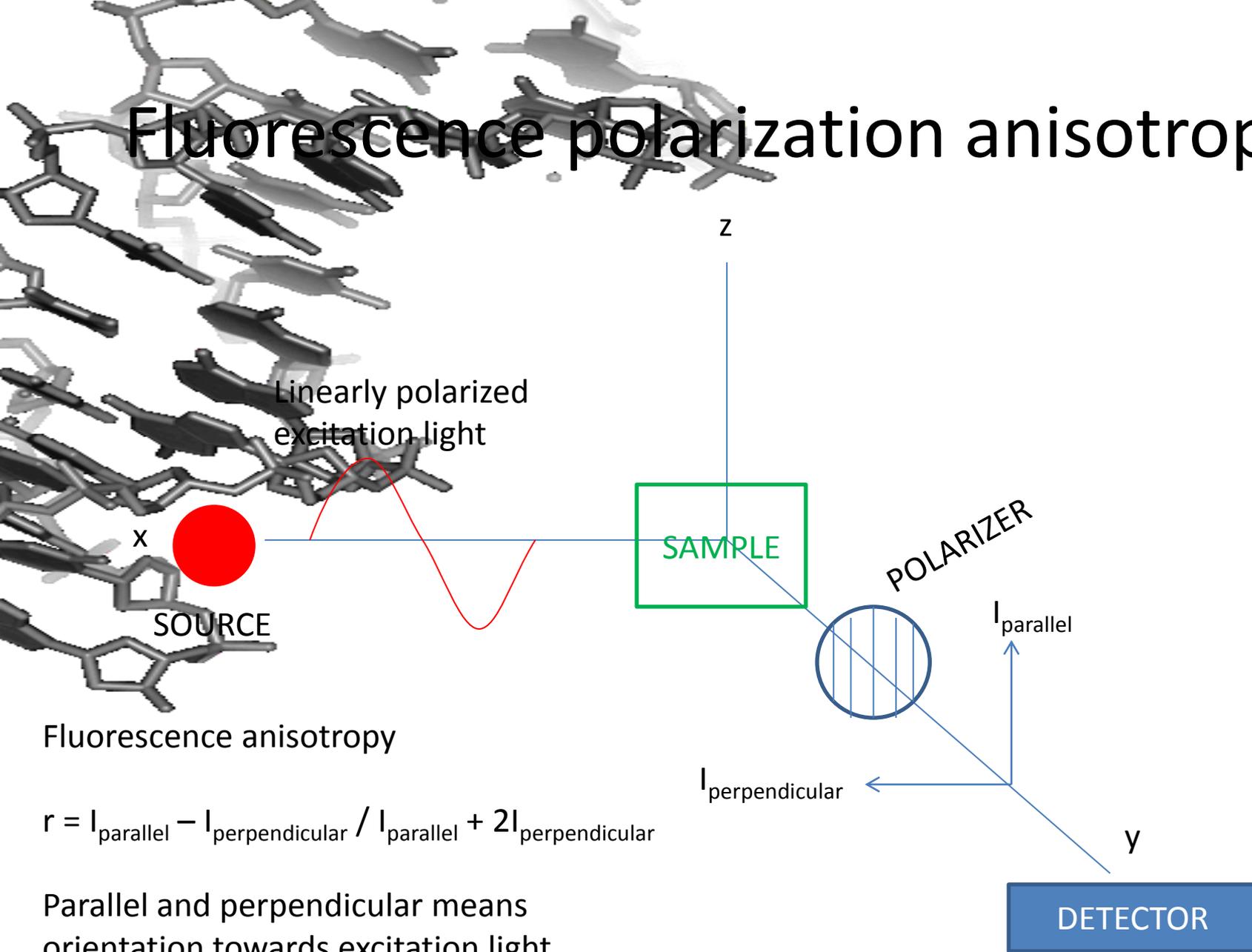
Difference in the intensity of the sample-emitted light with polarization parallel and perpendicular to the polarization of the excitation light.

- requirements: molecules are **fluorescent**

FA provides information on molecular size (monomer x dimer) and shape, local viscosities of a fluorophore's environment and allows measurement of kinetics parameters of reactions.

- often as a **time-resolved** method for rotational velocities measurement— short pulse of light (10^{-9} sec) followed by fluorescence measurement over time
- in this case the molecule must be **spherical** to avoid various rotational velocities in different directions and the fluorophore must be **firmly attached** to prevent rotation of the fluorophore only

Fluorescence polarization anisotropy

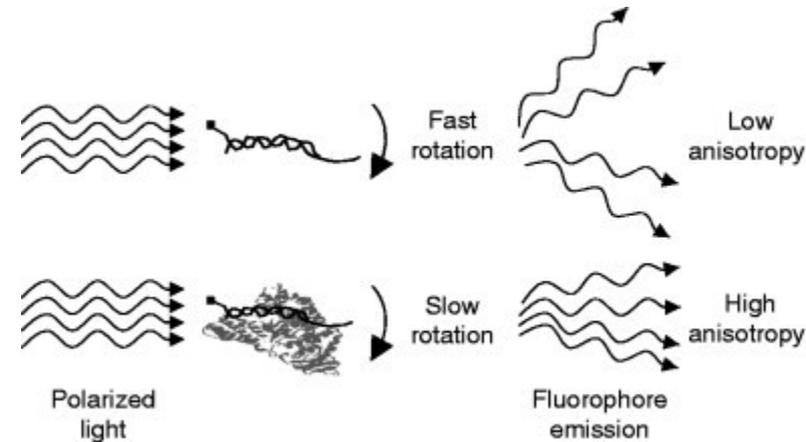
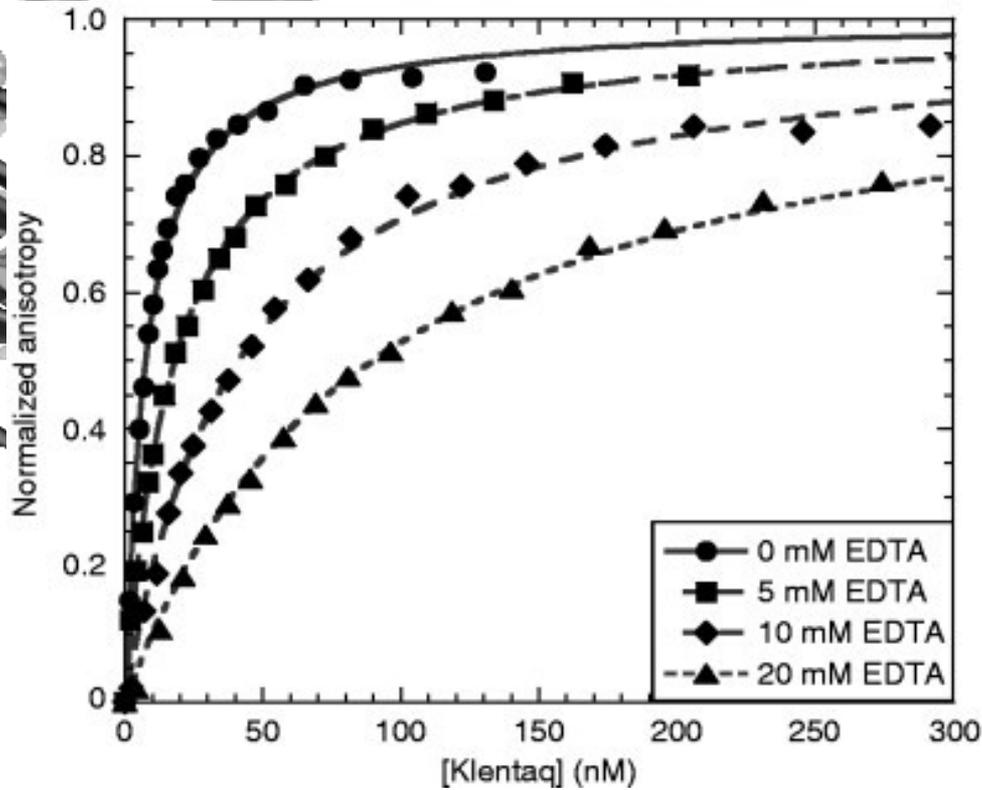


Fluorescence anisotropy

$$r = \frac{I_{\text{parallel}} - I_{\text{perpendicular}}}{I_{\text{parallel}} + 2I_{\text{perpendicular}}}$$

Parallel and perpendicular means orientation towards excitation light

Fluorescence polarization anisotropy



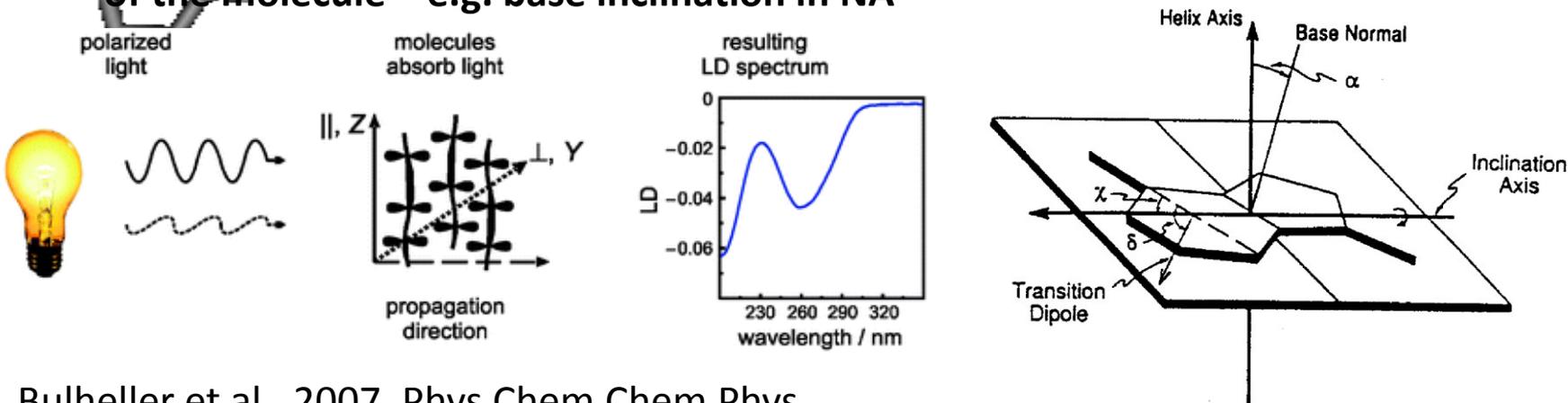
The effects of EDTA on the binding of Klentaq DNA polymerase to primed-template DNA (13/20-mer DNA)

Linear dichroism (LD)

Difference in absorption of the light linearly polarized parallelly and perpendicularly to the orientation of the molecules

- requirements: molecules are **oriented** and molecules **absorb** in the region of interest
- orienting the molecules: gel, electric field, flow (rotation)

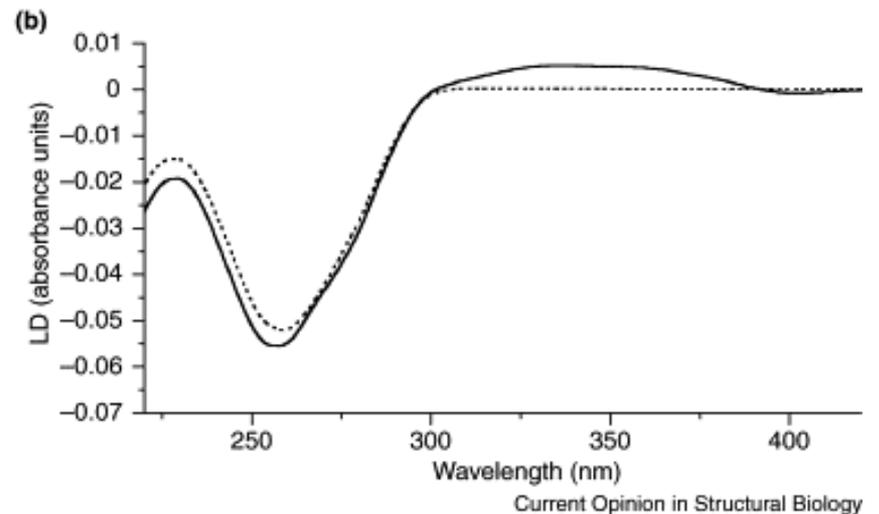
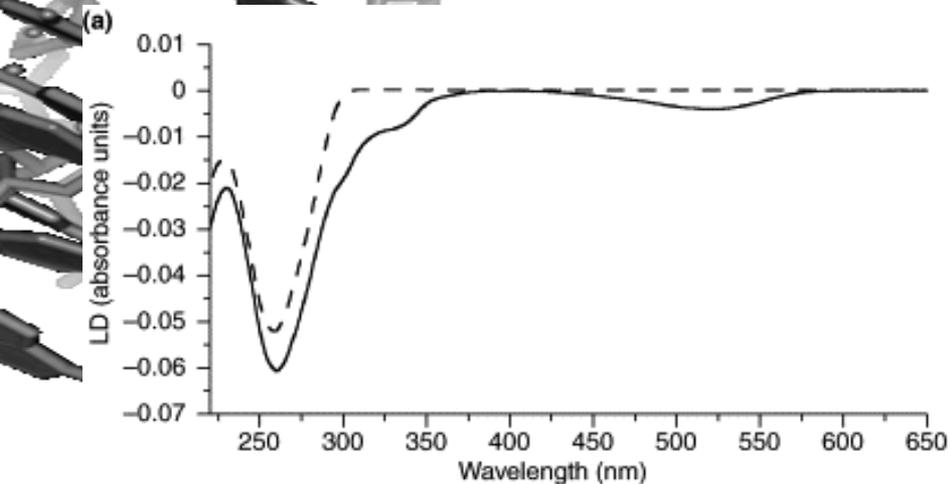
LD is sensitive to the orientation of absorbing parts (nucleobases) towards the orientation of the molecule – e.g. base inclination in NA



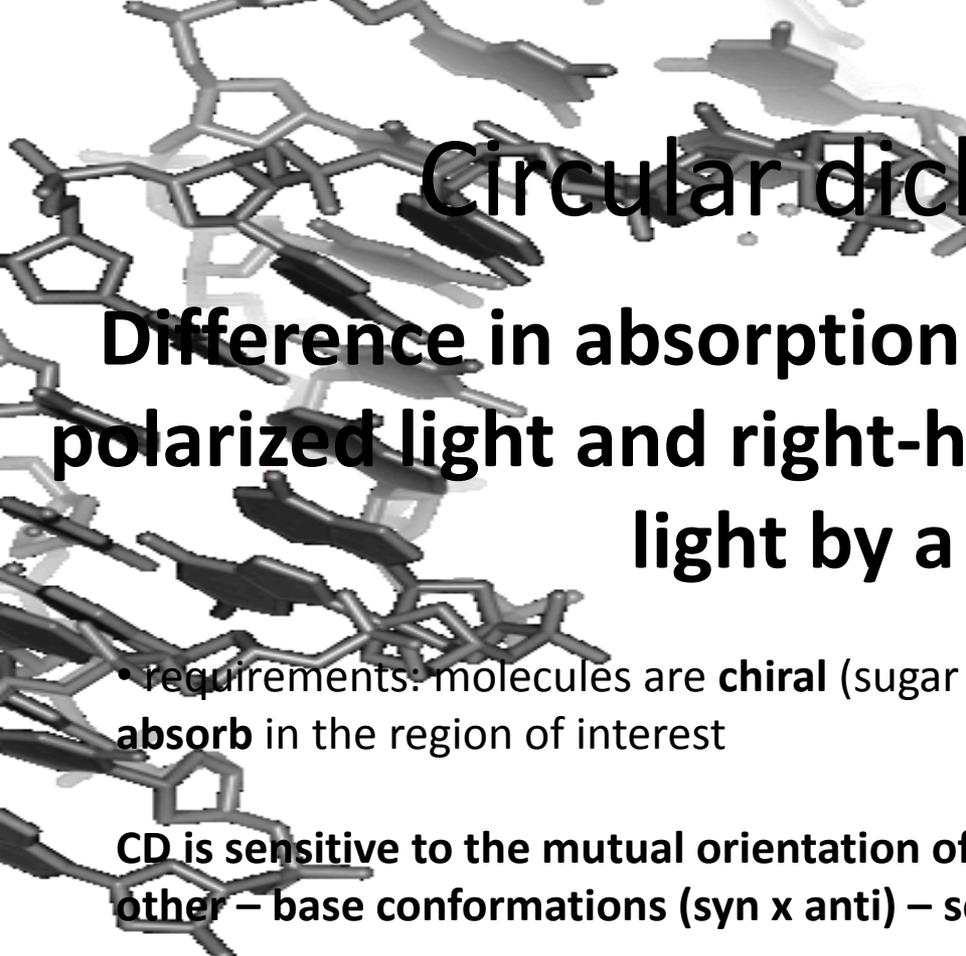
Bulheller et al., 2007, Phys Chem Chem Phys

Rodger et al., 2006, Phys Chem Chem Phys

LD DNA + ligand



LD of DNA and DNA–ligand systems. **(a)** LD of calf thymus DNA (1000 μM base, dashed line) and the DNA plus an ethidium bromide intercalator (50 μM, solid line). **(b)** LD of calf thymus DNA (1000 μM base, dashed line) and the DNA plus a minor groove binder (diaminophenyl indole, 50 μM, solid line)



Circular dichroism (CD)

Difference in absorption of left-handed circularly polarized light and right-handed circularly polarized light by a molecule

- requirements: molecules are **chiral** (sugar in NA), thus optically active and molecules **absorb** in the region of interest

CD is sensitive to the mutual orientation of absorbing parts (nucleobases) towards each other – base conformations (syn x anti) – secondary structure of DNA

- **optical activity** = ability of the molecule to differentially interact with left-handed and right-handed circularly polarized light
- **Optical rotatory dispersion (ORD)** – angle of rotation of the linearly polarized light after passing through the optically active molecule – ORD in whole range of wavelengths, with anomalous ORD, where molecule absorbs – more difficult interpretation than CD
- **Cotton effect** – CD / ORD band – positive x negative

Circular dichroism (CD)

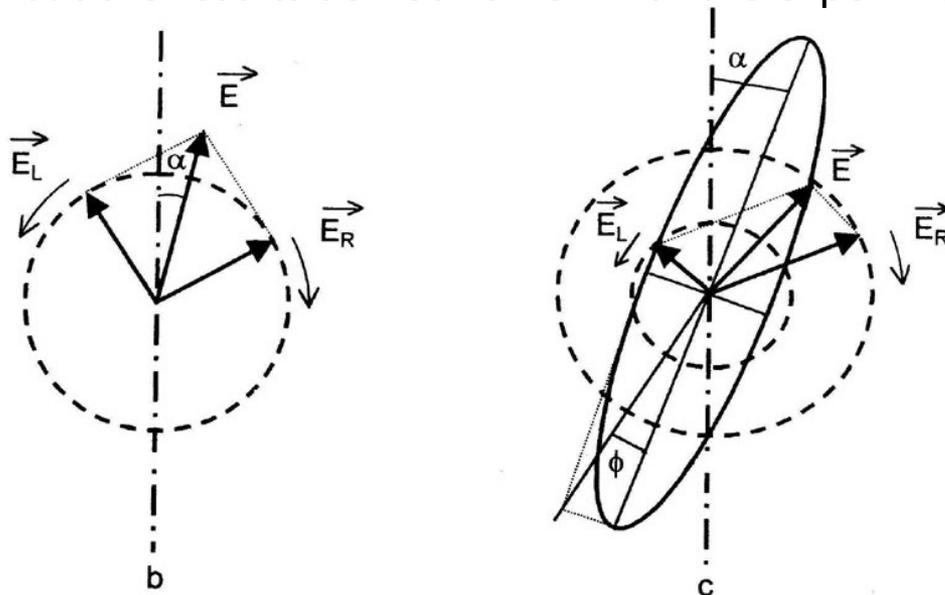
- Difference in absorbance: $\Delta A = A_L - A_R$

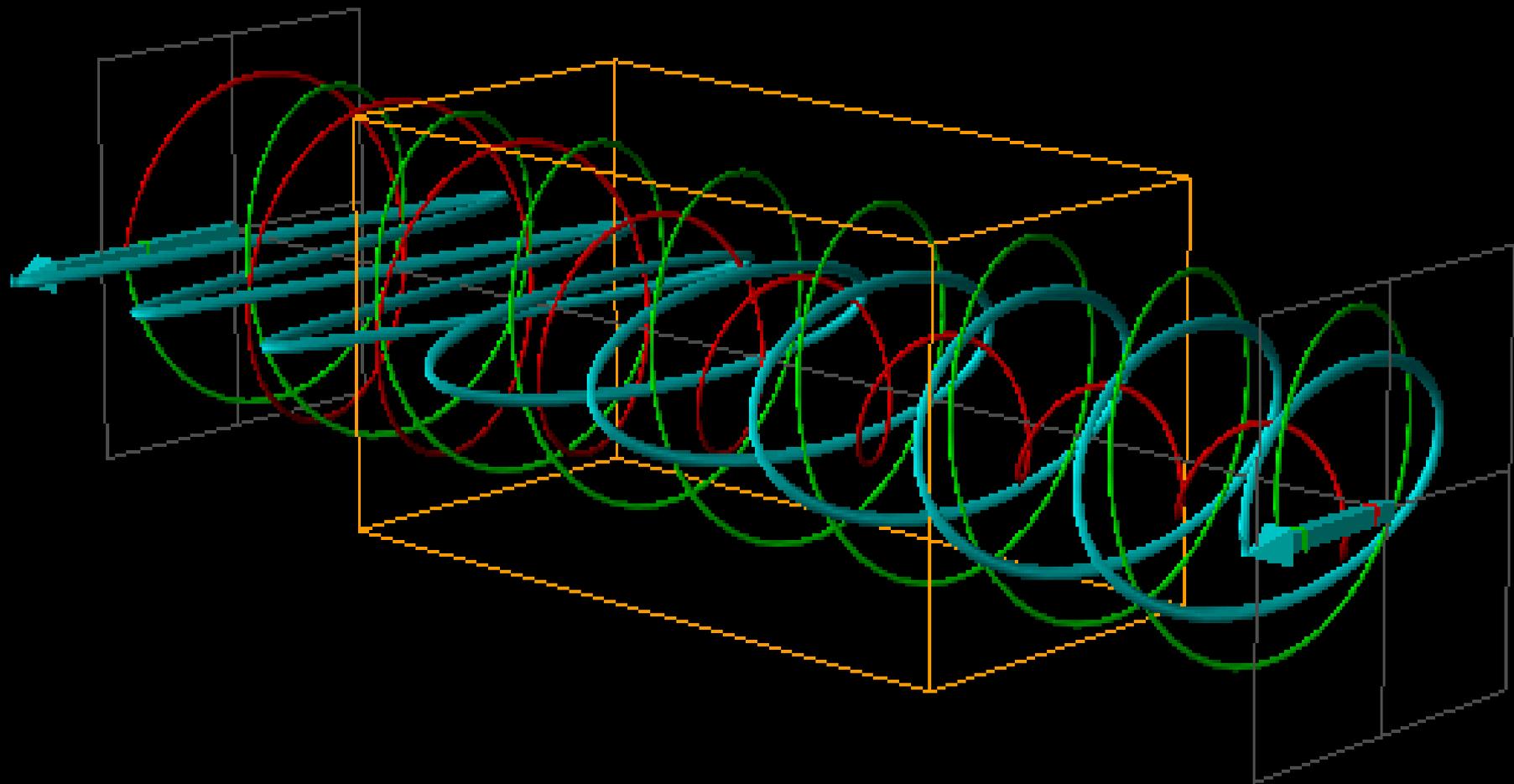
- When known concentration, difference in molar absorption $\Delta \epsilon = \epsilon_L - \epsilon_R = \Delta A / lc$
(Beer-Lambert law)

- Ellipticity – the angle that describes the extent of change of the linearly polarized light into a elliptically polarized light (0 for linearly polarized, 45° for circularly polarized)

$$\tan \phi = (E_L - E_R) / (E_L + E_R) = 3298 * \Delta \epsilon$$

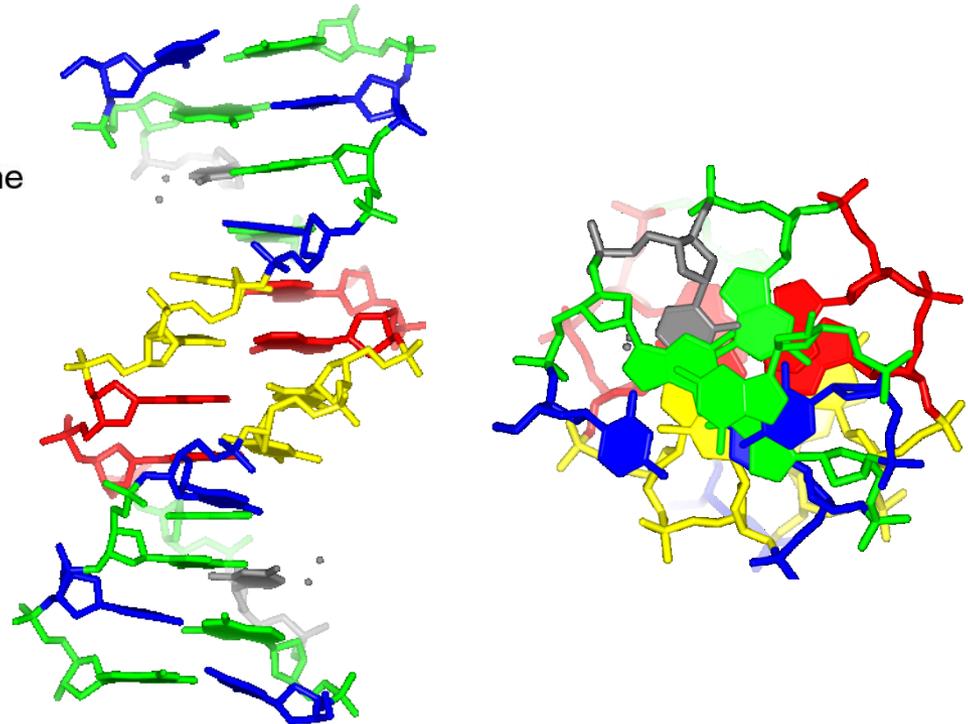
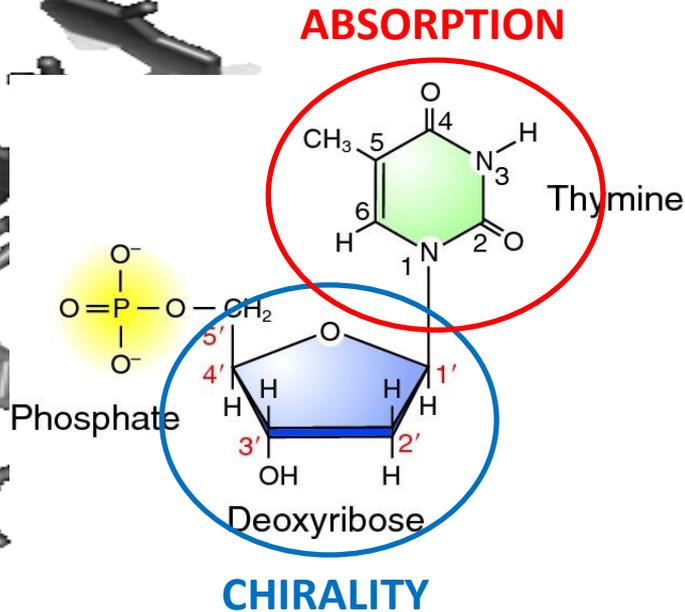
- CD can be calculated but the results do not fit well with the experiment





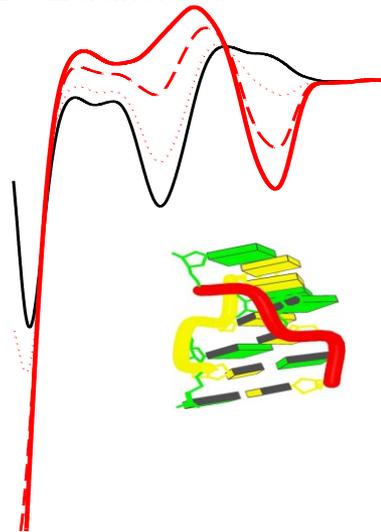
Circular dichroism – DNA / RNA

MUTUAL ORIENTATION OF BASES

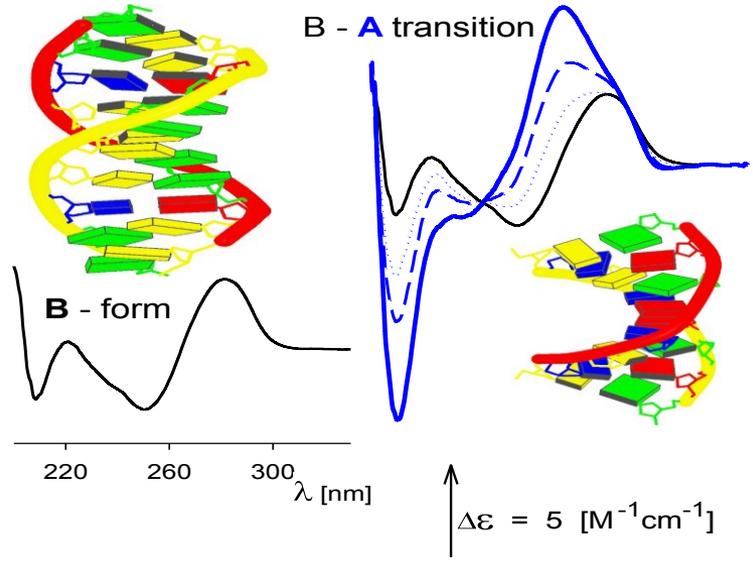




B - Z transition



B - A transition

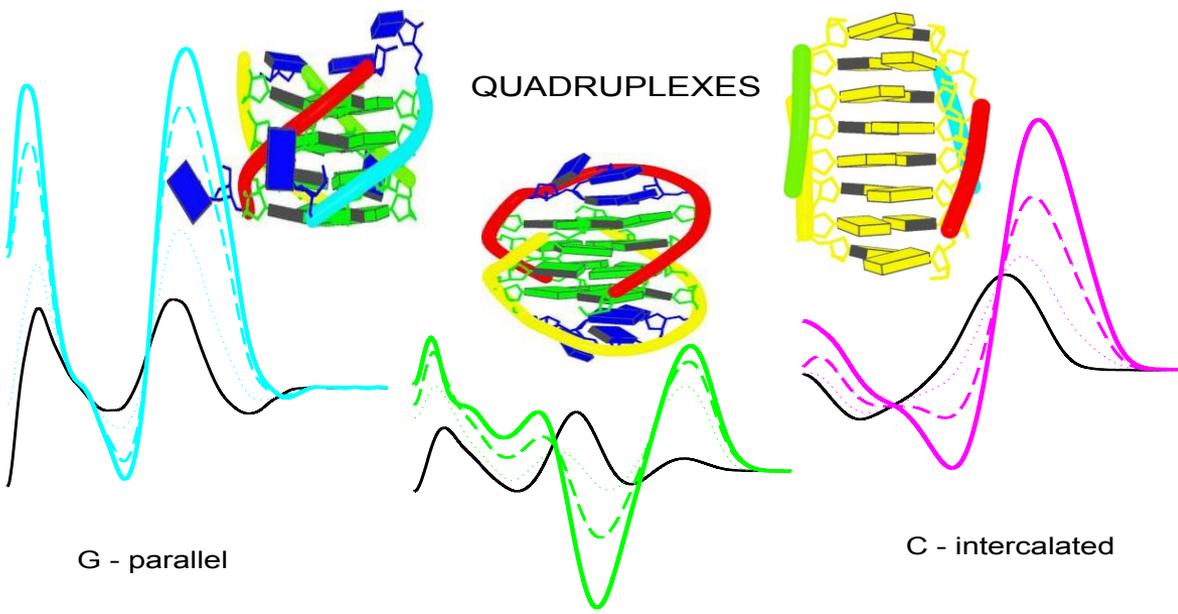


B - form

220 260 300 λ [nm]

$\Delta\epsilon = 5 \text{ [M}^{-1}\text{cm}^{-1}\text{]}$

QUADRUPLEXES



G - parallel

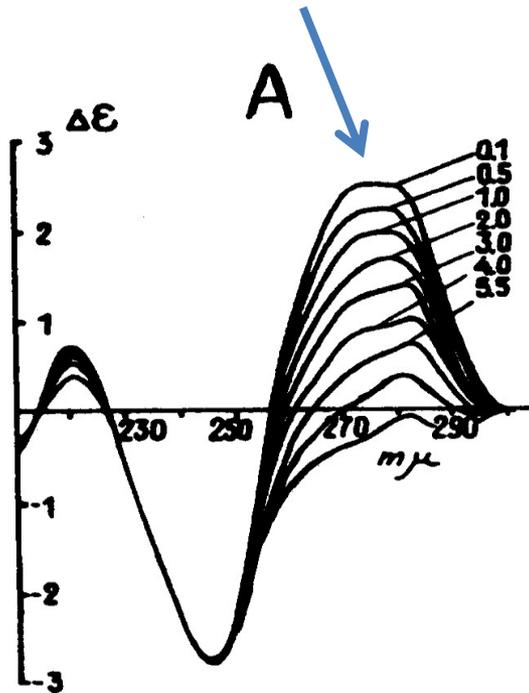
G - antiparallel

C - intercalated

Transition cooperativity

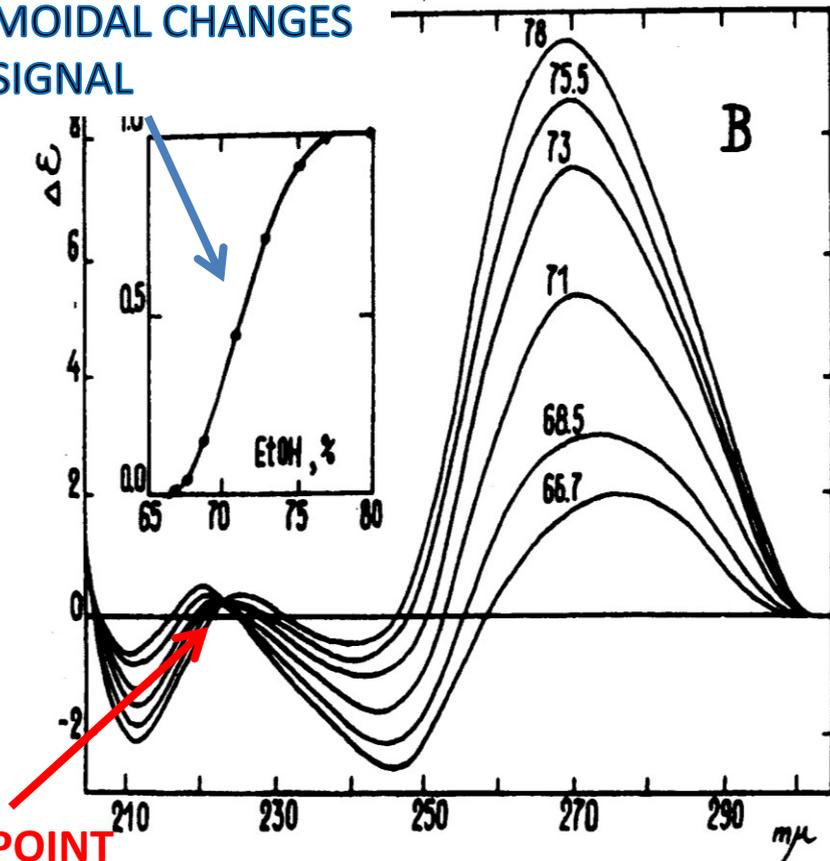
NON-COOPERATIVE
TRANSITION

LINEAR CHANGES
OF SIGNAL

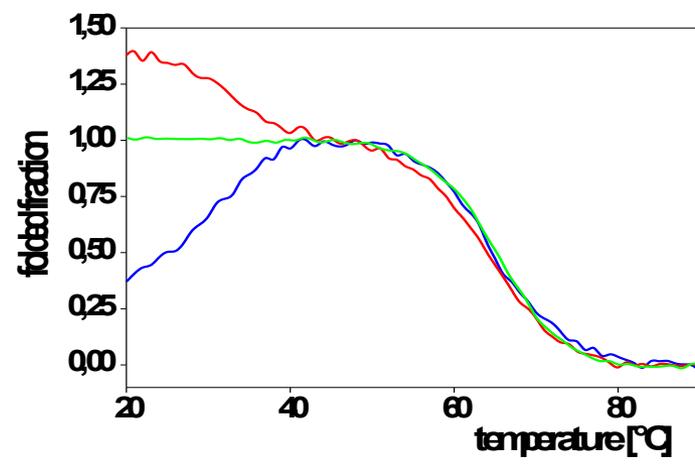
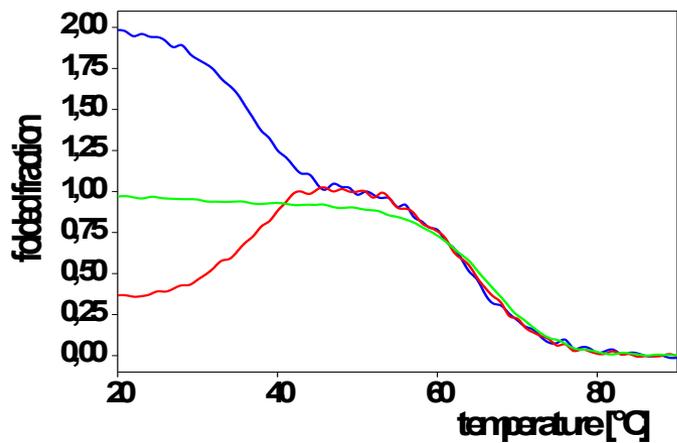
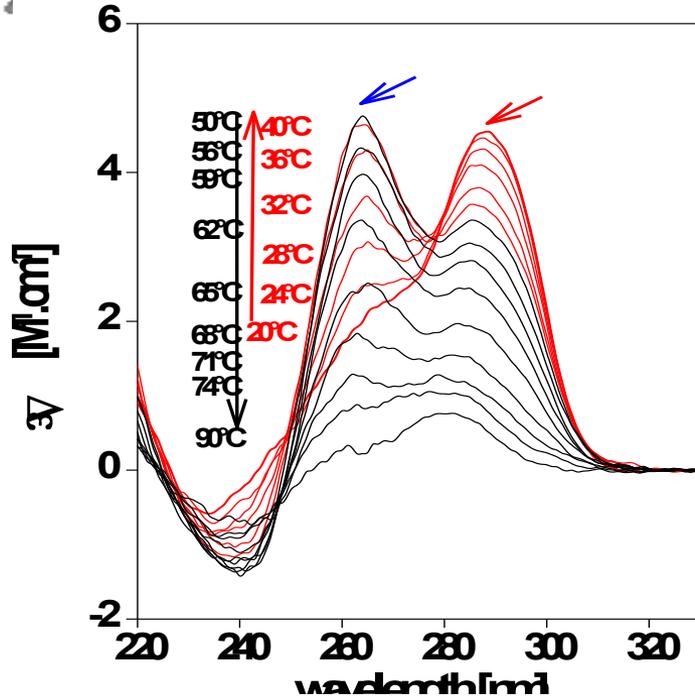
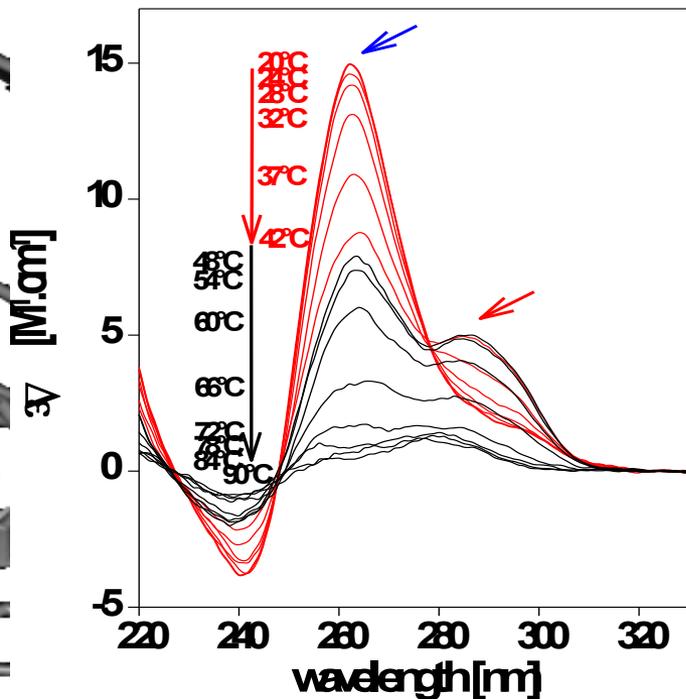


COOPERATIVE TRANSITION

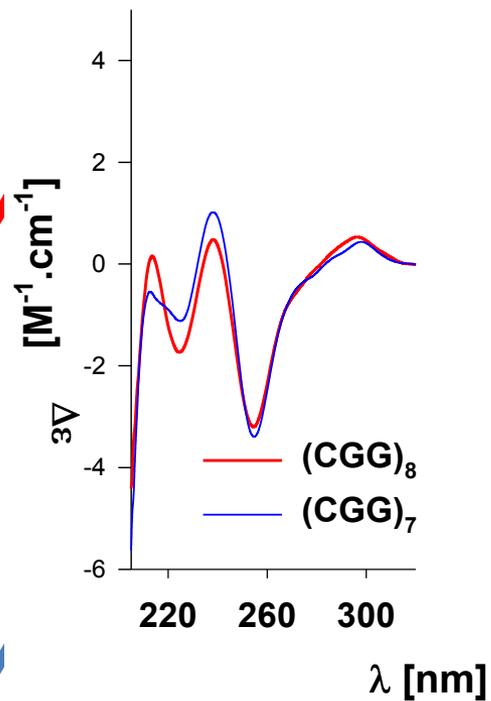
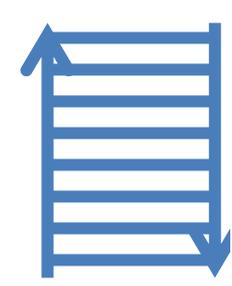
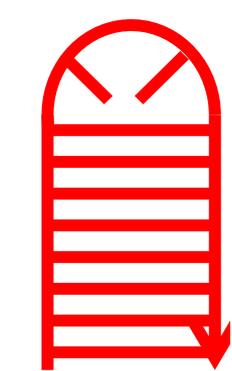
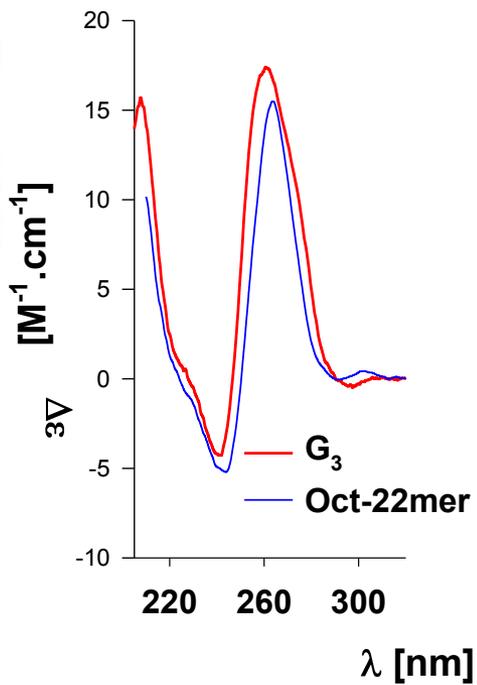
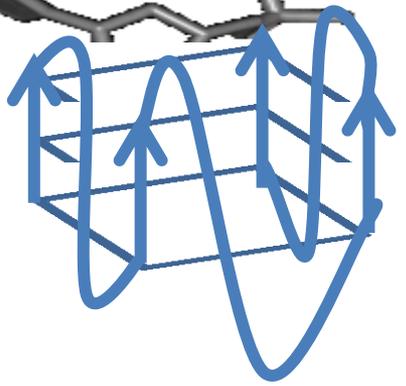
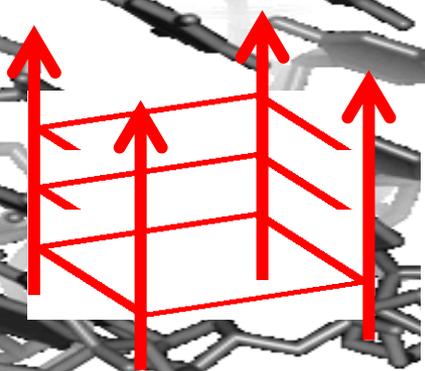
SIGMOIDAL CHANGES
OF SIGNAL



CD – NA melting



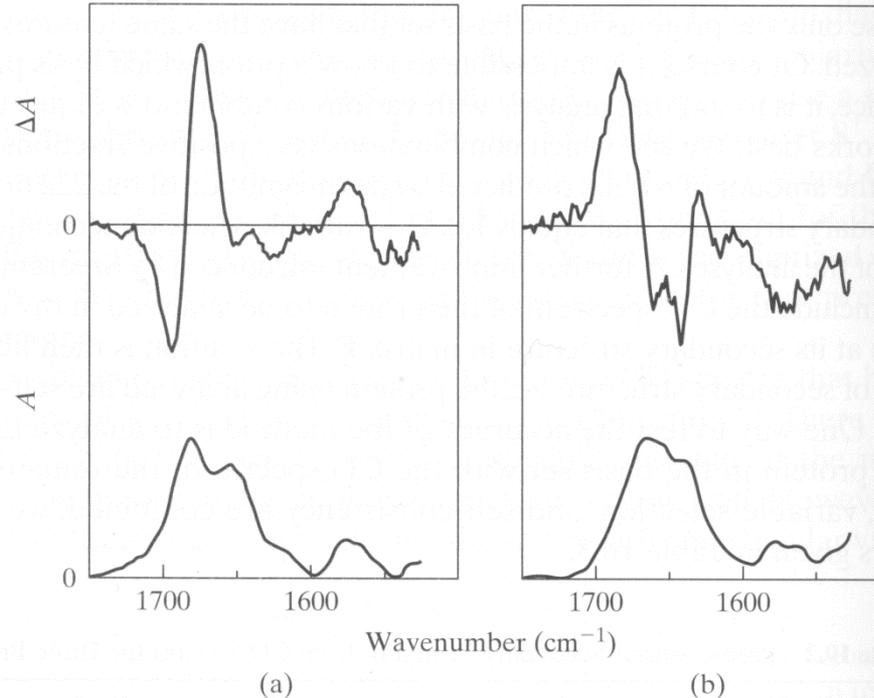
Moleculararity



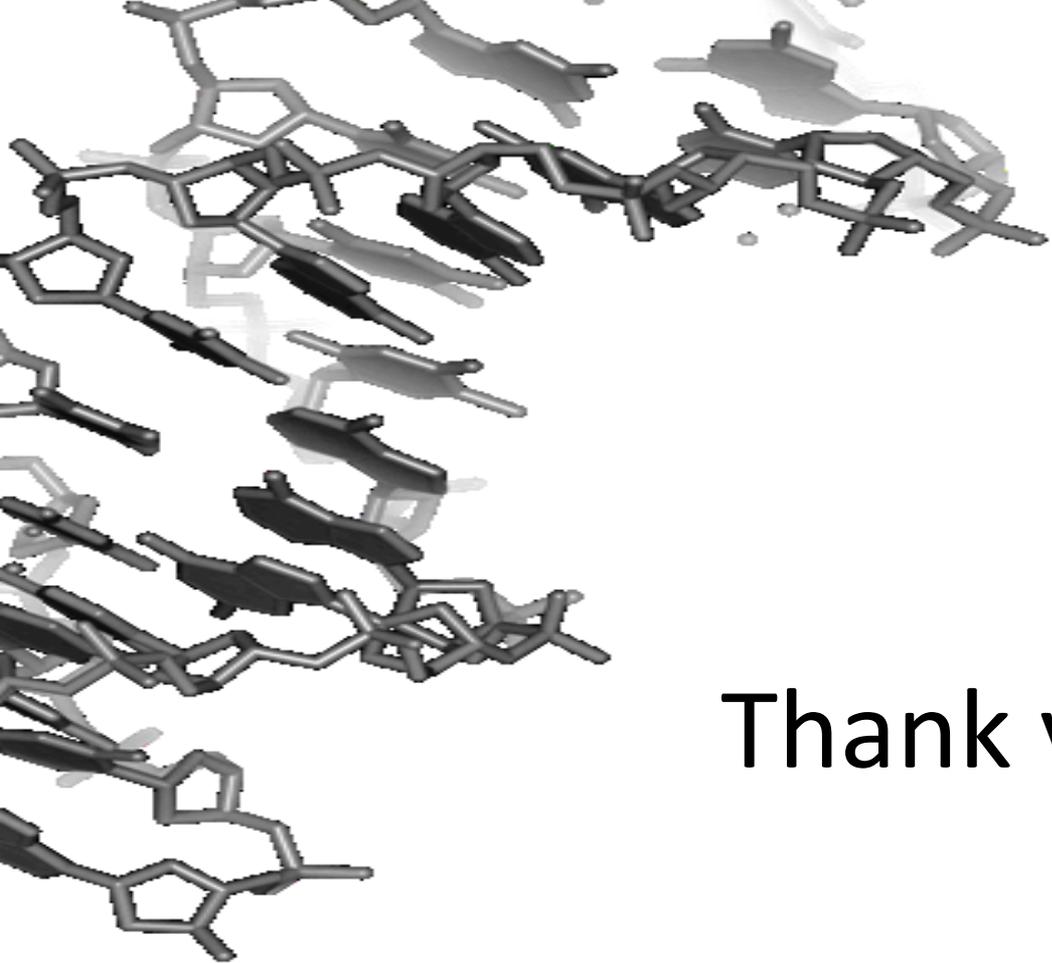
Vibrational / infrared CD (VCD/IRCD)

Difference in absorption of left-handed circularly polarized light and right-handed circularly polarized light in a region of vibrational transitions ($\lambda = 1-5 \mu\text{m}$).

- compared to eCD, IRCD shows well differentiated bands belonging to specific functional groups



The vibration CD and absorption spectra of homoduplex of $d(\text{GC})_{10}$ as the right-handed B-form and the left-handed Z-form.



Thank you