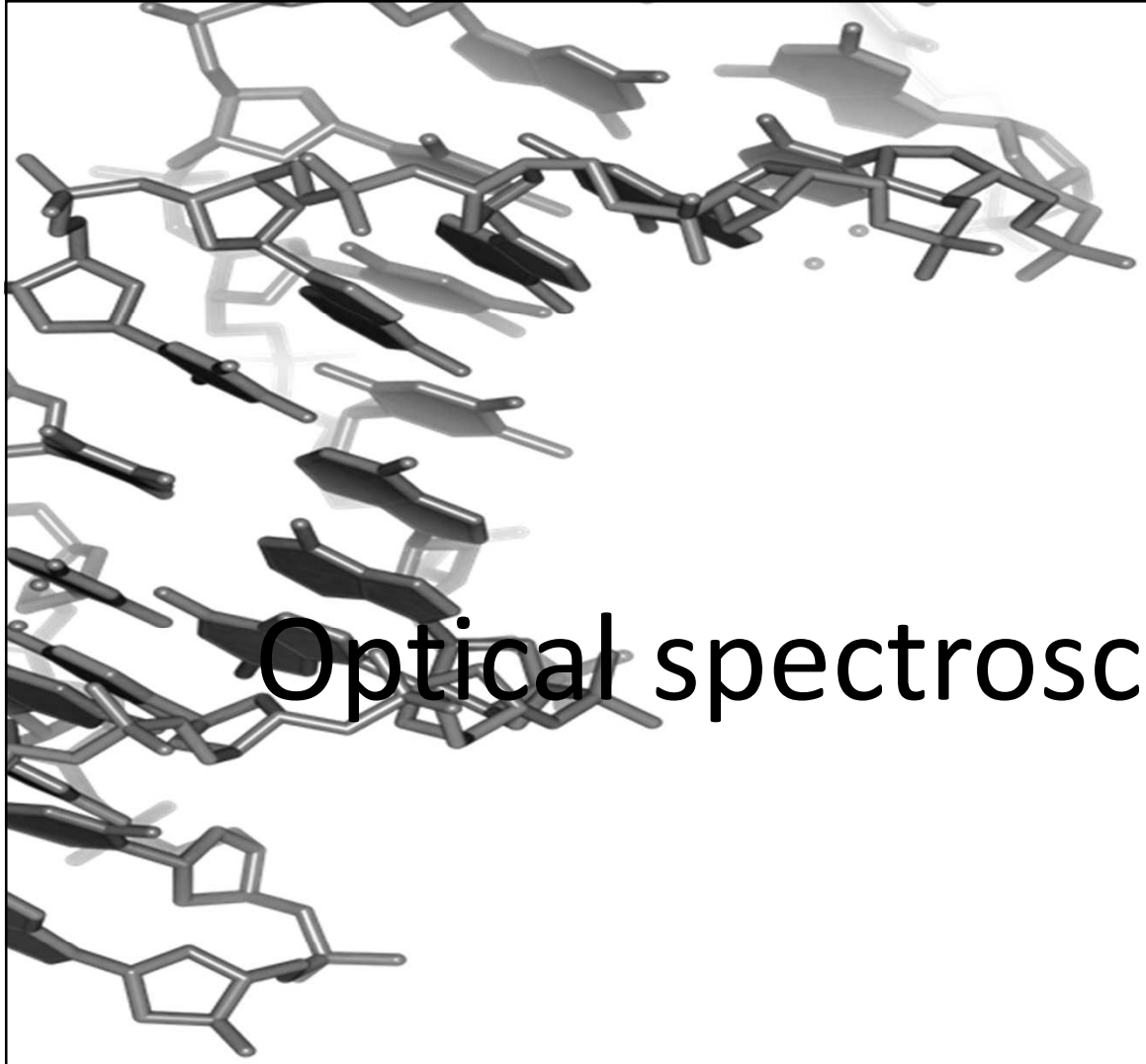
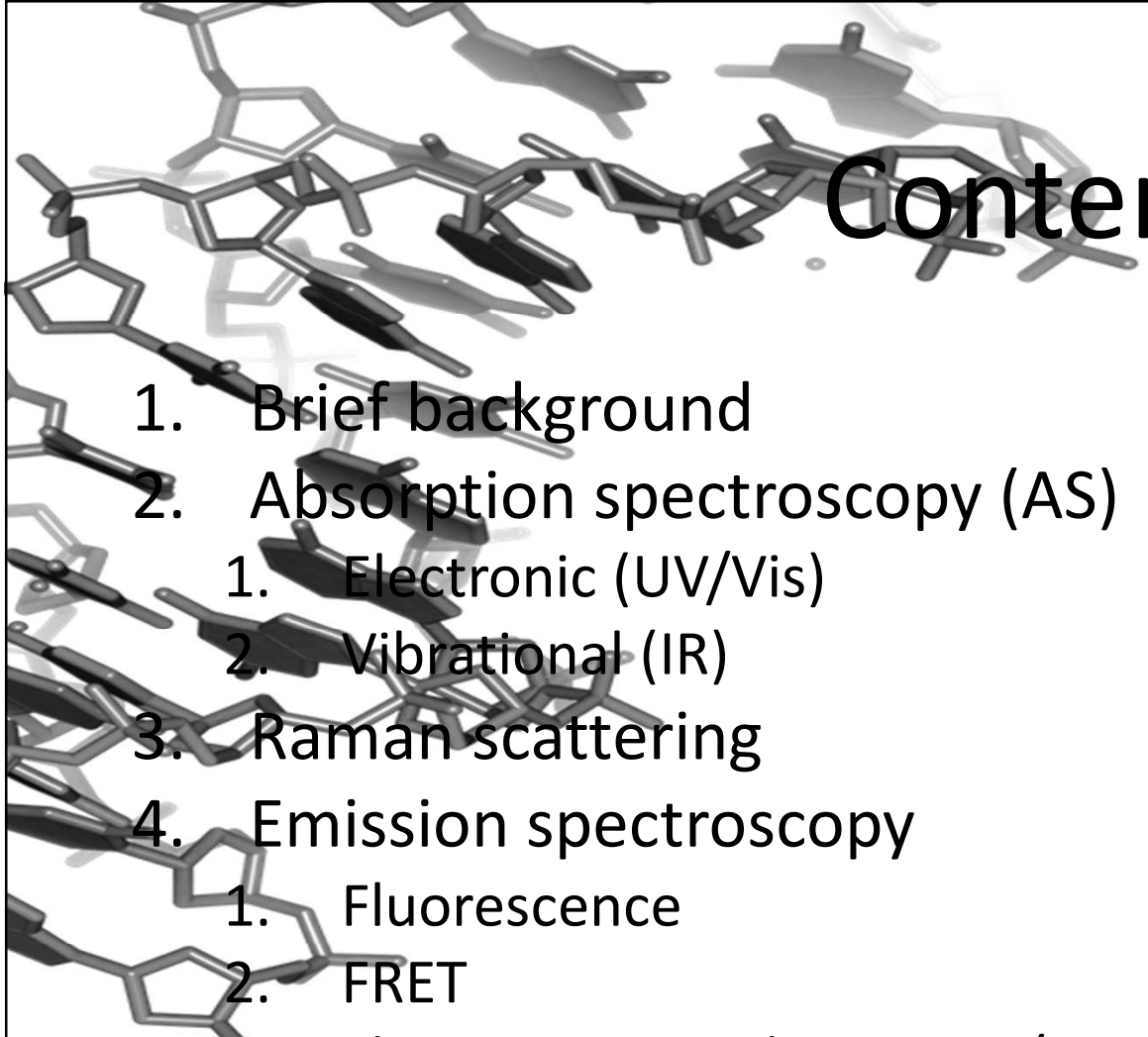


# Optical spectroscopic methods

Daniel Renčiuk
IBP AS CR

S1001 / 2016 - CEITEC



# 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. Chirooptical methods
    1. Linear dichroism
    2. Circular dichroism

# Interaction of mass with EM radiation

Theory

QUANTUM MECHANICS

Wavefunction describes states of the molecule.

Experiment

SPECTROSCOPY

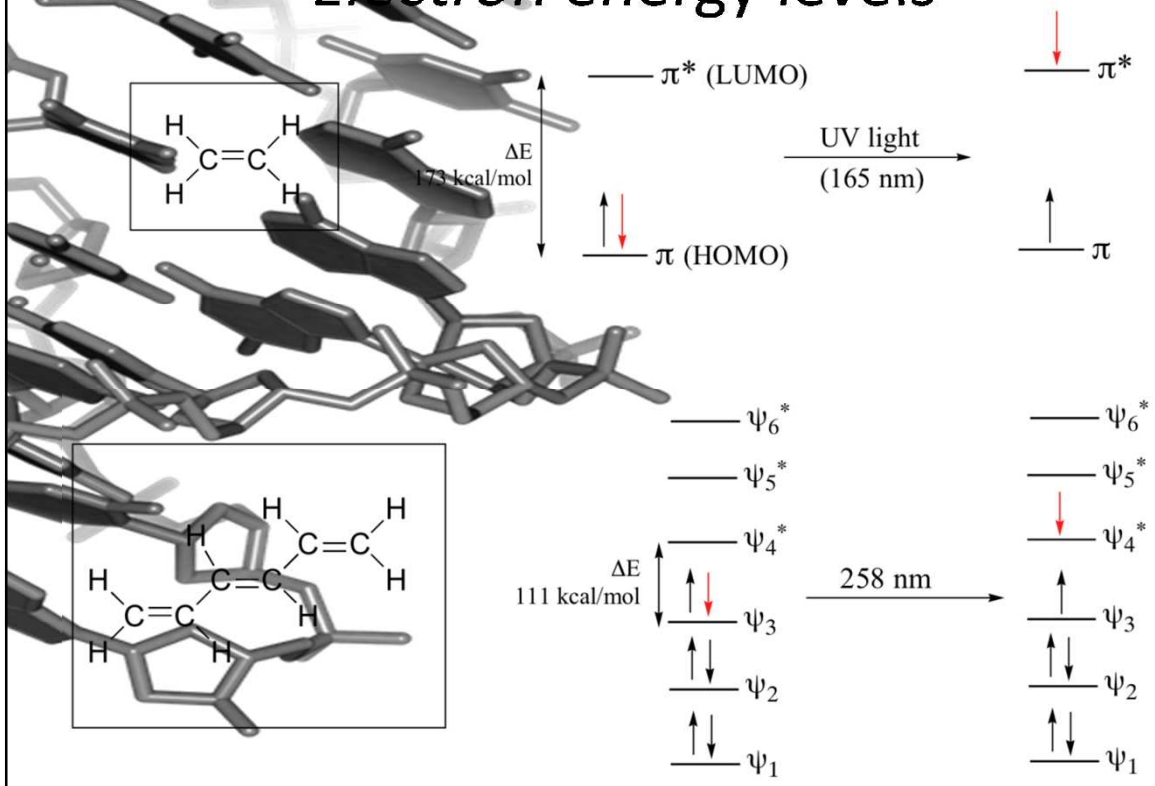
Position of absorption and emission peaks correspond to differences in E between states.

# Phenomena X EM spectral regions

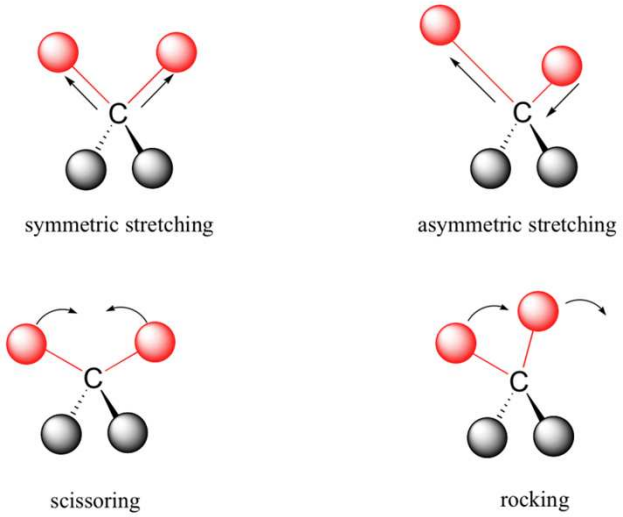
| Phenomenon | Spectral region | Wavelength |
|---|---|---|
| Nuclea | Gamma | 0.1 nm |
| Inner elect | | - 1.0 nm |
| Ionisation | UV | 0 - 200 nm |
| Valency electrons | near UV / VIS | 200 - 800 nm |
| Molecular vibrations | near IR / IR | 0.8 - 25 µm |
| Rotation and electron spin orientation in mag. fields | Microwaves | 400 µm – 30 cm |
| Nuclear spin orientation in mag. fields | Radiowaves | > 100 cm |

**OPTICAL SPECTROSCOPY**

# Electron energy levels

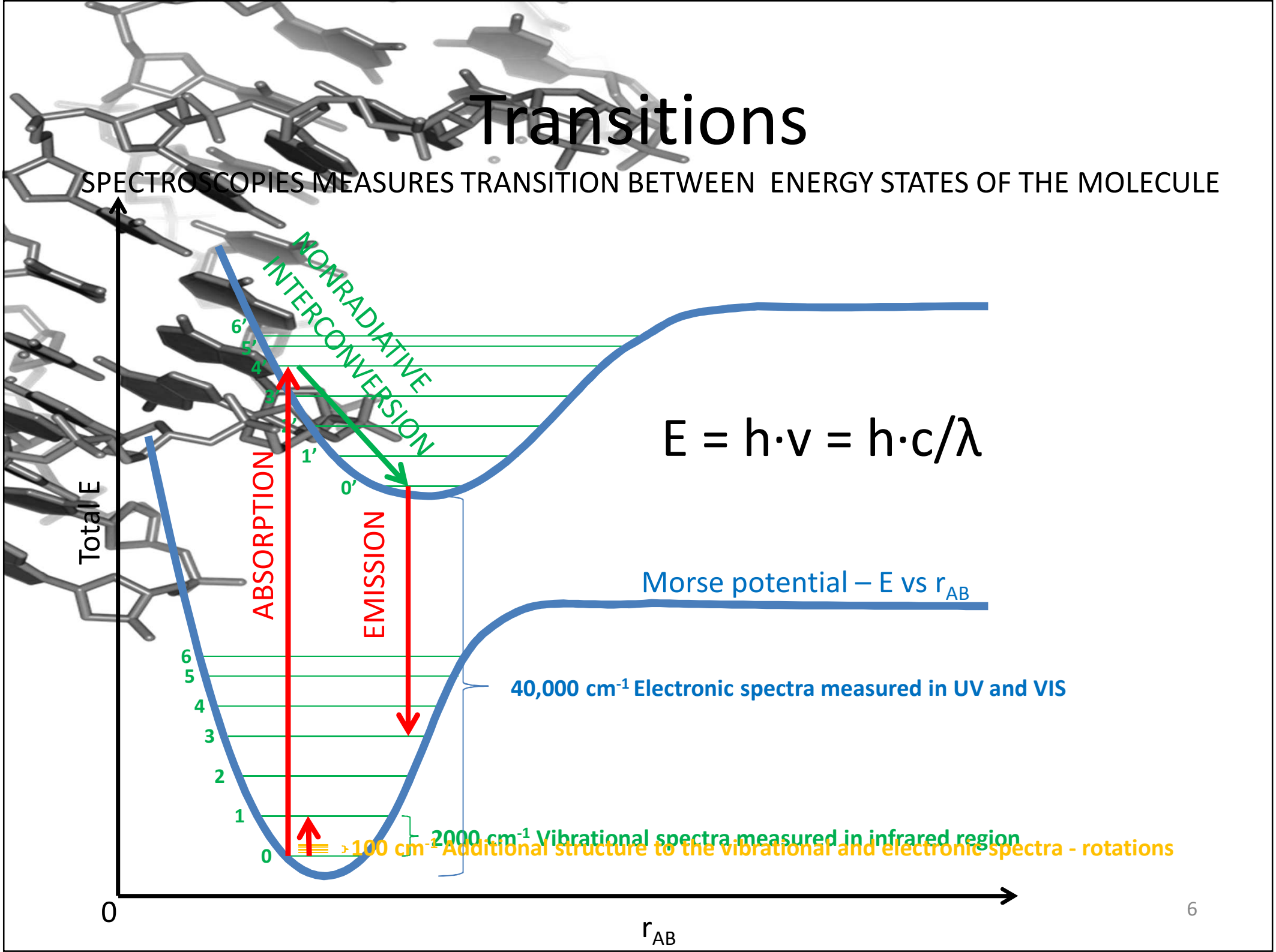



# Molecular vibrations



# Transitions

SPECTROSCOPIES MEASURES TRANSITION BETWEEN ENERGY STATES OF THE MOLECULE




$$E = h \cdot v = h \cdot c/\lambda$$

# 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 v0-vn' characterized by different energy and probability (intensity of the peak)

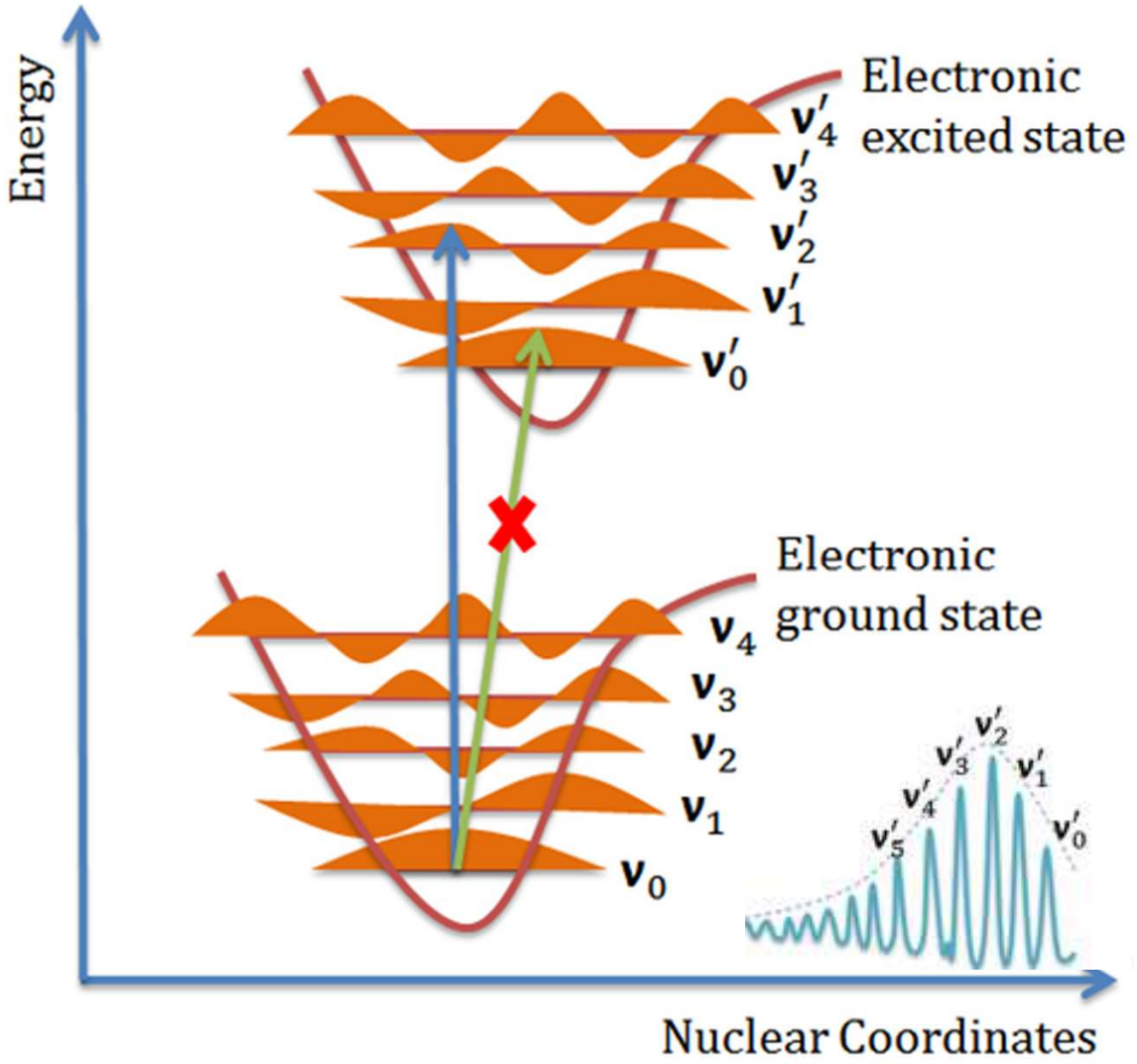
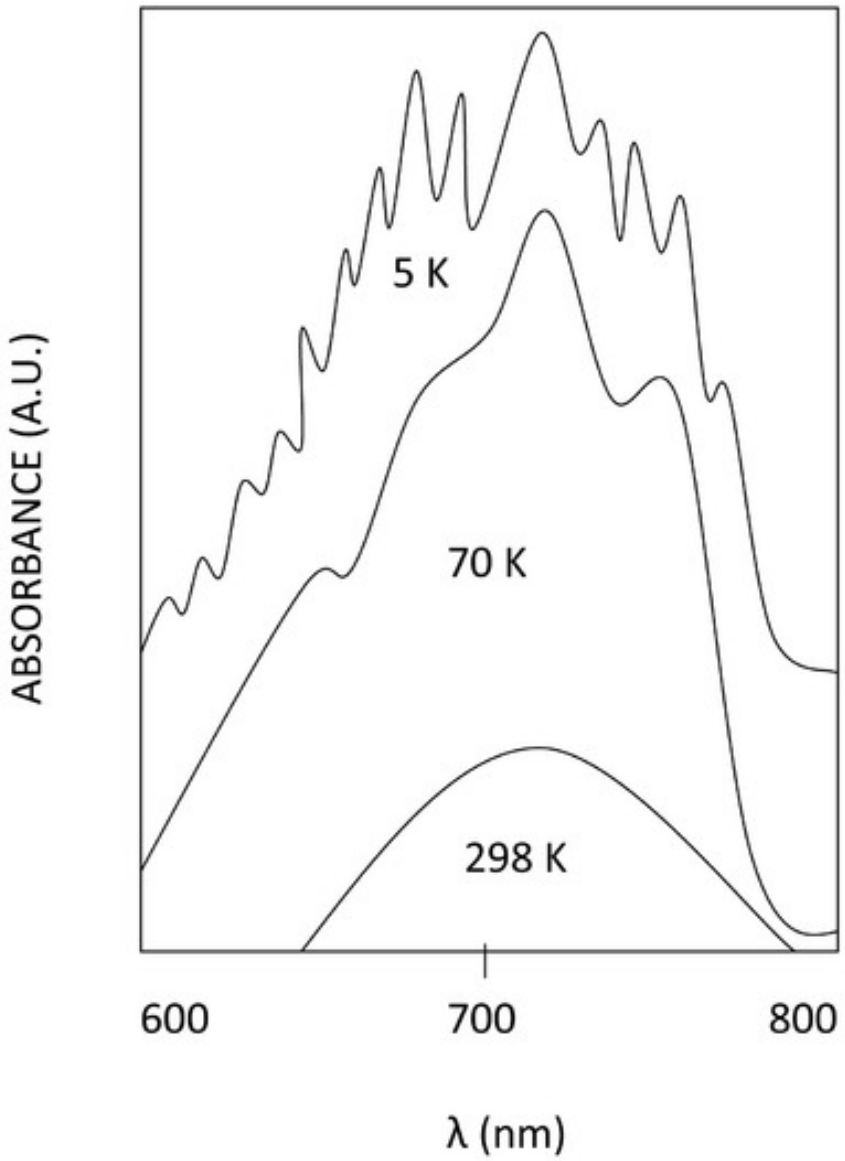
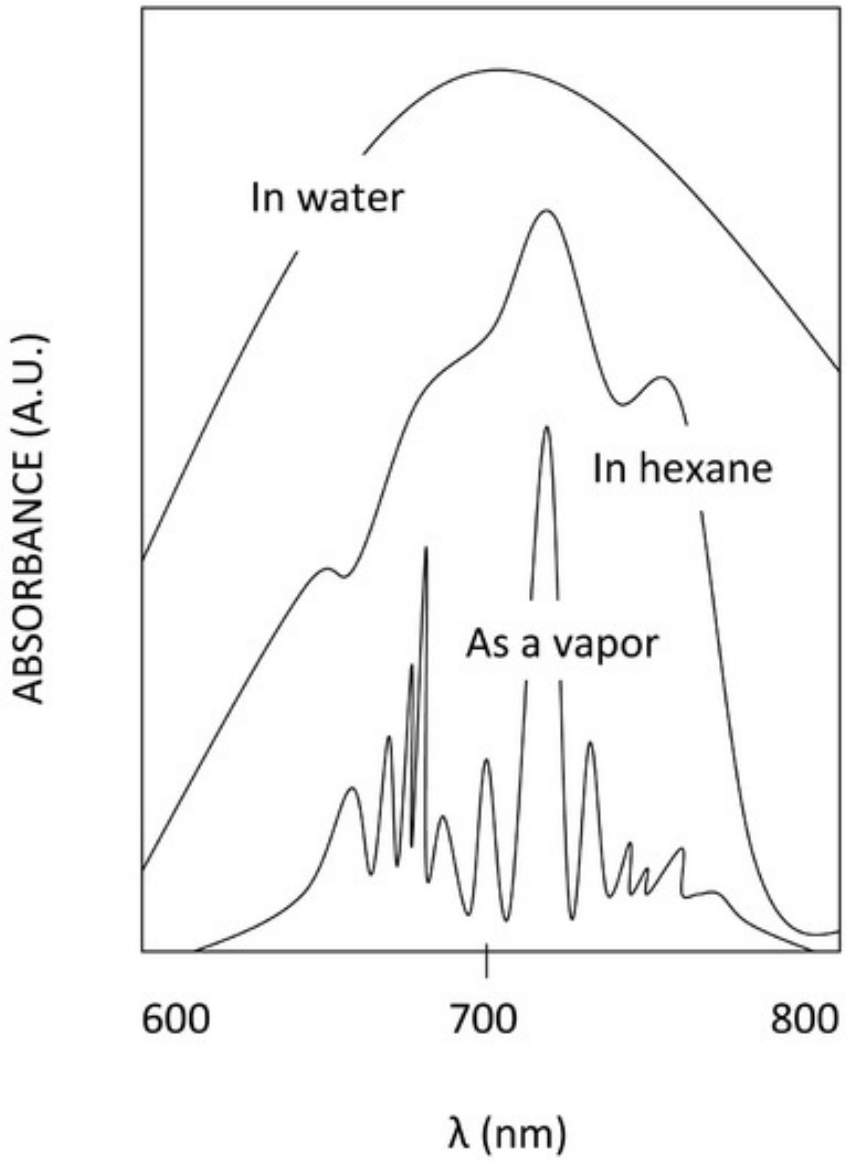


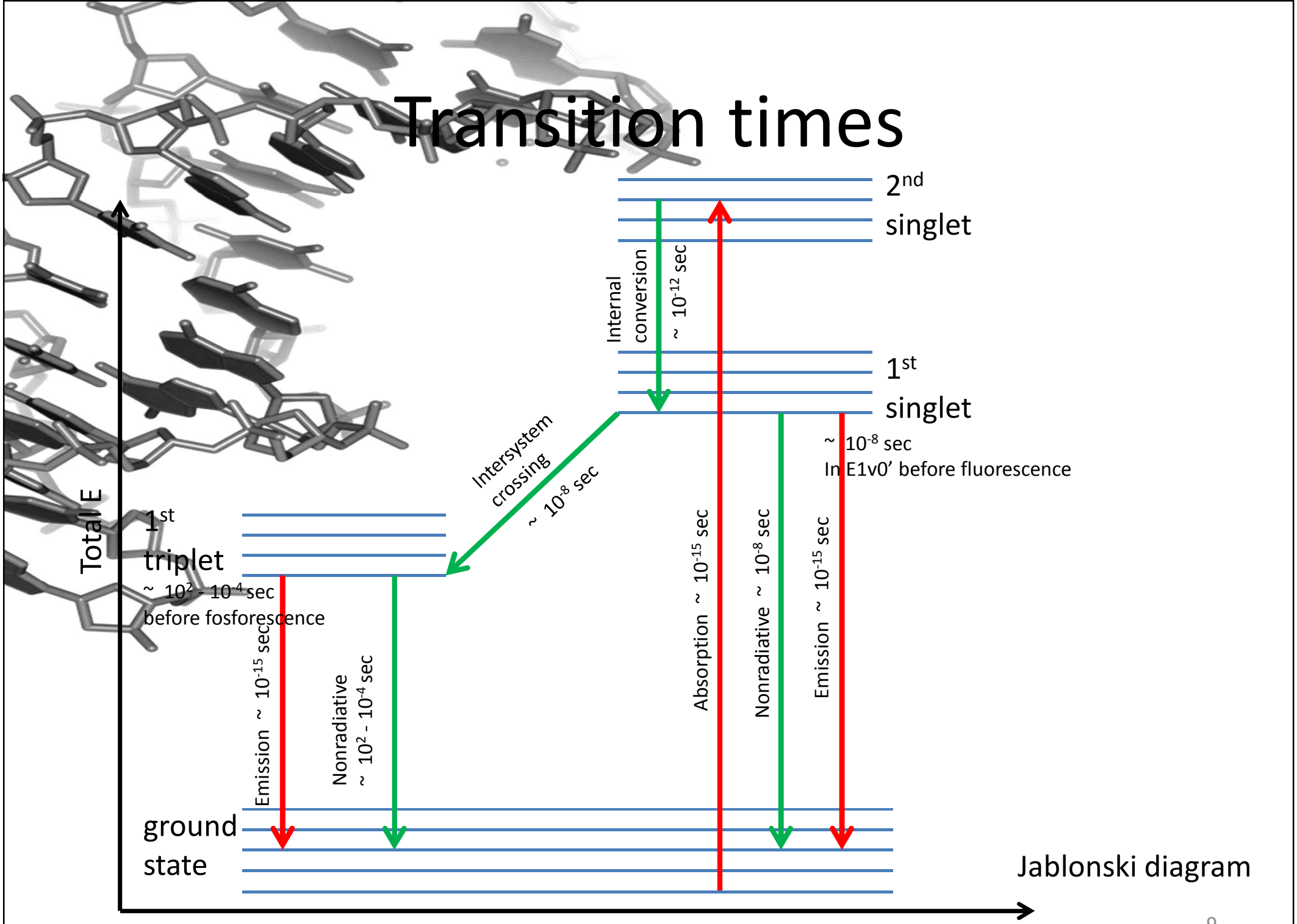

Fig. 2 Franck-Condon energy diagram

# Vibronic structure of absorption spectra



# Transition times



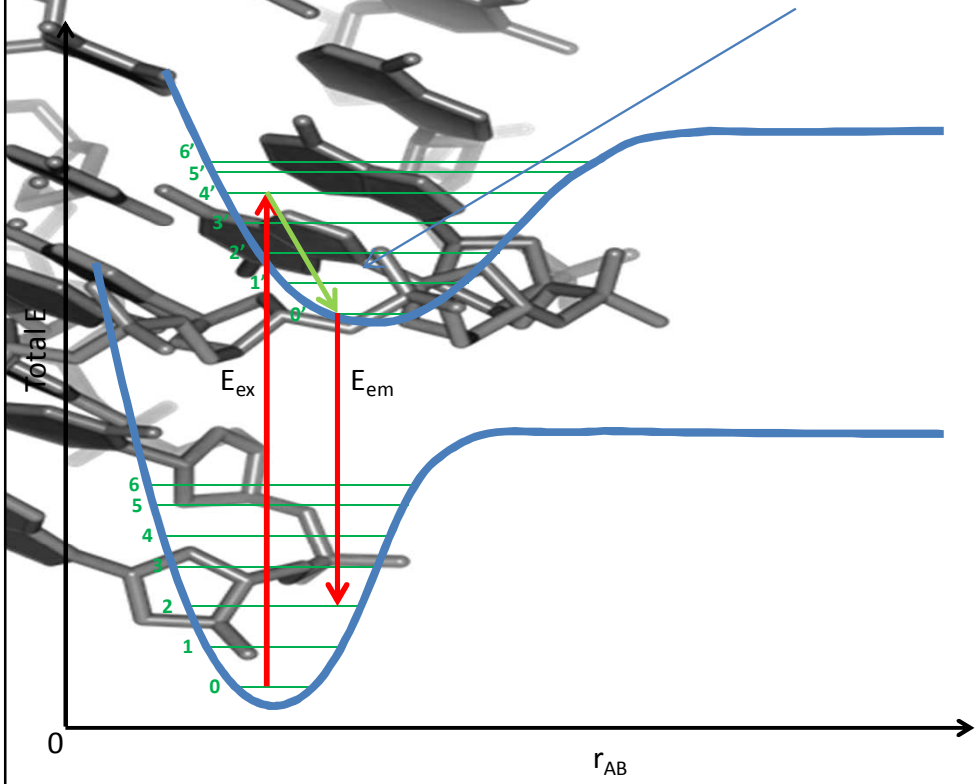

Van Holde et al., Principles of Physical Biochemistry, 2nd ed., 2006

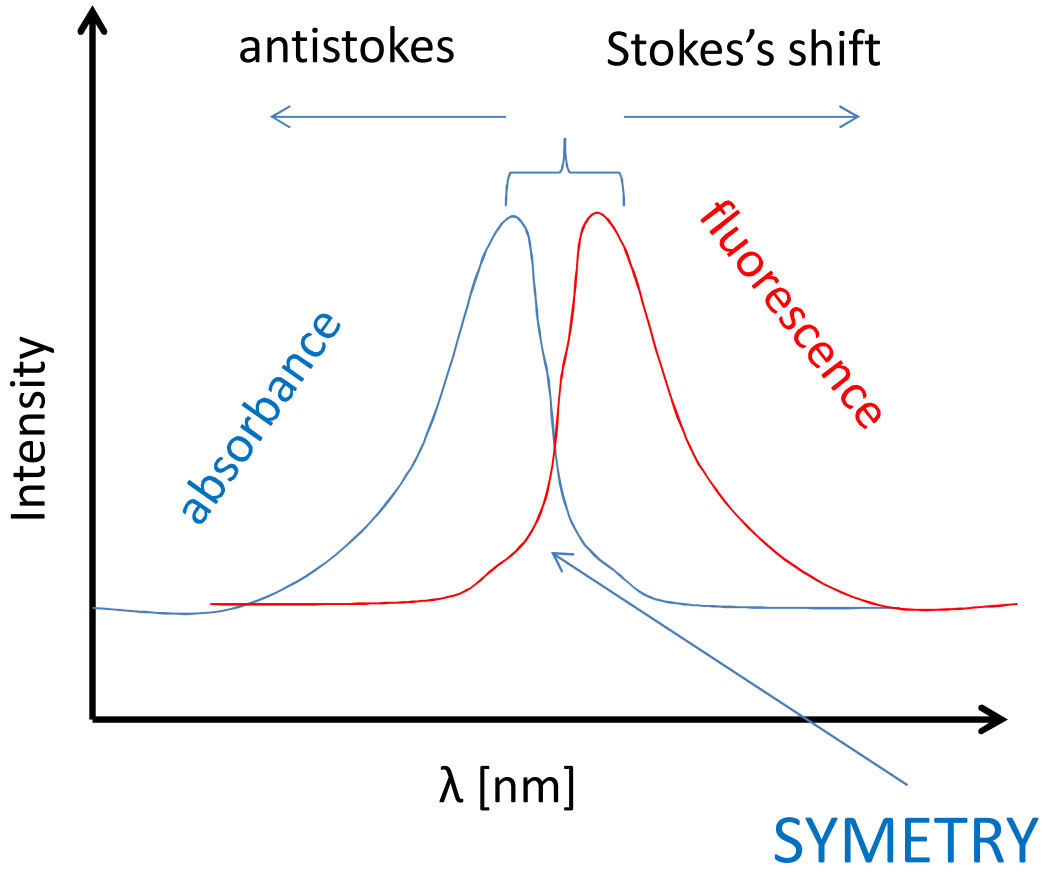
# Background – Kasha's rule

- 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

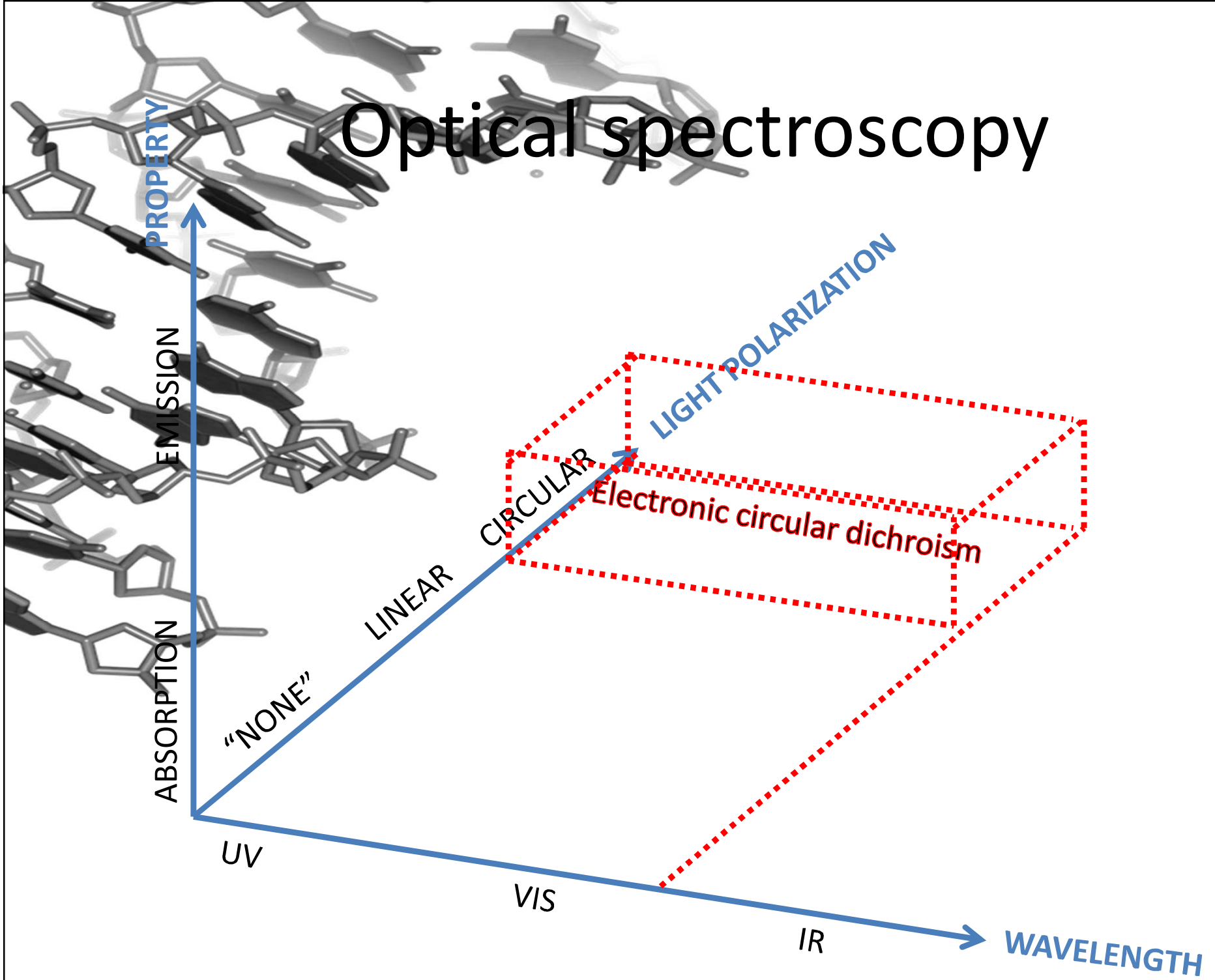



SYMETRY

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

# Optical spectroscopy



# Type of spectroscopy

These types require different instrumentation and are used for different purposes.

**Steady-state**

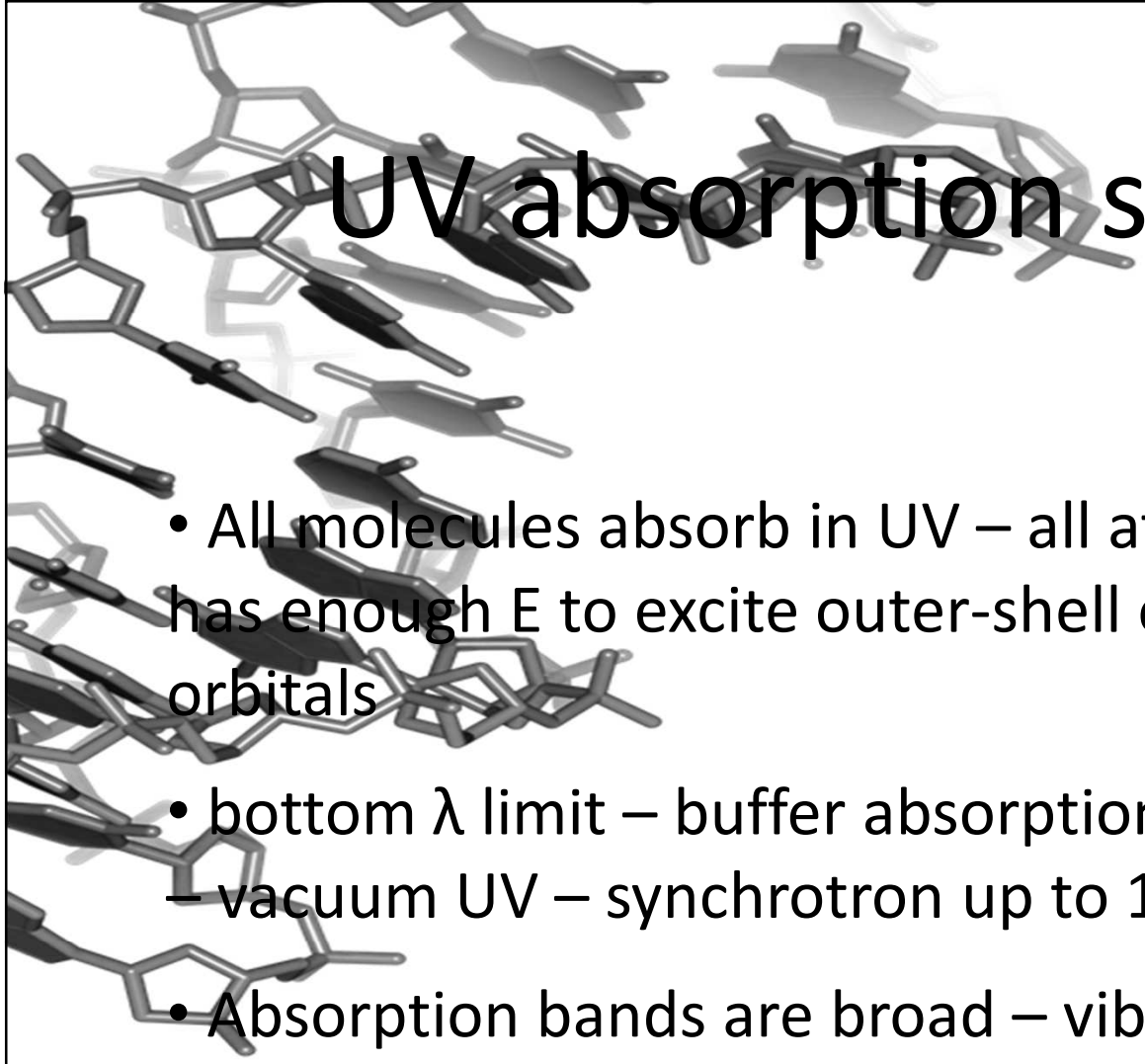
- continuous excitation
- weak intensities of excitation light
- highly populated ground state

**Time-resolved experiment**

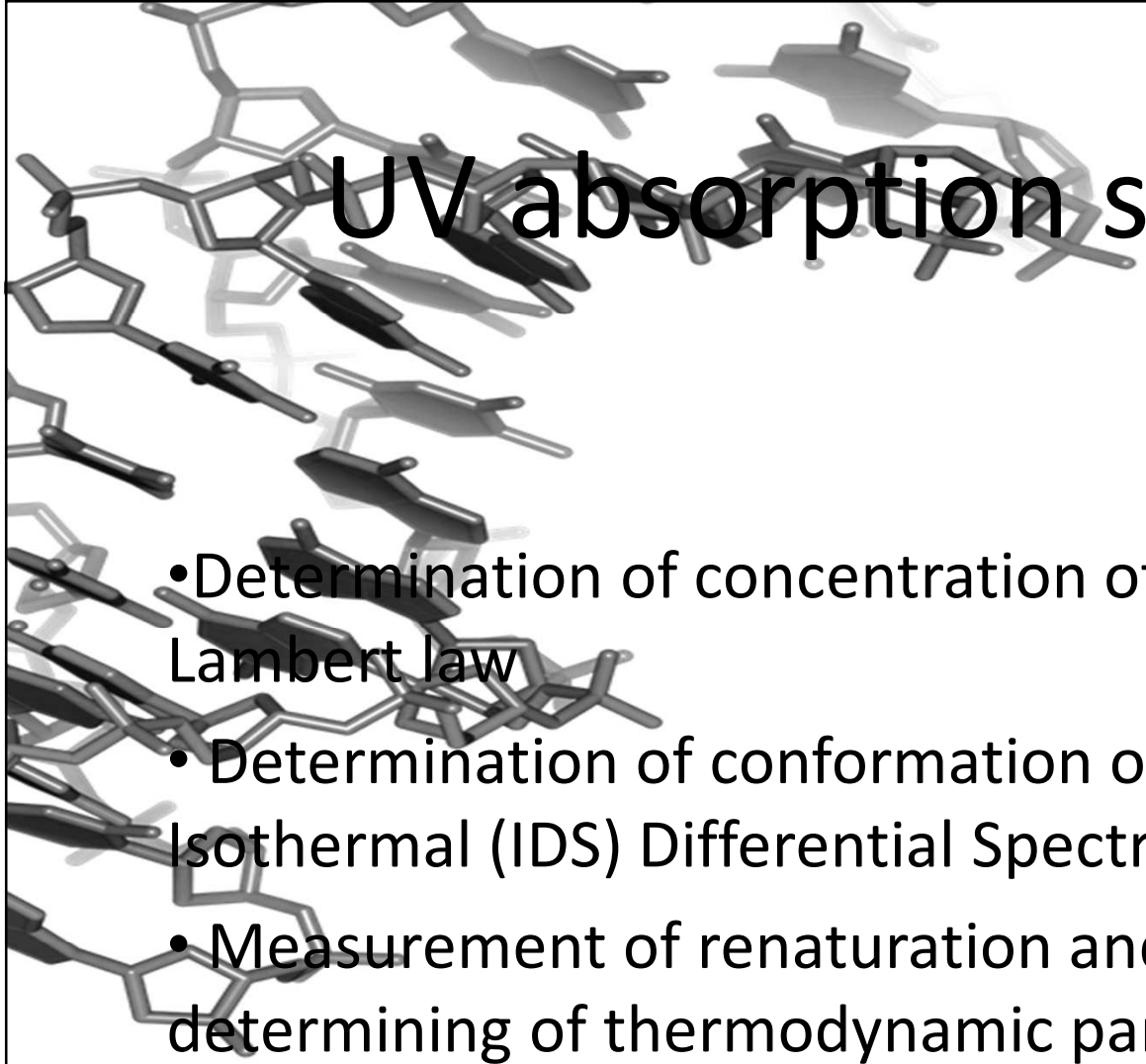
- short excitation pulse (fs,ps)
- higher intensities of excitation light (laser)
- significantly populated excited states

"pump" – light for excitation
"probe" – light for measurement
Either the same source or different.

# UV absorption spectroscopy



- All molecules absorb in UV – all atoms have electrons + UV has enough E to excite outer-shell electrons to higher energy orbitals
- 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
- **Chromophore** – part of the molecule that strongly absorbs in the desired region (UV/Vis)



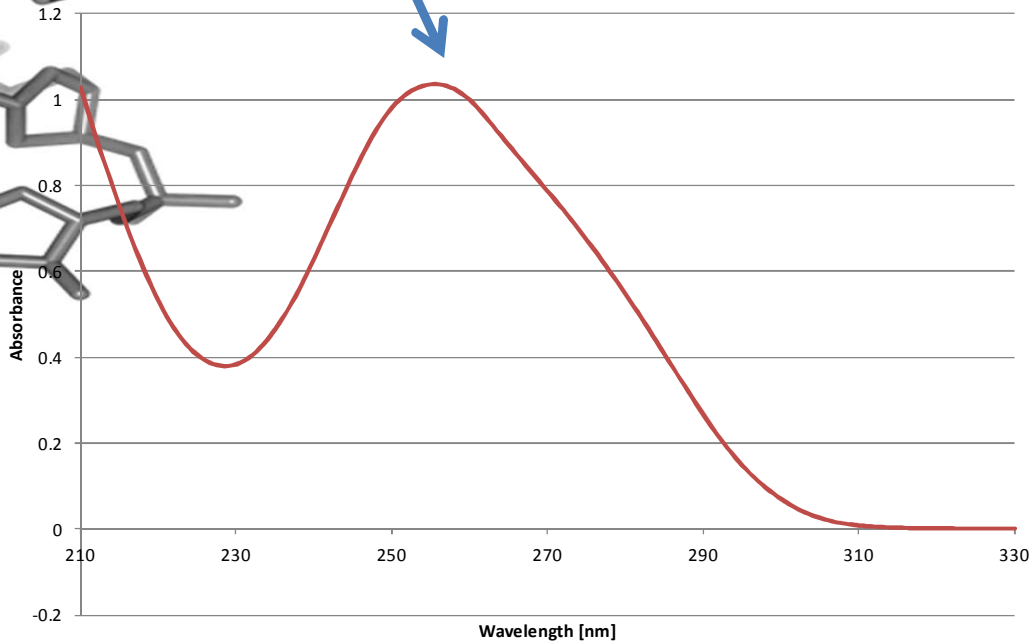
# UV absorption spectroscopy

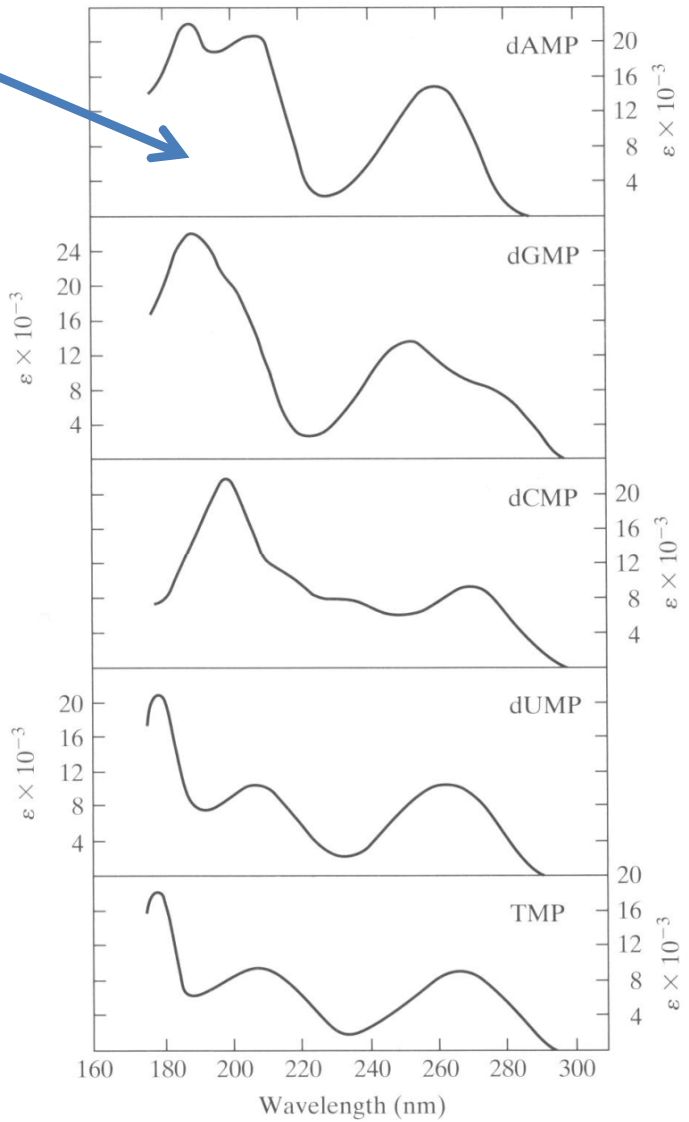
- Determination of concentration of nucleic acids – Beer-Lambert law
- Determination of conformation of DNA – Thermal (TDS) and Isothermal (IDS) Differential Spectra
- Measurement of renaturation and denaturation processes – determining of thermodynamic parameters using van't Hoff equation
- Following interactions of nucleic acids with ligands
- Protonation of bases

# DNA absorption spectrum

Spectra of particular nucleotides depend on transition dipole moments of the bases.

Peak around 260 nm due to a conjugated π-bonding system (bases).

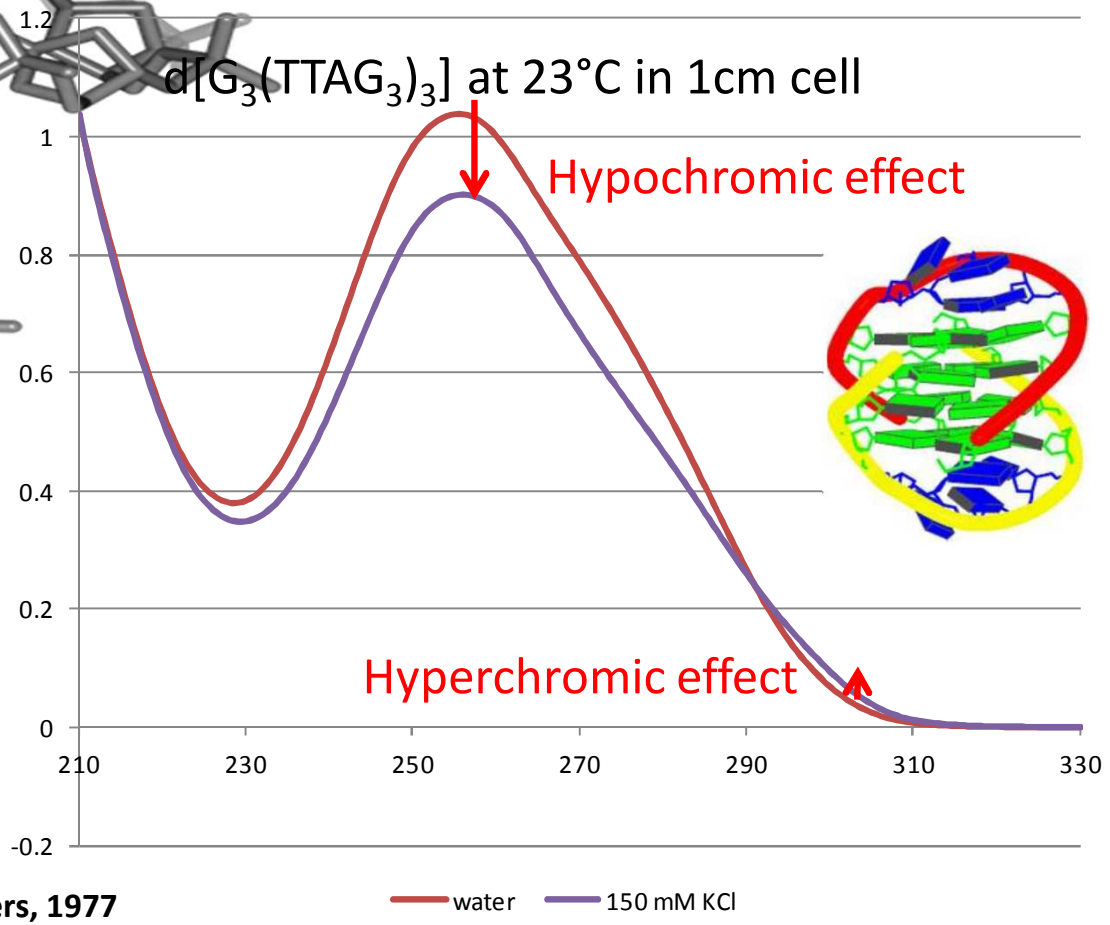



Final spectrum of unstructured DNA depends on primary sequence.

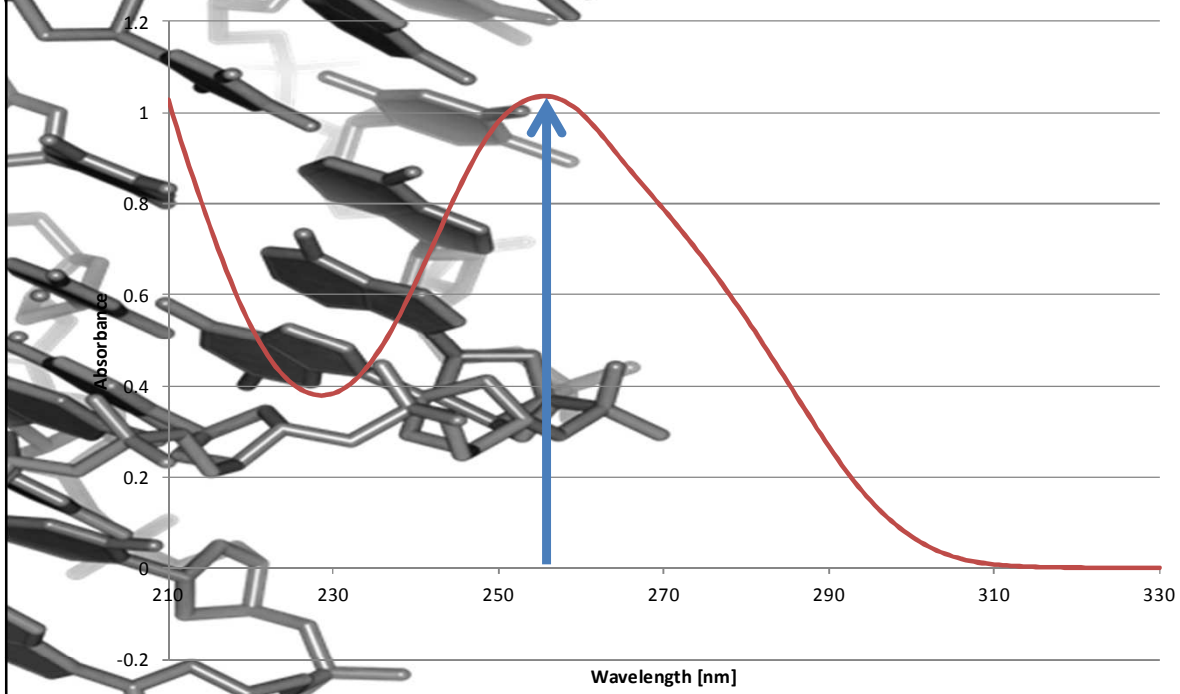
# Effect of structure

- 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)




**Sprecher et al., Biopolymers, 1977**

# UV absorption – NA concentration




Beer-Lambert law

$A = c \cdot \varepsilon \cdot l = \log_{10} I_0/I$

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

$I_0$ – 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

# Molar absorption coefficient - ε

$$A = c \cdot \varepsilon \cdot l$$

ε – molar absorption coefficient [$M^{-1}.cm^{-1}$]

- specific for each NA primary sequence
- can be either:
  - calculated – 2*sum of ε of dimers minus sum of ε of monomers except the two terminal ones (Gray et al., 1995, Methods Enzymol)

$$\varepsilon = \left( \sum_{1}^{n-1} 2^{*} \varepsilon_{dinucl.} - \sum_{2}^{n-1} \varepsilon_{mononucl.} \right)$$

  - analytically determined – amount of phosphorus vs absorbance
- usually calculated by DNA provider
- http://eu.idtdna.com/calc/analyzer

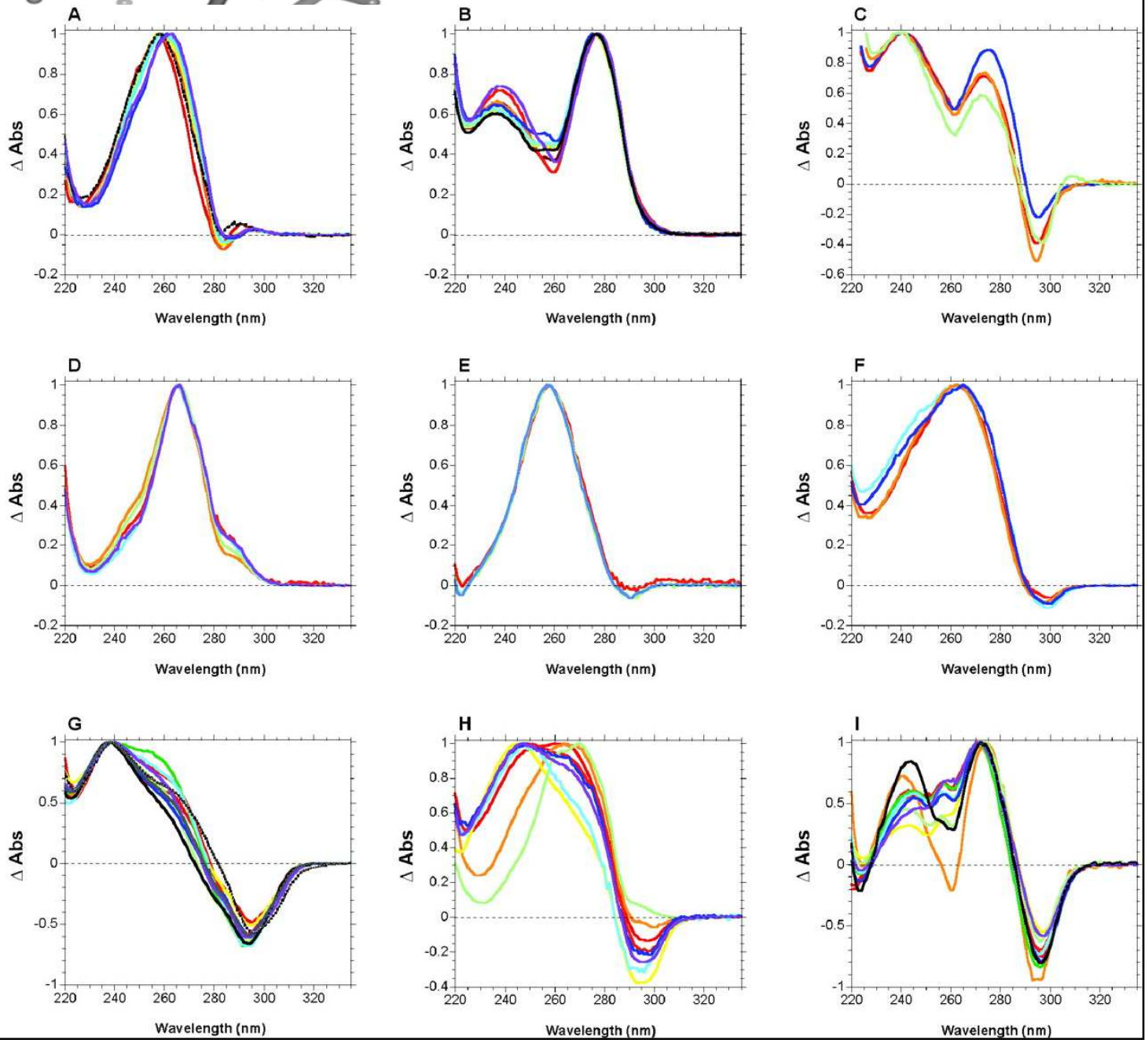
TABLE I

MOLAR EXTINCTION COEFFICIENTS OF NUCLEOTIDES AND DINUCLEOSIDE PHOSPHATES[a,b,c]

| Phosphates | ε (260), $M^{-1}$ cm$^{-1}$ RNA | DNA |
|---|---|---|
| Monomer | | |
| Ap | 15,340 | 15,340 |
| Cp | 7,600 | 7,600 |
| Gp | 12,160 | 12,160 |
| Up (dT) | 10,210 | 8,700 |
| Dimer | | |
| ApA | 13,650 | 13,650 |
| ApC | 10,670 | 10,670 |
| ApG | 12,790 | 12,790 |
| ApU (ApT) | 12,140 | 11,420 |
| CpA | 10,670 | 10,670 |
| CpC | 7,520 | 7,520 |
| CpG | 9,390 | 9,390 |
| CpU (CpT) | 8,370 | 7,660 |
| GpA | 12,920 | 12,920 |
| GpC | 9,190 | 9,190 |
| GpG | 11,430 | 11,430 |
| GpU (GpT) | 10,960 | 10,220 |
| UpA (TpA) | 12,520 | 11,780 |
| UpC (TpC) | 8,900 | 8,150 |
| UpG (TpG) | 10,400 | 9,700 |
| UpU (TpT) | 10,110 | 8,610 |

# UV Absorbance – TDS (or IDS)

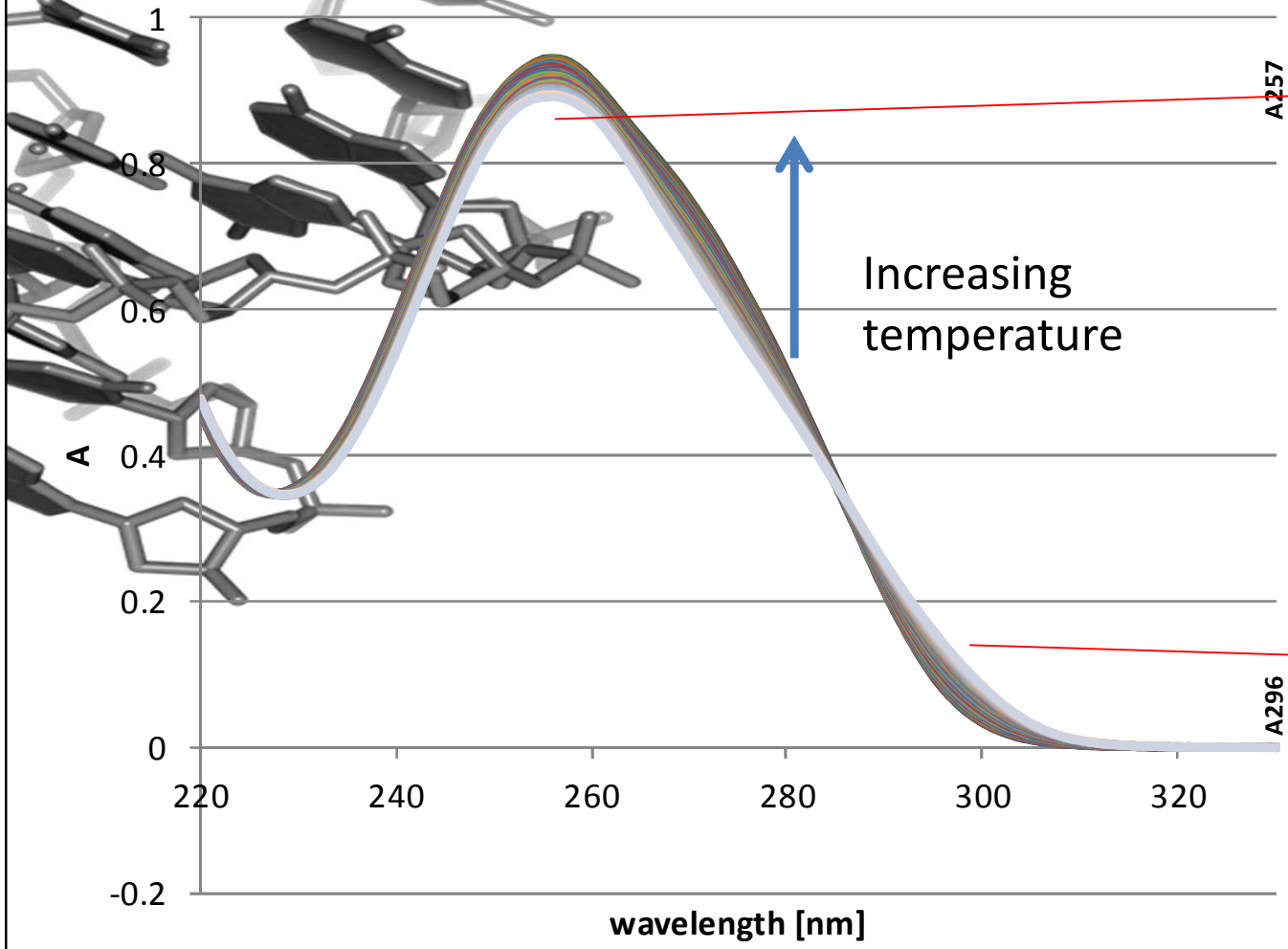
Normalized differential absorbance signatures: (A) DNA self-complementary duplexes, 100% AT; (B) DNA self-complementary duplexes 100% GC; (C) Z-DNA; (D) Parallel-stranded DNA; (E) GA DNA duplexes; (F) Hoogsteen DNA duplexes; (G) i-DNA; (H) Pyrimidine triplexes; (I) DNA G-quadruplexes in Na+.

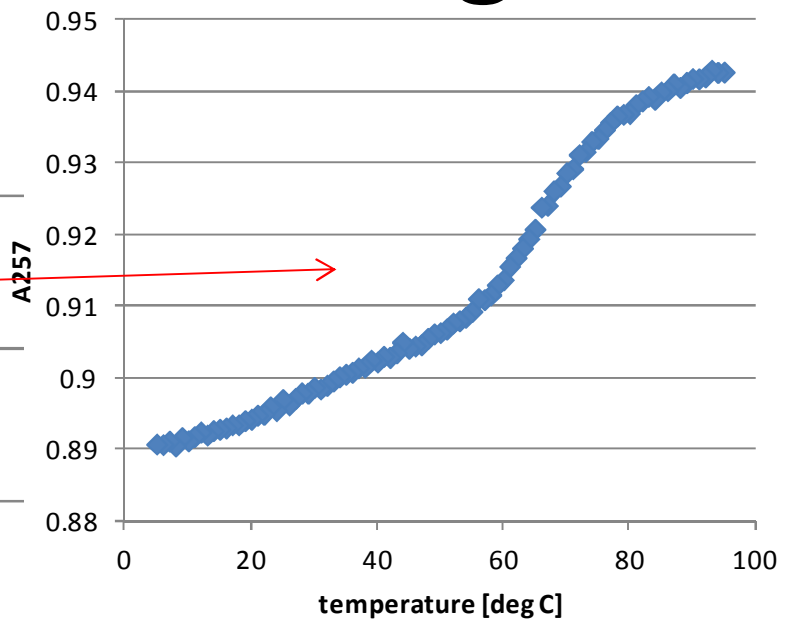


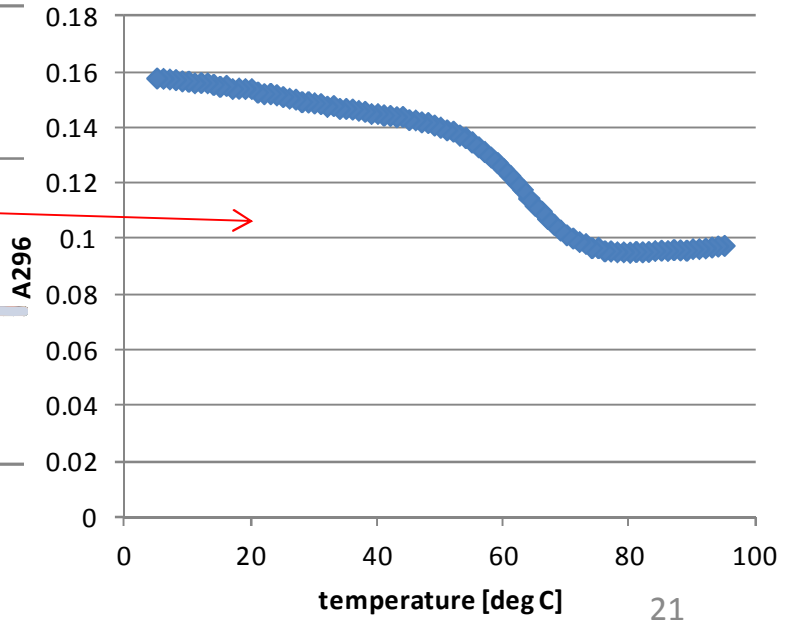

Mergny et al. Nucl. Acids Res. 2005

# UV Absorbance – NA melting

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

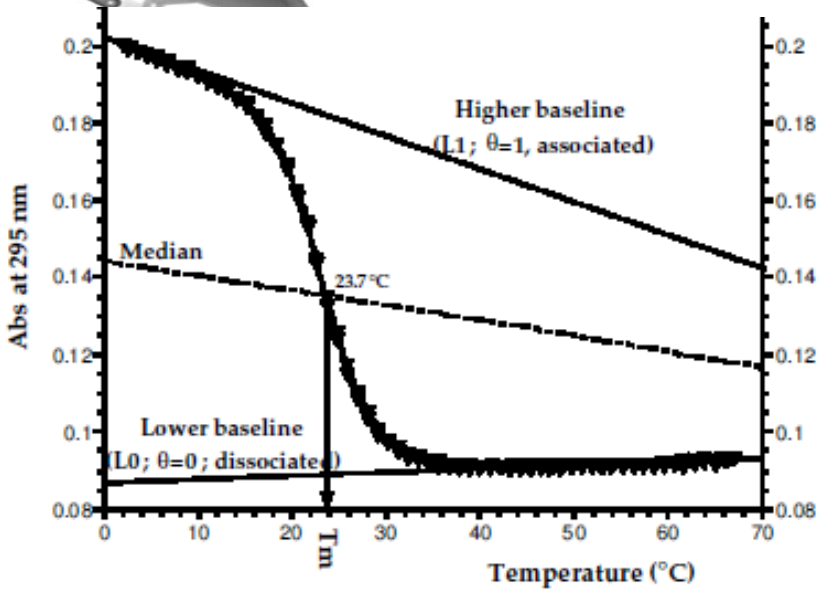


# Thermodynamic parameters - Van't Hoff

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ .$$




$lnK_{eq}$

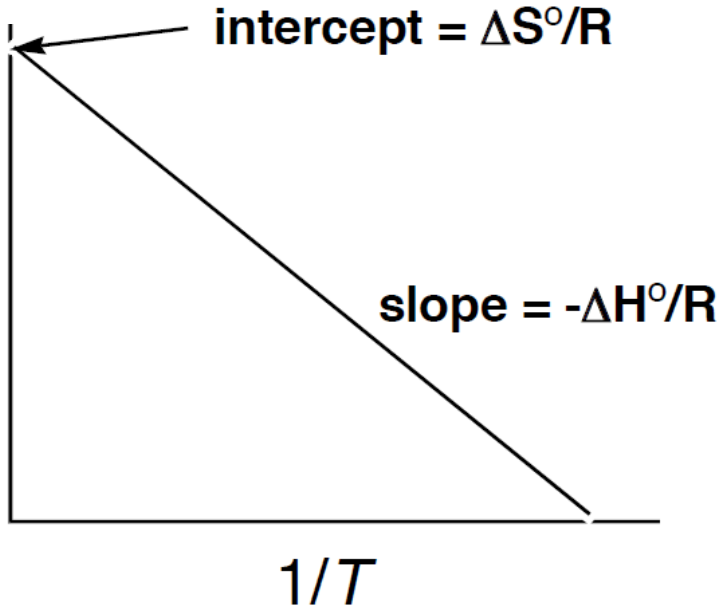



TABLE 2. EXAMPLES OF EQUILIBRIA

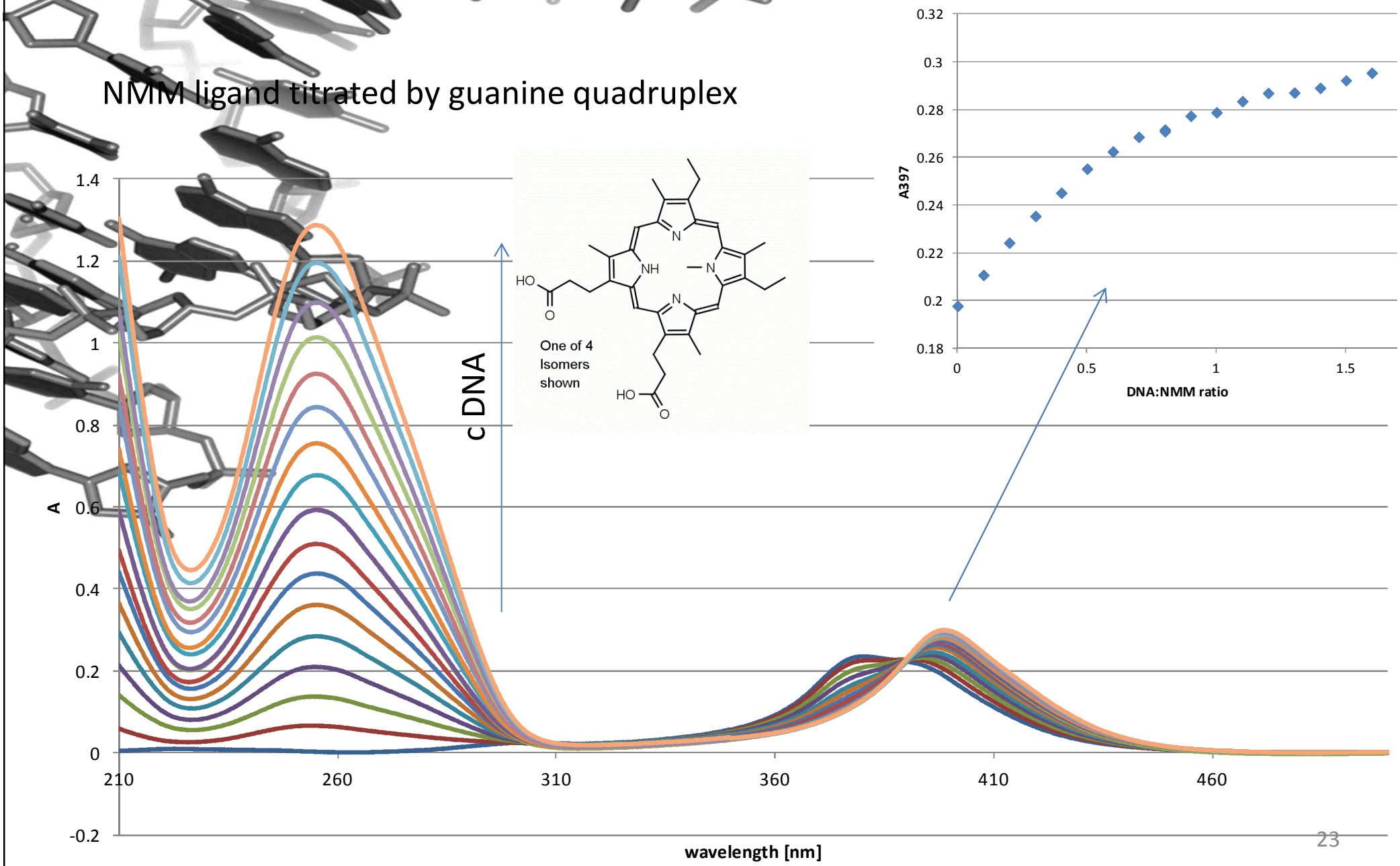
| *Equilibrium* | $A \Leftrightarrow C$ | $2A \Leftrightarrow C$ | $A + B \Leftrightarrow C$ [a,b] |
|---|---|---|---|
| Type | Intramolecular | Bimolecular | Bimolecular |
| Example | Hairpin | Oligo autocomplementary | 2 complementary oligos |
| Concentration effect | Independent | Dependent | Dependent |
| Affinity constant $K_a$ | $\theta/(1-\theta)$ | $\theta/2.C_o.(1-\theta)^2$ | $\theta/C_o.(1-\theta)^2$ |
| $\Delta G°$ at $T_m$ | 0 | $+RT_m\ln(C_o)$ | $+RT_m\ln(C_o/2)$ |
| $1/T_m$ [c] | $\Delta S°/\Delta H°$ | $\Delta S°/\Delta H° + R/\Delta H°.\ln C_o$ | $(\Delta S° - R.\ln 2)/\Delta H° + R/\Delta H°\ \ln C_o$ |
| Max[d] $d\theta/dT^{-1}$ for $\theta=$ | 0.5 | $\sqrt{2}-1$ [e] | $\sqrt{2}-1$ [e] |



Mergny and Lacroix, Oligonucleotides, 2003

# UV Absorbance – ligand interaction

NMM ligand titrated by guanine quadruplex



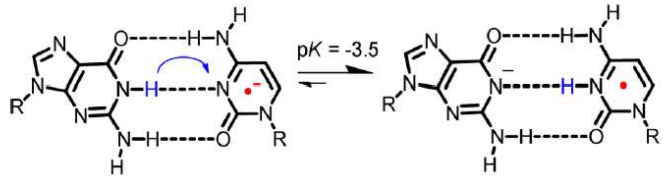
# Time-resolved absorption

THE JOURNAL OF PHYSICAL CHEMISTRY B — Article — pubs.acs.org/JPCB

How Does Guanine–Cytosine Base Pair Affect Excess-Electron Transfer in DNA?

Shih-Hsun Lin, Mamoru Fujitsuka,* and Tetsuro Majima*

**Scheme 1. Proposed Proton-Transfer Reaction Pathway for G:C$^{\bullet-}$ Base Pair**[27]



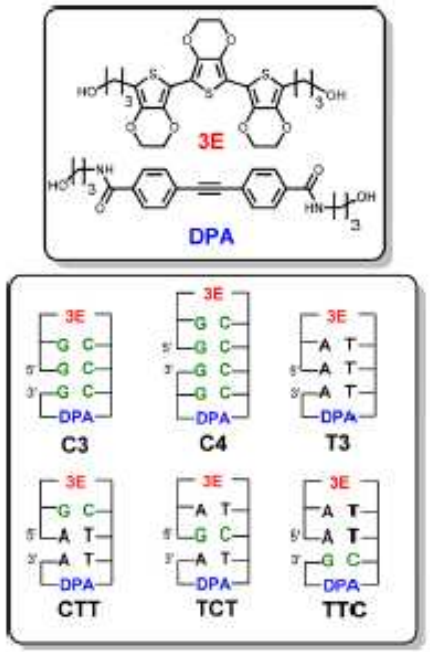




**Figure 1.** Structures of **3E**, **DPA**, and DNA oligomers (**C3**, **C4**, **T3**, **CTT**, **TCT**, and **TTC**). The gap between the 5′ and 3′ indicates a missing phosphate linker between two nucleobases in nicked dumbbell structure.

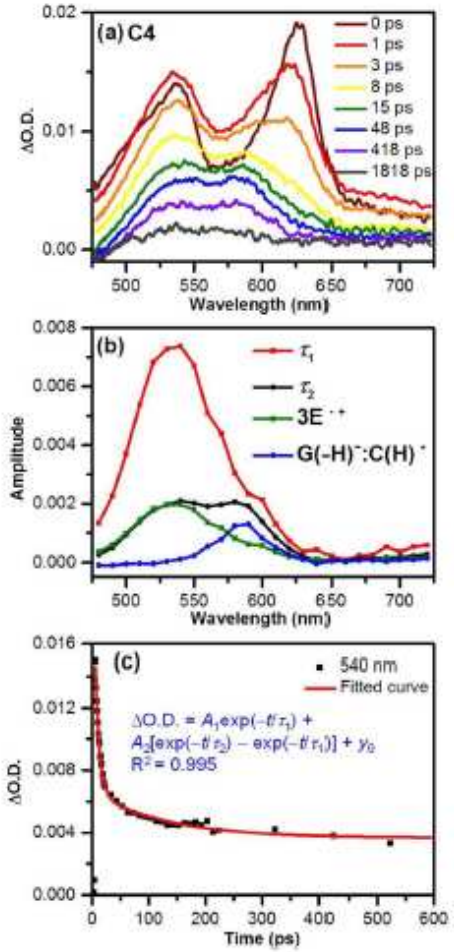
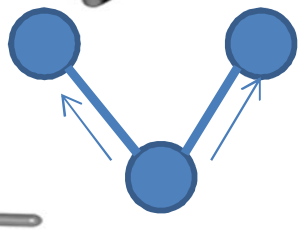


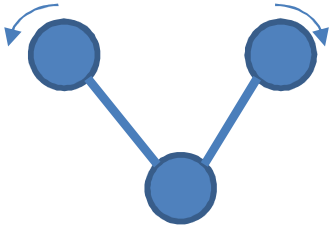

**Figure 4.** (a) Transient absorption spectra during the laser flash photolysis of **C4** upon excitation with 400 nm femtosecond laser pulse. (b) Species-associated spectra obtained by global fitting using a double exponential function for **C4** (red: $\tau_1$ ($3E^{\bullet+}$–$G:C^{\bullet-}$), black: $\tau_2$ ($3E^{\bullet+}$–$G(-H)^{\bullet}:C(H)^{\bullet+}$) and normalized by intensity at 540 nm (green: $3E^{\bullet+}$, blue: $G(-H)^{\bullet}:C(H)^{\bullet+}$). (c) The kinetic traces of global fitting for **C4** at 540 nm. $\tau_1$ and $\tau_2$ correspond to $(k_{CR1} + k_{PT})^{-1}$ and $k_{CR2}^{-1}$, respectively. Red curve is fitted curve.

# 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



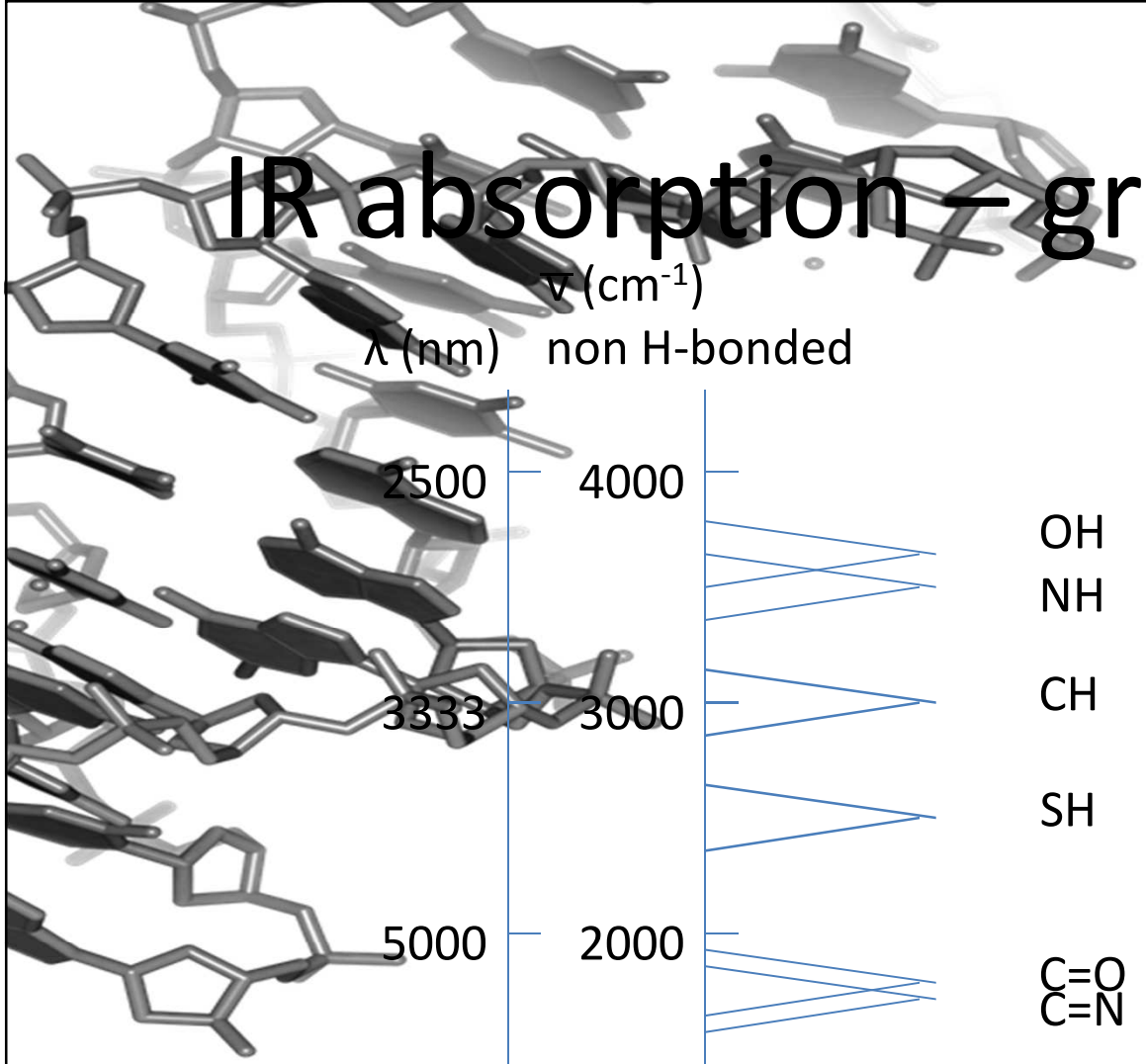
stretching



In-plane bending

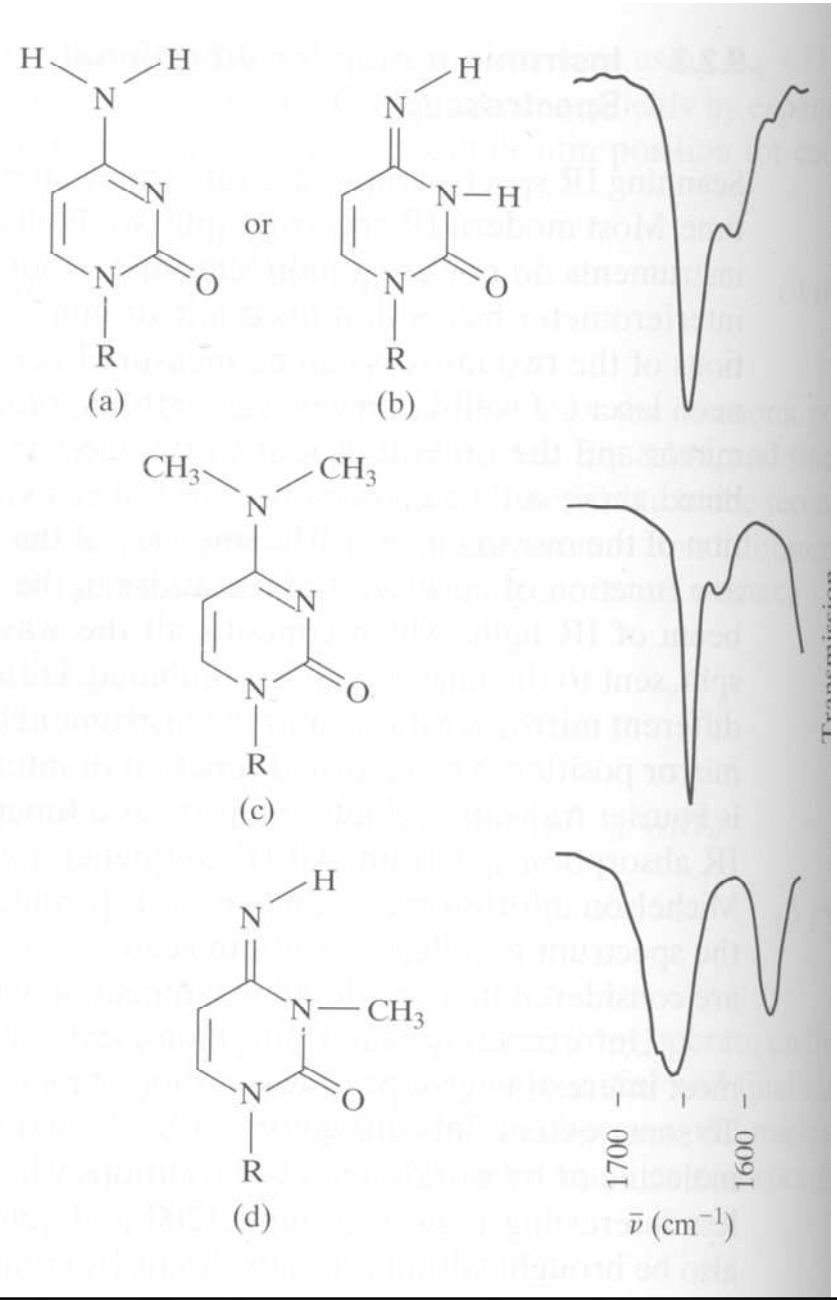
- modern IR spectrofotometers are Fourier transform instruments – Michelson interferometer + FT transformation of intensity to frequency – all frequencies taken simultaneously
- water absorption in interesting IR regions – $D_2O$ (peak in other regions, films

# IR absorption – group vibrations



# IR absorption – Miles experiment

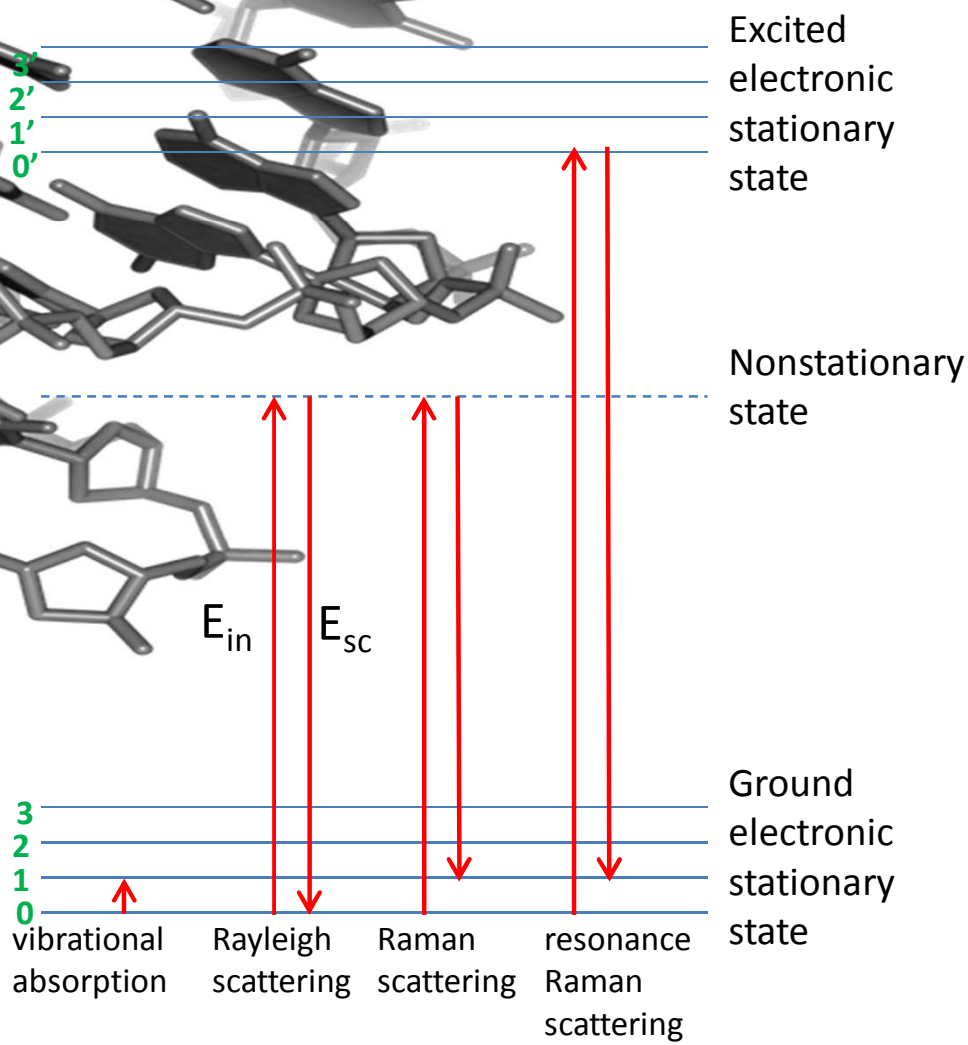
IR spectra in the 1750 to 1550 $cm^{-1}$ region for two nontautomerizing methyl derivatives (c) and (d), and cytidine, now known to be in the first tautomeric form shown (a).




Miles, 1961, PNAS

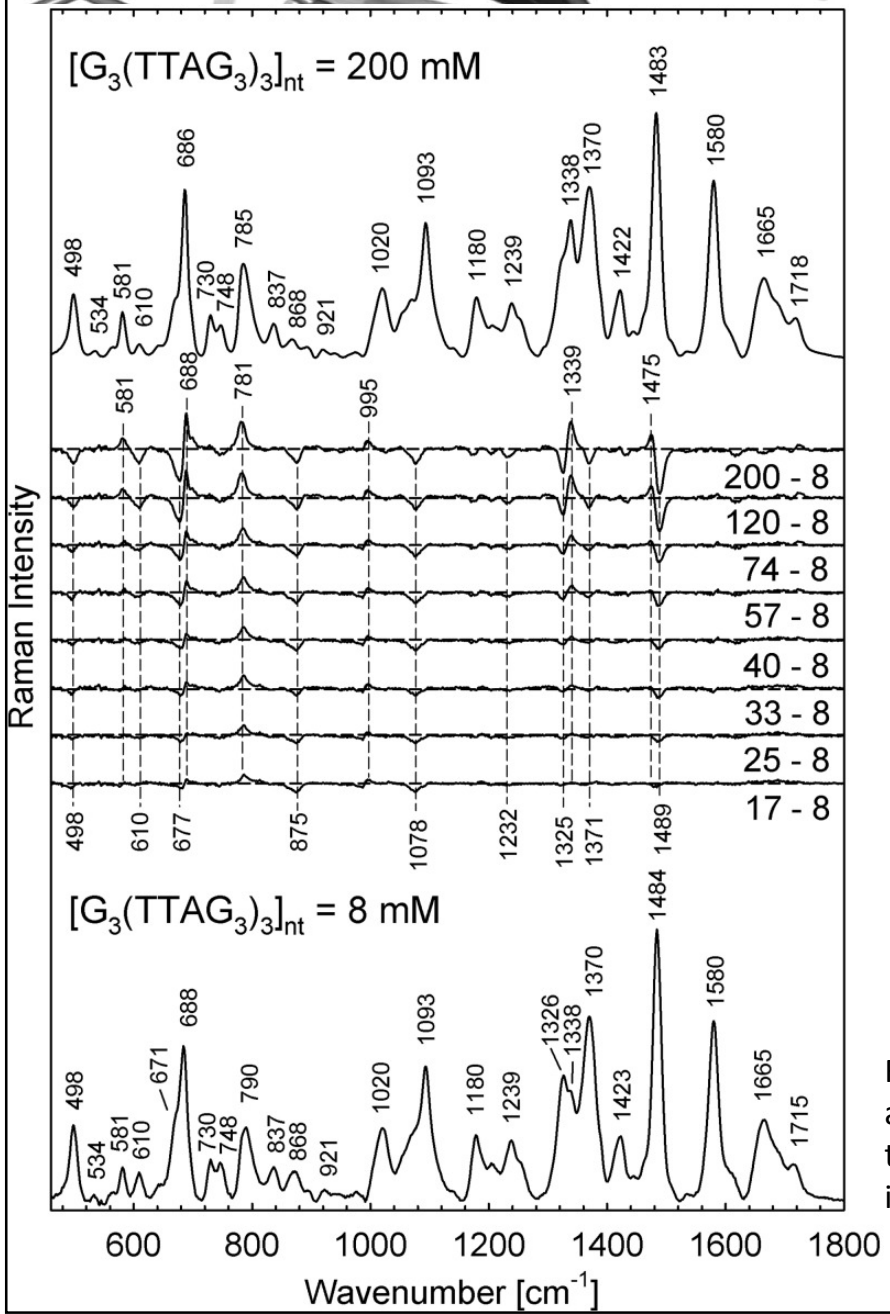
# Raman spectroscopy

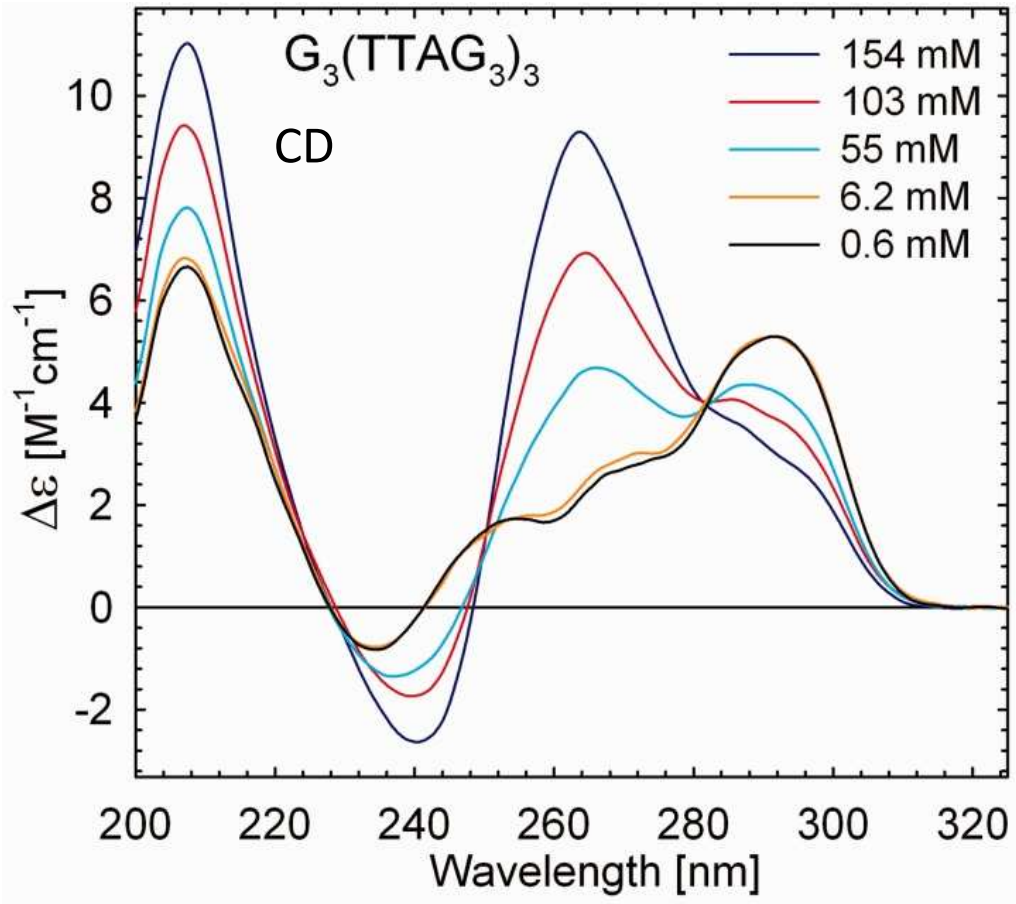
Band position: $v_{01} = (E_{in}-E_{sc})/hc$




- when used light with E < E0-E1 – scattering
- in most cases $E_{in} = E_{sc}$ – Rayleigh scattering
- sometimes $E_{in} <> E_{sc}$ – Raman scattering
  - $E_{in} > E_{sc}$ – Stokes
  - $E_{in} < E_{sc}$ – antistokes
- Raman photon incidence around $10^{-8}$
- Raman band position: $v_{01} = (E_{in}-E_{sc})/hc$
- 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

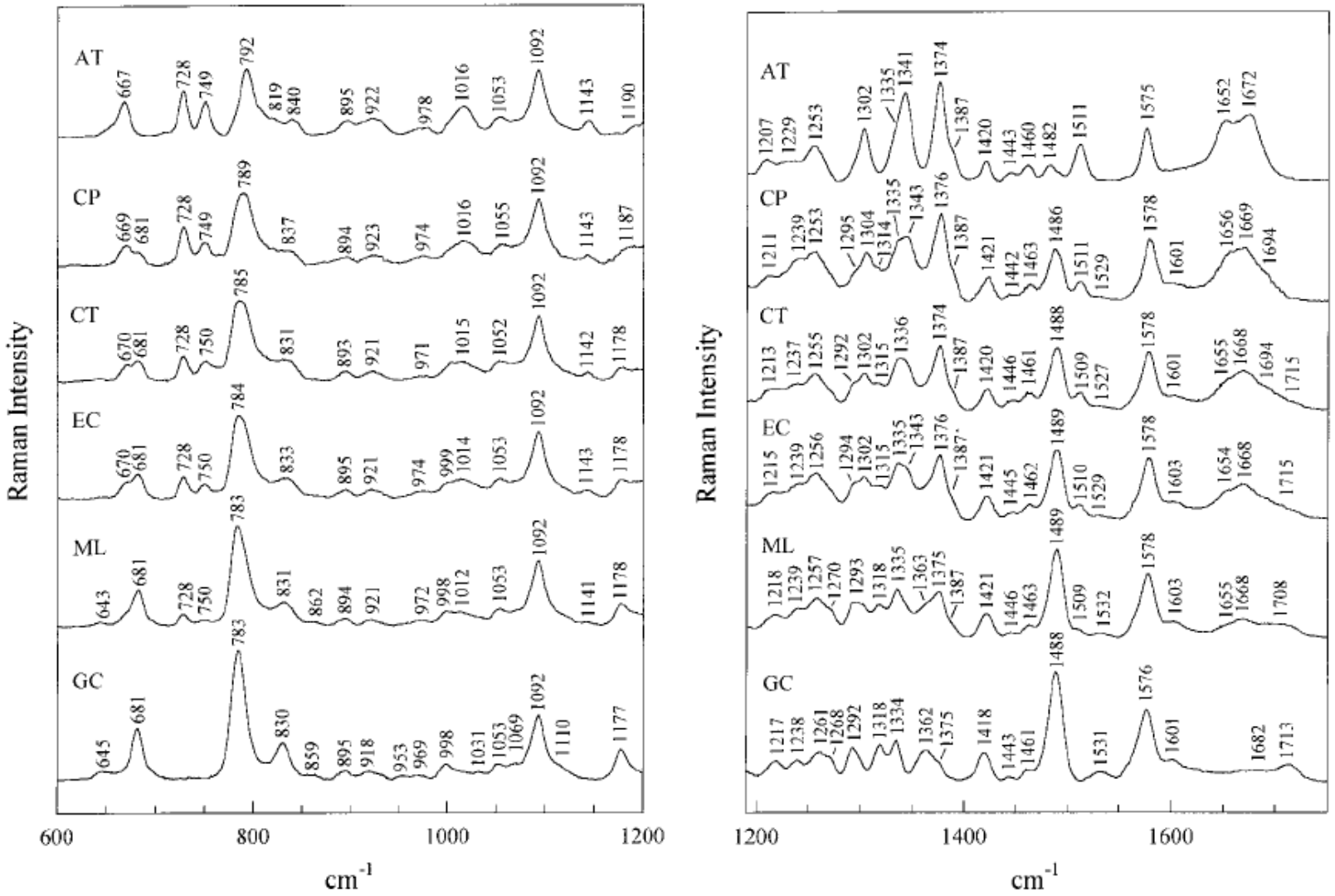



Raman spectra of $G_3(TTAG_3)_3$ in 200 mM $K^+$ (30 mM of PBS, pH 6.8, t = 5°C) at the nucleoside concentrations of 8 mM (bottom trace) and 200 mM (top trace). Intermediate traces show the differences between the spectra at indicated concentration and that of the lowest one

Palacky et al., 2013, NAR

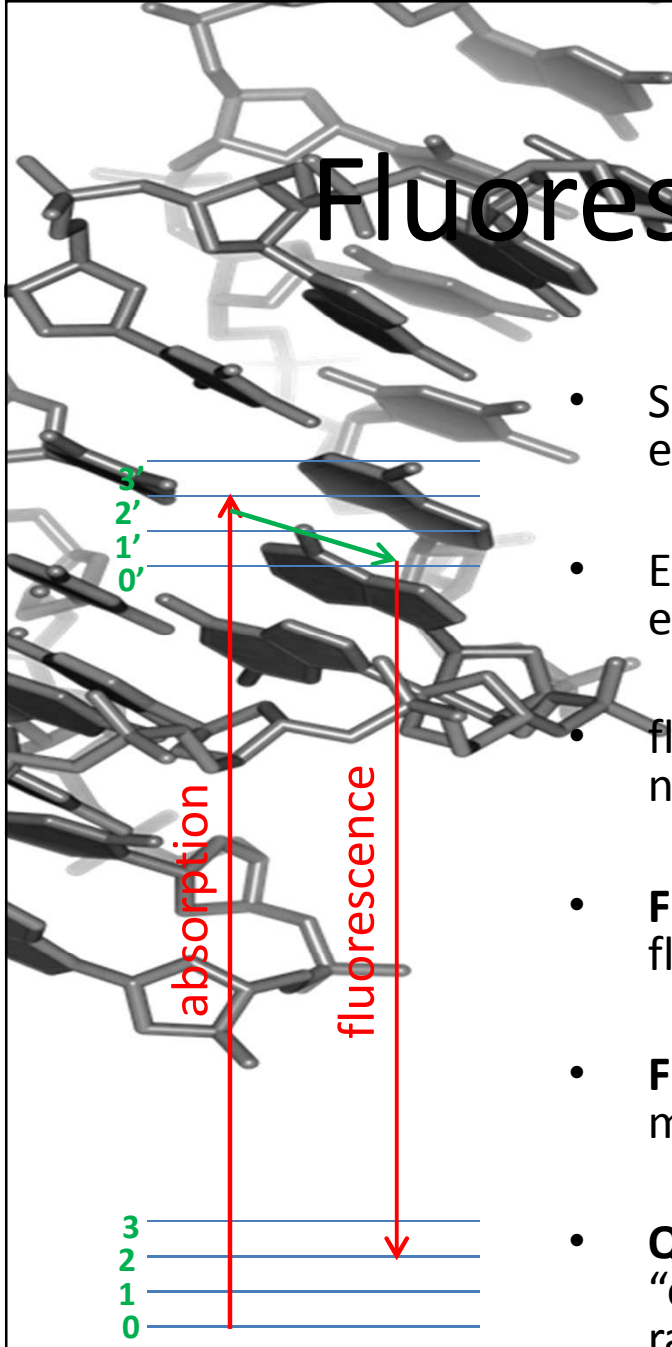
# Raman spectroscopy




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).

Deng et al., 1999, Biopolymers

# Fluorescence in nucleic acids

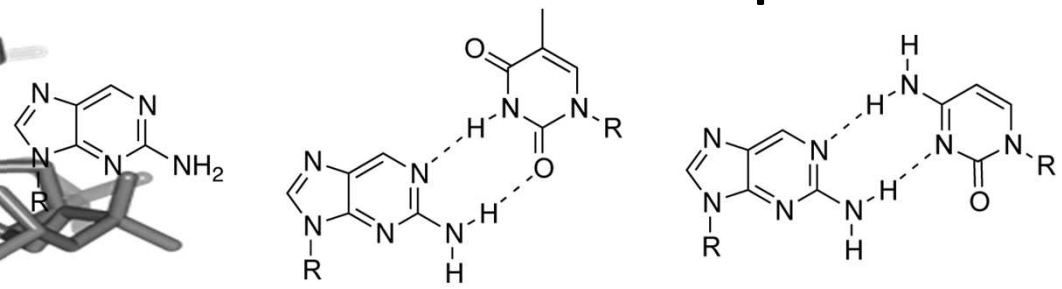



- Spontaneous emission of the photon followed by transition to electronic ground state (any vibrational state – Franck-Condon)
- Emission always from the vibrational ground state of the electronic excited state (Kasha's rule)
- fluorescence itself very fast ($10^{-15}$ s), but some time takes nonradiative conversion to v0'
- **Fluorophores** – molecules/parts of the molecule that exhibit fluorescence
- **Fluorescence lifetime – τ** – average time from excitation of the molecule to emission of light [ns]
- **Quantum yield** – ratio between emitted and absorbed photons – "efficiency" of the fluorescence - max = 1, but usually lower (non-radiative transitions)

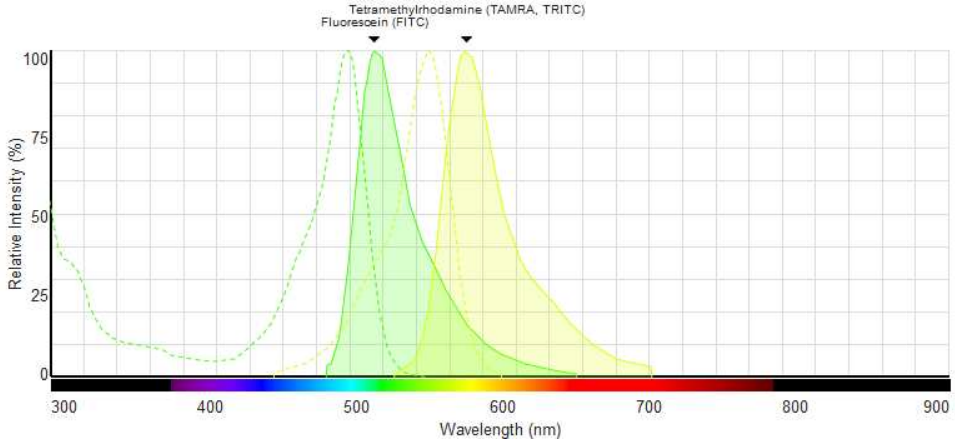
# Fluorescence in nucleic acids

- Very low intrinsic fluorescence, thus:

1. Fluorescent base – 2-aminopurine



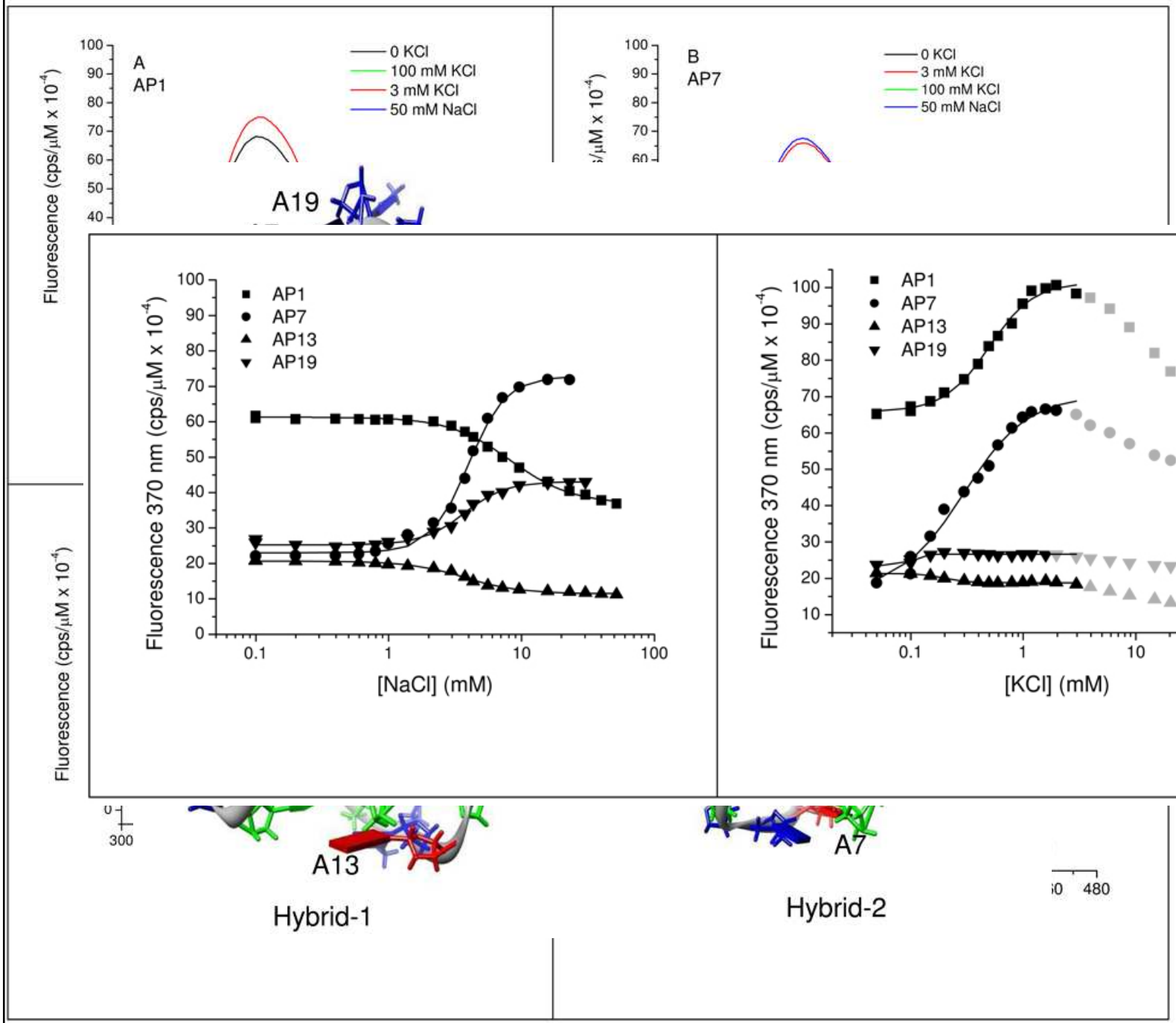

2. Fluorescent labels – FITC, TAMRA, …




**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.

Gray et al., 2010, Biochemistry

# Time-resolved fluorescence

THE JOURNAL OF PHYSICAL CHEMISTRY A LETTERS
2009, 113, 9585–9587
Published on Web 08/12/2009



**Conformational Analysis of DNA Repair Intermediates by Time-Resolved Fluorescence Spectroscopy**

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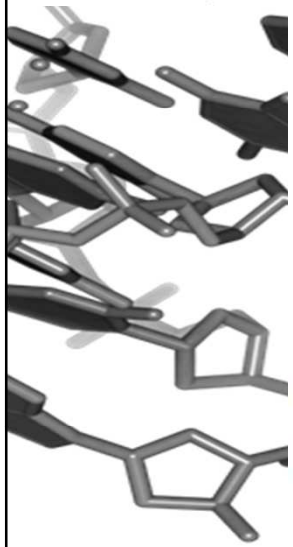
*Center for BioOptical Nanotechnology, The Biodesign Institute, and Department of Chemistry and Biochemistry, Arizona State University, Tempe, Arizona 85287-5201, and Howard Hughes Medical Institute and Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114*

*Received: July 16, 2009*



```
5'-GCTGCCAGTGTGGAACTCTAC      FBP
3'-CGACGGTCACPCCTTGAGATG

5'-GCTGCCAGTGT GGAACTCTAC     NICK
3'-CGACGGTCACP-CCTTGAGATG

5'-GCTGCCAGTG GGAACTCTAC      GAP
3'-CGACGGTCACPCCTTGAGATG

5'-GCTGCCAGT  GGAACTCTAC      2NT
3'-CGACGGTCACPCCTTGAGATG

5'-GCTGCCAGTG---GGAACTCTAC    BLG
3'-CGACGGTCAC-P-CCTTGAGATG
```

**Figure 1.** DNA repair intermediates analyzed in this study. Structures with a gap in their sequence have discontinuous phosphodiester backbones. Dashes indicate continuous DNA strands. The 2AP residue is shown as **P**.

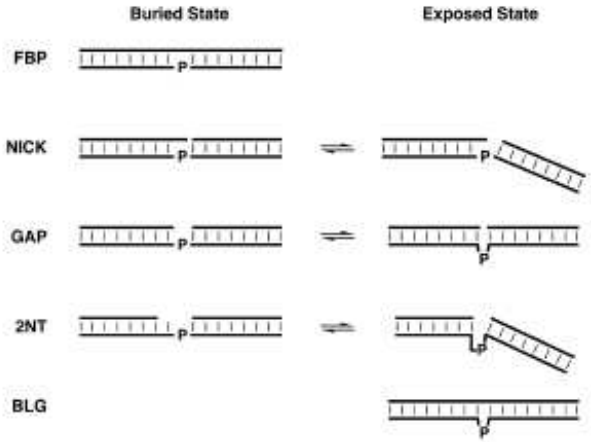



**Figure 2.** Schematic view of the DNA conformations. Damaged DNA structures are expected to equilibrate between several different conformations. The 2AP residue is indicated by the letter **P**.

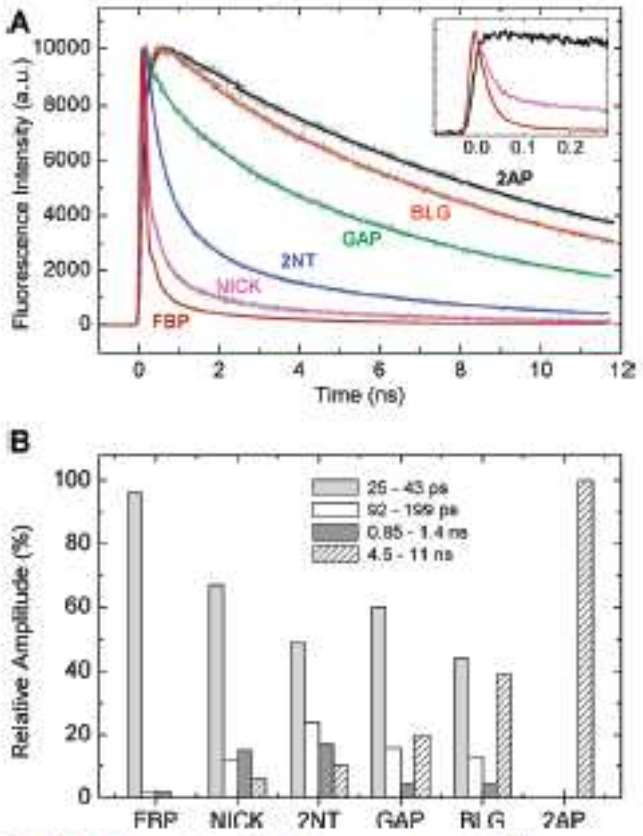
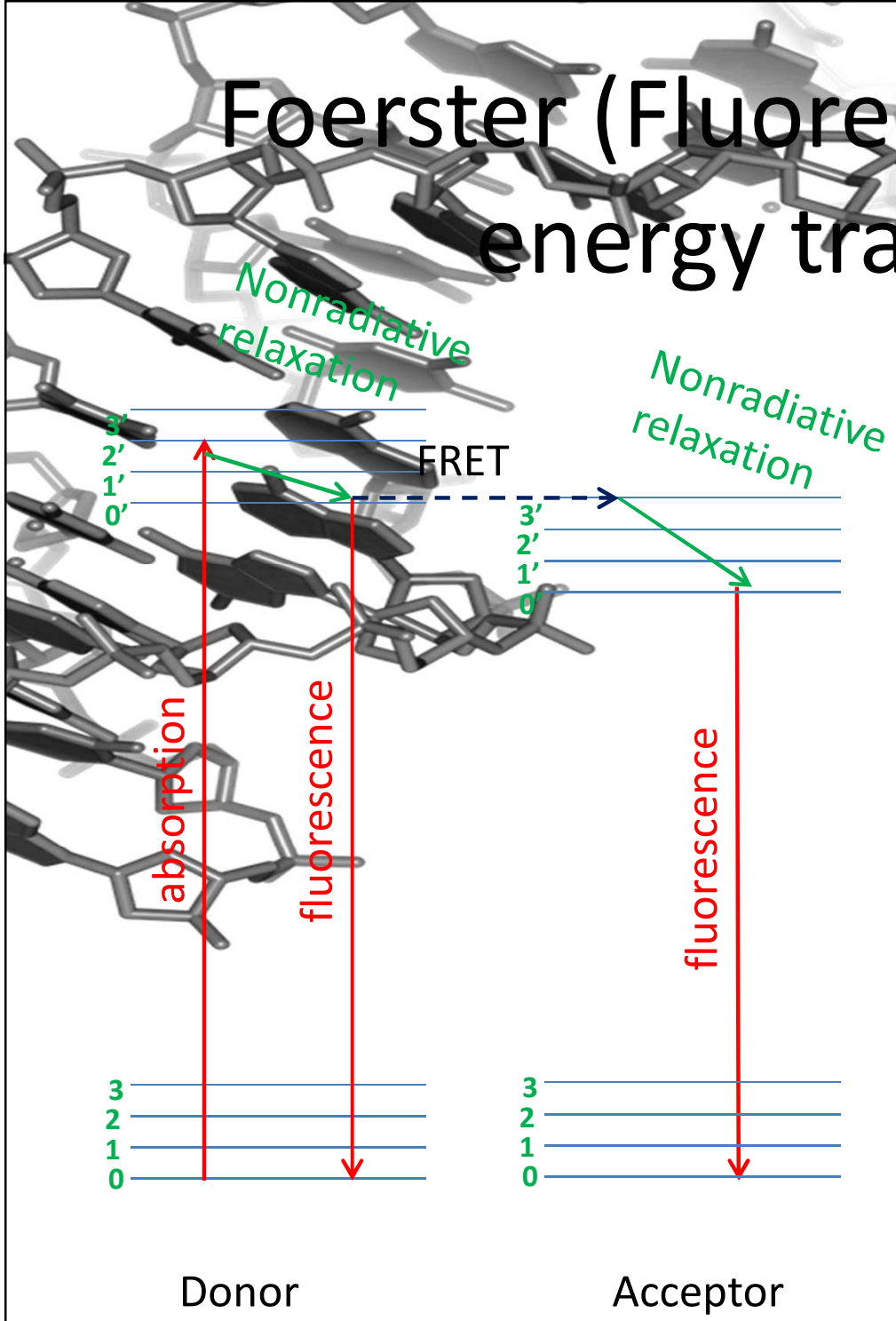


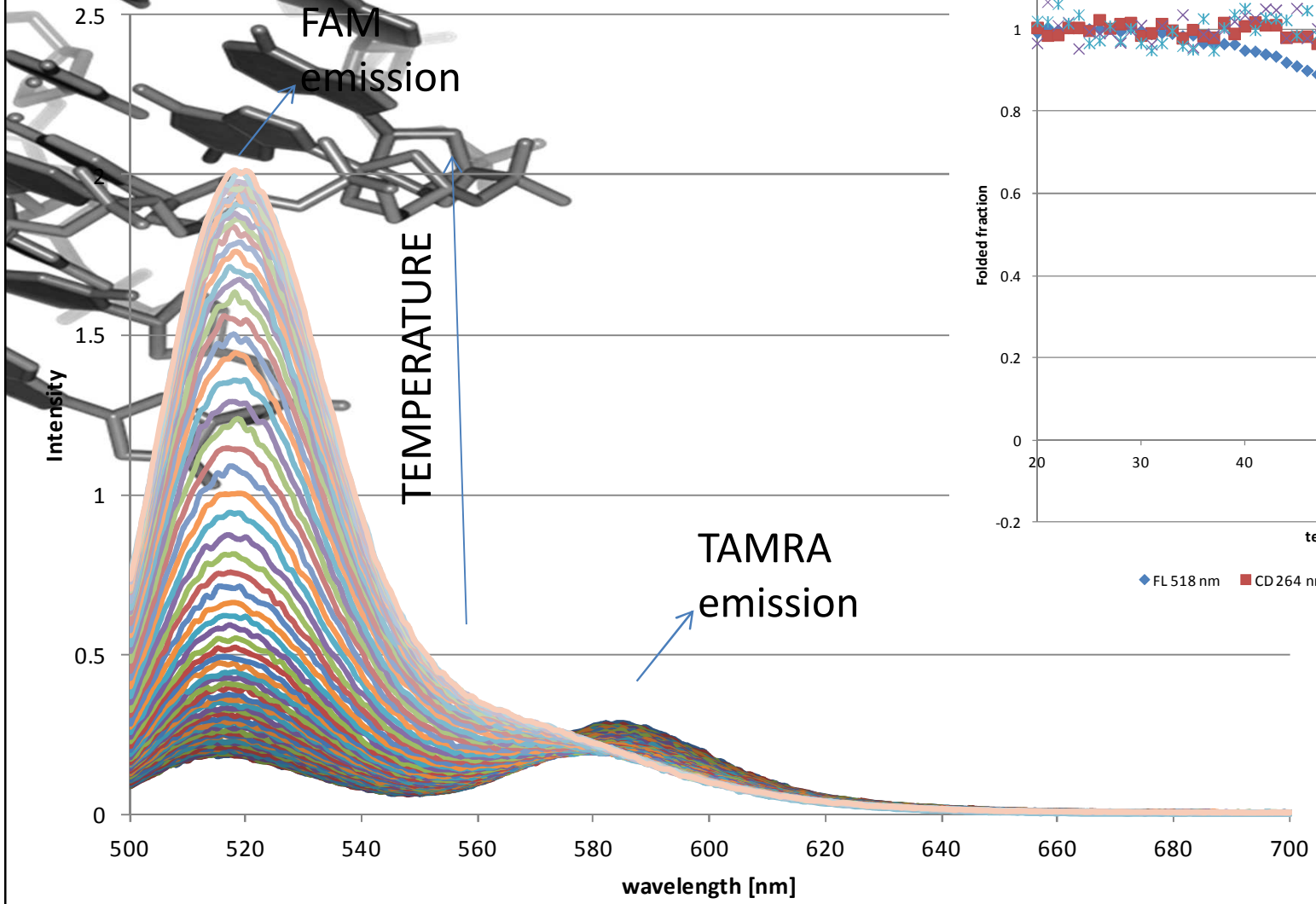

**Figure 3.** Kinetic analysis of DNA structures. (A) Fluorescence decay curves obtained by TCSPC were recorded at 390 nm with a time per step of 6.28 ps. The inset shows the early time kinetics of NICK, FBP, and free 2AP samples recorded on a streak camera system with 2 ps time resolution. (B) The relative amplitude of each lifetime obtained from a four-term exponential fit.

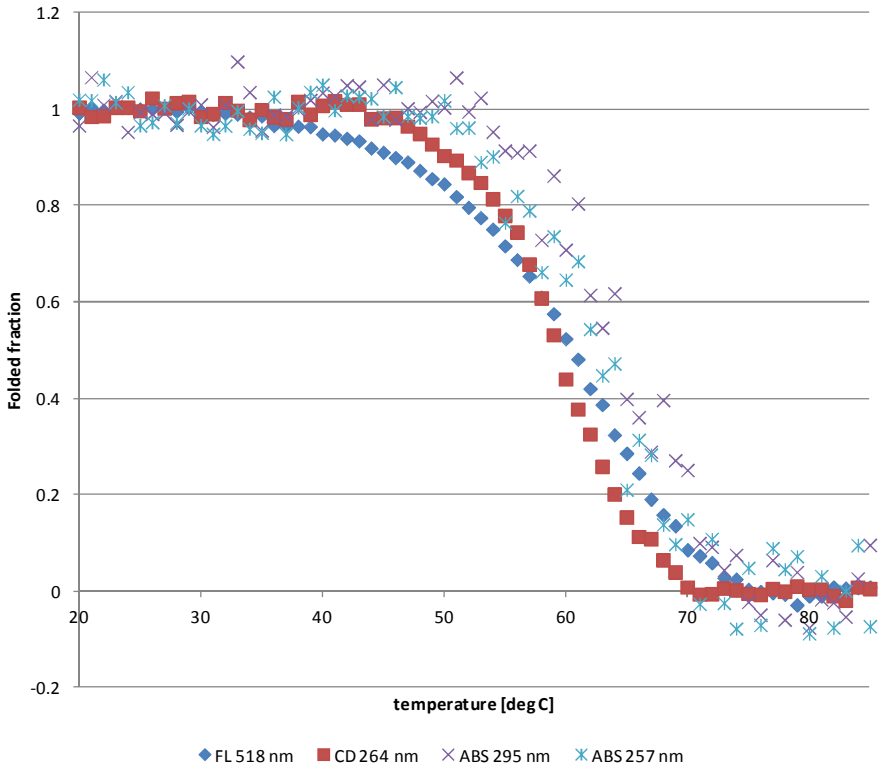
# Foerster (Fluorescence) resonance energy transfer (FRET)




- 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)

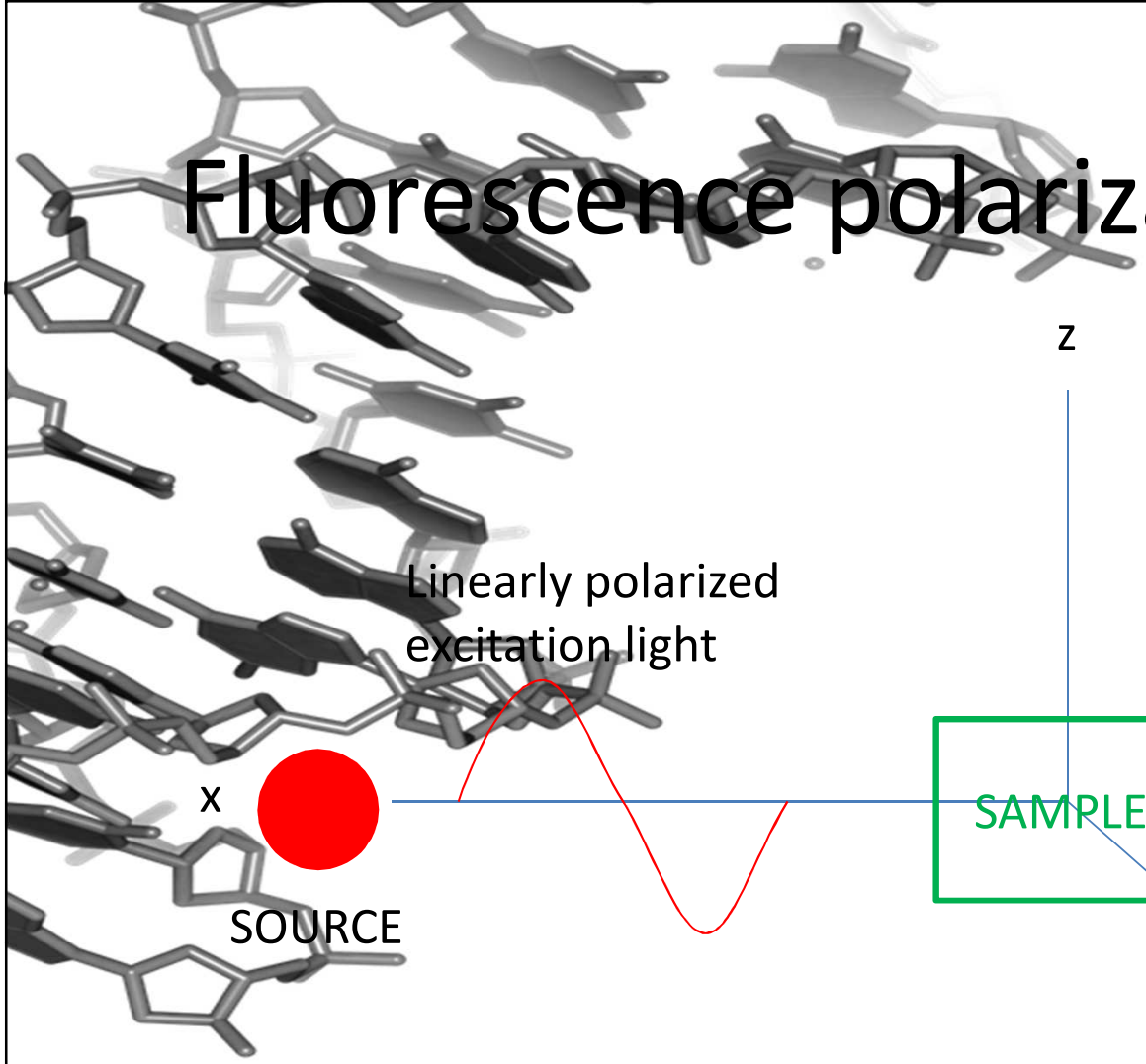
**Difference in the intensity of the sample-emitted light with polarization parallel and perpendicular to the polarization of the excitation light.**

• 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





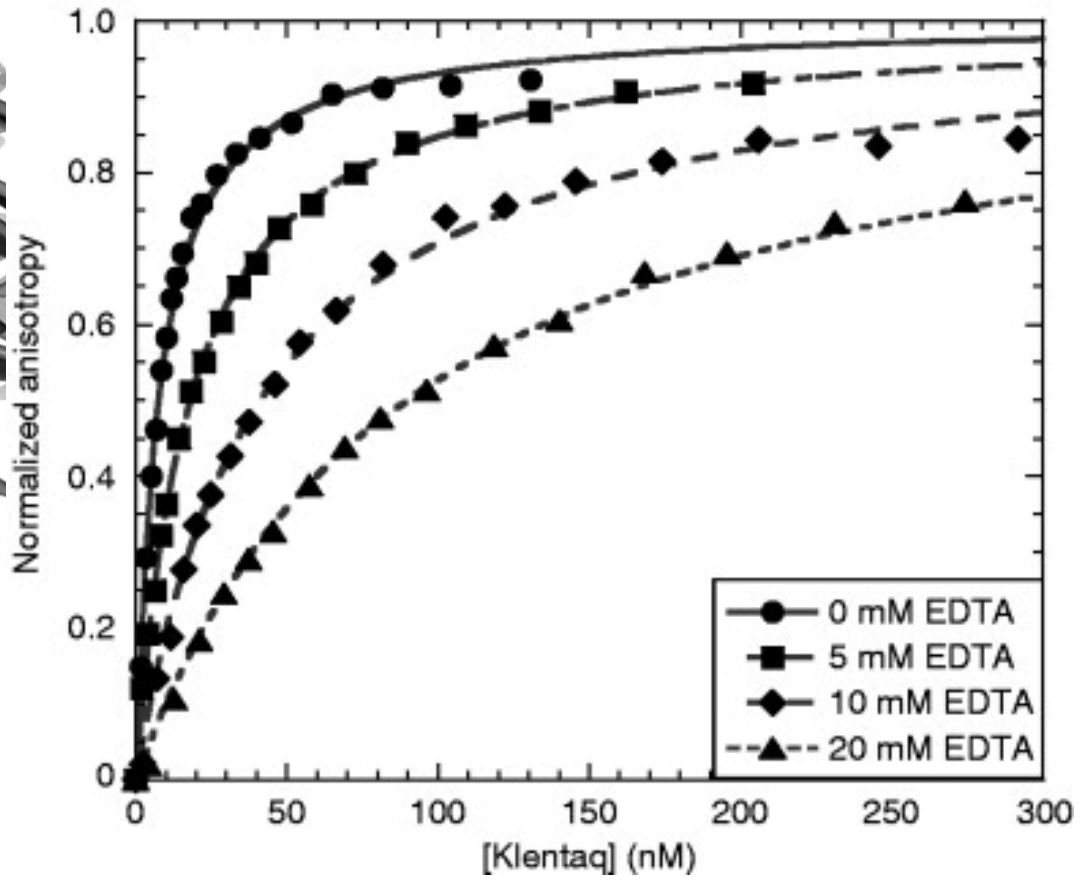

• significantly excited are only the molecules whose transition dipoles are parallel to the polarization of excitation light

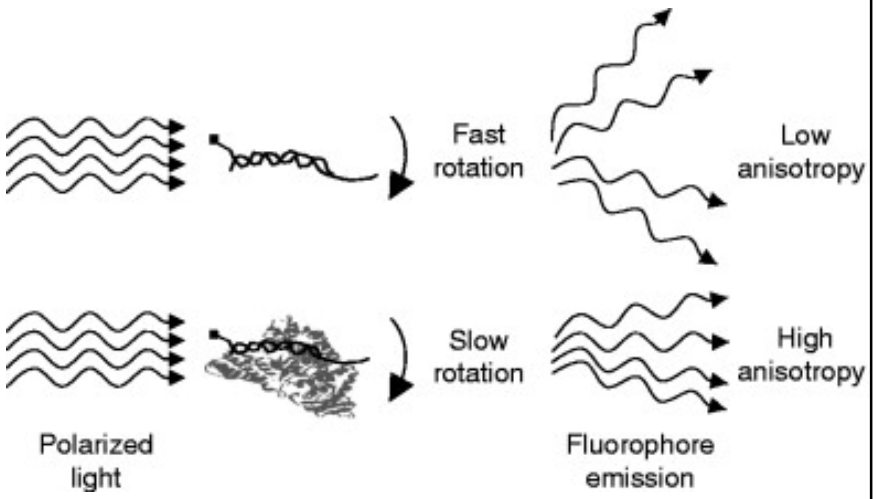
Fluorescence anisotropy

$$r = I_{parallel} - I_{perpendicular} / I_{parallel} + 2I_{perpendicular}$$

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)

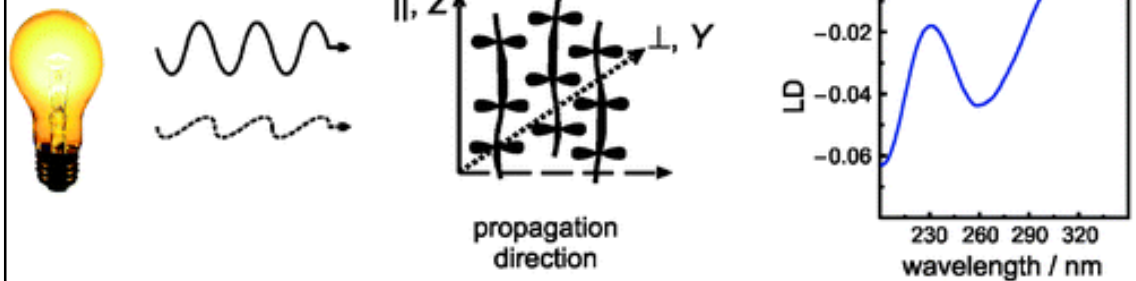
LiCata et al., 2007, Methods Cell Biol

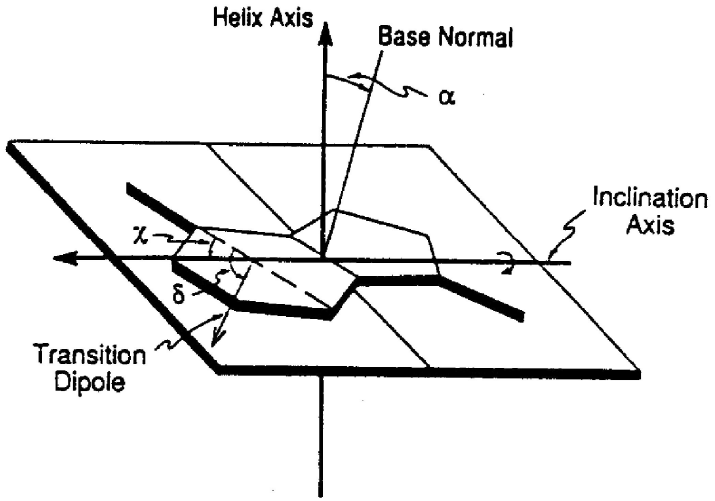
# Linear dichroism (LD)

## 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**

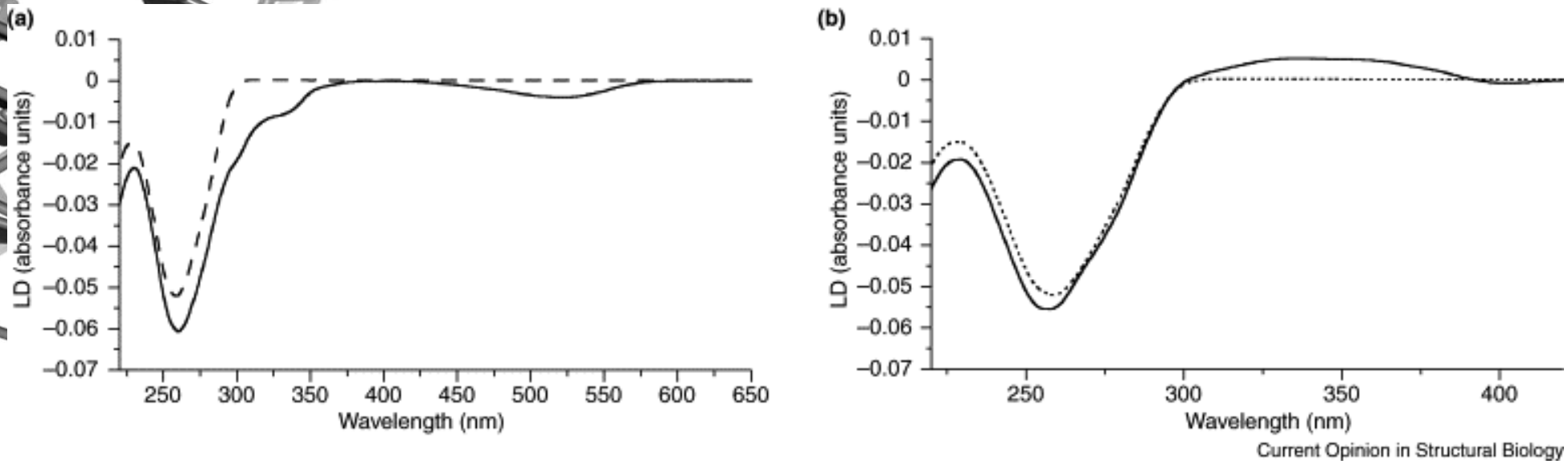




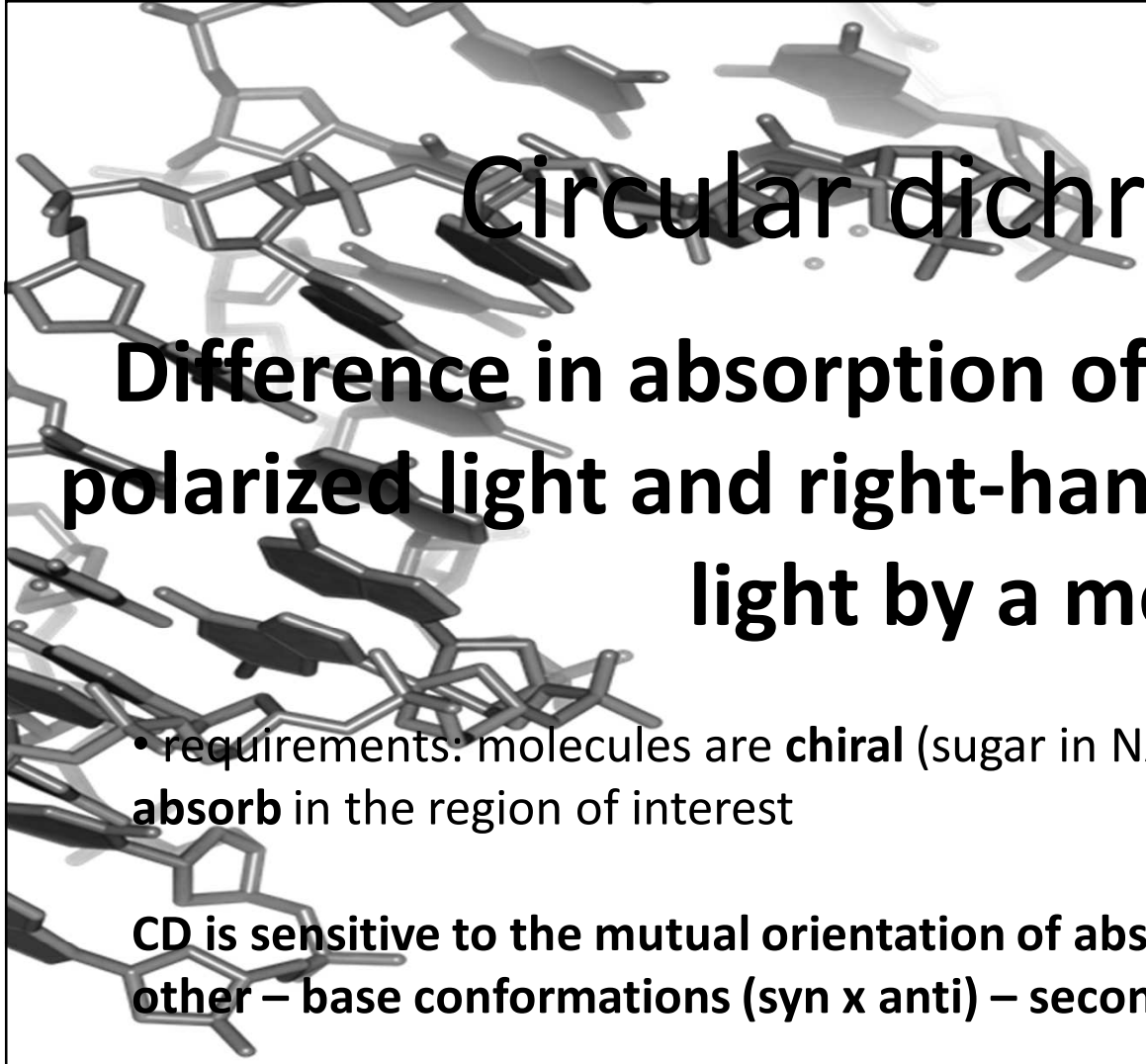
Bulheller et al., 2007, Phys Chem Chem Phys
Rodger et al., 2006, Phys Chem Chem Phys

# 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)

Dafforn et al., 2004, Curr Opin Struct Biol



# 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 wavelenghts, with anomalous ORD, where molecule absorbs – more difficult interpretation than CD
• **Cotton effect** – CD / ORD band – positive x negative

# Circular dichroism (CD)

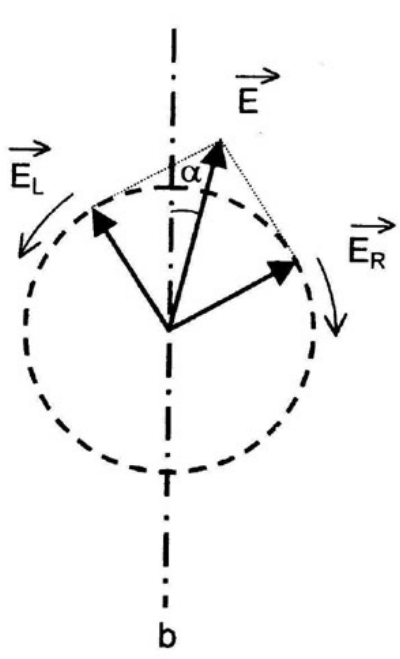
- Difference in absorbance: $\Delta A = A_L - A_R$

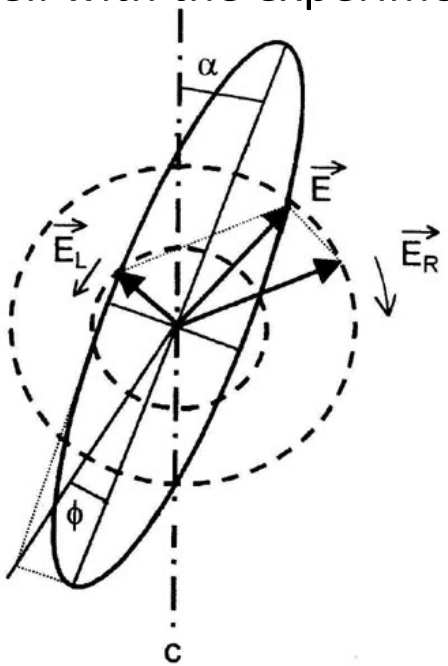
- When known concentration, difference in molar absorption $\Delta\varepsilon = \varepsilon_L - \varepsilon_R = \Delta A / lc$ (Beer-Lambert law)

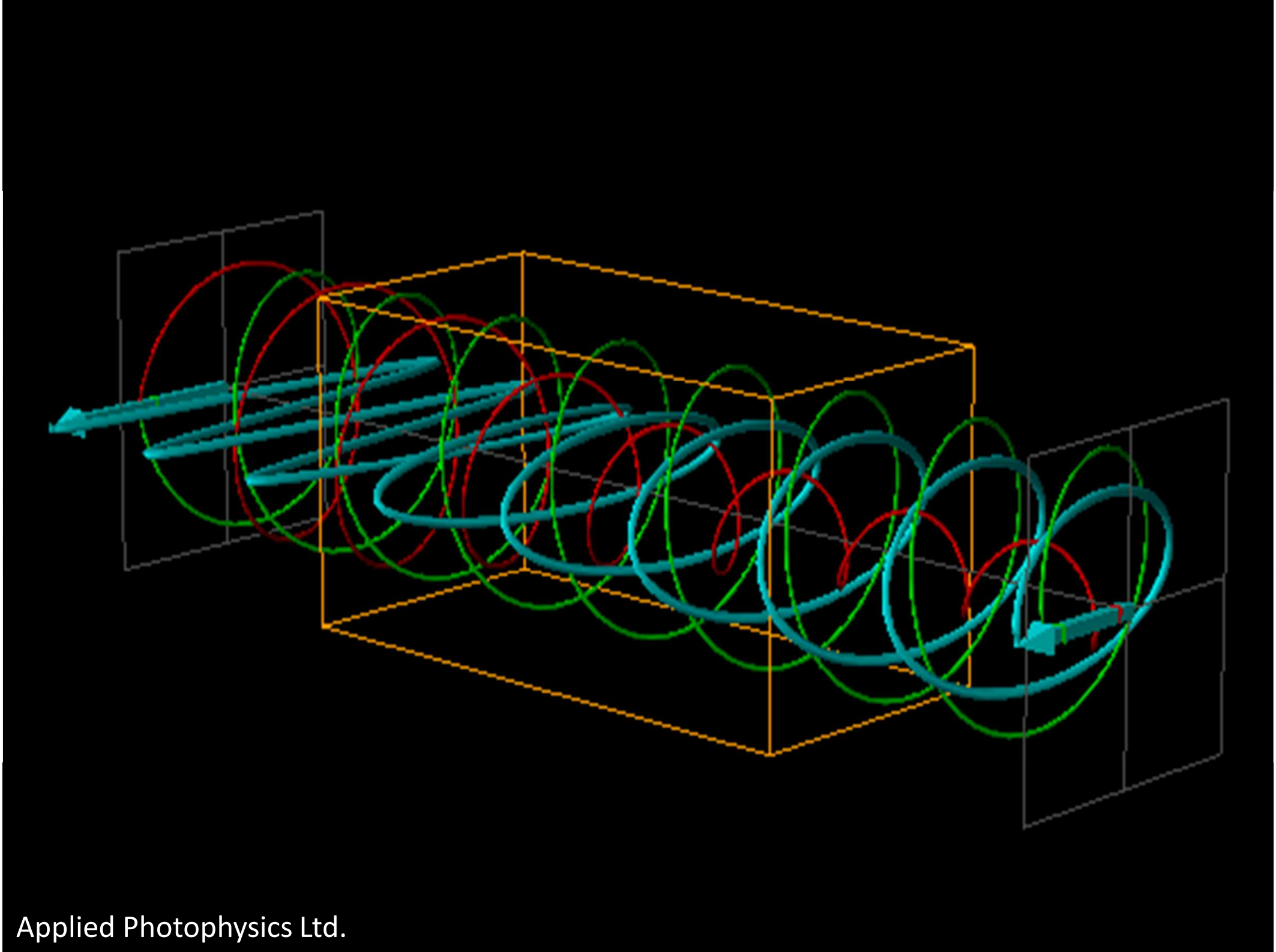
- Ellipicity – the angle that describes the extent of change of the linearly polarized light into a elliptically polarized light (0 for linearly polarized, 45° for circularly polarized)

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

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



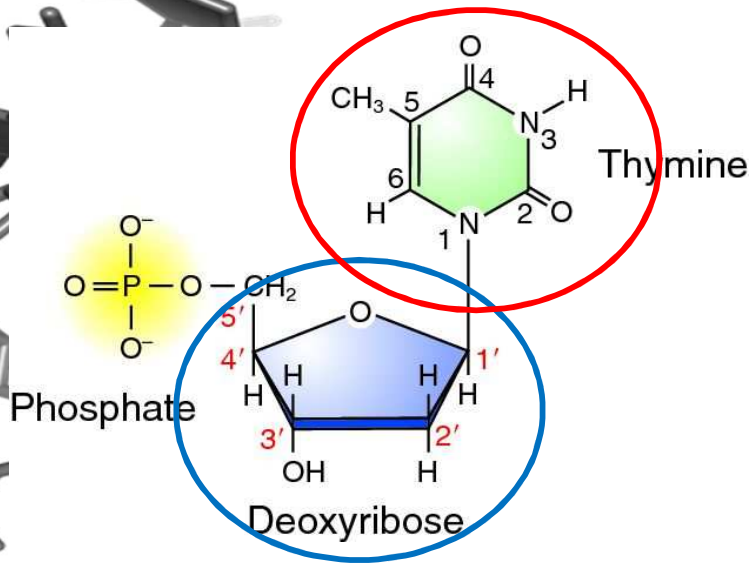





Applied Photophysics Ltd.

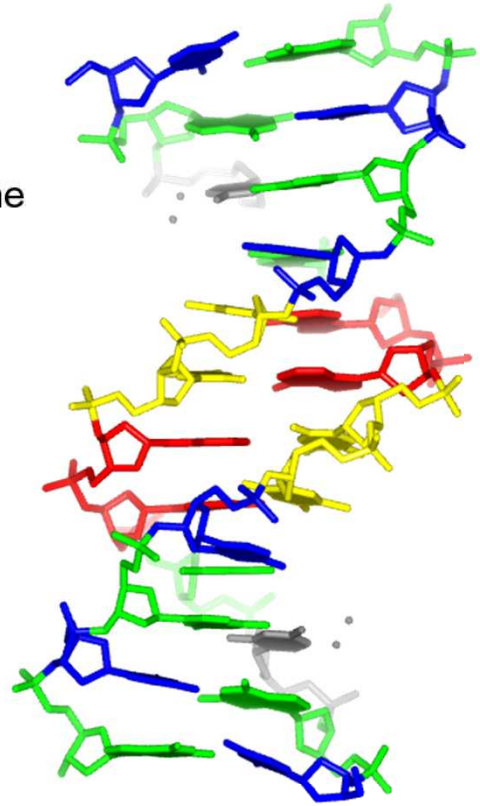
# Circular dichroism – DNA / RNA

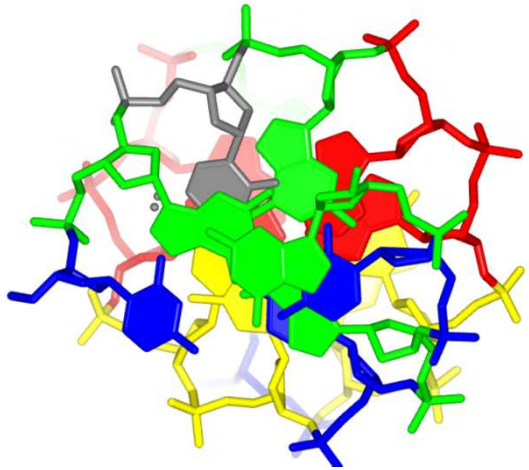
**ABSORPTION**

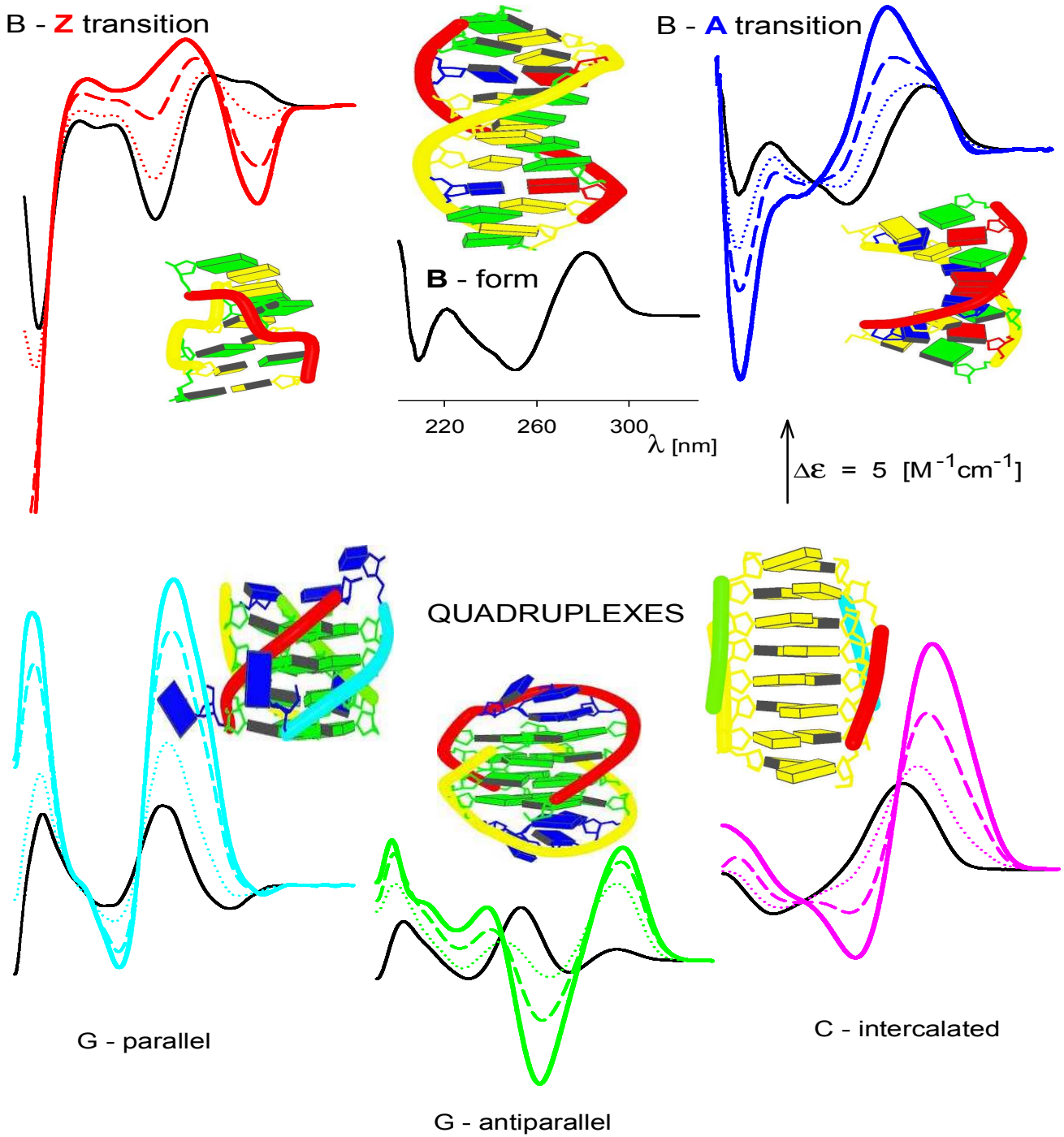



**CHIRALITY**

**MUTUAL ORIENTATION OF BASES**



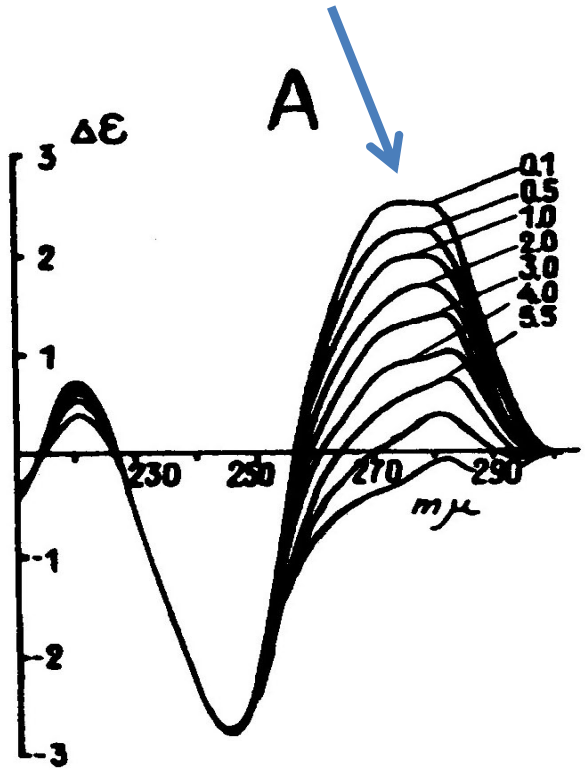



B - Z transition
B - A transition
B - form
220
260
300
λ [nm]
Δε = 5 [M-1cm-1]
QUADRUPLEXES
G - parallel
G - antiparallel
C - intercalated

# Transition cooperativity

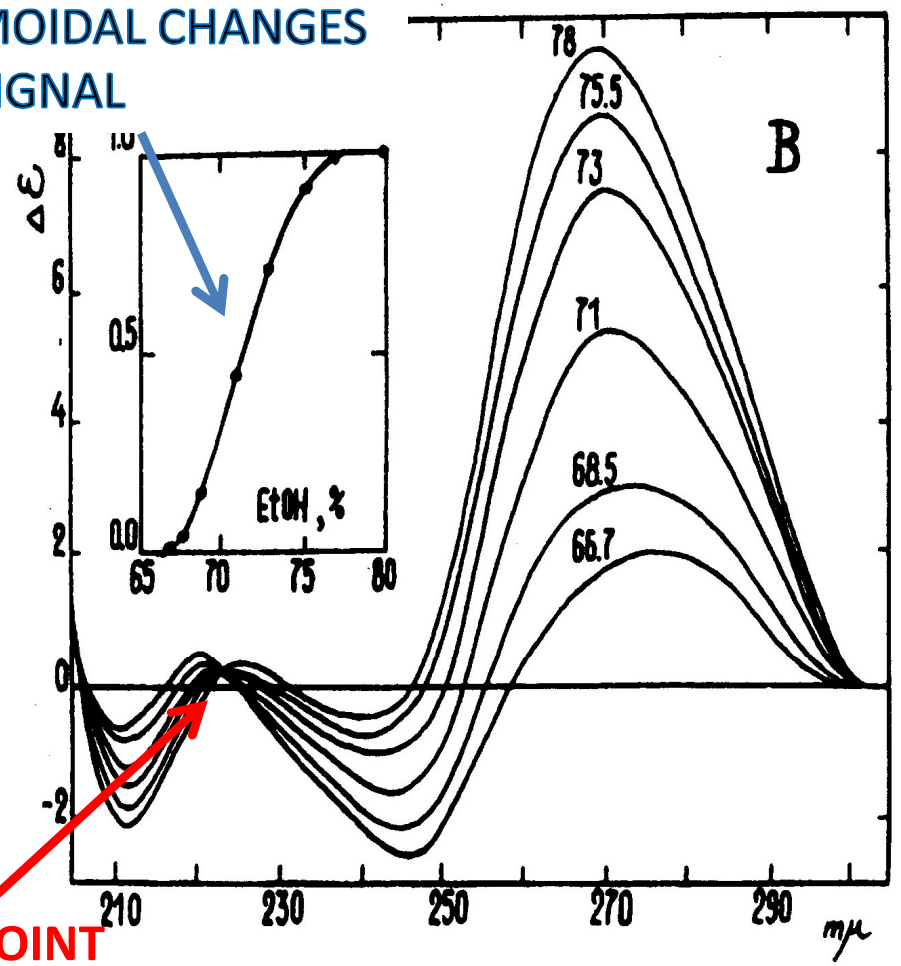
NON-COOPERATIVE TRANSITION

LINEAR CHANGES OF SIGNAL



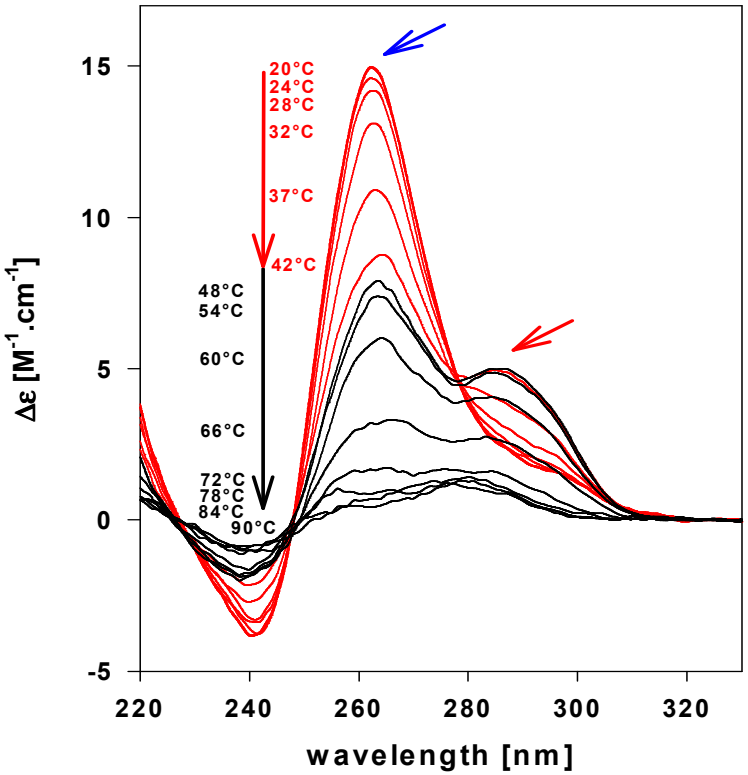

COOPERATIVE TRANSITION

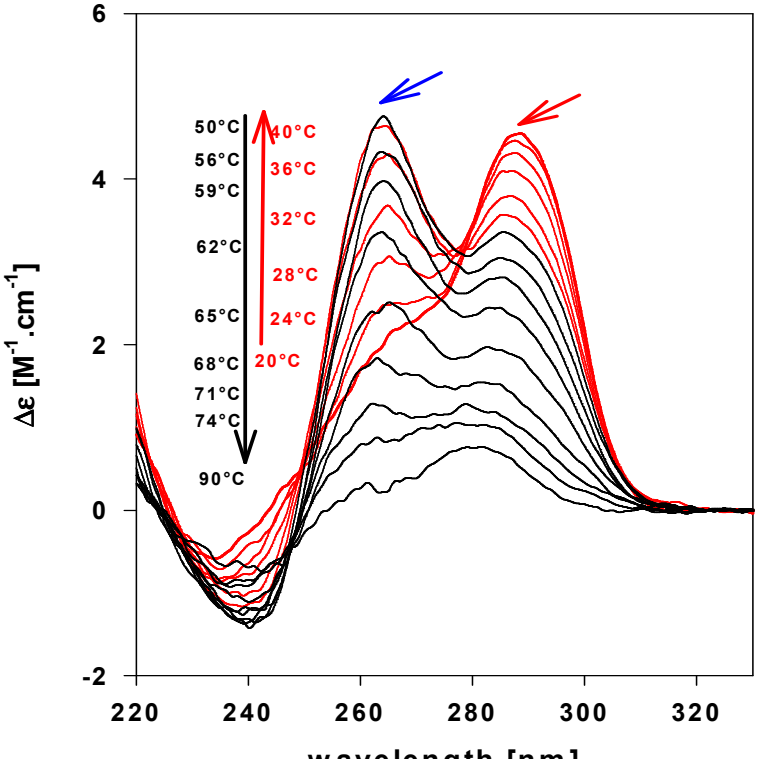
SIGMOIDAL CHANGES OF SIGNAL

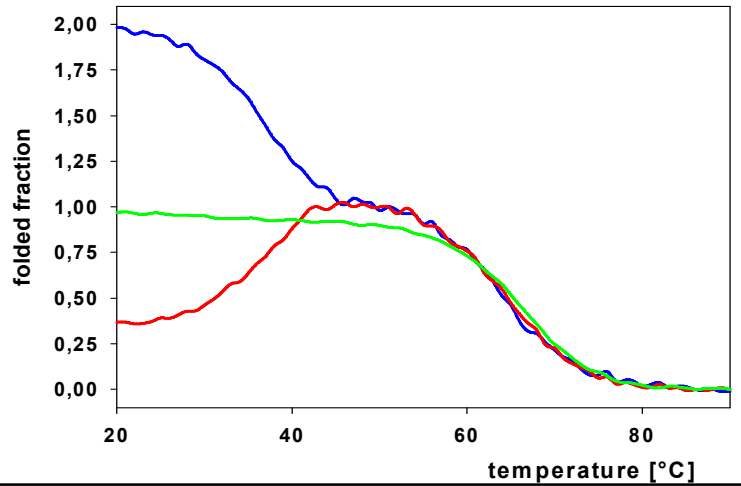


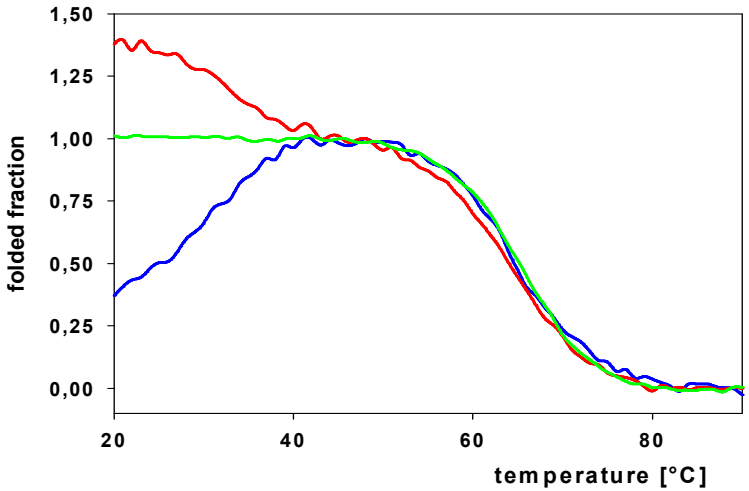

ISODICHROIC POINT

# CD – NA melting

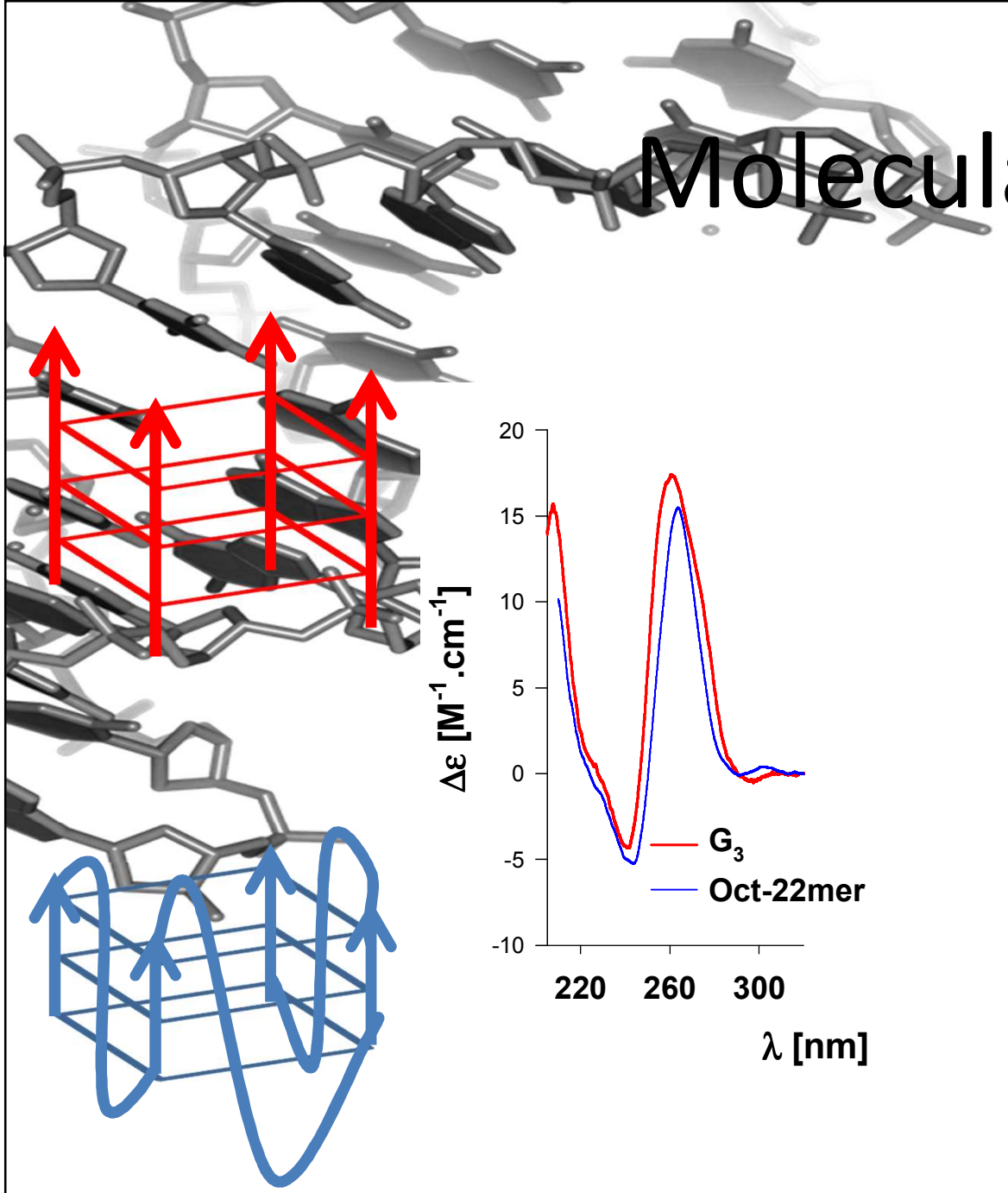


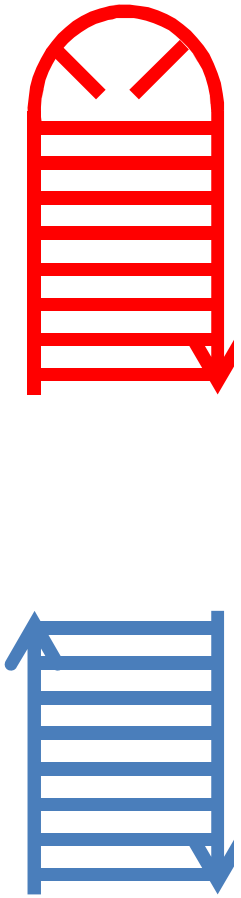



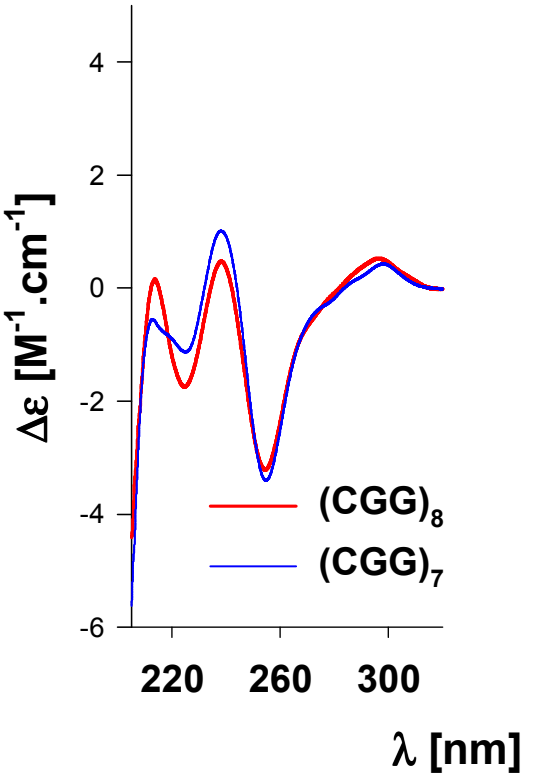



# Molecularity





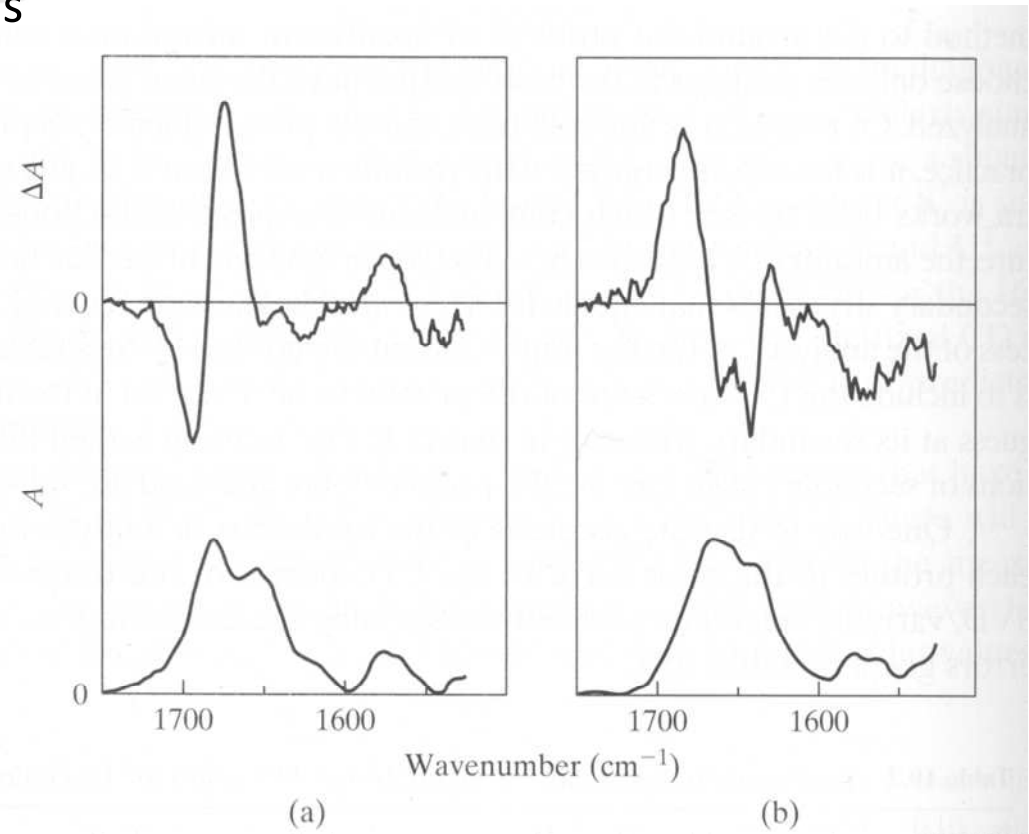





# Vibrational / infrared CD (VCD/IRCD)

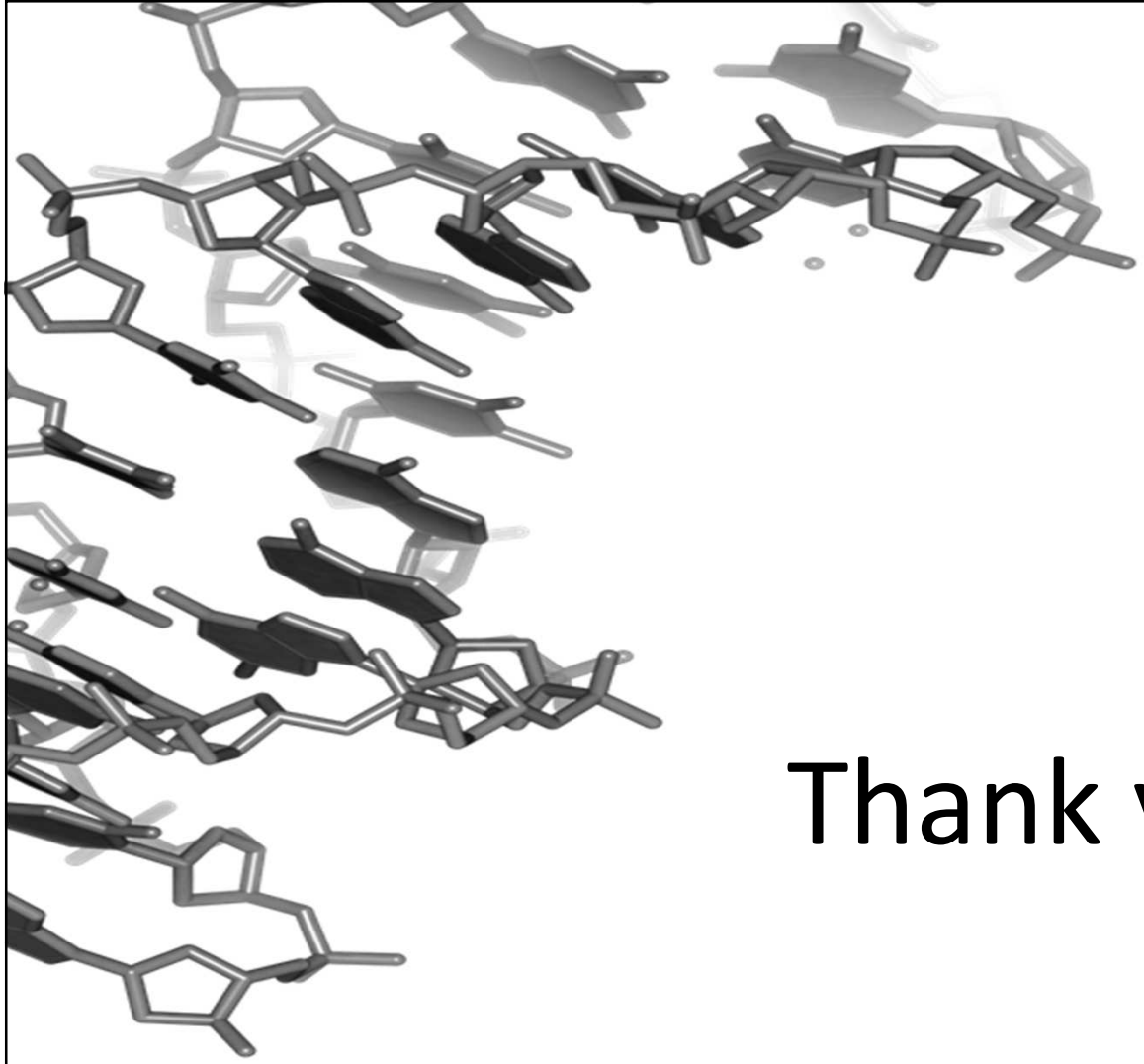
**Difference in absorption of left-handed circularly polarized light and right-handed circularly polarized light in a region of vibrational transitions (λ = 1-5 um).**

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




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

Keiderling et al., 1989, Biomol Spec



# Thank you