XXV IUCr Satellite Workshop

Spectroscopic methods to study biomolecular structure and dynamics

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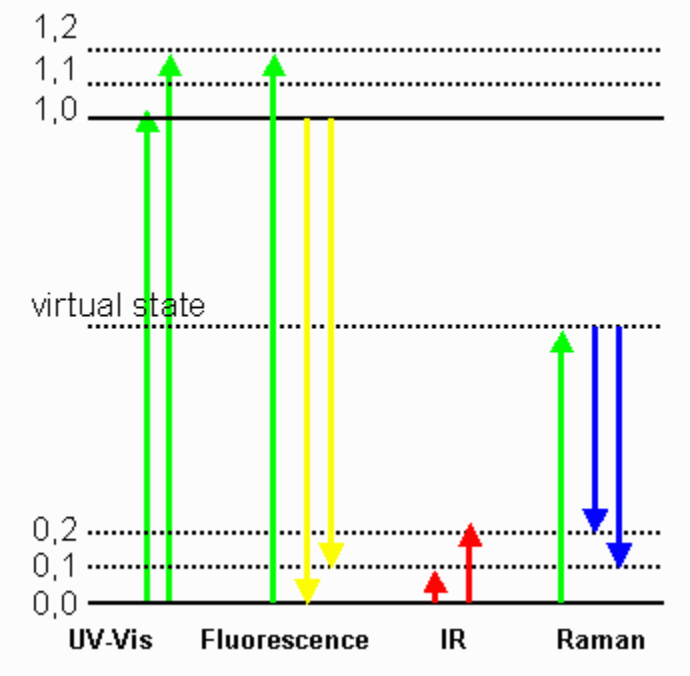
CMS Workshop: Methods of Structural Biology
13/08/2021

Outline

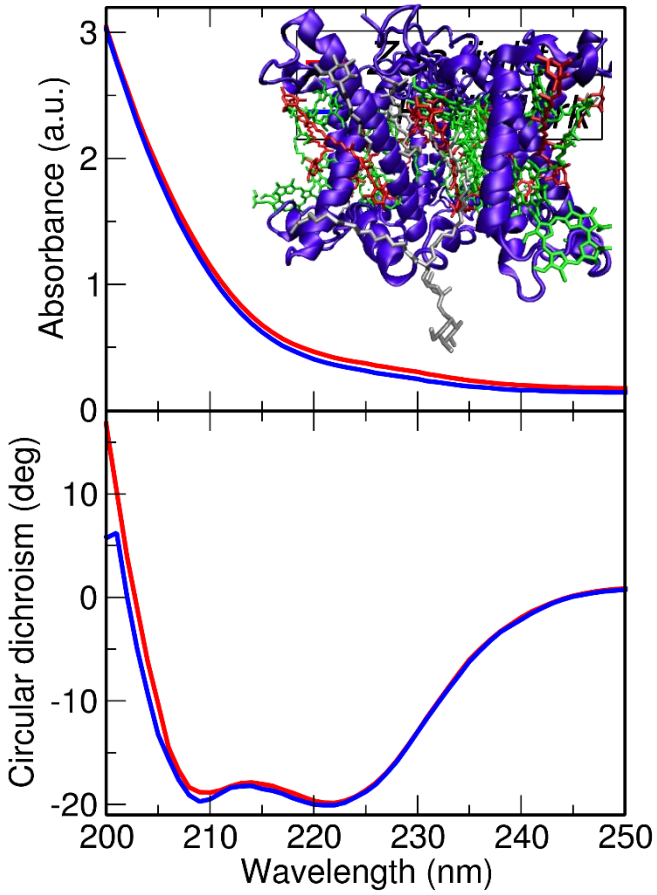
1. UV/Visible and circular dichroism spectroscopy
2. Fluorescence spectroscopy
   - (Single-molecule) resonance energy transfer
   - Correlation
   - Anisotropy
3. Infrared spectroscopy
4. Raman spectroscopy
5. Protein engineering & spectroscopy



UV-Vis & CD

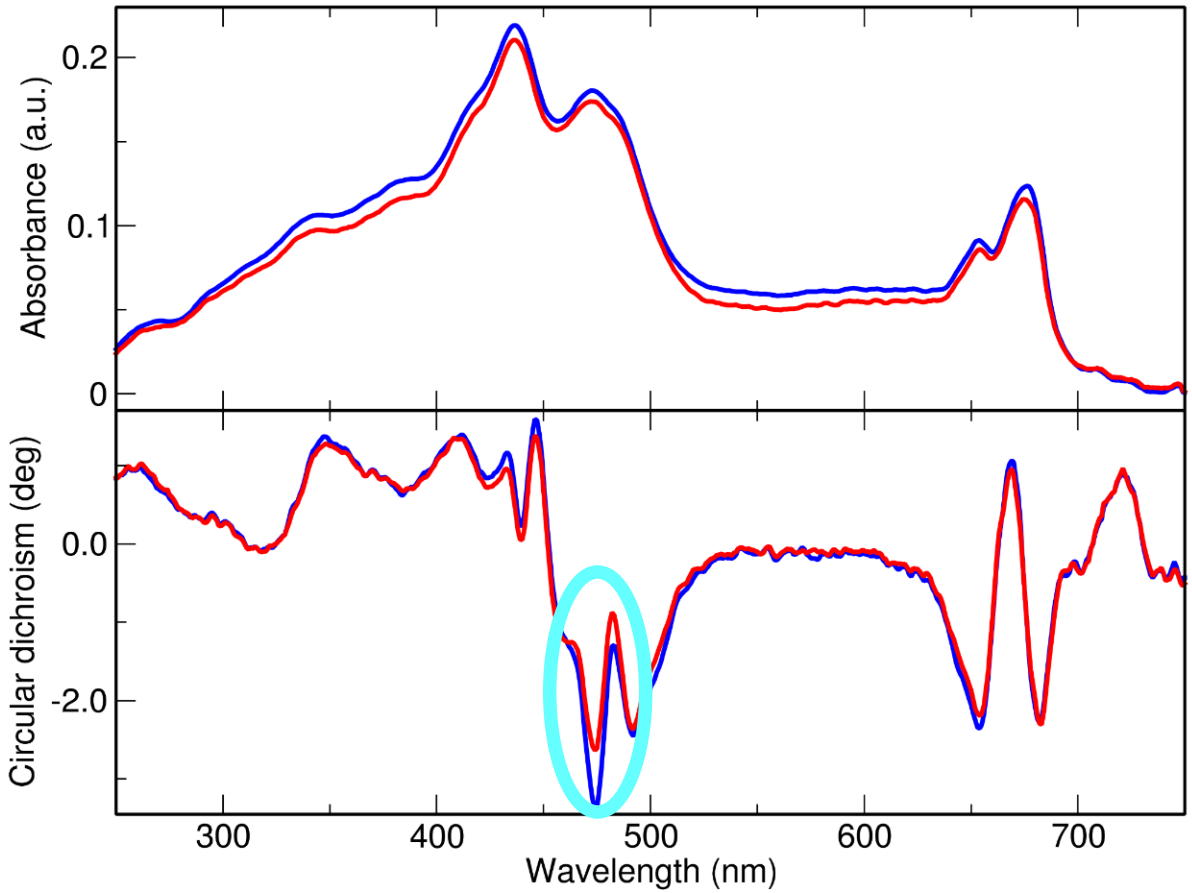
Far-UV

Secondary structure



Near-UV & visible

Aromatic residues & chromophore microenvironment

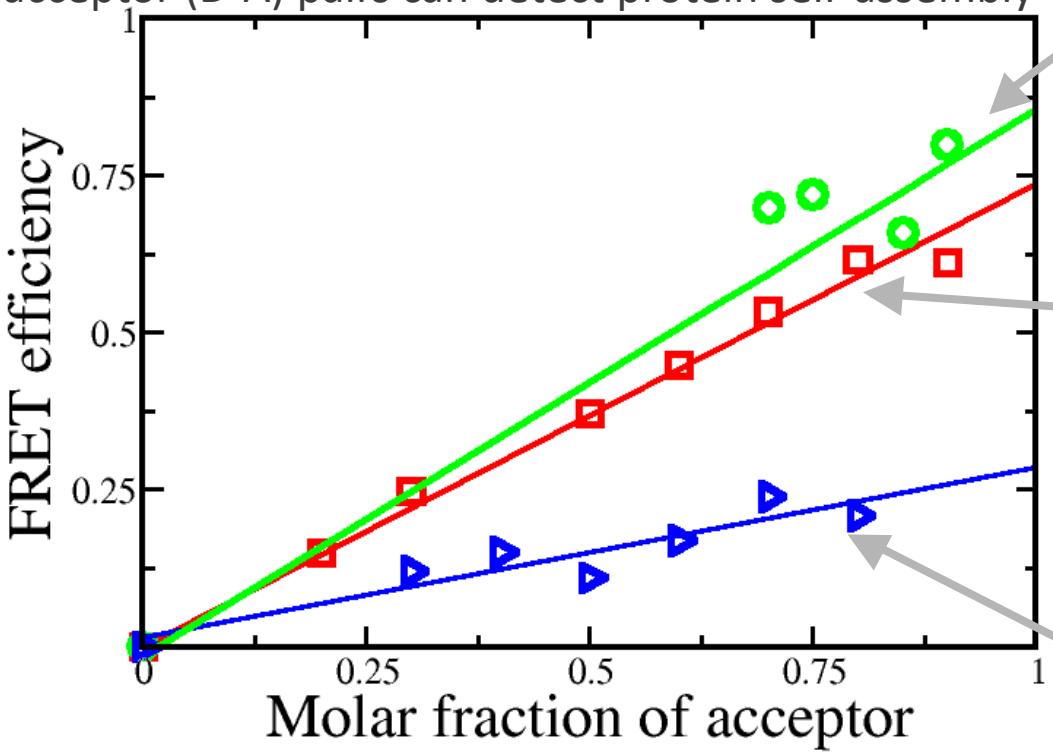


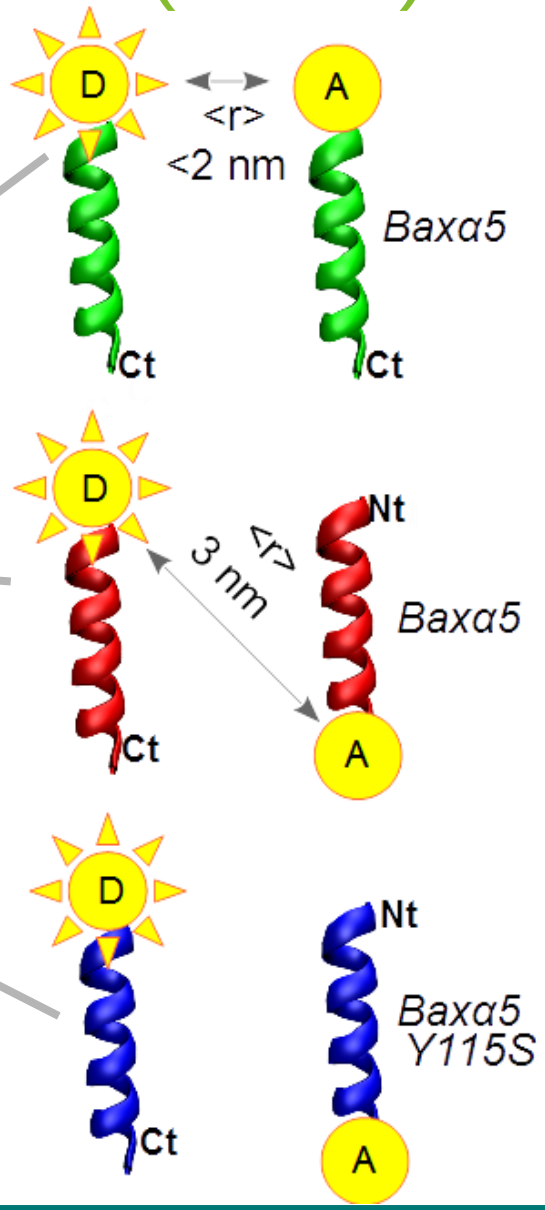
- *CD spectroscopy reveals local changes around chlorophylls/carotenoids in LHCII in the absence of major changes in protein conformation*

Förster Resonance Energy Transfer (FRET)

Molecular ruler: $E_{FRET} = \frac{R_0^6}{R_0^6 + r_{DA}^6}$

Intermolecular distance measurements between donor-acceptor (D-A) pairs can detect protein self-assembly





- *Ensemble FRET suggests the formation of parallel (head-to-head) Baxα5 dimers*

Fuertes G. (2011) *Ph.D. thesis*

Single molecule FRET (smFRET)

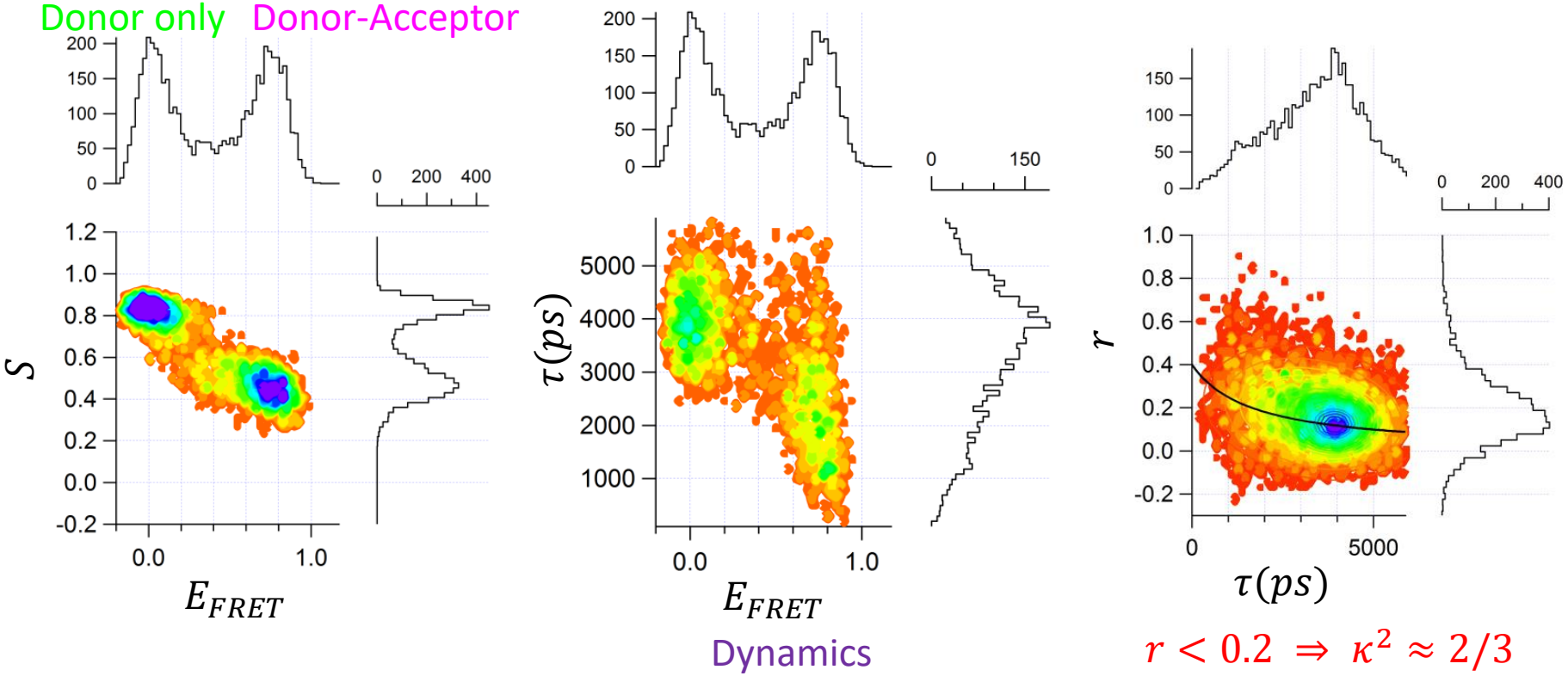
Multiparameter fluorescence detection (MPD) + Pulsed interleaved excitation (PIE/ALEX)

- *Number*: FRET efficiency (E_{FRET})
- *Color*: Stoichiometry (S)
- *Arrival time*: Lifetime (τ)
- *Polarization*: Anisotropy (r)

$$R_0 \propto \{\kappa^2, n, Q_D, J(\lambda)\}$$

FRET histogram → FRET efficiency (E_{FRET}) → Interdye distance (R_E)

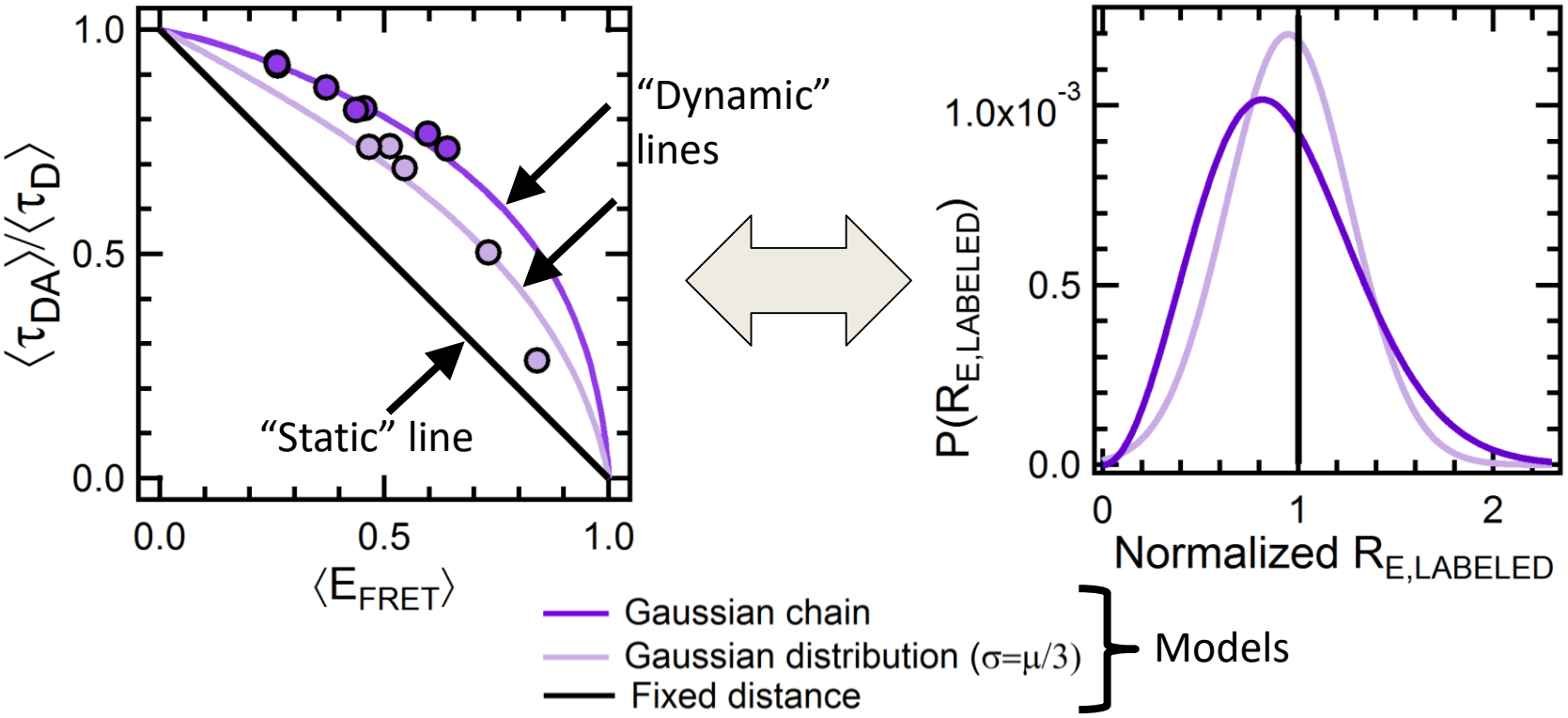
Donor only Donor-Acceptor



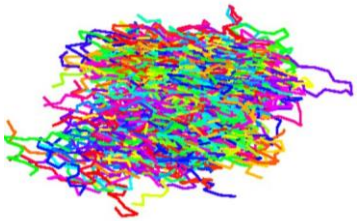
$r < 0.2 \Rightarrow \kappa^2 \approx 2/3$

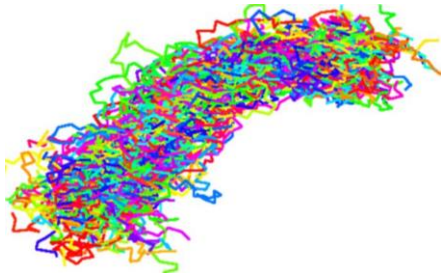
- *MFD enables accurate measurements of FRET efficiencies*

Inferring protein dynamics with smFRET



- *Folded proteins*
- *Native IDPs*
- *Denatured IDPs*

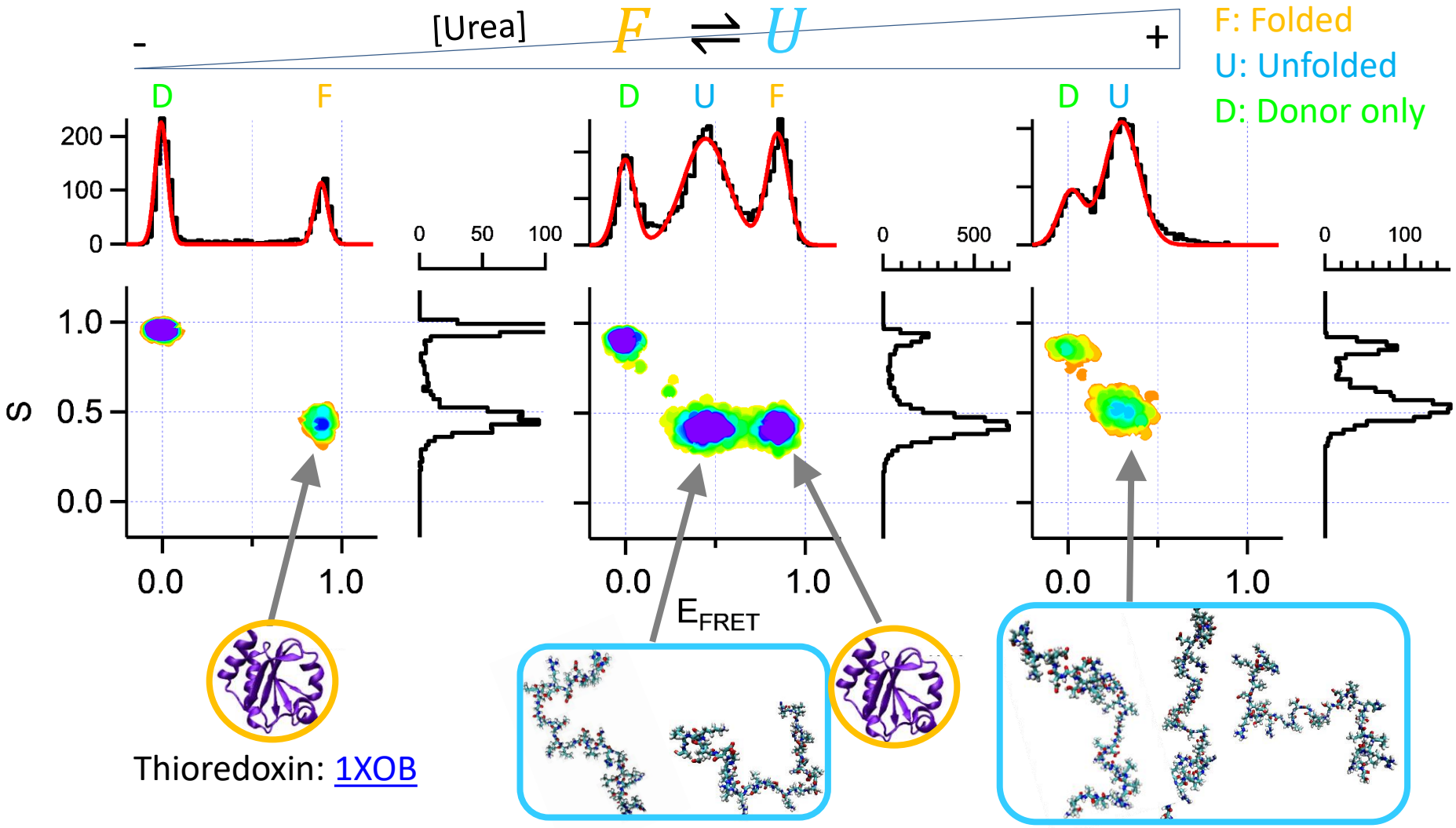




- *Differential dynamics of intrinsically disordered vs foldable proteins*

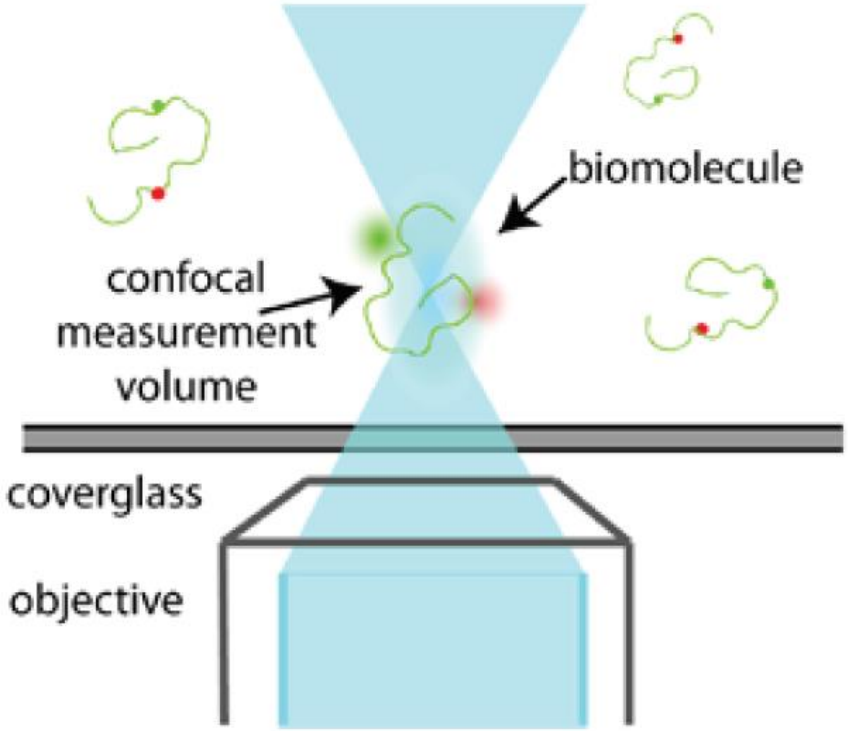
Fuertes G. et al (2017) *PNAS*
Fuertes G. et al (2018) *Science*

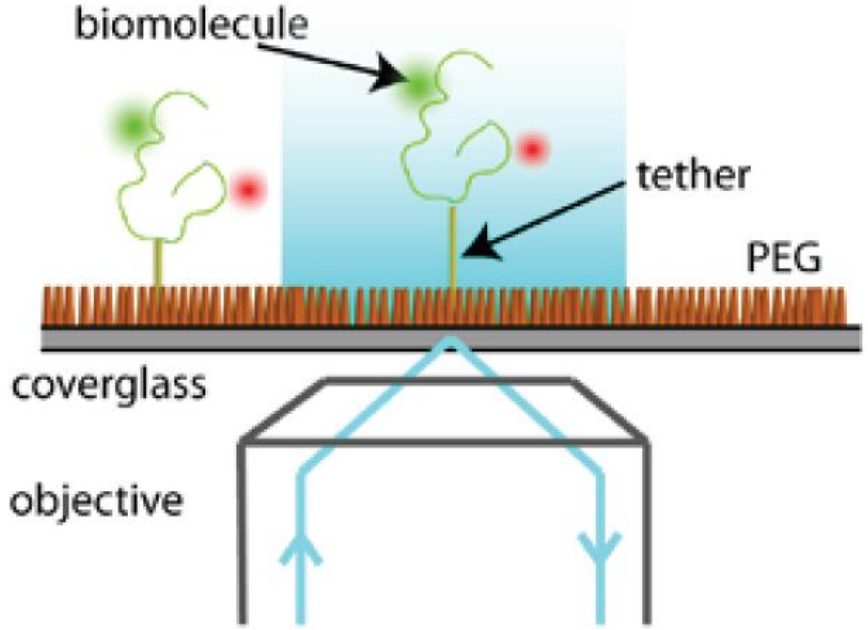
Separating multiple populations by smFRET



- *Co-existing folded and unfolded populations at low denaturant concentrations can be characterized individually.*

Confocal vs total internal reflection fluorescence (TIRF)





Confocal microscopy

✓ Freely diffusing

X Observation time limited to diffusion (~ms)

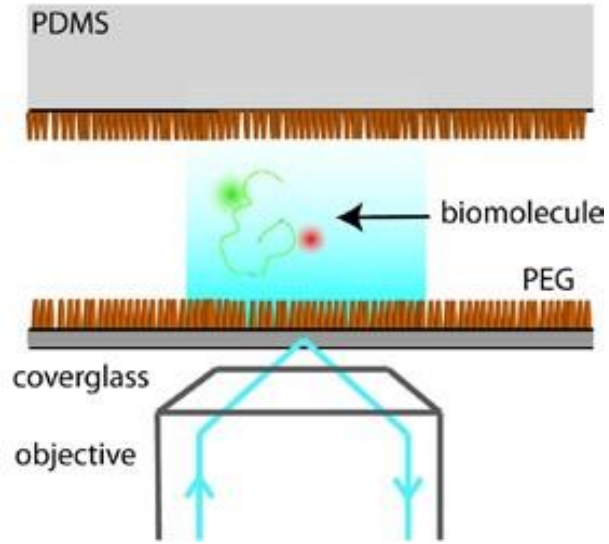
TIRF microscopy

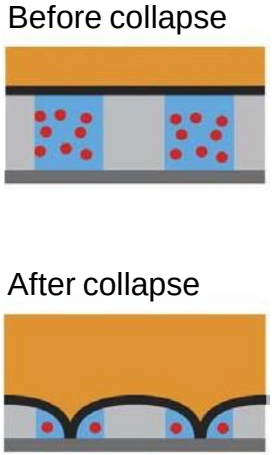
✓ Long observation time (~s)

X Need for immobilization

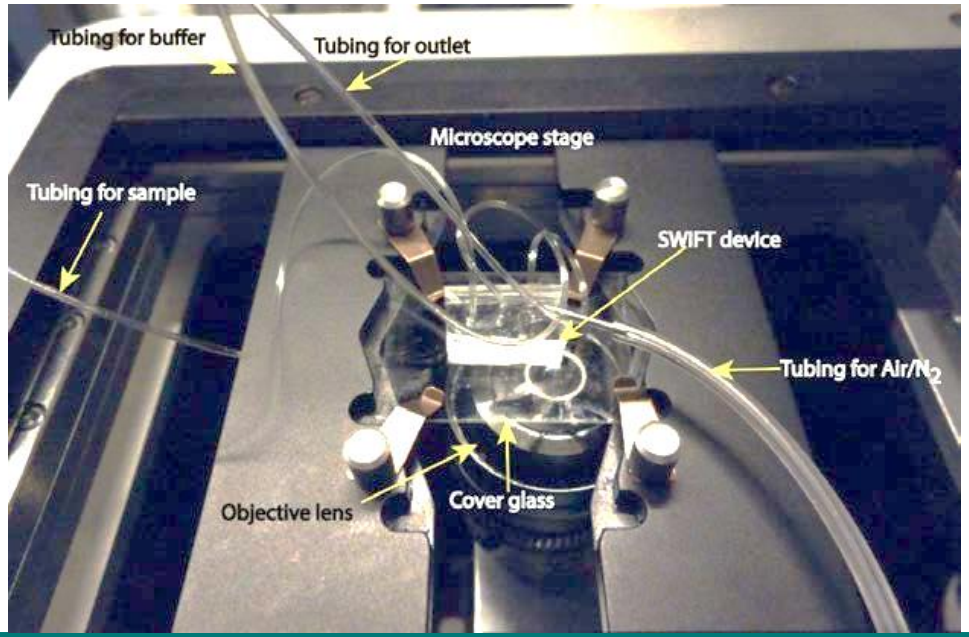
- *Can we combine the best of these two worlds?*

SWIFT: Single Molecules Without Immobilization for TIRF





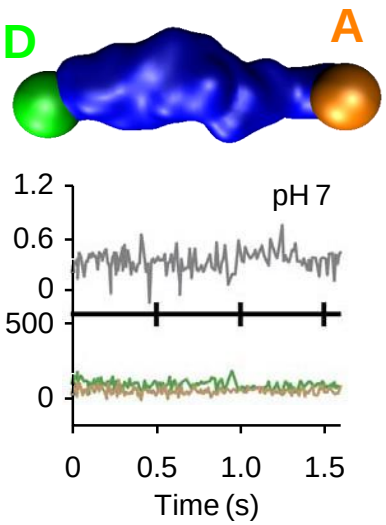
- Upon pressurization, the microfluidic channels collapse to < 100 nm ≈ evanescent field
- Multisecond observation
- Millisecond time resolution



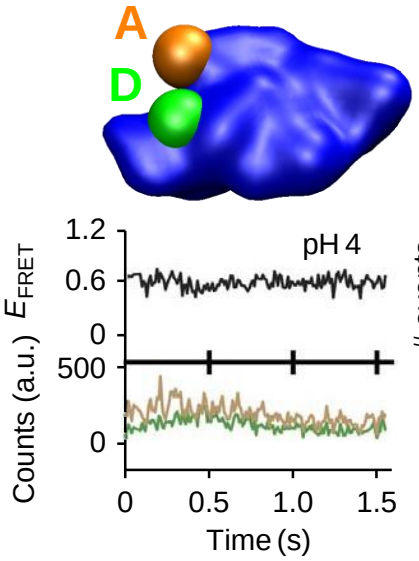
- *SWIFT is complementary to TIRF and confocal microscopies, especially for observing long trajectories of large biomolecules and complexes which are difficult to immobilize.*

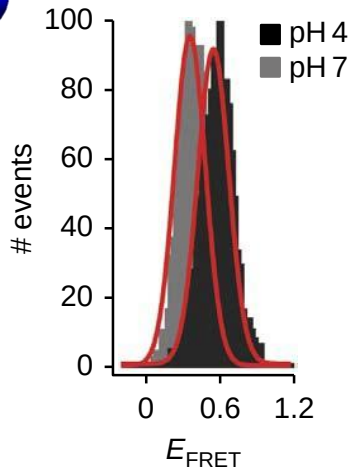
Measuring dynamics of biomolecules by SWIFT

2Q3Z

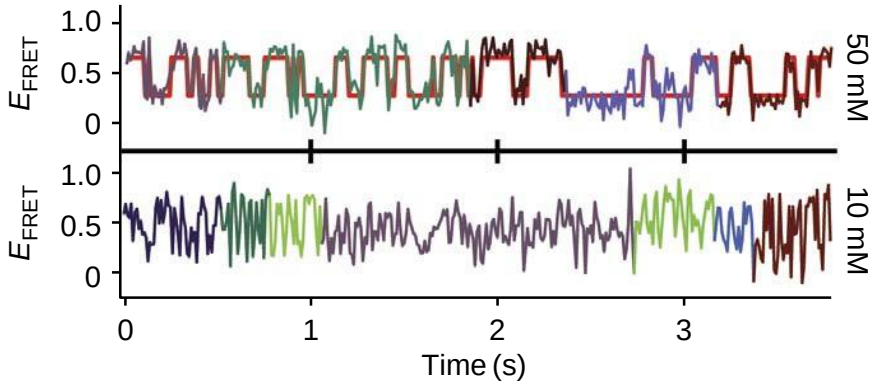


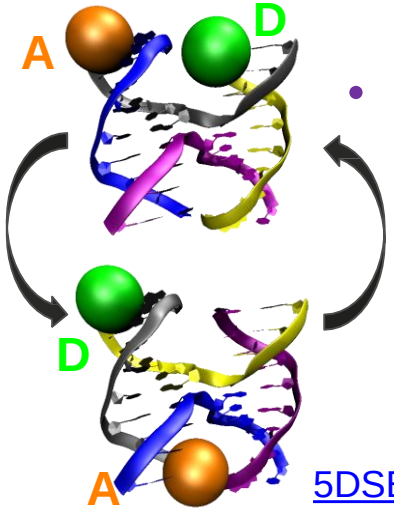
1KV3





- Transglutaminase 2:
  - 2-state protein
    - Open (active): pH=7
    - Close (inactive): pH=4



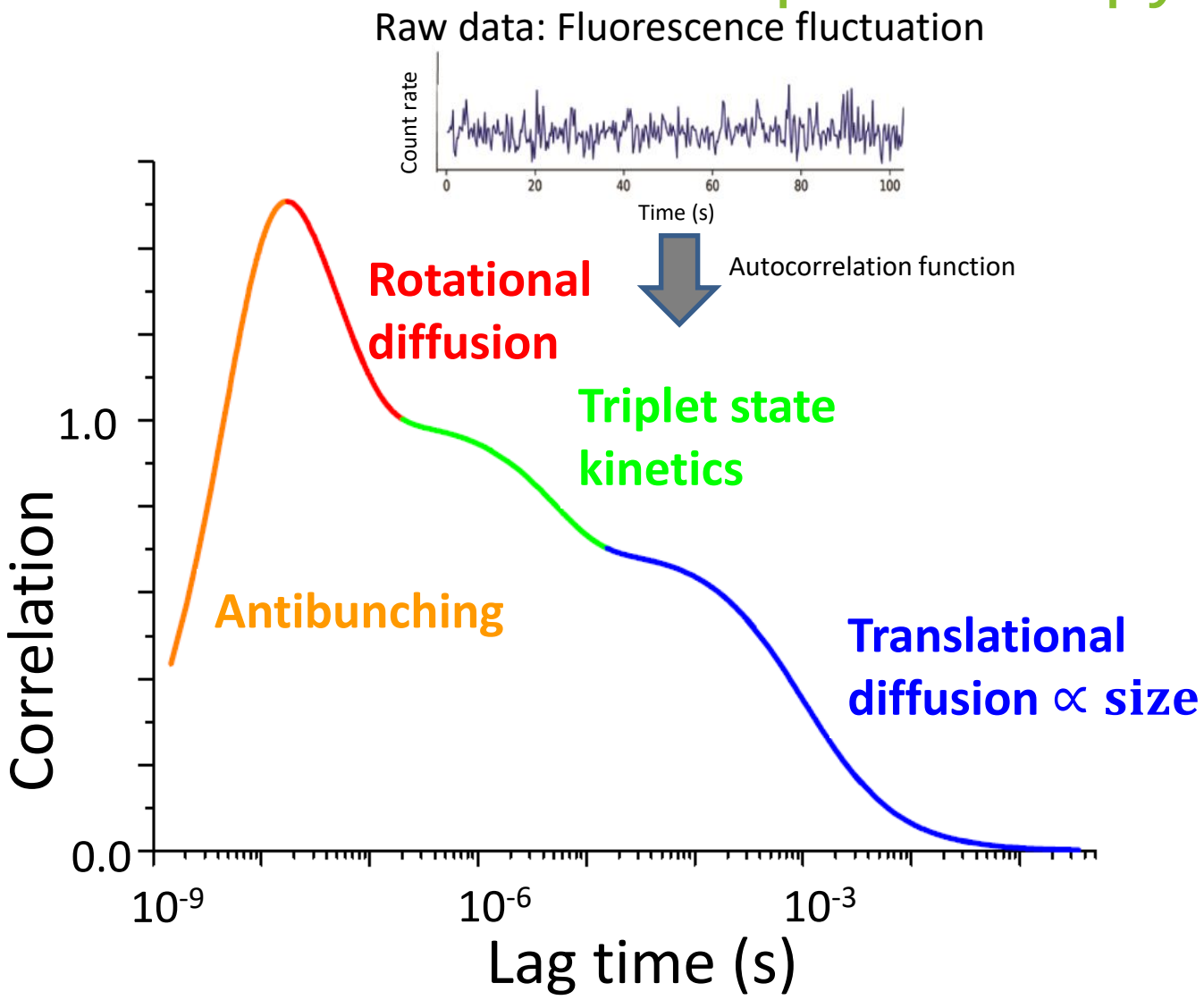


5DSB

- Holliday junction:
  - At least 2 different folding states
  - The exchange is $[Mg^{2+}]$- dependent
    - 200 ms (50 mM $MgCl_2$)

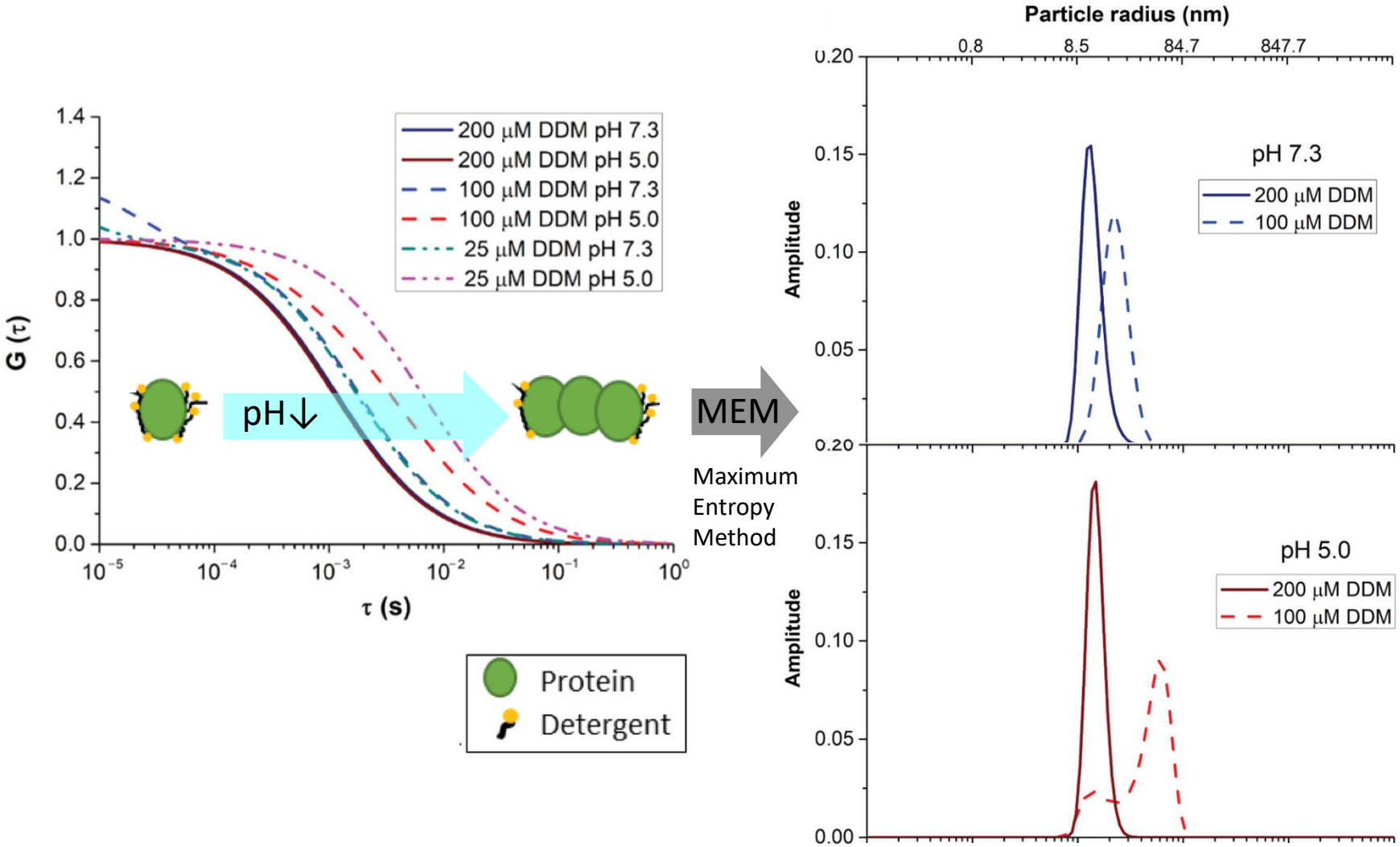
Tyagi S. et al (2014) *Nat Methods*

Fluorescence correlation spectroscopy (FCS)



- *FCS is the fluorescent counterpart to dynamic light scattering (DLS)*

Measuring protein aggregation with FCS

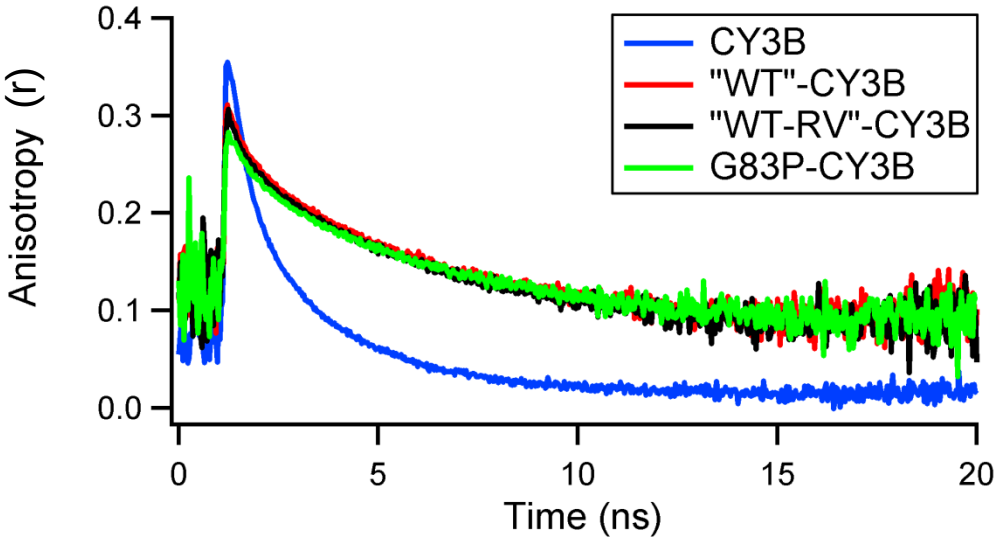


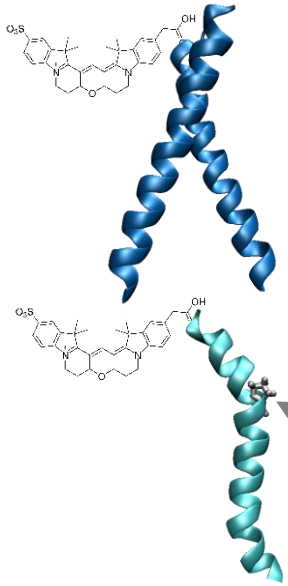
- *FCS suggests the formation of LHCII clusters at low pH and low [detergent]*

Time-resolved anisotropy

Revealing the ability of molecules to rotate while being in the excited state

$$r(t) = \frac{I_{VV}(t) - G\, I_{VH}(t)}{I_{VV}(t) + 2\, G\, I_{VH}(t)} \qquad G = \frac{I_{HV}(t)}{I_{HH}(t)}$$





Glycophorin A WT (GPA, 1AFO)

Glycophorin A G83P (model)

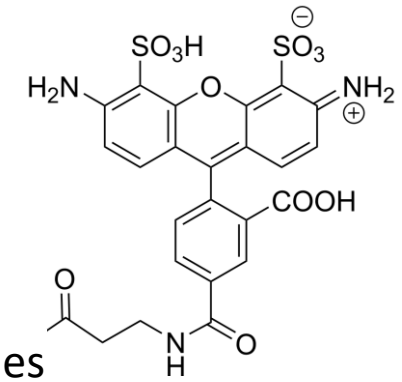
Sample	ϕ_{DYE} (ns)	ϕ_{HELIX} (ns)	r_∞
GPA-WT-P71C-CY3B	3.3	33	0.195
GPA-WT-I99C-CY3B	3.5	31	0.203
GPA-G83P-P71C-CY3B	2.9	25	0.190
CY3B	2.2	-	0.097

- *Internal dynamics (orientational fluctuations) are faster in proline-kinked GPA*

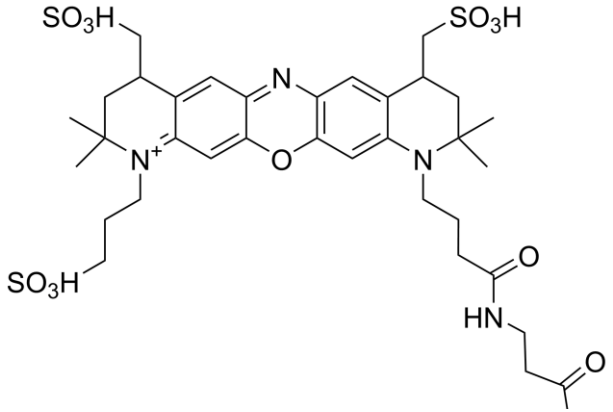
Labeling of proteins with fluorophores

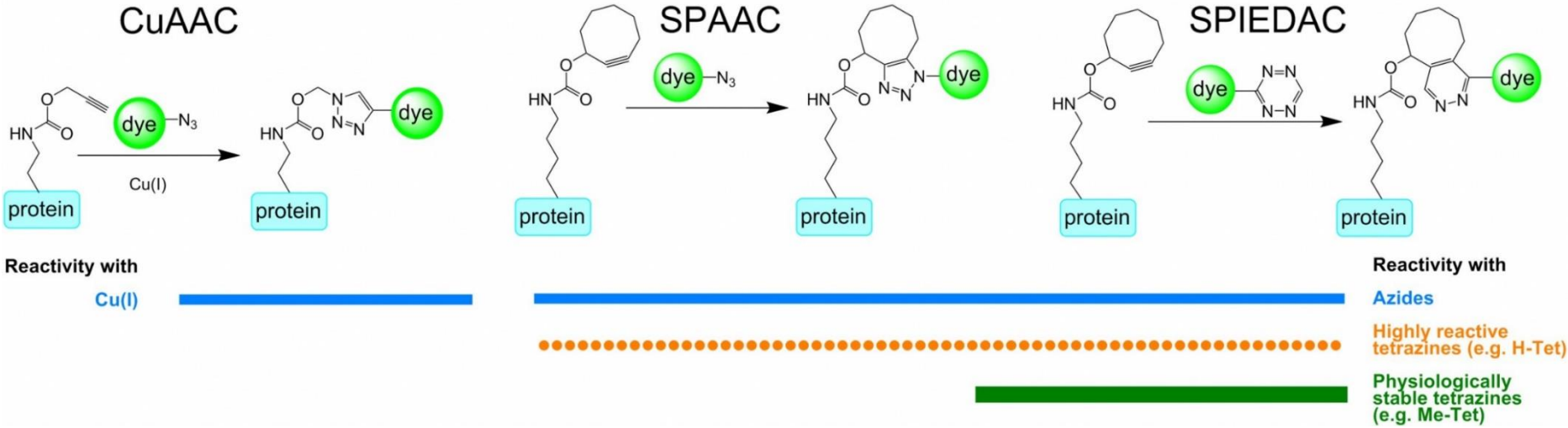
- Non bio-orthogonal reactions:
  - Cysteine + maleimide
- Bio-orthogonal reactions:
  - Ketone + hydroxylamine
  - Azide + alkyne
  - Tetrazine + strained alkenes/alkynes

AFDye488 (DONOR)



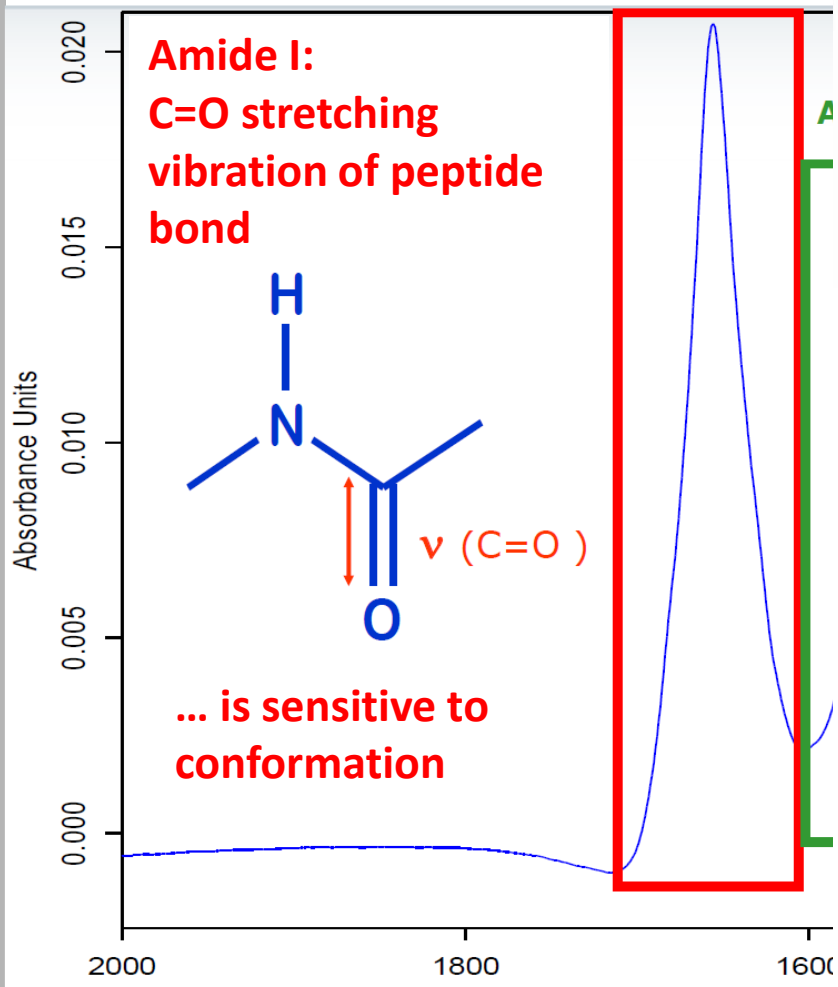
MB660R (ACCEPTOR)

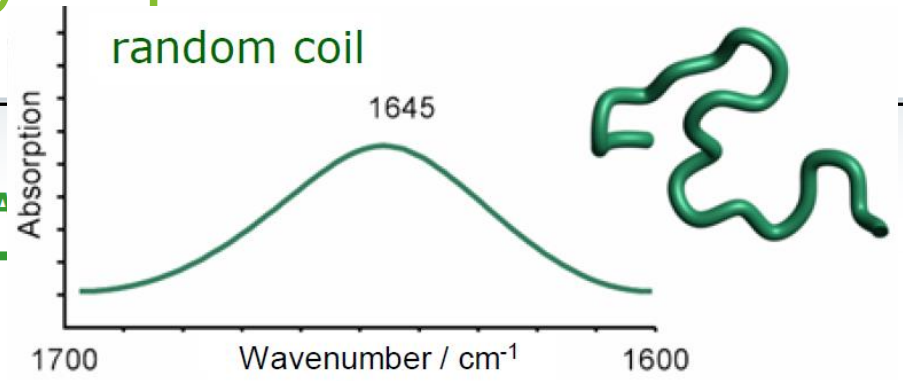


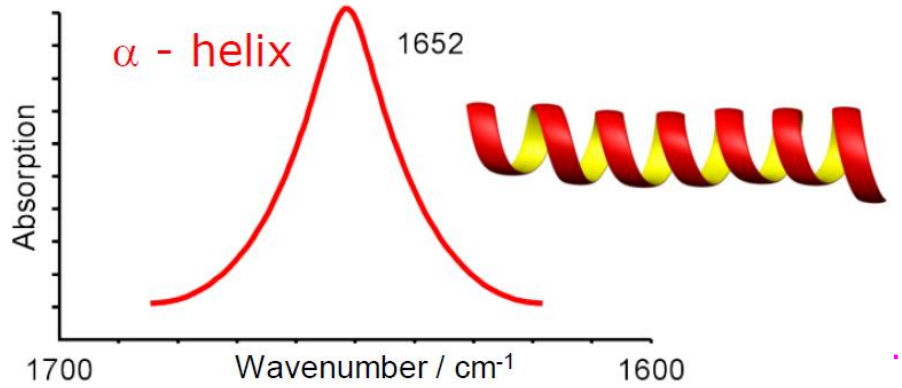


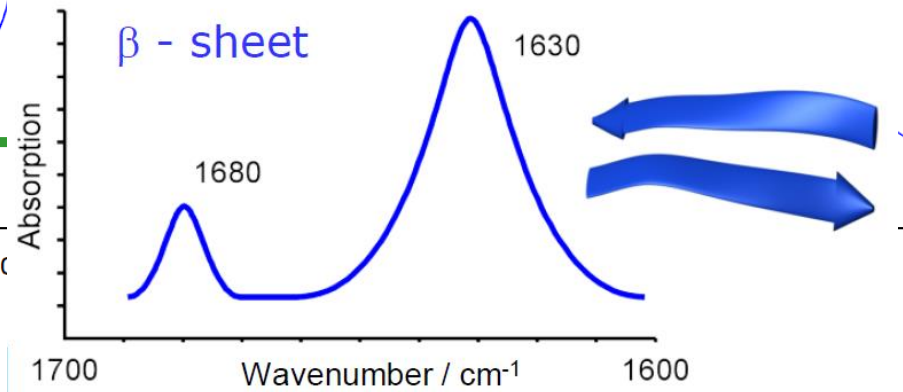
- *Genetic code expansion technology can be used to incorporate non-canonical amino acids bearing reactive handles which are subsequently reacted with suitable dyes*

Infrared spectroscopy of proteins

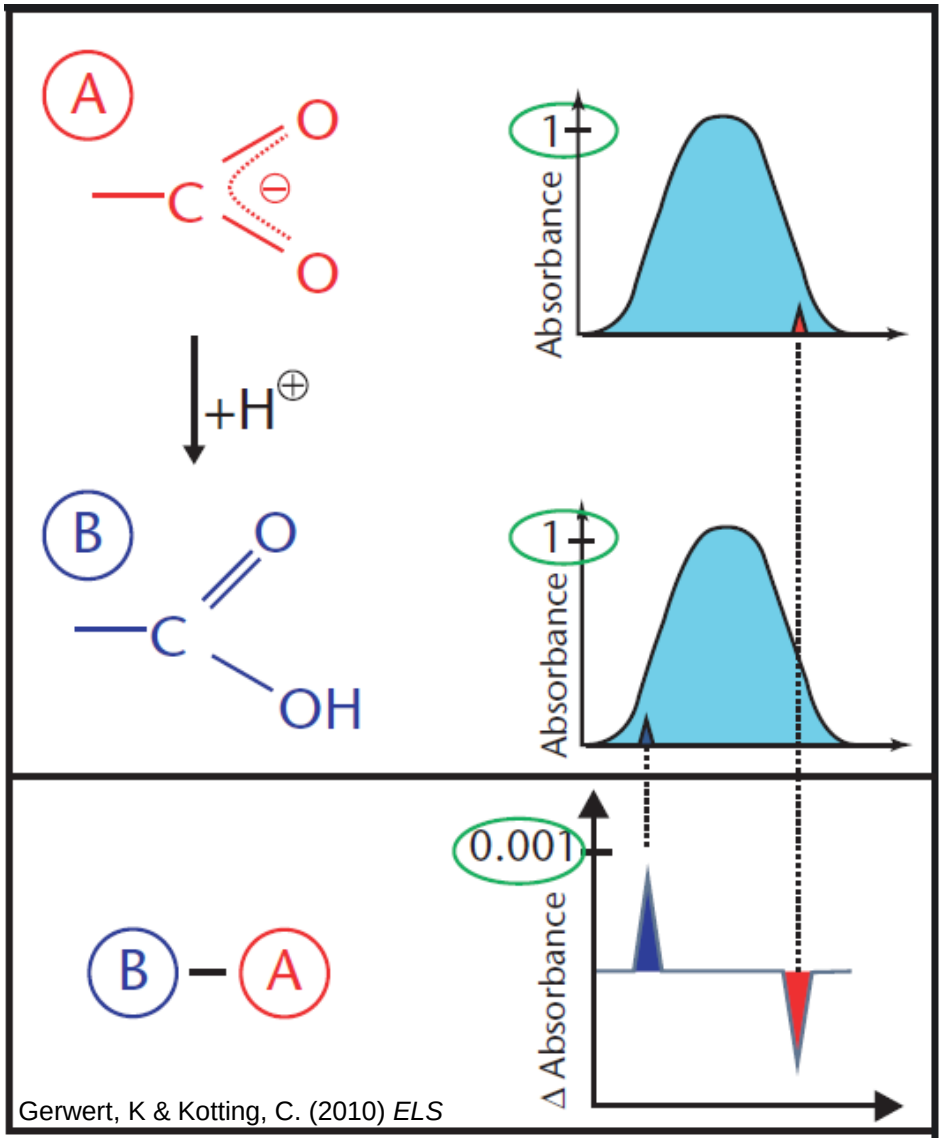








Infrared difference spectroscopy

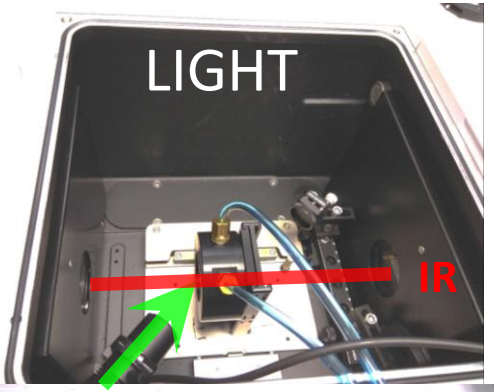


Gerwert, K & Kotting, C. (2010) *ELS*

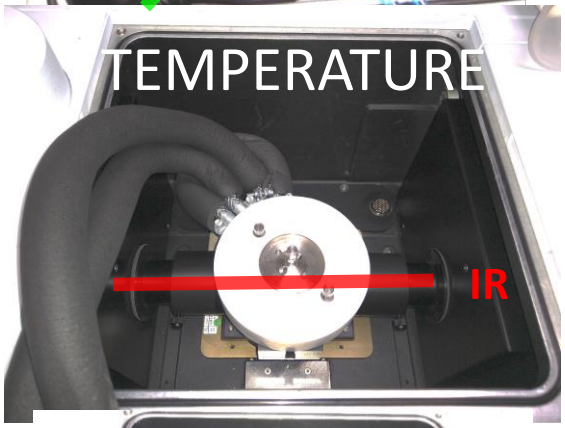
ATR: Attenuated Total Reflection

PERTURBATION METHODS:

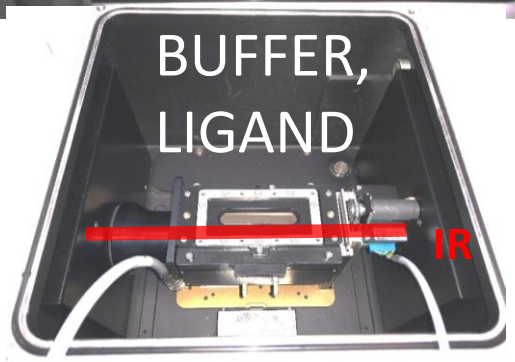
Transmision cell



ATR cell



ATR with dialysis

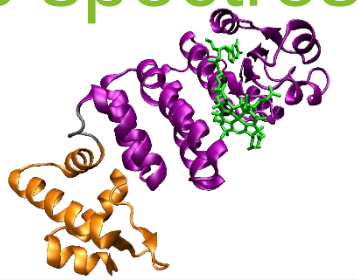


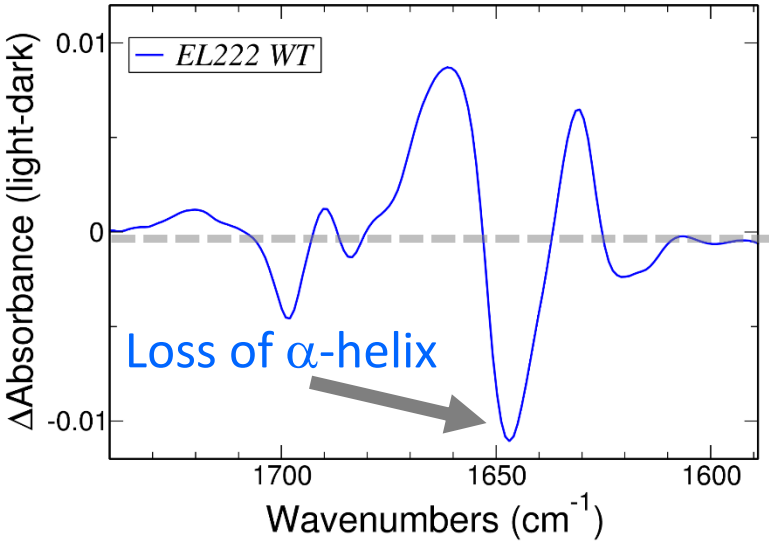
Light-induced infrared difference spectroscopy

?

EL222-lit

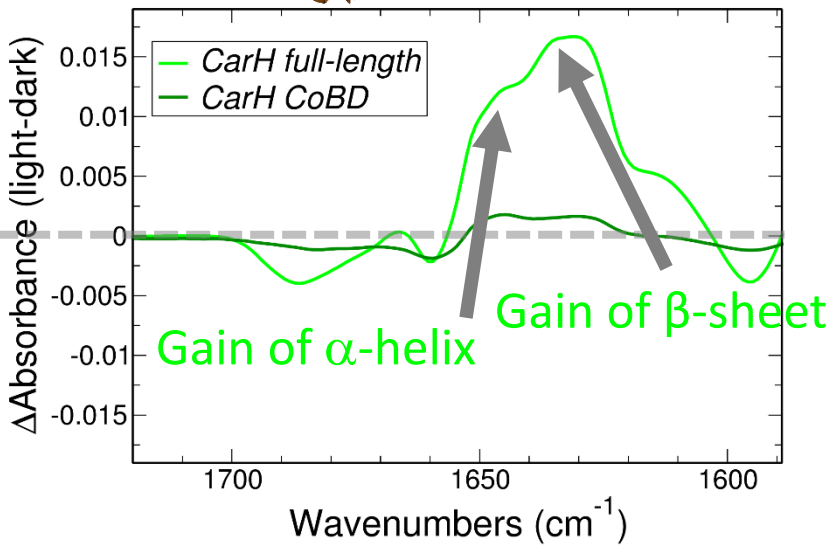
CarH-lit (5C8F)



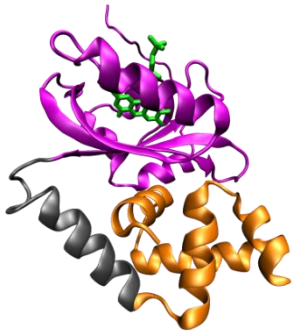


LIGHT

DARK

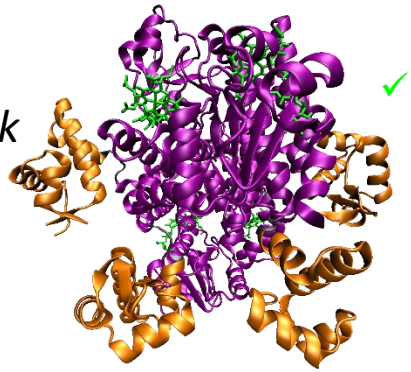


✓ The protein partially unfolds … but where?



EL222-dark (3P7N)

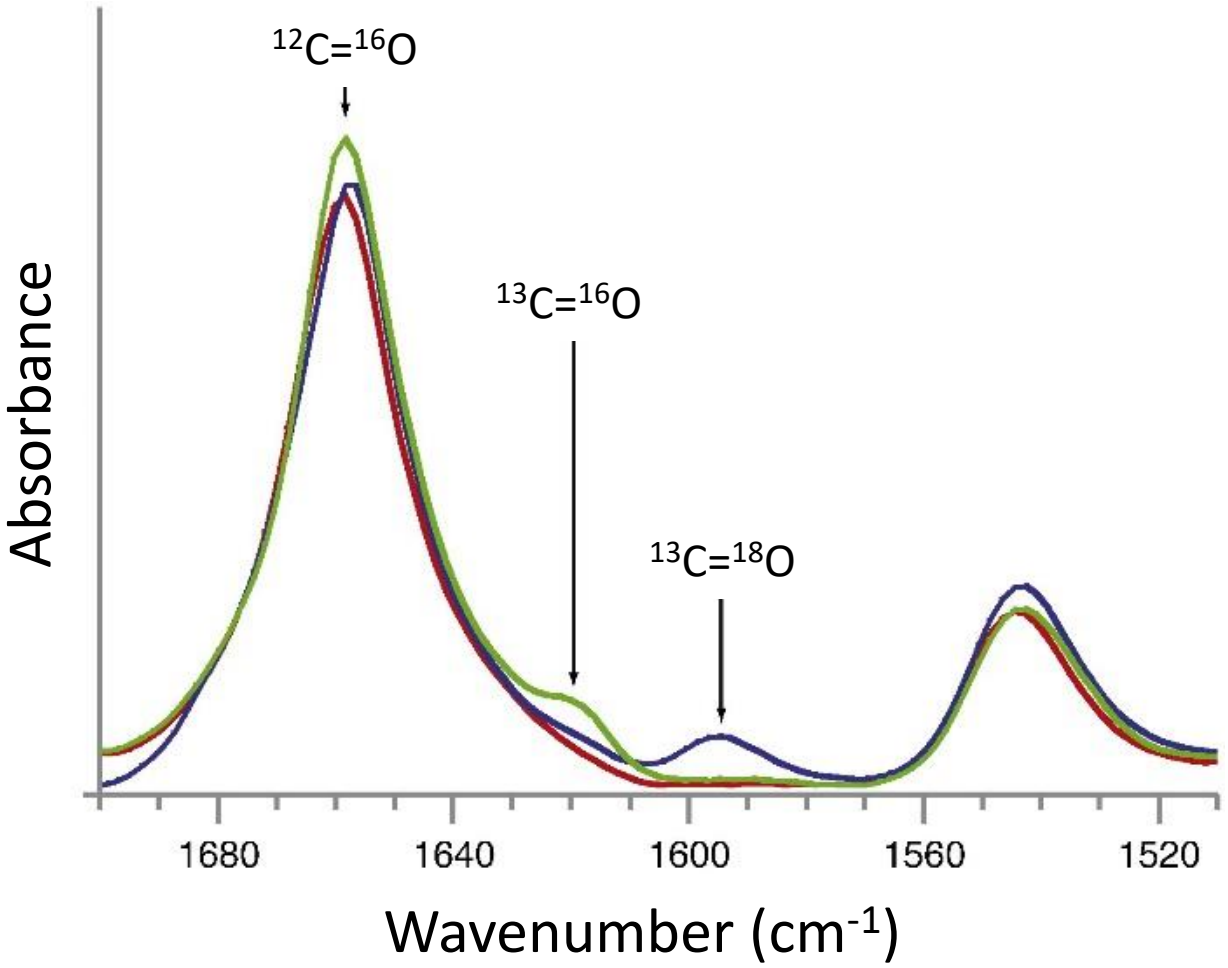
CarH-dark (5C8D)



✓ The DNA-binding domain folds as α-helix + β-sheet

Chaudhari A.S. Domingos, C.O. et al, *in preparation*
Nash, A.I. et al (2011) *PNAS*
Jost, M. et al (2015) *Nature*

Isotope-edited IR spectroscopy of proteins

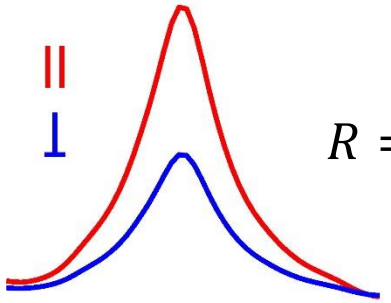


- *With stable isotope labeling, FTIR can be used to derive accurate information on single amino acids*

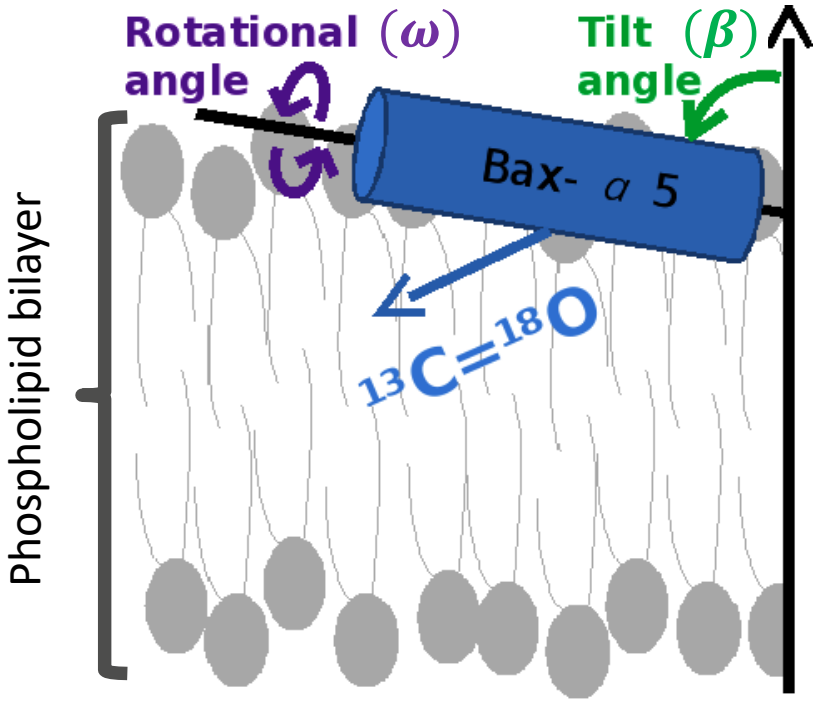
Manor J. & Arkin I.T.(2013) *BBA*

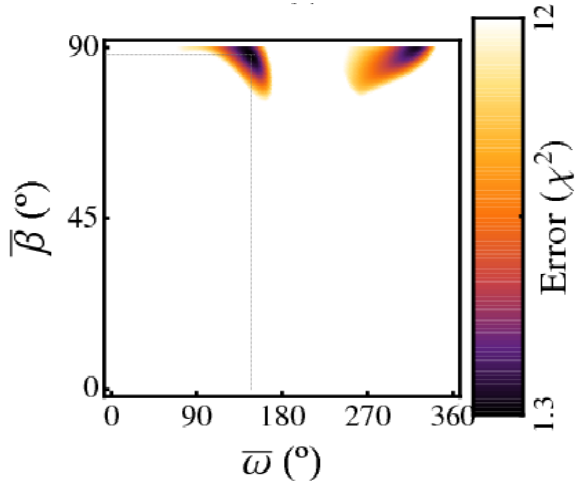
Structure & dynamics of peptide/lipid complexes

Orientation of Baxα5 in supported membranes by polarized **ATR-FTIR**



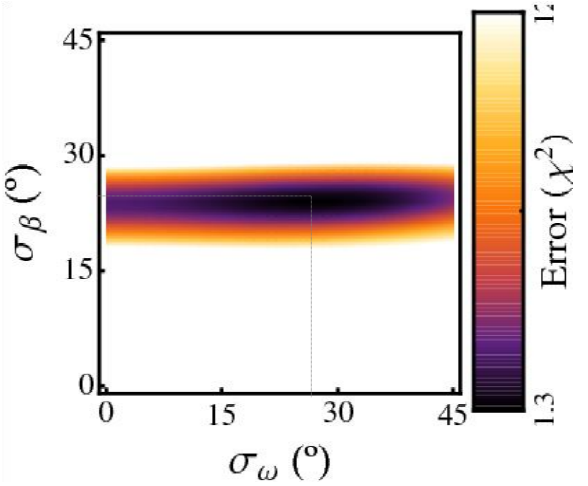
$$R = \frac{I_{\parallel}}{I_{\perp}} \propto (\beta, \omega)$$





Average values

Rigid body modeling

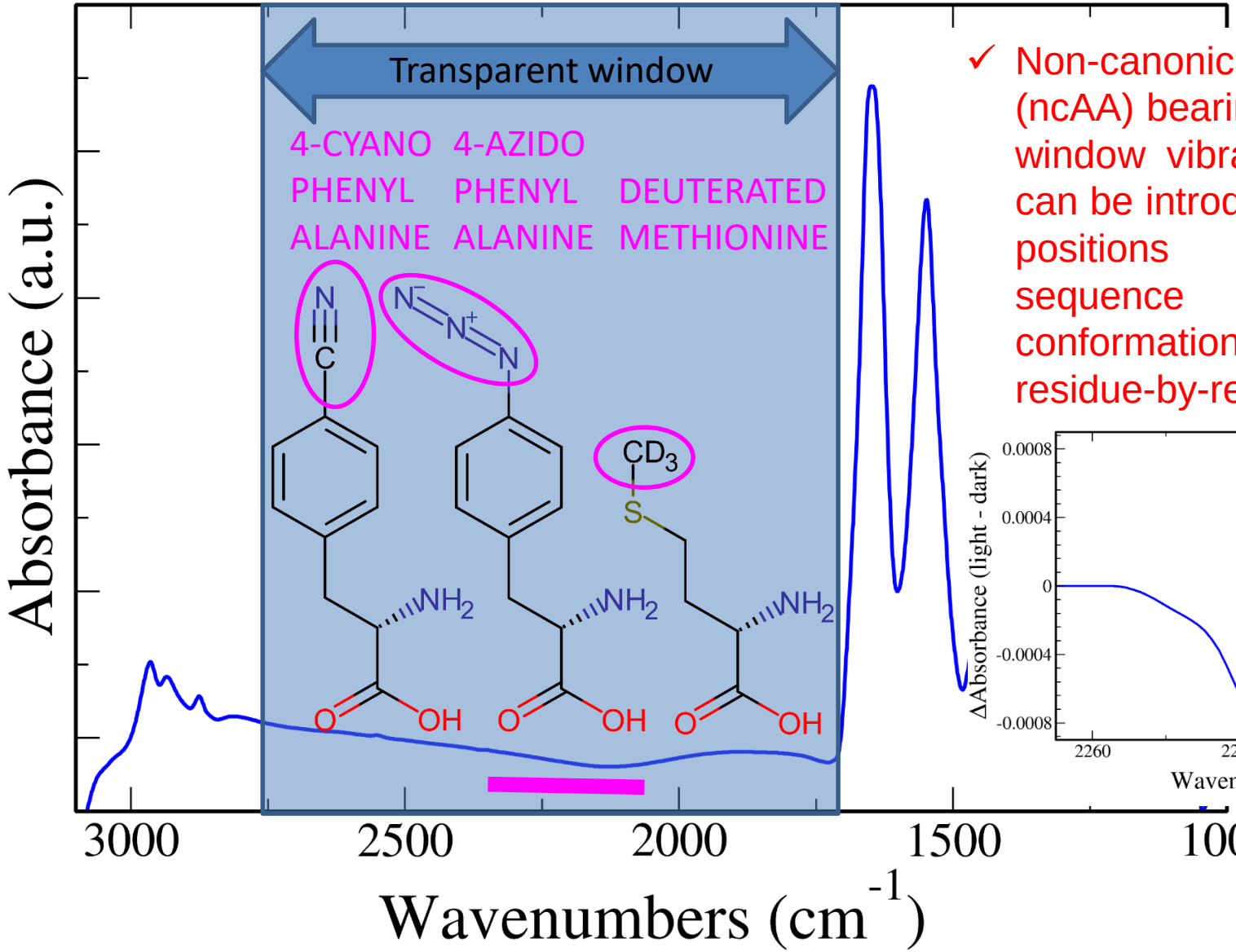


Gaussian Fluctuations

- *Baxα5 lays flat at the level of the membrane interface*

Fuertes G. et al (2009) *Biophys J*

“Transparent window” vibrational probes



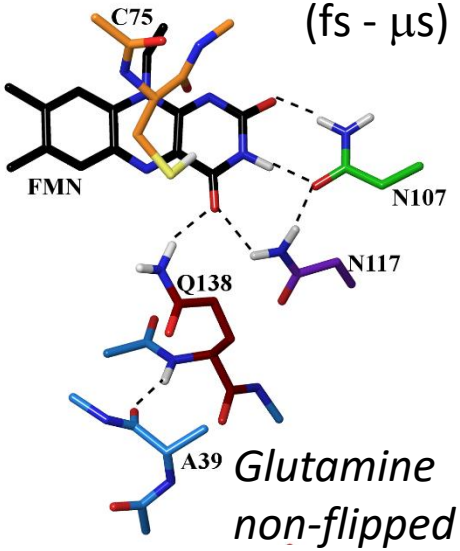
- ✓ Non-canonical amino acids (ncAA) bearing transparent window vibrational probes can be introduced at single positions within the sequence to monitor conformational changes residue-by-residue

Chaudhari A.S. et al (2021) *Biophys J*

Time-resolved IR spectroscopy of photoreceptors

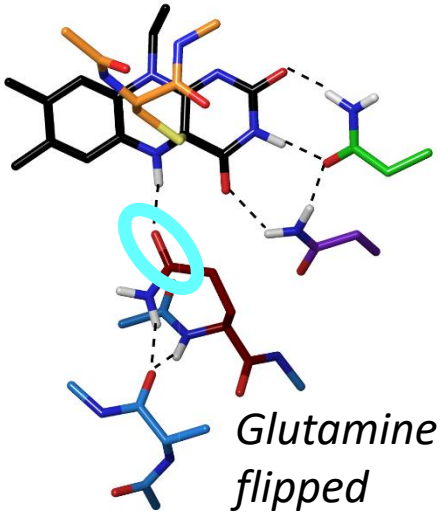
Ground state, Singlet, Triplet

(fs - μs)

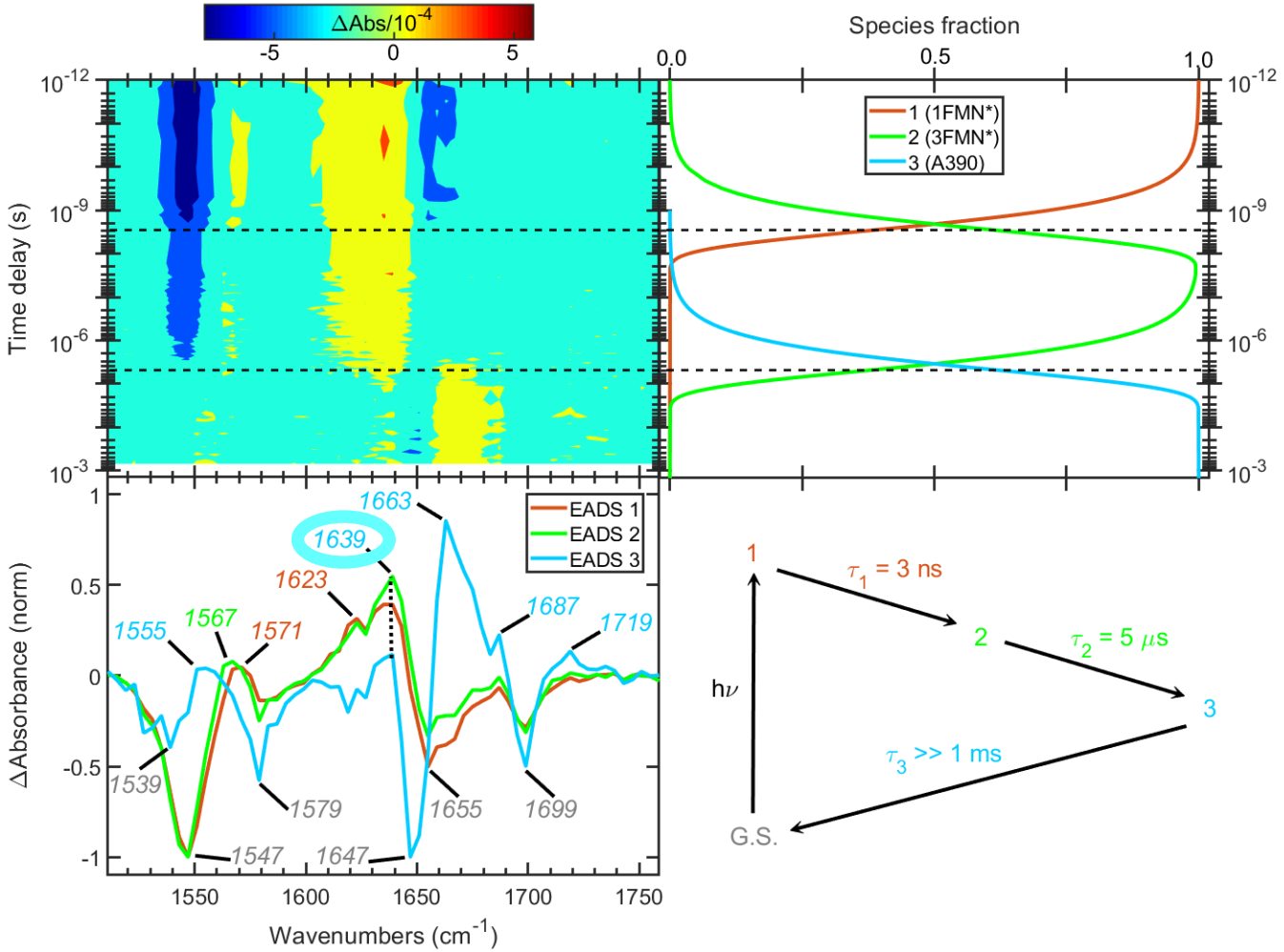


Glutamine non-flipped

Adduct (μs – s)



Glutamine flipped



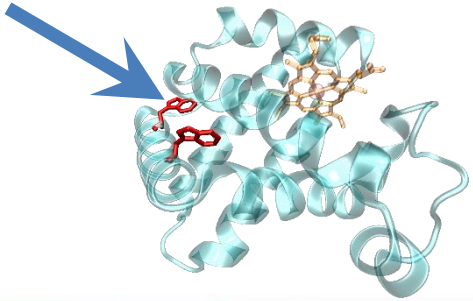
- Monitoring the early steps in signal transduction of LOV (light-oxygen-voltage) proteins with IR + quantum chemistry

Andrikopoulos P.C. et al (2021) *PCCP*

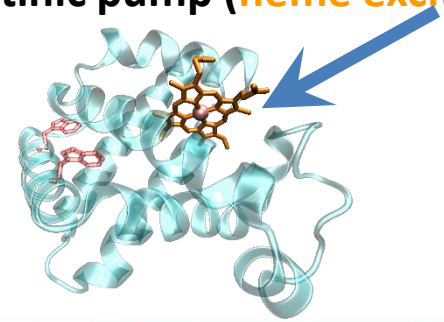
Time-resolved Raman spectroscopy

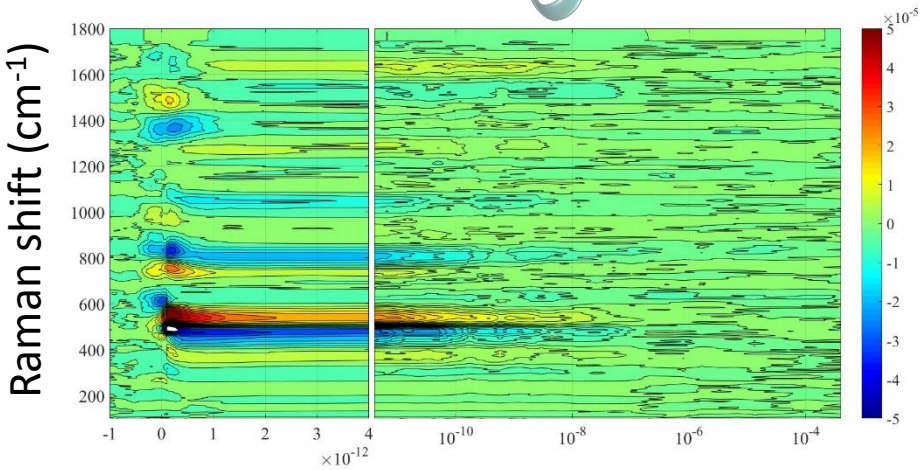
280 nm actinic pump (tryptophan excitation)

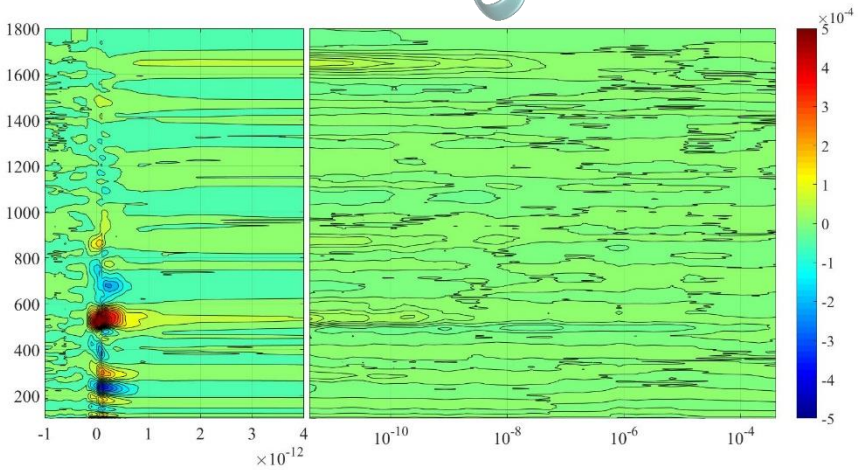
400 nm actinic pump (heme excitation)



Fe^{3+}-myoglobin (1A6K)







Time delay (s)

280 nm pump	400 nm pump	Assignment
	310 fs	Heme relaxation
	14 ps	Heme relaxation
3 ps		W15 fluorescence lifetime
170 ps		W8 fluorescence lifetime
64 ns	7 ns	Photo-reduction (Fe^{2+})
180 µs	30 µs	Protein quake

- *Mapping energy flow in myoglobin with femtosecond-stimulated Raman spectroscopy (FSRS) from fs to ms*

Liu Y. et al, *in preparation*

Conclusions

Spectroscopy	Pros	Cons	Ideal biomolecules
UV/VIS & CD	Medium sensitivity (µM), **fs time resolution**	Low information content	All in far-UV, need of a chromophore otherwise
FLUORESCENCE	High sensitivity (pM, nM), in vivo applications, ps time resolution	Potential perturbation by the dyes	All, particularly intrinsically disordered proteins
INFRARED	Global & local information, **fs time resolution**	Low sensitivity (mM), water is a nuisance	All, particularly membrane proteins
RAMAN	Excellent for cofactors, **fs time resolution**	Low sensitivity (mM)	Best for cofactor-containing proteins

+ high-resolution methods (MX, EM, NMR)
+ protein engineering tools (GCE)
+ computer simulations (QM, MD)

Acknowledgments

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Thank you for your attention

Questions