FRET ~~and FLIM~~ Applications: Single Pair FRET

Don C. Lamb

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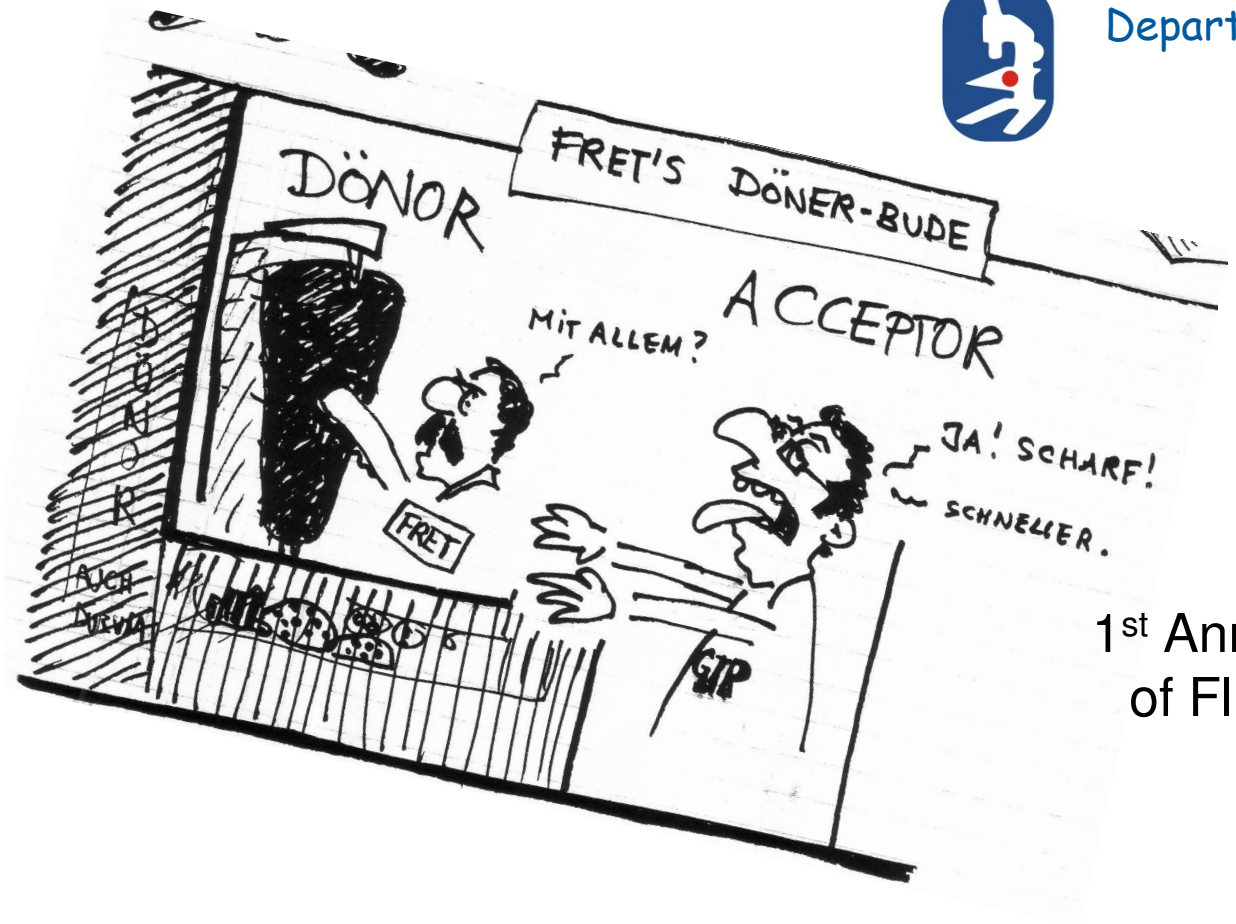
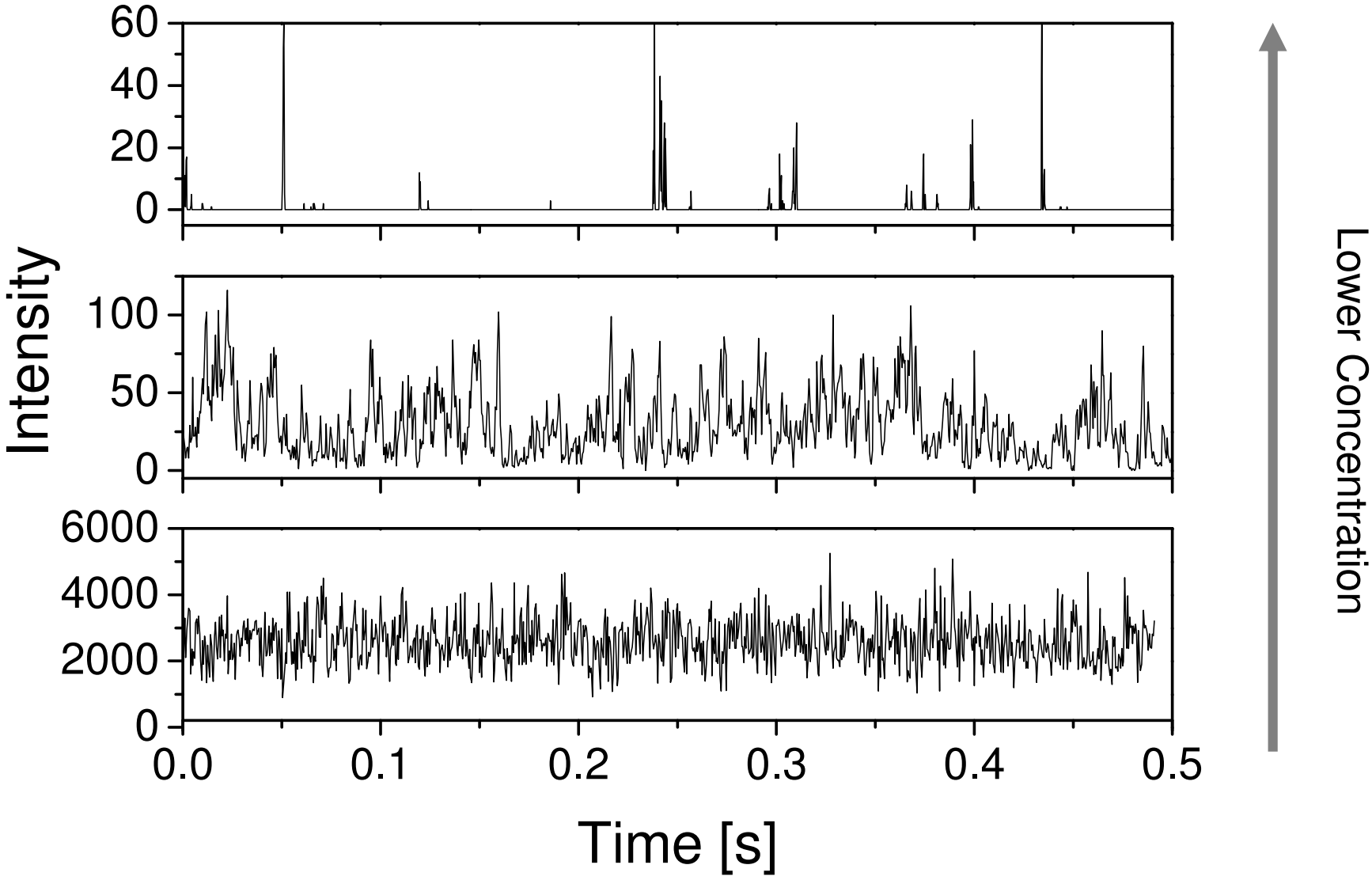


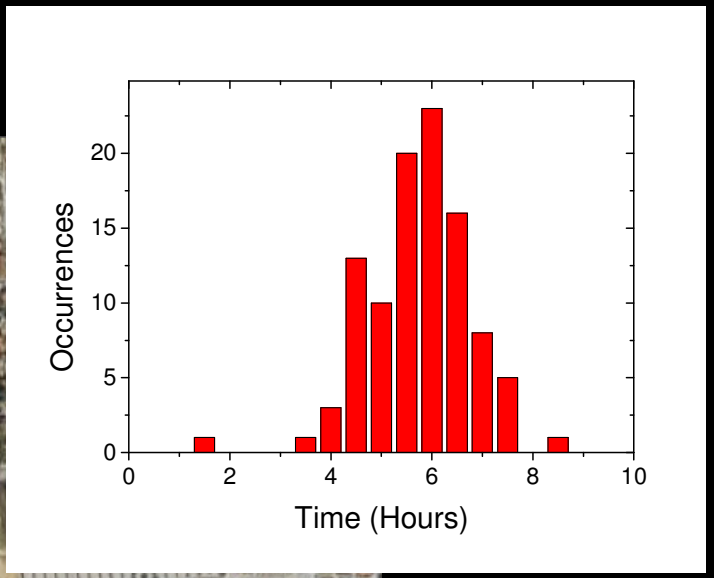
1st Annual Course on Principles of Fluorescence Techniques
Madrid, Spain

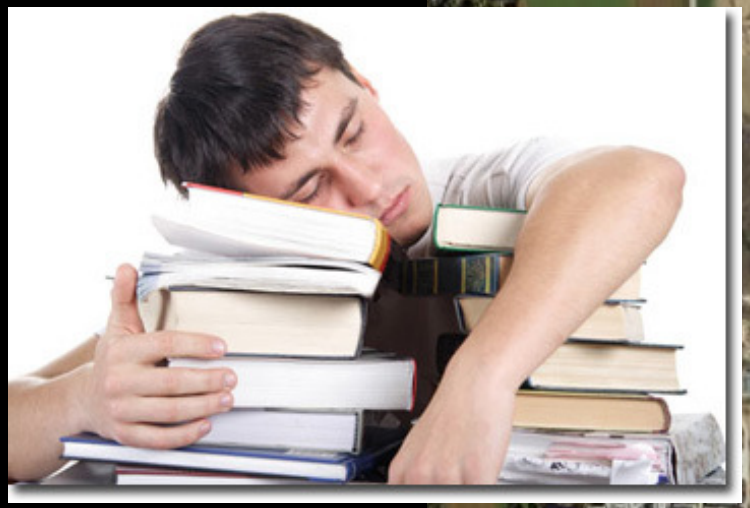
Single Molecule Measurements

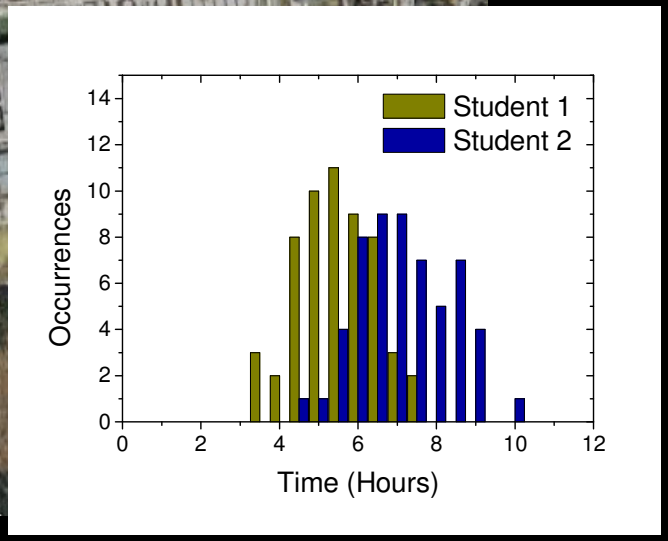


Ensemble versus Single Molecule

1. Detect individual events
2. Resolve rare events
3. Measure dynamics of non-synchonized events
4. Detect subpopulations
5. Determine Statistics (Distributions)



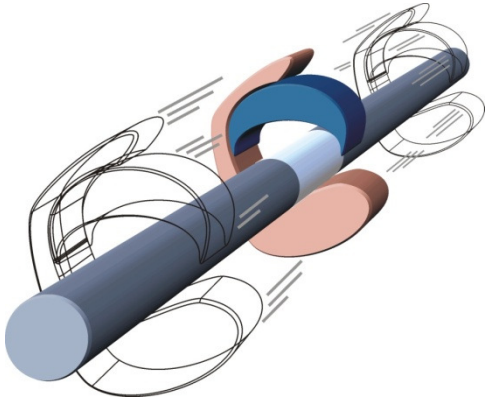




Overview

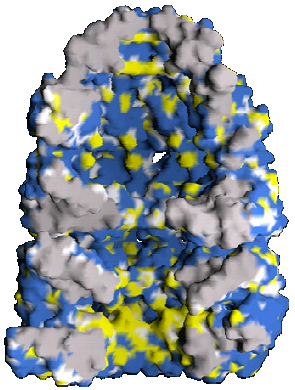
Dynamics of TBP-NC2

A Mechanistic Model for Gene Regulation



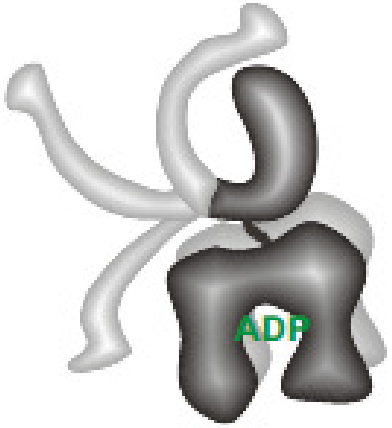
Chaperon Assisted Protein Folding

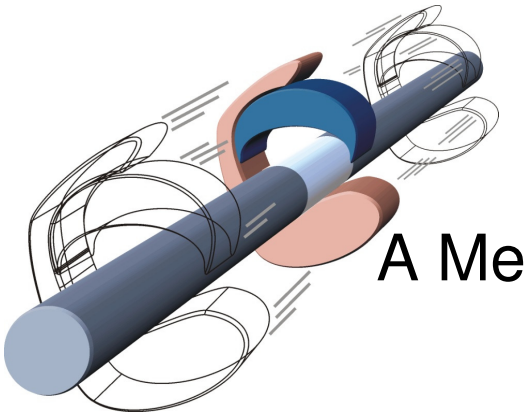
GroEL is a Strict Chaperon



Hsp70

Probing the Conformation of Chaperons





Protein Dynamics *in vitro*:

A Mechanistic Model for Gene Regulation

Christine Göbel

Dr. Peter Schlüsche

Prof. Michael Meisterernst

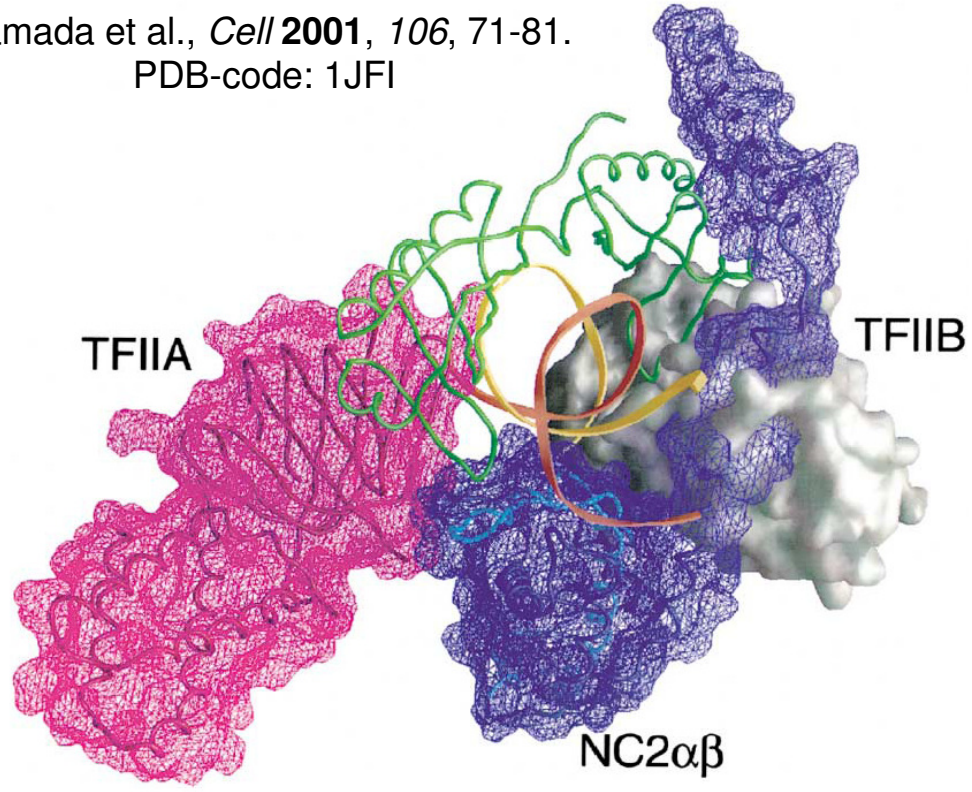
Gertraud Stelzer

Transcription regulation

In vitro: DNA-Transcription-Inhibition caused by Negative Cofactor 2 (NC2)

NC2 forms a complex with the DNA-bound TBP

Kamada et al., *Cell* **2001**, *106*, 71-81.
PDB-code: 1JFI



- When NC2 is bound, DNA transcription cannot occur

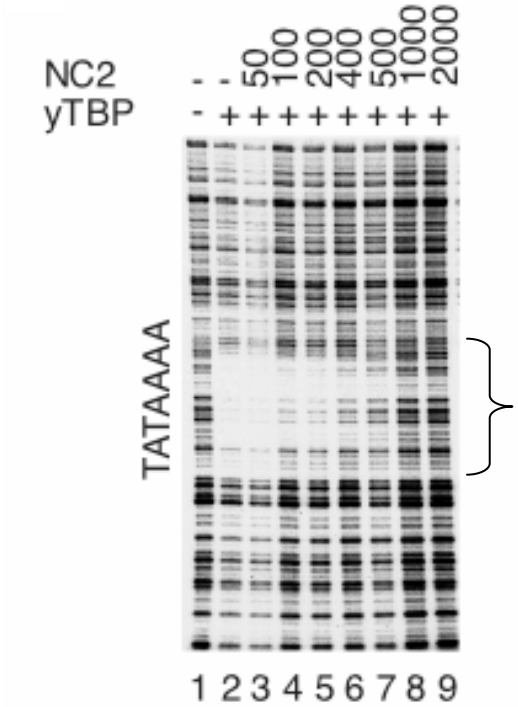
What is the **mechanism** of gene inhibition?

⇩

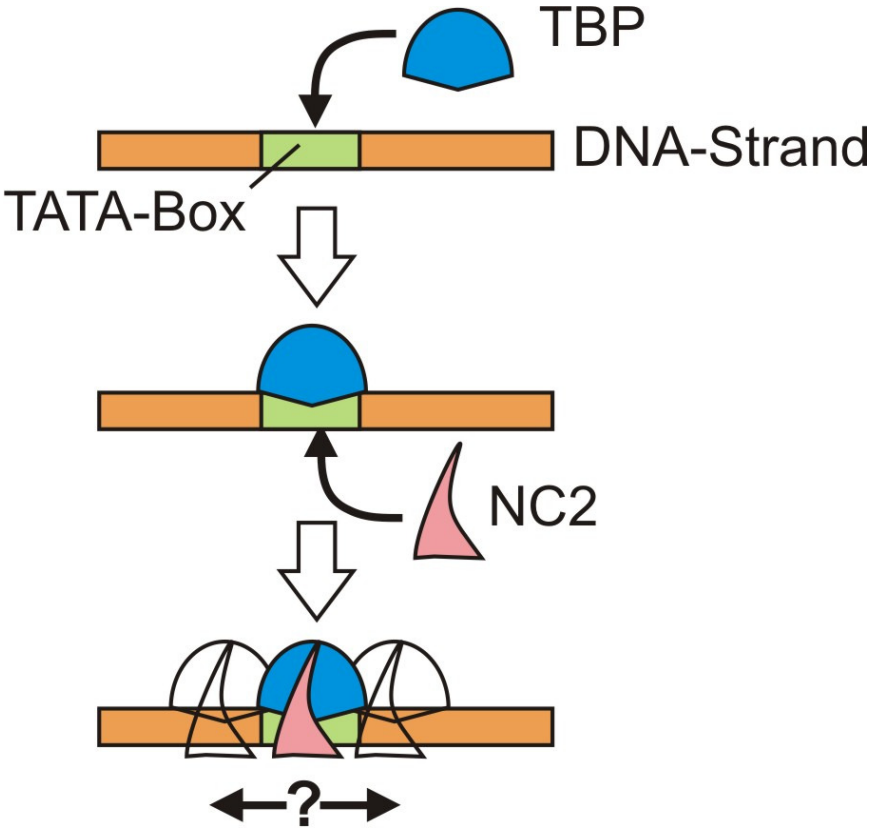
Current belief: Inhibition by NC2 is only due to sterical hindrance

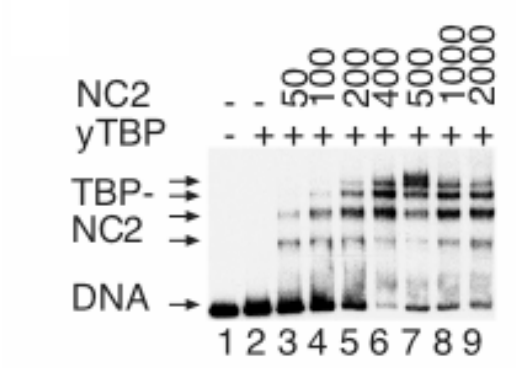
Footprinting

Footprintings of NC2 addition



Deprotection of the TATA-Box by NC2 binding onto the TBP-DNA complex





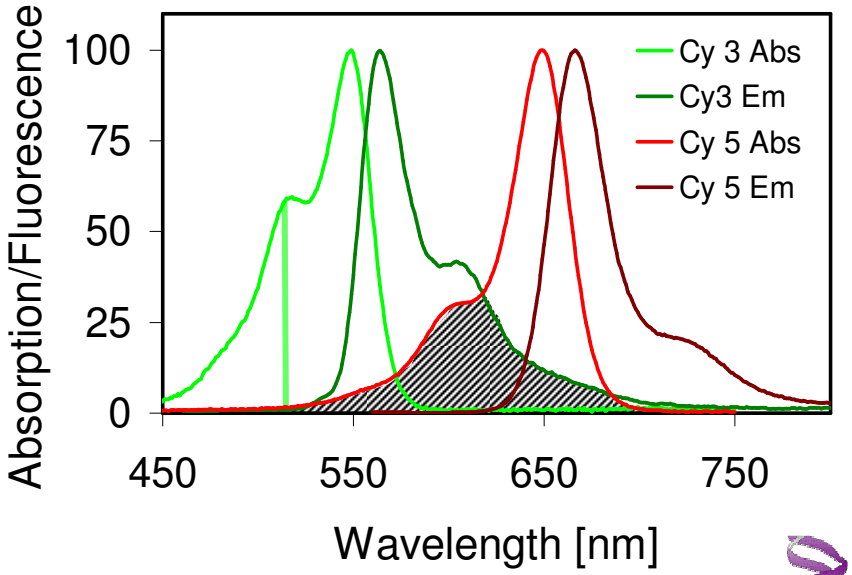
New Hypothesis:

TBP/NC2 complex is mobile along the DNA.

Förster Resonance Energy Transfer

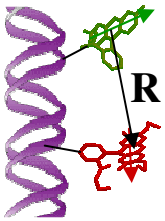
$$k_T = \frac{9000(\ln 10)\,\phi_D\,\kappa^2 J}{128\pi^5 N_A n^4\,\tau_D\,R^6}$$

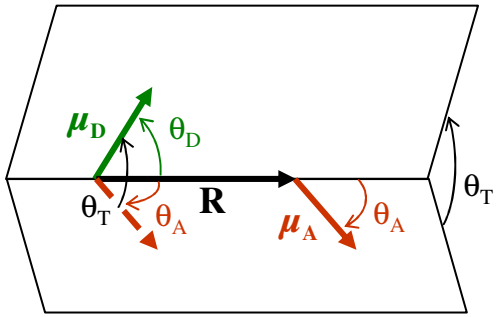
- spectral properties of D and A



- relative orientation of D and A

$$\kappa^2 = (\cos\theta_T - 3\cos\theta_D \cos\theta_A)^2$$

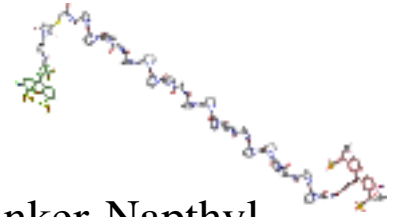




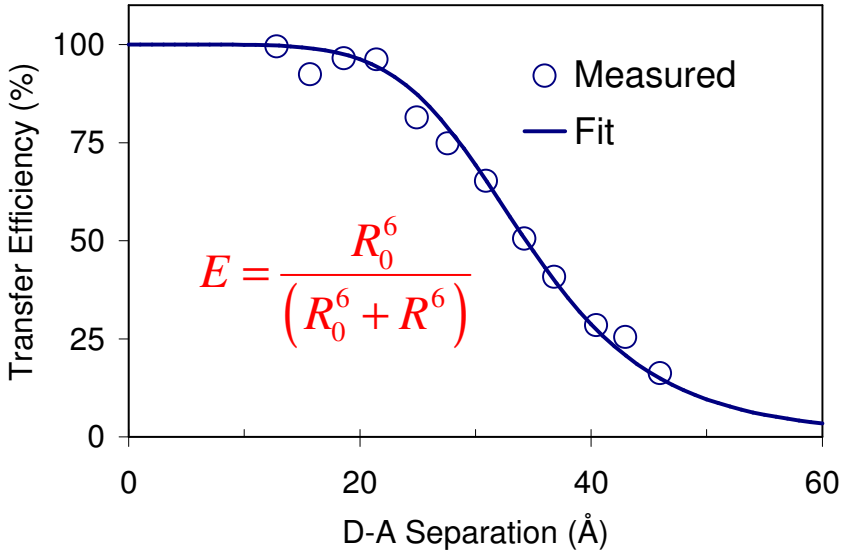
For flexible dyes averaged over all orientations

$\kappa^2 = 2/3$

- D-A separation



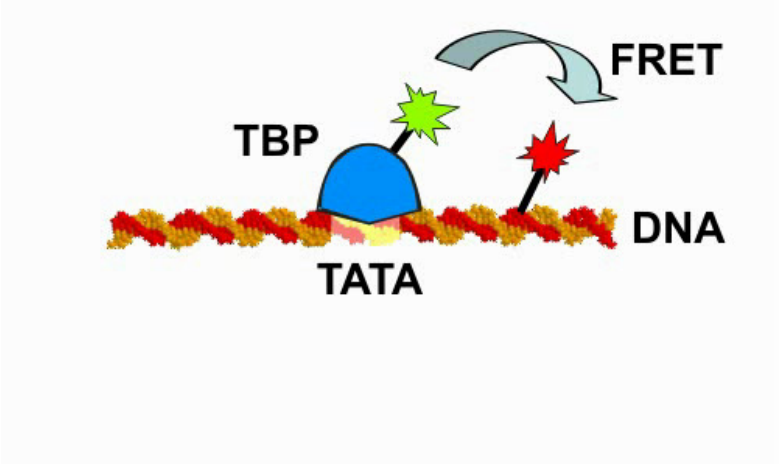
Dansyl-(Pro)$_n$-Linker-Napthyl

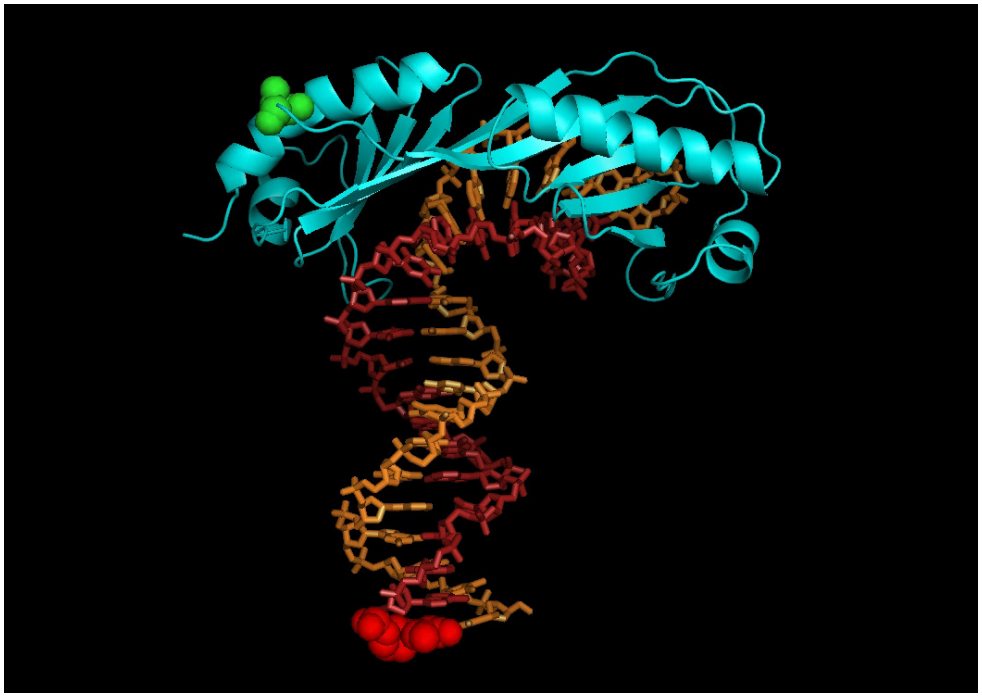


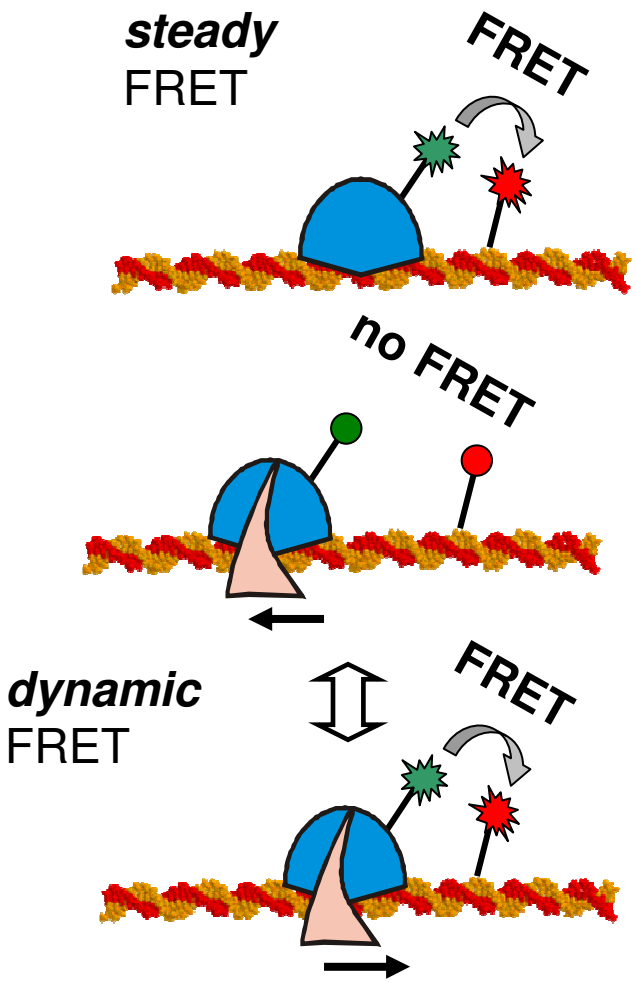
Data taken from: Stryer and Haugland (1967) *PNAS* **98**:719

The Experiment

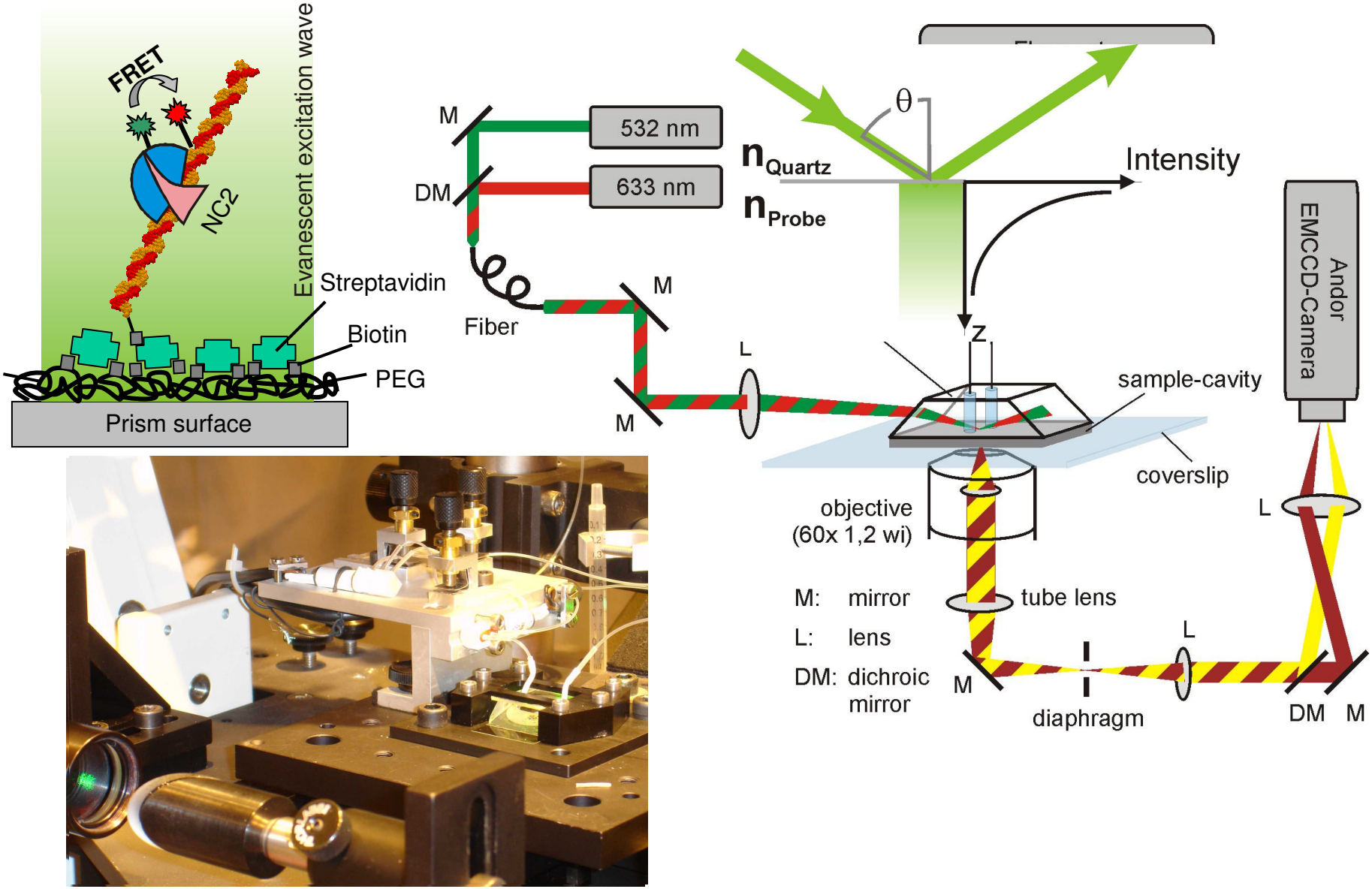
Visualize movement using in vitro FRET measurements on **single molecules**







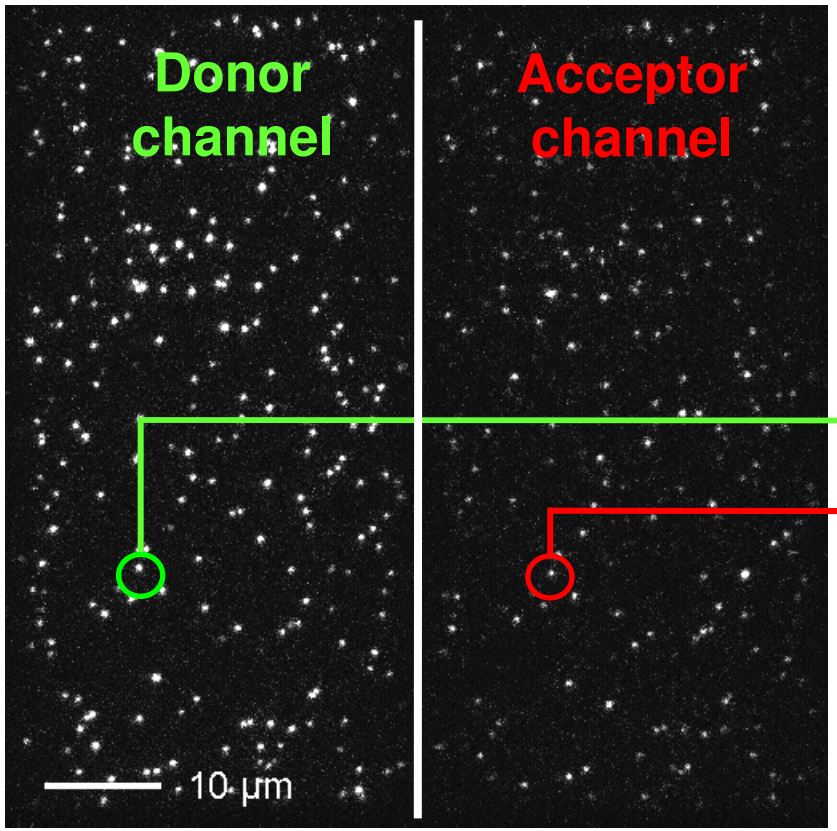
Prism-type TIRFM setup



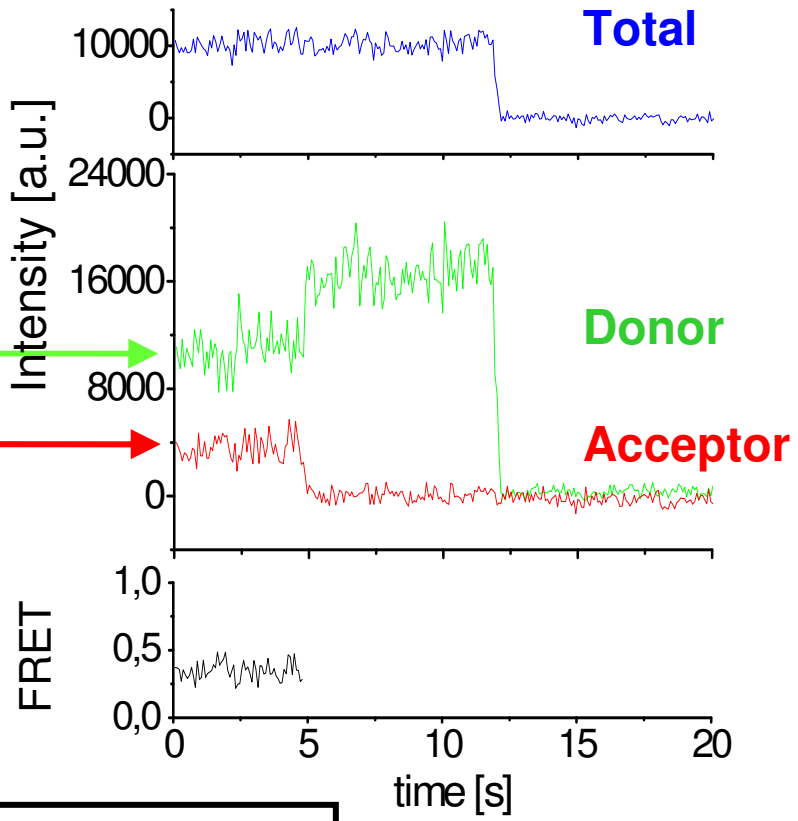
The Measurement

Raw data: 70 x 35 µm² per channel

(original movie speed)

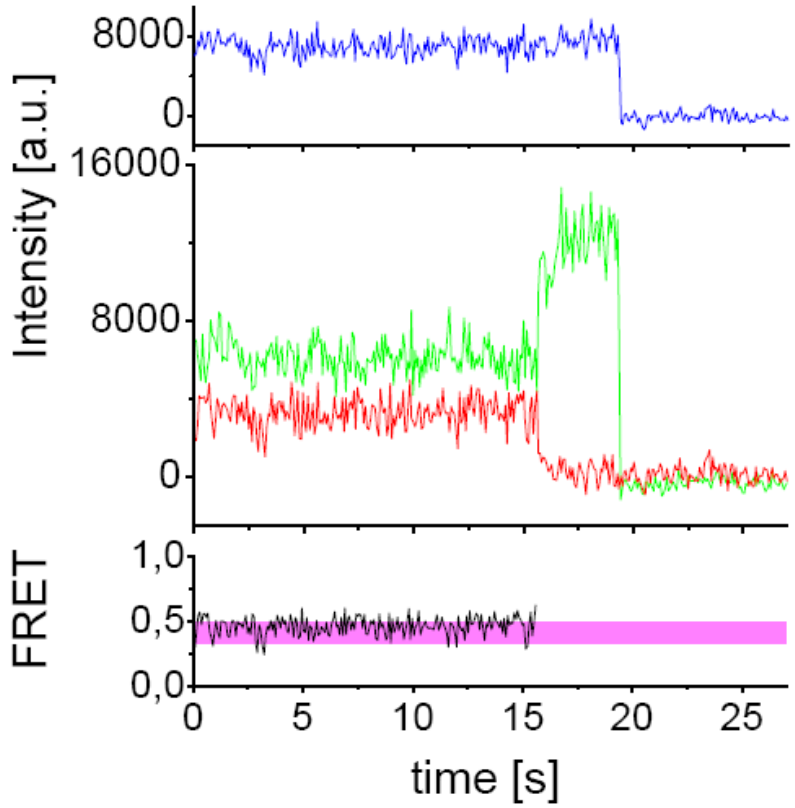


75 ms/frame



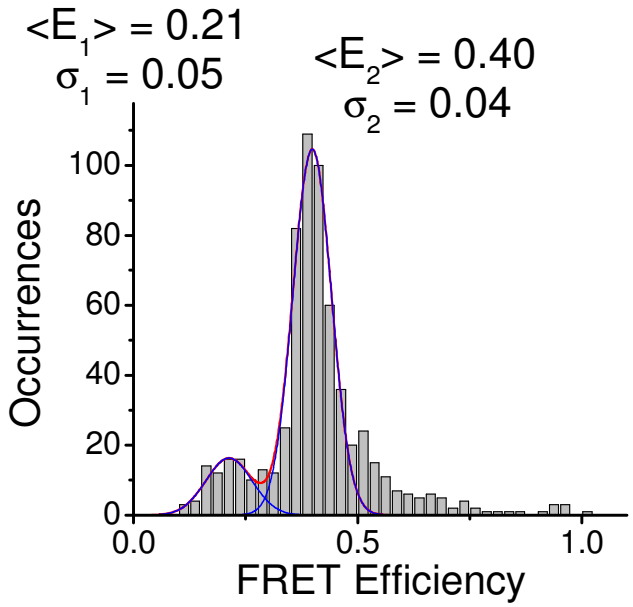
$$E_{FRET} = \frac{I_A}{I_A + \gamma I_D}$$

Steady FRET before NC2 addition



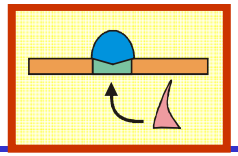
- **> 90 %** of all molecules show steady FRET

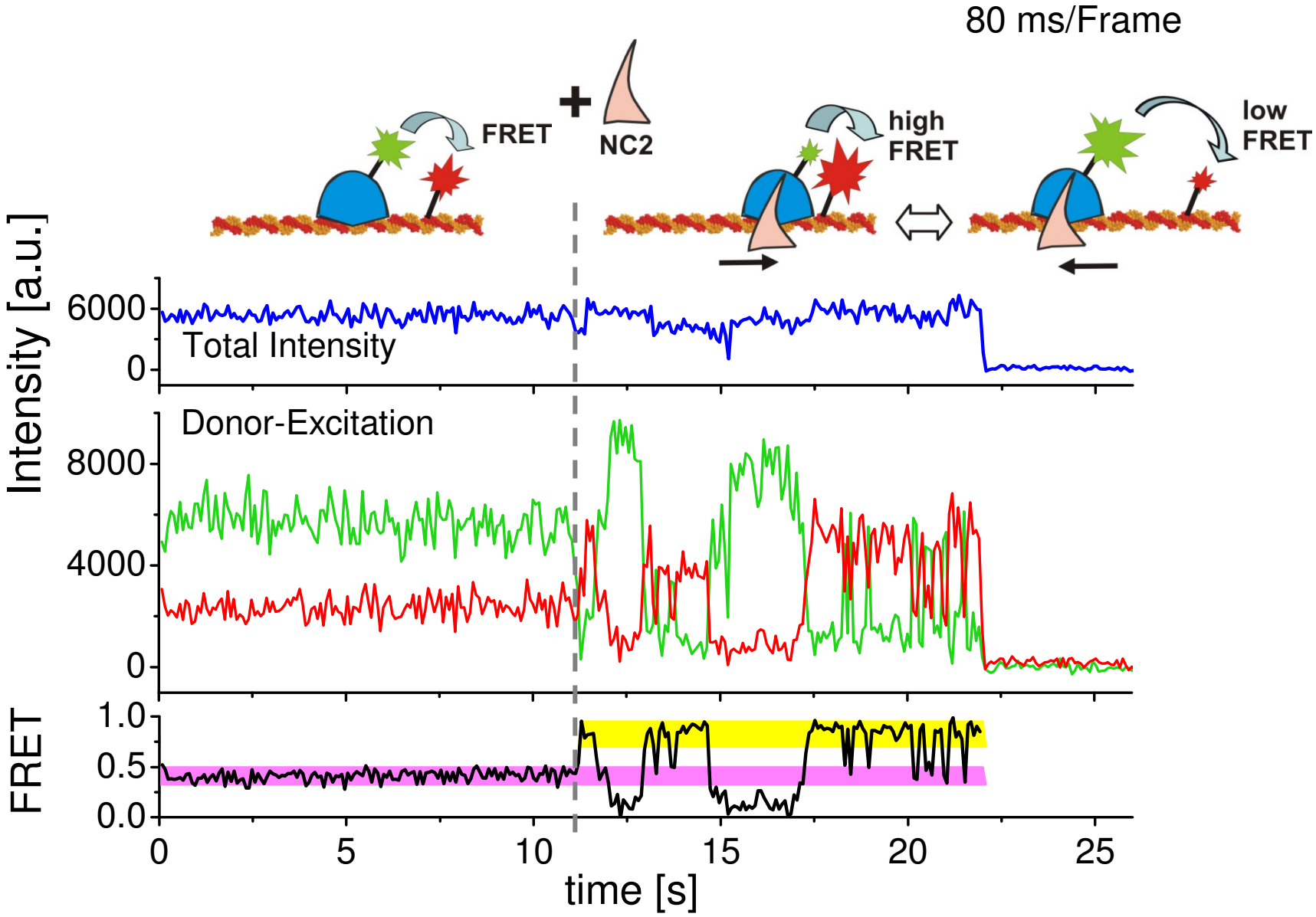
N = 631



- Final binding positions / conformation of TBP on DNA.

Addition of NC2



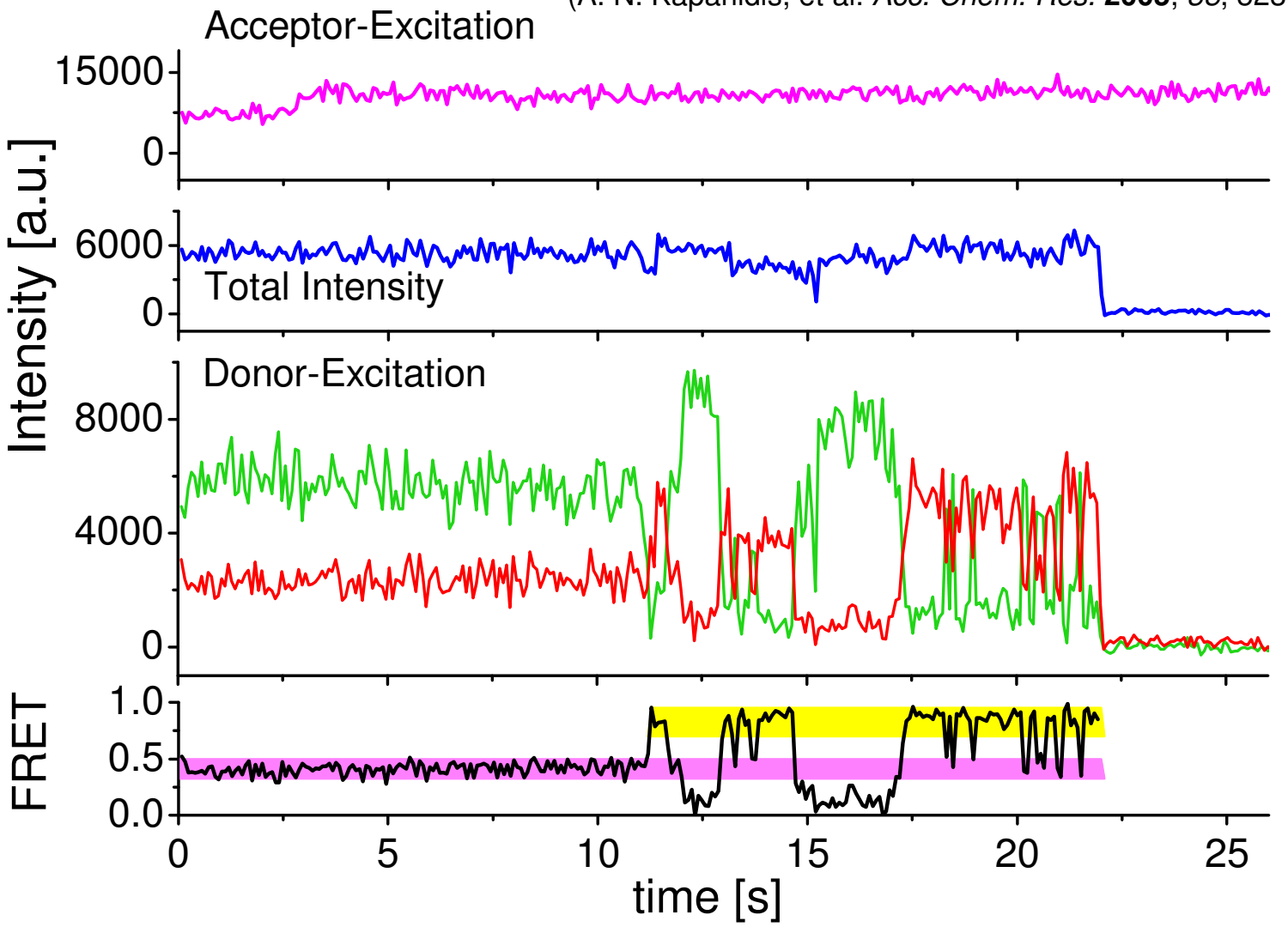


Addition of NC2

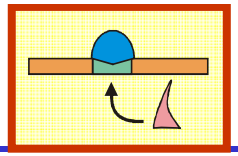
80 ms/Frame

msAlternating**L**aser**EX**citation

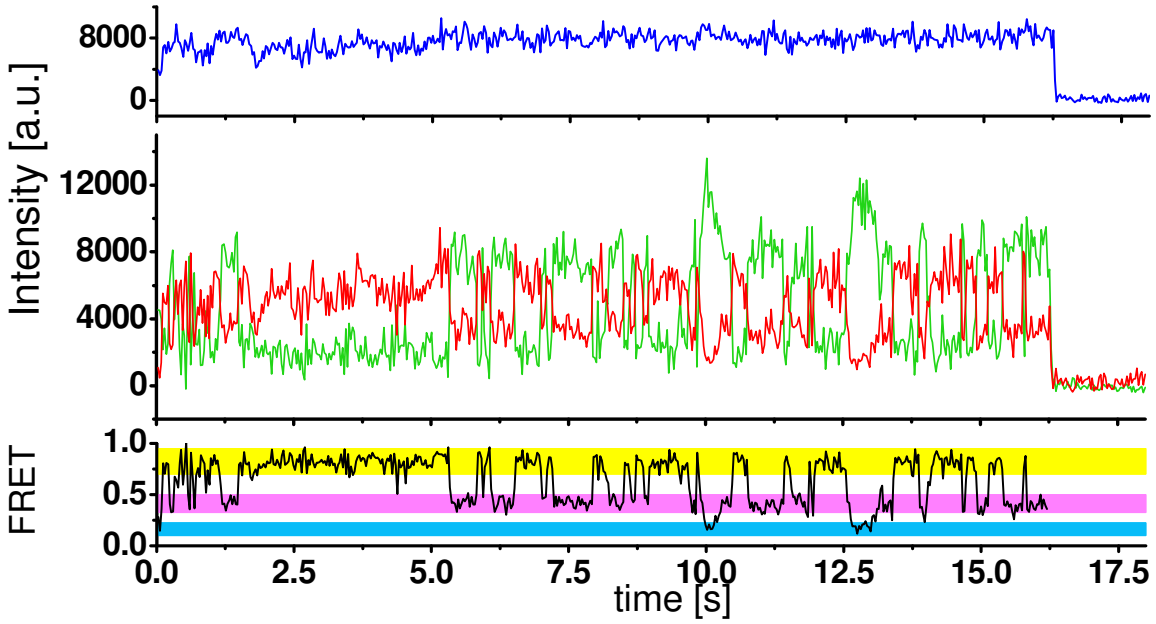
(A. N. Kapanidis, et al. *Acc. Chem. Res.* **2005**, *38*, 523-533.)

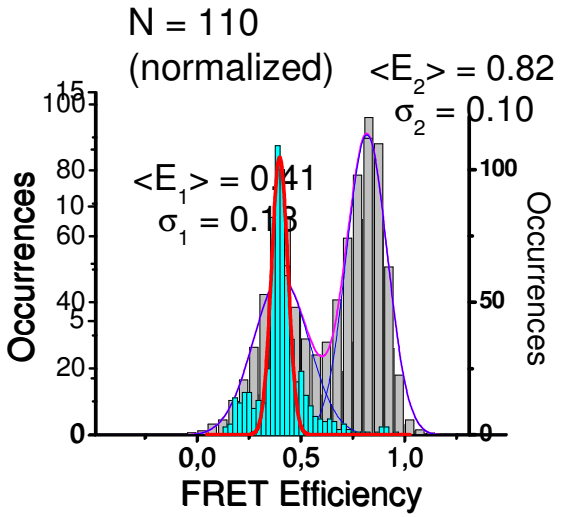


Dynamics at 30 ms/Frame

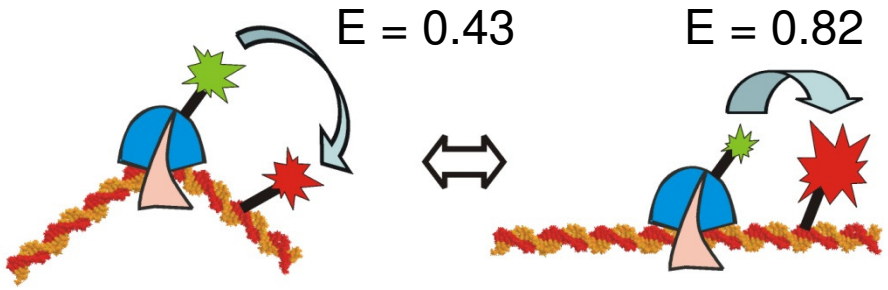


Schluesche et al 2007 *NSMB* ***14:****1196*

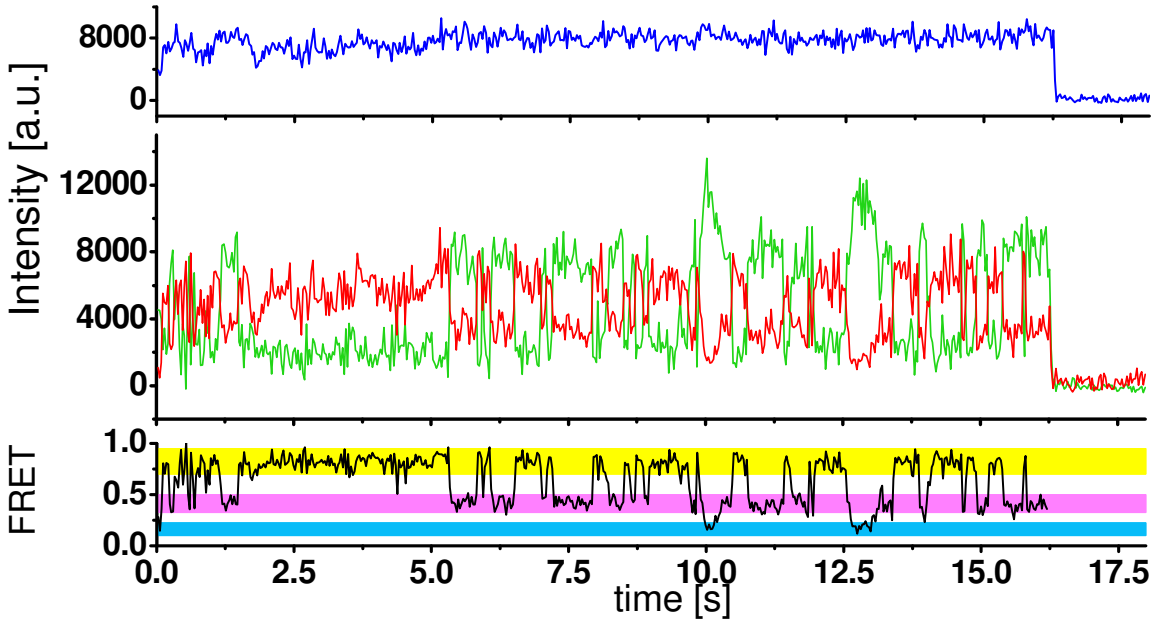




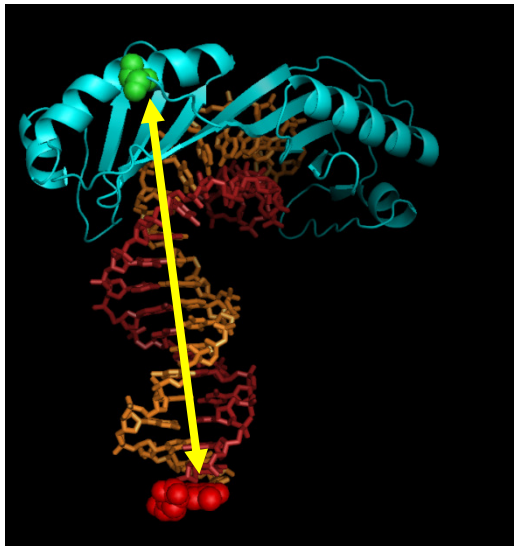
- The first conformation (E = 0.41) is the **initial steady state**
- 2nd conformation is DNA-unbending



Dynamics at 30 ms/Frame



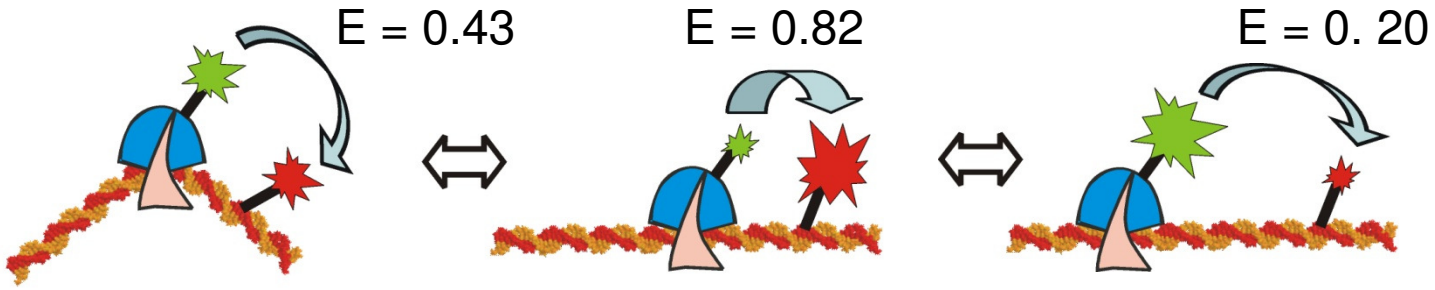
Schluesche et al 2007 *NSMB* ***14:****1196*



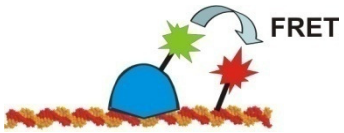
modified PDB-File 1RM1

- 3rd conformation is movement of TBP along the DNA

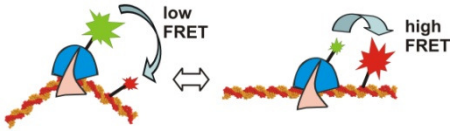
FRET-States << 0.40 cannot be described by conformational changes of DNA



Summary I

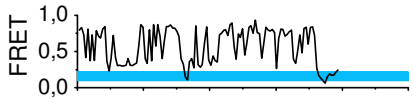


TBP-DNA complexes exhibit constant FRET-traces before the addition of NC2.

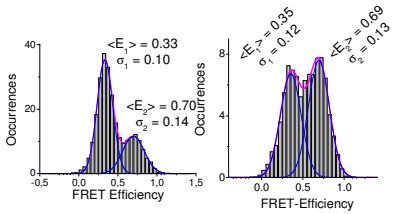


Upon binding of NC2, the FRET-traces converts to a dynamic behavior

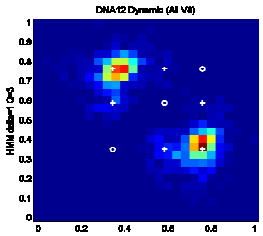
Discrete steps between two dominant populations are observed. The populations correspond to DNA in the bent and stretched conformation



A low FRET population is also observed which can only be explained by movement of TBP-NC2 along the DNA complex.



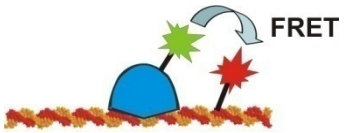
Dynamic properties vary with promoter

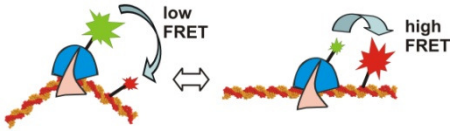


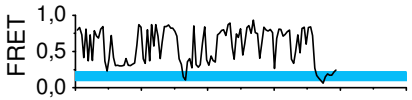
The kinetic information can be extracted using a Hidden Markov Model

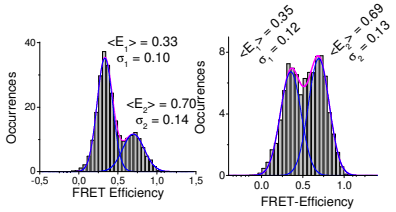
Schluesche et al 2007 *NSMB* **14**:1196

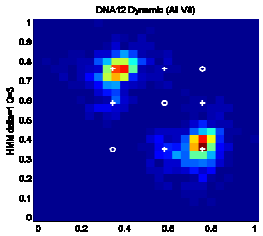
Summary I

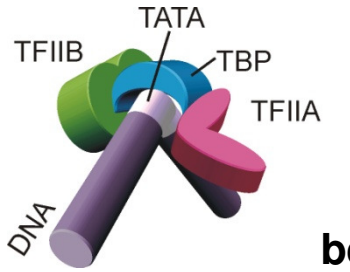




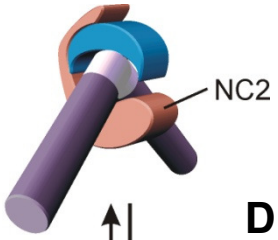








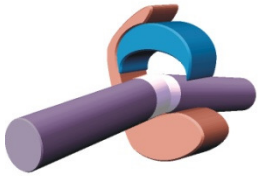
NC2-bending



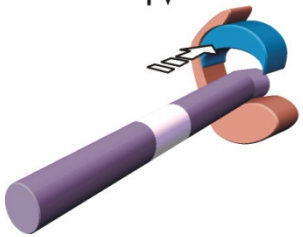
Transcription repression through NC2

DNA-unbending

Active PIC



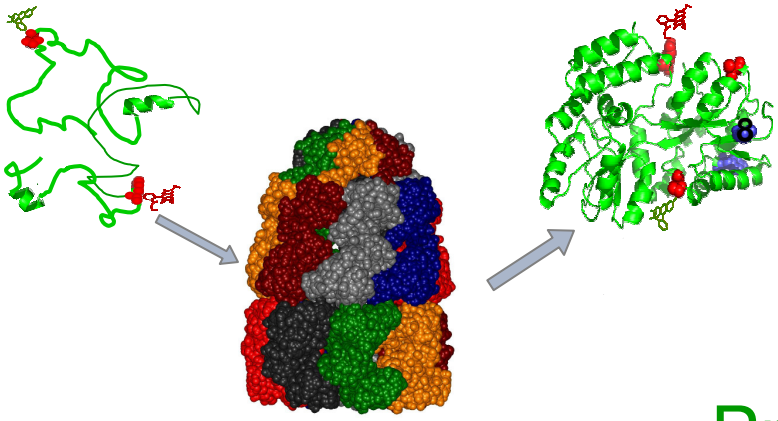
relocalization



Alternative Initiation positions

Model of TBP-NC2-Dynamics

Schluesche et al 2007 *NSMB* **14**:1196



MAX-PLANCK-GESELLSCHAFT

Prof. F. Urlich Hartl
Manajit K. Hayer-Hartl

Protein Folding:

The Role of Chaperonins in Protein Folding

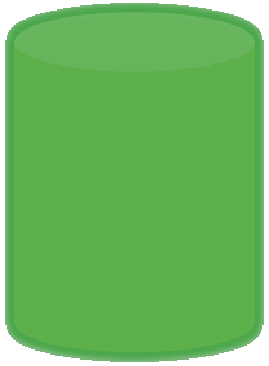
LMU Physical Chemistry

Dr. Barbara K. Müller

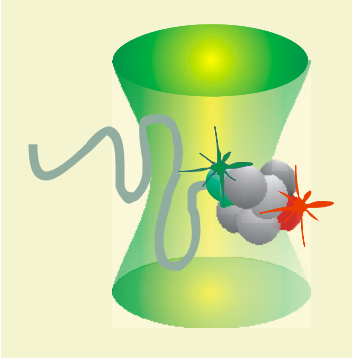
Dr. Shruti Sharma

Dr. Kausik Chakraborty

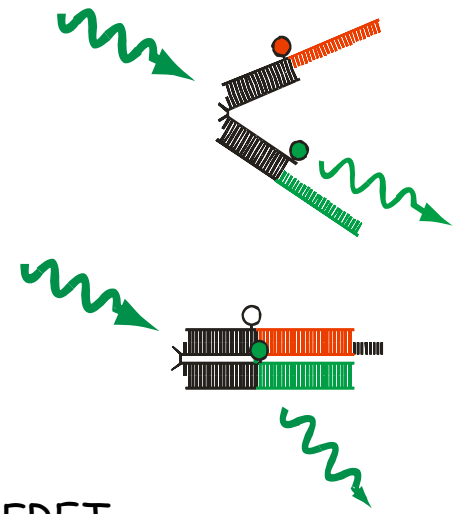
Burst Analysis

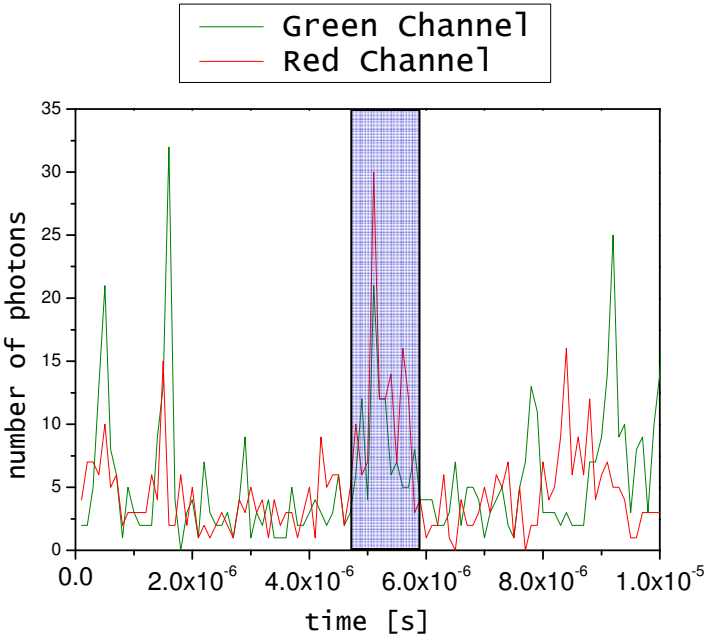


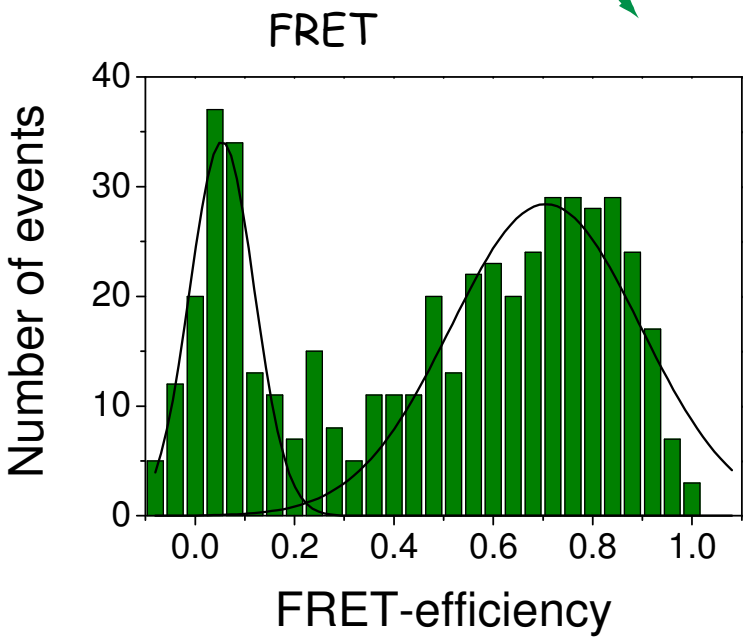
Burst Analysis



$$E = \frac{I_A}{\gamma I_D + I_A}$$

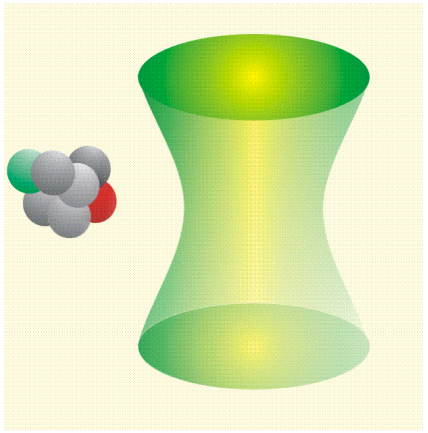


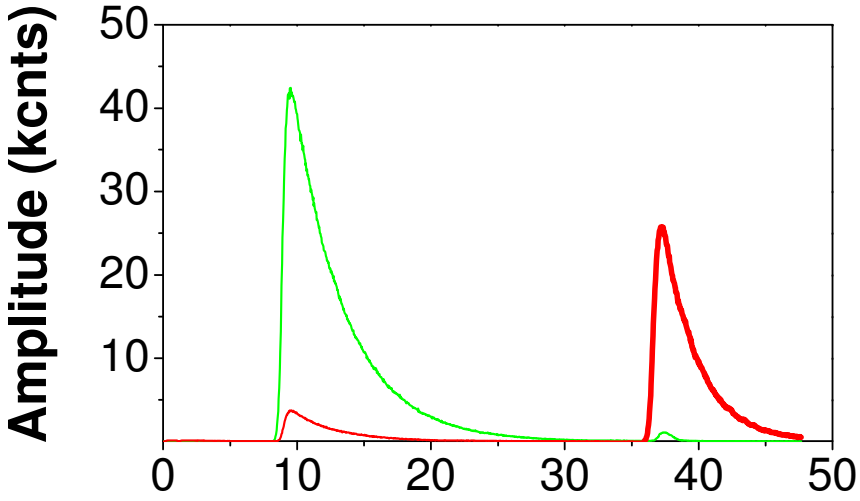


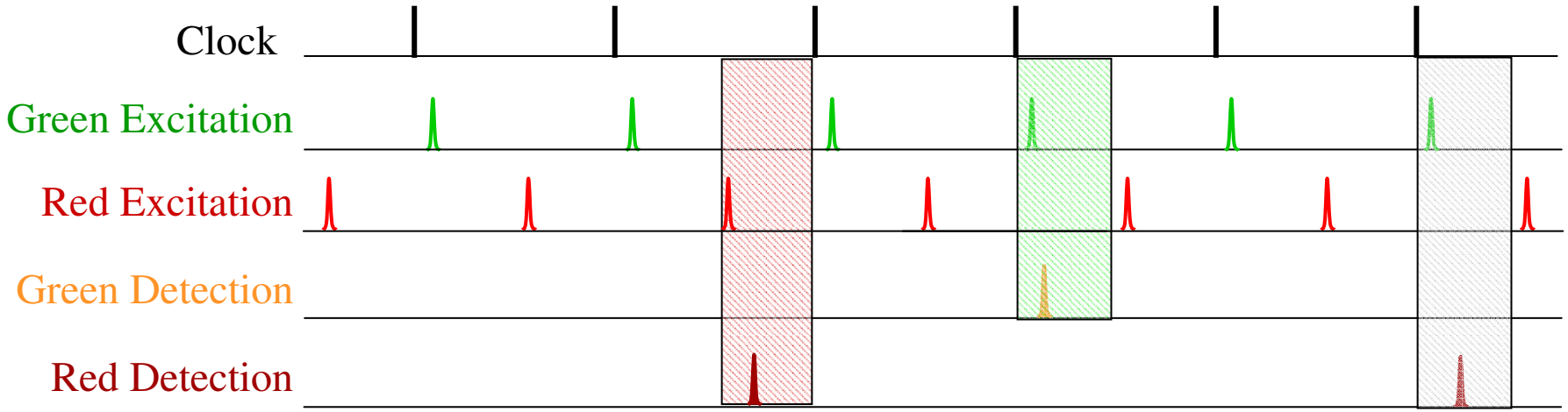


PIE: Pulsed Interleaved Excitation

Additional Information: Excitation Source of Each Photon







Application PIE to spFRET Measurements

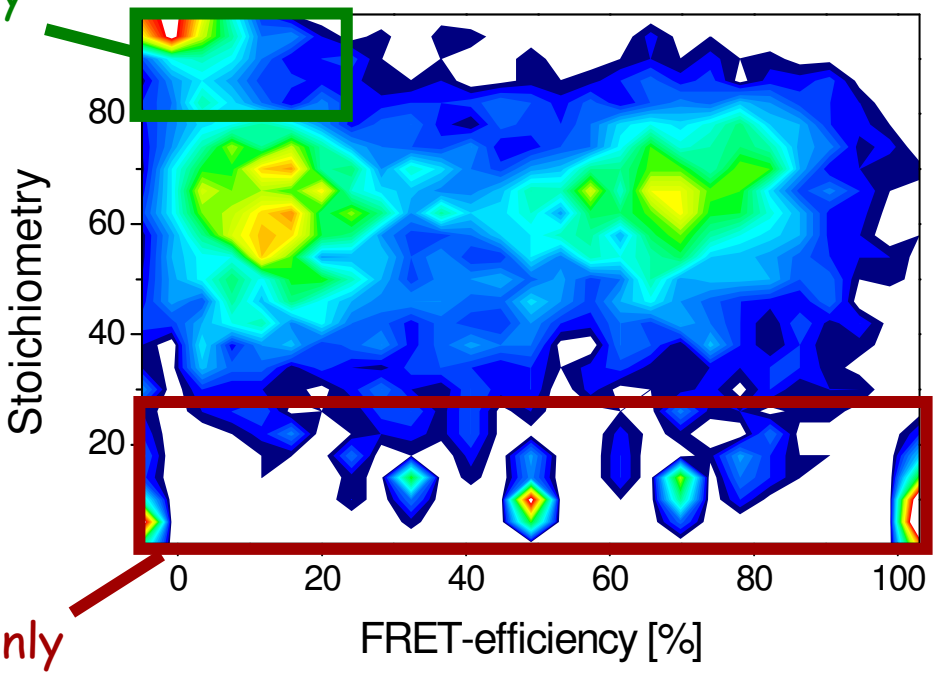
PIE in spFRET can be used to:

- Determine the stoichiometry of donor and acceptor labeled complexes
- Lifetime and intensity information can be used for determining FRET efficiency
  - Changes in fluorescence intensity of the dye can be monitored

Stoichiometry Factor

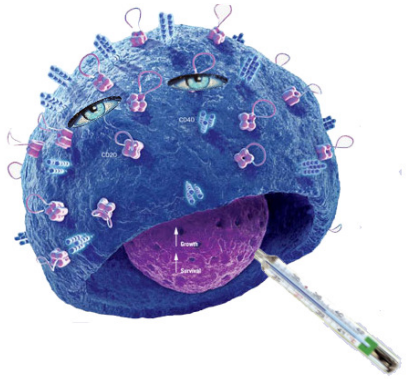
$$S = \frac{I_{GG} + I_{RG}}{I_{GG} + I_{RG} + I_{RR}}$$

$$E = \frac{I_A}{\gamma I_D + I_A}$$



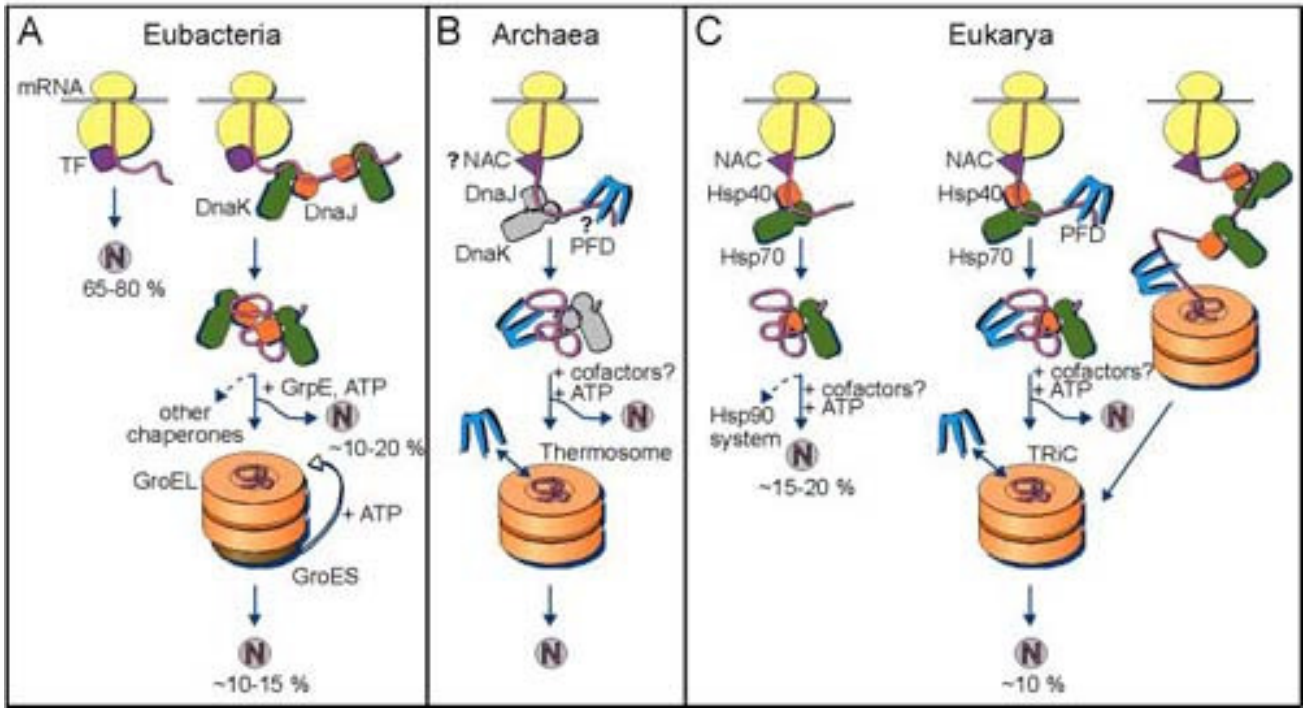
Muller et al. 2005 *Biophys J* **89**:3508

Heat shock proteins



Increased production if cell undergoes heat shock or other stress

Ubiquitous in virtually all living organisms



Classification according to molecular weight:

Hsp70: ATP-dependent stabilization of hydrophobic segments, motor protein

Hsp60: ATP-dependent facilitation of folding to the native state

Hsp90: Protein folding, cell signalling, and tumor repression

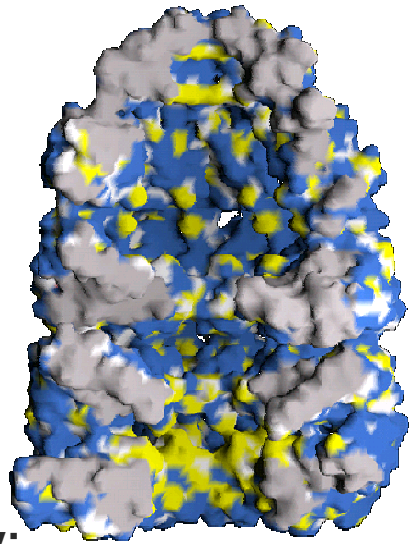
Hsp110: ATP-dependent disaggregation and unfolding for degradation

Small Hsps: Stabilization against aggregation during heat-shock

Chaperonin protein GroEL

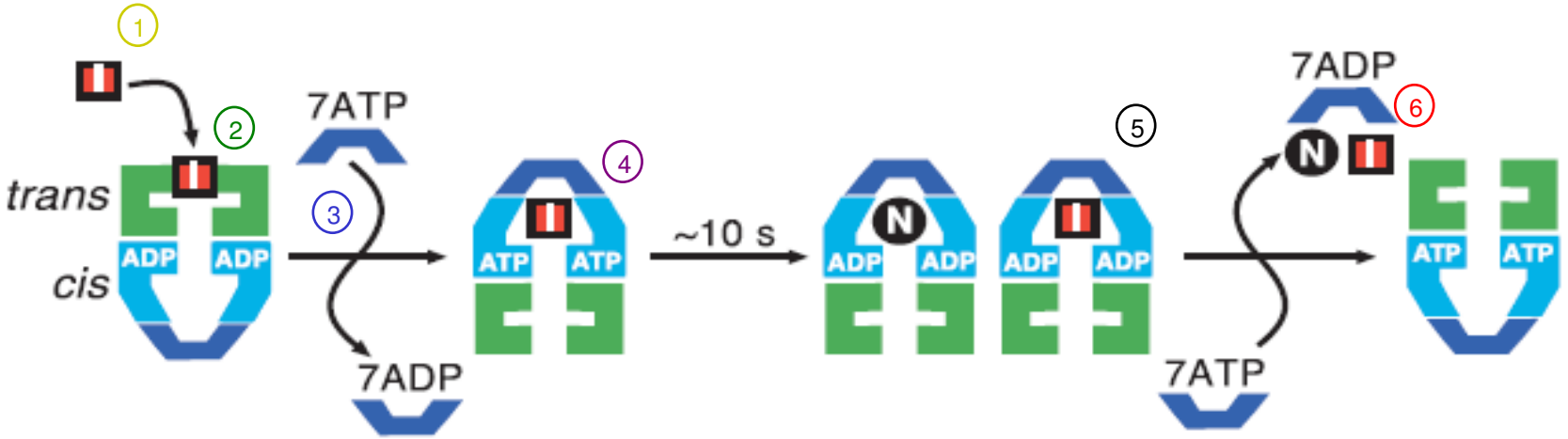
GroEL helps other proteins to fold correctly

- The folding pathway is
  1) Unfolded protein
  2) Binding protein to GroEL
  3) Binding of ATP and GroES to GroEL
  4) Release of the substate into the cavity of GroEL
  5) Folding of the substrate
  6) Release of folded protein from GroEL



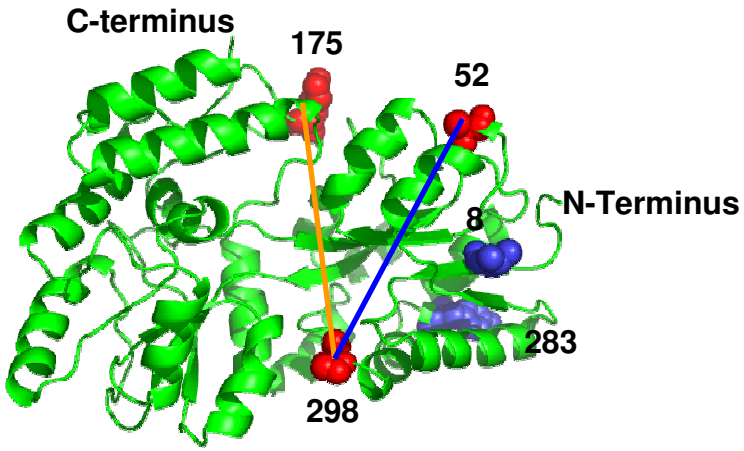
***cis* cavity: hydrophilic**

***trans* cavity: hydrophobic**



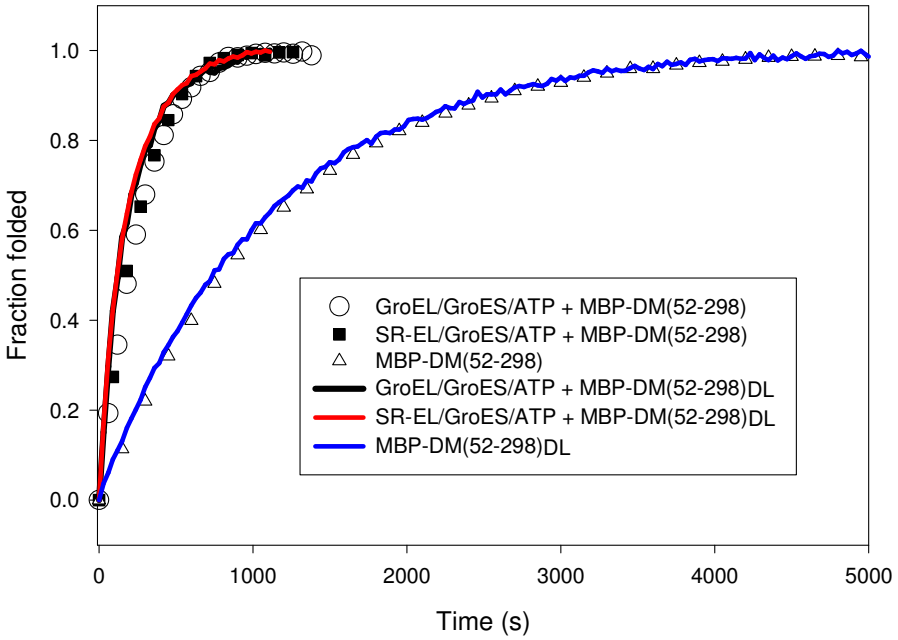
Hartl F.U and Hayer-Hartl M, 2002 *Science* 259:1852

MBP Folding

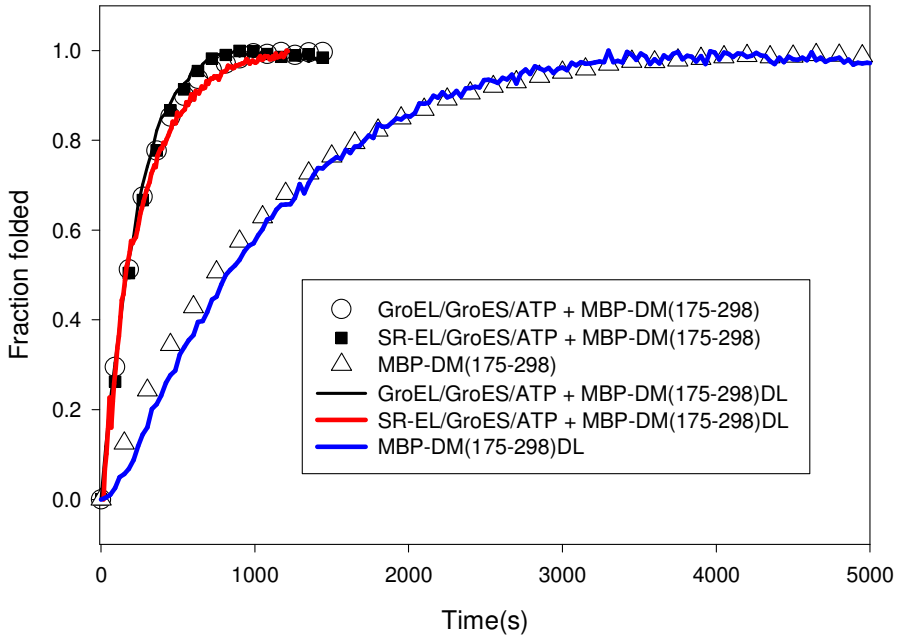


V8G
Y283D

MBP(52C-298C)

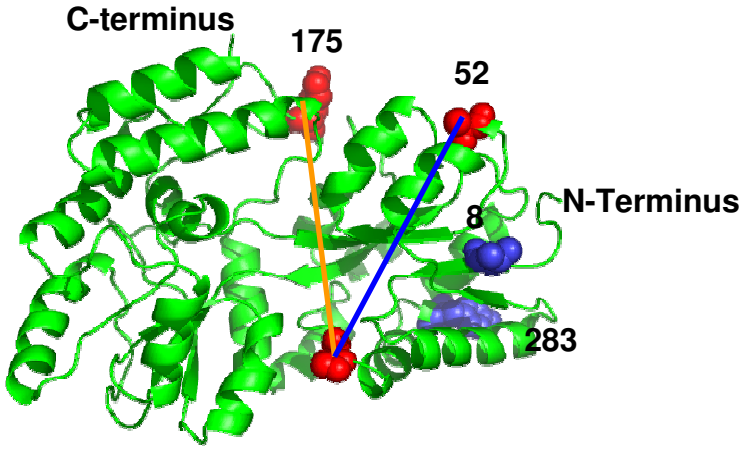


MBP(175C-298C)



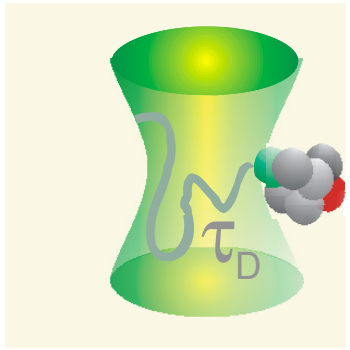
- MBP Folds Spontaneously
- MBP folding is accelerated by a factor **13** in the prescence of GroEL

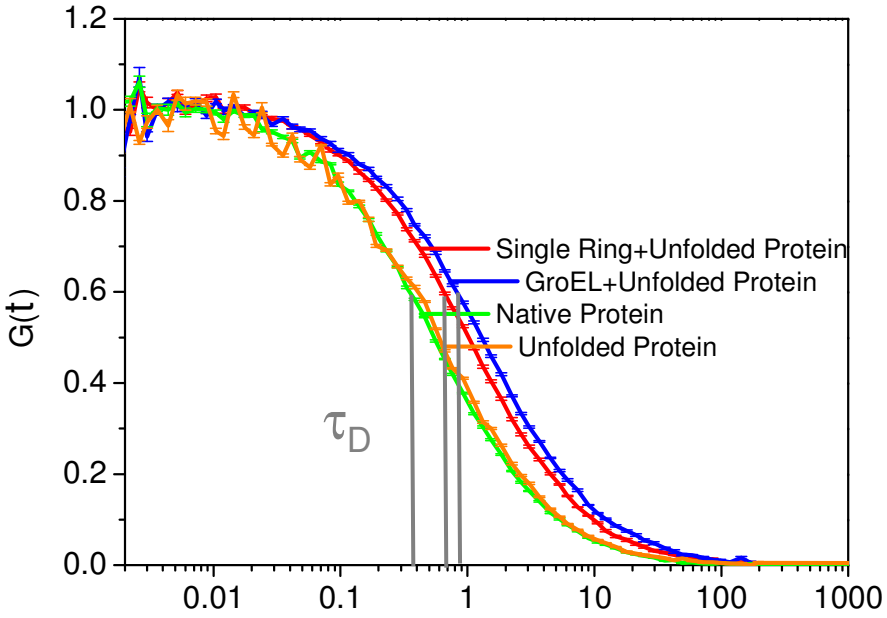
MBP Binding to GroEL at [pM]



V8G
Y283D

Fluorescence Correlation Spectroscopy



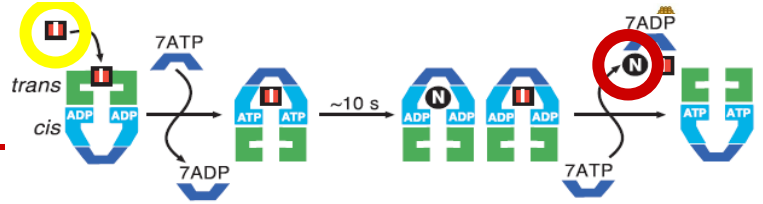


D_{MBP}= 48 µm²/s

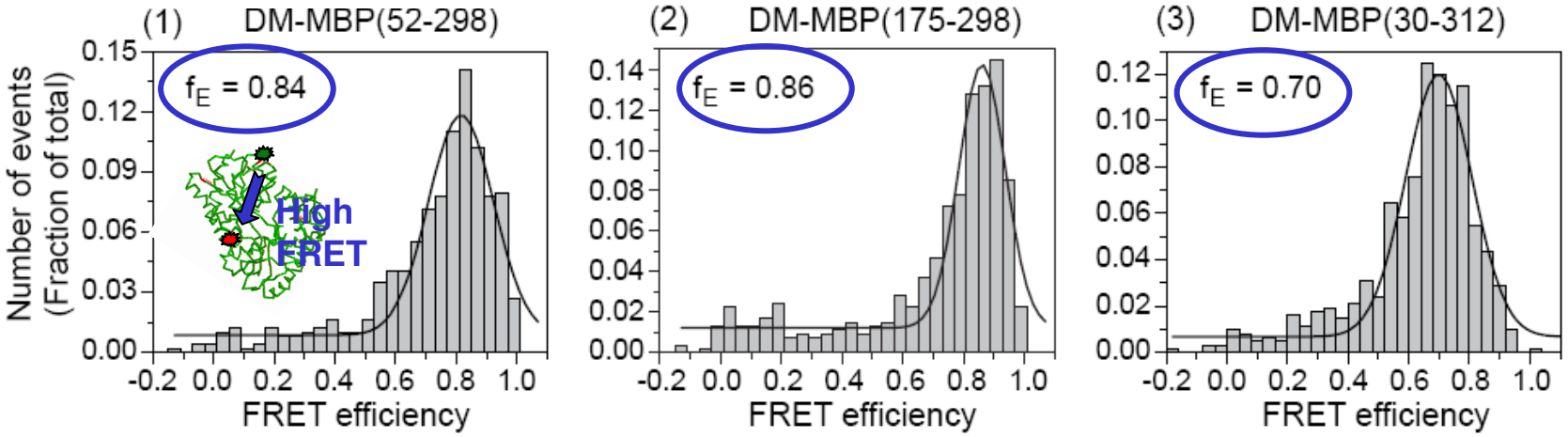
D_{SR}= 26 µm²/s

D_{GroEL}=20 µm²/s

Native and Denatured MBP

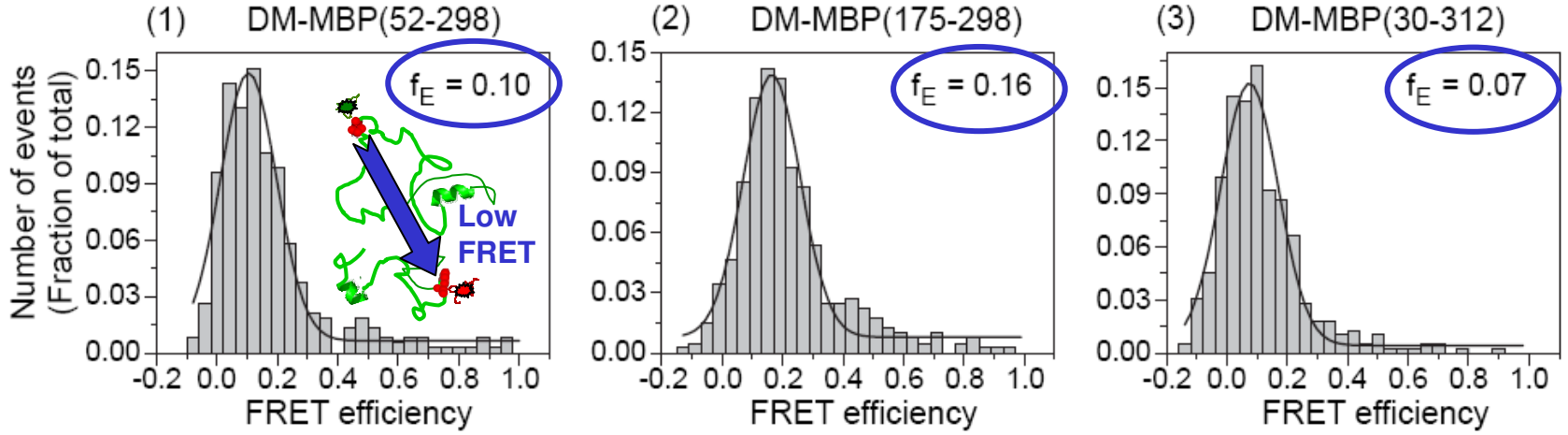


Spontaneously Refolded



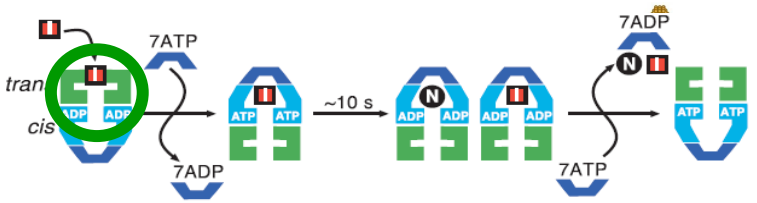
➢ Compact State

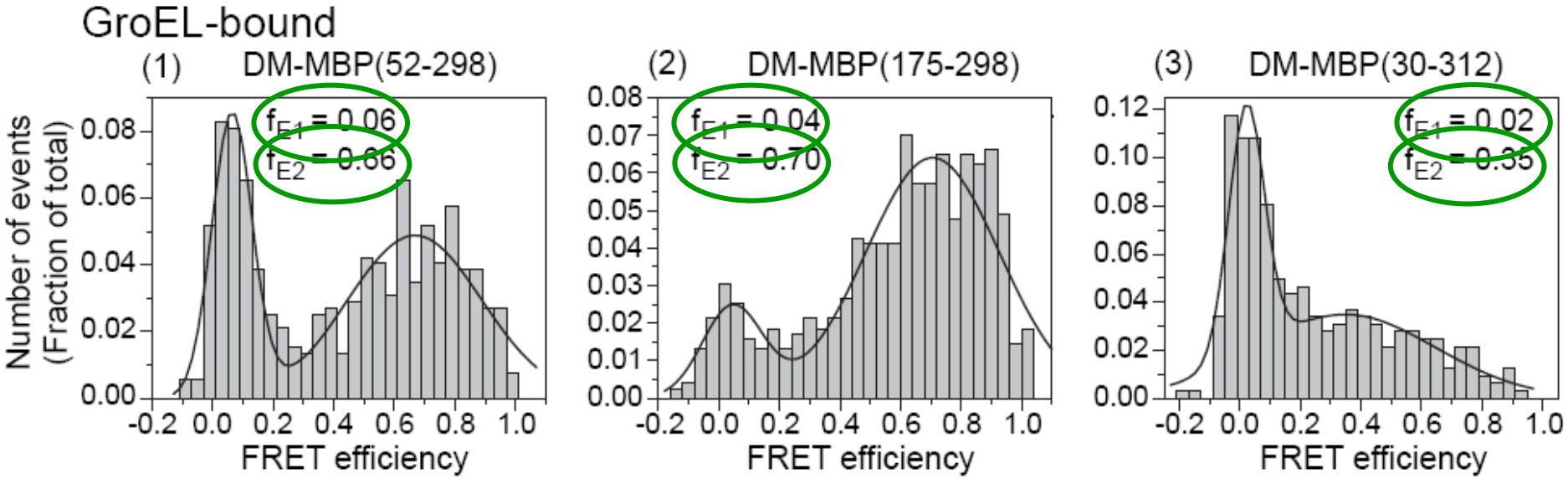
Denatured



➢ Extended (unfolded) State

Chaperonin-Substrate-Complex



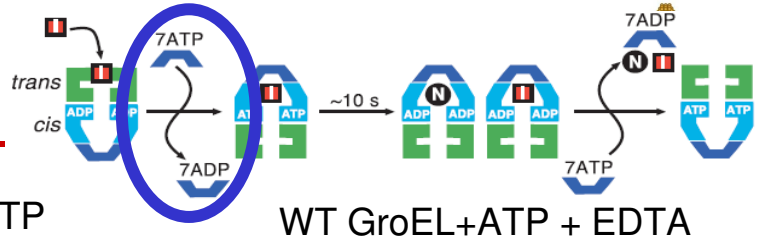


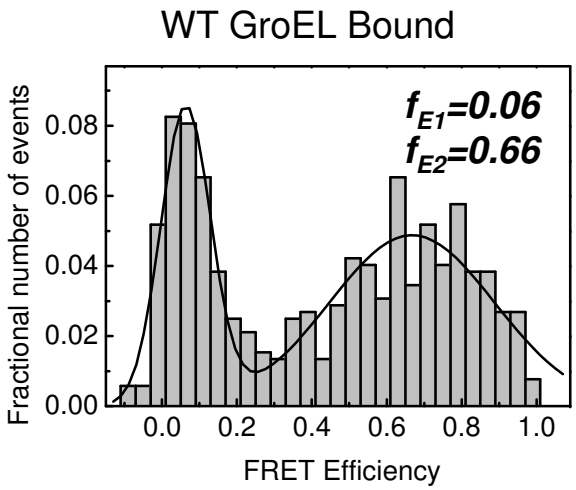
- A bimodal distribution is observed

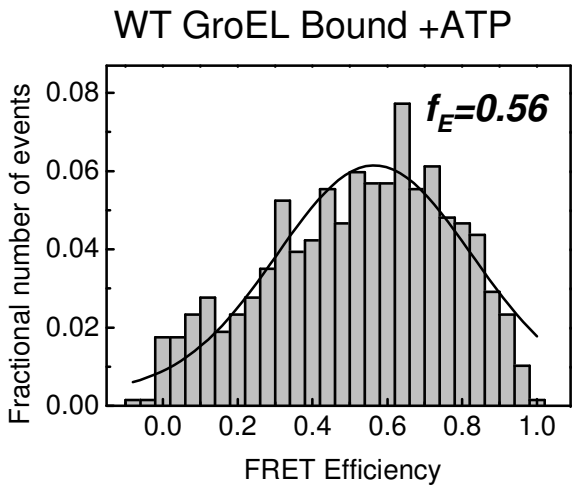
  The low FRET state has a similar donor-acceptor separation as the denatured state

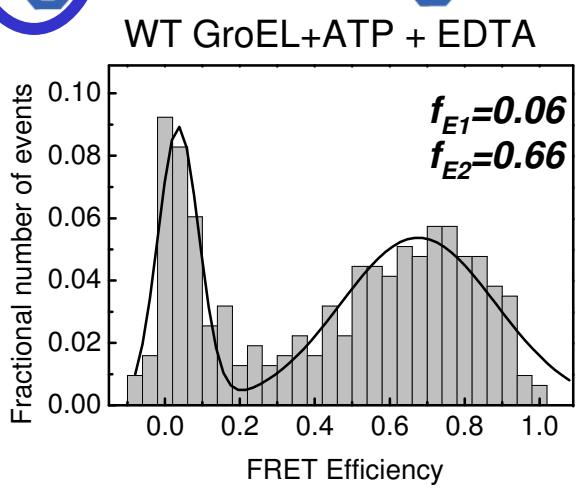
  The high FRET state is compact, but broadly distributed

ATP Dependence

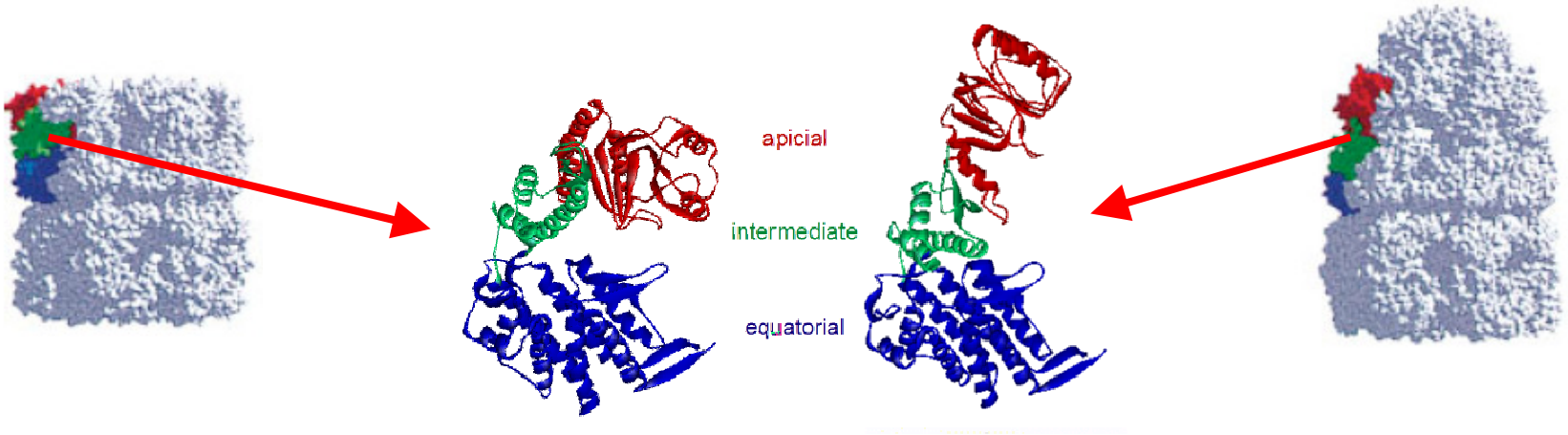






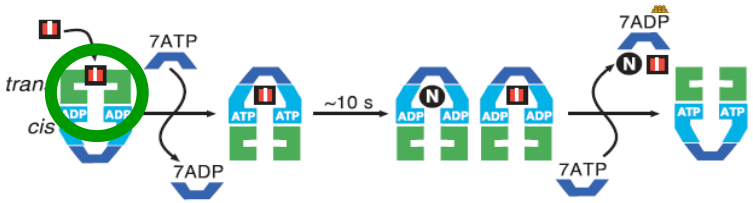


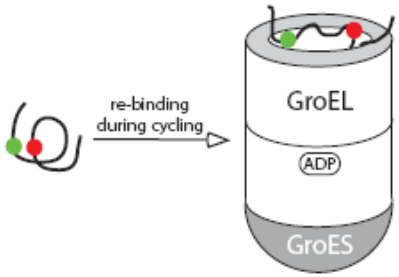
- The low FRET state disappears upon addition of ATP



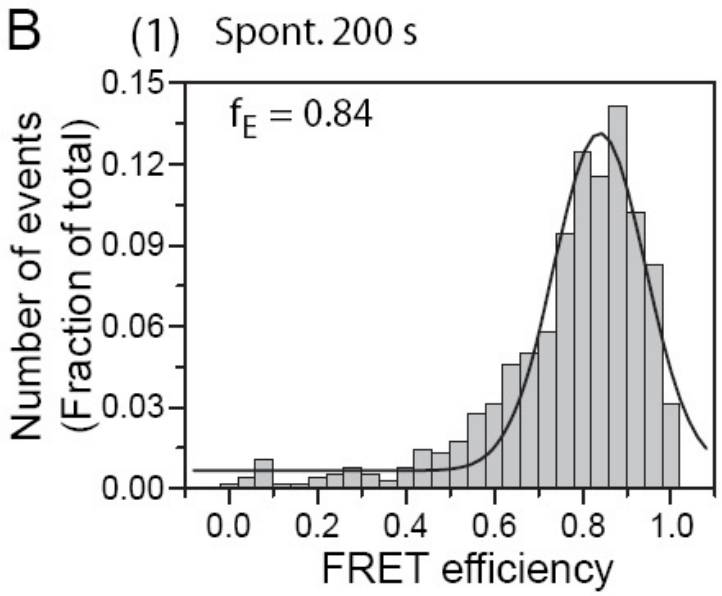
- The ATP-induced conformational change in MBP-GroEL is reversable

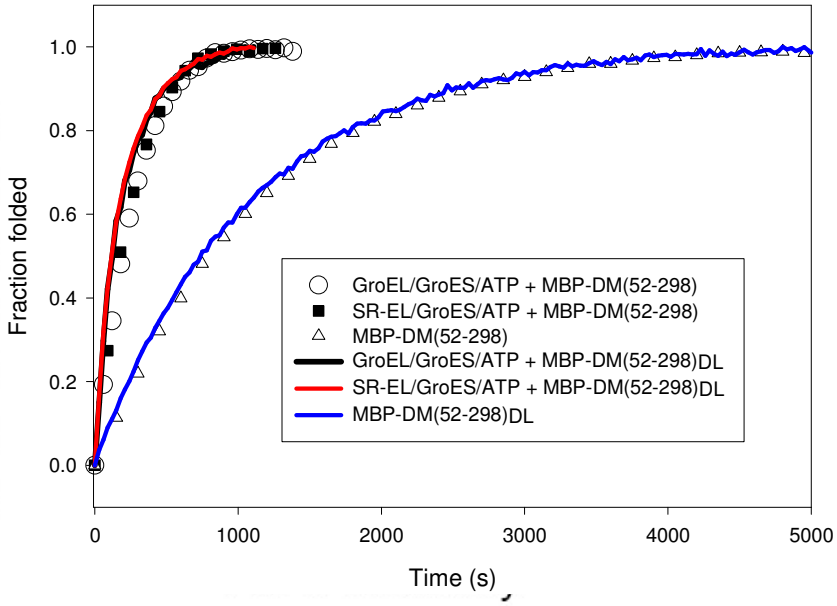
Chaperonin-Substrate-Complex





- MBP binding to GroEL after 200s spontaneous folding



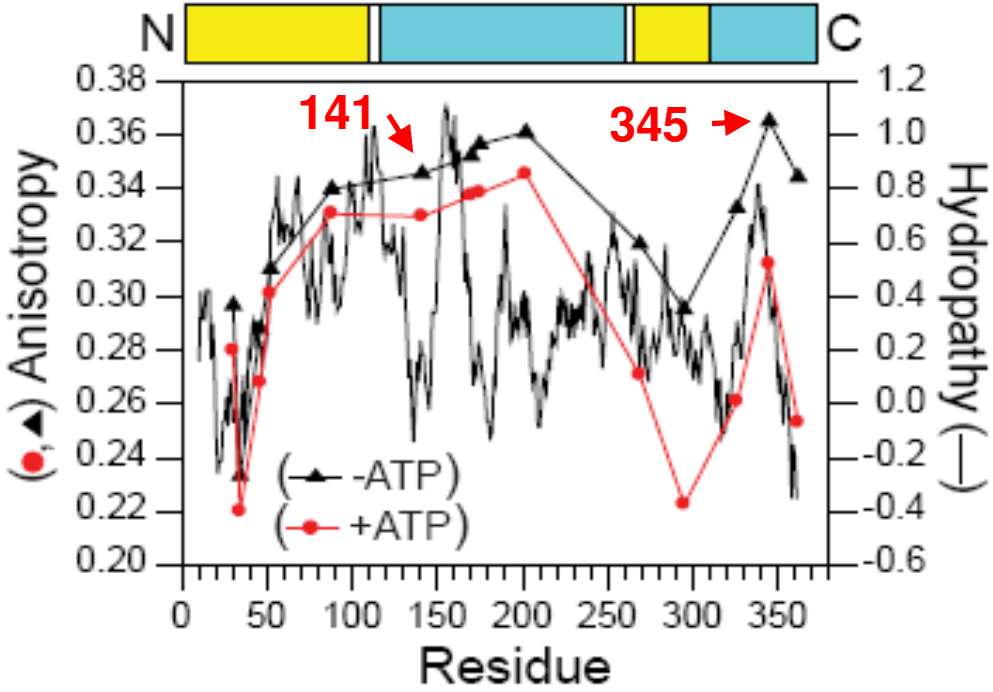


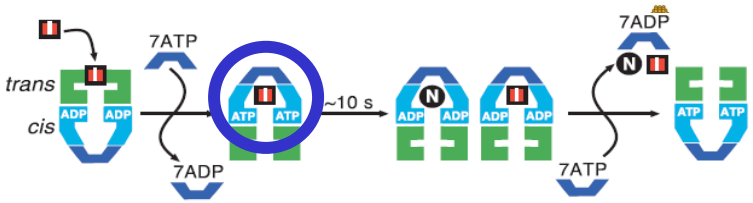
The low FRET population is present in GroEL / MBP after 200s spontaneous folding

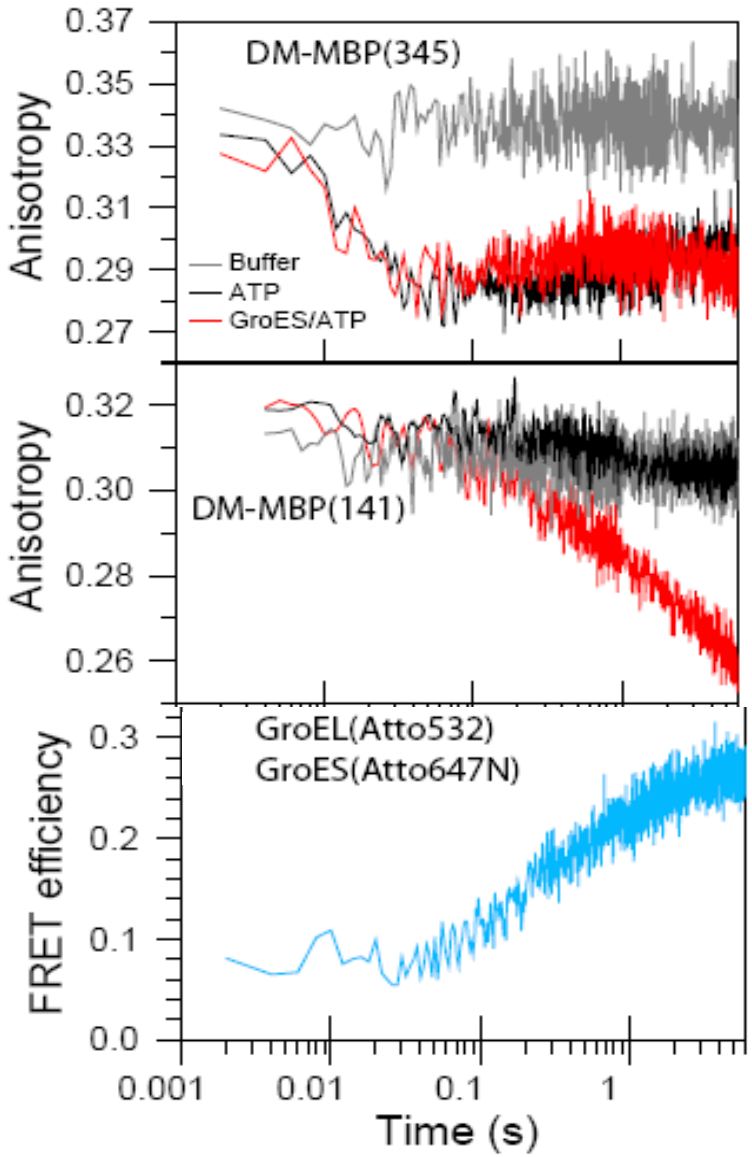
- A fraction of the substrates are stretched upon binding to GroEL

Sharma *et al*, *Cell* **133**:142-153

Controlled Release of Substrate





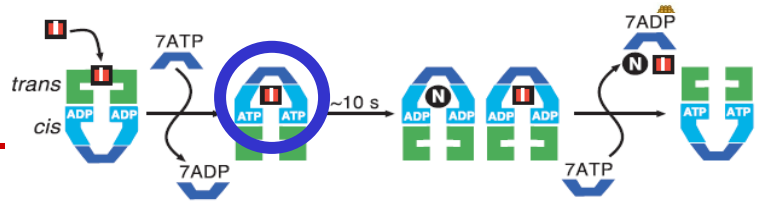


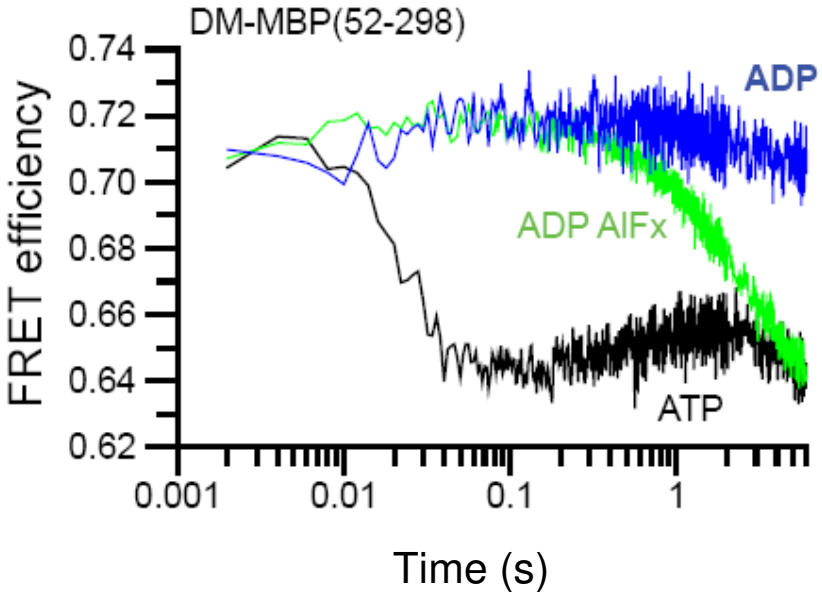
- Hydrophilic regions of the protein are released upon ATP binding
- Hydrophobic regions of the protein are released upon GroES binding.

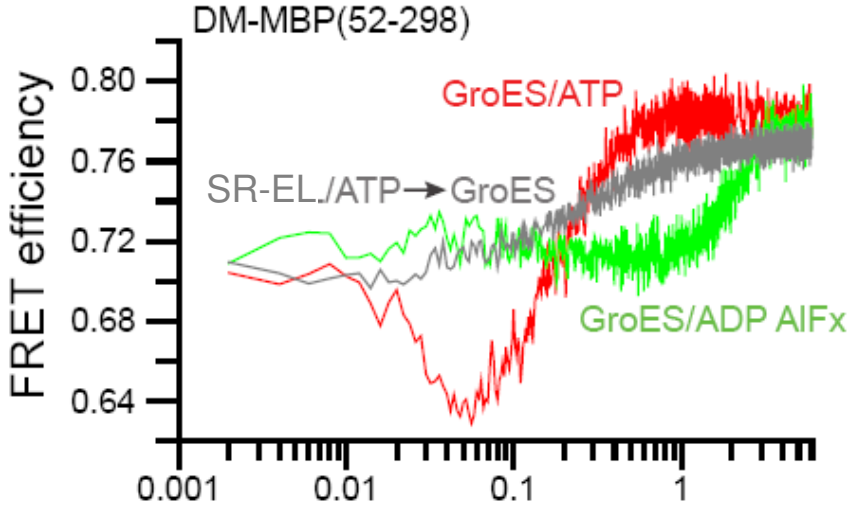
- This is opposite to hydrophobic collapse

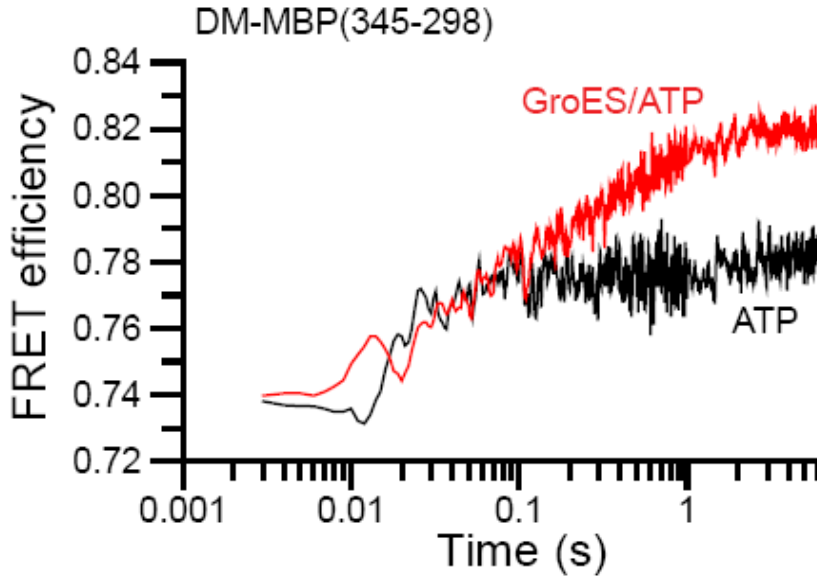
Sharma *et al* 2008 *Cell* ***133:****142*

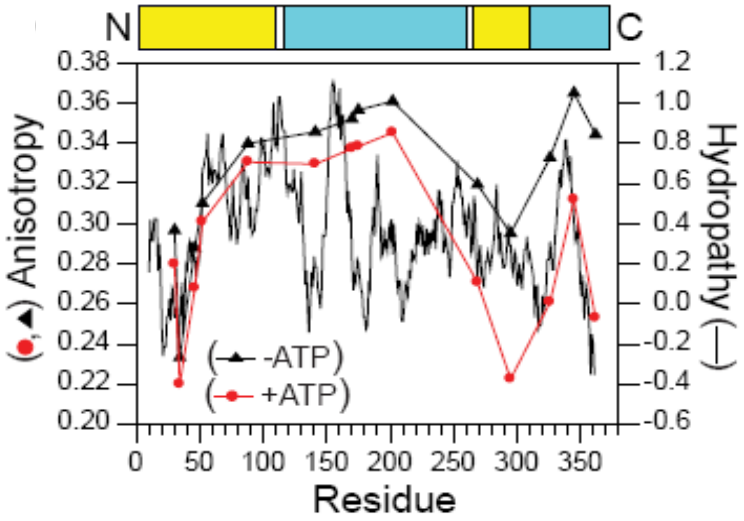
ATP-Dependent Stretching of MBP Bound to GroEL











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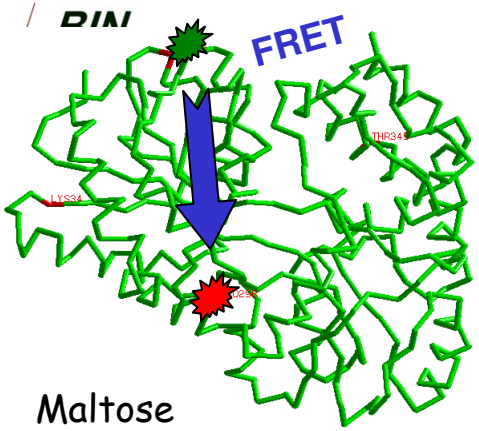
Summary II



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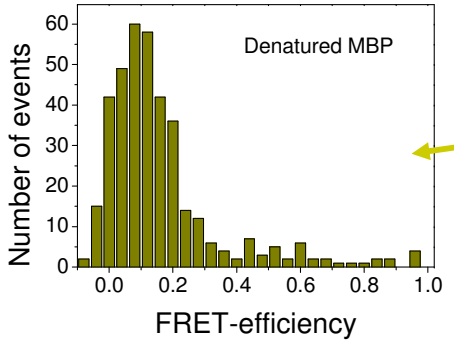
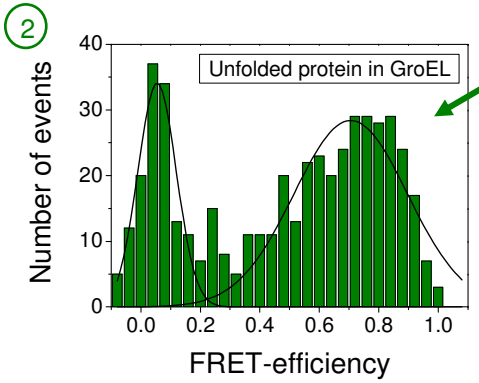
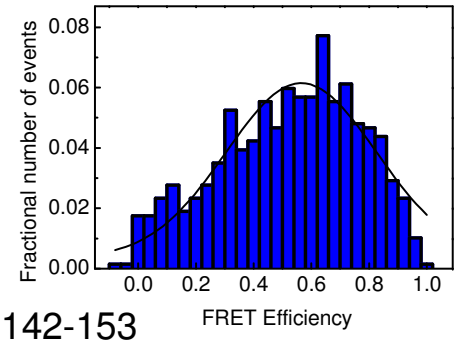
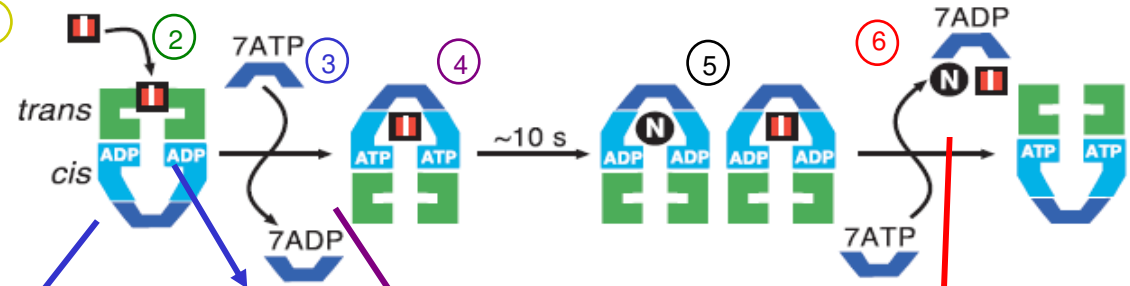
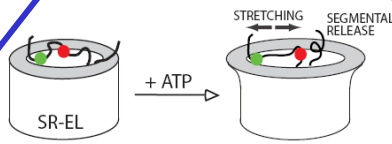
- Bimodal FRET peak
  - Possible removal of kinetic traps / ATP independent
- Transient streching upon ATP binding
- Controlled release of substrate into GroEL Cavity
- No cyclic dependence of MBP conformation bound to GroEL

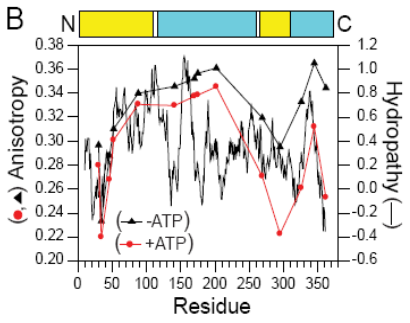


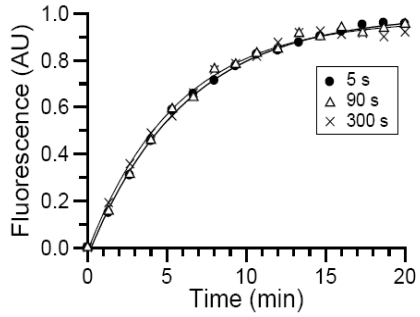












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Acknowledgments

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- CeNS
- BaCaTec