FRET and FLIM Applications:  
Single Pair FRET

Don C. Lamb

Department of Physical Chemistry  
Munich, Germany

1st Annual Course on Principles  
of Fluorescence Techniques  
Madrid, Spain
Ensemble versus Single Molecule

1. Detect individual events
2. Resolve rare events
3. Measure dynamics of non-synchronized 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

- 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

- 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

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

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)

Donor channel

Acceptor channel

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
- \( N = 631 \)
- \( \langle E_1 \rangle = 0.21 \)
- \( \sigma_1 = 0.05 \)
- \( \langle E_2 \rangle = 0.40 \)
- \( \sigma_2 = 0.04 \)
- Final binding positions / conformation of TBP on DNA.
Addition of NC2

80 ms/Frame

Intensity [a.u.]

Total Intensity

Donor-Excitation

FRET

0 5 10 15 20 25

0.0

0.5

1.0

0

4000

8000

6000

0

292x162

15000

222x162

262x133

187x123

438x161

388x132

338x132

349x119

80 ms/Frame
Addition of NC2

80 ms/Frame

Alternating Laser Excitation

The first conformation (E = 0.41) is the **initial steady state**.

The 2nd conformation is DNA-unbending.

Schluesche et al 2007 *NSMB* 14:1196

\[ \begin{align*}
\sigma_1 &= 0.13 \\
\langle E_1 \rangle &= 0.41 \\
N &= 110 \\
\langle E_2 \rangle &= 0.82 \\
\sigma_2 &= 0.10
\end{align*} \]
3rd conformation is movement of TBP along the DNA

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

E = 0.43

E = 0.82

E = 0.20

modified PDB-File 1RM1

Schluesche et al 2007 NSMB 14:1196
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

**Model of TBP-NC2-Dynamics**

**Transcription repression through NC2**

**DNA-unbending**

**relocalization**

**Alternative Initiation positions**

Schluesche et al 2007 *NSMB* 14:1196
Protein Folding:
The Role of Chaperonins in Protein Folding

Dr. Barbara K. Müller
Dr. Shruti Sharma
Dr. Kausik Chakraborty
Burst Analysis

\[ E = \frac{I_A}{\gamma I_D + I_A} \]

Green Channel

Red Channel

number of photons

0.0 2.0 \times 10^{-6} 4.0 \times 10^{-6} 6.0 \times 10^{-6} 8.0 \times 10^{-6} 1.0 \times 10^{-5}

time [s]

FRET-efficiency

Number of events

0.0 0.2 0.4 0.6 0.8 1.0

FRET
PIE: Pulsed Interleaved Excitation

Additional Information: Excitation Source of Each Photon

Clock
Green Excitation
Red Excitation
Green Detection
Red Detection
**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

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

Increased production if cell undergoes heat shock or other stress

Ubiquitous in virtually all living organisms
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

Hartl F.U and Hayer-Hartl M, 2002 Science 259:1852
- MBP Folds Spontaneously
- MBP folding is accelerated by a factor 13 in the presence of GroEL
MBP Binding to GroEL at [pM]

Fluorescence Correlation Spectroscopy

\[ D_{\text{MBP}} = 48 \, \mu m^2/s \]
\[ D_{\text{SR}} = 26 \, \mu m^2/s \]
\[ D_{\text{GroEL}} = 20 \, \mu m^2/s \]
Native and Denatured MBP

**Spontaneously Refolded**

1. DM-MBP(52-298) $f_E = 0.84$
   - Compact State

2. DM-MBP(175-298) $f_E = 0.86$

3. DM-MBP(30-312) $f_E = 0.70$
   - Extended (unfolded) State

**Denatured**

1. DM-MBP(52-298) $f_E = 0.10$
   - Compact State

2. DM-MBP(175-298) $f_E = 0.16$

3. DM-MBP(30-312) $f_E = 0.07$
   - Extended (unfolded) State
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
The low FRET state disappears upon addition of ATP.

The ATP-induced conformational change in MBP-GroEL is reversible.
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
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

Sharma et al. 2008 *Cell* **133**:142
Summary II

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

Sharma et al, 2008 Cell 133:142

Shama et al, 2008 Cell 133, 142-153
Acknowledgments

LMU, München

Fablab: Current Members
Dr. Aurélie Dupont
Gregor Heiss
Matthias Höller
Dr. Sergey Ivanchenko
Dr. Volodymyr Kudryavtsev
Nikolaus Naredi-Rainer
Martina Preiner
Stefan Riegelsberger
Dorothee Schupp
Martin Sikor

Former Members
Dr. Ondrej Burkacky
Dr. Yoshihiko Katayama
Dr. Barbara K. Müller
Stefan Paternoster
Dr. Peter Schlüsch

Gene Center
Prof. Dr. Ralf-Peter Jansen
Susanna Lange

Pharm. Biology
Prof. Dr. Ernst Wagner
Dr. Manfred Ogrsis
Martin Meyer

Physiological Chemistry
Prof. Dr. Dr. Walter Neupert
PD Dejana Mokranjac
Koyeli Mappa

Organic Chemistry
Dr. Stephan A. Sieber
Thomas Botter

Biology
Prof. Dr. Heinrich Leonhardt
Carina Frauer

Funding
• DFG – SFB 646
• DFG – SFB 749
• DFG – Schwerpunkt 1175
• LMUinnovativ BIN
• LMUexcellent
• NIM
• CiPSM
• CeNS
• BaCaTeC

Max Planck Institute, Biochemie
Prof. F. Urlich Hartl
Dr. Manajit K. Hayer-Hartl
Dr. Kausik Chakraborty
Dr. Shruti Sharma

Research Center for Environment and Health
Prof. Michael Meisterernst
Gertraud Stelzer
Christine Göbel

University of Heidelberg
Prof. Hans-Georg Kräusslich
PD Dr. Barbara Müller
Prof. Roland Eils
Prof. Karl Rohr
William Godinez

Universität Düsseldorf
Prof. Dr. Claus A. M. Seidel
Stanislaw Kalinin

University of Stuttgart
Dr. Micheal Börsch
Nawid Zarrabi

University of California, Irvine
Prof. Enrico Gratton
Dr. Michele Digman

University of California LA
Prof. Shimon Weiss
Kambiz Hamadani