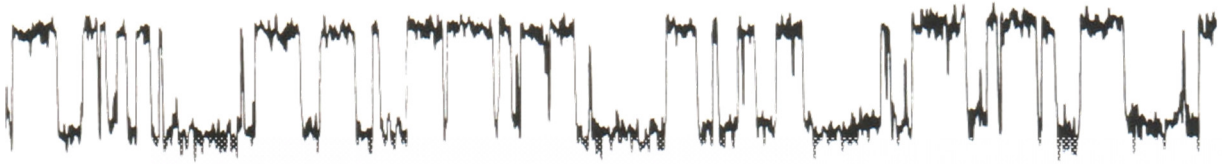
Introduction to Single-Molecule Spectroscopy



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September 2021

Introduction to Single-Molecule Spectroscopy

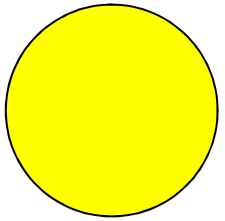
- Basics of single-molecule detection:
  - Förster Resonance Energy Transfer (FRET)
  - Fluorophore labeling
  - Confocal single-molecule detection
  - Free-diffusion measurements
- Single-molecule kinetics:
  - Surface-immobilized molecules
  - Principles of single-molecule kinetics
  - Simple and complex kinetics
- Outlook and summary:
  - In-cell single-molecule spectroscopy
  - Timescales
  - Literature

Averaging and Heterogeneity

Classical measurements yield values averaged over the entire ensemble of molecules in the observation volume.

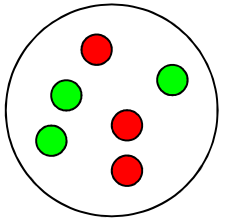
Consequences:

- Distributions of molecular properties are often averaged out
  $\Rightarrow$ some information is lost
- for kinetic experiments, the molecules usually need to be synchronized (perturbation)

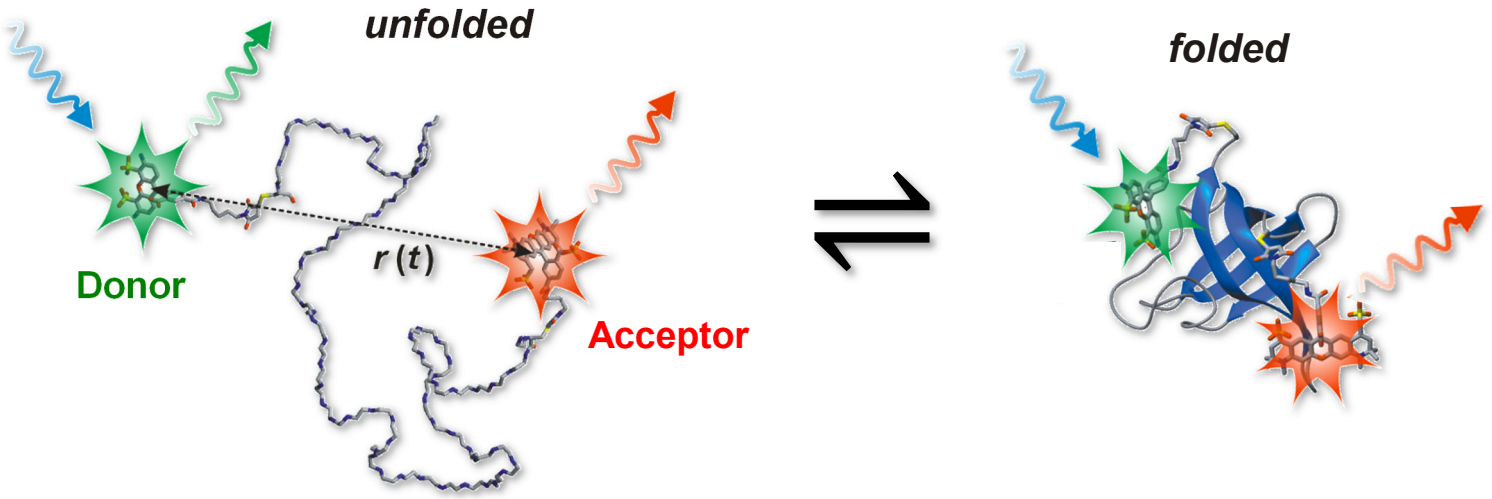


Single molecule experiment:

- Signals are recorded individually for every molecule
- Kinetics can be obtained from equilibrium measurements

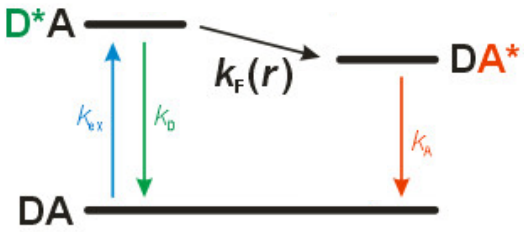


Förster Resonance Energy Transfer (FRET)



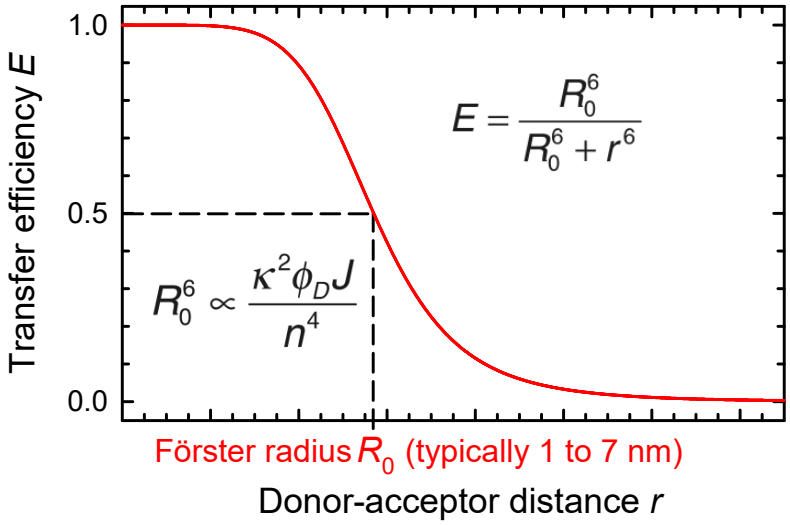
$$k_F = k_D \left(\frac{R_0}{r}\right)^6$$

$$= \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6$$

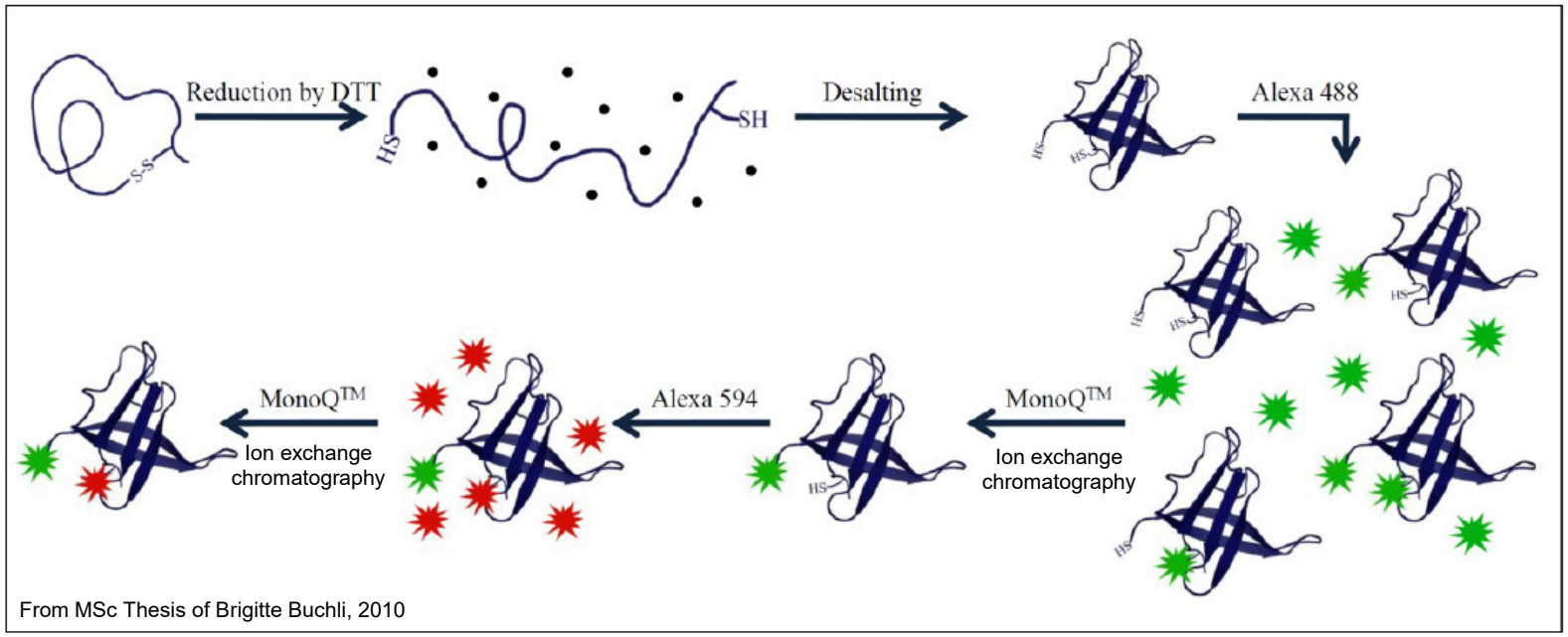


$$E = \frac{k_F}{k_F + k_D}$$

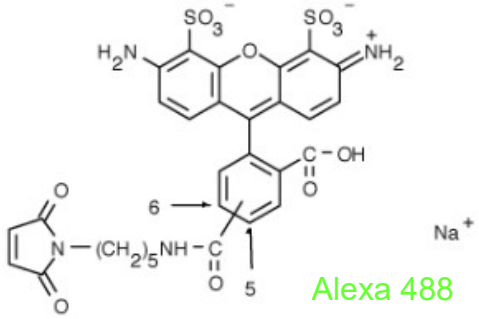
$$= \frac{n_A}{n_A + n_D} = 1 - \frac{\tau_{DA}}{\tau_D}$$

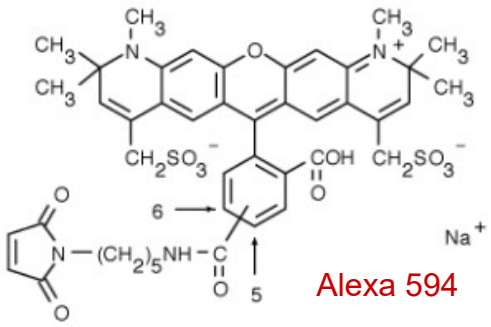


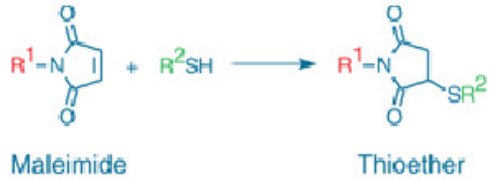
Protein labeling for single-molecule FRET



Example of fluorescence labeling of a protein for FRET with maleimide chemistry in combination with anion exchange chromatography







Principles of optical single molecule detection in solution

The challenge:

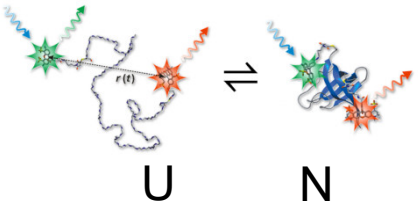
Detecting a single molecule in the presence of a huge excess of solvent molecules (e.g. ~10^{22} water molecules in 1 ml) that contribute to the background, especially by scattering

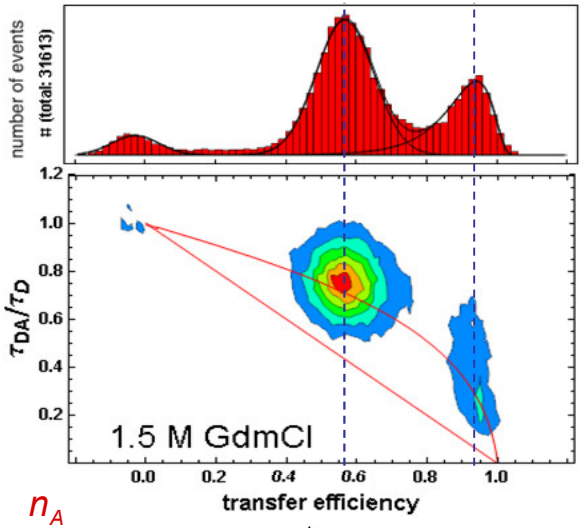
The solution:

1. Reduce the observation volume as much as possible (background will be proportional to the number of illuminated molecules)
   → **spatial selection**

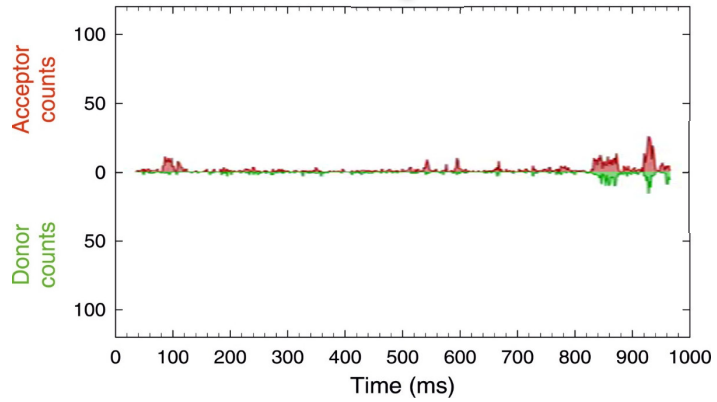
2. Choose a detection method with high slectivity for the molecule of interest: fluorescence allows selection of molecules by specific absorption and Stokes-shifted emission
   → **spectral separation**

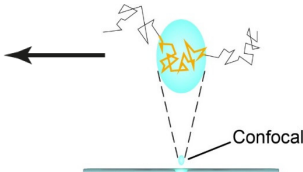
Confocal single-molecule fluorescence detection





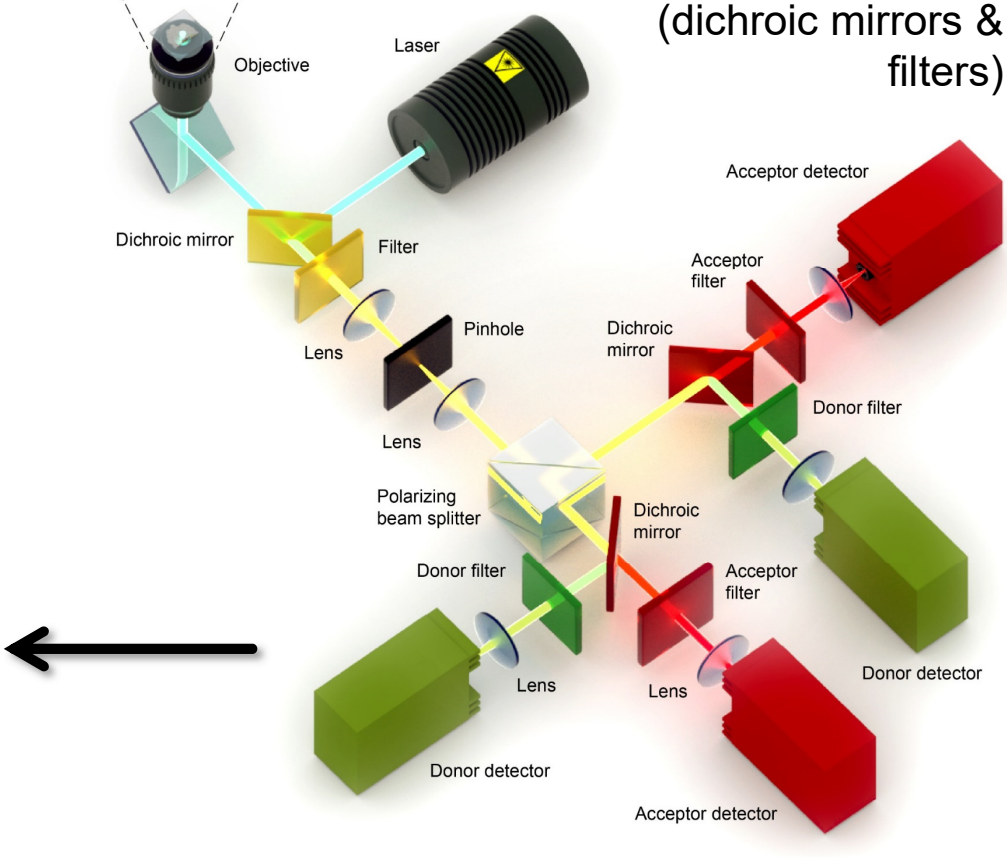
$$\langle E \rangle = \frac{n_A}{n_A + n_D}$$



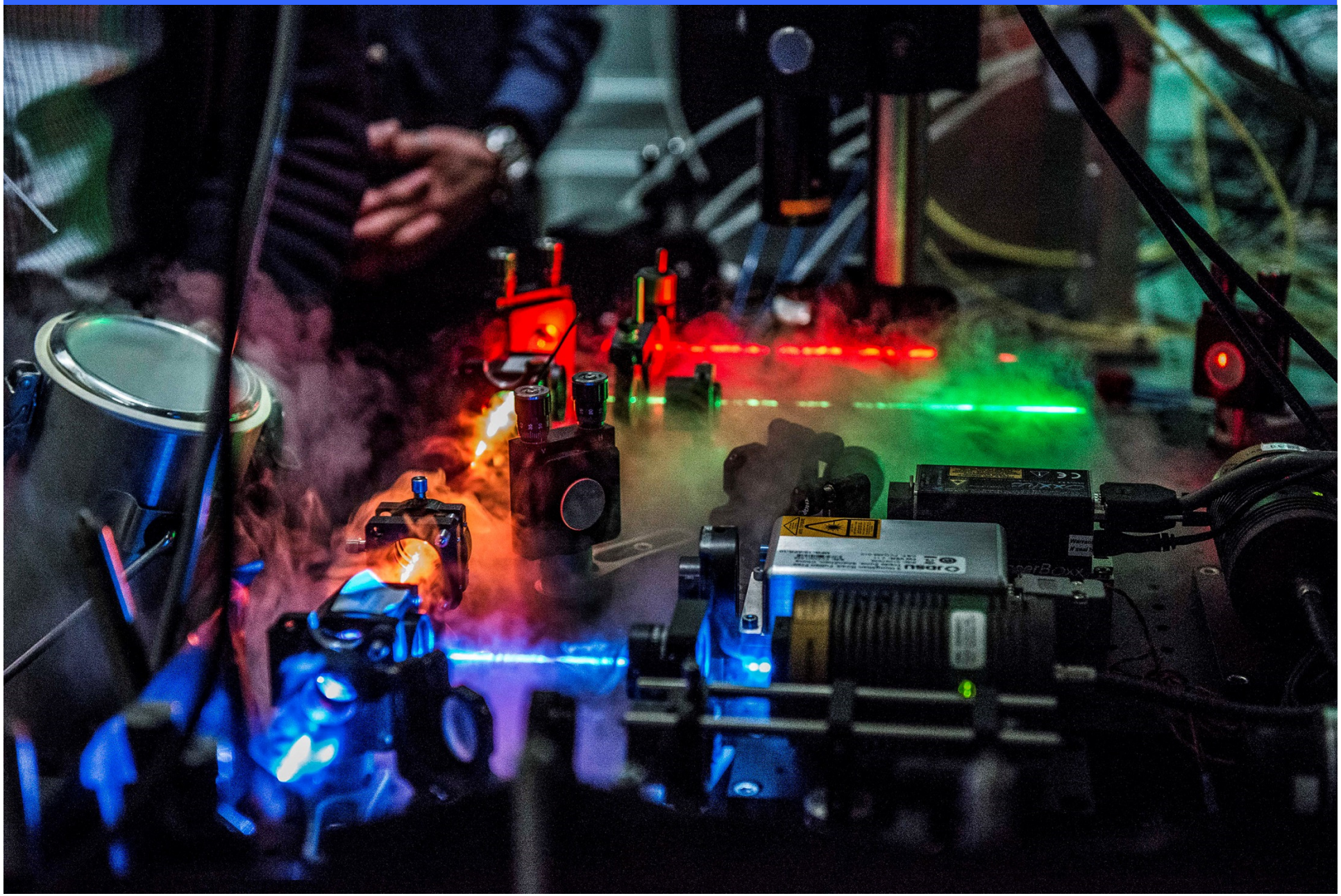


Spatial selection
(confocal observation volume, ~1 fl, sample conc. ~10 pM)

Spectral separation
(dichroic mirrors & filters)



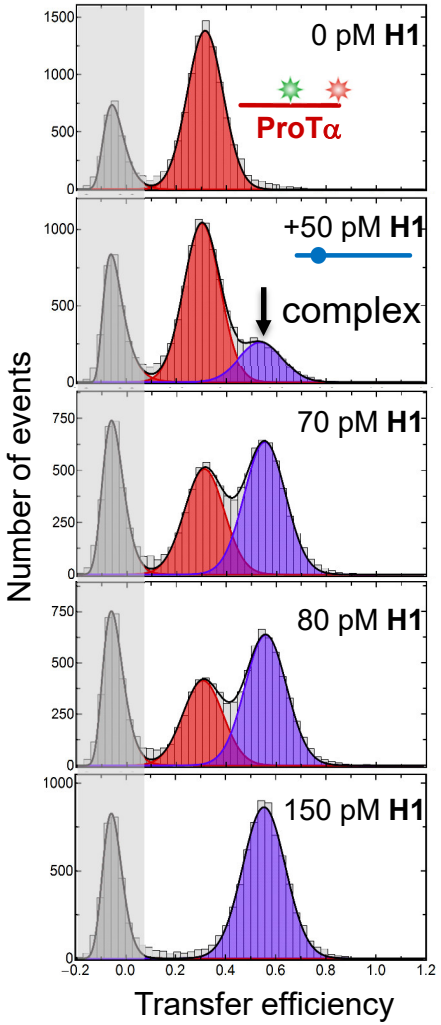
Instrumentation



Probing IDP interactions: conformations & dynamics

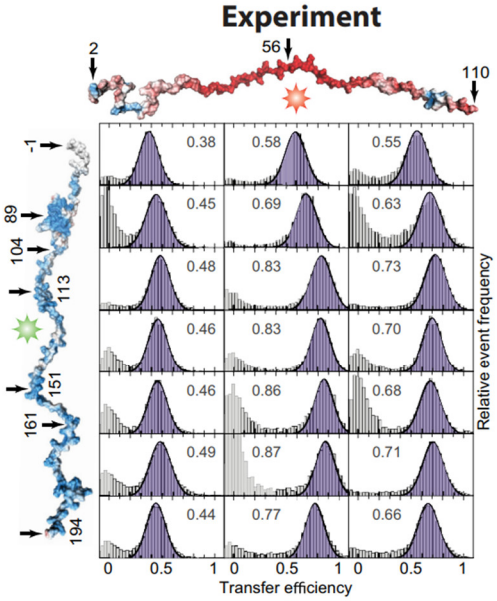
Borgia *et al.*,
Nature 555, 61-66 (2018)

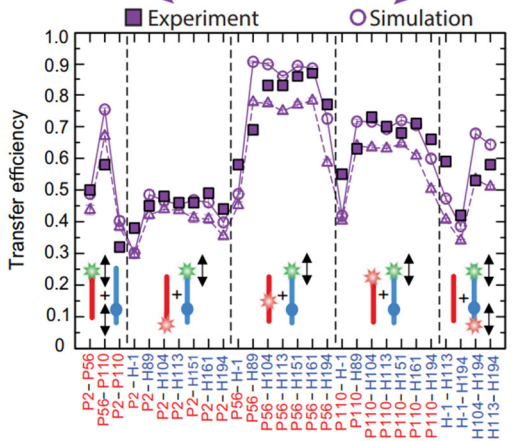
Binding titration



→ Picomolar affinity
(ionic strength 165 mM)

FRET-based mapping





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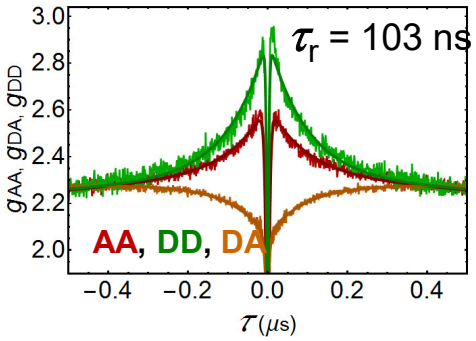
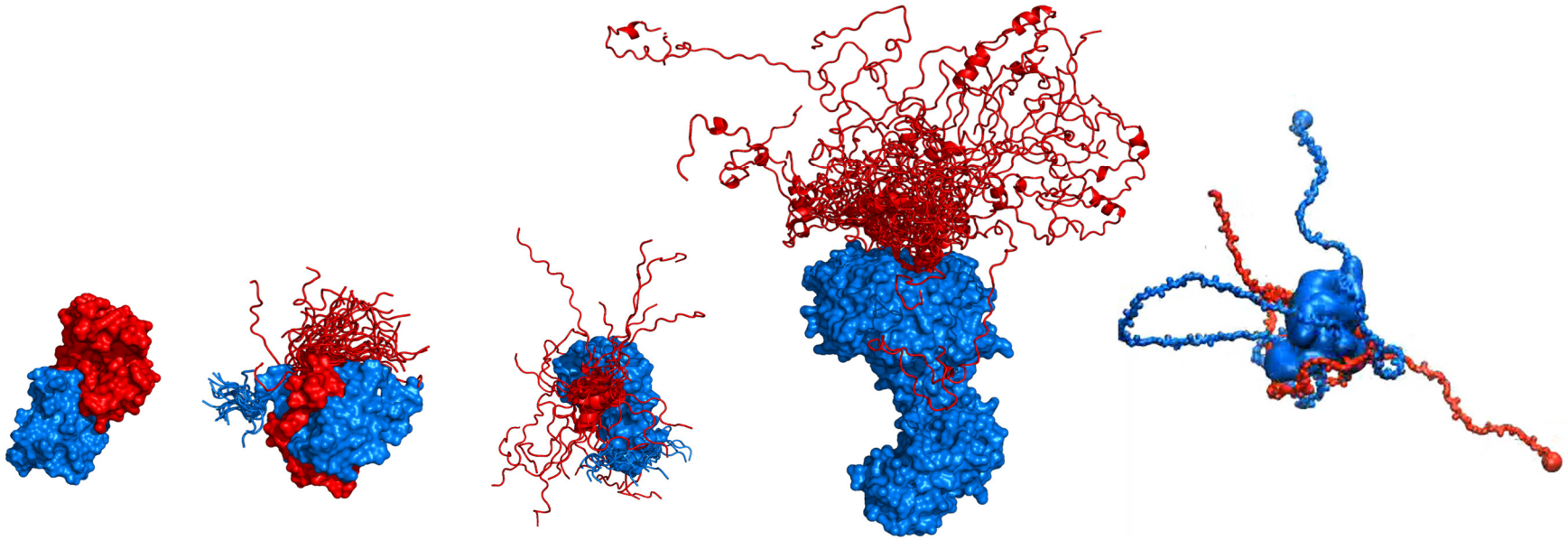


→ Complex remains
highly dynamic

The spectrum of disorder in protein complexes



Increasing disorder in the complex

Schuler *et al.*, *Curr. Opin. Struct. Biol.* 60, 66-76 (2020)

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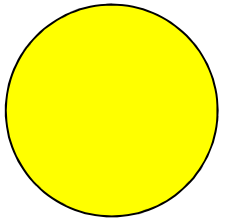

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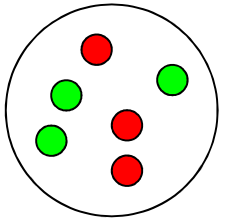
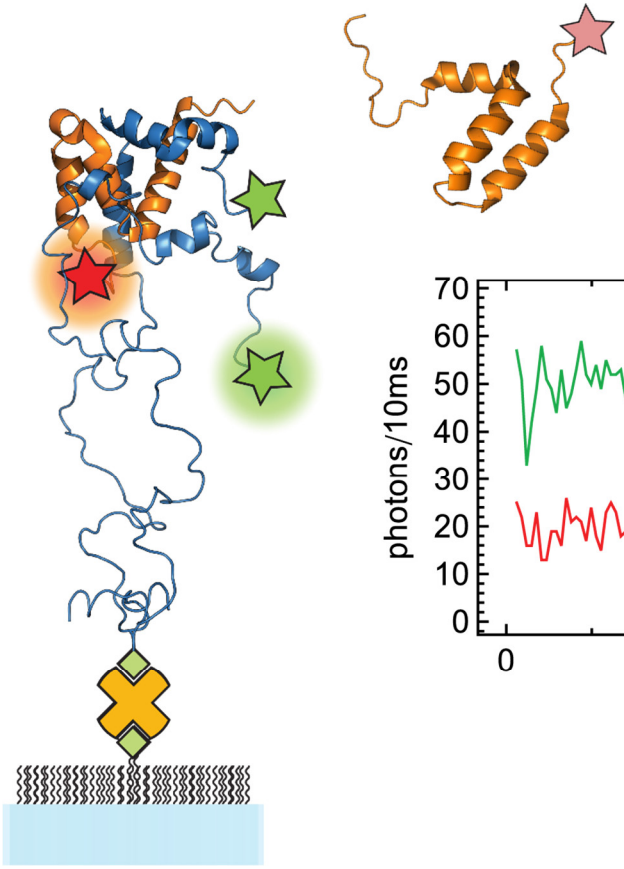
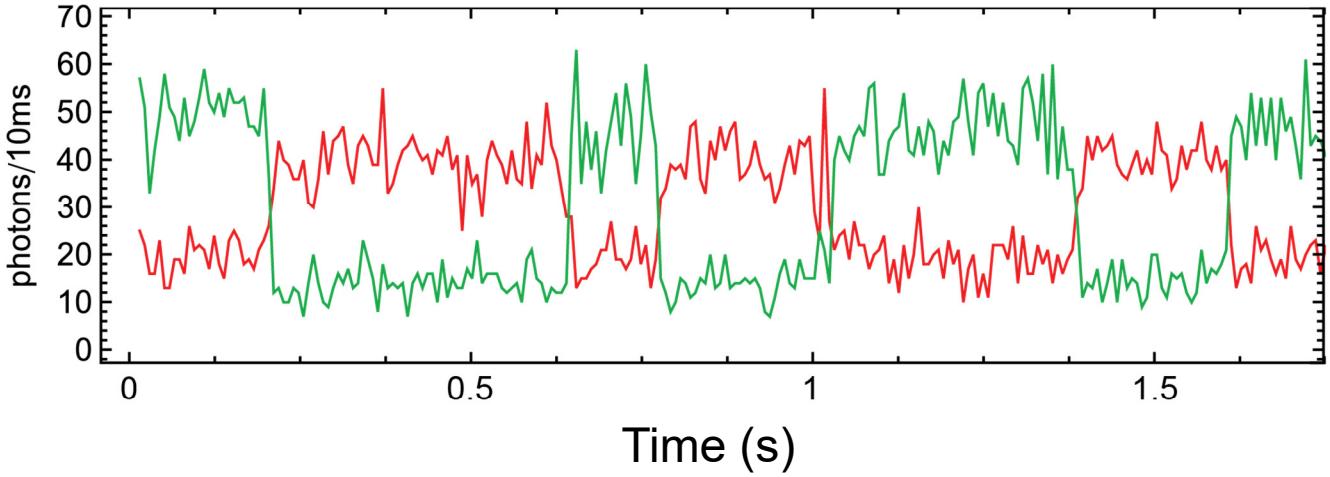


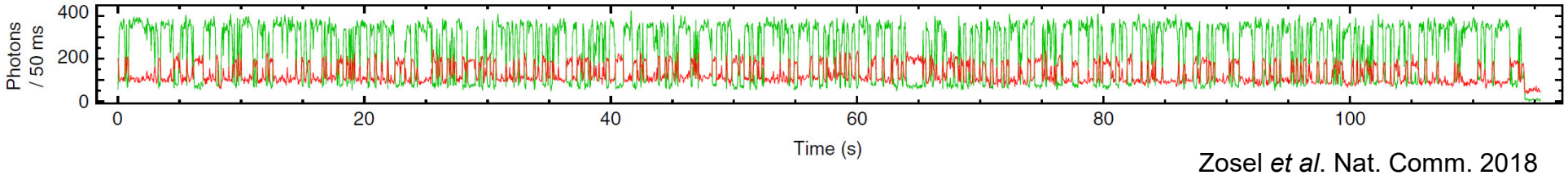
Protein binding kinetics at equilibrium



Example: coupled folding and binding
of two intrinsically disordered proteins:
ACTR + **NCBD**

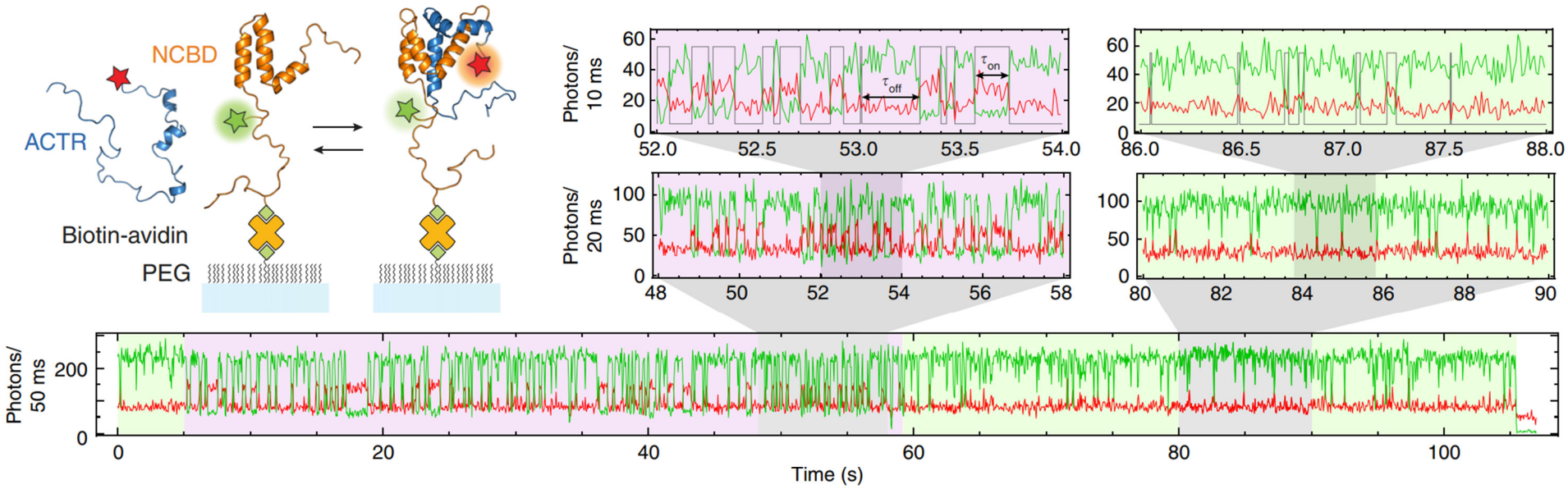


Recordings with hundreds of association/dissociation events:



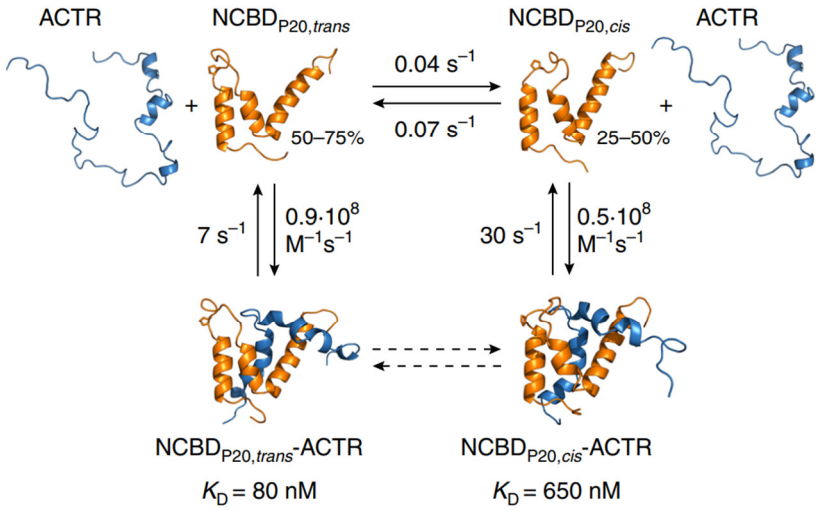
Zosel *et al*. Nat. Comm. 2018

Resolving kinetic heterogeneity



- Dwell-time distributions
- Hidden Markov modeling

→ Peptidyl-prolyl cis/trans isomerization results in two slowly interconverting subpopulations with different affinites



Zosel *et al*. Nat. Comm. 2018

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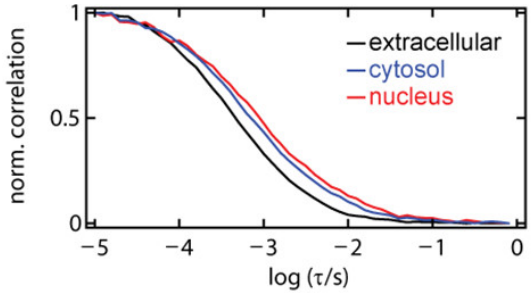
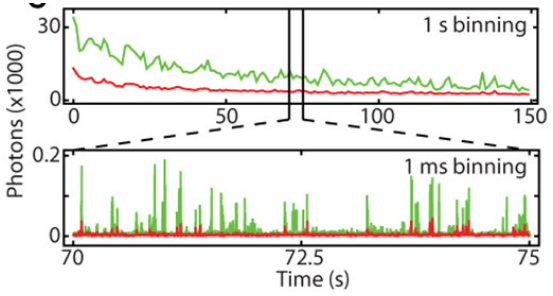
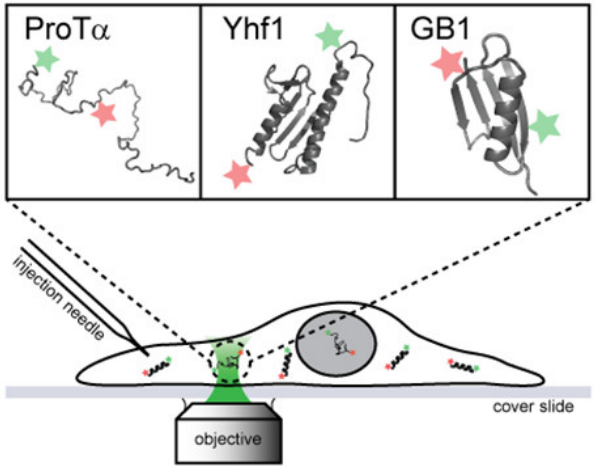
In-cell single-molecule spectroscopy

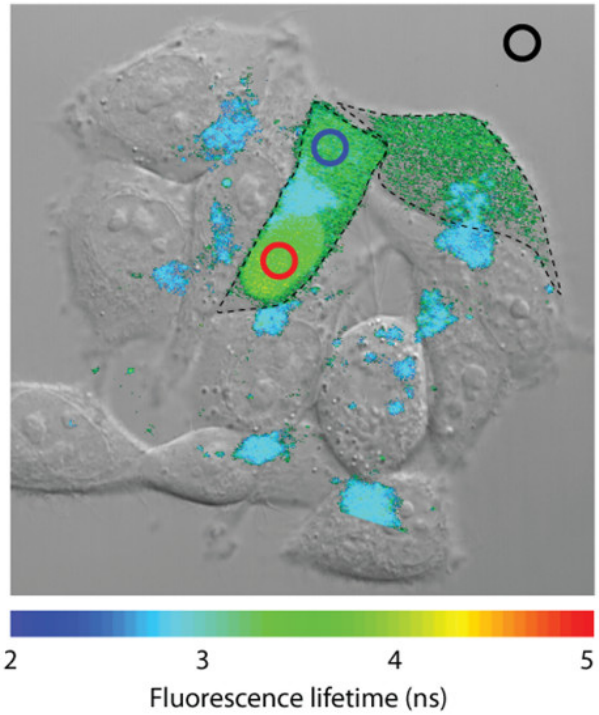
Translational diffusion



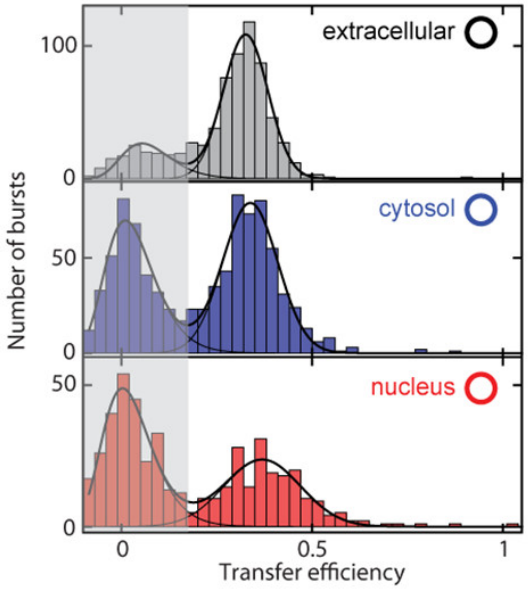
Fluorescence burst detection







Single-cell *E* histograms



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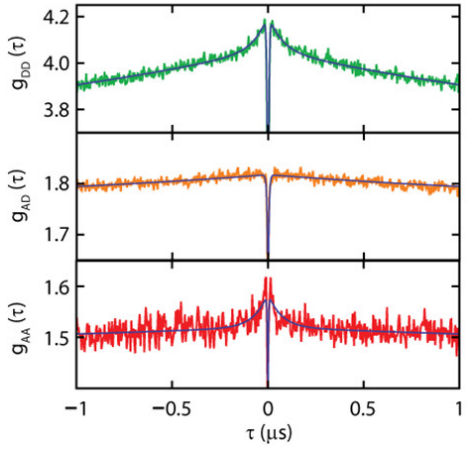
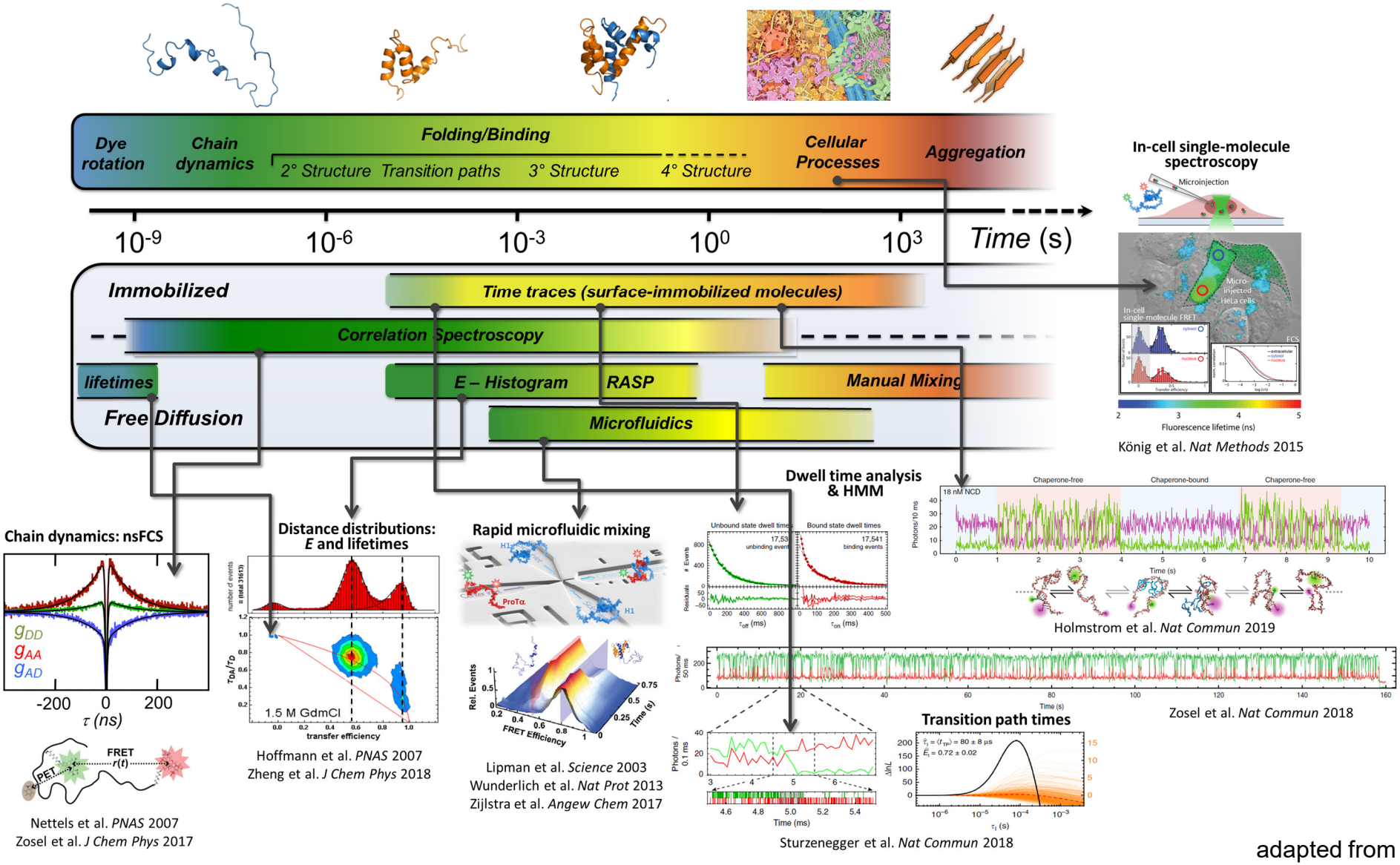


König et al., *Nature Methods* 12, 773–779 (2015)

The single-molecule toolbox for disordered proteins



adapted from
Schuler & Hofmann (2013) *Curr Opin Struct Biol*

Summary Single-Molecule Spectroscopy

- **Conformational heterogeneity** can be resolved by avoiding ensemble averaging
- **Kinetic properties** can be extracted from **equilibrium** measurements
  → rates correspond to **probabilities**, stochastic processes!
- Dynamics are accessible on a **wide range of time scales** (~nanoseconds to hours), even in complex environments
- Often useful or necessary: complementation by other biochemical and biophysical methods

Further reading

- **Single Molecule Techniques: A Laboratory Manual**
  Paul R. Selvin, Taekjip Ha (Cold Spring Harbor, 2007)
  → Optical single molecule methods and data analysis

- **Single-Molecule Detection in Solution: Methods and Applications**
  Christoph Zander, Richard R. Keller, Jörg Enderlein (Wiley 2002)
  → Correlation spectroscopy and single molecule methods

- **Single-molecule FRET of protein structure and dynamics - a primer.**
  Benjamin Schuler
  J. Nanobiotechnology 11 (S1), S2 (2013)