Centre de Biochimie Structurale
Montpellier

CNRS UMR 5048 - UM - INSERM U 1054
MONTPELLIER - France

Emmanuel Margeat
CBS Montpellier
Team « Integrative Biophysics of Membranes »

Single molecule FRET : a new tool for structural biology

Renofobis - 27/06/2019

Fluorescence in structural biology

Structures, dynamics et molecular interactions, *in vitro* & *in cellulo*

SAXS / SANS

Cryo-EM

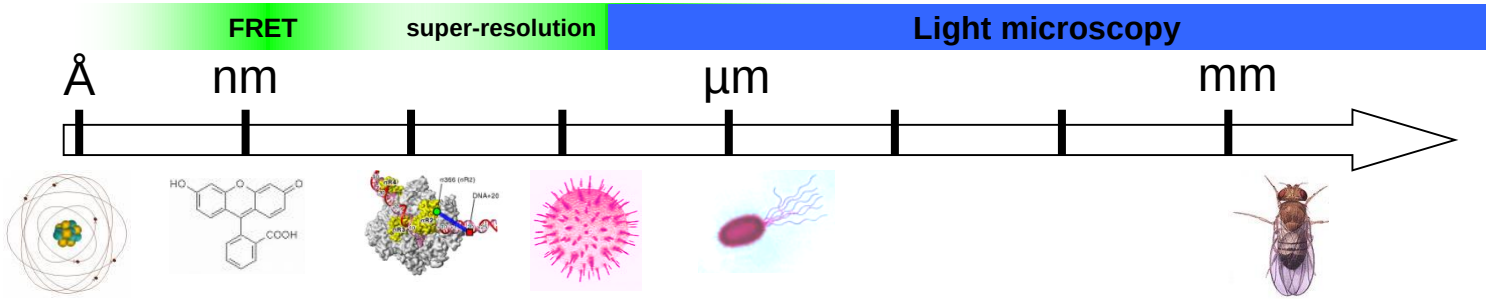
X-ray cristallography

NMR

ESR

Structural information

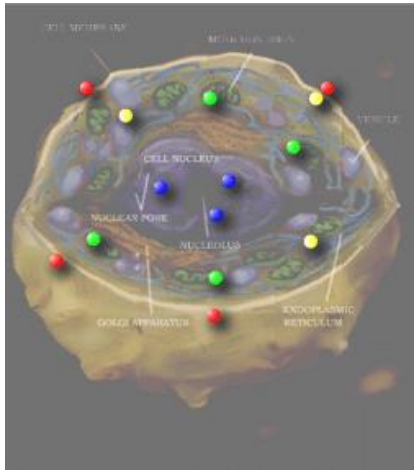
Enveloppe
Electron density
Distance between specific positions



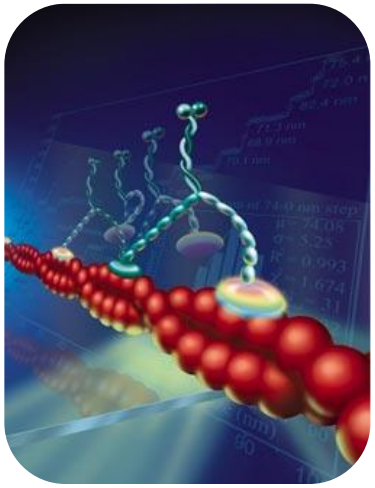
→ Méthods based on the detection of single fluorescent molecules

Single molecule detection

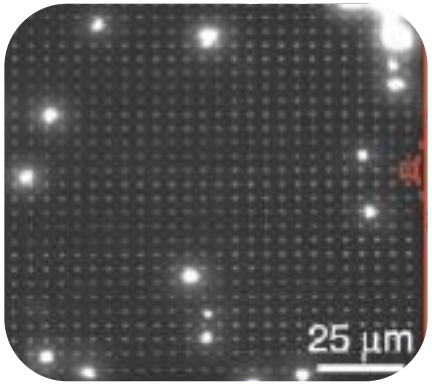
Single molecule tracking



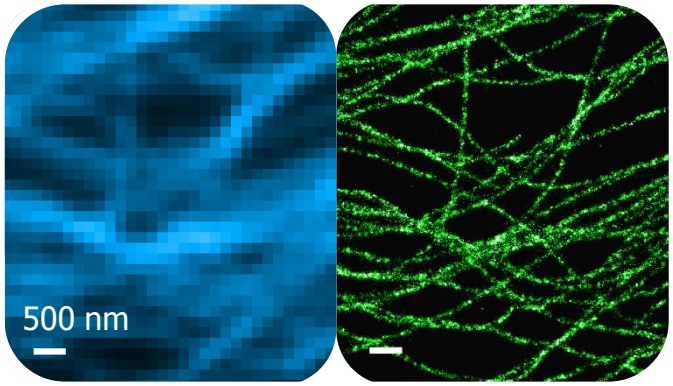
« Motility assays » *in vitro*



DNA sequencing



Structure & dynamics of biomolecules (FRET)



Super-resolution

Single molecule detection & superresolution

The Nobel Prize in Chemistry 2014

Photo: A. Mahmoud

Eric Betzig

Prize share: 1/3

Photo: A. Mahmoud

Stefan W. Hell

Prize share: 1/3

Photo: A. Mahmoud

William E. Moerner

Prize share: 1/3

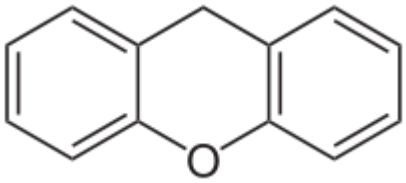
The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, Stefan W. Hell and William E. Moerner *"for the development of super-resolved fluorescence microscopy"*.

Outline

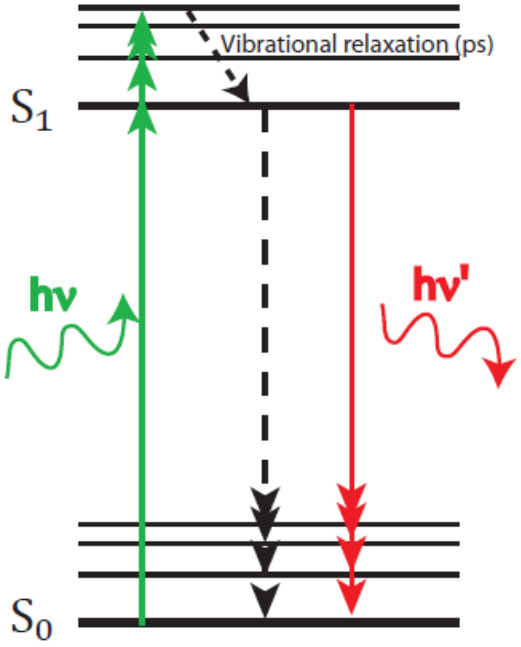
- *Basis of fluorescence & the FRET phenomenon*
- *Observation of single molecules : Why ? How ?*
- *Labeling your molecule of interest*
- *Measurement of distances with smFRET*
- *Measurement of dynamics with smFRET*

Fluorescence basics

Fluorophore : group of atoms that can absorb a photon (electronic excitation), then return to its electronic ground state by emitting a photon photon of lower energy



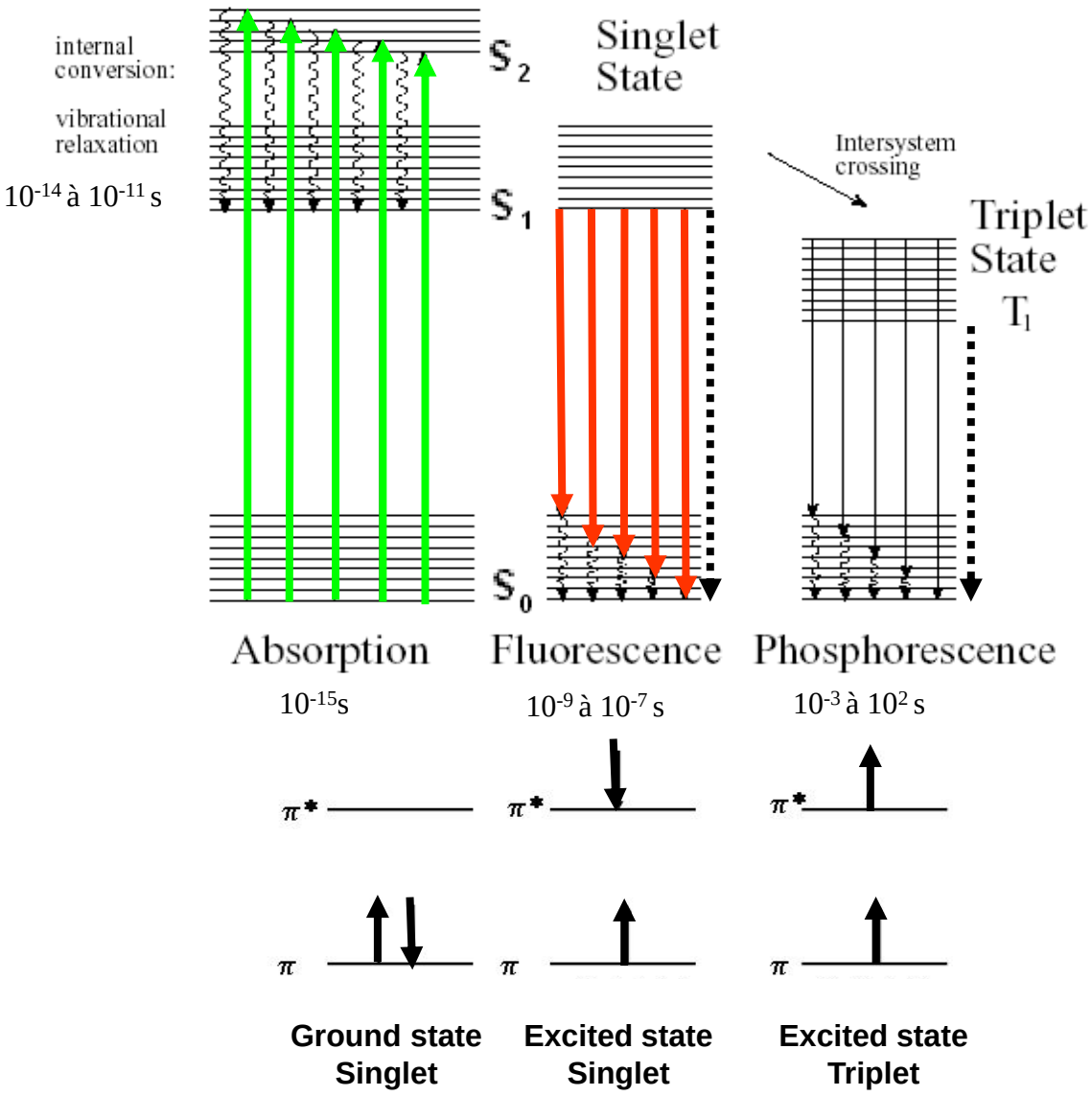
$$E = \frac{hc}{\lambda}$$



(ultra)-simplified Jablonski diagram

Fluorescence basics

JABLONSKI DIAGRAM

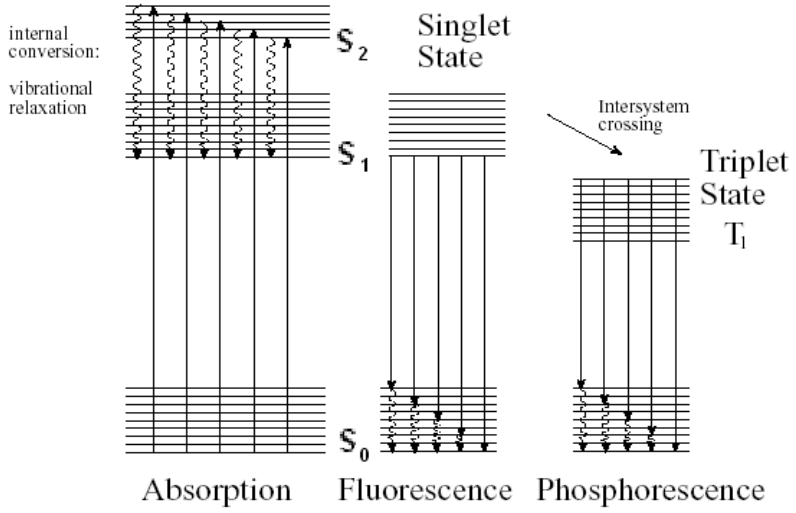


Photon emission

Non radiative relaxation
(no photon emission)

Fluorescence basics

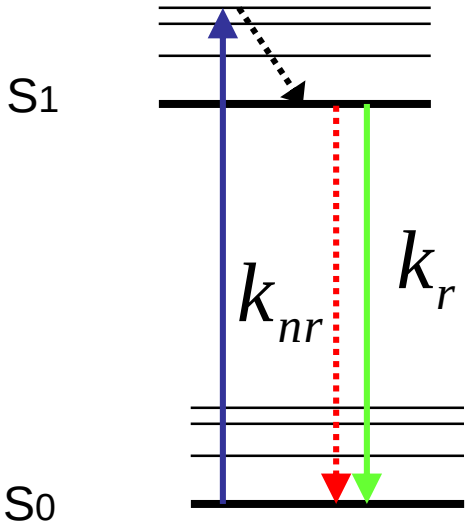
A fluorophore is highly sensitive to its local environment.
Many parameters can influence the properties of these transitions, leading to many different useful observables



- *Color of the emission*
- *Efficiency of the emission (Quantum Yield)*
- *Kinetics of the emission process (Lifetime)*
- *Resonant energy transfer*
- *Quenching*
- *Polarization of the emission*
- *Change in the fluorophore structure*

When the fluorescent molecule interacts with a partner, one or several of these parameters can change…

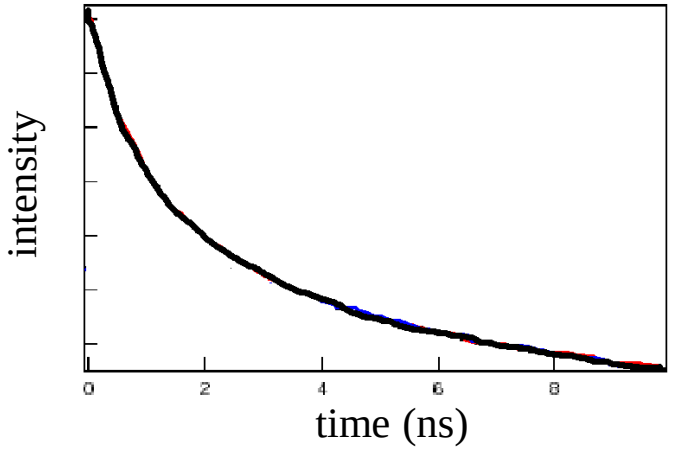
Efficiency of the emission



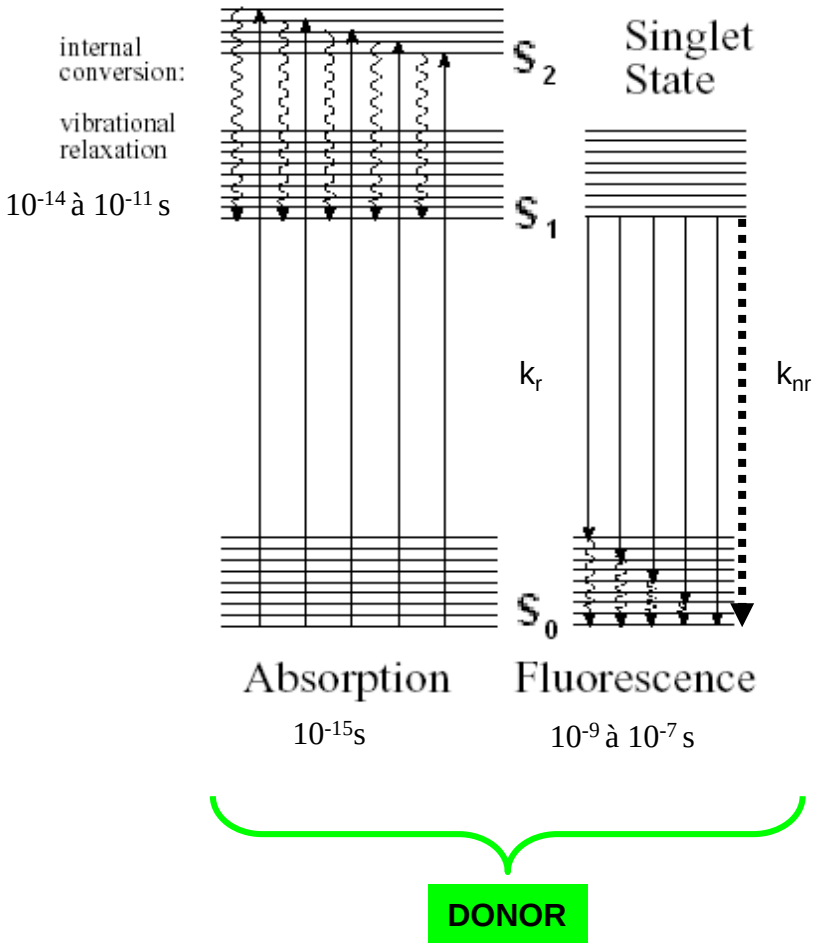
Quantum Yield $$QY = \frac{k_r}{k_r + k_{nr}}$$

Excited state lifetime $$\tau = \frac{1}{k_r + k_{nr}}$$

Excited state lifetime can be measured with pulsed excitation and time resolved detection :
time-resolved fluorescence



Jablonski diagram for FRET

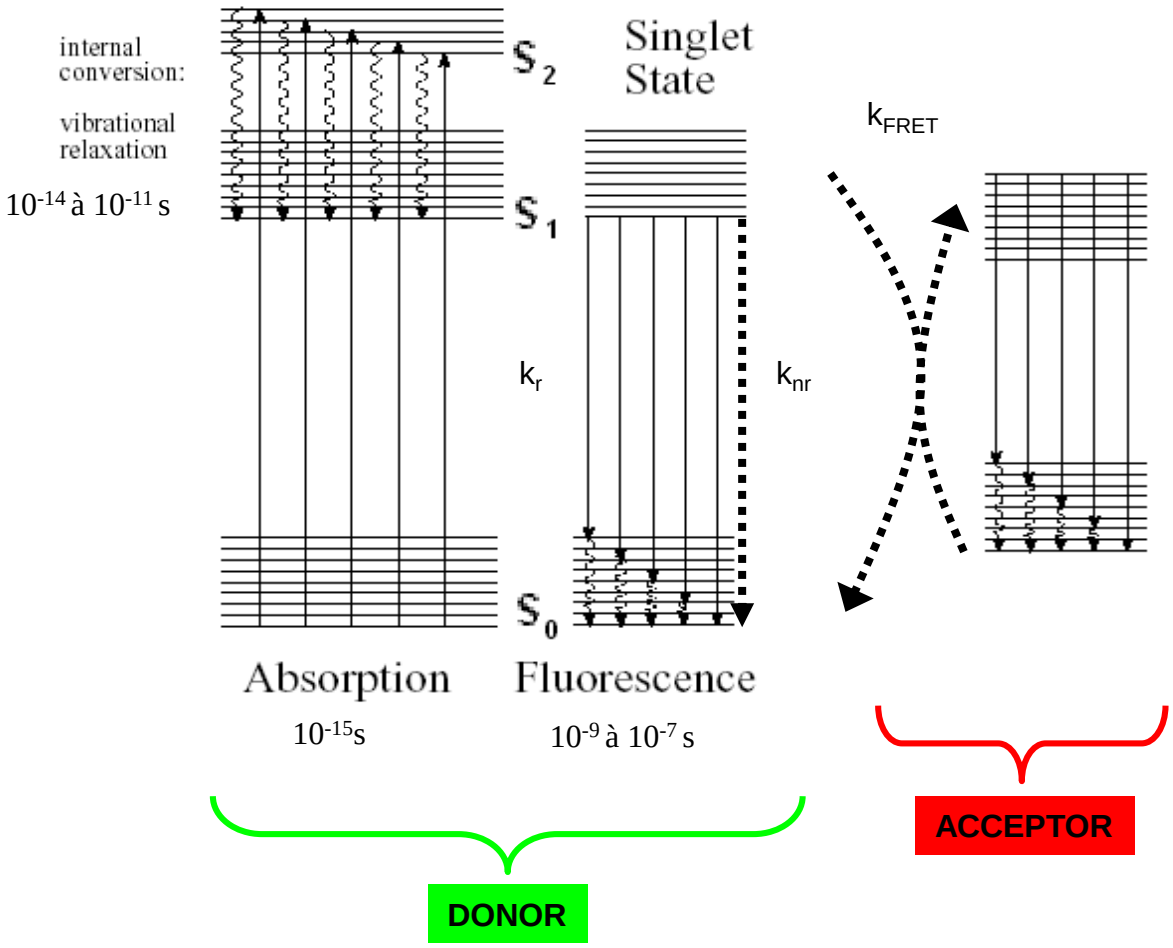


Radiative transition

Non radiative transition
(no emitted photon)

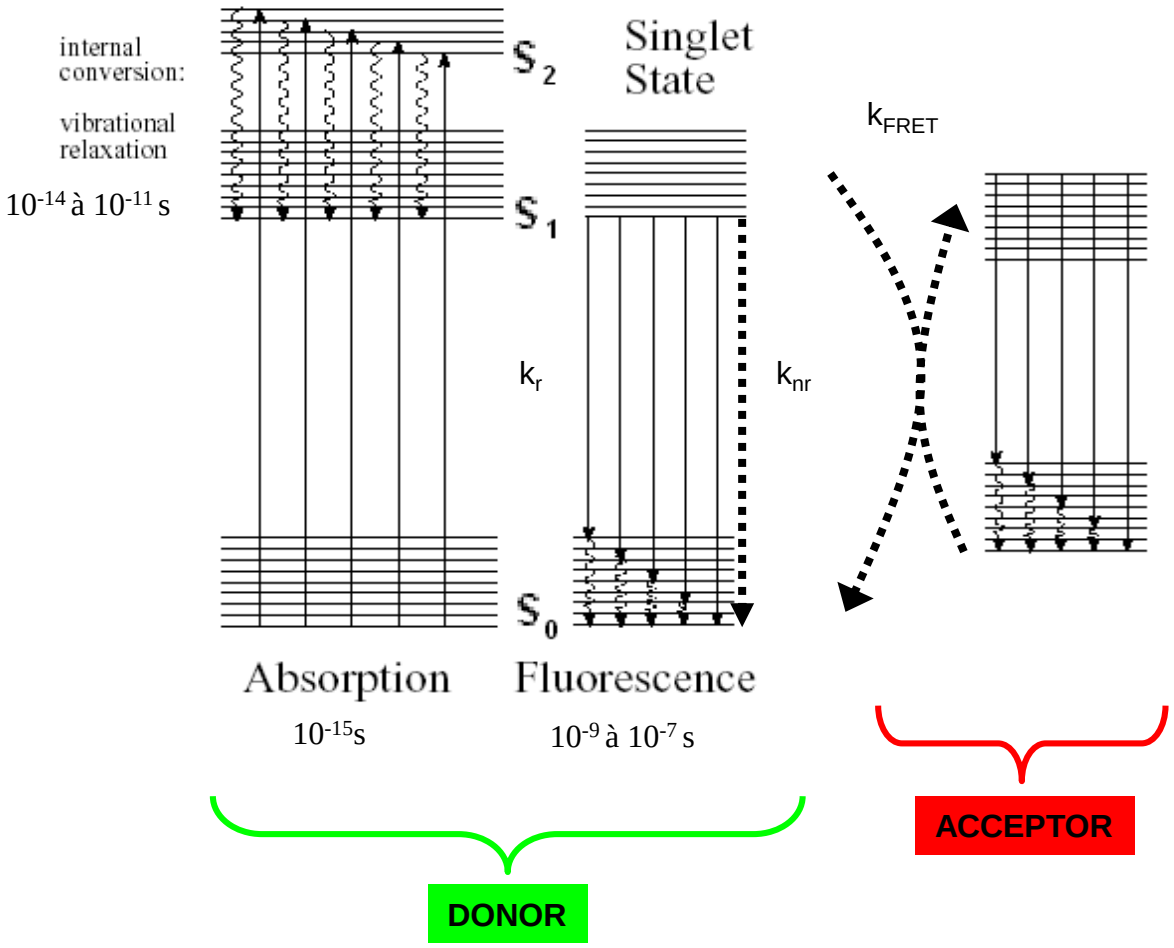
$$\tau_D = \frac{1}{k_r + k_{nr}}$$

Jablonski diagram for FRET



$$\tau_D = \frac{1}{k_r + k_{nr}}$$

Jablonski diagram for FRET

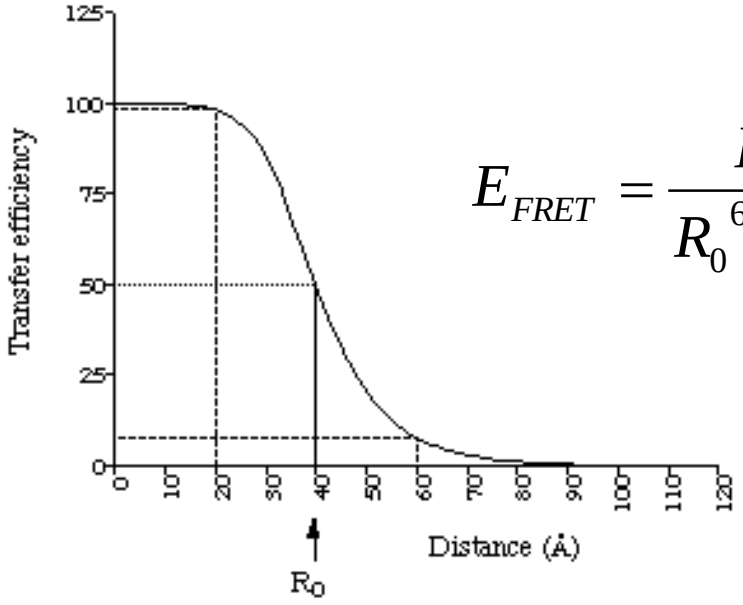


$$\tau_D = \frac{1}{k_r + k_{nr}} \qquad \tau_{D,A} = \frac{1}{k_r + k_{nr} + k_{FRET}}$$

Jablonski diagram for FRET

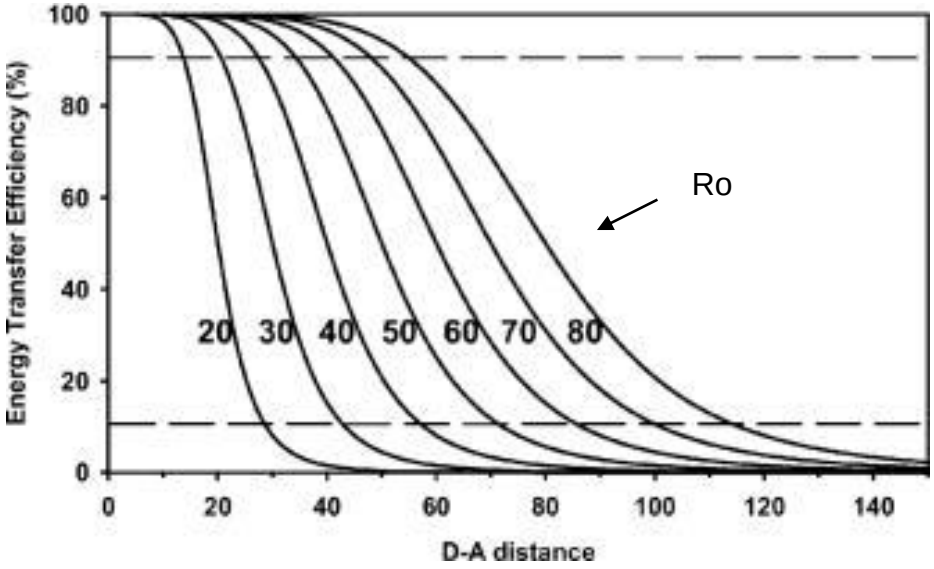
$$\tau_D = \frac{1}{k_r + k_{nr}} \qquad \tau_{D,A} = \frac{1}{k_r + k_{nr} + k_{FRET}} \qquad E = \frac{k_{FRET}}{k_r + k_{nr} + k_{FRET}} = 1 - \frac{\tau_{D,A}}{\tau_D}$$

FRET as a molecular ruler



$$E_{FRET} = \frac{R_0^{\,6}}{R_0^{\,6} + R^6}$$

$$R = R_0 \sqrt[6]{\frac{1-E}{E}}$$



→ FRET measurements are quantitative between 0.5 and 1.5 Ro

FRET : calculation of R_0

$$R_0 = 9780.\sqrt[6]{\Phi_D.\kappa^2.n^{-4}.J(\upsilon)}$$ (in Å)

Il est nécessaire de mesurer ou d'estimer plusieurs facteurs :

→ Φ_D donor quantum yield in the absence of acceptor

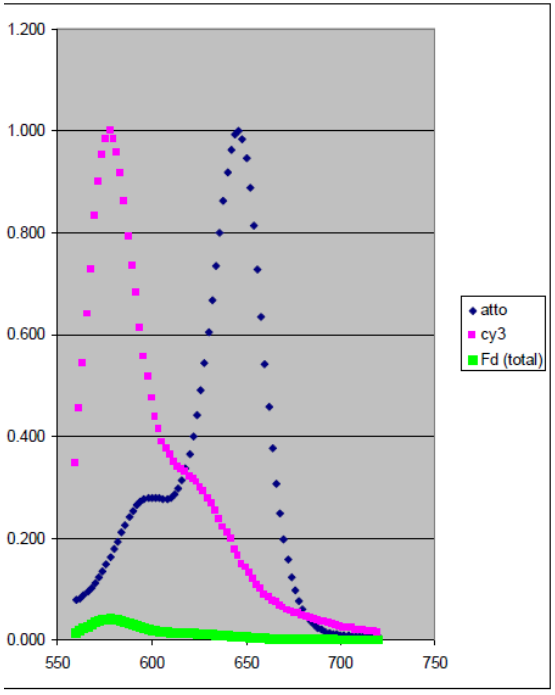
→ *n* = medium refraction index. For biological macromolecular, we estimate n=1.4

→ *J*(υ) : spectral overlap

→ κ^2 : orientation factor

FRET : calculation of R_0

$$J(\upsilon) = \int_0^{\infty} \left(\frac{\varepsilon^A(\upsilon).\phi(\upsilon)}{\upsilon^4} \right) .d\upsilon$$



$\varepsilon^A(\upsilon) =$ **Normalized excitation spectrum of A x** $\varepsilon^A_{\max}$

$\phi(\upsilon)$ **= Normalized fluorescence spectral shape function**
= Normalized emission spectrum of D, whose integral is normalized to 1

$\upsilon = \lambda^{-1}$ (cm^{-1})

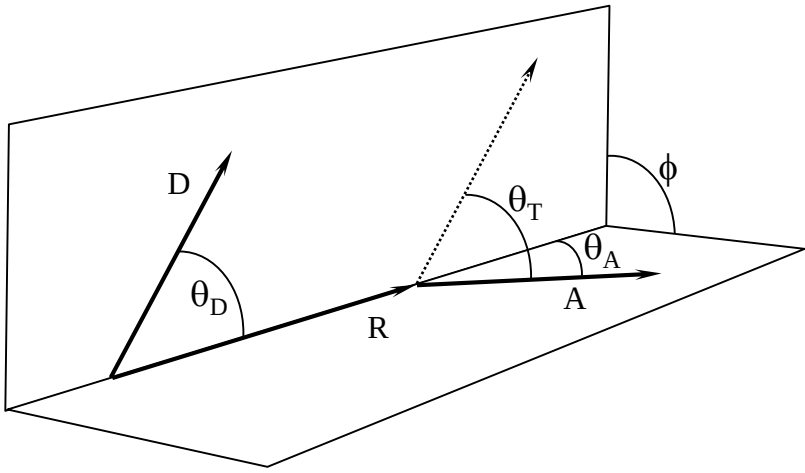
For each wavelength, calculate $\dfrac{\varepsilon^A(\upsilon).\phi(\upsilon)}{\upsilon^4}$

And sum over all wavelengths

Here (cy3 / Atto647N) : $J = 6.0972.10^{-13}$

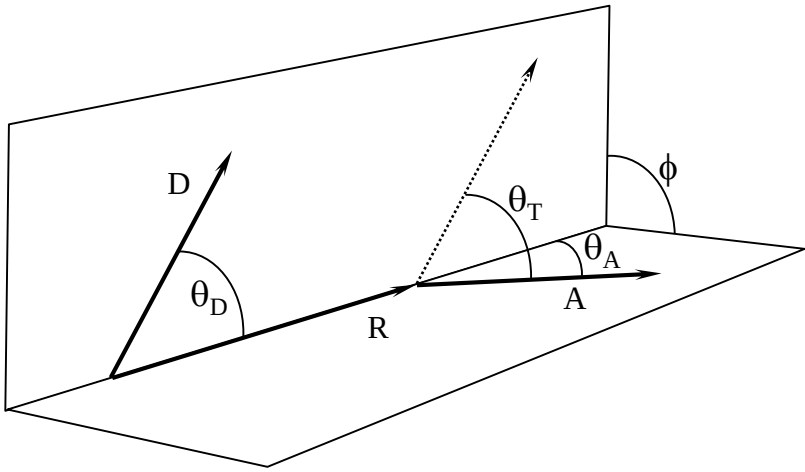
FRET : calculation of R_0

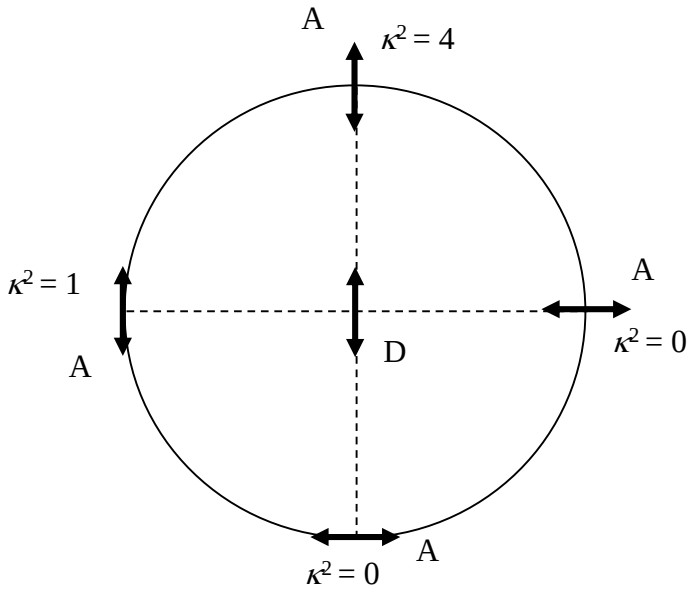
$$\kappa^2 = (\cos\theta_T - 3\cos\theta_D \cos\theta_A)^2$$



FRET : calculation of R_0

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





κ^2 varies from 0 to 4.
The value 4 is obtained when transition moments are colinear with R.
0 is obtained for many orientations.

FRET : calculation of R_0

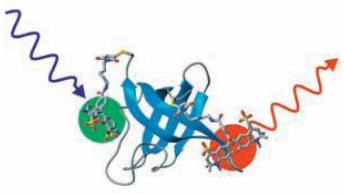
κ^2 = 2/3 for rapid orientational randomization of one of the probes during the donor lifetime

- We use in general 6-carbon linkers to give flexibility

- We can measure the fluorescence anisotropy of the donor and acceptor to check for this rotational diffusion. An anisotropy value <0.2 for a large complex is a good indication of rotational mobility

- We can also use time resolved anisotropy to measure d (or a) , the axial depolarization factor of the donor (or acceptor) transition moment

Dale, R.E., Eisinger, J., Blumberg, W.E., 1979. The orientational freedom of molecular probes: the orientation factor in intramolecular energy transfer. Biophys. J. 26, 161194.



If at least one of the fluorophores is free to rotate, κ^2 = 2/3 is a good approximation

Moreover, the lowest the FRET efficiency, the better the approximation *(Wu and Brand, Biochemistry, 1992, vol. 31, no34, pp. 7939-7947)*

How to measure FRET - I

Measure the donor excited state lifetime in the presence & absence of acceptor

$$E = 1 - \frac{\tau_{DA}}{\tau_D}$$

Absolute measurement
→ FLIM
→ Ensemble
→ Single molecules

Measure the donor quantum yield in the presence & absence of the acceptor

$$E = 1 - \frac{\phi_{DA}}{\phi_D}$$

Mesurement is harder to perform
→ In imaging mode, this the basis of the FRET measurement by photobleaching the acceptor

How to measure FRET - II

Measurement of the number of photons F' emitted by the donor and the acceptor, and their quantum yields :

$$E = \frac{\frac{F'_A}{\Phi_A}}{\frac{F'_D}{\Phi_D} + \frac{F'_A}{\Phi_A}}$$

In practice, we measure a number of detected photons F , related to the emiitted photons $F = \delta F'$

$$E = \frac{F_A}{F_A + \gamma . F_D}$$

Need to estimate / measure γ, that takes into account the quantum yields ϕ and the detection efficiencies

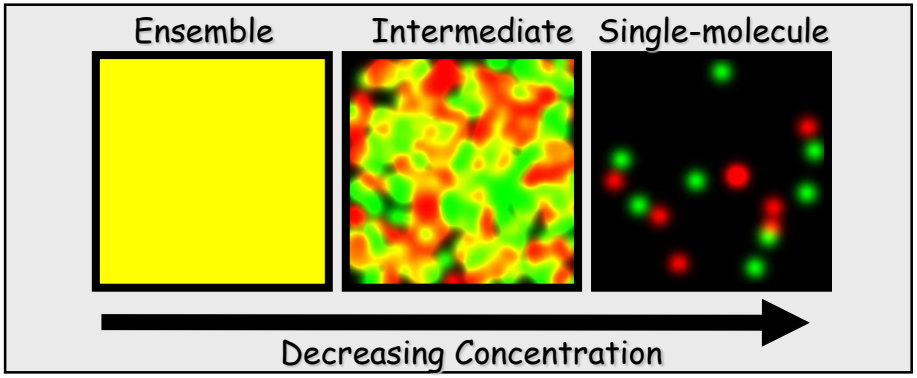
$$\gamma = \frac{\Phi_A . \delta_A}{\Phi_D . \delta_D}$$

Outline

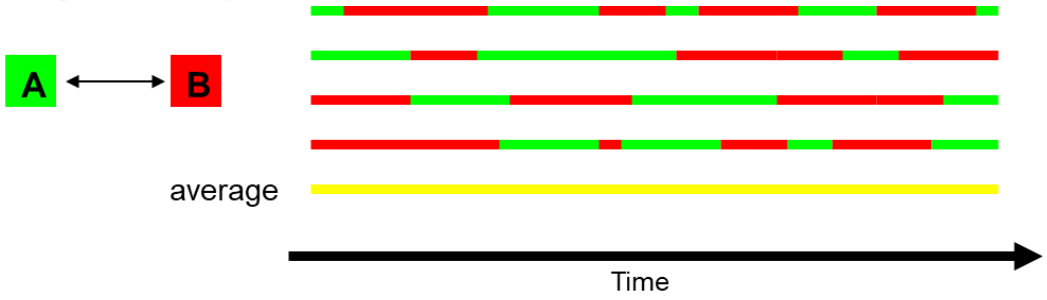
- *Basis of fluorescence & the FRET phenomenon*
- *Observation of single molecules : Why ? How ?*
- *Labeling your molecule of interest*
- *Measurement of distances with smFRET*
- *Measurement of dynamics with smFRET*

Why look at single molecules *in vitro* ?

- Looking at ensemble of molecules generally leads to average values
- In particular, you cannot see static heterogeneities, i.e. the presence of subpopulations :

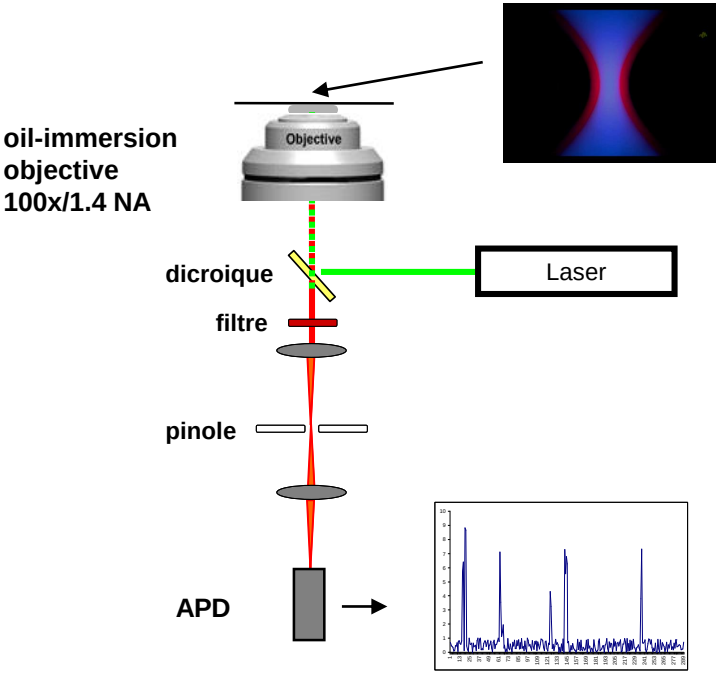


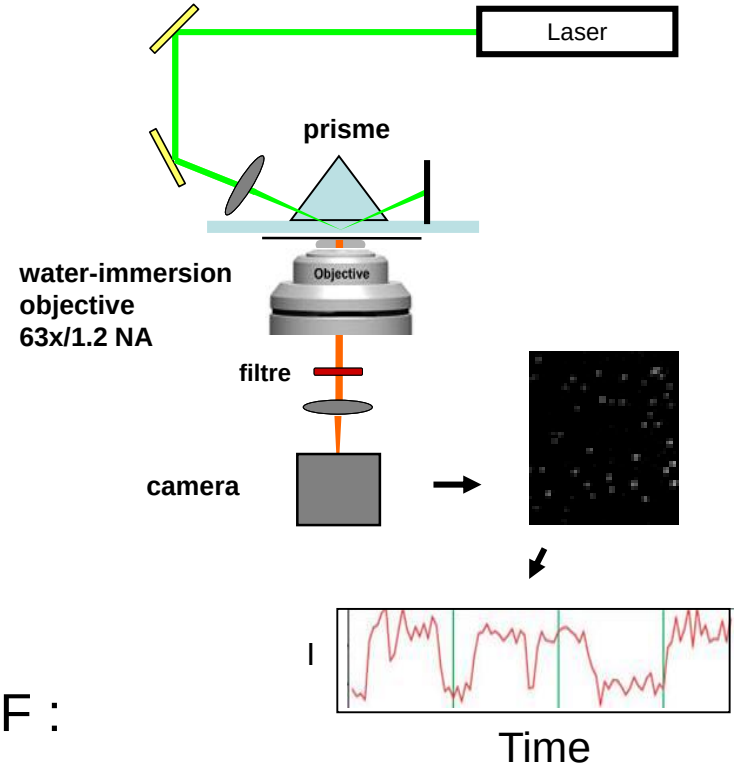
- Ensemble measurement do not allow to see the dynamics of unsynchronized molecules (dynamic heterogeneities)



- In the case of FRET, detect / correct for photophysical effects

How do we look at single molecules *in vitro ?*





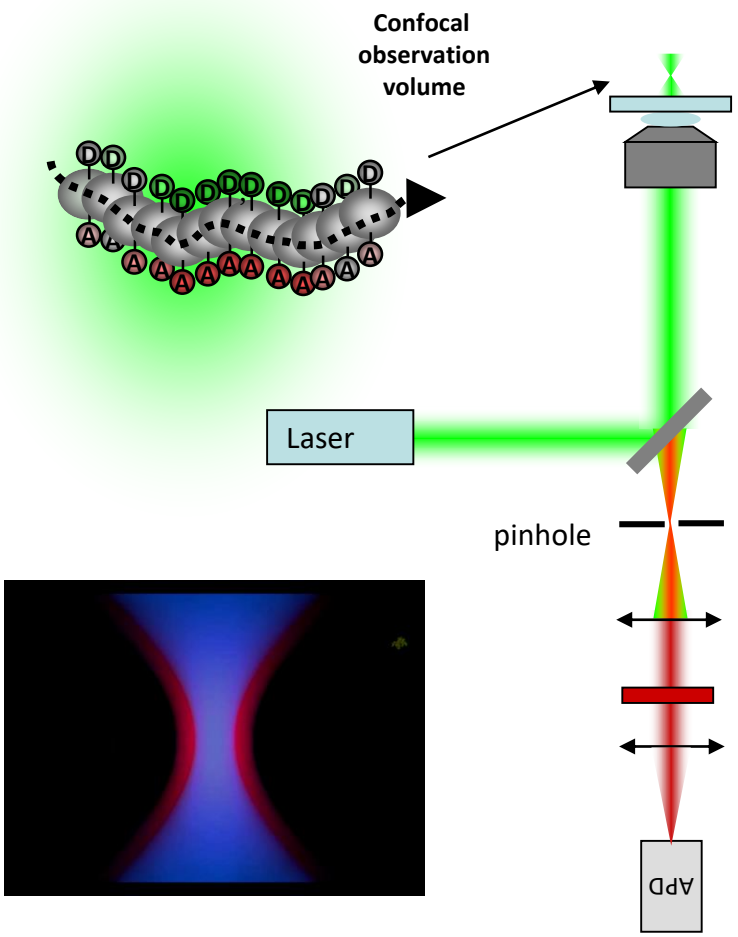
Confocal :

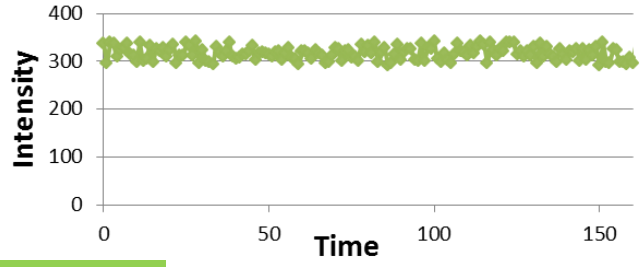
- 2D or 3D diffusing molecules
- 1 molecule at the time
- detection of subpopulation
- High temporal resolution (ns-µs)

TIRF :

- immobilized of 2D diffusing molecules
- hundreds of molecules in parallel
- long time traces for each molecule
- lower temporal resoltion (ms)

Observation regimes in fluorescence

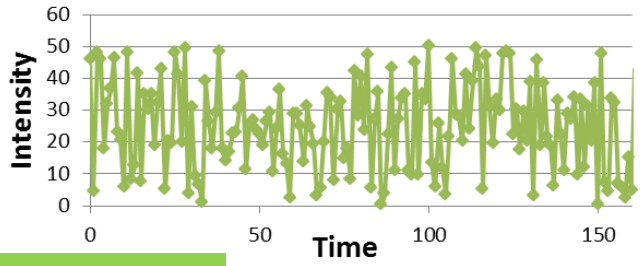




N>1000

[fluo] > µM

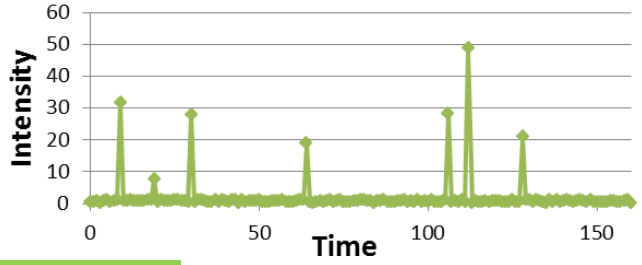
Ensemble regime



N<1000

nM < [fluo] < µM

« Small ensemble » regime

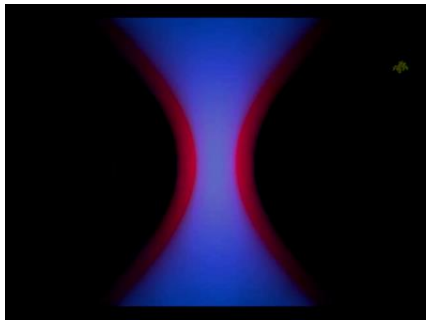


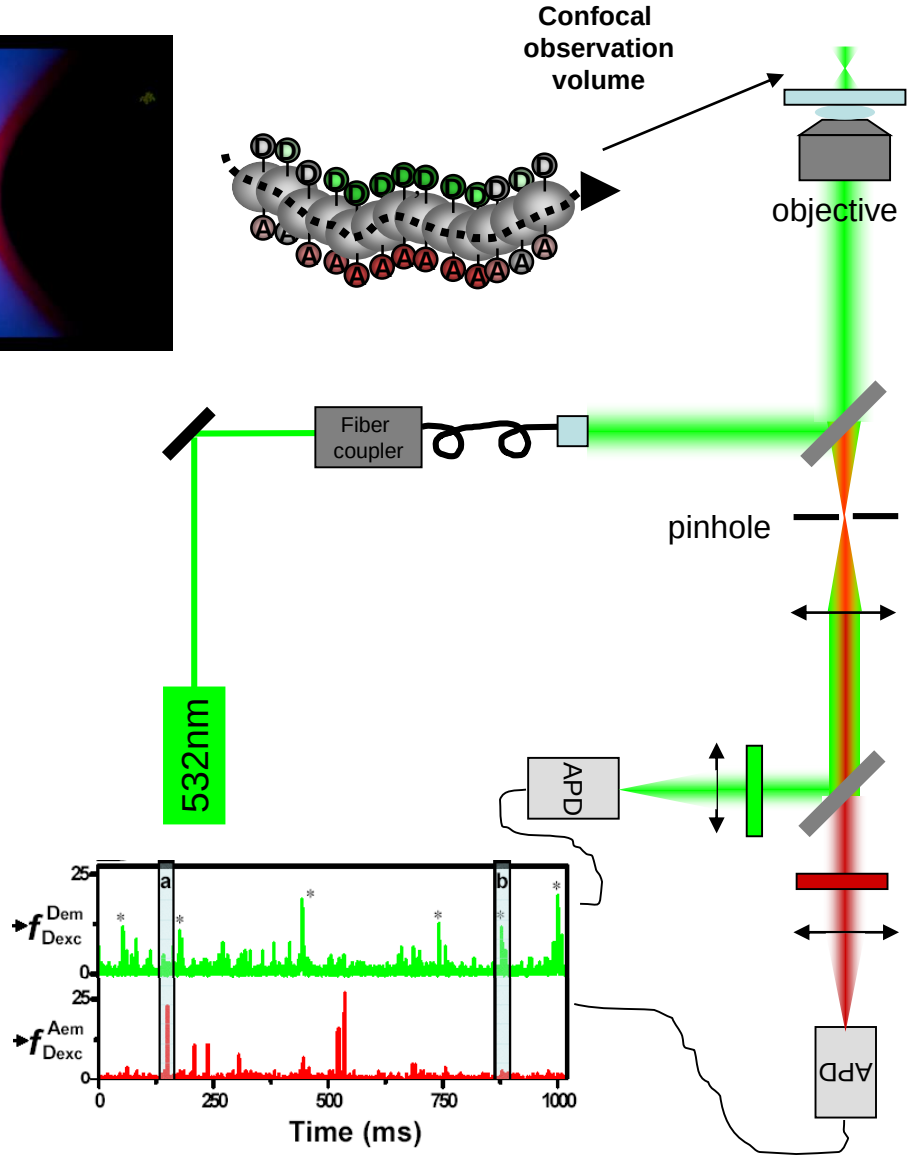
N<1

[fluo] < nM

Single molecules regime

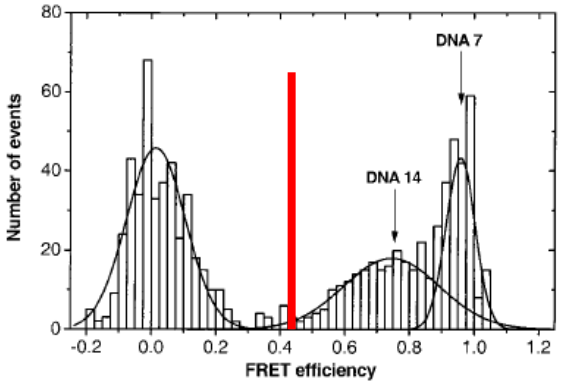
smFRET on diffusing molecules





For each molecule :

$$E_{PR} = \frac{F_{Aem}}{F_{Dem} + F_{Aem}}$$



→ resolve subpopulations

Deniz et al, *PNAS*, 1999

smFRET on diffusing molecules

- ⊕ Not necessary to have a high yield of labeling, or to know it exactly
- ⊕ Ability to recover subpopulations

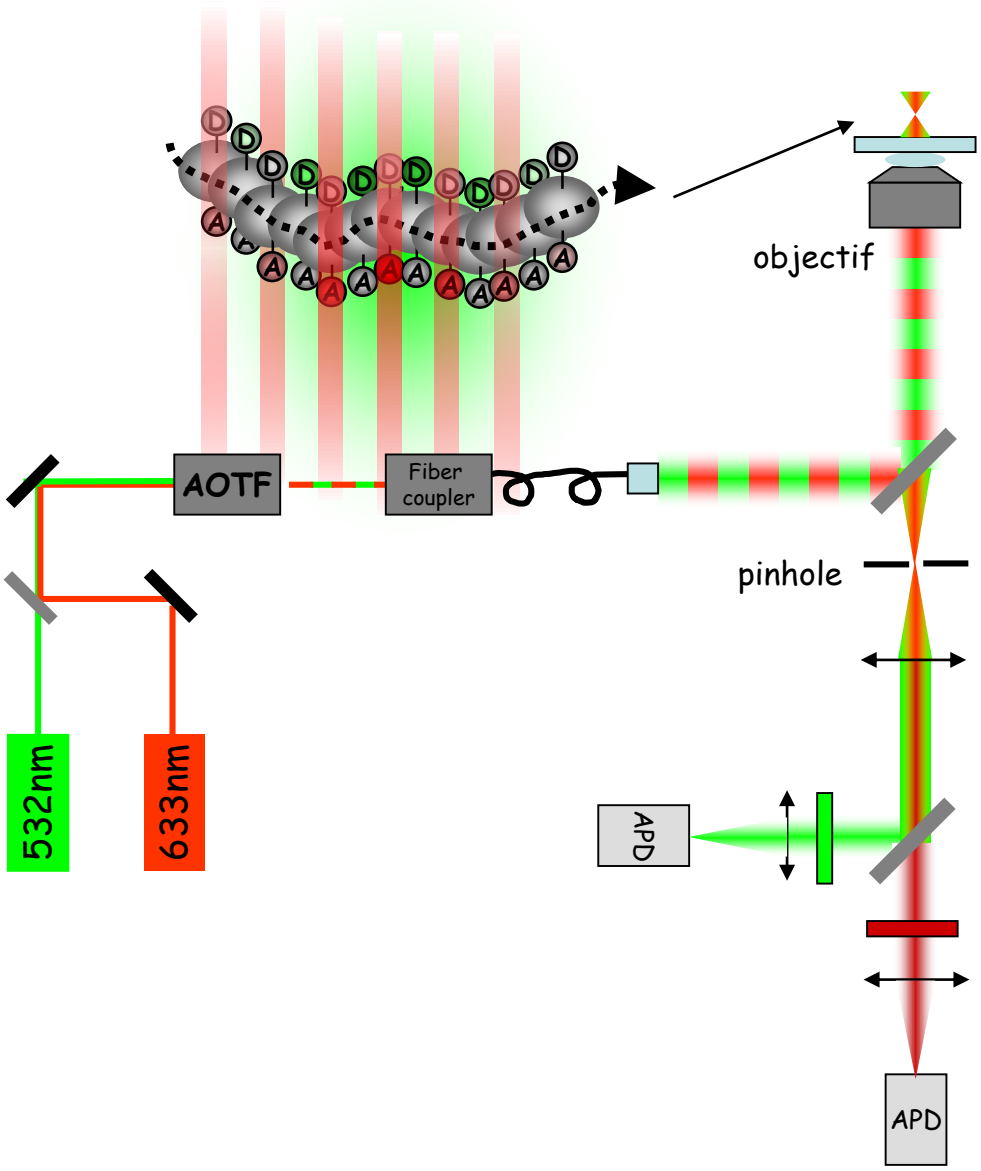
- ⊖ Acceptor photophysics : blinking, bleaching : D-only species
- ⊖ Sensitive to fluorescent contamination, appears as D-only
- ⊖ Unable to recover the correction factors needed to extract quantitative FRET efficiency, and thus distances

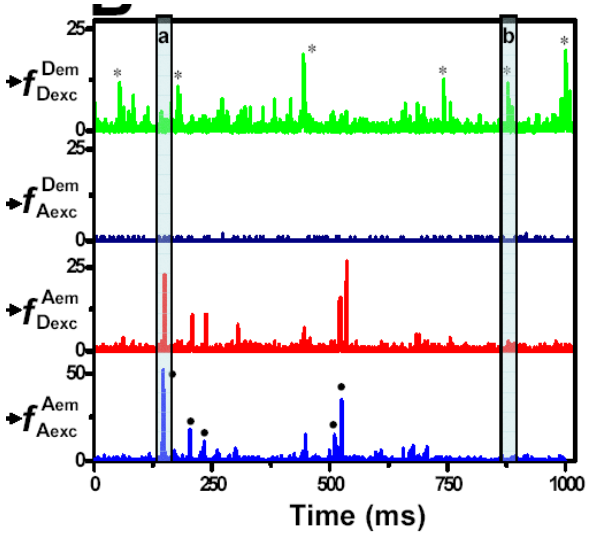
→ Addition of a second laser to directly probe the emission of the acceptor

Alternating Laser EXcitation

Kapanidis et al, *PNAS*, 2004

smFRET with ALEX



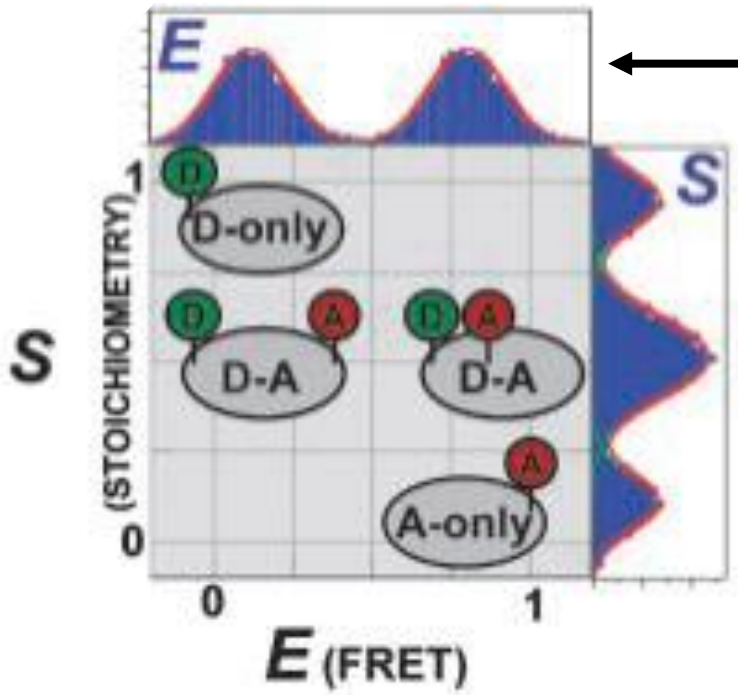


Kapanidis et al, *PNAS*, 2004

Identifying the species with ALEX

For each molécule

$$E_{PR} = \frac{F_{Dex,\,Aem}}{F_{Dex,\,Dem} + F_{Dex,\,Aem}} \qquad S = \frac{F_{Dex}}{F_{Dex} + F_{Aex}}$$



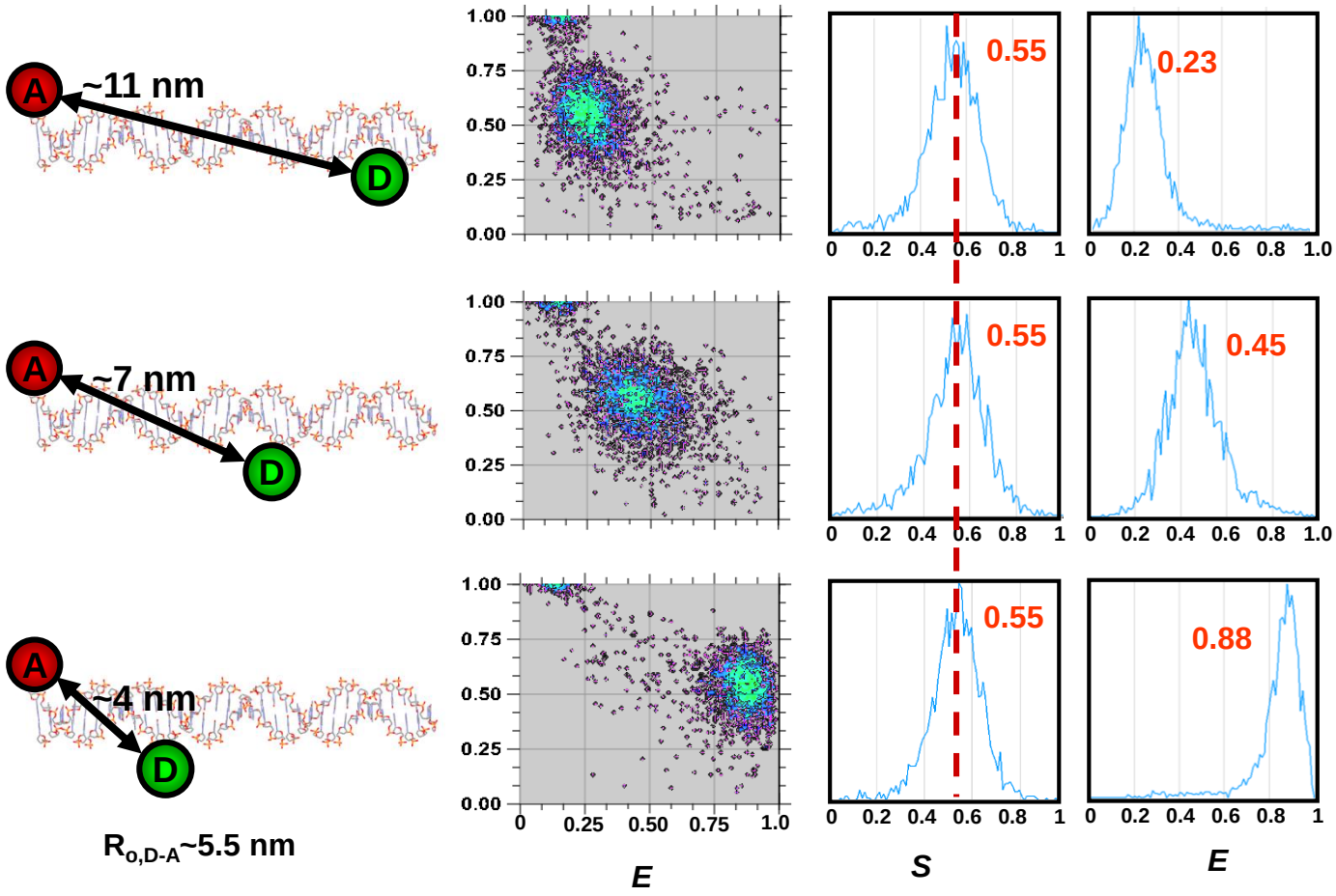
E_{PR} : distance between fluorophores

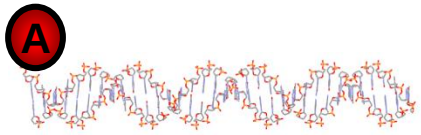
S : Presence of each fluorophores → Interaction

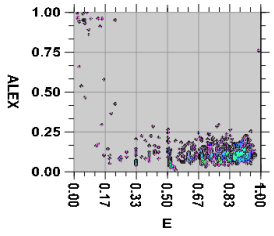
If D and A are on different molecules

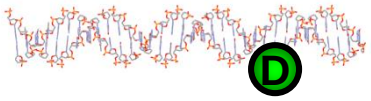
Kapanidis et al, *PNAS*, 2004

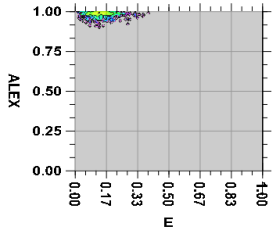
Identifying the species with ALEX



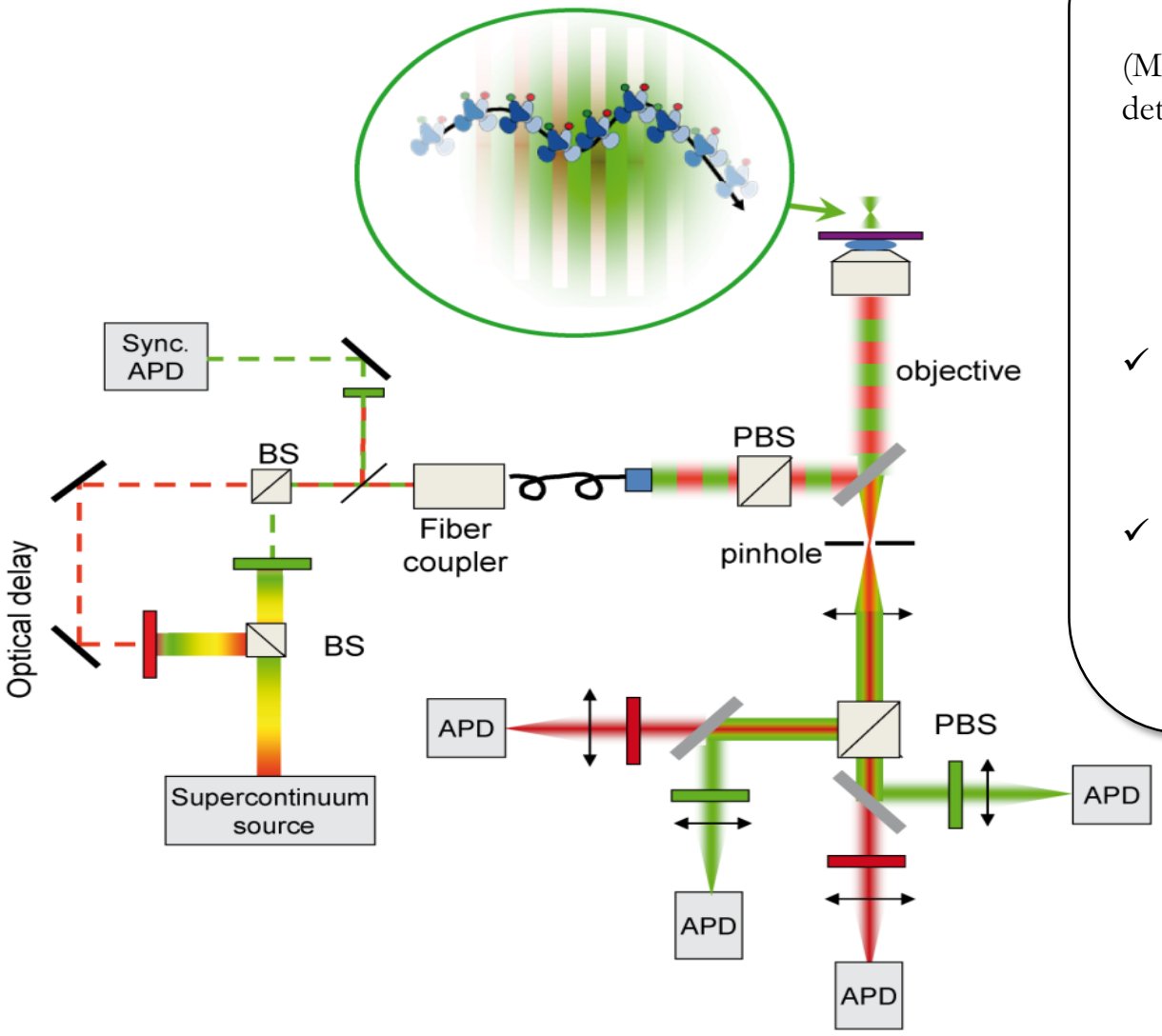








Multiparametric fluorescence spectroscopy



- ✓ Pulsed excitation, TCSPC detection mode and 4 SPADs

(MFD : Multiparametric fluorescence detection)

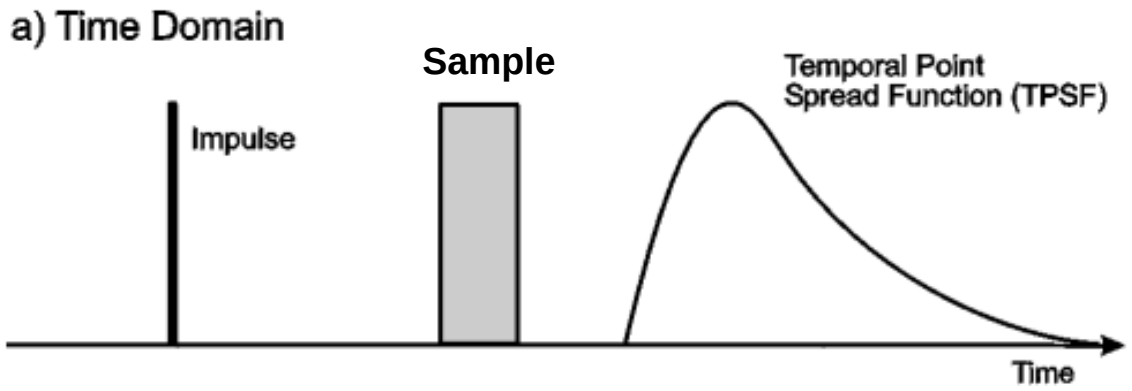
  - Excited state lifetime ($\tau_{D(A)}$)
  - Anisotropy (r)
  - ratiometric FRET (E_{PR})

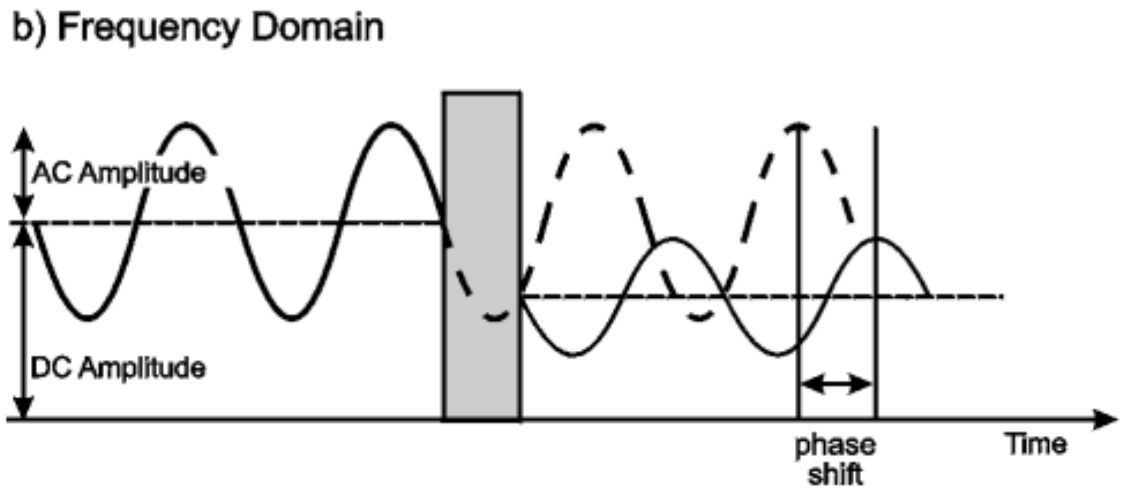
- ✓ PIE (~ ALEX)
  - Stoichiometry and photophysical artifacts
- ✓ Supercontinuum source
  - 100ps pulses
  - Broad spectrum

Seidel, Weiss, Lamb labs

Olofsson & Margeat , *Optics Express*, 2013

Measuring excited-state lifetimes



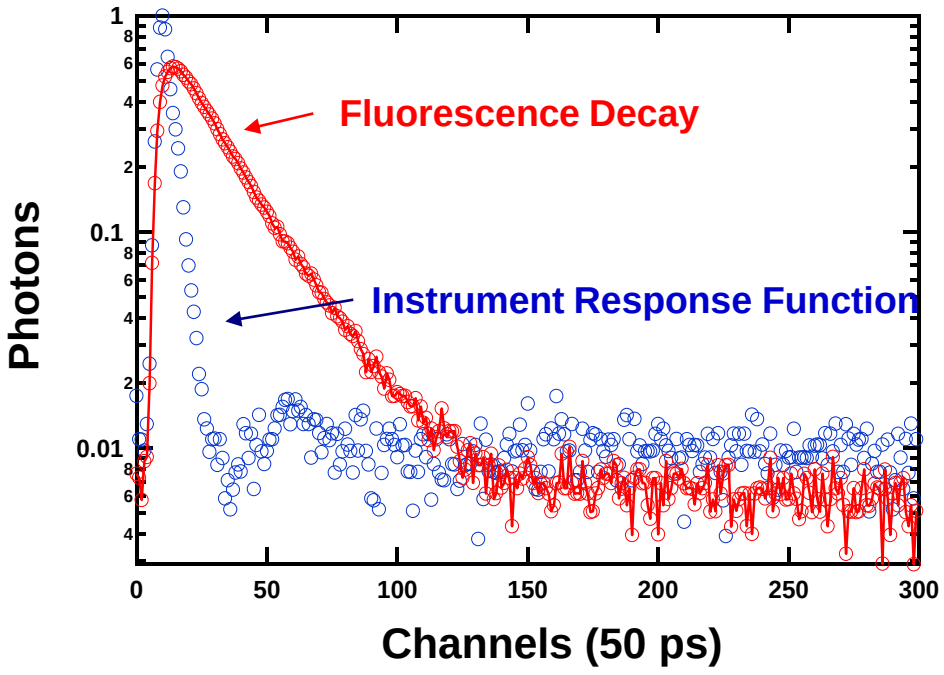


For single molecules

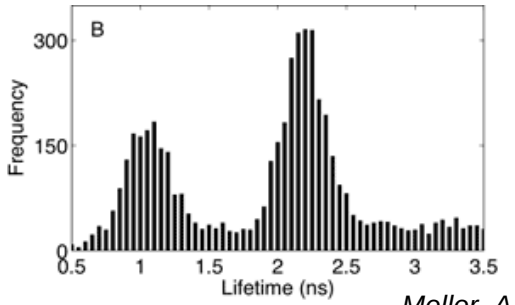
OK

X

Measuring excited-state lifetimes

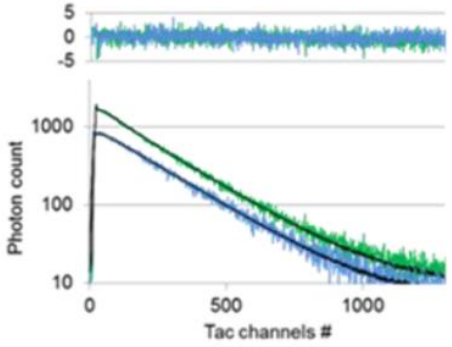


Assuming a single exponential decay
► approximate excited state lifetime for each single molecule

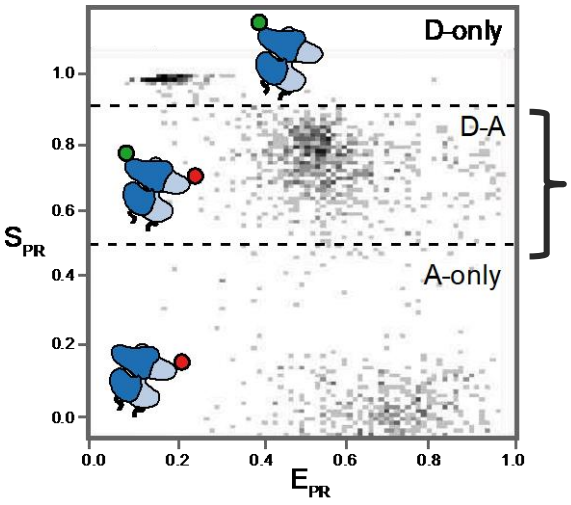


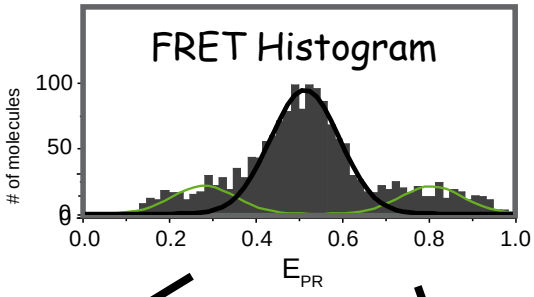
Meller, A.

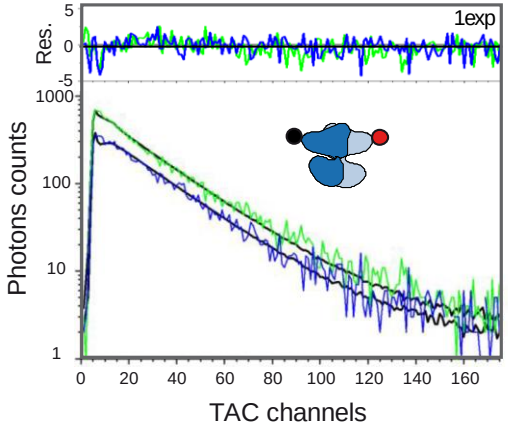
Photons from hundreds of molecules can be combined and fitted with a more accurate model



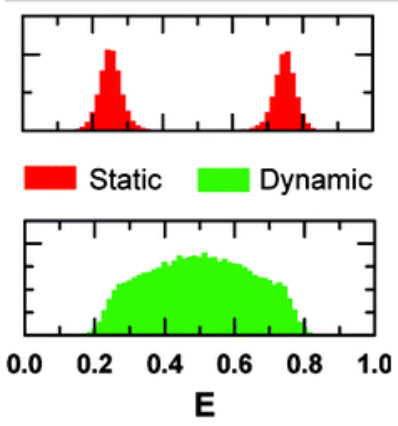
smFRET on diffusing molecules

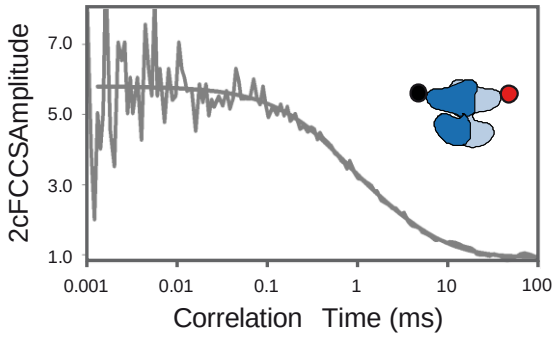






Excited state lifetime





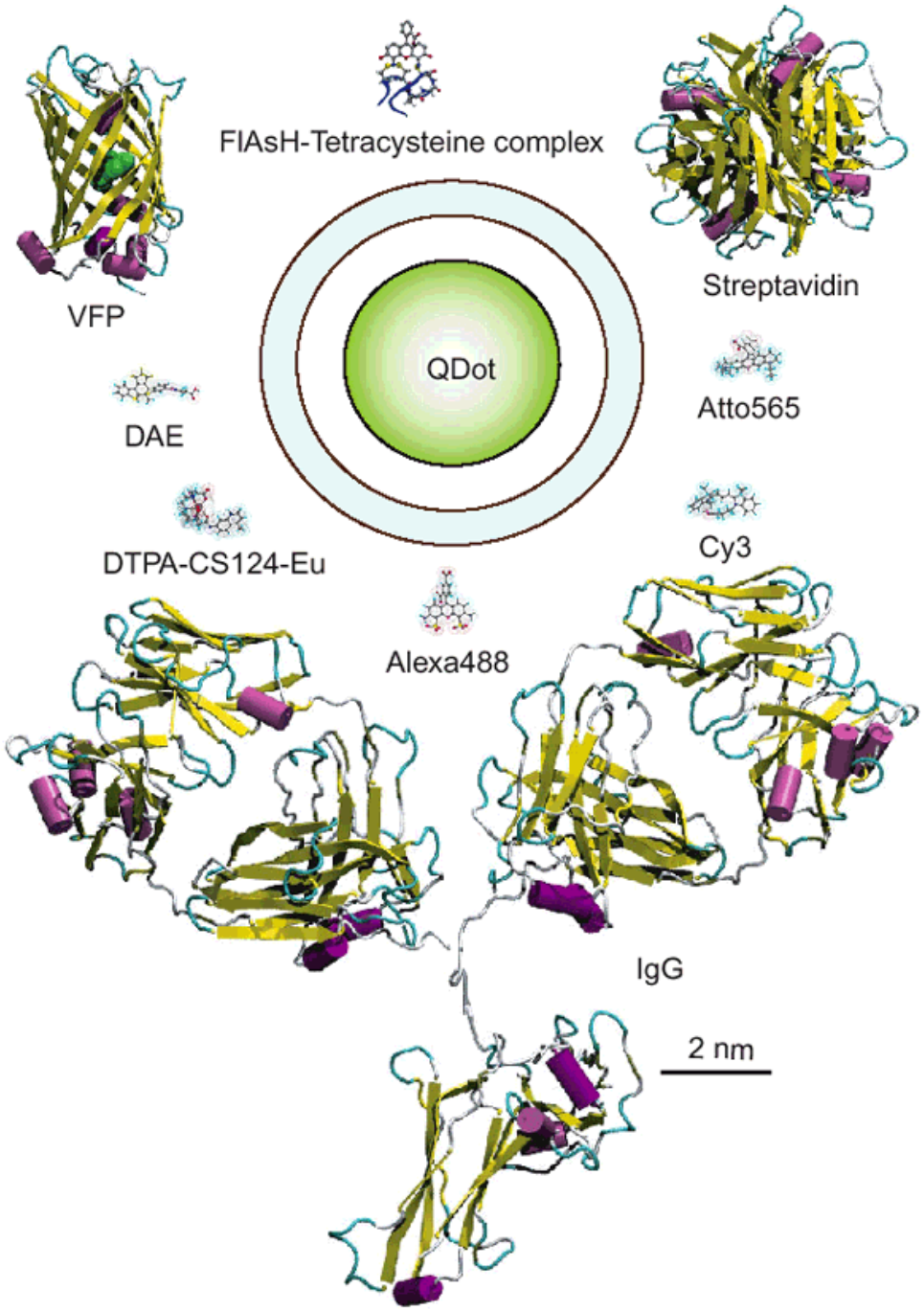
Correlation analysis (~FCS)

+ For each molecule :
- Lifetimes
- Anisotropy
- Photon flux
- Diffusion time
- Stoichiometry ….

Outline

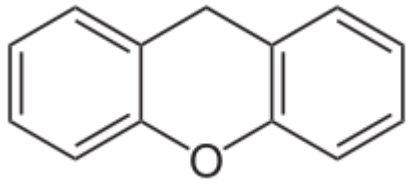
- *Basis of fluorescence & the FRET phenomenon*
- *Observation of single molecules : Why ? How ?*
- *Labeling your molecule of interest*
- *Measurement of distances with smFRET*
- *Measurement of dynamics with smFRET*

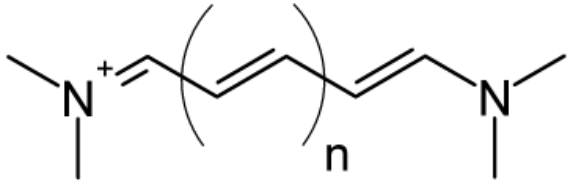
Tailles comparees de differents marqueurs fluorescents

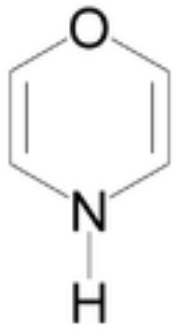


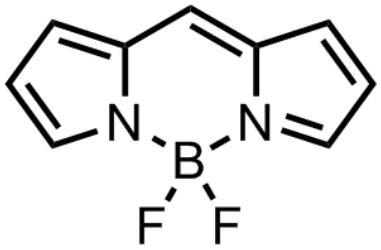
Organic fluorophores

- Various chemical structures
  - Xanthenes
  - Cyanines
  - Oxazine
  - Bodipy
  - Perylene
  - Napthalene
  - Coumarine
  - Acridine
  - ....









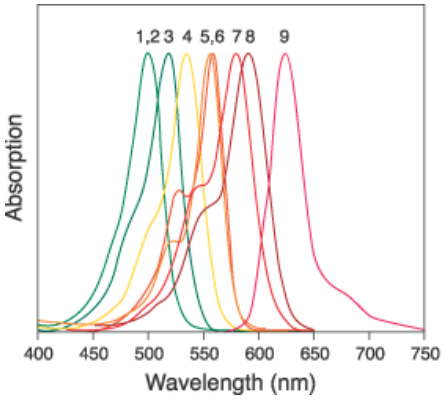
Organic fluorophores

- Various brand names
  - Cy dyes (Cy 3, Cy5) : Amersham
  - Alexa fluor (Invitrogen)
  - DyLight (Thermo / Pierce)
  - Atto dyes (Atto-Tec)
  - Fluoprobes (Interchim)
  - Abberior
  - Dy and megastockes (Dyomics)
  - .....

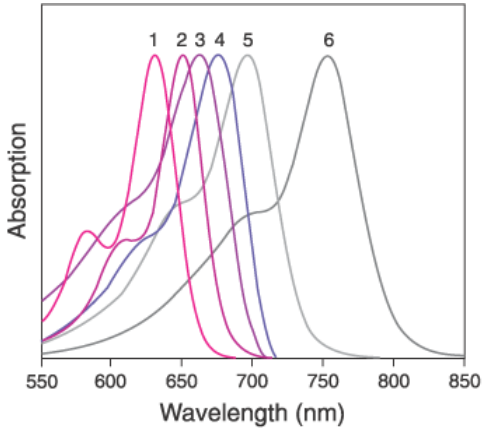
THERE IS NO CORRELATION BETWEEN THE NAME AND THE STRUCTURE, AND THUS, THE PROPERTIES !!!

Example of Alexa dyes

Derivatization of existing dyes to make them mores soluble, more photostables, or to circumvent patents...

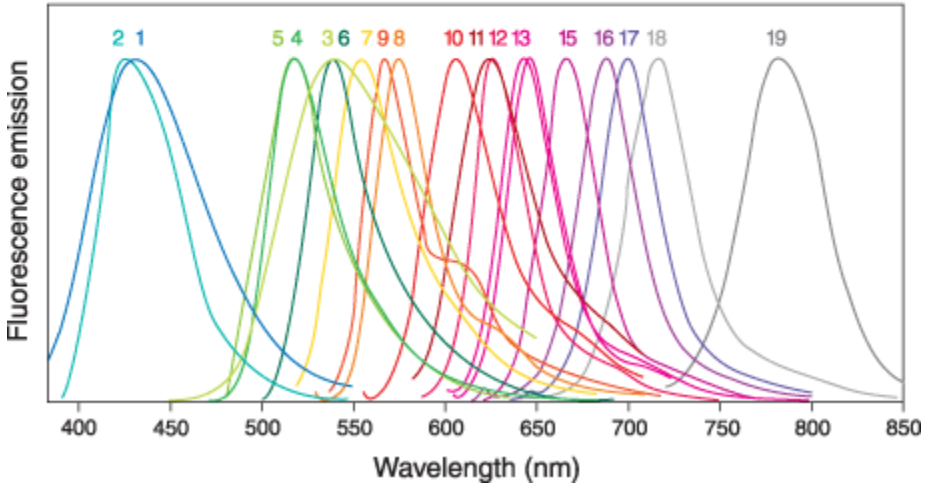


1 Alexa Fluor 488
2 Alexa Fluor 500
3 Alexa Fluor 514
4 Alexa Fluor 532
5 Alexa Fluor 555
6 Alexa Fluor 546
7 Alexa Fluor 568
8 Alexa Fluor 594
9 Alexa Fluor 610



1 Alexa Fluor 633
2 Alexa Fluor 647
3 Alexa Fluor 660
4 Alexa Fluor 680
5 Alexa Fluor 700
6 Alexa Fluor 750

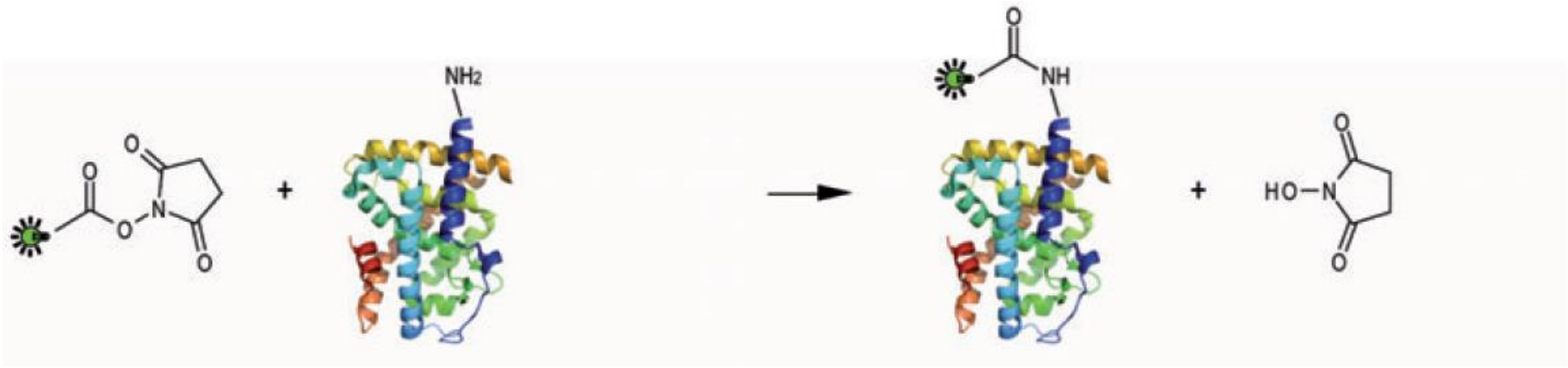
1. Alexa Fluor 350
2. Alexa Fluor 405
3. Alexa Fluor 430
4. Alexa Fluor 488
5. Alexa Fluor 500
6. Alexa Fluor 514
7. Alexa Fluor 532
8. Alexa Fluor 546
9. Alexa Fluor 555
10. Alexa Fluor 568
11. Alexa Fluor 594
12. Alexa Fluor 610
13. Alexa Fluor 633
14. Alexa Fluor 635
15. Alexa Fluor 647
16. Alexa Fluor 660
17. Alexa Fluor 680
18. Alexa Fluor 700
19. Alexa Fluor 750



How to label your molecule of interest with fluorophores

REACT ON PRIMARY AMINES

Proteines : N-terminal or lyzine
Oligonucleotides : NH_2 added during sythesis



N-hydroxy-succinimidyl ester (NHS)

Around pH7, the lyzines are protonated, and thus only the N-terminus is reactive.

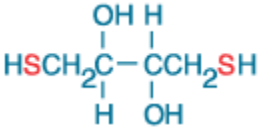
How to label your molecule of interest with fluorophores

REACT ON THIOLS

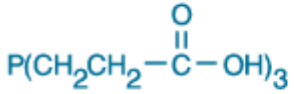
Proteins : cysteins

Maleimide

TO REDUCE CYSTEINS, USE TECP



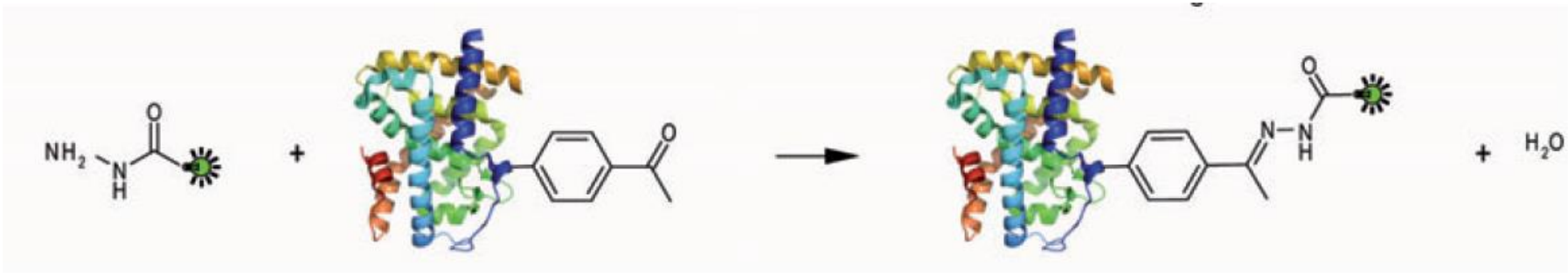
(A) dithiothreitol (DTT)



(B) tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP)

How to label your molecule of interest with fluorophores

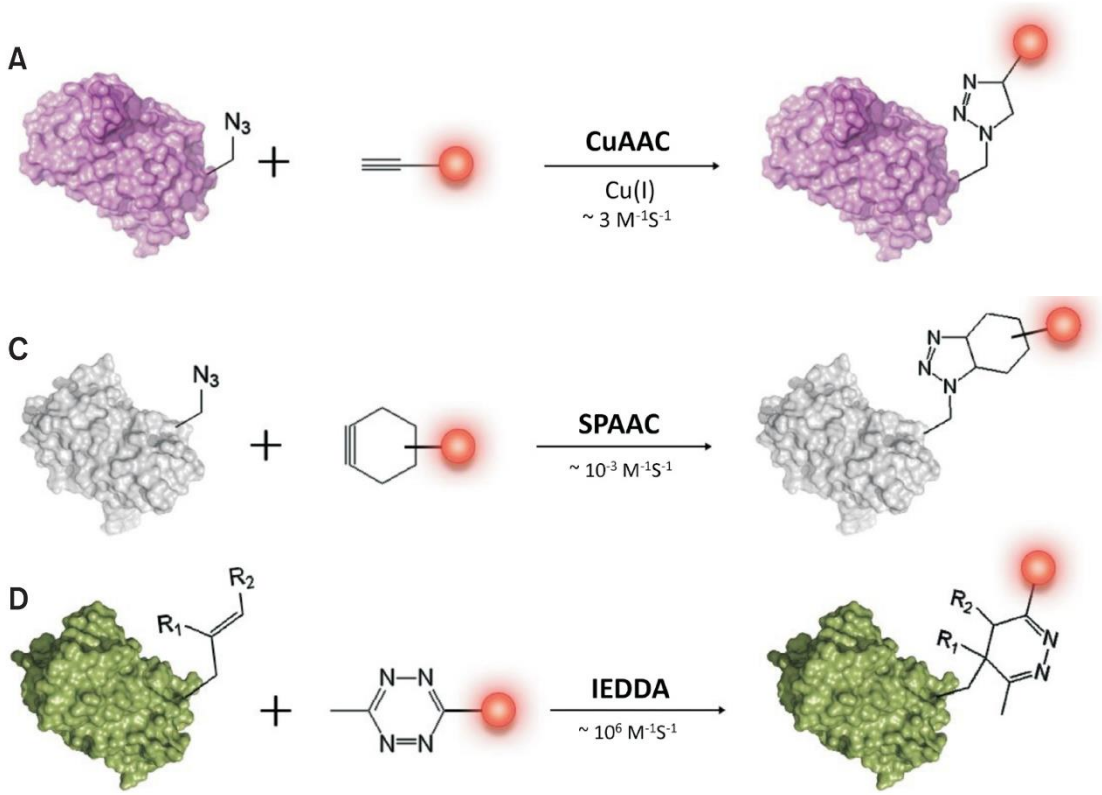
REACT ON UNNATURAL AMINO ACIDS



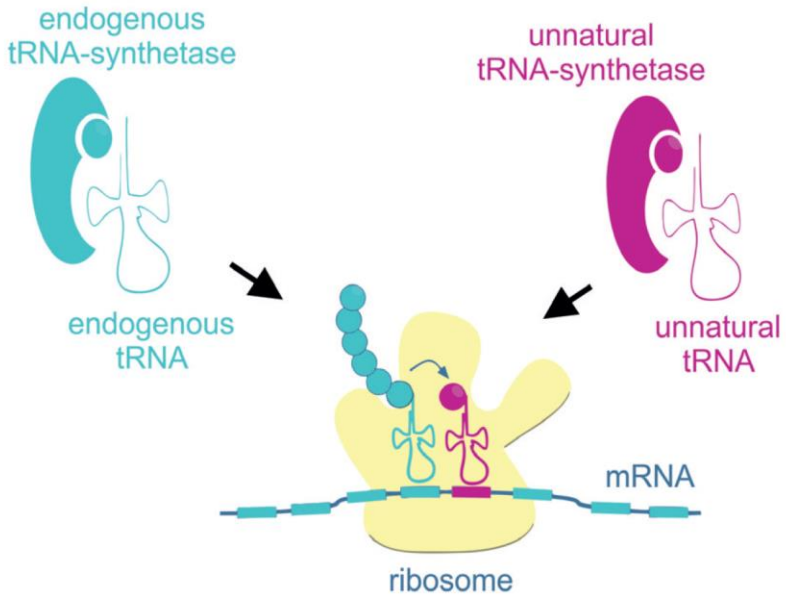
One of the first reactions described : fluorophore hydrazide on p-acetyl phenylalanine

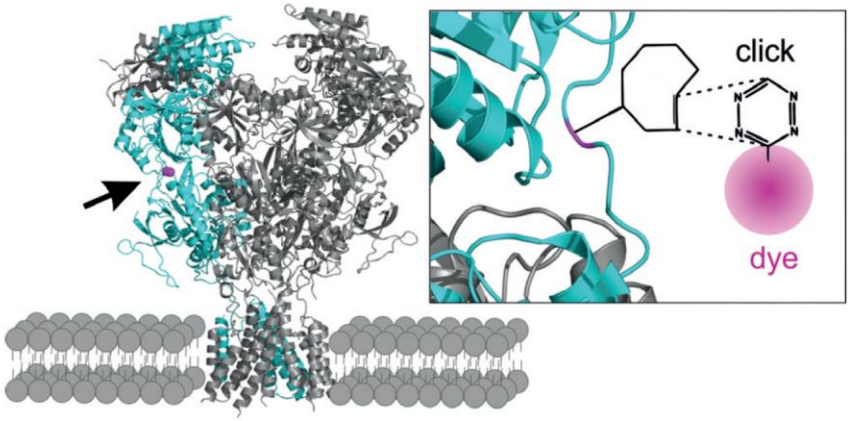
NOW : Development of click-chemistry

Labeling unnatural aminoacids



Labeling unnatural aminoacids

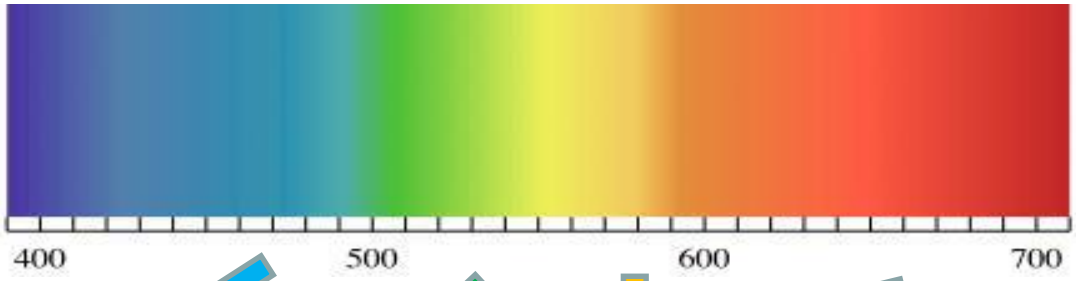




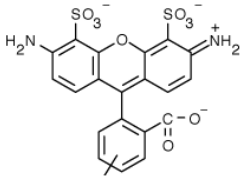
- Any position can be labeled
- Native cysteins are kept
- Vast (and increasing) choice of fluorophores
- Orthogonal labeling possible
- Live cell labeling (beware of Cu^{2+}

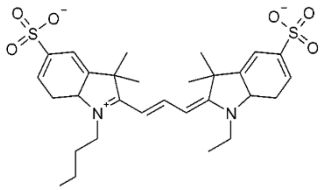
- Non specific incorporation of the UAA
- Reagents are expensive

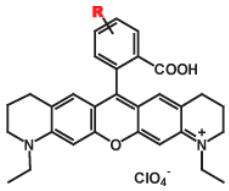
Popular dyes for single molecule FRET

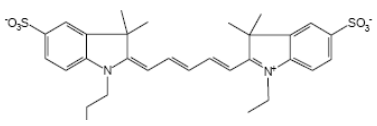


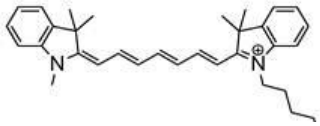
Alexa 488 Atto 488	TMR Cyanine 3 derivatives	Alexa 594 Atto 610	Cyanine 5 derivatives Atto 647N Atto 655	Cyanine 7 derivatives











Outline

- *Basis of fluorescence & the FRET phenomenon*
- *Observation of single molecules : Why ? How ?*
- *Labeling your molecule of interest*
- *Measurement of distances with smFRET*
- *Measurement of dynamics with smFRET*

How to measure a distance ?

$$R_0 = 9780.\sqrt[6]{\Phi_D.\kappa^2.n^{-4}.J(\upsilon)}$$ (in Å)

$$R = R_0 \sqrt[6]{\frac{1-E}{E}}$$

$$E = \frac{F_{A(FRET)}}{F_{A(FRET)} + \gamma . F_D}$$

$F_D = F_{Dex}^{Dem}$ is straighforward to obtain

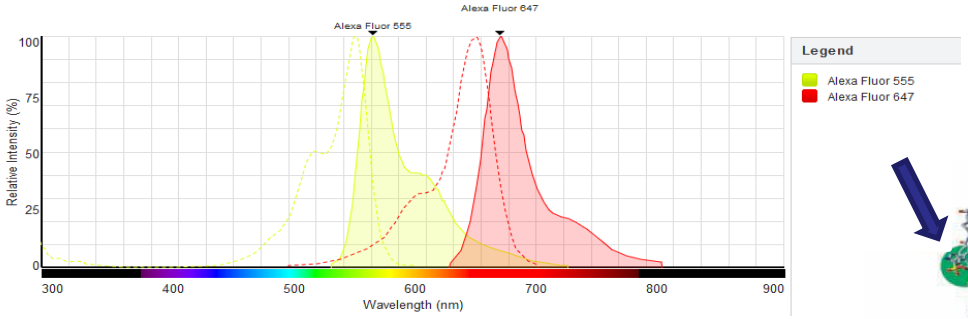
$F_{A(FRET)}$ Crosstalk factors need to be accounted for

γ Needs to be partly calculated, partly determined empirically

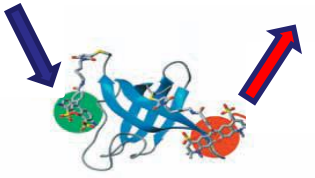
Correcting for spectral crosstalk

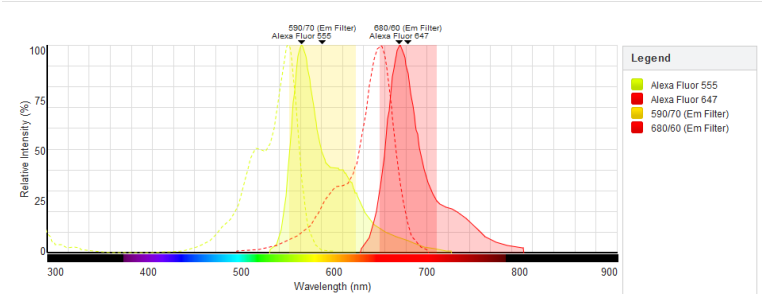
$$F_{FRET} = F_{Dex}^{Aem} - Lk - Dir$$

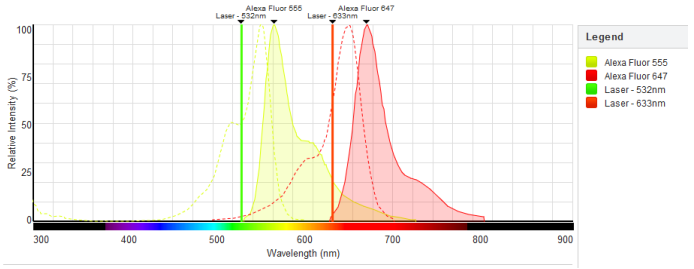
F_{Dex}^{Aem} is the observable

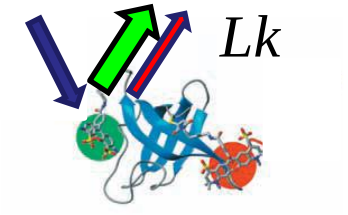


F_{FRET}





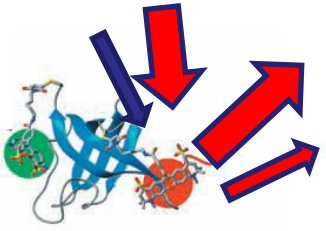




Lk

Donor leakage in acceptor channel

Dir



Direct excitation of the acceptor

Determination of the γ factor

$$\gamma = \frac{\Phi_A . \delta_A}{\Phi_D . \delta_D}$$

Φ

- The quantum yields can be measured in solution (cuvette) for a D-only and A-only labeled sample
- Alternative methods based on FCS exist, but have not been evaluated

$\frac{\delta_A}{\delta_D}$

The absolute detection efficiency measurement is extremely difficult

but what matters is the ratio

This ratio is instrument & dyes dependant. You can use a « standard » to determine it once.

CellPress

Structure
Meeting Review

Outcome of the First wwPDB Hybrid/Integrative Methods Task Force Workshop

Andrej Sali,[1,*] Helen M. Berman,[2] Torsten Schwede,[3] Jill Trewhella,[4] Gerard Kleywegt,[5] Stephen K. Burley,[2,6] John Markley,[7] Haruki Nakamura,[8] Paul Adams,[9,10] Alexandre M.J.J. Bonvin,[11] Wah Chiu,[12] Matteo Dal Peraro,[13] Frank Di Maio,[14] Thomas E. Ferrin,[15] Kay Grünewald,[16] Aleksandras Gutmanas,[5] Richard Henderson,[17] Gerhard Hummer,[18] Kenji Iwasaki,[19] Graham Johnson,[20] Catherine L. Lawson,[2] Jens Meiler,[21] Marc A. Marti-Renom,[22] Gaetano T. Montelione,[23,24] Michael Nilges,[25,26] Ruth Nussinov,[27,28] Ardan Patwardhan,[5] Juri Rappsilber,[29,30] Randy J. Read,[31] Helen Saibil,[32] Gunnar F. Schröder,[33,34] Charles D. Schwieters,[35] Claus A.M. Seidel,[36] Dmitri Svergun,[37] Maya Topf,[32] Eldon L. Ulrich,[7] Sameer Velankar,[5] and John D. Westbrook[2]

Structure *23*, July 7, 2015

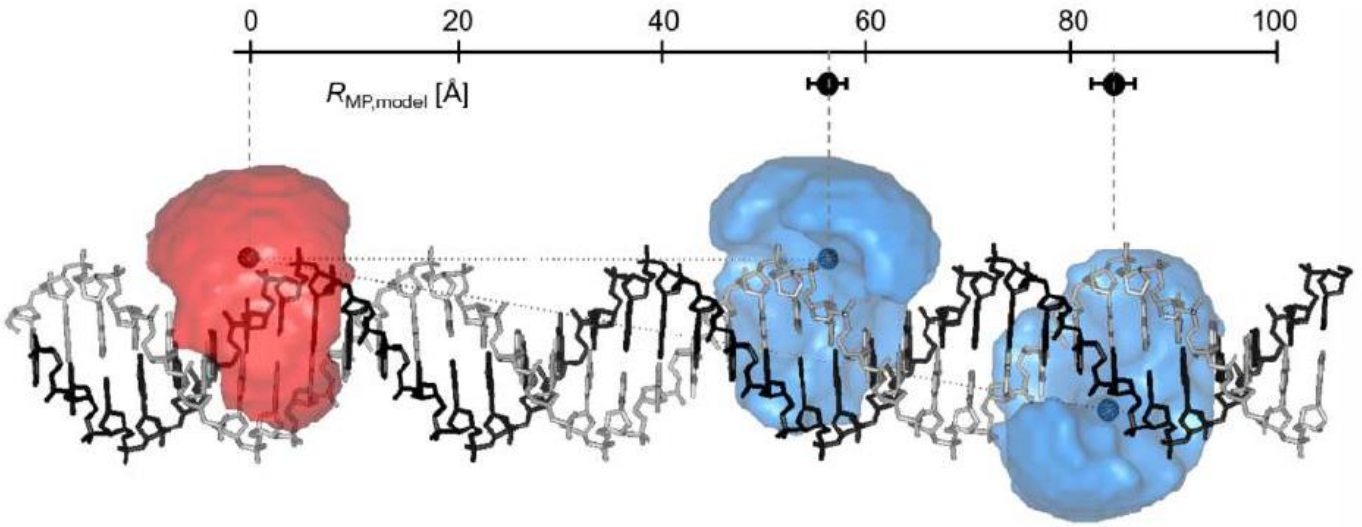
Precision and accuracy of single-molecule FRET measurements – a worldwide benchmark study

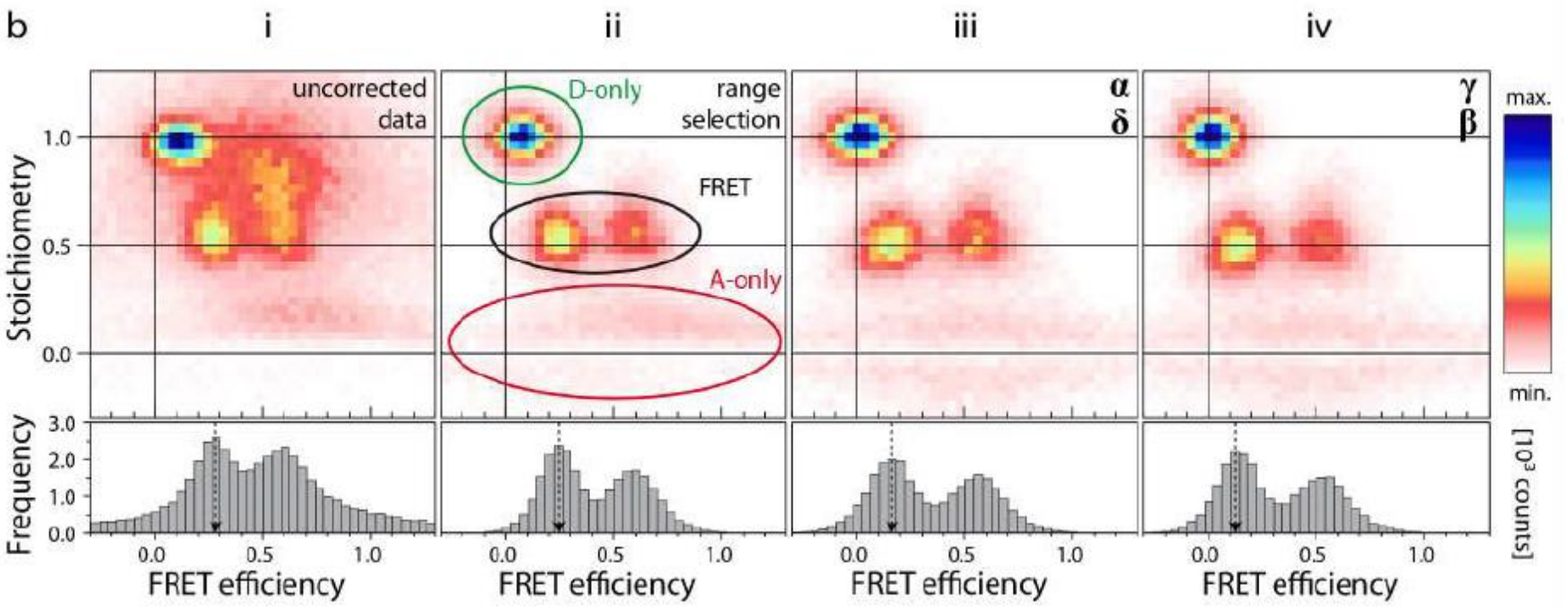
arXiv:1710.03807v1 [q-bio.QM] 10 Oct 2017

NATURE METHODS | VOL 15 | SEPTEMBER 2018 | 669–676

Here we report the results of a worldwide, comparative, blind study, in which 20 labs determined the FRET efficiencies of several dye-labeled DNA duplexes. Using a unified and straightforward method, we show that FRET efficiencies can be obtained with a standard deviation between ΔE = ±0.02 and ±0.05

Result of the benchmark study on nucleic acids





i : raw data

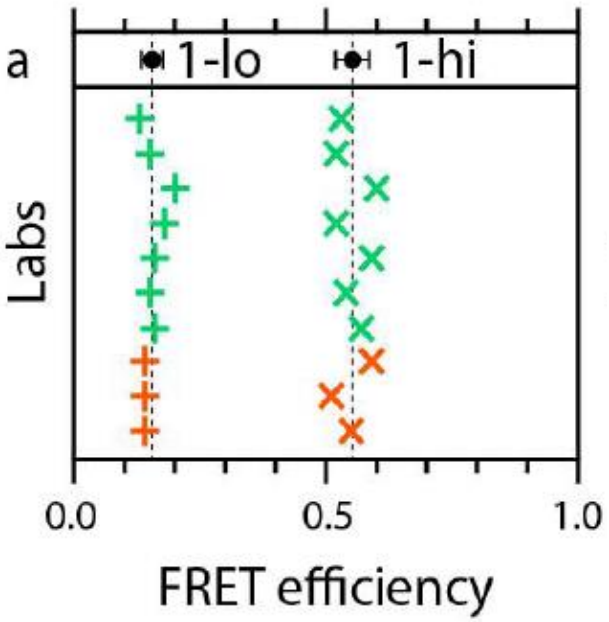
ii : Background correction : determined from Buffer-only sample

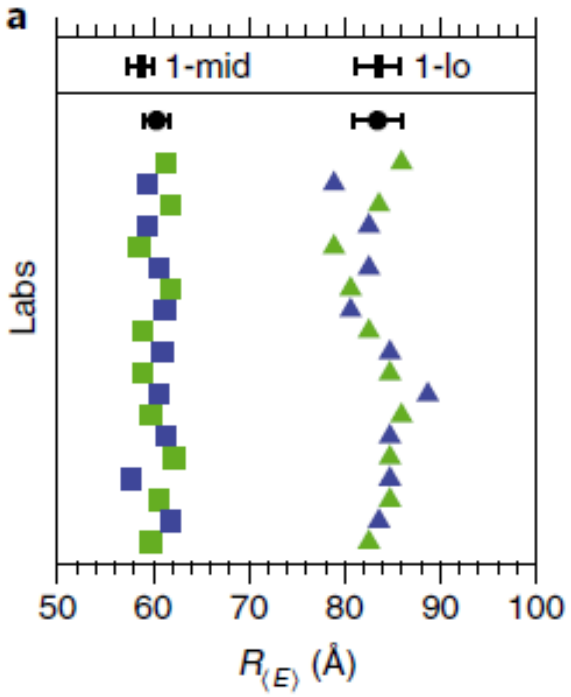
iii : Correction for Leakage (α) and Direct Excitation (δ)

- This is obtained thanks to ALEX

iv : Correction for relative laser intensities and the γ factor

Result of the benchmark study on nucleic acids





Static model
Exp. mean

Confocal

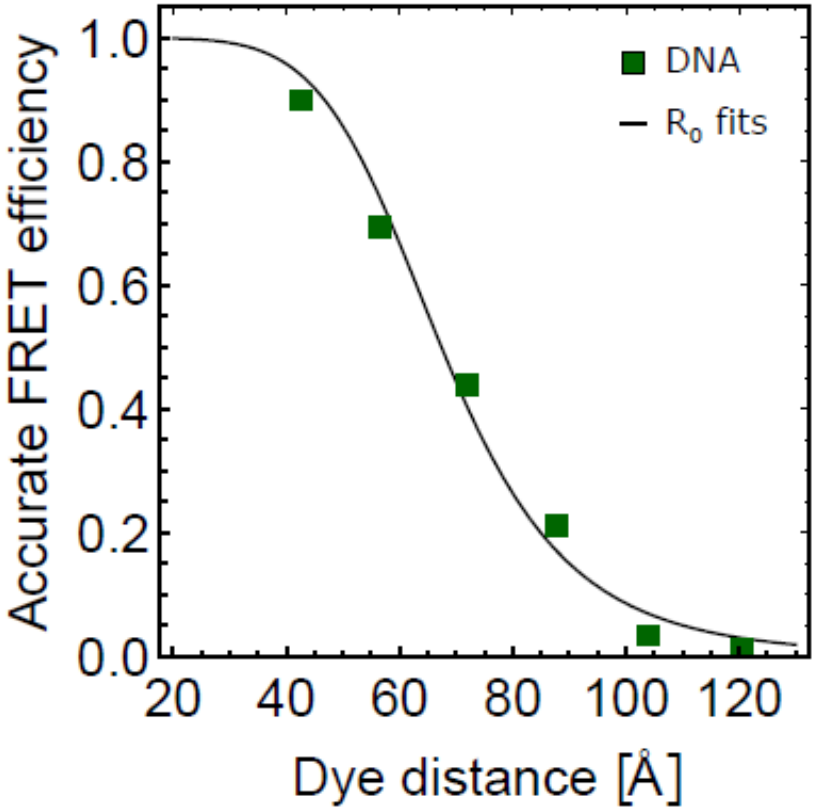
TIRF

● mean confocal

low/high FRET:
+/× global γ
+/× single species γ

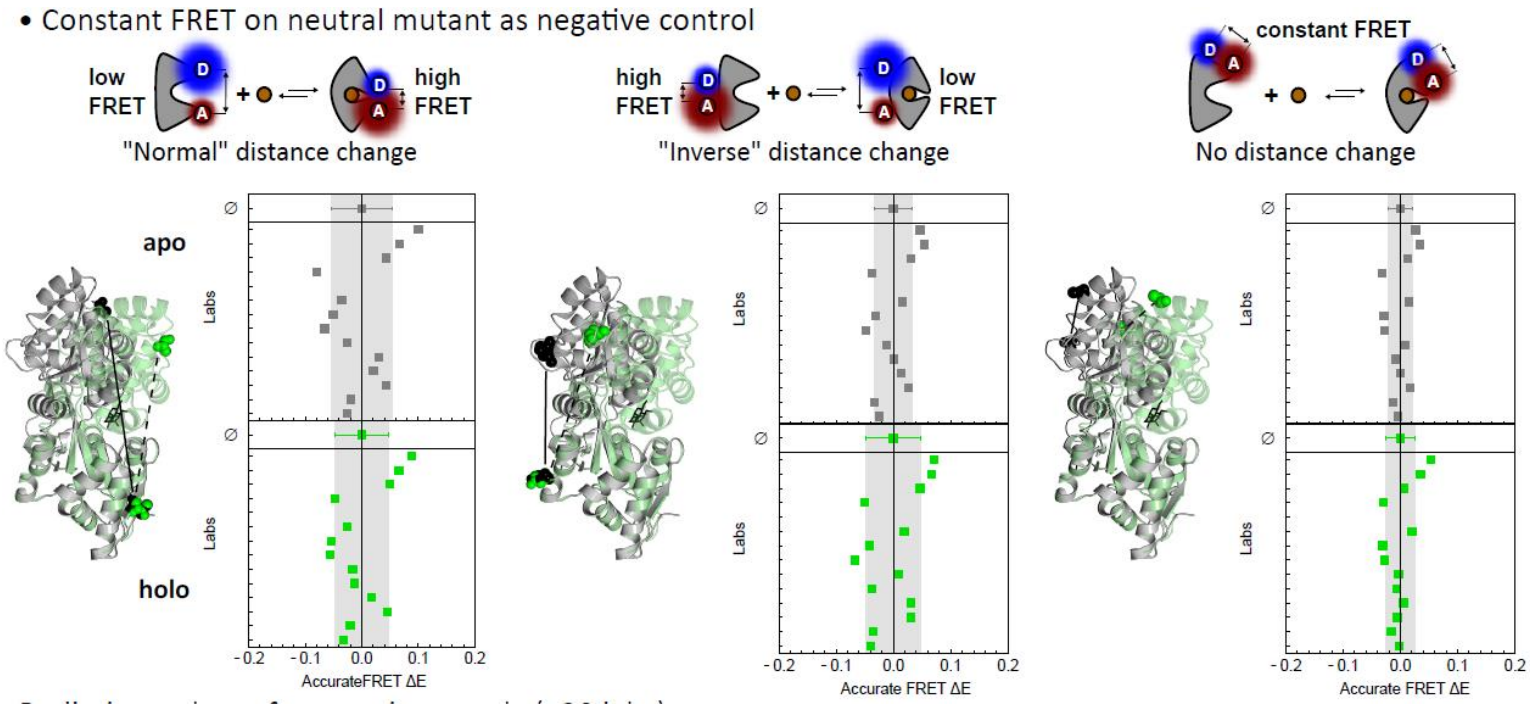
ΔE = ±0.02 and ±0.05

Result of the benchmark study on nucleic acids



Thorben Cordes, LMU Munich, personal communication

Second benchmark study : proteins



• 15 participating labs (current state): Cordes, Lamb, and Tinnefeld (LMU Munich), Kapanidis (Oxford), Cordes (Groningen), Hugel (Freiburg), Craggs (Sheffield), Seidel (Düsseldorf), Michaelis (Ulm), Schlierf (Dresden), Grohmann (Regensburg), Hübner (Lübeck), Margeat (Montpellier), Ha (Baltimore), Hendrix (Leuven)

• Results indicate FRET accuracy comparable to static DNA structures in study of reference [4]

Thorben Cordes, LMU Munich, personal communication

Outline

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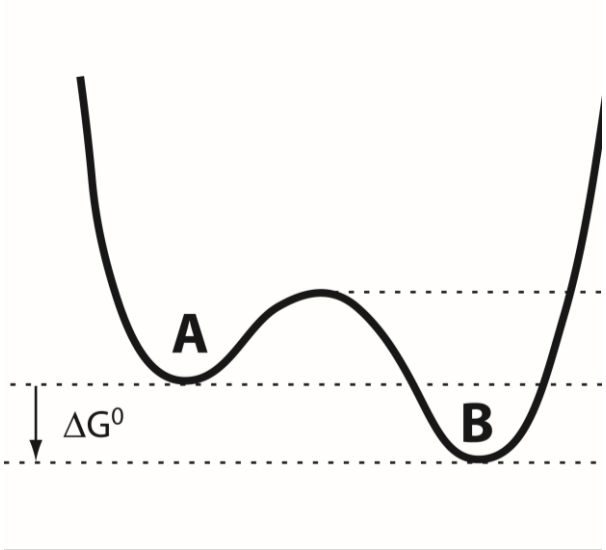
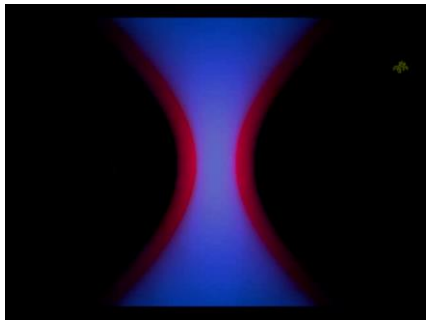
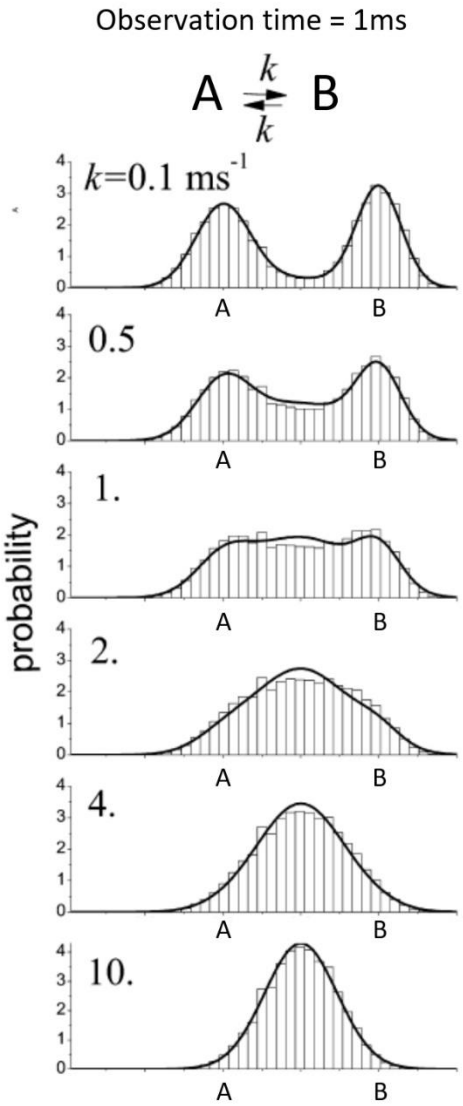
- *Measurement of distances with smFRET*
- *Measurement of dynamics with smFRET*

Looking at dynamics in proteins



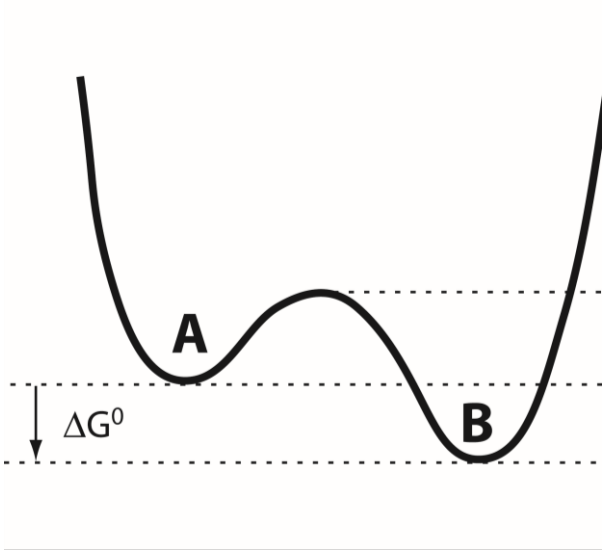


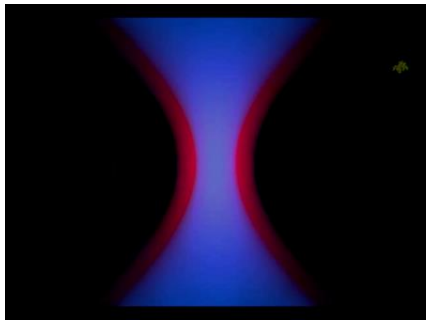


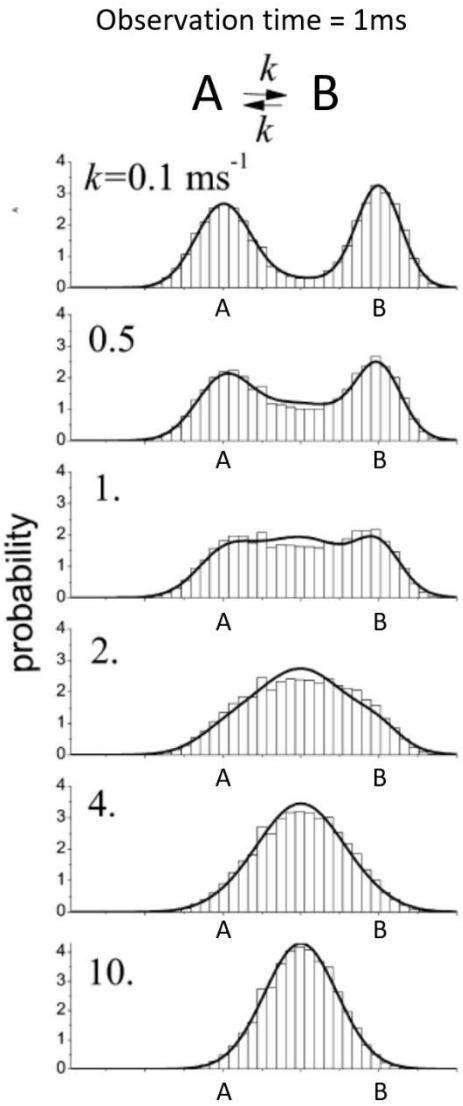
SLOW DYNAMICS

FAST DYNAMICS

Looking at dynamics in proteins



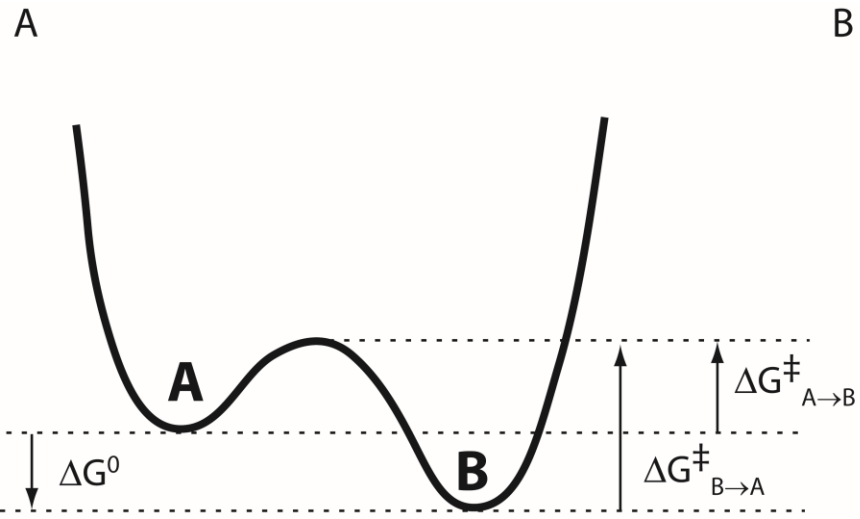


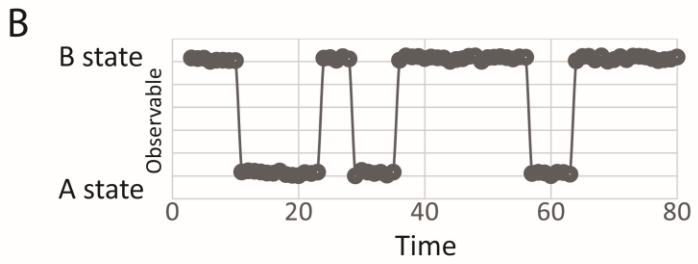


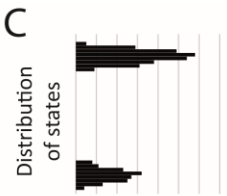
SLOW DYNAMICS

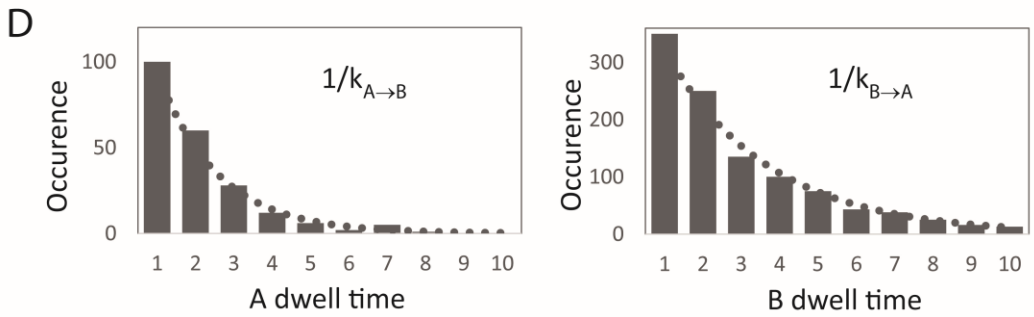
FAST DYNAMICS

The case of slow dynamics





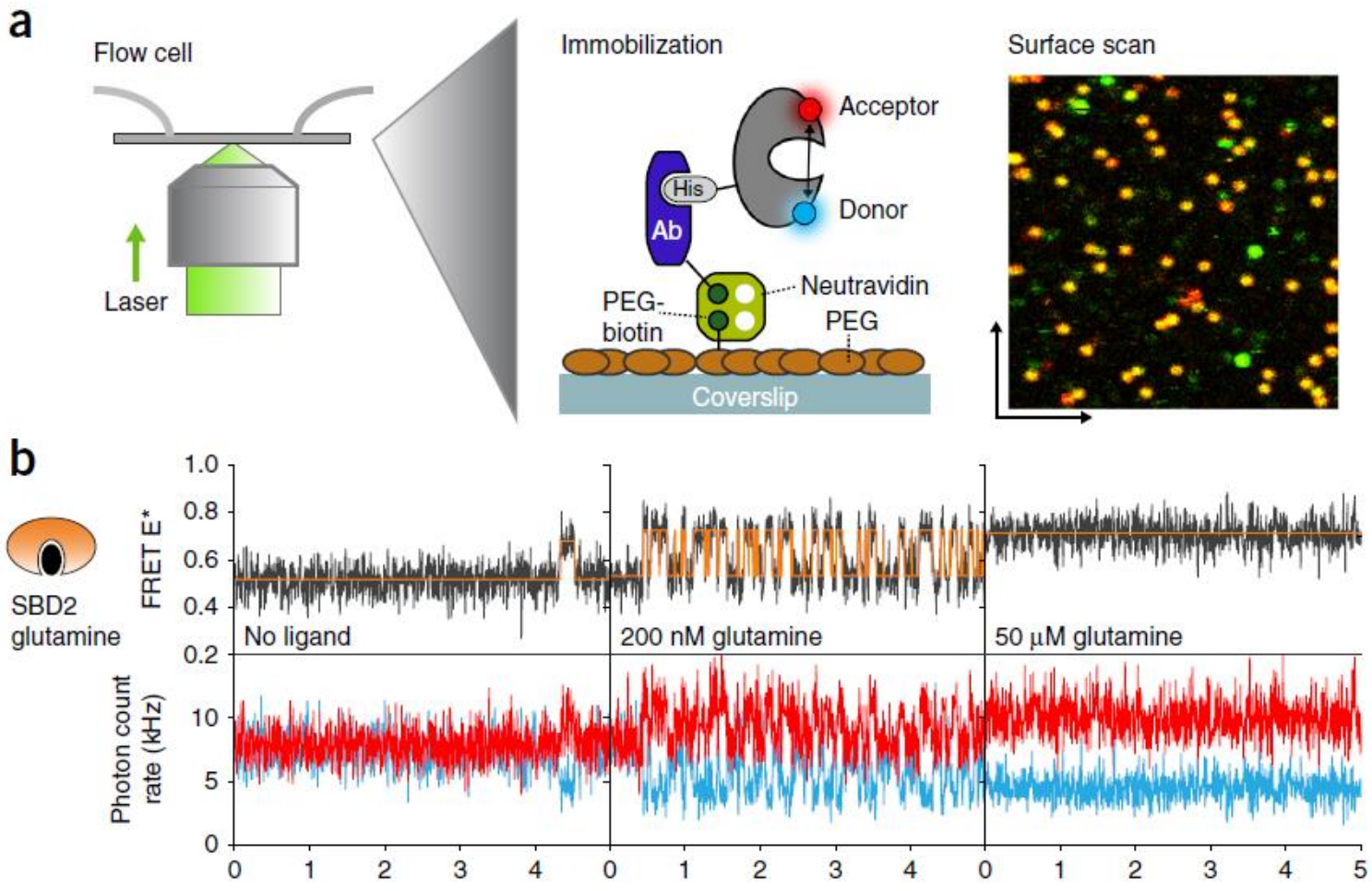




Molecules are immobilized on a surface

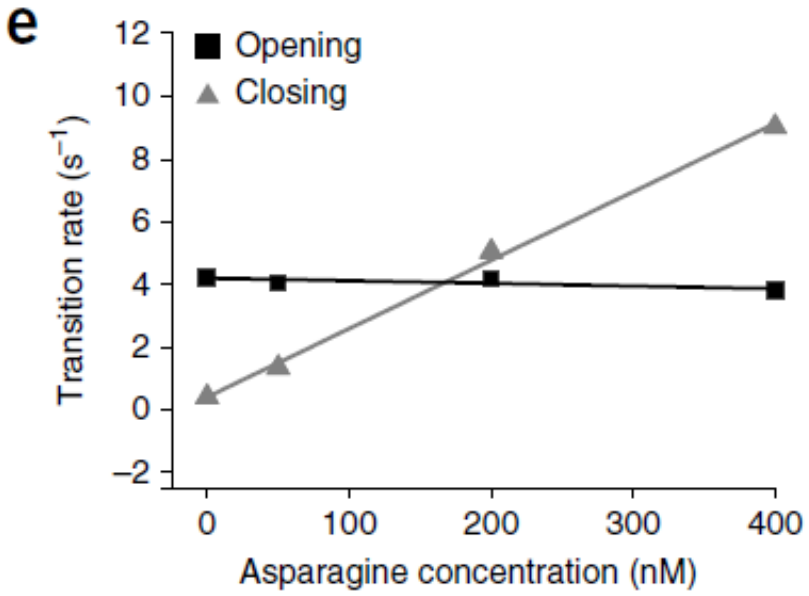
Transition rates and states characteritics are directly extracted from « time traces »

Substrate binding domain of an ABC transporter



Gouridis et al., NSMB, 2014

Substrate binding domain of an ABC transporter



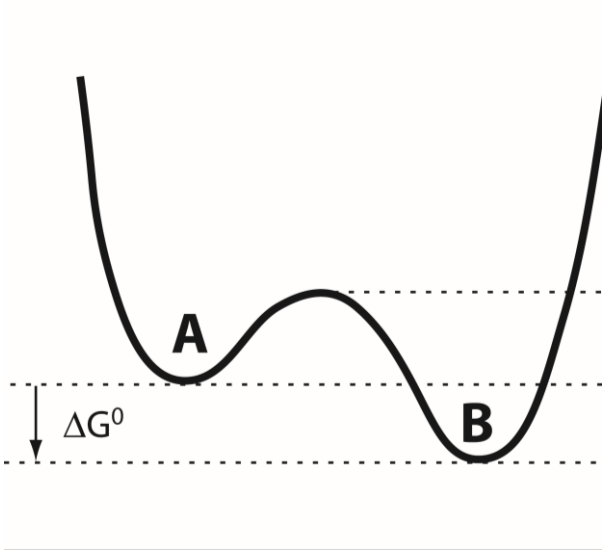
First order kinetics for closure

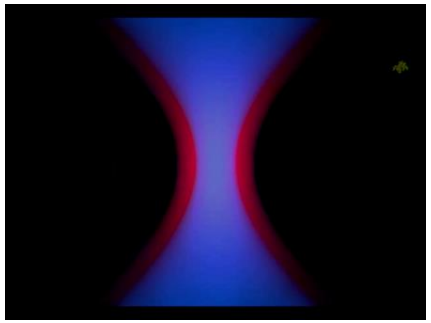
Zero order kinetics for opening

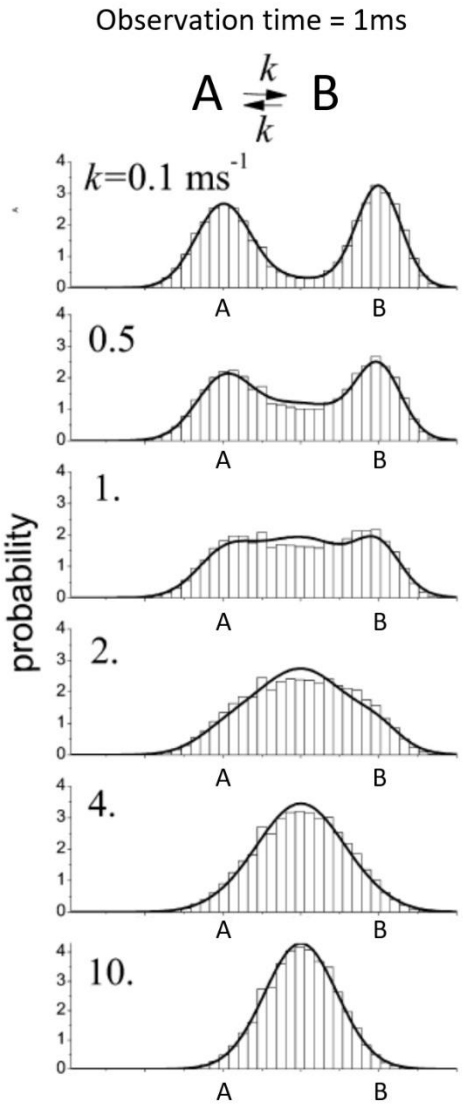
Binding of the ligand enhances the transition to the closed state, but without stabilizing this conformation

Gouridis et al., NSMB, 2014

Looking at dynamics in proteins



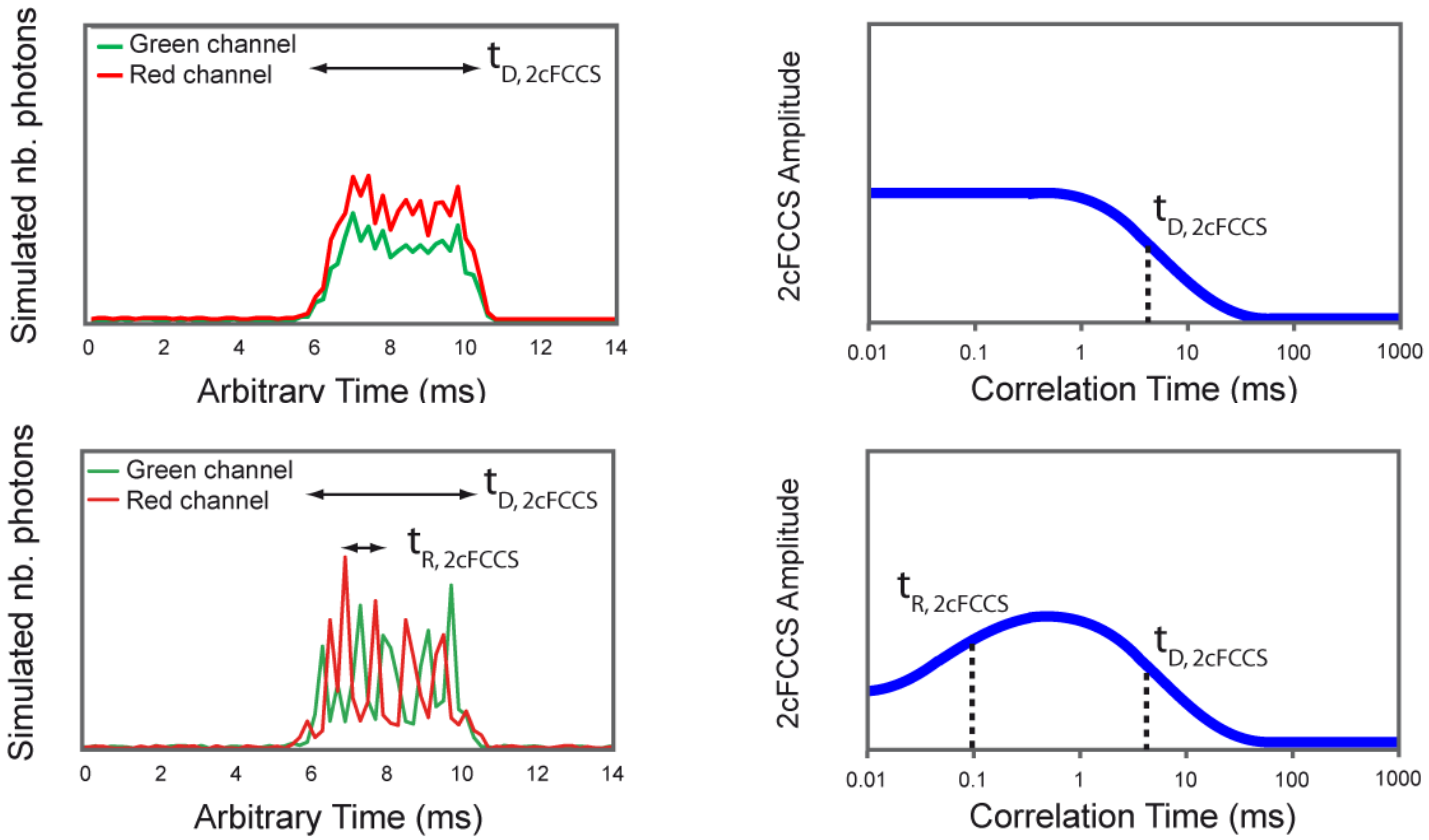




SLOW DYNAMICS

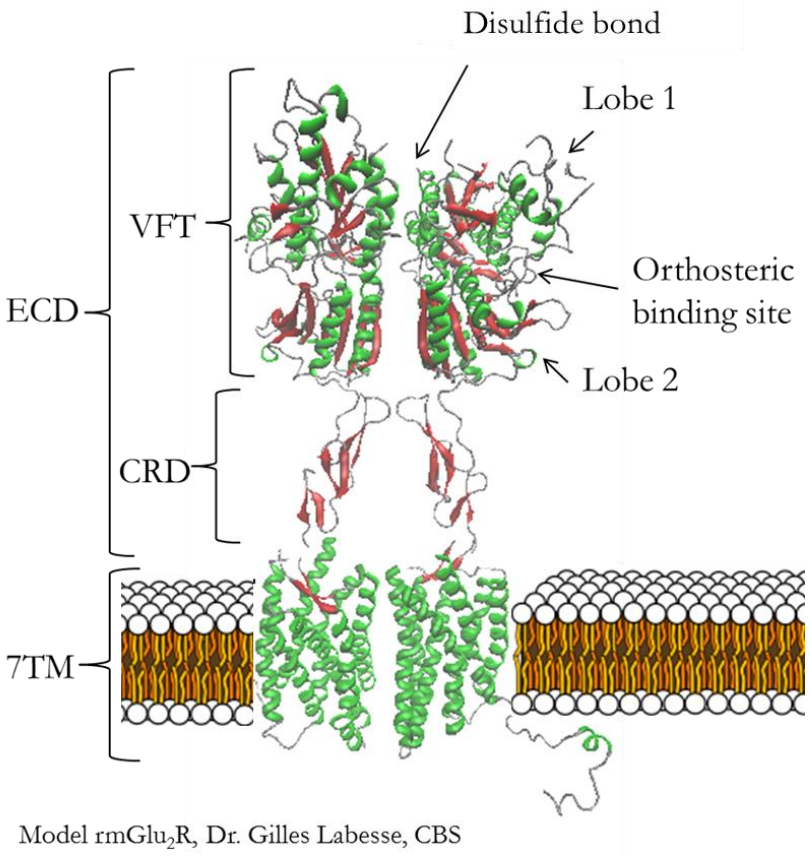
FAST DYNAMICS

Looking at fast dynamics with Fluorescence cross correlation spectroscopy

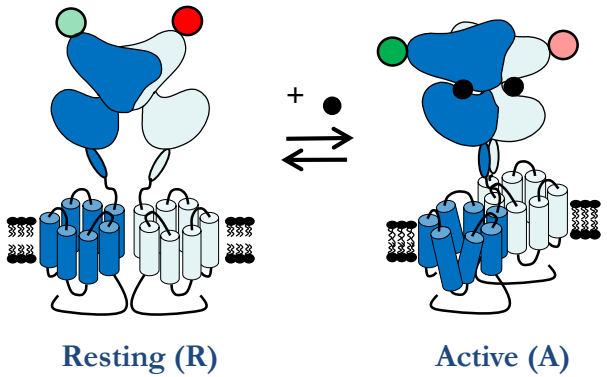


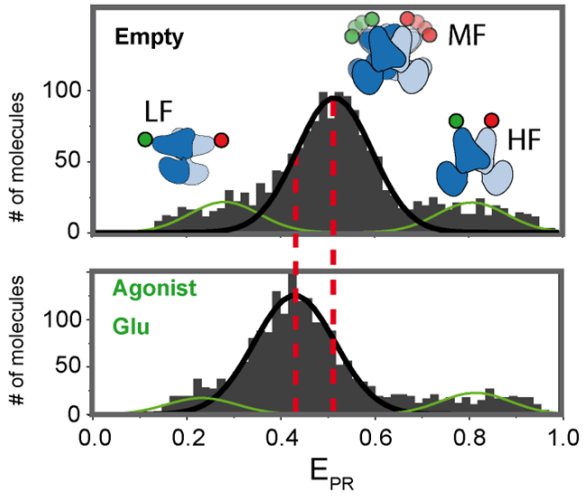
- ✓ Fluorescence Cross Correlation Spectroscopy (FCCS) :
  - ▶ a positive $t_{D,FCCS}$ term indicates the presence of doubly-labeled molecules
  - ▶ the $t_{R,FCCS}$ anticorrelation term represents the timescale of the transition between two FRET states

Structural dynamics of metabotropic glutamate receptors

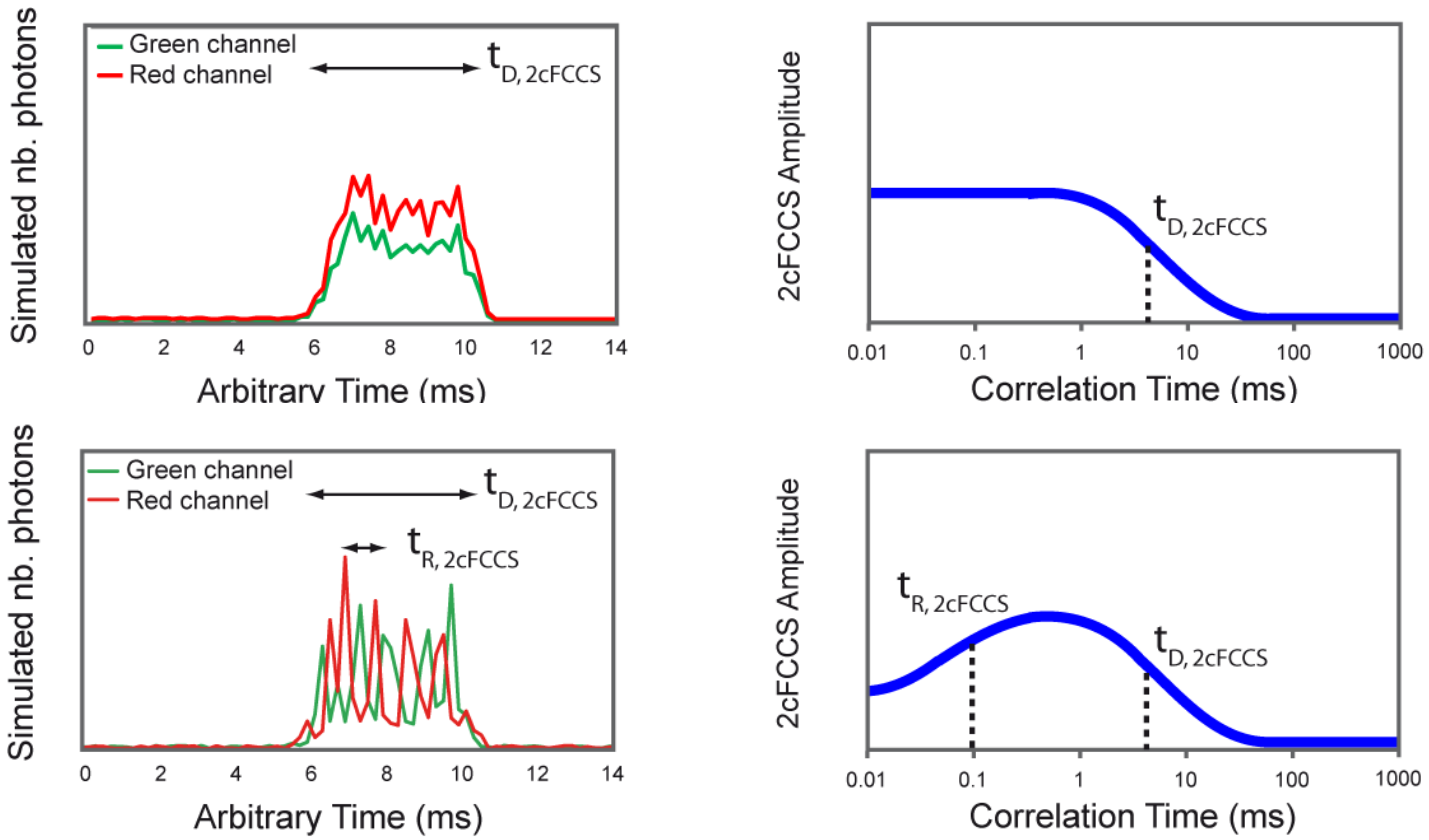


Model rmGlu$_2$R, Dr. Gilles Labesse, CBS





Looking at fast dynamics with Fluorescence cross correlation spectroscopy



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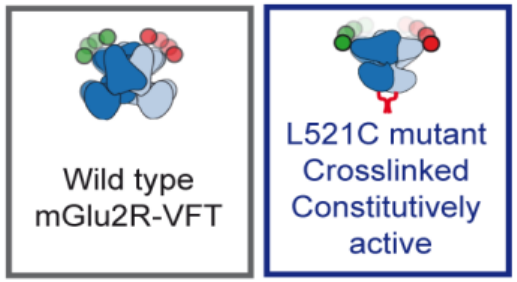
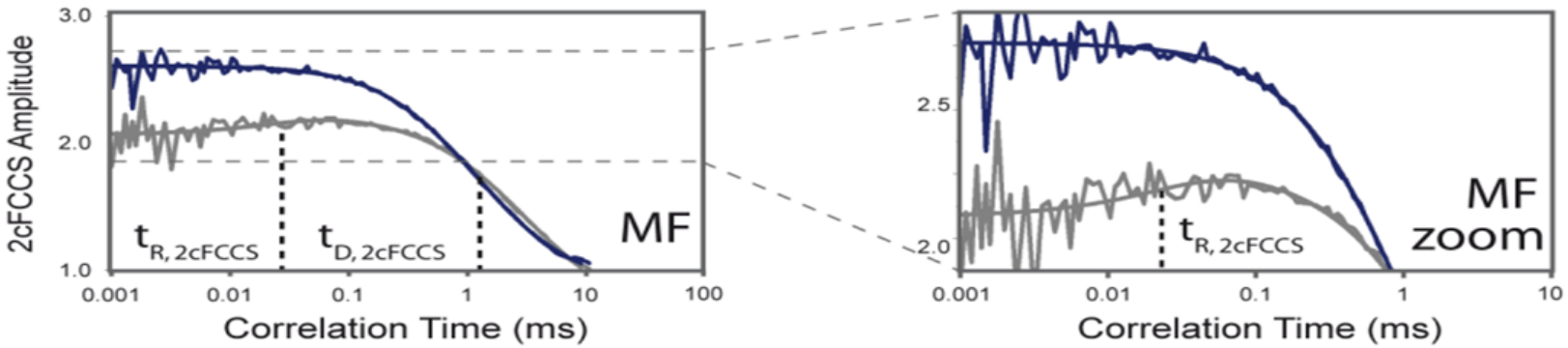
  - ▶ the $t_{R,FCCS}$ anticorrelation term represents the timescale of the transition between two FRET states

Looking at fast dynamics with Fluorescence cross correlation spectroscopy

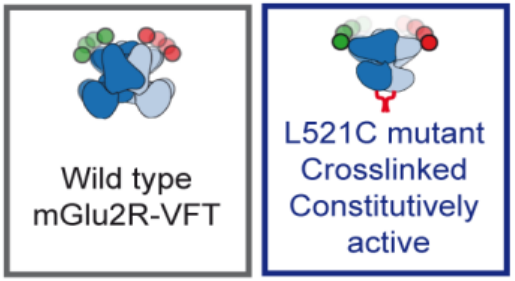
- ✓ **Anticorrelation (dynamics) in the 50-100µs range, for all ligands tested**
- ✓ Constitutively active mutant : no anticorrelation
- ✓

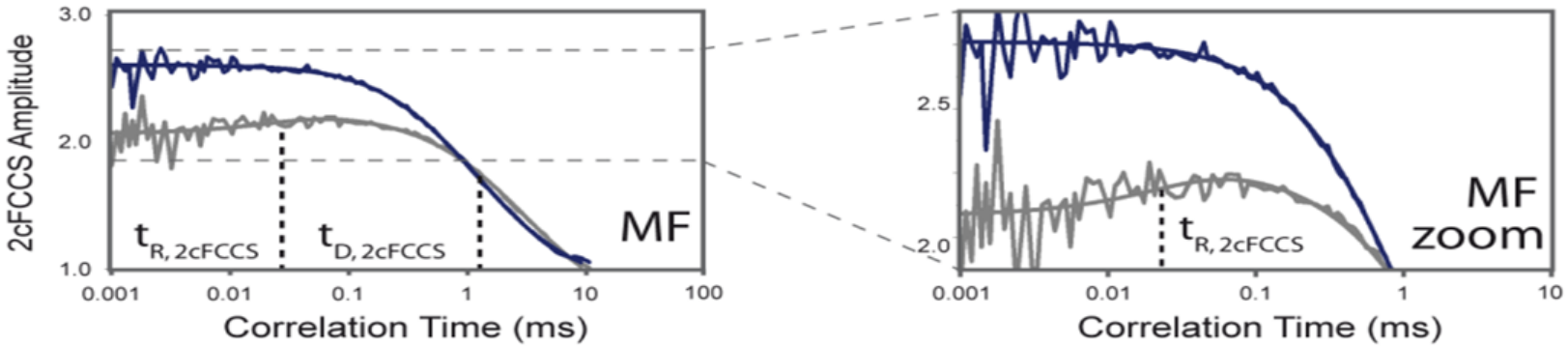


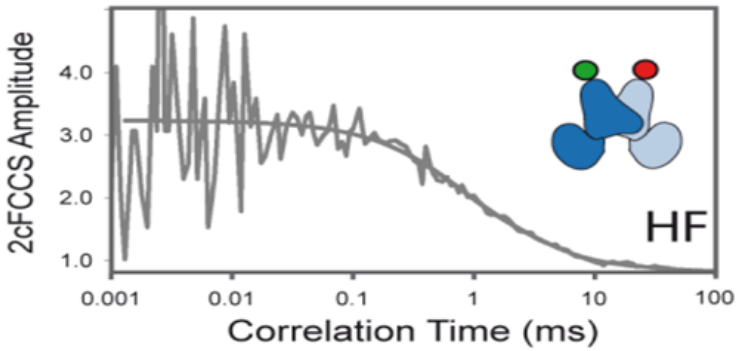


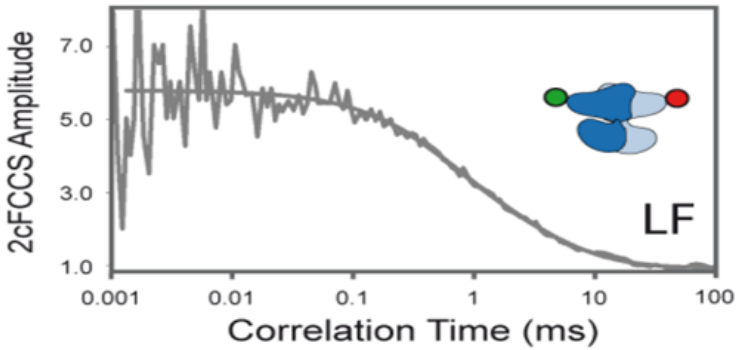
Looking at fast dynamics with Fluorescence cross correlation spectroscopy

- ✓ **Anticorrelation (dynamics) in the 50-100µs range, for all ligands tested**
- ✓ Constitutively active mutant : no anticorrelation
- ✓ No anticorrelation for LF & HF



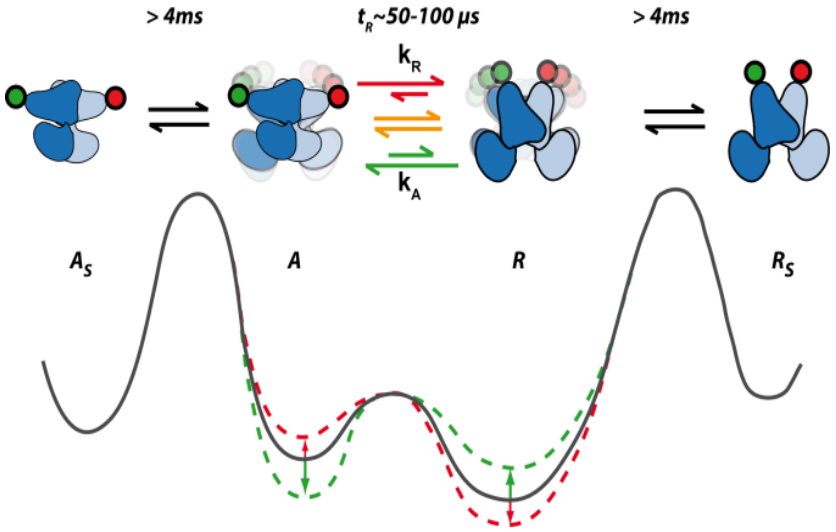






Activation model for metabotropic glutamate receptors

✓ *First observation of GPCR activation by single molecule FRET*



- ✓ **Excited state lifetime measurement**
- ✓ **FCCS**
- ✓ **Filtered FCS**
- ✓ **Photon Distribution Analysis**

✓ Evidence for a ***conformational selection process :***

- The ***mGluR ECD oscillates between two boundary states even in the presence of ligands***
- The ***ligands regulate the transition rate between the Active and the Resting state***

Olofsson et al., Nature Comm., 2014

Single molecule FRET in structural biology

- *Ability to perform single molecule measurements*
- *Accuracy >5A in distance measurements*
- *Distance constrains can be used to build molecular models (multidomain proteins)*
- *Dynamic information can be obtained, from the µs to the minute timescales*
- *Proper labeling of your molecule is crucial*

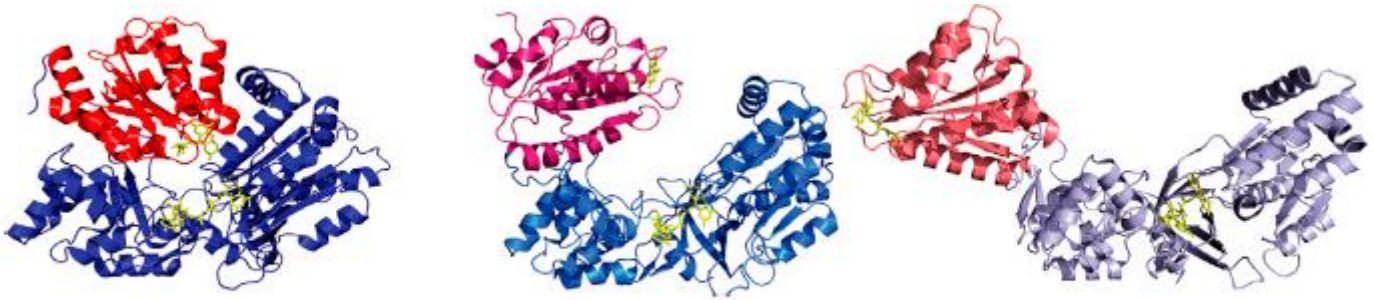
Application of the procedure for rigorous determination of E

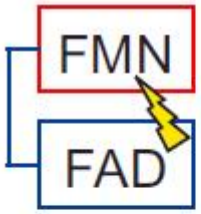
Demonstration of the relationship between dye labeling position, quantum yield, and E

Structural dynamics of CPR

Robert Quast
Gilles Truan
INSA TOULOUSE

H Cytochrome P450 reductase (CPR)



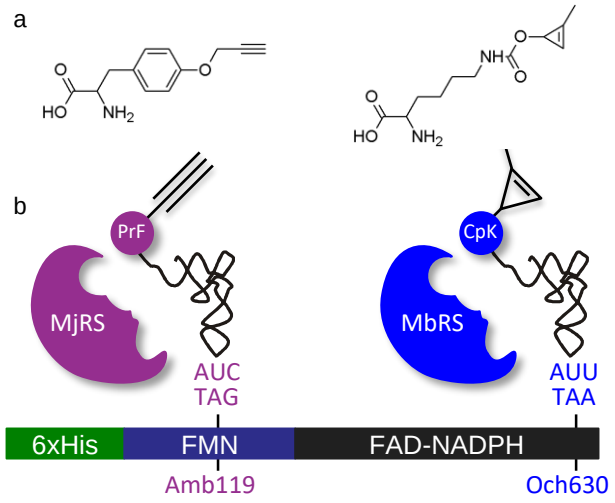


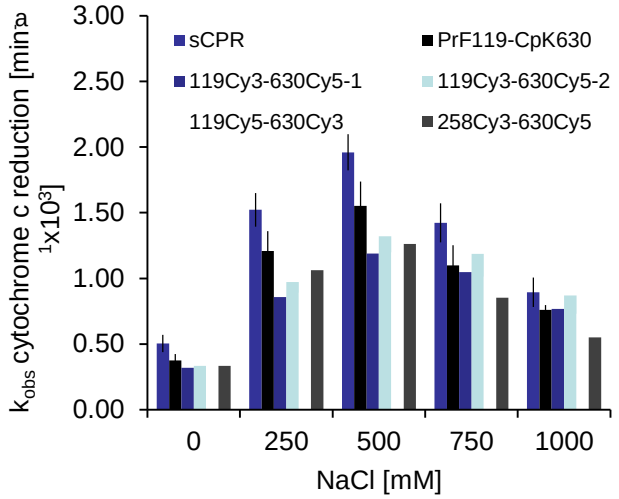
Closed conformation

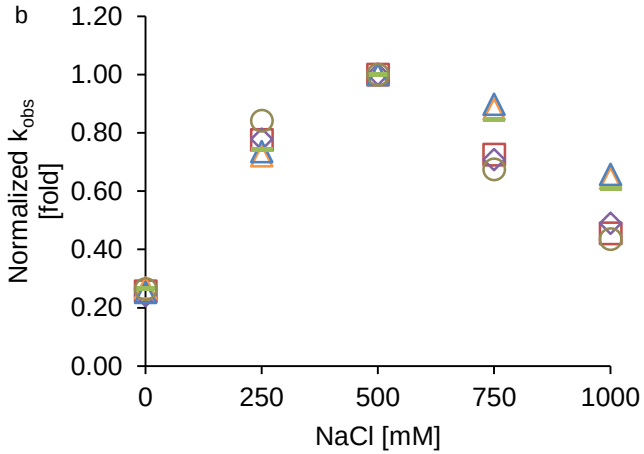
Open conformations

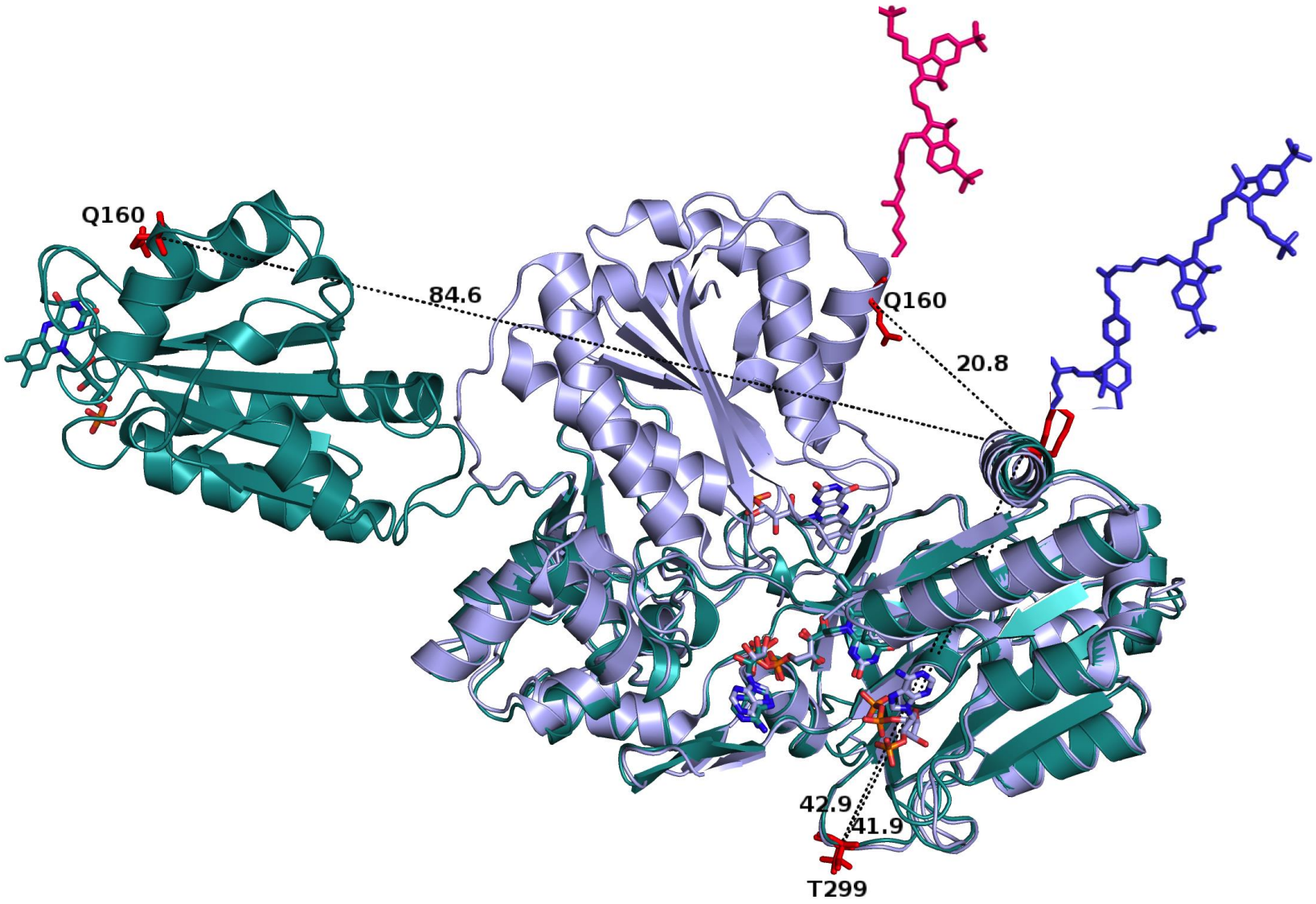
NADPH → FAD → FMN → acceptor

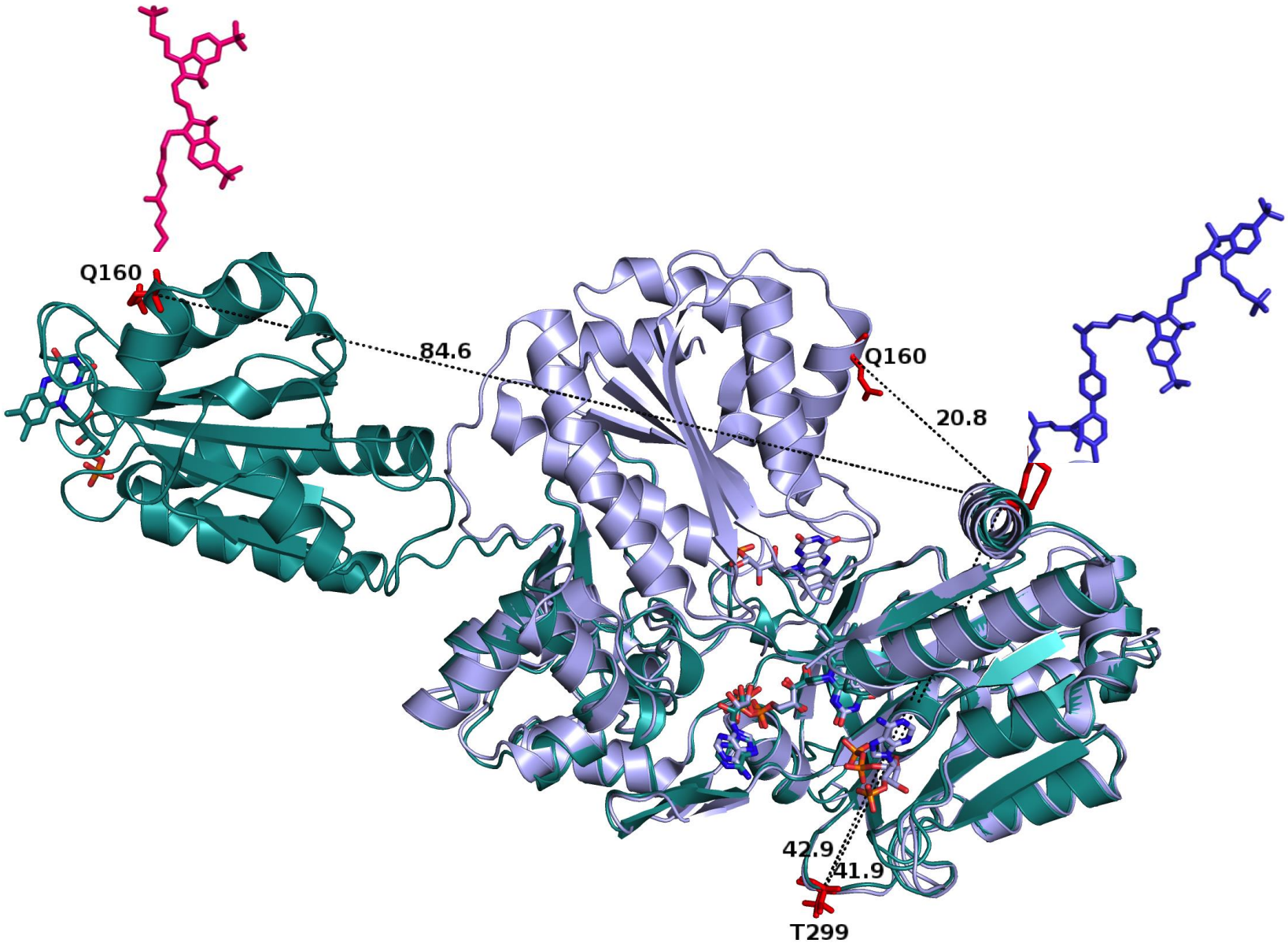
Site specific labeling with 2 UAAs








Q160
84.6
Q160
20.8
42.9
41.9
T299


Q160
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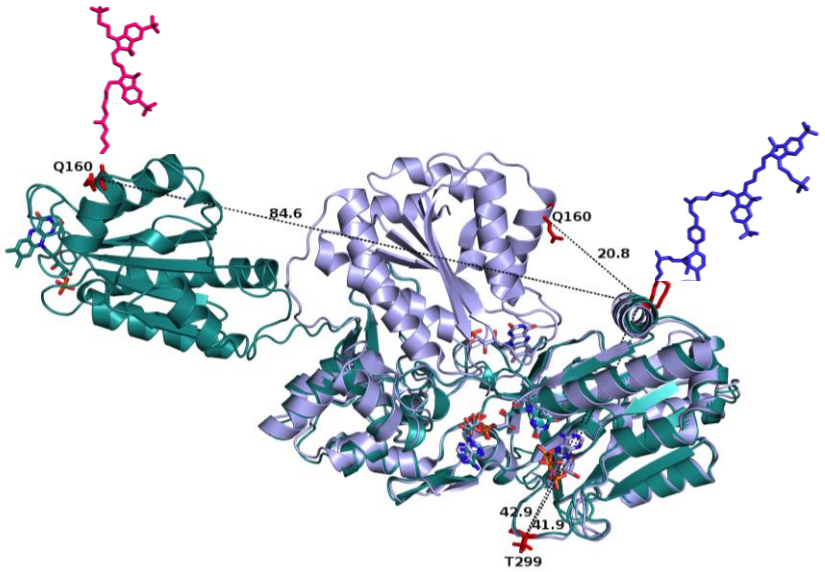
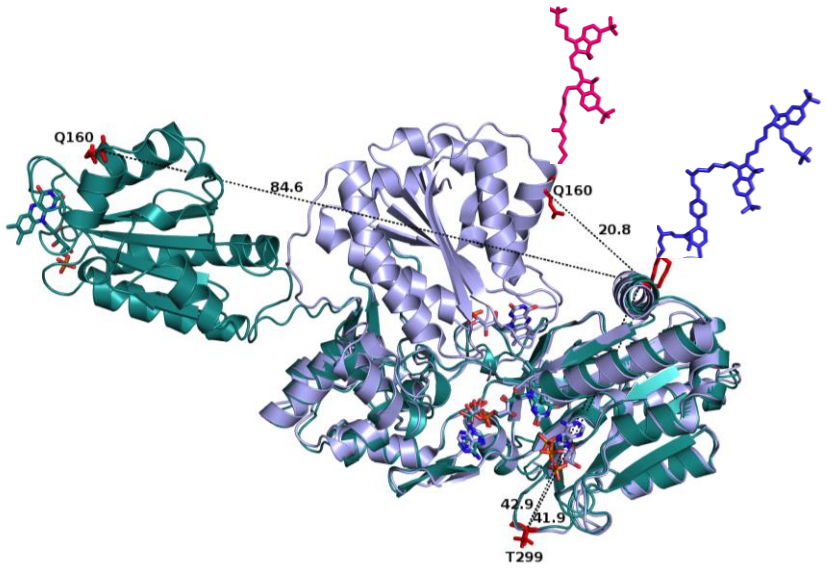
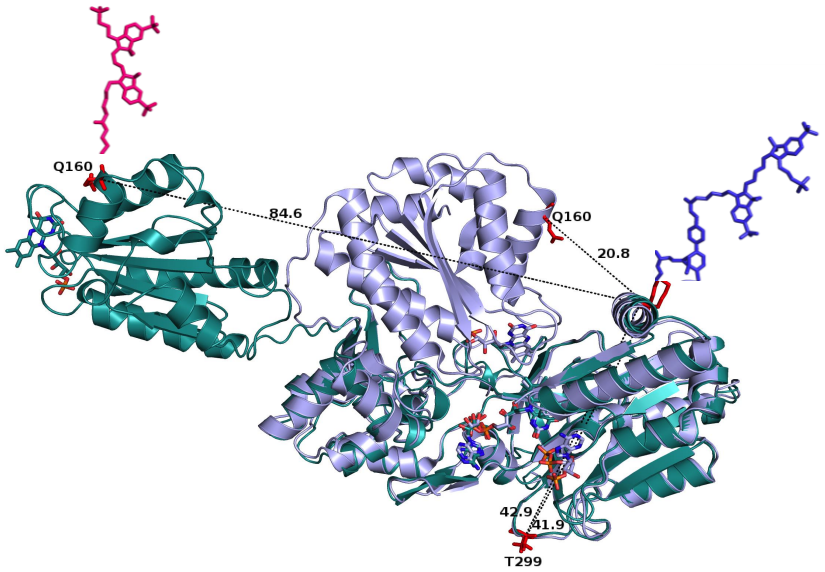
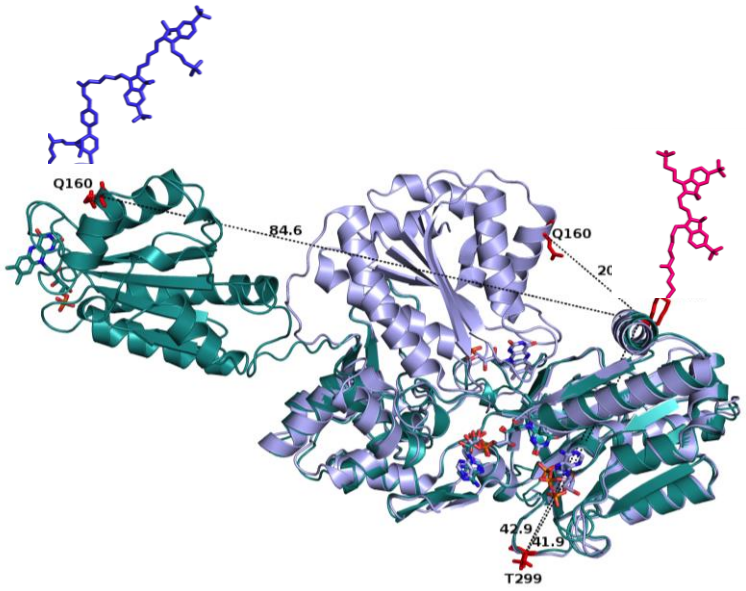




Can we see / quantify the open / closed transition ?

Which factor influence the equilibirum ?

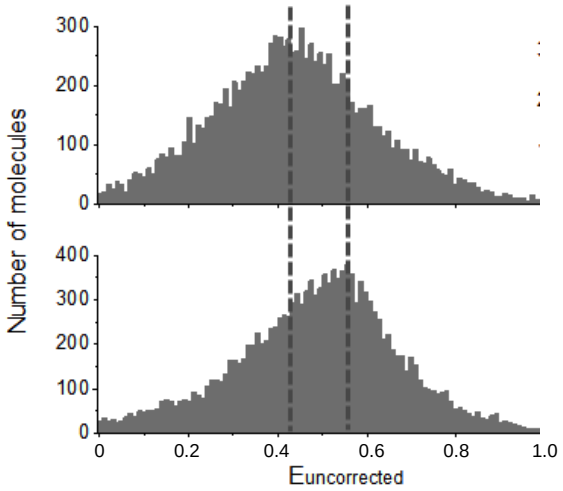


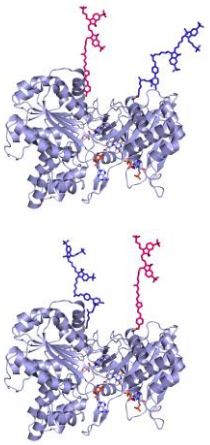


Do the position of the dye matter on the result ?

How does the local environment influence the QY ? And thus E determination ?

Correction for γ





$$\gamma = \frac{\Phi_A.\delta_A}{\Phi_D.\delta_D}$$

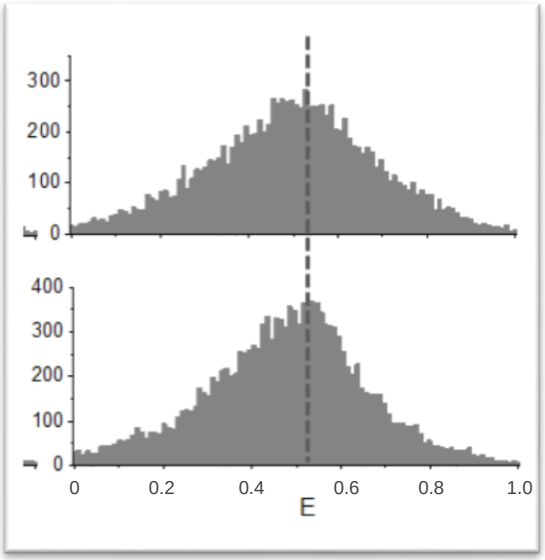
Cy3-Cy5	0.18	0.15
Cy5-Cy3	0.17	0.2

$\Delta\gamma = 41\%$

$$\gamma_1 = 0.833\frac{\delta_A}{\delta_D}$$

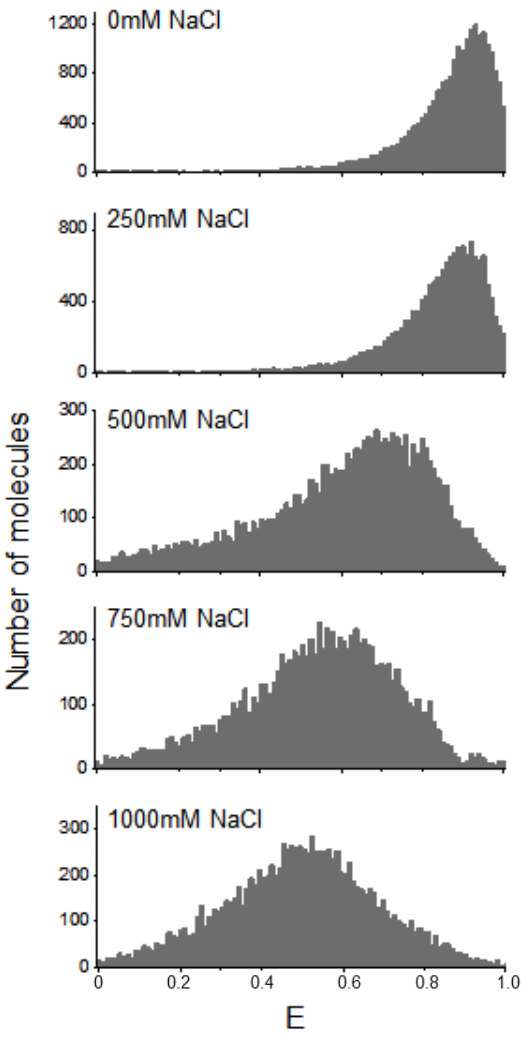
$$\gamma_2 = 1.18\frac{\delta_A}{\delta_D}$$

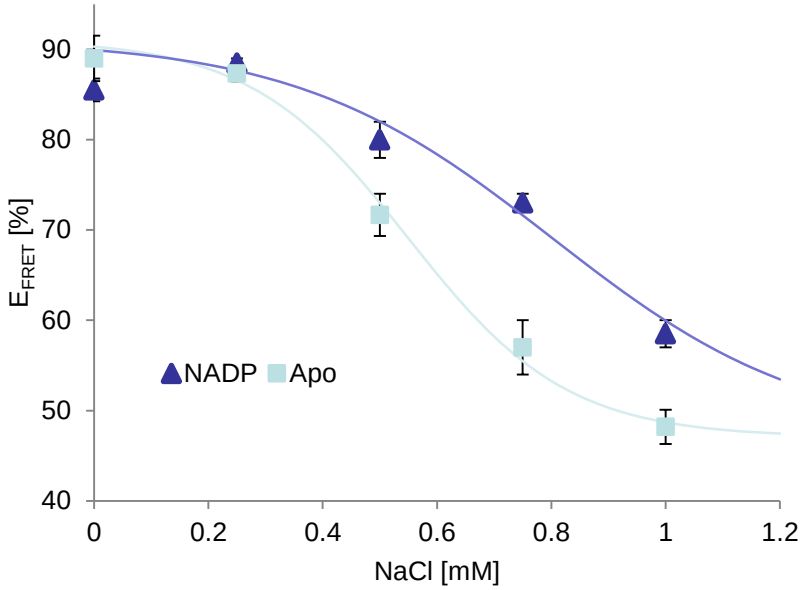
$$\gamma = \frac{\Phi_A.\delta_A}{\Phi_D.\delta_D}$$



- Correct FRET efficiencies via γ correction
- Important to have site-specific labeling

Open / closed equilibrium



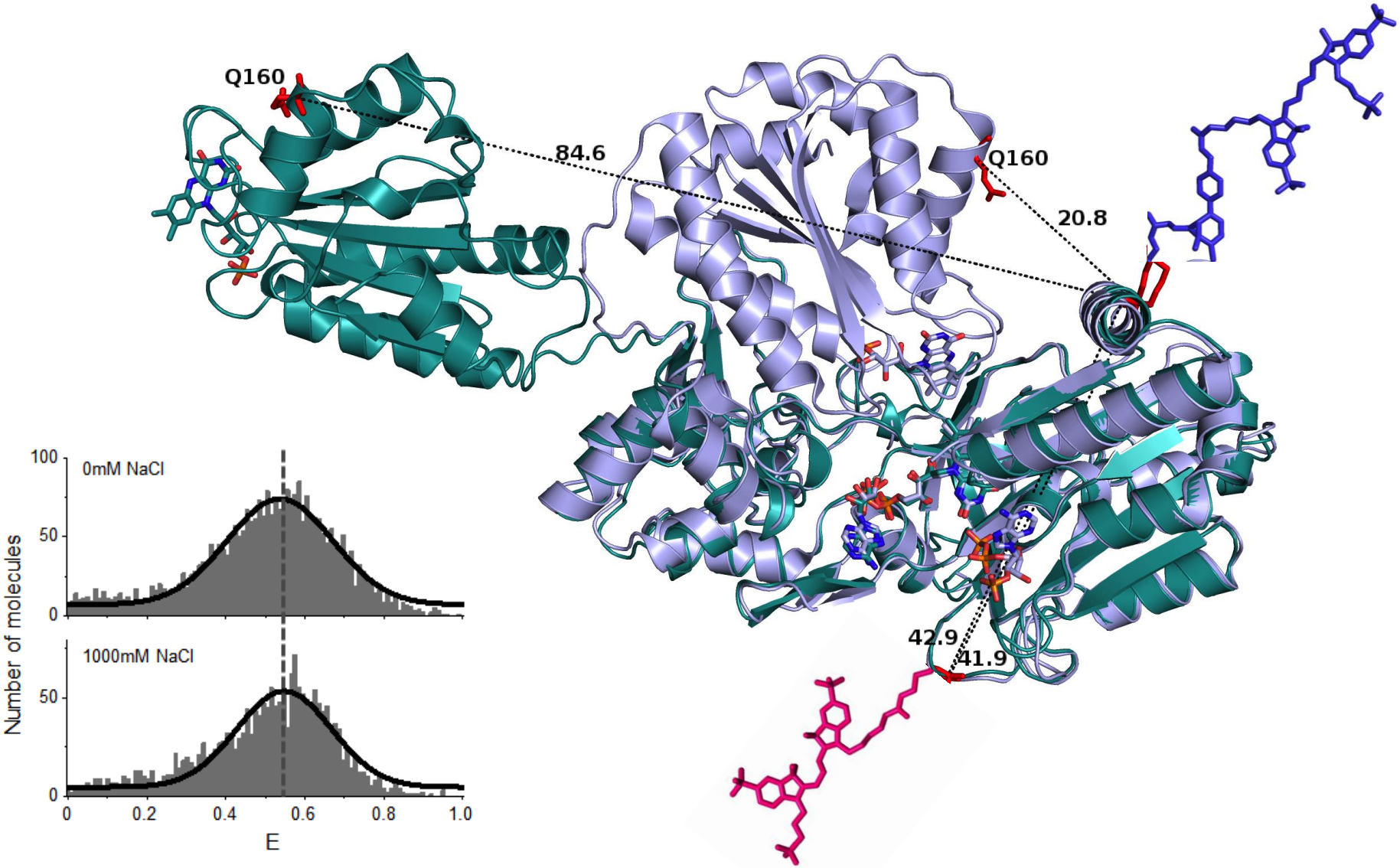


Salt-dependant opening of CPR
Half transition around 500mM

NADP binding promotes the closed form

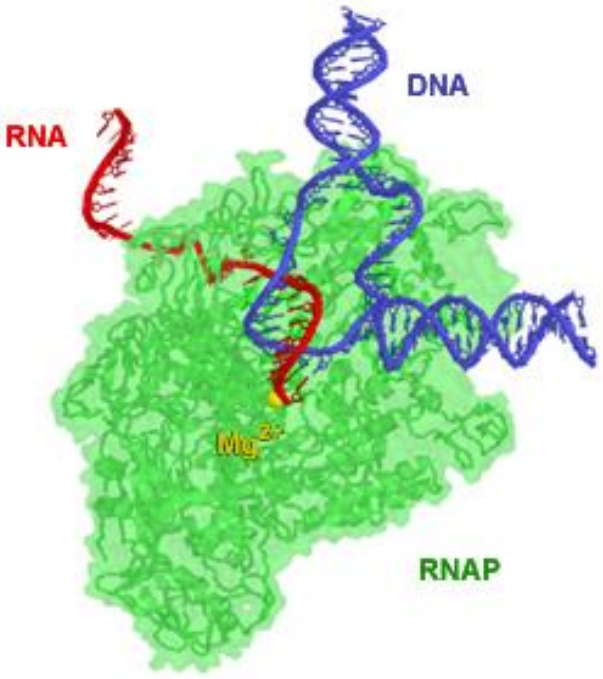
Figure 6. Influence of NaCl concentration on the equilibrium between open and closed states. Histograms show the FRET efficiency distribution at different final NaCl concentrations in 20 mM Tris buffer at pH7.4.

Negative control



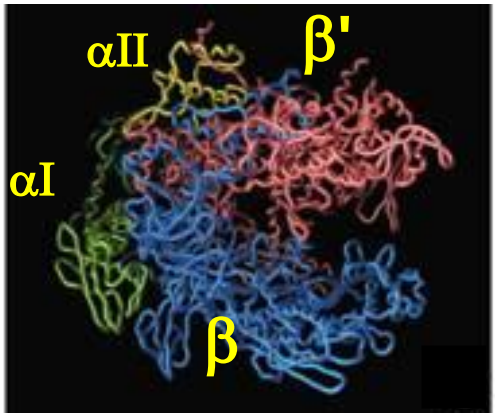
L'ARN polymérase

- Enzyme responsable de la transcription de l'ADN en ARN
- Bactéries : plusieurs sous – unités
- Modèle de l'enzyme eucaryote



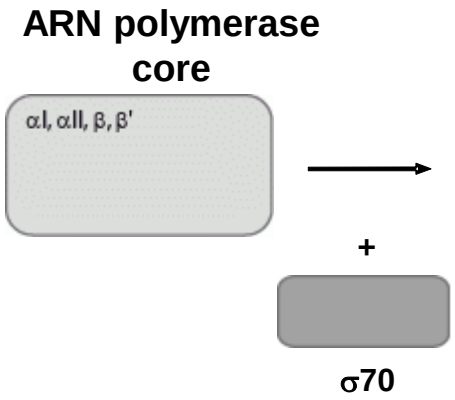
Core : Cœur catalytique, permet l'élongation

Pour la reconnaissance du promoteur et l'initiation, elle doit s'associer à un facteur de transcription (σ_{70})

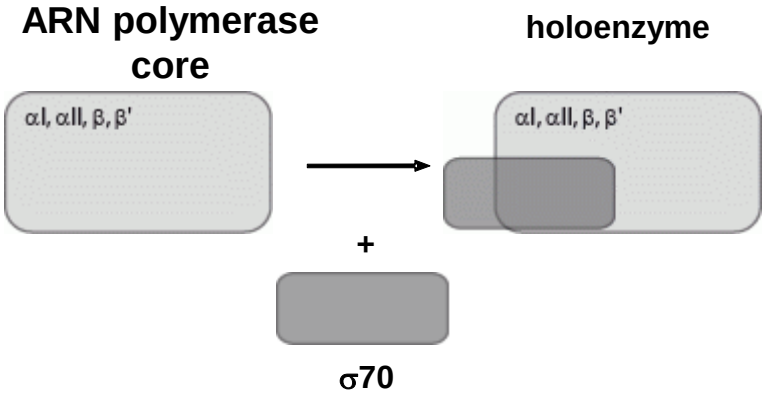


RNAP core ($\alpha_2\beta\beta'$)

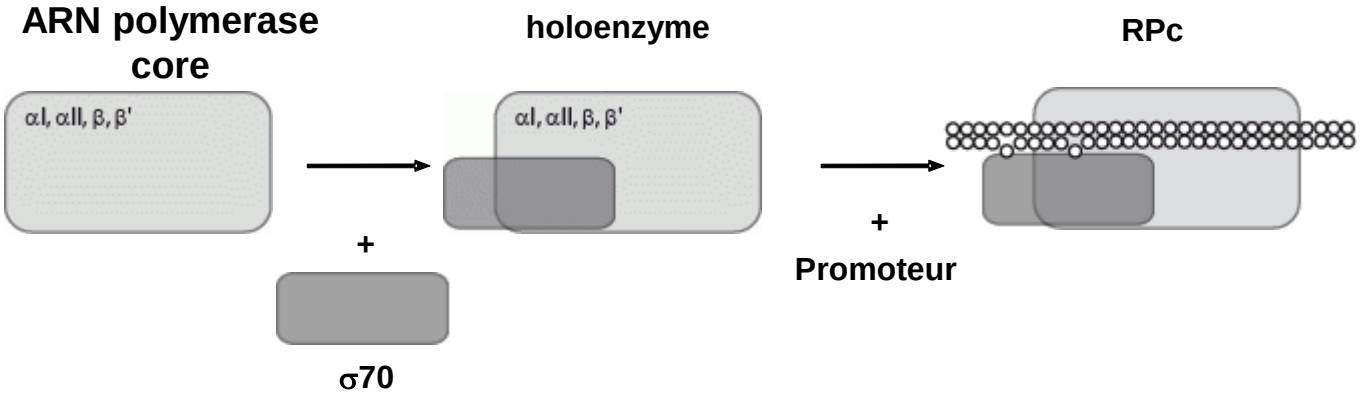
Le cycle de transcription



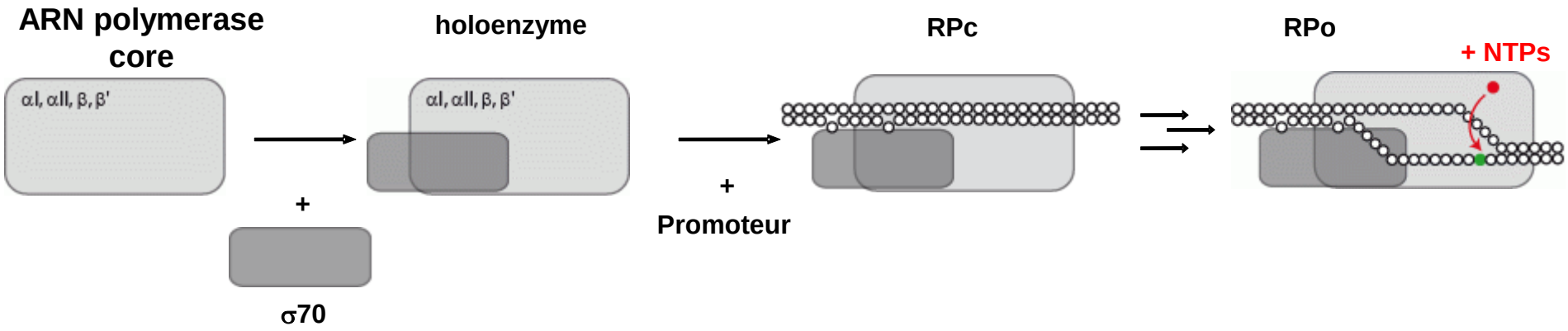
Le cycle de transcription



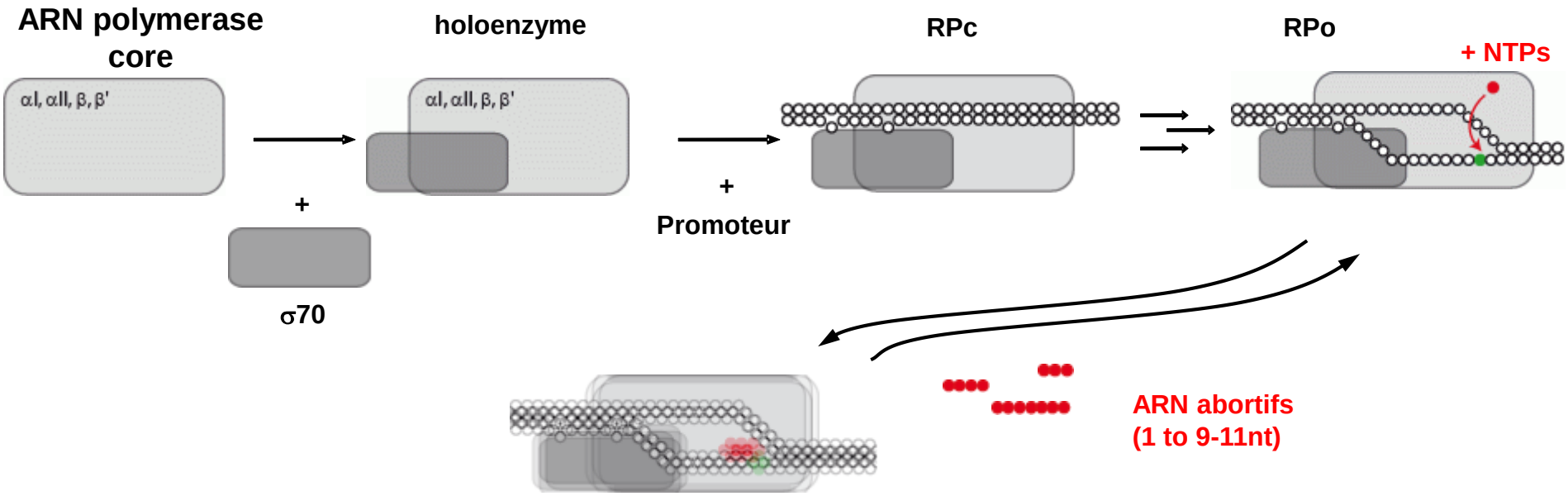
Le cycle de transcription



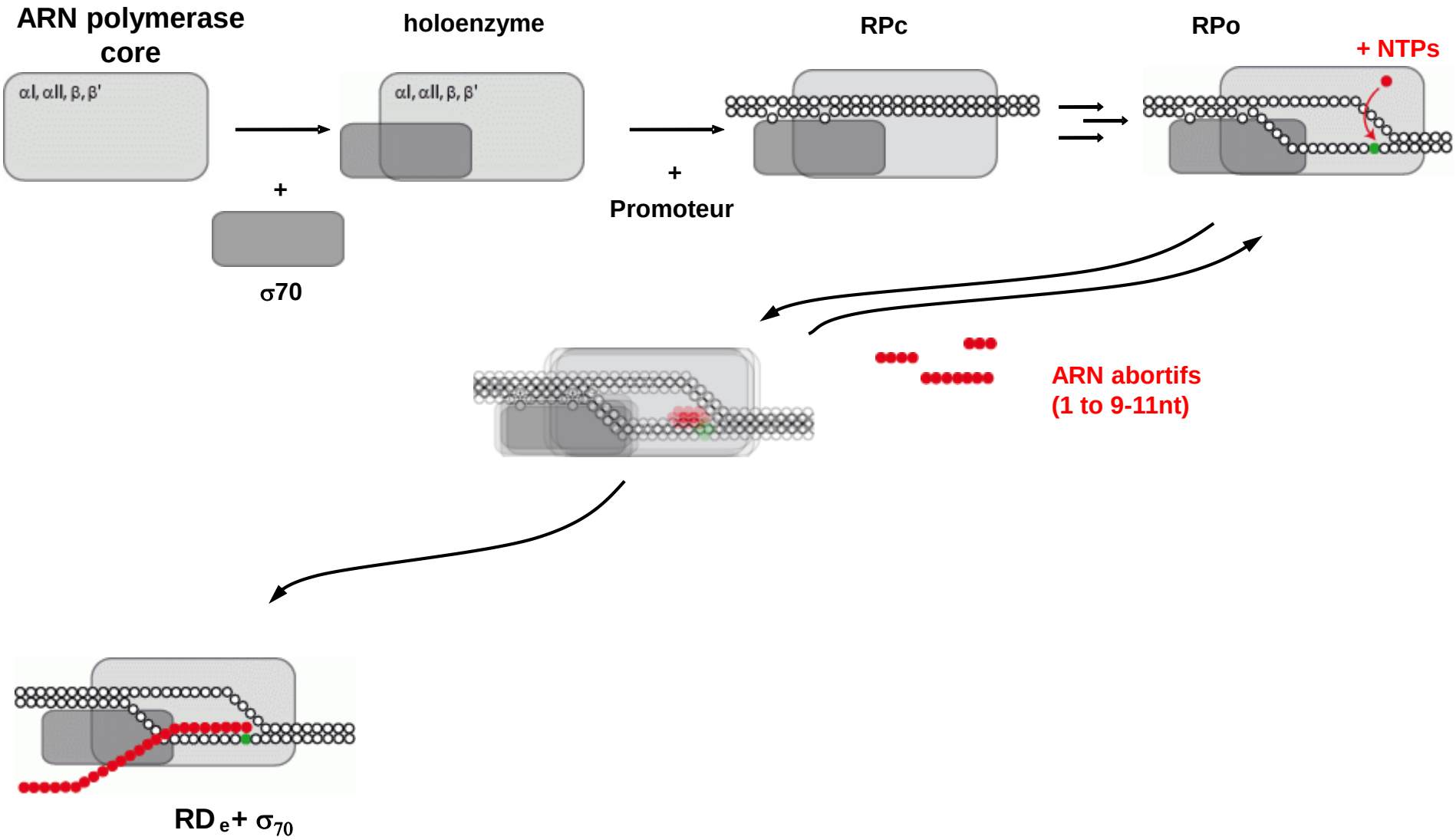
Le cycle de transcription



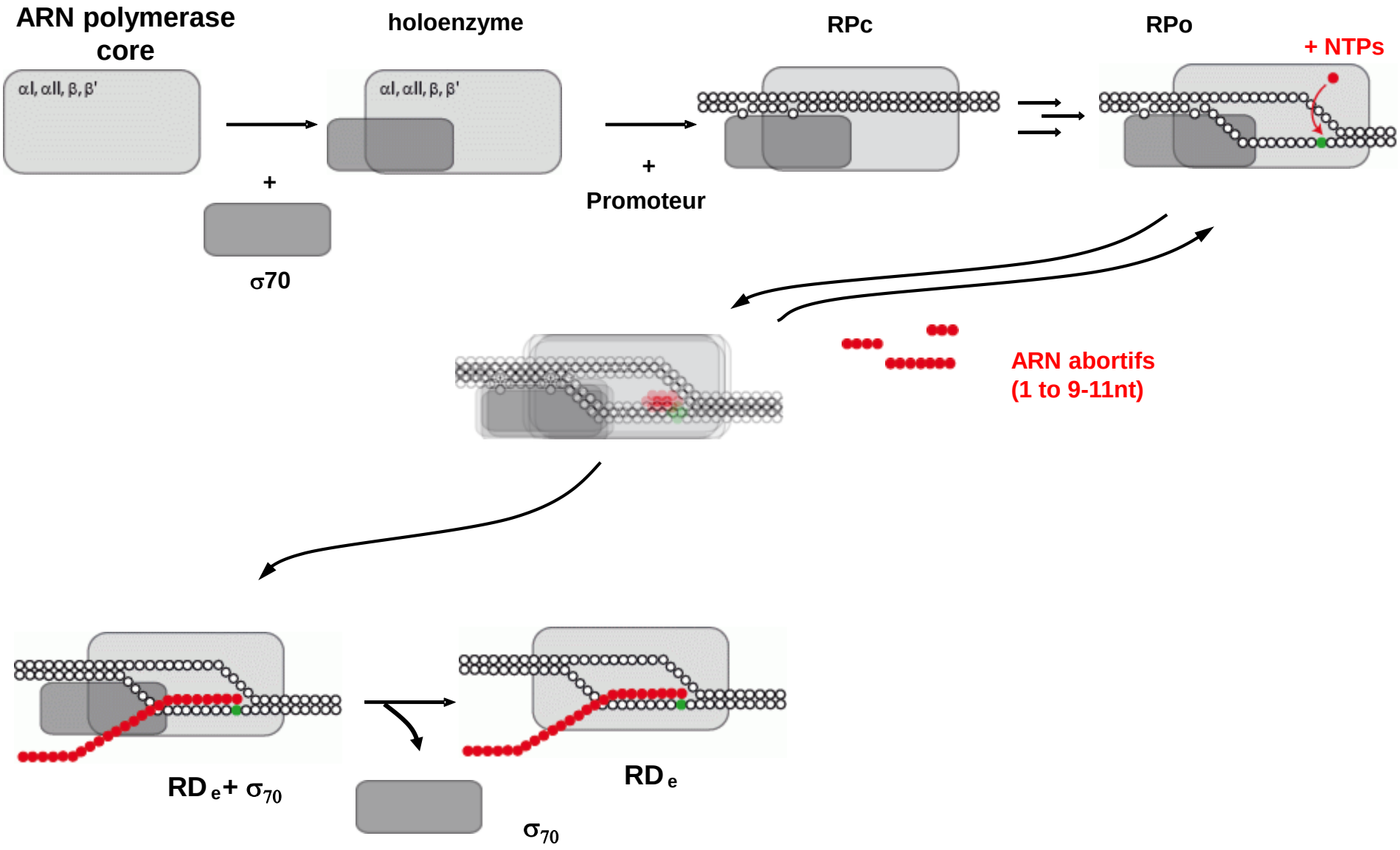
Le cycle de transcription



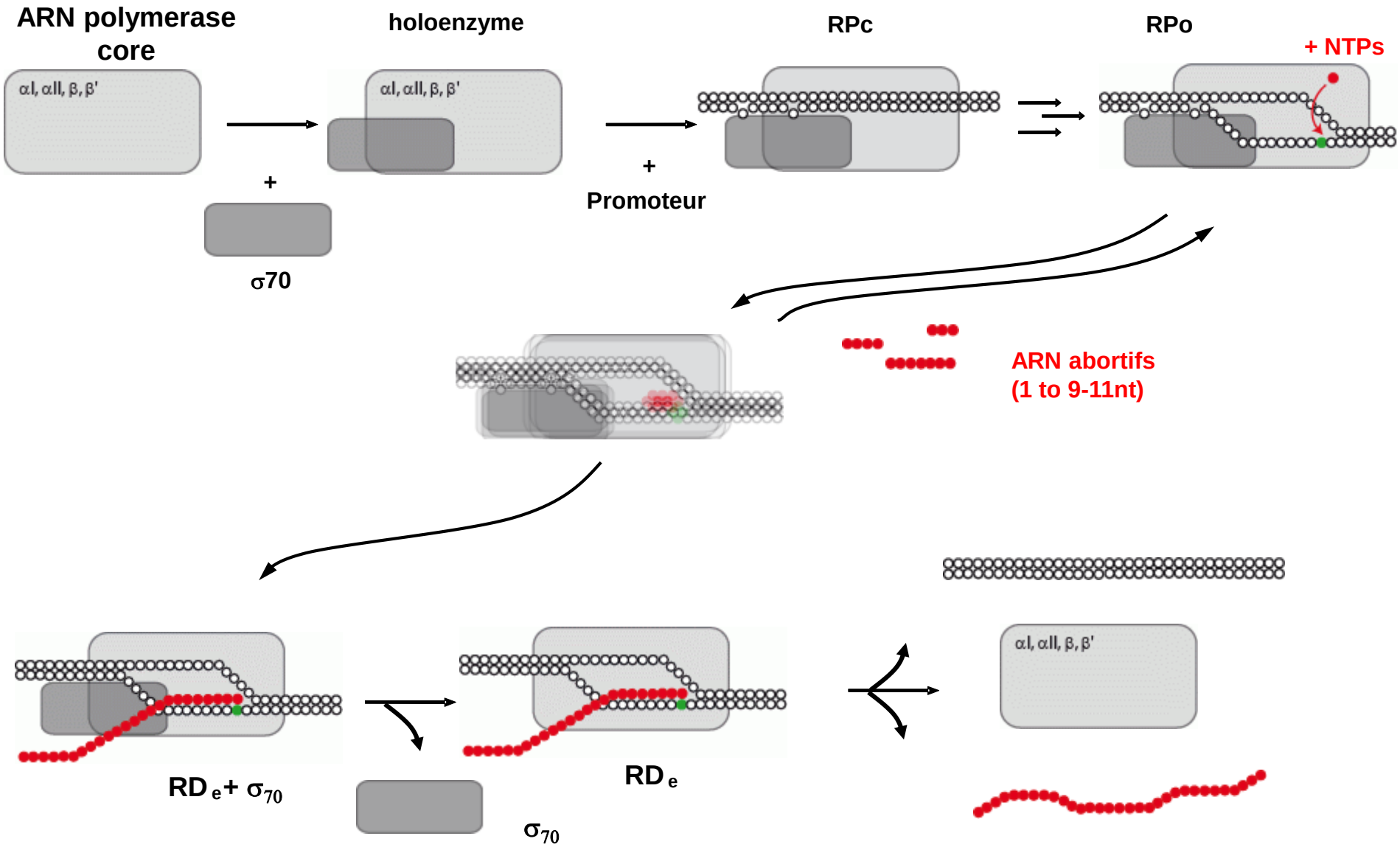
Le cycle de transcription



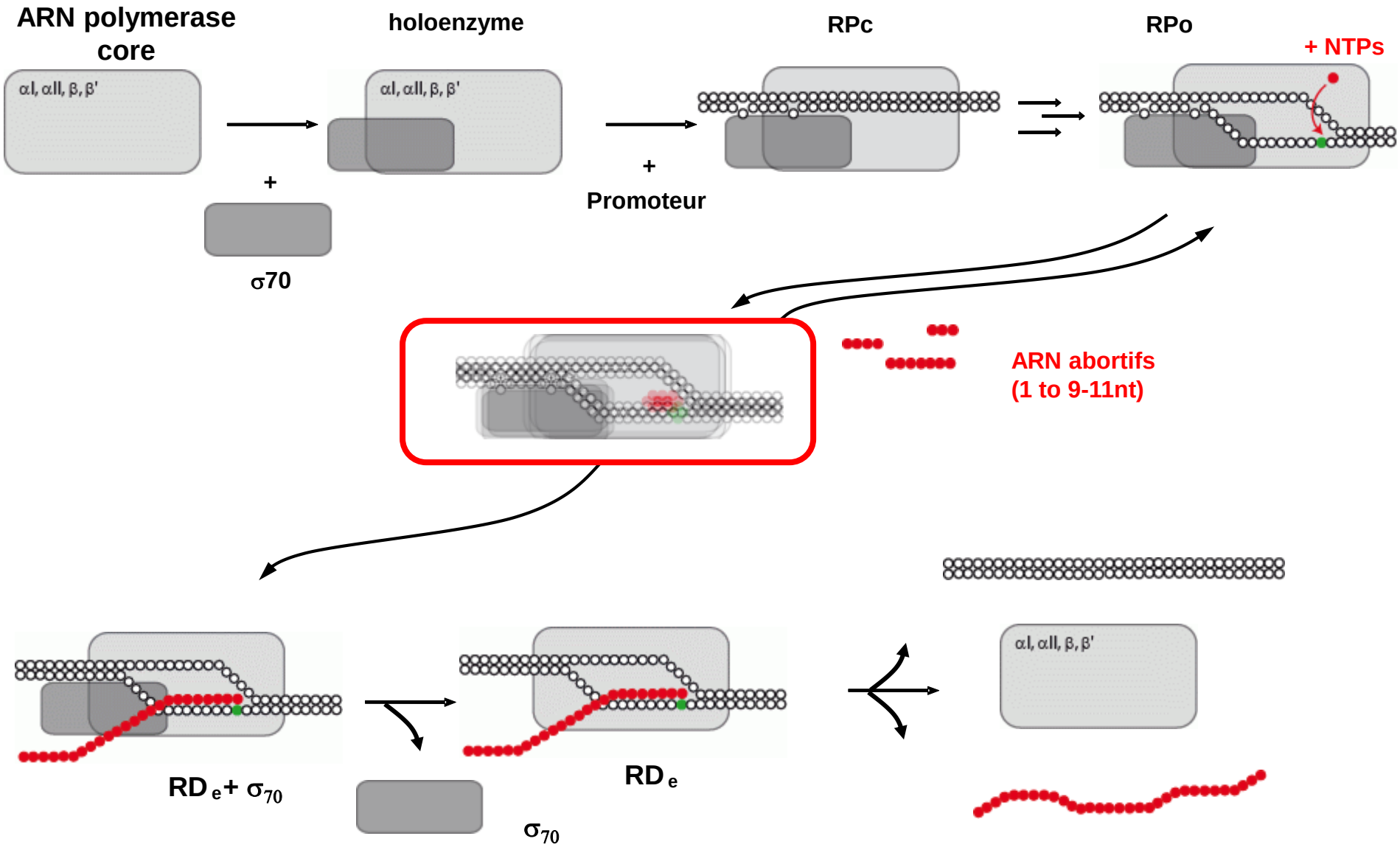
Le cycle de transcription



Le cycle de transcription



Le cycle de transcription



Les 3 modèles proposés

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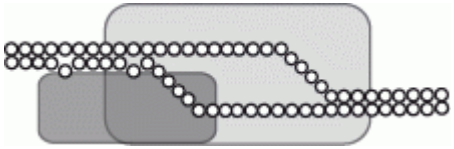
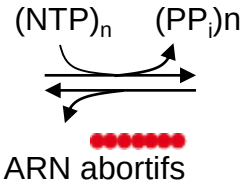
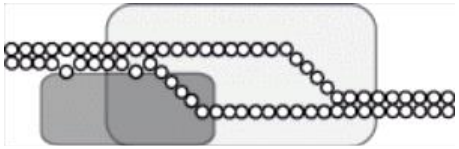
Translocation de l'ARNP (Excursions transitoires)

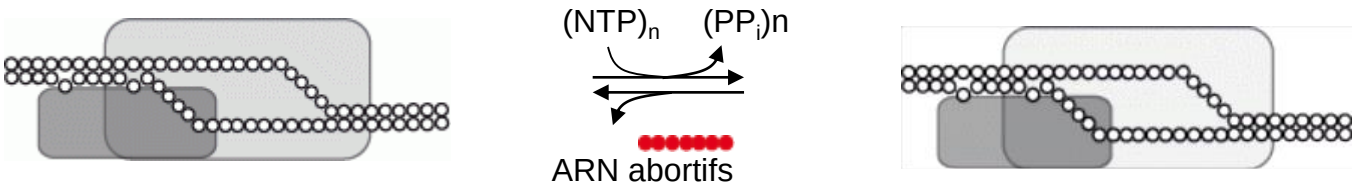




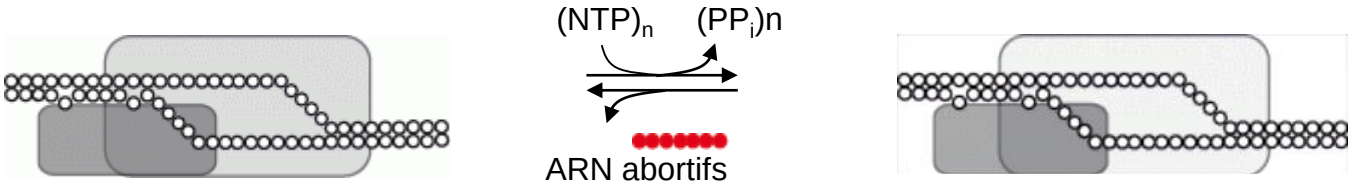


Les 3 modèles proposés

Translocation de l'ARNP (Excursions transitoires)

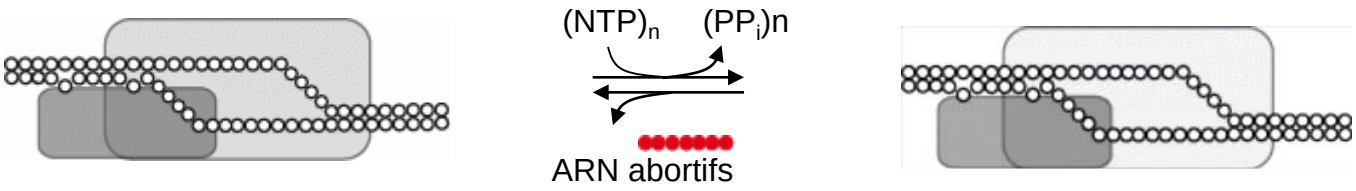


Changement conformationnel de l'ARNP (ver de terre)

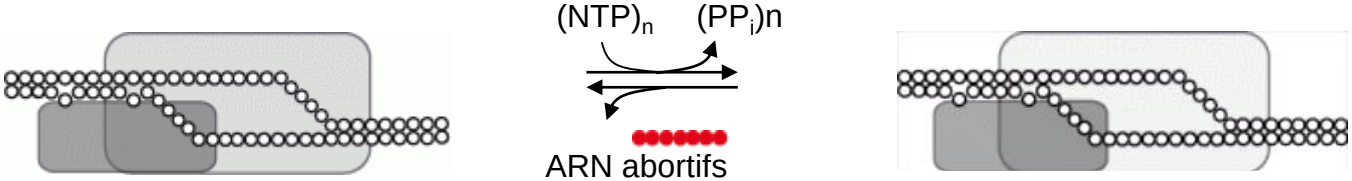


Les 3 modèles proposés

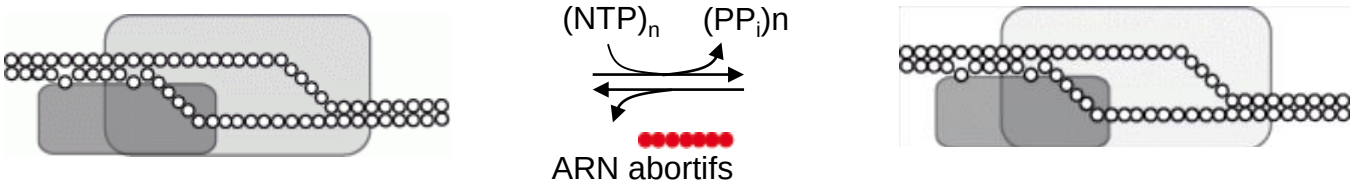
Translocation de l'ARNP (Excursions transitoires)



Changement conformationnel de l'ARNP (ver de terre)

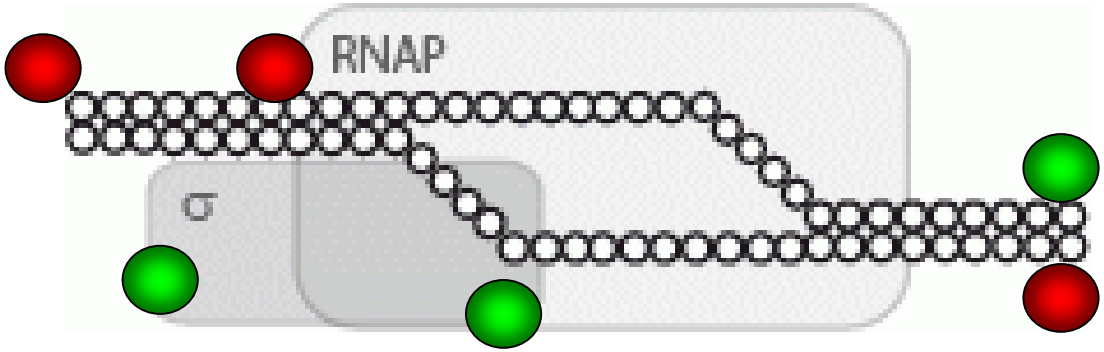


Compaction de l'ADN (« scrunching »)



Mécanisme de l'initiation abortive

Stratégie

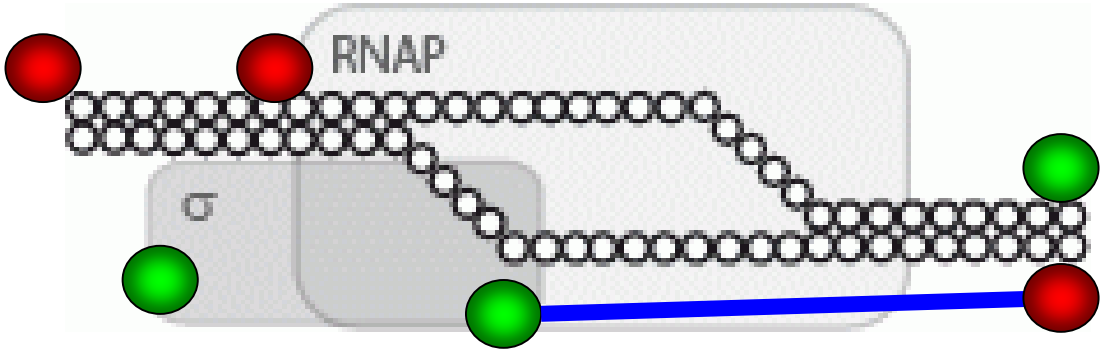


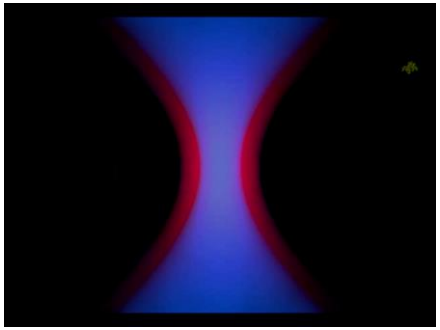
Marquage du complexe d'initiation à différentes positions

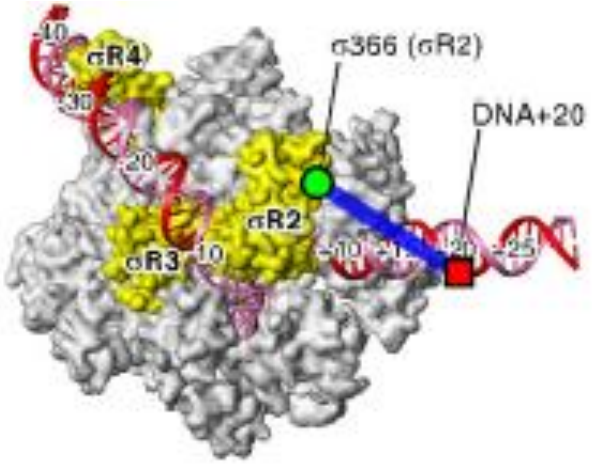
Mesure des changements de distances entre le complexe ouvert, et differents complexes abortifs

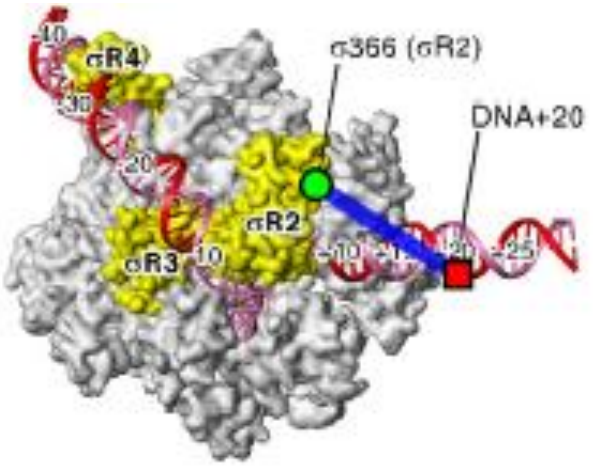
Mécanisme de l'initiation abortive

Stratégie

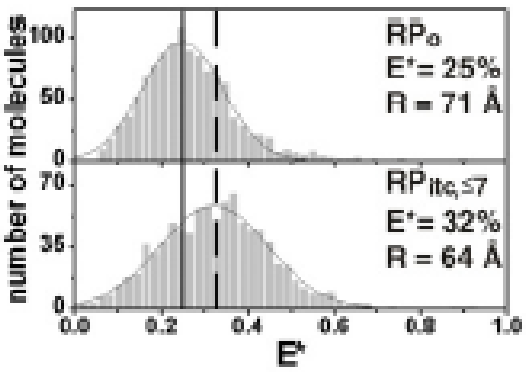


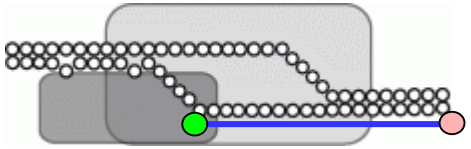






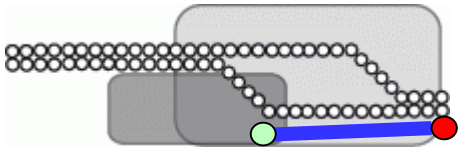
Distance diminue





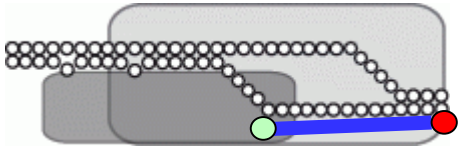
NTP subset →

Excursions transitoires



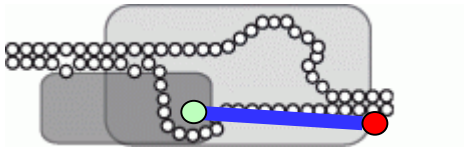
Distance diminue

Ver de terre

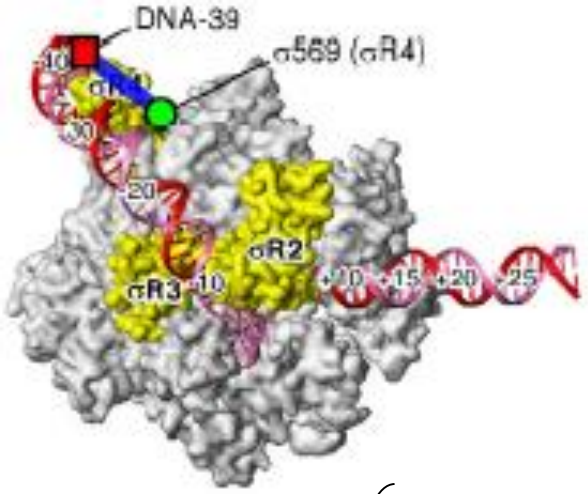


Distance diminue

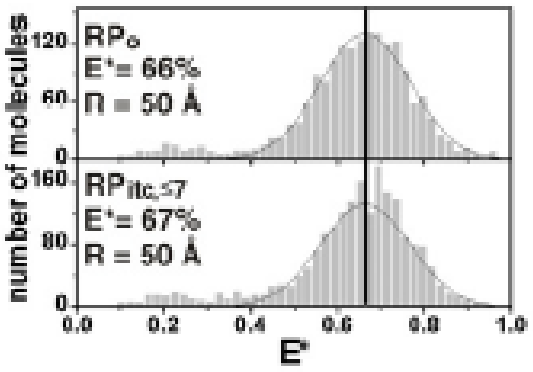
Scrunching

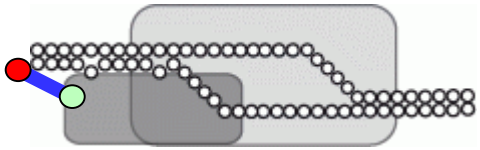


Distance diminue



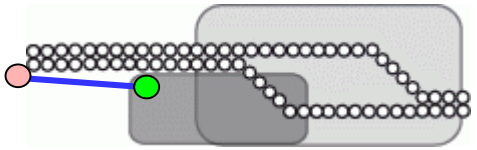
Pas de changement





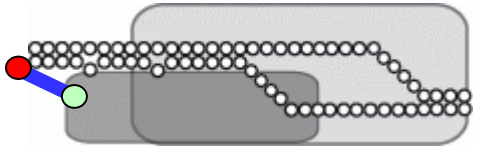
NTP subset →

Excursions transitoires



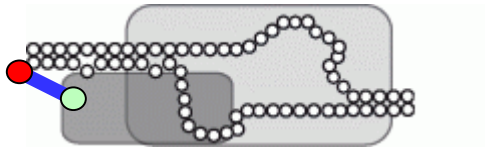
Distance augmente

Ver de terre

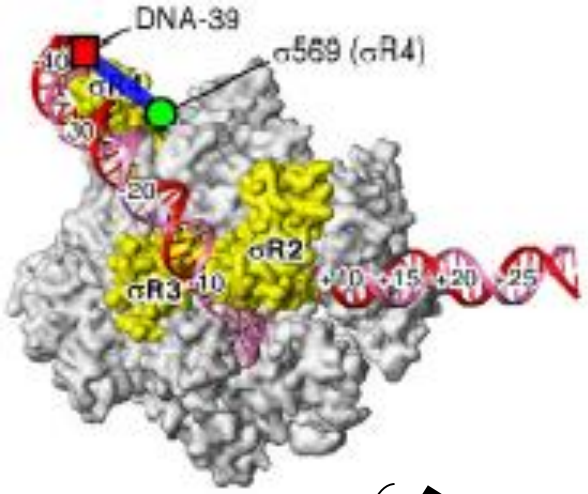


Pas de changement

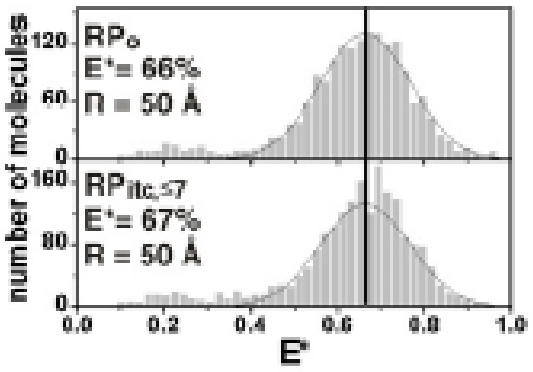
Scrunching

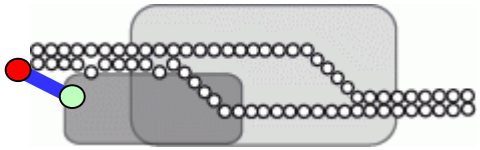


Pas de changement



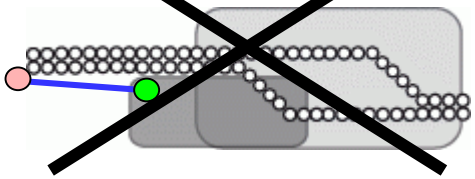
Pas de changement





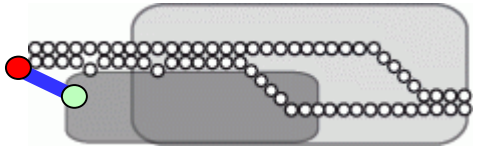
NTP subset →

Excursions transitoires



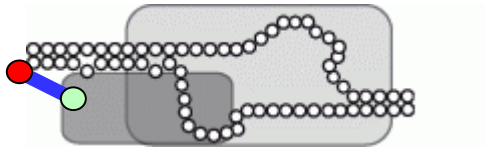
Distance augmente

Ver de terre

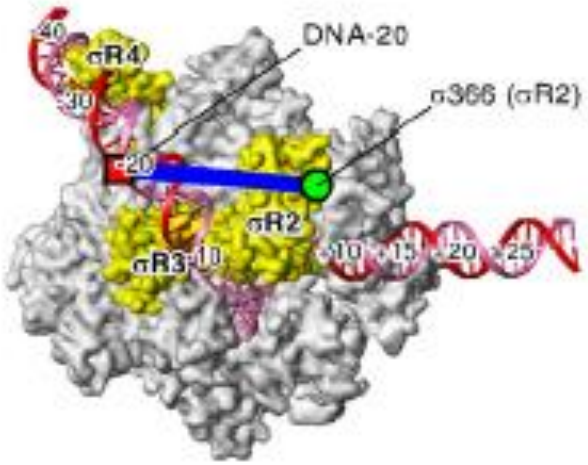


Pas de changement

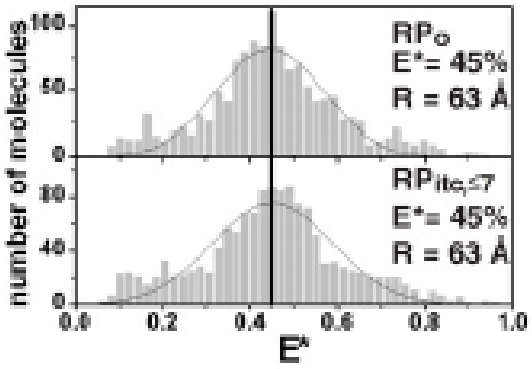
Scrunching

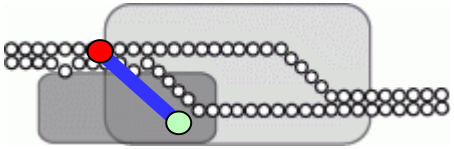


Pas de changement



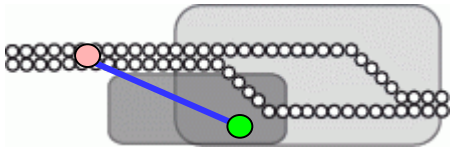
Pas de changement





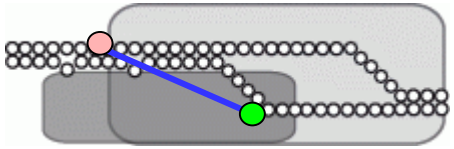
NTP subset →

Excursions transitoires



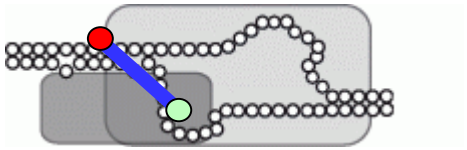
Distance augmente

Ver de terre

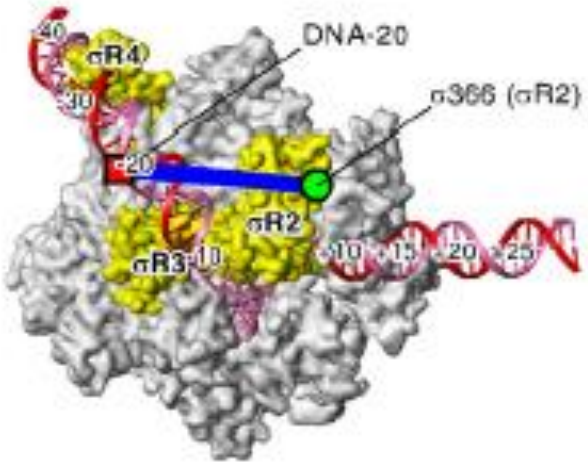


Distance augmente

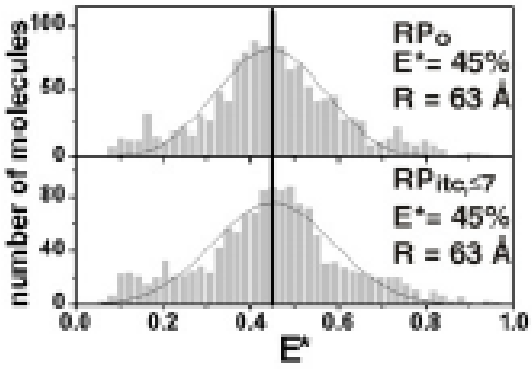
Scrunching

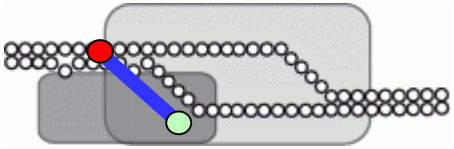


Pas de changement



Pas de changement



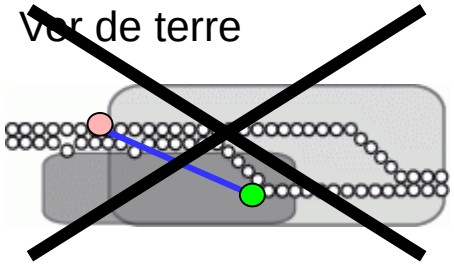


NTP subset →

Excursions transitoires

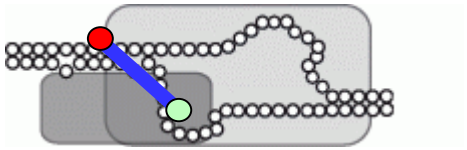
Distance augmente

Ver de terre

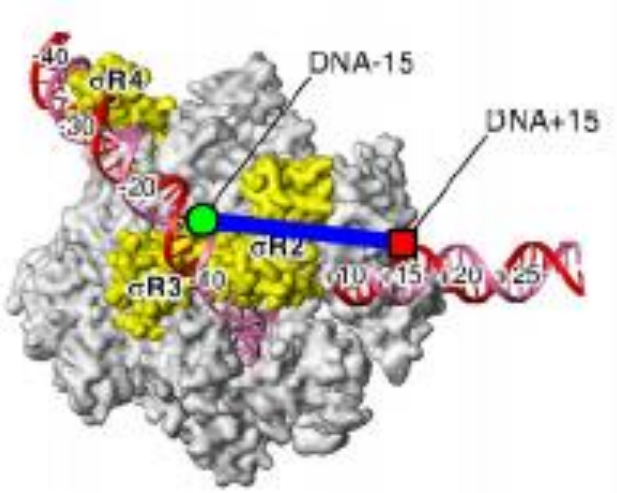


Distance augmente

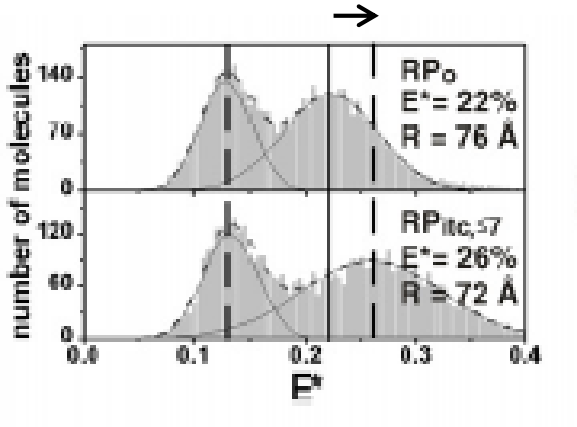
Scrunching

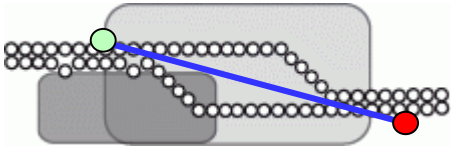


Pas de changement



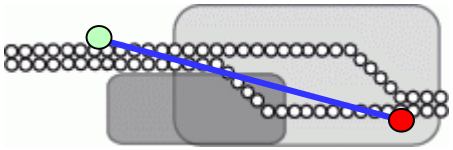
Distance diminue





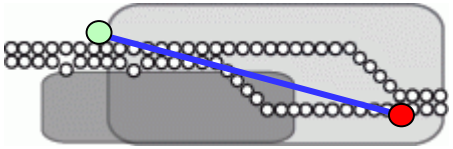
NTP subset →

Excursions transitoires



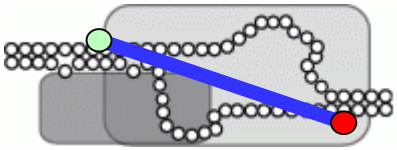
Pas de changement

Ver de terre

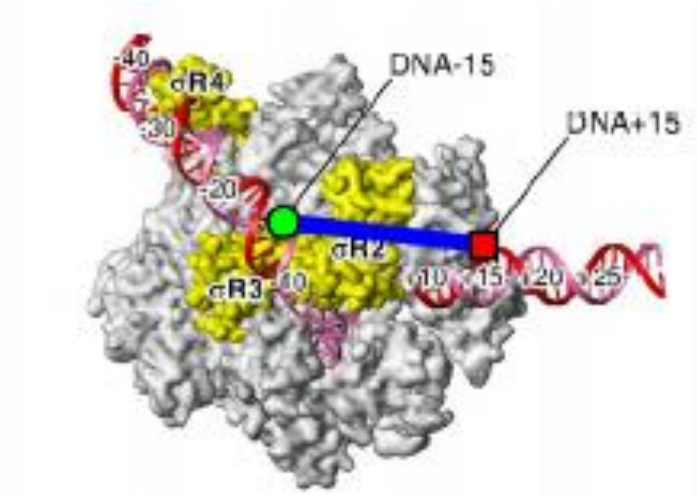


Pas de changement

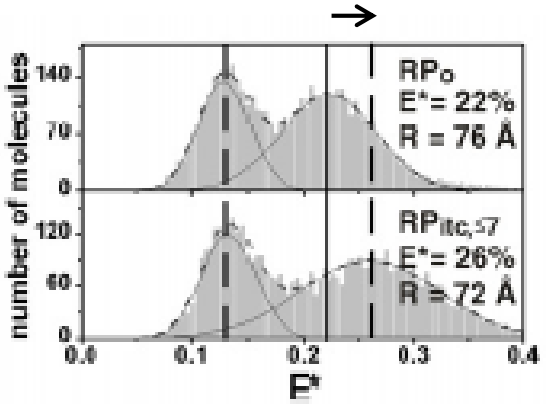
Scrunching

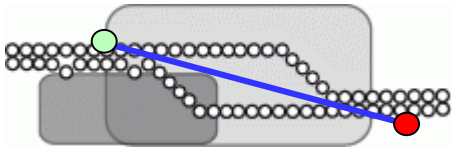


Distance diminue



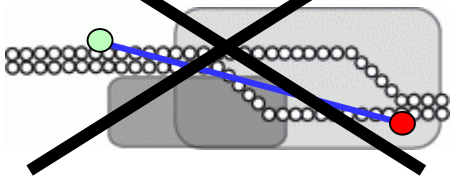
Distance diminue





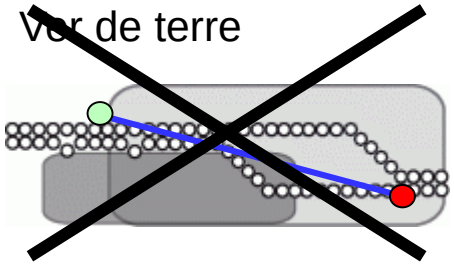
NTP subset →

Excursions transitoires



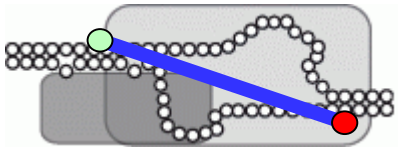
Pas de changement

Ver de terre



Pas de changement

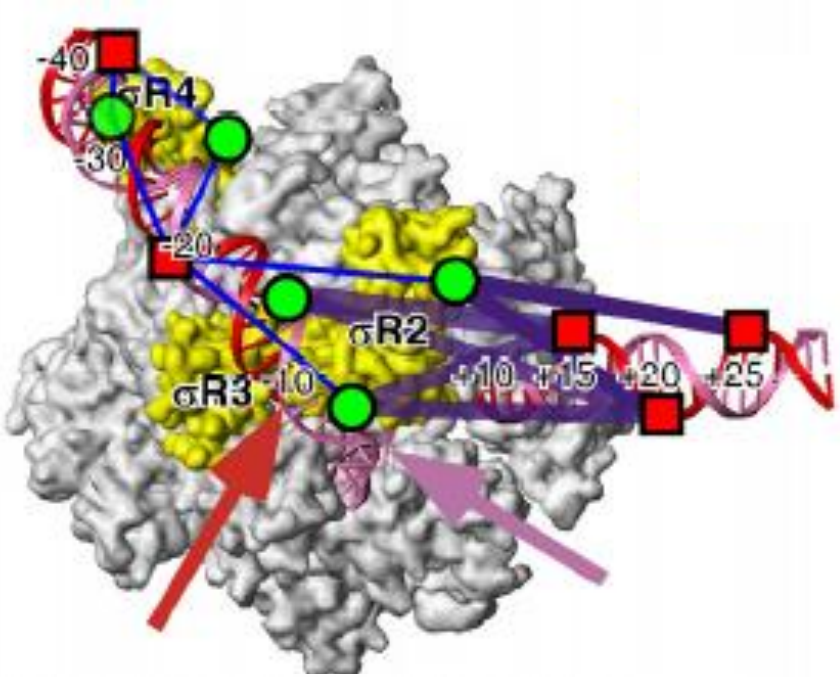
Scrunching



Distance diminue

Mécanisme de l'initiation abortive - Résultats

Toutes les données montrent une compaction de l'ADN au cours de l'initiation abortive



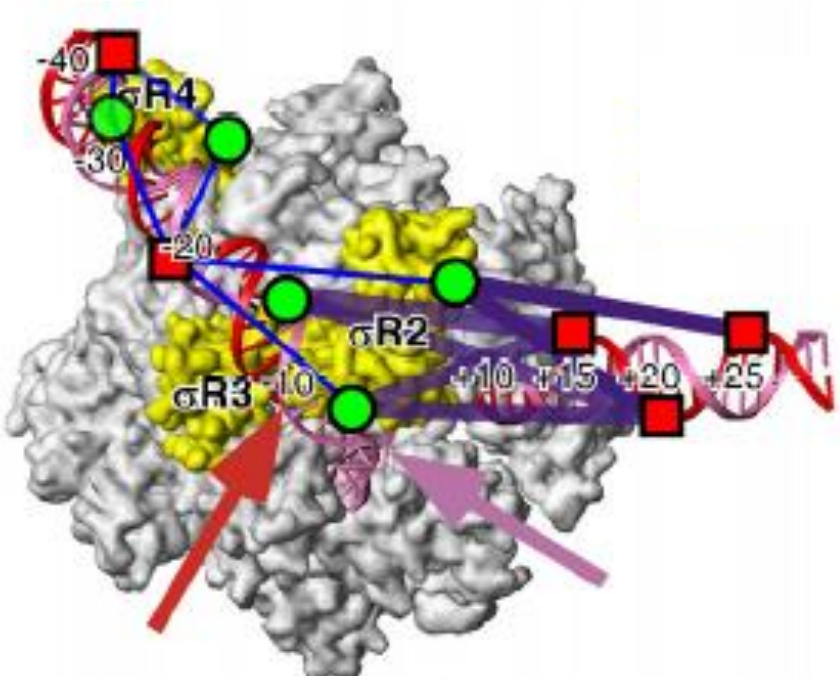
Pas de changement

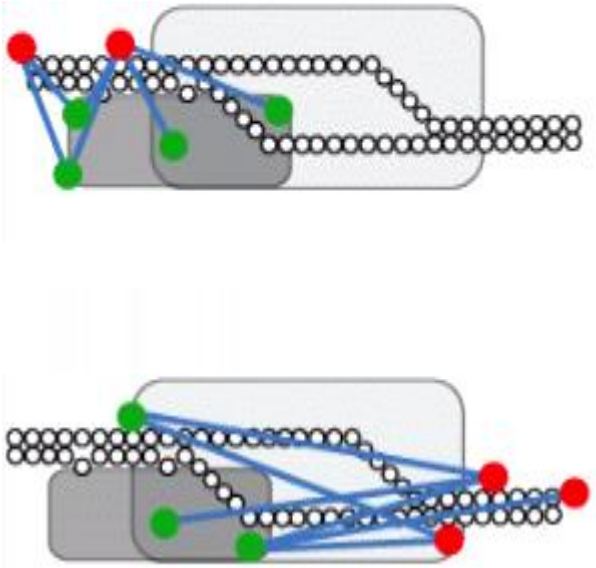
Distances diminuées

Margeat E., et al. ***Biophysical Journal*, 2006**
Kapanidis A., et al. ***Science,* 2006.**

Mécanisme de l'initiation abortive - Résultats

Toutes les données montrent une compaction de l'ADN au cours de l'initiation abortive





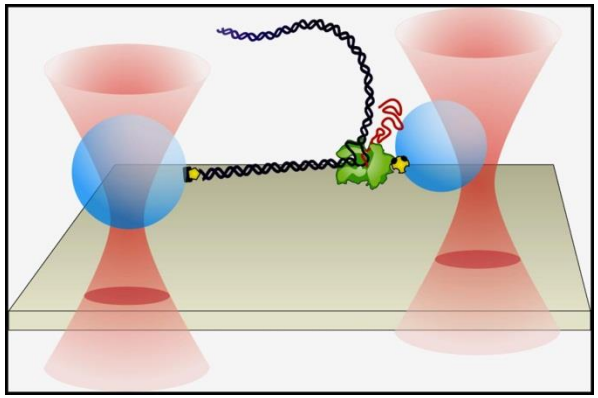
Pas de changement

Distances diminuées

Margeat E., et al. ***Biophysical Journal*, 2006**
Kapanidis A., et al. ***Science,* 2006.**

Comparaison entre élongation et initiation

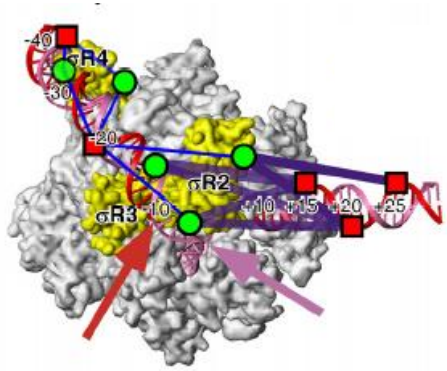
Elongation

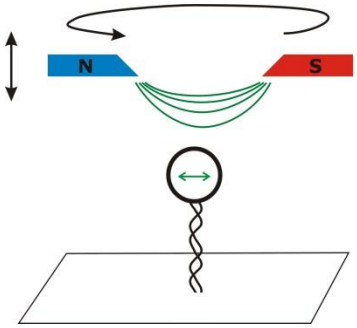


Pour chaque NTP
- translocation de 1 pb

Abbondanzieri et al. (2005) *Nature* 438, 460.

Initiation





Pour chaque NTP
- Compaction de l'ADN
-Pas de translocation

Kapanidis et al. (2006) *Science, 314, 1144*
Revyakin et al. (2006) *Science, 314, 1139*

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Team Structure and Dynamics of Nucleoproteic and Membrane Assemblies

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Further reading

Toward dynamic structural biology: Two decades of single-molecule Förster resonance energy transfer.
Science. 2018 Jan 19;359(6373)

Photophysics of fluorescent probes for single-molecule biophysics and super-resolution imaging.
Annu Rev Phys Chem. 2012;63:595-617

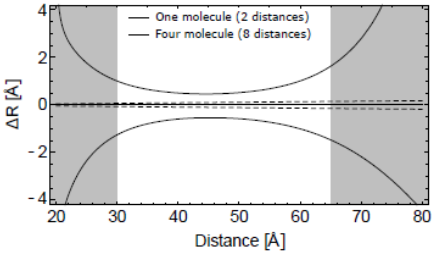
Precision and accuracy of single-molecule FRET measurements – a worldwide benchmark study.
arXiv:1710.03807v1. 10 Oct 2017

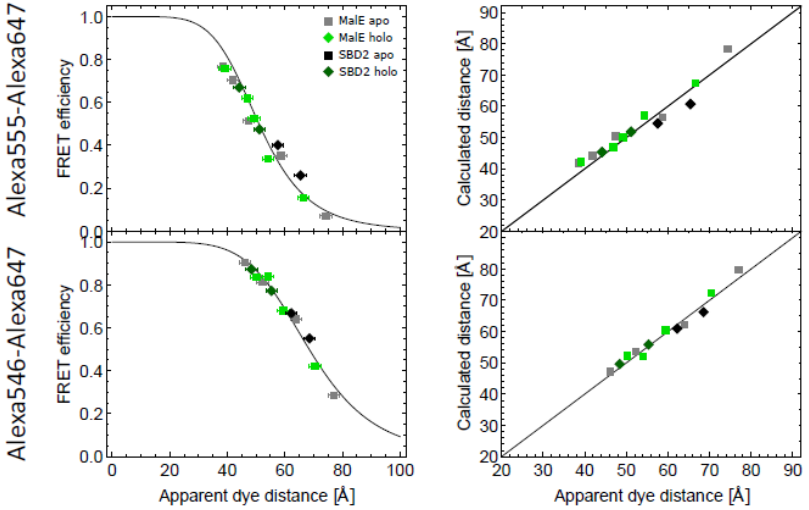
Taking the ruler to the jungle: single-molecule FRET for understanding biomolecular structure and dynamics in live cells.
Curr Opin Struct Biol. 2015 Oct;34:52-9.

Static Ruler in Dynamic Proteins

Angstrom precision distance determination:

- Comparison of two dye pairs Alexa555-Alexa647 and Alexa546-Alexa647
- Precision measurement uncertainty of lower than 5 Å for smFRET distance measurements
- Tunable sensitive range depending on Förster radius
- Error simulation indicates a better precision is theoretically possible



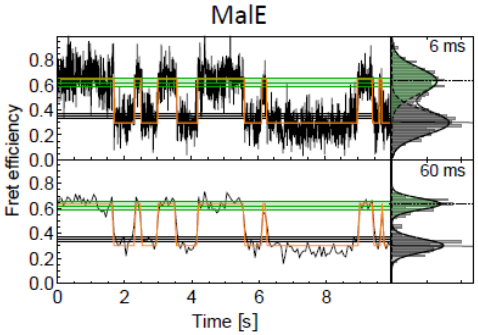


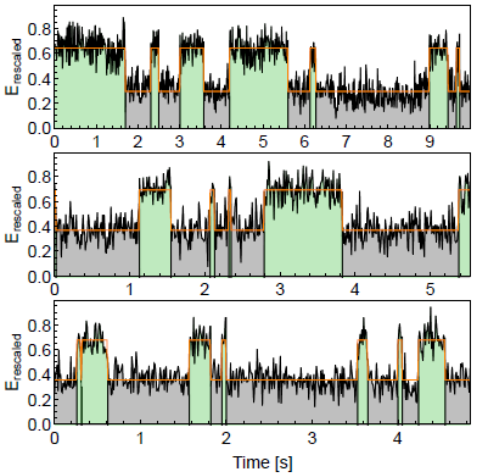
Thorben Cordes, LMU Munich, personal communication

Dynamic Ruler

Time traces of FRET signal on immobilized proteins:

- Measurement close to $K_d \approx 1$ µM with apo and holo states equally populated
- Dynamic population agrees with static population





Bottom Line:

→ **smFRET achieves angstrom precision and accuracy in static and dynamic biomolecules**

Thorben Cordes, LMU Munich, personal communication