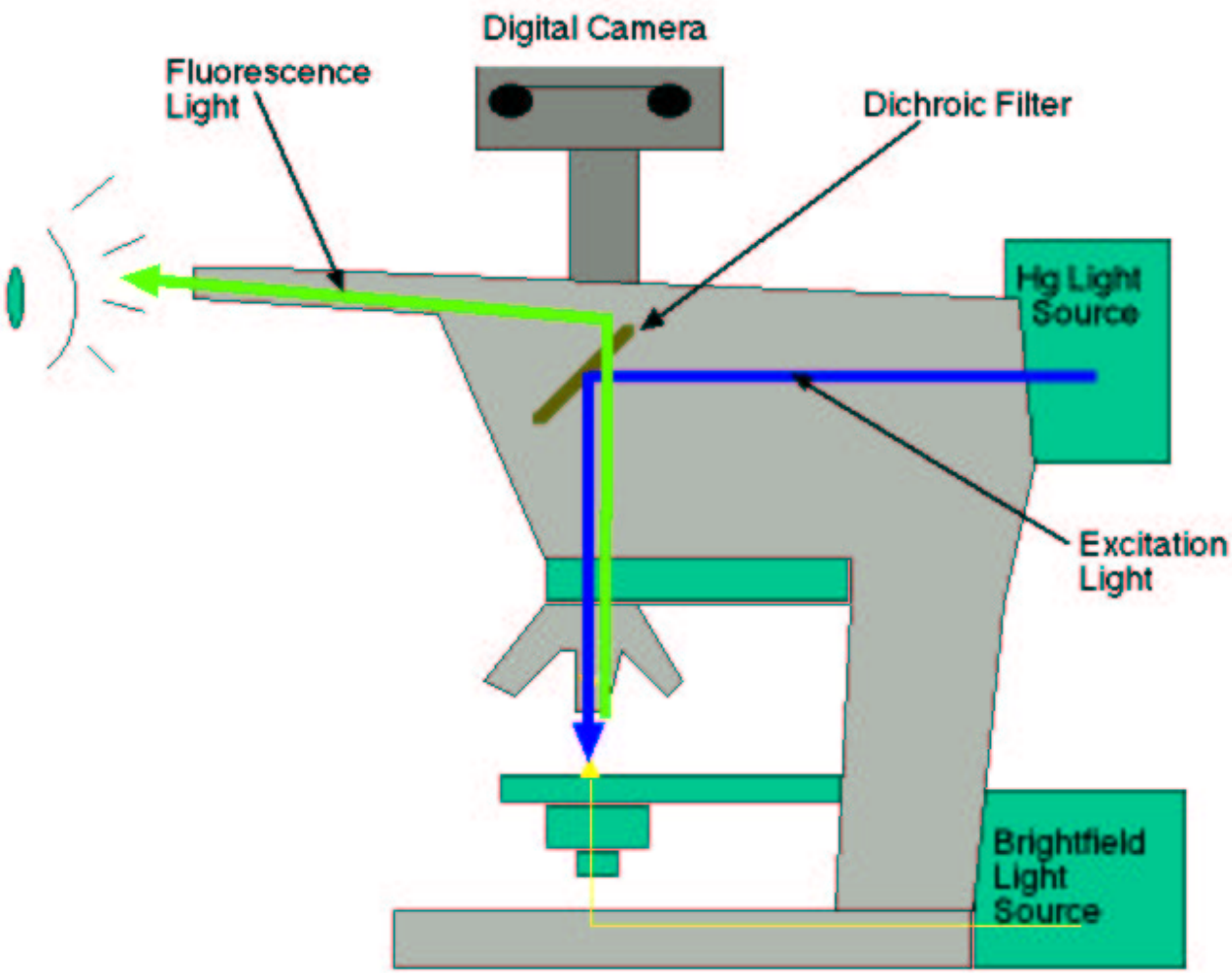
Microscopie par fluorescence

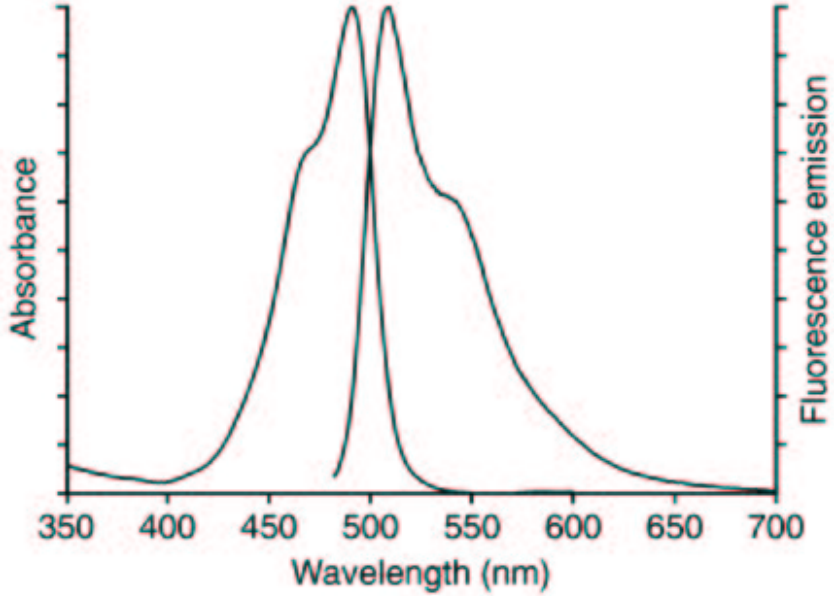


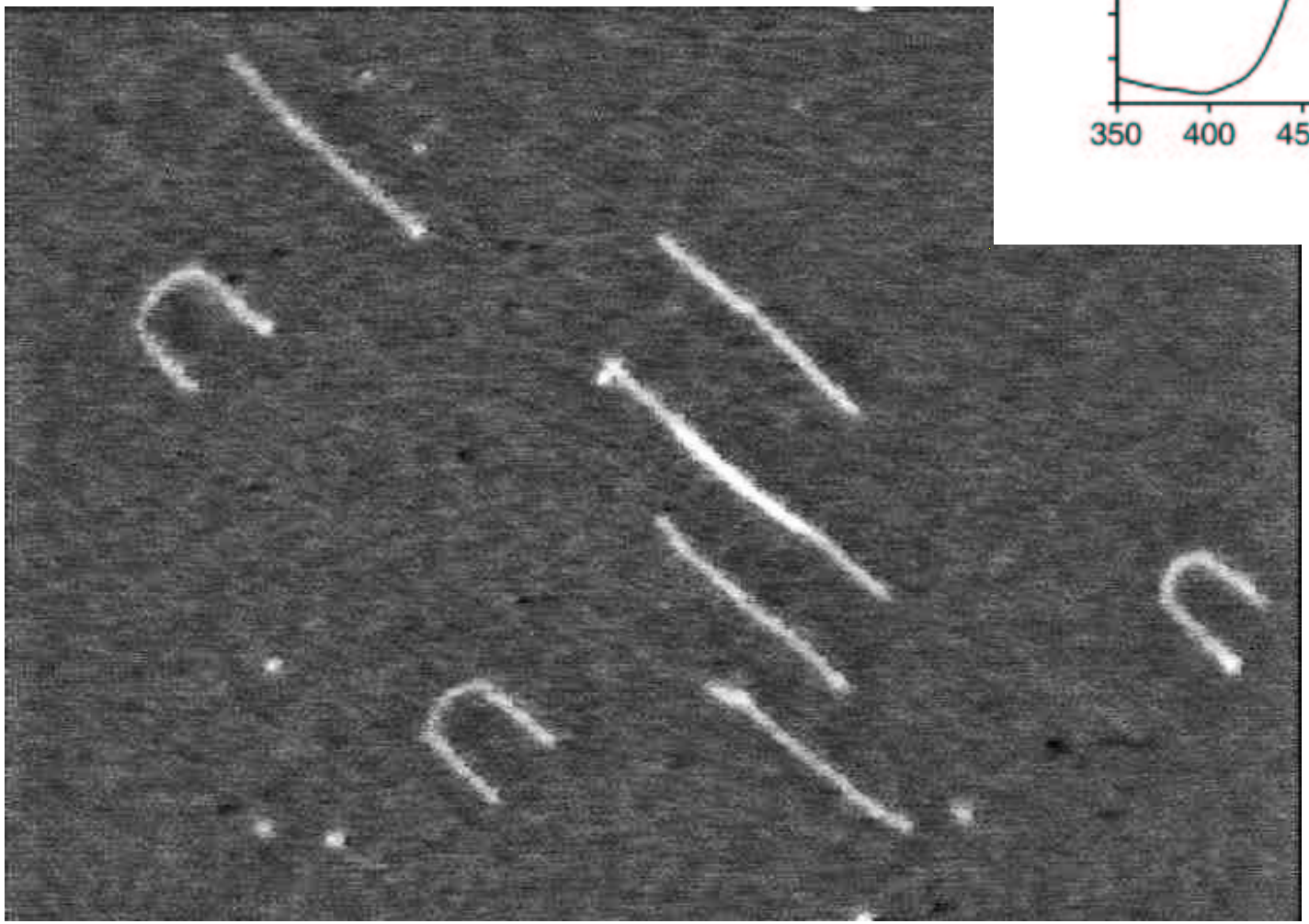
- Source intense dans le bleu
- Miroir dichroique très sélectif
- Fluorophores spécifiques

→ Problèmes en molécules uniques

Séparation difficile de la lumière de fluoresence des réflections parasites de l'éclairage

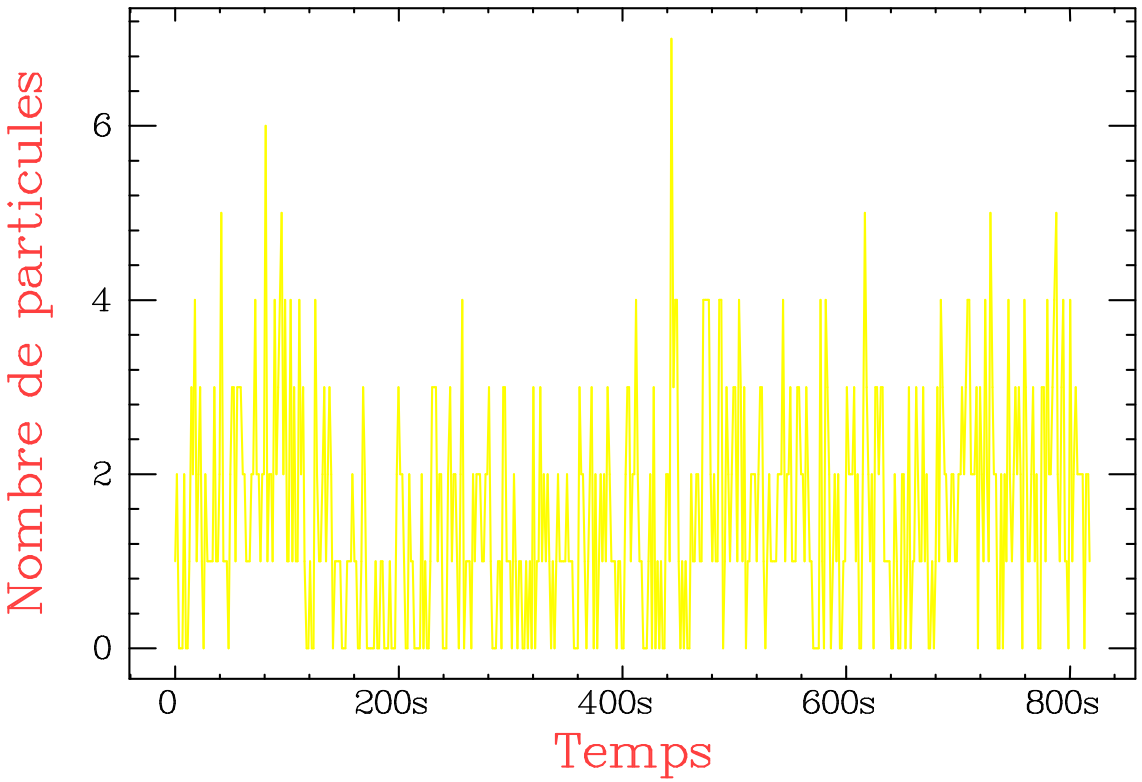
Spectres d'émission et d'absorbtion du YOYO1 accroché à l'ADN ——————————→

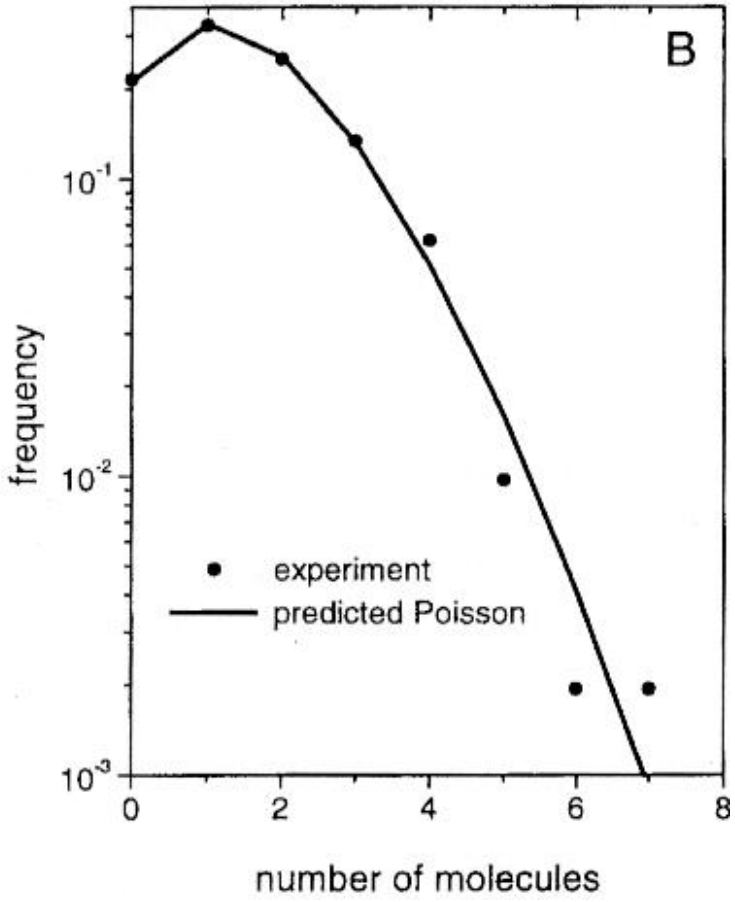




○ Visualisation de molécules d'ADN peignées avec le YOYO1

Particules d'or diffusant dans un petit volume

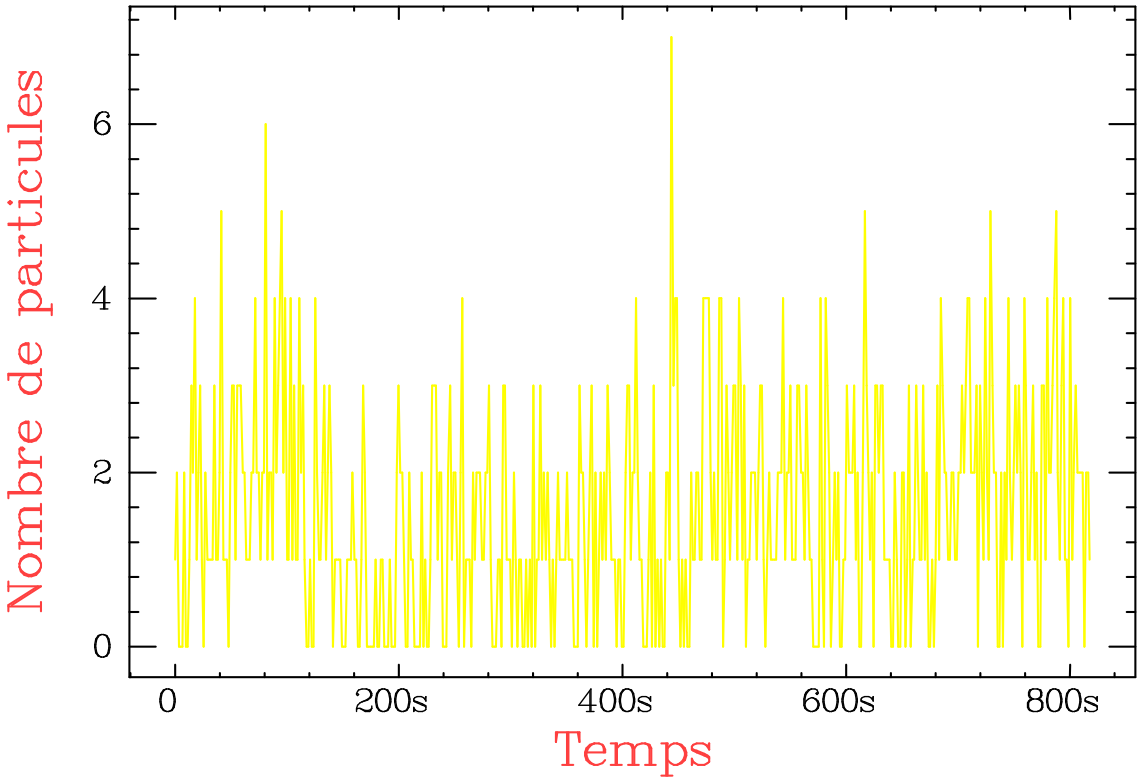




T. Svedberg and K. Inouye, Z. Phys. Chem. 77, 145–191, (1911)

$$\rho(N) = \frac{N^N \exp(-N)}{N!}$$

Distribution de probabilité des particules dans un petit volume.



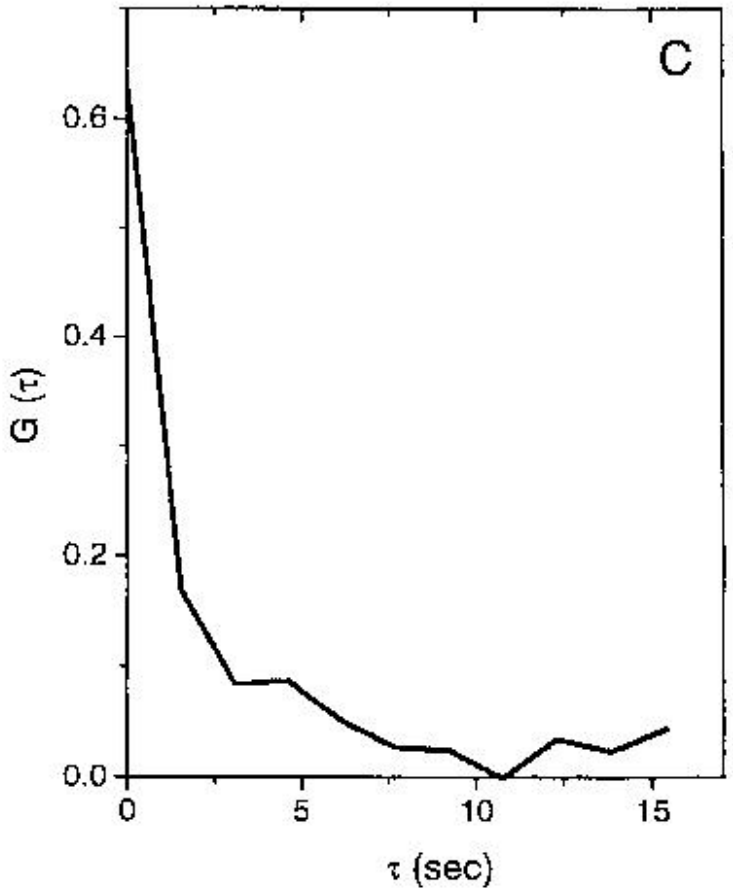
Le temps de décroissance de la corrélation τ est égal au temps de résidence dans le volume éclairé.

$$\tau = \frac{l^2}{D}$$

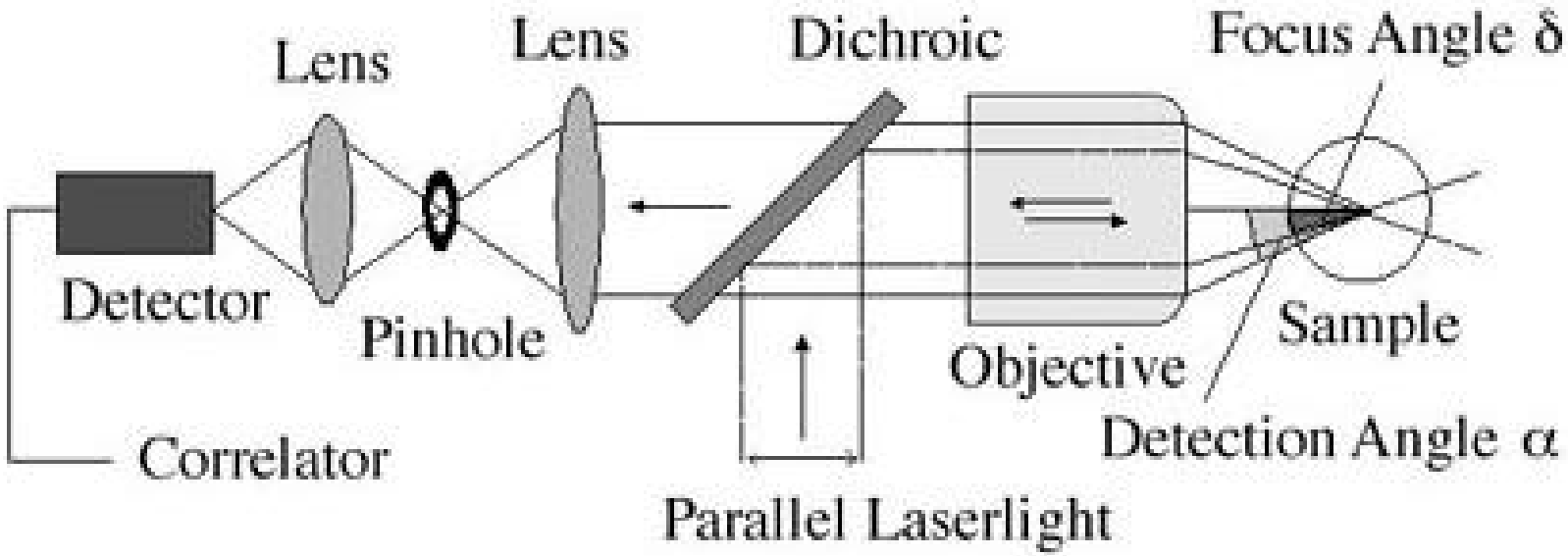
$$G(\tau) = \frac{<N(t).N(t+\tau)> - <N(t)>^2}{<N(t)^2>}$$

Fonction d'autocorrélation

$$G(0) = \frac{1}{N} \rightarrow \text{Nombre moyen de particules}$$

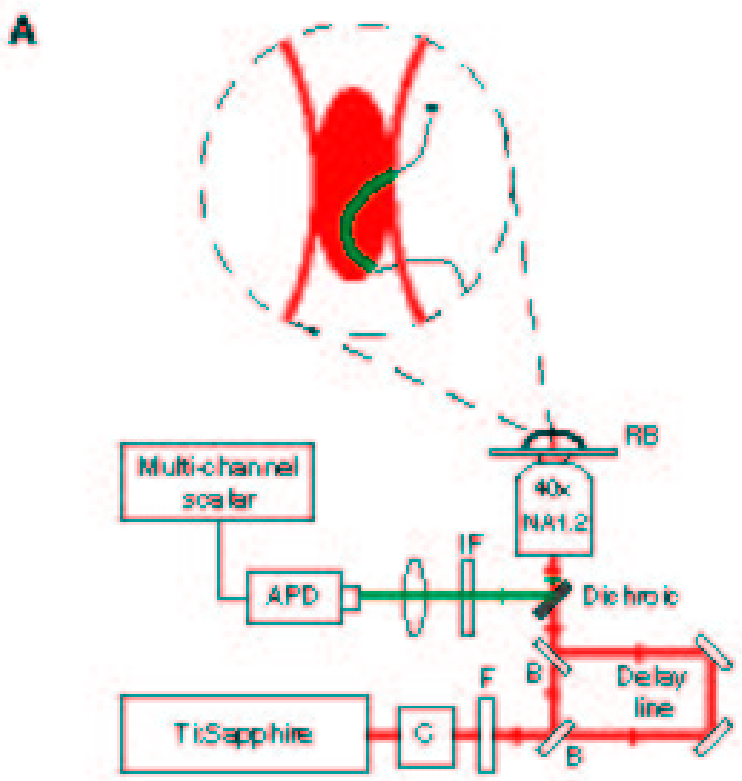


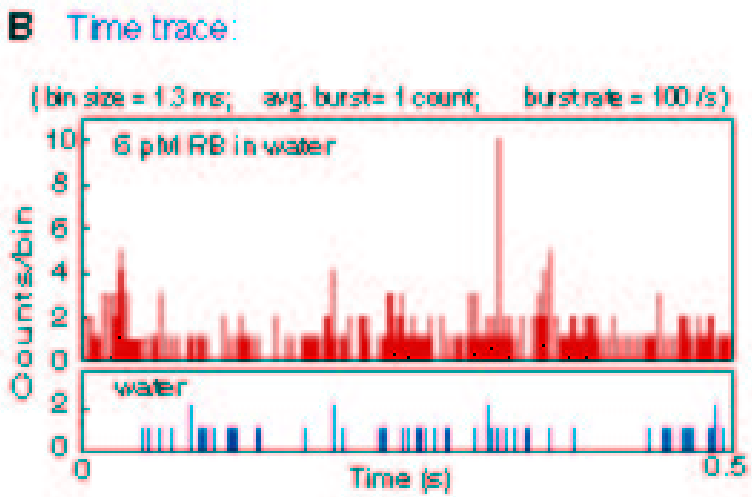
Montage confocal

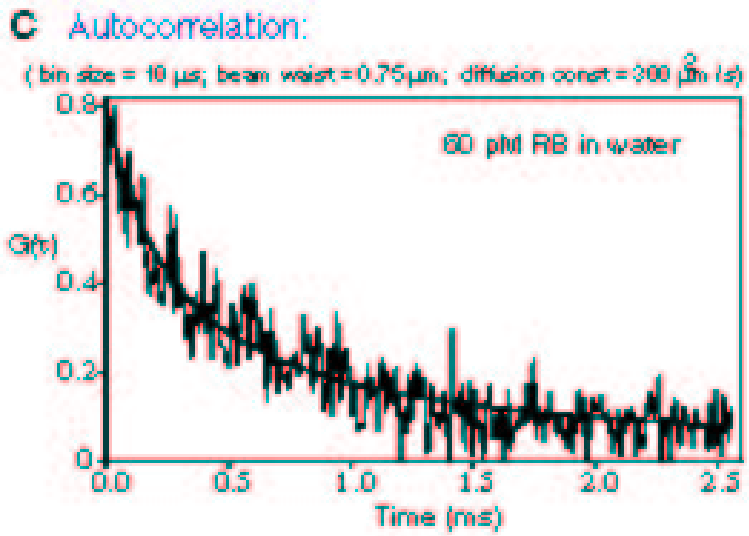


- Le diaphragme est placé au point focal image de l'objectif
- Le diaphragme permet de définir un petit volume dont vient la fluorescence
- permet de faire de la FCS avec un petit volume (0.5 μm)^3
- Permet de faire de la microscopie 3D d'un échantillon : il faut bouger le point focal et le diaphragme simultanément.

Fluoresence Correlation Spectroscopy (FCS)





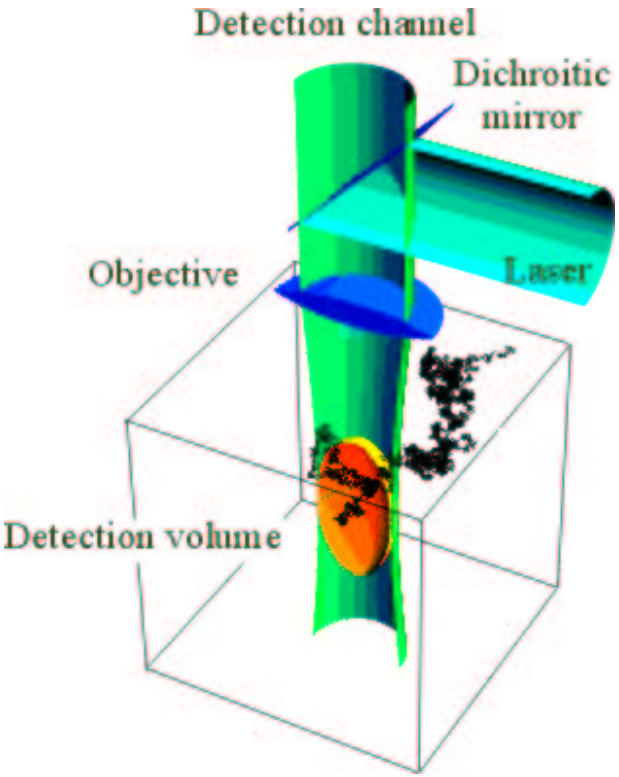


Un domaine d'excitation très petit ($< 1\ \mu m^3$), permet d'avoir très peu de molécules fluorescentes excitées simultanéement.

Diffusion

Cinétique d'une réaction

Diffusion moléculaire



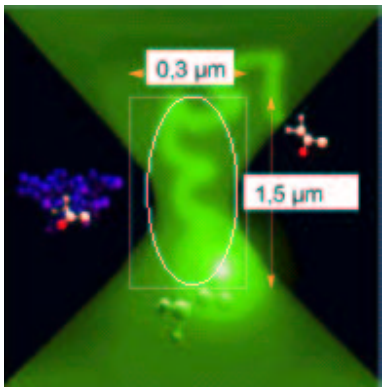
Fonction d'autocorrélation

$$G(t) = \frac{1}{N}\left(1 + \frac{1}{\tau_D}\right)^{-1}\left(1 + \frac{1}{\omega^2 \tau_D}\right)^{-1/2}$$

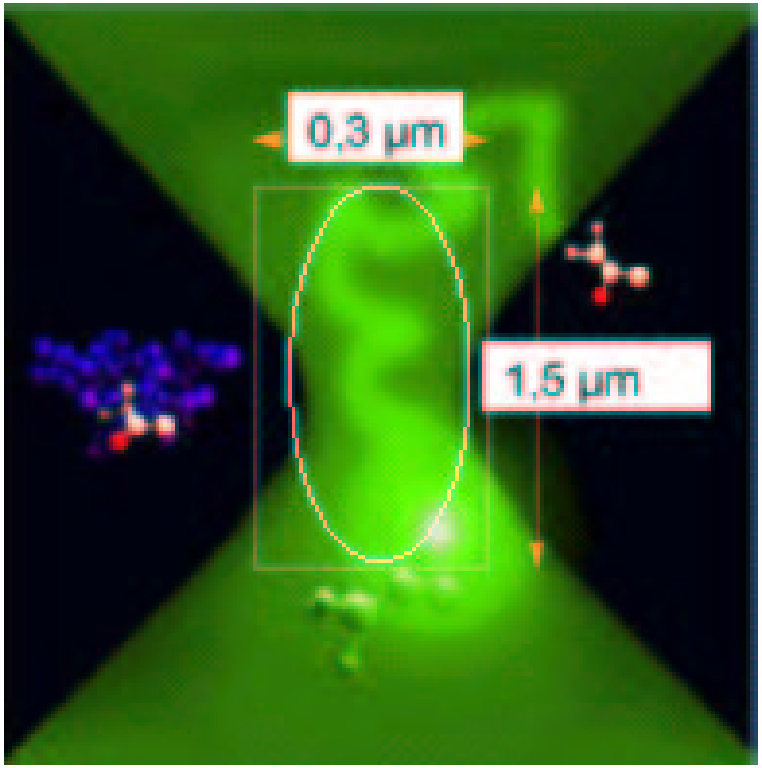
$V = \pi^{3/2}\ w_{xy}^2\ w_z$ Volume effectif d'analyse

$\tau_D = w_{xy}^2/4D$, $\tau'_D = w_z^2/4D$ et $\omega = w_z/w_{xy}$

$N = V[C]$ nombre moyen de molécule dans le volume d'analyse



Cinétique d'une réaction chimique



$$A \underset{k_{BA}}{\overset{k_{AB}}{\rightleftarrows}} B$$

A fluorescent, B non

A et B même coefficient de diffusion

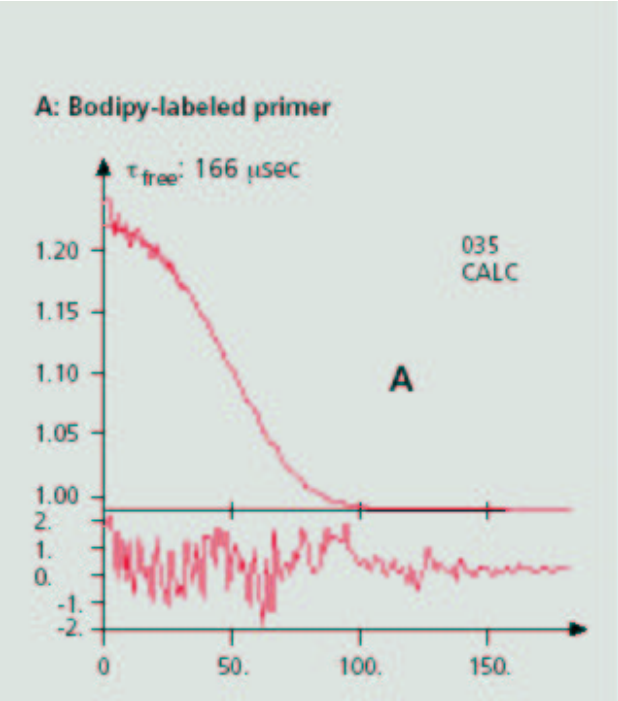
Fonction de corrélation

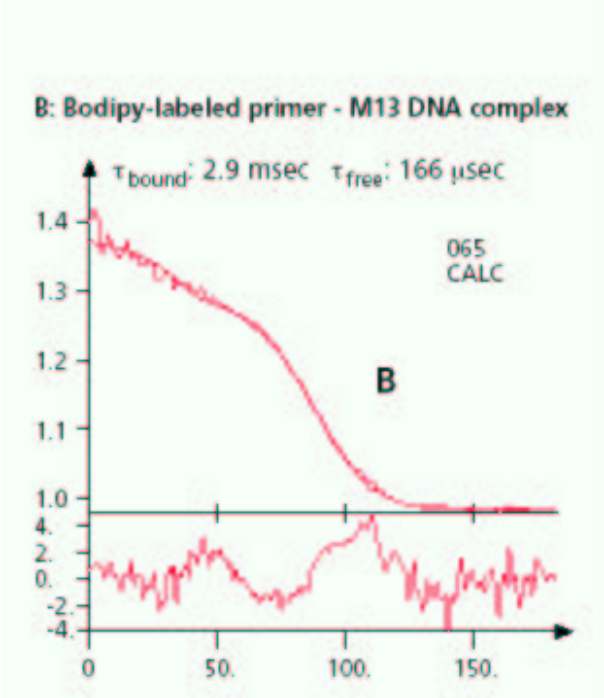
$$G(t) = \frac{1}{N}\left(1 + \frac{1}{\tau_D}\right)^{-1}\left(1 + \frac{1}{\omega^2\tau_D}\right)^{-1/2}\left(1 + K\exp(t/\tau)\right)^{-1}$$

Avec $M = V([A] + [B])$, $K = k_{AB}/k_{BA} = [B]/[A]$ et $\tau = (k_{AB} + k_{BA})^{-1}$

Modification des propriétés de diffusion

Hybridation d'un oligonucléotide fluorescent

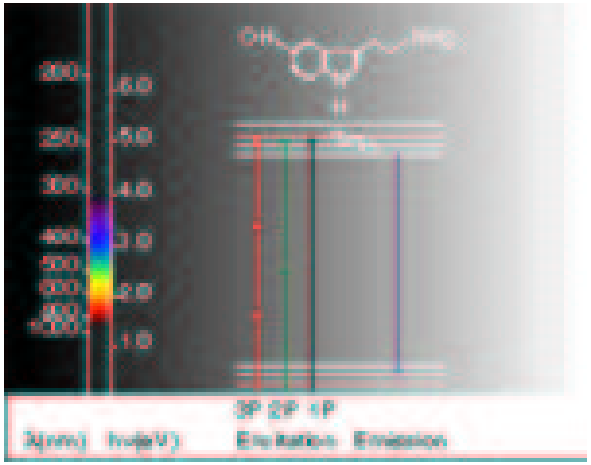


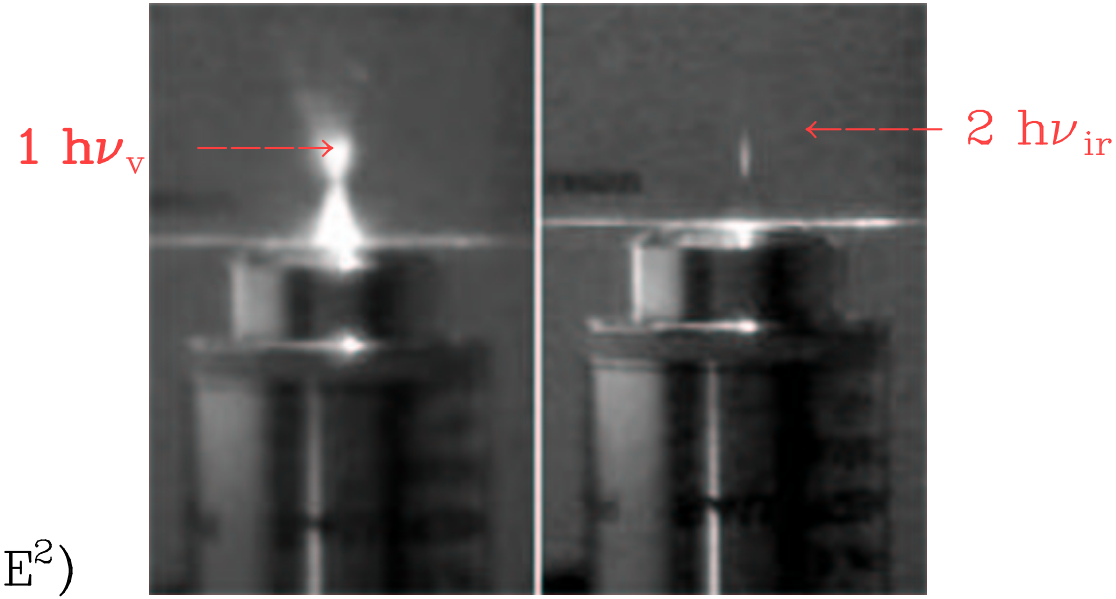


Anisotropie de fluorescence

Fluorescence multiphotoniques (W.W. Webb)

Avec une source laser très intense mais très brève, on atteint le régime non–linéaire d'excitation par la lumière, on stimule une molécule fluorescente avec l'harmonique deux ou trois de la fréquence incidente. Deux photons IR sont absorbés pour provoquer une fluorescence dans le visible.



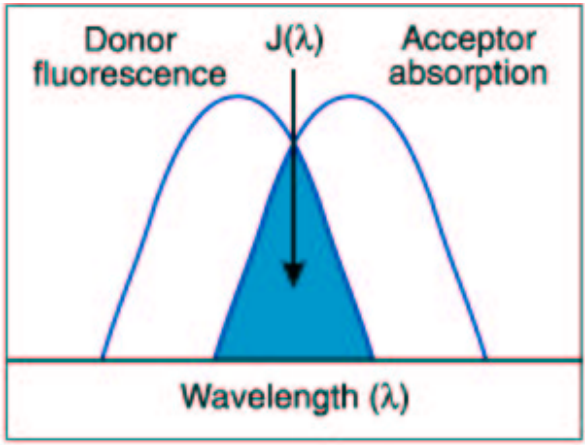


- Excitation très ponctuelle (en E^2)
- Très bonne séparation chromatique de l'exitation et de la fluorescence
- Plusieurs fluorophores peuvent être excités en même temps
- Peut travailler dans des milieux diffusants

– laser femtoseconde (cher)

Fluorescence Resonance Energy Transfert (FRET)

Deux molécules fluorescentes différentes une donneur et l'autre accepteur peuvent transférer l'excitation de la fluorescence avec une éfficacité qui dépend de leur distance distance respective R comme $(R_0/R)^6$



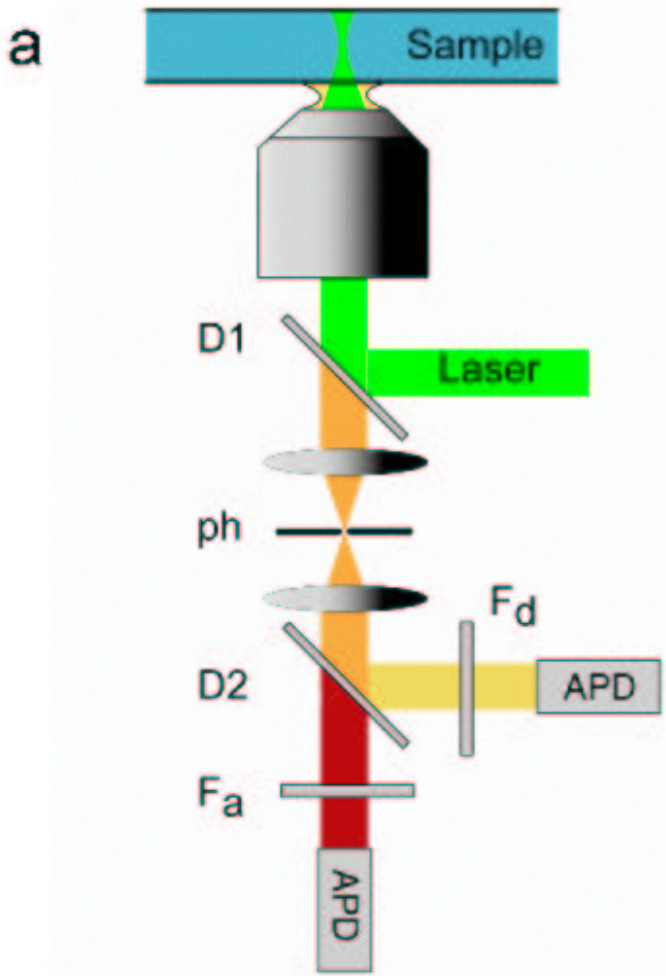
- Il faut que les spectres d'émission du donneur recouvre le spectre d'adsorbtion de l'accepteur
- Permet de mesurer la distance entre les deux molécules (de 1 à 10 nm).
- Il faut que les dipoles des deux molécules soient parallèles.

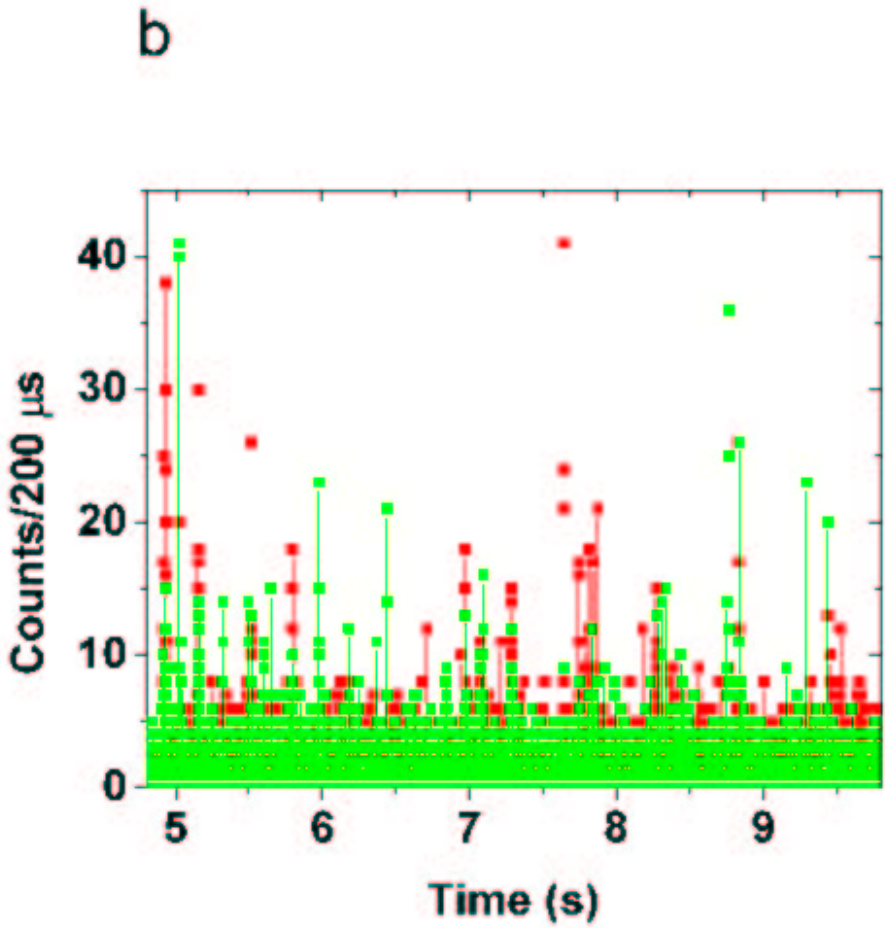
R_0 rayon de Foerster dépend des paires donneur/accepteur

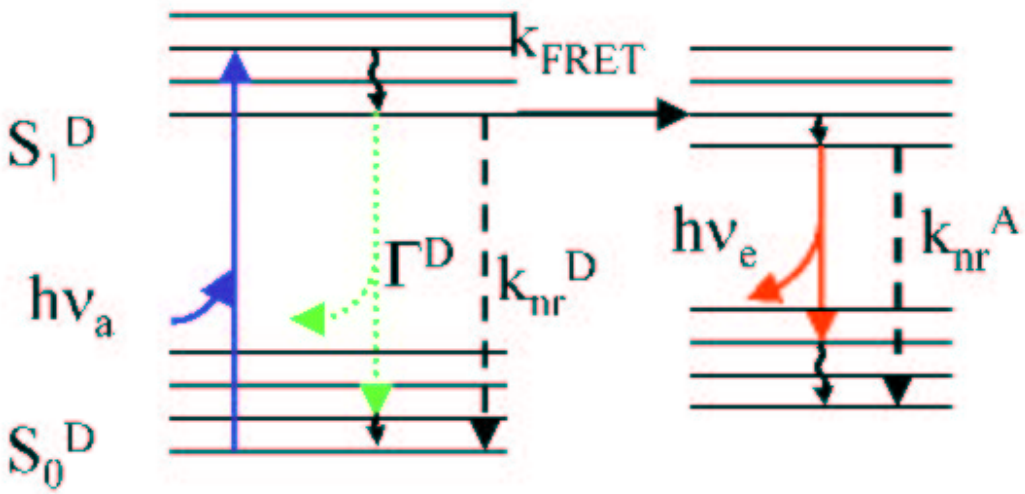
Exemple : Fluorescein / Tetramethylrhodamine R_0 = 5.5 nm

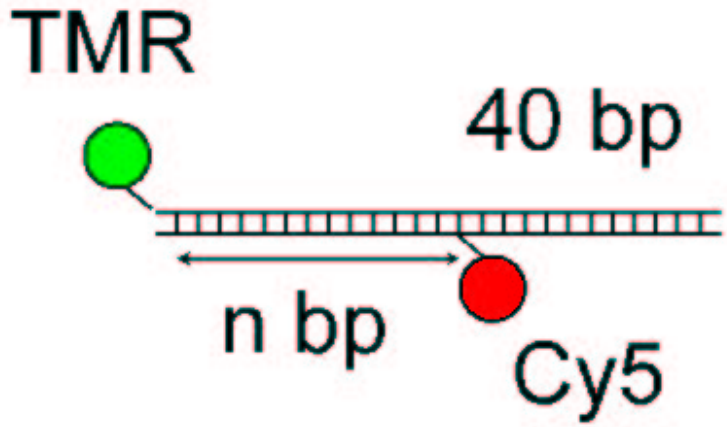
Application à l'ADN dénaturation d'une protéine

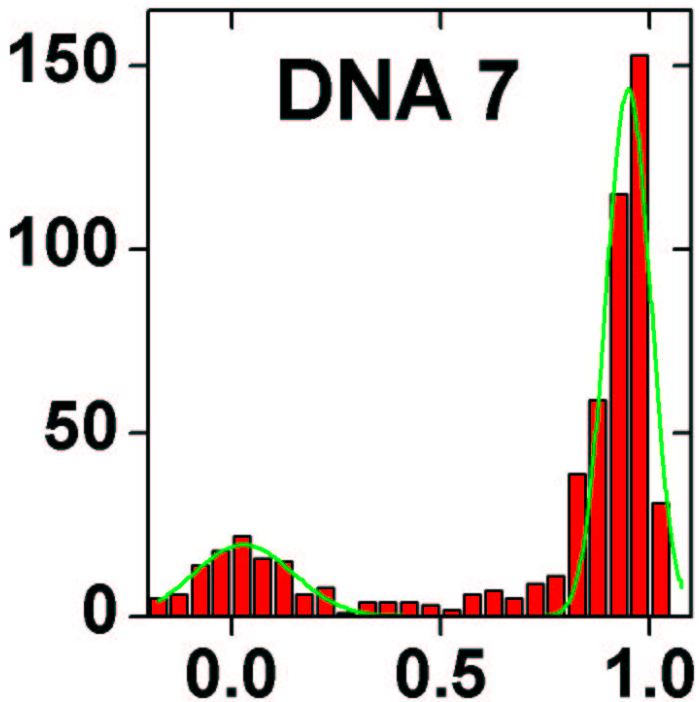
FRET appliqué à la molécule d'ADN

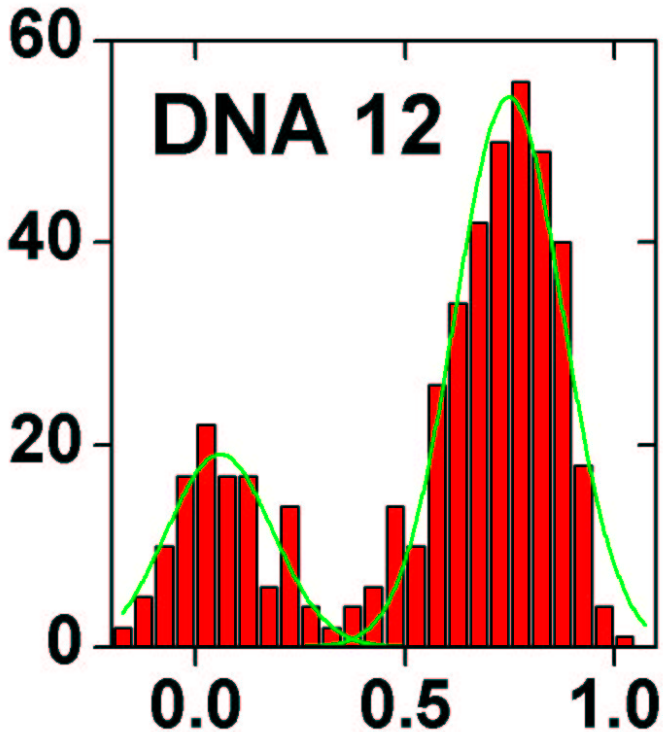


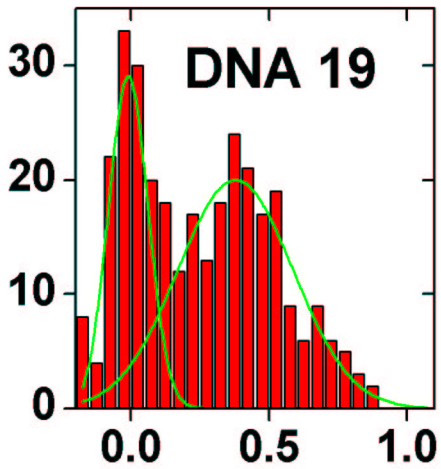



k_{FRET}
S_1^D
$h\nu_a$
Γ^D
k_{nr}^D
$h\nu_e$
k_{nr}^A
S_0^D

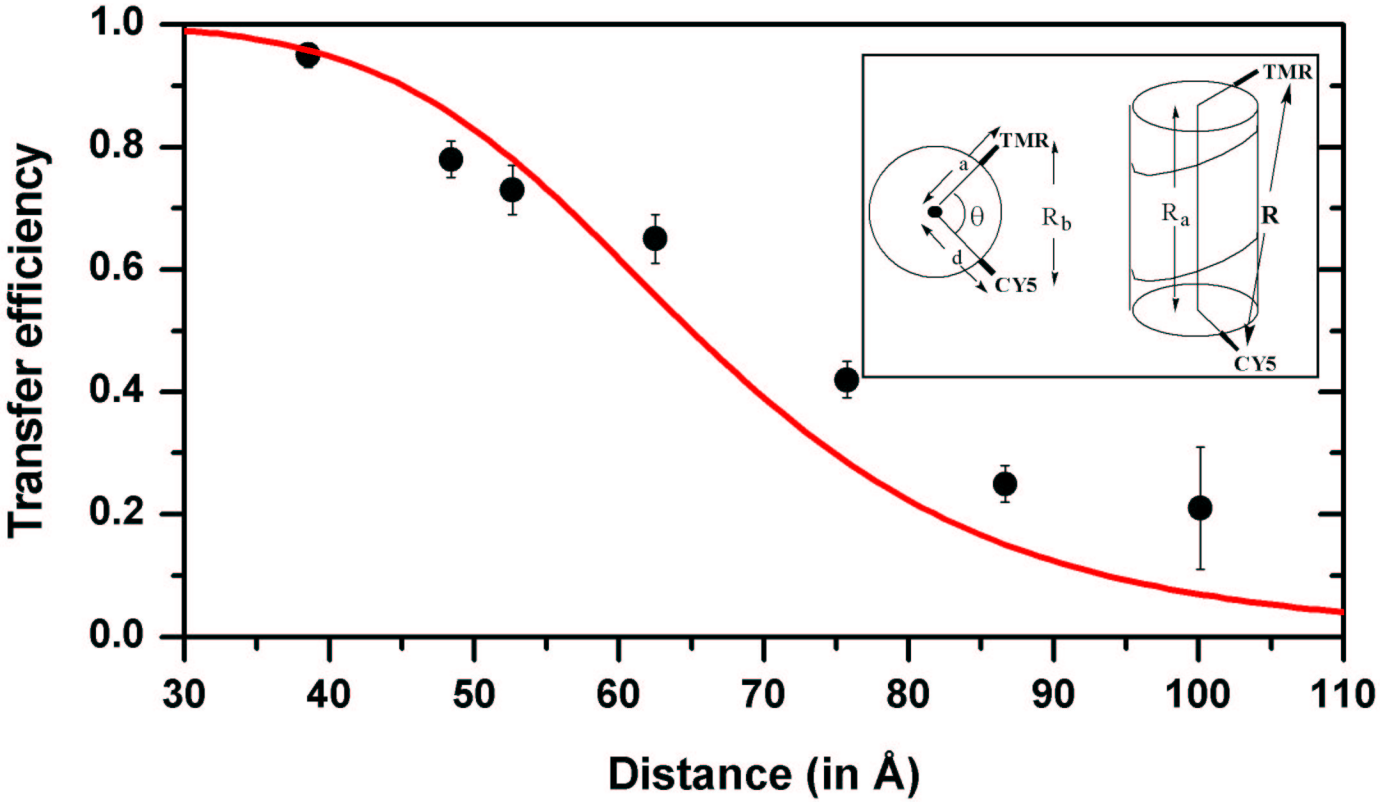

TMR
40 bp
n bp
Cy5


DNA 7
150
100
50
0
0.0
0.5
1.0

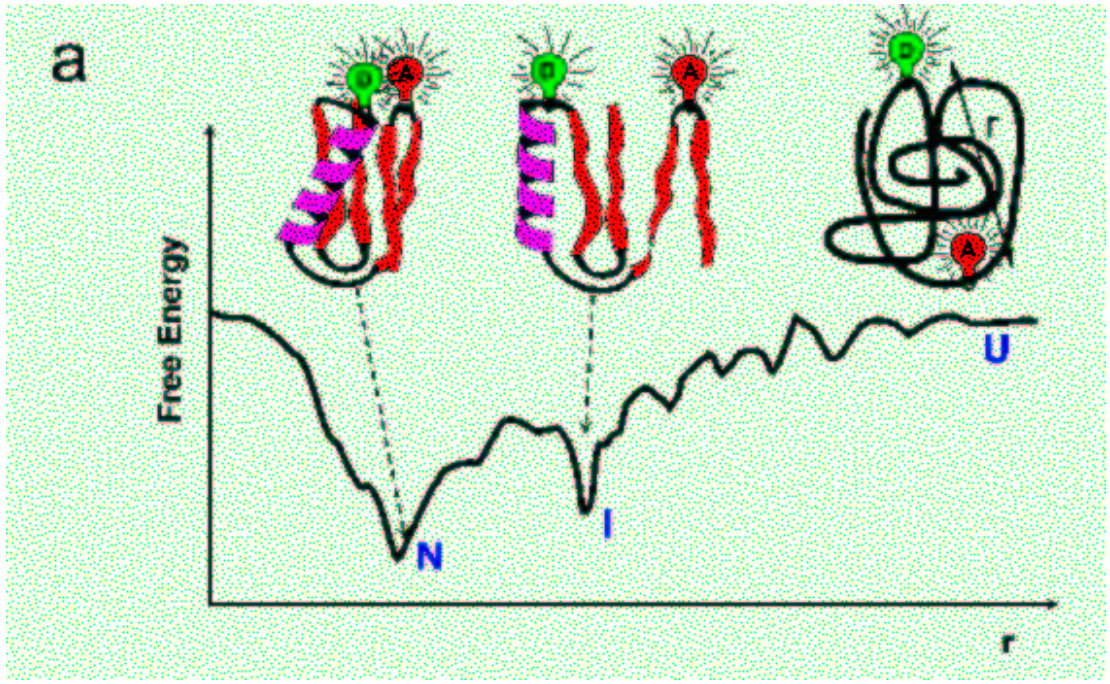

DNA 12
60
40
20
0
0.0
0.5
1.0



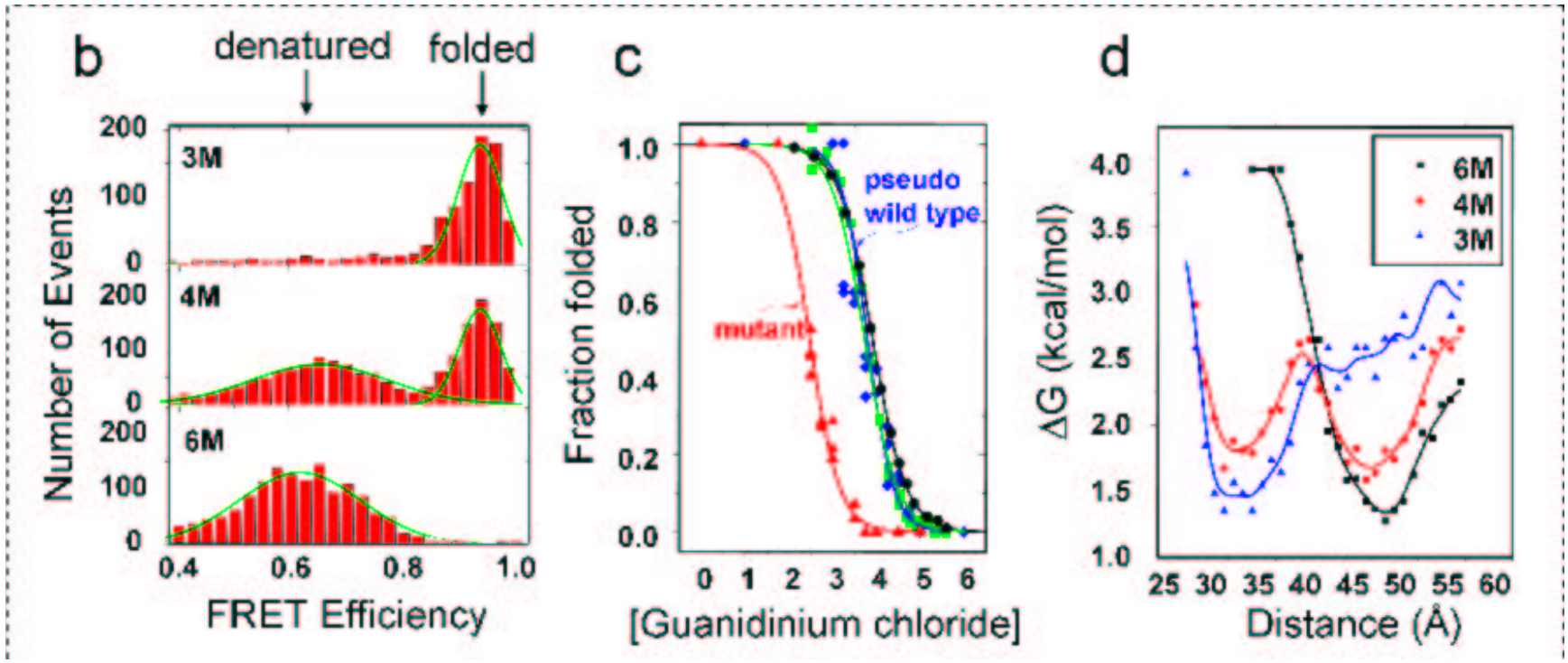
A.A. Deniz, M. Dahan, J.R. Grunwell, T. Ha, A.E. Faulhaber, D.S. Chemla, S. Weiss and P.G. Schultz, Single-pair fluorescence energy transfer on freely diffusing molecules: Observation of Froster distance depence and subpopulations. Proc. Nat. Acad. Acad. Sci. USA 96, (1999) 3670-3675



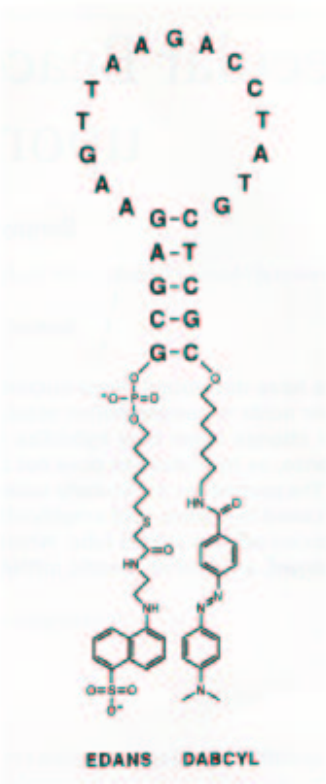
Cinétique de repliement d'une protéine



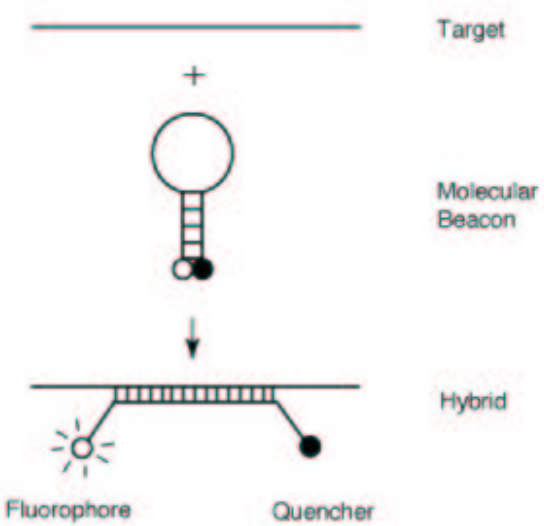
Inhibiteur de la Chymotrypsine

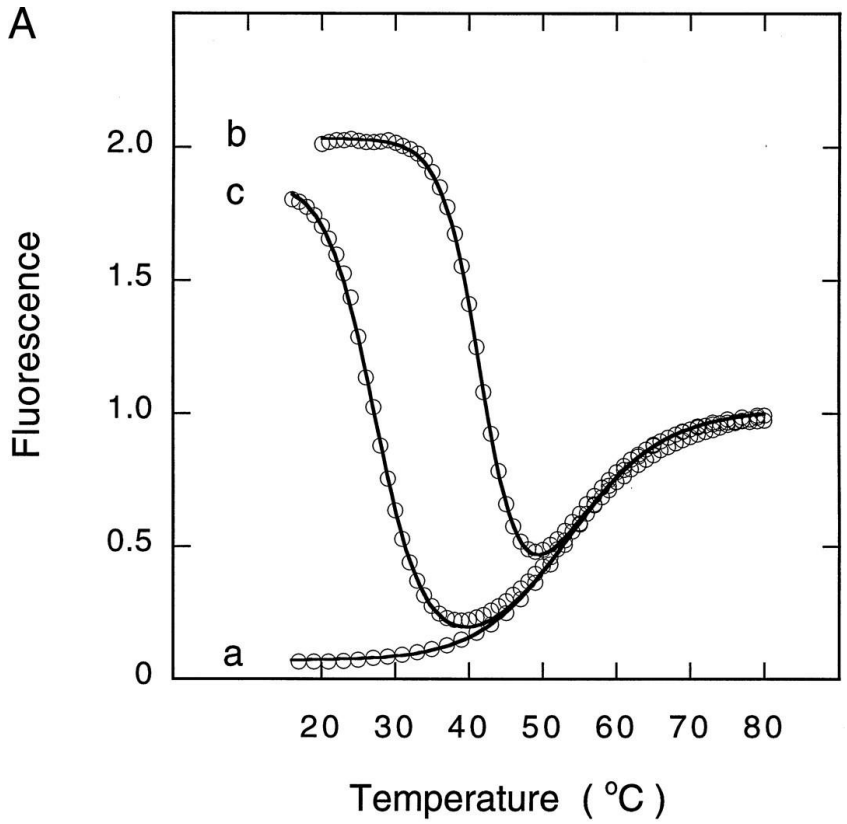


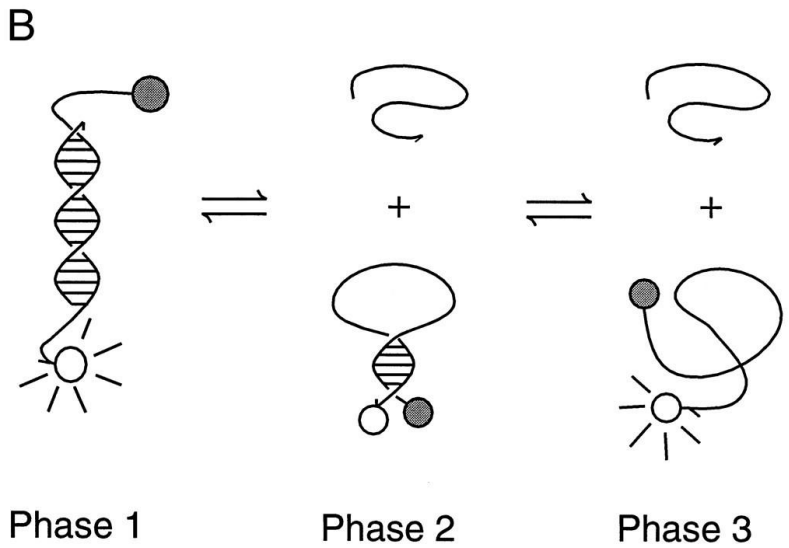
Molecular Beacon



- Application du FRET



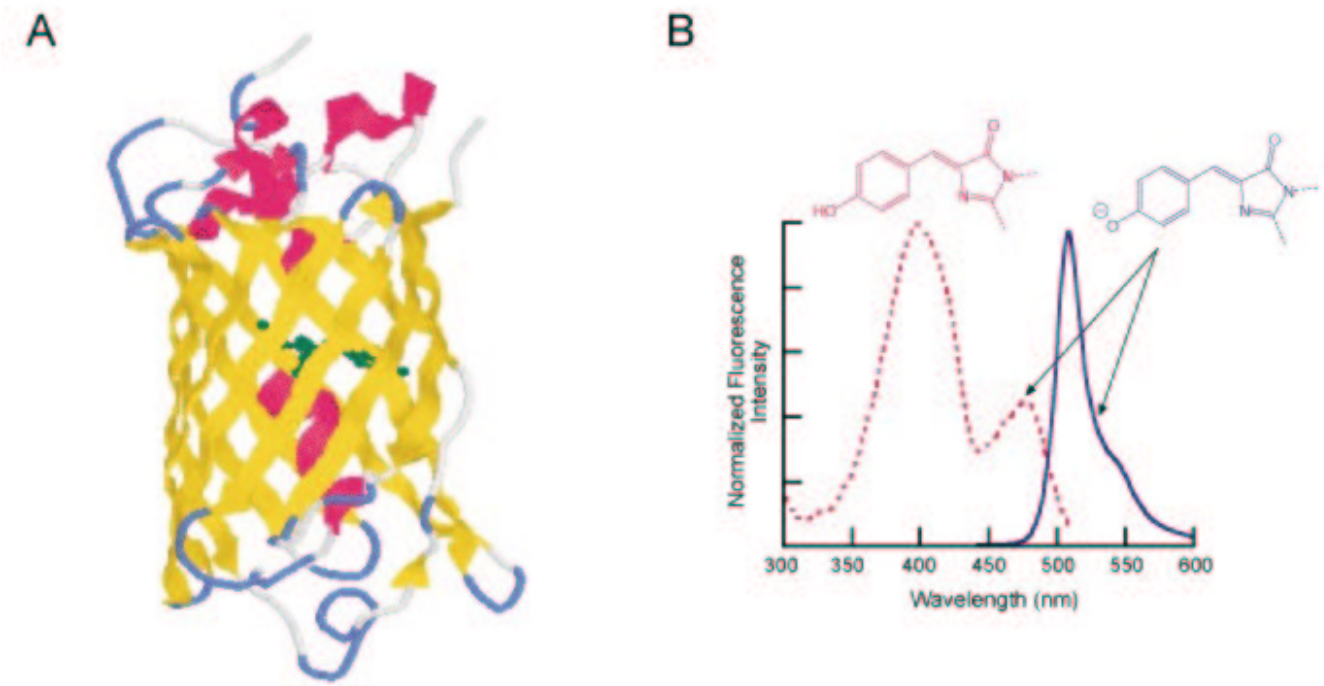




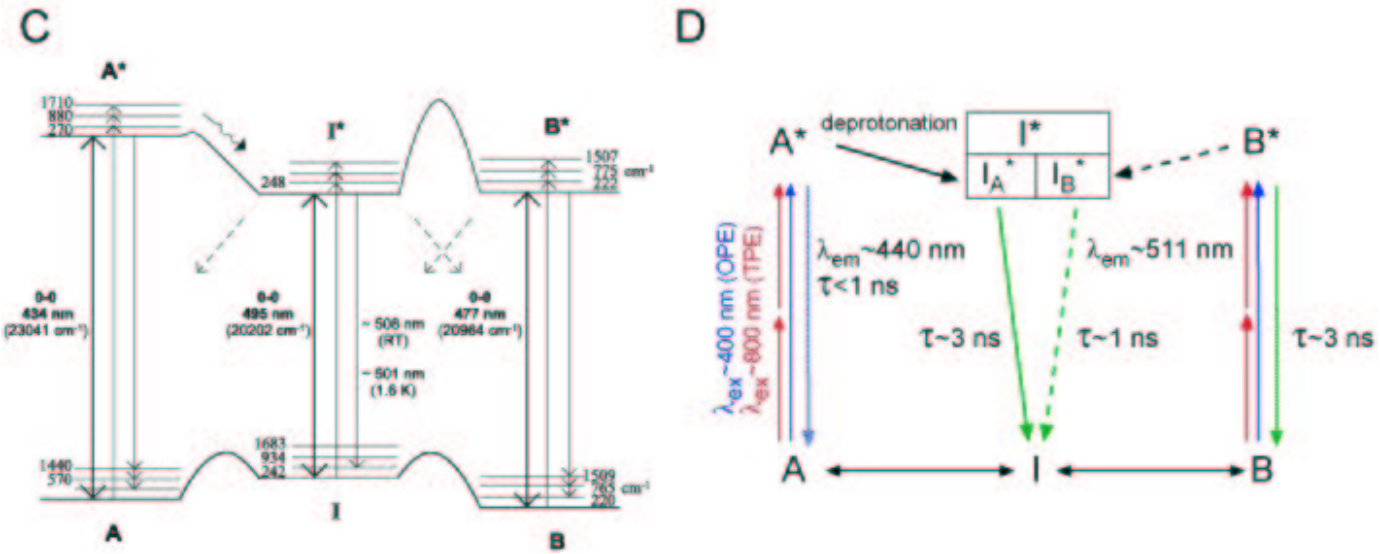
Grégoire Bonnet, Sanjay Tyagi, Albert Libchaber, and Fred Russell Kramer; Thermodynamic basis of the enhanced specificity of structured DNA probes, PNAS 96: 6171–6176. Grégoire Bonnet, Oleg Krichevsky, and Albert Libchaber, Kinetics of conformational fluctuations in DNA hairpin–loops PNAS 95: 8602–8606.

Green Fluorescent Protein (GFP)

- Protéine naturellement fluorescente

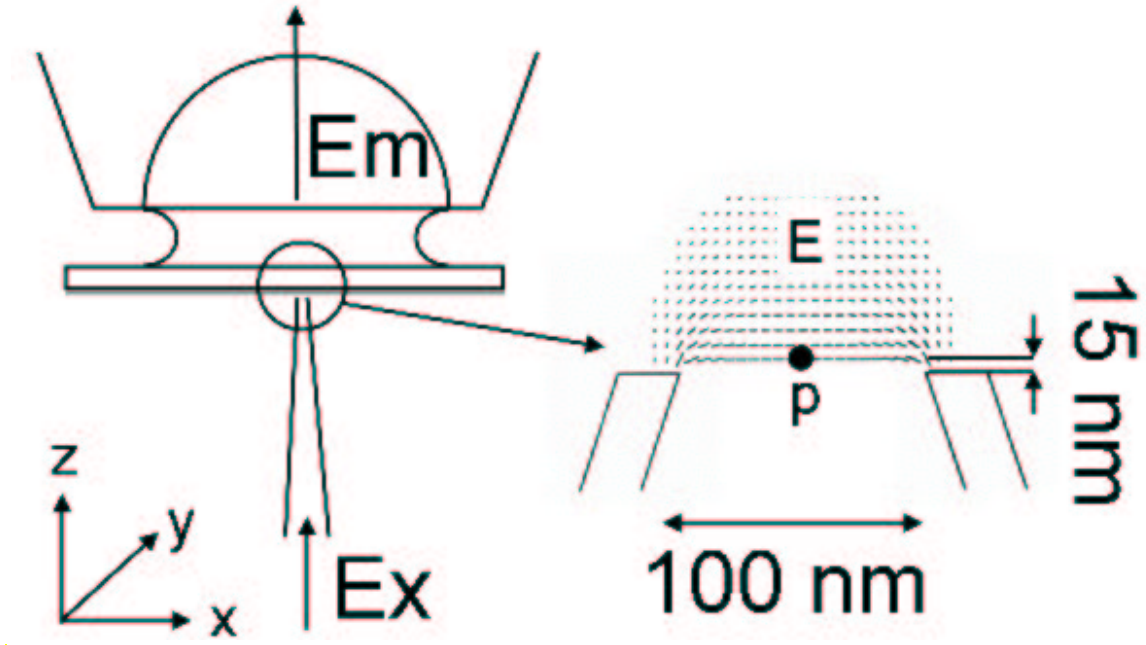


- Nouvelles protéines DsRed, eYFP, eGFP



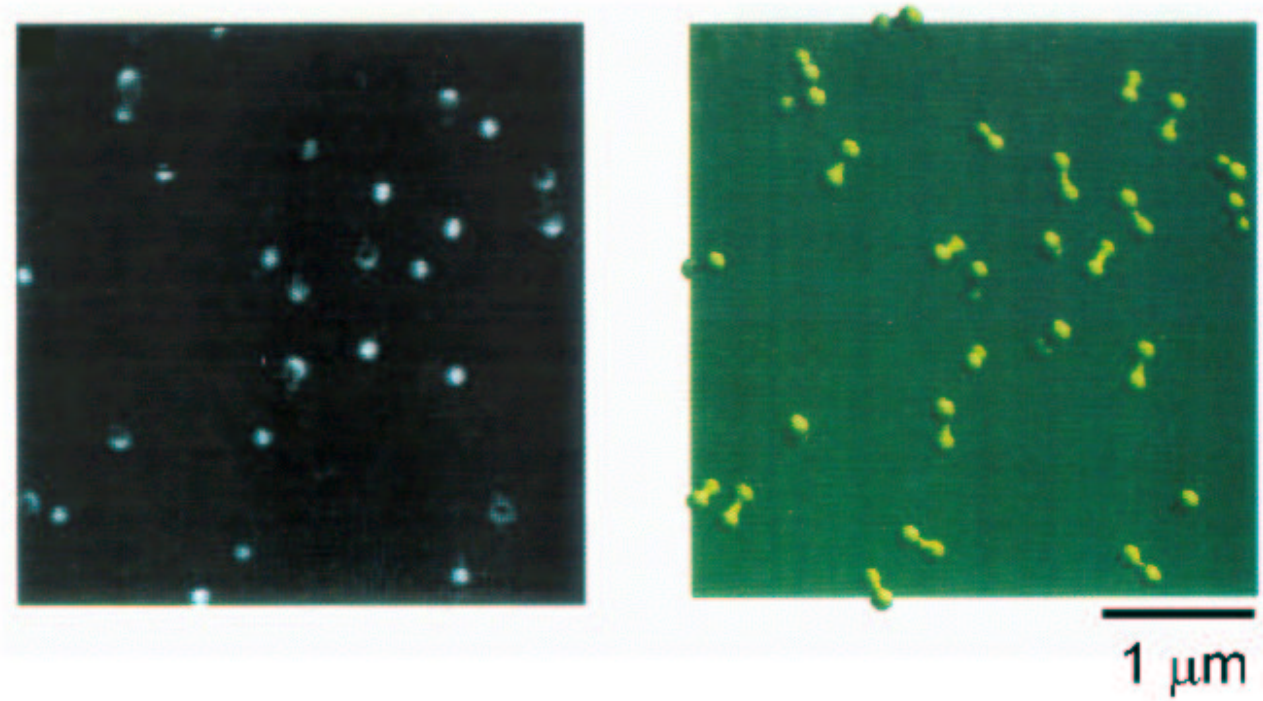
SNOM

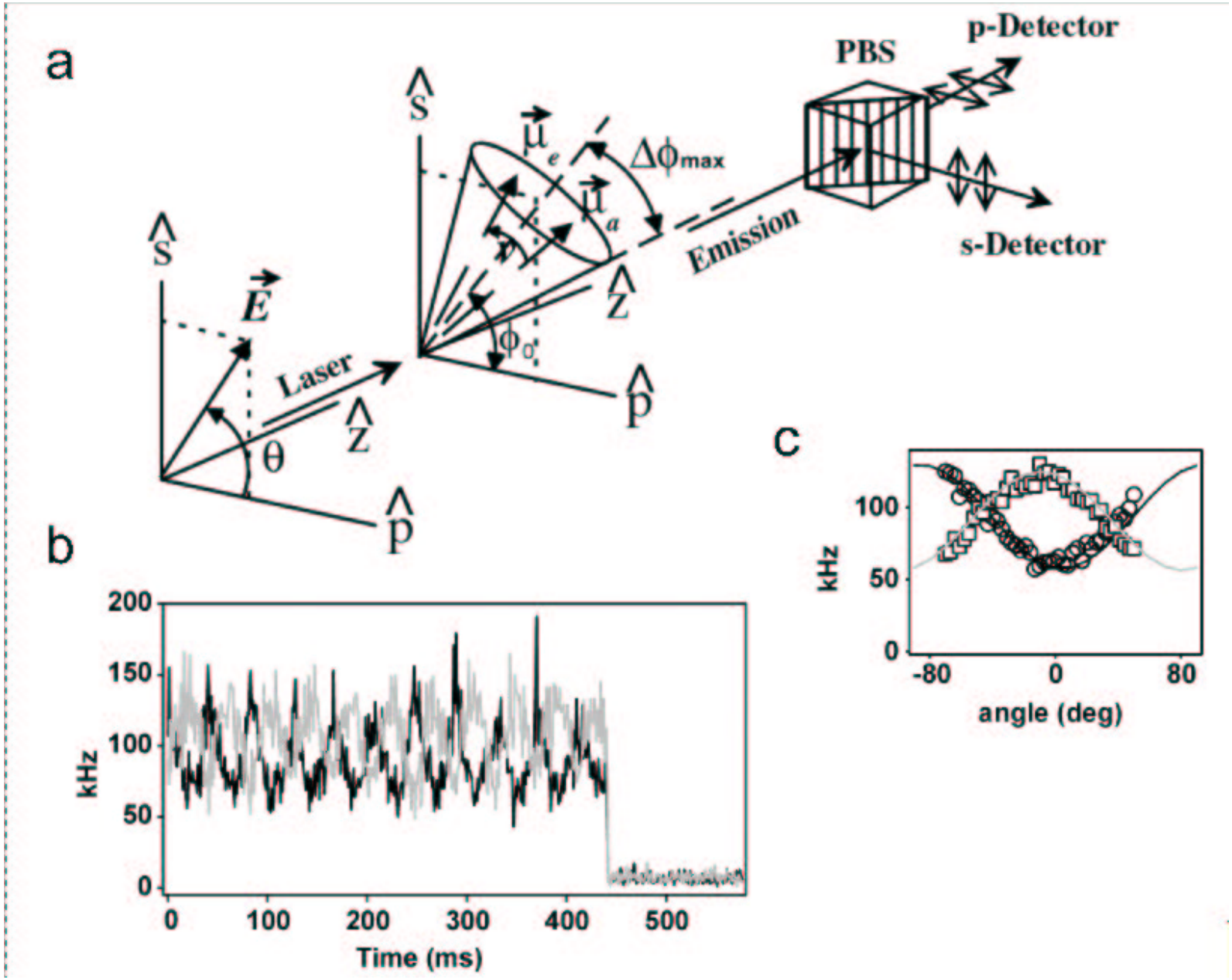
Scanning Near–field Optical Microscopy



E. Bertzig and R.J. Chichester, Single Molecules Observed by Near–field Scanning Optical Microscopy, Science 262, (1993) 1422–1425

Lipophilic carbocyanine fluorophore ($dilC_{12}$) dilué dans du PMMA couvrant une lamelle couvre objet.(gauche expérience, droite simulation)

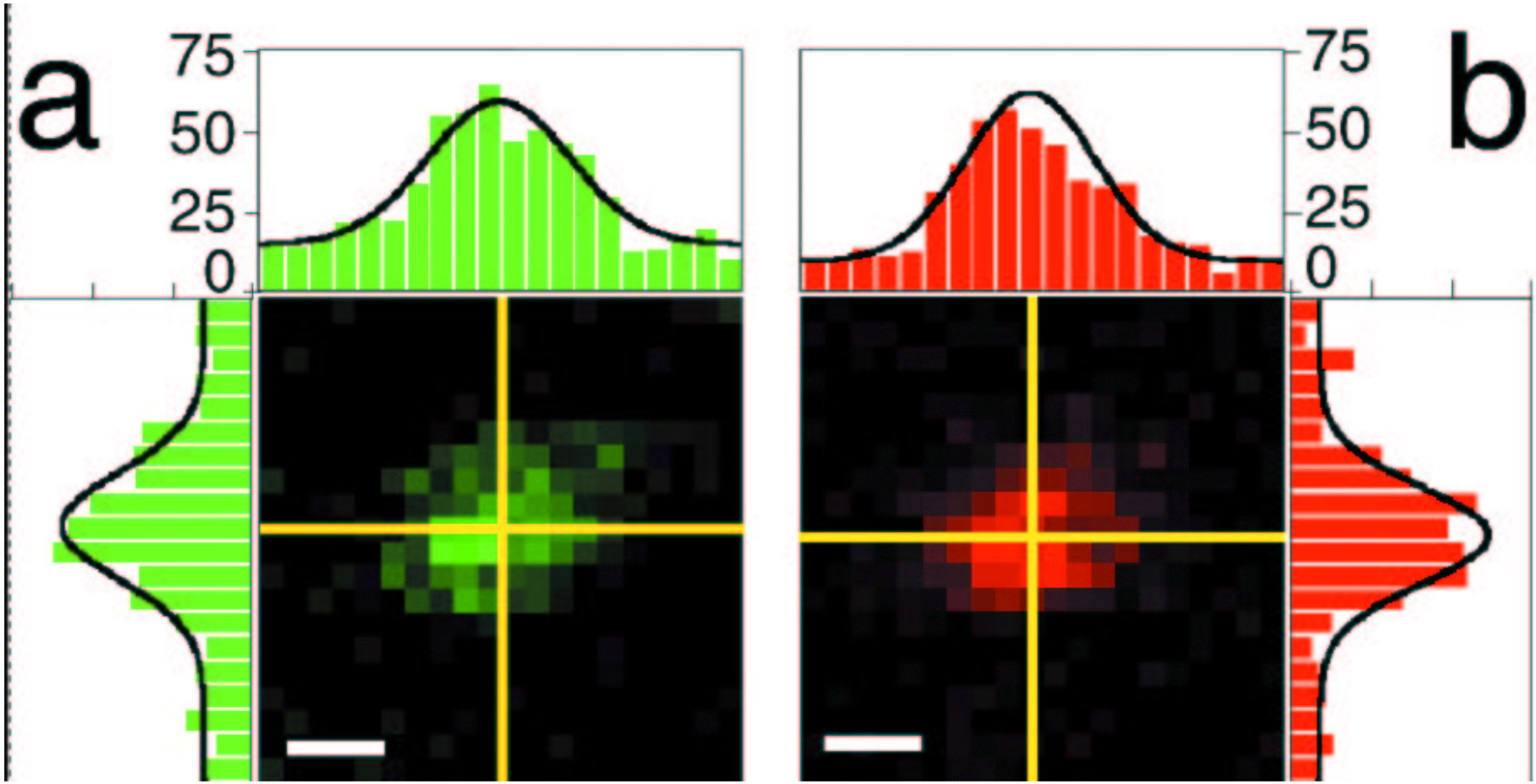



a
PBS
p-Detector
s-Detector
Emission
Laser
Δϕmax
θ
b
kHz
Time (ms)
c
kHz
angle (deg)

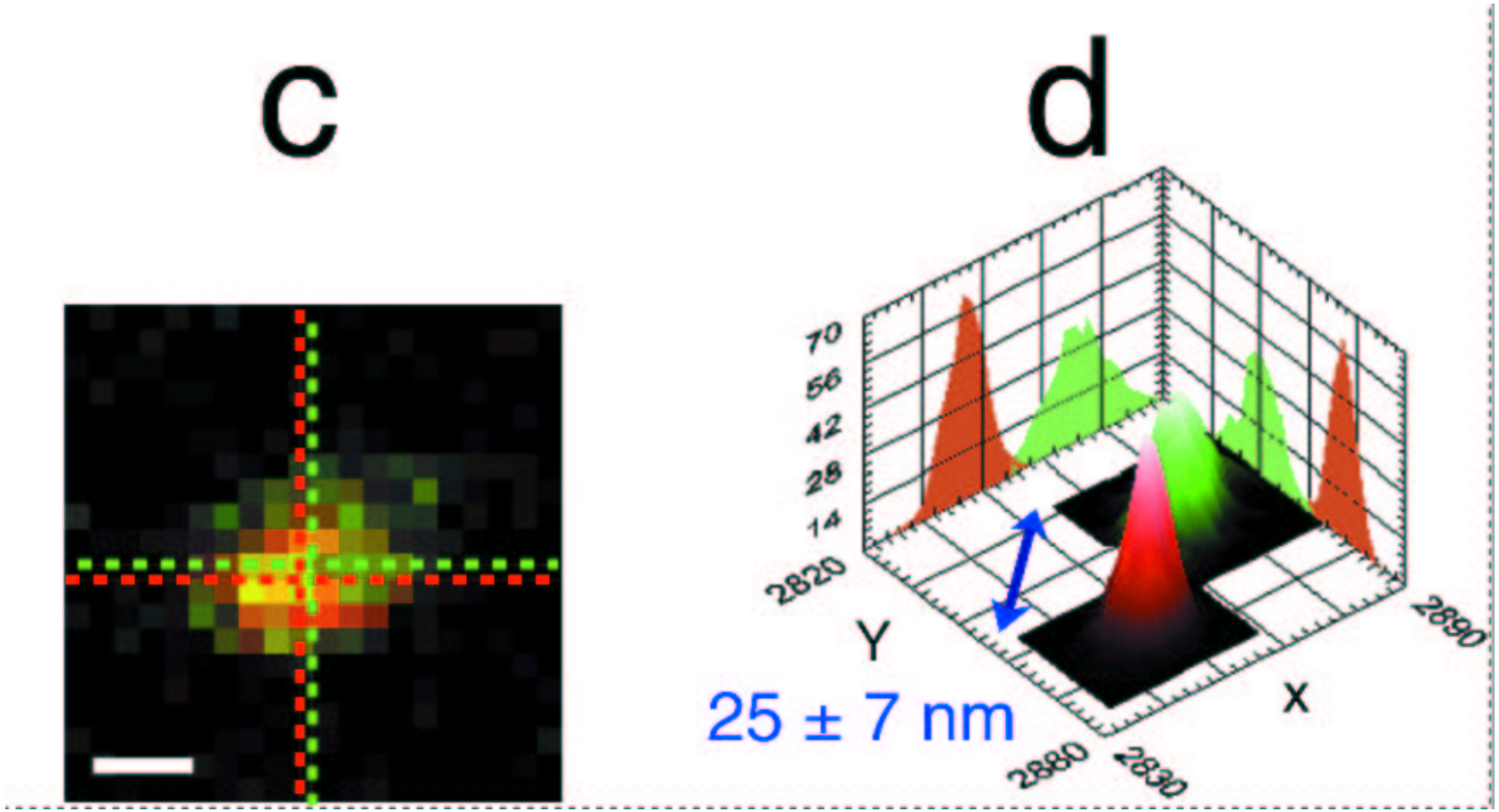
Co–localisation de nanocristaux

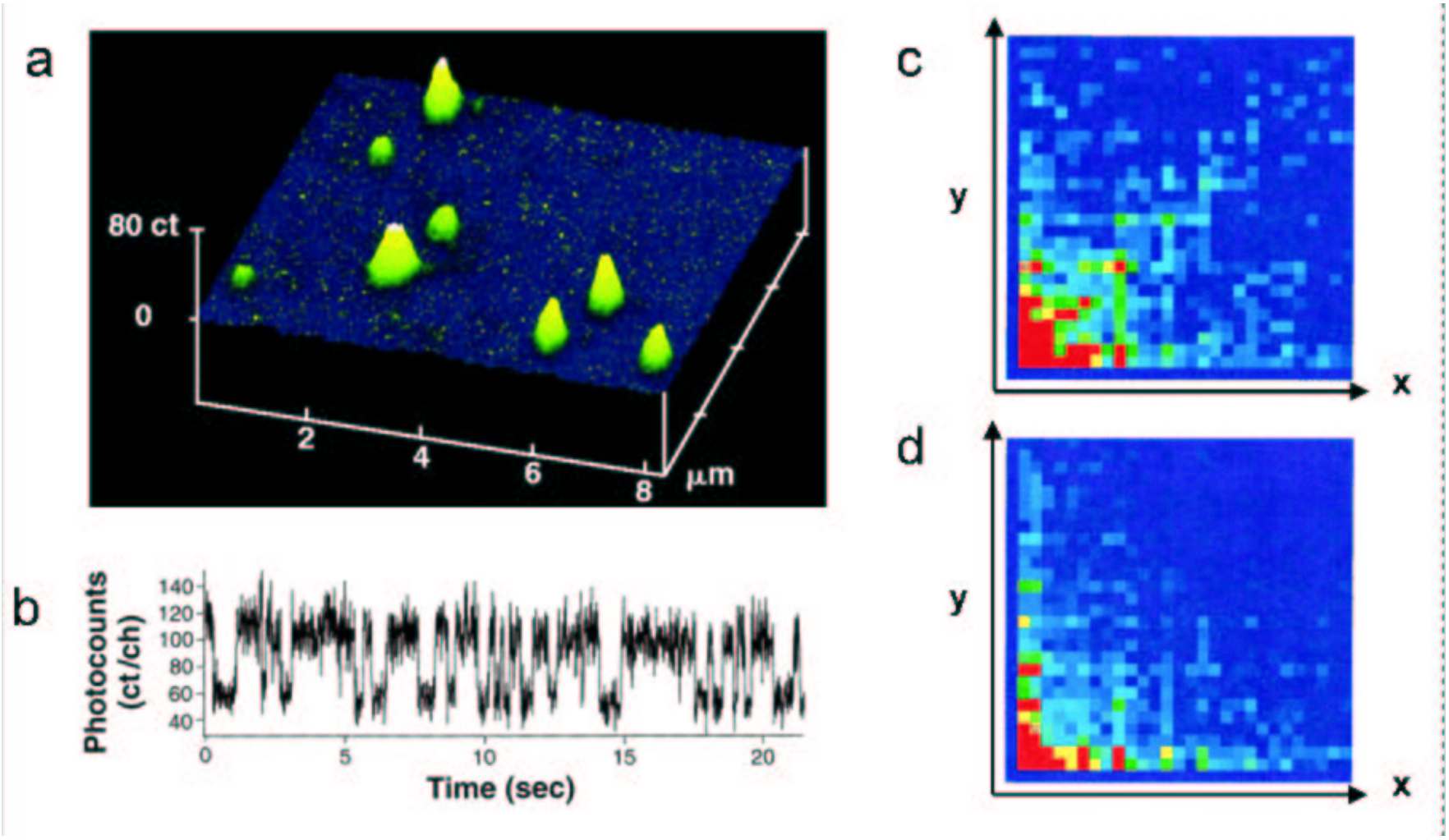
λ = 540 nm

λ = 620 nm



1×1 μm (bar = 200 nm)


c
d
70
56
42
28
14
2820
Y
2880
2830
X
2890
25 ± 7 nm


a
80 ct
0
2
4
6
8
µm
b
Photocounts (ct/ch)
140
120
100
80
60
40
0
5
10
15
20
Time (sec)
c
y
x
d
y
x