

Micro-manipulation de l'ADN

Vers une visualisation directe par microscopie de fluorescence

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Outline

- 1 Introduction to single-molecule experiments
- 2 Experimental Setup
- 3 RNA Pol
- 4 FtsK, a molecular motor
- 5 Results
- 6 Conclusions

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Seeing and manipulating biomolecules

Micro-manipulation

Technique	AFM	OT	MT
Measure nanometric changes	++	++	+
Generate force	++	++	+
Generate torque	-	+	++
Parallelize observations	-	+	++
Force and fluo colocalization	+	+	++
Previous experience in the lab	-	-	+

Observation

- Fluorescent labelling \Rightarrow direct visualization
- Evanescent wave \Rightarrow spatial positioning
- Objective illumination \Rightarrow compatible with MT setup

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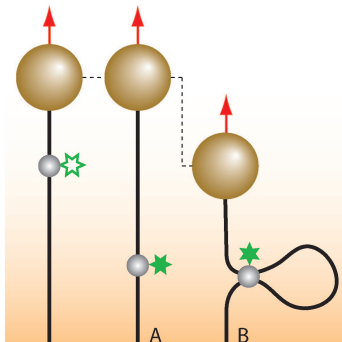
The idea

Characteristics

- specific surface-DNA & DNA-bead attachment
- relevant force scale :
 $1 k_B T / \text{nm} = 4 \text{ pN}$
- mm-wide fluo field of view
- $\sim 100 \text{ nm}$ EW z scale

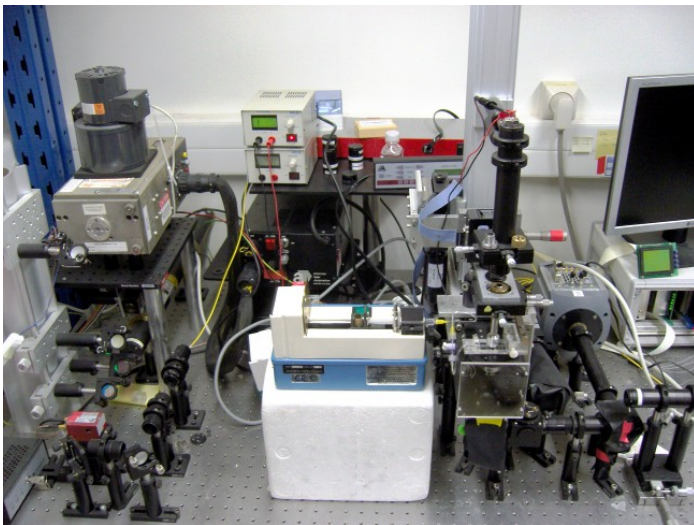
Objectives

- force and torsion generation
- direct fluorescence observation
- multi-color excitation/detection

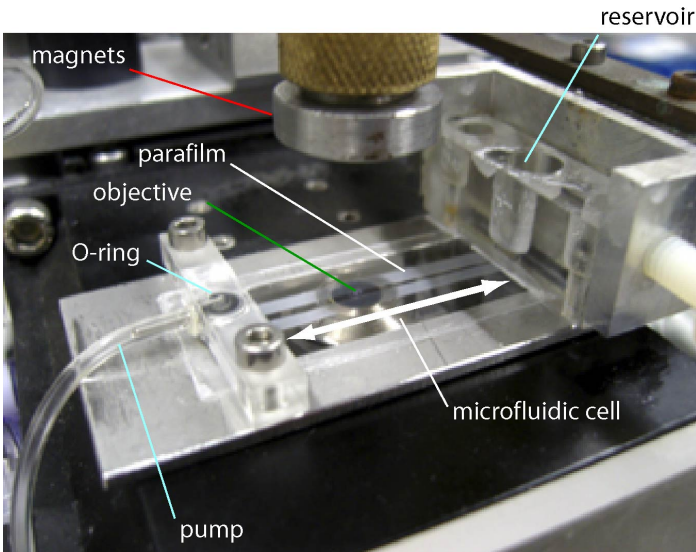


Effect	A	B
Fluo activity	Yes	Yes
MT activity	No	Yes

What the setup looks like : the general setup



What the setup looks like : the chamber



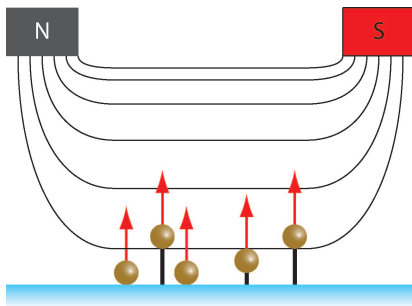
The MT setup

Key facts

- μm -sized DNA & super-paramagnetic bead
- $100 \mu\text{m}$ $F \sim \partial_{\parallel} B_{\perp}$ variation in z : 0.05 - 40 pN
- $\partial_{\parallel} B_{\perp}$ uniform over field of view

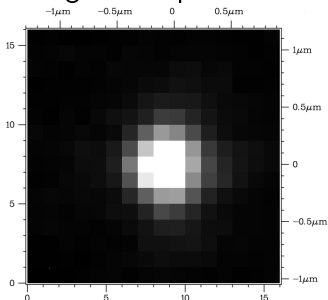
Conclusion

- Constant force and rotation
- Parallel experiments over the field
- 5 nm z tracking accuracy



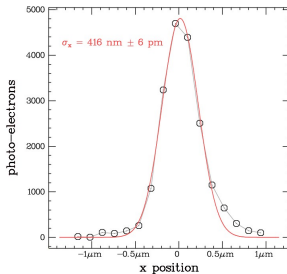
An application of fluorescence microscopy : fluorophore nm-accuracy positioning

A single fluorophore



- Quantum dot
- $T = 30ms$

Positioning

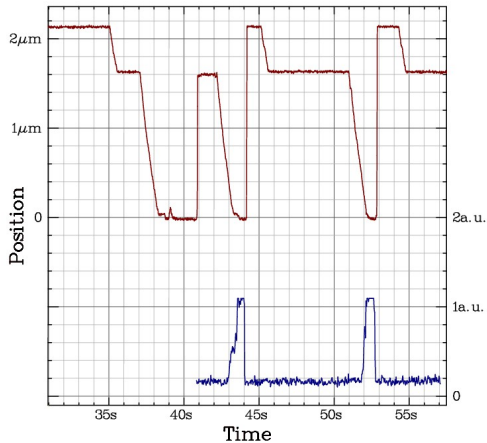
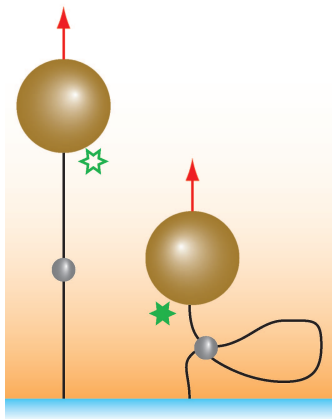


- $\xi = 400 \text{ nm}$ (gaussian)
- $x = 10 \pm 1 \text{ nm}$

Conclusion

Single fluorophore (x, y) positioning : $\sigma = 5-10 \text{ nm}$ at $T = 30ms$

A proof of principle



Observation

The motor MT and fluo activities are synchronous

Conclusion

Conclusions

- fully functional MT and TIRF setup
- 5-10 nm (x, y) fluorophore positioning accuracy
- 5 nm z MT accuracy
- force and torsion generation
- simultaneous observation of activity in MT and fluo

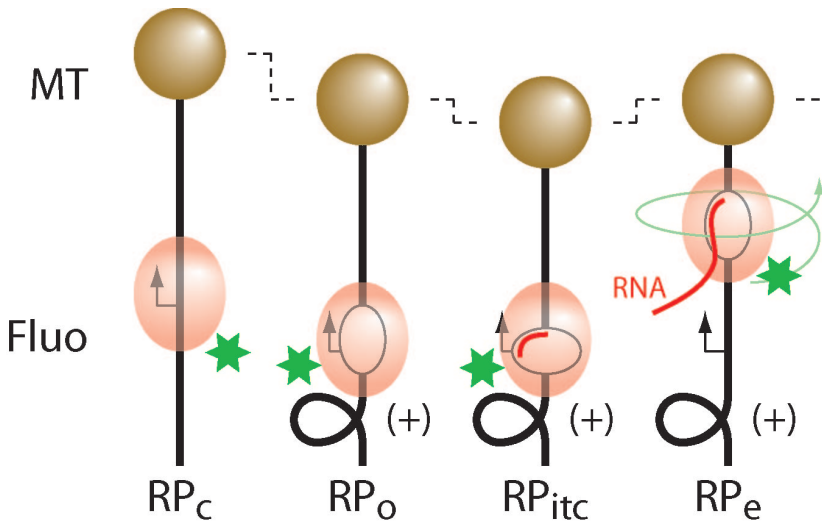
Objectives

- apply this setup on key experiments on 2 DNA translocases : T7 RNAP and FtsK
- begin with mechanistic studies

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The RNA Polymerase



RNAP studies

Strong structural basis on T7RNAP [Cheetham 99, Tahirov 02, Yin 04, Datta 06], bacterial RNAP [Vassylyev 02, Murakami 02] and yeast RNA Pol II [Cramer 01, Lehmann 07]

Single-molecule observations : mainly in *coli*

- T7 RNAP $\mathbf{RP}_{itc} \rightarrow \mathbf{RP}_e$ transition by FRET [Tang 09]
- T7 RNAP \mathbf{RP}_e kinetics by OT [Thomen 02, 05, 08]
- *E. coli* RNAP $\mathbf{RP}_c \rightleftharpoons \mathbf{RP}_o$ equilibrium by MT [Revyakin 04]
- *E. coli* RNAP \mathbf{RP}_{itc} scrunching by MT [Revyakin 06] and FRET [Kapanidis 06, Tang 08]
- *E. coli* RNAP \mathbf{RP}_e step size, kinetics, pausing by OT [Wang 98, Neuman 03, Abbondanzieri 05, Herbert 06, Mejia 08]
- yeast RNA Pol II \mathbf{RP}_e pausing and backtracking in OT [Galburt 07, Mejia 08]
- yeast RNA Pol II \mathbf{RP}_e kinetics by *in vivo* RNA labeling [Darzacq 07]

Planned experiments

Planned experiments

- fluo-labelled T7 RNAP :
monomeric, strong promoter
[Chen 00], biotin tag
[Thomen 02]
- RNAP-promoter interaction
(not available in MT)
- RP_e rotation (not available
in MT)

⇒ need good fluo (x, y)
resolution to reach bp resolution
and compare to Block

Outcome

- fluo-labelled RNAP
functional in bulk
- fluo-labelled RNAP active in
MT
- simultaneous MT and fluo
activity (e.g. promoter
unwinding) **never** observed

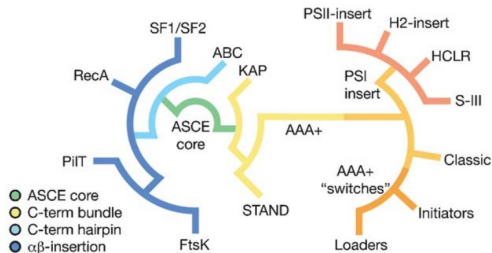
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FtsK and the ASCE ATPases

Characteristics

- *E. coli* cell cycle coordination
- chromosome dimer recombinase activation
- chromosome positioning at division septum
- Hints on mechanism : strong structural and functional homologies with ASCE ATPases



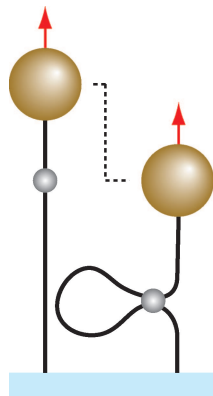
Reproduced from [Erzberger 06]

- FtsK/HerA superfamily : **SpolIIE**, TrwB, $\phi 29$ **gp16**
- RecA ATPases : RecA/Rad51, **Rho**, dnaB, UvrD, T4 gp41, T7 gp4
- AAA+ supergroup : SV40, **ClpX**

FtsK structural and functional features

Related proteins structures

- FtsK_{50C} forms *blobs* on DNA [Pease 05]
- FtsK_{C $\alpha\beta$} -DNA complexes are **hexameric** [Massey 06]
- FtsK/HerA superfamily proteins are multimeric : ϕ 29 gp16 portal motor [Morais 08], *E. coli* conjugation protein TrwB [Gomis-Rüth 01, Hormaeche 02], *P. abyssi* dsDNA helicase MlaA [Manzan 04]
- many other ASCE proteins are multimeric : dnaB helicase [Bailey 07], E1 replicative helicase [Enemark 06], T7 gp4 replicative helicase [Egelman 95], ClpX proteasome helicase [Grimaud 98]

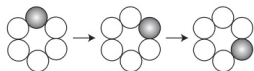


ATP hydrolysis models

a Concerted hydrolysis

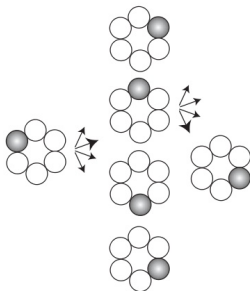


b Sequential hydrolysis



● ATP hydrolysis

c Probabilistic hydrolysis



Alternative

Direct observation
of fluorescent ATP
analogue

Related proteins

- probabilistic in bacterial ClpX [Martin 05]
- sequential in T7 helicase gp4 [Hingorani 97]
- strictly sequential in *E. coli* helicase Rho [Stitt 97]
- coordinated in ϕ 29 gp16 [Moffitt 09]

Issues in MT observation of FtsK

Key experiment

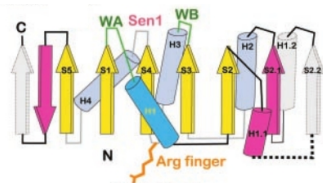
- use the MT + fluo setup
- fluo-labelled FtsK
- DNA force/torsion control by magnetic tweezers

Issue	Experiment
multimeric state of FtsK _C	quantization by fluorescence
translocation direction	fluorescence tracking
ATP hydrolysis mechanism	fluorescent ATP hydrolysis point mutations
clean SM FtsK complex	covalent multimers

ATP hydrolysis mechanism

Strategy

- covalent n-mers : MCM (natural) [Moreau 07], ClpX (artificial) [Martin 05]
- point mutations on monomers
- WA prevents ATP interaction
- WB prevents ATP hydrolysis
- RF (Arginine Finger) inhibit trans ATP hydrolysis coupling



Key experiment

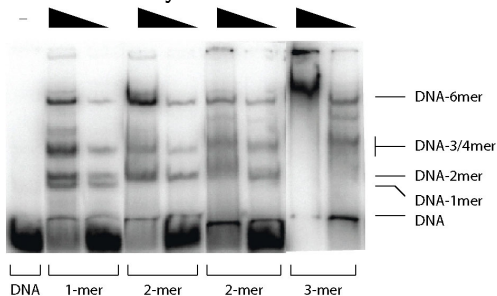
- **local** wt/mutant monomer state
- **global** activity effect at complex level

Covalent FtsK multimers

Covalent multimers

- FtsK_C prepared in n-mers
- auto-organize in **discrete** complexes on DNA
- 3-mers used for convenience (high multimerization)
- possible C-term biot

Gel shift assay

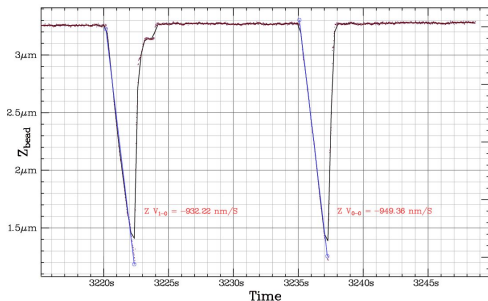


35 bp dsDNA oligomer (Ian Grainge, [Lowe 08])

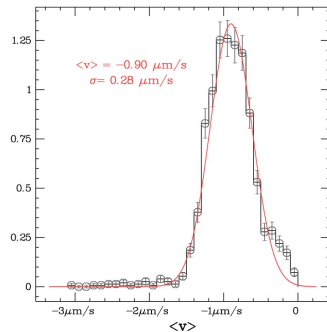
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FtsK DNA translocase activity

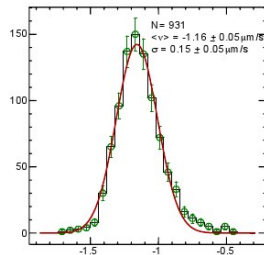
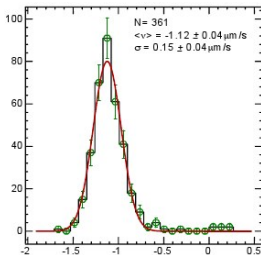
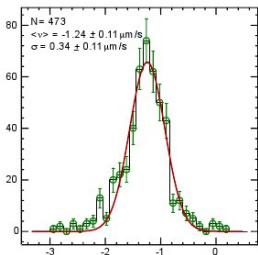


- automatic measurement tests at high (2-5 mM) ATP
- benchmarking on known data : FtsK_{50C} [Saleh 04, Pease 05]



- 2 mM ATP
- 1509 events
- monomodal
- $\langle v \rangle = 0.9 \pm 0.3 \mu\text{m/s}$
- consistent with literature

Translocation speed of variants

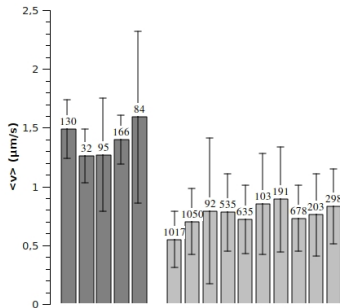
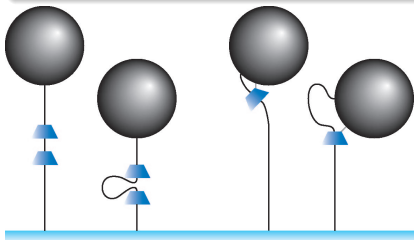


- wt-X-wt FtsK trimer variants (left to right : X = wt, WA, WB)
- similar translocation pattern for all variants and FtsK_{50C} at 2 mM ATP & F = 20 pN
- similar DNA translocation speed for wt, WA, WB, **no loop formation activity for RF**

The behavior of wt-RF-wt

Key observation

biot-wt-RF-wt exhibits DNA looping activity
⇒ consequence of DNA translocase activity



- biot-wt-WA-wt (black) vs. biot-wt-RF-wt (grey)
- 5 mM ATP

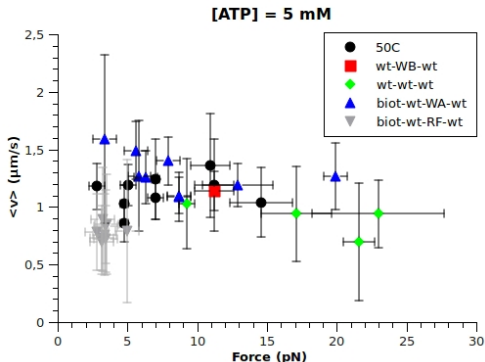
Force dependence studies

Bulk observations suggests

- roadblock displacement impaired by mutations
- different mechanisms for roadblock displacement and DNA translocase

Conclusion

- no force dependence on wt and WA up to 25 pN
- to be tested on WB, RF and 3+/6 mutants
- constant position OT may be better suited [Pease 05]



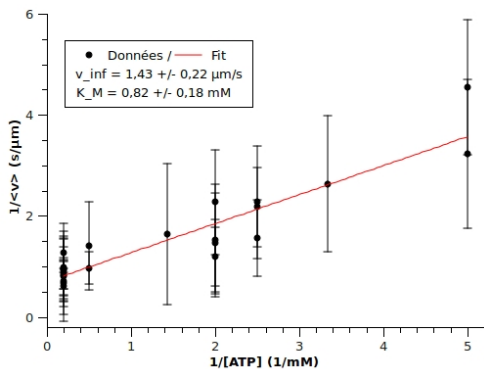
Cofactor dependence : ATP

Rationale

Study of the ATPase reaction :
 $\text{ATP} \rightarrow \text{ADP} + \text{P}_i$

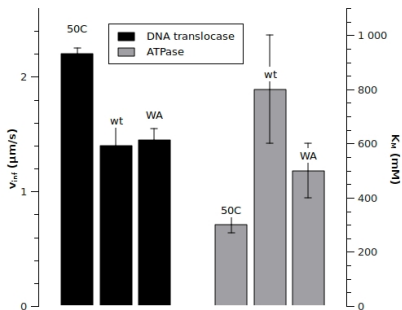
Observations

- $v_{\infty} = 1.4 \pm 0.2 \mu\text{m}/\text{s}$
- $K_M = 0.8 \pm 0.2 \text{mM}$
- v_{∞} for FtsK_{50C} :
2.3 $\mu\text{m}/\text{s}$ [Saleh 04]
1.7 $\mu\text{m}/\text{s}$ [Pease 05]
- K_M for FtsK_{50C} :
0.3 mM [Saleh 04]



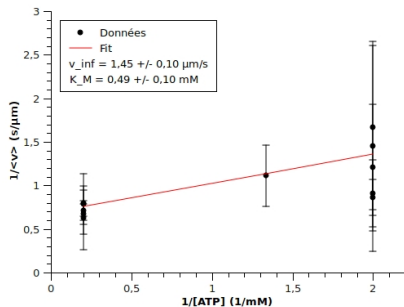
Set of data on **biot-wt-wt-wt** (for future use)

Cofactor dependence : ATP



Conclusion

- no effect of 2/6 WA on loop formation limiting step
- strikingly, no effect on ATP binding constant

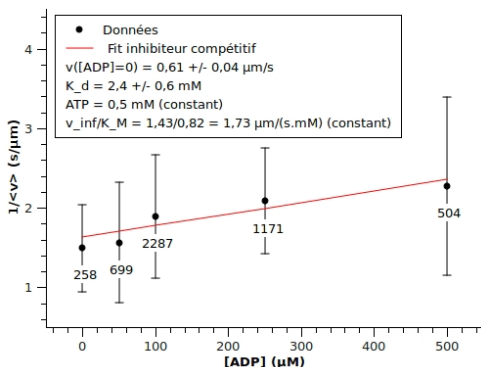


Set of data on biot-wt-WA-wt

Cofactor dependence : ADP

Observations

- ADP acts as a competitive inhibitor
- **but** processivity increases with [ADP]
- $v([ADP] = 0)$ consistent with MM
- ADP
 $K_d = 2.4 \pm 0.6 \text{ mM}$
- acquired on a **single** FtsK/DNA complex



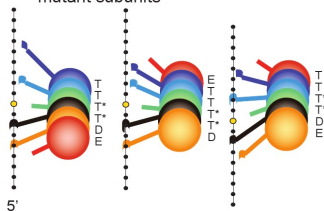
Data on a single biot-wt-wt-wt/DNA complex

Conclusions and perspectives

Conclusions

- different activities have different cooperativities
- concerted and stochastic mechanisms ruled out for DNA translocase
- concerted mechanism ruled out, but some cooperativity for DNA looping
- 2/6 RF does not form loops on its own

Escort model (3 contacts) with 2 mutant subunits



Perspectives

- activity as a function of $n/6$
⇒ quantize cooperativity (under investigation)
- wt-wt-wt-wt-WA-WA behaviour (under investigation)
- fluorescent ATP studies

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General conclusions

Conclusions

Setup

- fully functional MT and fluorescence setup
- proof of principle of simultaneous protein activity observation

FtsK

- wt-X-wt trimer MT DNA translocase activity validation
- identification of different types of cooperativity for different activities

Future work

Perspectives

- direct protein labelling protocols for RNAP and FtsK
- direct observation of RNAP-promoter interaction and rotation
- direct visualization of FtsK translocation
- FtsK quantization
- discrimination between ATPase cooperativity mechanisms
- higher-order FtsK multimers study
- application of fixed complexes to protein ageing

Outline

7 Photoelectron yield

8 FtsK activity

9 RNAP

10 DNA phases

The photoelectron yield

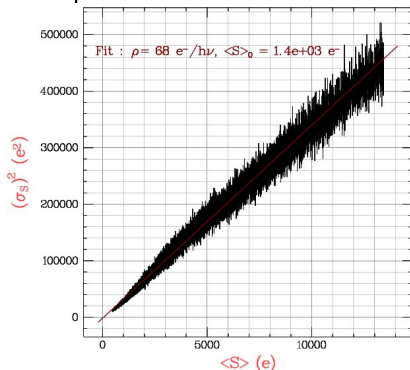
The problem

- For every photon hitting the sensor, ρ electrons are created and detected
- Must know ρ to measure the number N of photons from the electron signal S

The usual solution For an uncorrelated source of photons and $S = \rho \cdot N$:

$$\frac{\sigma_S^2}{\langle S \rangle} = \frac{\rho}{2} \quad (1)$$

Example : iXon EMCCD @ -80°C



On this camera
 $\rho = 68 \pm 21 e^-/h\nu$

Imaging a point source

Ideal case (hypotheses)

- lots of photons
- perfect spatial resolution on the detector
- cylindrical symmetry of optics

What would be observed

- spatial distribution of photons : Bessel function
- center (x,y)
- width ξ

A simulated ideal observation

Imaging a point source

Real world

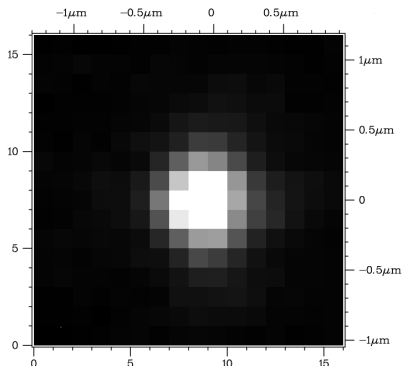
- time series of frames $(i, \Delta t)$
- finite number of photons N_i
- detector size a
- shot noise b

Questions

- what are (x_i, y_i) ?
- how much is ξ_i ?
- how much is N_i ?

An actual observation

- single QDot, $\Delta t = 30$ ms
- $a = 16 \mu\text{m}$



Point source measurements

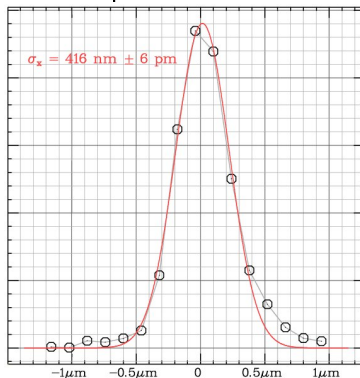
Questions

- what are (x_i, y_i) ?
- how much is ξ_i ?
- still, how much is N_i ?

Measurements

- Gaussian approximation
- $x_i = -140$ nm
- $\xi_i = 420$ nm

An example of measurement



Single QDot, $\Delta t = 30$ ms

Point source measurements

Questions

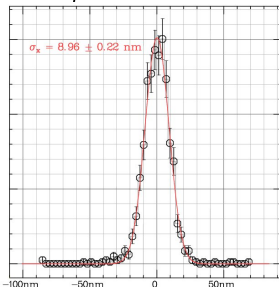
- still, how much is N_i ?

The position error σ_x

- Derived in [Thompson, 2002]
- a is known
- b , σ and ξ are measured
- $N = \langle N_i \rangle$ can be measured

$$\frac{\sigma}{\xi} = \frac{1}{\sqrt{N}} \sqrt{1 + \frac{1}{12} \left(\frac{a}{\xi}\right)^2 + \frac{8\pi b^2}{N} \left(\frac{\xi}{a}\right)^2} \quad (2)$$

The x_i distribution



Single QDot, 1024 frames

Source position error $\sigma_x = 9$ nm

Back to the photoelectron yield

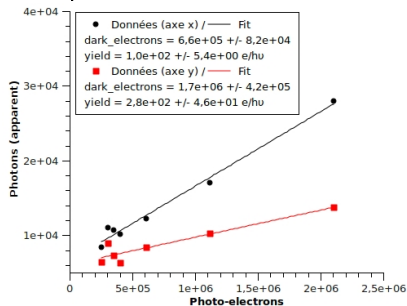
Method

- Vary Δt (or Laser intensity)
- Measure $\sigma(\Delta t)$
- Calculate $N(\Delta t)$
- Plot $S(\Delta t)$ vs. $N(\Delta t)$

Results

- σ as a function of Δt , maximum around 20 ms
- Fluorophore photon flux $\Phi = N/\Delta t = 100$ kHz
- Photoelectron yield $\rho = 100$ $e^-/h\nu$

Example : iXon EMCCD @ -80°C



Single QDot, 1024 frames/point

Conclusion

- Equally long than standard method (varying Δt)
- Requires more complex operations (fits), but have to be implemented anyway for tracking
- Direct access to setup parameters, most notably $\sigma(\Delta t)$
- Direct access to fluorophore parameter Φ , hence ability to count multiple QDs
- Independant measurement of ρ

Outline

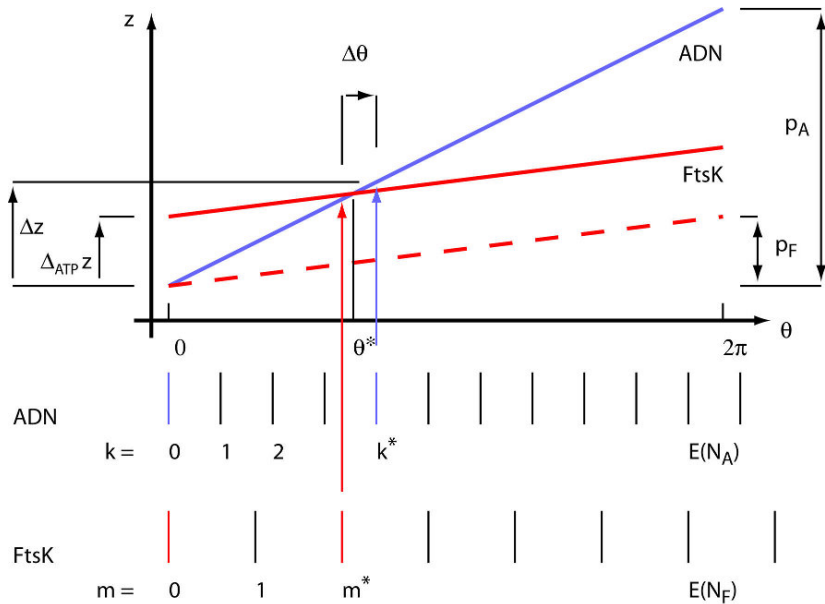
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Translocation-rotation coupling



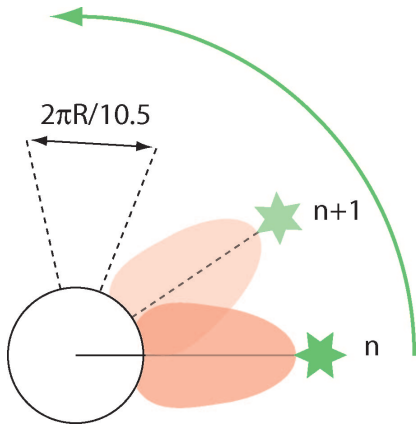
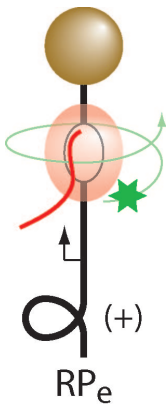
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$$\frac{2\pi R}{10.5} > \sigma_{xy} \Rightarrow R > 9 \text{ nm} \quad (3)$$

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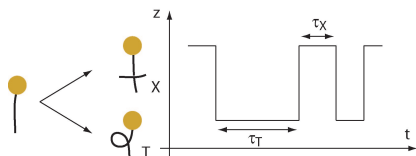
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Plectoneme - cruciform transition



$$\Delta E_X^T = -k_B T \ln \left(\frac{\langle \tau_T \rangle}{\langle \tau_X \rangle} \right) \quad (4)$$

$$E = \frac{1}{2} C \sigma^2 + \dots \quad (5)$$

$$\Delta \Gamma_X^T \propto \frac{\partial \Delta E_X^T}{\partial \sigma} = \Delta C_X^T \sigma \quad (6)$$

