

# Single-molecule fluorescence & membrane proteins

wallace.chem.ox.ac.uk

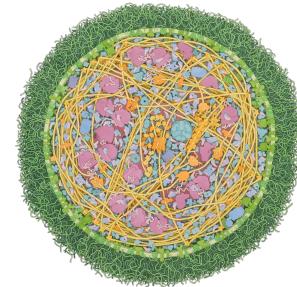
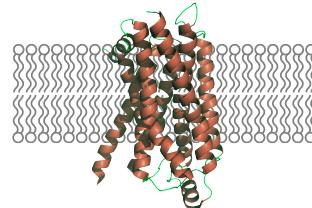


## Resources

- Slides
  - [wallace.chem.ox.ac.uk/teaching](http://wallace.chem.ox.ac.uk/teaching)
- Books
  - Lakowicz, Principles of Fluorescence Spectroscopy.
  - Leake, Single-molecule cellular biophysics.

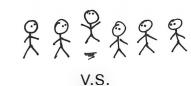


Cells are a complex environment



## Why single molecules?

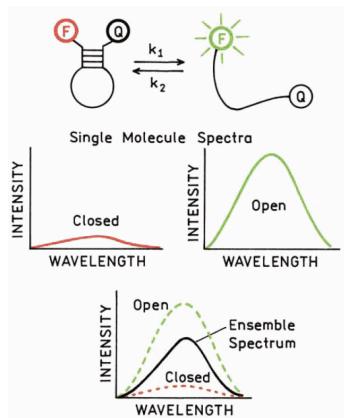
- No ensemble averaging.
  - Measure the distribution of a property.
  - Useful if population is heterogeneous.
- Kinetics at equilibrium.
  - Measure the time trajectory.
  - Useful if dynamics is difficult to synchronize.
- Detect rare events.



V.S.

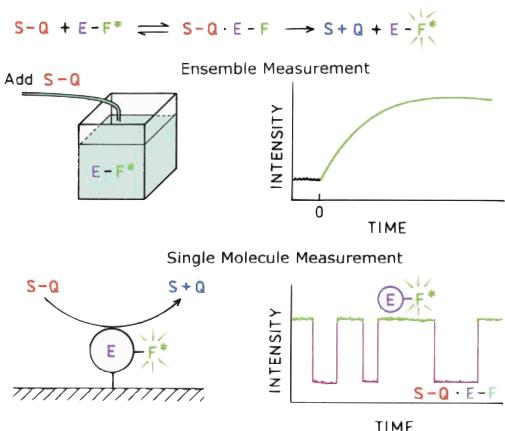


## Ensemble averaging



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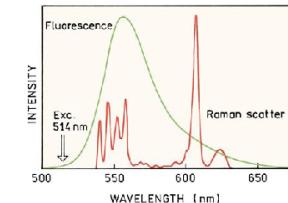
## Kinetics



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## Why Fluorescence?

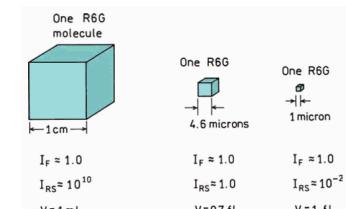
- Fluorescence provides amplification.
- Easier to detect 10,000's of photons (per second per molecule) than detecting the molecule itself.
- We can exploit Stoke's shift to separate excitation light from emission.
- One molecule vs. a lot of background!
- Impurities
- Scattered excitation light
- Detector noise
- Raman scattering



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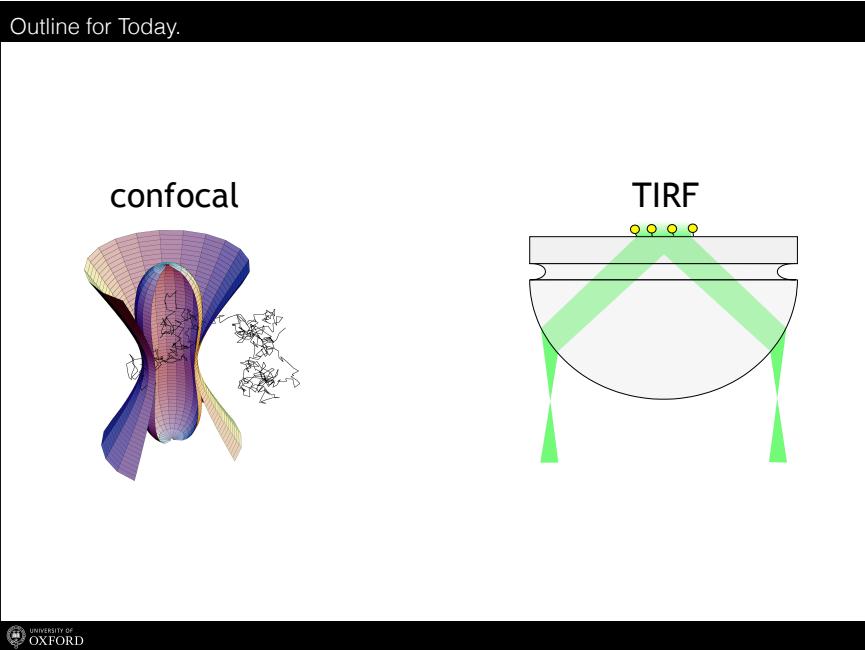
## Detection from small volumes

- Consider the relative cross sections for absorption v.s. Raman scattering.
- To make this easy, let's assume fluorescence quantum yield = 1 (Intensity then proportional to absorption cross-section and the number of molecules).
- Raman cross section for water is  $\sim 10^{-28} \text{ cm}^2$
- Absorption cross section of a typical fluorescent dye  $\sim 10^{-16} \text{ cm}^2$
- Calculate relative signal intensities as our volume of water changes

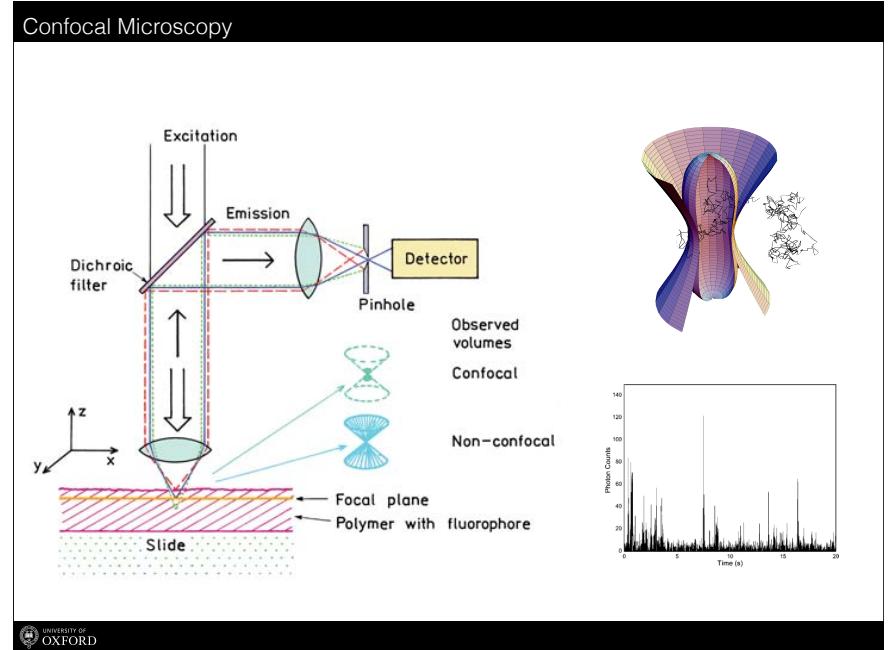


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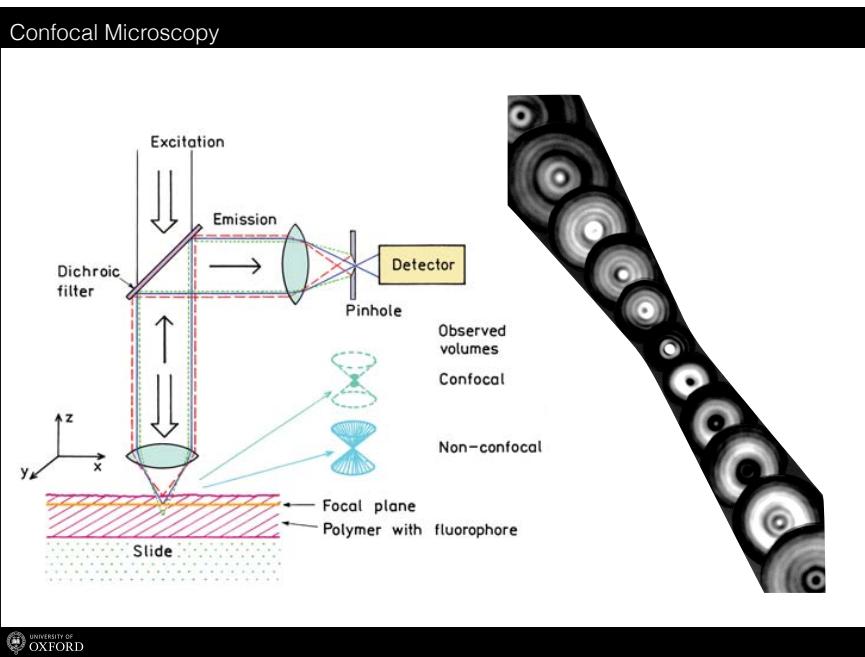
Outline for Today.



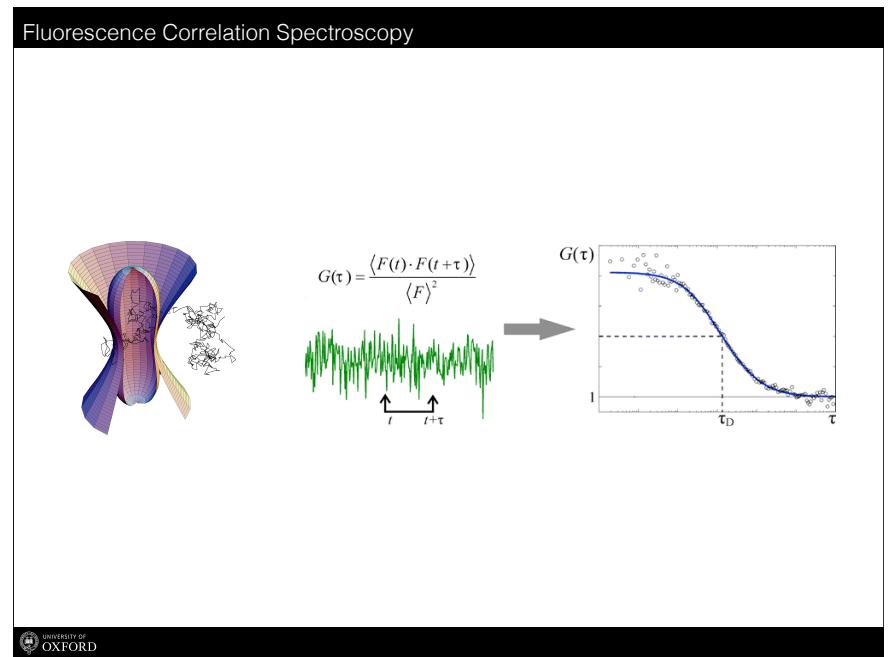
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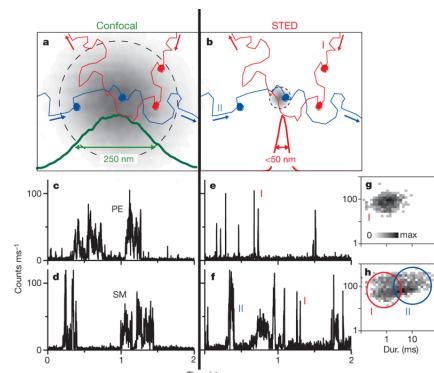


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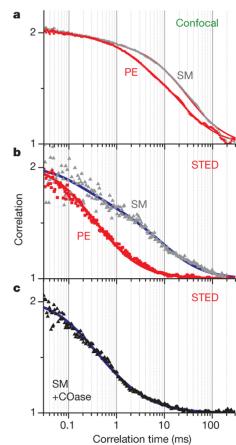
## Nanoscale dynamics of membrane lipids in a living cell



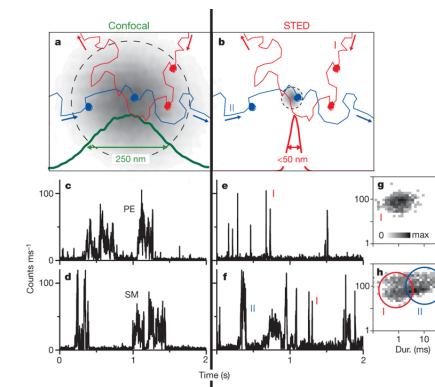
FCS of Atto647N-labelled phosphoethanolamine and sphingomyelin plasma membrane diffusion.



Egling et al. Nature. 2009, 457(7233):1159-62.



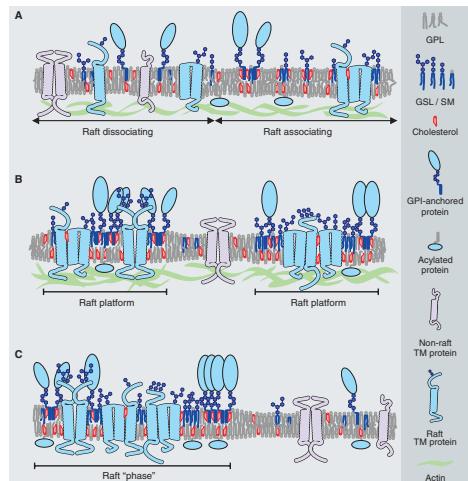
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Lingwood, D. & Simons, K. Science 327, 46-50 (2010).

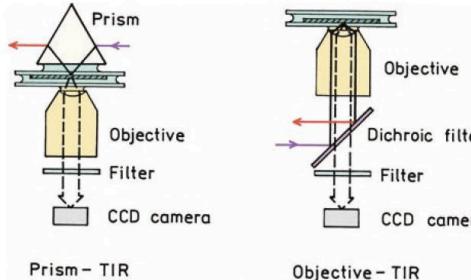


## Total Internal Reflection Fluorescence

### Introduction

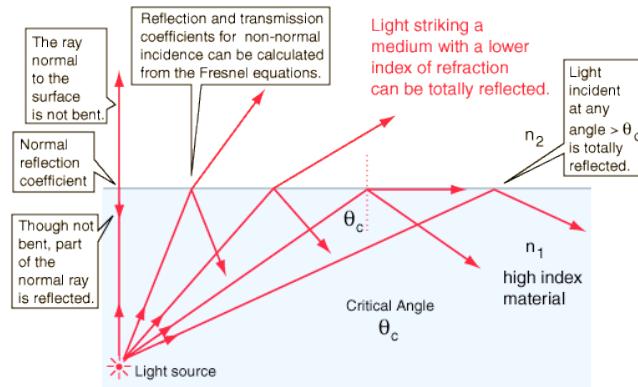


## Total Internal Reflection Fluorescence



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## TIR



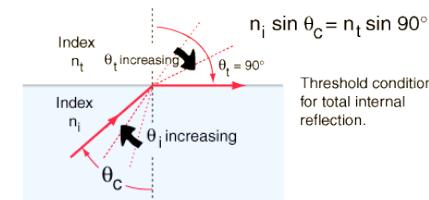
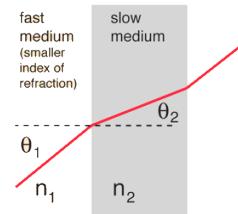
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[hyperphysics.phy-astr.gsu.edu](http://hyperphysics.phy-astr.gsu.edu)

## TIR

$$\text{Snell's Law}$$

$$\frac{n_1}{n_2} = \frac{\sin \theta_2}{\sin \theta_1}$$



Threshold condition  
for total internal  
reflection.

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## Total Internal Reflection Fluorescence

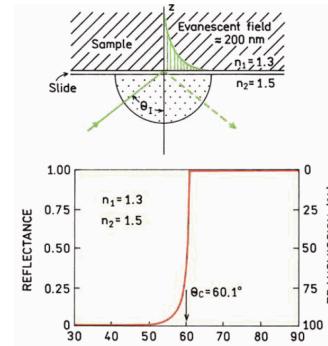


Figure 23.5. Top: Optical geometry for total internal reflection (TIR). Bottom: Calculated reflectance and transmittance for  $n_2 = 1.5$  and  $n_1 = 1.3$ .

## Critical Angle

$$\theta_c = \sin^{-1} \left( \frac{n_1}{n_2} \right)$$

## Evanescence Intensity

$$I(z) = I(0) \exp \left( -\frac{z}{d} \right)$$

## Penetration Depth

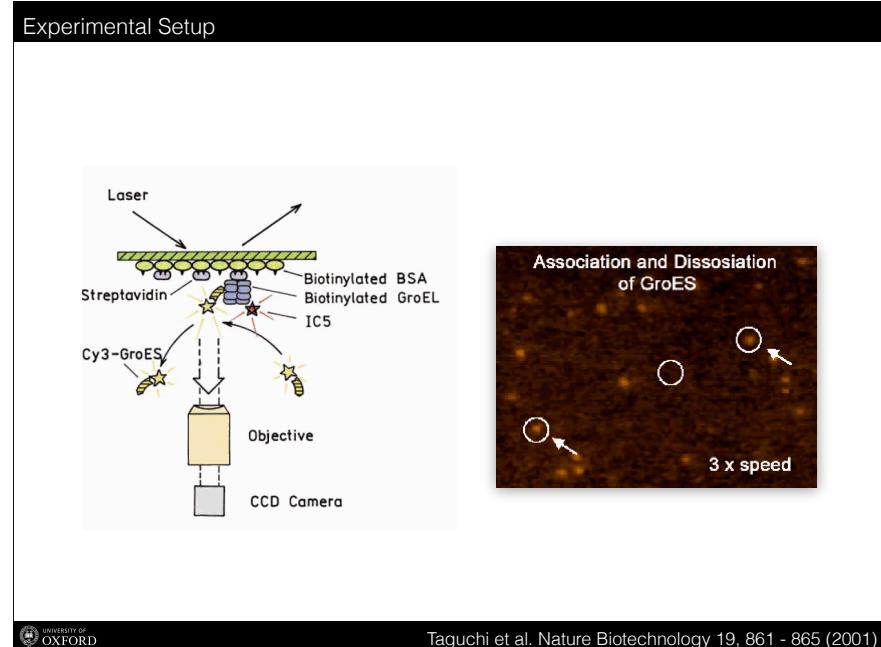
$$d = \frac{\lambda_0}{4\pi} (n_2^2 \sin \theta_2 - n_1^2)^{-1/2}$$

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Annual Review of Biophysics and Bioengineering Vol. 13: 247-268 (1984)

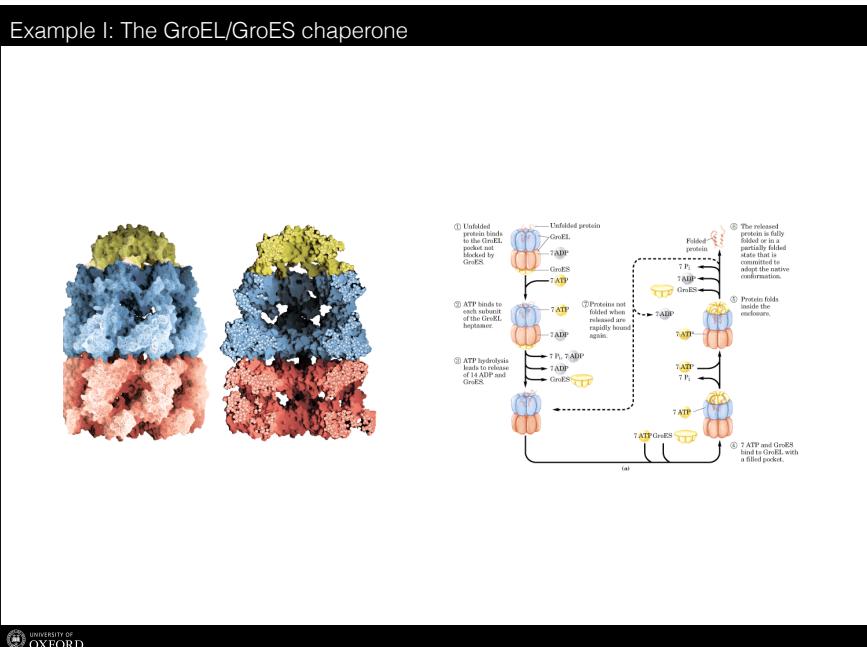


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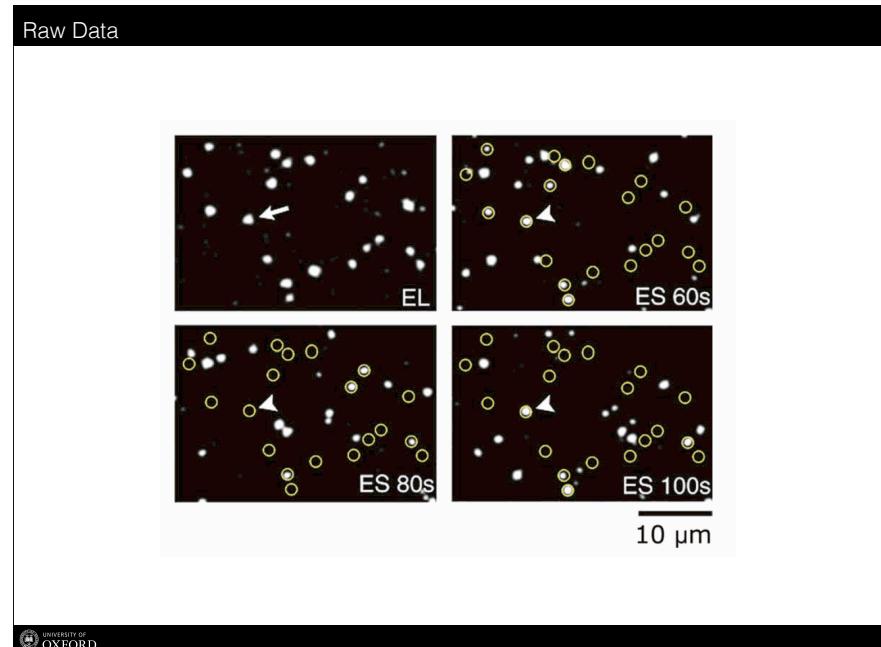


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Taguchi et al. Nature Biotechnology 19, 861 - 865 (2001)

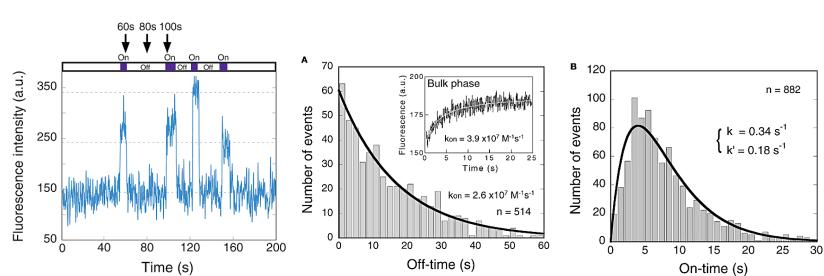


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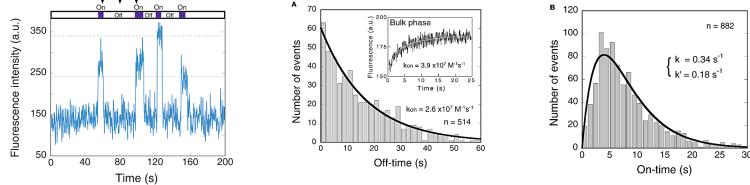


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## Analysis



## Single-molecule kinetics

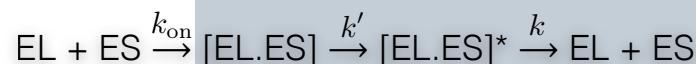
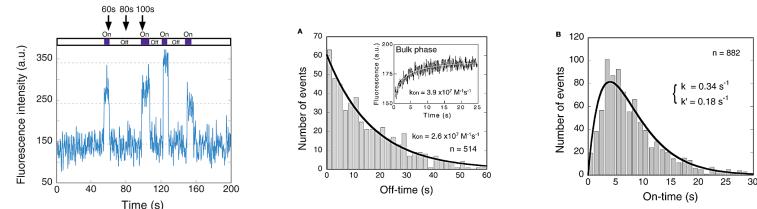


$$\frac{d[\text{EL} \cdot \text{ES}]}{dt} = k_{on}[\text{EL}][\text{ES}]$$

$$-\frac{d[\text{ES}]}{dt} = k''[\text{ES}] \quad \frac{N}{N_0} = e^{-k''t}$$



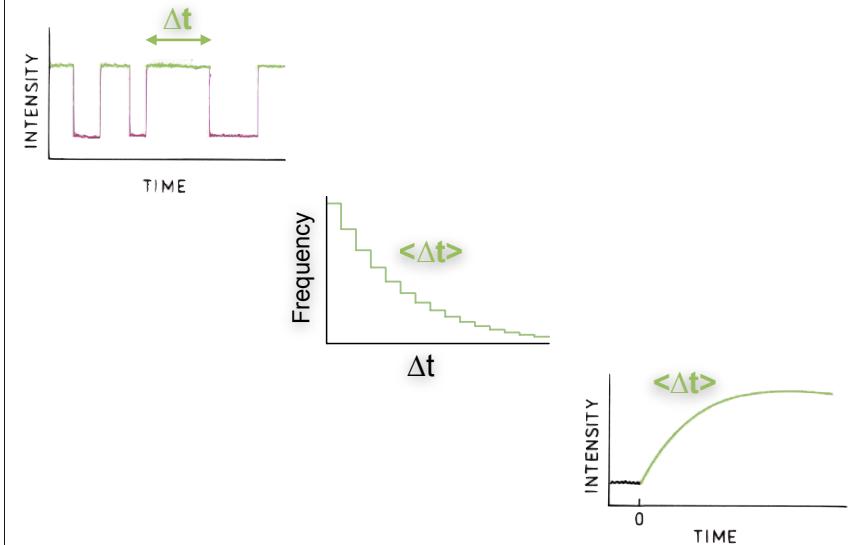
## Single-molecule kinetics

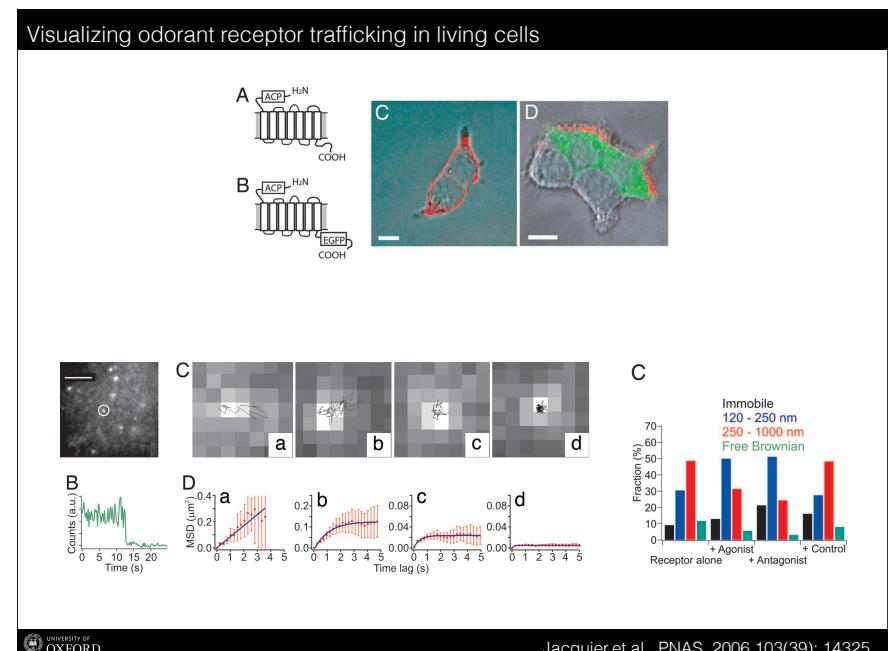
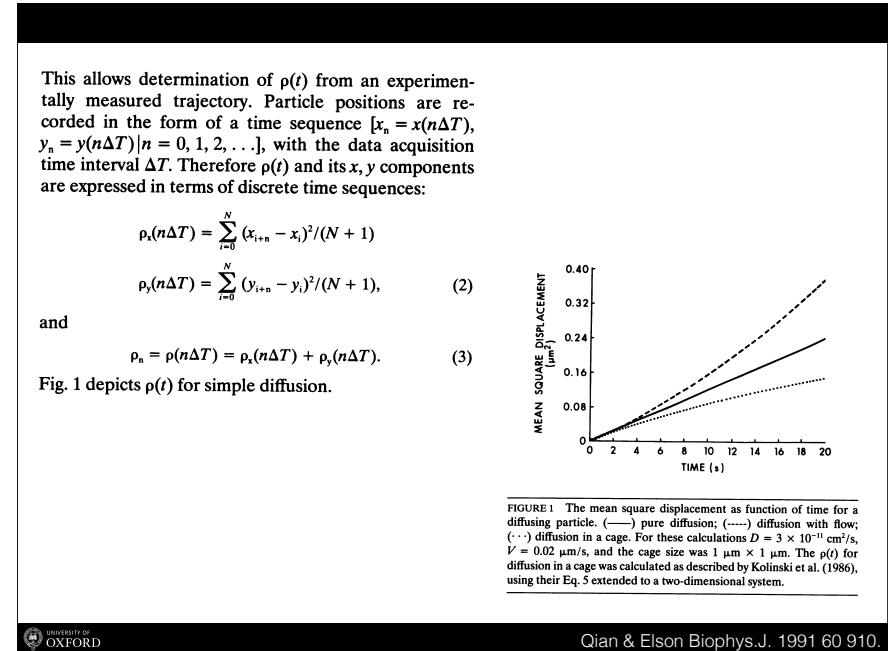
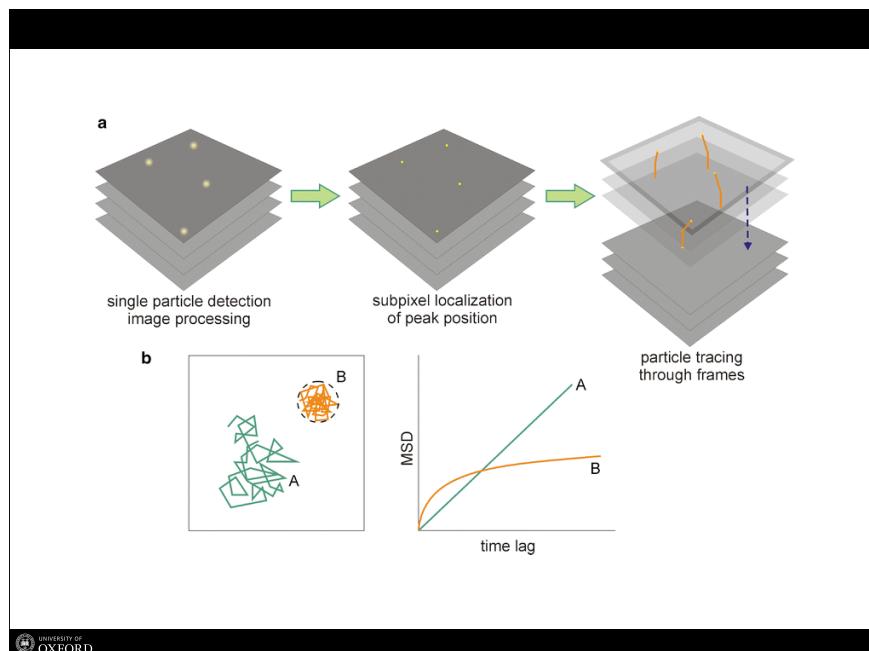
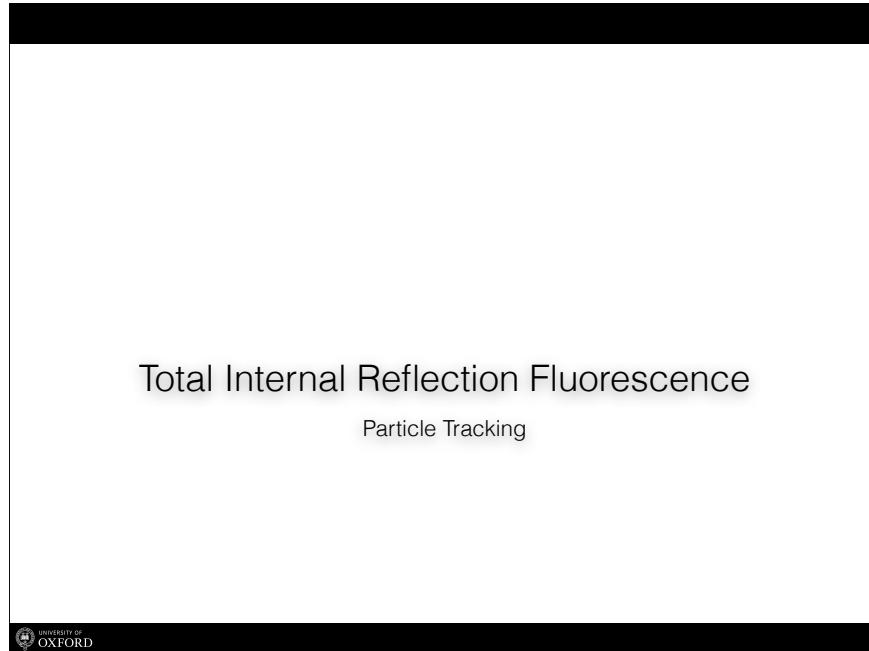


$$\frac{N}{N_0} = \frac{kk'}{k' - k} \{e^{-kt} - e^{-k't}\}$$



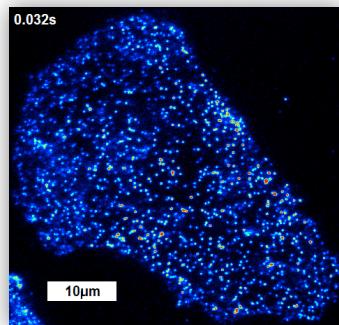
## Linking single-molecule & ensemble





## M1 muscarinic receptor dimers seen by TIRF imaging of single molecules

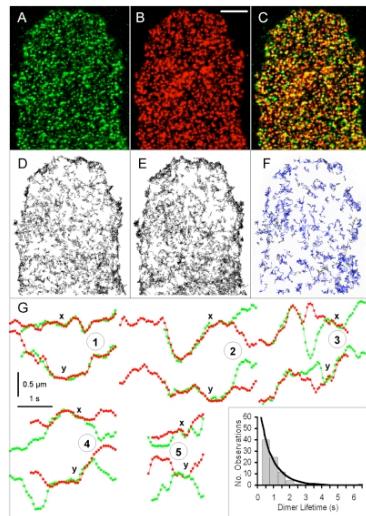
- Muscarinic acetylcholine receptor
- GPCR with important role in cognition and memory
- M1 receptors moving on a single CHO cell.
- Fluorescent telenzepine agonist binds receptors



Hern, et al. PNAS. 2010 107(6): 2693–2698.



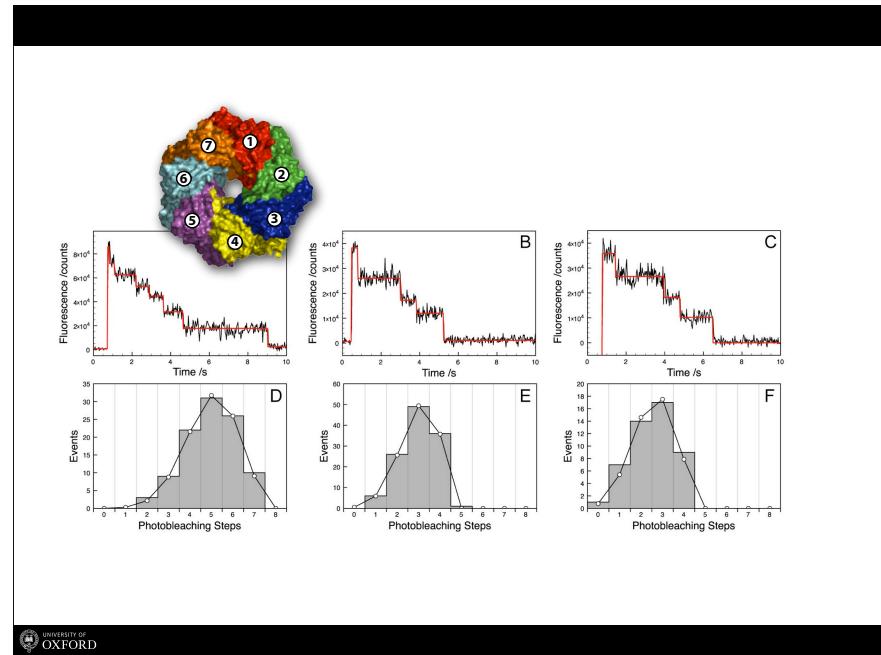
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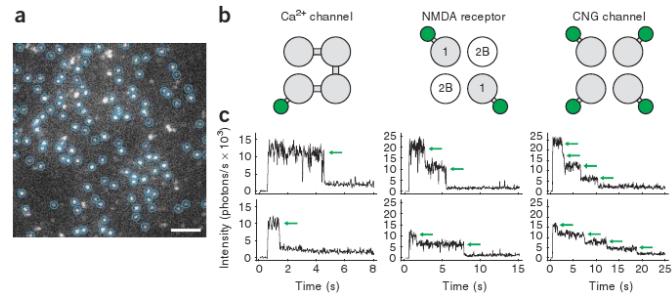
Hern, et al. PNAS. 2010 107(6): 2693–2698.



## Total Internal Reflection Fluorescence Photobleaching



## Subunit counting in membrane-bound proteins



Ulbrich, M. H. & Isacoff, E. Y.. Nat Methods 4, 319–321 (2007).

## Binomial distributions

Event	Number of heads	Probability
TTTT	0	1/16
HTTT		
THTT	1	4/16
TTHT		
TTTH		
HHTT		
HTHT		
HTTH	2	6/16
THHT		
THTH		
TTHH		
THHH	3	4/16
HTHH		
HHTH		
HHHT		
HHHH	4	1/16

$$P(X=x) = \frac{n!}{x!(n-x)!} p^x (1-p)^{n-x}$$

