Bottom-up systems biology using optical proteomics

Systems biology option, Biochemistry

Dr. Mark Leake, Oxford University

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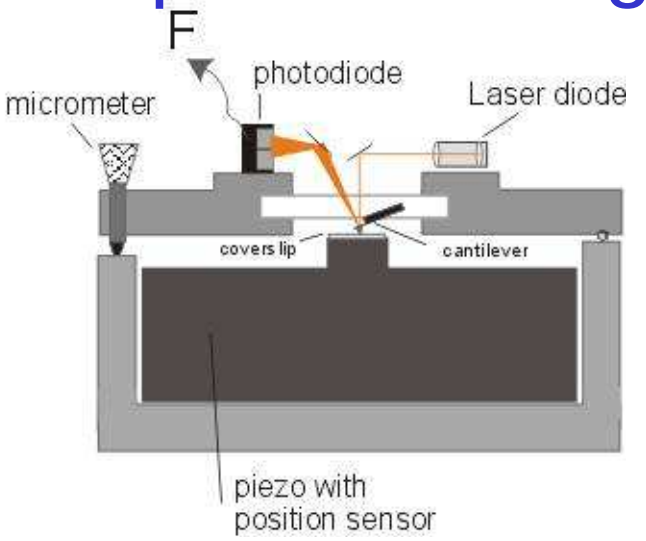
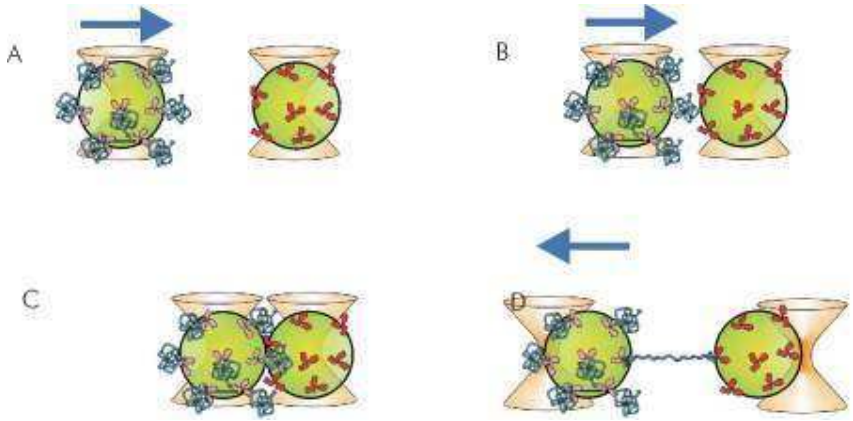
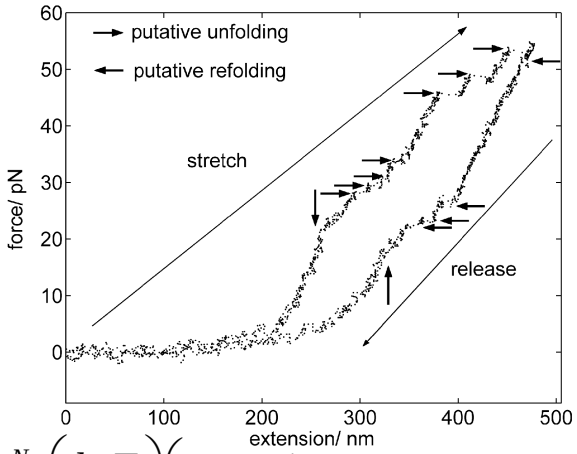
Some previous single-molecule techniques …





Leake et al. (2003) ***FEBS Lett.*** **535, 55.**

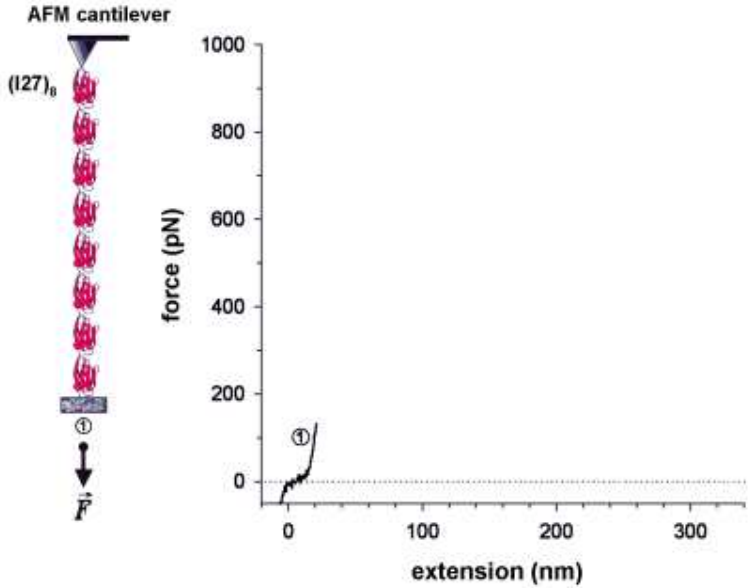


$$F = \sum_{i=1}^{N} \left(\frac{k_B T}{L_{pi}} \right) \left(\frac{1}{4\left(1 - x/L_{ci}\right)^2} - 1/4 + x/L_{ci} \right)$$

Linke & Leake (2004) *Phys. Med. Biol.* 49, 3613.

Opitz, Kulke, Leake et al. (2003) *PNAS* 100, 12688.

Leake et al. (2003) *Biophys. J.* 87, 1112.

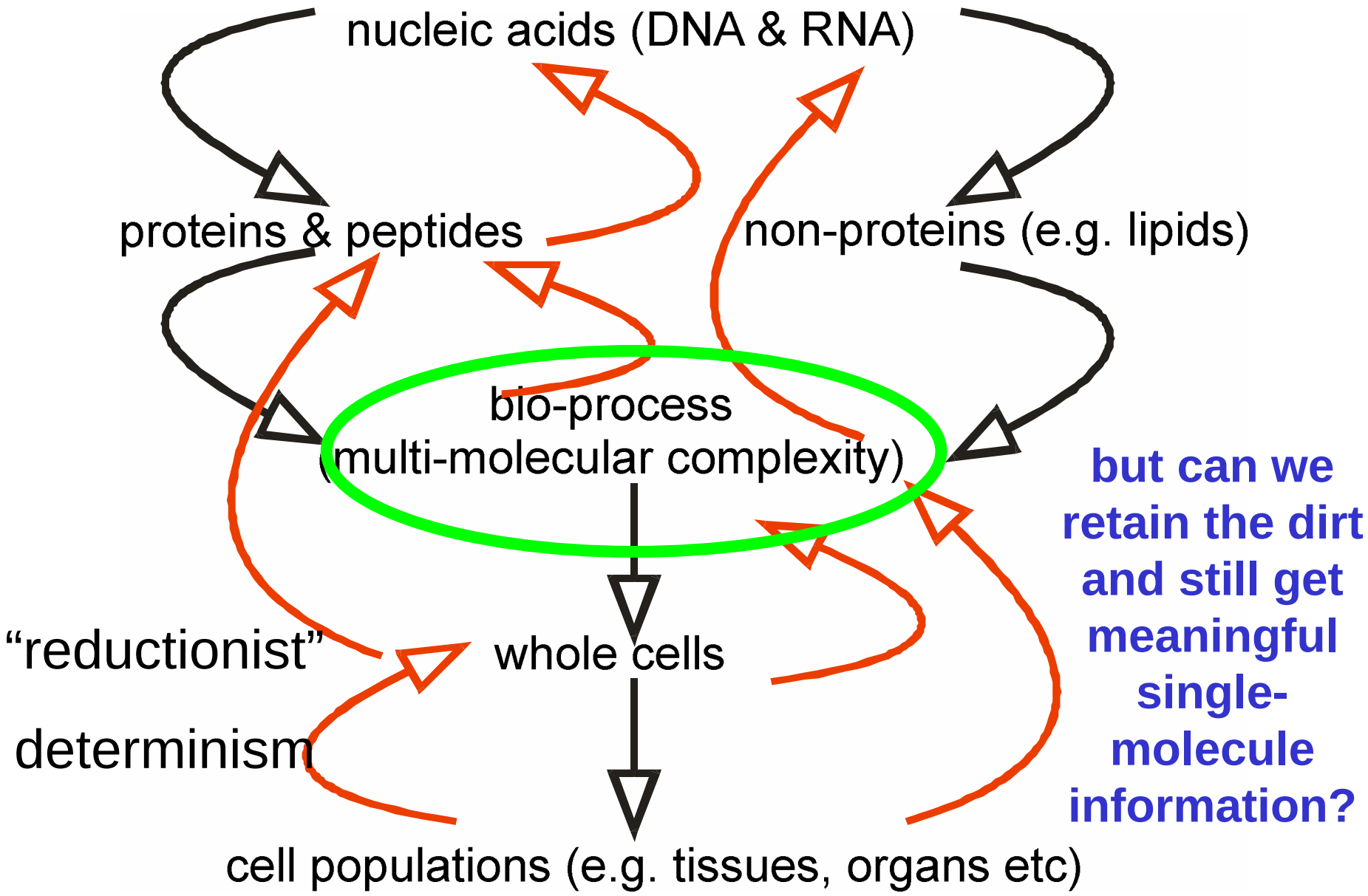


Leake et al. *J. Struct. Biol.* 155, 263 (2006)

Bullard, Benes, Tzintzuni, Leake et al. (2006) *PNAS* 101, 4451.

…molecular “signature”

Living organisms are “dirty”…



but can we retain the dirt and still get meaningful single-molecule information?

- Is it possible to correlate cellular and molecular levels?
- Can we have a systems biology approach, but still be reductionist experimentally?

1. Total Internal Reflection Fluorescence Microscopy (TIRFM)

2. Calibrations using Green Fluorescent Protein *in vitro*

3. Low-light measurements *in vivo*

different biological systems:

↓

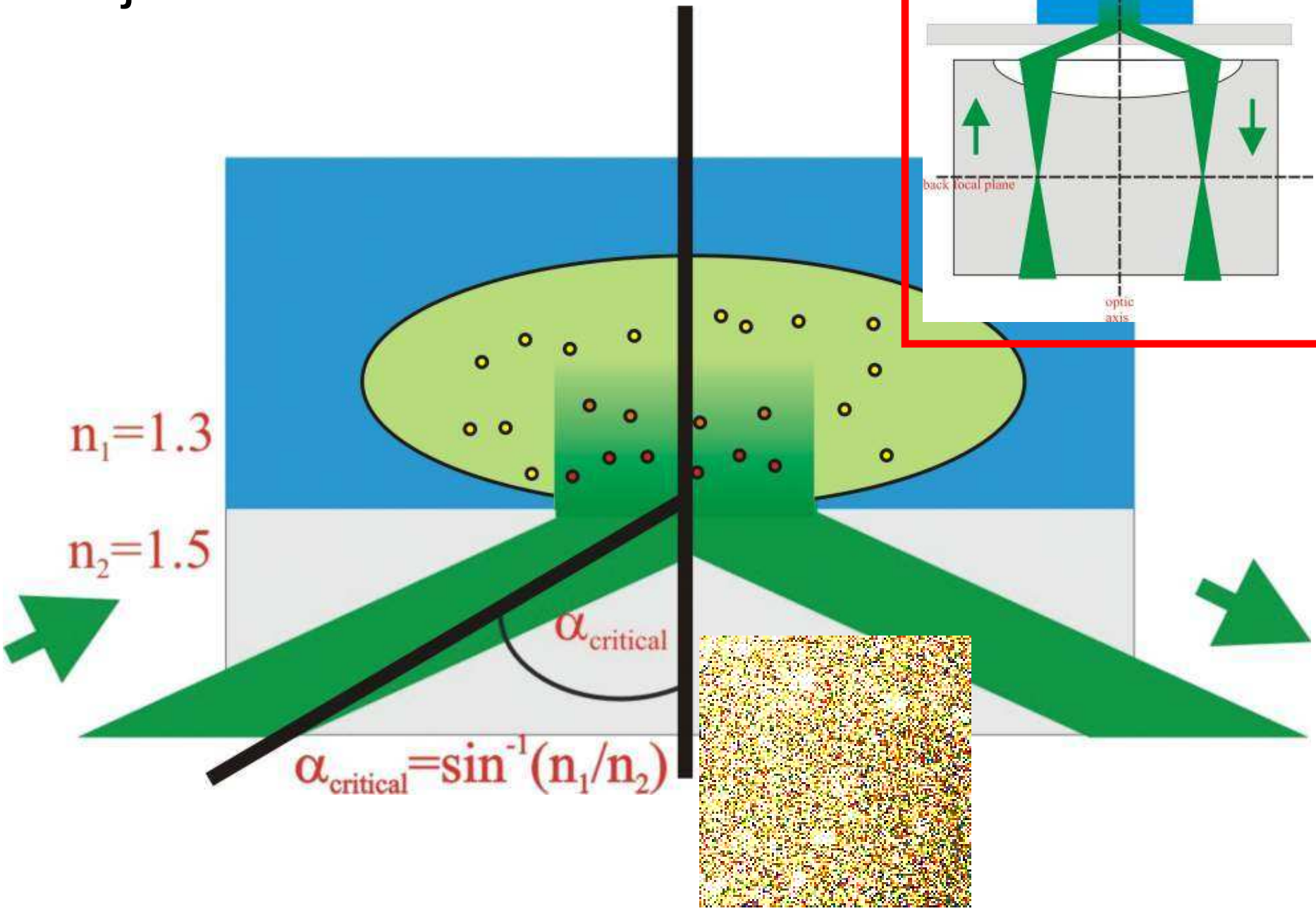
- motility
- protein transport
- chemotaxis
- cell shape and structure

additional microscopy techniques:

↓

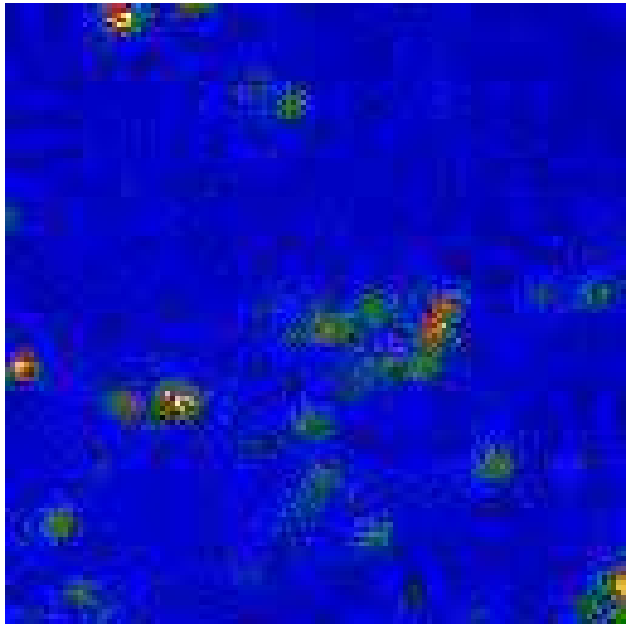
- Fluorescence Recovery After Photobleaching (FRAP)
- Fluorescence Loss In Photobleaching (FLIP)
- Fluorescence Imaging with One Nanometre Accuracy (FIONA)
- Foerster Resonace Energy Transfer (FRET)

Objective-method TIRF:

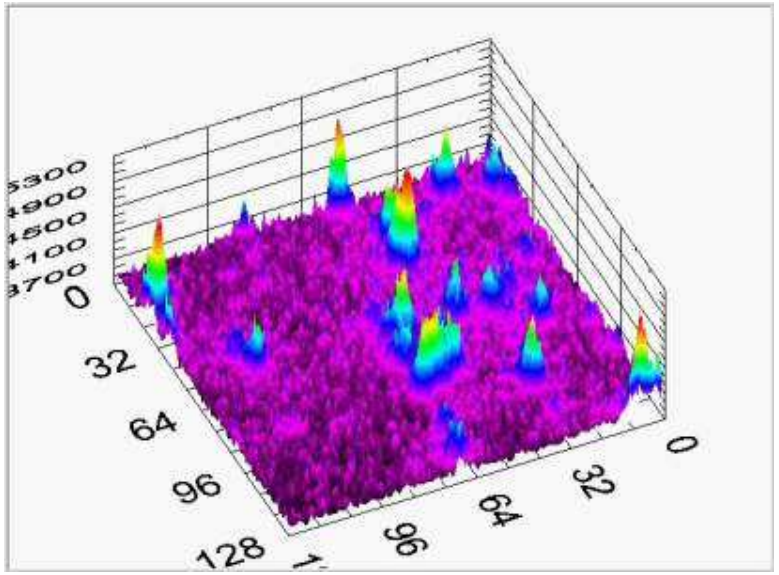


In vitro detection of single fluorophores using purified green-fluorescent protein [GFP]:

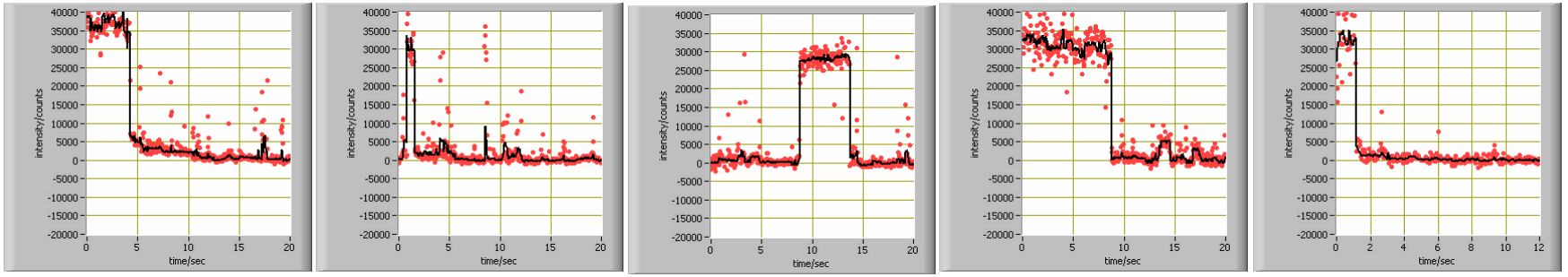
a)



b)



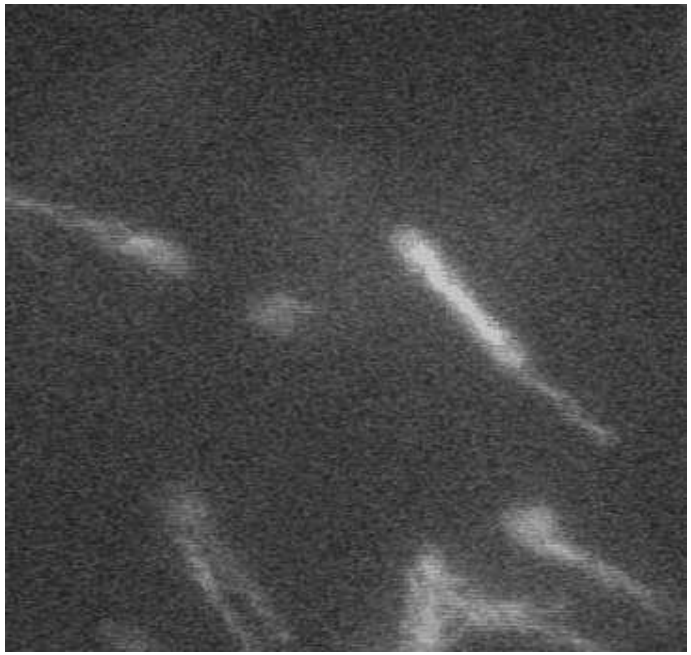
c)

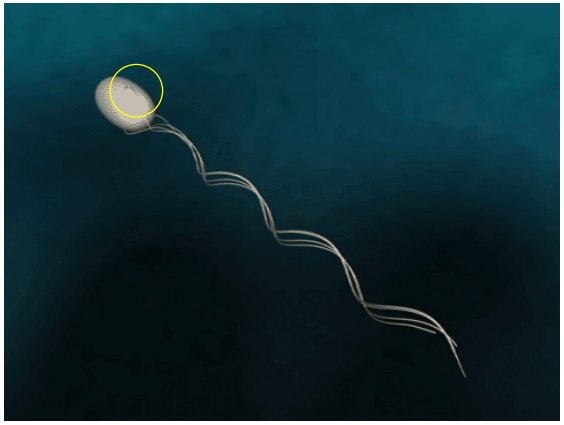


(a) 2D and (b) 3D raw pixel intensities from CCD array for GFP binding transiently to glass (white bar=1mm). (c) examples of raw (red) and Chung-Kennedy [CK] filtered (black) summed intensities for 6x6 pixel (300x300 nm) regions of interest centred over intensity peaks.

Bacterial motor proteins:

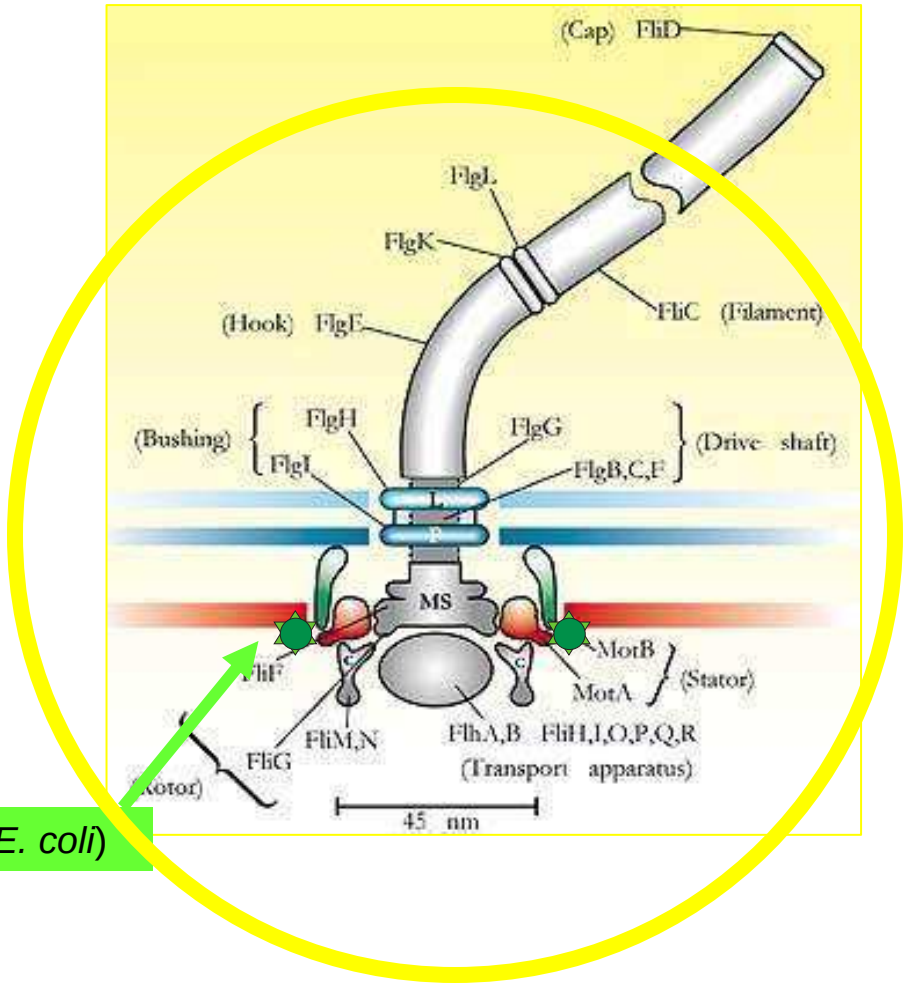
Courtesy of Howard Berg, Rowland Institute, Harvard University, USA:





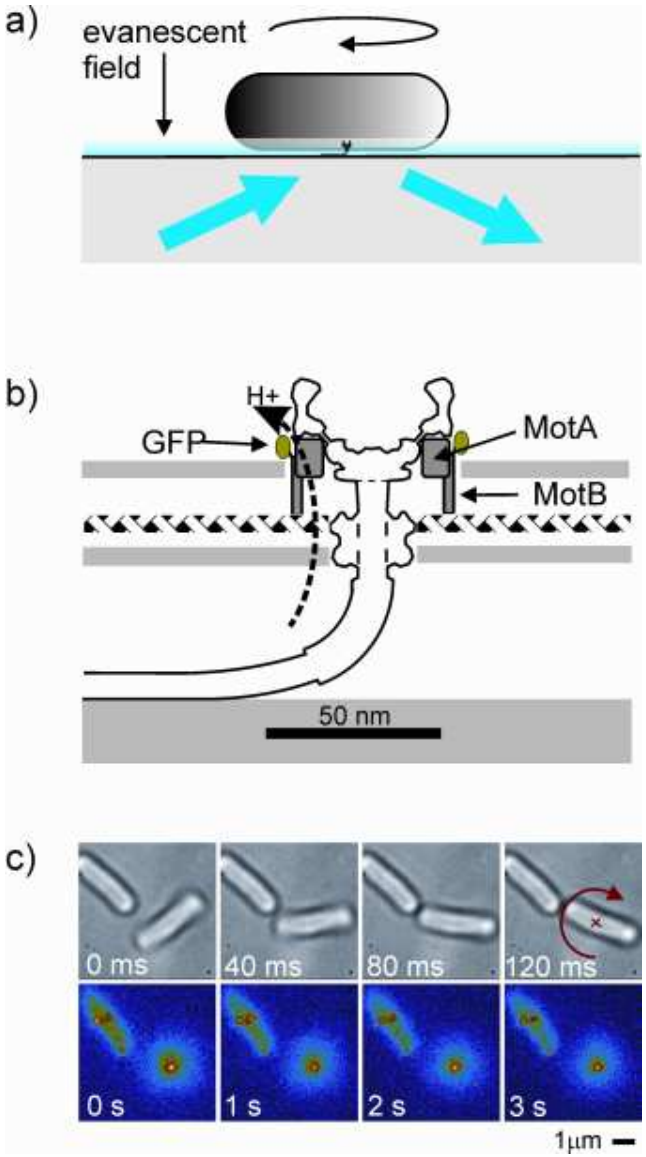
Courtesy of Keiichi Namba, Protonic Nanomachine Project, Osaka University, Japan

Leake et al. *Nature* 443, 355-8, *(*2006).

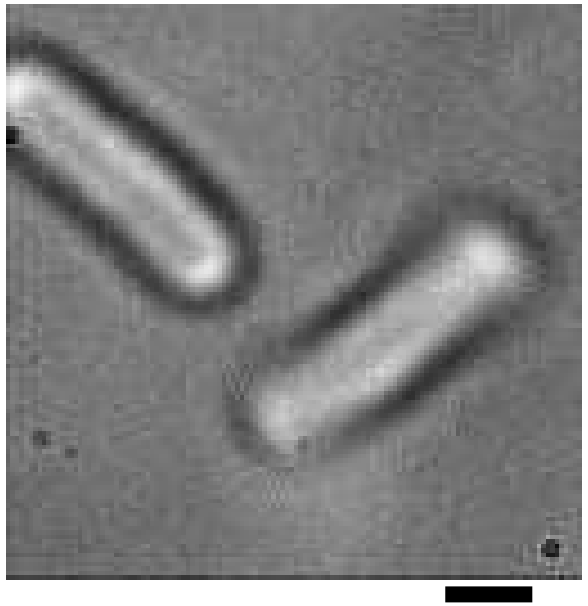


GFP-MotB (*E. coli*)

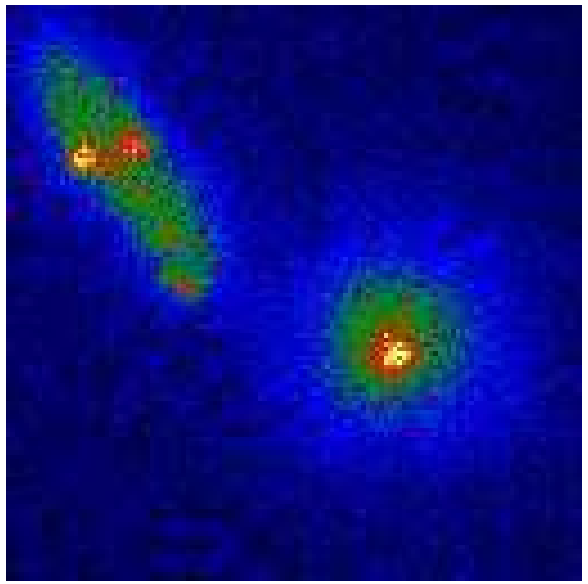
Antibody-tethered cell-rotation assay:



d)



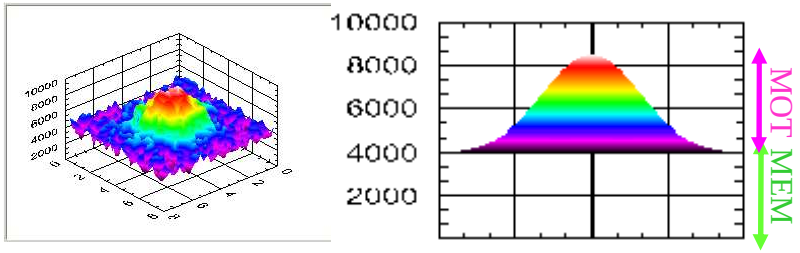
e)



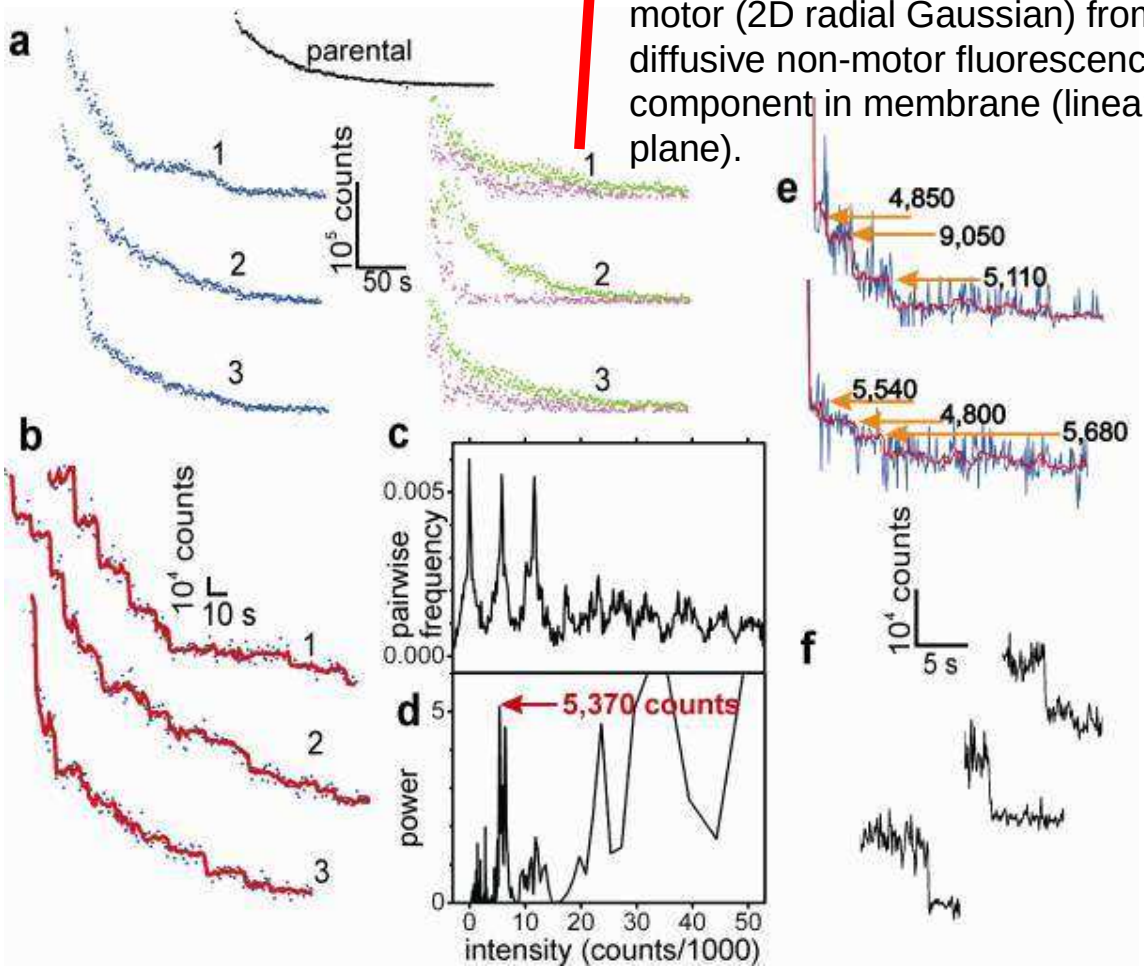
(d) Brightfield and (e) TIRF images of GFP-MotB *E. coli* mutant. Black bar=1μm

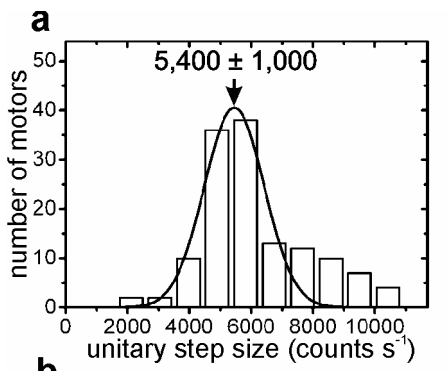
Estimating protein number…

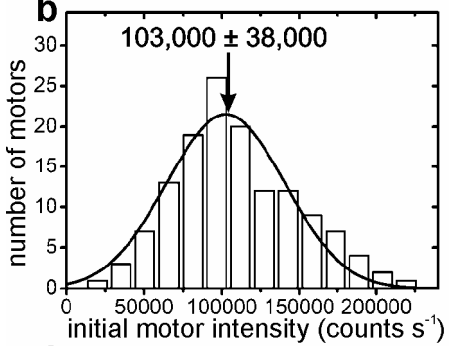
Quantifying the number of fluorophores at the motor:

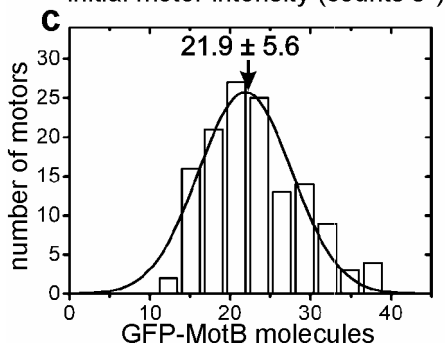


Separate fixed fluorescence at motor (2D radial Gaussian) from diffusive non-motor fluorescence component in membrane (linear plane).





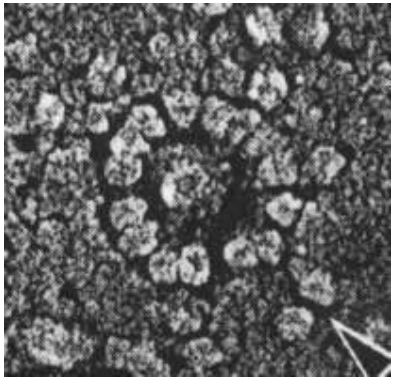




~22 MotB molecules in each motor complex

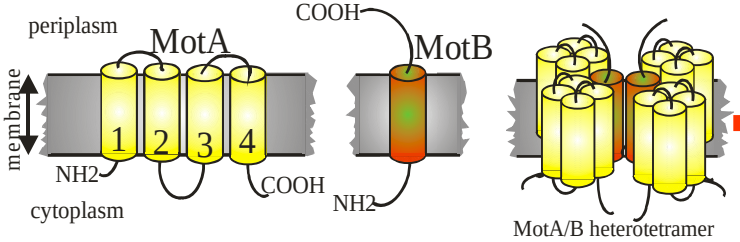
A plausible interpretation…

A Freeze-fracture electron microscopy:
J Mol Biol, 202(3):575-84, 1988.



10-16 studs around rotor axis… stators?

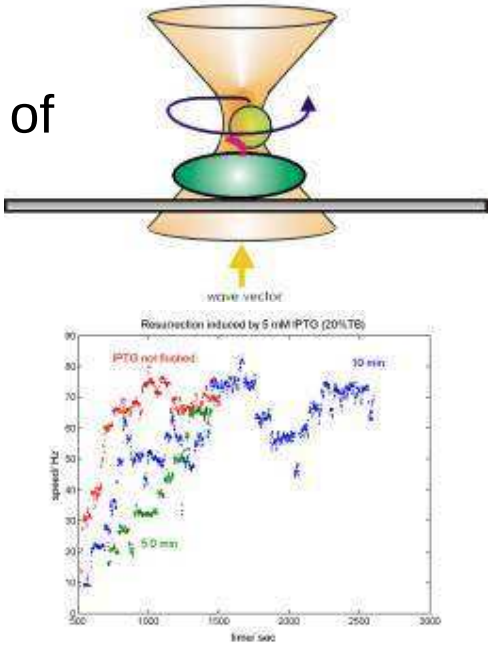
B Solubilisation and purification of the MotA/MotB complex:
Biochemistry. 43(1):26-34, 2004



MotA:MotB =2:1

C Induced motor resurrection of *E.coli* mutant WSR8:

Reid S, Leake MC, Chandler JH, Lo CJ, Armitage JP & Berry RM. *PNAS* 103, 8066-71, 2006.

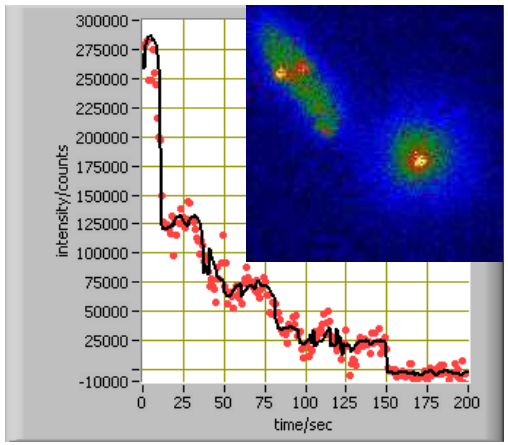


~11 distinct speed levels

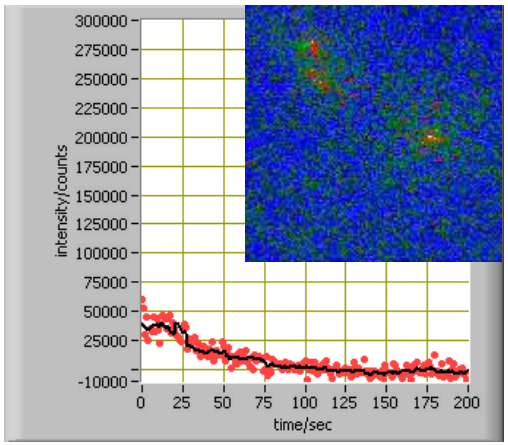
A, B and C are consistent with the current fluorescence data if there are 11 stator units with a $(MotA)_4$:$(MotB)_2$ heterotetramer conformation

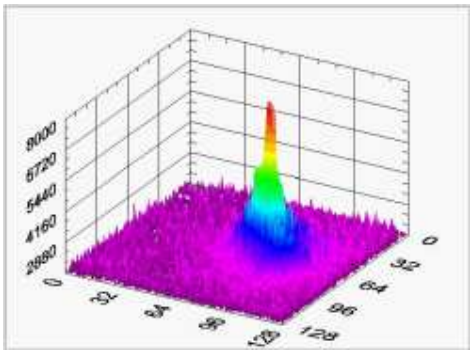
Estimating protein mobility and turnover…

TIRF-Fluorescence recovery after photobleaching [TIRF-FRAP]:



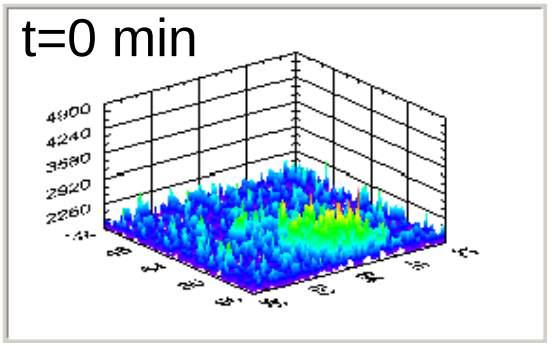
30 min

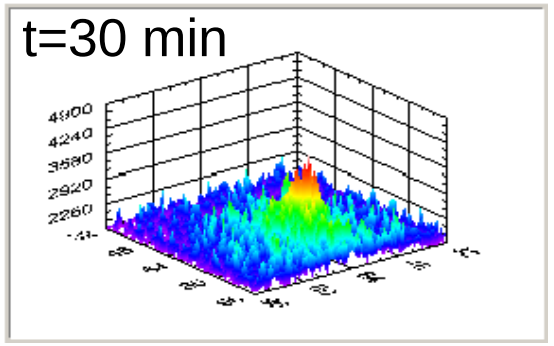


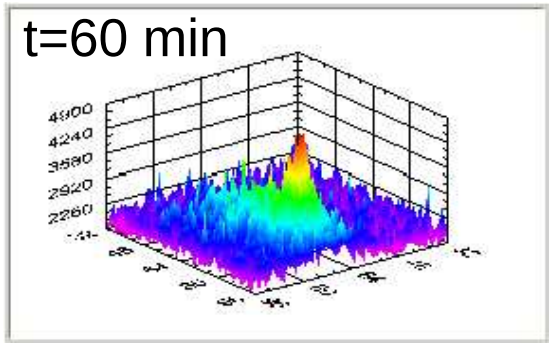


(A) Bleach-wait-bleach protocol

(B) Bleach-wait-snapshot-wait… protocol

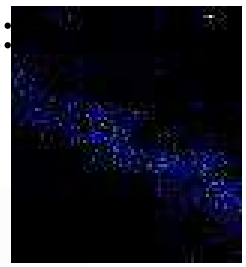


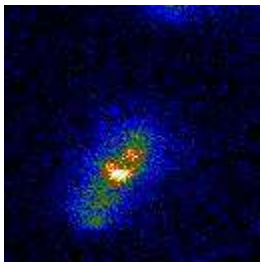


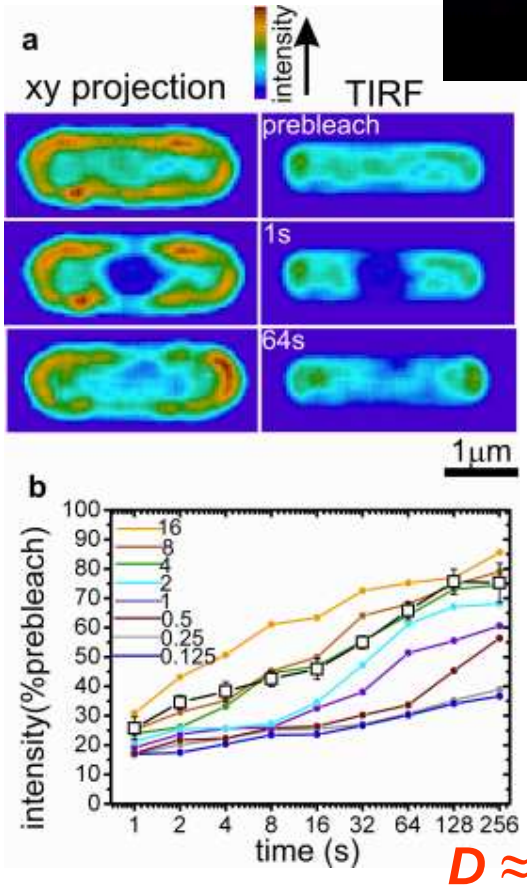


Focussed Laser FRAP/FLIP (…*Loss In* Photobleaching):

Either track inidividual particles directly, or apply Monte-Carlo 2D simulations to estimate diffusion coefficient:







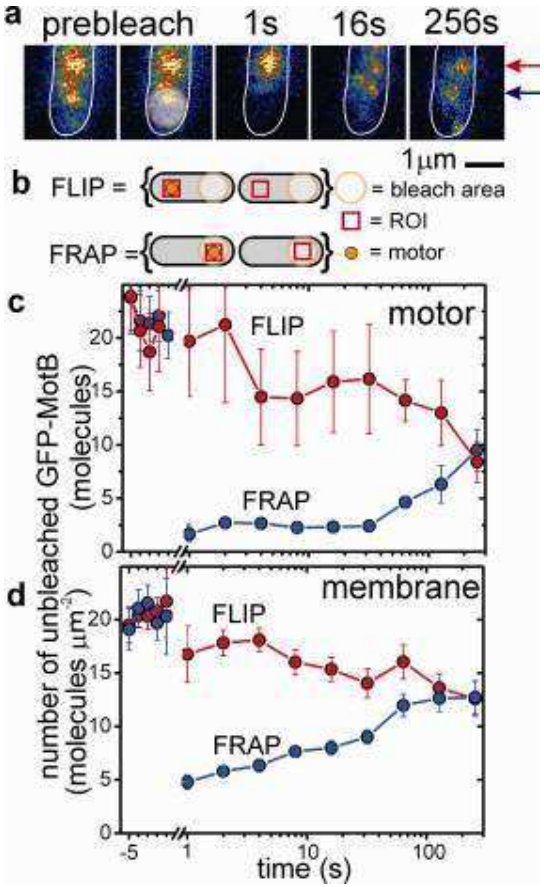
Components in biological machines may need to be replaced frequently in much the same way as those for man-made machines

Diffusion-to-capture model

$D \approx 0.008\ \mu m^2\ s^{-1}$

Stator dwell-time ~30s

Separating out motor and membrane components to measure turnover:

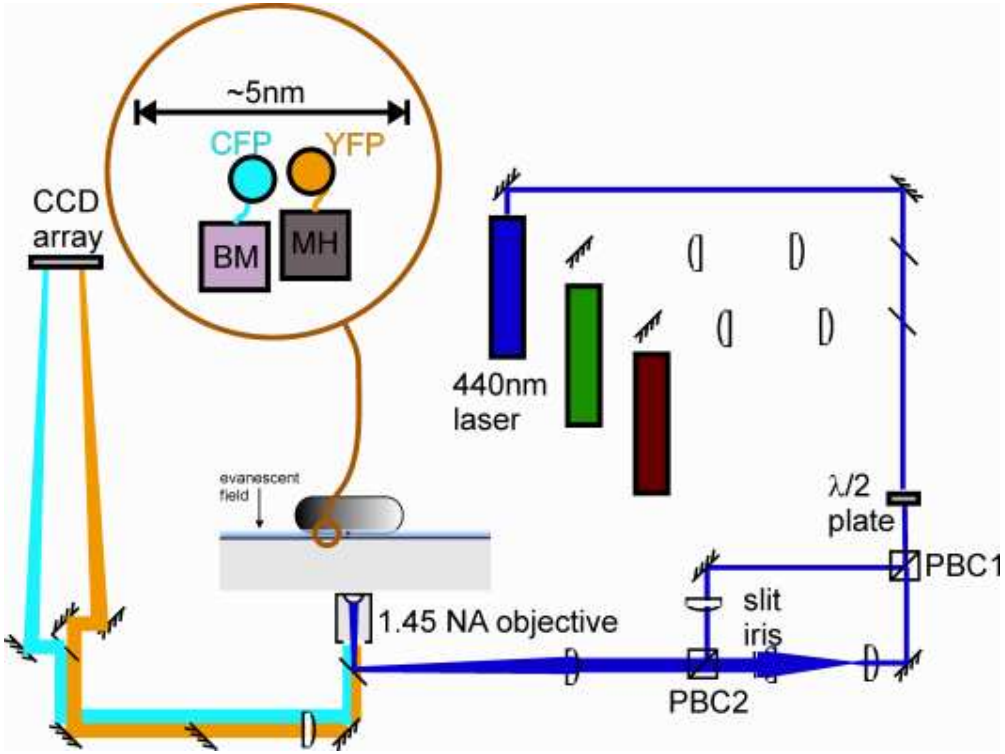


Conclusions…

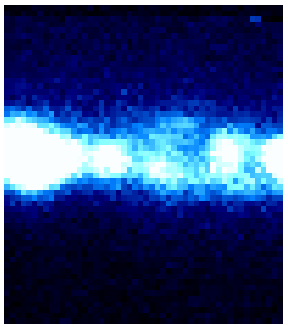
- We observe step-like photobleaching of single GFP fluorophores *in vivo*
- Can estimate *in vivo* concentrations of proteins to unprecedented accuracy
- Able to measure mobility and turnover of proteins within a functioning membrane complex in a living cell in real-time to the same single-molecule precision
- Bacterial cell motility exhibits ROBUST qualities

The future…

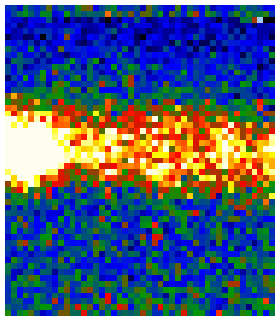
Observing two different proteins from the same system in the same cell at the same time…



Leake & Formstone et al. *In Prep*. (2008).



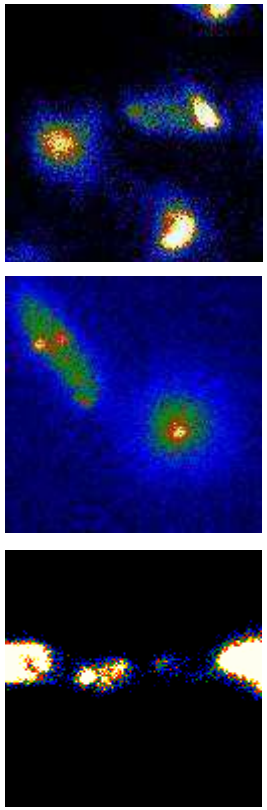
CYAN channel
(Donor emission)



YELLOW channel
(Acceptor emission)

Bacillus subtilis, dual-labelled with CFP-BM and YFP-BH

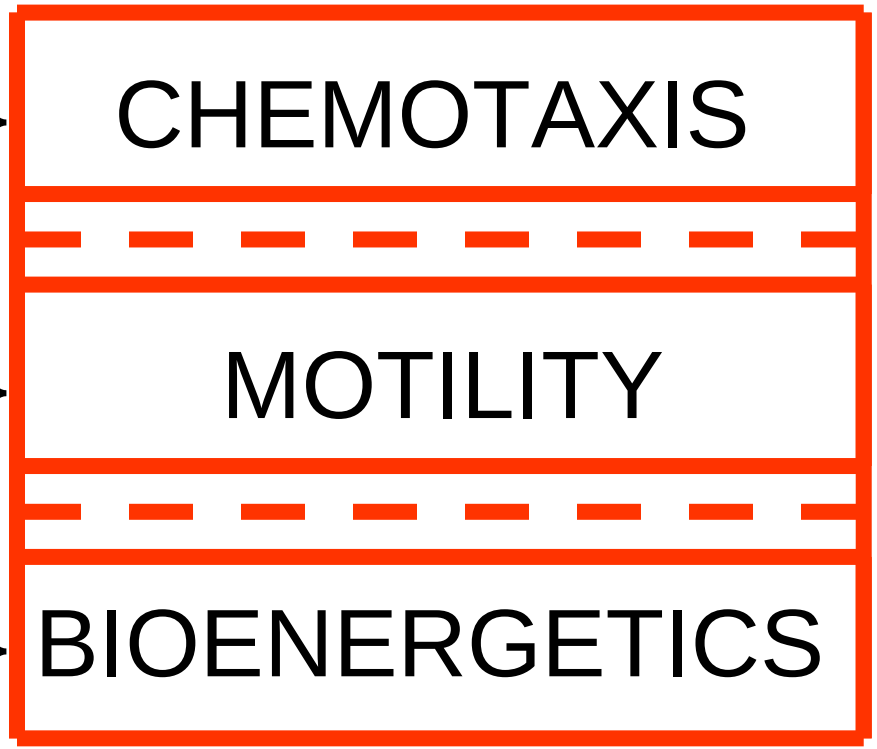
Many different proteins in many different systems…



GFP-MCPG

GFP-MotB

GFP-CytD

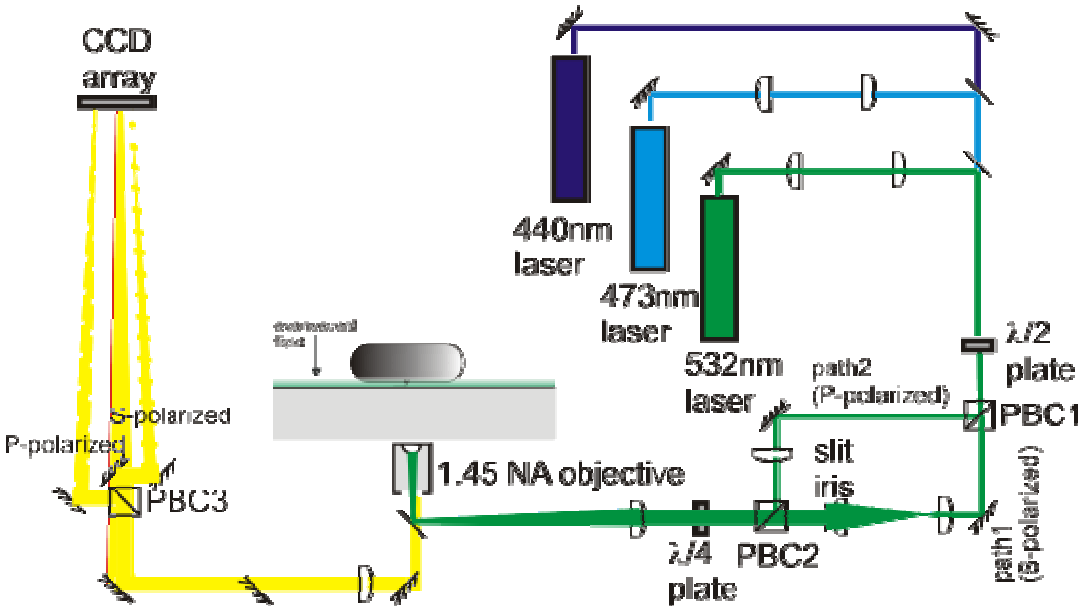


Leake & Lenn et al. *In Prep.* (2008).

But "different" systems may be functionally dependent, and have in effect shared elements…

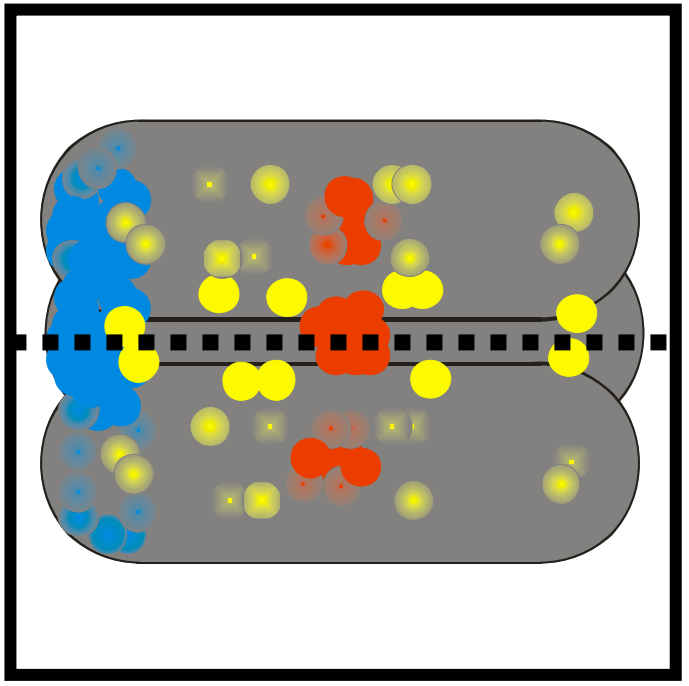
Future plan: to address biological complexity we need simultaneous single-molecule information from several systems in the same living cell…

ms-ALEX



<1ms possible frame-rate

CCD array:



bioenergetics-yellow

…polarisation control adds an extra dimension of data, providing dynamic information on conformational changes…

Futher developments…

- Brighter, longer-lived tags e.g. quantum dots, 40nm gold beads… but cell uptake still a challenge
- Smaller tags, such as FLAsH-peptides
- Metabolomics as well as proteomics e.g. fluorescent probes such as acrylodan to fatty acids
- Several simultaneous single-molecule techniques e.g. SERS-fluorescence + AFM imaging, or optical-trapping + TIRF microscopy
- More complex *eukaryotic* cells e.g. yeast and trypanosomes

Summary…

- single-molecule measurements generate more meaningful information than ensemble averages (no masking of heterogeneity etc)

- *in vivo* experiments provide far more relevant information than *in vitro*

- multiple simultaneous measurements from different processes is a key tool towards unravelling biological complexity, and should be at the forefront of experimental systems biology

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Randall Division, KCL: D. Wilson, M. Gautel & R. Simmons.

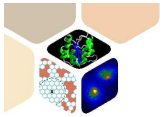
Ruprect-Karls-Universität Heidelberg: C. Opitz, M. Kulke & C. Neagoe.

Sealy Center, Texas University: T. Garcia & A. Oberhauser.

Universität Bielefeld: H. Hinssen.

Universität Münster: A. Grützner, M. Krüger & W. Linke.

York University: B. Bullard.



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Useful articles:

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Total internal reflection fluorescence. *Annu. Rev. Biophys. Bioeng.* 13, 247–268.

•Berg H C (2003) The rotary motor of bacterial flagella.
Annu. Rev. Biochem. 72, 19-54.

•Leake, MC. et al (2006) Stoichiometry and turnover in single, functioning membrane protein complexes. *Nature* 443, 355-8.

•Tsien, R. (1998) The green fluorescent protein. *Annu. Rev. Bioch.* 67, 509-44.

Useful websites:

•http://www.olympusmicro.com/primer/techniques/fluorescence/tirf/tirfhome.html

•http://www.physics.ox.ac.uk/users/leake

•http://www.sysbio.ox.ac.uk