# Single molecule optical proteomics *in vivo*

Using optics to study established bionanotechnology at the single molecule level in living cells

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## Lecture summary

Here, we will discuss two real experimental case studies investigating "established bionanotechnology" in living bacterial cells which use "optical proteomics" in the form of advanced fluorescence microscopy to monitor functional molecular machines

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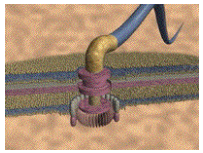
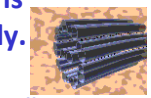
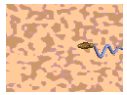
## What is it to be "established bionanotechnology"?

**Machines in the living cell built from a few, discrete molecular components – so-called "molecular machines"**

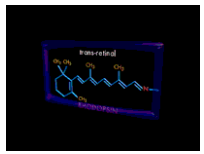


**They're small – length scale of single cell is ~1 million times smaller than human body. Length scale of these machines is ~1000 times smaller a single cell. "Nanomachines"**





**They work by transducing energy in response to biological stimuli in order to perform "useful" work**



**They are the drivers of the fundamental processes in a living cell**

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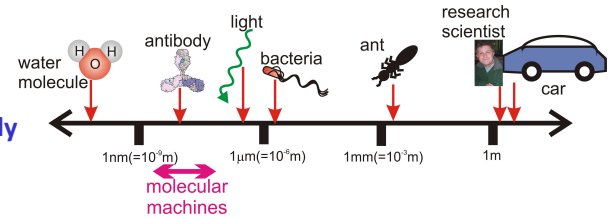
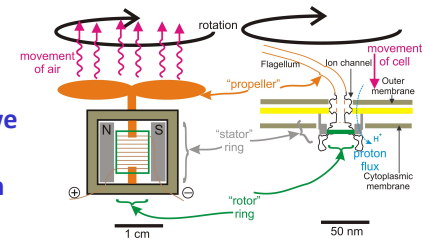
## Size matters

**The "nanometre" length scale of these machines, ~100 times smaller than the wavelength of light, means that they are highly sensitive to motions of surrounding molecules, notably water in living cells**




**Molecular machines have similarities to everyday "macro" scale machines we are familiar with, but are fundamentally different in being immersed in a "thermal bath"**

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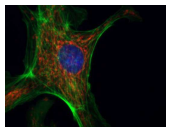
## Why study molecular machines in living cells when we can use the test-tube?

The test-tube system is a much reduced version of the living cell

Cells have spatial and temporal localization



A cell's physical and chemical environment is difficult to replicate

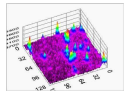
In the living cell, copy numbers often low

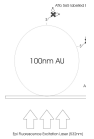
**Ultra-sensitive fluorescence microscopy can be minimally perturbative, may use highly specific tags and offers single molecule precision**



## Lecture outline

"Test-tube" imaging:





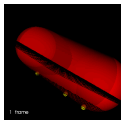

"Live-cell" imaging:

Imaging time scale:

Measuring molecular composition

~100-1000ms

Characterizing molecular dynamics



~10-100ms

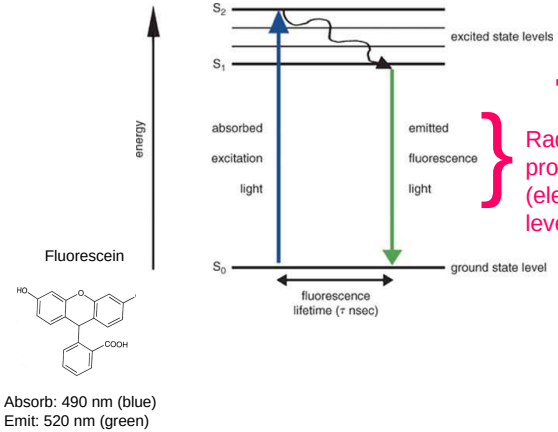
Faster imaging: DNA replication


~1-10ms



## What is "fluorescence"?

**Fluorescence is the process of absorption of electromagnetic radiation followed by rapid emission light of *longer* wavelength (within nanoseconds):**



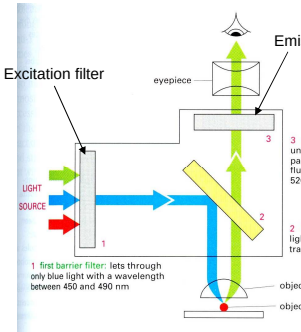

Absorb: 490 nm (blue)
Emit: 520 nm (green)

Absorb: 550 nm (green-yellow)
Emit: 580 nm (orange-red)



## Practical fluorescence microscopy

Key component in the microscope is a special cube containing usually two coloured filters plus a special beam-splitting ("dichroic") mirror



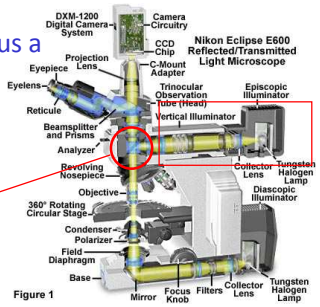
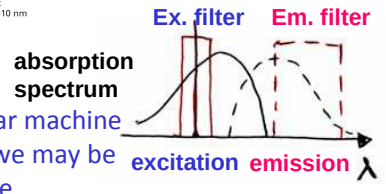
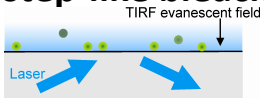



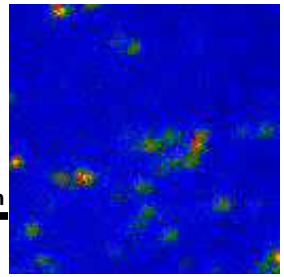

Figure 1



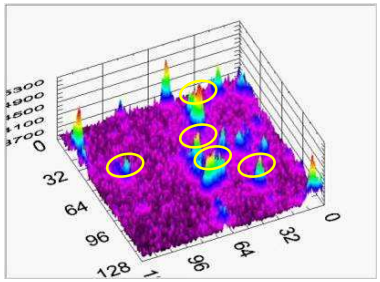

If we can specifically label a molecular machine with a fluorescent compound, then we may be able to see it using such a microscope

# Single fluorophores can undergo step-like bleaching

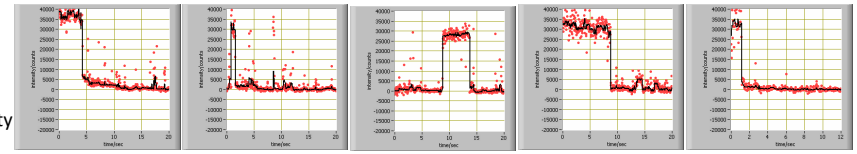



2D camera image



3D intensity plot

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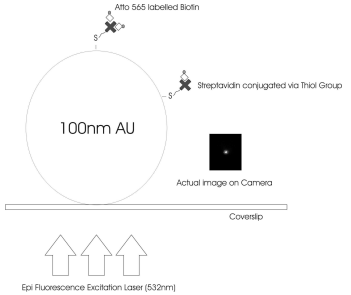
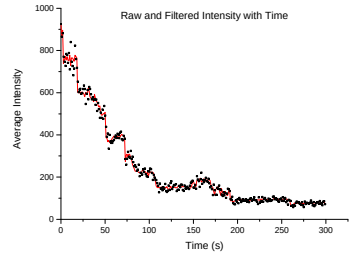
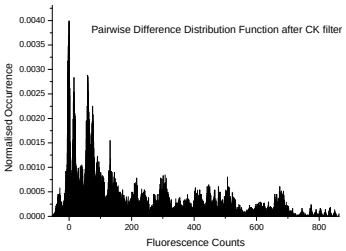
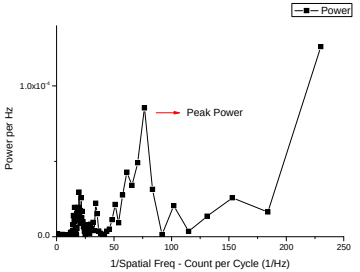
# A stoichiometry assay based on stepwise photobleaching

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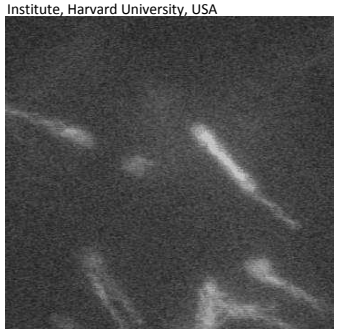
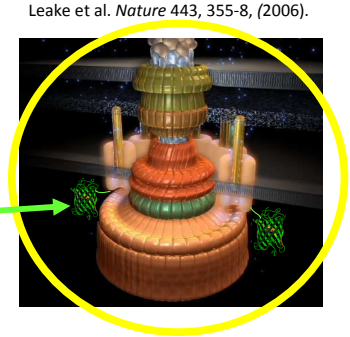
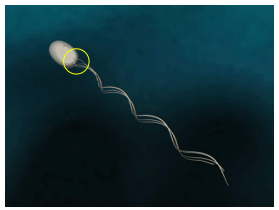
# Imaging at 100s of milliseconds per frame: bacterial motor proteins

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

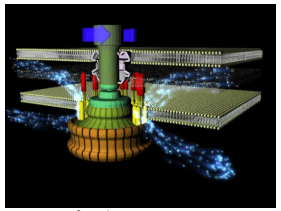


Leake et al. *Nature* 443, 355-8, (2006).



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



Courtesy of Michio Homma, Nagoya University, Japan

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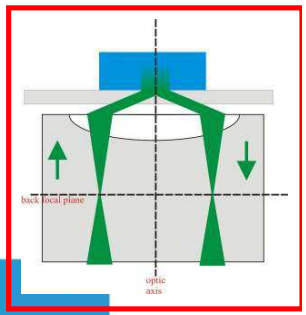
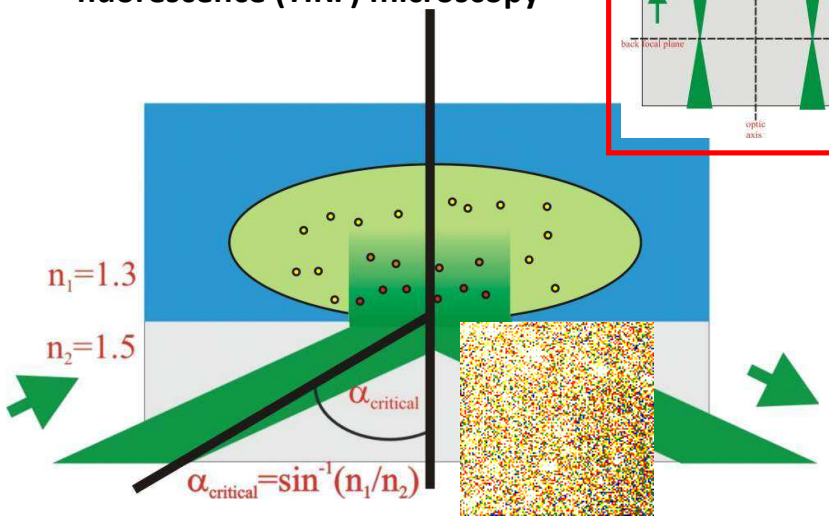
# "Bespoke" microscopy can help us "see" single molecules: Total-internal-reflection-fluorescence (TIRF) microscopy

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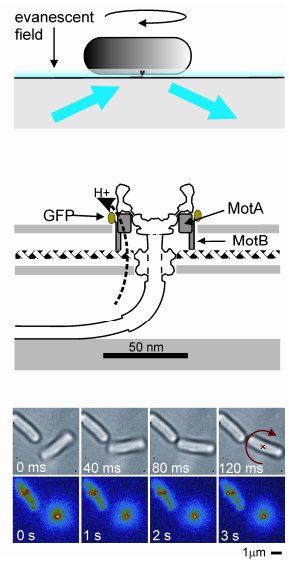
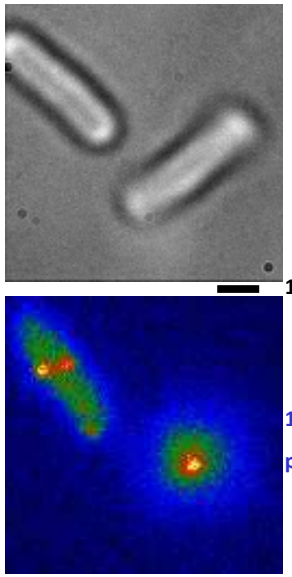
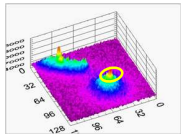
# Visualizing functional machines in the cell membrane

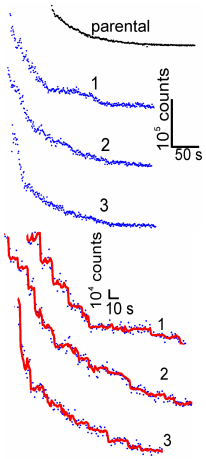


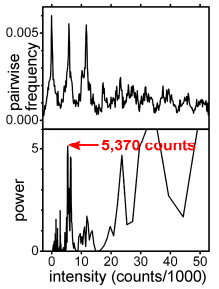



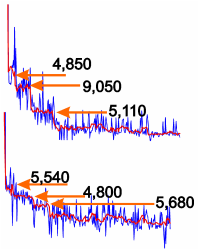


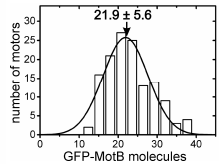

# Quantifying stoichiometry in molecular machines





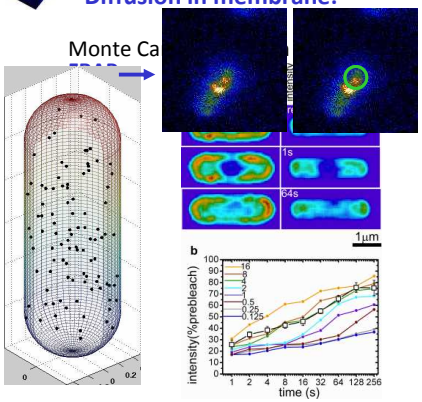




**~22 MotB molecules in each motor complex**

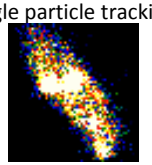


# Measuring dynamics

**Diffusion in membrane:**

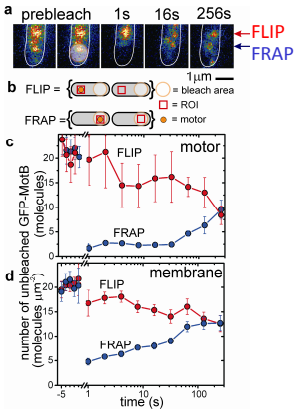



Single particle tracking



***D*** **≈ 0.008 μm² s⁻¹**

**Molecular turnover in motor:**

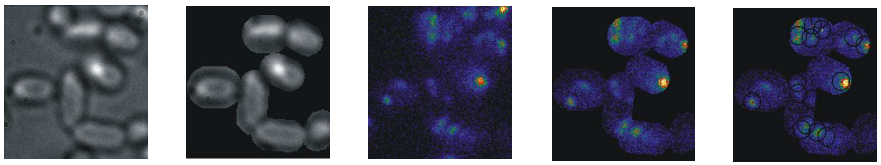



**Stator dwell-time ~30s**

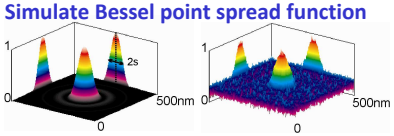
**Components in molecular machines replaced in much the same way as those for man-made machines?**

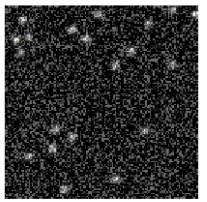


# Detection and tracking




**Simulate Bessel point spread function**



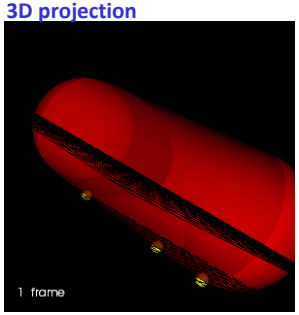



$$\left\langle (\Delta x)^2 \right\rangle = \frac{s^2 + a^2/12}{N} + \frac{4\sqrt{\pi} s^2 b^2}{aN^2}$$

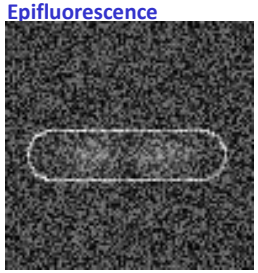
Thompson, Larson, Webb *Biophys J* 82, 2775 (2002).

# Diffusion of membrane machines: simulation

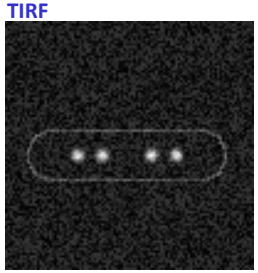
3D projection

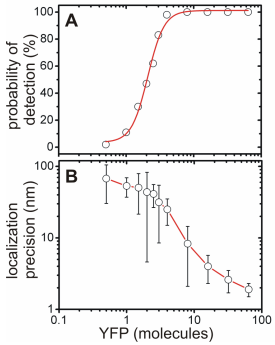



Epifluorescence



TIRF





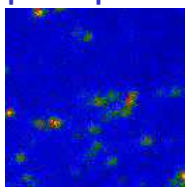


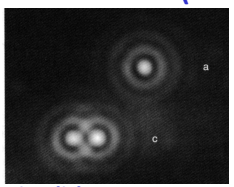

# In pursuit of "real time" imaging *inside* the cell

**What do we mean by "real time"?**

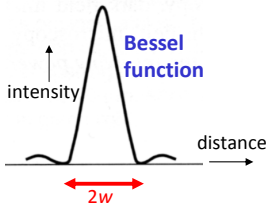
sampling data at least as fast as the process we are studying

**In far-field a static emitter seen as a spot of intensity of point spread function width *w* (~0.61λ/NA), or ~250-300nm**




Airy disks




If emitters are *mobile* and move distance *d* during image frame of time Δ*t,* we will only see them "unblurred" if ~*d*<*w*



# What is the maximum allowed Δt?

***d*** **~ √(mean-squared displacement, $R^2$)**

1-dimensional diffusion $R^2 = 2D\Delta t$

2-dimensional diffusion $R^2 = 4D\Delta t$

3-dimensional diffusion $R^2 = 6D\Delta t$

**Typical *membrane* protein (2D) diffusion *D* ~(1-20) x $10^{-3}$ μm²/s…**

**maximum Δ*t* ~100ms**

***Cytoplasmic*** **protein (3D) diffusion *D* ~5 μm²/s…**

**maximum Δ*t* ~3ms.**

**Video-rate microscopy (Δ*t*=40ms) OK for imaging machines in membranes, but not inside cells**



# DNA replication in bacteria is performed by the "replisome": cytoplasmic imaging needed

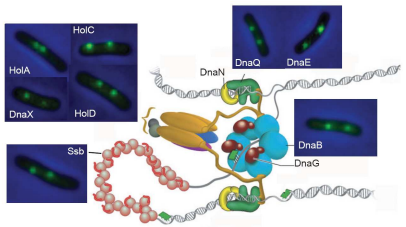
**The replisome is a complex molecular machine bound to the nucleoid in the cytoplasm, and a good example of established bionanotechnology**

**It replicates DNA by copying the leading-strand template continuously and the lagging-strand template discontinuously (in Okazaki fragments).**


**It couples the activities of more than 11 proteins during genome replication**

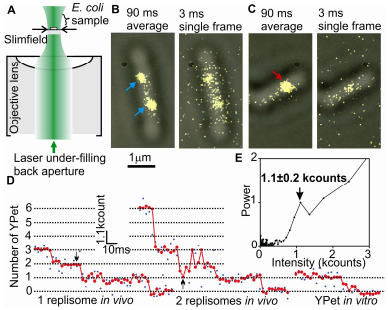
**The sub-units include in effect "sub-machines": primases and helicases, clamp loaders and sliding clamps, DNA polymerases, and components to stabilize single-stranded DNA**

# Using “slimfield” to monitor DNA replication: imaging at the millisecond level




Reyes-Lamothe et al, *Cell* (2008)

Reyes-Lamothe, Sherratt & Leake *Science* 328, 498-501(2010).

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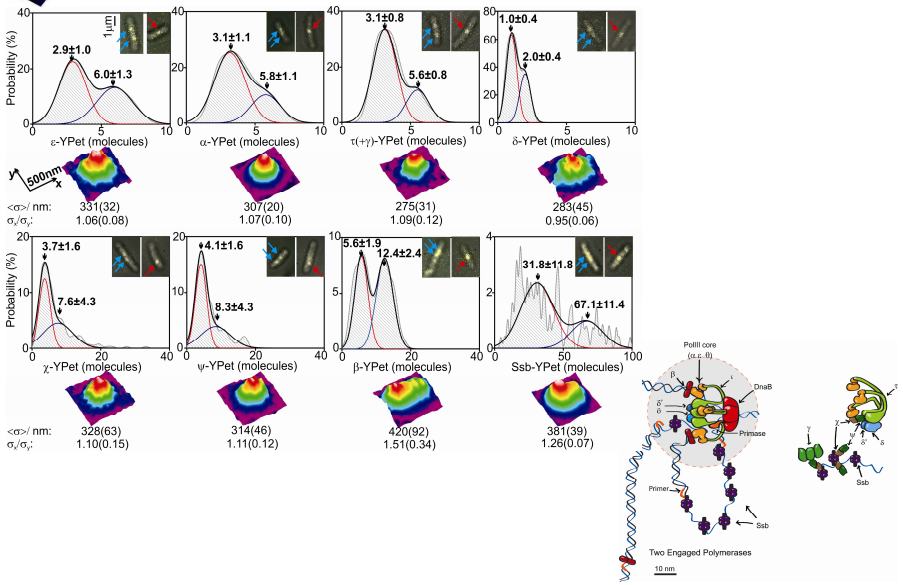
# Stoichiometry and shape in the replisome machine

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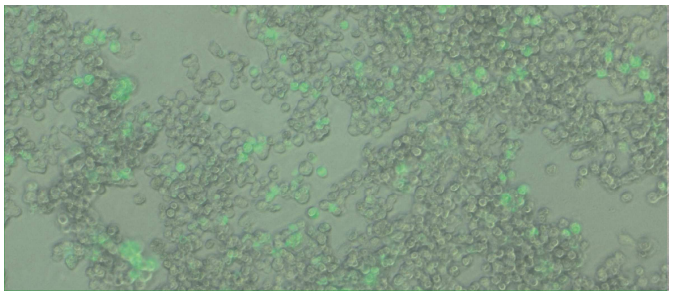
# Future challenges: machines in more complex cells – can we address more directly biomedically relevant examples of established bionanotechnology, or cases where this technology has gone wrong?

**Putting GFP into colorectal cancer cells**



**Tagging a molecular machine called the “EGF receptor” implicated in cancer formation**

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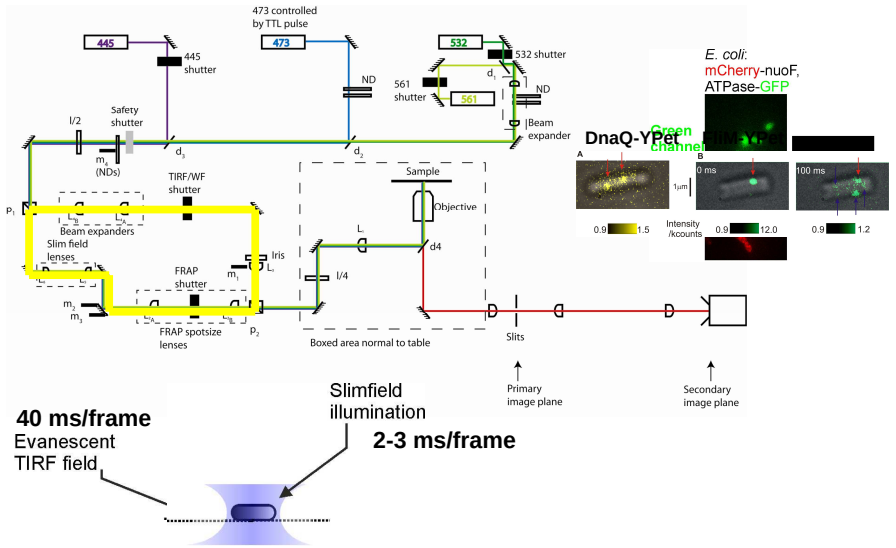
# Future challenges: multi-colour imaging sees different parts of a molecular machine at the same time

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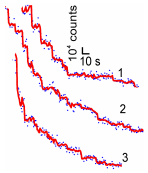
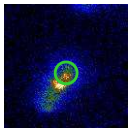
# Conclusions

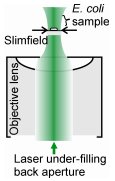
**The optical properties of single fluorescent protein molecules can be used to measure the composition of molecular machines in living cells**




**Molecular mobility and turnover can be measured as it actually happens**



**We can achieve very fast millisecond imaging using simple microscopy modifications, fast enough to follow very fine mechanistic details of these molecular machines**



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