Single molecule total internal reflection fluorescence microscopy (smTIRF):

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MicroSpectroscopy Centre (MSC)

Wageningen, 2013

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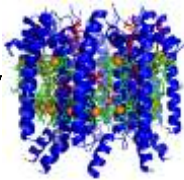
Overview (MicroSpectroscopy Centre)

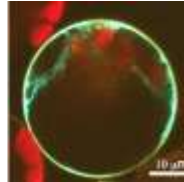
Protein spectroscopy



Techniques:
- Absorption spectroscopy (incl. polarization)
- Ultrafast fluorescence spectroscopy
- **Single molecule spectroscopy**

Typical applications:
- Early events in photosynthesis
- **Protein folding and dynamics**
- **Protein/DNA interactions**

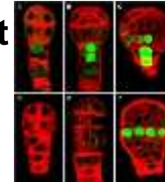
Subcellular imaging



Techniques:
- Fluorescence lifetime imaging
- Fluorescence correlation spectroscopy
- **Total internal reflection fluorescence microscopy**

Typical applications:
- **Protein/protein interactions in cells (FRET)**
- Protein dynamics in cells

Imaging plant tissues



Techniques:
- Confocal laser scanning microscopy
- Multi-photon microscopy combined with lifetime imaging

Typical applications:
- **Imaging plant embryo and root development**
- Imaging photosynthetic activity in intact leaves and algae

Food structure

Techniques:
- Confocal laser scanning microscopy
- Fluorescence spectroscopy

Typical applications:
- Imaging protein and lipid content in dairy products
- Structural modifications in proteins in food processing

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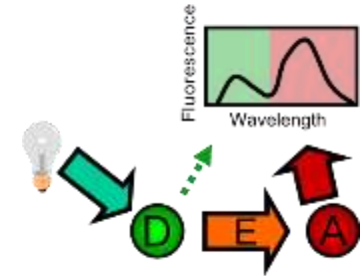
Overview

Why study single molecules?

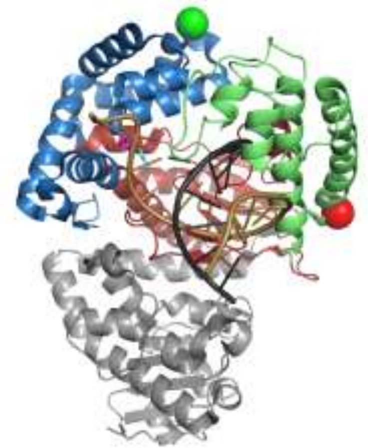
How?

- Total-Internal-Reflection (TIRF) and smFRET
- Super-resolution microscopy: STORM, PALM



What are we currently working on?

- Conformational dynamics of DNA Pol I (*in-vitro*)
- Nanofluidics
- *Arabidopsis Thaliana* (*in-vivo*)



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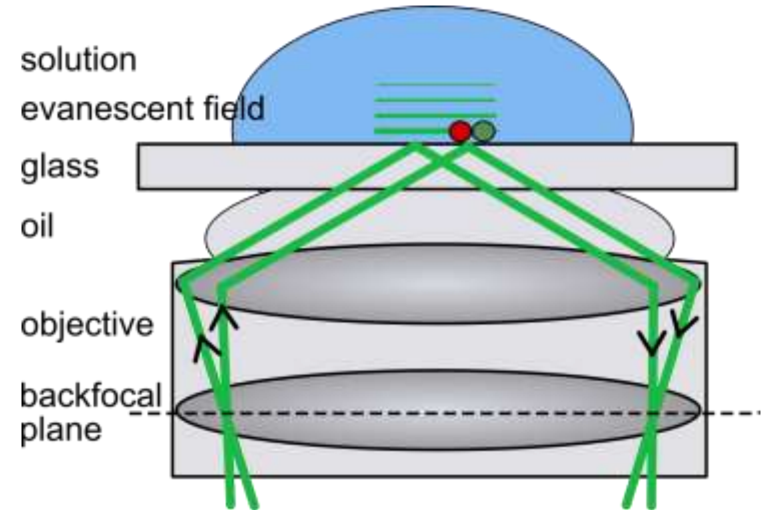
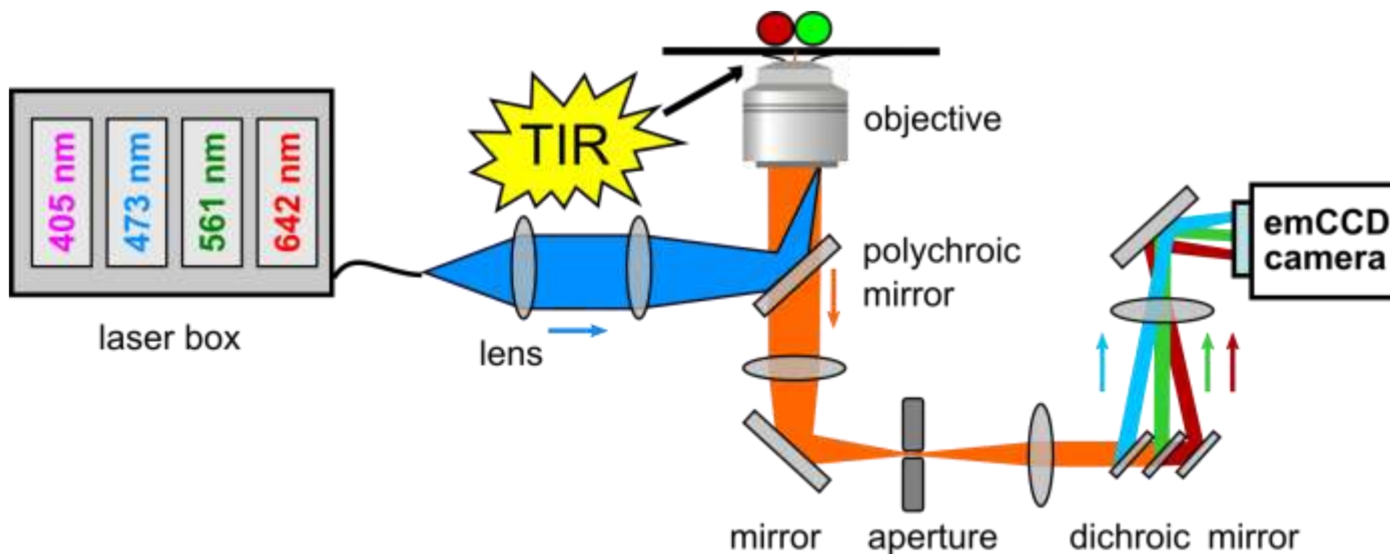
Total-Internal-Reflection Microscopy

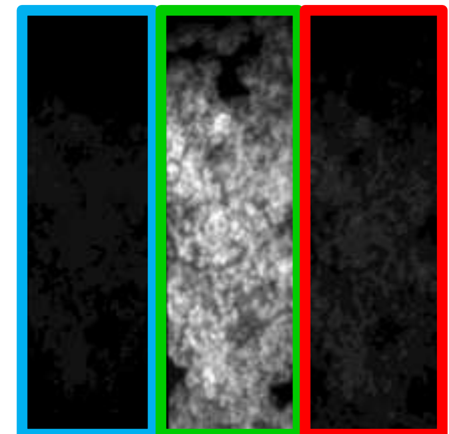
Experimental requirements

- Observation of single molecules
- Detection of many molecules in parallel



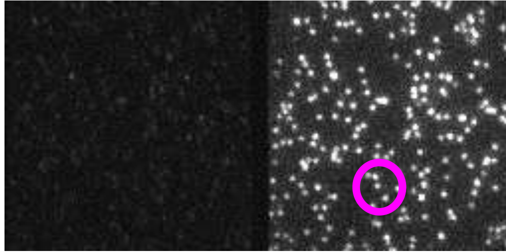
TIRF: intensity of the evanescent field decays exponentially within ~100 nm!



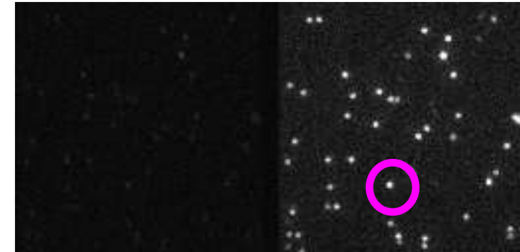


Stability of single fluorophores (here ALEX mode)

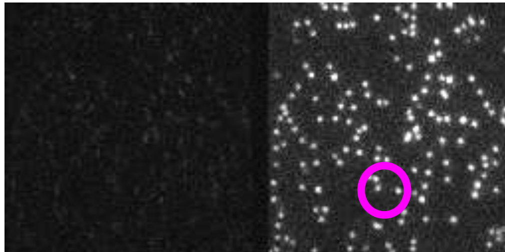
0 minutes



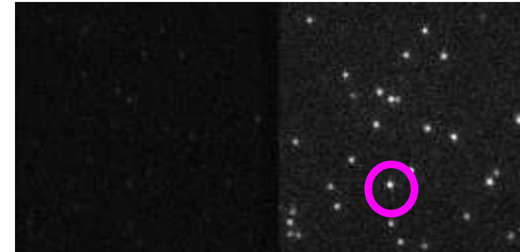
28 minutes



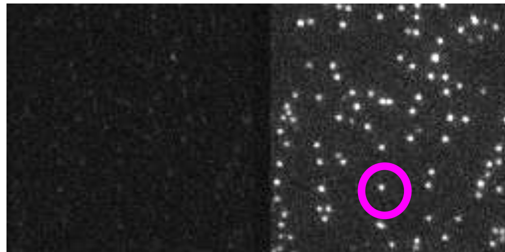
4 minutes



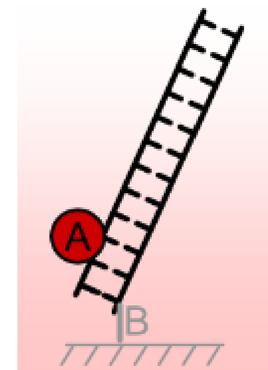
36 minutes



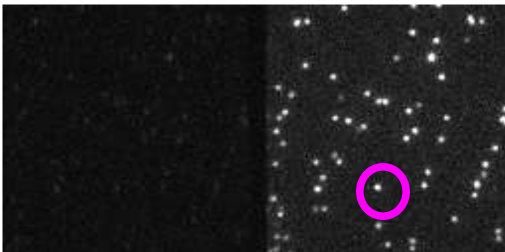
12 minutes



ATTO647N: quite stable…

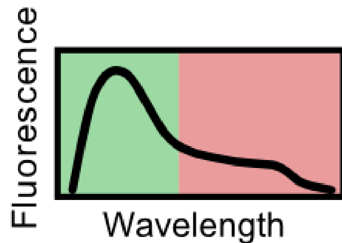


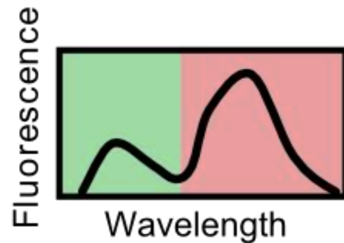
20 minutes

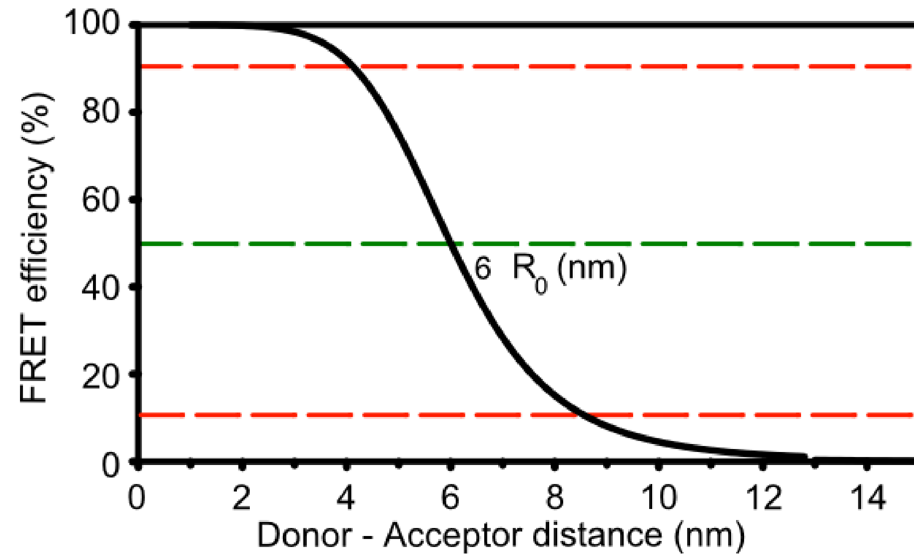


Foerster Resonance Energy Transfer (FRET)...

FRET: Distance-dependent energy transfer from a donor fluorophore (D) to an acceptor fluorophore (A)







$$E = \frac{R_0^6}{R_0^6 + R^6}$$

R **= D-A distance**

$\boldsymbol{R_0}$ **= Förster radius**

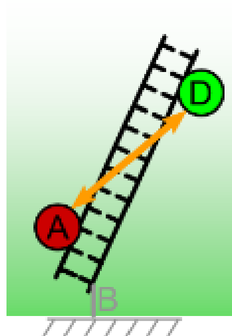
(D-A distance where *E* = 50%)

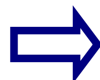
Foerster, Anal. Physik, 1948

Foerster Resonance Energy Transfer (FRET)

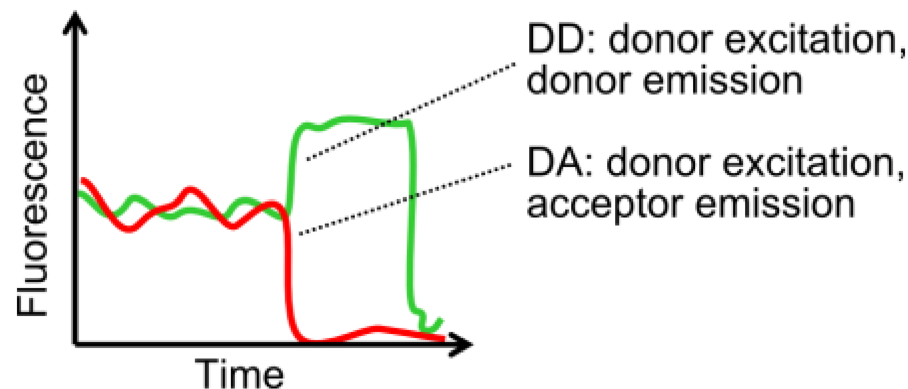
SmFRET on immobilised molecules

Many particles | Many frames | Time traces

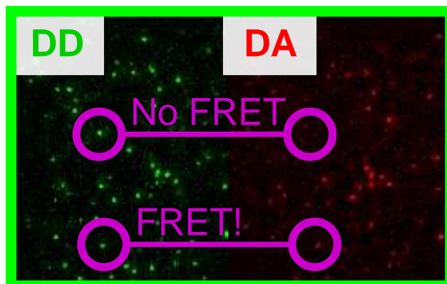




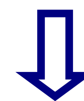
Data extraction



Green excitation



Green Channel **Red Channel**



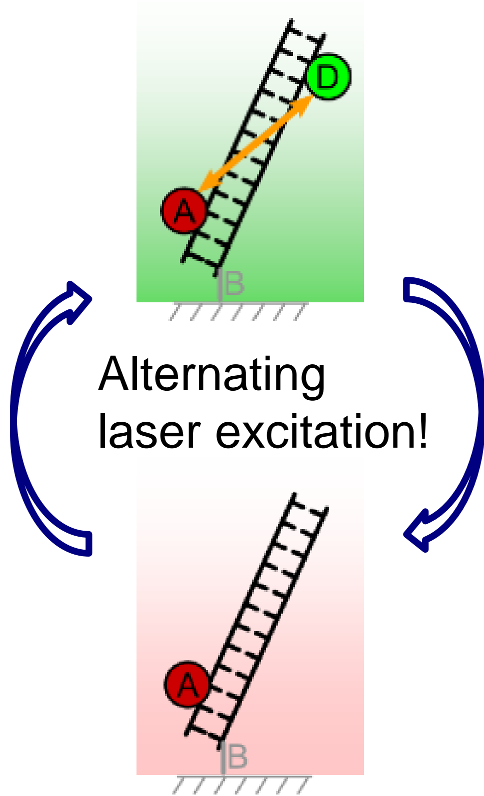
Analysis

$$E^* = \frac{DA}{DD + DA}$$

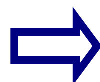
Alternating-laser excitation (ALEX)

SmFRET on immobilised molecules

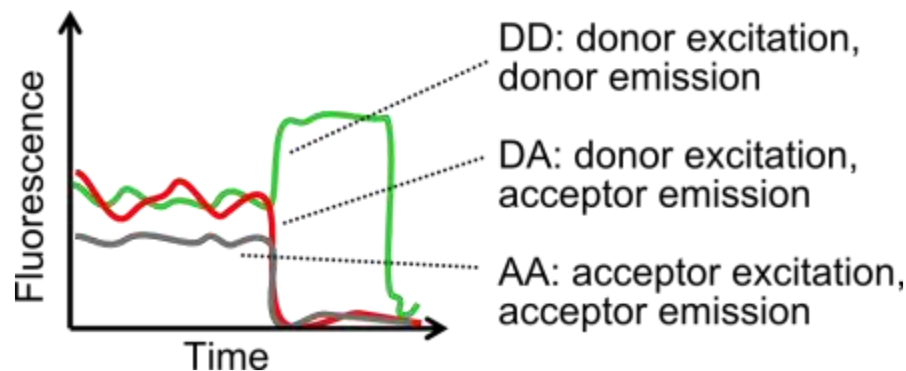
Many particles | Many frames | Time traces

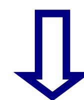


Alternating laser excitation!



Data extraction





Analysis

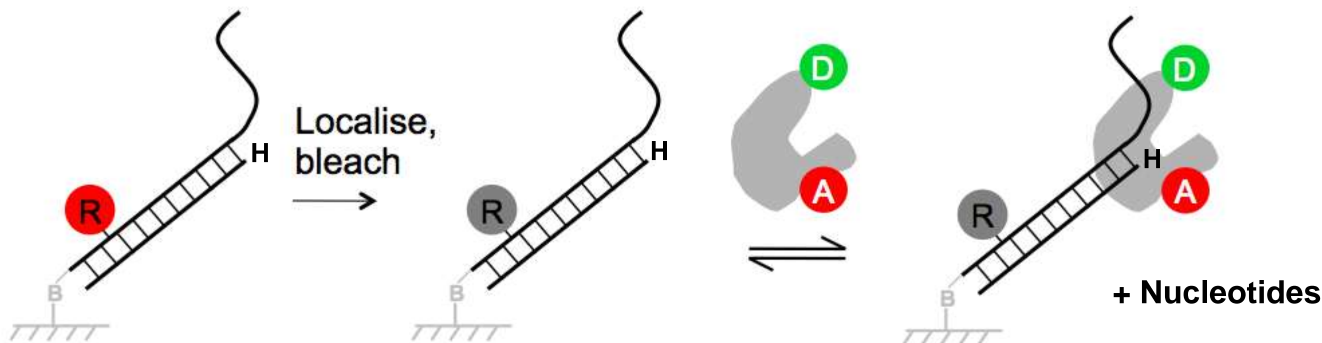
$$E^* = \frac{DA}{DD + DA}$$

$$S = \frac{DD + DA}{DD + DA + AA}$$

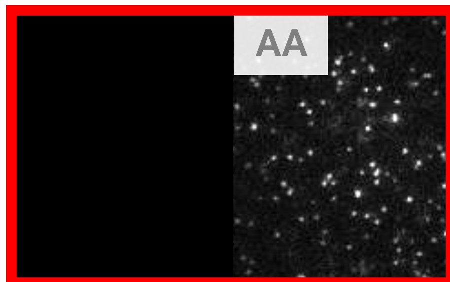
Kapanidis et al., PNAS, 2004

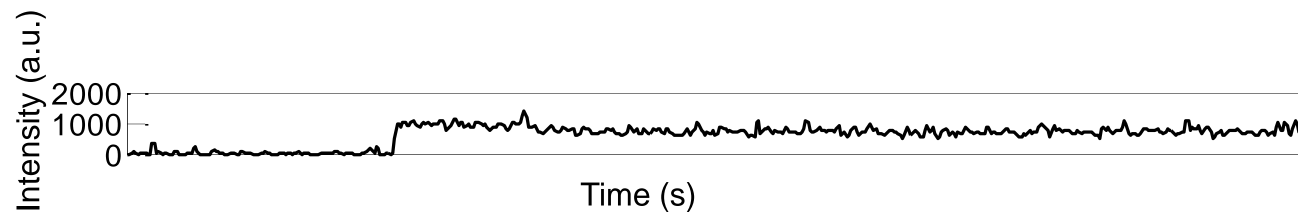
Surface immobilisation and TIRF

Oxford

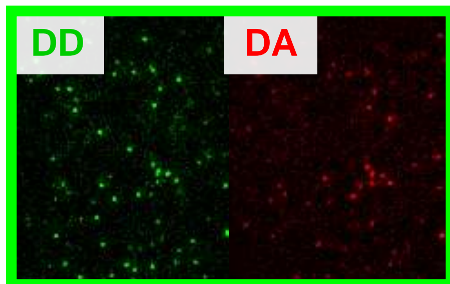


Red excitation

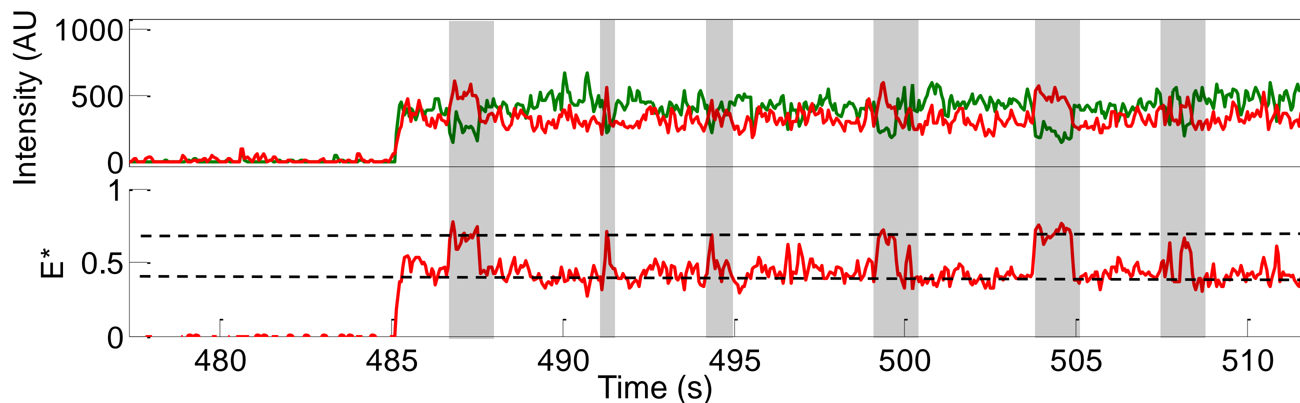




Green excitation



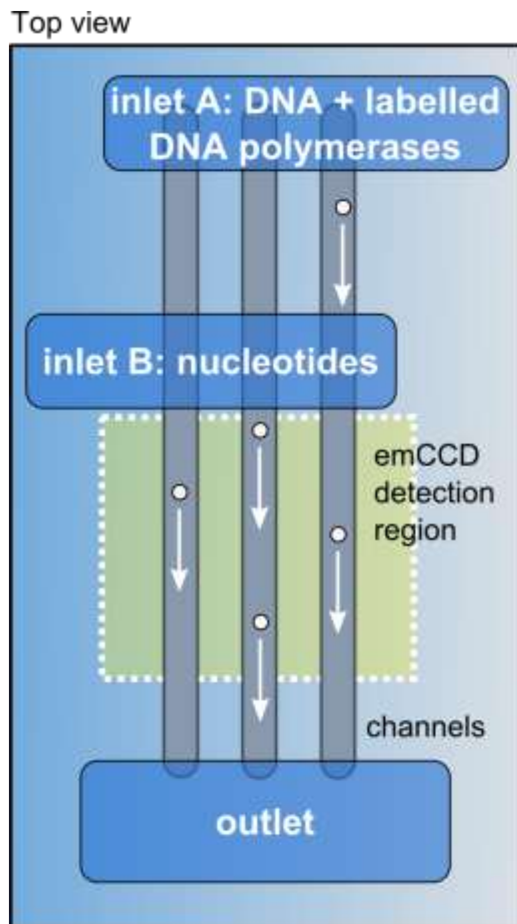
Green Channel **Red Channel**

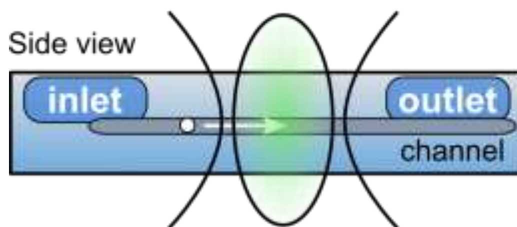


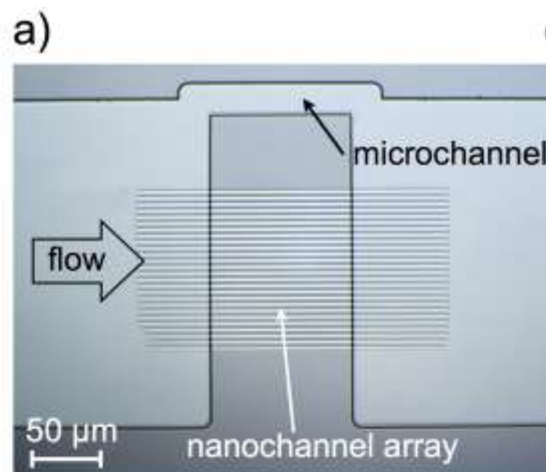
Evans, JH et al., *in prep.*

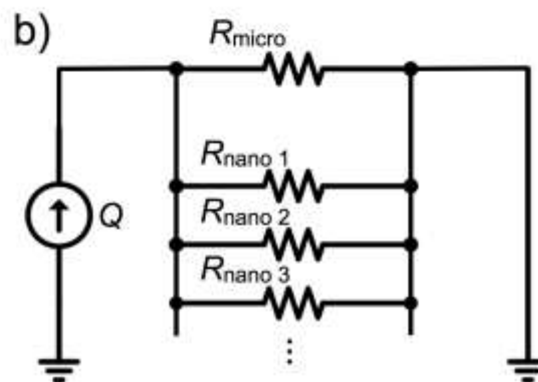
Nanofluidics (with Klaus Mathwig, University of Twente)

Twente





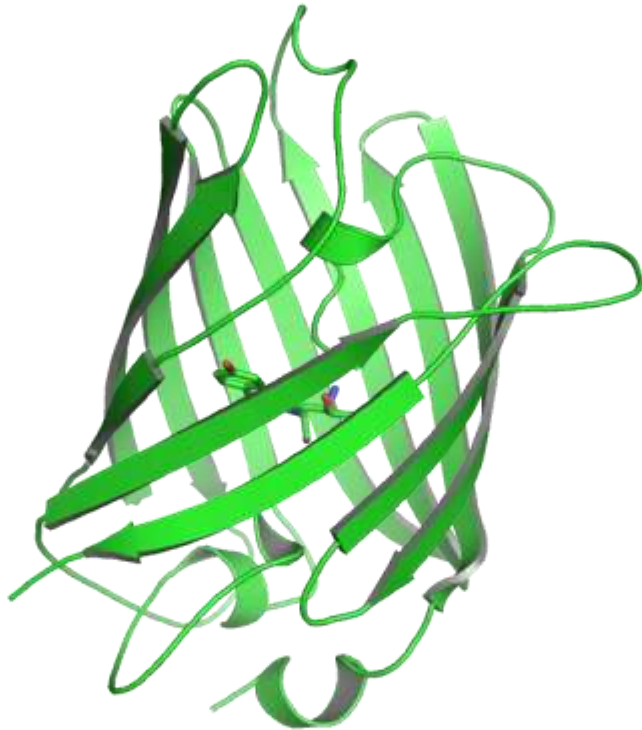


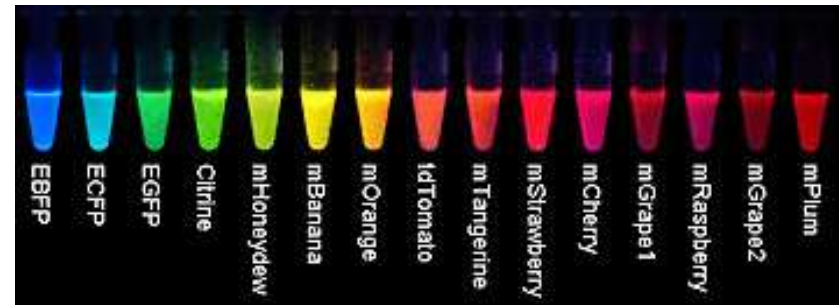


Imaging Microscopy

Green fluorescent protein and its variants

- Genetically encodeable fluorophore
- Many variants available (colour, photo-activation,...)





Ormoe et al, Science, 1996

Tsien lab

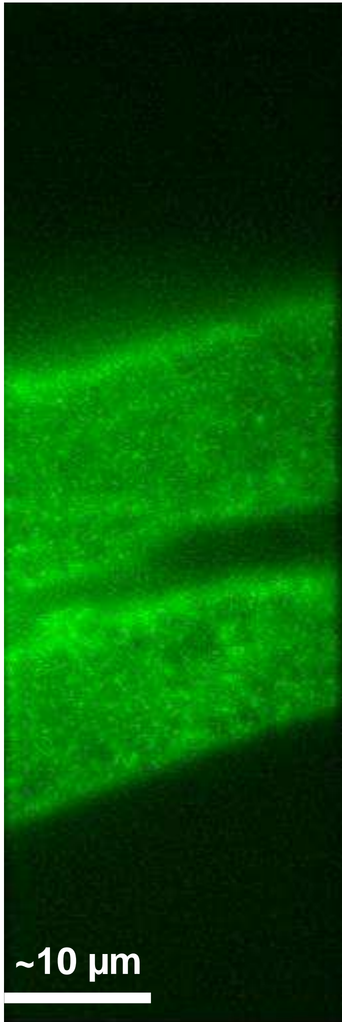
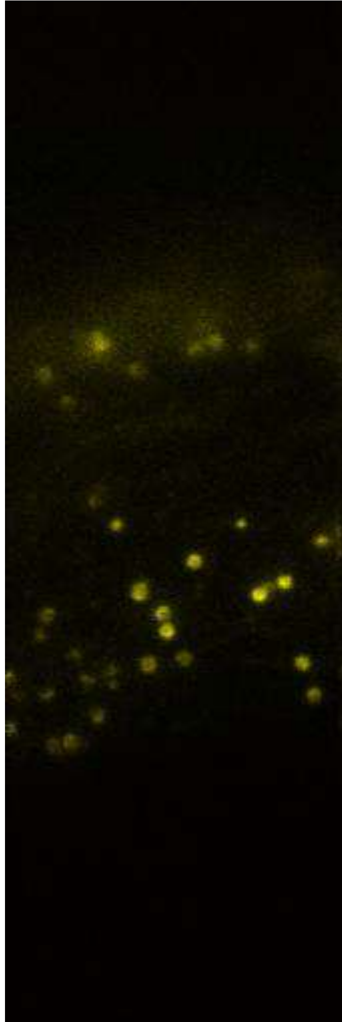
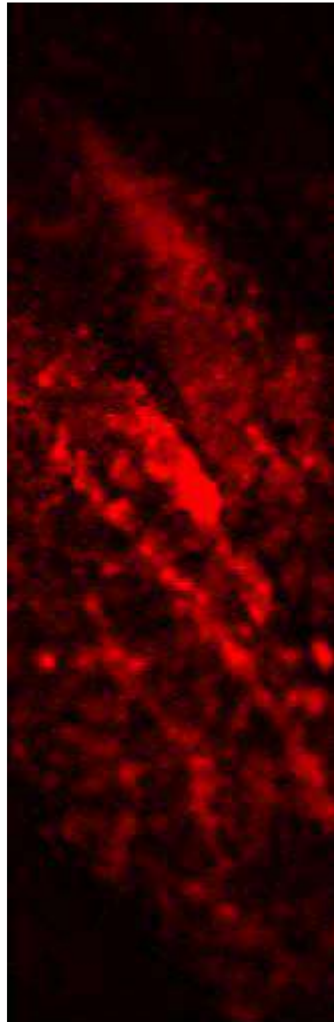
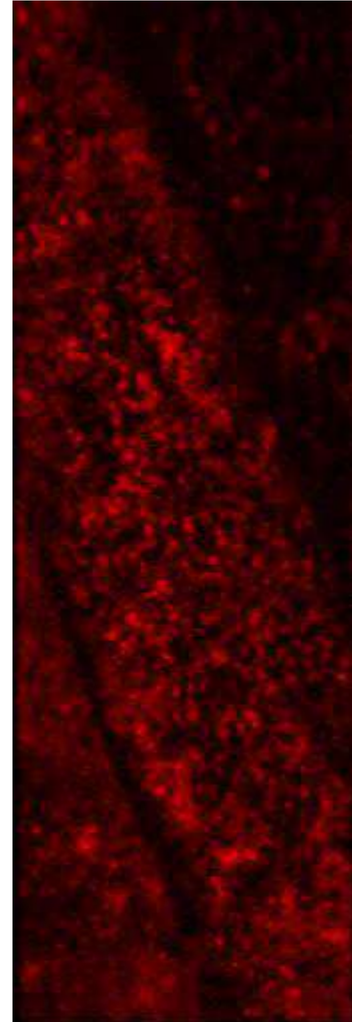
Arabidopsis thaliana (*in-vivo*)

Wageningen

~40 µm

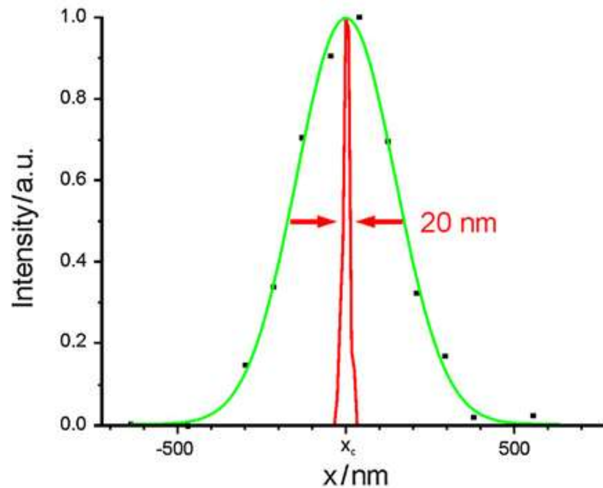
Early and late endosomes tagged with RFP

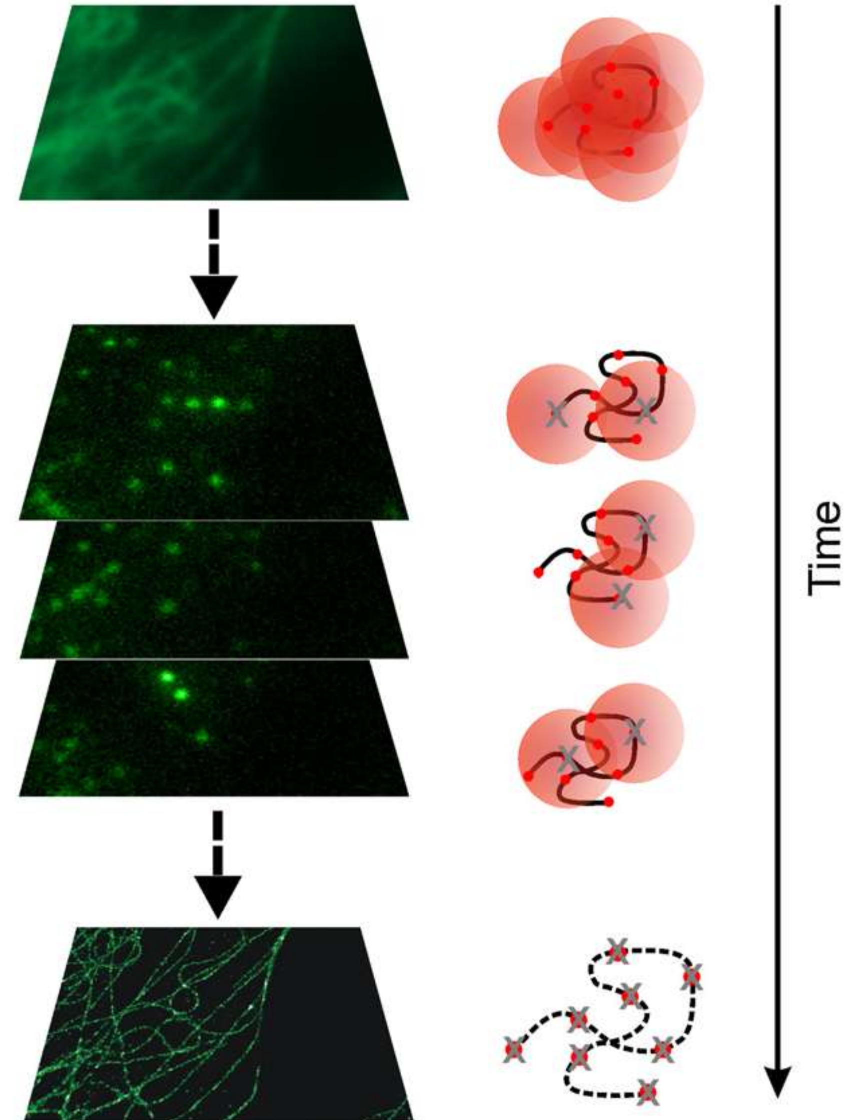
Arabidopsis thaliana (*in-vivo*)

Plasma Membrane	Golgi	ER	Early and late endosomes	Serk3-mCherry (PM)
				
static	moves slowly	dynamic	moving along lines	mainly static

Super-resolution Microscopy (STORM/PALM)

- To resolve structures < $\lambda/2$
- Either by blinking organic fluorophores (dSTORM) or by using fluorescent proteins

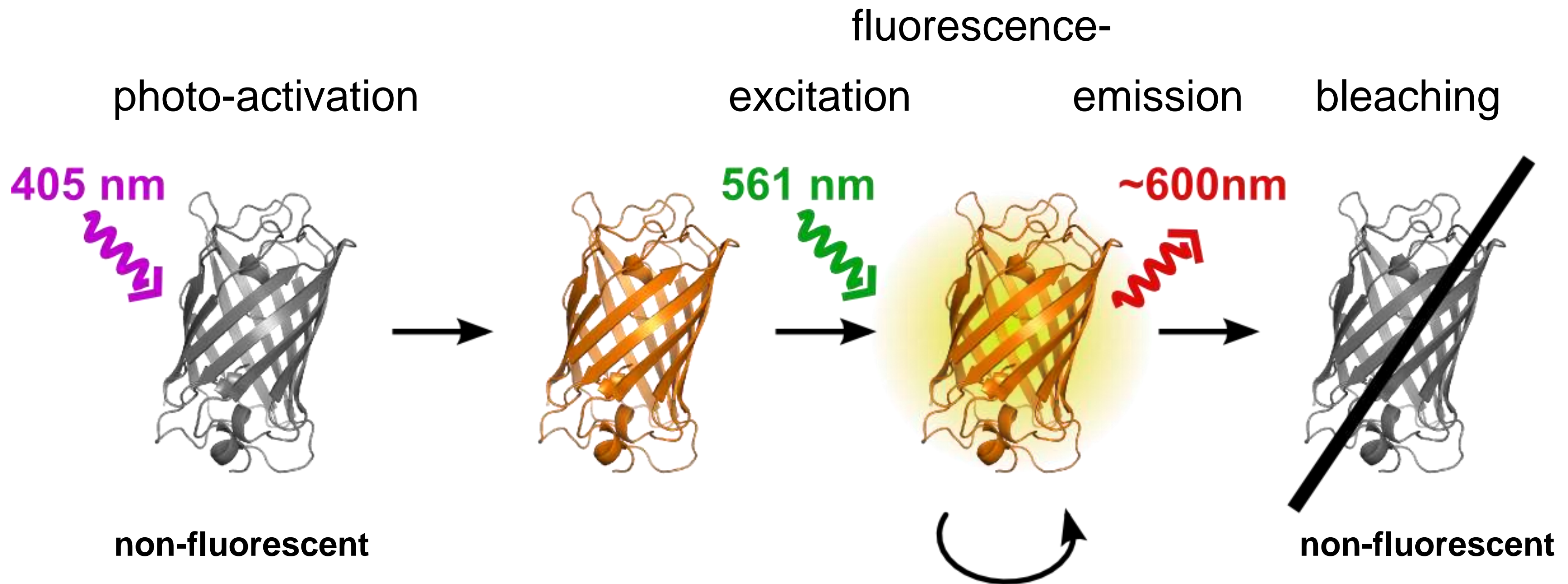




⇨ increase in resolution by a factor of ~10 is achievable!

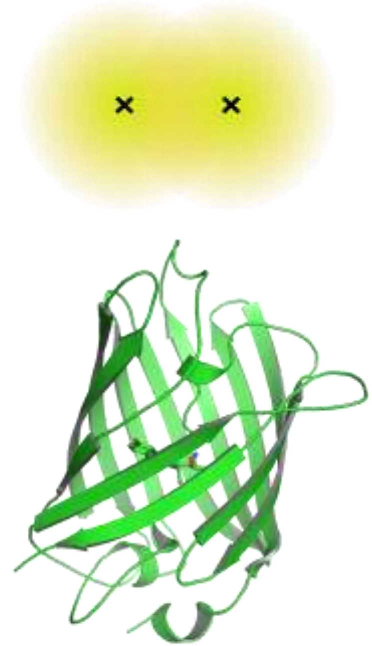
Super-resolution Microscopy (PALM)

Photo-activation turns non-fluorescent protein into a fluorescent one



Sumarry imaging

- Simultaneous multi-colour detection for studying (dynamic) interactions *in-vivo*
- Photoactivatable proteins will provide structural information with ~20-50 nm resolution



Thank you for you attention!

Oxford:	Dr. Tim Craggs, Dr. Louise Aigrain, Geraint Evans, Dr. Achillefs Kapanidis
Yale:	Dr. Catherine Joyce, Prof. Dr. Nigel Grindley
Twente:	Prof. Dr. Serge Lemay, Dr. Klaus Mathwig
Wageningen:	Shazia Farooq, Stefan Hutten, Stef van der Krieken, Members of the Laboratory of Biophysics
Wageningen:	Prof. Dr. Dolf Weijers, Prof. Dr. Sacco de Vries, Members of the Laboratory of Biochemistry