Monitoring protein dynamics and interactions in living cells

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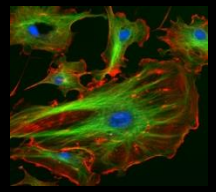
OIC Course Functional Imaging and Super Resolution Rotterdam 2026

Microspectroscopy

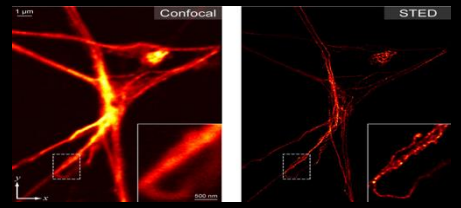
Application of fluorescence spectroscopy on microscopic objects

- Fluorescence imaging

  Confocal microscopy



  PALM, Storm, STED



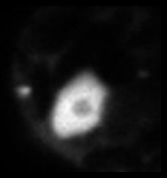
- Dynamics

  FCS, FRAP

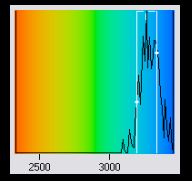


- Visualization and quantification of biomolecular interactions

  FRET -FLIM

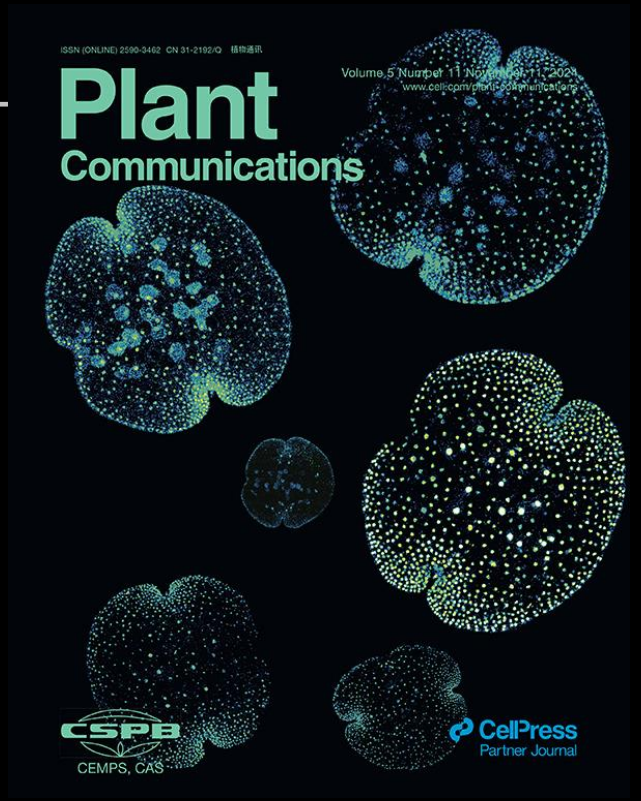






Quantitative imaging approaches

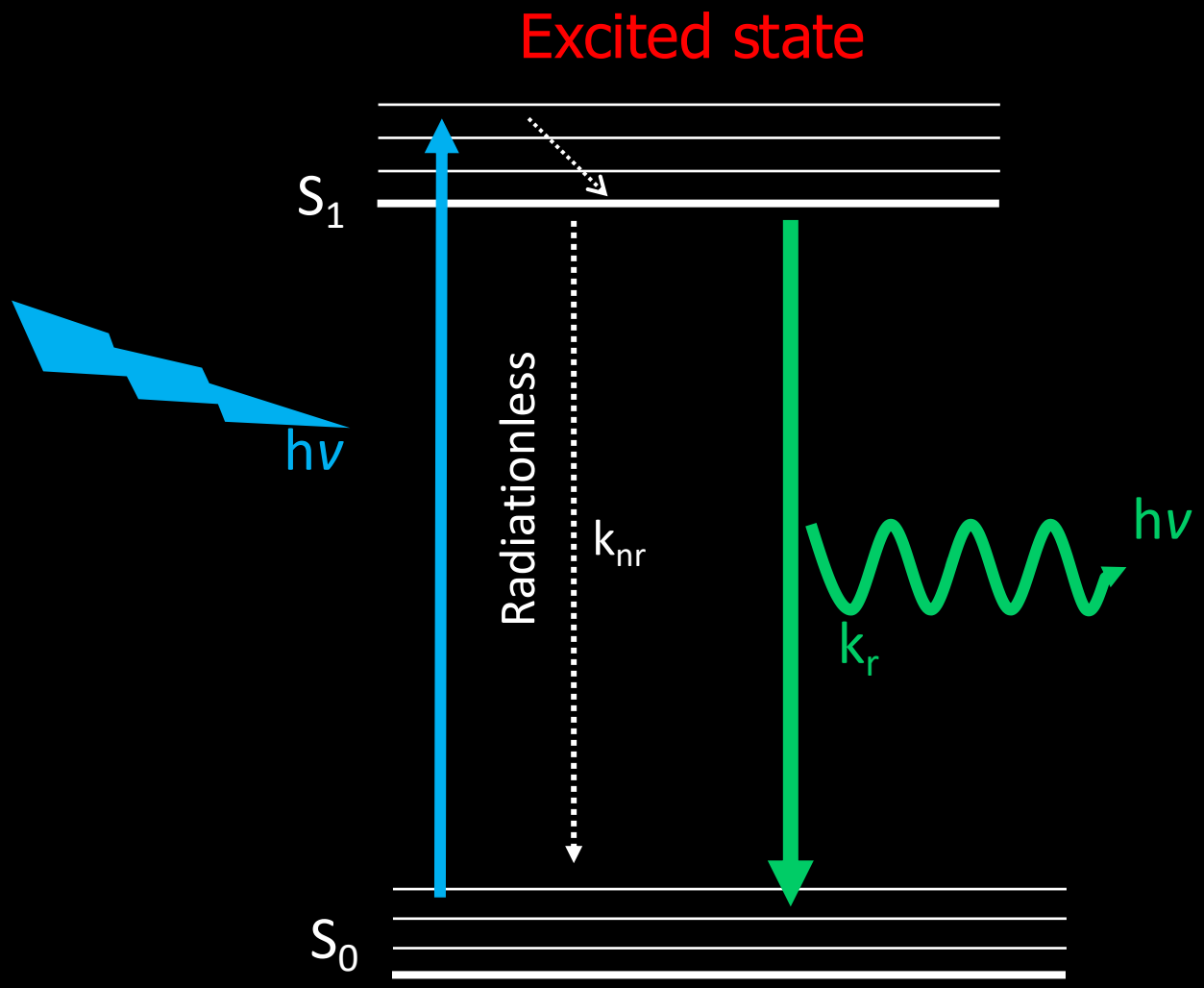
Confocal imaging photon counting mode



Fluorescence lifetime Imaging (FLIM)



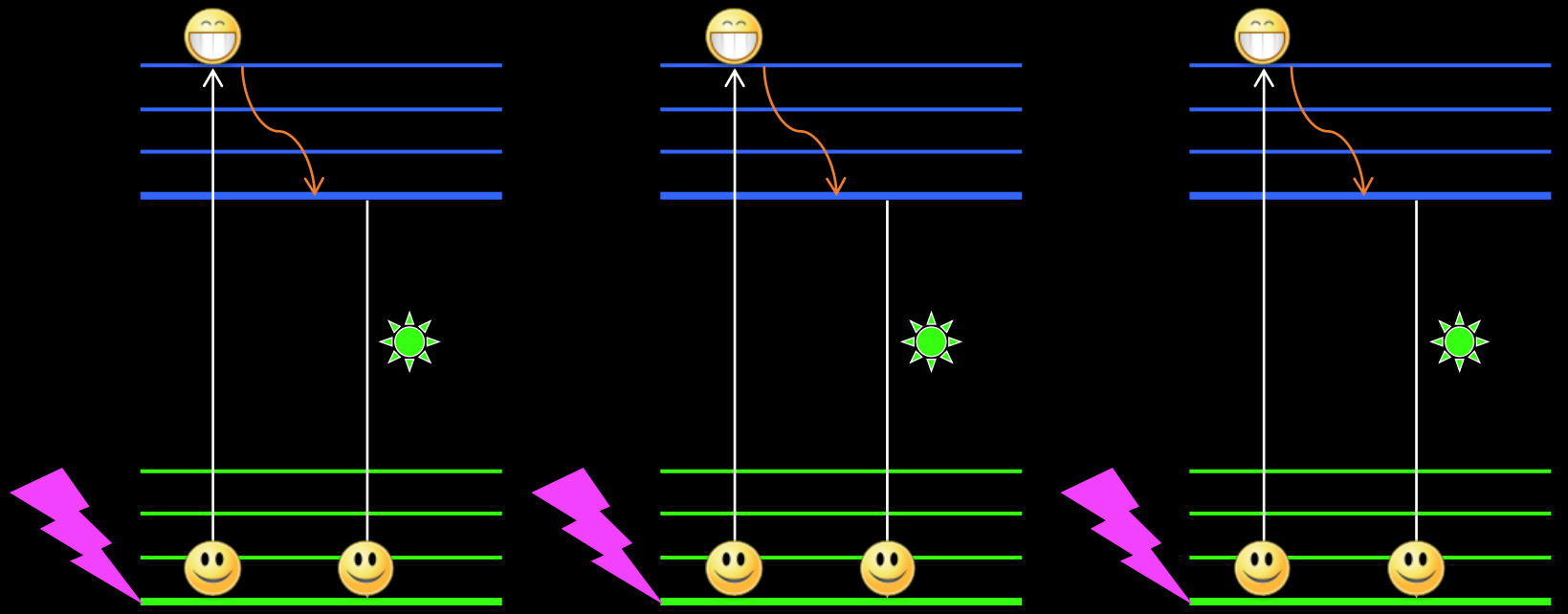
What is a fluorescence lifetime?



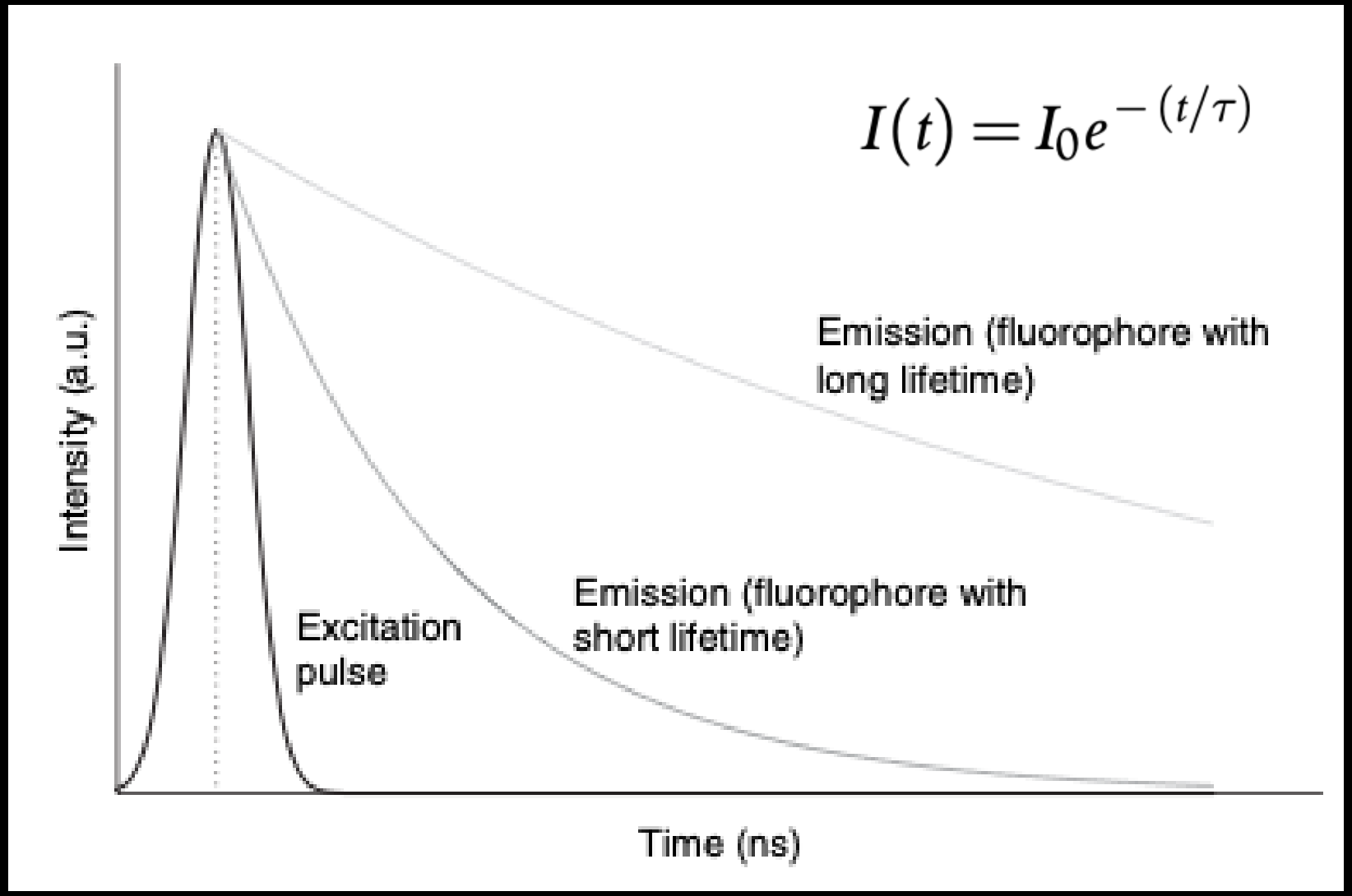
Average time a fluorophore remains in the excited state before returning to the ground state by emitting a photon

Fluorescence lifetime

Relaxation of an excited molecule takes place with a certain probability based on the radiative and non-radiative decay rates



Fluorescence lifetime



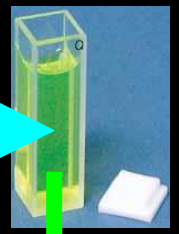
Fluorescence Lifetime

- ✓ Absolute measure, fluorescence intensity is a relative quantity
- ✓ Intrinsic property of fluorophore
- ✓ Independent of probe concentration or laser excitation intensity
- ✓ Sensitive to the environment
  - viscosity, pH, polarity, solvation
  - size and shape of molecules
  - molecular interactions

Time-Correlated Single Photon Counting



Pulsed laser



Sample



Photomultiplier



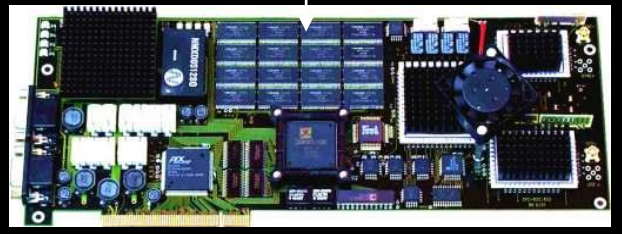
Photodiode

Timer



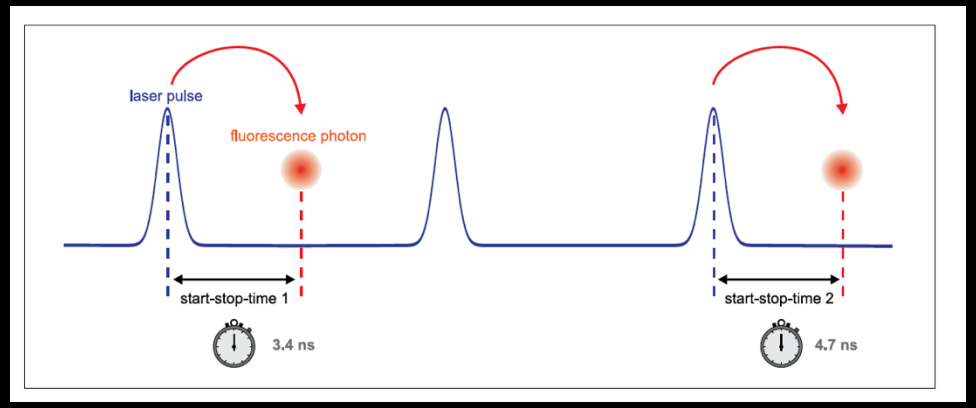
start

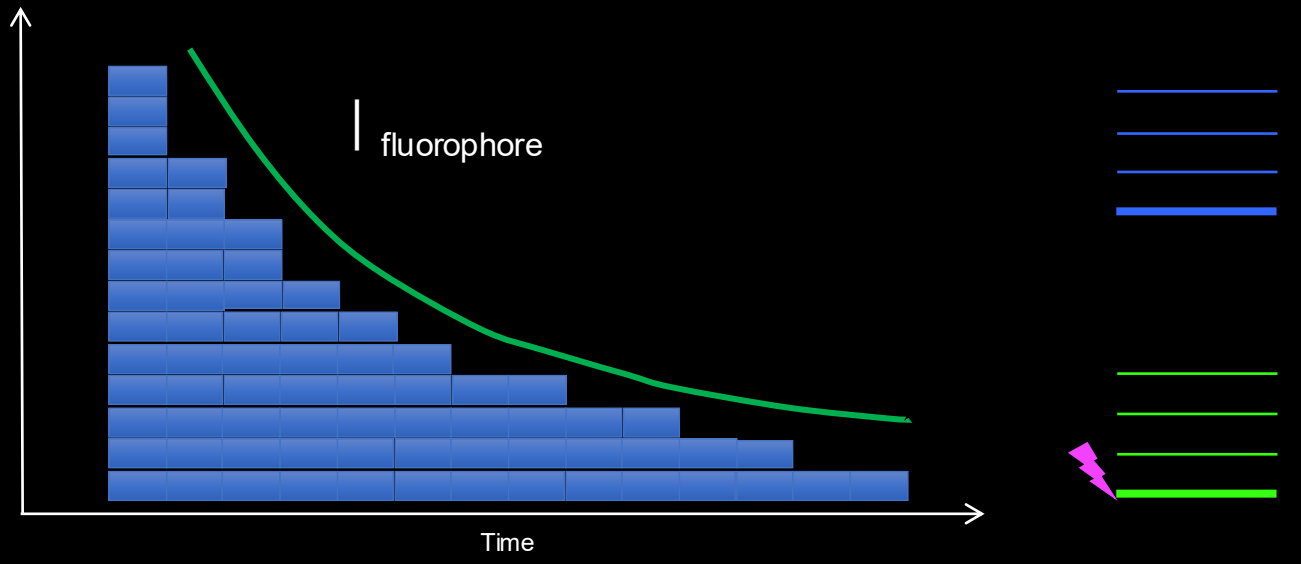
stop



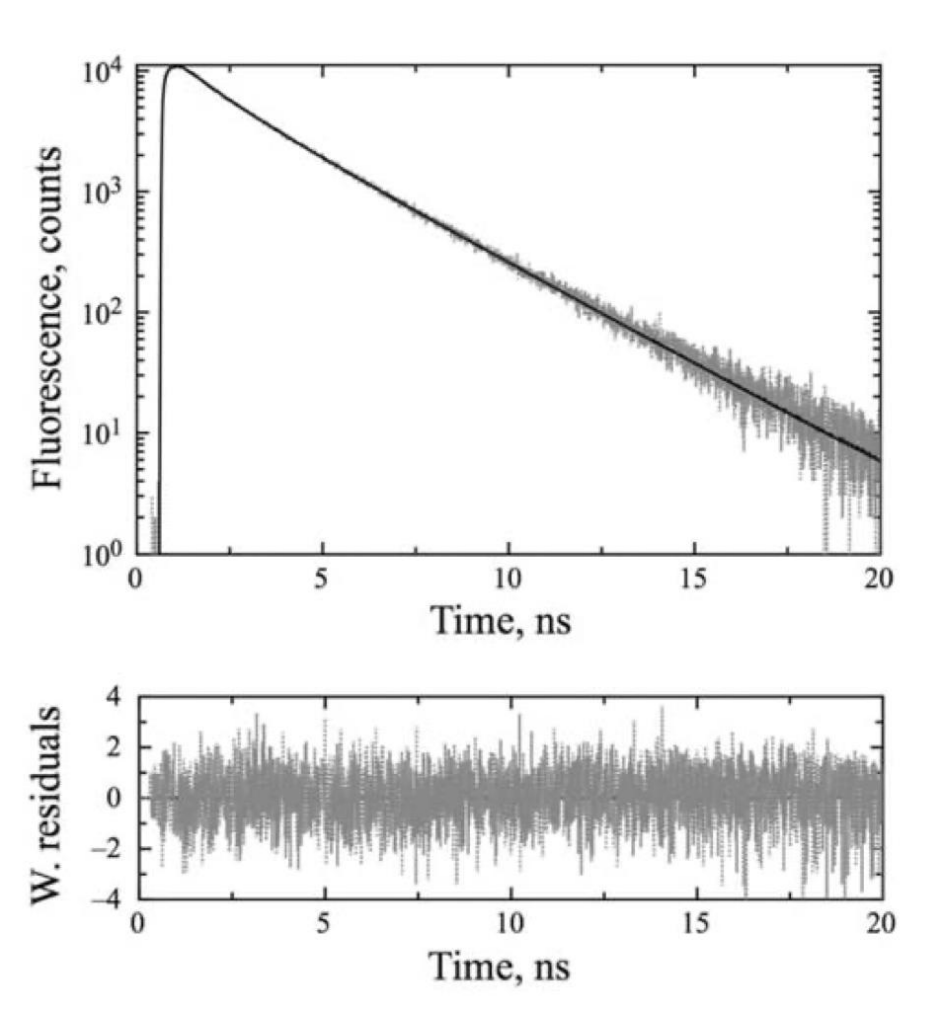
Photon counting card

Counting photons; fluorescence lifetime





Fluorescence decay curve of EGFP

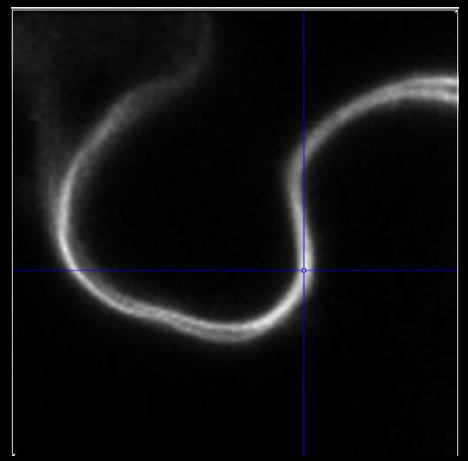


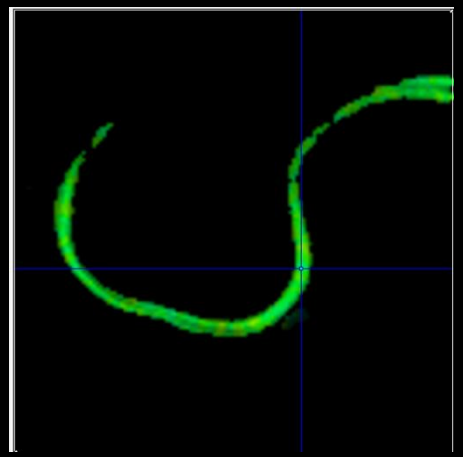
Tau: 2.7 ns

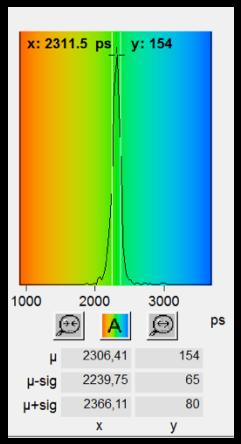
Visser *et al* 2010 Eur Biophys J

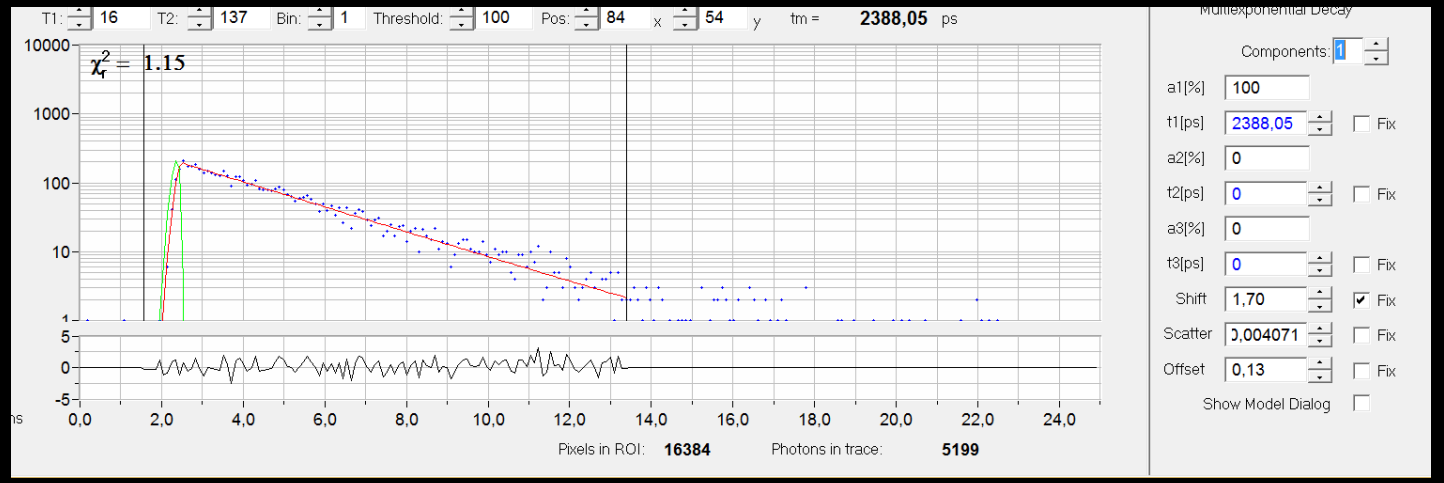
Fluorescence lifetime imaging (FLIM)

Fluorescence lifetime image is based on differences in excited state decay rates from a fluorescent sample.

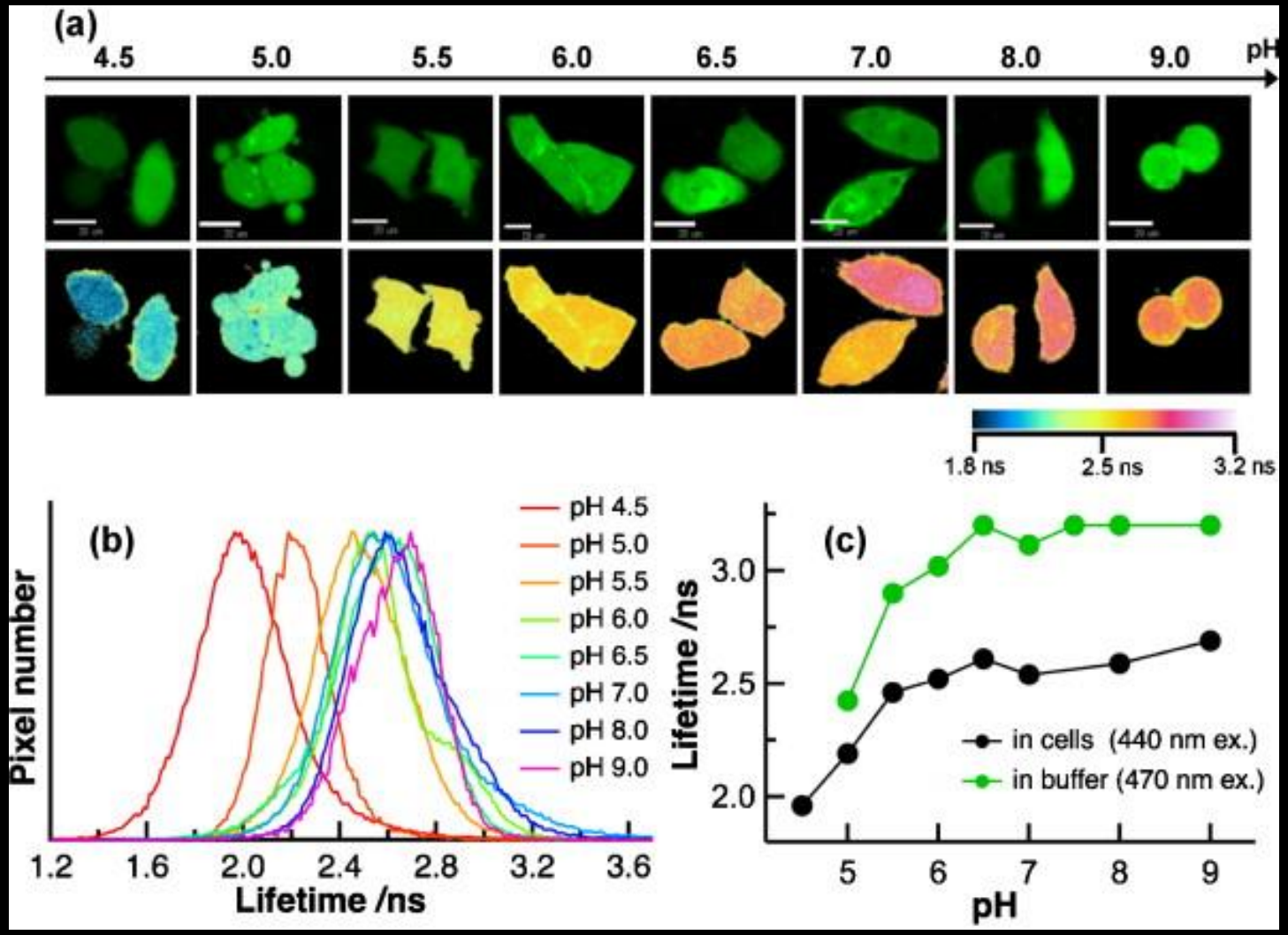






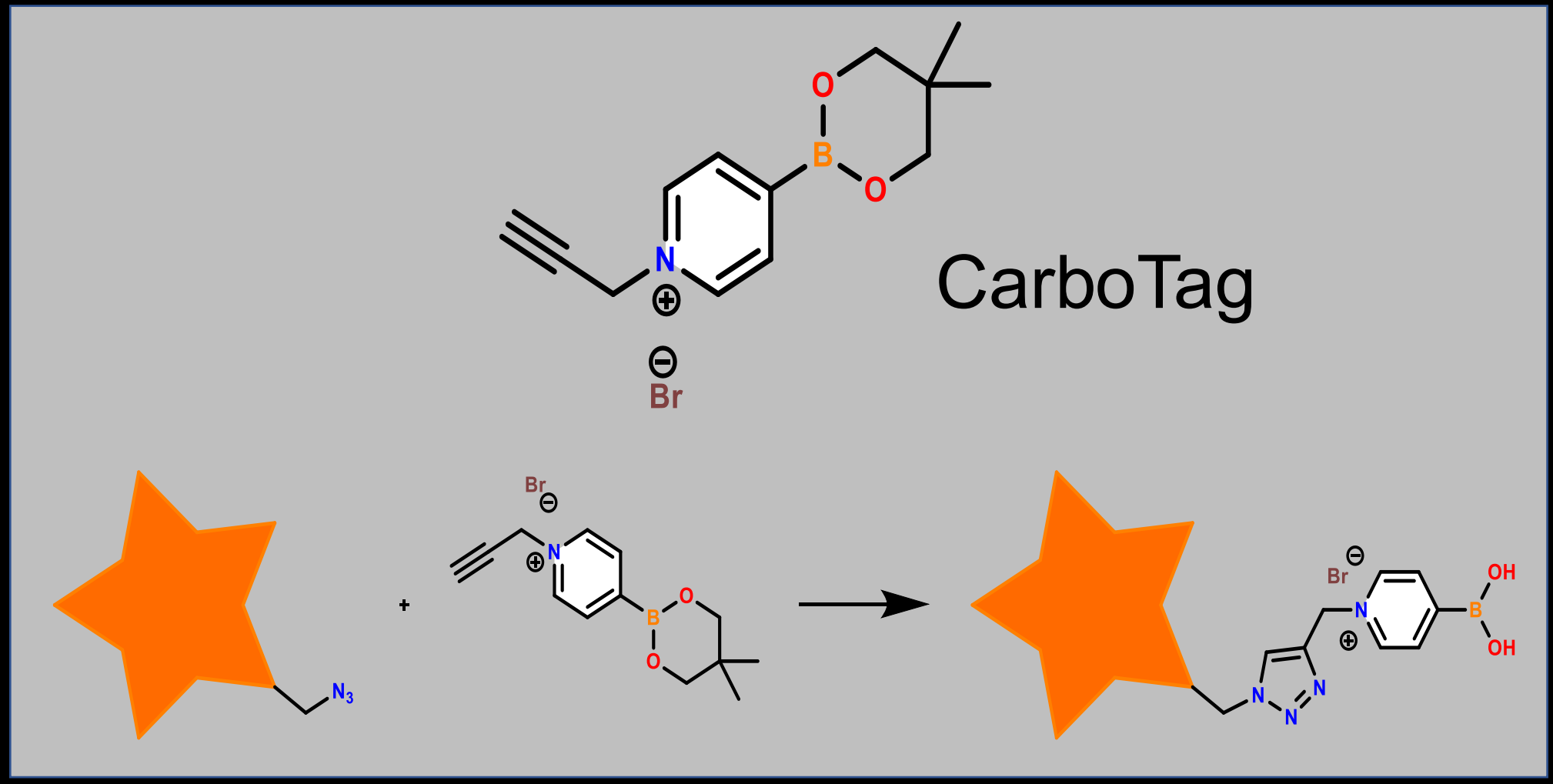


Monitoring pH in live cells



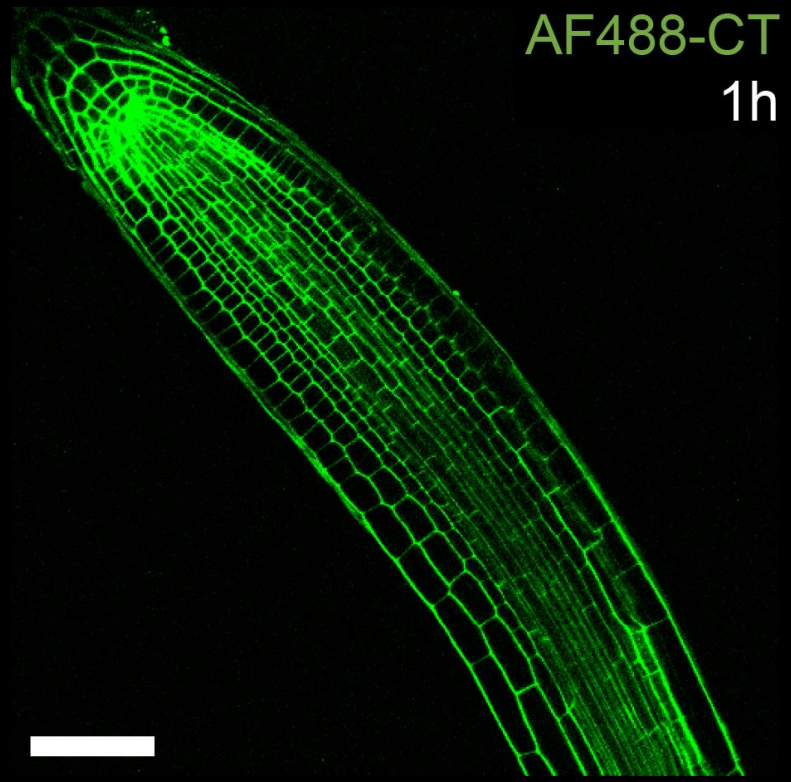
Nakabayashia J. Photochem.Photobiol. 235 (2012) 65– 71

A cell wall targeting boronic acid: CarboTag

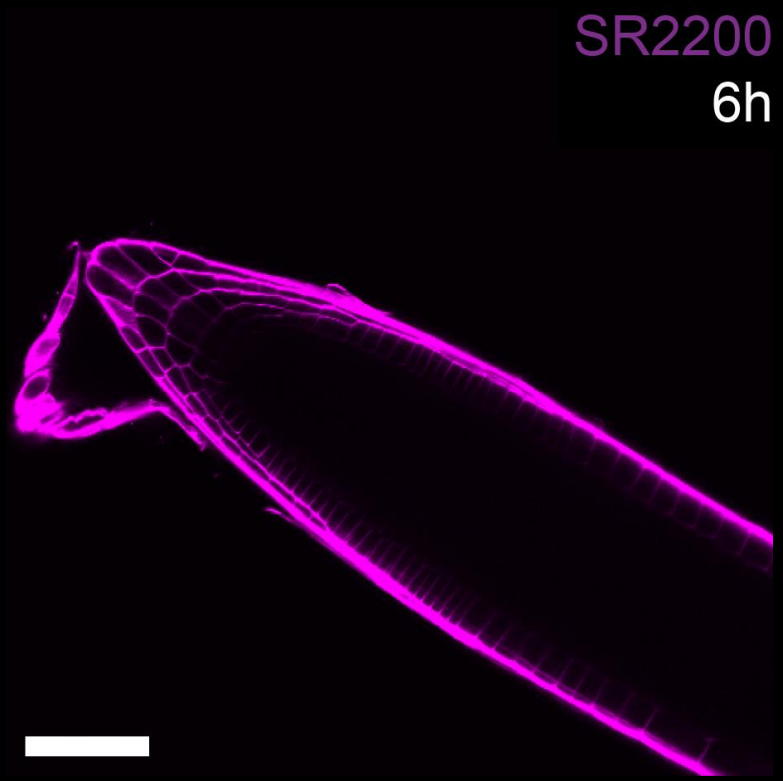


CarboTag excellent target for live imaging

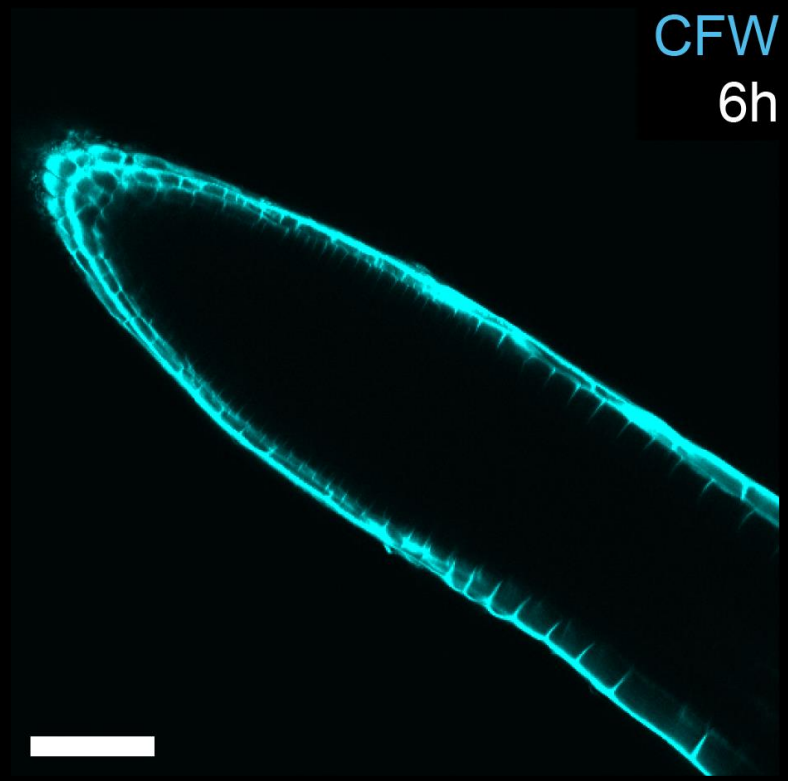
Alexafluor488-CarboTag



Renaissance 2200

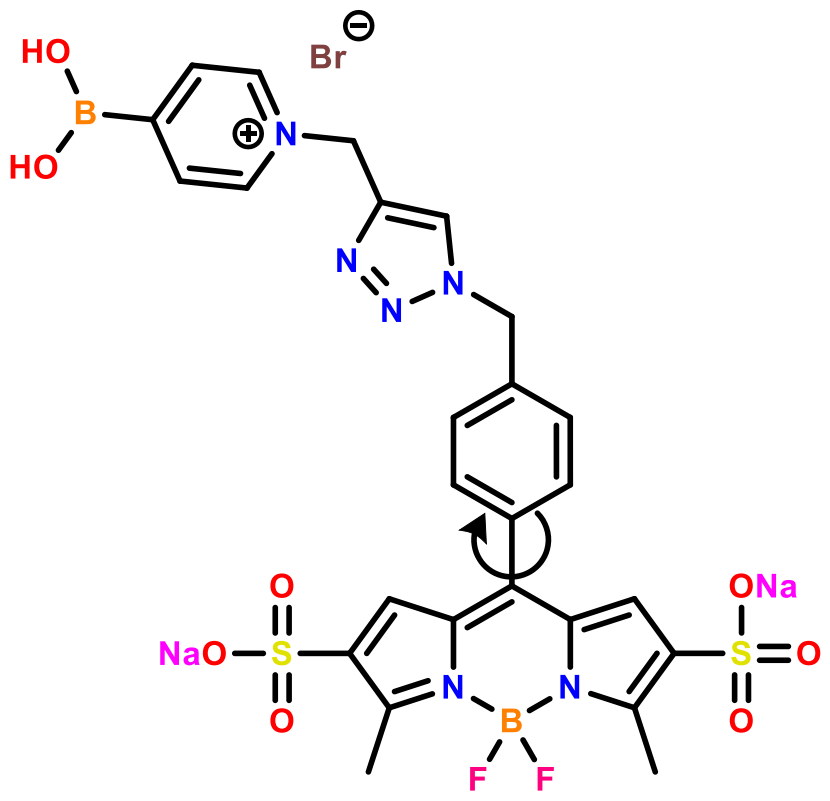


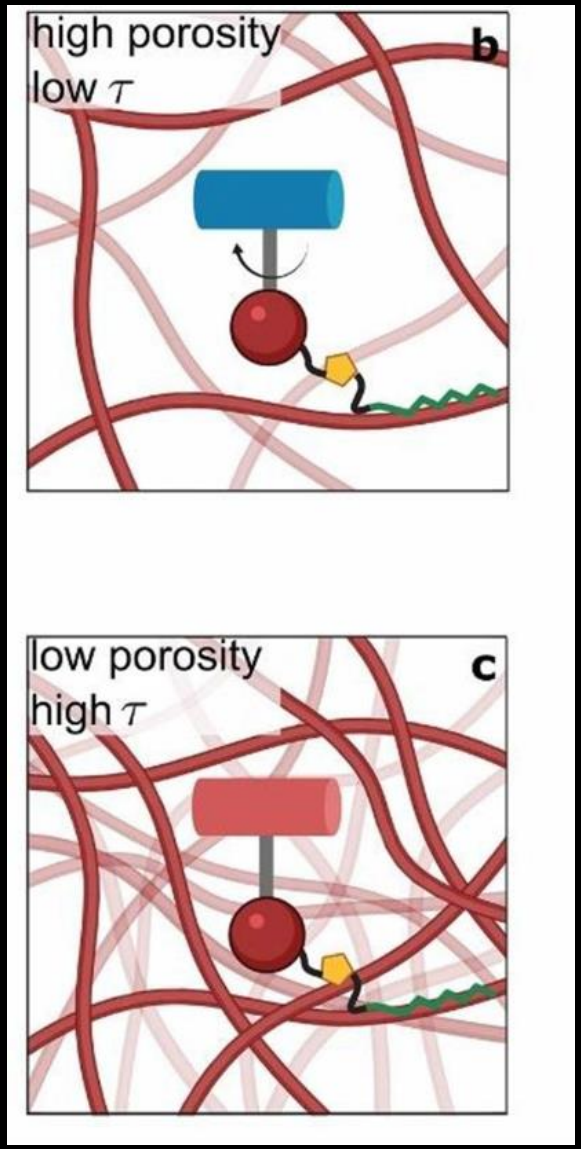
Calcofluor white



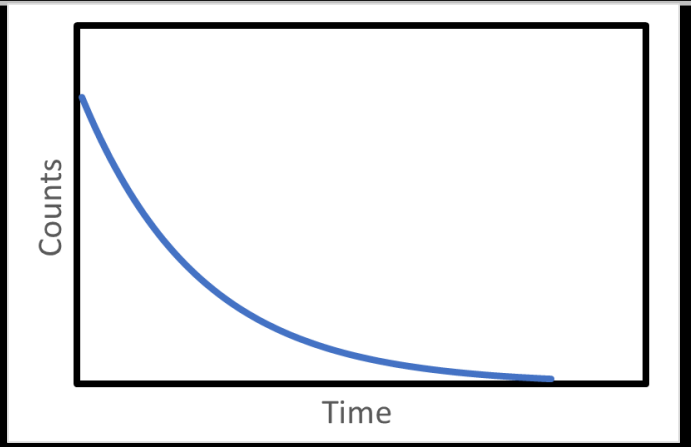
Scale bar: 50 um

Cell wall porosity probe

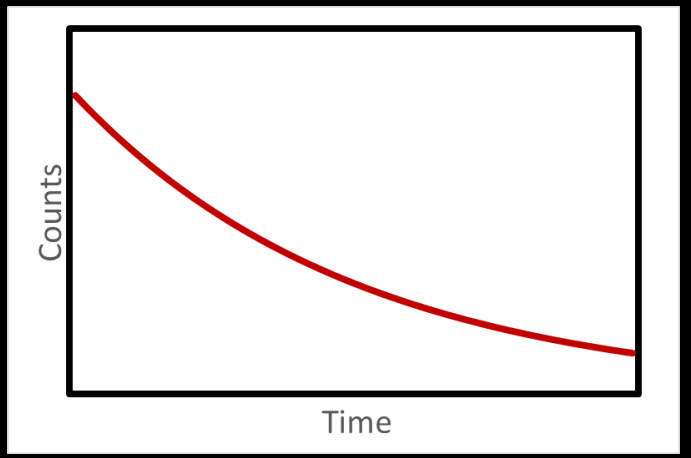




Fast decay



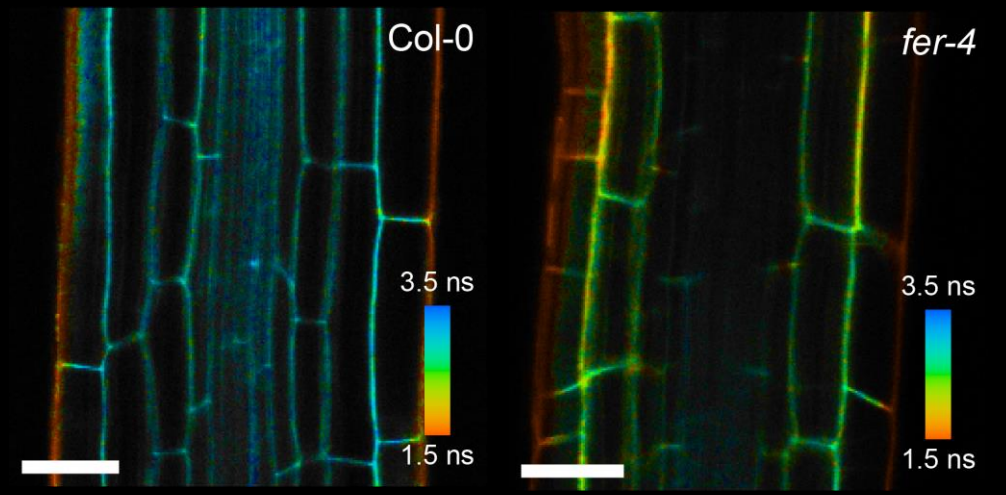
Longer lifetimes possible

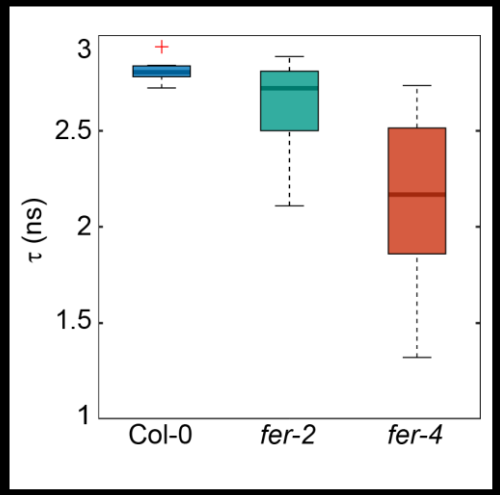


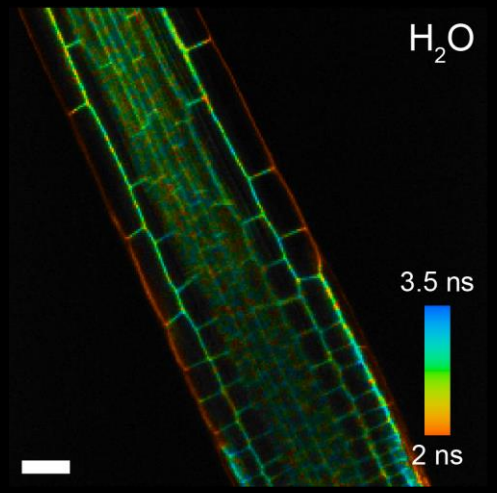
Bronkhorst & Michels et al, the Cell Surface, 2022

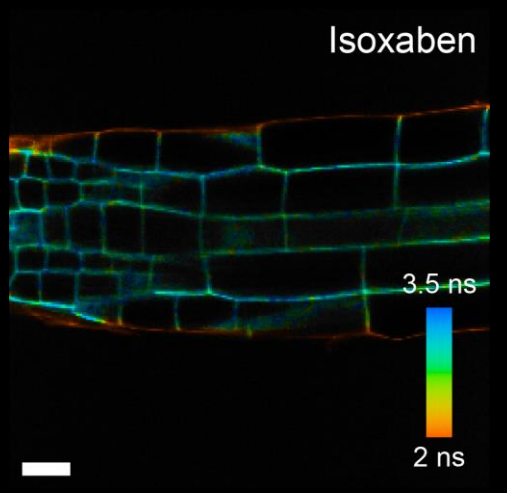
Cell wall porosity probe

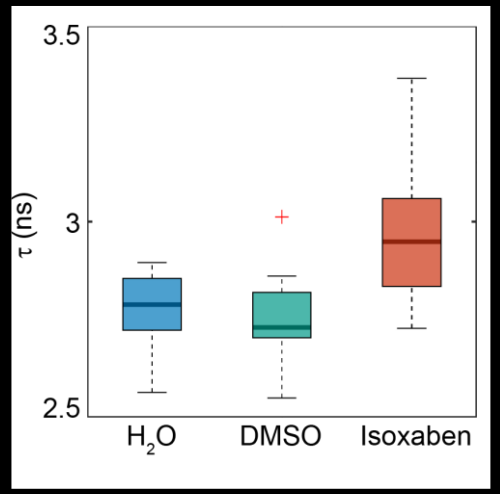
- Higher porosity in FERONIA mutants
- Lower porosity after isoxaben treatment





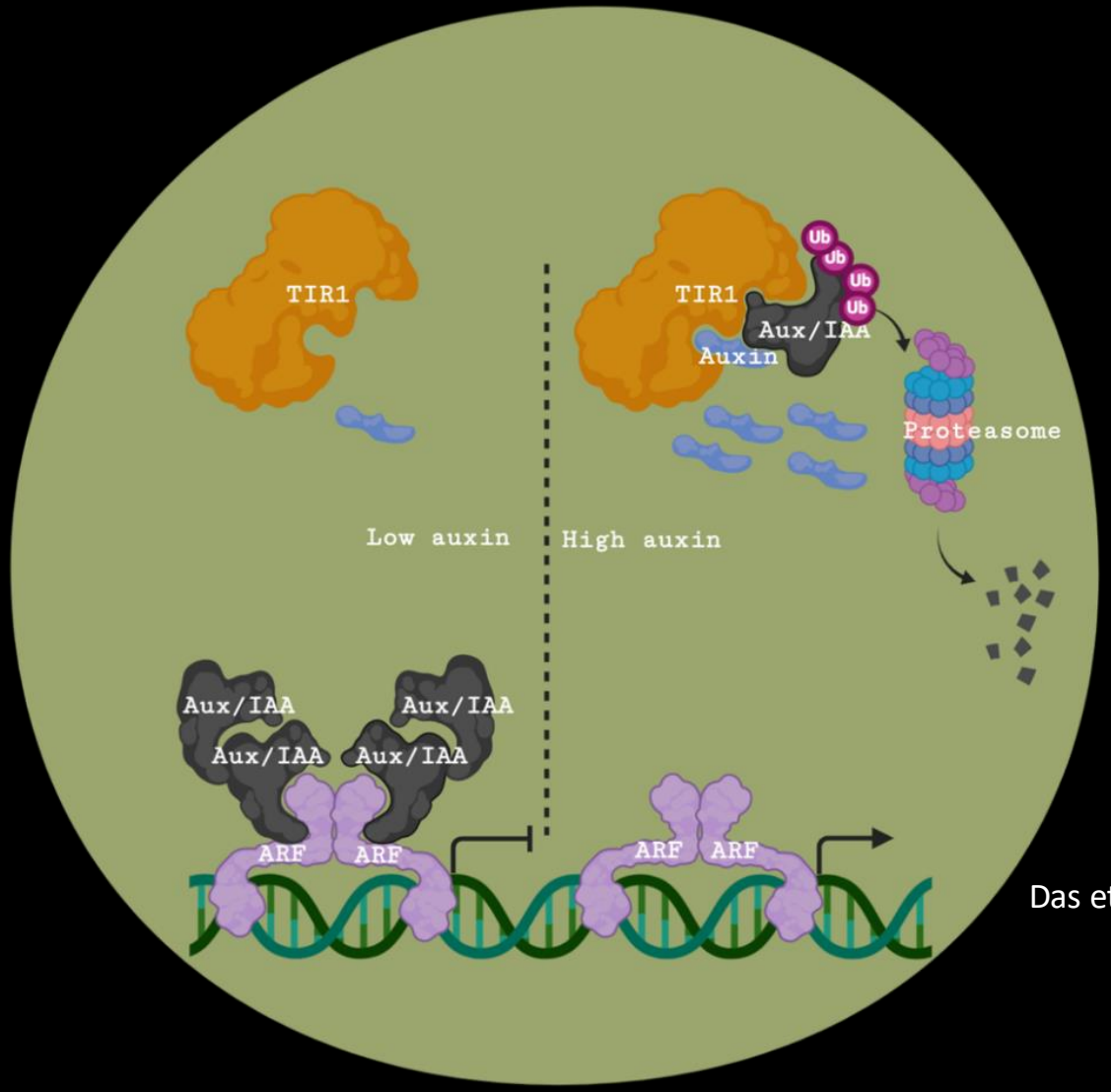






Auxin Biology:
Quantitative understanding of a dynamic protein network

The nuclear auxin signaling pathway



Das et al TIPs 2021

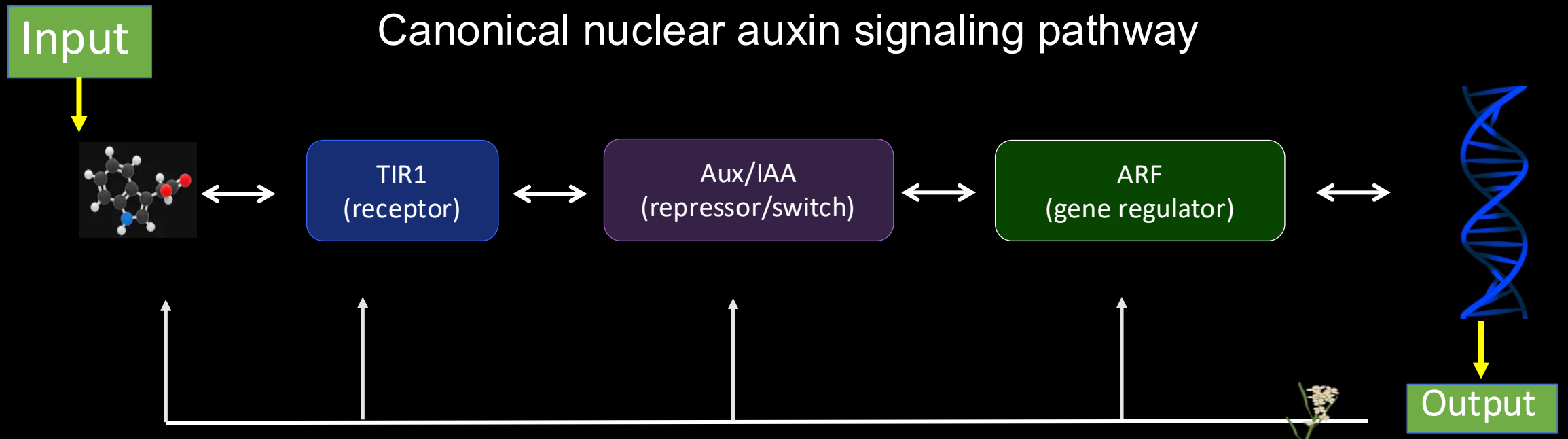
ARF: Auxin response factor

TIR1/AFB: TRANSPORT INHIBITOR RESISTANT 1/AUXIN SIGNALING F-BOX

Aux/IAA: AUXIN/INDOLE-3- ACETIC ACID transcriptional coregulators

Auxin signaling

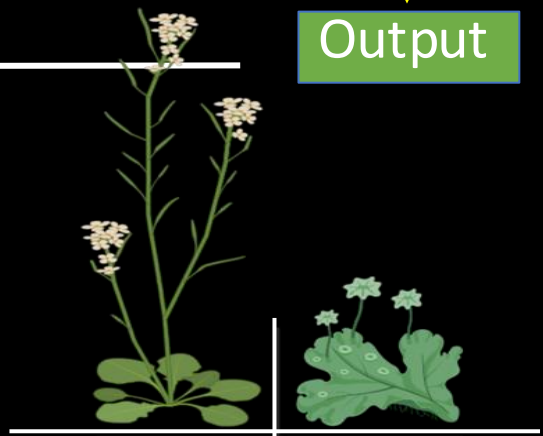
Canonical nuclear auxin signaling pathway





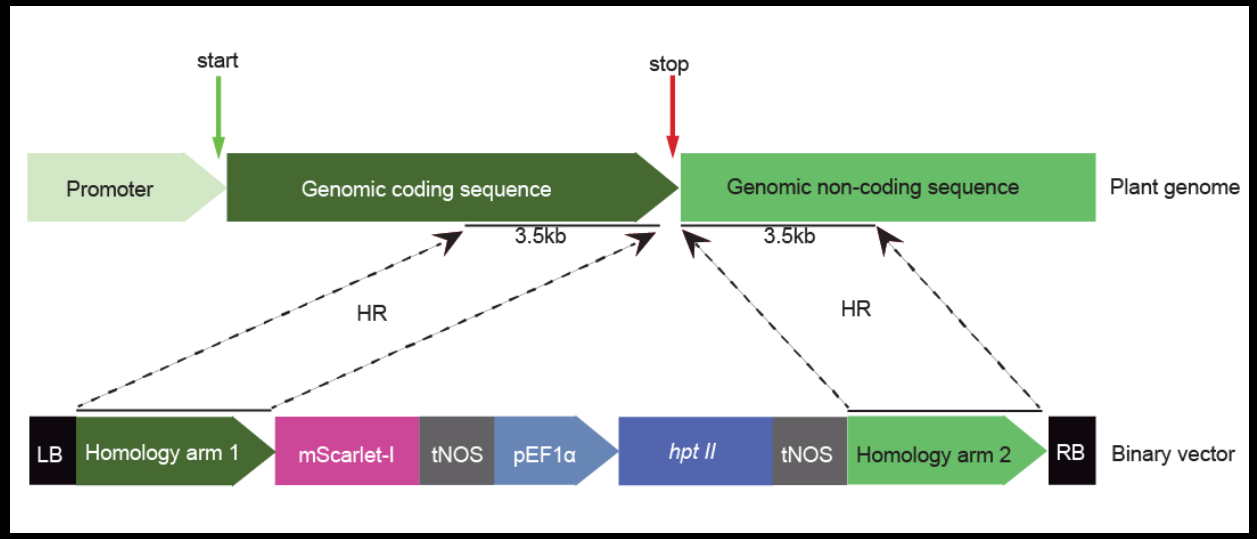




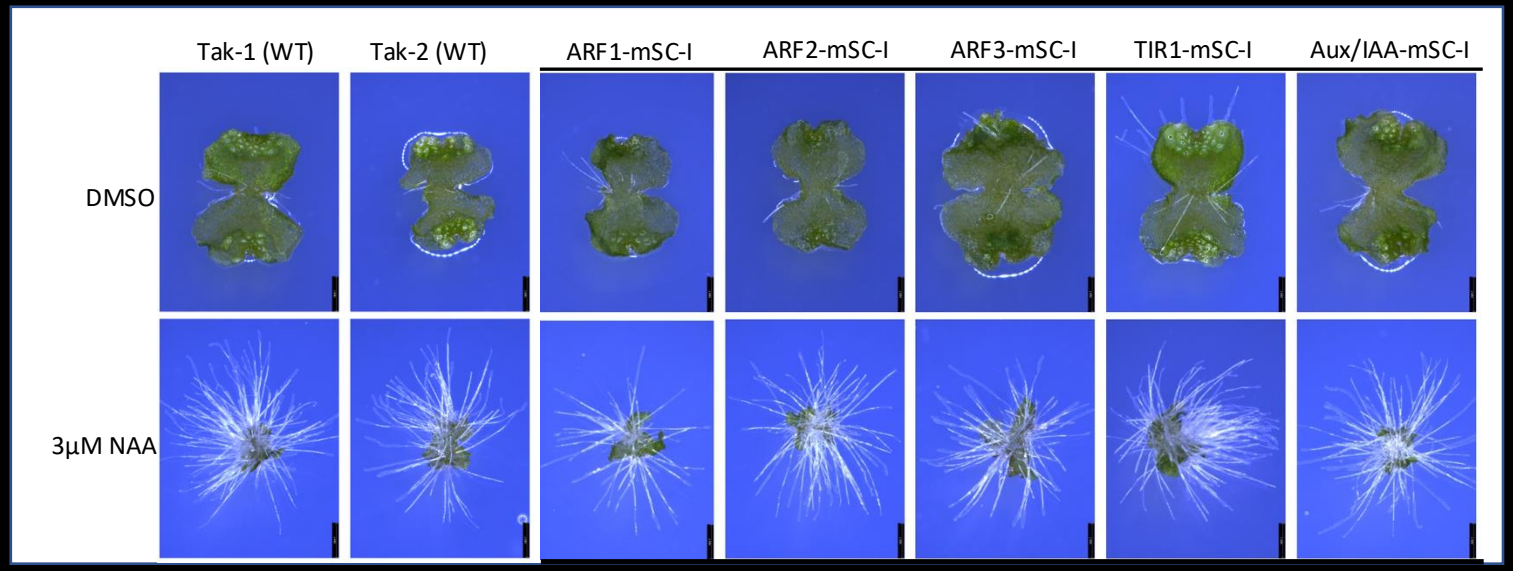


TIR1/AFB	6	1
Aux/IAAs	29	1
ARFs	23	3

Towards quantitative analysis of nuclear auxin signaling

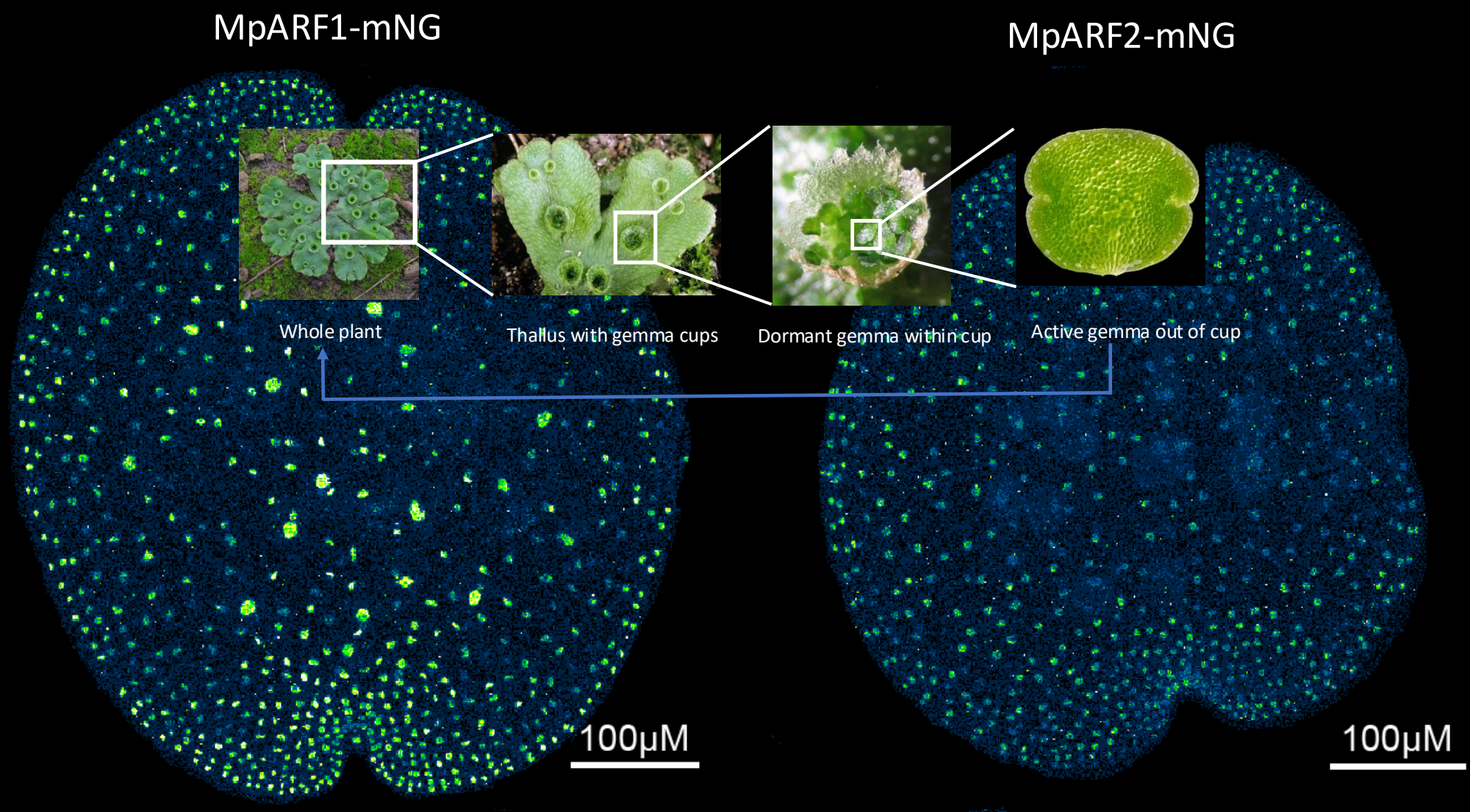


- No addition of extra gene copy
- Reports native protein concentrations



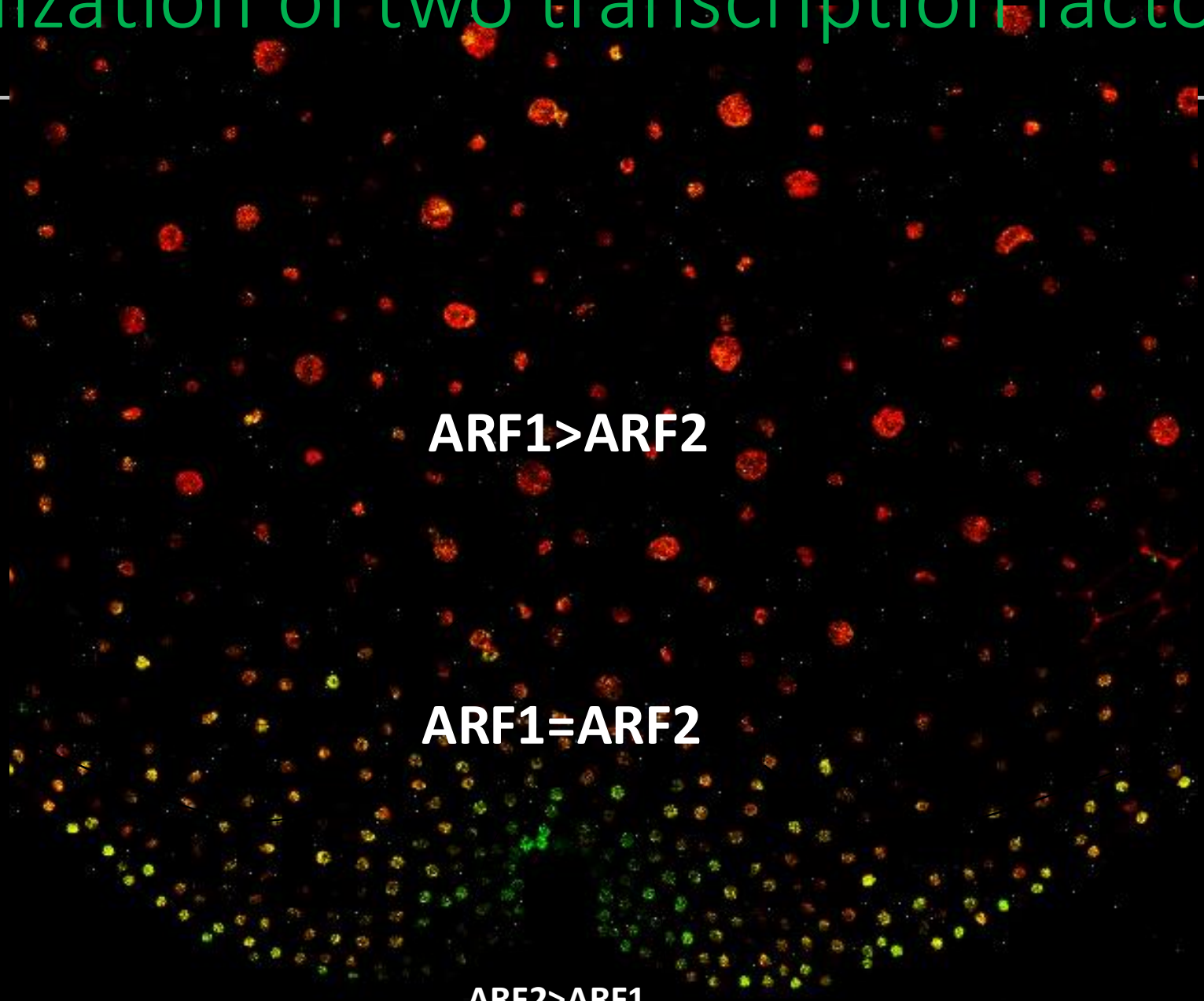
mScarlet-I	mNeonGreen
✓ MpARF1	✓ MpARF1
✓ MPARF2	✓ MPARF2
✓ MpARF3	✓ MpARF3
✓ MpTIR1	✓ MpTIR1
✓ MpAux/IAA	✓ MpAux/IAA
✓ MpncARF	✓ MpncARF
✓ MpncIAA	✓ MpncIAA
✓ MpTPL	✓ MpTPL

MpARF1/2-mNeonGreen expression in gemma



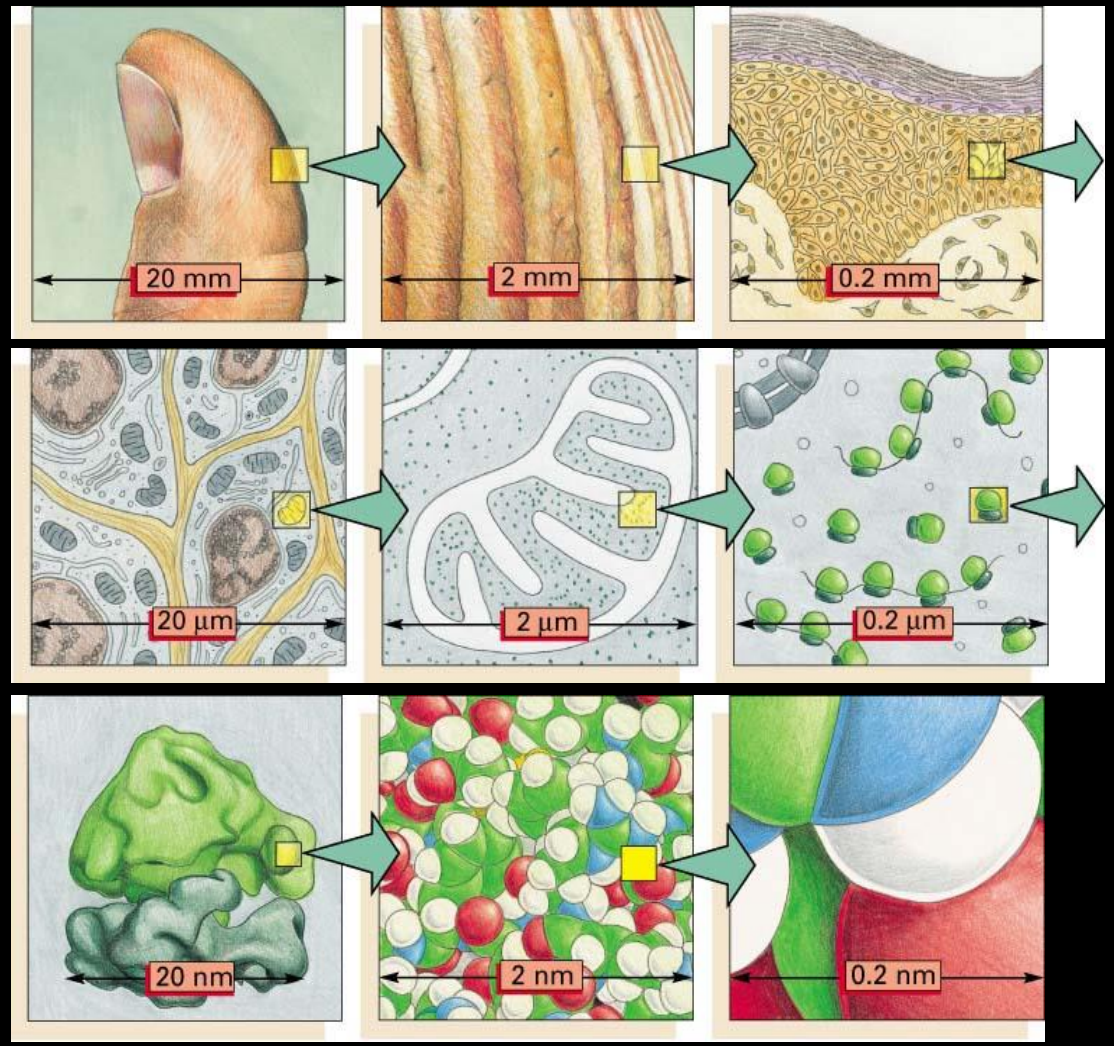
Shubhajit Das

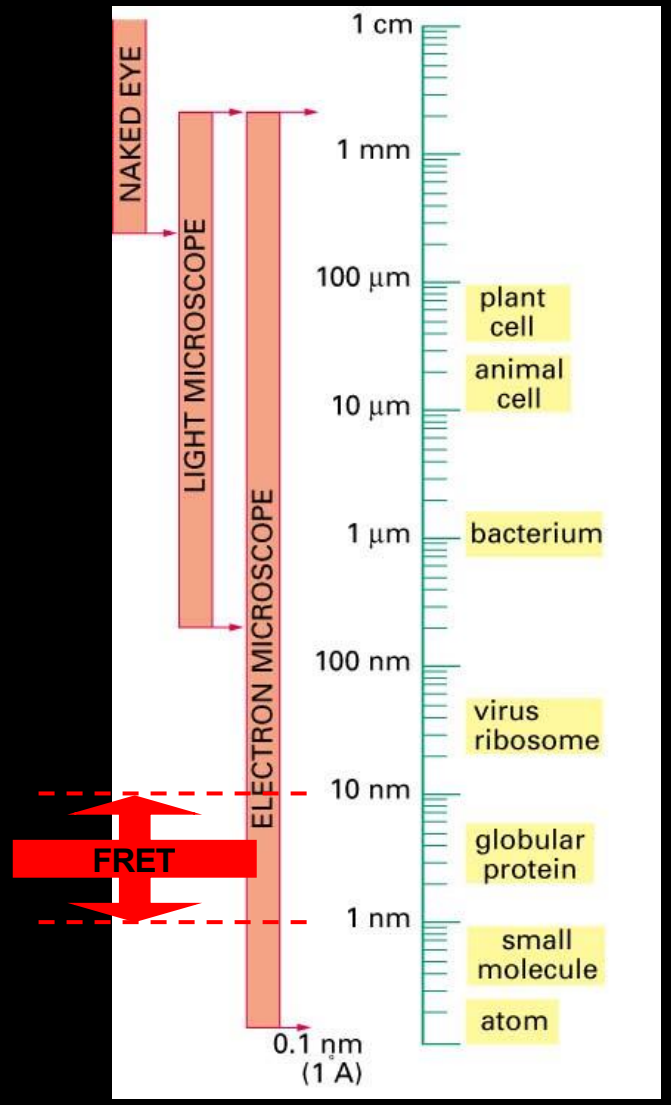
Colocalization of two transcription factors



Das et al 2024

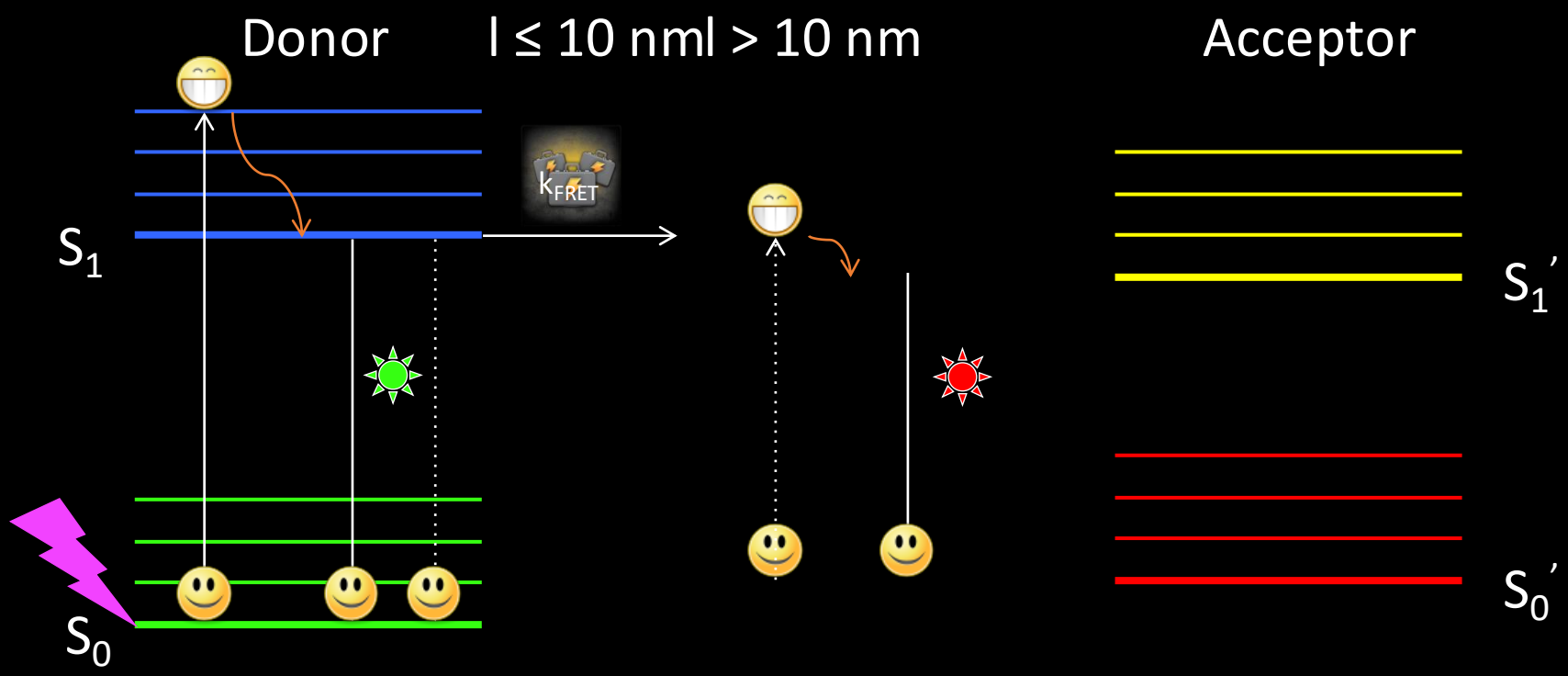
Sense of scale





Adapted from Molecular Biology of the Cell, 4th Ed. (2004) Chapter 1

Förster Resonance Energy Transfer (FRET)



Förster Resonance Energy Transfer (FRET)

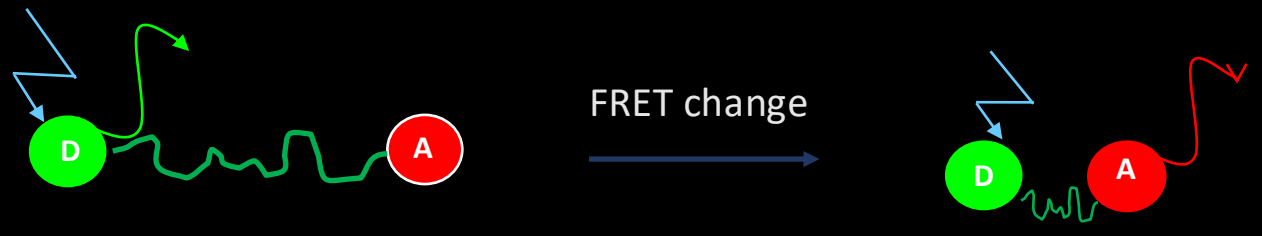
- FRET: transfer of energy from an excited fluorophore to a neighboring chromophore
- Donor: molecule that is excited and transfers energy
- Acceptor: molecule that receives energy
- Energy transfer is non-radiative and through space
- Dipole dipole interaction (resonance)

FRET for monitoring

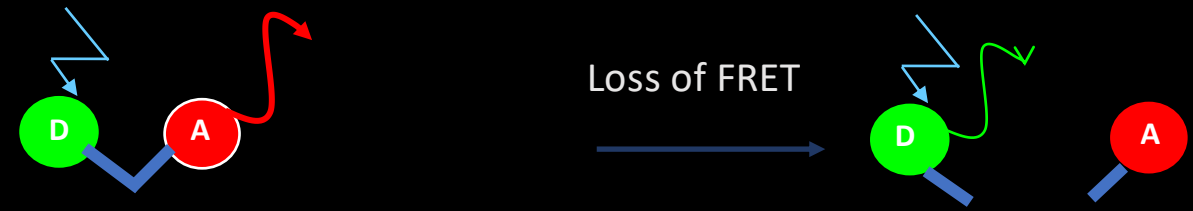
Intermolecular interactions



Intramolecular interactions



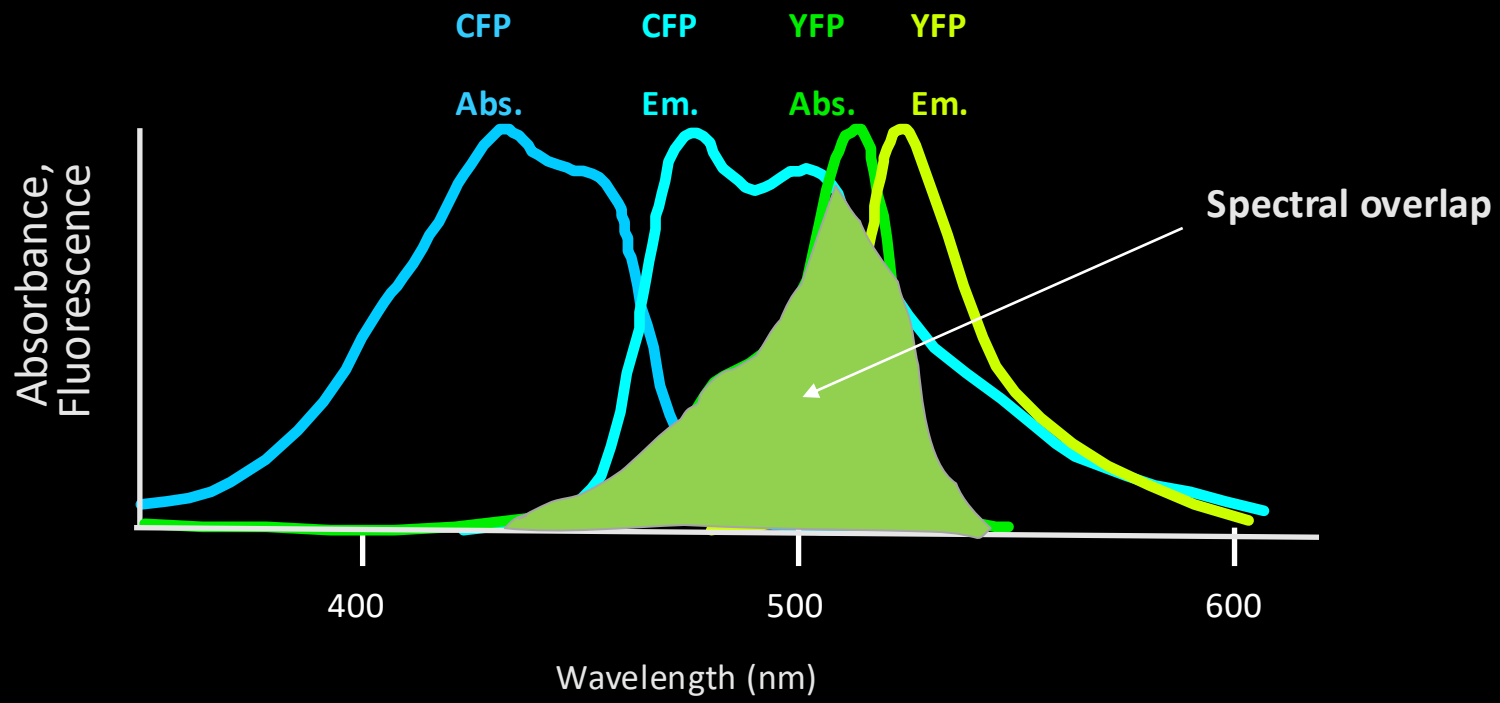
Proteolytic processing



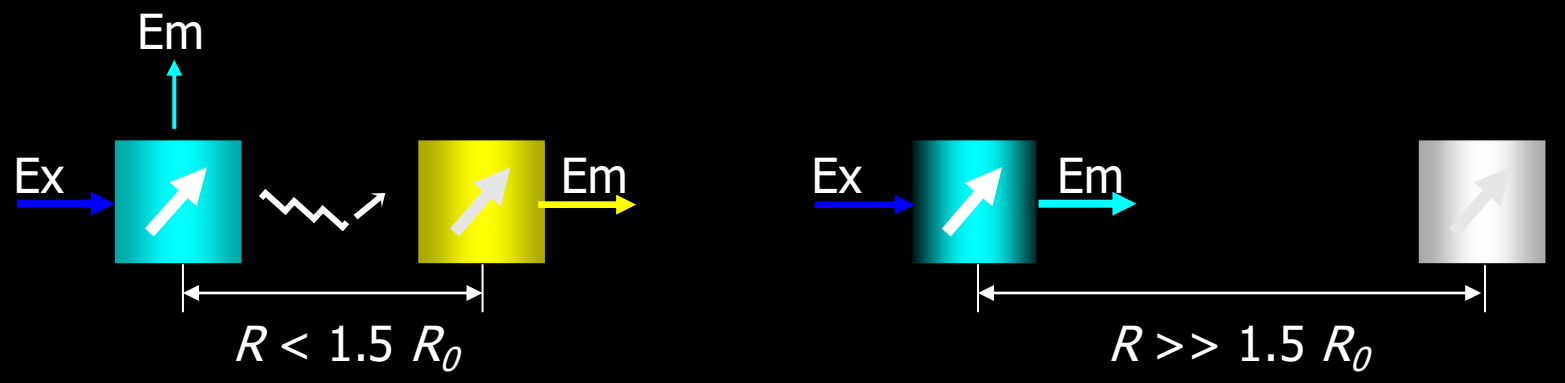
Selvin, *Nature Struct. Biol.* 7, 730 (2000) Ha, *Methods* 25, 78 (2001)

Prerequisites for FRET

1. **Donor must be fluorescent**
2. **There must be a spectral overlap between Donor fluorescence and Acceptor absorbance**



Prerequisites for FRET



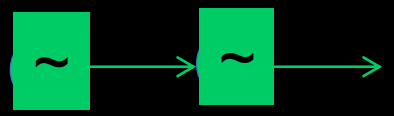
R_0 = critical transfer distance between donor and acceptor (50% energy transfer)

Quantitative description of R_0 (Förster radius)

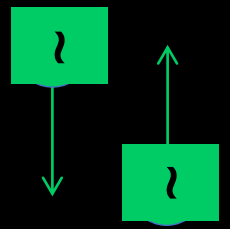
$$R_0 = 0.0211 \times (\kappa^2 Q_D J n^{-4})^{1/6} \quad \text{(nm)}$$

κ^2 = Dipole orientation factor $(0 < \kappa^2 < 4)$

Orientation matters …

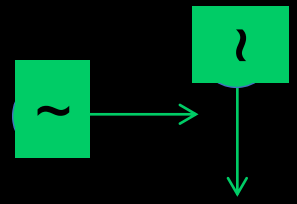


$\kappa^2 = 4$



$\kappa^2 = 1$

Random Orientation:
$\kappa^2 = 2/3$



$\kappa^2 = 0$

Quantitative description of R_0 (Förster radius)

$$R_0 = 0.0211 \times (\kappa^2 Q_D J n^{-4})^{1/6} \quad \text{(nm)}$$

Q_D = Quantum yield of the Donor ($0 < Q_D < 1$)

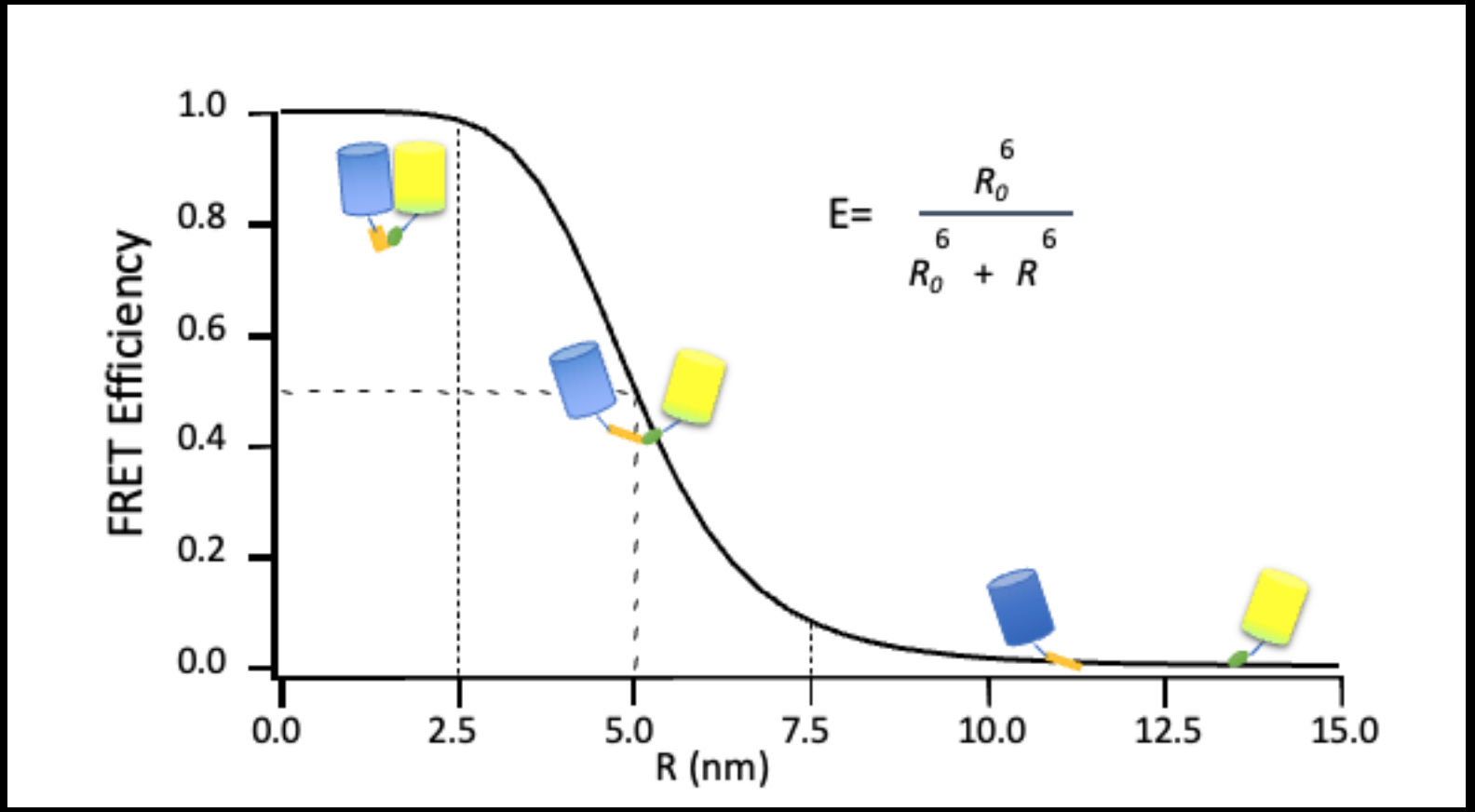
J = Spectral overlap between Donor emission (F_D) and Acceptor absorbance (ε_A)

$$J = \{ F_D(\lambda)\, \varepsilon_A(\lambda)\, \lambda^4\, d\lambda \} / \{ F_D(\lambda)\, d\lambda \} \quad (nm^4 M^{-1} cm^{-1})$$

n = Refractive index (=1.36 living cells)

FRET Efficiency

The efficiency transfer inversely proportional to the **sixth** power over the distance R between the Donor and the Acceptor



FRET between fluorescent proteins

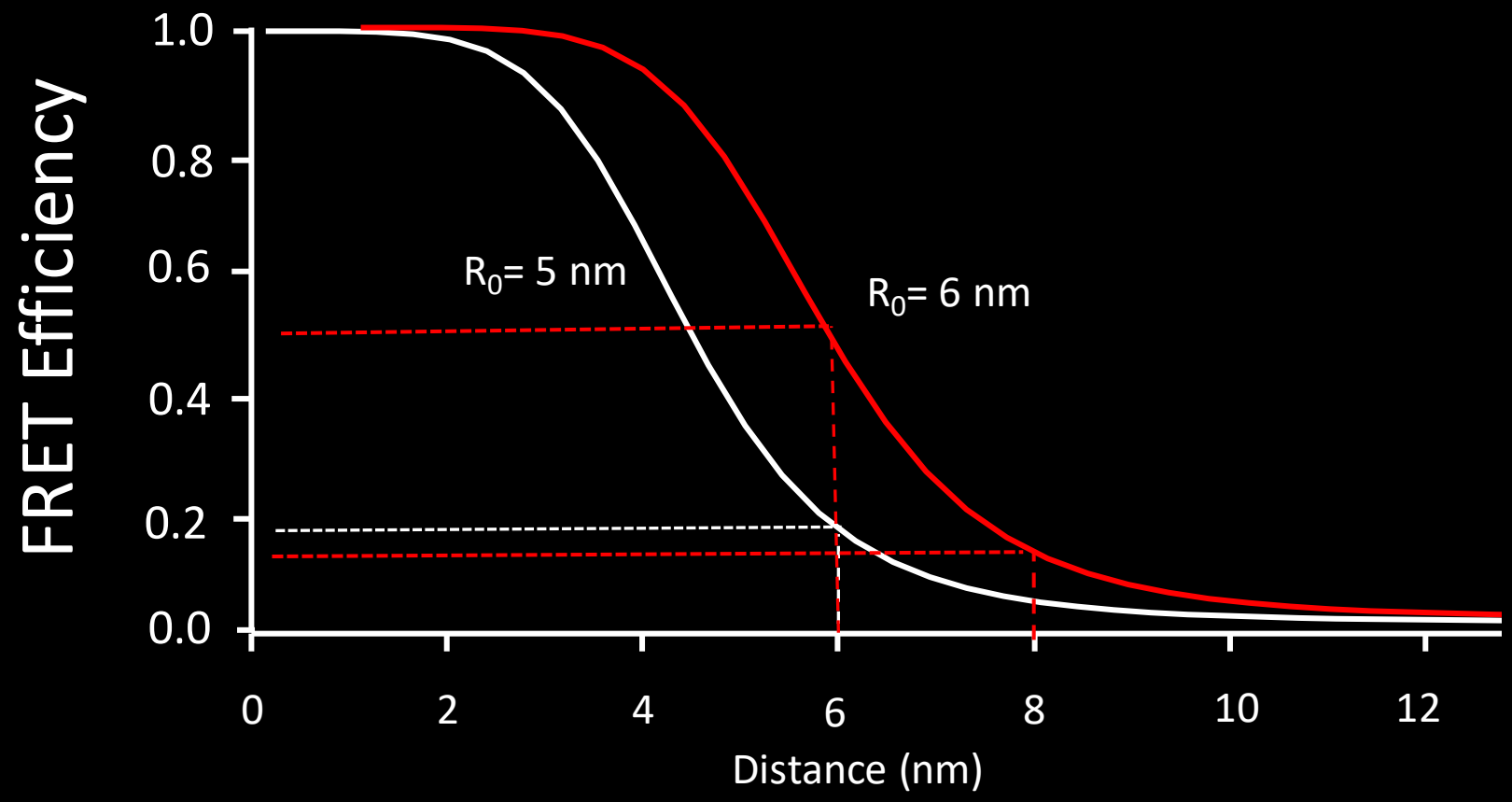
R_0 = The Förster radius for energy transfer, generally between 30-60 Å and is defined by the choice of Donor and Acceptor pair.

DA-couple	R_0 (nm)
EBFP => EGFP	4.1
ECFP => EGFP	4.6
ECFP => EYFP	4.6
EGFP => EGFP	4.6
EGFP => EYFP	5.3
EGFP => mCherry	5.6
EYFP => mCherry	5.7

R_0 is calculated assuming:

κ^2=2/3 (random orientation dipole moments)
n =1.36 (water environment)

Right FRET pair



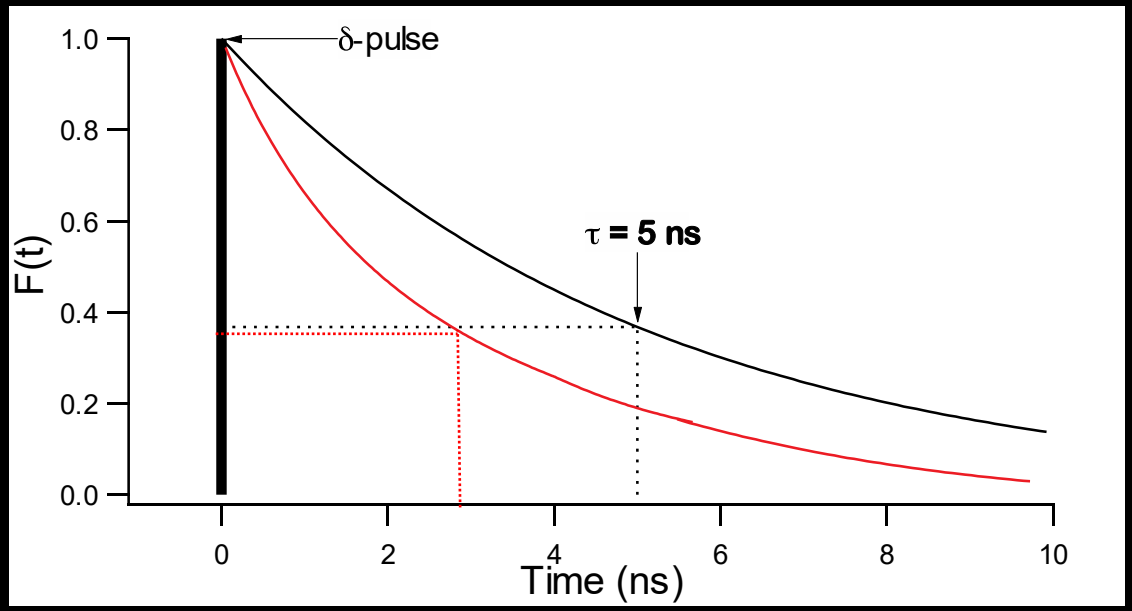
R_0 determined by spectral properties of donor and acceptor:

Quantum yield and molar extinction coefficient

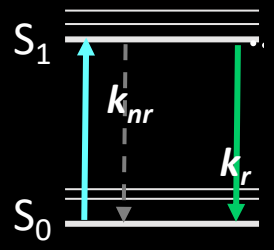
How can FRET be determined?

- FRET imaging
  - Donor fluorescence intensity
  - Sensitized emission of acceptor fluorescence
- Acceptor Photo bleaching
- Donor fluorescence lifetime

Fluorescence Lifetime and FRET



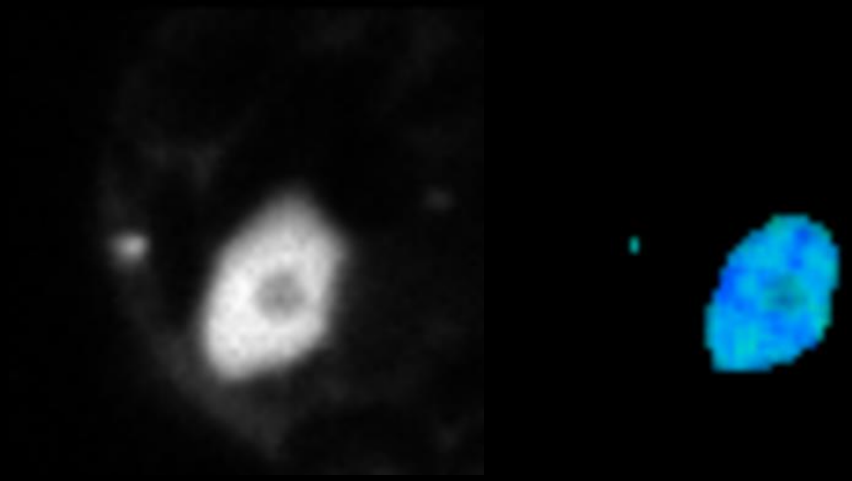
Donor

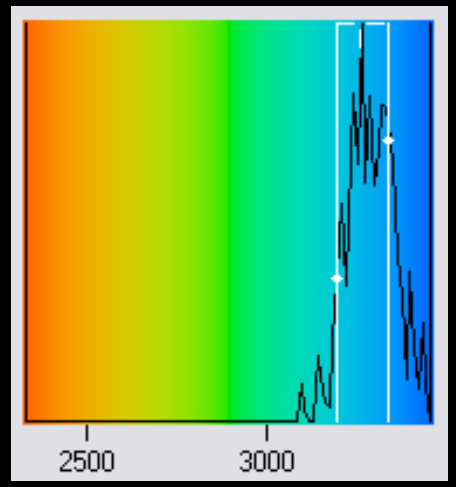


$$\tau_D = \frac{1}{k_r + k_{nr}}$$

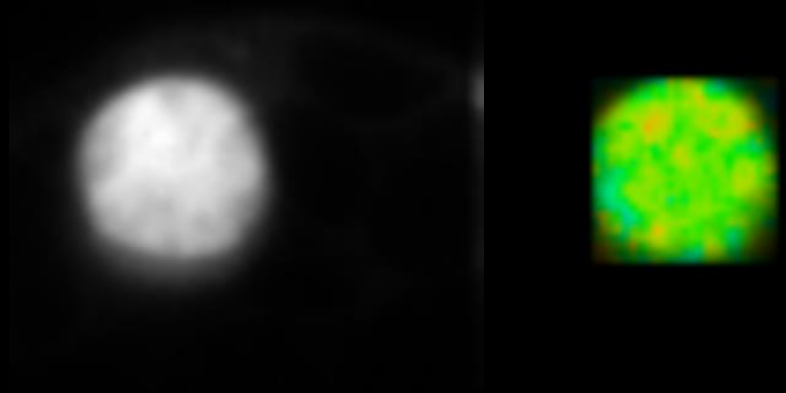
FLIM to visualize the interaction of transcription factors

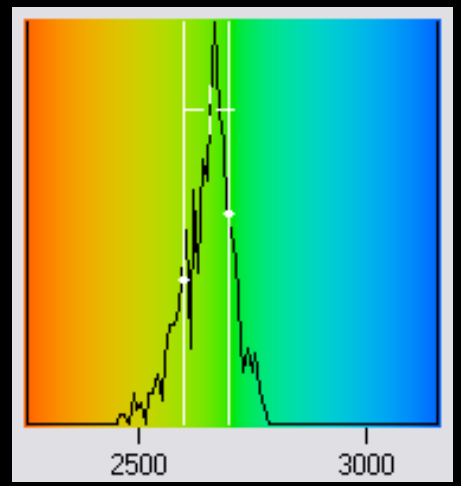
LHW-sCFP3A





LHW-sCFP3A +TMO5-sYFP2





De Rybel et.al *Dev Cell* 2013

Analogy Donor Fluorescence Lifetimes

Donor only

Donor + acceptor

1/4

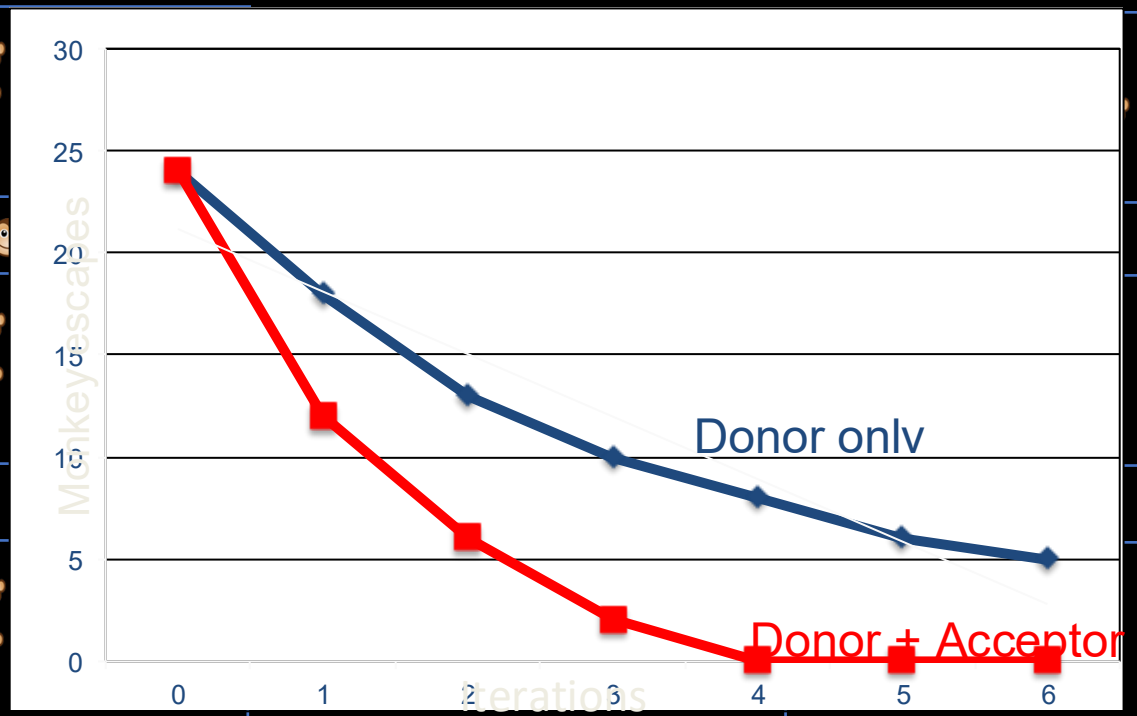
1/4

1/4

1/4

1/4

1/4

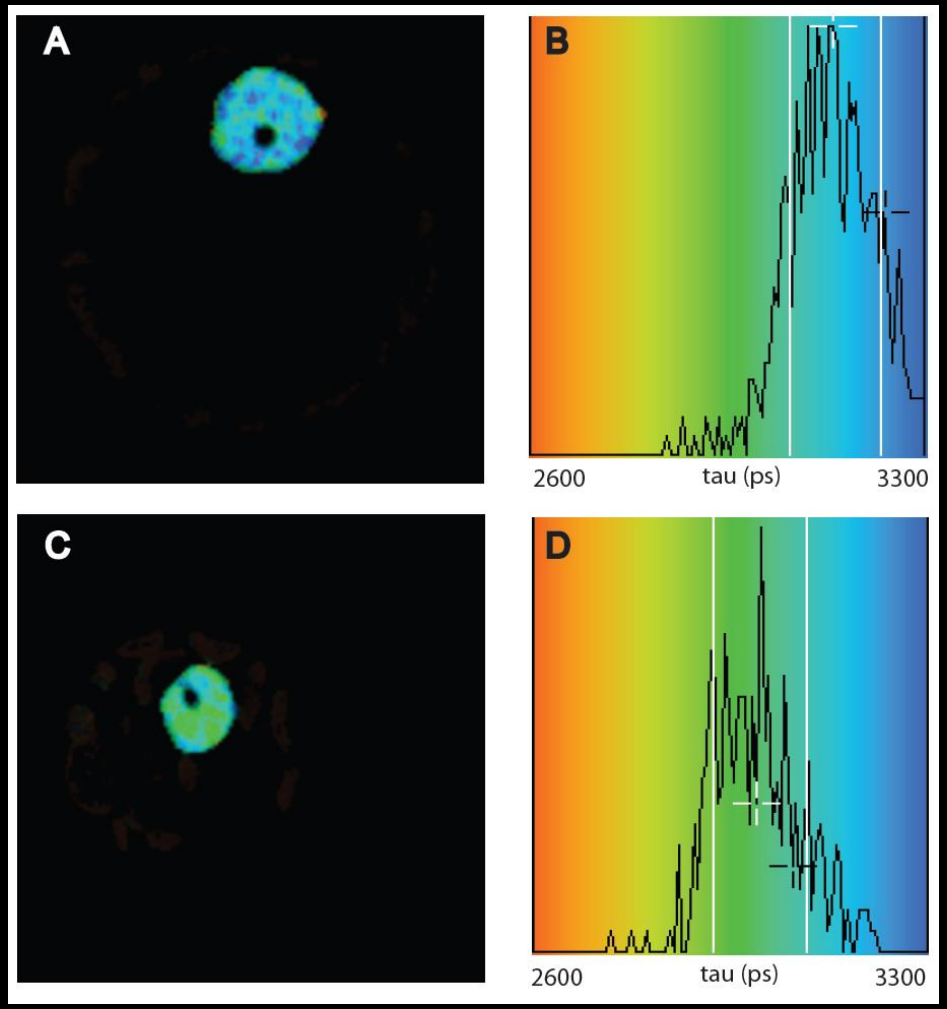


AtARF5 homo-dimers determined by FRET-FLIM

ARF5-sCFP3A

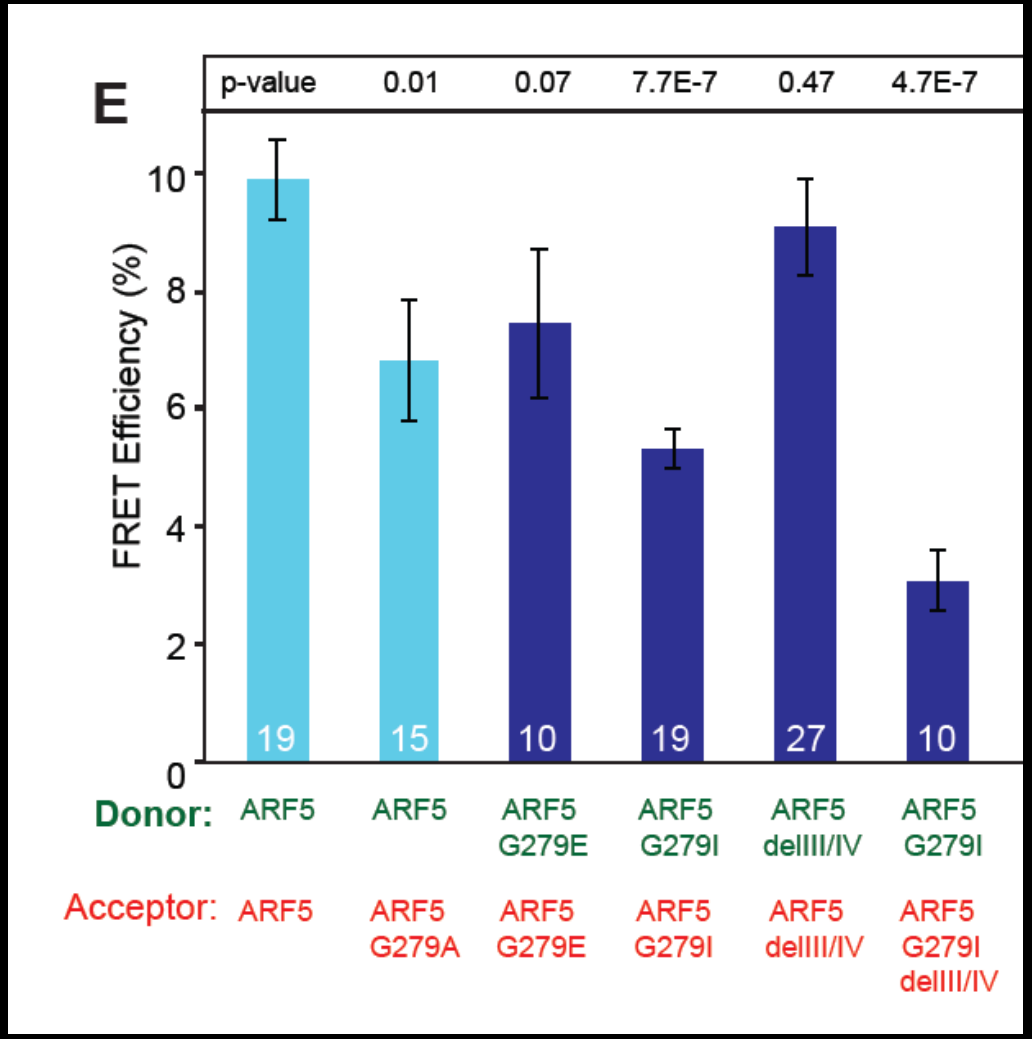
ARF5-sCFP3A

ARF5-sYFP2



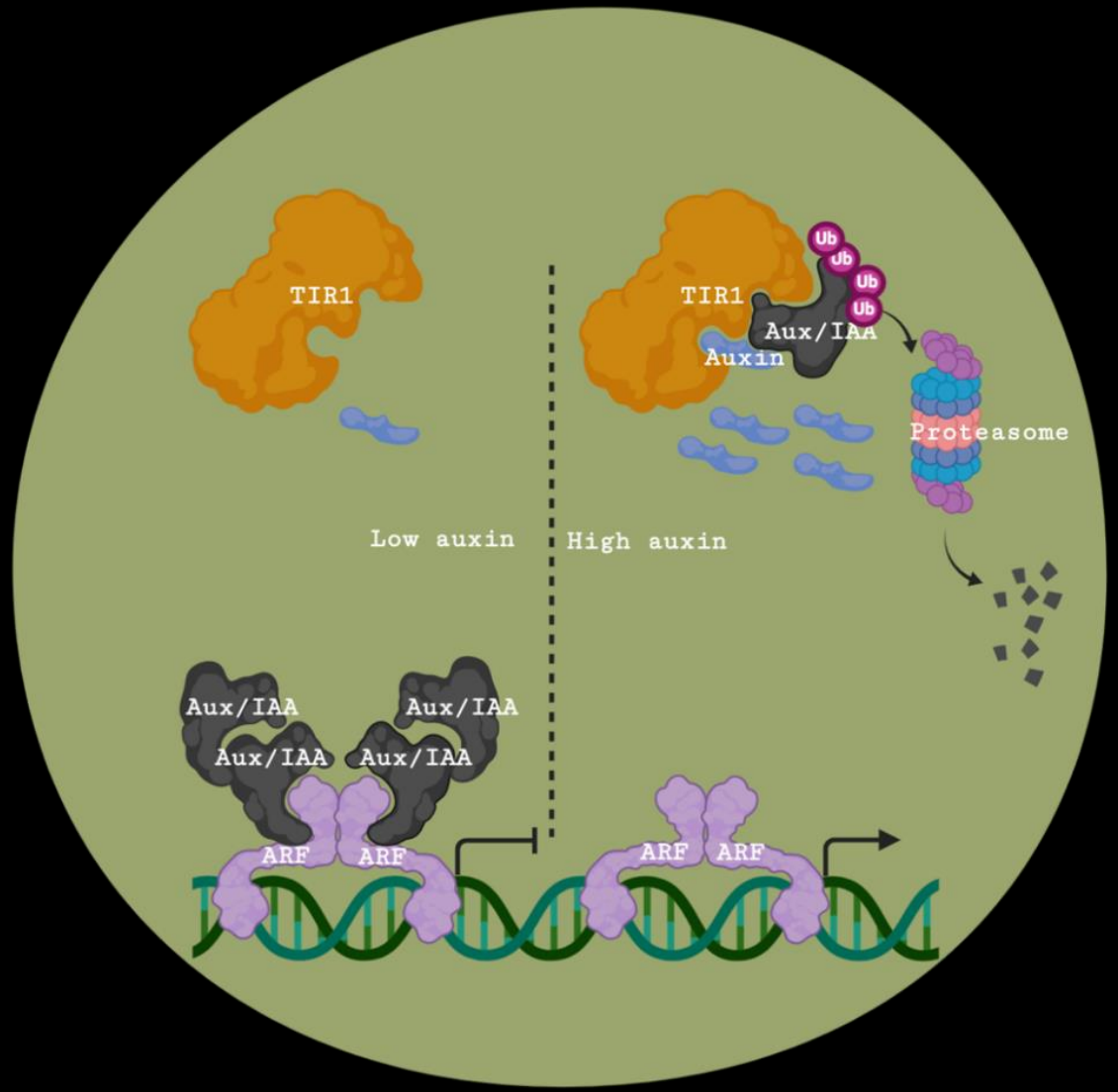
Freire Rios, et al 2016 MMB

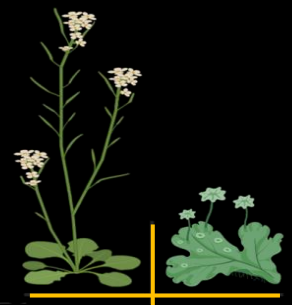
AtARF5 homo-dimers determined by FRET-FLIM



Boer, Freire-Rios, van den Berg *et al, Cell* (2014)

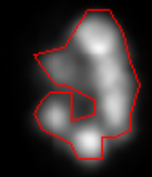
Nuclear auxin signaling pathway in *Marchantia polymorpha*

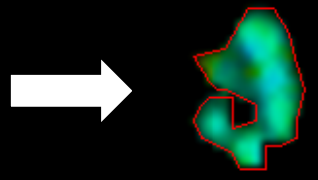




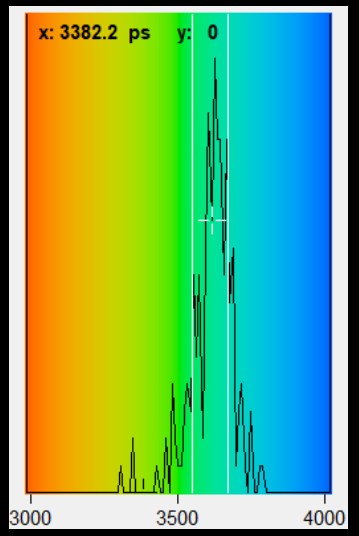
TIR1/AFBs	6	1
ARF	23	3 (A,B,C)
Aux/IAA	29	1

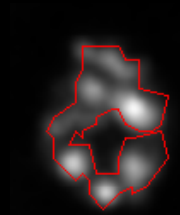
Quantifying MpARF interactions

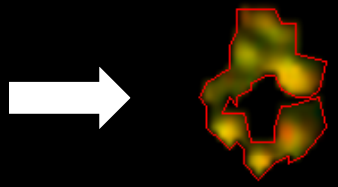


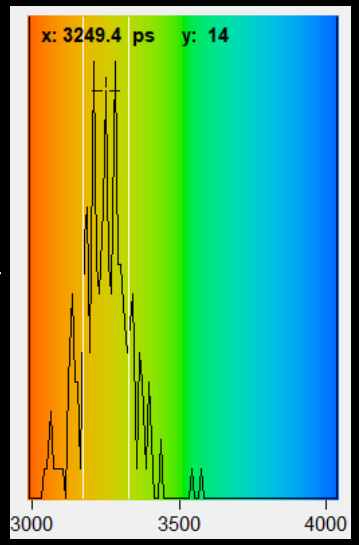


MpARF1-mT2

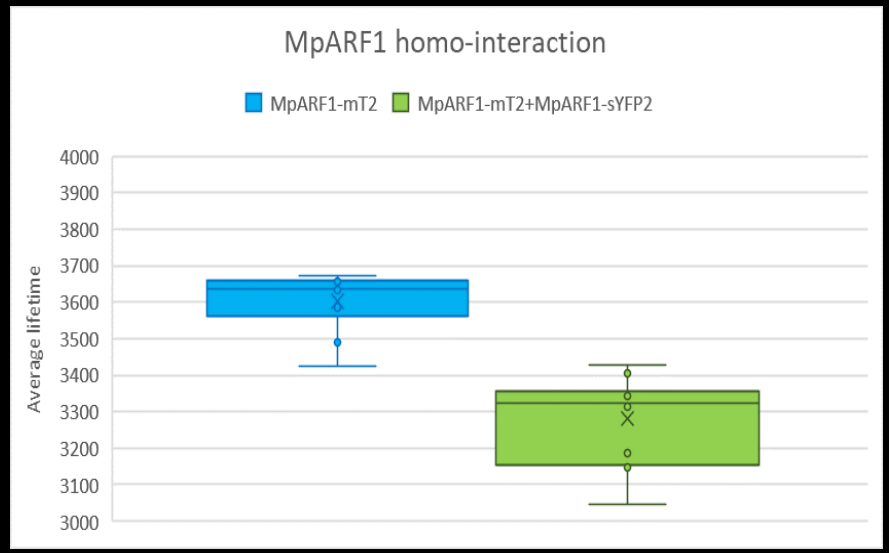






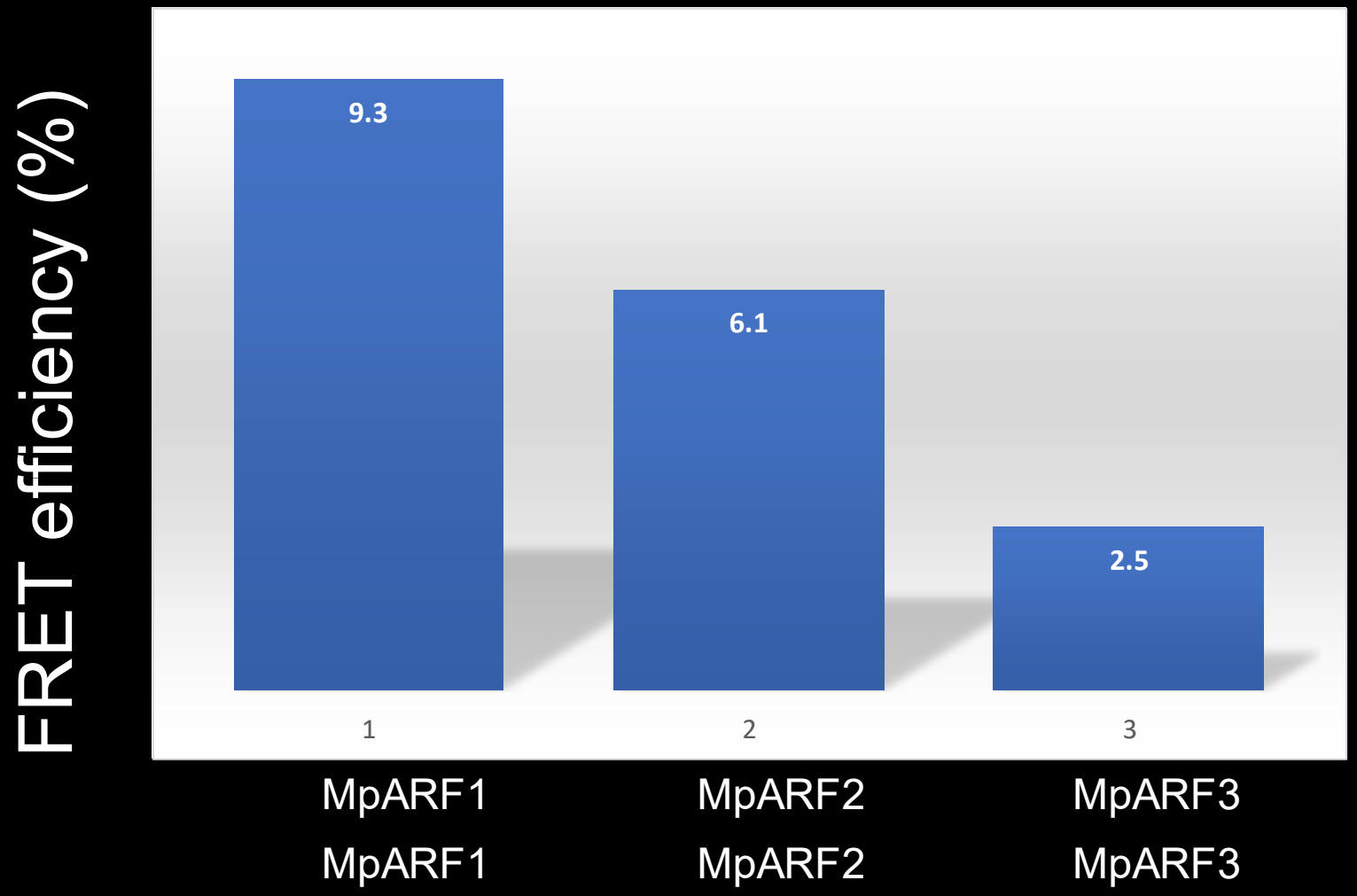


MpARF1-mT2 + MpARF1-sYFP2



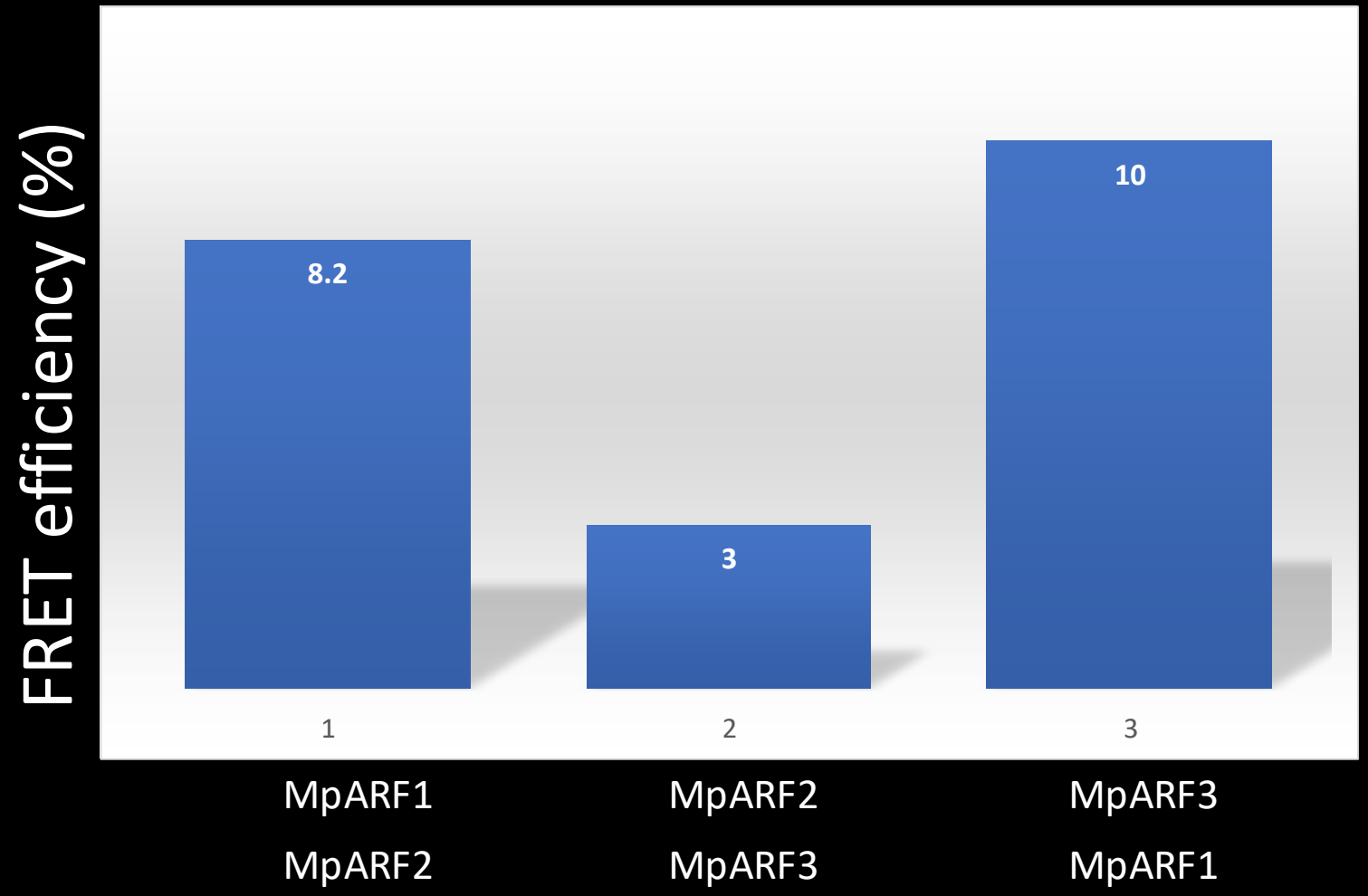
$$E = 1 - \frac{\tau da}{\tau d}$$

FRET efficiencies MpARF homo-interactions



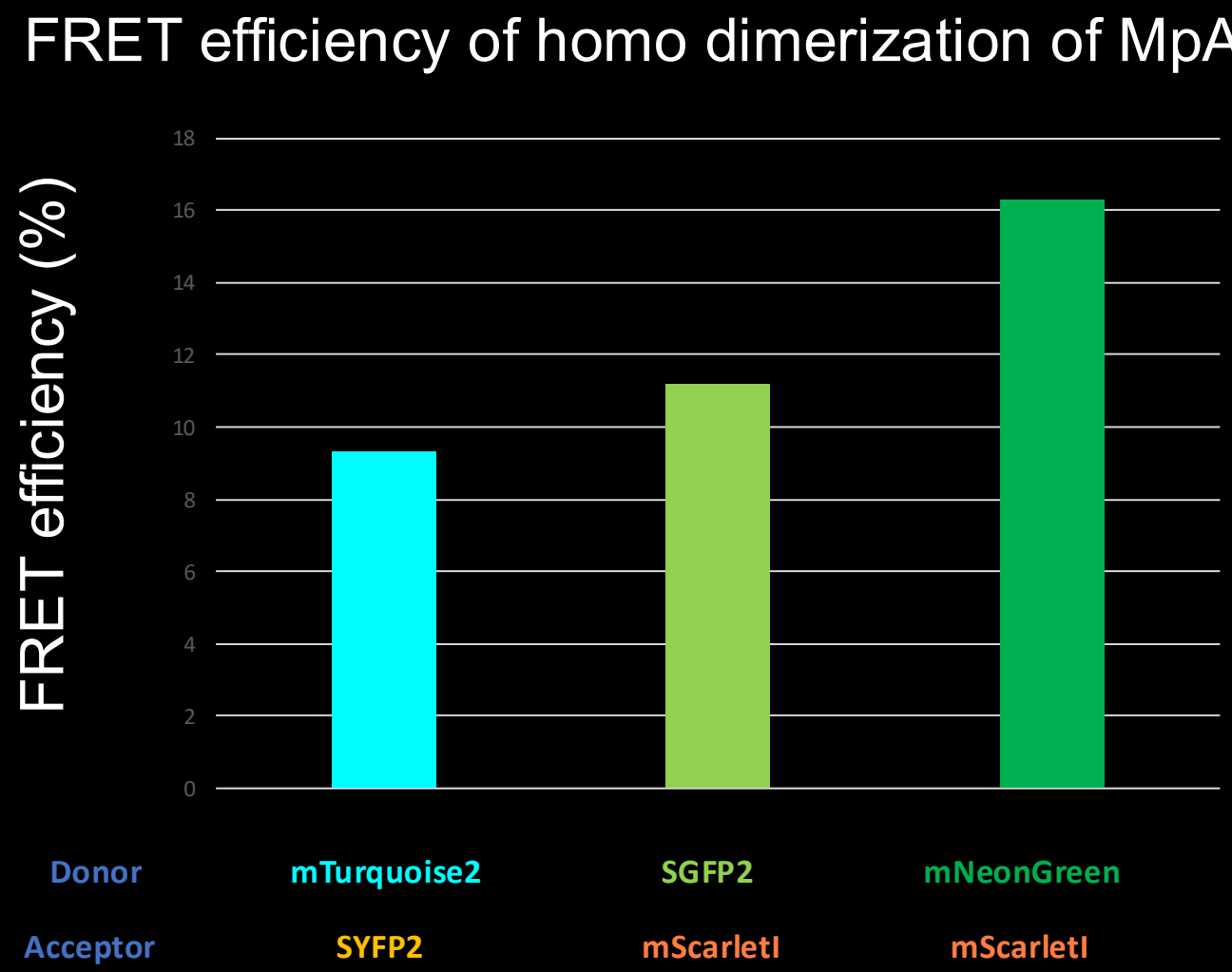
Sanne van Doorn

Hetero complexes between different MpARFs

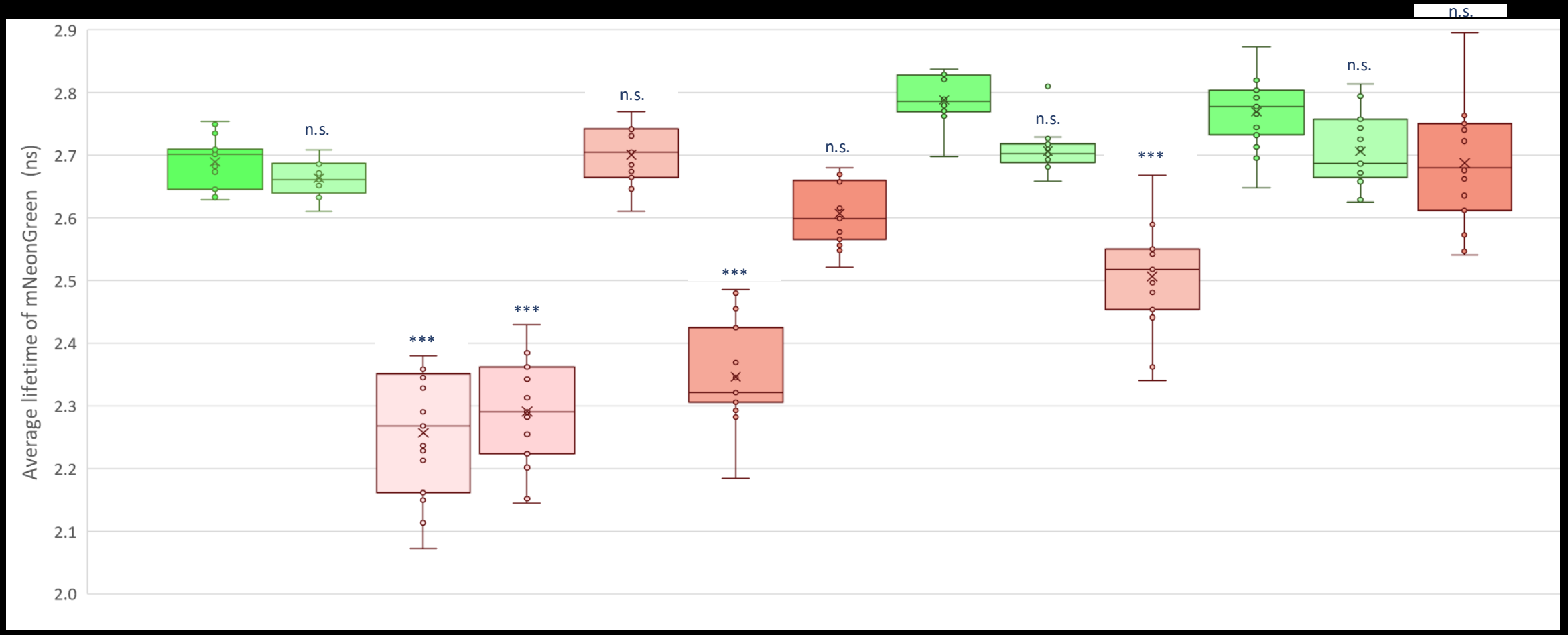


Sanne van Doorn

Optimal fluorophore combinations



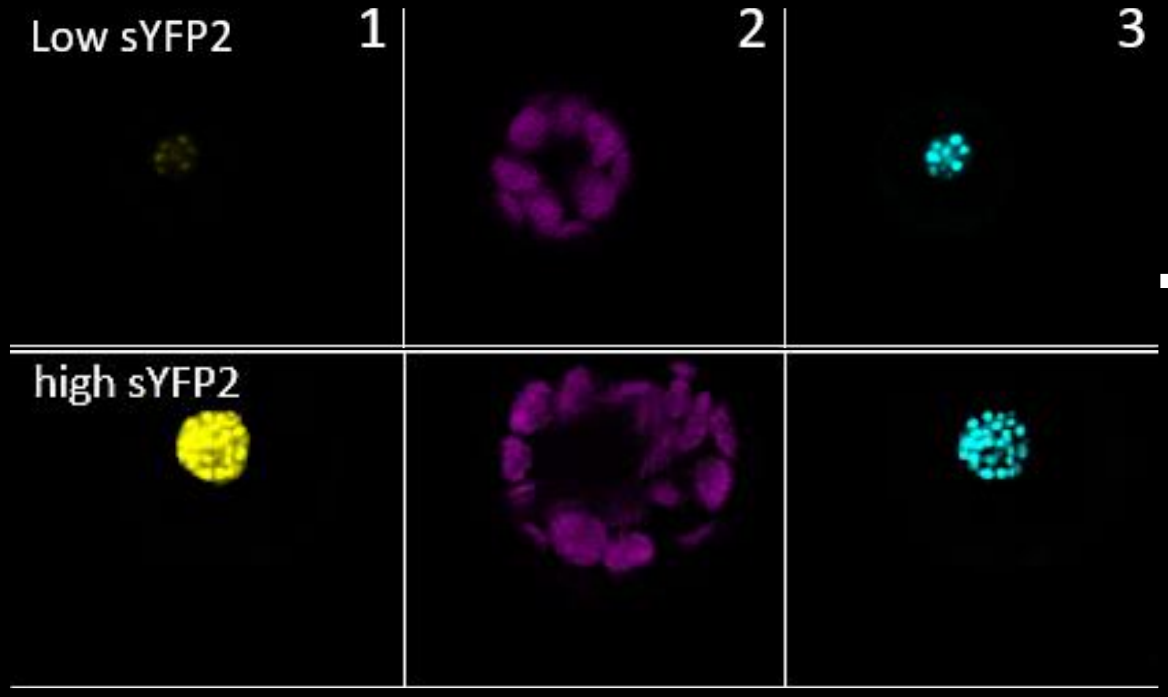
Auxin response protein interactions

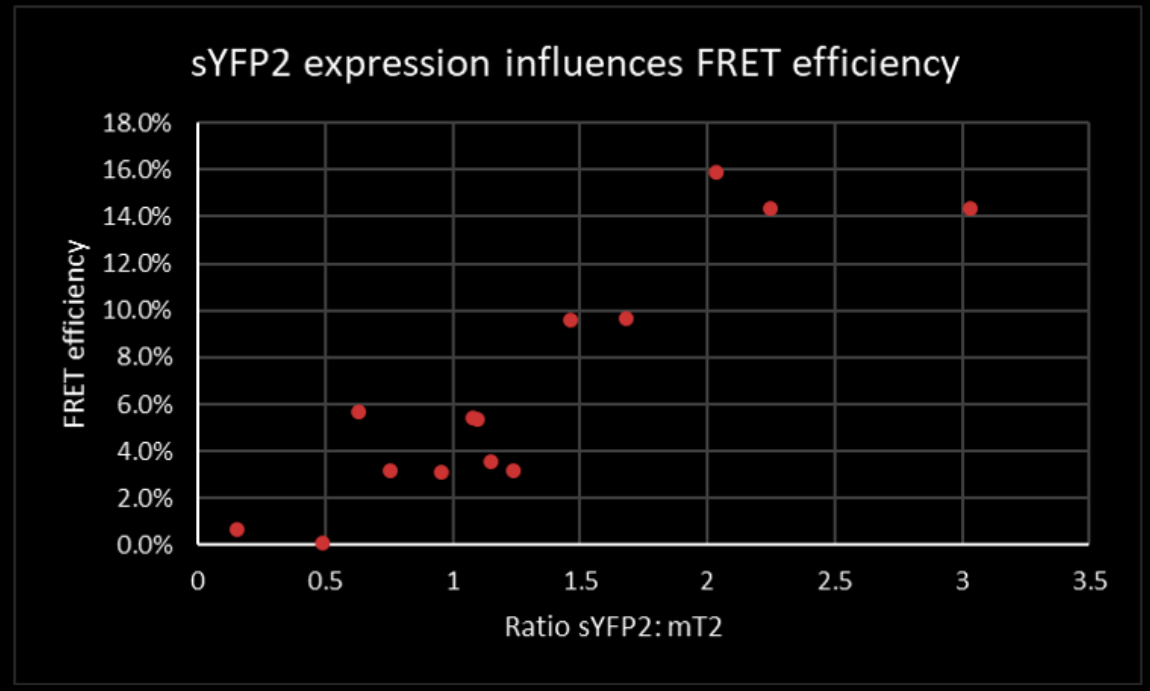


Vanessa Polet Carrillo Carrasco

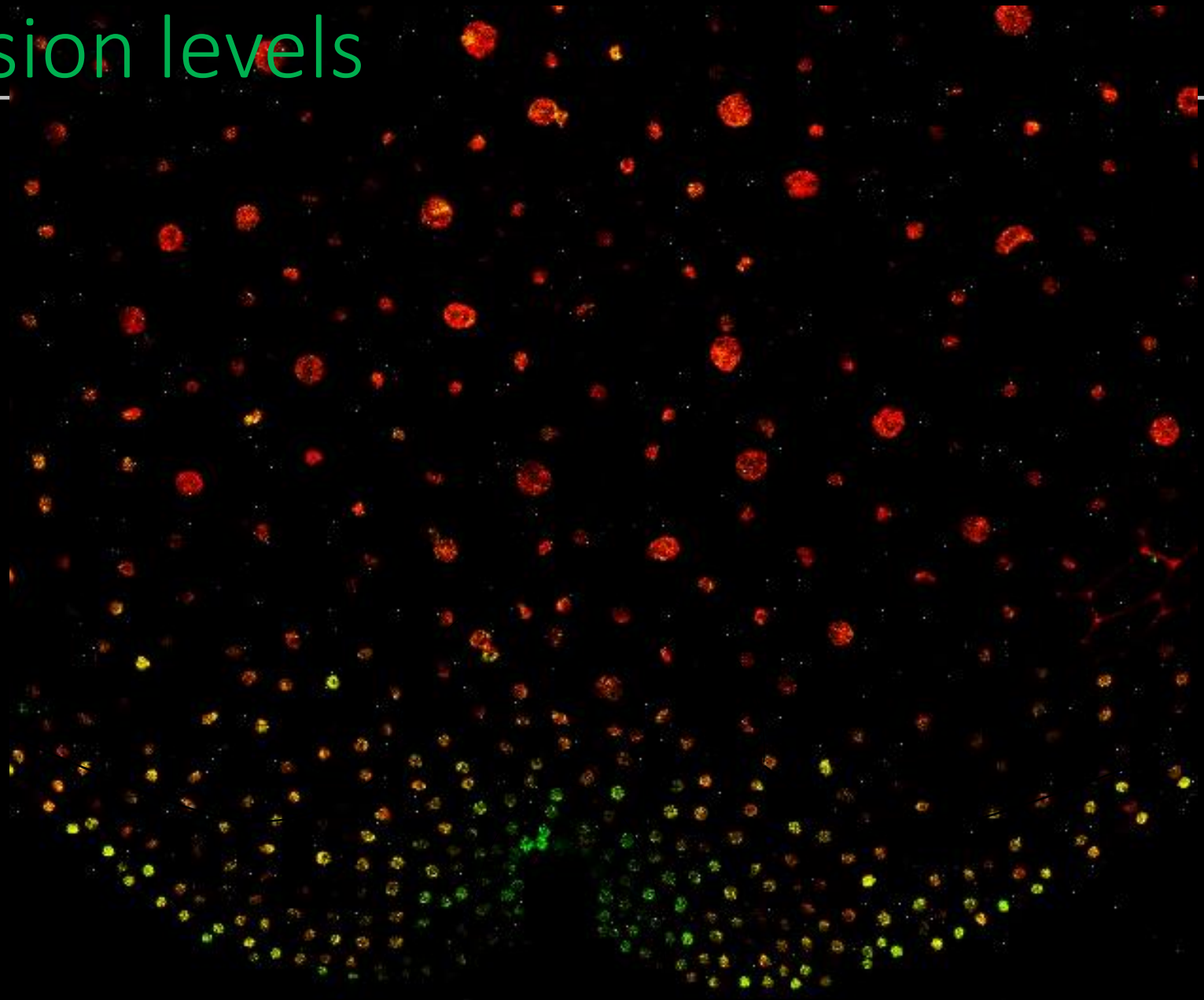
FRET efficiency is dependent on donor acceptor stoichiometry

FRET-FLIM combined with confocal analysis MpARF1-mTurquoise2 + MpARF3-sYFP2

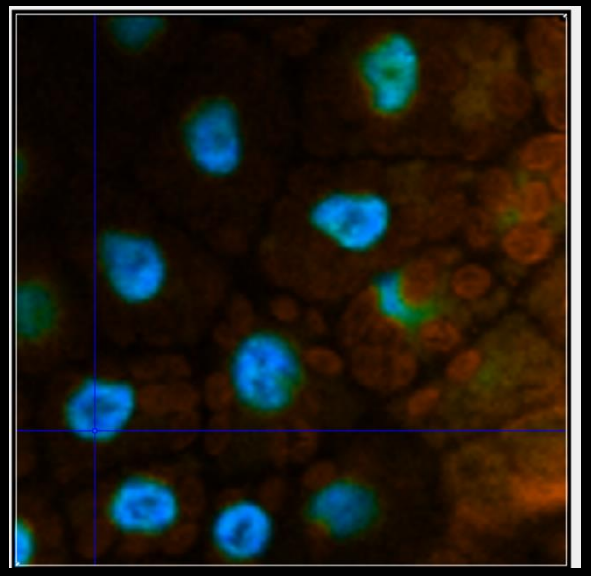




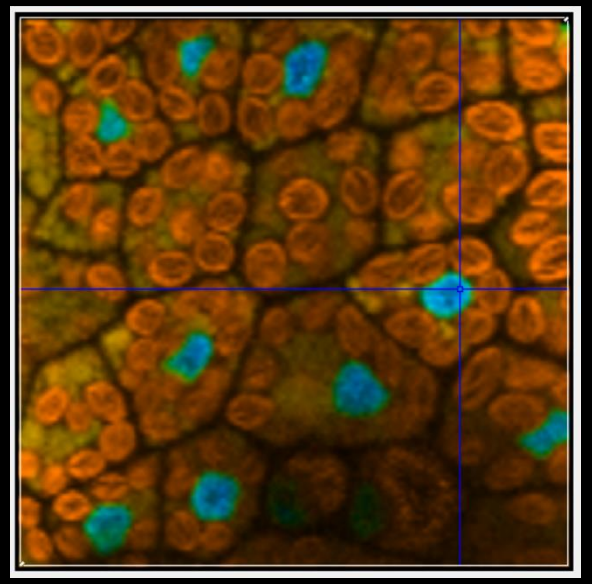
MpARF interactions determined at native expression levels



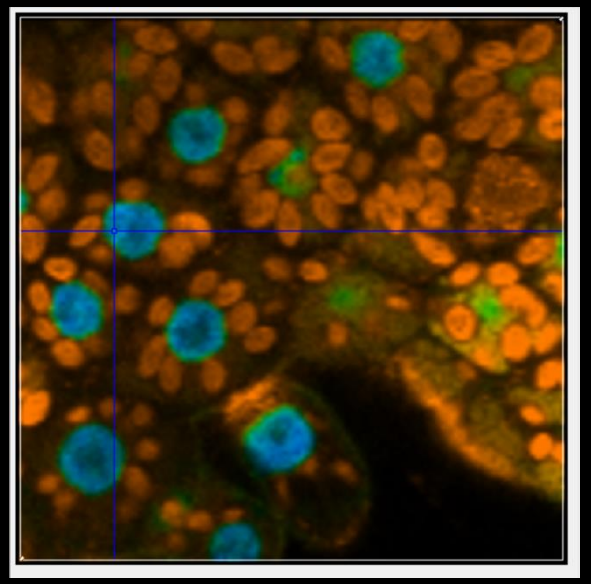
Quantifying MpARF1-MpARF2 interactions in gemmae



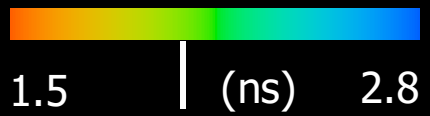
MpARF1-mNG



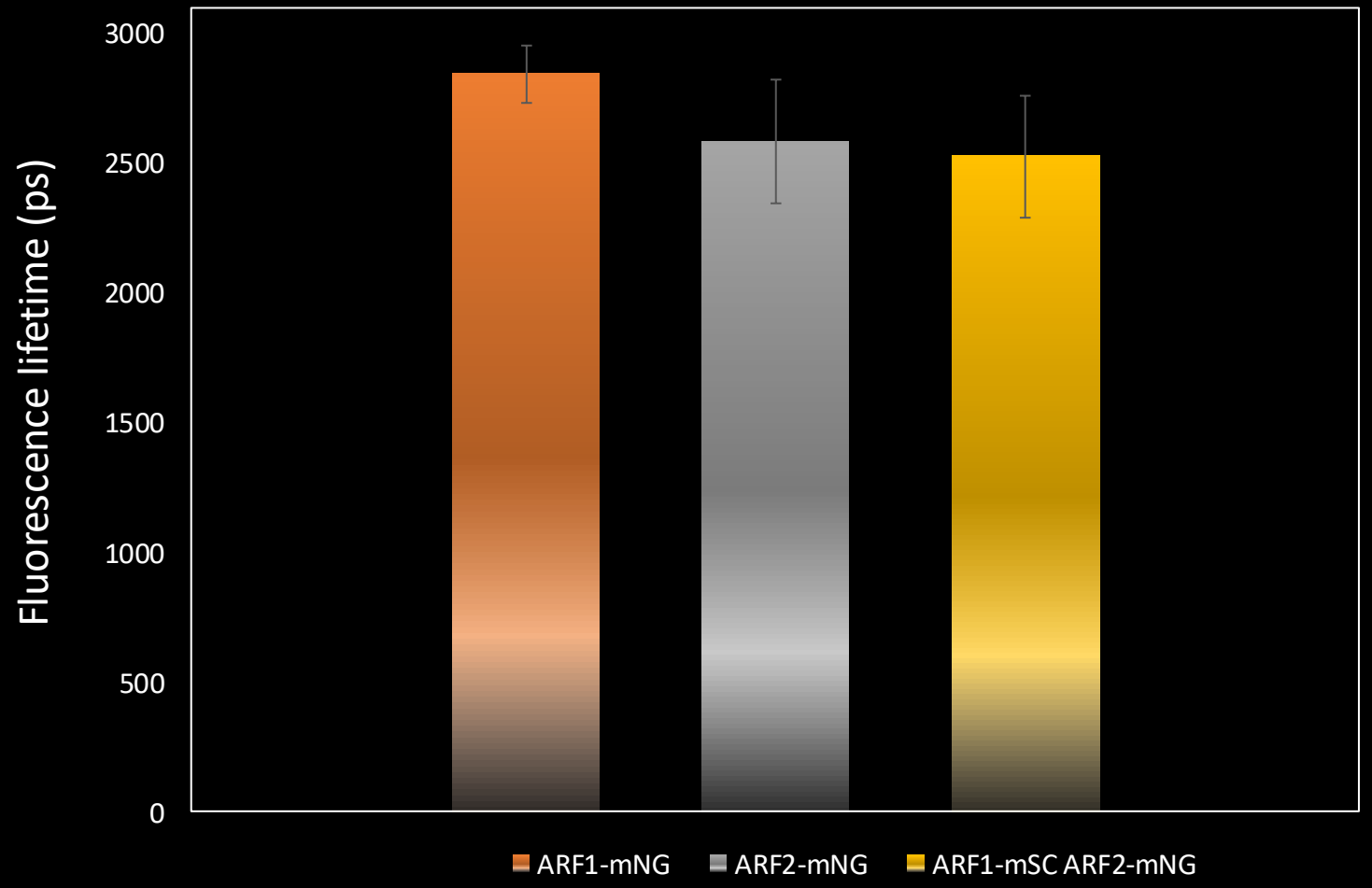
MpARF2-mNG



MpARF2-mNG-
MpARF1-mScarlet-I

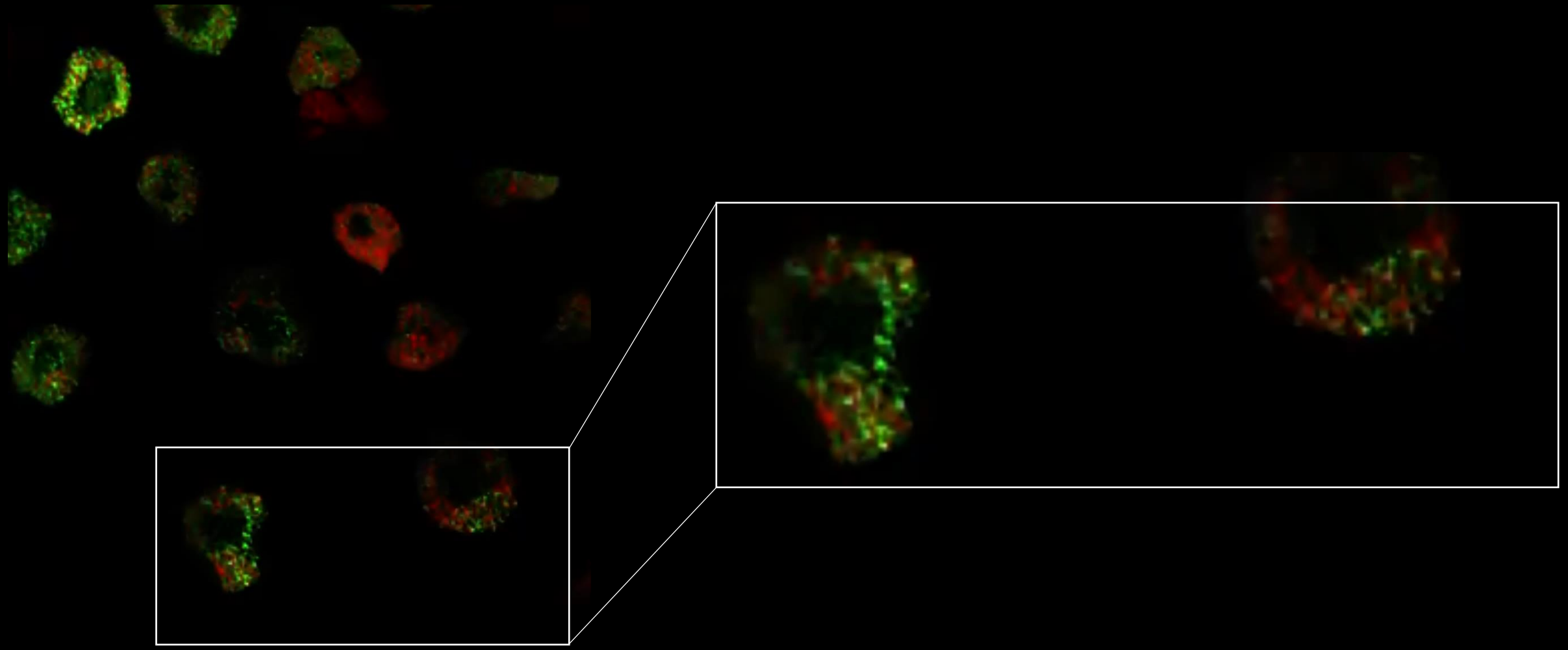


No interaction between MpARF2 –MpARF1



Renske Blok

Minimal colocalisation of MpARF1 and MpARF2



Summary

FRET-FLIM:

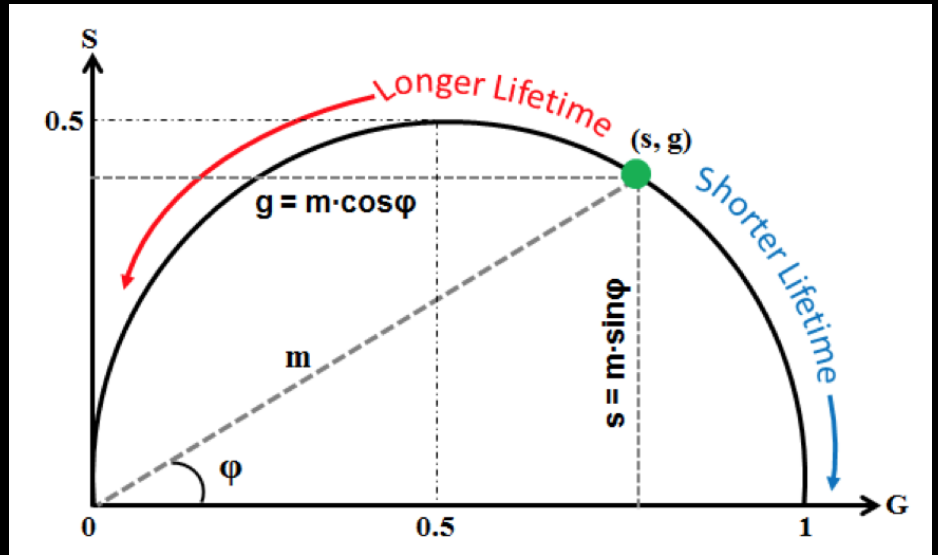
- FRET measured by FLIM is a quantitative method to spatially resolve molecular interactions *in vivo*
- Independent of donor concentration, dependent of donor acceptor stoichiometry
- FRET efficiency : ratio Tau Donor-Acceptor/Tau donor

- mNeonGreen-mScarlet-I is very good FRET pair.

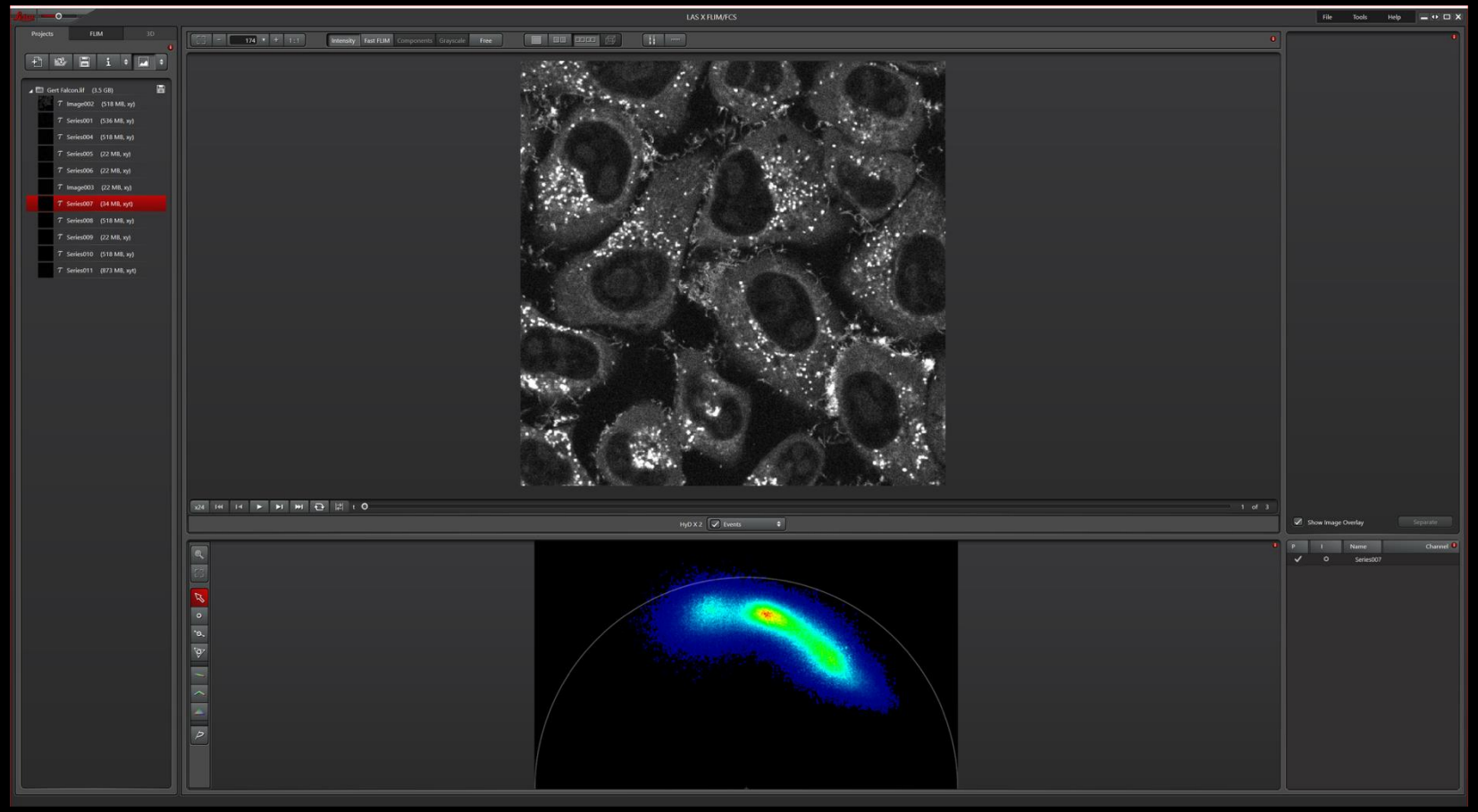
Alternative visualisation of FLIM data

Phasor plot analysis

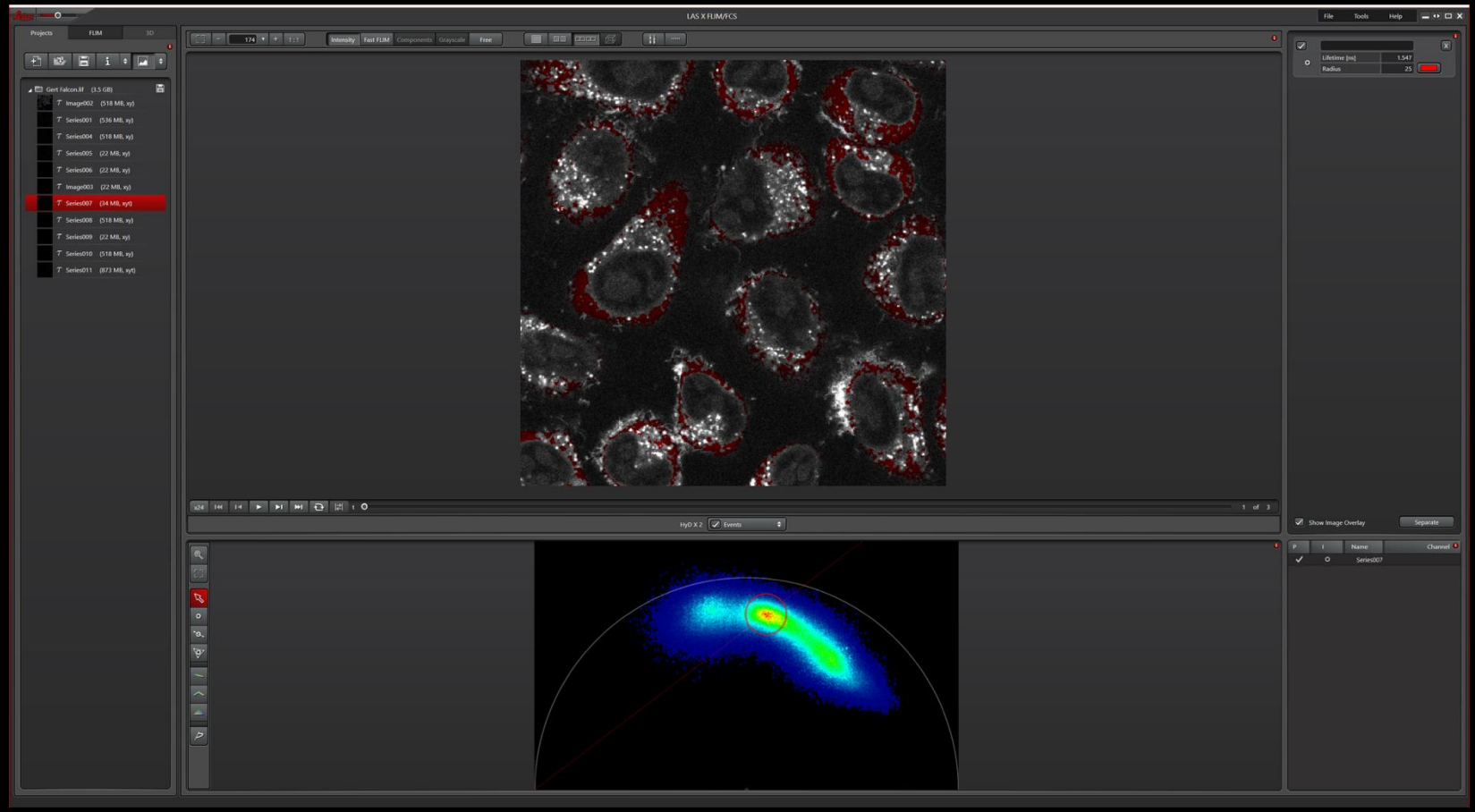
- A phasor plot is a graphical representation of fluorescence lifetimes.
- Each pixel (or decay curve) is represented by a point in the phasor (g, s) plane.
- Coordinates are derived from the Fourier transform of the fluorescence decay.



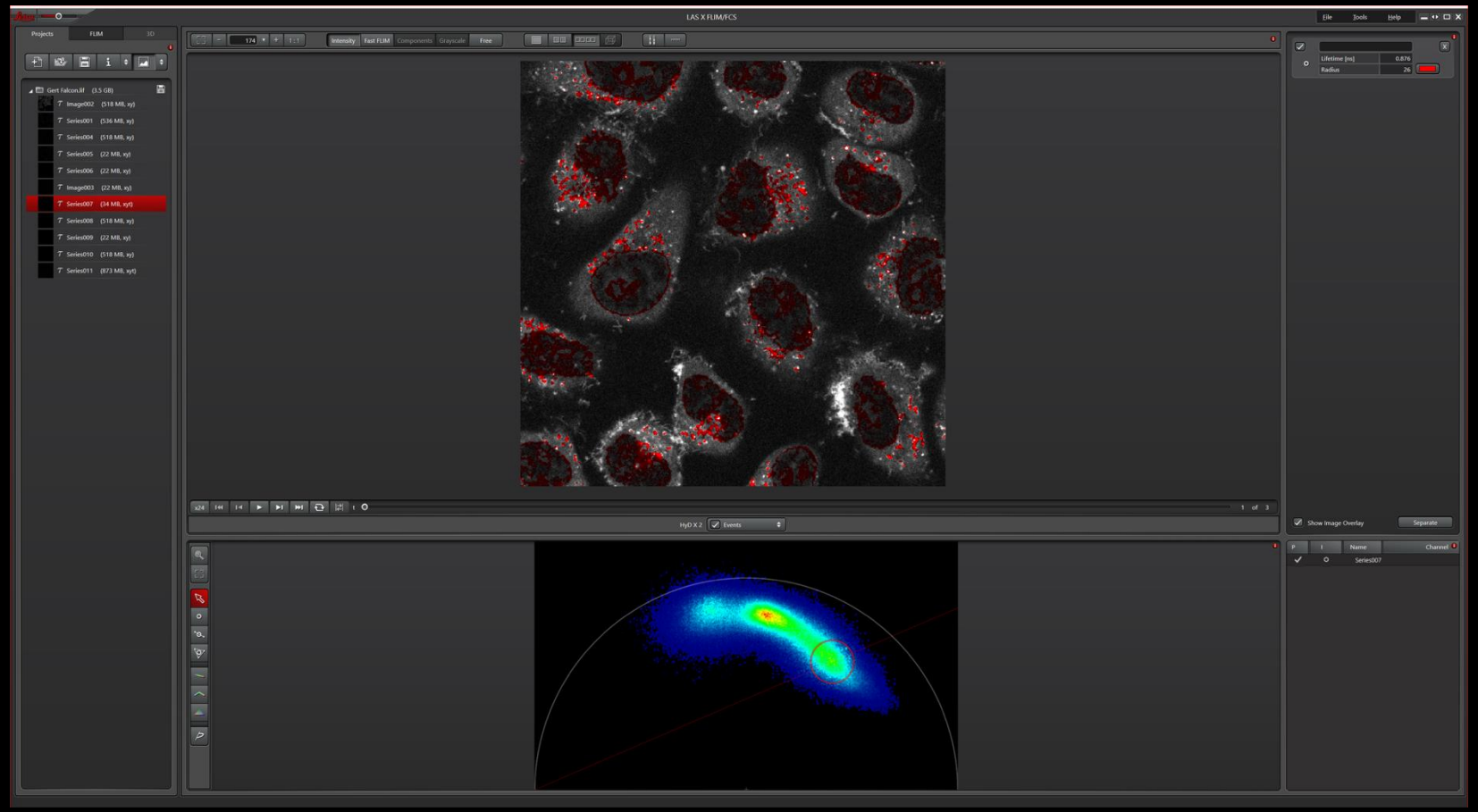
Phasor plot analysis



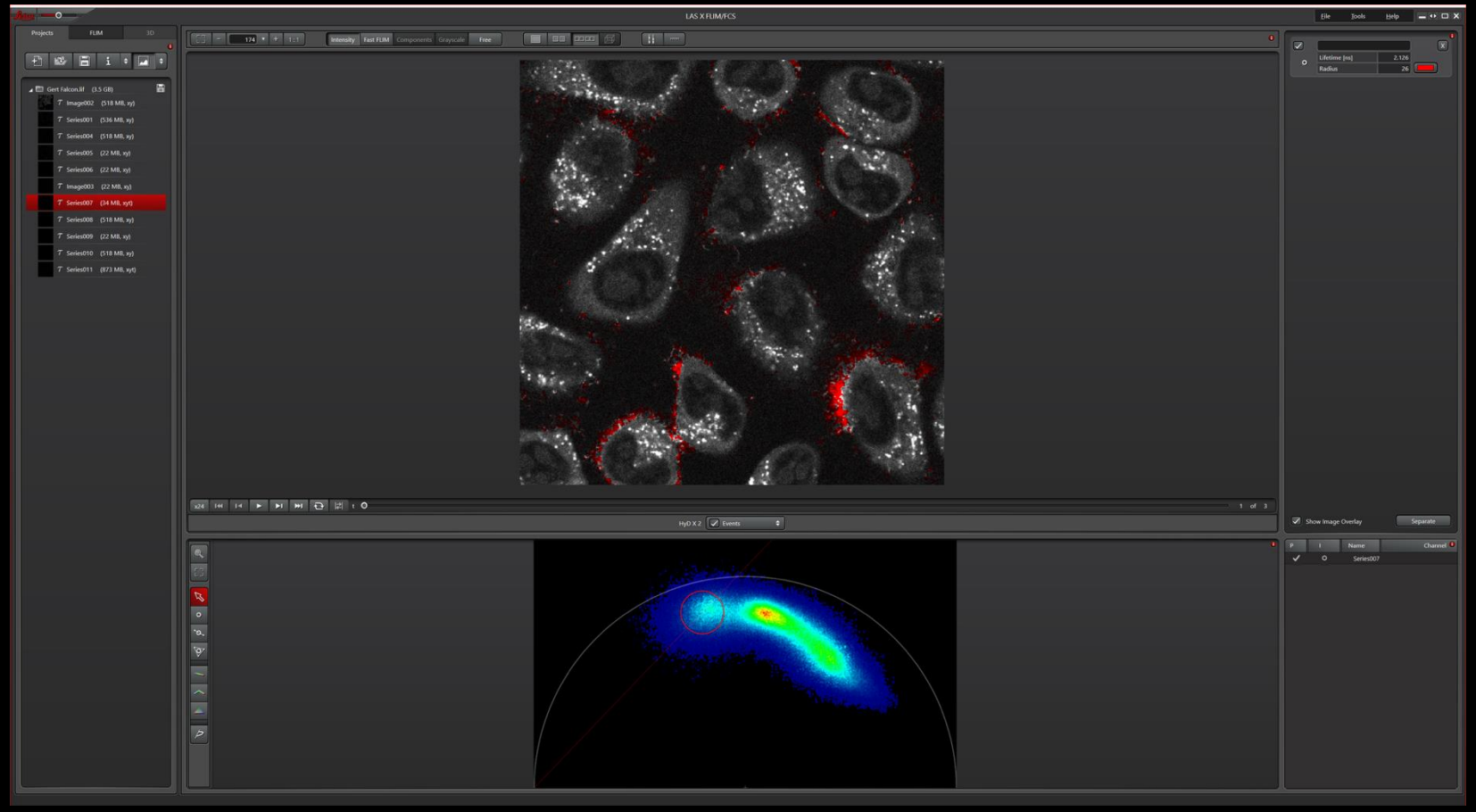
Phasor plot analysis



Phasor plot analysis

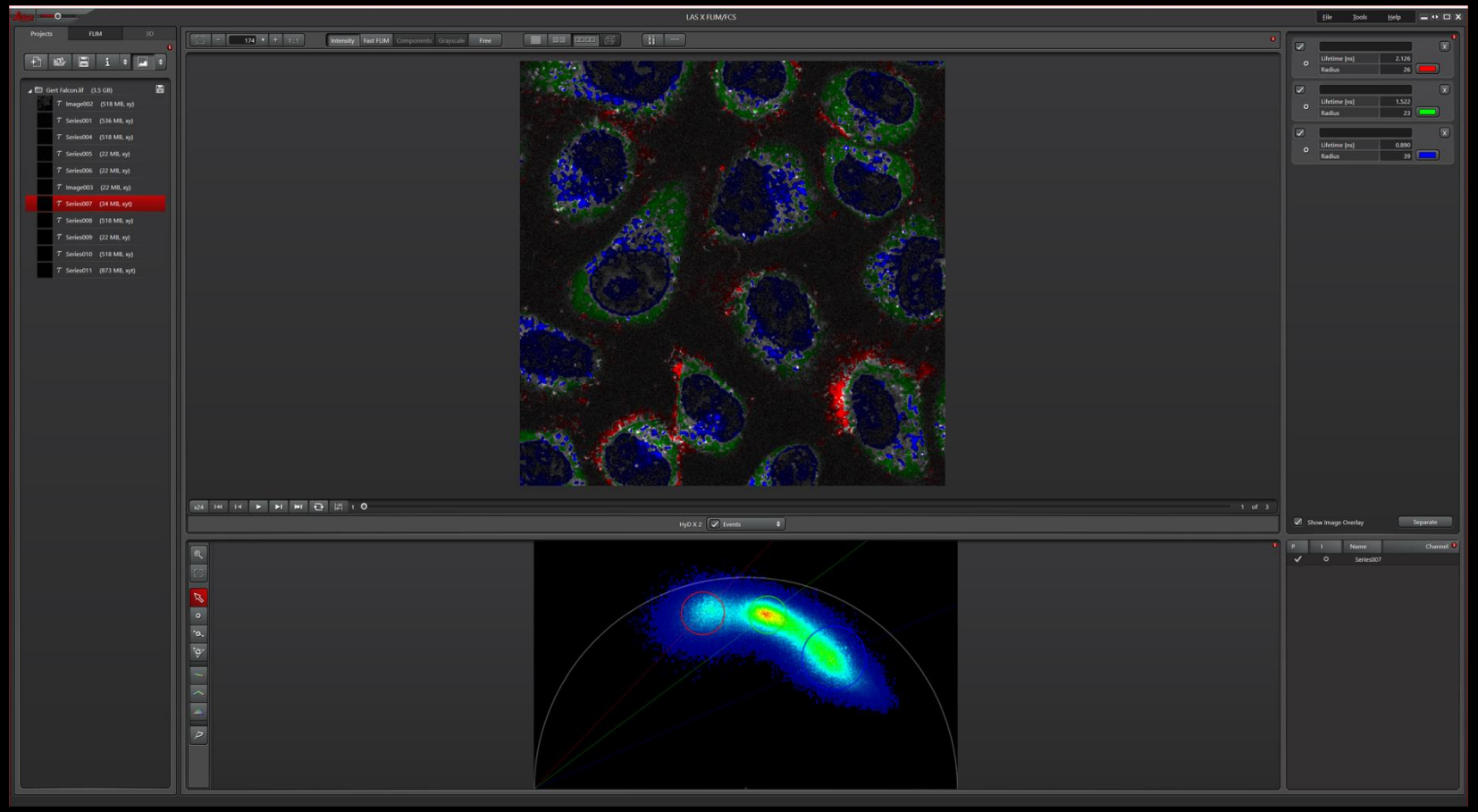


Phasor plot analysis



Phasor plot analysis

Model-free analysis of complex mixtures and visualization of different species based on their unique "fingerprint" or position in the plot.





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Infectious diseases



Nektarios Tavernarakis
Molecular Systems Biology



Matthew Vander Heiden
Precision Cancer Medicine



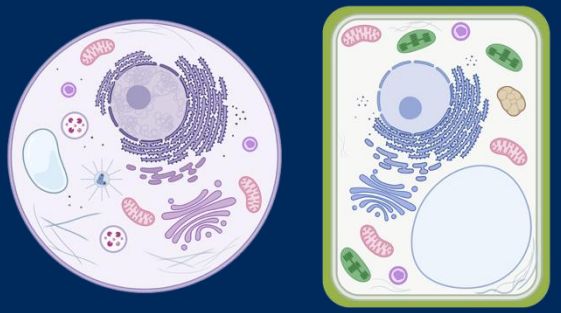
Alexander Wlodawer
Structural Biology



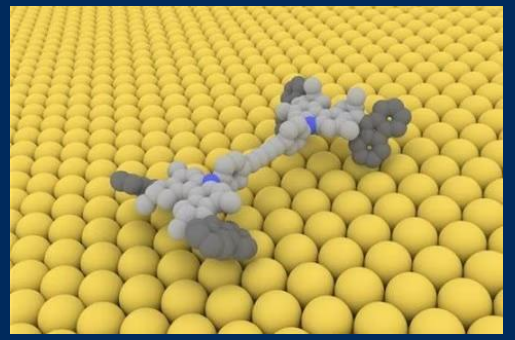
Peter Ratcliffe
Nobel Prize 2019
Oxygen sensing of cells

PROGRAMME TOPICS

INTRACELLULAR LANDSCAPES: DECODING THE CELL'S INNER ORGANIZATION

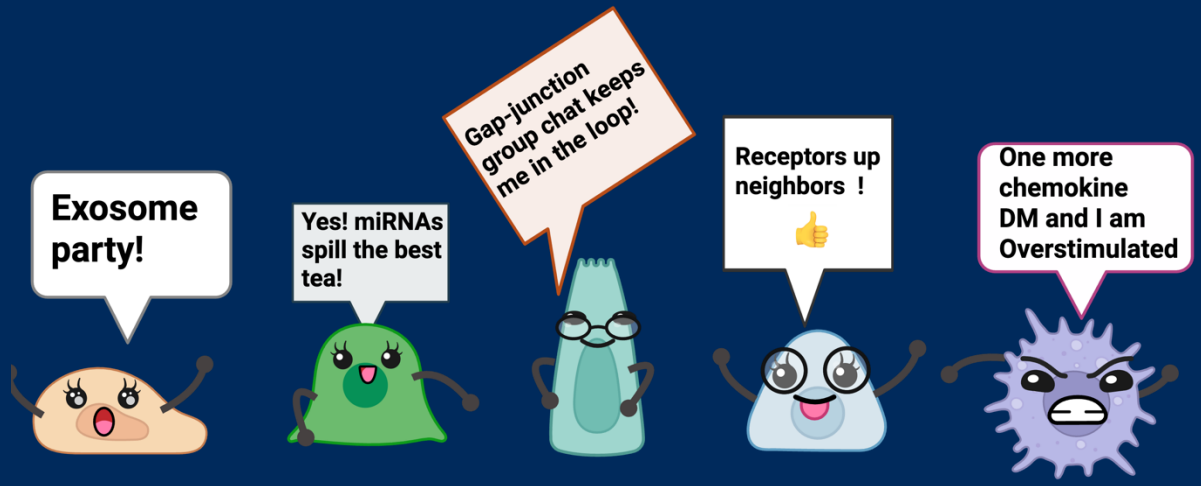


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Acknowledgements



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Sanne van Doorn

Jente Stouthamer

Vanessa Polet Carrillo Carrasco

Mariska Twaalfhoven

Mervin de Jong

Peter Bos

Dippayan Sauta

Isabella Sanders

NWO
Netherlands Organisation for Scientific Research

$$g = \frac{\int_0^\infty I(t)\cos(\omega t)dt}{\int_0^\infty I(t)dt}$$

$$s = \frac{\int_0^\infty I(t)\sin(\omega t)dt}{\int_0^\infty I(t)dt}$$

- $\omega = 2\pi f$ (modulation angular frequency)
- g: real (cosine) component
- s: imaginary (sine) component