MAX PERUTZ LABS VIENNA

HDYDI

Visualization of Protein Interactions in Living Cells by Homo- and Hetero-FRET

Paula Dlugosz
Johannes Nimpf Lab

A joint venture of

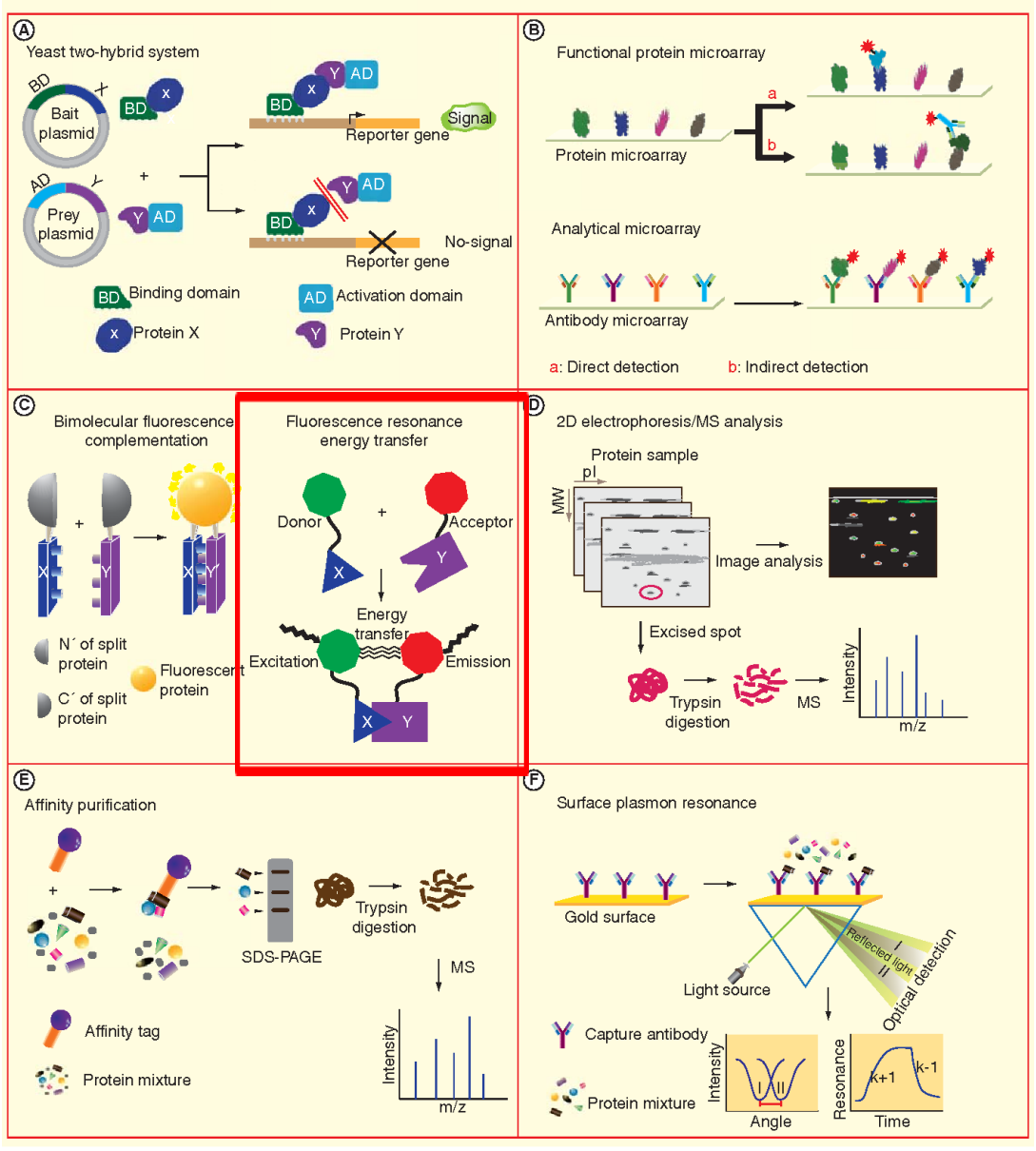
universität wien

MEDIZINISCHE UNIVERSITÄT WIEN

Part of

Vienna BioCenter

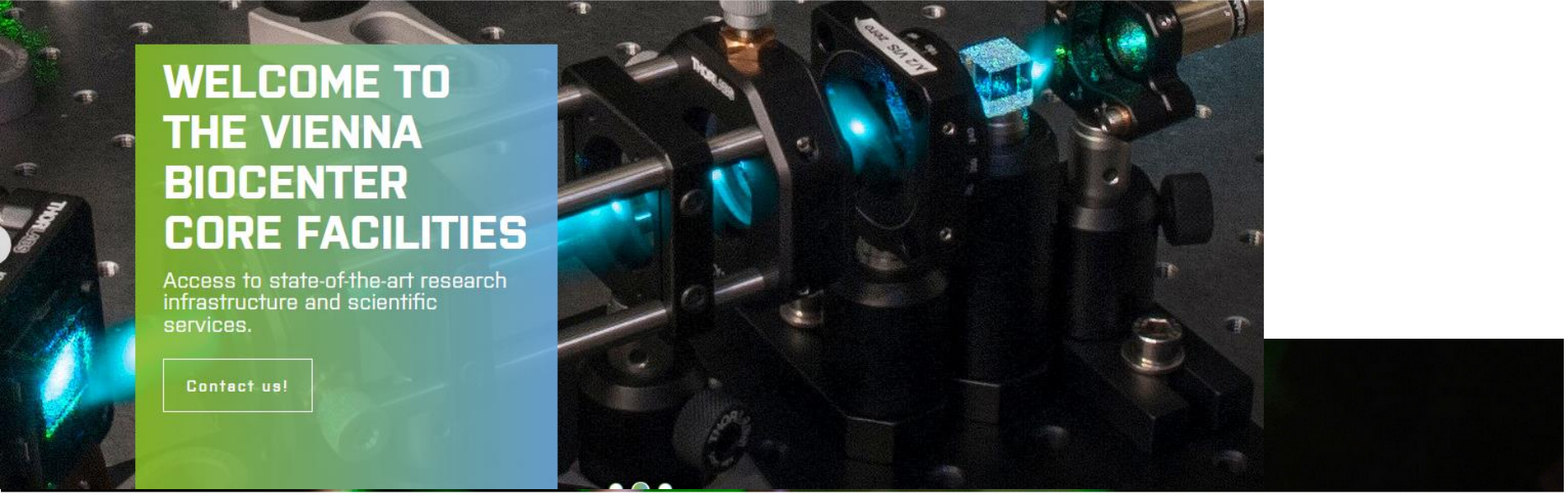
Methods to investigate protein-protein interactions



- Bimolecular fluorescence complementation (BiFC)
- Fluorescence cross-correlation spectroscopy (FCCS)
- Number&Brightness (N&B)
- MicroScale Thermophoresis (MST)
- Isothermal Titration Calorimetry (ITC)
- Proximity Ligation Assay (PLA)

Direct vs. Indirect

Live vs. fixed/lysed



Advanced Microscopy

Microscopes that let you see things no one has ever seen before!

Optical microscopy has historically played an important role in life-science discovery. Advances in imaging technology are rapidly expanding the boundaries of what is possible, allowing for studies of live cells with unprecedented spatio-temporal resolution and the extraction of diverse biophysical parameters intrinsic to the processes of life.

The **Advanced Microscopy Facility** offers a range of cutting-edge, novel and custom optical microscopy techniques, along with associated expertise and assistance, for life science researchers.

Interested in using one of our techniques? Need additional information? Got a specific project question?

Get in touch with us!

Contact

Kareem Elsayad
Core Facility Head
+43 1 796 2324 7110
+43 664 808 47 7110
E-Mail
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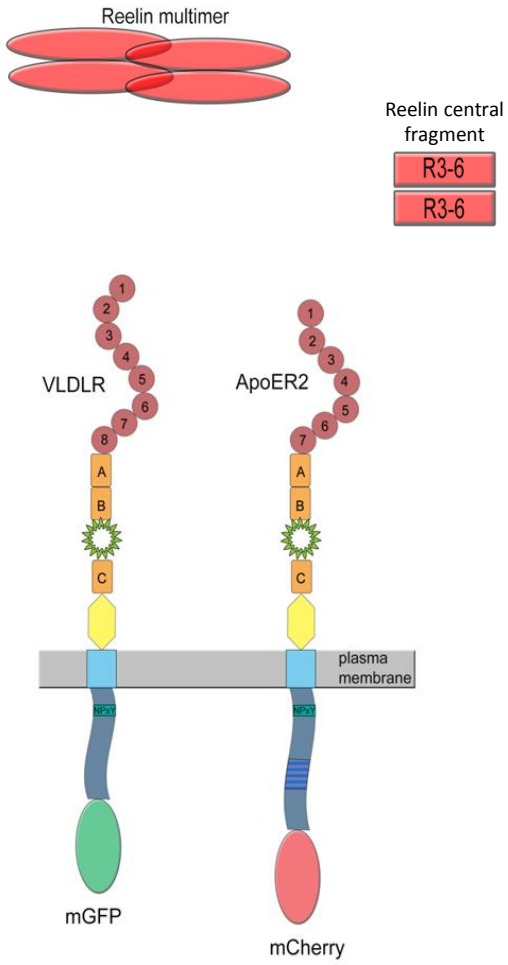
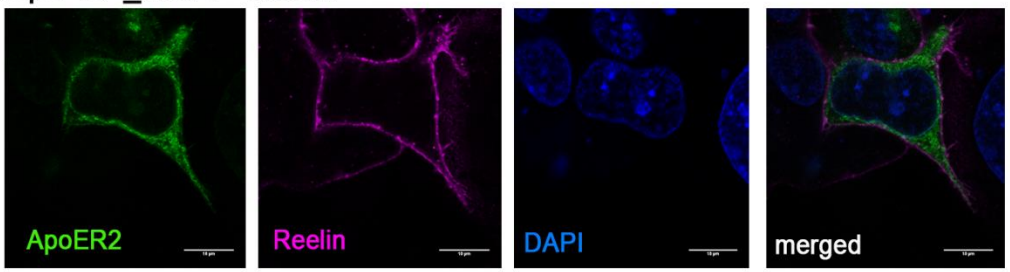
- Team
- MyVBCF

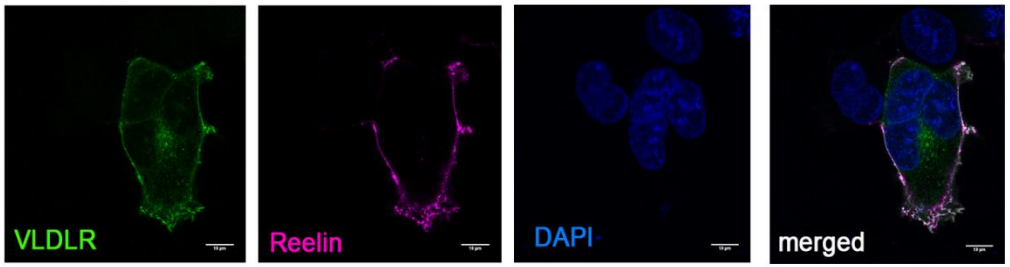
Localization and functionality of fluorescently tagged proteins of interest



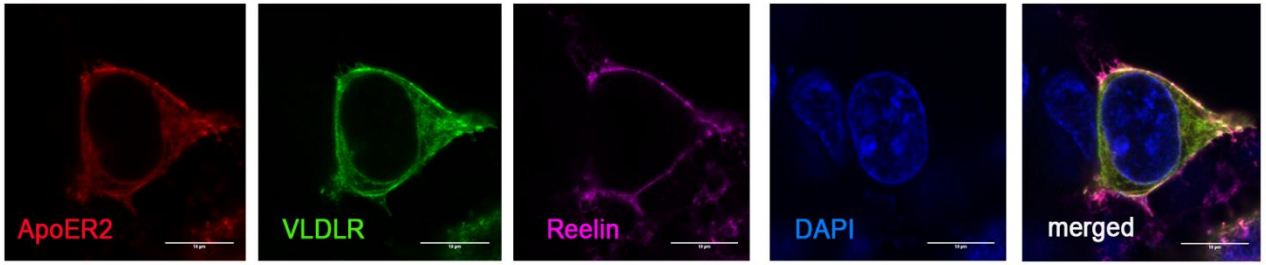
D ApoER2_mGFP+Reelin



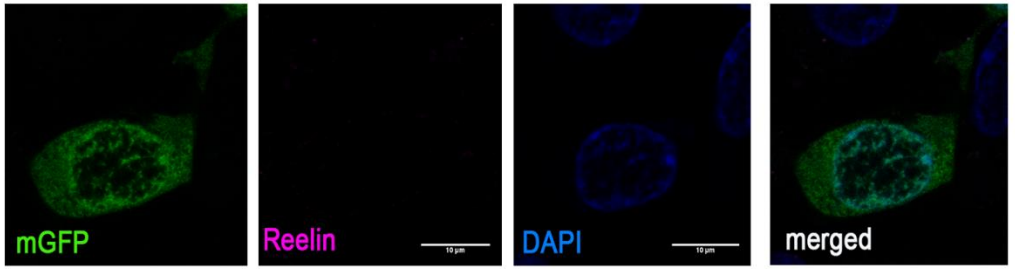
E VLDLR_mGFP+Reelin



F ApoER2_mCherry+VLDLR_mGFP+Reelin



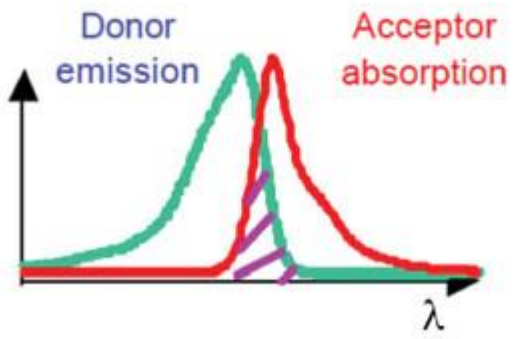
G mGFP+Reelin



HEK293 cells

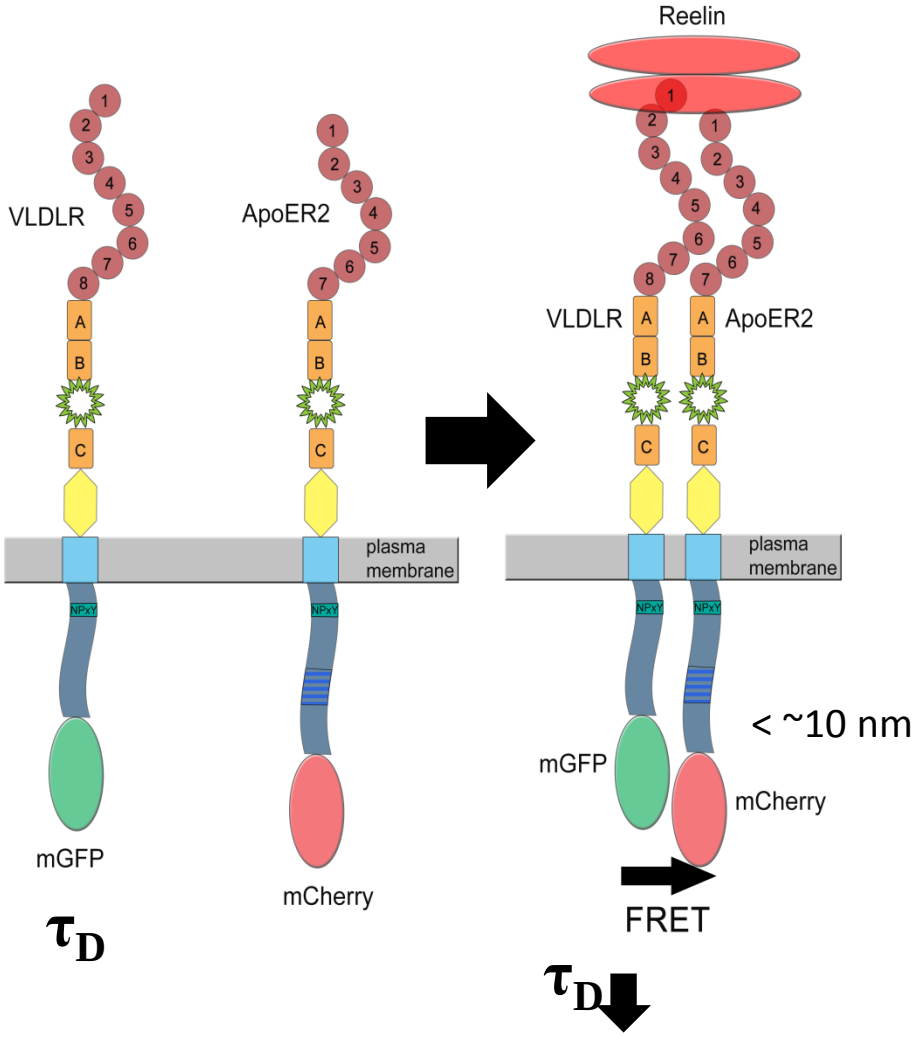
Hetero-FRET (Förster resonance energy transfer between a pair of different species of fluorophores) by FLIM (Fluorescence Lifetime Imaging Microscopy)

1) Spectral overlap

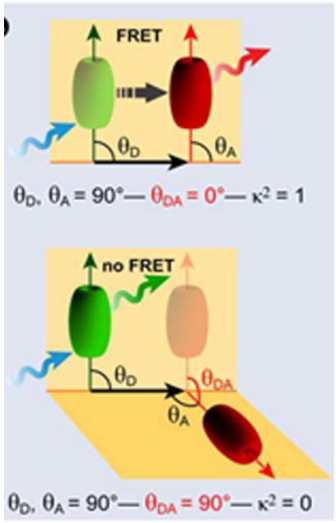


(Padilla-Parra & Tramier, 2012)

2) Proximity

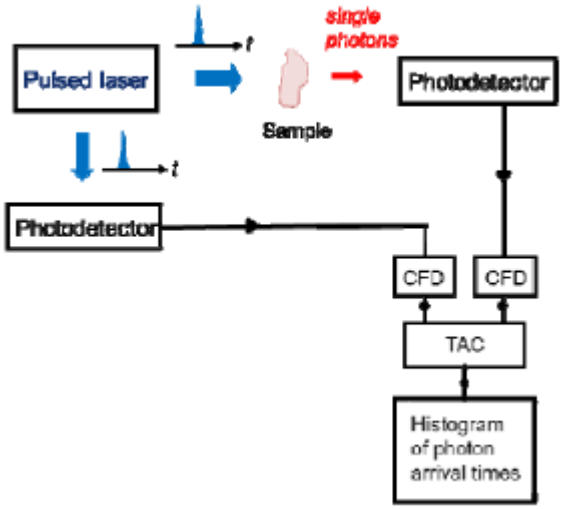


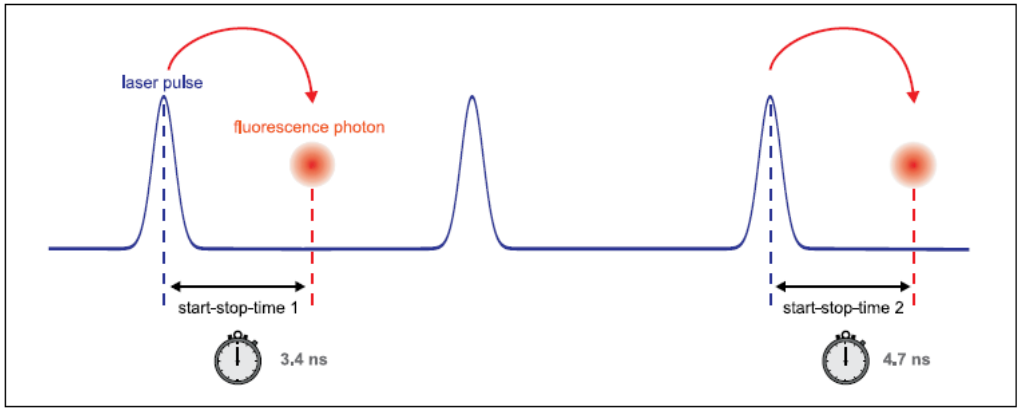
3) Orientation of fluorophores



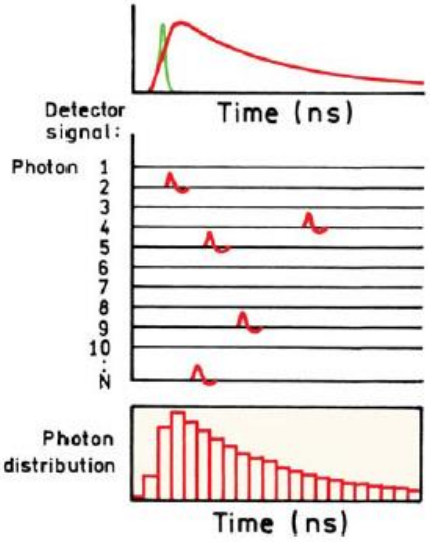
(Preus & Wilhelmsson, 2012)

Time-Correlated Single Photon Counting

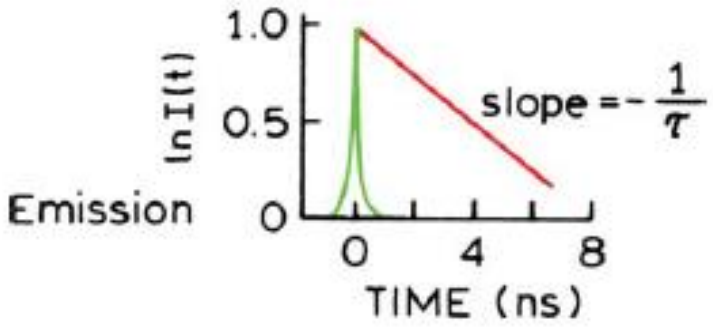




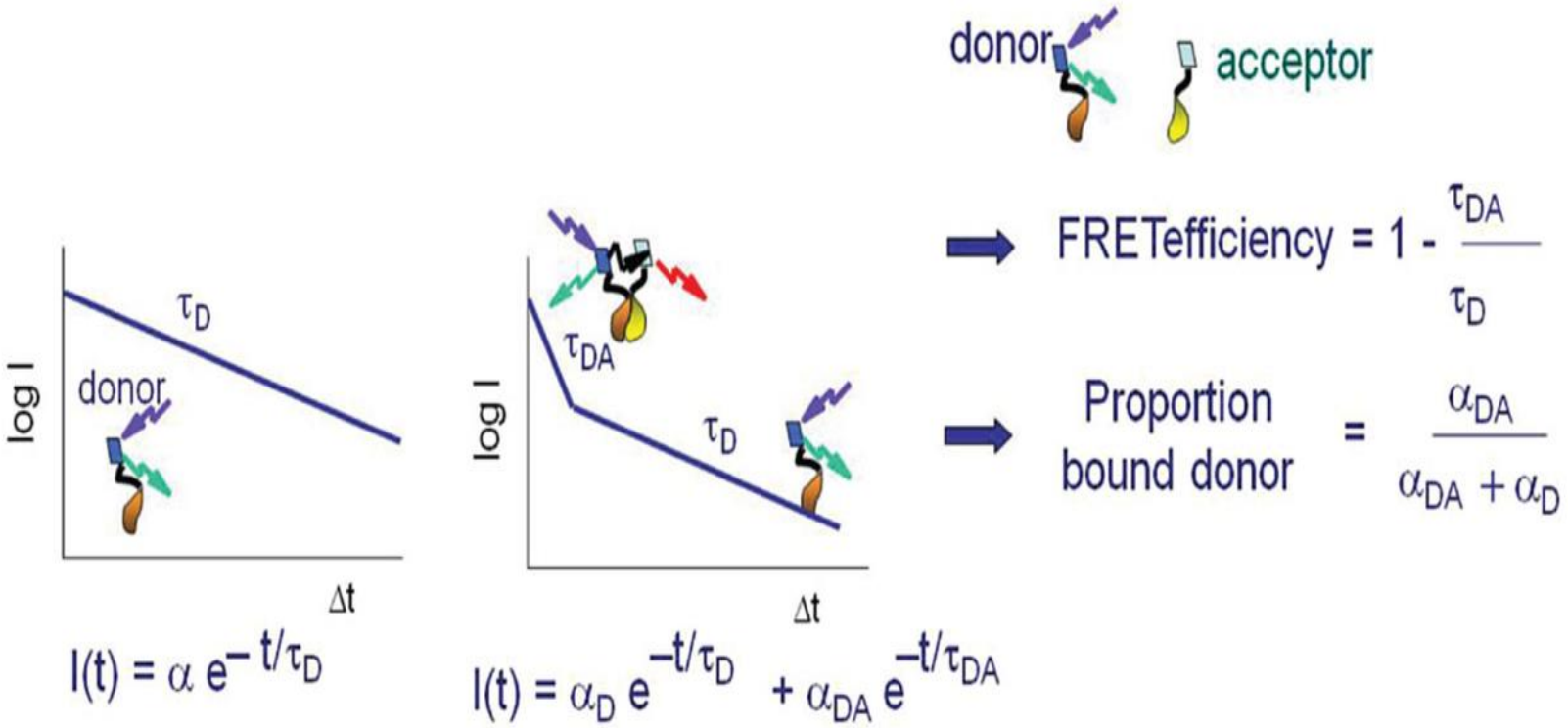
(Picoquant, Germany)



(Principles of Fluorescence Spectroscopy, Lakowicz)

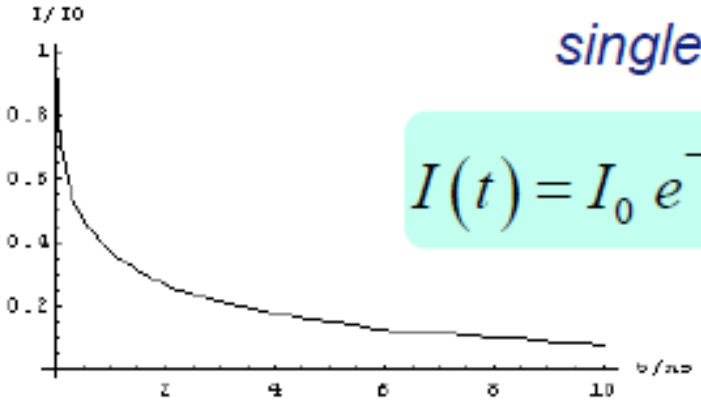


$$I(t) = I_0 \exp(-t/\tau)$$



(Padilla-Parra & Tramier, 2012)

Analysis of fluorescence decay profiles



single *multiple*

$$I(t) = I_0\, e^{-t/\tau} \rightarrow I(t) = I_0 \sum_{j=1}^{J} \alpha_j\, e^{-t/\tau_j}$$

Fitting complex decay profiles requires more detected photons

- achieving ~10% accuracy for single exponential fit requires 100's photons
- to fit double exponential decay model requires 1000's photons

***BUT** # photons/pixel is constrained in many biological experiments by photobleaching/toxicity, time resolution …*

⇒ *fit to single exponential decay model anyway*

⇒ *segment ROI, bin pixels and fit to (complex) model*

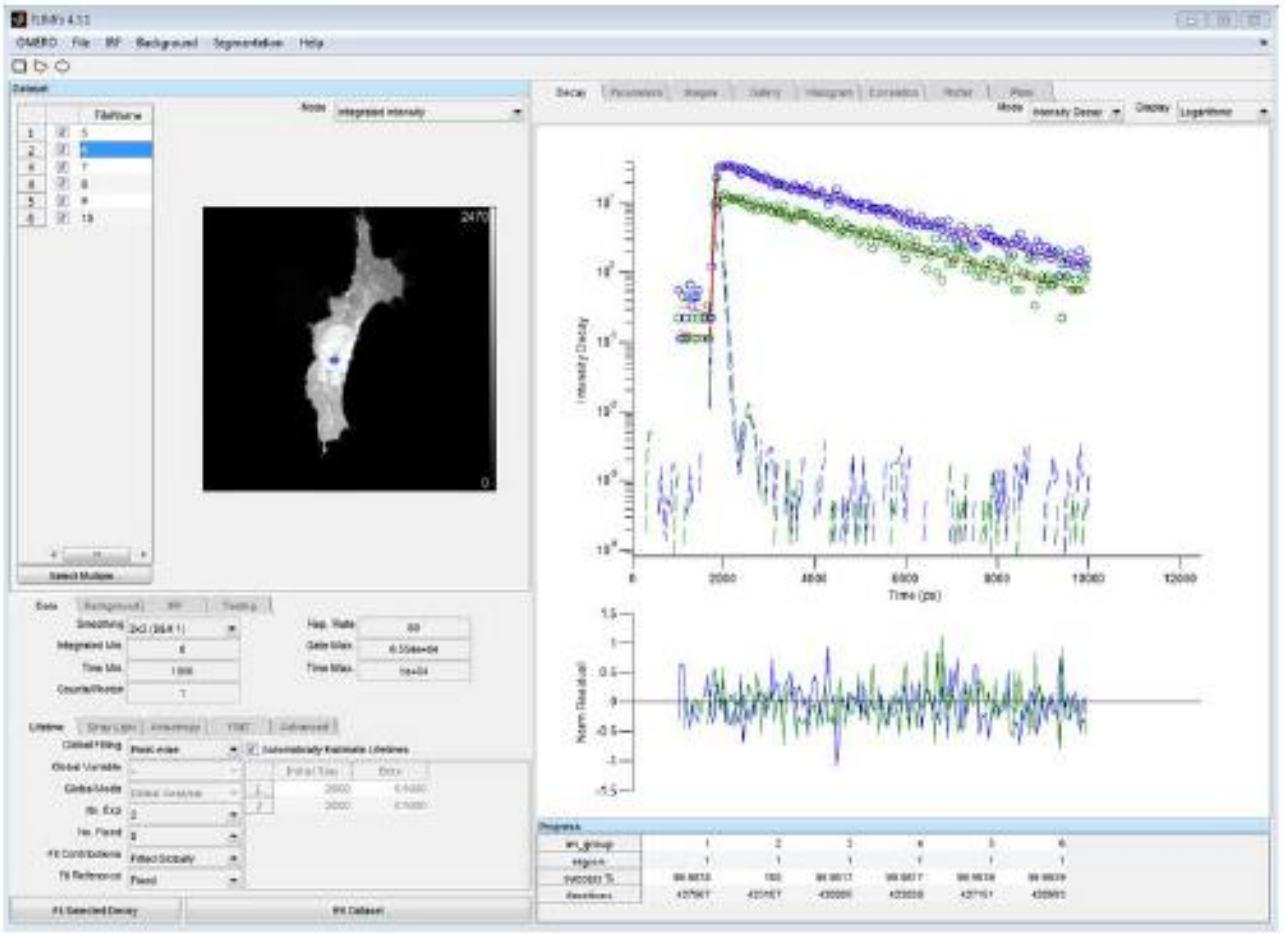
⇒ *global fitting to complex model*

⇒ *no fitting (phasor analysis)*

Imperial College London

"*FLIMfit*" by Sean Warren

OME



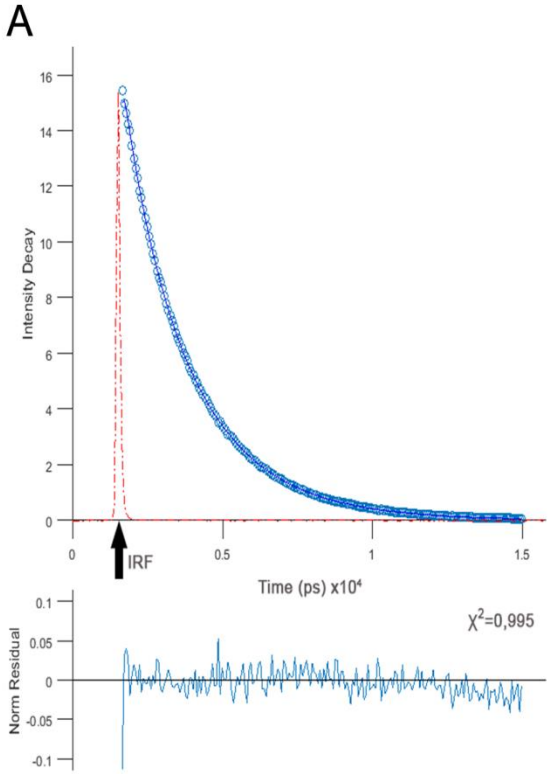
Open-source software available via Open Microscopy Environment

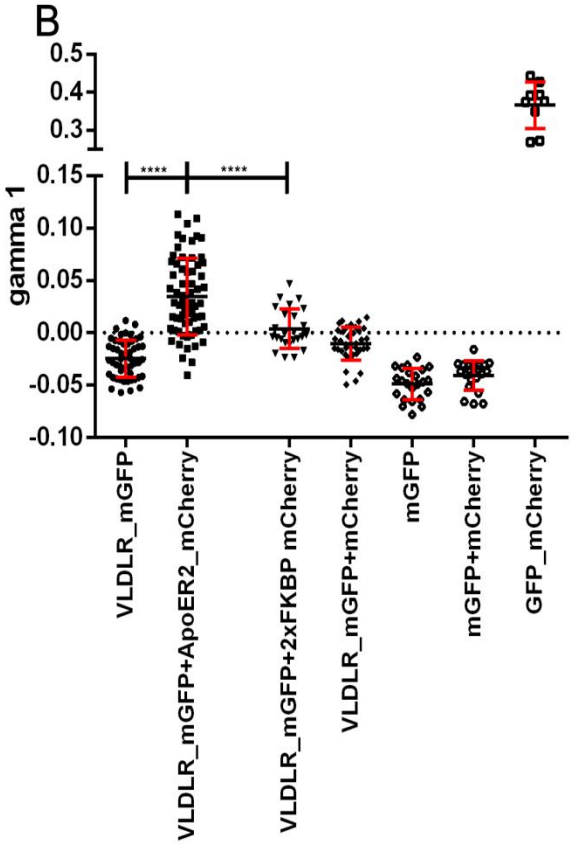
http://downloads.openmicroscopy.org/latest/flimfit

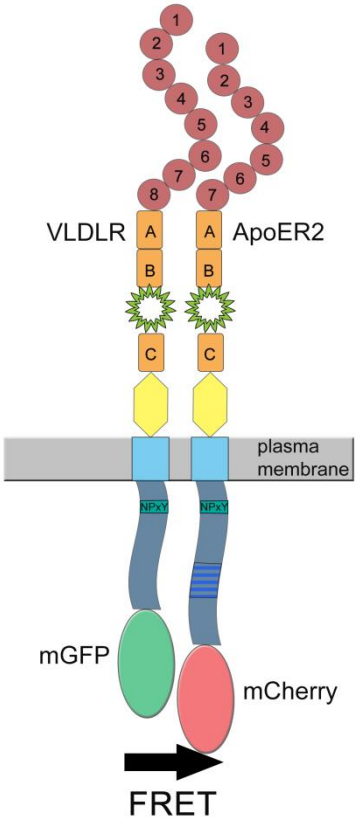
wellcome trust

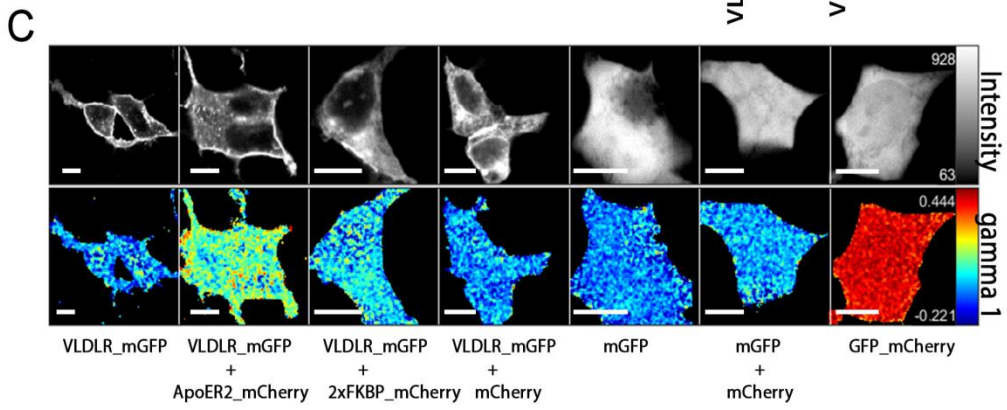
OMERO

ApoER2 and VLDLR form hetero-oligomers as assessed by fluorescence-lifetime imaging microscopy





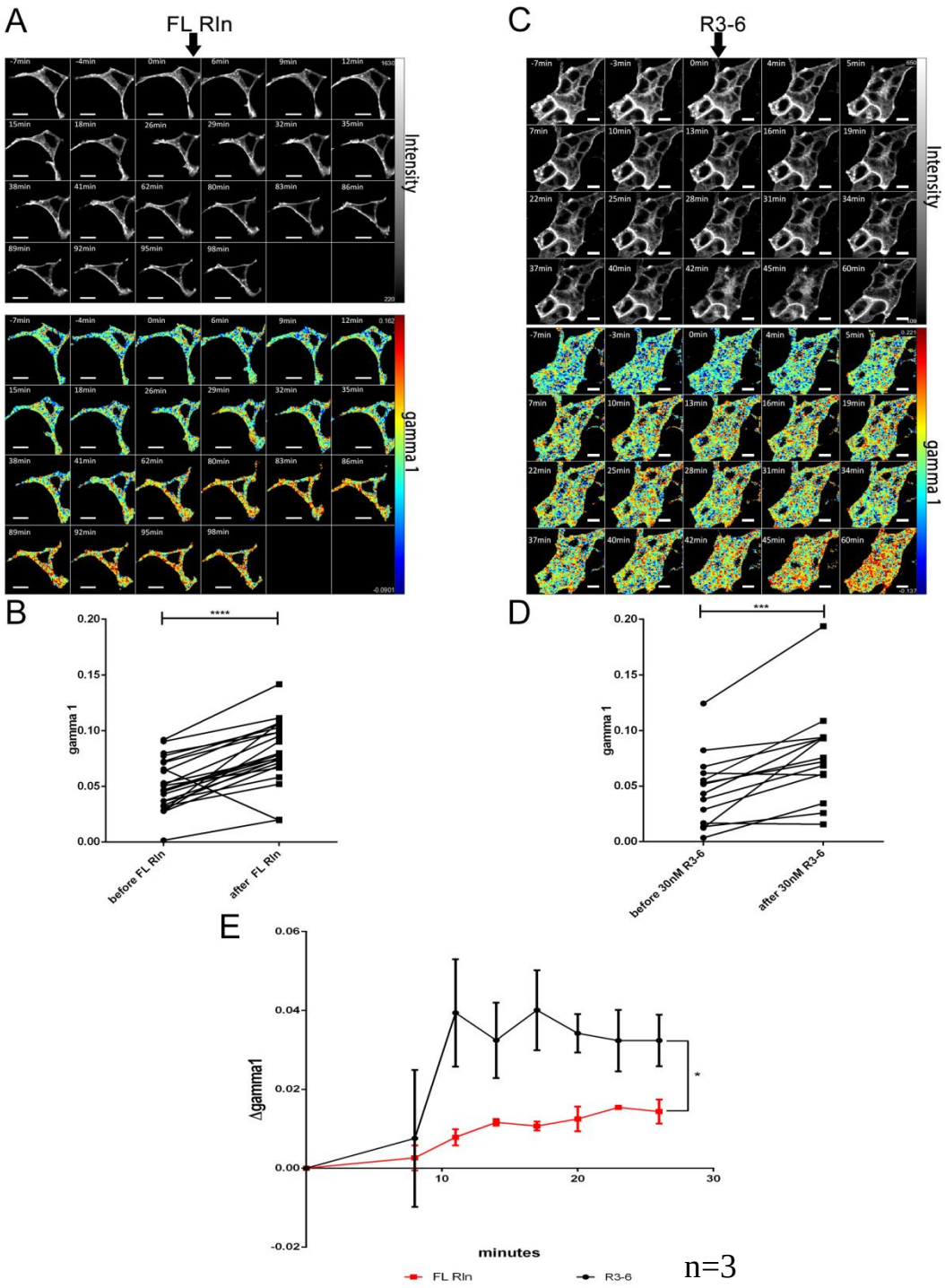




Gamma 1 = contribution of FRET with globally fitted efficiency (E) = 0.5

IRF= instrument response function

Differential effect of full length Reelin and R3–6 on the increase of cluster size of VLDLR/ApoER2 hetero-oligomers

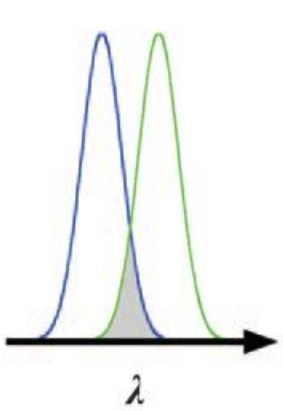


Homo-FRET Fluorescence Anisotropy Imaging as a Tool to study Molecular Self-Assembly in Live Cells

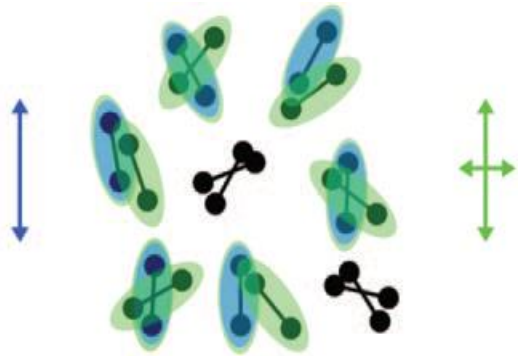
Using only one fluorophore brings advantages over hetero-FRET

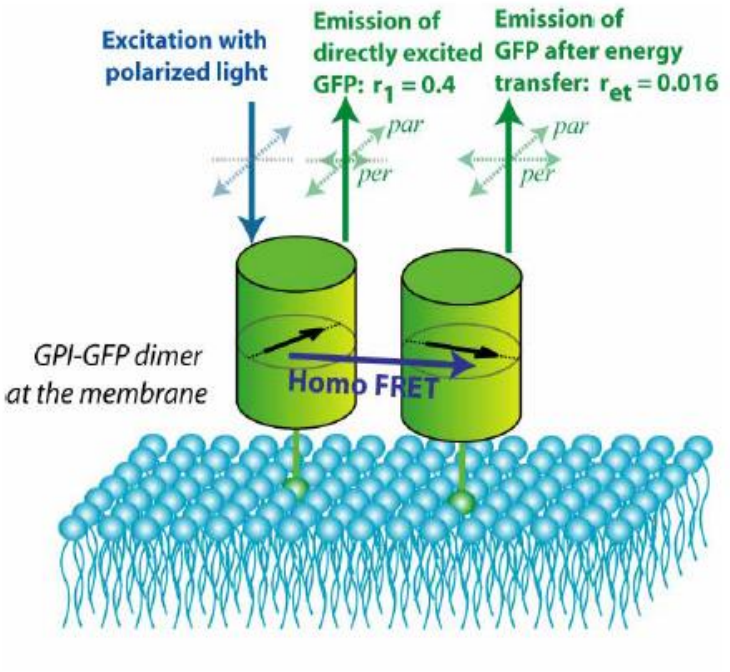
1) all the fluorescent molecules are analyzed by this method comparing to hetero-FRET where dimers composed of donor-donor or acceptor-acceptor cannot be identified;
2) easier fluorescent labelling as only one fluorescent probe is required;
3) problem with differential expression levels of donor and acceptor-tagged proteins does not have to be taken into consideration;
4) possibility to perform multiplexed imaging which allows to follow spatio-temporal correlation of signaling pathways.

Homo-FRET Fluorescence Anisotropy Imaging as a Tool to study Molecular Self-Assembly in Live Cells



GFP excitation and emission spectra

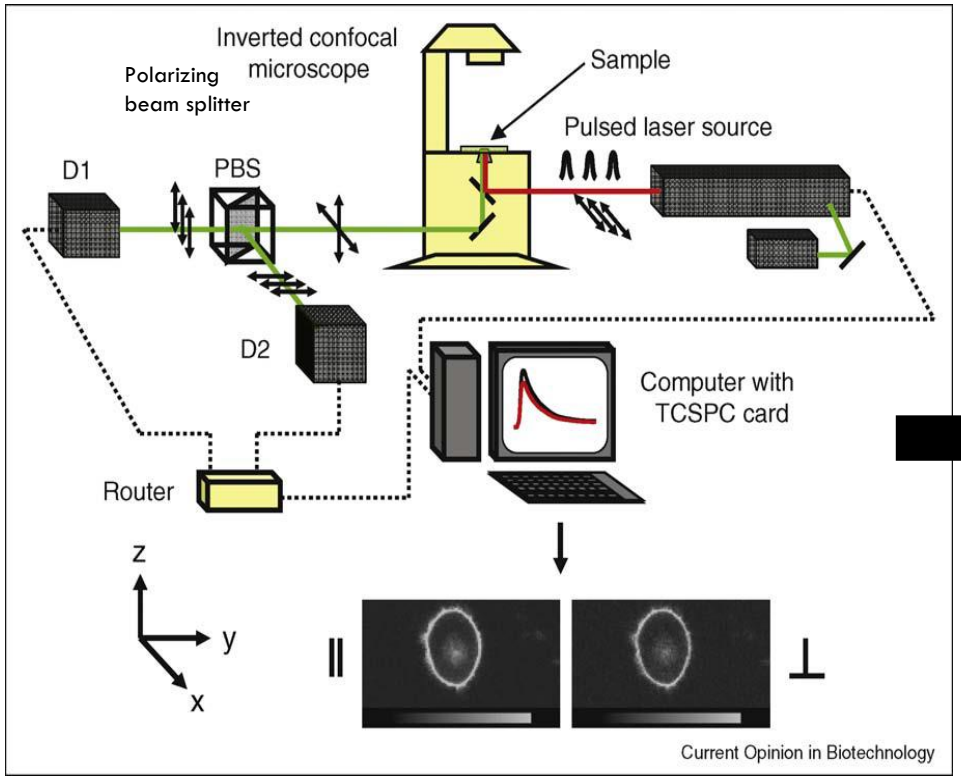


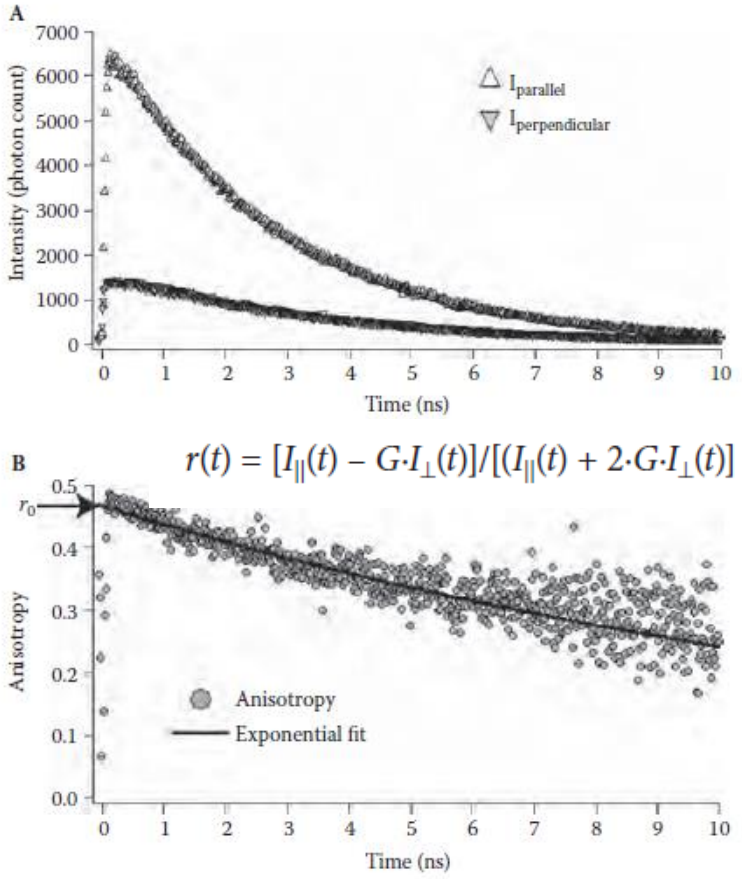


(Bader, Hofman et al., 2007)
(Chan, Kaminski et al., 2011)

HomoFRET **depolarisation increases with the number of participating fluorophores**.
This effect presents an opportunity for cluster size quantification by measuring the degree of depolarisation.

Time Resolved Fluorescence Anisotropy



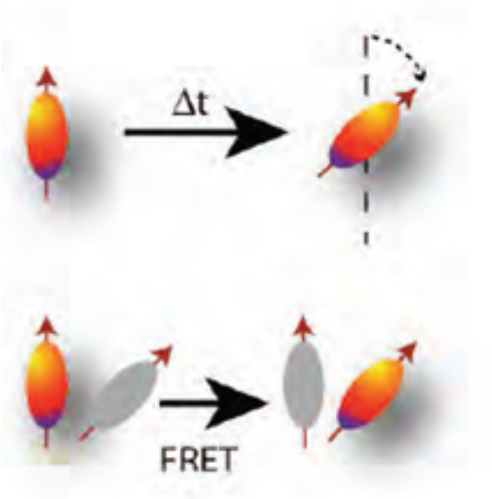


(Chan, Kaminski, and Kaminski Schierle, 2011)
(Vogel, Thaler et al. 2009)

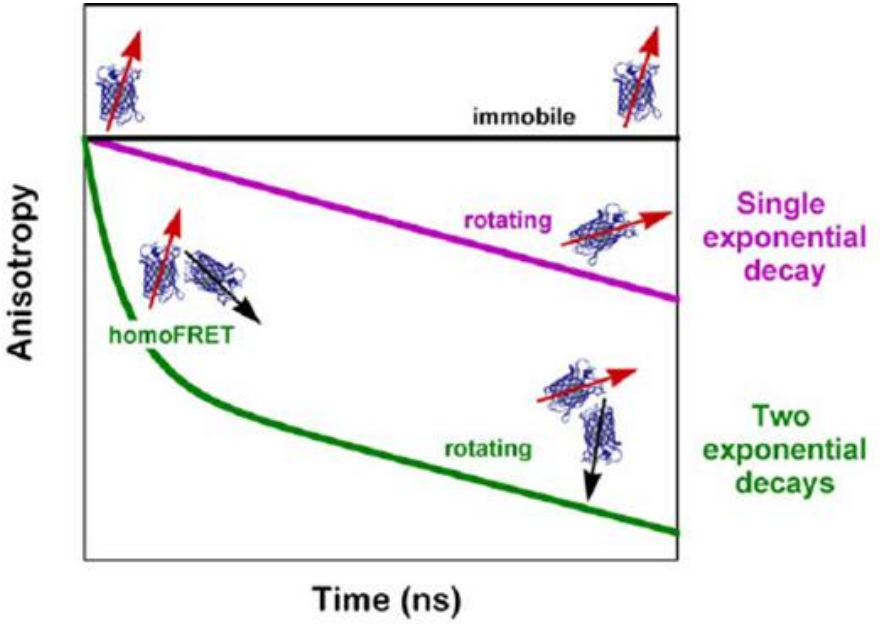
Depolarization of emitted light can arise from

rotation

energy transfer



(Vogel, Thaler et al., 2009)

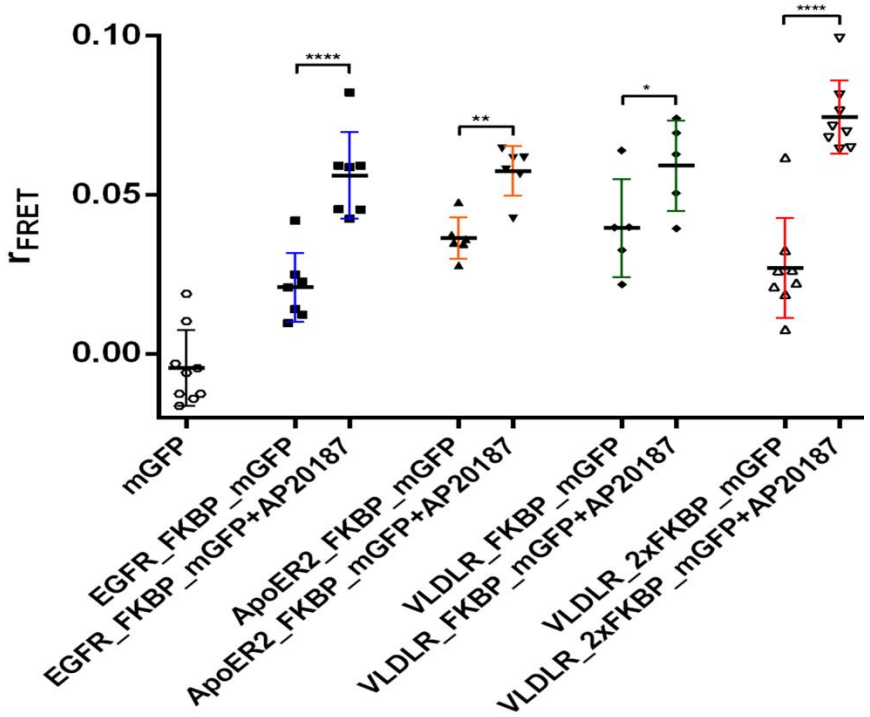


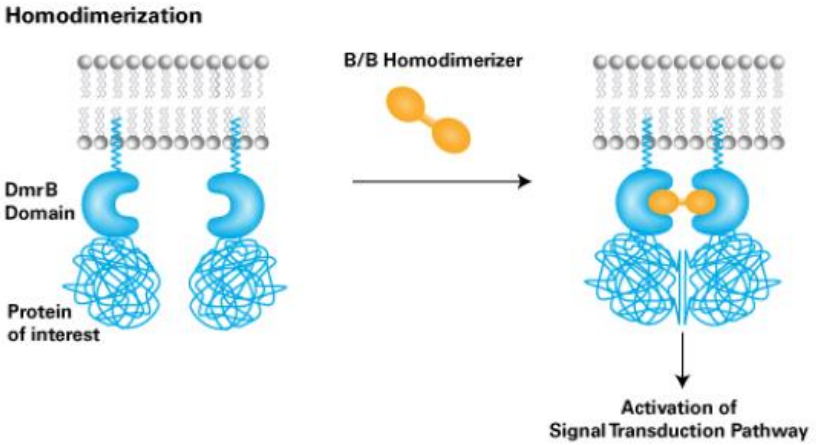
(Altman, Goswami et al., 2007).

Depolarization due to FRET by forced oligomerization of receptor molecules

Figure S2

A





B

HEK293	ApoER2_FKBP_mGFP		EGFR_FKBP_mGFP		VLDLR_FKBP_mGFP	
Parameter	before Homodim.	after Homodim.	before Homodim.	after Homodim.	before Homodim.	after Homodim
tau_1 [ns]	2,453					
tau_2 [ns]	1,309					
beta_1	0,685					
beta_2	0,315					
theta_1 [ns]	102,600					
theta_2 [ns]	1,944					
r_2	0,036	0,0574	0,0209	0,0561	0,0395	0,0591
Number of cells	n=6	n=6	n=7	n=7	n=5	n=5

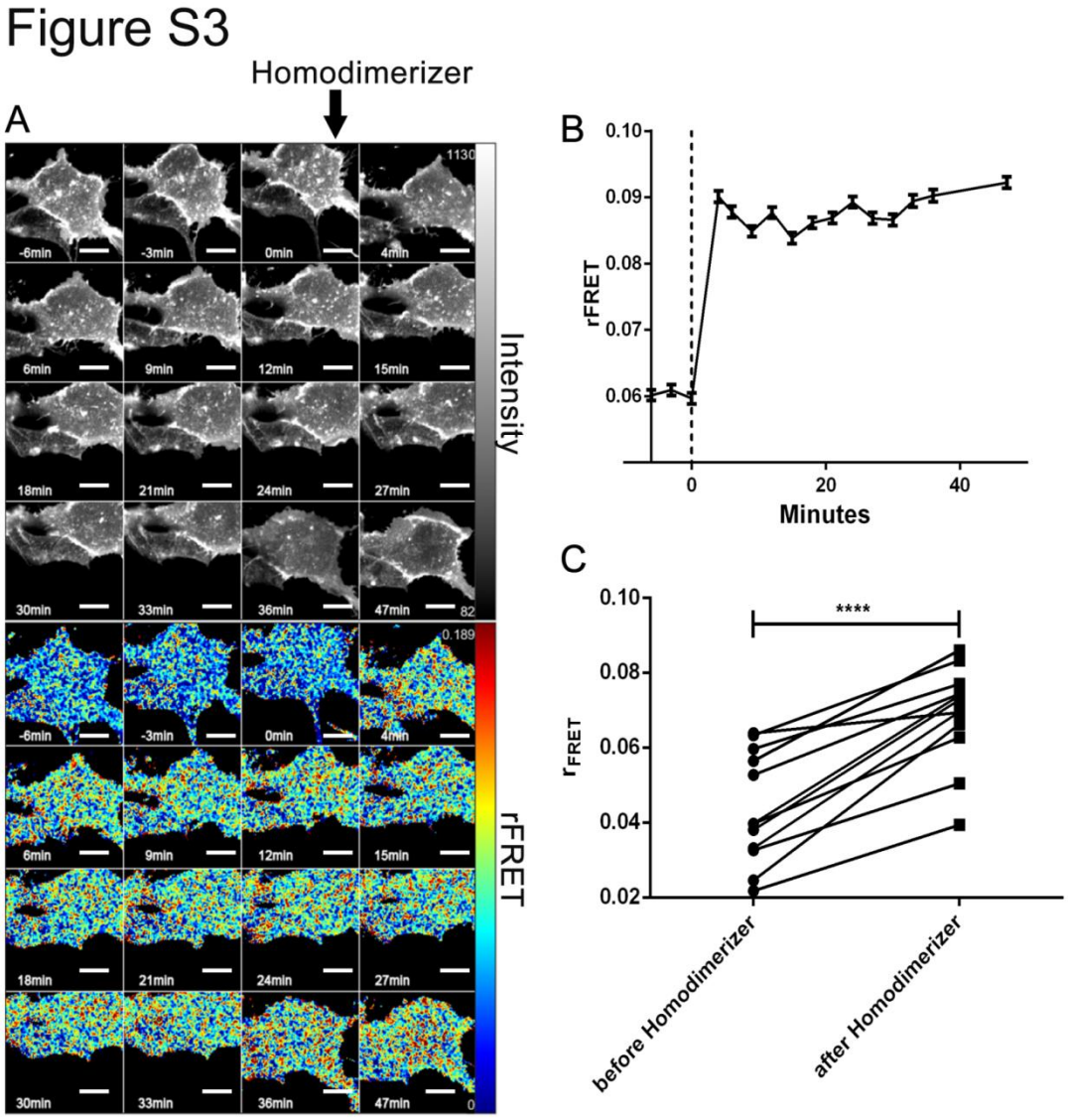
C

Parameter	mGFP
tau_1 [ns]	2,6708
tau_2 [ns]	1,552
beta_1	0,527
beta_2	0,473
theta_1 [ns]	18,825
theta_2 [ns]	2,503
r_2	-0,0044
Number of cells	n=9

From Clontech company

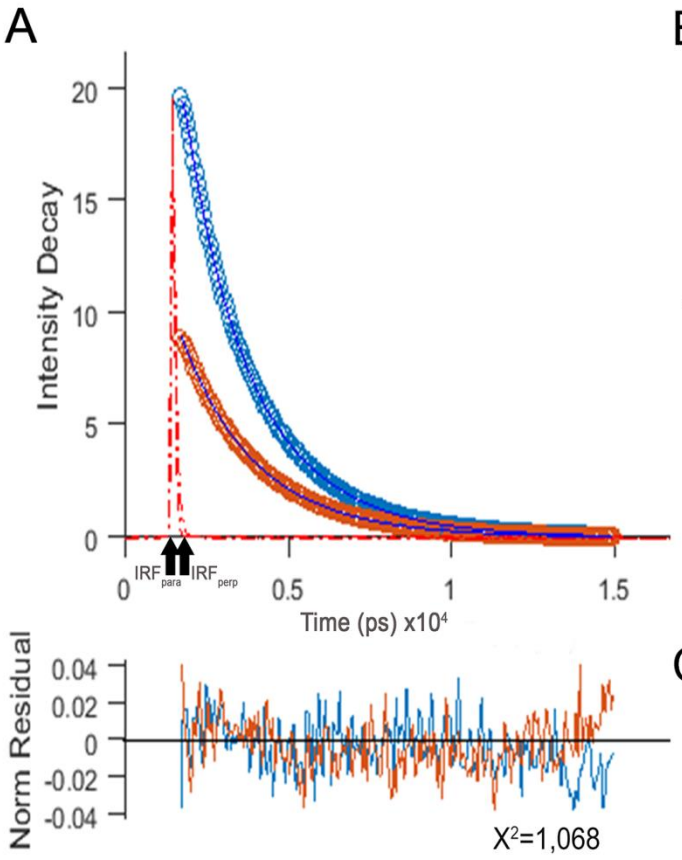
DmrB domain=FKBP domain
B/B Homodimerizer= AP20187

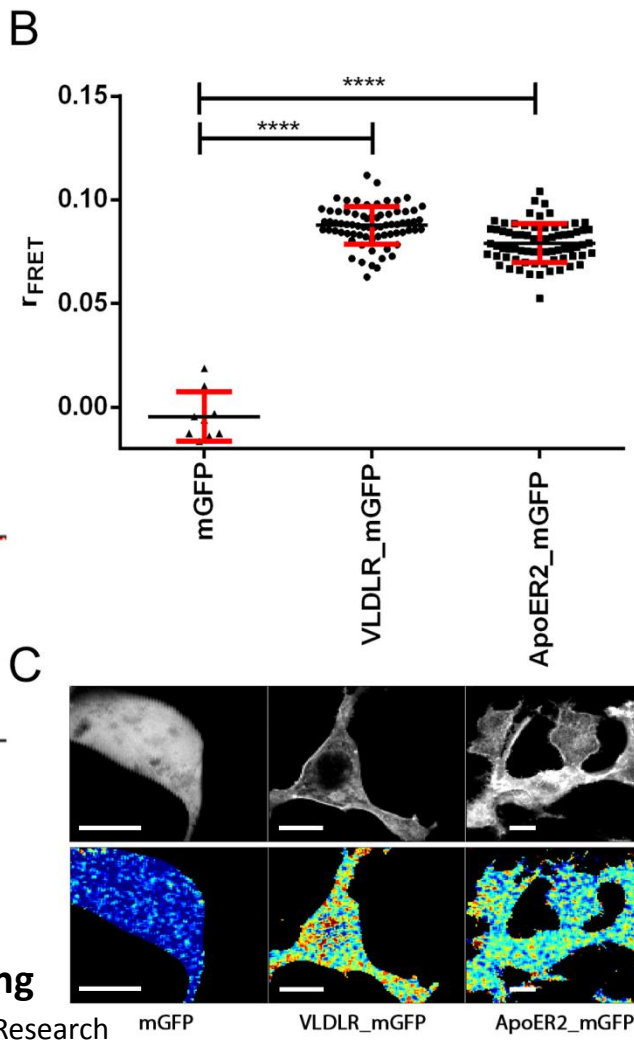
Depolarization due to FRET by forced oligomerization of receptor molecules

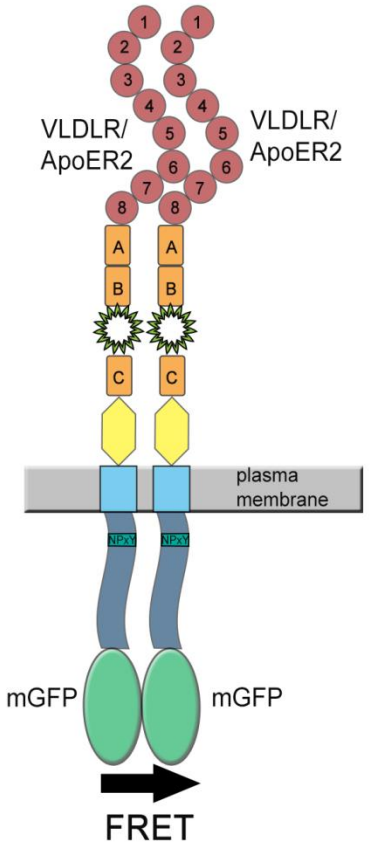


VLDLR_FKBP_mGFP was expressed in HEK293 cells and anisotropy associated with homo-FRET was measured

ApoER2 and VLDLR form homo-oligomers as assessed by time-resolved fluorescence anisotropy imaging





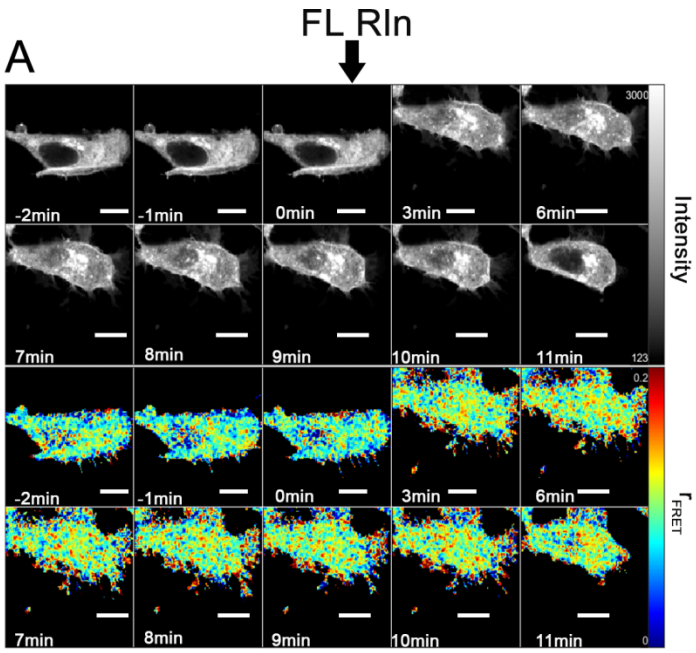


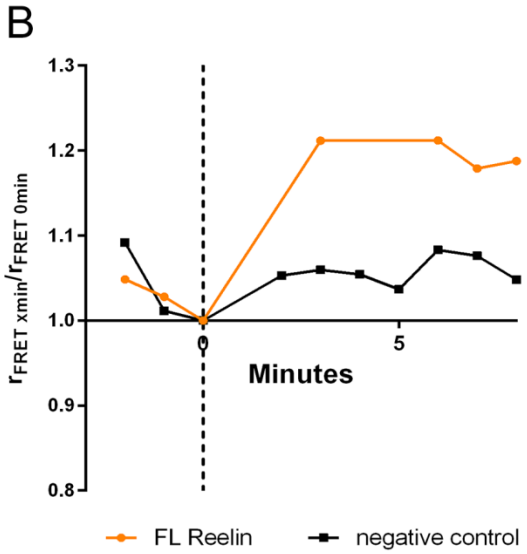
FLIMfit software =global fitting

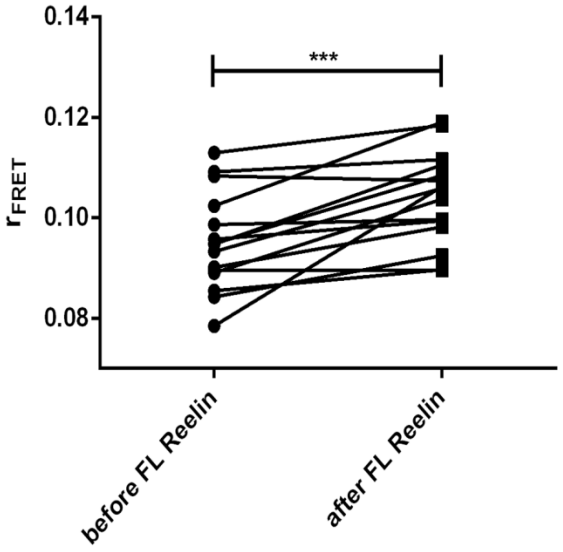
Sean Warren, Garvan Institute of Medical Research

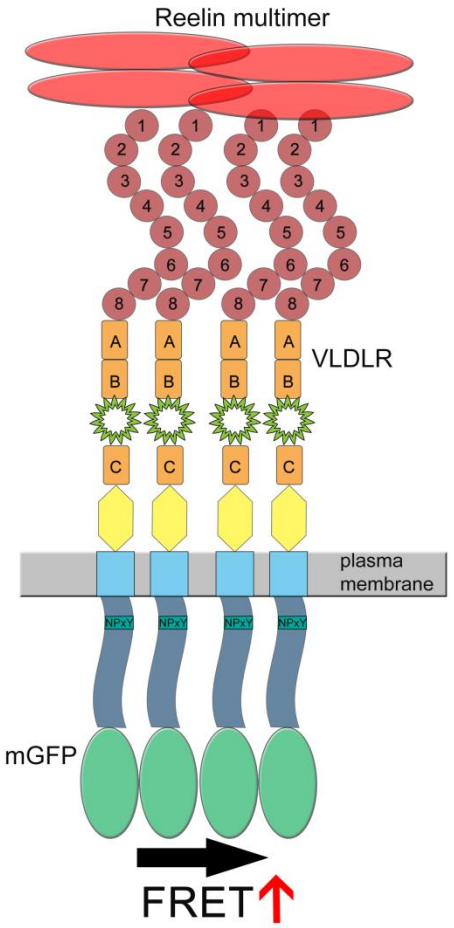
"Homo-FRET Based Biosensors and Their Application to Multiplexed Imaging of Signalling Events in Live Cells" , *Int. J. Mol. Sci.* **2015**

Full length Reelin increases cluster size of VLDLR homo-oligomers

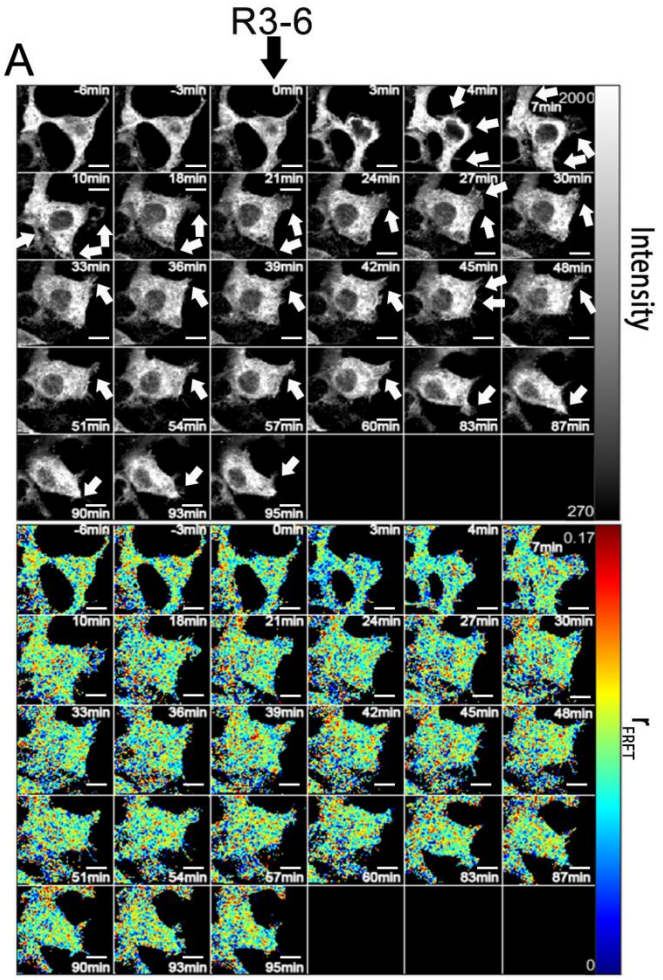


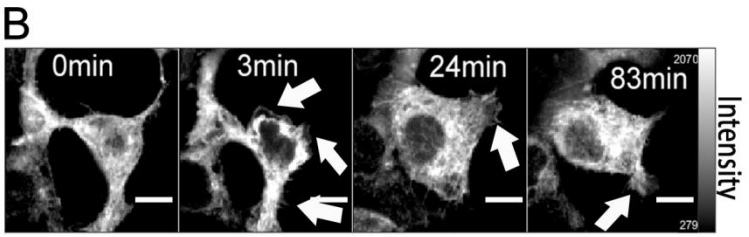


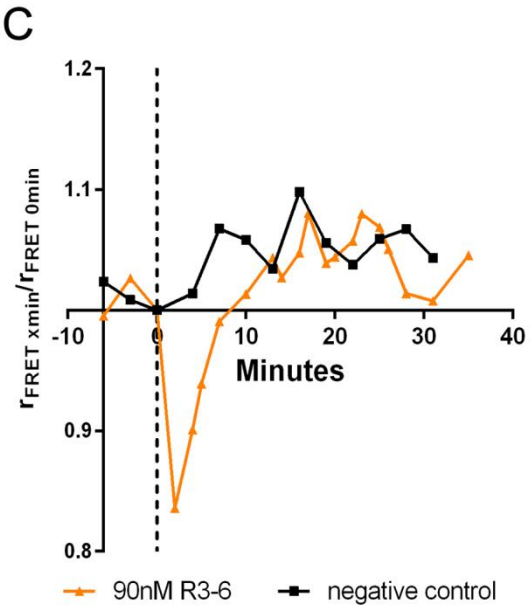


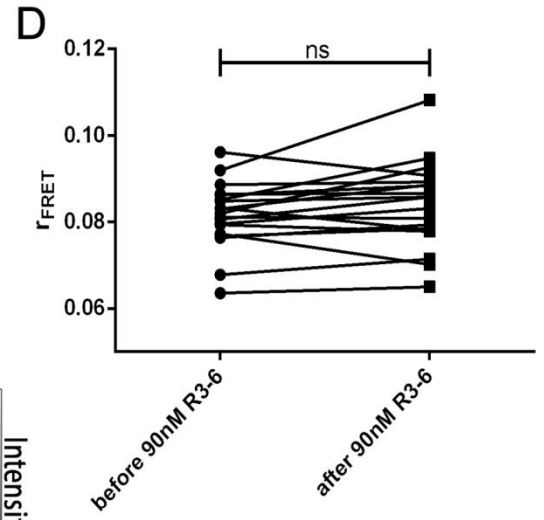


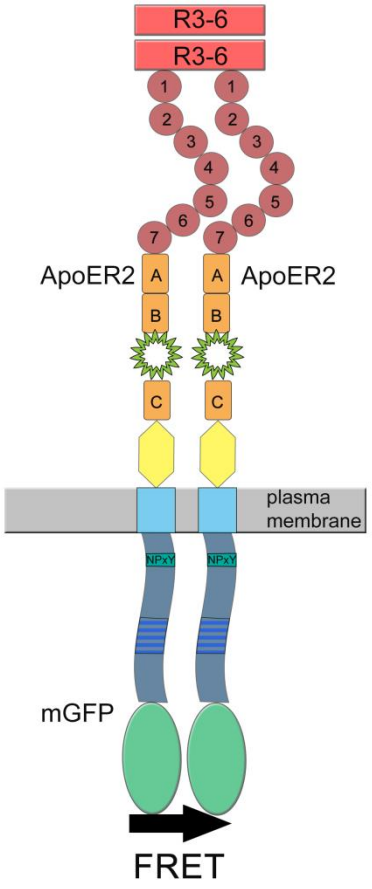
Central fragment of Reelin (R3–6) does not increase cluster size of ApoER2 homo-oligomers



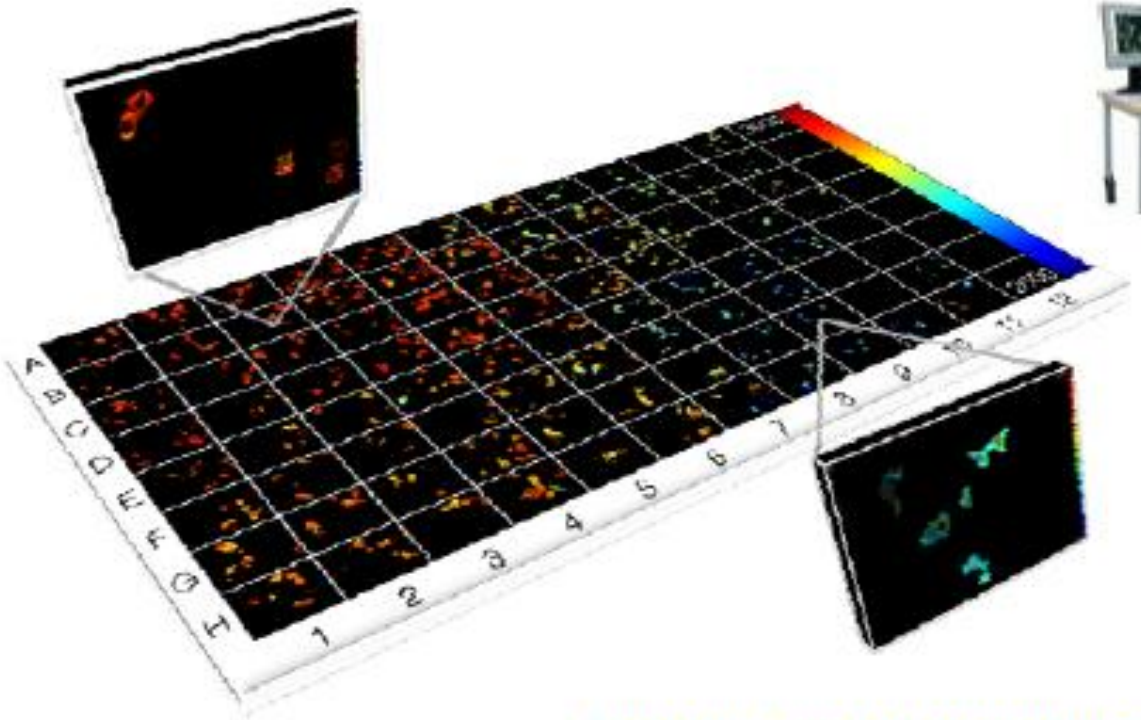








HCA: automated FLIM multiwell plate reader



Screening protein interactions for drug discovery

Technology Strategy Board
Driving Innovation

Healthcare

AstraZeneca

gsk

Imperial College London

Kentech Instruments Ltd

From Paul French, Imperial Collage London

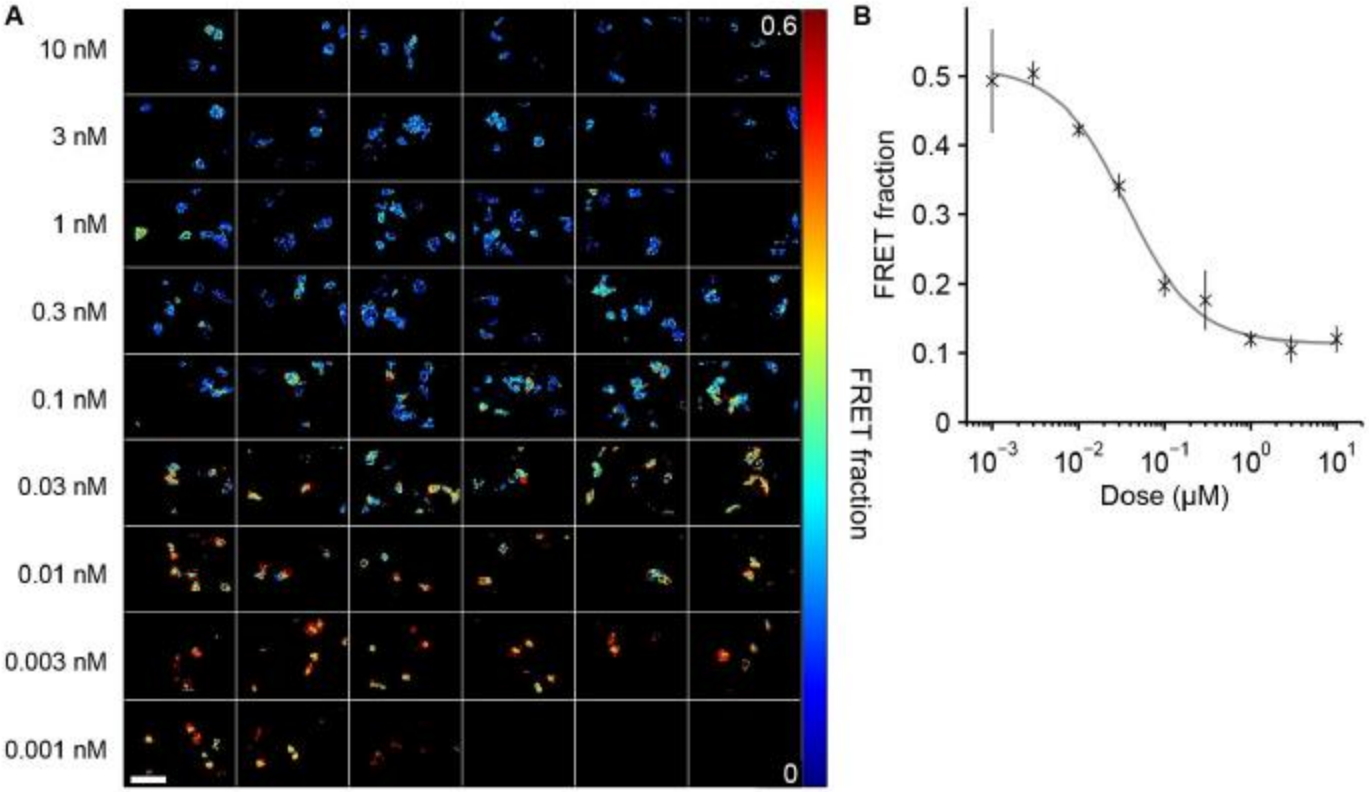
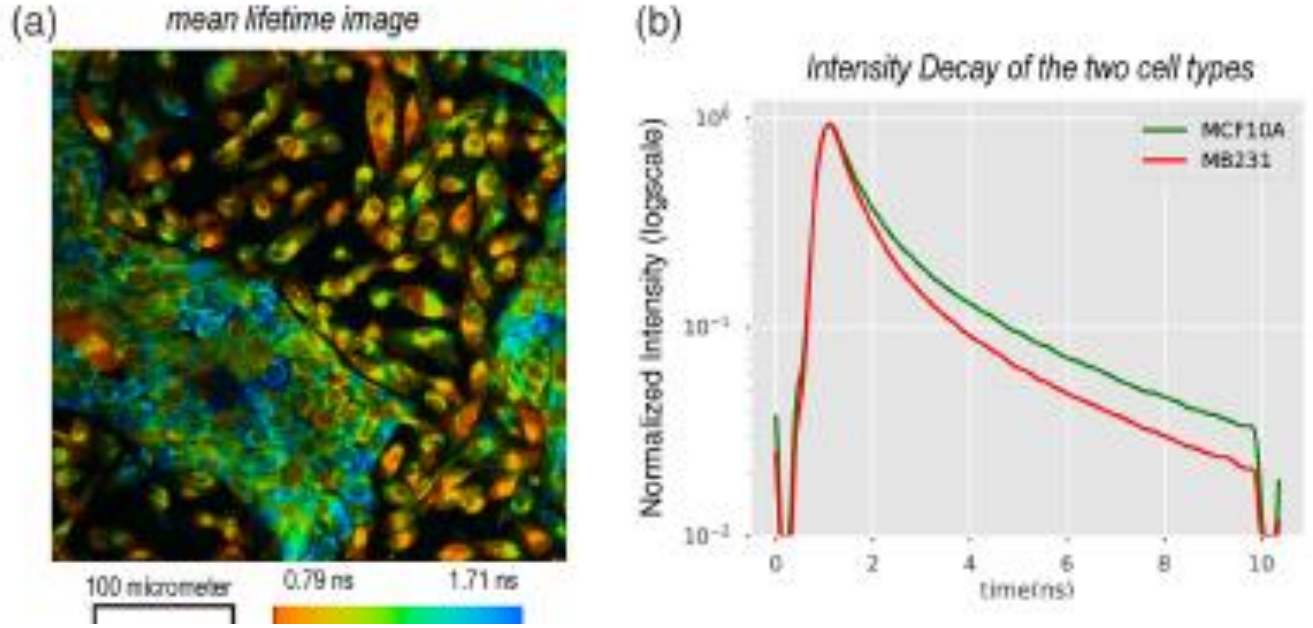


Figure 4. Global analysis of an NMT inhibitor dose-response dataset modulating Gag aggregation. Global analysis was performed across a multiwell plate dataset with HeLa cells expressing ECFP-Gag and EYFP-Gag with increasing levels of a NMT inhibitor using a bi-exponential donor FRET model. A) representative images from each inhibitor dose showing distribution of fraction Gag-CFP undergoing FRET. B) plot of fraction of Gag-CFP undergoing FRET against inhibitor concentration, averaged across wells with fitted dose-response curve. Error bars indicate 95% confidence intervals across wells. This dataset was collected as part of a previous study [23]. White scale bar represents 100 µm.
doi:10.1371/journal.pone.0070687.g004

Rapid global fitting of large fluorescence lifetime imaging microscopy datasets. Warren SC at el., 2013
„We fitted a dose-response curve to the average FRET population across wells using nonlinear fitting to the Hill equation, giving an **EC50 value of 0.037 µM.**

Label-free FLIM

Fluorescence lifetime imaging microscopy (FLIM) has emerged as one of the more powerful autofluorescence imaging tools for NADH and FAD characterization due to its unique ability to noninvasively detect metabolite bound and free states and quantitate cellular redox ratio.



Co-culture of MCF10A and MD-MB231
MCF 10A cell line is a non-tumorigenic epithelial cell line.
MB231 cancerous cell line

From Chacko JV an d Eliceiri KW, 2019

Thank you for attention!

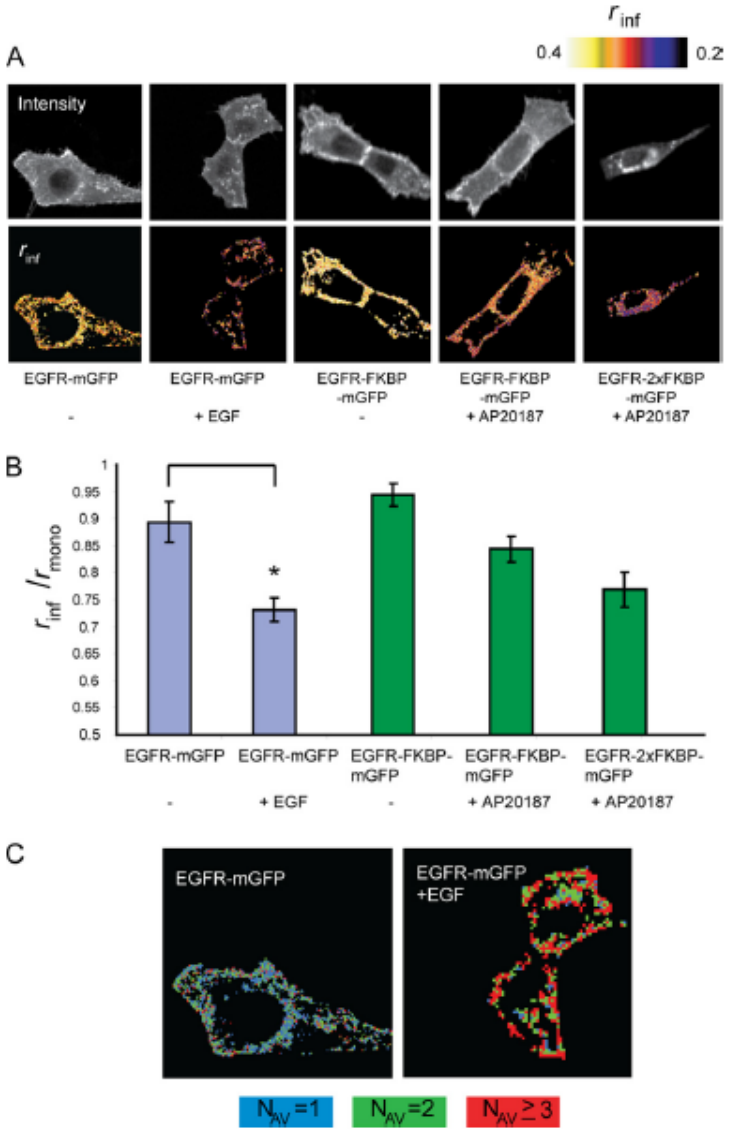


FIGURE 2. **EGFR-mGFP is oligomerized after EGF stimulation.** *A*, cellular distribution of GFP intensities and anisotropy values. NIH 3T3 2.2 cells expressing the indicated EGFR-mGFP constructs were left untreated or were stimulated with 8 nM EGF for 10 min or with 1 μM AP20187 for 2 h. Limiting anisotropy values (r_{inf}) were measured as described under "Experimental Procedures" and are expressed in false colors. *B*, average anisotropy values expressed as fraction of r_{mono} of the indicated constructs (S.E.). *, $p < 0.005$. *C*, representation of the cluster size values of EGFR-mGFP before and after EGF stimulation in false colors. The anisotropy values of the cells shown in *A* were classified as monomers ($N_{AV} = 1$ (*blue*)), dimers ($N_{AV} = 2$ (*green*)), and oligomers ($N_{AV} \geq 3$ (*red*)) as described under "Experimental Procedures."

(Hofman, Bader et al., 2010)

Using only one fluorophore brings advantages over hetero-FRET

1) all the fluorescent molecules are analyzed by this method comparing to hetero-FRET where dimers composed of donor-donor or acceptor-acceptor cannot be identified;
2) easier fluorescent labelling as only one fluorescent probe is required;
3) problem with differential expression levels of donor and acceptor-tagged proteins does not have to be taken into consideration;
4) possibility to perform multiplexed imaging which allows to follow spatio-temporal correlation of signaling pathways.