MOSBRI
Molecular-Scale Biophysics Research Infrastructure

End User Short Course

Quality control for Integral Membrane Proteins

12rd-14th September 2022

EMBL Hamburg (c/o DESY), Hamburg, Germany (EMBL-SPC)

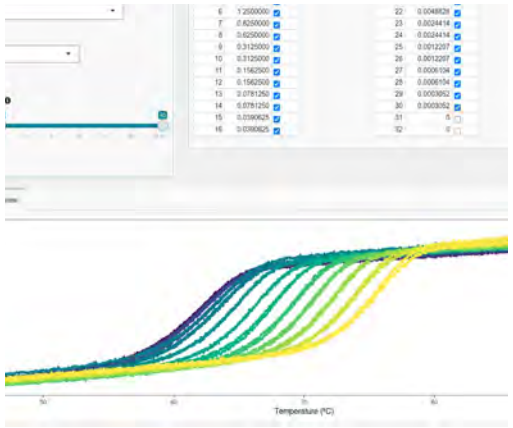
EMBL

EMBL SPC Services enabling structural biology research

Expertise in Sample preparation & Biophysics *Consulting, Training, Data analysis, Remote access*

Biophysical platform

HTX Laboratory



Data analysis platform

Angelica Struve

Stephan Niebling

Katharina Veith

David Ruiz Carrillo

Osvaldo Burastero

High-throughput Crystallization Facility

Screens | Robots | Plates | Imaging

CRIMS

- Initial screening 16
- Optimization 13
- LCP
- Additive
- Customized screens

- 2 TTP mosquito
- Scorpion

- CrystalDirect
- Swissci
- Intelli
- LCP
- Hanging drop
- Microbatch

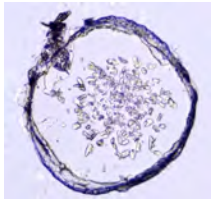
- Formulatrix
- 19 ºC and 4 ºC

Crystal Direct plate

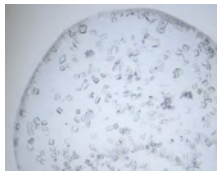
Imaging system

CRIMS

LCP



soluble

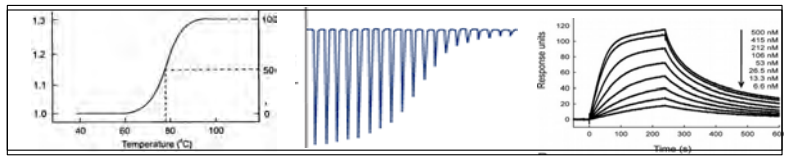


Harvester

Molecular Biophysics Platform

Characterization **Interaction** **Kinetics**

iLAB



1. MS 5. CD
2. TF 6. FT-IR
3. nDSF 7. UV/Vis
4. DLS 8. MP
9. ITC
10. MST
11. SPR
12. Interferometry
13. Stopped-flow

1)

MALDI/TOF: Bruker/CovalX

2)

Thermofluor

3)

Differential Scanning Fluorimetry (DSF)
Prometheus 48 Nanotemper

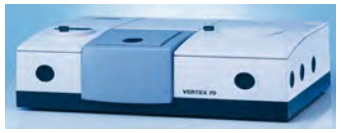
4)

Dynamic light scattering
Wyatt

5)

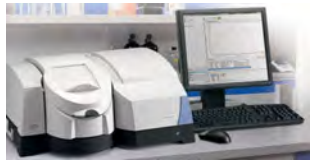
Circular Dichroism
AppliedPhotophysics

6)



Infrared spectroscopy (FT-IR)
Bruker Vertex 70

7)



Photometer
(UV/Vis)
Fischer Scientific

8)

Mass photometry
Refeyn One

9)

Isothermal titration calorimetry
MicroCal

10)

Microscale Thermophoresis
Monolith NT.115 and NT.LabelFree
Nanotemper

11)

Biacore T200 GE

12)

Bioforte Octet Red96

13)

Stopped-flow
AppliedPhotophysics

Integrated Biophysical pipeline services

Interactions

Scientific Advisory & Project Management

Biophysics & Biochemistry

Protein production collaborations

Binding | Kinetics | Characterization | Data Analysis | MD simulations

Binding affinity

- KD
- Thermodynamics (ΔS, ΔH, ΔG)
- Stoichiometry
- Competition assays

Dynamic kinetics

- Kon/Koff/KD
- Quantification
- Competition assays

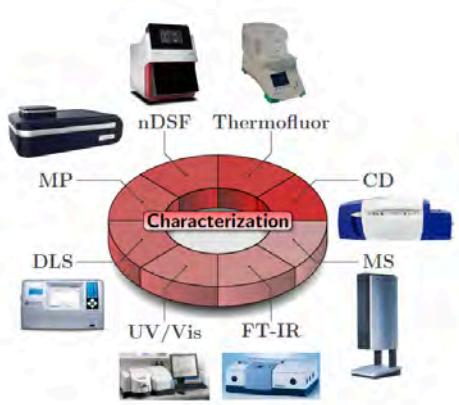
QC of target for structural studies

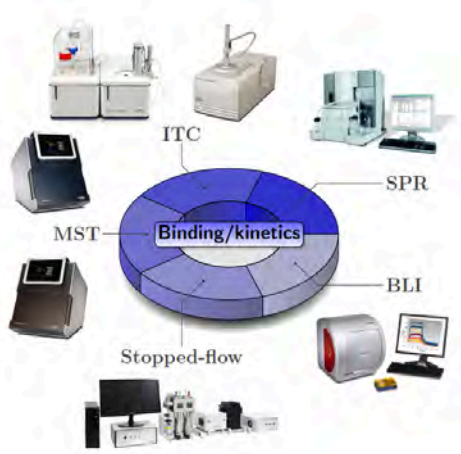
- Folding/Unfolding
- Purity/impurity profile
- Homogeneity
- Stability

eSPC

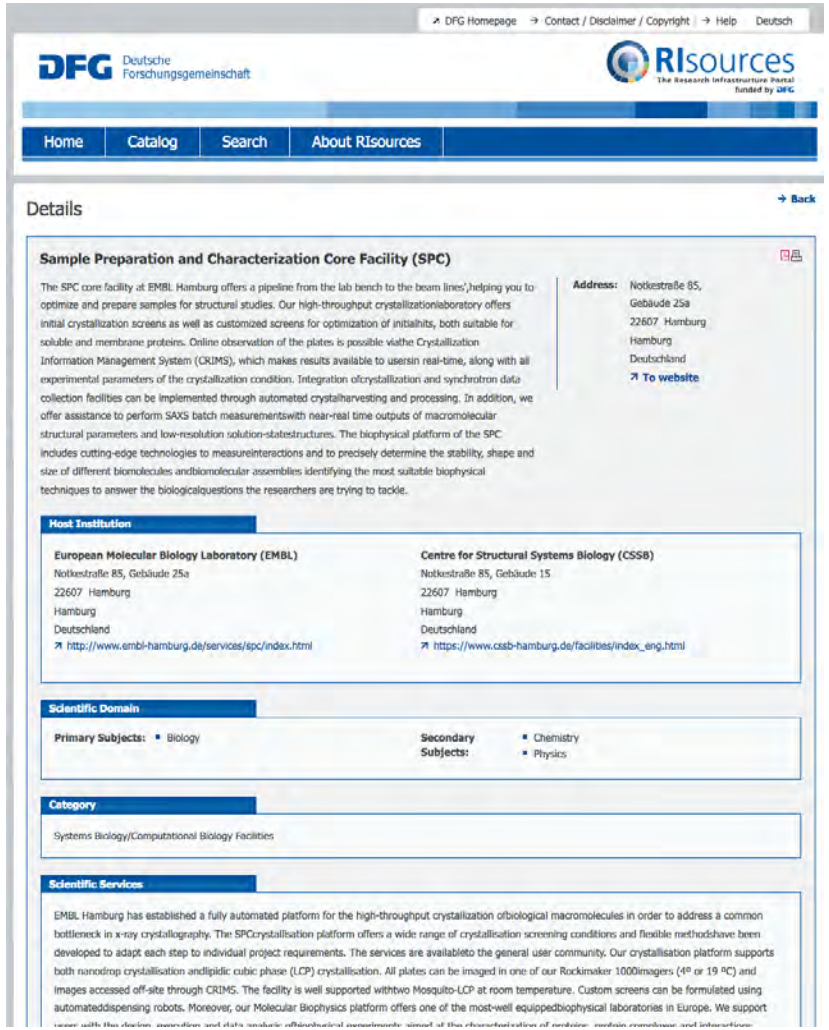
- online biophysical web server platform to analyze biophysical data

- Collaboration only





Funding to access the facilities



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DFG Deutsche Forschungsgemeinschaft

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The Research Infrastructure Portal
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Sample Preparation and Characterization Core Facility (SPC)

The SPC core facility at EMBL Hamburg offers a pipeline from the lab bench to the beam lines',helping you to optimize and prepare samples for structural studies. Our high-throughput crystallizationlaboratory offers initial crystallization screens as well as customized screens for optimization of initialhits, both suitable for soluble and membrane proteins. Online observation of the plates is possible viathe Crystallization Information Management System (CRIMS), which makes results available to usersin real-time, along with all experimental parameters of the crystallization condition. Integration ofcrystallization and synchrotron data collection facilities can be implemented through automated crystalharvesting and processing. In addition, we offer assistance to perform SAXS batch measurementswith near-real time outputs of macromolecular structural parameters and low-resolution solution-statestructures. The biophysical platform of the SPC includes cutting-edge technologies to measureinteractions and to precisely determine the stability, shape and size of different biomolecules andbiomolecular assemblies identifying the most suitable biophysical techniques to answer the biologicalquestions the researchers are trying to tackle.

Address: Notkestraße 85, Gebäude 25a, 22607 Hamburg, Hamburg, Deutschland
↗ To website

Host Institution

European Molecular Biology Laboratory (EMBL)
Notkestraße 85, Gebäude 25a
22607 Hamburg
Hamburg
Deutschland
↗ http://www.embl-hamburg.de/services/spc/index.html

Centre for Structural Systems Biology (CSSB)
Notkestraße 85, Gebäude 15
22607 Hamburg
Hamburg
Deutschland
↗ https://www.cssb-hamburg.de/facilities/index_eng.html

Scientific Domain

Primary Subjects: ▪ Biology
Secondary Subjects: ▪ Chemistry ▪ Physics

Category

Systems Biology/Computational Biology Facilities

Scientific Services

EMBL Hamburg has established a fully automated platform for the high-throughput crystallization ofbiological macromolecules in order to address a common bottleneck in x-ray crystallography. The SPCcrystallisation platform offers a wide range of crystallisation screening conditions and flexible methodshave been developed to adapt each step to individual project requirements. The services are availableto the general user community. Our crystallisation platform supports both nanodrop crystallisation andlipidic cubic phase (LCP) crystallisation. All plates can be imaged in one of our Rockimaker 1000imagers (4° or 19 °C) and images accessed off-site through CRIMS. The facility is well supported withtwo Mosquito-LCP at room temperature. Custom screens can be formulated using automateddispensing robots. Moreover, our Molecular Biophysics platform offers one of the most-well equippedbiophysical laboratories in Europe. We support

iNEXT

instruct ERIC

iNEXT DISCOVERY

Interreg
Öresund-Kattegat-Skagerrak
European Regional Development Fund

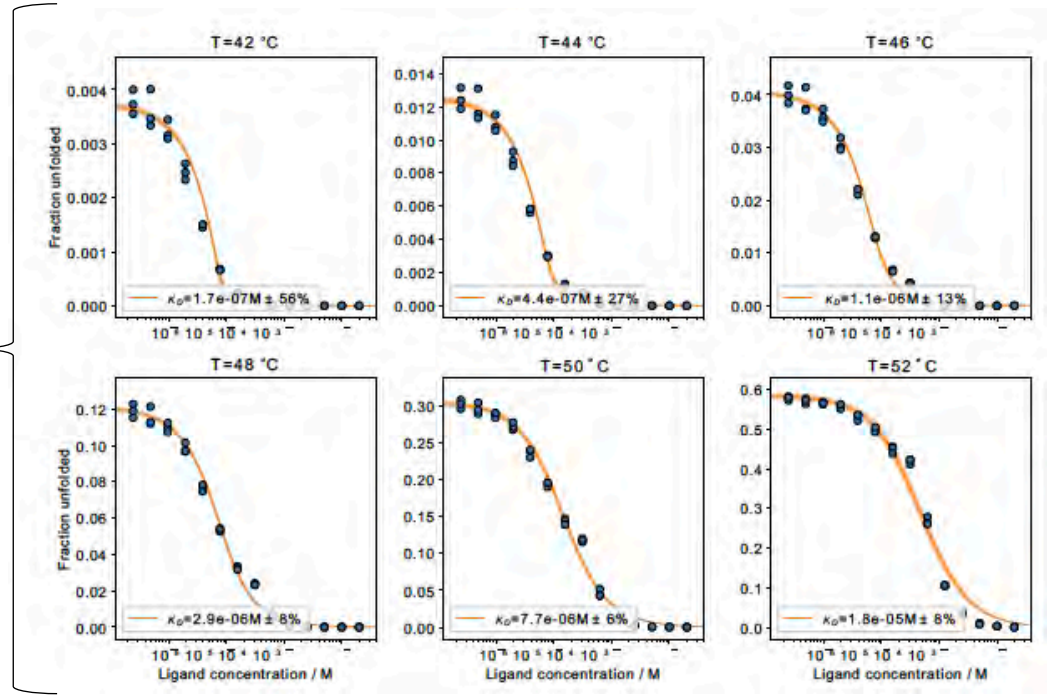
EUROPEAN UNION

Hanseatic League of Science
Interconnecting infrastructures
for life science research and innovation

MOSBRI
Molecular-Scale Biophysics
Research Infrastructure

Methods development

- High-throughput stability screening for detergent-solubilized membrane proteins. Kotov V, et al. *Sci Rep*. 2019
- Cyclohexyl-α maltoside as a highly efficient tool for membrane protein studies. Missel JW, et al. *Curr Res Struct Biol.* 2021
- FoldAffinity: binding affinities from nDSF experiments. Niebling S, et al. *Sci Rep*. 2021.
- In-depth interrogation of protein thermal unfolding data with MoltenProt. Kotov V, et al. *Protein Sci.* 2021
- eSPC: an online data-analysis platform for molecular biophysics. Burastero O, et al. Acta Crystallogr D. 2021
- Biophysical screening pipeline for cryo-EM grid preparation of membrane proteins. Niebling et al. Front. Mol. Bio. 2022



Stephan Niebling
STO

Osvaldo Burastero
ARISE Fellow

Quality control of purified protein
Best practice recommendations

Guideline

Minimal quality control parameters that should be tested on protein sample

- Purity & integrity
- Homogeneity (aggregation state)
- Identity

Extended quality control parameters

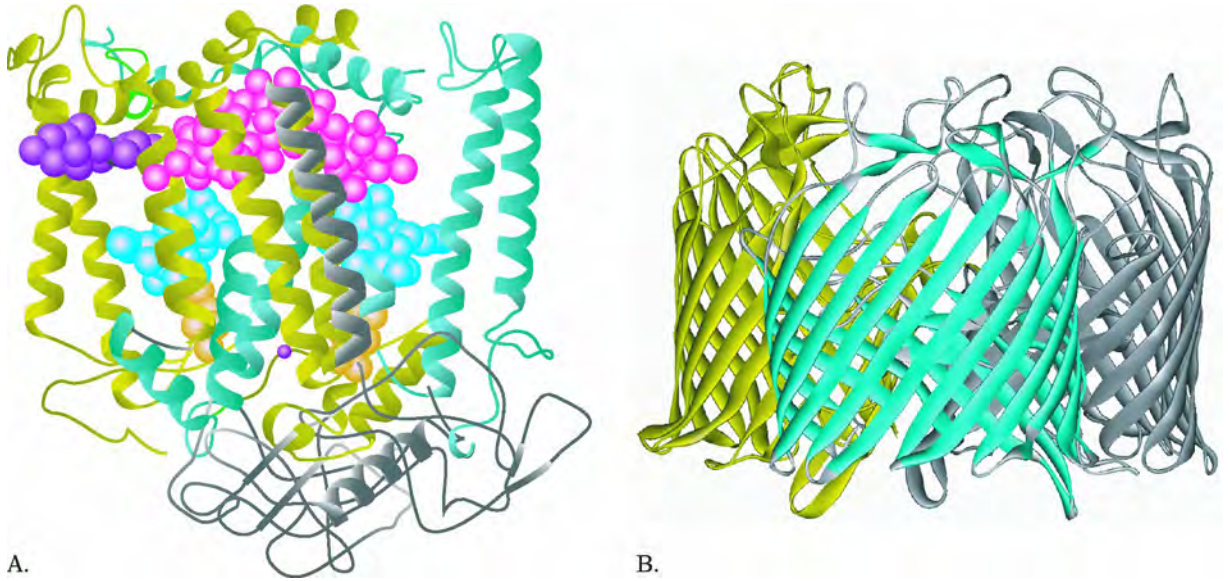
- General quality test by UV spectroscopy
- Homogeneity Conformational stability/folding state
- Optimization of storage conditions
- Batch-to-batch consistency

ARBRE-MOBIEU (Association of Resources for Biophysical research in Europe – MOlecular BIophysic in EUrope) and P4EU (Protein Production and Purification Partnership in EUrope)

De Marco et al. Nat Commun. 2021

IMPs: helical bundle and β-barrel membrane proteins

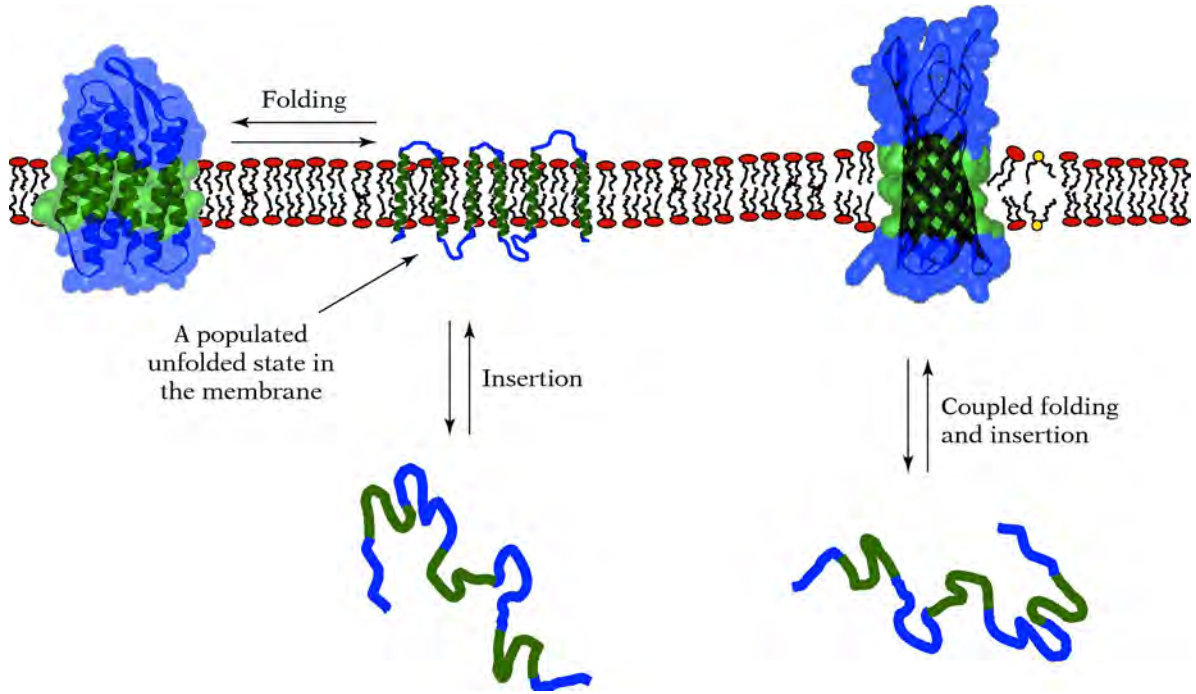
α-helical bundles



β-barrels

- TM segments of proteins utilize secondary structure to satisfy the hydrogen bond needs
- Two general classes of integral membrane proteins (IMPs):
- α-helical bundles: e.g. photosynthetic reaction centre (left)
- β-barrels: e.g. maltoporin trimer (right)

Folding and insertion of helical bundle and β-barrel membrane proteins utilize different mechanisms



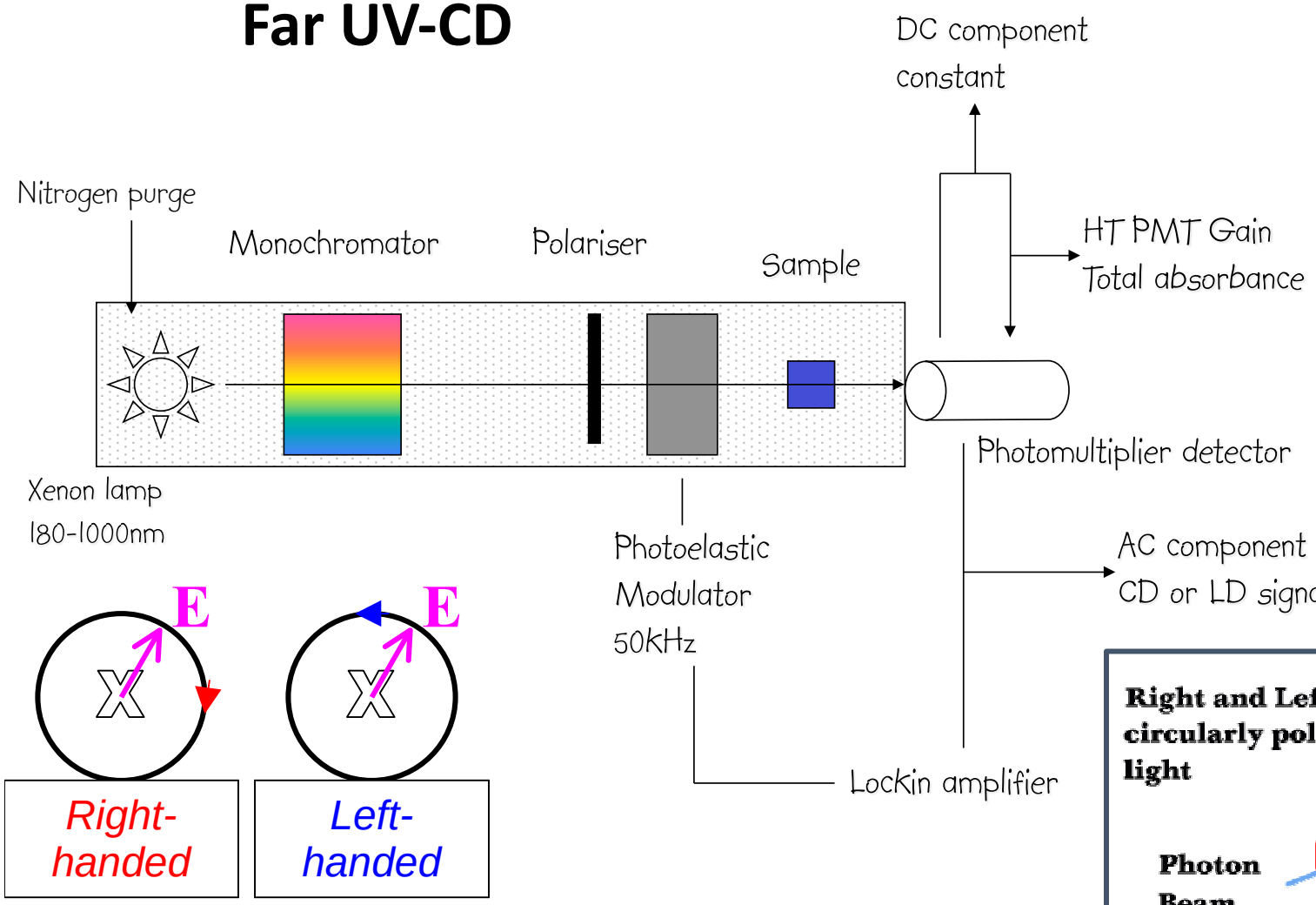
- Membrane protein folding *in vitro*
- Complex cellular process of translocation and integration of nascent proteins into the membrane (*in-vivo)*

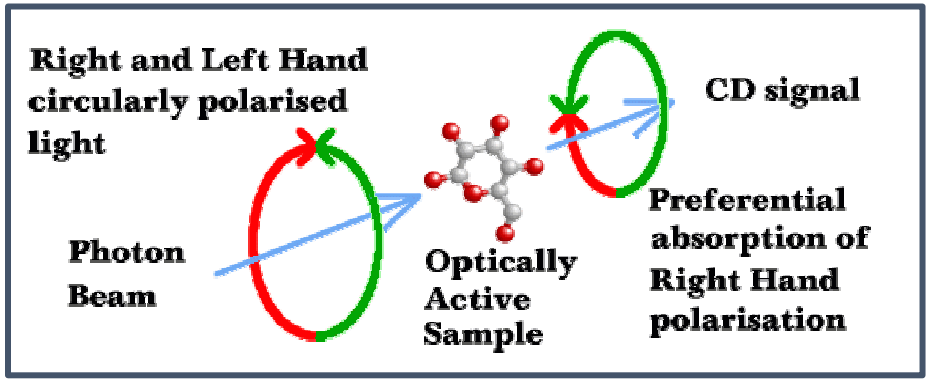
Biophysical methods for protein characterisation of membrane proteins…



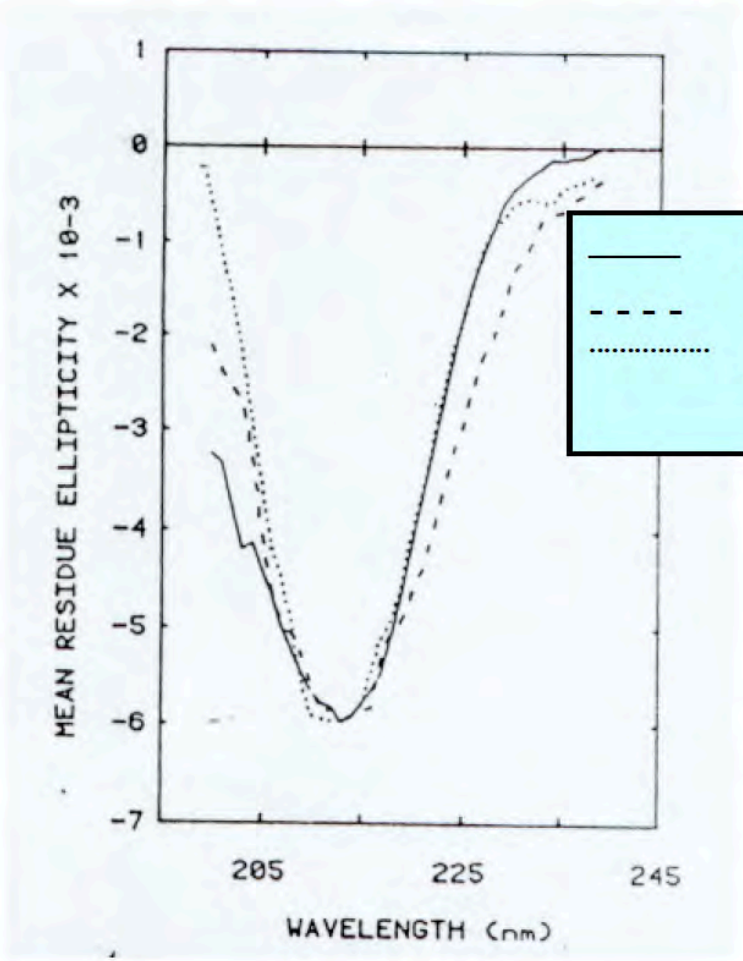


Far UV-CD


DC component
constant
Nitrogen purge
Monochromator
Polariser
Sample
HT PMT Gain
Total absorbance
Xenon lamp
180-1000nm
Photomultiplier detector
Photoelastic
Modulator
50KHz
AC component
CD or LD signal
Lockin amplifier
E
E
Right-handed
Left-handed

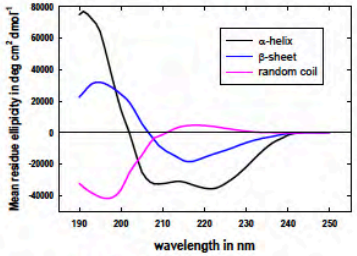

Right and Left Hand circularly polarised light
CD signal
Photon Beam
Optically Active Sample
Preferential absorption of Right Hand polarisation

α-Hemolysin CD spectra



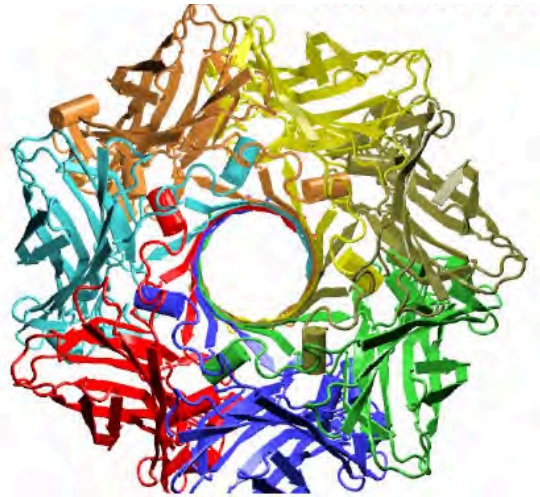
soliud monomer
lipid monomer
assembled hexamer or heptamer

- Tobkes et al. (1985) Biochemistry 24:1915

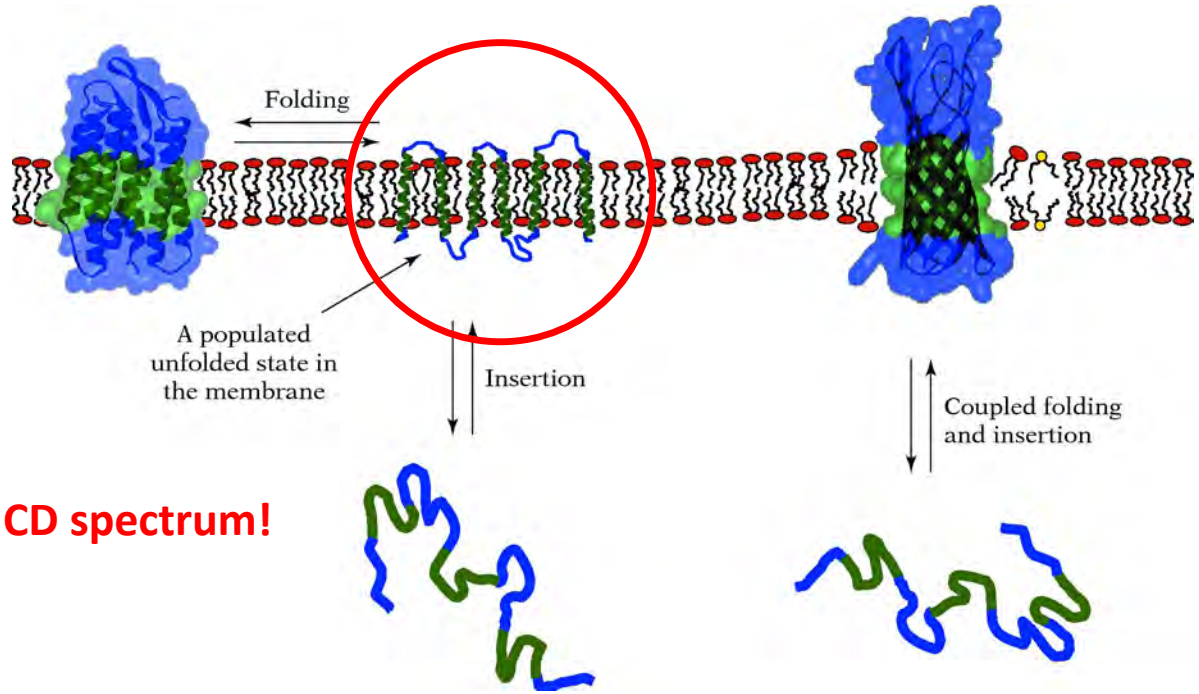


- The 3 spectra are similar

  So the 3 conformations have similar 2ndry structure
- What is the 2ndry structure?
- Is this normal for a membrane protein?



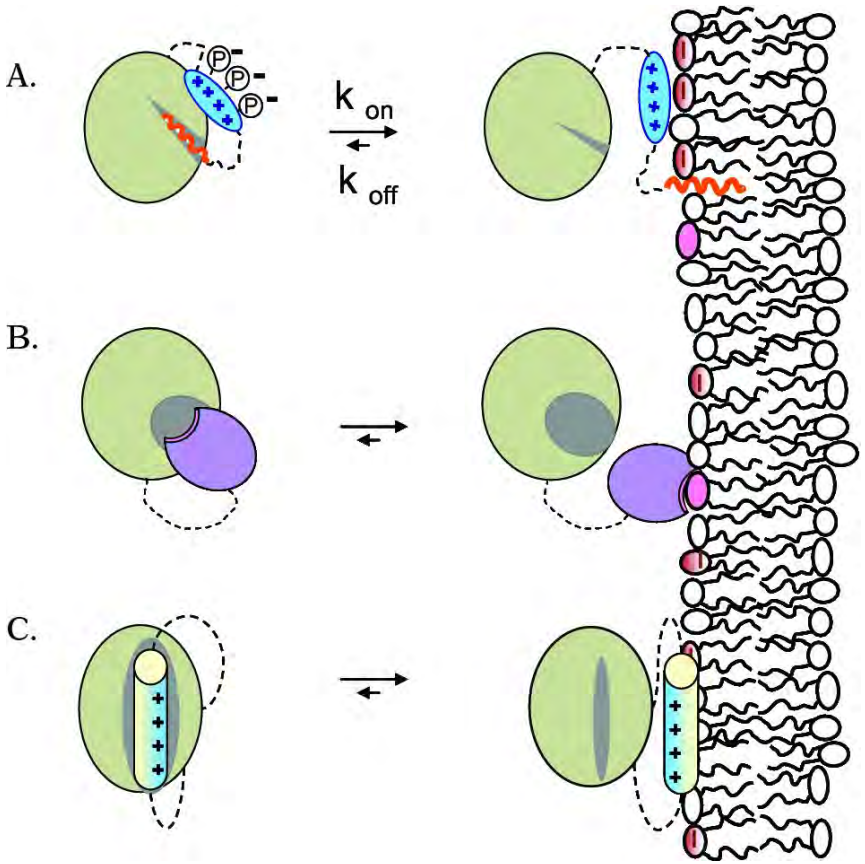
Folding and insertion of helical bundle and β-barrel membrane proteins utilize different mechanisms



A ”good” alpha CD spectrum!

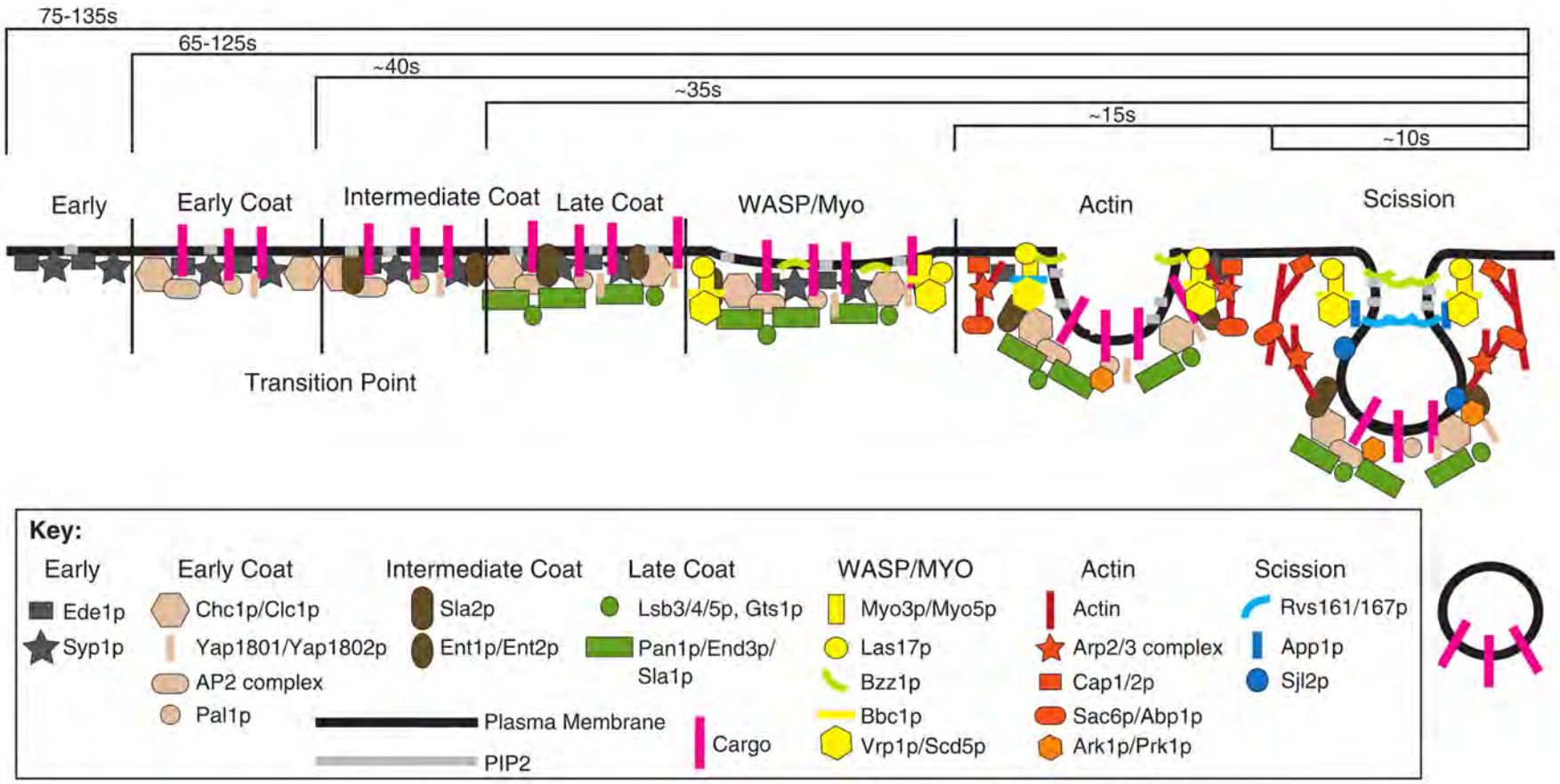
- Membrane protein folding *in vitro*
- Complex cellular process of translocation and integration of nascent proteins into the membrane (*in-vivo)*

Different modes of binding amphitropic proteins to membranes to regulate their activities



- Electrostatic interaction between polybasic protein motif and anionic lipid; coupled with lipid covalent anchor insertion (e.g. cytochrome c, myelin basic protein)
- Binding via lipid clamp, a binding pocket for a specific lipid headgroup (e.g. PLA_2)
- Insertion into the bilayer of an amphipathic α-helix (can be autoinhibitory in soluble form)

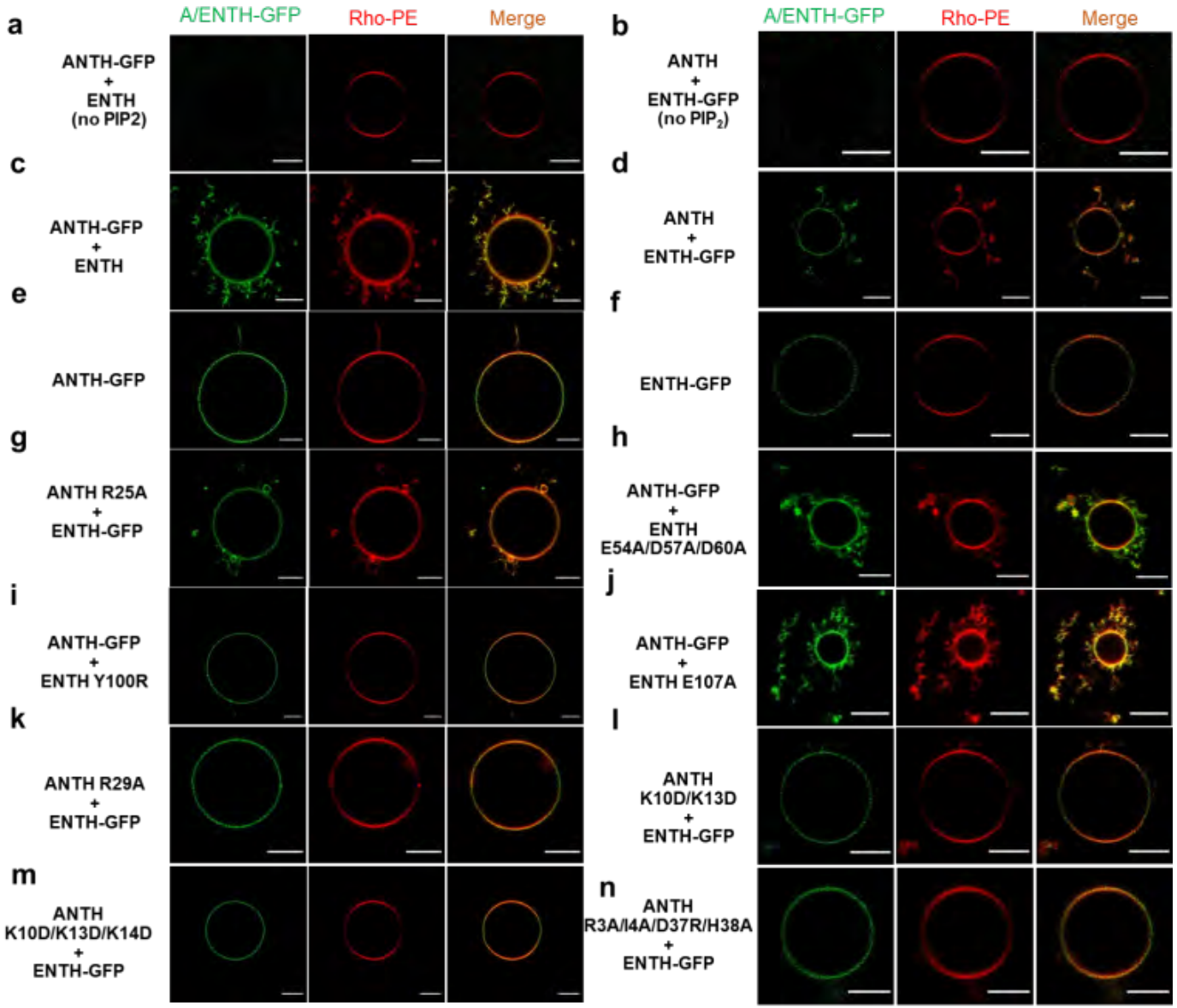
Clathrin Mediated Endocytosis in Yeast



Weinberg, J, et al. Trends in Cell Biology, 2012

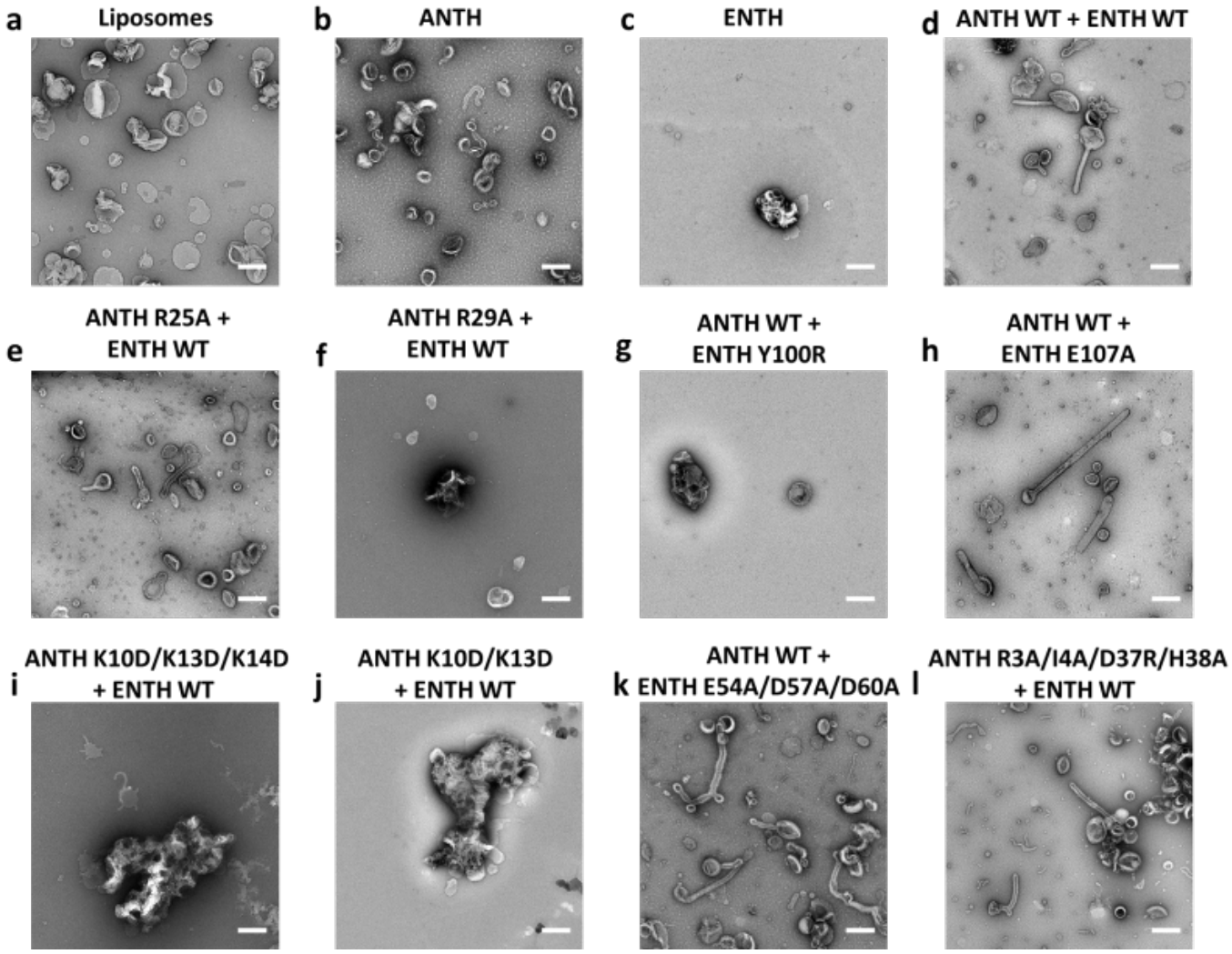
The role of an Adaptor complex in membrane remodelling

GUVs



Lizarrondo et al. Nat Commun. 2021

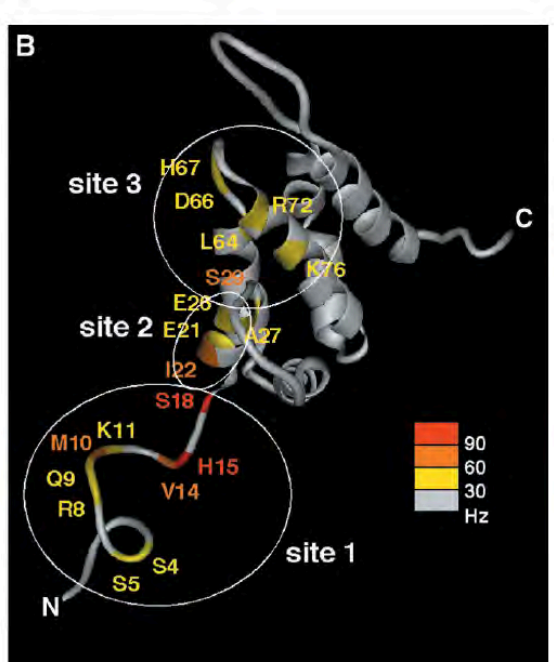
The role of the AENTH complex in membrane remodelling

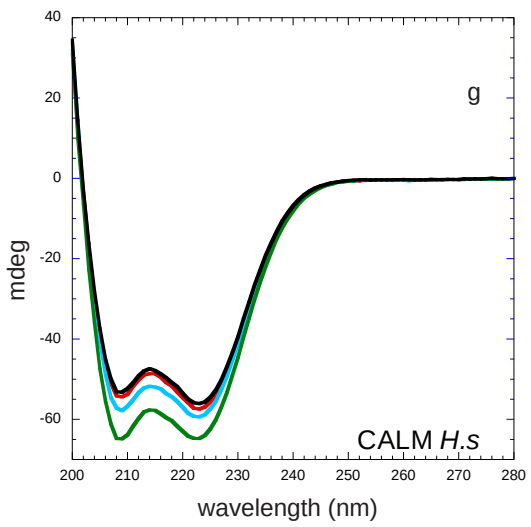


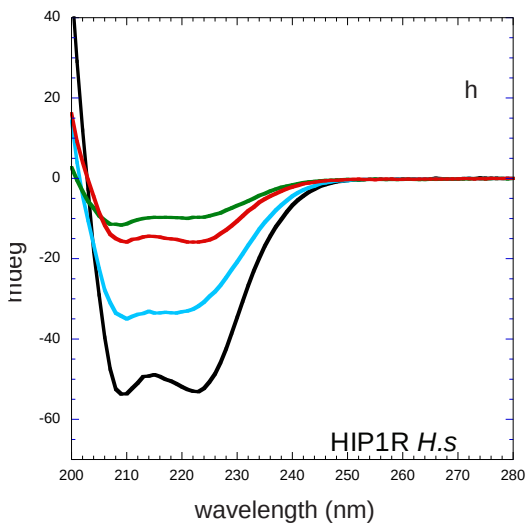
Conclusion:
Mutations impairing complex formation are unable to induce tubulation.

Lizarrondo et al. Nat Commun. 2021

Insertion into the bilayer: Induction of an amphipathic α-helix

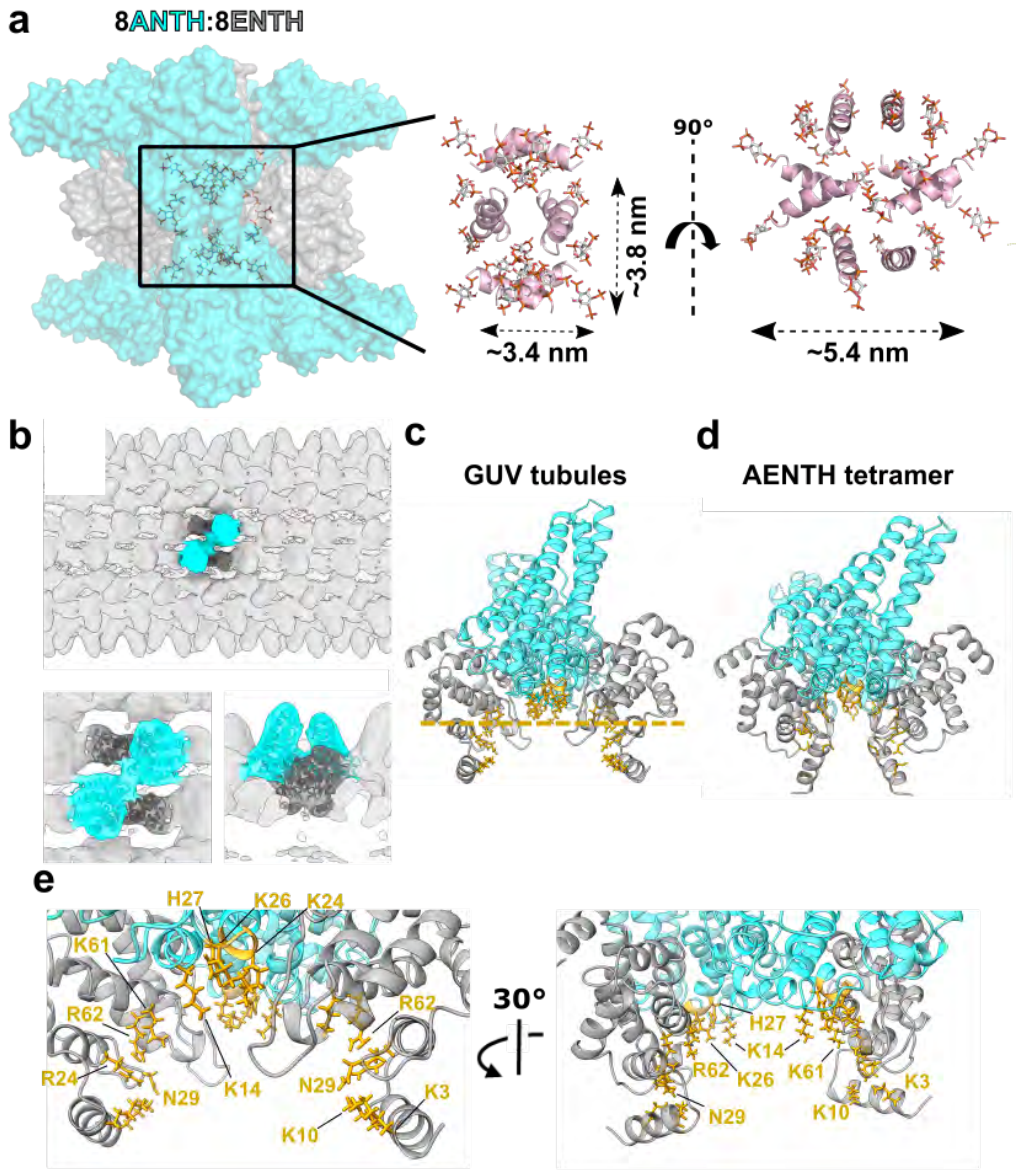


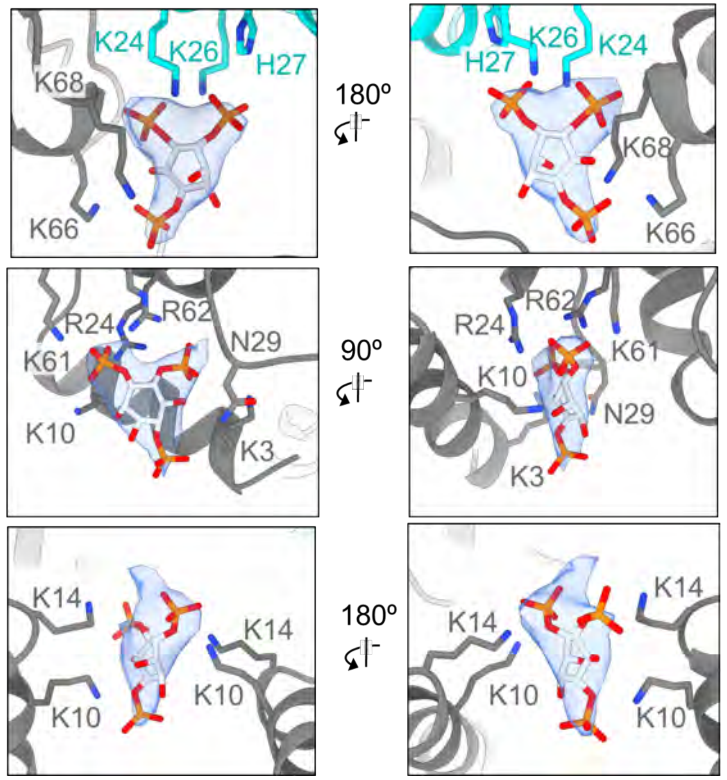




Garcia-Alai et al. Nat Commun. 2018

Protein-lipid-protein interfaces

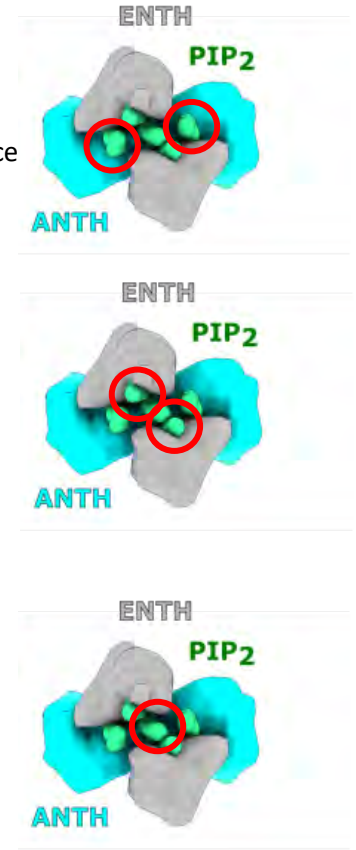




2x PIP_2
ANTH-ENTH Interface

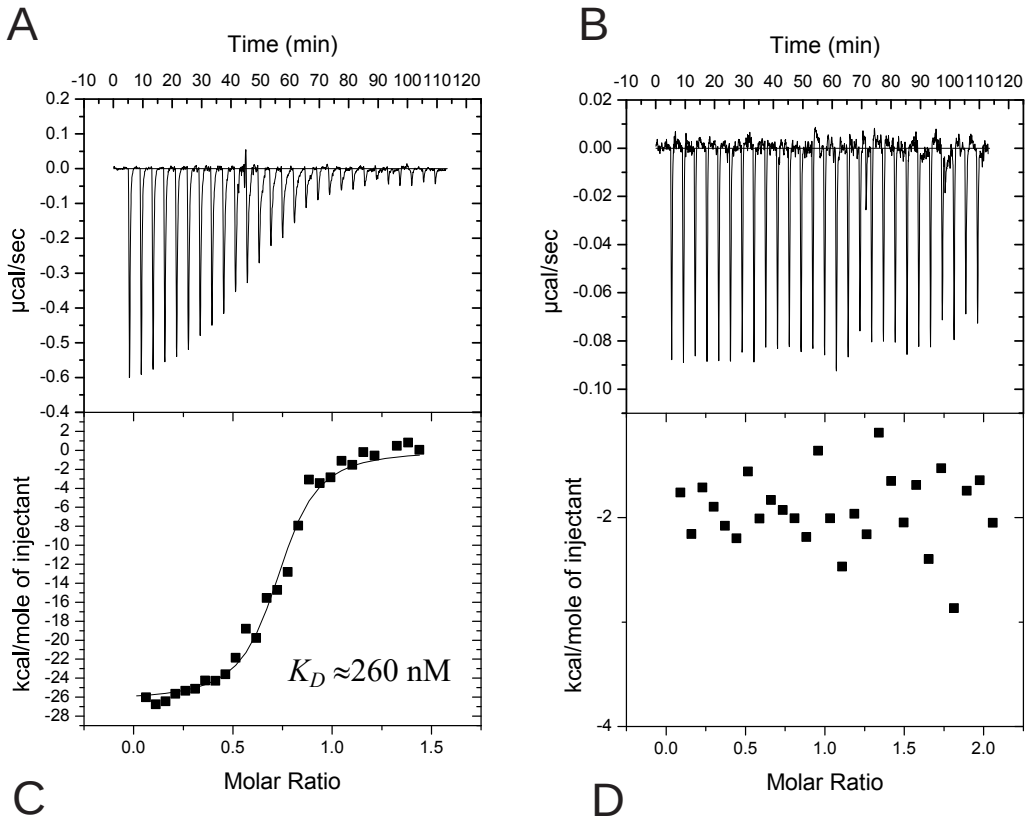
2x PIP_2
ENTH domain

1x PIP_2
ENTH-ENTH
Interface



Lizarrondo et al. Nat Commun. 2021

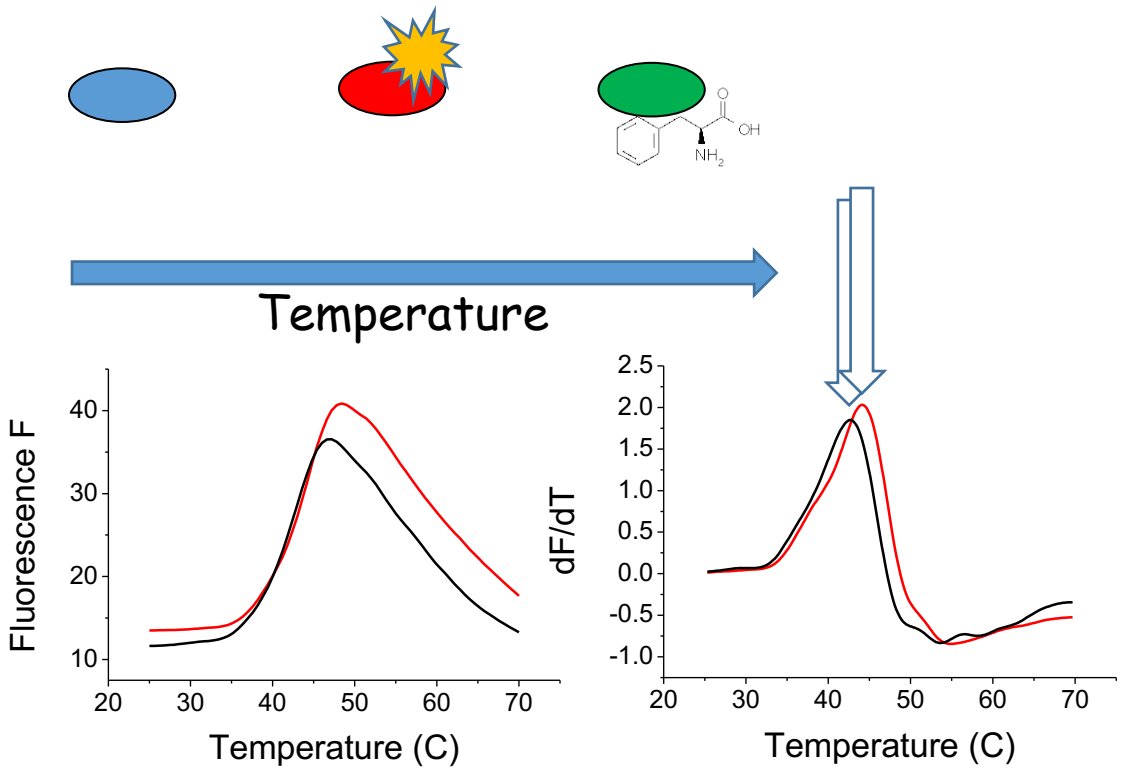
Isothermal Titration Calorimetry



ITC of ANTH-ENTH2 interaction in the presence of 0.15 mM DDM and 50 uM PIP2 (A) and without PIP2 (B).

Garcia-Alai et al. Nat Commun. 2018

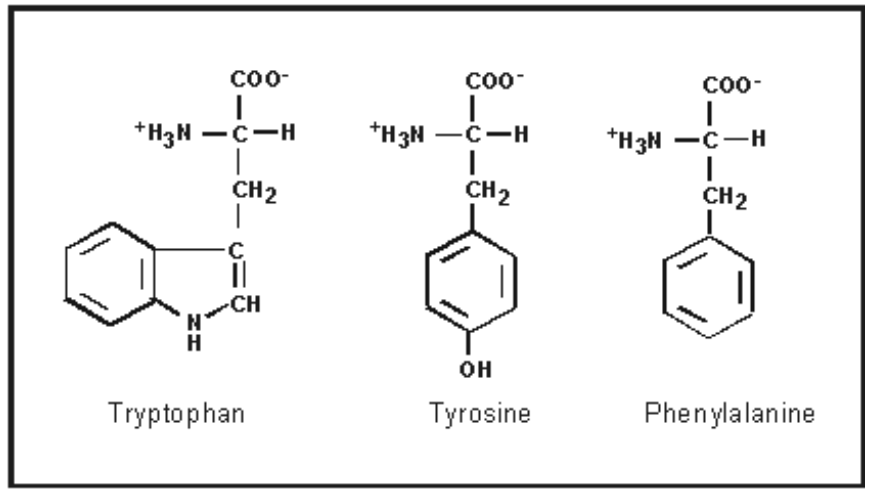
Differential Scanning Fluorimetry

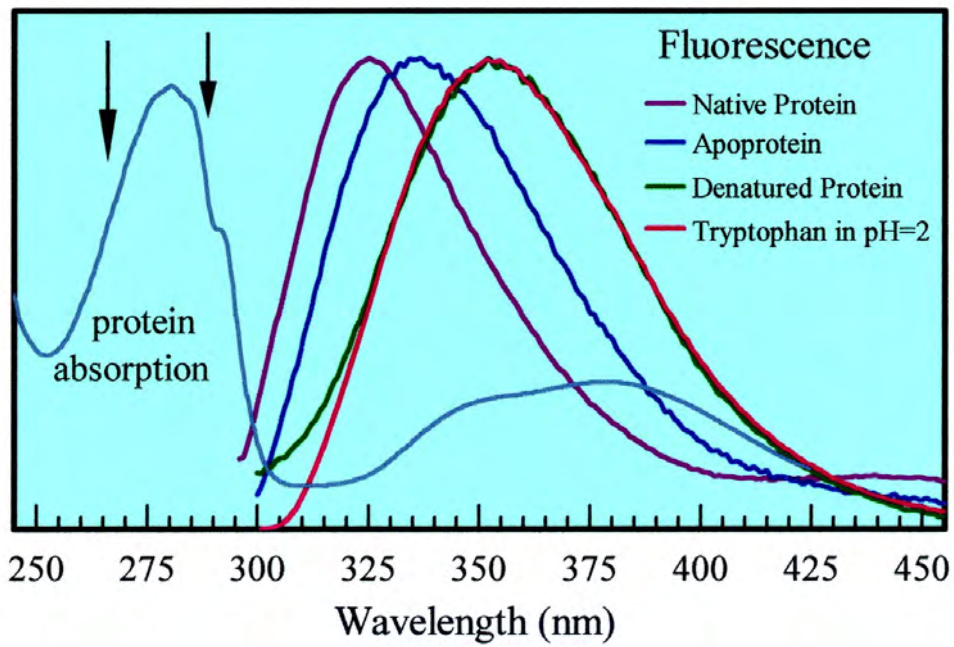


Fluorescence
Solubility
Turbidity
Aggregation
Hydrophobic interfaces

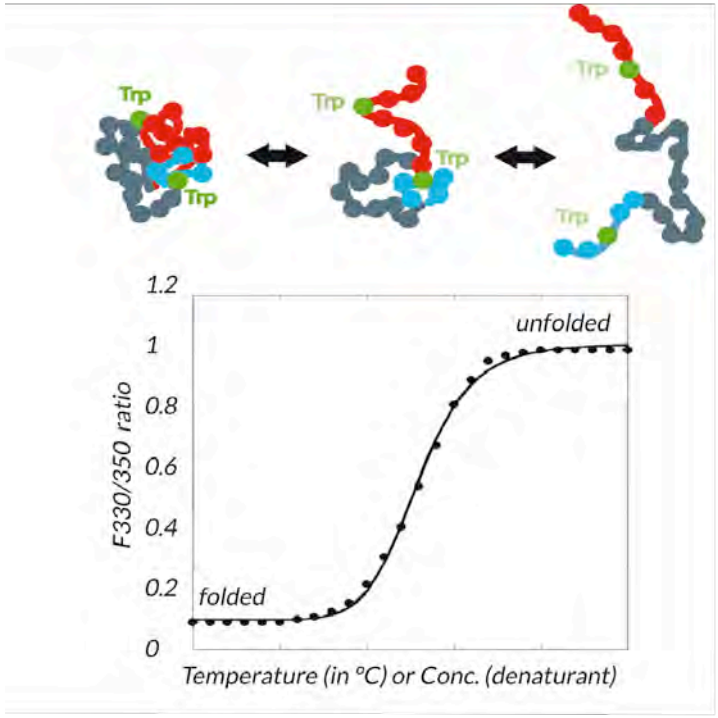
48- 96 samples

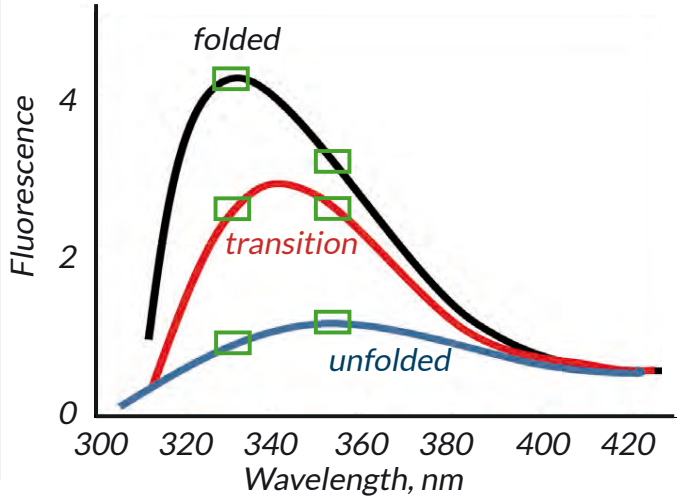
Intrinsic Fluorescence



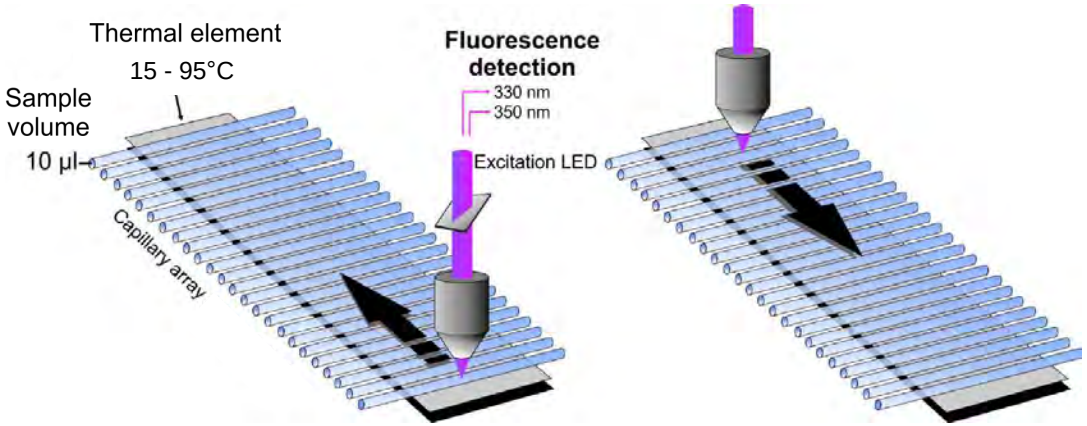


nanoDSF



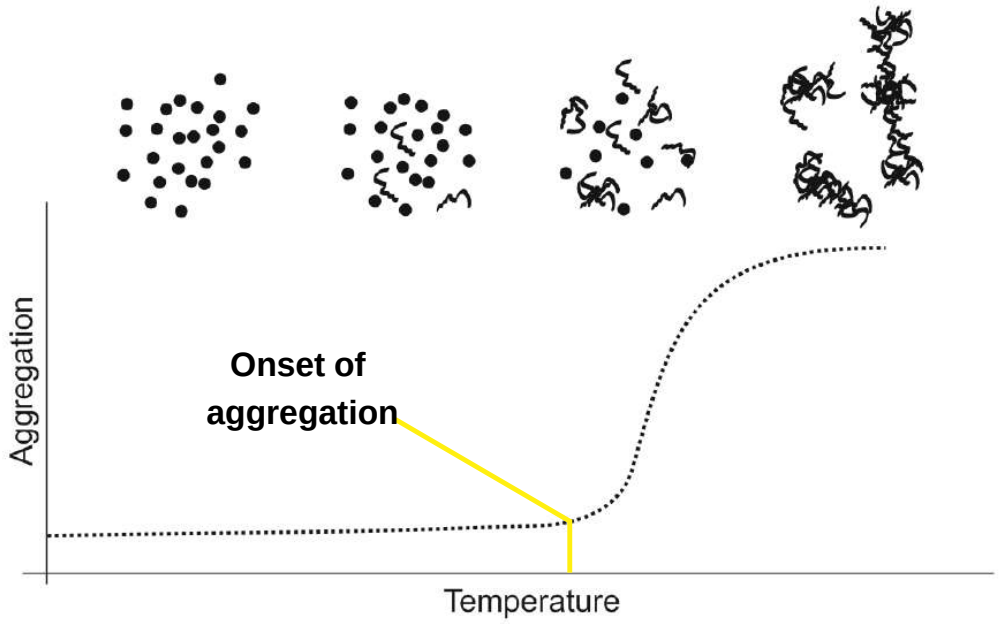


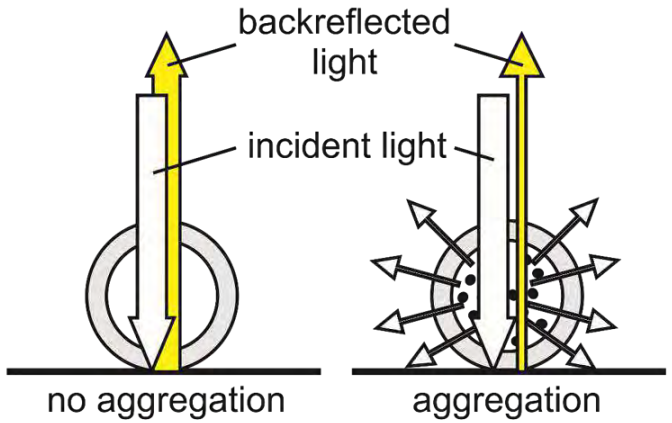
NanoTemper Technologies Prometheus NT.48



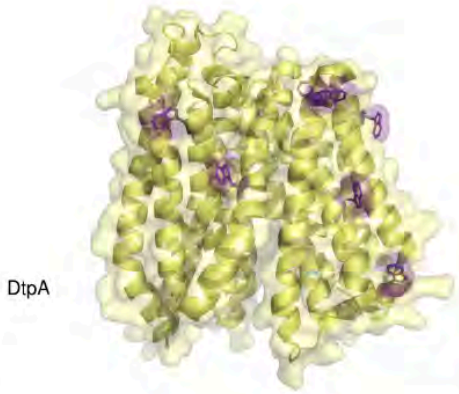
- **Principle behind the nanoDSF**. Increasing temperature causes protein unfolding that can be assessed by monitoring changes of tryptophan fluorenscence at 330nm and 350nm wavelength.

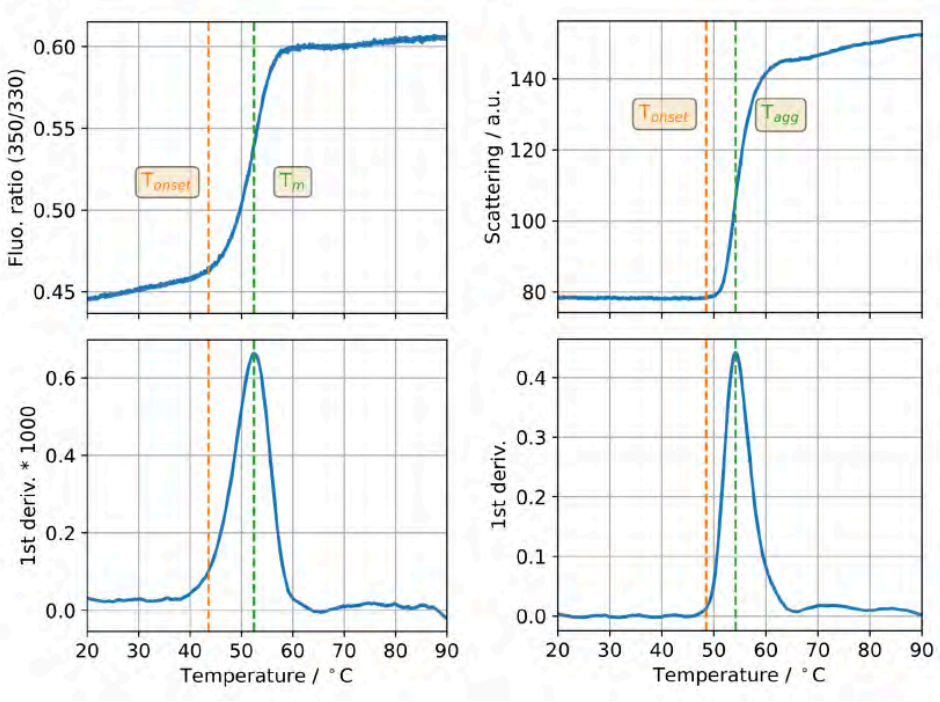
Backreflection Optics





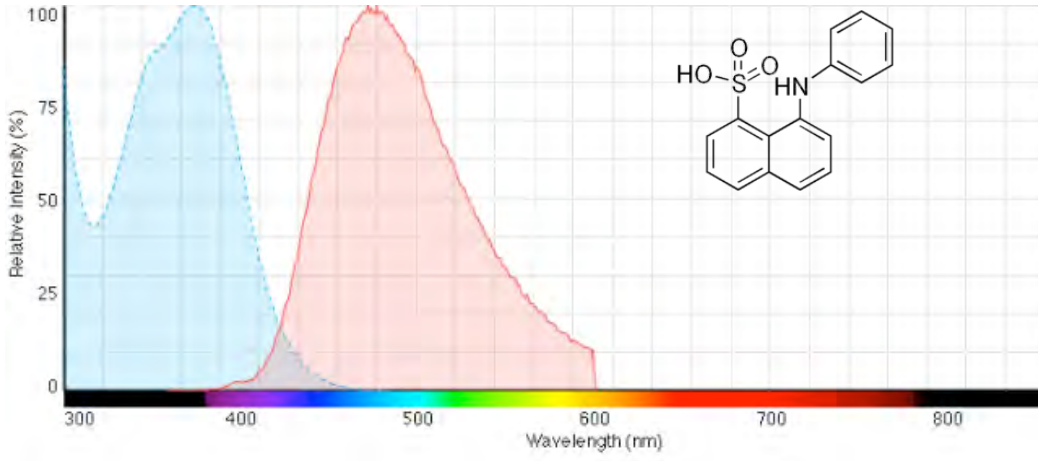
Tm & Tagg


DtpA


Fluo. ratio (350/330)
0.60
0.55
0.50
0.45
T_{onset}
T_m
Scattering / a.u.
140
120
100
80
T_{onset}
T_{agg}
1st deriv. * 1000
0.6
0.4
0.2
0.0
20 30 40 50 60 70 80 90
Temperature / °C
1st deriv.
0.4
0.3
0.2
0.1
0.0
20 30 40 50 60 70 80 90
Temperature / °C

Sypro Orange/ ANS fluorescent properties will change as it binds to hydrophobic regions on the protein surface

8-anilino, 1-naphthalene sulfonate



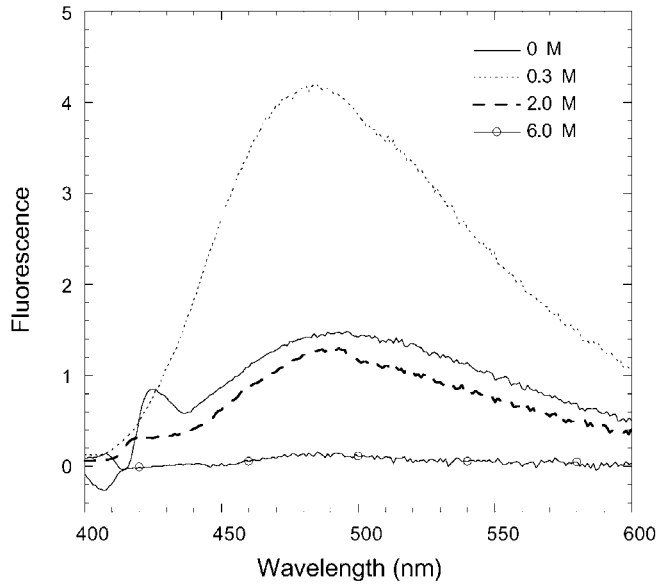
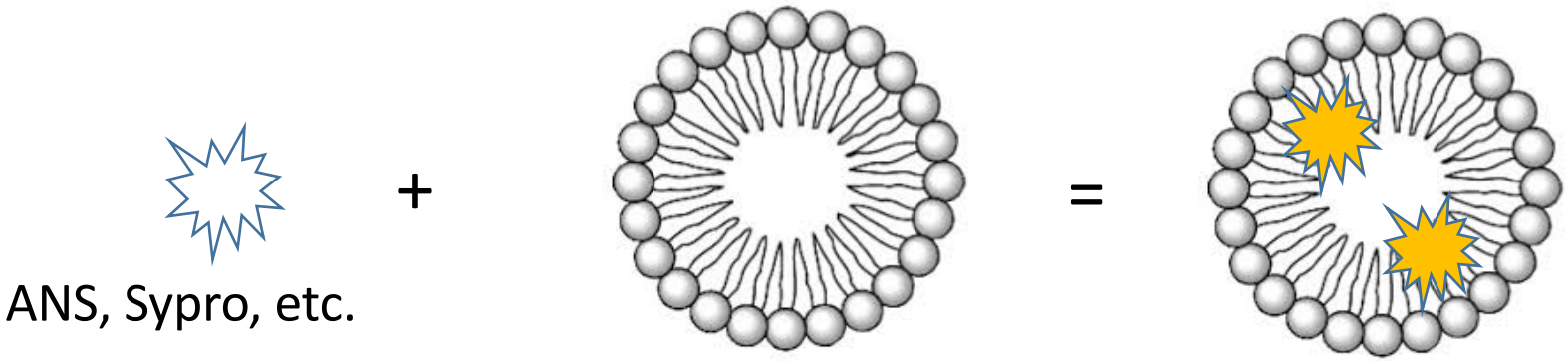


FIGURE 7: ANS binding of E7 after the GdmCl-induced conformational transition at the diferent denaturant concentrations indicated.

Not compatible with detergents!!!

What happens with our “unfolding reporters” in the presence of detergents?



Structure
Ways & Means

Microscale Fluorescent Thermal Stability Assay for Membrane Proteins

Alexander I. Alexandrov,[1] Mauro Mileni,[1] Ellen Y.T. Chien,[1] Michael A. Hanson,[1] and Raymond C. Stevens[1,*]

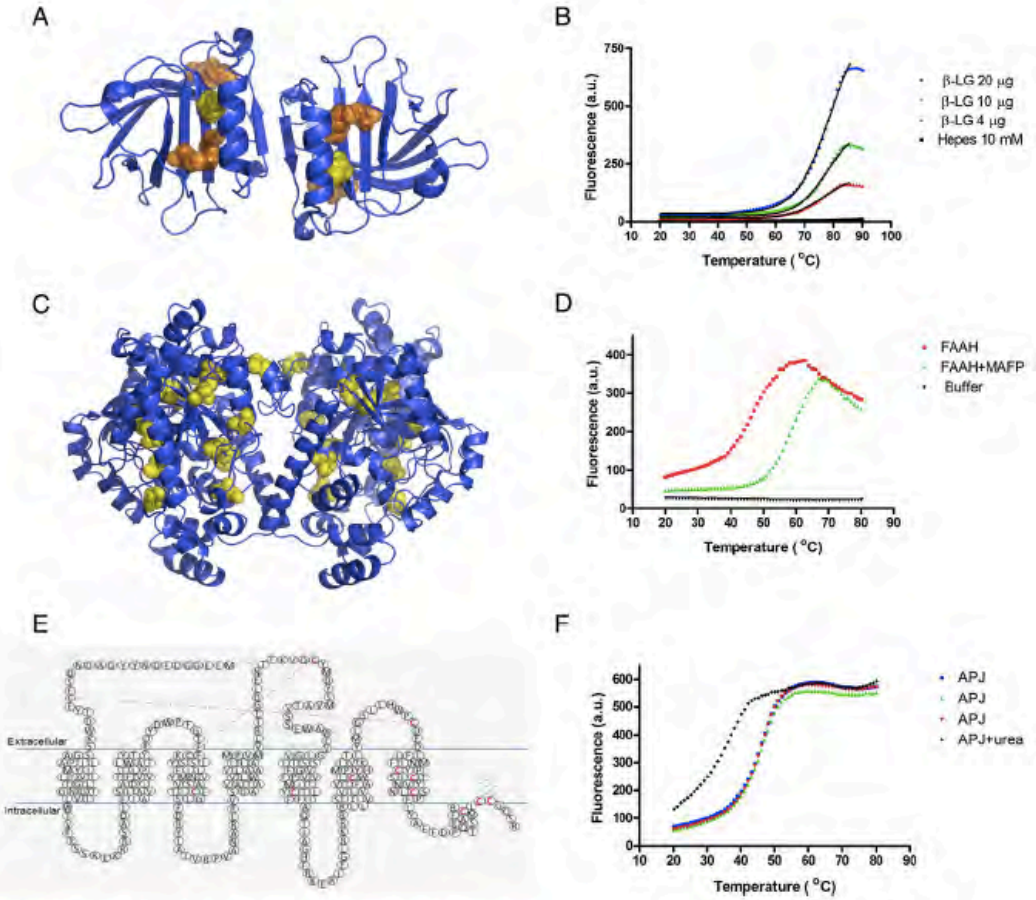
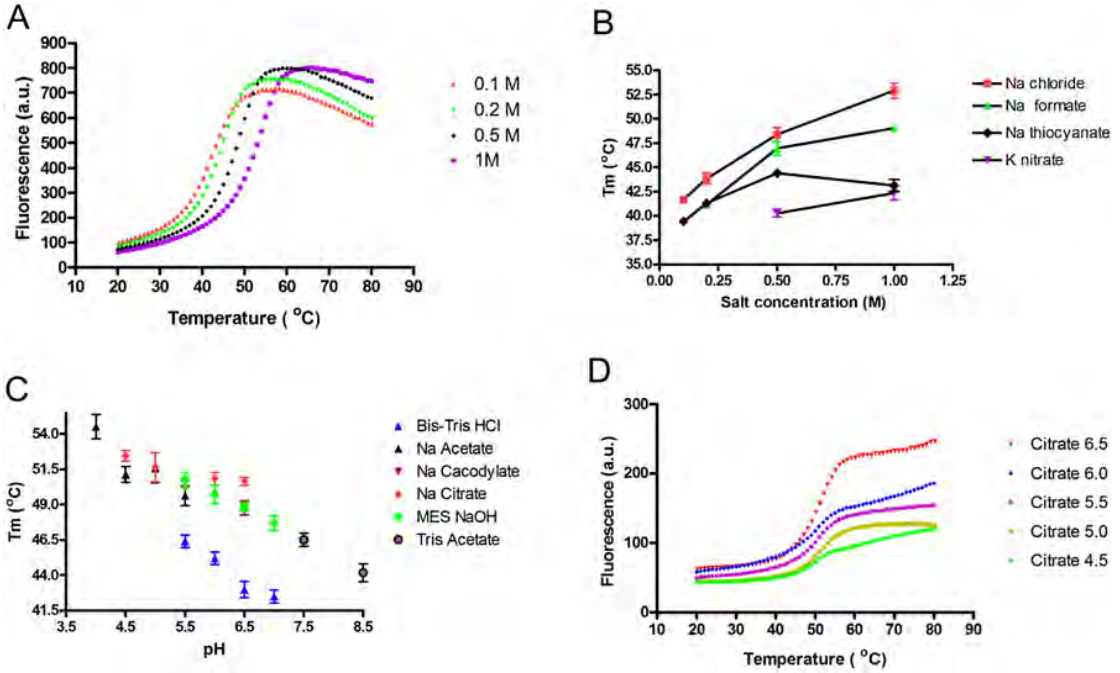


Figure 1. Thermal Stability Profiles of β-LG, FAAH, and APJ Receptor

- thiol-specific fluorochrome (CPM)
- The screen uses the chemical reactivity of the native cysteines embedded in amphipathic helices as a sensor for the overall integrity of the folded state.
- CPM is nonfluorescent in its unbound form

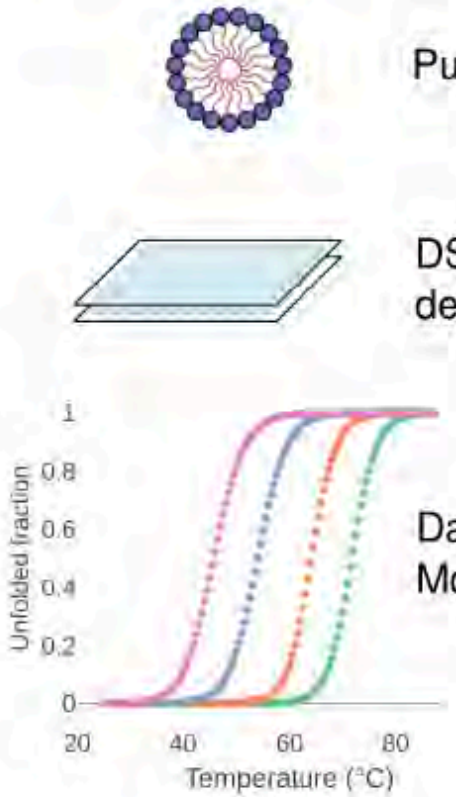


Thermofluor screen

	1	2	3	4	5	6
A	**water (ctrl)**	**10mM Hepes pH 7.5**	**50mM Hepes pH 7.5**	**100mM Hepes pH 7.5**	**150mM Hepes pH 7.5**	**250mM Hepes pH 7.5**
B	**50mM NaCl,** 50mM Hepes pH 7.5	**100mM NaCl,** 50mM Hepes pH 7.5	**250mM NaCl,** 50mM Hepes pH 7.5	**500mM NaCl,** 50mM Hepes pH 7.5	**750mM NaCl,** 50mM Hepes pH 7.5	**1000mM NaCl,** 50mM Hepes pH 7.5
C	**100mM Magic Buffer pH 4.0**	**100mM Magic Buffer pH 5.0**	**100mM Magic Buffer pH 6.0**	**100mM Magic Buffer pH 7.0**	**100mM Magic Buffer pH 8.0**	**100mM Magic Buffer pH 9.0**
D	**100mM MES pH 6.0**	**100mM Bis-Tris pH 6.5**	**100mM Na Phosphate pH 7.0**	**100mM PBS pH 7.4**	**100mM Tris-HCl pH 7.5**	**100mM Bicine pH 8.0**
E	**100mM imidazole,** 50mM Hepes pH 7.5	**250mM imidazole,** 50mM Hepes pH 7.5	**500mM imidazole**, 50mM Hepes pH 7.5	**5% (v/v) glycerol,** 50mM Hepes pH 7.5	**10% (v/v) glycerol,** 50mM Hepes pH 7.5	**15% (v/v) glycerol**, 50mM Hepes pH 7.5
F	**100mM KCl,** 50mM Hepes pH 7.5	**100mM NH_4Cl,** 50mM Hepes pH 7.5	**100mM LiCl,** 50mM Hepes pH 7.5	**10mM $MgCl_2$,** 50mM Hepes pH 7.5	**10mM $CaCl_2$,** 50mM Hepes pH 7.5	**1mM EDTA,** 50mM Hepes pH 7.5

“Magic Buffer” = does not exist, you need to screen!

DSF workflow



Purification with initial detergent

↓

DSF based detergents screening

- Dilution into excess detergent
- 1 hour equilibration
- Measurement of temperature induced unfolding and aggregation

↓

Data analysis in MoltenProt

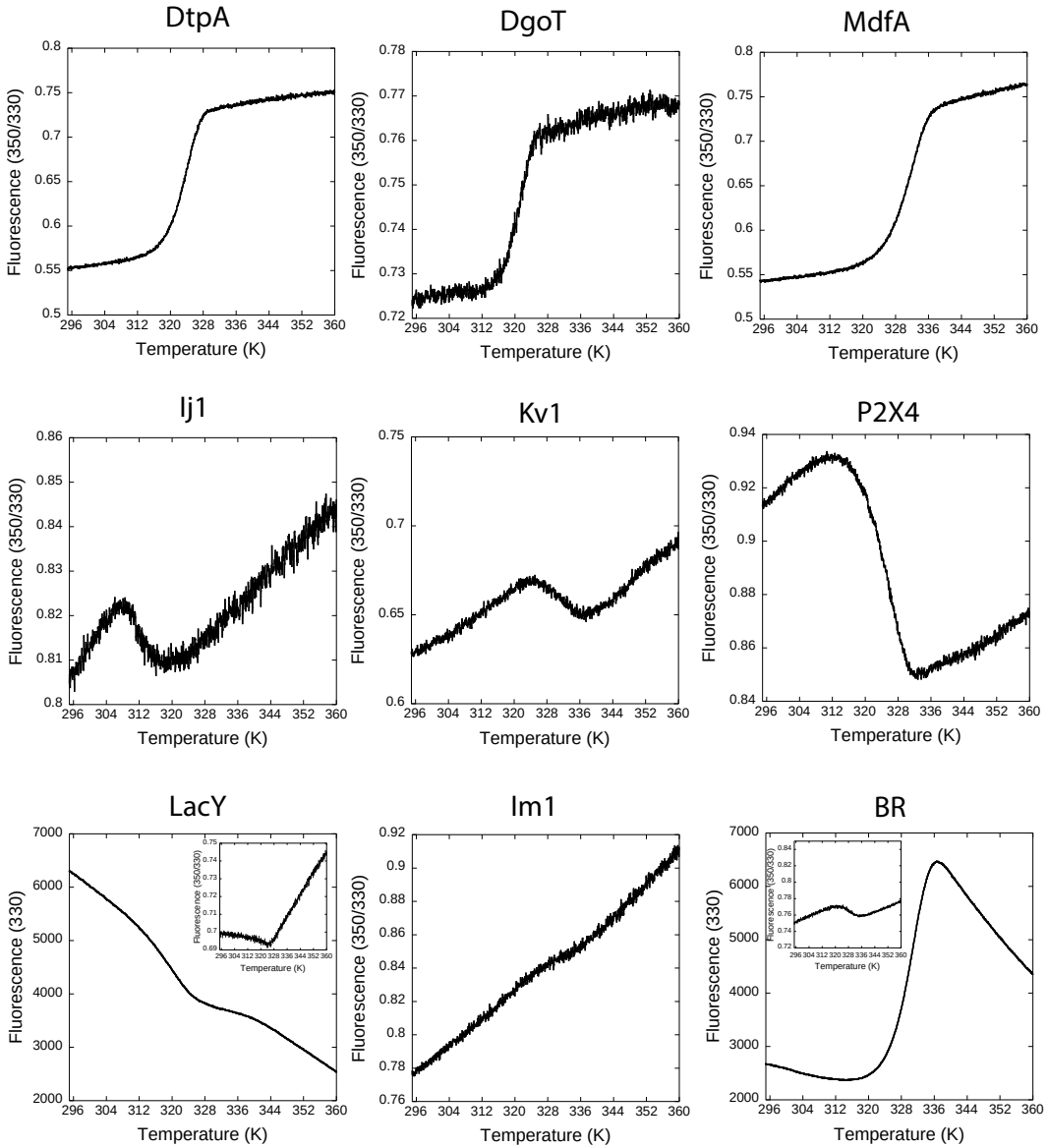
- Signal and temperature selection, normalization
- Melting curve fitting (T_m and T_{agg} estimation)
- Filtering of results and creation of PDF report
- Selection of the best condition(s)

Niebling & Burastero, under revision

Fluorescence raw data

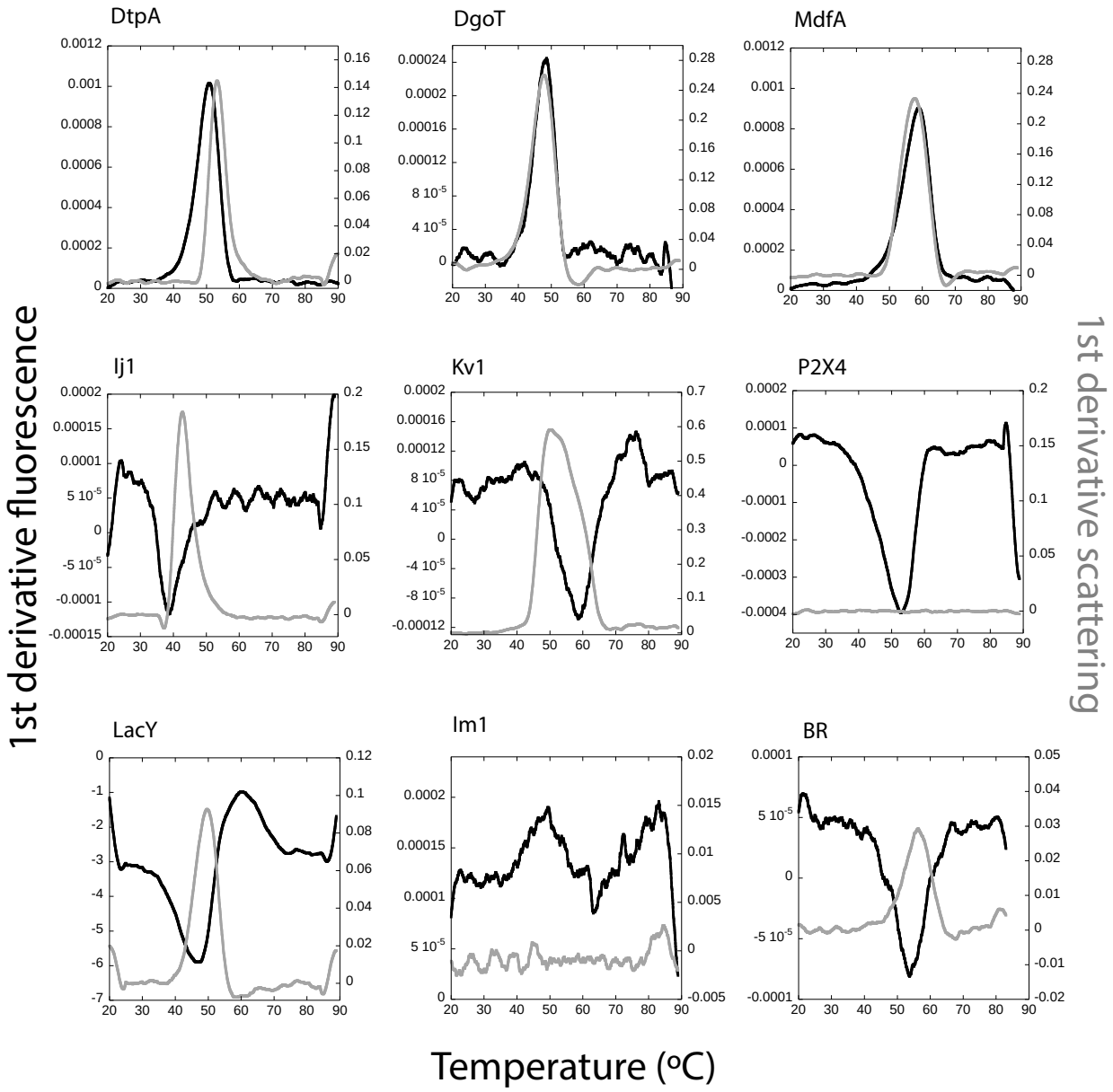
S curves

Z curves



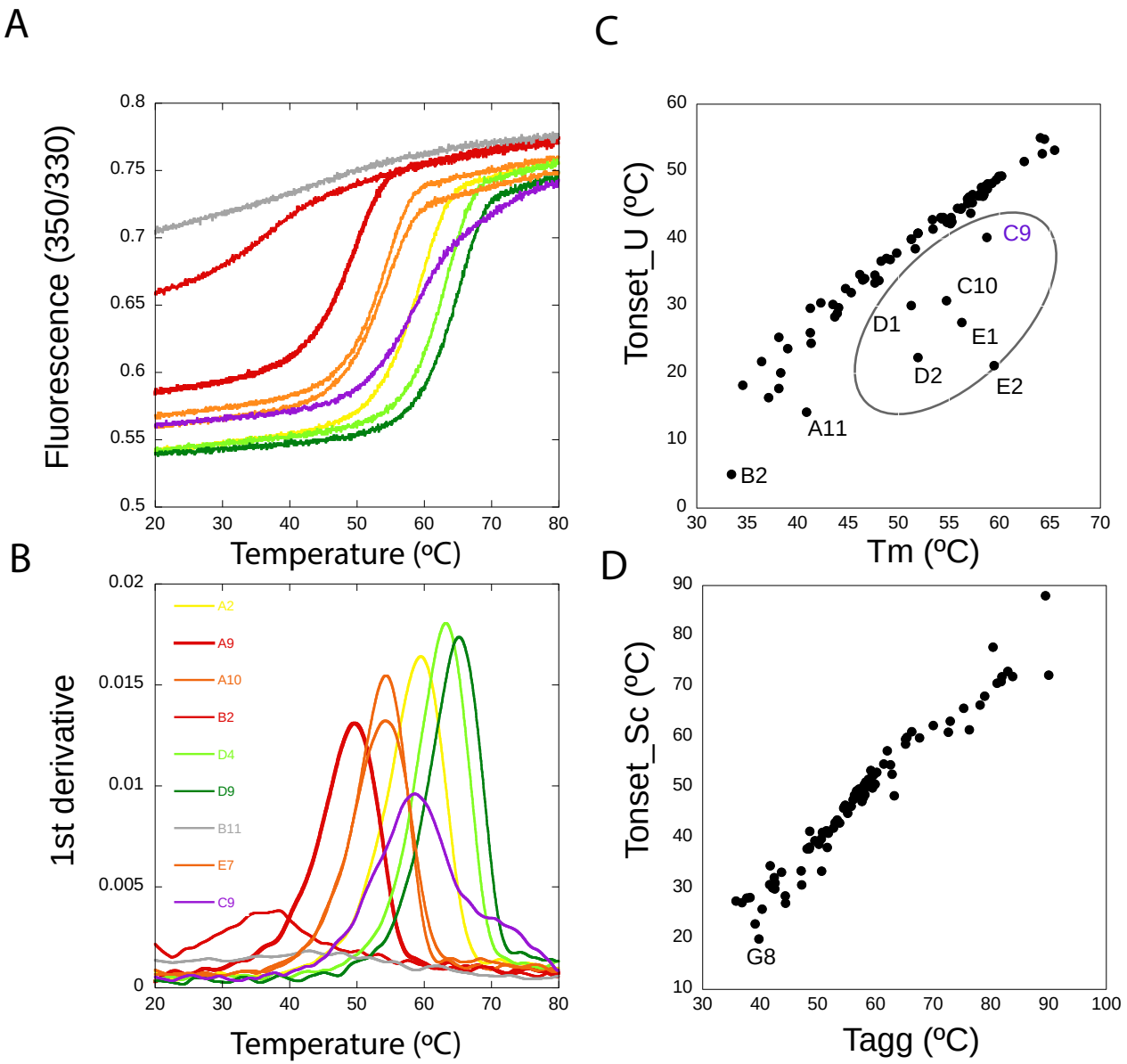
Kotov et al. Sci Rep. 2019

First derivative



Kotov et al. Sci Rep. 2019

Tm vs Tonset



Kotov et al. Sci Rep. 2019

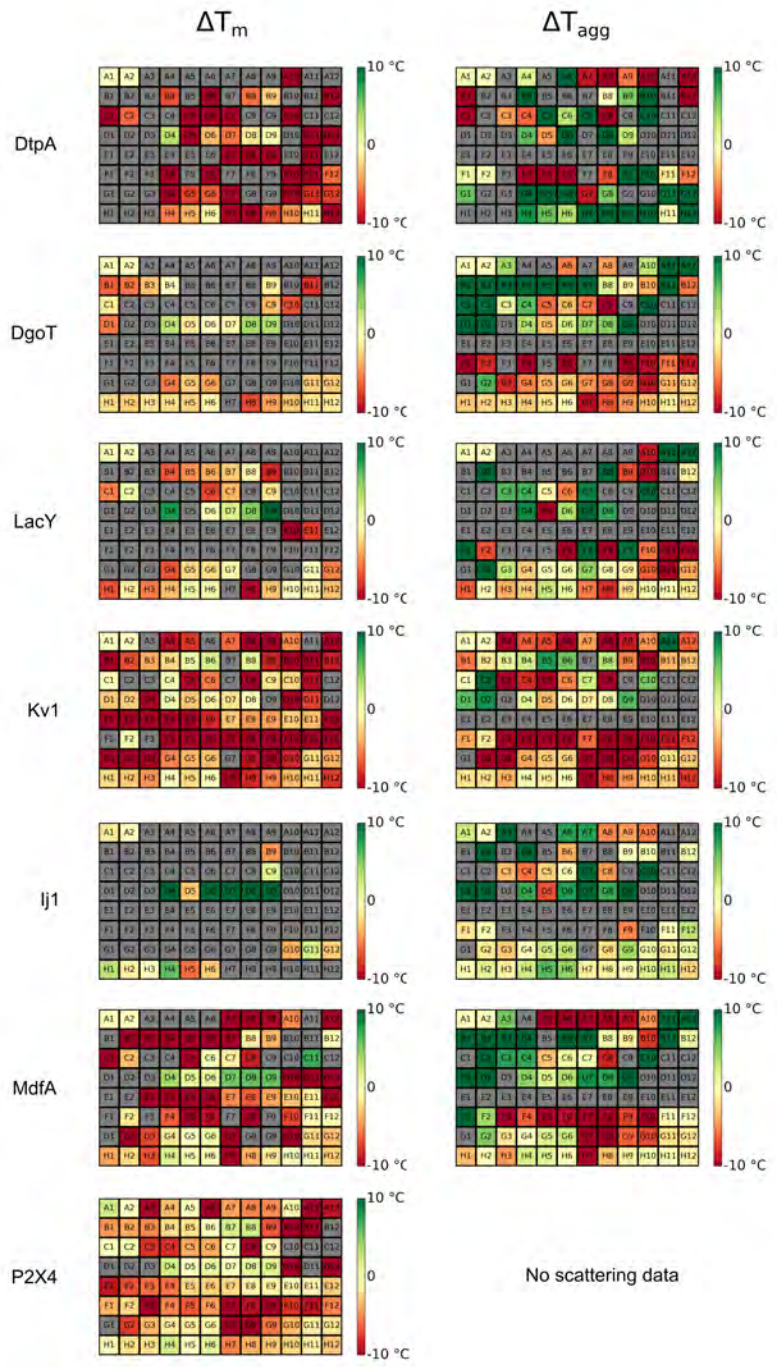
Data Analysis

- screening
96 detergents

- A2 is the starting condition, in our case DDM

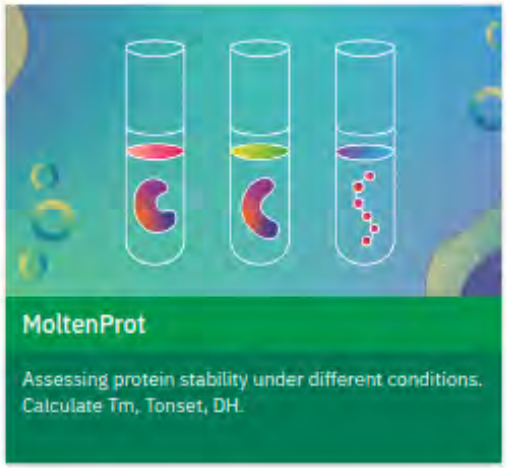
- Calculation of Tm

- **Stable**
- **Unstable**
- **No fit**



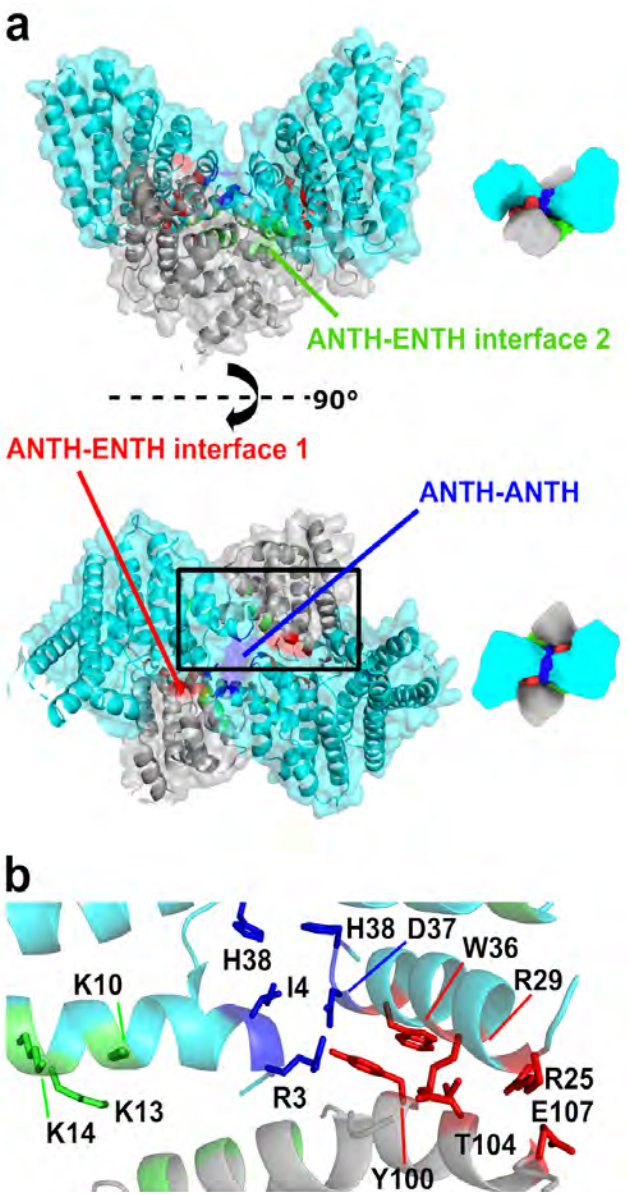
Kotov *et al.* Scientific Reports 2019

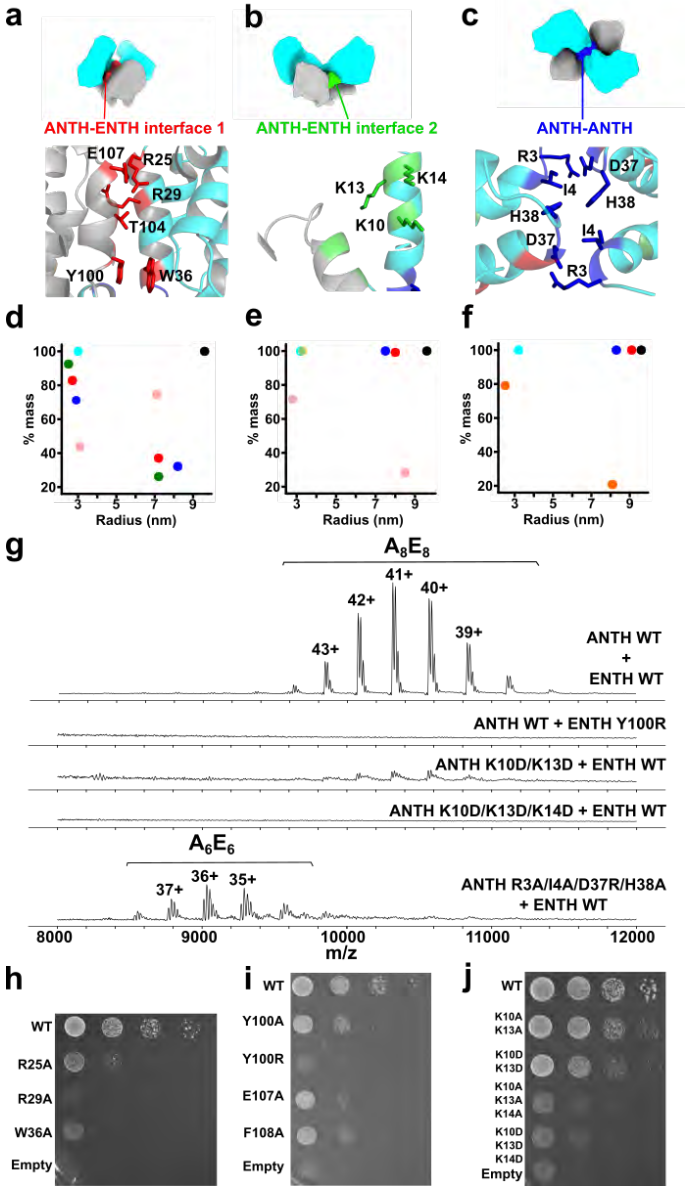
Online Server: eSPC

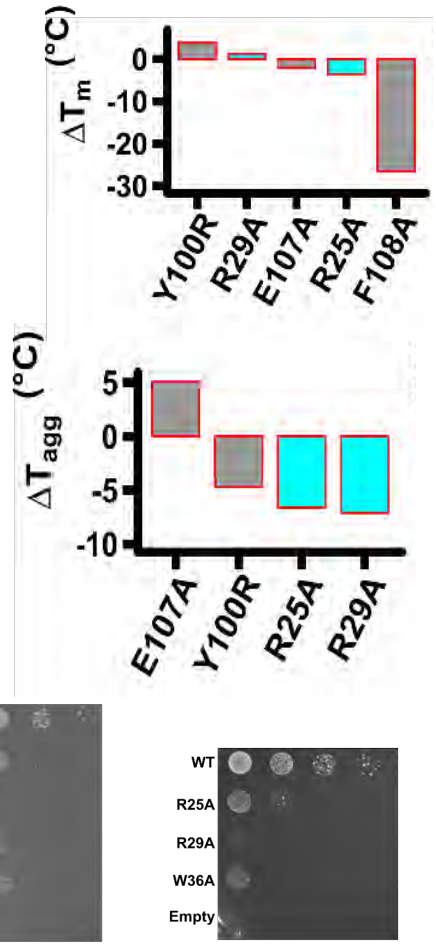


Burastero, et al. Acta Crystallogr D. 2021

Biophysical characterisation of a membrane remodelling complex

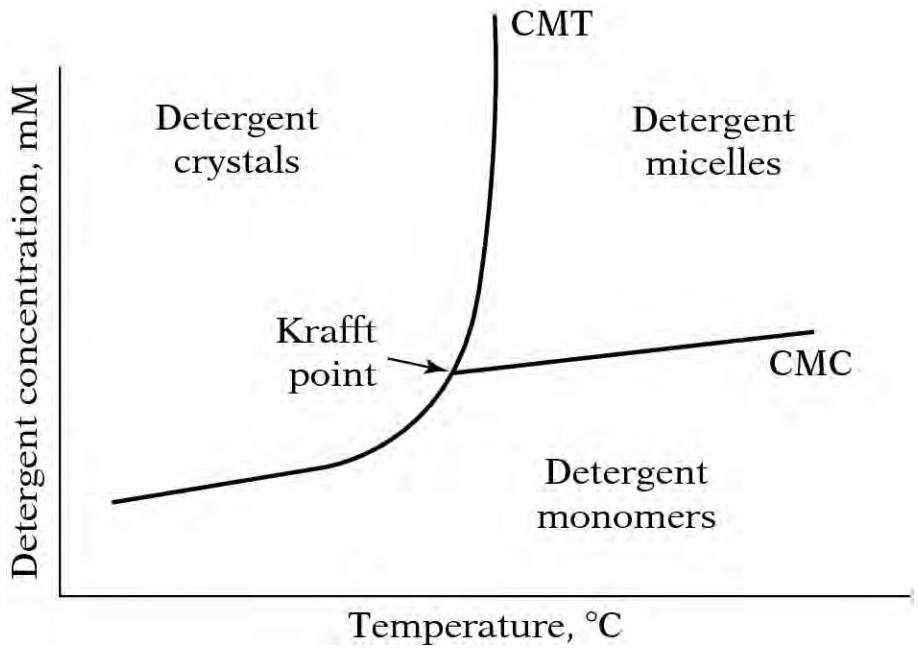






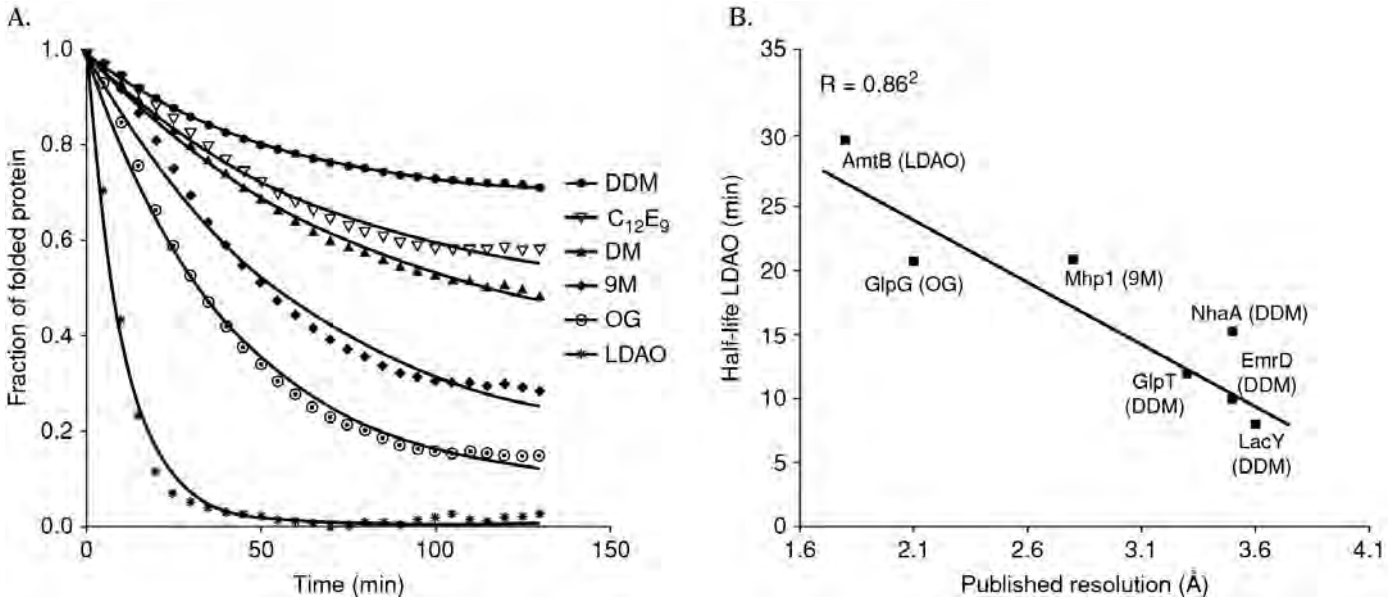
Lizarrondo et al. Nat Commun. 2021

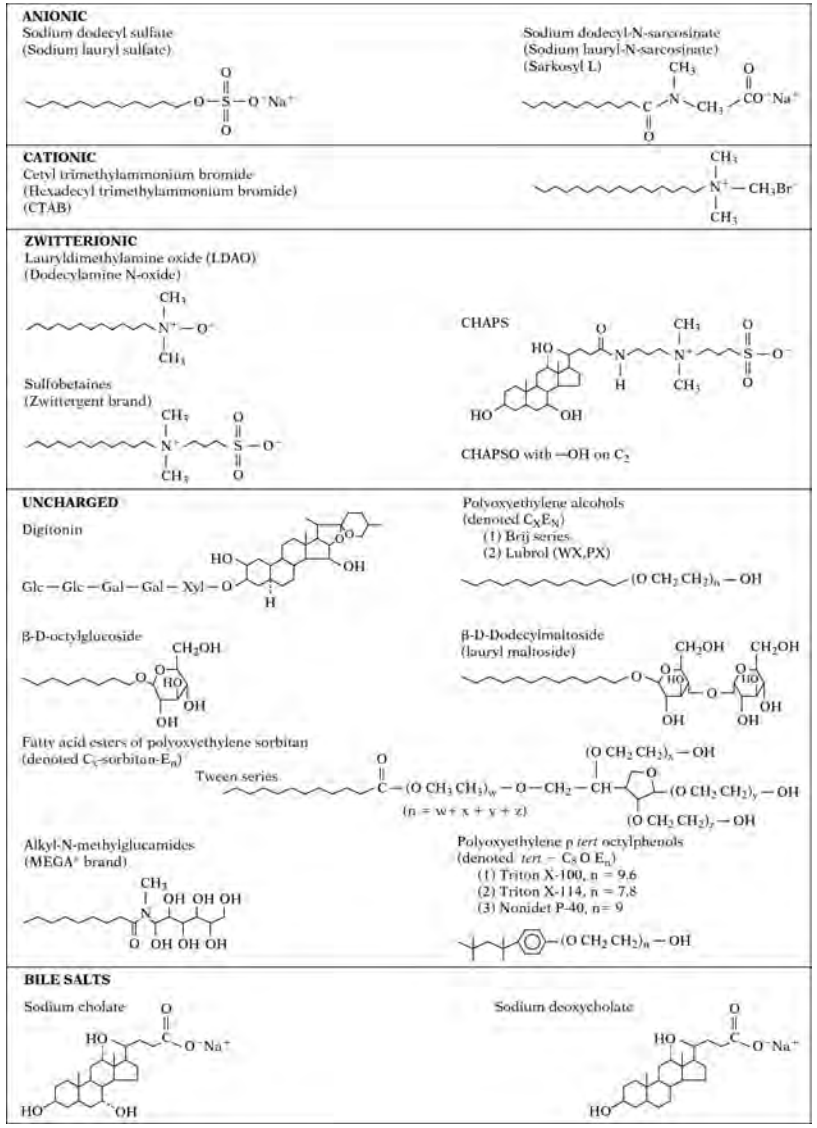
Detergent phase diagram



- Micelle formation dependent on temperature
- CMT: temperature above which micelles form
- Krafft point (cloud point): temperature at which a turbid solution becomes clear due to micelle formation
- Krafft point: intersection of lines for CMC and CMT
- Example: precipitation of SDS below 4° C

Choice of detergent for protein stability and success in crystallization





- Sometimes detergent used for purification is not optimal for crystallization (screening required)
- A) membrane protein stability is assayed by measuring unfolding at 40ºC
- B) stability judged by unfolding rates in LDAO correlates to resolution of membrane protein structures

Dynamic Light Scattering

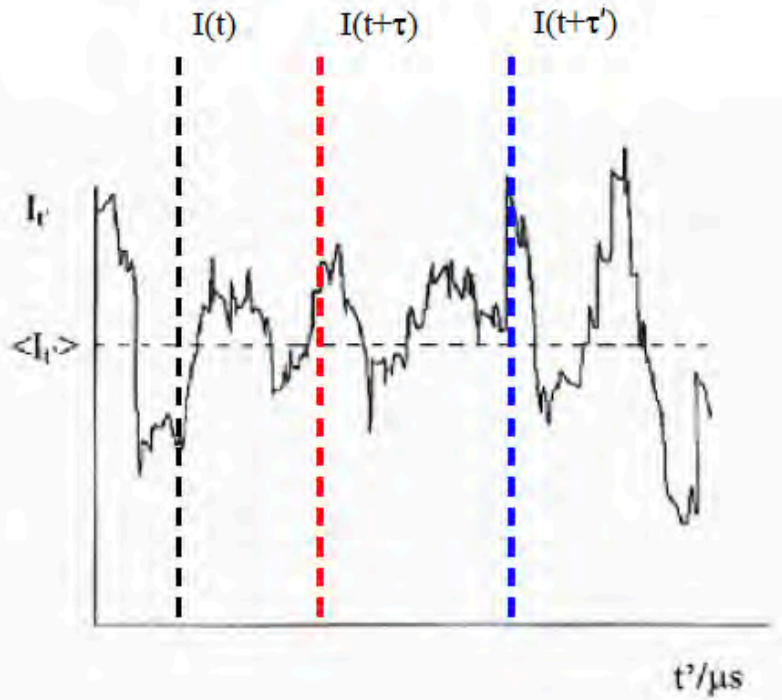
First, the Intensity Correlation Function, $G_2(\tau)$

Describes the rate of change in scattering intensity by comparing the intensity at time t to the intensity at a later time (t + τ), providing a quantitative measurement of the flickering of the light

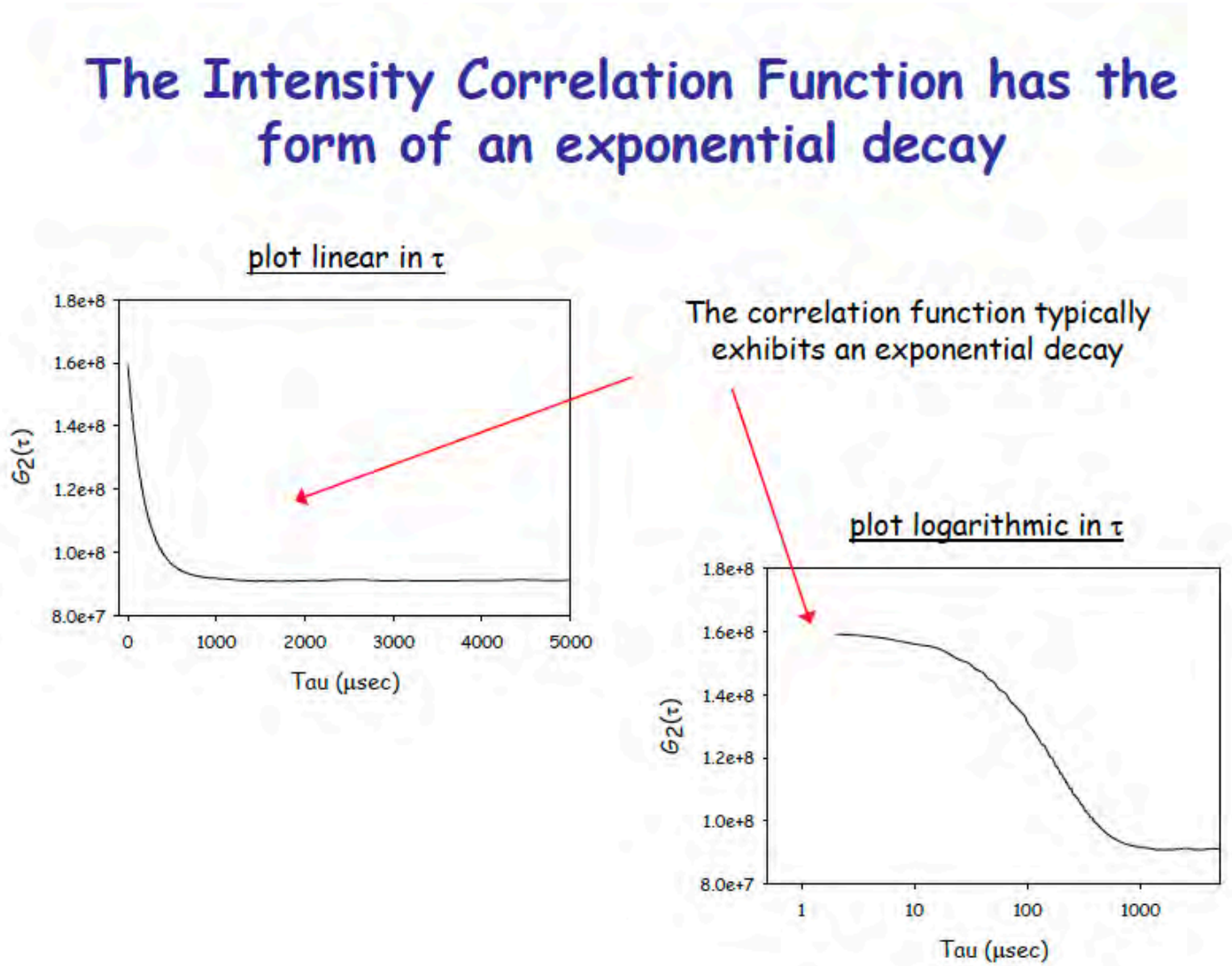
Mathematically, the correlation function is written as an integral over the product of intensities at some time and with some delay time, τ

$$G_2(\tau) = \frac{1}{T}\int_0^T I(t)I(t+\tau)d\tau$$

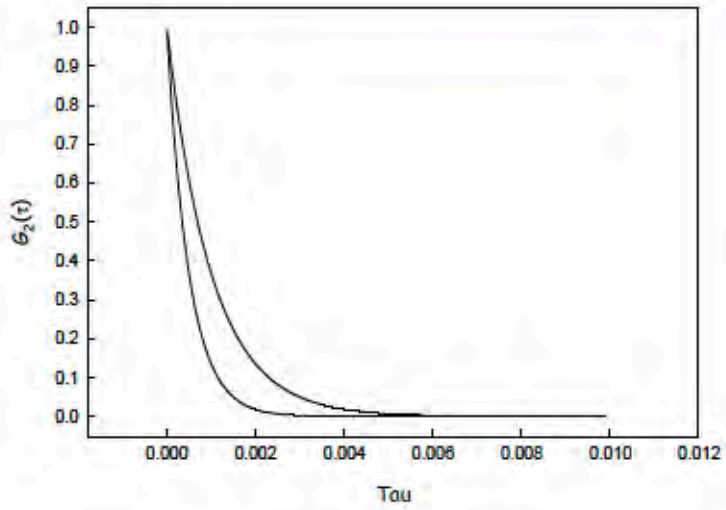
Which can be visualized as taking the intensity at I(t) times the intensity at I(t+τ)- red), followed by the same product at I(t+t')- blue, and so on…



The auto correlation function

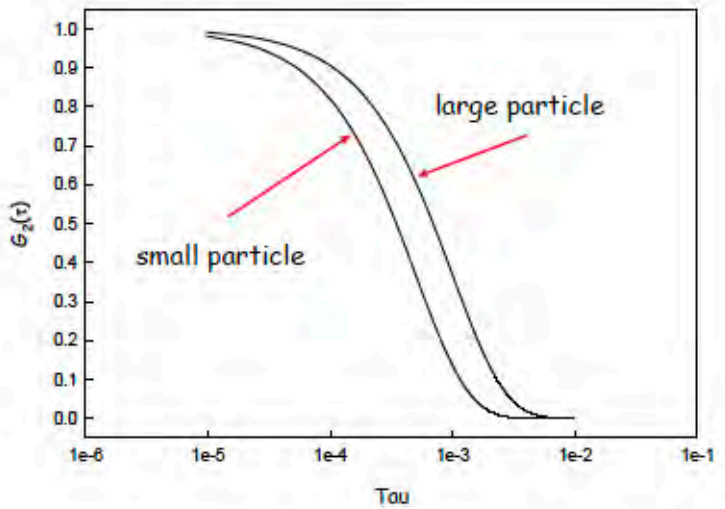


Rate of the decay depends on the particle size



large particles diffuse slower than small particles, and the correlation function decays at a slower rate.

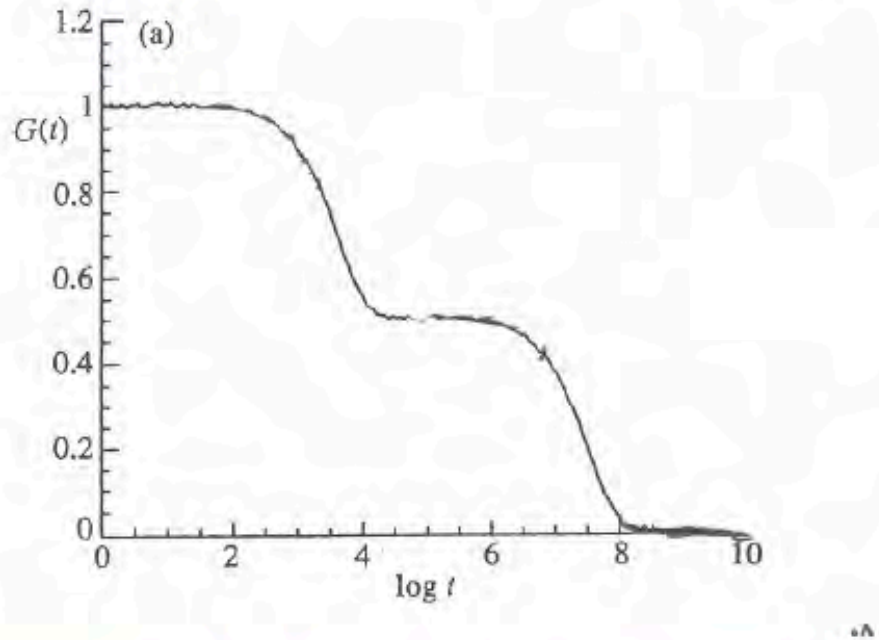
and the rate of other motions (internal, rotation…)



Bimodal distribution

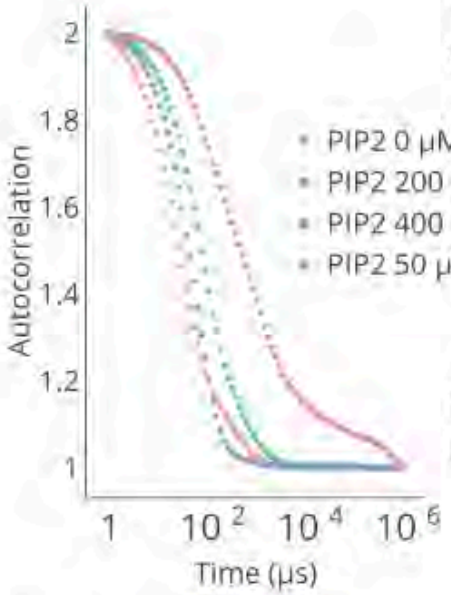
$$g_1(\tau) = \sum a_i e^{-\Gamma_i \tau}$$

Assume a finite number of particles, each with their own decay



To be reliable the sizes must be ~5X different

DLS workflow

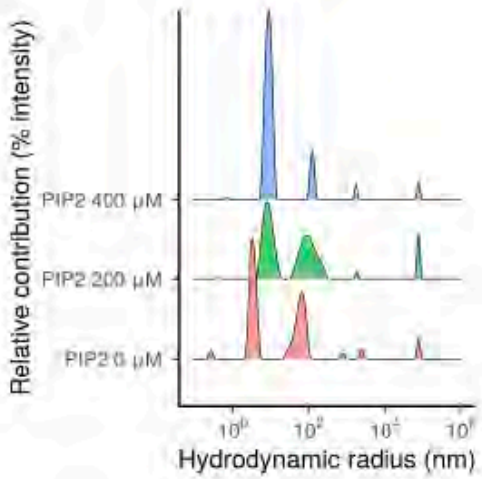


Initial setup & sample preparation

- Check buffer AC curves
- Protein concentration at 0.5 - 2 mg/ml
- 10' max speed spinning

Membrane protein DLS measurements

- 30 curves averaged, 1-5 sec acquisition time
- Try different lipids concentration (i.e., below and above the CMC)



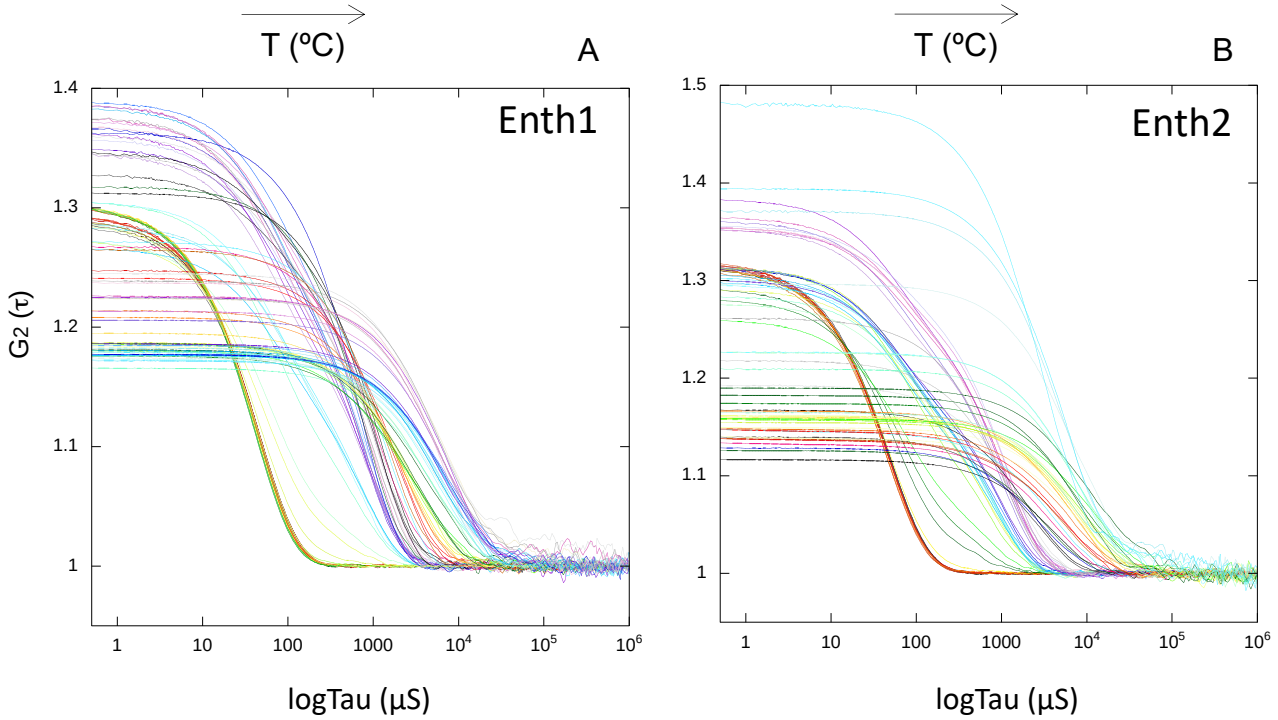
Data analysis

- Filtering of the AC curves
- Non-parametric distribution fitting
- Use peak width & height to assess sample quality

Niebling & Burastero, under revision

Dynamic Light Scattering at different Temperatures

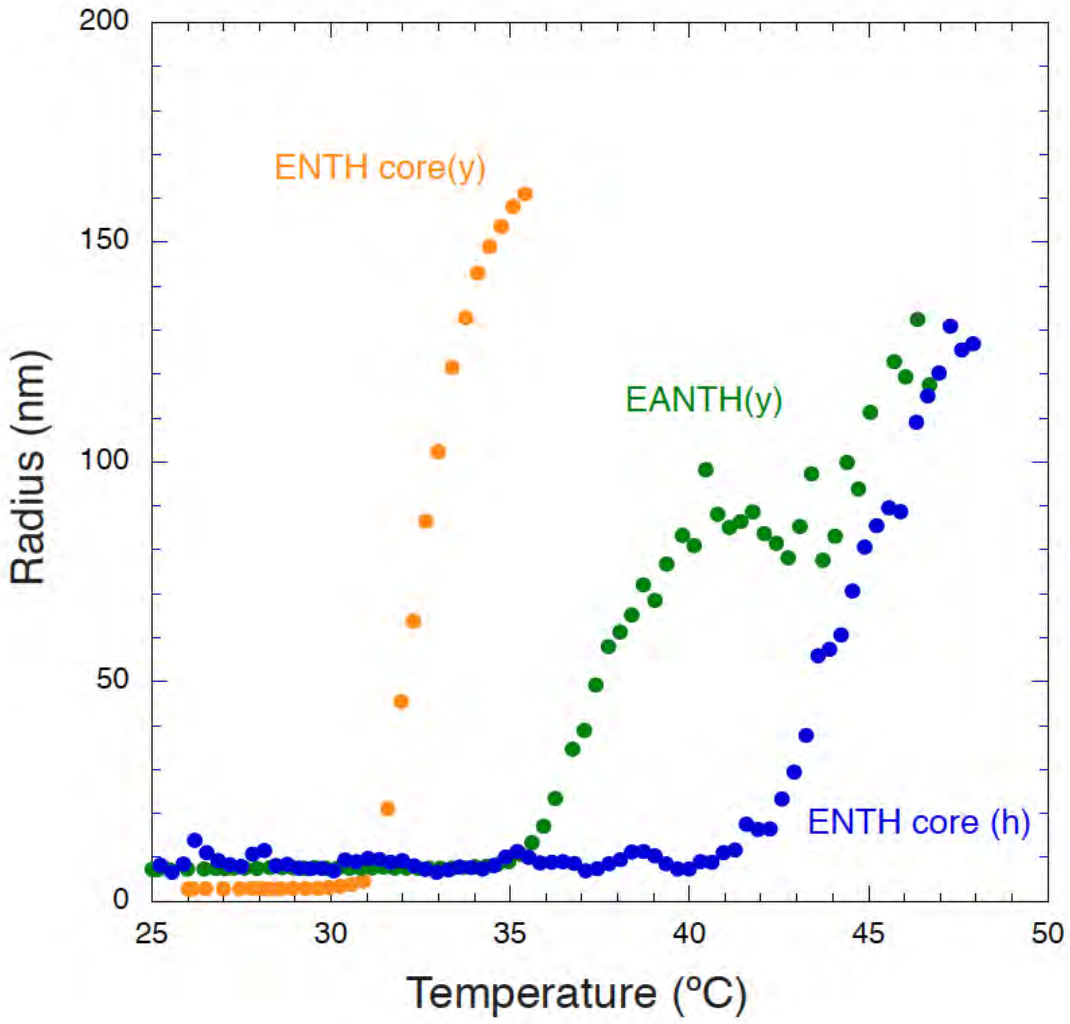
Intensity Correlation Function



[ENTH: SLA2: PiP2]≅ 1:1:20

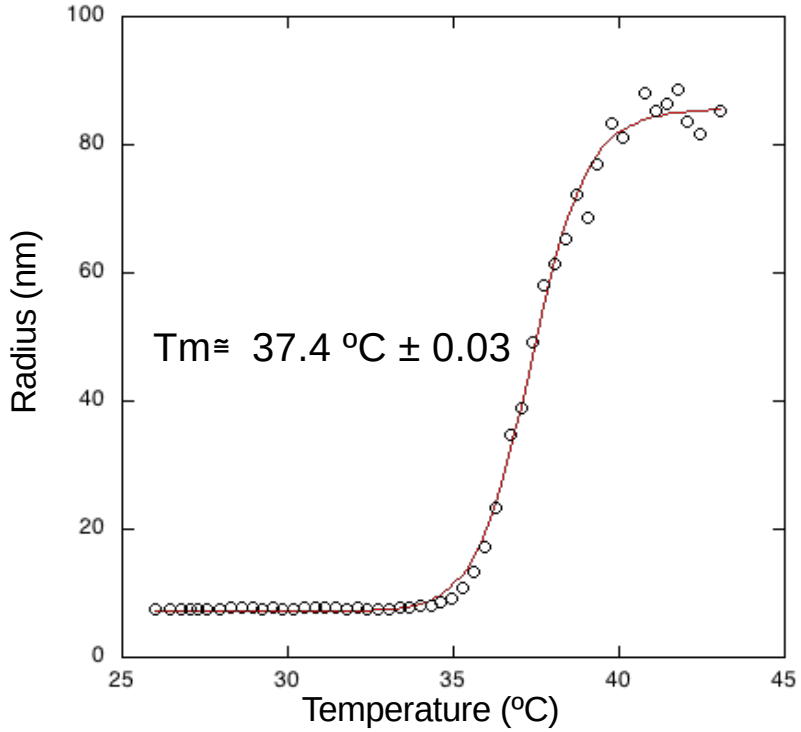
Thermal stability of the AENTH complex

Following protein aggregation by DLS



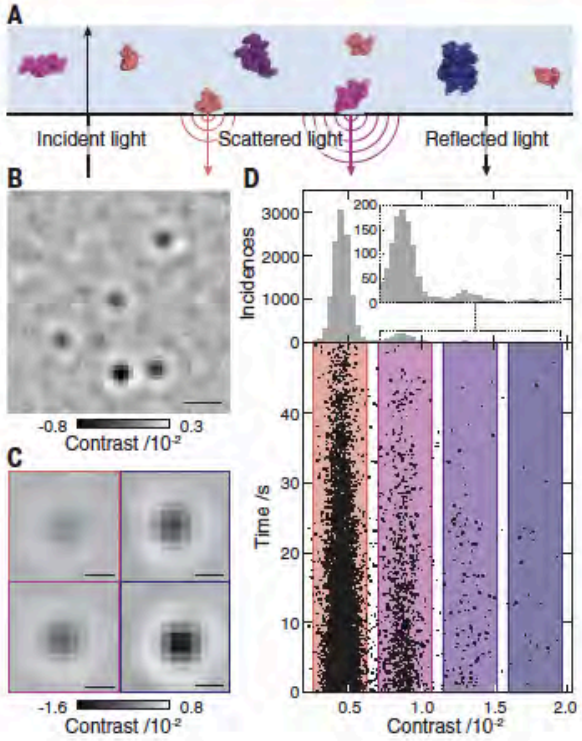
Increase in Rh as a function of temperature monitored by DLS

The AENTH complex is more stable than the ENTH-PIP2



Garcia-Alai et al. Nat Commun. 2018

Mass Photometry on Membrane proteins



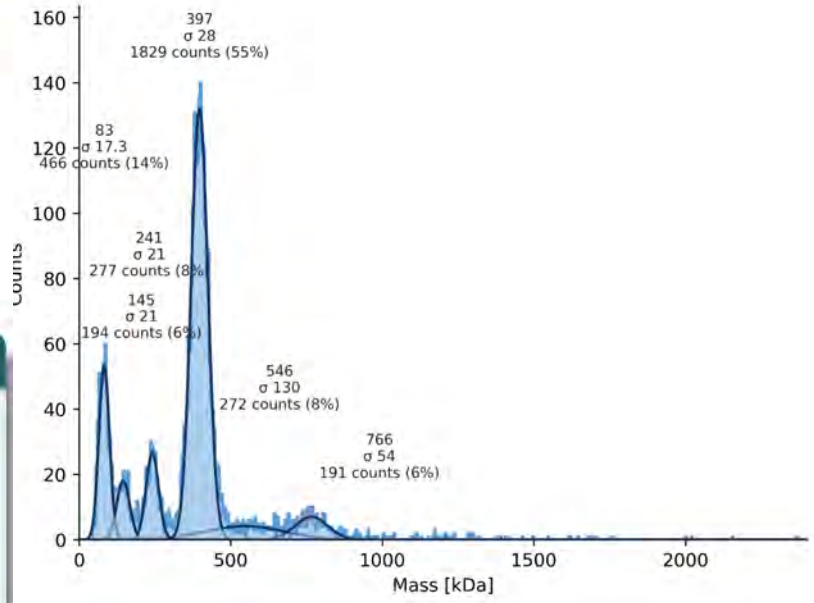
Science 2018, 360(6387), 423-427

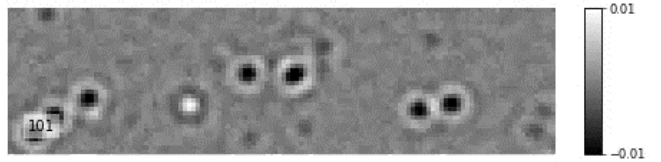
Advantages

- Measurement in solution
- Low sample consumption
- MDa range is no problem
- Easy measurement

Sample requirements

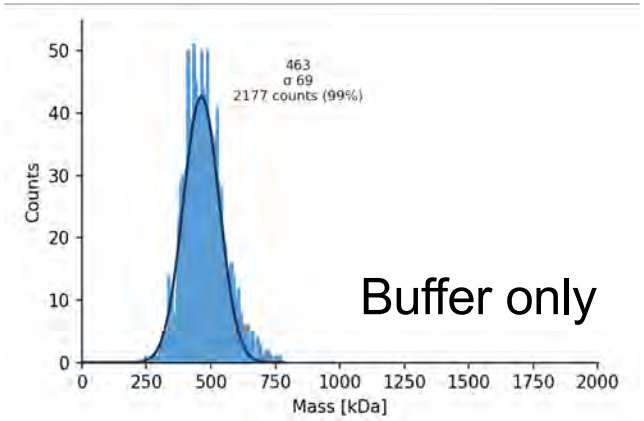
- Protein mass > 40kDa
- Conc. ≈30–100 nM
- For complexes: K_d in nM range or lower
- Buffer: Fresh and filtered (0.22 μm)



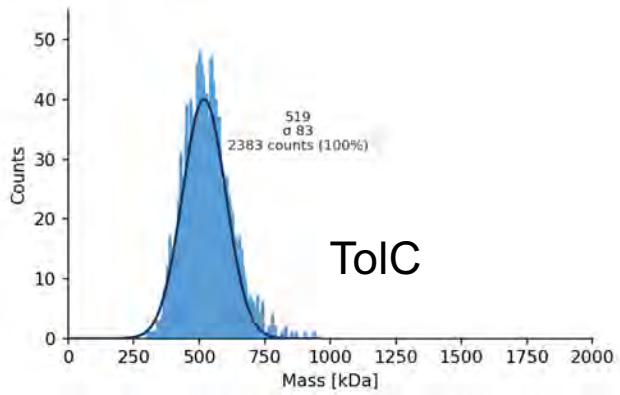


Expected MW of monomer 72 kDa + saposins + lipids

Sample – TolC in 0.3% DDM (theor. mass: 161 kDa)



Buffer only

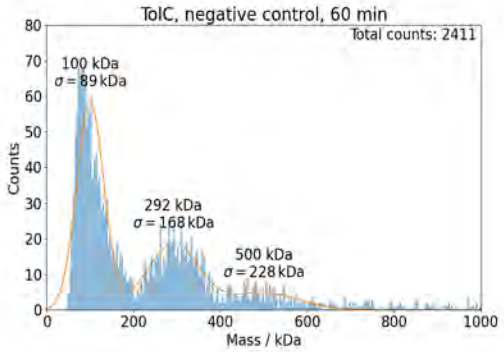


TolC

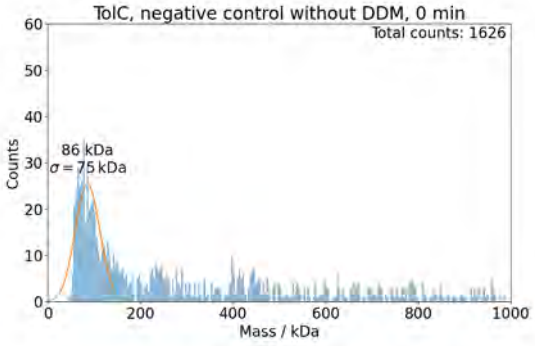
Critical micelle concentration

Relative concentration	1	5	20	100	500	2000	
SDS	0.082	0.41	1.64	8.2	41	164	mM
	N/A	70	70	170	180	180	kDa
DDM	0.0012	0.006	0.024	0.12	0.6	2.4	mM
	N/A	N/A	N/A	560	560	560	kDa
OG	0.23	1.15	4.6	23	115	460	mM
	N/A	N/A	N/A	220	460	760	kDa
Digitonin	0.004	0.02	0.08	0.4	2	8	mM
	N/A	60	240	900	910	1170	kDa
NP-40	0.0008	0.004	0.016	0.08	0.4	1.6	mM
	N/A	50	90	260	430	430	kDa
Tween®20	0.00059	0.00295	0.0118	0.059	0.295	1.18	mM
	90	120	240	430	430	430	kDa

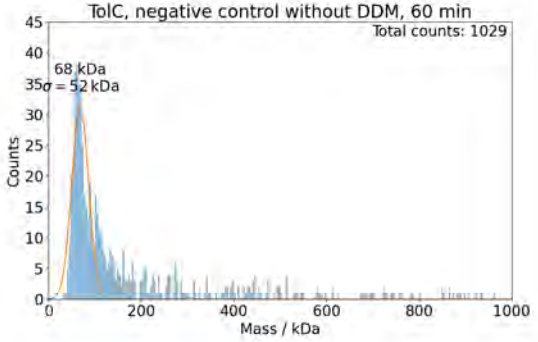
dilution in buffer without DDM 0.0003 % DDM



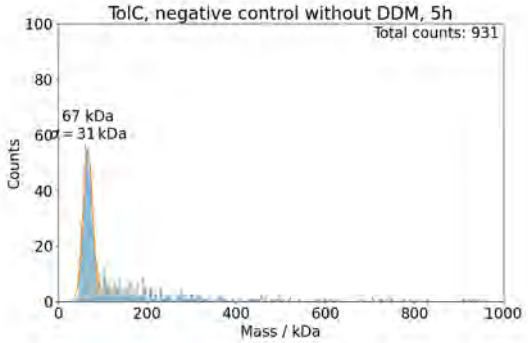
with 0.03% DDM



without DDM 0 min

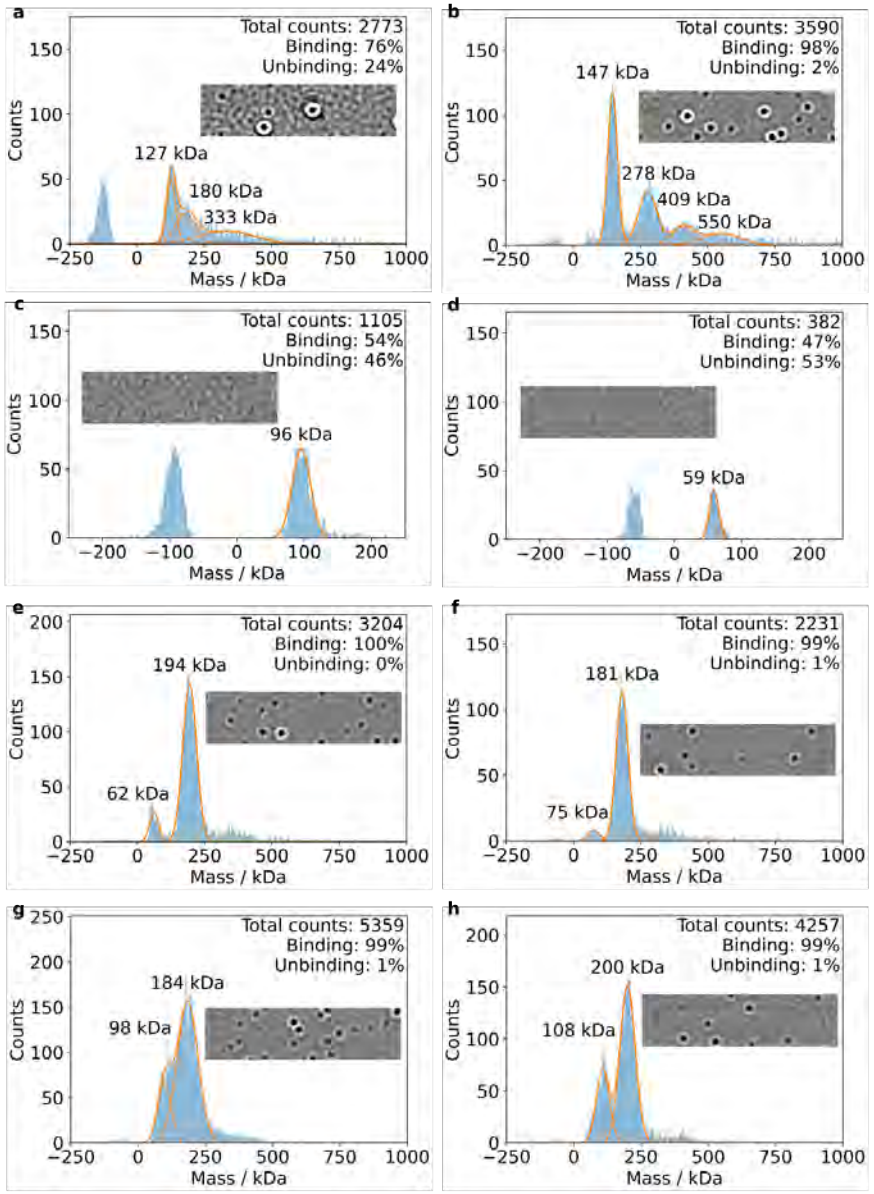


without DDM 60 min



without DDM 5 h

Mass Photometry on Membrane proteins



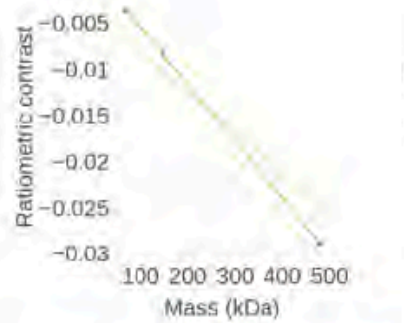
IJ1 (abc transporter) in “detergent-free” buffer

a. Measurement using a protein
final DDM concentration is 0.0009% and 40 nM protein.
b. final DDM concentration is 0.00015% and 80 nM protein.
c. and d. Control experiments using similar DDM concentrations and no protein.
e. and f. show the mass histograms different concentrations of LMNG as detergent at a final protein concentration of 40 nM.
g. and h. Amphipol solubilized

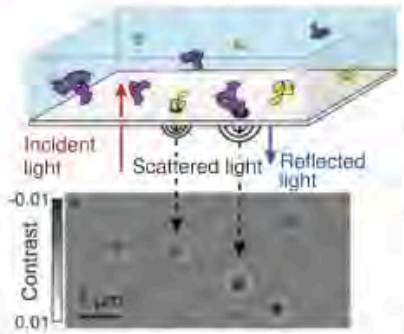
Ij1 65 KDa
dimer 130 KDa

Niebling *et al.* Front. Mol. Bio. 2022

Mass Photometry workflow

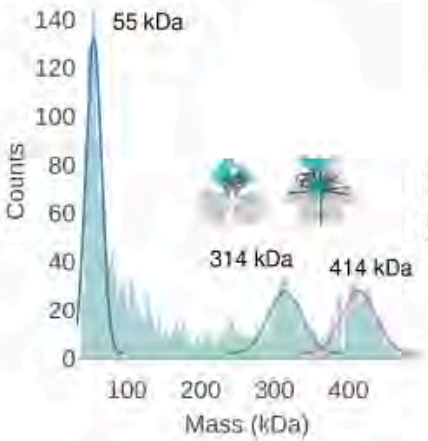


Calibration of the MP instrument
(3 protein standards, at least).



Membrane protein
MP experiment

- Initial protein stock at 1 µM
- 1:10 dilution (to decrease the detergent concentration)
- Measurement of binding and unbinding events

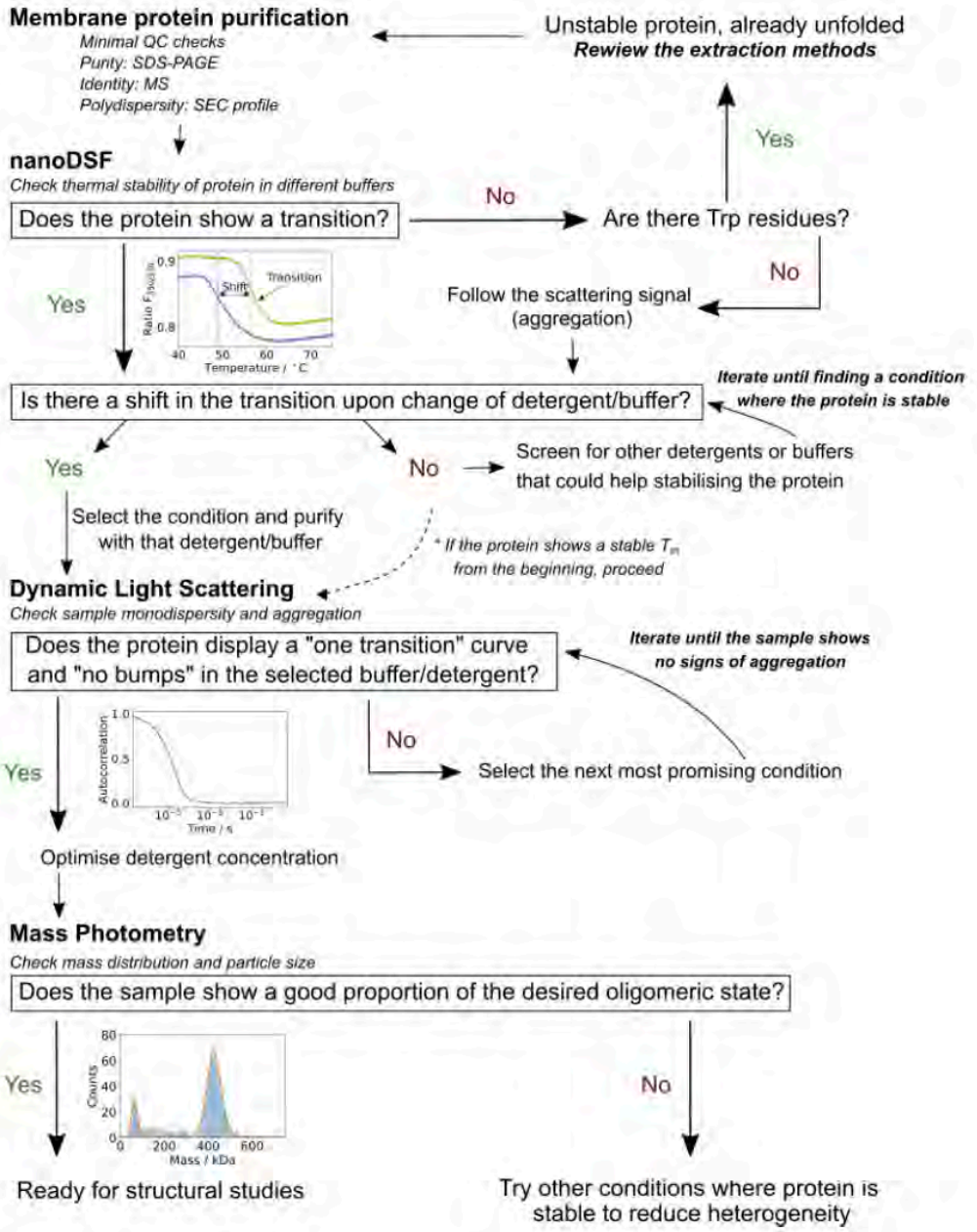


Data analysis
in PhotoMol

- Generation of the masses histogram
- Gaussian fitting and estimation of the sample species masses

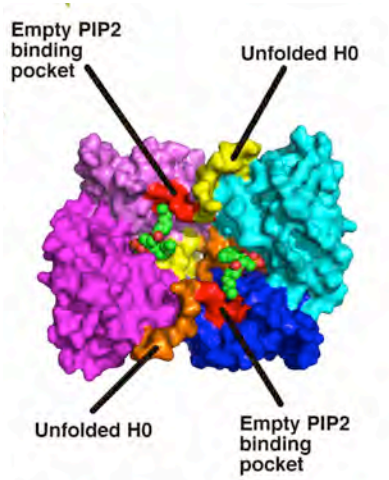
Niebling & Burastero, under revision

The complete pipeline

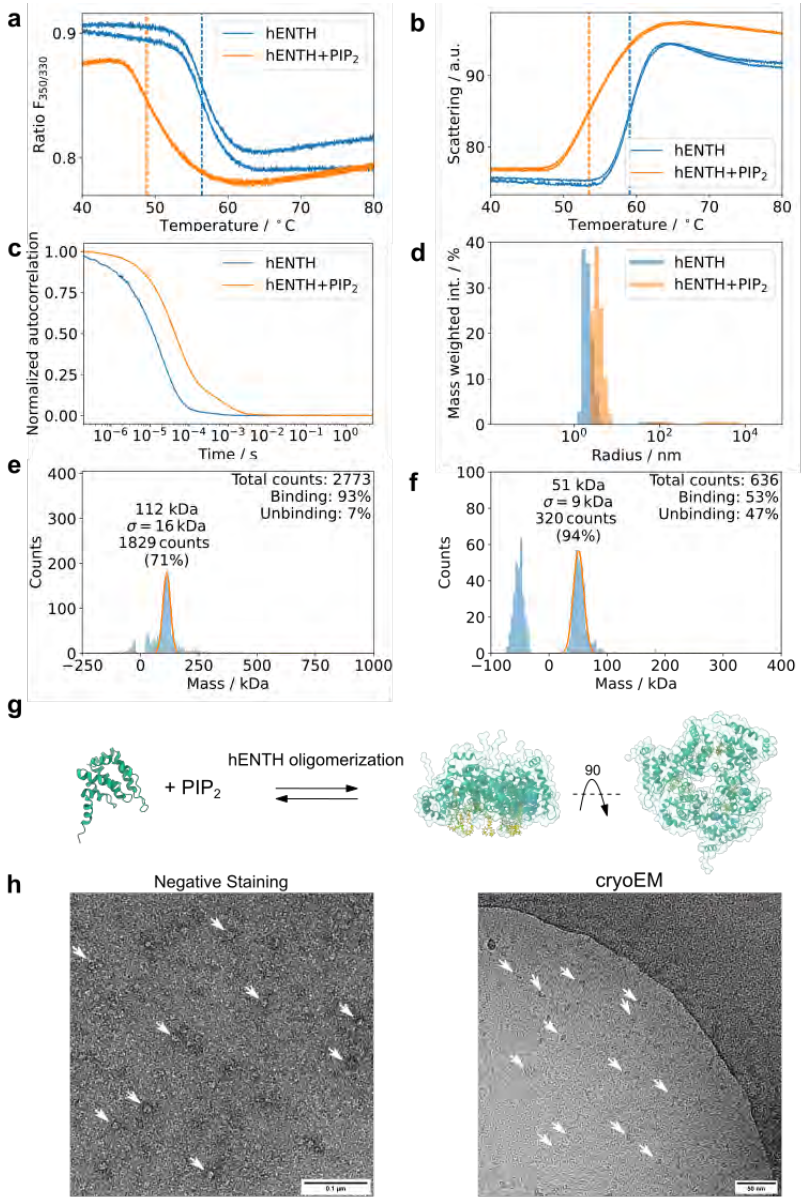


Niebling *et al.* Front. Mol. Bio. 2022

Following oligomerisation by DSF, DLS and Mass Photometry



Garcia-Alai et al. Nat Commun. 2018



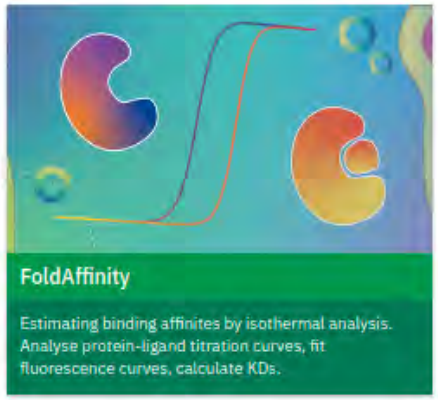
Niebling *et al.* Front. Mol. Bio. 2022

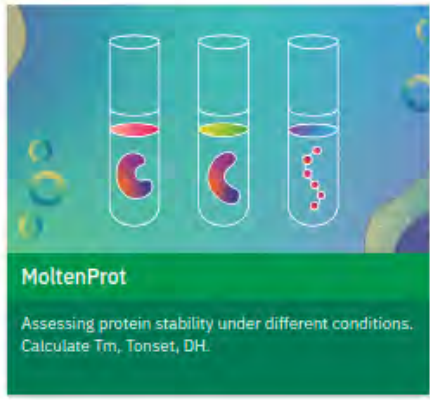
eSPC Online Data Analysis Platform for molecular biophysics

The EMBL Sample Preparation and Characterisation (SPC) Data Analytics Webserver provides easy to use software for the understanding of biophysical experiments.

spc.embl-hamburg.de

Differential Scanning Fluorimetry





MicroScale Thermophoresis

Research in Germany
@ResearchGermany

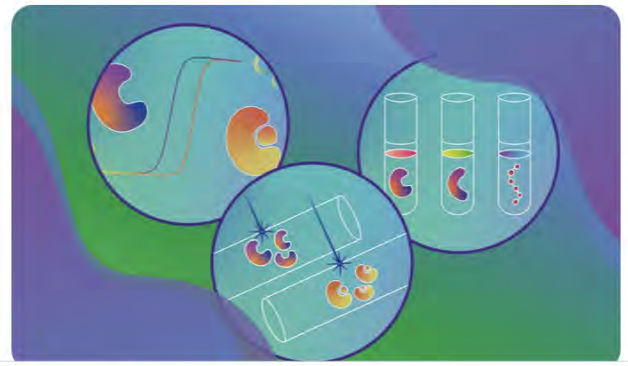
A New free online tool released by @embl Hamburg enables scientists around the world to easily analyse their data without the need to travel to the laboratory where the data was generated 👉ow.ly/tluP50Gyc8J #OpenScience #MolecularInteractions

EMBL
@embl

The @SPC_EMBL_HH has developed eSPC – a free online platform for analysing data from diverse biophysics experiments.

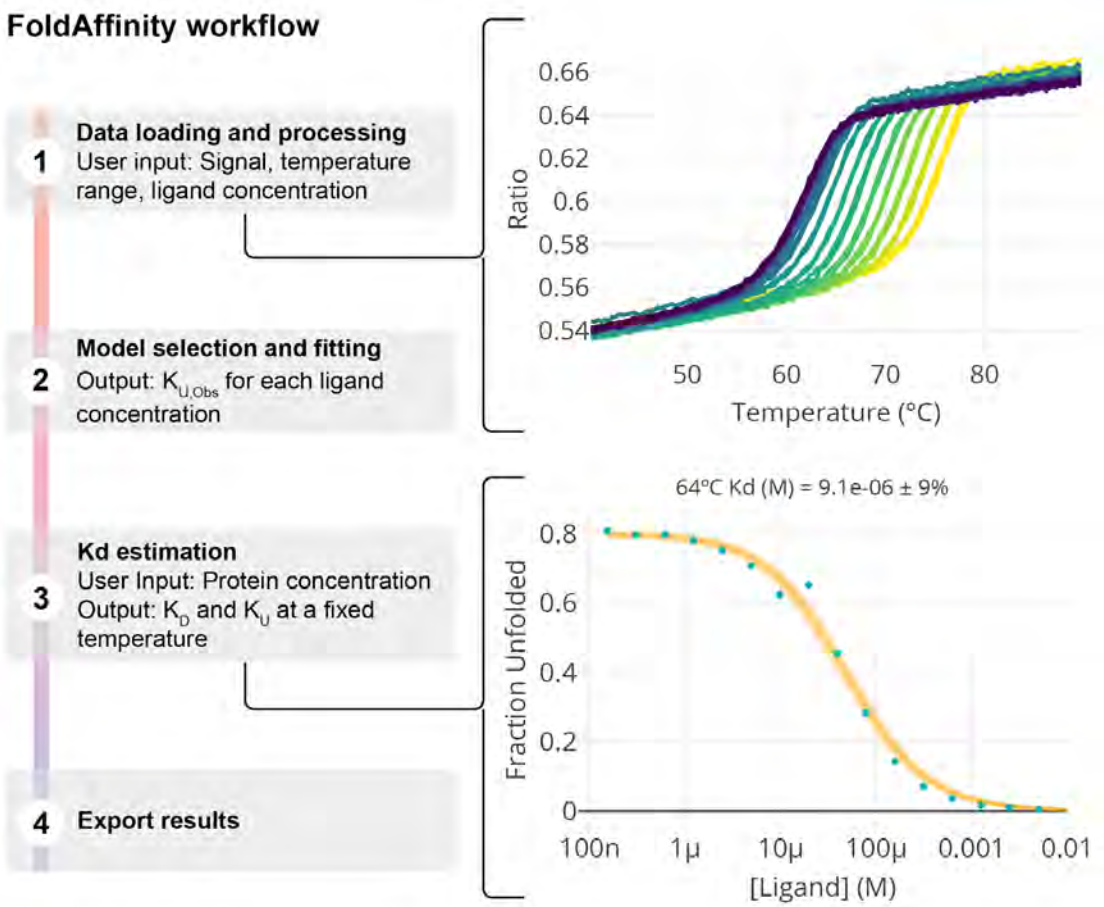
It enables scientists across fields to analyse their data much easier than before, and remotely without the need to travel.

embl.org/news/science/b...



Kotov *et al.*, Sci. Rep. **2019**
Kotov *et al.*, Prot. Sci. **2020**
Burastero et al. Acta Crystallogr D. **2021**
Niebling et al. Sci Rep. **2021**

Estimating binding affinities by Isothermal analysis



Screenrecord of FoldAffinity, an online application to estimate protein-ligand binding affinity.

Niebling et al, *in review*

Niebling et al. Sci Rep. **2021**

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