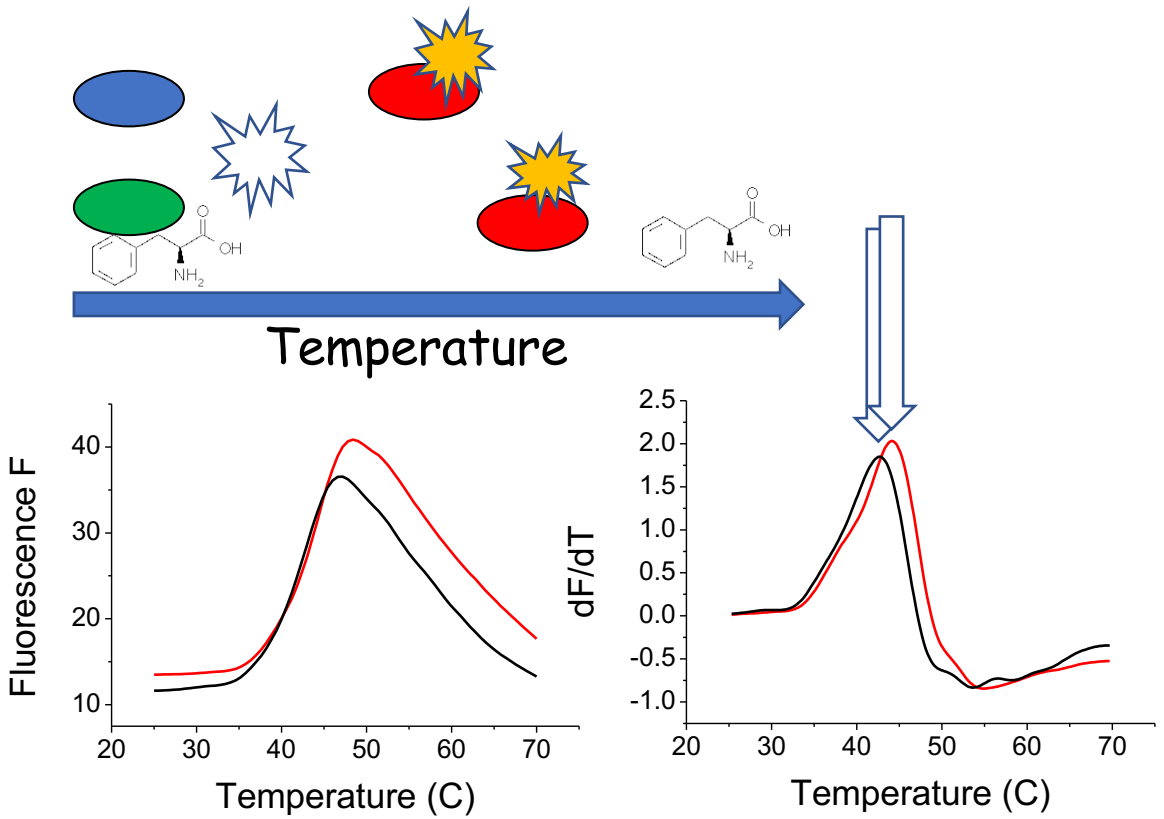
DSF Lecture
QC4B Pasteur 2022

Maria Garcia Alai
EMBL Team Leader

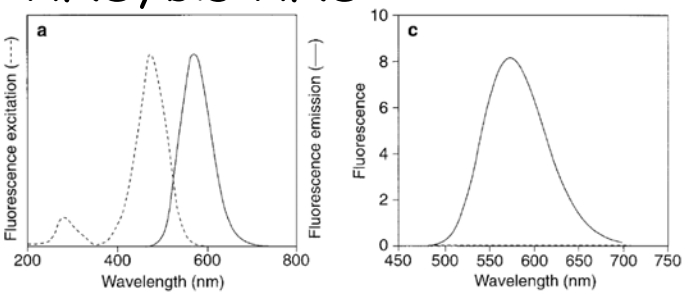
EMBL

6th April 2022

Differential Scanning Fluorimetry



ANS, bis-ANS



SYPRO Orange, Mol. Probes,
Steinberg et al Anal. Biochem. 1996

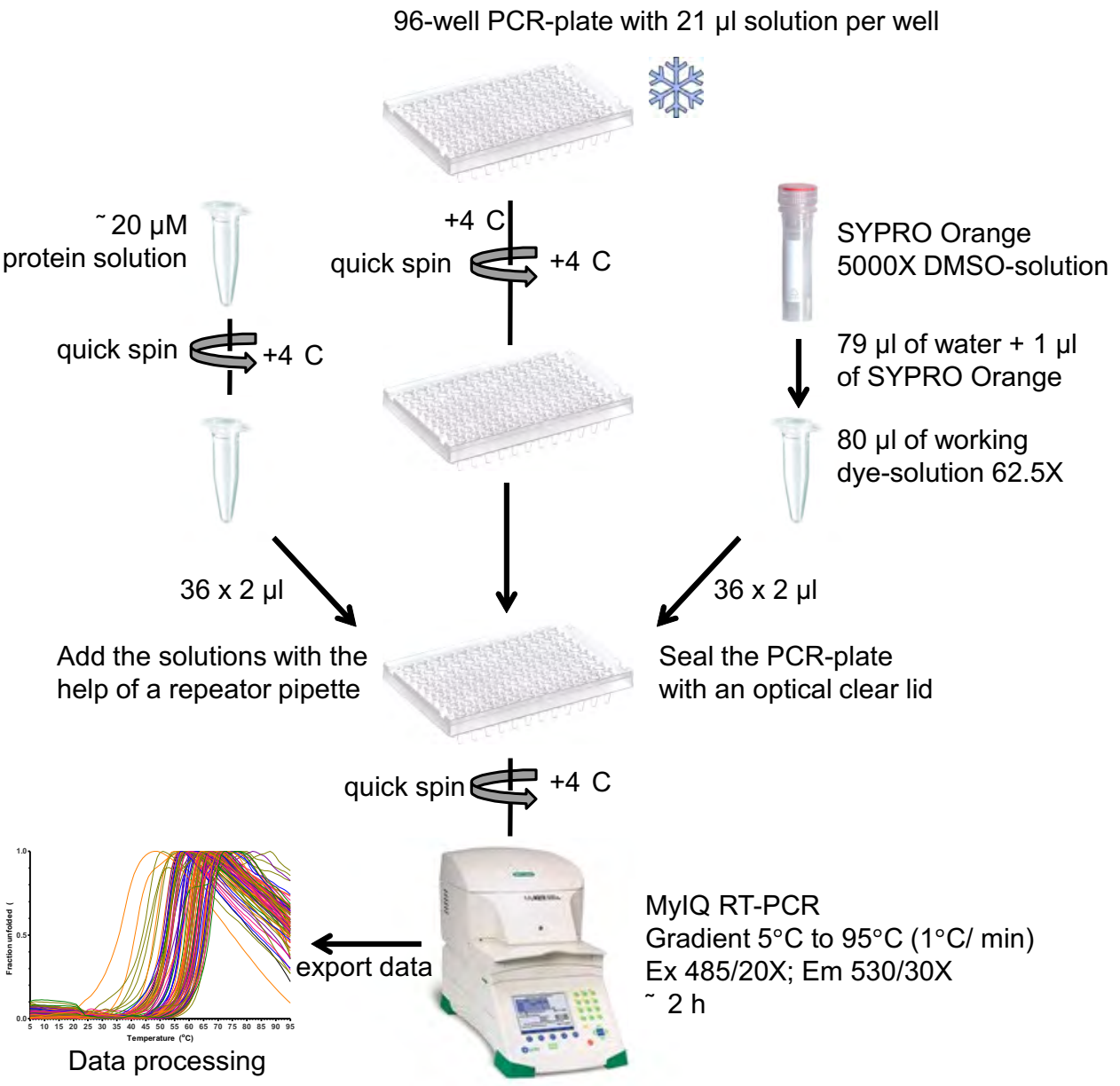
Pantoliano et al J Biomol Screen. 2001

Hydrophobic interfaces

Solubility
Turbidity
Aggregation
Fluorescence
Absorbance

72/100 samples
96 ul samples
10-40 uM

Setting up a Thermofluor experiment

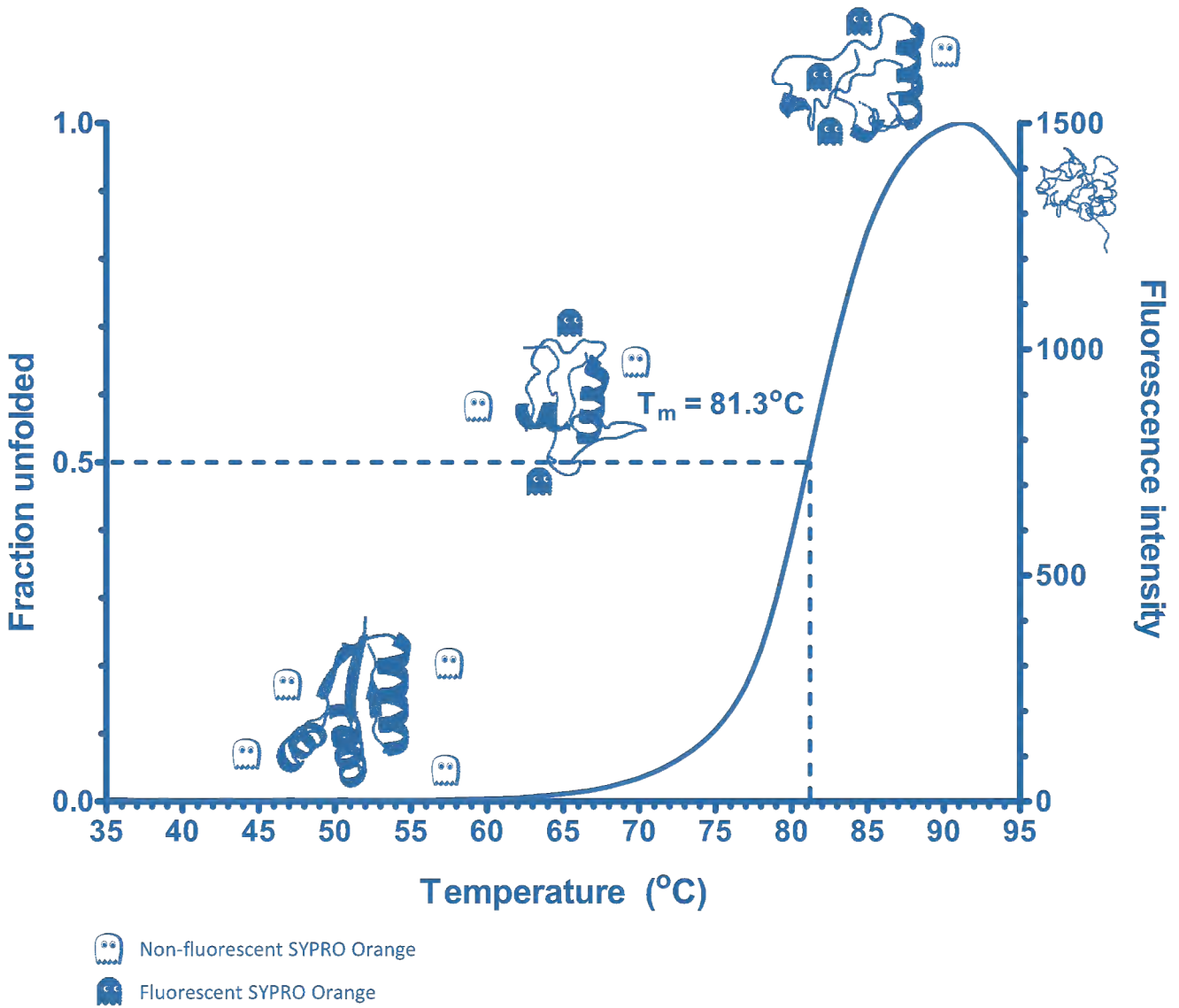


The 36-well Thermofluor screen

	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

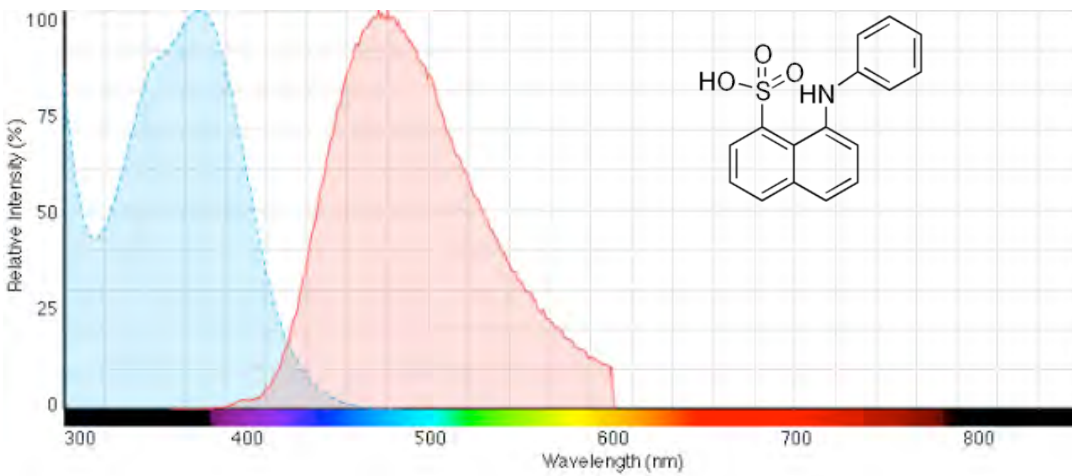
"Magic Buffer" = Succinic Acid / NaHepes / Glycine [2:7:7]

Principle of Thermofluor



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

8-anilino, 1-naphthalene sulfonate



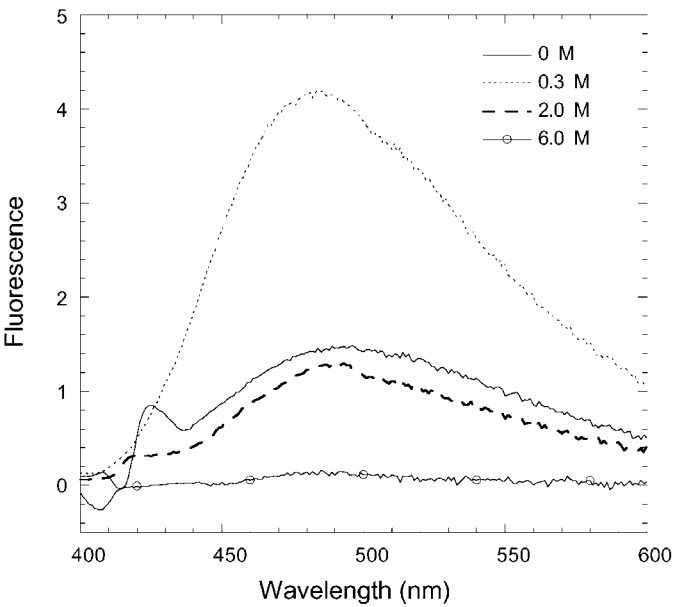
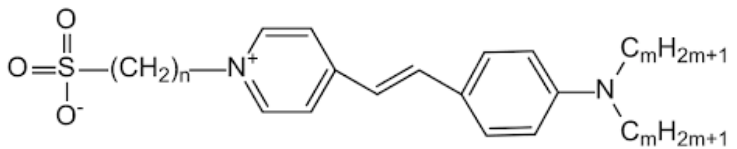


FIGURE 7: ANS binding of E7 after the GdmCl-induced conformational transition at the diferent denaturant concentrations indicated.

Alonso LG1, García-Alai MM, Nadra AD, Lapeña AN, Almeida FL, Gualfetti P, Prat-Gay GD. Biochemistry. 2002 Aug 20;41(33):10510-8.

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



S5692 Supelco

SYPRO® Orange Protein Gel Stain

(0)

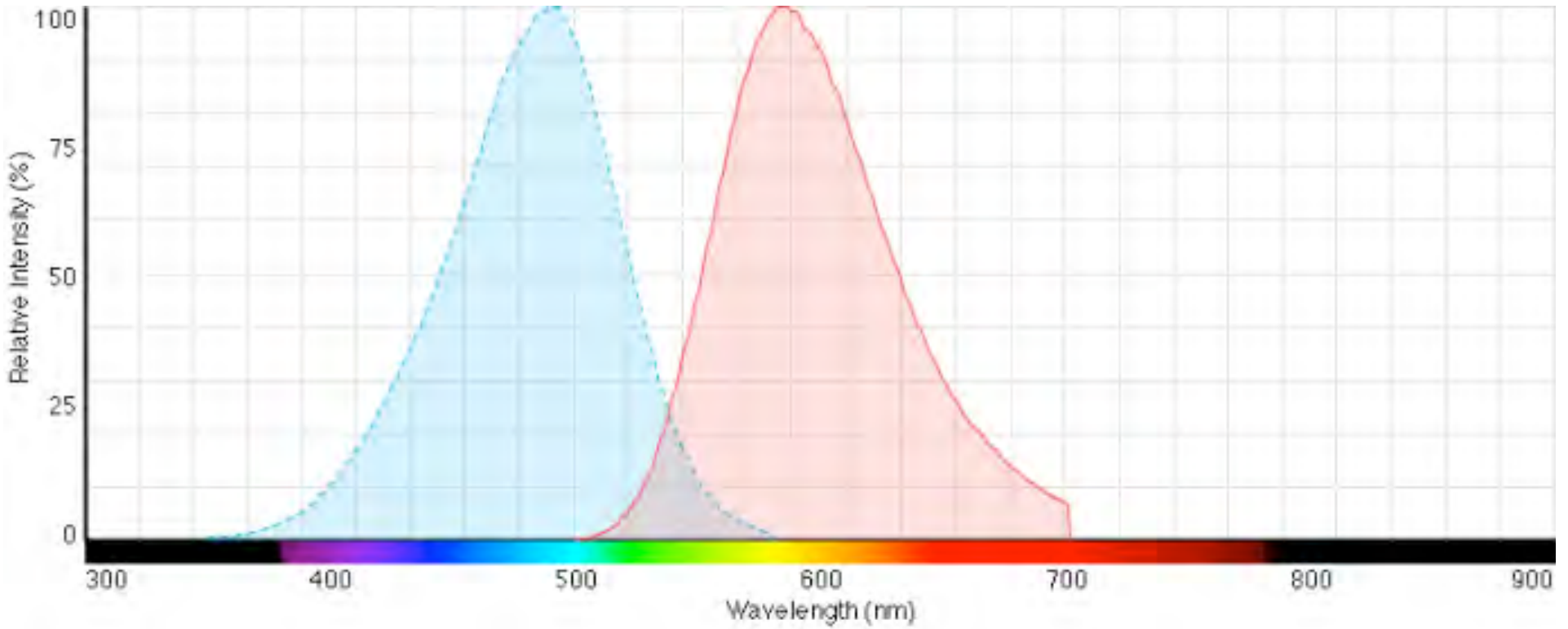
EC Number: 200-664-3

All Photos (1)

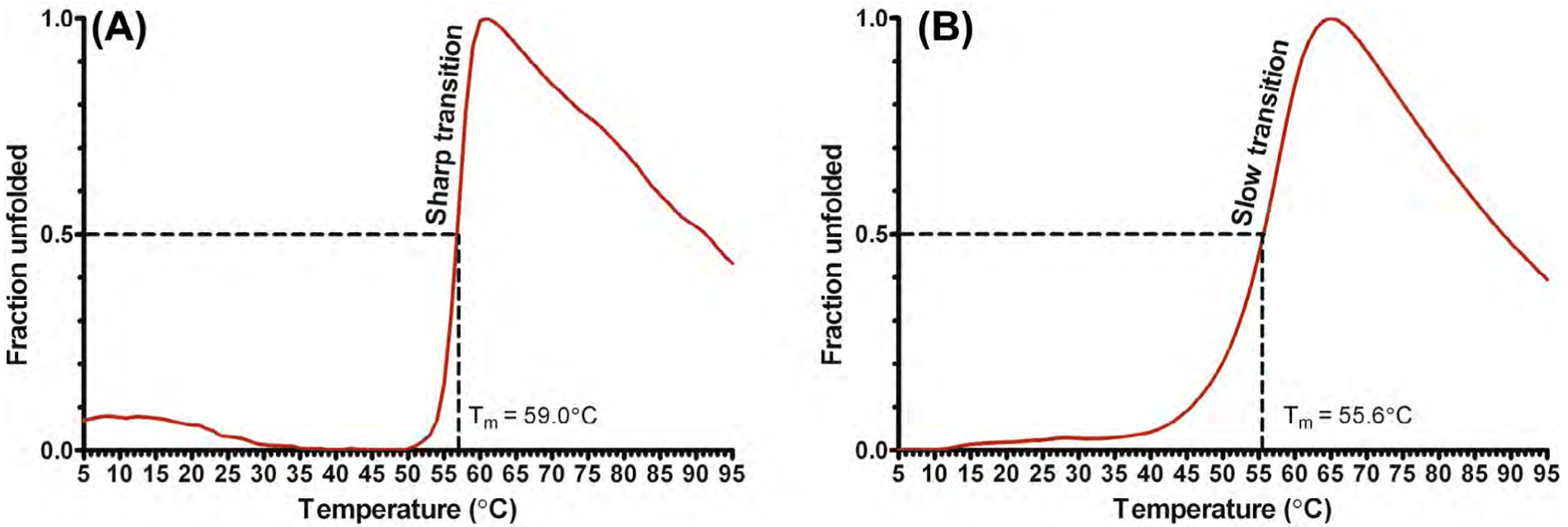
Documents

SKU	Pack Size	Availability	Price
S5692-50UL	50 µL	Estimated to ship on April 25, 2022	€79.40
S5692-500UL	500 µL	Only 1 left in stock (more on the way) Details...	€398.00

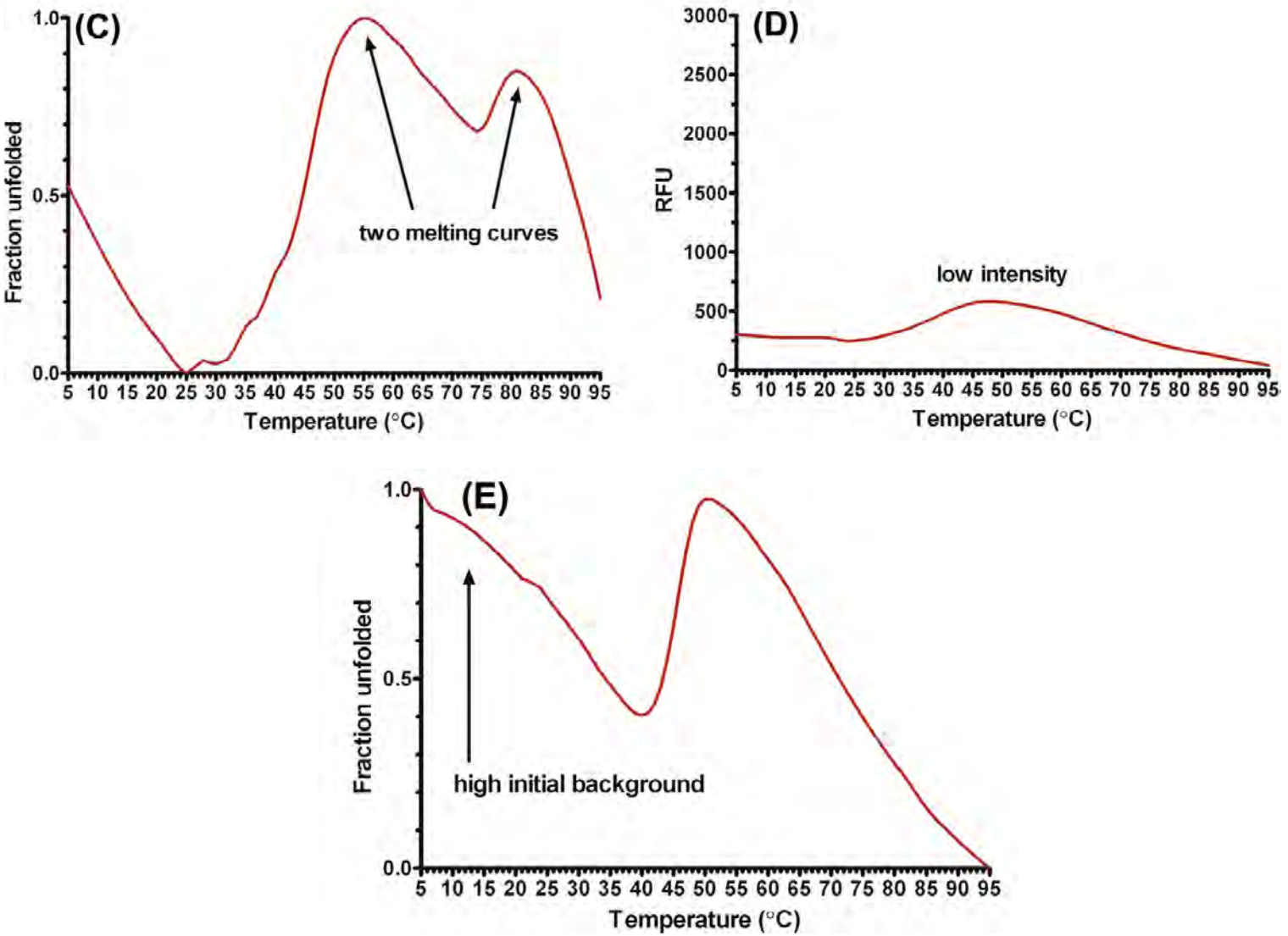
~1ul per plate



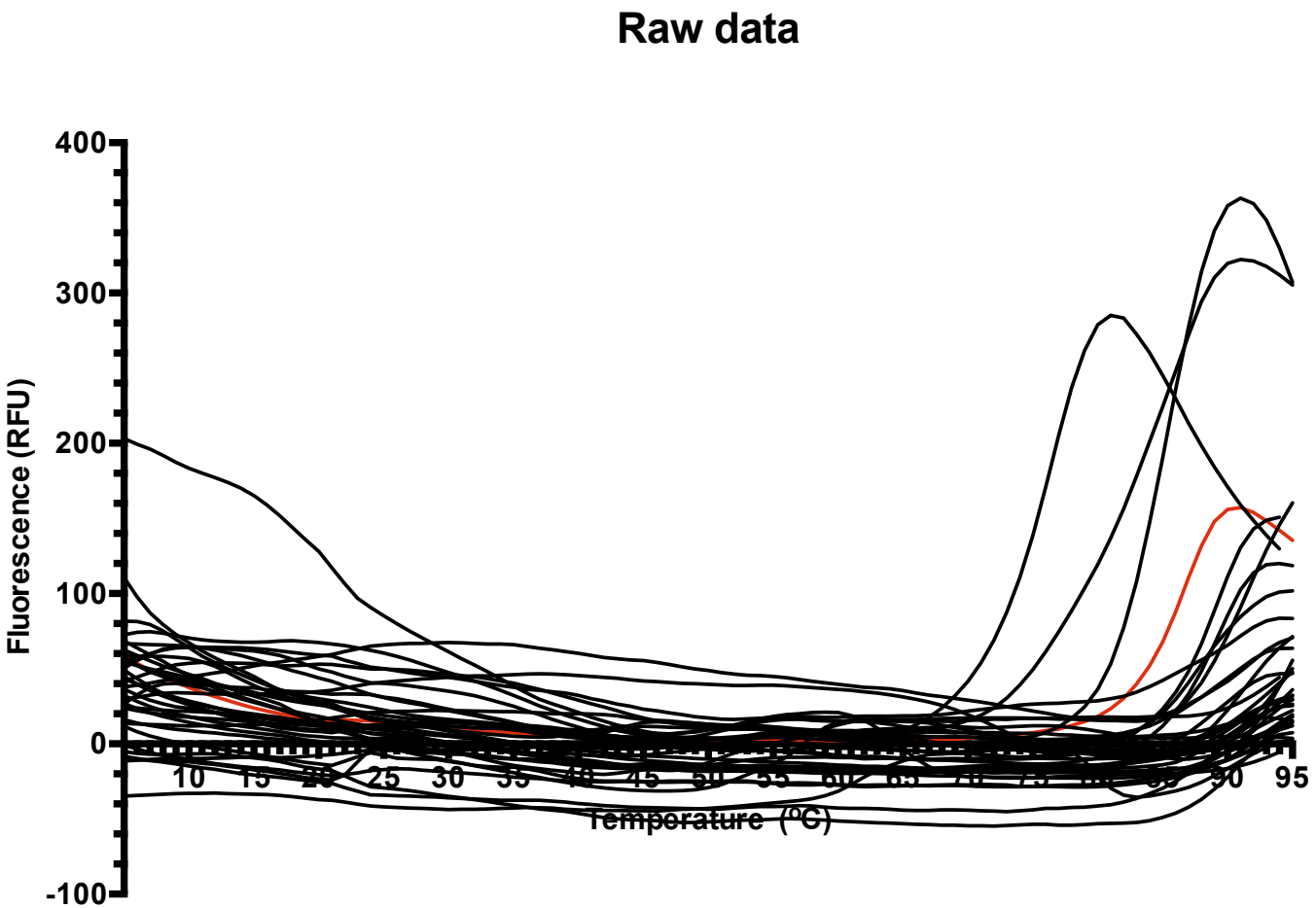
Curve shape and transition



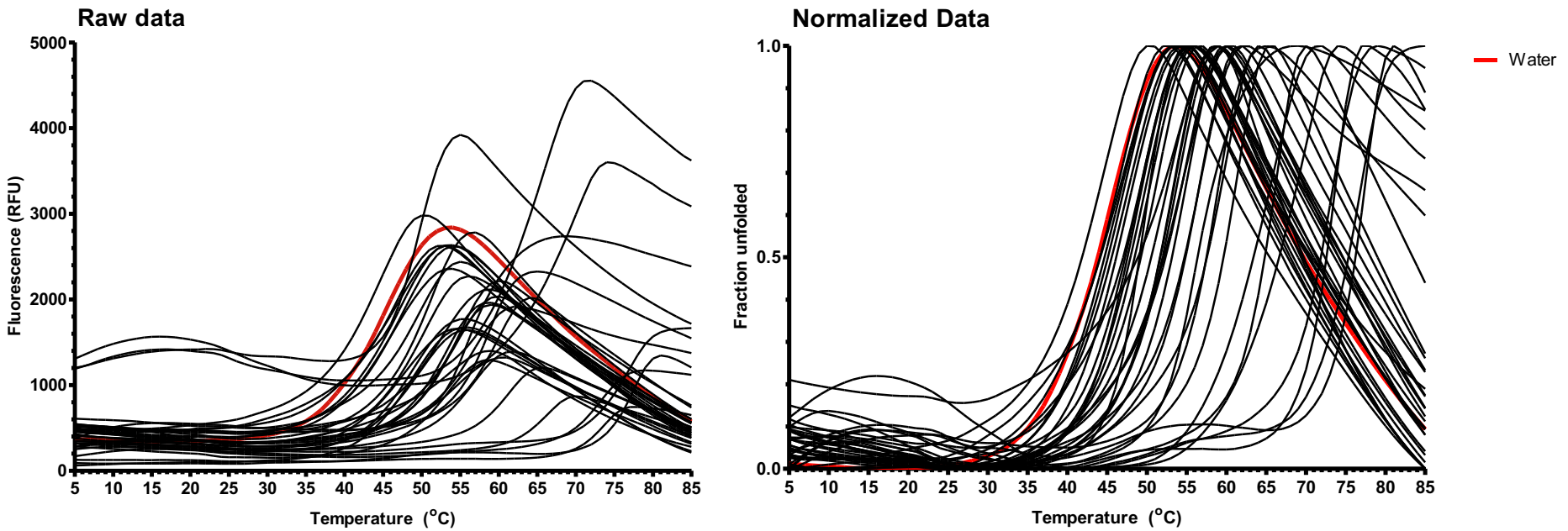
Curve shape – trouble shooting



What do you think about this?

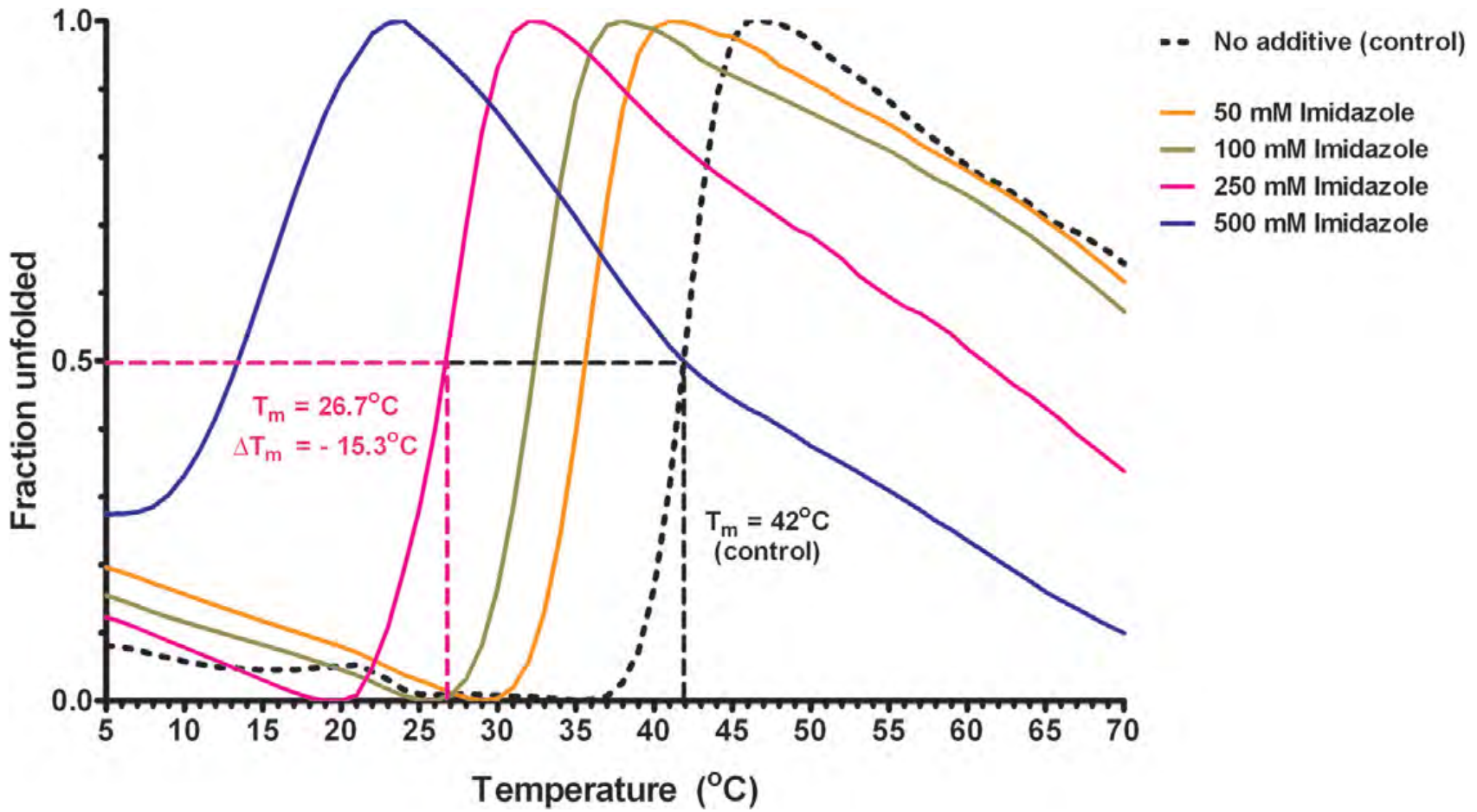


Normalization of the data

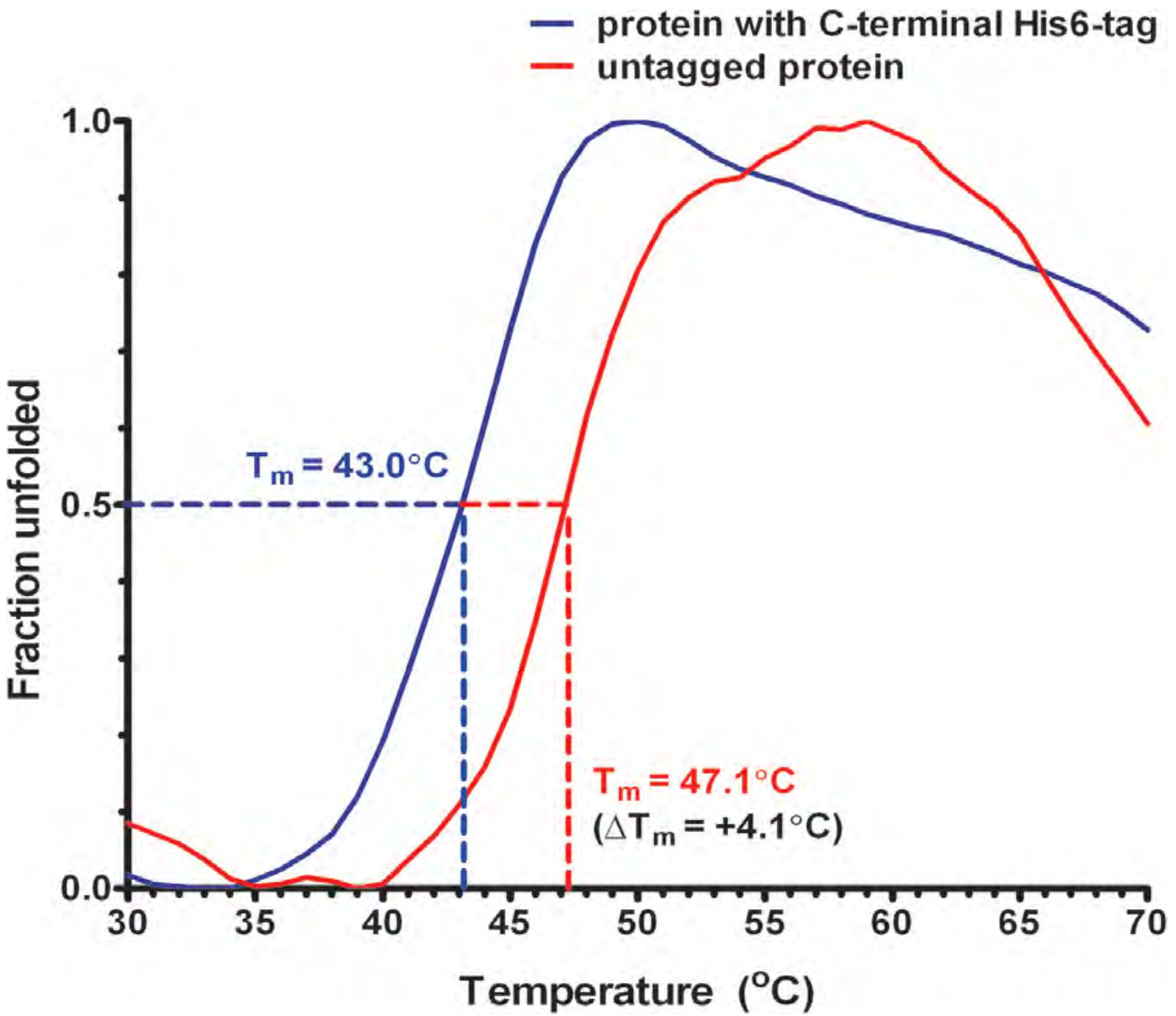


$$\text{Fraction unfolded} = \frac{[I_{measured} - I_{min}]}{[I_{max} - I_{min}]}$$

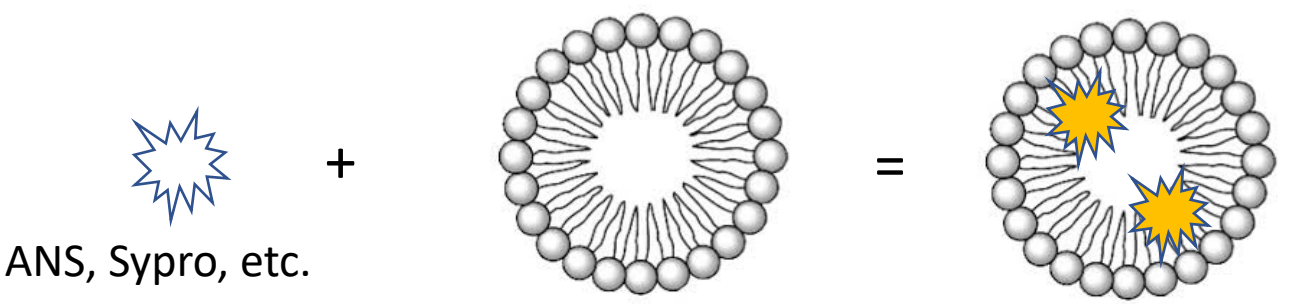
Typical effect *of imidazole*



What *to do with the His_6-tag?*



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



What to do with membrane proteins?

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,*]

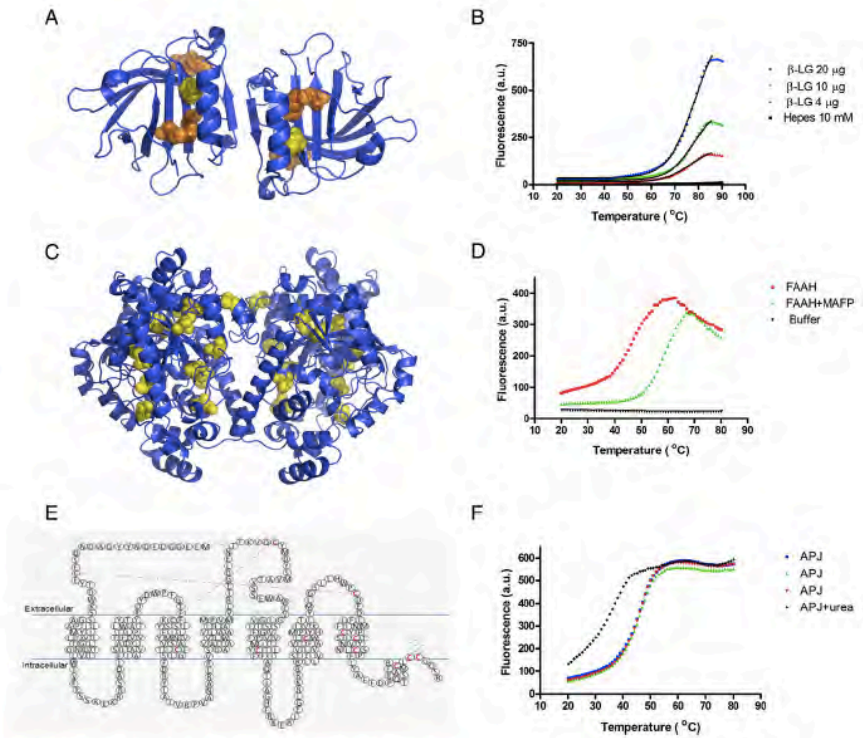
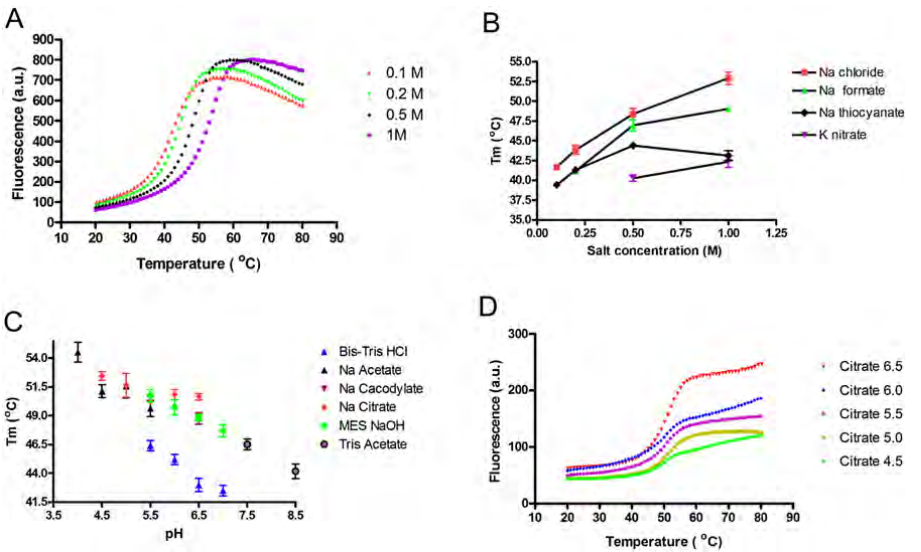
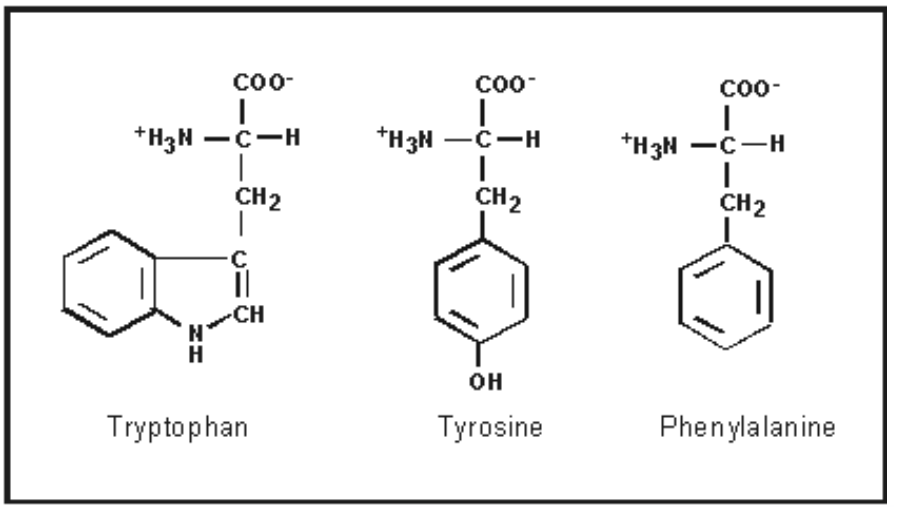


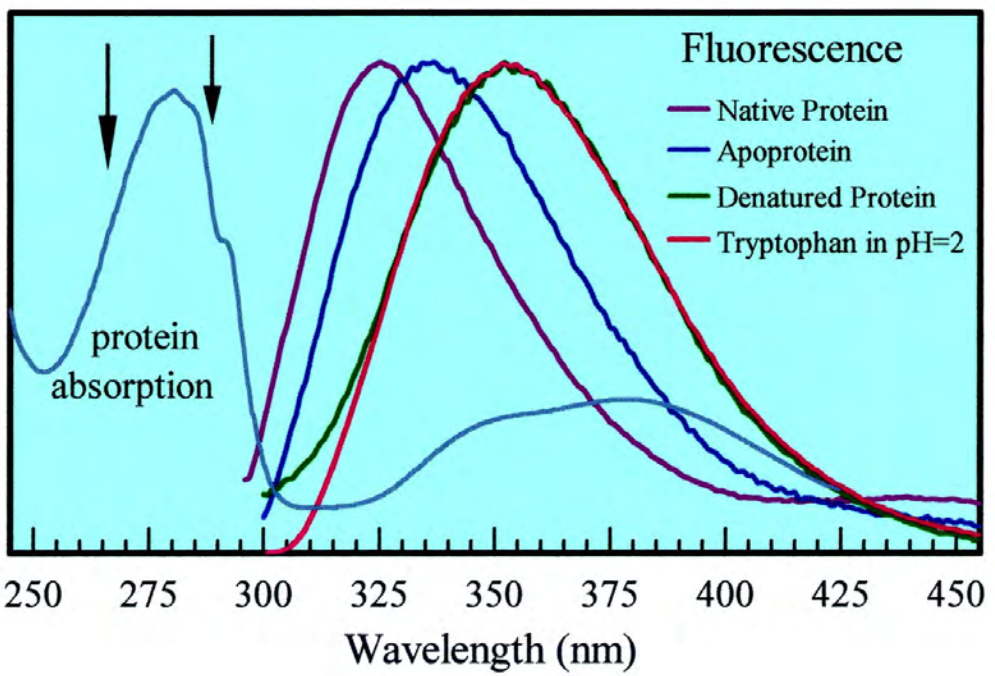
Figure 1. Thermal Stability Profiles of β-LG, FAAH, and APJ Receptor

- 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

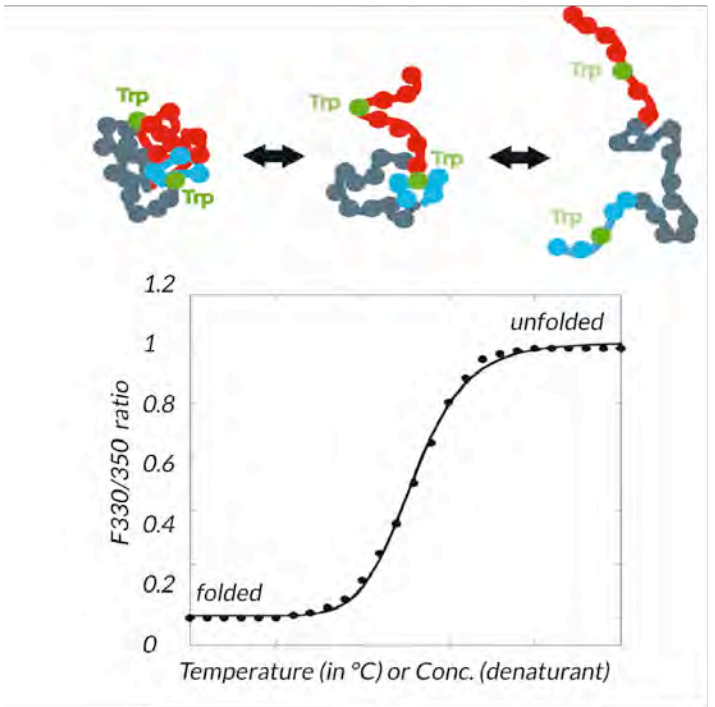


Intrinsic Fluorescence

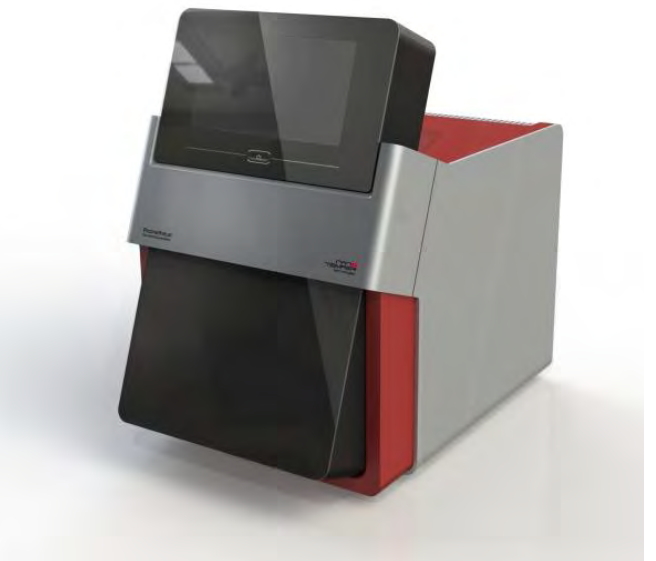




nanoDSF

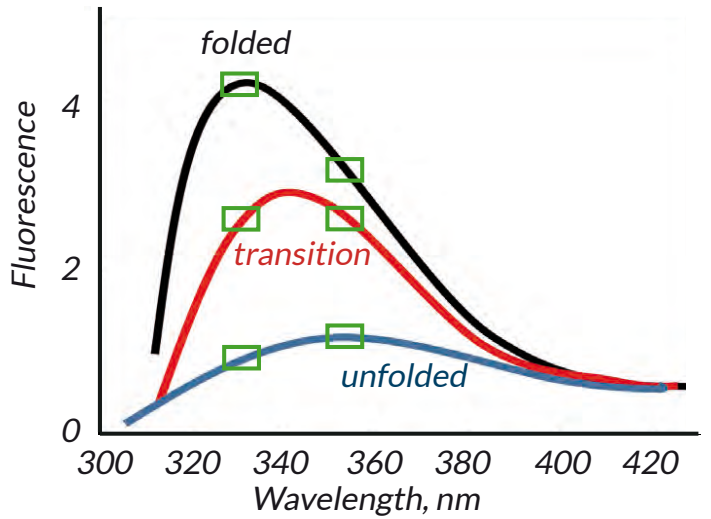


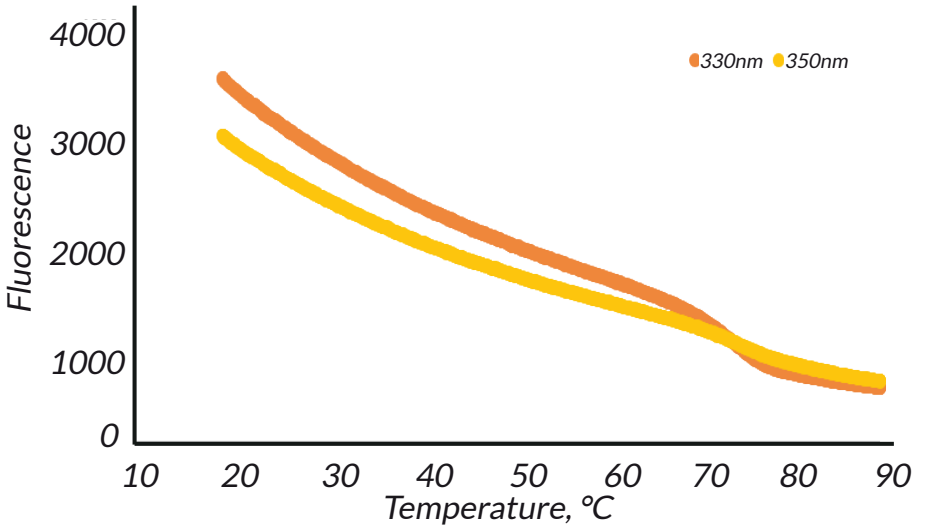
NanoTemper Technologies Prometheus NT.48

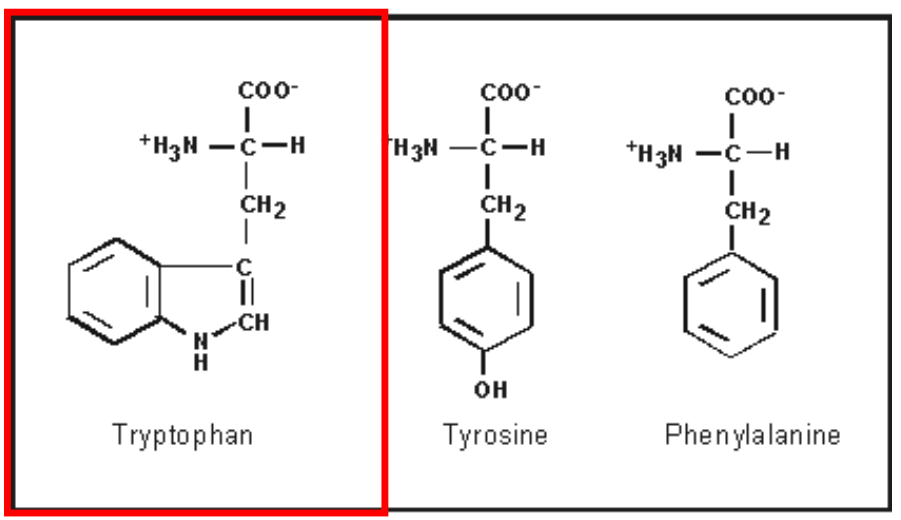


Principle behind the nanoDSF. Increasing temperature causes protein unfolding that can be assessed by monitoring changes of tryptophan fluorenscence at 330nm and 350nm wavelength.

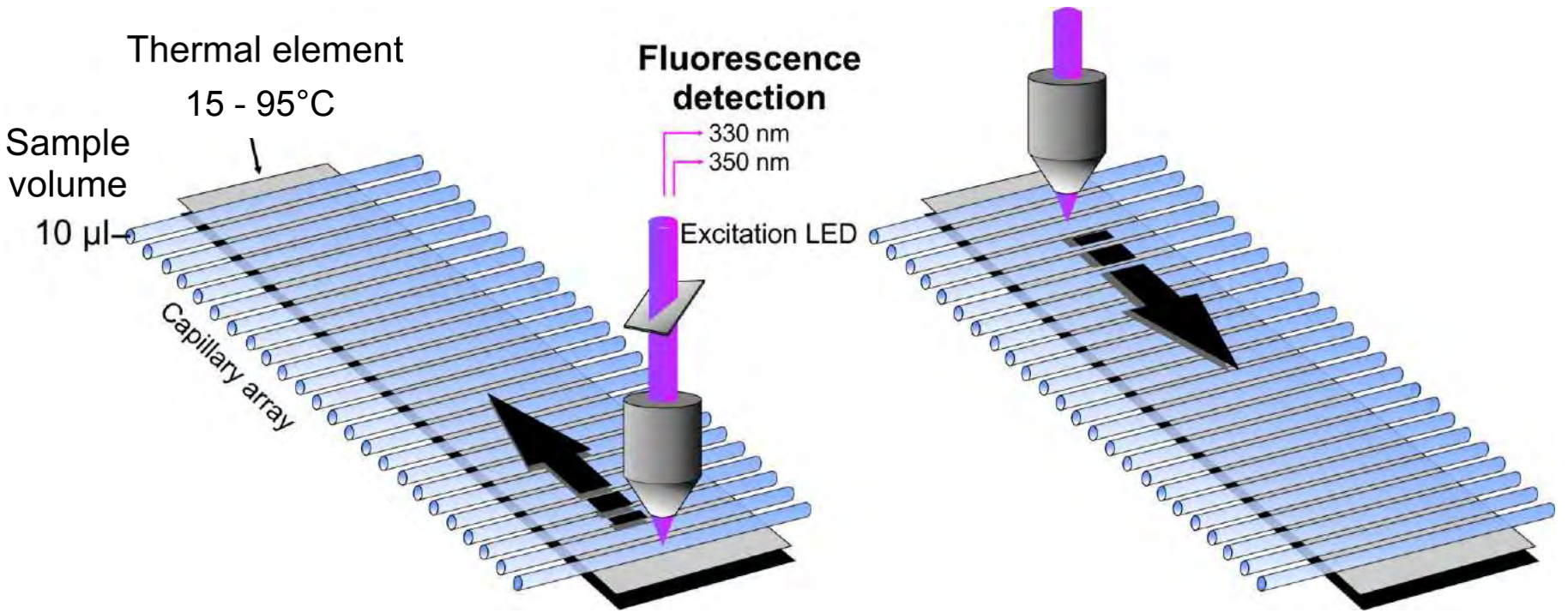
The thermal unfolding transition midpoint (Tm)



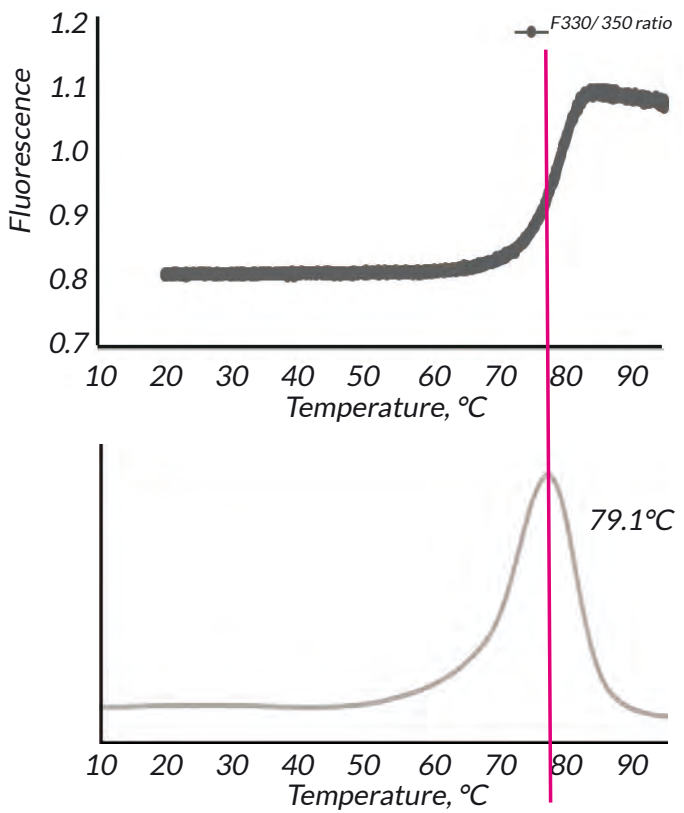




-Dual UV-detection system of the nanoDSF. The folded state of a protein presents a higher tryptophan fluorescence intensity than the unfolded state (left). Intrinsic tryptophan fluorescence is measured at 330nm and 350nm wavelength and plotted against the temperature from 15- 95°C (right).

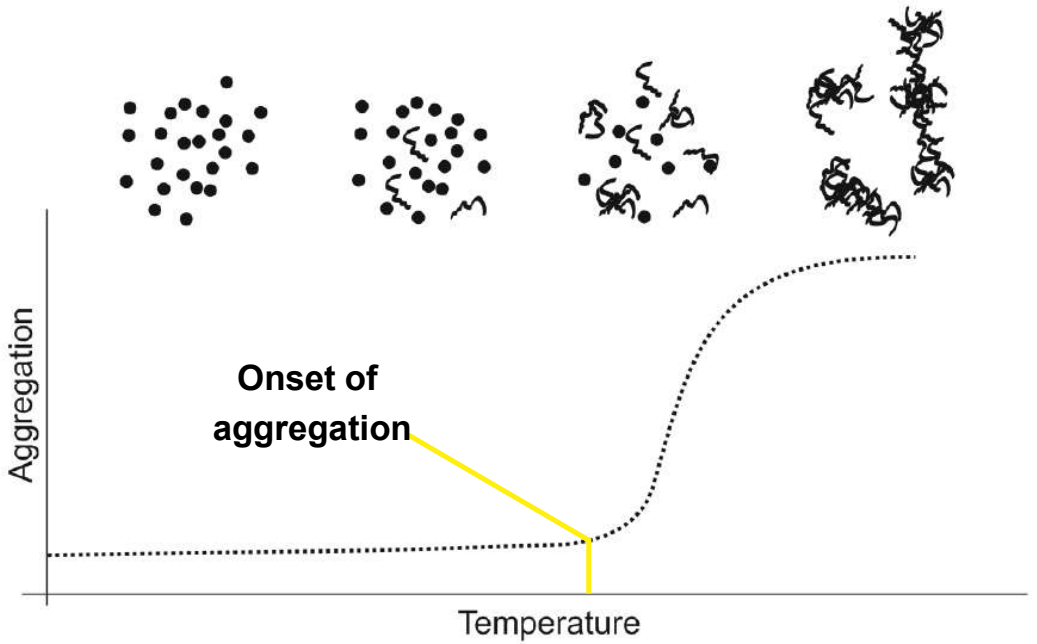

Thermal element
15 - 95°C
Sample volume
10 µl
Capillary array
Fluorescence detection
330 nm
350 nm
Excitation LED

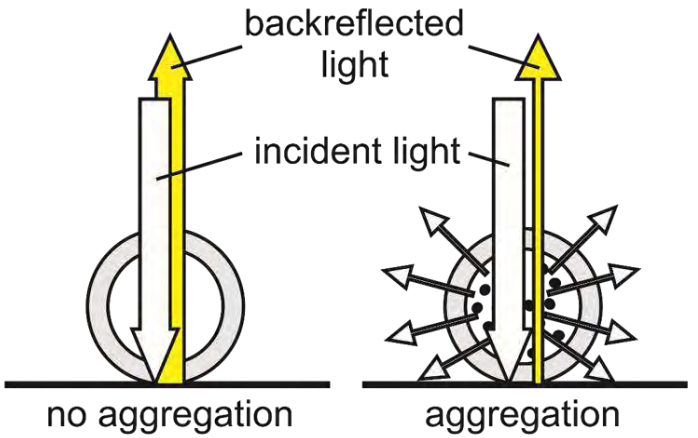
The thermal unfolding transition midpoint (Tm)



- After plotting the fluorescence ratio F330/350 against the temperature, the melting temperature Tm is determined by first derivate analysis
- The unfolding transition point and the Tm is determined to be 79.1°C.

Backreflection Optics





Experimental consideration

- **Temperature range**

nanoDSF analyses are usually performed in a temperature gradient of 15 - 95°C, with a heating rate of 1°C per min. However, these settings can be adapted for the specific protein.

- **Capillaries**

Depending on the fluorescence intensity, two different types of capillaries can be chosen for nanoDSF assays to ensure optimal signal to noise ratios.

- **Buffers**

nanoDSF offers free choice of buffers. There are no restrictions to buffer substances or salt concentrations. nanoDSF is the optimal tool to determine the buffer conditions providing optimal thermal stability.

- **Detergents**

nanoDSF assays can be performed using any kind of detergent. This is of special interest for membrane protein characterization.

What experiments could we do?

1. **Stability screening assays:**

– optimization of formulation conditions (also viscous solutions)
– buffer screening assays to identify the optimal conditions
– detergent screening assay to determine the optimal conditions for membrane proteins

2. Biophysical characterization assays:

– antibody + antibody-drug conjugate characterization
– determination of multiple domain unfolding transitions

3. Quality control assays:

– long term stability of proteins – forced degradation of proteins

4. Ligand binding screening assays

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High-throughput screening for IMPs stability

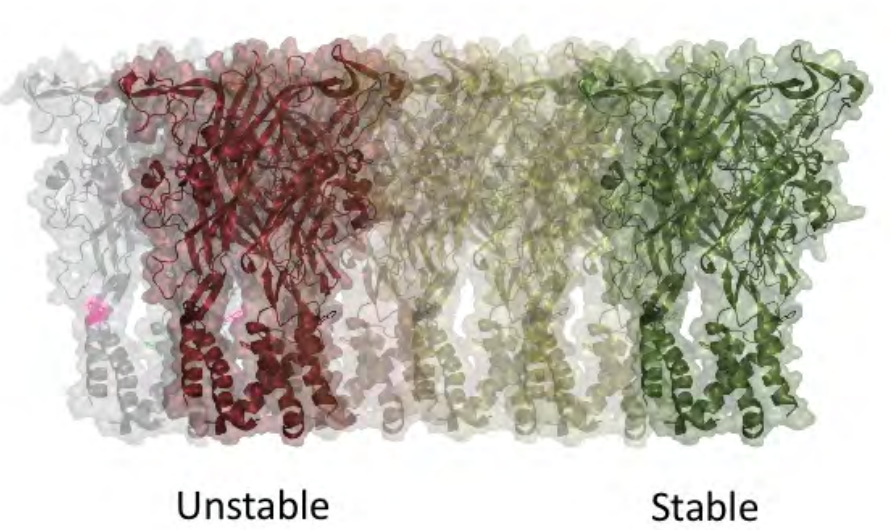
40% samples processed in the SPC are membrane proteins

- IMP stability in detergent or membrane-like environments is the bottleneck for structural studies
- Detergent solubilization from membranes is usually the first step in the workflow
- Looking for a simple high-throughput screening method to identify optimal conditions for membrane protein stabilization

High-throughput screening for IMPs stability

- following **nDSF and scattering** upon thermal denaturation
- (de-)stabilization **effects of detergents**
- find **suitable conditions** for downstream handling during purification
- **thermodynamic parameters (Tm, Tagg, Tonset)**
- We selected 9 IMPs to benchmark our protocol

Objective



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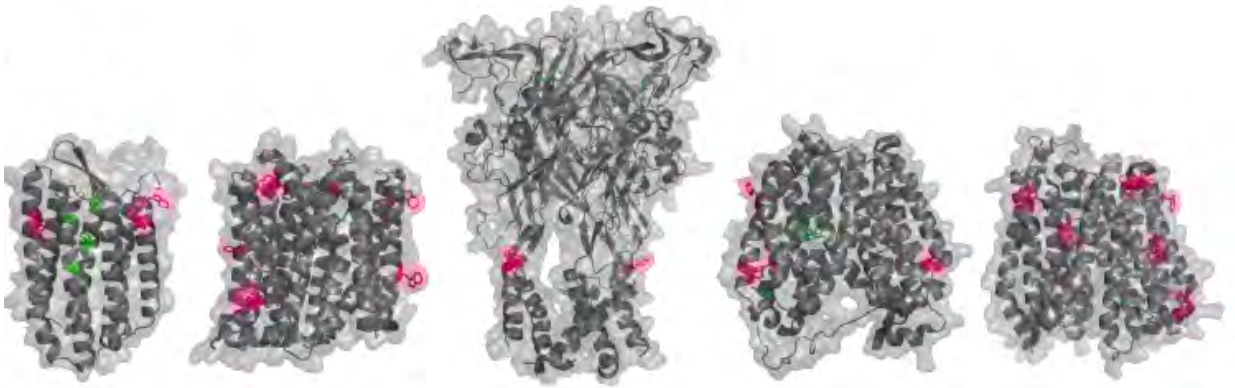
We selected 9 Integral membrane proteins (targets)



BR MdfA P2X4 LacY DtpA

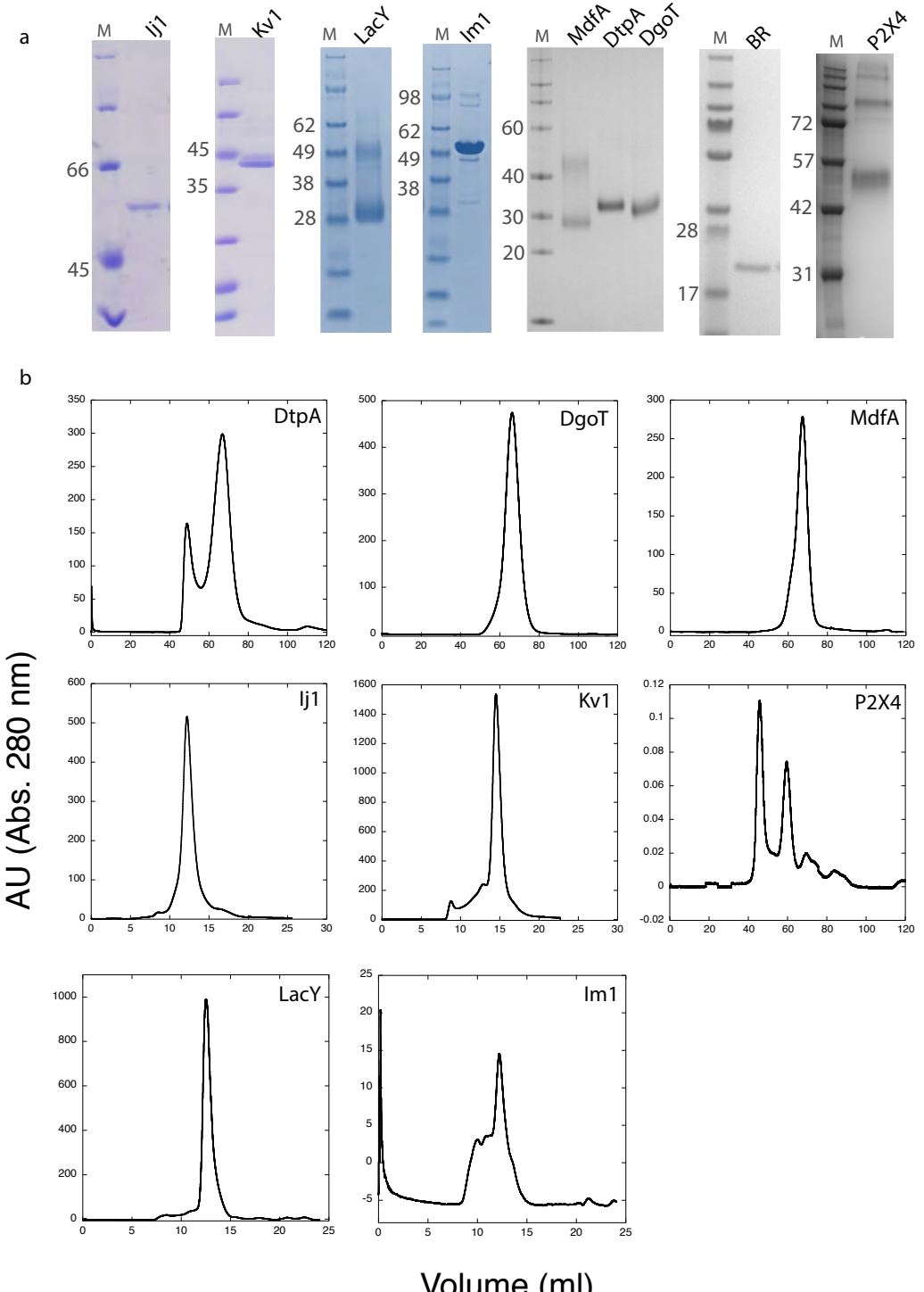
? ? ? ?

MFS transporter | 7TM pathogen | ABC transporter | kinase

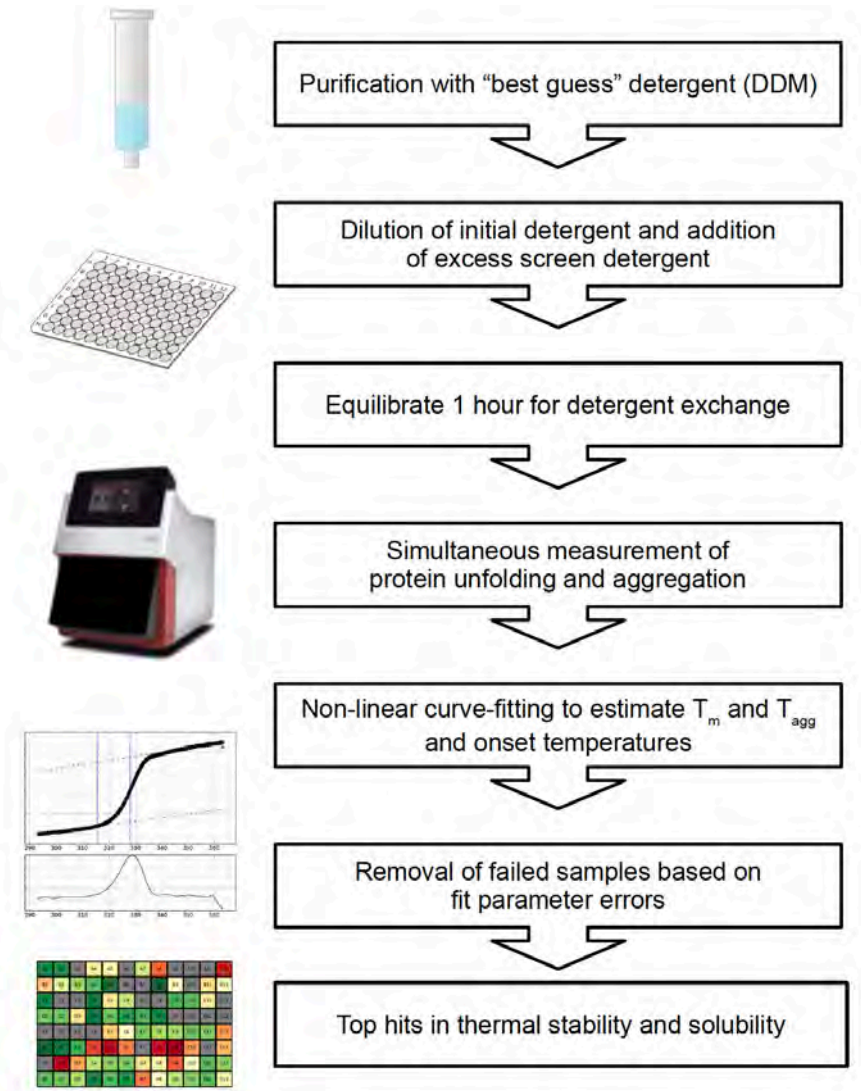
DgoT Kv1 Ij1 Im1

Protein	Organism	Family	Function	Number of Trp residues	PDB ID
DgoT	*E. coli*	MFS transporters	putative galactonate transporter	14	6E9N, 6E9O
MdfA	*E. coli*	MFS transporter	multi drug resistance	9	4ZP0, 4ZOW, 4ZP2, 6GV1, 6EUQ
DtpA	*E. coli*	MFS transporter	peptide transporter	10	6GS1, 6GS4, 6GS7
Kv1	*Pseudomonas aeruginosa*	unknown	unknown	17	—
Ij1	*E. coli*	ABC-Transporter	ion transport	22	—
P2X4	*Homo sapiens*	P2X ionotropic receptors	regulator in mediating neuropathic pain	6	4DW0, 4DW1 (zebrafish)
BR	*Halobacterium salinarum*	7TM receptor	proton pump	8	4MD1, 4MD2, 4XXJ
LacY	*E. coli*	MFS transporter	transport of beta-galactosides	5	1PV6
Im1	*E. coli*	HisKA	Kinase	2	—

- Membranes solubilized in 1–2% DDM
- DDM as starting detergent in SEC



Our pipeline



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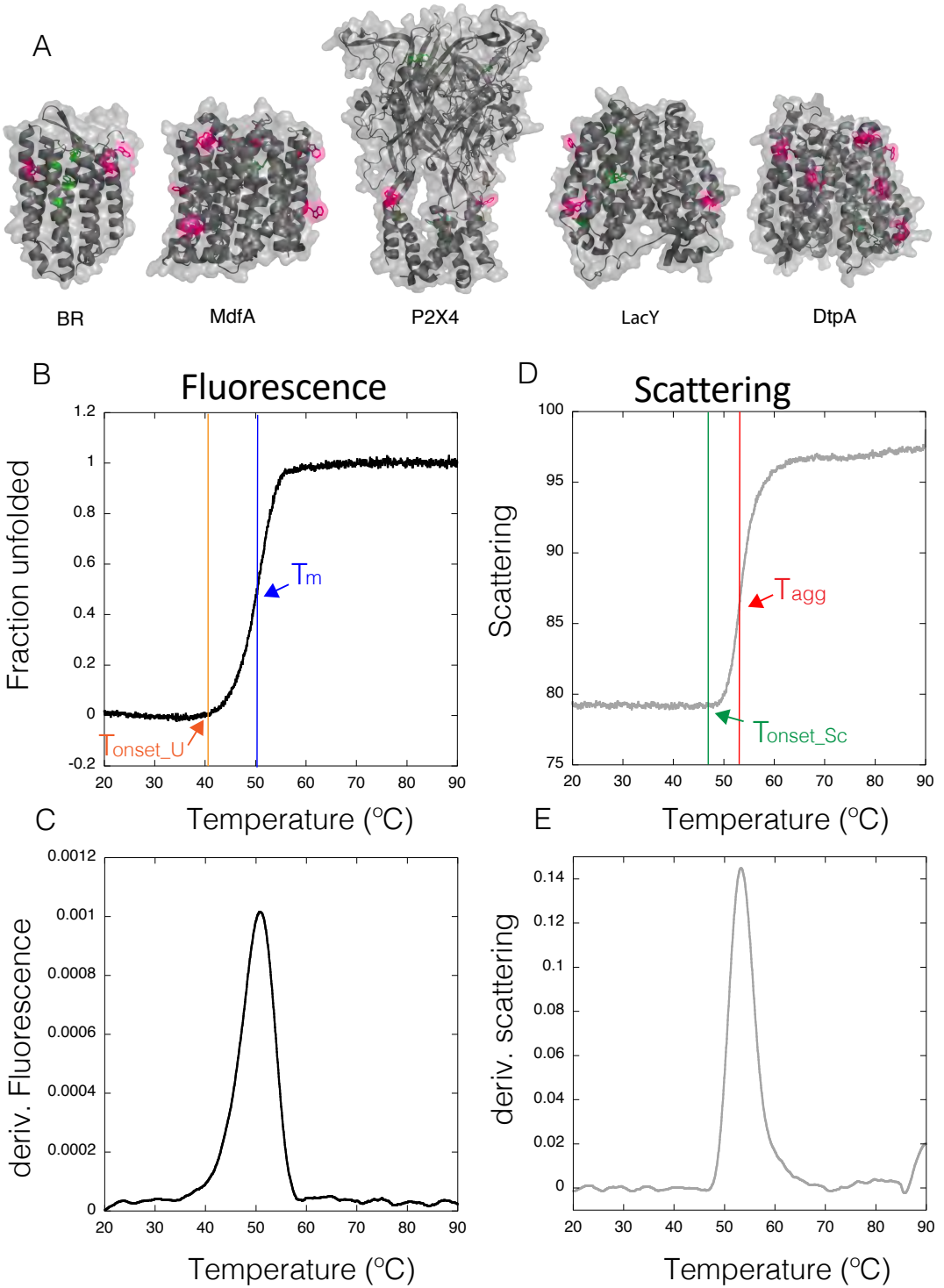
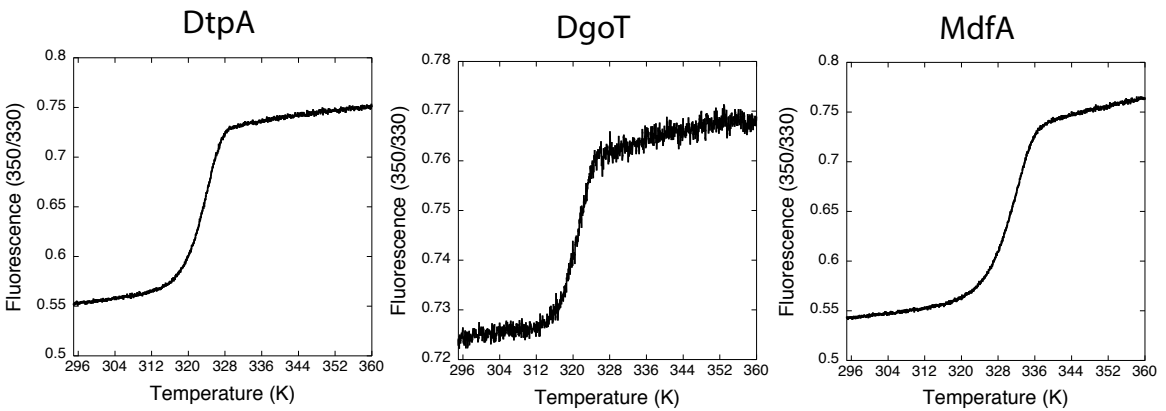
nDSF measurements

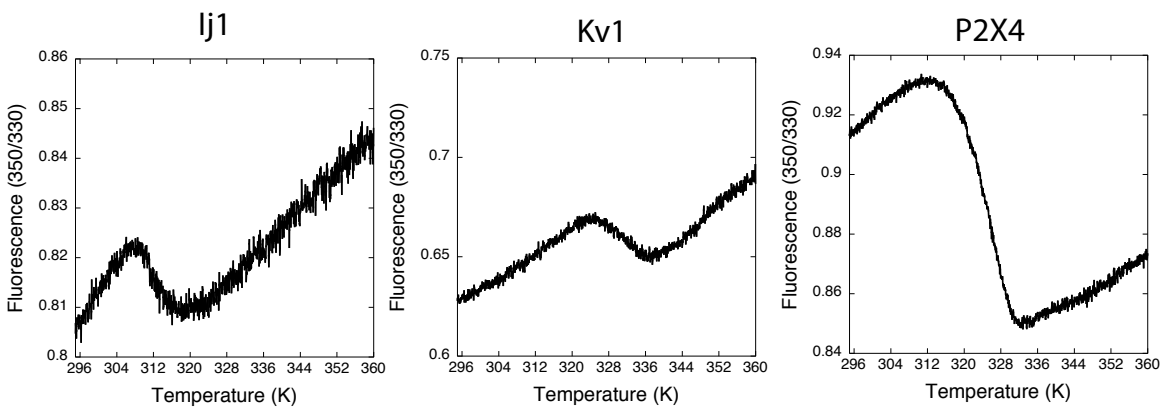


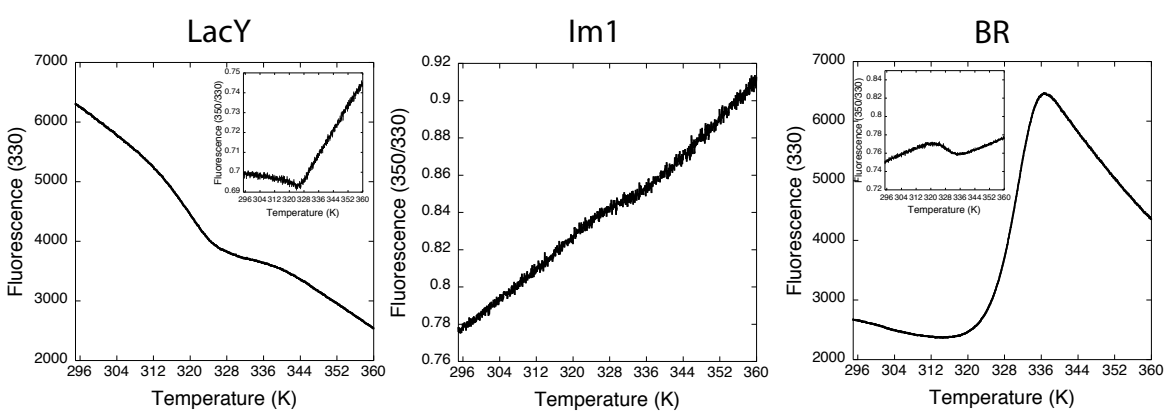
Fluorescence raw data

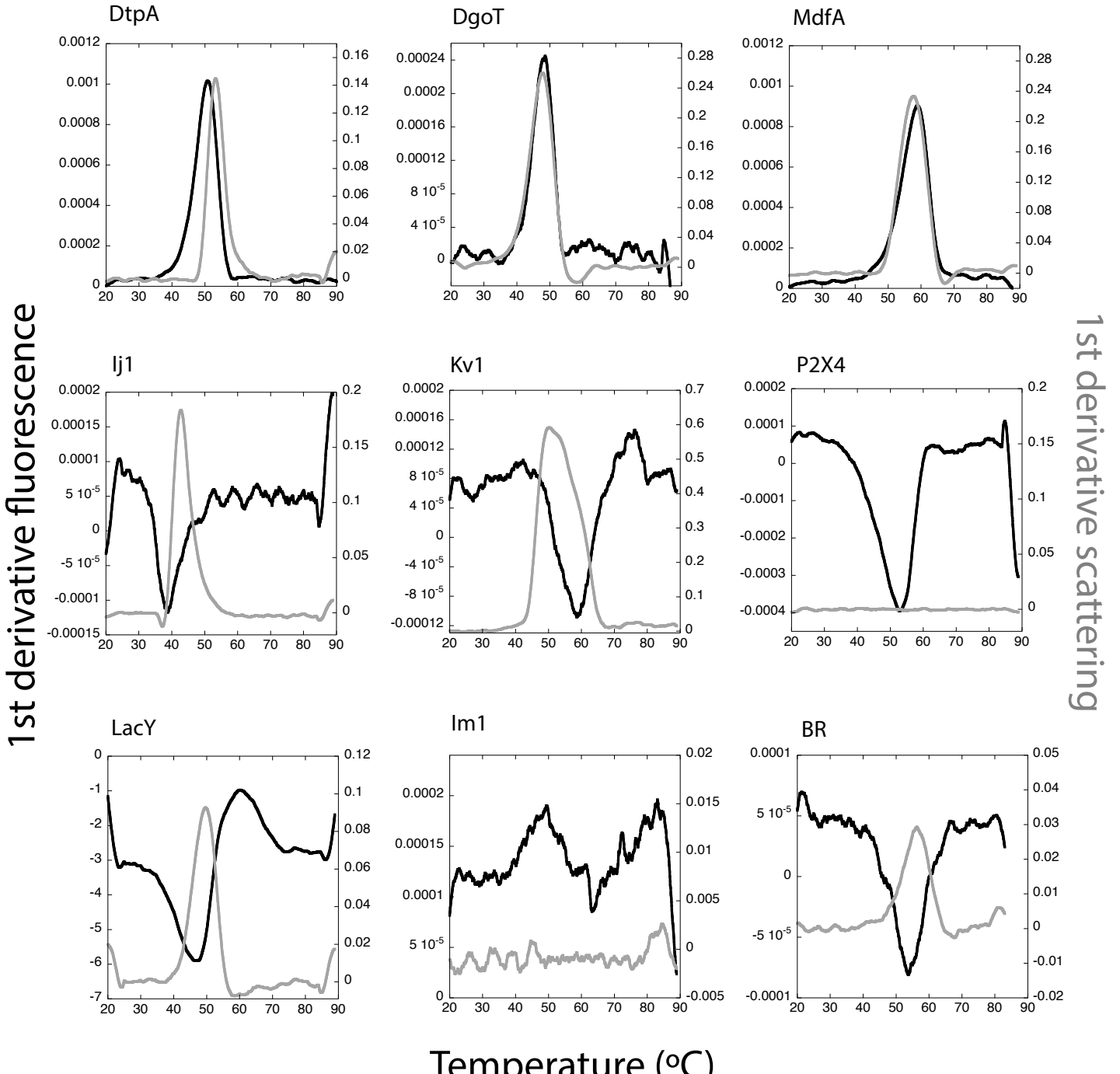
S curves



Z curves

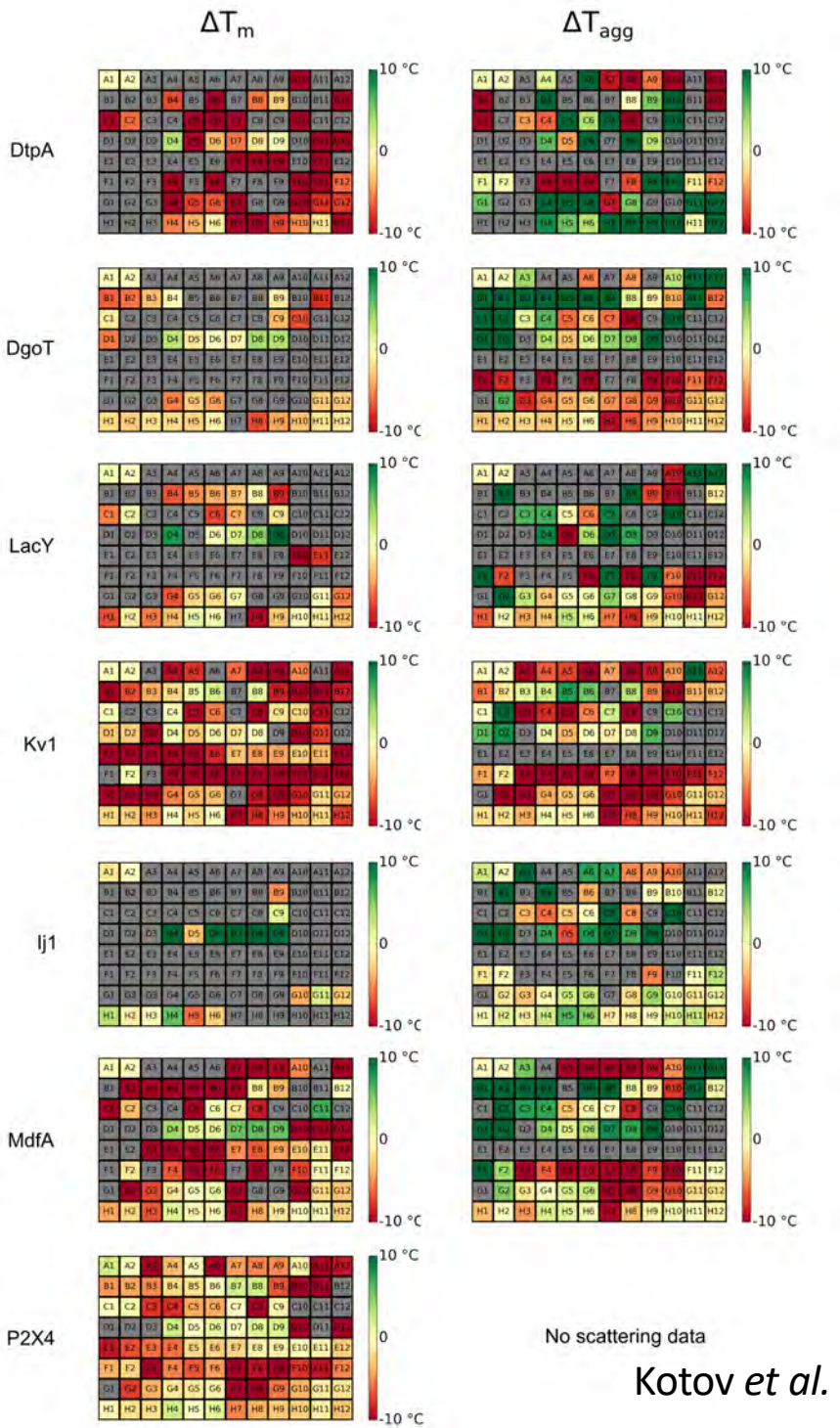





DtpA
DgoT
MdfA
Ij1
Kv1
P2X4
LacY
Im1
BR
1st derivative fluorescence
1st derivative scattering
Temperature (°C)

- screening 96 detergents
- A2 is the starting condition, in our case DDM
- Calculation of Tm

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



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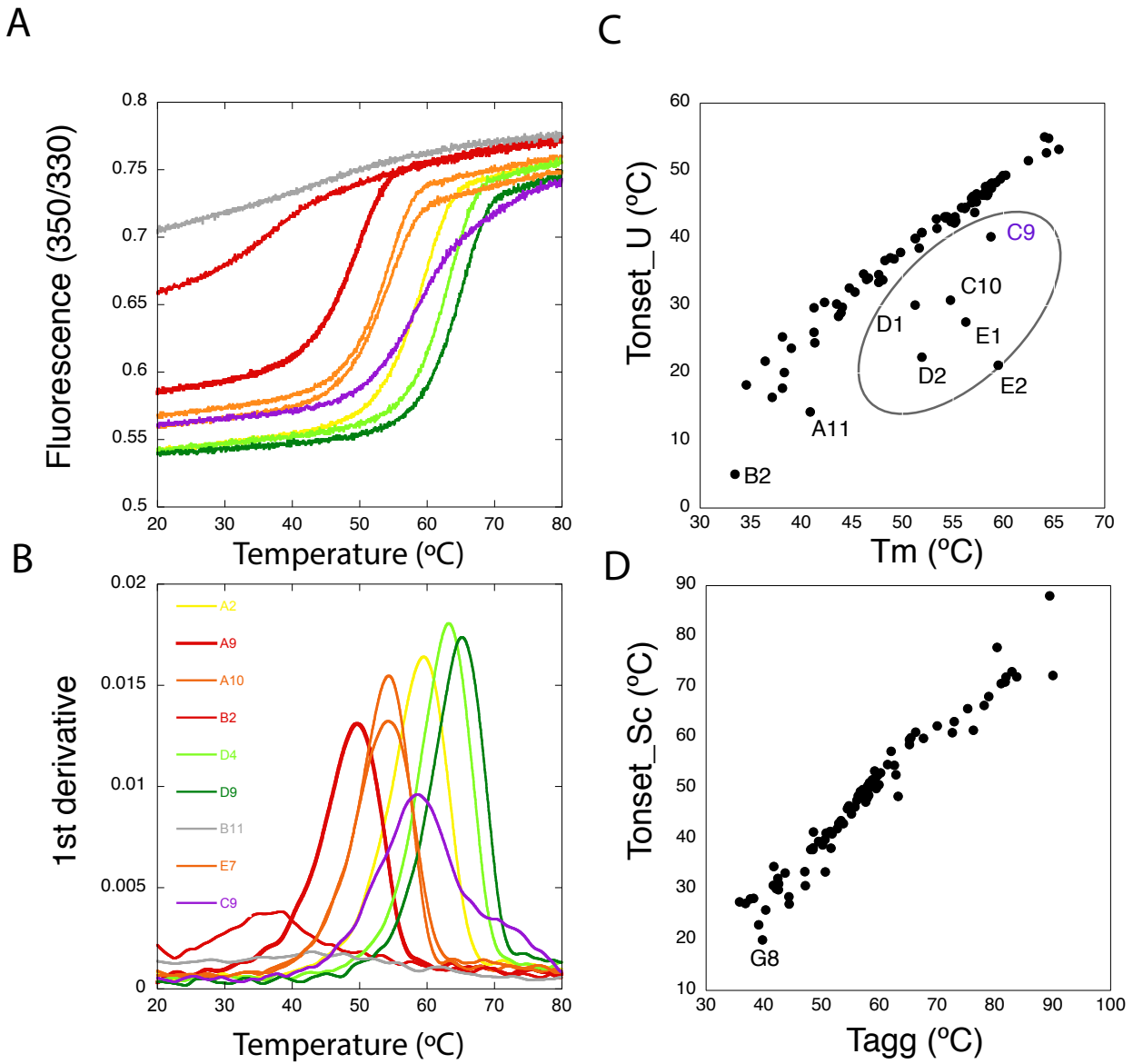
Tm vs Tonset


A
Fluorescence (350/330)
Temperature (ºC)
B
1st derivative
A2
A9
A10
B2
D4
D9
B11
E7
C9
Temperature (ºC)
C
Tonset_U (ºC)
C9
C10
D1
E1
D2
E2
A11
B2
Tm (ºC)
D
Tonset_Sc (ºC)
G8
Tagg (ºC)

Statements by Nanotemper

- nanoDSF is especially useful in antibody engineering, membrane protein characterization, formulation development and protein quality control.

- nanoDSF monitors these fluorescence changes with high resolution and is even capable of revealing multiple unfolding transition points.

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– **determination of multiple domain unfolding transitions**

3. Quality control assays:

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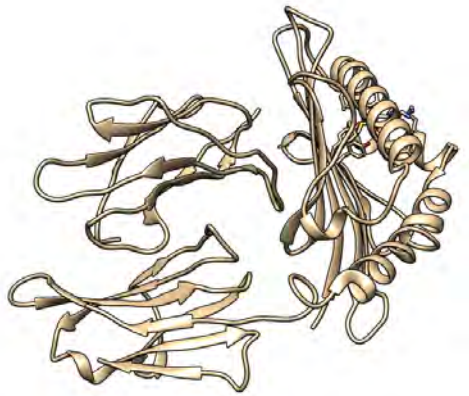
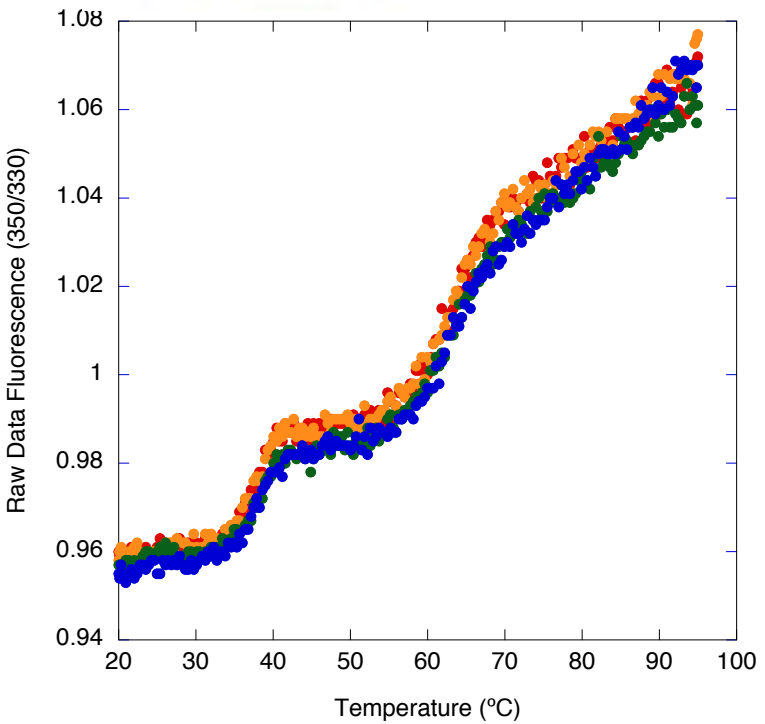
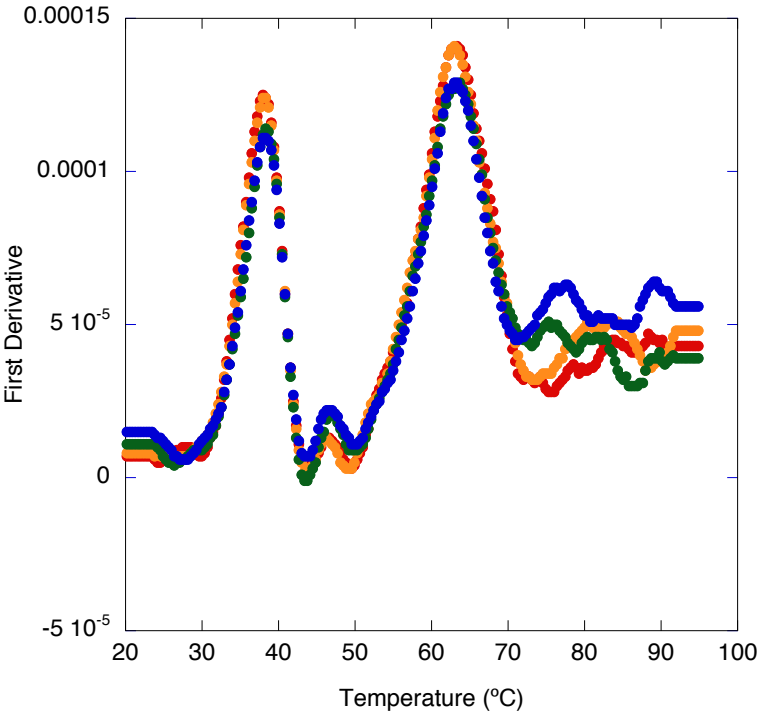
4. Ligand binding screening assays

Determination of multiple domain unfolding transitions



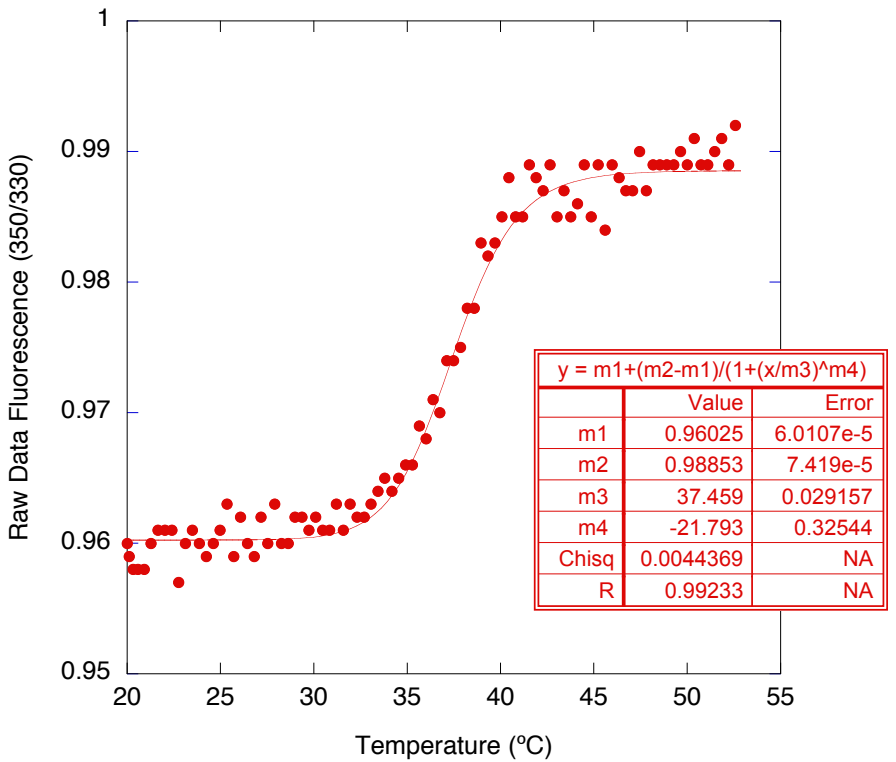
MHC class I

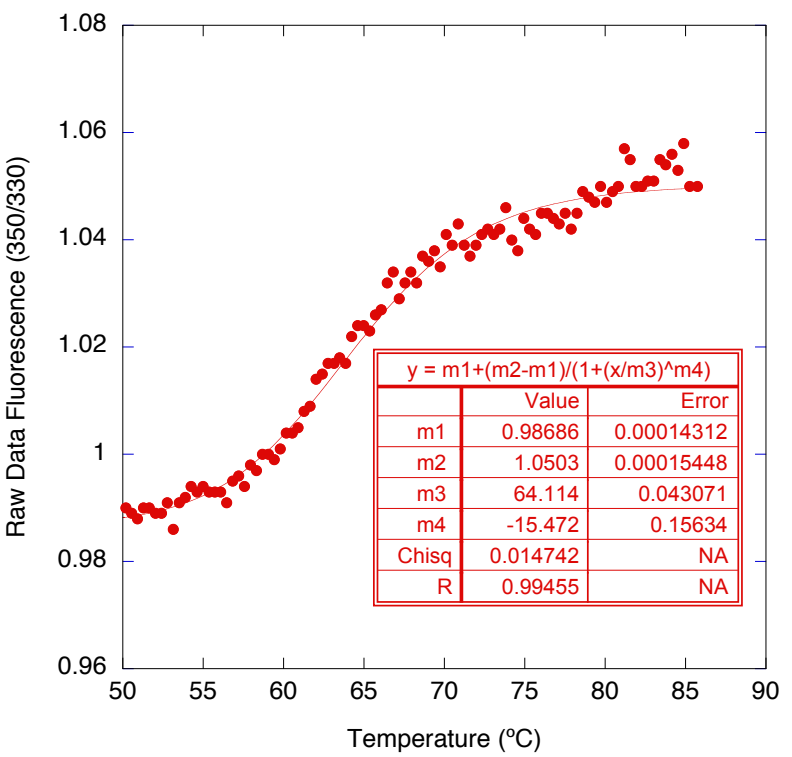




Anjanappa *et al.*, **Nat Commun. 2020** Mar 11;11(1):1314.

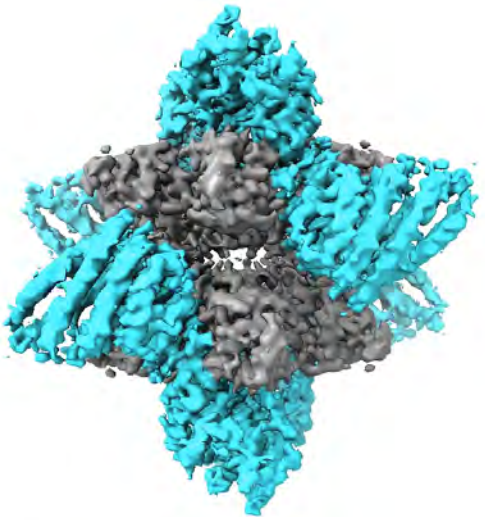
Fitting to a simple two-state model Two events of unfolding

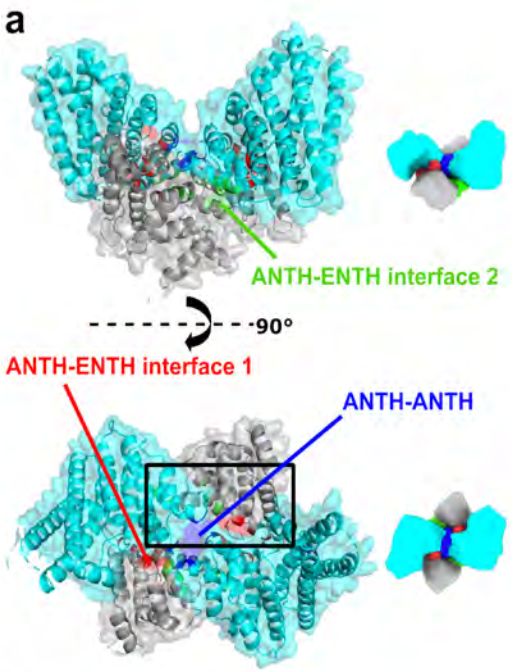


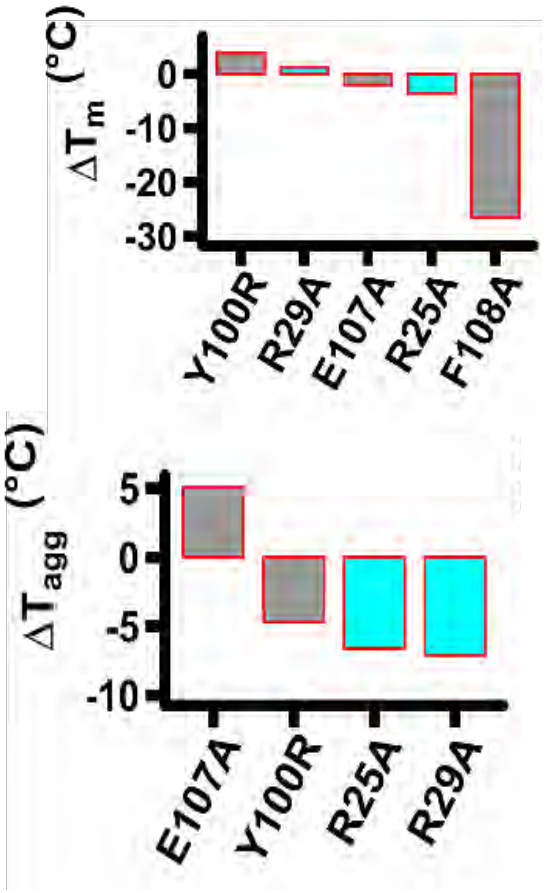


Tm ≅ m3

The AENTH endocytic complex

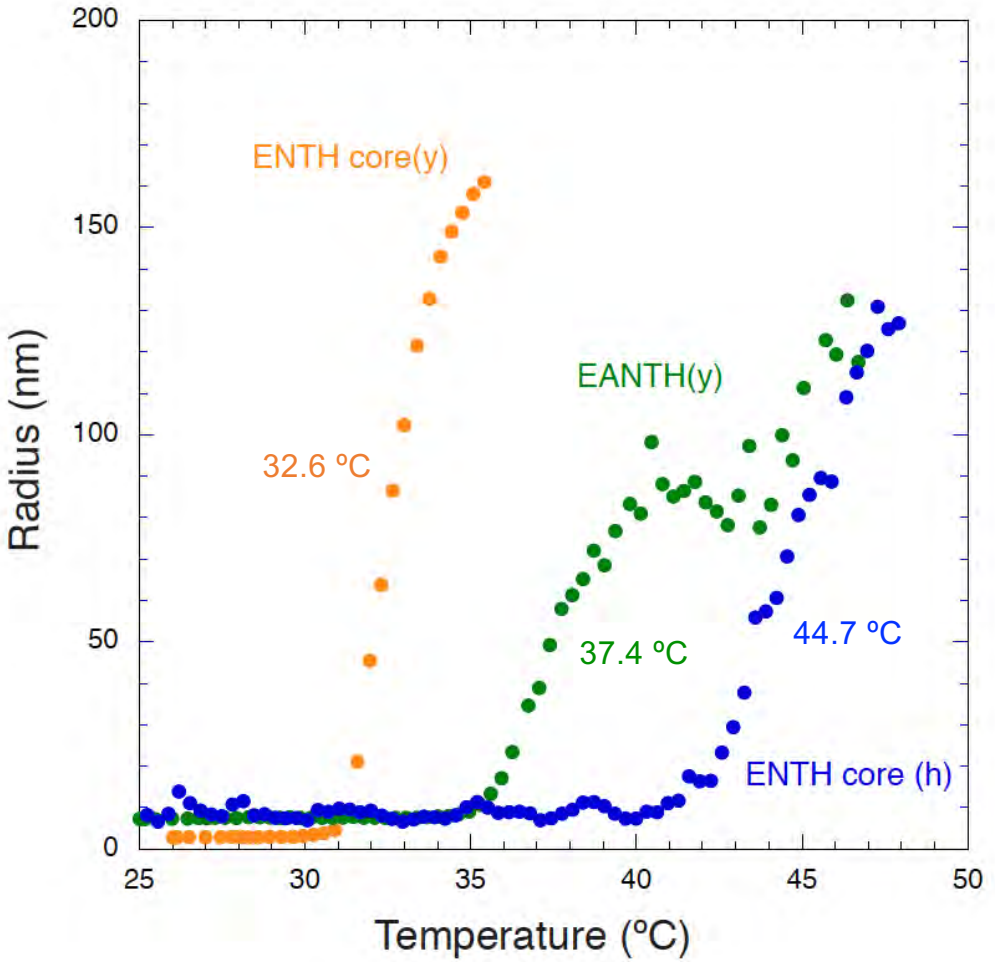




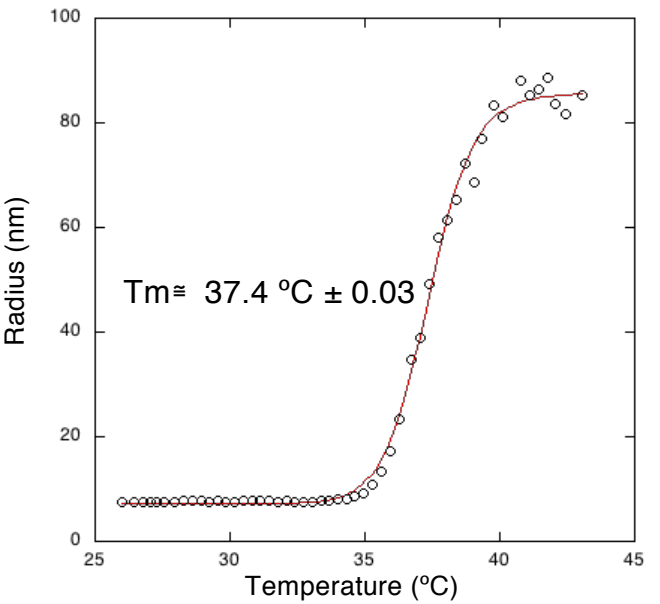


Lizarrondo et al, *Nat. Commun. 2021*

An alternative is following aggregation



The AENTH complex is more stable than the ENTH-PIP2



Increase in Rh as a function of temperature monitored by DLS

Garcia-Alai et al, *Nat. Commun. 2018*

What experiments could we do?

1. **Stability screening assays:**

– optimization of formulation conditions (also viscous solutions)
– buffer screening assays to identify the optimal conditions
– detergent screening assay to determine the optimal conditions for membrane proteins

2. Biophysical characterization assays:

– antibody + antibody-drug conjugate characterization
– determination of multiple domain unfolding transitions

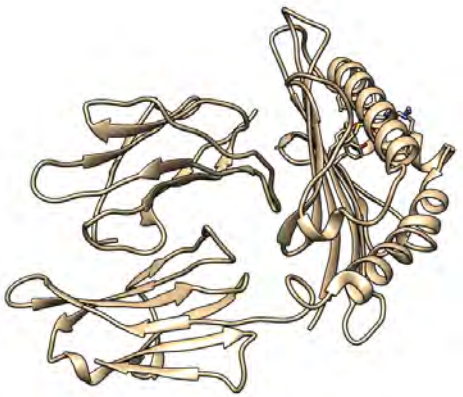
3. Quality control assays:

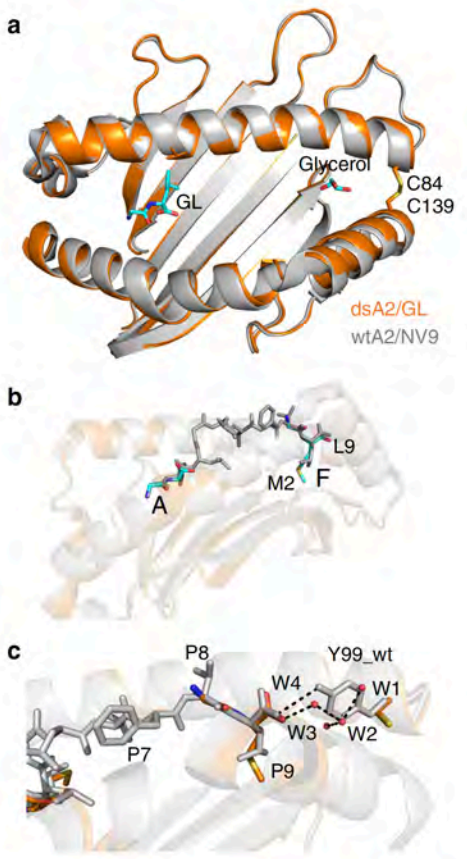
– long term stability of proteins – forced degradation of proteins

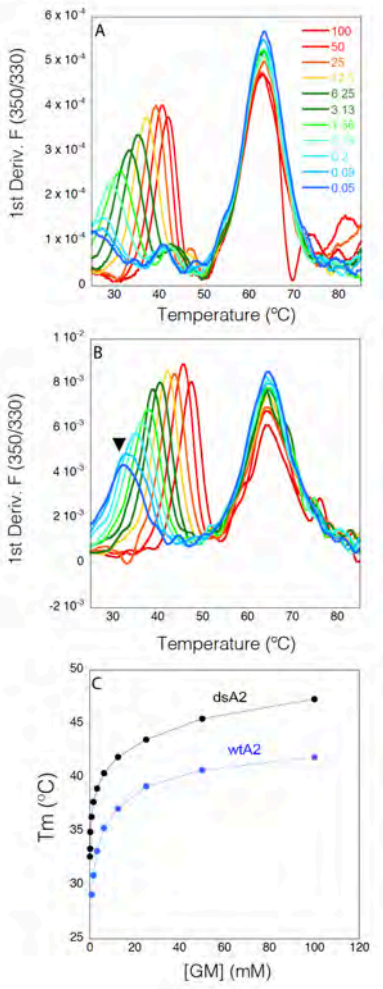
4. Ligand binding screening assays

Determination of multiple domain unfolding transitions

MHC class I



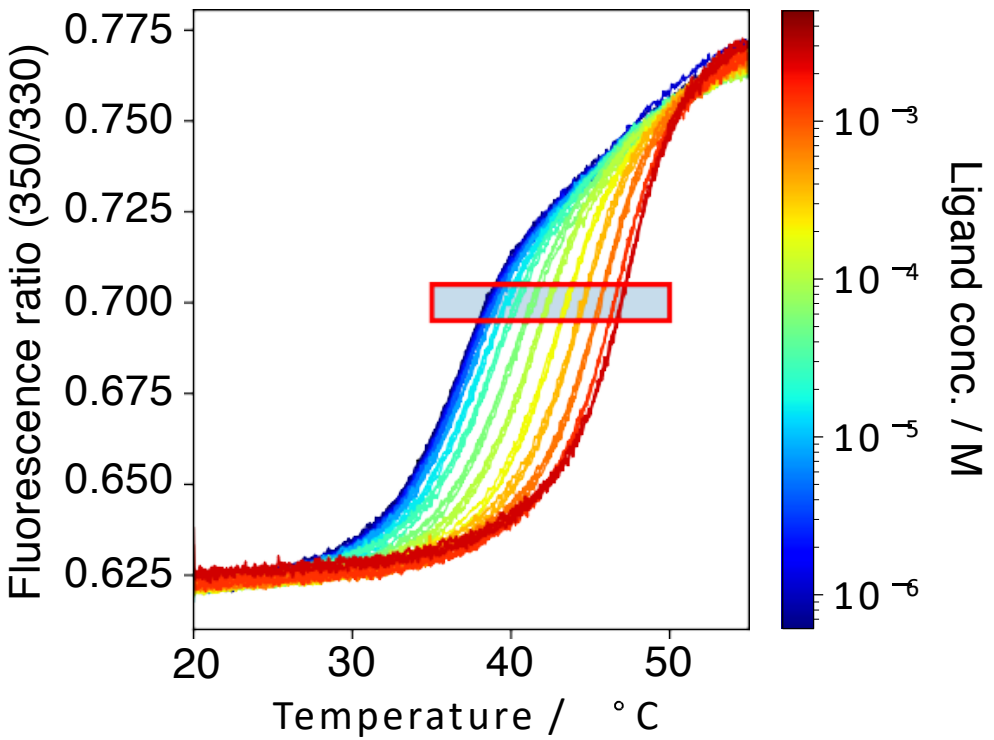




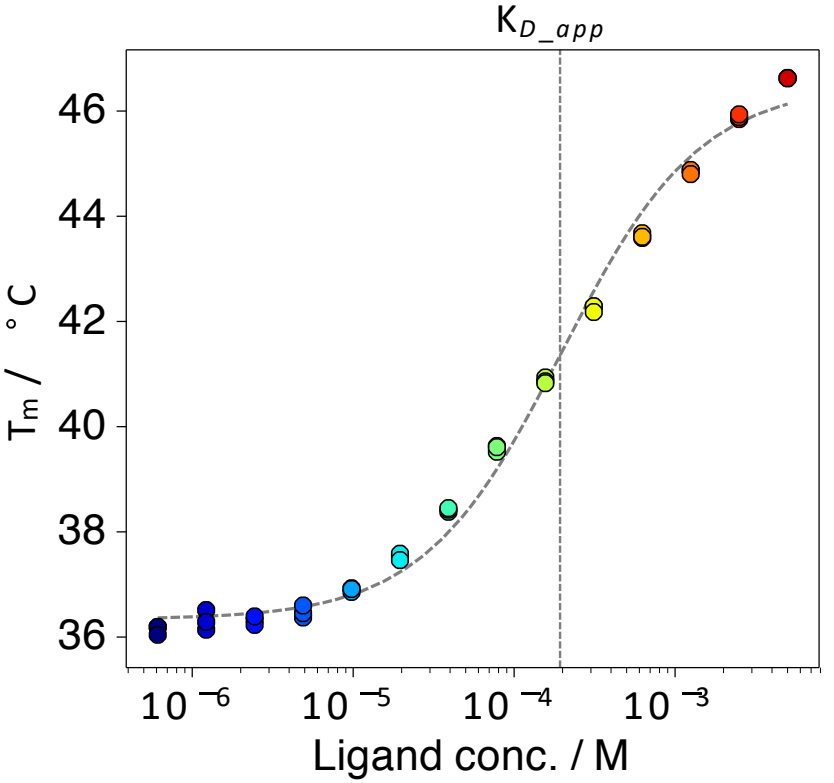
Anjanappa *et al.*, **Nat Commun. 2020** Mar 11;11(1):1314.

Apparent K_D from melting temperatures

nDSF binding study



Melting temperatures T_m

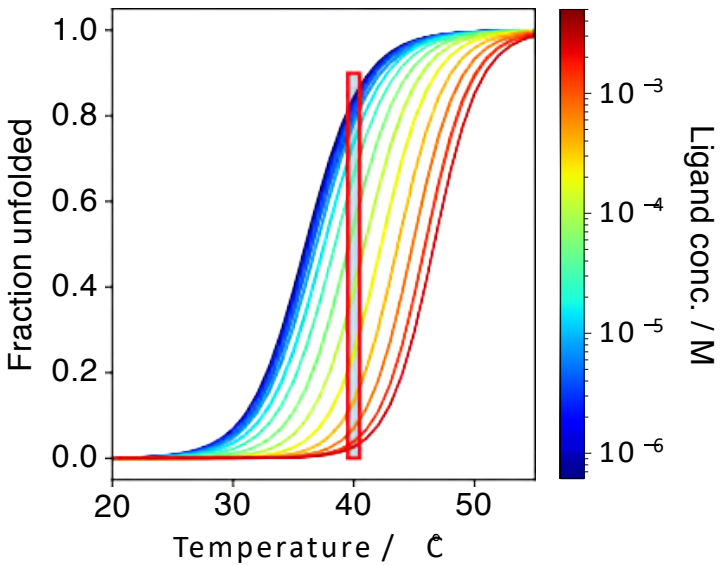


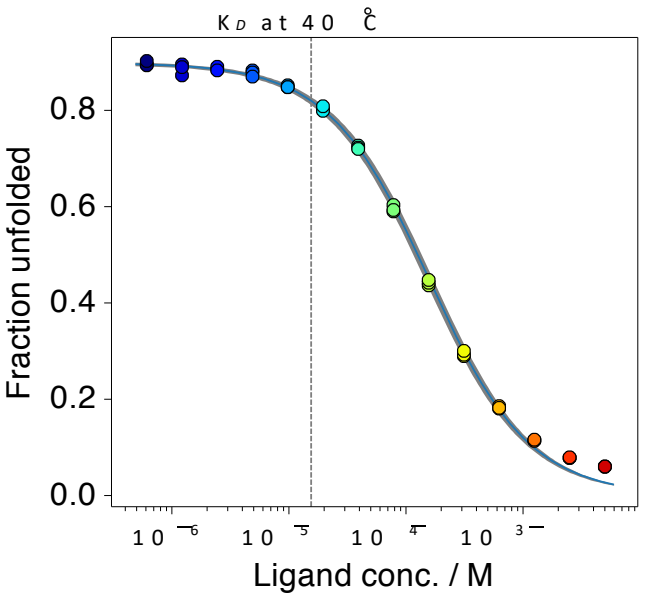
Niebling et al. Sci Rep. **2021**

Estimating binding affinities by Isothermal analysis

Steps

1. Fit melting curves → Fraction unfolded $f_u(T)$ for each lig. conc.
2. Extract $f_u([L])$ for each ligand concentration at a **defined temp.**
3. Fit binding model to $f_u([L])$ to obtain K_D

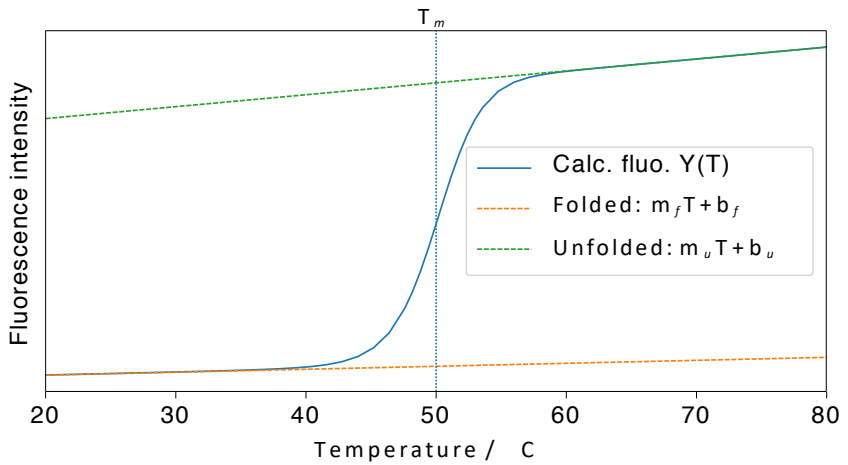




Isothermal analysis: General approach

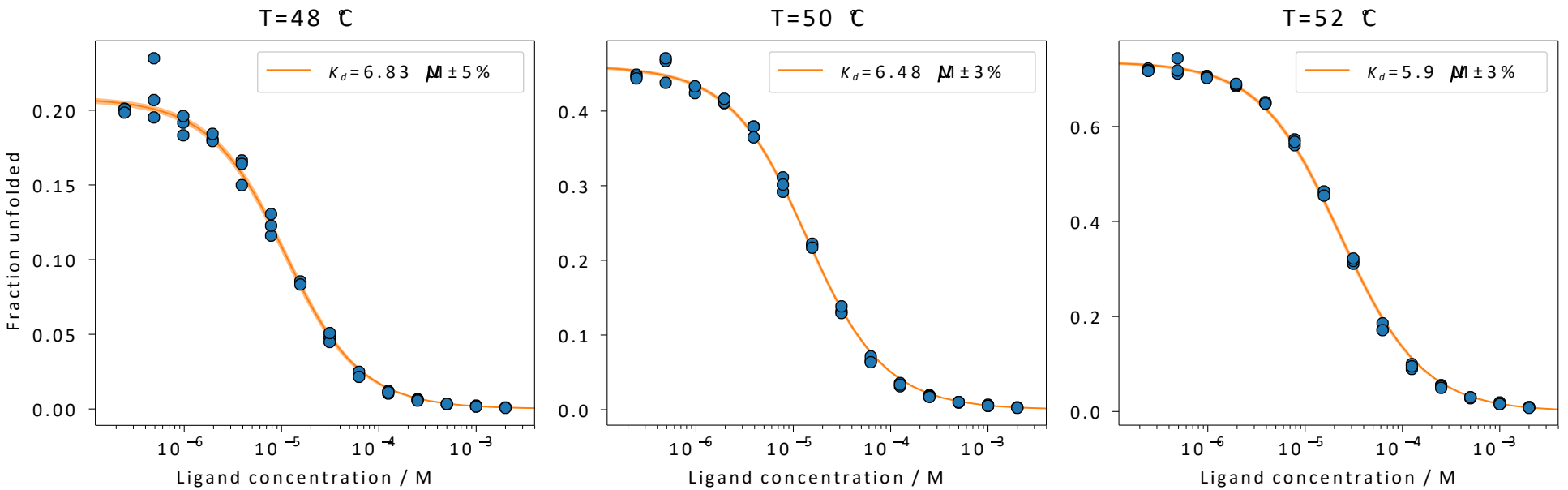
Steps

1. Fit melting curves → Fraction unfolded $f_u(T)$ for each lig. conc.



$$Y(T) = f_u(T) * (m_u * T + b_u) + (1 - f_u(T)) * (m_f * T + b_f)$$

Selecting a temperature around the Tm (empty state)

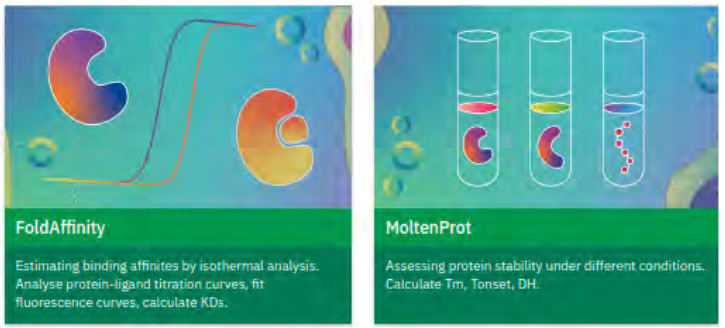


Comparison with ITC value: K_d = 3.5μM (25°C)

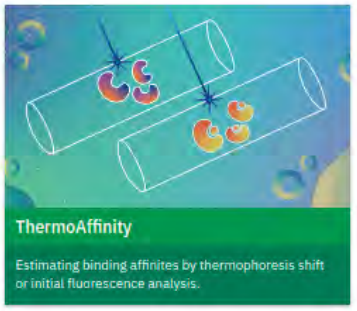
eSPC Online Data Analysis Platform for molecular biophysics

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

Differential Scanning Fluorimetry



MicroScale Thermophoresis



spc.embl-hamburg.de

Webserver by Osvaldo Burastero (ARISE)

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**

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

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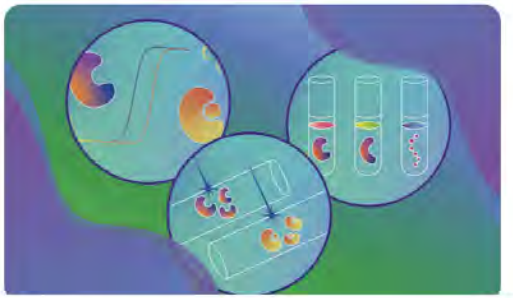
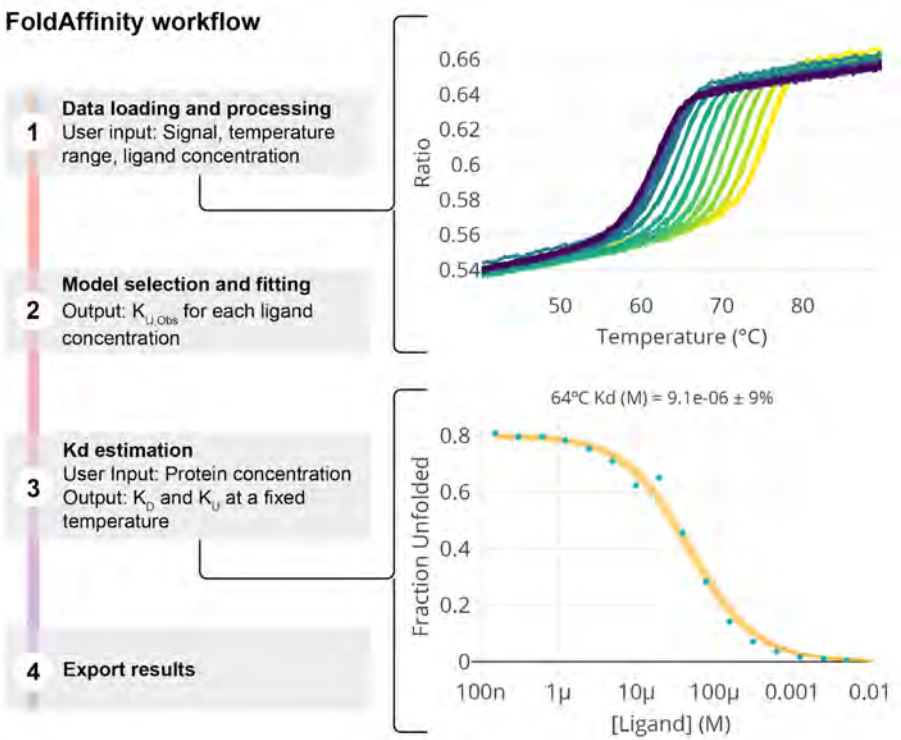
The @SPC_EMBL_HH has developed eSPC – a free online platform for analysing data from diverse biophysics experiments.

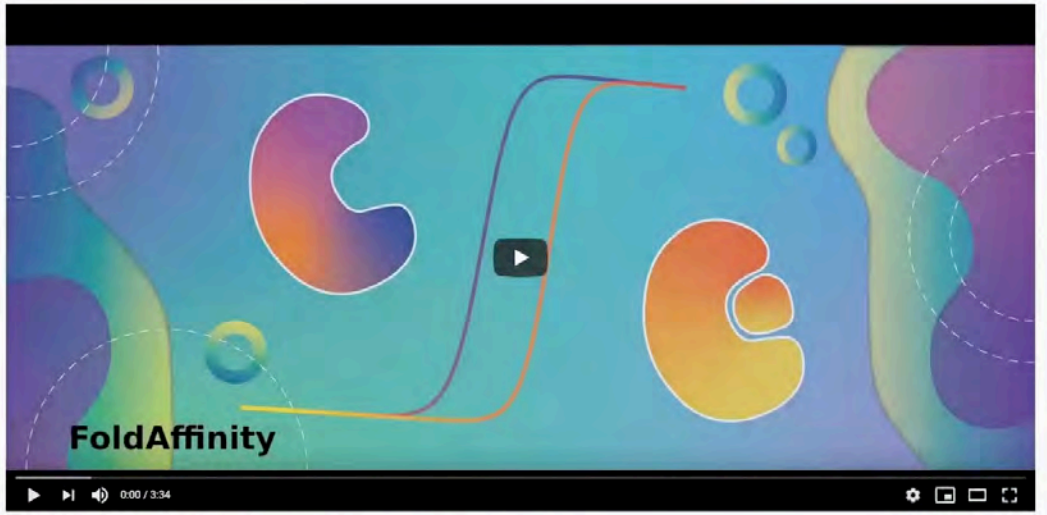
It enables scientists across fields to analyse their data much easier than before, and remotely without the need to travel.

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



Estimating binding affinities by Isothermal analysis





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