Interactions from stability of biomolecules
-
Differencial scanning fluorimetry

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

Stability

Resistance in environment

- Capability to retain **native structure** (stay folded)
- Capability to retain **activity**

no structure	⇨	no activity
structure		? activity ?

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Stability

Quick reminder – structure hierarchy

	Protein	DNA
Primary	Sequence (aminoacids, N-term - C-term)	Sequence (nucleotides, 5`- 3`end)
Secondary	α-helix, β-sheet, turns, loops (rotation along torsion angels Ψ and Φ)	Watson-Crick base pairing (A-T, C-G)
Tertiary	3D organization of secondary motives	A-form, B-form, Z-form
Quarternary	oligomerization	nucleosomes

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Stability

Quick reminder – structure hierarchy

	Protein	DNA
Primary	Sequence (aminoacids, N-term - C-term)	Sequence (nucleotides, 5`- 3`end)
Secondary	Upon unfolding (= denaturation) these structures are lost. The primary structure remains ⇩ The covalent bonds are not broken, only the non-covalent	
Tertiary		
Quarternary		

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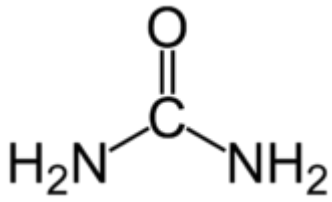
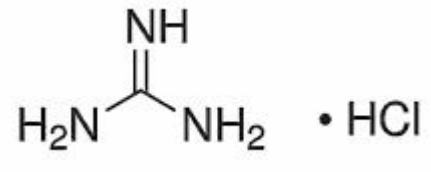
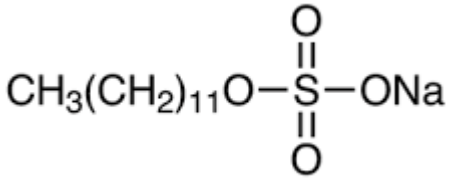
Denaturing conditions

- Chemicals
  - Urea (around 8M)
  - Guanidium chloride (around 6M)
  - High salts concentration
  - SDS







- Extreme pH
  - Proteins are most stable near isoelectric point (pI)
  - Extreme pH affects H-bonds and ionic interactions

- **Temperature**

$$\Delta G = \Delta H - T\Delta S$$

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Temperature as denaturant

T_m = melting temperature

- Temperature at which 50% of the sample is unfolded
- The most reliable indicator of thermal stability

- Influenced by:
  - Enviroment (buffer, pH, salts): in different condition, ΔG of unfolding is different
  - **Presence of ligand**: Complex protein-ligand is usually more stable than protein itself
  - Heating rate of experiment: slower heating → lower T_m
    standard is 1 °C/min

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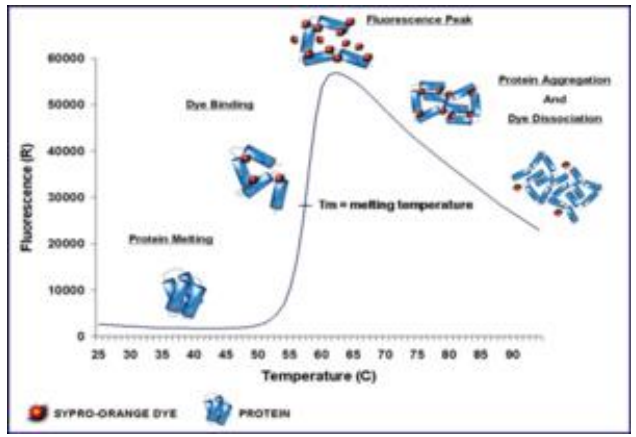
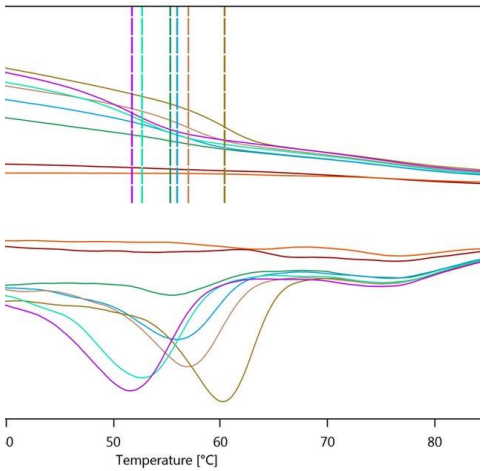
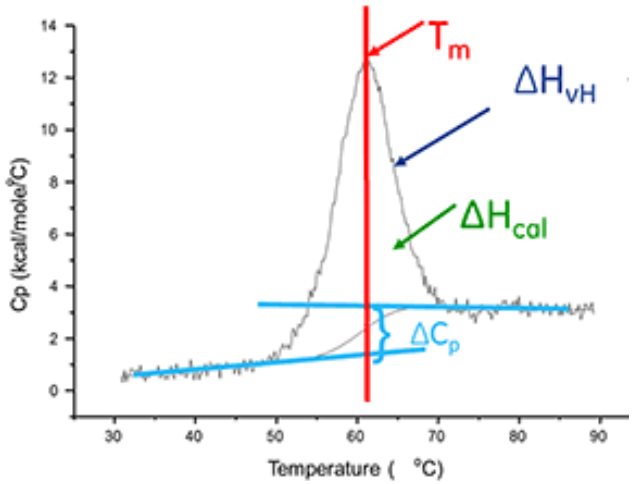
Methods

- Differential scanning calorimetry (DSC)
- Differential scanning fluorimetry (DSF) – Thermal shift assay (TSA)
- Nano-differential scanning fluorimetry (nanoDSF)
- Circular dichroism (CD)







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DSC = Diferential Scanning Calorimetry

Measures the energy absorbed or released by a sample as it is heated or cooled

Gold standard for T_m determination

Directly measures the thermodynamic of unfolding

- Suitable for all kinds of molecules
- Low throughput – one sample at a time

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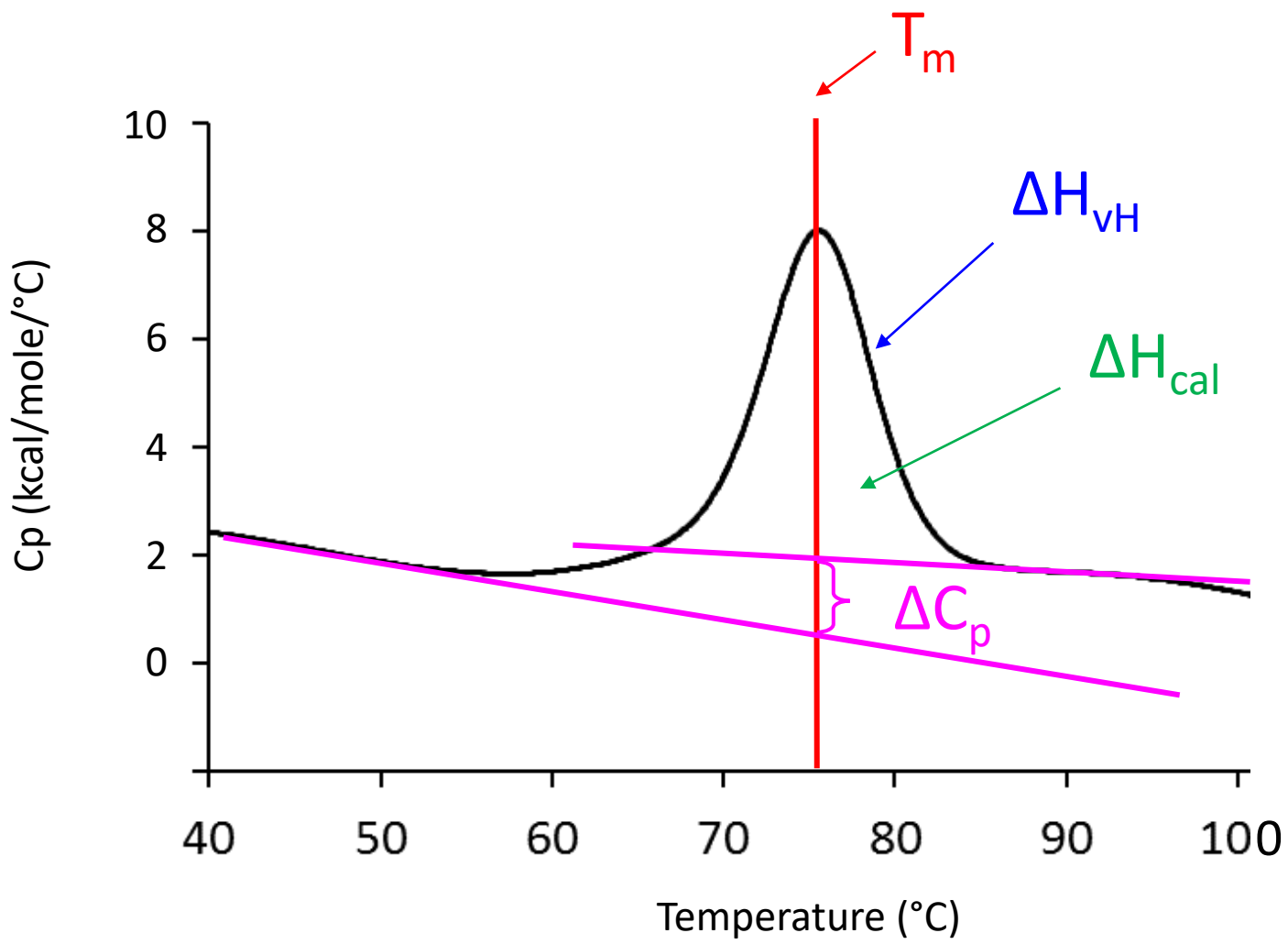
DSC = Diferential Scanning Calorimetry



T_m
the peak of the transition

ΔC_p
change of heat capacity
folded-unfolded sample
(difference between baselines)

ΔH_{cal}
Area of the peak (integration)

ΔH_{vH}
The slope of the peak

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TSA = Thermal Shift Assay

Also known as differential scanning fluorimetry (= DSF)

High-throughput (96 well plates)

No specialized machine – uses termocycler for RT-PCR

Measures changes of fluorescence of the added dye

- Commercial dyes (e.g. SYPRO Orange, bis-ANS, Nile Red)
- Suitable for average proteins with hydrophobic core

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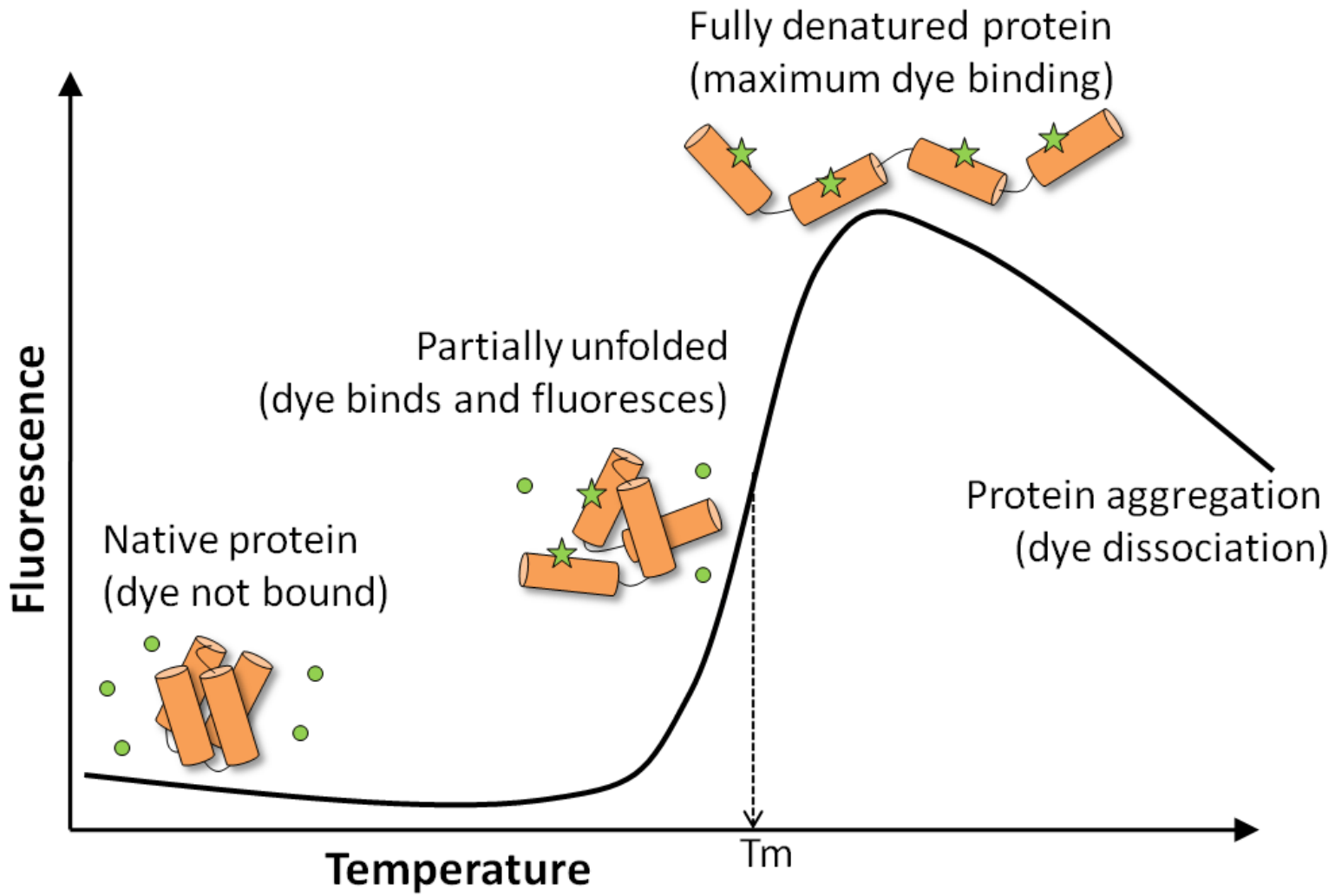
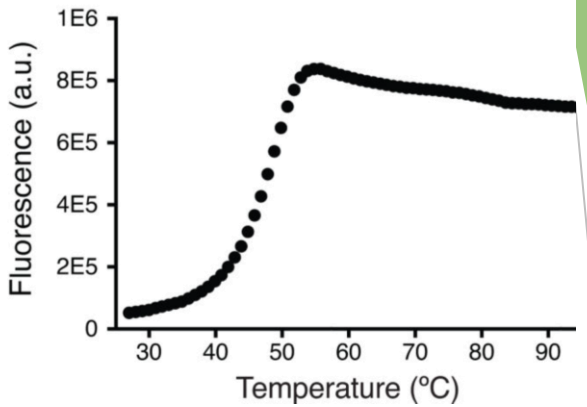
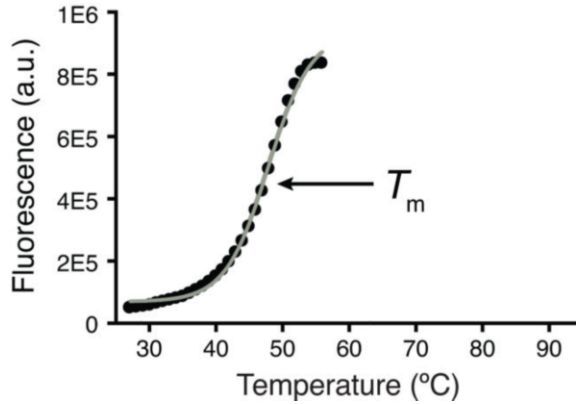
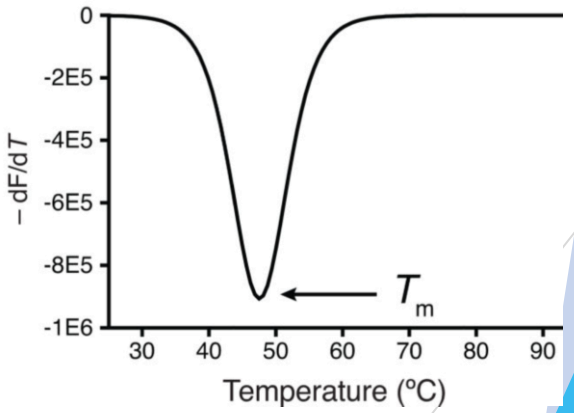
TSA









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nanoDSF = nano Diferential Scanning Fluorimetry

Measures changes of **intrinsic fluorescence** of the sample in temperature gradient

High-throughput (48 samples in 1 run)

Low sample consumption (10 µl)

Ideal for optimal condition screening

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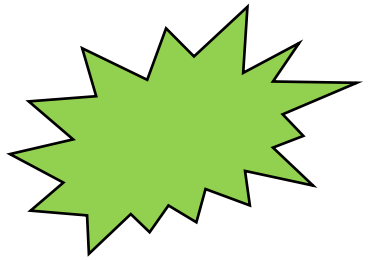
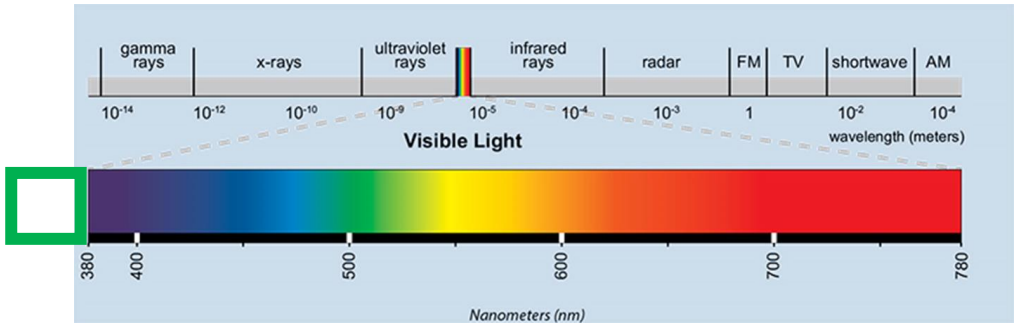
Intrinsic fluorescence of proteins

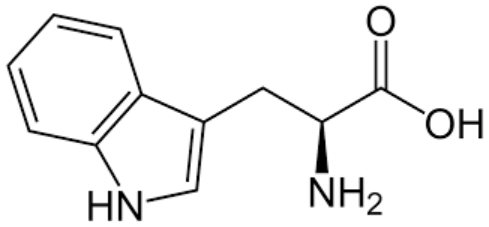


fluorophore

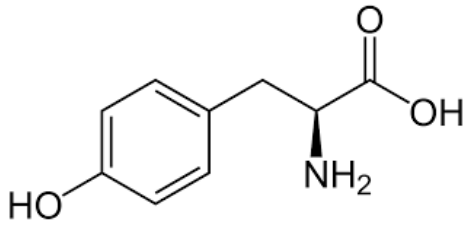
in **UV region** (λ = 300-360 nm)



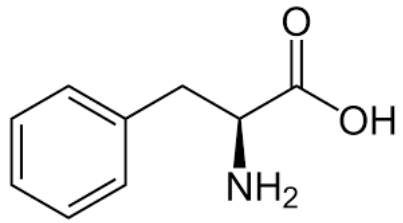
Tryptophan (Trp, W)



Tyrosine (Tyr, Y)



Phenylalanine (Phe, F)



Aromatic amino acids

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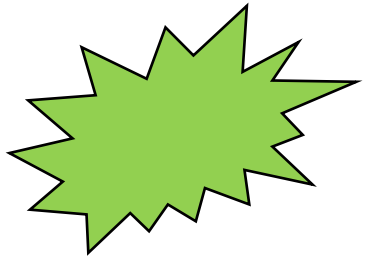
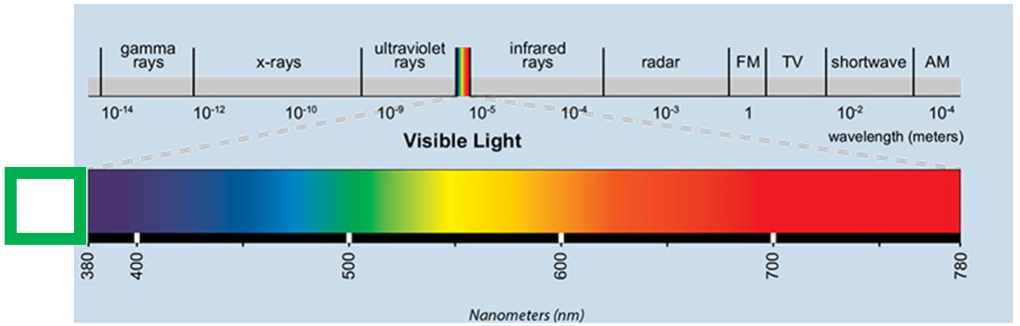
Intrinsic fluorescence of proteins

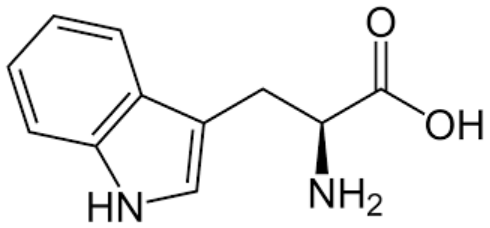


fluorophore

in **UV region** (λ = 300-360 nm)



Tryptophan (Trp, W)



Tyrosine (Tyr, Y)

Phenylalanine (Phe, F)

Aromatic amino acids

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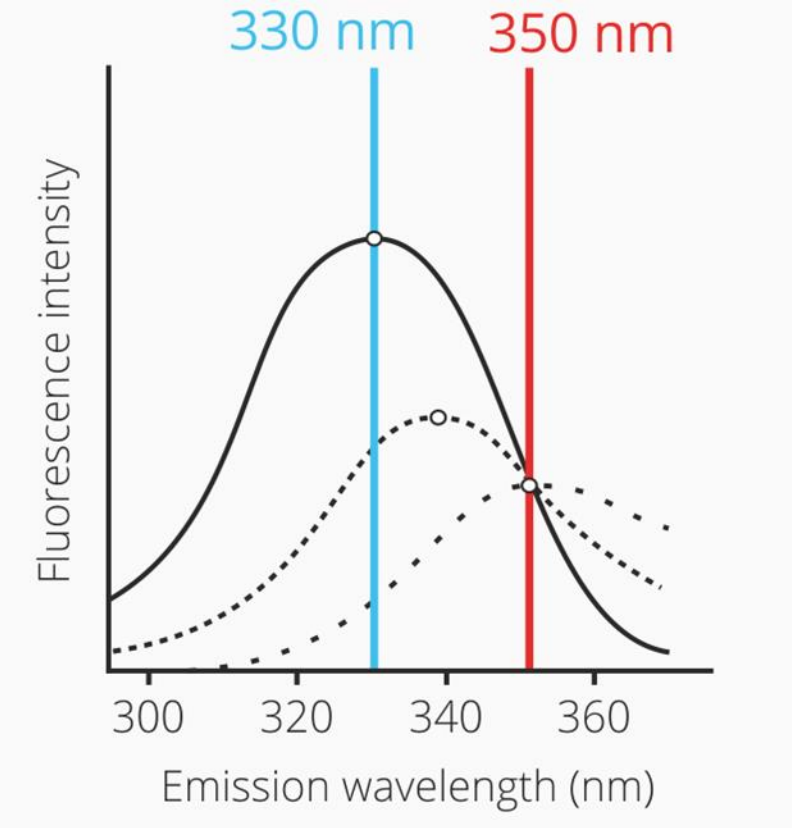
nanoDSF

Intrinsic fluorescence of proteins (UV region, λ = 300-360 nm) changes according to the local environment



——— hydrophobic
maximum at 330 nm

- - - - - hydrophilic
maximum at 350 nm

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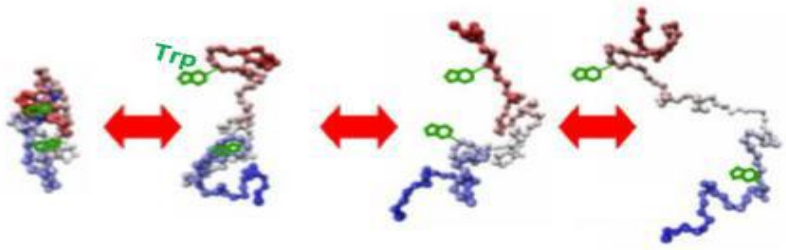
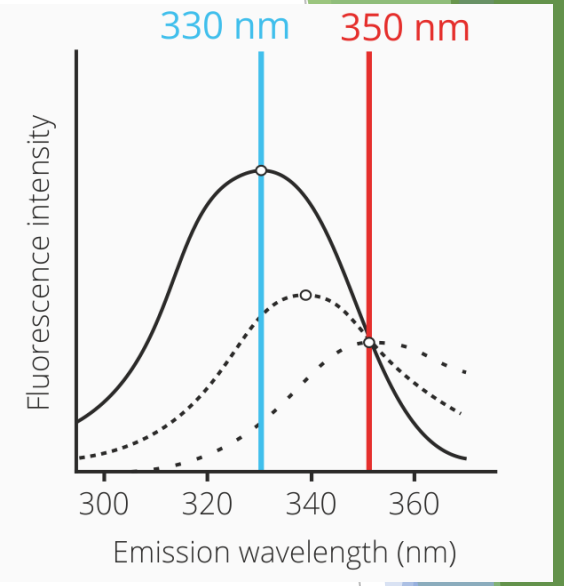
nanoDSF





Aromatic aminoacids (W, Y, F) are hydrophobic and are typicaly located inside the folded protein

⇩

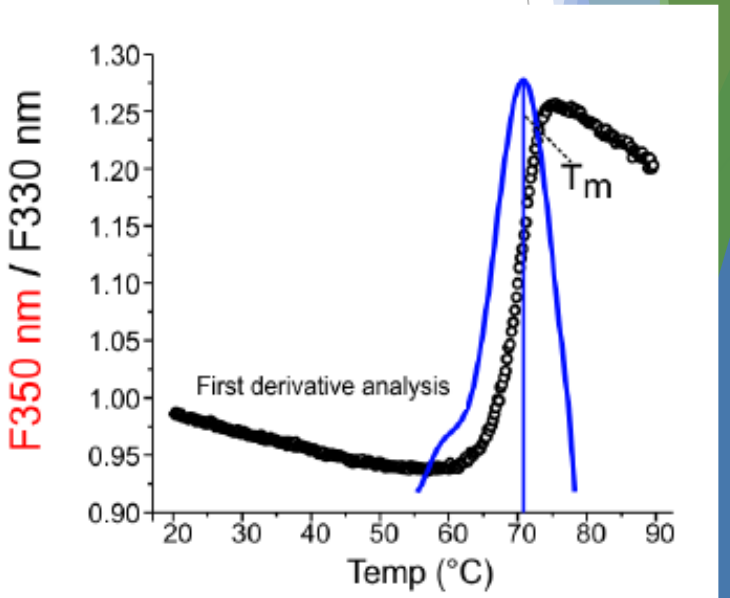
With increasing temperature the protein is unfolded

⇩

W, Y, F are exposed on the protein surface

⇩

Changes in fluorescence



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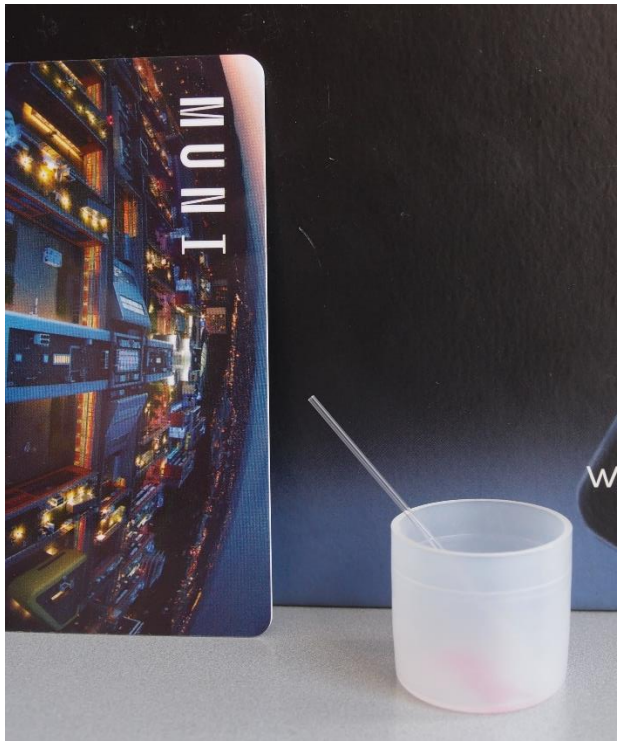
nanoDSF in practice

Protein samples → Put into a capillary or Pipette into plate → Measurement



Temperature gradient 20 – 110 °C

Heating rate 1 °C/min

quicker	higher Tm
slower	lower Tm

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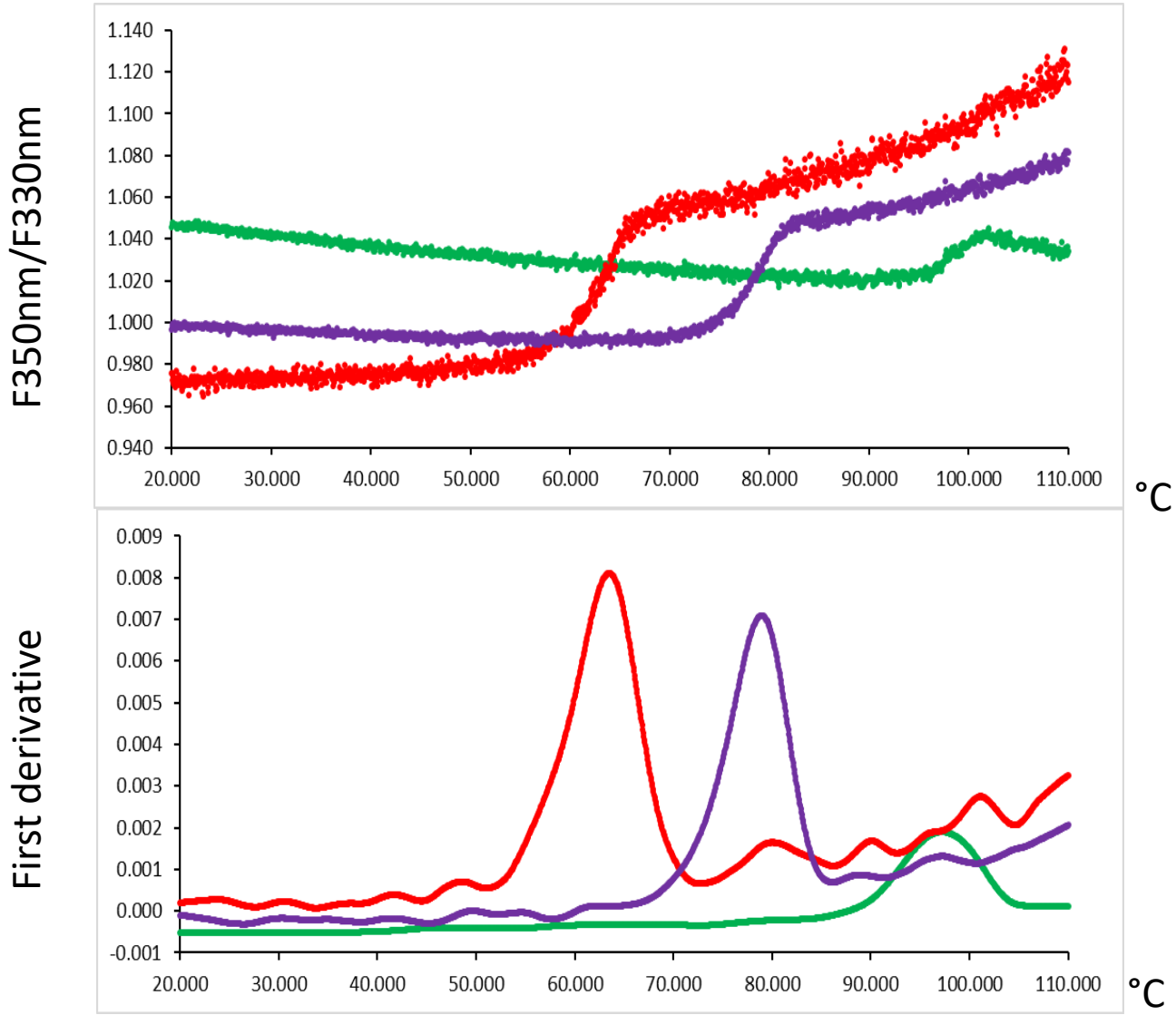
nanoDSF in practice



pH = 2 Tm = 63.3 °C

pH = 3 Tm = 78.8 °C

pH = 7.5 Tm = 97.1 °C

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nanoDSF

Pros:

Quick

High-throughput

Low sample consumption (10 µl)

Low concentration (0.1 – 1 mg/ml)

No labelling

Excelent for sample comparison

Machine is almost unbreakable

Cons:

Only for proteins

W (Y, F) in sequence necessary

Senzitive to capillary purity

Delicate manipulation with capillaries

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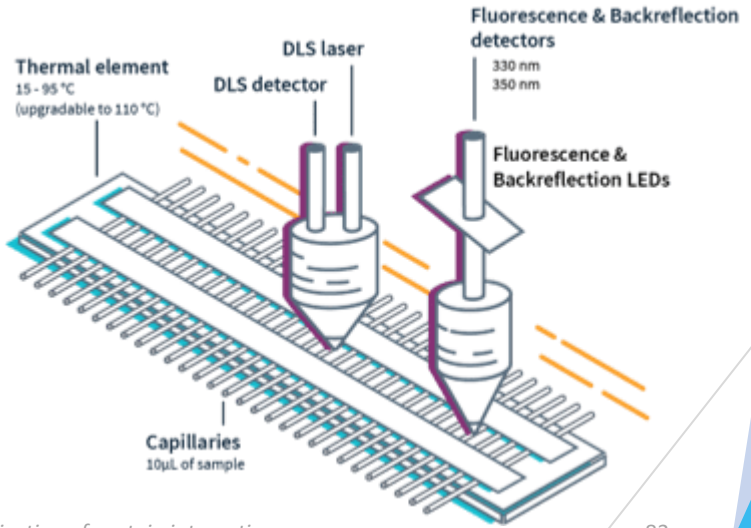
nanoDSF in combination

Combination with **Dynamic light scattering** (DLS)

- “Backreflection” measurement – decrease in light corresponds to aggregation
- “True DLS” – observation of particle size upon heating

Prometheus Panta



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Applications

- Thermal stability determination
- Buffer optimization for purification and storage
- Optimization of crystallization conditions
- Batch to batch comparison – quality control
- Ligand screening – affinity analysis

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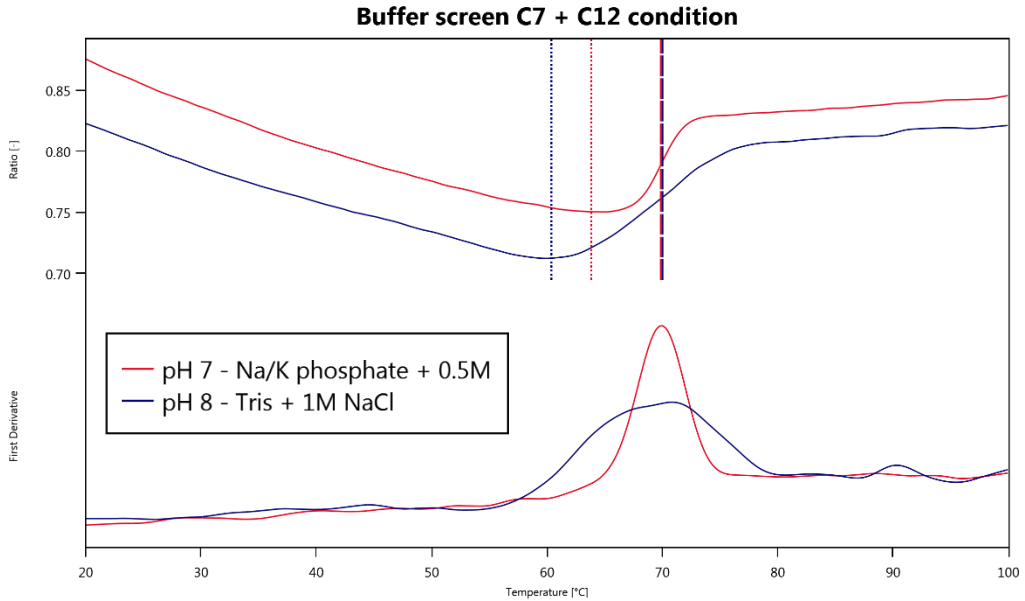
Buffer screening

	1	2	3	4	5	6	7	8	9	10	11	12
A	59.2°C	-	43.6°C	37.7°C	55.0°C	61.3°C	59.8°C	62.1°C	55.5°C	59.0°C	33.4°C	33.2°C
B	36.5°C	42.1°C	48.3°C	52.2°C	55.0°C	58.5°C	66.2°C	66.4°C	58.7°C	59.4°C	63.1°C	63.3°C
C	57.2°C	59.2°C	62.7°C	62.1°C	67.0°C	68.1°C	69.9°C	66.5°C	60.2°C	61.8°C	66.5°C	70.0°C
D	60.6°C	58.5°C	69.4°C	63.4°C	46.2°C	55.2°C	58.2°C	54.5°C	59.2°C	59.5°C	-	59.2°C

Thermal stability using 48-well buffer screen

Houser et al 2021



Original buffer **59.2**

vs.

Best buffer **69.9**

> 10°C difference !!!

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85

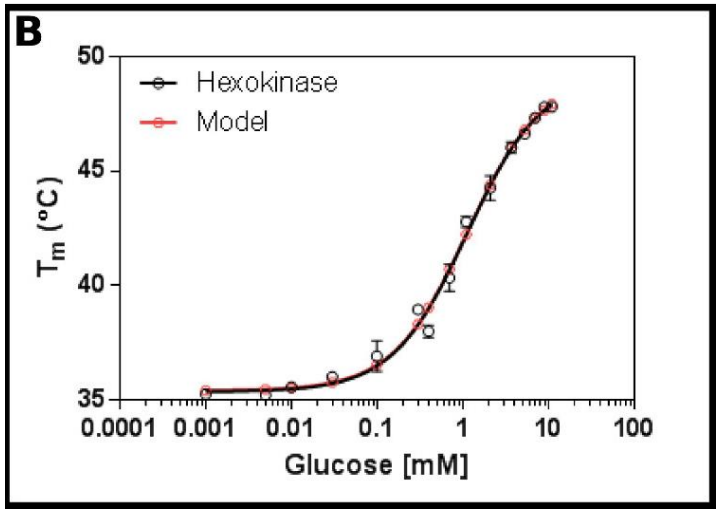
K_d from thermal stability

T_m at different ligand concentrations

Calculation of K_d from T_m shift

Various fitting models possible

Result is K_d at T_m (!) – difference from other methods



Vivoli et al 2014

$$L_t = \left(e^{-(\Delta_U H_{T_r} + \Delta_U C_p (T_m - T_r) - T_m(\Delta_U S_{T_r} + \Delta_U C_p \ln(T_m/T_r)))/RT_m} - 1\right) \times \left[\frac{P_t}{2} \frac{1}{e^{-(\Delta_U H_{T_r} + \Delta_U C_p (T_m - T_r) - T_m(\Delta_U S_{T_r} + \Delta_U C_p \ln(T_m/T_r)))/RT_m}} + \frac{1}{e^{-(\Delta_b H_{T_0} + \Delta_b C_p (T_m - T_0) - T_m(\Delta_b S_{T_0} + \Delta_b C_p \ln(T_m/T_0)))/RT_m}}\right]$$

Zubriene et al 2009

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Comparison

	DSC	TSA	**nanoDSF**
Sample	protein, nucleic acids, lipids, polymers	Some proteins	Proteins with Trp
Sample consumption	high	low	low
High-throughput	no	yes	yes
Automation	yes	(yes)	yes
Enthalpy	direct	indirect	indirect
Fluorescent dye	no	yes	no

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DSF Summary

- DSF is a high-throughput method for **protein stability**
- It is suitable for sample **characterization** and **optimization**
- **Stability-based methods** (DSF, DSC) offer an alternative approach to determine affinity parameters

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Biomolecular
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MUNI | CEITEC

instruct Integrating Biology

ARBRE Association of Resources for Biophysical Research in Europe

CIISB Czech Infrastructure for Integrative Structural Biology

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