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PEGS EUROPE
Protein & Antibody Engineering Summit

TROUBLESHOOTING AND ENGINEERING OF ANTIBODY CONSTRUCTS – PART II

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www.bioc.uzh.ch/plueckthun

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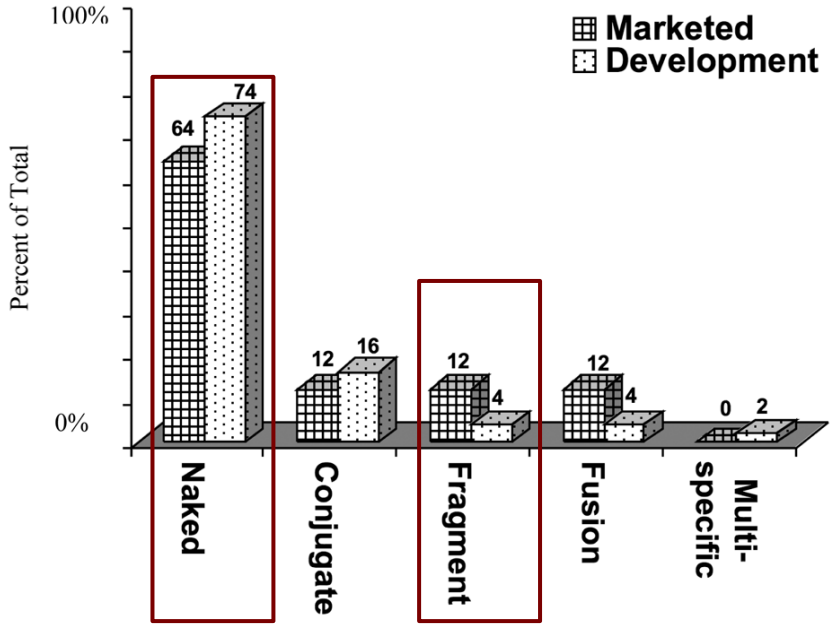
Antibody therapeutics vs. engineering



Total of 165 anti-cancer antibodies currently in clinical studies:

- **84 unmodified IgG (51%)**
- 25 ADC (15%)
- 10 bispecific (6%)
- **17 engineered (10%)**
- **16 fragments (10%)**

➡ while most antibodies on the market / in R&D are **full-length IgGs**, most of the antibody engineering is performed using **small fragments**

Zider and Drakeman, *Mabs* (2010)

Reichert and Dhimolea, *Drug Discovery Today* (2012)

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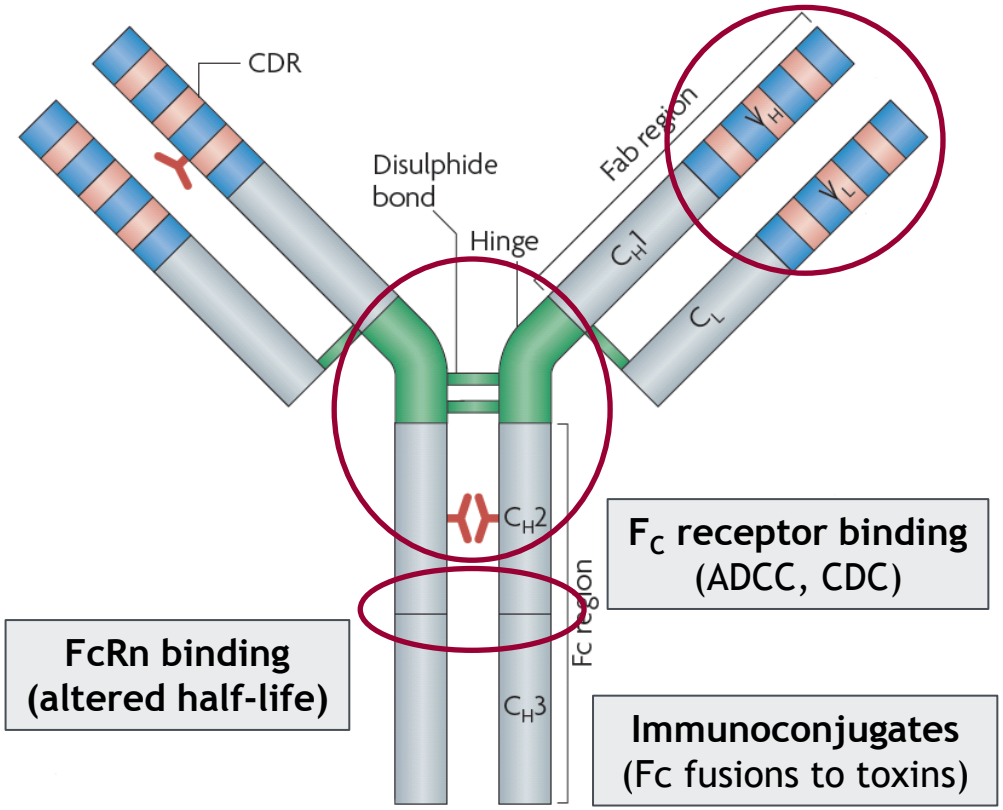
Full-length IgG engineering

Optimization antigen binding
(affinity, humanization, decreased elimination)



F_C receptor binding
(ADCC, CDC)

FcRn binding
(altered half-life)

Immunoconjugates
(Fc fusions to toxins)

➡ variable domains mainly used for **individual optimization**

➡ most "transferable" engineering is focused on **F_C region**

adapted from Beck *et al.*, *Nat. Rev. Immunol.* (2010)

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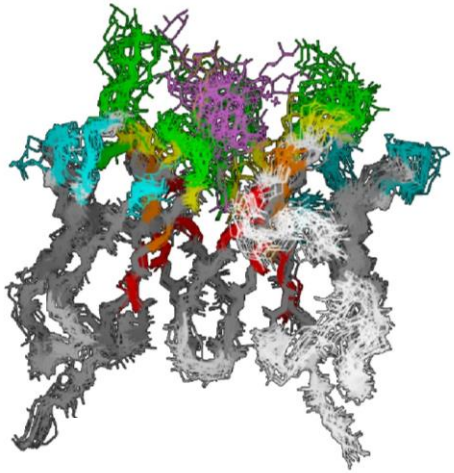
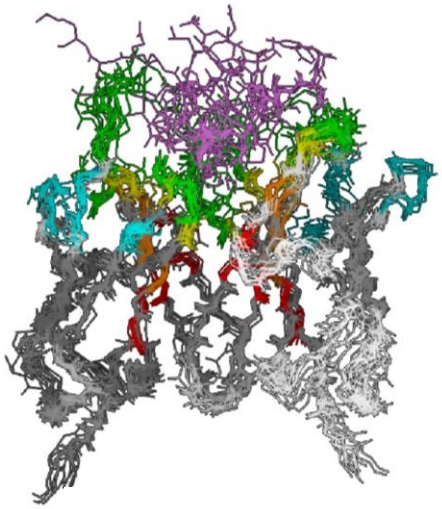
Why not just one "perfect" framework?

seven V_H germline families with different biophysical properties

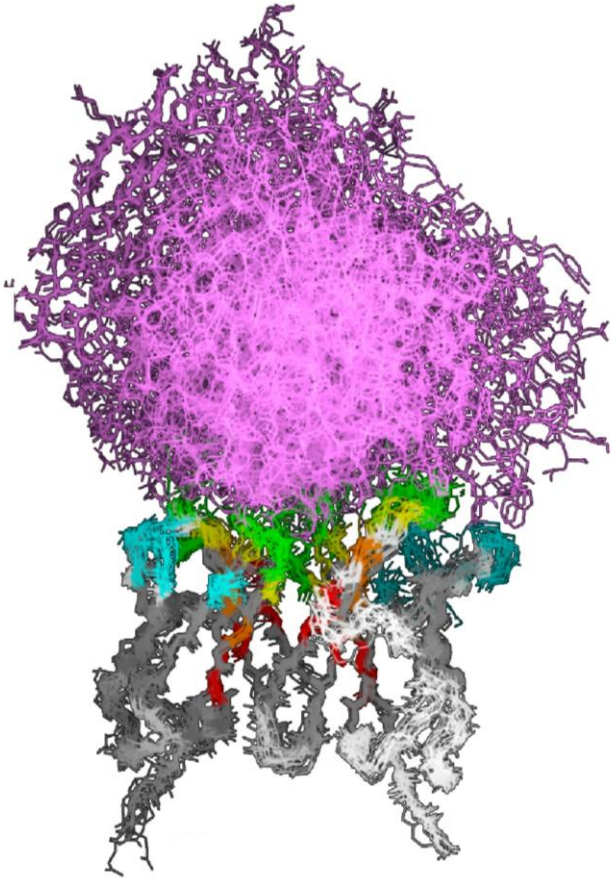
➡ variability in subfamilies increases **binding diversity**



Hapten



Peptides



Proteins

Ewert *et al.*, *Methods* (2004)

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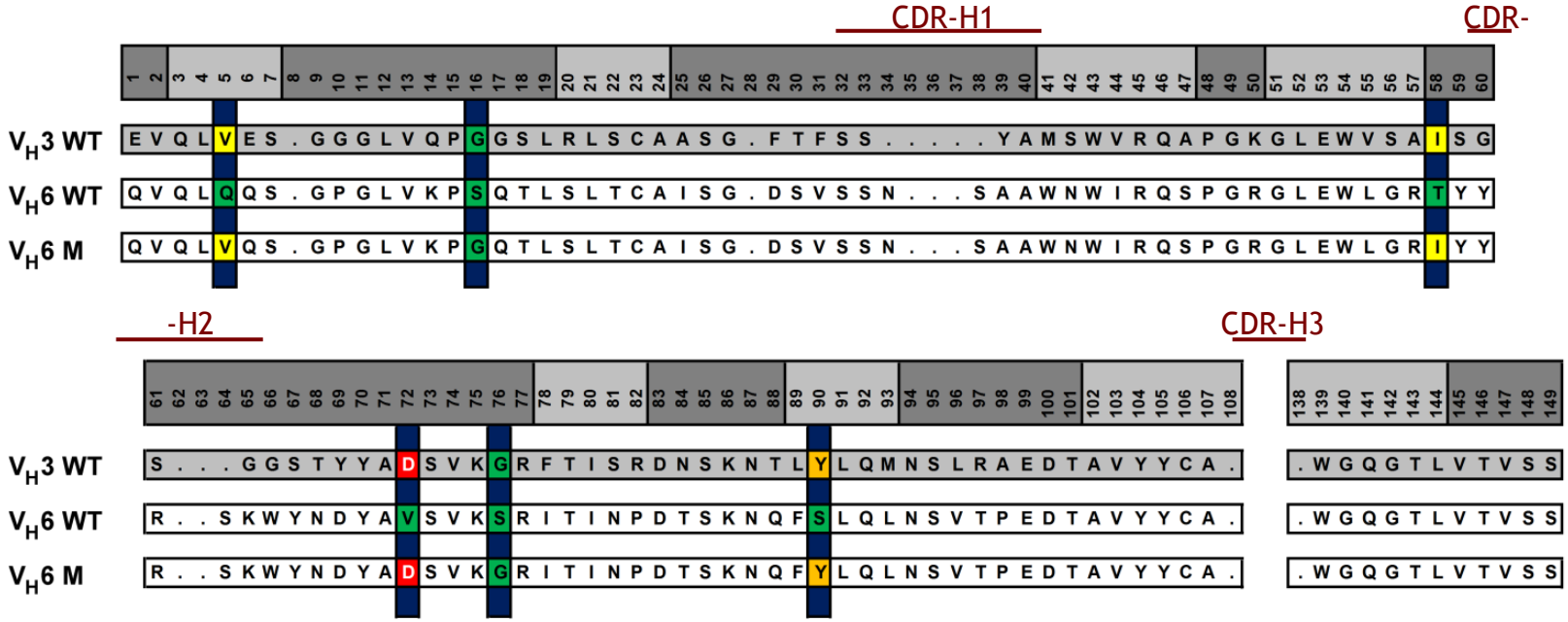
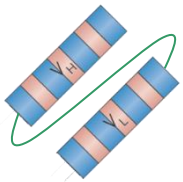
Engineering of unstable V_H6 domain

comparison of the human consensus V_H domains (germinal)



improved biophysical properties of scFv fragments expressed in *E. coli*:

- **increased stability: $\Delta\Delta G_{N-U}$ = 20.9 kJ/mol**
- **4-fold increase in expression levels**



Ewert *et al.*, *Biochemistry* (2003)

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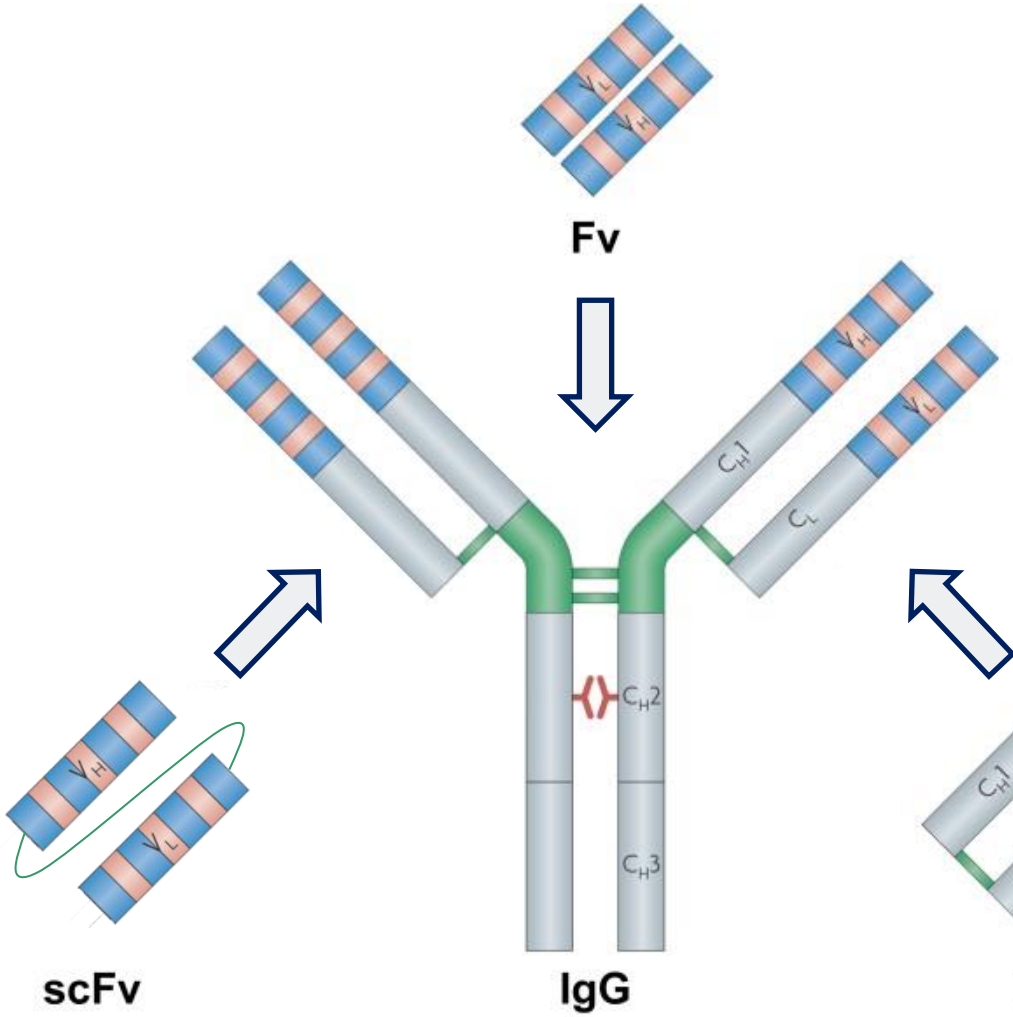
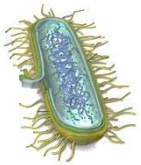
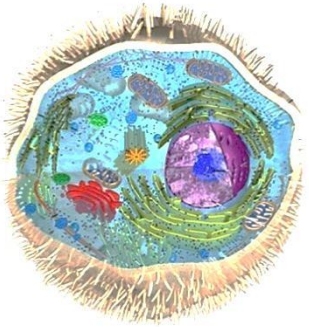
Are previous findings transferable?





prokaryote



eukaryote

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Model antibodies

	IgG 6B3	IgG 2C2
heavy chain (HC)	V_H6	V_H6
antigen	protein	peptide
light chain (LC)	**$V_\lambda3$** (lambda)	**$V_\kappa3$** (kappa)

➡ chosen model IgGs differ in

- Fab stability: rather unstable (6B3) vs. extremely stable (2C2)
- pI: 6.9 (6B3) vs. 8.7 (2C2)
- antigen: protein vs. peptide

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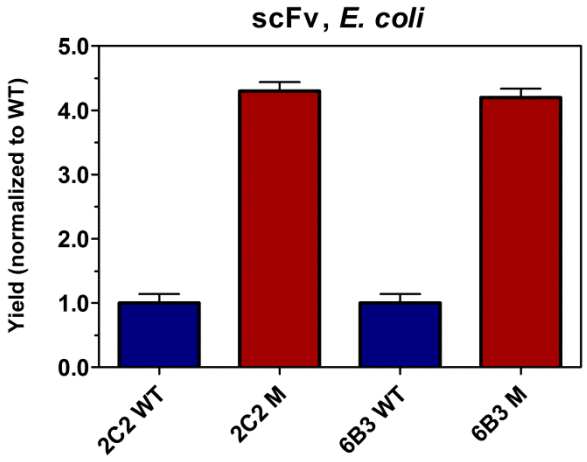
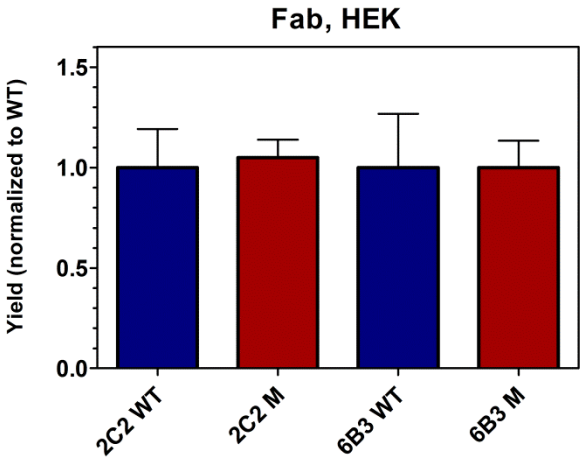
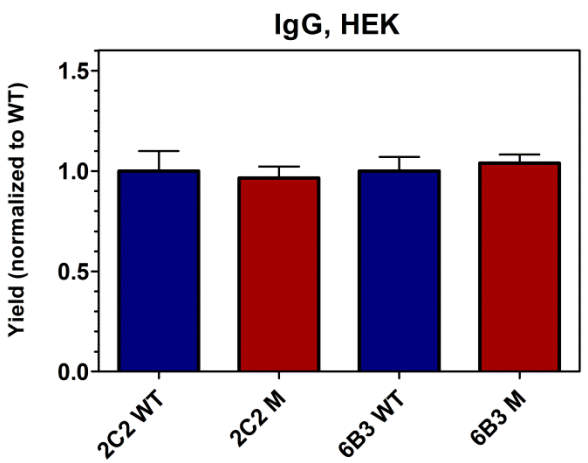
Comparison of expression levels

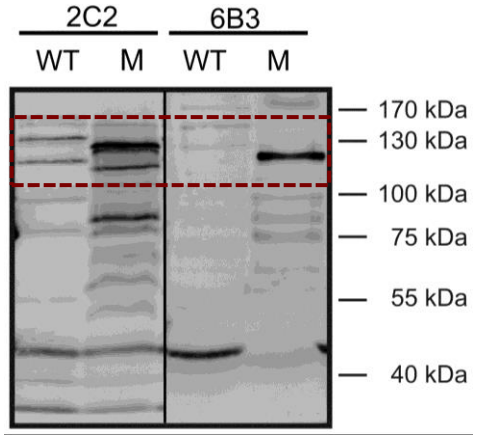






- eukaryotic **chaperons** and **quality control** systems equalize the expression yield between WT and stabilized V_H6

- **prokaryotic expression** of IgGs indicates increased periplasmatic levels of the M variants



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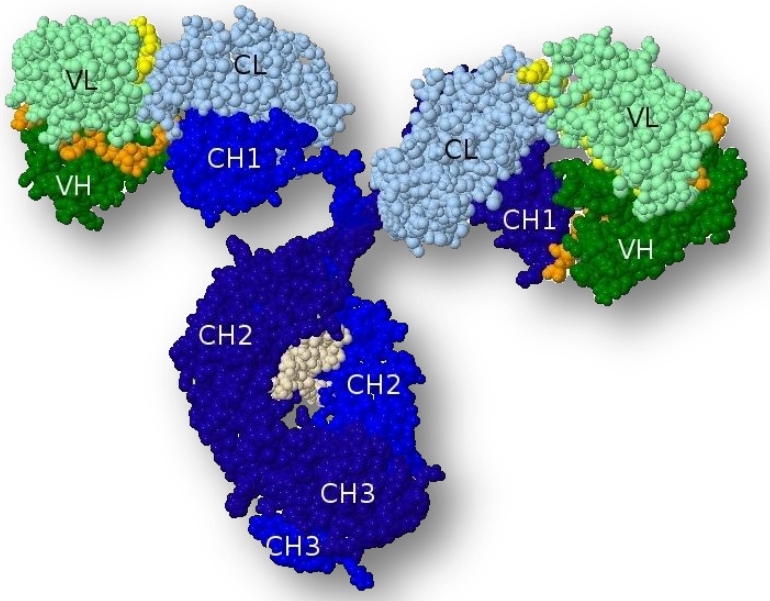
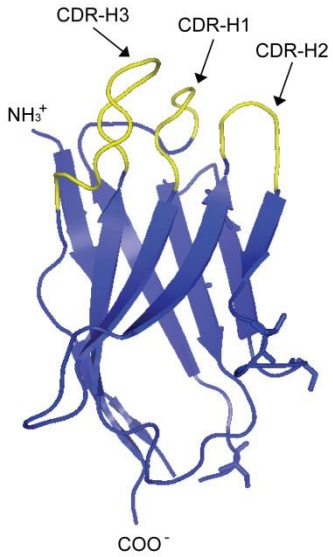
Analytical challenge: Multidomains

- IgGs consist of **six individual domains (each in duplicates)**, all having similar folds





- with most experimental setups, only **overall average** of biophysical features will be analyzed

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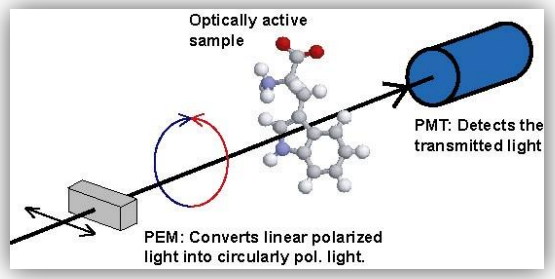
Biophysical analyses (methodology)

Circular Dichroism (CD)

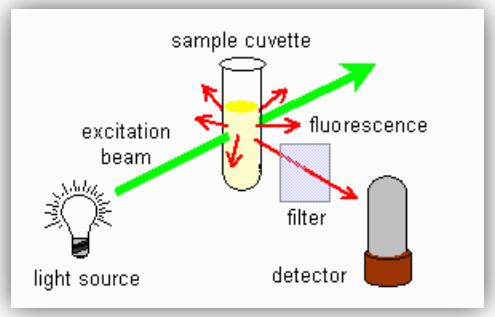


(2ry structure composition)

thermal denaturation

(aggregation analysis)

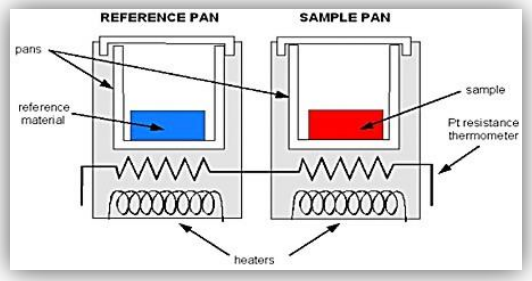
Intrinsic Tryptophan Fluorescence (ITF)

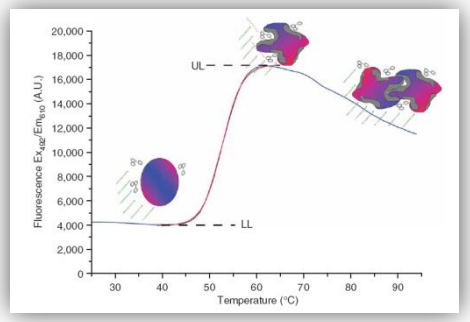


thermal denaturation

chemical denaturation

Differential scanning calorimetry / fluorimetry





analysis of individual domains

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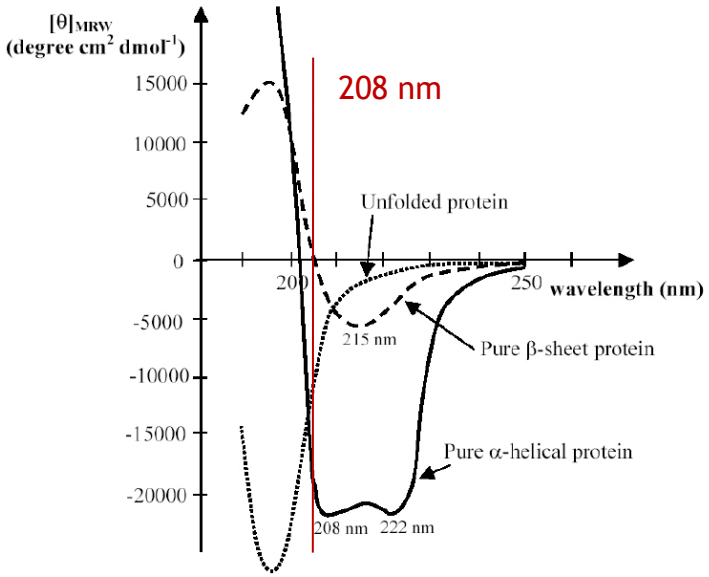
Circular Dichroism (CD)

Lambert-Beer derivative: $\Delta A = A_L - A_R = \varepsilon_L \times l \times C - \varepsilon_R \times l \times C = \Delta\varepsilon \times l \times C$

elipticity: $\theta = \frac{2.303\ (A_L - A_R)}{4l}$

MRE: $[\theta] = \frac{\theta \times 100 \times M}{C \times l \times n}$

amide chromophore of peptide bond has 2 electronic transitions of low energy: **n → π*** (signals at 222 nm and 215 nm) and **π →π*** (signals at 208 nm and 198 nm)



- at ~ **208 nm** intensity due to ß-sheets is essentially zero
- measuring ellipticity at 208 nm monitors **changes in structure** (negative shift caused by random coil formation)

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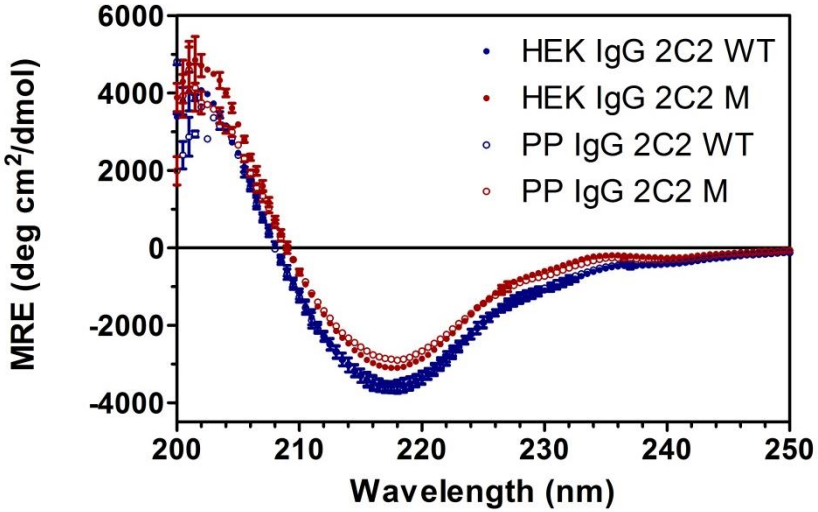
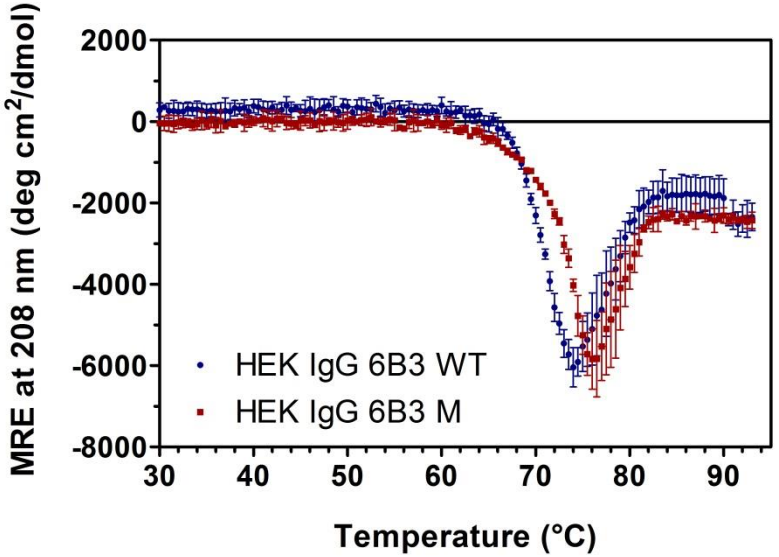
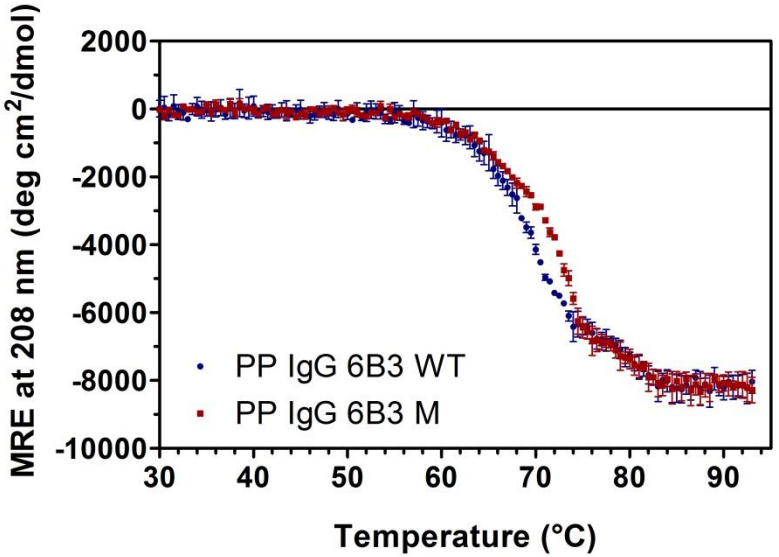
CD: real examples



➡ unfolding detectable, however sheaded by aggregation





Schaefer and Plückthun, *J. Mol. Biol.* (2012)

Schaefer and Plückthun, *Protein Eng. Sel. Des* (2012)

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Intrinsic Tryptophan Fluorescence (ITF)

Trp fluorescence is very sensitive to local conformation and environment

Quantum yields:

Phe – 0.02
Tyr – 0.13
Trp – 0.12

IgG 2C2: 24 Trp per IgG

IgG 6B3: 26 Trp per IgG

IgG 6B3

Domain	# of Trp	% of all Trp
V_H	5	38.5
CH_1	1	7.7
CH_2	2	15.4
CH_3	2	15.4
V_L	1	7.7
CL	2	15.4

IgG 2C2

Domain	# of Trp	% of all Trp
V_H	5	41.7
CH_1	1	8.3
CH_2	2	16.7
CH_3	2	16.7
V_L	1	8.3
CL	1	8.3

➡ **majority of Trp residues are located within V_H domain**

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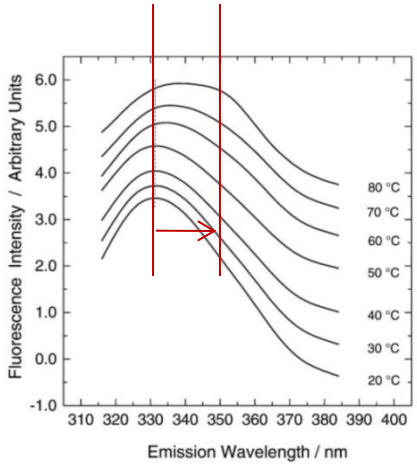
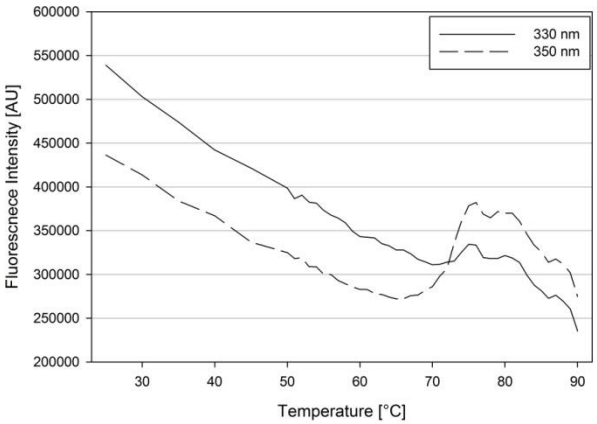
Intrinsic Tryptophan Fluorescence (ITF)

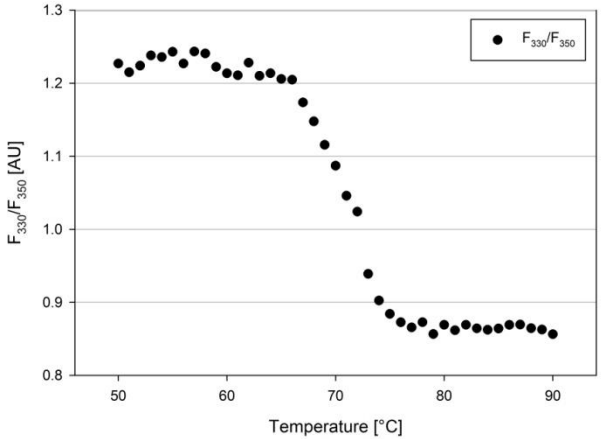
Trp fluorescence is very sensitive to local conformation and environment

wavelength maximum shifts upon heating due to changes of polarity in vicinity of Trp (**red-shift** of Trp emission spectrum)

red shift can be monitored by **ratio of intensities at 330 and 350 nm**







➡ **<u>benefit over other methods</u>:**

- aggregation doesn't cover unfolding reaction
- can easily be performed in 96well format

Method described in Garidel *et al.*, *Biotechnol. J.*(2008) **3**, 1201-11

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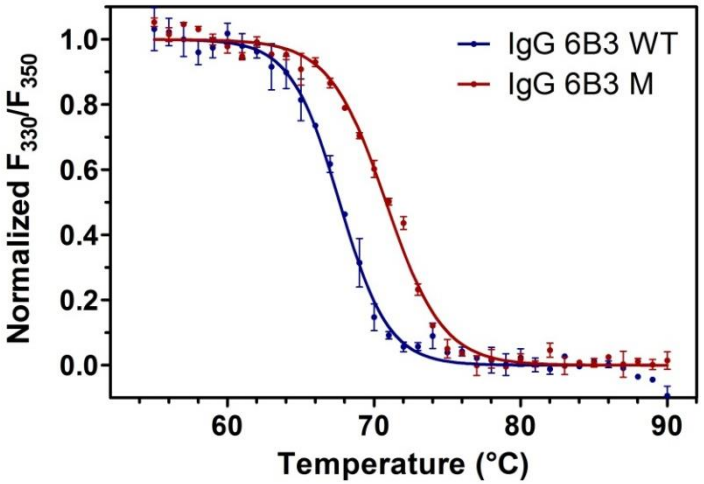
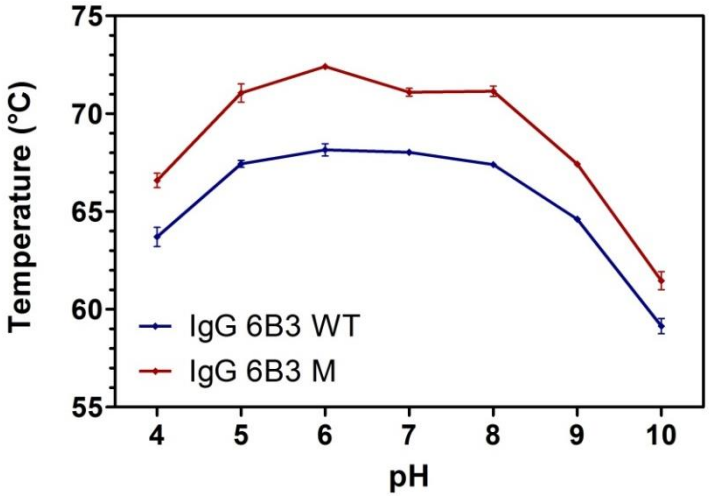
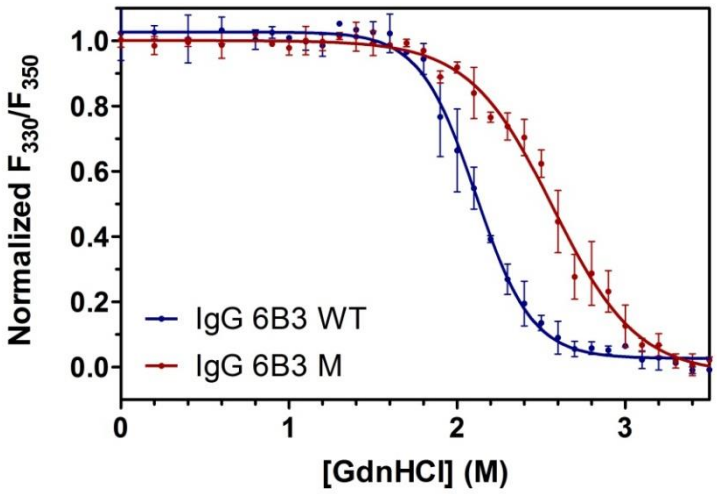
ITF: real examples

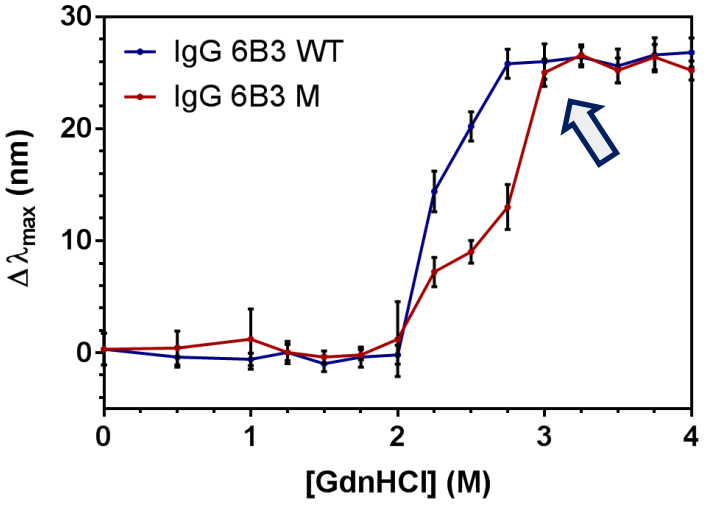
Temperature-unfolding





GdnHCl-unfolding





Schaefer and Plückthun, *Protein Eng. Sel. Des* (2012)

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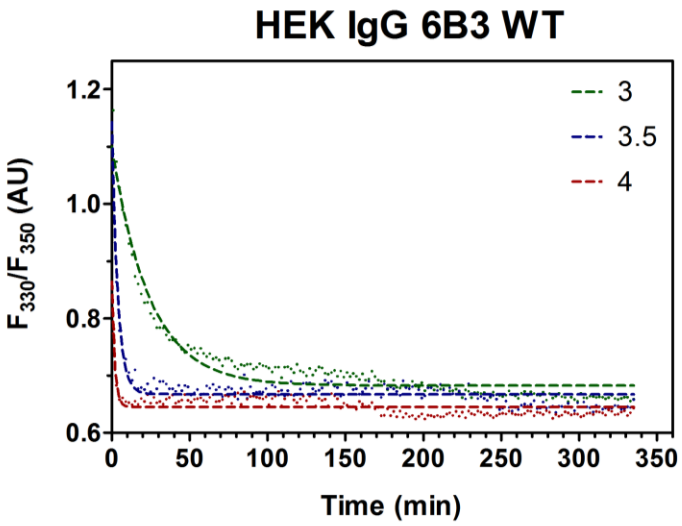
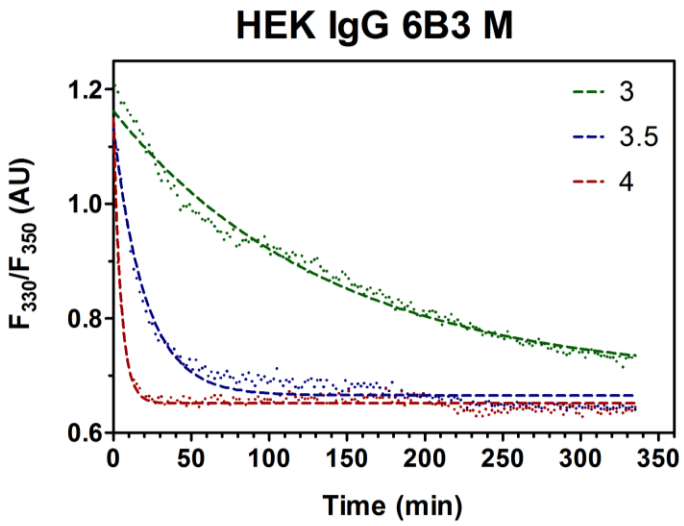
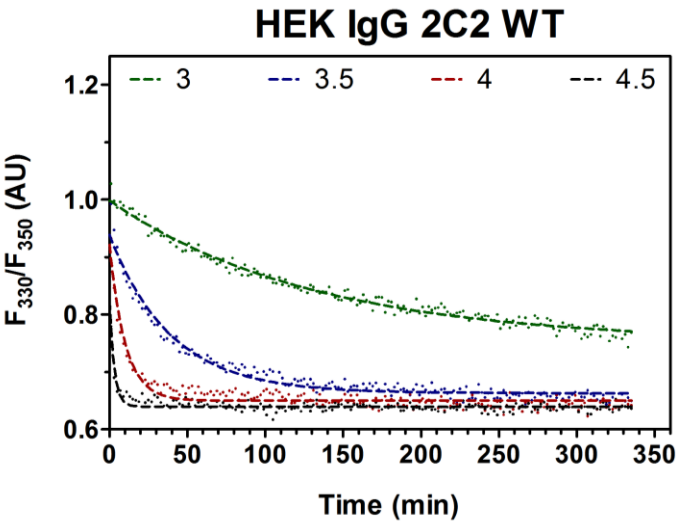
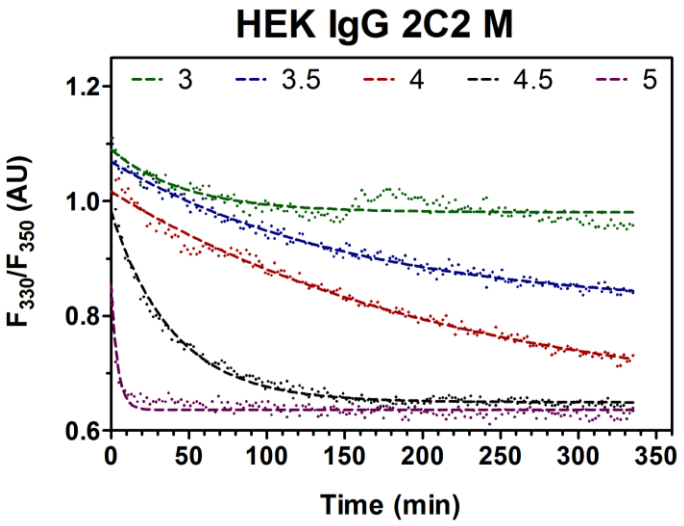
Real-time GdnHCl denaturation



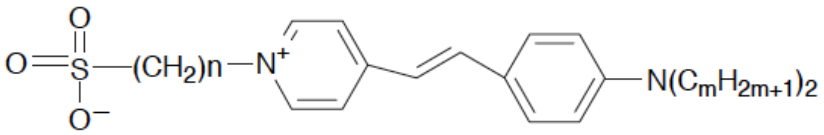




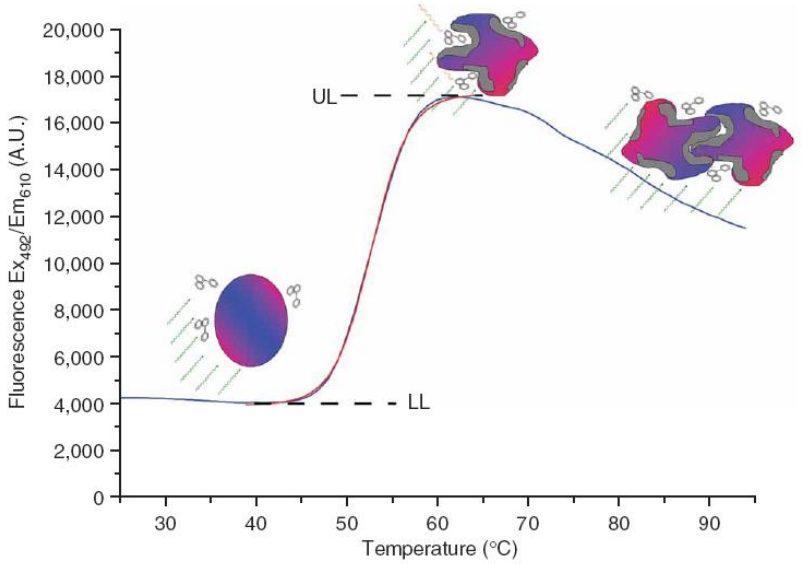


Differential Scanning Fluorimetry (DSF)

melting temperature detected by increased fluorescence of dye with **affinity for hydrophobic parts** of the protein

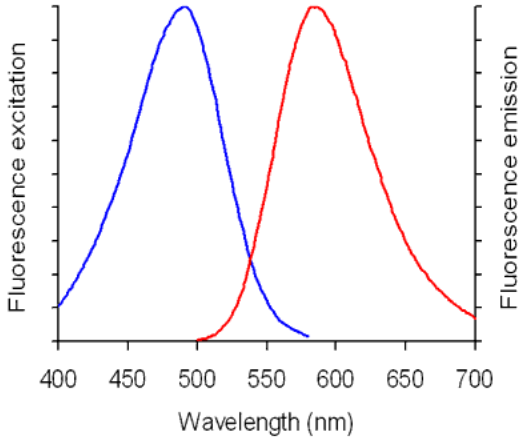


Sypro-Orange (Molecular Probes)



in aqueous solution: quenched fluorescence; highly fluorescent in non-polar environment

relatively **high excitation wavelength** decreases likelihood of small molecules interfering with optical properties of dye, causing quenching of fluorescence intensity



Method described in Niesen *et al.*, *Nature Protocols* (2007)

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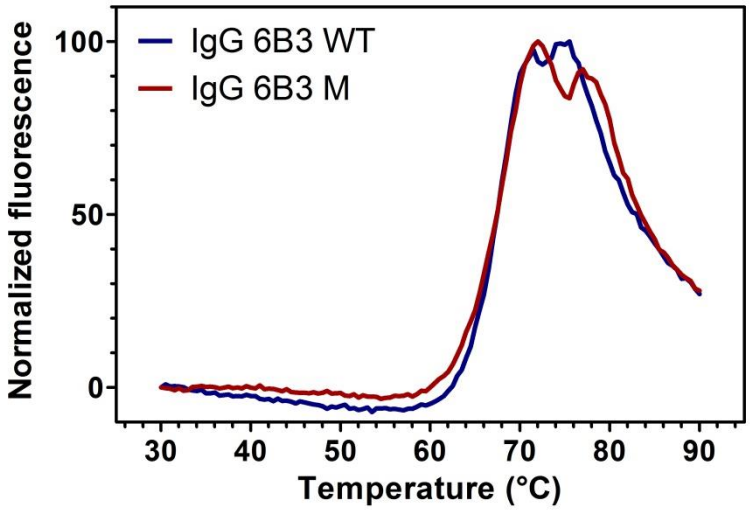
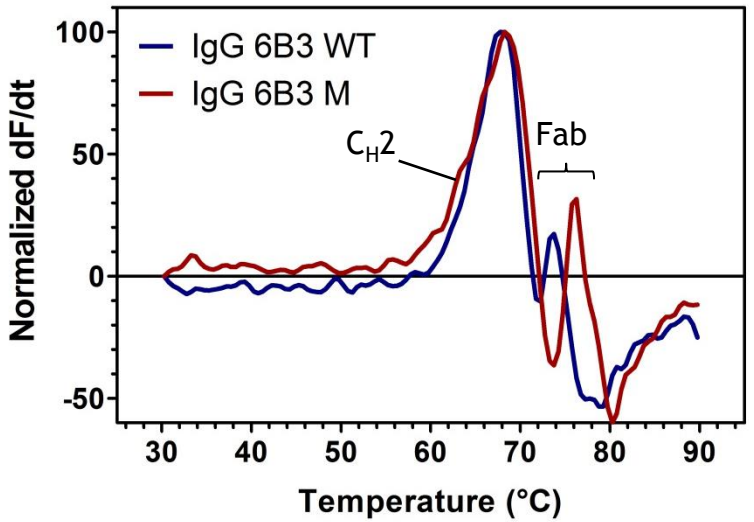
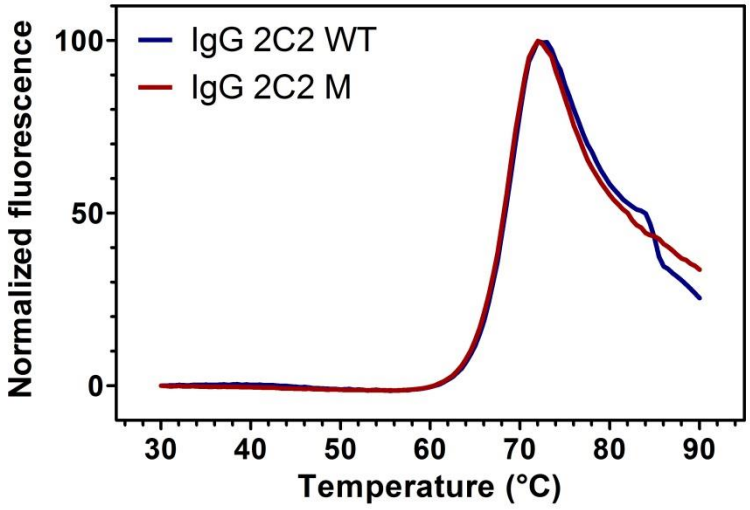
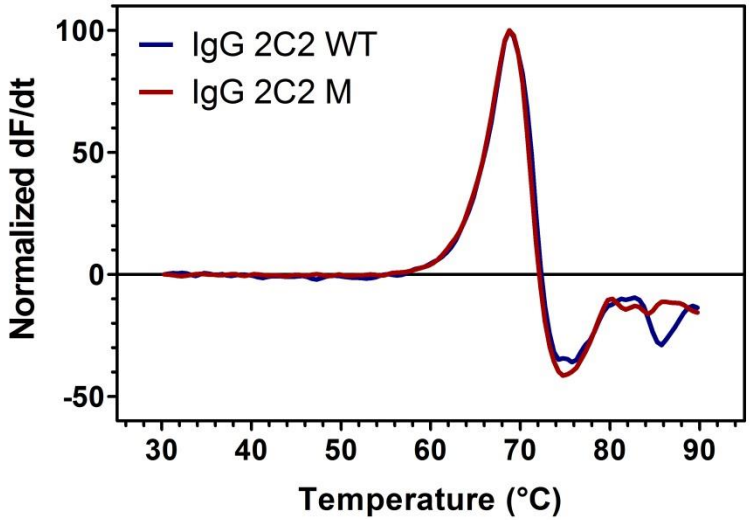
DSF: real examples





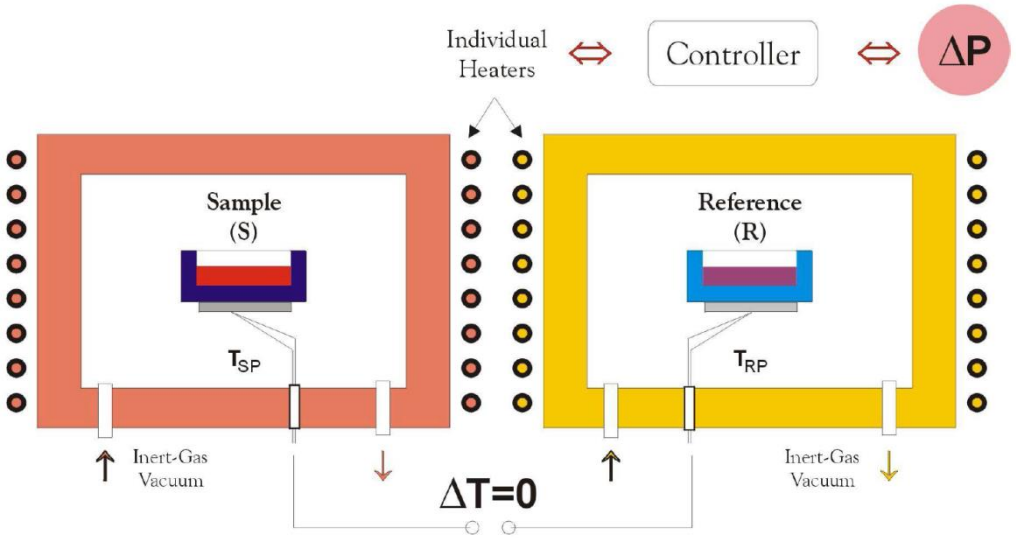




Schaefer and Plückthun, *Protein Eng. Sel. Des* (2012)

Differential Scanning Calorimetry (DSC)

Power-compensation DSC (not Heat-flux DSC)



continuously self-adjustment of heating power for keeping sample and reference at same temperature

difference of required **power [J/sec]** divided by the **scan rate [°C/sec]** leads to **heat capacity [J/°C]**

Integration of heat capacity *vs.* temperature yields the **enthalpy (ΔH)**

$$\Delta H = \int_{T1}^{T2} C_p \, dT$$

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

(Gibbs Free Energy equation)

Methods described in Ionescu *et al.*, *J. Pharm. Sci.* (2008)

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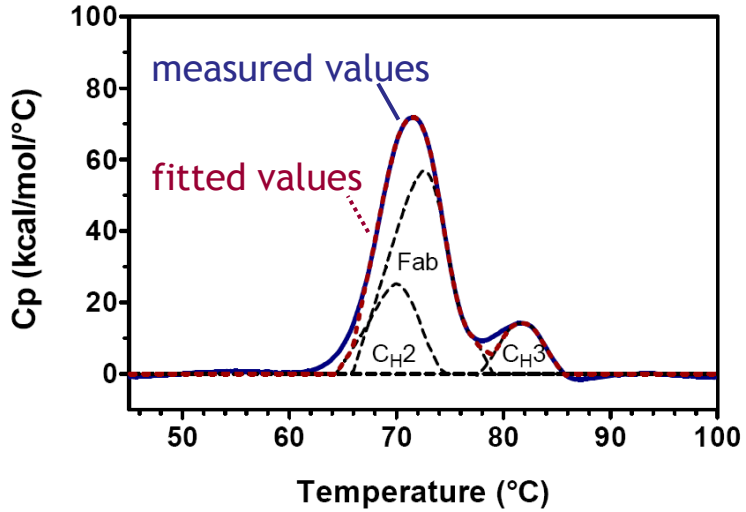
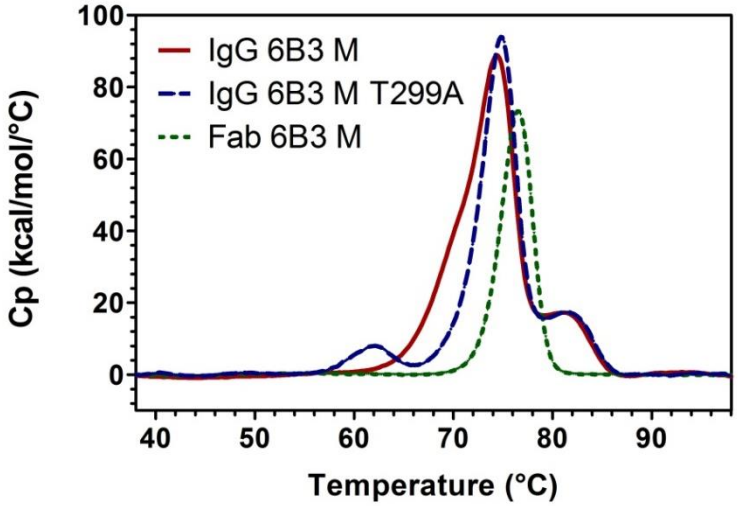
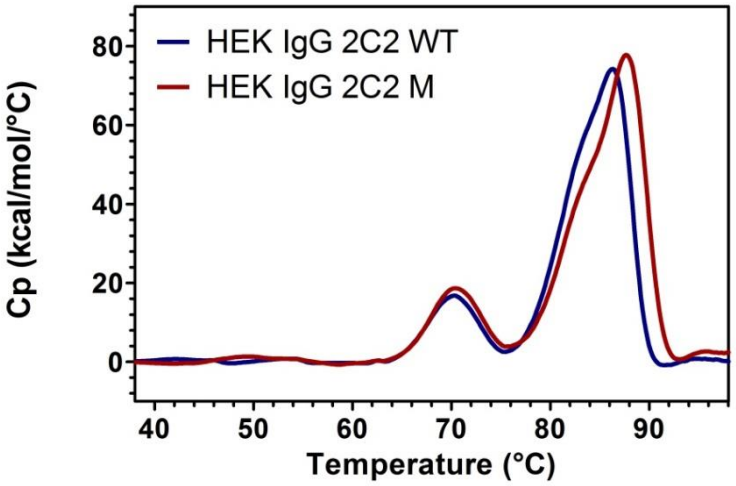
DSC: real examples







➡ DSC is only setup detecting small differences of very stable transition

Schaefer and Plückthun, *Protein Eng. Sel. Des* (2012)

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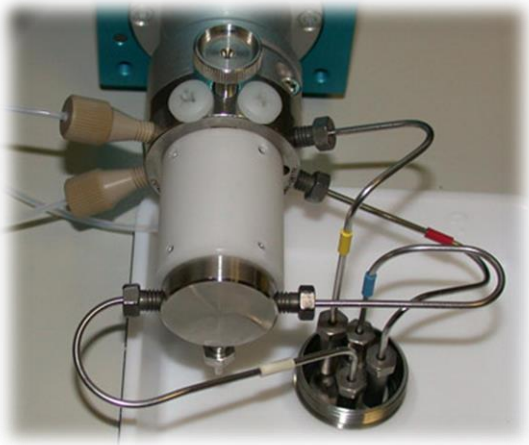
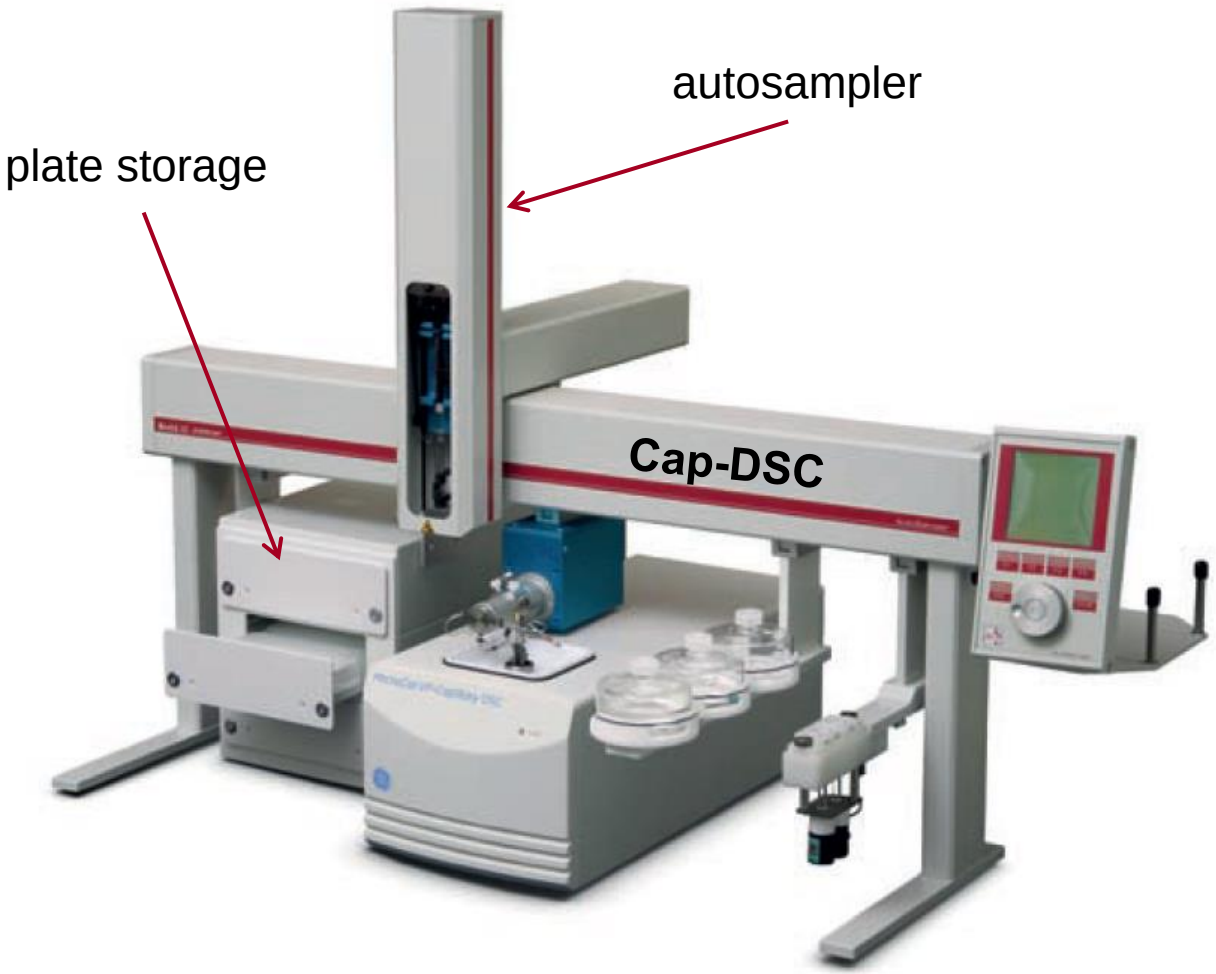
VP-DSC vs. VP-Capillary DSC





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VP-DSC vs. VP-Capillary DSC

	VP-DSC	VP-Capillary DSC
analyzed volume	510 µl	130 µl
sample volume	1´200 µl	400 µl
scan rates	0.5 - 1.5 °C/min	0.16 - 4°C/min
sample cell	coin shaped	capillary
samples	1	up to 288
measuring time	1 day	4 hrs
cleaning	manual	automatic

major advances: sensitivity, throughput, reproducibility, stability and ease of use (smaller sample requirements)

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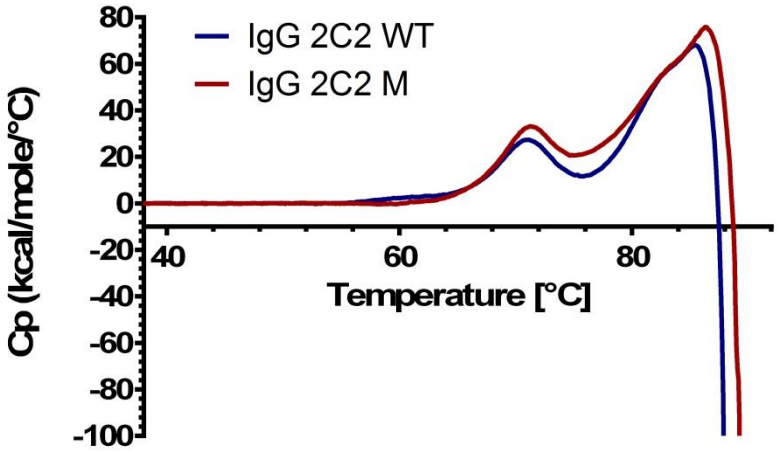
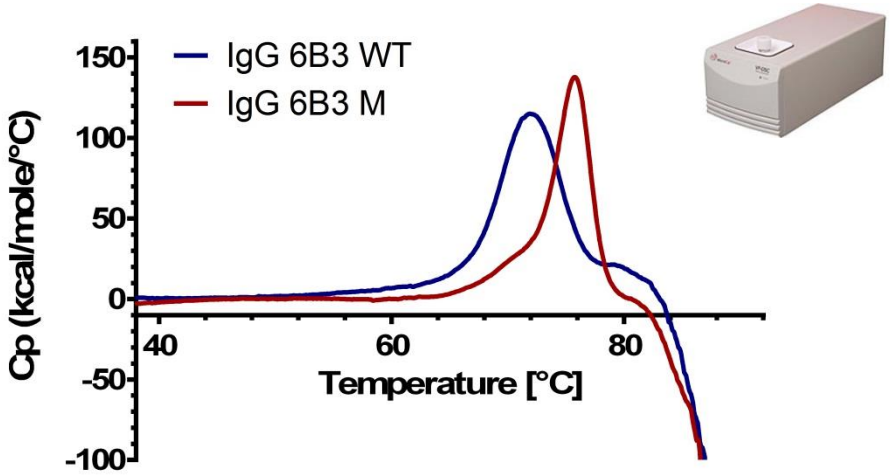
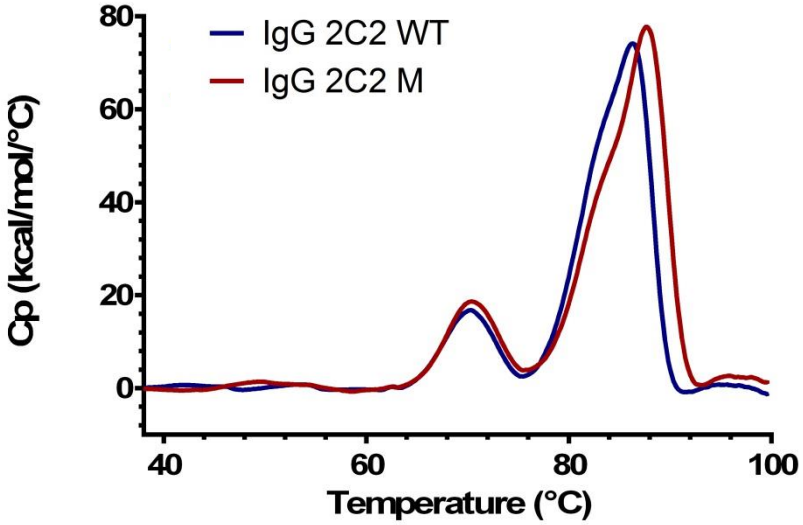
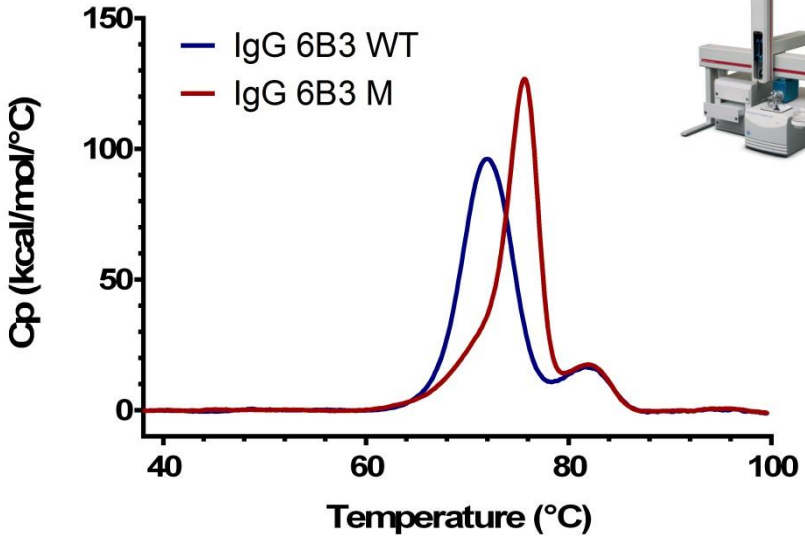
VP-DSC vs. VP-Capillary DSC









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Convection at aggregation

protein aggregation: heat signal detected by DSC is sum of both **endothermic unfolding** and **exothermic aggregation**

convection appears

once sample aggregates, interferance and baseline drop

molecules are located in small confined space

very little convection

due to small diameter of capillaries

molecules are separated with enough space (aggregation delayed)

➡ signals derived from Capillary-DSC are **less sensitive to aggregation**

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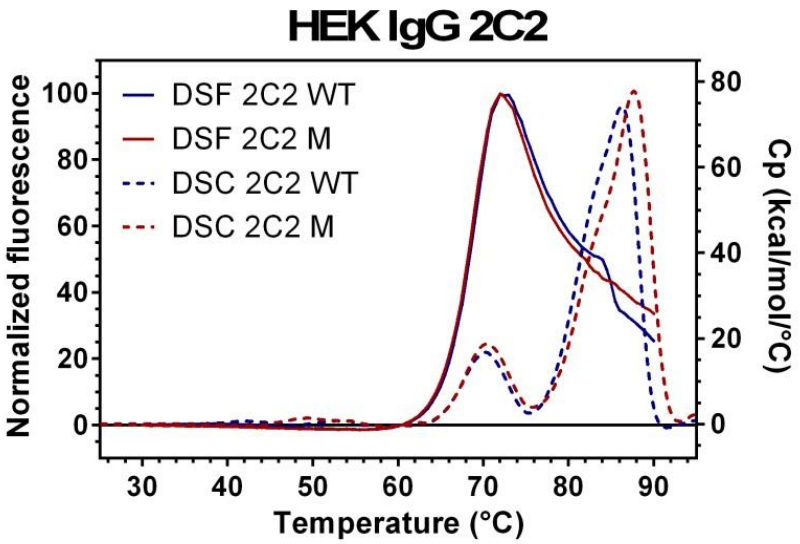
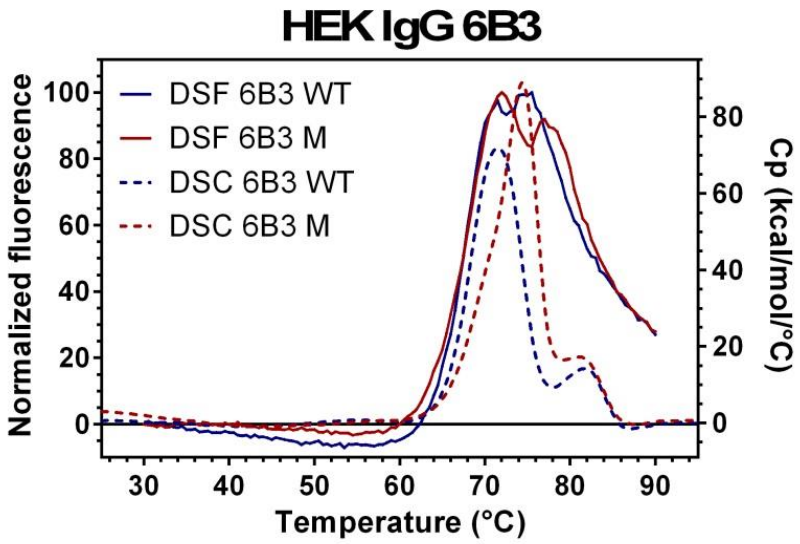
Comparison DSF vs. DSC





compared to DSC, DSF lacks "resolution" of individual domains, however is **much faster** (2-3 hrs vs. 48-72 hrs), can be **performed in parallel** and **requires much less protein** (20 µg vs. ~1 mg)

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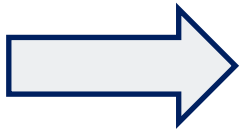
Stabilizing effects of V_H6 mutations

			ITF	GdnHCl	DSF	DSC
IgG 2C2	WT		70.4°C*	2.5 M	n.d.	86.0°C
	M		71.8°C*	3.8 M	n.d.	87.8°C
		Δ =	**1.4°C**	**1.3 M**	**-**	**1.8°C**
IgG 6B3	WT		67.6°C	2.0 M	74.5°C	72.1°C
	M		70.8°C	2.6 M	77.0°C	74.3°C
		Δ =	**3.2°C**	**0.6 M**	**2.5°C**	**2.2°C**
Fab 6B3	WT		69.7°C	2.0 M	76.5°C	72.6°C
	M		74.2°C	2.6 M	80.0°C	76.6°C
		Δ =	**4.5°C**	**0.6 M**	**3.5°C**	**4.0°C**

* – determined in presence of 1 M GdnHCl n.d. – not determined

Schaefer and Plückthun, *Protein Eng. Sel. Des* (2012)

IgG stability analyses



IgG expression systems

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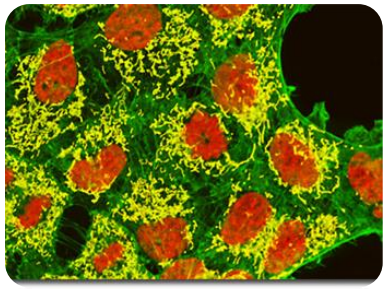
Eukaryotic expression systems

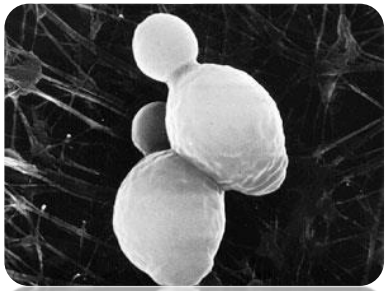
Mammalian cell culture



stable HEK293 (Flp-In)

CMV promoters (**constitutive**)

Yeast *Pichia pastoris*



stable SMD1163 (his4 pep4 prb1)

GAP promoters (**constitutive**)

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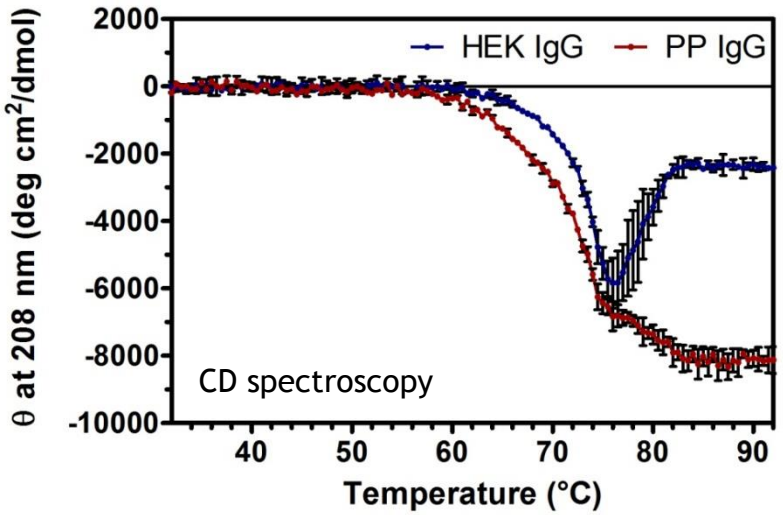
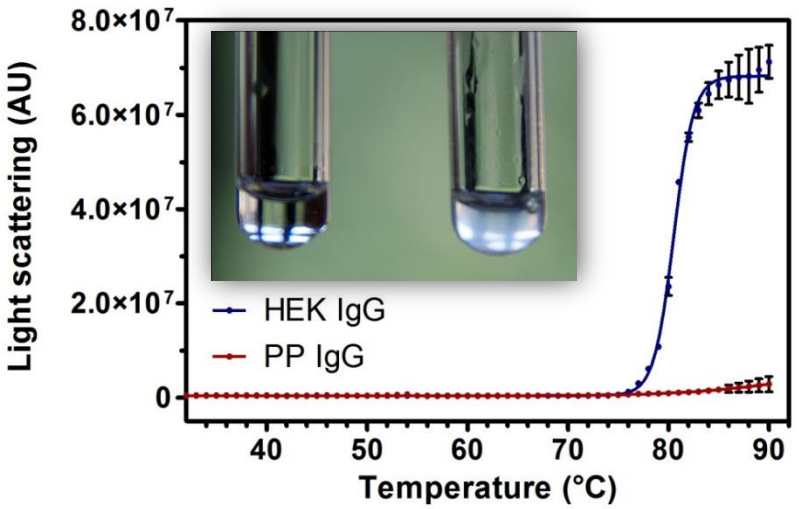
Difference in aggregation susceptibility





- ***Pichia*-derived glycans** reduce aggregation tendency
- **peptide remaining from yeast signal sequence** decreases aggregation susceptibility of HEK-IgG upon N-terminal addition

Schaefer and Plückthun, *J. Mol. Biol.* (2012)

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Expression system *Pichia pastoris*

Expression of full-length IgGs in **methylotrophic yeast *Pichia pastoris***

➡ **<u>advantages of expression system:</u>**

- disulfide bond formation / isomerization
- posttranslational modification (glycosylation)
- very high cell densities
- high expression levels (up to 30%)

➡ **<u>different promoters available:</u>**

- MeOH-inducible AOX1 (alcohol oxidase 1)
- constitutive GAP (glyceraldehyde-3-phosphate dehydrogenase)

➡ **only low-level secretion of endogenous proteins**, being advantageous for protein purification and downstream processing

➡ **> 50 reports describing antibody expression**
(mainly scFvs, several Fabs, only handful full-length IgG)

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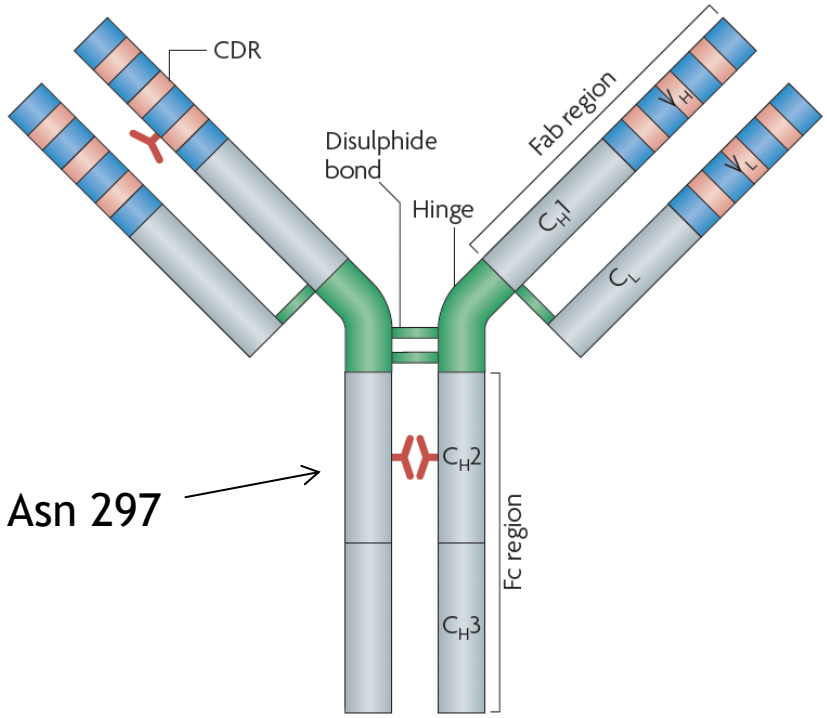
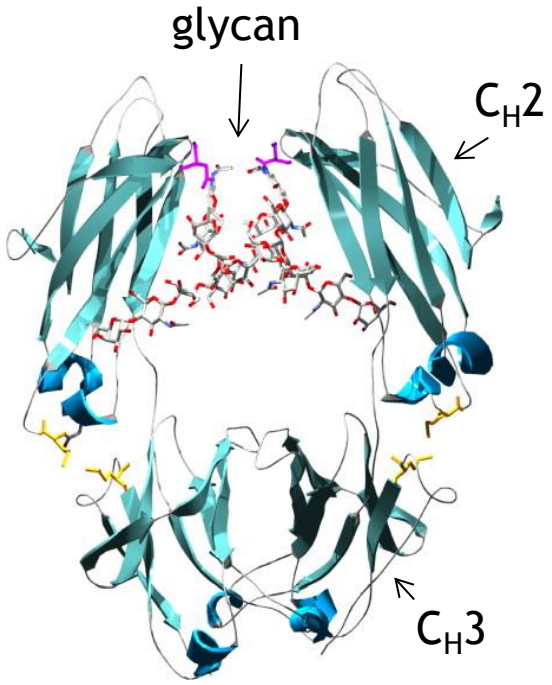
Difference in expression systems

mayor difference in expression systems: glycosylation

- yeast system processes same sugar precursor differently (in Golgi complex), resulting in a **different glycan**





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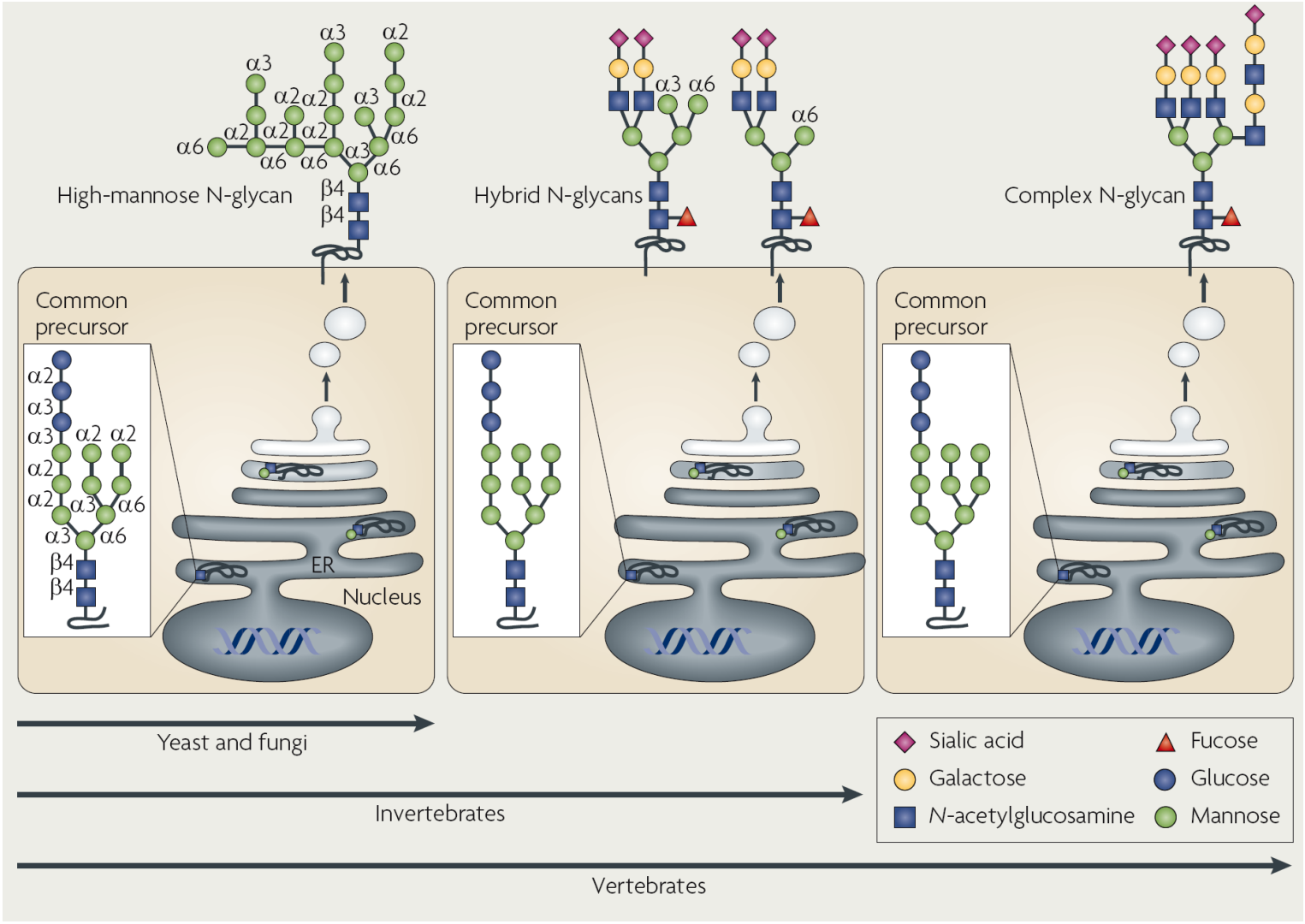
N-linked glycosylation



Marth and Grewal, *Nat Rev Immunol*. (2008)

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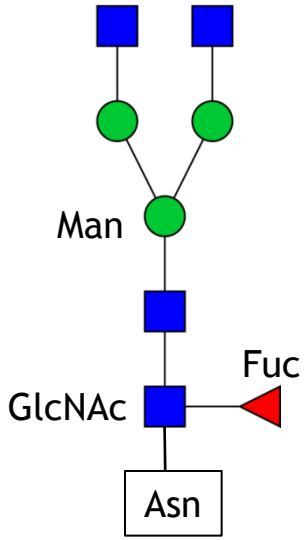
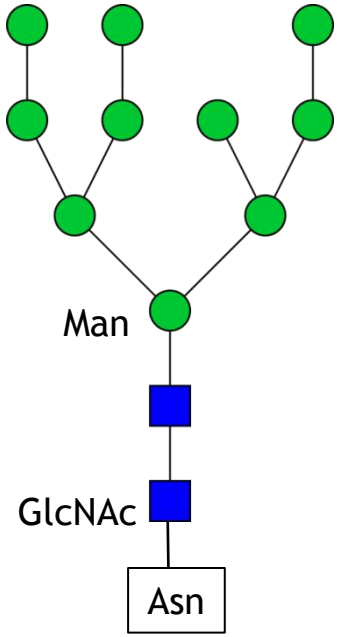
N-linked glycan processing

HEK293 cells

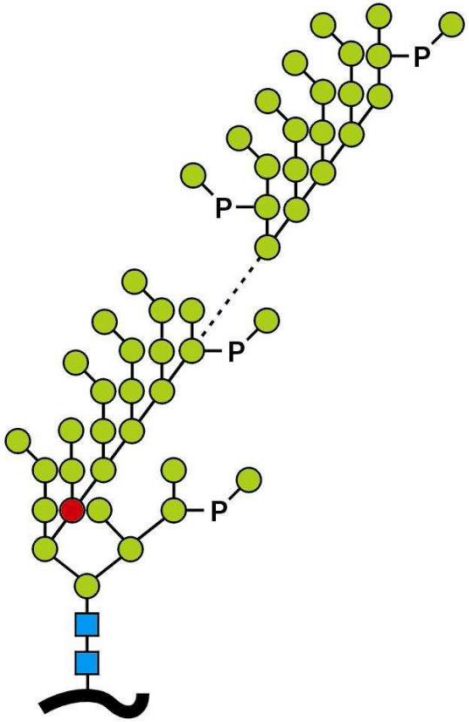


$Gal(GlcNAc)_2(Man)_3(GlcNAc)_2Fuc$

Pichia pastoris



$(Man)_{9\text{-}10\text{-}18}(GlcNAc)_2$



➡ *Pichia* glycan cause difficulties interacting with **Fcγ receptors (FcγR)** important for effector functions

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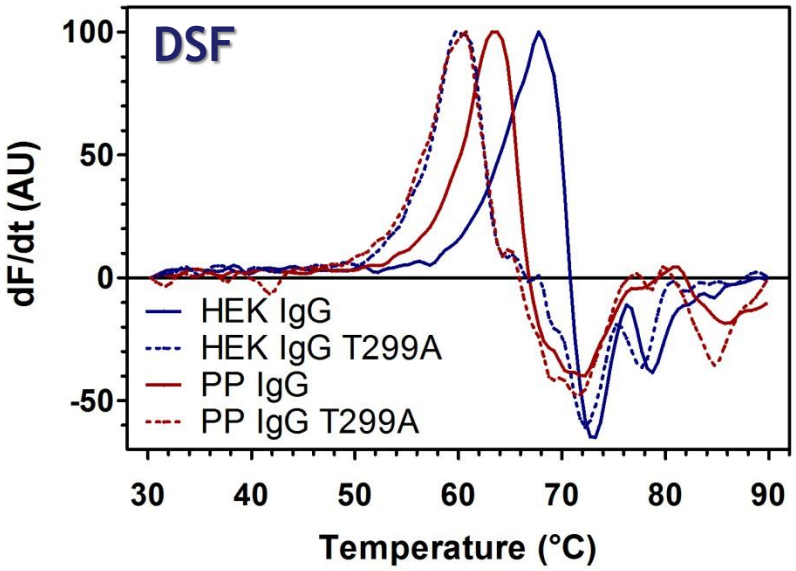
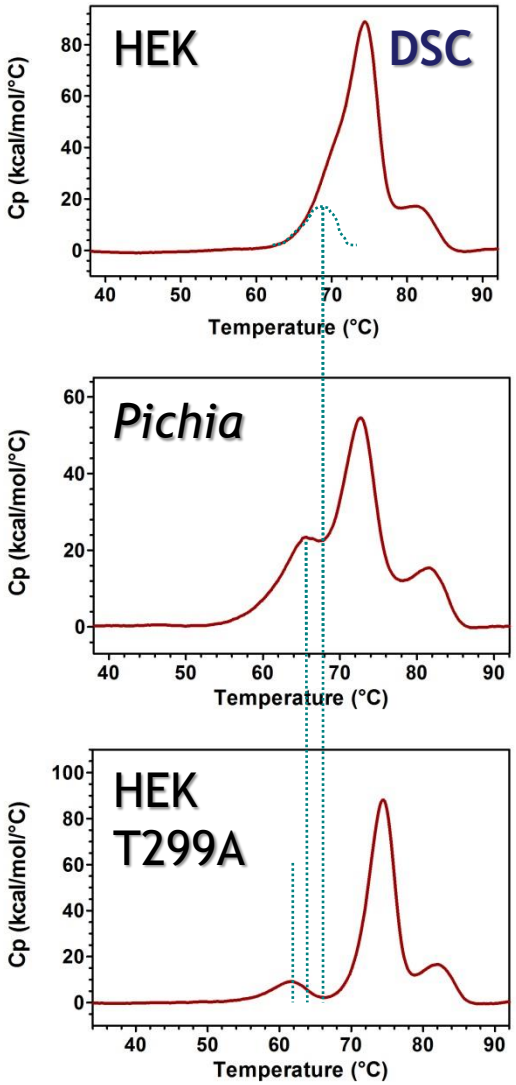
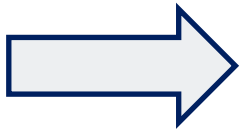
Influence of glycosylation on stability

- *Pichia* produced IgGs have decreased **C_H2 stability**, compared to mammalian expression
- different C_H2 stabilities are caused by **different glycan moieties**





IgG stability analyses



IgG homogeneity

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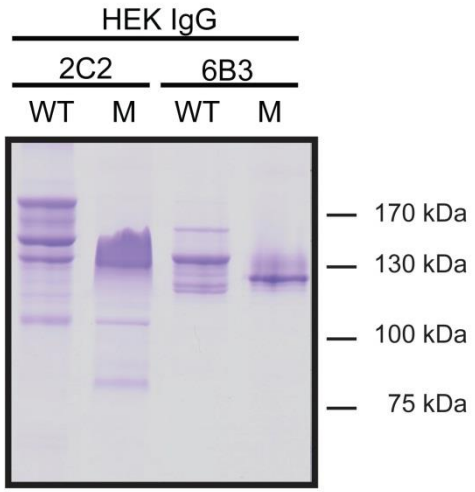
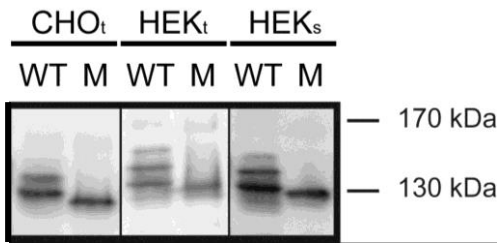
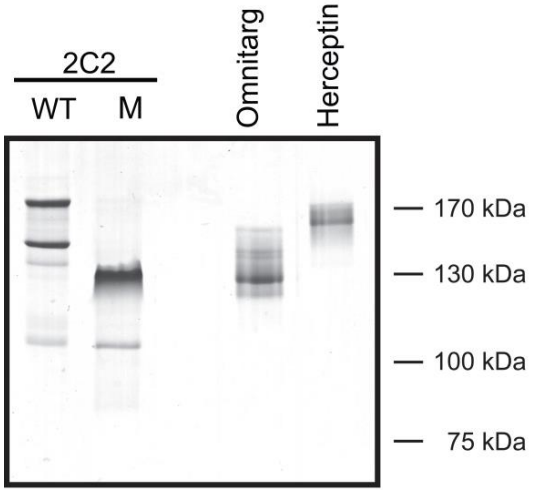
Electrophoretic analyses of IgGs



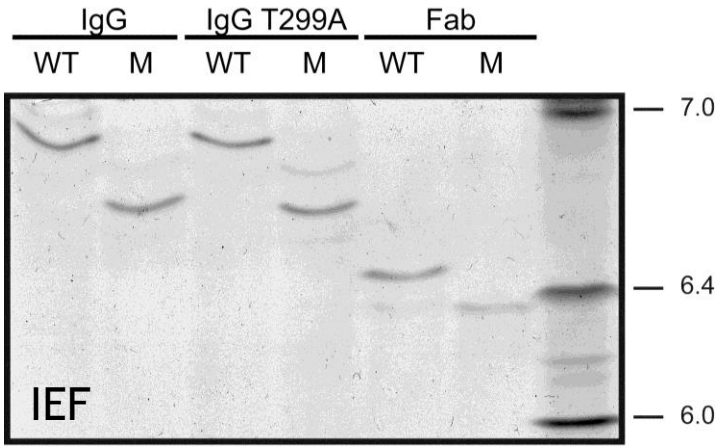




➡ non-reducing SDS-PAGE reveals **inhomogeneity of WT, but not of M variants**

➡ banding pattern is not caused by:

- **glycosylation**
- **proteolysis**
- **charge heterogeneity**



Schaefer and Plückthun, *Protein Eng. Sel. Des* (2012)

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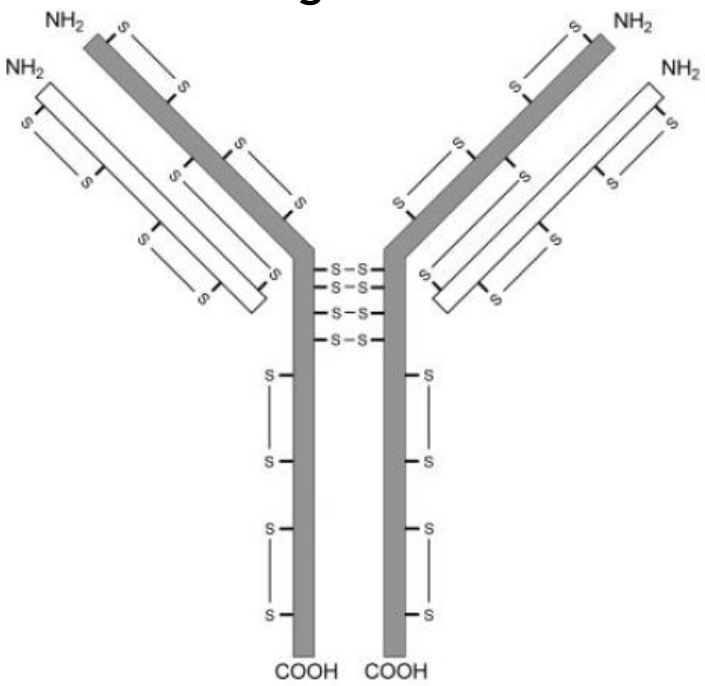
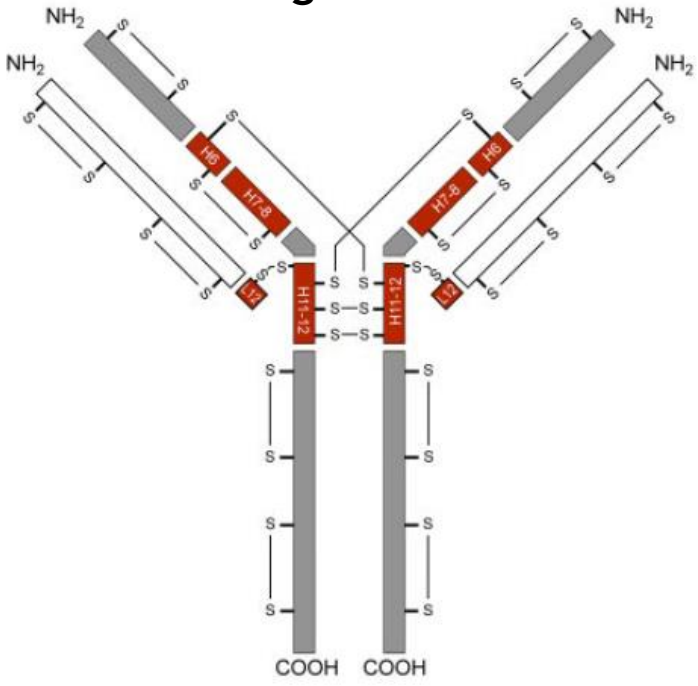
Disulfide bond scrambling in IgG2

IgG2-A



IgG2-B



➡ **Disulfide shuffling** as source of multiple bands?

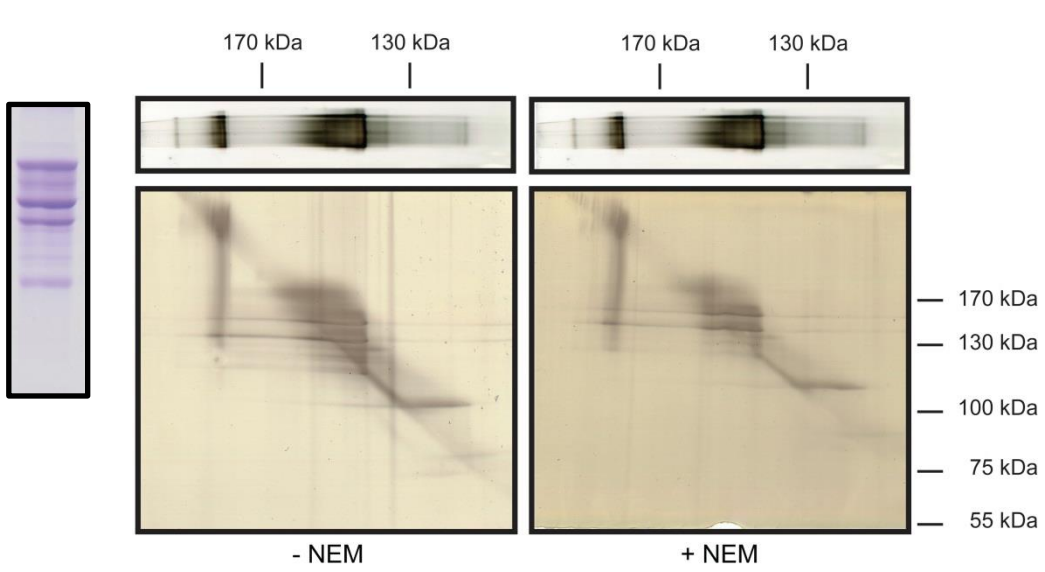
Wypych *et al.*, *J. Biol. Chem.* (2008)

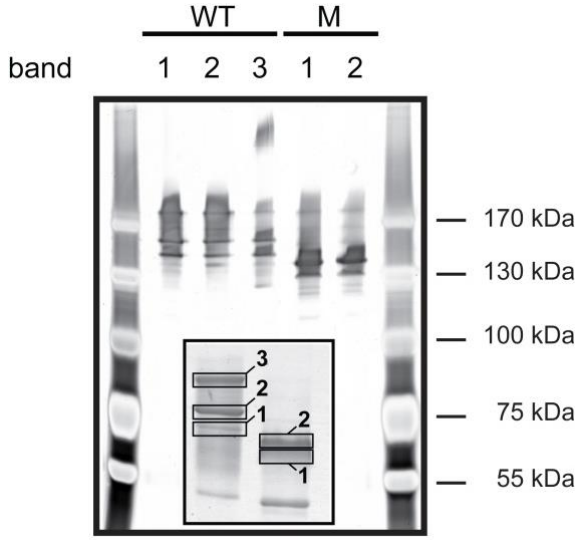
University of Zurich[UZH]

2D-electrophoresis

Analysis of IgG variants on "non-conventional" 2D-SDS-PAGE

HEK IgG 2C2 WT





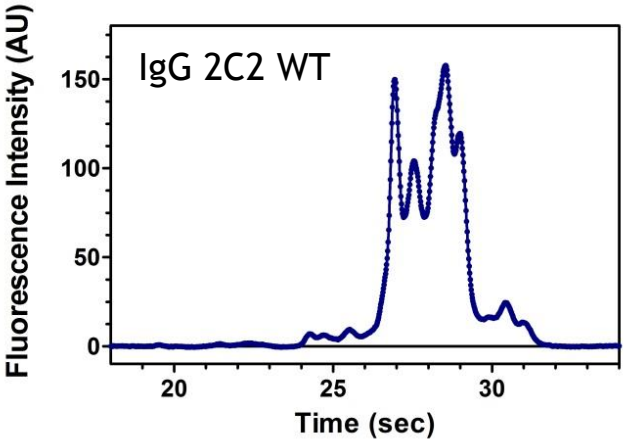
- distinct bands of 1st dimension resolve again in the 2nd dimension
- multiple bands are **not caused by disulfide heterogeneity / shuffling** (confirmed by MS analyses and determination of unpaired cysteines)

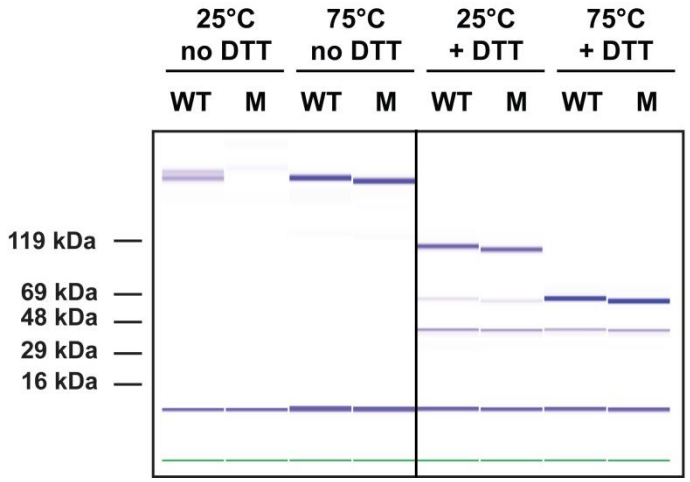
Schaefer and Plückthun, *Protein Eng. Sel. Des* (2012)

University of Zurich[UZH]

Stability probed by dye binding

Analysis by capillary electrophoresis (performed in microfluid chip)





➡ M variant seems **more densely packed** (less SDS-micelles can bind)

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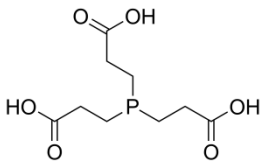
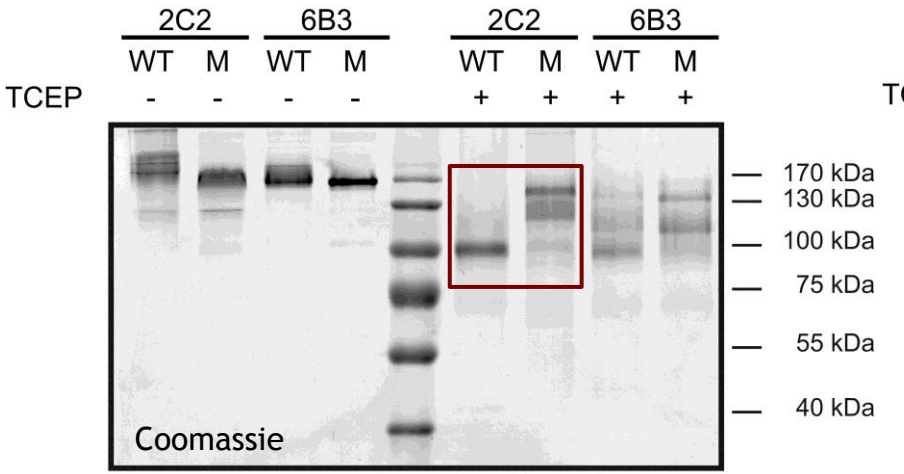
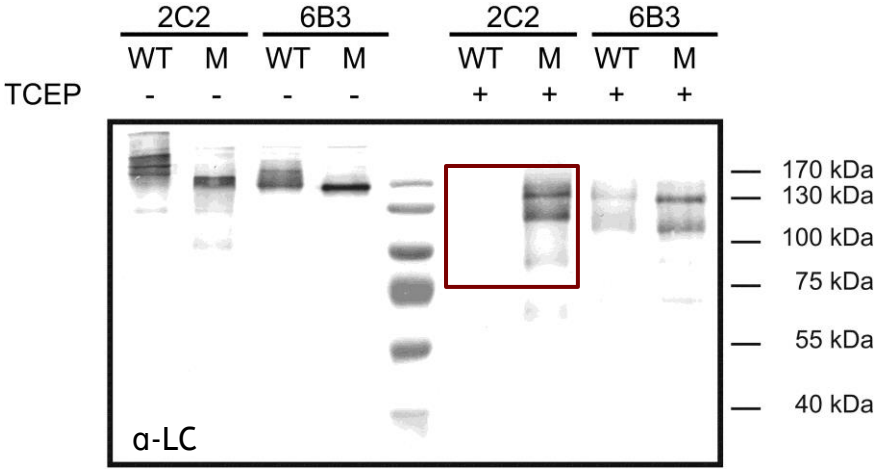
Stability probed by partial reduction

Partial reduction of IgG by hydrophilic TCEP

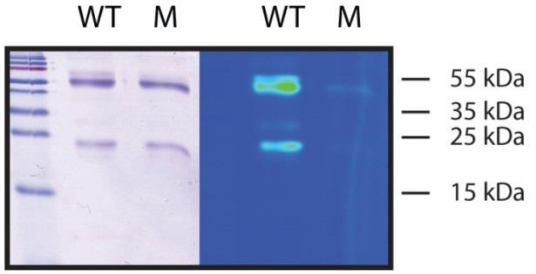






➡ TCEP treatment reduces **inter-molecular disulfide bond** only in WT IgGs

➡ labeling of free Cys with fluorescent 5-IAF confirms improved structural integrity / compactness

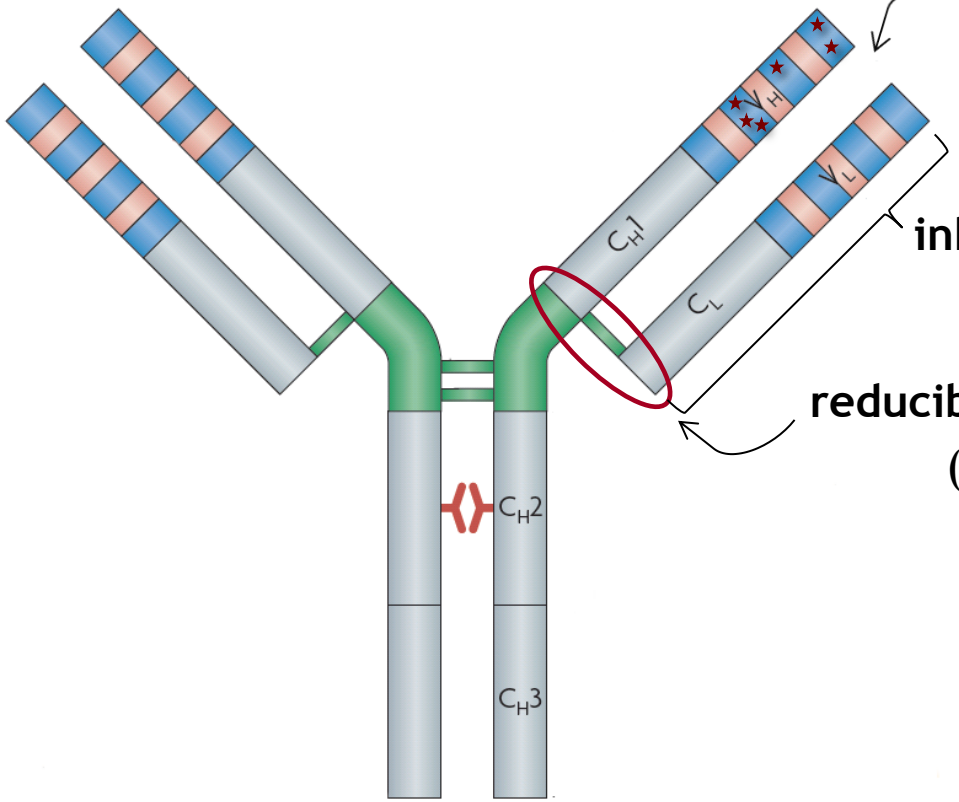


Schaefer and Plückthun, *Protein Eng. Sel. Des* (2012)

University of Zurich[UZH]

Conclusions

mutations affect **structural integrity and homogeneity**



packing of V_H-V_L interface
(dye accessibility)

inhomogeneous banding pattern
(in equilibrium)

reducibility of disulfide bond
(TCEP reduction)

Schaefer and Plückthun, *Protein Eng. Sel. Des* (2012)

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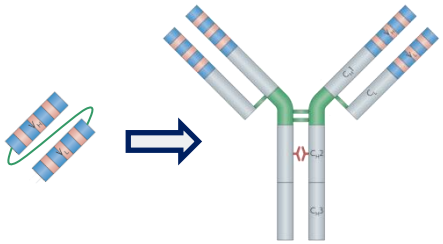
Conclusions

- variable domain mutations: effects on **expression level**
  - strong influence in *E. coli*
  - moderate influence in *Pichia pastoris*
  - no influence in HEK293

- mutations influence the biophysical properties of the IgG: **thermal and denaturant-induced unfolding**

- increased stability independent of the expression system used

- **transferability of improvements implemented in smaller fragments onto full-length IgG**



Schaefer and Plückthun, *Protein Eng. Sel. Des* (2012)

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Acknowledgements

Dept. of Biochemistry, UZH

Andreas Plückthun

Birgit Dreier

Annemarie Honegger

Peter Lindner

all present and former lab members

Academic partners

Ilian Jelezarov (UZH)

Paolo Cinelli (UZH)

Functional Genomics Center (UZH)

Shaikh Rafeek (ZHAW)

Manfred Heller (University of Bern)

Yuguang Zhao (Welcome Trust, Oxford)

Margaret Jones (Welcome Trust, Oxford)

general / financial support

FNSNF

FCI FONDS DER CHEMISCHEN INDUSTRIE

SSB Swiss Society for Biochemistry

p3

Industrial parters

Peter Gimeson (GE Healthcare)

Daniel Weinfurtner (MorphoSys)

Thomas Müller-Späth (ChromaCon)

Stefan Duhr (NanoTemper)

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Questions & Answers

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