TROUBLESHOOTING AND ENGINEERING OF ANTIBODY CONSTRUCTS – PART II
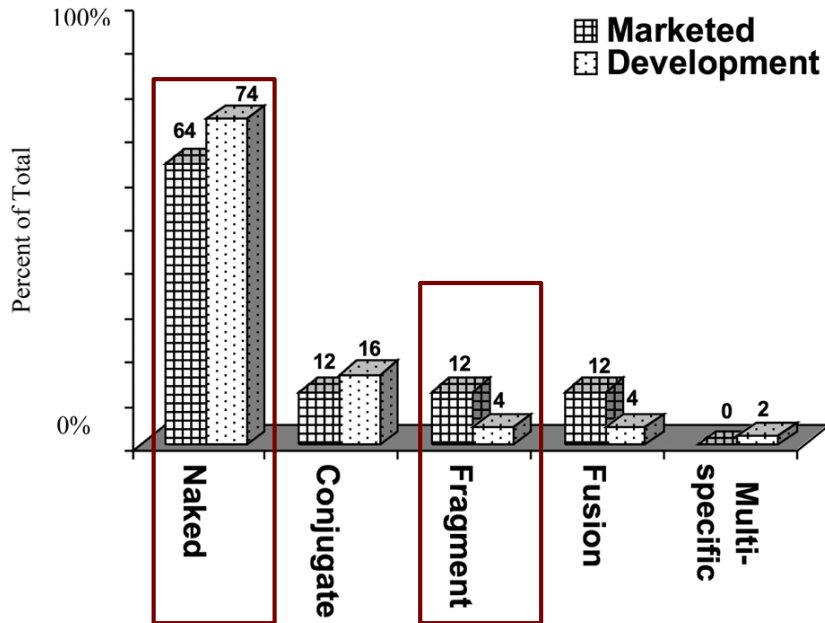
Jonas V. Schaefer, PhD

Head of High-Throughput Laboratory
Department of Biochemistry, University of Zurich

www.bioc.uzh.ch/plueckthun

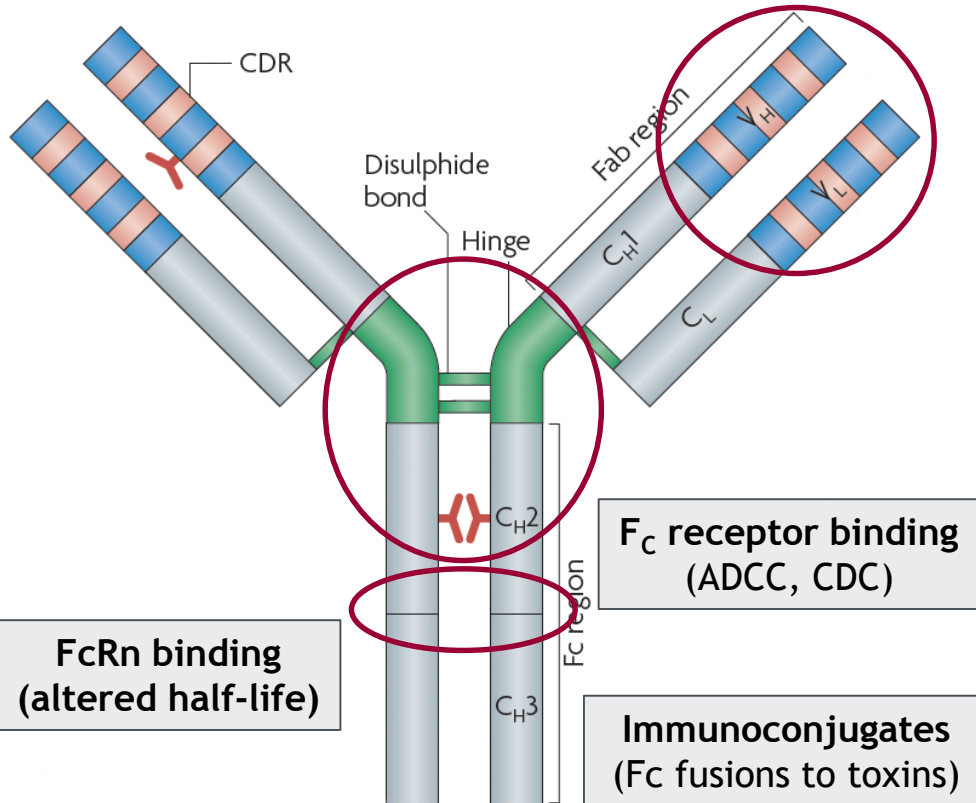# Antibody therapeutics vs. engineering

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)

Full-length IgG engineering

Optimization antigen binding
(affinity, humanization, decreased elimination)

CDR

Disulphide bond

Hinge

V_H

V_L

Fab region

C_H1

C_L

C_H2

Fc region

C_H3

F_C receptor binding
(ADCC, CDC)

FcRn binding
(altered half-life)
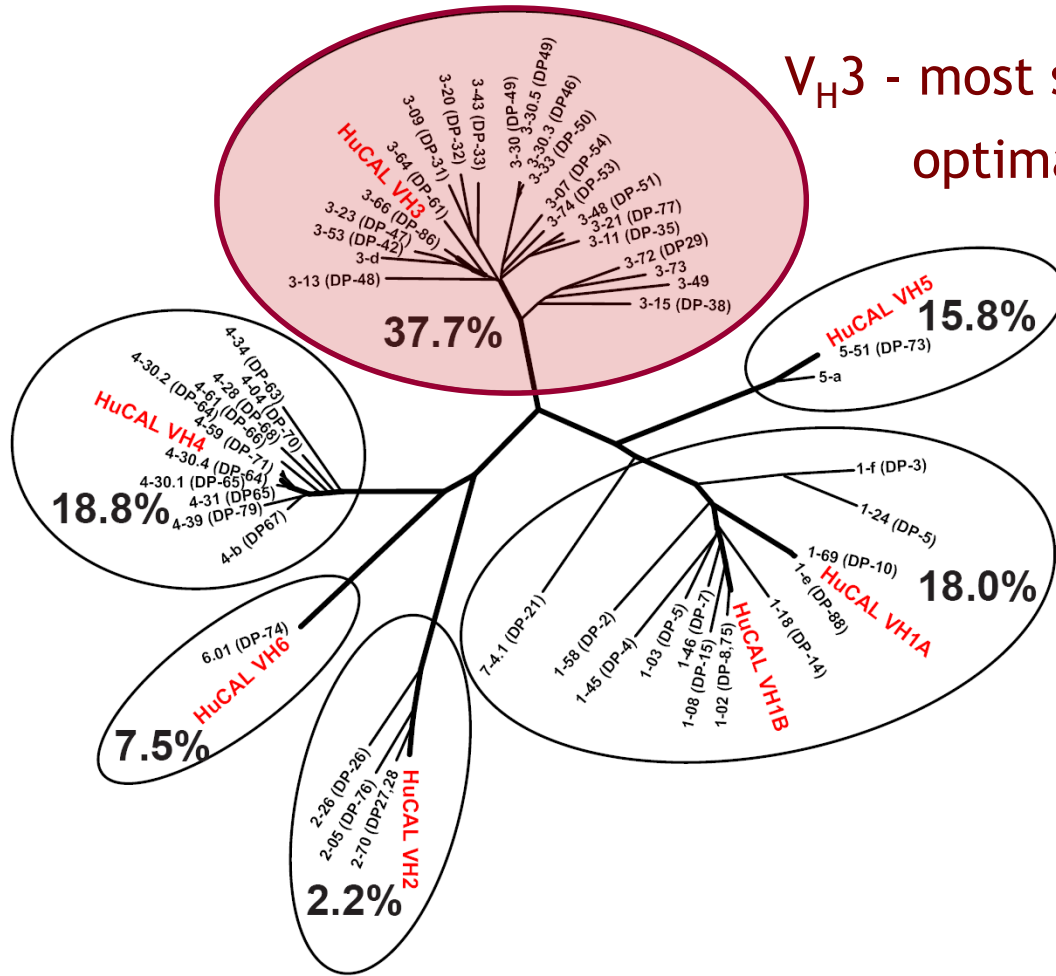
Immunoconjugates
(Fc fusions to toxins)

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

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

Why not just one "perfect" framework?

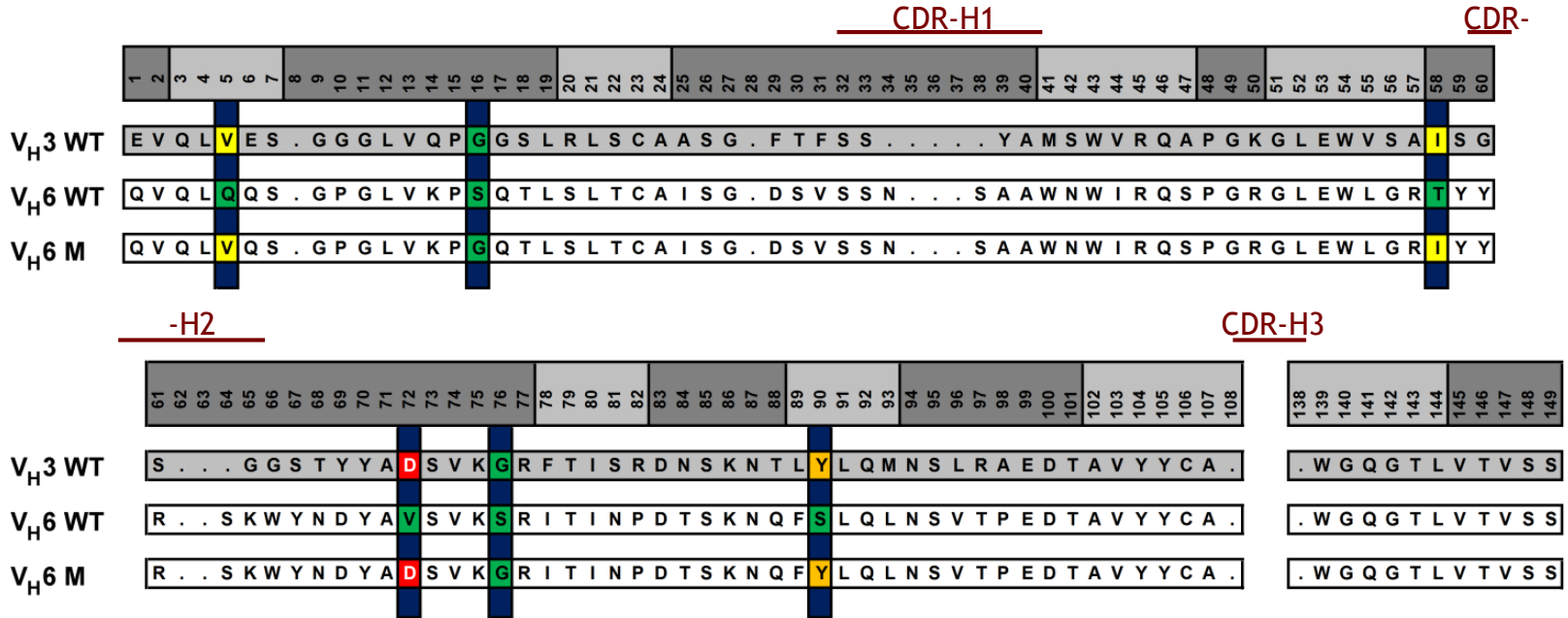**seven V$_H$ germline families** with different biophysical properties

V$_H$3 - most stable V$_H$ domain, optimal packing

Engineering of unstable V$_H$6 domain

comparison of the human consensus V$_H$ domains (germinal)
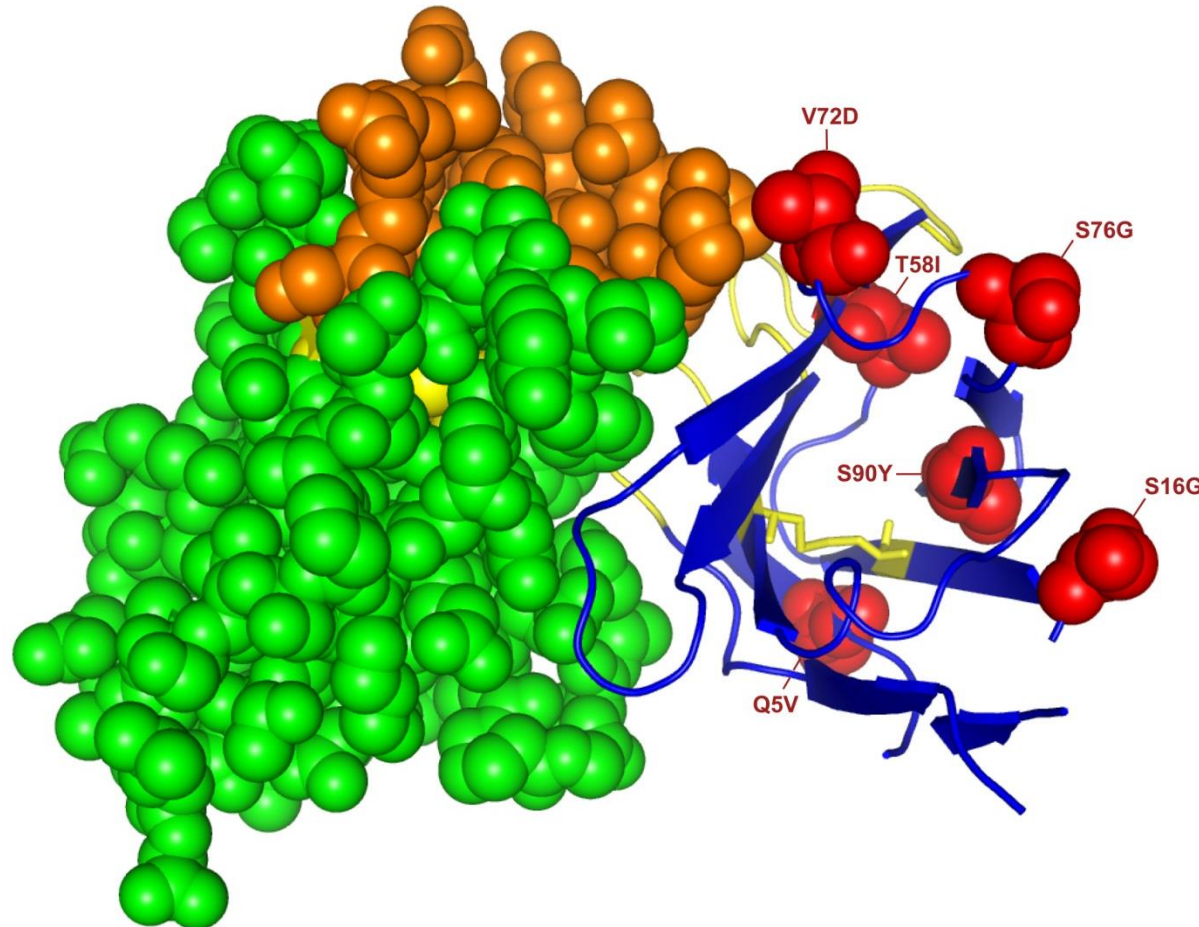

V$_H$3 - most stable V$_H$ domain, optimal packing

V$_H$6 - lowest midpoint of denaturation

Engineering of unstable V$_H$6 domain

exposed hydrophobic
Val 72 Asp

unsatisfied H-bond
Thr 58 Ile
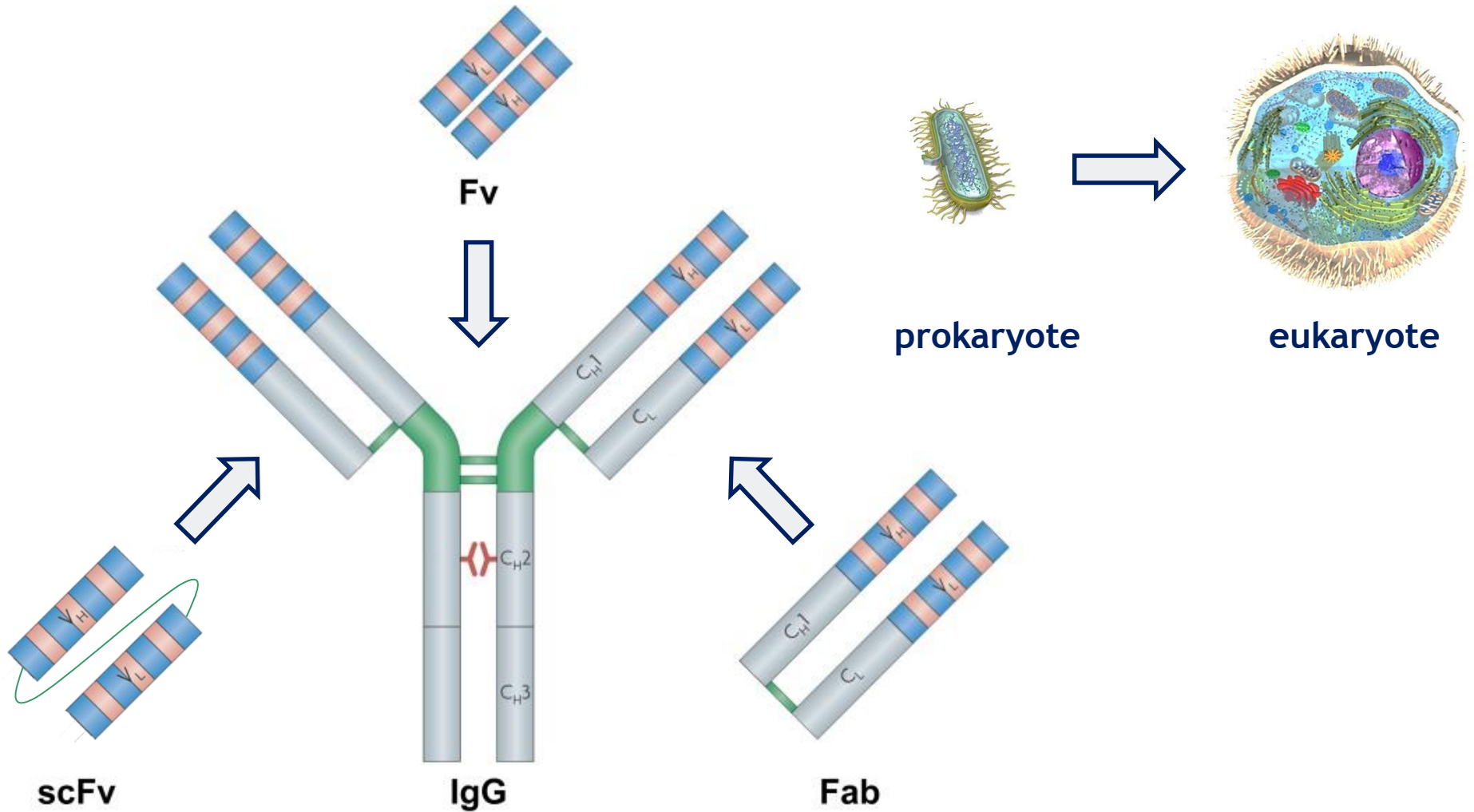
positive φ angle
Ser 76 Gly

β-propensity
Ser 90 Tyr

positive φ angle
Ser 16 Gly

β-propensity
Gln 5 Val

➡ mutations either influence **stability** (T58I), **folding yield** (V72D and S90Y) or **both** (Q5V, S16G and S76G)
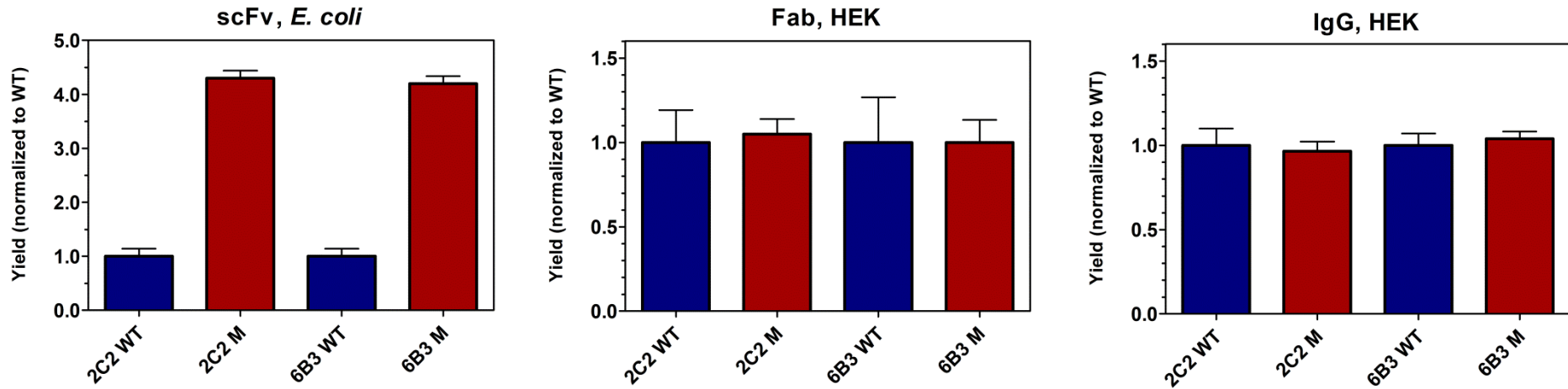
Fv

scFv

IgG

Fab

prokaryote

eukaryote

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)

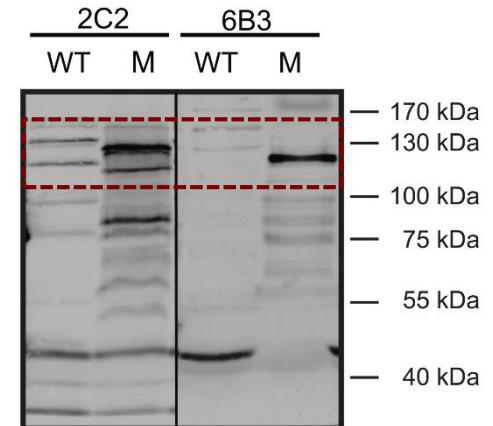- pI: 6.9 (6B3) vs. 8.7 (2C2)

- antigen: protein vs. peptide

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→ 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

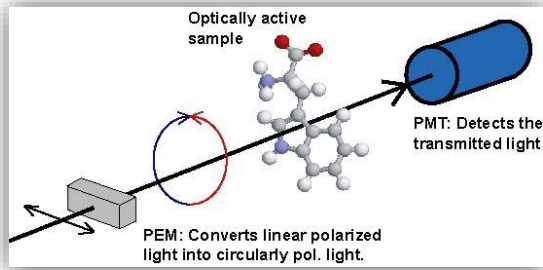➡️ IgGs consist of **six individual domains (each in duplicates)**, all having similar folds
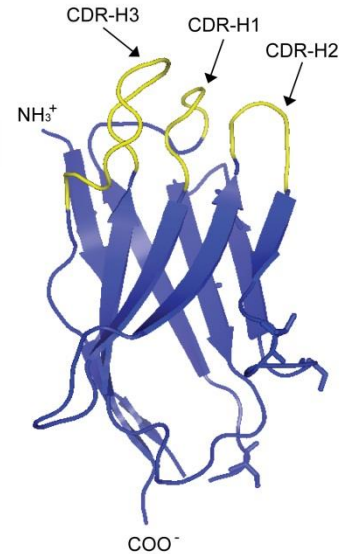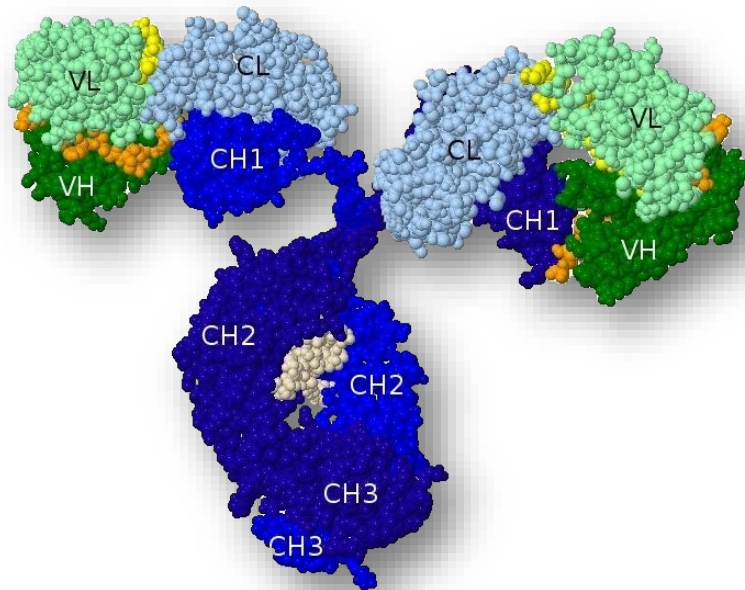
➡️ with most experimental setups, only **overall average** of biophysical features will be analyzed
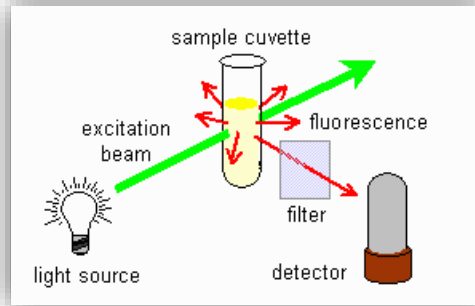
Biophysical analyses (methodology)

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Circular Dichroism (CD)

(2[ry] structure composition)

thermal denaturation

(aggregation analysis)

Intrinsic Tryptophan Fluorescence (ITF)

thermal denaturation

chemical denaturation

Differential scanning calorimetry / fluorimetry
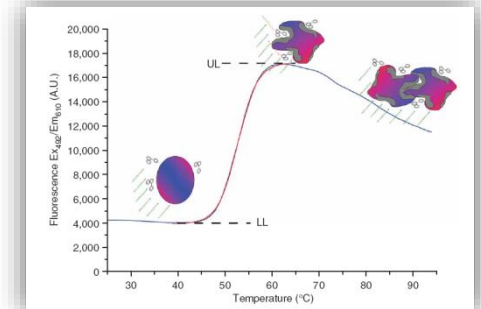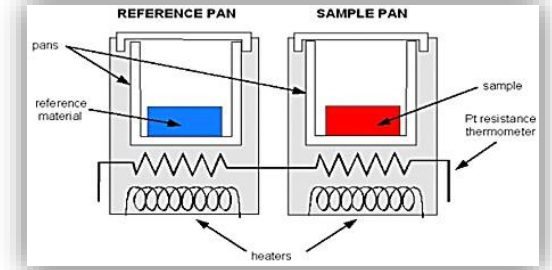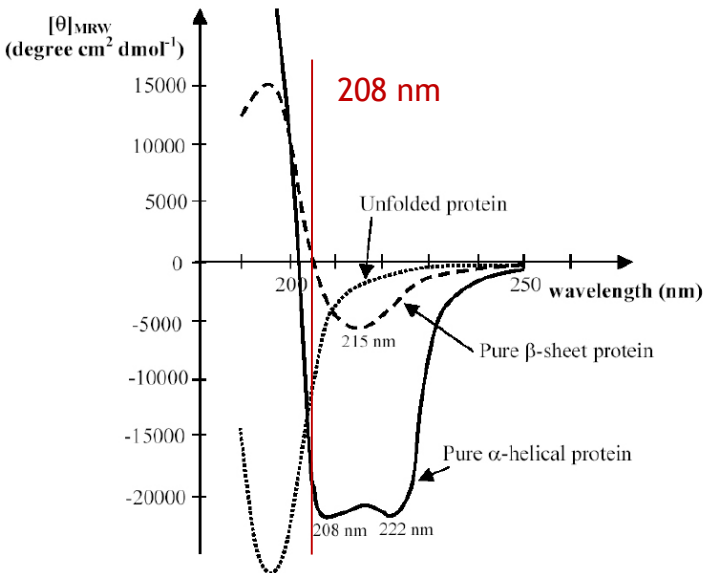

analysis of individual domains

Circular Dichroism (CD)

University of Zurich UZH

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: $\theta = \dfrac{2.303\,(A_L - A_R)}{4l}$ MRE: $[\theta] = \dfrac{\theta \times 100 \times M}{C \times l \times n}$
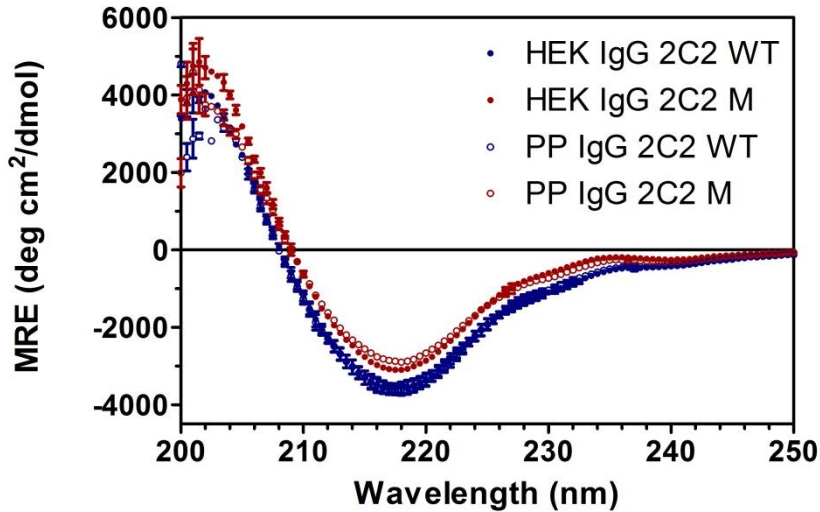
amide chromophore of peptide bond has 2 electronic transitions of low energy:
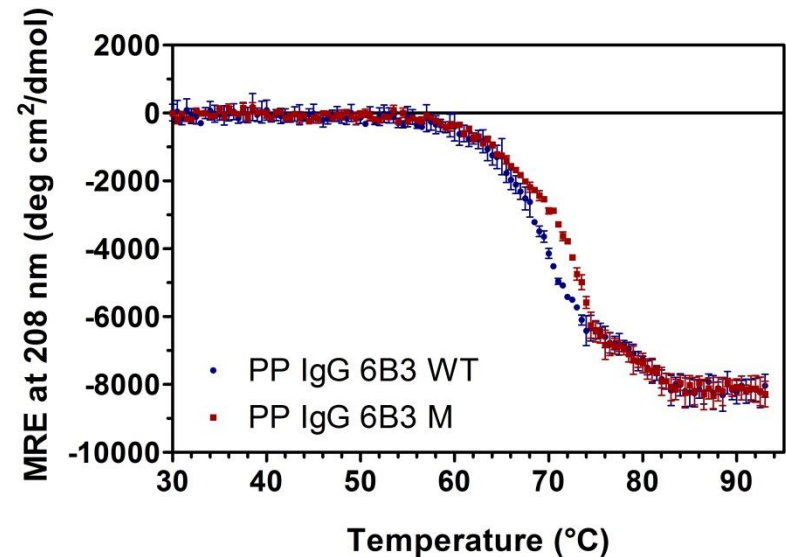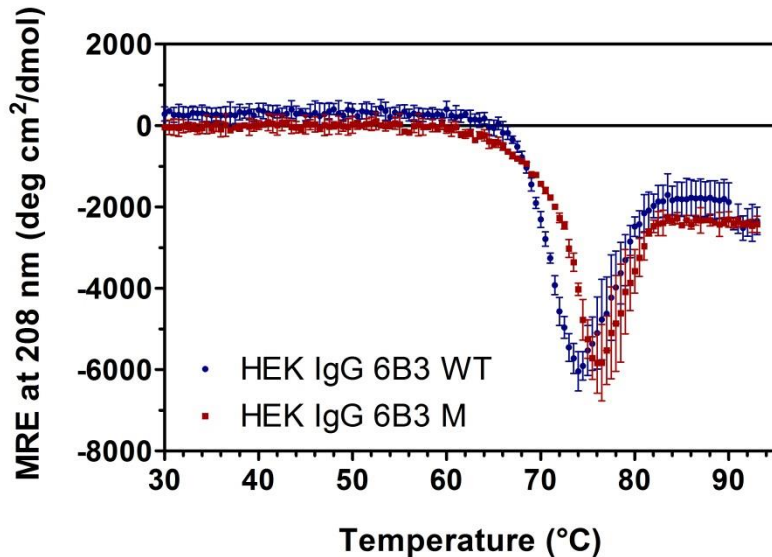**n → π*** (signals at 222 nm and 215 nm) and **π →π*** (signals at 208 nm and 198 nm)

➡ at ~ **208 nm** intensity due to β-sheets is essentially zero

➡ measuring ellipticity at 208 nm monitors **changes in structure** (negative shift caused by random coil formation)

CD: real examples

➡ unfolding detectable, however sheaded by aggregation

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

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**

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)

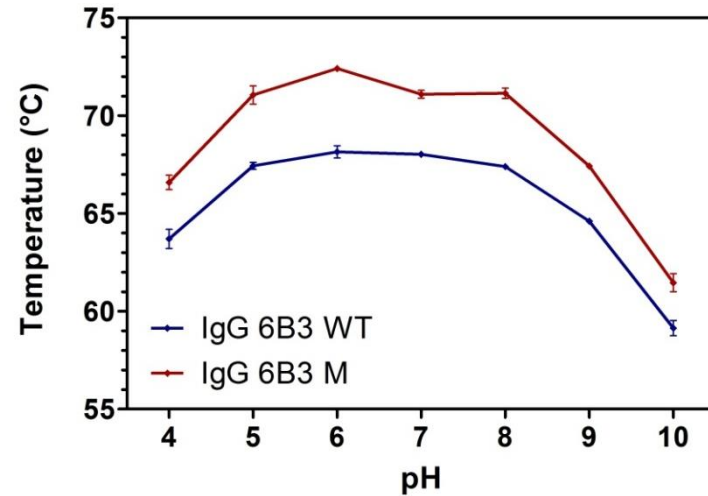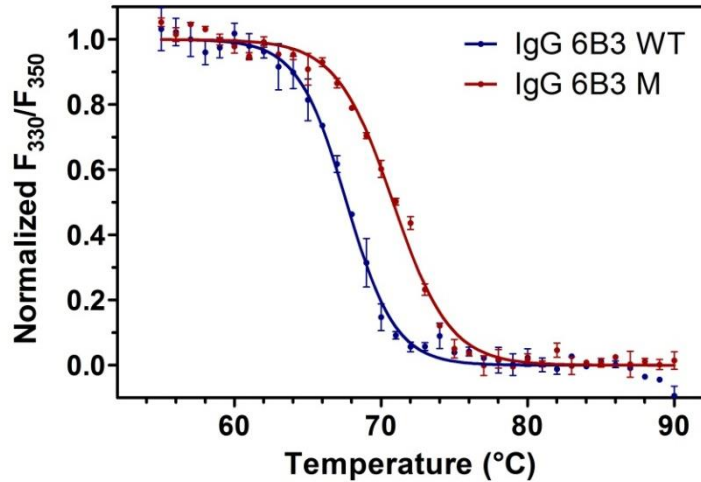red shift can be monitored by **ratio of intensities at 330 and 350 nm**
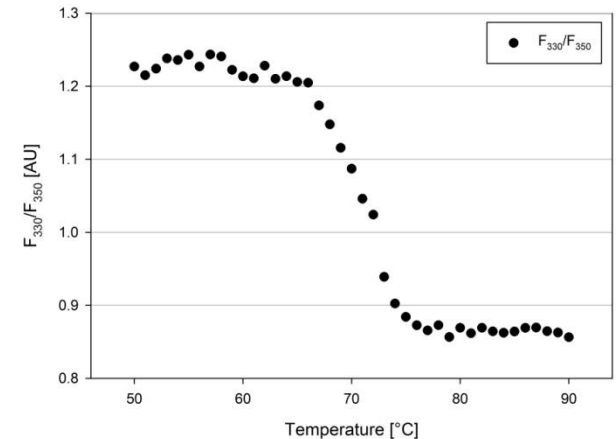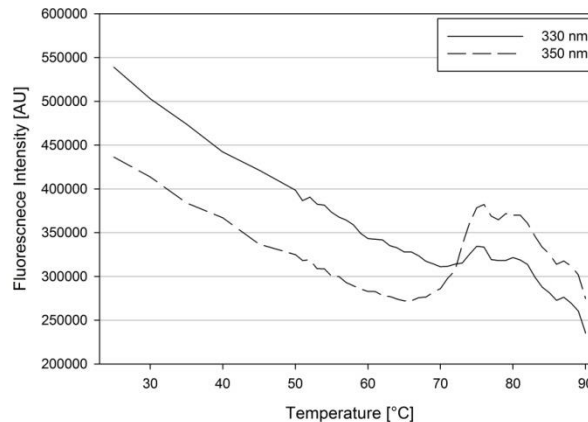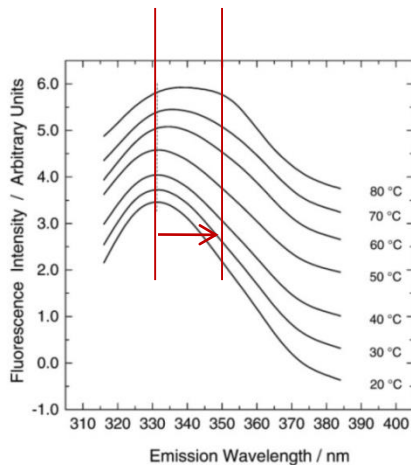
➡ **benefit over other methods:** • aggregation doesn't cover unfolding reaction
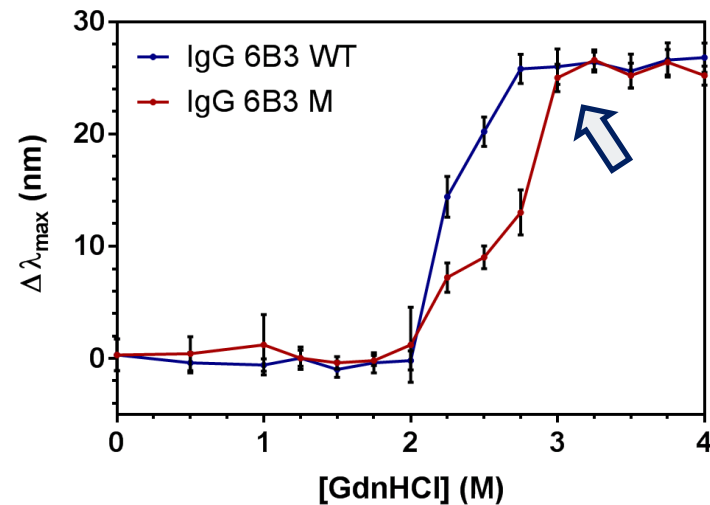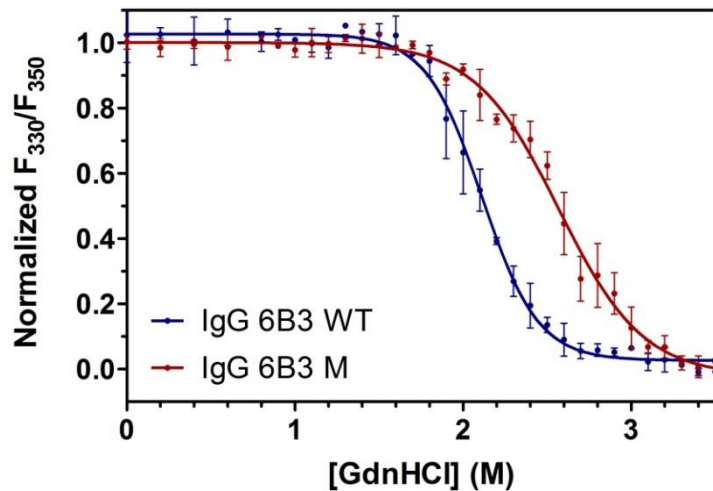• can easily be performed in 96well format
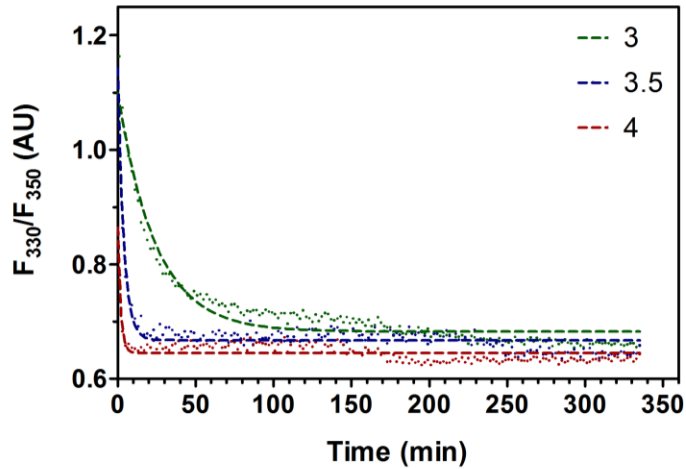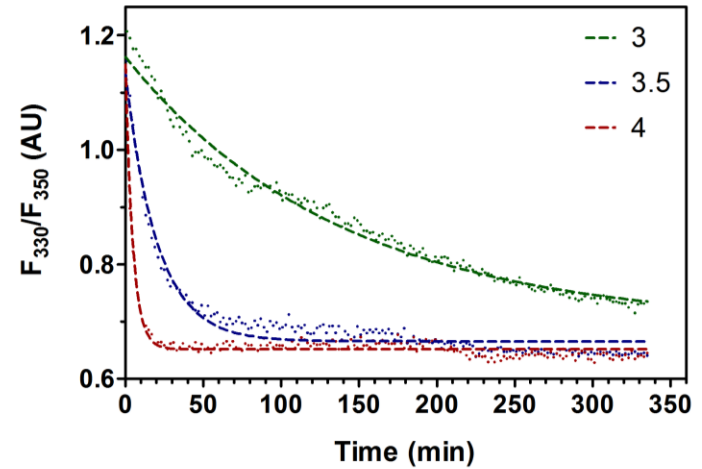
ITF: real examples

Temperature-unfolding

GdnHCl-unfolding

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

Real-time GdnHCl denaturation

University of Zurich[UZH]
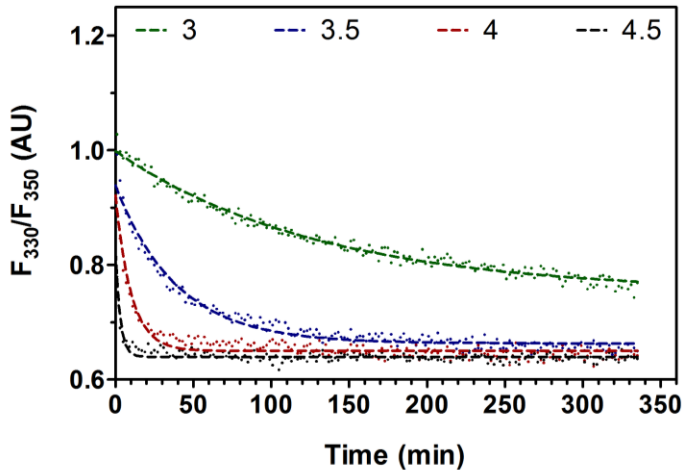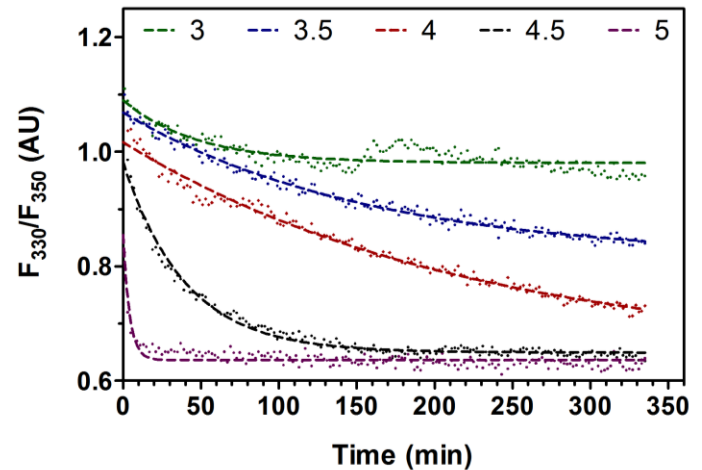
melting temperature detected by increased fluorescence of dye with **affinity for hydrophobic parts** of the protein
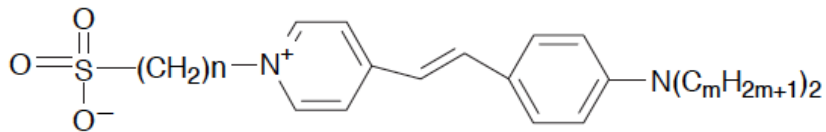

Sypro-Orange (Molecular Probes)

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
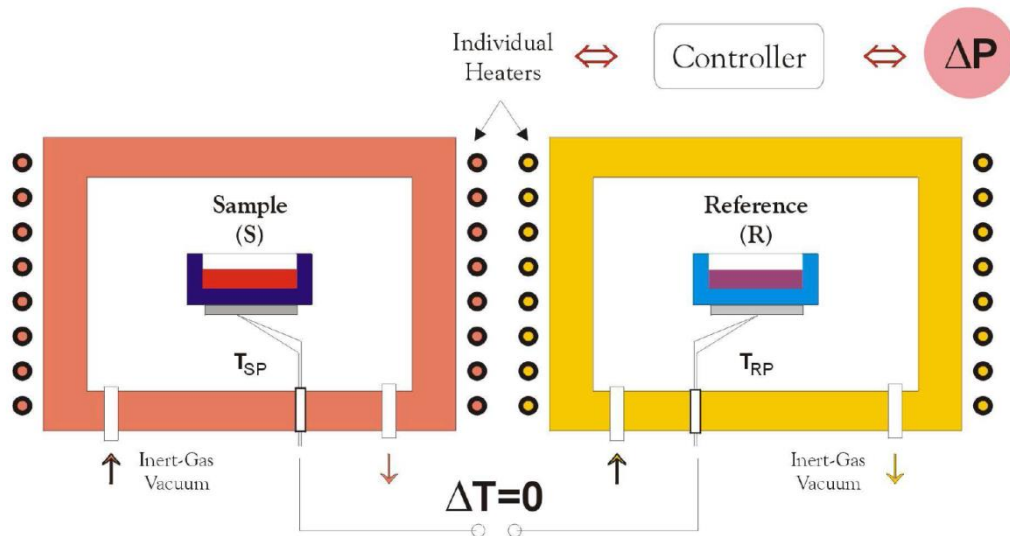

Method described in Niesen *et al.*, *Nature Protocols* (2007)

DSF: real examples

Differential Scanning Calorimetry (DSC)
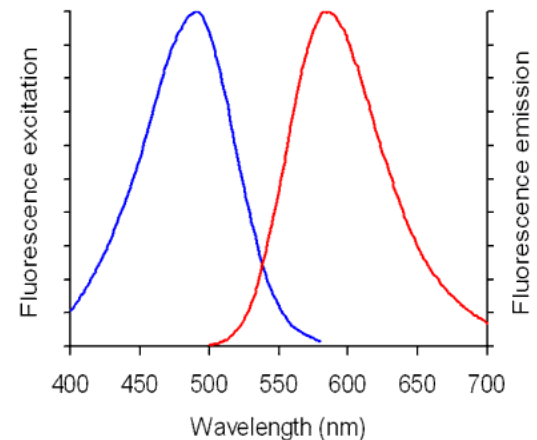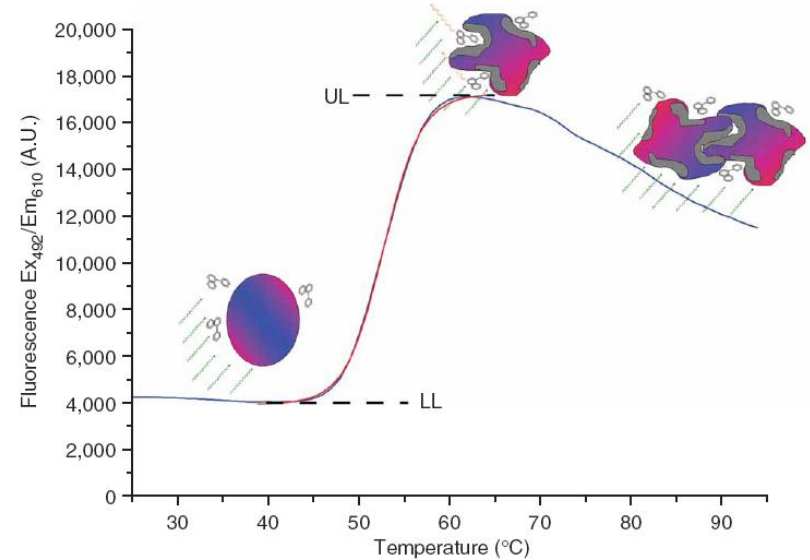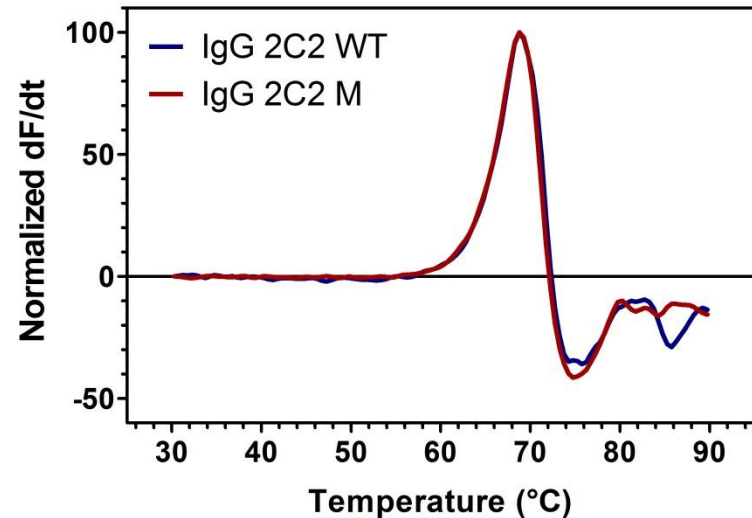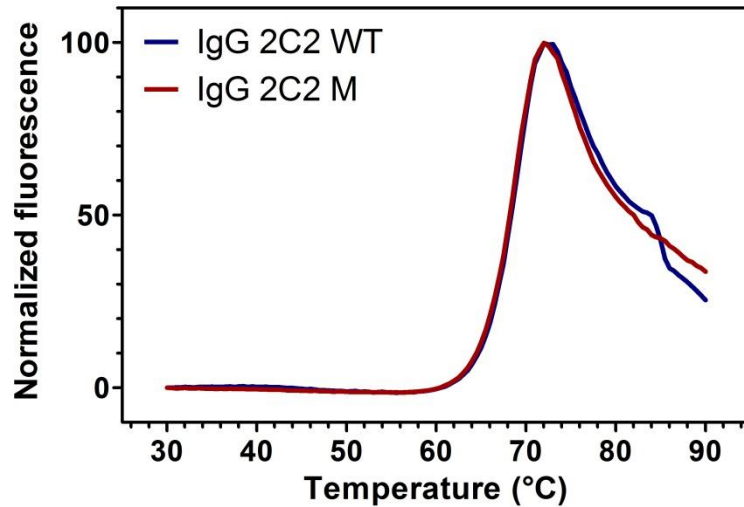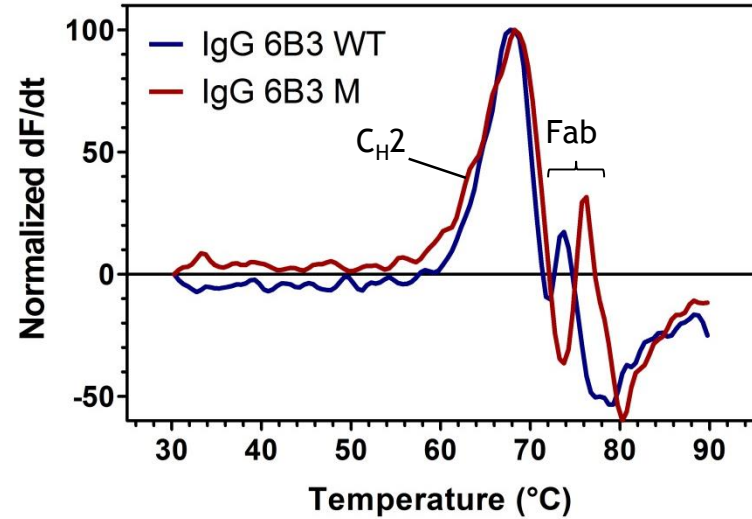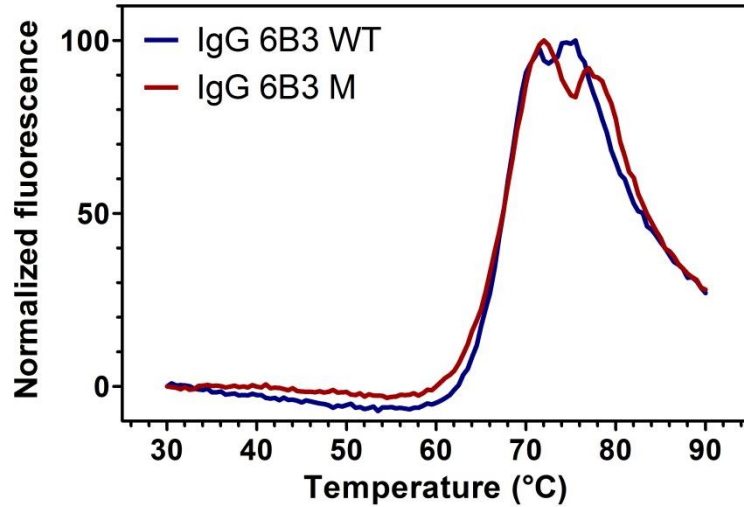
Power-compensation DSC (not Heat-flux DSC)

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]**

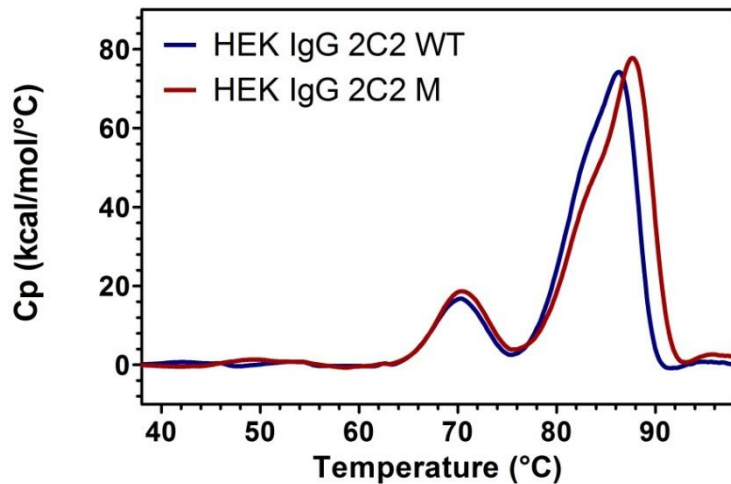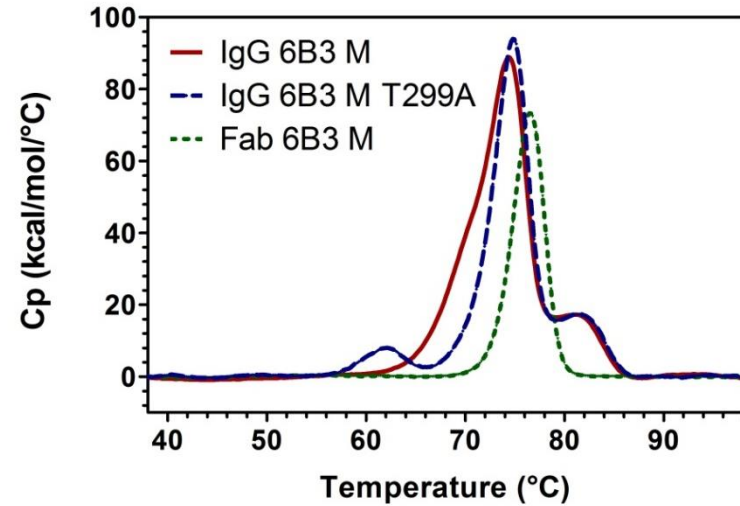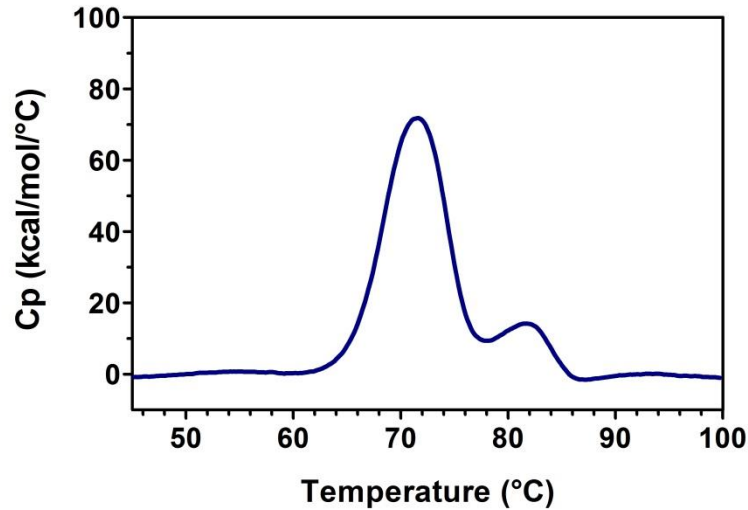Integration of heat capacity *vs.* temperature yields the **enthalpy (ΔH)**

$$\Delta H = \int_{T1}^{T2} C_p \, dT \qquad \Delta G = \Delta H - T \cdot \Delta S$$

(Gibbs Free Energy equation)

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

DSC: real examples

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

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

University of Zurich UZH

VP-DSC

autosampler

plate storage

Cap-DSC

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)
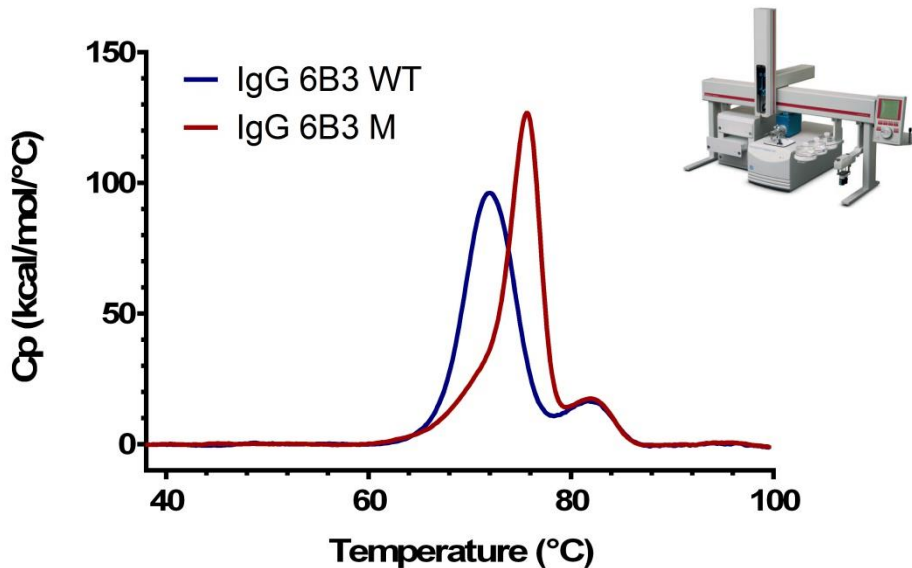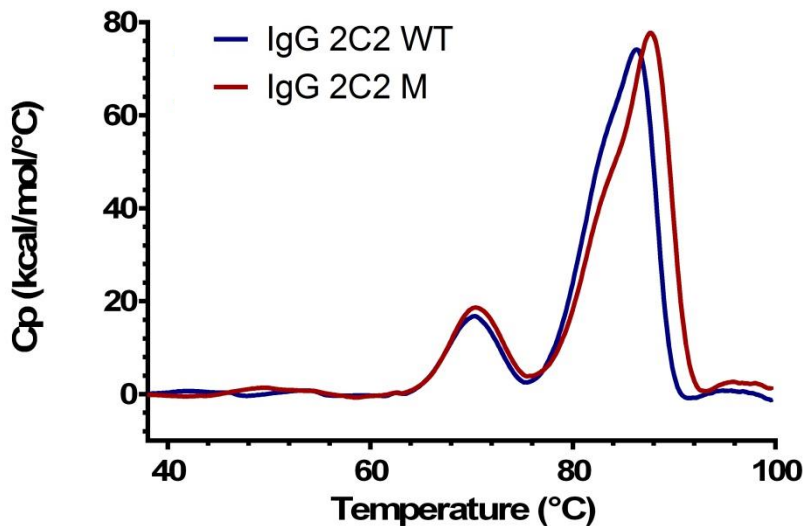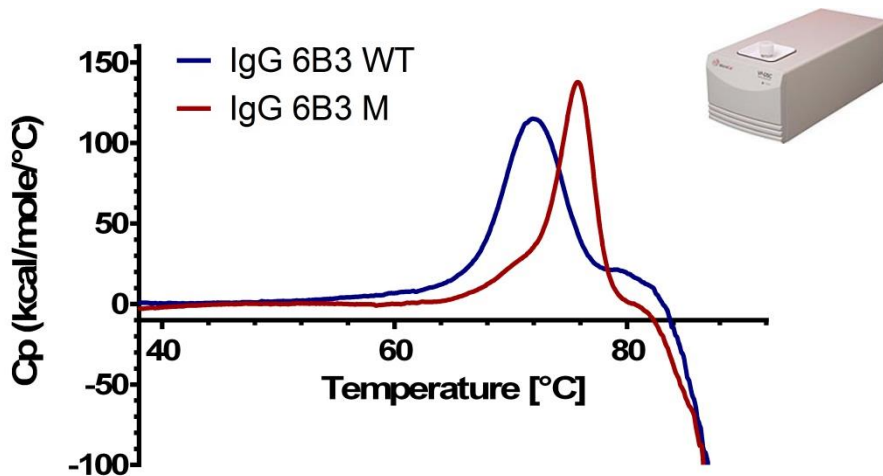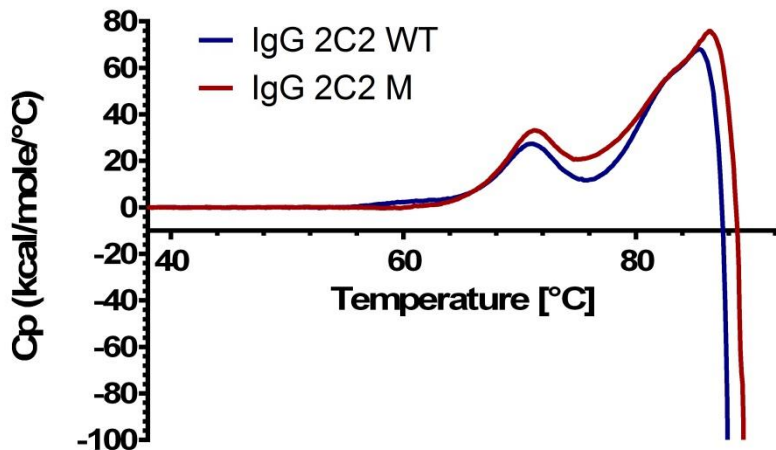
Convection at aggregation

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
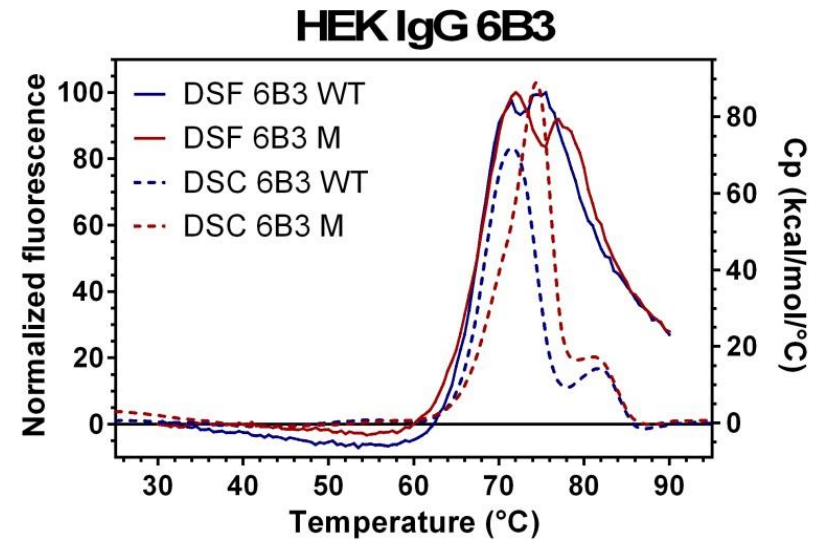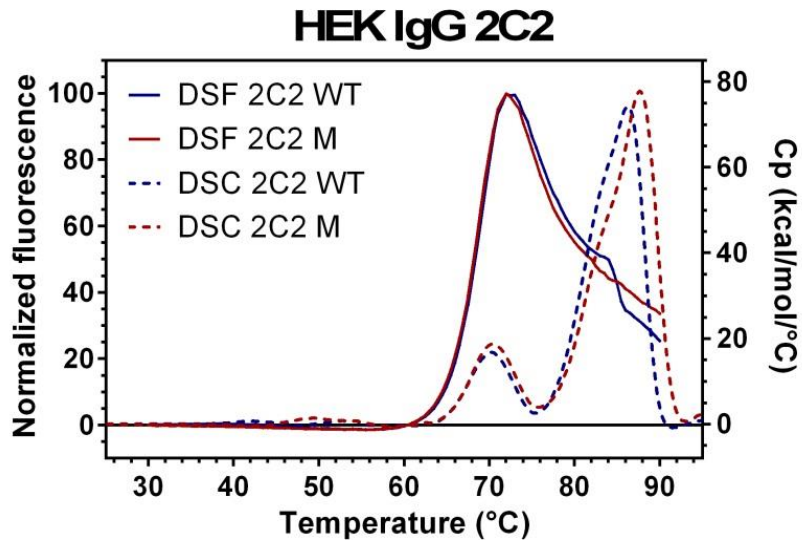
very little convection

due to small diameter of capillaries

molecules are separated with enough space (aggregation delayed)

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

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)

Stabilizing effects of V_H6 mutations

		ITF	GdnHCl (ITF)	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

IgG stability analyses → **IgG expression systems**
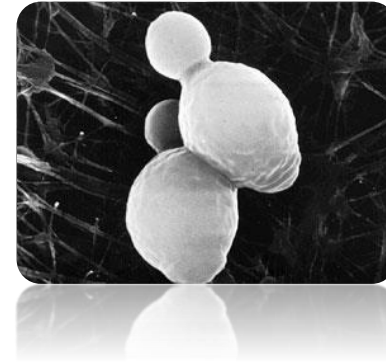
Eukaryotic expression systems

Mammalian cell culture

Yeast *Pichia pastoris*
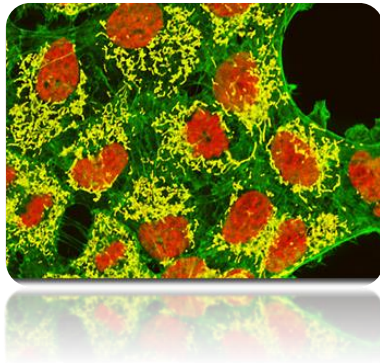
stable HEK293 (Flp-In)

CMV promoters (**constitutive**)

stable SMD1163 (his4 pep4 prb1)

GAP promoters (**constitutive**)

Expression system *Pichia pastoris*

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

➡ **advantages of expression system:**

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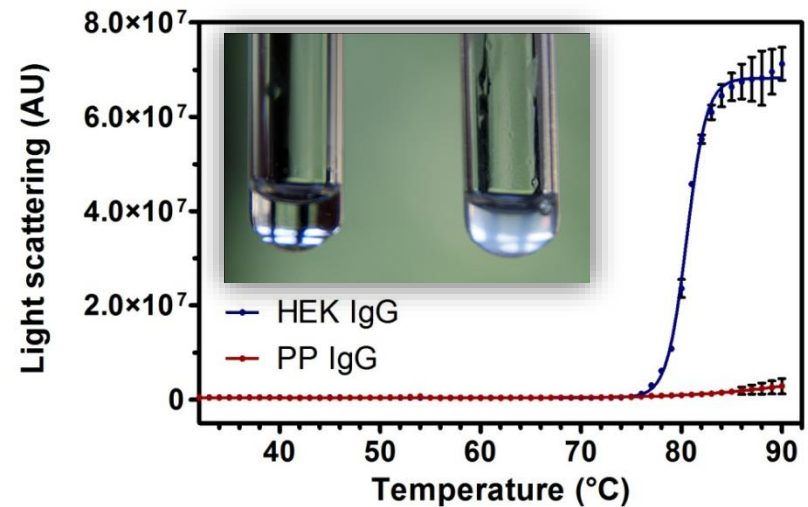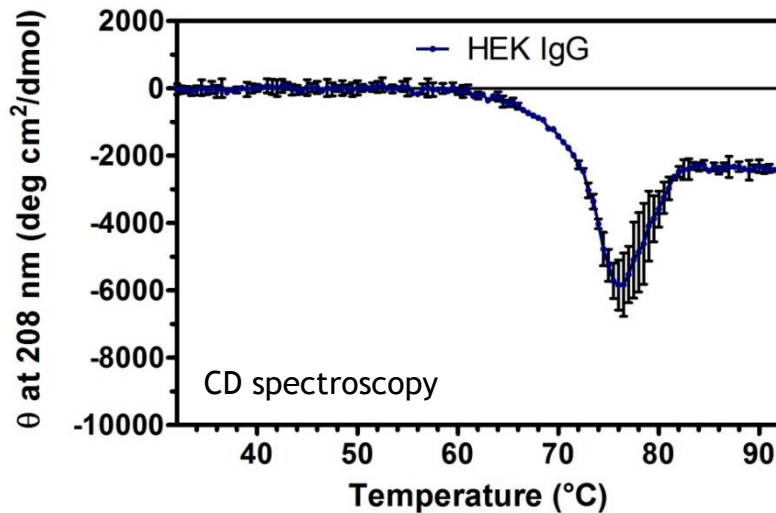- high expression levels (up to 30%)

➡ **different promoters available:**

- 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**
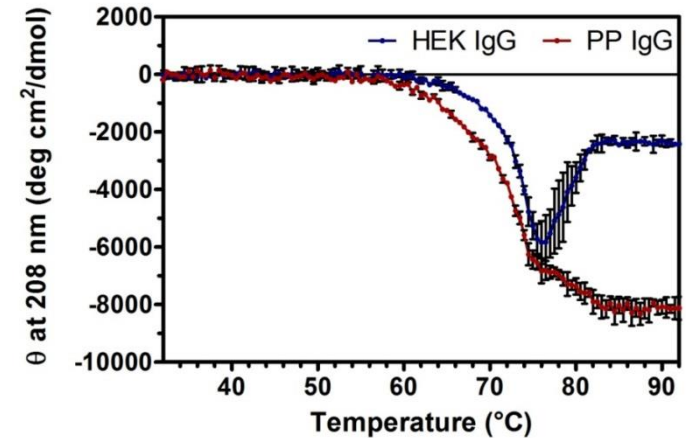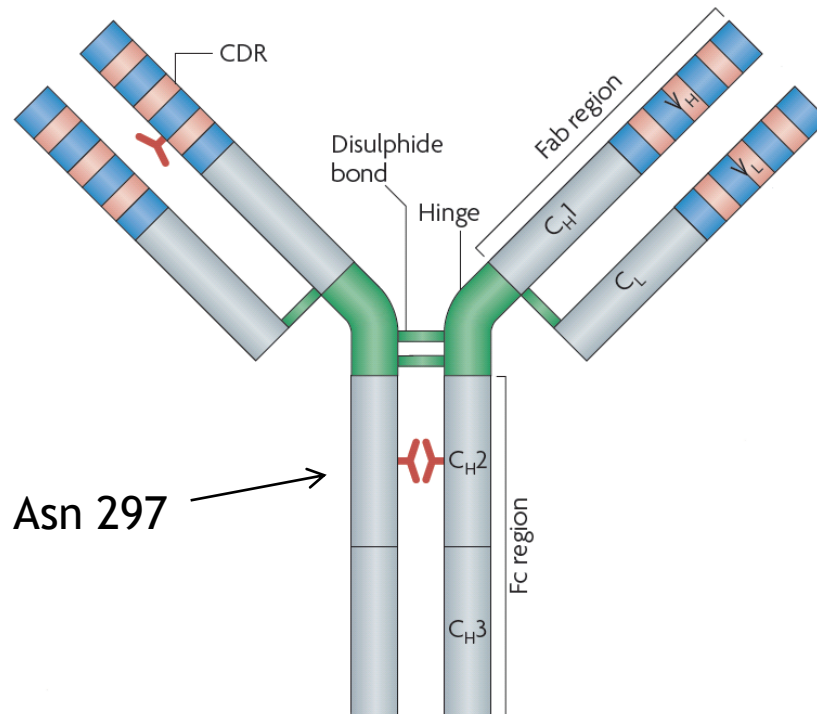(mainly scFvs, several Fabs, only handful full-length IgG)

➡️ *Pichia*-**derived glycans** reduce aggregation tendency

➡️ **peptide remaining from yeast signal sequence** decreases aggregation susceptibility of HEK-IgG upon N-terminal addition
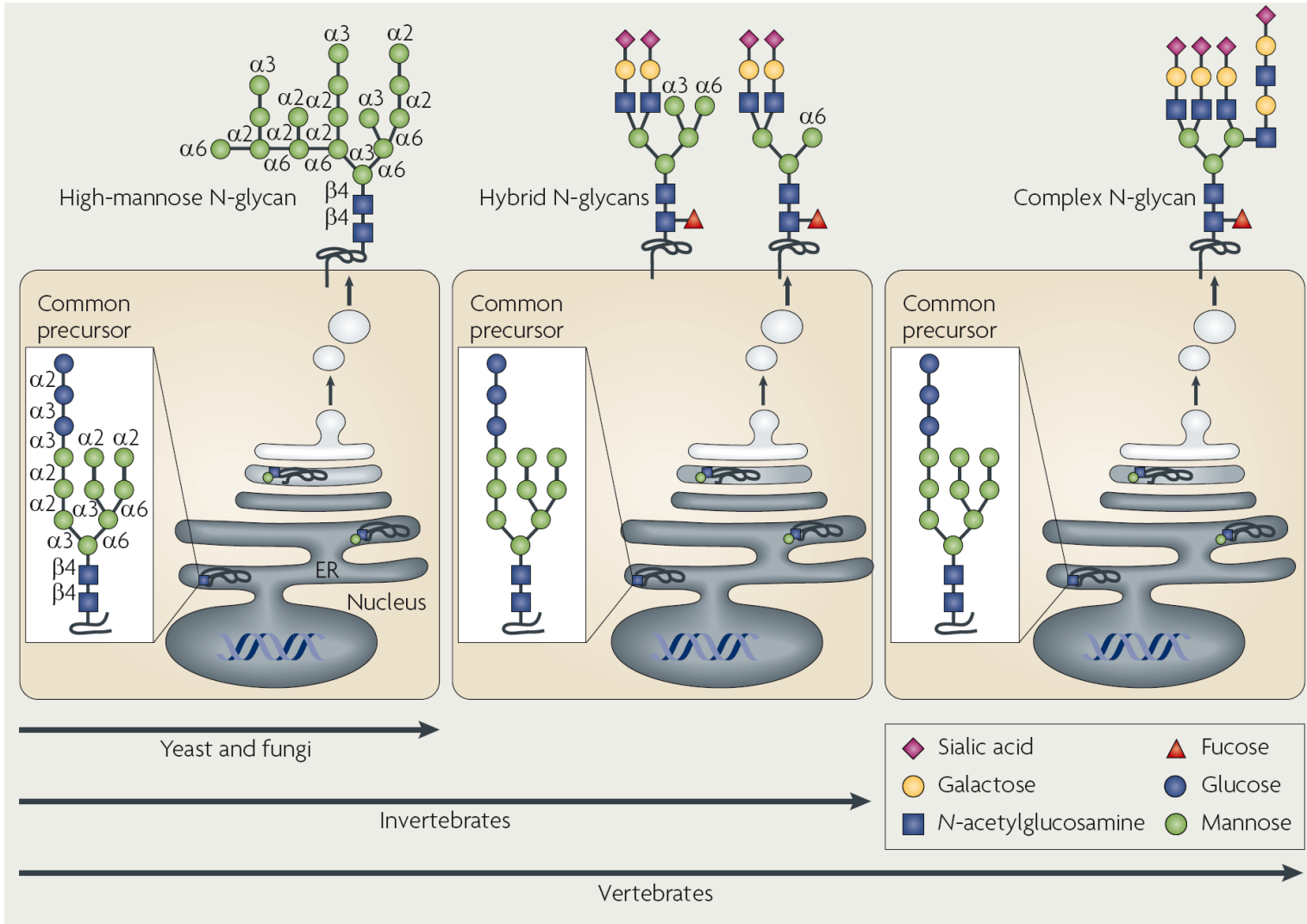
Difference in expression systems

mayor difference in expression systems: glycosylation

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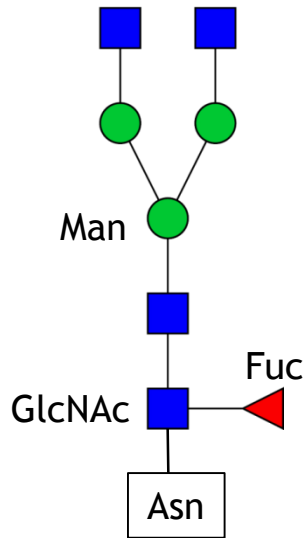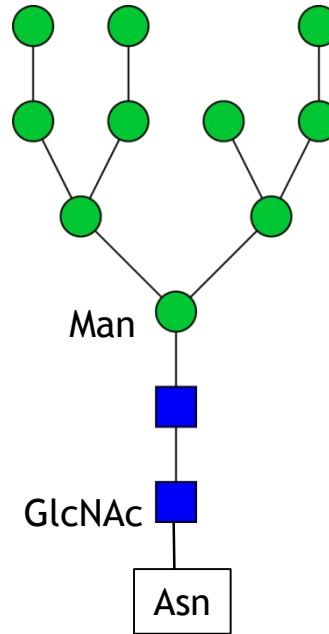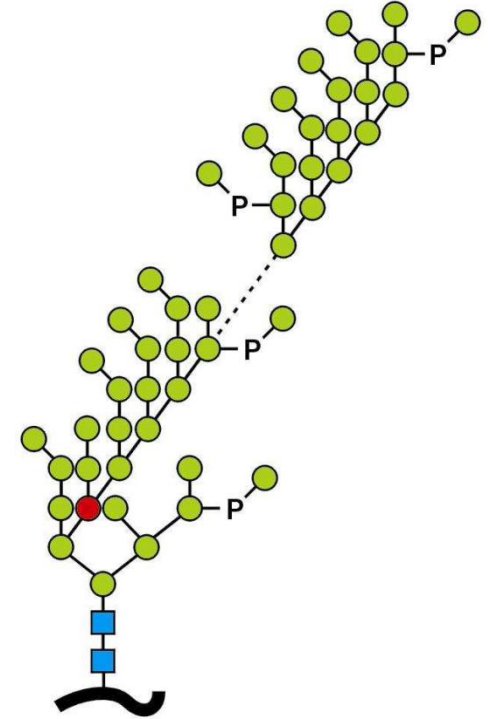
N-linked glycosylation

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

N-linked glycan processing

HEK293 cells

Pichia pastoris

Man

Man

GlcNAc

Fuc

GlcNAc

Asn

Asn

$Gal(GlcNAc)_2(Man)_3(GlcNAc)_2Fuc$
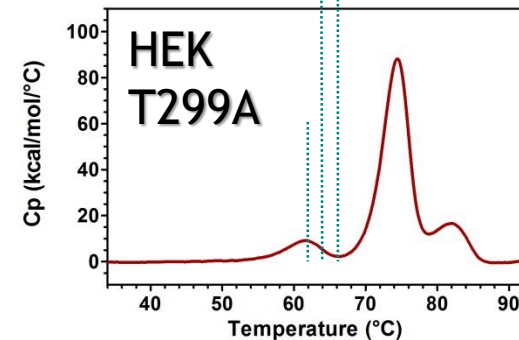
$(Man)_{9-10-18}(GlcNAc)_2$

➡ *Pichia* glycan cause difficulties interacting with **Fcγ receptors (FcγR)** important for effector functions
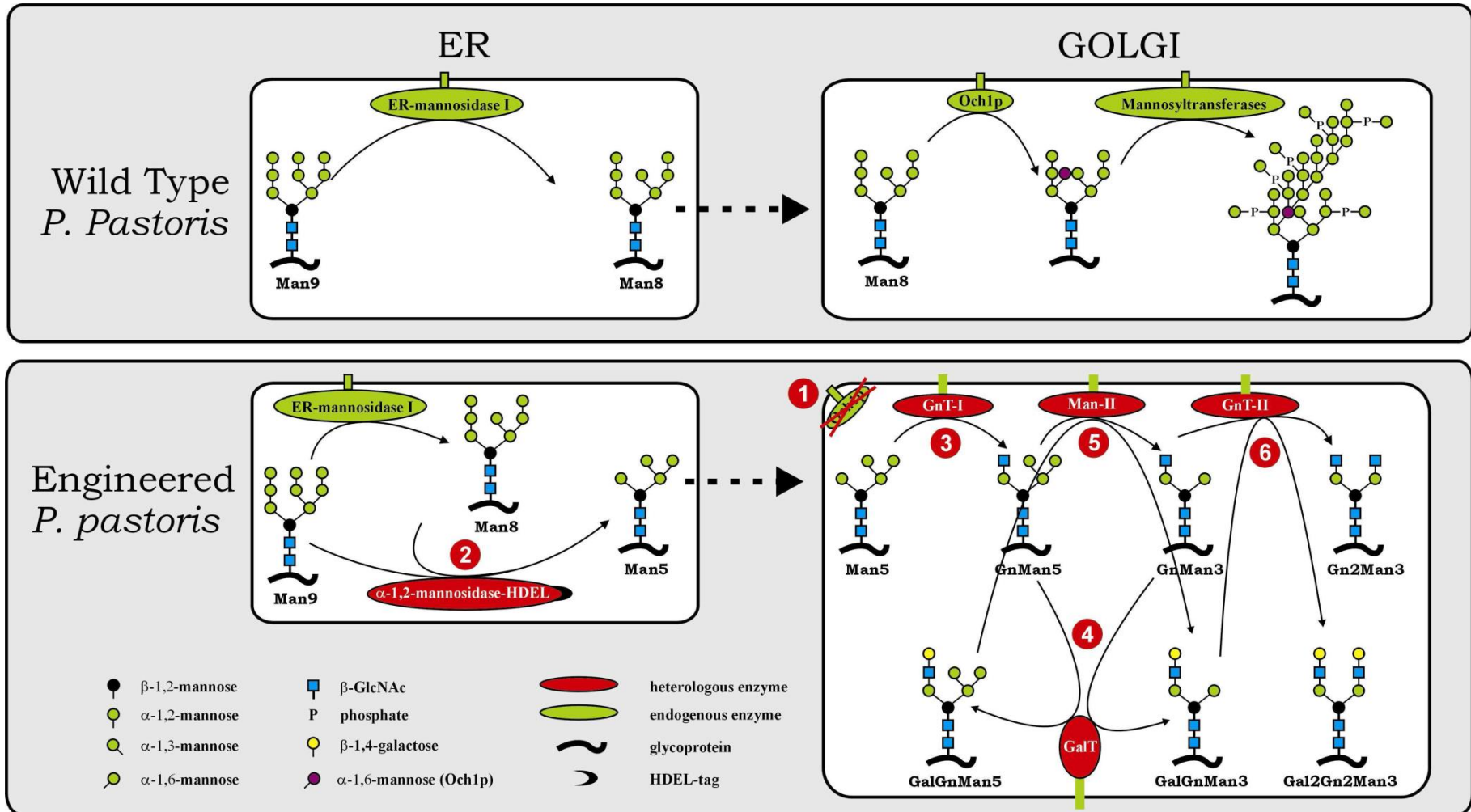
University of Zurich[UZH]

➡ *Pichia* produced IgGs have decreased **C$_H$2 stability**, compared to mammalian expression

➡ different C$_H$2 stabilities are caused by **different glycan moieties**
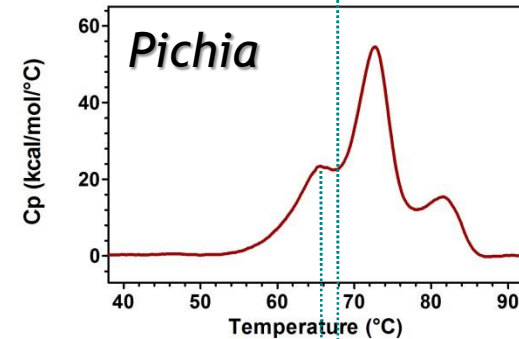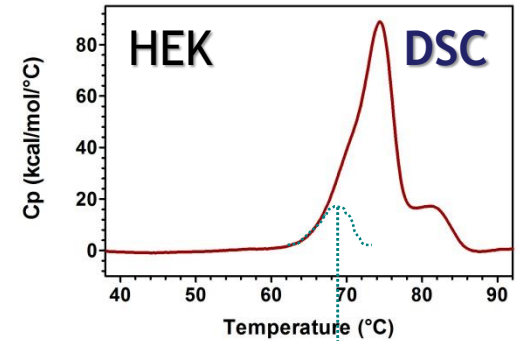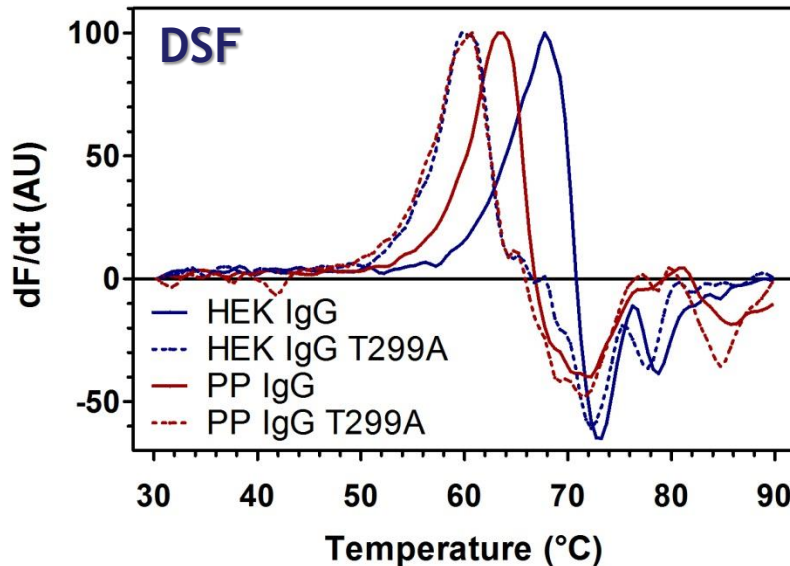
Glyco-engineering of *Pichia*

Pichia GlycoSwitch®: introducing complex, human-like glycosylation

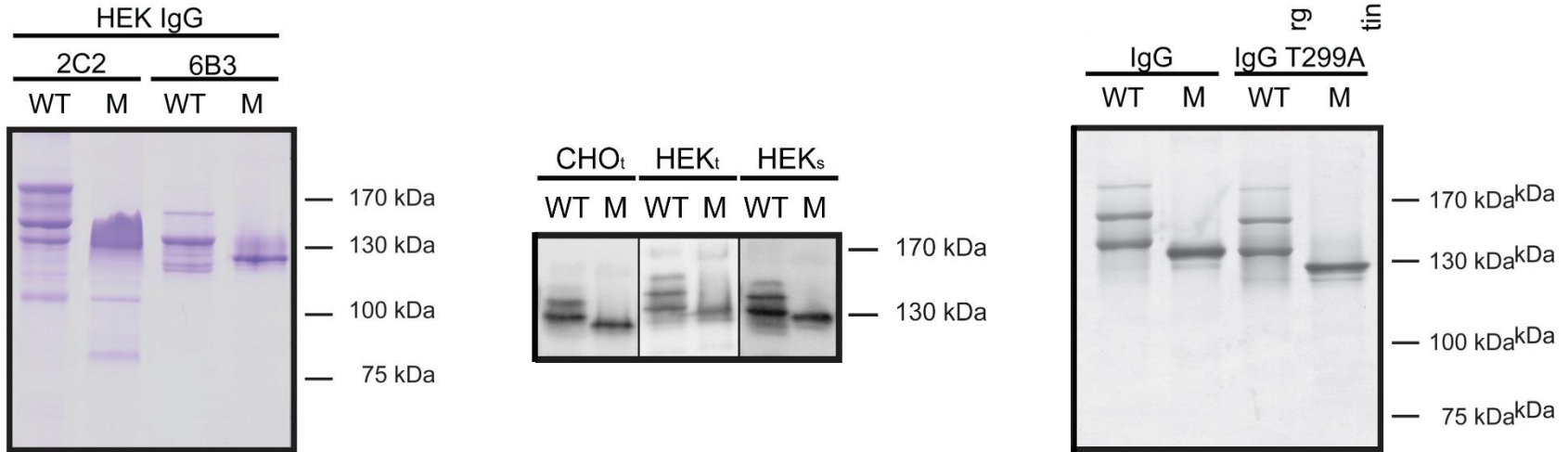Jacobs, Geysens, Vervecken, Contreras and Callewaert, *Nat Protoc*. 2009

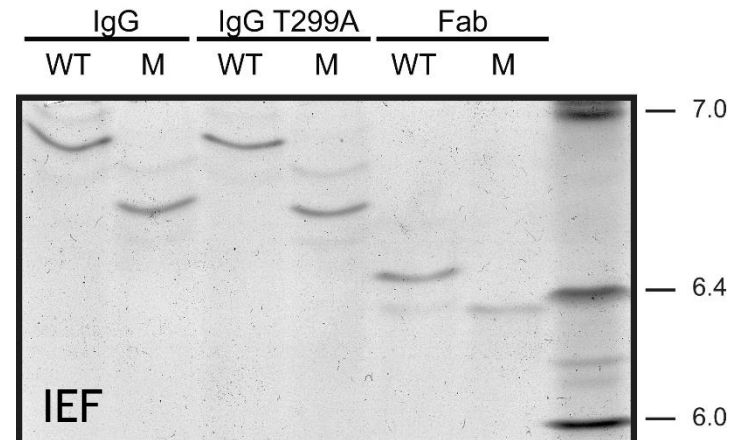**IgG stability analyses** → **IgG homogeneity**

Electrophoretic analyses of IgGs

➡ non-reducing SDS-PAGE reveals **inhomogeneity of WT, but not of M variants**
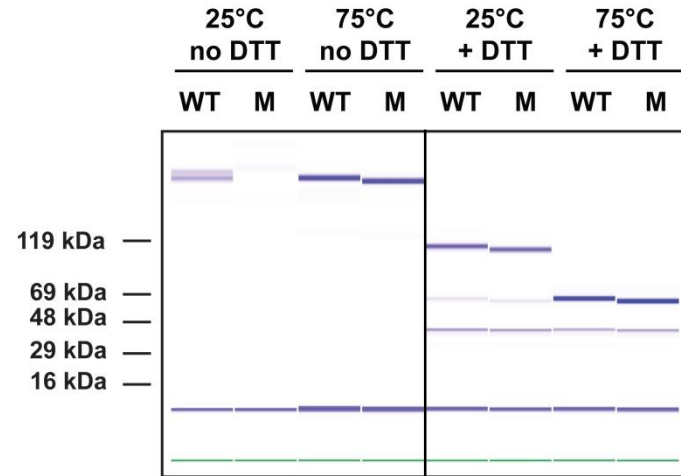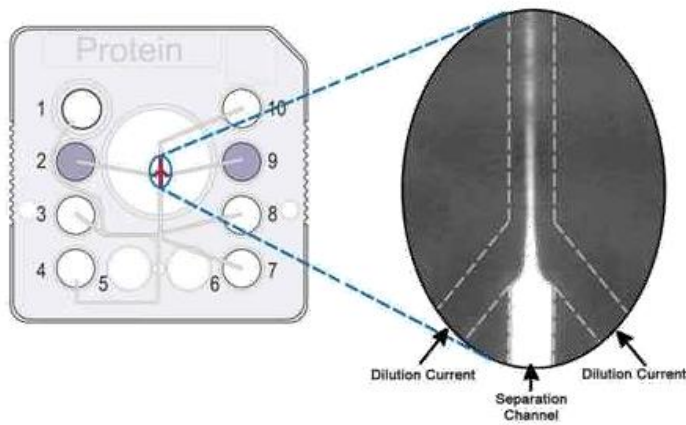
➡ banding pattern is not caused by:

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

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

University of Zurich UZH

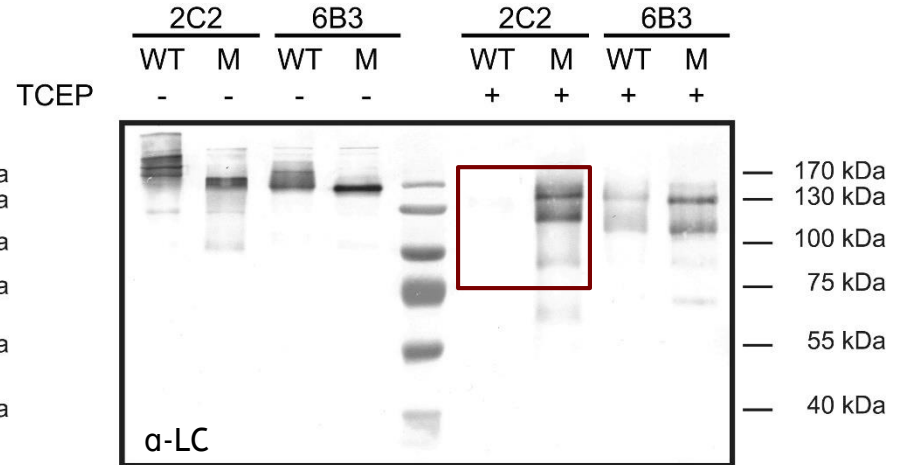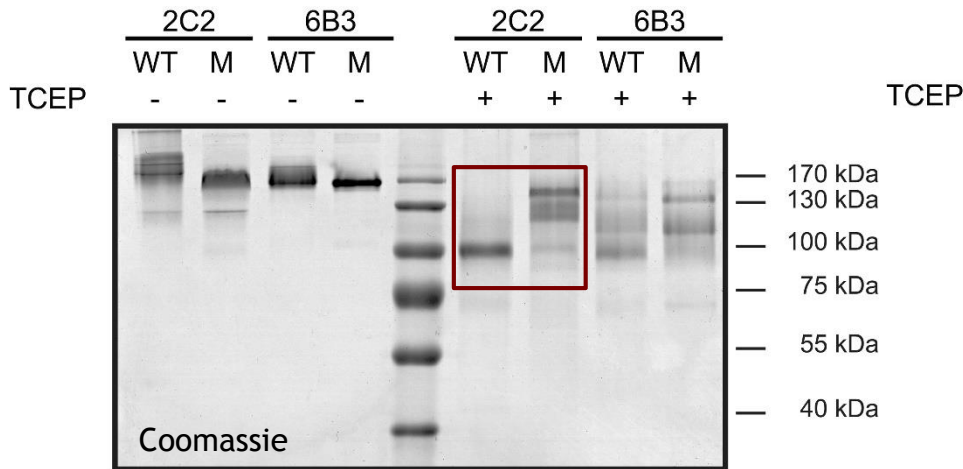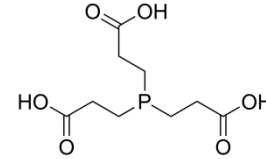**Analysis by capillary electrophoresis** (performed in microfluid chip)

fluorescently labeled stain binds to protein non-covalently

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

University of Zurich

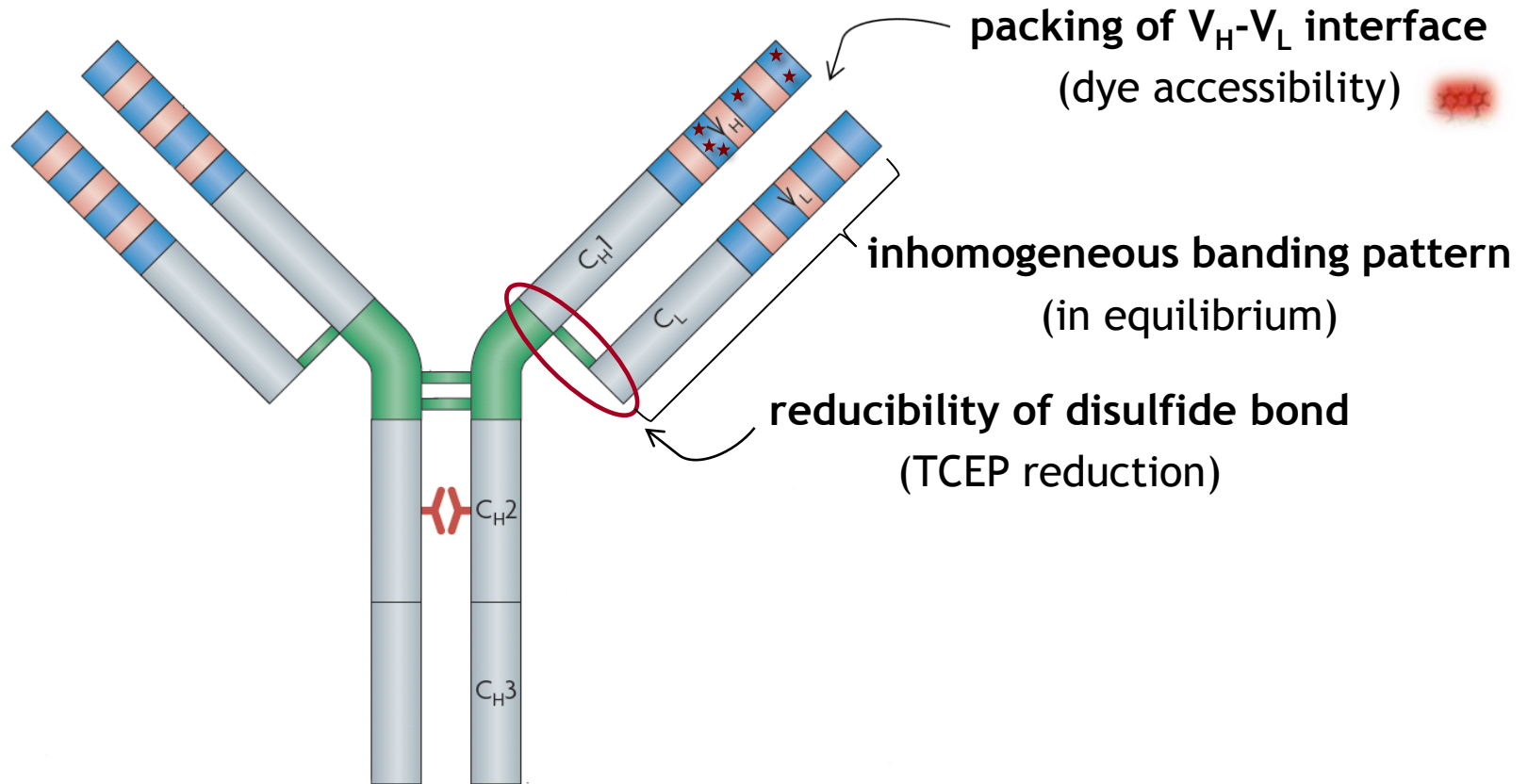## Partial reduction of IgG by hydrophilic TCEP

Coomassie

α-LC

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. Des. Sel.* (2012)

University of Zurich UZH
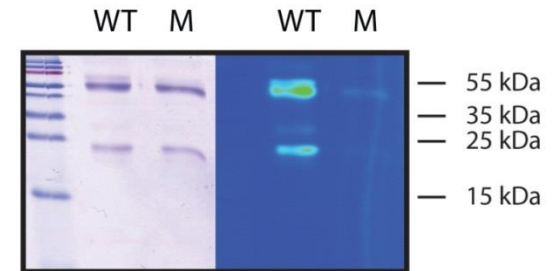
mutations affect structural integrity and homogeneity

packing of V_H-V_L interface
(dye accessibility)

inhomogeneous banding pattern
(in equilibrium)

reducibility of disulfide bond
(TCEP reduction)

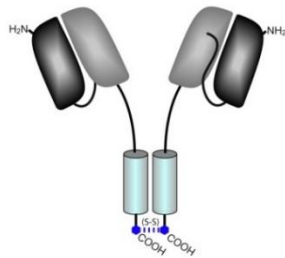**Schaefer and Plückthun**, *Protein Eng. Des. Sel.* (2012)

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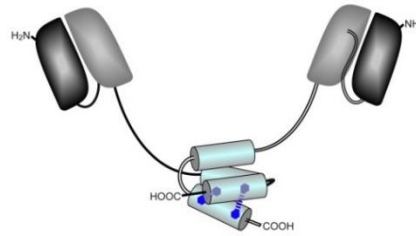
Schaefer and Plückthun, *Protein Eng. Des. Sel.* (2012)

University of Zurich UZH
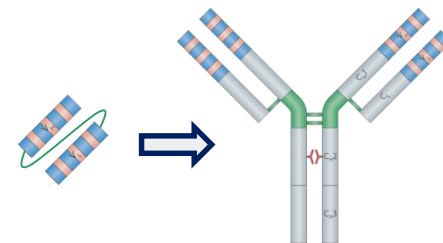
Dimeric miniantibodies
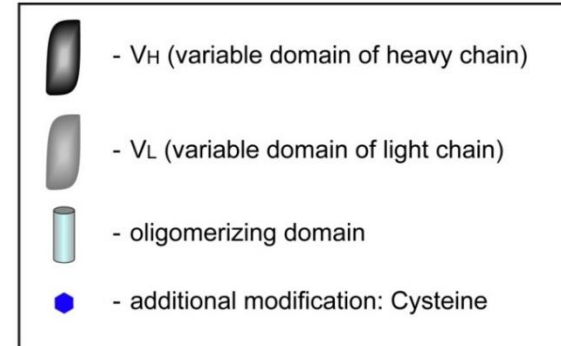
(A) scFv-ZIP(c)
(GCN4 leucin zipper)

(B) scFv-dHLX (-SS)
(Helix1-turn-Helix2)

Legend:
- V_H (variable domain of heavy chain)
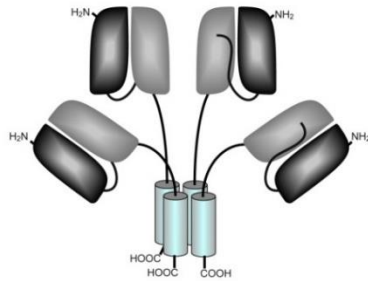- V_L (variable domain of light chain)
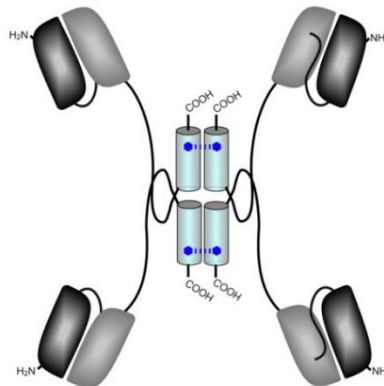- oligomerizing domain
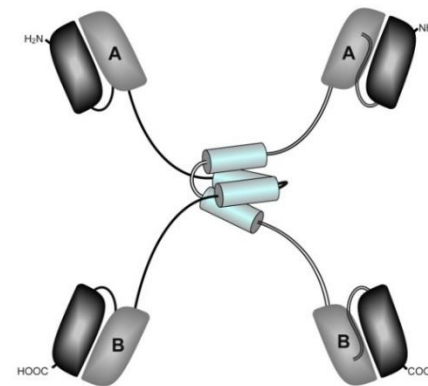- additional modification: Cysteine

Tetrameric miniantibodies

(C) scFv-TETRAZIP
(modified GCN4: 9 mutations)

(D) scFv-p53 (-SS)
(p53 oligomerization domain)

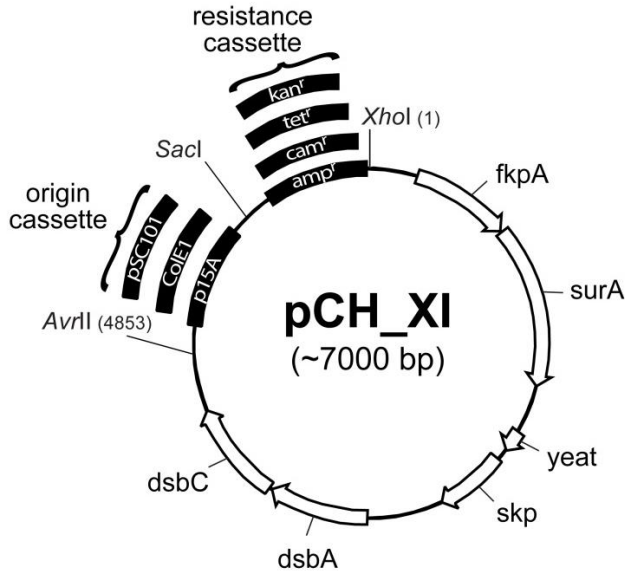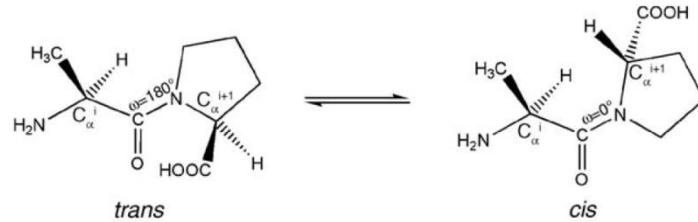(E) di-bi-miniantibody
(bispecificity & bivalency)

➡ scFv fused to ologomerization domain: **rotational freedom and flexibility**

Schaefer, Lindner and Plückthun (2010) in Antibody Engineering: **Miniantibodies**

Modular co-expression of chaperons

peptidyl-prolyl cis/trans-isomerases (PPIs) with chaperone activity, **FkpA** and **SurA**

chaperone protein **Skp** precursor

thiol-disulfide oxidoreductases **DsbA** and **DsbC**

➡ **different origin of replication:** **ColE1 (E)**, **p15A (A)** and **pSC101 (S)**

copy numbers: 50-70   20-30   ~10

➡ **modular system:**

compatibility with virtually all expression vectors; level of chaperone co-expression can be controlled; safeguards against plasmid incompatibility

Schaefer and Plückthun (2010) in Antibody Engineering: **Improving expression of scFv fragments by co-expression of periplasmic chaperones**

Acknowledgements

Questions & Answers

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Schaefer and Plückthun (2012) *Protein Eng. Des. Sel.* 25(10):485-506

Schaefer and Plückthun (2012) *J Mol Biol*. 417(4):309-35