Follow-on Biologics Workshop

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Field-Flow Fractionation (FFF) in Protein Purification and Characterization

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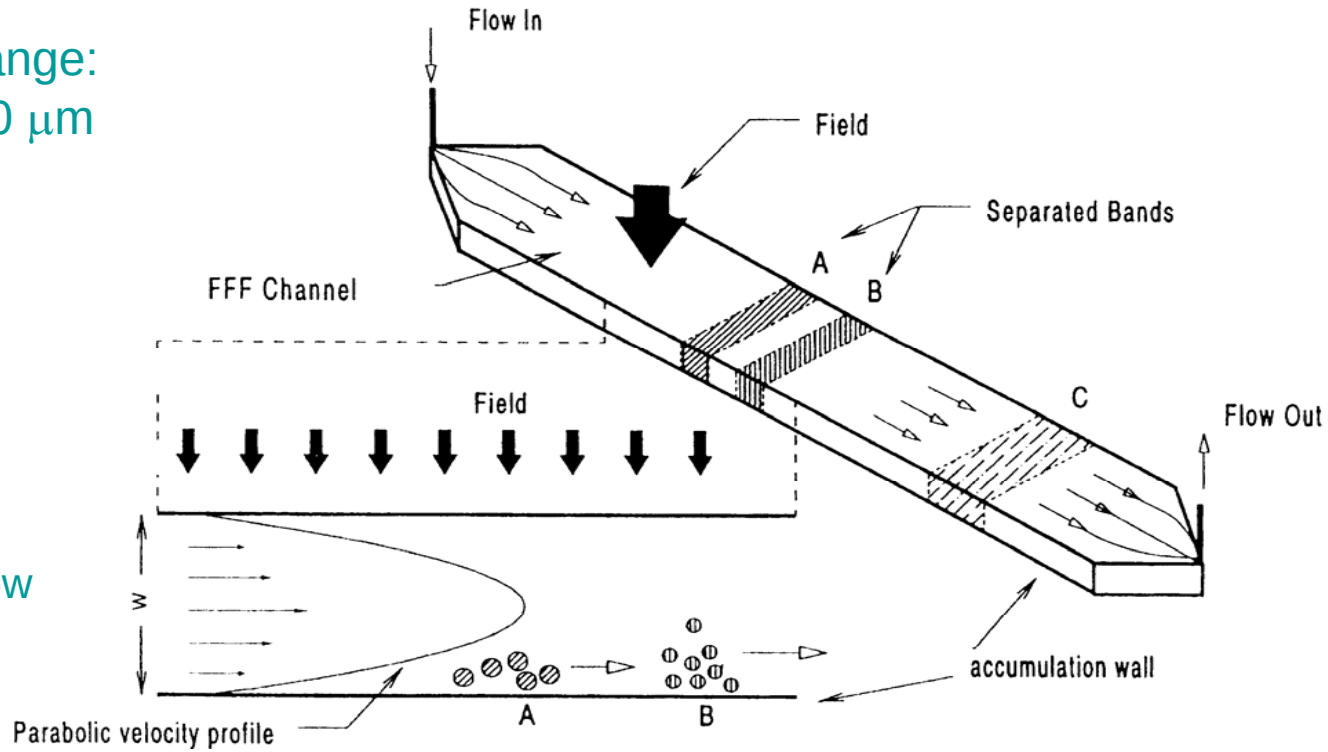
CONTENTS

- What is Field-Flow Fractionation?
- Asymmetrical flowFFF- Analytical advantages
- FFF-MALS – an informative combination
- Resolution of protein aggregates
- FFF – SEC in comparison
- Analysis of a complex protein sample
- Sedimentation FFF in glycosylation analysis – a method under development

FIELD-FLOW FRACTIONATION:
An Alternative to Chromatography ideal for Characterization of Colloids and Macromolecules

Size Range:
5nm- 50 μm

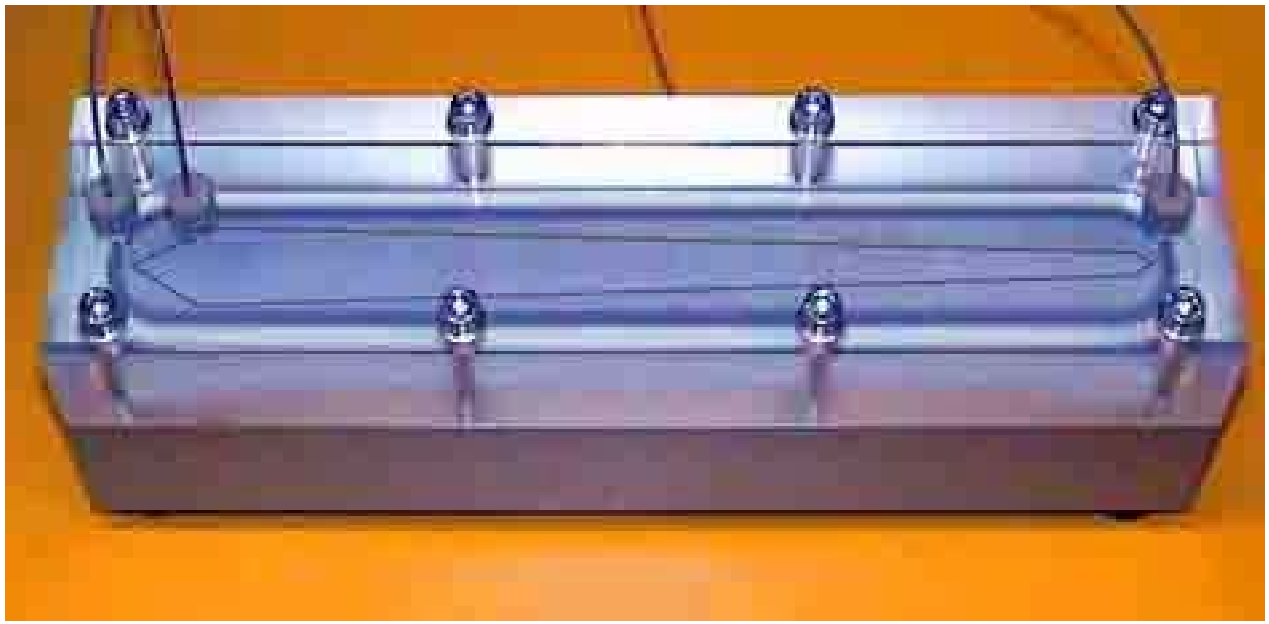
Mobile phase Under low shear



Flow field: elution time t_r prop. to size

Sedimentation field: t_r prop. to mass

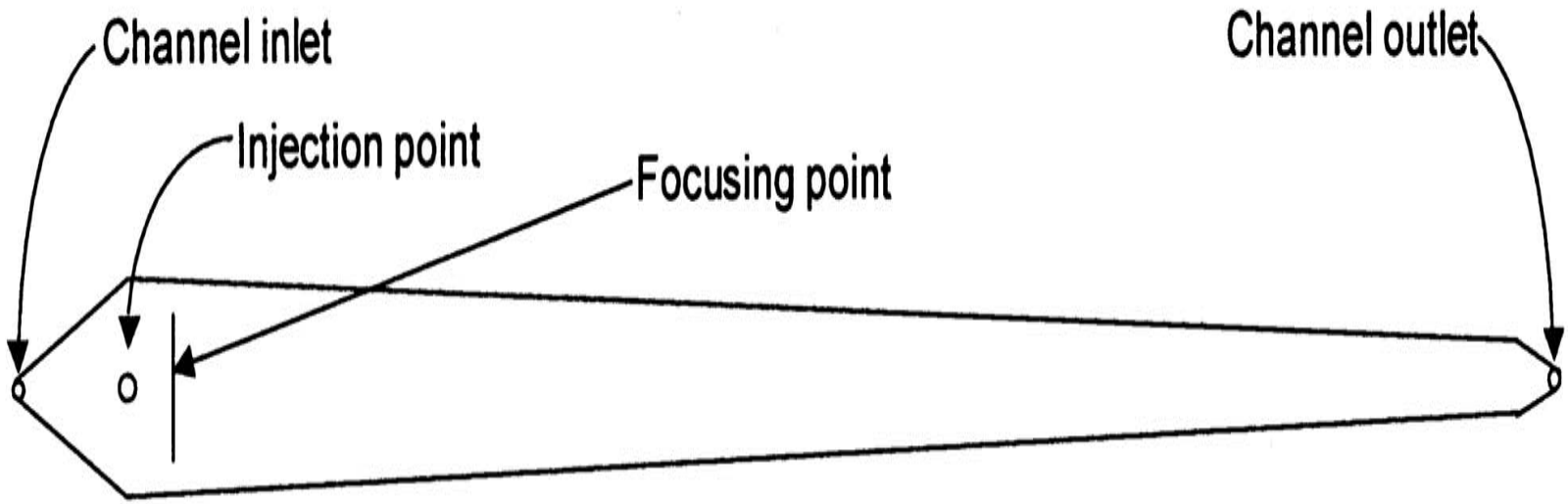
Asymmetrical Flow FFF channel with downstream central injection (DCI) and trapezoidal outline



Upper block non-permeable for the crossflow (but permeable for light!)

Wahlund, Giddings *Anal.Chem*.1987; Wahlund, Litzén *J. Chromatogr.*1989; Litzén, Wahlund *Anal. Chem*. 1991

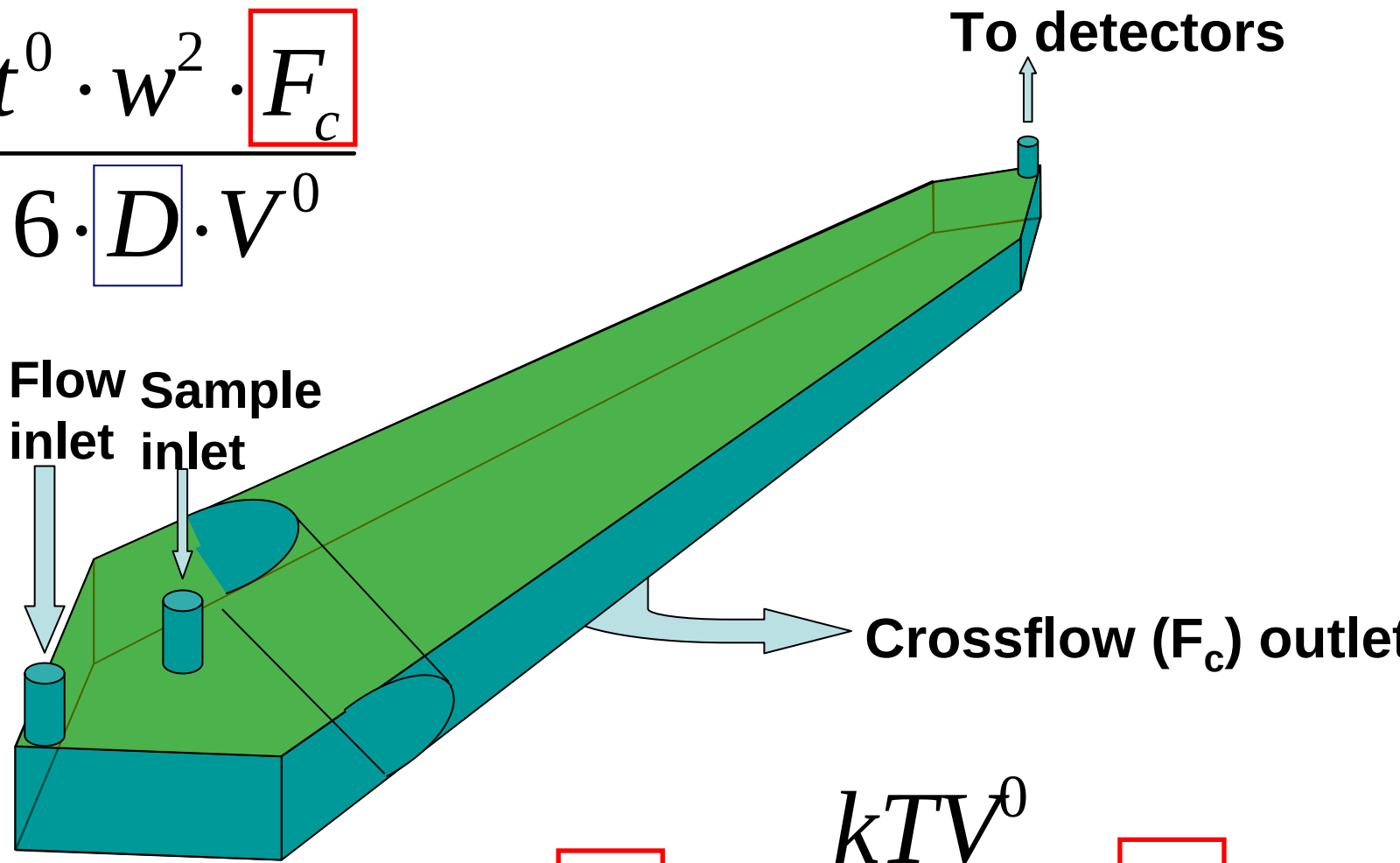
Illustration of the tapered trapezoidal asymmetrical flow FFF channel



Wahlund, Litzén *J. Chromatogr.*1989 ; Litzén, Wahlund *Anal. Chem*. 1991

Asymmetrical Flow FFF

$$t_r = \frac{t^0 \cdot w^2 \cdot F_c}{6 \cdot D \cdot V^0}$$



$$r_H = \frac{kTV^0}{\pi \eta F_c w^2 t^0} t_r$$

K-G Wahlund & G Modig 2005

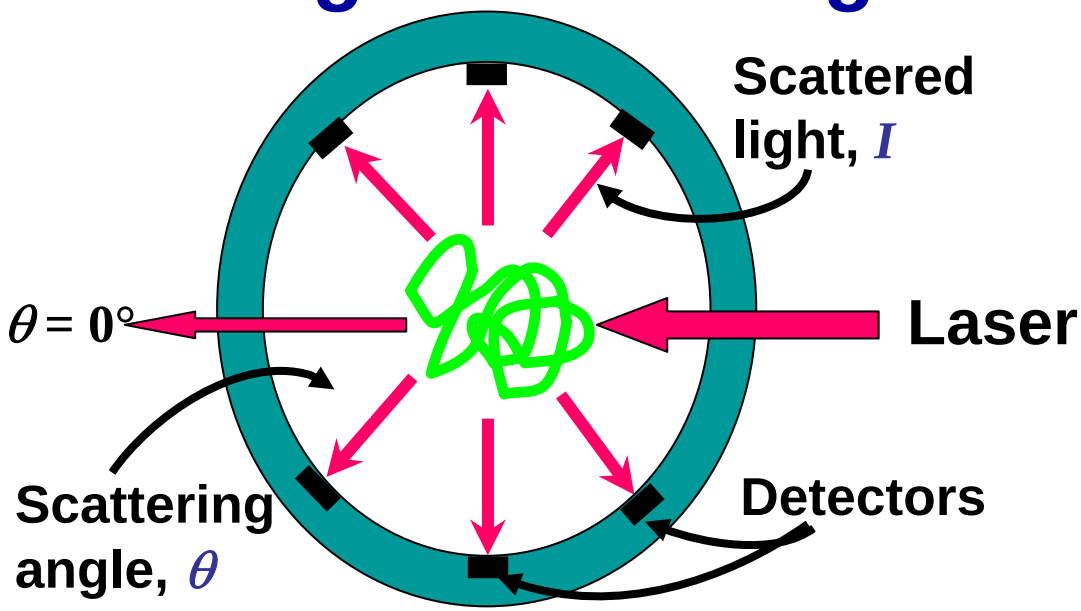
FFF – and multiangle light scattering

(FFF - MALS)

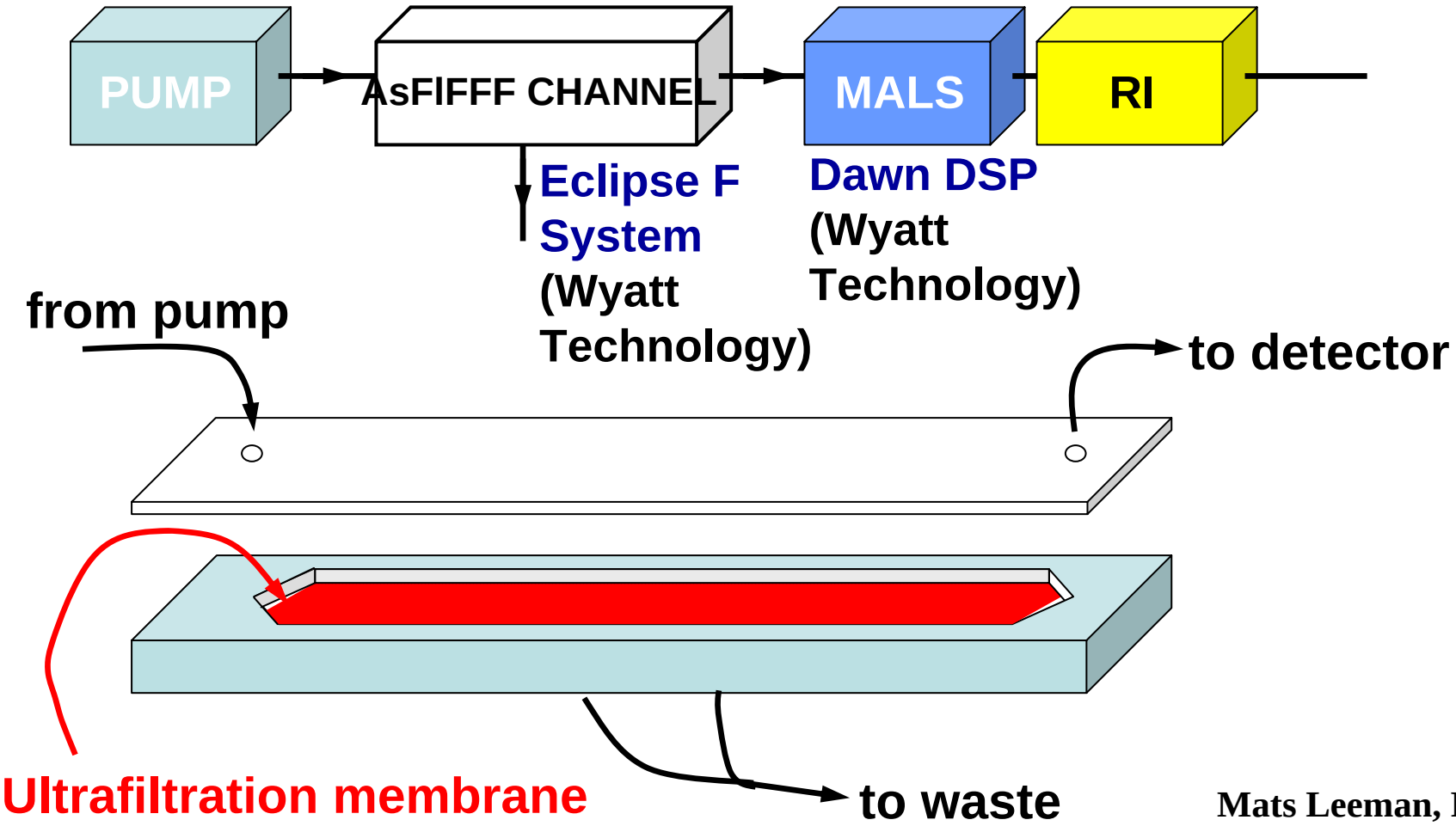
Provides directly (without calibration) the molar mass and radius distributions

From $I_{scattered}$ and θ the molar mass and root-mean-square radius of the sample can be directly determined

Light scattering



FFF - MALS - RI



Mats Leeman, PhD thesis
Lund University 2003

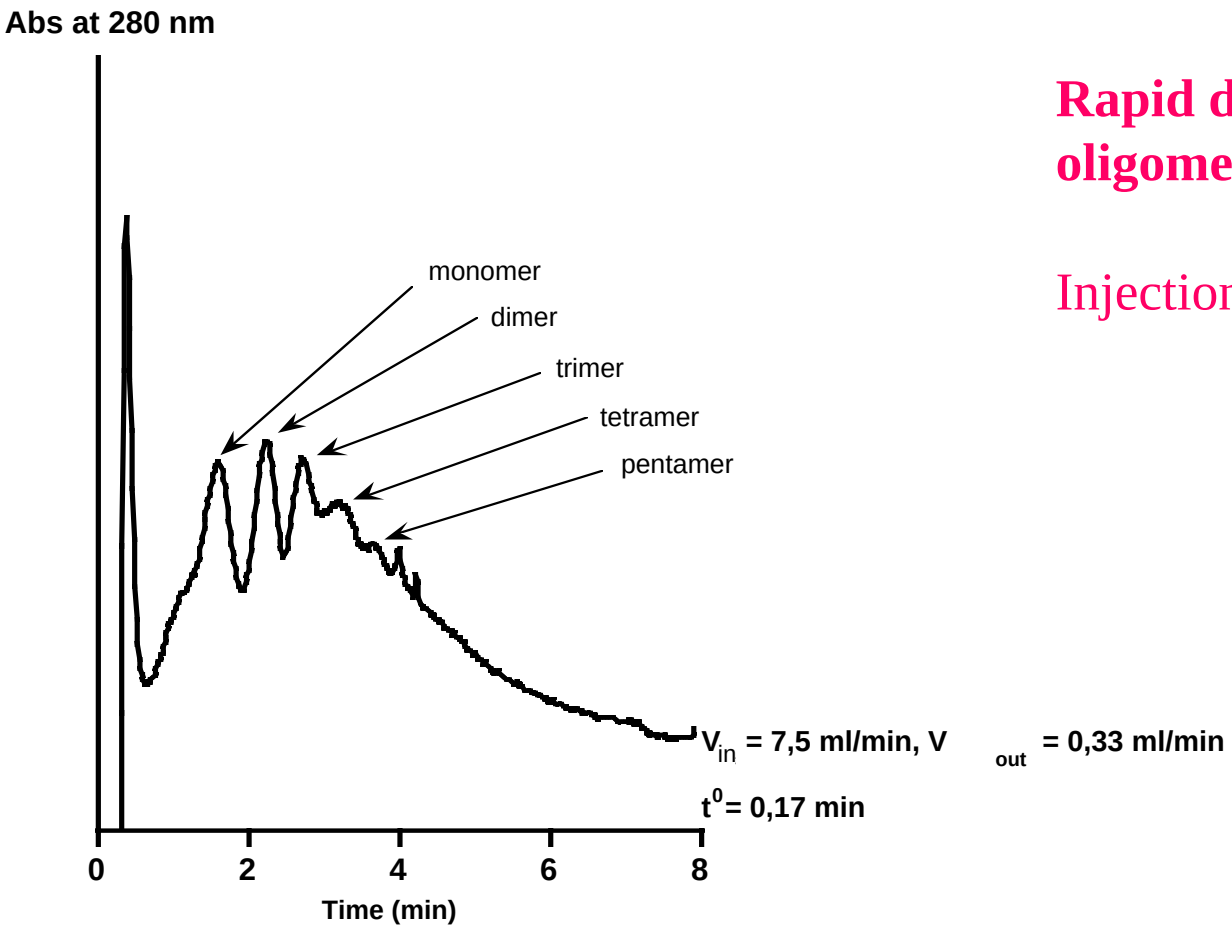
Channel thickness 100-300 µm, length ~ 30 cm, width ~ 2 cm.

The *Eclipse 2*: Accommodating needs of the pharmaceutical industry

- High degree of integration with Wyatt detectors and Agilent 1100 get the AF4 system accepted
- IQ/OQ available for the complete system

The Eclipse is manufactured in Germany by Wyatt Technology Europe

Oligomeric aggregates of the 52-kDa protein α1-antitrypsin



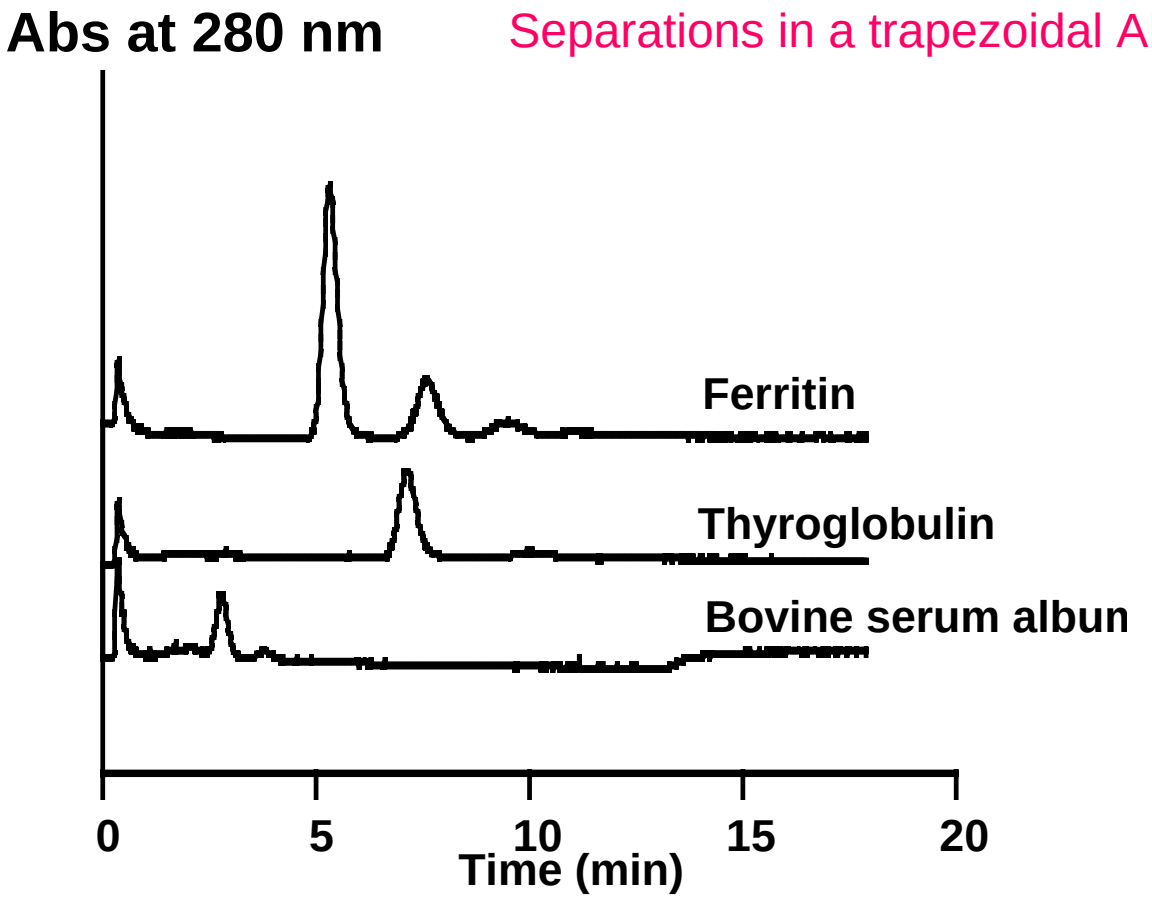
Rapid detection of oligomeric aggregates

Injection: 20μL, 0.1 mg/mL

Field-Flow Fractionation Handbook (2000); Courtesy of Mikael Nilsson, Technical Analytical Chemistry, Lund University, Sweden; (1997)

Standard proteins containing aggregates

Separations in a trapezoidal AF4 channel

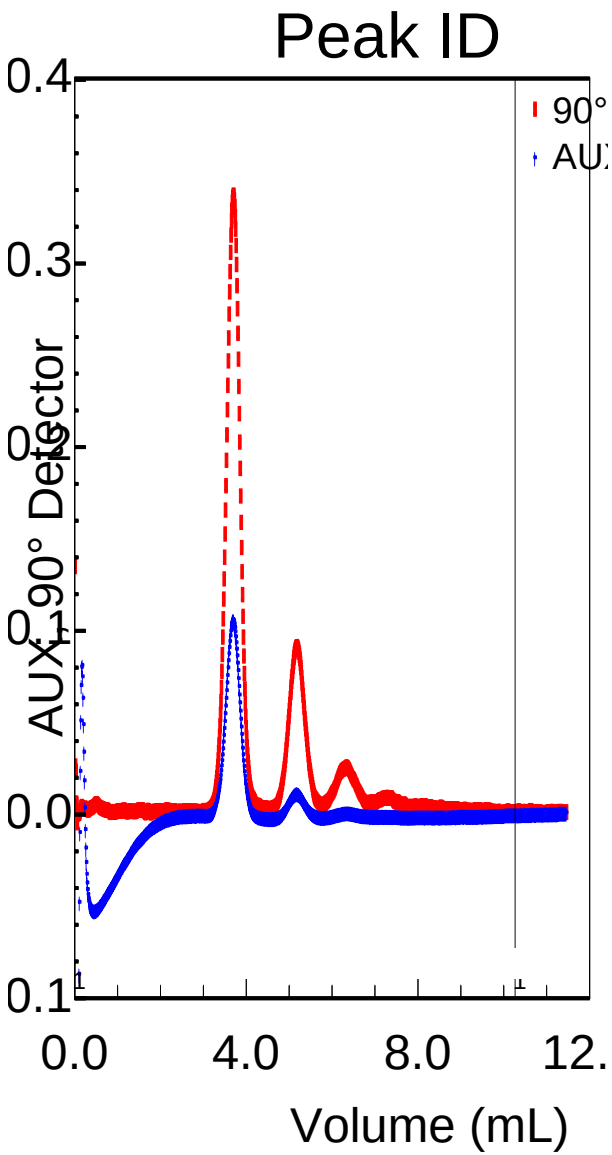


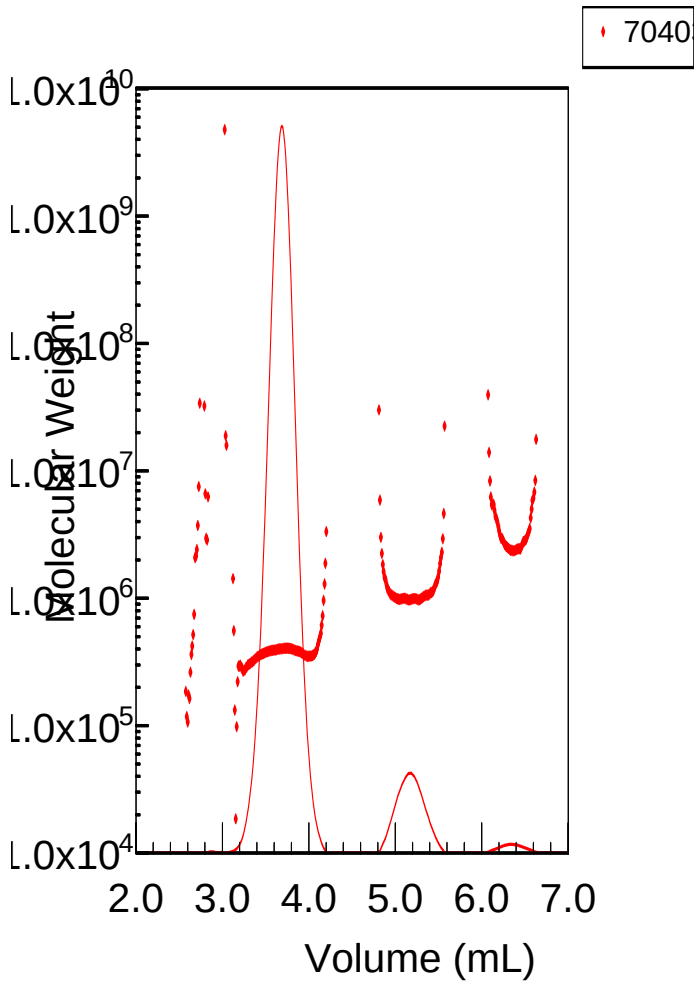
The carrier was 0.025 M phosphate buffer at pH 6.5. *V*in = 7.9 mL/min; *V*out = 0.5 mL/min; *V*c = 7.4 mL/min. Temperature: 25 °C. Channel thickness: 122 μm. Ultrafiltration membrane: Hoechst NADIR UF10-C regenerated cellulose.

Field-Flow Fractionation Handbook (2002); Courtesy of Mikael Nilsson, Technical Analytical Chemistry, Lund University,(1997)

Protein aggregates analysed by
flow FFF-TALS-RI

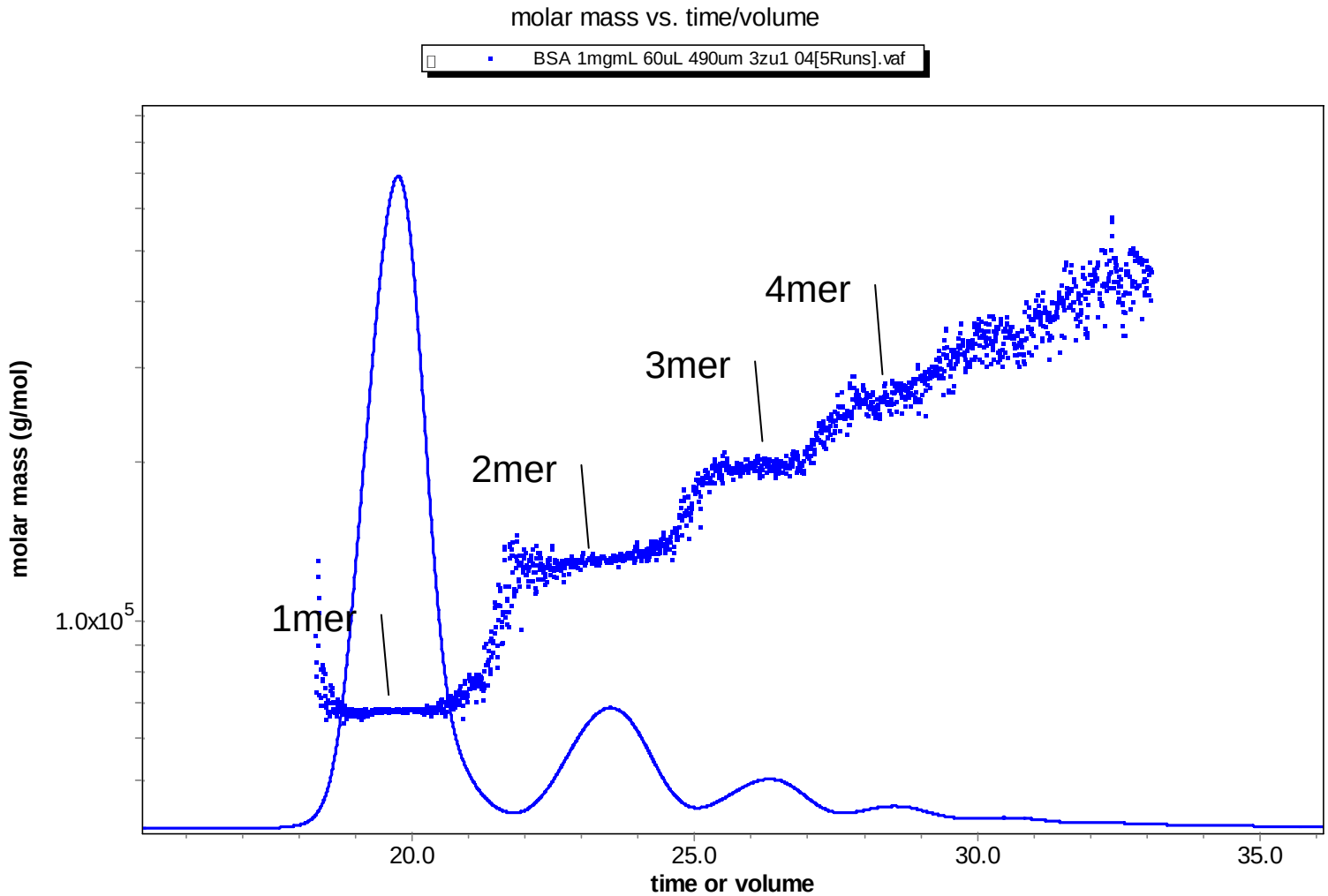
Eluent outlet from asymmetrical flow field-flow fractionator coupled on-line to a flow-through triangle laser light scattering (TALS) detector and a refractive index (RI) (or UV) detector.
Protein: ferritin (440 kDa); flowrate: 1.28 ml/min





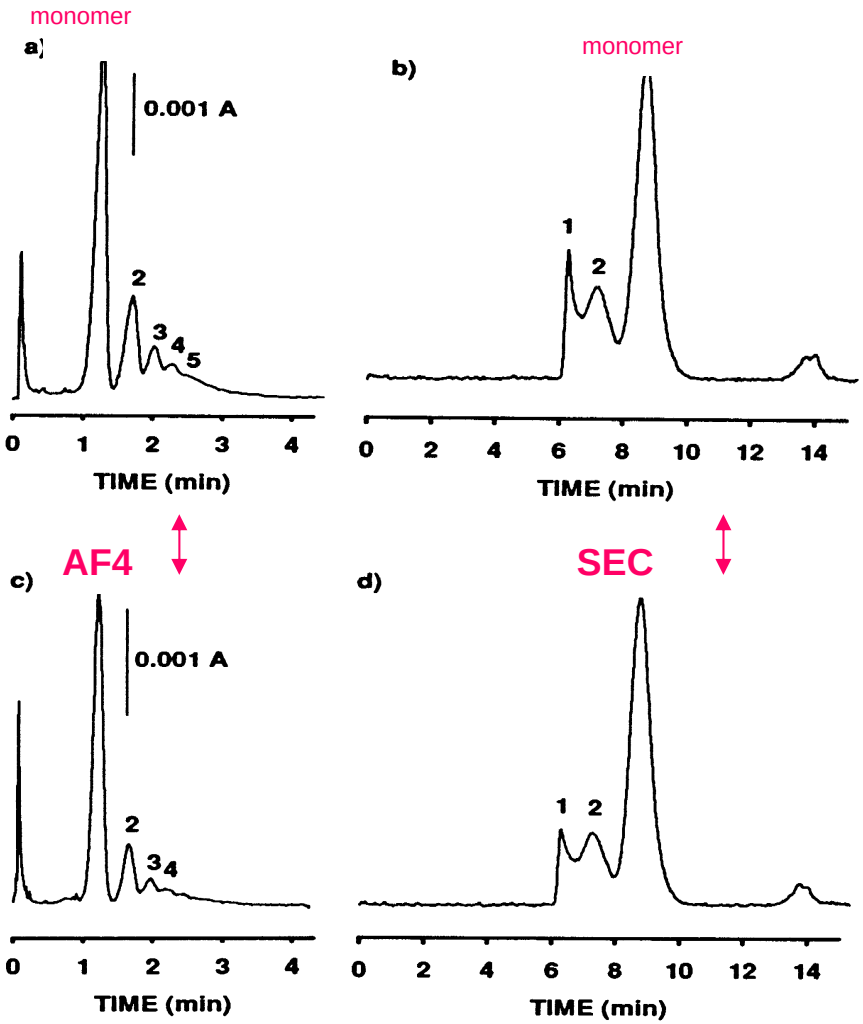
The strong size dependence of the scattering signal facilitates aggregate detection

Measurement on BSA



Courtesy Dr. Christoph Johann, Wyatt Technology Europe.

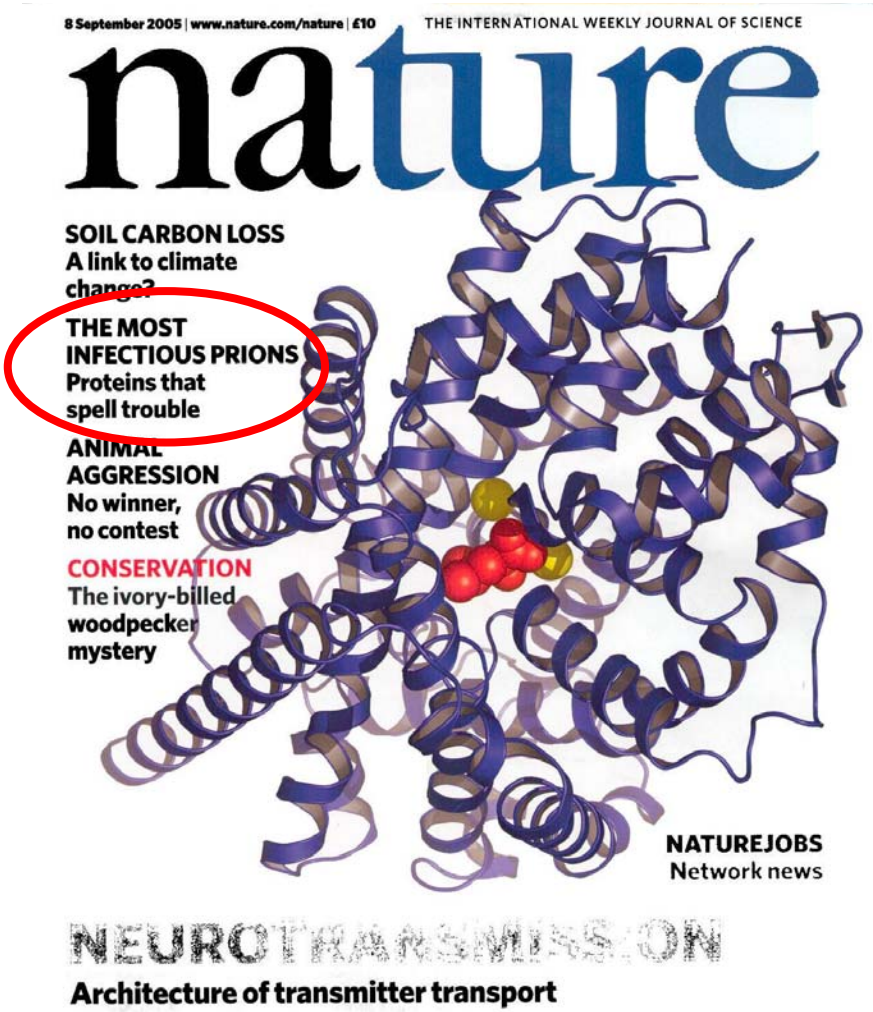
AF4 AND SEC
Analysis of two Monoclonal Antibodies

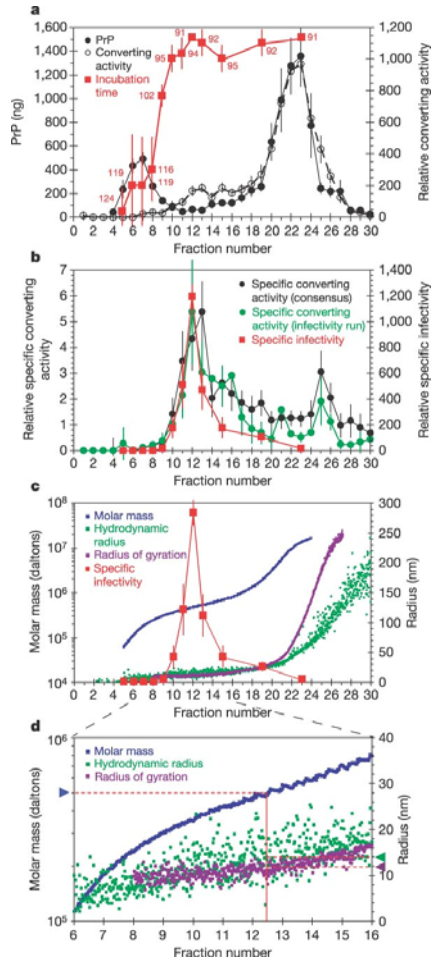


SEC: TSK Column, G 3000 SW. Flow rate: 1.0 mL/min.

Reproduced from:
Litzén, Walter, Krischollek and Wahlund,
Anal. Biochem. 212 (1993) 469-480.

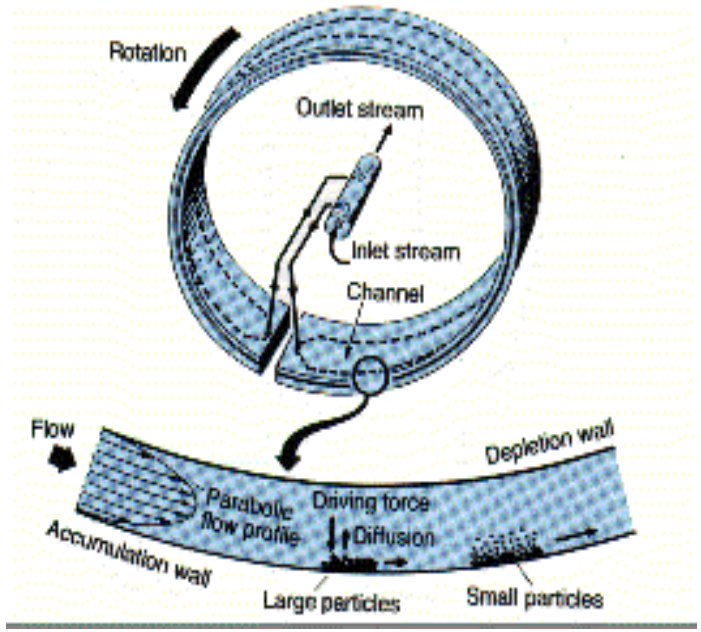
RECENT HIGH PROFILE USE OF AF4





J.R. Silveira et al., Nature **437** (2005) 257-261

The SdFFF system

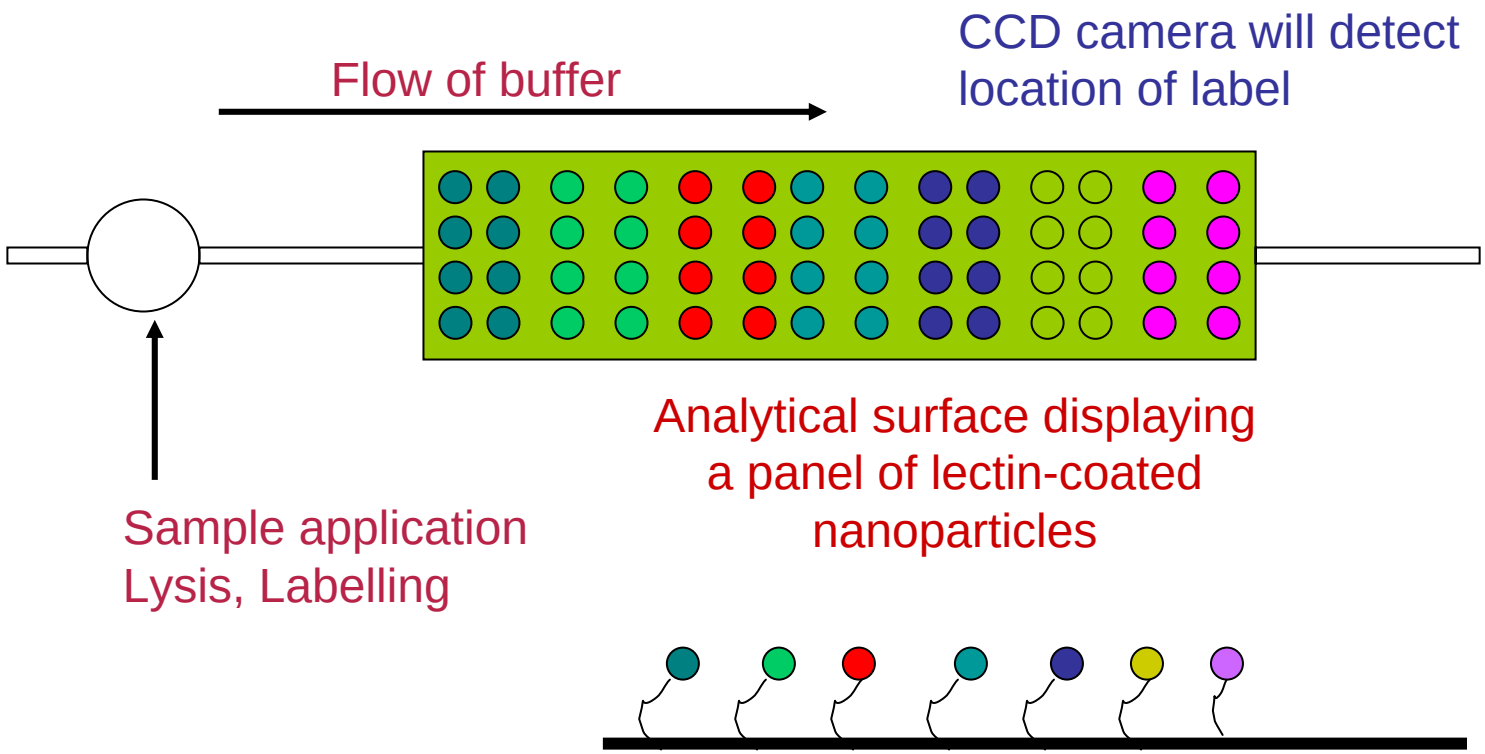


$R = t^0 / te \approx 6\,\lambda$

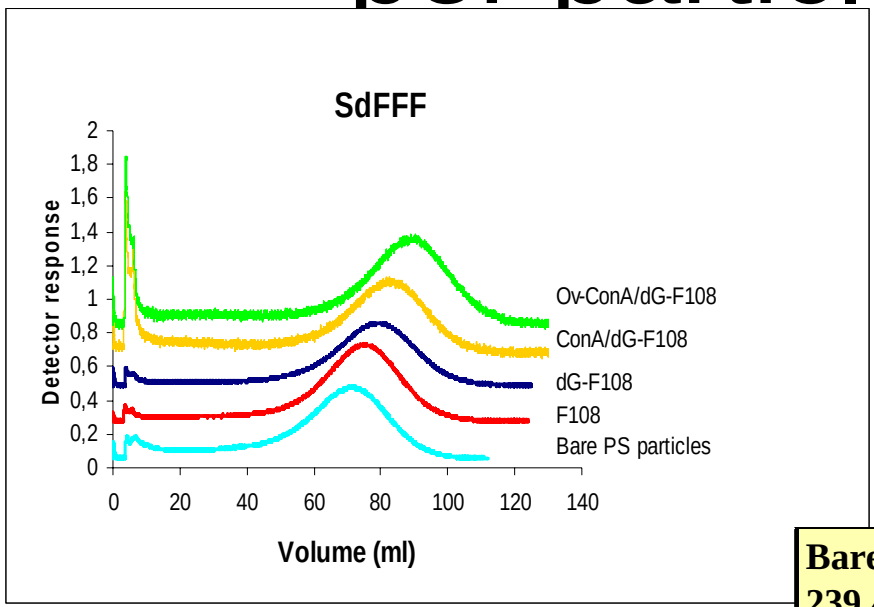
Homogenous: $\lambda_{SFFF} = kT/[m(\Delta\rho/\rho_p)\,Gw] = 6kT/[d^3\Delta\rho\pi Gw]$

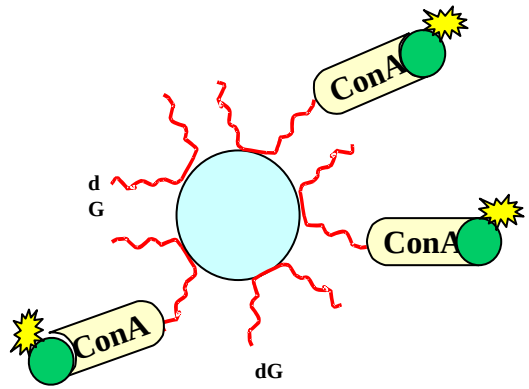
Layered: $\lambda_{SFFF} = kT/[m_A(1-\rho_{car}/\rho_A) + m_B(1-\rho_{car}/\rho_B) + + +]Gw$

Aim:
The Nanoparticle Microarray Platform for Glycoprotein Mapping



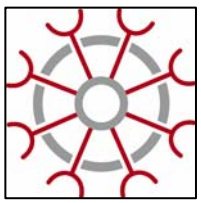
Quantification of mass uptake per particle





Precision: 9.4×10^{-18} g/PS particle

= 55 ConA molecules/PS particle



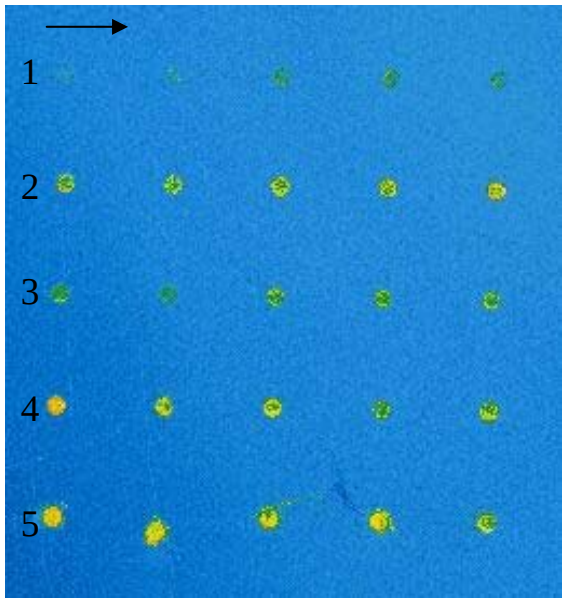
2.8 Ovalbumin/ConA ⇦

ConA has retained high activity

Bare PS: 239.4 ±1 nm	**Surface conc (g/particle)**	**No of Molecules/ particle**
F108-PDS	$3,5\times10^{-16}$	14400 ± 400
dG	$5,2\times10^{-17}$	6200 ± 1000
ConA	$1,2\times10^{-16}$	700 ± 55
Ovalbumin	1.5×10^{-16}	2000 ± 125

Fromell et al.*Colloids and Surfaces B. Biointerfaces*, (2005) **46,** 84-91.

Glycoprotein binding to latex-ConA



1.3-5 ng protein/sample

Row	*Sample*	*Intensity*
1	HSA	171 ± 108
2	Ovalbumin	1301 ± 540
3	Fetuin	714 ± 144
4	Thyroglobulin	1311 ± 468
5	Man-BSA	3765 ± 1157

SUMMARY

- Field-Flow Fractionation takes place in unobstructed channels with minimal shear stress on sample components
- Migration is well described by existing theoretical models
- The working field strength is selected by the operator for optimal resolution of a given sample
- FFF-LS provides real-time evidence for mass/size selectivity, especially suited for detection of aggregates
- Through sedFFF analysis of lectin-decorated particles and their interactions with glycoproteins we can gather information on glycosylation patterns