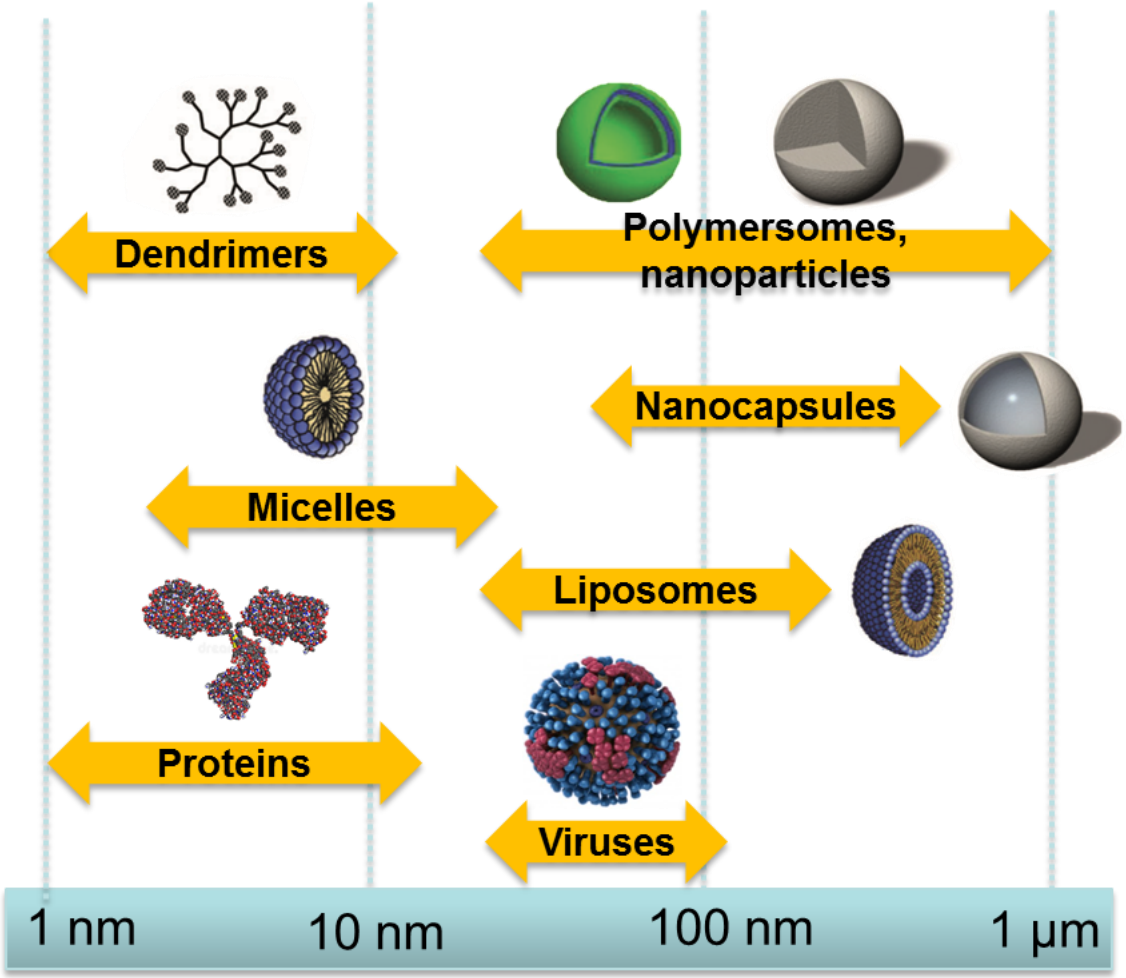
FFF 2.0: Field Flow Fractionation for the comprehensive Characterization of Proteins and Nanoparticles

Reaching the next level to characterize nanoparticles and proteins with Field-Flow Fractionation coupled to Multi-Angle Light Scattering

Dr. Christoph Johann, Wyatt Technology

WYATT TECHNOLOGY

Proteins conjugated with Nanoparticles …

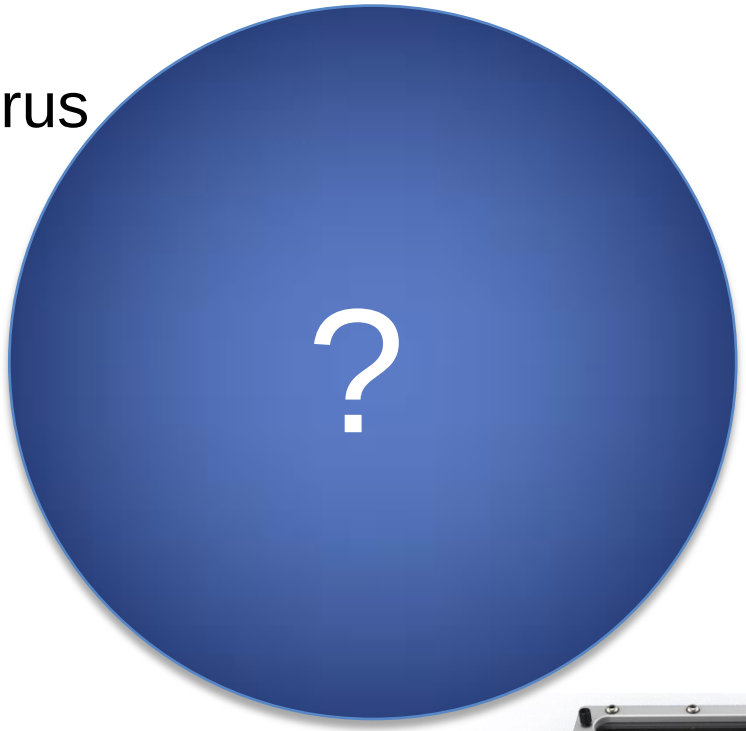


- Drug Delivery
- Molecular Biology
- Industrial applications

… need new analytical methods

Peptide Antibody Virus

HPLC-MS SEC
UHPLC-MS U-SEC



Field-Flow Fractionation

Eclipse FFF: Why use Field-Flow Fractionation

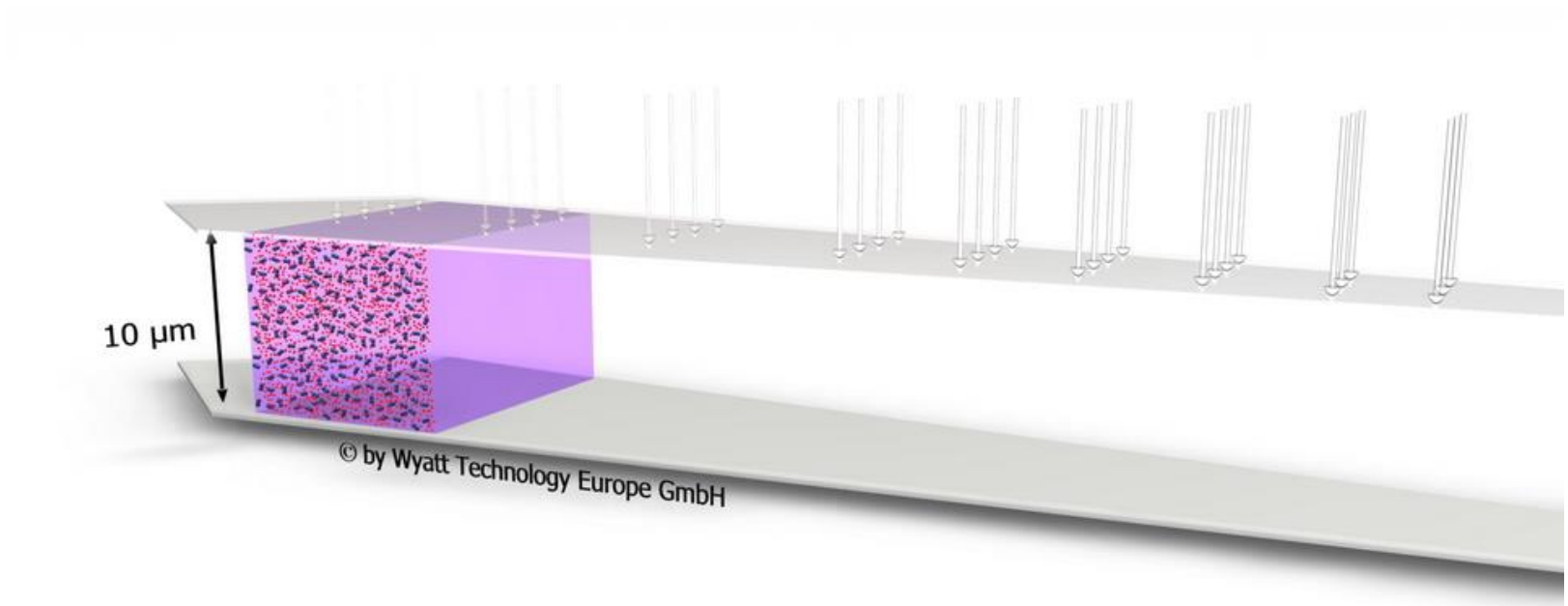
- True particle size distribution, can produce fractions
- High resolution
- Wide separation range from small macromolecules (nm) to large particles (µm)
- Powerful for analysis complex samples
- Gentle, low shear and non-destructive
- On-line absolute characterization from MALS
- Determines charge and charge distribution
- Robust and easy to use

WYATT TECHNOLOGY

Wyatt Eclipse – Complete System

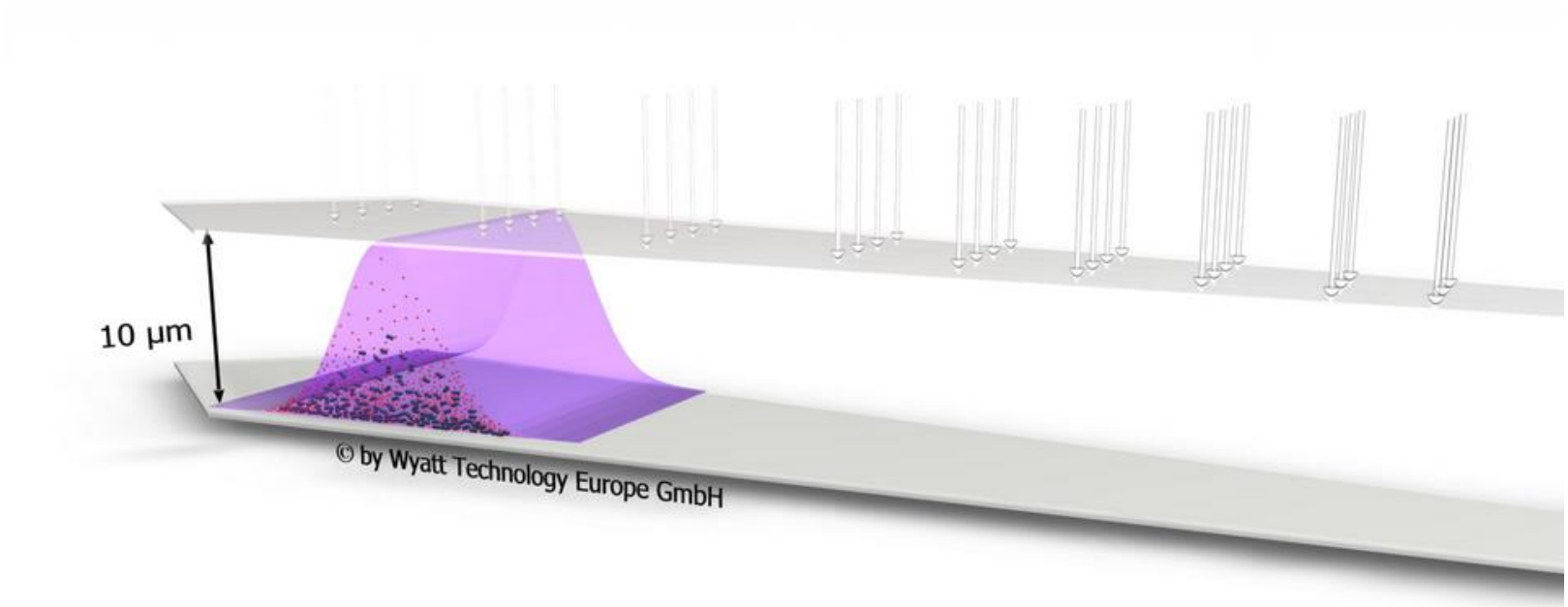
- Seamlessly integrated and automated system
- Sophisticated data processing of shape, conformation, charge distribution
- Software supported method development

How Flow-FFF Separation Works



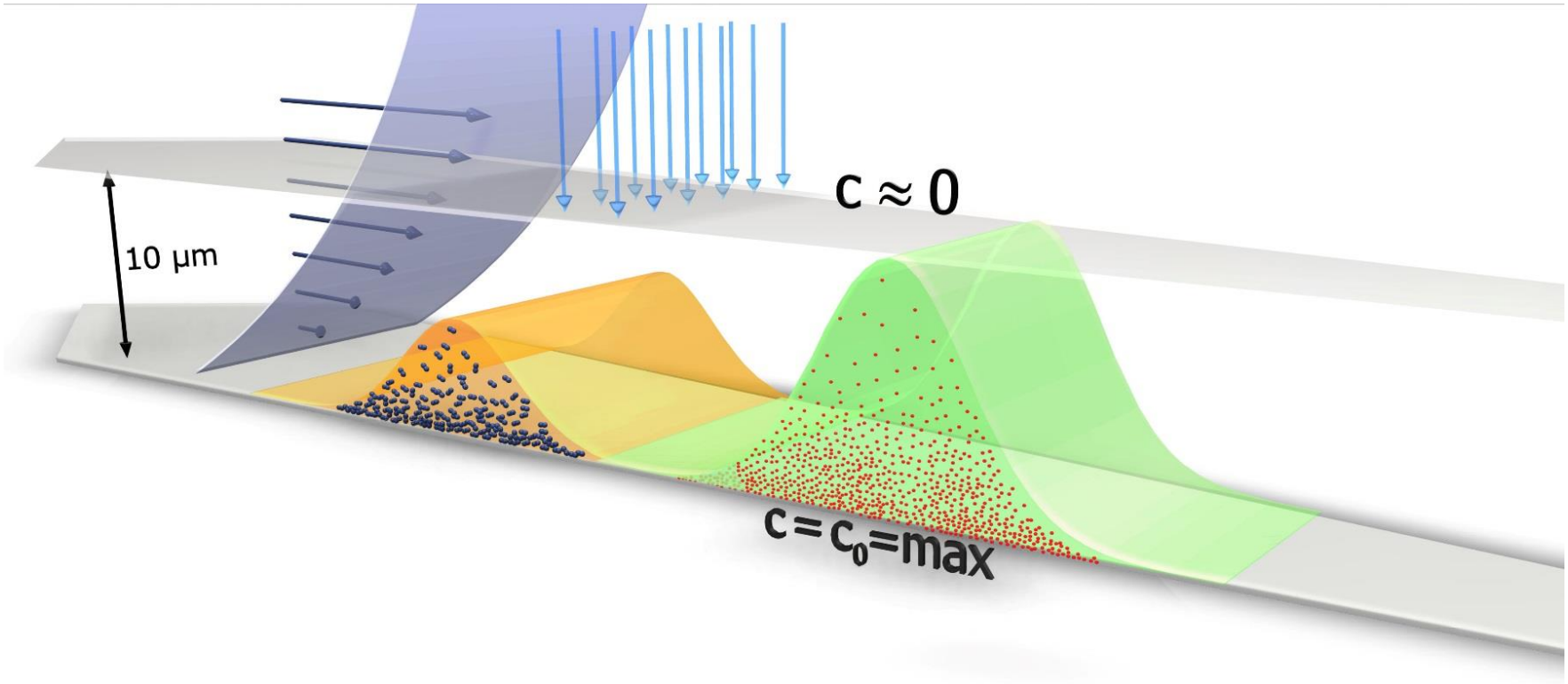
- Sample injected – by interaction with the cross-flow it is concentrated against the porous bottom wall

How Flow-FFF Separation Works



- The electrical field is added after focusing has taken place

How Flow-FFF Separation Works



- Separation based on diffusion against a cross-flow in a laminar flow stream

Flow-FFF - Retention Equation

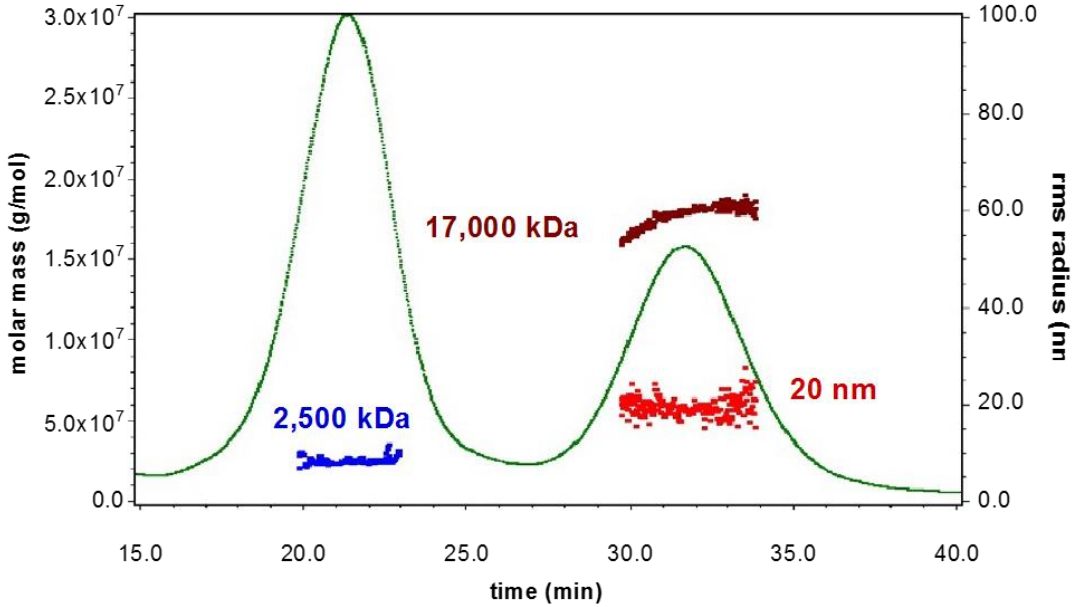
$$t_R = \frac{w^2}{6D_i} \ln\left(1 + \frac{F_x}{F_c}\right)$$

t_r retention time
w channel thickness
F_x cross-flow rate
F_c flow rate to the detector
D_i diffusion coefficient

[1] R. N. Qureshi, Wim Th. Kok, LCGC Europe Jan 2010

Retention depends on the flow rate ratio only, not the length or width of the channel (with given D_i and channel height)

Human Polyoma VPL particles for gene delivery



- Analysis is critical for optimizing VLP reassembly

Citkowicz, A., et al. (2008). "Characterization of virus-like particle assembly for DNA delivery using asymmetrical flow field-flow fractionation and light scattering." Anal Biochem **376**(2): 163-172, **2008**

VISION – integrated software suite

Method development with SCOUT

- Define a set of sizes which represent the sample

Sample Definition

Time Table & Flows | Separation Device | Sample & Experiment

Components

	ID	Description	Mass Fraction [%]	Hydrodynamic Radius [nm]	Electrophoretic Mobility [10^{-8} $m^2/(V \cdot s)$]
	1	3020A	30,00	11	
▶	2	3050A	50,00	23	
	3	3100A	20,00	51	
*					

Solvent Properties

Solvent Water

Temperature 25,00 °C Conductivity 0 S/m

Inject

Volume 20 µL Concentration 3,4 mg/mL

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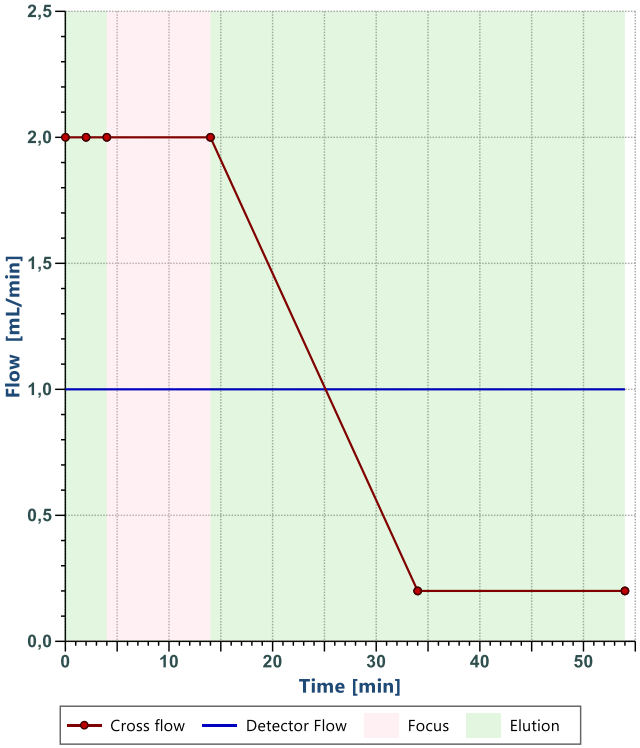
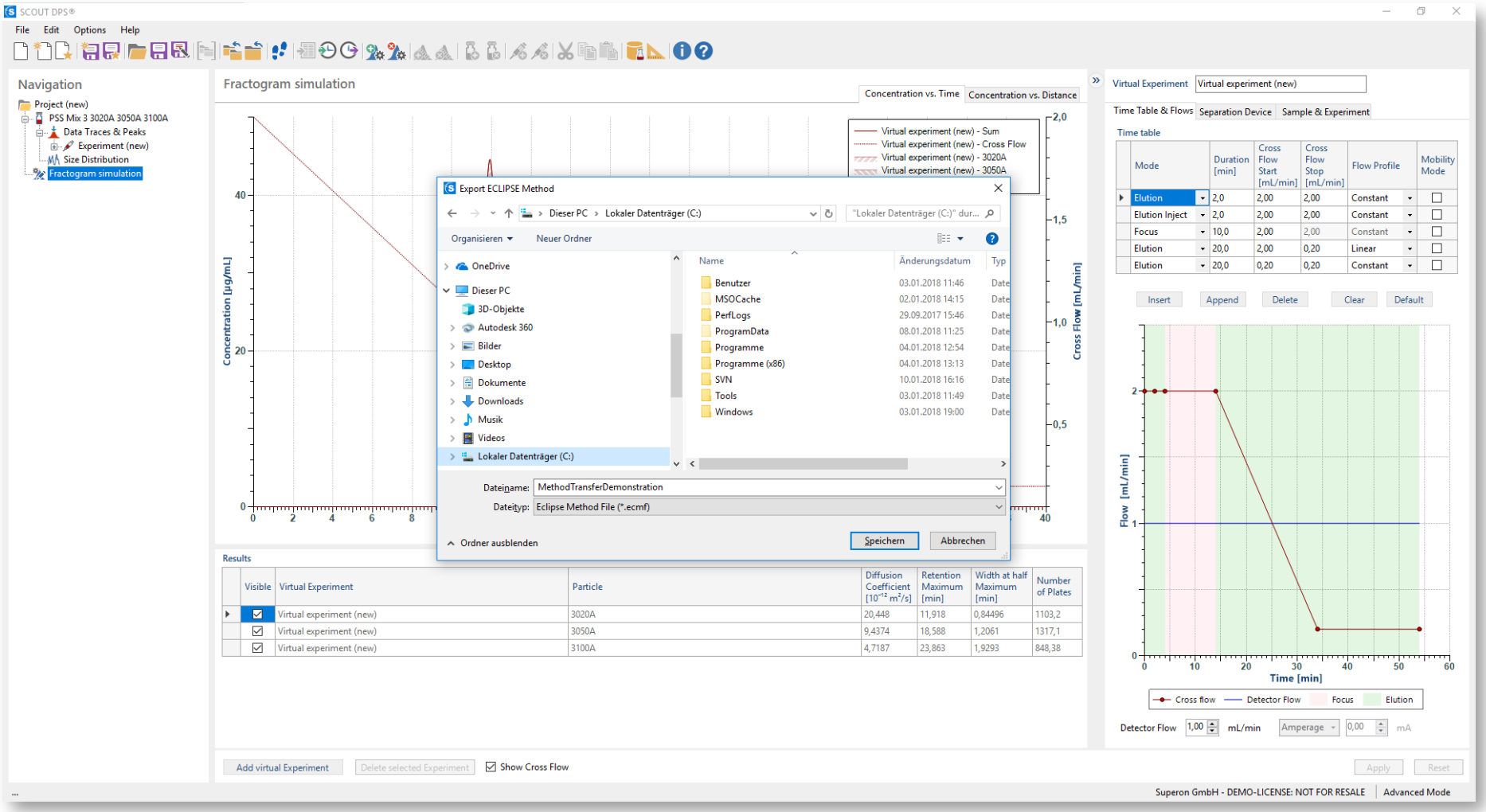
Method development with SCOUT

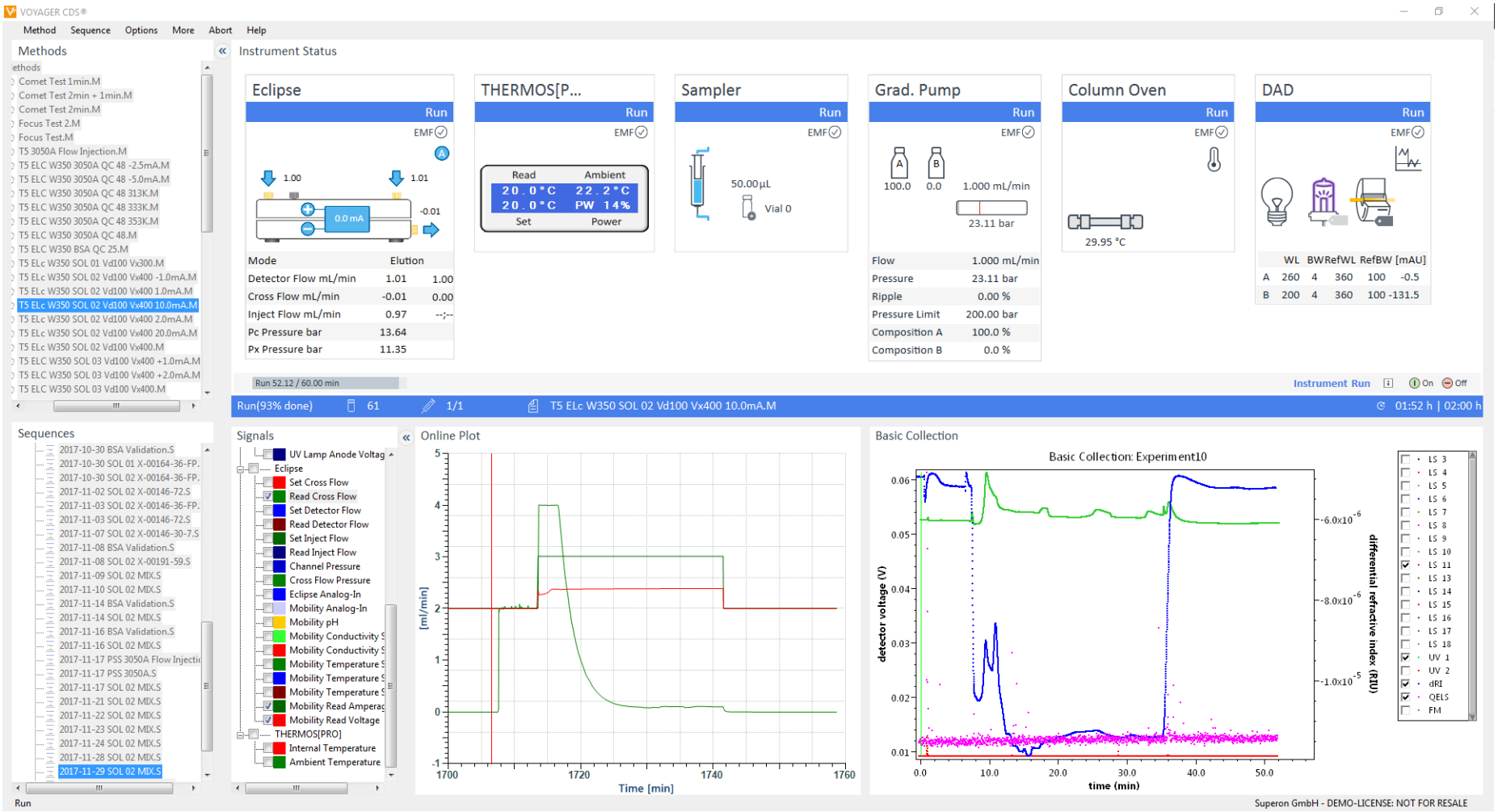
- Define a set of sizes which represent the sample
- Select a channel geometry
- Develop the flow program to show a satisfactory separation
- Export the method to VOYAGER and run it



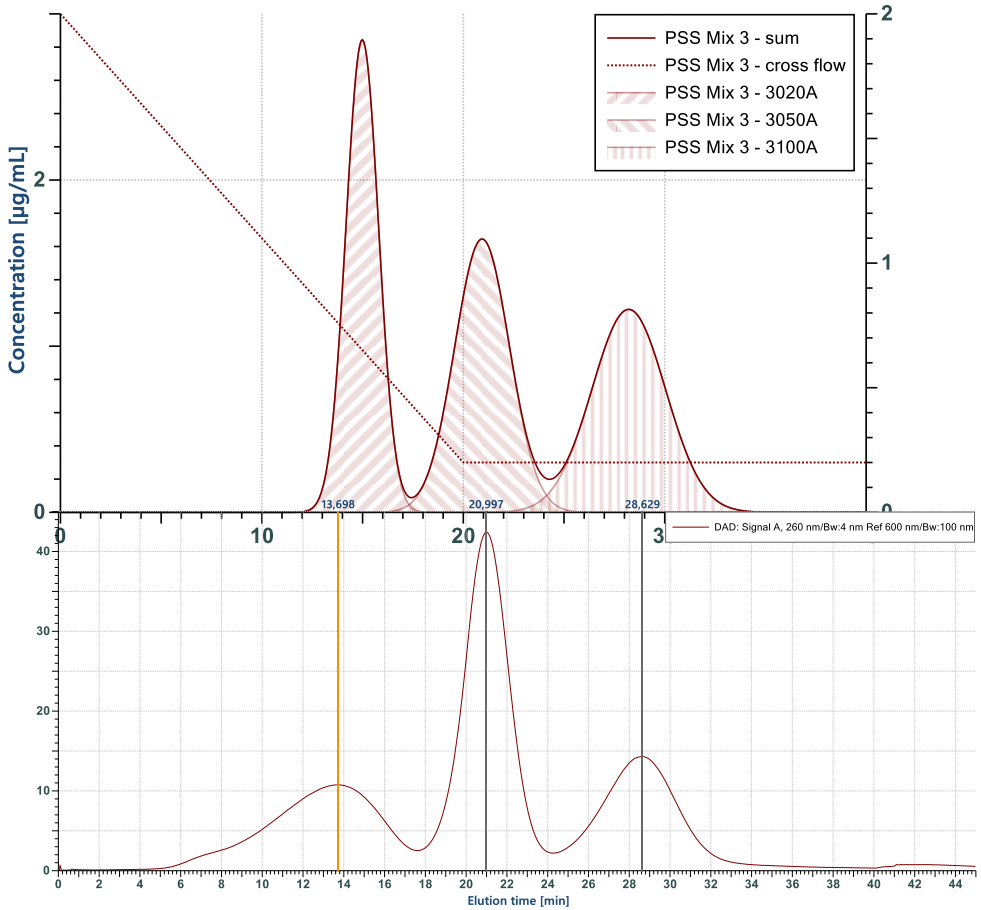
Method development in SCOUT II



Running a sample sequence in VOYAGER

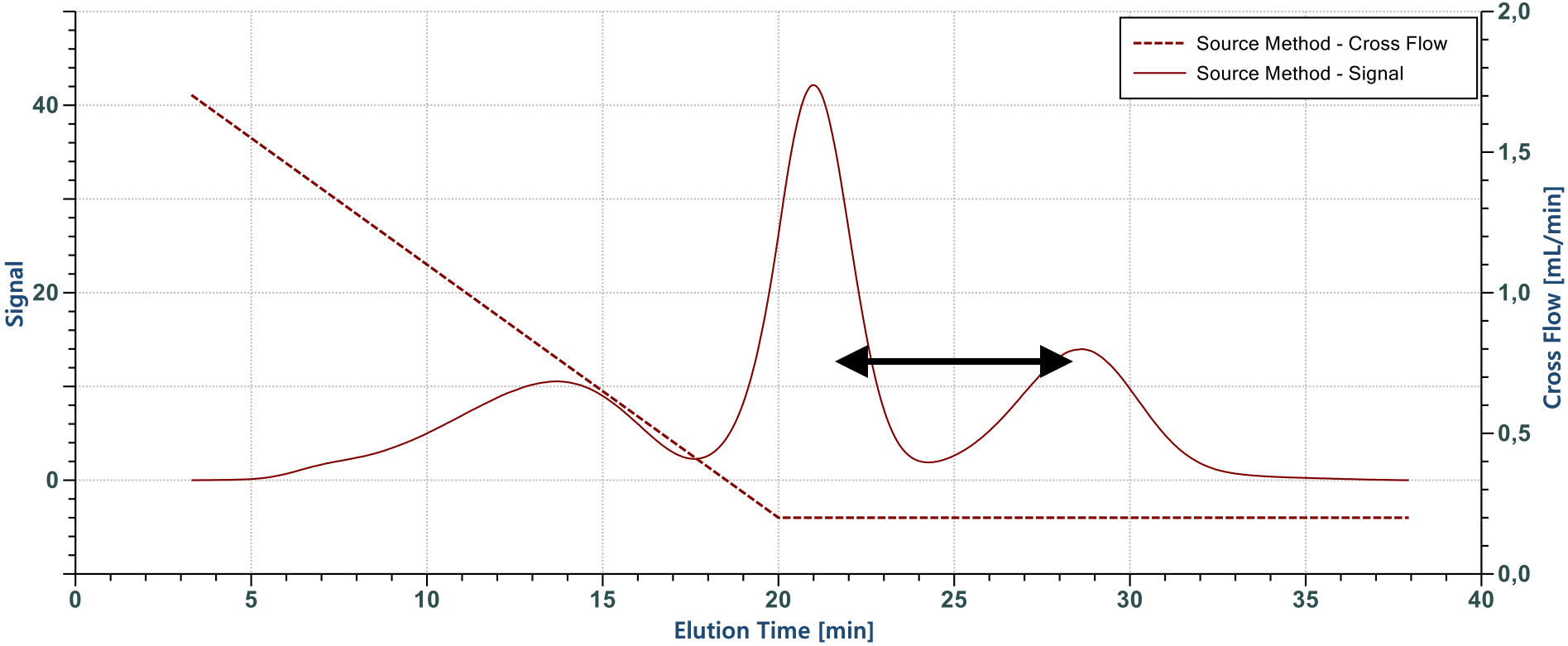


Comparison of simulation and experiment

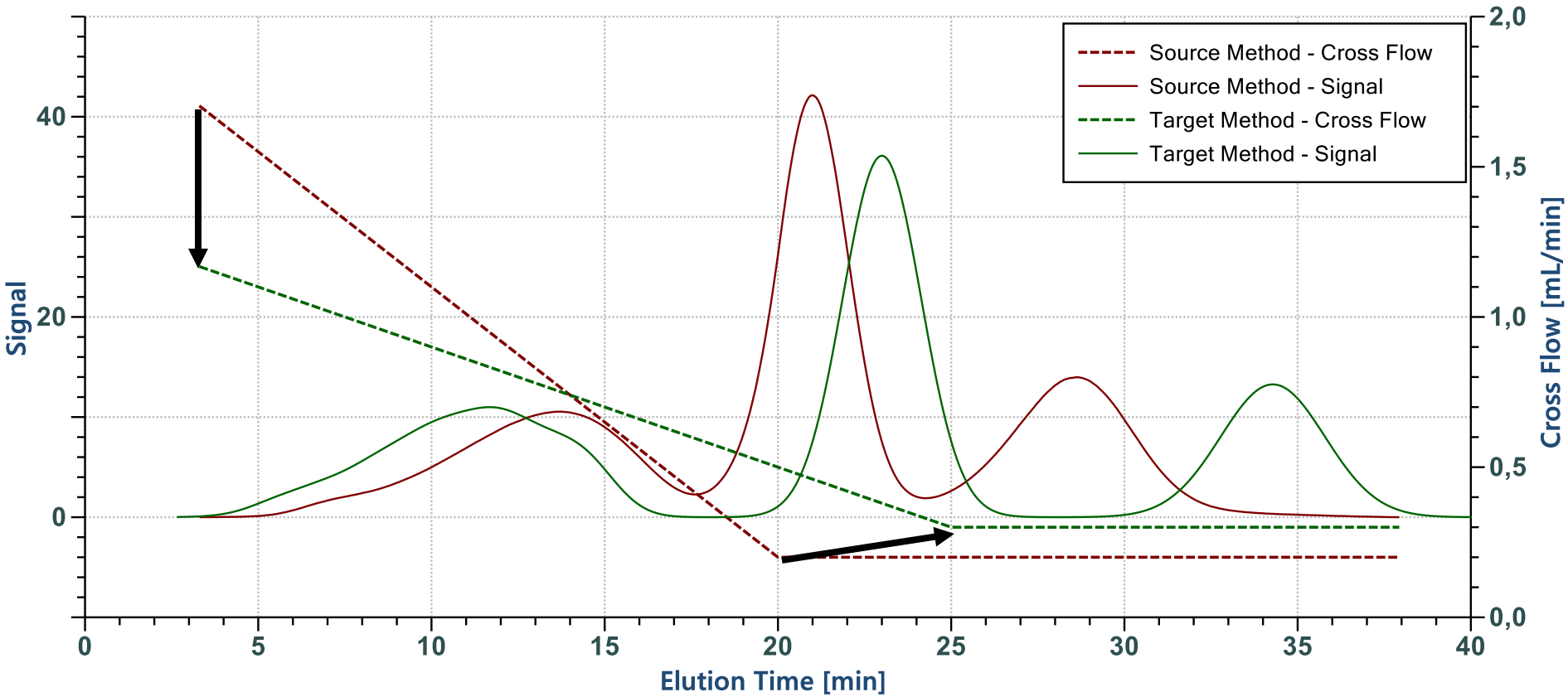


- Simulation for the three polystyrene latex samples
- Experiment with excellent agreement to the simulation

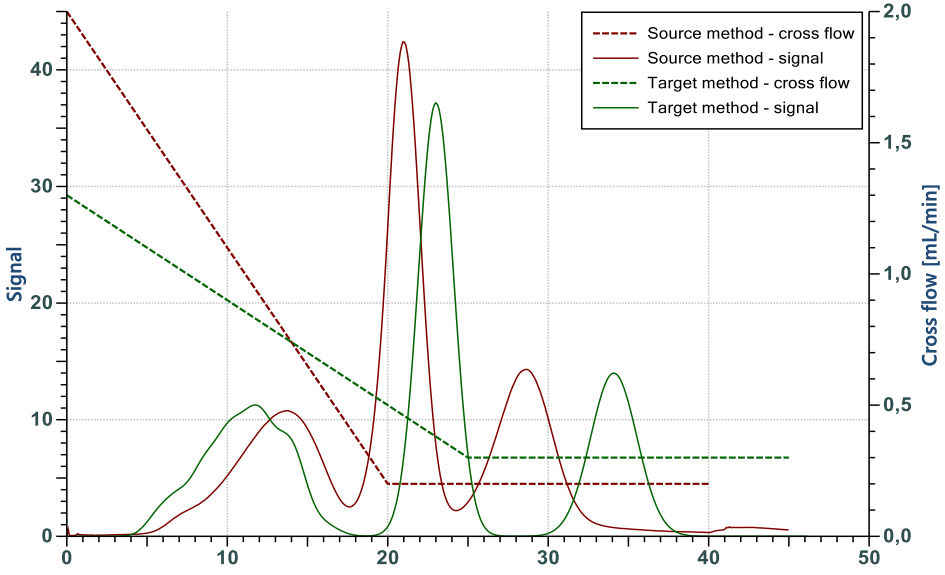
Method Optimization in SCOUT

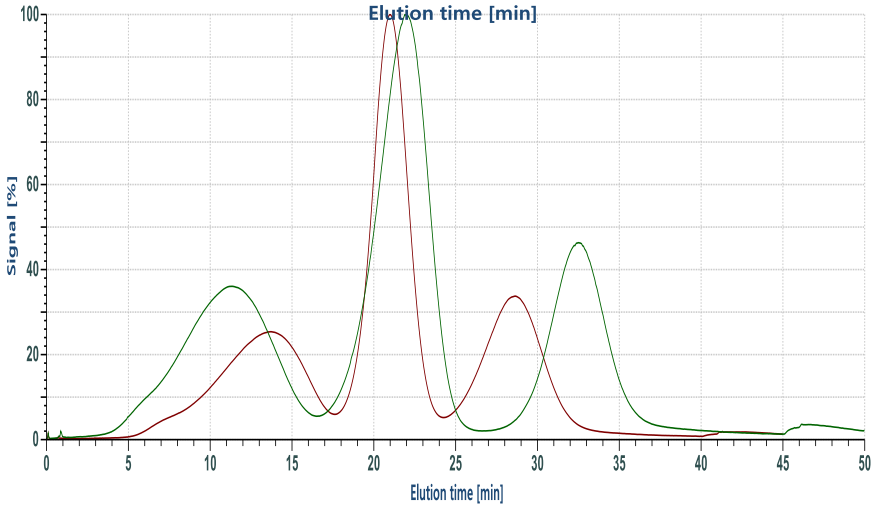


Method Optimization in SCOUT



Comparison simulation and experiment





- Overlay of experiment and simulation
- Overlay of the initial and optimized experiment

Size distribution based on calibration

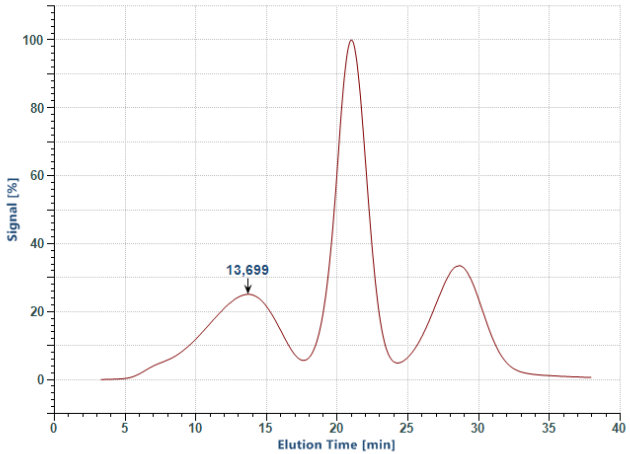
SCOUT DPS® - C:\Users\Public\Documents\SCOUT DPS®\Examples\PS mix.scpf

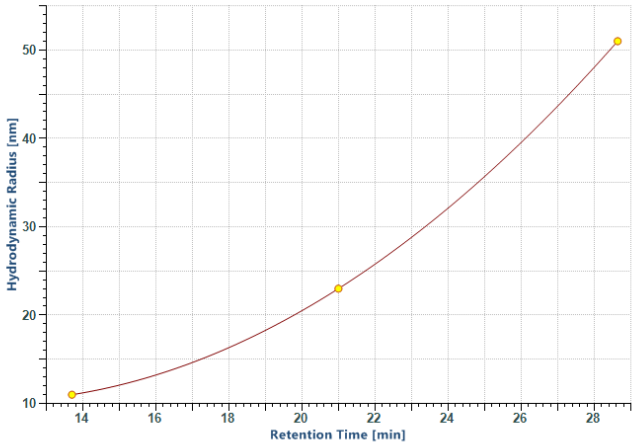
File Edit Options Help

Navigation

- Latex particles
  - PS Size Standard
    - Data Traces & Peaks
    - Size Distribution
  - No1
    - Data Traces & Peaks
    - Size Distribution
    - Electrophoretic Mobility
  - No2
    - Data Traces & Peaks
    - Size Distribution
    - Electrophoretic Mobility
  - No3
    - Data Traces & Peaks
    - Size Distribution
    - Electrophoretic Mobility
  - Size Distribution
  - Electrophoretic Mobility Comparision
  - Fractogram Simulation
- Work Flow
  - PSS Mix 3 3020A 3050A 3100A
    - Data Traces & Peaks
      - PSS Mix 3
        - ECLIPSE® Method
        - Signal Alignments
        - Components
        - Effective Height
        - Hydrodynamic Radius
    - Size Distribution
    - Fractogram Simulation

Particle Size Calibration





Name: Hydrodynamic Radius

Type
- Effective Height
- Molecular Weight
- Hydrodynamic Radius

Edit Values used for Calibration

Peak ID	Retention Time [min]	Hydrodynamic Radius [nm]	Enabled
1	13,699	11	☑
2	20,994	23	☑
3	28,638	51	☑
*			☐

Calibrate with Function

Regression Order 2

☐ Force through Origin

☐ Add (0, 0)

$Y = a + b X + c X^2$ $r^2 = 1$

a = 27,32 ± 0

b = -3,042 ± 0

c = 0,1351 ± 0

Reference from File | Reference from Experiment PSS Mix 3 | Apply | Reset

Wyatt Technology Europe GmbH - DEMO-LICENSE: NOT FOR RESALE | Dongle expires in 125 days | Advanced Mode

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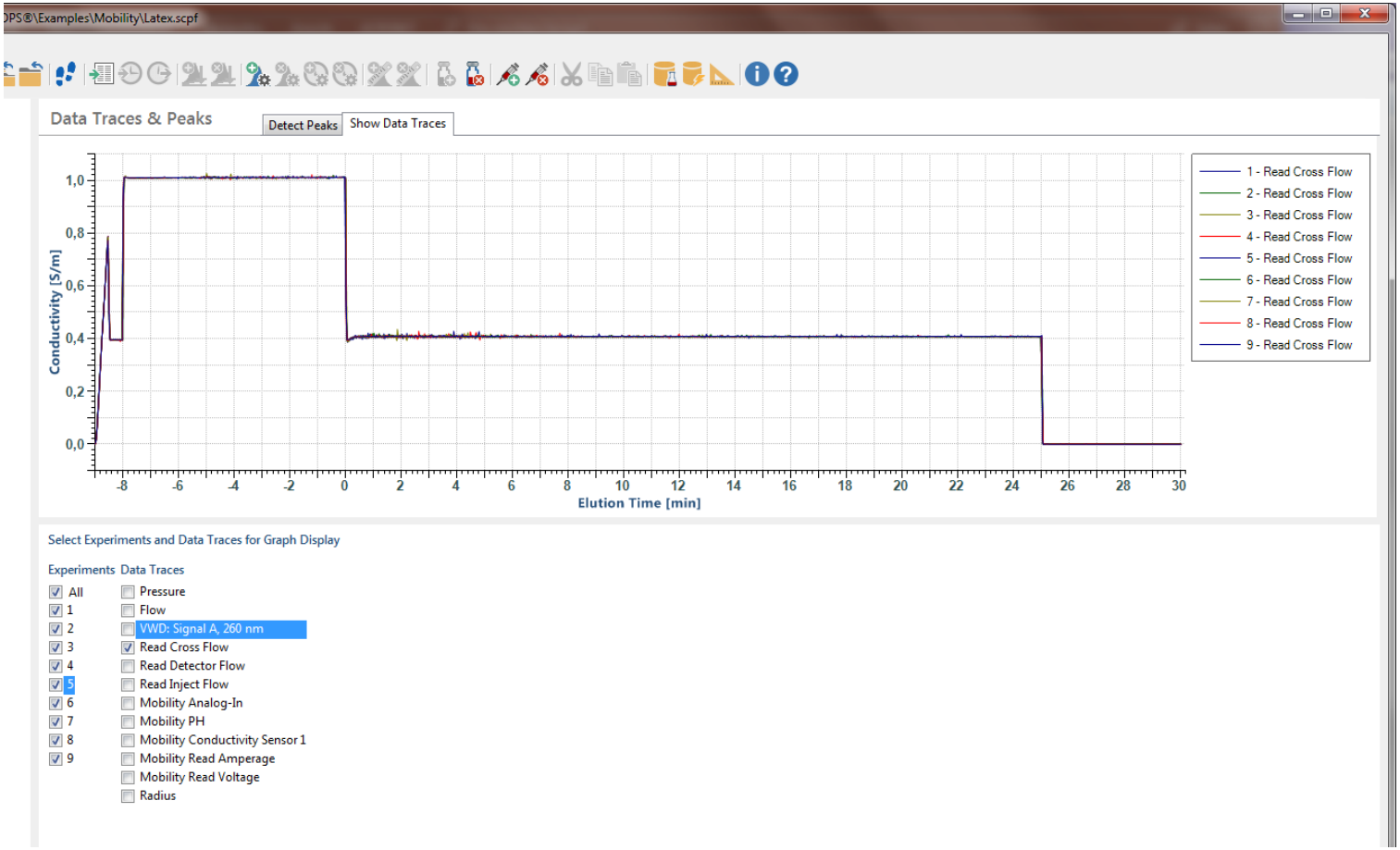
Post run view of system traces

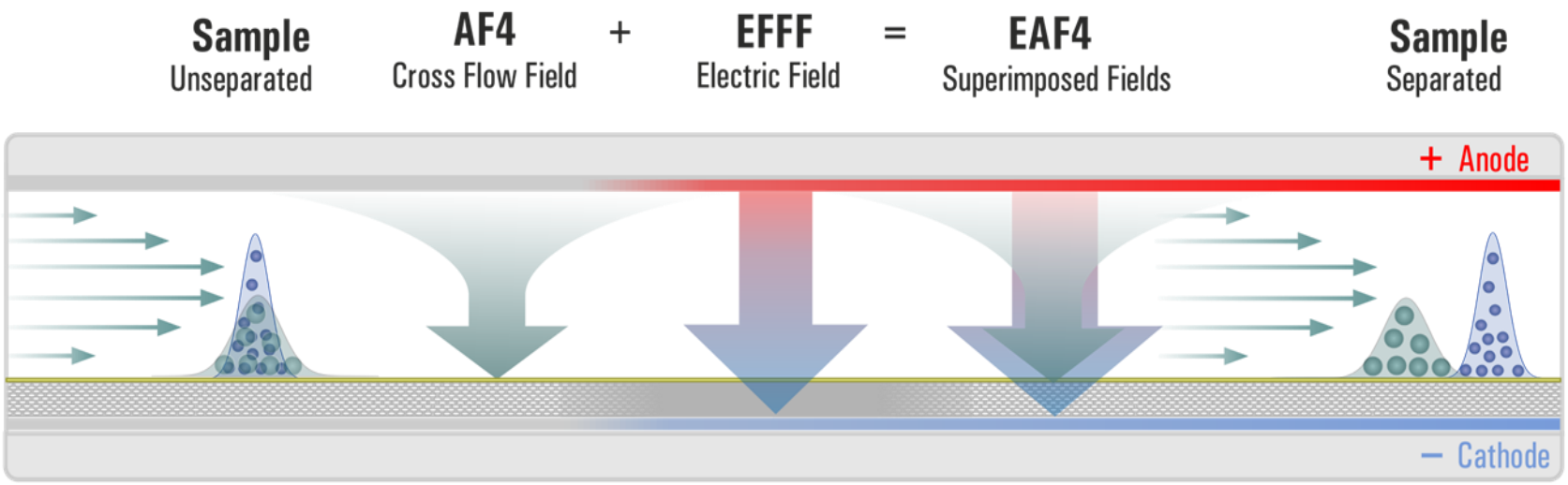


Comprehensive set of results

- Hydrodynamic radius
- Electrophoretic mobility
- Zeta potential
- Conductivity
- pH values
- Temperature

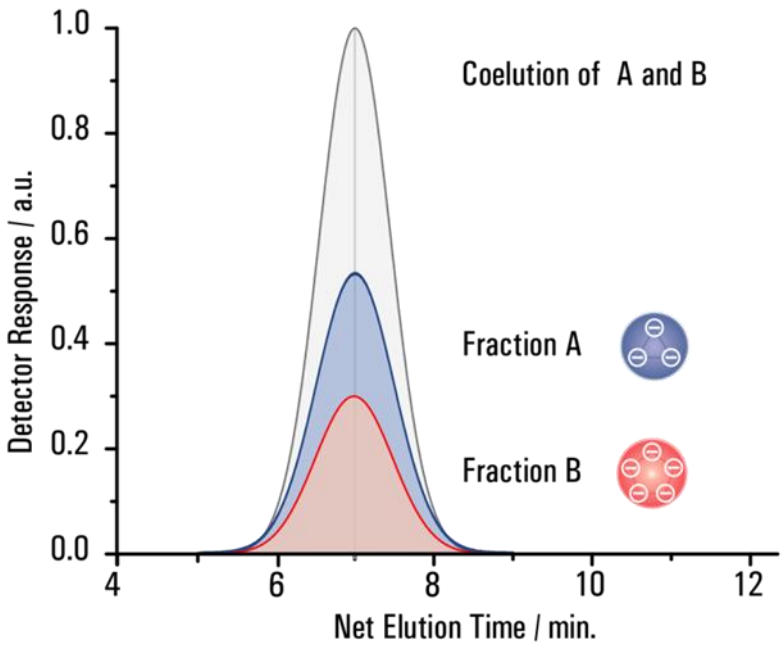
VISION
+
MOBILITY

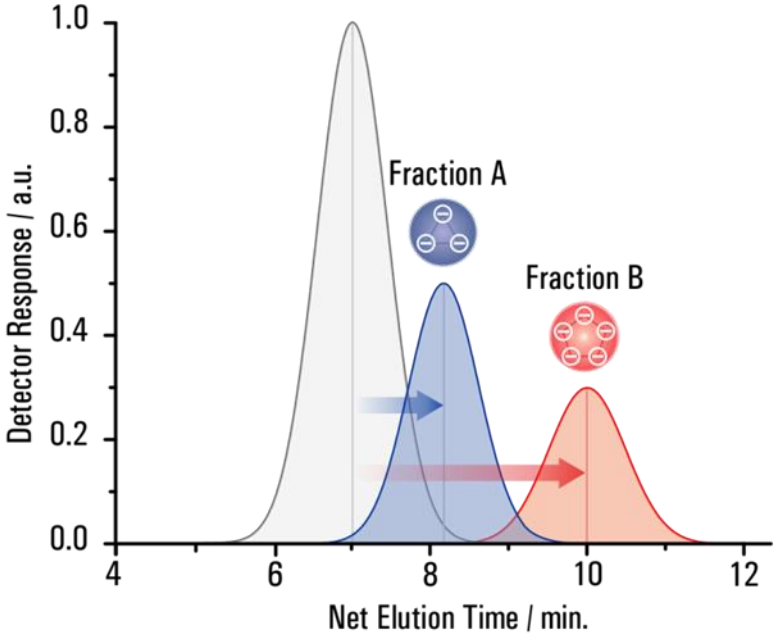
Principle of Electrical Flow-FFF



- EAF4 uses both force fields to generate a separation plus measurement of electrophoretic mobility

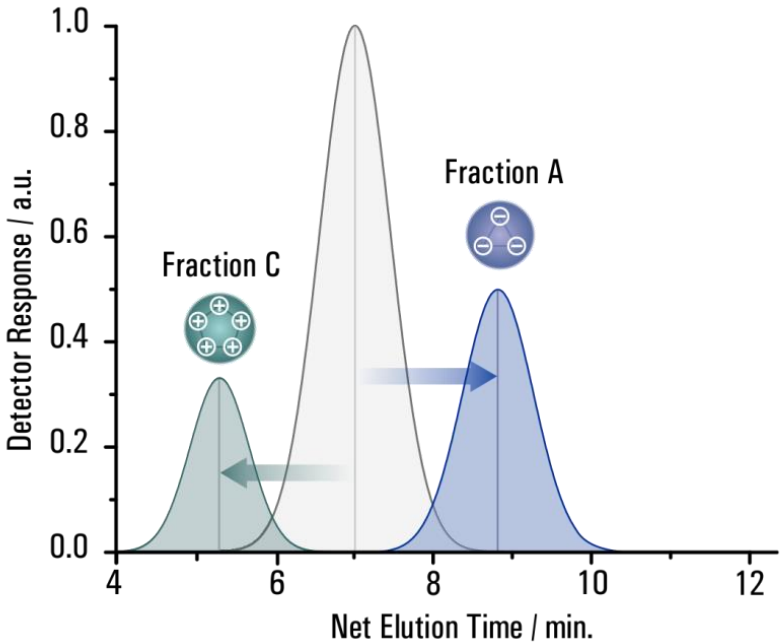
Improving Separation I

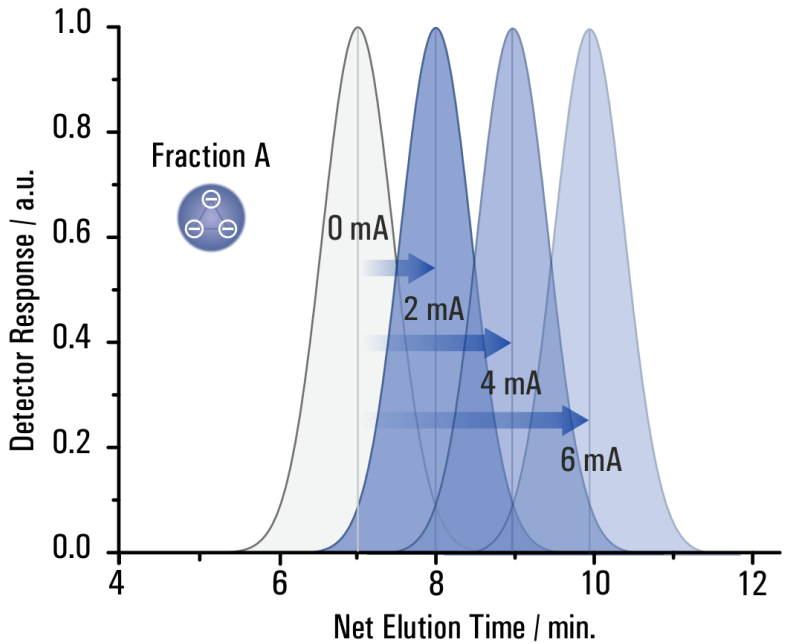




- Species of same size but different charge can be separated

Improving Separation II





- Different charge will move to shorter and longer retention time
- With a series of increasing electrical field, the shift will increase proportionally

Theory I

$$t_R = \frac{w^2}{6D} \cdot \ln(1 + \frac{f \cdot F_c}{F_{out}})$$

- Retention in A4F depends on the ratio of flow rates
- Consider mobility and electrical field

$$\mu = \frac{v_{EP}}{E} \qquad E = \frac{U}{d} = \frac{I \cdot R}{d} \qquad E = \frac{I}{A_{el} \cdot k}$$

- μ can be determined, if the drift velocity v_{EP}, electrical current I and the conductivity k are known, therefore conductivity has to be measured
- Under the influence of a cross-flow and the electrical field E the drift velocity has two components

$$v = v_c + v_{EP} \qquad v_c = \frac{F_c}{A_{el}}$$

Theory II

$$v_{EP} = \left(e^{\frac{t_{Ri} \cdot \ln\left(1+\frac{f \cdot F_c}{F_{out}}\right)}{t_R}} - \left(1 + \frac{f \cdot F_c}{F_{out}}\right)\right) \cdot \frac{F_{out}}{A_{el} \cdot f}$$

- At constant cross-flow the drift velocity is given by a simple equation
- For cross-flow gradients, a discretization algorithm has to be used
- Knowing size from MALS, DLS or FFF retention time, the zeta potential can be calculated

analytical chemistry

Article

pubs.acs.org/ac

Instrument and Method to Determine the Electrophoretic Mobility of Nanoparticles and Proteins by Combining Electrical and Flow Field-Flow Fractionation

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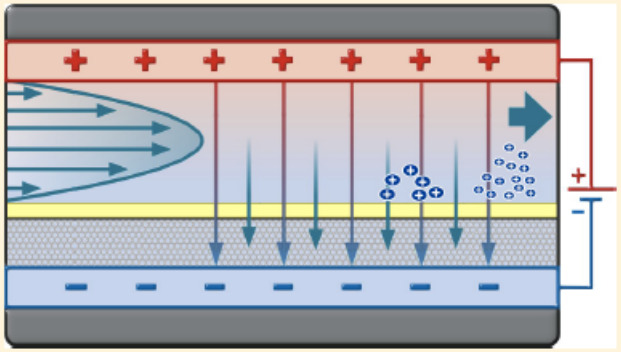
Supporting Information

ABSTRACT: A new FFF method is presented which combines asymmetrical flow-FFF (AF4) and electrical FFF (ElFFF) in one channel to electrical asymmetrical flow-FFF (EAF4) to overcome the restrictions of pure ElFFF. It allows for measuring electrophoretic mobility (μ) as a function of size. The method provides an absolute value and does not require calibration. Results of μ for two particle standards are in good agreement with values determined by phase analysis light scattering (PALS). There is no requirement for low ionic strength carriers with EAF4. This overcomes one of the main limitations of ElFFF, making it feasible to measure proteins under physiological conditions. EAF4 has the capability to determine μ for individual populations which are resolved into separate peaks. This is demonstrated for a mixture of three polystyrene latex particles with different sizes as well as for the monomer and dimer of BSA and an antibody. The experimental setup consists of an AF4 channel with added electrodes; one is placed beneath the frit at the bottom wall and the other covers the inside of the upper channel plate. This design minimizes contamination from the electrolysis reactions by keeping the particles distant from the electrodes. In addition the applied voltage range is low (1.5–5 V), which reduces the quantity of gaseous electrolysis products below a threshold that interferes with the laminar flow profile or detector signals. Besides measuring μ, the method can be useful to improve the separation between sample components compared to pure flow-FFF. For two proteins (BSA and a monoclonal antibody), enhanced resolution of the monomer and dimer is achieved by applying an electric field.



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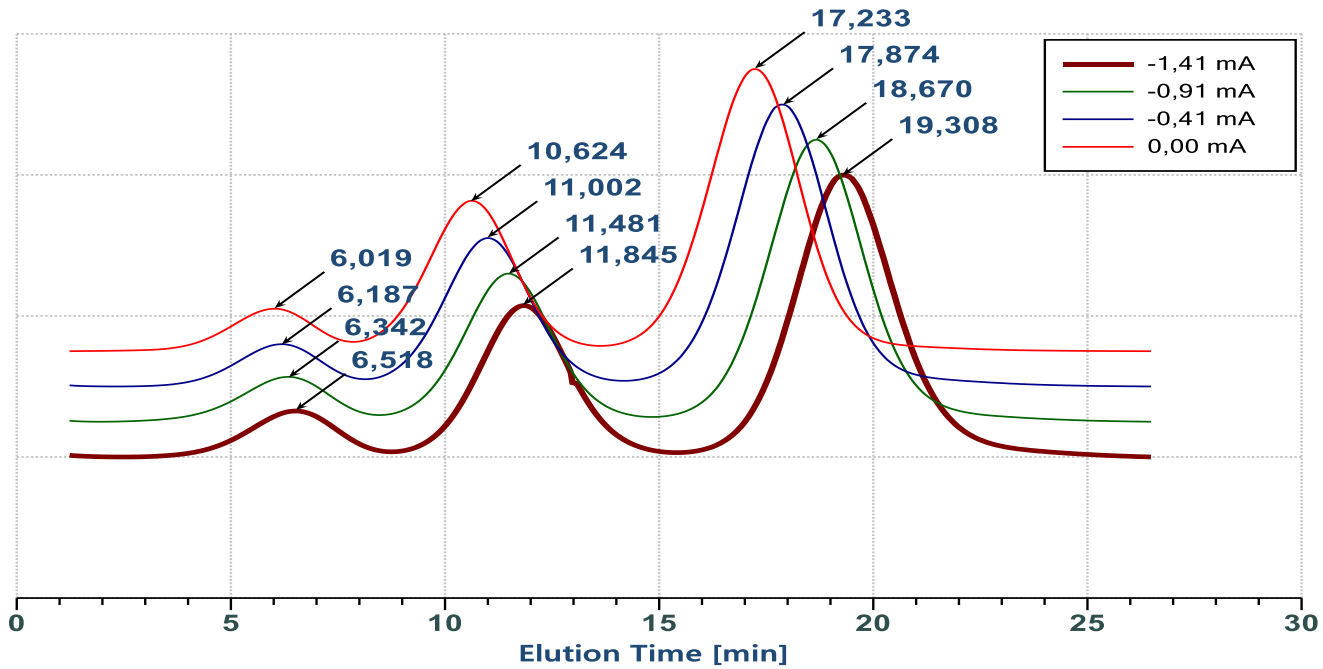
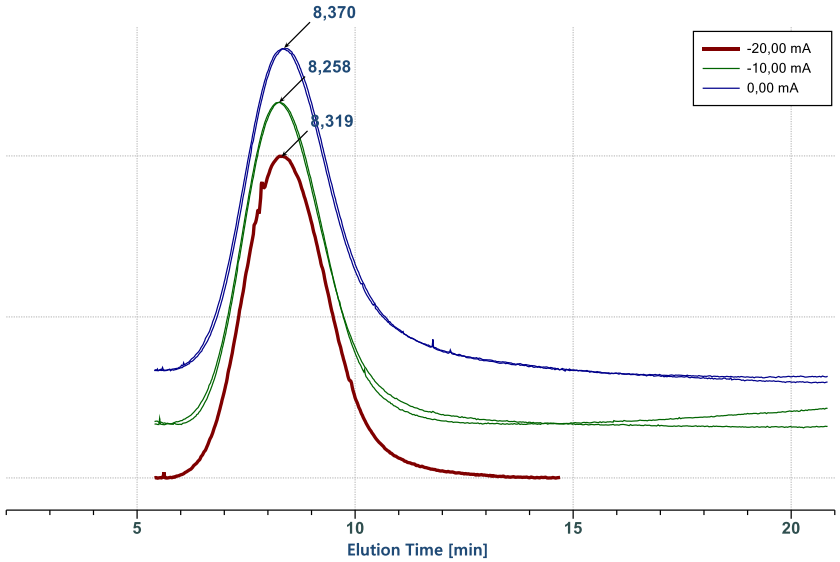
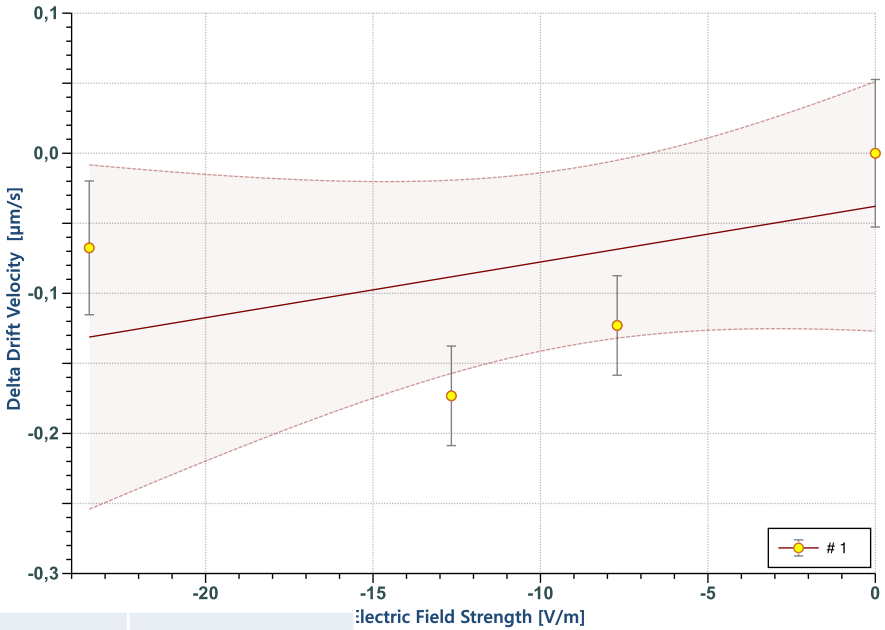
Mix of 3 Duke Latex 23, 46, 102 nm



Hydrodynamic Radius [nm]	Diffusion Coefficient [10^{-12} m²/s]	Electrophoretic Mobility [10^{-8} m²/(V·s)]	Net Charge	Zeta Potential [mV]
21,603 ± 0	10,5 ± 0	-4,2603 ± 0,14	-127,7 ± 4,11	-86,68 ± 2,79
42,669 ± 0	5,315 ± 0	-5,8125 ± 0,17	-405,6 ± 11,6	-117,6 ± 3,37
82,379 ± 0	2,753 ± 0	-5,385 ± 0,13	-925,9 ± 22,9	-107,3 ± 2,65

Native MAb

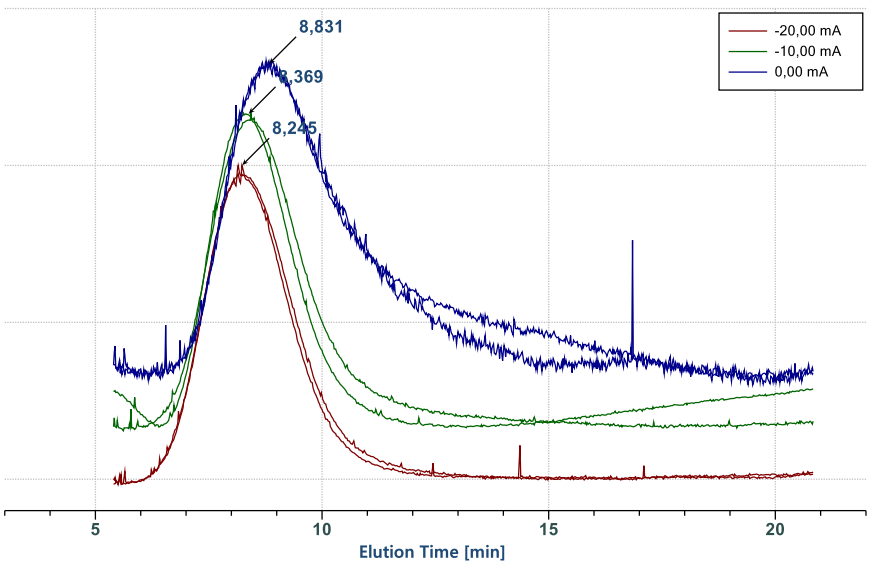


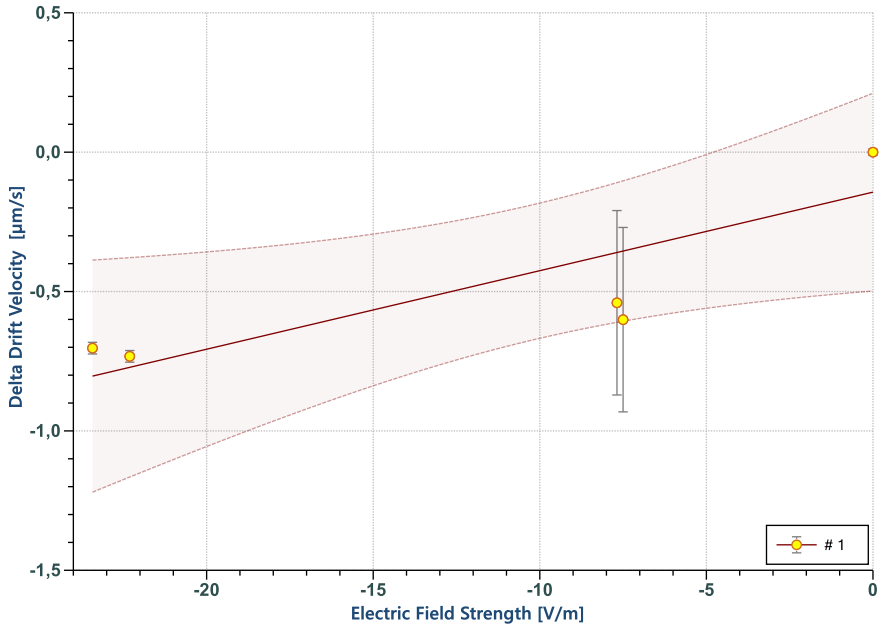


Hydrodynamic Radius [nm]	Diffusion Coefficient [10^{-12} m²/s]	Electrophoretic Mobility [10^{-8} m²/(V·s)]	Net Charge	Zeta Potential [mV]
5,0492 ± 0,0242	48,6 ± 0,23	0,39779 ± 0,38	2,657 ± 2,57	7,628 ± 7,37

- Very small positive charge, minor influence of the field on the peak shape

Stressed MAb

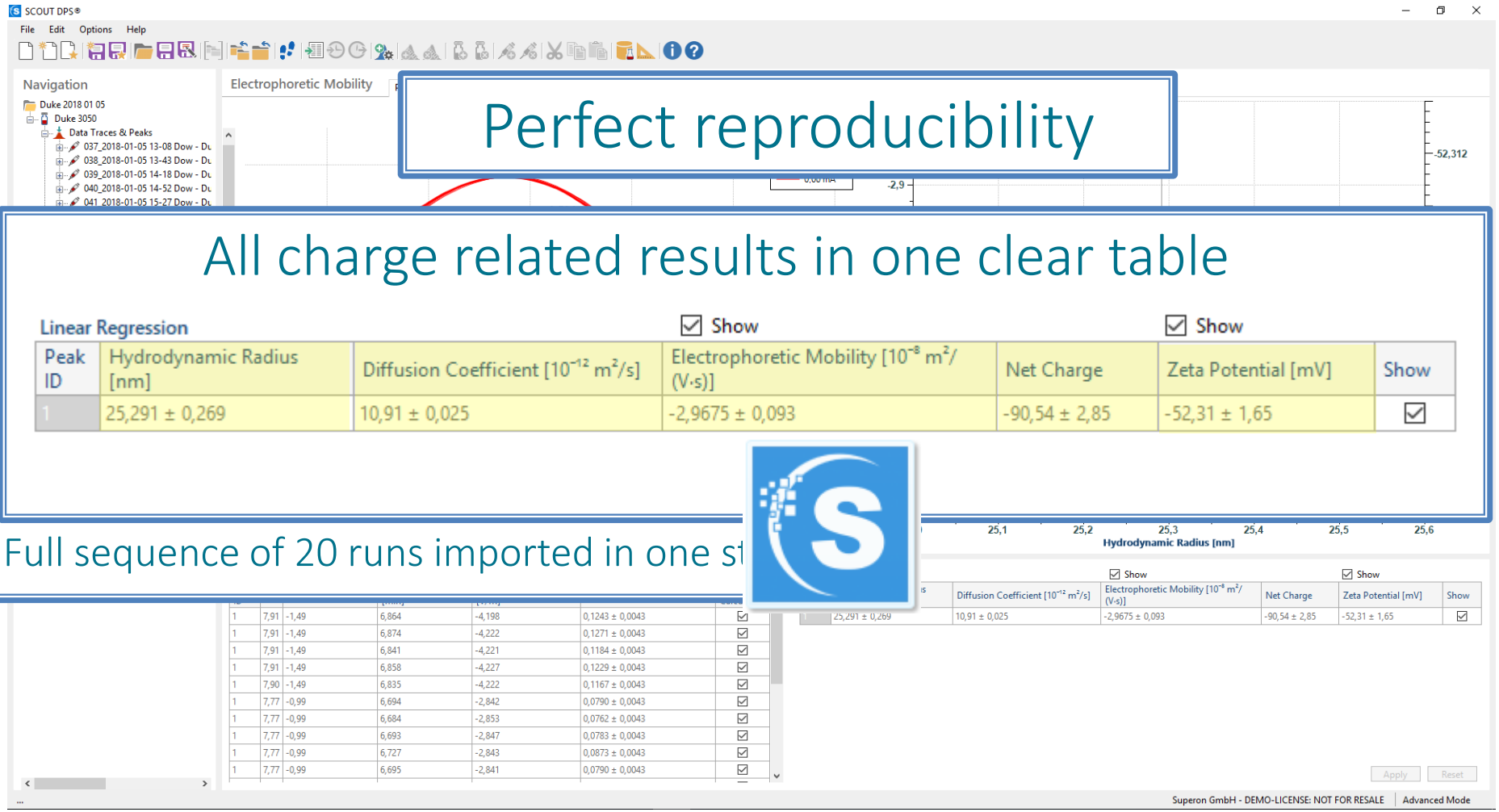




Hydrodynamic Radius [nm]	Diffusion Coefficient [10^{-12} m²/s]	Electrophoretic Mobility [10^{-8} m²/(V·s)]	Net Charge	Zeta Potential [mV]
5,338 ± 0,00812	45,97 ± 0,07	2,8179 ± 0,82	25,17 ± 7,32	53,48 ± 15,6

- Significant higher positive charge, striking strong influence of the field on the peak shape, at -20mA the peak tailing has disappeared

One-Click-Analysis – From template to instant results



Perfect reproducibility

All charge related results in one clear table

Linear Regression

Peak ID	Hydrodynamic Radius [nm]	Diffusion Coefficient [10^{-12} m^2/s]	Electrophoretic Mobility [10^{-8} $m^2/(V \cdot s)$]	Net Charge	Zeta Potential [mV]	Show
1	25,291 ± 0,269	10,91 ± 0,025	-2,9675 ± 0,093	-90,54 ± 2,85	-52,31 ± 1,65	☑

Full sequence of 20 runs imported in one st

Exosome and exomere isolation and characterization

nature cell biology

RESOURCE

https://doi.org/10.1038/s41556-018-0040-4

Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation

- Eclipse AF4 + fraction collector: Isolation of different exosome subpopulations
- DAWN HELEOS MALS with embedded DLS: Identification by size

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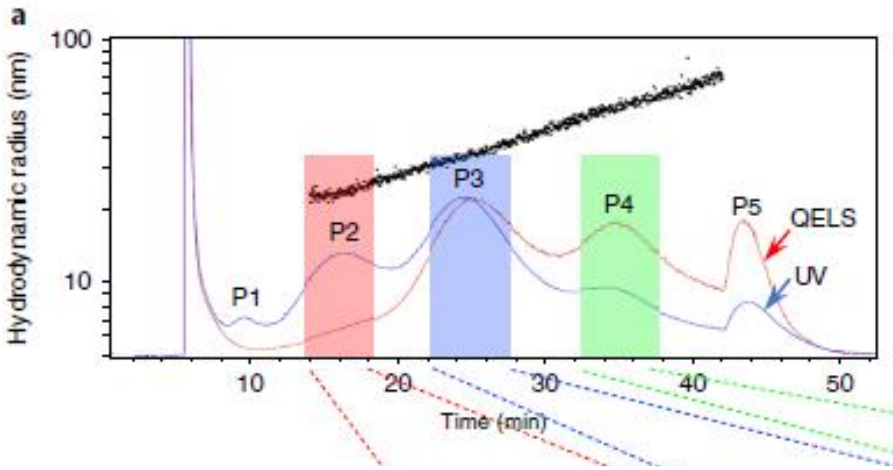
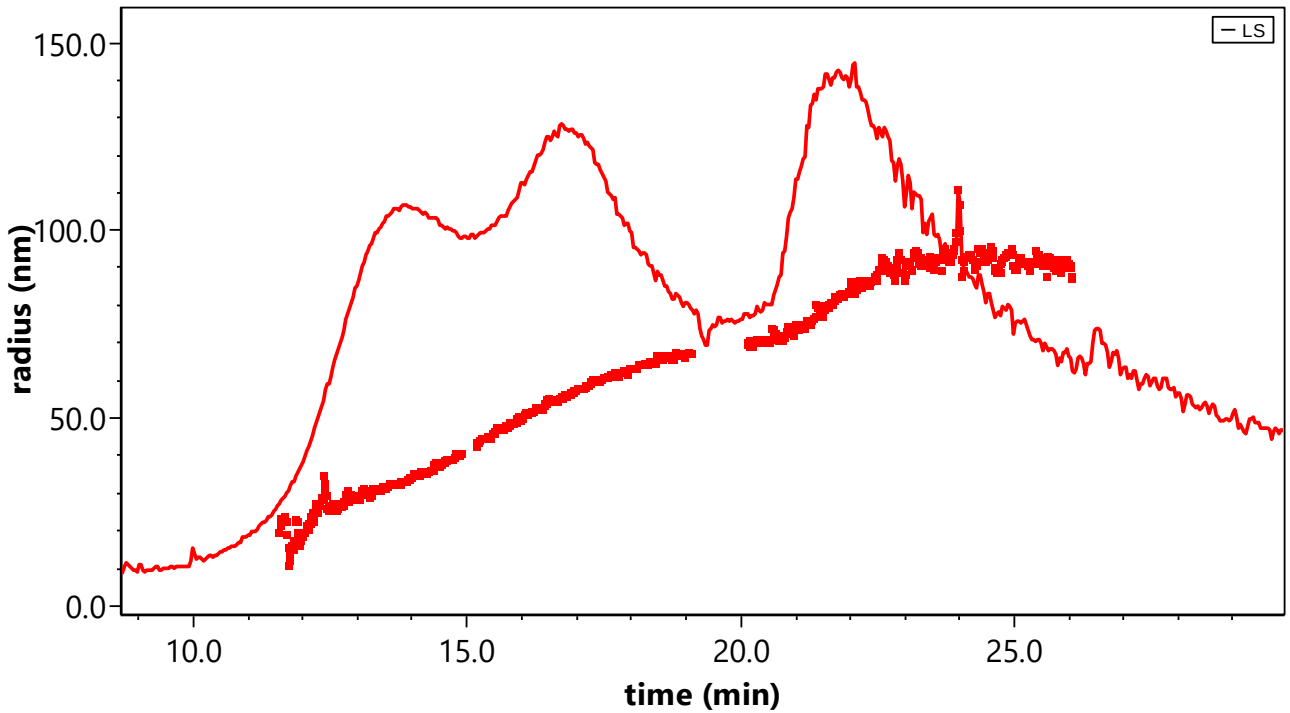
Identification: Radius by online DLS (low sensitivity)



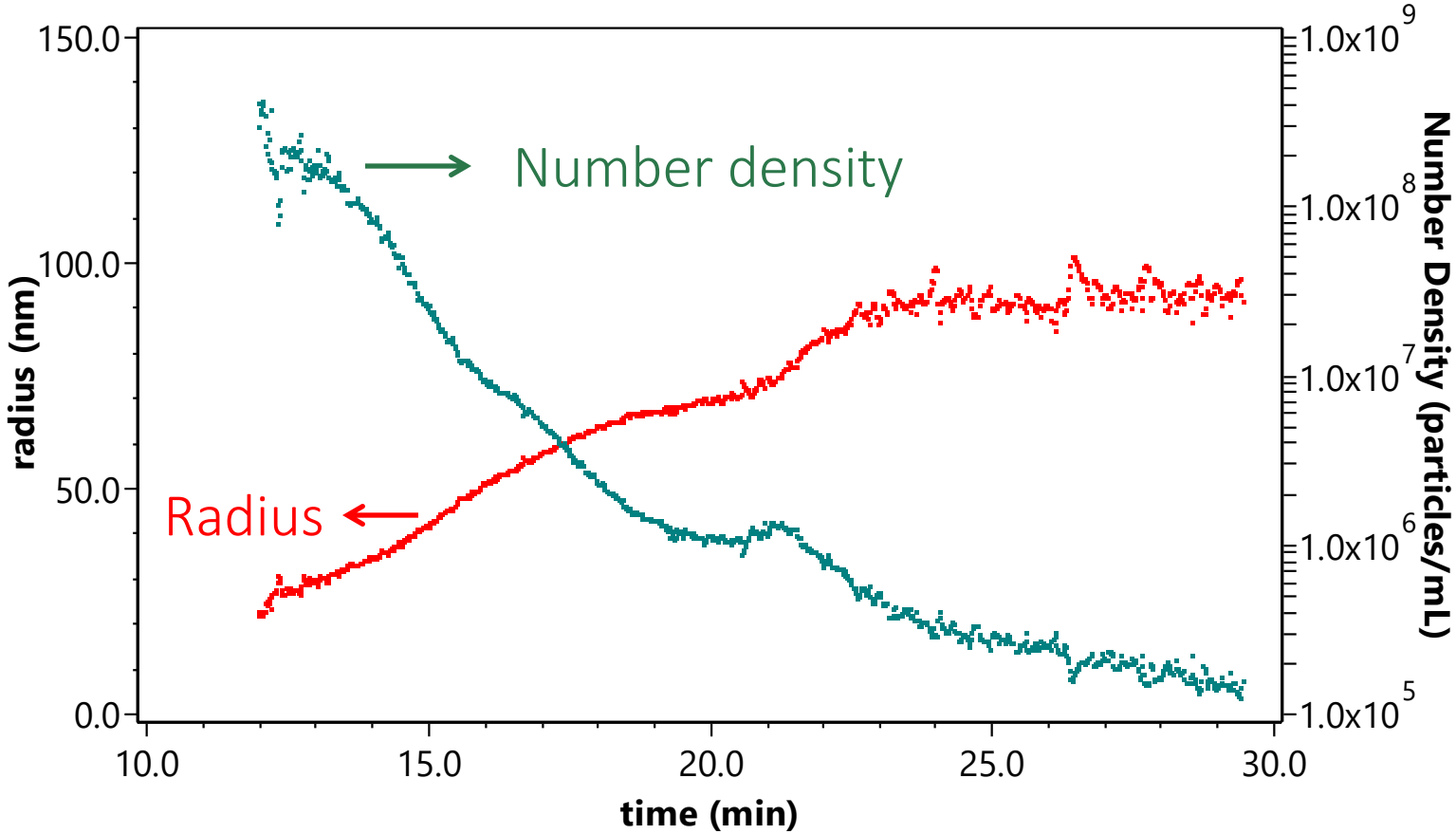
Zhang, Haiying, et al. "Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation." *Nature cell biology* (2018): 1. doi:10.1038/s41556-018-0040-4

Identification: Radius by MALS (low load, high sensitivity)



- Size of all three subpopulations could be determined by online MALS even at low peak concentration due to low loading.

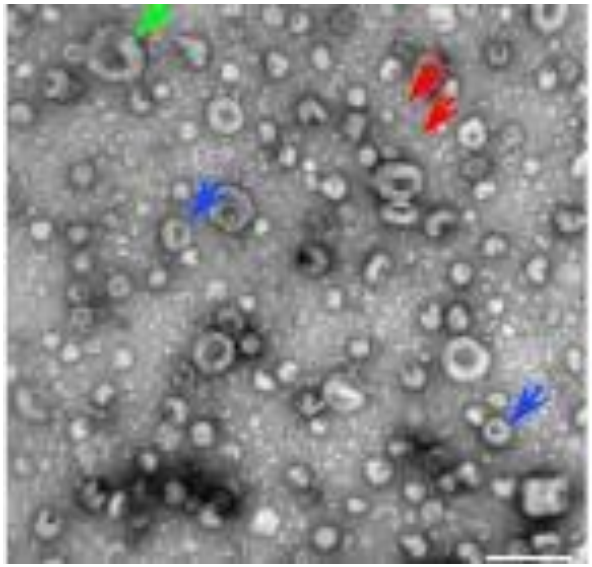
Quantitation: particle concentration by MALS



- Both size and particle counts can be measured online by Wyatt DAWN HELEOS MALS detector

EAF4-MALS-DLS: advanced platform for exosome and exomere isolation and characterization

- Isolation and fraction collection by size (Exo-S, Exo-L, exomeres)
- Online biophysical characterization:
  - Size (Rg and Rh), shape
  - Quantitative, high-resolution concentration vs. size
  - Charge & zeta potential
  - Relative protein, lipid, RNA/DNA content



Summary

- The Wyatt Eclipse Flow-FFF system is a powerful tool to characterize complex nanomaterials
- It is based on efficient separation coupled to online molar mass and size measurement with a Wyatt MALS detector
- Eclipse Mobility allows to determine charge and charge distribution in complex samples
- The system is fully integrated with the new software VISION and provides a seamless workflow from method development to final result
- We have seen application examples for polymer latex, proteins and exosome isolation and characterization

Thank you for your attention

Questions?

