Advancing Low flow LC/MS for single cell proteomics with variable flow and 50 cm microfabricated pillar array columns

HPLC 2025 Bruges
Jeff Op de Beeck

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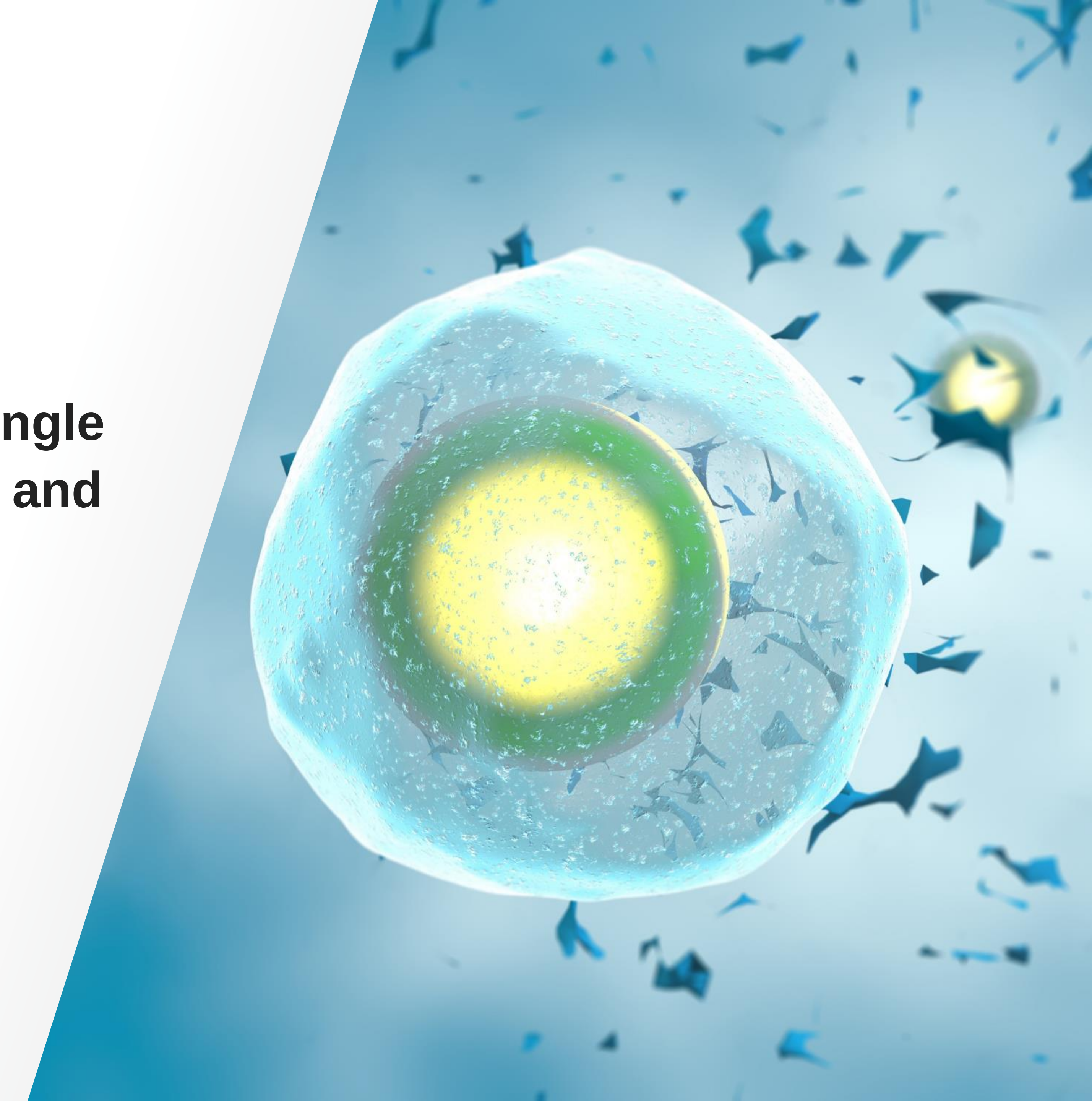
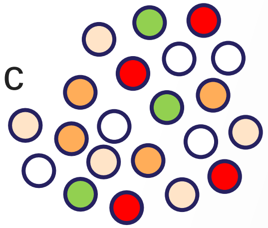
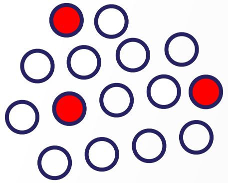
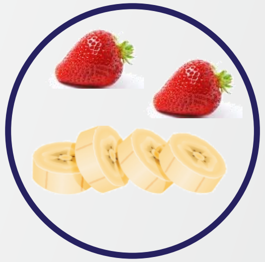
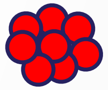
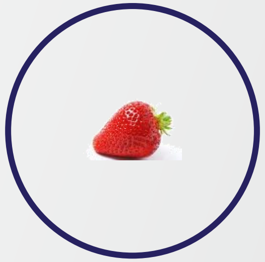
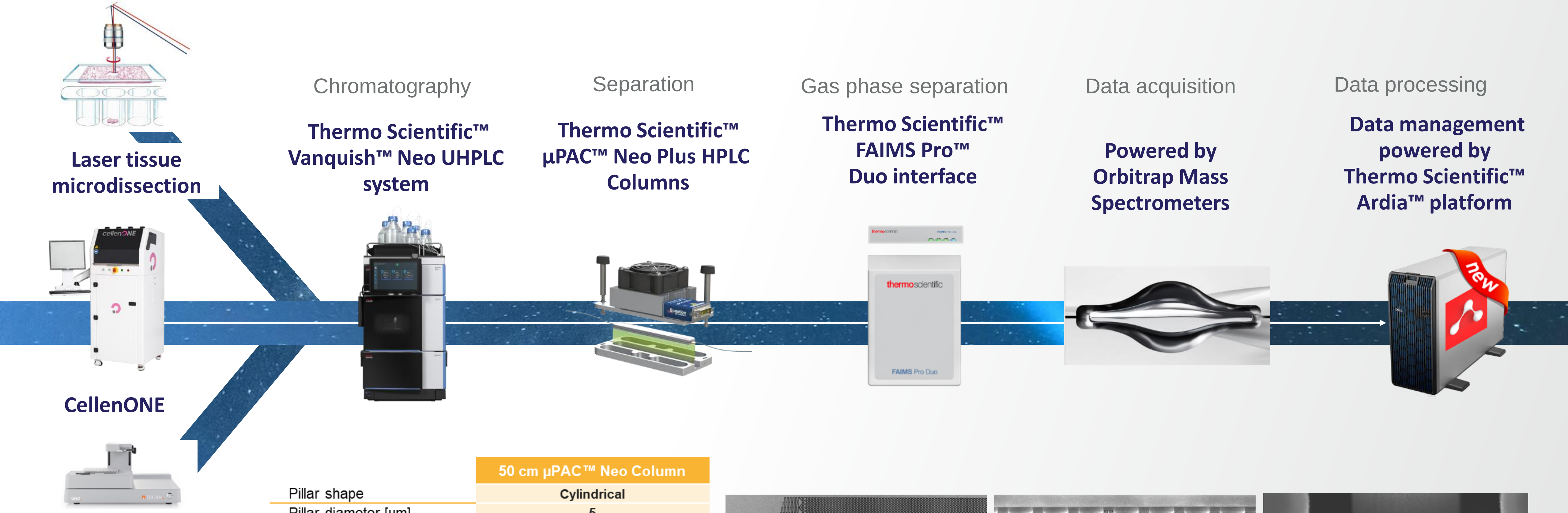


Why single cell proteomics

Application	Bulk result	Single cell data
Identification of cell subpopulations based on protein expression or metabolic profiles (tumors, tissues, immune cells, cell cultures) 		
Detection and analysis of rare cells (*i.e.,* CTCs from liquid biopsies) 		
Analysis of limited sample material (exosomes, needle aspirates, biopsies) 	No Data	

Single-cell analysis challenges all the steps in the proteomics workflow

Single cell proteomics workflow



Laser tissue microdissection

CellenONE

Tecan Uno Single Cell Dispenser

Chromatography

Thermo Scientific™ Vanquish™ Neo UHPLC system

Separation

Thermo Scientific™ µPAC™ Neo Plus HPLC Columns

Gas phase separation

Thermo Scientific™ FAIMS Pro™ Duo interface

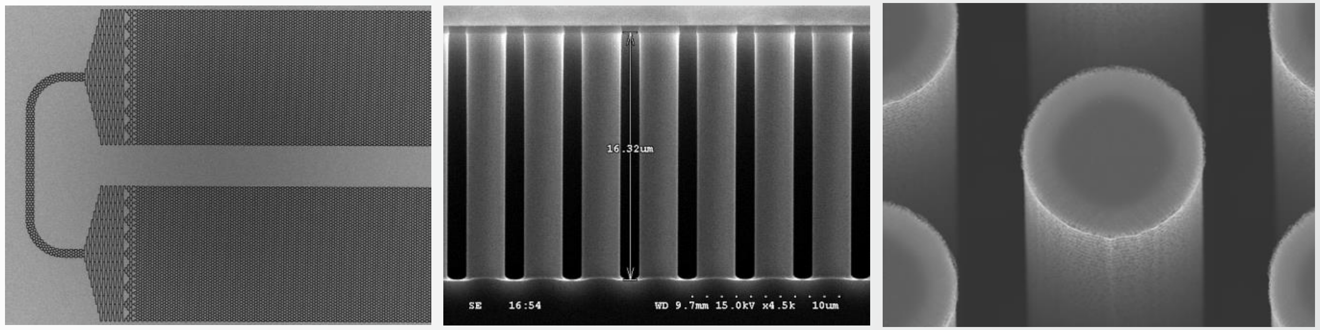
Data acquisition

Powered by Orbitrap Mass Spectrometers

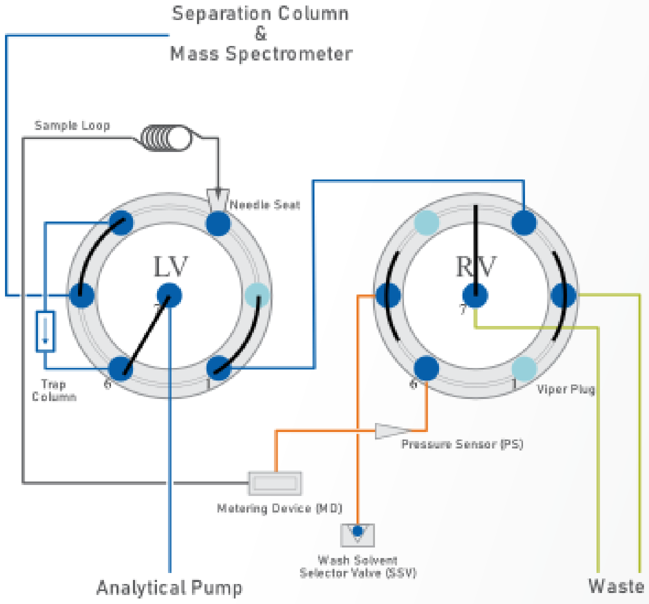
Data processing

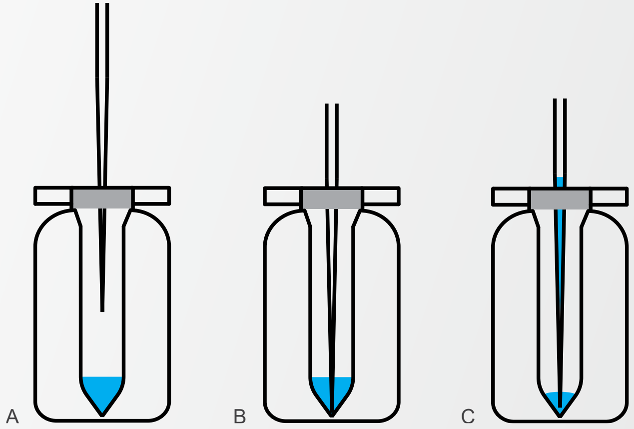
Data management powered by Thermo Scientific™ Ardia™ platform

	50 cm µPAC™ Neo Column
Pillar shape	Cylindrical
Pillar diameter [µm]	5
Interpillar distance [µm]	1,25
Channel width [µm]	180
Channel depth [µm]	16
Column length [cm]	50
Column volume [µl]	1,5
Surface morphology	Core shell
Porous layer thickness [nm]	400
Pore size range [A]	100 - 300
Surface functionalization	C18 + HMDS



Injection of single cell samples with Vanquish Neo UHPLC





Back Flush trap and elute workflow

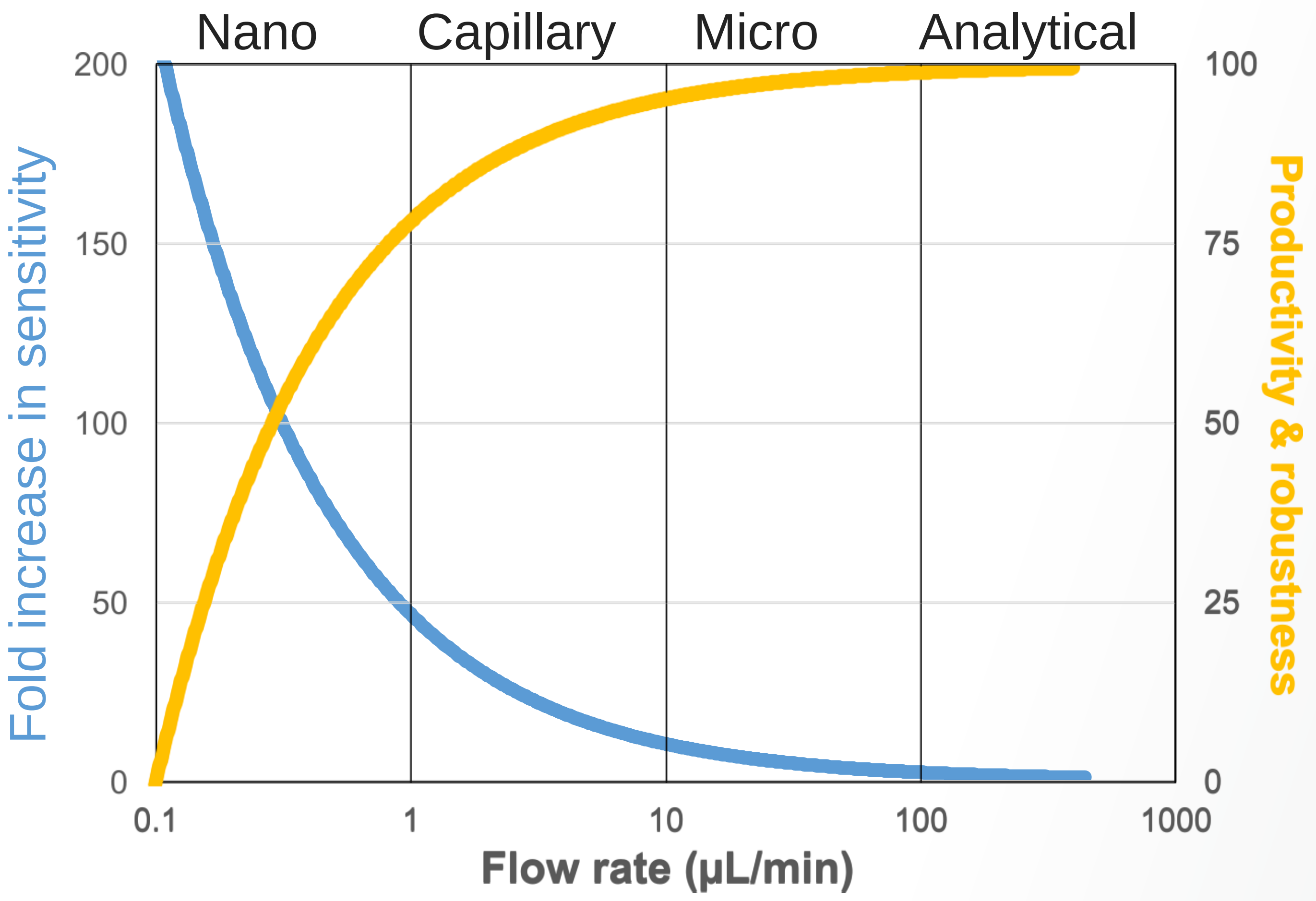
- Single cell samples vary in volume (between and within experiments) – sample volume range 1-10 µL
- Sample uptake + loading can be reduced to 2.4min
- Additional air drawn into sample loop will not affect ionization / analytical column performance

Vial bottom detection

- Samples can be fully aspirated by setting injection volume with some margin (1-2µL added)
- Compatible with conventional sample vials, 96 and 384 well plates

Sensitivity versus throughput

Impact on instrument productivity and sensitivity (in LC-ESI-MS)



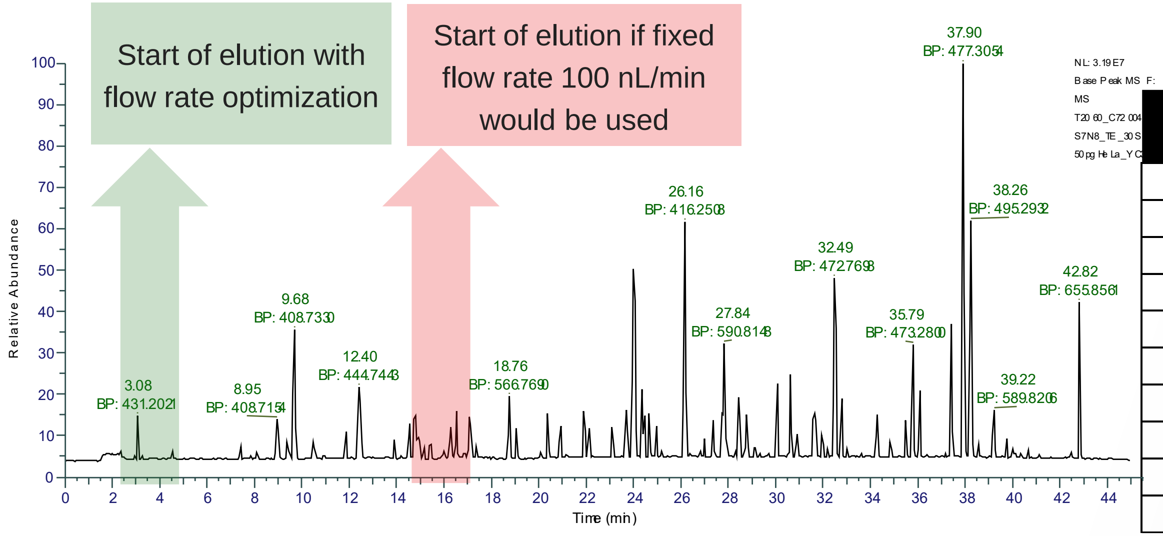
Low flow LC = reduced instrument productivity & robustness?

- Sample loading (µL volumes) has a much bigger impact at low flow rates
- Small dead volumes can have a huge impact on performance

Why does miniaturization improve sensitivity?

- Increase of analyte concentration with decrease of column ID (lower sample dilution)
- Improved ionization efficiency at lower flow rates (ESI)

Ultra Low Flow sensitivity with increased instrument productivity


Start of elution with flow rate optimization

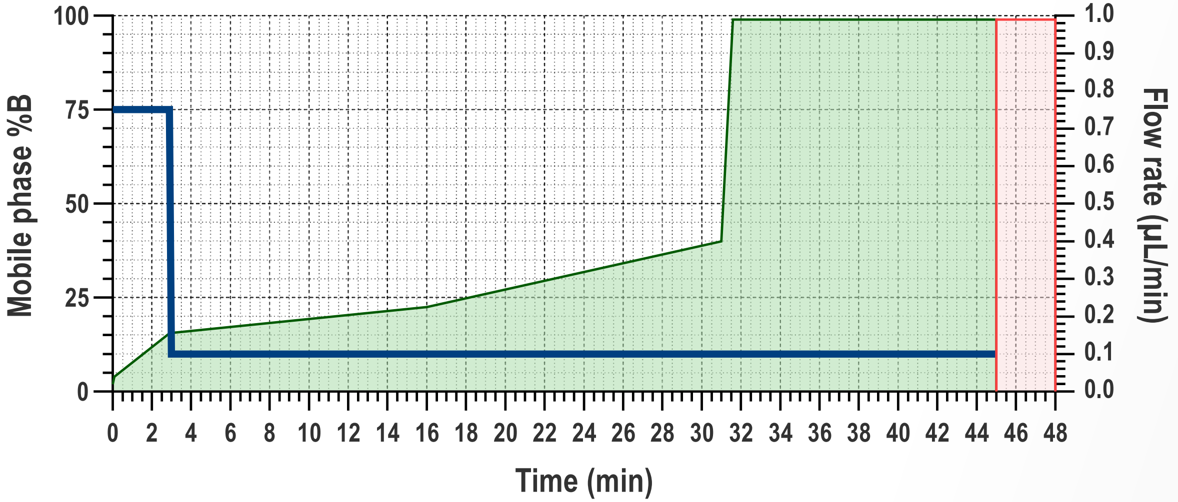
Start of elution if fixed flow rate 100 nL/min would be used

Gradient profile

Duration min	Time min	%B /	Flow rate µL/min	Volume µL	Total volume µL
0	0	2	0.75	0	0
0.1	0.1	4	0.75	0.075	0.075
2.8	2.9	15.5	0.75	2.1	2.175
0.1	3	15.6	0.1	0.01	2.185
13	16	22.5	0.1	1.3	3.485
15	31	40	0.1	1.5	4.985
0.5	31.5	99	0.1	0.05	5.035
13.5	45	99	0.1	1.35	6.385

83% instrument productivity

(including sample uptake, sample loading and column equilibration)



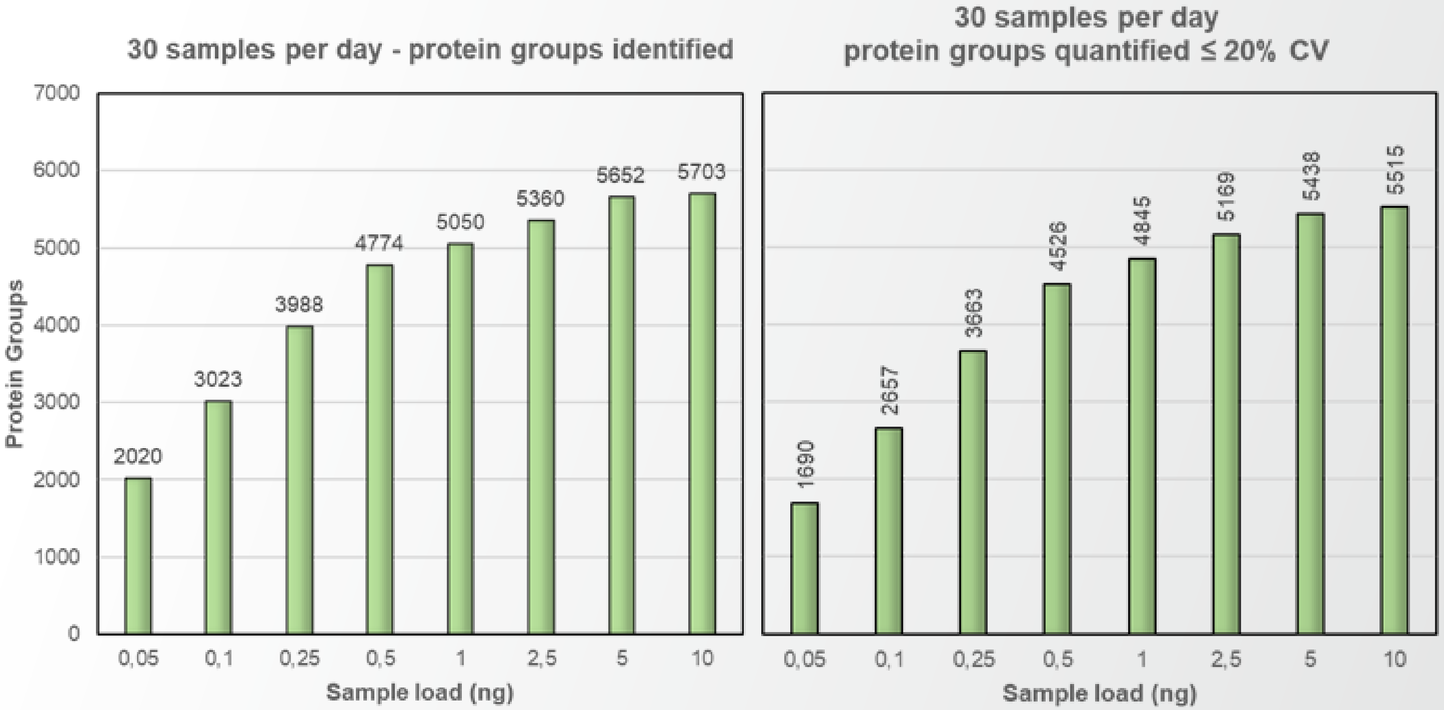
- “High” Flow rate (750 nL/min) is used in initial part of gradient to push analytes forward
- At the point where analytes start eluting, the flow rate is reduced to “low” flow rate (100 nL/min) to boost sensitivity
- Caution as **2.9 min gradient at 750 nL/min will give 21.75 min elution at 100 nL/min**

Single cell by dilution – Bulk HeLa

Vanquish Neo UHPLC

- Precise injection of sample volumes down to 10 nL
- Full dilution series using concentrated sample stock
- Ideal sample conditions (minimal handling & adsorption)

- “true” 30SPD Trap & Elute – 100 nL/min elution
- Bulk HeLa dilution (from 5 ng/µL sample)
- Orbitrap Exploris 240 MS without FAIMS interface
- 120k MS2 – 246ms IT – 80Th windows – 400-800 m/z
- Processing in Spectronaut 19 – triplicates together (MBR)



Consistent LC-MS method and workflow optimization...

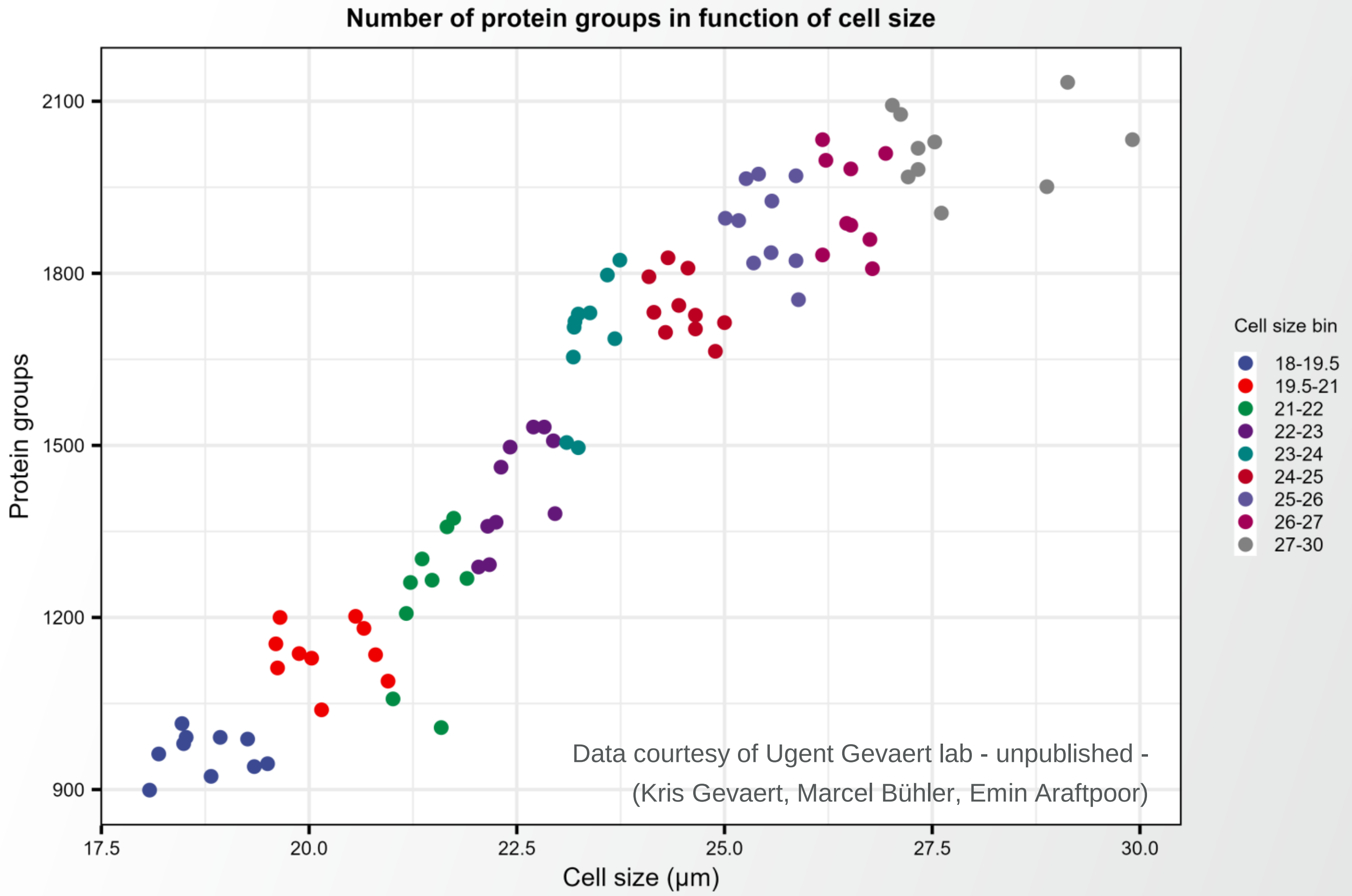
How about real single cell samples?

Effect of HeLa cell size on proteome depth

Cellenion cellenONE

- Single HeLa cells isolated within narrow size bins (1 - 1.5 – 3 µm)
- One-pot single cell digestion into LoBind Eppendorf 384 well plate – proper silicone seal needed!

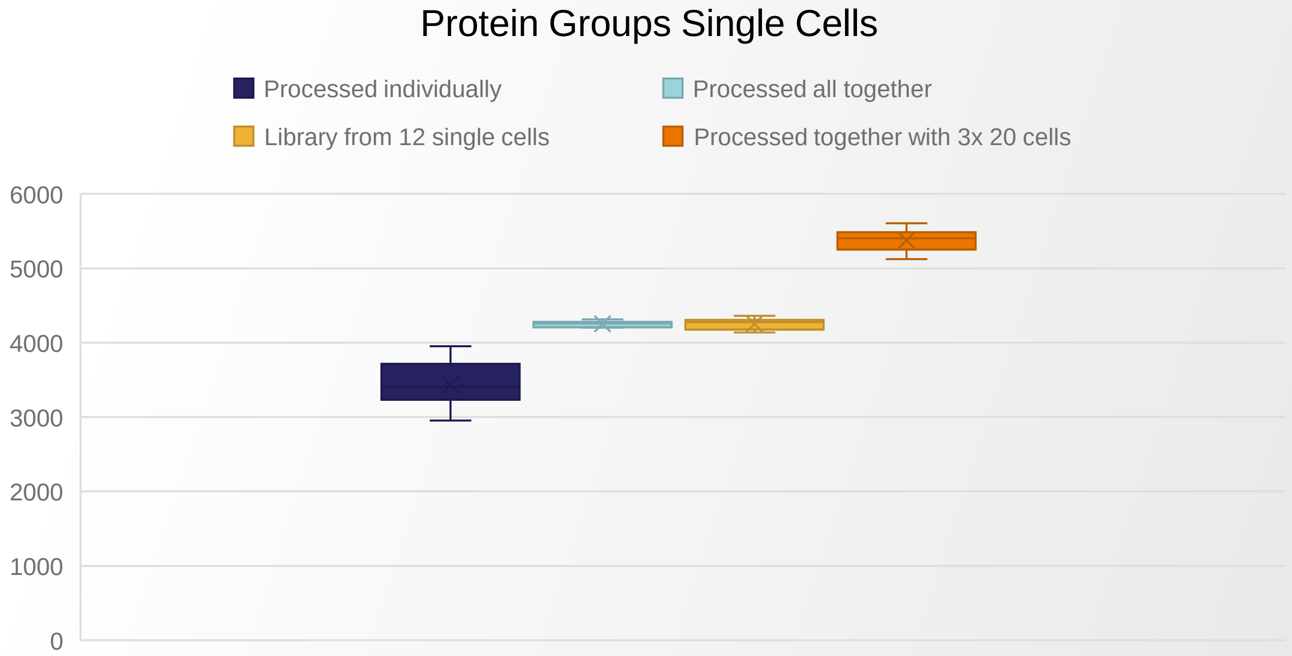
- “true” 30SPD Trap & Elute – 100 nL/min elution
- Thermo Scientific™ Orbitrap Exploris™ 240 without FAIMS
- 120k MS2 – 246ms IT – 60Th windows – 400-800 m/z
- Processing in Spectronaut 19 – cells within size bins (n=10) together (MBR)



Data courtesy of Ugent Gevaert lab - unpublished -
(Kris Gevaert, Marcel Bühler, Emin Araftpoor)

- Good correlation between cell size and proteome coverage observed
- Selection of single cells in narrow size bins increases data consistency

Ultimate sensitivity with Orbitrap Astral analyzer



- 72SPD – 200 nL/min elution
- Single HeLa cells
- Thermo Scientific™ Orbitrap Astral™ mass spectrometer with FAIMS interface
- Processing in Spectronaut 19

2-fold Increase in throughput

2-fold Increase in depth

Summary single cell proteomics workflow optimization

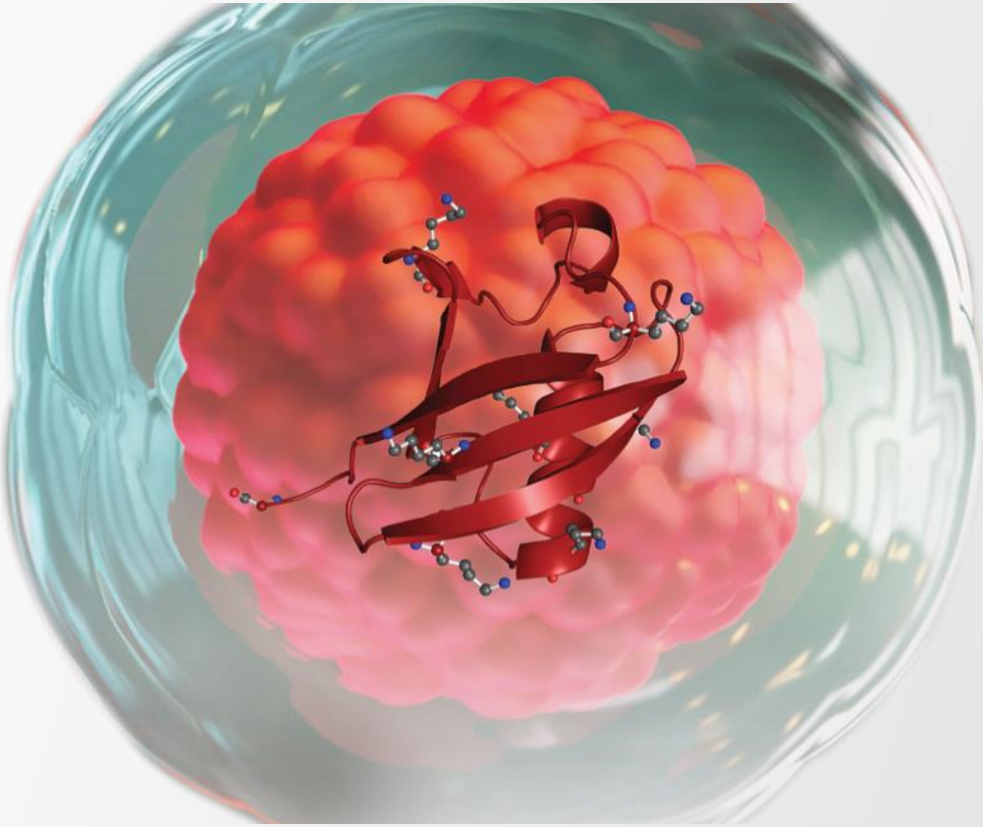
- **Careful Selection of workflow components**:
  Ensure minimal sample loss and maximum sensitivity in all steps.

- **Vanquish Neo UHPLC**:
  Ideal for method optimization and true single cell experiments.
  - Precise injection range: 10 nL to 100 µL.
  - Trap and elute with full aspiration of precious samples.

- **Ultra Low Flow NanoLC**:
  Implement without impacting instrument productivity using variable flow rate methods and micro Pillar Array Column technology.

- **Comprehensive Coverage**:
  Achieve study of biological processes (2k protein groups/cell) on Orbitrap Exploris MS platforms at 30 samples/day.

- **Ultimate Sensitivity**:
  Orbitrap Astral MS provides deeper insights with increased throughput.



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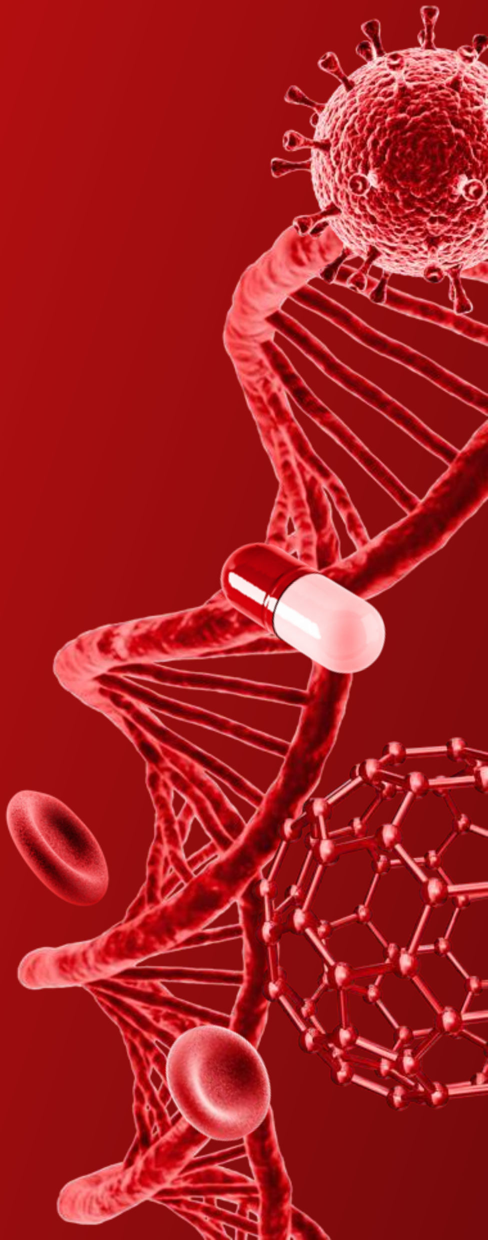

Thank you