Mastering Single Cell Proteomics: Daily Excellence with µPAC Neo Plus Trap-and-Elute Workflow

Jeff Op de Beeck
Staff Scientist R&D
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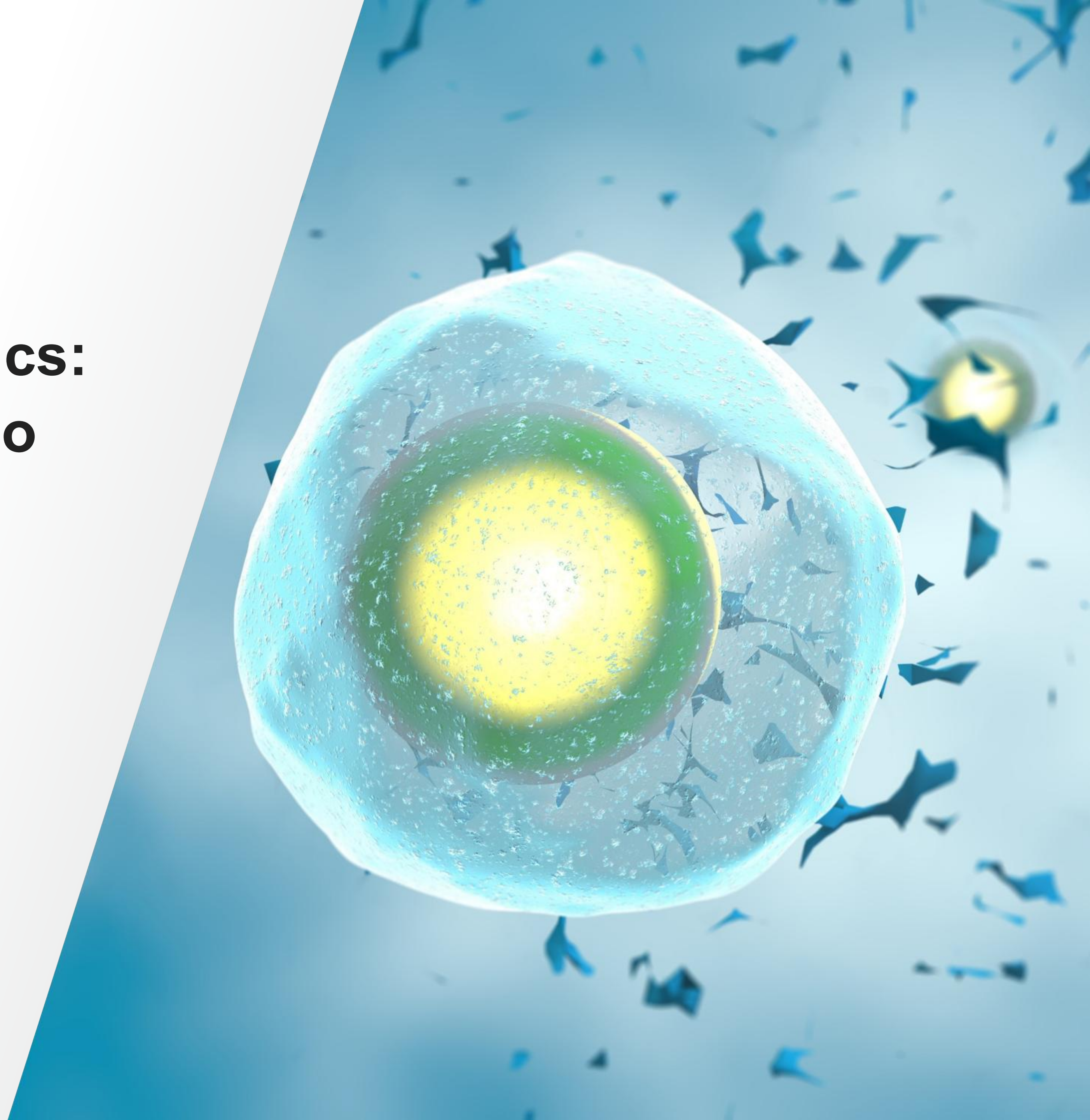
The world leader in serving science



Thermo Fisher SCIENTIFIC

Omics Innovations for the biopharmaceutical industry

Recordings from the seminar on December 11, 2025 at LSMBO in Strasbourg

DATE & TIME	TOPICS & PRESENTERS
Jan 27 • 11:00 CET	**Mastering Single-Cell Proteomics: Daily Excellence with µPAC Neo Plus Trap-and-Elute Workflow** Jeff Op de Beeck, Thermo Fisher Scientific
Feb 12 • 11:00 CET	**Introducing the Orbitrap Excedion Pro Hybrid MS** Claire Dauly, Thermo Fisher Scientific
Feb 12 • 11:00 CET	**Driving the process development of recombinant therapeutic products with automated MS-based target peptide mapping** Diego Bertaccini, Merck Group
Feb 19 • 11:00 CET	**Single cell and immunopeptidomics explorations on Orbitrap Astral MS** Farida Salimova and Jeewan Babu Rijal, CNRS
Feb 26 • 11:00 CET	**Toward early detection of congenital disease using Orbitrap Astral DIA MS/MS** / Joëlle Vinh, ESPCI

Workshop details and registration: https://bit.ly/4qKEi3k

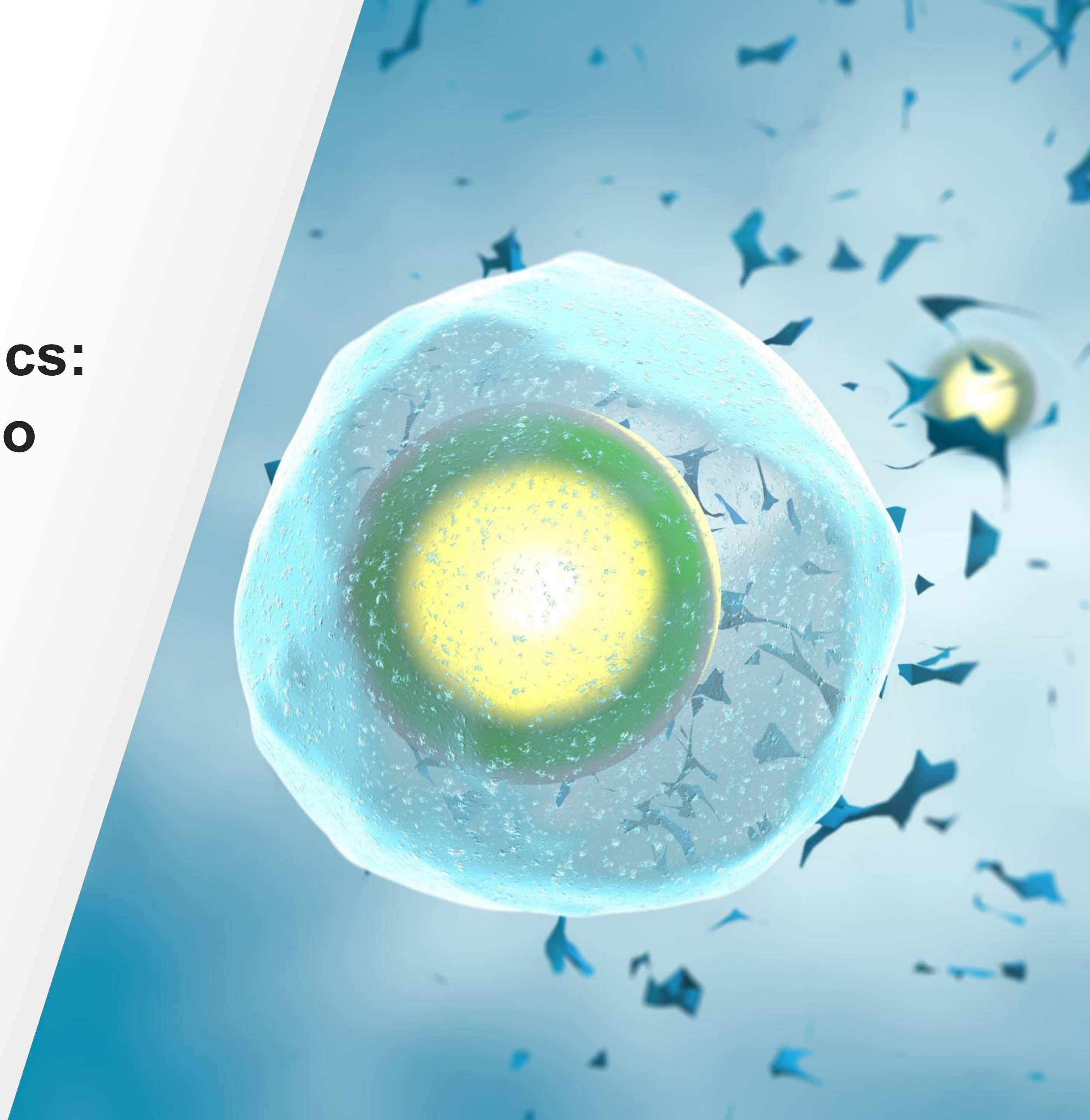
Thermo Scientific™ Proteome Discoverer™ Software

Free online workshops | Upcoming sessions and recordings

Date & Time	Topic
Mar 3 • 14:00 CET • 2h	SureQuant Hybrid DIA - Instrument method setup, data processing, result review
Feb 24 • 14:00 CET • 2h30	Targeted MS2 Hybrid DIA - Instrument method setup, data processing, result review
Feb 10 • 14:00 CET • 1h30	Understanding quantification algorithms
On-demand • 2h24m	Proteome Discoverer Phosphopeptides Workshop
On-demand • 1h32m	Quantitation study design in Proteome Discoverer software
On-demand • 1h23m	Introducing statistics insights node
On-demand • 2h36m	Understanding quantitation in Proteome Discoverer software
On-demand • 2h45m	Proteome Discoverer 3.2 TMTpro 32plex training workshop

Workshop details and registration: https://bit.ly/3LVh2Aq

Mastering Single Cell Proteomics: Daily Excellence with µPAC Neo Plus Trap-and-Elute Workflow



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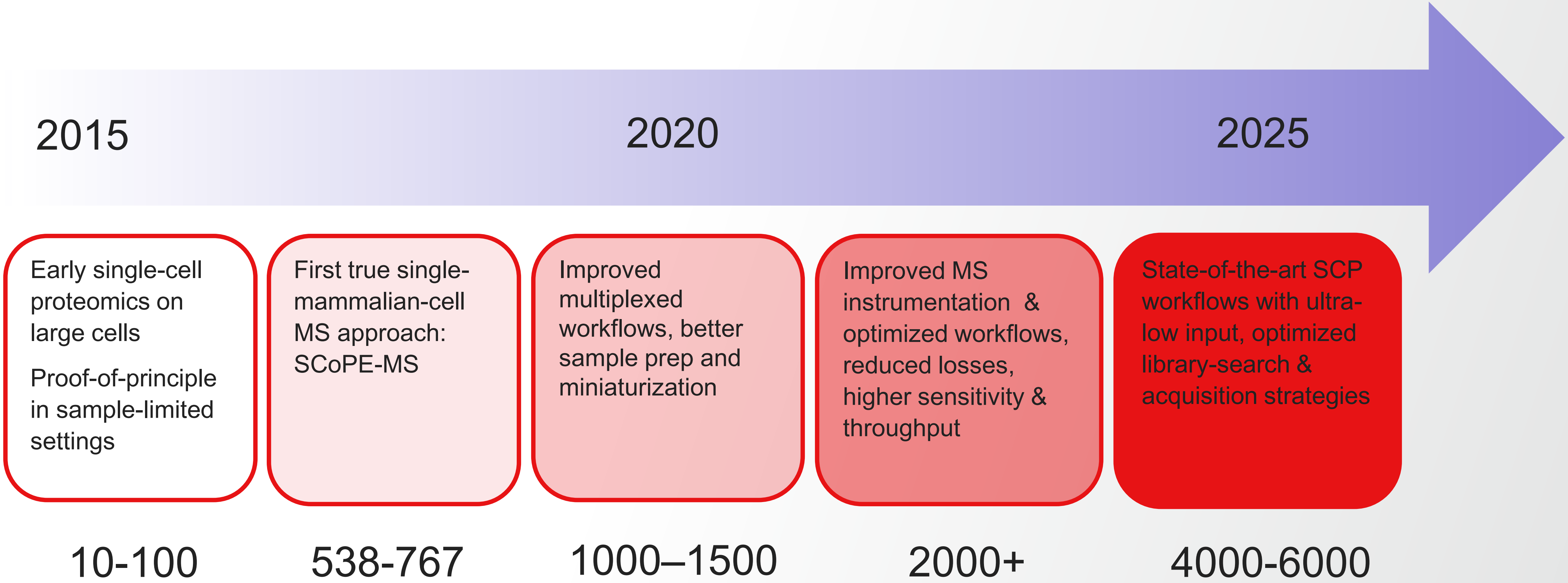
Evolution of MS-based single cell proteomics



2015

2020

2025

Early single-cell proteomics on large cells

Proof-of-principle in sample-limited settings

First true single-mammalian-cell MS approach: SCoPE-MS

Improved multiplexed workflows, better sample prep and miniaturization

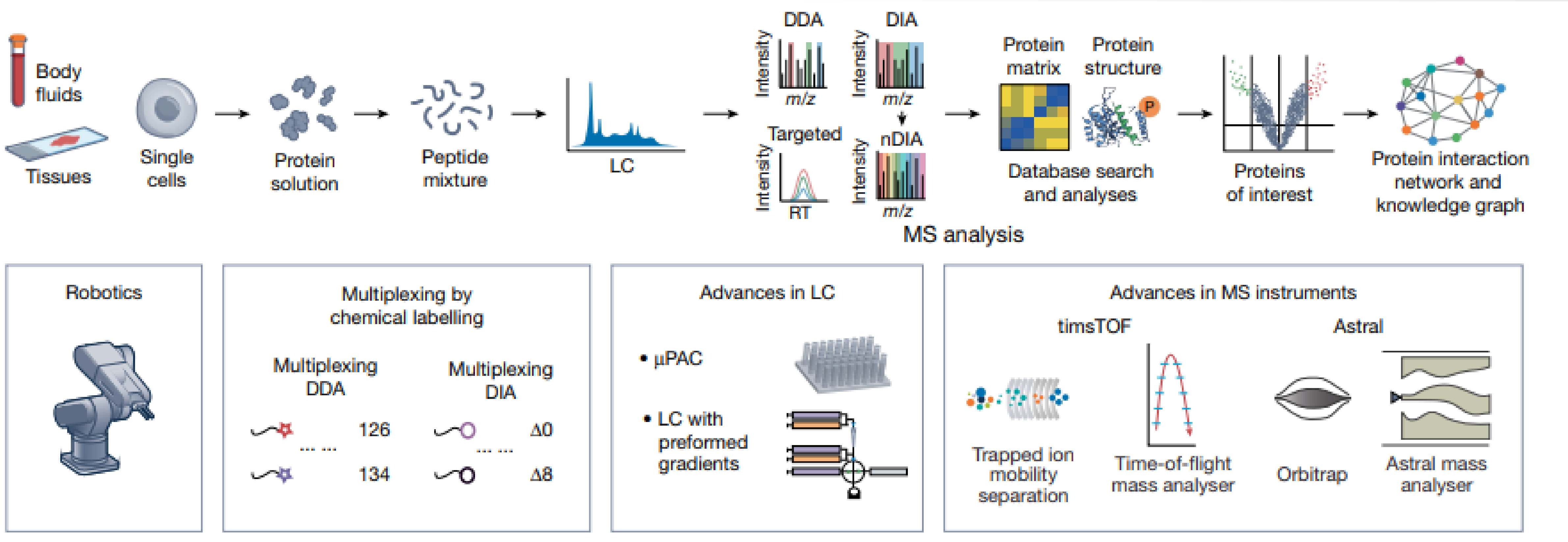
Improved MS instrumentation & optimized workflows, reduced losses, higher sensitivity & throughput

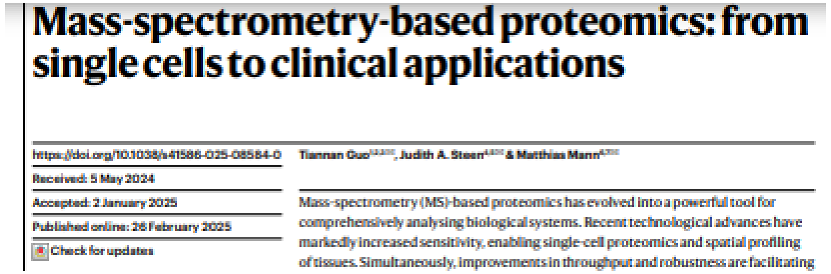
State-of-the-art SCP workflows with ultra-low input, optimized library-search & acquisition strategies

10-100	538-767	1000–1500	2000+	4000-6000

Proteins identified per cell

Single cell proteomics workflow





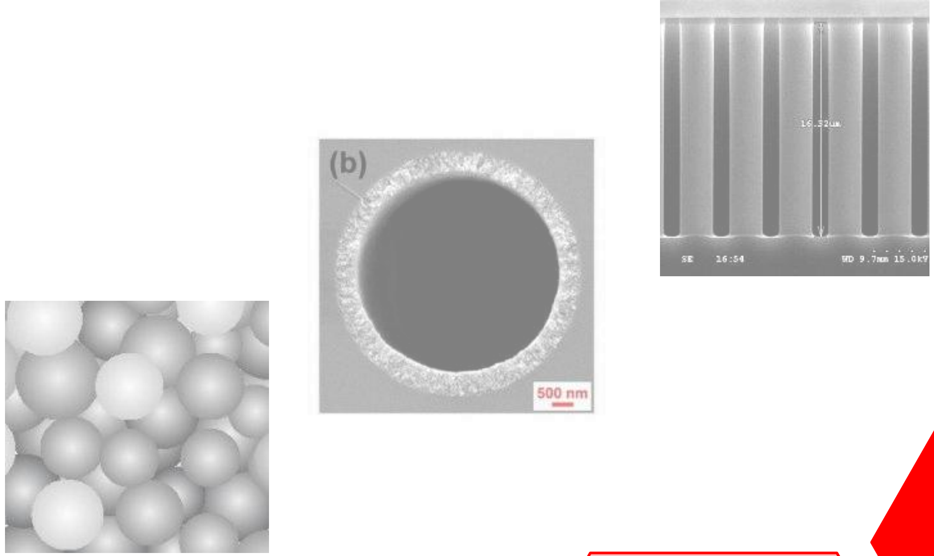
Mass-spectrometry-based proteomics: from single cells to clinical applications

https://doi.org/10.1038/s41586-025-08584-0
Received: 5 May 2024
Accepted: 2 January 2025

Tiannan Guo, Judith A. Steen & Matthias Mann

Mass-spectrometry (MS)-based proteomics has evolved into a powerful tool for comprehensively analysing biological systems. Recent technological advances have markedly increased sensitivity, enabling single-cell proteomics and spatial profiling of tissues. Simultaneously, improvements in throughput and robustness are facilitating

Optimization of all steps in the workflow towards minimal sample loss and maximum sensitivity have led to the current state of the art



Ideal LC column for single-cell proteomics?

Maximum sensitivity

- Low cross section
- Good chromatography at low nanoLC flow rates (sub 200 nL/min)

Throughput

- Low volume
- Wide flow rate range to reduce instrument overhead

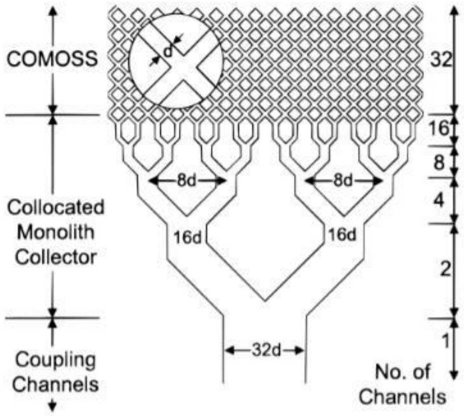
Performance

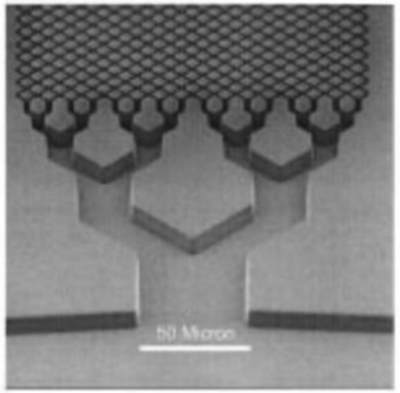
- Essential to deconvolve complex peptide mixtures at low abundance
- Sharper peaks give higher intensity

Robustness

- Samples are dilute but lack prior clean-up
- Ensures finishing a full study without interruption or batch effects

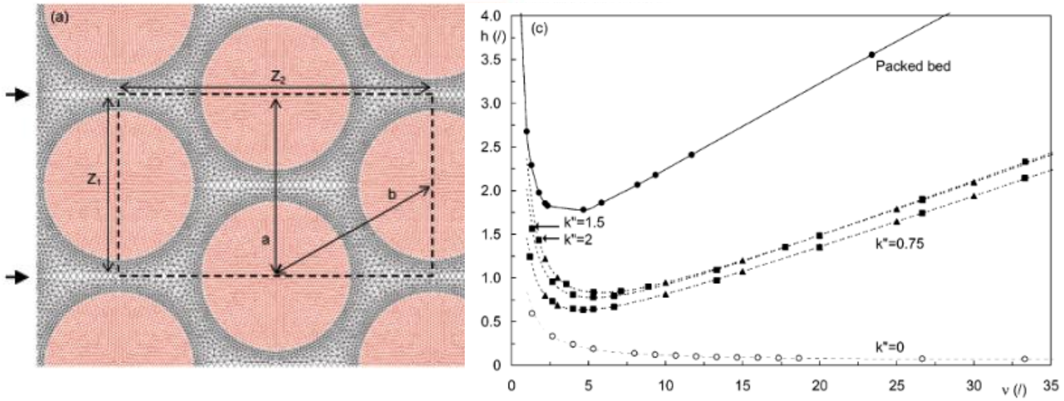
The pillar array LC column concept





Fabrication of Nanocolumns for Liquid Chromatography

- B. He, N. Tait and **F. Regnier**, Anal. Chem. **1998**, 70, 3790-3797
- 18 nL volume channels – not compatible with HPLC instrumentation
- On-chip performance measured for Capillary electrochromatography (CEC)

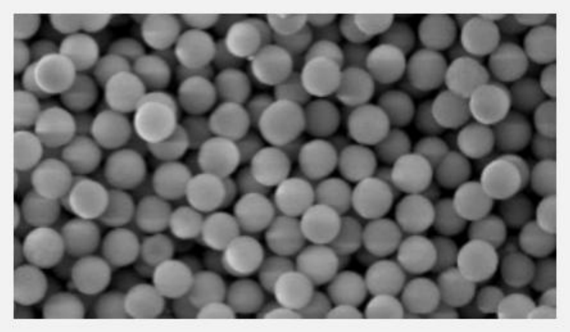


Advantages of perfectly ordered 2-D porous pillar arrays over packed bed columns for LC separations: a theoretical analysis

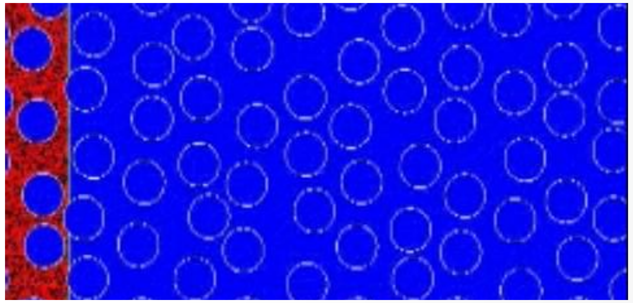
- P. Gzil, N. Vervoort, G. V. Baron, and **G. Desmet**, Anal. Chem. **2003**, 75, 6244-6250
- Computational fluid dynamics (CFD) simulation on performance of pillar array format
- Quantification of LC separation efficiency improvements by introducing perfect order in the chromatographic bed

µPAC Neo technology overview

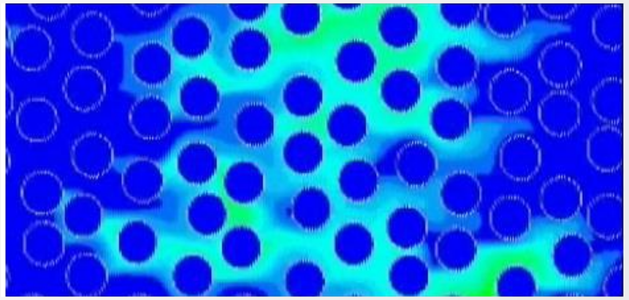
Pillar array – Order vs Disorder

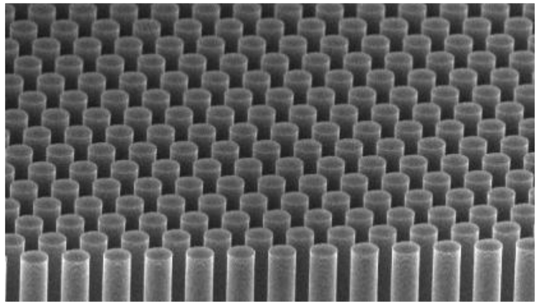


Disorder - Packed Bed

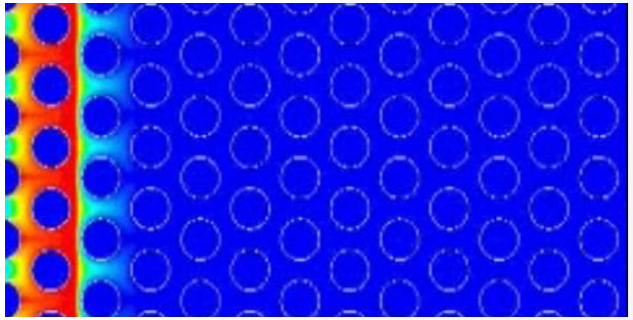


Disorder - Packed Bed

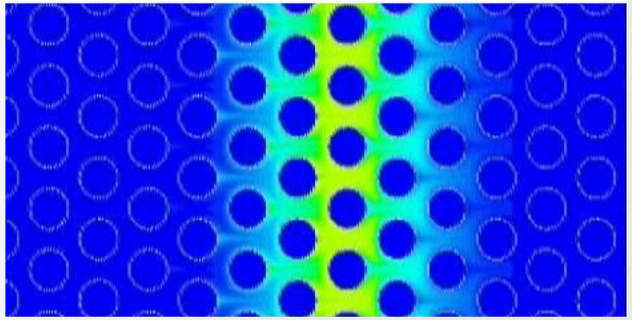




Order – Pillar Array



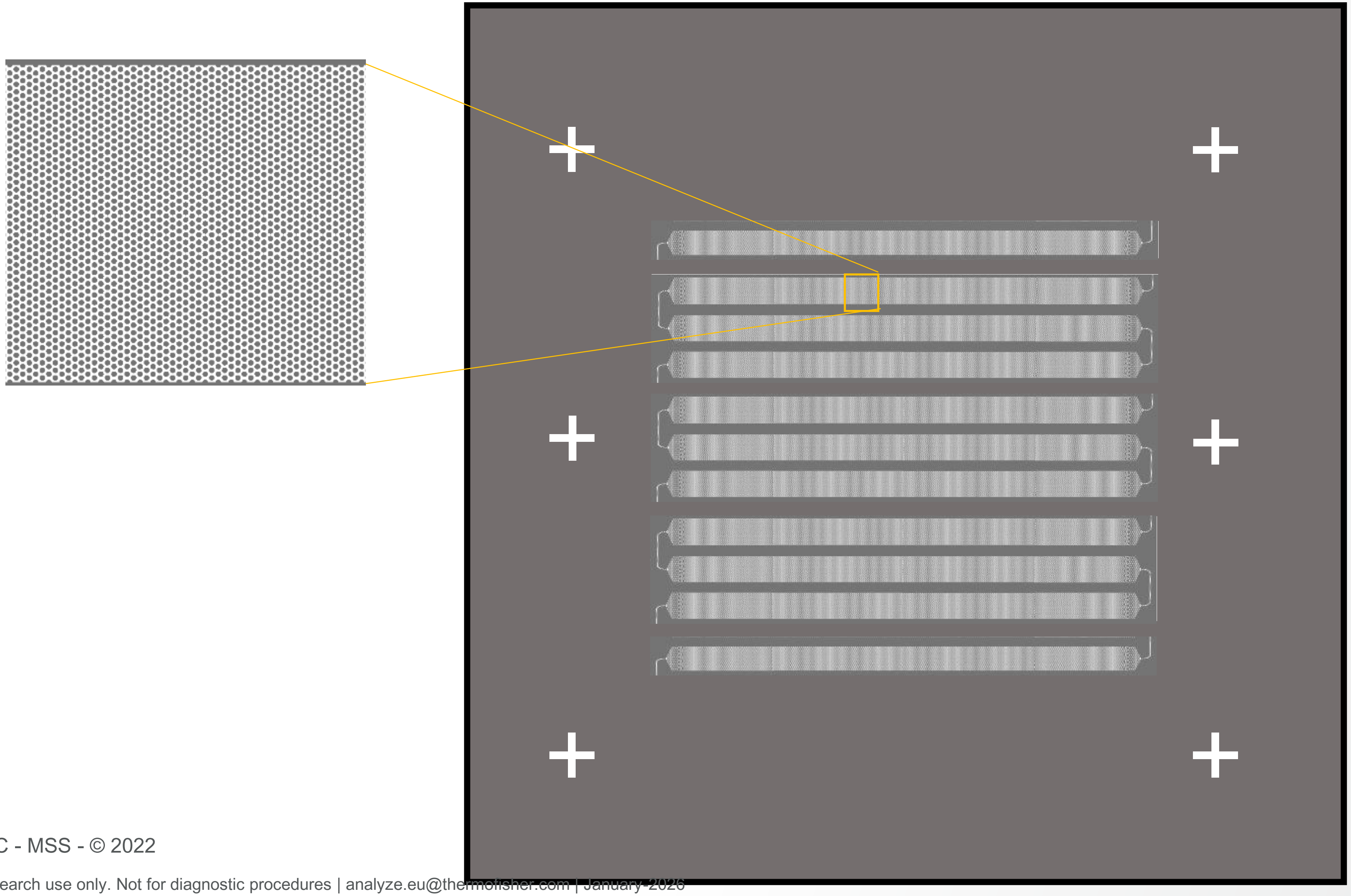
Order – Pillar Array



µPAC Technology

Outsourced

Means: Silicon Micromachining as an Enabler



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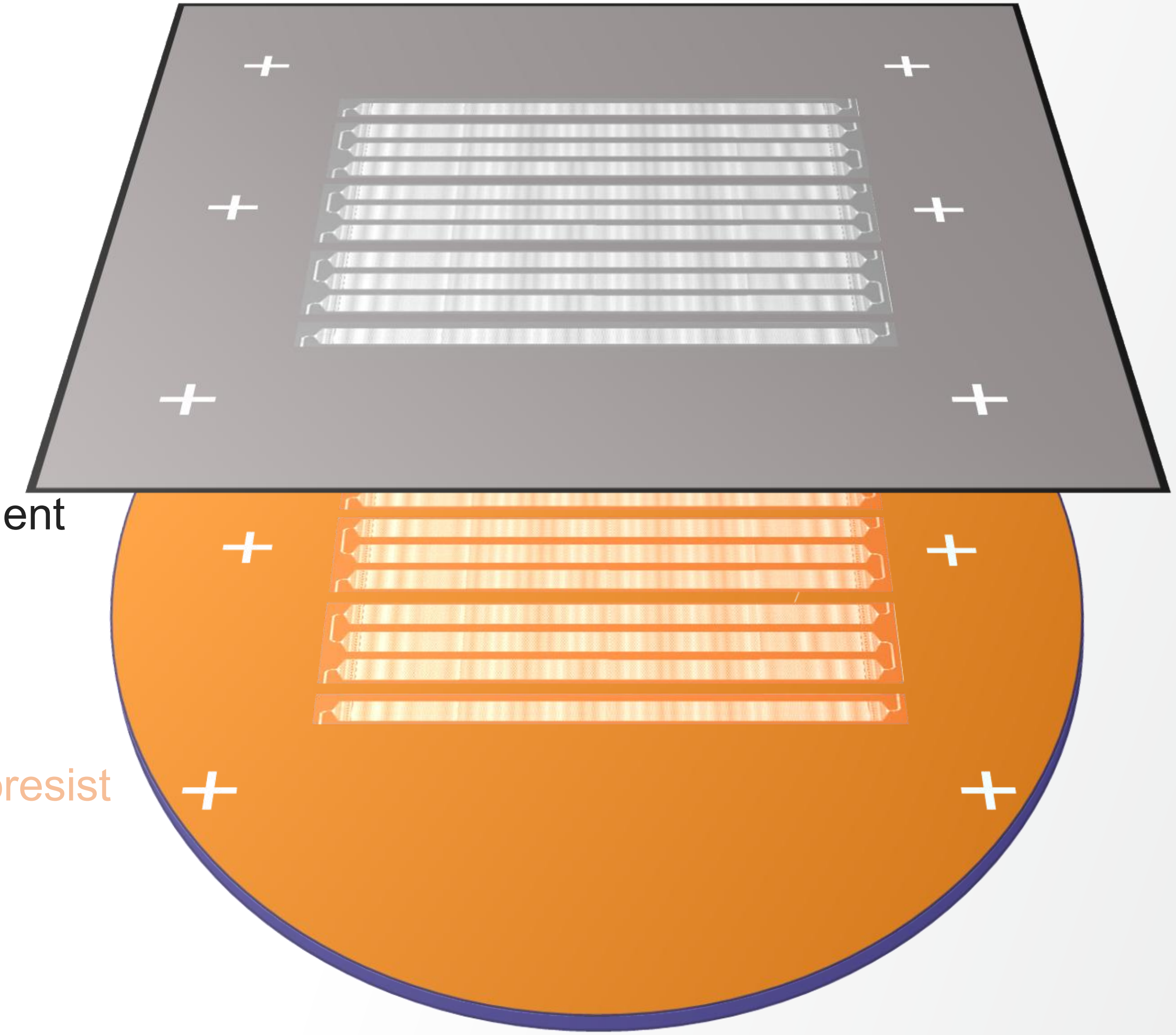
µPAC Technology

Outsourced

Means: Silicon Micromachining as an Enabler



Photomask or stepper

Illumination and development

Protective layer and photoresist

Silicon wafer

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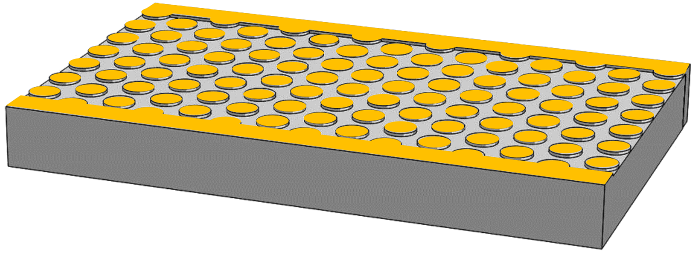
µPAC Technology

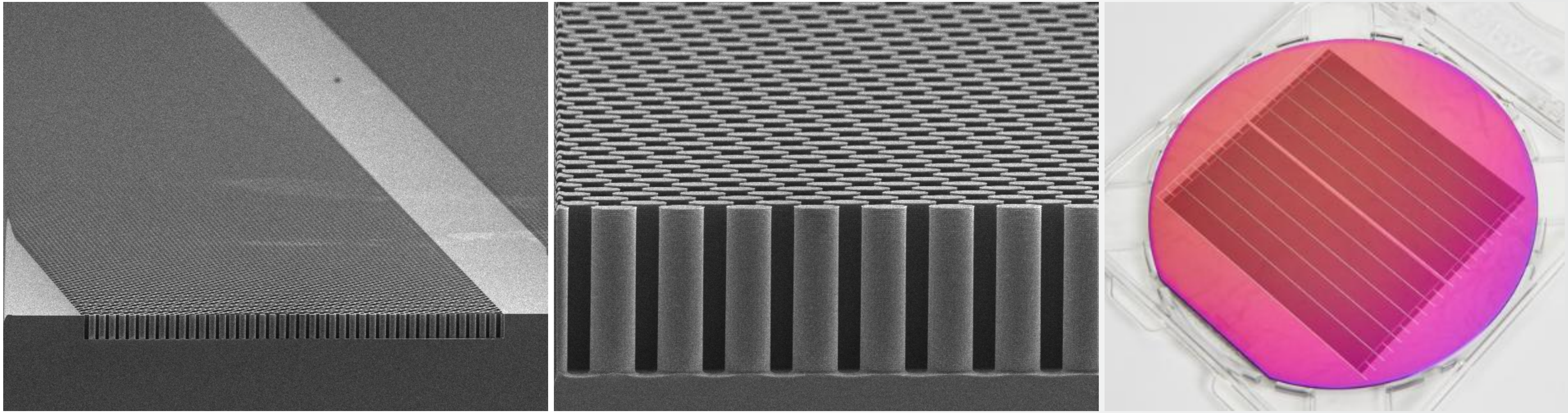
Means: Deep Reactive Ion Etching (DRIE)

Outsourced

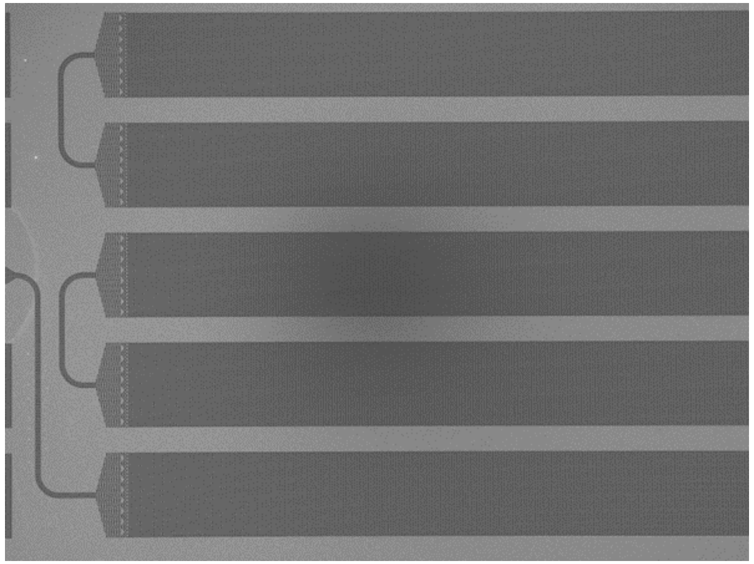


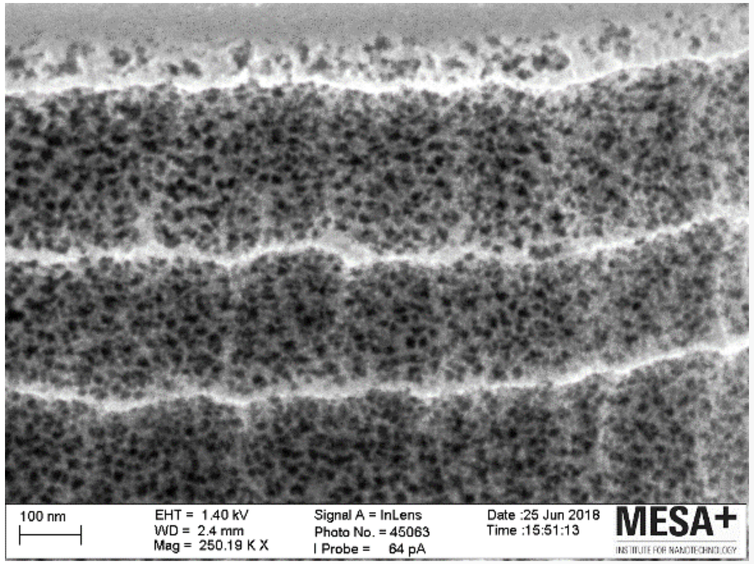
And each time exactly the same amount of pillars are formed…

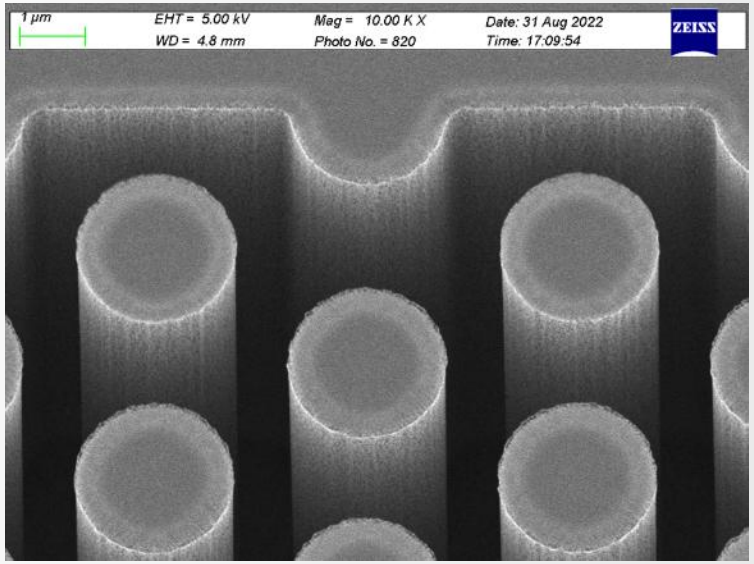
Each time at exactly the same position…



Key developments towards current performance







Low dispersion turn-structures

- Enable interconnection of parallel separation lanes without performance loss
- Allows creating “long” separation channels on a limited SI wafer footprint

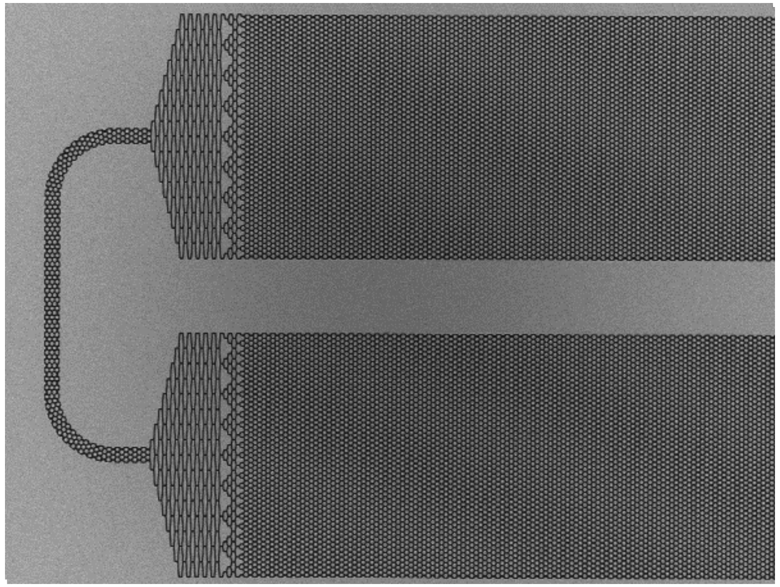
Porosification of silicon

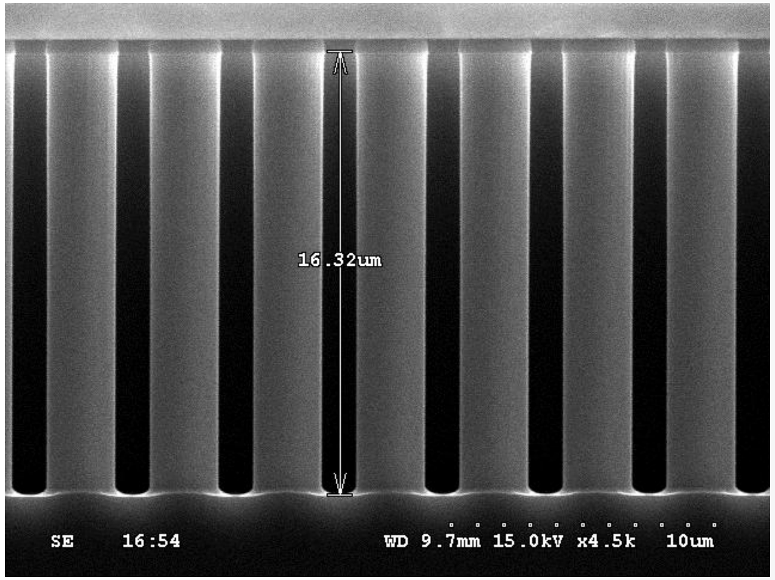
- The outer mantle of SI pillars can be rendered mesoporous (100-300Å)
- Increase in surface to volume needed for loadability and proper focussing of analytes at the onset of the gradient

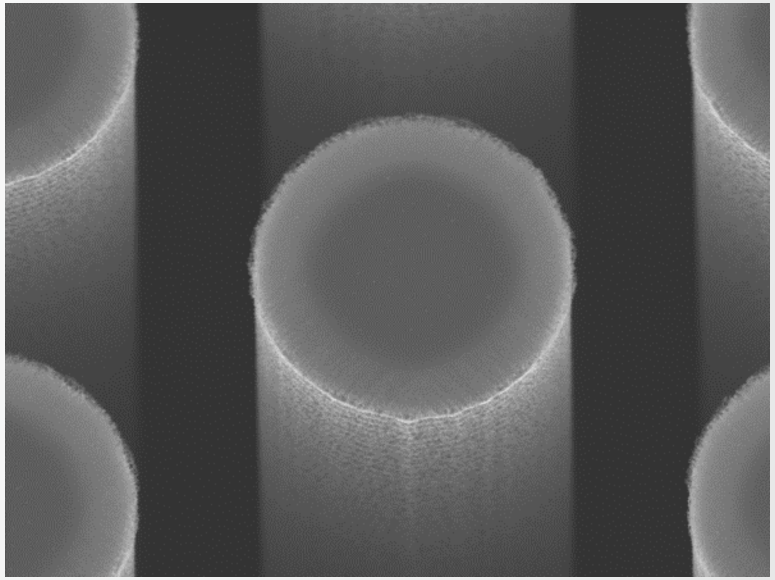
Dimension downscaling

- Reduction of the distance between the pillars increases separation performance
- True generations defined by smallest dimension in design
- Generation 1 (µPAC) : 2.5 µm distance
- Generation 2 (µPAC Neo and µPAC Neo Plus) : 1.25 µm distance

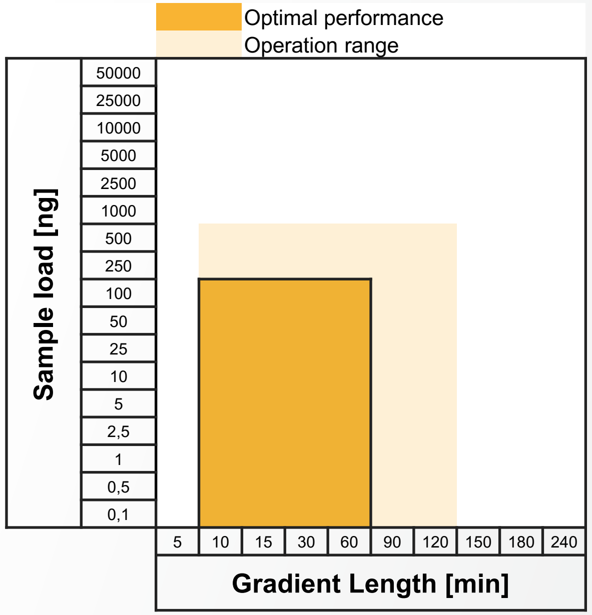
50 cm µPAC Neo Plus column







	50 cm µPAC™ Neo Plus 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**



Low input & Routine Proteomics

Highest sensitivity

Shorter gradients

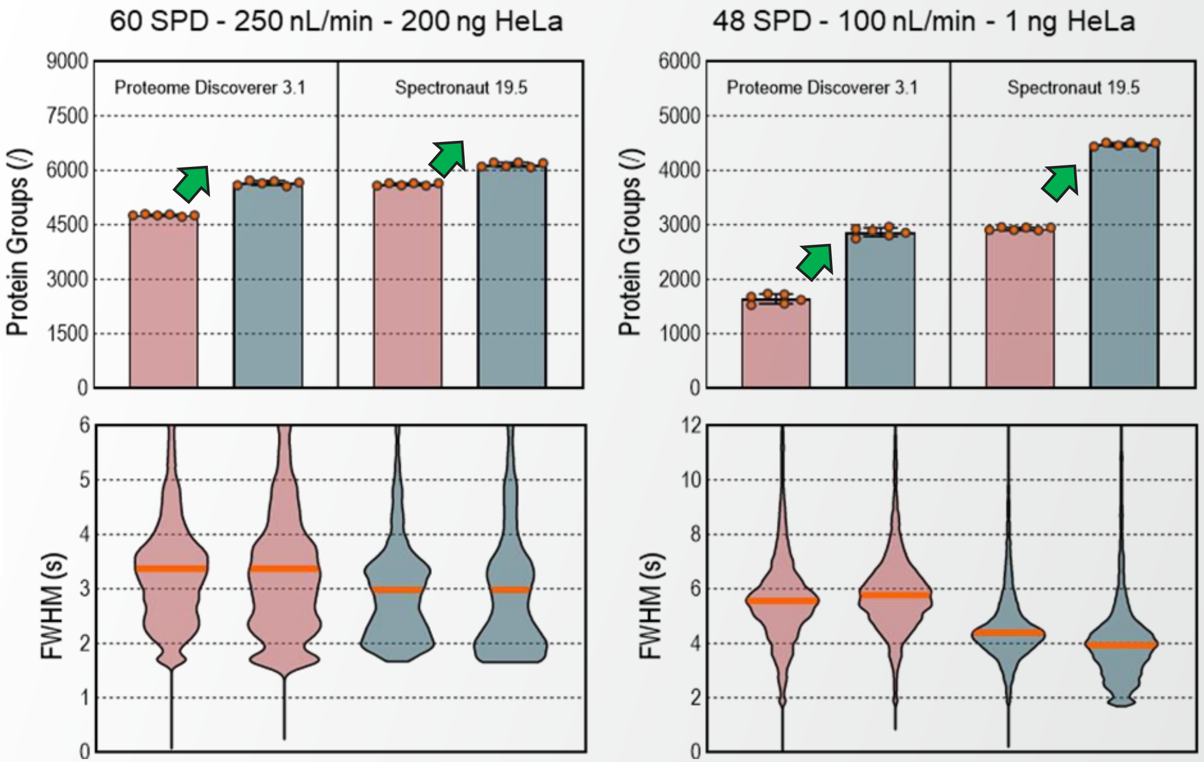
Optimal conditions

- Sample load ≤ 100 ng
- Flow rate 100-750 nL/min
- Gradient length ≤ 60 min

Optimized connectivity with µPAC Neo Plus format

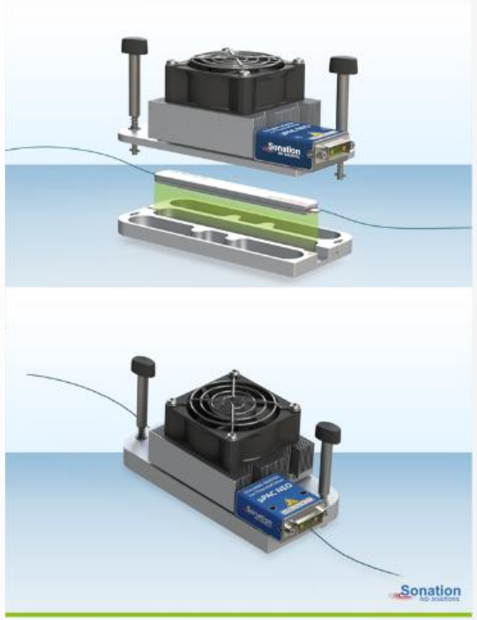
50 cm µPAC Neo

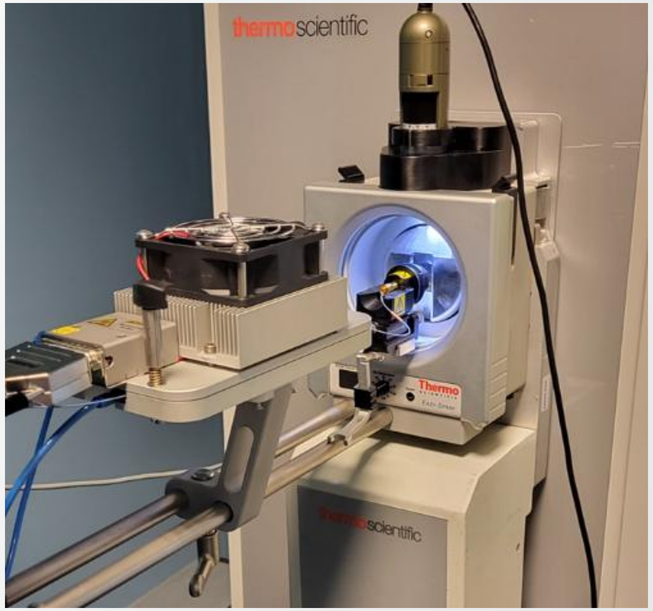
50 cm µPAC Neo Plus



- NanoViper fittings directly mounted onto 90 µm O.D. 20 µm I.D. fused silica capillary
- Grounding with precision engineered accessory
- Perfect bidirectionality
- 250 nL/min : Up to 10% decrease in peak width – **5-10% increase in proteome coverage**
- 100 nL/min : Up to 30% decrease in peak width - **25-40% increase in proteome coverage**

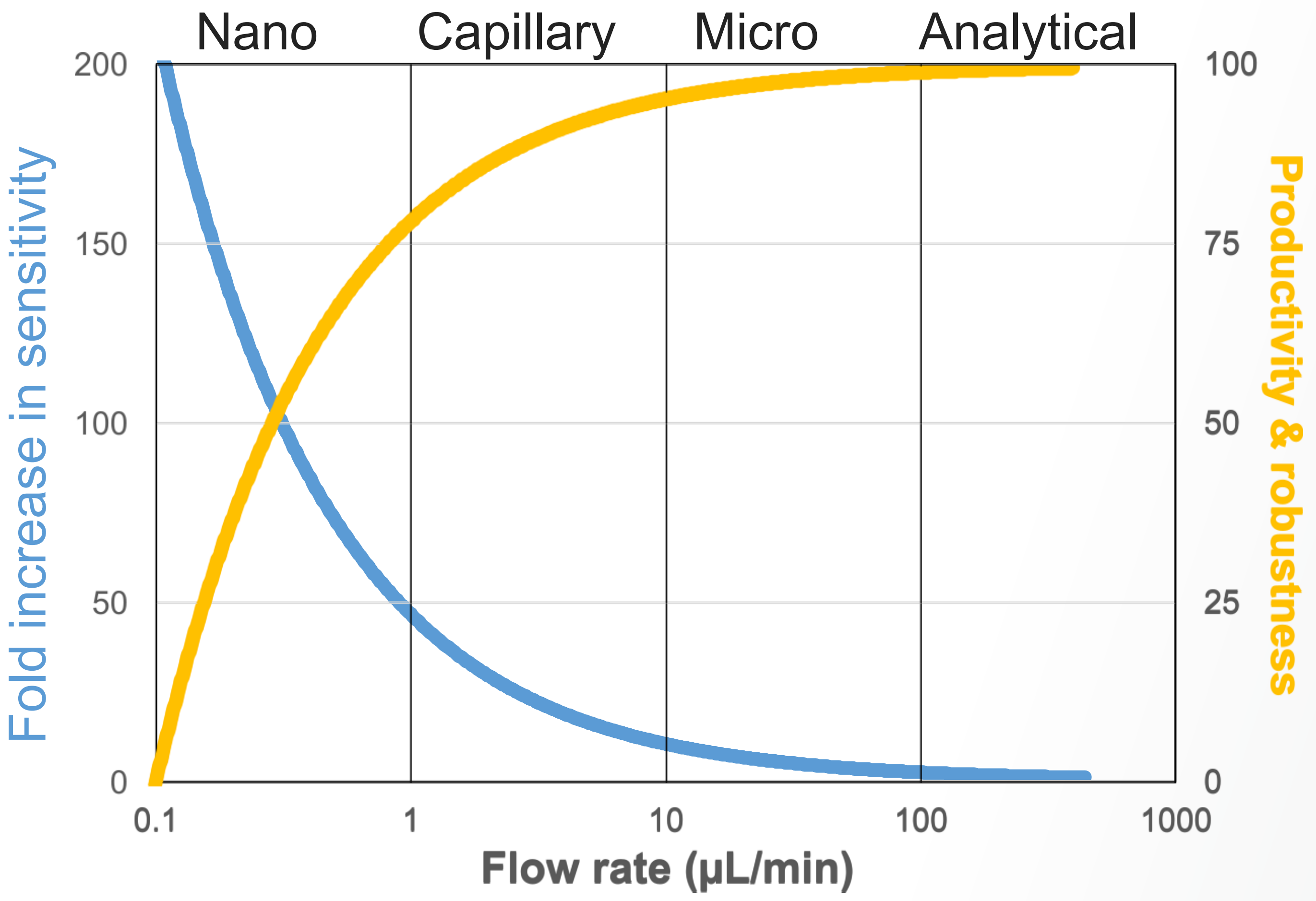
On-source mounting required for full benefits





Sensitivity versus throughput

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



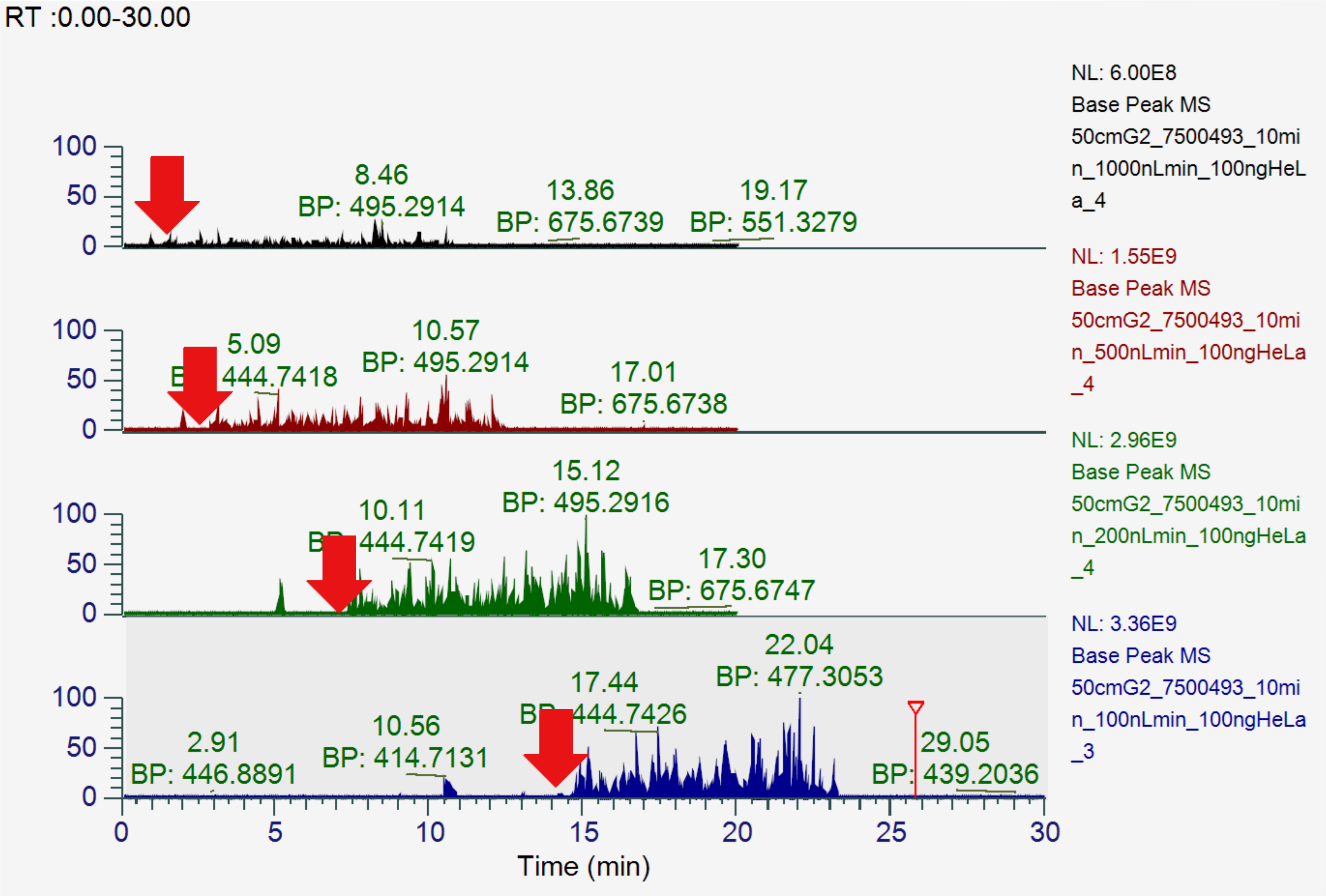
Low flow LC = reduced instrument productivity & robustness?

- 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)

Flow rate versus intensity in low flow LC-MS



1 µL/min

X 2.5

0.5 µL/min

X 2

0.2 µL/min

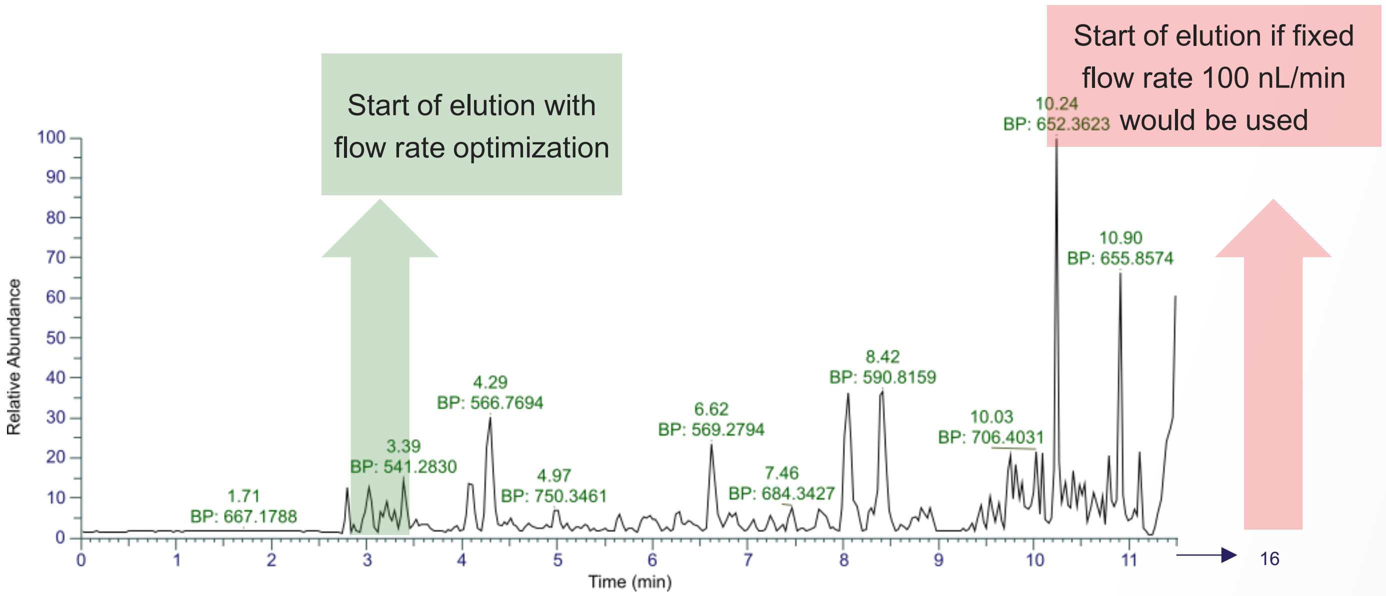
X1.5

X 1.2

0.1 µL/min

X 6!!

Option 1: Gradient completely formed at high flow rate



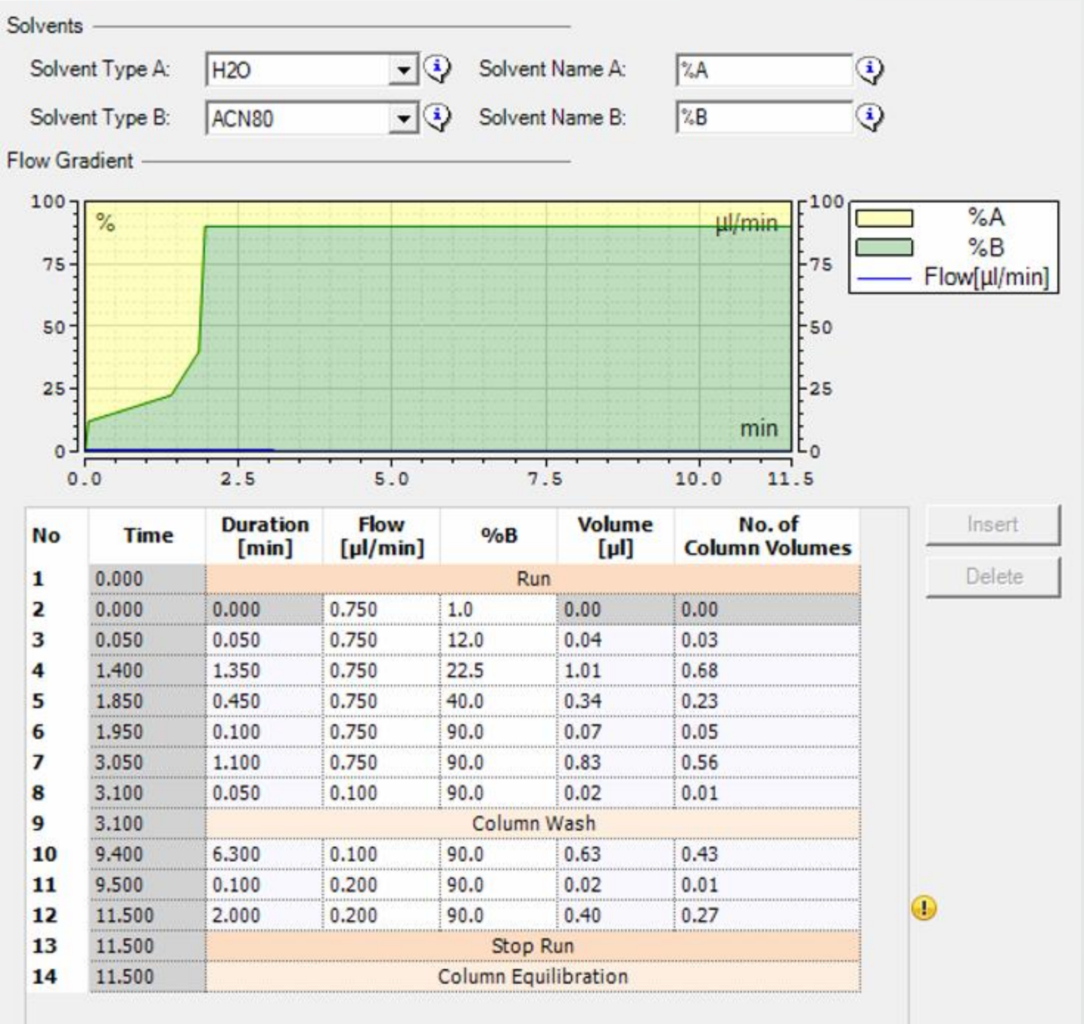
Start of elution with flow rate optimization

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

55% instrument productivity

(including sample uptake, sample loading and column equilibration)

- "High" Flow rate (750 nL/min) is used to form **full gradient** and pushes gradient with analytes towards detector
- At the point where analytes start eluting, the flow rate is reduced to "low" flow rate (100 nL/min) to boost sensitivity



Solvent Type A: H2O

Solvent Name A: %A

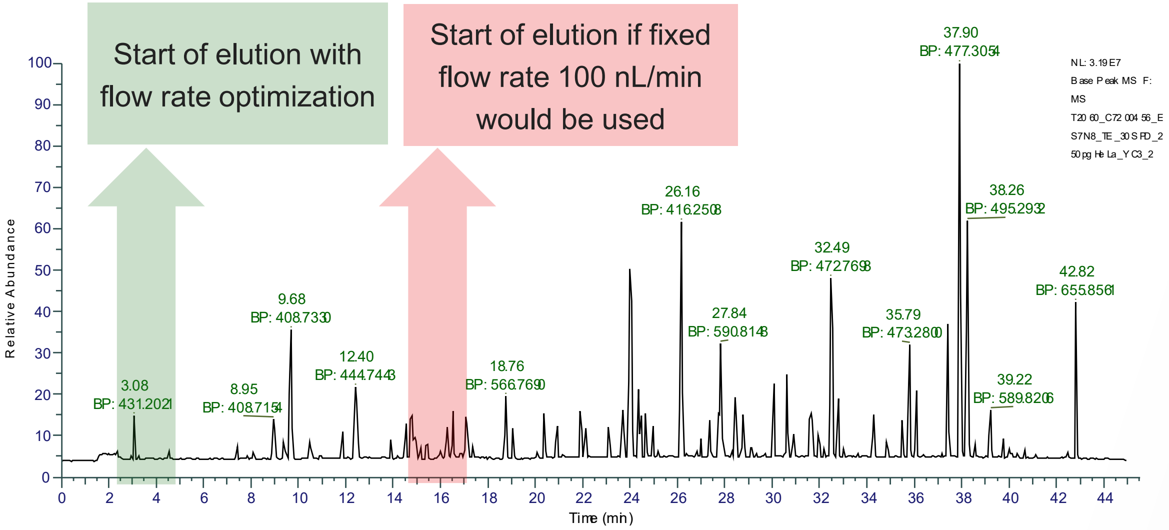
Solvent Type B: ACN80

Solvent Name B: %B

No	Time	Duration [min]	Flow [µl/min]	%B	Volume [µl]	No. of Column Volumes
1	0.000	Run				
2	0.000	0.000	0.750	1.0	0.00	0.00
3	0.050	0.050	0.750	12.0	0.04	0.03
4	1.400	1.350	0.750	22.5	1.01	0.68
5	1.850	0.450	0.750	40.0	0.34	0.23
6	1.950	0.100	0.750	90.0	0.07	0.05
7	3.050	1.100	0.750	90.0	0.83	0.56
8	3.100	0.050	0.100	90.0	0.02	0.01
9	3.100	Column Wash				
10	9.400	6.300	0.100	90.0	0.63	0.43
11	9.500	0.100	0.200	90.0	0.02	0.01
12	11.500	2.000	0.200	90.0	0.40	0.27
13	11.500	Stop Run				
14	11.500	Column Equilibration				

This strategy can be employed as long as $V_{gradient} \leq V_{void}$ (trap+column+tubing+…)

Option 2: Gradient partly formed at high flow rate



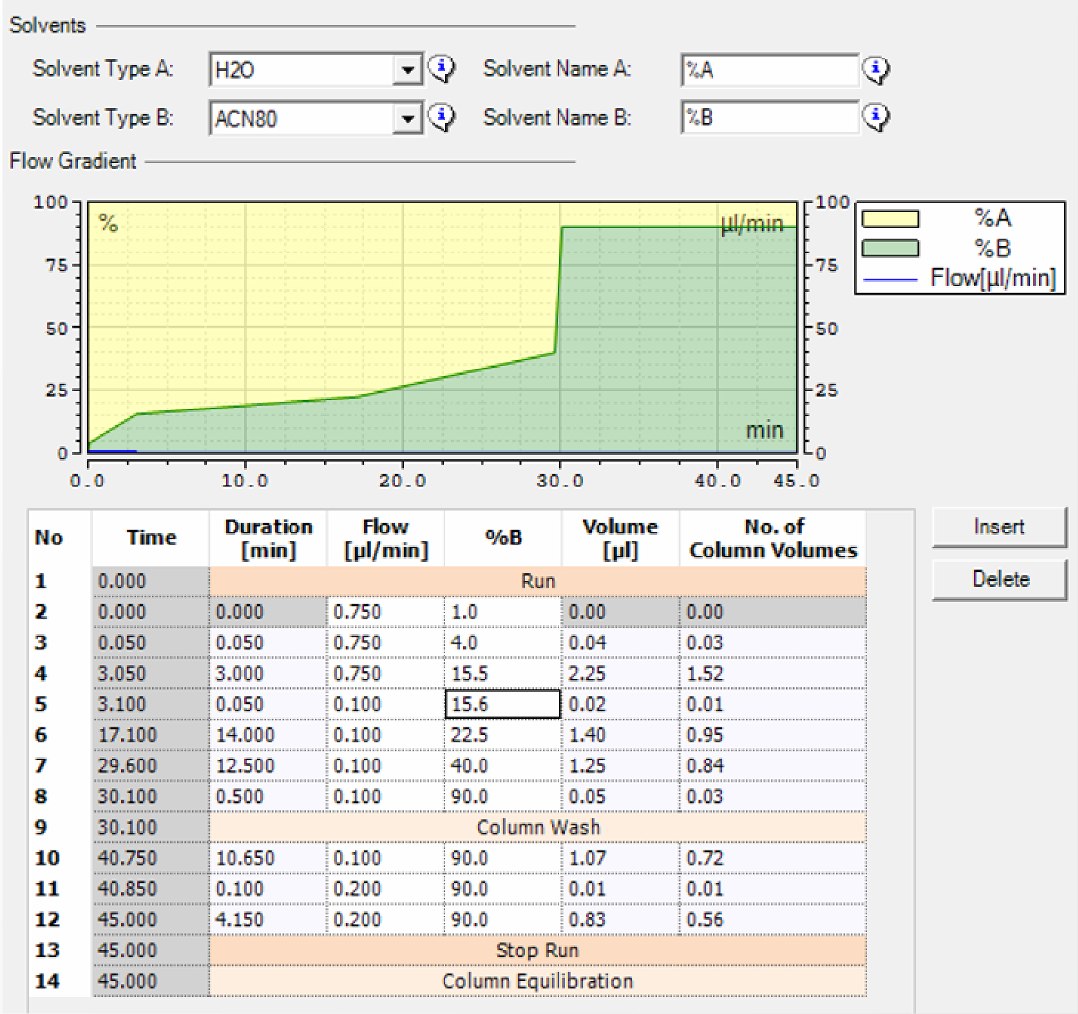
Start of elution with flow rate optimization

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

83% instrument productivity

(including sample uptake, sample loading and column equilibration)

- "High" Flow rate (750 nL/min) is used to form the gradient up to the point where analytes start eluting
- At the point where analytes start eluting, the flow rate is reduced to "low" flow rate (100 nL/min) to boost sensitivity


Solvents

Solvent Type A: H2O

Solvent Name A: %A

Solvent Type B: ACN80

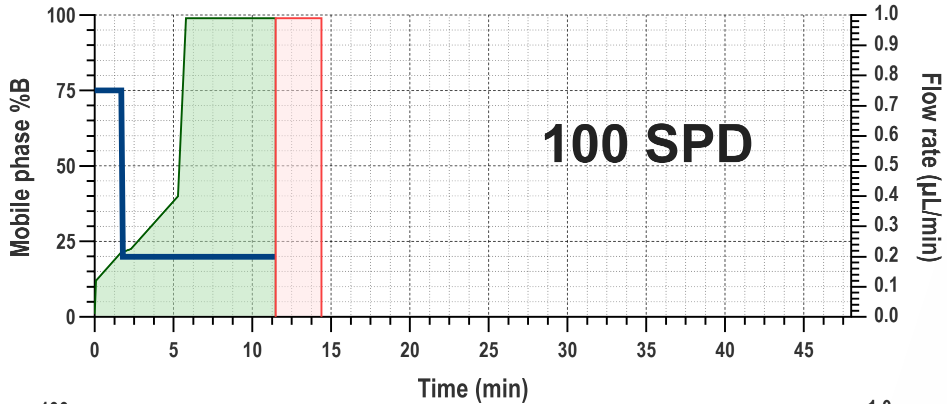
Solvent Name B: %B

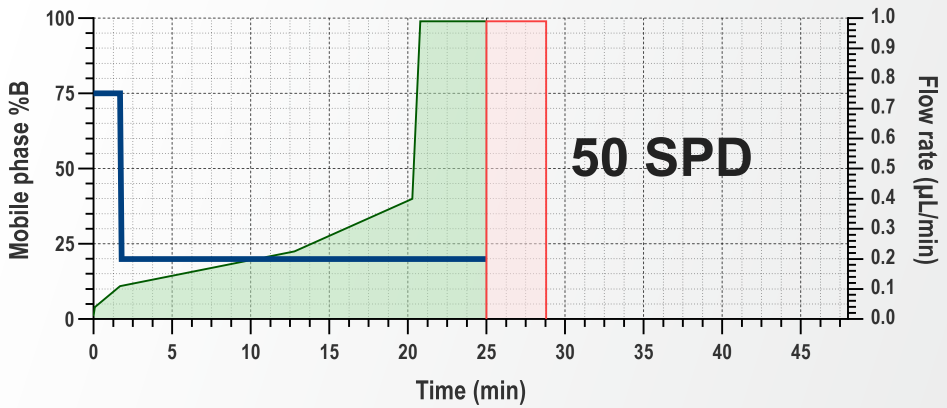
Flow Gradient

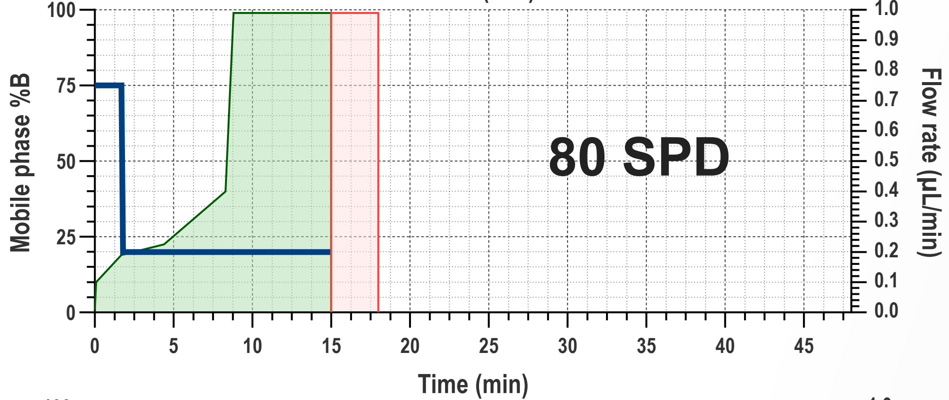
No	Time	Duration [min]	Flow [µl/min]	%B	Volume [µl]	No. of Column Volumes
1	0.000	Run				
2	0.000	0.000	0.750	1.0	0.00	0.00
3	0.050	0.050	0.750	4.0	0.04	0.03
4	3.050	3.000	0.750	15.5	2.25	1.52
5	3.100	0.050	0.100	15.6	0.02	0.01
6	17.100	14.000	0.100	22.5	1.40	0.95
7	29.600	12.500	0.100	40.0	1.25	0.84
8	30.100	0.500	0.100	90.0	0.05	0.03
9	30.100	Column Wash				
10	40.750	10.650	0.100	90.0	1.07	0.72
11	40.850	0.100	0.200	90.0	0.01	0.01
12	45.000	4.150	0.200	90.0	0.83	0.56
13	45.000	Stop Run				
14	45.000	Column Equilibration				

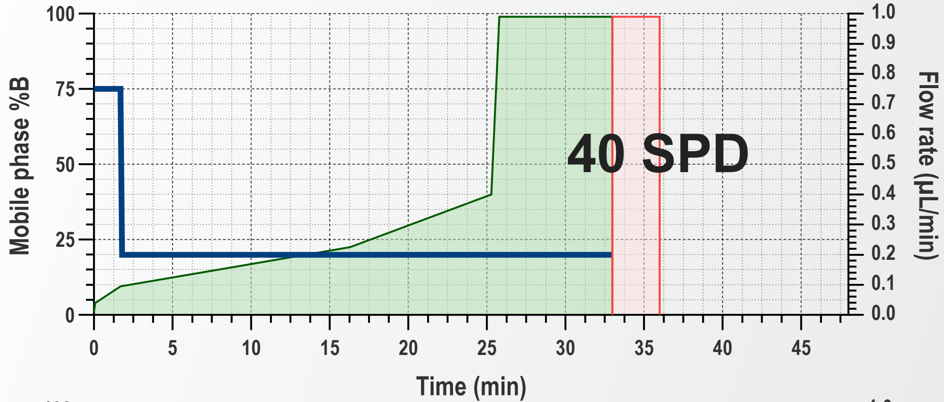
This strategy can be employed $V_{gradient} > V_{void}$ (trap+column+tubing+…)

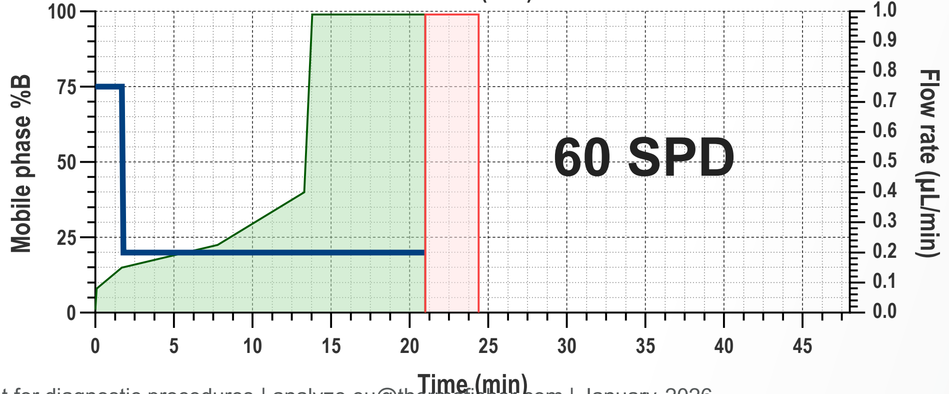
Gradient lengths evaluated

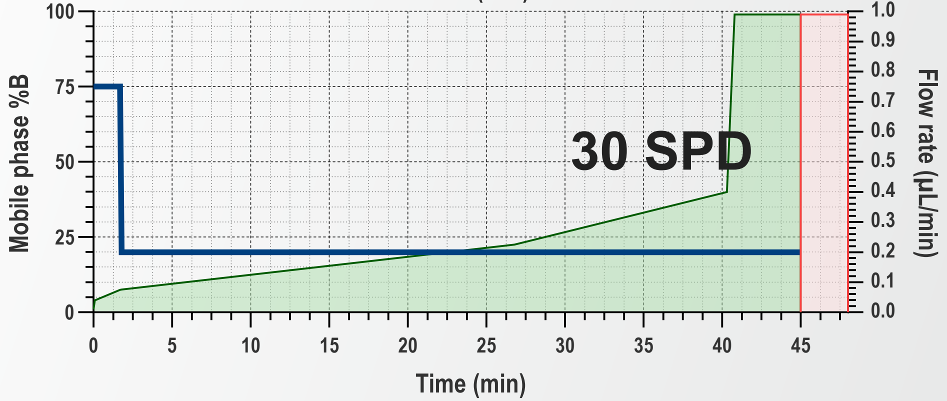




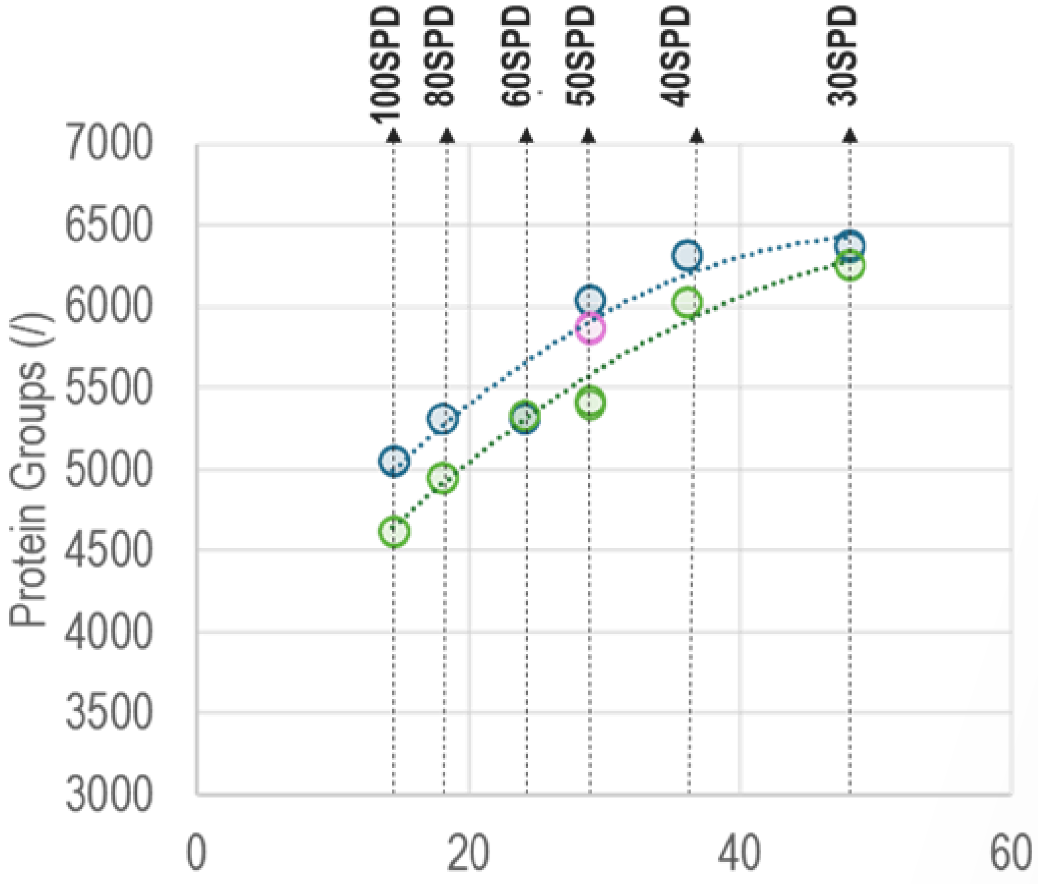


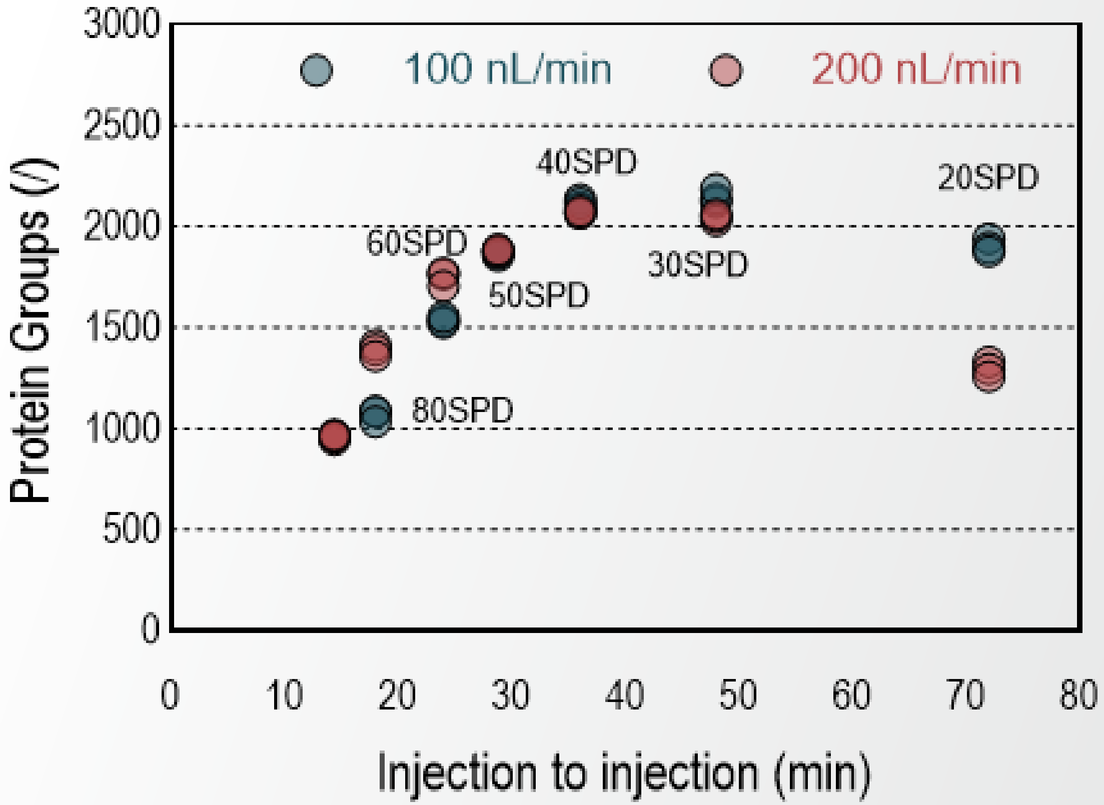






Flow rate comparison (100 vs 200 nL/min)



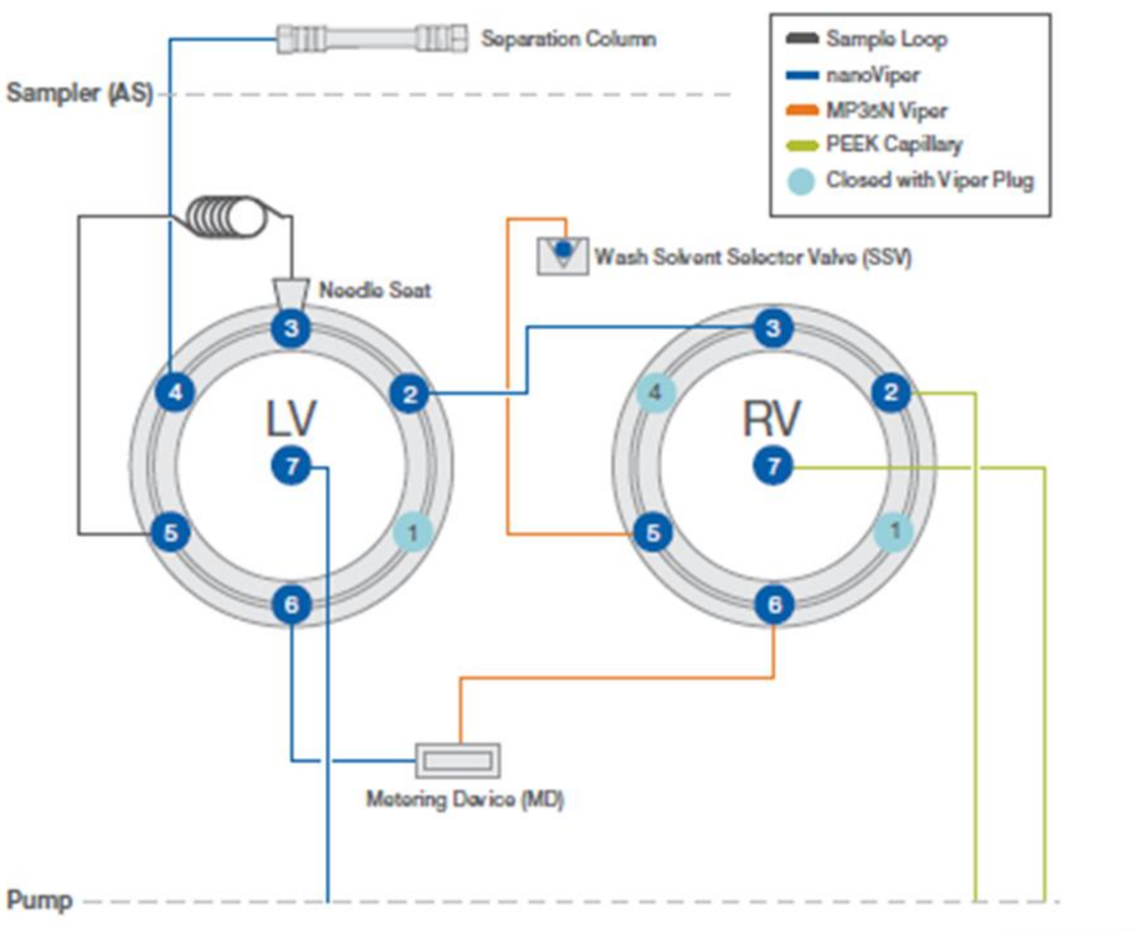


- Single cell by dilution (250 pg HeLa digest)
- 5 ng/µL – 50 nL sample volume
- Thermo Scientific™ Orbitrap™ Astral™ MS

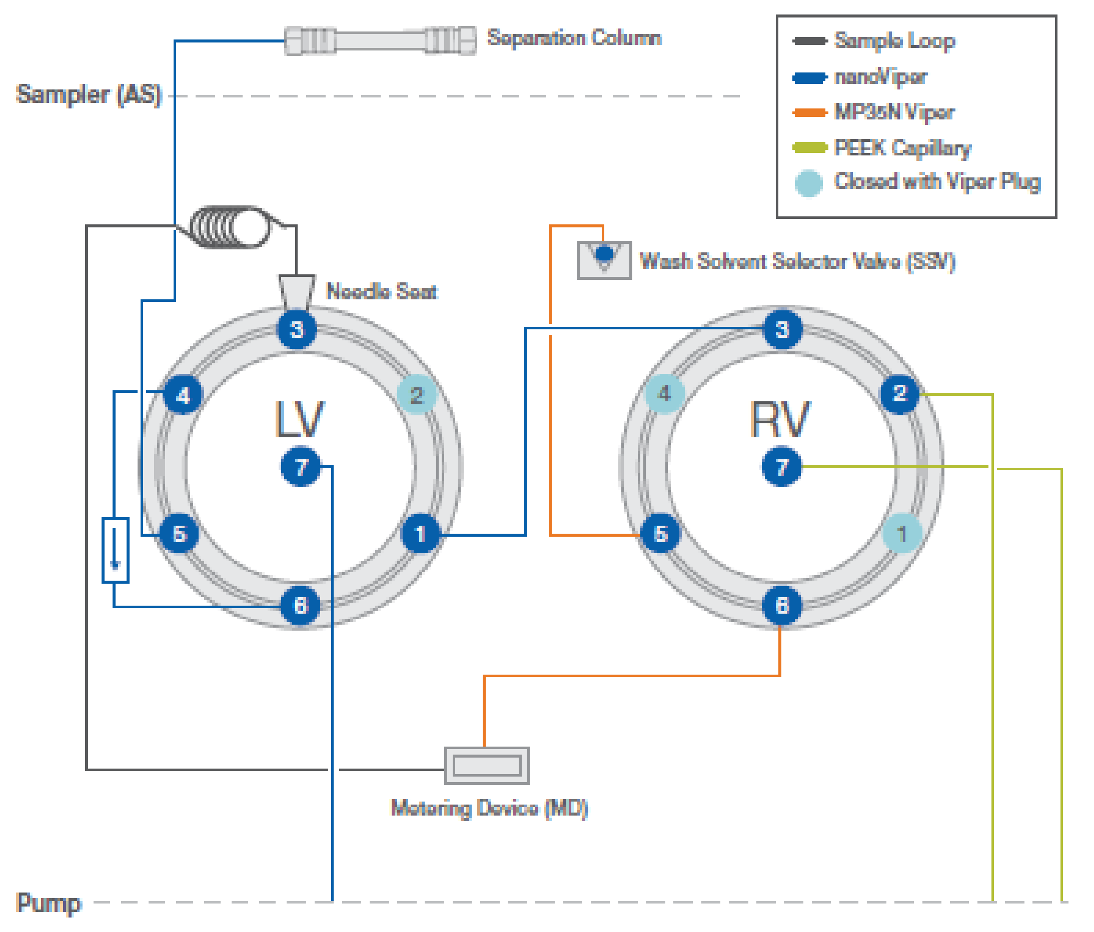
- Single HeLa cells
- Ca 0.01-0.05 ng/µL – 5 µL sample volume
- Thermo Scientific™ Orbitrap™ Exploris 240 MS

Direct injection versus trap and elute (backward and forward flush)

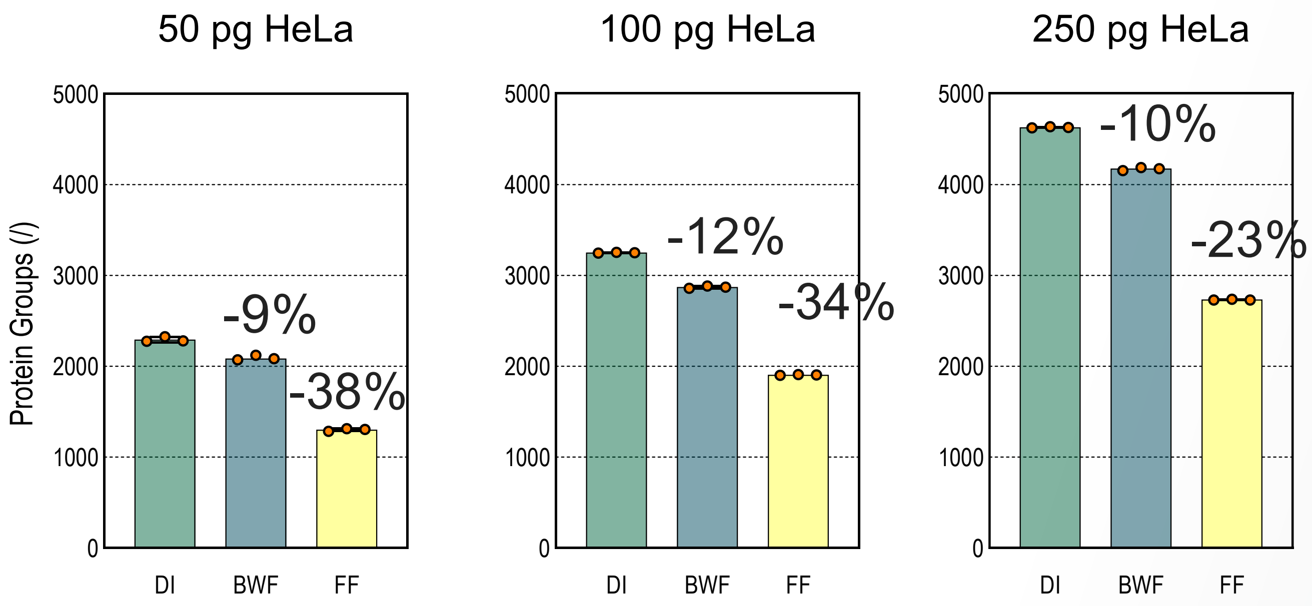
Direct injection

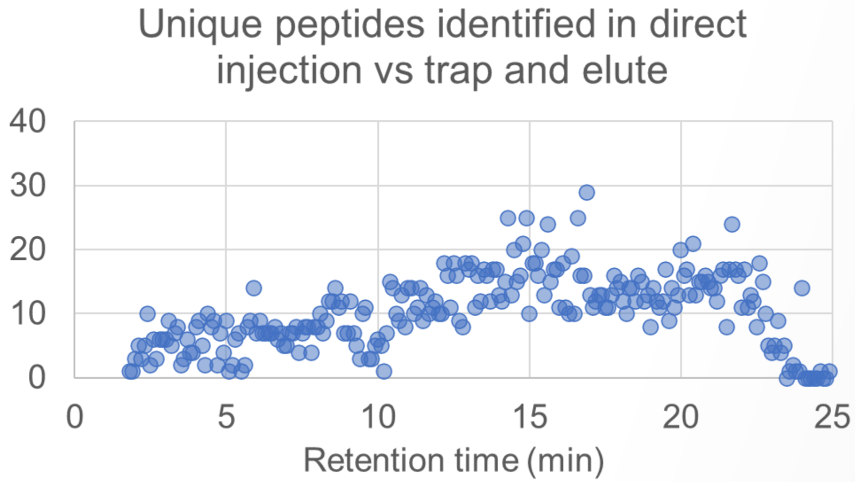


Trap and elute



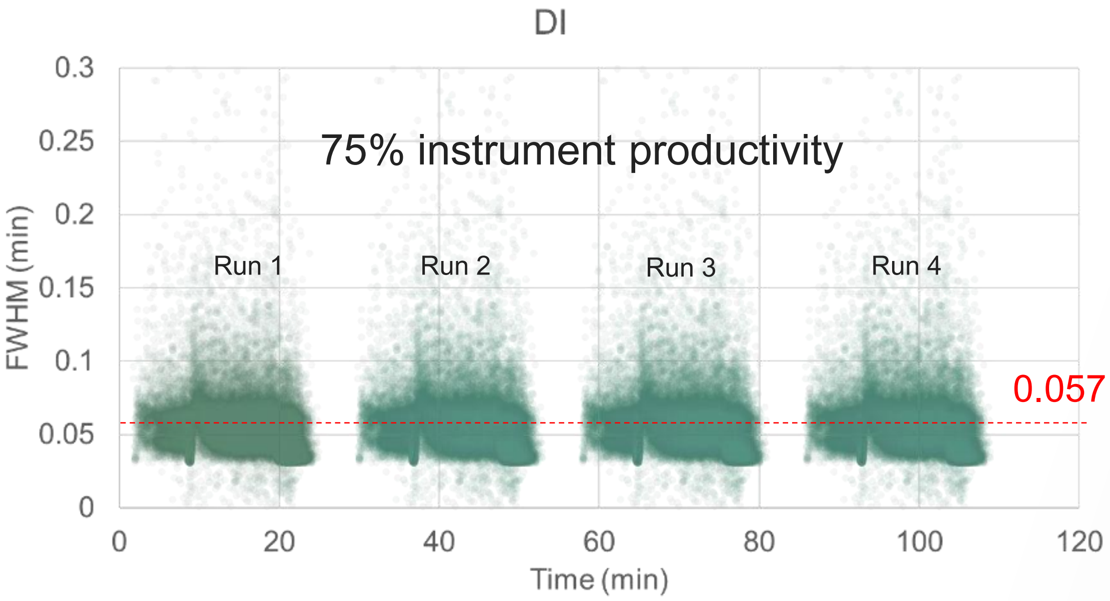
Direct injection vs trap and elute – single cell by dilution

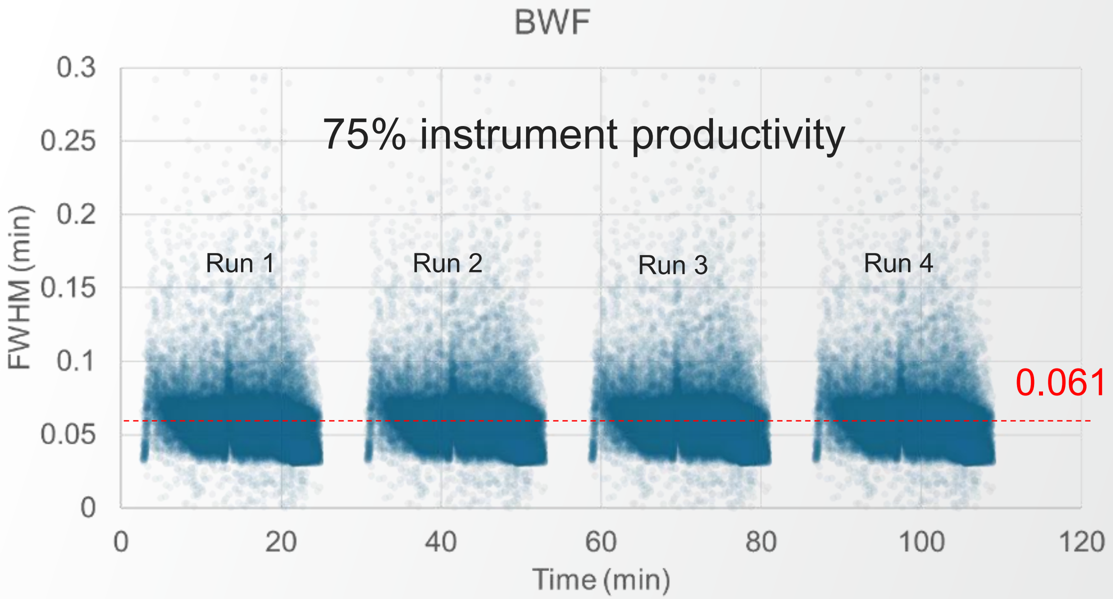


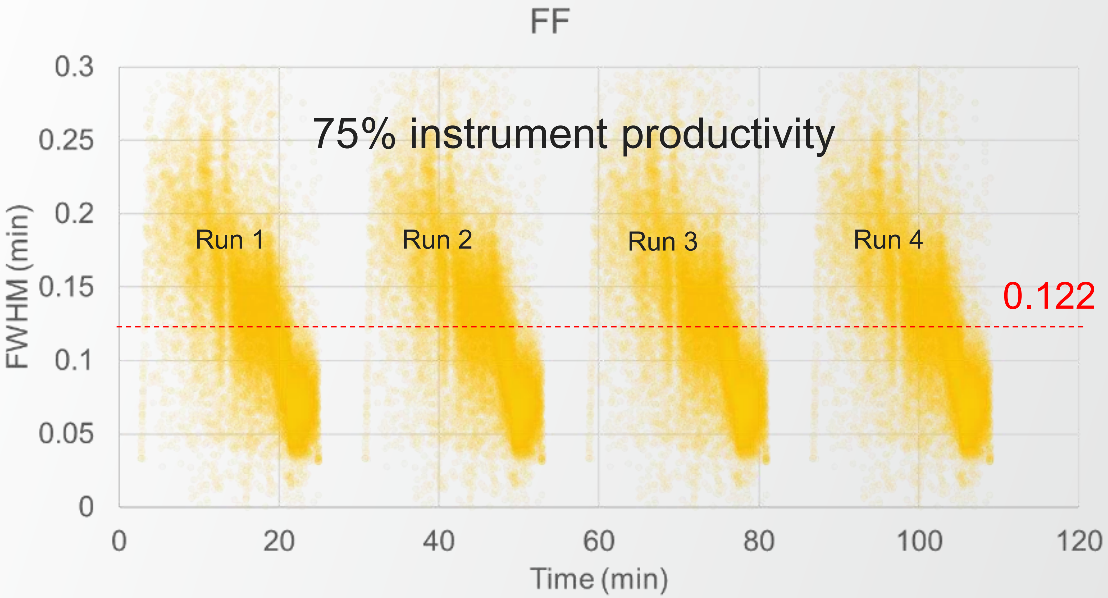


- 10-50 nL volume injections of concentrated HeLa digest
- 1.5 µL Weak wash used to push sample onto column or trap column (=trapping/washing volume)
- +-10% loss in proteome depth when switching from direct injection to backward flush trap and elute
- 20-40% loss in proteome depth when switching from backward flush trap and elute to forward flush trap and elute
- Loss attributed to increased peak width rather than loss of hydrophilic peptides

Impact of workflow on performance and productivity



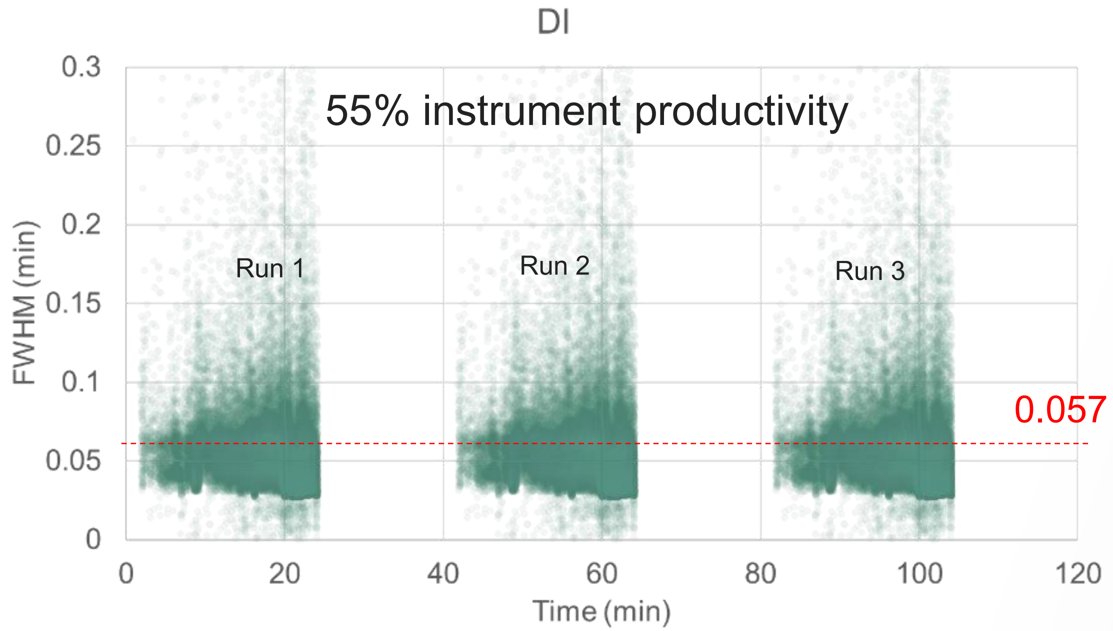


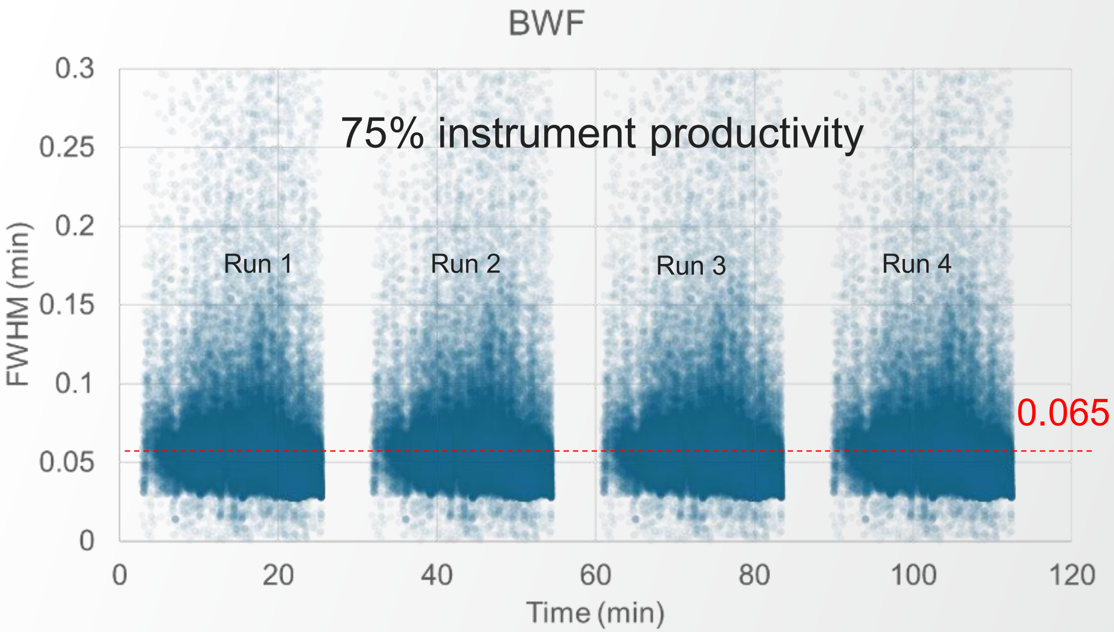


“Single cell by dilution”

- 10-50 nL volume injections of concentrated HeLa digest
- 1.5 µL Weak wash used to push sample onto column or trap column (=trapping/washing volume)
- Under these “ideal” conditions Direct injection sample loading only takes +- 2 min
- Column equilibration is performed during sample uptake

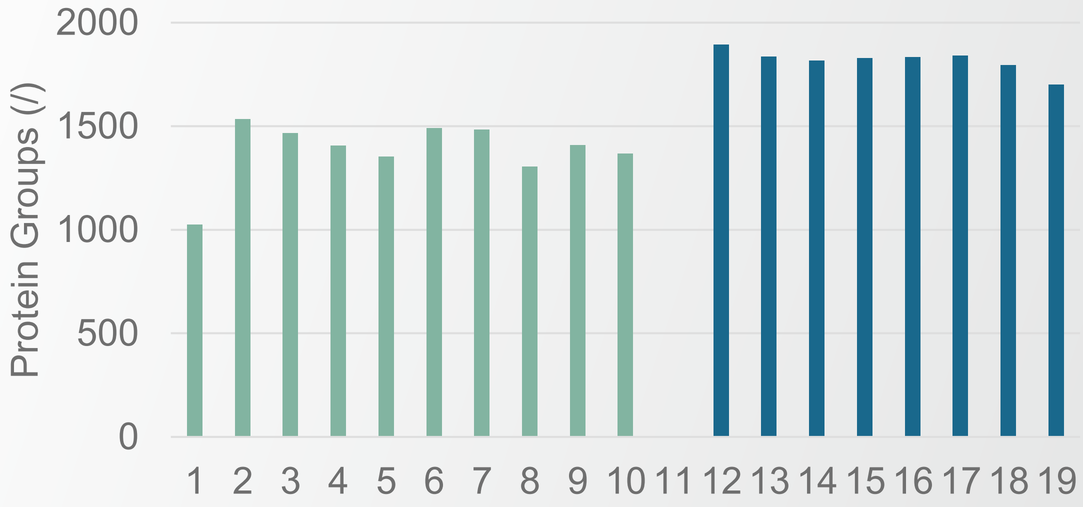
Impact of workflow on performance and productivity





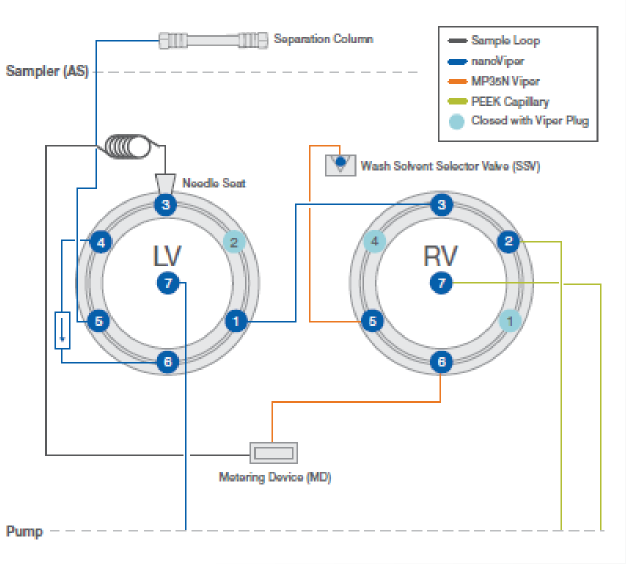
“True single cell”

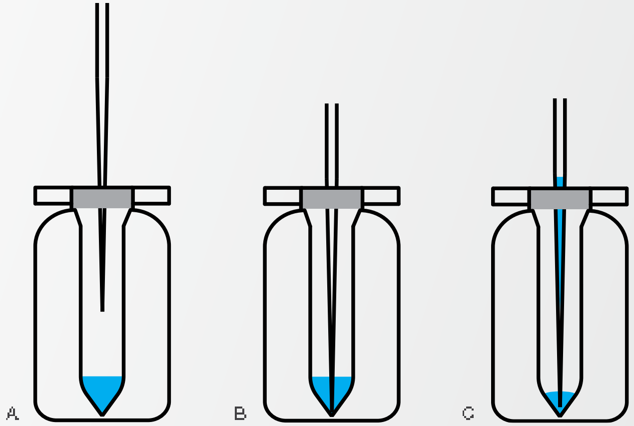
- 5µL volume injections of dilute single cell samples (7µL injected to ensure full aspiration)
- 5 µL Weak wash used to push sample onto column or trap column (=trapping/washing volume)
- Under these “realistic” conditions Direct injection sample loading takes +- 16 min
- Column equilibration is performed during sample uptake



Injection of single cell samples with Vanquish Neo UHPLC

Thermo Scientific™ Vanquish™ Neo UHPLC System





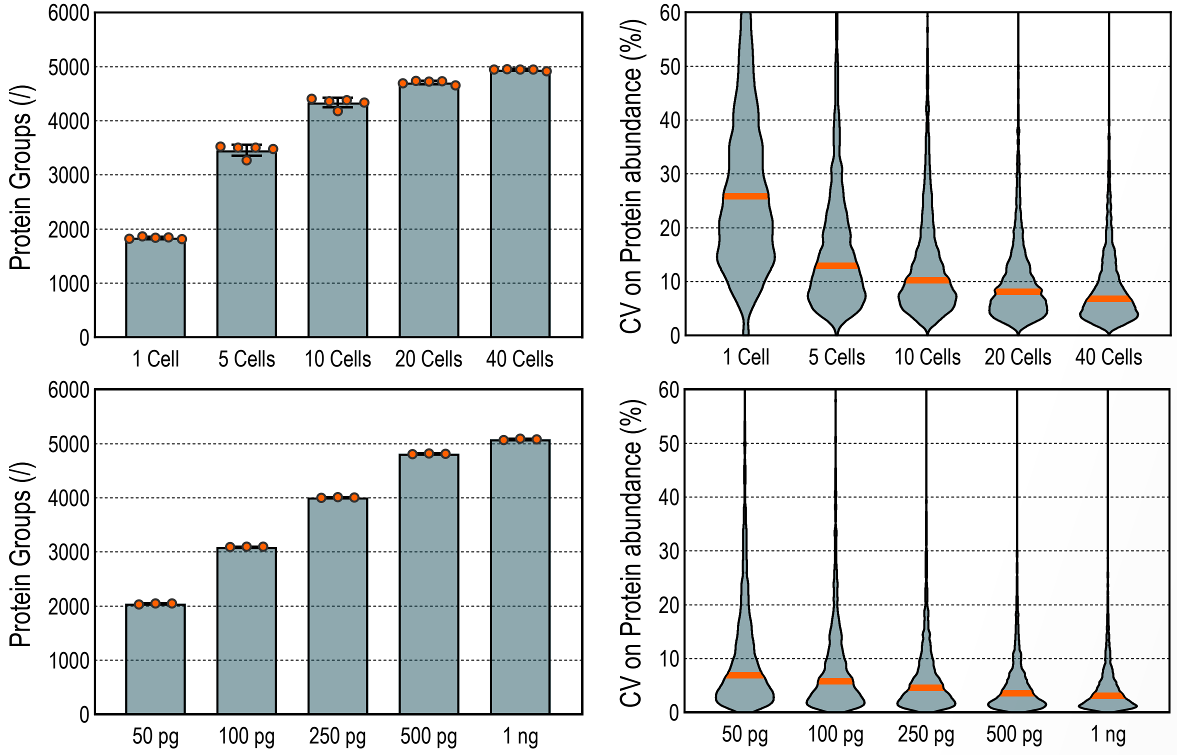
Back Flush trap and elute workflow

- 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

Single cell by dilution verus true single cells



Single HeLa cells isolated and digested in LoBind Eppendorf 384 well plate (+- 5µL volume)
7 µL Injection
5 µL loading volume

Bulk HeLa dilution (from 5 ng/µL sample)
10-20-50-100-200 nL Injection
1.5 µL loading volume

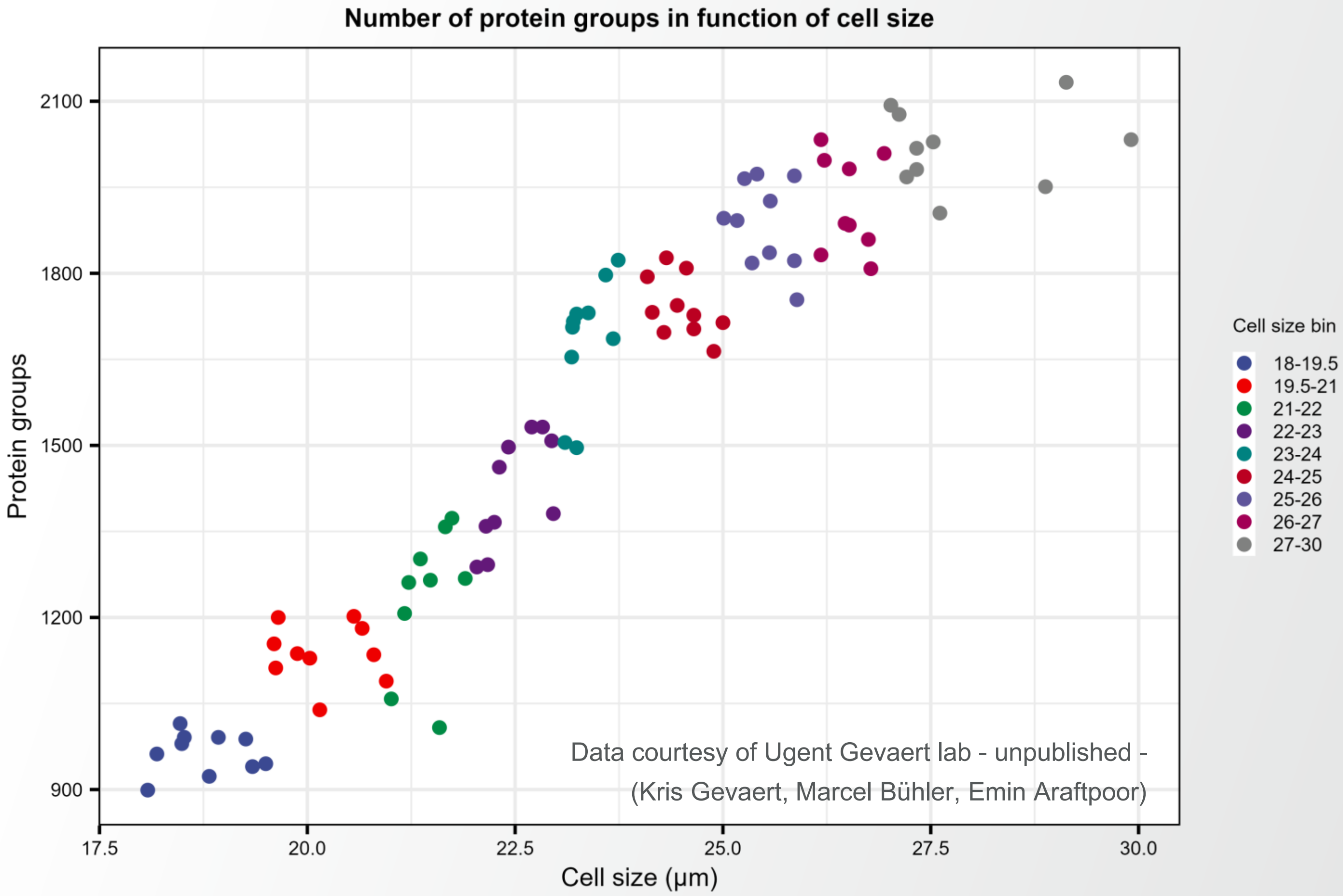
- 30SPD Trap & Elute – 100 nL/min elution
- Orbitrap Exploris 240 MS without FAIMS interface
- 120k MS2 – 246ms IT – 80Th windows – 400-800 m/z
- Processing in Spectronaut 19 – triplicates or quintuplicates together (MBR)

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!

- “30SPD Trap & Elute – 100 nL/min elution
- Thermo Scientific™ Orbitrap Exploris™ 240 without FAIMS
- 120k MS2 – 246ms IT – 80Th windows – 400-800 m/z
- Processing in Spectronaut 19 – cells within size bins (n=10) together (MBR)

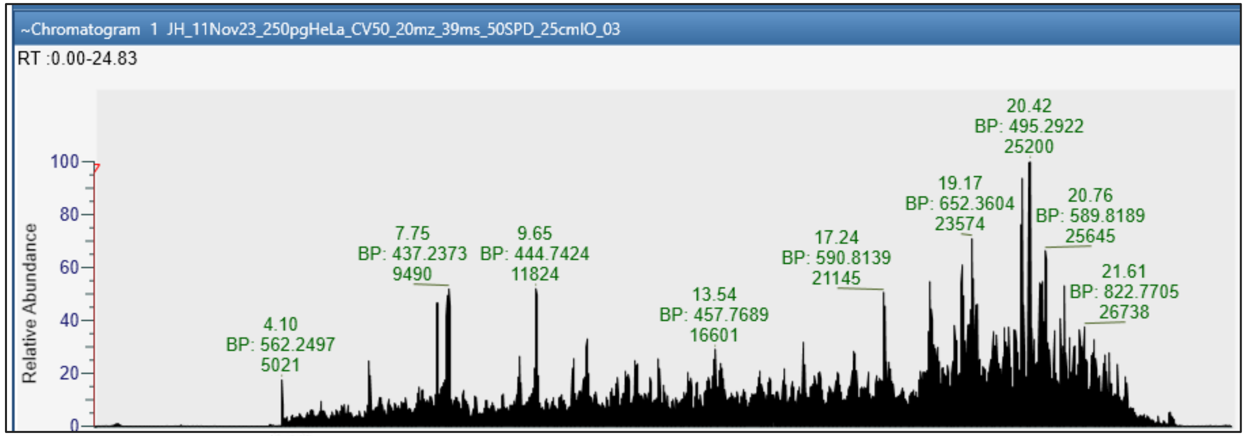


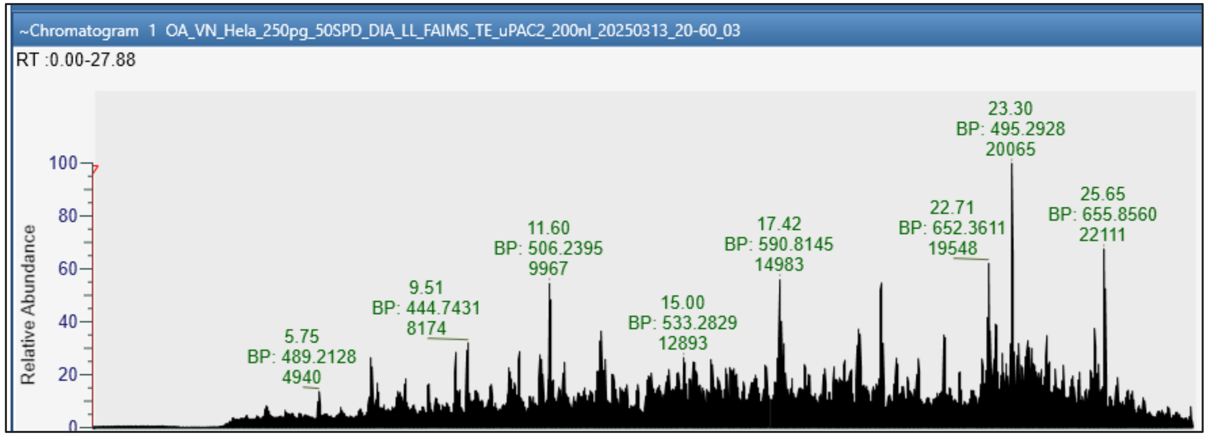
Data courtesy of Ugent Gevaert lab - unpublished - (Kris Gevaert, Marcel Bühler, Emin Araftpoor)

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

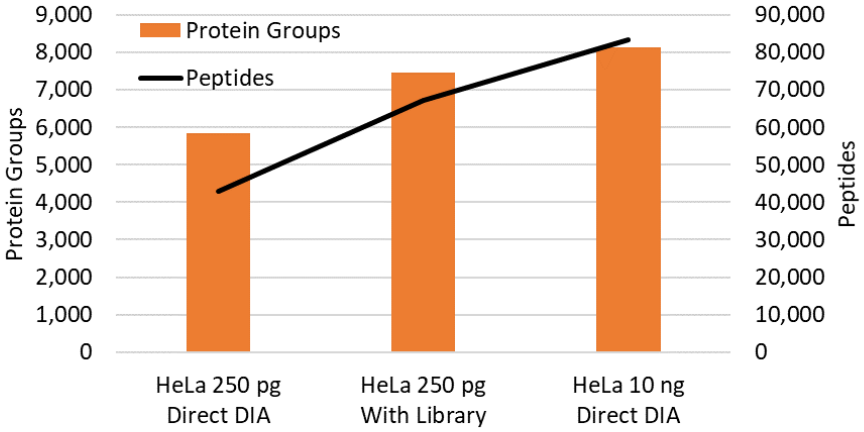
IDs Benchmark – *vs the 75 µm x 25 cm leader in performance for ultra low-load*

The general observation is at averaged 250pg level , the uPAC Neo2 in Trap and Elute setup with no DDM samples, allows recovering the same IDs as the Di setup + DDM, eg ~6000PG 40000 Peptides at 1% FDR at all levels (SN19)

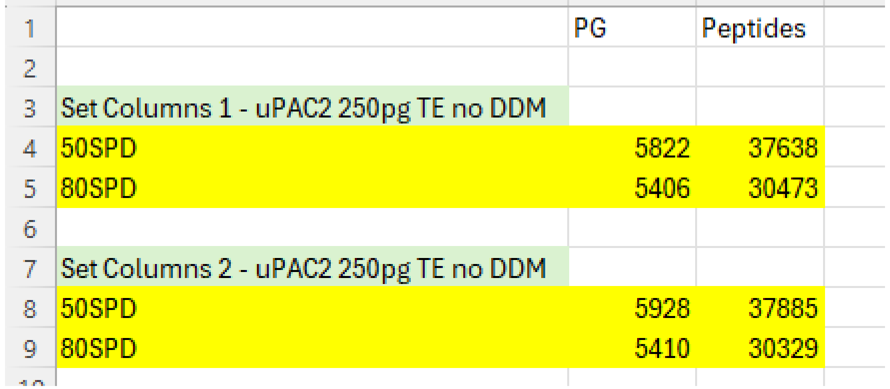




Direct Injection, DDM



Trap and Elute



		PG	Peptides
1		PG	Peptides
2			
3	Set Columns 1 - uPAC2 250pg TE no DDM		
4	50SPD	5822	37638
5	80SPD	5406	30473
6			
7	Set Columns 2 - uPAC2 250pg TE no DDM		
8	50SPD	5928	37885
9	80SPD	5410	30329

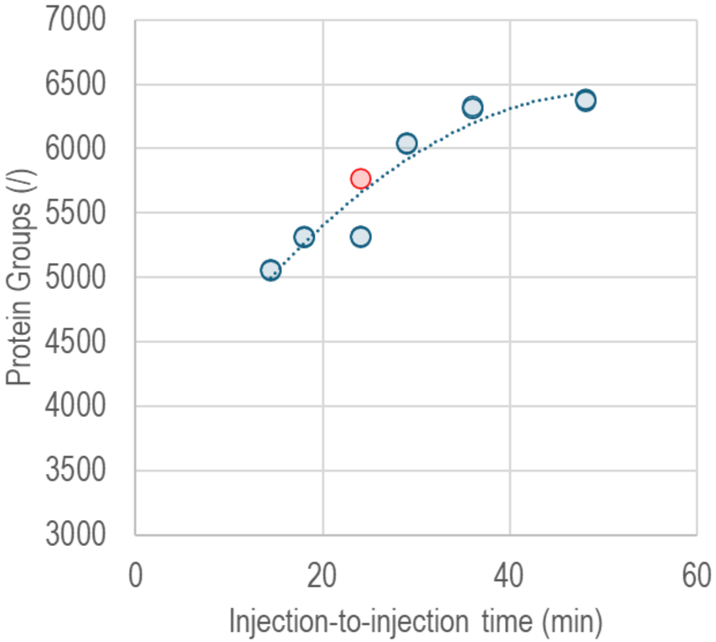
Betatesting and early adopters

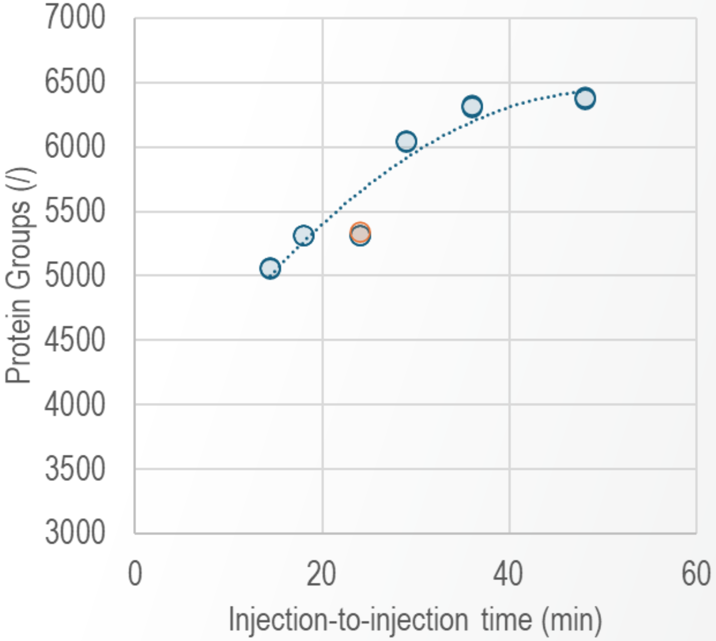
250 pg HeLa – single cell by dilution – installation runs (all trap and elute BWF)

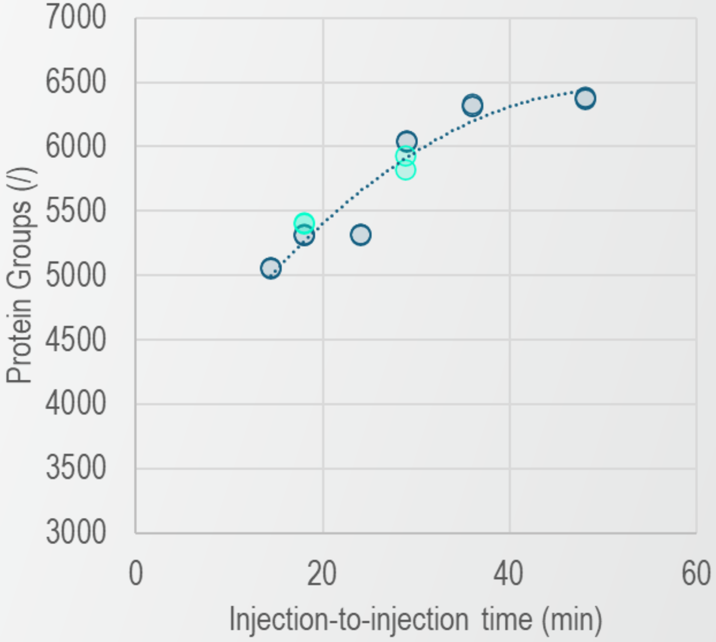
LSMBO

Curie CORE TECH

VIB-KU LEUVEN CENTER FOR BRAIN & DISEASE RESEARCH







Thermo Scientific™ OptiSpray™ Technology

Thermo Scientific™ OptiSpray™ Ion Source

Thermo Scientific™ OptiSpray™ Cartridges

An intelligent ESI interface that simplifies the acquisition of high-quality, reproducible nano and capillary LC-MS data.

Performance for deep, reproducible coverage

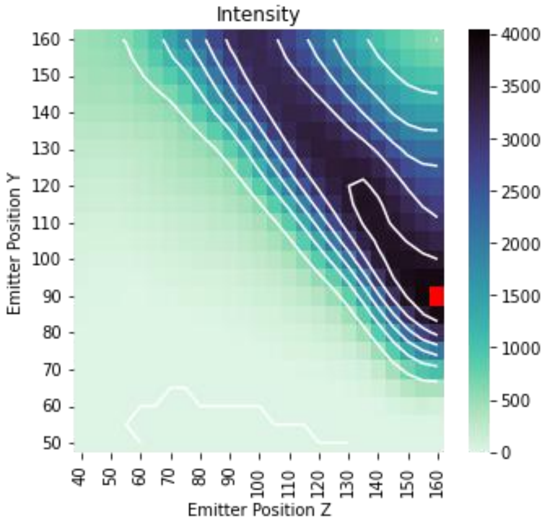
Simplicity for all users

Reliability across columns, users, and LC-MS systems

OptiSpray Source

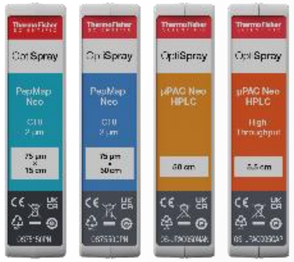
Performance

- All-in-one source for MS calibration, compound optimization, and acquisition
- Automated 3D emitter positioning



Simplicity

- Plug-and-play cartridge installation and spray optimization

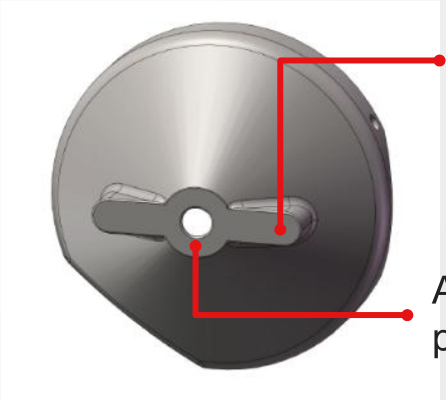


- Accessory cartridges for calibration, direct infusion, and source diagnostics and troubleshooting
- Visualization of emitter and spray

Reliability

- Automated installation and optimization yield highly-reproducible performance
- Inactive spray position between injections keeps MS system clean for longer

Sweep Cone

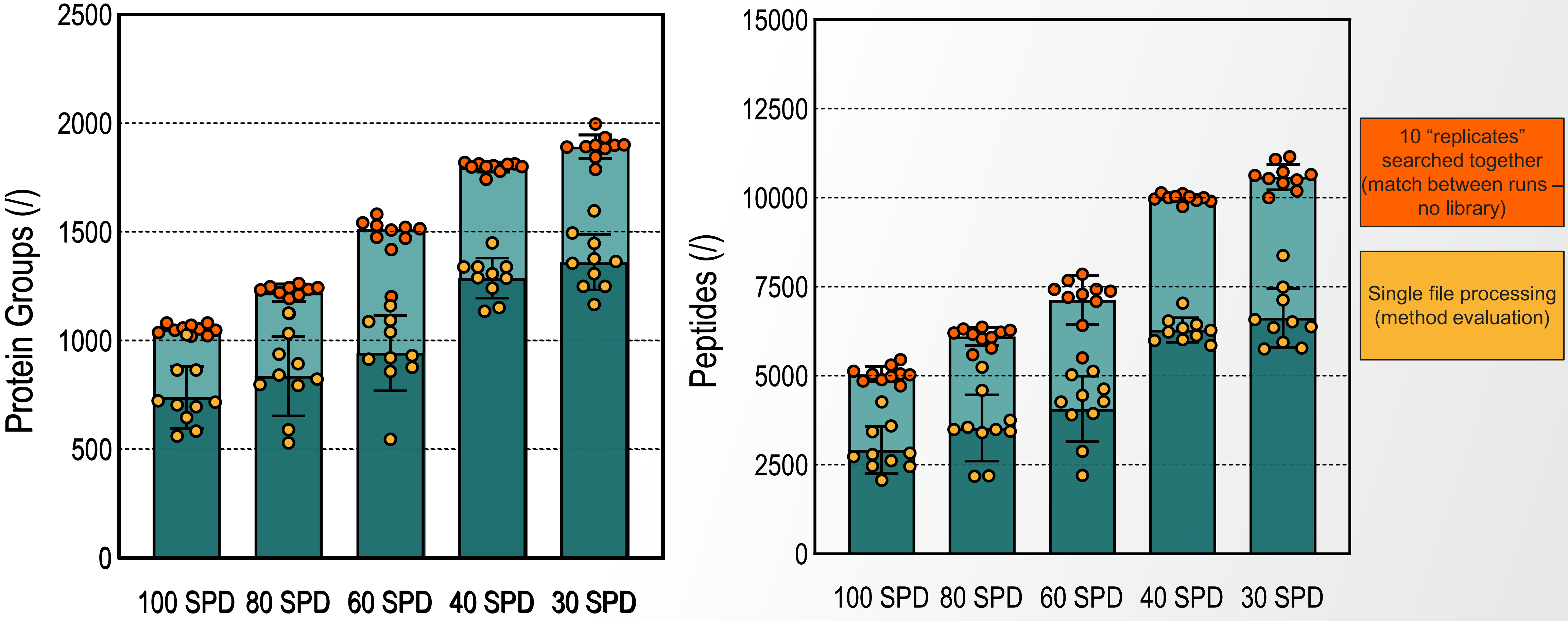


Inactive spray position

Active spray position

Reliable performance for all users

Single HeLa cells with 50 cm µPAC Optispray cartridge



10 "replicates" searched together (match between runs – no library)

Single file processing (method evaluation)

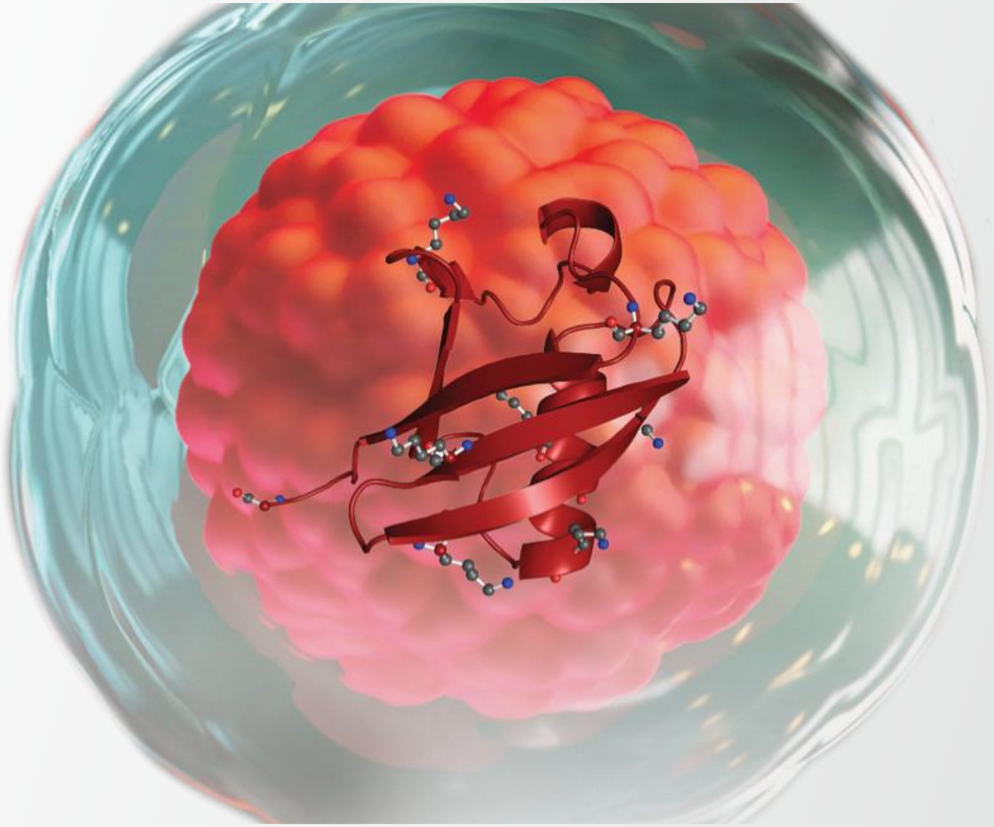
- Sample loading/initial transfer 750 nL/min
- Elution at 200 nL/min
- 2.1kV

- Data independent acquisition
- m/z 375-675 – 60 Th windows (N=5)
- 120k resolution MS2 – maxIT 246 ms

- Data processed with Spectronaut v19

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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- An Staes

Thank you