Supplementary methods

Centrifugal microfluidic automation of the protein aggregation capture workflow for robust mass spectrometry-based proteomics

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Michelle Hinrichs[a,b]‡, *Johanna Wallner*[c,d]‡, *Johanna Thiery*[e,f], *Carolin Kleber*[b], *Manuel Metzger*[a,b], *Stephan A. Sieber*[c], *Tobias Hutzenlaub*[a,b], *Oliver Schilling*[e], *Nils Paust*[a,b], *Hannes Hahne*[d], *Jan Muntel*[d], *Jan-Niklas Klatt*[a,b]*

Abstract text goes here. The abstract should be a single paragraph that summarises the content of the article

Supplementary materials and methods

M1 – Cell culture and cell analysis

HEK293 cells were cultured in DMEM Glutamax phenolred medium (Gibco, Thermo Fisher, 31966-021) with 10 % fetal bovine serum (Pan Biotech, P30-3031) and 1 % penicillin/streptomycin (Gibco, Thermo Fisher, 15140-122) in 10 cm cell culture dishes (Greiner Bio-One GmbH, 664160) under standard cell culture conditions (37 °C, 5 % CO_2). The cells were subcultured at 70 % confluency. For cell harvesting, the medium was removed, cells were washed with Dulbecco's phosphate-buffered saline (DPBS) (Gibco, Thermo Fisher, 14190-094), collected by scraping in 1 mL DPBS, and transferred to a 2 mL reaction tube. The cells were pelleted (1400 g, 3 min) and the supernatant removed. Cell pellets were stored at -80 °C. For lysis, cell pellets were thawed and transferred in 1 mL covaris tubes (520128, Covaris) filled with lysis buffer (1 % SDS (Sigma Aldrich, 327311000), 100 mM HEPES (pH 8.5) (Apollo Scientific, BI8181). The samples were sonicated in the M220 focused-ultrasonicator (Covaris, 500295) for 2 times 180 s, 6 °C, 200 CPB, followed by heating the samples for 1 h, 95 °C, 900 rpm and another round of sonication (180 s, 6 °C, 200 CPB). The lysed cells were centrifuged (10 min, 14000 rpm). The supernatants were pooled, and protein concentration was determined using a BCA assay (Thermo Fisher Scientific, 23227). Hep G2 (HB-8065, ATCC) cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) (PAN-Biotech, P04-04500) with 10 % (fetal bovine serum) (PAN-Biotech, P30-3031) under standard cell culture conditions (37 °C, 5 % CO_2) in T75 flasks (Greiner, 658175). When cells reached 70 % confluency, they were washed with warm PBS(-) (phosphate-buffered saline) (PAN-Biotech, P04-36500) and split by incubating them with accutase (PAN-Biotech, P10-21500) at 37 °C until they detached. Warm PBS(-) was added, and the cell suspension was transferred to a falcon. After spinning the cells down (200 g, 5 min), the supernatant was removed, and the cells were resuspended in their culture medium and cultured again in T75 flasks. For harvesting the cells, they were washed twice with cold PBS(-). Lysis buffer (5 % SDS (sodium dodecyl sulfate, Sigma, 05030-500ML-F), 50 mM TEAB (tetraethylammonium bromide, Sigma, T7408-500ML) pH 7.55) was added, and cells were removed from the flask using a cell scraper. The cell suspension was transferred to a falcon, and cells were lysed using a sonication stick (2 rounds, 15 s/round, 60 %, Bandelin Sonoplus mini20). The lysate was centrifuged (14,000 g, 10 minutes, 10 °C), the supernatant transferred to a new reaction tube, the protein concentration determined using a BCA assay (Thermo Fisher Scientific 23227), and the lysate stored at -80 °C. Proteins were reduced and alkylated before the experiment with 10 mM tris(2-carboxyethyl)phosphine (TCEP) (Sigma Aldrich, C4706-2G) and 40 mM chloroacetic acid (CAA) (Sigma Aldrich, 8024120100) for 30 min at 37 °C.

[a.] *Hahn Schickard, Georges-Koehler-Allee 103, 79110 Freiburg, Germany*
[b.] *Laboratory for MEMS Applications, IMTEK-Department of Microsystems Engineering, University of Freiburg, Georges-Koehler-Allee 103, 79110 Freiburg, Germany*
[c.] *Chair of Organic Chemistry II, Center of Functional Protein Assemblies (CPA), TUM School of Natural Sciences, Technical University of Munich, Ernst-Otto-Fischer Strasse 8, 85748 Garching, Germany*
[d.] *OmicScouts, a Momentum Biotechnologies Company, Lise-Meitner-Strasse 30, 85354 Freising, Germany*
[e.] *Institute for Surgical Pathology, Medical Center, University of Freiburg, Breisacher Strasse 115a, 79106 Freiburg, Germany*
[f.] *Faculty of Biology, University of Freiburg, Schaenzlestrasse 1, 79104 Freiburg, Germany*
‡, Contributed equally
*, Corresponding Author

M2 – Bead testing experiments

Based on the calculated bead surface area, the PureCube bead amount was first adjusted to match the 1:10 (w/w) SeraMag reference condition (corresponding to approximately 1:1 protein to bead (w/v)). For bead testing experiments, Hep G2 lysates were processed using the KingFisher Apex robot (Thermo Fisher Scientific, 5400930). 50 µg protein lysate (1 µg/µL) with protein-to-bead ratios of 1:0.5, 1:0.8, 1:1, and 1:2 (w/v) were systematically evaluated and benchmarked against the 1 µm SpeedBeads reference (1:10, w/w) on the KingFisher Apex (Thermo Fisher Scientific, 5400930). The samples were precipitated for 30 minutes with 70 % ethanol (Carl Roth, 9065.2) on magnetic carboxylate modified beads (protein:beads 1:10, w/w) (SpeedBeads Cytiva; GE65152105050250, GE45152105050250). The beads were washed three times and resuspended in their original volume with ddH_2O beforehand. The magnetic beads with the precipitated proteins were washed two times with 80 % ethanol (Carl Roth, 9065.2) and once with 100 % acetonitrile (Th. Geyer, 2697-2.5L) for 5 minutes each. Captured proteins were reduced for 45 minutes at 37 °C (f.c. 10 mM 1,4-dithiothreitol (Roth, 6908.2), 50 mM tris(hydroxymethyl)aminomethane pH 7.5 (VWR, 103156X), 50 % acetonitrile (Carl Roth, 9065.2)), alkylated for 30 minutes at 22 °C (f.c. 55 mM 2-choloroacetamide (Merck, 8. 02412)) and washed with 100 % acetonitrile. Proteins were digested in 50 mM ammonium-bicarbonate (Roth T817) with trypsin (trypsin/protein 1:25 (w/w), Promega, V5517) at 37 °C overnight on a thermomixer at 650 rpm. Beads were washed with 2 % trifluoroacetic acid (Th. Geyer, 044630.AP) to retrieve remaining peptides, and both elution fractions were combined to a f.c. of 1 % trifluoroacetic acid. Acidified peptides were directly desalted using C18 cartridges (Agilent, 5190-6532) on the BRAVO liquid handling platform (Agilent), following the manufacturer's instructions. Afterwards, samples were dried down again and resuspended in 0.1 % fluoroacetic acid (Roth, 1EHK.1) with 2 % acetonitrile (Chemsolute, 2697) for nLC-MS/MS measurements.

M3 – Organic solvent and stick pack experiments

For organic solvent and stick-pack experiments, HEK293 cells were used. 20 µg protein lysate was reduced and alkylated in 50 µL (100 mM HEPES, 10 mM TCPE, 40 mM CAA) at 37 °C for 30 min. The remaining workflow was performed using the KingFisher robot (ThermoFisher Scientific). Proteins were precipitated on magnetic PureCube Carboxy Magbeads (Cube Biotech, 50201) (protein:beads, 1:0.8 w/V) using 30 %, 50 %, or 70 % acetonitrile or ethanol. Beads were washed three times in either 80 % ethanol or 100 % acetonitrile, depending on the solvent used for precipitation. For the standard workflow, which was used as a reference, proteins were precipitated using 70 % ethanol and were washed twice with 80 % ethanol and once with 100 % acetonitrile. Proteins were digested in 50 mM ABC with trypsin (trypsin/protein 1:25 (w/w)) at 37 °C overnight on a thermomixer at 650 rpm. Beads were washed with 2% TFA to retrieve remaining peptides, and both elution fractions were combined to a final concentration of 1 % TFA. Acidified peptides were directly desalted using C18 cartridges (Agilent, 5190-6532) on the BRAVO liquid handling platform (Agilent), following the manufacturer's instructions. Peptides were eluted twice with 50 µL elution buffer. Afterwards, samples were dried down again and resuspended in 0.1 % FA with 2 % acetonitrile for nLC-MS/MS measurements.

M4 – Deparaffinisation and lysis of formalin-fixed paraffin-embedded (FFPE) prostate tumor sections

For the application experiment of the AutoPAC-Disk for clinical relevant samples sections from a single FFPE protstate tumor block was used. This material originated from study approved by the Ethics Committee of the University Medical Center Freiburg (20-1083) and conducted in compliance with institutional ethical standards.

FFPE tissue microdissection, deparaffinization and lysis

Prostate carcinoma tissue was fixed in 4% formalin (Sigma-Aldrich) for 12–24 h, dehydrated via graded ethanol series, cleared in xylene (Merck), embedded in paraffin by standard procedures, and stored at ambient temperature. FFPE blocks were sectioned at 10 µm thickness into reaction tubes. Deparaffinization involved addition of 1.2 mL xylene (L13317.AU, ThermoFisher Scientific) to each (10 sections) 10 µm slide, followed by two 30-min incubations at 75 °C with shaking (600 rpm). Samples were centrifuged (10 min, 18,000 × g), supernatant discarded, then washed twice with 1 mL absolute ethanol (5054.3, Roth) for 5 min at room temperature (500 rpm), with centrifugation (10 min, 18,000 × g) and supernatant removal after each wash. Pellets were air-dried prior to lysis. Each of the resulting ten pellets was resuspended in 140 µL lysis buffer (1 % SDS (327311000, ThermoFisher Scientific), 100 mM HEPES pH=8 (BI8181, Apollo Scientific)). The suspensions were pooled into two 1 mL Covaris tubes (520128, Covaris) and sonicated using a M220 Covaris instrument (500295, Covaris) under following conditions: 2x 180 seconds at 6 °C, 200 CPB. Samples were then incubated for 1 hour at 95 °C with shaking at 900 rpm in a thermoshaker. Sonication was repeated using identical parameters, followed by transfer to a fresh tube, centrifugation at maximum speed for 10 minutes, and collection of the supernatant into a fresh tube. Protein concentration was determined by Pierce™ BCA Protein Assay (23225, Thermo Scientific).

Post-processing and nLC-MS/MS measurement

The AutoPAC-Disk and manual workflow were performed as described in the main article. After processing the reaction was stopped by acidification to a final concentration of 1 % TFA (Merck, 1082180050). Acidified supernatants from AutoPAC-Disk and manual samples were transferred to fresh tubes and centrifuged (full speed, 10 minutes). The resulting supernatant was again transferred to a fresh tube, followed by drying in a vacuum concentrator (Eppendorf) at 45 °C and storage at -

80 °C. All samples from the AutoPAC and manual workflow were cleaned via the stage-tip protocol[31].

Samples were measured on an Orbitrap Astral Mass Spectrometer (ThermoFisher Scientific) coupled to a Vanquish Neo UHPLC-system (ThermoFisher Scientific) equipped with a trap cartridge (Pepmap 100 C18 5 µm 0.3x5 mm, Thermo Katalog Dreieich, 174500) and an analytical column (Aurora Elite, Generation 4, IonOpticks). Peptides were separated on the analytical column using a gradient ranging from 6 % to 32 % buffer B (0.1 % fluoracetic acid (1EHK.1 Roth) in HPLC-MS grade acetonitrile (2697, Th Geyer)) in buffer A (0.1 % fluoracetic acid (1EHK.1 Roth) in HPLC-MS grade water (455, Th Geyer)).

Full proteomes were acquired using a data-independent acquisition (DIA) MS/MS method with 27 min active gradient (MS1 orbitrap resolution 240,000, MS1 scan range 360-1,000 m/z, MS1 RF lens 40, MS1 normalized AGC target 300 %, MS1 maximum injection time 5 ms, MS2 Astral resolution 30,000, MS2 scan range 150-2,000 m/z, HCD collision energy 24 %, MS2 normalized AGC target 300 %, MS2 maximum injection time 3 ms, MS2 isolation window 2 m/z, DIA window coverage 360-1,000 m/z).