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Comprehensive and Robust Proteome Profiling using Online-2D NanoLC coupled to the Orbitrap Exploris 480 MS

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INTRODUCTION

Although high proteome coverages have been obtained with single shot liquid chromatography−tandem mass spectrometry (LC-MS/MS) analysis, multiple dimensional separation are necessary for deeper proteome profiling. Offline high-pH reversed-phase LC (high-pH RPLC) followed by low-pH reversed-phase LC (low-pH RPLC) has gradually replaced the strong cation exchange (SCX) coupled with low-pH reversed-phase LC-MS/MS. This is due to better efficiency of peptide separation under RP conditions, better nano LC-MS compatibility, and lack of need for an extra desalting step after the first-dimension sample fractionation. However, an offline high-pH RP fractionation method still has potential for improvement. For example, it is time-consuming, affording low throughput, and requires large amounts of sample. It is also prone to sample loss during fraction collection and transfer between fractionation devices and analytical instruments. Here we present a simple-to-use online low-flow high-pH RP x low-pH RP separation platform (termed online 2D-nanoLC) for deep proteome profiling using a Thermo Scientific™ UltiMate™ 3000 RSLCnano system coupled to a Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer.

MATERIALS AND METHODS

Sample Preparation

HeLa protein digest (Thermo Scientific™ Pierce™ HeLa protein digest, 20 µg/vial) was reconstituted to a final concentration of 800 ng/µL with 0.1% formic acid (FA) in water.

Serum samples from SeraLab (UK) were trypsin digested using a simple procedure that does not include reduction nor alkylation of cysteine residues. Briefly, 200 µL methanol was added to 50 µL serum aliquots in an Eppendorf™ LoBind™ 96-well plate for protein precipitation. The plate was vortexed for 5 minutes and subsequently centrifuged (1500 g, room temperature). After discarding the supernatant, the plate was inverted and allowed to dry for 15 minutes. 200 µL digestion mix (pH 7.0) was added to each well and the plate vortexed until the pellet was re-dissolved. Another 250 µL digestion mix was added and digestion allowed to proceed for 3 hours at 37 ° C and 1250 rpm. The digestion was quenched using 50 µL of a 10% FA solution. 100 µL of the digest was loaded onto a Thermo Scientific™ HyperSep™ C18 cartridge (100 mg bed weight, 1 mL capacity), washed with 400 µL 0.1% FA, eluted with 400 µL 0.1% FA in 50% ACN, dried and resuspended in 200 µL of 0.1% FA. Subsequently, 160 µL was transferred to a polypropylene vial with a glass insert prior to measurement.

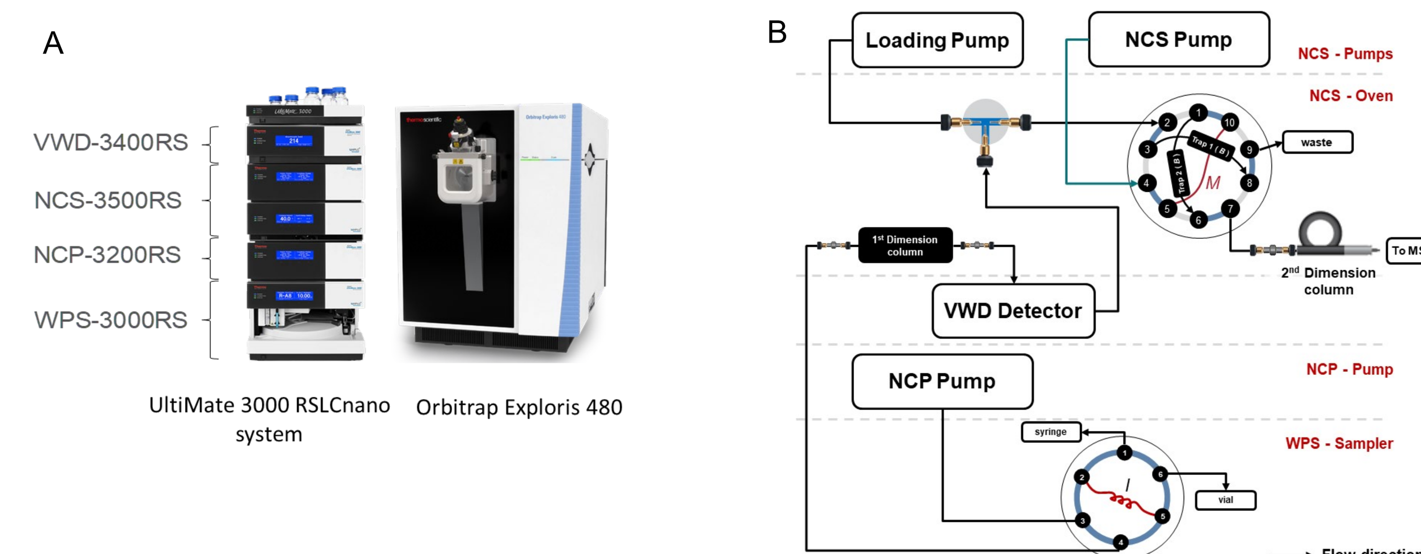
Online 2D-nanoLC-MS system

The high pH RP (pH 8.0, 50 mM NH4HCO3) peptide separation was accomplished on a monolithic capillary Thermo Scientific™ PepSwift™ column (100 µm x 250 mm). A loading pump was used to dilute and acidify the eluate from the first dimension. Eluted peptides were subsequently concentrated onto one of two trap columns every 45 min and eluted from the trap column onto an EASY-Spray (75 µm x 15 cm) column using a nano-flow pump for low pH RP (pH 3.0, 0.1% FA) peptide separation. The column was connected to an Orbitrap Exploris 480 mass spectrometer operated in Data Dependent Acquisition (DDA) mode using an EASY-Spray source.

Data Analysis

Raw data files were processed using Thermo Scientific™ Proteome Discoverer™ 2.2 software. The data were searched against the Homo sapiens (SwissProt TaxID=9606) fasta. The following settings were applied: precursor mass tolerance of 10 ppm, fragment mass tolerance of 20 mmu. Trypsin was specified as digesting enzyme and 2 missed cleavages were allowed. Cysteine carbamidomethylation was defined as fixed modifications and methionine oxidation and deamidation were variable modifications. Only high confident peptides with FDR < 1% and first ranked peptides were included in the results.

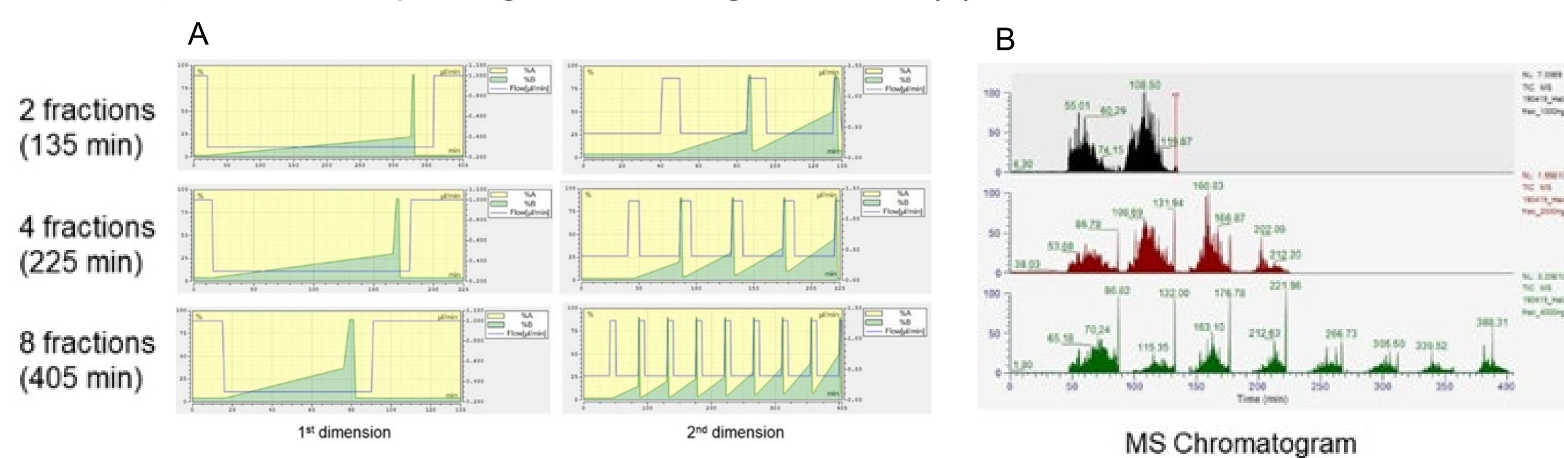
Figure 1. Modules for assembling the online 2D-nanoLC-MS platform (A) and its fluidic connection scheme (B).



RESULTS

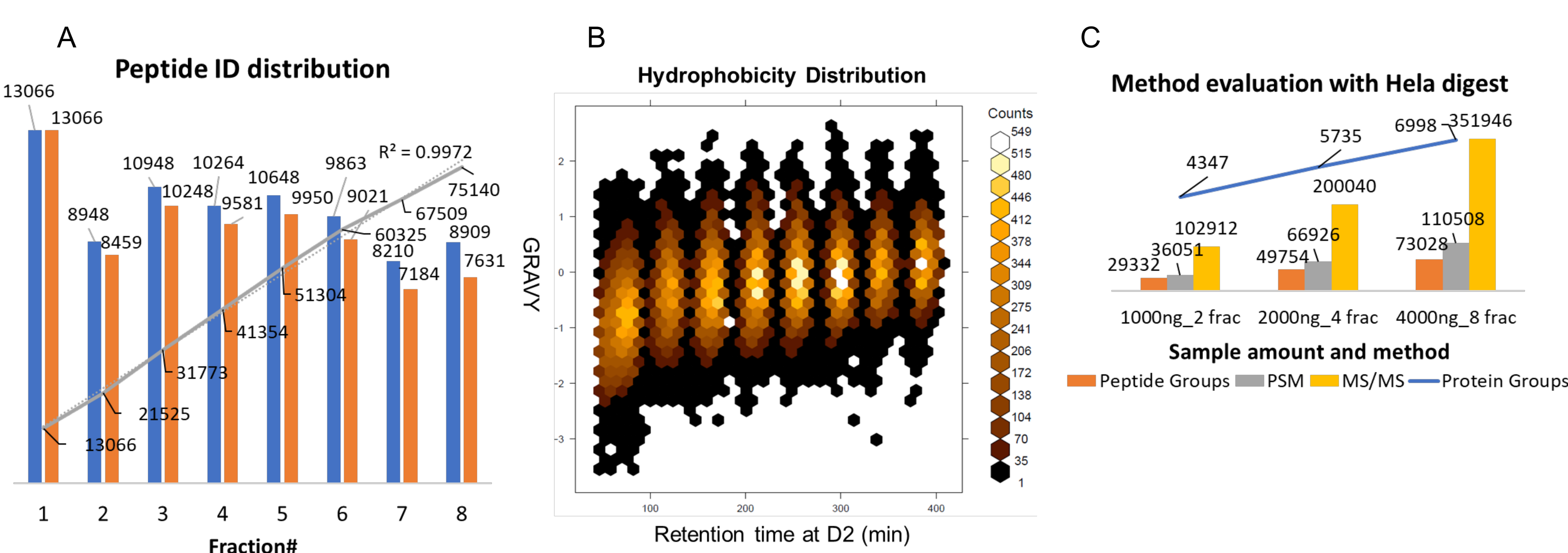
With the low-flow online high-pH RP × low-pH RP method, fractions from the first dimension are captured alternatively on one of two trapping columns and sequentially analyzed using a high-throughput low-pH RP gradient with an Orbitrap Exploris 480 mass spectrometer. As both the first- and second-dimension separations are driven by peptide hydrophobicity, it follows that the fractions collected at later time points from dimension one would also elute at higher %B values in dimension two. Therefore, to enable a better peptide distribution for each fraction and improve MS utilization, we programmed the 2nd dimension gradients with a staggered increase of mobile phase B portion at the start and end of the gradient for each fraction (Figure 2 A). The developed method was evaluated with HeLa cell digest and body fluid (crude serum prepared without depletion, reduction, and alkylation steps) samples. In figure 2 B the MS chromatogram for HeLa digest are displayed for 2-, 4-, and 8-fraction methods.

Figure 2. Gradients (A) for high-pH RP (1st dimension) and low-pH RP (2nd dimension) of 2-, 4-, 8-fraction methods, and the corresponding MS chromatogram in HeLa (B).



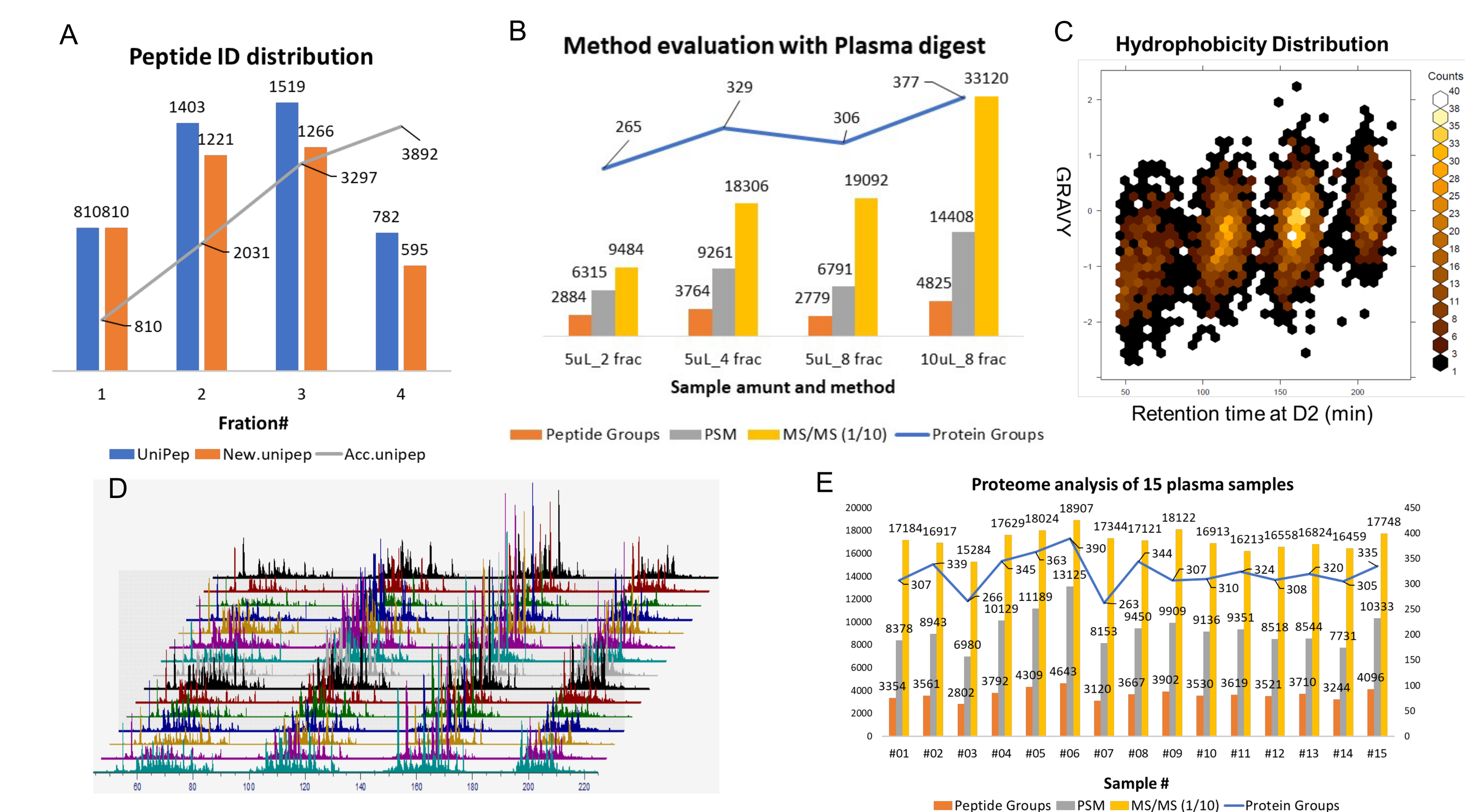
As exemplified for the 8-fraction method in figure 3A, the identified peptides are well distributed across eight fractions, with an average of 91% of unique peptides identified in each. The orthogonality plot of the 8-fraction data (based on peptide hydrophobicity calculation using the R package[2-4]) demonstrates good 1st dimension and 2nd dimension orthogonality (Figure 3b). Furthermore, the correlation (r=0.9985) in the accumulative curve (Figure 3a) reveals a linear gain in peptide identification with an increasing number of fractions. As expected, as the total number of identified peptides\proteins increases with increasing number of faction (Figure 3 C).

Figure 3. Peptide identification with the 8-fraction method shows even peptide distribution among fractions and feasibility of method extension for deeper proteome coverage; (A) orthogonality presented as peptide hydrophobicity distribution through eight fractions (B). Protein identification for 2-, 4-, 8-fractions.



The positive relationship between increased peptide and protein group identifications with an increased number of fractions was also observed for a serum sample analysis (Figure 4A). Increasing the number of fractions from two to four resulted in 329 protein group identifications, a 30% increase compared to the 2-fraction method. In a quest to dig deeper in the plasma proteome, more samples can be loaded on column as well as increase the number of fractions. This leads to an increase of 28% and 14% protein and peptide identifications, respectively(Figure 4B). Again, good orthogonality of the two dimensions is demonstrated by the broad peptide hydrophobicity distribution (Figure 4C), which displayed similar features to the HeLa digest. This platform was then employed to profile the proteome of 15 serum samples with the 4-fraction method in around 57 hours (Figure 4D). The MS1 intensity distribution in each fraction across 15 samples is highly similar, facilitating a reach to a proteome depth of 321 protein and 3658 peptide groups on average (Figure 4E).

Figure 4. Peptide identification in each fraction(A) ; Peptide and protein group numbers for 2, 4, and 8 fraction methods (equivalent to 0.25 µL crude serum and 0.5 µL serum, respectively) (MS/MS count is shown as 1/10 scale (B); Orthogonality presented as peptide hydrophobicity distribution through four fractions (C). Deep proteome profiling of 15 serum samples with the online 2D-nanoLC-MS platform, showing TIC intensity (D) and peptides, PSM, MS/MS, and protein identifications for further bioinformatic analysis (E) (MS/MS count is shown as 1/10 scale) and reproducibility.



CONCLUSIONS

We developed a novel and simple-to-use online 2D-nanoLC-MS approach for automated proteome profiling by coupling the versatile UltiMate 3000 RSLCnano system to the state-of-art Orbitrap Exploris 480 mass spectrometer. This approach yields the following attributes:

- No requirement for manual sample manipulation after enzymatic digest
- High orthogonality between 1st and 2nd dimension separations
- High MS utilization (up to 88% in the 8-fraction method)
- Compatibility with challenging sample matrices, e.g. human serum
- A powerful alternative to long one-dimensional separations in shotgun proteomics

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PO65780-EN 0422S

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