Complex Peptide Mixture Fractionation via Parallel Isoelectric Focusing for Direct LC-MS/MS Analysis

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Introduction

Charge-based separation of peptides prior to LC-MS analysis has been hampered by long run times and the presence of components such as detergents and carrier ampholytes that interfere with MS (1). Cell Biosciences has developed a workflow using the digital ProteomeChip® (dPC®), that captures peptides in acrylamide gel plugs according to charge, with run times less than one hour (2,3) (figure 1). This workflow employs buffers and conditions that have been designed to be "MS-friendly," eliminating the need for post-separation sample clean-up and dramatically reducing artifacts introduced by the separation processes. Cell Biosciences ProteomeChips are available in three pH ranges: 3.5-4.5, 4.2-6.2 and 6.0-8.0, providing an overall pH range of 3.5-8.0.

Discussion

Novel separation and pre-fractionation of samples for "bottom-up" proteomic screening is accomplished utilizing Cell Biosciences digital ProteomeChip technology. Encompassing mass spec friendly solutions, complex mixtures of peptides are separated in less than 1 hour, with LC-MS ready peptide solutions processed in less than 3 hours. These findings demonstrate the reproducibility and speed of the ProteomeChip for peptide fractionation and identification. The technology easily integrates into standard mass spec workflow and establishes a foundation for improved reliability and more rapid throughput in areas such as discovery proteomics and biomarker screening.

Conclusions

- More than 3,800 unique peptides (17,066 total) were found using three dPCs with a combined pH range of 3.5-8.0
- 1,873 unique proteins (2,025 total) identified across the entire working pH range
- 1,132 unique proteins (1,189 total) identified on at least two dPCs
- Targeted analysis results of specific peptides identified on the LTQ MS showed high reproducibility

Workflow

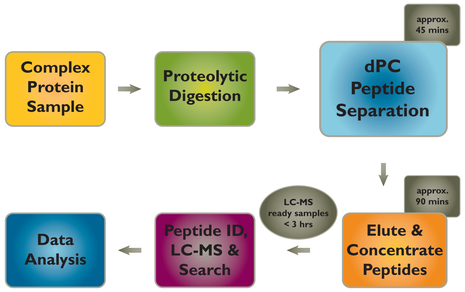


Figure 1. Overview of integrated peptide separation workflow using the dPC.

Technology

The ProteomeChip employs a parallel focusing geometry (figure 2a); 41 gel plugs, each at a specific pH are positioned between a pair of high voltage electrodes immersed in pH controlled buffers. During a run, peptides that are far from their isoelectric point (pI) become charged causing them to rapidly migrate across the ProteomeChip and into the opposing buffer chamber. Once in the opposing buffer, the peptide may reverse its charge causing it to re-enter the chip through a different plug (figure 2b). When a peptide enters a gel plug that is near its pI, the peptide loses its charge and becomes trapped. Otherwise, the process is repeated until all peptides are sorted into individual plugs according to their pI. Because all 41 plugs are presented in parallel, the entire sorting process is completed in less than an hour.

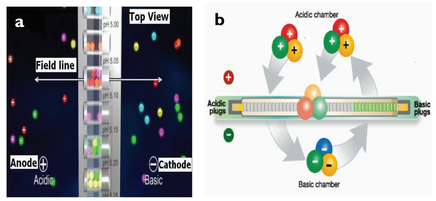


Figure 2. (a) Peptides migrate in an electrical field until they encounter a gel plug near their pI. (b) Fractionation by parallel isoelectric focusing on the dPC.

Methods

Sample Preparation and dPC Fractionation

Frozen human A431 cell lysates were reduced with tributylphosphine and alkylated with iodoacetamide. Proteins were precipitated in methanol/chloroform and dissolved in 50 mM ammonium bicarbonate. Trypsin was added and the sample was allowed to digest overnight at 37°C. Following proteolysis, 50 µg aliquots were diluted with running buffer and run on ProteomeChips in each of the three available pH ranges: 3.5-4.5, 4.2-6.2 and 6.0-8.0. ProteomeChips were run for a maximum of 45 minutes. After the run, each chip was briefly rinsed with water and gel plugs were harvested into microcentrifuge tubes.

LC-MS/MS Analysis

Gel plugs were pooled into groups of 5, corresponding to 0.25 pH units, and extracted with 200 µL of 0.2% formic acid in 50% acetonitrile at 37°C for 1 hour. Extracts were collected in fresh microcentrifuge tubes and concentrated to a volume of 50 µL via SpeedVac. Samples were then loaded onto a Thermo Scientific Micro AS, equipped with a 25 µL syringe, 2.4 µL sample needle, 200 µL buffer tubing and 10 µL sample loop. The wash solvent used was 0.1% formic acid in 30% acetonitrile. The pump was a Surveyor MS pump plus equipped with a static split at a ratio of 800:1 at 250 µL/min at 95% A (0.1% formic acid in water), 5% B (0.1% formic acid in acetonitrile) with a 75 µm x 15 cm column packed with Michrom Bioresources Magic 5 µm C18 media. A Thermo LTQ™ linear ion trap mass spectrometer was used in a "Top 8" configuration.

Data Analysis

Raw files from the LTQ mass spectrometer were collated using SEQUEST® through Thermo Scientific Bioworks 3.3.1 SR1 software package against the Refseq Human database, with static carbamidomethyl modified cysteines and differentially modified oxidized methionines. SEQUEST data was exported and analyzed further by the Cell Biosciences Mass Spectrometry Research Analysis Tool (MSRAT®).

Results

Figure 3 demonstrates dPC-based fractionation using 2 fluorescently tagged peptides mixed with A431 tryptic digested cell lysate on two pH 4.2-6.2 ProteomeChips. Studies performed using this A431 cell lysate, identified 3,876 unique (17,066 total) peptides using 3 ProteomeChips with a combined pH range of 3.5-8.0. Reproducibility was evaluated by comparing identity and position of specific peptides in multiple dPC runs (figure 4), leading to strong agreement in protein IDs between 2 ProteomeChips (figure 5). On the basis of these peptide IDs, 1,873 unique proteins (2,025 total) were identified across the entire pH range (figure 6), with 1,132 unique proteins (1,189 total) identified on at least two ProteomeChips.

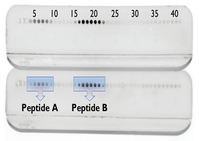


Figure 3. Focusing of two fluorescently-tagged peptides in a matrix of a tryptic digested A431 cell lysate using a pH 4.2-6.2 dPC.

Results

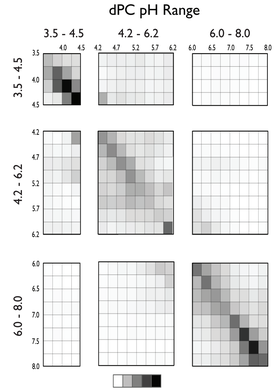


Figure 4. Reproducibility between two dPC runs across the current working range. Each ProteomeChip was loaded with a 50 µg aliquot of an A431 tryptic digest. Each box represents pools of plugs in which the same peptides were found in each of the two ProteomeChips. The number of peptides observed is represented by the degree of intensity towards black (scale shown below).

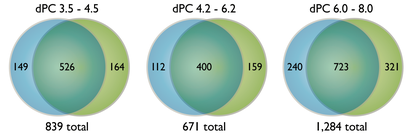
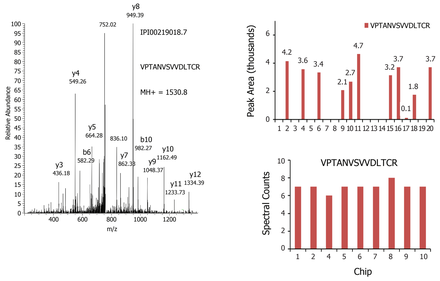


Figure 5. Number of proteins identified in each pH range and reproducibility observed between two technical replicates.

Figure 6. Reproducibility of the dPC using targeted versus non-targeted mode on an LTQ. Using non-targeted analysis, we saw this moderately abundant peptide approximately 50% of the time. Using a targeted mode where a particular precursor ion is followed, we reliably find the peptide in all chips with a comparable spectral count for each chip (rsd = 7%).



References

(1) B. Cargile et al. Immobilized pH Gradient Isoelectric Focusing as a First-Dimension Separation in Shotgun Proteomics; Journal of Biomolecular Techniques 2005,16, 181–189.
(2) G. Zilberstein et al. Parallel isoelectric focusing chip; Proteomics 2004, 4, 2533–2540.
(3) S. Buhskpan et al. Matrixes, Arrays, Systems and Methods; USPT 7,166,202, 2007.