Integration of HPLC-FTICR MS and HPLC-QIT MS² to Achieve Enhanced Proteome Characterization

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Overview

- **Objectives**

  Proteome characterization generally consists of peptide separation by high performance liquid chromatography (HPLC) and peptide identification by mass spectrometry (MS). We aim to enhance the specificity and sensitivity of peptide identification by *in silico* integrating nanoLC-FTICR-MS and nanoLC-QIT-MS/MS.

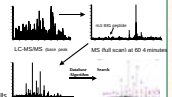
- **Methods**
  - **nanoLC-FTICR-MS:** 1D reverse phase LC coupled online to IonSpec 9.4T FTICR with MS scan
  - **nanoLC-QIT-MS/MS :** 1D reverse phase LC coupled online to LCQ with data dependent MS/MS scan
  - ***in silico* integration:** Retention time normalization; peptide correlation by retention time and mass

- **Results**
  - Development of a robust nanoLC-FTICR-MS system
  - Development of retention time normalization and peptide correlation
  - Demonstration of enhanced peptide identification from simple protein mixture digest

Introduction

- Proteomics
  - Systematic analysis of protein complement in a given cell, tissue or organism for their identity, quantity and function.
  - Major challenges: proteome coverage, low abundance proteins, post-translationally modified proteins,
  - General procedures: Gel-based or LC-based separation, MS-based characterization, informatics-based identification.

- Current approaches to proteomics
  - MudPIT technology (Ref. 1)
    1. Biphasic column separation of tryptic digested proteome, integrating a SCX resin and RP resin in one column
    2. Introduction of eluent directly to QIT through ESI interface; data-dependent MS² scan of eluting peptides
    3. Identification of peptides by SEQUEST database searching (Ref. 2)
    4. Assemble of peptide identification to protein identification by DTAselect (Ref. 3)
  - Switching LC technology (Ref. 4)
    1. Autosampler loading of tryptic digested proteome onto a trapping cartridge
    2. 1D RP-LC separation or 2D switching SCX/RP LC separation
    3. QIT data-dependent MS² scan of eluting peptides, possible multiple mass range scan
    4. SEQUEST/MASCOT database searching and protein identification



    Advantages:
    - HPLC separation of peptides are relatively unbiased.
    - MS²scans are informative of peptide sequence

    Disadvantages:
    - The score from MS² database searching algorithm is the only base for identification calls
    - The selection of cutoff is a compromise between identification specificity and sensitivity
    - Common cutoff for SEQUEST Xcorr (+1) > 1.8, (+2) > 2.5, (+3) > 3.5

  - AMT technology (Ref. 5)

    "An AMT tag is a peptide with a sufficiently distinctive mass and LC elution time to act as a biomarker for a given protein."
    1. Construction of *potential mass tag* (PMT) library
       1.1 Capillary HPLC / QIT MS² characterization of a given organism's proteome under a variety of conditions
       1.2 Establishment of PMT for a peptide using a very liberal cutoff (SEQUEST Xcorr (+1, +2, +3)= 2.0)
    2. Construction of *accurate mass and time tag* (AMT) library
       2.1 Capillary HPLC / FTICR MS characterization of those proteome samples
       2.2 Establishment of AMT for a peptide matched with its PMT's retention time and calculated mass
    3. Proteome examination by capillary HPLC / FTICR MS
       3.1 Capillary HPLC / FTICR MS characterization of the proteome samples of interest
       3.2 Identification of protein by searching AMT library with measured mass and retention time

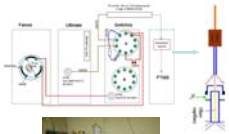
    Advantages:
    - High throughput proteome examination once AMT library is constructed
    - High proteome coverage given a confident AMT library

    Disadvantages:
    - Large initial effort investment in constructing AMT library
    - Multiple dimensional separation not straight forward
    - False positive protein identification due to insufficiently distinctive mass and LC elution time of an AMT tag

NanoLC-FTICR MS Methods and Results

Methods:

- 1D-RPLC
  - Autosampler injection, LC packings FAMOS 50 ul
  - Preconcentration: 300 um x 5 mm C18 PepMap
  - RP-LC: Vydac 75 um id x 25 cm C18 nanocolumn

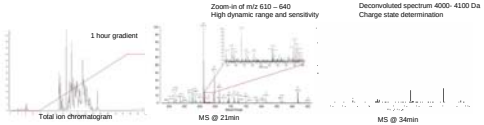


- Nanospray
  - Tip: 10 um ID NewObjective Picotip
  - $V_{nanospray}$: -1800V V_{lig}: 0V $V_{entrance}$: -1800V
  - Distance: 3mm

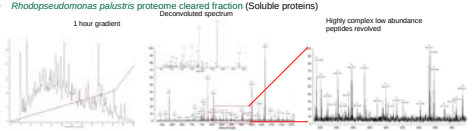
- FT ICR MS
  - 9.4 Tesla IonSpec
  - 2 sec. hexapole ion accumulation
  - 256 data point @ 1M ADC
  - 2-scan accumulation, ~ 9 sec. per spectrum

Results

- Standard protein mixture digest
  - Constituents: ALCOHOL DEHYDROGENASE, CARBONIC ANHYDRASE, HEMOGLOBIN, MYOGLOBIN, LYSOZYME, SERUM ALBUMIN



- *Rhodopseudomonas palustris* proteome cleared fraction (Soluble proteins)



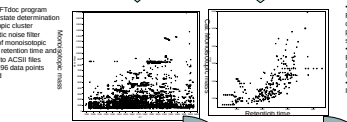
- 3 consecutive nanoLC- FTICR MS runs with proteome samples

Automatic 3 repetitive sample injections
Highly reproducible results (mass spectra)
A robust LC/MS system

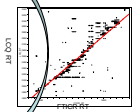
Integration Methods and Results

1. **Parallel LC/MS experiments**

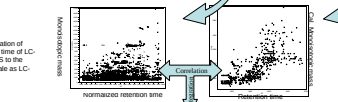
2. **Data process and extraction**



3. **Normalization of retention time**
   1. Selection of high confident peptide hits from LCQ data Xcorr (+1) > 2.3; (+2) > 3.0; (+4) > 3.5 Total 206 I.D.
   2. Search against FT ICR data with calculated monoisotopic masses Mass error < 0.05 Da
   3. Data representation and visual inpection to remove spurious hits Deletion of the obvious outliers
   4. Linear regression: $RT_{LCQ} = 0.9669*RT_{FT} - 11.5872$ correlation coefficient = 0.94

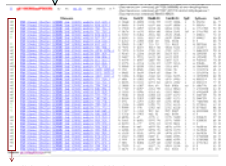


4. **Correlation and Integration**



Mass tolerance < 0.05 Da
RT tolerance < 3 min
Xcorr (+1) > 1.3, (+2) > 2.0, (+3) > 3.0)

Export of results to DTASelect readable format. Use of manual validation flag to flag the presence of FTICR data hits



"V", if there is an FTICR hit within the mass and RT tolerance

LC QIT MS² Methods

- Sample preparation and protein digestion
  1. The protein sample is denatured with 6-8 M Guanidine or Urea, and reduced with DTT or some other reducing agent at 60oC for 10-60 minutes.
  2. The denaturant concentration is lowered by dilution with Tris; sequencing grade trypsin is added to the sample and incubated overnight
  3. The sample is desalted by solid phase extraction and organic solvent is removed by SpeedVac
- 1D LC / QIT MS2
  - One-dimensional LC-MS/MS experiments were performed with an Ultimate HPLC (LC Packings, a division of Dionex, San Francisco, CA) coupled to an LCQ-DECA ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with an electrospray source. Injections were made with a Famos (LC Packings) autosampler onto a 50ul loop. Flow rate was ~4ul/min with a 240min gradient for each run.
  - A VYDAC 218MS5.325 (Grace-Vydac, Hesperia, CA) C18 column (300µm id x 15cm, 300Å with 5µm particles) or a VYDAC 238EV5.325 monomeric C18 (300µm id x 15cm, 300Å with 5µm particles) was directly connected to the Finnigan electrospray source with 100µm id fused silica.
  - For all 1D LC/MS/MS data acquisition, the LCQ was operated in the data dependent mode with dynamic exclusion enabled, where the top four peaks in every full MS scan were subjected to MS/MS analysis..

- SEQUEST and DTASelect
  - The resultant MS/MS spectra from the sample were searched with SEQUEST against the six constituent protein sequence and all predicted ORFs from R. palustris. The ORFs from R. palustris serves as indication of false position rate.
  - The raw output files were filtered and sorted with DTASelect. The filter criteria include the minimal Xcorr, the validation flag for FT ICR data hits and the FTICR mass measurment error

Conclusion

- Development of a robust nanoLC-FTICR-MS system
  - Stable electrospray and good sensitivity in highly aqueous solution
  - Low sample consumption
  - Good mass accuracy and mass resolution
- Development of retention time normalization and peptide correlation
  - Use of linear regression to offset gradient start time and adjust gradient slope
  - High correlation coefficient in retention time correlation
  - Demonstration of comparability of FTICR data and QIT data
- Demonstration of enhanced peptide identification from simple protein mixture digest
  - FTICR data as a validation method for SEQUEST identification
  - Improved specificity and sensitivity in peptide identification
- Future directions
  - Improve the mass measurement accuracy of FTICR and retention time reproducibility of LC system
  - Devise a rigorous method to integrate FTICR accurate mass measurement and QIT MS/MS measurement

Reference

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