Application of FoxWare® Software for Resolving Isomeric Heterogeneity and Retention Time Drift in Oxidized Peptides from Hydroxyl Radical Protein Footprinting

Jiana Duan[1], Robert Egan[1], Calyx Liu[1], Tyler Fletcher[1], Emily E. Chea[1], Sandeep K. Misra[2], Joshua S. Sharp[1,2], Scot R. Weinberger[1]

1. *GenNext Technologies, Inc., Half Moon Bay, CA, United States*
2. *University of Mississippi, Oxford, MS, United States*

HRPF Introduction

The higher order structure (HOS) of a protein plays a critical role in a drug's stability, safety and biological function. Incorrect HOS or protein interactions are linked to adverse drug reactions which can result in further sickness or death. Advance techniques are required to robustly address the HOS of proteins. One such method is Hydroxyl Radical Protein Footprinting (HRPF). The Fox® Protein Footprinting System is a novel HRPF method that uses a proprietary flash oxidation system to generate hydroxyl radicals (•OH) that irreversibly modify solvent exposed amino acid side chains. As solvent accessibility changes, the •OH modification concordantly changes.

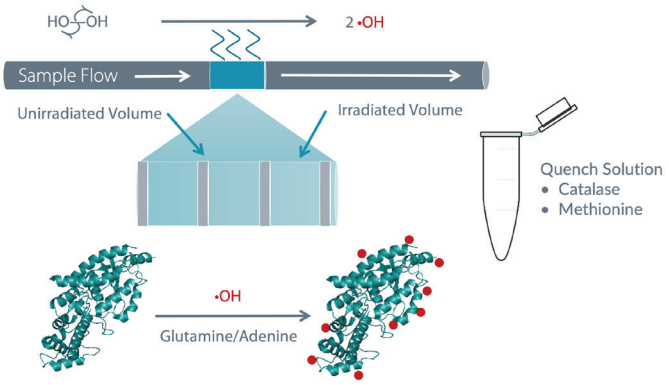
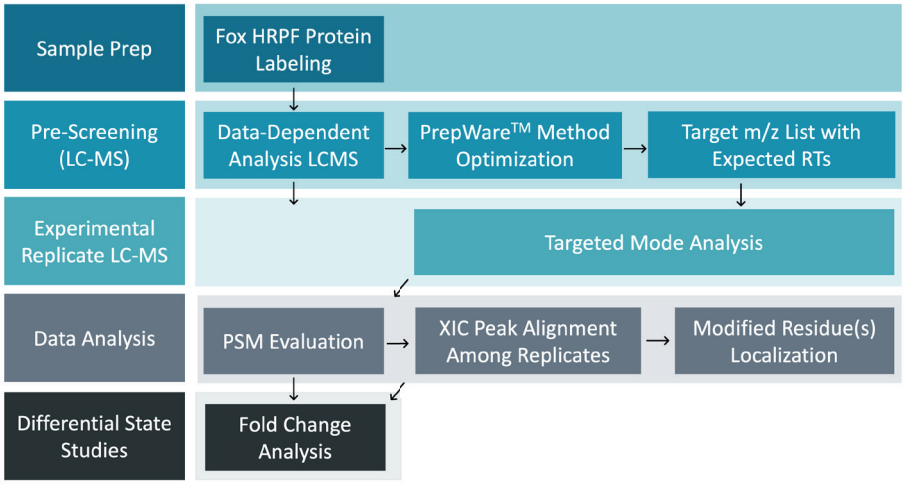


Figure 1: Schematic of an HRPF method, fast Photochemical Oxidation of Proteins (FPOP).

With FPOP, protein is mixed with hydrogen peroxide and flowed passed a pulsing light source which photolyzes the hydrogen peroxide into two •OH and modifies solvent exposed amino acids. Following labeling, the sample is deposited into a quench solution of catalase and methionine.

FoxWare Data Analysis Software Workflow

FoxWare Data Analysis Software is used to expedite and automate the analysis of native peptides and modified peptides of HRPF experiments. Software is data format agnostic (.raw, .d, .wiff, etc.) and operates under the following workflow.



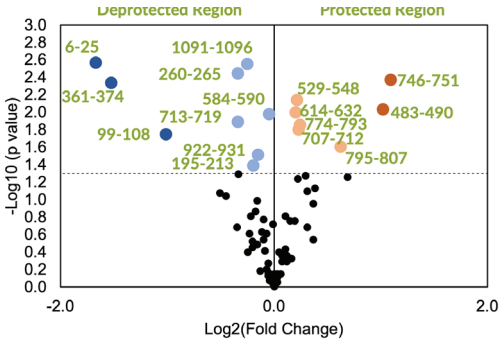


Figure 2: (Left) Workflow for automated HRPF analysis using FoxWare Data Analysis software.

A modified-peptide inclusion list and optimal retention time ranges are created based on peptide-spectrum match quality and native peptide retention times in PrepWare Software. Then targeted-mode experiments are performed in replicate. Data analysis at the residue level is performed prior to identifying statistically significant fold change (Top).

Laser-Free Flash Oxidation (Fox®) HRPF System

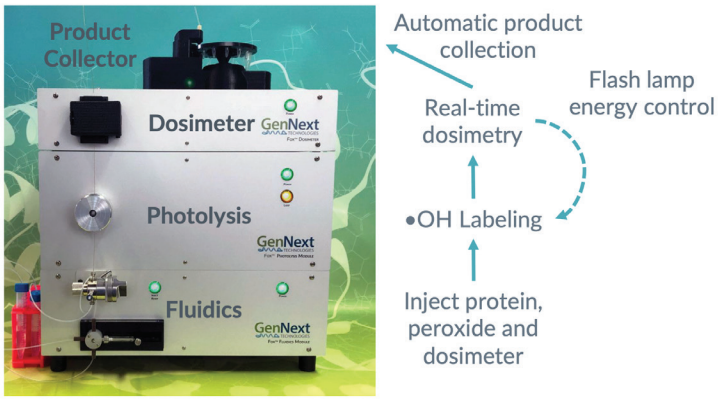
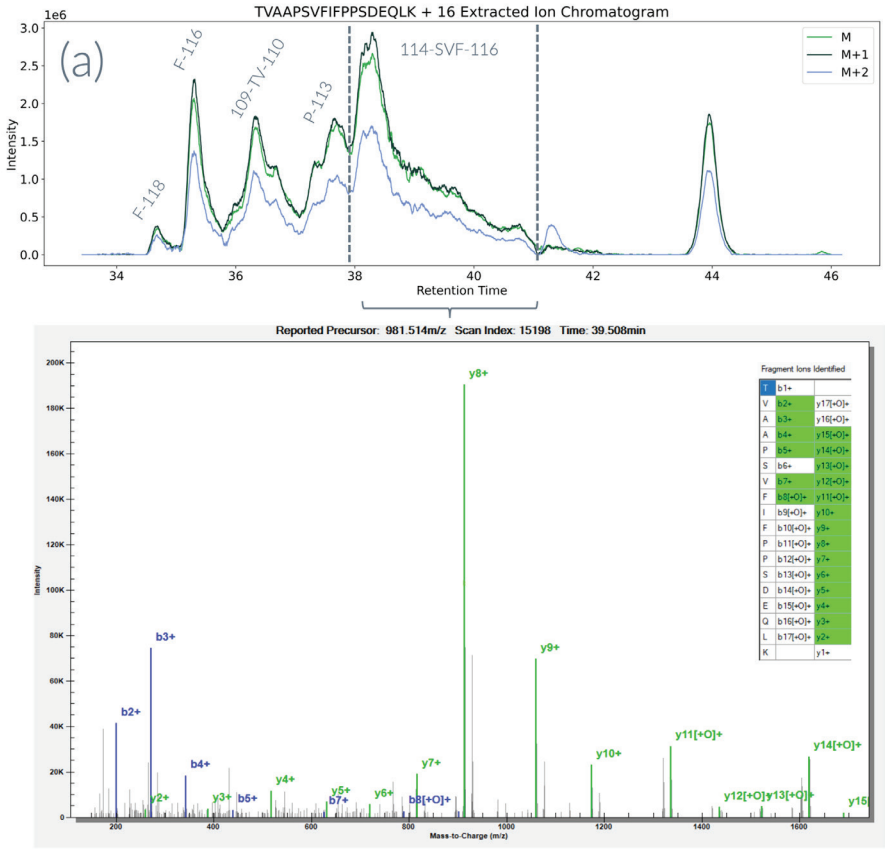


Figure 3: Schematic of Fox system with the four modules (fluidics, photolysis, dosimeter, and product collector).

Challenges in Automated HRPF Analysis

Oxidized peptides in HRPF can often elute in LC with incomplete separation between different modified residues (isomers), resulting in inaccurate quantitation. Nuances in tandem MS fragmentation and defining a modified "peak" in the XIC in current commercial proteomics software is often overly optimistic about localizing the modified residues. Alignment of peaks across replicates is also challenging with conventional approaches, resulting in poor statistical robustness when looking at solvent accessibility. FoxWare Software's algorithm is designed to focus on key challenges in HPRF, with a novel approach to managing oxidation ambiguity and mitigate over-interpretation of LC-MS results.

Chimeric XIC Peaks of Modified Peptides



Identifying Ideal Oxidized Peptide Retention Times

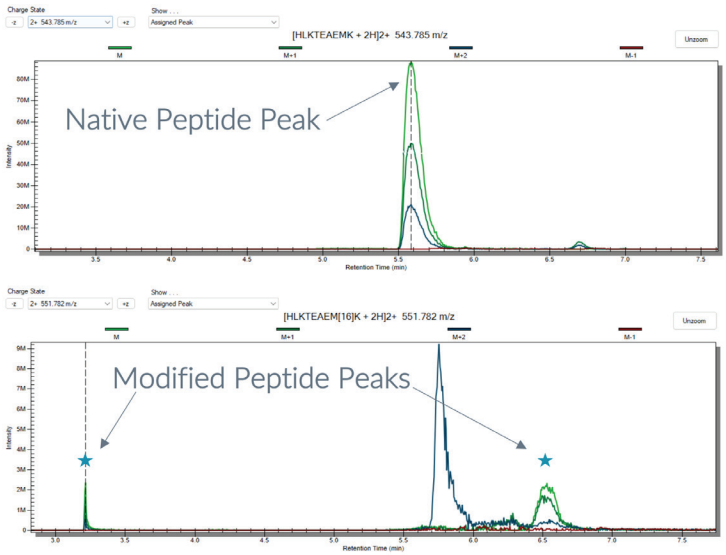


Figure 4: Optimized inclusion-list generation from cursory data-dependent analysis.

(Left) Identification of a native tryptic peptide with valid PSMs provide an approximate viable retention time range for putative OH modified forms of the same peptide. PSM evaluation of DDA data of the modified peptide are used to solidify boundaries when generating a target-mass list (below). Identification of a unique chromatographic elution (i.e. XIC peak) for modified species is automated based on cursory evaluation of DDA MS/MS quality. Multiple modified peaks of interest are detected using a combination of PSMs and isotopic validation.

Sequence	Rank	Start Position	Stop Position	z	Selected Charge State	Main Peak Intensities	Average Intensity	Intensity RSD	Main Peak RTs (min)	Average Main Peak RT (min)	Main Peak RT RSD	Present in ___ Files	Include?
R.TPSDKPVAHVVANPQAEGQLQWLNR.R	1	7	31	2		1.40E+006, 2.04E+006, 1.19E+006	1.54E+006	23.34%	24.54, 24.56, 24.50	24.53	0.10%	3	☐
R.TPSDKPVAHVVANPQAEGQLQWLNR.R	1	7	31	3	*	6.45E+007, 6.49E+007, 5.44E+007	6.13E+007	7.91%	24.53, 24.57, 24.48	24.52	0.14%	3	☑
R.RANALLANGVELR.D	1	32	44	2		6.06E+006, 5.47E+006, 2.40E+006	4.64E+006	34.54%	22.42, 22.43, 22.42	22.42	0.03%	3	☐
R.RANALLANGVELR.D	1	32	44	3	*	8.48E+006, 1.37E+007, 5.89E+006	9.37E+006	34.91%	22.42, 22.43, 22.39	22.41	0.08%	3	☑
R.ANALLANGVELR.D	1	33	44	2	*	3.09E+008, 3.46E+008, 3.11E+008	3.22E+008	5.34%	24.90, 24.95, 24.90	24.92	0.09%	3	☑
R.DNQLVVPSEGLYLIYSQVLFK.G	1	45	65	2		6.52E+006, 9.09E+006, 1.29E+007	9.52E+006	27.77%	38.36, 38.38, 38.30	38.34	0.09%	3	☐
R.DNQLVVPSEGLYLIYSQVLFK.G	1	45	65	3	*	2.43E+007, 2.89E+007, 6.59E+007	3.97E+007	46.89%	38.33, 38.36, 38.30	38.33	0.07%	3	☑
K.GQGCPSTHVLLTHTISR.I	1	66	82	3	*	5.41E+006, 4.35E+006, 5.52E+006	5.09E+006	10.31%	20.89, 20.89, 20.86	20.88	0.08%	3	☑
R.IAVSYQTK.V	1	83	90	2	*	3.04E+007, 2.15E+007, 2.02E+007	2.40E+007	18.81%	19.16, 19.21, 19.17	19.18	0.13%	3	☑
K.VNLLSAIK.S	1	91	98	2	*	1.87E+008, 1.77E+008, 1.54E+008	1.73E+008	8.12%	25.48, 25.52, 25.46	25.48	0.11%	3	☑
R.ETPEGAEAKPWYEPIYLGGVFQLEK.G	1	104	128	2		9.25E+005, 9.45E+005, 1.22E+006	1.03E+006	12.96%	32.71, 32.73, 32.57	32.67	0.21%	3	☐
R.ETPEGAEAKPWYEPIYLGGVFQLEK.G	1	104	128	3	*	9.90E+006, 2.32E+007, 2.13E+007	1.81E+007	32.37%	32.70, 32.77, 32.60	32.69	0.22%	3	☑
R.ETPEGAEAKPWYEPIYLGGVFQLEKGDR.L	1	104	131	3	*	No PKS, No PSMs, No PKS	N/A	N/A	No PKS, No PSMs, No PKS	N/A	N/A	0	☐
R.LSAEINRPDYLDFAESGQVYFGIIAL.-	1	132	157	2		1.41E+006, 2.17E+006, 3.18E+006	2.26E+006	32.17%	37.62, 37.61, 37.53	37.59	0.11%	3	☐
R.LSAEINRPDYLDFAESGQVYFGIIAL.-	1	132	157	3	*	4.86E+006, 6.57E+006, 1.35E+007	8.31E+006	45.04%	37.60, 37.61, 37.56	37.59	0.06%	3	☑

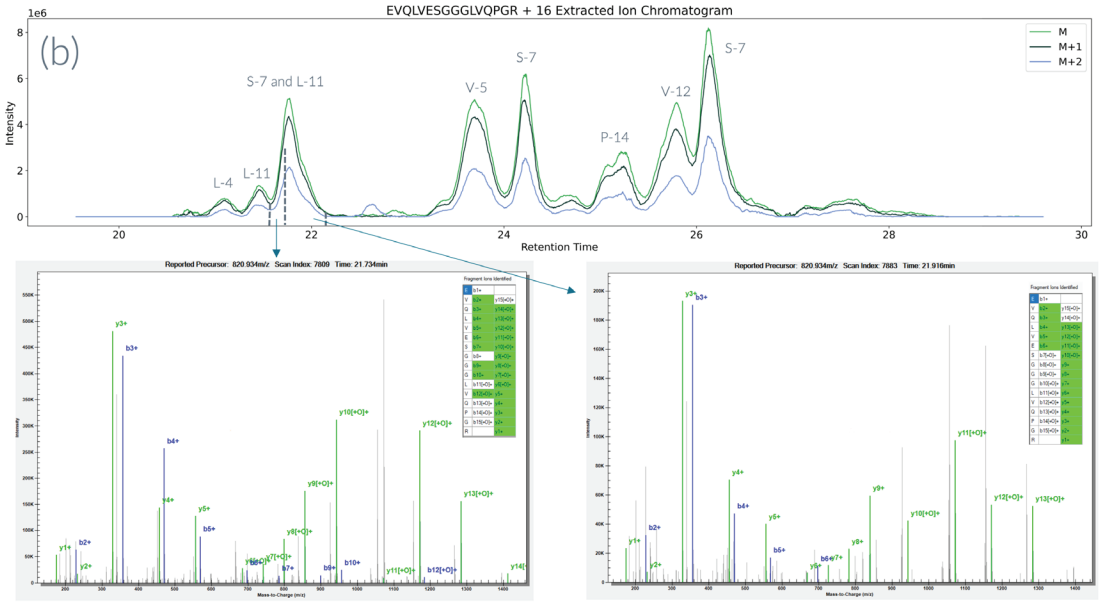


Figure 5: XIC peaks.

XIC peaks have the potential to: (a - Left) be chimeric at the tandem MS level where more than 1 residue could potentially contain a modified residue. Low-confidence fragmentation suggests a range of residues that can be oxidized. (b - Above) Have multiple definitive positions that can be localized based on fragmentation patterns, in which case more than 1 site must be localized. Both can be recognized and distinguished in FoxWare Software.

Alignment of XIC Peaks and Automated PSM Crossover

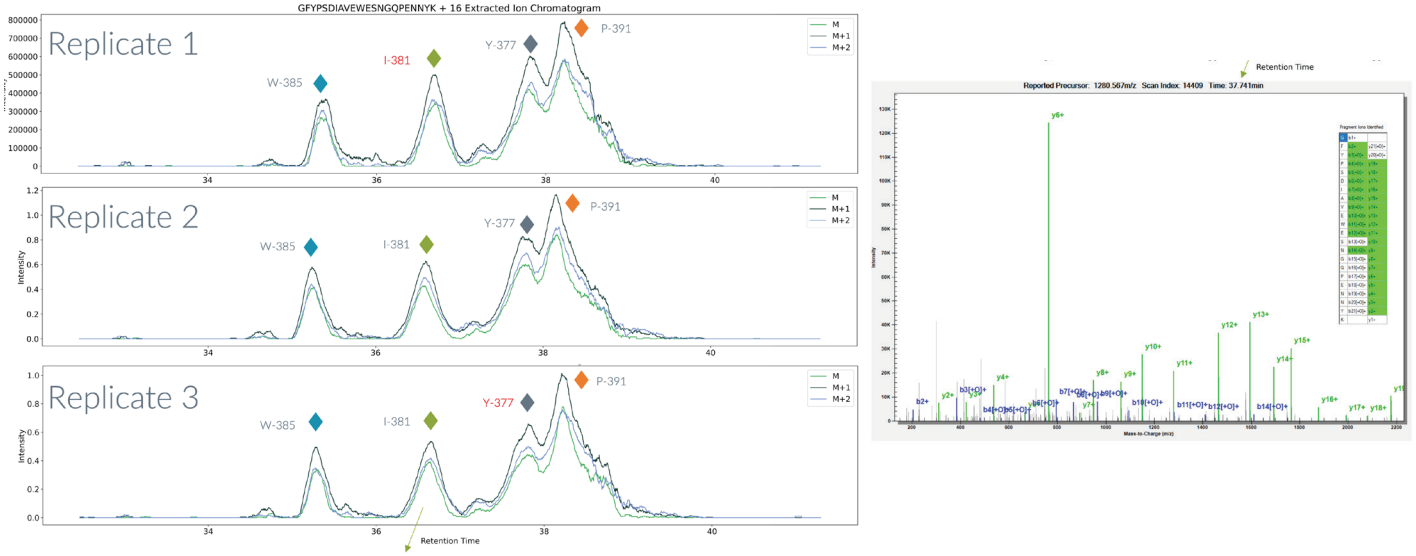


Figure 6: XIC Peaks.

(Left) Modified peptide XIC peaks are aligned across replicates based on retention time (relative to the native peak) and peak widths. Colored diamonds indicate aligned peaks of each XIC across a triplicate run. Residues in red were assigned using borrowed PSMs from other replicates.

(Right) Residue-level PSM analysis can further assist in maintaining consistency. Deleterious effects of tandem MS ambiguity (e.g. poor fragmentation or uncertain modification site) can be mitigating by borrowing higher-quality PSM(s) from other replicates.

Conclusions

- FoxWare® Software is uniquely positioned to handle key issues with HRPF analysis while not over-interpreting results, increasing statistical robustness of quantitation.
- FoxWare Software's PrepWare module is a unique pre-screening tool to analyze DDA data and provide users a valuable inclusion-list dataset for optimized targeted-mode analysis.
- Complicated elution profiles common to HRPF-LC-MS can be readily and easily managed within FoxWare Software without the need for user-intervention.

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