FoxWare™ Protein Footprinting Software—

An Integrated Package for Hydroxy Radical Protein Footprinting Featuring Radical Dosimetry, Data Processing, and Data Analysis

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Introduction

The Higher Order Structure (HOS) of a protein plays a critical role in drug 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). HRPF relies upon irreversible labeling solvent exposed amino acid side chains with hydroxyl radicals (•OH). As a protein changes structure or interactions, the surface exposed amino acids can change in solvent accessibility resulting in a change in •OH modification.

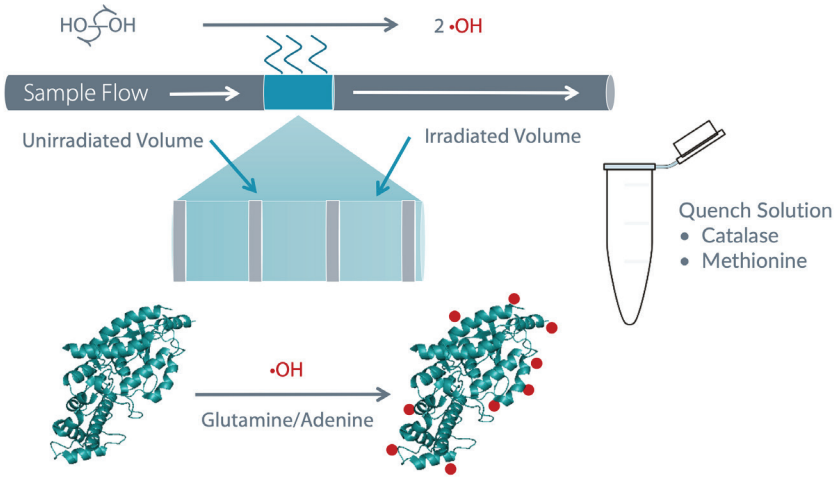


Figure 1: Schematic of an HRPF method, fast photochemical oxidation of proteins (FPOP).

With FPOP, protein is mixed with hydrogen peroxide and flown passed a pulsing UV light source which photolyzes the hydrogen peroxide into two •OH, oxidizing solvent exposed amino acids. Following labeling, the sample is deposited into a quench solution of catalase and methionine, digested, and analyzed using high resolution LC-MS/MS.

FoxWare Protein Footprinting Software

Data processing for HRPF historically required several programs to analyze low-level MSn signals from multiple oxidative products for each peptide and to process and collate the large volume of replicate data. FoxWare Protein Footprinting Software addresses these problems with •OH dosimetry calculations for optimal experimental conditions, complete and automated HRPF data processing of average peptide oxidation (APO) (Equation 1), and integrated data analysis software.

Equation 1: $$APO = \frac{\sum_{n=1}^{MaxOxLevel}(\text{XIC peak area for OxLevel}_n)(n)}{(\text{XIC peak area unmod peptide})+(\sum_{n=1}^{Max\ OxLevel}(\text{XIC peak area for OxLevel}_n)}$$

FoxWare Radical Dosimetry Module

The FoxWare Radical Dosimetry Module processes replicate LC/MS data from a dosimetry calibration experiment (Δ mAU vs APO). Individual peptide dosimetry response curves for all peptides are generated, allowing for selection of optimal experimental •OH concentration, ensuring adequate oxidation of all peptides while avoiding artifactual conformational changes.

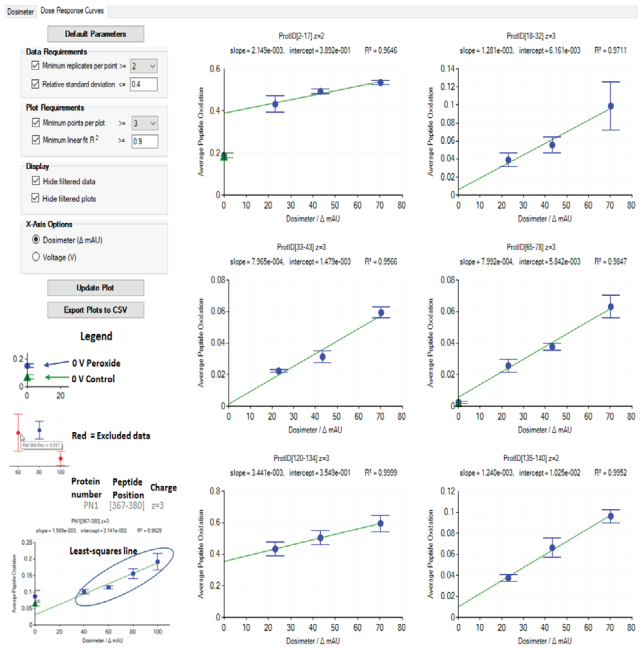


Figure 2: FoxWare Radical Dosimetry Software with apomyoglobin dose response data.

Error bars represent one standard deviation from a triplicate measurement. Apomyoglobin was labeled at 5 µM with 1 mM adenine (the dosimeter) and 50 mM H_2O_2 using three increasing Fox Flash Lamp voltages. As more •OH were generated, the change in absorbance and APO linearly increased.

FoxWare Data Processing Module

The Data Processing module processes replicate data from multiple experimental states and automatically calculates the average peptide oxidation (APO) for all detected peptides. Differential chromatographic retention time limits from the unmodified peptide are utilized in the analysis of modified peptides. A highly interactive data browser allows the user to easily display low-level supporting data to investigate and adjust APO as required.

Decrease Processing Time

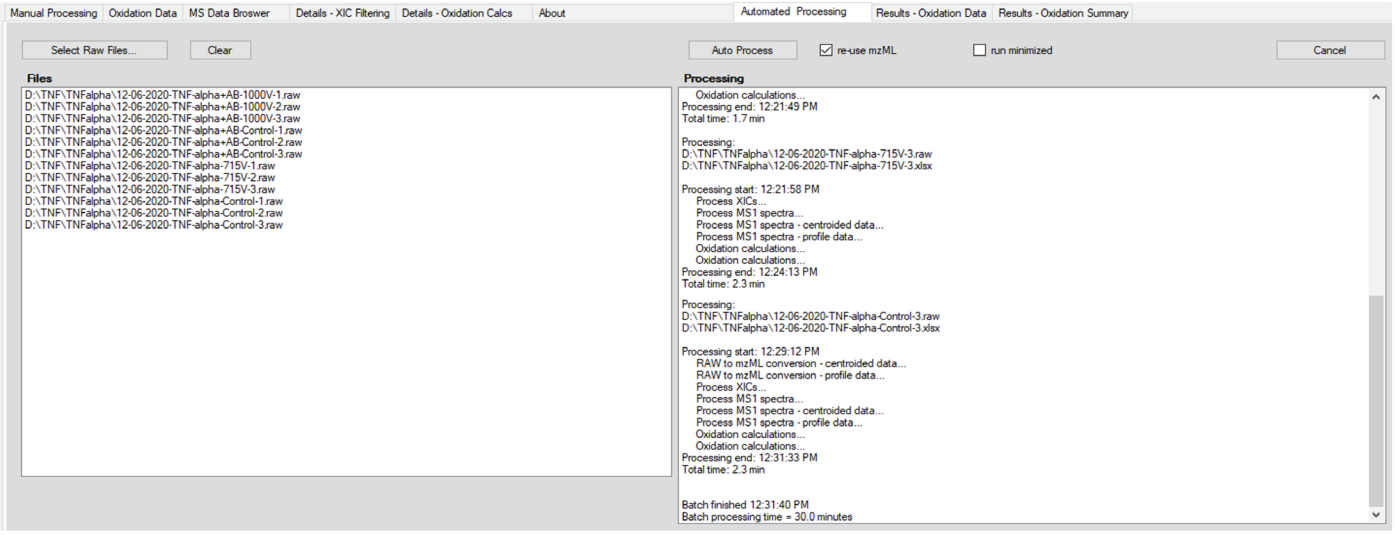


Figure 3: FoxWare Software automated data processing.

Manual analysis for 12 samples would typically take ~30 hours for an FPOP expert. With FoxWare Software, a full epitope experiment's data processing can take as little at 30 minutes.

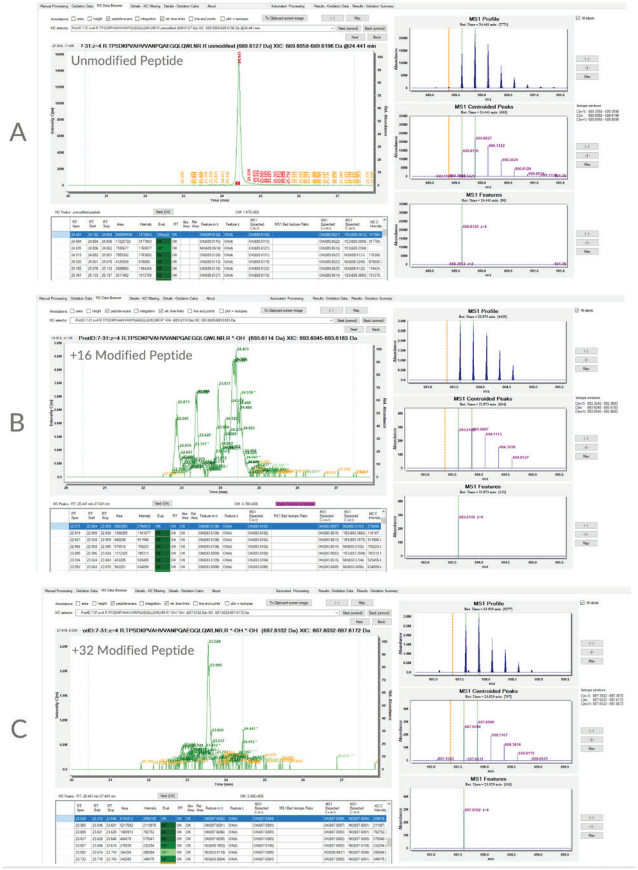
XIC Peak Evaluation

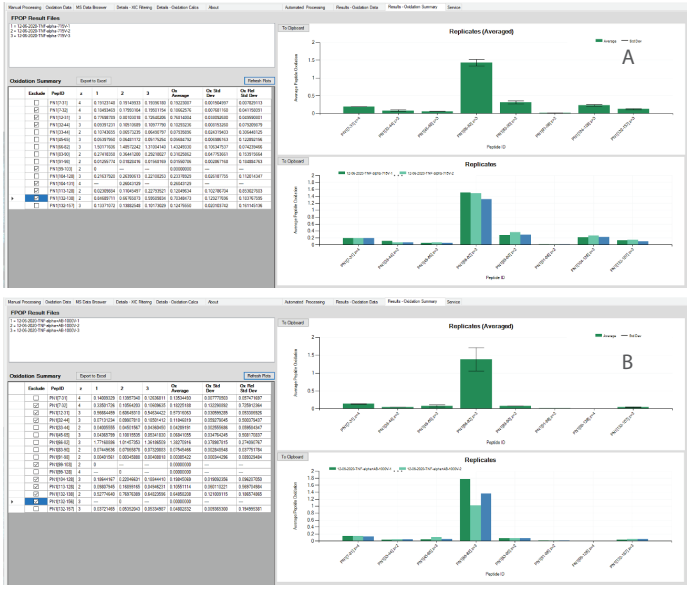
Figure 4: FoxWare XIC Data Browser feature.

FoxWare Data Processing Software uses the monoisotopic mass of an unmodified peptide and calculates the monoisotopic masses for modified peptides and generates XICs for all masses within a given mass error.

A) Unmodified peptide 7-31 in TNFa. The primary unmodified peak is chosen using an algorithm that searches for the most intense peak with the highest number of MS2 peptide identifications. To compensate for intermittent MS2 identifications, adjacent XIC peaks are evaluated and selected based on their MS1 spectra. Red annotation above the peak indicates the peaks selected for the APO calculation.

B) Modified peptide 7-31 with one oxidation event (+16 Da) and C) two oxidation events (+32 Da). When using reverse phase chromatography, the addition of oxygen(s) will shift the retention time of the peptide. Typically, this causes the peptide to elute immediately prior to the corresponding unmodified peptide. Due to this phenomenon, modified peptides are searched within a retention time window of four minutes prior and three minutes after the primary unmodified peak. To confirm the XIC peaks are from the modified peptide, each peak is evaluated using the MS1 spectra data at the XIC peak apex. Green annotation above the peak indicates the peaks selected for the APO calculation.





Averaged Peptide Oxidation Across Replicate Samples

Figure 5: APO results for triplicate FPOP labeled samples.

FoxWare calculates the APO average and standard deviation for all peptides across the selected replicates. A) Histograms generated for replicate and average APO for TNFa alone or B) for TNFa + adalimumab.

FoxWare Data Analysis Module

The FoxWare Data Analysis Module allows comparison of results among multiple experimental states with optional control subtraction. Results are displayed in data tables, histograms, volcano plots, and 3-D interactive models.

Data Table for an Epitope Experiment

Samples & Controls | Average Peptide Oxidation | Summed Peak Areas

QC

Toggle

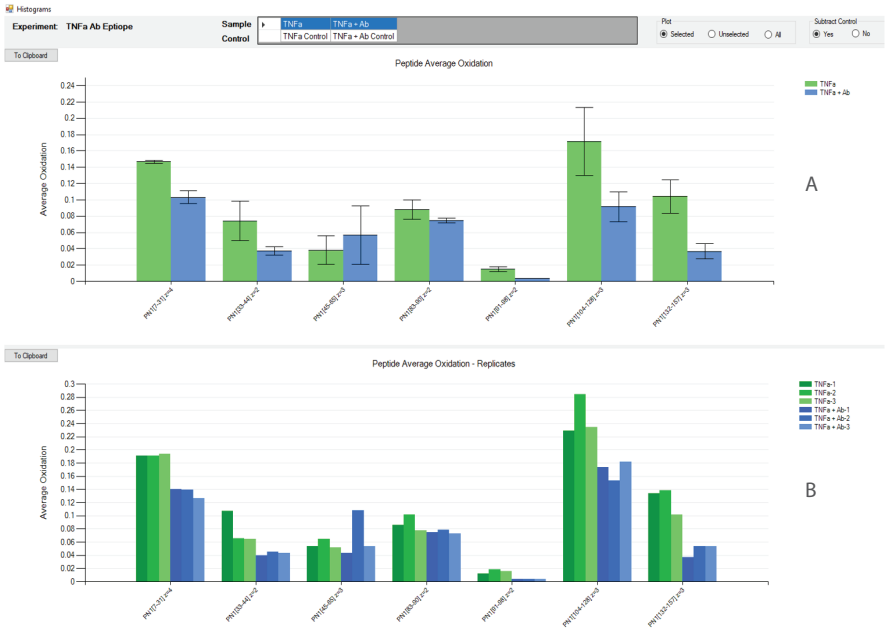
TNFa Ab Eptiope

	☑	☑
Sample	TNFa	TNFa + Ab
Control	TNFa Control	TNFa + Ab Control

Select	Sample	Peptide	z	S1	S2	S3	Sample APO	Sample Std Dev	Sample Rel Std Dev	Control	C1	C2	C3	Control APO	Control Std Dev	Control Rel Std Dev	FPOP APO	FPOP Std Dev	FPOP Rel Std Dev
☑	TNFa	PN1[7-31]	4	0.191	0.191	0.194	0.192	0.0015	0.0078	TNFa Control	0.047	0.045	0.044	0.045	0.0012	0.0271	0.147	0.0019	0.0132
☑	TNFa + Ab	PN1[7-31]	4	0.140	0.140	0.126	0.135	0.0078	0.0575	TNFa + Ab Control	0.033	0.031	0.031	0.032	0.0012	0.0370	0.103	0.0079	0.0762
☐	TNFa	PN1[7-32]	3	---	---	---	---	---	---	TNFa Control	---	---	---	---	---	---	---	---	---
☐	TNFa + Ab	PN1[7-32]	3	---	---	---	---	---	---	TNFa + Ab Control	---	---	0.000	0.000	---	---	---	---	---
☐	TNFa	PN1[7-32]	4	0.185	0.180	0.195	0.187	0.0077	0.0412	TNFa Control	0.024	0.122	0.094	0.080	0.0506	0.6307	0.106	0.0511	0.4805
☐	TNFa + Ab	PN1[7-32]	4	0.335	0.106	0.106	0.182	0.1323	0.7259	TNFa + Ab Control	0.066	0.084	---	0.075	0.0129	0.1729	0.108	0.1329	1.2362
☐	TNFa	PN1[12-31]	3	0.777	0.801	0.726	0.768	0.0381	0.0496	TNFa Control	---	---	---	---	---	---	---	---	---
☐	TNFa + Ab	PN1[12-31]	3	0.567	0.606	0.546	0.573	0.0306	0.0534	TNFa + Ab Control	---	---	---	---	---	---	---	---	---
☐	TNFa	PN1[32-44]	3	0.094	0.105	0.110	0.103	0.0082	0.0792	TNFa Control	0.038	0.029	0.062	0.043	0.0170	0.3980	0.060	0.0188	0.3127
☐	TNFa + Ab	PN1[32-44]	3	0.071	0.099	0.185	0.118	0.0593	0.5004	TNFa + Ab Control	0.091	0.025	0.035	0.050	0.0353	0.7023	0.068	0.0690	1.0108
☑	TNFa	PN1[33-44]	2	0.107	0.066	0.065	0.079	0.0243	0.3064	TNFa Control	0.007	0.004	0.004	0.005	0.0014	0.2606	0.074	0.0244	0.3284
☑	TNFa + Ab	PN1[33-44]	2	0.040	0.045	0.044	0.043	0.0026	0.0596	TNFa + Ab Control	0.011	0.003	0.003	0.005	0.0045	0.8369	0.038	0.0052	0.1377
☑	TNFa	PN1[45-65]	3	0.054	0.065	0.052	0.057	0.0070	0.1229	TNFa Control	0.009	0.037	0.009	0.018	0.0161	0.8707	0.038	0.0175	0.4567
☑	TNFa + Ab	PN1[45-65]	3	0.044	0.108	0.053	0.068	0.0348	0.5082	TNFa + Ab Control	0.007	0.021	0.006	0.011	0.0078	0.6832	0.057	0.0356	0.6260
☑	TNFa	PN1[66-82]	3	1.502	1.486	1.310	1.432	0.1063	0.0742	TNFa Control	0.294	0.249	0.405	0.316	0.0801	0.2536	1.117	0.1332	0.1193
☑	TNFa + Ab	PN1[66-82]	3	1.772	1.015	1.362	1.383	0.3790	0.2741	TNFa + Ab Control	0.251	0.111	0.216	0.193	0.0727	0.3772	1.190	0.3859	0.3243
☑	TNFa	PN1[83-90]	2	0.086	0.101	0.077	0.088	0.0122	0.1382	TNFa Control	0.000	0.000	0.000	0.000	0.0001	0.3810	0.088	0.0122	0.1386
☑	TNFa + Ab	PN1[83-90]	2	0.074	0.079	0.073	0.075	0.0028	0.0378	TNFa + Ab Control	0.000	0.000	0.002	0.001	0.0008	0.9361	0.075	0.0029	0.0395
☑	TNFa	PN1[91-98]	2	0.013	0.018	0.016	0.016	0.0029	0.1849	TNFa Control	0.000	0.001	0.000	0.000	0.0006	1.7321	0.015	0.0029	0.1923

Figure 6: Table view of TNFa epitope experiment.

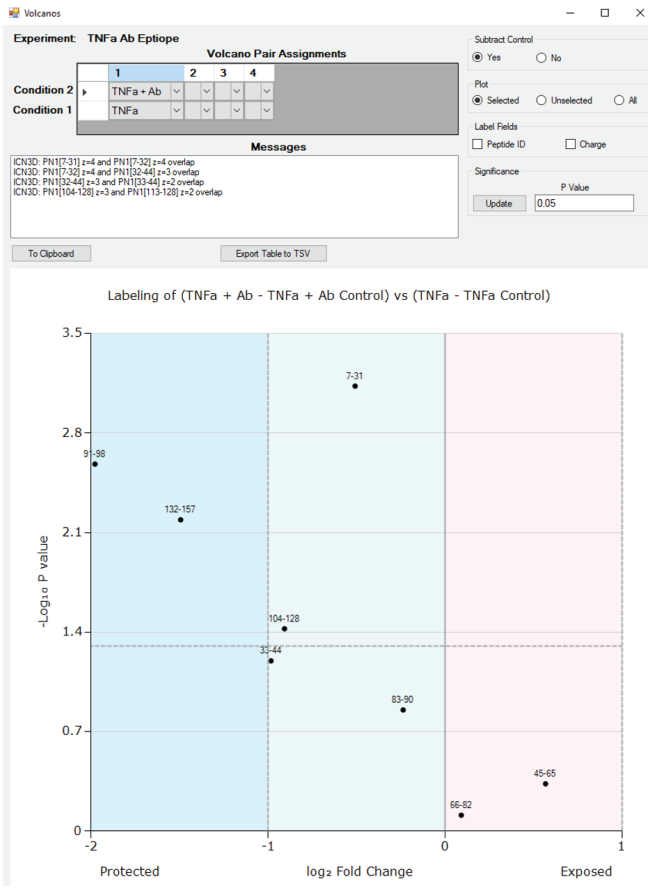
The replicate and averaged APO is displayed for all selected conditions. The control APO is subtracted from the Sample APO to calculate the FPOP APO. This removes endogenous oxidation, H_2O_2 induced oxidation, and ESI induced oxidation.



Histograms for an Epitope Experiment

Figure 7: Histograms of the A) averaged APO or B) replicate APO across multiple conditions can be plotted together.

The Sample APO or FPOP APO can be plotted. Histograms provide both absolute magnitude APO and relative magnitude of change between conditions, but it is poor at showing statistical confidence.



Volcano Plot for an Epitope Experiment

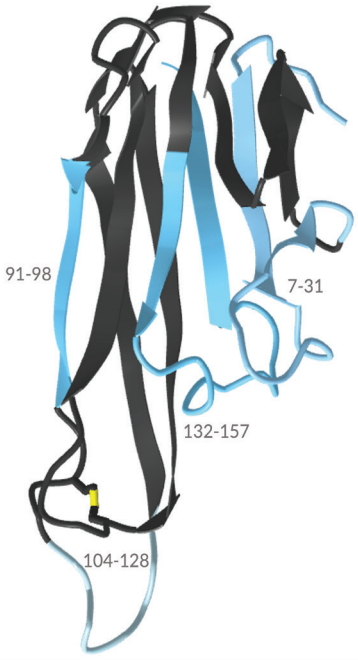
Figure 8: Volcano plot comparing the APO of TNFa ± adalimumab.

Peptides with a significant change in oxidation are above the dotted line (p-value ≤ 0.05). Peptides with a negative fold change were protected following the addition of adalimumab. Peptides with an APO fold change less than two are in the green region and peptides in the blue region have a fold change greater than two. The significance cut off can be adjusted as required. Volcano plots provide relative magnitude of change and statistical confidence.

3-D Interactive Model from Epitope Experiment

Figure 9: FoxWare data to iCn3D, a 3-D structural viewer, to highlight peptides with a significant change in APO between two conditions.

TNFa (PDB: 3WD5) peptides in black have no change in APO, peptides in blue have a significant decrease in APO following adalimumab incubation, and peptides that significantly increase in oxidation are colored red (not shown). 3-D models can also be generated showing absolute and control-adjusted peptide level APO.



Conclusions

- FoxWare Software's Radical Dosimetry Module quickly generates dosimetry response curves for all detected peptides providing optimal oxidation level while avoiding artifactual oxidation.
- FoxWare Software's Data Processing Module saves significant time calculating the APO.
- FoxWare Software provides a platform to quickly evaluate and verify peptide chromatographic area selected for the APO calculation.
- FoxWare Software's Data Analysis Module organizes full experiments and subtracts background oxidation.
- FoxWare Software's Data Analysis Module visualizes APO using histograms, volcano plots, and 3-D interactive models.

GenNext has pioneered a superior, compact, cost-effective, and safe means of performing advanced FPOP HRPF analysis.
By replacing expensive, complicated, and hazardous lasers with our proprietary Flash Oxidation System, you can easily perform HRPF with a convenient benchtop instrument.

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