Laser-Free Flash Oxidation (Fox™) Hydroxyl Radical Protein Footprinting System Accurately Maps the Epitope of TNFα Bound to Adalimumab

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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 irreversibly 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.

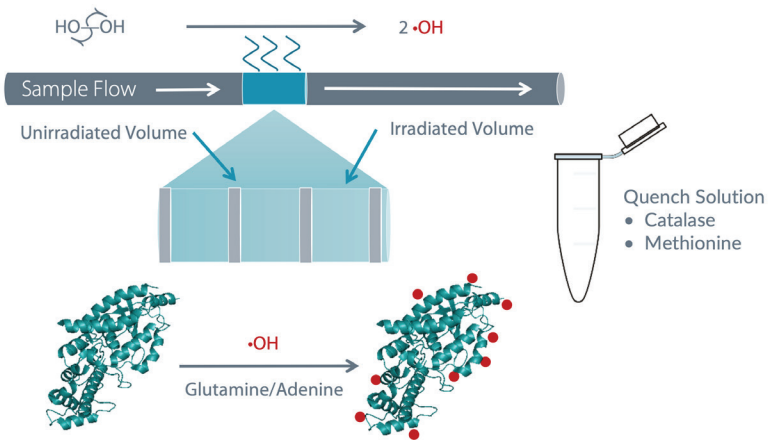


Figure 1: Schematic of an HRPF method, fast photochemical oxidation of proteins (FPOP).

In FPOP, protein is mixed with hydrogen peroxide and flown 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.

Laser-Free Fox System

Previously, FPOP required a hard-to-maintain and hazardous laser as the irradiation source to photolyze H_2O_2. The laser and laser gas requires specialized training and substantial safety equipment. Furthermore, laser optic adjustments and alignments can be very challenging and tedious. To overcome the laser obstacles, a high-pressure flash lamp was developed, creating the Flash oxidation (Fox) Protein Footprinting System.

The Fox System is composed of the fluidics module, photolysis module, dosimeter module, and the product collector (Figure 2A). Protein pre-mixed with adenine and H_2O_2 is injected on the sample loop connected to the fluidics module. Running buffer is used to push the protein sample pass the photolysis module which contains the fully enclosed high-pressure flash lamp (Figure 2B). The flash lamp has a pulse width <10 µs (Figure 2C) and reliably produces a broad-band spectral output from 200-300 nm wavelength along with some visible light (Figure 2D). The broad spectrum of the lamp matches the UV absorbance spectrum of H_2O_2, resulting in effective production of •OH.

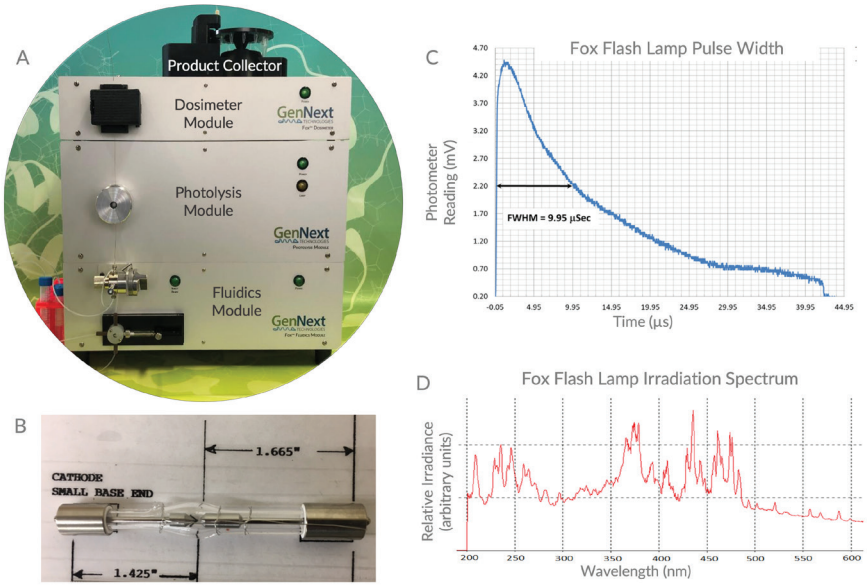


Figure 2: Fox System and flash lamp.

A) The Fox System with its four modules (fluidics, photolysis, dosimeter, and product collector). B) High-pressure plasma lamp housed in the photolysis module. The plasma lamp withstands high peak energy, generates very small plasma arc for optimum focusing, and stable over many thousands of flashes. C) Fox flash lamp pulse FWHM is 9.95 µs. D) Emission spectrum of the Fox flash lamp. The high-pressure Nobel gas blend lamp emits broad spectrum UV irradiation from 200-300 nm wavelength along with some visible light.

Fox™-Based Epitope Mapping

Tumor necrosis factor α (TNFα) is a pro-inflammatory cytokine. Adalimumab (Ab) is a monoclonal antibody that binds to TNFα and is prescribed to treat inflammatory diseases. TNFα and Ab were reconstituted in PBS and pre-mixed with adenine and H_2O_2 to a final concentration of 3 µM TNFα with or without 1.5 µM Ab, 1 mM adenine, and 100 mM H_2O_2. TNFα ± Ab was labeled in triplicates with and without flash oxidation. Samples were introduced to the Fox System using a 12 µL injection loop. Running buffer pushed the sample passed the Fox flash lamp, efficiently photolyzing H_2O_2 to form •OH. Downstream of the photolysis module, a dosimeter module accurately determines the effective •OH concentration by detecting the change in adenine's absorbance at 265 nm. Finally, samples are collected in 20 µL of quench, 0.3 mg/mL catalase and 35 mM methionine, and underwent a tryptic digestion for bottom-up proteomics.

Dosimetry

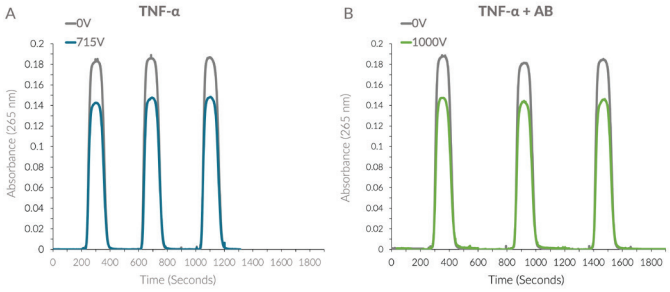


Figure 3: Radical dosimetry of TNFα ± Ab.

A) Real-time adenine dosimetry for triplicate injections of TNFα at 0 V (gray) or 715 V (blue). The decrease in absorbance between the gray and blue plot is proportional to the effective •OH concentration. B) Real-time adenine dosimetry for triplicate injections of TNFα + Ab at 0 V (gray) or 1000 V (green). To compensate for Ab •OH scavenging, the lamp voltage was increased from 715 V to 1000 V. With this lamp voltage, the same change in absorbance was reached, i.e., same effective •OH concentration.

Average Peptide Oxidation (APO)

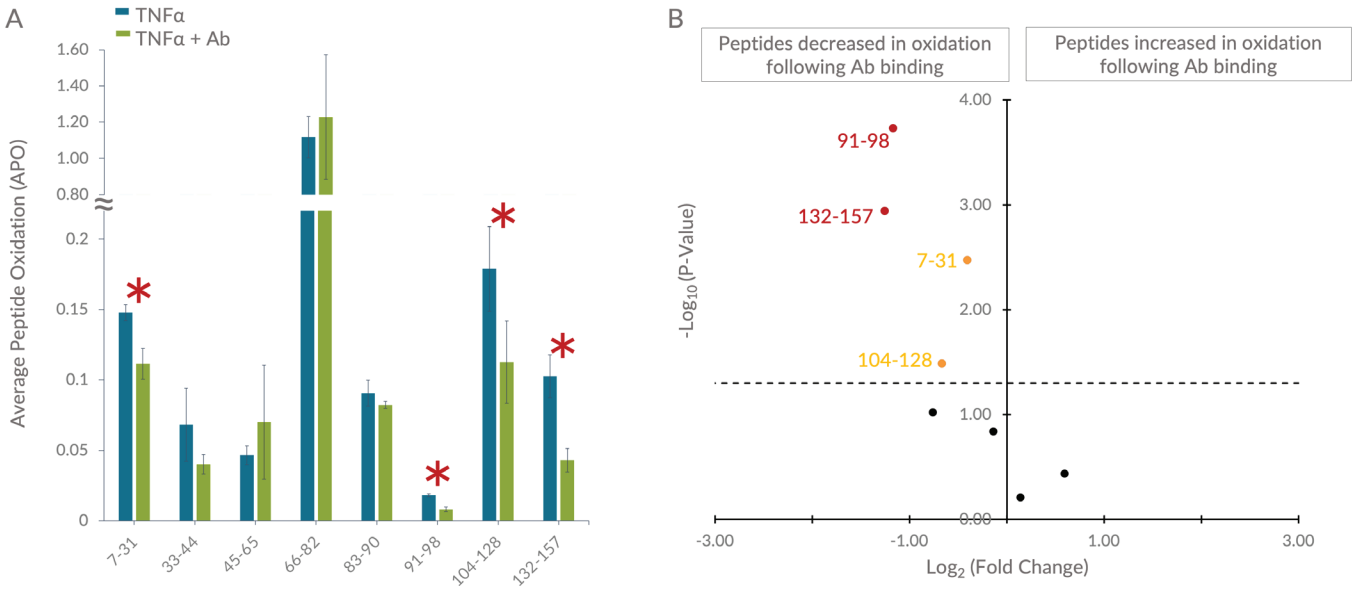


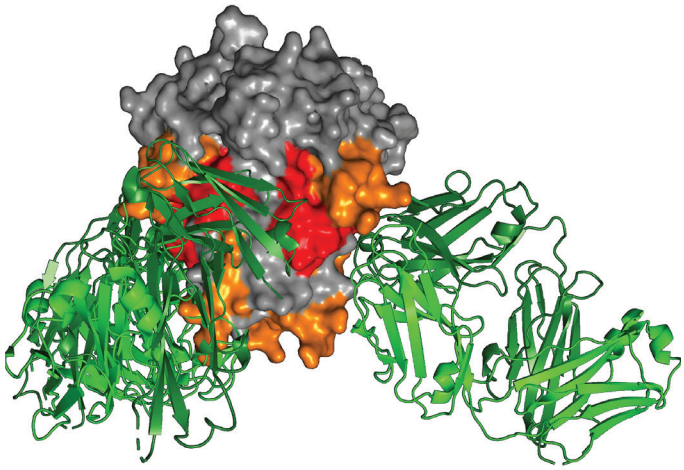
Figure 4: Fox™ System FPOP of TNFα ± Ab.

A) Histogram of Fox System FPOP average peptide oxidation (APO) for TNFα alone (blue) or with Ab (green). Error bars represent one standard deviation from a triplicate measurement. Peptides that showed statistically significant protection upon Ab binding are marked with a red asterisk ($p \leq 0.05$). B) Volcano plot of changes in the TNFα Fox System FPOP results upon Ab binding. Peptides above the dotted line have a p-value less than 0.05. Peptides with a statistically significant change with an APO fold change less than two are highlighted in orange and peptides with an APO fold change greater than two are highlighted in red.

TNFα-Ab Crystal Structure Comparison

Figure 5: Fox system FPOP protection of TNFα plotted on an X-ray crystal structure of the TNFα-Ab complex (PDB: 3WD5 and 1TNF).

Ab Fab heavy chain is colored dark green, and the light chain is colored light green. Regions of TNFα that show no significant change in oxidation are colored gray. TNFα peptides with a significant decrease in oxidation are highlighted in red (fold change greater than two) and orange (fold change less than two). All four peptides with a significant decrease in oxidation are involved in the TNFα-Ab interface. One peptide (66-82) involved in the interface was not detected with a significant decrease in oxidation. Residues apart of the interface have low reactivity towards •OH, while outside of the interface is a cysteine. Cysteine has the highest •OH rate constant and can account for most of the oxidation detected on peptide 66-82. Importantly, no region outside of the epitope interface was detected with a significant change in oxidation.



Conclusions

- The Fox™ System is an easy and robust FPOP technique that replaces the difficult-to-use and maintain laser-based FPOP technique.
- Real-time •OH dosimetry improves reproducibility and provides a platform to quickly adjust for varying background •OH scavenging.
- Fox FPOP detected 4 TNF-α peptides with a significant decrease in oxidation following Ab binding.
- The four peptides with a significant decrease in oxidation are known to be a part of the TNFα-Ab interface.

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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