In Vitro and In-Cell Radical Dosimetry for Reproducible Hydroxyl Radical Protein Footprinting Experiments

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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.

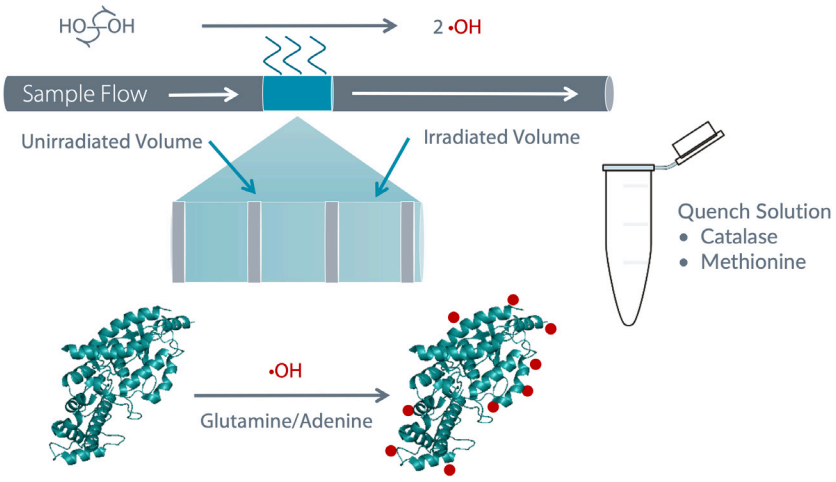

Figure 1: Schematic of an HRPF method, fast photochemical oxidation of proteins (FPOP).

With FPOP, protein is mixed with hydrogen peroxide and flown passed a pulsing 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.

In Vitro Radical Dosimetry

•OH have varying reactivity towards a broad range of molecules. With changes in the buffer composition, such as ligands/small molecules and changing protein concentration, the •OH will be scavenged differently.

This background scavenging will result in compromised reproducibility and decreased confidence in differential studies. With radical dosimetry, the effective •OH yield is monitored, and changes in background scavenging can be compensated for in real time.

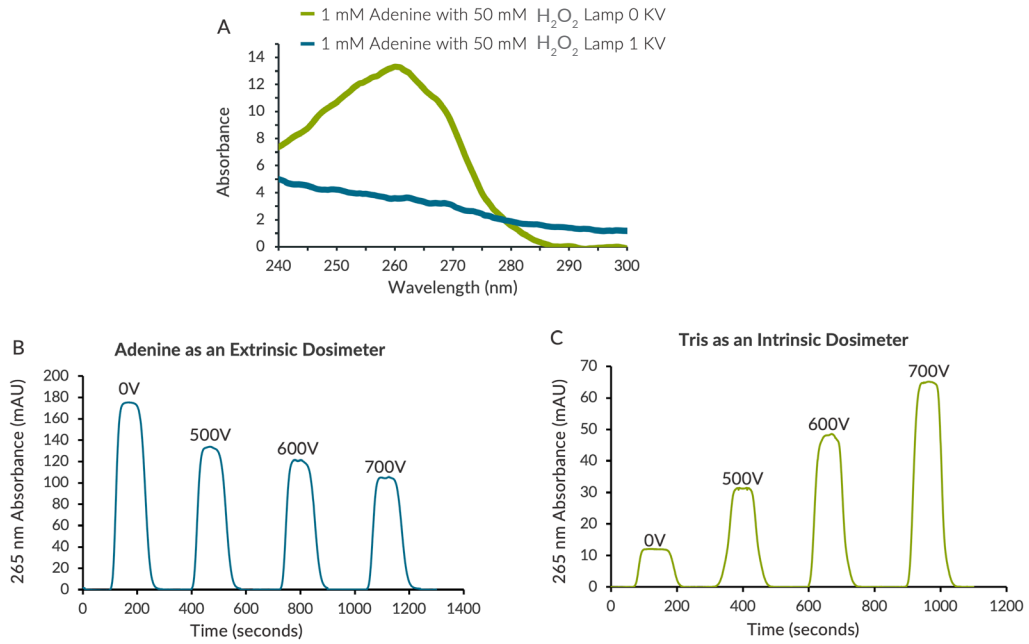

Figure 2: Monitoring the •OH concentration by monitoring the change in UV absorbance at 265 nm.

A) Unoxidized adenine strongly absorbs at 265 nm (green trace), but upon oxidation it decreases in absorbance (blue trace). B) As the lamp voltage increases, more •OH are generated and adenine linearly decreases in absorbance. C) Tris buffer is the opposite. Unoxidized tris does not absorb at 265 nm, but as the •OH concentration increases, the absorbance linearly increases.

Myoglobin Protein Radical Dose Response

Apomyoglobin at 5 µM, 1 mM adenine, and 50 mM H_2O_2 was labeled with three increasing lamp voltages (i.e., three •OH concentrations) for a protein dose response experiment. As the •OH concentration increased, the average peptide oxidation (APO) linearly increased. A linear increase in the APO confirms peptide oxidation stops before any artifactual structural changes that might take place following protein oxidation.

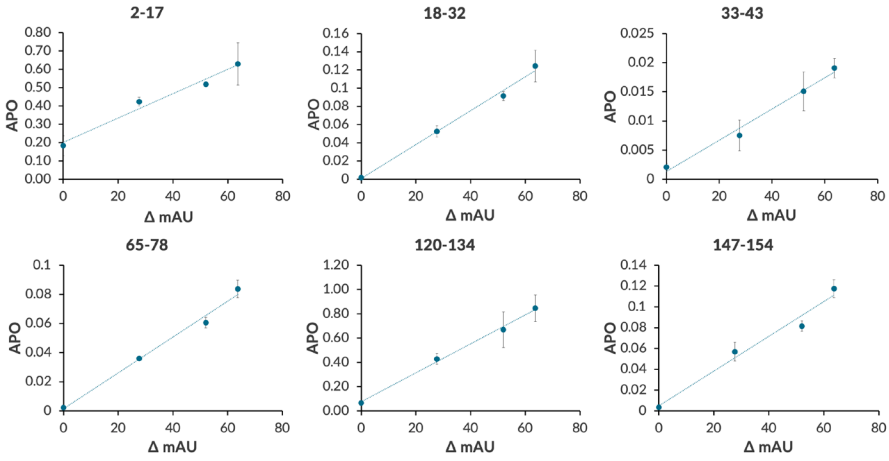

Figure 3: Apomyoglobin dose response experiment.

The APO of six peptides versus the change in 265 nm absorbance. The ΔmAU represents the effective •OH concentration.

Real-time Radical Dosimetry

Protein is mixed with 1 mM adenine and H_2O_2 and injected in the sample loop. The fluidics module pumps running buffer through the injection loop, pushing the protein sample passed the photolysis module. Once the sample reaches the photolysis module, the pulsing high pressure plasma lamp produces radicals to oxidize the protein and adenine. Following the photolysis module is the real-time dosimetry module, monitoring the absorbance change of adenine. Finally, sample is pushed to the product collector and collected in the quench solution. With real-time radical dosimetry, background scavenging can be compensated quickly and easily, so all conditions are labeled with the same effective •OH concentration.

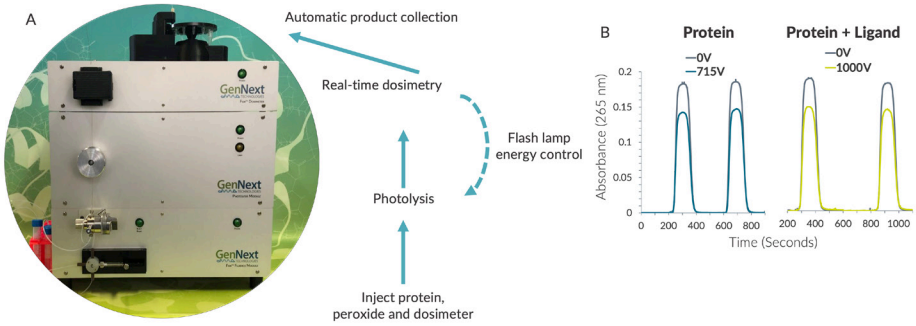

Figure 4: Real-time radical dosimetry using the laser-free FPOP Fox® System.

A) The Fox System is composed of four modules (fluidics, photolysis, dosimetry, and product collector). Arrows describe the basic workflow and sample flow direction. B) Example of absorbance change for a differential study. Gray plots represent the background absorbance from 1 mM adenine. Blue trace is the absorbance of an FPOP labeled protein in its native state. The decrease in absorbance between the gray and blue plot is proportional to the effective •OH concentration. The green trace is the absorbance of an FPOP labeled protein with a ligand. To compensate for the •OH scavenged by the ligand, the lamp voltage was increased from 715 V to 1000 V. With this lamp voltage, the same change in absorbance was reached.

In-Cell FPOP Radical Dosimetry

In-cell FPOP (IC-FPOP) provides a snapshot of the Higher Order Structure of the full proteome. Studying the Higher Order Structure in cells accounts for structural changes or protein interactions resulting from molecular crowding and quinary structures, and it is an ideal solution to study membrane proteins.

Like *in vitro* FPOP, IC-FPOP is challenged by variability in background scavenging. However, unlike *in vitro* radical dosimetry, UV absorbance is not sensitive enough for real time IC-FPOP dosimetry. Fluorescence detection provides the required sensitivity and dynamic range for in-cell dosimetry.

CellROX® Red was used as the dosimeter molecule due to its ease in cellular uptake and significant fluorescent signal after radical attack. Cells were treated with 50 µM CellROX Red for one hour then rinsed with PBS. Cells were collected and concentrated to 5e6 cells/mL and aliquoted so that each sample contained 7e5 cells. Cells were labeled using the single cell flow system developed by Espino et al. Immediately downstream of the on-capillary laser irradiation is the in-line radical fluorescence detector that utilizes a highly stable and compact UV diode laser at 640 nm. The •OH induced fluorescence of CellROX Deep Red at 665 nm is directed through a dichroic mirror and further processed by collection optics to strike a high speed, high gain, compact photomultiplier and is converted into electrical current that is proportional to the detected fluorescence. CellROX Red is solvated in DMSO, a known radical scavenger. Accordingly, CellRox limited in-cell protein oxidation. We are collaborating with ThermoFisher Scientific to create new and improved IC-FPOP dosimeter dyes to overcome CellROX limitations.

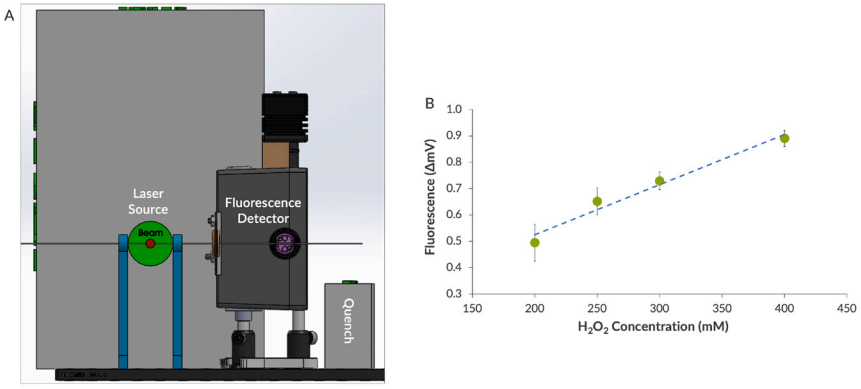

Figure 5: Real time IC-FPOP radical dosimetry.

A) Schematic of the on-capillary fluorescence detector immediately downstream of the laser irradiation source and before quench collection. B) Fluorescence dose response of 50 µM CellROX Red during IC-FPOP. As the H_2O_2 concentration increased, the fluorescence signal from CellROX Red linearly increased. The fluorescent signal of 7e5 cells was averaged as a single sample. The plotted points and error bars represent the average and standard deviation of triplicate samples. Major source of variation was a result of unstable laser energy.

Conclusions

- *In vitro* radical dosimetry can use an extrinsic dosimeter (1 mM adenine) or an intrinsic dosimeter (10 mM Tris).
- Protein dose response experiments utilize the radical dose response and the APO to confirm the native in-solution confirmation of the protein is probed.
- Real-time radical dosimetry can quickly and easily compensate for varying levels of background •OH scavenging. This improves experimental confidence and reproducibility.
- Real-time IC-FPOP radical dosimetry has been performed using fluorescent detection. Our collaboration with Thermo Fisher Scientific will provide new and improved IC-FPOP radical dosimetry internal standards.

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