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Title
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Permalink
https://escholarship.org/uc/item/17b9x1nn

Journal
Nature Methods, 23(6)

ISSN
1548-7091

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Publication Date
2026-06-01

DOI
10.1038/s41592-026-03083-0

Peer reviewed

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Author manuscript

Nat Methods. Author manuscript; available in PMC 2026 June 12.

Published in final edited form as:

Nat Methods. 2026 June ; 23(6): 1089–1099. doi:10.1038/s41592-026-03083-0.

Recommendations and Considerations for Hydroxyl Radical Protein Footprinting - Mass Spectrometry

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Abstract

Protein oxidative footprinting—using hydroxyl radical labeling detected by bottom-up proteomics—has progressed from an emerging method to a widely used approach in structural biology.

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Contributions

All authors participated in regular meetings from 2023–2025 to develop the community guidelines. A.T.W. conceived the project and led the overall effort. L.J.B. and J.S.S. developed the proposed statistical framework. M.L.G., R.Y.C.H., L.M.J., F.S., L.W., X.C.K., and S.K.M. contributed to the single-dose HRPF best practices. M.R.C., M.B., C.Y.R., E.R.F., S.G., L.G.K., and Y.S. contributed to the dose-response best practices. P.N. provided comments and assisted with manuscript editing and revisions.

Ethics Declaration

J.S.S., L.M.J., M.L.G and M.R.C. disclose an interest in GenNext Technologies, Inc., a growth-stage company seeking to commercialize technologies for protein higher-order structure analysis.

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Hydroxyl radicals generated from hydrogen peroxide (via photolysis, Fenton chemistry, or electrochemistry) or directly from water (via X-rays, plasma, or gamma rays) irreversibly encode structural information within protein side chains, which is read out using standard LC–MS workflows. Quantitative changes in labeling report on solvent accessibility and reveal effects of protein–protein interactions, ligand binding, protein folding, conformational changes, or applied stress. Comparing labeling patterns between states provides detailed maps of structural changes and interaction sites. Over the past decade, oxidative footprinting has proven valuable as a solution-phase and in-cell method for protein structure analysis. This perspective summarizes best practices for experimental design, sample processing, data analysis, interpretation, and integration with orthogonal data, offering a consensus framework to guide application of oxidative footprinting in academic and biopharmaceutical research.

Keywords

hydroxyl radical protein footprinting-mass spectrometry (HRPF); protein oxidative footprinting; bottom-up mass spectrometry; fast photochemical oxidation of proteins (FPOP); epitope mapping

Introduction

‘Footprinting’ refers to in-solution structural analysis in which a macromolecule is exposed to a chemical or enzymatic reagent that generates stably modified or cleaved products [1]. The resulting sites correlate with solvent-accessible regions on the macromolecule and can be used to infer structural information about the molecule and its complexes. Although this article focuses on hydroxyl radicals (·OH) as the modifying reagent, the principles apply to most irreversible chemical labeling reagents (e.g., other radicals, carbenes) and slower-reacting agents. The high reactivity and small size of ·OH provide excellent properties for irreversible labeling [2], and multiple strategies exist for generating ·OH from water or hydrogen peroxide, making these reagents widely accessible (Figure 1). Hydroxyl radical footprinting was first used to map DNA structure and solvent accessibility of protein binding sites with single base resolution [3, 4] and later to characterize RNA structures [5, 6]. It was subsequently adapted for proteins and coupled to LC-MS to map reactive and solvent-accessible residues [7], laying the groundwork for hydroxyl radical protein footprinting (HRPF)[8–10].

Currently, the term “hydroxyl radical protein footprinting” (or HRPF) has been applied to mapping protein-protein interactions [11, 12] including epitope mapping [13–17]; to probing protein dynamics in systems ranging from small globular proteins to multi-subunit complexes [18–21]; to the structure of intrinsically disordered proteins [22, 23]; to small-molecule ligand binding to protein targets for fundamental and drug-discovery applications [24 25–27]; and to protein folding/unfolding [28, 29]. A range of ·OH-generation methods have been used, including Fe(II)-EDTA Fenton chemistry [30–33], irradiation using synchrotron X-ray [7, 10], gamma [2, 34], Van de Graaff electron sources [35], plasma-based methods [36, 37], and UV lasers [38] and flash lamp photolysis of H_2O_2 [39]. HRPF provides unique structural insights when used alone or with complementary structural approaches.

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Common to all HRPF approaches is that ·OH covalently modifies amino acid sidechains (in preference to the backbone) based on the residue solvent accessibility and the variable intrinsic reactivity of the side chains to oxidation by ·OH [2, 40]. A review of the apparent rates of each amino acid under simplified conditions can be found in the work of Xu and Chance [2]; however, intrinsic reactivities of amino acids can change based on primary sequence [41] making interpretation of HRPF results much easier when comparing the same (or highly similar) polypeptide sequence in two or more conformational states. While this article focuses on the most common application of protein oxidative footprinting, that of mapping the interface between a protein and another molecule, the principles and practices articulated herein are generally relevant to all applications of the method.

The following minimum community standards and recommendations for HRPF data acquisition and analysis emerged from extensive discussions among active contributors to HRPF from 2023–2025, including the authors of this perspective. Our aim is to harmonize HRPF practices to support rigor and reproducibility. Although not all HRPF applications will conform to these guidelines, they provide a foundation for robust application of HRPF to questions in biological and biomedical research, as well as therapeutic development. These guidelines represent a community-accepted minimum; continued methodological advances are expected and encouraged as they are substantiated.

General Experimental Design

Many HRPF applications have been described in the literature. For this perspective, we focus on a well-defined high-affinity 1:1 protein–protein interaction as a prototypical example. Stable complexes like these generally require minimal biochemical characterization before HRPF analysis. Antibody–antigen interactions exemplify this type of complex, where one or two regions interact, leading to reduced radical labeling at the interface owing to lower solvent accessibility. Saturation of the binding site is achieved by using a small excess of ligand. New HRPF users are advised to validate their protocol on a well-characterized 1:1 system before studying more complex or dynamic assemblies.

This perspective focuses on peptide-level HRPF analysis, comparable to HDX [42], which is generally sufficient to map binding interfaces. Protocols for residue-level resolution are emerging using approaches such as HPLC- or ion-mobility–separable isomeric peptides, multiple reagents, and proteases [25, 43, 44]. One workable approach occurs when isomeric peptides representing different labeling sites are separable by HPLC (or ion mobility), in which case integration of extracted ion chromatograms can provide quantitation at the residue level. As consensus on residue-level analysis has not yet emerged, recommendations here are limited to peptide-level oxidation.

Radical Generation

As discussed in the introduction, multiple methodologies have been developed for ·OH generation, either by splitting hydrogen peroxide or through the ionization/dissociation of water (Table 1 summarizes the most widely used of these technologies). While a detailed comparison is beyond the scope of this perspective (see the cited references and [45, 46]), we briefly highlight key considerations for new HRPF users when selecting a radical generation

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method. A common recommendation across all technologies is to use a biologically compatible buffer that minimizes ·OH scavenging by excipients. Common scavengers in biological systems include buffer salts, glycerol, dimethyl sulfoxide, dithiothreitol, and detergents [47]. Although scavengers can be accommodated to some extent by all methods, best practice is to limit reactions of generated ·OH with molecules other than the protein of interest.

Two related considerations when choosing the appropriate method for ·OH generation are whether the HRPF study is a one-off experiment or part of a larger series, and the availability of the required technology. For single experiments, collaboration with an academic laboratory, national facility, or commercial provider is recommended. Laboratories aiming to develop HRPF capabilities in-house should carefully assess the resources required for each approach. For example, synchrotron X-ray radiolysis is accessed through national laboratory facilities; FPOP requires a custom-built laser and fluidics system; FOX is a commercially available fluidics-based system; and PLIMB can be accessed through a contract research organization (Table 1). When studying rapid processes on sub-millisecond timescales—such as protein folding—the most suitable options include FPOP with a nanosecond pulsed laser at 248 nm, synchrotron X-ray radiolysis using liquid jets with sub-100 μs exposures [48], and FOX with sub-50 μs exposure times [39]. Fenton chemistry (Fe^{2+}-EDTA), although implementable with standard laboratory equipment, exposes proteins to ·OH over seconds to minutes, compared with the millisecond-to-microsecond timescales of FPOP, FOX, and radiolysis. Nonetheless, Fenton-based approaches are suitable for mapping protein-protein interactions. Overall, the availability, technical characteristics, and timescale requirements of each method should be carefully evaluated when developing in-house HRPF capabilities.

A third consideration involves the two quantitative oxidation strategies described in the HRPF literature: single-radical dose measurements and multiple-radical dose (“dose–response”) measurements. Developers of each radical generation technology have typically adopted one strategy or the other (Table 1). For both approaches, experimental replicates are recommended. Replicates should be designed to capture all significant sources of variability that could introduce artifactual differences between samples. The nature of the replicates used must be clearly documented to satisfy community minimum standards.

Single-Dose Measurement

Single-dose HRPF is typically performed by photolysis of hydrogen peroxide by using approaches such as laser or flash-lamp photolysis (FPOP and FOX, Table 1), followed immediately by quenching of all secondary oxidants [38, 39, 49, 50]. Measurements have at least three experimental replicates of samples that are irradiated immediately after hydrogen peroxide addition. Experiments include at least one control (no radicals are produced) and three experimental replicates per condition. For a 1:1 complex, this entails three replicates each for free and bound protein (six samples total). If identifying the binding interface on both protein partners is of interest, then labeling of both proteins in the free state (unbound) would be required, for a total of nine irradiated samples Control samples that are exposed to hydrogen peroxide but not irradiation will allow subtraction of any background oxidation,

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and minimum community standards require at least two control replicates for each sample state.

In laser/photon-based HRPF experiments, radical scavengers are added to limit radical lifetimes and prevent over-oxidation that can trigger unfolding, aggregation, or other artifacts [51]. Background (pre-irradiation) oxidation can substantially influence hydroxyl-radical labeling quantitation, so minimizing it through careful sample preparation and handling is essential. Elevated background oxidation can affect protein conformation and saturate susceptible HRPF targets, complicating interpretation. Likewise, excessive on-target labeling should be avoided because it can (1) perturb native structure, (2) produce non-linear modification levels, and (3) introduce errors in percent-modification calculations due to altered ionization efficiencies of modified versus unmodified peptides. Investigators should explicitly describe how both background oxidation and overall modification levels influence their data interpretation.

A single-sided interface analysis of a 1:1 complex requires at least 10 samples: two negative-control replicates in the unbound state, two in the bound state, three experimental replicates unbound, and three experimental replicates bound. Determining a double-sided interface requires the corresponding control and irradiated samples for the second protein in the complex.

Multi-Dose (Dose-Response) Measurement Strategies

For multi-dose measurements, samples are exposed to progressively increasing doses of ·OH to generate a dose-response plot. The experimental deliverable is the change in oxidation as a function of ·OH dose. The multi-dose approach surmounts challenges with the 1000x range in amino acid side chain intrinsic reactivity towards ·OH by allowing each side chain type to be optimally oxidized. Furthermore, one can construct a plot of labeling yield vs. dose, akin to a kinetic plot in HDX, which provides more confidence in interpretation. In radiolysis, the dose can be controlled by sample geometry, such as speed of the sample in a capillary as it passes through the X-ray beam [52], or use of a shutter to control exposure time on a fixed target [53]. Similar considerations apply to FPOP where laser energy, flow rate, and concentration of scavenger can be employed as variables [54].

A recent implementation of Fenton chemistry uses a multi-channel pipette to generate a dose-response series simultaneously by adding increasing concentrations of Fe(II)-EDTA and hydrogen peroxide (at a constant catalyst-to-substrate ratio) to a set of samples [31, 33]. Calibration of the ·OH dose is typically carried out using a dosimeter strategy described in the next section. Dose-response plots typically start with a control sample (e.g., no applied radical generation) and at least three different radical doses. At least two (three is preferred) replicates are collected at each dose. Comparable to single-dose experiments, a quencher is added to stop further secondary oxidation after exposure (Table 1). Because the ·OH reactivity is a combination of the intrinsic reactivity and solvent accessibility of each residue, the data are presented as ratios of rate constants to highlight peptides (and/or residues) showing the average solvent accessibility changes from one state to another, such as in antibody binding to an antigen. Thus, a five-dose triplicate dose-response analysis of

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a 1:1 complex would require oxidation of 30 samples, 15 each for the free target and the complex.

Single-dose experiments generally use less sample and require less time than multi-dose strategies. However, multi-dose strategies generally should provide more precise results than single-dose measurements owing to the more thorough measurement of oxidation kinetics. Investigators should choose their strategy based on available instrumentation, sample, and the needs of the analysis. Moreover, if there is any uncertainty in which type of approach should be used, the authors invite investigators to consult with experts who are proficient with these two types of strategies to ensure they obtain optimal HRPF-MS data for their system of interest.

Dosimeter Considerations

HRPF is unusual among bottom-up MS methods because the impact of scavengers on ·OH availability cannot be predicted before experimentation. The effective radical dose experienced by the protein is determined by sample composition and the amount of radical generated, and must be equivalent across samples to enable direct comparison. To address scavenging effects, several empirical dosimetry approaches have been developed. [53, 55–59]. The initial radical concentration depends on photolysis or radiolysis energy (and beam characteristics) and, when applicable, hydrogen peroxide concentration. Radical scavengers in the sample (e.g., buffer components, membrane lipids, solvent, reactive amino acids) further reduce the available radical through non-productive consumption. Any change in these factors alters the effective radical concentration and can affect assessments of protein conformation. In-line dosimetry provides a real-time measurement of delivered radical dose, enabling adjustment of labeling conditions to match scavenging environments across samples [39]. Internal dosimeters offer a quantitative readout of dose, and details are available in the cited references.

When dosimetry is not available, users may employ reporter peptides to assess relative dose changes [54] or external dosimetry (e.g., GC/MS) to calibrate selected conditions [55]. Critically, all samples must receive equivalent effective doses. To meet community standards, both the dosimetry method and results must be clearly reported, and alternative approaches are acceptable if supported by sound scientific rationale.

Downstream Sample Processing

As with all bottom-up MS technologies, samples labeled by oxidative footprinting are subjected to proteolysis (peptide mapping) and LC-MS/MS analysis. The general sample processing workflow requires that the protein be denatured and reduced (cysteine alkylation is common) for protein digestion. The resulting desalted peptide digest is the analyzed by LC-MS/MS analysis to identify peptides modified by ·OH and to quantitate the extent of labeling. The extent of labeling is proportional to the average local solvent accessibility of the oxidation target (discussed below) within the three-dimensional structure of the protein.

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Peptide Mapping Considerations

In general, the ideal protease should be highly specific and provide 100% digestion efficiency and sequence coverage of the target protein. This enables the most accurate quantitation of peptide labeling and ensures coverage of any unknown sites of potential interest. However, there are practical limitations and considerations for peptide mapping, such as enzyme specificity/efficiency, availability of proteolytic sites, and resulting peptide length. It is suggested that the analyte of interest be analyzed by the peptide mapping strategy of interest to evaluate the sequence coverage, specificity, and efficiency prior to HRFP-MS analysis. Deglycosylation is also commonly employed to reduce the quantitation complication that is inherent for glycopeptides [60].

Trypsin is the enzyme of choice for most footprinting studies as this enzyme is highly selective, highly efficient, and the distribution of proteolytic cleavage sites (C-terminal arginine/lysine) provides excellent coverage and good peptide ionization in the usual positive-ion mode MS [61]. Further, arginine and lysine are not extensively modified by ·OH, and cleavage is relatively resistant to effects of oxidation, which can be more problematic for other enzymes (e.g., chymotrypsin). However, it should be noted that heavily oxidized proteins can cause the formation of hydroxy-lysine, leading to potential digestion efficiency concerns [62]. Other specific proteases that can be considered as effective alternatives are shown in Table 2.

Regardless of the protease used, the investigator should demonstrate that the cleavage event has proceeded as close to completion as practically possible [61]. Completion can be determined by evaluating if there are any large signals observed (at high *m/z*-values) during column cleaning/regeneration at the end of the organic-phase gradient. Table 2 provides an overview of common proteases and reagents used for HRPF analysis.

Glycoproteins make up an estimated 50% of all proteins, and thus, analytes of interest may contain glycans [63, 64]. The resulting glycopeptides can be difficult to identify and quantitate in the MS analysis following HRPF [60]. Deglycosylation of peptides is common to remove the glycans and reduce the resulting peptide to a single amino acid sequence. The use of PNGaseF is used to remove N-linked glycosylation in which the resulting peptide will have a sequence change from Asn to Asp, and is particularly common for monoclonal antibody peptide mapping [65]. It is important to understand the composition of the analyte of interest with regards to potential glycosylation sites and the occupancy thereof, and in most cases the community recommends deglycosylation of N-linked glycopeptides prior to LC-MS/MS analysis.

LC-MS/MS Considerations

The cited literature provides detailed guidance on LC-MS/MS parameters, but several general recommendations apply. Peptide separations are typically performed by high-pressure reversed-phase LC using water/acetonitrile with formic acid. Both nano-LC and micro-LC are suitable: nano-LC offers higher sensitivity but lower robustness, whereas micro-LC requires more material but provides greater stability and reproducibility. When sample quantity allows, micro-LC is recommended. High resolving-power MS detection

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for both MS1 and MS2 is preferred. For most digests containing more than two peptides, precursor ions should generally be measured at ≥10 ppm resolution and fragment ions at ≥20 ppm. These values are guidelines rather than strict requirements, as resolution, sensitivity, and acquisition time must be balanced against sample complexity. Low resolving-power MS/MS may be justified when appropriate. For fragmentation, HCD or CID is typically sufficient, though ETD, EAD, or UVPD may also be used.

Three MS/MS acquisition strategies are commonly applied. The most widely used is data-dependent acquisition (DDA), where the instrument selects ions for fragmentation (e.g., the top N precursors), optionally assisted by an inclusion list. DDA can miss low-abundance modified peptides or be impacted by coeluting species, even with dynamic exclusion. Data-independent acquisition (DIA) avoids this limitation by fragmenting all precursors, but includes isotopologues and requires more complex data analysis [66]. When needed, targeted MS/MS is recommended. A preliminary MS or DDA run identifies the peptides of interest (including oxidation products), which are then selectively fragmented based on retention time and *m/z*. Targeted MS/MS enables reliable detection of low-abundance oxidation products and ensures full coverage of positional isomers that may elute across a broad window and otherwise be missed due to dynamic exclusion.

Each approach has strengths and limitations, and the choice should reflect the goals and constraints of the study. Targeted MS/MS is particularly advantageous when sample quantity is limited, as it focuses acquisition time on ions directly relevant to the analysis.

Data Analysis

This section provides a brief overview of the expected protein modifications observed from HRPF analysis, traditional processing strategies for the corresponding LC-MS data, and the recommended statistical approaches and data presentations.

Quantitation of Peptide Modifications

Quantifying the extent of modification at the peptide-level is commonly based on ion intensity in the extracted ion chromatogram (XIC) for the modified peptide(s), relative to the unmodified (or precursor) peptide. The intensities of the peptide signals are determined at the MS^1-level for quantitative purposes, whereas validation of the identity of the modified peptide is performed at the MS^2-level. When MS^2 is not sufficient to positively identify either the precursor or modified peptide, additional factors (e.g., accurate mass, isotopic distribution, retention time shift of the precursor relative to the unmodified peptide) or the multi-digestion approach, may be used in validation. However, these factors may not compensate for poor data quality, low sequence coverage, or low peptide abundance. A combination of these factors should be provided for any identified peptides used to make scientific conclusions using HRPF data.

The most common peptide modification from ·OH labeling is the direct incorporation of oxygen (i.e., addition of 17 Da and loss of 1 Da = +16 Da nominal), which typically modifies the most reactive residues (e.g., sulfur-containing (e.g., Cys, Met), aromatics (Phe, Tyr, His, Trp), etc.). However, depending on the radical source and time of labeling, many

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modifications can occur (Table 3). At a minimum, the +16 Da modification must be utilized for quantitation as that is the dominant modification. Peptides with high susceptibility to oxidation (e.g., Met-containing) should be searched for +32 Da modifications (a detailed recommendation for quantitation of modified peptides is in Supplemental Information).

Quantitation of the extent of modification at the peptide-level takes into consideration all the modified forms of the peptide and can be determined by two different approaches as described in Supplemental Information. The most common method for calculating oxidation is percent oxidation, where all XIC signals from all modified versions of a peptide are summed and divided by the sum of all XIC signals from both modified and unmodified versions of that peptide. This ratio is then multiplied by 100% (See Eq. S1). Oxidation that occurs from the electro-spray ionization process (i.e., in-source oxidation) should not be included in these calculations. In-source oxidation can be readily identified by having an identical LC elution profile as the unmodified peptide, while HRPF products will usually show a shift in retention time. In-source oxidation should be minimized by ensuring proper maintenance and tuning of the LC-MS instrument (e.g. spray voltage, emitter location, ESI emitter changes) and by thoroughly cleaning and desalting before introduction to the LC-MS.

Similarly, quantitation of labeling at the residue-level can be done if homogeneous XIC peaks and a definitive MS/MS are obtained. This is best determined for peptides containing a single, highly reactive residue (e.g. Met, Trp etc.) in which there is only a single modified form of the peptide detected. In this situation, the peptide-level modification reflects residue-level modification, in which accurate MS^2 is used to determine the modification position. Quantitation of residue-level modification for peptides that exhibit multiple modification sites is beyond the scope of this perspective.

Software Processing

There are many different approaches to processing HRPF data, ranging from manual identification and XIC integration, to the use of freeware and commercially available software. Several software packages are available for data processing; they include Xcalibur, Byos, Skyline, ProtMapMS, MassMatrix, FoxWare, Mascot, and FragPipe.

Window widths for creating the XIC should be based on MS mass error, and ideally should not include peptides or peptide oxidation products from more than one peptide sequence. The XIC integration should include all the isomeric peptide modification products that are HPLC separable, and if possible, the identity of these modified peptides should be validated by a method other than measuring intact mass (e.g. by MS/MS, relative retention time, carbon isotope distribution pattern, etc.) XIC of peptides that are partially separated (appear as composite chromatographic peaks) can be integrated together for peptide-level quantitation.

It is also important to understand the impact of different charge states, isotopic distributions, retention time and digestion efficiency on the quantitation of the extent(s) of modification. Although the mass spectrometer responds differently to unmodified vs. modified peptides, this problem is moderated by doing the experiment in a differential/comparative mode

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(e.g., bound vs. unbound, wild type vs. mutant) where ratios of ratios are used. Software that takes into consideration all the charge states and isotopes for quantitation limits the concerns for misquantitation of these fundamental mass spectrometric features of peptides. Furthermore, choosing the most abundant charge state and the most abundant isotopic peak may provide the precision of quantitation needed to have confidence in determining the solvent accessibility change between two states [67]. Minimum community standards require that comparison of unmodified and modified versions of the same peptide use the same charge state and isotopologue for measurement of both the modified and unmodified versions of the same peptide. It is important to note that measurement errors may come from using isotopologues with different relative abundances.

Statistical Approach

Traditionally, the statistical approach for single-point HRPF data has been to generate bar graphs to visualize the data across all peptides and to perform a series of separate analyses, one each peptide, comparing two or more states (e.g., unbound and bound states) by using Student's t-test at the $\alpha = 0.05$ significance level) [13, 14, 16]. The statistical significance (p-value < 0.05) can be used to conclude that a difference in the <SASA> had been detected. Although the traditional approach is based on bar graphs with error bars (aka. dynamite detonator plots) and individual Student's *t*-tests per peptide (or per residue), which may suffice for a quick and simple assessment of <SASA> change, this approach may not be appropriate for all applications. Plots of average and standard deviation may not accurately convey the distribution of the data, especially at the small numbers of replicates typically used in HRPF experiments. Therefore, community minimum standards require each replicate data point be reported in addition to the mean and standard deviation for each peptide (or residue) for single-point measurements as indicated in the *Data Presentation* section (and Supplementary Information). A more detailed discussion of statistical approaches for single-dose HRPF data analysis can be found in the Supplemental Information.

For multi-point (dose response) measurements, following quantification of fractional modification for each sample, the points are assembled into a dose-response plot per peptide (or per individual residue). For synchrotron X-ray radiolysis, the principle of steady state approximation for the determination of a rate constant is applied on the dose response plot, which represents fraction of unmodified vs exposure time. Since the concentration of ·OH remains constant at all exposure times under a fixed dose rate, under the assumption of a pseudo-unimolecular reaction between protein and ·OH, the data are fitted to a first-order exponential $y = e^{-kt}$, and a ·OH reactivity rate constant '*k*' is determined for the peptide (or amino acid side chain) [2]. The results from the Fenton multi-point oxidation protocol are analogous to X-ray footprinting except that the ratio of the extent of oxidation as a function of oxidant concentration, rather than exposure time, is calculated. Community minimum standards require calculating the standard error of the first-order exponential fit to the data. Ideally, dose-response rates are calculated separately for each replicate of each sample. The standard deviation of the ratio of rate constants (or abundances) for each residue is then calculated, and the resulting error bars are depicted on the bar graph.

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Data Presentation & Transparency

Regardless of whether descriptive statistics alone or hypothesis testing is used, it is often appropriate to discriminate further between peptides based on the effect size as measured by fold-change in modification, as this difference is correlated to the magnitude of change in <SASA> [56, 68, 69]. Data should be minimally provided in bar graph or dot plot representation, or as tabulated data. Background oxidation of control samples should be measured and disclosed for all peptides. A representation of variability should be provided for each measured peptide or residue with an associated description (e.g., standard deviation, confidence interval). All replicate results should be made available to allow for a complete understanding of variability, preferably in the primary results visualization.

Currently, there is no shared public database specifically for oxidative footprinting results. We recommend the use of an established international database for mass spectrometry data (e.g., proteomexchange.org); however, the authors acknowledge that proprietary information such as non-public protein sequences will likely be restricted. Archived raw data will consist of LC-MS/MS datasets, search parameters (e.g., modified amino acid, type of modification), and metadata including irradiation parameters or chemical reagents, exposure times, dosimetry standards, sample preparation protocols, protease digestion parameters and efficiency (enzyme specificity, number of missed cleavages), and LC gradients. Processed data will consist of validated peptide and/or residue specific oxidation/accessibility as a function of the extent of oxidation. These latter data may be further processed to include linear fits, with the slope of the fits for each peptide/residue reported in tabular or bar-dot graph form.

Data Interpretation

Measuring Changes in <SASA>

The key to accurate data interpretation is to understand that there are at least two contributing factors governing the extent of radical labeling in footprinting. One is the specific residue's intrinsic reactivity with ·OH or other radicals; the other is the solvent accessibility of each residue. The latter is the foundation for footprinting because it reflects the exposure of a protein residue to ·OH diffusing through solution. As mentioned earlier, traditional HRPF data analyses often focus on comparing the differences in the footprinting rate or modification extent of a specific site to characterize conformational changes across different protein states apo vs. holo states [27, 70, 71], unbound vs. protein-bound states [15, 60], mutant vs. wild-type [72], one oligomer state vs. another [23, 40] and/or kinetics of structural changes [24]. A comparison of differences in footprinting between identical amino acid sequences in two or more conformational states normalizes the contribution of the intrinsic reactivity of each peptide to the rate of oxidation, allowing fold-changes in oxidation to reflect only changes in solvent accessibility with the magnitude of change being correlated directly with the magnitude of change in <SASA>.

For example, a significant decrease in modification extent going from an unbound to a bound state, may indicate that the region is involved in the ligand-binding interface. Likewise, an increase in modification for the same system likely represents structural

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changes resulting in an increase in the <SASA>. Thus, the changes in HRPF between protein states are conceptually reporting the change in the <SASA> between the two states. This relative comparison is quite reliable as all experimental workflows (and thus any inefficiencies) are common across the states compared. Studies demonstrating the absolute correlation between <SASA> and HRPF data (e.g., protection factor analysis) suitable for modeling were previously published [68]. This correlation is well-established for soluble proteins, but it is not perfect due to other factors affecting the footprinting yield, such as the presence of nearby reactive residues, protein dynamics, and potentially other factors that have not yet been identified.

Structural Changes vs. Binding Interfaces

Another aspect complicating the interpretation of protein-folding and protein-interaction data is the distinction between binding and binding-induced remote conformational changes (allostery). While binding generally reduces solvent accessibility at the interaction interface, local conformational and dynamic perturbations, as well as distant allosteric effects, may result in either decreases or increases in SASA and, consequently, in HRPF labeling. This is widely recognized as a challenge in HDX-MS [73, 74], but also extends to any reversible or irreversible MS-based footprinting strategy [75].

Our guidance focuses on protein-protein interactions as the example application for HRPF; however, there are many examples that elucidate structural changes vs. direct binding interactions. For example, HRPF has been used to investigate myoglobin-heme binding interactions, characterizing not only the binding pocket but also the changes induced by the binding [38]. Another study compared the structures of unbound and peptide-bound calmodulin, identifying several residues involved in ligand-induced structural changes [71]. Similarly, HRPF probed the interaction of Robo1 Ig1–2 with unfractionated heparin, and the findings suggest that heparin interactions induce modest conformational change that is not captured in the X-ray structure [76]. Caution is suggested, however, in distinguishing binding from binding-induced conformational changes [77]. In such cases, a more comprehensive approach using other methods to assign a fuller picture of protein binding vs. remote conformational changes would be warranted, as discussed in the following section.

The use of Orthogonal and/or Complementary Data

Characterizing protein structures and their dynamics, particularly in protein complexes, requires detailed information from various perspectives, including static protein structures, dynamic changes, and binding stoichiometry. Structural characterization is even more challenging when dealing with flexible proteins. Therefore, it is recommended that different structural elucidation techniques be combined, as well as different biophysical analytics be implemented, for characterization of protein complexes to infer correct mechanisms of action [75, 78, 79]. HRPF provides an ensemble average of solvent-accessibility information, sometimes at the residue level, making “protection” a foundational computational parameter that complements other methods of structural analysis.

For example, HRPF offers both direct and accurate measures of solvent accessibility and the relation to conformational change, but HRPF does not enable direct visualization

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of structures. In contrast, when combined with suitable computational methods, HRPF and other MS-based footprinting methods may be used to predict protein structure[80]. CryoEM and X-ray crystallography allow for direct visualization of structures but often lack information on dynamics and disordered regions of a protein [78]. Further, since HRPF is a solution-based method it provides structural information that is not visualized in static, solid-state states. Combining multiple methods reveals not only the detailed structure of complexes, but also the dynamic changes proximal to the binding region or distal to binding interfaces. HRPF, in combination with other biophysical techniques like surface plasmon resonance (SPR), can provide a detailed understanding of binding events from both structural and kinetic perspectives [76]. Further, mutational analysis, biological assays, and computational predictions/simulations frequently utilize HRPF data to support their hypotheses [60, 76, 81, 82]. This practice underscores the utility of HRPF in providing useful and complementary structural analysis within biologically relevant settings. Overall, integrating biophysical methods with structural biology approaches provides greater understanding of biological systems [79, 83, 84]. Table 4 summarizes orthogonal and complementary methods used in combination with HRPF.

Conclusion

HRPF is an in-solution method for mapping protein structure that can be applied alone or alongside other structural approaches. Here, we provide best-practice guidelines for mapping interfaces formed during protein assembly, including experimental design, sample preparation and oxidation, processing of oxidized samples, and analysis and interpretation of the resulting data. Minimum community requirements and recommendations are summarized in Table 5 to support consistency across studies and help new users adopt the method.

Hydroxyl radicals can be generated by X-rays or plasma irradiation of water, or by UV photolysis or Fenton chemistry of H_2O_2. Each approach can provide protein- or residue-level information on solvent accessibility, and the choice of method depends on the system and available resources. Detailed protocols and timing considerations are available in the primary literature referenced herein. All HRPF experiments require LC-MS/MS and appropriate software for identification and quantification of oxidative products. Automated tools can generate peptide-level results when signal-to-noise is sufficient, but residue-level analysis requires targeted data acquisition and careful validation of the resulting oxidative products.

What other method developments are needed for the continued improvement of HRPF? A key limitation of current methods is sparse labeling, especially in regions with highly reactive residues such as methionine, which consume a significant fraction of radicals. Unlabeled regions provide no information on conformation, limiting interpretive power. Complementary reagents, such as trifluoromethyl radicals ($\bullet CF_3$), can expand coverage. Recent studies combining •OH and $\bullet CF_3$ labeling in a single reaction achieved roughly twice the labeling, with CF_3 preferentially targeting residues poorly labeled by ·OH [85–87]. Continued improvements in MS and LC instrumentation and HRPF methodology will

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enable deeper coverage and detection of lower-abundance footprints beyond current MS limits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

MLG acknowledges support from the NIH, National Institute of General Medical Sciences (1R01GM131008) and the National Institute on Aging (R01AG079283). NSLS-II XFP beamline development was funded by NSF-MRI award (DBI-1228549, M.R.C) and Case Western Reserve University. NSLS-II is a U.S. DOE User Facility operated for the DOE Office of Science by Brookhaven National Laboratory under Contract No. DE-SC0012704. J.S.S. acknowledges support from the National Institute of General Medical Sciences (R01GM127267) for HRPF technology development. LMJ acknowledges support from the National Institute of General Medical Sciences (R35GM144324). X-ray hydroxyl radical footprinting development and operations at Advanced Light Source beamline 3.3.1 are supported by National Institutes of Health (1R01GM126218, C.Y.R and P30GM124169, C.Y.R). User operation at Advanced Light Source beamline 3.3.1 and Molecular Foundry is supported by the Office of Science of the U.S. Department of Energy (DOE) under contract DE-AC02-05CH11231.

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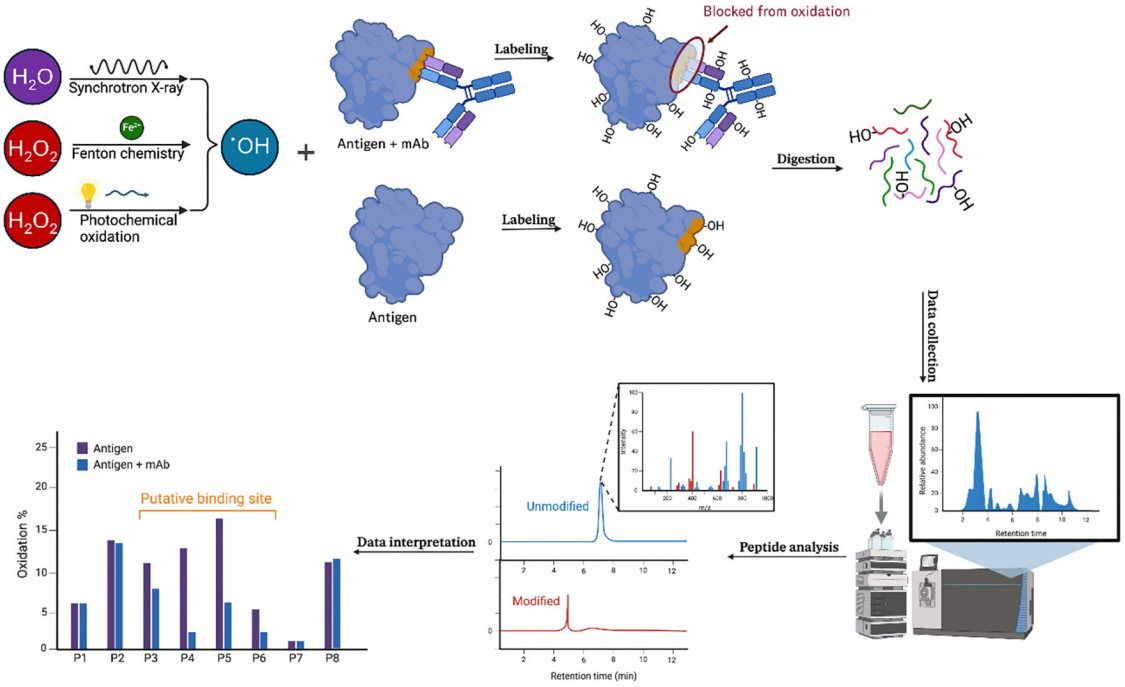

Figure 1.

Overview of hydroxyl radical protein footprinting – mass spectrometry workflow

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Table 1:

Examples of HRPF Technologies and Components

Generating Hydroxyl Radicals	Dose Type	Dosimeter	Scavenger/Quench	Recommended Buffer	Reference(s)
FPOP (Laser-Based)	Single (typically)	LeuEnk, Adenine, Tris	His, Arg, Gln, Met, Catalase, DMTU	Phosphate	38
FOX© (Flash Lamp)	Single (typically)	Adenine, His, Tris	Adenine, His, Catalase, DMTU	Phosphate	39
Fenton (Fe^{2+}-EDTA)	Multiple	Fluorescein	Thiourea, Methionine amide, catalase	HEPES	31, 32, 33
Fenton (Electrochemical)	Multiple	None Indicated	Met, catalase	Phosphate	88
Synchrotron X-Ray (XFMS)	Multiple	Alexa488	Met-amide	Phosphate, Acetate	53, 59, 48
Plasma Induced Modification of Biomolecules (PLIMB)	Multiple	None Indicated	None Indicated	Phosphate	36

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Table 2:

Example HRPF Sample Processing Reagents

Reducing Agent	Cysteine Capping	Desalting	Protease
Dithiothreitol	Iodoacetate, Iodoacetamide	NAP-5, PD-10, Zeba, Spin SEC, C18, R2, HLB	Trypsin, Asp-N, Glu-C, Lys-C Chymotrypsin

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Table 3:

Typical HRPF Modifications

Type	Mass Change (Da)	Residue
Typical	+16	A,C,D,E,F,H,I,K,L,M,N,P,Q,R,T,V,W,Y
	+32	C,F,M,W,Y
	+48	C,F,W,Y,
Rare	+4	W
	−30	D,E
	−43	R

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Table 4:

Examples of Technology Combination with HRPF

MS-based methods	HDX, Covalent Labeling-MS, Cross-linking MS, Native MS, Ion Mobility	Epitope mapping of an antibody/IL-23 interaction[13] Epitope mapping of PLBL2 Sandwich ELISA[60] Epitope mapping of IL-6R[44]
Structure-based methods	Electron Microscopy, Crystallography, SAXS	Defined the binding interface between H3K14Cr and BAF45D[89] Confirmed the key residues in the interaction of 15-PGDH and its inhibitor[90] Validated the cross-talk between DBD and LBD in hERα[20] Confirming X-Ray structure of ABC Transporter MsbA[27] Epitope determination and allostery in nanobody binding to SARS-Cov-2 Spike protein[91]
Spectroscopy-based methods	NMR, FT-IR	Validated the contact residues in the disordered form of the transactivation domain in estrogen receptor[92] Identified interface residues in CCL5 oligomer[93] Defined membrane-bound state of KRAS[94] Provided detailed aggregation curves for amyloid (Aβ) proteins[95]
Computational-based methods	Molecular Dynamics, Docking, Homology Modeling	Impact of Fab-FcγRIIIa interactions on ADCC[11] Measured structural effects of PTMs[82] Determined the Ig-like domain of NRG1[81] Characterized the interaction of gp120-bNAb complex[96] Combined with Boltzman docking, located hidden regions of enzyme that causes increased activity[72]
Biophysical methods	SPR, FRET, ITC, CD	Ultra-low affinity lipase-antibody interactions[12] Confirmed the key residues in Robo1-Heparin interaction[76] Characterized the interaction of CopR-DNA complex[97] Engineering protein interactions[98]

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Table 5:

Key Community Guidelines for HRPF

	Minimum Community Requirements	Recommendations
Radical Exposure	• Dosimetry measurements establish all samples receive equivalent radical doses • Two negative control replicates of each experimental state • Three replicates in each experimental state (single dose) • Six data points for each experimental state (multi-dose)	• On-line radical dosimetry • Two replicates for at least three radical doses (multi-dose)
Sample Processing	• Use of specific protease for digestion • Digestion pushed as far to completion as possible	• Trypsin for digestion • Use of reversed phase chromatography for LC-MS/MS • <10 ppm MS • <20 ppm MS/MS • Deglycosylation of N-linked glycopeptides
Data Analysis	• +16 modifications must be quantified for all peptides • XIC windows defined by instrument method mass accuracy • Validation of identity of all peptide oxidation products by accurate mass and at least one other factor • Quantitation by either percent oxidized or average peptide oxidation • Quantitation uses the same isotopologue and same charge state for both modified and unmodified versions of the same peptide	• All detectable oxidative modifications quantified for all peptides • Only one peptide sequence present for each XIC window analyzed • Validation of all peptide oxidation products by MS/MS, when possible • Most abundant isotopologue used • Most abundant charge state or all charge states used
Statistical Analysis and Data Presentation	• Determine a statistical analysis plan prior to acquisition of final data • Provide *t*-values for each replicate data point. • Single Dose: Mean and standard deviation reported. Multi Dose: rate estimate and standard error for rate reported, and standard error of the first order exponential fit (for multi-dose) reported • Publish results from all oxidized peptides, not just selected peptides of interest • Background subtraction (if performed) uses the mean of control oxidation replicates • Definitions of any error bars presented must be given in figure legend • Both oxidation difference and fold change must be reported for all comparisons • Fold change must be considered in all interpretations of results • Analysis plans provide the probability of a false positive outcome.	• Consult a statistician • Estimate the number of replicates you will need; use a pilot experiment if necessary • Consider statistical approaches other than hypothesis-testing • Plot individual replicates t • Welch *t*-test (if *t*-tests are used) • Adjust experimental replicate values by subtracting the mean of non-irradiated controls, or (preferred) via statistical modeling. • Post LC-MS/MS data and metadata on public repository