

retention. Through a combination of studies, Dickinson *et al.* demonstrate that the trappable probe does indeed exhibit increased cellular uptake and retention. These properties endow the new H₂O₂-specific fluorescent probe with enhanced sensitivity, thereby expanding the arsenal of chemical tools useful for analyzing this oxygen metabolite in cells.

With PF6 in hand, the authors perform live-cell imaging studies on freshly isolated AHPs and demonstrate that these CNS stem/progenitor cells produce H₂O₂ upon stimulation with FGF-2. Given these findings, the authors examined the relationship between endogenous H₂O₂ production and PI3 kinase-dependent activation of the Akt kinase, which is required for growth and proliferation of AHPs and characterized by several potentially redox-regulated components, including PTEN. These studies show that treatment with exogenous H₂O₂ or FGF-2 triggers an increase in Akt phosphorylation. Importantly, they go on to demonstrate that the FGF-dependent increase in phosphorylation of Akt could be blocked by antioxidants or the Nox inhibitor diphenyliodonium (DPI). On the basis of the robust expression of Nox2 in the CNS and the observed inhibitory effect of DPI on Akt phosphorylation, Dickinson *et al.* next evaluated Nox2 as a possible H₂O₂ source in AHPs. RNAi knockdown of Nox2, but not Nox3, significantly reduced endogenous

H₂O₂ generation in response to FGF-2 stimulation and was also accompanied by a decrease in the phosphorylation of Akt. Finally, bromodeoxyuridine (BrdU) incorporation experiments in wild-type or Nox2 knockout mice show that Nox2 deficiency greatly decreases the number of proliferating AHP cell populations. Collectively, these and other data presented by Dickinson *et al.* reveal that AHPs produce H₂O₂ via Nox2 to regulate intracellular growth signaling pathways, which are essential to maintaining their normal proliferation *in vitro* and *in vivo* (Fig. 1c).

The study by Dickinson *et al.* represents an important advance in chemical tools available for selective imaging of H₂O₂ in living cells. Future synthetic directions include enhancing the photostability of the dyes, extending the utility of trappable H₂O₂ probes to multicolor imaging experiments and devising chemical strategies to improve our ability to visualize endogenous ROS generation with spatial and temporal resolution. Additionally, the findings presented in this report open up several new lines of biological inquiry. Given the recent discovery that AHPs have substantial plasticity⁸, questions arise as to whether Nox-derived ROS also regulate neural cell differentiation in the adult brain and as to the identity of the biomolecular targets of H₂O₂ along the Akt pathway and other signaling cascades. By analogy to phosphorylation, increased H₂O₂ production can lead to

oxidation of specific redox-sensitive cysteine residues within signaling proteins and constitute a facile switch for modulating their function⁴. New chemical reporters of cysteine oxidation have recently enabled selective *in situ* detection of sulfenic acid (–SOH; the direct protein product of cysteine modification by H₂O₂) and improved proteomic analysis of redox-regulated proteins⁹. Lipids may also represent another important target of Nox-generated H₂O₂, as the resulting oxidation products can react with protein nucleophiles¹⁰. With these questions and many others, oxidative biochemistry and its relationship to human health and disease should be fertile scientific ground in the years to come.

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Competing financial interests

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PROTEOMICS

Mapping reactive cysteines

A new quantitative proteomic approach can identify reactive cysteine residues in native proteins and distinguish them on the basis of reactivity. This resource-rich study offers a useful new technology and is a significant step toward understanding the reactivity and functions of cysteines in cells.

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Cysteine is one of the least abundant amino acids in proteins, but it ranks among the most frequently found in protein functional sites¹. Cysteine functions range from metal binding to stabilization of protein structure to enzyme catalysis, and cysteines are also involved in a variety of post-translational modifications and associated regulatory roles. For example, the reversible oxidation of cysteine thiols is important in redox regulation via the formation of intramolecular and mixed disulfides, sulfenic acid intermediates and

overoxidation products^{2,3}. Additionally, cysteine is a target of nitrosative stress, leading to the formation of reversible S-nitrosothiols⁴. The susceptibility of cysteine to these modifications largely depends on the characteristics of each thiol, as exposure and pK_a play a significant role in determining cysteine reactivity (cysteine thiolates are more nucleophilic than their protonated forms)^{1,3}.

In recent years, much effort has been dedicated to the identification of reactive cysteines at a genome-wide level, with

both experimental^{5–7} and computational approaches⁸. Several useful methods identifying redox cysteines have been developed, such as the OxICAT⁷. However, these studies mostly focused on specific subsets of cysteine function (for example, disulfides, sulfenic acid cysteine, catalytic cysteine or redox thiols); they did not address the broader questions of how widespread reactive cysteines are in native proteomes and how cysteine reactivity can be quantified.

Along with the detection of reactive cysteines, desired capabilities for future

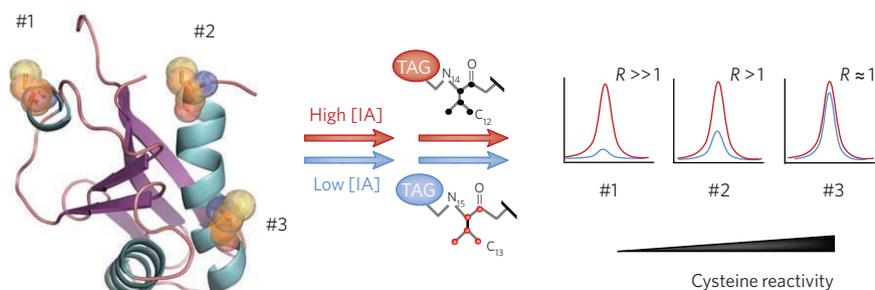


Figure 1 | The isoTOP-ABPP method. A protein with three cysteines (#1, #2, #3) is analyzed. Protein samples are subjected to differential labeling using two isotopic variants of a functionalized-biotin tag. The 'light' tag (red circle) is used to label samples treated with high [IA]. The 'heavy' tag (blue circle) contains isotopic variants of some of its atoms and is used to label samples treated with low [IA]. Tagged proteins are then enriched on a streptavidin affinity column and released by TEV cleavage, and tryptic peptides are analyzed by MS. By comparing the amount of labeling for 'light' (red curves in the diagrams on the right) and 'heavy' (blue curves) peptides, R values for each cysteine are derived. R values close to 1 describe hyper-reactive cysteine (#3), and higher R values describe less reactive cysteine (#1).

applications include the capacity to study native proteins and to quantify the extent of reactivity of different thiols, allowing cysteine reactivities in proteomes to be ranked. A paper by Weerapana *et al.*⁹ describes a new proteomic approach that takes a significant step in this direction. The method isoTOP-ABPP (isotopic tandem orthogonal proteolysis—activity-based protein profiling) relies on (i) the reactivity of cysteine residues in native proteins with electrophilic iodoacetamide probes and (ii) the ability of the probe to conjugate with an azide-functionalized TEV protease-recognition peptide bearing a biotin tag. The tag contains a site for the alternative introduction of either a standard (light) or an isotopically labeled (heavy) valine; this feature in combination with mass spectrometry allows for precise quantification of differentially labeled peptides (**Fig. 1**).

In experiments to validate the method, the authors observed that at low iodoacetamide concentration (low [IA], **Fig. 1**), highly reactive cysteines are promptly labeled, whereas cysteines with low reactivity are not, as they require higher amounts of iodoacetamide (high [IA], **Fig. 1**) or more time. Weerapana *et al.* then identified numerous cysteine residues reacting with the probe and assigned each cysteine a reactivity score, designated the isoTOP-ABPP ratio (R), derived from the

comparison of the labeling under different conditions (**Fig. 1**). For example, paired samples were treated with a series of isotopically labeled iodoacetamide probes, ranging from low [IA]_{light} with low [IA]_{heavy} to high [IA]_{light} with low [IA]_{heavy}, and they quantified $R_{\text{light};\text{heavy}}$ for each cysteine in each combination. Consistently low R values (~ 1) describe highly reactive cysteine, as they quickly react with iodoacetamide and can be labeled even at low [IA] (#3 in **Fig. 1**). In contrast, cysteines with low reactivity show higher R values and concentration-dependent iodoacetamide reactivity (#1 in **Fig. 1**). Therefore, the isoTOP-ABPP method allows the scientist to rank each reactive cysteine in native proteins by means of its R value. Notably, because the readout is a ratio and not an absolute measurement, these scores represent a direct measure of reactivity with the iodoacetamide probe independent of protein abundance.

By manual analyses of their results, the authors validated the R values in scoring reactive (and biologically functional) cysteines in proteomes. Both known catalytic residues (for example, active-site nucleophiles of glutathione-*S*-transferase and acetyl-CoA acetyltransferase) and highly reactive regulatory cysteines were found. Furthermore, the authors could infer functional activities for previously uncharacterized proteins. Lastly, the R values for two hyper-reactive,

non-natural, computer-designed hydrolases establish cysteine nucleophilicity as an important feature for hydrolase design.

The isoTOP-ABPP approach could be applied in many ways, ranging from comparative analysis of cysteine reactivities in different samples to functional prediction of uncharacterized proteins. In this regard, the method is particularly well suited for the detection of highly nucleophilic thiols. This feature should make it a useful tool for the identification of novel catalytic cysteine-containing enzymes.

Additionally, the direct quantification of cysteine reactivity via R scores offers a range of future applications in computational analyses of thiol reactivity and regulation. For example, an immediate application could be to observe how R scores correlate with theoretical values for cysteine pK_a , derived from different methods. A major point here would be to establish the features responsible for chemical activation of reactive cysteine residues, as well as to explore the link between high reactivity and specific microenvironmental features.

Finally, the output of isoTOP-ABPP can be easily digitalized (via protein IDs, cysteine number, R values), thus providing a valuable source of information that could be used to improve existing theoretical approaches for prediction of reactive cysteines¹⁰; indeed, this application could significantly benefit the development of effective computational tools for *in silico* prediction of different types of reactive cysteine residues. ■

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