Letter

# Chemical Bias in Cysteine-Reactive Probe Profiling: In Vivo Perfusion versus Postmortem Lysis and Cells

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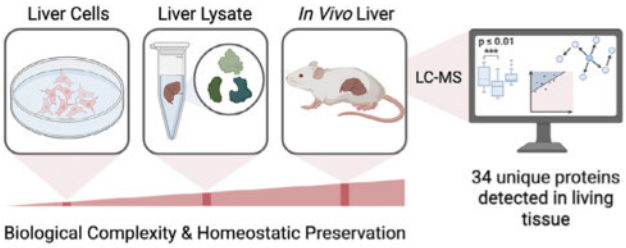
## GRAPHICAL ABSTRACT

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## ABSTRACT

Chemoproteomic probes are typically screened in postmortem lysates or cell cultures, assuming these models accurately reflect native protein reactivity. However, the impact of the reaction medium, intact tissues versus homogenate, remains poorly defined. Here we utilize iodoacetamide-alkyne to benchmark probe performance across three distinct reaction environments, in vivo perfusion, postmortem lysate, and live cell culture. Contrary to the assumption that lysate offers comprehensive access, we show that homogenization systematically suppresses the labeling of ATP-dependent ligases and integral membrane proteins, likely due to the rapid collapse of active-site energetics and membrane depolarization. Postmortem lysate showed greater nuclear access and enrichment of oxidoreductase/stress-response signatures, consistent with processing effects, whereas the cell model overlapped least with the tissue and displayed culture-specific pathway biases. Furthermore, we address the confounding variable of probe distribution by demonstrating that perfusion achieves equivalent saturation to lysate dosing, confirming that observed differences stems from native-state reactivity rather than accessibility. These findings provide a critical correction factor for synthetic chemists: probes validated solely in lysate may suffer from predictable false negatives against metabolically active targets.

**Keywords** Chemoproteomics, Iodoacetamide-alkyne, In vivo perfusion, Tissue lysate, Chemical bias

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## Introduction

Proteomics, the large-scale study of proteins and their functions, has revolutionized our understanding of biological systems by revealing insights into cellular processes, disease mechanisms, and therapeutic targets that go beyond what genomic information alone can provide.[1] Unlike the relatively static genome, the proteome is dynamic and context-dependent, reflecting the functional state of cells and tissues in real time. Modern mass spectrometry workflows now enable sensitive biomarker discovery and patient stratification, supporting early detection, prognosis, and therapy response.[2,3] They can dissect disease phenotypes at unprecedented resolution.[4]

Tissue proteomics plays a central role in biomedical research by enabling comprehensive characterization of protein expression patterns across disease states. This approach is particularly valuable in cancer research, where protein profiles often provide more direct insights into tumor pathophysiology than genomic data alone.[5] However, practical constraints in sample collection including how and when samples are obtained limit access to truly native biological material.[6]

The majority of proteomic studies analyze postmortem, flash-frozen tissue samples, which preserve much of the proteome but may fail to capture proteins that depend on active circulation, intact cellular architecture, and ongoing metabolic processes. Recent investigations have demonstrated significant, tissue-specific proteomic alterations in postmortem samples, including rapid changes in protein abundance and degradation that vary according to subcellular compartment and postmortem interval.[7]

As an alternative approach, some studies employ specific probes that mimic cellular metabolites to monitor probe incorporation, particularly as posttranslational modifications.[8] Similar methodologies have been applied to identify protein targets of pharmaceuticals and drugs for monitoring off-target effects.[9] However, these probe-based approaches are heavily dependent on enzymatic activity and metabolic incorporation and lack the comprehensive coverage necessary for global proteomic profiling, since they only target specific protein subsets.[8,9]

Despite these insights, comprehensive proteomic profiling of intact, living tissues remains largely unexplored. Herein, we evaluate how dosing context shapes the chemoproteomic landscape across live organs, postmortem tissue, and cultured cells. Using iodoacetamide alkyne (IAA), a standard cysteine-reactive probe[10], we performed matched labeling of mouse livers by cardiac perfusion, postmortem lysate, and live BNL CL.2 cells, then applied an identical LC–MS/MS pipeline. Our goal is to define when postmortem analysis suffices, when in vivo labeling adds unique value, and to provide practical guidance for choosing the right context for chemoproteomic studies.

## Results and Discussion

We initially evaluated two methods for in vivo proteomic labeling in mice: tail injection and perfusion. Tail injection would provide the most physiologically relevant dosing, as it is not a terminal process, whereas perfusion results in sacrifice during the dosing process. As a pilot study, we utilized a tail vein injection to introduce IAA to a live mouse (100 µL, 1 mM in saline with 2% DMSO) versus cardiac perfusion (30 mL, 1 mM in saline with 2% DMSO).

After a 10-minute IAA exposure, mice were euthanized, and livers were rapidly excised and snap-frozen. In parallel, a control mouse was perfused with PBS and liver was processed identically. The liver tissue was mechanically homogenized in RIPA buffer, total protein content was quantified by Bradford assay, and unlabeled lysate was then dosed ex vivo with 1 mM IAA for 10 min (**Fig. 1A**).

To visualize protein labeling, all samples, including a negative control without IAA labeling, were subjected to copper-catalyzed click chemistry with Cy5-azide and analyzed by SDS-PAGE (**Fig. 1B**). In-gel fluorescence showed much stronger protein labeling after cardiac perfusion than after tail-vein injection, consistent with higher effective probe delivery during perfusion and probable reactivity of probe with serum proteins during tail dosing. Notably, perfused liver and ex vivo lysate dosing produced comparable overall fluorescence, supporting the use of perfusion for in vivo experiments and ex vivo lysate dosing as a practical benchmark.

A critical consideration in comparing in vivo and ex vivo labeling is distinguishing between biological availability and probe distribution kinetics. It could be argued that the unique signatures

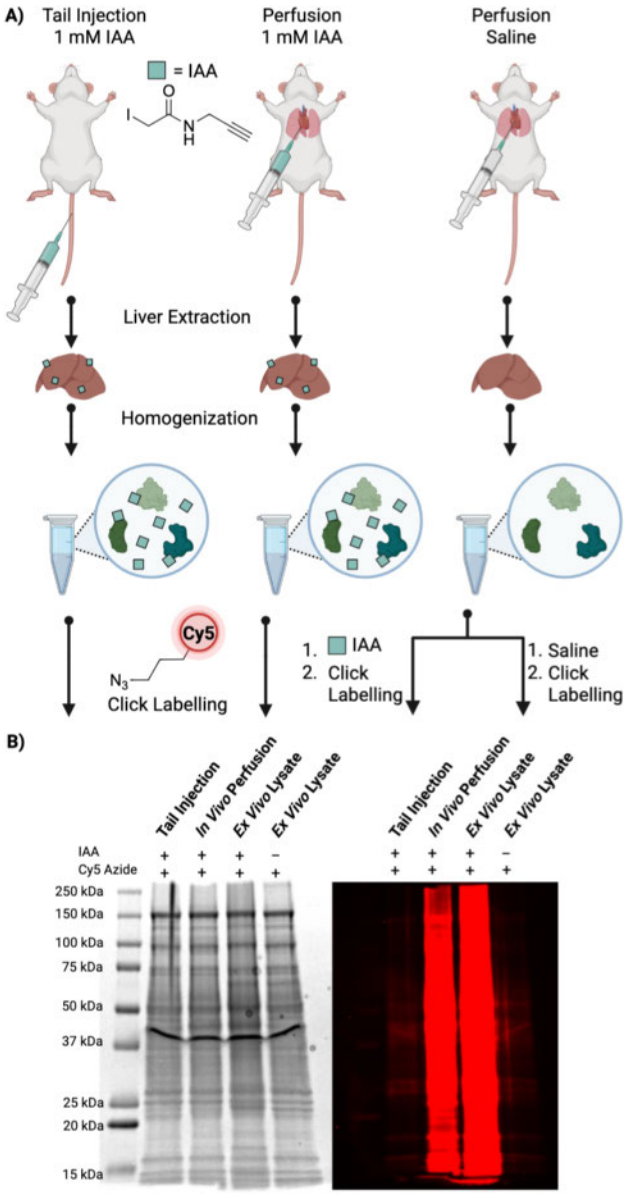


**Fig. 1** Exploration of in vivo proteomic labeling protocols. (**A**) Schematic of three proteomic labeling protocols with 10 mM iodoacetamide alkyne (IAA) in saline with 2% DMSO for 10 min. Tail injection and in vivo perfusion mice were treated with IAA in vivo while ex vivo lysate was labeled after tissue homogenization and cell lysis. (**B**) Coomassie and fluorescence (Cy5) gel of mouse liver lysate from each dosing condition (tail injection, in vivo perfusion, ex vivo lysate) and control lysate without IAA labeling. Created in BioRender. *Raj, M. (2025) https://BioRender.com/tmf4m7i [rerif]*

observed in perfusion are simply due to transport differences. However, our fluorescence benchmarking (**Fig. 1B**) demonstrates that perfusion dosing yields a global protein labeling intensity comparable to, and in some molecular weight ranges exceeding, that of ex vivo lysate mixing. This confirms that the perfusion protocol effectively overcomes the diffusion limits often associated with immersion or tail-vein injection.

With this preliminary study, we proceeded with the cardiac perfusion dosing ($n$ = 3 mice) along with matched PBS perfused

controls and ex vivo lysate dosing ($n$ = 3 mice). We also labeled live BNL CL.2 mouse liver cells with 1 mM IAA for 10 min before lysis ($n$ = 3). BNL CL.2 cell line is an embryonic liver cell line derived from the BALB/c mouse, offering a promising cellular comparison (**Fig. 2A**).

After removing proteins found multiple times in the same sample, and removing proteins not found in ≥2 replicates, we identified 56 proteins in perfused in vivo labeled liver, 365 proteins in postmortem ex vivo labeled lysate, and 882 proteins in BNL CL.2 cells. These sets were used for gene ontology (GO) enrichment analysis[11] and subsequent visualization (Supplementary Fig. 1).[12]

GO cellular component (CC) analysis: in vivo perfusion and ex vivo-lysate datasets showed very similar coverage, with comparable enrichment of organelle-membrane and envelope proteins, indicating that both dosing formats capture cellular compartmentalization despite different preparation methods. By contrast, in vivo labeling strongly enriched proteins associated with extracellular vesicles. BNL CL.2 cells shared several pathways (e.g., myelin sheath, organelle-membrane-associated, and cytosolic proteins) but were uniquely enriched for lumen-associated proteins, consistent with the limited higher-order architecture of monolayer cultures relative to organs (**Fig. 2B**, Supplementary Fig. 1).[13]

GO molecular function (MF) analysis: in vivo perfusion and ex vivo lysate were both enriched for broad catalytic and binding activities. Although notably, translation activity and ligase activity, was selectively enriched in in vivo perfusion samples

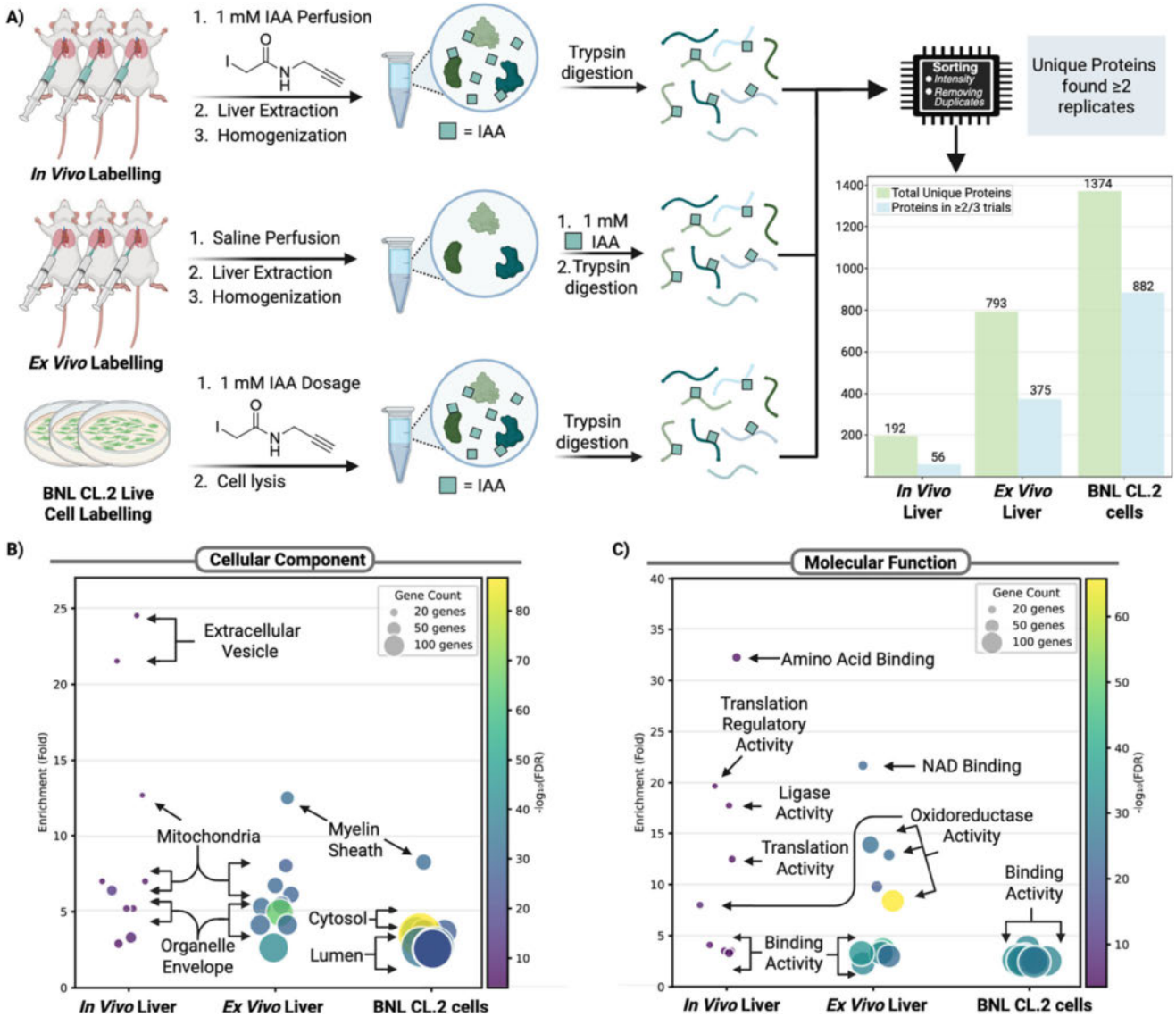


**Fig. 2** Labeling and proteomic analysis of abundant proteins found in ≥2 trials of each dosing cohort. (**A**) Schematic of mouse perfusion in vivo IAA dosing, ex vivo lysate IAA dosing, and live BNL CL.2 cell IAA dosing and subsequent proteomic analysis revealing protein labeling. All IAA dosing was 1 mM in saline with 2% DMSO for 10 min. Proteins found in ≥2 samples were retained for further analysis. (**B**) Cellular compartment pathway analysis of the three dosing conditions. (**C**) Molecular function analysis of the three dosing conditions. *Created in BioRender. Raj, M. (2025) https://BioRender.com/nrwugup. [rerif]*

(Fig. 2C, Supplementary Fig. 1). This pattern is consistent with preserved ATP and cellular architecture in vivo, whereas postmortem lysates suffer rapid loss of ligases due to metabolic collapse to preserve energy and eventual proteolysis.[14,15] The loss of specific protein families (e.g., ligases) in the lysate condition is not an artifact of low probe accessibility, but rather a result of the chemical collapse of the proteome, specifically the loss of ATP-dependent reactive conformations that occurs immediately upon tissue homogenization.

The prominence of ligases and associated translation proteins in the in vivo perfusion labeling samples highlights the value of live tissue dosing for capturing dynamic, ATP-dependent enzymes. However, the strong pathway overlap between perfusion and ex vivo lysate suggests that postmortem lysis remains effective for many stable protein families, while the distinct enrichment in BNL CL.2 cells underscores that cell lines do not fully recapitulate tissue biology. Together, these results guide sample-format selection by balancing biological fidelity with practicality.

To more comprehensively evaluate the proteomic landscape across the different experimental approaches, we conducted a systematic comparison of the similar and unique proteins identified within each cohort (Fig. 3A). Our analysis revealed a substantial degree of protein overlap across all three experimental cohorts.

Across the total of 1829 proteins, 87 were detected in all three cohorts (in vivo liver labeling, ex vivo liver labeling, and live BNL CL.2 liver cells), representing a conserved hepatic core (Fig. 3A). The majority of proteins found in the in vivo perfusion labeling were also found in the ex vivo labeling (141/192, ~73.4%) as shown in the containment heat map (Fig. 3B) whereas the live cells contained a smaller portion (104/192, ~54.2%). This tissue–tissue proximity confirms that organ-level formats preserve more physiologic protein expression than immortalized cells.[16] The reduced overlap likely reflects the loss of tissue-specific architecture, cell–cell interactions, and the artificial nature of immortalized cell culture conditions, which collectively contribute to altered protein expression profiles that may not accurately represent in vivo hepatic biology.

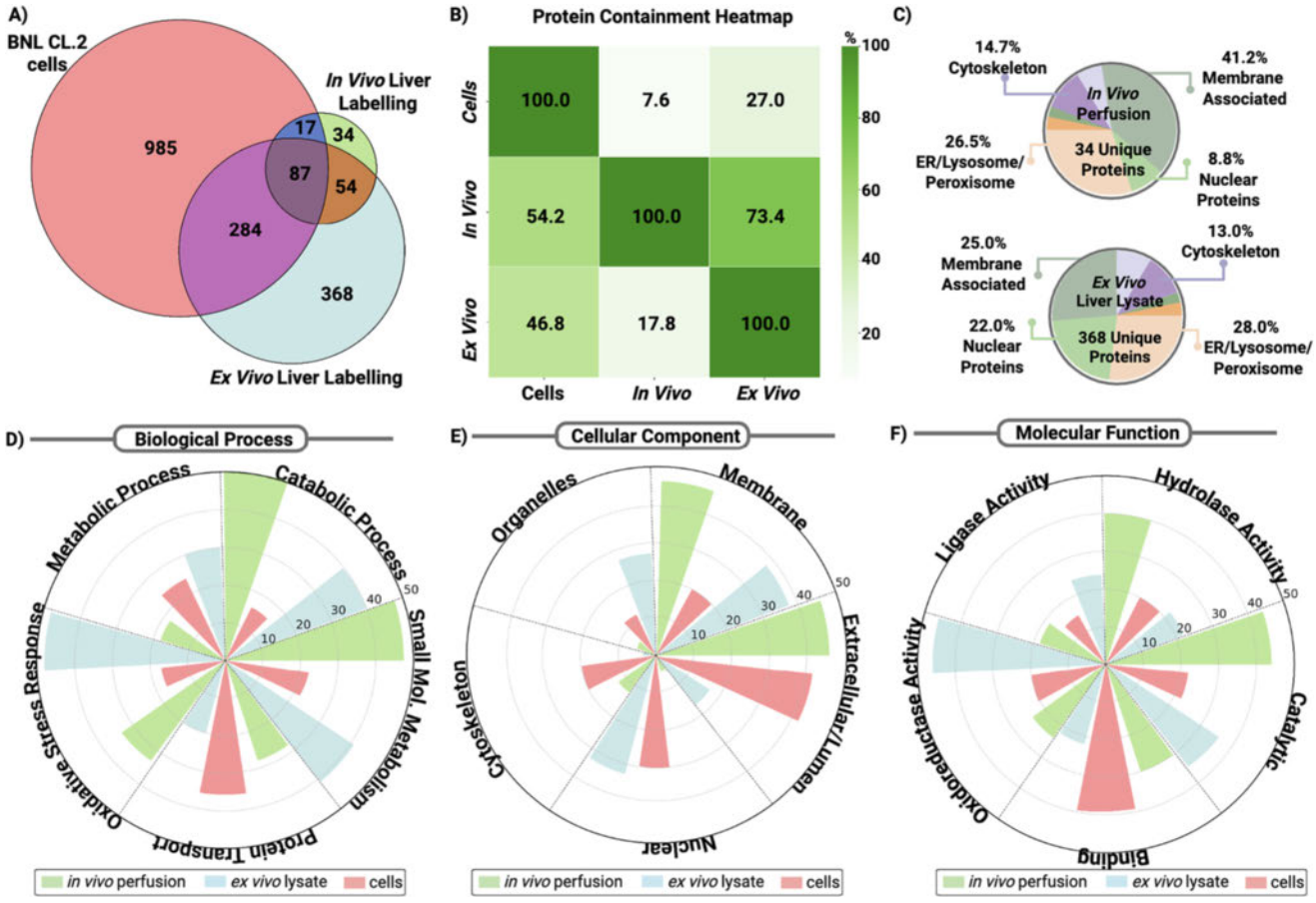


**Fig. 3** Analysis of unique proteins found in liver after in vivo perfusion of IAA, ex vivo IAA labeling of liver lysate, or live BNL CL.2 cell labeling with IAA. (**A**) Venn diagram of proteins found in each sample cohort (in vivo liver, ex vivo liver, or live cells) identifying overlapped and unique proteins. (**B**) Protein containment heat map. Values represent the percentage of proteins from the sample in each row that were found in the other sample in the column. (**C**) Unique proteins identified in the in vivo liver dosing cohort overrepresent membrane, organelle, and cytoskeleton cellular components compared to the ex vivo liver lysate dosing cohort. (**D–F**) GO biological process, cellular component, and molecular function pathway analysis of the three dosing conditions. The radial axis represents the fold enrichment (FDR) of proteins found in these categories. Created in BioRender. *Raj, M. (2025) https://BioRender.com/qfwssp2. [rerif]*

A detailed examination of the unique proteins identified in each experimental approach revealed striking differences in cellular compartmentalization patterns. Unique proteins labeled in the in vivo perfusion ($n$ = 34) were enriched for membrane (41.2%), intracellular organelle (ER/lysosome/peroxisome; 26.5%), and cytoskeleton (14.7%) components, consistent with intact architecture and complexes at the time of labeling (**Fig. 3C**).

In unique proteins found in ex vivo liver labeling ($n$ = 368 proteins), the fraction of membrane proteins (25.0%) was lower, indicating some degree of structural protein degradation or loss during the tissue processing and lysis procedures. This distribution pattern suggests that the perfusion methodology maintains cellular architecture and organelle integrity more effectively than postmortem tissue processing.

A particularly notable difference was observed in nuclear protein representation. The number of nuclear proteins increased (22.0% in ex vivo liver labeling vs. 8.8% in in vivo labeling, **Fig. 3C**). This discrepancy may reflect differential protein extraction efficiency between the two methodologies, with the lysate approach potentially providing enhanced access to nuclear compartments due to the tissue homogenization and cellular lysis that occurred before IAA labeling.

To gain deeper insight into the functional implications of the observed proteomic differences, we performed comprehensive GO pathway analyses examining biological processes, cellular compartments (CCs), and molecular functions (MFs) associated with unique proteins across experimental conditions.

Analysis of biological and metabolic process signatures revealed distinct physiological states across experimental models. In vivo labeled-unique proteins were strongly enriched in catabolic processes, suggesting that the perfusion methodology captures hepatocytes in a more physiologically representative state with maintained cellular machinery for normal metabolic turnover and energy production. In contrast, ex vivo labeled unique proteins were enriched in oxidoreductase activity and pathways related to eicosanoid, fatty-acid, and small-molecule metabolism, together with proteolysis signatures expected from early ischemia/hypoxia (**Fig. 3D**, Supplementary Fig. 2).[17]

Examination of cellular architecture and compartmentalization through subcellular localization analysis revealed architecture-dependent protein signatures. In vivo labeled-unique proteins mapped to extracellular components including endocytic vesicles and the endoplasmic reticulum, consistent with intact tissue architecture and preserved metabolism at the moment of labeling (**Figs. 2B** and **3E**). Conversely, unique proteins found in the BNL CL.2 samples showed over-representation of lumen-associated components characteristic of 2D monolayers that lack native hepatic architecture (e.g., organized bile canaliculi). This architectural simplification represents one of the key challenges inherent in using monocellular proteomic models for studying complex organ biology (**Fig. 3E**, Supplementary Fig. 2).[13] In contrast, ex vivo lysate labeling lacked these regulatory components, likely because the homogenization process during cell lysis disrupts normal protein organization and accessibility patterns. This disruption appears to promote labeling of proteins that would normally be less accessible in intact liver, as evidenced by the increased capture of nuclear proteins (**Fig. 3E**, Supplementary Fig. 2).

MF analysis confirmed the experimental validity of different approaches through enzymatic activity profiles that reinforced the physiological relevance of each method. The in vivo labeling model demonstrated significant enrichment of proteins with hydrolase activity, supporting maintained healthy, metabolically active cellular states. Hydrolase enzymes are essential for protein degradation, lipid metabolism, and carbohydrate processing, and their robust representation suggests intact and functional cellular enzymatic machinery. The ex vivo labeling model showed major overrepresentation of oxidoreduction proteins, indicating cellular attempts to counteract hypoxic conditions developing during sacrifice and liver isolation. This enrichment reflects tissue adaptive responses to experimental procedural stress (**Fig. 3F**, Supplementary Fig. 2).[18]

While it is intuitive that living and dead tissues differ, this study quantifies the specific chemoproteomic blind spots introduced by standard lysate screening. Specifically, we identified that ex vivo lysis serves as a negative selection filter for ATP-dependent ligases and translation machinery. In a synthetic context, this implies that electrophilic probes designed to target these enzyme classes will appear inactive if screened in standard lysates, not due to poor molecular design, but due to the absence of the requisite co-factors and active-site geometry in the homogenate. Conversely, lysate screening artificially amplifies oxidoreductase signatures, potentially skewing target identification toward stress-response proteins. Thus, we propose that in vivo perfusion is not merely a “better” model, but a chemically distinct reaction condition required to validate probes against energy-dependent enzymatic cysteines.

These findings have important implications for the design and interpretation of proteomics experiments, particularly those aimed at understanding normal physiology or evaluating therapeutic interventions. Researchers should carefully consider the potential impact of their chosen methodology on the resulting proteomic data, with particular attention to how sample preparation procedures might introduce artifacts that could obscure biologically relevant findings or lead to misinterpretation of results.

## Conclusion

In summary, we demonstrate that the choice of labeling environment introduces a deterministic chemical bias into chemoproteomic datasets. For synthetic chemists developing novel electrophiles, reliance on lysate screening risks overlooking targets that require membrane integrity or ATP flux for nucleophilic competence. We therefore recommend that in vivo perfusion be adopted not just for biological validation, but as a necessary chemical benchmarking step for probes targeting metabolic or membrane-associated cysteine nucleophiles. These steps will sharpen interpretation, reduce artifacts, and increase the translational value of chemoproteomic studies.

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## Statements and Additional Information

**Conflict of Interest** The authors declare that they have no conflict of interest.

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**Contributors' Statement** J.M.T.: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. T.B.V.: Formal analysis, Methodology, Writing – review & editing. M.R.: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – review & editing. J.M.T. and M.R. designed the study. J.M.T. carried out all experiments. T.B.V. performed proteomic data analysis. J.M.T., T.B.V., and M.R. edited the manuscript.

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