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An Integrated, High-Throughput Strategy for Multiomic Systems Level Analysis

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Abstract

Proteomics, metabolomics, and transcriptomics generate comprehensive data sets, and current biocomputational capabilities allow their efficient integration for systems biology analysis. Published multiomics studies cover methodological advances as well as applications to biological questions. However, few studies have focused on the development of a high-throughput, unified sample preparation approach to complement high-throughput omic analytics. This report details the automation, benchmarking, and application of a strategy for transcriptomic, proteomic, and

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ASSOCIATED CONTENT

Supporting Information

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metabolomic analyses from a common sample. The approach, sample preparation for multi-omics technologies (SPOT), provides equivalent performance to typical individual omic preparation methods but greatly enhances throughput and minimizes the resources required for multiomic experiments. SPOT was applied to a multiomics time course experiment for zinc-treated HL-60 cells. The data reveal Zn effects on NRF2 antioxidant and NFkappaB signaling. High-throughput approaches such as these are critical for the acquisition of temporally resolved, multicondition, large multiomic data sets such as those necessary to assess complex clinical and biological concerns. Ultimately, this type of approach will provide an expanded understanding of challenging scientific questions across many fields.

Graphical Abstract

Keywords

mechanism of action; metabolomics; multiomics; proteomics; sample preparation; systems biology; transcriptomics; zinc

INTRODUCTION

Modern technologies for analyzing the molecular components of cells, including metabolites, proteins, and RNA, are now able to generate comprehensive data sets useful for understanding complex cellular processes.^{1–5} Recent increases in both the speed and sensitivity of these analytical approaches not only enable the identification of important biomolecules but can also illuminate important dynamic changes in molecular expression that may result from exposure to outside stimuli like drugs and toxins.⁶ Advances in biocomputational tools now make it feasible to analyze large data sets such as these in a time-efficient manner. Through the application of a suite of omics technologies to the study of a single biological system, one can examine the way in which complex cellular processes work together across all molecular domains (e.g., metabolite/lipid, protein, and gene) to, for example, mitigate the effects of an environmental stimulus or respond to a therapeutic intervention. In recent years, numerous multiomics studies have been published across a wide range of fields, attesting to the power and utility of such a unified approach. For example, multiomics strategies are utilized in microbiology,^{7,8} in microbial ecology to better understand intra- and intercommunity heterogeneity,^{9–11} and in the field of plant biology.^{12–15} Multiomics strategies have also been established for clinical purposes¹⁶ to improve drug development⁶ and to investigate drug toxicities.^{17,18}

The utilization of advanced omics technologies has been largely a specialized endeavor; thus, relatively little work has been done to optimize the methods for sample procurement and processing in a way that is compatible across platforms. Many published methodologies for preparing samples for proteomic analysis by mass spectrometry are entirely incompatible with the analysis of metabolites or RNA from the same sample due to the presence of specific buffer components or detergents. To proceed with metabolomics analysis and/or RNA sequencing from such a sample would require additional biological experiments. This can be costly to the laboratory, in terms of time and money, and may introduce error because there may exist batch-to-batch variations in cell cultures that must be considered in the final analysis.^{19–22} Furthermore, if the biological phenomenon under investigation requires the examination of two time points in close succession, aligning observations from multiple analytical approaches in time may be challenging or impossible if the analysis cannot be accomplished from a single batch of cells. It is also important to consider that biologically meaningful measurements within each modality (e.g., transcriptomics, proteomics, and phosphoproteomics) may occur on different time scales within the cell.²¹ Therefore, the acquisition of data across a comprehensive time-scale (seconds to days) is ideal,²¹ although this may be impractical if multiple samples per omics modality are required. For moving toward the acquisition of systems-level data sets, where reliable insights can be drawn among the metabolome, proteome, and genome, it is ideal to measure these molecular components from a single preparation of cells or tissue.

In recent years, there have been some notable efforts to develop multiomics approaches that incorporate an optimized unified sample preparation approach for biomedical samples. A pair of recent publications from different groups demonstrate the unified analysis of lipids, metabolites, and proteins.^{19,20} However, these strategies incorporate several manual steps that are not easily amenable to automation, making large-scale analyses impractical. Thus, there remains a significant gap between the efficiency of high-throughput omic analysis strategies and current sample preparation approaches. Establishing a robust, unified, and efficient sample preparation strategy for multiomics studies will reduce the time and cost of sample preparation/data generation and will facilitate the adoption of multiomic technologies into new areas of application.

This study presents a simple high-throughput process that has been optimized to provide high quality specimens for metabolomics, proteomics, and transcriptomics from a common cell culture sample. The protocols that are presented were designed to be performed manually or using laboratory automation. Furthermore, we demonstrate that this approach can be accomplished efficiently: 16–24 samples can be processed from a cell pellet to a desalted sample ready for mass spectrometry analysis within 9 h. Furthermore, this automated workflow is compatible with 96-well plate throughput if sample limitations are overcome.

EXPERIMENTAL PROCEDURES

Cell Culture

Human acute promyelocytic leukemia HL-60 cells and human lung carcinoma A549 cells were obtained from ATCC (Manassas, VA). The HL-60 cells were cultured in Isocove's

modified Dulbecco's medium (IMDM, Gibco) supplemented with 10% v/v heat-inactivated fetal bovine serum (Atlanta Biologicals) at 37 °C with 5% CO₂ atmosphere and treated with 225 μ M Zn or deionized water. The A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% v/v heat-inactivated fetal bovine serum (Atlanta Biologicals) at 37 °C with 5% CO₂ atmosphere and treated with either 10 pM of the *Clostridium difficile* toxin TcdB²³ or 20 mM Hepes/50 mM NaCl buffer.

Unified Sample Preparation

An aliquot of approximately one million cells was removed from samples for RNA analysis (1 and 6 h time points only), and the remaining cells were kept for proteomic and transcriptomic analysis. RNA was isolated using the RNeasy Mini Kit (Qiagen). For each time point, untreated and zinc- treated samples were isolated in triplicate and analyzed by the Genomics Services Lab at HudsonAlpha. RNA-seq was performed using poly(A) selection on an Illumina HiSeq v4 sequencing platform. Reads were paired-end with a read length of 50 bp and 20 million reads per sample.

The remaining HL-60 cells (approximately one million) were lysed in 100 μ L of 1:1:2 CH₃CN:CH₃OH:NH₄HCO₃ (50 mM, pH 8.0) followed by one freeze–thaw cycle (3 min each) and a 10 min sonication in an ice bath. Protein concentration was obtained using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). An aliquot of 100 or 150 μ g of each sample in a total of 100 μ L of lysis buffer was precipitated with 300 μ L of ice cold 75:25 CH₃COCH₃:CH₃OH for 2 h at –80 °C. Samples were spun for 15 min at 6,800g, and the supernatant was removed and utilized for metabolomics analysis. The pellets were rinsed with 300 μ L of ice cold acetone and spun as above. Acetone was removed, and the pellet was allowed to dry briefly. Metabolite supernatants were dried and reconstituted in 50 μ L of appropriate reverse-phase liquid chromatography (0.1% formic acid in 98:2 H₂O:CH₃CN) or hydrophilic interaction chromatography (80:20 CH₃CN: H₂O) compatible buffers prior to analyses.

Individual Omic Modality Preparations

Proteomics.—A549 cells grown on indium tin oxide-coated glass slides were lysed in 50 mM Tris pH 8, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, HALT Protease Inhibitor Cocktail. Lysis buffer (300 μ L) was added to the slide on ice and allowed to sit for 5 min. Cells were harvested by scraping, transferred to cold tubes, and then sonicated in an icy slurry for 10 min. HL-60 cell pellets were lysed in 100 μ L of 50 mM Tris pH 8, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, HALT Protease Inhibitor Cocktail and then vortexed for 30 s. Samples were centrifuged at a maximum speed of 25,830g, and the supernatant was retained for experiments. Samples were assayed for protein concentration using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). An aliquot of 100 or 150 μ g of each sample in a total of 100 μ L of lysis buffer was precipitated with 300 μ L of ice cold 75:25 CH₃COCH₃:CH₃OH for 2 h at –80 °C. Samples were spun for 15 min at 6,800g, and the supernatant was removed. The pellets were rinsed with 300 μ L of ice cold acetone and spun as above. Acetone was removed, and the pellet was allowed to dry briefly.

Metabolomics.—For the traditional-based metabolomics approach, A549 cells grown on slides were lysed in 500 μL 1:1:2 $\text{CH}_3\text{CN}:\text{CH}_3\text{OH}:\text{NH}_4\text{HCO}_3$ (50 mM, pH 8.0). Lysis buffer was added to the slide on ice and allowed to sit for 5 min. Cells were harvested by scraping, transferred to cold tubes, and then frozen in a dry ice with ethanol slurry for 3 min. Samples were defrosted over ice over a 10 min period. Following one freeze—thaw cycle, samples were sonicated individually (three times) using a probe tip sonicator. Samples were assayed for protein concentration using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). One hundred microgram protein aliquots from each sample in a total of 200 μL of lysis buffer were precipitated with 600 of ice-cold methanol overnight at -80°C . Samples were spun for 15 min at 6,800g at 4°C , and the supernatant was removed and dried in vacuo. Prior to analyses, supernatants were dried and reconstituted in 50 μL of appropriate reverse-phase liquid chromatography (0.1% formic acid in 98:2 $\text{H}_2\text{O}:\text{CH}_3\text{CN}$) or hydrophilic interaction chromatography (80:20 $\text{CH}_3\text{CN}:\text{H}_2\text{O}$) compatible buffers.

Transcriptomics.—Samples for transcriptomics were only collected from samples prepared by the unified sample preparation.

Tryptic Digestion for Proteomic Samples

Tryptic digestion and desalting were automated using the Agilent AssayMAP Bravo (with the exception of the 1 h time point, which was digested manually). A protein pellet of 100 μg was resuspended in 10 μL of neat trifluoroethanol (TFE) and 10 μL of 100 mM Tris (pH 8.0) and then shaken at 2,000 rpm using the AssayMAP Bravo T-shake for 2 min. The AssayMAP Bravo In-solution Digestion Single Plate v 1.0 Protocol was followed for digestion. Samples were reduced with 5 μL of 100 mM tris(2-carboxyethyl)phosphine (TCEP) at room temperature for 30 min and alkylated with 5 μL of 200 mM of iodoacetamide in the dark at room temperature for 30 min. For digestion, 65 μL of Rapid Trypsin Digestion Buffer (Promega) was added to each sample followed by 5 μL of Rapid-Digestion Trypsin/Lys-C (Promega) at 0.4 $\mu\text{g}/\mu\text{L}$ for an enzyme/protein ratio of 1:50. The samples were incubated at 70°C for 30 min, and then digestion was stopped by adding 5 μL of 60% HCOOH to each sample. Samples were desalted using the AssayMAP Bravo Peptide Cleanup v 2.0 protocol. C18 cartridges were primed with 100% CH_3CN , 0.1% CF_3COOH and then equilibrated with 100% H_2O , 0.1% CF_3COOH . A 15 μg aliquot of sample was loaded onto a cartridge, washed with 100% H_2O , 0.1% CF_3COOH , and eluted in 70% $\text{CH}_3\text{CN}:\text{H}_2\text{O}:\text{HCOOH}$ to equal a concentration of 1 $\mu\text{g}/1 \mu\text{L}$. Desalted samples were dried via vacuum centrifugation and stored at -80°C . Prior to mass spectrometry analysis, samples were reconstituted in 15 μL of 0.1% HCOOH .

Proteomic Data Acquisition and Analysis

Label-free proteomic samples were analyzed on a Thermo Scientific Orbitrap Fusion Tribrid mass spectrometer in line with a Thermo Scientific Easy-nLC 1000 UHPLC system. Samples (2 μL) were injected via the autosampler and loaded onto a pulled-tip C_{18} UHPLC column (75 $\mu\text{m} \times 450 \text{ mm}$) packed with Phenomenex Jupiter resin (3 μm particle size, 300 Å pore size) with 0.1% HCOOH in H_2O (mobile phase A). Peptides were separated over a 130 min two-step gradient with initial conditions set to 100% mobile phase A for 2 min before ramping to 20% mobile phase B, 0.1% HCOOH in CH_3CN , over 100 min and then 32%

mobile phase B over 20 min. The remainder of the gradient was spent washing at 95% mobile phase B and returning to initial conditions. Eluted peptides were ionized via positive mode nanoelectrospray ionization (nESI) using a Nanospray Flex ion source (Thermo Fisher Scientific). The mass spectrometer was operated using a top 17 data-dependent acquisition mode. Fourier-transform mass spectra (FTMS) were collected using 120,000 resolving power, an automated gain control (AGC) target of $1e^6$, and a maximum injection time of 100 ms over the mass range of 400–1600 m/z . Precursor ions were filtered using mono-isotopic precursor selection of peptide ions with charge states ranging from 2 to 6. Previously interrogated precursor ions were excluded using a 30 s dynamic window (± 10 ppm). Precursor ions for tandem mass spectrometry (MS/MS) analysis were isolated using a 2 m/z quadrupole mass filter window and then fragmented in the ion-routing multipole via higher energy dissociation (HCD) using a normalized collision energy of 35%. Ion trap fragmentation spectra were acquired using an AGC target of 10,000 and maximum injection time of 35 ms, and 120 m/z was set for the first scan mass to enable detection of the lysine residue fragmented ion. Data were analyzed against the UniProt human database via Proteomizer (Vulcan Analytical, Inc.) to identify proteins and determine a fold change in proteins common to the treated and control samples. Search parameters were set to include carbamido- methyl, phosphorylation, and oxidation modifications as well as methionine-containing and miscleaved peptides (maximum of two miscleavages). Both peptide and protein target FDR rates were set to 1%. For the Orbitrap-LTQ, data precursor tolerance was set to 6 ppm. Changes in protein abundance were considered statistically significant at a value of greater than 1.5 or less than -1.5 and a p -value of 0.1 .

Metabolomic Acquisition and Analyses

Dried extracts were collected from the unified approach, SPOT, and reconstituted in 50 μL of appropriate reverse-phase liquid chromatography (0.1% formic acid in 98:2 $\text{H}_2\text{O}:\text{CH}_3\text{CN}$) or hydrophilic interaction chromatography (80:20 $\text{CH}_3\text{CN}:\text{H}_2\text{O}$) compatible buffers prior to analyses. Quality control samples were prepared by pooling equal volumes from each experimental sample. Global untargeted analyses were performed on a Waters Synapt G2 (Waters Corporation, Manchester, UK) traveling wave ion mobility- mass spectrometer equipped with a Waters NanoAcquity UPLC system. Analyses were performed in positive mode with simultaneous analysis of molecular fragmentation (MS^e) with a scan time of 0.5 s and a ramp transfer collision energy of 10–50 V. For RPLC analyses, extracts (5 μL injected volume) were separated on an HSS C18 precolumn (1.8 μm , 2.1 mm $\times$ 5 mm) followed by an HSS T3 column (1.8 μm , 1 mm $\times$ 100 mm column; Waters, Milford, MA, USA) held at 45 $^\circ\text{C}$. Reverse-phase liquid chromatography was performed across a 30 min gradient at 75 $\mu\text{L min}^{-1}$ using 0.1% HCOOH in H_2O (mobile phase A) and 0.1% HCOOH in CH_3CN (mobile phase B) (see Supporting Information for chromatography details). For HILIC analyses, extracts (5 μL injected volume) were separated on a Kinetix HILIC column (1.7 μm , 2.1 mm $\times$ 100 mm column; Phenomenex, Torrance, CA, USA) held at 40 $^\circ\text{C}$. HILIC chromatography was performed across a 40 min gradient at 250 $\mu\text{L min}^{-1}$ using 90:10 $\text{H}_2\text{O}:\text{CH}_3\text{CN}$ 10 mM HCO_2NH_4 (pH 6.9) (mobile phase A) and 10:90 $\text{H}_2\text{O}:\text{CH}_3\text{CN}$, 10 mM HCO_2NH_4 (pH 6.9) (mobile phase B). Chromatographic ramps and other instrumental parameters can be found in the Supporting Information.

UPLC-MS/MS raw data were imported, processed, normalized, and reviewed using Progenesis QI v.2.1 (Non-linear Dynamics, Newcastle, UK). All sample runs were aligned against a QC pool reference run, and peak picking was performed on individual aligned runs to create an aggregate data set. Features (retention time and m/z pairs) were grouped using both adduct and isotope deconvolution to generate unique compounds (retention time and m/z pairs) representative of metabolites. Data were normalized to all compounds. Pair-wise comparisons were used to assess significance between groups and filtered on the basis of significance ($p < 0.1$, fold change $> |2|$). Tentative and putative annotations were determined using accurate mass measurements (<10 ppm error), isotope distribution similarity, and fragmentation spectrum matching (when applicable, <20 ppm) of the Human Metabolome Database (HMDB), METLIN, Mass- Bank, NIST, and an internal curated library.

Computational Analysis and Data Mining

Metadata from transcriptomics, metabolomics, and proteomics were uploaded into a central in-house database and organized by identifiers such as project, analysis type, and experimental information (e.g., exposure time and treatment). Data from each experiment were arranged into an exportable file that included, for each analyte, measured fold changes between treated and control samples as well as an identifier (e.g., UniProt gene symbol) and descriptor (e.g., protein function or metabolite description). Where available, metabolites were tagged with UniProt gene symbols based on the Human Metabolome Database associations to help integrate metabolomics and proteomic data sets. The in-house database generated integrated reports and enabled user queries based on protein function, gene symbol, metabolite, and fold change across all omics data. Project data were exported from this central database into two custom data analysis and visualization tools. The first tool provided enrichment analysis and data driven network construction with empirical data overlaid onto interacting partners based on literature annotation for each time point over the course of the experiment. The details of these tools will be published elsewhere. The second tool provided visualization (through the use of Cytoscape²⁴) of the constructed data networks.

Phosphoenriched SILAC Sample Preparation

As a supplement to the label-free data, results from the analysis of 1 and 24 h Zn-treated SILAC labeled and phosphopeptide-enriched HL-60 cells were incorporated into the computational network analysis. These samples were prepared in a similar manner as previously described for A549 cells⁶ with the exception that they were analyzed on a QExactive HF mass spectrometer (Thermo Scientific) in line with a Dionex Ultimate 3000 NanoLC and autosampler equipped with a nanoelectrospray ionization source. Phosphopeptides were loaded on a MudPIT column. An 8-step salt pulse gradient (25, 50, 75, 100, 150, 250, 500, and 1000 mM ammonium acetate) was performed. Following each salt pulse, peptides were gradient-eluted from the reverse phase analytical column at a flow rate of 350 nL/min, and the mobile phase solvents consisted of 0.1% HCOOH in H₂O (solvent A) and 0.1% HCOOH in CH₃CN (solvent B). A 95 min reverse-phase gradient was used that consisted of 2–50% solvent B in 83 min followed by a 12 min equilibration at 2% solvent B for the peptides from the first seven SCX fractions. For the last fraction, the peptides were eluted from the reverse-phase analytical column using a gradient of 2–98%

solvent B in 83 min. The instrument method consisted of MS1 using an MS automatic gain control target value of 3e6 followed by up to 15 MS/MS scans of the most abundant ions detected in the preceding MS scan. A maximum MS/MS ion time of 40 ms was used with an MS2 automatic gain control target of 1e5. Dynamic exclusion was set to 30 s; high energy collision dissociation was set to 27% of the normalized collision energy, and peptide match and isotope exclusion were enabled.

Data Availability

The proteomics data from this publication have been deposited to the ProteomeXchange Consortium²⁵ [<http://www.proteomexchange.org/>] via the PRIDE partner repository²⁶ and assigned the identifier PXD009149. The phospho-proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository and assigned the identifier PXD009367. The RNA sequencing data from this publication have been deposited to the Sequence Read Archive²⁷ [<https://www.ncbi.nlm.nih.gov/sra/>] and assigned the submission identifier: SRP136044 (accessible via: <https://www.ncbi.nlm.nih.gov/sra/SRP136044>). Metabolomics data have been deposited to the NIH Metabolomics workbench [<http://www.metabolomicsworkbench.org/>] and assigned the identifier 1401. The metabolomics data is presently under manual curation by the metabolomics workbench team with an editable go-live date.

RESULTS AND DISCUSSION

Workflow and Validation of Methodology

To maximize the power and utility of multiomic analyses, we developed an integrated and automated sample preparation platform: sample preparation for multi-omic technologies (SPOT). Figure 1 demonstrates the SPOT workflow and compares it to a common multiomic workflow. The SPOT strategy facilitates an integrated, high-throughput sample preparation approach that saves time and resources and is amenable to automation. Figure 1A illustrates the unified structure of the SPOT workflow: each omic analysis stems from a common sample. The platform is demonstrated with cultured cells but should be applicable to a range of sample types, e.g., tissues, serum, and so forth. The first step in this workflow uses an aliquot of sample for RNA sequencing, and the remaining sample is lysed in a metabolomics-compatible buffer. Next, a protein assay determines the sample concentration, and the protein is precipitated from an aliquot of sample. The supernatant is further processed for metabolomics analysis, and the protein pellet is digested for proteomics analysis. Figure 1B presents a common sample preparation approach for multiomics analyses, where separate samples are processed for each analytical platform. This represents a lower throughput and more costly experimental design. Figure S1 illustrates unified preparations for lipidomics, metabolomics, and proteomics (S1-A) or for genomics, transcriptomics, and proteomics (S1-B). Although these approaches have the benefit of being unified, they are labor intensive and not easily amenable to automation.

For facilitating the use of a single sample for metabolomic and proteomic analyses, a lysis buffer comprised of 1:1:2 CH₃CN:CH₃OH:NH₄HCO₃ was used. Typical proteomic preparation strategies employ detergents that are incompatible with metabolomics analyses.

Therefore, the SPOT approach was developed from a combination of solvents previously demonstrated to preserve metabolomics integrity and minimize MS background while still meeting the needs of proteomics analyses. A combination of water, methanol, and acetonitrile are commonly used for metabolomics extraction^{28,29} (herein described as “traditional metabolomics approach”). Ammonium bicarbonate, a metabolomics-friendly quenching neutralizer and LC additive, was added to solubilize proteins. The ratio of acetonitrile, methanol, and ammonium bicarbonate was selected to maintain compatibility with the BCA protein concentration assay. A single freeze/thaw and sonication step in the solvent-based lysis buffer yielded the most reproducible protein concentration and resulted in protein concentrations that were nearly equivalent to two or three freeze/thaw and sonication steps (Figure S2, panel A). Lysis in 100 μL of buffer provided the most reproducible protein concentrations for 0.25×10^6 to 4.0×10^6 cells (Figure S2, panel B). Analysis of the cells under these conditions demonstrated nearly 100% lysis. Ideal conditions for metabolomics analyses were obtained with supernatant from 100 to 150 μg of precipitated protein. To determine the minimum required number of HL-60 cells and an accurate measure of protein content per cell, cells were counted by flow cytometry, collected, and assayed for protein content (in triplicate). A starting amount of 1×10^6 cells supplied $\sim 100 \mu\text{g}$ of protein (Figure S2, panel C). The inclusion of transcriptomics analyses required additional cells. In the case of HL-60 cells, an additional 1 million cells were cultured; however, the minimal amount necessary for transcriptomics analyses from HL-60 cells approximates $0.25\text{--}0.5 \times 10^6$ cells.

For validating the proteomics aspect of this workflow, the SPOT approach was compared to a detergent-based proteomics preparation for HL-60 control cells (Figure 2). Both preparation methods identified 24,304 of the same peptides, representing 60% of the total unique peptides identified overall. SPOT identified 9,053 peptides (27%), and the detergent approach identified 6,731 peptides (17%) that were not found using the alternative approach (Figure 2A). At the protein level, the two approaches shared 3,377 proteins (75%), whereas the SPOT approach quantified 671 distinct proteins (15%) and the detergent approach quantified 471 distinct proteins (10%) (Figure 2B). Additionally, the distribution of estimated copies per cell (cpc) for each approach was similar, spanning a range of 1×10^2 to 1×10^8 and with similar numbers of proteins in each cpc bin (Figure 2C).

The physiochemical properties of the proteins uniquely extracted by each method were determined to further understand differences in the two methods. The distribution of the 671 proteins uniquely extracted by the SPOT approach is slightly more hydrophobic than the distribution of the 471 proteins uniquely extracted by the detergent-based lysis approach (Figure 2D). In addition, a slightly higher percentage of the proteins uniquely extracted by the proteomics approach have isoelectric points in the range of 4–8 compared to the proteins uniquely extracted by SPOT, which have a greater percentage of proteins with higher isoelectric points in the range of 8–11 (Figure 2E). The SPOT method extracts a lower percentage of proteins in the range of 20–90 kDa and a higher percentage of proteins at molecular weights greater than 100 kDa. Characterization of these properties provides insight into which method may be better to use if particular proteins are targets of interest. The PROMPT tool³⁰ used here to determine these distributions for hundreds of proteins is of

great utility in comparing different sample preparation methods and could even be used as a measure of quality assessment in the case when hundreds of samples are run.

A subsequent experiment tested the SPOT approach for a quantitative experimental design: investigation of proteomic changes in a control versus treated scenario. Table 1 shows the proteomic results for the SPOT preparation method for the comparison of untreated and 250 μ M Zn-treated HL-60 cells.

Additional experiments were performed using A549 human lung carcinoma cells (an adherent cell line). The SPOT approach consistently produced results similar to a detergent-based proteomics approach in untreated and treated A549 cells, and trends in physiochemical properties were similar to those described for HL-60 cells (Tables S1 and S2, Figures S3 and S4, panels A-B). One distinction observed for the A549 data set was the low overlap for significantly changed proteins between each data set (Figure S4, panel C). Further investigation showed that 69% of the significantly changed proteins in the SPOT data set were quantified in the detergent-based lysis data set. Many of these had fold change values close to the significance threshold but had higher *p*-values (Figure S4, panel D). Likewise, 78% of the significantly changed proteins in the detergent-based lysis approach were found in the SPOT data set. These had fold change values near the significance threshold but had higher *p*-values overall (Figure S4, panel E). In some cases, the slight difference in fold change dropped the value below the significance threshold. In other cases, the *p*-value was too high to meet significance criteria. Additionally, the nature of data-dependent acquisition often leads to differences in peptide detection among replicates, affecting protein quantification and variance and, therefore, significance of the fold change. A key benefit of this multiomic approach is the use of multiple lines of evidence to determine important biological trends. Cellular processes that are supported by molecular changes from multiple omic platforms provide a strong indication of biological significance.

Another difference observed using the A549 cells that was not observed for the HL-60 cells was a shift toward quantifying more proteins at lower copies per cell using the SPOT approach (Figure S4, panel F). It is likely that this difference is due to the combination of the method for estimating copies per cell and the differences in physiochemical extraction properties of the SPOT and detergent-based lysis approaches. The proteomic ruler method³¹ for estimating copies per cell in A549 cells relates protein intensity to the summed histone intensity and the DNA content, which is estimated to be 6.5 pg per diploid cell.³¹ As shown by the physiochemical plots (Figure S4, panels G—I), the detergent-based lysis approach used here extracts a lower percentage of proteins compared to the SPOT approach in the pI range of 8—12. The isoelectric points of 54 histones span the pI range of 9—13 with 91% at pI 11 (Figure S4, panel J), indicating that the SPOT approach is more likely to extract histones than the detergent-based lysis approach. A comparison of the two data sets shows that, in the SPOT data set, histones comprised 4% of the total MS signal (similar to published extraction percentages³¹), whereas in the detergent-based lysis data set, histones represented 0.08% of the MS signal. Therefore, the copies per cell plotted for the detergent-based approach are likely overestimated due to an underrepresentation of histones extracted from these samples. This difference was not observed for HL-60 cells (Figure 2C) because protein content per cell was estimated using a standard curve of cell number (accurately

determined by flow cytometry) and protein content (determined as described in the methods).

The next step in assessing the utility of the SPOT approach for a unified omics platform was to evaluate its performance for metabolomics analysis (Figure 4). A reproducible number of metabolite compounds was detected across six sample process replicates of untreated cells (normalized to 75 μg protein/ sample), which illustrates consistency and reproducibility in metabolomics analyses (Figure 4A). Results from control and 225 μM Zn-treated HL-60 cells prepared using the SPOT approach illustrate numerous metabolites affected by treatment; specifically, 3,033 of 25,068 measured features met significance criteria (fold change ≥ 2 and p -value ≤ 0.1) (Figure 4B). Lastly, control and 6 h Zn-treated HL-60 samples separate in principal component 1, indicating that the sample preparation and metabolomic recovery are sufficient to distinguish control and treated samples (Figure 4C). Overall, these results demonstrate that application of the SPOT approach for metabolomics analyses provides reproducible results, extracts significantly changing metabolites, and is amenable to integrated omics analysis in one sample. Additionally, Figure S5 demonstrates the utility of the SPOT approach on an additional cell line and shows that the SPOT strategy generates more unique metabolites and more consistency across replicates, which is important toward building a comprehensive and reliable picture of biochemical cellular response.

Application of SPOT to Zn-Treated HL-60 Cells for Multiomics Mechanism Discovery

As a proof-of-principal, the SPOT strategy was applied to an omics investigation of Zn intoxication. Table S3 reports proteomics metrics for all time points, and Table S4 provides the number of metabolites detected and significantly changed as well as the number of tentative IDs for 6, 18, and 24 h. Figure 5 displays a broad overview of the data collected by each platform. The overlap of unique significantly changed proteins for the 6, 24, and 48 h time points is only 6.2% (Figure 5A), highlighting the critical role of temporal resolution in determining the mechanism of action. For the transcriptomics data, a greater number of changes were found at 6 h (5,671) compared to 1 h (171) (Figure 5B). Figure 5C demonstrates the molecular breadth obtained by analysis of metabolomics extracts when both HILIC and RPLC chromatographic platforms are utilized. Although there is substantial overlap, a significant increase in metabolomic measurements may be obtained by utilizing both platforms in this approach.

These data were further interrogated for Zn mechanism of action to verify that the SPOT strategy captures biologically meaningful results. Significant proteomic changes for 6–48 h were sorted into the top ten increasing abundance changes and grouped by gene ontology (GO) function. Nine of the top ten changing proteins are related to Zn and/or metal ion binding (Figure 6A, Table S5). Examination of these ten proteins and their interacting partners (according to literature-based annotations) identified upregulation of sequestosome-1 (SQSTM1/p62) and its association with receptor-interacting serine/threonine-protein kinase 1 (RIPK1; Figure S6), a well-known regulator of inflammation through Nuclear factor kappa beta (NFkappaB) signaling. In accordance with this finding,

enrichment analysis of the data set suggested neutrophil degranulation starting 18 h after exposure (Table S6).

Further interrogation of these interactions, with the inclusion of phosphoenriched data collected at 1 and 24 h, revealed a complex molecular landscape surrounding Zn exposure. SQSTM1 stood out as a central molecule for mediation of NRF2 and NFkappaB signaling in response to Zn intoxication. Zn treatment also induced metal regulatory transcription factor 1 (MTF1), metallothioneins, and Zn transporter 1 (SLC30A1) expression. Elucidation of these mechanisms of action are described below. Capture of these mechanisms in a global manner from one data set exemplifies the power of the SPOT approach to contribute to biologically relevant data and demonstrates the power of high-throughput, automated, temporally resolved multiomic platforms.

Zn Exposure Mediates Oxidative Stress and NRF2 Signaling in HL-60 Cells

Zn does not induce reactive oxygen species (ROS) through electron transfer as do transition metals such as Fe and Cu. However, it can stimulate ROS as a result of impaired mitochondrial function.^{32,33} A recent publication reported the Zn-induced production of H₂O₂ in cultured human airway epithelial cells and BEAS-2B lung epithelial cells,³² suggesting the induction of ROS via H₂O₂ in HL-60 cells treated with Zn. Several proteins with functional roles in the management of superoxide production were upregulated in this data set, including C3a anaphylatoxin chemotactic receptor (C3AR1) and key components of NADPH oxidase: putative neutrophil cytosol factor 1B (NCF1B), putative neutrophil cytosol factor 1C (NCF1C), neutrophil cytosol factor 2 (NCF2), and NADPH oxidase activator 1 (NOXA1; transcript only) (Table S7). Furthermore, superoxide production was observed in Zn- treated A549 cells (Figure S7, panel A).

NRF2 signaling is an established antioxidant response pathway,³⁴ and it is known to regulate expression of antioxidant proteins such as NAD(P)H dehydrogenase [quinone] 1 (NQO1) and heme oxygenase 1 (HMOX1).³⁵ Previously, the upregulation of HMOX1 mRNA expression resulted directly from increased H₂O₂ and additional H₂O₂-independent means, possibly via the Keap1/Nrf2 pathway.³² In the present study, HMOX1 was one of the top ten proteins with increased abundance; NQO1, which has multiple roles including detoxification and protection against oxidative stress, was upregulated by RNA-seq at 6 h and by label-free proteomics at 18 and 24 h. The copper—zinc superoxide dismutase (SOD1), another significantly increased transcript in this data set, also has an antioxidant response element (ARE) in the promoter region.³⁶ In some conditions, SOD1 expression can be induced by NRF2 signaling, although basal levels are not affected.³⁵

These data indicate that Zn induces oxidative stress through H₂O₂ and superoxide and that the cell initiates an antioxidant response. Given the additional evidence of NFkappaB signaling and neutrophil degranulation at later time points, it is likely that the antioxidant response does not lead to recovery. A summary network of 201 species constructed using SQSTM1, SOD1, and HMOX1 revealed that 86% of the species in the expanded network were detected and that 52% of these were significantly changed (Figure S8).

Zn Intoxication Mediates NFkappaB Signaling in HL-60 Cells

NFkappaB signaling has been extensively studied, and two pathways have been established: the canonical NFkappaB signaling pathway that mediates its effects through TNF receptors, interleukin, and toll like receptors and the alternate or noncanonical NFkappaB pathway that exerts its effects via tumor necrosis factor receptor superfamily members (TNFRSF): TNFRSF3 (LTBR), TNFRSF5 (CD40), TNFRSF13C (BAFFR), and TNFRSF12A (FN14).^{37,38} Extensive cross talk between these two pathways was measured in this data set over the time course of the experiment.

SQSTM1 is a mediator of nuclear factor kappa beta 1 (NFkappaB1) signaling through interaction with TNF receptor-associated factor 6 (TRAF6).³⁹ Phosphorylation of SQSTM1 at T269 and S272 promotes the binding of TRAF6 to SQSTM1 and maintains polyubiquitinated TRAF6, leading to NFkappaB signaling.^{39–41} In the current work, SQSTM1 phosphorylated at one or both of these residues was upregulated at 18 and 24 h. In addition, ubiquitin carboxyl-terminal hydrolase CYLD (CYLD) phosphorylated at serine 418 and either serine 422 or threonine 424 was significantly increased at 24 h. CYLD is a deubiquitinase that affects NFkappaB signaling through regulation of the ubiquitin state of its targets, including TRAF6 and TRAF2.⁴² Phosphorylation of CYLD at S418 has been shown to reduce its deubiquitinase activity, leading to increased NFkappaB signaling.⁴²

Proteins that suppress NFkappaB signaling were also found to be significantly changed. Transcription factor jun-B (JUNB) is a negative regulator of SQSTM1.⁴³ Phosphorylation of JUNB at S259 promotes phosphorylation at S251 and S255, which results in its ubiquitination by FBXW7 and subsequent degradation.⁴⁴ Thus, phosphorylation at these sites interferes with JUNB suppression of NFkappaB signaling. In the current data set, Zn treatment of HL-60 cells resulted in upregulation of JUNB and also of JUNB phosphorylated at S251, S255, and S259. Leukocyte-associated Ig-like receptor-1 (LAIR1) has been shown to prevent NFkappaB translocation to the nucleus. Here, LAIR1 was downregulated according to RNA-seq analysis at 6 h and by proteomic analysis at 18, 24, and 48 h.

RIPK1 is a well-known regulator of NFkappaB signaling through the TNFR signaling complex. As a scaffold protein in complex I, RIPK1 recruits key downstream targets leading to the activation of NFkappaB signaling. However, RIPK1 can form complexes, termed complex II a or b with alternative downstream targets, which lead to either apoptosis or necroptosis.⁴⁵ At 1 h, RIPK1 phosphorylated at serine 320 (pS320) was significantly upregulated. This modification has been shown to reduce RIPK1-induced cell death through complex II, allowing for the cell to proceed with an inflammatory response⁴⁶ through NFkappaB signaling.

Taken together, these avenues suggest Zn intoxication leads to overall increased NFkappaB signaling (Table S9). In support of this, NFkappaB2 showed a trend of upregulation over time. For increasing the molecular understanding surrounding these interactions, a time-series network was built using RIPK1, SQSTM1, TRAF6, and CYLD as seed species and expanding these to the nearest neighbor of interacting partners (Figure S9). The network expanded to 432 nodes (species) with 94% detected and 54% significantly changed.

Inflammatory Response

These initial findings indicated an inflammatory response. Further interrogation of the data along this avenue revealed S100A9 as a significantly changed protein. S100A9 interacts with S100A8 to form the calprotectin heterodimer that binds calcium, manganese, iron, nickel, and zinc.^{47–49} Calprotectin acts as an antibacterial protein through metal sequestration,^{50–52} and S100A9 is released as a danger signal to potentiate the inflammatory response.⁵³ It is known to increase the release of interleukin-8 (CXCL8), interleukin-1 beta (IL1B), interleukin-17 (IL17), and IFN-gamma.^{53,54} CXCL8 is an established marker of inflammatory response, and its production in Zn-treated cells has been reported.^{55–58} In the current data set, it was upregulated at 6 h by RNA-seq and label-free proteomics analysis. A separate assay also showed upregulation of CXCL8 in Zn-treated HL-60 cells compared to control at 6 and 18 h (Figure S7, panel B).

In addition, CXCL8 is an activating factor for neutrophils; it is one of several signals that initiates the process of degranulation.⁵⁹ Neutrophil degranulation was ranked as the number one process from 18 to 48 h by enrichment analysis. The process of neutrophil degranulation leads to cells expelling granules that contain antimicrobial molecules and pro-inflammatory molecules and involves the molecules required for intracellular vesicle transport to the plasma membrane and neutrophil transportation to the site of perturbation.^{59,60} Examination of the genes associated with neutrophil degranulation through enrichment analysis showed 11 genes that were found in at least three of the time points. Among these, three genes had functions closely associated with an inflammatory response and/or neutrophil degranulation: complement C3 (C3), ras-related protein Rab-27A (RAB27A), and alpha-1-antitrypsin (SERPINA1). C3 is a component of the complement system that acts as a modulator of inflammation during the innate immune response.^{61,62} Rab proteins mediate trafficking of intracellular vesicles along with Rab effector proteins and proteins of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) family to coordinate an inflammatory response by neutrophils.^{60,63,64} A number of Rab family members were detected in this data set. Those with trending significant upregulation included RAB14, RAB1B, RAB27A, RAB2A, RAB2B, and RAB8B. Additionally, members of the SNARE family were detected with significant upregulation: synaptosomal-associated protein 23 (SNAP23) and vesicle-associated membrane protein 7 (VAMP7). Table S10 lists the changes associated with inflammatory signaling and vesicular transport.

The 11 genes determined through enrichment analysis were used to seed a network to further examine the molecular complexity of neutrophil degranulation. A network of 449 species was constructed with 84% of the species detected in the empirical data set and 46% of these significantly changed (Figure S10).

Metabolomics of Zn Intoxication

Over 2,000 unique compounds were measured across six time points. In this study, we performed network activity prediction analysis⁶⁵ to predict potential pathways that were affected by Zn treatment. Briefly, network activity prediction analysis allows for accurate mass of *m/z* features to map candidate metabolites to metabolic networks and calculate a local enrichment of metabolites to distinguish those networks from a stochastic distribution

of metabolites.^{66–68} In these analyses, there were two main pathways implicated as significant pathways ($p < 0.1$) at numerous time points, including purine metabolism at the 1, 6, 18, and 24 h time points and tryptophan metabolism at the 6, 24, and 36 h time points (Figure 7). These data agree with previously published results by Mocchengiani et al.⁶ in which genomic data were generated from a patient cohort that received dietary Zn treatment to investigate the impact of zinc on modulation of gene and protein functional activities. Briefly, genomic network activity analysis revealed that tryptophan metabolism, eicosanoid signaling, and purine metabolism signaling were proposed as pathways significantly modified with zinc treatment.⁶⁹

Regulation of these metabolic pathways corroborates the results derived from proteomic and transcriptomic experiments and reveals additional cellular responses not initially realized through investigation of the other modalities. Tentatively identified metabolites in the tryptophan metabolic pathway that were upregulated or produced include glutathione (at 6 and 18 h), glutamate (at 24, 36, and 48 h), and deoxyguanosine monophosphate (dGMP)/adenosine triphosphate (ATP) (at 24, 36, and 48 h). Tryptophan catabolism into kynurenine leads to an anti-inflammatory response via the aryl hydrocarbon receptor (AHR).^{70,71} Both kynurenine 3-monooxygenase (KMO, a member of the kynurenine pathway) and AHR were upregulated at 6 h by RNA-seq analysis. Neither indoleamine 2,3-dioxygenase 1 or 2 nor tryptophan 2,3-dioxygenase 2 were significantly changed in abundance.

The fold change ratios for metabolites in the purine metabolism pathway (hypoxanthine, glutamate, adenosine, deoxyguanosine, adenosine diphosphate-ribose (adp-ribose), deoxyguanosine monophosphate (dGMP), adenosine triphosphate (ATP) guanine, inosine monophosphate (IMP), and inosine) were assessed across all time points (Figure S11). In these data, hypoxanthine, adenosine/deoxyguanosine, and guanine had consistent fold change ratios throughout Zn exposure (exhibiting fold changes <1.5 throughout the treatment), whereas glutamate, dGMP/ATP, ADP-ribose, IMP, and inosine metabolites showed changes in consumption and/or production rates throughout the course of the experiment. Specifically, IMP and inosine were consumed or not produced in later time points (6–48 h), whereas glutamate and ADP ribose were produced or not consumed. Taken together, these data add metabolomics support to the genomic-based reported impact on Zn in purine metabolism.⁶⁹

Peroxisomal metabolism was implicated by network activity analysis of the metabolomic data, and this was supported by transcriptomics results (PPAR gamma coactivator 1 beta (PPARGC1B) was downregulated by RNA-seq at 6 h). However, evidence from these modalities was limited. PPAR signaling has been shown to inhibit NFkappaB activity,^{72,73} and modulation of zinc levels has been linked to the regulation of inflammation via PPAR.^{69,74}

Mechanism Summary

Overall, this multiomics time-resolved data set indicates that Zn intoxication of HL-60 cells perturbs NRF2 and NFkappaB signaling, leading to an inflammatory response culminating in neutrophil degranulation (Figure 1) and changes in several metabolism and purine metabolism. Although these conclusions require additional validation, the described

signaling pathways have been reported previously, although not in a comprehensive publication specific to Zn intoxication of HL-60 cells. The comprehensive capture of these mechanisms exemplifies the power of the temporally resolved multiomics approach. SPOT fills a critical gap by providing an automated, high-throughput, and reproducible sample preparation strategy that facilitates the collection of multicondition, time-resolved, large-scale comprehensive data sets.

CONCLUSIONS

Multimic studies are emerging as powerful tools to investigate disease state and cellular response. However, studies incorporating these platforms can be time and resource intensive. Although acquisition of data via omics technologies can be high-throughput, the sample preparation and data analysis workflows often paired with these technologies limit overall experimental throughput. The workflow presented here is automated and high-throughput, capable of analyzing 16–24 samples in 8–10 h. Furthermore, this sample preparation approach is optimal for obtaining high-quality data from all omics platforms; thus, the quality of one particular omics approach is not sacrificed by unification of the sample preparation process. This efficiency in sample preparation facilitates the acquisition of large data sets, such as those necessary to unravel complex systems biology issues in a reasonable time frame while permitting the use of replicates and appropriate time scales and/or conditions.

These types of data sets provide insight into global mechanisms of action. Furthermore, the untargeted nature of these analyses provide the opportunity for greater understanding of additional molecules associated with cellular processes, the interplay of various pathways, and novel mechanisms. Sample preparation methods, such as SPOT, that enable multiomics analysis of comprehensive data sets will positively impact our understanding of biology across a wide range of fields. High-throughput time and condition-resolved multiomics data acquisition is a powerful approach to greatly advance our understanding of challenging issues such as drug development, host-microbe interactions, drug resistance, and precision medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- (1). Aviner R; Shenoy A; Elroy-Stein O; Geiger T Uncovering Hidden Layers of Cell Cycle Regulation through Integrative Multi-Omic Analysis. *PLoS Genet.* 2015, 11 (10), No. e1005554.
- (2). Tisoncik-Go J; Gasper DJ; Kyle JE; Einfeld AJ; Selinger C; Hatta M; Morrison J; Korth MJ; Zink EM; Kim Y-M; et al. Integrated Omics Analysis of Pathogenic Host Responses during Pandemic H1N1 Influenza Virus Infection: The Crucial Role of Lipid Metabolism. *Cell Host Microbe* 2016, 19 (2), 254–266. [PubMed: 26867183]
- (3). Bouslimani A; Porto C; Rath CM; Wang M; Guo Y; Gonzalez A; Berg-Lyon D; Ackermann G; Moeller Christensen GJ; Nakatsuji T; et al. Molecular Cartography of the Human Skin Surface in 3D. *Proc. Natl. Acad. Sci. U. S. A.* 2015, 112 (17), E2120–2129. [PubMed: 25825778]
- (4). Breitkopf SB; Yuan M; Helenius KP; Lyssiotis CA; Asara JM Triomics Analysis of Imatinib-Treated Myeloma Cells Connects Kinase Inhibition to RNA Processing and Decreased Lipid Biosynthesis. *Anal. Chem.* 2015, 87 (21), 10995–11006. [PubMed: 26434776]
- (5). Kershaw CJ; Costello JL; Talavera D; Rowe W; Castelli LM; Sims PFG; Grant CM; Ashe MP; Hubbard SJ; Pavitt GD Integrated Multi-Omics Analyses Reveal the Pleiotropic Nature of the Control of Gene Expression by Puf3p. *Sci. Rep.* 2015, 5, srep15518.
- (6). Norris JL; Farrow MA; Gutierrez DB; Palmer LD; Muszynski N; Sherrod SD; Pino JC; Allen JL; Spraggins JM; Lubbock ALR; et al. Integrated, High-Throughput, Multiomics Platform Enables Data-Driven Construction of Cellular Responses and Reveals Global Drug Mechanisms of Action. *J. Proteome Res.* 2017, 16 (3), 1364–1375. [PubMed: 28088864]
- (7). The Integrative Human Microbiome Project: Dynamic Analysis of Microbiome-Host Omics Profiles during Periods of Human Health and Disease. *Cell Host Microbe* 2014, 16 (3), 276–289. [PubMed: 25211071]
- (8). Lo AW; Moriel DG; Phan M-D; Schulz BL; Kidd TJ; Beatson SA; Schembri MA ‘Omic’ Approaches to Study Uropathogenic Escherichia Coli Virulence. *Trends Microbiol.* 2017, 25 (9), 729–740. [PubMed: 28550944]
- (9). Roume H; Heintz-Buschart A; Muller EEL; Wilmes P Sequential Isolation of Metabolites, RNA, DNA, and Proteins from the Same Unique Sample. *Methods Enzymol* 2013, 531, 219–236. [PubMed: 24060123]
- (10). Roume H; El Muller EEL; Cordes T; Renaut J; Hiller K; Wilmes P A Biomolecular Isolation Framework for Eco-Systems Biology. *ISMEJ.* 2013, 7 (1), 110–121.
- (11). Muller EEL; Glaab E; May P; Vlassis N; Wilmes P Condensing the Omics Fog of Microbial Communities. *Trends Microbiol.* 2013, 21 (7), 325–333. [PubMed: 23764387]
- (12). Fukushima A; Kusano M; Redestig H; Arita M; Saito K Integrated Omics Approaches in Plant Systems Biology. *Curr. Opin. Chem. Biol.* 2009, 13 (5–6), 532–538. [PubMed: 19837627]
- (13). Yuan JS; Galbraith DW; Dai SY; Griffin P; Stewart CN Plant Systems Biology Comes of Age. *Trends Plant Sci.* 2008, 13 (4), 165–171. [PubMed: 18329321]
- (14). Weckwerth W; Wenzel K; Fiehn O Process for the Integrated Extraction, Identification and Quantification of Metabolites, Proteins and RNA to Reveal Their Co-Regulation in Biochemical Networks. *Proteomics* 2004, 4 (1), 78–83. [PubMed: 14730673]
- (15). Mostafa I; Zhu N; Yoo M-J; Balmant KM; Misra BB; Dufresne C; Abou-Hashem M; Chen S; El-Domiaty M New Nodes and Edges in the Glucosinolate Molecular Network Revealed by Proteomics and Metabolomics of Arabidopsis Myb28/29 and Cyp79B2/B3 Glucosinolate Mutants. *J. Proteomics* 2016, 138, 1–19. [PubMed: 26915584]
- (16). Yoo BC; Kim K-H; Woo SM; Myung JK Clinical Multi-Omics Strategies for the Effective Cancer Management. *J. Proteomics* 2017, DOI: 10.1016/j.jprot.2017.08.010.
- (17). García-Sevillano MÁ; García-Barrera T; Abril N; Pueyo C; López-Barea J; Gómez-Ariza JL Omics Technologies and Their Applications to Evaluate Metal Toxicity in Mice *M. Spretus* as a Bioindicator. *J. Proteomics* 2014, 104, 4–23. [PubMed: 24631825]
- (18). Cho Y-E; Moon P-G; Lee J-E; Singh TSK; Kang W; Lee H-C; Lee M-H; Kim S-H; Baek M-C Integrative Analysis of Proteomic and Transcriptomic Data for Identification of Pathways Related to Simvastatin-Induced Hepatotoxicity. *Proteomics* 2013, 13 (8), 1257–1275. [PubMed: 23322611]

- (19). Coman C; Solari FA; Hentschel A; Sickmann A; Zahedi RP; Ahrends R Simultaneous Metabolite, Protein, Lipid Extraction (SIMPLEX): A Combinatorial Multimolecular Omics Approach for Systems Biology. *Mol. Cell. Proteomics* 2016, 15 (4), 1453–1466. [PubMed: 26814187]
- (20). Nakayasu ES; Nicora CD; Sims AC; Burnum-Johnson KE; Kim Y-M; Kyle JE; Matzke MM; Shukla AK; Chu RK; Schepmoes AA; et al. MPLEX: A Robust and Universal Protocol for Single-Sample Integrative Proteomic, Metabolomic, and Lipidomic Analyses. *mSystems* 2016, 1 (3), e00043–16. [PubMed: 27822525]
- (21). Kopczynski D; Coman C; Zahedi RP; Lorenz K; Sickmann A; Ahrends R Multi-OMICS: A Critical Technical Perspective on Integrative Lipidomics Approaches. *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids* 2017, 1862 (8), 808–811. [PubMed: 28193460]
- (22). Goh WWB; Wang W; Wong L Why Batch Effects Matter in Omics Data, and How to Avoid Them. *Trends Biotechnol.* 2017, 35(6), 498–507. [PubMed: 28351613]
- (23). Yang G; Zhou B; Wang J; He X; Sun X; Nie W; Tzipori S; Feng H Expression of Recombinant Clostridium Difficile Toxin A and B in Bacillus Megaterium. *BMC Microbiol.* 2008, 8, 192. [PubMed: 18990232]
- (24). Shannon P; Markiel A; Ozier O; Baliga NS; Wang JT; Ramage D; Amin N; Schwikowski B; Ideker T Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. *Genome Res.* 2003, 13 (11), 2498–2504. [PubMed: 14597658]
- (25). Deutsch EW; Csordas A; Sun Z; Jarnuczak A; Perez- Riverol Y; Ternent T; Campbell DS; Bernal-Llinares M; Okuda S; Kawano S; et al. The ProteomeXchange Consortium in 2017: Supporting the Cultural Change in Proteomics Public Data Deposition. *Nucleic Acids Res.* 2017, 45 (D1), D1100–D1106. [PubMed: 27924013]
- (26). Vizcaíno JA; Csordas A; del-Toro N; Dianas JA; Griss J; Lavidas I; Mayer G; Perez-Riverol Y; Reisinger F; Ternent T Update of the PRIDE Database and Its Related Tools. *Nucleic Acids Res.* 2016, 44 (D1), D447–456. [PubMed: 26527722]
- (27). Leinonen R; Sugawara H; Shumway M The Sequence Read Archive. *Nucleic Acids Res.* 2011, 39 (suppl_1), D19–D21. [PubMed: 21062823]
- (28). Sellick CA; Hansen R; Stephens GM; Goodacre R; Dickson AJ Metabolite Extraction from Suspension-Cultured Mammalian Cells for Global Metabolite Profiling. *Nat. Protoc.* 2011, 6(8), 1241–1249. [PubMed: 21799492]
- (29). Abdel Rahman A. M.; Pawling J; Ryczko M; Caudy AA; Dennis JW Targeted Metabolomics in Cultured Cells and Tissues by Mass Spectrometry: Method Development and Validation. *Anal. Chim. Acta* 2014, 845, 53–61. [PubMed: 25201272]
- (30). Schmidt T; Frishman D PROMPT: A Protein Mapping and Comparison Tool. *BMC Bioinf.* 2006, 7, 331.
- (31). Wi niewski JR; Hein MY; Cox J; Mann MA “Proteomic Ruler” for Protein Copy Number and Concentration Estimation without Spike-in Standards. *Mol. Cell. Proteomics* 2014, 13 (12), 3497–3506. [PubMed: 25225357]
- (32). Wages PA; Silbajoris R; Speen A; Brighton L; Henriquez A; Tong H; Bromberg PA; Simmons SO; Samet JM Role of H2O2 in the Oxidative Effects of Zinc Exposure in Human Airway Epithelial Cells. *Redox Biol.* 2014, 3, 47–55. [PubMed: 25462065]
- (33). Wu W; Bromberg PA; Samet JM Zinc Ions as Effectors of Environmental Oxidative Lung Injury. *Free Radical Biol. Med.* 2013, 65 (Supplement C), 57–69. [PubMed: 23747928]
- (34). Nguyen T; Nioi P; Pickett CB The Nrf2-Antioxidant Response Element Signaling Pathway and Its Activation by Oxidative Stress. *J. Biol. Chem.* 2009, 284 (20), 13291–13295. [PubMed: 19182219]
- (35). Zhu H; Itoh K; Yamamoto M; Zweier JL; Li Y Role of Nrf2 Signaling in Regulation of Antioxidants and Phase 2 Enzymes in Cardiac Fibroblasts: Protection against Reactive Oxygen and Nitrogen Species-Induced Cell Injury. *FEBS Lett.* 2005, 579 (14), 3029–3036. [PubMed: 15896789]
- (36). Milani P; Gagliardi S; Cova E; Cereda C SOD1 Transcriptional and Posttranscriptional Regulation and Its Potential Implications in ALS. <https://www.hindawi.com/journals/nri/2014/58427/> (accessed Jan 5, 2018).

- (37). Cildir G; Low KC; Tergaonkar V Noncanonical NF-KB Signaling in Health and Disease. *Trends Mol. Med.* 2016, 22 (5), 414–429. [PubMed: 27068135]
- (38). Chen ZJ Ubiquitin Signalling in the NF-KB Pathway. *Nat. Cell Biol.* 2005, 7 (8), 758. [PubMed: 16056267]
- (39). Taniguchi K; Yamachika S; He F; Karin M P62/SQSTM1—Dr. Jekyll and Mr. Hyde That Prevents Oxidative Stress but Promotes Liver Cancer. *FEBS Lett.* 2016, 590 (15), 2375–2397. [PubMed: 27404485]
- (40). Martin P; Diaz-Meco MT; Moscat J The Signaling Adapter P62 Is an Important Mediator of T Helper 2 Cell Function and Allergic Airway Inflammation. *EMBO J.* 2006, 25 (15), 3524–3533. [PubMed: 16874300]
- (41). Duran A; Linares JF; Galvez AS; Wikenheiser K; Flores JM; Diaz-Meco MT; Moscat J The Signaling Adaptor P62 Is an Important NF-KB Mediator in Tumorigenesis. *Cancer Cell* 2008, 13(4), 343–354. [PubMed: 18394557]
- (42). Hutti JE; Shen RR; Abbott DW; Zhou AY; Sprott KM; Asara JM; Hahn WC; Cantley LC Phosphorylation of the Tumor Suppressor CYLD by the Breast Cancer Oncogene IKKf Promotes Cell Transformation. *Mol. Cell* 2009, 34 (4), 461–472. [PubMed: 19481526]
- (43). Zhang X; Jin JY; Wu J; Qin X; Streilein R; Hall RP; Zhang JY RNA-Seq and ChIP-Seq Reveals SQSTM1/P62 as a Key Mediator of JunB Suppression of NF-KB-Dependent Inflammation. *J. Invest. Dermatol.* 2015, 135 (4), 1016–1024. [PubMed: 25501661]
- (44). Pérez-Benavente B; García JL; Rodríguez MS; Pineda-Lucena A; Piechaczyk M; Font de Mora J; Farrás R GSK3- SCF(FBXW7) Targets JunB for Degradation in G2 to Preserve Chromatid Cohesion before Anaphase. *Oncogene* 2013, 32 (17), 2189–2199. [PubMed: 22710716]
- (45). Christofferson DE; Li Y; Yuan J Control of Life-or-Death Decisions by RIP1 Kinase. *Annu. Rev. Physiol.* 2014, 76 (1), 129–150. [PubMed: 24079414]
- (46). Jaco I; Annibaldi A; Lalaoui N; Wilson R; Tenev T; Laurien L; Kim C; Jamal K; Wicky John S.; Liscardi G; et al. MK2 Phosphorylates RIPK1 to Prevent TNF-Induced Cell Death. *Mol. Cell* 2017, 66 (5), 698–710.e5. [PubMed: 28506461]
- (47). Corbin BD; Seeley EH; Raab A; Feldmann J; Miller MR; Torres VJ; Anderson KL; Dattilo BM; Dunman PM; Gerads R; et al. Metal Chelation and Inhibition of Bacterial Growth in Tissue Abscesses. *Science* 2008, 319 (5865), 962–965. [PubMed: 18276893]
- (48). Nakashige TG; Zygiel EM; Drennan CL; Nolan EM Nickel Sequestration by the Host-Defense Protein Human Calprotectin. *J. Am. Chem. Soc.* 2017, 139 (26), 8828–8836. [PubMed: 28573847]
- (49). Nakashige TG; Zhang B; Krebs C; Nolan EM Human Calprotectin Is an Iron-Sequestering Host-Defense Protein. *Nat. Chem. Biol.* 2015, 11 (10), 765–771. [PubMed: 26302479]
- (50). Moore JL; Becker KW; Nicklay JJ; Boyd KL; Skaar EP; Caprioli RM Imaging Mass Spectrometry for Assessing Temporal Proteomics: Analysis of Calprotectin in *Acinetobacter Baumannii* Pulmonary Infection. *Proteomics* 2014, 14 (7–8), 820–828. [PubMed: 23754577]
- (51). Kehl-Fie TE; Chitayat S; Hood MI; Damo S; Restrepo N; Garcia C; Munro KA; Chazin WJ; Skaar EP Nutrient Metal Sequestration by Calprotectin Inhibits Bacterial Superoxide Defense, Enhancing Neutrophil Killing of *Staphylococcus Aureus*. *Cell Host Microbe* 2011, 10 (2), 158–164. [PubMed: 21843872]
- (52). Sohnle PG; Hunter MJ; Hahn B; Chazin WJ Zinc- Reversible Antimicrobial Activity of Recombinant Calprotectin (Migration Inhibitory Factor-Related Proteins 8 and 14). *J. Infect. Dis.* 2000, 182 (4), 1272–1275. [PubMed: 10979933]
- (53). Lee T-H; Chang HS; Bae D-J; Song HJ; Kim M-S; Park JS; Jun JA; Lee SY; Uh ST; Kim SH; et al. Role of S100A9 in the Development of Neutrophilic Inflammation in Asthmatics and in a Murine Model. *Clin. Immunol.* 2017, 183 (Supplement C), 158–166. [PubMed: 28847516]
- (54). Simard J-C; Noël C; Tessier PA; Girard D Human S100A9 Potentiates IL-8 Production in Response to GM-CSF or FMLP via Activation of a Different Set of Transcription Factors in Neutrophils. *FEBS Lett.* 2014, 588 (13), 2141–2146. [PubMed: 24796790]
- (55). Vandebriel RJ; De Jong WH A Review of Mammalian Toxicity of ZnO Nanoparticles. *Nanotechnol., Sci. Appl.* 2012, 5, 61–71. [PubMed: 24198497]

- (56). Saptarshi SR; Feltis BN; Wright PF; Lopata AL Investigating the Immunomodulatory Nature of Zinc Oxide Nanoparticles at Sub-Cytotoxic Levels in Vitro and after Intranasal Instillation in Vivo. *J. Nanobiotechnol.* 2015, 13, 6.
- (57). Kim Y-M; Reed W; Wu W; Bromberg PA; Graves LM; Samet JM Zn²⁺-Induced IL-8 Expression Involves AP-1, JNK, and ERK Activities in Human Airway Epithelial Cells. *Am. J. Physiol. - Lung Cell. Mol. Physiol.* 2006, 290 (5), L1028–L1035. [PubMed: 16373669]
- (58). Johnston H; Brown DM; Kanase N; Euston M; Gaiser BK; Robb CT; Dyrinda E; Rossi AG; Brown ER; Stone V Mechanism of Neutrophil Activation and Toxicity Elicited by Engineered Nanomaterials. *Toxicol. In Vitro* 2015, 29 (5), 1172–1184. [PubMed: 25962642]
- (59). Lacy P Mechanisms of Degranulation in Neutrophils. *Allergy, Asthma, Clin. Immunol.* 2006, 2 (3), 98–108. [PubMed: 20525154]
- (60). Ramadass M; Catz SD Molecular Mechanisms Regulating Secretory Organelles and Endosomes in Neutrophils and Their Implications for Inflammation. *Immunol. Rev.* 2016, 273 (1), 249–265. [PubMed: 27558339]
- (61). Lubbers R; van Essen MF; van Kooten C; Trouw LA Production of Complement Components by Cells of the Immune System. *Clin. Exp. Immunol.* 2017, 188 (2), 183–194. [PubMed: 28249350]
- (62). Coulthard LG; Woodruff TM Is the Complement Activation Product C3a a Proinflammatory Molecule? Re-Evaluating the Evidence and the Myth. *J. Immunol.* 2015, 194 (8), 3542–3548. [PubMed: 25848071]
- (63). Chen YA; Scheller RH SNARE-Mediated Membrane Fusion. *Nat. Rev. Mol. Cell Biol.* 2001, 2 (2), 98. [PubMed: 11252968]
- (64). Ungar D; Hughson FM SNARE Protein Structure and Function. *Annu. Rev. Cell Dev. Biol.* 2003, 19, 493–517. [PubMed: 14570579]
- (65). Li S; Park Y; Duraisingham S; Strobel FH; Khan N; Soltow QA; Jones DP; Pulendran B Predicting Network Activity from High Throughput Metabolomics. *PLoS Comput. Biol.* 2013, 9(7), No. e1003123.
- (66). Ravasz E; Somera AL; Mongru DA; Oltvai ZN; Barabasi A-L Hierarchical Organization of Modularity in Metabolic Networks. *Science* 2002, 297 (5586), 1551–1555. [PubMed: 12202830]
- (67). Newman MEJ; Girvan M Finding and Evaluating Community Structure in Networks. *Phys. Rev. E Stat Nonlin Soft Matter Phys.* 2004, 69 (2), 026113. [PubMed: 14995526]
- (68). Newman MEJ Modularity and Community Structure in Networks. *Proc. Natl. Acad. Sci U. S. A.* 2006, 103 (23), 8577–8582. [PubMed: 16723398]
- (69). Mazzatti DJ; Malavolta M; White AJ; Costarelli L; Giacconi R; Muti E; Cipriano C; Powell JR; Mocchegiani E Differential Effects of in Vitro Zinc Treatment on Gene Expression in Peripheral Blood Mononuclear Cells Derived from Young and Elderly Individuals. *Rejuvenation Res.* 2007, 10 (4), 603–620. [PubMed: 17985944]
- (70). Opitz CA; Litzenburger UM; Sahm F; Ott M; Tritschler I; Trump S; Schumacher T; Jestaedt L; Schrenk D; Weller M; et al. An Endogenous Tumour-Promoting Ligand of the Human Aryl Hydrocarbon Receptor. *Nature* 2011, 478 (7368), 197–203. [PubMed: 21976023]
- (71). Cervenka I; Agudelo LZ; Ruas JL Kynurenines: Tryptophan's Metabolites in Exercise, Inflammation, and Mental Health. *Science* 2017, 357 (6349), No. eaaf9794.
- (72). Wahli W; Michalik L PPARs at the Crossroads of Lipid Signaling and Inflammation. *Trends Endocrinol. Metab.* 2012, 23 (7), 351–363. [PubMed: 22704720]
- (73). Eisele PS; Salatino S; Sobek J; Hottiger MO; Handschin C The Peroxisome Proliferator-Activated Receptor γ Coactivator 1 α/β (PGC-1) Coactivators Repress the Transcriptional Activity of NF- κ B in Skeletal Muscle Cells. *J. Biol. Chem.* 2013, 288 (4), 2246–2260. [PubMed: 23223635]
- (74). Shen H; Oesterling E; Stromberg A; Toborek M; MacDonald R; Hennig B Zinc Deficiency Induces Vascular Pro- Inflammatory Parameters Associated with NF- κ B and PPAR Signaling. *J. Am. Coll. Nutr.* 2008, 27 (5), 577–587. [PubMed: 18845708]
- (75). Braun F; Rinschen MM; Bartels V; Frommolt P; Habermann B; Hoeijmakers JHJ; Schumacher B; Dollé MET; Müller R-U; Benzing T; et al. Altered Lipid Metabolism in the Aging Kidney Identified by Three Layered Omic Analysis. *Aging* 2016, 8 (3), 441–457. [PubMed: 26886165]

- (76). Zhao P; Li J; Li Y; Tian Y; Yang L; Li S Integrating Transcriptomics, Proteomics, and Metabolomics Profiling with System Pharmacology for the Delineation of Long-Term Therapeutic Mechanisms of Bufeijianpi Formula in Treating COPD. *BioMed Res. Int.* 2017, 2017, 1.

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Figure 1. Sample preparation workflows. (A) The SPOT workflow: an integrated, unified sample preparation strategy where data acquired from each omic platform stems from the same sample. (B) Separate preparations are performed independently of the others for each omic technology, requiring multiple sets of samples (e.g., refs 6, 18, 75, and 76).

Figure 2.

Comparison of the SPOT approach to a detergent-based lysis approach using untreated HL-60 cells. Comparisons are made for (A) unique peptides, (B) unique, quantified proteins, (C) estimated copies per cell for quantified proteins, (D) hydrophobicity, (E) isoelectric point, and (F) protein molecular weight (MW). (D-F) SPOT (orange line) represents the 671 proteins uniquely extracted by the SPOT method, and Proteomics (purple line) represents the 471 proteins uniquely extracted using the detergent-based approach (see panel B Venn diagram). For reference, UniProt (gray dashed line) shows these distributions for the 20,360 reviewed proteins downloaded from the human UniProt database on June 25th, 2018. Distributions were obtained using the PROMPT Protein Mapping and Comparison Tool, version 0.9.8.³⁰.

Figure 3.

Metabolomics assessment of the SPOT approach using HL-60 cells. (A) A reproducible number of metabolite compounds were detected using the SPOT preparation approach for a control sample set normalized to 75 μg of protein/sample. (B) Global metabolomics volcano plot analysis of all measured metabolites in HILIC and RPLC analyses observed upon Zn treatment comparing untreated and treated HL-60 cells; 3,033 measured metabolites of 25,068 were significant (fold change ≥ 2 and $p \leq 0.1$). (C) Principal component analysis of control (black) undifferentiated HL-60 cells and cells treated for 6 h with 250 μM Zn (red, 6 replicates).

Figure 4. Omics data collected for MOA analysis for Zn intoxication of HL-60 cells. (A) Proteomic data (unique, significant changes) over time. (B) Transcriptomic data (across time). (C) Metabolomic data were aligned across all time points and compared across HILIC and RPLC analyses, demonstrating an increase in molecular breadth when using both chromatographic platforms.

Figure 5.

Assessment of the highest abundance fold changes with Zn treatment. A pie chart shows the distribution of GO function assignments for the top ten proteins with the highest increasing abundance changes from 6 to 48 h.

Figure 6. Top proposed pathways resulting from network activity analysis using metabolomics measurements with Zn treatment. These data are supported by previously described genomic results.⁶⁹

Figure 7. Global mechanism summary. Gray up/down arrows depict findings from Zn-treated HL-60 cells. Pathway interactions are based on literature annotations.

Table 1.

SPOT Preparation of HL-60 Cells Using a Quantitative Experimental Design with Each Experiment Comparing Untreated to 6 h, 250 μ M Zn-Treated HL-60 Cells

note	instrument	amt. injected (μ g)	reps (treated, cont.)	unique peptides ^b	unique proteins ^b
<i>a</i>	Thermo Fusion Tribid	0.66	3, 3	23,760	3,873
	Thermo Fusion Tribid	0.66	3, 3	22,973	3,970
<i>a</i>	Bruker QTOF Impact II	0.66	3, 3	17,869	3,267
<i>a</i>	Bruker QTOF Impact II	1.32	3, 3	21,087	3,611

^aData come from the same set of samples analyzed on different instruments or with different injection amounts. Digestion was not automated for these samples.

^bValues represent quantified protein species that are unique by gene symbol and unique identified peptides.