Bead Assisted Mass Spectrometry (BAMS): A Robust Affinity Capture, MS Method for Multiplexed Biomarker Profiling

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INTRODUCTION

Proteomic studies that monitor protein and PTM abundance often employ multi-dimensional analytical methods such as nano-LC-ESI-MS/MS to simplify the inherent sample complexity and wide dynamic range of target molecules within a typical biological specimen.[2, 3] The sophistication of these analytical platforms can inhibit the technology adoption in programs for drug development or biomarker screening due to throughput limitations, data management thresholds and informatics processing needs required to adequately support these efforts. In this study, we present a method that integrates immunoaffinity purification (IAP) with MALDI TOF MS, which is capable of monitoring hundreds of target analytes in a microarray format, with as little as 5 µg of total protein, in a fraction of the time it takes for one LC-MS/MS acquisition.

METHODS

Cell Culture and Serum: MKN-45 and HeLa cells were prepared in RPMI media with 10% fetal bovine serum (FBS) and 1X Pen-Strep (Sigma, #P4333) to 75% confluence at 37 °C with 5% CO_2. Prior to chemical treatment, cells were serum starved in RPMI media with 0.2% FBS and 1% Pen/Strep for 12 hrs. SU11274 (SU) and staurosporine (ST) were used at a final concentration of 1 µM and 0.2 µM, respectively in 0.05% DMSO. Hydrogen peroxide (H_2O_2) was used at a final concentration of 2 mM with a 30 m pre-treatment of 0.1 mM sodium orthovanadate. Rapamycin treatment was carried out using 2 hours at 1 mM. Serum samples were obtained from commercial sources.

Western Blot Analysis: Protein concentrations for lysate supernatants were determined by Bradford assay using Coomassie Plus Protein Assay Reagent (Life Technologies, Carlsbad, CA, #23236), and protein amounts were normalized between samples. Samples were mixed with Laemmli sample buffer (BioRad, #1610747) and run on 4-15% Mini-Protean TGX precast gels (BioRad, #4561083). Proteins were transferred to mini nitrocellulose membranes (BioRad, #1704158) and blocked for 1 h at room temperature in 5% nonfat dry milk (Carnation) in TBST. Primary antibodies were incubated in 5% BSA in TBST overnight at 4°C. Membranes were washed 4 times with TBST, incubated with anti-rabbit secondary antibody conjugated with HRP (Cell Signaling Technology) for 1 h at room temperature in 5% milk TBST, washed 4 times with TBST and developed on the BioRad ChemiDoc Touch Gel Imaging System. Clarity Western ECL blotting substrate (BioRad, #1705060) was used as substrate for blot development.

Preparation of Protein Lysates and Digested Peptides: Cells were washed twice with cold PBS. PBS was removed and cells were scraped in Urea Lysis Buffer (9 M sequanal grade Urea, 20 mM HEPES pH 8.0, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 2.5 mM sodium pyrophosphate). Cells were sonicated 3 times for 20 s each at 15 W output power with a 1-minute cooling on ice between each burst. Sonicated lysates were centrifuged 15 min at 4 °C at 20,000× g. An aliquot of each supernatant was reserved for Western blotting and stored at −80 °C. Supernatants were collected and reduced with 4.5 mM DTT for 30 min at 40 °C. Reduced lysates were alkylated with 10mM iodoacetamide for 15 min at room temperature in the dark. Samples were diluted 1:4 with 0.2% ammonium bicarbonate (pH 8.0) and digested overnight with 10 µg/mL trypsin-TPCK (Promega) in 1 mM HCl. Digested peptide lysates were desalted over 360 mg SEP PAK Classic C18 columns (Waters, Richmond, VA, USA, #WAT051910). Peptides were eluted with 40% acetonitrile in 0.1% TFA, dried under vacuum, and stored at −80 °C.

Immunoaffinity Enrichment & MALDI Analysis: Protein A/G beads were prepared using NHS-activated XL magnetic agarose beads (400 micron, Cube Biotech) with Protein A/G (Abcam, 1 mg/ml) in PBS buffer. Antibodies (2 µg) were conjugated to 5 µL slurry of Protein A/G beads by overnight incubation in PBS with 0.1% BSA. Unbound antibody was removed with three 400 µL washes of PBS with 0.1% BSA. Individual target peptide enrichment was performed using 40 – 1000 µg of purified peptides with 1 – 5 beads. Multiplex target peptide enrichment was performed using 40 – 1000 µg of purified peptides with 1 – 5 beads/target. Peptides were incubated overnight at 4 °C. Beads were washed three times in PBS to remove non specific bound peptides. Removed all liquid from last wash and add 1.5 – 2.0 µL of matrix (10 mg/mL CHCA in 50% ethanol/water, 0.1% formic acid) to elute bound peptide(s) for 15 min at 25 °C. Spot 1uL of eluted peptides (in matrix) onto the MALDI plate. Allow to dry completely before MS analysis using a MALDI TOF instrument (Autoflex Speed, Bruker & SimulTOF ONE, SimulTOF).

METHODS

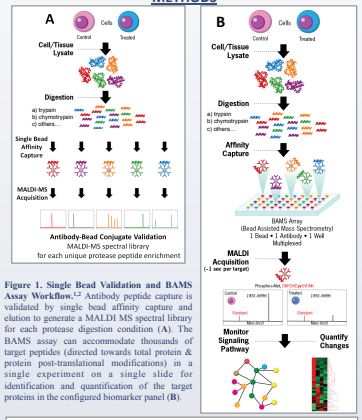

Figure 1. Single Bead Validation and BAMS Assay Workflow.[4,5] Antibody peptide capture is validated by single bead affinity capture and elution to generate a MALDI MS spectral library for each protease digestion condition (**A**). The BAMS assay can accommodate thousands of target peptides (directed towards total protein & protein post-translational modifications) in a single experiment on a single slide for identification and quantification of the target proteins in the configured biomarker panel (**B**).

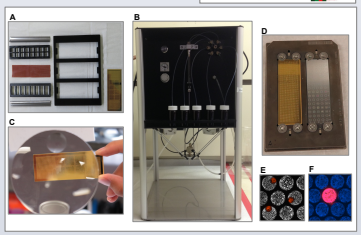

Figure 2. Apparatus & Components for BAMS Assay. The BAMS assay components include: ITO coated or gold slides, pico-well gasket, sample chamber gaskets, clamps and centrifuge adapter (**A**) Antibody beads are provided separately. The matrix sprayer provides optimized elution conditions for MALDI MS measurement (**B**). Eluted peptides on gold BAMS slide with 2,286 available assays (**C**). Slide adapter for BAMS slide and standard MALDI slide (**D**). Fluorescent labeled peptide on bead (**E**) & eluted peptide in pico-well (**F**).

RESULTS

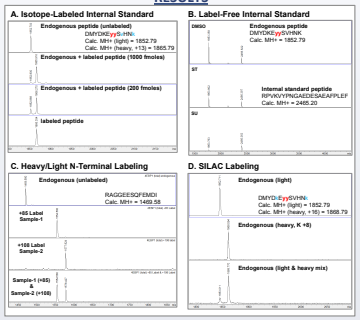

Figure 3. Multiple Modes of Quantitation for BAMS Assay. A) Internal Standard – Isotope Labeled Synthetic Peptide – AQUA, **B)** Internal Standard – Label-Free Synthetic Peptide (conserved epitope sequence with engineered DELTA MASS); **C)** Heavy/Light-End Labeling (fixed DELTA MASS), **D)** Stable Isotope Labeling by Amino Acids in Cell Culture, SILAC.

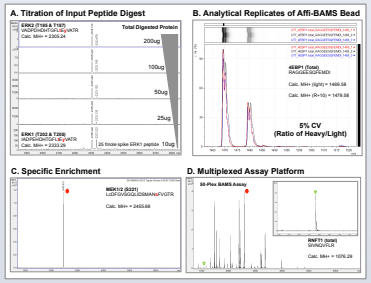

Figure 4. Sensitivity, Reproducibility, Specificity & Multiplexing of BAMS. A) Dilution of total input material with IP of endogenous target protein using BAMS, **B)** Analytical triplicate of Affi-BAMS assay for 4EBP1 (total) from cell lysate with heavy spiked control peptide, **C)** highly specific enrichment of MEK1/2 peptide from 200ug of digested cell lysate, **D)** Overlay of MALDI MS signal from triplicate 50-Plex BAMS assay from 200 ug of digested cell lysate, highlighting MEK1/2 (red) and RNFT1 (green).

Rapamycin Treatment of Gastric Carcinoma

Figure 5. Targeted Proteomics to Monitor Rapamycin Inhibition in MKN45 Cells using Affi-BAMS with SILAC quantitation. A) Control western blots for RPS6 & 4EBP1, known to be inhibited by rapamycin, **B)** Subset of targeted peptides/proteins from a 50-Plex BAMS assay for control (DMSO, light) and rapamycin (heavy, R+10 & K+8) treatment, **C-F)** MALDI MS signal of light-heavy SILAC pairs for a subset of the 50-plex BAMS assay along with observed fold-changes between control and rapamycin treatment. Expected mass difference between light & heavy pair is shown for the expected arginine and lysine composition.

Kinase Inhibition of Gastric Carcinoma[5]

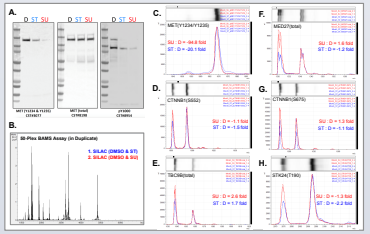

Figure 6. Targeted proteomics using Affi-BAMS & MALDI MS with SILAC labeling to Probe Different Kinase Inhibitors. A) Control western blots for total, pMET and global phosphotyrosine, **B)** Overlay of targeted peptides/proteins from a 50-Plex BAMS assay for control (DMSO, heavy) and SU (light) and ST (light) in duplicate, **C-H)** MALDI MS signal of light-heavy SILAC pairs for a subset of the 50-plex BAMS assay along with observed fold-changes between control and kinase inhibitor treatment (normalized to heavy control). An average CV of 6.5% was observed from the replicate fold-changes.

Targeted BAMS Assay in Serum[6]

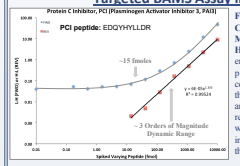

Figure 7. Forward and Reverse Curves for the PCI Peptide Measured in Pooled Normal Human Serum using BAMS. The endogenous concentration of the PCI peptide is revealed by the concentration of the plateau shown by the forward curve (FWD) and the analytical sensitivity of the assay is revealed by the reverse curve (REV) when conducted using SIS standards in triplicate. The average %CV for the analytical replicates was 15%.

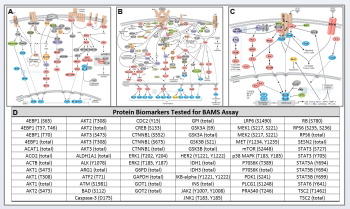

Figure 8. List of BAMS assays to monitor a wide variety of signaling networks. A sample of signaling pathways represented by proteins that have been configured for BAMS assays are shown in **Panels A – C**. A partial list of proteins, including total & phosphorylated targets, that have been configured for BAMS assays are shown in **Panel D**.

CONCLUSIONS

- BAMS assays have been configured to efficiently monitor close to 75 protein biomarkers from a variety of biological samples.
- The binding capacity of a single bead is sufficient to measure the abundance of a target protein-site within at least 2.5 orders of magnitude from as little as 5 ug of total protein.
- MALDI MS data acquisition of the BAMS assay enables one to rapidly survey key protein-site targets within critical signaling nodes.

REFERENCES

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