Multiplexed Analytical Platform using Affinity Capture and MALDI MS Enables Novel Assay Development for Screening Biomarkers in Neurological Diseases

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INTRODUCTION

Proteomic studies critically rely on multi-dimensional separation methods such as 2D-LC to simplify the complexity of protease digested biological specimens before subsequent MS & MS/MS to efficiently monitor multiple proteins of interest. The time and expertise required to implement LCMS methods can often be a barrier to using targeted proteomic applications in translational research. Here we present further development of a microarray analytical platform called Bead Assisted Mass Spectrometry (BAMS), which integrates multiplex immuno-affinity capture with MALDI MS to create customized targeted proteomic assays for translational biology research. We demonstrate efficient monitoring of several core Alzheimer's disease pathological biomarkers including tau and amyloid beta peptides with a sensitivity greater than that of LCMS as well as histone epigenetic modifications.

METHODS

CSF and Tissue Samples: Human brain and serum/plasma samples were obtained from Maine Medical Center Research Institute BioBank (Scarborough, ME). Human CSF samples were obtained from Johns Hopkins School of Medicine, Alzheimer's Disease Research Center (Baltimore, MD).

Preparation of Protein Lysates and Digested Peptides: Tissue was pulverized under liquid nitrogen using a a Bessman press, and transferred into Urea Lysis Buffer (ULB, 8 M Urea, 20 mM HEPES pH 8.0, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 2.5 mM sodium pyrophosphate). The tissue slurry was homogenized using a mini-beadbeater and sonicated 3 times for 20 s each at 15 W output power with a 1 min cooling on ice between each burst. Lysates were centrifuged 15 min at 4 °C at 20,000× g to remove insoluble debris. CSF was added to dry aliquots of RapiGest (Waters) and ammonium bicarbonate (pH8.0) to a final concentration of 0.1% and 50 mM, respectively. Soluble protein was reduced with 4.5 mM DTT (30 min, 40 °C) and alkylated with 10 mM iodoacetamide (15 min, RT) in the dark. Samples were diluted 1:4 with 200 mM ammonium bicarbonate (pH 8.0) and digested overnight with trypsin-TPCK (1:75, w:w, Promega) in 1 mM HCl. Other protease digestions were performed using the manufacturer's recommended protocol (LysC, AspN, GluC, ArgC, Promega and NEB). Digested peptide lysates were desalted over 360 mg SEP PAK Classic C18 columns (Waters, Richmond, VA, USA, #WAT051910). Peptides were eluted with 50% acetonitrile in 0.1% TFA, dried under lyophilization conditions, and stored at −80 °C in 0.1 – 1.0 mg aliquots. TAU and H3K9acetyl BAMS assays were performed using 100 µg of protein from human brain. Beta-amyloid BAMS assay was performed using 100 µL of undigested CSF.

BAMS Assay - Bead Preparation: Each antibody was conjugated to NHS-activated magnetic agarose beads in a 10 µL slurry (7 µg of antibody/100 beads, 375 - 420 micron diameter) in PBS buffer, for 3-12 h (4 °C), and quenched with Tris HCl (100 mM, pH 8.0) for 1 h at RT. Unbound antibody was removed with three 400 µL washes of cold PBS (2 min, 4 °C). Beads were stored in PBS and 0.02% sodium azide (4 °C).

BAMS Assay – Target Peptide Binding: Target peptide enrichment was performed using 10 – 1000 µg of digested peptides (or soluble protein) with typically 3 replicate beads/target. Multiplex peptide enrichment was performed using 10 – 1000 µg of purified peptides (or soluble protein) with typically 3 replicate beads/target in a volume of 50 – 200 µL. Peptides and affinity capture beads were incubated in binding buffer (1M KCl, 100 mM Tris HCl in deionized water, pH 8.0) for a period of between 3 – 12 hrs at 4 °C in Thermomixer (Eppendorf). Beads were washed sequentially in PBS (700 uL), ammonium bicarbonate (700 uL, 10 mM, pH 8.0) and deionized water (700 uL) to remove any nonspecific bound peptides (2 min, 4 °C).

BAMS Assay – Target Peptide Elution: Washed BAMS beads are transferred to the hydrated wells to settle into the micro-wells of the BAMS plate assembly with gentle agitation and centrifugation (5 min, 200 x g). After centrifugation, the sample chamber gasket is removed, leaving the micro-well gasket fixed in place on the slide. The bead array is exposed to an aerosol of elution buffer using a Matrix Sprayer, containing 0.5 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile and 0.4% trifluoroacetic acid (TFA) for approximately 15 min. Once the matrix is dry, the silicone gasket is lifted off the slide and any remaining dry agarose beads are removed by compressed air leaving an array of spots containing purified and concentrated target peptides for subsequent MALDI MS measurement.

MALDI TOF Linear Instrument Settings: Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) MS data was acquired on Bruker Daltonics (Billerica MA) Autoflex Speed or rapifleX MALDI-TOF/TOF mass spectrometer using FlexControl software. Unless otherwise indicated, the autoflex speed acquisition conditions in the positive linear mode were, 750-7000 m/z mass range (2 kHz, 10000 spectra/spot using the random walk method). The voltage settings were 19.50 kV (ion source 1), 18.35 kV (ion source 2) and 6.0 kV (lens). The pulsed ion extraction was 130 ns. The detector gain voltage was 4.0X or 2910 V. The acquisition settings on the rapifleX for automated runs in positive linear mode were 700-7000 m/z mass range, 10 kHz laser repetition rate, 4000 laser shots.

MALDI TOF Analysis: Mass spectra were processed and analyzed using FlexAnalysis software. Peaks were detected that had a signal-to-noise ratio of at least 3. In some cases, baseline subtraction procedure was applied to individual mass spectra. Unless otherwise indicated, peaks in the mass spectra are labeled using either average or monoisotopic m/z values using the peak picking algorithms available in the software. mMass was utilized for data review.

[1] G.M.Hamza, V.B.Bergo, S.Mamaev, D.M.Wojchowski, P.Toran, C.R.Worsfold, M.P.Castaldi & J.C.Silva, *International Journal of Molecular Sciences*, (2020) 21(6):1-36 (https://www.mdpi.com/1422-0067/21/6/2016).

[2] US patent 9,618,520 by inventor V.Bergo, titled *Devices and methods for producing and analyzing microarrays*

[3] US patent 10,101,336 by inventor V.Bergo, titled *Eluting analytes from bead arrays*

[4] US patent application 16/125164 by inventor V.Bergo, titled *Multiplexed bead arrays for proteomics*

METHODS

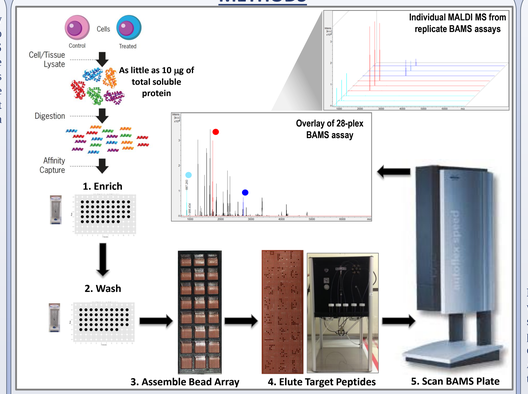
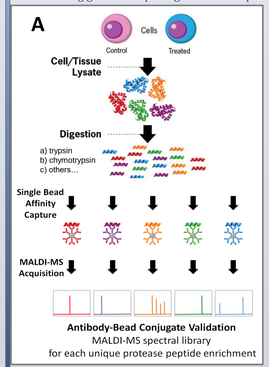


Figure 1. BAMS Workflow for Targeted Proteomics. Standard bottom-up methods are used to generate peptides for targeted monitoring of proteins using BAMS (lysis, reduction, alkylation, digestion). Digested peptides are incubated overnight with BAMS affinity capture beads in Eppendorf tube or 96-well plate. For middle-down applications, such as CSF, beads are incubated directly for peptide binding (**1**). Magnetic agarose beads are transferred, sequentially into wash buffers (PBS, ammonium bicarbonate, DDW) before placed onto the BAMS slide (**2**). Washed beads are transferred onto the BAMS slide to settle beads into pico-wells (**3**), captured peptides from each BAMS bead are eluted into the pico-well using a matrix sprayer (**4**), eluted, dry peptides are analyzed after disassembling gaskets and placing into slide adapter for MALDI MS measurement (**5**).



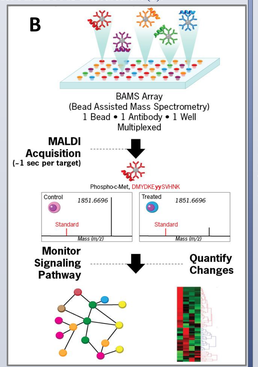
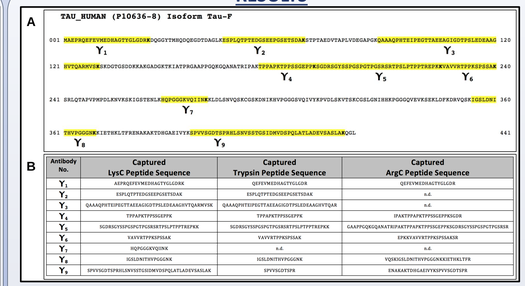


Figure 2. BAMS Assay Validation. BAMS affinity beads are individually validated by localized peptide elution and direct, MALDI MS measurement of the target peptide. Targeted MS/MS acquisition can be performed to verify the sequence of the captured peptide(s). A MALDI MS spectral library is generated for each protease digestion condition with each Affi-BAMS bead (**A**). The BAMS assay can accommodate thousands of target peptides (unmodified & protein PTM) in a single experiment on a slide for identification and quantification of the target proteins in the configured assay panel (**B**).

RESULTS


A
TAU_HUMAN (P10636-8) Isoform Tau-F
001 MAEPRQEFEVMEDHAGTYGLGDRKDQGGYTMHQDQEGDTDAGLKESPLQTPTEDGSEEPGSETSDAK
Y1 Y2 Y3
121 KVTQAKVVDAVSEKIDQTGSDDKKAKGADGKTKIATPRGAAPPQGQKGANATRIPAKTPPAPKTPPSSGEPPKSGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSAK
Y4 Y5 Y6
241 SRLQTAPVPMPDLKNVKSKIGSTENLKHQPGGGKVQIINKKLDLSNVQSKCGSKDNIKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSLDNI 360
Y7
361 TEVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYKSPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAKQGL
Y8 Y9 441

B

Antibody No.	Captured LysC Peptide Sequence	Captured Trypsin Peptide Sequence	Captured ArgC Peptide Sequence
Y_1	AEPRQEFEVMEDHAGTYGLGDR	QEFEVMEDHAGTYGLGDR	QEFEVMEDHAGTYGLGDR
Y_2	ESPLQTPTEDGSEEPGSETSDAK	ESPLQTPTEDGSEEPGSETSDAK	n.d.
Y_3	GAAADPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQAR	GAAADPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQAR	n.d.
Y_4	TPPAPKTPPSSGEPPK	TPPAPKTPPSSGEPPK	IPAKTPPAPKTPPSSGEPPKSGDR
Y_5	SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKK	SGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKK	GAAPPGQKGANATRIPAKTPPAPKTPPSSGEPPKSGDRSGYSSPGSPGTPGSRSR
Y_6	VAVVRTPPKSPSSAK	VAVVRTPPKSPSSAK	EPKKVAVVRTPPKSPSSAKSR
Y_7	HQPGGGKVQIINK	n.d.	n.d.
Y_8	IGSLDNITHVPGGGNK	IGSLDNITHVPGGGNK	VQSKIGSLDNITHVPGGGNKKSITHKLTFR
Y_9	SPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAK	SPVVSGDTSPR	EKAAKATDHGAEIVYKSPVVSGDTSPR

Figure 3. Regions of TAU Protein Configured for BAMS Assay. TAU protein sequence (isoform Tau-F) with yellow highlighted areas corresponding to LysC peptide sequences captured by BAMS assay (**A**) shown in Figure 4. List of 8 antibodies configured for BAMS assays for the TAU protein. Alternative proteases can be used to monitor the designated regions of TAU as specified by the antibody. Validated captured peptide sequences are shown for LysC, Trypsin and ArgC protease digestion conditions (**B**). Alternative digestions conditions can be used to monitor areas of the protein outside the epitope region if the resulting proteolytic peptide preserves the epitope sequence for efficient antibody binding.

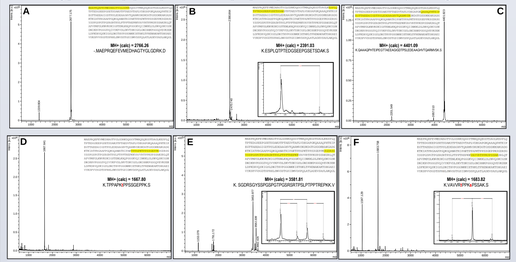


Figure 4. TAU BAMS Assays with LysC Digested Brain Sample. A) N-terminus of Tau (aa1-24) with loss of methionine and acetylation at the n-terminal alanine, **B)** N-terminus of Tau (aa45-67), including a phosphorylated site within the amino acid region 45-67, **C)** Internal TAU (aa88-130), **D)** Phosphorylated TAU (pT181) containing amino acids 175-190. **E)** Unmodified and phosphorylated TAU within amino acids 191-224, a region containing multiple sites of phosphorylation (pS198, pS199, pS202 & pT205). **F)** Phosphorylated TAU (pT231) with nonphosphorylated and phosphorylated LysC peptide readout.

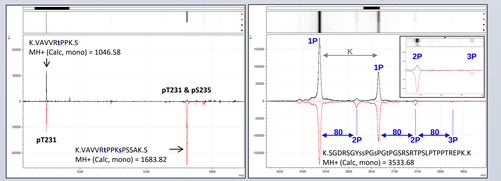


Figure 5. TAU BAMS Assays with Normal (BLACK) and Disease (RED) Brain. A) Mono- and Di-phosphorylated TAU (singly pT231 and doubly pT231 & pS235 phosphorylated), Normalized to pT231, **B)** Phosphorylated TAU containing amino acids 191-224 containing multiple sites of phosphorylation (pS198, pS199, pS202 & pT205), normalized to singly phosphorylated pS199.

RESULTS

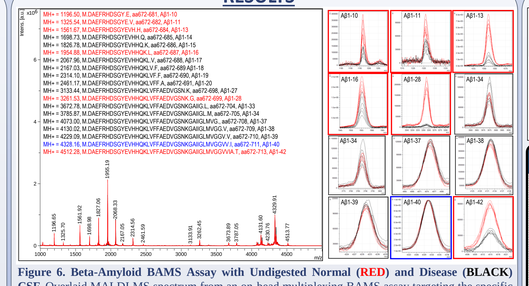


Figure 6. Beta-Amyloid BAMS Assay with Undigested Normal (RED) and Disease (BLACK) CSF. Overlaid MALDI MS spectrum from an on-bead multiplexing BAMS assay targeting the specific region of beta amyloid spanning from aa672-aa713 from triplicate bead assays of four patients (2 normal & 2 disease). The multiplex assay retrieves 19 specific beta-amyloid peptides between aa672-aa713. Normalizing MS spectra to the Aβ1-40 peptide (**BLUE BOX**), clear separation of NORMAL and DISEASE patient samples is seen for specific beta-amyloid peptides (**RED BOX**).

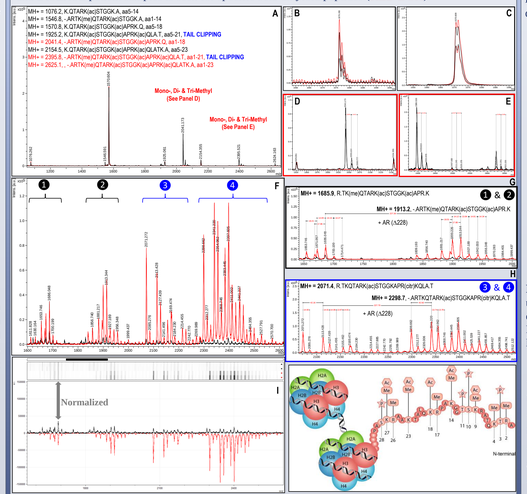


Figure 7. Histone H3 K9acetyl BAMS Assay with Normal (BLACK) and Disease (RED) Brain. MS spectra from the H3K9acetyl BAMS assay of LysC digested normal and disease brain (**TOP, PANELS A-E**). Captured targets include H3K9-acetylated peptides within aa1-23 of the H3 tail. Absence of mono-, di- and tri-methylated H3K9acetyl peptides (2041, 2395 & 2625) are observed in diseased brain (**D & E**). MS spectra from H3K9acetyl BAMS assays with equal quantities (100 µg) ArgC digested normal and disease brain (**BOTTOM, PANELS F-I**). Captured targets include H3 K9-acetylated peptides within aa3-21 of the H3 tail, including combinatorial H3 tail peptides with acetylation, methylation and deimination of arginine (from conversion of arginine to citrulline), all containing lysine acetylation at K9 as directed by the site specific affinity capture of the BAMS assay.

RESULTS

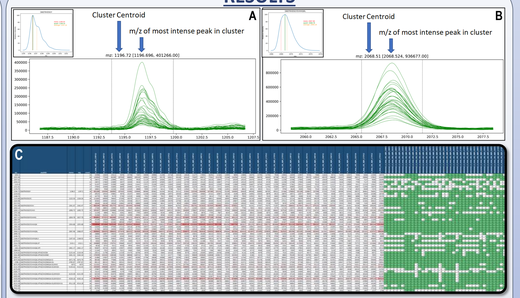


Figure 8. Optimized Assay Data Processing. Spectral features are extracted from each MALDI MS as centroids and clustered into mass bins. A reference library for the specific BAMS assay is then generated by assigning each peptide identification to the corresponding measured mass and quantitation is performed using the apex centroid within each clustered centroid group (**A-B**). Unassigned clustered centroids can be flagged for further characterization, (**C**).

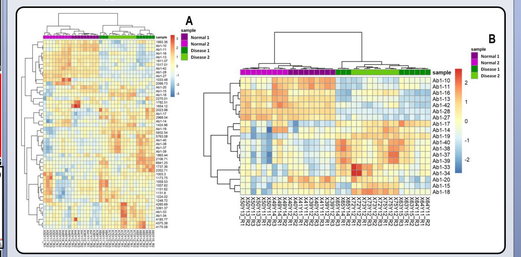


Figure 9. Hierarchical Clustering of Beta-Amyloid BAMS Assay Features. Hierarchical clustering using all standardized features (regardless of identification) from the multiplexed BAMS assays yields an overall grouping by disease status (Normal vs. Disease) with internal grouping by biological replicate (**A**). Focusing on features with known assignments to Beta-amyloid fragments further improves the separation of Normal vs. Disease groups (**B**).

CONCLUSIONS

- BAMS assays have been configured to enable multiplexed MALDI MS monitoring of both unmodified and phosphorylated sites within TAU in human brain & CSF samples.
- A single on-bead, multiplex BAMS assay has been configured monitor up to 18 Beta-Amyloid fragments in undigested CSF.
- BAMS assays have been configured for epigenetic applications to monitor Histone H3 K9-acetylation and associated combinatorial PTMs.
- Data processing of BAMS assays is facilitated using software that performs peak detection, clustering, and export of spectral features for library matching and statistical analysis.
- BAMS assays can be utilized to perform a wide range of targeted proteomic applications and is amenable for high-throughput screening.

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