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Activity-Based Protein Profiling (ABPP) and Click Chemistry (CC)-ABPP by MudPIT Mass Spectrometry

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Abstract

Activity-based protein profiling (ABPP) is a chemical proteomic method for functional interrogation of enzymes within complex proteomes. This Unit presents a protocol for in vitro and in vivo labeling using ABPP and Click Chemistry (CC)-ABPP probes for in-depth profiling using the Multi-dimensional Protein Identification Technology (MudPIT) analysis platform.

Keywords

Activity-Based Protein Profiling; ABPP; Click Chemistry; Mass Spectrometry; MudPIT; Activity-Based Probes; Biotin; Alkyne; Azide

INTRODUCTION

The field of proteomics aims to characterize and assign function to the tens of thousands of eukaryotic and prokaryotic proteins annotated by genome sequencing efforts. In contrast to global platforms such as 2D gel electrophoresis (Patton et al. 2002), shotgun LC-MS analysis (Gygi et al. 1999; Washburn et al. 2001), yeast two-hybrid screening (Ito et al. 2002), and protein microarrays (MacBeath 2002) that analyze proteins based on abundance, ABPP (Cravatt et al. 2008; Jessani and Cravatt 2004) is a chemical proteomic strategy for the analysis of enzyme function within complex biological systems. ABPP utilizes active site-directed chemical probes consisting of two elements: 1) an active-site-directed reactive group for binding and covalently labeling a specific subset (or family) of catalytically-related enzymes, and 2) a reporter tag (e.g., fluorophore or biotin) for detection/quantification and/or enrichment/identification of labeled enzymes. Because ABPP probes selectively label active enzymes, but not their inactive forms (Jessani and Cravatt 2004; Jessani et al. 2002), they allow monitoring of changes in enzyme activities resultant from post-translational modification and/or protein-protein/protein-small molecule interactions that occur without corresponding changes in protein abundance or mRNA expression (Kobe and Kemp 1999). To date, ABPP probes have been generated for more than a dozen enzyme classes (Cravatt et al. 2008; Evans and Cravatt 2006; Paulick and Bogoy 2008).

Because most ABPP probes have limited cell permeability due to their bulky reporter tag, ABPP protocol typically involves homogenization of the proteomic source (e.g., cells, tissue) prior to labeling, which disrupts the native cellular environment, potentially compromising the endogenous activity of certain enzymes. For applications requiring the interrogation of enzyme activities in living cells or organisms, the reporter group is substituted with a small, latent chemical handle (alkyne or azide), which does not impede

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cell permeability. An orthogonally-functionalized reporter tag is then appended to the probe post homogenization using click chemistry – specifically, the Cu(I)-catalyzed stepwise version of Huisgen's azide-alkyne cycloaddition (Kolb and Sharpless 2003).

Following labeling, proteins are enriched using immobilized streptavidin and subject to on-bead trypsin digestion. As outlined in Figure 1, the resulting peptides are then separated by multi-dimensional liquid chromatography and analyzed by tandem mass spectrometry (MudPIT), which provides both protein identifications and an estimation of labeled (active) protein abundance using semi-quantitative methods such as spectral counting (Alexander and Cravatt 2006; Jessani et al. 2005; Sieber et al. 2006). The biotinylated active-site peptides still bound to the beads can also be eluted and analyzed by MudPIT to identify sites of probe labeling (Figure 1: box).

For standard ABPP using biotinylated probes, follow Basic Protocol 1; for profiling enzyme activities in living cells or mice using alkyne probes, use Alternate Protocol 1. Both of these protocols will generate labeled proteomic samples for enrichment, digestion and MS analysis according to Basic Protocol 2. Support Protocol 1 details a general method for preparation of membrane and soluble proteomic samples from cultured cells or tissues. Support Protocol 2 provides a detailed method for methanol-chloroform precipitation following the CC reaction, which can be used if incomplete precipitation of proteins is observed.

Basic Protocol 1: LABELING ENZYMES IN VITRO BY ABPP

The following is a standard protocol for in vitro labeling of cell or tissue homogenates using biotinylated ABPP probes. A no-probe control sample should always be prepared for comparison to the experimental sample. Note: this protocol specifies the use of Tris buffer, however, PBS may be substituted. If using other buffer conditions, ensure compatibility of ABPP probe labeling reaction and enrichment protocol.

Materials

Biotinylated ABPP probe
Proteome source (e.g., cells or tissue)
50 mM Tris-HCl, pH 8.0 (store 4 °C for months)
1x PBS, pH 7.4 (Gibco, store 4 °C for months)
100x probe stock (0.5–2 mM) in DMSO (store –20 or –80 °C for years)
DMSO
Triton X-100
10DG disposable chromatography columns (BioRad)
10% (w/v) SDS in water

Sample Labeling

- 1 Aliquot 2×1 mL (for experimental and control samples) of 1 mg/mL cell or tissue homogenate (see Support Protocol 1) into microcentrifuge tubes.
- 2 To the experimental sample add 10 μ L of 100x probe stock, giving a final concentration of 5–20 μ M. To the control sample add 10 μ L of DMSO.

Optimal probe concentration depends on proteome source and probe reactivity. Unless otherwise noted, all subsequent steps are the same for both samples.

- 3 Vortex samples and incubate for 1 hr at rt.

Removal of Excess Probe

- 4 If samples are membrane fractions, add 100 μ L of 10% Triton X-100, giving a final concentration of 0.9%, and rotate for 1 hr at 4 $^{\circ}$ C.
- 5 Transfer each sample to a 15 mL conical and bring volume up to 2.5 mL with Tris.
- 6 Apply sample to 10DG column (pre-equilibrated with 25 mL Tris) and discard flow-through. Elute proteins with 3.5 mL Tris and collect eluate in 15 mL conical.
- 7 Add 184 μ L of 10% SDS, giving a final concentration of 0.5%.
- 8 Divide each sample eluate into 5x $\sim$ 750 μ L aliquots in microcentrifuge tubes. Heat aliquots for 8 min at 90 $^{\circ}$ C.

Caution: loosen caps before heating.

- 9 Recombine respective aliquots and allow samples to cool to rt. Proceed directly to Basic Protocol 2 or freeze samples at -20° C.

Alternate Protocol 1: LABELING ENZYMES IN LIVING CELLS OR MICE BY CC-ABPP

This protocol details general methods for the in situ labeling of cells in culture or the in vivo labeling of mice via i.p. injection using a CC-ABPP probe bearing a bio-orthogonal alkyne; following homogenization (Support Protocol 2) a biotin-azide tag is then appended using CC. Notes: 1) A no-probe control sample should always be prepared for comparison to the experimental sample; 2) if labeling enzymes with an azide probe and conjugating to a biotin-alkyne tag, the same protocol may be followed; 3) if labeling cell homogenates using CC probes, label experimental sample aliquoted in step 5 (below) with 5–20 μ M probe-alkyne for 1 hour (using the same volume of DMSO for the control), and proceed directly to step 6.

Additional Materials

Cells in culture or laboratory mice

1–5 mg/mL probe-alkyne in vehicle (see recipe)

1000 $\times$ probe-alkyne stock (5–25 mM) in DMSO (store at -20° C for years)

5 mM biotin-azide (PEG4 carboxamide-6-azidohexanyl biotin, Invitrogen) in DMSO (store at -20° C for years)

1 mM TCEP in water (Fluka, prepare fresh prior to use)

1.7 mM TBTA (Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine; Aldrich) in 4:1 t-butanol/DMSO, see recipe)

50 mM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in water (store at rt for months, remake if ppt forms)

2.5% (w/v) SDS in PBS (store at rt for months)

MeOH

Probe sonicator

Proteome Labeling

In Cells

1. Grow cells to 80% confluency in appropriate medium (e.g., RPMI-1640 supplemented with 10% fetal calf serum).
For a typical experiment use 1–2 15 cm plates for each experimental and control sample; before labeling, wash cells and add 5 mL fresh media.
2. To experimental sample add 5 μ L of 1000x probe-alkyne stock, giving a final concentration of 5–25 μ M; swirl plate to mix. To control sample add 5 μ L of DMSO, swirl plate to mix.
3. After incubating for 1 hr (or longer, as desired, typically not exceeding 24 hrs) at 37 °C, remove media, wash cells 2 $\times$ PBS, and harvest cells in ~5 mL PBS using a cell scraper. Collect cells in a 15 mL conical.
4. Centrifuge cells for 5 min at 1000 $\times$ g, 4 °C or rt, to pellet. Remove supernatant. Process cells (see Support Protocol 1) for biotin-azide conjugation, or store pellet at –80 °C (for months).

In Mice

- 1 Determine desired dosage (typically 10–50 mg/kg) and prepare the corresponding solution (1–5 mg/mL) probe-alkyne in vehicle.
- 2 Weigh mice. Administer experimental mouse an intraperitoneal (i.p.) injection of 10 μ L/g probe-alkyne in vehicle. Administer control mouse an i.p. injection of 10 μ L/g vehicle only.
- 3 After one hour, sacrifice mice according to approved animal protocol and harvest tissue.
- 4 Snap-freeze tissue on dry ice. Process tissue (see Support Protocol 1) for biotin-azide conjugation, or store samples at –80 °C (for months).

Biotin-azide Conjugation via CC

- 5 For each experimental and control sample, aliquot 2 $\times$ 0.5 mL of 2 mg/mL cell/tissue homogenate (prepared in PBS, see Support Protocol 1) into microcentrifuge tubes.

The CC reaction works best in PBS. Avoid using buffers containing amines as they interfere with the Cu(I)-stabilizing TBTA ligand. The CC reaction is less efficient with starting reaction volumes greater than ~0.5 mL.

- 6 Add the following reagents and vortex sample after each addition:
11.3 μ L of 5 mM biotin-azide, giving a final concentration of 100 μ M.
11.3 μ L of 50 mM TCEP, giving a final concentration of 1 mM.
34.0 μ L of 1.7 mM TBTA, giving a final concentration of 100 μ M.
11.3 μ L of 50 mM CuSO₄·5H₂O, giving a final concentration of 1 mM.
- 7 Incubate at rt for 1 hr, vortexing after 30 min.
The majority of proteins will precipitate during this reaction.

Removal of Excess CC Reagents

- 8 Combine respective experimental and control aliquots, and centrifuge 4 min at $6500 \times g$, 4°C to pellet protein. Remove supernatant.
- 9 Add 750 μL cold MeOH and sonicate for 3–4 sec using a probe sonicator (~30% power level) at 4°C to re-suspend protein. Centrifuge for 4 min at $6500 \times g$ at 4°C and remove supernatant. Repeat methanol wash $2\times$.

If protein loss is observed after washes, perform a methanol:chloroform precipitation (see Support Protocol 2) and continue with next step.
- 10 Add 0.65 mL of 2.5% SDS in PBS to the protein pellet and sonicate (3×5 sec).

At this point, may store sample at -20°C or proceed to next step of protocol.
- 11 Heat sample for 5 min at 60°C and repeat sonication to re-suspend pellet. Centrifuge for 4 min at $6500 \times g$ to pellet any un-solubilized protein, and remove supernatant to clean 15 mL conical.

If pellet larger than ~20% of original pellet is observed (indicating a substantial amount of protein has not been re-solubilized), repeat heating and sonication steps. If necessary, heat for 2–5 min at 90°C .
- 12 Bring volume up to 3.5 mL with PBS, giving a final SDS concentration of ~0.5%. Freeze sample overnight at -20°C or proceed to Basic Protocol 2.

Support Protocol 1: PREPARATION OF CELLS/TISSUE HOMOGENATES

The following is a general protocol for preparing membrane and soluble fractions from cells or tissue.

Additional Materials

Harvested cells/tissue

Dounce homogenizer

Probe sonicator

Ultracentrifuge

1. For preparation of cells, resuspend cell pellet in ~4x volume of PBS on ice and homogenize using a Douncer (10–15 strokes) or probe sonicator ($2-4 \times 10$ sec at ~30% power) on ice. For preparation of tissue, mince tissue with razor blade on ice (e.g., on ice-cold glass plate or large tissue culture dish) and Dounce homogenize (15–20 strokes) in ~4x volume $1\times$ PBS on ice.
2. Centrifuge for 5 min at $1000 \times g$, 4°C , to pellet nuclei and unbroken cells.
3. Remove supernatant to clean ultracentrifuge tube and centrifuge for 1 hr at $100,000 \times g$, 4°C , to separate membrane (pellet) and soluble (supernatant) fractions. Remove supernatant to clean tube. Wash pellet with cold PBS and resuspend in cold PBS. Quantify protein concentration in membrane and soluble fractions and adjust to desired concentration (1–2 mg/mL).

For example, quantification may be accomplished using the DC protein assay (Bio-Rad) according to manufacturer's protocol.
4. Aliquot samples and store at -80°C (for 6–9 months, avoid freeze-thaw cycles).
5. Prior to ABPP probe labeling, thaw sample on ice and briefly allow to warm to rt.

Support Protocol 2: METHANOL/CHLOROFORM PRECIPITATION

If incomplete protein precipitation is observed following the CC reaction (e.g., if a protein of interest remains in solution or if detergents are used during the CC reaction), this protocol can be used where indicated in Alternate Protocol 1.

Additional Materials

MeOH

CHCl₃

1. At the end of the CC reaction, combine aliquots for respective samples into 15 mL conicals, giving ~1 mL final volume.
2. Add 4 mL of MeOH and 1 mL of CHCl₃ and vortex the sample. Add 3 mL of water and vortex the sample again. Centrifuge for 15 min at 4000 × g, 4 °C. Remove top and bottom layers, leaving behind the precipitated protein.

After centrifugation, protein will precipitate at the interface of the aqueous and organic layers.

3. Transfer protein to microcentrifuge tube in MeOH (2 × 300 μL). Add 150 μL CHCl₃ and vortex the sample. Add 600 μL of water and vortex the sample again. Centrifuge for 5 min at 9000 × g and remove top and bottom layers.
4. Add 600 μL of MeOH and sonicate for 5–10 sec with probe sonicator to resuspend (but not resolubilize) protein.

Once fully precipitated, addition of methanol is much less likely to result in protein resolubilization.

5. Centrifuge for 5 min at 9000 × g to pellet protein. Remove supernatant.
6. Proceed with Step 10 of Alternate Protocol 1 (addition of 0.65 mL 2.5% SDS in PBS)

Basic Protocol 2: ENRICHMENT AND DIGESTION OF PROBE-LABELED PROTEINS

This protocol is used to generate streptavidin enriched tryptic digests and probe-labeled peptides for MudPIT analysis using the labeled proteomes obtained from Basic/Alternate Protocol 1. Note: it is not necessary to complete the section entitled *elution of labeled peptides* unless analysis of probe labeling sites is desired.

Materials

1x PBS, pH 7.4 (Gibco)

Immunopure immobilized streptavidin (Pierce)

1% (w/v) SDS in PBS (store at rt for months)

6 M Urea in PBS (make fresh prior to use)

Micro Bio-Spin Chromatography Columns (Bio-Rad)

Low-adhesion screw-top microcentrifuge tubes (Sarstedt)

200 mM DTT in water (make fresh daily, or store aliquots at –20 °C for months)

500 mM IAA in water (make fresh daily, or store aliquots at –20 °C for months)

2 M Urea in PBS (make fresh prior to use)

- 100 mM CaCl₂ in water (store at rt for months)
- Sequence-grade modified trypsin (Promega)
- 90% formic acid (Sigma-Aldrich)
- 50% (v/v) MeCN/0.1% TFA in water (make fresh prior to use)
- 5% (v/v) FA in water

Streptavidin Enrichment

- 1 To the ~3.5 mL volume of SDS-solubilized protein from Basic Protocol 1 or Alternate Protocol 1, add 5 mL PBS to bring volume to 8.5 mL, giving a final SDS concentration of 0.2%.
- 2 Add a 50 μ L aliquot (equal to 100 μ L of a 50% slurry) of streptavidin beads that have been pre-washed in a biospin column with PBS (3 $\times$ 1 mL).

Use a clean razor blade to cut off end of standard pipette tip to generate a wide-bore tip for transferring beads.
- 3 Rotate samples for 1–1.5 hrs at rt. Centrifuge sample for 2 min at 1400 $\times$ g to pellet beads. Remove ~95% of supernatant. With remaining supernatant, transfer beads to biospin column. Use a small volume of PBS to transfer any remaining beads to biospin column.

Make sure to label columns before transferring beads. Beads should not be vortexed, as they may break apart.
- 4 Wash the beads with the following solutions:

1% SDS (3 $\times$ 1 mL)

6 M Urea (3 $\times$ 1 mL)

PBS (5 $\times$ 1 mL)
- 5 Transfer beads in PBS to a low-adhesion screw-top tube.

Screw-top prevents sample loss during overnight incubation at elevated temperature, low-adhesion minimizes sample adhesion.
- 6 Centrifuge 2 min at 1400 $\times$ g (or pulse in minifuge) to pellet beads. Remove supernatant with a gel-loading tip.

Use a gel-loading tip or other fine-bore tip to minimize bead loss.

On-bead Reduction, Alkylation, and Digestion

- 7 Resuspend beads in 500 μ L of 6 M Urea in PBS. Add 25 μ L of 200 mM DTT, giving a final concentration of 10 mM, and heat at 65 $^{\circ}$ C for 15 min.

May substitute TCEP (25 μ L of 200 mM solution, made fresh prior to use) for DTT and rotate at rt for 30 min (do not heat).
- 8 Add 25 μ L of 500 mM IAA, giving a final concentration of 25 mM, and rotate for 30 min at rt under foil. Centrifuge for 2 min at 1400 $\times$ g. Remove supernatant. Wash bead with PBS (1 $\times$ ~1 mL).
- 9 Add the following reagents to the sample:

200 μ L of 2 M Urea in PBS

2 μ L of 100 mM CaCl_2 , final $[\text{CaCl}_2]$ of 1 mM

4 μ L of 0.5 mg/mL trypsin (2 mg total) in re-suspension buffer

- 10 Rotate or shake overnight at 37 °C.

To rotate, use incubator with rotisserie or plug table-top rotator into incubator with internal outlet; if using an enclosed shaker, set on medium speed and lay tube flat rather than upright.

Elution of Tryptic Peptides for MS analysis

- 11 Centrifuge samples for 2 min at $1400 \times g$ and transfer beads and supernatant to a biospin column. Elute solution (containing tryptic peptides) into clean microcentrifuge tube. Transfer any remaining solution/beads into the biospin column with PBS (100 μ L) and elute into the same collection tube, giving a total volume of 300 μ L. Save the experimental sample beads if following the *elution of labeled peptides* protocol below.
- 12 To the eluate containing the tryptic peptides, add 17 μ L of 90% FA, giving a final concentration of 5%. Proceed directly to MS analysis or store tryptic digest samples at -80°C (will keep for several months or more).
- 13 Analyze by MudPIT. For additional details, see Current Protocols in Protein Science, Unit 23.1, and (Weerapana et al. 2007), protocol for analysis of tryptic peptides.

Elution of Labeled Peptides for MS analysis (optional)

- 14 Wash beads in biospin column with PBS (5×1 mL) and water (5×1 mL). Transfer beads to clean low-adhesion tube in water, spin briefly using a minifuge, and remove supernatant.
- 15 To elute labeled peptides, add 150 μ L of 50% MeCN/0.1% TFA and heat for 2 min at 65°C . Transfer beads back to biospin column, and elute into clean low-adhesion tube. Add an additional 100 μ L of 50% MeCN/0.1% TFA to biospin column and elute into same collection tube. Speedvac sample to dryness.
- 16 Resuspend peptides in 100 μ L of 5% FA and vortex 5 min. Proceed directly to MS analysis or store samples at -80°C (will keep for several months or more).
- 17 Analyze by MudPIT. For additional details, see Current Protocols in Protein Science, Unit 23.1, and (Weerapana et al. 2007), protocol for analysis of labeled peptides.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for all aqueous solutions used in this unit; for suppliers, see SUPPLIERS APPENDIX.

1–5 mg/mL probe-alkyne in vehicle (prepare fresh prior to use)

1. Prepare a solution of 18:1 (v/v) saline:emulphor as follows: Prepare a saline solution of 1% (w/v) NaCl in water. Add emulphor to give a ratio of 18:1 (v/v) saline:emulphor (store at rt for days or 4°C for weeks).
2. Determine dosage of probe-alkyne (typically 10–50 mg/kg) to be administered. Dissolve probe-alkyne in EtOH at a concentration of X mg/mL, where $X = \# \text{ mg/kg} \times 2$ (e.g., if desired dosage is 10 mg/kg, then concentration would be 20 mg/mL).

Assuming a maximum mouse weight of 30 g, prepare 15 μL of EtOH solution per mouse.

3. Dilute EtOH solution 20x with 18:1 saline:emulphor, giving a final probe-alkyne concentration of X/20 mg/mL (e.g., 1 mg/mL from a 20 mg/mL EtOH stock) in 18:1:1 saline:emulphor:EtOH.

10 μL of this solution should be injected per gram of mouse.

1.7 mM TBTA in 4:1 t-butanol/DMSO (store at rt for months, remake if crystallization or ppt is observed)

Prepare a 50 $\times$ (83.5 mM) stock of TBTA by dissolving 8.85 mg TBTA in 200 μL DMSO (store at rt for years). Prepare a 1.7 mM TBTA solution by adding 20 μL of 50 $\times$ stock to a glass vial containing 180 μL of DMSO, mix thoroughly. Add 800 μL of t-butanol.

The CC reaction is aided by presence of t-butanol. This solvent is included in the TBTA stock rather than added to the CC reaction by itself because it has a melting point of 25.5 $^{\circ}\text{C}$, and thus tends to freeze at rt. Mixing with DMSO prevents freezing, and including with the TBTA minimizes the number of solutions that need to be added to the CC reaction. Note: thoroughly mix the diluted TBTA before adding the t-butanol to avoid ppt.

COMMENTARY

Background Information

ABPP experiments can be analyzed using a variety of different platforms besides the MudPIT approach described in this Unit (Bachovchin et al. 2009; Cravatt et al. 2008). Originally, probe-labeled samples were separated by 1D SDS-PAGE and visualized by in-gel fluorescence scanning (for fluorophore-conjugated proteins) or avidin blotting (for biotinylated proteins) (Greenbaum et al. 2002; Kidd et al. 2001; Liu et al. 1999; Patricelli et al. 2001). For target identification, biotin-labeled proteins were enriched prior to SDS-PAGE, and bands corresponding to labeled proteins excised for in-gel digestion and MS analysis. Gel-based ABPP offers a robust and high throughput platform, capable of analyzing hundreds of proteomes per day, and is still the preferred method for the comparative analysis of many samples in parallel. In contrast, ABPP-Mudpit is significantly more time-intensive, and is most applicable to the in-depth analysis of dozens, rather than hundreds, of samples. An additional consideration is the amount of sample required, which is 0.5 to 1.0 mg for a MudPIT experiment vs. 0.01–0.02 mg for gel-based ABPP. As such, ABPP-MudPIT may not be applicable in certain situations of limited protein quantity, such as clinical biopsy samples.

The principle drawback of gel-based analysis is the limited resolution of SDS-PAGE. As such, the MudPIT LC-MS-platform has been adopted for complementary in-depth profiling of individual proteomic samples, allowing profiling of 50–100+ enzyme activities per experiment versus ~10–20 enzyme activities per gel-lane. ABPP-MudPIT has been implemented for the analysis of a variety of enzyme activities, including serine hydrolases in human breast tumors (Jessani et al. 2005), histone deacetylases (Salisbury and Cravatt 2007), and metalloproteases (Sieber et al. 2006), the latter targeted using a cocktail of ABPP probes. Kinases (Patricelli et al. 2007) have also been analyzed using a variant of ABPP-MudPIT that only analyzes the probe-labeled peptides. Rather than enriching ABPP probe-labeled proteins, the labeled proteome is digested in solution, and the biotinylated peptides are streptavidin enriched, eluted, and analyzed by LC-MS.

In terms of quantitation, both gel-based ABPP and ABPP-Mudpit can be used to provide estimates of active enzyme abundance, using fluorescence intensity for the former and semi-quantitative methods such as spectral counting for the latter. It should be noted that protein spectral counts in the tryptic dataset (rather than the labeled-peptide dataset) are used to estimate active protein abundance, as labeled peptides typically give too few (<10) spectral counts for reliable estimates of active protein.

In contrast to gel-based ABPP, implementation of an MS-based platform allows for direct analysis of site of labeling, which can be helpful for confirming the activity-based nature of probe labeling (i.e., modification of a catalytic residue). This Unit describes a basic protocol for obtaining probe-labeled peptides by denaturing the streptavidin upon heating in a MS-compatible aqueous solution of acetonitrile and trifluoroacetic acid (Okerberg et al. 2005). Alternative methods for elution of labeled peptides involve enzymatic (Weerapana et al. 2007) or chemical (Cravatt et al. 2008) cleavage of tags with labile linkers. These methods have been suggested to provide a cleaner elution, and have the added advantage of removing the bulky tag, a step which should facilitate LC separation and MS ionization (Hansen et al. 2003). However, direct elution of biotinylated peptides can be implemented with a high degree of success. For example, the kinase profiling experiment mentioned above resulted in the identification of over a hundred specifically-labeled peptides (Patricelli et al. 2007).

Critical Parameters and Troubleshooting

If the CC reaction yield appears to be sub-optimal, ensure that the TCEP solution is prepared fresh using Fluka TCEP that has been properly stored at 4 °C; try another lot of the reagent if necessary. The CC reaction works best in a buffer free of amines at pH 7.4 or above, but is tolerant to a wide range of salt and phosphate concentrations. Inclusion of detergents (e.g., SDS) at concentrations greater than 0.1–0.5% can also impede the CC reaction and protein precipitation. As such, keep detergent concentrations at a minimum and perform the methanol-chloroform precipitation described in Support Protocol 2 if inhibition of precipitation is observed.

Streptavidin beads can show significant non-specific protein binding, so results should always be evaluated with relation to controls. Filter the protein IDs obtained from the tryptic digest experimental sample by comparison to the tryptic digest control sample; proteins identified in both datasets likely indicate abundant proteins that bind non-specifically to the streptavidin beads. Filter labeled peptide identifications by comparison to proteins identified in the tryptic digest experimental sample; proteins selectively identified in the labeled peptide dataset (and not in the trypsin digest experimental dataset) should be discarded as false positives.

Anticipated Results

The number and type of protein identifications obtained from analyzing tryptic and labeled peptide samples will vary from dozens to hundreds depending on the proteome source and the type of probe(s) utilized. For example, analysis of human tumor specimens by ABPP-MudPIT using a biotinylated fluorophosphonate probe that specifically targets serine hydrolases identified over 50 enzymes of that superfamily (Jessani et al. 2005). Analysis of labeled peptides obtained from a human cancer cell line homogenate treated with a ATP-mimetic biotinylated kinase probe resulted in identification of over 100 ATP and other nucleotide-dependent enzymes (Patricelli et al. 2007).

Time Considerations

Sample preparation using the described protocols requires two days, with the trypsin digest run overnight at the end of day 1. It is suggested that 6–12 samples be prepared in parallel.

Samples may be stored frozen at the end of Basic Protocol 1, Alternate Protocol 1, Support Protocol 1 or at the penultimate step of Basic Protocol 2, prior to MS analysis. MudPIT analysis of each sample requires 10–12 hours, plus 2–8 hours (depending on the MS instrument and computing infrastructure) for data analysis. Overall, it should take approximately 4 days to complete the full analysis of a tryptic sample, a control tryptic sample, and a labeled peptide sample.

Note the following abbreviations are used throughout this unit

ABPP	activity-based protein profiling
biotin-azide	biotin tag bearing an azide
CC	click-chemistry
CHCl₃	chloroform
DMSO	dimethylsulfoxide
DTT	dithiothreitol
FA	formic acid
IAA	iodoacetamide
i.p.	intraperitoneal
LC	liquid chromatography
TBTA	Tris-[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
MeCN	acetonitrile
MeOH	methanol
MS	mass spectrometry
MudPIT	Multi-dimensional Protein Identification Technology
PBS	1x Dulbecco's phosphate buffered saline, pH 7.4 (Gibco)
ppt	precipitate
probe	biotinylated activity-based probe
probe-alkyne	activity-based probe bearing an alkyne
rt	room temperature
SDS	sodium dodecyl sulfate
TCEP	tris(2-carboxyethyl)phosphine hydrochloride
TFA	trifluoroacetic acid
Tris	50 mM Tris-HCl, pH 8.0

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Figure 1.

The ABPP-MudPIT method for high-content proteomic analysis of enzyme activities. A proteome is labeled with a biotinylated ABPP probe, and labeled proteins are affinity enriched on streptavidin beads. After on-bead trypsin digest, the tryptic peptides are analyzed by MudPIT for identification and quantification (e.g., via spectra counting). Box: The probe labeled peptides can also be eluted from the streptavidin beads for MS analysis of labeling sites.