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Abstract

The identification of bona fide protein-protein interactions and the mapping of proteomes was greatly enhanced by protein tagging for generic affinity purification methods and analysis by mass spectrometry (AP-MS). The high quality of AP-MS data permitted the development of proteomic navigation by sequential tagging of identified interactions. However AP-MS is laborious and limited to relatively high affinity protein-protein interactions. Proximity labeling, first with the biotin ligase BirA, termed BioID, and then with ascorbate peroxidase, termed APEX, permits a greater reach into the proteome than AP-MS enabling both the identification of a wider field and weaker protein-protein interactions. This additional reach comes with the need for stringent controls. Proximity labeling also permits experiments in living cells allowing spatiotemporal investigations of the proteome. Here we discuss proximity labeling with accompanying methodological descriptions for E.coli and mammalian cells.

Keywords	biotin labelling; mass spectrometry; affinity purification; protein-protein interactions.
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There are no linked research data sets for this submission. The following reason is given:
The data generated during the development of these methods will be published elsewhere.

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The Editor
Methods

Dear Editor,

We submit a manuscript “Proteomic navigation using proximity-labeling” to the special Methods issue edited by Profs Harald von Melchner and myself.

This is a review article with accompanying detailed methods sections.

Please note that the first author, Marc Gentzel and the last (fifth) author, Jyoti Choudhary are both corresponding authors and I am not a corresponding author. I was not able to implement this change during submission.

Regards,

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The Editor
Methods

Dear Editor,

Our manuscript “Proteomic navigation using proximity-labeling” has been revised according to the reviewer’s requests. The changes are highlighted in yellow in the revised text.

Please note that the first author, Marc Gentzel and the last (fifth) author, Jyoti Choudhary are both corresponding authors and I am not a corresponding author. I was not able to implement this change during submission.

Regards,

Proteomic navigation using proximity-labeling

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Abstract

The identification of *bona fide* protein-protein interactions and the mapping of proteomes was greatly enhanced by protein tagging for generic affinity purification methods and analysis by mass spectrometry (AP-MS). The high quality of AP-MS data permitted the development of proteomic navigation by sequential tagging of identified interactions. However AP-MS is laborious and limited to relatively high affinity protein-protein interactions. Proximity labeling, first with the biotin ligase BirA, termed BioID, and then with ascorbate peroxidase, termed APEX, permits a greater reach into the proteome than AP-MS enabling both the identification of a wider field and weaker protein-protein interactions. This additional reach comes with the need for stringent controls. Proximity labeling also permits experiments in living cells allowing spatiotemporal investigations of the proteome. Here we discuss proximity labeling with accompanying methodological descriptions for *E.coli* and mammalian cells.

Introduction

Specific protein-protein interactions mediate assemblies that range from simple homo- and hetero-dimers to complex cellular machines including the spliceosome, ribosome or proteasome to the greater complexities of phase transitions and amorphous cellular compartments. Moreover, protein assemblies can be dynamic and may be continuously

built, deconstructed or remodeled during operation. An important step towards the systematic characterization of protein complexes was taken when genetic engineering enabled the addition of specific tags to endogenous proteins thereby permitting standardized native affinity purification protocols for the generic isolation of different proteins and their interactions partners. The interaction partners were then identified by generic affinity purification and mass spectrometry (AP-MS) [1–3]. Notable examples of this approach included tandem affinity purification (TAP-tagging) and proteomic navigation [4–6]. Systematic applications of high throughput genetic engineering for AP-MS led to protein interaction networks in yeast [7,8], *E.coli* [9] and mammalian cells [10–12].

AP-MS has been the most successful approach for proteomic mapping and navigation [11]. However only high affinity components that remain stably bound during sample preparation and washing can be identified. Advances in mass spectrometric instrumentation, the acceleration of and improvements to sample preparation and improved quantitative approaches to distinguish true interacting proteins from background have further increased the reach and sensitivities of proteomic analyses [10,12–16]. However, as soon as cells are lysed the native environment around a protein complex is lost and the assemblies start to decay. Consequently, weak and transiently interacting proteins are likely to be missed by AP-MS approaches. This is a critical technical omission because proteins are usually found in highly crowded circumstances such as within organelles or phase separated compartments. Macromolecular crowding provides the venue for the functional importance of weak protein-protein interactions, which are unlikely to be detected by AP-MS. Affinity capture of membrane protein complexes has also been particularly challenging, requiring use of detergents to solubilize hydrophobic proteins whilst balancing conditions to preserve their native protein interactions. A long this line *in vivo* chemical cross-linking prior to cell lysis freezes interactions aiding their characterization by mass spectrometry [17,18].

Proximity-labeling methodology aims to reach into the proteome beyond the limits of AP-MS or crosslinking approaches. Proximity labeling in live cells employs an enzyme to covalently modify nearby proteins with a chemical adduct, usually biotin, that facilitates generic purification after cell lysis, followed by mass spectrometric identification. Here we focus on protein tagging approaches and the two main types of enzymes used for proximity labeling. The first, termed BioID, employs relaxed specificity

mutants of the 35 kDa *E. coli* BirA biotin ligase as the protein tag. Stimulated by the addition of exogenous biotin, mutant BirA* will biotinylate lysines on nearby proteins in living cells [19,20]. The biotin label provides the opportunity for high affinity enrichment of labeled proteins through binding to streptavidin or streptavidin analogs for affinity purification. The strong biotin-streptavidin interaction permits stringent washing of the affinity resin thereby reducing unspecific background. After elution from the resin, the identification of the biotinylated proteins by mass spectrometry, compared to appropriate controls, profiles the proteomic environment surrounding the tagged protein. An improved version of smaller size and higher efficacy, BioID2, has been developed [21]. BioID has been used extensively for proteomic mapping including the characterization of protein complexes and organelles [22–24]. A major limitation of BioID and BioID2 is the long labeling period required, often for many hours, which precludes the study of dynamic processes. More recently, two further versions, TurboID and miniTurbo, have been engineered for faster labeling [25].

A different proximity approach, termed APEX, employs ascorbate peroxidase as the enzyme tag. APEX utilizes hydrogen peroxide and phenolic compounds to generate free radicals that react with various amino acids, primarily aromatics. Biotin derivatives of tyramine or other phenolic compounds are used to biotinylate the local proteomic environment around the tagged protein [26,27]. An improved version termed APEX2 was developed by molecular evolution to generate a more active enzyme that does not need to be expressed at high levels, thereby facilitating a wider range of applications [28].

BioID2/TurboID and APEX2 have different advantages and disadvantages that potentially provide a complementary access to the mapping of proteomic environments. BioID attaches biotin to lysine whereas APEX modifies several amino acids predominantly tyrosine and tryptophan. Because lysines are characteristically presented on protein surfaces whereas the aromatics are usually buried in hydrophobic protein folds, these two systems differ with respect to the accessibility of the substrate amino acid. Biotinylation by APEX2 is fast (after addition of phenol-biotin and hydrogen peroxide, labeling for 1 minute or less is typical) whereas BioID is slower. Even TurboID usually requires 10 minutes or more after biotin addition to the culture medium. Consequently APEX2 is more suited to the rapid dynamics of intracellular protein diffusion. For this reason, we opted for the application of APEX2 to mapping proteomic

environments and protein dynamics in *E.coli* and mammalian cells. However the diverse successes of BioID should be considered if rapid dynamics are not a priority.

Quantitative Analysis of APEX - Experiments by Mass Spectrometry

The challenge for proximity labeling experiments is the identification of specific interacting proteins. Unspecific background can arise from the affinity materials or 'random' vicinity to the protein or protein complex under investigation. In addition, the experimental conditions might alter the global cellular proteome, e.g. by stress response, and hence can alter both sources of background. The random labeling of unspecific proteins by chance as they come into close vicinity of the bait protein by diffusion depends on their abundance in global proteome or, if specific organelles are targeted, the organelle. Normalization of the enrichment of these proteins by their relative abundance in the proteome can address this challenge and help to identify true interacting proteins. However, knowledge of the relative proteome abundance by proteomic analysis of the cell lysate used for biotin-affinity purification is required for this adjustment, which may be beyond the scope of the experiment. Defining valid protein interaction partners using proximity labeling presents greater challenges than AP-MS and stringent experimental design to include high value controls is necessary. Minimally, two controls are required. First, expression of the untagged biotinylating enzyme (here APEX2) from the identical expression context in cells that are processed in parallel to the tagged cells. Second, a control to identify the unspecific protein background that is presented by the affinity resin. In the case of unbounded cellular compartments or domains, the extent of biotinylation by reference APEX constructs can be used to control for spatial specificity [29]. Parallel experiments with APEX2 fusions that act as spatial references in combination with quantitative mass spectrometry is a powerful strategy for spatio-temporal resolution of neighbouring proteins [30]. With these controls, quantitative mass spectrometric analysis can circumvent the challenges and enable the specific, reproducible, determination of proteomic environments including weak protein-protein interactions (Figure 1).

Methods

Expression of APEX2-tagged proteins in *E.coli*

For proximity-labeling in *E.coli,* the untagged and tagged APEX2 proteins were expressed from the low copy plasmid, pSC101 in *E.coli* DH10B, which is a standard host for recombinant DNA molecular biology. We used the DH10B derivative, GB2005 (F-mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZ ΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK ΔrpsL nupG fhuA::IS2 recET redα, phage T1-resistent). Transformation of the bacteria was performed by electroporation and selection of transformed cells is achieved by suitable antibiotics.

The pSC101 expression plasmids for APEX2 and the APEX2 fusion proteins were cloned under the arabinose-inducible pBAD promoter using recombineering methods previously described [31,32].

Materials

Luria Bertani (LB) medium pH 7.0 is prepared by dissolving 10grams Tryptone, (BD Biosciences #211705), 10g NaCl (VWR #27810.364) and 5g yeast extract (BD Biosciences #212750) in 500ml sterile distilled water. Once solids have dissolved, add water to 1000ml final volume and autoclave to sterilize. For LB agar plates, dissolve 1.5g agar (Amresco# J637-1KG) in 100ml LB medium by bringing to boiling in a microwave. Cool down to 35^{o}C and then add streptomycin sulfate to a final concentration of 80μg/ml, stir well and pour 25ml into 10cm petridishes. The preparation of electrocompetent cells requires ~60ml ice cold sterile water and ~1ml pre-cooled 10% (v/v) glycerol solution. The later is prepared by weight, as glycerol is viscous. Add 12.6g glycerol (Merck Millipore #1.04092.1000) and sterile distilled water to 100ml final volume. Autoclave the solution. Antibiotic stock solutions: 10mg/ml tetracycline hydrochloride (Sigma# T-3383) concentration in 75% (v/v) ethanol and sterile water and 50mg/ml streptomycin in sterile water, Sigma #S6501504. Sterile 10% (w/v) L-arabinose (Sigma #A3256_100G) in water is prepared by filtration through 0.22μm sterile filter (Merck Millipore #SLGS033SS). Store 500μl aliquots at minus 20^{o}C. For cell lysis a sonicator from Diagenode type Bioruptor UCD-200TM was used.

Methods

To generate electrocompetent bacteria, streak out *E.coli* from -80°C glycerol stocks onto LB agar with 80µg/ml streptomycin and incubate at 37°C overnight. The following day, pick a single colony and inoculate into a conical flask containing 50ml LB medium supplemented with 80µg/ml streptomycin and incubate overnight at 37°C with shaking at 200rpm. Inoculate 1ml of the fresh overnight culture into a conical flask containing 50ml LB medium supplemented with 80µg/ml streptomycin and incubate for 2 hours at 37°C with shaking at 200rpm. Meanwhile prepare 200ml ice cold sterile water and 1ml 10% glycerol (on ice for at least 1hour). Cells are harvested when the optical density at 600nm is between 0.4-0.6. Split the 50ml culture into two ice cold 50ml Falcon tubes (25ml each) and centrifuge with a swinging-rotor centrifuge at 1600rcf (Beckman Coulter JLA-16.250 rotor, 4000 rpm) for 30 minutes at +4°C. Discard the supernatant and resuspend each pellet in 10ml ice cold sterile distilled water then fill the tube to 50ml with ice cold sterile distilled water and centrifuge again at at 1600rcf for 30 minutes at +4°C. Repeat the wash step once more. Subsequently, using cold pipette tips, resuspend the pellets in 1ml ice cold 10% glycerol and pipette 50µl aliquots into sterile ice cold 1.4ml Eppendorf tubes and store at -80°C until use.

The required number of tubes of electrocompetent cells and electroporation cuvettes (VWR #76102-576, square cap 1mm) are placed on ice for 10 minutes. Then add ~2µl of ice cold DNA, usually ~10ng (here the expression plasmids) to 50µl electrocompetent cells and keep on ice. Electroporation is performed at 1350V (should be less than 5.0ms). Add 1ml LB medium at room temperature to the cuvette and transfer into 1.4ml reaction tube with a hole punched using a wide gauge sterile needle. Incubate at 30°C for 1 hour with shaking at 950rpm on a thermoshaker. Plate 10µl, 100µl (and more if needed) of the culture on LB plate supplemented with with 5µg/ml tetracycline or a suitable antibiotic for the plasmid(s). Culture at 30°C overnight.

Affinity enrichment of *E. coli* biotin-labeled proteins

Each proximity-labeled experiment should be performed in duplicate along with duplicates of the controls, all performed in parallel. Small bacterial cultures can be sustained in standard 1.4ml and 2.0ml Eppendorf tubes when small holes are punched into the caps by a large (e.g. 23) gauge needle for aerobic exchange.

Materials

Stock solutions for bacterial culture, protein expression and the APEX2 labeling reaction are detailed below. Here we included also treatment with bleomycin to induce double stranded DNA breaks as an example experimental strategy. All solutions should be sterilized by autoclaving (AC) or sterile filtration (SF): 10% glycerol (AC, glycerol: Merck Millipore #1.04092.1000), 100mM bleomycin (SF, Cayman Chemical #9041-93-4), 50mM biotin-phenol (SF, R&D Systems #6241/25), 500mM Trolox (Cayman Chemical #53188-07-1, dissolve 0.125g in 1ml 100% DMSO, Sigma #D8418), 1M stock hydrogen peroxide (30% solution, Merck Millipore #1.07209.0250) and 1M stock sodium ascorbate (SF, Sigma#A7631-100G) and 50mM Tris-HCl pH7. The cell lysis buffer consists of 100mM Tris-HCl pH 8.0 (Fisher Scientific #BP152-5), 150mM NaCl 1mM MgCl2, 1mM EDTA (Sigma #ED3SS-500G), the IP buffer of 100mM Tris-HCl pH 8.0; 150mM NaCl , 10% Glycerol and for the detergent containing IP buffer 1% Triton X100 are added. Dynabeads MyOne Streptavidin C1 (Invitrogen #65001) are used as affinity resin.

Methods

For each expression plasmid, pick two single colonies of transfected cells and transfer each into 1.8ml LB medium supplemented with 5µg/ml tetracycline in a 2.0ml reaction tube with a hole in the cap. Incubate the tubes in a thermoshaker at 30°C 950rpm overnight. On following day inoculate 1ml into 50ml LB medium supplemented with 5µg/ml tetracycline in conical 250ml flasks and culture at 30°C, 200rpm. After 2 hours, induce protein expression by adding L-arabinose to a final concentration 0.2g/ml. Continue growth for 40 minutes at 30°C, 200rpm. Transfer two 7ml aliquots into 50ml tubes and pellet for 10min at 3000rpm at room temperature. Discard supernatant and suspend pellets in 1/20th volume LB medium (here: 350µl) and transfer to 2.0ml tubes. Depending on experimental design, here for example to induce DNA damage (add Bleomycin to a final concentration of 2mM) or buffer control and incubate for 30min at 37°C. Pellet cells at room temperature by centrifugation at 16,100rcf for 1min, discard the supernatant (with bleomycin, carefully to chemical waste!) and resuspend the pellet in 1ml fresh LB, centrifuge again and resuspend in 1ml LB. Prime the APEX2-labeling by addition of 10µl 50mM stock Biotin-phenol and incubate for 30min at 37°C to allow uptake of the reagent. Labeling is initiated by addition of 10µl 1M hydrogen peroxide and incubation for 5min at 37°C. Quench the reaction with 10µl 1M sodium ascorbate, 10µl 0.5M Trolox and 5min on ice. Pellet cells and discard supernatant to chemical waste.

Resuspend the pellet in ice cold cell lysis buffer supplemented with bacterial protease inhibitor cocktail and DNaseI. Place the eppendorf tubes in a eppendorf tube holder, tightly close the lid and place the tube holder embedded in an ice bath in the rotatory slot of the sonicator. Sonicate for 10minutes with alternating pulses of 30s sonication, 30s rest (Diagenode Bioruptor UCD-200TM). Clear the lysates by centrifugation at 16,100rcf for 1min at +4°C. Carefully transfer the supernatant to fresh tubes on ice.

In parallel, add 80µl of magnetic Dynabeads to a fresh tube and wash with 1ml IP buffer. Separate the beads and wash on the magnetic stand for 5 minutes and discard the supernatant. Repeat this step three times. Then add 120µl cell lysis supernatant to the beads and dilute with 380µl IP buffer. Insert the tubes in a rotary mixer and allow binding for 30 minutes at room temperature. Subsequently, wash the beads once with IP containing detergent and thrice with detergent-free IP buffer and resuspend the beads finally in 100µl detergent-free IP buffer and transfer them into a fresh small reaction tube (0.65ml, Sorenson BioScience #15160). Note that the proteins are digested on the beads and that this buffer should not contain any protease inhibitors.

Proteolytic Digestion on Affinity-Beads and Preparation for LC-MS/MS

Materials

The following materials are required for protein digestion and desalting: trypsin sequencing grade (Promega V5280, Roche #11418475001), Lys-C (Roche #11420429001) or rLys-C (Promega V1671), Ultramicro Spin Columns (3-30µg capacity, Nest Group SSV18 or Harvard Apparatus 74-7206). Solutions of and 2.0% (v/v) TFA (trifluoroacetic acid, Merck #1.08262.025), 0.1% (v/v) TFA in 2% acetonitrile in HPLC-grade water (Merck #1.13358.2500, #1.15333.2500), 0.1% (v/v) formic acid in HPLC-grade water (Merck #1.00264.1000), 0.1% (v/v) formic acid in 60% (v/v) acetonitrile in water are required for stage tip desalting of the On-Bead digests. The eluents of for liquid chromatography are 0.1% (v/v) formic acid in water and 0.1% formic acid in 60% acetonitrile in water respectively. The LC-MS/MS analysis are conducted with a nanoflow HPLC system (Thermo Dionex Ultimate3000, ThermoScientific) with C18 Acclaim Pepmap 100 column 75µm x 2cm (trap column, ThermoScientific #164535) and C18 Acclaim Pepmap RSLC 75µm x 15cm (separation column, ThermoScientific #164534). An isotope-labeled peptide retention time standard (Thermo Pierce #88321) for technical quality control is

diluted to 25fmol/µl peptide in 30% (v/v) formic acid (Merck #1.00264.1000) in HPLC-grade water (Merck #1.00264.1000) and used to recover the dried digests before injection into LC system. The MS/MS analysis utilized a LTQ-Orbitrap XL ETD mass spectrometer (ThermoScientific). Raw LC-MS/MS data is interpreted with the MaxQuant and Perseus software packages (MPI Biochemistry, https://www.biochem.mpg.de).

Methods

Proteolytic Digestion on Affinity-Beads and Preparation for LC-MS/MS

Add 100ng trypsin directly to the 80µl bead suspension from (2.) and incubate overnight at 37°C with agitation. Beads should not settle during digestion. To complete digestion, add additional 100ng trypsin, incubate for 8-16h and subsequently add 50ng Lys-C and incubate 8-16h.

Acidify the digest solution with 2% TFA to pH <2. Wet spin columns by passing through 200µl acetonitrile (0.1rcf, 30s) and equilibrate the columns with 200µl 0.1% TFA, 2% acetonitrile in water thrice (0.1-0.2rcf, 1min). Add 100µl acidified digest and 100µl 0.1% TFA, 2% acetonitrile in water and spin slowly at 0.1-0.2rcf for 2min. Washed bound peptides thrice with 200µl 0.1% TFA, 2% acetonitrile in water and elute the peptides into a fresh tube by elution twice with 100µl with 0.1% formic acid, 60% acetonitrile in water. Dry the peptides in vacuum and store at -20°C. For LC-MS/MS analysis recover peptides in 3µl 30% formic acid supplemented with 25fmol/µl peptide retention time standard and dilute with 20µl HPLC-grade water. Retention time standards can be added to 30% formic acid solution for quality control.

Expression of APEX2-tagged proteins in mammalian cells

Tagging of genes with APEX2 at the endogenous locus can be achieved through CRISPR/Cas9 targeting [33,34]. Alternatively, APEX2 gene fusions can be expressed from BAC transgenes [32] ideally introduced as BAC transposons [35] or introduced into cells by transient transfection or lentiviral infection [30]. High expression of APEX2 fusions in membranes can induce aggregation or morphological aberrations [29] and thus is best avoided. Proximity-labeling experiments should be performed in triplicate, in parallel with negative controls. Negative controls can include APEX2-tagged bait cell line without addition of biotin-phenol and H_2O_2, or without induction in the case of inducible constructs. Labeling is usually carried out by incubation with biotin-phenol for 30 min

followed by activation of the labeling reaction by H_2O_2. A longer incubation time with biotin-phenol (2 hours) may be advantageous to achieve labeling of nuclear proteins in mammalian cells [34]. The amount of starting material can range between 1-10 mg of protein, depending on the expression level of the target protein. After labeling, cells can be stored as frozen pellets at -80°C.

Affinity enrichment of mammalian biotin-labeled proteins

Materials

Stock solutions for labeling are as follows: 500 mM Trolox (Sigma #238813), 1 M hydrogen peroxide (Merck Millipore #1.07209.0250) and 1 M sodium ascorbate (Sigma #A7631). RIPA buffer for cell lysis consists of 50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Igepal CA-630 (Sigma #I8896), 0.1% SDS, 0.5% sodium deoxycholate (Sigma #30970), Halt Protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific #78440). Benzonase® is from Sigma (#E8263). Strep-Tactin® Sepharose bead slurry (IBA Lifesciences #2-1201-002) is used as streptavidin affinity resin.

Method

Incubate cells expressing the APEX2-tagged target in culture medium containing 0.5 mM biotin-phenol for 30 min at 37°C. Add H_2O_2 for a final concentration of 1 mM and gently shake cells for 1-2 min at room temperature. Remove the culture medium and quench the reaction by washing cells thrice in a solution containing 5 mM Trolox, 10 mM sodium ascorbate and 10 mM sodium azide in DPBS (Dulbecco's Phosphate Buffered Saline). Wash cells twice with ice-cold PBS to remove excess reagents, then scrape cells from culture dishes in ice-cold PBS and pellet at 120rcf for 10 min. Discard the supernatant.

Resuspend the cell pellet in ice-cold RIPA buffer (1 ml of buffer to 1.0xE7 cells) containing 5 mM Trolox, 10 mM sodium ascorbate, 10 mM sodium azide and protease and phosphatase inhibitors. Incubate on ice for 10 min, then lyse the cells in a Dounce homogeniser using 20 strokes with a tight pestle. Clarify the cell lysate by centrifugation at 18,000rcf for 15 min at 4°C.

When working with nuclear target proteins, it might be beneficial to isolate nuclei [36,37] and prepare nuclear lysates rather than use whole cell lysates [34]. To prepare nuclear lysates, resuspend the nuclei pellet in RIPA lysis buffer containing 450 mM NaCl and incubate in ice for 10 min. Dilute the lysate back to 150 mM NaCl RIPA and lyse in a

Dounce homogeniser with tight pestle using 20 strokes. Add 2 mM MgCl and 1 µl of benzonase per ml of lysate. Incubate for 20 min at room temperature. Centrifuge the nuclear lysate at 18,000rcf for 15 min at 4°C. Transfer the supernatant (nuclear lysate) to a fresh tube on ice and determine protein concentration.

Wash 10-100 µl of streptavidin bead slurry thrice with RIPA buffer. Settle the beads and remove the supernatant. Mix 1-10 mg lysate with the streptavidin beads and incubate with gentle rotation for 60 min at 4°C. Remove lysate and wash beads five times with 10 bead volumes of ice-cold RIPA buffer. The concentration of sodium chloride in RIPA washing buffer can be increased up to 1M to reduce non-specific binding.

On-bead tryptic digestion of mammalian streptavidin affinity-purified proteins and preparation for mass spectrometry analysis

Materials

The following materials are required for on-bead trypsin digestion and peptide clean-up: 50 mM ammonium bicarbonate (from Sigma #40867, should be freshly prepared), 5% formic acid (v/v, Sigma #A117-50), 500 mM ammonium bicarbonate, trypsin (sequencing grade, Roche #11 047 841 001), acetonitrile HPLC grade, 60% acetonitrile HPLC grade (v/v), 40 mM TCEP (from 0.5 M TCEP stock, Sigma #646547), MultiScreen HTS plates (Millipore #MSDVN6550). Prepare all reagents with HPLC grade bottled water.

Method

Wash beads thrice with 10 bead volumes of cold 50 mM ammonium bicarbonate to remove detergents from the RIPA buffer. Transfer beads to a new cold tube with the last wash and remove all supernatant. Resuspend beads in 90 µl of 50 mM ammonium bicarbonate. Add 10 µl of trypsin stock solution (0.1 µg/µl in 0.5% formic acid) and incubate at 37°C overnight with shaking. Settle the beads and collect the supernatant, which now contains the peptides, and add 10 µl of 5% formic acid to it. Resuspend the beads in 100 µl of 500 mM ammonium bicarbonate. Settle the beads, collect the supernatant and add 10 µl of 5% formic acid. Pool with the supernatant containing the peptides.

Clean the peptide samples by filtering [38]. To do this, reduce the volume of the peptide solution to approximately 50 µl. Add acetonitrile to the peptide solution for a final concentration of 60% acetonitrile. Wet the filters in a Multiscreen HTS plate by passing

200 µl of 60% acetonitrile three times (by centrifuging at 2000 g for 1 min). Do not let the filters dry out. Add the peptide solution onto filter plate, spin into a fresh standard 96-well plate and transfer the filtered peptides to a glass vial. Add 100 µl of 60% acetonitrile onto filter, spin at 2000 g for 1 min, collect the filtrate and pool with the filtered peptide solution in the vial. Dry peptides under vacuum and store at -20 °C until LC-MS/MS analysis.

Resolubilise the peptides in 36 µl of 40 mM TCEP by vortexing vigorously, sonicating in a water bath for 10 sec and shaking for 15 min. Add 4 µl of 5% formic acid, vortex and proceed to LC-MS/MS analysis.

Quantitative Proteomics of Biotin-Labeled Samples

The main challenge for proximity labeling experiments is the determination of specific interacting proteins. The first unspecific background arises from *in vivo* 'noise' and the second from the affinity materials during the *in vitro* steps. Also, the experimental conditions might alter the global proteome, e.g. by stress response, and hence alter vicinity, abundance or affinity material-dependent backgrounds. Thoughtful choice of accurate controls is essential.

The main control for the expression of the APEX- or BioID-tagged protein is the expression of untagged APEX or BioID tag from the identical expression venue. A complementary control involves the omission of the biotin tagging reagent(s). For APEX, omit both the biotin tagging reagent and hydrogen peroxide. Background from random labeling of unspecific proteins by chance as they to come into close vicinity of the bait protein by diffusion depends on their abundance in global proteome or, if specific organelles are targeted, the organelle. Normalization of the enrichment of these proteins by their relative abundance in the proteome eludes this challenge and helps to identify true interacting proteins. However, knowledge of relative proteome abundance profiles is required. Although advisable, determination of these values requires dedicated proteomic analysis that may be more demanding than achievable in practice.

Label-free Quantitative LC-MS/MS Data Analysis

Peptide mixtures are separated on (U)HPLC systems with trap-separation setup of C18 trap columns and separation columns at common flow rates of 2-3µl/min for trapping and 200-300nl/min for separation. Linear gradients of 0.1% formic acid in water (eluent A) and 0.1% formic acid, 60% acetonitrile in water (eluent B) from 0% A to 60% B within

90-120min are well suited for reproducible label-free LC-MS/MS analyses. Operation of the mass spectrometer in either data-dependent acquisition (DDA) or data-independent acquisition mode is suitable for analysis of complex peptide mixtures and parameters depend on manufacturer and capabilities of the instrument. In a classical setup we operate a LTQ-Orbitrap XL ETD in Top8 data-dependent acquisition mode with high resolution (R60000 at m/z 400) for MS1 and fragmentation of the 8 most intense multiply charged ions by CID in the linear ion trap.

We use the MaxQuant and Perseus package (MPI Biochemistry, Martinsried, Germany) for data analysis. MaxQuant permits the use of multiple, independent sequence databases and provides a database for common contaminants. For *E. coli* APEX2 experiments we use the reviewed entries for *E. coli* (K12) from the Uniprot database and a separate database in FASTA format for all custom sequences such as cloned constructs. For mammalian experiments we recommend using the relevant taxonomy entries in SwissProt only or the full Uniprot. A database of contaminants is included in MaxQuant. Common search parameters comprise protein N-terminal acetylation and oxidation of methionines with no fixed modifications, as commonly we do not reduce and alkylate cysteines in case of on bead digests. The MaxQuant software itself has been described in detail [39–42]. Raw data is imported directly into the software and peak detection, quantification and identification. The protein quantification is exported by default in the file proteingroups.txt and is imported to Perseus for data evaluation and processing. Data sets from different analysis can be joined into one data matrix based on a shared unique identifier, commonly the protein accession number/FASTA ID. Thereby, quantified proteins from affinity enrichment analysis are associated with protein quantification results of the ‘global’ proteome analysis of the corresponding lysates and an enrichment factor of a protein enrichment by APEX2-labeling can be calculated.

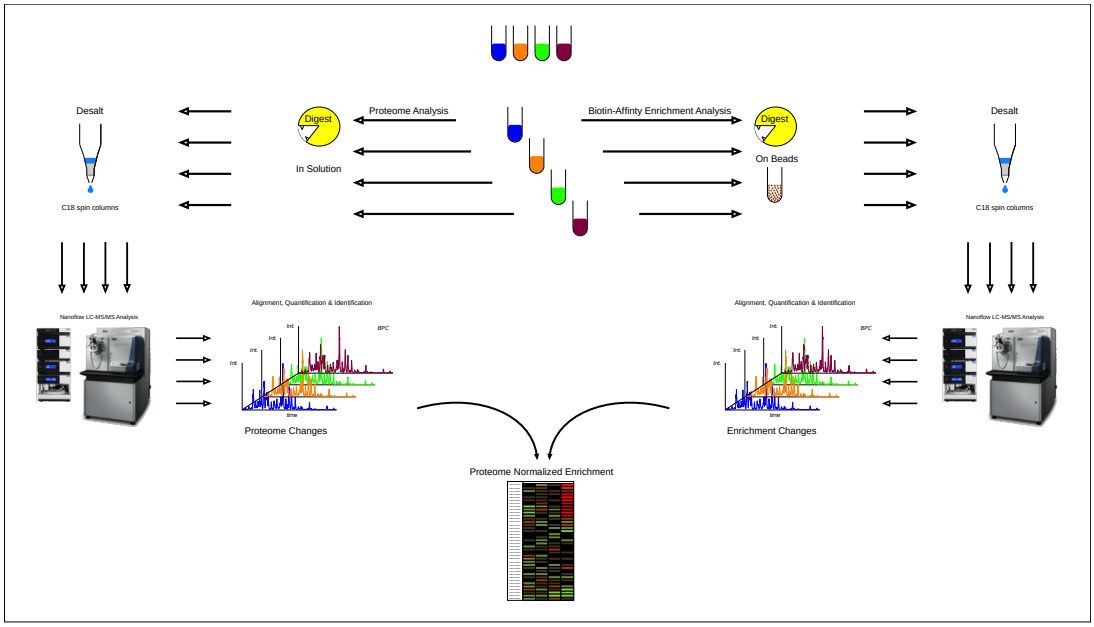

Figure 1: Scheme of an APEX2 - LFQ Proteomic Analysis. For simplicity, only 4 samples that represent two biological conditions with respective controls are depicted. Samples are split for proteomic and affinity enrichment analysis. After sample processing the experimental is merged on different levels *in silico* for quantitative comparisons: changes in the proteome, changes in proximity-label profiles and proximity-label profiles normalized to the global proteome.

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Highlights

Review of proteomic navigation and the emergence of proximity labeling

Detailed methods for proteomic navigation in *E.coli* and mammalian cells

Proteomic navigation using proximity-labeling

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Abstract

The identification of *bona fide* protein-protein interactions and the mapping of proteomes was greatly enhanced by protein tagging for generic affinity purification methods and analysis by mass spectrometry (AP-MS). The high quality of AP-MS data permitted the development of proteomic navigation by sequential tagging of identified interactions. However AP-MS is laborious and limited to relatively high affinity protein-protein interactions. Proximity labeling, first with the biotin ligase BirA, termed BioID, and then with ascorbate peroxidase, termed APEX, permits a greater reach into the proteome than AP-MS enabling both the identification of a wider field and weaker protein-protein interactions. This additional reach comes with the need for stringent controls. Proximity labeling also permits experiments in living cells allowing spatiotemporal investigations of the proteome. Here we discuss proximity labeling with accompanying methodological descriptions for *E.coli* and mammalian cells.

Highlights

Review of proteomic navigation and the emergence of proximity labeling

Detailed methods for proteomic navigation in *E.coli* and mammalian cells

Introduction

Specific protein-protein interactions mediate assemblies that range from simple homo- and hetero-dimers to complex cellular machines including the spliceosome, ribosome or proteasome to the greater complexities of phase transitions and amorphous cellular compartments. Moreover, protein assemblies can be dynamic and may be continuously built, deconstructed or remodeled during operation. An important step towards the systematic characterization of protein complexes was taken when genetic engineering enabled the addition of specific tags to endogenous proteins thereby permitting standardized native affinity purification protocols for the generic isolation of different proteins and their interactions partners. The interaction partners were then identified by generic affinity purification and mass spectrometry (AP-MS) [1–3]. Notable examples of this approach included tandem affinity purification (TAP-tagging) and proteomic navigation [4–6]. Systematic applications of high throughput genetic engineering for AP-MS led to protein interaction networks in yeast [7,8], *E.coli* [9] and mammalian cells [10–12].

AP-MS has been the most successful approach for proteomic mapping and navigation [11]. However only high affinity components that remain stably bound during sample preparation and washing can be identified. Advances in mass spectrometric instrumentation, the acceleration of and improvements to sample preparation and improved quantitative approaches to distinguish true interacting proteins from background have further increased the reach and sensitivities of proteomic analyses [10,12–16]. However, as soon as cells are lysed the native environment around a protein complex is lost and the assemblies start to decay. Consequently, weak and transiently interacting proteins are likely to be missed by AP-MS approaches. This is a critical technical omission because proteins are usually found in highly crowded circumstances such as within organelles or phase separated compartments. Macromolecular crowding provides the venue for the functional importance of weak protein-protein interactions, which are unlikely to be detected by AP-MS. Affinity capture of membrane protein complexes has also been particularly challenging, requiring use of detergents to solubilize hydrophobic proteins whilst balancing conditions to preserve their native protein interactions. A long this line *in vivo* chemical cross-linking prior to cell lysis freezes interactions aiding their characterization by mass spectrometry [17,18].

Proximity-labeling methodology aims to reach into the proteome beyond the limits of AP-MS or crosslinking approaches. Proximity labeling in live cells employs an enzyme to covalently modify nearby proteins with a chemical adduct, usually biotin, that facilitates generic purification after cell lysis, followed by mass spectrometric identification. Here we focus on protein tagging approaches and the two main types of enzymes used for proximity labeling. The first, termed BioID, employs relaxed specificity mutants of the 35 kDa *E. coli* BirA biotin ligase as the protein tag. Stimulated by the addition of exogenous biotin, mutant BirA* will biotinylate lysines on nearby proteins in living cells [19,20]. The biotin label provides the opportunity for high affinity enrichment of labeled proteins through binding to streptavidin or streptavidin analogs for affinity purification. The strong biotin-streptavidin interaction permits stringent washing of the affinity resin thereby reducing unspecific background. After elution from the resin, the identification of the biotinylated proteins by mass spectrometry, compared to appropriate controls, profiles the proteomic environment surrounding the tagged protein. An improved version of smaller size and higher efficacy, BioID2, has been developed [21]. BioID has been used extensively for proteomic mapping including the characterization of protein complexes and organelles [22–24]. A major limitation of BioID and BioID2 is the long labeling period required, often for many hours, which precludes the study of dynamic processes. More recently, two further versions, TurboID and miniTurbo, have been engineered for faster labeling [25].

A different proximity approach, termed APEX, employs ascorbate peroxidase as the enzyme tag. APEX utilizes hydrogen peroxide and phenolic compounds to generate free radicals that react with various amino acids, primarily aromatics. Biotin derivatives of tyramine or other phenolic compounds are used to biotinylate the local proteomic environment around the tagged protein [26,27]. An improved version termed APEX2 was developed by molecular evolution to generate a more active enzyme that does not need to be expressed at high levels, thereby facilitating a wider range of applications [28].

BioID2/TurboID and APEX2 have different advantages and disadvantages that potentially provide a complementary access to the mapping of proteomic environments. BioID attaches biotin to lysine whereas APEX modifies several amino acids predominantly tyrosine and tryptophan. Because lysines are characteristically presented on protein surfaces whereas the aromatics are usually buried in hydrophobic protein folds, these two systems differ with respect to the accessibility of the substrate amino

acid. Biotinylation by APEX2 is fast (after addition of phenol-biotin and hydrogen peroxide, labeling for 1 minute or less is typical) whereas BioID is slower. Even TurboID usually requires 10 minutes or more after biotin addition to the culture medium. Consequently APEX2 is more suited to the rapid dynamics of intracellular protein diffusion. For this reason, we opted for the application of APEX2 to mapping proteomic environments and protein dynamics in *E.coli* and mammalian cells. However the diverse successes of BioID should be considered if rapid dynamics are not a priority.

Quantitative Analysis of APEX - Experiments by Mass Spectrometry

The challenge for proximity labeling experiments is the identification of specific interacting proteins. Unspecific background can arise from the affinity materials or 'random' vicinity to the protein or protein complex under investigation. In addition, the experimental conditions might alter the global cellular proteome, e.g. by stress response, and hence can alter both sources of background. The random labeling of unspecific proteins by chance as they come into close vicinity of the bait protein by diffusion depends on their abundance in global proteome or, if specific organelles are targeted, the organelle. Normalization of the enrichment of these proteins by their relative abundance in the proteome can address this challenge and help to identify true interacting proteins. However, knowledge of the relative proteome abundance by proteomic analysis of the cell lysate used for biotin-affinity purification is required for this adjustment, which may be beyond the scope of the experiment. Defining valid protein interaction partners using proximity labeling presents greater challenges than AP-MS and stringent experimental design to include high value controls is necessary. Minimally, two controls are required. First, expression of the untagged biotinylating enzyme (here APEX2) from the identical expression context in cells that are processed in parallel to the tagged cells. Second, a control to identify the unspecific protein background that is presented by the affinity resin. In the case of unbounded cellular compartments or domains, the extent of biotinylation by reference APEX constructs can be used to control for spatial specificity [29]. Parallel experiments with APEX2 fusions that act as spatial references in combination with quantitative mass spectrometry is a powerful strategy for spatio-temporal resolution of neighbouring proteins [30]. With these controls, quantitative mass spectrometric analysis can circumvent the challenges and enable the specific, reproducible, determination of proteomic environments including weak protein-protein interactions (Figure 1).

Methods

Expression of APEX2-tagged proteins in *E.coli*

For proximity-labeling in *E.coli,* the untagged and tagged APEX2 proteins were expressed from the low copy plasmid, pSC101 in *E.coli* DH10B, which is a standard host for recombinant DNA molecular biology. We used the DH10B derivative, GB2005 (F-mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZ ΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK ΔrpsL nupG fhuA::IS2 recET redα, phage T1-resistent). Transformation of the bacteria was performed by electroporation and selection of transformed cells is achieved by suitable antibiotics.

The pSC101 expression plasmids for APEX2 and the APEX2 fusion proteins were cloned under the arabinose-inducible pBAD promoter using recombineering methods previously described [31,32].

Materials

Luria Bertani (LB) medium pH 7.0 is prepared by dissolving 10grams Tryptone, (BD Biosciences #211705), 10g NaCl (VWR #27810.364) and 5g yeast extract (BD Biosciences #212750) in 500ml sterile distilled water. Once solids have dissolved, add water to 1000ml final volume and autoclave to sterilize. For LB agar plates, dissolve 1.5g agar (Amresco# J637-1KG) in 100ml LB medium by bringing to boiling in a microwave. Cool down to 35^{o}C and then add 120μl streptomycin sulfate (50mg/ml in sterile water, Sigma #S6501504), stir well and pour 25ml into 10cm petridishes. The preparation of electrocompetent cells requires ~60ml ice cold sterile water and ~1ml pre-cooled 10% (v/v) glycerol solution. The later is prepared by weight, as glycerol is viscous. Add 12.6g glycerol (Merck Millipore #1.04092.1000) and sterile distilled water to 100ml final volume. Autoclave the solution. Other stock solutions; 10mg/ml tetracycline hydrochloride (Sigma# T-3383) concentration in 75% (v/v) ethanol and sterile water. Sterile 10% (w/v) L-arabinose (Sigma #A3256_100G) in water is prepared by filtration through 0.22μm sterile filter (Merck Millipore #SLGS033SS). Store 500μl aliquots at minus 20^{o}C.

Methods

To generate electrocompetent bacteria, streak out *E.coli* from -80^{o}C glycerol stocks onto LB agar with 80μg/ml streptomycin and incubate at 37^{o}C overnight. The following day, pick a single colony and inoculate into a conical flask containing 50ml LB medium

supplemented with 60μl 50mg/ml streptomycin and incubate overnight at 37°C with shaking at 200rpm. Inoculate 1ml of the fresh overnight culture into a conical flask containing 50ml LB medium supplemented with 60μl 50mg/ml streptomycin and incubate for 2 hours at 37°C with shaking at 200rpm. Meanwhile prepare 200ml ice cold sterile water and 1ml 10% glycerol (on ice for at least 1hour). Cells are harvested when the optical density at 600nm is between 0.4-0.6. Split the 50ml culture into two ice cold 50ml Falcon tubes (25ml each) and centrifuge with a swinging-rotor centrifuge at 4000rpm (Beckman Coulter JLA-16.250 rotor, 1600 rcf) for 30 minutes at +4°C. Discard the supernatant and resuspend each pellet in 10ml ice cold sterile distilled water then fill the tube to 50ml with ice cold sterile distilled water and centrifuge again at 4000rpm for 30 minutes at +4°C. Repeat the wash step once more. Subsequently, using cold pipette tips, resuspend the pellets in 1ml ice cold 10% glycerol and pipette 50μl aliquots into sterile ice cold 1.4ml Eppendorf tubes and store at -80°C until use.

The required number of tubes of electrocompetent cells and electroporation cuvettes (VWR #76102-576, square cap 1mm) are placed on ice for 10 minutes. Then add ~2μl of ice cold DNA, usually ~10ng (here the expression plasmids) to 50μl electrocompetent cells and keep on ice. Electroporation is performed at 1350V (should be less than 5.0ms). Add 1ml LB medium at room temperature to the cuvette and transfer into 1.4ml reaction tube with a hole punched using a wide gauge sterile needle. Incubate at 30°C for 1 hour with shaking at 950rpm on a thermoshaker. Plate 10μl, 100μl (and more if needed) of the culture on LB plate supplemented with 5ml of 10mg/ml tetracycline or a suitable antibiotic for the plasmid(s). Culture at 30°C overnight.

Affinity enrichment of *E. coli* biotin-labeled proteins

Each proximity-labeled experiment should be performed in duplicate along with duplicates of the controls, all performed in parallel. Small bacterial cultures can be sustained in standard 1.4ml and 2.0ml Eppendorf tubes when small holes are punched into the caps by a large (e.g. 23) gauge needle for aerobic exchange.

Materials

Stock solutions for bacterial culture, protein expression and the APEX2 labeling reaction are detailed below. Here we included also treatment with bleomycin to induce double stranded DNA breaks as an example experimental strategy. All solutions should be sterilized by autoclaving (AC) or sterile filtration (SF): 10% glycerol (AC, glycerol: Merck

Millipore #1.04092.1000), 100mM bleomycin (SF, Cayman Chemical #9041-93-4), 50mM biotin-phenol (SF, R&D Systems #6241/25), 500mM Trolox (Cayman Chemical #53188-07-1, dissolve 0.125g in 1ml 100% DMSO, Sigma #D8418), 1M stock hydrogen peroxide (30% solution, Merck Millipore #1.07209.0250) and 1M stock sodium ascorbate (SF, Sigma#A7631-100G) and 50mM Tris-HCl pH7. The cell lysis buffer consists of 100mM Tris-HCl pH 8.0 (Fisher Scientific #BP152-5), 150mM NaCl 1mM MgCl2, 1mM EDTA (Sigma #ED3SS-500G), the IP buffer of 100mM Tris-HCl pH 8.0; 150mM NaCl , 10% Glycerol and for the detergent containing IP buffer 1% Triton X100 are added. Dynabeads MyOne Streptavidin C1 (Invitrogen #65001) are used as affinity resin.

Methods

For each expression plasmid, pick two single colonies of transfected cells and transfer each into 1.8ml LB medium supplemented with 0.72µl of tetracycline in a 2.0ml reaction tube with a hole in the cap. Incubate the tubes in a thermoshaker at 30°C 950rpm overnight. On following day inoculate 1ml into 50ml LB medium supplemented with 20µl tetracycline in conical 250ml flasks and culture at 30°C, 200rpm. After 2 hours, induce protein expression with 1.1ml of 10% L-arabinose. Continue growth for 40 minutes at 30°C, 200rpm. Transfer two 7ml aliquots into 50ml tubes and pellet for 10min at 3000rpm at room temperature. Discard supernatant and suspend pellets in 1/20th volume LB medium (here: 350µl) and transfer to 2.0ml tubes. Depending on experimental design, here for example to induce DNA damage (add 7µl Bleomycin) or buffer control and incubate for 30min at 37°C. Pellet cells at room temperature by centrifugation at 13,200 rpm (16,100 rcf) for 1min, discard the supernatant (with bleomycin, carefully to chemical waste!) and resuspend the pellet in 1ml fresh LB, centrifuge again and resuspend in 1ml LB. Prime the APEX2-labeling by addition of 10µl 50mM stock Biotin-phenol and incubate for 30min at 37°C to allow uptake of the reagent. Labeling is initiated by addition of 10µl 1M hydrogen peroxide and incubation for 5min at 37°C. Quench the reaction with 10µl 1M sodium ascorbate, 10µl 0.5M Trolox and 5min on ice. Pellet cells and discard supernatant to chemical waste. Resuspend the pellet in ice cold cell lysis buffer supplemented with bacterial protease inhibitor cocktail and DNaseI. Place the eppendorf tubes in a eppendorf tube holder, tightly close the lid and place the tube holder embedded in an ice bath in the rotatory slot of the sonicator. Sonicate for 10minutes with alternating pulses of 30s sonication, 30s rest. Clear the

lysates by centrifugation at 13,200rpm (16,100 rcf) for 1min at +4°C. Carefully transfer the supernatant to fresh tubes on ice.

In parallel, add 80µl of magnetic Dynabeads to a fresh tube and wash with 1ml IP buffer. Separate the beads and wash on the magnetic stand for 5 minutes and discard the supernatant. Repeat this step three times. Then add 120µl cell lysis supernatant to the beads and dilute with 380µl IP buffer. Insert the tubes in a rotary mixer and allow binding for 30 minutes at room temperature. Subsequently, wash the beads once with IP containing detergent and thrice with detergent-free IP buffer and resuspend the beads finally in 100µl detergent-free IP buffer and transfer them into a fresh small reaction tube (0.65ml, Sorenson BioScience #15160). Note that the proteins are digested on the beads and that this buffer should not contain any protease inhibitors.

Proteolytic Digestion on Affinity-Beads and Preparation for LC-MS/MS

Materials

The following materials are required for protein digestion and desalting: trypsin sequencing grade (Promega V5280, Roche #11418475001), Lys-C (Roche #11420429001) or rLys-C (Promega V1671), Ultramicro Spin Columns (3-30µg capacity, Nest Group SSV18 or Harvard Apparatus 74-7206). Solutions of and 2.0% (v/v) TFA (trifluoroacetic acid, Merck #1.08262.025), 0.1% (v/v) TFA in 2% acetonitrile in HPLC-grade water (Merck #1.13358.2500, #1.15333.2500), 0.1% (v/v) formic acid in HPLC-grade water (Merck #1.00264.1000), 0.1% (v/v) formic acid in 60% (v/v) acetonitrile in water are required for stage tip desalting of the On-Bead digests. The eluents of for liquid chromatography are 0.1% (v/v) formic acid in water and 0.1% formic acid in 60% acetonitrile in water respectively. The LC-MS/MS analysis are conducted with a nanoflow HPLC system (Thermo Dionex Ultimate3000, ThermoScientific) with C18 Acclaim Pepmap 100 column 75µm x 2cm (trap column, ThermoScientific #164535) and C18 Acclaim Pepmap RSLC 75µm x 15cm (separation column, ThermoScientific #164534). An isotope-labeled peptide retention time standard (Thermo Pierce #88321) for technical quality control is diluted to 25fmol/µl peptide in 30% (v/v) formic acid (Merck #1.00264.1000) in HPLC-grade water (Merck #1.00264.1000) and used to recover the dried digests before injection into LC system. The MS/MS analysis utilized a LTQ-Orbitrap XL ETD mass

spectrometer (ThermoScientific). Raw LC-MS/MS data is interpreted with the MaxQuant and Perseus software packages (MPI Biochemistry, https://www.biochem.mpg.de).

Methods

Proteolytic Digestion on Affinity-Beads and Preparation for LC-MS/MS

Add 100ng trypsin directly to the 80µl bead suspension from (2.) and incubate overnight at 37°C with agitation. Beads should not settle during digestion. To complete digestion, add additional 100ng trypsin, incubate for 8-16h and subsequently add 50ng Lys-C and incubate 8-16h.

Acidify the digest solution with 2% TFA to pH <2. Wet spin columns by passing through 200µl acetonitrile (centrifuge 800 rcf, 30s) and equilibrate the columns with 200µl 0.1% TFA, 2% acetonitrile in water thrice (1000-1200rpm, 0.1-0.2 rcf, 1min). Add 100µl acidified digest and 100µl 0.1% TFA, 2% acetonitrile in water and spin slowly at 1000-1200rpm (0.1-0.2 rcf) for 2min. Washed bound peptides thrice with 200µl 0.1% TFA, 2% acetonitrile in water and elute the peptides into a fresh tube by elution twice with 100µl with 0.1% formic acid, 60% acetonitrile in water. Dry the peptides in vacuum and store at -20°C. For LC-MS/MS analysis recover peptides in 3µl 30% formic acid supplemented with 25fmol/µl peptide retention time standard and dilute with 20µl HPLC-grade water. Retention time standards can be added to 30% formic acid solution for quality control.

Expression of APEX2-tagged proteins in mammalian cells

Tagging of genes with APEX2 at the endogenous locus can be achieved through CRISPR/Cas9 targeting [33,34]. Alternatively, APEX2 gene fusions can be expressed from BAC transgenes [32] ideally introduced as BAC transposons [35] or introduced into cells by transient transfection or lentiviral infection [30]. High expression of APEX2 fusions in membranes can induce aggregation or morphological aberrations [29] and thus is best avoided. Proximity-labeling experiments should be performed in triplicate, in parallel with negative controls. Negative controls can include APEX2-tagged bait cell line without addition of biotin-phenol and H_2O_2, or without induction in the case of inducible constructs. Labeling is usually carried out by incubation with biotin-phenol for 30 min followed by activation of the labeling reaction by H_2O_2. A longer incubation time with biotin-phenol (2 hours) may be advantageous to achieve labeling of nuclear proteins in mammalian cells [34]. The amount of starting material can range between 1-10 mg of

protein, depending on the expression level of the target protein. After labeling, cells can be stored as frozen pellets at -80°C.

Affinity enrichment of mammalian biotin-labeled proteins

Materials

Stock solutions for labeling are as follows: 500 mM Trolox (Sigma #238813), 1 M hydrogen peroxide (Merck Millipore #1.07209.0250) and 1 M sodium ascorbate (Sigma #A7631). RIPA buffer for cell lysis consists of 50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Igepal CA-630 (Sigma #I8896), 0.1% SDS, 0.5% sodium deoxycholate (Sigma #30970), Halt Protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific #78440). Benzonase® is from Sigma (#E8263). Strep-Tactin® Sepharose bead slurry (IBA Lifesciences #2-1201-002) is used as streptavidin affinity resin.

Method

Incubate cells expressing the APEX2-tagged target in culture medium containing 0.5 mM biotin-phenol for 30 min at 37°C. Add H_2O_2 for a final concentration of 1 mM and gently shake cells for 1-2 min at room temperature. Remove the culture medium and quench the reaction by washing cells thrice in a solution containing 5 mM Trolox, 10 mM sodium ascorbate and 10 mM sodium azide in DPBS (Dulbecco's Phosphate Buffered Saline). Wash cells twice with ice-cold PBS to remove excess reagents, then scrape cells from culture dishes in ice-cold PBS and pellet at 1,100 rpm (120 *g*) for 10 min. Discard the supernatant.

Resuspend the cell pellet in ice-cold RIPA buffer (1 ml of buffer to 1e7 cells) containing 5 mM Trolox, 10 mM sodium ascorbate, 10 mM sodium azide and protease and phosphatase inhibitors. Incubate on ice for 10 min, then lyse the cells in a Dounce homogeniser using 20 strokes. Clarify the cell lysate by centrifugation at 13,000 rpm (18,000 *g*) for 15 min at 4°C.

When working with nuclear target proteins, it might be beneficial to isolate nuclei [36,37] and prepare nuclear lysates rather than use whole cell lysates [34]. To prepare nuclear lysates, resuspend the nuclei pellet in RIPA lysis buffer containing 450 mM NaCl and incubate in ice for 10 min. Dilute the lysate back to 150 mM NaCl RIPA and lyse in a Dounce homogeniser using 20 strokes. Add 2 mM MgCl and 1 µl of benzonase per ml of lysate. Incubate for 20 min at room temperature. Centrifuge the nuclear lysate at 13,000

rpm (18,000 *g*) for 15 min at 4°C. Transfer the supernatant (nuclear lysate) to a fresh tube on ice and determine protein concentration.

Wash 10-100 µl of streptavidin bead slurry thrice with RIPA buffer. Settle the beads and remove the supernatant. Mix 1-10 mg lysate with the streptavidin beads and incubate with gentle rotation for 60 min at 4°C. Remove lysate and wash beads five times with 10 bead volumes of ice-cold RIPA buffer. The concentration of sodium chloride in RIPA washing buffer can be increased up to 1M to reduce non-specific binding.

On-bead tryptic digestion of mammalian streptavidin affinity-purified proteins and preparation for mass spectrometry analysis

Materials

The following materials are required for on-bead trypsin digestion and peptide clean-up: 50 mM ammonium bicarbonate (from Sigma #40867, should be freshly prepared), 5% formic acid (v/v, Sigma #A117-50), 500 mM ammonium bicarbonate, trypsin (sequencing grade, Roche #11 047 841 001), acetonitrile HPLC grade, 60% acetonitrile HPLC grade (v/v), 40 mM TCEP (from 0.5 M TCEP stock, Sigma #646547), MultiScreen HTS plates (Millipore #MSDVN6550). Prepare all reagents with HPLC grade bottled water.

Method

Wash beads thrice with 10 bead volumes of cold 50 mM ammonium bicarbonate to remove detergents from the RIPA buffer. Transfer beads to a new cold tube with the last wash and remove all supernatant. Resuspend beads in 90 µl of 50 mM ammonium bicarbonate. Add 10 µl of trypsin stock solution (0.1 µg/µl in 0.5% formic acid) and incubate at 37°C overnight with shaking. Settle the beads and collect the supernatant, which now contains the peptides, and add 10 µl of 5% formic acid to it. Resuspend the beads in 100 µl of 500 mM ammonium bicarbonate. Settle the beads, collect the supernatant and add 10 µl of 5% formic acid. Pool with the supernatant containing the peptides.

Clean the peptide samples by filtering [38]. To do this, reduce the volume of the peptide solution to approximately 50 µl. Add acetonitrile to the peptide solution for a final concentration of 60% acetonitrile. Wet the filters in a Multiscreen HTS plate by passing 200 µl of 60% acetonitrile three times (by centrifuging at 2000 g for 1 min). Do not let the filters dry out. Add the peptide solution onto filter plate, spin into a fresh standard 96-

well plate and transfer the filtered peptides to a glass vial. Add 100 µl of 60% acetonitrile onto filter, spin at 2000 g for 1 min, collect the filtrate and pool with the filtered peptide solution in the vial. Dry peptides under vacuum and store at -20 °C until LC-MS/MS analysis.

Resolubilise the peptides in 36 µl of 40 mM TCEP by vortexing vigorously, sonicating in a water bath for 10 sec and shaking for 15 min. Add 4 µl of 5% formic acid, vortex and proceed to LC-MS/MS analysis.

Quantitative Proteomics of Biotin-Labeled Samples

The main challenge for proximity labeling experiments is the determination of specific interacting proteins. The first unspecific background arises from *in vivo* 'noise' and the second from the affinity materials during the *in vitro* steps. Also, the experimental conditions might alter the global proteome, e.g. by stress response, and hence alter vicinity, abundance or affinity material-dependent backgrounds. Thoughtful choice of accurate controls is essential.

The main control for the expression of the APEX- or BioID-tagged protein is the expression of untagged APEX or BioID tag from the identical expression venue. A complementary control involves the omission of the biotin tagging reagent(s). For APEX, omit both the biotin tagging reagent and hydrogen peroxide. Background from random labeling of unspecific proteins by chance as they to come into close vicinity of the bait protein by diffusion depends on their abundance in global proteome or, if specific organelles are targeted, the organelle. Normalization of the enrichment of these proteins by their relative abundance in the proteome eludes this challenge and helps to identify true interacting proteins. However, knowledge of relative proteome abundance profiles is required. Although advisable, determination of these values requires dedicated proteomic analysis that may be more demanding than achievable in practice.

Label-free Quantitative LC-MS/MS Data Analysis

Peptide mixtures are separated on (U)HPLC systems with trap-separation setup of C18 trap columns and separation columns at common flow rates of 2-3µl/min for trapping and 200-300nl/min for separation. Linear gradients of 0.1% formic acid in water (eluent A) and 0.1% formic acid, 60% acetonitrile in water (eluent B) from 0% A to 60% B within 90-120min are well suited for reproducible label-free LC-MS/MS analyses. Operation of the mass spectrometer in either data-dependent acquisition (DDA) or data-independent

acquisition mode is suitable for analysis of complex peptide mixtures and parameters depend on manufacturer and capabilities of the instrument. In a classical setup we operate a LTQ-Orbitrap XL ETD in Top8 data-dependent acquisition mode with high resolution (R60000 at m/z 400) for MS1 and fragmentation of the 8 most intense multiply charged ions by CID in the linear ion trap.

We use the MaxQuant and Perseus package (MPI Biochemistry, Martinsried, Germany) for data analysis. MaxQuant permits the use of multiple, independent sequence databases and provides a database for common contaminants. For *E. coli* APEX2 experiments we use the reviewed entries for *E. coli* (K12) from the Uniprot database and a separate database in FASTA format for all custom sequences such as cloned constructs. For mammalian experiments we recommend using the relevant taxonomy entries in SwissProt only or the full Uniprot. A database of contaminants is included in MaxQuant. Common search parameters comprise protein N-terminal acetylation and oxidation of methionines with no fixed modifications, as commonly we do not reduce and alkylate cysteines in case of on bead digests. The MaxQuant software itself has been described in detail [39–42]. Raw data is imported directly into the software and peak detection, quantification and identification. The protein quantification is exported by default in the file proteingroups.txt and is imported to Perseus for data evaluation and processing. Data sets from different analysis can be joined into one data matrix based on a shared unique identifier, commonly the protein accession number/FASTA ID. Thereby, quantified proteins from affinity enrichment analysis are associated with protein quantification results of the 'global' proteome analysis of the corresponding lysates and an enrichment factor of a protein enrichment by APEX2-labeling can be calculated.

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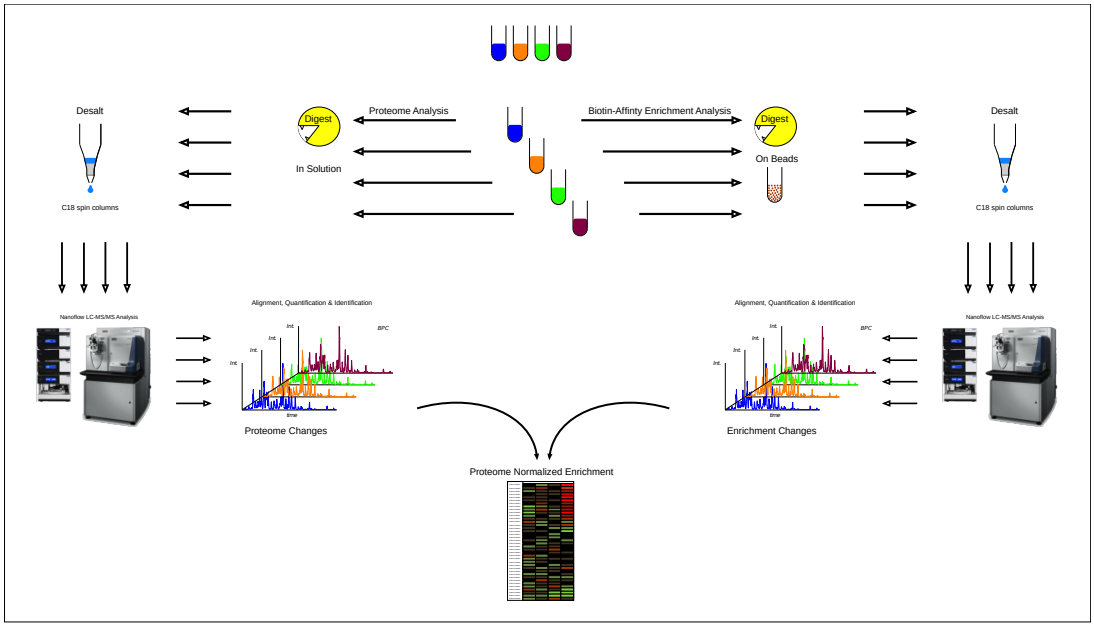

Figure 1: Scheme of an APEX2 - LFQ Proteomic Analysis. For simplicity, only 4 samples that represent two biological conditions with respective controls are depicted. Samples are split for proteomic and affinity enrichment analysis. After sample processing the experimental is merged on different levels *in silico* for quantitative comparisons: changes in the proteome, changes in proximity-label profiles and proximity-label profiles normalized to the global proteome.

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