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Spatially resolved proteomic mapping in living cells with the engineered peroxidase APEX2

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Abstract

This protocol describes a method to obtain spatially resolved proteomic maps of specific compartments within living mammalian cells. An engineered peroxidase, APEX2, is genetically targeted to a cellular region of interest. Upon the addition of hydrogen peroxide for 1 min to cells preloaded with a biotin-phenol substrate, APEX2 generates biotin-phenoxyl radicals that covalently tag proximal endogenous proteins. Cells are then lysed, and biotinylated proteins are enriched with streptavidin beads and identified by mass spectrometry. We describe the generation of an appropriate APEX2 fusion construct, proteomic sample preparation, and mass spectrometric data acquisition and analysis. A two-state stable isotope labeling by amino acids in cell culture (SILAC) protocol is used for proteomic mapping of membrane-enclosed cellular compartments from which APEX2-generated biotin-phenoxyl radicals cannot escape. For mapping of open cellular regions, we instead use a 'ratiometric' three-state SILAC protocol for high spatial specificity. Isotopic labeling of proteins takes 5–7 cell doublings. Generation of the biotinylated proteomic sample takes 1 d, acquiring the mass spectrometric data takes 2–5 d and analysis of the data to obtain the final proteomic list takes 1 week.

INTRODUCTION

To arrive at a molecular understanding of a specific cellular compartment or signaling pathway, one first needs a 'parts list' of proteins found there. Mass spectrometry (MS)-based proteomics is a powerful tool for elucidating the protein components of specific organelles and signaling complexes. In a typical MS proteomic experiment, the cellular entity of interest is first purified from live cells or tissue. For example, an organelle such as the mitochondrion can be purified via a series of density centrifugations[1]. For a more focused analysis of a single protein complex, an antibody can be used to immunoprecipitate a specific protein, along with its stable interaction partners[2]. The purified sample is then

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digested into peptides and analyzed by tandem MS (MS/MS). The resultant individual peptide sequences identify the parent proteins that were present in the purified sample.

Although this approach is exceptionally powerful, the quality of the resulting MS data is fundamentally limited by the quality of the purified protein sample. Current purification strategies are highly imperfect. For example, density centrifugation of organelles often carries over contaminants[3], which results in false positive assignment of nonspecific proteins to the organelle of interest. Immunoprecipitation of protein complexes may only capture stable interaction partners and miss transient or weaker binders, resulting in false negatives[2]. Most importantly, many cellular regions of interest (ROIs), such as the synaptic cleft and the mitochondrial intermembrane space (IMS), cannot be purified at all, and hence they are inaccessible to traditional MS proteomic analysis.

To address this limitation, our laboratory has developed an enzymatic biotinylation approach that bypasses the need for organelle or protein complex purification (**Fig. 1a**)[4,5]. Instead, proteomes of interest are covalently tagged with biotin while cells are still alive, membranes are intact and protein complexes are undisrupted. After the 1-min tagging reaction, the cells are lysed and the biotinylated endogenous proteins are collected using streptavidin-coated beads. The proteins are identified using conventional MS/MS techniques. As described below, it is essential to use an isotopic encoding strategy such as SILAC[6], isobaric tags for relative and absolute quantitation (iTRAQ[7]) or tandem mass tags (TMT[8]) in order to confidently assign biotinylated proteins and to distinguish these from nonspecific binders that make it through the streptavidin enrichment step (**Fig. 1b,c**). Here, we illustrate the approach using SILAC and briefly highlight the key steps where the iTRAQ procedure differs from the SILAC procedure.

The enzyme we use to perform the in-cell biotinylation is an engineered ascorbate peroxidase called APEX2 (ref. 9). APEX2 is the improved, second-generation enzyme[9], and we recommend this one over the original, first-generation APEX[10] for all proteomic applications (and electron microscopy (EM) applications as well[4,5,9,10]). The first-generation APEX is a triple mutant of wild-type soybean ascorbate peroxidase derived by structure-guided mutagenesis and screening[4,10]. APEX2 has one additional mutation, and it was evolved by yeast display selections[9]. APEX2 has a molecular weight of 27 kDa (the same size as GFP), and it is monomeric, free of disulfide bonds and contains a noncovalently bound heme cofactor in its active site[9].

APEX2 uses hydrogen peroxide (H_2O_2) as an oxidant to catalyze the one-electron oxidation of a diverse set of small-molecule substrates. The substrate that is relevant for proteomics is biotinphenol (BP) (**Fig. 1a**). The oxidized product, biotin-phenoxyl radical, is a highly reactive, short-lived (< 1 ms (refs. 11,12)) species that conjugates to endogenous proteins that are proximal to the APEX2 active site where it was generated. We have detected covalent adducts of BP to tyrosine side chains[4,5], and we believe that other electron-rich amino acids, such as tryptophan[13], cysteine[4,14] and histidine[15], may be labeled as well. The 'biotinylation radius' is not a fixed value, but it is better described as a 'contour map' in which the extent of biotinylation falls off nanometer by nanometer from APEX2 (**Fig. 1c**). This means that endogenous proteins that are many nanometers away from APEX2 may still

be biotinylated, although weakly, and detected by MS. This is why the ratiometric SILAC approach described below is crucial for achieving high spatial specificity in non-membrane-enclosed cellular regions. Note that the ratiometric approach is *not* required for membrane-enclosed compartments (such as the mitochondrial matrix) because endogenous membranes act as an impenetrable barrier to the BP radical[4] (**Fig. 1a**). Furthermore, it is important to note that the shape of the biotinylation contour map will be influenced by the local environment, including glutathione concentration, endogenous protein density and other factors.

The first demonstration of the APEX proteomic method used the first-generation APEX enzyme to map the mitochondrial matrix proteome in human embryonic kidney (HEK) 293T cells[4]. A two-state SILAC scheme was used to distinguish between biotinylated proteins and nonspecific streptavidin bead binders (**Fig. 1b**). A proteome of 495 proteins was obtained, with > 94% mitochondrial specificity, > 97% mitochondrial matrix specificity and 85% coverage of known mitochondrial matrix proteins[4]. The second application was to a more challenging compartment: the mitochondrial IMS[5], which cannot be biochemically purified. The IMS lies in between the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). Because the OMM contains porins that allow the free exchange of molecules < 5 kDa (ref. 16), it does not act as a barrier to the BP radical, and undesired biotinylation of cytosolic proteins can result from IMS-APEX activity (**Fig. 1c**). Hence, it was essential to apply the ratiometric SILAC approach (**Fig. 1c**) to achieve high spatial specificity in this compartment. Our resulting IMS proteome of 127 proteins had a mitochondrial specificity of > 94% and IMS specificity of > 99%. The coverage of known IMS proteins was 65% (ref. 5). Since these two publications, the APEX proteomic method has also been used to map the mitochondrial matrix proteome in live *Drosophila* muscle tissue[17], the proteome of endoplasmic reticulum (ER)-plasma membrane contact sites in HEK 293 cells[18] and the proteome of the primary cilium in inner medullary collecting duct epithelial cells[19]. We have also successfully used this methodology to map proteomes of cultured rat neurons (K.H.L., N.D.U., S.A.C. and A.Y.T., unpublished results; K.J.C., N.D.U., S.A.C. and A.Y.T., unpublished results).

This protocol describes both the two-state SILAC approach (**Fig. 1b**) that can be used to map membrane-enclosed cellular regions (such as the mitochondrial matrix) and the three-state ratiometric SILAC approach (**Fig. 1c**) that can be applied to open cellular compartments. Our protocols are specific for cultured mammalian cells. We have not yet developed APEX proteomics for other cell types, including yeast and bacteria.

Experimental design

Targeting APEX2 to the ROI—The first step in an APEX proteomic experiment is to target the APEX2 enzyme to the cellular region or protein complex of interest. This can be achieved by genetic fusion of APEX2 to a protein known to reside in this compartment, or to a targeting peptide, if one is available[4,5,9,10]. To target APEX to the mitochondrial matrix and IMS, we used 24– and 68–amino acid N-terminal targeting peptides, which were derived from the endogenous mitochondrial proteins COX4 and LACTB, respectively[10,20]. It is advisable to generate and test a few different fusion constructs at different expression levels

to select the one that gives the most specific targeting and minimal perturbation of the ROI. We have observed, for example, that overexpression of APEX2 on the OMM or ER membrane (with APEX2 facing the cytosol) induces severe aggregation of each organelle[9]. Such aggregation is not seen when the same constructs are expressed at lower levels via lentiviral transduction. An epitope tag such as V5 should be appended to each APEX2 fusion construct to facilitate its detection in cells via immunofluorescence staining. The hemagglutinin (HA) epitope tag should be avoided because the biotin-phenoxyl radical is reactive toward the tyrosines in the HA sequence.

By immunostaining and comparison with a trusted marker protein (such as mitochondrially localized GFP, in our case), check that the fusion construct is localized correctly. If there is a substantial pool of the construct outside the ROI, one should try to reduce its expression level, change the APEX2 insertion site or use a fusion to a different gene or peptide altogether. In addition to fluorescence microscopy, the localization of the APEX2 construct can be examined at higher resolution by EM because APEX2 also functions as a genetically encoded EM tag[9,10]. The protocol for EM visualization involves transfecting or transducing cells with the APEX2 construct, fixing, reacting with diaminobenzidine (DAB) and H_2O_2 for 5–40 min, staining with electron-dense osmium and then embedding and sectioning the cells for EM. Because of the narrow diameter of mitochondria, EM was necessary to distinguish between matrix and IMS localization for our mitochondrially targeted APEX fusion constructs[5] (**Fig. 2a**).

Characterizing the activity of the APEX2 construct—After confirming correct localization of the APEX2 fusion construct, the next step is to check its activity. This is done in two ways: by immunofluorescence staining of biotinylated proteins and by streptavidin blot analysis of whole-cell lysate. For the first assay (**Fig. 2b**), cells expressing the APEX2 construct are preincubated with BP for 30 min and treated live with H_2O_2 for 1 min. Under these conditions, we have not noticed any cell toxicity due to BP. Note that for cells or organisms other than mammalian cells, the preincubation time with BP may need to be optimized. The labeling reaction is then quenched, and the cells are fixed and stained with streptavidin- or NeutrAvidin-Alexa Fluor conjugate to visualize the biotinylated proteins. The cells are simultaneously stained with anti-V5 antibody to visualize the expression of the APEX2 construct. Negative controls are performed alongside, with APEX2 omitted, BP omitted or H_2O_2 omitted. The objective is to see APEX2- and H_2O_2-dependent biotinylation, as evidenced by positive streptavidin-Alexa Fluor staining. The biotinylation pattern should encompass the V5 staining pattern (i.e., be present wherever APEX2 is expressed). However, if the biotinylation pattern extends beyond the localization of APEX2, this is not necessarily a reason for concern. As shown in our IMS proteomic study[5], even when the biotinylation pattern is quite diffuse, the proteomic data can still be highly spatially specific. Diffuse labeling patterns may result from migration of biotinylated proximal endogenous proteins away from APEX2 in these live cells during the 1-min reaction window before quenching and cell fixation.

If the imaging assay is positive, it is next necessary to perform a western blot assay (**Fig. 2c**). As the imaging assay cannot differentiate between signal resulting from APEX2 self-biotinylation and biotinylation of endogenous neighboring proteins, the streptavidin–

horseradish peroxidase (HRP) blot analysis of the whole-cell lysate provides additional information about endogenous proteins biotinylated by APEX2. To perform this assay, live cells expressing the APEX2 construct are preincubated with BP for 30 min and then treated with H_2O_2 for 1 min. Next, the cells are lysed and resolved by SDS-PAGE. After transfer to a nitrocellulose membrane, the blot is incubated with streptavidin-HRP conjugate. Negative controls, in which APEX2, BP or H_2O_2 is omitted, are prepared and run in parallel. The expected result is a collection of bands that spans a large molecular-weight range and is dependent on the presence of both APEX2 and H_2O_2 (**Fig. 2c**). In the negative control lanes, there should only be three bands corresponding to endogenous biotinylated proteins (at 130, 75 and 72 kDa)[21]. If these bands are not visible, your streptavidin-HRP blotting and development conditions may not be adequately sensitive.

In addition to blotting with streptavidin-HRP, it is also helpful to blot the same lysates with V5-specific antibody to visualize the APEX2 construct itself and to confirm that the APEX2 construct is expressed at the same level in different samples and that it has not been partially proteolyzed.

APEX2 constructs localized to different cellular regions may give distinct banding patterns on the streptavidin-HRP blot. For instance, biotinylation catalyzed by IMS-APEX and mitochondrial matrix-APEX produce very distinct streptavidin-HRP blot 'fingerprints' (**Fig. 2c**)[4,5]. However, cytosolic APEX2 and OMM-anchored APEX2 (facing the cytosol) produce very similar banding patterns to each other with only subtle differences (V.H., S.S.L., N.D.U., S.A.C. and A.Y.T., unpublished results), probably because of shared accessibility to cytosolic protein pools. Nevertheless, after application of the ratiometric SILAC approach, the resulting proteomes mapped by each construct are completely distinct (V.H., S.S.L., N.D.U., S.A.C. and A.Y.T., unpublished results). Therefore, identical-looking banding patterns from different APEX2 constructs localized to overlapping compartments are not necessarily a reason for concern. The main purpose of this gel-based assay is to show that the APEX2 construct of interest biotinylates a large collection of endogenous proteins, and that the biotinylation depends on the presence of both the enzyme and H_2O_2.

The next step in the workflow is to perform a small-scale enrichment of APEX2-biotinylated proteins using streptavidin-coated beads. This is necessary before the large-scale proteomic experiment because several parameters need to be optimized. Our laboratory has commonly encountered problems at this step that require troubleshooting. Many factors affect both the capture efficiency and the background caused by nonspecific bead binding. For example, incomplete washout of unused BP probe from cells before cell lysis can drastically reduce the recovery of biotinylated proteins because of competition for binding to streptavidin sites. The use of more streptavidin beads than necessary can increase the background by providing more surface area for nonspecific binders. One should perform a bead titration experiment to determine the minimal amount of beads that can be used to capture all of the biotinylated material. The streptavidin bead binding and subsequent bead washing steps must be performed under harsh, denaturing conditions to ensure that only proteins directly and covalently tagged by APEX2 are enriched, and not interaction partners of those proteins or other nonspecific proteins that stick tightly to the partially unfolded biotinylated proteome that is anchored to the streptavidin beads.

To perform the small-scale enrichment, whole-cell lysate is generated using denaturing lysis buffer. After mixing with streptavidin beads, a series of high-salt, high-pH and high-urea washes are performed to thoroughly remove nonbiotinylated proteins. **Figure 2d** shows data from the mitochondrial matrix study[4], but since that publication we have increased the stringency of our streptavidin bead washes to further reduce background (our new protocol is described below). After the washes, the biotinylated proteome is eluted from the beads by boiling in SDS protein loading buffer in the presence of free biotin and analyzed by gel. Negative control samples omit APEX2, BP or H_2O_2 from the live-cell labeling. The gel is developed in Coomassie stain, or silver stain for greater sensitivity, to visualize the amount of enriched protein material. The expected result is many bands across a large molecular-weight range in the experimental sample and substantially less protein material in the negative controls (**Fig. 2d**). These streptavidin-enriched samples and the flow-through material (that contains all proteins not captured by the streptavidin beads) can also be analyzed by streptavidin-HRP blotting to ensure that the bead volume was sufficient to capture all biotinylated proteins (i.e., there are no biotinylated proteins remaining in the flow-through). If biotinylated proteins remain in the flow-through, or if there is a large amount of proteins eluted from the negative control samples, the enrichment protocol should be optimized by varying the bead volume, the number of washes and the types of washes to maximize capture of biotinylated proteins and to minimize nonspecific sticking to the streptavidin beads.

At this point, it is possible to proceed to the large-scale proteomic experiment. However, an optional assay is to probe the streptavidin-enriched material (i.e., the biotinylated proteome) for the presence or absence of endogenous marker proteins via western blotting. For instance, in Lam *et al.*[9], we showed that the biotinylated proteome generated using APEX2-OMM was enriched in the endogenous OMM proteins Tom70 and Tom20 and de-enriched in both the mitochondrial matrix protein ATP5B and the ER membrane (ERM) protein BCAP31 when compared with whole-cell lysate analyzed in the same way[9]. This analysis can help give confidence that the biotinylation is spatially specific. However, we routinely proceed to MS proteomics without performing this step because we trust the spatial specificity of the biotinylation reaction.

SILAC labeling and MS—For the MS proteomic experiment, it is essential to use a quantitative approach to obtain the best results. Previously, we used SILAC to quantify relative protein abundance. However, some biological samples, such as nondividing primary cells, do not efficiently incorporate exogenously supplied amino acids (as required by SILAC) into their proteomes; in these cases, it may be necessary to use iTRAQ or TMT for quantification instead. When iTRAQ or TMT is used, biotinylated proteins are captured with streptavidin beads and then digested on-bead with trypsin. The released peptides are then chemically labeled using the isobaric mass tag reagents. This procedure leaves the biotinylated peptides on the beads, preventing their identification. For this reason, it can be advantageous to use SILAC. Note that the sole use of spectral counting for estimating protein abundance will not work nearly as well or give the same precision as these quantitative, ratio-based methods.

For a SILAC setup, we typically culture the experimental sample in medium containing heavy isotopes of lysine and arginine (**Fig. 1b**). The negative control sample, which may omit APEX2, for example, is cultured in light-isotope medium. To provide additional confidence, a replicate sample set is prepared, usually with a different negative control (such as the omission of H_2O_2). In parallel, we plate cells to repeat the fluorescence imaging assay above as a quality control. After performing the live-cell biotinylation, quenching and cell lysis, a small amount of each lysate is also reserved for quality analysis via streptavidin blotting and silver staining, as described above. The heavy and light lysates are then combined, and streptavidin bead enrichment is performed on the mixture. Biotinylated proteins are eluted from the beads, run on a gel, and then 12–16 gel bands that span the entire gel lane are excised per sample. For each gel band, in-gel trypsin digestion is performed, followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to sequence all the peptides. Because of the mixing of heavy and light lysates, each peptide detected by MS will have two precursor ion peaks: one derived from the heavy sample and one derived from the light sample. Nonspecific binders should bind comparably to the beads in the presence or absence of APEX2 biotinylation, and hence they should produce peptides that have similar heavy and light peak intensities. A protein that has been biotinylated by APEX2, however, will be enriched by streptavidin beads over its nonbiotinylated counterpart. Therefore, markedly higher intensity should be observed for the heavy version of its peptides compared with the negative control light version that was not exposed to APEX2 labeling chemistry.

Data analysis—The raw MS data are searched against a UniProt database of known human proteins (or other species, depending on the cell type one uses). A protein is considered ‘detected’ only if two or more unique peptides derived from that protein are found in the MS data. For each detected protein, a heavy/light (H/L) SILAC ratio is calculated from the median of its peptide SILAC ratios[22]. We then rank all the proteins from high to low SILAC ratio. To assess whether the proteomic experiment succeeded in enriching ROI-resident proteins, we generate comprehensive, carefully curated lists of true positive (TP) and false positive proteins, as reported by literature. We cross these lists with the subset of detected proteins with known annotations to determine which hits are true positives and false positives. We plot the distribution of the subset of true positives versus the distribution of false positives on the same graph to determine whether our proteomic experiment succeeded in enriching the known true positives over the false positives (**Fig. 3a**). From our analysis of this smaller subset of proteins, we then select a SILAC ratio cutoff for our entire data set of detected proteins (**Fig. 3b**), above which we consider the proteins to be genuinely biotinylated by APEX2, and below which we consider the proteins to be possible nonspecific bead binders. This analysis is repeated for each independent replicate, and the resulting protein lists are intersected to obtain the final proteome.

The quality of the proteome can be assessed by two metrics: specificity and depth of coverage. Specificity is determined by calculating the percentage of the proteome that consists of TP proteins. Proteins without prior annotation or literature support for localization in the ROI are so-called ‘orphans’ that can be assayed by imaging or western blotting to determine whether these are indeed in the ROI. If any orphans can be assigned to

the ROI, the actual specificity of the proteome is higher than what was previously calculated. Depth of coverage is calculated as the percentage of the list of TP proteins detected in the final proteome.

If SILAC is used, one may examine the list of biotinylated peptides generated by MaxQuant. The sites of modification may yield information on the topology of a protein complex or a transmembrane protein. Note that, using current methods, we do not typically obtain sequences for a large number of biotinylated peptides. For example, in our mitochondrial matrix study[4], biotinylated peptides were sequenced for only 96 of the 495 proteins in our final proteomic list.

Ratiometric APEX tagging

The two-state SILAC protocol described above is suitable for mapping the proteomes of membrane-enclosed cellular compartments from which the APEX2-generated biotin-phenoxyl radicals cannot escape. For mapping of 'open' or 'partially open' cellular regions, it is essential to instead use a 'ratiometric' three-state SILAC protocol to achieve high spatial specificity (**Fig. 1c**). This approach was used to successfully map the proteome of the partially enclosed mitochondrial IMS[5], and it has also been applied by us to obtain highly specific proteomic maps of the cytosolic faces of the OMM and ERM in human cells (V.H., S.S.L., N.D.U., S.A.C. and A.Y.T., unpublished results). As with the two-state scheme, SILAC can be replaced by other quantitative MS techniques such as iTRAQ or TMT.

In a ratiometric three-state SILAC experiment (**Fig. 1c**), the heavy- and light-state samples are the same as in the two-state SILAC experiment. However, the medium state is new, and it is a sample in which live-cell biotinylation is performed by an APEX2 fusion construct immediately outside the ROI. For mapping of the IMS, OMM and ERM proteomes, we used a whole-cytosol-filling construct APEX2-NES (NES, nuclear export sequence) for the medium state. If one is mapping a chromatin domain, for example, a suitable medium state construct would be APEX2-NLS (NLS, nuclear localization sequence), which fills the entire nucleus.

The rationale for the three-state SILAC approach is that although a protein distal to the ROI might still be detectably biotinylated in the heavy sample, such distal proteins should be biotinylated to a greater extent by APEX2 outside the ROI than by APEX2 inside the ROI. Therefore, the heavy/medium (H/M) SILAC ratio can be used as a measure of relative proximity to the ROI; proteins with high H/M ratios are the ones that should be retained in the final proteome.

Apart from the addition of a third SILAC state, the workflow is identical to that described above for the two-state SILAC experiment. The final analysis of the MS data requires additional cutoff analysis based on the H/M ratio in addition to the H/L ratio. These analysis protocols are described below.

Comparison with BioID and HRP

BioID is an alternative live-cell enzymatic biotinylation technique based on a promiscuous variant of *Escherichia coli* biotin ligase ($BirA^{R118G}$) (refs. 23,24). The proposed labeling

chemistry involves the generation of an activated biotin adenylate ester (biotin-AMP) in the BirA active site, followed by release into solution[23]. The biotin-AMP then covalently reacts with lysine side chains of endogenous proteins. There are two potential disadvantages of BioID compared with APEX2 tagging. First, $BirA^{R118G}$ has slow kinetics, and it requires labeling times of 18–24 h to achieve appreciable levels of biotinylation[24–27]. This prevents the study of shorter-lived cell states or of a cell or organelle's acute response to a stimulus or drug. Furthermore, the accumulation of a biotinylated proteome over time could potentially be toxic to cells or affect the protein composition of the region under study. Second, biotin-AMP has a half-life of minutes in water[28], which implies that the labeling radius should be considerably greater than that of the biotin-phenoxyl radical, which has a half-life of <1 ms (refs. 11,12). Unfortunately, there are no reports of BioID performed using a quantitative technique such as SILAC or iTRAQ, and hence we cannot compare the spatial specificity and coverage directly with that of the APEX2 approach. Nevertheless, APEX2 proteomics is worth exploring for applications that require high spatial specificity and short labeling windows.

HRP is a ubiquitously used reporter for western blot detection and ELISAs, and it can be recombinantly expressed in mammalian cells[29,30]. However, because HRP contains four structurally essential disulfide bonds and two Ca^{2+} ion binding sites, HRP is inactive when it is expressed in the reducing environment of the mammalian cytosol, or when it is expressed in organelles such as the mitochondrion that receive proteins translated in the cytosol[31].

However, HRP is active in the oxidizing environments found in the lumen of the ER and Golgi, as well as on the cell surface. We have found that HRP catalyzes the same labeling chemistry as APEX2 with faster kinetics and greater resistance to H_2O_2-induced inactivation[9]. Hence, for proteomic mapping applications on the cell surface and in the secretory pathway, we often prefer HRP to APEX2. However, note that HRP is considerably larger than APEX2 (44 kDa with glycosylation, compared with 27 kDa for APEX2), and it can impair the trafficking of some proteins that tolerate APEX2 at the same fusion site.

Other laboratories have also used HRP to perform proteomics on the live-cell surface, not via genetic targeting of HRP as we do, but via antibody-based targeting to specific proteins[32]. This approach is limited by the specificity, affinity and binding sites of available antibodies, and it is also restricted to extracellular sites. However, antibody-based targeting can be useful when working with cells that are difficult to transfect.

Limitations of APEX proteomics

A major limitation of the APEX approach arises when using the ratiometric three-state SILAC protocol (but not the two-state protocol for membrane-enclosed compartments). Because this protocol improves specificity by removing proteins with low H/M SILAC ratios, it will inevitably also remove dual-localized proteins: proteins that reside both inside and outside the ROI. This can result in a substantial reduction in the depth of coverage of the proteome. For example, our IMS proteome had a coverage of only 65%, compared with 85% for the mitochondrial matrix proteome[4,5], a difference that we primarily attribute to the loss of dual-localized proteins caused by the use of the three-state SILAC protocol. Note that for

the mitochondrial matrix proteome, whose coverage was 85%, we believe that most of the missed detections are due to steric shielding: some proteins are buried in the center of large macromolecular complexes or by membranes in the context of living cells, and hence they are not accessible to the biotin-phenoxyl radical.

A second limitation of the current methodology is that some regions or complexes of interest cannot be targeted with high specificity using an APEX2 genetic fusion construct. This is the case for cellular regions in which most of the known occupants also reside elsewhere in the cell (such as contact sites between mitochondria and ER). The specificity of the resulting proteomic list can only be as clean as the localization of the APEX2 fusion construct.

Third, we have only demonstrated the methodology on mammalian cells. We have not attempted proteomic mapping in yeast or bacteria. We do know that APEX2 is active in these cell types. However, a potential concern is delivery of the BP probe, particularly in yeast. BP does not have high membrane permeability, and different protocols or structural derivatives may be needed to deliver the probe effectively to other cell types or into tissue.

METERIALS

REAGENTS

- Lipofectamine 2000 (Life Technologies, cat. no. 11668-019) or other preferred transfection reagent
- Cell line of interest (human embryonic kidney (HEK) 293T cells (ATCC, cat. no. CRL-11268) were used in our published studies[4,5]) **! CAUTION** Cell lines should be regularly checked to ensure that they are authentic and are not infected with *Mycoplasma*.
- DMEM (VWR International, cat. no. 45000-312)
- DMEM deficient in l-arginine and l-lysine (Thermo Fisher Scientific, cat. no. 89985)
- Dialyzed FBS (Sigma-Aldrich, cat. no. F0392)
- Penicillin, streptomycin and glutamine (Invitrogen, cat. no. 10378-016)
- d-(+)-Glucose solution (Sigma-Aldrich, cat. no. G8644-100ML)
- l-arginine (Arg-0; Sigma Aldrich, cat. no. A5006)
- l-lysine (Lys-0; Sigma Aldrich, cat. no. L5501)
- l-arginine [$^{13}C_6$]HCl (Arg-6; Cambridge Isotope Laboratories, cat. no. CLM-2265-H-PK)
- l-lysine-4,4,5,5-d_4 (Lys-4; Cambridge Isotope Laboratories, cat. no. DLM-2640-PK)
- l-arginine [$^{13}C_6$,$^{15}N_4$]HCl (Arg-10; Sigma-Aldrich, cat. no. 608033)

- l-lysine [$^{13}C_6$,$^{15}N_2$]HCl (Lys-8; Cambridge Isotope Laboratories, cat. no. CNLM-291-H-PK)
- iTRAQ reagents, 4-plex (Sciex, cat. no. 4352135)
- Ponceau S HCl (Sigma-Aldrich, cat. no. P-3504)
- Pierce 660-nm protein assay reagent (Thermo Fisher Scientific, cat. no. 22660)
- Coomassie stain: for mass spectrometry (MS) samples, we use SimplyBlue SafeStain from Life Technologies, cat. no. LC6065
- Silver stain (Pierce, cat. no. 24612) **! CAUTION** Silver stain is toxic. Wear protective gloves and clothing.
- Sequencing-grade modified trypsin (Promega, cat. no. V5111)
- Mouse anti-V5 antibody (Life Technologies, cat. no. R960-25)
- Streptavidin-horseradish peroxidase (streptavidin-HRP; Life Technologies, cat. no. S-911) ▲ **CRITICAL**Other streptavidin-HRP conjugates may give higher background.
- Streptavidin magnetic beads (Pierce, cat. no. 88817)
- BSA (Fisher Scientific, cat. no. BP1600-1)
- NeutrAvidin biotin-binding protein (Invitrogen, cat. no. A-2666)
- Alexa Fluor 647 succinimidyl ester (Invitrogen, cat. no. A-20006)
- Dulbecco's PBS (DPBS; Sigma-Aldrich, cat. no. D5773-10L)
- Tween-20 (Sigma-Aldrich, cat. no. P1379-1L)
- Triton X-100 (Sigma-Aldrich, cat. no. T9284-500ML)
- Sodium deoxycholate (Sigma-Aldrich, cat. no. D6750-100G)
- SDS (VWR International, cat. no. 97064-496)
- Bromophenol blue (Sigma-Aldrich, cat. no. B0126)
- Glycerol (VWR International, cat. no. BDH1172-4LP)
- Urea (VWR International, cat. no. EM-UX0065-1)
- Biotin (Sigma-Aldrich, cat. no. B4501)
- Protease inhibitor cocktail, 100× (Sigma-Aldrich, cat. no. P8849)
- PMSF (G-Biosciences, cat. no. 786-055) **! CAUTION** PMSF is toxic. Wear protective gloves and clothing.
- Hydrogen peroxide (H_2O_2), 30% (wt/wt) (Sigma-Aldrich, cat. no. H1009-100ML) **! CAUTION** Hydrogen peroxide is corrosive. Wear protective gloves and clothing.

- Formaldehyde, 10% (wt/wt), methanol free (Polysciences, cat. no. 04018) **! CAUTION** Formaldehyde is toxic and corrosive. Wear protective gloves and clothing. Use it in a chemical fume hood.
- DMSO (VWR International, cat. no. EM-MX1458-6)
- Methanol (Sigma-Aldrich, cat. no. 34966-4L) ▲ **CRITICAL** Purchase LC- and MS-grade reagents.
- Acetonitrile (J.T. Baker, cat. no. 9829-03) ▲ **CRITICAL** Purchase LC- and MS-grade reagents.
- Acetic acid (VWR International, cat. no. MKV193-45)
- Trifluoroacetic acid (TFA; Sigma-Aldrich, cat. no. 91707) ▲ **CRITICAL** Purchase LC- and MS-grade reagents.
- Formic acid (FA; Sigma-Aldrich, cat. no. 56302) ▲ **CRITICAL** Purchase LC- and MS-grade reagents.
- Iodoacetamide (Sigma-Aldrich, cat. no. A3221)
- DTT (Pierce, cat. no. 20291)
- Biotin-phenol (BP; IUPAC name (3aS,4S,6aR)-hexahydro-*N*-[2-(4-hydroxyphenyl)ethyl]-2-oxo-1H-thieno[3,4-d]imidazole-4-pentanamide): synthesize or purchase from Iris-Biotech (CAS no.: 41994-02-9)
- Ammonium bicarbonate (Sigma-Aldrich, cat. no. A6141-500G)
- Sodium ascorbate (VWR International, cat. no. 95035-692)
- Trolox (Sigma-Aldrich, cat. no. 238813-5G)
- Sodium azide (VWR International, cat. no. AA14314-22) **! CAUTION** Sodium azide is toxic. Wear protective gloves and clothing.
- Tris(hydroxymethyl)aminomethane (Tris; Sigma-Aldrich, cat. no. T6066-5KG)
- Sodium chloride (NaCl; VWR International, cat. no. 470302-512)
- Potassium chloride (KCl; Sigma-Aldrich, cat. no. P3911-500G)
- Sodium carbonate (Na_2CO_3; Mallinckrodt, cat. no. 7527-04)

EQUIPMENT

- 18-gauge needle for picking up coverslips
- Tweezers
- Number 1 glass coverslips, 22 × 22 mm: cut glass coverslips into 7 × 7 mm squares. UV-sterilize for at least 20 min
- Standard equipment for mammalian cell culture
- 48-well plate for imaging cells

- Six-well plate for preparing western blot samples
- Tabletop centrifuge
- Floor centrifuge
- Fluorescence microscope with appropriate filter sets
- Magnetic rack for streptavidin beads (Invitrogen, cat. no. CS15000)
- NuPAGE Novex 4–12% Bis-Tris Protein Gels, 1.5 mm, 10 well (Thermo Fisher Scientific, cat. no. NP0335PK2)
- Standard UV-visible spectrophotometer
- SpeedVac vacuum concentrator
- Solid-phase extraction disk for StageTips: Empore C18 extraction disk (3M, cat. no. 98060402181 or cat. no. 98060402173)
- Nanospray column: Self-pack PicoFrit column, 360 μm outer diameter (o.d.) × 75 μm inner diameter (i.d.), 10 μm i.d. tip, 50 cm length (New Objective, cat. no. PF360-75-10-N-5); ReproSil-Pur 120 Å, C18-AQ, 1.9 μm (Dr. Maisch)
- Q Exactive mass spectrometer (Thermo Fisher Scientific)
- Proxeon Easy-nLC 1000 (Thermo Fisher Scientific)
- Nanospray column heater, 20 cm (Phoenix S&T, cat. no. PST-CH-20U) and column heater controller (Phoenix S&T, cat. no. PST-CHC)
- A protein/peptide identification and quantification software tool such as MaxQuant (freely available; can be downloaded from http://www.coxdocs.org/doku.php?id=maxquant:common:download_and_installation)

REAGENT SETUP

SILAC medium—DMEM deficient in L-arginine and L-lysine is supplemented with 10% (vol/vol) dialyzed FBS, penicillin, streptomycin, glutamine, 4.5 g/liter glucose and isotopes of lysine (final concentration 146 mg/liter) and arginine (final concentration 84 mg/liter). For heavy SILAC medium, add L-lysine [$^{13}C_6$,$^{15}N_2$]HCl (Lys-8) and L-arginine [$^{13}C_6$,$^{15}N_4$]HCl (Arg-10). For medium SILAC medium, add L-lysine-4,4,5,5-d_4 (Lys-4) and L-arginine [$^{13}C_6$]HCl (Arg-6). For light SILAC medium, add L-lysine (Lys-0) and L-arginine (Arg-0). Filter-sterilize with a 0.2-μm filter. After filtering, this can be stored at 4 °C for several months.

NeutrAvidin–Alexa Fluor 647 conjugate—This reagent is prepared as per Invitrogen's instructions.

Tris-HCl, 1 M (pH 8.0)—Dissolve Tris into dH_2O, and adjust the pH to the appropriate value with hydrochloric acid (HCl). This can be stored at room temperature (20–25 °C) for several months.

Formaldehyde fixation solution, 4% (wt/wt)—Dilute 10% (wt/wt) formaldehyde into the appropriate buffer system for the cell line of interest. The 4% (wt/wt) formaldehyde solution can be stored at 4 °C for several months.

Protein loading buffer, 6×—Combine 10.5 ml of dH_2O, 10.5 ml of 1 M Tris-HCl (pH 6.8), 10.8 ml of glycerol, 3.0 g of sodium dodecylsulfate, 2.79 g of DTT and 3.6 mg of bromophenol blue into a conical tube. Mix well, and then divide the buffer into 1-ml aliquots and store them at − 20 °C. Frozen aliquots can be stored for several months.

Tween-20 in Tris-buffered saline, 0.1% (vol/vol) (1× TBST)—We typically first make a 20× TBST stock. Mix 48.4 g of Tris, 160 g of NaCl and 20 ml of Tween-20 in a 1-liter bottle; adjust the pH to 7.6 with HCl and bring the volume to 1 liter with dH_2O. This solution can be stored at room temperature for several months. To make 1× TBST, dilute the 20× TBST stock with dH_2O. This can be stored at room temperature for several months.

Ponceau S stain—Combine 50 mg of Ponceau S powder with 2.5 ml of acetic acid in a 50-ml conical tube. Adjust dH_2O to 50 ml. This solution can be stored at room temperature for several months. Ponceau S stain can be reused several times.

RIPA lysis buffer—Combine 50 mM Tris, 150 mM NaCl, 0.1% (wt/vol) SDS, 0.5% (wt/vol) sodium deoxycholate and 1% (vol/vol) Triton X-100 in Millipore water. Adjust the pH to 7.5 with HCl. This solution can be stored at 4 °C for many months, although for proteomic experiments it is best to make it fresh.

PMSF, 100 mM—Dissolve PMSF in isopropanol. Divide the solution into aliquots and store them at − 20 °C. Frozen aliquots can be stored for several months.

Stock of BP, 500 mM—Dissolve BP in DMSO. The 500 mM stock may need to be sonicated. Divide the solution into 10-μl aliquots in the BP stock and store them at − 80 °C. These aliquots can be stored for several months.

Stock of H_2O_2, 100 mM—Dilute the 30% (wt/wt) H_2O_2 reagent (~10 M H_2O_2 in water) into Dulbecco's PBS (DPBS) immediately before using this to label cells. ▲ **CRITICAL** Do not store this solution.

Sodium ascorbate, 1 M—Store sodium ascorbate powder in a desiccator. Dissolve sodium ascorbate in Millipore water immediately before making solution. ▲ **CRITICAL** Do not store this stock.

Trolox, 500 mM—Dissolve Trolox in DMSO. Sonicate it well. Prepare this stock immediately before making quencher solution. ▲ **CRITICAL** Do not store this stock.

Sodium azide, 1 M—Dissolve sodium azide in Millipore water. Aliquots can be stored at − 20 °C or below for several months.

Quencher solution—Dilute each of the stocks above to make a 10 mM sodium ascorbate, 5 mM Trolox and 10 mM sodium azide solution in DPBS. ▲ **crItIcal** Make this solution immediately before it is to be used to quench the biotinylation reaction. Do not store this solution.

KCl, 1 M—Dissolve KCl in Millipore water. This can be stored at room temperature for several months.

Na_2CO_3, 0.1 M—Dissolve Na_2CO_3 in Millipore water. This can be stored at room temperature for several months. Note that this is not buffered, and it will give a pH of 11.5.

Urea, 2 M in 10 mM Tris-HCl (pH 8.0)—Dissolve urea in the Tris buffer, and adjust the pH with HCl. Make this solution fresh on the day of use.

Biotin stock, 100 mM—Dissolve biotin in DMSO. Make 1-ml aliquots and store at –20 °C. This can be stored for several months.

DTT stock, 1 M—Dissolve DTT in Millipore water. Freshly prepare this solution before use.

Ammonium bicarbonate, 100 mM (pH 8.0)—Dissolve ammonium bicarbonate in Millipore water. The pH should be close to 8.0. This is best if made fresh, but it can be stored at room temperature for a couple of weeks.

50:50 acetonitrile: 100 mM ammonium bicarbonate (pH 8.0)—Mix equal volumes of 100% acetonitrile and 100 mM ammonium bicarbonate, pH 8.0. This is best if made fresh.

DTT, 10 mM in 100 mM ammonium bicarbonate (pH 8.0)—Dissolve DTT into 100 mM ammonium bicarbonate. Freshly prepare this solution before use.

Iodoacetamide, 55 mM in 100 mM ammonium bicarbonate (pH 8.0)—Dissolve iodoacetamide in 100 mM ammonium bicarbonate, pH 8.0. Freshly prepare this solution before use. Keep the solution in the dark.

Solution of trypsin, 10–20 ng/μl in 100 mM ammonium bicarbonate (pH 8.0—Dissolve the lyophilized, sequencing-grade trypsin in 100 mM ammonium bicarbonate, pH 8.0. Freshly prepare this solution before use.

Gel extraction solvent—Gel extraction solvent is 60% (vol/vol) acetonitrile/0.1% (vol/vol) trifluoroacetic acid. This can be made in advance and is stable at room temperature for at least 2 weeks.

StageTip solvents—StageTip solvents are 0.1% (vol/vol) formic acid (FA), 0.1% (vol/vol) FA/50% (vol/vol) acetonitrile and 0.1% (vol/vol) FA/50% (vol/vol) acetonitrile. These can be made in advance and stored at room temperature for several weeks.

LC-MS/MS solvents—Solvent A is 0.1% (vol/vol) FA/3% (vol/vol) acetonitrile. Solvent B is 0.1% (vol/vol) FA/90% (vol/vol) acetonitrile. These solvents are stable for at least two weeks at room temperature.

EQUIPMENT SETUP

Liquid chromatography (LC) settings—Solvents A and B are given in Reagent Setup. These settings are specific to a nanospray column packed up to 24 cm with ReproSil-Pur 1.9 μm, C18 resin. If another LC system is used, it may be necessary to adjust these parameters.

Time (min)	%B	Flow rate (nl/min)
0	2	200
1	6	200
85	30	200
94	60	200
95	90	200
100	90	200
101	50	500
110	50	500

Mass spectrometer (MS) settings—MS settings are as tabulated below. The parameters listed below are specific for analysis on a Q Exactive Plus MS system. If another MS system is used for the analysis, it may be necessary to adjust these parameters.

Method parameter	Value
Polarity	Positive
Full MS	
Microscans	1
Resolution	70,000
Automatic gain control (AGC) target	3×10^6 ion counts
Maximum ion time	5 ms
Scan range	300–1800 *m/z*
dd-MS^2	
Microscans	1
AGC target	5×10^4 ion counts
Maximum ion time	120 ms
Loop count	12
Isolation window	1.6 *m/z*
Fixed first mass	100 *m/z*
Normalized collision energy	25
dd settings	
Underfill ratio	5%

Method parameter	Value
Charge exclusion	Unassigned, 1, 7
Peptide match	Preferred
Exclude isotopes	On
Dynamic exclusion	20

Parameters for searching the MS data—Typical parameters for searching data with MaxQuant are as tabulated below

Parameter	Value
Variable modification	Oxidation (M)
	Acetyl (protein N-term)
	Biotinylation by BP (Y; (C18H23N3O3S))
Fixed modification	Carbamidomethyl (C)
Maximum missed cleavages	2–4
Maximum charge	6
First search p.p.m.	20
Main search p.p.m.	6
MS/MS tolerance (p.p.m.)	20
Peptide false discovery rate (FDR)	0.01
Site FDR	0.01
Protein FDR	0.01
Minimum peptide length	7

PROCEDURE

Generation of APEX2 fusion constructs ● TIMING variable

1| See **Box 1**.

? TROUBLESHOOTING

characterization of the APEX2 fusion constructs by imaging ● TIMING 3–4 d; 8–9 d for step 8 (optional)

2| For imaging, plate the cells on 7 × 7 mm glass coverslips that are placed inside the wells of a plastic 48-well plate. If needed, precoat the coverslips with 50 μg/ml human fibronectin to improve cell adherence. Include wells for negative controls that omit APEX2, BP or H_2O_2 to assess the background signal contributed by nonspecific sticking of the secondary antibody or streptavidin/NeutrAvidin-fluorophore conjugates, as well as by endogenously present H_2O_2 and endogenous peroxidase activity.

3| Introduce the APEX2 plasmid to the cells, typically via transfection with Lipofectamine 2000 or infection with lentivirus. If you are using a fluorescent protein

organelle marker such as mitochondrial-GFP, then transfect the marker together with the APEX2 gene. Alternatively, immunostain for an endogenous organelle marker later.

4| About 24 h after transfection with Lipofectamine 2000 or 48 h after induction with lentivirus, incubate the cells in 200 μl of 500 μM BP in complete medium for 30 min at 37 °C.

▲ **CRITICAL STEP**The medium should be prewarmed to 37 °C to facilitate dissolution of the BP. The solution may need to be sonicated to fully dissolve the probe.

5| Make a fresh 100× stock of 100 mM H_2O_2 in DPBS. Next, add the appropriate amount of H_2O_2 directly to the BP solution and briefly agitate to achieve a final concentration of 1 mM. Incubate the cells at room temperature for 1 min.

6| Quickly aspirate the labeling solution and wash all cells three times in quencher solution. Fix with 4% (wt/wt) formaldehyde using standard protocols[5]. For one well of a 48-well plate, each quencher wash will typically be 200 μl.

▲ **CRITICAL STEP** Quencher solution should be made immediately before use.

▲ **CRITICAL STEP** Do not omit the washes because they remove excess, unused BP probe left inside the cells. Make sure that the washes are performed using the quencher solution and not merely DPBS, so that BP radicals are quenched.

7| After cell fixation, immunostain for the APEX2 fusion protein via its epitope tag. For V5 detection, use a 1:1,000 dilution of mouse anti-V5 antibody. Compare this immunostaining pattern with a fluorescent protein marker (e.g., mitochondrial-GFP) or endogenous marker (e.g., anti-Tom20 to visualize mitochondria) to determine the localization of the construct. In addition, stain for biotinylated proteins using a streptavidin- or NeutrAvidin-fluorophore conjugate during incubation with the secondary antibody.

? TROUBLESHOOTING

8| (Optional) Prepare EM samples to analyze the APEX2 construct at higher spatial resolution[10] (such as in **Fig. 2a**). The protocol for EM visualization of APEX2 or APEX is given in Lam *et al.*[9] and Martell *et al.*[10].

Box 1

Generating APEX2 fusion constructs

We recommend APEX2 over APEX for all proteomic (and EM[9,10]) applications because of its higher activity and resistance to H_2O_2-induced inhibition[9]. When considering how to fuse APEX2 to target it to a cellular ROI, previous examples of GFP targeting to the same region can be informative. Most proteins and peptides that tolerate GFP tags will also tolerate APEX2, which is the same size (27 kDa) and monomeric. APEX2 is active as an N-terminal fusion[5,9,10], as a C-terminal fusion[4,5,9,10] and also as an internal fusion[4]. We typically insert flexible Gly- and Ser-rich linkers up to ten amino acids in length between APEX2 and the targeting protein or peptide in order to minimize the effect on both the targeting moiety and APEX2. The linker can also incorporate an epitope tag

such as V5 in order to facilitate detection of the APEX2 fusion construct. For example, the fusion construct that we used for mapping the mitochondrial matrix was (24–amino acid leader sequence from COX4)-(V5 epitope tag)-APEX[4]. The construct used for IMS proteomic mapping was (68–amino acid leader sequence from LACTB)-(V5 epitope tag)-APEX[5]. It is crucial to avoid HA and other tyrosine-containing epitope tags because these may be irreversibly destroyed by APEX2 labeling chemistry.

APEX2-encoding DNA plasmids can be introduced into mammalian cells by transfection or by viral transduction. Using clonal cells that stably express APEX2 or changing the promoter of the construct may be necessary to achieve the desired balance between high enough expression for sufficient APEX2 activity on the one hand and low enough expression to enable clean targeting with minimal perturbation of organelle properties on the other. If the APEX2 fusion construct is transfected, we recommend a transfection efficiency of at least 50% so that proteins biotinylated by the APEX2-catalyzed reaction are not swamped out by endogenous biotinylated proteins during the streptavidin bead enrichment. If the APEX2 fusion construct is expressed at a low level, higher transfection efficiency is needed to generate more biotinylated material.

The localization of the APEX2 fusion construct should be tested by immunofluorescence staining and compared with a marker that is known to reside in the organelle or ROI. For certain organelles such as mitochondria and the ER, imaging will be clearer in flat cells such as COS-7 or HeLa cells, rather than HEK cells. However, it is more common to observe high activity in HEK cells because of their ability to overexpress protein. Ultimately, the localization of the APEX2 construct should be visualized in the cell type used for the proteomic experiment.

characterization of the apeX2 fusion constructs by western blotting ● TIMING 5 d; 3–4 d for step 19

9| For each condition, plate fresh cells into one well of a six-well plate and introduce the APEX2 construct via lipofection or lentiviral transduction. As in the imaging experiments, plate additional wells for negative controls—i.e., omit APEX2, BP or H_2O_2 samples. Label, quench and wash the live cells as described in Steps 4–6 with 2-ml volumes for each step.

10| Use fresh quencher solution to gently pipette the cells off of the bottom of the well. More adherent cells such as HeLa may need to be scraped from the bottom of the well. Pellet the cells by centrifugation for 10 min at 3,000*g* at 4 °C, and then remove the supernatant.

■ **PAUSE POINT** The pellets can be stored at − 80 °C for several months.

11| Lyse the cell pellets by gentle pipetting in RIPA lysis buffer supplemented with 1× protease inhibitor cocktail, 1 mM PMSF and quenchers (10 mM sodium azide, 10 mM sodium ascorbate and 5 mM Trolox). For HEK 293T cells, we use 100 µl of lysis buffer for each pellet derived from one well of a six-well plate. After resuspension, leave the sample on ice for ~2 min. Clarify the lysates by centrifuging at 15,000*g* for 10 min at 4 °C. Keep the lysates on ice throughout the procedure.

■ **PAUSE POINT** The samples can be flash-frozen and stored at − 80 °C. Typically, the protein concentration of the clarified sample is ~4 mg/ml.

12| For each sample, combine 5 μl of whole-cell lysate with 1 μl of 6× protein loading buffer, and then boil it at 95 °C for 10 min.

13| Cool the boiled samples on ice, centrifuge them briefly to bring down condensation and separate the proteins on a 9% (wt/vol) SDS gel.

14| Transfer the samples to a nitrocellulose membrane using standard equipment and protocols[5].

15| Stain the membrane with Ponceau S to check the quality of the transfer, and compare the protein content across lanes. Next, remove the Ponceau S stain by rinsing the membrane with deionized water several times.

16| Block the membrane with 3% (wt/vol) BSA in 1× TBST overnight at 4 °C. Note that this step can also be performed for only 1 h at room temperature, but we find that overnight blocking gives the best results.

17| Rock the membrane in 10 ml of 0.3 μg/ml streptavidin-HRP in 3% (wt/vol) BSA in 1× TBST at room temperature for 1 h, and then wash it with 1× TBST four times for 5 min each time.

18| Develop the membrane using standard protocols[5]. In the negative control lanes, only the endogenous biotinylated proteins should be visible at 130, 75 and 72 kDa (ref. 21) (**Fig. 2c**).

? TROUBLESHOOTING

19| To visualize the APEX2 construct by blot, run the lysates in a new gel, as described in Steps 12 and 13, and transfer them to a nitrocellulose membrane. After blocking as above, incubate the membranes with mouse anti-V5 antibody at a 1:5,000 dilution in 3% (wt/vol) BSA in 1× TBST for 1 h at room temperature. Wash the membrane by rocking it in 1× TBST four times for 5 min each time. Next, proceed with incubation in a secondary antibody solution and subsequent development according to standard protocols.

optimization of streptavidin pull-down of biotinylated proteins before a full-scale Ms experiment ● TIMING 4–5 d; 4–5 d for step 27 (optional)

20| Prepare fresh biotinylated cell lysate samples as described above in Steps 9–11. Prepare cells for two replicate samples in six-well plates for each condition so that one replicate can be analyzed by western blotting and one replicate can be analyzed by silver staining. Be particularly careful to perform the postlabeling cell washes with quencher solution thoroughly, as described in Step 6, using 2-ml volumes each time.

21| Quantify the amount of protein in each clarified whole-cell lysate by using the Pierce 660-nm assay. If necessary, dilute the clarified whole-cell lysate first so that the concentrations fall in the linear range of the assay. Prepare triplicate samples for each condition.

▲ **CRITICAL STEP** The bicinchoninic acid (BCA) assay cannot be used for protein quantification because of the presence of ascorbate and Trolox in the quencher solution. If you must use the BCA assay, then after the labeling and quenching steps, separate 10% of the cells into another tube and pellet them. The cell pellet should be resuspended in 1 ml of DPBS instead of quencher solution. Pellet again, resuspend the pellet in fresh DPBS, and then pellet yet again to wash the cells. Lyse the final pellet in RIPA lysis buffer (without quenchers added) and assay by using a BCA kit.

22| For each sample, take 30-µl aliquots of streptavidin magnetic beads. *Note:* when you are handling the streptavidin magnetic beads, use 200-µl or larger pipette tips whose tips have been cut off using a clean razor. Wash each aliquot of beads twice with 1 ml of RIPA lysis buffer. Then, incubate 360 µg (~90 µl) of each whole-cell lysate sample with 30 µl of streptavidin magnetic beads for 1 h at room temperature on a rotator. We add an additional 500 µl of RIPA buffer to each sample to facilitate rotation. *Note:* this step can also be done at 4 °C overnight. Save the remaining whole-cell lysate for gel and western blot analysis.

23| Pellet the beads using a magnetic rack and collect the supernatant. This is the 'flow-through,' which should be saved on ice for subsequent analysis. Wash each bead sample with a series of buffers (1 ml for each wash) to remove nonspecific binders: twice with RIPA lysis buffer, once with 1 M KCl, once with 0.1 M Na_2CO_3, once with 2 M urea in 10 mM Tris-HCl, pH 8.0, and twice with RIPA lysis buffer. The wash buffers are kept on ice throughout the procedure. Note that this washing protocol is more extensive than that described in Rhee *et al.*[4] and Hung *et al.*[5].

24| Elute biotinylated proteins from the beads by boiling each sample in 30 µl of 3× protein loading buffer supplemented with 2 mM biotin and 20 mM DTT for 10 min. Vortex the beads briefly, and then cool the samples on ice and briefly spin down to bring down condensation. Place the samples on a magnetic rack to pellet the beads and to collect the eluate. Place the eluates on ice. Use the first replicate set for western blot analysis (Step 25) and set aside the second replicate set for silver stain analysis (Step 26).

25| For western blot analysis, prepare and boil the whole-cell lysate and flow-through samples from the first replicate set in 1× protein loading buffer. Cool the samples on ice and spin them briefly to bring down condensation. Load and run the whole-cell lysate, streptavidin enrichment eluate and flow-through samples on a 9% (wt/vol) SDS gel. For this analysis, we usually load all of the streptavidin enrichment eluate. Perform a streptavidin-HRP western blot as in Steps 14–18. Check that there is no biotinylated material left in the flow-through.

? TROUBLESHOOTING

26| Load the streptavidin enrichment eluates from the second replicate set onto another 9% (wt/vol) SDS gel and separate the proteins. Use Coomassie or silver stain to visualize the total protein in each lane. More protein material should be eluted from the experimental sample than from the negative controls (such as in **Fig. 2d**). Depending on your results, repeat with higher or lower amounts of streptavidin beads.

? TROUBLESHOOTING

27| (Optional) If desired, prepare fresh streptavidin-enriched samples to determine whether endogenous protein markers are enriched via western blotting. See **Box 2** for the protocol.

preparing sllac samples for Ms proteomics ● TIMING variable; for HeK 293t cells: 10–13 d

28| Design at least two replicate experimental sample sets, in which the heavy state is the experimental flask, the medium state is the flask in which APEX2 is targeted outside the ROI and the light state is a negative control in which biotinylation does not occur (**Fig. 1c**). Include a different negative control for each experimental sample set—for example, one in which APEX2 is not expressed (to control for possible biotinylation by endogenous peroxidases) and one in which H_2O_2 is omitted (to control for biotinylation caused by endogenous H_2O_2).

29| Culture mammalian cells in SILAC medium for 5–7 cell doublings (we cultured HEK 293T cells in SILAC medium for 8–10 d). All of the cultures should be passaged in parallel so that there is minimal variation in the incorporation of the isotopic amino acids. Expand cultures to anticipate sufficient cellular material so that the amount of biotinylated proteins collected at the end is visible by Coomassie staining and so there is enough whole-cell lysate to reserve for quality control streptavidin-HRP and silver stain analysis. For our mitochondrially targeted APEX2 constructs, we expanded our HEK 293T cultures into one T150 flask per condition (~20 million cells when fully confluent). When splitting the cells for the last time before labeling, plate some cells in separate wells for performing quality control fluorescence imaging. These cells should be labeled concurrently using the same reagents as the proteomic flasks. *Note:* for iTRAQ and TMT, special medium is not needed.

30| If you are not using stable cells, transfect or transduce cells with the APEX2 constructs of interest. Use previously optimized conditions that give maximum transfection or transduction efficiency while achieving the proper level of APEX2 expression per cell.

31| After waiting for the appropriate amount of time for APEX2 expression (also previously optimized in Steps 9–19), incubate the cells in 500 μM BP in complete SILAC medium prewarmed to 37 °C (30 ml for a T150 flask).

32| After 30 min of BP incubation, invert the flask so that the cells are on the ceiling and the BP medium is pooled on the bottom of the flask. Quickly add the H_2O_2 to the BP medium for a final concentration of 1 mM, and agitate the flask to mix. Invert the flask so that the cells are once again exposed to the BP medium, which is now supplemented with 1 mM H_2O_2. Allow the reaction to proceed for 1 min. Next, invert the flask and pour out the medium. Immediately wash the cells twice with quencher solution, twice with DPBS and once with quencher solution for 1 min each time using the same inversion technique. To do this, invert the flask and gently pour each solution onto the bottom face of the flask and then invert the flask so that the cells are covered with the

solution. After 1 min, invert the flask and pour out the wash solution. This ensures that cells such as HEK 293T, which are loosely adherent, are not lost during the washes. For a T150 flask, we use 25 ml for each wash. Alternatively, quencher solution can be used for all five washes.

▲ **CRITICAL STEP** Freshly prepare the 1 M sodium ascorbate stock in water and 500 mM Trolox stock in DMSO, and then assemble the quencher solution immediately before use. We also freshly prepare 100 mM stock of H_2O_2 in DPBS.

▲ **CRITICAL STEP** After incubation and labeling, the cells may be prone to lifting. Gentle technique is crucial to successfully completing the labeling and wash protocol without loss of cellular material.

33| Collect the cells in fresh quencher solution (10 ml for a T150 flask) as in Step 10. Pellet the cells by centrifugation at 3,000*g* for 10 min at 4 °C. Remove the supernatant, flash-freeze the pellet in liquid nitrogen and store the pellet at − 80 °C.

■ **PAUSE POINT** The pellet can be stored at − 80 °C until you are ready to proceed.

34| Lyse the cells in fresh RIPA lysis buffer supplemented with 1× protease inhibitor cocktail, 1 mM PMSF, 5 mM Trolox, 10 mM sodium azide and 10 mM sodium ascorbate (800 μl for a pellet from a T150 flask) by gentle pipetting. Incubate the lysates on ice for ~2 min. Clarify the lysates by centrifugation at 15,000*g* for 10 min at 4 °C. Keep the lysates on ice throughout the procedure.

35| Use the Pierce 660-nm assay to measure the amount of protein in each clarified whole-cell lysate, as described in Step 21.

▲ **CRITICAL STEP** The BCA assay cannot be used for protein quantification; see Step 21 for details.

36| Set aside enough of each whole-cell lysate (determined by the optimization experiments leading up to the proteomic experiment) for quality control analysis by streptavidin blotting to ensure that biotinylation worked, as well as for SDS-PAGE and silver staining after a small-scale streptavidin bead enrichment to ensure that enrichment worked and that there is minimal nonspecific binding to the streptavidin beads for the negative control samples. These are performed to check the quality of the cells, reagents and protocols used for preparation of the proteomic samples.

37| Mix the whole-cell lysates from the heavy (H), medium (M) and light (L) samples in a 1:1:1 H:M:L ratio by protein mass (determined using the Pierce 660-nm assay). Use at least 2 mg of total protein per state; 3–4 mg per state is preferable. ▲ **CRITICAL STEP** If samples are for iTRAQ or TMT rather than SILAC, do not mix the protein samples at this step.

38| Wash streptavidin magnetic beads for each experimental set two times with 1 ml of RIPA lysis buffer each time. The amount of streptavidin-coated magnetic beads should be scaled appropriately based on the results from the bead titration performed on a small scale. For SILAC samples, add the 1:1:1 H:M:L protein mixture to the beads. For iTRAQ or TMT samples, the proteins for each condition are separately rotated with

prepared streptavidin-coated magnetic beads. Rotate the samples for 1 h at room temperature or 4 °C overnight.

39| Pellet the beads on a magnetic rack. Save the supernatant ('flow-through') for further analysis. This can be stored at 4 °C for a few days, or it can be flash-frozen and stored indefinitely at −80 °C.

40| Wash the beads twice with RIPA lysis buffer, once with KCl, once with 0.1 M Na_2CO_3, once with 2 M urea in 10 mM Tris-HCl (pH 8.0) and twice with RIPA lysis buffer for each sample. Use 1-ml volumes at 4 °C to wash. For each wash, thoroughly suspend the beads, rotate a few times and then pellet on the magnetic rack. Cleanly remove all of the liquid before adding the next wash buffer. All buffers are kept on ice during the procedure.

41| For SILAC samples, elute the biotinylated proteins by boiling the beads in 60 µl of 3× protein loading buffer supplemented with 2 mM biotin and 20 mM DTT for 10 min. Vortex briefly, and then cool the tubes and briefly spin them down to bring down condensation. Pellet the beads using a magnetic rack and collect the eluate.

▲ **CRITICAL STEP** The volume of the elution buffer is limited by the volume that can be loaded into the NuPAGE Novex Bis-Tris 4–12% (wt/vol) gel, and it may need to be reduced if one is using a gel with smaller wells.

▲ **CRITICAL STEP** For iTRAQ or TMT samples, Hubner *et al.*[33] and Sancak *et al.*[34] provide a protocol that can be used after the beads are washed as in Step 38. Briefly, instead of eluting the biotinylated proteins, move the beads to a clean tube and proceed to on-bead trypsin digestion. After trypsin digestion, peptides are labeled with iTRAQ or TMT reagents, differentially labeled samples are mixed and the mixed sample is desalted before injection on the MS. This protocol has been successfully used to map the mitochondrial matrix proteome in *Drosophila* muscle tissue[17].

■ **PAUSE POINT** The eluate can be flash-frozen and stored at − 80 °C for several weeks if needed.

Box 2

Blotting the biotinylated proteome for endogenous markers

The streptavidin bead–enriched cellular material can also be blotted for proteins that reside within or outside the ROI to ensure that APEX2-catalyzed biotinylation is spatially specific. We find that in order to perform this assay successfully, we need to scale up 2.5-fold, from six-well plates to T25 flasks. The labeling method for these larger flasks is slightly different. We find that we can control timing more accurately using this method compared with conventional pipetting and aspirating out of T25 flasks.

1. Preincubate the cells for 30 min in 4 ml of 500 µM BP in full cell culture medium, and then invert the flask upside-down such that the labeling solution is now pooled on the ceiling of the flask.

2. Quickly add 40 μl of 100 mM H_2O_2 to this solution and agitate to mix. Labeling commences when the flask is inverted right-side up, exposing the cells to the BP and H_2O_2 solution.
3. After 1 min, invert the flask again, and pour out the labeling solution.
4. Directly pour 5 ml of fresh quencher solution to the upside-down flask, and invert it. Repeat this step two more times.
5. Collect the cells in 5 ml of quencher solution and pellet the cells as described in Step 10. Lyse the cell pellet as described in Step 11, but use 250 μl of RIPA lysis buffer supplemented with 1× protease inhibitor cocktail, 1 mM PMSF and quenchers. Clarify the cell lysate as in Step 11, and quantify the amount of protein in the clarified lysates, as described in Step 21.
6. Perform the streptavidin enrichment as described in Step 22 with 2.5-fold more streptavidin-coated magnetic beads and 2.5-fold more whole-cell lysate. Remove the flow-through and wash the beads as described in Step 23. Elute the biotinylated proteins as in Step 24 using 75 μl of 3× protein loading buffer supplemented with 2 mM biotin and 20 mM DTT.
7. Combine 20 μg of each whole-cell lysate sample with 1× protein loading buffer and boil the samples at 95 °C for 10 min. Cool the samples on ice and spin them briefly. Load and run the whole-cell lysate samples and 25 μl of each streptavidin bead eluate on a 9% (wt/vol) SDS gel. Transfer the proteins to a nitrocellulose membrane and proceed with blotting for endogenous protein markers using standard equipment and protocols[5,9].

Fractionation of biotinylated proteome, digestion to peptides and Ms ● TIMING 2–5 d

42| Load each SILAC sample into one well of a NuPAGE Novex Bis-Tris 4–12% (wt/vol) gel and separate for 1 h at 130 V.

43| Stain the gel overnight with SimplyBlue SafeStain stain and record an image of the gel. Remove the SimplyBlue stain, and destain the gel with H_2O for ~1 h.

44| Excise 12–16 gel slices per lane of the gel. The number of slices will vary depending on the complexity of the labeled proteome. For best results, separate the gel in such a way that smaller slices, which include fewer proteins, are created for intensely stained regions and larger slices, which include more proteins, are created for regions that show moderate- to low-intensity staining.

45| Use a razor to cut the gel slices into 1-mm pieces. Put all the pieces derived from a single gel slice into a single Eppendorf tube. Use a different tube for each gel slice. Next, to each of the 12–16 tubes, add destaining solution with 50:50 acetonitrile:100 mM ammonium bicarbonate, pH 8.0 to destain, and shake until the gel pieces lose most of their blue color. Briefly spin down the gel pieces, pipette off the destaining solution and wash the pieces once with 100 mM ammonium bicarbonate, pH 8.0. Next, briefly

spin down the gel pieces, pipette off the ammonium bicarbonate solution, and dehydrate the pieces by adding 100–300 µl of acetonitrile. Let the gel pieces incubate in acetonitrile for 5 min at room temperature, and then spin down the gel pieces and pipette off the acetonitrile.

46| Add 100 µl of 10 mM DTT in 100 mM ammonium bicarbonate, pH 8.0, to each tube and shake them for 1 h at room temperature. Briefly spin down the tubes, pipette off the DTT solution and add 100 µl of 55 mM iodoacetamide in 100 mM ammonium bicarbonate to each sample. Let this react in the dark for 1 h at room temperature. Next, briefly spin down the tubes and pipette off the iodoacetamide solution.

47| To digest the proteins to peptides in-gel, add just enough of a 10–20 ng/µl solution of trypsin in 100 mM ammonium bicarbonate, pH 8.0, to cover the gel pieces. Allow the digestion to proceed overnight at room temperature while shaking. During this time, the gel pieces will absorb the trypsin solution.

48| To extract the peptides, briefly spin down the gel pieces and cover the pieces with 15–20 µl of 60% (vol/vol) acetonitrile/0.1% (vol/vol) trifluoroacetic acid. Let this solution sit on the gel pieces for 10 min at room temperature, and then collect the extracted peptide solution in a clean tube. Repeat this extraction procedure two additional times, and then add 100% acetonitrile for the final extraction. Dry down the extracted peptide solution by vacuum centrifugation at room temperature until completely dry.

49| Desalt the peptides using C18 Stage Tips. Prepare Stage Tips for each sample as described by Rappsilber *et al.* [35]. We pack two plugs of Empore C18 material into each StageTip. Place StageTips on top of 2-ml microcentrifuge tubes using StageTip adapters. Complete steps for StageTip desalting at 3,000–4,000*g* for 3 min at room temperature or until all of the top-loaded solution has traversed the StageTip. Wash StageTips with 50 µl of methanol, followed by equilibration with 2 × 50 µl of 0.1% (vol/vol) FA. Load the sample onto the equilibrated StageTip and subsequently desalt with 2 × 50 µl of 0.1% (vol/vol) FA. Elute peptides into a clean microcentrifuge tube with 50 µl of 50% (vol/vol) acetonitrile/0.1% FA (vol/vol). Dry the eluted peptide solution by vacuum centrifugation at room temperature until completely dry.

50| Reconstitute each sample in 9 µl of 0.1% (vol/vol) FA/3% (vol/vol) acetonitrile and place it in the LC autosampler.

51| Set up a nanospray column packed up to 24 cm with ReproSil-Pur 1.9 µm, C18 resin. Use the column heater to heat the column to 50 °C.

52| Set up the Q Exactive Plus MS using the parameters given in Equipment Setup. Run the LC with the solvents and gradient described in Equipment Setup. For each analysis, we typically inject 4 µl of each sample.

analysis of Ms data ● TIMING 1 week

53| We typically analyze MS data from SILAC experiments using MaxQuant[22,36]. Typical parameters used for searching data with MaxQuant are listed in Equipment

Setup. MaxQuant identifies proteins that are common contaminants, as well as proteins that are identified only by peptides that match to a decoy database of reversed peptide sequences. Remove these proteins from your data set.

54| Analyze the data from each replicate experiment independently through Step 63. Filter the protein list by unique peptide count. Remove any protein that has been identified by fewer than two unique peptides. We require two or more unique peptides to consider a protein 'detected'.

55| Starting with the un-normalized H/M and H/L SILAC ratios provided by MaxQuant, normalize the values against the distribution of nonbiotinylated false positive proteins (FP-A proteins from **Box 3**). Do not use the normalized ratios provided by MaxQuant. To normalize, first calculate the median of the distribution of ratios (H/M and H/L) for the subset of FP-A proteins. Divide the ratio for each detected protein by this median to normalize. Next, take the $\log_2$ of those values. Work with $\log_2$(normalized ratios) for the rest of the analysis. This normalization serves to center the distribution of nonspecific binders around $\log_2$(H/L) or $\log_2$(H/M) = 0.

56| Check the quantitative correlation for each SILAC/iTRAQ ratio across replicate experiments by generating separate scatter plots. Plot the $\log_2$(H/L) or $\log_2$(H/M) ratio values for each protein from the two different replicates and calculate the Pearson correlation coefficient for each ratio.

? TROUBLESHOOTING

57| Plot histograms that show the distribution of $\log_2$(H/L) ratios and $\log_2$(H/M) ratios within each replicate. We usually choose a bin size of 0.1 $\log_2$ units for each ratio. If the data are sparse, use a bin size of 0.2 $\log_2$ units to see a more continuous distribution.

58| For the $\log_2$(H/L) ratios (or the equivalent ratio of enrichment in the experimental sample compared with the negative control for iTRAQ), plot a histogram depicting the distribution of $\log_2$(H/L) ratios for TP proteins. Plot a second histogram of $\log_2$(H/L) ratios for nonbiotinylated false positive proteins (FP-A proteins). An example of such a plot is shown in **Figure 3a**.

? TROUBLESHOOTING

59| For the $\log_2$(H/M) ratios (or the equivalent ratio of enrichment for the sample with APEX2 inside the ROI compared with that for the sample with APEX2 outside the ROI in iTRAQ), plot a histogram depicting the distribution of detected TP proteins. Plot a second histogram depicting the distribution of potentially biotinylated but distal false positive proteins (FP-B proteins from **Box 3**). Typically, the right shift of the TP distribution is not as marked as with the $\log_2$(H/L) TP and FP-A histograms.

? TROUBLESHOOTING

60| Determine quantitative cutoffs for $\log_2$(H/L) and $\log_2$(H/M) SILAC ratios. First, plot separate receiver operating characteristic (ROC) curves for each SILAC ratio in each replicate, which depict how well APEX2 enriches for TP proteins over false positive proteins. Rank proteins by the SILAC ratio of interest in descending order. Then, for each cutoff (i.e., first protein, first two proteins, first three proteins and so on), calculate

the TPR and FPR. TPR = (# detected TP proteins above the cutoff)/(total # of detected TP proteins in that replicate). FPR = (# detected false positive proteins above the cutoff)/ (total # of detected false positive proteins in that replicate). Plot TPR values on the *y* axis, and plot the corresponding FPR values on the *x* axis. Determine whether the curve arcs above the diagonal, which indicates that the proteomic experiment enriched the TP proteins over false positive proteins. Also make sure that the curve is smooth.

? TROUBLESHOOTING

61| To determine the cutoffs, plot TPR-FPR versus $\log_2$(ratio) for each SILAC ratio. Take the $\log_2$(ratio) value corresponding to the maximum TPR-FPR value as the cutoff. A smooth ROC curve usually means that one should be able to get a single maximum. Do this analysis for both $\log_2$(H/L) and $\log_2$(H/M) ratios (or equivalent iTRAQ ratios).

? TROUBLESHOOTING

62| For each replicate, retain only the proteins with $\log_2$(H/L) and $\log_2$(H/M) ratios higher than the specific cutoffs determined in Steps 60 and 61.

63| Intersect the protein lists from all independent replicates to obtain the final proteome. If SILAC was used, one may also examine the list of biotinylated peptides from these proteins to find the sites of modification.

64| Calculate the specificity of the final proteome. Specificity is defined as the percentage of the proteome with prior annotation or literature connection to the ROI. For prior annotation, make use of Gene Ontology Cell Component (GOCC) annotations, UniProt annotations and literature. Different levels of specificity can be calculated; for example, if one is mapping the proteome for the mitochondrial matrix, one can calculate the specificity for mitochondria, as well as the specificity for the mitochondrial matrix subcompartment.

65| Calculate the depth of coverage of the final proteome. Depth of coverage is equal to the percentage of your TP list that was detected in the final proteome.

? TROUBLESHOOTING

Troubleshooting advice can be found in **table 1**.

Box 3

Creating lists of TP and false positive proteins

TP proteins should be proteins that are known to be in the ROI. We draw from the Gene Ontology Cell Component (GOCC)[37] database, UniProt and literature to create this list. Use AmiGO (http://amigo.geneontology.org/amigo) to find all of the gene products associated with a particular Gene Ontology (GO) term. Make this list as comprehensive as possible without sacrificing accuracy, as it will be used to determine cutoffs for SILAC ratios and to estimate the depth of coverage of the final proteome.

For analysis of three-state SILAC data, two different false positive protein lists will be necessary (FP-A and FP-B). The first, FP-A, is a list of proteins that should not be biotinylated to any degree by the APEX2 fusion construct. These proteins should reside

in a cellular location separated from the ROI by a biotin-phenoxyl radical-impermeant membrane. For example, if one is mapping the nuclear proteome using APEX2-NLS, then this FP-A list could consist of soluble mitochondrial matrix proteins, as provided in Rhee *et al.*[4]. If one is mapping the cytosolic proteome, one could also use soluble mitochondrial matrix proteins for the FP-A list, or soluble ER lumen proteins, which are separated from the cytosol by the ER membrane. It is crucial to choose soluble proteins because transmembrane proteins could be partially accessible and thus labeled. This list will be used to determine cutoffs for $\log_2$(H/L) to separate biotinylated proteins from nonbiotinylated proteins (i.e., nonspecific streptavidin bead binders).

The second false positive list, FP-B, consists of proteins that could be biotinylated by your APEX2 fusion construct (because they are not separated by an impermeable membrane) but are not localized to your ROI. For example, IMS-targeted APEX can biotinylate cytosolic proteins that never enter the IMS because the OMM is porous. Therefore, the FP-B list used for the IMS proteomic experiment consisted of cytosolic proteins lacking mitochondrial annotation. This list is used to determine cutoffs for $\log_2$(H/M), which distinguishes proteins within the ROI from proteins outside the ROI.

●TIMING

Step 1, generation of APEX2 fusion constructs: variable

characterization of the apeX2 fusion constructs by imaging

Steps 2–3: 2–3 d

Steps 4–6: 45 min

Step 7: 3.5 h–overnight

Step 8 (optional): 8–9 d

characterization of the apeX2 fusion constructs by western blotting

Step 9: 2–3 d

Step 10: 15–20 min

Steps 11 and 12: 40 min

Step 13: 1.5 h

Step 14: 3 h

Step 15: 20 min

Step 16: overnight

Steps 17 and 18: 1.75–3.5 h

Step 19: 3–4 d

optimization of streptavidin pull-down of biotinylated proteins before a full-scale Ms experiment

Steps 20–26: 4–5 d

Step 27 (optional): 4–5 d

preparing sIlac samples for Ms proteomics

Steps 28 and 29: variable; for HEK 293T cells: 8–10 d Step 30 (if not using stable cells): 1–2 d

Steps 31–33: 3–6 h (more if preparing more samples) Steps 34–37: 1–1.5 h

Steps 38–41: 2 h

Steps 42–52, fractionation of biotinylated proteome, digestion to peptides and MS: 2–5 d

Steps 53–65, analysis of MS data: 1 week

ANTICIPATED RESULTS

An appropriate APEX2 construct should target correctly and cleanly to the ROI. Imaging results of cells expressing APEX2 that were fixed and stained with a NeutrAvidin-fluorophore conjugate are shown in **Figure 2b**. Biotinylation should be dependent on the expression of APEX2 and the addition of H_2O_2 and BP. Representative streptavidin-HRP blots and a typical streptavidin enrichment gel are shown in **Figure 2c,d**.

In a successful proteomic experiment, TP proteins will have high $\log_2$(H/L) and $\log_2$(H/M) ratios, whereas false positive proteins will have low SILAC ratios (**Fig. 3**). Thus, a histogram of the distribution of SILAC ratios should show a right-shifted population of TP proteins relative to false positive proteins (**Fig. 3a**). For a three-state ratiometric SILAC experiment, a scatter plot analysis of $\log_2$(H/M) versus $\log_2$(H/L) ratios for each protein should show that known TP proteins reside in the upper right hand quadrant of the graph (**Fig. 3c**).

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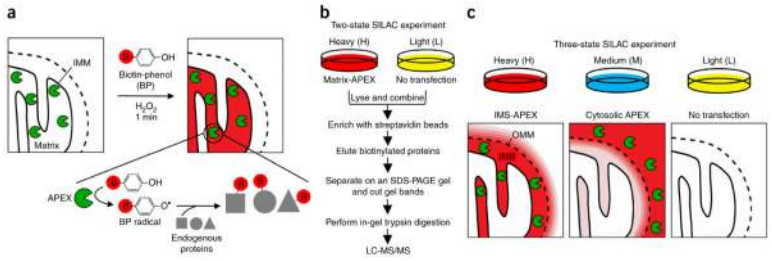

Figure 1.

Live-cell proteomics using APEX. (**a**) Scheme showing APEX-catalyzed biotinylation. The mitochondrial matrix is enclosed by the IMM. This example shows APEX (green Pac-Man) targeted to the mitochondrial matrix[4,10]. Live cells are incubated with biotin-phenol probe (red B = biotin) for 30 min and then treated for 1 min with 1 mM H_2O_2 to initiate biotinylation. APEX and APEX2 catalyze the one-electron oxidation of biotin-phenol into a biotin-phenoxyl radical, which covalently tags proximal endogenous proteins. The biotin-phenoxyl radical does not cross the IMM. (**b**) Two-state SILAC experiment used to map the proteome of the mitochondrial matrix. The experimental sample was cultured in heavy (H) arginine and lysine amino acids. The negative control sample, with APEX omitted, was cultured in light (L) arginine and lysine amino acids. Each dish of cells was separately treated with biotin-phenol and H_2O_2 and then lysed, and streptavidin enrichment was performed on the combined lysates. Subsequent processing steps are shown. For each detected peptide, the H/L intensity ratio reflects the extent to which that peptide was biotinylated by APEX. Nonspecific binders have H/L ratios close to 1. (**c**) Three-state SILAC experiment used to map the proteome of the IMS. The IMS lies in between the IMM and the OMM, which contains porins that allow the exchange of molecules < 5 kDa (ref. 16). Consequently, biotin-phenoxyl radicals generated by IMS-localized APEX can escape into the cytosol, leading to background (biotinylation contour map outside the OMM shown in drawing). To subtract this background, we used a three-state rather than two-state SILAC experiment. The heavy sample expresses APEX targeted to the IMS[5,20]. The light sample is a nonbiotinylated negative control, just as in **b**. The medium (M) sample expresses APEX outside the ROI (in this case, the cytosol, via fusion to a nuclear export sequence peptide). Streptavidin enrichment is performed on the mixture of H, M and L lysates, as shown in **b**. For each MS-detected peptide, the H/L intensity ratio reflects the extent of biotinylation by IMS-APEX. The H/M ratio reflects the extent to which that peptide is preferentially biotinylated by IMS-APEX versus cytosolic APEX. Adapted from *Science* **339**, 1328–1331 (2013). Reprinted with permission from AAAS; and adapted from ref. 5 with permission.

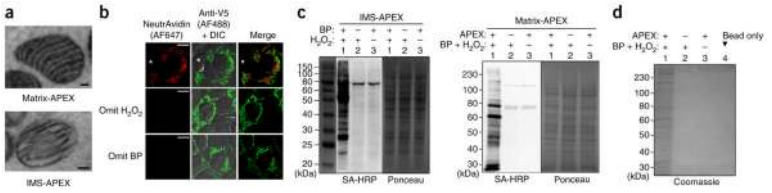

Figure 2.

Sample data showing characterization of APEX localization and activity leading up to a proteomic experiment. (**a**) EM images of cells expressing APEX targeted to the mitochondrial matrix (top) and intermembrane space (bottom). Dark regions indicate the presence of APEX[4,5,9,10]. Scale bars, 100 nm. The IMS-APEX EM image was reproduced with permission from ref. 5. (**b**) Confocal fluorescence imaging of matrix-APEX labeling in cells. Human embryonic kidney (HEK) 293T cells were transfected with matrix-APEX and labeled live as in **Figure 1a**. In parallel, samples in which either H_2O_2 or biotin-phenol (BP) was omitted were prepared as negative controls (rows 2 and 3). Cells were fixed and stained with a NeutrAvidin–Alexa Fluor 647 (AF647) conjugate to visualize biotinylated proteins and anti-V5 antibody to visualize matrix-APEX localization. The untransfected cell in the top row (starred, *) shows that biotinylation is dependent on APEX expression. DIC, differential interference contrast image. Scale bars, 10 μm. (**c**) Characterization of APEX-mediated biotinylation of endogenous proteins by streptavidin blotting. HEK 293T cells were transfected with IMS-APEX or matrix-APEX (>50% transfection efficiency) and labeled as in **b**. Cells were then lysed, separated by SDS-PAGE and analyzed by blotting with streptavidin-horseradish peroxidase (SA-HRP). The right shows Ponceau S staining of the same membrane. Negative controls in which BP, H_2O_2 or APEX was omitted are shown in lanes 2 and 3 of each blot. The IMS-APEX streptavidin-HRP blot was reproduced from ref. 5 with permission. The matrix-APEX streptavidin-HRP blot was reproduced from *Science* **339,** 1328–1331 (2013). Reprinted with permission from AAAS. (**d**) Streptavidin enrichment of proteins biotinylated by matrix-APEX. Whole-cell lysates were prepared as in **c**. Biotinylated proteins within lysates were then enriched using streptavidin-coated magnetic beads and eluted by boiling in SDS and biotin and analyzed by SDS-PAGE and Coomassie staining. Negative controls in which APEX was omitted (lane 2) or substrates were omitted (lane 3) are shown. Elution of beads that were not incubated with lysate is shown in lane 4. Reproduced from *Science* **339**, 1328–1331 (2013). Reprinted with permission from AAAS.

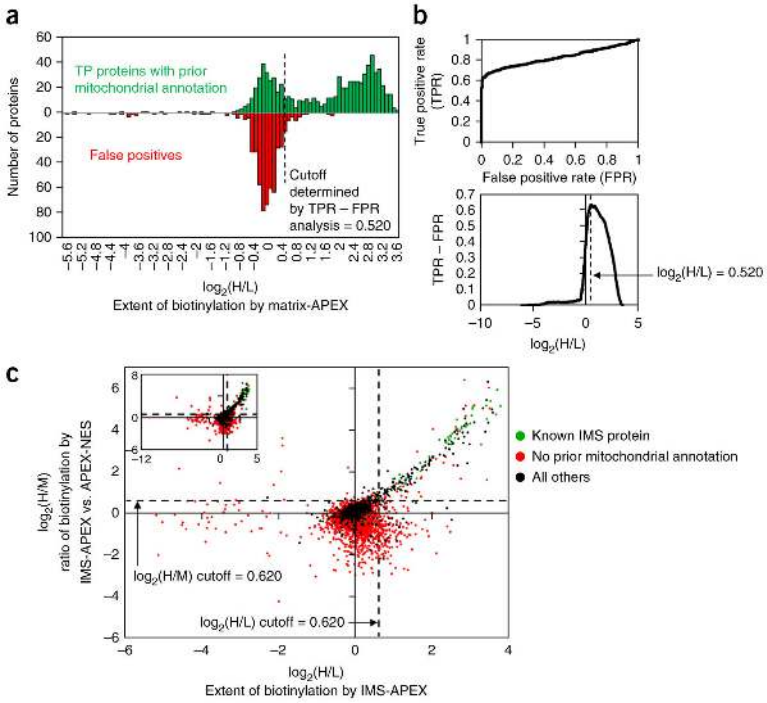

Figure 3.

Filtering the mass spectrometric data to obtain a final proteomic list. (**a**) Analysis of two-state SILAC data from the mitochondrial matrix proteomic experiment. In total, 3,430 proteins were identified by three or more unique peptides in replicate 1 of this experiment[4]. To plot the true positive distribution, we took the subset of 808 proteins with prior mitochondrial annotation in the Gene Ontology Cell Component (GOCC) database[37], MitoCarta[38] or literature (green histogram). The false positive distribution (red histogram) is given by the 521 proteins from this data set that were found in a hand-curated list of 2,410 non-mitochondrial proteins[38]. The green population has a right-shifted distribution compared with the red population, which indicates that the $\log_2$(H/L) ratio allows us to distinguish bona fide mitochondrial proteins from non-mitochondrial proteins. Adapted from *Science* **339,** 1328–1331 (2013). Reprinted with permission from AAAS. (**b**) Determination of the optimal SILAC ratio cutoff for the data in **a**. For every possible SILAC cutoff, the true positive rate (TPR) was plotted against the false positive rate (FPR) in a receiver operating characteristic (ROC) curve (top). TPR is defined as the fraction of detected true positive proteins above the cutoff. FPR is defined as the fraction of detected false positive proteins above the cutoff. The bottom graph plots the difference between the TPR and FPR at every SILAC ratio cutoff. The dashed line indicates the $\log_2$(H/L) ratio at which TPR-FPR is maximal. This SILAC ratio is used as our cutoff, and it is depicted in the histogram in **a** as a dashed line. (**c**) Analysis of three-state SILAC data from the mitochondrial IMS proteomic experiment. Here, the data are presented as a scatter plot rather than histogram, because two rather than one SILAC ratios are used for the data filtering. In this plot, the $\log_2$(H/M) value is plotted against the $\log_2$(H/L) value for each of the 4,868 proteins detected in this experiment[5]. Known IMS-exposed proteins (true positives) are colored in green, proteins that lack prior mitochondrial annotation (possible false positives) are colored in red and all

other proteins are colored in black. The dashed lines indicate the SILAC ratio cutoffs that were calculated via TPR-FPR analysis performed as in **b**. The inset shows the complete data. Adapted from ref. 5 with permission.

TABLE 1

Troubleshooting table.

step	problem	possible reason	solution
1	APEX2 fusion construct is mislocalized	Targeting sequence does not work	Try another targeting sequence Use a different fusion site
		Expression level of the construct is too high	Reduce the expression level by changing the promoter or transfection method (e.g., use lentivirus), or make stable cells
	APEX2 expression disrupts the organelle of interest	Expression level of the construct is too high	Reduce the expression level by changing the promoter, transfection method (e.g., use lentivirus), or make stable cells
		Targeting sequence introduces toxicity	Instead of fusing APEX2 to a full, functional protein for targeting purposes, try to fuse it to just a peptide sequence or inactive protein fragment that retains targeting ability but does not have biological activity Alternatively, introduce a mutation in the carrier protein (not the APEX2 part) that eliminates its activity
		Wrong APEX2 gene is used	Make sure that you are using the soybean-derived APEX2 with the following mutations: K14D, W41F, E112K and A134P. If you are using pea-derived APEX, this has some residual dimerization at high expression levels. Soybean-derived APEX and APEX2 are more monomeric and should give less perturbation
7	APEX2 fusion construct is active by NeutrAvidin-fluorophore staining, but localization cannot be confirmed by immunostaining	You are observing nonspecific sticking of the NeutrAvidin-fluorophore	Always compare the signal that you observe with a negative control to determine whether this is true signal. If it is, then the expression of your construct may be too low to be detected by immunostaining
		Expression level is too low to be detected by immunostaining	Use the NeutrAvidin-fluorophore staining as an indirect readout for the localization of your construct
	Streptavidin-fluorophore staining has high background—i.e., there is a strong signal in the negative control or outside the ROI	The streptavidin-fluorophore conjugate is nonspecifically sticking	We find that NeutrAvidin-fluorophore conjugates give lower background sticking than streptavidin-fluorophore conjugates Unhealthy cells can result in more nonspecific sticking. Repeat this experiment with lower-passage, healthier cells Try using a different blocking reagent, such as 1% (wt/vol) casein in PBS
		Your APEX2 fusion construct is not sufficiently active	Use mitochondrial matrix-APEX (available from Addgene) in parallel as a positive control. This construct is very active, and it can help determine whether your construct is not active or if there is a technical problem with the assay. If your construct has poor activity, re-design the construct (**Box 1**)
	APEX2-catalyzed biotinylation pattern looks diffuse by imaging	You are biotinylating an 'open' cellular compartment, and endogenous proteins biotinylated by APEX2 can diffuse away from APEX2 during the 1-min live-cell labeling window	None needed. This is not a problem, and your proteomic data can still be spatially specific
		If you are biotinylating in a membrane-enclosed compartment, then your cells may be unhealthy, and your membranes may be rupturing during the course of the labeling	Try to culture healthier, earlier-passage cells Make sure that the H_2O_2 treatment window is limited to only 1 min Immediately quench the labeling after 1 min with quenching buffer
7,18	APEX2 fusion construct is not active	Construct is not expressed	Stain for the epitope tag to confirm expression. If no expression is detected, try fusing APEX2 to a different site in the targeting protein, or use a different targeting protein or peptide
		Expression level of the construct is too low	Increase the expression level by changing the plasmid amount, method of transduction, promoter and so on Alternatively, change to a cell type that may give higher expression
		The BP labeling protocol and/or the streptavidin staining protocol	Check that labeling works with a positive control construct, such as mitochondrial matrix-APEX from our Addgene site

step	problem	possible reason	solution
		are not correct	
		Reagents have degraded	Use a positive control construct such as mitochondrial matrix-APEX from our Addgene site. Remake or acquire fresh reagents
		BP does not efficiently enter cells	Make sure that BP is sufficiently dissolved within the medium by warming the medium before attempting to dissolve BP. If necessary, sonicate the solution. No precipitate should be observed Test for APEX2 activity using a different, membrane-permeable substrate, Amplex Red. This is a fluorogenic substrate that is converted into fluorescent resorufin upon reaction with APEX2 in the presence of H_2O_2. Detect resorufin generation by either fluorescence plate reader measurement or by fluorescence microscopy[9,10]. If Amplex Red shows activity but the BP assay still fails, try higher concentrations of BP or longer incubation times Fix the cells, permeabilize, perform biotinylation reaction and assay by immunofluorescence to assess whether APEX2 is active within the cell. APEX2 survives paraformaldehyde fixation but not methanol fixation
18	Biotinylation 'fingerprint' by streptavidin blot looks the same for two different APEX2 fusion constructs	The two constructs are in overlapping compartments (for example, OMM and cytosol)	None needed. We often observe this
		The quencher solution is not working	Use fresh components to remake the quencher solution as described in Reagent Setup
		If the two constructs are in nonoverlapping compartments (for example, the mitochondrial matrix and cytosol), perhaps your western blot quality could be improved so that you see the bands more sharply	Ensure that your streptavidin-HRP reagent is fresh for maximum detection sensitivity on the blot Run the gel out far enough that you can resolve bands of different molecular weights Use our mitochondrial matrix-APEX and cytosolic-APEX2 constructs on Addgene as positive controls for this assay
25,26	Poor enrichment of biotinylated proteome by streptavidin beads	Too much excess BP remains in the cell lysate (competes for binding to streptavidin beads)	Increase the number of washes and the duration of the washes with quencher solution Do empirical optimizations to reduce the amount of BP probe used for the live-cell labeling step Ensure that BP remains well-dissolved at all times, including after addition to cell culture medium
		Too few streptavidin beads are used	Use more beads to capture more biotinylated proteins and account for any residual BP probe
		Elution is not effective Biotin contamination	Increase the boiling time Check that you are adding the right amount of free biotin Remake buffers that come into contact with the streptavidin beads (e.g., RIPA lysis buffer) to ensure that they are free of trace biotin that could compete for binding to streptavidin beads
26	Too much material eluted from the streptavidin beads from negative control samples (i.e., the silver stain gel looks similar for both experimental and negative control samples when analyzing streptavidin-coated bead-enriched material)	Too many nonspecific binders come through the streptavidin-enrichment step	Increase the number of bead washes, wash volume and wash duration. One can also try more stringent washing conditions, such as higher urea concentrations Reduce the amount of streptavidin beads used (to reduce surface area available for nonspecific binding) Ensure that the biotinylated proteome is fully denatured
		Insignificant quantities of protein are being enriched from all samples. You are merely seeing background signal in all lanes	Improve the signal using suggestions above (under 'poor enrichment' guidelines)
56	The correlation between the replicates is poor	One or more of the experiments failed	Try to determine the cause of failure—e.g., the APEX2 fusion construct was not expressed, enrichment was poor and so on—from the controls performed in parallel. Use this information to repeat or re-design the experiment starting from Step 28
		The proteome of the ROI is very	This is not a problem. If only a small number of proteins

step	problem	possible reason	solution
		small	is expected in the ROI, this may appear to result in poor correlation between replicates because the number of non-biotinylated proteins is much larger compared with the number of proteins biotinylated by APEX2
58	The histogram of $\log_2$(H/L) values for true positives is not right-shifted compared with the histogram of $\log_2$(H/L) values for FP-A false positives	The biotinylation reaction failed	This hypothesis must be tested on a smaller scale before re-doing the full proteomic experiment. See TROUBLESHOOTING for Steps 7 and 18
		High nonspecific binding to streptavidin beads, or the streptavidin beads fail to enrich biotinylated proteins	These hypotheses must be tested on a smaller scale before re-doing the full proteomic experiment. See TROUBLESHOOTING for Step 26
59	The histogram of $\log_2$(H/M) values for true positives is not right-shifted compared with the histogram of $\log_2$(H/M) values for FP-B false positives	Spatial specificity of this experiment is poor	Make sure that you are using quencher solution to wash the proteomic samples and are using quenchers in the lysis buffer (Steps 32–34). APEX2 remains active after cell lysis, and it can continue to biotinylate endogenous proteins in lysate; hence, peroxidase activity and any remaining BP radicals must be quenched in order to achieve high spatial specificity of labeling Test whether the APEX2 fusion construct inside the ROI biotinylates proteins that are known to reside in the ROI more strongly than the APEX2 fusion construct outside the ROI biotinylates those same marker proteins (**Box 2**) A small APEX2 population may reside outside the ROI. Create different APEX2 fusion constructs and use the assay detailed in **Box 2** to determine whether marker proteins are more heavily biotinylated by an APEX2 fusion construct inside the ROI than by an APEX2 fusion outside the ROI
		The expected size of the proteome of interest may also affect the separation; it is harder to visualize the separation of small numbers of proteins from a large background population of non-enriched proteins	This is not necessarily a problem. Proteins that are strongly enriched in the proteomic experiment may still be in the ROI but will need further validation
60	Resulting ROC curve is not smooth	The true positive and/or false positive lists are too small	When building your true positive and false positive lists, make them as large as possible. Alternatively, one may be able to obtain rough cutoffs for initial analysis by choosing true positive and false positive lists for a cellular compartment that encompasses the ROI—e.g., the entire mitochondrion versus a mitochondrial subcompartment. Although this may allow one to define a proteome, this proteome may include proteins that are not localized specifically to the ROI, and may instead be localized to the cellular compartment that encompasses the ROI
61	Resulting TPR-FPR curve is multimodal	There is a systematic bias in the true positive and/or false positive lists	Examine the true positive and false positive protein lists for some systematic error. For example, your false positive list may contain a whole class of proteins that was misannotated by databases
		Your APEX2 construct may have a small population outside the ROI	If your construct appears cleanly targeted by immunostaining, use a different peptide or protein to target APEX2 to your ROI. You may want to perform the optional assay described in **Box 2** to gain confidence that your construct is cleanly targeted