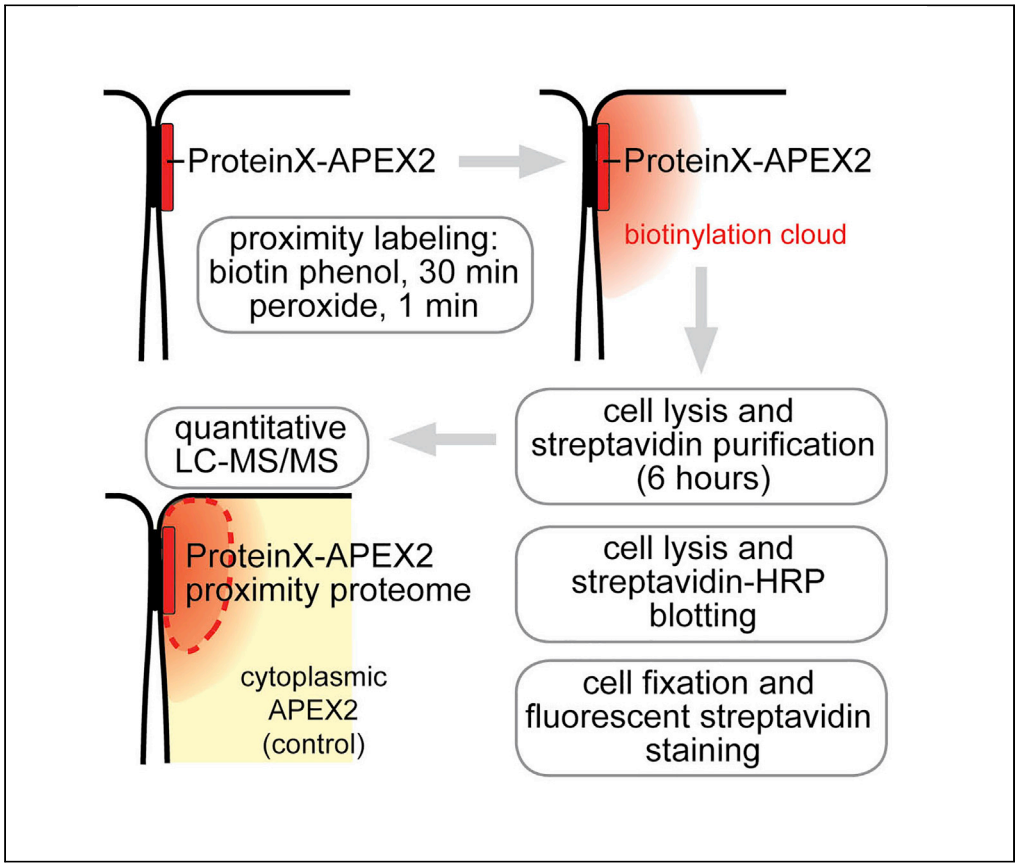
STAR Protocols

CellPress
OPEN ACCESS

Protocol

# An Optimized Protocol for Proximity Biotinylation in Confluent Epithelial Cell Cultures Using the Peroxidase APEX2




The peroxidase APEX2 has been widely used for proximity biotinylation and subsequent proteomics analyses. However, the poor membrane permeability of the biotin phenol substrate and the inhibitory effect of peroxide on the enzyme's activity has hampered proximity labeling in certain cell culture systems and tissues. Here, we describe an APEX2 protocol that uses alternative peroxide and biotin phenol concentrations. The protocol permits robust proximity biotinylation in confluent epithelial cell cultures and may be applicable to other cell cultures and tissues.

Benedict Tan, Suat Peng, Siti Maryam J.M. Yatim, Jayantha Gunaratne, Walter Hunziker, Alexander Ludwig

aludwig@ntu.edu.sg

HIGHLIGHTS

APEX2 permits proximity biotinylation in confluent cell cultures

Biotin phenol and peroxide concentrations are critical

Spatial controls are required to generate specific proximity proteomes

Tan et al., STAR Protocols 1, 100074
September 18, 2020 
https://doi.org/10.1016/j.xpro.2020.100074

STAR Protocols

CellPress
OPEN ACCESS

Protocol

# An Optimized Protocol for Proximity Biotinylation in Confluent Epithelial Cell Cultures Using the Peroxidase APEX2

Benedict Tan,[1,3] Suat Peng,[2] Siti Maryam J.M. Yatim,[5,6] Jayantha Gunaratne,[2,4] Walter Hunziker,[1,3] and Alexander Ludwig[5,6,7,8,*]

[1]Epithelial Cell Biology Laboratory, Institute of Molecular and Cell Biology, Agency for Science, Technology and Research (A*STAR), 61 Biopolis Drive, Singapore 138673, Singapore

[2]Quantitative Proteomics Group, Institute of Molecular and Cell Biology, Agency for Science, Technology and Research (A*STAR), 61 Biopolis Drive, Singapore 138673, Singapore

[3]Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, 2 Medical Drive, Singapore 117593, Singapore

[4]Department of Anatomy, Yong Loo Lin School of Medicine, National University of Singapore, 4 Medical Drive, Singapore 117594, Singapore

[5]School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551, Singapore

[6]NTU Institute of Structural Biology, Nanyang Technological University, 59 Nanyang Drive, Singapore 636921, Singapore

[7]Technical Contact

[8]Lead Contact

*Correspondence: aludwig@ntu.edu.sg
https://doi.org/10.1016/j.xpro.2020.100074

## SUMMARY

**The peroxidase APEX2 has been used widely for proximity biotinylation and subsequent proteomics analyses. However, the poor membrane permeability of the biotin phenol substrate and the inhibitory effect of peroxide on the enzyme's activity has hampered proximity labeling in certain cell culture systems and tissues. Here, we describe an APEX2 protocol that uses alternative peroxide and biotin phenol concentrations. The protocol permits robust proximity biotinylation in confluent epithelial cell cultures and may be applicable to other cell cultures and tissues.**

**For complete details on the use and execution of this protocol, please refer to Tan et al. (2020).**

## BEFORE YOU BEGIN

APEX2 tagging permits proteins to be localized with high spatial precision by transmission electron microscopy (TEM) and the molecular environment of a given protein to be probed by proximity biotinylation followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Figure 1) (Martell et al., 2017; Hung et al., 2016; Lam et al., 2015). APEX2 proximity labeling has been used, for instance, for the proteomic mapping of mitochondria (Hung et al., 2014), primary cilia (Mick et al., 2015), stress granules (Markmiller et al., 2018), autophagosomes (Le Guerroue et al., 2017), lipid droplets (Bersuker et al., 2018), and chromatin domains (Qiu et al., 2019; Gao et al., 2018). Due to its rapid labeling kinetics (< 1 min), the technique also permits the interrogation of dynamic cellular processes (Paek et al., 2017; Lobingier et al., 2017).

The enzymatic activity of APEX2 is largely determined by the intracellular availability of the biotin phenol substrate as well as the hydrogen peroxide concentration applied during the labeling reaction. Current protocols recommend biotin phenol and hydrogen peroxide concentrations of 0.5 mM and 1 mM, respectively, as well as a 30 min pre-incubation with biotin phenol prior to the addition of

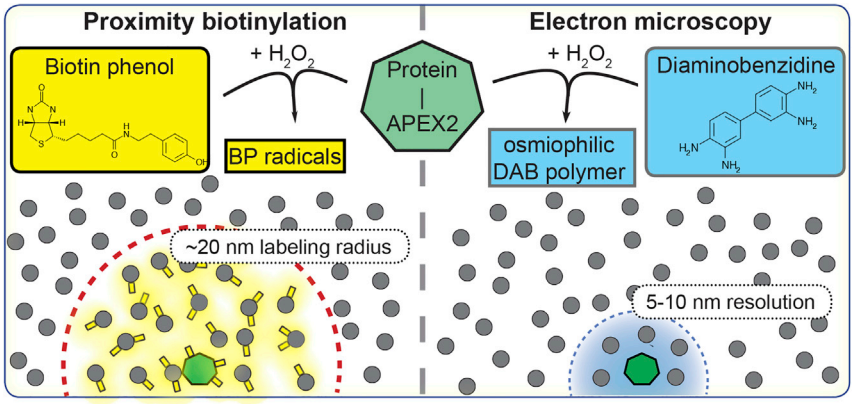



**Figure 1. APEX2 as a Genetically Encoded Reporter for Proximity Biotinylation and Electron Microscopy**

For proximity biotinylation live cells are bathed in buffer containing biotin phenol (BP) and hydrogen peroxide ($H_2O_2$) for < 1 min to produce highly reactive and short-lived (< 1 ms) biotin phenoxyl radicals that biotinylate proteins in an estimated radius of ~20 nm. For electron microscopy (EM) imaging, fixed cells are incubated with diaminobenzidine (DAB) and $H_2O_2$ to produce an osmiophilic DAB polymer that provides a discrete electron-dense stain in transmission EM.

hydrogen peroxide (Hung et al., 2016; Lam et al., 2015). Although this protocol appears to work well in many sub-confluent mammalian cell culture models, several reports have highlighted that the poor membrane permeability of biotin phenol, and potentially the inhibitory effect of hydrogen peroxide on the enzyme's activity, hamper proximity labeling in certain other cell types and tissues (Mannix et al., 2019; Hwang and Espenshade, 2016; Chen et al., 2015). In these cases, chemical or physical manipulations were required to facilitate entry of biotin phenol into the sample. Such manipulations, however, can cause artifacts, calling for alternative strategies to render the APEX2 technique applicable to a wider range of cell and tissue samples.

We recently found that confluent MDCK-II cell cultures (a commonly used cell culture system in epithelial research) are also relatively impermeable to biotin phenol (Tan et al., 2020). This prompted us to establish a modified labeling protocol, which employs higher biotin phenol (2.5 mM) and lower hydrogen peroxide (0.1–0.5 mM) concentrations. These amendments to the protocol permitted us to generate specific proximity proteomes of the cell junction-associated polarity proteins Par3 and Pals1 and to resolve their spatial and molecular organization at the epithelial cell cortex in intact and fully polarized MDCK-II cells (Tan et al., 2020). This protocol is likely of significant value to proximity labeling experiments in other confluent cell culture systems, 3D cultures, tissue samples, and live animals.

## Clone APEX2 Fusion Constructs and Generate Stable Cell Lines

Timing: at least 4 weeks

1. Clone APEX2 fusion constructs by appending genetically the enzyme to the N- or C-terminus of the cDNAs of choice. An appropriate linker sequence should be included.
2. Generate clonal cell lines stably expressing the APEX2 fusion proteins of interest.

⚠ CRITICAL: The APEX2 fusion protein should be expressed at levels comparable to that of the corresponding endogenous protein. Excessive over-expression should be avoided whenever possible. In addition, both N- and C-terminal tagged versions of the protein of interest should be generated and their subcellular localization be assessed carefully by fluorescence microscopy using antibodies against APEX2, and/or by TEM via the

APEX2 enzyme (Lam et al., 2015; Martell et al., 2017; Ludwig et al., 2017; Ludwig et al., 2016; Ludwig, 2020).

*Alternatives:* Proteins of interest can be expressed as tandem APEX2-GFP fusion proteins. The addition of GFP in the same polypeptide facilitates the selection of stable cell lines by fluorescence activated cell sorting, it permits direct visualization of the fusion protein by fluorescence microscopy, and it provides a means to isolate the fusion protein by immunoprecipitation using anti-GFP antibodies. Note that APEX2 should immediately follow (in the case of C-terminal tags) or precede (in the case of N-terminal tags) the respective cDNA of choice.

*Note:* To generate specific proximity proteomes, it is advisable to compare the APEX2 fusion protein of interest with one or more APEX2 fusion proteins targeted to the same or a related subcellular compartment. Peptide abundance should be quantified using label-free quantification approaches or isotope labeling (e.g., using stable isotope labeling of amino acids in cell culture (SILAC)). We have successfully determined proximity proteomes of cell junction-associated APEX2 fusion proteins using pairwise SILAC-LC-MS/MS against APEX2 expressed in the cytoplasm (i.e. by fusing APEX2 to the nuclear export signal (NES)) (Tan et al., 2020). A membrane-targeted APEX2 (e.g., using a CAAX motif or PH domain fusion protein) may also serve as an appropriate control. Such spatial controls largely eliminate non-specific bystanders from the proximity proteomes and therefore facilitate the identification of proteins that are truly associated with the APEX2 bait, including known or novel interaction partners.

## Optimize Conditions for Proximity Labeling

Timing: 2 weeks

3. Carefully determine the optimal $H_2O_2$ and biotin phenol concentrations in the cell culture system and cell lines used.
4. Evaluate the extent and spatial specificity of the labeling reaction by probing cell lysates with streptavidin-HRP, and by light microscopy with fluorescently conjugated streptavidin.

⚠ CRITICAL: Always include a negative control in which $H_2O_2$ or biotin phenol is omitted.

5. If two or more cell lines expressing different APEX2 fusion proteins are to be compared (qualitatively or quantitatively), attempts should be made to match their expression levels and/or to equalize the levels of biotinylation by adjusting the $H_2O_2$ concentration. The extent of biotinylation in control cell lines (e.g., in cell lines expressing cytoplasmic APEX2 as a proximity control for membrane-associated and cytosol-facing proteins) can be slightly higher than in the experimental cell line, yet excessive biotinylation should be avoided as it can interfere with the identification of true proximity partners.

*Note:* The protocol described below is optimized for proximity biotinylation in confluent MDCK-II cells grown on plastic supports, glass coverslips, or Transwell filters, and differs from previously published labeling protocols (Hung et al., 2016; Lam et al., 2015). To achieve efficient and spatially restricted proximity biotinylation in MDCK-II cell cultures we lowered the $H_2O_2$ concentration to 0.1–0.5 mM and increased the biotin phenol concentration to 2.5 mM (Figure 2). We note that $H_2O_2$ concentrations of 0.1–0.5 mM also resulted in more efficient proximity labeling in retinal pigment epithelial (RPE-1) cells and enhanced the sensitivity and intensity of the APEX2 EM labeling reaction in a number of cell lines. In addition, although higher concentrations of biotin phenol were needed for efficient proximity biotinylation in confluent MDCK-II cells, labeling in RPE-1 cells was optimal at 0.5 mM biotin phenol.

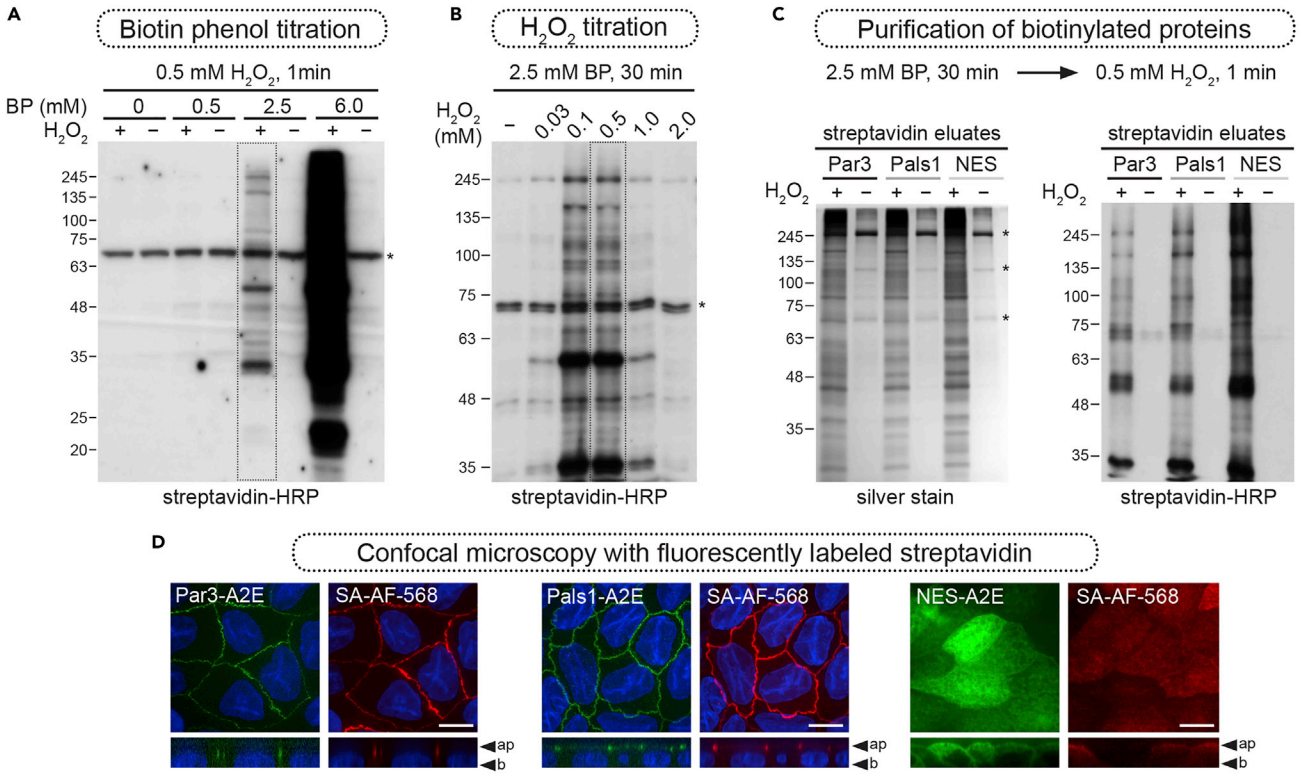



**Figure 2. APEX2-Mediated Proximity Biotinylation in Confluent and Filter-Grown MDCK-II Cells**

(A) Titration of biotin phenol (BP). MDCK-II cells stably expressing Par3-APEX2-EGFP (Par3-A2E) were pre-incubated for 30 min with the indicated concentrations of BP, followed by the addition of 0.5 mM $H_2O_2$ for 1 min. Cell lysates were probed with Streptavidin-HRP. Proximity biotinylation is optimal at a BP concentration of 2.5 mM. Note that the use of 6 mM biotin phenol resulted in non-specific biotinylation as determined by confocal microscopy of cells stained with fluorescently labeled streptavidin (data not shown).

(B) Titration of $H_2O_2$. Par3-A2E cells were pre-incubated for 30 min with 2.5 mM biotin phenol, followed by the addition of $H_2O_2$ at the indicated concentrations for 1 min. Cell lysates were probed with Streptavidin-HRP. Note that proximity biotinylation is optimal at $H_2O_2$ concentrations of 0.1 - 0.5 mM.

(C) Proximity labeling in Par3-A2E, Pals1-A2E, and NES-A2E cell lines. Cells were pre-incubated with 2.5 mM biotin phenol for 30 min followed by the addition of 0.5 mM $H_2O_2$ for 1 min. Biotinylated proteins were purified using streptavidin Sepharose, eluted, separated by SDS-PAGE, and either silver-stained (left) or blotted and probed with streptavidin-HRP (right).

(D) Confocal micrographs of filter-grown Par3-A2E, Pals1-A2E, and NES-A2E cell lines after proximity biotinylation as in C. Cells were fixed and stained with fluorescently labeled streptavidin (SA-AF-568). Maximum intensity projections are shown. Scale bar is 10 μm.

## Adapt Cell Lines to SILAC Medium (Optional)

Timing: 2 weeks

6. For quantitative proximity proteomics by SILAC, adapt your cell lines to heavy [H], medium [M], or light [L] SILAC medium, and ascertain by MS that the incorporation rate is >95%.

   *Note:* For pairwise SILAC experiments grow the respective cell lines in [H] or [L] medium. For triple SILAC, include cells grown in [M] medium. Triple SILAC provides a means to include a "no $H_2O_2$" control, which is useful to identify and subtract endogenously biotinylated proteins as well as proteins that non-specifically interact with the streptavidin beads matrix.

   *Alternatives:* Peptide quantification in MS can also be achieved using tandem mass tagging (TMT) or label-free quantification (LFQ) approaches.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Chemicals, Peptides, and Recombinant Proteins | | |
| Biotin phenol | Iris Biotech | Cat# LS-3500 |
| Biotin | Sigma-Aldrich | Cat# 1071508 |
| Trolox | Sigma-Aldrich | Cat# 238813 |
| 3% $H_2O_2$ | Milipore | Cat# 88597 |
| Sodium Ascorbate | Sigma-Aldrich | Cat# A4034 |
| Protease Inhibitor Cocktail | Thermo Fisher Scientific | Cat# A32965 |
| 4× LDS loading dye | Thermo Fisher Scientific | Cat# NP0007 |
| FBS (dialyzed) for SILAC | Gibco | Cat# 26400-044 |
| DMEM (dialyzed) for SILAC | Thermo Fisher Scientific | Cat# 88364 |
| L-arginine (R0) | Sigma-Aldrich | Cat# A8094 |
| L-arginine (R10) | Cambridge Isotope Laboratories | CNLM-539 |
| L-lysine (K0) | Sigma-Aldrich | Cat# L8662 |
| L-lysine (K8) | Cambridge Isotope Laboratories | CNLM-291 |
| Streptavidin Sepharose | GE Healthcare | Cat# 17-5113-01 |
| Streptavidin Alexa Fluor 568 | Thermo Fisher Scientific | Cat# S11226 |
| Paraformaldehyde (32%) | Electron Microscopy Sciences | Cat# 100504-858 |
| Bradford assay | Biorad | Cat# 5000006 |
| Vectashield Antifade Mounting medium H1000 | Thermo Fisher Scientific | Cat# NC9265087 |
| Experimental Models: Cell Lines | | |
| MDCK.2 cells | ATCC | ATCC CRL-2936 |
| Other | | |
| Transwell filter inserts (6.5 mm) | Corning | Cat# 3413 |
| Transwell filter inserts (12 mm) | Corning | Cat# 3401 |
| Transwell filter inserts (75 mm) | Corning | Cat# 3419 |
| Glass coverslips (12 mm, #1.5) | Electron Microscopy Sciences (EMS) | Cat# 72230 |

## MATERIALS AND EQUIPMENT

| Reagent | Final Concentration | Amount | Storage/Comments |
|---|---|---|---|
| 100× stock of biotin phenol (BP) | 250 mM | MW BP: 363.5 g/mol<br>Dissolve 90.875 mg/mL in DMSO<br>Note: Volume of dissolved BP is 0.71 µL/mg | Prepare 150 µL aliquots<br>Store at −80°C |
| 200× stock of $H_2O_2$ from 3% (w/w) $H_2O_2$ stock | 100 mM | Density of 3% (w/w) stock of $H_2O_2$ is 100 g/ 0.1 $dm^3$<br>100 g of stock contains 3 g $H_2O_2$ → 0.088 mol<br>Concentration of 3% $H_2O_2$ = 0.088 mol/ 0.1 $dm^3$ = 0.88 M<br>Prepare 200× stock of $H_2O_2$ (100 mM) in milli Q (MQ) water | Prepare fresh<br>Keep on ice |

(*Continued on next page*)

*Continued*

| Reagent | Final Concentration | Amount | Storage/Comments |
|---|---|---|---|
| 100× stock of Trolox | 500 mM | MW Trolox: 250.3 g/mol<br>Dissolve 125.15 mg/mL in DMSO<br>Note: Volume of dissolved Trolox is 0.76 µL/mg | Prepare fresh<br>Keep at 20°–25°C until use |
| 100× stock of sodium ascorbate | 1 M | MW sodium ascorbate: 198 g/mol<br>Dissolve 198 mg/mL in MQ water<br>Note: Volume of dissolved ascorbate is 0.38 µL/mg | Prepare fresh<br>Keep on ice |
| 100× stock of sodium azide | 1 M | MW Sodium azide: 65 g/mol<br>Dissolve 65 mg/mL in MQ water | Store at 4°C<br>Note: Sodium azide is toxic! |
| biotin stock | 50 mM | MW Biotin: 244 g/mol<br>Dissolve 12.2 mg/mL in 10% DMSO/MQ water<br>Adjust to pH 6 with NaOH<br>Stir at 60°C until fully dissolved | Store at 4°C |

**10× PBS, pH 6.9 [1 L]**

| | | | |
|---|---|---|---|
| $Na_2HPO_4 \cdot 2H_2O$ (177.99 g/mol) | 100 mM | 18 g | |
| $KH_2PO_4$ (136.1 g/mol) | 18 mM | 2.4 g | |
| KCl (74.55 g/mol) | 27 mM | 2g | |
| NaCl (58.44 g/mol) | 1.4 M | 80 g | |

Weigh in salts, add 800 mL MQ water, adjust pH to 6.9 using HCl (if needed), add to 1 L with MQ water.
Autoclave and store at RT.
Note that pH will be 7.3–7.4 at 1×.

**1× $PBS^{++}$ pH 7.4 [50 mL]**

| | | | |
|---|---|---|---|
| 10× PBS, pH 6.9 | 1×, pH 7.4 | 5 mL | Store stock at 20°C–25°C |
| 50 mM $MgCl_2$ | 0.5 mM | 500 µL | Store stock at 20°C–25°C |
| 100 mM $CaCl_2$ | 1 mM | 500 µL | Store stock at 20°C–25°C |
| MQ water | | 44 mL | |

Prepare fresh as $Ca_3(PO_4)_2$ precipitates if cold.
Store at RT.

**STOP/Wash Buffer [50 mL]**

| | | | |
|---|---|---|---|
| 10× PBS, pH 6.9 | 1×, pH 7.4 | 5 mL | Store stock at 20°C–25°C |
| 50 mM $MgCl_2$ | 0.5 mM | 500 µL | Store stock at 20°C–25°C |
| 100 mM $CaCl_2$ | 1 mM | 500 µL | Store stock at 20°C–25°C |
| 100× Trolox | 5 mM | 500 µL | Keep at 20°C–25°C until use |
| 100× sodium ascorbate | 10 mM | 500 µL | Keep on ice |
| 100× sodium azide | 10 mM | 500 µL | Store at 4°C<br>Note: Sodium azide is toxic! |
| MQ water | | 22.5 mL | |

Prepare fresh, store on ice.

**RIPA Buffer [30 mL]**

| | | | |
|---|---|---|---|
| 1 M Tris pH 8 | 50 mM | 1.5 mL | Store stock at 20°C–25°C |
| 5 M NaCl | 150 mM | 900 µL | Store stock at 20°C–25°C |
| 0.5 M EDTA | 5 mM | 300 µL | Store stock at 20°C–25°C |
| Sodium deoxycholate | 0.5% | 150 mg | |
| 10% SDS | 0.1% | 300 µL | Store stock at 20°C–25°C |
| 10% Triton X-100 | 1% | 3 mL | Store stock at 20°C–25°C in the dark |
| MQ water | | 24 mL | |

Prepare fresh and store on ice.

**Lysis Buffer [5 mL]**

| | | | |
|---|---|---|---|
| RIPA buffer | 1× | 4.75 mL | Keep on ice |
| 50× Protease inhibitor cocktail | 1× | 100 µL | Aliquot stock and store at −20°C |
| 100× Trolox | 5 mM | 50 µL | Keep on ice |
| 100× sodium ascorbate | 10 mM | 50 µL | Keep on ice |
| 100× sodium azide | 10 mM | 50 µL | Store at 4°C<br>Note: Sodium azide is toxic! |

Prepare fresh and store on ice.

**Wash Buffers [10 mL]**

| | | | |
|---|---|---|---|
| KCl (74.55 g/mol) | 1 M | 745.5 mg | Store at 4°C |
| $Na_2CO_3$ (106 g/mol) | 0.1 M | 106 mg | Store at 4°C |
| Urea (60 g/mol) in 10 mM Tris pH 8 | 2 M | 1.2 g<br>Add 100 µL 1M Tris pH 8 | Store at 4°C |

Prepare in MQ water and store on ice.

**Elution Buffer [1 mL]**

| | | | |
|---|---|---|---|
| 4× LDS loading buffer | 2× | 500 µL | Store at 20°C–25°C |
| 1M DTT | 200 mM | 200 µL | Store stock at −20°C |
| 50 mM biotin solution | 15 mM | 300 µL | Store stock at 4°C |

Store at −20°C.

**1% PFA [for 8 mL] in PBS, pH 7.4**

| | | | |
|---|---|---|---|
| 10× PBS, pH 6.9 | 1×, pH 7.4 | 800 µL | Store at 20°C–25°C |
| 50 mM $MgCl_2$ | 0.5 mM | 80 µL | Store stock at 20°C–25°C |
| 100 mM $CaCl_2$ | 1 mM | 80 µL | Store stock at 20°C–25°C |
| 32% PFA | 1% | 250 µL | Aliquot and store stock at −80°C |
| MQ Water | | 6.79 mL | |

Prepare fresh and store at RT.

## STEP-BY-STEP METHOD DETAILS

Figure 3 summarizes the general sample preparation workflows for APEX2-mediated proximity biotinylation and electron microscopy imaging. Biotinylated proteins can be affinity purified from whole cell lysates using streptavidin Sepharose or visualized in fixed cells using fluorescently labeled streptavidin and light microscopy. For a detailed protocol on how to prepare APEX2 samples for EM see (Martell et al., 2017; Ludwig et al., 2017; Ludwig et al., 2016; Ludwig, 2020).

### Pre-incubation of Cells with Biotin Phenol and Proximity Biotinylation

Timing: 45 min

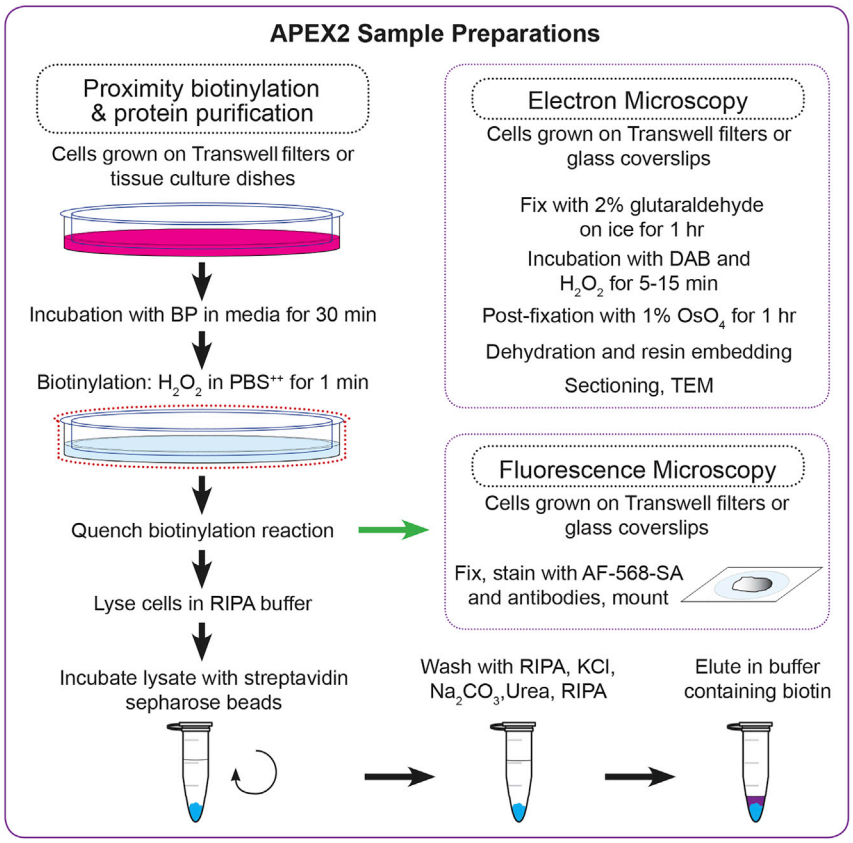



**Figure 3. Overall Workflow for APEX2-Mediated Proximity Biotinylation and Electron Microscopy Imaging**

Biotinylated proteins can be visualized in fixed cells by fluorescently labeled streptavidin or affinity purified using streptavidin Sepharose. Eluted proteins can be analyzed by LC-MS/MS. See (Martell et al., 2017; Ludwig et al., 2017; Ludwig et al., 2016; Ludwig, 2020) for detailed protocols on APEX2-EM.

The purpose of this 30 min pre-incubation step is to allow biotin phenol to enter the cells. Pre-incubation for up to 2 h can slightly increase the cellular biotin phenol availability.

1. Add BP to warm growth medium in a Falcon tube to a final concentration of 2.5 mM and mix well. Use minimal volume of medium to save on BP (see Table 1)

2. Replace medium in tissue culture dish/filters with medium supplemented with 2.5 mM BP
3. Place cells back into the incubator set to 37°C/5% $CO_2$ for 30 min
4. In the meantime, dissolve the pre-weighed quenchers and mix them into the lysis buffer and STOP/Wash buffers

   ⚠ CRITICAL: Handle at most 2 dishes at a time and work quickly!

   ***Note:*** Just before use, add 200× $H_2O_2$ to $PBS^{++}$ to make up the Biotinylation buffer

   ***Note:*** If working with Transwell filters, transfer filters between dishes. If working with plastic dishes, decant or aspirate old solution and add fresh.

5. Wash 3 times with $PBS^{++}$

**Table 1. Volumes of Buffers and Beads Used for Proximity Biotinylation and Protein Purification**

| | Pre-Incubation with BP | Biotinylation ($PBS^{++}$ + $H_2O_2$) | STOP/Wash (×3) | Cell Lysis | Bead Slurry | Elution |
|---|---|---|---|---|---|---|
| 100 mm dish | 7 mL | 12 mL | 12 mL | 1 mL | 50 µL | ~50 µL |
| 6-well plate[a] | 2 mL | 4 mL | 4 mL | 500 µL | 10 µL | ~20 µL |
| 75 mm filter insert (Dish; Insert) | 7 mL; 7 mL | 10 mL; 10 mL | 8 mL; 8 mL | 1.2 mL | 50 µL | ~50 µL |
| 24 mm filter insert (Well; Insert) | 3 mL; 2 mL | 4 mL; 3 mL | 4 mL; 3 mL | 600 µL | 10 µL | ~20 µL |

[a]For quenching of the reaction in multi-well plates, add 2× STOP solution to all wells to quench immediately, followed by two additional washes with 1× STOP solution.

6. Decant or aspirate and add biotinylation buffer for 20 s to 1 min (maximum 2 min). See Table 1 for appropriate volumes.
7. Decant biotinylation buffer and immediately wash three times with STOP/Wash buffer. See Table 1 for appropriate volumes.

⚠ CRITICAL: Work quickly and time the reaction accurately

*Note:* Direct comparison of different APEX2 fusion proteins requires the overall levels of biotinylation be matched. This can theoretically be achieved by adjusting the biotinylation time within the first 1–2 minutes. Although this offers some degree of control over the labeling reaction, we recommend adjusting different samples by a) matching the expression levels of the APEX2 fusion proteins to be compared, and b) by varying the $H_2O_2$ concentration across the samples. In MDCK-II cells $H_2O_2$ concentrations of 0.1–0.5 mM resulted in optimal proximity labeling in the presence of 2.5 mM BP, with $H_2O_2$ concentrations below and above these values resulting in less efficient labeling (Figure 2).

## Preparation of Cell Lysate

Timing: 1 h

The purpose of this step is to prepare a whole cell lysate using RIPA buffer

8. Aspirate STOP/Wash buffer thoroughly, and add Lysis Buffer. See Table 1 for appropriate volumes.
9. Scrape cells with lysis buffer into microfuge tube. If working with filters, rest filter on the inside surface of the dish cover as a hard backing for scraping.
10. Incubate lysate on ice for 30 min.

**Pause Point:** At this point lysates can be snap-frozen in liquid nitrogen and stored at −80°C

⚠ CRITICAL: Addition of Trolox, sodium ascorbate, and sodium azide to the lysis buffer is critical in order to inactivate the APEX2 enzyme and therefore to prevent non-specific biotinylation in the cell lysate.

11. Sonicate lysate on Medium intensity for 30 s, pause 30 s, and repeat once.
12. Clear lysate by centrifugation at 20,000 × *g* for 30 min at 4°C.
13. Collect supernatant and dispose pellet.

### Measure and Equalize Protein Concentrations of Cell Lysates

Timing: 1 h

The purpose of this step is to determine the protein concentrations of the cell lysates and, if required, equalize their concentration, which is critical for SILAC-LC-MS/MS.

14. The protein concentration of a cell lysate from a 75 mm filter dish is ~3.5 mg/mL
15. Dilute a small volume of lysate in MQ for measurement. Typically, a 1:10 and 1:20 dilution are prepared and the average measured concentration of the two is taken
16. Incubate with Bradford's dye 1:50 5 min RT. Measure $A_{595}$
17. Equalize the protein concentrations of the lysates with RIPA buffer and use equal volumes for the subsequent streptavidin purification
18. Save 5% of the lysate for SDS-PAGE and Western blotting with streptavidin-HRP

### Streptavidin Purification

Timing: 4 h

The purpose of this step is to isolate biotinylated proteins from whole cell lysates using streptavidin beads. Captured proteins are washed extensively and eluted for subsequent in-gel digestion with trypsin and LC-MS/MS analysis.

19. Add 1 mL RIPA buffer to streptavidin Sepharose beads, spin at 500 × *g* for 2 min. See Table 1 for appropriate volumes of streptavidin beads.
20. Wash with 1 mL RIPA twice.
21. Remove all RIPA, and add clarified cell lysate to Sepharose beads.
22. Rotate sample for 1–2 h at 4°C.
23. Spin, wash with 1 mL RIPA. 2 times.
24. Spin, wash with 1 mL 1 M KCl.
25. Spin, wash with 1 mL 0.1 M $Na_2CO_3$.
26. Spin, wash with 1 mL 2 M Urea in 10 mM Tris-HCl pH 8.
27. Spin, wash with 1 mL RIPA.

To produce individual eluates for Western blotting with streptavidin-HRP, proceed as follows:

28. Spin, wash with 1 mL RIPA without detergents
29. Spin, aspirate supernatant, spin again, and remove as much supernatant as possible using a 20 μL tip, leaving behind as little buffer as possible. Avoid aspiration of beads!
30. Add 50% of final elution buffer volume to beads, e.g., 2 × 75 mm filter inserts correspond to a final elution volume of 100 μL, so add 50 μL elution buffer at this step
31. Incubate at 95°C for 15 min
32. Spin while hot, transfer supernatant to fresh tube
33. Add second half of elution buffer, incubate at 95°C for 15 min, spin while hot and pool supernatants

*Note:* To combine two eluates for subsequent SILAC-LC-MS/MS, proceed after step 27 as follows:

34. Spin, aspirate supernatant, and add 400 μL RIPA without detergents to resuspend beads
35. Combine resuspended beads of the [H] and [L] samples into a fresh microfuge tube
36. Add 300 μL RIPA without detergents to previous tubes to collect remaining beads left on the walls of the tubes. Add this to the tube in step 35

37. Spin, aspirate, leaving a meniscus. Spin again and remove as much supernatant as possible using a 20 µL tip. Avoid aspiration of beads!
38. Add half of elution buffer volume to beads, i.e. 2 × 75 mm filter inserts correspond to a final elution volume of 100 µL, so add 50 µL elution buffer at this step
39. Incubate at 95°C for 15 min
40. Spin while hot, transfer supernatant to fresh tube
41. Add second half of elution buffer, incubate at 95°C for 15 min, spin while hot and pool supernatants

*Note:* In our experience a 1–2 h incubation with streptavidin beads is sufficient to capture most (up to 90%) biotinylated proteins from the cell lysate. While incubation times of up to 4 h may facilitate binding and therefore identification of low abundant proteins, extended incubation times should be avoided, as this may result in the non-specific binding or retention of non-biotinylated proteins.

*Note:* This protocol describes the use of streptavidin Sepharose beads and subsequent elution of the captured proteins using LDS-based sample buffer supplemented with biotin. This is followed by SDS-PAGE of the eluted proteins and in-gel digestion with trypsin.

*Alternatives:* Use magnetic streptavidin beads, which are compatible with on bead digestion protocols.

⚠ CRITICAL: The washing steps 24–26 are critical and should not be omitted. These stringent washing steps remove non-biotinylated proteins from the streptavidin beads, but do not affect the streptavidin-biotin interaction.

### Fluorescence Streptavidin Labeling

**Timing: 5 h**

The purpose of this step is to assess the spatial specificity of the proximity biotinylation reaction in fixed cells using fluorescently labeled streptavidin and light microscopy. Cells can be grown on glass coverslips or Transwell filters. The protocol below describes the processing of cells grown on Transwell filters.

⚠ CRITICAL: Cell fixation, permeabilization and blocking (steps 43–47) should be performed immediately following the biotinylation reaction described in steps 6–7.

42. Decant STOP/Wash buffer from the Transwell insert and quickly but gently wash cells once with $PBS^{++}$
43. Fix cells with either 1% or 4% PFA in $PBS^{++}$ for 20 or 10 min at RT, respectively
44. Wash 3 times with $PBS^{++}$
45. Permeabilize cells with 0.5% Triton X-100 in PBS for 10 min at RT
46. Wash 3 times with PBS
47. Block cells with 10% FBS in PBS (+0.1 mM sodium azide) for 1–12 h at 4°C

**Pause Point:** At this point cells can be stored for up to 3 days at 4°C in the dark.

*Note:* Steps 48–63 describe antibody and fluorescent streptavidin staining.

*Optional:* Prepare primary and secondary antibody solutions (if desired) (at least 25 µL of solution is required per sample) in antibody incubation buffer (0.1% BSA, 0.01% Tween in PBS).

48. Prepare a humidified dark chamber with a piece of parafilm for the incubation of filter pieces with antibody solution.
49. Pipette the primary antibody solutions onto the parafilm just prior to cutting the filters.
50. Cut out filter from the Transwell insert using a sharp razor blade.
51. Each 24-well filter piece can be cut into a maximum of 6 small pieces.
52. Ensure filter pieces are kept wet with blocking buffer during this time.
53. With sharp forceps, place the small filter pieces into the droplets of primary antibody solution, with the monolayer facing up.
54. Incubate filter pieces with primary antibody for 1–4 h.
55. Wash 3 times 5 min with antibody incubation buffer by aspirating droplets and adding at least 25 μL of fresh buffer.
56. Incubate filter pieces with appropriate secondary antibodies for 1 h. AlexaFluor 568 streptavidin (SA-AF-568) is used at 1:1000 (1 μg/mL stock) together with the secondary antibody.
57. Wash 3 times with PBS.
58. Stain with DAPI at 1:100 (~1 μg/mL) for 5 min at RT.
59. Wash 3 times with PBS.
60. Place up to 3 small filter pieces on a glass slide in ~6 μL of Vectashield mounting media, with the monolayer facing up.
61. Cover the filter pieces with a glass coverslip. Avoid bubbles.
62. Let settle for 1 h at RT in the dark.
63. Seal glass coverslip with transparent nail polish.

## EXPECTED OUTCOMES

The APEX2 fusion protein of interest should be expressed stably and relatively homogenously in the clonal cell population, and should localize correctly as judged by light and/or electron microscopy. The fusion protein should be expressed at the expected molecular weight, with no sign of degradation, and its abundance should ideally not exceed that of the corresponding endogenous protein. Expression of the APEX2 fusion protein should not alter cell morphology, behavior, or protein function. Immunoprecipitations should be used to ascertain that the fusion protein interacts with known binding partners. Streptavidin-HRP blotting of cell lysates should reveal clear differences in the biotinylation patterns between samples treated with $H_2O_2$ and samples in which $H_2O_2$ was omitted (Figures 2A–2C). The biotinylation reaction should be spatially restricted and biotinylated proteins should colocalize with the APEX2 fusion protein, as judged by fluorescent microscopy with fluorescently labeled streptavidin and an antibody against APEX2 (or an alternative way of visualizing the fusion protein, e.g. via a tag or an EGFP tandem fusion) (Figure 2D). Biotin labeling should not be observed in cellular compartments devoid of the APEX2 fusion protein. If two or more APEX2 fusion proteins are to be compared, the abundance of biotinylated proteins should be relatively equal upon elution from streptavidin Sepharose beads (Figure 2C).

## LIMITATIONS

The poor membrane permeability of biotin phenol appears to be the major limitation in APEX2-mediated proximity biotinylation (Mannix et al., 2019; Hwang and Espenshade, 2016; Chen et al., 2015). In addition, $H_2O_2$-mediated inhibition of APEX2 might limit the sensitivity of the technique (Lam et al., 2015). Our titration experiments in MDCK-II cell cultures revealed that proximity biotinylation can be enhanced significantly by a) increasing the biotin phenol concentration in the medium and b) by lowering the $H_2O_2$ concentration during the induction of the labeling reaction (Figure 2) (Tan et al., 2020). We attribute this affect to a) an increase in the intracellular availability of biotin phenol and b) to an increase in APEX2 activity due to reduced $H_2O_2$-mediated autoinhibition. Our protocol, therefore, provides a potential new strategy for proximity labeling experiments in more complex cell and tissue samples. Moreover, our work indicates that APEX2 fusion proteins need to be expressed at a certain critical level (or be locally concentrated above a certain threshold) for proximity labeling to proceed at rates above background (Tan et al., 2020). Hence, proximity

labeling of low abundant proteins is challenging, and may necessitate expression of the protein of interest at non-physiological levels.

## TROUBLESHOOTING

### Problem

Based on streptavidin-HRP blotting and fluorescence streptavidin labeling the APEX2 fusion protein of interest does not produce sufficient protein biotinylation under any $H_2O_2$ and biotin phenol concentrations tested.

### Potential Solution

In such cases we recommend generating cell lines expressing the fusion protein at levels above that of the endogenous protein. Alternatively, a more abundantly expressed protein should be tagged to probe the subcellular compartment or biological process of interest.

### Problem

The localization of the APEX2 fusion protein only partially overlaps with the staining pattern produced by fluorescence streptavidin labeling.

### Potential Solution

Ascertain by Western blotting that the fusion protein is stable and not partially degraded. If too much cytoplasmic background is observed, lower the biotin phenol concentration. Further note that labeling of highly dynamic compartments (e.g., intracellular vesicles) can produce what appears to be "off-target biotinylation" due to continued trafficking during the 1 min biotinylation period. This can be advantageous or a drawback, depending on the question that is being addressed. Conducting the biotinylation reaction on ice (which will minimize or halt movement) and/or reducing the biotinylation time should produce a more complete colocalization of the APEX2 fusion protein and the biotinylated proteins.

### Problem

The intensity of fluorescence streptavidin labeling is inhomogeneous across cells in the culture, and does not correlate well with the expression levels of the APEX2 fusion protein.

### Potential Solution

This is a common phenomenon we have observed both in MDCK-II and in RPE-1 cells (even in clonal cell populations expressing relatively homogeneous levels of the APEX2 fusion protein, see Figure 2D, NES-A2E). We speculate this to be related to the poor membrane permeability of biotin phenol. In our experience there is no ultimate solution to this problem at this point in time. We note that extended pre-incubation with biotin phenol (up to 2–4 h) tends to enhance the overall extent of biotinylation, suggesting an increase in biotin phenol uptake over time. However, the level of biotinylation within the culture remained variable, suggesting that penetration of biotin phenol into the cell is a stochastic process that is largely dictated by the biochemical properties of the cell membrane and/or other unknown factors.

## RESOURCE AVAILABILITY

### Lead Contact

Further information and requests for resources should be directed to the Lead Contact Alexander Ludwig (aludwig@ntu.edu.sg).

### Materials Availability

All DNA constructs and cell lines generated in this study are available from the lead author upon request.

## Data and Code Availability

The data generated during this study are available at DR-NTU: https://doi.org/10.21979/N9/TFWJFO.

## ACKNOWLEDGMENTS

This work was supported by an NTU Singapore start-up grant to A.L. and by funding from the Agency for Science and Technology (A*STAR), Singapore, to W.H. and J.G. We thank Sara Sandin and Tom Carney for supporting this work through research grants RG39/14 and NIM/03/2016. B.T. was a recipient of a PhD scholarship from the A*STAR Graduate Academy. We further thank the NTU Institute of Structural Biology (NISB) for support.

## AUTHOR CONTRIBUTIONS

B.T. generated cell lines and established and optimized the proximity labeling protocol. S.P. performed MS measurements. S.M.J.M.Y. helped in preparing the manuscript. J.G. and W.H. supervised S.P. and B.T., respectively. A.L. conceived the project, supervised B.T. and S.M.J.M.Y., and wrote the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests

## REFERENCES

Bersuker, K., Peterson, C.W.H., To, M., Sahl, S.J., Savikhin, V., Grossman, E.A., Nomura, D.K., and Olzmann, J.A. (2018). A proximity labeling strategy provides insights into the composition and dynamics of lipid droplet proteomes. Dev. Cell *44*, 97–112.e7.

Chen, C.L., Hu, Y., Udeshi, N.D., Lau, T.Y., Wirtz-Peitz, F., He, L., Ting, A.Y., Carr, S.A., and Perrimon, N. (2015). Proteomic mapping in live Drosophila tissues using an engineered ascorbate peroxidase. Proc. Natl. Acad. Sci. U S A *112*, 12093–12098.

Gao, X.D., Tu, L.C., Mir, A., Rodriguez, T., Ding, Y., Leszyk, J., Dekker, J., Shaffer, S.A., Zhu, L.J., Wolfe, S.A., and Sontheimer, E.J. (2018). C-BERST: defining subnuclear proteomic landscapes at genomic elements with dCas9-APEX2. Nat. Methods *15*, 433–436.

Hung, V., Udeshi, N.D., Lam, S.S., Loh, K.H., Cox, K.J., Pedram, K., Carr, S.A., and Ting, A.Y. (2016). Spatially resolved proteomic mapping in living cells with the engineered peroxidase APEX2. Nat. Protoc. *11*, 456–475.

Hung, V., Zou, P., Rhee, H.W., Udeshi, N.D., Cracan, V., Svinkina, T., Carr, S.A., Mootha, V.K., and Ting, A.Y. (2014). Proteomic mapping of the human mitochondrial intermembrane space in live cells via ratiometric APEX tagging. Mol. Cell *55*, 332–341.

Hwang, J., and Espenshade, P.J. (2016). Proximity-dependent biotin labelling in yeast using the engineered ascorbate peroxidase APEX2. Biochem. J. *473*, 2463–2469.

Lam, S.S., Martell, J.D., Kamer, K.J., Deerinck, T.J., Ellisman, M.H., Mootha, V.K., and Ting, A.Y. (2015). Directed evolution of APEX2 for electron microscopy and proximity labeling. Nat. Methods *12*, 51–54.

Le Guerroue, F., Eck, F., Jung, J., Starzetz, T., Mittelbronn, M., Kaulich, M., and Behrends, C. (2017). Autophagosomal Content Profiling Reveals an LC3C-Dependent Piecemeal Mitophagy Pathway. Mol. Cell *68*, 786–796.e6.

Lobingier, B.T., Huttenhain, R., Eichel, K., Miller, K.B., Ting, A.Y., von Zastrow, M., and Krogan, N.J. (2017). An approach to spatiotemporally resolve protein interaction networks in living cells. Cell *169*, 350–360.e12.

Ludwig, A. (2020). Selective Visualization of Caveolae by TEM Using APEX2. Methods Mol. Biol. *2169*, 1–10.

Ludwig, A., Nguyen, T.H., Leong, D., Ravi, L.I., Huan, T.B., Sandin, S., and Sugrue, R.J. (2017). Caveolae provide a specialized membrane environment for respiratory syncytial virus assembly. J. Cell Sci. *130*, 1037–1050.

Ludwig, A., Nichols, B.J., and Sandin, S. (2016). Architecture of the caveolar coat complex. J. Cell Sci. *129*, 3077–3083.

Mannix, K.M., Starble, R.M., Kaufman, R.S., and Cooley, L. (2019). Proximity labeling reveals novel interactomes in live Drosophila tissue. Development *146*, dev176644.

Markmiller, S., Soltanieh, S., Server, K.L., Mak, R., Jin, W., Fang, M.Y., Luo, E.C., Krach, F., Yang, D., Sen, A., Fulzele, A., Wozniak, J.M., Gonzalez, D.J., Kankel, M.W., Gao, F.B., Bennett, E.J., Lecuyer, E., and Yeo, G.W. (2018). Context-dependent and disease-specific diversity in protein interactions within stress granules. Cell *172*, 590–604.e13.

Martell, J.D., Deerinck, T.J., Lam, S.S., Ellisman, M.H., and Ting, A.Y. (2017). Electron microscopy using the genetically encoded APEX2 tag in cultured mammalian cells. Nat. Protoc. *12*, 1792–1816.

Mick, D.U., Rodrigues, R.B., Leib, R.D., Adams, C.M., Chien, A.S., Gygi, S.P., and Nachury, M.V. (2015). Proteomics of primary cilia by proximity labeling. Dev. Cell *35*, 497–512.

Paek, J., Kalocsay, M., Staus, D.P., Wingler, L., Pascolutti, R., Paulo, J.A., Gygi, S.P., and Kruse, A.C. (2017). Multidimensional tracking of GPCR signaling via peroxidase-catalyzed proximity labeling. Cell *169*, 338–349.e11.

Qiu, W., Xu, Z., Zhang, M., Zhang, D., Fan, H., Li, T., Wang, Q., Liu, P., Zhu, Z., Du, D., Tan, M., Wen, B., and Liu, Y. (2019). Determination of local chromatin interactions using a combined CRISPR and peroxidase APEX2 system. Nucleic Acids Res. *47*, e52.

Tan, B., Yatim, S., Peng, S., Gunaratne, J., Hunziker, W., and Ludwig, A. (2020). The mammalian crumbs complex defines a distinct polarity domain apical of epithelial tight junctions. Curr. Biol. https://doi.org/10.1016/j.cub.2020.05.032.