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Protocol

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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 Guerroué 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
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 H₂O₂ 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 H₂O₂ 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 H₂O₂ 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 H₂O₂ concentration to 0.1–0.5 mM and increased the biotin phenol concentration to 2.5 mM (Figure 2). We note that H₂O₂ 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.
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 H2O2” 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% H₂O₂ | 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 | 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  
Dissolve 90.875 mg/mL in DMSO  
Note: Volume of dissolved BP is 0.71 μL/mg | Prepare 150 μL aliquots  
Store at −80°C |
| 200× stock of H₂O₂ from 3% (w/w) H₂O₂ stock | 100 mM | Density of 3% (w/w) stock of H₂O₂ is 100 g/0.1 dm³  
100 g of stock contains 3 g H₂O₂ → 0.088 mol  
Concentration of 3% H₂O₂ = 0.088 mol/0.1 dm³ = 0.88 M  
Prepare 200× stock of H₂O₂ (100 mM) in milli Q (MQ) water | Prepare fresh  
Keep on ice |

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Continued

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

10× PBS, pH 6.9 [1 L]

|                      | 100 mM     | 18 g   |
|----------------------|------------|--------|
| Na₂HPO₄⋅2H₂O (177.99 g/mol) | 100 mM     | 18 g   |
| KH₂PO₄ (136.1 g/mol)     | 18 mM      | 2.4 g  |
| KCl (74.55 g/mol)        | 27 mM      | 2 g    |
| 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× 3.10 PBS++ pH 7.4 [50 mL]

|                     | 1 x, pH 7.4 | 5 mL  | Store stock at 20°C–25°C |
|---------------------|-------------|-------|-------------------------|
| 10× PBS, pH 6.9     | 1 x, pH 7.4 | 5 mL  | Store stock at 20°C–25°C |
| 50 mM MgCl₂         | 0.5 mM      | 500 μL| Store stock at 20°C–25°C |
| 100 mM CaCl₂        | 1 mM        | 500 μL| Store stock at 20°C–25°C |
| MQ water            | 44 mL       |       |                         |

Prepare fresh as Ca₃(PO₄)₂ precipitates if cold.

Store at RT.

STOP/Wash Buffer [50 mL]

|                     | 1 x, pH 7.4 | 5 mL  | Store stock at 20°C–25°C |
|---------------------|-------------|-------|-------------------------|
| 10× PBS, pH 6.9     | 1 x, pH 7.4 | 5 mL  | Store stock at 20°C–25°C |
| 50 mM MgCl₂         | 0.5 mM      | 500 μL| Store stock at 20°C–25°C |
| 100 mM CaCl₂        | 1 mM        | 500 μL| Store stock at 20°C–25°C |
| 100x Trollox        | 5 mM        | 500 μL| Store stock at 20°C–25°C |
| 100x sodium ascorbate | 10 mM   | 500 μL| Keep at 20°C–25°C until use |
| 100x sodium azide   | 10 mM       | 500 μL| Store at 4°C             |
| MQ water            |             | 22.5 mL | Note: Sodium azide is toxic! |

Prepare fresh, store on ice.
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

**RIPA Buffer [30 mL]**

| Component               | Concentration | Volume | Storage Temperature |
|-------------------------|---------------|--------|---------------------|
| 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]**

| Component                   | Concentration | Volume | Storage Temperature |
|-----------------------------|---------------|--------|---------------------|
| RIPA buffer                 | 1 x           | 4.75 mL| Keep on ice         |
| 50× Protease inhibitor cocktail | 1 x      | 100 μL | Aliquot stock and store at –20°C |
| 100× Trolax                 | 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        |

Note: Sodium azide is toxic!

Prepare fresh and store on ice.

**Wash Buffers [10 mL]**

| Component                   | Concentration | Volume | Storage Temperature |
|-----------------------------|---------------|--------|---------------------|
| KCl (74.55 g/mol)           | 1 M           | 745.5 mg| Store at 4°C        |
| Na2CO3 (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  | Store at 4°C        |

Add 100 μL 1M Tris pH 8

Prepare in MQ water and store on ice.

**Elution Buffer [1 mL]**

| Component                    | Concentration | Volume | Storage Temperature |
|------------------------------|---------------|--------|---------------------|
| 4× LDS loading buffer       | 2 x           | 500 μL | Store at 20°C–25°C   |
| 1 M DTT                     |               | 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**

| Component                    | Concentration | Volume | Storage Temperature |
|------------------------------|---------------|--------|---------------------|
| 10× PBS, pH 6.9             | 1 x, pH 7.4   | 800 μL | Store at 20°C–25°C   |
| 50 mM MgCl2                 | 0.5 mM        | 80 μL  | Store stock at 20°C–25°C |
| 100 mM CaCl2                | 1 mM          | 80 μL  | Store stock at 20°C–25°C |
| 32% PFA                     | 1%            | 250 μL | Aliquot and store at –80°C |
| MQ Water                    |               | 6.79 mL|                     |

Prepare fresh and store at RT.
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% CO2 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 200x H2O2 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++
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 H₂O₂ concentration across the samples. In MDCK-II cells H₂O₂ concentrations of 0.1–0.5 mM resulted in optimal proximity labeling in the presence of 2.5 mM BP, with H₂O₂ 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.

| Table 1. Volumes of Buffers and Beads Used for Proximity Biotinylation and Protein Purification |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|
|                                  | Pre-Incubation | Biotinylation (PBS++ + H₂O₂) | STOP/Wash (x3) | Cell Lysis | Bead Slurry Elution |
| 100 mm dish                      | 7 mL           | 12 mL                  | 12 mL          | 1 mL       | 50 μL           | −50 μL         |
| 6-well plate*                   | 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         |

*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.
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 Na₂CO₃.
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 homogeneously 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 H2O2 and samples in which H2O2 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, H2O2-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 H2O2 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) an increase in APEX2 activity due to reduced H2O2-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$_2$O$_2$ 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**

Ascertaining 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.

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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

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