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1 Supplementary Information
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3 **Proximity labeling expansion microscopy (PL-ExM) evaluates interactome labeling**
4 **techniques**
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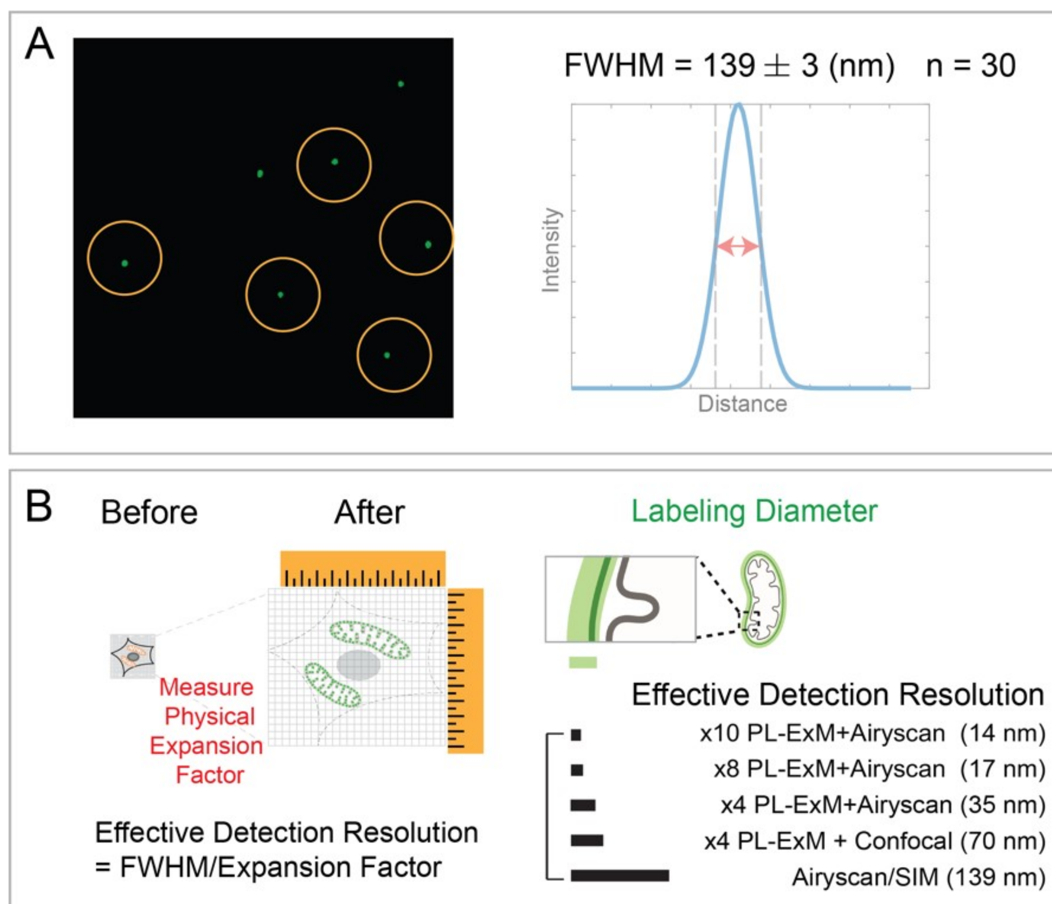
32 **Image resolution measurement**

33 0.1µm size fluorescent beads (TetraSpeck Microspheres, Invitrogen; T7279) were used
34 to measure the resolution of the Airyscan LSM980 resolution with 63x water immersion
35 objective (NA1.15). 30 different beads were sampled to obtain the average full width half
36 maximum (FWHM) with standard error. The effective resolution of PL-ExM was measured
37 by calculating FWHM divided by the physical length expansion factor of the hydrogel.



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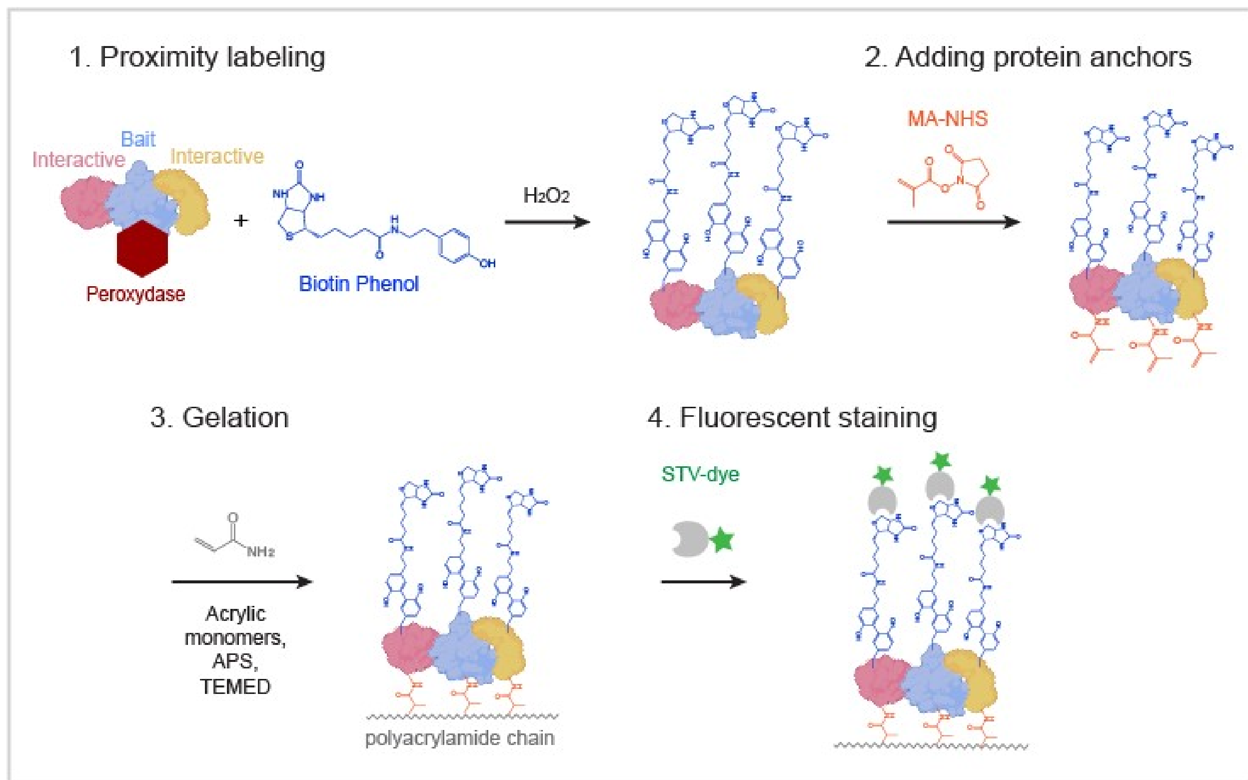
39 **Figure S1. Resolution measurement.** (A) A 0.1µm size fluorescent bead slide is used
40 to measure the resolution of the Airyscan LSM980 with 63x water immersion objective
41 (NA1.15), which is the system used for this paper. 30 different beads are sampled to
42 obtain average full width half maximum (FWHM) value with standard error. Measured
43 value is 139 ± 3 nm (mean ± standard error). (B) The effective resolution of PL-ExM after
44 different times of expansion. The measured effective resolution of PL-ExM in this paper
45 was in the range of 14 – 70 nm depending on the length expansion factors and the type
46 of microscope.

47 **Chemical reactions in PL-ExM**



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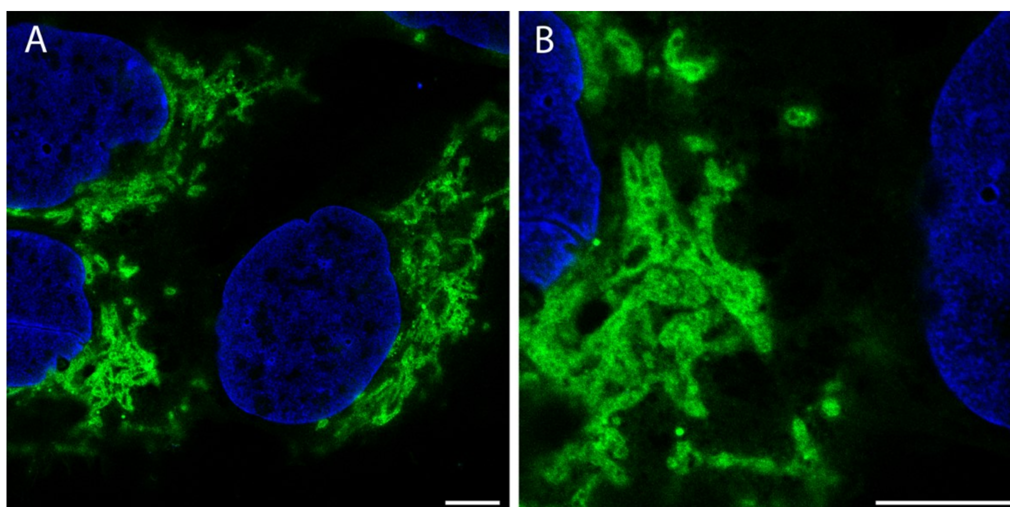
50 **Figure S2. Chemical reactions involved in the PL-ExM workflow, including**
51 **proximity labeling, protein anchoring, gelation, and fluorescent staining steps.**

52 **PL-ExM imaging using confocal microscopy**



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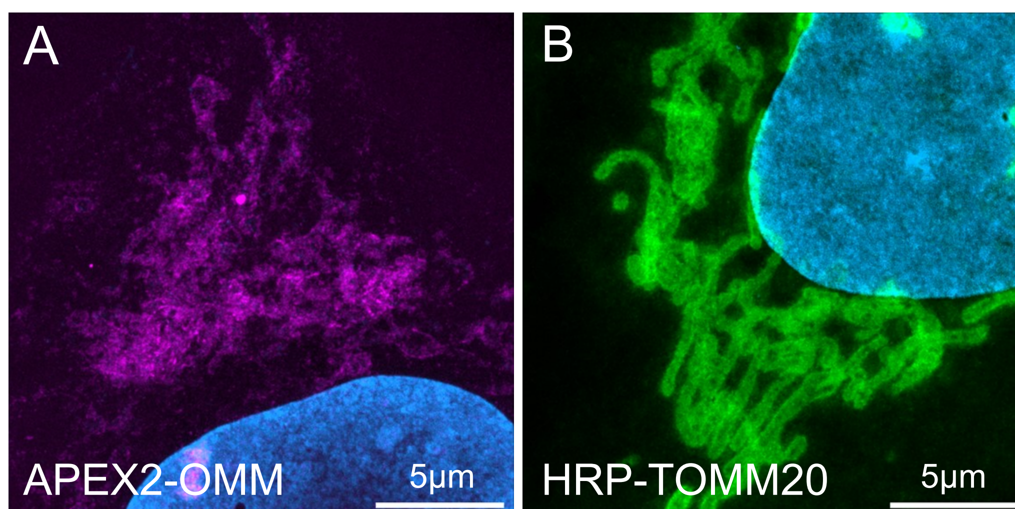
54 **Figure S3. Confocal PL-ExM images of mitochondria in U2OS cells.** Proteins in the
55 proximity of TOMM20 were labeled with antibodies conjugated with HRP (green). DNA
56 was stained with DAPI (blue). The images were taken on a laser scanning confocal
57 microscopy LSM780 with 63x water immersion objective (NA1.15). Length expansion
58 factor: 4.1. Scale bars: 5 μm (physical size post-expansion, 20.5 μm).

59

60 **APEX and HRP approaches of PL-ExM**



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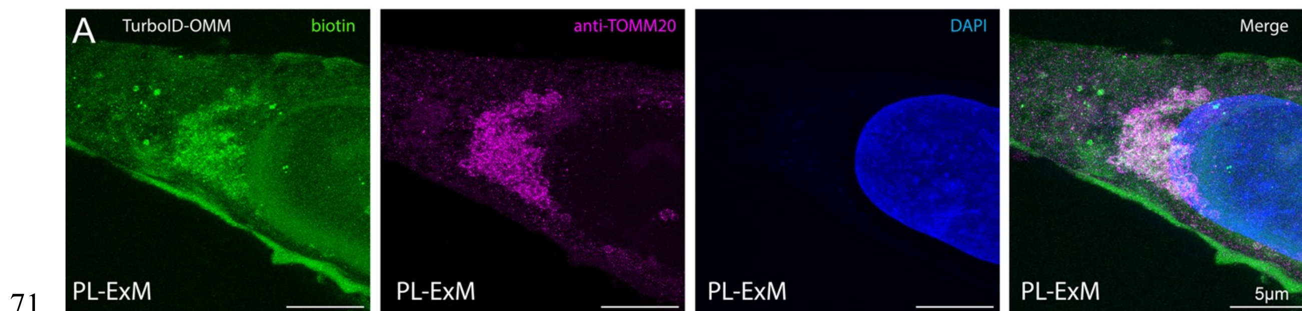
62 **Figure S4. Airyscan PL-ExM images of mitochondria labeled with APEX2 or HRP as**
63 **the peroxidase.** (A) Proteins in the proximity of APEX2 expressed with the outer
64 mitochondrial membrane were labeled (magenta). DNA was stained with DAPI (blue). (B)
65 Proteins in the proximity of TOMM20 were labeled with antibodies conjugated with HRP
66 (green). DNA was stained with DAPI (blue). The images were taken on an Airyscan LSM

67 980 with 63x water immersion objective (NA1.15). Length expansion factor: 4.0. Scale
68 bars: 5 μm (physical size post-expansion, 20 μm).

69

70 **TurboID approach of PL-ExM**

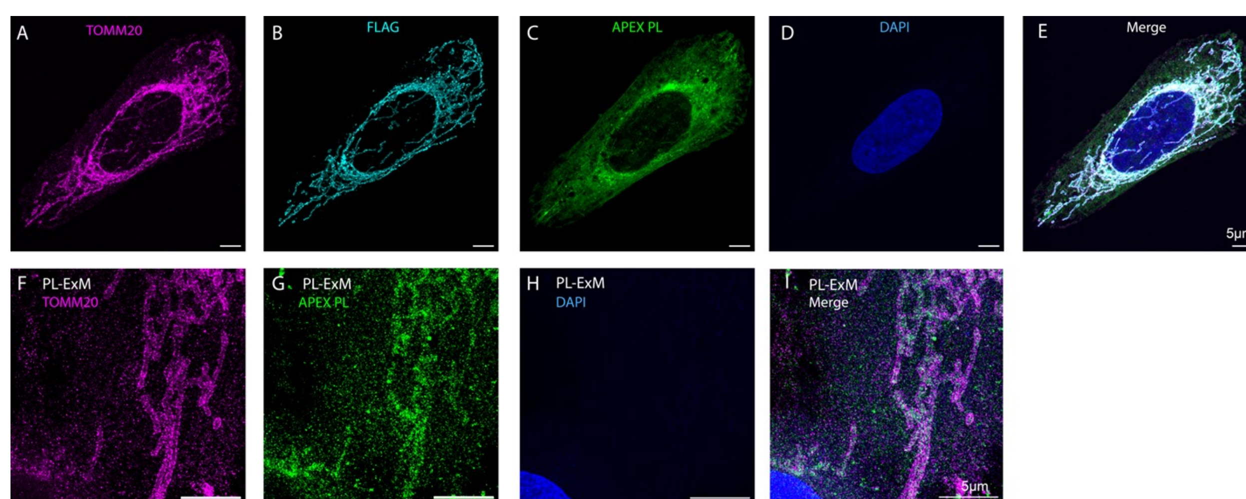
71 

72 **Figure S5. Airyscan PL-ExM images of mitochondria labeled with TurboID**
73 **approach**. Live cell PL was initiated for 30 min in cells stably expressing the TurboID-
74 OMM fusion construct. (A) Biotinylated proteins were visualized by post-gelation staining
75 with streptavidin- Alexa Fluor 488 and (B) Immunostaining of TOMM20 localized
76 mitochondria. DAPI is a nuclear marker. Length expansion factor: 4.4. Scale bars, 5 μm
77 in pre-expansion unit. All images in this figure were taken on an Airyscan microscope.

78

79 **Validation of APEX2-OMM cells shows negligible overexpression artifact**

80 We are careful about the potential artifact that overexpressing APEX2-OMM could cause. As
81 stated in the Methods, we made an APEX2-OMM plasmid with a moderate promoter SFFV rather
82 than CMV to transfect U2OS cells. The strong CMV promoter can easily introduce
83 overexpression. However, we didn't observe noticeable artifacts in our cell lines expressing
84 APEX2-OMM with the SFFV promotor. In our SFFV APX2-OMM cell line, a FLAG- tag is fused
85 on the N terminus of APEX2. We immunostained TOMM20 and FLAG-tag and performed APEX2-
86 catalyzed PL. The colocalization of the signals from TOMM20, FLAG-tag, and APEX PL (Figures
87 S6A-C) confirms that the APX2 was expressed specifically in mitochondria. The stringy and
88 tubular morphology of mitochondria in our APEX2-OMM cells (Figure S6A) is similar to the typical
89 mitochondrial morphology in U2OS cells (Figures 2A&G). This similarity indicates negligible
90 overexpression artifact. More structural details are visualized in the multicolor PL-ExM images of
91 APEX PL and immunostained TOM20 (Figures S6F-I).



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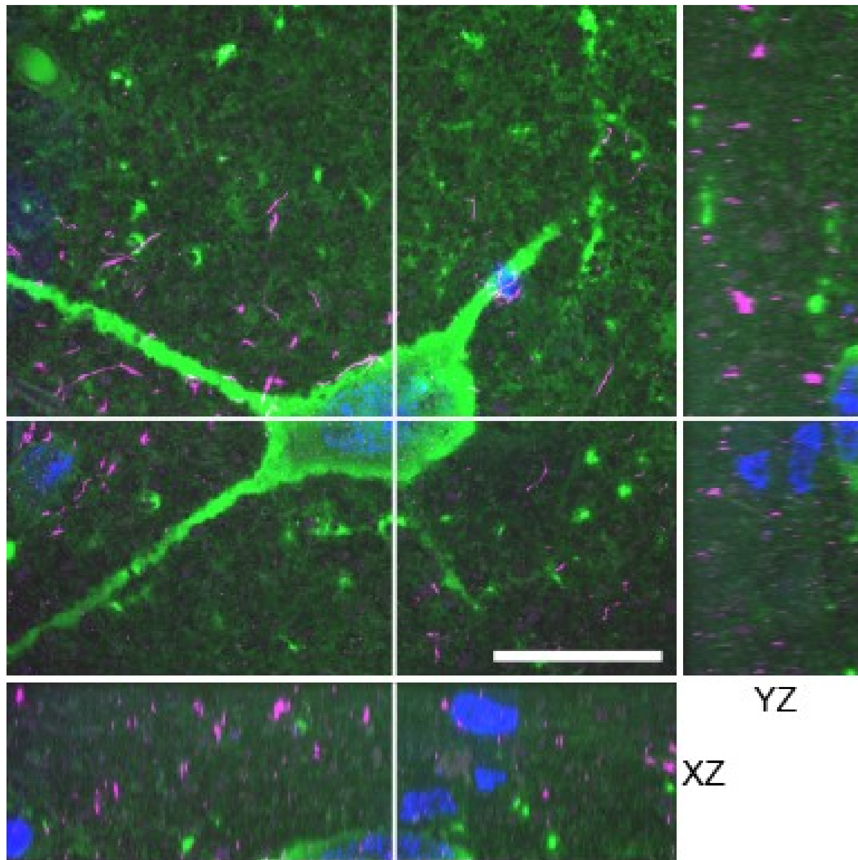
93 **Figure S6. Validation of APEX2-OMM cells with Airyscan and PL-ExM.** (A-E)
94 Fluorescence imaging of APEX2-mitochondria localization and biotinylation activity in a
95 cell line stably expressing APEX2-OMM with SFFV promotor. A FLAG-tag is fused on the
96 N terminus of APEX2 Live cell PL was performed for 1 min. Mitochondria (A) and FLAG-
97 APEX2 (B) expression were visualized with antibody staining, while biotinylation was
98 visualized with streptavidin-Alexa Fluor 488 (C). DAPI is a nuclear marker (D). Scale bars,
99 5 um.

100 (F-I) Multicolor PL-ExM images of APEX2-OMM cells. Live cell PL was performed for 1
101 min in activity in the APEX2-OMM, followed by expansion microscopy. Mitochondria were
102 marked with anti-TOMM20 antibodies (F), and biotinylated proteins were visualized with
103 streptavidin- Alexa Fluor 488 (G). DAPI is a nuclear marker (H). Length expansion factor:
104 4.4. Scale bars, 5 μm in pre-expansion unit.

105 All images in this figure were taken on an Airyscan microscope.

106 **3D PL-ExM imaging of brain tissues**

107 

108 **Figure S7. Orthogonal views of PL-ExM imaging of mouse brain section.** The images,
109 captured using Airyscan PL-ExM, depict a 20-μm mouse brain section expressing Thy1-YFP.
110 Proximity labeling was applied to Thy1-YFP (green) and GFAP was immunostained (magenta).
111 Antibodies permeated tissue thoroughly for proximity labeling to occur robustly along the z-
112 axis of the tissue. The tissue was expanded by a factor of 4.0. Scale bar: 20 μm in pre-expansion
113 units. All images in this figure were taken on an Airyscan microscope.

Negative Controls for HRP-, APEX2-, and TurboID-catalyzed PL.

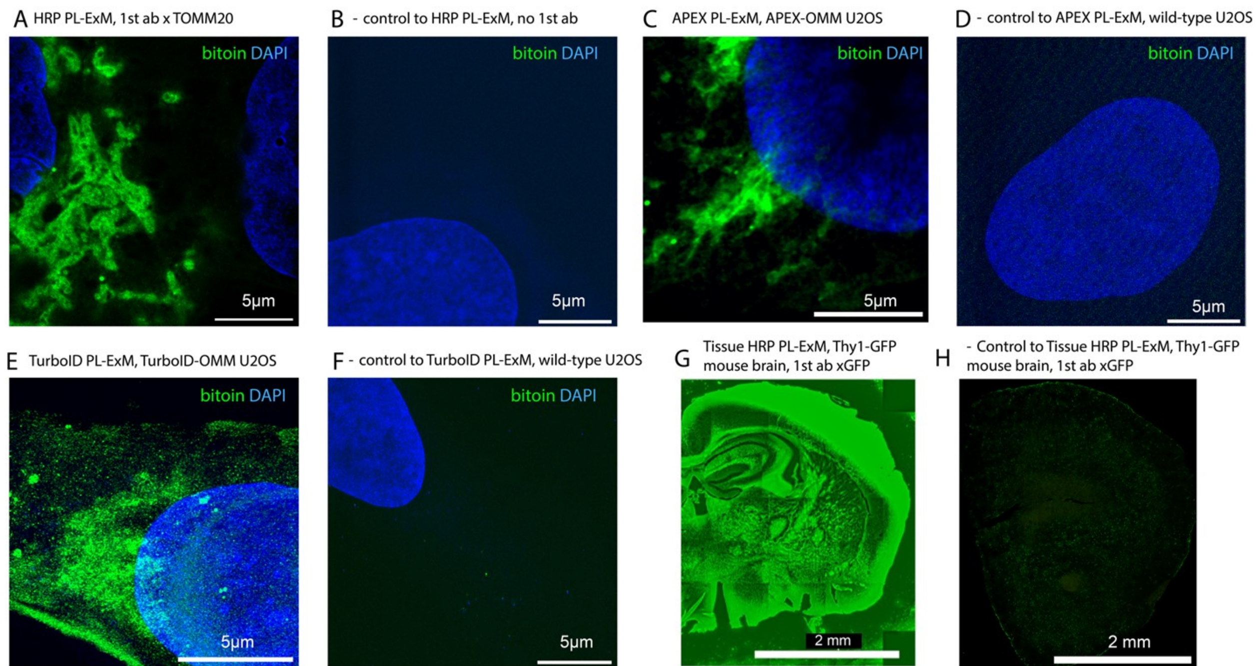


Figure S8. Three approaches of PL-ExM with negative controls in cells, and tissues. (A-B) HRP-catalyzed PL-ExM. PL was initiated for 30s. Biotinylation was visualized with streptavidin- Alexa Fluor 488 (A), while no primary antibody was used in the negative control (B). Length expansion factors: 4.0(A) and 4.4(B). Scale bars, 5 μm in pre-expansion unit.

(C-D) APEX2-catalyzed PL-ExM. Live cell PL was initiated for 1 min, and biotinylation was visualized with streptavidin- Alexa Fluor 488 (C), while wild-type U2OS cells without APEX2 were used in the negative control (D). Length expansion factors: 4.3(C) and 4.4(D). Scale bars, 5 μm in pre-expansion unit.

(E-F) TurboID-catalyzed PL-ExM. Live cell PL was initiated for 30 min, and biotinylation was visualized with streptavidin- Alexa Fluor 488 (E), while wild-type U2OS cells without TurboID were used in the negative control (F). Length expansion factors: 4.4(E) and 4.4(F). Scale bars, 5 μm in pre-expansion unit.

(G-H) HRP-catalyzed PL-ExM in a 20 μm tissue section of a mouse brain expressing Thy1-YFP. The samples were immunostained with Rabbit anti-GFP antibodies, followed by Donkey x Rabbit and HRP Horse x Goat antibodies. PL duration was 1 min. Biotinylation was visualized with streptavidin- Alexa Fluor 488 (G), while no primary antibody was used in the negative control (H). Length expansion factors: 4.0 (G) and 4.1(H). Scale bars, 2 mm in pre-expansion unit.

All images in this figure were taken on an Airyscan microscope.