
Figures and figure supplements

High-quality ultrastructural preservation using cryofixation for 3D electron microscopy of genetically labeled tissues

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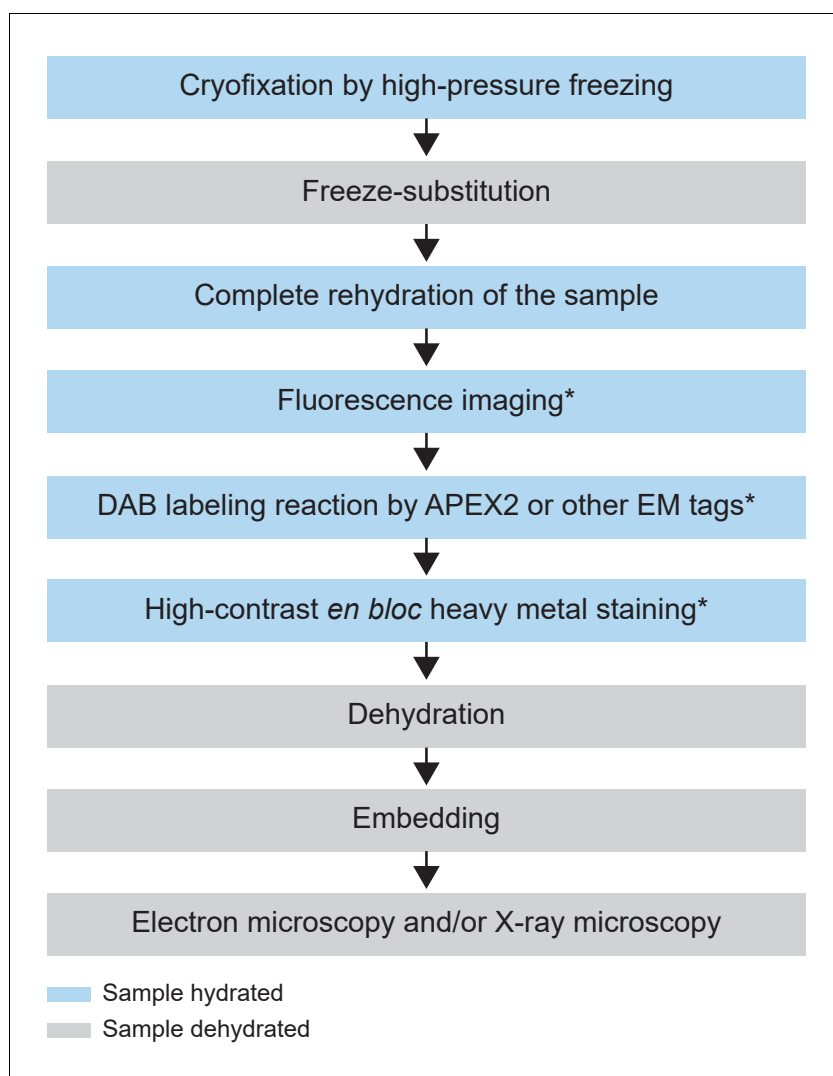


Figure 1. Flowchart of the CryoChem method. After cryofixation by high-pressure freezing and freeze-substitution, cryofixed samples are rehydrated gradually. Rehydrated samples can then be imaged for fluorescence, subjected to DAB labeling reaction or *en bloc* stained with a substantial amount of heavy metals. The protocol is modular; the first three processes are the core steps of CCM and the starred steps are optional depending on the experimental design. The samples are then dehydrated for resin infiltration and embedding, followed by imaging with any EM technique of choice. Blue and grey denote hydrated and dehydrated states of the sample, respectively.

DOI: <https://doi.org/10.7554/eLife.35524.003>

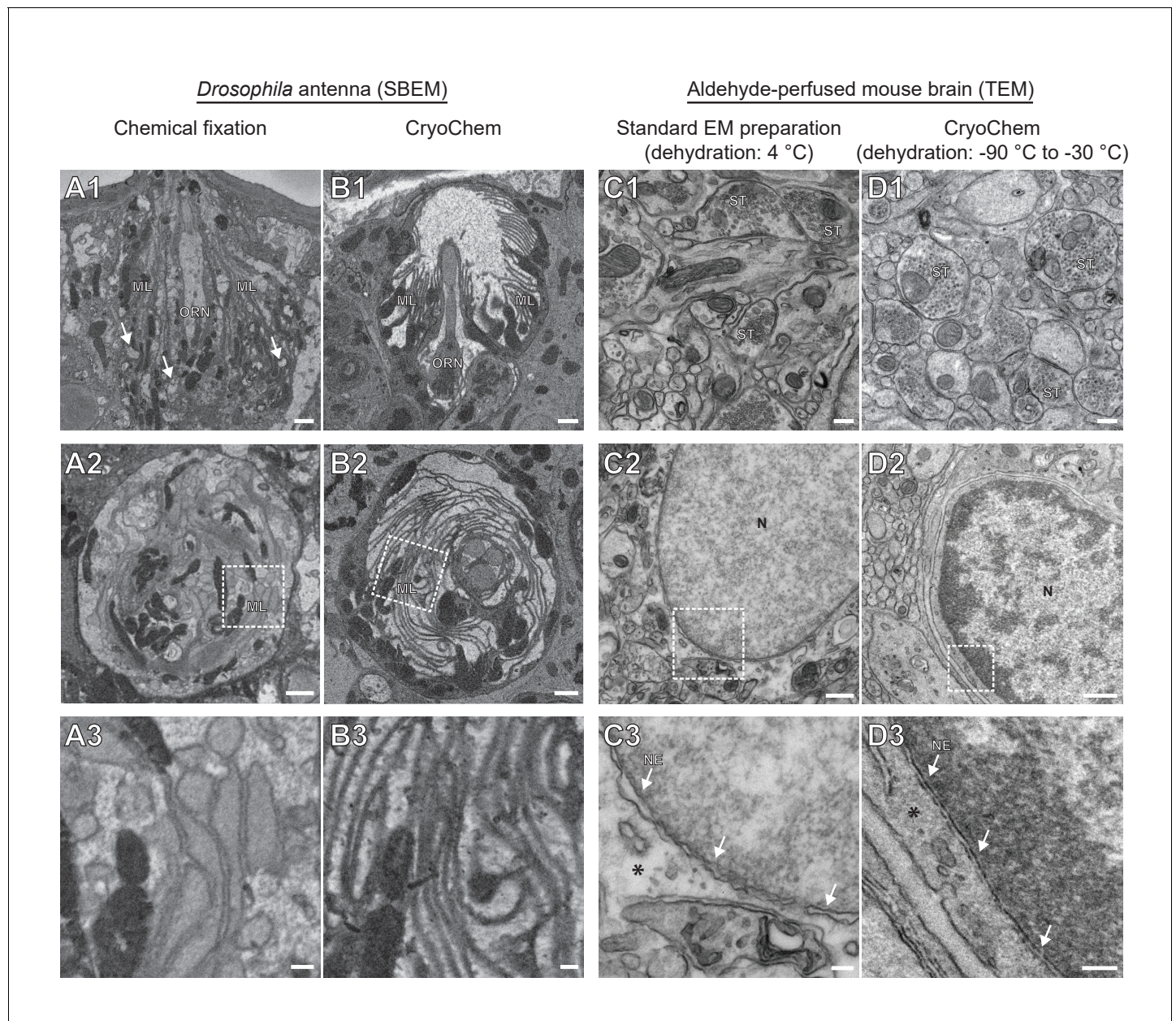


Figure 2. CryoChem method offers high-quality ultrastructural preservation and sufficient *en bloc* staining for SBEM. EM images were acquired to assess the morphology of CCM-processed tissues. (A-B) The quality of preservation was markedly improved in the CCM-processed *Drosophila* antenna compared to the chemically fixed counterpart. Pixel resolution of SBEM images (x,y): 6.5 nm. (A1 and B1) Unlike the CCM-processed antenna, the chemically fixed antenna showed signs of extraction (arrow) and disorganized membranes. ORN: olfactory receptor neuron; ML: microlamella. Scale bars: 1 μ m. (A2 and B2) The microlamellae were well-preserved in the CCM-processed antenna, compared to the chemically fixed samples. Scale bars: 1 μ m. (A3 and B3) In the enlarged views of the boxed regions, the microlamellae in the CCM-processed antenna appeared uniform in size and shape, unlike the chemically fixed ones which were distorted. Scale bars: 200 nm. (C-D) CCM enhanced the morphological preservation of aldehyde-perfused mouse brain. The initial dehydration in standard EM preparation took place on ice for 1 hr, but it occurred during freeze-substitution at -90 °C to -30 °C for over 5 days in CCM processing. (C1 and D1) The smoothness of membranes was improved by CCM processing. ST: synaptic terminal. Scale bars: 200 nm. Pixel resolution of TEM images (x,y): 1.92 nm. (C2 and D2) The preservation of nuclear envelope was improved by CCM processing. N: nucleus. Scale bars: 500 nm. Pixel resolution (x,y): 2.88 nm. (C3 and D3) In the enlarged views of the boxed regions, the nuclear envelope (NE; arrows) appeared smoother and the cytoplasmic density (asterisk) was increased with CCM processing. We note that the chromatin was more heavily stained in the CCM-processed specimen, likely due to the additional exposure to uranyl acetate during freeze-substitution. Scale bars: 100 nm. Pixel resolution (x,y): 1.14 nm.

DOI: <https://doi.org/10.7554/eLife.35524.004>

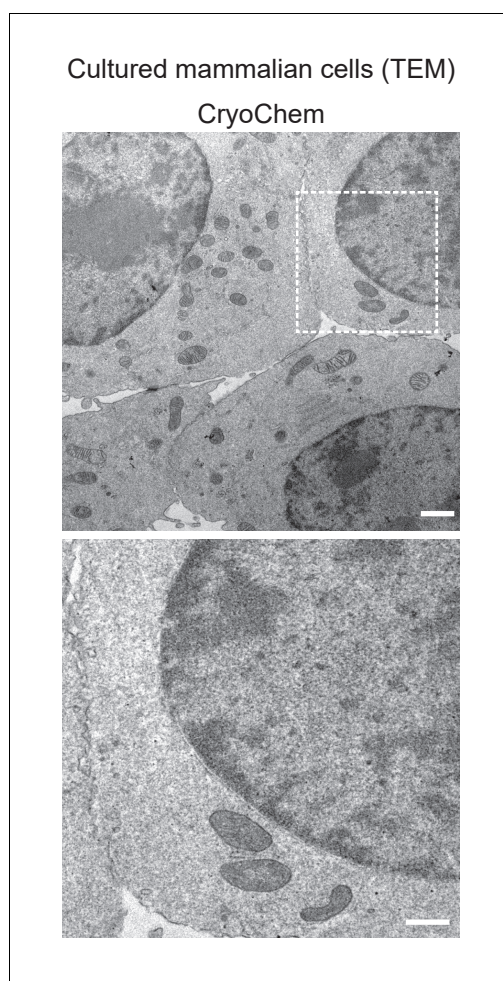


Figure 2—figure supplement 1. TEM images showed well-preserved ultrastructures in the CCM-processed HEK 293T cells. In the enlarged view of the boxed region (bottom panel), smooth nuclear membranes were observed. Scale bars: 1 μm for the top panel, 500 nm for the bottom panel. Pixel resolution (x,y): 6.01 nm.
DOI: <https://doi.org/10.7554/eLife.35524.005>

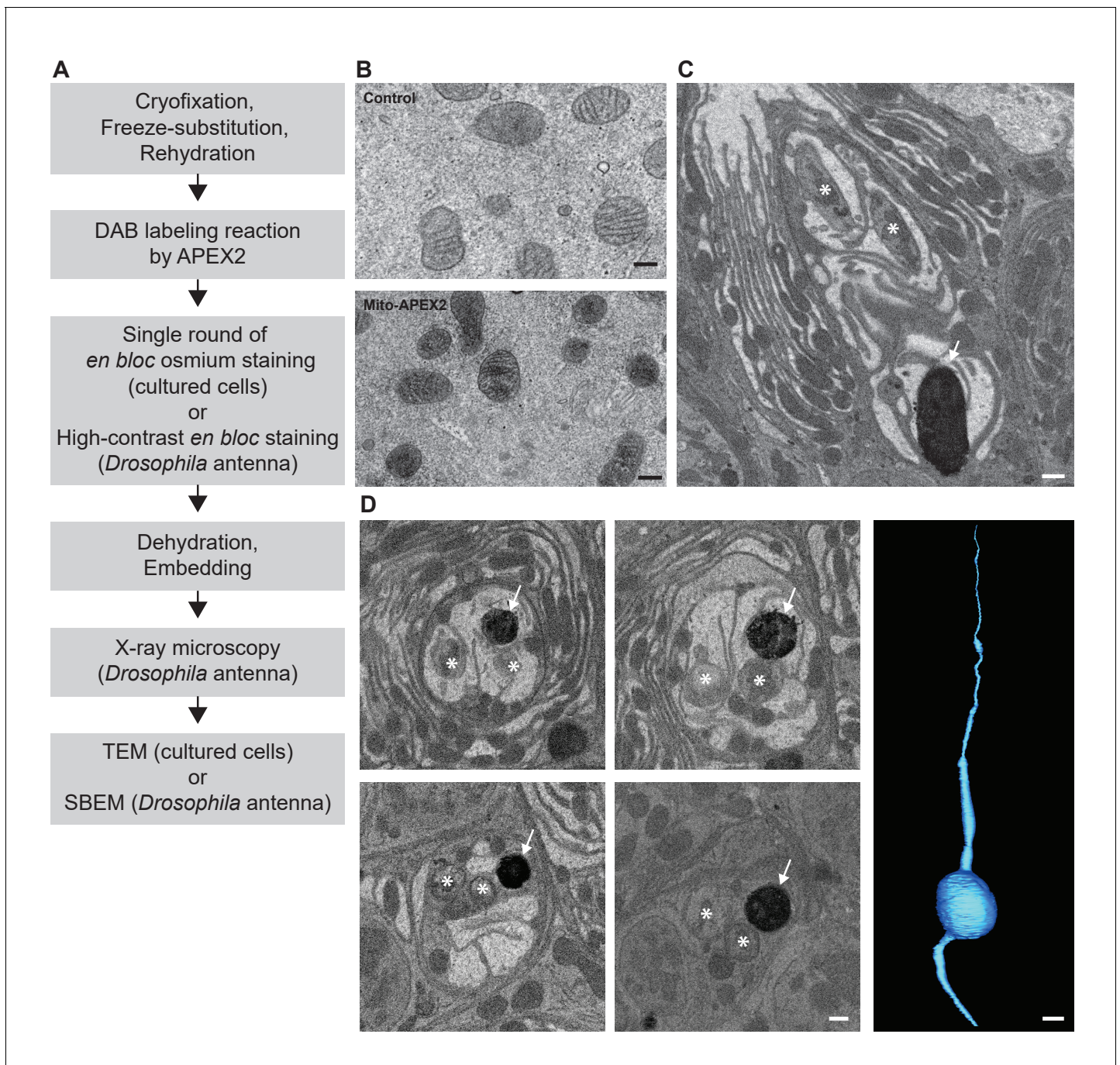


Figure 3. CryoChem method enables DAB labeling by APEX2 in cryofixed tissues. In the CCM-processed cultured cells and *Drosophila* antennae, DAB labeling was observed in cells expressing APEX2. (A) Flowchart for DAB labeling of target structures expressing APEX2 in CCM-processed samples. In our experiments, the cultured cells were imaged with TEM and the *Drosophila* antennae were imaged with X-ray microscopy, followed by SBEM. (B) Mitochondria in the HEK 293T cell transfected with Mito-APEX2 were labeled with DAB (bottom panel), in contrast to the untransfected control cell (top panel). Scale bars: 200 nm. Pixel resolution (x,y): 3.97 nm (top panel); 2.88 nm (bottom panel). (C) An APEX2-expressing olfactory receptor neuron (ORN) was labeled with DAB (arrow) in the *Drosophila* antenna (*10XUAS-myc-APEX2-Orco*; *Or47b-GAL4*). Asterisks denote ORNs without APEX2 expression. Scale bar: 500 nm. Pixel resolution of SBEM images (x,y): 6.5 nm. (D) A series of SBEM images showing the same DAB labeled *Drosophila* ORN (arrow) in different planes of section. Asterisks denote ORNs without APEX2 expression. The images were acquired using standard imaging methods without charge compensation by nitrogen gas injection (Deerinck et al., 2018). These images, together with the rest of the EM volume acquired using SBEM, enabled semi-automatic segmentation and 3D reconstruction of the labeled ORN (right panel). Scale bars: 500 nm for SBEM images, 2 μ m for the 3D model of ORN. SBEM imaging parameters: Z step: 50 nm; Z dimension: 1200 sections; raster size: 12k \times 9k; pixel size: 3.8 nm.

DOI: <https://doi.org/10.7554/eLife.35524.007>

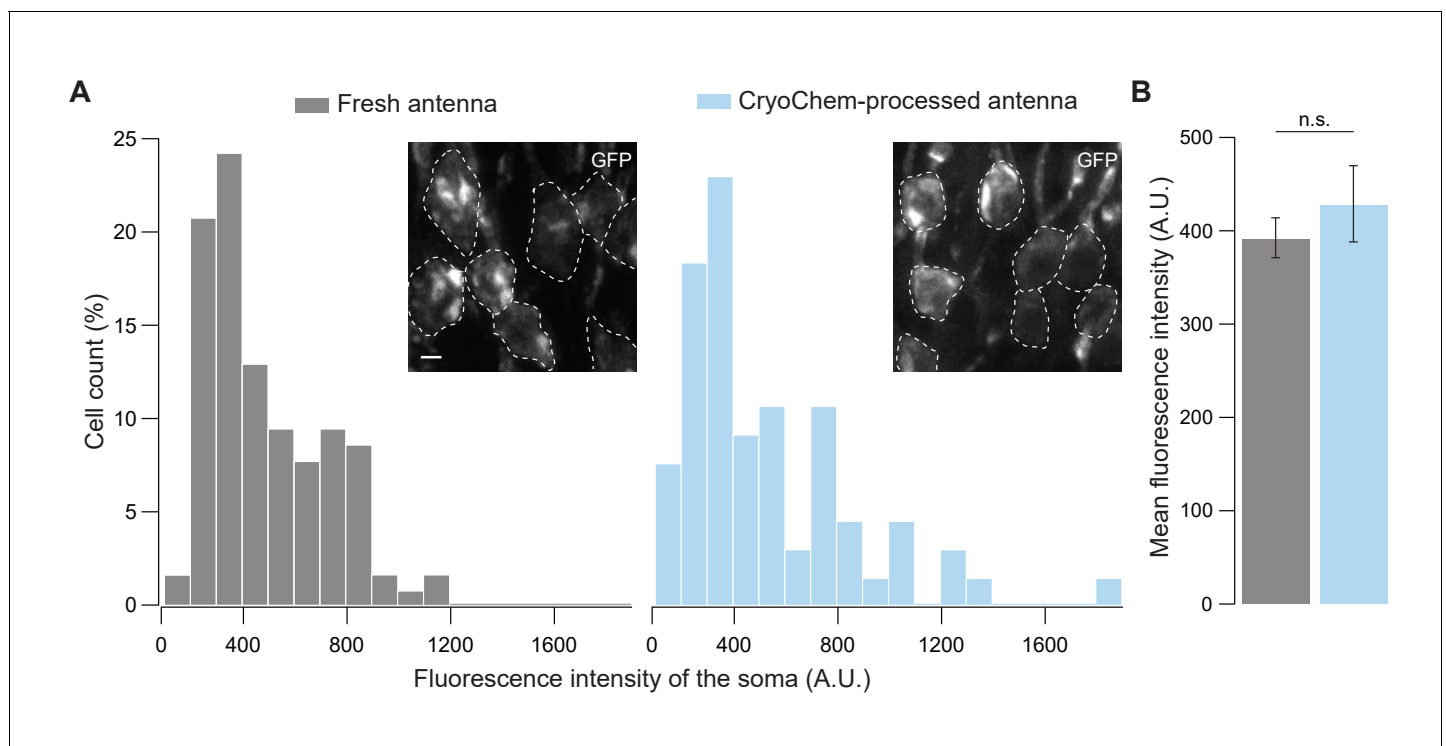


Figure 4. GFP fluorescence is well-preserved in CryoChem-processed samples. Confocal images were taken to quantify the level of GFP fluorescence in *Drosophila* ORNs. Antennae were collected from transgenic flies expressing GFP in a subset of ORNs. **(A)** GFP fluorescence intensity distributions of the ORN soma in the freshly-dissected, unfixed antennae (left panel) and the CCM-processed antennae (right panel) are not significantly different. $p=0.810$, Kolmogorov-Smirnov test. Insets show representative images, with ORN soma outlined. Scale bar: 2 μm . **(B)** Comparison of the average fluorescence intensities. GFP intensities are virtually identical between neurons in the unfixed antennae and the frozen-rehydrated antennae. $n = 3$ antennae, Error bars denote SEM, $p=0.950$, Mann-Whitney U Test.

DOI: <https://doi.org/10.7554/eLife.35524.009>

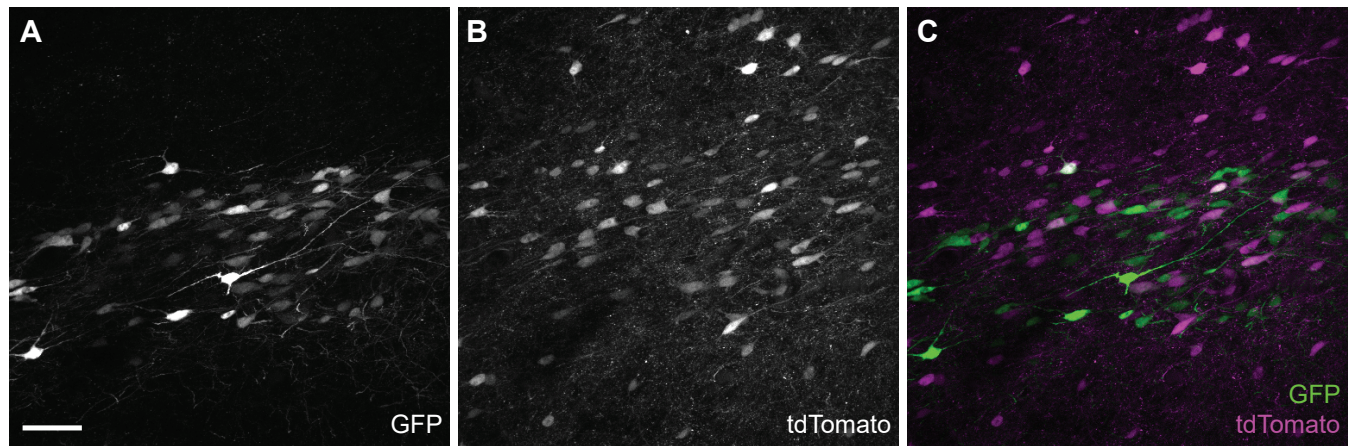


Figure 4—figure supplement 1. GFP and tdTomato fluorescence in a cryofixed-rehydrated mouse brain. (A) GFP- and (B) tdTomato-positive neurons (C) Co-expression of GFP and tdTomato fluorescence was detected in the cryofixed-rehydrated mouse brain. Scale bar: 50 μ m.

DOI: <https://doi.org/10.7554/eLife.35524.011>

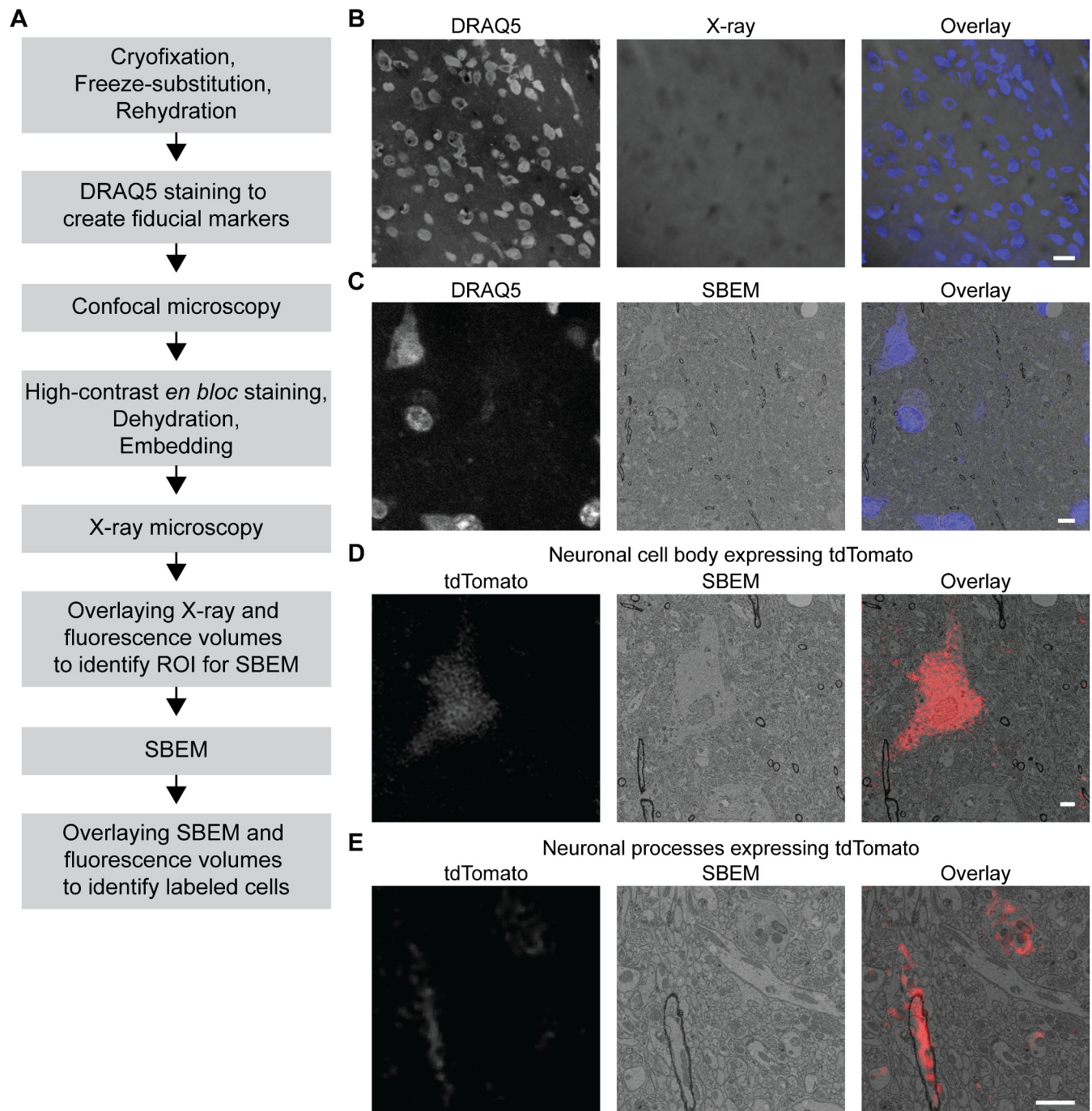


Figure 5. 3D correlated light and electron microscopy (CLEM) in CCM-processed mouse brain. Mouse brain slices with fluorescently labeled neurons were processed with CCM, imaged with confocal microscopy, X-ray microscopy and SBEM for 3D CLEM. **(A)** Flowchart for performing 3D CLEM with CCM-processed samples. Similar to a typical CCM protocol, cryofixed samples are first freeze-substituted and rehydrated. The frozen-rehydrated sample is then stained with DRAQ5 to label DNA in the nuclei. Next, the region of interest (ROI) is identified using confocal microscopy based on fluorescent signals, while the DRAQ5 signals are also acquired to serve as fiducial markers. Subsequently, the sample is stained, dehydrated and embedded for X-ray microscopy and SBEM. Using the DRAQ5 signals as fiducial markers, the confocal volumes can be registered to the X-ray volume such that the ROI for SBEM can be identified. Once the SBEM volume is acquired, it can be registered to the confocal volumes based on the positions of the nuclei for 3D CLEM. **(B)** An example of the DRAQ5 fluorescence signals (left), the corresponding ROI in X-ray volume (middle) and the overlay

Figure 5 continued on next page

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(right). This image registration process facilitates ROI identification in SBEM. Scale bar: 20 μm . (C) DRAQ5 fluorescence labeling served as fiducial points for registering the confocal volume to the SBEM volume. Scale bar: 5 μm . (D) The cell body of a tdTomato-expressing neuron (left) was identified in the SBEM volume (middle) through CLEM (right). (E) Neuronal processes expressing tdTomato (left) were also identified in the SBEM volume (middle) through CLEM (right). Scale bars: 2 μm , for both (D) and (E). SBEM imaging parameters: Z step: 70 nm; Z dimension: 695 sections; raster size: 10k \times 15k; pixel size: 6.8 nm.

DOI: <https://doi.org/10.7554/eLife.35524.012>

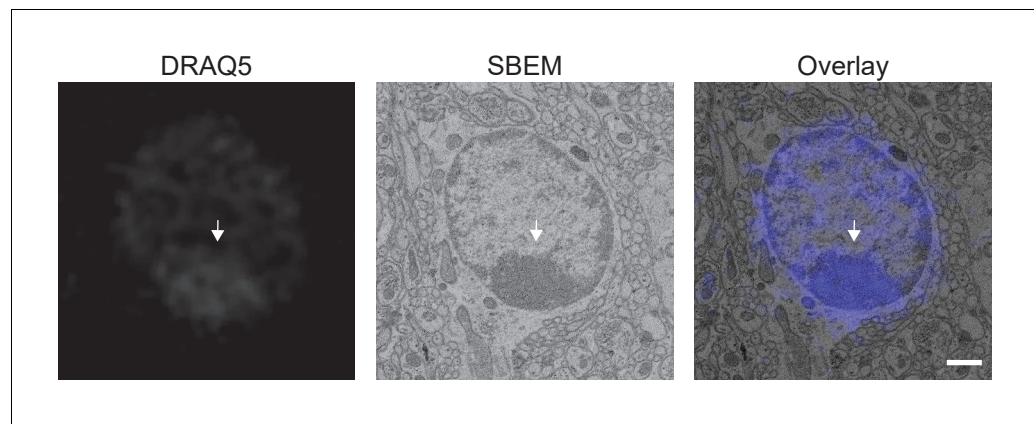


Figure 5—figure supplement 1. A correlation of subcellular structure can be achieved using 3D CLEM performed on the CCM-processed mouse brain. A heterochromatin structure (arrow) labeled by DRAQ5 in fluorescence microscopy was identified in the SBEM volume through CLEM. This heterochromatin structure was not used as a fiducial marker for image registration. Scale bar: 1 μm .

DOI: <https://doi.org/10.7554/eLife.35524.013>