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

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26 **ABSTRACT**

27 Electron microscopy (EM) offers unparalleled power to study cell substructures at the
28 nanoscale. Cryofixation by high-pressure freezing offers optimal morphological preservation, as
29 it captures cellular structures instantaneously in their near-native states. However, the
30 applicability of cryofixation is limited by its incompatibilities with diaminobenzidine labeling using
31 genetic EM tags and the high-contrast *en bloc* staining required for serial block-face scanning
32 electron microscopy (SBEM). In addition, it is challenging to perform correlated light and
33 electron microscopy (CLEM) with cryofixed samples. Consequently, these powerful methods
34 cannot be applied to address questions requiring optimal morphological preservation and high
35 temporal resolution. Here we developed an approach that overcomes these limitations; it
36 enables genetically labeled, cryofixed samples to be characterized with SBEM and 3D CLEM.
37 Our approach is broadly applicable, as demonstrated in cultured cells, *Drosophila* olfactory
38 organ and mouse brain. This optimization exploits the potential of cryofixation, allowing quality
39 ultrastructural preservation for diverse EM applications.

40 INTRODUCTION

41 The answers to many questions in biology lie in the ability to examine the relevant
42 biological structures accurately at high resolution. Electron microscopy (EM) offers the
43 unparalleled power to study cellular morphology and structure at nanoscale resolution
44 (Leapman, 2004). Cryofixation by high-pressure freezing (hereafter referred to as cryofixation) is
45 the optimal fixation method for samples of thicknesses up to approximately 500 μm (Dahl and
46 Staehelin, 1989; McDonald, 1999; Moor, 1987; Shimoni et al., 1998). By rapidly freezing the
47 samples in liquid nitrogen ($-196\text{ }^{\circ}\text{C}$) under high pressure ($\sim 2100\text{ bar}$), cryofixation immobilizes
48 cellular structures within milliseconds and preserves them in their near-native states. In contrast,
49 cross-linking based chemical fixation takes place at higher temperatures ($\geq 4\text{ }^{\circ}\text{C}$) and depends
50 on the infiltration of aldehyde fixatives, a process which takes seconds to minutes to complete.
51 During chemical fixation, cellular structures may deteriorate or undergo rearrangement
52 (Korogod et al., 2015; Steinbrecht and Müller, 1987; Szczesny et al., 1996) and enzymatic
53 reactions can proceed (Kellenberger et al., 1992; Sabatini, 1963), potentially resulting in
54 significant morphological artifacts.

55 Cryofixation is especially critical, and often necessary, for properly fixing tissues with cell
56 walls or cuticles that are impermeable to chemical fixatives, such as samples from yeast, plant,
57 *C. elegans*, and *Drosophila* (Ding, 1993; Doroquez et al., 2014; Kaeser et al., 1989; Kiss et al.,
58 1990; McDonald, 2007; Müller-Reichert et al., 2003; Shanbhag et al., 1999, 2000; Winey et al.,
59 1995). As cryofixation instantaneously halts all cellular processes, it also provides the temporal
60 control needed to capture fleeting biological events in a dynamic process (Hess et al., 2000;
61 Watanabe et al., 2013; Watanabe et al., 2013; Watanabe et al., 2014).

62 Despite the clear benefits of cryofixation, it is incompatible with diaminobenzidine (DAB)
63 labeling reactions by genetic EM tags. For example, APEX2 (enhanced ascorbate peroxidase)
64 is an engineered peroxidase that catalyzes DAB reaction to render target structures electron
65 dense (Lam et al., 2015; Martell et al., 2012). Despite the successful applications of APEX2 to

66 three-dimensional (3D) EM (Joesch et al., 2016), there has been no demonstration that APEX2
67 or other genetic EM tags can be activated following cryofixation. Conventionally, cryofixation is
68 followed by freeze-substitution (Steinbrecht and Müller, 1987), during which water in the sample
69 is replaced by organic solvents. However, the resulting dehydrated environment is incompatible
70 with the aqueous enzymatic reactions required for DAB labeling by genetic EM tags.

71 EM structures can also be genetically labeled with fluorescent markers through
72 correlated light and electron microscopy (CLEM). Yet, performing CLEM with cryofixed samples
73 also presents challenges. Fluorescence microscopy commonly takes place either before
74 cryofixation (Brown et al., 2009; Kolotuev et al., 2010; McDonald, 2009) or after the sample is
75 embedded (Kukulski et al., 2011; Nixon et al., 2009; Schwarz and Humbel, 2009). However, if
76 the specimen is dissected from live animals, the time taken to acquire fluorescence images
77 delays cryofixation and could cause ultrastructural deterioration. In order for fluorescence
78 microscopy to take place after embedding, special acrylic resins need to be used (Kukulski et
79 al., 2011; Nixon et al., 2009; Schwarz and Humbel, 2009) and only a low concentration of
80 osmium tetroxide stain can be tolerated (De Boer et al., 2015; Watanabe et al., 2011). These
81 constraints limit the applicability of CLEM for cryofixed samples.

82 Another disadvantage of cryofixation is that *en bloc* staining during freeze-substitution is
83 often inadequate. As a result, post-staining of ultramicrotomy sections is frequently needed for
84 cryofixed samples (Shanbhag et al., 1999, 2000; Takemura et al., 2013). However, post-staining
85 could be labor-intensive and time-consuming, especially for volume EM (Ryan et al., 2016;
86 Zheng et al., 2017). Critically, on-section staining is impossible for samples imaged with block-
87 face volume EM techniques (Briggman and Bock, 2012), such as serial block-face scanning
88 electron microscopy (SBEM) (Denk and Horstmann, 2004). A large amount of heavy metal
89 staining is necessary for SBEM to generate sufficient back-scatter electron signal and prevent
90 specimen charging (Deerinck et al., 2010; Kelley et al., 1973; Tapia et al., 2012). Therefore, it

91 remains impossible to image cryofixed samples with SBEM or other techniques that require
92 high-contrast staining.

93 To overcome these limitations of cryofixation, here we present a robust approach,
94 named the CryoChem Method (CCM), which combines key advantages of cryofixation and
95 chemical fixation. This technique enables labeling of target structures by genetically encoded
96 EM tags or fluorescent markers in cryofixed samples, and permits high-contrast *en bloc* heavy
97 metal staining sufficient for SBEM. Specifically, we rehydrate cryofixed samples after freeze-
98 substitution to make the specimen suitable for subsequent aqueous reactions and fluorescence
99 imaging. We further show that 3D CLEM can be achieved by combining SBEM with confocal
100 microscopy imaging of the frozen-rehydrated specimen. We successfully apply CCM to multiple
101 biologically significant systems with distinct ultrastructural morphologies, including cultured
102 mammalian cells, *Drosophila* olfactory organ (antenna) and mouse brain. By overcoming critical
103 technical barriers, our method exploits the potential of cryofixation, making it compatible with
104 genetically encoded EM tags, fluorescence imaging before resin embedding, and any EM
105 techniques that require substantial heavy metal staining.

106

107 **RESULTS**

108 Given that a key limitation of cryofixation arises from the dehydrated state of the
109 samples after freeze-substitution (Figure 1), it is imperative that our approach delivers a
110 cryofixed specimen that is fully hydrated and can then be processed at higher temperatures (4
111 °C or room temperature) for enzymatic reactions and/or high-contrast *en bloc* heavy metal
112 staining. It has been demonstrated that cryofixed samples can be rehydrated for immunogold
113 labeling following cryosectioning (van Donselaar et al., 2007), but the method only yields
114 modest EM contrast and is not compatible with genetic labeling using APEX2 nor volume EM
115 techniques.

116

117 **The CryoChem Method**

118 To achieve the ultrastructural preservation of cryofixation and the versatility of chemical
119 fixation, we developed a hybrid protocol which we refer to hereafter as the CryoChem Method
120 (CCM) (Figure 1). Importantly, we devised a freeze-substitution cocktail (see below) that allows
121 preservation of APEX2 enzymatic activity and signals from fluorescent proteins. CCM begins
122 with high-pressure freezing of the sample, followed by freeze-substitution in an acetone solution
123 with glutaraldehyde (0.2%), uranyl acetate (0.1%), and water (1%), to further stabilize the cryo-
124 preserved structures at low temperatures. After freeze-substitution, the sample is rehydrated
125 gradually on ice with a series of acetone solutions containing an increasing amount of water or
126 0.1M HEPES. Once completely rehydrated, the cryofixed sample is amenable for imaging with
127 fluorescence microscopy, DAB labeling reactions using genetically encoded tags, and the high-
128 contrast *en bloc* staining (e.g. osmium-thiocarbohydrazide-osmium and uranyl acetate) normally
129 reserved only for chemically fixed samples. Afterwards, samples are dehydrated through a
130 series of ethanol solutions and acetone, then infiltrated with epoxy resin and cured using
131 standard EM procedures. These resin embedded samples may be sectioned or imaged directly
132 with any desired EM technique (Figure 2, see *Materials and Methods* for details).

133

134 **CryoChem Method offers high-quality ultrastructural preservation and sufficient *en bloc*** 135 **staining for SBEM**

136 To determine whether CCM provides high-quality ultrastructural preservation, we first
137 tested the method in a mammalian cell line. Using transmission electron microscopy (TEM),
138 well-preserved mitochondria and nuclear membranes were observed in the CCM-processed
139 cells (Figure 3—figure supplement 1). Given that cryofixation is often necessary for properly
140 fixing tissues surrounded by a barrier to chemical fixatives, we next tested CCM in a *Drosophila*
141 olfactory organ, the antenna, which is encased in a waxy cuticle (Figure 3A). A hallmark of
142 optimally preserved antennal tissues prepared by cryofixation is the smooth appearance of

143 membrane structures (Shanbhag et al., 1999, 2000; Steinbrecht, 1980; Steinbrecht and Müller,
144 1987). In the insect antenna, auxiliary cells extend microlamellae to surround the olfactory
145 receptor neurons (ORNs), forming the most membrane-rich regions in the antenna. We
146 therefore focused on this structure to evaluate the quality of morphological preservation afforded
147 by our method. In CCM-processed antennal tissues, we found that the delicate structures of the
148 microlamellae were well-preserved (Figure 3A and Figure 3—video supplement 1), unlike the
149 chemically fixed counterparts in which microlamellae were distorted (Figure 3A) (Steinbrecht,
150 1980). Importantly, the overall ultrastructural preservation achieved through CCM resembles
151 that obtained by standard cryofixation and freeze-substitution protocols (Shanbhag et al., 1999,
152 2000).

153 In contrast to fly antennae, which can be dissected expeditiously and frozen in the live
154 state, certain tissues (e.g., mouse brain) are difficult to cryofix from life without tissue damage
155 caused by anoxia or mechanical stress associated with dissection. In these cases, cryofixation
156 can be performed after aldehyde perfusion and still produce quality morphological preservation
157 (Sosinsky et al., 2008). To test whether CCM can improve morphological preservation of
158 aldehyde-perfused samples, we cryofixed vibratome sections (100 μm) from an aldehyde-
159 perfused mouse brain and processed the sample with CCM. As a control, we used standard
160 chemical fixation procedures to process the vibratome sections of the same brain (see *Materials*
161 *and Methods* for details). Compared to chemically fixed controls, the membranes of the CCM-
162 processed samples were markedly smoother, indicating an improvement in morphological
163 preservation (Figure 3B). This result agrees with our previous observation that cellular
164 morphology can be markedly improved even when cryofixation is performed after aldehyde
165 perfusion (Sosinsky et al., 2008).

166 Of note, we adopted a high-contrast *en bloc* staining protocol (Deerinck et al., 2010;
167 Tapia et al., 2012; West et al., 2010; Williams et al., 2011) when processing the *Drosophila*
168 antennae and mouse brain. An adequate level of heavy metals was incorporated into these

169 cryofixed samples to allow for successful imaging by SBEM (Figure 3A and 4), even without
170 nitrogen gas injection for charge compensation (Deerinck et al., 2017) (Figure 3-- video
171 supplement 1 and Figure 4D). This *en bloc* staining protocol is normally reserved only for
172 chemically fixed tissues, but is now made compatible with cryofixed samples by CCM.

173

174 **CryoChem Method enables DAB labeling in cryofixed samples expressing APEX2**

175 Next we determined if DAB labeling reaction can be performed in cryofixed samples with
176 CCM. Using CCM-processed cultured cells expressing APEX2, we observed DAB labeling in
177 the targeted organelles (mitochondria) in the transfected cells (Figure 4A), compared to the
178 untransfected controls (Figure 4B). We further validated this approach in CCM-processed
179 *Drosophila* antenna; successful DAB labeling was also detected in genetically identified ORNs
180 expressing APEX2 with X-ray microscopy (Figure 4—video supplement 1). This imaging
181 technique facilitates the identification of the region of interest for SBEM (Figure 4C), as we and
182 others reported previously (Bushong et al., 2015; Ng et al., 2016). Crucially, we demonstrated
183 that an EM volume of a genetically labeled, cryofixed ORN can be acquired with SBEM, which
184 allows for an accurate 3D reconstruction of the ORN through semi-automated segmentation
185 (Figure 4D). Taken together, these results demonstrate that CCM can reliably generate DAB
186 labeling by genetically encoded EM tags in cryofixed samples.

187

188 **Fluorescence is well-preserved in CryoChem-processed samples**

189 To determine whether CCM is compatible with fluorescence microscopy, we first
190 evaluated the degree to which fluorescence level is affected after CCM processing. Using
191 confocal microscopy, we quantified GFP fluorescence in the soma of unfixed *Drosophila* ORNs
192 and that from CCM-processed samples after rehydration (Figure 5). Remarkably, GFP
193 fluorescence intensities of fresh and CCM-processed ORNs are essentially indistinguishable
194 with respect to their distributions (Figure 5A) and average levels (Figure 5B), indicating that

195 CCM processing has little effect on GFP fluorescence in fly ORNs. Similarly, we observed
196 strong GFP signals in the mouse brain after the cryofixed sample was rehydrated (Figure 5—
197 figure supplement 1A).

198 Next, we asked whether this observation also applies to another type of fluorescence
199 protein. To this end, we examined tdTomato fluorescence in the mouse brain (Figure 5—figure
200 supplement 1B). We note that tdTomato is not a variant of GFP and is instead derived from
201 *Discosoma sp.* fluorescence protein ‘DsRed’ (Shaner et al., 2004). Confocal images of the
202 CCM-processed mouse brain showed that the tdTomato fluorescence was also well-preserved
203 (Figure 5—figure supplement 1B) and we were able to detect the co-expression of GFP and
204 tdTomato in a subpopulation of neurons (Figure 5—figure supplement 1C). Taken together, our
205 results indicate that CCM-processed sample can serve as a robust substrate for fluorescence
206 imaging.

207

208 **3D correlative light and electron microscopy (CLEM) in CCM-processed samples** 209 **expressing fluorescent markers**

210 Finally, we took advantage of the fact that fluorescence microscopy can now take place
211 in a cryofixed sample before resin embedding to develop a protocol for 3D CLEM in CCM-
212 processed specimens (Figure 6A, see *Materials and Methods* for details). The protocol first uses
213 the core CCM steps to deliver a frozen-rehydrated sample. Subsequently, DRAQ5 DNA stain is
214 introduced to the sample to label the nuclei, which can then serve as fiducial markers for CLEM.
215 Next, the region containing target cells expressing fluorescent markers is imaged with confocal
216 microscopy, during which signals from DRAQ5 and fluorescent markers are both acquired. After
217 confocal microscopy, the sample is *en bloc* stained with multiple layers of heavy metals
218 (Deerinck et al., 2010; Tapia et al., 2012; West et al., 2010; Williams et al., 2011), then
219 dehydrated and embedded as in a typical CCM protocol. Subsequently, the embedded sample
220 is imaged with X-ray microscopy. The resulting micro-computed tomography volume can be

221 registered to the confocal volume using the nuclei as fiducial markers, so that the region of
222 interest (ROI) for SBEM can be identified. After SBEM imaging, the EM volume can be
223 registered to the confocal volume in a similar fashion for 3D CLEM.

224 As a proof of principle, we performed 3D CLEM in an aldehyde-perfused, CCM-
225 processed mouse brain expressing tdTomato in a subset of neurons. To this end, we first
226 determined if DRAQ5 staining can be performed in a frozen-rehydrated specimen. Using
227 confocal microscopy, we were able to observe DRAQ5 labeling of the nuclei in a cryofixed brain
228 slice after rehydration (Figure 6B). We used the labeled nuclei as fiducial markers to register the
229 X-ray volume with the confocal data (Figure 6B) and thereby target a ROI with tdTomato-
230 expressing neurons for SBEM imaging.

231 Similarly, we were able to register the confocal volume to the SBEM volume (Figure 6C).
232 Of note, the CLEM accuracy was ensured by using the bright heterochromatin labeling by
233 DRAQ5 and their corresponding structures in EM as finer fiducial points (Figure 6C).
234 Furthermore, the fluorescent markers made it possible to identify the target cell bodies (Figure
235 6D and Figure 6—video supplement 1) and small neuronal processes (Figure 6E and Figure
236 6—video supplement 1) in the SBEM volume. Lastly, we note that with CCM, fluorescence
237 microscopy in cryofixed specimens can take place before *en bloc* EM staining. Therefore, our
238 protocol does not require special resins for embedding and permits high-contrast staining with
239 high concentrations of osmium tetroxide.

240

241 **DISCUSSION**

242 We described here a hybrid method, named CryoChem, which combines key
243 advantages of cryofixation and chemical fixation to substantially broaden the applicability of the
244 optimal fixation technique. With CCM, it is now possible to label target structures with DAB by a
245 genetically encoded EM tag, image cells expressing fluorescent markers before resin
246 embedding and deposit high-contrast *en bloc* staining in cryofixed tissues. In addition, with

247 CCM, one can also perform 3D CLEM in cryofixed specimens. Our method thereby provides an
248 important alternative to conventional cryofixation and chemical fixation methods.

249 The modular nature of CCM (Figure 2) makes it highly versatile as researchers can
250 modify the modules to best suit their needs. For instance, to prevent over-staining, one can
251 replace the high-contrast *en bloc* staining step (osmium-thiocarbohydrazide-osmium and uranyl
252 acetate) (Deerinck et al., 2010; Tapia et al., 2012; West et al., 2010; Williams et al., 2011) with a
253 single round of osmium tetroxide staining for thin section TEM (Figures 3B, 4A and 4B) or
254 electron tomography. In addition, CCM is essentially compatible with a wide range of reactions
255 catalyzed by EM tags other than APEX2 (Ellisman et al., 2015). For example, the protein
256 labeling reactions mediated by miniSOG (Shu et al., 2011) and the tetracysteine-based methods
257 using FIAsH and ReAsH (Gaietta et al., 2002), or the non-protein biomolecule labeling reactions
258 using Click-EM (Ngo et al., 2016) or ChromEM (Ou et al., 2017). The versatility of CCM will
259 likely expand the breath of biological questions that can be addressed using cryofixed samples.

260 In addition to using EM tags, we have also developed a 3D CLEM protocol (Figure 6A)
261 that allows optimally and rapidly preserved EM structures to be genetically labeled with
262 fluorescent markers in CCM-processed tissues. In contrast to EM tags, fluorescent markers do
263 not generate electron-dense products (e.g. DAB polymers) that can obscure the subcellular
264 structures. Moreover, with multicolor CLEM, one can utilize multiple readily available genetically
265 encoded fluorescent markers to label different target structures or cells. Using the 3D CLEM
266 protocol, one could also pinpoint labeled subcellular structures (e.g., microtubules) or proteins
267 (e.g., ion channels) in an EM volume with super-resolution microscopy.

268 The advantages of CCM makes it particularly suited for addressing biological questions
269 that require optimal and rapid preservation of a genetically labeled structure. For example, to
270 construct an accurate model to describe the biophysical properties of a neuron, it is essential to
271 acquire morphological measurements based on faithfully preserved ultrastructures. CCM
272 processing provides such an opportunity; we were able to obtain a 3D reconstruction of a

273 genetically labeled *Drosophila* ORN at nanoscale resolution with quality morphological
274 preservation (Figure 4D). In addition, by combining CCM with Flash-and-Freeze EM (Watanabe
275 et al., 2014) and electron tomography, it is possible to capture the fast morphological changes
276 of genetically labeled vesicles in 3D during synaptic transmission.

277 Furthermore, CCM is applicable to addressing questions in diverse tissue types, as
278 demonstrated here with cultured mammalian cells or tissues of *Drosophila* antennae and mouse
279 brains. Notably, identical solutions and experimental conditions were used for these different
280 tissues in all core steps (Figure 2). Thus, the protocol described here can likely be readily
281 adapted to cells and tissues of other biological systems. In addition, we demonstrated that CCM
282 can further improve the ultrastructure of an aldehyde-perfused brain compared to chemically
283 fixed counterparts (Figure 3B). Given that aldehyde perfusion is often required for the dissection
284 of deeply embedded or fragile tissues, the compatibility of CCM with aldehyde fixation further
285 broadens the applicability of the method.

286 **MATERIALS AND METHODS**

287 **Cultured cells preparation**

288 HEK 293T cells were grown on 1.2 mm diameter punches of Aclar (2 mil thick; Electron
289 Microscopy Sciences, Hatfield, PA) for 48 hours, in a humidified cell culture incubator with 5%
290 CO₂ at 37 °C. The culture medium used was DMEM (Mediatech Inc., Manassas, VA)
291 supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA). The
292 cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with a plasmid
293 carrying APEX2 targeted to mitochondria (pcDNA3-Mito-V5-APEX2, Addgene #72480) (Lam et
294 al., 2015). At 24 hours after transfection, the cells were used for CCM processing.

295

296 **DNA constructs and *Drosophila* transgenesis**

297 Orco cDNA was a gift from Dr. Aidan Kiely, and APEX2 cDNA was acquired from
298 Addgene (APEX2-NES, #49386). Membrane targeting of APEX2 was achieved by fusing the
299 marker protein to the C-terminus of mCD8GFP or to the N-terminus of Orco. Briefly, gel-purified
300 PCR fragments of mCD8GFP, APEX2, and/or Orco were pieced together with Gibson Assembly
301 following manufacturer's instructions (New England Biolabs, Ipswich, MA). A linker (SGGGG)
302 was added between APEX2 and its respective fusion partner. In the APEX2-Orco construct, a
303 myc tag was included in the primer and added to the N-terminus of APEX2 to enable the
304 detection of the fusion protein by immunostaining. To facilitate Gateway Cloning (ThermoFisher
305 Scientific, Waltham, MA), the attB1 and attB2 sites were included in the primers and added to
306 the ends of the Gibson assembly product by PCR amplification. The PCR products were then
307 purified and cloned into pDONR221 vectors via BP Clonase II (Life Technologies, Carlsbad,

308 CA). The entry clones were recombined into the pBID-UASC-G destination vector (Wang, Beck
309 and McCabe, PloS ONE, 2012) using LR Clonases II (Life Technologies, Carlsbad, CA).

310 *Drosophila* transgenic lines were derived from germline transformations using the Φ C31
311 integration systems (Groth, Fish, Nusse, & Calos, 2004; Markstein, Pitsouli, Villalta, Celniker, &
312 Perrimon, 2008). All transgenes described in this study were inserted into the attP40 landing
313 site on the second chromosome (BestGene Inc., Chino Hills, CA). Target expression of APEX2
314 in the ORNs was driven by the Or47b-GAL4 driver (#9984, Bloomington *Drosophila* Stock
315 Center, Figures 3-5) or the Or22a-GAL4 driver (Dobritsa et al. 2003, Figure 4—video
316 supplement 1). Flies were raised on standard cornmeal food at 25 °C in a 12:12 light-dark cycle.

317

318 ***Drosophila* antennae preparation**

319 Six to eight days old flies were cold anesthetized and then pinned to a Sylgard dish. The
320 third segments of the antennae were removed from the head of the fly with a pair of fine forceps
321 and then immediately transferred to a drop of 1X PBS on the dish. With a sharp glass
322 microelectrode, a hole was poked in the antenna to facilitate solution exchange. It is critical that
323 the tissue remained in PBS at all times to prevent deflation. The antenna should remain plump
324 and maintain its shape prior to cryofixation.

325

326 **Chemical fixation of *Drosophila* antenna**

327 Antennae were dissected as described above, and then incubated in Karnovsky fixatives
328 (2% paraformaldehyde/2.5% glutaraldehyde/2 mM CaCl₂ in 0.1 M sodium cacodylate) at 4°C for
329 18 hours. Next, samples were washed in 0.1 M sodium cacodylate for 10 minutes and in a

330 solution of 100 mM glycine (Bio-Rad Laboratories, Hercules, CA) in 0.1 M sodium cacodylate for
331 another 10 minutes, and twice more in 0.1 M sodium cacodylate. All washing steps were
332 performed on ice. The following *en bloc* heavy metal staining, dehydration and resin embedding
333 steps were carried out as described in the CryoChem Method section below.

334

335 **Transgenic mice and virus-mediated gene transfer**

336 Animals were handled in accordance with the guidelines established by the *Guide for*
337 *Care and Use of Laboratory Animals* of the National Institutes of Health and approved by the
338 Animal Care and Use Committee of University of California, San Diego. To introduce GFP and
339 tdTomato fluorescent markers in a mouse brain (Figure 5—figure supplement 1), GFP was
340 expressed in the tyrosine hydroxylase (TH)-expressing neurons and tdTomato in the
341 corticotropin releasing factor (CRF)-expressing neurons. A CRF driver mouse line (B6.Cg-
342 $Crh^{tm1(cre)Zjh}/J$, Jackson laboratory) expressing CRE recombinase under the control of the *Crh*
343 promoter/enhancer elements was first crossed to a tdTomato reporter line (B6.Cg-
344 $Gt.ROSA.26Sor^{tm14(CAG-tdTomato)Hze}/J$, Jackson Laboratory). The progeny was then crossed to a
345 TH-GFP mouse line (Kessler, Yang, Gollomp, Jin, & Iacovitti, 2003), obtaining a transgenic
346 model stably expressing GFP in dopaminergic (TH⁺) neurons and CRE/tdTomato in CRF-
347 releasing neurons. To test the 3D CLEM protocol and the morphological preservation offered by
348 CCM (Figure 3B, Figure 6 and Figure 6—video supplement 1), a mouse brain from a tdTomato
349 reporter line (B6.Cg- $Gt.ROSA.26Sor^{tm14(CAG-tdTomato)Hze}/J$, Jackson Laboratory) was used.

350

351

352 **Mouse brain preparation**

353 Mice were anesthetized with ketamine/xylazine and then transcardially perfused with
354 Ringer's solution followed by 0.15 M sodium cacodylate containing 4% paraformaldehyde/0.2%
355 (Figure 5—figure supplement 1) or 0.5% (Figure 3B, Figure 6 and Figure 6—video supplement
356 1) glutaraldehyde/2 mM CaCl₂. The animal was perfused for 10 minutes with the fixatives and
357 then the brain was removed and placed in ice-cold fixative for 1 hour. The brain was then cut
358 into 100- μ m thick slices using a vibrating microtome. Slices were either processed for chemical
359 fixation (Figure 3B) or stored in ice-cold 0.15 M sodium cacodylate for around 4 hours until used
360 for high-pressure freezing (Figure 3B, Figure 5—figure supplement 1, Figure 6 and Figure 6—
361 video supplement 1).

362

363 **Chemical fixation of mouse brain**

364 The aldehyde-perfused mouse brain slices were post-fixed in 2.5% glutaraldehyde for 20
365 minutes, then washed with 0.15 M sodium cacodylate five times for 5 minutes on ice. Next, the
366 samples were incubated in 0.15 M sodium cacodylate with 100 mM glycine for 5 minutes on ice,
367 then washed in 0.15 M sodium cacodylate similarly. The following *en bloc* heavy metal staining,
368 dehydration and resin embedding steps were carried out as described in the CryoChem Method
369 section below.

370

371

372 **CryoChem Method:**

373 **(I) Cryofixation by high-pressure freezing**

374 *Cultured cells:* Aclar disks were placed within the well of a 100 μm -deep membrane
375 carrier. The cells were covered with the culture medium and then high pressure frozen with a
376 Leica EM Pact 2 unit.

377 *Drosophila antennae:* The third antennal segment was dissected as described above.
378 Antennae from the same fly were transferred into the 100 μm -deep well of a type A planchette
379 filled with 20% BSA (Sigma-Aldrich, St. Louis, MO) in 0.15 M sodium cacodylate. The well of the
380 type A planchette was then covered with the flat side of a type B planchette to secure the
381 sample. The samples were immediately loaded into a freezing holder and frozen with a high-
382 pressure freezing machine (Bal-Tec HPM 010). Planchettes used for cryofixation were pre-
383 coated with 1-hexadecene (Sigma-Aldrich, St. Louis, MO) to prevent planchettes A and B from
384 adhering to each other so as to allow solution to reach the samples during freeze-substitution.

385 *Mouse brain slices:* A 1.2 mm tissue puncher was used to cut a portion of hypothalamus
386 expressing tdTomato (Figure 3B, Figure 5—figure supplement, Figure 6 and Figure 6—video
387 supplement 1) and GFP (Figure 5—figure supplement) from a tissue slice. The tissue punch
388 was placed into a 100 μm -deep membrane carrier and surrounded with 20% BSA in 0.15 M
389 sodium cacodylate. The specimen was high-pressure frozen as described for the *Drosophila*
390 antennae.

391 All frozen samples were stored in liquid nitrogen until further processing.

392

393 **(II) Freeze-substitution**

394 Frozen samples in a planchette were transferred to cryo-vials containing the following
395 freeze-substitution solution: 0.2% glutaraldehyde (#18426, Ted Pella, Redding, CA), 0.1%
396 uranyl acetate (Electron Microscopy Sciences, Hatfield, PA), and 1% water in acetone
397 (#AC326800010, ACROS Organics, USA) in a liquid nitrogen bath. The sample vials were then
398 transferred to a freeze-substitution device (Leica EM AFS2) at -90 °C for 58 hours, from -90 °C
399 to -60 °C for 15 hours (with the temperature raised at 2 °C/hr), at -60 °C for 15 hours, from -60
400 °C to -30 °C for 15 hours (at +2 °C/hr), and then at -30 °C for 15 hours. In the last hour at -30°C,
401 samples were washed three times in an acetone solution with 0.2% glutaraldehyde and 1%
402 water for 20 minutes. The cryo-tubes containing the last wash were then transferred on ice for
403 an hour.

404 **(III) Rehydration**

405 The freeze-substituted samples were then rehydrated gradually in a series of nine
406 rehydration solutions (see below). The samples were transferred from the freeze-substitution
407 solution to the first rehydration solution (5% water, 0.2% glutaraldehyde in acetone) on ice for
408 10 minutes. The rehydration step was repeated in a stepwise manner until the samples were
409 fully rehydrated in the final rehydration solution (0.1 M and 0.15 M sodium cacodylate for cells
410 and antennae or mouse brain slices, respectively) (van Donselaar et al., 2007):

- 411 1) 5% water, 0.2% glutaraldehyde in acetone
- 412 2) 10% water, 0.2% glutaraldehyde in acetone
- 413 3) 20% water, 0.2% glutaraldehyde in acetone
- 414 4) 30% water, 0.2% glutaraldehyde in acetone
- 415 5) 50% 0.1M HEPES (Gibco, Taiwan), 0.2% glutaraldehyde in acetone

- 416 6) 70%, 0.1M HEPES, 0.2% glutaraldehyde in acetone
- 417 7) 0.1 M HEPES
- 418 8) 0.1 M / 0.15 M sodium cacodylate with 100 mM glycine
- 419 9) 0.1 M / 0.15 M sodium cacodylate

420 After rehydration, samples were removed from the planchettes using a pair of forceps
421 under a stereo microscope to a 0.1 M / 0.15 M sodium cacodylate solution in a scintillation vial
422 on ice. It is important that subsequent DAB labeling and *en bloc* heavy metal staining are
423 carried out in scintillation vials instead of the planchettes because metal planchettes may react
424 with the labeling or staining reagents.

425 **(IV) DRAQ5 staining**

426 Mouse brain slices were incubated in DRAQ5 (1:1000 in 0.15 M sodium cacodylate
427 buffer; ThermoFisher Scientific, Waltham, MA) on ice for 60 minutes. Then the samples were
428 washed in 0.15 M sodium cacodylate three times for 10 minutes on ice before fluorescence
429 imaging.

430 **(V) Fluorescence imaging**

431 *Drosophila antenna*: Freshly dissected or cryofixed-rehydrated antennae (*10x UAS-*
432 *APEX2-mCD8GFP; Or47b-GAL4*) were mounted in FocusClear (elExplorer, Taiwan) between
433 two cover glasses (#1.5 thickness, 22 mm x 22 mm, Fisher Scientific, Hampton, NH) separated
434 by two layers of spacer rings. Confocal images were collected on an Olympus FluoView 1000
435 confocal microscope with a 60X water-immersion objective lens. The 488 nm laser was used to
436 excite GFP and all images were acquired at the same laser power and gain to enable
437 comparison between the fresh vs cryofixed-rehydrated samples.

438 *Mouse brain slices*: Confocal images of mouse brain slices were collected on a Leica
439 SPE II confocal microscope with a 20X water-immersion objective lens using 488 nm and 561
440 nm excitation. After freeze-substitution and rehydration, the specimens were placed in ice-cold
441 0.15 M sodium cacodylate for imaging. Confocal volumes of DRAQ5 and tdTomato signals were
442 collected on an Olympus FluoView 1000 confocal microscope with 20X air and 60X water
443 objectives.

444 **(VI) DAB labeling of target structures by APEX2**

445 *Cultured cells*: Samples were transferred to a 0.05% DAB (#D5637, Sigma-Aldrich, St.
446 Louis, MO) solution in 0.1 M sodium cacodylate for 5 minutes on ice to allow DAB to diffuse into
447 the tissue. To label the mitochondria in the APEX2-expressing cells, samples were then
448 transferred to a 0.05% DAB solution with 0.015% H₂O₂ (Fisher Scientific, Hampton, NH) in 0.1
449 M sodium cacodylate until DAB labeling was visible under a microscope (~5 minutes on ice).
450 After the reaction, samples were washed three times with 0.1 M sodium cacodylate on ice for 10
451 minutes.

452 *Drosophila antennae*: Samples were first placed into a 0.05% DAB solution in 0.1 M
453 sodium cacodylate for an hour on ice to allow DAB to access target neurons underneath the
454 cuticle in the antenna. To label APEX2-expressing ORNs, antennae were then transferred into a
455 0.05% DAB solution with 0.015% H₂O₂ in 0.1 M sodium cacodylate for an hour on ice. After the
456 reaction, samples were washed three times with 0.1 M sodium cacodylate on ice for 10 minutes.

457 **(VII) *En bloc* heavy metal staining for TEM and SBEM**

458 For TEM: Cultured cells and mouse brain slices were incubated in 2% OsO₄ (Electron
459 Microscopy Sciences, Hatfield, PA) /1.5% potassium ferrocyanide (Mallinckrodt, Staines-Upon-
460 Thames, UK) /2 mM CaCl₂ in 0.1 M (cells) or 0.15 M (brain) sodium cacodylate for an hour on

461 ice. Then samples were washed in water five times for 5 minutes on ice prior to the dehydration
462 step detailed below.

463 For SBEM: *Drosophila* antennae and mouse brain slices were incubated in 2%
464 OsO₄/1.5% potassium ferrocyanide/2 mM CaCl₂ in 0.1 M (antennae) or 0.15 M (brain) sodium
465 cacodylate for an hour at room temperature. Then samples were washed in water five times for
466 5 minutes and transferred to 0.5% thiocarbohydrazide (filtered with 0.22 µm filter before use;
467 Electron Microscopy Sciences, Hatfield, PA) for 30 minutes at room temperature. Samples were
468 washed in water similarly and incubated in 2% OsO₄ for 30 minutes at room temperature.
469 Afterwards, samples were rinsed with water, then transferred to 2% aqueous uranyl acetate
470 (filtered with 0.22 µm filter) at 4 °C overnight. In the next morning, samples were first washed in
471 water five times for 5 minutes and then subjected to the dehydration steps detailed below.

472 **(VIII) Dehydration**

473 Samples were dehydrated with a series of ethanol solutions and acetone in six steps of
474 10 minutes each: 70% ethanol, 90% ethanol, 100% ethanol, 100% ethanol, 100% acetone,
475 100% acetone. All ethanol dehydration steps were carried out on ice, and the acetone steps at
476 room temperature. The first acetone dehydration step was carried out with ice-cold acetone, and
477 the second one was with acetone kept at room temperature.

478 **(IX) Resin infiltration**

479 *Cultured cells*: Samples were transferred to a Durcupan ACM resin/acetone (1:1)
480 solution for an hour on a shaker at room temperature. The samples were then transferred to
481 fresh 100% Durcupan ACM resin overnight and subsequently placed in fresh resin for four
482 hours. While in 100% resin, samples were placed in a vacuum chamber on a rocker to facilitate

483 the removal of residual acetone. Finally, the samples were embedded in fresh resin at 60 °C for
484 two days.

485 *Drosophila antennae and mouse brain slices*: Samples were transferred to a Durcupan
486 ACM resin/acetone (1:1) solution overnight on a shaker. The next day, samples were
487 transferred into fresh 100% Durcupan ACM resin twice, with six to seven hours apart. While in
488 100% resin, samples were placed in a vacuum chamber on a rocker to facilitate the removal of
489 residual acetone. After the overnight incubation in 100% resin, samples were embedded in fresh
490 resin at 60 °C for at least two days.

491 Durcupan ACM resin (Sigma Aldrich, St. Louis, MO) composition was 11.4 g component
492 A, 10 g component B, 0.3 g component C, and 0.1 g component D.

493

494 **X-ray Microscopy (microcomputed tomography)**

495 *Drosophila antenna*: Microcomputed tomography (microCT) was performed on resin-
496 embedded specimens using a Versa 510 X-ray microscope (Zeiss). Flat-embedded specimens
497 were glued to the end of an aluminum rod using cyanoacrylic glue. Imaging was performed with
498 a 40X objective using a tube current of 40 kV and no source filter. Raw data consisted of 1601
499 projection images collected as the specimen was rotated 360 degrees. The voxel dimension of
500 the final tomographic reconstruction was 0.4123 µm.

501 *Mouse brain slices*: X-ray microscopy scan was collected of a resin-embedded sample
502 at 80 kVp with a voxel size of 0.664 µm prior to mounting for SBEM imaging. A second scan
503 was collected of the mounted specimen at 80 kVp with 0.7894 µm voxels.

504

505 **Transmission Electron Microscopy**

506 Ultrathin sections (70 nm) were collected on 300 mesh copper grids. Samples were
507 post-stained with either Sato's lead solution only (cultured cells) or with 2% uranyl acetate and
508 Sato's lead solution (mouse brain slices). Sections were imaged on an FEI Spirit TEM at 80 kV
509 equipped with a 2k x 2k Tietz CCD camera.

510

511 **Serial Block-face Scanning Electron Microscopy**

512 *Drosophila antenna*: Following microcomputed tomography to confirm proper orientation
513 of region of interest, specimens were mounted on aluminum pins with conductive silver epoxy
514 (Ted Pella, Redding, CA). The specimens were trimmed to remove excess resin above ROI and
515 to remove silver epoxy from sides of specimen. The specimens were sputter coated with gold-
516 palladium and then imaged using a Gemini scanning electron microscope (Zeiss) equipped with
517 a 3View2XP and OnPoint backscatter detector (Gatan). Images were acquired at 2.5 kV
518 accelerating voltage with a 30 μm condenser aperture and 1 μsec dwell time; Z step size was
519 50 nm. Volumes were either collected in variable pressure mode with a chamber pressure of 30
520 Pa and a pixel size of 3.8 nm (Figure 3—video supplement 1 and Figure 4D) or using local gas
521 injection (Deerinck et al., 2017) set to 85% and a pixel size of 6.5 nm (Figures 3A and 4C).
522 Volumes were aligned using cross correlation, segmented, and visualized using IMOD.

523 *Mouse brain slices*: SBEM was performed on a Merlin scanning electron microscope
524 (Zeiss) equipped with a 3View2XP and OnPoint backscatter detector (Gatan). The volume was
525 collected at 2 kV, with 6.8 nm pixels and 70 nm Z steps. Local gas injection (Deerinck et al.,
526 2017) was set to 15% during imaging. The raster size was 10k x 15k and the Z dimension was
527 659 sections.

528

529 **Semi-automated segmentation of DAB-labeled *Drosophila* olfactory receptor neuron**

530 The DAB-labeled *Drosophila* ORN was segmented in a semi-automated fashion using
531 the IMOD software (Kremer, Mastronarde, & McIntosh, 1996) to generate the 3D model. The
532 IMOD command line 'imodauto' was used for the auto-segmentation by setting thresholds to
533 isolate the labeled cellular structures of interest. Further information about the utilities of
534 'imodauto' can be found in the IMOD manual
535 (<http://bio3d.colorado.edu/imod/doc/man/imodauto.html>). Auto-segmentation was followed by
536 manual proofreading and reconstruction by two independent proofreaders. The proofreaders
537 used elementary operations in IMOD, most commonly the 'drawing tools' to correct the contours
538 generated by 'imodauto'. Where 'imodauto' failed to be applied successfully, the proofreaders
539 also used the 'drawing tools' to directly trace the outline of the labeled structure. The contours of
540 ORNs generally do not vary markedly between adjacent sections. Therefore, alternate sections
541 were traced for the reconstruction of some parts of the ORN dendrite.

542

543 **Quantification of fluorescence intensity**

544 To quantify GFP fluorescence intensity shown in Figure 5, maximum intensity Z-
545 projections were generated using ImageJ (NIH). Average fluorescence intensity in the
546 background was subtracted from the fluorescence intensity of each cell body measured. Only
547 non-overlapping cell bodies were quantified. Kolmogorov-Smirnov Test was performed on
548 <http://www.physics.csbsju.edu/stats/KS-test.html> and Mann-Whitney *U* Test was performed
549 using SigmaPlot 13.0 (Systat Software, San Jose, CA).

550

551 **Light and electron microscope volume registration**

552 To target tdTomato-expressing cells in the mouse brain for SBEM imaging, the confocal
553 volumes collected in the frozen-rehydrated specimen was registered with the microCT volume
554 of the resin-embedded sample, using a software tool developed in our lab. The resin-embedded
555 specimen was then mounted and trimmed for SBEM based on the microCT volume. A second
556 microCT scan of the mounted specimen allowed for precise targeting of the cells of interest with
557 the Gatan stage for SBEM. After the SBEM volume was collected, the confocal and SBEM
558 volumes were registered using the landmark tool of Amira 6.3 (ThermoFisher, Waltham, MA).
559 Heterochromatin structures revealed by DRAQ5 labeling and visible in the SBEM volume were
560 used as landmark points for the registration.

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777 **FIGURE LEGENDS**

778 **Figure 1. Comparison of the advantages and limitations of different sample preparation**
779 **methods for electron microscopy.** The CryoChem Method (CCM) combines the advantages
780 of chemical fixation and cryofixation. With CCM, samples are fixed with high-pressure freezing
781 and freeze-substitution to achieve quality ultrastructural preservation. This approach allows
782 preservation of tissues with cuticle or cell wall and captures biological events with high temporal
783 resolution. A rehydration step is introduced to enable fluorescence imaging, DAB labeling by
784 genetically encoded EM tags and high-contrast *en bloc* heavy metal staining of the cryofixed
785 sample. The high-contrast *en bloc* heavy metal staining permitted by CCM reduces the need for
786 post-staining on sections, and makes CCM compatible with serial block-face scanning electron
787 microscopy (SBEM). Common limitations of chemical fixation and cryofixation are denoted in
788 red.

789
790 **Figure 2. Flowchart of the CryoChem Method.** After cryofixation by high-pressure freezing
791 and freeze-substitution, cryofixed samples are rehydrated gradually. Rehydrated samples can
792 then be imaged for fluorescence, subjected to DAB labeling reaction or *en bloc* stained with a
793 substantial amount of heavy metals. The protocol is modular; the first three processes are the
794 core steps of CCM and the starred steps are optional depending on the experimental design.
795 The samples are then dehydrated for resin infiltration and embedding, followed by imaging with
796 any EM technique of choice. Blue and grey denote hydrated and dehydrated states of the
797 sample, respectively.

798
799 **Figure 3. CryoChem Method offers high-quality ultrastructural preservation and sufficient**
800 ***en bloc* staining for SBEM.** TEM and SBEM images were acquired to assess the morphology
801 of CCM-processed tissues. (A) The microlamella structures were well-preserved in CCM-

802 processed *Drosophila* antenna (top right panel), compared to chemically fixed samples (top left
803 panel). In the enlarged views of the boxed regions (bottom panels), the microlamellae in the
804 CCM-processed antenna appeared uniform in size and shape, unlike the chemically fixed ones
805 which were distorted. Scale bars: 1 μm for top panels, 200 nm for bottom panels. (B) CCM
806 enhanced the morphological preservation of aldehyde-perfused mouse brain (top right panel),
807 compared to the chemically fixed control (top left panel). In the enlarged views of the boxed
808 regions (bottom panels), the nuclear membranes (arrows) are smoother in the CCM-processed
809 sample. We note that the chromatin was more heavily stained in the CCM-processed specimen,
810 likely due to the additional exposure to uranyl acetate during freeze substitution. Scale bars: 500
811 nm for top panels, 100 nm for bottom panels.

812 **Figure supplement 1 for Figure 3.** TEM images showed well-preserved ultrastructures in the
813 CCM-processed HEK 293T cells. In the enlarged view of the boxed region (bottom panel),
814 smooth nuclear membranes were observed. Scale bars: 1 μm for the top panel, 500 nm for the
815 bottom panel.

816 **Video supplement 1 for Figure 3.** A SBEM volume from a CryoChem-
817 processed *Drosophila* antenna. Scale bar: 500 nm.

818

819 **Figure 4. CryoChem Method enables DAB labeling by APEX2 in cryofixed tissues.** In
820 CCM-processed cultured cells and *Drosophila* antennae, DAB labeling was observed in cells
821 expressing APEX2. (A) Mitochondria expressing APEX2 were labeled with DAB in a transfected
822 HEK 293T cell. (B) An untransfected control cell. Scale bars: 200 nm. (C) An APEX2-expressing
823 olfactory receptor neuron (ORN) was labeled with DAB (arrow) in the *Drosophila* antenna.
824 Asterisks denote ORNs without APEX2 expression. Scale bar: 500 nm. (D) A series of SBEM
825 images showing the same DAB labeled *Drosophila* ORN (arrow) in different planes of section.
826 Asterisks denote ORNs without APEX2 expression. The images were acquired using standard
827 imaging methods without charge compensation by nitrogen gas injection (Deerinck et al., 2017).

828 These images, together with the rest of the EM volume acquired using SBEM, enabled semi-
829 automatic segmentation and 3D reconstruction of the labeled ORN (right panel). Scale bars:
830 500 nm for SBEM images, 2 μm for the 3D model of ORN.

831 **Video supplement 1 for Figure 4.** An X-ray micro-computed tomography volume from a CCM-
832 processed *Drosophila* antenna showing DAB labeling in subsets of ORNs expressing APEX2.
833 The damaged region on the opposite side of the labeled cells indicates the hole poked in the
834 antenna to facilitate solution exchange. Scale bar: 30 μm .

835

836 **Figure 5. GFP fluorescence is well-preserved in CryoChem-processed samples.** Confocal
837 images were taken to quantify the level of GFP fluorescence in *Drosophila* ORNs. Antennae
838 were collected from transgenic flies expressing GFP in a subset of ORNs. (A) GFP fluorescence
839 intensity distributions of the ORN soma in freshly-dissected, unfixed antennae (left panel) and
840 CCM-processed antennae (right panel) are not significantly different. $p=0.810$, Kolmogorov-
841 Smirnov test. Insets show representative images, with ORN soma outlined. Scale bar: 2 μm . (B)
842 Comparison of the average fluorescence intensities. GFP intensities are virtually identical
843 between neurons in unfixed antennae and frozen-rehydrated antennae. $n=3$ antennae for each
844 condition, Error bars denote SEM, $p=0.950$, Mann-Whitney U Test.

845 **Figure supplement 1 for Figure 5. GFP and tdTomato fluorescence in a cryofixed-**
846 **rehydrated mouse brain.** (A) GFP- and (B) tdTomato-positive neurons. (C) Co-expression of
847 GFP and tdTomato fluorescence was detected in the cryofixed-rehydrated mouse brain. Scale
848 bar: 50 μm .

849

850 **Figure 6. 3D correlated light and electron microscopy (CLEM) in CCM-processed mouse**
851 **brain.** Mouse brain slices with fluorescently labeled neurons were processed with CCM, imaged
852 with confocal microscopy, X-ray microscopy and SBEM for 3D CLEM. (A) Flowchart for
853 performing 3D CLEM with CCM-processed samples. Similar to a typical CCM protocol, the

854 cryofixed sample is first freeze-substituted and rehydrated. The frozen-rehydrated sample is
855 then stained with DRAQ5 to label DNA in the nuclei. Next, the region of interest (ROI) is
856 identified using confocal microscopy based on fluorescent signals, while the DRAQ5 signals are
857 also acquired to serve as fiducial markers. Subsequently, the sample is stained, dehydrated and
858 embedded for X-ray microscopy and SBEM. Using the DRAQ5 signals as fiducial markers, the
859 confocal volumes can be registered to the X-ray volume such that the ROI for SBEM can be
860 identified. Once the SBEM volume is acquired, it can be registered to the confocal volumes
861 based on the positions of the nuclei for 3D CLEM. (B) An example of the DRAQ5 fluorescence
862 signals (left), the corresponding ROI in X-ray volume (middle) and overlay (right). This image
863 registration process facilitates ROI identification in SBEM. Scale bar: 20 μm . (C) DRAQ5
864 fluorescence labeling served as fiducial points for registering the confocal volume to the SBEM
865 volume. Scale bar: 5 μm . (D) The cell body of a tdTomato-expressing neuron (left) was
866 identified in the SBEM volume (middle) through 3D CLEM (right). (E) Neuronal processes
867 expressing tdTomato (left) were also identified in the SBEM volume (middle) through 3D CLEM
868 (right). Scale bars: 2 μm , for both (D) and (E).

869 **Video supplement 1 for Figure 6.** A volume showing 3D CLEM in a CCM-processed mouse
870 brain. tdTomato-expressing neurons were clearly identified in the SBEM volume. Scale bar: 10
871 μm .

872 **SOURCE DATA FILES**

873 **Figure 5-Source Data 1** GFP fluorescence intensities in fresh and CCM-processed *Drosophila*

874 antennae.

Figure 1

	Chemical fixation	Cryofixation	CryoChem
Fixation	Aldehyde fixatives (4°C)	1) High pressure freezing (-196°C, ~2100 bar) 2) Freeze-substitution in organic solvents	1) High pressure freezing (-196°C, ~2100 bar) 2) Freeze-substitution in organic solvents
Ultrastructural preservation	Fair	Excellent	Excellent
Tissues with cuticle or cell wall	Incompatible	Compatible	Compatible
Temporal resolution of events captured	Low	High	High
Hydration state of the sample	Hydrated	Dehydrated after freeze substitution	Hydrated after rehydration
Fluorescence imaging after fixation	Compatible	Generally incompatible	Compatible
DAB labeling by genetic EM tags	Compatible	Incompatible due to dehydration	Compatible due to rehydration
High-contrast <i>en bloc</i> heavy metal staining	Compatible	Limited	Compatible due to rehydration
Post-staining on sections	Optional	Often required	Optional
SBEM compatibility	Compatible	Incompatible	Compatible

Figure 2

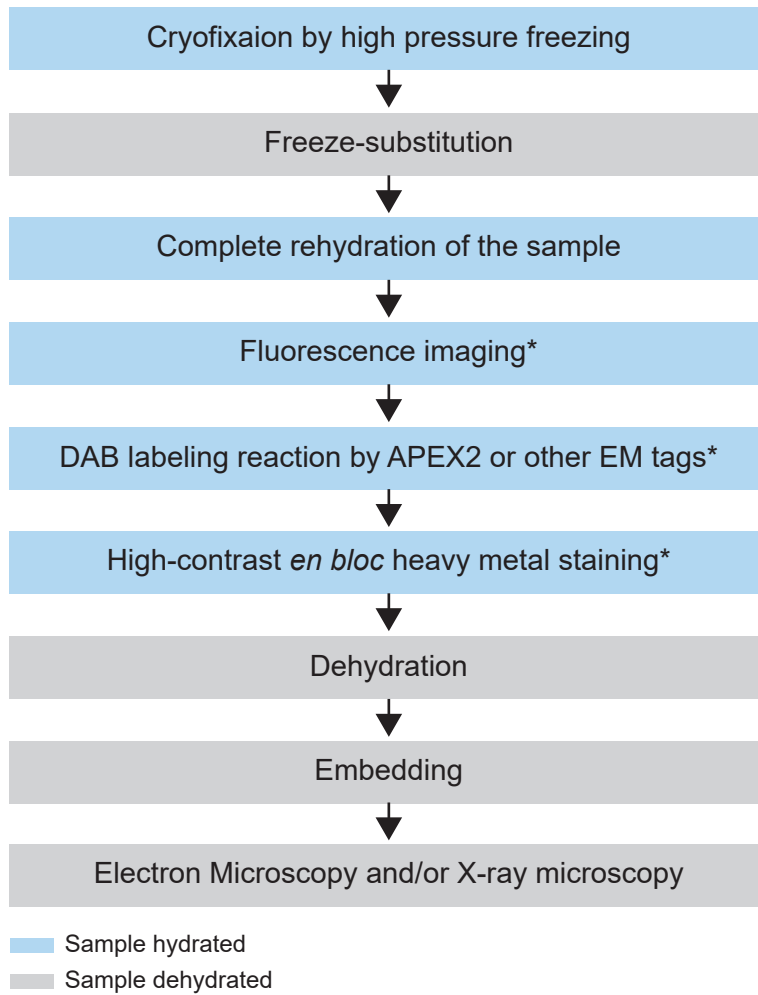


Figure 3

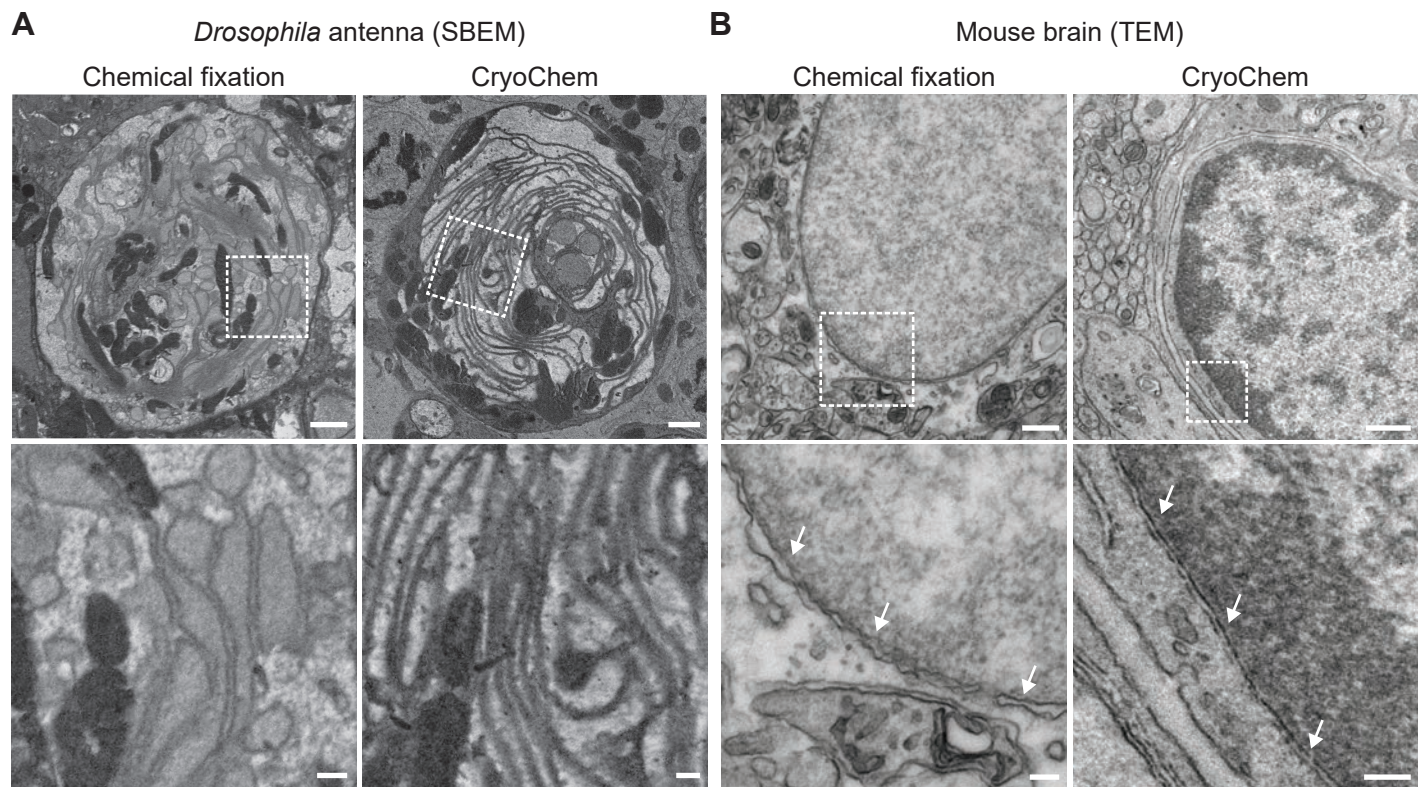


Figure 3-figure supplement 1

Cultured mammalian cells (TEM)

CryoChem

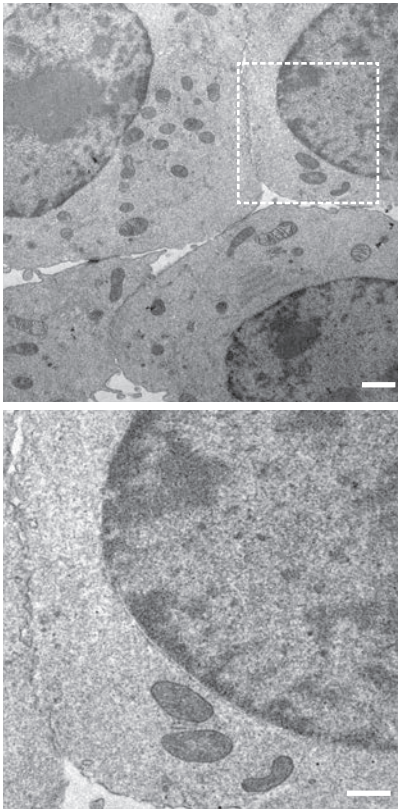


Figure 4

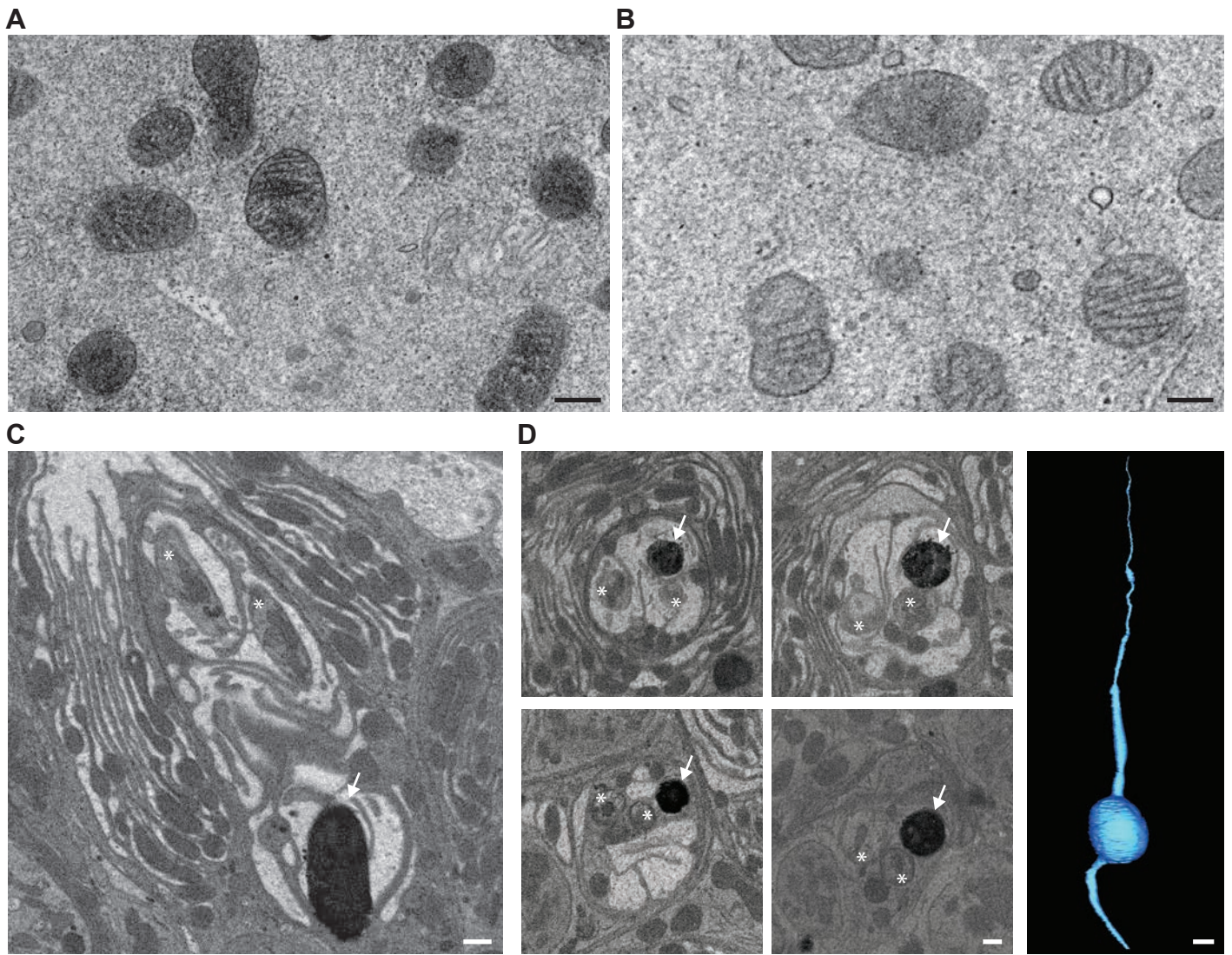


Figure 5

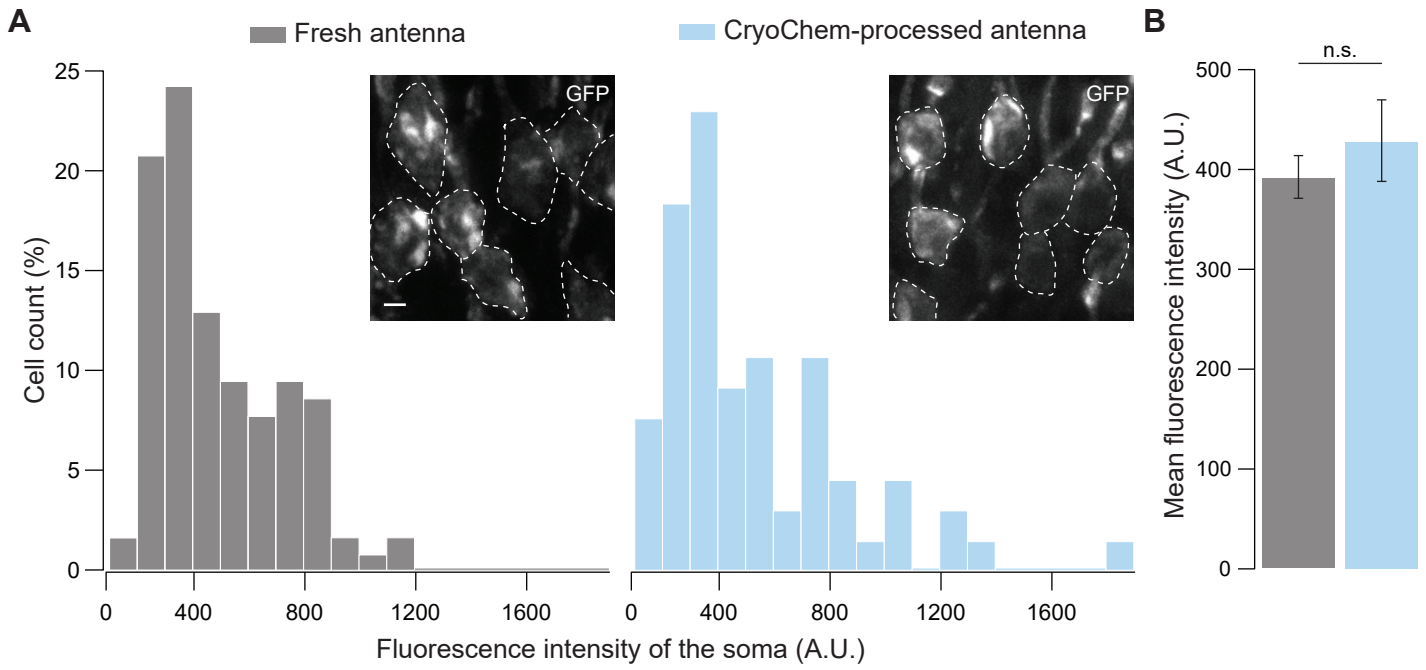


Figure 5-figure supplement 1

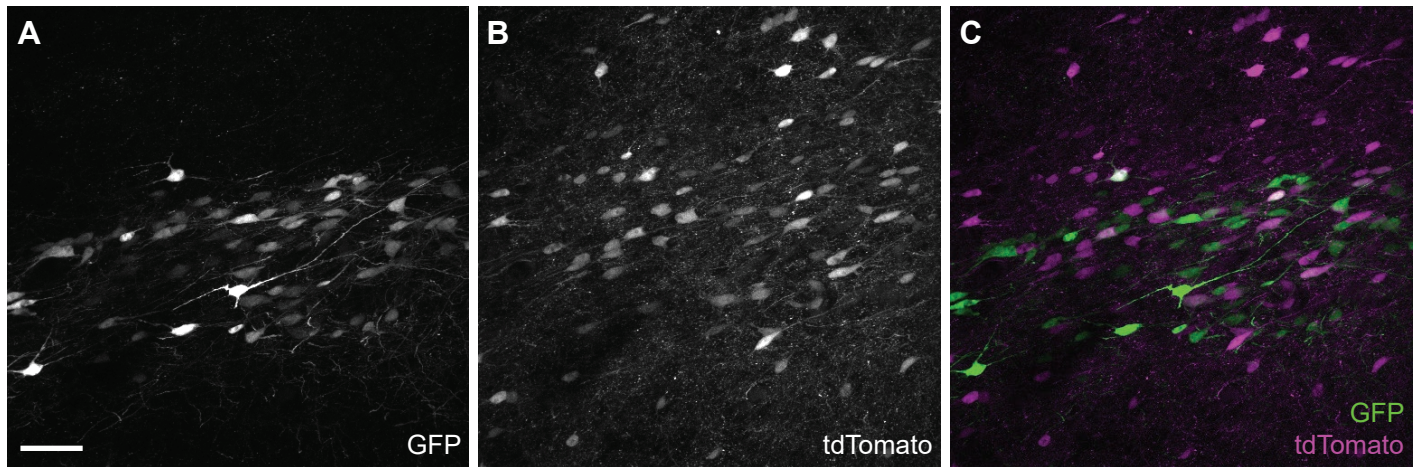


Figure 6

