The impact of chemical fixation on the microanatomy of mouse organotypic hippocampal slices

This Early Release article has been peer-reviewed and accepted, but has not been through the composition and copyediting processes. The final version may differ slightly in style or formatting and will contain links to any extended data.

Alerts: Sign up at www.eneuro.org/alerts to receive customized email alerts when the fully formatted version of this article is published.

eNeuro Accepted Manuscript

1 **The impact of chemical fixation on the microanatomy of mouse organotypic**

2 **hippocampal slices**

3 **Abbreviated title:** Anatomical impact of PFA fixation on brain tissue

4 Agata Idziak[1], V.V.G. Krishna Inavalli[1], Stephane Bancelin[1], Misa Arizono[1,2*], U.
5 Valentin Nägerl[1*]

6 [1] Univ. Bordeaux, CNRS, IINS, UMR 5297, F-33000 Bordeaux, France
7 [2] Department of Pharmacology, Kyoto University Graduate School of Medicine/The
8 Hakubi Center for Advanced Research, Kyoto University
9 *Co-corresponding authors (arizono.misa.7s@kyoto-u.ac.jp and valentin.nagerl@u-
10 bordeaux.fr)

11 **Number of pages:** 21

12 **Number of figures:** 4

13 **Number of tables:** 1

14 **Number of words: Abstract (235), Introduction (663), Discussion (734)**

15 **Conflict of interest statement:** The authors declare no competing financial interests.

28 **Author contributions:** AI carried out all experiments and analysis. MA provided
29 supervision, technical and intellectual input. VVGKI and SB provided technical support
30 for the STED microscopy. UVN conceived the study and provided supervision. The
31 paper was written by AI, MA and UVN with input from all authors.

32

33

34

35

Abstract

Chemical fixation using paraformaldehyde (PFA) is a standard step for preserving cells and tissues for subsequent microscopic analyses such as immunofluorescence or electron microscopy. However, chemical fixation may introduce physical alterations in the spatial arrangement of cellular proteins, organelles and membranes. With the increasing use of super-resolution microscopy to visualize cellular structures with nanometric precision, assessing potential artifacts - and knowing how to avoid them - takes on special urgency.

We addressed this issue by taking advantage of live-cell super-resolution microscopy that makes it possible to directly observe the acute effects of PFA on organotypic hippocampal brain slices, allowing us to compare tissue integrity in a 'before-and-after' experiment. We applied super-resolution shadow imaging to assess the structure of the extracellular space (ECS) and regular super-resolution microscopy of fluorescently labeled neurons and astrocytes to quantify key neuroanatomical parameters.

While the ECS volume fraction and micro-anatomical organization of astrocytes remained largely unaffected by the PFA treatment, we detected subtle changes in dendritic spine morphology and observed substantial damage to cell membranes. Our experiments show that PFA application via immersion does not cause a noticeable shrinkage of the ECS in hippocampal brain slices maintained in culture, unlike the situation in transcardially perfused animals *in vivo* where the ECS typically becomes nearly depleted.

Our study outlines an experimental strategy to evaluate the quality and pitfalls of various fixation protocols for the molecular and morphological preservation of cells and tissues.

Significance Statement

Chemical fixation of biological samples using PFA is a standard step routinely performed in neuroscience labs. However, it is known to alter various anatomical parameters ranging from protein distribution to cell morphology, potentially affecting our interpretation of anatomical data. With the increasing use of super-resolution microscopy, understanding the extent and nature of fixation artifacts is an urgent concern.

Here, we use live STED microscopy to monitor in real time the impact of PFA on the microanatomy of organotypic hippocampal brain slices. Our results demonstrate that while PFA has little impact on the extracellular space and astrocytes, it compromises cell membranes and dendritic structures. Our study provides a strategy for a direct characterization of fixation artifacts at the nanoscale, facilitating the optimization of fixation protocols.

Introduction

Chemical fixation is a commonly used preservation step for electron microscopy (EM) and super-resolution microscopy techniques, such as Stimulated Emission Depletion microscopy (STED), single-molecule localization microscopy and expansion microscopy. These techniques permit structural and molecular analyses of cells and tissues at a sub-microscopic level. Chemical fixatives like paraformaldehyde (PFA) covalently cross-link proteins, which has the effect of physically hardening the cellular and molecular structure of the sample (Fox et al., 1985). This procedure is to protect the sample from decay and damage during subsequent processing steps, such as tissue slicing, dehydration or embedding in resin.

However, it is known that even the most carefully executed fixation protocol can introduce structural artifacts that compromise data quality and interpretation (Ebersold et al., 1981; Ryter, 1988; Wyffels, 2001; Schnell et al., 2012). While these problems are perhaps not noticeable at a macroscopic level, they might appear at the microscopic subcellular scale. Indeed, organelles such as endosomes and lysosomes become deformed by chemical fixation (Murk et al., 2003), while cellular proteins can still move substantially and reposition after chemical fixation, potentially casting doubts over conclusions based on this approach (Tanaka et al., 2010).

As the spatial resolution of modern microscopy techniques keeps improving, allowing researchers to make ever more detailed and discriminating observations, concerns about fixation artifacts become more relevant. Recent super-resolution techniques can now reach into the low nanometer range (Arizono et al., 2023), where fixation artifacts may abound. In turn, these gains in spatial resolution necessitate the development of

95 more stringent ways to assess the quality of fixation protocols and how well they can

96 preserve cellular elements at this finer spatial scale.

97 The question of how much the micro-architecture and ultrastructure of brain tissue is

98 affected by chemical fixation was addressed in two EM studies that compared the

99 effects of chemical and cryogenic fixation protocols on tissue fine structure. Cryogenic

100 fixation is based on rapid high-pressure freezing of the sample, which produces

101 amorphous ice instead of ice crystals, that otherwise would destroy the ultrastructure.

102 These studies clearly showed that chemical fixation via transcardial perfusion leads to a

103 strong depletion of the extracellular space (ECS) as well as changes in astrocytic

104 (Korogod et al., 2015) and dendritic spine morphology (Tamada et al., 2020), raising

105 serious concerns about the use of chemical fixation protocols in high-resolution

106 anatomical studies of brain tissue. However, due to differences in sample preparation

107 required for either fixation method (transcardial perfusion of the brain *in vivo* with

108 buffered PFA versus rapid cryo-genic freezing of acute brain slices with subsequent

109 storage in liquid nitrogen) and the inability to compare the EM samples with their live

110 originals, the reason of the observed differences remains elusive.

111 To directly compare nanoscale neuroanatomical structures before and after chemical

112 fixation, we took advantage of the super-resolution shadow imaging (SUSHI) technique,

113 which is based on 3D-STED microscopy and fluorescence labeling of the interstitial fluid

114 (Tonnesen et al., 2018). SUSHI allows for visualization of tissue anatomy, including the

115 ECS, projecting all cellular structures as sharply contoured 'shadows', providing a

116 comprehensive and non-biased view of the cellular architecture of the tissue. Using this

117 technique, we imaged organotypic brain slices and analyzed the impact of PFA on the

118 ECS. In addition, we imaged fluorescently labeled astrocytes and neurons, and

119 analyzed the effect of PFA on their nanoscale morphology in a before-and-after manner.

120 We observed that PFA does not induce major changes in the shape and size of the

121 ECS and astrocytes. However, we detected subtle changes in dendritic spine

122 morphology as well as a widespread disruption of cellular membranes and cellular

123 blebbing.

124 The study gives a 'real time' and nanoscale view of the effects of PFA on tissue micro-

125 architecture of cultured hippocampal slices, revealing the extent and type of fixation

126 artifacts, which had remained inconclusive. Applying this approach to other brain areas

127 and tissue preparations (acute brain slices, intact brain *in vivo*) may help the

128 development of fixation protocols to preserve the native structure of the tissue as well

129 as possible.

130

131 **Materials and Methods**

132 **Mouse line**

133 Animal experimental procedures were in accordance with the French National Code of

134 Ethics on Animal Experimentation and approved by the Committee of Ethics of

135 Bordeaux. All procedures were performed according to the guidelines of the European

136 Directive 2010/63/UE.

137 Mice were housed under a 12 h light/12 h dark cycle at 20-22 °C with *ad libitum* access

138 to food and water in the animal facility of the Interdisciplinary Institute for Neuroscience

139 (University of Bordeaux/CNRS) and monitored daily by trained staff. All animals used

6

140 were free of any disease or infection at the time of experiments. Pregnant females and

141 females with litters were kept in cages with one male. We did not distinguish between

142 males and females among the perinatal pups used for organotypic cultures, as potential

143 anatomical and/or physiological differences between the two sexes were considered

144 irrelevant in the context of this study.

145 C57Bl/6J wild-type mice were used for all experiments in this study.

146 **Organotypic brain slices**

147 Organotypic hippocampal slices (Gahwiler, 1981) were dissected from 5 to 7 days old

148 mice, and were cultured for 2 to 5 weeks in a roller drum at 35°C (for details, see

149 (Tonnesen et al., 2018)). Once a week, 500 μl of medium was exchanged in the tubes.

150 For experiments, a given coverslip with a slice was mounted in an imaging chamber,

151 and the slice was imaged from below through the glass coverslip, while PFA-containing

152 solutions were added to the imaging chamber from above.

153 **Viral injections**

154 In order to fluorescently label neurons or astrocytes, we have introduced either Sindbis-

155 Citrine or AAV2/1.gfaABC1D-Clover viruses to the brain slices via microinjections using

156 a glass pipette connected to a Picospritzer (Parker Hannifin). Briefly, the virus was

157 injected via a pipette positioned into the CA1 area of the slice by brief pressure pulses

158 (30 ms; 15 psi). For imaging of the neurons, Sindbis-Citrine virus was injected into 2-

159 week old wild-type slices one day before the experiments. To image astrocytes, 2-week

160 old wild-type slices were injected with AAV2/1.gfaABC1D-Clover two weeks before the

161 experiments.

162

Extracellular labeling

164 Extracellular labeling of organotypic slices was performed as described before
165 (Tonnesen et al., 2018). In brief, once the slice was transferred to the imaging chamber,
166 it was immersed in HEPES-buffered artificial cerebrospinal fluid (ACSF), containing 200
167 μM of the fluorescent dye Calcein (Dojindo Laboratories).

Chemical fixation

169 After acquiring a live image of a slice (where either the ECS, neurons or astrocytes
170 were labeled), the Calcein/ACSF or ACSF-only solutions were carefully removed with a
171 pipette, making sure to keep drift of the slice to a minimum. Subsequently, solutions
172 containing 4% PFA diluted in HEPES-based ACSF with or without Calcein were
173 pipetted on top of the slice. To minimize the evaporation of PFA, the imaging chamber
174 was covered with a lid.

175 For overnight chemical fixation, the organotypic slices on a glass coverslip were
176 transferred from the roller drum tube to a 6-well plate and instantly immersed in 4% PFA
177 diluted in 1x PBS solution. The 6-well plate was placed at 4 °C overnight, followed by 3
178 washes in PBS. Finally, the fixed slices on the glass coverslip were mounted onto the
179 imaging chamber containing Calcein/ACSF solution.

3D-STED microscopy

181 We used a home-built 3D-STED setup (for details, see (Inavalli et al., 2019))
182 constructed around an inverted microscope body (DMI 6000 CS, Leica Microsystems),

183   which was equipped with a TIRF oil objective (100X, 1.47 NA, HXC APO, Leica

184   Microsystems) and contained within a heating box (Cube and Box, Life Imaging

185   Services) to maintain a stable temperature of 32°C. A pulsed-laser (PDL 800-D,

186   PicoQuant) was used to deliver excitation pulses (90 ps at 80 MHz) at 485 nm and a

187   synchronized de-excitation laser (Onefive Katana 06 HP, NKT Photonics) operating at

188   592 nm was used to generate the STED light pulses (500-700 ps). The STED beam

189   was reflected on a spatial light modulator (SLM, Easy3D Module, Abberior Instruments)

190   to generate a mixture of doughnut- and bottle-shaped beams for 2D and 3D-STED,

191   respectively. Image acquisition was controlled by the Imspector software (Abberior

192   Instruments). The performance and spatial resolution of the microscope was checked

193   and optimized by visualizing and overlapping the PSFs of the laser beams using 150

194   nm gold nano-spheres and 40 nm fluorescent beads and correcting the main optical

195   aberrations with the SLM. The spatial resolution was 175 nm (lateral) and 450 nm

196   (axial) in confocal mode and 60 nm (lateral) and 160 nm (axial) in STED mode.

197   **Image acquisition**

198   For imaging, slices were transferred on their glass coverslip to the imaging chamber

199   and immersed in ACSF consisting of (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 1

200   NaH_2PO_4 x $2H_2O$, 2.5 $CaCl_2$ x $2H_2O$, 20 D-Glucose x H_2O and 10 HEPES (all from

201   Sigma Aldrich); 300 mOsm; pH 7.4. We acquired confocal image stacks with the

202   following parameters: 100 x 100 x 4 μm^3 with a Δz size of 1 μm and a pixel size of 48.8

203   nm. For STED imaging, we either acquired 100 x 100 μm^2 single sections or z-stacks

204   (15 x 15 x 1 μm^3 or 25 x 25 x 1 μm^3 with a Δz size of 200 nm). All STED images were

205   acquired with a pixel size of 19.53 nm and a pixel dwell time of 30 μs. The excitation

206   power was 0.5 μW and STED power was 30 mW at the entrance pupil of the objective.

207   We adjusted the z focus and the xy stage to keep sample drift to a minimum between

208   image acquisitions. Images were cropped at the edges if needed to keep the field of

209   view the same.

210   **Image processing and analysis**

211   *ECS volume fraction and widths*

212   SUSHI images are single sections taken from z-stacks or time-lapse series for Fig. 1B,

213   selected to match the x-y-z planes. Brightness and contrast were adjusted for each

214   individual image using ImageJ (NIH) and the look-up table (LUT) was 'grays'. To

215   measure the ECS volume fraction (VF) and widths, images were binarized using a

216   wavelet-based software SpineJ (Levet et al., 2020) (**Fig. 1A**). For the VF (α), we

217   computed the ratio of pixels representing ECS over the total number of pixels (α =

218   N_{ECS}/N_{total}) in a region of interest. For ECS widths, we analyzed 3-pixel-wide line profiles

219   drawn across the binarized images.

220   *Measurements of astrocytic structures and dendritic spines*

221   Images of astrocytes and dendrites are shown as maximum intensity z-projections with

222   the LUT 'orange hot'. Areas of astrocytic cell bodies and main branches were measured

223   by drawing a contour line around the structures and calculating their surface areas in

224   ImageJ. The widths of astrocytic fine processes were measured on raw images in

225   ImageJ, using the 'Plot Line Profile' function after drawing a 3-pixel-wide straight line

226   across the structure of interest. 'Dendritic vacuoles' were visually identified and

227   quantified by counting them in the images acquired before and after PFA application.

228 Morphological parameters of dendritic spines were measured using the SpineJ

229 software. The software identifies the neck region and places lines that are orthogonal to

230 the neck axis at regular intervals of 75 nm. It calculates the FWHM of the neck

231 diameter, returning the minimum, maximum and median values for each analyzed

232 spine. We limited the morphology analysis to dendritic spines with clear neck and head

233 compartments, commonly referred to as mushroom spines.

234 **Experimental design and statistical analysis**

235 Statistical tests were performed using Graphpad Prism software. Normality tests were

236 performed to confirm data were normally distributed. Normally distributed data are

237 presented as mean with standard deviation, while non-normal data are presented as

238 median with interquartile range. Paired t-tests were performed for normally distributed

239 data and Wilcoxon tests for non-normally distributed data. The size and type of

240 individual samples, n, for given experiments is indicated and specified in the results

241 section and figure legends. Asterisks in figures indicate p values as follows: * $p < 0.05$,

242 ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Image analysis and statistical tests were

243 performed blind to the experimental condition.

244

245 **Results**

246 *30 minutes of PFA fixation has no detectable effects on ECS volume fraction*

247 To assess whether chemical fixation using PFA has an effect on hippocampal ECS

248 structure, we established an experimental workflow that allowed us to compare the

249 same sample before and after PFA fixation in a paired manner (**Fig. 1A**). As a readout

11

250   of the ECS structure, we analyzed the volume fraction (α) and geometric widths of the

251   ECS. The volume fraction α is an important structural parameter of brain tissue

252   (Nicholson and Hrabetova, 2017), which is defined by the ratio of the ECS volume over

253   the total volume in a region of interest.

254   We performed time-lapse confocal shadow imaging at 5-minute intervals before and

255   during PFA application using Calcein to label the ACSF that the slices were maintained

256   in (**Fig. 1B**). The images were binarized using SpineJ software (Levet et al., 2020)

257   based on wavelet filtering, allowing us to calculate the ratio of the number of 'ECS

258   pixels' over the total number of pixels as an estimate of the volume fraction in a region

259   of interest. We found that 30 minutes of PFA incubation did not cause any significant

260   changes in the ECS volume fraction (**Fig. 1C**; n_{PFA} = 6; p= 0.3962; paired t-test).

261   This result was confirmed by shadow-imaging with 3D-STED (SUSHI) (**Fig. 1D & E**; n =

262   6; p= 0.3066; paired t-test), indicating that 30 minutes of PFA incubation has little impact

263   on the volume fraction of the ECS in organotypic hippocampal slices.

264   ***Prolonged PFA incubation introduces pronounced artifacts***

265   As brain slices are often maintained in fixative for more than one hour or even

266   overnight, we investigated the effects of longer incubation times on ECS structure (**Fig.**

267   **2A**). We maintained the slices for 90 minutes under PFA or normal ACSF conditions

268   and compared SUSHI images before and after this period. Even 90 minutes of PFA

269   fixation appeared to affect neither ECS volume fraction (**Fig. 2B** left; n = 6; p> 0.05;

270   Wilcoxon matched-pairs test) nor widths measured in line profiles of binarized images

271   using SpineJ (**Fig. 2C, D, E;** n_{cntrl} = 12 lines, n_{PFA} = 16 lines; p_{cntrl} = 0.1281; p_{PFA} =

272   0.7249; paired t-test).

273   However, after 90 minutes under PFA conditions we observed dye-free, cellular blebs in

274   the immediate vicinity of cell bodies (**Fig. 2A, C** white arrows; **Fig. 2B** right, n_{live} = 6,

275   n_{PFA} = 6; p= 0.0043; Mann-Whitney test).

276   Incubation for 180 minutes with PFA caused even more pronounced changes, such as

277   dye accumulation around cell bodies and dye permeation into the cells (**Fig. 2F**),

278   indicating that PFA by itself permeabilized cell membranes, even in the absence of

279   detergents, like Triton, that are typically used in immunofluorescence protocols for

280   antibodies to reach intracellular epitopes (Goldenthal et al., 1985). Indeed, after

281   overnight PFA incubation the extracellular dye had entered into the cells (**Fig. 2G**),

282   indicating disruption of cellular membranes. This made assessing the impact of PFA on

283   the ECS impossible, because the inside-outside contrast, which is required for the

284   shadow imaging approach, was lost. Thus, more than 90 minutes of PFA fixation

285   appears to seriously damage the integrity of cellular membranes.

286   ***90 minutes of PFA fixation does not affect the morphology of astrocytes***

287   In addition to the effects of PFA on ECS structure, we set out to determine the impact of

288   PFA fixation on different cell types. We first focused on astrocytes, whose morphology

289   is known to be very sensitive to environmental changes, such as osmotic challenges

290   (Arizono et al., 2021) and transcardial perfusion of fixatives (Korogod et al., 2015). In

291   order to label astrocytes, we micro-injected AAV-GFAP-Clover viral particles into

292   organotypic hippocampal slices. Confocal microscopy did not turn up any significant

293 changes in the size of the major branches and cell bodies of astrocytes after 90 minutes

294 of PFA fixation (**Fig. 3A, B**; $n_{branches}$ = 12; n_{bodies} = 11; $p_{branches}$ = 0.0952; p_{bodies} =

295 0.1322; paired t-test). Likewise, STED microscopy did not detect any significant

296 changes in the widths of astrocytic fine processes (**Fig. 3C, D**; n_{cntrl} = 26; n_{PFA} = 28; p=

297 0.9589; paired t-test). These results suggest that PFA incubation by itself has

298 surprisingly little impact on astrocytic morphology.

299 ***90 minutes of PFA fixation leads to changes in dendritic spine morphology***

300 Finally, we performed similar experiments with neurons by virally labeling them with

301 Citrine as fluorescent protein. Using STED microscopy, we imaged dendrites and

302 dendritic spines before and after 90 minutes of PFA fixation (**Fig. 4A**). Unlike astrocytes,

303 many neurons formed 'dendritic vacuoles' after exposure for 90 minutes to PFA, which

304 could be extracellular holes in the dendrites or intracellular vacuoles free of the

305 fluorescent label. As we did not observe any dye-filled holes in dendrites in SUSHI

306 images, these structures most likely reflect Citrine-free spaces formed by coalescing

307 intracellular organelles such as mitochondria. In control experiments, i.e., in the

308 absence of PFA, we rarely observed such structures (**Fig. 4B**; n_{cntrl} = 23, n_{PFA} = 31; n =

309 number of analyzed dendritic segments; p = 0.0074; Mann-Whitney test).

310 Quantitative analysis of dendritic spine morphology revealed that there were no

311 significant changes in spine neck lengths **(Fig. 4D)**; however, spine head areas became

312 significantly smaller **(Fig. 4C, D)** and the median spine neck diameter (measured along

313 the length of the neck, see Methods for details) became wider after 90 minutes of PFA

314 treatment (**Fig. 4E, F**; $p_{h.area}$ = 0.0157; p_{width}< 0.0001; Wilcoxon matched-pair test).

315 While this effect was statistically highly significant for the median neck diameter, it was

14

316   not for the thinnest parts of the spine necks (**Fig. 4F**; p_{width} = 0.1565; Wilcoxon matched-

317   pair test).

318   These results show that dendrites and spines are affected by PFA fixation, suggesting

319   that neurons are more sensitive than astrocytes to PFA treatment in organotypic brain

320   slices within the limits of the resolution of our STED imaging approach.

321

322   **Discussion**

323   In this study, we describe the impact of chemical fixation with buffered PFA on tissue

324   architecture in organotypic hippocampal slices, focusing on the ECS and cellular

325   morphology. Our SUSHI approach indicates that immersion of organotypic brain slices

326   in PFA up to 90 minutes does not affect ECS volume fraction and widths. Similarly,

327   STED imaging showed that astrocytic morphology remains unaffected by PFA even at

328   the level of the fine processes. However, subtle changes in spine morphology appeared

329   within these relatively brief applications of PFA.

330   Previous studies have shown that transcardial perfusion of PFA reduces ECS widths

331   (Thorne and Nicholson, 2006) and causes major ECS shrinkage and astrocytic swelling

332   (Korogod et al., 2015). The fact that our experiments did not reveal such changes

333   indicates that they were not a direct result of PFA by itself on the tissue, but rather a

334   consequence of the anomalies subsequent to transcardial PFA perfusion, such as

335   anoxia (Tao-Cheng et al., 2007), or dehydration. This is in line with the observation that

336   acute slices fixed either chemically or cryogenically did not appear different in terms of

337   tissue quality and ECS distribution (Korogod et al., 2015). Given the absence of major

338   remodeling of the ECS under our conditions, it is perhaps not surprising that the

339   astrocytes did not show any changes either, suggesting that their morphology and ECS

340   topology are closely linked (Korogod et al., 2015).

341   Transcardial fixation was also shown to impact the spatial and molecular organization of

342   synaptic vesicles (Maus et al., 2020). In the same vein, a recent study showed that PFA

343   affects the behavior of proteins in liquid–liquid phase separation experiments,

344   underscoring the importance of understanding better the artifacts induced by PFA

345   fixation (Irgen-Gioro et al., 2022).

346   PFA fixation over 90 minutes considerably disturbed membrane integrity as indicated by

347   the penetration of the extracellular dye into the cells, preventing us from acquiring

348   SUSHI images. The membrane permeabilization was accompanied with cellular

349   blebbing, which has already been observed in cell cultures (Zhao et al., 2014),

350   suggesting that this is a common effect of PFA. Immunofluorescence experiments with

351   and without membrane permeabilization showed that PFA permeabilizes the membrane

352   to an extent that an antibody can pass into the cells (data not shown). Such loss of

353   membrane integrity needs to be considered in experiments focused on membrane

354   proteins, including ion channels or surface receptors (Ichikawa et al., 2022), as well as

355   many intracellular proteins that are linked to these proteins, such as components of the

356   postsynaptic density (Chen et al., 2008). A loss of membrane integrity may thus distort

357   our view of the synapse.

358   90 minutes of PFA fixation also induced 'dendritic vacuoles', which were virtually absent

359   in control experiments. Morphometric analysis of dendritic spines revealed that PFA

360   application leads to wider spine necks, in line with previous findings, where spine necks

16

361 were 30% thinner in cryogenically than chemically fixed samples (Tamada et al., 2020).

362 While we did not see any changes in spine neck length, we observed slightly decreased

363 spine head sizes.

364 To summarize, the time-lapse super-resolution approach allowed us to compare the

365 same tissue sample at the nanoscale under live and fixed conditions. Whereas short-

366 lasting fixation (< 30 minutes) is largely innocuous to tissue structure, longer PFA

367 applications unmistakably lead to artifacts. However, as our study was carried out in an

368 artificial system (organotypic brain slices), we do not know what happens *in vivo*, where

369 other factors (e.g., anoxia) come into play. In principle, STED imaging *in vivo* (Pfeiffer et

370 al., 2018) during transcardial perfusion of fixatives could address this knowledge gap.

371 With the proliferation of super-resolution studies based on chemical fixation, it is crucial

372 to reveal the effects of chemical fixatives, ambient conditions (e.g., temperature) and

373 other sample preparation steps on the native molecular and structural organization of

374 the samples, in order to optimize fixation protocols (Whelan and Bell, 2015; Pereira et

375 al., 2019; Laporte et al., 2022). Single-molecule imaging, ideally in combination with

376 STED (Inavalli et al., 2019), could provide a very sensitive readout to check protein

377 arrangements and cell morphology in parallel, helping to determine effective and

378 practical solutions to increase the preservation of fixed cells and tissues and the fidelity

379 of their microscopic analysis.

380 Finally, our study also presents a case for the development and use of live-cell super-

381 resolution microscopy, delivering information on the natural and dynamically evolving

382 state of the biological system free of concerns over fixation artifacts of whatever

383 provenance.

17

384 **References**

385 Arizono M, Idziak A, Quici F, Nagerl UV (2023) Getting sharper: the brain under the spotlight of super-
386   resolution microscopy. Trends Cell Biol 33:148-161.
387 Arizono M, Inavalli V, Bancelin S, Fernandez-Monreal M, Nagerl UV (2021) Super-resolution shadow
388   imaging reveals local remodeling of astrocytic microstructures and brain extracellular space
389   after osmotic challenge. Glia 69:1605-1613.
390 Chen X, Winters C, Azzam R, Li X, Galbraith JA, Leapman RD, Reese TS (2008) Organization of the core
391   structure of the postsynaptic density. Proc Natl Acad Sci U S A 105:4453-4458.
392 Ebersold HR, Cordier JL, Luthy P (1981) Bacterial mesosomes: method dependent artifacts. Arch
393   Microbiol 130:19-22.
394 Fox CH, Johnson FB, Whiting J, Roller PP (1985) Formaldehyde fixation. J Histochem Cytochem 33:845-
395   853.
396 Gahwiler BH (1981) Organotypic monolayer cultures of nervous tissue. J Neurosci Methods 4:329-342.
397 Goldenthal KL, Hedman K, Chen JW, August JT, Willingham MC (1985) Postfixation detergent treatment
398   for immunofluorescence suppresses localization of some integral membrane proteins. J
399   Histochem Cytochem 33:813-820.
400 Ichikawa T, Wang D, Miyazawa K, Miyata K, Oshima M, Fukuma T (2022) Chemical fixation creates
401   nanoscale clusters on the cell surface by aggregating membrane proteins. Commun Biol 5:487.
402 Inavalli V, Lenz MO, Butler C, Angibaud J, Compans B, Levet F, Tonnesen J, Rossier O, Giannone G,
403   Thoumine O, Hosy E, Choquet D, Sibarita JB, Nagerl UV (2019) A super-resolution platform for
404   correlative live single-molecule imaging and STED microscopy. Nat Methods 16:1263-1268.
405 Irgen-Gioro S, Yoshida S, Walling V, Chong S (2022) Fixation can change the appearance of phase
406   separation in living cells. Elife 11.
407 Korogod N, Petersen CC, Knott GW (2015) Ultrastructural analysis of adult mouse neocortex comparing
408   aldehyde perfusion with cryo fixation. Elife 4.
409 Laporte MH, Klena N, Hamel V, Guichard P (2022) Visualizing the native cellular organization by coupling
410   cryofixation with expansion microscopy (Cryo-ExM). Nat Methods 19:216-222.
411 Levet F, Tonnesen J, Nagerl UV, Sibarita JB (2020) SpineJ: A software tool for quantitative analysis of
412   nanoscale spine morphology. Methods 174:49-55.
413 Maus L, Lee C, Altas B, Sertel SM, Weyand K, Rizzoli SO, Rhee J, Brose N, Imig C, Cooper BH (2020)
414   Ultrastructural Correlates of Presynaptic Functional Heterogeneity in Hippocampal Synapses.
415   Cell Rep 30:3632-3643 e3638.
416 Murk JL, Posthuma G, Koster AJ, Geuze HJ, Verkleij AJ, Kleijmeer MJ, Humbel BM (2003) Influence of
417   aldehyde fixation on the morphology of endosomes and lysosomes: quantitative analysis and
418   electron tomography. J Microsc 212:81-90.
419 Nicholson C, Hrabetova S (2017) Brain Extracellular Space: The Final Frontier of Neuroscience. Biophys J
420   113:2133-2142.
421 Pereira PM, Albrecht D, Culley S, Jacobs C, Marsh M, Mercer J, Henriques R (2019) Fix Your Membrane
422   Receptor Imaging: Actin Cytoskeleton and CD4 Membrane Organization Disruption by Chemical
423   Fixation. Front Immunol 10:675.
424 Pfeiffer T, Poll S, Bancelin S, Angibaud J, Inavalli VK, Keppler K, Mittag M, Fuhrmann M, Nagerl UV (2018)
425   Chronic 2P-STED imaging reveals high turnover of dendritic spines in the hippocampus in vivo.
426   Elife 7.
427 Ryter A (1988) Contribution of new cryomethods to a better knowledge of bacterial anatomy. Ann Inst
428   Pasteur Microbiol 139:33-44.

429   Schnell U, Dijk F, Sjollema KA, Giepmans BN (2012) Immunolabeling artifacts and the need for live-cell
430   imaging. Nat Methods 9:152-158.
431   Tamada H, Blanc J, Korogod N, Petersen CC, Knott GW (2020) Ultrastructural comparison of dendritic
432   spine morphology preserved with cryo and chemical fixation. Elife 9.
433   Tanaka KA, Suzuki KG, Shirai YM, Shibutani ST, Miyahara MS, Tsuboi H, Yahara M, Yoshimura A, Mayor S,
434   Fujiwara TK, Kusumi A (2010) Membrane molecules mobile even after chemical fixation. Nat
435   Methods 7:865-866.
436   Tao-Cheng JH, Gallant PE, Brightman MW, Dosemeci A, Reese TS (2007) Structural changes at synapses
437   after delayed perfusion fixation in different regions of the mouse brain. J Comp Neurol 501:731-
438   740.
439   Thorne RG, Nicholson C (2006) In vivo diffusion analysis with quantum dots and dextrans predicts the
440   width of brain extracellular space. Proc Natl Acad Sci U S A 103:5567-5572.
441   Tonnesen J, Inavalli V, Nagerl UV (2018) Super-Resolution Imaging of the Extracellular Space in Living
442   Brain Tissue. Cell 172:1108-1121 e1115.
443   Whelan DR, Bell TD (2015) Image artifacts in single molecule localization microscopy: why optimization
444   of sample preparation protocols matters. Sci Rep 5:7924.
445   Wyffels JT (2001) Principles and Techniques of Electron Microscopy: Biological Applications, Fourth
446   Edition, by M. A. Hayat. Microsc Microanal 7:66.
447   Zhao S, Liao H, Ao M, Wu L, Zhang X, Chen Y (2014) Fixation-induced cell blebbing on spread cells
448   inversely correlates with phosphatidylinositol 4,5-bisphosphate level in the plasma membrane.
449   FEBS Open Bio 4:190-199.
450

451 **Figure legends**

452 **Figure 1. Brief PFA incubation does not affect ECS volume fraction.**

453 **(A)** Graphical overview of the workflow of experiments and analysis. **(B)** Time-lapse

454 shadow imaging of ECS in live and buffered PFA conditions. The live condition is

455 represented both with a raw and inverted LUT. **(C)** ECS volume fraction α does not

456 change during 30 minutes incubation with PFA. The images were analyzed either as a

457 whole ('global') or divided into 'neuropil' or 'cell bodies' areas (n = 6). **(D)** Representative

458 images of ECS live and after 30 minutes of incubation with PFA. Blue squares indicate

459 the magnified area that is shown below with an inverted LUT. **(E)** Paired analysis of

460 ECS volume fraction live and after 30 minutes of incubation with PFA (n = 5; ns: not

461 significant; p < 0.05; in Wilcoxon matched-pairs test). Scale bars: 10 μm.

Figure 2. Prolonged PFA incubation introduces artifacts into ECS structure.

(A) Representative images of ECS live and after 90 minutes of incubation with PFA. The inset and white arrow indicate 'cell blebbing'. **(B) Left:** Paired analysis of ECS volume fraction between live and 90 minutes after PFA fixation. The images were analyzed either as a whole ('global') or divided into 'neuropil' or 'cell bodies' areas (n_{cntrl} = 6; n_{PFA} = 6; ns: not significant; p< 0.05; in Wilcoxon matched-pairs test). **Right:** Comparison of the 'cell blebbing' between live and 90 minutes PFA conditions (n = 6; ns: not significant; p< 0.05; Mann-Whitney test). **(C)** Representative STED images of ECS live and after 90 minutes of PFA fixation. **(D)** SUSHI-based analysis of ECS widths. The blue lines indicate an example of the analyzed width. The line profiles are shown together with measured FWHMs. **(E)** Paired analysis of ECS widths live and 90 minutes after PFA fixation (n_{cntrl} = 12; n_{PFA} = 16; ns: not significant; p< 0.05; paired t-test). **(F)** Representative images of ECS live and 180 minutes after PFA fixation. Inset and white arrows indicate examples of dye accumulation around cell bodies. **(G)** A representative confocal shadow image after fixation overnight with PFA. Scale bars (A, F, G): 10 µm; (C, D): 5 µm.

Figure 3. PFA fixation has no detectable effects on astrocytic fine structure. (A) Representative confocal images of a brain slice expressing GFAP-Clover in astrocytes, live and 90 min after PFA fixation. White arrows indicate a representative astrocytic main branch and cell body. **(B)** Paired analysis of astrocytic areas of main branches and cell bodies live and 90 minutes after PFA fixation ($n_{branches}$ = 12; n_{bodies} = 11; ns: not significant; p< 0.05; paired t-test). **(C)** Representative STED images of astrocytic

485   structures in spongiform domain expressing GFAP-Clover, live and 90 min after PFA

486   fixation. The blue lines show representative line profiles of astrocytic structures to

487   determine their width. The line profiles are shown on the right together with calculated

488   FWHMs. **(D)** Paired analysis of widths of astrocytic fine processes, live and 90 minutes

489   after PFA fixation (n_{cntrl} = 26; n_{PFA} = 28; ns: not significant; $p < 0.05$; paired t-test). Scale

490   bars: 10 µm.

491

492   **Figure 4. PFA fixation affects spine morphology.**

493   **(A)** Representative STED maximum intensity z-projections of a dendritic segment

494   cytosolically filled with Citrine, live and 90 minutes after PFA fixation. White arrow

495   indicates a 'dendritic vacuole'. **(B)** Bar graph showing appearance of 'dendritic vacuoles'

496   after 90 minutes under live or fixed conditions (expressed as appearances over total

497   number of experiments: control: 2 out of 23; PFA: 15 out of 31; Mann-Whitney test). **(C)**

498   Representative STED images of dendritic spines and an example of head and neck

499   analysis using SpineJ. **(D)** Paired analysis of spine head area and neck length (n_{cntrl} =

500   79; n_{PFA} = 86; ns: not significant; $p < 0.05$; Wilcoxon matched-pairs test). **(E)** An example

501   of a dendritic spine with a wider neck after 90 minutes of PFA fixation. **(F)** Paired

502   analysis of spine neck width (smallest and median values) between live and 90 min

503   PFA-fixed conditions (n_{cntrl} = 79; n_{PFA} = 86; ns: not significant; $*p < 0.05$; $***p < 0.001$;

504   Wilcoxon matched-pairs test). Scale bars (A): 10 µm; (C, E): 500 nm.

505

506 **Table. PFA fixation-induced effects on brain tissue microstructure.** Summary of

507 observed changes in various tissue structures (ECS, astrocytes and dendrites) for

508 different fixation times. VF: volume fraction; NE: not examined.

Figure 1

Figure 2

A Live AAV-GFAP-Clover | Fixed 90 min
main branch
cell body

B Main branches | Cell bodies
ns
ns

C Live/STED | Fixed 90 min/STED | Line profiles
FWHM
134 nm
142 nm

D Astrocytic widths
ns
ns
0 min 90 min | 0 min 90 min
live | PFA

Figure 3

A Live/STED · Sindbis-Citrine · Fixed 90 min/STED · 'dendritic vacuole'

B 'Dendritic vacuoles' · Fraction of observation · ** · 90 min live · 90 min PFA

C Live/STED · Raw · SpineJ · 0.461 µm² · Ø 205 nm · Fixed 90 min/STED · Raw · SpineJ · 0.39 µm² · Ø 240 nm

D Spine head area · Head area [µm²] · ns · * · 0 min 90 min live · 0 min 90 min PFA · Spine neck length · Neck length [nm] · ns · ns · 0 min 90 min live · 0 min 90 min PFA

E Live/STED · 158 nm · Fixed 90 min/STED · 234 nm

F Spine neck width (smallest) · Neck width [nm] · ns · ns · 0 min 90 min live · 0 min 90 min PFA · Spine neck width (median) · Neck width [nm] · ns · **** · 0 min 90 min live · 0 min 90 min PFA

Figure 4

Time of fixation / Structure	30 minutes	90 minutes	> 90 minutes
Extracellular space	No change in VF	No change in VF No change in width	NE (dye uptake)
Global tissue	No visible changes	**Cell blebbing**	**Membrane permeabilization**
Astrocytes	NE	No changes in areas of cell bodies and main branches No changes in widths of fine processes	NE
Dendrites **Dendritic spines**	NE	**'Dendritic vacuoles'** No changes in spine neck length **Decreased spine head area** **Increased spine neck width**	NE