Revealing intact neuronal circuitry in centimeter-sized formalin-fixed paraffin-embedded brain

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Abstract

Tissue clearing and labeling techniques have revolutionized brain-wide imaging and analysis, yet their application to clinical formalin-fixed paraffin-embedded (FFPE) blocks remains challenging. We introduce HIF-Clear, a novel method for efficiently clearing and labeling centimeter-thick FFPE specimens using elevated temperature and concentrated detergents. HIF-Clear with multi-round immunolabeling reveals neuron circuitry regulating multiple neurotransmitter systems in a whole FFPE mouse brain, and is able to be used as the evaluation of disease treatment efficiency. HIF-Clear also supports expansion microscopy and can be performed on a non-sectioned 15-year-old FFPE specimen, as well as a 3-month formalin-fixed mouse brain. Thus, HIF-Clear represents a feasible approach for researching archived FFPE specimens for future neuroscientific and 3D neuropathological analyses.

1. Introduction

Clinical brain tissues play a critical role in unraveling the molecular mechanisms underlying neurological diseases and facilitating the development of effective treatments,[1–3] and also represent an invaluable resource for retrospective studies on rare diseases or conditions with long-term outcomes.[4] Currently, the majority of archived clinical specimens are stored in the form of formalin-fixed paraffin-embedded (FFPE) blocks. Advanced molecular analysis tools—such as chromatin immunoprecipitation (ChIP), DNA/RNA sequencing (DNA/RNA-seq), and Luminex assays—have been performed on FFPE specimens to enable comprehensive analysis from genomic, transcriptional, and proteomic perspectives.[3,5–7] However, image-based qualification and quantification of FFPE samples remains limited to performing chemical staining and immunohistochemistry on thin sections. Although whole-slide imaging enables unbiased analyses of entire tissue sections,[3,8–10] its two-dimensional (2D) nature cannot provide the 3D information required for accurate quantification. Such quantification in neurobiological contexts is crucial for evaluating neuronal damage and neuroprotection, including neuronal cell counts and the extent of necrosis. Moreover, such 2D-based techniques also fail to reveal structural changes within target tissues.

Tissue clearing protocols,[11–14] in combination with deep-tissue immunolabeling [15–17] and optical sectioning microscopy techniques,[18–20] represent powerful tools for collecting 3D information at a subcellular resolution from centimeter-scale specimens. Tissue clearing techniques render biological tissues transparent by removing components that induce refraction index mismatching, which is mainly attributable to two factors, i.e., light absorption due to pigments or biological chromophores and light scattering mainly caused by lipids.[11,21] In current tissue clearing protocols, samples are fixed with 4% paraformaldehyde (PFA) for approximately 24 hours before undergoing delipidation steps that involve solvents,[22–24] aqueous solutions with denaturing reagents or detergents,[25–28] or protection with chemicals that create crosslinks to withstand harsh electrophoretic delipidation.[29–31] Such protocols now enable imaging of whole-rodent bodies.[32,33] and even entire

26 human organs at the cellular or subcellular level,[34] facilitating detailed and comprehensive analyses
27 of intact biological systems. However, FFPE specimens have not reaped the benefits of these advanced
28 techniques. For instance, although tissue clearing approaches such as CUBIC, iDISCO, CLARITY,
29 and passive SDS treatment have been applied previously to FFPE specimens,[35–40] they have only
30 yielded immunolabeling depths of 1-2 mm, underscoring the need for a procedure tailored to FFPE
31 samples in order to achieve centimeter-scale tissue clearing and labeling.

32 Our objective was to develop a protocol capable of rendering centimeter-scale FFPE specimens
33 optically transparent and suitable for deep immunolabeling. To this end, we took account of two critical
34 issues. First, successful immunohistochemistry of FFPE tissue sections requires antigen retrieval to
35 restore target conformation and expose masked epitopes.[41,42] Secondly, despite xylene treatment
36 during paraffin processing and dewaxing protocols removing a substantial proportion of FFPE tissue
37 lipids, certain phospholipids persist within the lipid bilayer, impeding antibody penetration and
38 maintaining opacity.[38,43] Therefore, we aimed to devise a procedure that achieves efficient antigen
39 retrieval and delipidation for centimeter-scale FFPE specimens. Here, we present Heat-Induced FFPE
40 based tissue Clearing (HIF-Clear), a pipeline that employs an optimized heat-induced antigen retrieval
41 technique to achieve that goal. We validated the effectiveness of HIF-Clear using FFPE human brain
42 tissues and entire mouse brains. Using HIF-Clear, we demonstrate the possibility of conducting
43 multiple rounds of immunolabeling, which allowed characterization of the same brain tissue using six
44 different neuronal markers and revealing neuronal circuitry. We further demonstrate volumetric
45 quantification by means of HIF-Clear on intact FFPE mouse brains, with statistical analysis of the
46 FFPE samples resulting in identical trends to the results determined for fresh brains. We also showcase
47 the broad applications of HIF-Clear by successfully conducting volumetric imaging of mouse brain
48 archived in FFPE blocks for a period of 15 years, as well as the compatibility of this apporach with
49 expansion microscopy.

50

2. Results

2.1. Optimized antigen retrieval: Delipidation and epitope recovery of FFPE specimens

SDS is a detergent commonly used for lipid removal in tissue clearing methods. It is also known as an antigen retrieval agent and has been used to enhance immunolabeling signals in cells,[44] cryosections,[45] formalin-fixed human brains,[46] and epithelia of the pancreas, liver and lung.[47] Therefore, we began optimizing tissue clearing by applying two types of SDS-based approaches, passive and active, to FFPE mouse brain hemispheres and then evaluated their impacts on antigen retrieval/delipidation by means of electrophoretic immunolabeling.[15] For the passive approach, we applied a FLASH protocol published previously involving sample incubation in an SDS-based solution (4% SDS and 200 mM borate) at 54 °C for antigen retrieval and tissue clearing of epithelial cells and abdominal organs.[47] For the active approach, we employed SDS-based electrophoresis at 42 °C for 48 hours. We observed that both FLASH-processed and SDS-electrophoresed samples showed weak tyrosine hydroxylase (TH, a marker of dopaminergic neurons) signal (Figure S1a). Additionally, we noticed that the FLASH-processed samples had almost no signal of NeuN (a marker of neuronal nuclei, Figure S1b left), and exhibited strong non-specific background noise (Figure S1a left). The presence of this background noise is considered an indicator of inadequate antigen retrieval.[48]

We found that FLASH-processed samples displayed weak tyrosine hydroxylase (TH, a marker of dopaminergic neurons) signal and almost completely lacked signal of NeuN (a marker of neuronal nuclei) (Figure S1a left, 1b left). We also observed that the SDS-electrophoresed sample only possessed weak TH-positive signal, as well as strong non-specific labeling (Figure S1a right), with this latter considered an indicator of inadequate antigen retrieval.[48]

These results imply insufficient antigen retrieval using the SDS approaches, likely due to the low-temperature experimental conditions. In 1991, Shi et al. published a heat-induced epitope retrieval (HIER) method utilizing a temperature of 100 °C,[49] which now represents the most broad-spectrum

75 antigen retrieval protocol for FFPE tissue sections. Superheating (i.e., temperatures >100 °C) in
76 pressure cookers has been reported to enhance immunohistochemistry signal intensity and
77 stability.[50,51] However, heating with a classical antigen-retrieval citrate buffer (10 mM sodium citrate,
78 pH 6.0) at 121 °C for 10 minutes in a pressure cooker, a condition appropriate for routine FFPE sections,
79 only resulted in weak immunostaining signals of NeuN and SMI312 (a pan-neurofilament marker) in
80 the middle of a 2-mm-thick FFPE mouse brain block (Figure S1b middle). Since dewaxed and
81 rehydrated FFPE specimens still have residual phospholipids that prevent antibody penetration,[38,43]
82 we added 1% SDS into the citrate buffer to increase the delipidation capability of the HIER approach
83 and found that NeuN and SMI312 signals were significantly enhanced (Figure S1b right). However,
84 deploying SDS at such high temperatures also resulted in tissue expansion and fragility (Figure S1b,
85 gross view), prompting us to search for a detergent condition that could achieve sufficient delipidation
86 while retaining tissue integrity.

87 Accordingly, we tested four additional detergent conditions: 1% Tween 20; 1% Triton X-100; 1%
88 sodium cholate (NaC); and 1% CHAPS. Tween 20 and Triton X-100 are commonly used to disrupt
89 cell membranes in immunohistochemistry. NaC and CHAPS have higher critical micelle
90 concentrations and form smaller micelles than SDS, and both have been reported as being more
91 efficient than SDS with respect to active electrophoretic delipidation[52] and passive delipidation,[34]
92 respectively. All tests were performed on 2-mm-thick FFPE mouse brain blocks with citrate buffer (10
93 mM sodium citrate, pH 6.0) at 121 °C for 10 minutes in a pressure cooker. First, we assessed amounts
94 of residual lipids by staining with DiD (a lipophilic dye with an excitation wavelength at 649 nm).
95 Undelipidated and SDS-delipidated (hereafter referred to as PFA-SDS) mouse brain blocks, as well as
96 dewaxed FFPE mouse brain blocks without HIER, were also stained as controls. To prove that DiD
97 signal quantification could be used to reflect amounts of residual lipids in tissue sections, we compared
98 DiD signal across the FFPE group without HIER, the undelipidated group and the PFA-SDS group
99 (**Figure 1A**, left panel). The FFPE group without HIER exhibited a higher DiD signal intensity than

100 the PFA-SDS group but lower than that of the undelipidated group, supporting partial lipid removal
101 during the paraffin processing and dewaxing process. Among all of the test groups subjected to varied
102 heated detergent conditions, only the 1% Tween 20 condition presented significantly lower lipid
103 content compared to the FFPE without HIER group (p-value = 0.0035), achieving the same level of
104 delipidation as the PFA-SDS group (p-value = 0.999) (Figure 1A, right panel). We also performed Oil
105 red O staining to visualize the residual lipids and tissue morphology under bright-field microscopy
106 (Figure S2).

107 Next, we evaluated the efficiency of antigen retrieval by conducting immunolabeling with NeuN
108 and SMI312 antibodies on all of the test groups and examined the signals at the center of each FFPE
109 specimen. We found that the 1% Tween 20 group displayed the sharpest and clearest neuronal nuclei
110 and axon bundles with a high signal-to-background ratio, indicating both successful delipidation and
111 antigen retrieval (Figure 1B, panel 2). Interestingly, for the 1% Triton X-100, 1% NaC and 1% CHAPS
112 groups, the fluorescence intensity of neuronal nuclei were even lower than the background signal,
113 implying that heating these detergents might hinder nuclear epitopes (Figure 1B, panels 3, 4 and 5).
114 We also examined the gross appearance of each group upon heating, which confirmed that the 1%
115 Tween 20 group retained good sample integrity without apparent changes in size or color (Figure 1C).
116 Therefore, we employed 1% Tween 20 in citrate buffer with heating in a pressure cooker at 121 °C for
117 10 minutes as the critical step of our HIF-Clear pipeline for delipidation and antigen retrieval of
118 centimeter-scale FFPE specimens.

119 **2.2. The HIF-Clear pipeline**

120 We illustrate the complete HIF-Clear pipeline in Figure 1D. To retrieve a specimen from a FFPE
121 block, the paraffin is first melted at 65-70 °C, before dewaxing at room temperature using xylene and
122 sequentially rehydrating the tissue with alcohol. It is important to note that when working on a large
123 sample, the dewaxing time should be increased to avoid opacity caused by residual wax due to
124 insufficient dewaxing. Opacity can be eliminated through repeated dehydration and dewaxing steps

125 (Figure S3b), with extended dewaxing times or repeated dewaxing steps not affecting tissue structure
126 or antigenicity (Figure S3 and S4). A typical dewaxing timeframe for paraffin sections of less than 20
127 μm thickness is ~20 minutes, with complete dewaxing of an entire mouse brain or similarly-sized
128 sample achievable within 24 hours (see Materials and Methods). Optimal dewaxing times for tissue
129 sizes between these two extremes warrant further testing.

130 The rehydrated FFPE samples are then treated with epoxy[31] and immersed in the optimized
131 antigen retrieval solution (10 mM sodium citrate, 1% Tween 20, pH 6.0) at 37 °C for 24 hours, before
132 then being heated to 121 °C for 10 minutes. The samples are then subjected to active immunolabeling
133 (see Materials and Methods). After staining, the specimen is immersed in 4% PFA for 24 hours to fix
134 the antibodies bound to epitopes, matched for the refractive index (RI), and imaged by means of
135 lightsheet microscopy. Figure S5 shows the gross views of the same mouse brain after undergoing 4%
136 PFA fixation, paraffin processing, optimized antigen retrieval, and RI-matching, demonstrating
137 intactness of the brain shape and preservation of tissue integrity. To enable 3D phenotyping and
138 proteomic investigations, a multi-round staining protocol involving photobleaching and classic heat-
139 induced antigen retrieval can also be included. In this study, unless otherwise specified, all HIF-Clear-
140 processed whole mouse brains had been stored in paraffin blocks for two to six months before the
141 experiments and underwent active labeling after heating with 1% Tween 20.

142 To further verify HIF-Clear's efficacy in removing lipids from whole mouse brains, we employed
143 coherent anti-Stokes Raman scattering (CARS), a spectroscopic technique for detecting vibrational
144 responses of specific molecular structures (Figure 1E). In contrast to a dewaxed FFPE mouse brain
145 (Figure 1D, left) for which we observed CARS signals from axon bundles, thus indicating the presence
146 of myelinated lipids, both the FFPE-HIF-Clear-processed and PFA-SDS-treated mouse brain samples
147 displayed significantly lower CARS signals (Figure 1E, middle and right, respectively; quantification
148 in Figure 1F), confirming removal of myelinated lipids.

149 **2.3. The HIF-Clear pipeline enables immunolabeling and registration of intact FFPE mouse**
150 **brain**

151 Previous studies have confirmed that transcriptomic and proteomic data are equally preserved in
152 FFPE sections and fresh-frozen sections. To assess biomarker preservation within a spatial context, we
153 investigated patterns of anti-TH antibody labeling in FFPE-HIF-Clear-processed mouse brains (**Figure**
154 **2**A). We observed consistent distributions of dopaminergic neurons in key tissue regions, including
155 the striatum, nigrostriatal fiber tracts, substantia nigra, ventral tegmental area, pontine reticular nucleus,
156 and lateral reticular nucleus, aligning with the findings of a prior study[16] (Figure 2A, left) and
157 matching results from Allen Brain Atlas registration[53] (Figure 2A, right). Furthermore, we compared
158 the volume of each brain region between the left and right hemispheres, which revealed minimal
159 sample distortion (Figure 2B). Our investigation extended to a 2-mm-thick mouse brain that had been
160 preserved in an FFPE block for 15 years. By applying HIF-Clear, we unveiled clear clusters of
161 dopaminergic neurons at a cellular resolution in that specimen (Figure 2C). These results confirm the
162 feasibility of deploying HIF-Clear for long-term stored FFPE blocks, even without the need for
163 sectioning, enabling effective retrieval of information on spatial biomarkers comparable to that
164 obtained from freshly fixed samples. We also applied HIF-Clear to 3-month fixed mouse brain
165 hemispheres (**Figure 3**). Although the long-term fixed specimens exhibited reduced TH intensity and
166 S/N ratio, the major dopaminergic regions were labeled, and magnified images revealed clear details
167 of cell bodies and neuronal fibers. These results suggest that HIF-Clear has the potential to be applied
168 to long-term fixed specimens.

169 **2.4. A tailored HIF-Clear+ pipeline enables multi-round immunolabeling of human brain tissues**
170 **and whole mouse brains**

171 Next, we investigated if HIF-Clear-processed whole-brain FFPE samples could be subjected to a
172 multi-round staining process, a strategy commonly used for multiplexed immunolabeling. Chemical
173 elution techniques utilizing detergents, reducing reagents, and denaturing reagents have been

174 developed previously and tested on thin paraffin sections or cryosections. For thick sections, the
175 CLARITY protocol published in 2013 involves incubation in 4% SDS solution, which was first
176 demonstrated on a 1-mm-thick mouse hippocampus specimen. However, based on our preliminary
177 experiments, we found that post-staining fixation is required to maintain signals in a centimeter-sized
178 sample (i.e., a whole mouse brain) (Figure S6) and the post-fixed antibodies cannot be completely
179 stripped using SDS-based stripping protocols (Figure S7). We also observed that fixation after staining
180 hindered subsequent rounds of staining, although this impediment could be overcome by traditional
181 heat-induced epitope retrieval (referred to hereafter as classical HIER to avoid confusion with the
182 aforementioned optimized antigen retrieval protocol) (Figure S8). To overcome the difficulty of
183 antibody stripping due to post-fixation, which is required for centimeter-sized specimens, we
184 developed HIF-Clear+ to include a multi-round immunolabeling protocol utilizing photobleaching and
185 classical HIER (Figure 1D). After imaging, we photobleached transparent RI-matched samples using
186 a 100W LED white light to quench the previously labeled fluorophores (Figure S9). The selected
187 duration of bleaching is dependent on sample thickness; overnight bleaching is sufficient for tissue
188 samples of <500 μm thickness, whereas ~72 hours are required for thicker samples such as whole
189 mouse brains (a thickness of 0.8-1 cm). Bleached specimens are then subjected to classical HIER to
190 restore the antigenicity hindered by post-staining PFA fixation. The brain shape and structural integrity
191 remained after 4 rounds of immunolabeling, and there is no cross-reactivity in subsequent rounds of
192 immunostaining following bleaching (Figure S10).

193 To explore the potential of revealing multiple neuronal circuitry in the whole FFPE mouse brain
194 using HIF-Clear+, we performed a six-round immunolabeling procedure for TH, tryptophan
195 hydroxylase 2 (TPH2), calbindin, parvalbumin (PV), choline acetyltransferase (ChAT) and SMI312,
196 respectively, on one intact FFPE mouse brain (**Figure 4**). For each round, the brain was labeled not
197 only with the antibody specific for the target protein, but also for lectin as a structural reference. Image
198 datasets from each immunolabeling round were combined into a single multichannel image (**Figure**

199 **5**A), with lectin-labeled blood vessels deployed as a reference channel using Elastix,[54] demonstrating
200 the ability of HIF-Clear+ to reveal the spatial distribution of different neuronal markers. We conducted
201 a more detailed examination of the habenula-interpeduncular circuitry (Hbn-IPN circuitry) and its
202 connection with these biomarkers. We visualized the expression of ChAT, calbindin, TH, and TPH2
203 in the IPN and the neighboring regions (Figure 5B). Previous studies have shown that these neurons
204 are regulated by the Hbn-IPN circuitry through their connections with the IPN region, which plays a
205 role in controlling emotion, addiction, and the reward process.[55,56] To demonstrate that HIF-Clear+
206 can also be applied to human brain FFPE blocks, we performed three consecutive rounds of
207 immunolabeling on a 1-mm-thick FFPE human brain specimen collected surgically from the temporal
208 lobe of a brain tumor patient. For each round, the brain tissue was labeled with GFAP/lectin,
209 SMI312/lectin, or MAP2/lectin, respectively (**Figure 6**A). Labeling homogeneity throughout the entire
210 block was confirmed by imaging at depths of 100, 500 and 900 μm (Figure 6B, S11)

211 **2.5. Application of FFPE-HIF-Clear to mouse disease models**

212 In disease research, volumetric imaging offers precise microenvironmental information and
213 reveals structural changes that are essential for evaluating injuries and therapeutic effects.[13,35,57] As a
214 first assessment of HIF-Clear applicability to disease models, we subjected an intact FFPE brain from
215 an astrocytoma mouse model (see Materials and Methods) to the HIF-Clear pipeline to label tumor
216 cells (ASTS1CI-GFP positive astrocytes) and GFAP-positive astrocytes (**Figure 7**A, C). Accordingly,
217 we could segment GFAP-positive astrocytes surrounding the tumor (Figure 7B, D, and E) and classify
218 them according to their distances from the tumor cells. Statistical analysis (Figure 7F) revealed that
219 nearly half of the GFAP-positive astrocytes were within the tumor, with 63.9% being located near the
220 tumor surface (±200 μm). These results demonstrate that FFPE-HIF-Clear-derived image datasets can
221 reveal cell distributions and the spatial architecture of disease microenvironments.

222 Next, we employed FFPE mouse brains from a traumatic brain injury (TBI) model to showcase
223 the feasibility of employing HIF-Clear for 3D quantification of neuronal circuit recovery in disease

research. Reduced dopamine levels are known to be a major cause of cognitive impairment after TBI.[58] To assess the impact on dopaminergic neural regeneration across four treatment groups (sham, sMN, aNORO@sMN, and aNORO@sMN+AMF; see Materials and Methods for details), we conducted immunolabeling using anti-TH antibodies (Figure 7G) and compared quantitative changes in the dopaminergic system among these groups. We calculated quantitative ipsilateral-to-contralateral changes for three structural features: striatum volume (Figure 7H), nigrostriatal fiber tract volume (Figure 7I), and the number of dopaminergic cells in the substantia nigra (SN) (Figure 7J). The sham group displayed a decrease (~24%) in the ratio of dopaminergic neurons on the injured side to the uninjured control side (Figure 7K), indicative of dopaminergic system malfunction or imbalance. Treatment with aNORO@sMN rescued this disease phenotype (from 76% to 94%) (Figure 7K), a positive impact on dopamine production and transmission recovery post-TBI.

Next, we evaluated the therapeutic effect of aNORO@sMN on angiogenesis. To quantify angiogenesis, we selected three regions of interest around brain injury sites and segmented blood vessels (Figure 7L). The aNORO@sMN treatment group exhibited a substantial increase in blood vessel length, surface area, volume, and branch numbers (244%, 301%, 186%, and 220%, respectively) compared to the sham group (Figure 7M), indicating enhanced angiogenesis. Thus, overall, HIF-Clear facilitates the 3D quantification critical for assessing neuronal damage and recovery in FFPE specimens.

We also performed identical TBI procedures and 3D quantification using the SHIELD protocol and SDS-based electrophoresis for brain clearing and labeling (Figure S12), which resulted in comparable quantitative outcomes compared to those determined for FFPE-HIF-Clear brains (Figure S13). These results indicate that FFPE-HIF-Clear recovers biomarker signals as effectively as for freshly processed samples, even after the samples have been subjected to prolonged storage as FFPE blocks.

2.6. HIF-Clear enables expansion microscopy on thick FFPE samples

249 Expansion microscopy is a super-resolution microscopy technique that overcomes the limitations
250 of optical diffraction by enlarging biological structures. It is achieved by synthesizing a polymer
251 network within the specimen to anchor protein molecules, causing the specimen to physically expand
252 and enabling analysis of sub-cellular structures.[59,60] It is generally difficult to uniformly expand
253 heavily formaldehyde-fixed specimens, such as FFPE tissues, due to the formaldehyde occupying the
254 binding sites of anchoring reagents. ExPath, a modified version of protein-extension expansion
255 microscopy (ProExM) in which the EDTA concentration in the proteinase K digestion buffer is
256 increased, was developed to overcome this issue.[61] However, although ExPath has been successfully
257 deployed to expand 5-μm-thick FFPE sections, it cannot uniformly expand samples beyond 1 mm even
258 with a prolonged incubation time (**Figure 8**A). We discovered that tissues embedded in paraffin and
259 subjected to the optimized antigen retrieval step in the HIF-Clear pipeline can be directly expanded
260 using the original ProExM method without extended incubation, yielding clear staining signal for
261 abundant targets such as SMI312 or MAP2 (Figure 8B and C).

262

263 **3. Discussion**

264 In this study, we propose HIF-Clear, which utilizes a heat-induced antigen retrieval protocol optimized
265 with 1% Tween 20, to eliminate residual lipids and recover masked antigens from FFPE specimens.
266 Our method enables labeling agents to penetrate to a depth of centimeters within such specimens,
267 enabling large-scale neuronal circuitry analyses of archived brain tissues and providing comprehensive
268 information that previously could not typically be obtained from conventional 3-5 μm FFPE sections.
269 We have demonstrated herein that FFPE samples subjected to the HIF-Clear pipeline can be repeatedly
270 stained without compromising their integrity. Moreover, in evaluating therapeutic efficacy for a TBI
271 mouse model, we found that HIF-Clear-treated FFPE mouse brains provided as much statistical
272 information as shortly PFA-fixed mouse brains.

273 **3.1. HIF-Clear facilitates retrospective 3D analyses of neuronal diseases**

274 FFPE processing and sectioning are standard procedures in pathology departments, and FFPE sections
275 are routinely subjected to targeted chemical staining or immunohistochemistry for morphological
276 observations, biomarker validations, or clinical diagnoses.[62,63] However, it is challenging to obtain
277 brain-wide information from conventional 3-5 μm-thick FFPE sections for studies of
278 neurodegenerative diseases.[64] Moreover, biomarker quantifications and structural observations of 2D
279 sections can be biased by sectioning positions and orientations. By applying HIF-Clear/HIF-Clear+,
280 3D histological information can be collected from FFPE specimens, thereby providing more accurate
281 biomarker quantifications, brain-wide neuronal circuitry and cell analyses. Moreover, HIF-Clear/HIF-
282 Clear+ may be performed on archived patient samples hosted in neuronal disease brain banks to
283 establish a 3D biomarker atlas for neurological diseases, maximizing the utility of archived specimens.
284 Such an atlas would represent an invaluable resource for retrospective research on biomarker
285 distributions throughout the entire neural network, enabling tracking of their changes and interactions.
286 The resulting comprehensive imaging datasets could also be deployed to train deep learning models,
287 explore potential therapeutic targets, and to predict early diagnostic markers.

288 **3.2. FFPE-HIF-Clear as an archival and delipidation strategy for clearing centimeter-sized tissue**
289 **samples**

290 The development of tissue clearing has revolutionized neuroscientific research. However, current
291 tissue-clearing protocols impose a 24-hour fixation limit on mouse brains, requiring immediate tissue
292 clearing and labeling upon animal sacrifice. HIF-Clear offers a solution to this predicament by enabling
293 long-term preservation of mouse brains in FFPE blocks. Accordingly, clinical samples can be
294 conveniently transported to imaging facilities and staining decisions can be postponed until essential
295 analytical results, such as RNA sequencing, become available. Additionally, HIF-Clear minimizes
296 excessive use of experimental mice in time-course studies by allowing brain samples in FFPE blocks
297 after sacrifice, adhering to 3Rs principles. Consequently, researchers can decide on whole-brain

298 staining approaches upon completing experiments, such as for toxicological or pharmacological targets,
299 thereby avoiding the need to repeat animal experiments.

300 Another possible application of FFPE-HIF-Clear is to use it as a tissue clearing method for removing
301 lipids. Since paraffin embedding, dewaxing, and heat-induced antigen retrieval are commonly used
302 pathological procedures, FFPE-HIF-Clear can be quickly, easily, and efficiently executed in the
303 pathology department of hospitals or research institutions, reinforcing its potential utility in clinical
304 research.

305 **3.3. Microscopy options for imaging centimeter-sized specimens**

306 Optical sectioning techniques are crucial for obtaining high-quality volumetric images. Techniques
307 such as confocal microscopes, multi-photon microscopy, and light-sheet microscopy filter out-of-focus
308 signals, resulting in sharp images of individual planes. In our study, we used light-sheet microscopy
309 and multi-point confocal (i.e., spinning disc) for imaging centimeter-sized specimens because of their
310 scanning speeds. While two-photon and confocal microscopy offer high-resolution imaging of smaller
311 volumes, they are not ideal for scanning entire tissues because of their prolonged scanning times.

312 Non-optical sectioning wide-field fluorescence microscopes, like the Olympus BX series or ZEISS
313 Axio imager series, can also be used to scan samples up to about 3.5mm thick with long working
314 distance objective lenses. In these cases, deconvolution algorithms are required to eliminate out-of-
315 focus signals. However, it should be noted that the epifluorescence system might reduce fluorescent
316 intensity in deeper regions within the samples.

317 **3.4. Heating source and buffer choices for heat-induced antigen retrieval in HIF-Clear procedure**

318 Early efforts to clear and label archived FFPE specimens have only achieved limited labeling depths,
319 [35–40] likely due to a lack of effective antigen retrieval. Protein denaturants employed in hydrophilic
320 tissue clearing protocols, such as SDS (in CLARITY, PACT, FLASH) and urea (in CUBIC and Scale),
321 enhance signal intensity and specificity for some types of specimens, such as cryosections or well-

322 controlled formalin-fixed but non-paraffin-embedded tissues. However, their utility for FFPE tissues
323 is less apparent, with temperature being considered a primary factor in their applicability in such
324 instances.

325 Heat-induced antigen retrieval has been widely used since first being reported by Shi et al. in 1991,
326 with many protocol variations since being developed, including the use of different heat sources (e.g.,
327 water baths, steamers, microwave ovens, autoclaves, and pressure cookers) and buffer choices (low
328 molarity buffers of acid or alkaline pH).[65] Nevertheless, a unifying principle is to heat the fixed tissue
329 sections in buffer solutions at or above 100 °C for up to 30 minutes without boiling.[51,65] In our study,
330 we used a pressure cooker as the heat source to achieve a temperature of ~121 °C, thereby ensuring
331 temperature stability and time efficiency. Any heating condition that is effective for FFPE sections
332 should also work for centimeter-scale FFPE specimens (e.g., using water baths at 95-99 °C), but their
333 effects on delipidation require further testing.

334 The choice of antigen retrieval solutions depends on the antibody being deployed. For example, EDTA
335 solutions are favorable for antibodies against phosphorylated tyrosine.[65,66] Generally, alkaline pH
336 solutions exhibit higher antigen retrieval efficiency, but they are also more tissue-destructive and result
337 in higher background signal compared to lower pH solutions.[41,65] In our study, we tested the addition
338 of 1% Tween 20 to two common antigen retrieval solutions, citrate buffer (pH 6.0) and Tris-EDTA
339 (pH 9.0), both of which yielded satisfactory staining results (Figure S14), demonstrating that HIF-
340 Clear facilitates the choice between acidic or alkaline antigen retrieval solutions based on antibody
341 preference without compromising the delipidation effect.

342 **3.5. Staining strategies and Epoxy/hydrogel pre-treatments in HIF-Clear**

343 In Figure 1B, we propose two staining strategies for samples with thicknesses less than 500 um and
344 greater than 1 mm: passive immunolabeling and active immunolabeling. In passive immunolabeling,
345 antibodies penetrate and reach their targets solely through diffusion, without any additional force. It

346 takes approximately two months to passively stain a whole mouse brain.[26,28] Compared to passive
347 immunolabeling, active immunolabeling uses an external force, such as pressure, electrophoresis, etc.,
348 to facilitate antibody penetration and therefore significantly speed up the staining process, reducing
349 the required staining time for a whole mouse brain to one day. However, the harsh conditions, such as
350 pressure and heat, caused by external forces might damage specimens. To protect specimens from the
351 harsh conditions caused by active staining, specimens could be strengthened by treatment with epoxy
352 or acrylamide monomer to form a tissue-epoxy or tissue-hydrogel hybrid.[29,31] Laboratories that do
353 not have adequate devices or handle small specimens could use passive immunolabeling instead and
354 skip the step of epoxy or hydrogel pretreatment.

355 **3.6. Limitations**

356 We acknowledge that there are some limitations to HIF-Clear application, which we wish to address.
357 Firstly, the FFPE tissues we tested in this study have been limited to mouse and human brain tissues
358 fixed under well-controlled conditions. Differences in tissue fixation procedures at different hospitals
359 and tissue banks may affect the clarity and immunostaining efficiency of dewaxed FFPE tissues. In
360 the demonstration of HIF-Clear to 3-month fixed specimens, we observed that pontine reticular nucleus
361 (Figure 3A, yellow arrowheads) lose TH-positive signals after long-term fixation. The fluorescence
362 intensity was more affected by fixation with formalin, which is methanol-stabilized and stronger, than
363 with PFA. The results indicate that a stronger antigen retrieval method may be a possible solution.
364 However, achieving the right balance between antigen retrieval efficiency and tissue integrity will
365 require additional testing and investigation. Secondly, HIF-Clear has not yet been extensively tested
366 on tissues other than the brain. Thus, its applicability and effectiveness for other tissues remain to be
367 established. Third, the size of the samples used in this study is mostly in the range of 2-3 cm^3. Larger
368 samples may require a prolonged incubation time and/or increased Tween 20 concentration. Fourth,
369 HIF-Clear is not compatible with endogenous fluorescence due to a reduction in fluorescence intensity
370 caused by xylene and alcohol used in paraffin processing. Researchers who need to directly observe

371 genetically encoded fluorescent proteins can utilize tissue-clearing methods such as 3DISCO, X-
372 CLARITY, CUBIC, etc., which have been shown to minimize the decrease in fluorescence intensity.
373 On the other hand, if researchers need to visualize transgenic fluorescent proteins along with other
374 biomarkers, they can use HIF-Clear for delipidation and boost-immunolabeling to visualize the
375 transgenic fluorescent proteins. Fifth, HIF-Clear+ may not be suitable for cases necessitating iterative
376 staining with the same antibody. In addition, the applicability of HIF-Clear+ to antibodies not tested
377 in the study requires further testing and validation. Finally, whether HIF-Clear-treated tissues retain
378 sufficient molecular features and structural integrity for omics analysis warrants verification. Further
379 research and testing will help to address these limitations and improve the applicability and
380 effectiveness of the HIF-Clear pipeline we present herein.

381

382 **4. Conclusions**

383 In conclusion, as a method enabling multi-round immunolabeling of centimeter-scale archived FFPE
384 specimens, HIF-Clear/HIF-Clear+ presents new possibilities for researching neural disease and could
385 serve as a powerful tool for neuronal circuitry analyses and pathology. Its integration with conventional
386 pathological procedures also renders it readily applicable in clinical settings. Through further
387 validation across various organs and testing of its compatibility with spatial omics techniques, HIF-
388 Clear could potentially catalyze broader and more comprehensive investigations in both the
389 fundamental and clinical research domains.

390 **Materials and Methods**

391 *Experimental animals and human samples*

392 We used 8-week-old CD-1 male mice to develop the HIF-Clear pipeline. All animal procedures and
393 handling complied with guidelines from the Institutional Animal Care and Use Committee of
394 Academia Sinica (IACUC protocol No.: 12-05-370), Taiwan. Information on the astrocytoma and TBI
395 mouse models is provided in the respective subsections below. The analyses involving human
396 participants were reviewed and approved by the Institutional Review Board of National Taiwan
397 University Hospital (IRB No.: 202203079RINC), and all participants provided signed informed
398 consent.

399 *Astrocytoma animal model*

400 All animal procedures and handling complied with guidelines from the Institutional Animal Care and
401 Use Committee of National Tsing Hua University (IACUC protocol No.: 109067), Taiwan. ALTS1C1-
402 GFP cells (1×10^5 cells) were implanted into the brains of 8-week-old C57BL/6 mice according to a
403 previously published procedure.[67] In brief, 1×10^5 ALTS1C1-GFP cells were injected into the
404 C57BL/6 mice under anesthesia at a depth of 2.5 mm and 2 mm lateral to the midline and 1 mm
405 posterior to the bregma. After injection, bone wax (ETHICON, W810, Somerville, NJ, USA) was
406 applied to seal the drilled hole. Eighteen days after tumor implantation, mice were sacrificed after
407 cardiac perfusion.

408 *TBI mouse model*

409 All animal procedures and handling complied with guidelines from the Institutional Animal Care and
410 Use Committee of National Tsing Hua University (IACUC protocol Number: 110081). We divided 7-
411 week-old C57BL/6 female mice into four groups: (1) untreated (sham), (2) treated with a paracetamol-
412 coated silk-based microneedle (sMN), (3) treated with alternating magnetic field-responsive NO-
413 release octahedrons (aNORO@sMN), and (4) treated with aNORO@sMN and an alternating magnetic
414 field (aNORO@sMN+AMF). To perform traumatic brain injury (TBI), we used an electric drill to

415 create a hole in the skull at the left motor cortex (M1 & M2) and used a 2-mm-diameter punch to cause
416 a 1.5-mm-deep injury. For all treatments except sham, the microneedle was implanted one day post-
417 injury to avoid excessive swelling in the injured area. For the aNORO@sMN and
418 aNORO@sMN+AMF groups, the NO-release octahedrons (aNORO) were embedded in a silk
419 microneedle (sMN). For the AMF-treated group, a magnetic field at a power of 3.2 kW and a frequency
420 of 1 MHz was applied for 5 min per day until the mice were sacrificed. Additional experimental details
421 are as described in Chan et al..[68] Mice were sacrificed 45 days after TBI induction.

422 *Sample collection and preparation*

423 For mouse brain samples, the mice were euthanized by injecting them with 0.2 mL of a 30% Urethane
424 saline solution. The euthanized mice were perfused with 20 mL of cold phosphate-buffered saline
425 (PBS) and 20 mL of 4% PFA. The collected brains were further fixed using 4% PFA at 4 °C for 24
426 hours. Human brain tissue specimens were surgically collected from the temporal lobe of patients and
427 fixed with 4% PFA at 4 °C for 24 hours. For paraffin embedding, fixed specimens were dehydrated
428 and processed in a tissue processor (Tissue-Tek VIP 5 Jr., Sakura Finetek Japan Co. Ltd., Japan) under
429 vacuum conditions and then embedded in paraffin wax.

430 *SHIELD processing, SDS-electrophoretic delipidation and FLASH delipidation*

431 PFA-fixed specimens were incubated in SHIELD-OFF solution at 4 °C for 96 hours, followed by
432 incubation for 24 hours in SHIELD-ON solution at 37 °C. All reagents were prepared using SHIELD
433 kits (LifeCanvas Technologies, Seoul, South Korea) according to the manufacturer's instructions. For
434 SDS-electrophoretic delipidation, SHIELD-processed specimens were placed in a stochastic electro-
435 transport machine (SmartClear Pro II, LifeCanvas Technologies, Seoul, South Korea) running at a
436 constant current of 1.2 A for 5-7 days. For FLASH delipidation, the SHIELD-processed specimens
437 were placed in FLASH reagent (4% w/v SDS, 200 mM borate) and then incubated at 54 °C for 18
438 hours.[47] The delipidated specimens were washed with PBST at room temperature for at least 1 day.

439 *Evaluating the extent of delipidation*

440 Serial 20-μm-thick cryosections were cut from mouse brain slices (2-mm thick) of various treatment
441 conditions for subsequent DiD or Oil red O staining. For DiD staining, cryosections (that were of
442 approximately 0-40 μm depth) were post-fixed with 4% PFA at room temperature for 5 minutes. The
443 sections were then rinsed with distilled water and immersed in PBST (PBS with 0.1% Triton X-100)
444 for 10 minutes to remove OCT. These sections were stained with 0.0025 uM DiD/PBST solution at
445 room temperature for 1 hour, before rinsing them with distilled water and washing with 1% SDS for
446 several seconds to remove excess DiD. The slides were mounted in 50% glycerol and imaged under a
447 Zeiss 780 confocal microscope using a 10× 0.75NA Plan-Apochromat objective (Zeiss). For Oil red
448 O staining, the sections (that were of approximately 40-80 μm depth) were post-fixed with a 4%
449 formal-calcium solution for 5 minutes and then rinsed with distilled water. The fixed sections were
450 pre-incubated in 60% isopropanol for 5 minutes and then stained with fresh Oil red O working solution
451 (0.3% Oil red O in 60% isopropanol) for 15 minutes. The sections were briefly washed with 60%
452 isopropanol and mounted in glycerine jelly. Images were acquired using a 3DHISTECH Pannoramic
453 250 slide scanner with a 40× 0.95NA Plan-Apochromat objective (Zeiss). Details of statistical analyses
454 are described in the *Quantification and Statistics* section below.

455 *Comparison of brain region volume*

456 Autofluorescence-channel imaging datasets of whole mouse brains were aligned to the BrainGlobe
457 Atlas API[53] using the aMAP tool.[69] The volume of each brain subregion was then obtained and
458 organized based on anatomical hierarchy using the Allen Brain Atlas. The 679 subregions were merged
459 into 78 brain regions at the same anatomical hierarchy as the hippocampus. The brain region volume
460 of right and left hemisphere of the FFPE-HIF-Clear mouse brain was compared and plotted using
461 GraphPad Prism 9.5.1 (GraphPad Software Inc., San Diego, CA, USA).

462 *HIF-Clear pipeline*

463 The HIF-Clear pipeline is illustrated in Figure 1C and described in detail below.

464 *Deparaffination and rehydration*. FFPE blocks were incubated at 65-70 °C to melt the paraffin.
465 Residual paraffin was removed through immersion in xylene for 24 hours, with the xylene being
466 changed at least twice. Dewaxed brains were rehydrated with 100%, 100%, 95%, 85%, 75%, and 55%
467 alcohol diluted with distilled water. The rehydrated brains were then washed with PBS.

468 *Epoxy processing*. Specimens were processed with commercialized SHIELD kit as described above.

469 *Optimized antigen retrieval*.

470 Specimens were incubated overnight at 37 °C in a modified citrate buffer (10 mM sodium citrate, 1%
471 Tween 20, pH 6.0). The specimens and buffer were then heated for 10 minutes at 121 °C in a pressure
472 cooker (Bio SB, Santa Barbara, CA, USA) and kept in the pressure cooker until the temperature
473 dropped below 100 °C (for approximately 15 minutes). Next, the specimens were removed from the
474 cooker and cooled for 10 minutes at room temperature. The specimens were then washed three times
475 in PBST, with each wash lasting 1 hour.

476 *Electrophoretic immunolabeling (active staining)*. The procedure was modified from the previously
477 published eFLASH protocol[15] and was conducted in a SmartLabel System (LifeCanvas Technologies,
478 Seoul, South Korea). The specimens were preincubated overnight at room temperature in sample
479 buffer (240 mM Tris, 160 mM CAPS, 20% w/v D-sorbitol, 0.9% w/v sodium deoxycholate). Each
480 preincubated specimen was placed in a sample cup (provided by the manufacturer with the SmartLabel
481 System) containing primary, corresponding secondary antibodies and lectin diluted in 8 mL of sample
482 buffer. Information on antibodies, lectin and their optimized quantities is detailed in Supplementary
483 Table 1. The specimens in the sample cup and 500 mL of labeling buffer (240 mM Tris, 160 mM
484 CAPS, 20% w/v D-sorbitol, 0.2% w/v sodium deoxycholate) were loaded into the SmartLabel System.
485 The device was operated at a constant voltage of 90 V with a current limit of 400 mA. After 18 hours
486 of electrophoresis, 300 mL of booster solution (20% w/v D-sorbitol, 60 mM boric acid) was added,
487 and electrophoresis continued for 4 hours. During the labeling, the temperature inside the device was
488 kept at 25 °C. Labeled specimens were washed twice (3 hours per wash) with PTwH (1× PBS with

489 0.2% w/v Tween-20 and 10 μg/mL heparin),[23] and then post-fixed with 4% PFA at room temperature
490 for 1 day. Post-fixed specimens were washed twice (3 hours per wash) with PBST to remove any
491 residual PFA.

492 *Refractive index (RI) matching*. Before imaging, the specimens were RI-matched by being immersed
493 in NFC1 (RI = 1.47) and NFC2 (RI = 1.52) solutions (Nebulum, Taipei, Taiwan). Each immersion
494 lasted for one day at room temperature. Alternatively, RI-matching can also be accomplished by
495 immersing specimens in a 1:1 dilution of CUBIC-R[28] for one day, followed by pure CUBIC-R for an
496 additional day.

497 *Volumetric imaging and 3D visualization*. For centimeter-scale specimens, images were acquired using
498 a light-sheet microscope (SmartSPIM, LifeCanvas Technologies, Seoul, South Korea) with a 3.6x
499 customized immersion objective (NA = 0.2, working distance = 1.2 cm). For samples <3 mm thick,
500 imaging was performed using a multipoint confocal microscope (Andor Dragonfly 200, Oxford
501 Instruments, UK) with objectives that were UMPLFLN10XW (10x, NA = 0.3, working distance = 3.5
502 mm), UMPLFLN20XW (20x, NA = 0.5, working distance = 3.5 mm), UMPLFLN40XW (40x, NA =
503 0.8, working distance = 3.3 mm). 3D visualization was performed using Imaris software (Imaris 9.5.0,
504 Bitplane, Belfast, UK).

505 *Photobleaching*. Immunolabeled and imaged specimens were placed in a multi-well plate and
506 immersed in RI-matching solutions so they retained transparency. The plate was then sealed with
507 paraffin. A 100-W projection lamp with an LED array was placed on the plate to quench fluorescence
508 signals. A representative photobleaching apparatus constructed using off-the-shelf components is
509 shown in Supplementary Figure 10a. In our experience, approximately 18 hours photobleaching is
510 sufficient for a 2-mm-thick sample, whereas 3 days is required for a whole mouse brain (approximately
511 8 mm to 1 cm in thickness).

512 *Raman microscopy*

513 A Yb:KGW laser (Carbide, Light Conversion) emitting a 190 fs, 200 kHz, 20 W pulse train at a 1030
514 nm wavelength was used to generate a supercontinuum via a double-pass multiple-plate continuum
515 module. The resulting spectrum (600-1300 nm) was spectrally sliced by tunable color filters (3G
516 LVLWP, LVSWP, and LVFBP, Delta) to provide pump (725 nm) and Stokes (914 nm) beams, which
517 could address the Raman shift (2850 cm-1) of lipids. The two beams were temporally and spatially
518 overlapped, and then guided into a commercial upright microscope (Axio Examiner.Z1, Zeiss) with a
519 scanning unit (LSM7MP, Zeiss) to achieve raster scanning on the x-y plane. A single 20x water
520 immersion objective (UMPLFLN 20XW, Olympus) was used to focus the combined laser beams on
521 the mouse sample. Epi-CARS signal at 601 nm was spectrally separated from the incident radiation
522 with a bandpass filter (BP 565-610, Zeiss) and detected using a photomultiplier tube.

523 *Multichannel image registration*

524 For six-round immunolabeling of a single whole mouse brain, each dataset included one structural
525 reference channel stained with lectin and one antibody-stained channel. The first round of staining
526 served as the standard brain, with lectin channel images of each subsequent dataset being registered to
527 the lectin channel of the standard brain using the Elastix toolbox.[54] The transformation parameters
528 obtained from rigid and B-spline deformable registration of the lectin channels were then applied to
529 the antibody-stained channels. The transformed images were merged and visualized using Imaris
530 software. In the case of three-round immunolabeling of human specimens, the image datasets from the
531 three rounds were registered and resampled using Amira software (Thermo Fisher Scientific, Waltham,
532 MA, USA), and then merged and visualized using Imaris software.

533 *Expansion microscopy and ExPath*

534 Expansion microscopy was performed using the proExM protocol.[58] A 1-mm-thick HIF-Clear-
535 processed FFPE mouse brain slice was incubated in anchoring solution (0.01% w/v methacrylic acid
536 N-hydroxysuccinimide ester in 1×PBS) at 4 °C for 24 hours. After anchoring, the sample was washed
537 twice (5 minutes each time) with PBS at room temperature and then rinsed twice (5 minutes each time)

538 in acrylamide monomer solution (2 M NaCl, 8.625% w/w sodium acrylate, 2.5% w/w acrylamide,
539 0.15% w/w N,N'-methylene-bisacrylamide in 1×PBS) at 4 °C. For gelation, the specimen was pre-
540 incubated in gelation solution (0.02% w/w ammonium persulfate (APS), 0.02% w/w tetramethyl-
541 ethylenediamine (TEMED), and 0.01% w/w 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (4-
542 hydroxy-TEMPO) in monomer solution) for 5 minutes at 4 °C, and then moved into fresh gelation
543 solution for an additional 25 minutes. The specimen in gelation solution was then transferred to a
544 humidified 37 °C incubator for 2 hours. The gel was then immersed in a digestion solution (8 units/ml
545 proteinase K, 50 mM Tris, 1 mM EDTA, 0.5% w/v Triton X-100, 1M NaCl, pH 8.0) at 37 °C for 4
546 hours. After digestion, the gel was immersed in an excess of deionized water for 0.5-2 hours to expand.
547 This step was repeated 3−5 times with fresh water until the size of the sample ceased to increase.
548 For the ExPath experiment, a 1-mm-thick FFPE mouse slice was dewaxed, rehydrated and underwent
549 the same anchoring and gelation steps as described above. The gel was digested in ExPath digestion
550 solution (8 units/ml proteinase K, 50 mM Tris, 25 mM EDTA, 0.5% w/v Triton X-100, 0.8M NaCl,
551 pH 8.0) at 60 °C for 3 hours.[59]
552 *Post-expansion immunolabeling*
553 Digested gels were washed twice (20 minutes each time) in PBS, and then incubated in a primary
554 antibody cocktail containing anti-SMI312 (Biolegend, 837904, 1:500 dilution), anti-MAP2 (Cell
555 Signaling Technology, #8707, 1:500 dilution), 2% v/v normal goat serum (Jackson ImmunoResearch,
556 005-000-121, 1:500 dilution), and 0.1% w/v Triton X-100 in 1×PBS at 4 °C for 24 hours. The gels
557 were then washed three times (20 minutes each time) in wash buffer (30 mM sodium chloride, 0.1%
558 w/v Triton X-100 in 1×PBS) and incubated in a secondary antibody cocktail containing Alexa Fluor®
559 647 AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch, 115-605-003, 1:500 dilution),
560 Rhodamine Red™-X (RRX) AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch, 111-295-003,
561 1:500 dilution), 0.2% v/v normal goat serum, and 0.1% w/v Triton X-100 in 1×PBS at 4 °C for 24
562 hours. The antibody-labeled gels were washed three times (20 minutes each time) in wash buffer and

563 then placed in deionized water for expansion. For nuclei staining, propidium iodine was added to
564 deionized water at a final concentration of 1 μg/ml during expansion.

565 *Quantification and statistics*

566 To quantify the extent of delipidation (Figure 1A), DiD-stained sections were imaged at an excitation
567 wavelength of 642 nm. To quantify the fluorescence intensity, 10 regions of interest (ROI) of 0.8 × 0.8
568 mm were selected randomly within the cortex of each specimen. The gray (pixel) value of each ROI
569 was measured using Fiji,[70] Mean values and standard deviations (SD) were plotted using GraphPad
570 Prism 9.5.1 (GraphPad Software Inc., San Diego, CA, USA). The significance of the difference in
571 mean values was determined by means of one-way ANOVA with Tukey's multiple comparison tests
572 at an α level = 0.05 (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

573 For the Raman signal quantification shown in Figure 1F, gray values of 18 Raman images (0.4 × 0.4
574 mm) at a different depth for each group were measured using Fiji. The mean values and SD were
575 determined, and the significance of differences were also calculated by means of one-way ANOVA
576 using GraphPad Prism as described above.

577 For the astrocyte-tumor distance analysis shown in Figure 7F, the astrocytes were segmented using the
578 SPOT module of Imaris based on a fluorecence intensity threshold and point spread function (PSF)
579 size definition. GFP-expressing tumors were segmented using the Surface module of Imaris. Distances
580 of all segments were calculated in Imaris.

581 To quantify dopaminergic regions in TBI mouse brains, the volume of the striatum and nigrostriatal
582 fiber tracts was calculated using the Surface module of Imaris, and cell number in the substantia nigra
583 was determined using the SPOT module. To demonstrate the similarity of quantification results from
584 FFPE-HIF-Clear and PFA-SDS brains, the quantification data for the injured side of each brain was
585 normalized according to the results for the corresponding contralateral side. Mean values and SD were
586 plotted using GraphPad Prism (N = 3 in Figure 7K and Supplementary Figure 9a). The significance of

587 the difference in mean values was determined by means of one-way ANOVA with Tukey's multiple
588 comparison tests at an α level = 0.05 (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).
589 To evaluate angiogenesis, we selected three ROIs (each of 0.6 × 0.6 × 0.6 mm) close to the injury site.
590 The Surface module of Imaris was used to segment the blood vessels and calculate their total volume
591 and surface area. Their length and numbers of bifurcation points were calculated using Vessap.[71]
592 Erosion and dilation were performed to remove false-negative pixels and to avoid false centerline
593 detections. Next, the centerlines were extracted by means of a 3D thinning algorithm.[72] The
594 bifurcation points were detected using the surrounding pixels of each point to define a point that splits
595 into two or more vessels. Mean values, SD, and the significance of differences were calculated by
596 independent *t* test using GraphPad Prism.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The raw data that support the findings of this study are available upon request.

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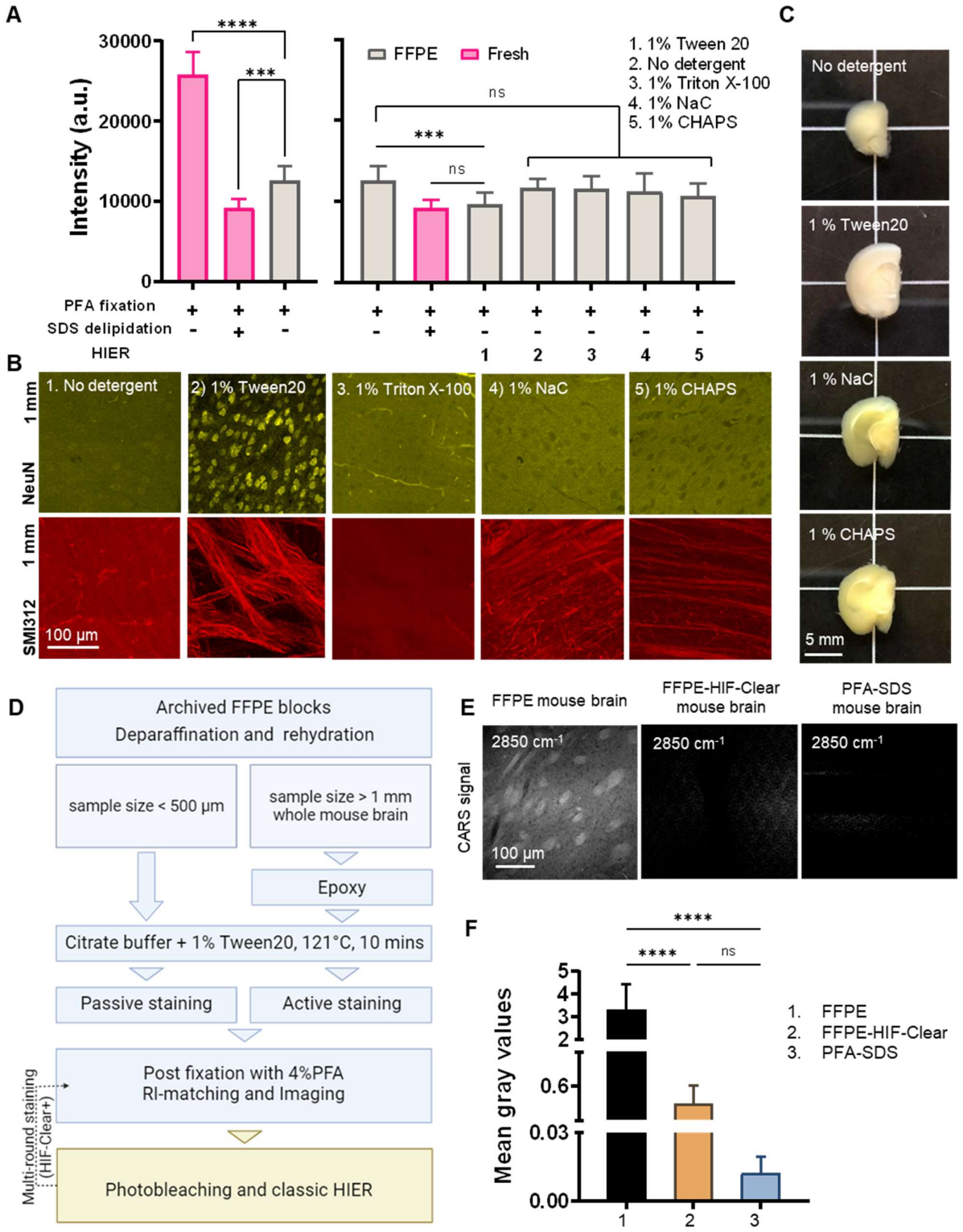
A
Intensity (a.u.)
30000
20000
10000
0
****
***
FFPE
Fresh
1. 1% Tween 20
2. No detergent
3. 1% Triton X-100
4. 1% NaC
5. 1% CHAPS
ns
***
ns
PFA fixation
SDS delipidation
HIER
1
2
3
4
5
B
1 mm
NeuN
1 mm
SMI312
1. No detergent
2) 1% Tween20
3. 1% Triton X-100
4) 1% NaC
5) 1% CHAPS
100 µm
C
No detergent
1 % Tween20
1 % NaC
1 % CHAPS
5 mm
D
Archived FFPE blocks
Deparaffination and rehydration
sample size < 500 µm
sample size > 1 mm
whole mouse brain
Epoxy
Citrate buffer + 1% Tween20, 121°C, 10 mins
Passive staining
Active staining
Post fixation with 4%PFA
RI-matching and Imaging
Photobleaching and classic HIER
Multi-round staining
(HIF-Clear+)
E
FFPE mouse brain
FFPE-HIF-Clear
mouse brain
PFA-SDS
mouse brain
CARS signal
2850 cm-1
2850 cm-1
2850 cm-1
100 µm
F
Mean gray values
5
4
3
2
0.6
0.03
0.00
****
****
ns
1
2
3
1. FFPE
2. FFPE-HIF-Clear
3. PFA-SDS

Figure 1. Development of the HIF-Clear pipeline.
(**A**) Comparison of DiD signal intensity in 2-mm-thick slices of undelipidated formaldehyde-fixed mouse brain, PFA-SDS mouse brain, and FFPE mouse brain treated under various detergent conditions.
(**B**) Multi-point confocal fluorescence images of neuronal nuclei in the cortex (top row) and axons in the striatum (bottom row) from 2-mm-thick FFPE mouse brain slices that had been treated under various detergent conditions. Images of optical sections captured at a depth of 1 mm are shown. Detergent types and concentrations are indicated. NeuN, a neuronal nuclear marker; SMI312, a pan-axonal marker.
(**C**) Gross views of 2-mm-thick slices of FFPE mouse brain treated under various detergent conditions.
(**D**) Schematic of the HIF-Clear pipeline.
(**E**) Images of coherent anti-Stokes Raman scattering (CARS) of dewaxed FFPE mouse brain, HIF-Clear-processed FFPE mouse brain, and PFA-SDS mouse brain. The images were taken at a Raman shift of 2850 cm^{-1} to address the CH2 vibration frequency.
(**F**) Statistical analysis of the gray values for the three treatment groups in (E). Gray values for 18 z-continuous images of each group were analyzed.
Statistical analysis: (A) n = 10; (E) n = 18. Mean ± SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA with Tukey's multiple comparison test.

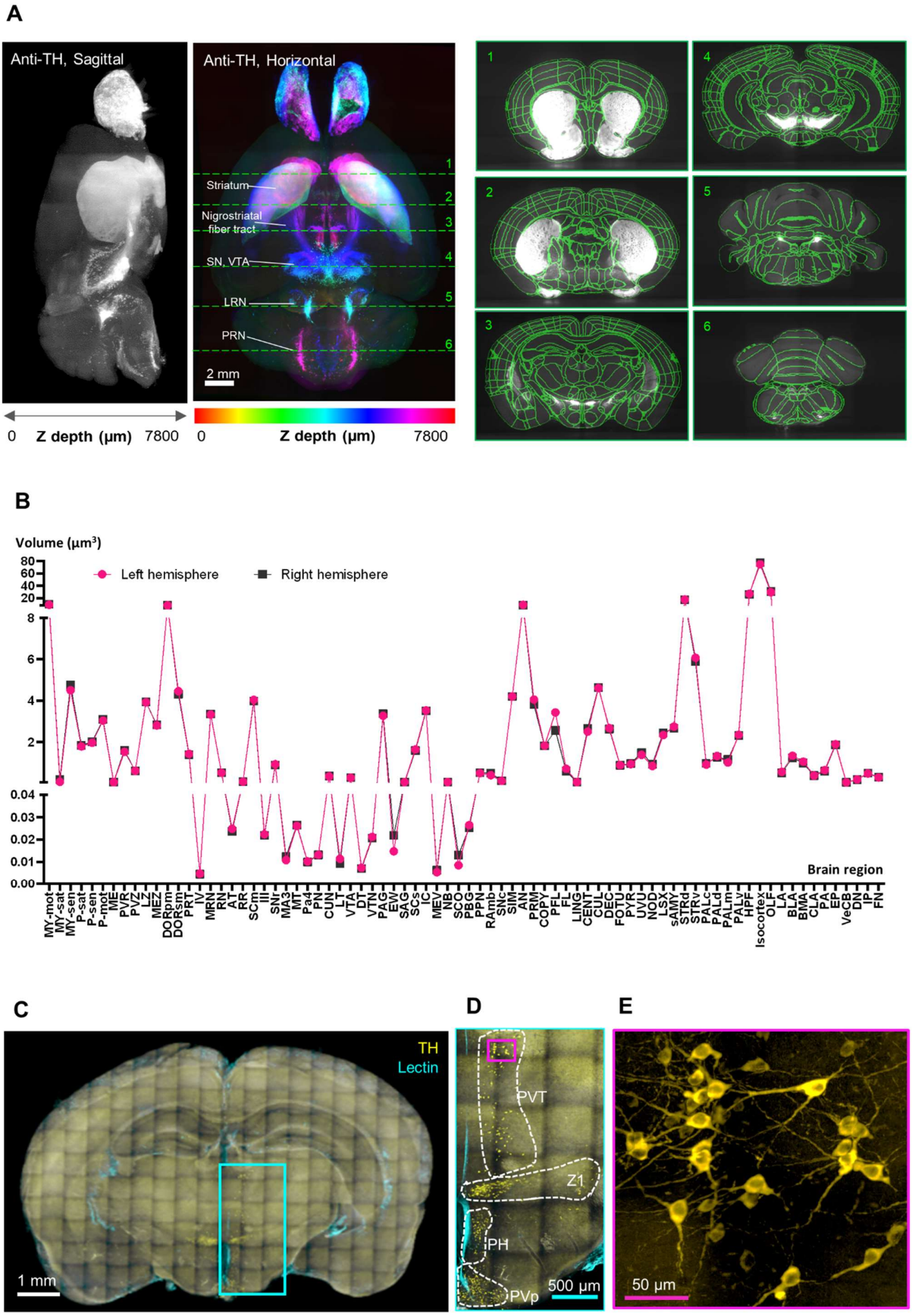
A
Anti-TH, Sagittal
Anti-TH, Horizontal
Striatum
Nigrostriatal fiber tract
SN, VTA
LRN
PRN
2 mm
0 Z depth (µm) 7800
0 Z depth (µm) 7800
B
Volume (µm³)
Left hemisphere
Right hemisphere
Brain region
MY-mot MY-sat MY-sen P-sat P-sen P-mot ME PVR PVZ LZ MEZ DORpm DORsm PRT IV MRN RN RT AR PR SCm III SNr MA3 MT Pa4 PN CUN LT VTA DT VTN PAG EW SAG SCs IC MEV NB SCO PBG PPN RAmb SNc SIM AN PRM COPY PFL FL LING CENT CUL DEC FOTU PYR UVU NOD LSX sAMY STRd STRv PALc PALd PALm PALv HPF Isocortex OLF LA BLA BMA CLA PA EP VeCB DN IP FN
C
TH
Lectin
1 mm
D
PVT
Z1
PH
PVp
500 µm
E
50 µm

Figure 2. Validation of the HIF-Clear pipeline.
(**A**) Brain-wide tyrosine hydroxylase (TH) expression (left panel) and registration (right panel) in FFPE-HIF-Clear C57BL/6 mouse brains. Whole mouse brain images were acquired using light-sheet microscopy, and the 3D-rendered images of the horizontal (XY) and sagittal (YZ) views are shown. Major dopaminergic regions are indicated: SN (substantia nigra), VTA (ventral tegmental area), LRN (lateral reticular nucleus), and PRN (pontine reticular nucleus). The results of brain registration for these regions (marked with green dotted lines and numbered in the XY view) are displayed from the coronal (XZ) view in the right panel.
(**B**) Comparison of brain region volumes between the left and right hemispheres of an FFPE-HIF-Clear mouse brain. All abbreviations are listed in Supplementary Table 2.
(**C** to **E**) Application of HIF-Clear to a 15-year-old FFPE block with TH-immunolabeling and multi-point confocal imaging. Specimen thickness: 2 mm.
(**C**) The projection image.
(**D**) Magnification of the cyan-lined region in (C). Dopaminergic regions are indicated. PVT, paraventricular nucleus of thalamus; PH, posterior hypothalamic nucleus; PVp, periventricular hypothalamic nucleus.
(**E**) Magnification of the magenta-lined region in (D). Scale bars are indicated in each panel.

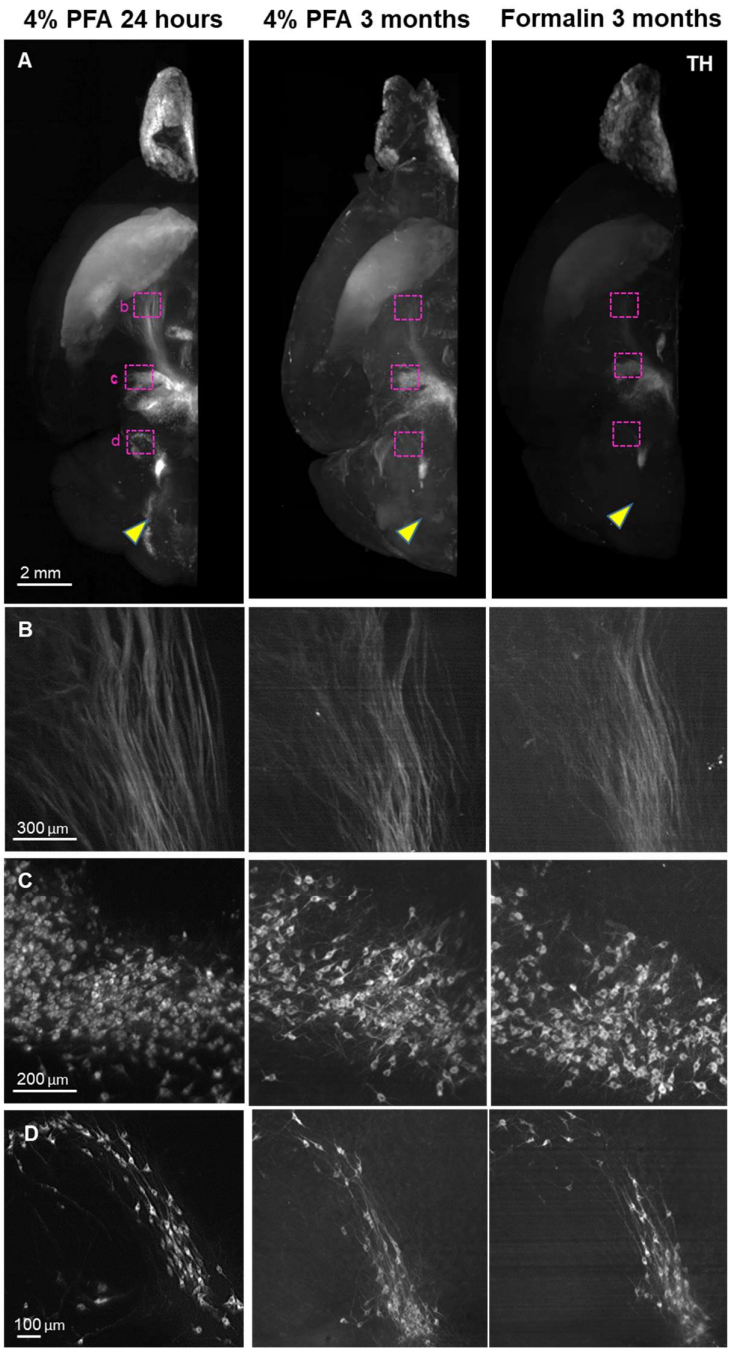

Figure 3. Application of HIF-Clear to long-term fixed mouse brain hemispheres.
Mouse brain hemispheres were fixed under various fixation conditions and then subjected to FFPE processing and HIF-Clear. The specimens were stained with TH antibodies and imaged with light-sheet microscopy.
(**A**) Projection images of the horizontal (XY) view. The magenta-lined regions indicating Nigrostriatal fiber tract, VTA, and LRN were magnified in (**B**), (**C**), and (**D**), respectively. The yellow arrowheads indicate PRN, which was clearly immunolabeled in the 4% PFA 24-hour-fixed specimen but not in the 3-month-fixed specimens.

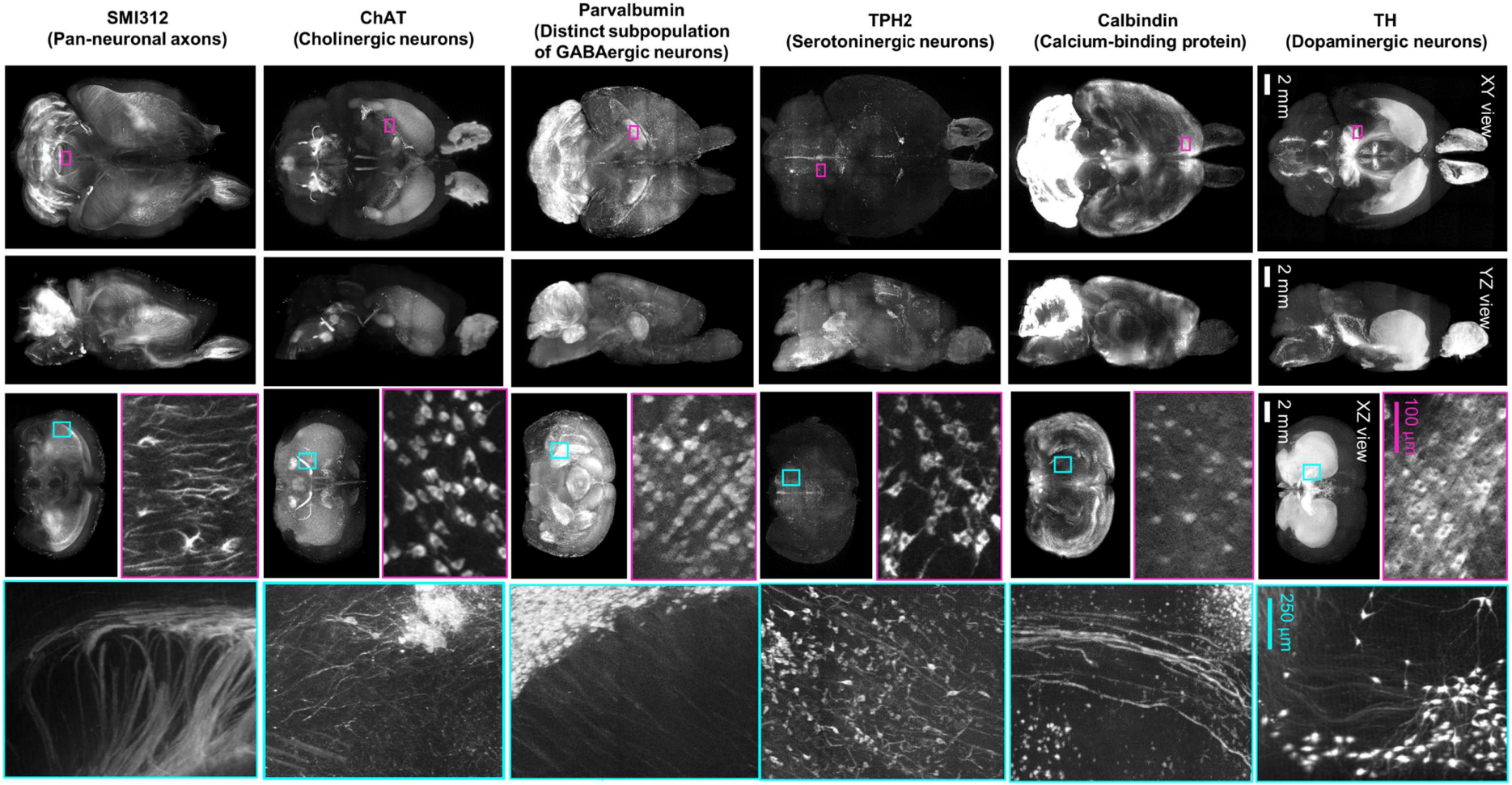
SMI312
(Pan-neuronal axons)
ChAT
(Cholinergic neurons)
Parvalbumin
(Distinct subpopulation
of GABAergic neurons)
TPH2
(Serotoninergic neurons)
Calbindin
(Calcium-binding protein)
TH
(Dopaminergic neurons)
XY view
YZ view
XZ view
2 mm
100 µm
250 µm

Figure 4. Multi-round immunostaining of FFPE-HIF-Clear whole mouse brain (HIF-Clear+). Light-sheet microscopy imaging datasets of six-round immunolabeling performed on one whole FFPE mouse brain. Projection images of the horizontal (XY), sagittal (YZ), and transverse (XZ) views are shown. Projection images (200-µm thick) of magenta-lined and cyan-lined regions marked in the XY and XZ views are magnified and displayed within correspondingly colored frames. Scale bars are indicated in the first row.

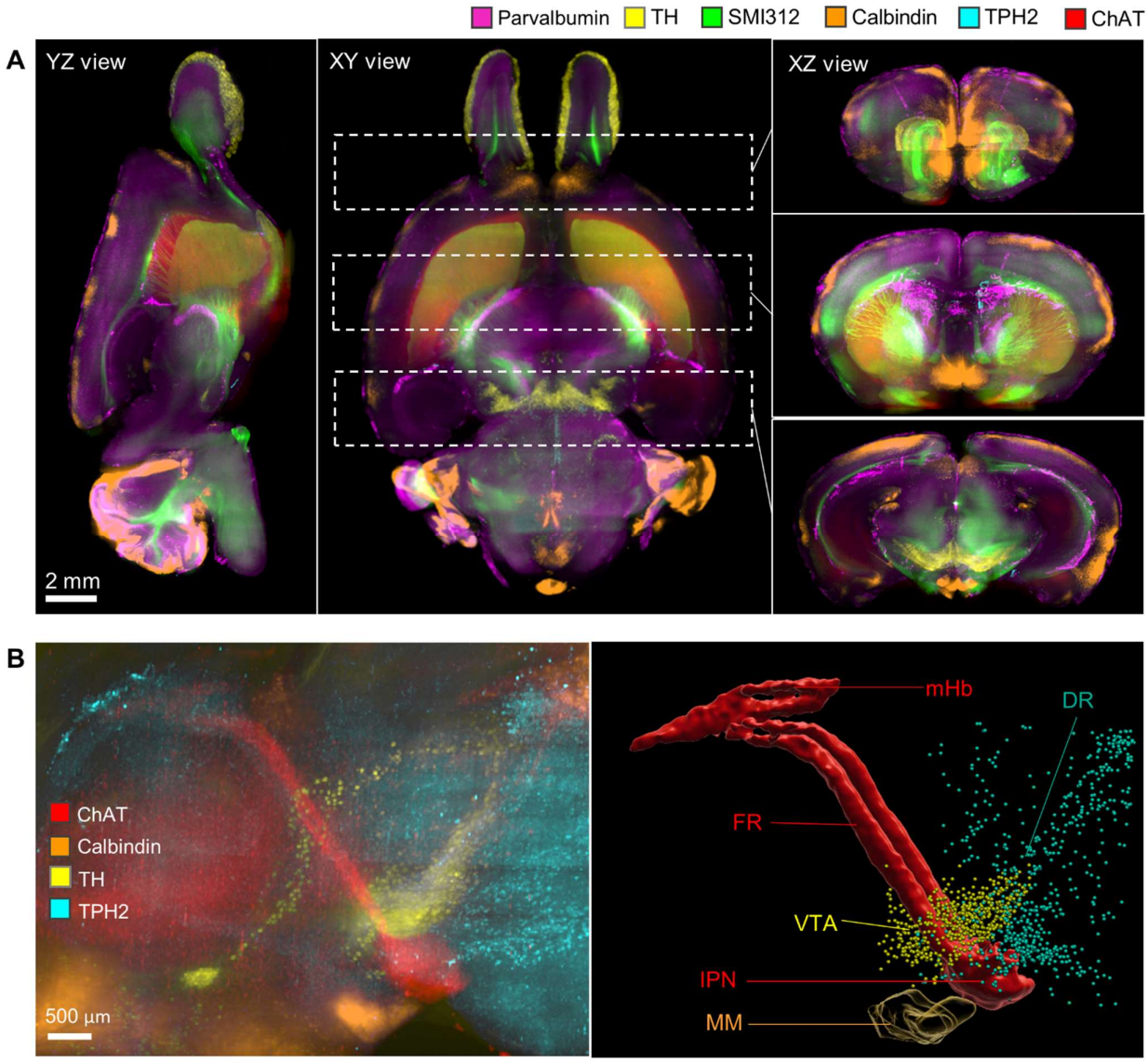

Figure 5. Merged multichannel neuron circuitry image generated by HIF-Clear+.
(**A**) Merged multichannel image generated using the datasets from Fig. 3. Optical sections from the horizontal (XY) and sagittal (YZ) perspectives are presented. For the transverse (XZ) views, projection images of the regions encompassed by the dotted lines in the XY view are displayed. (**B**) The spatial relationship of the Hbn-IPN circuitry, dopaminergic (TH), serotoninergic (TPH2), and calbindin+ GABAergic neurons. Fluorescence (right) and segmented (left) images are shown. mHb, medial habenula; FR, fasciculus retroflexus; IPN, interpeduncular nucleus; SuM, medial mammillary area of the hypothalamus; DR, dorsal raphe nuclei.

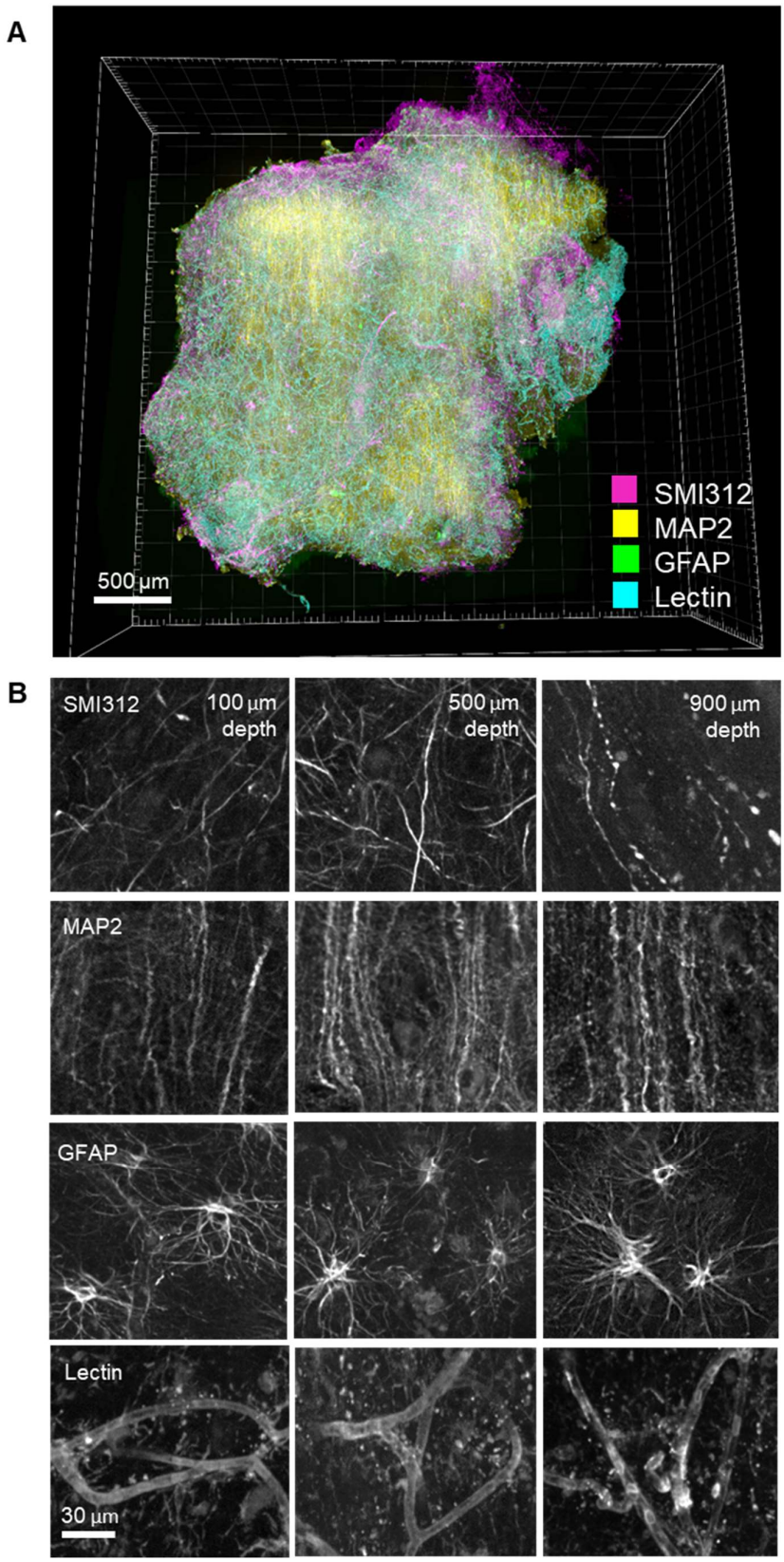
A
SMI312
MAP2
GFAP
Lectin
500 µm
B
SMI312
100 µm depth
500 µm depth
900 µm depth
MAP2
GFAP
Lectin
30 µm

Figure 6. Multi-round immunostaining of a FFPE-HIF-Clear human brain specimen.
Three-round immunolabeling was performed on a FFPE human brain specimen collected from a patient with cerebral hemorrhage. Images were acquired using multi-point confocal microscopy.
(**A**) 3D rendering of the entire specimen.
(**B**) Images depicting merged and single channels of an optical section from the selected area (white frame) in (A) at depths of 100 µm, 500 µm, and 900 µm. GFAP: Glial fibrillary acidic protein; SMI312: pan-axonal marker; MAP2: microtubule-associated protein 2 (a dendritic marker).

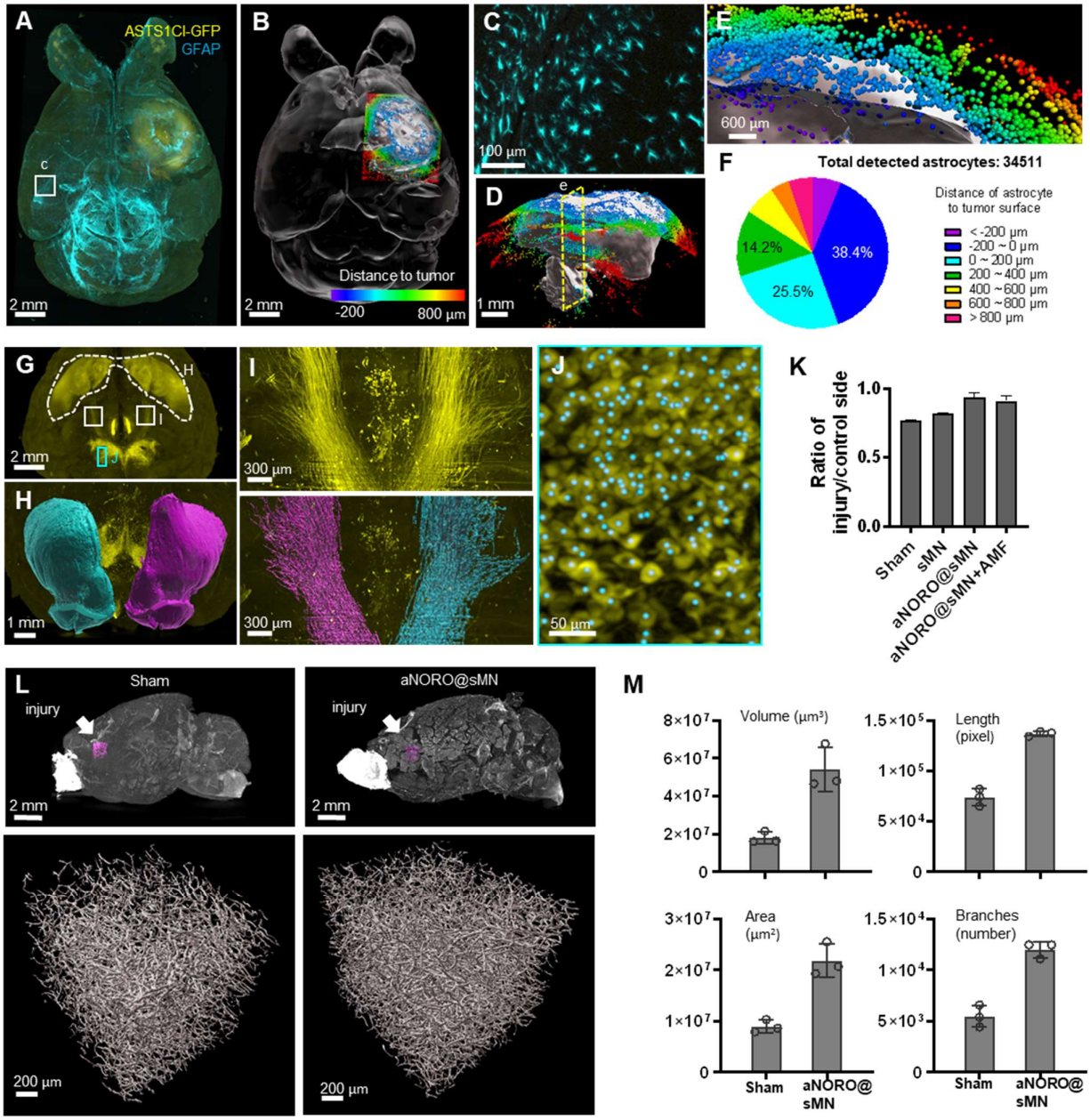
A
ASTS1CI-GFP
GFAP
c
2 mm
B
Distance to tumor
-200
800 μm
2 mm
C
100 μm
D
e
1 mm
E
600 μm
F
Total detected astrocytes: 34511
Distance of astrocyte to tumor surface
< -200 μm
-200 ~ 0 μm
0 ~ 200 μm
200 ~ 400 μm
400 ~ 600 μm
600 ~ 800 μm
> 800 μm
38.4%
25.5%
14.2%
G
H
I
J
2 mm
1 mm
300 μm
50 μm
K
Ratio of injury/control side
1.0
0.5
0.0
Sham
sMN
aNORO@sMN
aNORO@sMN+AMF
L
Sham
aNORO@sMN
injury
2 mm
200 μm
M
Volume (μm³)
Length (pixel)
Area (μm²)
Branches (number)
Sham
aNORO@sMN

Figure 7. Applications of HIF-Clear in disease models.
(**A** to **F**) HIF-Clear reveals cell-tumor relationships in an astrocytoma model.
(**A**) Projection light-sheet image of a FFPE-HIF-Clear mouse brain with a GFP-expressing astrocytoma xenograft (ALTS1C1 cells, see Materials and Methods) stained for GFP (yellow) and GFAP (cyan).
(**B**) Segmented tumor (white) and surrounding astrocytes (colored). The astrocytes are colored according to the color-coded scale of cell-to-tumor distance.
(**C**) Magnification of the region marked in (A), showing astrocyte morphology.
(**D**) Sagittal view of the segmented tumor and color-coded astrocytes.
(**E**) Magnification of the clipping plane marked in (D, yellow dashed line), showing astrocytes inside (dark blue, purple) and outside (red, orange, yellow, green, cyan, blue) the tumor.
(**F**) Quantification of detected astrocytes and their classification according to distance to the tumor surface.
(G to M) HIF-Clear reveals brain damage and the therapeutic effect of alternating magnetic field-responsive NO-release octahedrons (aNORO) administered using a paracetamol-coated silk-based microneedle (sMN) in a TBI mouse model.
(G to J) Segmentation of dopaminergic regions in FFPE-HIF-Clear TBI mouse brains.
(G) Whole-brain projection light-sheet image focusing on TH-positive regions.
(**H**) Frontal view of the segmented striatum (dashed white line in (G)). Magenta: the injured side; cyan: the contralateral side.
(**I**) Horizontal views of the nigrostriatal fiber tract (paired white boxes in (G)); fluorescence (top) and segmented (bottom) images are shown. Magenta: the injured side; cyan: the contralateral side.
(J) Detection of dopaminergic cells in the substantia nigra (SN). Magnification of the marked area in (G), including the TH fluorescence signal (yellow) and segmented cells (cyan dots).
(**K**) Comparison of the therapeutic effects of different treatments. $n = 3$ (three indicators: striatum volume, nigrostriatal fiber tract volume, SN cell number), mean ± SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA with Tukey's multiple comparison test.
(**L**) Reconstruction of blood vessels in TBI brains with or without treatments. The regions of interest (ROIs) selected to assess angiogenesis are indicated in the top row (magenta boxes, directly below the injury site). 3D reconstructions of blood vessels are shown in the bottom row.
(M) Quantification of blood vessel volume, blood vessel surface area, number of blood vessel branches, and blood vessel length. $n = 3$, mean ± SD have been plotted.

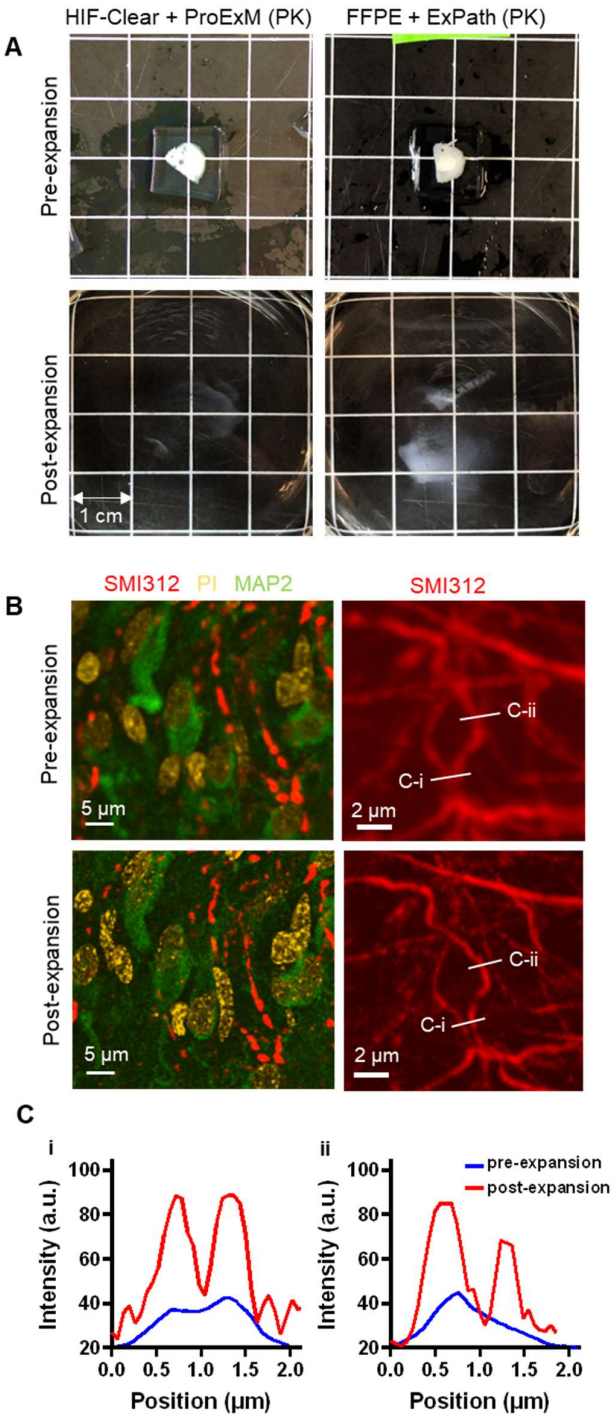

Figure 8. Expansion microscopy on a FFPE-HIF-Clear human brain specimen.
(**A**) Gross views of expansion of a 1-mm FFPE specimen.
(**B**) Fluorescence images of a FFPE human brain pre- (top row) and post-expansion (bottom row). Left: merged images of multiplexed staining; right: fluorescence images of SMI312 staining.
(**C**) Profiles of axon signal intensity taken along the white lines shown in the images of the right panel in (B). The scale bar has been divided by the expansion factor.