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Tracking Calcium Dynamics and Immune Surveillance at the Choroid Plexus Blood-Cerebrospinal Fluid Interface

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1 **Tracking calcium dynamics and immune surveillance at the choroid plexus**
2 **blood-cerebrospinal fluid interface**

3
4 Frederick B. Shipley,^{1,2,*} Neil Dani,^{1,*} Huixin Xu,¹ Christopher Deister,³ Jin Cui,¹ Joshua P.
5 Head,¹ Cameron Sadegh,^{1,4} Ryann M. Fame,¹ Morgan L. Shannon,¹ Vanessa I. Flores,⁵ Thomas
6 Kishkovich,³ Emily Jang,³ Eric M. Klein,³ Glenn J. Goldey,⁵ Kangmin He,^{6,9} Yong Zhang,⁷
7 Michael J. Holtzman,⁷ Tomas Kirchhausen,⁶ Claire Wyart,⁸ Christopher I. Moore,³
8 Mark L. Andermann,^{2,5,#} Maria K. Lehtinen^{1,2,#}

9
10 ¹Department of Pathology, Boston Children's Hospital, Boston, MA 02115, USA

11 ²Graduate Program in Biophysics, Harvard University, Cambridge, MA 02138, USA

12 ³Carney Institute for Brain Science, Brown University, Providence, RI 02912, USA

13 ⁴Department of Neurosurgery, Massachusetts General Hospital, Harvard Medical School,
14 Boston, MA 02114, USA

15 ⁵Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, Beth Israel
16 Deaconess Medical Center, Boston, MA 02115, USA

17 ⁶Department of Cell Biology and Department of Pediatrics, Harvard Medical School, Program in
18 Cellular and Molecular Medicine, Boston Children's Hospital, Boston, MA 02115, USA

19 ⁷Pulmonary and Critical Care Medicine, Department of Medicine, Washington
20 University, St. Louis, MO 63110, USA

21 ⁸Institut du Cerveau et de la Moelle épinière (ICM), Sorbonne Université, Inserm U1127, CNRS
22 UMR 7225, Paris 75013, France

23 ⁹Present Address: National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese
24 Academy of Sciences, Beijing 100101, China

25 * These authors contributed equally

26 # Co-senior authors

27 Lead contact: maria.lehtinen@childrens.harvard.edu

28 Corresponding authors: manderma@bidmc.harvard.edu; maria.lehtinen@childrens.harvard.edu

29 **SUMMARY**

30 The choroid plexus (ChP) epithelium is a source of secreted signaling factors in cerebrospinal
31 fluid (CSF) and a key barrier between blood and brain. Here, we develop imaging tools to
32 interrogate these functions in adult lateral ventricle ChP in wholemount explants and in awake
33 mice. By imaging epithelial cells in intact ChP explants, we observed calcium activity and
34 secretory events that increased in frequency following delivery of serotonergic agonists. Using
35 chronic two-photon imaging in awake mice, we observed spontaneous subcellular calcium events
36 as well as strong agonist-evoked calcium activation and cytoplasmic secretion into CSF. Three-
37 dimensional imaging of motility and mobility of multiple types of ChP immune cells at baseline
38 and following immune challenge or focal injury revealed a range of surveillance and defensive
39 behaviors. Together, these tools should help illuminate the diverse functions of this understudied
40 body-brain interface.

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43 **KEYWORDS**

44 Choroid plexus, cerebrospinal fluid, two-photon imaging, calcium activity, serotonin, secretion,
45 epithelial cells, immune cells

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51 INTRODUCTION

52 The choroid plexus (ChP) is a distinct, vital organ that extends into each ventricle in the
53 brain. It is composed predominantly of epithelial cells that envelop a network of stromal cell
54 types including immune, mesenchymal, and vascular cells (Dani et al., 2019). The epithelial cells
55 provide a source of cerebrospinal fluid (CSF) (Damkier et al., 2013) and associated growth-
56 promoting factors for neural stem cells (Lehtinen et al., 2011; Fame and Lehtinen, 2020; Silva-
57 Vargas et al., 2016). They also form a blood-CSF barrier that gates passage of nutrients, toxins,
58 and immune cells from body to brain (Gherzi-Egea et al., 2018; Reboldi et al., 2009; Schwartz
59 and Baruch, 2014; Shechter et al., 2013), and may regulate CSF composition via clearance of
60 toxins and waste (Crossgrove et al., 2005). Thus, the sensing, secretory, and transcytotic
61 functions of the ChP suggest diverse roles in regulating brain function. These roles may be
62 disrupted in neurologic conditions ranging from hydrocephalus (Karimy et al., 2017) to
63 Alzheimer's disease (Balusu et al., 2016a; Marques et al., 2013). Further, the ChP is an attractive
64 target for enhancing drug delivery to the brain (Gonzalez et al., 2011; Haddad et al., 2013; Hudry
65 and Vandenberghe, 2019).

66 Despite the importance of the ChP-CSF system, little is known about the behavior of
67 mammalian ChP cell types *in vivo*. *In vitro* approaches exist for culturing ChP cell lines (Zheng
68 and Zhao, 2002), dissociated ChP cells (Zheng et al., 1998), ChP epithelial cell sheets in
69 transwell models (Strazielle and Gherzi-Egea, 1999), and ChP organoids (Pellegrini et al., 2020;
70 Watanabe et al., 2012). Isolated ChP explants have also been used for analyzing secretion into
71 conditioned medium (Gudeman et al., 1987, 1989; Lun et al., 2015a; Silva-Vargas et al., 2016),
72 or for fixation and immunostaining (Dani et al., 2019; Lun et al., 2015a). Anatomical studies
73 using light and electron microscopy (EM) have provided clues as to the cellular architecture of

74 the ChP (e.g. Netsky and Shuangshoti, 1975). However, a major obstacle to progress in
75 understanding the roles of ChP cells has been the lack of available tools for stable visualization
76 and manipulation of specific ChP cell types in intact tissue *in vitro* and *in vivo* in a fluid
77 environment deep within the brain.

78 Here, we adapted a suite of modern neuroscience tools to target the lateral ventricle ChP,
79 providing optical access to this unexplored tissue in mice. We developed methods for volumetric
80 two-photon imaging and non-rigid alignment of the ChP in acute explant preparations, as well as
81 in awake mice across hours, days, and weeks. Dynamic cellular functions of other epithelia (e.g.
82 in retina, lung, and salivary gland) are typically associated with changes in intracellular calcium
83 (Ambudkar, 2016; Balaji et al., 2017; Concepcion and Feske, 2017; Narciso et al., 2017;
84 Samanta and Parekh, 2016). For example, calcium signaling in salivary gland is important for
85 on-demand secretion (Ambudkar, 2018, 2016). We found that ChP epithelial cells exhibited
86 spontaneous subcellular calcium activity *in vitro* and *in vivo*. Serotonergic agonists evoked
87 distributed increases in calcium activity, as well as secretory events measured using a sensor of
88 exocytosis. We then visualized the motility and mobility of ChP immune cells in relation to ChP
89 vasculature in awake mice at baseline and in response to peripheral immune stimulation and to
90 laser-induced, focal ChP injury. Together, these methods provide a novel imaging platform for a
91 wide range of studies imaging multiple genetically accessible ChP cell types in intact tissue at
92 unprecedented spatial and temporal resolution.

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97 RESULTS

98 Imaging ChP explants

99 We first optimized adult lateral ventricle (LV) ChP explant preparations (Dani et al.,
100 2019; Lun et al., 2015a) to enable histological analyses (**Figures 1A-1D**) and stable live-cell
101 imaging (**Figures 1E, 1F, and S1A**). Epithelial cells constitute the majority of adult ChP cells
102 (Dani et al., 2019). In addition, the ChP contains immune cells (labeled by *Cx3cr1^{+GFP}* (Jung et
103 al., 2000)) consisting mostly of monocytes/macrophages but also including a smaller number of
104 mast cells and dendritic cells (Dani et al., 2019; Van Hove et al., 2019). These immune cells
105 evenly tiled the entire tissue under baseline conditions (**Figures 1A-1C**). The ChP could be
106 divided into stereotyped zones defined by arterial and venous landmarks (**Figures 1D, S1B, and**
107 **S1C**) (Dani et al., 2019). This vascular pattern strongly resembles that observed for human ChP
108 (Hudson, 1960). As such, it provides an anatomical roadmap that allows specific subregions of
109 the lateral ventricle ChP to be identified and analyzed across mice within the same study, across
110 studies from different labs, and across species.

111 ChP explants were stabilized for acute *in vitro* imaging (**Figure 1E**). To visualize
112 calcium activity in epithelial cells, we gently dissected and stabilized the entire LV ChP from
113 one hemisphere. We expressed the calcium reporter GCaMP6f (using *Ai95D* mice; Madisen et
114 al., 2015) in ChP epithelial cells (using *FoxJ1-Cre* mice that selectively target this cell
115 population) (**Figure 1F**) (Lun et al., 2015a; Zhang et al., 2007). Using epifluorescence imaging,
116 we could visualize spontaneous calcium activity across thousands of epithelial cells
117 simultaneously (**Figure 1F; Video S1**). We focused on a subregion and performed activity-based
118 cell segregation (STAR Methods), resulting in time courses of spontaneous activity in individual
119 epithelial cells (**Figures 1F-1K; Video S1**). Most epithelial cells showed large, transient

120 elevations in calcium activity lasting several seconds (**Figures 1K and 1L**). Such events were
121 not synchronized across cells (**Figure 1M**). These findings suggest baseline regulation of
122 calcium levels and calcium-dependent signal transduction in ChP epithelial cells.

123

124 **Activation of serotonin receptors stimulates secretion via VAMP3-mediated exocytosis**

125 Elevated calcium regulates many cellular processes including gene transcription and
126 secretion in other body epithelia such as the salivary gland (Ambudkar, 2016). One factor
127 previously shown to elevate calcium levels in ChP cell lines in culture is serotonin (5-HT, 5-
128 hydroxytryptamine) (Esterle and Sanders-Bush, 1992; Sanders-Bush and Breeding, 1990).
129 Metabolites of the 5-HT signaling pathway are present in the CSF (Toda et al., 2013). CSF-5-HT
130 can originate from multiple sources, including direct release by dorsal raphe nucleus serotonergic
131 neurons that course along the ventricles and in close proximity to the ChP (Narboux-Neme et al.,
132 2008; Okaty et al., 2020; Tong et al., 2014), and via peripheral circulation, originating, for
133 example, from 5-HT secretion in the gut (Stasi et al., 2019) or platelets (Cloutier et al., 2012).
134 We found that 5-HT (Audhya et al., 2012; Toda et al., 2013) triggered coordinated waves of
135 calcium activity that recruited increasing numbers of ChP epithelial cells across the explant with
136 higher concentrations of 5-HT (**Figures 2A, 2B, and S2E; Video S2**).

137 The 5-HT_{2C} serotonin receptor (**Figure 2C**) (Lein et al., 2007), a Gq/G₁₁-coupled G-
138 protein coupled receptor (GPCR), is the most highly expressed GPCR in ChP epithelial cells
139 (Lun et al., 2015a). We found that subcutaneous (SC) injection of WAY-161503, a selective
140 agonist of 5-HT_{2C} (Rosenzweig-Lipson et al., 2006), drove robust immediate early gene
141 expression in ChP (**Figures 2D, S2A, and S2B**). Antibodies available for this receptor have
142 typically shown low signal quality. Thus, we used genome editing to generate a *Htr2c^{mRuby3}*

143 mouse line in which the fluorescent protein mRuby3 was inserted at the C-terminus of 5-HT_{2C}
144 (**Figures S2C and S2D**). 5-HT_{2C}-mRuby3 was functional in these mice, as subcutaneous
145 injection of WAY-161503 in *Htr2c^{mRuby3}* mice induced *c-fos* expression similar to that observed
146 in wild type mice (**Figure 2D**; fold increase in *c-fos* mRNA expression in *Htr2c^{mRuby3}* mice
147 receiving WAY-161503 [3 mg/kg] vs. vehicle: 80.6 ± 17.3 , mean \pm s.e.m., n = 4 heterozygous
148 male mice; *Htr2c* expressed from X chromosome). Fluorescence of the mRuby3 tag revealed
149 receptor localization throughout ChP epithelial cells, including at the apical and basal
150 membranes (**Figure 2E**). This localization is consistent with the prediction that ChP epithelial
151 cells can sense both central and peripheral sources of 5-HT (**Figure S1C**). Accordingly, using
152 higher-magnification two-photon calcium imaging, we obtained similar patterns of activation of
153 an increasing number of cells with increasing concentrations of the 5-HT_{2C} agonist, WAY-
154 161503 (**Figures 2F and S2E; Video S3**).

155 Application of 5-HT to dissociated ChP cells in culture can increase the transfer of water
156 and protein secretion (Conn and Sanders-Bush, 1986; Esterle and Sanders-Bush, 1992; Watson et
157 al., 1995). Our expression analyses confirmed that the secretory machinery commonly required
158 for calcium-dependent gene induction, protein secretion, vesicle trafficking/release machinery,
159 and/or homologs of proteins from other epithelia are expressed in ChP epithelial cells (e.g.
160 *Vamp3*, *Snap23*, *Stx12*, *Stxbp4*), implicating vesicular exocytosis as a mechanism of protein
161 secretion (**Figures S2F-2SJ**) (Dani et al., 2019; Lun et al., 2015a). In EM images of ChP
162 epithelial cells, a high density of vesicles was observed near the apical membrane (**Figure S2I**,
163 black arrows). VAMP3 showed the highest gene expression amongst vesicle proteins in the ChP
164 (**Figure S2G**, RNA-seq data from (Lun et al., 2015a)), and VAMP3 protein expression was
165 confirmed by immunoblotting and immunostaining (**Figures S2H and S2J**). These data led us to

166 investigate activity-dependent and VAMP3-mediated ChP exocytosis. Specifically, we used
167 AAV-VAMP3-pHluorin (Urbina et al., 2018), a pH-sensitive variant of GFP, to visualize
168 individual secretory events. pHluorin fluoresces upon plasma membrane fusion, when the lower
169 pH (~5.6) inside intact exocytic vesicles changes to a pH of 7.4 upon exposure to the
170 extracellular environment. The fluorescence signal disappears following endocytosis and re-
171 acidification of the vesicles (Sankaranarayanan et al., 2000).

172 We first expressed VAMP3-pHluorin in the Z310 ChP epithelial cell line (Zheng and
173 Zhao, 2002). Using total internal reflection fluorescence (TIRF) microscopy, a method with high
174 signal-to-noise ratio, fast frame rate (2 frames/s), and narrow fluorescence excitation and
175 emission ranges, we could capture numerous spontaneous vesicle fusion events (**Figure S2K**;
176 **Video S4**). To evaluate ChP secretion in a more naturalistic setting, we transduced ChP *in vivo*
177 with AAV-VAMP3-pHluorin, dissected ChP explants, and investigated vesicle fusion events *in*
178 *vitro*. Due to the elaborate apical structure of ChP epithelial cells including multiple microvilli
179 and cilia (**Figure S2I**), these cells were not amenable to TIRF microscopy (axial resolution <100
180 nm) without compressing the cells against a coverglass – a procedure that could compromise
181 cellular integrity or induce cellular responses to mechanical distortion. Instead, we used Airyscan
182 confocal microscopy (ZEISS LSM880) that afforded comparable signal-to-noise ratio and frame
183 rates (1.59 frames/s). We observed spontaneous VAMP3-mediated exocytosis in individual
184 epithelial cells in whole ChP explants (**Video S5**). We extracted fluorescence time courses of the
185 localized secretion events following spatial filtering of each image (**Figure 2G**). We then defined
186 regions with co-active pixels and extracted time courses (**Figures 2G, 2H, and S2L**). Strikingly,
187 5-HT_{2C} activation by WAY-161503 (delivered at levels similar to those that drove calcium
188 activity in **Figure 2F**) drove an increase in the rate of VAMP3-mediated exocytosis (**Figures 2H**

189 **and S2L; Video S5**). Our data using live imaging at subcellular resolution demonstrate that 5-
190 HT stimulates ChP exocytosis via activation of 5-HT_{2C}. More generally, our findings validate a
191 platform for fluorescence imaging in ChP explants, enabling high-resolution studies of calcium
192 activation, secretion, and other processes.

193

194 ***In vivo* imaging of ChP in awake mice**

195 Virtually nothing is known about the activity of ChP cell types *in vivo*. We developed a
196 deep-brain cannula implantation strategy that enables acute and longitudinal imaging of the ChP
197 over weeks and months in awake mice. A cannula and glass window were surgically implanted
198 above the lateral ventricle (**Figures 3A-3D**), similar to our recent approach for imaging in visual
199 thalamus (Liang et al., 2018). At 2-3 weeks post-surgery, windows were typically translucent,
200 allowing brightfield imaging of ChP (**Figure 3E**).

201 Similar to brain surgery in the clinical setting, insertion of the imaging cannula is an
202 invasive procedure. We performed additional control experiments to determine the extent of the
203 injury response and to verify the health of the preparation following recovery from surgery at the
204 time of imaging. As anticipated, GFAP-positive astrocytes and *Cx3cr1*-positive immune cells
205 were enriched in cerebral cortical tissue adjacent to the cannula (**Figures S3A-S3G**). The density
206 of glial cells (GFAP-positive) and immune cells (*Cx3cr1*-positive) dropped to baseline levels by
207 ~100 μm from the edge of the cannula (**Figures S3A-S3G**). The ventricular lining of the lateral
208 ventricle below and lateral to the implant did not show accumulation of GFAP- or *Cx3cr1*-
209 positive cells, and retained characteristic S100 β -positive ependymal cells (**Figure S3B**).
210 Importantly, immune cells from the ChP tissue located below the implant exhibited a ramified,
211 non-activated morphology with extended processes and a level of tiling of the ChP that was

212 indistinguishable from observations in contralateral ChP and in ChP from control mice that did
213 not undergo surgery (see **Figures 1A, 1B, 1C, S3H, and S3I**). Elevated CSF cytokine levels that
214 were evident in some mice one day following surgery also returned to undetectably low levels in
215 all mice by 3 weeks post-surgery (**Figure S3J**). These data demonstrate that, at the time that
216 imaging began several week post-surgery, our imaging preparation did not show signs of
217 persistent inflammation.

218 Epifluorescence images of lateral ventricle ChP from transgenic mice expressing
219 GCaMP6f in ChP epithelial cells (*FoxJ1-Cre::Ai95D*; **Figure 3F**; **Video S6**) demonstrated
220 consistently high image quality across mice. Notably, anatomical features of the ChP were stable
221 upon repeated imaging across weeks and months, with no evidence of substantial remodeling of
222 vasculature across imaging sessions beginning several weeks following surgery (**Figure 3G**).
223 While the location of the ChP in the lateral ventricle showed moderate mouse-to-mouse
224 variability following surgery (**Figure 3F**), identification of arterial and venous landmarks
225 allowed longitudinal imaging of a similar anatomical region of the ChP across mice, and within
226 the same mouse across sessions (**Figures 3 and S1B**).

227 To maximize spatial resolution and minimize bleaching during cellular imaging, we
228 performed two-photon imaging using a long working-distance, high numerical aperture objective
229 coupled to the imaging window (see STAR Methods). We targeted local regions of interest
230 within previously acquired epifluorescence images (**Figures 4A-4C**). In contrast to other brain
231 tissues that can be largely pressurized and stabilized for two-photon imaging (Goldey et al.,
232 2014; Liang et al., 2018), the ChP is only anchored at one edge near the base of the lateral
233 ventricle, and is otherwise suspended in CSF. Therefore, the ChP often exhibited large and non-
234 rigid motion in three dimensions during changes in behavior such as locomotion or adjustment of

235 body posture (**Video S6**). As described below, we used different imaging strategies and custom
236 registration algorithms to overcome these technical challenges.

237 First, video-rate two-photon imaging of a single plane allowed precise and high-speed
238 tracking of small numbers of cells following in-plane alignment, particularly during periods of
239 minimal brain motion while the mouse was stationary. For these analyses, occasional large tissue
240 movement could be stabilized or omitted from further analyses. Second, for longitudinal tracking
241 across hours, for which larger non-rigid motion and drift of the tissue out-of-plane were often
242 evident, we instead used a volumetric imaging strategy (0.25-0.5 volumes/s, 31-62
243 planes/volume, volume dimensions: 170 x 170 x 350 μm^3 or 355 x 230 x 100 μm^3 ; see STAR
244 Methods). This approach was important for achieving stable cell tracking following non-rigid
245 alignment in three dimensions (see **Figure 5**, below).

246

247 **Imaging calcium activity and apocrine secretion in ChP epithelial cells *in vivo***

248 We imaged ChP epithelial cell calcium activity using a transgenic mouse expressing
249 GCaMP6f, which provided similar expression levels across cells and stable expression across
250 days (**Figures 4A-4C**; see also **Figure 1F**). We first performed single-plane two-photon calcium
251 imaging (**Figure 4D**). High-speed imaging (33-41 frames/s) revealed spontaneous subcellular
252 calcium events lasting ~200 ms (**Figure 4G**; **Videos S7-S8**). To quantify this observation, we
253 manually outlined the borders and nuclei of individual cells (**Figures 4D-4F**). A typical
254 subcellular event from one example cell is shown in **Figure 4G**. We segmented each cell into 12
255 radial sectors extending from the center of the nucleus (**Figure 4E**, bottom), and “unwrapped”
256 the sectors to create a kymograph of averaged subcellular activity across frames (**Figure 4H**). A
257 maximum intensity projection across sectors revealed large subcellular events (**Figure 4I**) of a

258 consistent duration and characteristic exponential decay (**Figure 4J**). The consistent dynamics
259 and correlated changes across nearby pixels for this and other cells (**Figure S4**) further suggested
260 that these events were not due to photon noise or brain motion. In contrast to maximum-intensity
261 projections across sectors, median projections showed no significant fluctuations (**Figures 4K,**
262 **S4A, and S4B**), consistent with the subcellular nature of these events.

263 Our earlier findings demonstrated that application of 5-HT_{2C} agonist WAY-161503
264 evoked robust calcium responses in ChP epithelial cells *in vitro* and induced immediate early
265 gene expression following peripheral injection *in vivo* (**Figures 2D, 2F, S2A, S2B, and S2E;**
266 **Video S3**). Further, signatures of apocrine secretion (**Figures 4M and 4N; Video S9**) have
267 previously been reported to occur in ChP *ex vivo* (Agnew et al., 1980; Farkaš, 2015; Gudeman et
268 al., 1989). We therefore sought to define the dynamics of ChP calcium activity and apocrine
269 secretion upon WAY-161503 delivery *in vivo*. To obtain stable estimates of calcium transients
270 across tens of minutes (see above), we used volumetric imaging (0.32 volumes/s, 93
271 planes/volume, 3.8 μm spacing between planes). Subcutaneous injection of WAY-161503
272 resulted in robust increases in calcium activity that progressed along the epithelium over tens of
273 minutes (**Figure 4L; Video S9**). The large differences in timing of activation of various cells
274 may relate to cell-to-cell differences in 5-HT_{2C} expression (**Figure 2E**), to slow changes in the
275 concentration of WAY-161503, or to sequential sensing of signals release from activated
276 neighboring cells. Cellular increases in calcium activity culminated in apocrine secretion,
277 reflected by a release of cytoplasmic protrusions from the apical surface of the cell and cellular
278 release of cytoplasmic contents directly into the CSF (**Figures 4L-4N; Video S10**). The basal
279 portion of the cells including the nucleus remained intact. Taken together, these findings
280 highlight novel *in vitro* and *in vivo* approaches to test and visualize calcium activity and distinct

281 modes of exocrine signaling by ChP epithelial cells. Further, these data establish a platform for
282 testing how exogenous signals such as serotonin can stimulate calcium activation, gene
283 transcription, and exocrine secretion.

284

285 **ChP immune cells at baseline and in response to local or peripheral stimulation**

286 The ChP is not only important for secretion of water and proteins into the CSF, but is also
287 an essential barrier that protects the brain from harmful blood-borne factors (Gherzi-Egea et al.,
288 2018; Saunders et al., 2018) and is implicated as a site of immune cell entry into the brain (Fame
289 and Lehtinen, 2020; Gherzi-Egea et al., 2018; Reboldi et al., 2009; Schwartz and Baruch, 2014;
290 Shechter et al., 2013). However, the *in vivo* functions of ChP immune cells in physiological or
291 pathological conditions remain largely unexplored (Kierdorf et al., 2019). Thus, we investigated
292 ChP immune cells under homeostatic, immune-challenged, and injury conditions.

293 We repeated the surgical approach described above in transgenic mice expressing GFP in
294 *Cx3cr1*-positive immune cells (Jung et al., 2000). Following surgical recovery, we performed
295 intraperitoneal (IP) injection of Texas Red-conjugated dextrans that rapidly filled the major
296 vessels and fine capillary networks of the ChP. These large dextrans (70 kDa) did not
297 immediately leak into the ChP stromal space. We then performed two-color imaging of ChP
298 immune cells and vasculature (**Figure 5A**), focusing on regions of ChP that were oriented
299 parallel to the imaging plane, and thus amenable to time-lapse volumetric imaging across the
300 thickness of the tissue (**Video S11**).

301 For tracking of fine immune cell processes across seconds, minutes and hours in awake
302 mice, it was critical to develop a procedure for accurate alignment of the 3D imaging volumes
303 (see **Figure 5B** and legend). It was useful to estimate shifts in ChP using the stable, bright red

304 dextran signal, and then apply these shifts to both the imaged vasculature (red) and immune cells
305 (green). Given that the individual frames were acquired at 15.5 frames/s, there was minimal
306 within-plane non-rigid motion. However, brain motion could result in X and Y shifts in
307 successive imaging planes within a volume (**Video S12**). Thus, alignment of each Z-plane to a
308 reference plane within each volume was important (**Figures 5C and 5D**). We then performed
309 rigid-body 3D alignment. Following these corrections, images of static objects (e.g. vasculature)
310 could be effectively stabilized (**Figures 5E and 5F**). As a final step, we calculated the mean
311 intensity across Z-planes for each volume and ran a second translational alignment. Across all 20
312 sessions from 13 mice, estimated intra-volume and inter-volume shifts in X, Y and Z could be
313 quite large, reflecting ChP suspension in CSF (**Figures 5G-5J**).

314 We observed substantial exploratory movements of ChP immune cell bodies and/or distal
315 processes. In each of 26 fields of view from 14 mice, we observed large numbers of GFP-
316 positive immune cells. Some of the cells were located within the ChP stromal space, while others
317 were located on the apical surface of the ChP, in contact with lateral ventricle CSF (i.e.,
318 epiplexus or Kolmer cells) (**Figures 6A-6G**). Epiplexus cell bodies often exhibited substantial
319 mobility. For example, the cell in **Video S13** (*top left*) traveled 210 μm in 1 hour. Some
320 epiplexus cell bodies moved at a constant rate, while others displayed saltatory movements
321 (**Figure 6D; Video S13**). In contrast, the majority of GFP-positive immune cells located within
322 the stromal space showed minimal cell body mobility. However, these cells possessed highly
323 dynamic processes that extended and contracted (**Figures 6E-6G; Video S13**), similar to
324 microglia in other brain areas (Hierro-Bujalance et al., 2018). These processes appeared to serve
325 a surveillance function, as they frequently contacted vessels within the stromal space, and

326 retracted upon contact with other processes from the same or neighboring immune cells (**Figures**
327 **6E-6G; Video S13**).

328 These surveillance-like behaviors were reminiscent of immune cells in other parts of the
329 brain that play key roles in sensing environmental perturbations and protecting against injury
330 (Hickman et al., 2018; Kierdorf et al., 2019; Li and Barres, 2018). Indeed, we found that immune
331 cells in the ChP appear to partake in similar functions. First, we noted that the fluorescent
332 dextrans used to label vasculature were cleared from circulation over several days. In these
333 experiments, ChP immune cells in the stromal space, but not epiplexus cells, took up fluorescent
334 dextrans 30 minutes following injection (**Figures 6H and 6I; Videos S14 and S15**), and
335 dextran-labeled punctae could be observed even 26 days following injection (**Figures S5A-S5C**).
336 These data demonstrate that ChP immune cells participate in uptake of foreign material from the
337 peripheral circulation, consistent with the known housekeeping functions of immune cells in
338 other parts of the body and brain.

339 The ChP contributes to blood-brain communication during peripheral inflammation
340 (Balusu et al., 2016b), and the effects of immune challenges on the ChP have been implicated in
341 several neurologic conditions. For example, genetic markers of immune function and
342 inflammation are upregulated in ChP of schizophrenia patients (Kim et al., 2016). Thus, we next
343 considered the effects of peripheral administration of the bacterial endotoxin lipopolysaccharide
344 (LPS), which induces inflammatory responses in mouse ChP (Balusu et al., 2016b; Marques et
345 al., 2009), on ChP immune cell morphology *ex vivo* and *in vivo*. As expected, LPS induced an
346 inflammatory cytokine response in serum and CSF (**Figures 7A and S6A**). Using
347 immunohistochemistry, we found that while peripheral LPS administration did not affect tiling
348 of immune cells across the ChP (**Figures 1A-1C, S3H, and S3I**), it triggered a marked

349 repositioning of GFP-positive immune cell bodies and processes to regions surrounding the
350 vasculature within the ChP (**Figures 7B, 7C, and S6B**).

351 To define the morphological dynamics of individual immune cells in response to LPS, we
352 performed *in vivo* two-photon imaging during peripheral delivery of LPS. Many GFP-positive
353 ChP immune cell bodies and processes that were initially located distal to vessels prior to LPS
354 moved towards and spread along nearby vessels within ~45-60 minutes of LPS delivery (**Figure**
355 **7D; Video S16**). Using a custom algorithm to segment vasculature and define periluminal
356 regions (**Figures 7E, 7F, and S6C**; STAR Methods), we confirmed that immune cell
357 fluorescence increased in periluminal regions (**Figure 7F**). Not all *Cx3cr1*-expressing cells
358 responded to LPS, consistent with the multiplicity of *Cx3cr1*-expressing ChP immune cell types
359 that likely exhibit distinct responses to peripheral stimuli (Dani et al., 2019; Van Hove et al.,
360 2019). This repositioning of ChP immune cells along the periluminal region may provide an
361 extra layer of brain protection from harmful blood-borne signals during peripheral inflammation
362 (Mottahedin et al., 2019).

363 In addition to the robust response of ChP immune cells following peripheral
364 inflammation, we found that these cells often move towards sites of local injury. We induced a
365 focal injury by high-power two-photon heating of a small area in the center of the field of view
366 ($89 \times 57 \mu\text{m}^2$, **Figure 7G**). This triggered rapid recruitment of immune cells to the injury site from
367 nearby regions of the ChP. Immune cells initiated movement immediately following the laser
368 injury, transitioned to an apparently more activated state (retracted processes, larger cell bodies),
369 and continued moving until they stabilized in an aggregate surrounding the injury site (**Figure**
370 **7H; Video S17**). Across three mice, most but not all immune cells moved towards the injury site
371 (**Figure 7I**). The majority of the cells that did move towards the injury site were confirmed to be

372 epilexus cells (**Video S18**). Together, these findings reflect diverse contributions of different
373 types of resident ChP immune cells to host defense.

374

375 **DISCUSSION**

376 The scarcity of experimental tools for selectively targeting, monitoring, and manipulating
377 ChP cells has hindered progress in understanding this essential and distinct organ located deep
378 within the brain. Despite its principal roles in producing CSF, forming a brain barrier, and
379 secreting important health and growth promoting factors for the brain (Fame and Lehtinen, 2020;
380 Ghersi-Egea et al., 2018; Lun et al., 2015b; Saunders et al., 2018), remarkably little is known
381 regarding the functions of its cellular networks. Here, we developed imaging and analysis
382 approaches for monitoring and pharmacological manipulation of multiple ChP cell types in live
383 explants and in awake mice. Using a combination of epifluorescence, confocal, and two-photon
384 microscopy in ChP explants, we observed spontaneous calcium activity as well as spontaneous
385 exocytotic fusion events in individual epithelial cells. Both of these processes were enhanced by
386 application of agonists of the 5-HT_{2C} receptor, which is highly expressed in ChP epithelial cells.
387 Epifluorescence and two-photon microscopy in awake mice revealed subcellular spontaneous
388 calcium activity and 5-HT_{2C} agonist-evoked calcium activity and apocrine-type exocrine release.
389 By developing tools for volumetric, multi-color two-photon imaging of vasculature and immune
390 cells within and on the surface of the ChP *in vivo*, we uncovered spontaneous surveillance
391 behaviors of immune cells as well as profound immune cell activation and translocation
392 following peripheral or local perturbations. We hope this ChP imaging toolkit will accelerate the
393 pace of discoveries regarding the diverse functions of this vital deep brain tissue.

394

395 **Imaging the ChP *in vitro* and *in vivo***

396 We hope these methods for imaging ChP explants can be of broad utility, as this
397 approach is relatively simple and inexpensive, and allows tracking of tissue prior to and
398 following controlled delivery of multiple drugs to the apical surface of the ChP. Our description
399 of vascular landmarks should also improve repeatability within and across studies. The lateral
400 ventricle ChP tissue is thin and delicate, and not entirely flat (albeit much flatter than third and
401 fourth ventricle ChP). Thus, in order to obtain high-quality data, it was important to carefully
402 extract the explant, stably mount it, and adjust fluid flow and osmolarity to avoid undue
403 stretch/pressure (**Figure S1A**).

404 *In vivo* methods enabled monitoring of ChP in a largely natural environment during
405 systemic delivery of drugs or perturbations (**Figures 4, 6, and 7**). While *in vivo* imaging using a
406 cannula has been demonstrated in many deep brain areas (e.g., Dombeck et al., 2010; Liang et
407 al., 2018), motion of ChP tissue posed a particularly challenging problem, as the ChP is anchored
408 at the ventromedial aspect of the lateral ventricle, far from the dorsal ChP regions that we
409 imaged. This likely contributed to substantial non-rigid motion in three dimensions beyond what
410 is observed in other brain tissues that are pressurized and anchored by the imaging window.
411 Thus, while our use of a treadmill to minimize head torque applied by the limbs likely reduced
412 motion artifacts to some extent (Dombeck et al., 2007), it was critical to additionally use several
413 methods for 2D and non-rigid 3D co-registration of imaging datasets in order to attain
414 subcellular resolution (**Figure 5**). Another option to reduce coupling of body and brain motion
415 could be to anesthetize mice prior to imaging. While this may be particularly useful for structural
416 imaging studies, anesthesia could significantly alter the functional properties of the ChP.

417 We did not observe sustained inflammation of ChP for time points at which imaging was
418 performed, several weeks following implantation (**Figure S3**). Intracranial pressure also
419 normalized to baseline levels following this recovery period (data not shown). This recovery
420 period also improved imaging clarity in comparison to acute imaging immediately following
421 surgery (not shown), consistent with deep imaging in other brain regions (Goldey et al., 2014;
422 Liang et al., 2018). Nevertheless, additional improvements to our approach could further
423 minimize the invasive nature of the cannula implant. For example, with improved red and
424 infrared fluorescent indicators, window implants for two- and three-photon imaging of ChP can
425 be placed well above the dorsal surface of the lateral ventricle (e.g. Wang et al., 2018;
426 Weisenburger et al., 2019). Alternatively, lower-profile GRIN lenses may be used for
427 intraventricular imaging, albeit with a much smaller field of view and range of imaging depths.

428

429 **Spontaneous and evoked calcium activity and vesicle fusion in ChP**

430 We observed diverse rates of spontaneous calcium activity and diverse thresholds for
431 evoked activity across nearby cells. These differences may relate to differences in activity states
432 or to subtypes of epithelial cells. In future, such functional characterizations of epithelial cells
433 can be merged with single-cell transcriptomics (Dani et al., 2019) to better understand potential
434 divisions of labor across cells.

435 Spontaneous calcium transients were restricted to subregions of a cell. Future studies can
436 assess whether these subcellular events relate to the subcellular vesicle fusion events we
437 observed in explants, or to activation of a single protrusion among the many protrusions on the
438 apical surface of each epithelial cell (evident in EM images in **Figure S2I**). These events were

439 particularly fast (~200 ms) when measured *in vivo*, possibly due to calcium imaging at warmer
440 ambient temperatures *in vivo* vs. *in vitro*.

441 Application of a 5-HT_{2C} receptor agonist drove strong increases in calcium activity and
442 increased rates of vesicular fusion. This calcium sensitivity of epithelial tissue to serotonin and
443 associated agonists is consistent with previous reports using cultured, dissociated ChP cells
444 (Watson et al., 1995). Higher concentrations of 5-HT_{2C} agonist evoked large, apocrine-type
445 secretory events (**Figures 4L-4N; Video S9 and S10**) that have been reported in ChP and other
446 epithelia including sweat and mammary glands (Farkaš, 2015). While these secretory events
447 involve massive release of internal contents from an epithelial cell, they do not imply that cell
448 health is compromised. Rather, this process may represent an efficient and rapid means for
449 activity-dependent secretion of large amounts of cargo in response to an external stimulus,
450 possibly in conjunction with other rapid changes (e.g. rapid activation of water and ion
451 channels). Our studies set the stage for more in-depth investigations of how the ChP dynamically
452 regulates the molecular composition of the CSF that bathes the CNS.

453

454 **Immune surveillance at the ChP**

455 Immune cells have been proposed to enter the brain via the ChP (Gherzi-Egea et al.,
456 2018; Reboldi et al., 2009; Schwartz and Baruch, 2014; Shechter et al., 2013), but little is known
457 about the functions of resident ChP immune cells during baseline conditions or in response to
458 peripheral immune challenge or local injury. Tracking of *Cx3cr1*-expressing ChP immune cells
459 together with vascular labeling *in vivo* revealed vascular surveillance by stromal immune cell
460 processes, while cell bodies remained largely immobile. This surveillance points to active
461 maintenance and phagocytic roles at the blood-CSF barrier. Notably, we found that these stromal

462 immune cells still contained 70 kDa dextrans *weeks* after IP injection. In contrast, epilexus cells
463 on the apical surface of the ChP showed much greater cell body mobility but did not take up
464 dextrans.

465 ChP immune cells also responded to systemic LPS delivery by spreading their cell bodies
466 and processes along the periluminal region near blood vessels, a finding confirmed using
467 immunohistochemistry. This cellular response may reflect a means of protection against
468 peripheral insults, and differs from that of cortical microglia, which retract their processes and
469 adopt an amoeboid “activated” shape during inflammation (Pozner et al., 2015). Without access
470 to the time-lapse *in vivo* imaging, it would not have been possible to determine whether the same
471 local immune cells change their morphology and location or whether new immune cells had
472 entered the same region of ChP. Indeed, despite previous reports that immune cells cross at the
473 ChP (Gherzi-Egea et al., 2018; Reboldi et al., 2009; Schwartz and Baruch, 2014; Shechter et al.,
474 2013), our imaging sessions did not reveal arrival or departure of new immune cells from either
475 the CSF or the vasculature during baseline conditions or following LPS. Future studies should
476 examine deeper regions of the lateral ventricle across a broader range of conditions to more fully
477 assess potential subregions that mediate transit of immune cells to and from the brain.

478 We also noted rapid mobilization of nearby immune cells following deliberate heating of
479 a focal region of the field of view using transient, high-magnification and high-power two-
480 photon imaging. Many of these cells were epilexus cells, which acted as “first responders” by
481 accumulating at the injury site. This behavior is strikingly different from that of *Cx3cr1*-positive
482 microglia in cortex and other brain regions (Davalos et al., 2005; Pozner et al., 2015), which
483 extend their processes towards a laser-induced lesion to contain the injury while their cell bodies

484 remain stationary (Davalos et al., 2005). Our findings can inform surgical procedures involving
485 focal heating of ChP, such as during cauterization of ChP to treat hydrocephalus (Warf, 2005).

486 In future, it should be possible to use the *in vivo* imaging approach described here to
487 assess the role of changes in calcium and other intracellular signals in immune cells, epithelial
488 cells and other stromal cell types in the ChP during these and other immune challenges and brain
489 injuries. A better understanding of the dynamic roles of multiple ChP cell types in various barrier
490 functions in the intact brain should spark new ideas for penetrating this barrier for drug delivery
491 to the brain, as well as for fortifying this barrier across the lifespan. More generally, given that
492 repeated *in vivo* access and chronic imaging may be more amenable in the ChP than in most
493 other body epithelia other than skin epithelium (Mesa et al., 2015; Rompolas et al., 2016), this
494 platform may provide a unique window into the general functions of barrier epithelia in their
495 natural environments.

496

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519

520 **AUTHOR CONTRIBUTIONS**

521 F.B.S., N.D., M.L.A., C.I.M., and M.K.L. designed the study; N.D. and F.B.S. developed whole
522 tissue explants and imaging protocols; C.D. and M.L.A. developed the *in vivo* 2-photon calcium
523 imaging protocol; F.B.S. performed all computational analyses of all imaging data with advice
524 from M.L.A.; J.C. helped generate *Htr2c^{mRuby3}* mice; F.B.S., J.C., and M.L.S. analyzed peripheral
525 activation of immune cells; H.X. analyzed secretory pathways, and developed the vesicle
526 imaging assay with assistance from C.W., K.H., and T.K.; J.H. developed immediate early gene
527 assays; G.J.G. developed and T.K., E.J., E.K., V.I.F., and C.S. refined the imaging cannula
528 approach; J.C. and R.M.F. analyzed CSF cytokines; Y.Z. and M.J.H. shared *FoxJ1-Cre* mice;
529 F.B.S., M.L.A., and M.K.L. wrote the manuscript.

530

531 **DECLARATION OF INTERESTS**

532 The authors declare no competing interests.

533

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539

540 **FIGURE LEGENDS**541 **Figure 1. Isolation, immunostaining, and calcium imaging of lateral ventricle ChP explants.**

542 (A) *Left*: large leaf of LV ChP from a $Cx3cr1^{+/GFP}$ mouse immunostained with anti-GFP (green,
543 immune cells) and PECAM (red, vasculature). Scale, 500 μm . *Right*: zoom-in of small dashed
544 box. Scale, 100 μm . $Cx3cr1^{+/GFP}$ cells tile the ChP (confirmed in 8 other mice). (B) Positions of
545 1781 $Cx3cr1^{+/GFP}$ cells from A. (C) Cumulative distribution of nearest-neighbor distances of each
546 $Cx3cr1^{+/GFP}$ cell. Immune cells showed regular spacing ($\sim 30 \mu\text{m}$) relative to random Poisson
547 spacing (red trace; gray envelope: 1% acceptance interval). (D) PECAM (red) and ACTA2
548 (green) immunostains demarcate stereotyped LV ChP regions (confirmed in 3 other mice). Blue
549 arrowheads: veins. Scale, 500 μm . (E) Light path and setup for imaging LV ChP. (F)
550 Epifluorescence image containing a $FoxJ1-Cre::Ai95DLV$ ChP explant expressing GCaMP6f in
551 multiciliated ChP epithelial cells. Cells near stabilizing glue attachments at explant borders
552 (asterisks) showed elevated GCaMP6f fluorescence (indicating unhealthy cells) and were

553 excluded from subsequent analyses. Scale, 1 mm. **(G)** Zoom-in of 122 epithelial cells (dashed
 554 box in **F**). Scale, 50 μm . **(H)** Cell masks (see STAR Methods). **(I)** Twenty labeled cells
 555 corresponding to traces in **K**. **(J)** Pink: traces surrounding each calcium transient with a
 556 fractional change in fluorescence, $\Delta F/F > 5\sigma$ (235 events across 122 cells from **H**). Red: mean
 557 calcium transient across traces. **(K)** Five-minute time courses from cells in **I**. **(L)** 76% (93/122)
 558 of cells in **H** exhibited calcium events. **(M)** Average of all cross-correlations between binarized
 559 event time courses of all pairs of cells from **H** (computed at delays from -5 to +5 s),
 560 demonstrating that spontaneous events were uncorrelated across cells. We observed qualitatively
 561 similar results as in **G-M** in 25 other mice, not shown. See also **Figure S1; Video S1**.

562

563 **Figure 2. Evoked calcium activity and exocrine secretion in ChP epithelial cells.** **(A)**
 564 Epifluorescence calcium imaging of ChP epithelial cells from *FoxJ1-Cre::Ai95D* LV ChP
 565 explant. Mean baseline fluorescence (*left*) and changes in fluorescence from baseline in response
 566 to 0, 5, 50, and 500 nM 5-HT. Scale, 100 μm . **(B)** Time course of changes from baseline,
 567 averaged across the explant. Responses to at least one dose of 5-HT were observed in 18/19
 568 mice, and to all three doses in 10/19 mice, not shown. **(C)** *Htr2c* expression in LV ChP (from
 569 Allen Brain Atlas, Lein et al., 2007). Scale, 500 μm . **(D)** *c-fos* induction following injection of 5-
 570 HT_{2C} agonist WAY-161503 (****p < 0.0001, t-test, saline vs. 3 mg/kg SC; left to right: n =
 571 8,8,2,2,2). **(E)** *Htr2c^{mRuby3}* LV ChP labels 5-HT_{2C} receptors in epithelial cells. Axial (*left*) and
 572 side-on (*right*; from dashed box at left) maximum projections show preferential apical (apposed
 573 to the CSF) vs. basal (closer to vessels) localization. Scale, 10 μm . **(F)** Two-photon imaging of
 574 *FoxJ1-Cre::Ai95D* explants. Higher concentrations of WAY-161503 activated more cells (green
 575 arrowheads), and cells activated at lower concentrations are not reactivated later. Responses were

576 observed in 7/7 mice, and to each dose in 5/7 mice (not shown). Scale, 10 μ m. (G) Confocal
577 imaging of vesicle release from an example LV ChP epithelial cell following viral expression of
578 VAMP3-pHluorin. *Top left*: maximum intensity projection across baseline period shows
579 fluorescent vesicle release (white punctae). *Bottom left*: similar projection following Hessian-
580 based filtering. *Middle panels*: same as left but following application of WAY-161503 (500 nM).
581 *Right*: vesicle release event masks segmented from the filtered movie. Scale, 5 μ m. (H)
582 Cumulative number of VAMP3-pHluorin vesicle release events following application of WAY-
583 161503 (red) or aCSF (blue). See also **Figure S2; Videos S2-S5**.

584

585 **Figure 3. Imaging lateral ventricle ChP in awake mice.** (A,B) Schematic of cannula (gray
586 cylinder) with glass bottom, implanted above the LV ChP (green). (C) Headpost placement. (D)
587 Head-fixed mouse on a trackball. An immersion well attached to the headpost allowed imaging
588 via a high numerical aperture objective. (E) Brightfield image of ChP through the cannula, 27
589 days post-surgery. Dotted line outlines ChP. (F) Epifluorescence images of ChP (arrowheads)
590 from *FoxJ1-Cre::Ai95D* mice, 42-56 days after surgery. Scale, 1 mm. (G) Tracking the same
591 ChP (arrowheads) via a clear window across many days following surgery (similar results
592 observed in 9 other mice, not shown). Scale, 1 mm. See also **Figure S3; Video S6**.

593

594 **Figure 4. Two-photon calcium imaging of epithelial cells in awake mice.** (A) Epifluorescence
595 image of GCaMP6f-expressing ChP epithelial cells (diagonal vascularized sheet; *FoxJ1-
596 Cre::Ai95D* mouse). Scale, 1 mm. (B) Zoomed-in image (dashed red square in A). Scale, 100
597 μ m. (C) Maximum projection of two-photon imaging volume encompassing the ChP region in

598 **B.** Scale, 100 μm . **(D)** Average of images at a single plane. Scale, 50 μm . **(E)** Individual
 599 epithelial cell (red square in **D**), annotation of cell outline and nucleus, and division into 12
 600 sectors. **(F)** Annotation of all cell outlines and nuclei in **D**. **(G)** Time-lapse of a single subcellular
 601 calcium event. **(H)** Kymograph of activity across all 12 sectors of cell in **E,G**. Red arrowhead:
 602 event from **G**. **(I)** Time course of brightest-sector activity (black, maximum across sectors in **H**)
 603 and median activity (red). Asterisks: peaks of subcellular events exceeding 3 std (dashed blue
 604 line) above a running mean. **(J and K)** Brightest-sector (**J**) and median-sector (**K**) activity
 605 surrounding peak ($t=0$) of all events for cell in **E**. Thicker lines: mean traces. Similar results were
 606 observed in 3 other mice, not shown. **(L)** Images of cross-sections of two sheets of GCaMP6-
 607 expressing epithelial cells separated by stromal space, beginning 25 min after injection of WAY-
 608 161503 (3 mg/kg, SC; similar results observed in 2 other mice, not shown). Scale, 50 μm . **(M)**
 609 Zoom-in of a single epithelial cell reveals release of subcellular plumes (arrowheads) of
 610 intracellular contents including GCaMP6f into CSF. The basal side of the epithelium remained
 611 intact, consistent with apocrine secretion. Scale, 10 μm . Similar events were observed in a
 612 second mouse, not shown. See also **Figure S4; Videos S7-S10**. **(N)** Scanning EM of ChP, 15
 613 min following WAY-161403 (3 mg/kg, SC) reveals apocrine blebs (arrowheads). Scale, 5 μm .

614

615 **Figure 5. 3D imaging and registration of ChP in awake mice.** **(A)** Maximum projections
 616 across a time-averaged two-photon imaging volume of $Cx3cr1^{+/GFP}$ immune cells (green) and
 617 Texas Red dextran-labeled vasculature (red, IP injection). Projections from two mice are shown
 618 (similar results in 13 other mice, not shown). Scale, 100 μm . **(B)** Registration algorithm (see
 619 STAR Methods). Step 1: correct for depth-dependent magnification due to tunable lens. Step 2:
 620 intra-volume alignment of each plane to its neighbor. Step 3: 3D translation of each volume to a

621 local target. Steps 4-5: Z-projection and X-Y alignment. **(C)** Mean Z-projection of a single
 622 volume, before vs. after Step 2. Scale, 50 μm . **(D)** Estimated X and Y corrections for each plane
 623 of volume in **C**. **(E)** Z-profile time lapse of vasculature, before and after 3D registration.
 624 Columns: 600 volumes spanning ~63 min; rows: average fluorescence in the white box in **C** at
 625 each Z plane. White trace: estimated Z correction. **(F)** Index of motion artifact (sliding estimate
 626 of [standard deviation]/[mean] vasculature fluorescence across volumes; see STAR Methods).
 627 Registration reduced both large, transient motion artifacts (peaks in orange trace) and persistent,
 628 higher-frequency motion (see **J**). **(G-I)** Cumulative distributions of X and Y displacements of
 629 planes within each volume **(G)**, and XY displacements **(H)** and Z displacements **(I)** across
 630 consecutive volumes. Data in **G-J** from 20 sessions from 13 mice. **(J)** Mean motion artifact (see
 631 **F**) per session, pre- vs. post-registration. **** p < 0.0001; paired t-test.

632

633 **Figure 6. ChP immune cells perform local surveillance and housekeeping *in vivo*.** **(A)** Cross-
 634 section of ChP. Epiplexus immune cells (orange arrowhead) are located on apical (CSF-sensing)
 635 surface of epithelium (green sheet). Stromal immune cells (blue arrowheads) are located in
 636 stromal space between vasculature (red with purple endothelial cells) and epithelium. **(B) Top:**
 637 axial mean projection of $Cx3cr1^{+GFP}$ cells in LV ChP explant *ex vivo*. *Bottom:* side-on view.
 638 Arrowheads indicate stromal (blue) and epiplexus (orange) immune cells. Scale, 100 μm . **(C-G)**
 639 Similar to **C** but from *in vivo* two-photon imaging (see also **Videos S11 and S13**). Scale, 25 μm .
 640 **(C)** Example epiplexus cells from 4 mice. Side-on views (bottom) indicate locations outside
 641 vascular plane (likely outside the epithelium). **(D)** Example epiplexus cell pausing, then traveling
 642 across the ChP surface (colored dots: cell location at 1-min intervals). **(E-G)** Example stromal
 643 immune cells showed either stationary cell bodies with processes that survey nearby vessels (**E-**

644 **F**), and that retract following upon contacting a different immune cell (**F**), or, occasionally, cell
645 body movement constrained by surrounding vessels (**G**). (**H**) *Left, middle*: IP-injected red
646 dextran (70 kDa) fills the ChP vasculature. *Right*: 2 days later, dextran has leaked into stromal
647 space and accumulated within immune cells. Scale, 50 μm . (**I**) Snapshots of dextran punctae
648 accumulating within immune cell processes (arrowheads). See also **Figure S5**; **Video S14**.

649

650 **Figure 7. ChP immune cells respond to systemic and local insults.** (**A**) Higher CSF cytokine
651 levels 1-hr after IP injection of LPS vs. saline (mean \pm s.e.m.; n = 3 samples, each consisting of
652 25 μl pooled across 3-6 mice; t-test, IL-1 α , p = 0.0017; TNF- α , p = 0.0072; CCL2, p = 0.0260;
653 IL1 β , p = 0.0451; IFN- β , p = 0.0212). (**B-D**) Following LPS, immune cells flatten along vessels.
654 (**B**) LV ChP explants from *Cx3cr1^{+GFP}* mice that received IP saline (*left*) or LPS (*middle*).
655 Segmentation of immune cells (right panel, green), and periluminal region surrounding
656 vasculature (blue; **Figures S6B** and **S6C**, STAR Methods) allowed assessment of overlap
657 (yellow). Scale, 50 μm . (**C**) Percentage of periluminal region occupied by immune cell processes
658 following IP saline (n = 15 explants, 9 mice) or LPS (n = 20 explants, 10 mice). ****p < 0.0001,
659 Welch's t-test. Mean \pm s.e.m. (**D**) *In vivo* imaging of immune cells (green) and vasculature (red)
660 pre-LPS (left) and 3 hrs following IP LPS (right). Scale, 25 μm . Arrowheads: transitions of cell
661 bodies to splayed morphology (see **Video S16**). (**E**) Segmentation of periluminal region (STAR
662 Methods). (**F**) Fractional change in immune cell fluorescence ($\Delta F/F$) in periluminal region across
663 4 hrs, relative to pre-LPS baseline (red line). (**G**) Schematic of focal injury via brief, high-power
664 focusing of a laser on a small region of ChP during *in vivo* imaging. (**H**) Maximum projections
665 of immune cells and vasculature before, 6 min after, and 1 hr after a local burn of the region
666 within the white box. At 6 min, dextran leaks out of damaged vessels (see **Video S17**). Immune

667 cell bodies then migrate to the injury site. Scale, 50 μ m. **(I)** Average pre- and post-injury velocity
668 of immune cells towards (positive) or away from (negative) the injury site (n = 15 cells, 3 mice).
669 ** p = 0.0075, paired t-test.

670

671 **STAR METHODS**

672 ***RESOURCE AVAILABILITY***

673 **Lead Contact**

674 Further information and requests for resources and reagents should be directed to and will be
675 fulfilled by the Lead Contact, Maria Lehtinen (maria.lehtinen@childrens.harvard.edu).

676 **Materials Availability**

677 All unique/stable reagents generated in this study are available from the Lead Contact with a
678 completed Materials Transfer Agreement.

679 **Data and Code Availability**

680 The registration and vascular segmentation algorithms generated for this study are available at
681 <https://github.com/LehtinenLab/Shipley2020>. Original data is available from the corresponding
682 author upon request.

683

684 ***EXPERIMENTAL MODEL AND SUBJECT DETAILS***

685 All animal care and experimental procedures were approved by the Institutional Animal
686 Care and Use Committees of Beth Israel Deaconess Medical Center (Figures 3, 5-7), Boston
687 Children's Hospital (Figures 1-7), and Brown University (Figures 3, 4). Mouse lines used
688 include *FoxJ1-Cre* (Zhang et al., 2007), *Ai95D* (Jax# 024105; Madisen et al., 2015), *Cx3cr1^{+/GFP}*
689 (Jax# 005582; Jung et al., 2000), *Htr2C^{mRuby3}*, CD-1, and C57BL/6 (Figures 1, 2: male and

690 female mice; Figures 3-7 male mice). *Htr2C^{mRuby3}* mice were generated by the Gene
691 Manipulation & Genome Editing Core, IDDR, BCH. Best-ranked sgRNAs near the targeting
692 region in *Htr2C* genome were picked (Doench et al., 2016) and synthesized (Alt-R® CRISPR-
693 Cas9 crRNA, Integrated DNA Technologies). Donor plasmid was custom made at GeneScript,
694 prepared with EndoFree Plasmid Maxi Kit (Qiagen). Alt-R® S.p. HiFi Cas9 Nuclease (Integrated
695 DNA Technologies) was used for the editing. A mixture of crRNA (0.61 μM), Cas9 protein (10
696 ng/μl), and donor (10ng/μl) was injected into 0.5 dpc embryos harvested from C57BL/6NHsd
697 (Envigo) mating pairs. Embryos that survived the injection were implanted into recipient
698 pseudopregnant females and allowed to reach term. Tail biopsies from pups were genotyped to
699 identify founders. The line was maintained in C57BL/6J.

700

701 ***DETAILED METHODS***

702 **ChP explant preparation**

703 Whole ChP from the lateral ventricle was harvested using #5 forceps and fine-dissection
704 scissors. To collect the LV ChP, the hindbrain was separated from the mid- and forebrain
705 structures using a scalpel, followed by a bilateral cut along the midline to separate the cortex into
706 two hemispheres. Each hemisphere was stabilized with forceps and a third of the rostral end was
707 cut off, the developing hippocampus was rolled out using the flat surface of a scalpel, and the
708 attached LV ChP was gently separated from the hippocampus/fornix using forceps.

709 LV ChP was transferred onto round coverslips (15 mm, Warner Instruments, Cat. 64-
710 0733) that had been prepared as follows: briefly, coverslips were lightly coated with Silicone
711 (Kwik-sil, World Precision Instruments, Item. 600022), and while wet (1xCSF: 119 mM NaCl,
712 2.5 mM KCl, 26 mM NaHCO₃, 1 mM NaH₂PO₄, 11 mM glucose, with fresh 2.0 mM magnesium

713 chloride and 2.8 mM calcium chloride), a polycarbonate membrane (Whatman, Nucleopore, 13
 714 mm wide, 8.0 μm pore size, Cat. 110414) was placed on the cover slip. Edges of the
 715 polycarbonate membrane were attached to the coverslip using adhesive (3M, Vetbond). These
 716 glass coverslips were kept at room temperature and allowed to cure. The ChP was flattened onto
 717 the membrane and secured using 3M Vetbond. All samples were placed in a holding chamber
 718 with continuously oxygenated (95% O_2 / 5% CO_2) 1x CSF.

719

720 ***IN VITRO* EPITHELIAL CELL EXPERIMENTS**

721 ***In vitro* epifluorescence calcium imaging**

722 Epifluorescence calcium recordings were acquired from *FoxJ1-Cre::Ai95D* ChP explants (see
 723 above) using a 4x, 0.28 NA objective (Olympus). A halogen lamp and FITC filter set (Olympus)
 724 were used for excitation and emission filtering. Green fluorescence was collected using an
 725 sCMOS camera (Hamamatsu). Images (2048 x 2048 pixels, 3.30 x 3.30 mm^2) were acquired at
 726 10 frames/s using MicroManager (NIH).

727

728 **Segmentation of cell masks**

729 Epifluorescence calcium videos were cropped to a small region (161 x 161 μm^2) near the center
 730 of the sample, for ease of processing. We obtained cell masks based on cell shape, as follows:
 731 first, a mean image, I , was generated, and was locally contrast-normalized using:

$$732 \quad I_n = \frac{I - G(I; \sigma = 3)}{\sqrt{G(I; \sigma = 3)}}$$

733 Where G is the 2D Gaussian operation. The normalized image was then binarized and watershed
 734 to separate cells that were joined together. Objects smaller than 5 pixels were considered noise
 735 and discarded. The convex hulls of remaining objects were used as cell masks. Neuropil masks

736 were estimated as the annulus spanning the region between the cell perimeter and the perimeter
 737 obtained after dilating the cell by 5 pixels.

738

739 **Trace extraction**

740 A raw trace, $F(t)$, was extracted from each cell mask by calculating mean intensity across pixels
 741 in each mask, and for each movie frame. A neuropil activity trace, $F_{neuropil}(t)$, was calculated in
 742 the same way, using the corresponding neuropil mask. A neuropil-corrected signal, $F_{corrected}(t)$,
 743 was calculated by subtracting the neuropil trace from the raw trace, and adding back the mean of
 744 the signal:

$$745 \quad F_{corrected} = F - F_{neuropil} + \bar{F}$$

746 The signal was further normalized by:

$$747 \quad \Delta F/F = \frac{F_{corrected} - \text{median}(F_{corrected}, 500)}{\text{median}(F_{corrected}, 500)}$$

748 Where $\text{median}(F_{corrected}, 500)$ denotes a moving median filter with a window size of 500
 749 frames (50 seconds) surrounding the time t .

750 Calcium events were defined as peaks in which $\Delta F/F > (5 \times \text{std}(\Delta F/F))$. Cells with traces
 751 that never exceeded this threshold were considered “inactive”.

752

753 ***In vitro* two-photon calcium imaging**

754 Two-photon microscopy was used to record calcium activity in explants in which ChP epithelial
 755 cells express GCaMP6f (in *FoxJ1-Cre::Ai95D* mice, using an Olympus MPE-RS two-photon
 756 microscope; 30.0 frames/s; 512x512 pixels/frame). All imaging was performed with a 25x, 1.0
 757 NA objective (Olympus) at 4.5x digital zoom ($\sim 113 \times 113 \mu\text{m}^2$). Laser power measured below
 758 the objective at 940 nm was 55 mW using a Mai Tai DeepSee laser (Spectra-Physics). To

759 perform 3D recordings, the settings above were used in conjunction with a nPFocus250 piezo
760 microscope stage (nPoint) moving axially in a sawtooth pattern. 3D volume recordings were
761 acquired at ~0.25 Hz to capture baseline activity, during which time aCSF flowed through the
762 perfusion chamber (Warner; performed at room temperature) containing the ChP explant.
763 Subsequently, increasing concentrations of 5-HT or the 5-HT_{2C} selective agonist, WAY-161503
764 (Tocris), in aCSF were introduced for one minute per concentration, with ten minute aCSF
765 washouts in between drug deliveries. To measure bulk tissue fluorescence, a mean volume
766 projection along the axial (z) dimension was performed to flatten each 3D volume into a 2D
767 image, resulting in a 2D video across time. Average fluorescence across the 10 minutes baseline
768 period prior to the first drug delivery was used as a baseline image. A ΔF image stack was
769 constructed by subtracting this baseline image from each frame in the video. A trace of ΔF
770 activity was calculated as the mean pixel intensity of each frame of the image stack.

771

772 **VAMP3-pHluorin imaging**

773 Cultured cells: Z310 cells were cultured on glass coverslips and lipofectamine transfected with
774 pAAV-VAMP3-pHluorin. After 3 days, the cells were imaged using TIRF microscopy (Cocucci
775 et al., 2012) with a 100x objective (1.46 NA, Carl Zeiss) and a 2x magnification lens placed in
776 front of the CCD camera (QuantEM, Photometrics). This arrangement provided a final pixel size
777 of 80 nm. ChP explants: AAV2/5-VAMP3-pHluorin was injected *in utero* i.c.v. in E14.5
778 embryos. LV ChP explants were then harvested at P18-P24. Each ChP was attached directly onto
779 an imaging dish using Vetbond and immersed with 1.8 ml of aCSF. WAY-161503 was added
780 until the final bath concentration reached 500 nM. Individual epithelial cells from explants were

781 imaged using a ZEISS LSM880 Airyscan confocal microscope. The chamber, imaging dish
782 holder, and all buffers used were maintained at 37°C.

783 To detect secreted vesicles, each image frame was first smoothed with a two-pixel radius
784 Gaussian filter (160 nm). We then further filtered each image by calculating the determinate of
785 the Hessian matrix at every pixel, and this image stack was used to isolate VAMP3 fusion events
786 from cell background. Masks of regions involving a fusion event were obtained by PCA/ICA
787 segmentation (Mukamel et al., 2009). Fluorescent traces were extracted by averaging
788 fluorescence of all pixels within each mask. Each fluorescence trace was normalized to peak
789 fluorescence, and sorted the traces by the time at which this peak occurred, in order to generate a
790 heatmap of time courses of vesicle release events.

791

792 ***IN VIVO IMAGING EXPERIMENTS***

793 **Headpost and cranial window placement**

794 Mice used for *in vivo* two-photon imaging (8-20 weeks) were outfitted with a headpost
795 (titanium, 0.7 g, H.E. Parmer) and 3 mm cranial window using minor modifications of
796 techniques previously described (Goldey et al., 2014; Liang et al., 2018). Briefly, each cranial
797 window implant was first prepared by gluing a 3 mm x 2 mm (diameter x height) stainless steel
798 cylindrical cannula (MicroGroup) to a 3 mm diameter glass coverslip (Warner) using a UV-
799 cured optical adhesive (Norland, type 71). Approximately 3 hours prior to surgical implantation,
800 dexamethasone sodium phosphate (4 mg/ml, intramuscular) was administered in order to reduce
801 brain edema. Anesthesia was induced using isoflurane (1-3% in 100% O₂, with flow rate titrated
802 to a respiratory rate of 1 breath per minute). Using standard aseptic techniques and a
803 stereomicroscope, a 3-mm diameter craniotomy was performed over the left side of the skull,

804 centered using stereotactic coordinates (2.0 mm lateral and 0.2 mm posterior to Bregma). Next,
805 portions of neocortex, corpus callosum, and hippocampal tissue were carefully and slowly
806 aspirated to expose the lateral ventricle, with the specific purpose of preventing undue increase
807 in intracranial pressure. The ChP was visualized floating within the ventricle. Hemostasis was
808 achieved with copious irrigation using sterile phosphate-buffered saline and occasional use of
809 gelfoam. At this point, the cranial window implant was inserted through the craniotomy site and
810 lowered to a depth of approximately 2.0 mm below the skull where it pressed lightly on the
811 surface of the thalamus and preserved direct visualization of the intact ChP. The cannula was
812 temporarily affixed to the skull with Vetbond (3M) followed by a permanent seal with C&B
813 Metabond (Parkell). A custom two-pronged titanium headpost was then affixed to the skull and
814 again sealed with C&B Metabond (the headpost implantation can also be performed prior to the
815 craniotomy according to investigator preference).

816 To create a low-profile adaptor to accommodate the water-immersion objective and light
817 shielding, a custom 3D-printed imaging well (outer diameter of the base, inner diameter, height:
818 20 mm, 10 mm, 4 mm, or 7.5 mm, 5 mm, 1 mm) was then positioned around the cannula and
819 glued to the cement and headpost. Animals were given Meloxicam (0.5 mg/kg, s.c.), individually
820 housed, and allowed at least 2 weeks to recover before live imaging. The estimated success rate
821 in obtaining clear windows was ~80% for a trained surgeon. In the first post-operative week, the
822 mice were undisturbed and, during the second week, the mice were habituated to the imaging
823 environment. Each mouse was placed on a custom 3D-printed running wheel and the animal's
824 head was fixed using clamps (Thorlabs) that attach to each prong of the two-pronged titanium
825 headpost. The running wheel and associated flexible hinges were useful for decreasing brain
826 motion, by decreasing the degree to which hindlimb-related forces couple to brain motion.

827 During two-photon imaging sessions, the low-profile imaging well was covered with blackout
828 fabric (Thorlabs).

829

830 ***IN VIVO* EPITHELIAL CELL EXPERIMENTS**

831 **Epifluorescence imaging**

832 To initially localize the ChP and assess stability and orientation of the ChP post-surgery, an
833 epifluorescence video was recorded while scanning axially through the tissue. To account for
834 lensing effects from changing z planes, planes were registered with scaled rotations to each other
835 using the StackReg plugin in Fiji (NIH).

836

837 **Two-photon imaging of spontaneous activity**

838 To capture high-speed subcellular and cellular activity in epithelial cells *in vivo*, two-photon
839 microscopy was used to record calcium activity in a ~25 cells. Imaging of GCaMP6f-expressing
840 epithelial cells (in *FoxJ1-Cre::Ai95D* mice, see above) was performed using a resonant-scanning
841 two-photon microscope (Olympus, 512x512 pixels/frame; Bruker, 490x372 pixels/frame).
842 Spontaneous activity was recorded at a single imaging plane (Olympus, 30.0 frames/s; Bruker
843 41.5 frames/s). All imaging was performed with a 25x, 1.0 NA objective (Olympus) at 4.5x
844 digital zoom (~113 x 113 μm^2). Laser power measured below the objective was 55 mW using a
845 Mai Tai DeepSee laser at 940 nm (Newport Corp.).

846

847 **Registration/preprocessing of spontaneous activity**

848 To compensate for rapid ChP motion caused by mouse locomotion and changes in posture, each
849 frame was registered to a target image created by the mean of the first 500 frames. Registration

850 was performed by cross-correlating the Fourier transform of each image with this target image
 851 (i.e. rigid-body translation correction; Guizar-Sicairos and Fienup, 2008).

852

853 **Cellular and subcellular segmentation – spontaneous activity**

854 After registration, a mean image across the entire recording was generated. Cell outlines and
 855 outlines of cell nuclei were manually drawn for each cell in the field of view (~20-40 cells).
 856 Cytoplasm masks were generated from the difference between cell mask and nucleus mask.
 857 Cytoplasm masks were subdivided into 12 radially symmetric subsections from the center of the
 858 nucleus. The neuropil area was an annulus surrounding the cell, calculated by dilating the
 859 cytoplasm masks by 10 pixels, and excluding pixels in the original cell mask from this dilated
 860 cell mask.

861

862 **Subcellular trace extraction – spontaneous activity**

863 First, the aligned video was down-sampled by a factor of 4. For each cell, the raw sector activity
 864 (i.e. a pie slice of the cell), $F_{raw}(\theta, t)$, was calculated as mean activity across pixels inside each
 865 of the cytoplasm sectors (θ) for every timepoint, t . Neuropil activity, $F_{neuropil}(t)$ was calculated
 866 as mean pixel activity in the neuropil mask defined above, at every timepoint, t . Neuropil activity
 867 was subtracted from raw activity to generate a neuropil-corrected time course:

$$868 \quad F_{corrected}(\theta, t) = F_{raw}(\theta, t) - F_{neuropil}(t)$$

869 To normalize for different baseline section brightness, a rolling median of 6.67 s was subtracted
 870 from neuropil-subtracted signal:

$$871 \quad \Delta F(\theta, t) = F_{corrected}(\theta, t) - \text{median}_{\Delta t=6.67s}(F_{corrected}(\theta, t))$$

872 To find subcellular calcium events, the maximum signal across cell sectors was calculated, for
 873 each time t , by taking a maximum projection across sectors, and then subtracting the median
 874 across sectors:

$$875 \quad F_{max}(t) = \max_{\theta}(\Delta F(\theta, t))$$

$$876 \quad \Delta F_{max}(t) = F_{max}(t) - \text{median}_t(F_{max}(t))$$

877 This approach generated a single trace of the largest fluorescence deviation from median
 878 fluorescence across cell sectors at every timepoint.

879 To identify subcellular events, a peak detector was applied to the above trace using a threshold
 880 based on the trace of median activity across sectors ('median trace', F_{med}). First, F_{med} was
 881 calculated as:

$$882 \quad F_{med}(t) = \text{median}_{\theta}(\Delta F(\theta, t))$$

$$883 \quad \Delta F_{med}(t) = F_{med}(t) - \text{median}_t(F_{med}(t))$$

884 Subcellular calcium events were defined as local peaks of epochs in which $\Delta F_{max} > (5 \times$
 885 $\text{std}(\Delta F_{med}(t)))$.

886

887 ***In vivo* 3D imaging of epithelial cell responses to delivery of a serotonin agonist**

888 To perform 3D recordings, the same imaging settings described above for spontaneous *in vivo*
 889 calcium imaging were used, but with the addition of a nPFocus250 piezo microscope stage
 890 (nPoint) that moved the imaging plane axially in a sawtooth pattern (scanning of 93 planes per
 891 volume across 350 μm of depth with a scan rate of frame rate of 30.0 frames/s, 512x512
 892 pixels/frame, resulting in volume scanning of a 170x170x350 μm^3 volume at 0.32 volumes/s).

893 To register these volumes, we first averaged together every ten volumes in order to
 894 improve signal-to-noise ratio. Since the observed effects of WAY-161503 were slow and long

895 lasting, this approach did not overly compromise temporal resolution. Each plane of these
896 average volumes was registered to the center z plane (middle plane) of the volume using the
897 StackReg plugin in Fiji (NIH), creating a rectified volume. The maximum intensity projection of
898 each of these rectified volumes were used to correct for inter-volume motion X-Y motion. Using
899 the first volume as an anchor point, each volume was registered to the previous volume. X-Y
900 plane transverse shifts were calculated by cross-correlating the Fourier transformations of the
901 maximum intensity projection of a given volume and of the previous volume (Guizar-Sicairos
902 and Fienup, 2008).

903

904 ***IN VIVO* IMMUNE CELL EXPERIMENTS**

905 **Dextran injection**

906 Mice received intraperitoneal injections of dextran conjugated with Texas Red (70 kDa 0.2
907 mg/gm IP). ThermoFisher Scientific), delivered 30 minutes before imaging. Presence of dextran
908 in vasculature was confirmed by two-photon imaging.

909

910 **Two-photon imaging**

911 3D volume recording was necessary to robustly track the ChP across long timescales due to
912 mouse motion, changes in posture, and occasional axial drift of ChP. Two-photon imaging of
913 immune cells and vasculature was performed using a resonant-scanning two-photon microscope
914 (experiments were performed on two different microscopes: Olympus; 12.8 frame/s; 512x512
915 pixels/frame; 0.16 volumes/s, 81 planes/volume; volume size: 254x254x400 μm^3 . NeuroLabware:
916 15.5 frames/s; 796x512 pixels/frame; 0.25-0.5 volumes/s, 31-62 planes/volumes; volume size:
917 355x230x100 μm^3). Volume scanning on the Olympus was achieved by using a piezo

918 microscope stage (nPFocus250). Volume scanning on the Neurolabware microscope was
919 achieved using a tunable focus lens (Optotune). All imaging was performed with a 25x, 0.95 NA
920 objective (Olympus) at 2x zoom ($\sim 254 \times 254 \mu\text{m}^2$ (Olympus), $\sim 360 \times 230 \mu\text{m}^2$ (Neurolabware)).
921 Laser power at 940-960 nm (Mai Tai DeepSee laser, Spectra Physics) measured below the
922 objective was 30-40 mW. Immune cells were confirmed to be located within or on the outside of
923 the ChP based on colocalization with the fluorescent dextran-labeled vasculature pattern.

924

925 **3D registration**

926 Due to the rapid motility of immune cells across seconds, 3D registration of individual
927 volumes was necessary to properly account for ChP movement at these rapid timescales (see
928 **Figure 5**). To account for optical deformation and warping caused by the focus-tunable lens, a
929 counter-warping correction was calculated for each imaging session. The first 30 volumes were
930 averaged together to create a mean distorted volume. The affine transformation was used to
931 iteratively match each plane to its neighbor, beginning with the brightest plane of the volume and
932 moving up and down until the ends of the volume. Since affine transformations are linear
933 functions, the adjacent transformations could be combined by multiplication of the augmented
934 transformation matrix to generate the warp-correction of every focus-tunable lens plane to the
935 reference. These matrices are calculated using the MultiStackReg plugin in Fiji (NIH). Since
936 these deformations were due to the optical system, not motion of the sample, these corrections
937 were applied to every volume prior to subsequent motion correction (**Figure 5B**, “Step 1”). For
938 the Olympus microscope that uses a piezo microscope stage to scan axially, there is no
939 deformation, and this step is skipped.

940 Due to rapid motion caused by mouse movement, it was necessary to account for intra-
941 volume changes. Using the brightest plane as a stationary anchor plane, each plane was
942 registered to its neighbor, using Fourier cross-correlation to estimate the X and Y shifts. These
943 neighboring X and Y shifts were summed cumulatively so that each plane is aligned with the
944 anchor plane. (**Figure 5B**, “Step 2”) (Guizar-Sicairos and Fienup, 2008).

945 After intra-volume alignment, reference volumes were generated by averaging every 20
946 volumes. To account for inter-volume lateral and axial shifts, each volume was then registered to
947 its respective reference volume by cross-correlating the 3D Fourier transformation of the two
948 volumes to find the X, Y, and Z shifts. Each reference volume was registered to the first
949 reference volume using the same method (**Figure 5B**, “Step 3”).

950 Axial projections, such as mean, median, and maximum projections, were then performed
951 (**Figure 5B**, “Step 4”). Finally, the movie of these projected images was further stabilized in
952 three successive steps: (i.) matching each frame to the average of the first 50 frames, (ii.)
953 matching each frame of the resulting movie iteratively to its neighbor, (iii.) matching each frame
954 of the resulting movie to the average of the first 50 frames (**Figure 5B**, “Step 5”).

955 To estimate the degree of brain motion of ChP *in vivo*, we quantified the two kinds of
956 correction for brain motion that were applied (see above). The first correction involved intra-
957 volume XY displacements for each plane (**Figure 5B**, “Step 2”). The second involved inter-
958 volume displacements from 3D translational registration (**Figure 5B**, “Step 3”), together with
959 additional XY displacements common to all planes and derived from the registration of the 2D
960 image stack resulting from axial mean projections of each volume (**Figure 5B**, “Step 5”). The
961 intra-volume XY displacement reflected faster frame-to-frame motion within a given Z-scan
962 (12.8-31 frames per second; 31-81 frames per volume). We quantified the distribution of intra-

963 volume XY displacements using the Euclidean distance of intra-volume shifts in X and Y. We
964 also calculated inter-volume displacements between successive volumes (0.16-0.97 volumes per
965 second) to estimate the level of motion observed at these somewhat slower timescales (using the
966 using the Euclidean distance of X and Y displacements between consecutive volumes, ΔXY , and
967 using the absolute value of the shift in Z between consecutive volumes). These distributions were
968 then expressed as cumulative distribution functions for each recording (**Figures 5G and 5H**).
969 Overall, the degree of brain motion was substantially larger than what is observed for recordings
970 in other brain regions such as in the neocortex.

971

972 **Inter-volume motion artifacts**

973 To assess the efficacy of our 3D registration in removing motion artifacts, we considered sets of
974 five consecutive volumes of the red channel (vasculature), which was expected to be stable (i.e.
975 near-constant voxel intensity) in the absence of brain motion at this timescale. Thus, we used the
976 metric of local standard deviation as a proxy for inter-volume motion artifacts. A rolling standard
977 deviation across five neighboring volumes was calculated for each 3D voxel. These standard
978 deviation contributions were averaged to obtain a global estimate of image stability. To account
979 for global intensity changes within and across recordings, we normalized this mean standard
980 deviation signal by the mean fluorescence to obtain an estimate of inter-volume motion artifacts
981 over time.

982

983 **Re-registration of single-cell regions**

984 After 3D registration of the entire region, individual cell regions of interest (ROIs) were selected
985 for local re-registration. XYZ regions were determined manually. The selected regions were then

986 re-registered in XY with Fourier transformation-based cross-correlation, and individual plane
987 affine registration, as described in the “3D registration” section, above.

988

989 **Recording “physiological housekeeping” by immune cells**

990 To assess the uptake of dextran by immune cells, a 3D recording of the ChP was acquired for 1
991 hour before the injection of 70 kDa red dextran. Immediately after this recording, without
992 moving the recording field of view, the mouse was injected with 70 kDa red dextran, and
993 recorded for 1 hour, as the dextran filled the vessels. Another 48 hours later, using local vascular
994 features and tissue morphology, we imaged the same volume of ChP for an additional hour,
995 without injecting more red dextran. All three recordings used the same acquisition parameters.

996

997 **LPS RESPONSE**

998 To elicit a peripheral inflammatory response, 0.5 mg/kg lipopolysaccharide (LPS, Sigma) was
999 delivered IP (Monje et al., 2003). An equal volume of saline was used as a control.

1000

1001 **Quantifying *in vitro* “vessel coating” by immune cells**

1002 To quantify immune cell alignment with the periluminal region immediately adjacent to
1003 vessels, wholemount LV ChP explants of LPS- and saline-injected *Cx3cr1^{+GFP}* mice were
1004 isolated and immunostained for GFP to label immune cells, and PECAM to label vasculature.
1005 Using a 500 pixel x 500 pixel ROI (225 x 225 μm^2), the image pixel intensities were rescaled to
1006 the range 0-1 (20th percentile of pixel brightness rescaled to 0; 90th percentile of pixel brightness
1007 rescaled to 1). A first step to defining the periluminal region was to develop an automated
1008 algorithm to segment the vasculature. Segmenting vasculature involved identifying image

1009 regions that contain tube-like structures. Additionally, these tubes may be of different sizes (e.g.,
1010 capillaries vs. veins), and may also join together in junctions. Based on this structural
1011 description, a Jerman filter (Jerman et al., 2016) based on the local second-order derivative of the
1012 image (filter widths from 8 to 15 pixels; regularization factor $\tau = 1$) was used to enhance pixels
1013 that were part of tube-like structures. The resulting image was then binarized to separate
1014 vasculature from the background.

1015 To validate the automated vessel segmentation algorithm used as an initial step towards
1016 defining periluminal space surrounding vessels, the vasculature image was manually segmented
1017 as a ground truth comparison. Using the Selection Brush tool in Fiji, the vessels were hand traced
1018 and converted to a manual binary mask. The same region was automatically segmented, as
1019 above, to generate an automatic binary mask. The automated and binary masks were compared
1020 with the contour matching function, `bfscore.m`, in MATLAB. Briefly, the Boundary F1 score
1021 measures the how closely a predicted boundary matches a ground truth boundary. This algorithm
1022 was chosen over Dice or Jaccard similarity coefficients, as our principal goal involved defining
1023 the accuracy of the estimate of periluminal boundary.

1024 To obtain an estimate of periluminal space, the edge of the binary image of vasculature
1025 (see above) was then dilated with a disk kernel with a width of 5 pixels ($\sim 1.6 \mu\text{m}$). Pixels
1026 belonging to this dilated mask but not to the original vascular mask were considered to belong to
1027 the periluminal region.

1028 Pixels containing immune cell bodies or processes were defined as follows, from the
1029 green emission image. First, the image pixel intensities were rescaled to the range 0-1 (10th
1030 percentile of pixel brightness rescaled to 0; 98th percentile of pixel brightness rescaled to 1). The
1031 image was then binarized, and dilated using a disk kernel with a width of 5 pixels. The degree of

1032 immune cell process occupancy within the periluminal region (“vessel coating”) was estimated
1033 as the proportion of vessel edge that overlapped with the binarized and dilated immune cells.

1034

1035 ***In vivo* vessel coating**

1036 To quantify *in vivo* changes in immune cell occupancy of periluminal regions near
1037 vessels in response to LPS, a four-hour, four-dimensional (X Y x Z x T stack) dataset was mean-
1038 projected along the Z-axis to produce an XYT image stack. This image stack was downsampled
1039 to 120 volumes (by averaging successive sets of 30 volumes), to improve the signal-to-noise
1040 ratio. This image stack was split into two channels: immune cells and vasculature. To define the
1041 periluminal region, vascular image stack intensity levels were rescaled to the range 0-1 (20th
1042 percentile of pixel brightness rescaled to 0; 98th percentile of pixel brightness rescaled to 1), and
1043 registered using the MultiStackReg function in Fiji, based on motion estimates from the immune
1044 cell image stack (NIH). A median projection of the vascular channel was computed, and pixels
1045 belonging to vessels were enhanced relative to the background using a Jerman filter (Jerman et
1046 al., 2016)(filter width from 8 to 10 pixels; regularization factor, $\tau = 1$; see above). The gradient
1047 of the Jerman filtered image was calculated to find the vessel edges, and the gradient image was
1048 binarized, and morphologically closed (dilation, followed by erosion of a binary image, resulting
1049 in the filling of small holes; the structuring element was a disk with radius of 2 pixels), defining
1050 the periluminal regions of the image.

1051 The immune cell image stack intensity levels were rescaled to the range 0-1 (10th
1052 percentile of pixel brightness levels, rescaled to 0; 98th percentile of pixel brightness levels,
1053 rescaled to 1). For each time frame in the image stack, t , the fluorescence in the periluminal
1054 region, $F(t)$ was calculated as mean pixel intensity in the periluminal region defined above.

1055 GFP fluorescence in the periluminal region, $F(t)$, was measured at each frame in the
1056 image stack as the mean immune cell pixel intensity in the periluminal region defined above. The
1057 average fluorescence in the 1 hour prior to LPS injection was used as a baseline, F_0 . Change in
1058 fluorescence in response to LPS was expressed as: $\Delta F/F_0$.

1059

1060 **IMMUNE CELL IMAGING DURING ACUTE ChP INJURY**

1061 In this experiment, a local region of the ChP of an adult awake mouse was heated using the
1062 imaging laser, together with two-photon imaging of immune cells and vasculature prior to and
1063 following the heating. After one hour of imaging of 3D volumes of immune cells and vasculature
1064 (as detailed above) at 2x magnification and 30-40 mW power, the scan settings were changed to
1065 8x magnification and ~150 mW for two minutes to induce focal heating of a local region of the
1066 tissue. Immediately following this tissue manipulation, the previous laser scanning settings were
1067 returned to observe immune cell activity in response to the focal damage.

1068

1069 **Tracking immune cell acute response to laser burn injury**

1070 Immune cell body positions relative to the rectangular region targeted for focal laser heating
1071 were sampled every 5 minutes for 30 minutes before heating and in the one hour after heating. A
1072 distance transformation was used to calculate the closest distance from each cell to the
1073 rectangular injury site at each timepoint (using `bwdist.m` in MATLAB). Velocities of these cells
1074 toward the burn site ($\Delta distance / \Delta time$) were calculated for the 30-minute period before the
1075 injury, and separately for the 60-minute period after the injury. In cases where the cell entered
1076 the burn site prior to the end of the recording period, the velocity was calculated using the
1077 displacement that took place until the moment that the cell entered the burn site.

1078

1079

1080 OTHER SUPPORTING EXPERIMENTS**1081 Immunostaining and immunoblotting**

1082 ChP explants were dissected and fixed in 4% paraformaldehyde for 10 min at room temperature.

1083 Samples were incubated in primary antibodies overnight at 4°C and in secondary antibodies at

1084 room temperature for two hours. For ACTA2 staining, samples were permeabilized with 0.1%

1085 Tween 20 in PBS prior to primary antibody incubation. For GFP and CD31 staining, samples

1086 were blocked (0.3% TritonX-100, 5% goat serum in PBS) for 1 hour prior to primary antibody

1087 incubation. All samples were counterstained with Hoechst 33342 (Invitrogen, H3570, 1:10,000)

1088 and mounted onto slides using Fluoromount-G (SouthernBiotech). Standard protocols were used

1089 for immunoblotting.

1090

1091 Nearest-neighbor analysis

1092 Locations of cell centers were selected using the Cell Counter plugin in Fiji. The tissue

1093 outline was drawn manually and made into a binary mask using Fiji. The nearest-neighbor

1094 distance was calculated for each individual cell, and the cumulative distribution function was

1095 plotted using the spatstat package in R (Badedy et al., 2015).

1096 Simulated distributions based on a 2D Poisson distribution were generated iteratively 100

1097 times. The mean of these simulated distributions yielded the “Poisson” estimate, while their

1098 extrema yielded a $p = 0.01$ acceptance interval.

1099

1100 Cytokine FACS array

1101 Pure CSF samples were collected from the cisterna magna. Blood samples were collected by tail-
1102 nick one hour following saline or LPS injection. The samples were coagulated, centrifuged and
1103 diluted five-fold. Post-surgical CSF samples were diluted two- or three-fold; post-LPS CSF
1104 samples were not diluted. For the 13-plex cytokine FACS-ELISA analysis, all samples were
1105 processed according to the manufacturer's instructions. After resuspension, the beads were run
1106 on a FACS Celesta (BD Biosciences) and FACS results were analyzed by LegendPlex v7.1
1107 software.

1108

1109 **Quantitative RT-PCR**

1110 RNA samples were prepared using either the RecoverAll Total Nucleic Acid Isolation Kit
1111 (Ambion) or the mirVana miRNA isolation kit, following the manufacturer's specifications.
1112 Extracted RNA was quantified spectrophotometrically and 100 ng was reverse-transcribed into
1113 cDNA using the ImProm-II Reverse Transcription System (Promega) or ABI High Capacity
1114 cDNA Reverse Transcription Kit (4368813, Thermo Fisher). Primers were purchased from
1115 Thermo Fisher (Taqman Gene expression assays, informed by "Best Coverage") and q-PCR
1116 reactions were conducted performed in duplicate using Taqman Fast Univ. PCR Master Mix.
1117 Cycling was executed using the StepOnePlus Real-Time PCR System (Invitrogen) and analysis
1118 of relative gene expression was performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen,
1119 2001). Gene expression readouts were normalized to eukaryotic *18S* rRNA or *Gapdh* as internal
1120 controls.

1121

1122 **Transmission and Scanning EM**

1123 Lateral ventricle ChP tissue from adult mouse brain was micro-dissected and processed for EM
1124 using standard methods (Coulter et al., 2018).

1125

1126 ***QUANTIFICATION AND STATISTICAL ANALYSIS***

1127 To achieve robust and unbiased results while minimizing animal use, whenever possible, we
1128 focused on within-mouse comparisons (e.g. changes in immune cell motility before and after
1129 tissue injury, **Figure 7I**), which affords greater sensitivity. It was not possible for experimenter
1130 to be not blinded to experimental conditions, except in analysis of LPS vs. saline control (**Figure**
1131 **7C**). We attempted to adhere to principles of Good Laboratory Practice
1132 (www.who.int/tdr/publications/documents/glp-handbook.pdf). Unbiased results were obtained by
1133 prospectively defining exclusion criteria (e.g., acquisition criteria [alignment, image quality],
1134 viral expression/localization). All descriptions of statistical significance, statistical tests used,
1135 and exact values and representations of n can be found in the figure legends. Software packages
1136 used for statistical tests can be found in the STAR methods Key Resource Table under Software
1137 and Algorithms.

1138

1139 **SUPPLEMENTAL VIDEO LEGENDS**

1140 **Video S1. Imaging calcium activity in adult lateral ventricle ChP explant. Related to Figure**
1141 **1.** Widefield epifluorescence imaging of spontaneous calcium activity of epithelial cells in ChP
1142 explants (large leaf of LV ChP) in *FoxJ1-Cre::Ai95D* mice. This method allowed for
1143 simultaneous visualization of activity in many thousands of active epithelial cells (see also
1144 **Figure 1F**). *Inset*: zoom-in of cellular activity in the region outlined by a solid white square

1145 (same subregion as in **Figures 1G-1M**, spanning 161 x 161 μm^2). Note uncorrelated activity
1146 throughout the explant. Timestamps are in units of minutes:seconds.

1147

1148 **Video S2. Epithelial cells show spontaneous and evoked calcium activity in explants.**

1149 **Related to Figure 2.** Widefield epifluorescence imaging of spontaneous calcium activity of
1150 epithelial cells in ChP explants (large leaf of LV ChP) in *FoxJ1-Cre::Ai95D* mice during
1151 delivery of 5 nM, 50 nM and 500 nM doses of 5-HT, indicated at top of movie. *Left*: raw movie.
1152 *Right*: change in fluorescence from pre-drug baseline. Note the increasing magnitude of
1153 responses to progressively larger doses of 5-HT. Note also the progressive decline in
1154 spontaneous activity following drug administration. Movie data and dimensions are same as in
1155 **Figure 2A**. Timestamps are in units of minutes:seconds.

1156

1157 **Video S3. Epithelial cells show spontaneous and evoked calcium activity that can stimulate**

1158 **exocrine secretory events in explants. Related to Figure 2.** Two-photon calcium imaging of a
1159 LV ChP explant from a *FoxJ1-Cre::Ai95D* mouse during delivery of 5 nM, 50 nM, and 500 nM
1160 doses of the 5-HT_{2C}-specific agonist, WAY-161503. Movie data and dimensions are same as in
1161 **Figure 2F**. Timestamps are in units of minutes:seconds. The larger the dose, the more cells
1162 showed sustained evoked increases in calcium, followed by apocrine secretion events involving
1163 gradual pinching off and release of intracellular contents (see, for example, minutes 32-44 of the
1164 video).

1165

1166 **Video S4. Cultured ChP cell shows spontaneous vesicle fusion events. Related to Figure 2.**

1167 TIRF imaging of VAMP3-pHluorin from Z310 cultured ChP cell shows spontaneous vesicle
1168 fusion events (brief, bright dots). Movie data and dimensions are same as in **Figure S2K**. Scale
1169 in first frame, 10 μm . Timestamps are in units of minutes:seconds.

1170

1171 **Video S5. Epithelial cell shows spontaneous and evoked vesicle fusion events in explants.**

1172 **Related to Figure 2.** Confocal imaging of a single cultured LV ChP epithelial cell exhibiting
1173 increased frequency of VAMP3-related vesicle release events following delivery of the 5-HT_{2C}
1174 agonist WAY-161503 (500 nM). *Left*: raw confocal images. *Right*: spatially filtered images (see
1175 **Figure 2G** and associated legend for details). Movie data and dimensions are same as in **Figure**
1176 **2G**. Scale in first frame, 5 μm . Timestamps are in units of minutes:seconds.

1177

1178 **Video S6. Imaging lateral ventricle ChP in awake mice. Related to Figure 3.** Epifluorescence

1179 imaging from four awake mice (*FoxJ1-Cre::Ai95D*) with surgically implanted imaging cannulae
1180 and windows highlighting typical motion of LV ChP (bright tissue in center) over a 30-second
1181 period. These four mice were imaged at 134, 123, 325, and 349 days post-surgery, respectively.
1182 Timestamps are in units of minutes:seconds. Note that mice shown here are different than those
1183 in **Figure 3F**.

1184

1185 **Video S7. Spontaneous *in vivo* calcium activity of epithelial cells reveals subcellular activity.**

1186 **Related to Figure 4.** Two-photon imaging of spontaneous subcellular calcium activity of ChP
1187 epithelial cells in an awake mouse (*FoxJ1-Cre::Ai95D*) over 16 seconds. The movie was filtered

1188 using a 2D spatial Gaussian (sigma: 0.55 μm), and a 1-dimensional Gaussian in time (sigma:
1189 0.033 s). Dimensions of the movie are: 282 x 282 μm^2 . Timestamps are in units of
1190 minutes:seconds.

1191

1192 **Video S8. Spontaneous *in vivo* calcium activity of epithelial cells shows subcellular activity**
1193 **in a second mouse. Related to Figure 4.** Two-photon imaging of spontaneous subcellular
1194 calcium activity of ChP epithelial cells in an awake mouse (*FoxJ1-Cre::Ai95D*) over 6 seconds
1195 (similar to **Video S7**, but from a second mouse). Timestamps are in units of minutes:seconds.
1196 Movie data and dimensions are same as in **Figure 4D**.

1197

1198 **Video S9. Epithelial cells show spontaneous and evoked calcium activity that can stimulate**
1199 **exocrine secretory events *in vivo*. Related to Figure 4.** *In vivo* two-photon calcium imaging of
1200 LV ChP epithelial cells (*FoxJ1-Cre::Ai95D* mouse) prior to and following delivery of a 5-HT_{2C}-
1201 specific agonist, WAY-161503 (3 mg/kg, SC). Movie data and dimensions are same as in **Figure**
1202 **4L**. Timestamps in top left corner indicate times after injection of WAY-161503, in units of
1203 hours:minutes:seconds. Note the elevation in calcium in each cell, followed by apocrine
1204 secretion events involving release of intracellular contents including GCaMP6f protein into the
1205 CSF.

1206

1207 **Video S10. Individual epithelial cells show spontaneous and evoked calcium activity that**
1208 **can stimulate exocrine secretory events *in vivo*. Related to Figure 4.** Zoom-in from an *in vivo*
1209 two-photon imaging session involving three individual epithelial cells expressing GCaMP6f

1210 (from a *FoxJ1-Cre::Ai95D* mouse) following injection of a 5-HT_{2C}-specific agonist, WAY-
1211 161503 (3 mg/kg SC; see also **Video S9**). Note the apocrine secretion events involving release of
1212 intracellular contents including GCaMP6f into the CSF. Timestamps are in units of
1213 minutes:seconds. Movie data and dimensions for cell in left panel are same as in **Figure 4M**;
1214 other panels show two additional example cells (same dimensions).

1215

1216 **Video S11. *In vivo* imaging of *Cx3cr1*^{+GFP} ChP immune cells reveals diverse functions**
1217 **including local surveillance. Related to Figure 5.** Four hour imaging dataset (maximum
1218 intensity projections of two-photon imaging volumes, similar to **Figure 5A**) demonstrates
1219 immune cells (green, *Cx3cr1*^{+GFP} mouse) surveying LV ChP vasculature (red, labeled using
1220 subcutaneous injection of Texas Red dextran). Scale in first frame: 50 μm. Timestamps are in
1221 units of hours:minutes:seconds.

1222

1223 **Video S12. Illustration of within-volume brain motion. Related to Figure 5.** Pan through of a
1224 single imaging volume (duration of acquisition of each volume was 6.33 s, and each volume
1225 consisted of 81 planes spanning 400 μm in depth), from a volumetric imaging run collected in an
1226 awake head-fixed mouse running on a trackball (*top left*). We used multi-color two-photon
1227 imaging of Texas Red-labeled vessels and *Cx3cr1*^{+GFP} immune cells, with Z-scanning enabled by
1228 a piezoelectric objective stage. Note the X-Y shifts across planes of this volume. We thus
1229 sequentially aligned each frame to a common reference using a translational alignment procedure
1230 (see also **Figures 5B-5D**). The degree that we shifted each frame in X and in Y directions is
1231 illustrated at bottom. The resulting aligned volume is shown at top right (blue regions indicate

1232 areas where no data is available due to alignment shifts in the imaging plane). Each frame spans
1233 255 x 255 μm^2 .

1234

1235 **Video S13. Additional movies from *in vivo* imaging of individual $Cx3cr1^{+GFP}$ ChP immune**
1236 **cells indicates multiple cell subtypes. Related to Figure 6.** Individual $Cx3cr1^{+GFP}$ immune
1237 cells surveying the LV ChP across a 44-minute period. Timestamps are in units of
1238 minutes:seconds. Dimensions for movies in top left, top right, bottom left, and bottom right
1239 panels are same as in **Figures 6D-6G**, respectively. *Top left*: epiplexus immune cell that moves
1240 freely over neighboring vessels because it is on the apical surface of the ChP. *Top right*: stromal
1241 immune cell with cell body that remains in place, but with processes extending beyond
1242 neighboring vasculature and then retracting due to apparent contact mediated inhibition with a
1243 neighboring process. *Bottom left*: stromal immune cell whose processes travel along vasculature,
1244 exhibiting contact mediated inhibition after touching processes from neighboring cells, and
1245 contact with one of its own processes. *Bottom right*: stromal immune cell with mobile cell body
1246 whose mobility appears constrained by the surrounding vessels, but whose processes extend
1247 beyond these vessels.

1248

1249 **Video S14. Uptake of dextran by stromal ChP immune cells. Related to Figure 6.** Individual
1250 $Cx3cr1^{+GFP}$ immune cells surveying the LV ChP across a 61-minute period following IP
1251 injection of Texas Red dextran (70 kDa) in awake mice. Note the gradual uptake of dextran in
1252 immune cell processes in the periluminal region near vessels (**Figure 6I**). Scale, 25 μm .
1253 Timestamps are in units of hours:minutes:seconds.

1254

1255 **Video S15. Identification of stromal and epiplexus cells in the ChP during dextran uptake.**

1256 **Related to Figure 6.** 48 hours after IP injection of Texas Red dextran (70 kDa) (**Figure 6H**),

1257 some immune cells accumulated dextran in their processes and cell bodies while others did not.

1258 Here, we characterize the immune cells that did not uptake dextran by labeling with white circles

1259 in a scan through the tissue. We then considered a maximum intensity projection image from the

1260 same volume (middle of video), and inspected X-Z and Y-Z cross-section scans of the volume

1261 (rectangular movies at end of video; location of each cross section indicated by yellow and cyan

1262 lines through the maximum intensity image). Note that most immune cells that did not uptake

1263 dextran were located out of the central, vascular layer of the ChP and were classified as

1264 epiplexus cells. Scale, 50 μm .

1265

1266 **Video S16. ChP immune cells respond to systemic injury. Related to Figure 7.** *In vivo* two-

1267 photon imaging of LV ChP immune cells ($Cx3cr1^{+/GFP}$ mouse) during IP delivery of LPS (5

1268 mg/kg). Immune cells flatten in the periluminal region near vessels (red) in response to LPS at

1269 ~1 hour post-LPS (indicated at top of movie). Timestamps are in units of hours:minutes (13

1270 minutes/frame). Movie data are same as in **Figure 7D**. Dimensions of the movie are: 97 x 90

1271 μm^2 . Note that many but not all immune cells respond to LPS. White arrowhead tracks an

1272 immune cell that flattens into the periluminal region.

1273

1274 **Video S17. ChP immune cells respond to acute injury. Related to Figure 7.** *In vivo* two-

1275 photon imaging of LV ChP immune cells ($Cx3cr1^{+/GFP}$ mouse) during focal laser heating within

1276 the region outlined by a white square. Approximately 6 minutes post-burn, Texas Red dextran
1277 leaks out from damaged vessels. Some nearby immune cells change to an activated state with
1278 retracted processes and migrate to the site of injury immediately following focal laser heating.
1279 After reaching the site of injury, these cells aggregate and remain stationary. Other immune cells
1280 appear unaffected. Movie data are same as in **Figure 7H**. Timestamps indicate time since focal
1281 laser heating and are in units of hours:minutes:seconds. Scale, 50 μm . See also **Video S18**.

1282

1283 **Video S18. Identification of stromal and epiplexus immune cells in the ChP during a focal**
1284 **injury model. Related to Figure 7.** Following a focal ChP injury induced by laser heating
1285 (**Figures 7G and 7H**), some immune cell bodies moved towards the injury site while others did
1286 not. Here, we characterized the immune cells that did move towards the injury by labeling them
1287 with white circles in a flythrough of the imaging volume (beginning of video; snapshot at 25
1288 minutes following laser burn). We then considered a maximum intensity projection image from
1289 the same volume (middle of video), and inspected various X-Z and Y-Z cross-sections of the
1290 volume (rectangular movies at end of video; location of each cross-section indicated by yellow
1291 and cyan lines through the maximum intensity projection image). Note that most immune cells
1292 that later moved towards the site of injury (6/8 mobile cells) were located outside of the central,
1293 vascular layer of the ChP, and were thereby characterized as epiplexus cells. Scale, 50 μm .

1294

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Figure 1

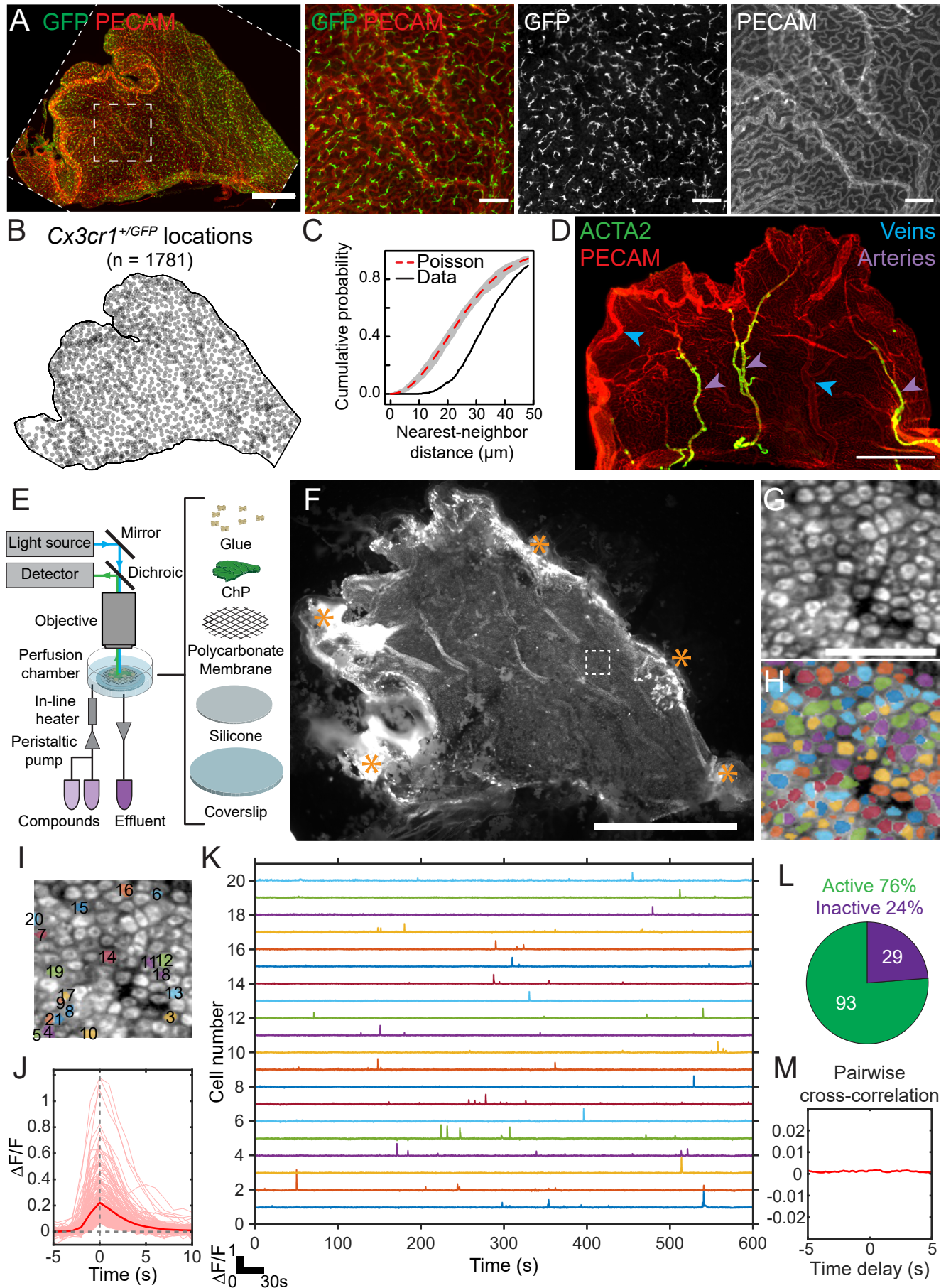


Figure 2

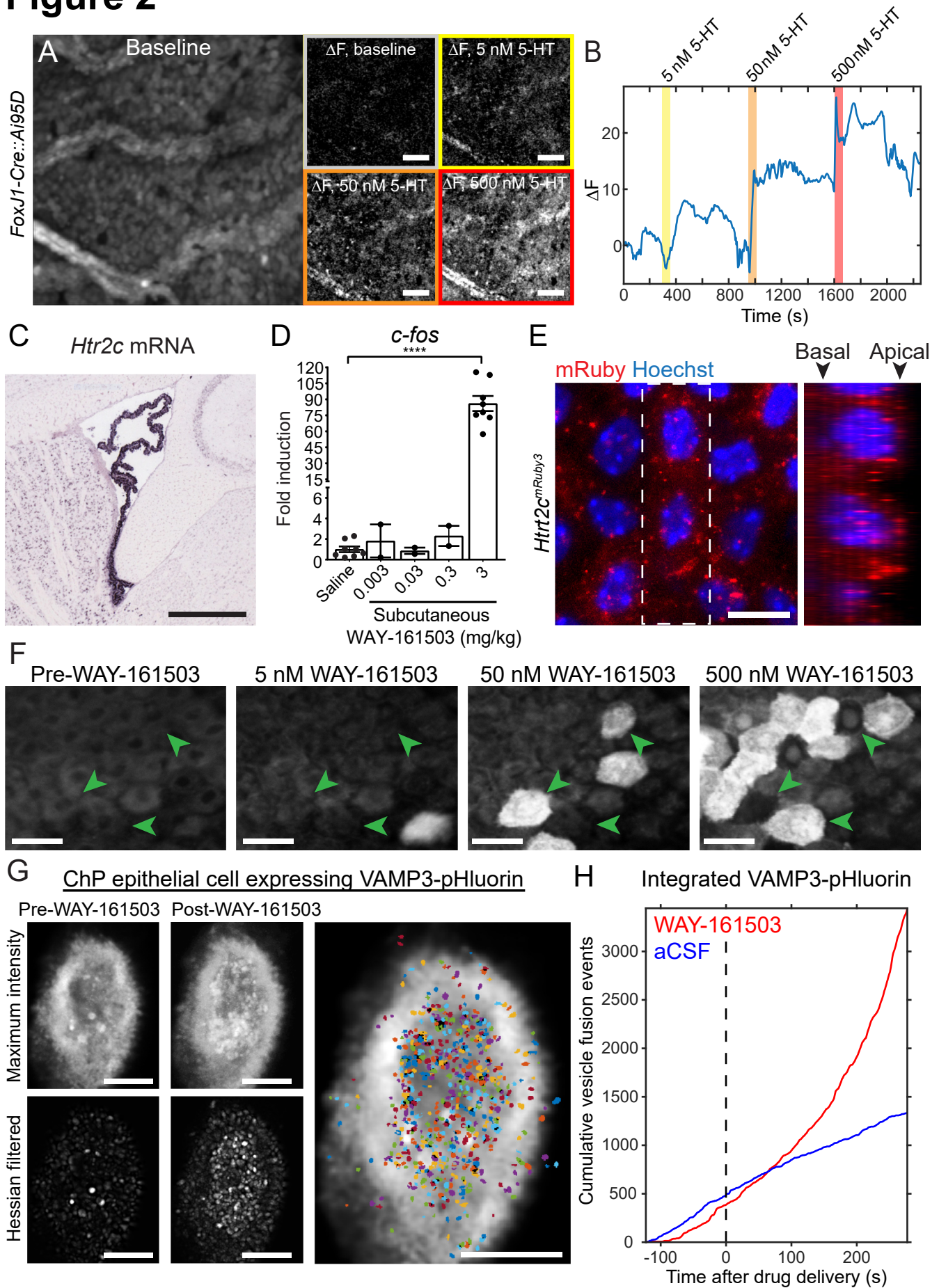


Figure 3

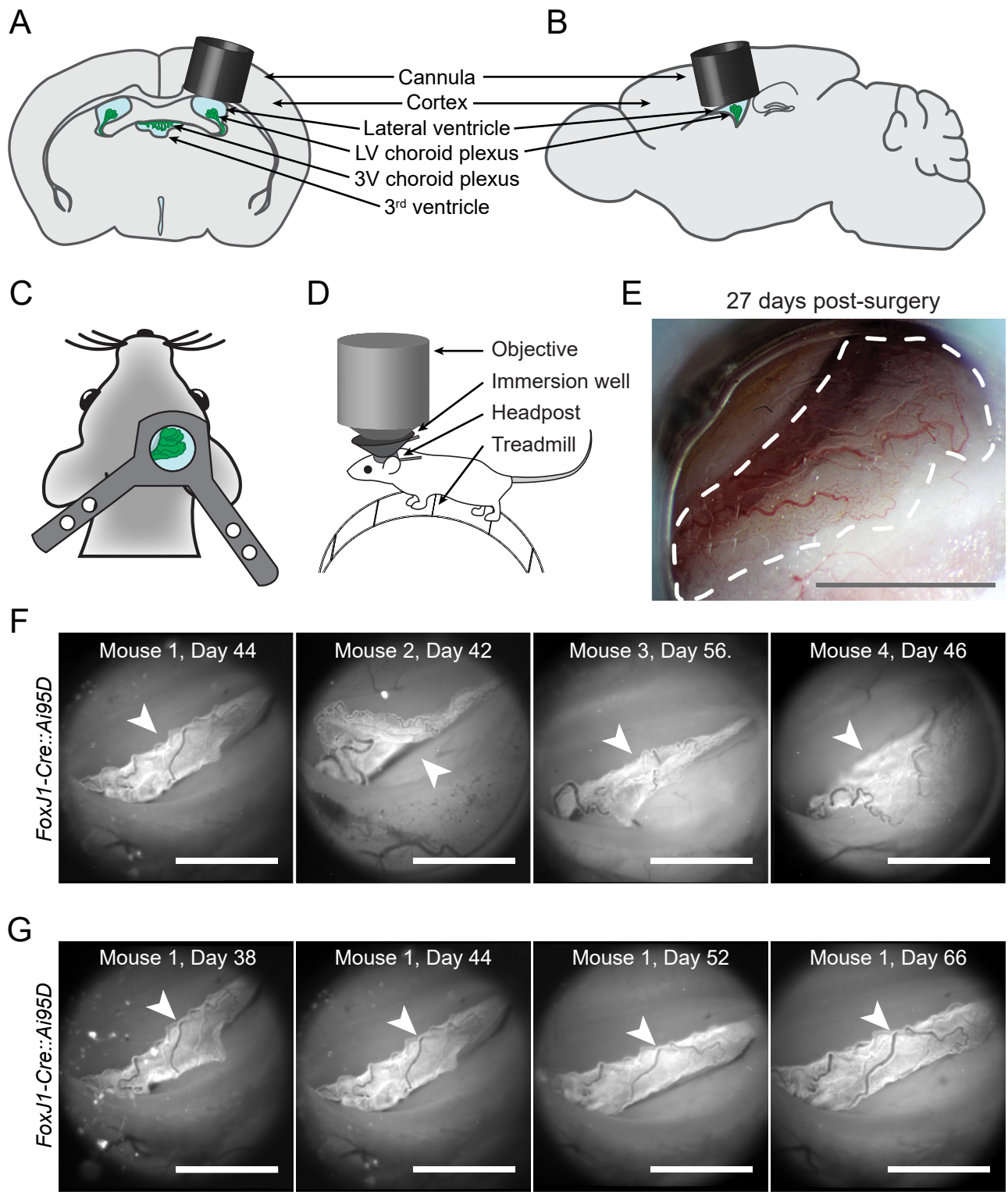


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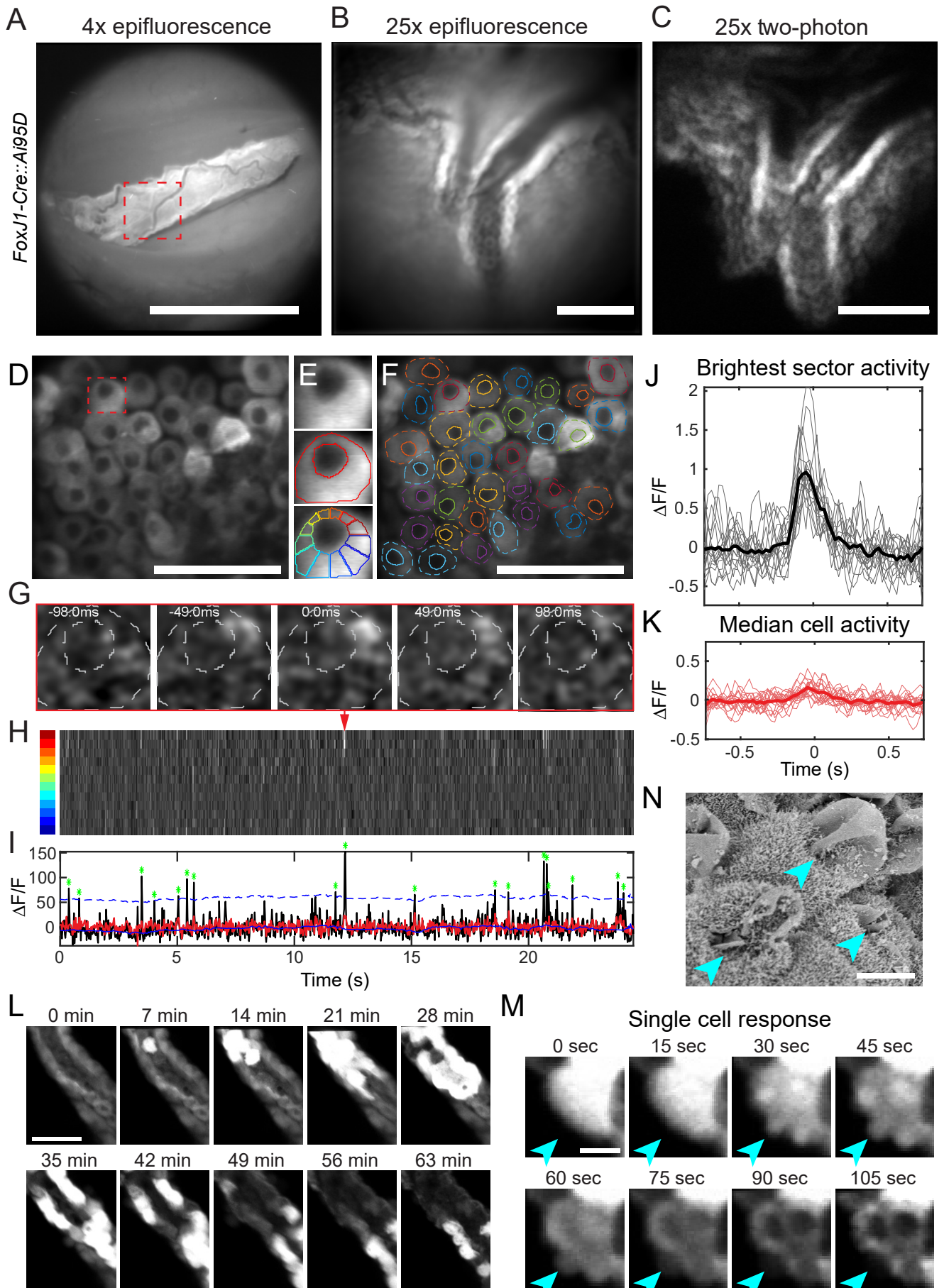
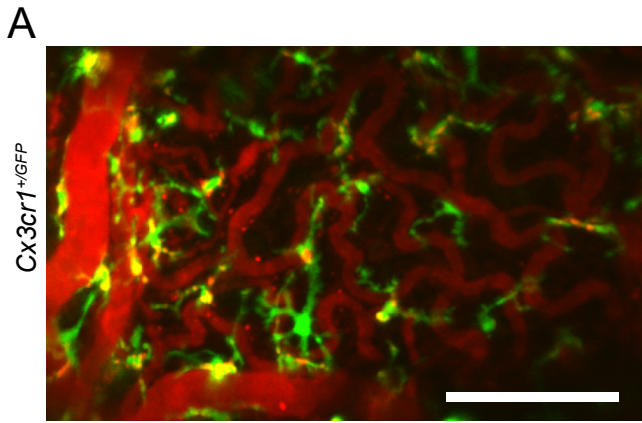


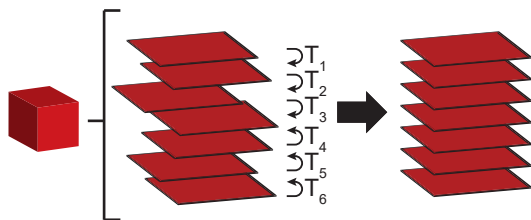
Figure 5



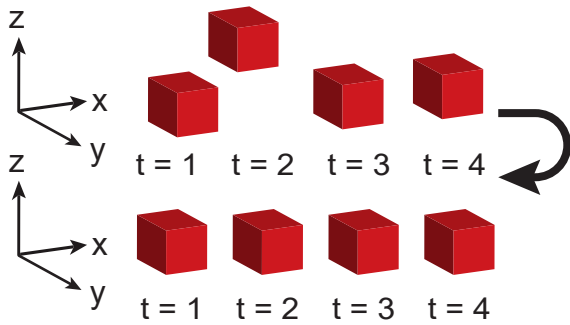
B Step 1: Distortion correction



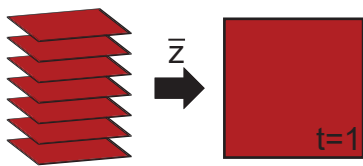
Step 2: Intra-volume alignment



Step 3: 3D translational registration



Step 4: Mean intensity projection



Step 5: 2D projection registration

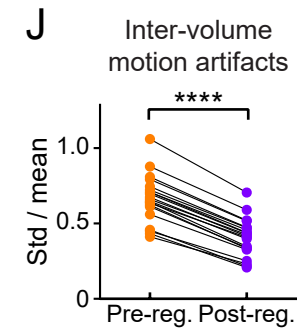
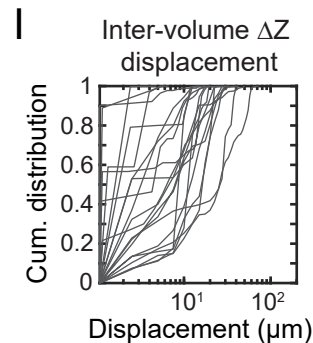
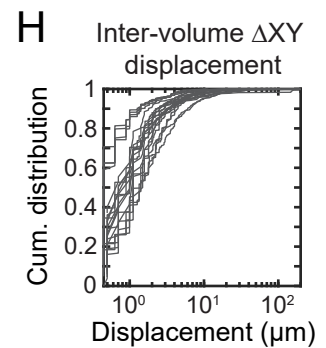
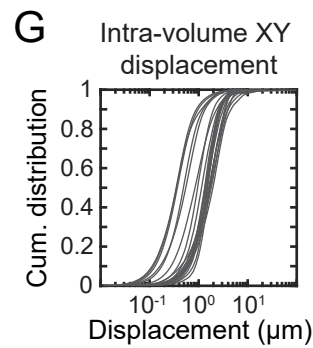
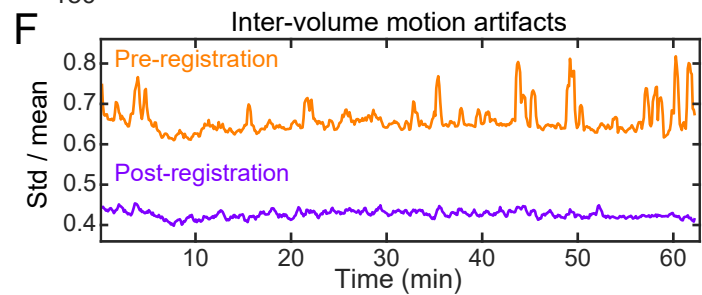
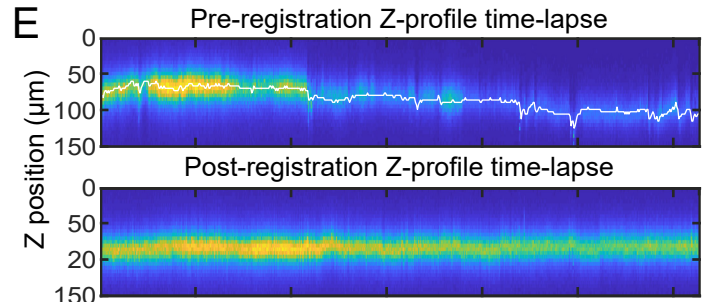
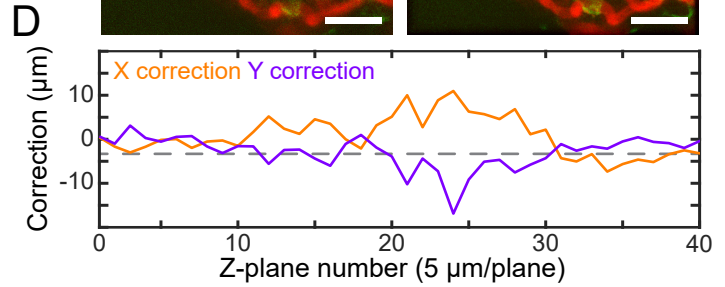
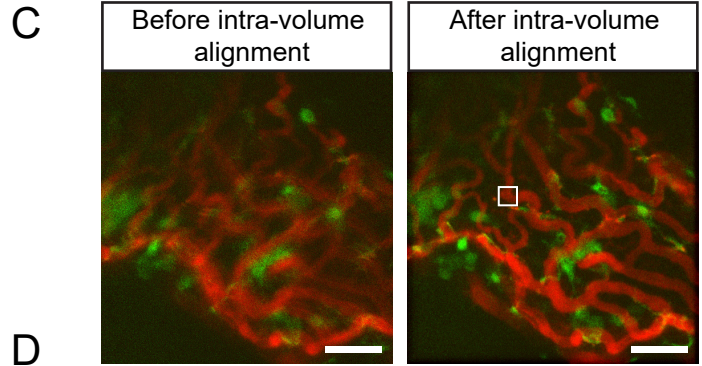
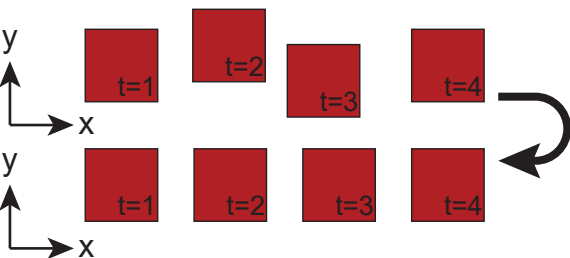


Figure 6

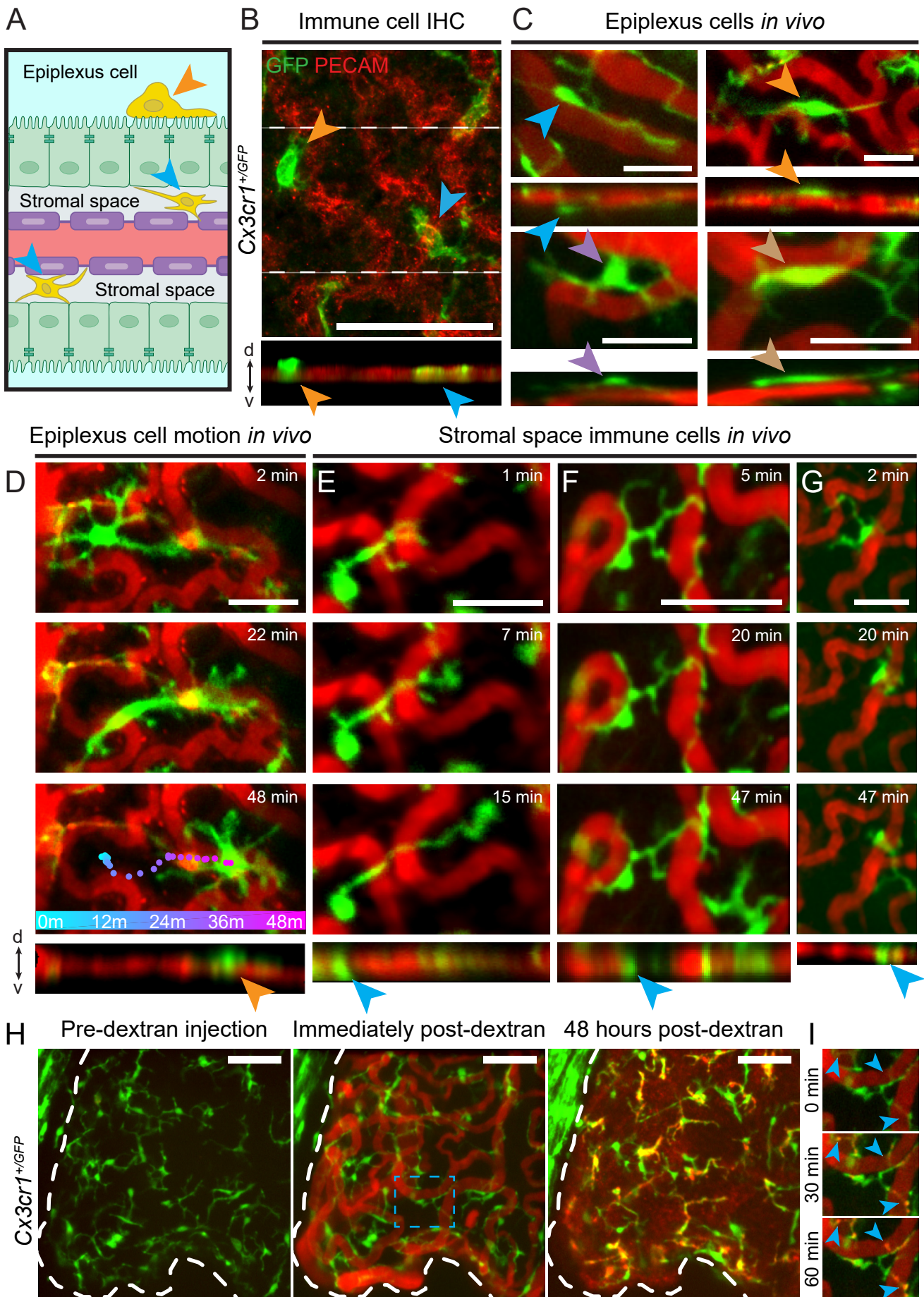


Figure 7

